CELL CYCLE-DEPENDENT, IN VITRO ASSEMBLY

OF MICROTUBULES ONTO THE

PERICENTRIOLAR MATERIAL OF HeLa CELLS

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ABSTRACT

A centriolar complex comprising a pair of centrioles and a cloud of pericentriolar material is located at the point of convergence of the microtubules of the mitotic apparatus. The in vitro assembly of microtubules was observed onto these complexes in the 1,400 g supernatant fraction of colcemid-blocked, mitotic HeLa cells lysed into solutions containing tubulin and Triton X-100. Dark-field microscopy provided a convenient means by which this process could be visualized directly. When this 1,400 g supernate was incubated at 30°C and centrifuged into a discontinuous sucrose gradient, a band containing centriolar complexes and assembled microtubules was obtained at the 50-60% sucrose interface. Ultrastructural analysis indicated that the majority of the microtubules assembled predominantly from the pericentriolar material but also onto the centrioles. When cells were synchronized by a double thymidine block, the assembly of microtubules onto centriolar complexes was observed only in lysates of mitotic cells; no assembly was seen in lysed material of interphase cells. Microtubule assembly occurred onto centriolar complexes in solutions of either 100,000 g brain supernate, 2 × cycled tubulin, or purified tubulin dimers. This study demonstrates that the pericentriolar material becomes competent as a microtubule-organizing center (MTOC) at the time of mitosis. With use of the techniques described, a method for the isolation of centriolar complexes may be developed.

KEY WORDS   microtubules · pericentriolar material · centrioles · cell cycle · dark-field microscopy

A centriolar complex comprising a pair of centrioles associated with an amorphous cloud of pericentriolar material is located at the point of convergence of the microtubules of the mitotic apparatus in a wide variety of cells. It has been hypothesized that the centrioles themselves may play a role in mediating the assembly of microtubules from the mitotic pole (29), although recent experimentation has suggested that it is the pericentriolar material that controls the assembly of these microtubules.

For example, electron microscope observation of dividing cells has demonstrated that the majority of the mitotic microtubules arise from the

pericentriolar material (8, 15, 20–22) and that the proliferation of the pericentriolar material is temporally coincident with the assembly of these microtubules (21). Furthermore, centrioles are absent at the poles of the meiotic spindles of mouse oocytes (30) as well as one pole of the mitotic apparatus of rat kangaroo cells undergoing meiotic-like reduction division in vitro (3); in these cases, microtubules arise from a cloud of pericentriolar-like material at the pole. Other experiments with rat kangaroo cells have shown that microtubules no longer assemble from the mitotic pole whose pericentriolar material has been destroyed by laser microbeam irradiation (2, 17), while similar irradiation of the centrioles has no effect on the assembly of the mitotic microtubules (1). Finally, studies investigating the in vitro assembly of tubulin onto the centriolar complexes of mammalian cells have demonstrated that the pericentriolar material has the ability to initiate the assembly of microtubules (7, 27, 33).

The mechanism by which a cell controls the assembly and disassembly of the mitotic microtubules only at a specific time during the cell cycle and assures that these microtubules arise only at specific microtubule-organizing centers (MTOCs), the kinetochores of chromosomes and the pericentriolar material at the mitotic pole, is not yet known. Experiments with colchicine binding (23) and vinblastine precipitation (11) have shown that tubulin is synthesized throughout the cell cycle, and additional work has demonstrated that the total amount of tubulin is the same in either unactivated or metaphase eggs of the surf clam (35). Thus, the assembly of the mitotic microtubules apparently is not due to an increased synthesis of tubulin immediately before mitosis, and the possibility remains that their assembly may be controlled by the mitotic MTOCs instead.

Accordingly, it would be of considerable interest to determine whether a mitotic MTOC such as the pericentriolar material can support the assembly of microtubules throughout the cell cycle, or whether, instead, it becomes competent as an MTOC only during a defined period such as mitosis. Experiments can be performed in which the in vitro assembly of microtubules onto centriolar complexes is observed under a wide variety of conditions, and these complexes can then be examined to determine the nature of the sites which initiated microtubule assembly. Finally, by assessing the ability of centriolar complexes isolated at all stages of the cell cycle to serve as sites for the

in vitro assembly of microtubules, some insight into the means by which a cell regulates the assembly of the mitotic microtubules may be obtained.

MATERIALS AND METHODS

Cell Cultures

HeLa cells were grown as a monolayer in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine and were maintained at 37°C in a humid atmosphere with 5% CO_2.

OBTAINING MITOTIC CELLS: Cells arrested in mitosis were obtained by the addition of colcemid to the culture medium at a final concentration of 0.015 μg/ml. After incubation for 1 h, mitotic cells were dislodged from the substrate into the medium which was then decanted and discarded. Fresh medium containing colcemid was added, and the flasks were allowed to incubate for 2–12 h. Mitotic cells were then dislodged into the medium by gentle shaking and were used in these studies. Routinely, mitotic cell populations of 95% were obtained. This procedure insured that the cells used were blocked at the initiation of mitotis before mitotic microtubules had assembled. Examination by electron microscopy revealed that no microtubules extended from the centriolar complexes in these cells.

CELL SYNCHRONIZATION: Synchronized populations of cells were obtained by the use of a double thymidine block (19). Thymidine (2 mM) was added to the medium of 60-mm diameter culture dishes containing nonconfluent, exponentially growing cells for 16 h, and the medium was then removed. The culture dishes were rinsed with 5 ml of medium without thymidine, and fresh medium was then added for 9 h before the second addition of thymidine (2 mM). After 14 h of further incubation the thymidine-containing medium was decanted, the dishes were rinsed with 5 ml of medium, and fresh medium was added. The cells began to enter mitosis after 6 h of further incubation, and a maximal number of mitotic cells was observed ~2 h later. Populations of cells synchronized in S phase were obtained 2 h after release of the second thymidine block.

To obtain cells synchronously entering but then blocked in mitosis, 0.015 μg/ml colcemid was added to the culture medium 2 h after the release of the second thymidine block.

Purification of Tubulin

A 100,000 g supernate containing tubulin was prepared from chick brain by a modification of the procedure of Weisenberg (36) as previously described (32). Partially purified tubulin was obtained from calf brain by two cycles of polymerization and depolymerization according to the method of Shelanski et al. (24) as modified by Sloboda et al. (26), and purified tubulin

dimers were obtained by molecular sieve chromatography according to the procedure described by Sloboda (26).

All tubulin samples (100,000 g supernate; 2 × cycled tubulin; and tubulin dimers) were either used immediately or lyophilized in 0.5-ml aliquots in a VirTis Automatic Freeze Dryer, model 10-010 (VirTis Co., Inc., Gardiner, N. Y.) and stored at −20°C. Before use, additional tubulin polymerization medium (PM, 100 mM piperazine-N-N'-bis[2-ethane sulfonic acid] [PIPES], pH 6.9, 1 mM MgSO₄, 1 mM GTP, and 2 mM EGTA) was added to each aliquot of fresh or reconstituted, lyophilized tubulin, and the absence of microtubules observed with either dark-field or electron microscopy when this preparation was incubated at 37°C for 10 min was taken as an indication that the tubulin concentration was low enough so that spontaneous microtubule assembly had not occurred. Under these conditions, microtubules would assemble only if an organizing center capable of stimulating microtubule polymerization was present.

Preparation of Centriolar Complexes

SPREADING OF MITOTIC CELLS ON A HYPOPHASE: Hypophase spreads of HeLa cells were obtained according to the procedure described by Moses and Counce (14). Mitotic cells were centrifuged at 40 g for 5 min in a table-top centrifuge, and the pellet was resuspended in 15 ml of RPMI 1640 medium without serum and recentrifuged. The supernatant fluid was carefully removed until a volume approximately two times that of the cell pellet remained. The cells were resuspended, drawn into a micropipette, and a small drop was carefully applied to a surface of 0.5% NaCl. After permitting the preparation to stabilize for 1 min, a carbon-over-Formvar, glow-discharged, copper grid was gently touched to the surface.

LYSIS OF CELLS INTO SOLUTIONS CONTAINING TUBULIN: Mitotic cells were sedimented at 1,000 g for 3 min, and the cell pellet was resuspended in 10 ml of PM without GTP. The suspension was centrifuged again, and the resulting pellet was resuspended in 50 vol of tubulin containing 0.2% Triton X-100. After incubation for 10 min at 4°C, the cells were lysed by passage four times through a 22-gauge needle, and the lysate was centrifuged at 1,400 g for 10 min at 4°C to sediment chromosomes and any unlysed cells. The supernatant fluid contained the centriolar complexes used in these studies.

1.5 ml of this suspension was carefully layered on a discontinuous sucrose gradient (0.5-ml aliquots of 40-50-60-70-80% sucrose (wt/vol) in PM in a 5 × 1.25 cm cellulose nitrate tube) and centrifuged at 35,000 g for 30 min at 4°C. Centrioles and pericentriolar material were obtained at the 70-80% sucrose interface.

Lysis of Synchronized Cells

Interphase cells were considerably more difficult to lyse than mitotic cells, and uniform cell lysis was not obtained under the conditions described in which mitotic cells were lysed. Accordingly, a different procedure was designed for studies in which centriolar complexes were obtained from cells in all stages of the cell cycle.

Medium in culture dishes containing cells exposed to a double thymidine block was removed and saved, and 2 ml of 0.25% trypsin was added. After 5-min incubation at 20°C, the cells were released from the substrate, and this cell suspension was combined with the original incubation medium which contained any mitotic cells that may have been dislodged from the substrate into the medium. Cells were sedimented at 1,000 g for 3 min at 4°C, and the pellet was resuspended with 0.35 ml of distilled water at 4°C. After being allowed to swell for 3-5 min, the cells were drawn through a 27-gauge needle into a 3-cm³ syringe containing 0.5 ml of tubulin in 0.34% Triton X-100, 0.17 M PIPES, pH 6.9, 1.7 mM MgSO₄, 1.7 mM GTP, and 3.4 mM EGTA (final buffer = 0.2% Triton X-100 in normal PM). Cells were lysed by rapid passage through the syringe six times, and nuclei from interphase cells remained. This suspension of lysed cells was centrifuged at 100 g for 5 min at 4°C. The pellet containing the nuclei was resuspended with 100 μl of tubulin, and the supernate was centrifuged at 1,400 g for 10 min at 4°C. The resulting supernatant fluid contained centrioles and pericentriolar material.

In Vitro Microtubule Assembly

ASSEMBLY ON GRIDS: Electron microscope grids containing material picked up from the hypophase upon which mitotic cells were spread were rinsed with four drops of PM and were floated on 75 μl of tubulin and incubated at 37°C for 6 min to permit microtubule assembly to occur. After this, grids were rinsed with four drops of PM and then were floated immediately on 4% paraformaldehyde, 0.1 M sucrose, and 0.1% dimethylsulfoxide, pH 6.9, for 5 min for fixation.

ASSEMBLY ON MICROSCOPE SLIDES: 4 μl of tubulin solution containing nuclei or tubulin-Triton X-100 solution containing the 1,400 g supernate with centriolar complexes was placed on a cleaned microscope slide and overlaid with a 22-mm square, No. 1¹/₂ cover slip. Slides were incubated at 37°C on a slide-warming tray for 10 min before observation.

To determine the cold stability of assembled microtubules, slides with centriolar complexes from which microtubules had assembled after incubation at 37°C were placed at 4°C for 5-60 min. Up to three cycles of alternate incubation at 37° and 4°C were performed.

ASSEMBLY IN SOLUTION: The 1,400 g supernatant fraction of mitotic cells lysed into tubulin-Triton X-100 was incubated at 30°C for 12 min to permit microtubule assembly to occur onto centrioles and pericentriolar material. This suspension was carefully layered onto a discontinuous sucrose gradient as described and centrifuged at 35,000 g for 30 min at 25°C. A band of material containing centriolar complexes from which

microtubules had assembled was removed at the 50–60% sucrose interface.

ASSEMBLY IN MIXED CELL LYSATES: To test for the presence in interphase cells of an inhibitor of the in vitro assembly of microtubules from mitotic centriolar complexes, 0.5 ml of the 1,400 g supernatant fractions of synchronized S-phase and colcemid-blocked mitotic cells lysed into tubulin-Triton X-100 were mixed together before incubation at 37°C on a microscope slide. In addition, 0.5 ml of the supernatant fractions of similarly lysed S-phase or mitotic cells was incubated directly on slides or were first diluted with 0.5 ml of additional tubulin-Triton X-100 before incubation.

Electron Microscopy

WHOLE MOUNT PREPARATIONS: Electron microscope grids were floated on a drop of material obtained from the sucrose gradient, rinsed with four drops of PM, and fixed as described. Grids were then stained in alcoholic phosphotungstic acid according to the procedure of Miller and Beatty (13) and Counce and Meyer (5) as previously described (32) and were examined in a Philips EM-201 electron microscope operated at 80 kV.

THIN SECTIONING: The band from the sucrose gradient containing centriolar complexes which nucleated microtubule assembly was centrifuged at 100,000 g for 30 min at 25°C, and the pellet was overlaid with 2% glutaraldehyde in PM for 1 h. Pellets were then rinsed three times with 50 mM sodium phosphate, pH 7.0 and rinsed in distilled water. After dehydration in acetone, the pellets were embedded in Spurr's resin. Thin sections were stained with 0.4% lead citrate in 0.1 N NaOH for 10 min and examined at 60 kV in the electron microscope.

Dark-Field Microscopy

Dark-field microscopy was performed according to the methods described by Macnab (10). The light source consisted of a 500 W xenon arc (Hanovia 959C-98) in an Oriel 6140 lamp housing with a 48-mm f1.0 fused silica condensing lens and powered by an Oriel 6242 power supply (Oriel Corp. of America, Stamford, Conn.). A heat filter (Optical Industries, 03FHA023, Optical Industries, Inc., Irvine, Calif.), UV filter (Oriel G-772-3900), and yellow filter (Corning 3-69, Corning Glass Works, Science Products Div., Corning, N. Y.) were used. Observations were made with a Zeiss microscope equipped with a Zeiss universal mirror, Zeiss oil immersion dark-field Ultracondenser (NA 1.2/1.4), Zeiss Neofluar 40×/0.75 objective, Zeiss 8× eyepieces, and Zeiss camera. Photographs were taken on Kodak Tri-X or Ilford HP-5 film at exposures of 1/8–1 s. The film was developed in Acufine ACU-1 developer at ASA 1200.

Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed

according to the method of Laemmli (9) as modified by Jarvik.[1] A stacker containing 3% acrylamide and no SDS was overlaid on a slab gel (20 × 15 × 0.15 cm) containing a 4–16% gradient of acrylamide, a 3–8 M gradient of urea, and no SDS. The gel was run at 20 MA for 12 h.

RESULTS

Assembly of Microtubules from Centriolar Complexes

Centriolar complexes obtained from hypophase spreads of cells preincubated in colcemid, picked up on grids, incubated in tubulin, and examined in the electron microscope are shown in Fig. 1. Two pairs of centrioles surrounded by an amorphous cloud of pericentriolar material are evident. Large numbers of microtubules have assembled from the pericentriolar cloud although microtubules can also be traced within the cloud to abut the centrioles. Typically, the pericentriolar material displays an asymmetric distribution around the centriolar duplex, with the majority of the material present on one side of the pair.

Subsequent experiments investigated the assembly of microtubules onto the centriolar complexes present in the 1,400 g supernatant fraction of colcemid-blocked, mitotic cells lysed directly into solutions containing tubulin. When this supernate was incubated at 37°C on a microscope slide and observed by dark-field microscopy, microtubule assembly occurred within 5 min onto discrete MTOCs as illustrated in Fig. 2.

To determine whether these organizing centers represented centriolar complexes capable of initiating microtubule assembly, a procedure was developed to separate these MTOCs from the 1,400 g supernate and to examine them by electron microscopy.

Accordingly, this supernatant fraction was incubated at 30°C and then centrifuged into a discontinuous sucrose gradient. A band of material containing organizing centers from which microtubules had assembled was obtained at the 50–60% sucrose interface. These structures, resembling asters, are illustrated in Fig. 3a. No other identifiable cellular component was present in this band although some material from the original supernate had become trapped within the meshwork of assembled microtubules. Additional microtubule-organizing centers recovered from the
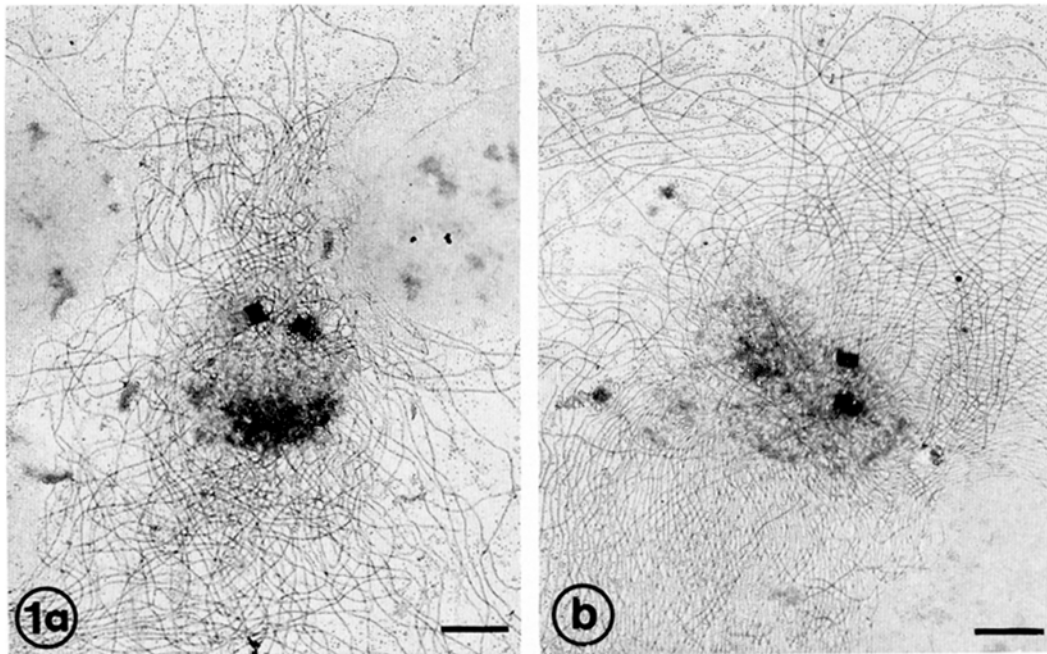
FIGURE 1 Electron micrographs of centriolar complexes from colcemid-blocked, mitotic cells that were spread on a hypophase of 0.5% NaCl, picked up on a grid, incubated in tubulin, and then fixed in formaldehyde and stained with alcoholic PTA. A cloud of pericentriolar material surrounds the pair of centrioles. Bars, 1 µm.

50-60% sucrose interface are illustrated in Fig. 3b–d. At the point of convergence of the assembled microtubules, a structure with the morphology of a centriole surrounded by amorphous material can be observed (Fig. 3b and c). Microtubules originate from the centriole and closely adhering material although specific initiation sites cannot be resolved because of the intensity of staining and close apposition of any pericentriolar material to the centriole. Occasionally, the two centrioles of the original duplex remained together during the isolation procedure and, with the surrounding material, served as a center for microtubule assembly (Fig. 3d).

If the 1,400 g supernatant fraction of cells lysed into tubulin was not incubated at 30°C but, instead, was directly centrifuged into a discontinuous sucrose gradient at 4°C so that microtubule assembly did not occur, centrioles with pericentriolar material could be recovered at the 70-80% sucrose interface. The individual centrioles of a duplex have often separated from each other (Fig. 4a) although an intact pair of centrioles could occasionally be recovered (Fig. 4b). Pericentriolar material can be observed around these centrioles.

Ultrastructural Analysis

To confirm that the MTOCs obtained at the 50-60% sucrose interface were, in fact, centriolar complexes and to determine what specific sites were capable of initiating microtubule assembly, ultrastructural analysis was undertaken as illustrated in Fig. 5a. A pair of centrioles in orthogonal arrangement is evident and the nine triplet blades of one of the centrioles are discernible. An amorphous cloud of pericentriolar material can be observed. Microtubules in both longitudinal and cross section are observed to arise from the pericentriolar material, and an assembled microtubule appears to be continuous with a microtubule within the wall of the centriole. Similar observations can be made on the complex illustrated in Fig. 5b. Interestingly, an assembled microtubule is observed within the lumen of one of the centrioles shown in Fig. 5b. In both Fig. 5a and b the majority of the pericentriolar material and assembled microtubules appear to encircle one centriole of each duplex, an observation also made in sections of dividing cells (15).
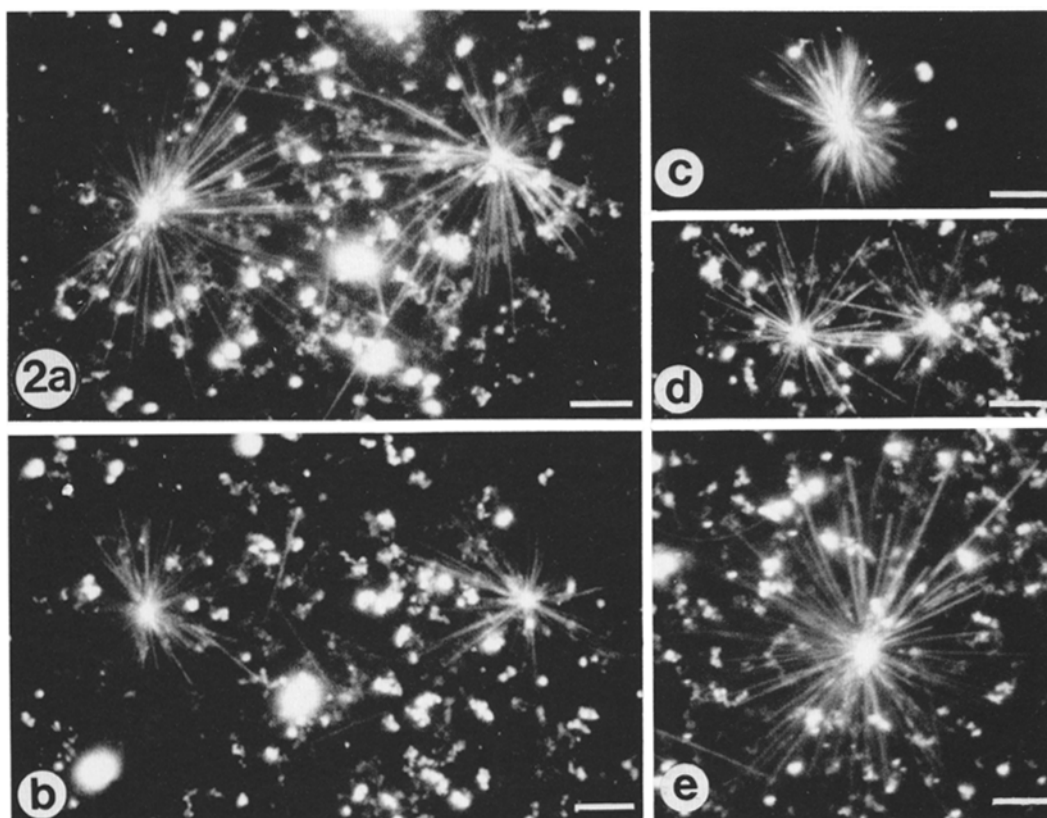
Illustrated in Fig. 6a and b are single centrioles

FIGURE 2 Dark-field micrographs of microtubules assembled onto the centriolar complexes from colcemid-blocked, mitotic cells lysed into solutions containing tubulin and incubated on a microscope slide. Bars, 5 μm.

of a duplex observed in longitudinal section. Microtubules have polymerized that are continuous with the microtubules constituting the triplet blades of the centriole, and, significantly, the assembly of these microtubules is polar with one end of the centriole favored as an assembly site. As observed before, the majority of the assembled microtubules arise from the pericentriolar material.

Cell Cycle Experiments

These studies confirmed that the in vitro assembly of microtubules from centriolar complexes of colcemid-blocked, mitotic HeLa cells could be observed directly by means of dark-field microscopy. Because the centriolar complex acts as an MTOC only within a defined period of the cell cycle, it was of interest to determine whether the complex could serve as a site for the in vitro assembly of microtubules when it was obtained

from cells at a period of the cell cycle other than mitosis. Accordingly, cells were synchronized at the initiation of S phase by means of a double thymidine block and were lysed into solutions containing tubulin at appropriate intervals after release of the block. Assembly of microtubules from centriolar complexes resulting in the formation of asterlike structures was assessed by means of dark-field observation of the 1,400 g supernatant fraction of cell lysates incubated at 37°C. Nuclei from the lysed cells were shown by phase contrast and electron microscopy to be free of cytoplasmic tags, and no centriolar complexes could be observed adhering to these nuclei. The nuclei were sedimented, resuspended in tubulin, and also observed with dark-field illumination to determine whether any material capable of initiating microtubule assembly had adhered to the nuclear membrane during the lysis procedure.

Cells were observed to enter mitosis ~6 h after release of the thymidine block, and the maximal
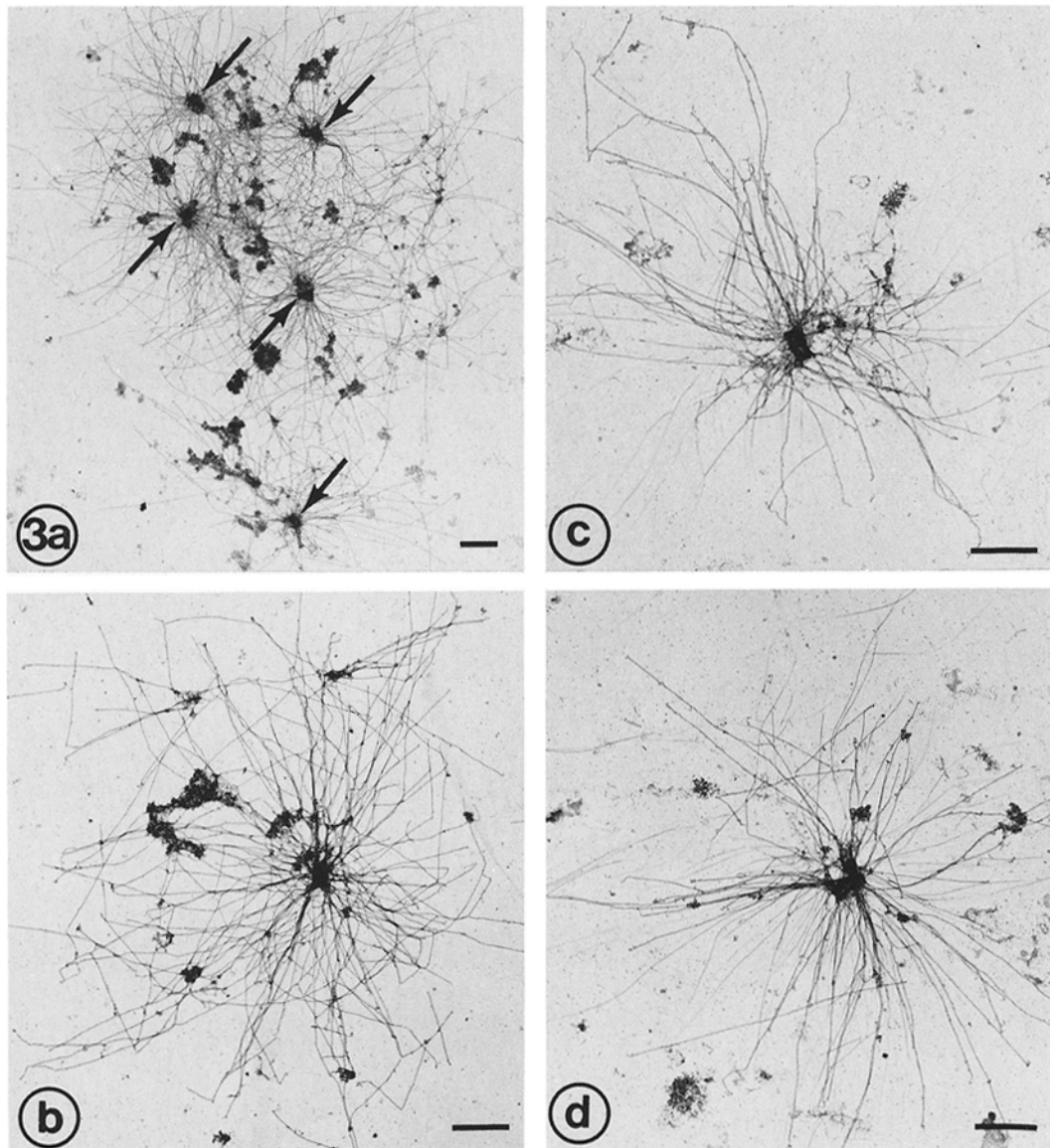
FIGURE 3 Microtubules assembled onto the centriolar complexes isolated from colcemid-blocked, mitotic cells lysed into solutions containing tubulin, incubated at 30°C, centrifuged into a discontinuous sucrose gradient, and then fixed in formaldehyde and stained with alcoholic PTA. Bars, 1 μm.

number of cells in all phases of mitosis was observed ~2 h later before the mitotic index then decreased as the cells re-entered interphase (Fig. 7 a). Significantly, assembly of microtubules onto centriolar complexes was seen only in lysates derived from populations containing mitotic cells. No microtubule assembly was observed onto the isolated nuclei obtained from cells at any other point in the cell cycle, indicating that no microtu-

bule initiation sites accessible to exogenous tubulin were adhering to these nuclei.

Additional experiments were conducted in which colcemid was added to the culture medium after release of the thymidine block. As indicated above, the cells were observed to enter mitosis after ~6 h, but they remained blocked in mitosis; assembly of microtubules onto centriolar complexes was observable only in cell lysates derived
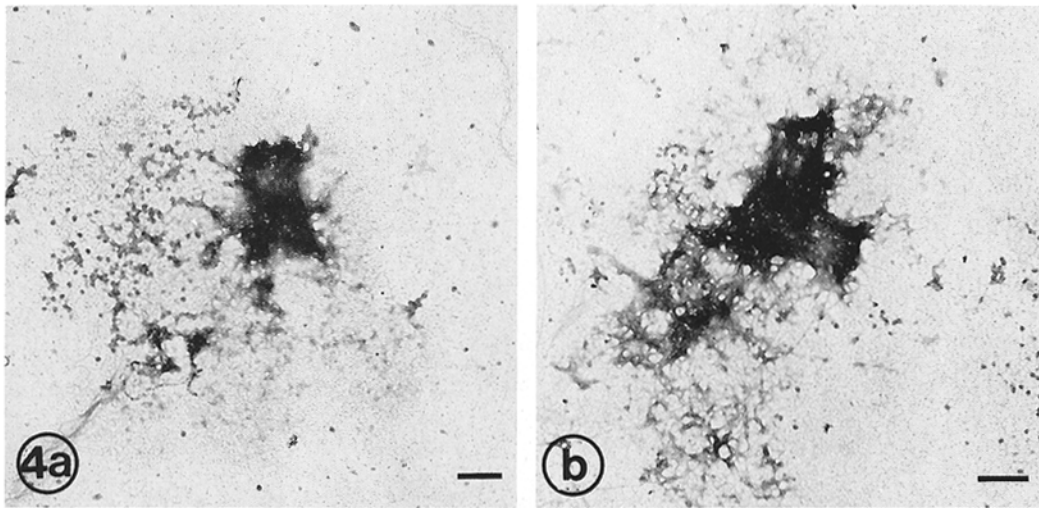
FIGURE 4 Centrioles isolated from colcemid-blocked, mitotic cells lysed into solutions containing tubulin, centrifuged into a discontinuous sucrose gradient at 4°C, and then fixed in formaldehyde and stained with alcoholic PTA. Pericentriolar material can be seen surrounding the centrioles. Bars, 0.2 μm.
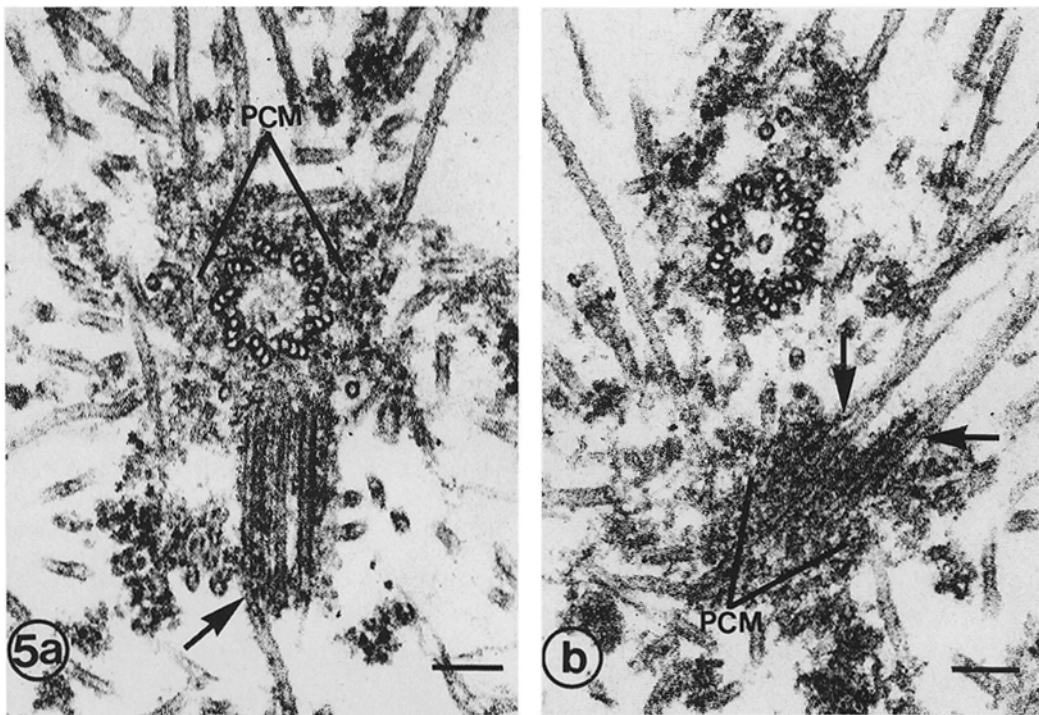

FIGURE 5 Sections of centriole duplexes isolated from colcemid-blocked, mitotic cells lysed into solutions containing tubulin, incubated at 30°C, and centrifuged into a discontinuous sucrose gradient. Microtubules have assembled predominantly onto the pericentriolar material (PCM) and also onto the distal ends of the centrioles (arrows). Bars, 0.1 μm.
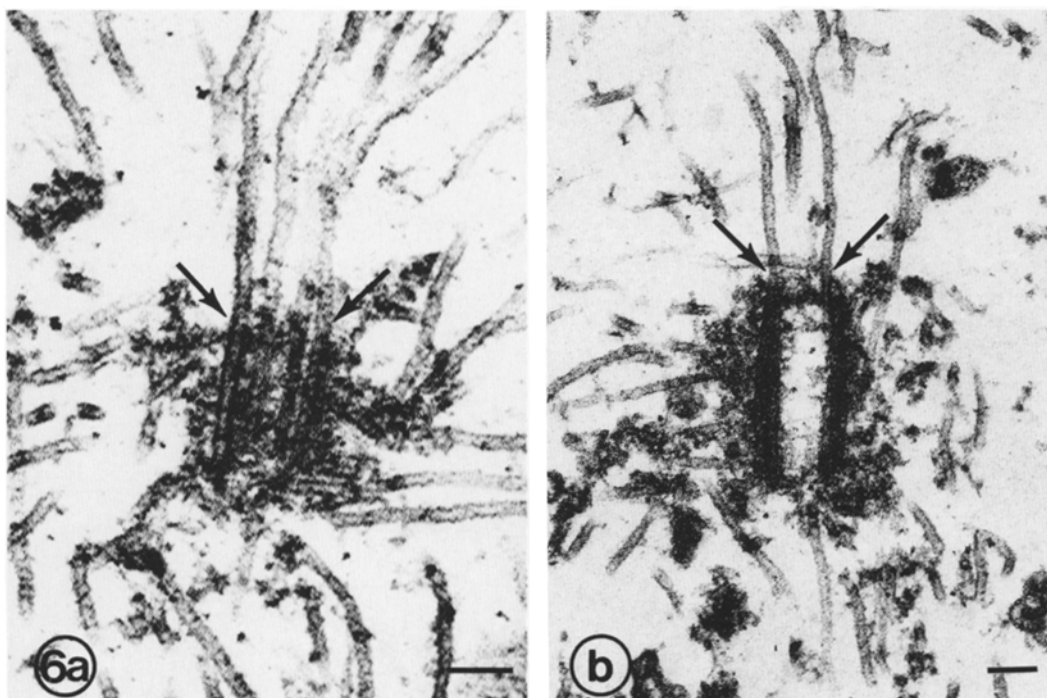
FIGURE 6 Sections of centrioles isolated from colcemid-blocked, mitotic cells. Microtubules have assembled predominantly from the pericentriolar material and onto the centrioles (arrows). Typically, microtubule assembly favored one end of the centriole. Bars, 0.1 μm.

from mitotic cell populations (Fig. 7b).

To determine whether an inhibitor capable of suppressing microtubule assembly from a competent mitotic MTOC was present in interphase cells, mixing experiments were performed using the lysates of mitotic and S phase cells. As illustrated in Table I, no assembly of microtubules onto centriolar complexes was observed in solutions of tubulin containing the 1,400 g supernatant fraction of lysed S phase cells. In contrast, microtubule assembly resulting in the formation of asterlike structures was seen in a similar preparation of lysed mitotic cells. This result is in agreement with the data presented in Fig. 6a and b. When 0.5 ml of tubulin containing the 1,400 g supernatant fraction of lysed mitotic cells was diluted with 0.5 ml of additional tubulin, approximately one-half the number of asters per microliter was observed as before dilution. The number of asters per microliter was also reduced by one-half when the mitotic cell lysate was diluted with an equal volume of the lysate of S phase cells in tubulin. These data indicate that no inhibitor was present in interphase cells which could suppress

microtubule assembly from a competent centriolar complex.

Assembly Studies using Different Preparations of Tubulin

All of the experiments previously described were performed using either a freshly prepared or a lyophilized 100,000 g supernatant fraction of brains of 1- to 3-d-old chicks. Tubulin constituted ~15% of the total protein complement in this preparation. Tubulin purified through two cycles of assembly/disassembly and purified tubulin dimers were also used in these assembly studies, and either type of tubulin was capable of assembly onto the mitotic centriolar complexes as shown in Fig. 8.

The effects of temperature shifts on the assembly of tubulin onto centriolar complexes were studied. Regardless of the source of tubulin, microtubules which assembled onto these complexes were observed to depolymerize when the preparation was incubated at 4°C for 2-5 min. Microtubule assembly reoccurred onto the complexes
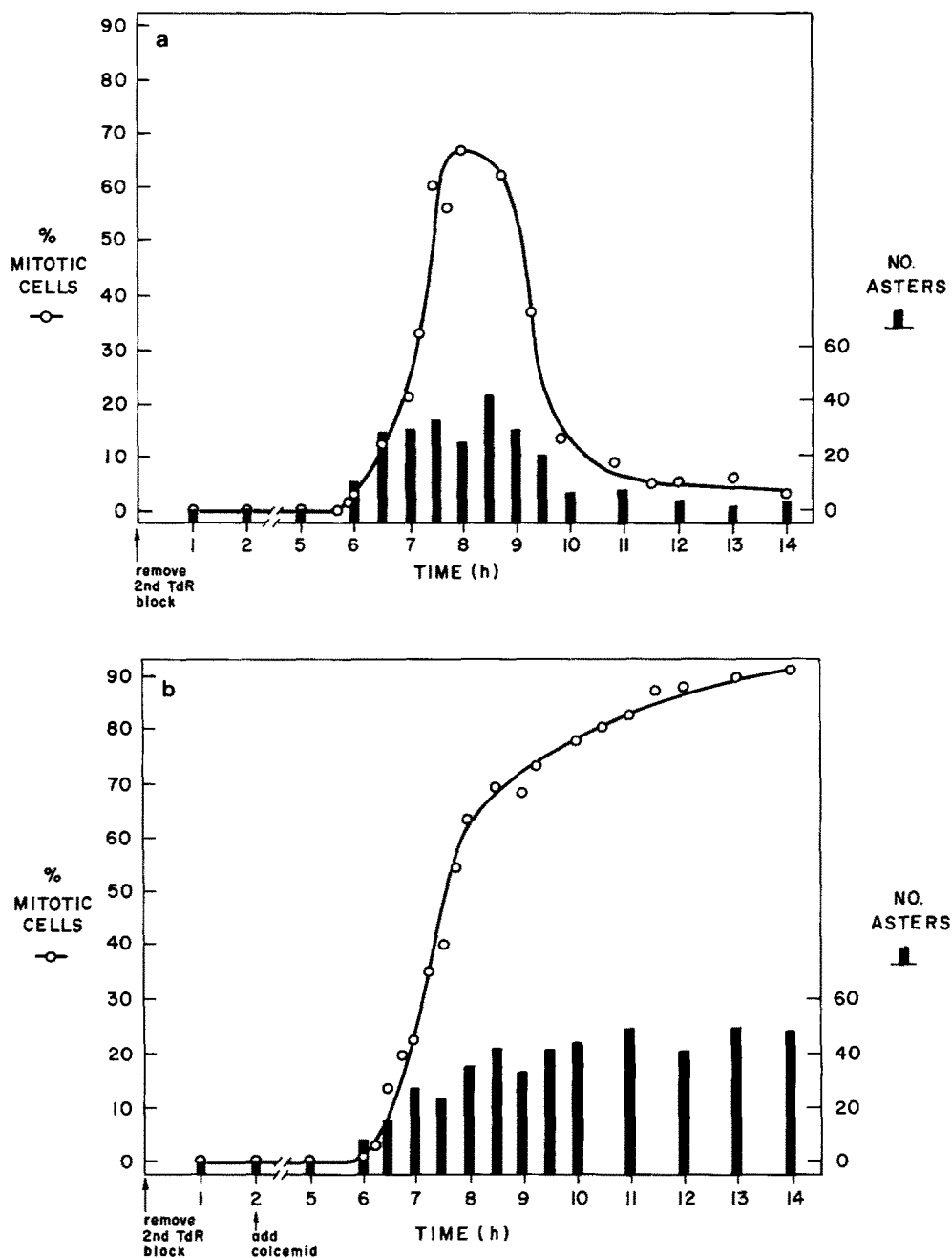
FIGURE 7 (a) The mitotic index of HeLa cells synchronized by a double thymidine block (left ordinate). (b) The mitotic index of HeLa cells synchronized by a double thymidine block followed by incubation in 0.015 μg/ml colcemid (left ordinate). Cells were lysed into solutions containing tubulin and centrifuged at 1,400 g. Microtubule assembly onto centriolar complexes was determined by counting the number of asterlike structures observed by dark-field microscopy in 1 μl of the supernate incubated at 37°C on a microscope slide (right ordinate).

TABLE I

*Microtubule Assembly in Mixed Cell Lysates*

Cells lysed into tubulin	No. of asters
S phase	0
M phase	39.1 ± 7.1
S phase + equal volume additional tubulin	0
M phase + equal volume additional tubulin	21.4 ± 5.0
M phase + equal volume S phase	22.7 ± 5.7

HeLa cells synchronized in either S phase or M phase were lysed into solutions containing tubulin and centrifuged at 1,400 g. Microtubule assembly onto centriolar complexes was determined by counting the number of asterlike structures observed by dark-field microscopy in 1 μl of the supernate incubated at 37°C on a microscope slide. The supernatant fractions of lysed S phase or M phase cells in tubulin were also diluted with an equal volume of additional tubulin, or equal volumes of the supernatant fractions of S phase and M phase cells lysed into tubulin were mixed together before incubation. The number of asterlike structures in 1 μl of these suspensions was determined.

upon reincubation at 37°C, and up to three cycles of assembly/disassembly could be observed. Apparently, no change in the ability of the centriolar complexes to initiate microtubule assembly occurred, and microtubule initiation sites were conserved despite repeated cycles of polymerization.

Lyophilization of aliquots of the various tubulin preparations provided a convenient means to store tubulin samples which could be reconstituted easily and used in the assembly studies when required. However, lyophilization could affect the nature of assembled microtubules. For example, the microtubules assembled from lyophilized 100,000 g supernate onto centriolar complexes were cold stable and were still evident after incubation at 4°C for up to 60 min although addition of 5 mM calcium resulted in their depolymerization. In contrast, microtubules assembled from lyophilized tubulin dimers were cold labile, and up to three cycles of assembly/disassembly from centriolar complexes could be observed in temperature shift experiments.

FIGURE 8 Dark-field micrographs of microtubules assembled onto the centriolar complexes when colcemid-blocked, mitotic cells were lysed into (a) 100,000 g brain supernate, (b) 2 × cycled tubulin, and (c) purified tubulin dimers. Gels of each tubulin preparation are illustrated. Bars, 5 μm. map, Microtubule-associated protein; tub, tubulin.
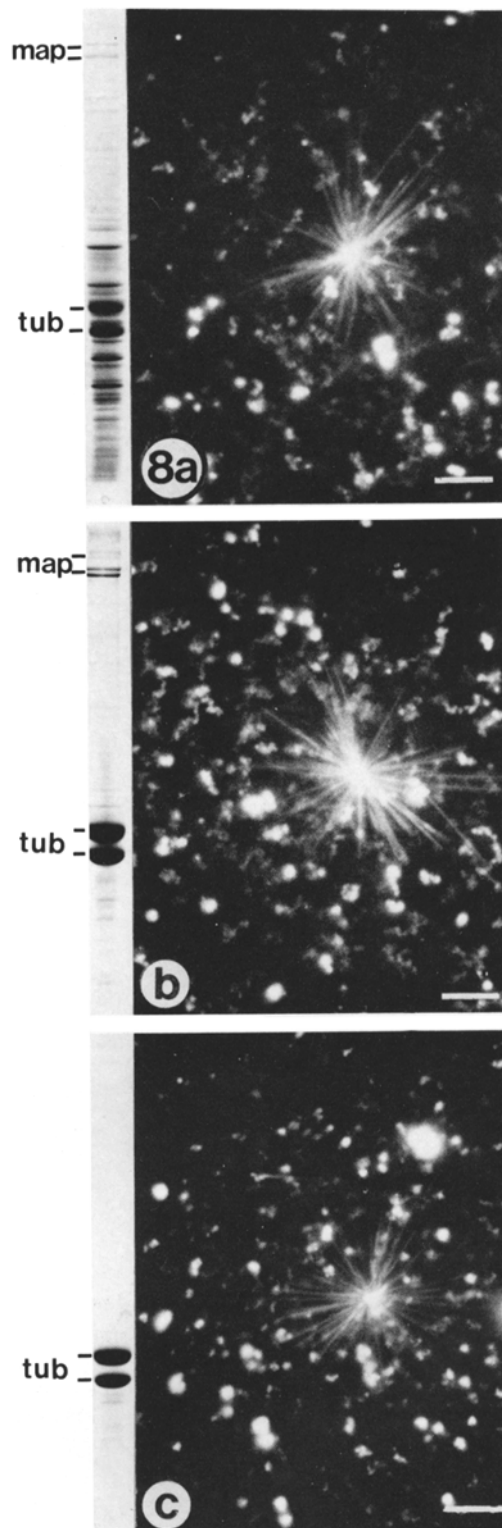
DISCUSSION

The results of this study indicate that centriolar complexes which initiate the in vitro assembly of microtubules can be observed by dark-field microscopy and obtained by centrifugation into a discontinuous sucrose gradient. The majority of the microtubules that assemble from these centriolar complexes do so from the pericentriolar material. Microtubules can assemble onto the centrioles themselves, but only as extensions of the microtubules present within the triplet blades of the organelle. The ability of the pericentriolar material to serve as a microtubule initiation site varies as a function of the cell cycle, and it becomes competent as an MTOC at the time of mitosis. That interphase centriolar complexes do not serve as initiating sites is not due to the presence of an inhibitor. Finally, the ability of centriolar complexes to initiate microtubule assembly is not dependent upon the presence of the microtubule-associated proteins (MAPs) in the tubulin solution which stimulate microtubule assembly.

Robbins et al. (22) have shown previously in HeLa cells that the amount of pericentriolar material is related to stages of the cell cycle. The pericentriolar cloud was not discernible during G1 but later developed in interphase as a weak, electron-dense zone surrounding the centrioles. The region underwent striking transitions during mitosis. The fact that the pericentriolar material became most prominent during prophase and prometaphase immediately before and during the period that spindle microtubules first appeared led Robbins and co-workers to propose a causal relationship. More recent work has shown that the number of microtubules able to polymerize in vitro from the pericentriolar material in rat kangaroo cells increased during the transition from prophase to late prometaphase (27).

The present findings support Robbins' original hypothesis. The studies performed with synchronized cells established that centriolar complexes capable of initiating microtubule assembly could be obtained only from populations containing mitotic cells. This result is similar to that of Weisenberg and Rosenfeld (37) who demonstrated that the in vitro assembly of microtubules onto the centriolar complexes of *Spisula* occurred only when the complexes were obtained from artificially activated eggs about to divide. It can be hypothesized that the pericentriolar material in

HeLa cells represents a specialized MTOC which becomes capable of initiating microtubule assembly only during or just before the initiation of mitosis.

It has been proposed that before prophase a compound must be synthesized which would lead to the initiation of mitosis (31). Although protein synthesis is completed at least 4 h before mitosis in L cells (18), other studies have shown that a specific protein must be synthesized 30-60 min before mitosis in human amnion cells (6, 25) or at a time virtually coincident with the initiation of mitosis in Chinese hamster ovary cells (34). It is conceivable that this requirement for protein synthesis before mitosis may be related to either the proliferation or activation of the microtubule-initiating sites within the pericentriolar material and may also be involved with the maturation of the kinetochore.

Studies with tubulin antibody immunofluorescence in fibroblasts such as 3T3 and rat kangaroo cells have suggested that the region surrounding the centrioles may play a role in mediating the assembly of cortical microtubules in interphase cells (4, 16). These fibroblasts are flattened and well spread out when grown in monolayer; they possess large numbers of cytoplasmic microtubules which are responsible for the maintenance of the fibroblastic shape and which react with tubulin antibody. The fact that these microtubules reappear in interphase cells reincubated in fresh medium after treatment with colchicine or colcemid and arise from the centriolar region suggests that the pericentriolar material has the capacity to nucleate the assembly of the cortical microtubules in these cells. HeLa cells, on the other hand, lack the flattened shape of fibroblasts, possess relatively few cytoplasmic microtubules, and exhibit greatly reduced immunofluorescent staining properties compared to fibroblasts. These observations may be related to the fact that during interphase in HeLa the pericentriolar material is diminished in size and does not support the in vitro assembly of microtubules. It would be of interest to test the ability of the centriolar complexes of fibroblasts in all stages of the cell cycle to initiate the in vitro assembly of microtubules using the technique described here.

Previous work has also demonstrated that the centriolar complexes of mammalian cells could serve as sites for the in vitro assembly of microtubules. McGill and Brinkley (12) and Snyder and McIntosh (27) examined in the electron micro-

scope the centriolar complexes of individual HeLa and rat kangaroo cells lysed into and then incubated in the presence of tubulin. In other experiments, Gould and Borisy (7) obtained centriolar complexes by lysing Chinese hamster ovary cells into tubulin solutions and centrifuging these whole cell lysates onto grids for examination in the electron microscope after incubation. The present study represents the first time that the process of microtubules assembling onto centriolar complexes has been visualized directly by means of dark-field microscopy and describes a procedure by which these complexes and assembled microtubules can be separated from crude cell lysates by centrifugation into a sucrose gradient.

The studies performed with rat kangaroo and Chinese hamster ovary cells showed that the majority of the microtubules assembling from a centriolar complex arose from the pericentriolar material (7, 27). However, both of these cell lines contain viruslike particles specifically associated with the pericentriolar material (7, 28, 38), and the possibility exists that these particles played some role in the assembly of microtubules. No such viruslike particles have been observed in HeLa cells.

It should be noted that McGill and Brinkley (12) observed by thin sectioning and electron microscopy the in vitro assembly of microtubules from the centriolar complexes present within individual interphase HeLa cells. The possibility cannot be precluded that the pericentriolar material in interphase HeLa cells may possess some residual capacity to act as an MTOC compared to mitotic cells. This would not be detectable if the integrity of the pericentriolar material were reduced as a result of the isolation procedures used in this study which involved the rapid syringing of cells into solutions containing detergent and tubulin.

The observation that centriolar complexes which initiated microtubule assembly could be recovered by centrifugation into a discontinuous sucrose gradient may be useful in developing a technique for the isolation of MTOCs. With the use of colcemid-blocked cells and the lysis conditions described, virtually no cellular material was obtained at the 50–60% sucrose interface where microtubules would normally sediment. The use of tubulin unable to self-assemble insured that in vitro microtubule polymerization would occur in this lysate after incubation at 30°C only if a suitable MTOC were present. These microtubules

could then be recovered at the 50–60% sucrose interface as well as the MTOCs which initiated their assembly, thus permitting these MTOCs to be separated from crude cell lysates. This observation may facilitate their purification in future studies.

The assembly characteristics of different classes of tubulin provide some insight into the mechanisms by which the pericentriolar material controls microtubule assembly. Purified tubulin dimers incapable of self-polymerization did assemble onto centriolar complexes as did 2 × cycled tubulin containing the MAPs which are capable of stimulating microtubule assembly (26) or the 100,000 g supernate containing not only the MAPs but many other proteins. Accordingly, the possibility may be excluded that it was first necessary for either the MAPs or some other protein within the 2 × cycled or 100,000 g tubulin preparations to associate with the pericentriolar material in order for it to initiate microtubule assembly. A more reasonable explanation is that some factor specifically stimulating microtubule assembly is present within the pericentriolar material during mitosis. The nature of this factor and the means by which it mediates microtubule assembly from the mitotic poles will be known only when the pericentriolar can be isolated and its biochemical nature determined.

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