Calcium-labile Mitotic Spindles Isolated
from Sea Urchin Eggs (*Lytechinus variegatus*)

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ABSTRACT   We isolated calcium-labile mitotic spindles from eggs of the sea urchin *Lytechinus variegatus*, using a low ionic strength, EGTA lysis buffer that contained 5.0 mM EGTA, 0.5 mM $MgCl_2$, 10-50 mM PIPES, pH 6.8, with 1% Nonidet P-40 (detergent) and 20-25% glycerol. Isolated spindles were stored in EGTA buffer with 50% glycerol for 5-6 wk without deterioration. The isolated spindles were composed primarily of microtubules with the chromosomes attached. No membranes were seen. Isolated spindles, perfused with EGTA buffer to remove the detergent and glycerol, had essentially the same birefringent retardation (BR) as spindles in vivo at the same mitotic stage. Even in the absence of glycerol and exogenous tubulin, the isolated spindles were relatively stable in the EGTA buffer: BR decayed slowly to about half the initial value within 30-45 min. However, both the rate and extent of BR decay increased with concentrations of Ca^{2+} above 0.2-0.5 μM as assayed using Ca-EGTA buffers (0.2 mM EGTA, 0.5 mM $MgCl_2$, 50 mM PIPES, pH 6.8, plus various amounts of $CaCl_2$). Microtubules depolymerized almost completely in <6 min at Ca^{2+} concentrations of 2 μM and within several seconds at 10 μM Ca^{2+}. Of several divalent cations tested, only Sr^{2+} caused comparable changes in BR. The absence of membranes in the isolated spindles appeared to be associated with a lack of calcium-sequestering ability. Our results suggest that calcium ions play an important role in the depolymerization of spindle microtubules and that membrane components may function within the mitotic apparatus of living cells to sequester and release calcium ions during mitosis.

Calcium ions may play a major role in the dynamic equilibrium assembly of spindle fiber microtubules and the generation of chromosome movement. One idea is that calcium ions (Ca^{2+}) trigger depolymerization of microtubules, thus allowing kinetochore fibers to shorten and pull the chromosomes poleward (7, 8, 13, 27, 47, 49-51, 64, 65). It has been postulated that an endoplasmic reticulum integral to the mitotic apparatus sequesters and locally releases Ca^{2+} (17, 18, 22, 44, 65).

Physiological studies have shown that cells maintain calcium ion levels below 0.1 μM (2, 10, 48, 55). Cellular processes regulated by Ca^{2+} are, in general, stimulated by local elevation of Ca^{2+} concentrations above 0.1 μM and are activated fully at 1.0-10.0 μM Ca^{2+}. In many cases a calcium-sensitizing protein, such as calmodulin or troponin, is required as a cofactor in the mechanism of calcium regulation (33).

Weisenberg (76) has demonstrated that astral microtubules can be reassembled in vitro in homogenates of sea urchin eggs, provided that the concentration of calcium is kept very low by high concentrations (5 mM or greater) of EGTA, a calcium chelator. In addition, Kiehart (27, 31) has recently shown that

microinjection of 1 mM Ca^{2+} into the spindle region of sea urchin eggs rapidly abolishes spindle fiber birefringence (BR) by depolymerizing the spindle microtubules. The effect is restricted to the injection region and reverses spontaneously within minutes, thus indicating the activity of a potent calcium-sequestering mechanism in the mitotic apparatus. Evidence from several sources indicates that membrane components of the mitotic apparatus resemble the sarcoplasmic reticulum of muscle and may actively sequester calcium ions (17, 22, 23, 31, 44, 51, 54, 69). Also, immunofluorescence analysis of fixed cell preparations reportedly indicates that calmodulin is localized within the mitotic apparatus (1, 7, 77, 78).

We became interested in calcium ions during a study investigating the minimum buffer conditions required to obtain relatively stable mitotic spindles during lysis of sea urchin eggs. In a preliminary report (64), Salmon and Jenkins described how mitotic spindles could be preserved with normal distribution of spindle BR and organization of microtubules by rapid lysis of *Strongylocentrotus droebachiensis* and *Lytechinus variegatus* sea urchin eggs into a simple calcium-chelating

(EGTA), low ionic strength, Triton X-100 detergent buffer. Critical requirements for preservation of the microtubules during cell lysis were strong calcium chelation and rapid membrane solubilization. Adding low micro+molar concentrations of Ca^{2+} afterwards to these lysed cell preparations caused the spindle BR to disappear rapidly.

Expanding on this earlier work, we describe in this paper a method for isolating and storing large quantities of calcium-labile mitotic spindles from eggs of the sea urchin L. variegatus. The term "isolated spindle" refers here to the central spindle fibers and the astral fibers, as well as the centrosome complexes (poles) and the chromosomes, but not the membranous material in between the fibers. Spindles isolated by our method contain no visible membranes. The isolated spindles are apparently structured primarily of microtubules embedded in a low density, filamentous matrix. Without membranes to act as diffusion barriers, changes in the ion concentration of solution buffers are expected to produce corresponding changes within the microenvironment of the isolated spindles.

We quantitated the kinetics and extent of microtubule depolymerization as a function of Ca^{2+} concentration primarily by measuring changes in the BR of spindle fibers (66) during perfusion experiments with Ca-EGTA buffers to regulate Ca^{2+} concentration. We verified our BR observations with electron micrographs. The sensitivity to other divalent cations and to changes in buffer conditions were examined also. Further aspects of the problem are currently being studied, such as factors that influence the calcium lability, the shortening of spindle fibers that occurs concurrently with the depolymerization of properly anchored spindles, and the establishment of a monomer-polymer equilibrium in vitro. In this report we describe the calcium lability of our isolated spindles, which is probably characteristic of spindles in vivo, and discuss the implications from our lysis procedure that membranes in the mitotic apparatus participate in mitosis by regulating the calcium levels in strategic locations of the mitotic spindle.

MATERIALS AND METHODS

Biological Material

Lytechinus variegatus were obtained primarily from Florida and maintained in artificial sea water (ASW) at 20°C. Some were collected near the Bermuda Biological Station, St. George's West, Bermuda, and were maintained there in tanks of natural filtered sea water (FSW). To collect gametes (12), we injected 3-5 ml of 0.56 M KCl into the urchin's body cavity. Sperm were collected dry and stored at 4°C. Eggs were shed into 100 ml of ASW or FSW at room temperature (~23°C) and were decanted eight times through a 150-μm Nitex screen (Tetko Inc., Elmsford, N. Y.) to remove the egg's jelly coat. Complete removal of the egg's jelly coat is critical for preservation of spindle BR during the isolation procedures using L. variegatus eggs.

Isolation and Storage of Mitotic Spindles

The isolation procedures, reported briefly elsewhere (64), were devised from information and results reported earlier by Rebhun and co-workers (52), Stephens (72), and Sakai and co-workers (57, 58).

Eggs were fertilized in ASW or FSW at 23°C. After 15-30 s, the eggs were pelleted in a hand-operated centrifuge. The supernate was quickly aspirated and replaced with 1 M glycerol, 5 mM Tris-HCl buffer, pH 8.3, to soften and remove the elevating fertilization membranes (72). The eggs were gently resuspended in Moore's calcium-free ASW (11) and developed in monolayers in large finger bowls at room temperature (~23°C). Development was checked with the polarization microscope. 5 min before the expected time of isolation (late metaphase, early anaphase), the eggs were collected, concentrated by manual centrifugation, and washed once with 1 M glycerol, 5 mM Tris-HCl, pH 8.3, to remove the calcium-free ASW salts. To lyse the cells, the pelleted eggs at 25°C were diluted rapidly into 50-100 vol of "EGTA buffer" (5 mM EGTA, 0.5 mM MgCl₂, 10-50

mM PIPES) (16), pH 6.8-7.0 (KOH), containing 0.5-1.0% Nonidet P-40 and 20-25% glycerol (vol/vol) to stabilize the microtubules. Nonidet P-40 (polyoxyethylene (9) p-t-oxtylphenol [21]) is a nonionic detergent chemically similar to Triton X-100, and was obtained from Particle Data Laboratories, Ltd., Elmhurst, Ill. 60126. Spindles were freed with difficulty from the cortex by vigorous pipetting. After 15-30 min in the lysis buffer, the isolated spindles were pelleted at 500 g for 10 min, resuspended in glycerol storage buffer (EGTA buffer with 0.1 mM DL-dithiothreitol [DTT] and 50% glycerol [vol/vol]), then stored at 4°C. We have kept spindles for as long as 5-6 wk without significant deterioration, but the maximum possible length of storage has not been determined.

Light Microscopy

A Zeiss photomicroscope I was equipped with a Zeiss differential interference contrast condenser, × 10 and × 20 Nikon rectified polarization objectives, Zeiss × 25 Neofluor phase contrast objective, and Zeiss × 16 and × 40 differential interference contrast objectives and prisms. The strain-free condenser lens permitted satisfactory observation of one specimen by any or all three image contrast methods. An HBO 200-W mercury arc lamp with heat-cut and 546-nm interference filters provided the illumination.

Photographs were taken using Kodak Plus X 35-mm negative film and processed in Microdol X. Polarization photographs were taken at 3-5 nm of compensation.

Birefringence retardation (BR) was measured midway between the chromosomes and the poles by visual compensation of the strongest fibers, using the Zeiss Brace-Köhler λ/30 compensator. Detailed methodology is described elsewhere (60, 63).

Experimental Protocol

In most experiments, isolated spindles in a drop of glycerol storage buffer were sandwiched between an ethanol-cleaned slide and a coverslip supported by parallel ridges of silicone high-vacuum grease (Dow Corning Corp., Midland, Mich.). The isolated spindles were allowed to settle, and some adhered to the slide. Fragments of no. 1 filter paper were used to draw buffers through the coverslip-slide sandwich. Preparations were perfused initially with 0.2-EGTA buffer (same as EGTA buffer, except only 0.2 mM EGTA was used) to remove the glycerol, lower the EGTA concentration, and locate attached spindles. One attached spindle was chosen from each preparation to be monitored in the light microscope during perfusion with experimental buffer. Ca^{2+} sensitivity was measured using Ca-EGTA experimental buffers: 0.2-EGTA buffer plus CaCl₂ at various concentration ratios to EGTA as described below. Divalent cation specificity was examined by substituting another divalent cation (strontium, manganese, magnesium, or barium) for calcium in the 0.2-EGTA buffer.

In some experiments, the isolated spindles were transferred from storage buffer to the Ca-EGTA buffers by centrifugation. The BR changes seen in spindles examined after 10-min incubations were typical of the changes seen after perfusions, and thus are not presented separately here.

Calculation of Free Ca^{2+} Concentrations in Ca-EGTA Buffer

The free Ca^{2+} concentration in the Ca-EGTA buffers was determined according to the methods of Portzehl et al. (46; see also description in reference 71). Computation was performed using an Apple II microcomputer (Apple Computer Inc., Cupertino, Calif. 95014) and a Houston Instrument Hi-Plot digital plotter (Houston Instruments, Austin, Tex. 78753). Fig. 1 shows a computer-generated plot of the calculated free Ca^{2+} concentration vs. the ratio (R) of total CaCl₂ to total EGTA in our Ca-EGTA buffer for total EGTA = 0.2 mM, Mg = 0.5 mM, and pH = 6.8. At pH 6.8, the apparent association constant (K_{app}) of Ca^{2+} for EGTA is ~2 × 10⁶ (M⁻¹). Strict monitoring of the pH of the Ca-EGTA buffers is necessary, because K_{app} is extremely sensitive to changes in pH (46). PIPES was chosen for the pH buffer because it does not significantly bind Ca^{2+} (18). Changes in Mg^{2+} concentration (0-5 mM) in the Ca-EGTA buffer have little effect on the free Ca^{2+} concentration because the ratio of the apparent association constant of EGTA for Ca^{2+} and Mg^{2+} is ~10⁵ (46). The curve shown in Fig. 1 is not significantly altered, for values of R = 0.9 or less, when 1 mM EGTA is substituted for 0.2 mM EGTA in the Ca-EGTA buffer. The addition of 1-2 mM nucleotide triphosphate with equimolar Mg^{2+} to the Ca-EGTA buffer is expected to reduce the free Ca^{2+} concentration about 10% or less below the levels shown in Fig. 1 for a given value of R (79).

In comparison to calcium (true association constant, $K = 10^{11}$), neither strontium ($K = 10^8$), magnesium ($K = 10^{5.4}$), nor barium ($K = 10^8$) is significantly chelated by EGTA (68). We have not yet found a value for the association constant (K) of manganese.
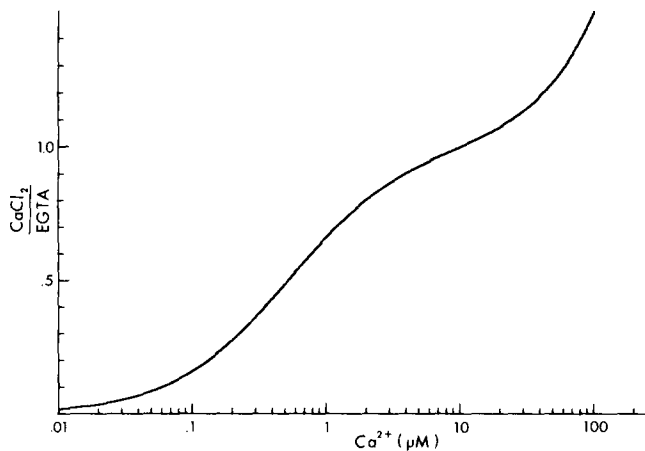
FIGURE 1   Free Ca^{2+} concentration as a function of the ratio of total $CaCl_2$ to total EGTA in the Ca-EGTA buffer containing 0.5 mM $MgCl_2$ at pH 6.8, calculated as described in Portzehl et al. (46).

Electron Microscopy

An aliquot of isolated spindles in glycerol storage buffer was warmed to 23°C and enough additional EGTA buffer was added to dilute the glycerol to 25%. The isolated spindles were then pelleted at 500 g for 10 min and resuspended in 0.2-EGTA buffer or 2 μM Ca^{2+} (Ca-EGTA) buffer to assay for calcium sensitivity. To fix the isolated spindles, they were pelleted and resuspended for 1 h at room temperature in the appropriate buffer (0.2-EGTA with or without Ca^{2+}) to which had been added 3% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa., no. 80127, 25% ampules) (3). The fixed isolated spindles were washed three times by centrifugation in EGTA buffer, postfixed for 30 min in 1% OsO_4 at 0°C, rinsed, stained with 1% uranyl acetate for 2 h at 22°C, then dehydrated in an ethanol series, embedded in Mollenhauer no. 2 Epon-Araldite, sectioned with a diamond knife, stained in lead citrate, and viewed in a Hitachi HU11B electron microscope at 75 kV.

Microtubule Protein

Microtubule protein was purified from porcine brain by two cycles of temperature-dependent polymerization-depolymerization as described by Borisy et al. (5). The reassembly buffer contained 0.1 M PIPES, pH 6.8, 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.5 mM GTP plus 25% glycerol (vol/vol) during the warm incubation cycle. Twice-repolymerized microtubules were stored as a pellet at −80°C. Protein concentrations were determined by the Lowry procedure (35). For experiments, a pellet containing ~5 mg of protein was homogenized into 2.5 ml of 4°C reassembly buffer without glycerol. After a 0.5-h incubation at 4°C, the preparation was clarified before use by centrifugation at 100,000 g for 30 min at 4°C.

The effect of 1% Nonidet P-40 on microtubule assembly was assayed by monitoring microtubule polymerization, using turbidity measurements (14) at 350 nm during two cycles of warming to 23°C and cooling to 4°C.

RESULTS

Structure

Treatment of *L. variegatus* eggs with EGTA lysis buffer preserved the fibrous structure of the mitotic spindle, which was retained in the center of the intact cortex. Vigorous pipetting released the spindle from the cortex (Fig. 2), but as a result the asters often broke away from the central spindle or anaphase spindles broke in half in the interzone. Consequently, our experimental preparations contain a mixture of isolated spindles (some incomplete), isolated cortices, and a small number of unbroken extracted eggs.

Spindles isolated by our procedure retain the magnitude and distribution of spindle fiber BR typical of spindles in living cells at the time of isolation (Fig. 2). In phase contrast or differential interference contrast microscopy, the chromosomes,

the centrosomes, and the spindle's fibrous elements are the prominent structures (Fig. 2). Globular material, typically <0.5 μm Diam, is seen radially aligned along the astral fibers, particularly distal from the centrosome region. Little globular material is seen among the central spindle fibers. Our isolated spindles appear distinctly different from the mitotic apparatus isolated from sea urchins by the standard hexylene glycol procedure developed earlier by Kane (15, 29).

Electron micrographs show that the isolated spindles contain no membranes and that they are highly extracted (Fig. 3 and 4). Clearly visible are microtubules, the centrosome-centriole complex at the poles (Fig. 4), and the chromosomes. The number and distribution of microtubules seen in electron micrographs correspond with the magnitude and distribution of spindle fiber BR seen in isolated spindles of the same mitotic stage. Most half-spindle microtubules appear to be nonkinetochore microtubules that extend from the pole to the region of the chromosomes or just beyond.

As seen in Fig. 4, there is a noticeable boundary zone where the central spindle microtubules end near the centrosome-aster complex. The scarcity of microtubules crossing this boundary may account for the ease with which asters were mechanically sheared from the central spindle without disrupting the spindle's morphology. This structural discontinuity in the spindles warrants further examination because it suggests that the centriole-centrosome complex may not be the organizing center for the central spindle microtubules.

Higher magnification electron micrographs show a low density, fine, filamentous material coating the walls of the microtubules and dispersed between the microtubules (Fig. 5). Particles, ≈20 nm Diam, adhere to the finely filamentous material. The particles, sparsely dispersed along the central spindle microtubules, occur in much higher concentrations in the centrosome-centriole complex, forming large clumps along the aster microtubules distally from the centrosome-centriole complex (Fig. 4). These particle complexes appear to correspond with the globular material seen in light micrographs of the isolated spindles (Fig. 2). The nature of the fine filamentous material and the particles is not yet known. Similar particles have been seen adhering to fine filamentous material on microtubules in spindles isolated by other methods. It has been postulated that the particles are ribosomes or some other complex of ribonucleic protein (15, 19).

Stability

Relatively stable spindles have been isolated in a simple lysis buffer that contained only 10 mM EGTA, pH 6.6, and 0.25% Triton X-100 (64). The pH of the lysis buffer was very important. Above pH 7.0, spindle preservation and stability in EGTA glycerol lysis buffer degenerated; at pH 7.6, no spindles were preserved. As found earlier by Kane (29), pH below 6.8 promoted microtubule stabilization, but lower pH also increased substantially the amount of amorphous material adhering to the the spindles' fibrous structures. We included 0.5 mM $MgCl_2$ in the EGTA lysis and storage buffers because it increased the stability of the spindle microtubules. Ionic strength was kept low because KCl concentrations above 200 mM rapidly abolished spindle fiber BR within seconds in the absence of glycerol.

Nonidet P-40 (21) was used to solubilize the membranes completely. Nonidet P-40 does not appear to have any direct effect on microtubule stability. Addition of 1% Nonidet P-40
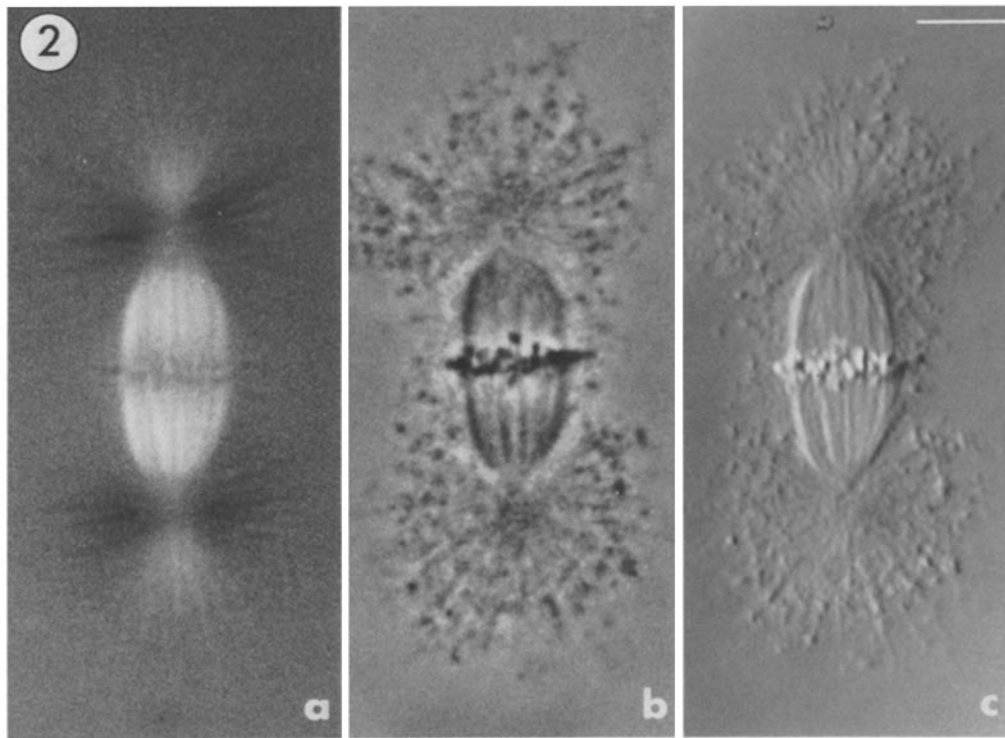
FIGURE 2 Isolated spindle at early metaphase viewed with (*a*) polarization, (*b*) phase-contrast, and (*c*) differential interference contrast microscopy. The polarization micrograph was taken with the compensator set for 3.5 nm positive BR with respect to the spindle interpolar axis. Bar, 10 μm. × 1,200.
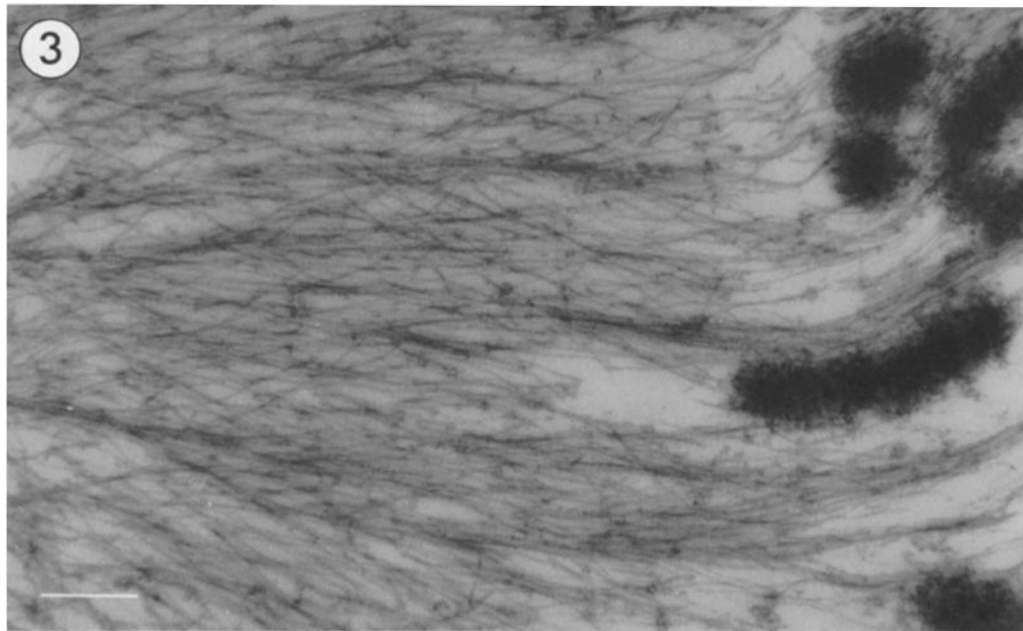

FIGURE 3 Microtubules and chromosomes in an isolated spindle viewed with electron microscopy. The 0.3-μm thick section cuts through the half-spindle from the metaphase plate (right) toward one spindle pole (at left, not in view). The spindles isolated by the detergent lysis procedure described in the text are highly extracted. Intertubular cytoplasm and membranes are not apparent. Most microtubules are longer than 1 μm and many appear to extend all the way from the pole region to the metaphase plate. This spindle has been distorted slightly at the metaphase plate by the preparative centrifugation techniques employed. Bar, 1 μm. × 12,600.

to the reassembly buffer had no measurable effect on the rate and extent of assembly and disassembly of purified porcine brain microtubules in vitro during two cycles of heating and cooling. A similar result has been reported for the detergent Triton X-100 (58).

When mitotic spindles were isolated in EGTA lysis buffer without glycerol, the initial spindle fiber BR was nearly identical to the BR of spindles at the same mitotic stage in living cells (e.g., at metaphase, measured BR of isolated spindles ranged from 2.4–3.0 nm at 25°C, depending on the batch of
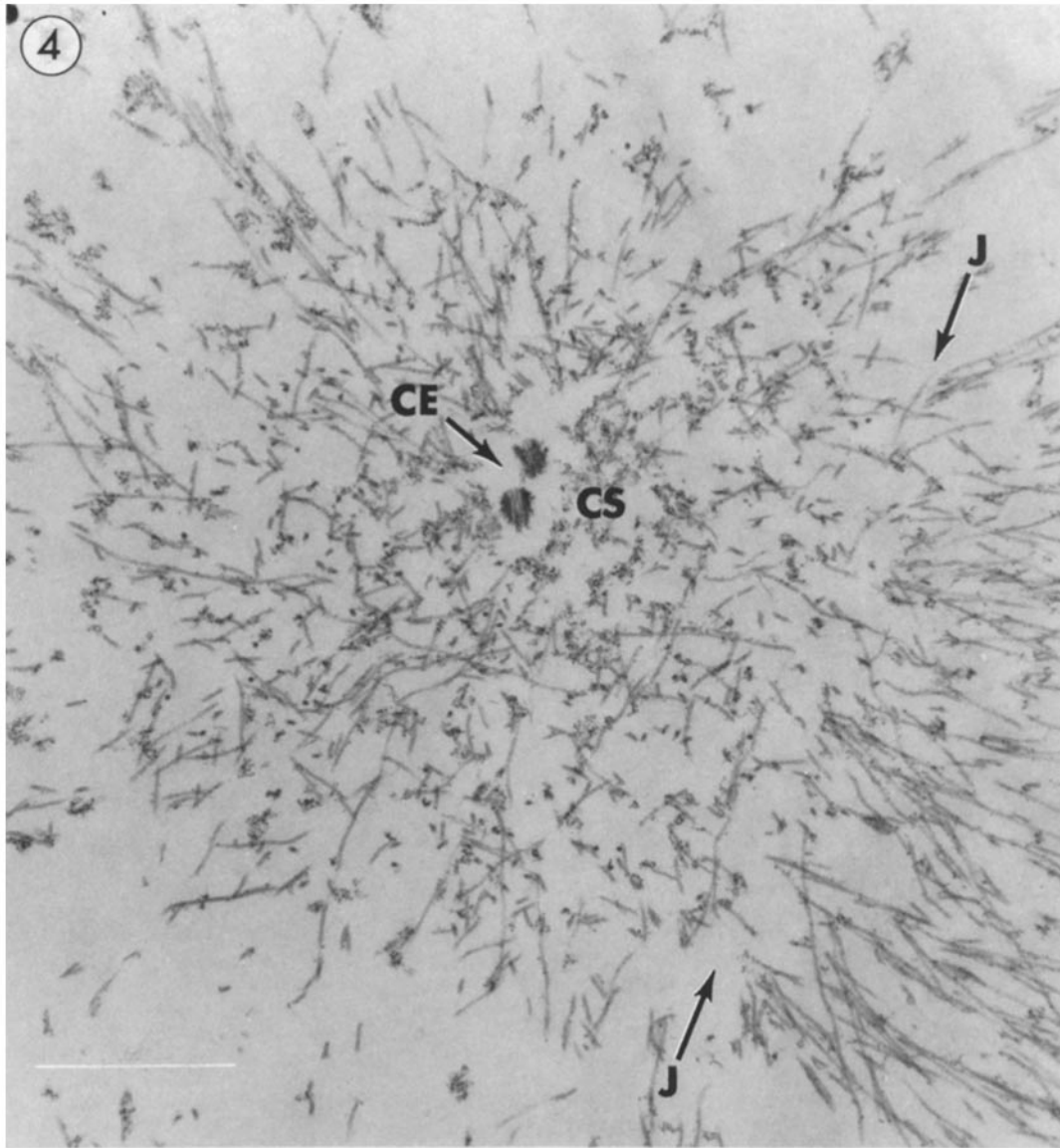
FIGURE 4 The centrosome-aster complex of an isolated metaphase spindle in a thin-section electron micrograph. Centrioles (CE) are contained within the centrosome complex (CS), which is a region where electron-dense material and 20-nm particles are concentrated. Astral microtubules appear to end in the centrosome complex and extend radially away from it. The central spindle microtubules do not appear to end in the centrosome complex, but terminate at a peripheral junction (J). Bar, 1 μm. × 16,333.

eggs). The BR of isolated spindles decayed slowly in EGTA buffer to about half the initial value over a period of ~45 min at 25°C. Addition of 20–25% glycerol to the EGTA buffer blocked the decay in spindle BR during the lysis and wash procedures. After washing out the detergent and raising the glycerol concentration in the EGTA buffer to 50%, the isolated spindles could be stored for long periods (4–6 wk) at 4°C.

When stored spindles were transferred to EGTA or 0.2-EGTA buffer (to remove the glycerol), their initial spindle fiber BR, the rate of BR decay, and their calcium lability (see below) became similar to those of spindles freshly isolated in the absence of glycerol. Cooling spindles to 4°C for 10 min, adding 100 μM colchicine, or adding 5 mM caffeine to the 0.2-EGTA buffer did not noticeably accelerate the BR decay of either stored spindles or freshly isolated spindles assayed 30 min after cell lysis.

Although the spindles isolated by the EGTA lysis buffer described here are not labile to cooling, pressure, or colchicine, as spindles are in vivo, these properties can be partially restored if the isolated spindles in the glycerol storage buffer are treated for 1 h with 10 mM EDTA, which chelates both Mg^{2+} and Ca^{2+} (Salmon et al., manuscript in preparation). Such treatment makes the stability of the isolated spindles directly dependent on the assembly characteristics of exogenous tubulin in the buffer after glycerol has been perfused out. The EDTA-treated spindles are depolymerized within several minutes by 4°C, 8,000 psi, or dilution of the microtubule protein concentration in the buffer, treatments that depolymerize or prohibit assembly of purified brain microtubule protein in vitro (32, 41, 47, 61).

Calcium Lability

Calcium in low micromolar concentrations had two distinct effects on these isolated spindles: (a) spindle BR decayed and microtubules depolymerized rapidly, the rate and extent of both increasing with increasing Ca^{2+} concentrations (Figs. 6–
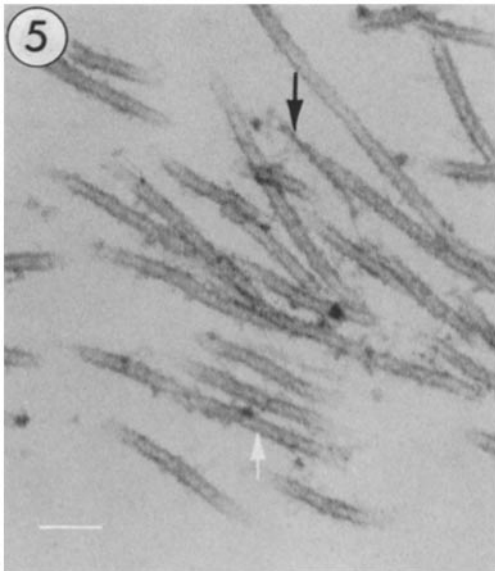
FIGURE 5 Microtubules of an isolated spindle seen in a thin-section electron micrograph at high magnification. The microtubule walls appear coated with a fine, fuzzy, filamentous material (<5 nm Diam) that can sometimes be seen to interconnect microtubules (white arrow). Thicker filaments also are seen occasionally (black arrow). Bar, 0.1 μm. $\times$ 81,700.

9); and (b) as the microtubules depolymerized, the spindle fibers shortened considerably, changing the spindle's morphology (Fig. 6). The fiber shortening induced by Ca^{2+} will be analyzed in detail elsewhere, so it will not be presented here.

Unbuffered Ca^{2+} at <10 μM concentration rapidly reduced spindle BR. When the glycerol storage buffer was replaced with a buffer containing only 0.5 mM $MgCl_2$ and 10 mM PIPES, pH 6.8, spindles lost almost all BR within several minutes. Addition of 10 μM EGTA to this perfusion buffer blocked the rapid decay in spindle BR by chelating the low concentration of calcium present as a contaminant in our chemicals, distilled water, and/or glassware.

With Ca-EGTA perfusion buffers, the rate and extent of decay of spindle BR (within 10 min) increased progressively with Ca^{2+} concentration (Figs. 7 and 8). The slow decay of spindle BR in EGTA buffer was accelerated by free Ca^{2+} concentrations above 0.2–0.5 μM (Fig. 8). Spindle BR was reduced from ~3 nm to <0.3 nm within several minutes by 2 μM Ca^{2+} (Figs. 6 and 7). As expected from the disappearance of spindle BR, microtubules were difficult to detect in electron micrographs of spindles that were fixed 10 min after the addition of 2 μM Ca^{2+} (Fig. 9). At low concentrations of Ca^{2+} (<2 μM), the BR of various spindle fibers could be seen to decay at different rates (Fig. 10). Astral BR disappeared first; what appeared to be kinetochore fiber BR persisted longest.

The kinetics of decay in spindle BR shown in Fig. 7 are typical of all the spindles observed. However, the sensitivity at a given calcium concentration could differ as much as 10-fold between different spindles (Fig. 8). This variation in calcium sensitivity was most noticeable between spindles from different isolation batches. Spindles from the same isolation batch generally displayed quantitatively similar responses.

Microtubule depolymerization in the isolated spindles is highly specific for calcium. Among the other divalent cations tested, only strontium was close to calcium in its effectiveness (Fig. 11; notice the change in time-scale from Fig. 7). Spindle

BR decayed to <0.5 nm within 10 min when 15–20 μM strontium was added to the 0.2-EGTA buffer. Manganese caused substantial reduction in spindle BR within 10 min only at concentrations >300 μM. Neither magnesium nor barium at 1 mM caused any BR changes within 10 min. In contrast to calcium, elevated magnesium concentration stabilized rather than depolymerized the spindle microtubules.

Calcium sensitivity was not noticeably altered if the Ca-EGTA perfusion buffer with 2 μM Ca^{2+} was made using 1 mM EGTA, or contained any of the following: 100 mM KCl, 100 mM PIPES, 2 mM phenylmethyl sulfonyl fluoride (PMSF), 0.1 mM DTT, 2 mM $MgCl_2$ + 2 mM ATP (or GTP, AMP-PNP, ADP), 5 mM caffeine, or 100 μM colchicine. Cooling to 4°C did not cause the spindle BR to disappear rapidly, nor did it inhibit the spindles' calcium response. On the other hand, glycerol concentrations above 15% (vol/vol) or 5 mM $MgCl_2$ in the 2 μM Ca^{2+} Ca-EGTA buffer inhibited depolymerization of the isolated spindles by calcium.

DISCUSSION

We have demonstrated that spindles with relatively stable microtubules can be isolated by using simple calcium-chelating detergent buffers to maintain low calcium concentrations during cell lysis. After isolation, elevation of the Ca^{2+} concentration to 2.0 μM causes the spindles' BR and microtubules to disappear rapidly. Our results support the hypothesis that calcium plays a major role in regulating the disassembly of spindle microtubules. Exposures of microtubules to Ca^{2+} above 0.5 μM within the microenvironment of the mitotic apparatus could be the mechanism by which spindle fibers are disassembled during mitosis.

Other investigators have also shown that low micromolar concentrations of Ca^{2+} depolymerize "native" cytoplasmic microtubules (as opposed to in vitro repolymerized microtubules). By microinjecting high concentrations (100 mM) of Ca-EGTA buffers to overcome the cell's apparent calcium-sequestering system, Kiehart (31) was able to estimate roughly that 4–10 μM or less was the effective Ca^{2+} concentration for microtubule depolymerization in vivo. Kiehart found with developing sea urchin eggs, as we found with isolated spindles, that Sr^{2+} substituted for Ca^{2+} in abolishing spindle BR, but that Ba^{2+} and Mg^{2+} were not effective. By using a Ca^{2+} ionophore, Schliwa (67) determined that 10 μM Ca^{2+} or less reversibly depolymerized cytoplasmic microtubules in the axopodia of a heliozoan. In another Ca^{2+} ionophore experiment, Fuller and Brinkley (13) demonstrated that elevated cytoplasmic Ca^{2+} concentrations reversibly disassembled the cytoplasmic microtubule complex in cultured mouse fibroblasts, but the effective Ca^{2+} concentration was not determined. The effective Ca^{2+} concentrations determined by Kiehart and Schliwa are very close to the range of Ca^{2+} concentrations that we found depolymerized microtubules in the isolated mitotic spindles.

The mechanism by which Ca^{2+} depolymerizes the spindle microtubules in our isolated spindles is not understood, but a microtubule-associated, calcium-sensitizing factor may be required. Although our results, with those of Kiehart (31) and Weisenberg (76), apparently demonstrate that native sea urchin spindle microtubules are depolymerized by micromolar concentrations of calcium, Keller and Rebhun (30) have found that 1–10 μM Ca^{2+} does not depolymerize the cold-labile microtubules that they assembled in vitro from tubulin purified from mitotic apparatus of the sea urchin Strongylocentrotus purpuratus. In 1972, Weisenberg (75) showed that micromolar
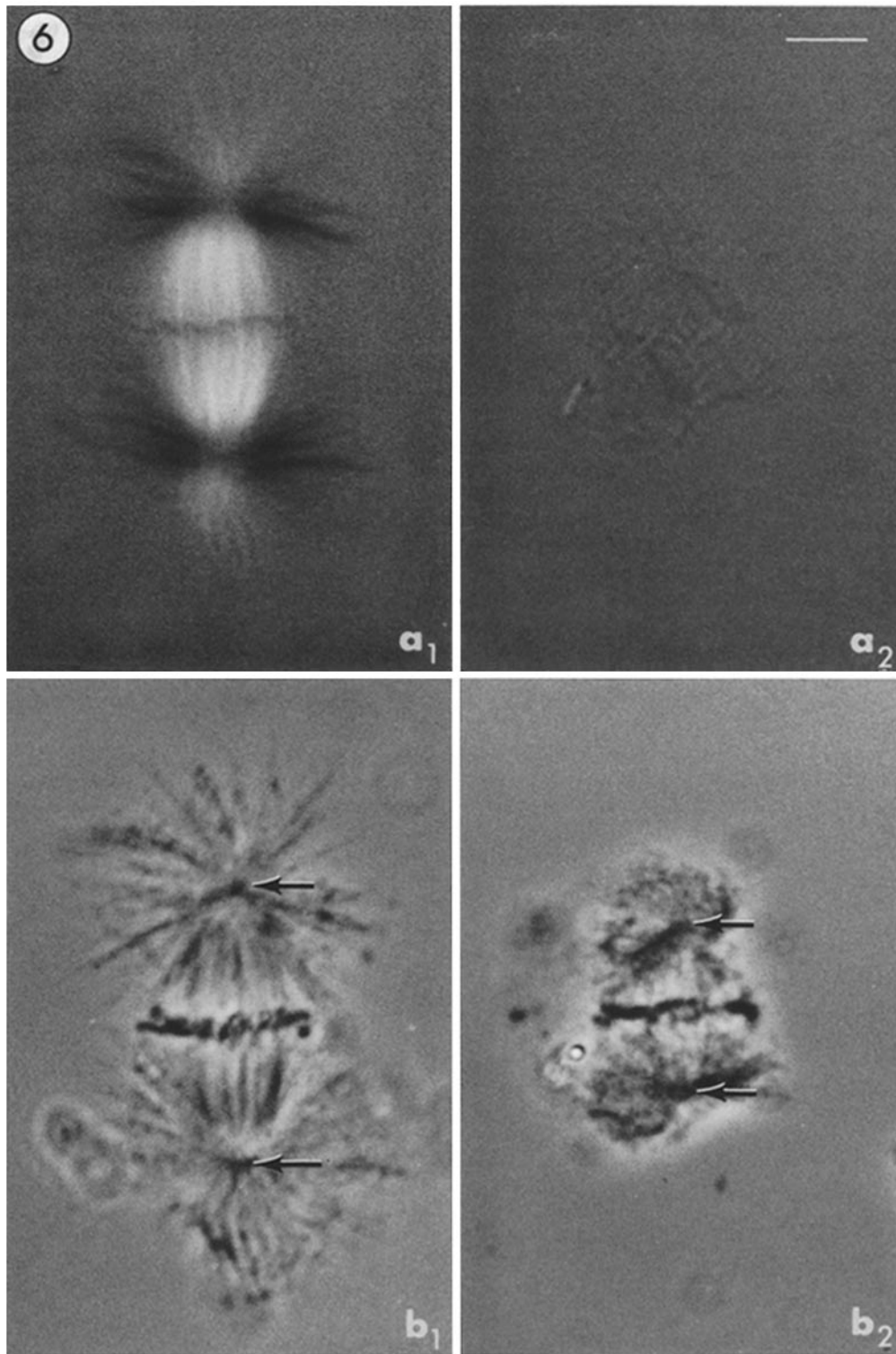
FIGURE 6   Changes in spindle fiber BR and morphology induced by 2 μM Ca^{2+}. Isolated metaphase spindle in 0.2-EGTA buffer before 2 μM Ca^{2+} treatment (a_1 and b_1) and 5 min after (a_2 and b_2) viewed with polarization (a_1 and a_2) and phase-contrast microscopy (b_1 and b_2). The centrosomes (arrows) can be seen as phase-dense regions abutting the spindle poles. Note the loss of spindle fiber BR (a_2), and the shortening of the interpolar, chromosomal, and astral fibers (b_2) that occurred after addition of 2 μM Ca^{2+}. The width of the metaphase plate remained unchanged. Polarization micrographs were taken with the compensator set for 3.5 nm positive BR with respect to the spindle interpolar axis. Bar, 10 μm. X 1,200.

Ca^{2+} levels inhibited repolymerization of microtubules from crude mammalian brain homogenates. However, later studies (41) showed that with tubulin purified from brain by the temperature-dependent assembly-disassembly method, repo-lymerization of microtubules is inhibited only by near milli-molar Ca^{2+} levels. Isolated spindles whose BR has been augmented by addition of purified brain microtubule protein to the solution buffer are not depolymerized by 2 μM Ca^{2+} in the
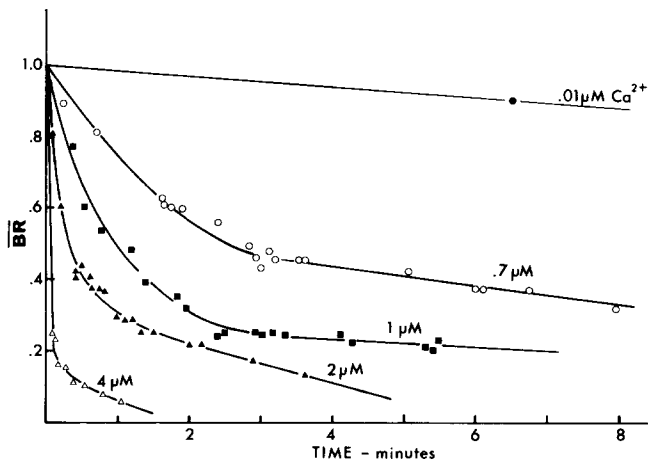
FIGURE 7 Changes in normalized half-spindle BR induced by perfusion with Ca-EGTA buffers having different free Ca^{2+} concentrations. For each of the five experiments shown, an attached metaphase isolated spindle was located during perfusion with 0.2-EGTA buffer; then the BR was measured before and repeatedly after perfusion with Ca-EGTA buffer. The free Ca^{2+} concentration of each buffer, calculated as described in the text, is indicated for all experiments. These values correspond to $CaCl_2$/EGTA ratios (R) of: R = 0 (●), R = 0.6 (○), R = 0.7 (■), R = 0.8 (▲), and R = 0.9 (△). The BR data were normalized by dividing by the initial spindle BR in 0.2-EGTA buffer before perfusion with buffer containing Ca^{2+}. The solid lines were drawn by eye through the data points. Data represent the response of spindles from one isolation batch.

reassembly buffer, probably because the purified brain microtubule protein by itself is not sensitive to 2 μM Ca^{2+} (E. D. Salmon, R. R. Segall, and G. Pape, unpublished observation).

The above observations indicate that the structure and/or the composition of native spindle microtubules differ significantly from that of microtubules purified by the temperature-dependent reassembly procedure. One possibility is that a calcium-binding protein, which ordinarily is complexed with the native spindle microtubules, does not co-purify with the microtubule protein during the reassembly purification procedures. The 10-fold variability that we found in the Ca^{2+} sensitivity of different batches of isolated spindles (Fig. 8) could well be accounted for by variations in the amount of a calcium-sensitizing factor preserved during lysis.

The calcium-binding protein calmodulin, which constitutes as much as 0.2% of the total protein of the sea urchin egg (20), may be the calcium-sensitizing factor for spindle microtubules, or one component of it. Marcum et al. (36) have shown that adding calmodulin to microtubules polymerized in vitro from purified brain tubulin enhanced the microtubules' Ca^{2+} lability. Immunofluorescence studies (1, 7, 77, 78) have located calmodulin in the poleward regions of the mitotic apparatus of fixed culture cells. Calmodulin staining was shown to persist along the stable kinetochore fibers in tissue culture cells cooled to 4°C and was abolished in cells treated with colchicine to depolymerize all the microtubules (78). It should be cautioned, however, that calmodulin has not yet been shown to be complexed with spindle microtubules, nor has it been demonstrated that calmodulin confers calcium sensitivity to purified spindle microtubules (40).

When our results are compared with those of Kiehart (31), there appear to be two significant differences between the mitotic spindles isolated in our EGTA lysis buffer and mitotic
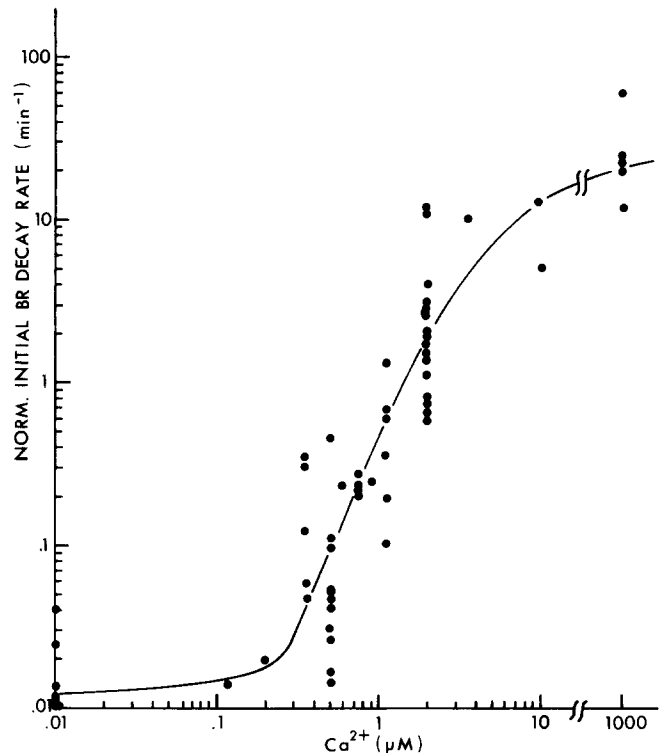

FIGURE 8 The normalized initial spindle BR decay rates as a function of Ca^{2+} concentration taken graphically from the initial slopes of kinetic curves such as shown in Fig. 7. Note that the rate of BR decay increases significantly above 0.2-0.5 μM Ca^{2+}. The rate of BR decay above 10 μM may be limited by the time required to exchange solution buffers during perfusion. The solid line was drawn by eye through the data points.
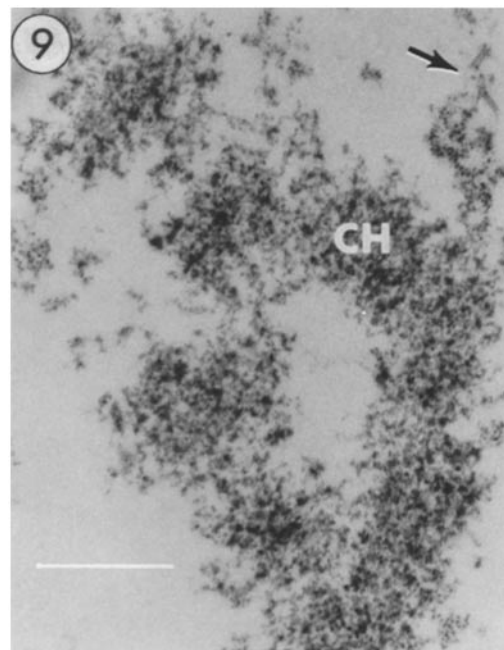

FIGURE 9 Cross section through a group of chromosomes (CH) in an isolated spindle treated for 10 min before fixation with Ca-EGTA buffer having 2 μM free Ca^{2+}. Few microtubules (arrow) are visible compared with the 220 microtubules counted in a similar region of a metaphase spindle treated with 0.2-EGTA buffer without Ca^{2+}. Bar, 1 μm. × 21,600.
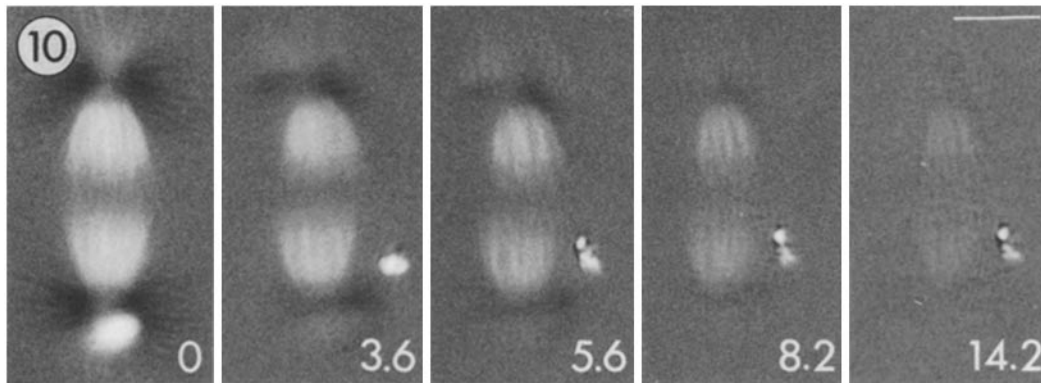
FIGURE 10 Polarization micrographs of the change in spindle fiber BR of an isolated anaphase spindle during perfusion with Ca-EGTA buffer having 1.2 μM Ca^{2+}. The time (in minutes) = 0 at the start of perfusion with Ca-EGTA buffer. The compensator was set for 3.5 nm positive BR with respect to the spindle interpolar axis. Note the differential stability of the various microtubules to Ca^{2+} as indicated by the persistence of BR. Astral microtubules appear most sensitive, whereas kinetochore microtubules appear most persistent. Bright spot at right of spindle is an uninvited birefringent particle. Bar, 10 μm. × 880.
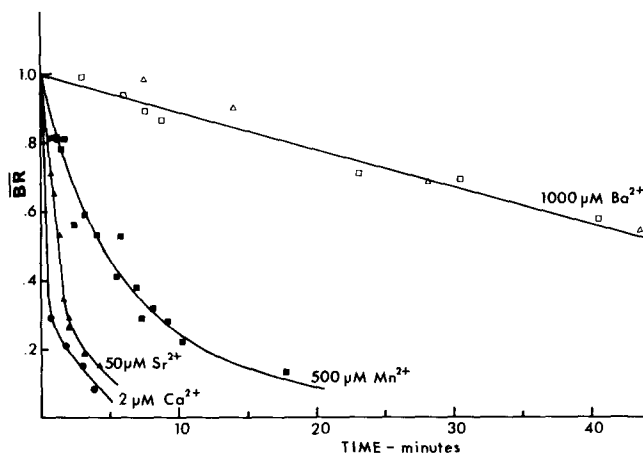

FIGURE 11 Lability of the isolated spindles to various divalent cations. Spindles were perfused and BR measured as described in Fig. 8. $SrCl_2$ (▲), $MnCl_2$ (■), or $BaCl_2$ (□) was added to the 0.2-EGTA perfusion buffer at the concentrations indicated on the graph. For reference, examples of BR decay in Ca-EGTA buffer with 2 μM Ca^{2+} (●) and in 0.2-EGTA perfusion buffer without Ca^{2+} (△) are also presented.

spindles in living cells. First, the mitotic apparatus in vivo apparently have a potent calcium-sequestering activity that is missing in the detergent-extracted membrane-free isolated spindles. Secondly, the isolated spindles are not in a labile equilibrium with a pool of tubulin subunits. Our isolated spindles depolymerized completely when exposed to un-buffered Ca^{2+} of 10 μM or less, but microinjections of un-buffered Ca^{2+} in vivo (31) abolished BR only within the region of injection and only with Ca^{2+} concentrations ≥ 1 mM. Furthermore, the BR of spindles in vivo returned spontaneously after the calcium injection, whereas the BR of our isolated spindles did not. When calcium depolymerizes the microtubules of our isolated spindles, apparently the tubulin subunits solubilize and are dispersed in the solution buffer (verified by loss of tubulin-antibody staining from the spindle fibers; E. D. Salmon and J. Fuseler, unpublished observation). Further evidence for lack of a monomer-polymer equilibrium in the isolated mitotic spindles is their insensitivity to cold, hydrostatic pressure, and colchicine, all of which normally depolymerize spindle microtubules in vivo (26).

These comparisons support the hypotheses that (a) the membrane components of the mitotic apparatus perform an important function in mitosis by regulating the calcium levels within strategic locations of the mitotic apparatus, and (b) calcium ions trigger depolymerization of spindle microtubules during mitosis, independently of a temperature-sensitive monomer-polymer equilibrium as defined by Inoué and his co-workers (26, 28, 59).

Harris has shown that membranes are normally a prominent component of the mitotic apparatus in fixed whole eggs of sea urchins (17, 18). It has been postulated that membrane vesicles, tubules, and cisternae, which are concentrated particularly in the spindle pole regions and within the centrospheres (17), sequester and release Ca^{2+} during mitosis, in a manner analogous to the function of sarcoplasmic reticulum in muscle (17, 18, 22). Recently, Hepler (23) has given cogency to this idea by demonstrating that in plant tissue fixed in the presence of ferricyanide membranes of the mitotic apparatus appear structurally similar to sarcoplasmic reticulum, and that calcium is concentrated within the membrane vesicles. Caffeine, which causes the sarcoplasmic reticulum to release calcium (53, 73, 74), dissolves spindle BR in vivo (31, 51), but 5 mM caffeine has no effect on our isolated spindles. Ca-ATPase, which has been localized within isolated sea urchin mitotic apparatus that contain membrane vesicles (37, 39, 43–45, 69, 70), is not present in membrane-free spindles isolated by the techniques described here (42). The absence of caffeine lability, of Ca-ATPase activity, and of calcium-sequestering activity in our membrane-free isolated spindles supports the contention that membranes in the mitotic apparatus normally have a calcium-regulatory function.

We would like to stress that the calcium-sensitivity of the isolated spindles does not depend on a labile monomer-polymer equilibrium of the microtubules. From in vivo spindle birefringence studies, Inoué and co-workers (24, 25, 26, 28) have characterized spindle microtuble assembly as a dynamic equilibrium with a pool of assembly-competent tubulin subunits. Assembly is thought to be temporally and spatially controlled by the activity of the mitotic centers–the kinetochores and the centrosome or centrosphere complexes–and by the activation-inactivation of tubulin subunits (4, 38, 47, 56). Our results suggest that this model needs to include a role for Ca^{2+} in producing microtubule depolymerization, or prohibit-

ing microtubule polymerization. Isolated spindles that we further treated with EDTA appeared to have equilibrium characteristics more similar to those of spindles in vivo, but microtubules in EDTA-treated and untreated isolated spindles were both rapidly depolymerized by Ca^{2+}. This becomes significant when considering the implications of experiments in which kinetochore fiber microtubules in spermatocytes (62), in tissue culture cells (6, 78), and in plant cells (34) were found to resist depolymerization by cooling. From thermal lability studies on living crane fly spermatocytes, Salmon and Begg (62) concluded that shifts in a monomer-polymer equilibrium could not adequately explain microtubule depolymerization and spindle fiber shortening during anaphase. On the other hand, a process based on calcium-induced depolymerization of spindle microtubules to produce fiber shortening is a plausible hypothesis. Membranes in the mitotic apparatus may function to sequester and release Ca^{2+} in strategic locations and at appropriate times to control the process of microtubule depolymerization and spindle fiber shortening during mitosis.

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