

# Cytoplasmic Microtubule Assembly-Disassembly from Endogenous Tubulin in a Brij-lysed Cell Model

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**ABSTRACT** We studied the characteristics of cytoplasmic microtubule reassembly from endogenous tubulin pools *in situ* using a Brij 58-lysed 3T3 cell system. Cells that were pretreated *in vivo* with colcemid retain endogenous tubulin in the depolymerized state after lysis. When lysed cells were removed from colcemid block and incubated in GTP-PIPES reassembly buffer at pH 6.9, microtubules repolymerized randomly throughout the cytoplasm, appeared to be free-ended and were generally not associated with the centrosomes. However, tubulin could be induced to polymerize in an organized manner from the centrosomes by increasing the pH to 7.6 in the presence of ATP and cAMP. Microtubules polymerized in ATP had significantly longer lengths than those assembled in GTP or UTP. When cells not treated with colcemid were lysed, the integrity of the cytoplasmic microtubule complex (CMTC) was maintained during subsequent incubation in reassembly buffer. However, in contrast to unlysed, living cells, microtubules of lysed cells were stable to colchicine. A significant fraction of the CMTC was stable to cold-induced disassembly whereas microtubules reassembled after lysis were extremely cold-sensitive. When cells not treated with colcemid were lysed and incubated in millimolar  $\text{Ca}^{++}$ , microtubules depolymerized from their distal ends and a much reduced CMTC was observed.  $\text{Ca}^{++}$  reversal with EGTA rapidly resulted in a reformation of the CMTC apparently by elongation of  $\text{Ca}^{++}$  resistant microtubules.

Most eucaryotic cells contain a network of microtubules that extends throughout the cytoplasm, commonly referred to as the cytoplasmic microtubule complex (CMTC) (for review, see reference 9). The microtubules are not arranged randomly, but, rather, assembly occurs at specific times in the cell cycle by regulated polymerization of tubulin in association with the centrosome or microtubule-organizing center (MTOC) and proceeds in an organized fashion to the cell periphery. This pattern can be observed in daughter cells following mitosis or in cells recovering from colcemid block (9). The factors responsible for the precise spatial distribution of microtubules and the intracellular location of these factors remain to be elucidated. With respect to the polymerization process, there are essentially two major intracellular levels at which the spatial organization and extent of microtubule assembly can be determined; the organizing center and the microtubule subunit protein tubulin and its associated proteins.

Ultrastructural studies of MTOCs have shown that they consist of one or two pairs of centrioles with microtubules radiating from an amorphous pericentriolar material. The latter may contain initiating factors required for assembly (4, 21, 50). Although the precise molecular composition of MTOCs re-

sponsible for directing site specific assembly remains to be determined, studies have indicated that intrinsic kinetochore and centrosomal nucleic acids (23, 40, 50, 59) as well as tubulin (38, 39) are involved in the initiating capacity of these organizing centers. Furthermore, evidence has been presented suggesting that cell cycle related changes in centrosomes may influence their capacity to direct microtubule assembly (30, 59).

Extensive tubulin polymerization studies *in vitro* have revealed several physiological factors that may be important in the control of intracellular microtubule assembly. Experiments using primarily brain tubulin purified by cycles of assembly-disassembly have shown a pH optimum for polymerization between 6.5–7.0; the capacity for nucleating assembly and the extent of polymer formation is progressively decreased above pH 7 (19, 42). Assembly is also affected by several nontubulin proteins. Microtubule nucleation and the rate and extent of assembly are greatly facilitated by microtubule-associated proteins (MAPs) which co-purify with tubulin and bind along the polymer wall (3, 34, 48, 55). In addition, MAPs stabilize microtubules under various depolymerizing conditions (16, 35, 49, 61). *In vitro* experiments have also demonstrated a nucleo-

tide triphosphate requirement for nucleation and that GTP at the exchangeable site (E site) on the tubulin dimer promotes polymerization, whereas GDP is inhibitory (5, 13, 56, 58, 61). Exogenous GTP can either bind directly to the E site or tubulin-bound GDP can be transphosphorylated to GTP by ATP or UTP (37, 58). In the presence of ATP, tubulin and/or MAPs can also be phosphorylated and evidence exists indicating that this influences polymerization both *in vitro* (26, 32, 53) and in a lysed cell system (53). Although MAPs have been localized with microtubules in cultured cells (11, 14, 25, 46), the physiological significance and function of these and other factors mentioned above remain speculative.

Originally, permeabilized mammalian mitotic cell systems were used to demonstrate that centrosomes and kinetochores could nucleate the assembly of purified exogenous tubulin (for review, see reference 51). Recently, a Triton X-100-lysed cell system developed in our laboratory has been used to study reassembly characteristics of exogenous brain tubulin in association with MTOCs (8, 53). When Triton X-100-extracted cells are incubated in tubulin dimer which lacks the ability to self-nucleate, microtubule assembly occurs predominantly from the organizing centers and is similar to that observed *in vivo*.

In the present study, we used another detergent system, Brij 58, in which the ultrastructure and permeability properties have been well characterized by Schliwa and co-workers (44, 45) and Cande et al. (12). This lysis system has been found to provide greater preservation of cytoplasmic and cytoskeletal components. Unlike the Triton X-100-extracted cells, Brij-lysed cells retain endogenous tubulin and this has allowed us to examine the characteristics of microtubule assembly and disassembly *in situ*, from endogenous, unpurified microtubule protein. Following Brij extraction, we observed profound differences in microtubule reassembly properties which are a function of the nucleotide triphosphate and pH. Using the Brij model system, we now report conditions favoring either random microtubule assembly which is not associated with the centrosome or a more organized, site-associated polymerization similar to that observed *in vivo*. Random assembly is favored at pH 6.9 while site-associated assembly requires a pH above neutrality and incubation in ATP and cAMP. These contrasting findings may have important implications in understanding the principles of organized microtubule assembly in cells.

## MATERIALS AND METHODS

**Cell Culture and Lysis:** Swiss mouse fibroblast (3T3) cells were grown on glass coverslips for 24–48 h to 90–95% confluency in plastic petri dishes containing Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 10% CO<sub>2</sub> and were used in all experiments.

Just prior to lysis, cells on coverslips were removed from petri dishes and washed for 15 s at room temperature in 0.08 M PIPES buffer, pH 6.9, containing 10 mM EGTA, 1 mM MgCl<sub>2</sub>, and 0.1 mM GTP (extraction buffer) essentially as described by Schliwa et al. (45). For experiments involving Ca<sup>++</sup> effects, we used 1 mM EGTA. For microtubule reassembly studies, cells were first treated with 0.3 μM colcemid in culture for 2–3 h whereas the extraction buffer contained 0.16 μM colcemid. When we studied reassembly with nucleotides other than GTP, GDP substituted for GTP. Cells were lysed in extraction buffer containing 0.15% Brij-58 for 2.5–3 min, followed by a 30-s wash in extraction buffer to remove the detergent. To remove the colcemid, lysed cells were either incubated for 5 min at 25°C in reassembly buffer (RB) containing 0.08 M PIPES, pH 6.9–7.6, 1 mM EGTA-MgCl<sub>2</sub>, and 0.1 mM GTP (or GDP) or incubated for 10 min at 25°C in PIPES buffer containing 2 × 10<sup>-5</sup> M 8-Bromo-cAMP, 1.5 mM ATP, and 2 mM MgCl<sub>2</sub>–1 mM EGTA. Microtubule reassembly was then studied by inverting the cells on coverslips over a drop of buffer in petri dishes at various reassembly conditions.

**Immunofluorescent Staining and Microscopy:** After cells

were lysed and exposed to various experimental conditions, they were fixed for 30 min at 25°C in 3% formaldehyde made up in phosphate-buffered saline (PBS) and processed for indirect immunofluorescence microscopy using sheep antibody to tubulin and fluorescein-tagged goat-anti-sheep IgG as described previously in detail (9). We examined cells with a Leitz orthoplan microscope equipped with epillumination and photographs were recorded on Tri-X Pan film (Kodak).

**Electron Microscopy:** For scanning electron microscopy, cells grown on glass coverslips were lysed, washed in 0.1 M PIPES buffer, and subsequently fixed in 3% glutaraldehyde-PIPES pH 7.35 at 25°C for 2 h with agitation. Cells were then rinsed several times in PIPES buffer to remove glutaraldehyde and fixed in 1% OsO<sub>4</sub>-PIPES for 45 min with agitation followed by a 7-min rinse in water and stepwise dehydration using 30%, 50%, 70% and 90% acetone for 5 min each and 100% acetone three times for 20 min each. Following critical point drying, the cells were sputter-coated with Au/Pd and examined in a JOEL JEM-100 CX electron microscope on scanning mode.

For transmission electron microscopy, lysed cell preparations were fixed in 3% glutaraldehyde-PIPES buffer for 15 min, washed with PIPES buffer and postfixed with 1% OsO<sub>4</sub>-PIPES buffer. The cell monolayers were then dehydrated in ethanol and processed for flat-embedding directly in the culture dishes by the procedure of Brinkley et al. (10). Cells were preselected from the embedded monolayers by phase-contrast microscopy, serially sectioned, collected on 200-mesh copper grids, and stained with a saturated solution of uranyl acetate in 50% ethanol and lead citrate. The grids were examined with a JOEL JEM-100 CX electron microscope.

**Gel Electrophoresis:** Cells near confluence in 60-mm plastic petri dishes were lysed as described above, collected in 50 μl of PBS with a rubber policeman and sheared by several passages through a 50-μl Hamilton syringe. The extract was then made 50 μg/ml DNase I and incubated for 15 min to digest DNA. Cell extracts were prepared for electrophoresis and applied to 0.1% SDS polyacrylamide slab gels consisting of a 5% pH-6.8 stacking gel and a 10% polyacrylamide pH-8.8 running gel as described by Ames (1). After staining and destaining, the gels were scanned at 525 nm in a Quick Scan Jr. (Helena Laboratories, Beaumont, TX) and the relative area under each peak was calculated by the gel scan integrator.

## RESULTS

### *Ultrastructure of Lysed Cell Membranes and Retention of Endogenous Tubulin*

To study the characteristics of microtubule assembly *in situ* from endogenous tubulin pools, the cells must be readily permeable to various molecules known to affect polymerization yet retain tubulin in an active state. After cells are exposed for 2.5 min to 0.08 M PIPES RB containing 0.15% Brij-58 and 1 mM Mg<sup>++</sup>–10 mM EGTA, holes are present throughout the plasma membrane with a mean diameter of 95 nm as determined by scanning electron microscopy (Fig. 1). It should be noted that this diameter is approximately four times that of a microtubule. Further evidence that the cells are indeed permeable to large molecules is indicated by the ready access of immunofluorescent antibody probes to microtubules.

The relative extent to which tubulin in the depolymerized state is maintained within the cytoplasm after lysis was examined by SDS gel electrophoresis. Cells were pretreated *in vivo* with 0.16 μM colcemid for 2.5 h to depolymerize most cytoplasmic microtubules and either were not lysed (Fig. 2A) or were lysed with 0.05% Triton X-100 for 90 s and maintained in a colcemid block for an additional 15 min (Fig. 2B) or with 0.15% Brij-58 for 2.5 min and maintained in a colcemid block for an additional 40 min (Fig. 2C), followed by gel electrophoresis. Using the protein band that migrates with the molecular weight characteristic of actin as an internal reference, the ratio of the apparent tubulin doublet band to actin is essentially the same in unlysed and Brij-lysed samples, whereas in the Triton X-100-lysed cells the ratio is reduced by 30%. These data indicate that the unpolymerized cytoplasmic tubulin remains associated with the cytoplasm and is not readily permeable to the Brij-lysed cell membranes.

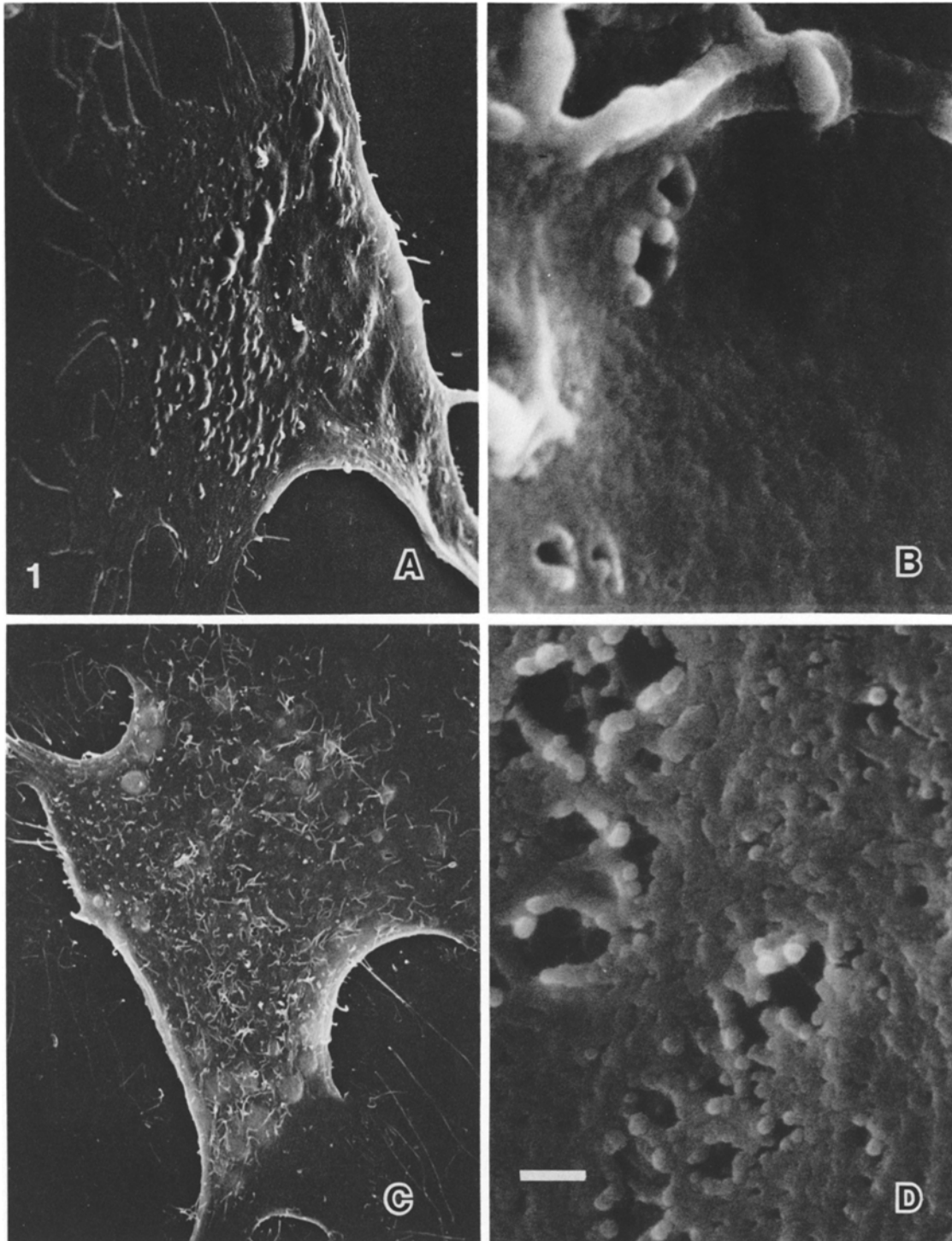


FIGURE 1 Scanning electron micrographs of 3T3 cells before and after lysis in 0.15% Brij 58. *A* and *B* show unlysed cells washed in PIPES-RB. *C* and *D* show cells lysed for 2.5 min. Bar, 0.2  $\mu$ m. (*A* and *B*)  $\times$  2,500. (*C* and *D*)  $\times$  50,000.

### *Characteristics and Nucleotide Requirements of Endogenous Tubulin Reassembly*

Since it appeared that the endogenous tubulin could be maintained within the lysed cells, we then wanted to examine the characteristics of microtubule reassembly following colcemid reversal. After colcemid treatment for 2–3 h *in vivo*, followed by lysis and incubation in colcemid-RB for 10–40 min, essentially no microtubules are present (Fig. 3*A*). When

these cells, which were exposed to colcemid for 10 min (Fig. 3*B*) or 40 min (Fig. 3*C*) after lysis, are reversed from colcemid block and incubated in 1 mM GTP-RB for 30 min, microtubules reassemble randomly throughout the cytoplasm. Although some assembly is associated with the centrosome (Fig. 4*A*), and possibly with the plasma membrane (2, 7) (Fig. 4*B*) as shown by transmission electron microscopy, for the most part, microtubules appear to be free-ended. Under these conditions a nucleotide triphosphate is required for initiation since

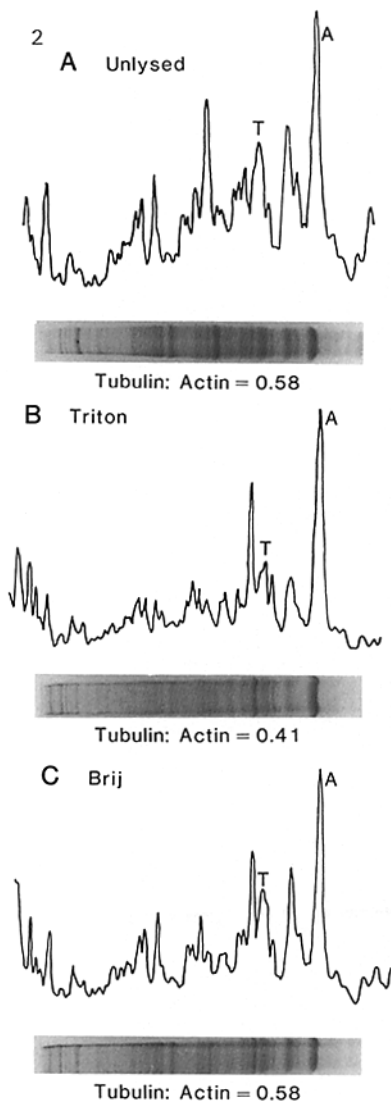


FIGURE 2 SDS PAGE of proteins from unlysed and lysed 3T3 cells. Unlysed cells (A), cells lysed for 1.5 min in 0.05% Triton X-100 (B), or cells lysed for 2.5 min in 0.15% Brij 58 (C) were prepared for PAGE as described in Materials and Methods.

submicromolar concentrations of GTP or millimolar GDP did not induce microtubule assembly (Fig. 3 D).

Although GDP inhibits microtubule assembly (5, 13, 56, 58, 61), *in vitro* studies have shown that tubulin-bound GDP can be transphosphorylated to GTP by ATP or UTP and a nucleoside diphosphate kinase (37, 58). Microtubule reassembly was induced *in situ* in the presence of millimolar concentrations of ATP (Fig. 5 A) or UTP (Fig. 5 B) plus GDP and, like GTP-induced assembly at pH 6.9, is primarily not associated with the centrosomes. Assembly in UTP is similar to that in GTP whereas microtubules are significantly longer in ATP.

The lengths of both free microtubules and microtubules associated with the centrosome were measured with a micrometer ocular as described elsewhere (8, 53). At least 10 microtubules were measured per cell, and in each case 10 cells were selected. The mean length of free-ended microtubules assembled in GTP or UTP plus GDP was  $\sim 5 \mu\text{m}$ , whereas microtubules assembled in ATP plus GDP have a mean length of  $17 \mu\text{m}$ . Microtubules associated with the centrosome in GTP and UTP were similar in length to free-ended tubules, whereas those assembled in ATP were approximately two times longer.

There is evidence that the increased lengths in ATP are due to the phosphorylation of a microtubule protein(s). Microtubules assembled in the nonhydrolyzable ATP analog, AMPPNP ( $[\beta, \gamma\text{-imidoadenosine-5'-triphosphate}]$ ) which is re-

ported to contain low levels [0.1%] of contaminating ATP), are much shorter and similar in average length to those assembled in GTP or UTP. When the chromatographically purified analog AMPPCP was used (1 mM), however, polymerization did not occur (Fig. 5 C) except when 1–5  $\mu\text{M}$  ATP was added. It should be noted that, in the presence of ATP or UTP, GDP stimulates rather than inhibits assembly; without added GDP, for example, in the presence of these triphosphates alone, no assembly is observed.

Microtubule assembly *in vivo* appears to occur not at random but in association with MTOCs. Therefore, an attempt was made at establishing conditions that would inhibit spontaneous, random nucleation and thus possibly shift microtubule assembly onto the centrosome as it occurs in the living cell. Because previous *in vitro* studies have indicated that microtubule nucleation can be retarded at pH values above neutrality (19, 42), we examined the effect of increasing the pH  $> 6.9$ . When lysed cells containing colcemid-depolymerized tubulin were incubated at  $37^\circ\text{C}$  between pH 7.0 and 7.6 in the presence of GTP, microtubule assembly continued to be predominately free of the centrosome. However, if the lysed cells were preincubated at  $25^\circ\text{C}$  for 10 min in PIPES buffer at pH 7.6 containing 0.02 mM 8-Bromo-cAMP/1.5 mM ATP and subsequently incubated at  $37^\circ\text{C}$  for 20–30 min in 0.01 mM 8-Bromo-cAMP, 1 mM ATP, 0.2 mM GDP, we observed a more organized pattern of assembly. As shown in Fig. 5 D, microtubules extended from a central focus (centrosome) outward to the cell periphery. It should be pointed out, however, that some microtubules continued to assemble randomly. Thus, in this system, a minimal condition for site-associated assembly requires both high pH and ATP.

#### Behavior of Microtubules to Depolymerizing Conditions

It has been shown that when various types of cells in culture are treated with submicromolar concentrations of colcemid or colchicine for a few hours the mitotic and cytoplasmic microtubules disassemble (for review, see reference 9). However, the sensitivity of microtubules to depolymerizing conditions varies when cells are lysed in different detergents. Following lysis in 0.05% Triton X-100 for 1.5 min, many microtubules are sensitive to dilution since significant disassembly is observed upon incubation of the lysed cells in RB (not shown). Disassembly also occurs if colchicine is present in the RB. In contrast, following Brij-lysis, there is a dramatic change in the sensitivity of microtubules to dilution and colchicine-induced disassembly. When cells are not treated with colcemid *in vivo*, a normal CMTC is observed after Brij-lysis (Fig. 6 A), provided a high concentration of EGTA (10 mM) is present as reported by Schliwa et al. (45). If these cells are subsequently incubated for 30 min in 0.2 mM colchicine-RB with or without 1 mM ATP, essentially no effect is observed on interphase CMTCs (Fig. 6 B) or on mitotic spindle microtubules (Fig. 6 C). We observed a similar stability with microtubules reassembled *in situ* (Fig. 6 D and E). This concentration of colchicine depolymerizes all microtubules *in vivo* within 20 min (not shown).

We also examined the effects of low temperature on microtubules. It has been previously reported (6) that certain microtubules of the mitotic apparatus of fibroblastic PtK cells are resistant to cold disassembly and, recently, that a population of microtubules from crude rat brain extracts are stable at  $0\text{--}4^\circ\text{C}$  (22, 27, 31, 54). The CMTC of 3T3 cells depolymerized by  $\sim 60\text{--}70\%$  when cells were incubated at  $0\text{--}4^\circ\text{C}$  for 30 min;  $30\text{--}40\%$  of the microtubules remained and are presumed to be cold

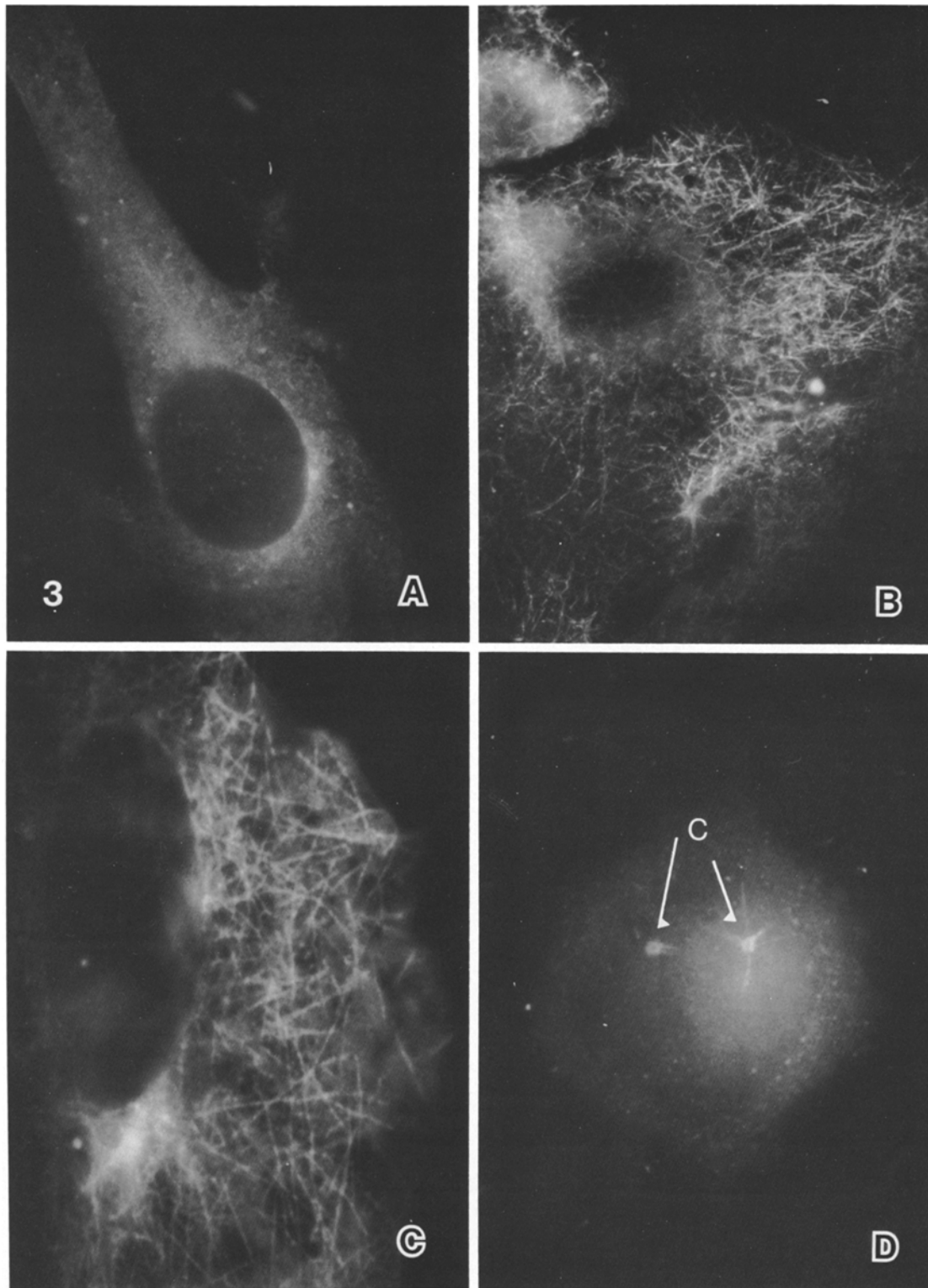


FIGURE 3 Microtubule reassembly from endogenous tubulin in the presence of GTP and inhibition of assembly by GDP. (A) Cells were pretreated with colcemid *in vivo*, lysed in Brij extraction buffer and exposed to colcemid-RB for 10–40 min at 25°C.  $\times 1,300$ . (B) Lysed cells were incubated for 25 min at 37°C in GTP-RB after a 10-min or (C) 40-min colcemid block following lysis.  $\times 1,000$  and  $\times 1,800$ , respectively. (D) Lysed cells were incubated in RB containing 1 mM GDP. C, centrosomes.  $\times 1,800$ .

stable (Fig. 7B). In contrast, microtubules reassembled *in situ* were extremely cold-sensitive and generally only a few microtubule fragments were observed after cold treatment (Fig. 7D). These observations are in contrast to those of colchicine stability and indicate that drug and cold stability can be determined by different factors and mechanisms.

When cells which were not treated with colcemid are lysed in low (1 mM) EGTA-RB and further incubated in 1–3 mM  $\text{Ca}^{++}$ -RB for 20 min, most microtubules appear to depolymerize from their distal ends toward the MTOCs as previously reported by Schliwa et al. (45) and a much reduced microtubule complex is observed around the centrosomes (Fig. 8A). In

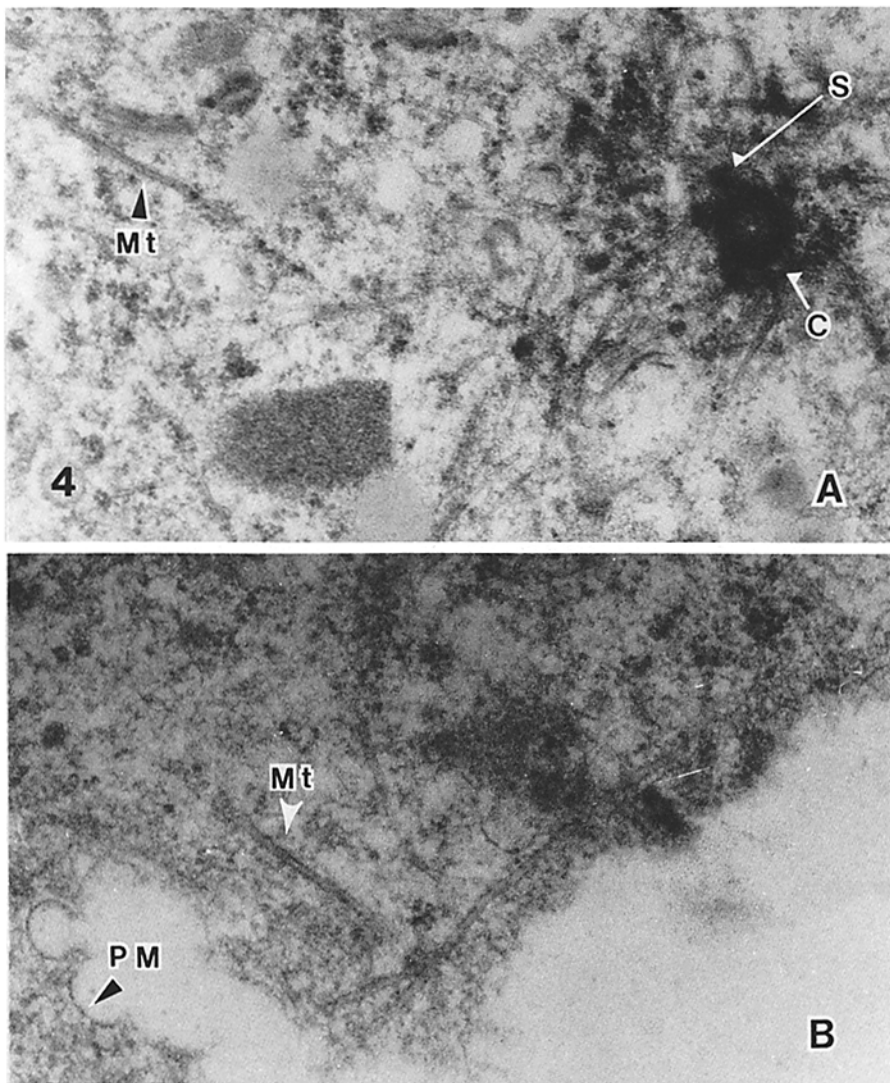


FIGURE 4 Electron micrographs of Brij-lysed cells following endogenous microtubule reassembly. (A) Lysed cells were incubated in GTP-RB as described in Fig. 3 and prepared for transmission EM. Attenuated microtubule assembly was observed associated with the centrosome. Note that the cytoplasm is well preserved upon Brij lysis. C, centriole; MT, microtubule; S, satellite.  $\times 32,000$ . (B) shows microtubules which appear to be associated with the plasma membrane of which little remains. PM, plasma membrane.  $\times 62,400$ .

contrast to the random reassembly observed following colcemid reversal,  $Ca^{++}$  reversal with high EGTA concentrations rapidly results in a reformation of the CMTC apparently by elongation of  $Ca^{++}$ -resistant microtubules (Fig. 8B). This reassembly does not require GTP and also occurs at millimolar concentrations of GDP.

#### DISCUSSION

Detergent-lysed cell systems allow one to subject various intracellular components to defined, controlled buffer conditions such that factors influencing the microtubule assembly process can be examined. Previous studies have shown that purified, exogenous tubulin will assemble from kinetochores and centrosomes in a fashion similar to that observed in vivo (for review, see reference 51). In the present study, we find that upon lysing 3T3 fibroblast cells with the non-ionic detergent Brij-58, the endogenous, depolymerized tubulin is retained within the cytoplasm and can be induced to repolymerize primarily independent of the organizing centers at pH values that are optimal for assembly in vitro. In contrast, incubation of the lysed cells at more alkaline pH and in the presence of ATP results in a more organized reassembly from the centrosome and is similar to that observed following colcemid reversal in vivo.

When cells are briefly incubated in buffer containing Brij-

58, holes are produced throughout the plasma membrane which are large enough for endogenous tubulin dimers to readily permeate. However, cytoplasmic tubulin in the unpolymerized state (in the presence of colcemid or  $Ca^{++}$ ) is well retained for a relatively long time after lysis. This strongly suggests that, at least following lysis, the tubulin exists in an aggregated state and/or may associate with the cytoplasmic matrix or microtubules (60). It is difficult to resolve at this time whether tubulin actually has these properties in vivo prior to lysis, in light of the potential differences observed in the reassembly pattern as well as the drug stability of microtubules in lysed cells vs. those in vivo, as will be discussed. However, it should be mentioned that several investigators have presented evidence for the presence of particulate tubulin or nonmicrotubular aggregates of tubulin in nondividing surf clam eggs (57), transformed 442 rat kidney cells (43), and neuroblastoma cells (36).

Consistent with in vitro studies using purified tubulin, endogenous tubulin polymerization in situ is induced by GTP as well as by ATP or UTP. Although GDP alone does not induce assembly, in the presence of ATP or UTP, GDP is required for polymerization. These observations combined with the inability of the nonhydrolyzable ATP analog AMPPCP plus GDP to promote assembly indicate that, as in vitro (37, 58),

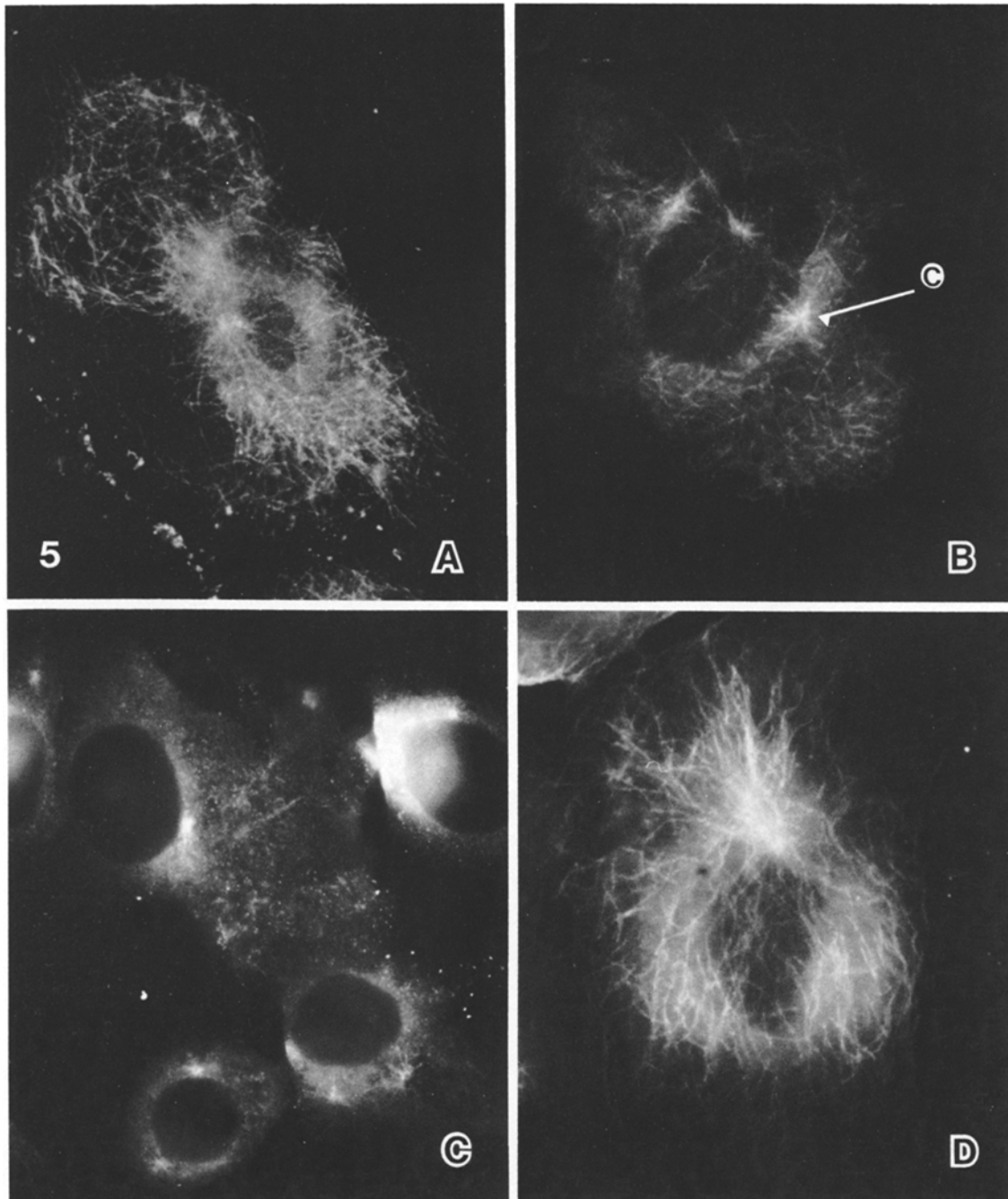


FIGURE 5 Effects of nucleotides and pH on the pattern of microtubule assembly. Lysed cells pretreated with colcemid as described in Fig. 3 were incubated for 25 min at 37°C in 1 mM ATP-0.3 mM GDP-RB, pH 6.9 (A); 1 mM UTP-0.3 mM GDP-RB, pH 6.9 (B); 1 mM AMPPCP-0.3 mM GDP-RB, pH 6.9 (C); or 1 mM ATP-0.3 mM GDP-0.01 mM 8-Bromo-cAMP-RB, pH 7.6 (D). C, centrosome. (A)  $\times 1,000$ . (B)  $\times 1,300$ . (C)  $\times 1,000$ . (D)  $\times 1,300$ .

GDP can be transphosphorylated by ATP or UTP in situ. At pH values which are optimal for polymerization in vitro, reassembly in all cases is attenuated at the centrosomes, occurs predominantly free of the centrosomes, and actually contrasts the organized reassembly of microtubules from MTOCs in vivo (9) or in Triton X-100-lysed cells using exogenous, MAP-depleted tubulin (8, 53). This suggests to us the possibility that, following lysis, endogenous tubulin may be associated with assembly-promoting factors such as MAPs which allows for spontaneous, self-nucleating processes to occur as described in vitro (3, 35, 48).

Recent reports by DeBrabander et al. (15) and Simone et al.

(47) suggest that a similar mechanism may influence the assembly of free microtubules in living cells following treatment with the microtubule assembly promotor, Taxol. When applied after nocodazole or colcemid disruption of endogenous microtubules, Taxol induced extensive assembly of short, randomly dispersed bundles of microtubules throughout the cytoplasm and blocked the organizing capacity of MTOCs. The Taxol studies along with our observations herein suggest that dispersed spontaneous nucleation can be induced by the presence of assembly-promoting factors such as Taxol or MAPs. These factors can effectively lower the critical concentration of tubulin for initiation and assembly at sites other than MTOCs.

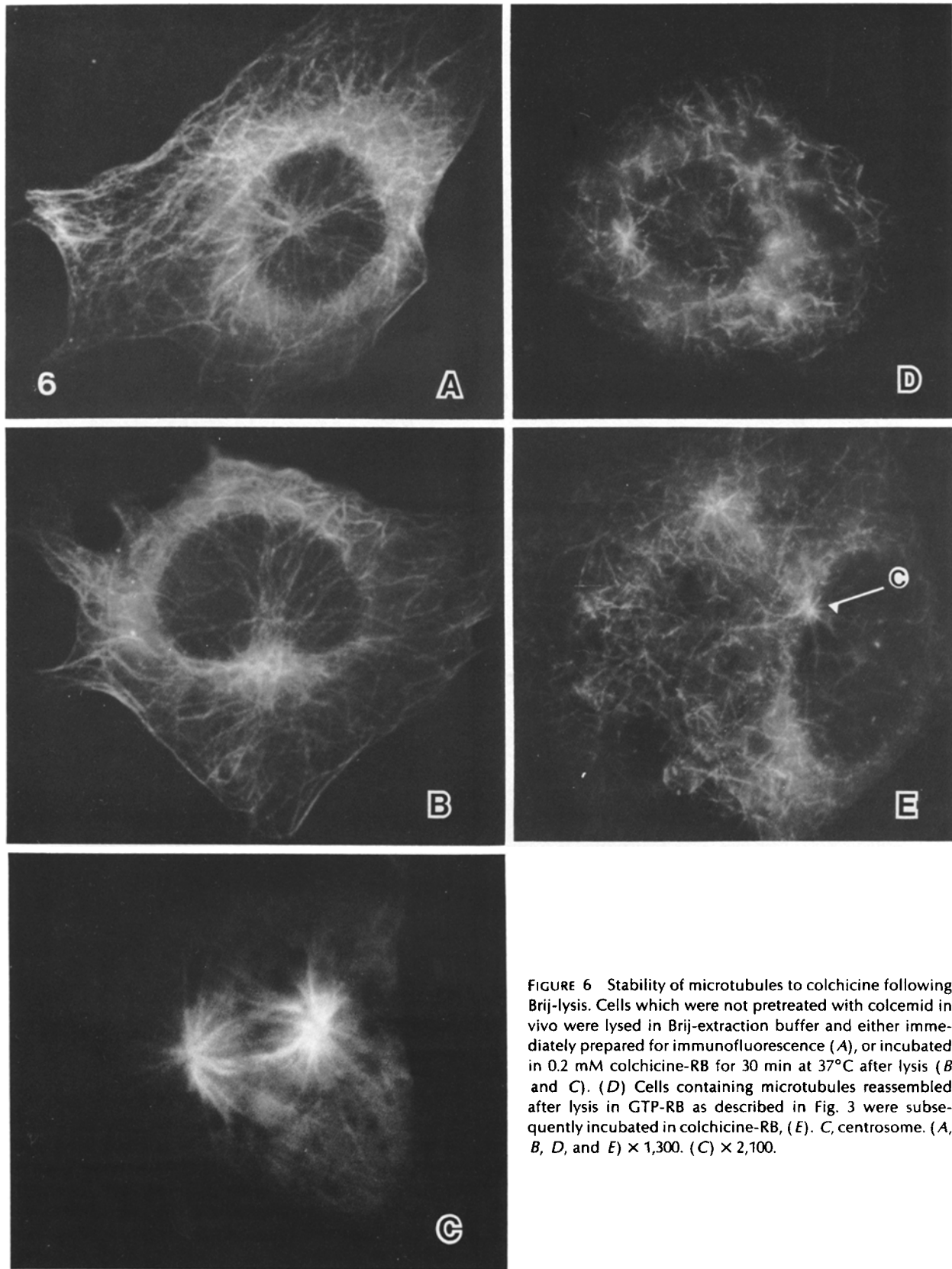


FIGURE 6 Stability of microtubules to colchicine following Brij-lysis. Cells which were not pretreated with colcemid *in vivo* were lysed in Brij-extraction buffer and either immediately prepared for immunofluorescence (A), or incubated in 0.2 mM colchicine-RB for 30 min at 37°C after lysis (B and C). (D) Cells containing microtubules reassembled after lysis in GTP-RB as described in Fig. 3 were subsequently incubated in colchicine-RB, (E). C, centrosome. (A, B, D, and E)  $\times 1,300$ . (C)  $\times 2,100$ .

We have found two conditions where the reassembly of tubulin in lysed cells is similar to the organized pattern observed *in vivo*: (a) alkaline pH plus ATP and (b) reversal from disassembly induced by high  $\text{Ca}^{++}$  concentrations. Although conditions were found that appeared to favor site-associated

assembly, we are not certain that all cytoplasmic microtubules grow from the MTOCs in these models. Indeed, some microtubules may assemble randomly and then become aligned as a result of association with either microtubules extending from MTOCs or other cytoskeletal components. Ultimately, such



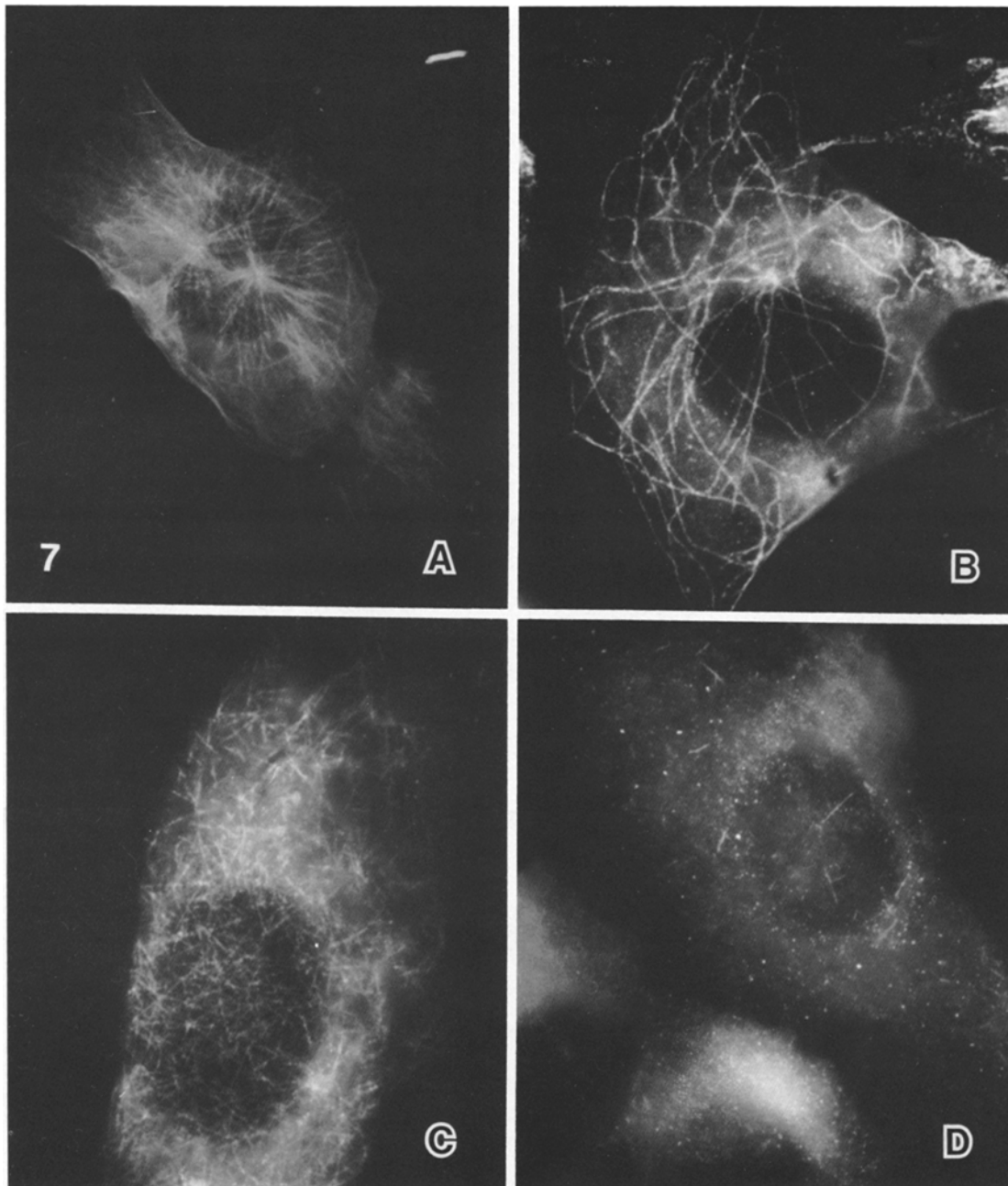


FIGURE 7 Stability of microtubules to low temperature. Cells which were not pretreated with colcemid *in vivo* were either lysed in extraction buffer and prepared for immunofluorescence (A), or preincubated for 30–45 min at 2°C in culture followed by lysis at 4°C (B). (C) Cells containing microtubules reassembled after lysis in GTP-RB as described in Fig. 3 were subsequently incubated in GTP-RB for 15 min at 2°C (D).  $\times 1,450$ .

interactions would lead to a highly organized CMTC. Consistent with the data of Schliwa et al. (45), we find that incubation of lysed cells in reassembly buffer containing millimolar concentrations of  $\text{Ca}^{++}$  results in a reduction of the CMTC primarily from disassembly of microtubules from their distal ends. The extent of the disassembly, however, is found to vary from cell to cell. Upon chelation of the  $\text{Ca}^{++}$  with excess EGTA, reassembly appears to occur predominantly from the ends of microtubules resistant to  $\text{Ca}^{++}$  and the CMTC is restored. Further evidence that reassembly under these conditions is via an elongation reaction is indicated by the inability of GDP to inhibit the restoration of the microtubule complex. Thus, under these conditions an energy source in the form of a nucleotide triphosphate is not required. This is consistent with *in vitro*

studies that have shown that, in the presence of glycerol or MAPs, GDP-tubulin can elongate preexisting microtubules (13, 28, 61), while MAP-depleted phosphocellulose-purified GDP-tubulin is not competent to elongate (personal unpublished results). These observations further support the idea that endogenous tubulin after lysis may be associated with a MAP.

We observed a significant increase in the average length of microtubules assembled in ATP compared to those polymerized in GTP or UTP. The increased lengths are reduced, however, when ATP-induced assembly occurs in the presence of nonhydrolyzable ATP analogs. Since ATP and UTP are similar in their ability to induce assembly via transphosphorylation of GDP (37), these observations strongly suggest that phosphorylation of tubulin and/or MAPs is involved in the

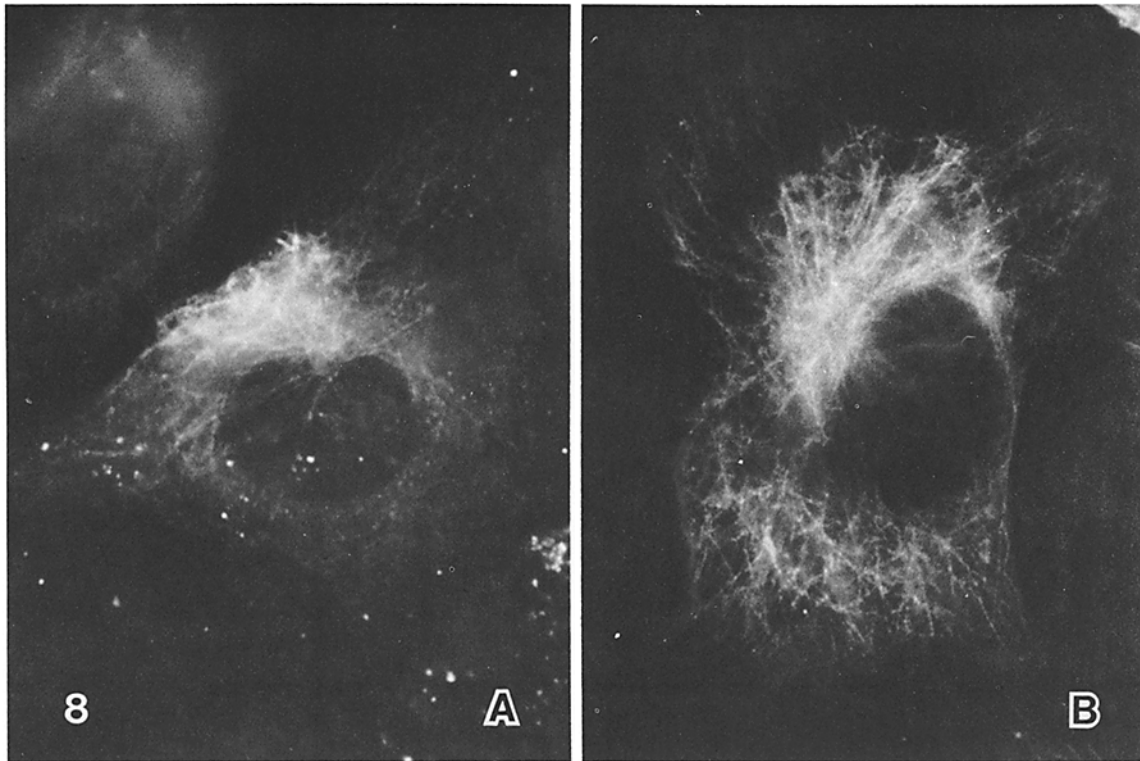


FIGURE 8  $\text{Ca}^{++}$ -induced disassembly of the CMTC and reformation of the CMTC following  $\text{Ca}^{++}$  chelation. Cells that were not pretreated with colcemid *in vivo* were lysed in extraction buffer containing 1 mM EGTA followed by incubation in 3 mM  $\text{Ca}^{++}$ -RB for 20 min at 25°C (A). After  $\text{Ca}^{++}$  treatment, cells were washed in 5 mM EGTA-RB and subsequently incubated in GTP-RB for 5–10 min at 37°C (B).  $\times 1,300$ .

length differences. Phosphorylation of tubulin and associated proteins *in vitro* by a MAP kinase has been reported (18, 41) and other studies have shown that the kinase is dependent on cAMP (20, 48, 52). Although the mechanism and significance of phosphorylation on microtubule assembly are not clear at this time, several studies have demonstrated a stimulatory effect of cAMP on microtubule growth in several transformed cell types in culture (17, 24) and in a lysed cell system (53). In light of these observations, one possible explanation for increased microtubule lengths in the presence of ATP is that phosphorylation may decrease the number of nuclei formed by inhibiting the nucleation reaction, while subsequently enhancing the kinetics of elongation.

After Brij lysis, we observe a dramatic change in the sensitivity of 3T3 cell microtubules to colchicine-induced disassembly. Normally, most mitotic and cytoplasmic microtubules of cells in culture are extremely labile to low concentrations of colcemid or colchicine. This sensitivity, however, has been found to vary depending on the cell type. Following Brij-lysis, both preexisting microtubules (CMTC) and microtubules reassembled from endogenous tubulin are extremely insensitive to high concentrations of colchicine. It should be noted that, although a subpopulation of microtubules assembled *in vivo* are cold-stable, the reassembled colchicine-stable microtubules depolymerize upon lowering the temperature to 4°C. Taken together, these observations indicate that colchicine and cold-stability can be determined by different factors and mechanisms. Since concentrations of colchicine that completely depolymerize microtubules *in vivo* do not depolymerize microtubules *in situ*, it is possible that some artifactual changes may occur in the molecular composition of microtubules and tubulin assembly species ensuing lysis.

Our present results from the Triton X-100 and Brij-lysed cell

systems combined have important implications concerning the process of site-specific microtubule assembly. Recently, Kirschner (29) has proposed a model to account for site-specific assembly and suppression of spontaneous polymerization based strictly upon opposite end assembly-disassembly and differences in the critical concentration at opposite ends of the polymer. Thermodynamically, microtubules with the higher critical concentration end anchored at MTOCs and unavailable for subunit exchange would predominate due to the lower critical concentration reaction at the free end. His model appears plausible under steady state conditions where the unpolymerized tubulin is at or near the critical concentration but becomes questionable for nonsteady state conditions where tubulin concentrations exceed the critical concentration for assembly. The model of Kirschner (29) becomes a problem when considering the regulation of early stages of site-specific spindle or cytoplasmic microtubule complex formation when tubulin concentrations are expected to be high, thus favoring spontaneous nucleation. Our data lead us to an alternative explanation involving several physiological factors which influence microtubule assembly. An important feature of our model is that it does not necessarily restrict subunit exchange from occurring at the end of the microtubule associated with the MTOC.

It is possible that certain assembly-promoting factors which lower the critical concentration (e.g., MAPs) may be localized mainly at MTOCs as has been recently reported for MAP<sub>1</sub> (33). Tubulin which is not competent to spontaneously self-nucleate would therefore be dependent upon these sites to initiate assembly. Indeed, this appears to be the case for the organized assembly of MAP-depleted tubulin from centrosomes in Triton-lysed cells (8, 53), whereas use of MAP-containing tubulin results in random assembly of free-ended mi-

crotubules. Thus, microtubules assembling *in vivo*, depending on their function and the cell type, may contain relatively low "stabilizing protein"/tubulin ratios which could also account for their drug sensitivity. Our findings suggest that, following lysis in Brij-58, assembly-promoting factors could diffuse away from the centrosome (also see reference 50) and associate with tubulin, thus resulting in a greater capacity for polymerization independent of MTOCs and increased drug stability (16). In this case, conditions which can be more inhibitory to nucleation than to elongation, for example, alkaline pH (19, 42) and phosphorylation (26), could account for diminished random assembly and the shift to more site-specific, elongated microtubules. Regardless of the degree to which tubulin may associate with MAP-like molecules, the data presented here indicate that intracellular pH and the phosphorylation state of proteins involved in tubulin polymerization should be considered in the control of organized microtubule assembly. It is also interesting to speculate that cell cycle-related changes in these parameters may facilitate the formation of discrete microtubule complexes.

The authors are grateful to Linda Wible and Frances Jackson for the excellent cell culturing, to Russell Edwards and Dr. George Smith for their help with the electron microscopy and to Pat Williams for typing the manuscript.

This research was supported by National Institutes of Health grant CA 23022.

Received for publication 22 March 1982, and in revised form 7 February 1983.

## REFERENCES

- Ames, G. F. L. 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. *J. Biol. Chem.* 249:634-644.
- Becker, J. S., J. M. Oliver, and R. D. Berlin. 1975. Fluorescence techniques for following interactions of microtubule subunits and membranes. *Nature (Lond.)* 254:152-154.
- Berkowitz, S. A., J. Katagiri, H. K. Binder, and R. C. Williams. 1977. Separation and characterization of microtubule proteins from calf brain. *Biochemistry* 16:5610-5617.
- Berns, M. W., J. B. Rattner, S. Brenner, and S. Meredith. 1977. The role of the centriolar region in animal cell mitosis. *J. Cell Biol.* 72:351-367.
- Borisy, G. G., and J. Olmsted. 1972. Nucleated assembly of microtubules in porcine brain extracts. *Science (Wash. DC)* 177:1196-1197.
- Brinkley, B. R., and J. Cartwright. 1975. Cold-labile and cold stable microtubules in the mitotic spindle of mammalian cells. *Ann. NY Acad. Sci.* 253:428-439.
- Brinkley, B. R., S. M. Cox, and D. A. Pepper. 1980. Microtubule-plasma membrane interactions: analysis of tubulin immunofluorescence and electron microscopy. In *Testicular Development, Structure and Function*. A. Steinberger and E. Steinberger, editors. Raven Press, New York. 305-314.
- Brinkley, B. R., S. M. Cox, D. A. Pepper, L. Wible, S. L. Brenner, and R. L. Pardue. 1981. Tubulin assembly sites and the organization of cytoplasmic microtubules in cultured mammalian cells. *J. Cell Biol.* 90:554-562.
- Brinkley, B. R., S. H. Fistel, J. M. Marcum, and R. L. Pardue. 1980. Microtubules in cultured cells: indirect immunofluorescent staining with tubulin antibody. *Int. Rev. Cytol.* 63:59-95.
- Brinkley, B. R., P. Murphy, and L. C. Richardson. 1967. Procedure for embedding in situ selected cells cultured *in vitro*. *J. Cell Biol.* 35:279-283.
- Bulinski, J. C., and G. G. Borisy. 1980. Immunofluorescence localization of HeLa cell microtubule-associated proteins on microtubules *in vitro* and *in vivo*. *J. Cell Biol.* 87:792-801.
- Cande, W. Z., K. McDonald, and R. L. Meeusen. 1981. A permeabilized cell model for studying cell division: a comparison of anaphase chromosome movement and cleavage furrow constriction in lysed PTK<sub>1</sub> cells. *J. Cell Biol.* 88:618-629.
- Carlier, M. F., and D. Pantaloni. 1978. Kinetic analysis of cooperativity in tubulin polymerization in the presence of guanosine di- or triphosphate nucleotides. *Biochemistry* 17:1908-1915.
- Connolly, J. A., V. I. Kalnins, D. W. Cleveland, and M. W. Kirschner. 1978. Intracellular localization of the high molecular weight microtubule accessory protein by immunofluorescence. *J. Cell Biol.* 76:781-786.
- DeBrabander, M., G. Geuens, R. Nuydens, R. Willebrords, and J. De Mey. 1981. Taxol induces the assembly of free microtubules in living cells and blocks the organizing capacity of the centrosomes and kinetochores. *Proc. Natl. Acad. Sci. USA.* 78:5608-5612.
- Deery, W. J., and R. C. Weisenberg. 1981. Kinetic and steady state analysis of microtubules in the presence of colchicine. *Biochemistry* 20:2316-2324.
- DiPasquale, A. M., J. McGuire, G. Moellmann, and S. J. Wasserman. 1976. Microtubule assembly in cultured green melanoma cells is stimulated by dibutyryl adenosine 3':5'-cyclic monophosphate or cholera toxin. *J. Cell Biol.* 71:735-748.
- Eipper, B. A. 1974. Rat brain tubulin and protein kinase activity. *J. Biol. Chem.* 249:1398-1406.
- Gaskin, F., C. R. Cantor, and M. L. Shelanski. 1974. Turbidimetric studies of the *in vitro* assembly and disassembly of porcine neurotubules. *J. Mol. Biol.* 89:737-758.
- Goodman, D. B. P., H. Rasmussen, F. DiBella, and C. E. Guthrow. 1970. Cyclic adenosine

- 3':5'-monophosphate-stimulated phosphorylation of isolated neurotubule subunits. *Proc. Natl. Acad. Sci. USA.* 67:652-659.
- Gould, R. R., and G. G. Borisy. 1977. The pericentriolar material in Chinese hamster ovary cells nucleates microtubule formation. *J. Cell Biol.* 73:601-615.
- Grisham, L. M. 1976. Griseofulvin inhibition of microtubule assembly: evidence for microtubule subclasses. Ph.D. thesis, Stanford University, Palo Alto, CA.
- Heidemann, S. R., G. Sander, and M. W. Kirschner. 1977. Evidence for a functional role of RNA in centrioles. *Cell* 10:337-350.
- Hsie, A., and T. Puck. 1971. Morphological transformation of Chinese hamster ovary cells by dibutyryl adenosine cyclic 3':5'-monophosphate and testosterone. *Proc. Natl. Acad. Sci. USA.* 68:358-361.
- Izant, J. G., and J. R. McIntosh. 1980. Microtubule-associated proteins: a monoclonal antibody to MAP<sub>2</sub> binds to differentiated neurons. *Proc. Natl. Acad. Sci. USA.* 77:4741-4745.
- Jameson, L., T. Frey, B. Zeeberg, F. Dalldorf, and M. Caplow. 1980. Inhibition of microtubule assembly by phosphorylation of microtubule-associated proteins. *Biochemistry* 19:2472-2479.
- Job, D., C. T. Rauch, E. H. Fischer, and R. L. Margolis. 1982. Recycling of cold-stable microtubules: evidence that cold stability is due to substoichiometric polymer blocks. *Biochemistry* 21:509-515.
- Karr, T. L., A. E. Podrasky, and D. L. Purich. 1979. Participation of guanine nucleotides in nucleation and elongation steps of microtubule assembly. *Proc. Natl. Acad. Sci. USA.* 76:5475-5479.
- Kirschner, M. W. 1980. Implications of treadmilling for the stability and polarity of actin and tubulin polymers *in vivo*. *J. Cell Biol.* 86:330-334.
- Kuriyama, R., and G. G. Borisy. 1981. Microtubule-nucleating activity of centrosomes in Chinese ovary cells is independent of the centriole cycle but coupled to the mitotic cycle. *J. Cell Biol.* 91:822-826.
- Margolis, R. L., and C. T. Rauch. 1981. Characterization of rat brain crude extract microtubule assembly: correlation of cold stability with the phosphorylation state of a microtubule-associated 64K protein. *Biochemistry* 20:4451-4458.
- Margolis, R. L., and L. Wilson. 1979. Regulation of the microtubule steady state *in vitro* by ATP. *Cell* 18:673-679.
- Mascardo, R. N., and P. Sherline. 1981. MAP<sub>2</sub> is found in microtubule-organizing centers of a wide variety of cells. *J. Cell Biol.* 91(2, Pt. 2):327a. (Abstr.)
- Murphy, D. B., and G. G. Borisy. 1977. Identity and polymerization-stimulatory activity of the nontubulin proteins associated with microtubules. *Biochemistry* 16:2598-2605.
- Murphy, D. B., K. A. Johnson, and G. G. Borisy. 1977. Role of tubulin-associated proteins in microtubule nucleation and elongation. *J. Mol. Biol.* 117:33-52.
- Olmsted, J. B. 1981. Tubulin pools in differentiating neuroblastoma cells. *J. Cell Biol.* 89:418-423.
- Penningroth, S. M., and M. W. Kirschner. 1977. Nucleotide binding and phosphorylation in microtubule assembly *in vitro*. *J. Mol. Biol.* 115:643-673.
- Pepper, D. A., and B. R. Brinkley. 1977. Localization of tubulin in the mitotic apparatus of mammalian cells by immunofluorescence and immunoelectron microscopy. *Chromosoma (Berl.)* 60:223-235.
- Pepper, D. A., and B. R. Brinkley. 1979. Microtubule initiation at kinetochores and centrosomes in lysed mitotic cells: inhibition of site-specific nucleation by tubulin antibody. *J. Cell Biol.* 82:585-591.
- Pepper, D. A., and B. R. Brinkley. 1980. Tubulin nucleation and assembly in mitotic cells: evidence for nucleic acids in kinetochores and centrosomes. *Cell Motility* 1:1-15.
- Rappaport, L., J. F. Le Terrier, A. Virion, and J. Nunez. 1976. Phosphorylation of microtubule-associated proteins. *Eur. J. Biochem.* 62:539-549.
- Regua, C. S., J. R. Pfeiffer, and R. D. Berlin. 1981. Microtubule assembly and disassembly at alkaline pH. *J. Cell Biol.* 89:45-53.
- Rubin, R. W., and R. H. Warren. 1979. Organization of tubulin in normal and transformed rat kidney cells. *J. Cell Biol.* 82:103-113.
- Schiwa, M. 1980. Structural organization of detergent-extracted cells. In *Proceedings of the 38th Meeting of the Electron Microscopy Society of America*. G. W. Bailey, editor. Claitor's, Baton Rouge, LA. 814-817.
- Schiwa, M., U. Euteneuer, J. C. Bulinski, and J. G. Izant. 1981. Calcium lability of cytoplasmic microtubules and its modulation by microtubule-associated proteins. *Proc. Natl. Acad. Sci. USA.* 78:1037-1041.
- Sherline, P., and K. Shavone. 1977. Immunofluorescence localization of high molecular weight proteins along intracellular microtubules. *Science (Wash. DC)* 198:1038-1040.
- Simone, L. D., S. L. Brenner, L. J. Wible, D. S. Turner, and B. R. Brinkley. 1981. Taxol-induced microtubule initiation and assembly in mammalian cells. *J. Cell Biol.* 91(2, Pt. 2):337a. (Abstr.)
- Sloboda, R. D., W. L. Dentler, and J. L. Rosenbaum. 1976. Microtubule-associated proteins and the stimulation of tubulin assembly *in vitro*. *Biochemistry* 15:4497-4505.
- Sloboda, R. D., and J. L. Rosenbaum. 1979. Decoration and stabilization of intact, smooth-walled microtubules with microtubule-associated proteins. *Biochemistry* 18:48-55.
- Snyder, J. A. 1980. Evidence for a ribonucleoprotein complex as a template for microtubule initiation *in vivo*. *Cell Biol. Int. Reports* 4:859-868.
- Snyder, J. A. 1981. Studies of mitotic events using lysed cell models. In *Mitosis/Cytokinesis*. A. M. Zimmerman and A. Forer, editor. Academic Press, Inc., New York. 301-325.
- Soifer, D., A. Laszlo, K. Mack, J. Scotto, and L. Siconolfi. 1975. The association of a cyclic AMP-dependent protein kinase activity with microtubule protein. *Ann. NY Acad. Sci.* 253:598-610.
- Tash, J. S., L. Lagace, D. R. Lynch, S. M. Cox, B. R. Brinkley, and A. R. Means. 1981. Role of cAMP-dependent protein phosphorylation in microtubule assembly and function. *Conf. Cell Proliferation. Cold Spring Harbor (Book A)* 8:1171-1185.
- Webb, B. C., and L. Wilson. 1980. Cold stable microtubules from brain. *Biochemistry* 19:1993-1998.
- Weingarten, M. D., A. H. Lockwood, S. Y. Hwo, and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA.* 72:1858-1862.
- Weisenberg, R. C. 1972. Microtubule formation *in vitro* in solutions containing low calcium concentrations. *Science (Wash. DC)* 117:1104-1105.
- Weisenberg, R. C. 1972. Changes in the organization of tubulin during meiosis in the eggs of the surf clam. *J. Cell Biol.* 54:266-278.
- Weisenberg, R. C., W. J. Deery, and P. Dickinson. 1976. Tubulin-nucleotide interactions during the polymerization and depolymerization of microtubules. *Biochemistry* 15:4248-4254.
- Weisenberg, R. C., and A. C. Rosenfeld. 1975. *In vitro* polymerization of microtubules into aster and spindles in homogenates of surf clam eggs. *J. Cell Biol.* 64:146-158.
- Wolosewick, J. J., and K. R. Porter. 1979. Microtubular lattice of the cytoplasmic ground substance: artifact or reality? *J. Cell Biol.* 82:114-139.
- Zackroff, R. V., R. C. Weisenberg, and W. J. Deery. 1980. Equilibrium and kinetic analysis of microtubule assembly in the presence of guanosine diphosphate. *J. Mol. Biol.* 139:641-659.