GROWTH AND LABILITY OF *CHAETOPTERUS* OOCYTE MITOTIC SPINDLES ISOLATED IN THE PRESENCE OF PORCINE BRAIN TUBULIN

SHINYA INOUÉ, GARY G. BORISY, and DANIEL P. KIEHART

From the Marine Biological Laboratories, Woods Hole, Massachusetts 02543, Program in Biophysical Cytology, Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin 53715

ABSTRACT

Purified tubulin solutions stabilized and augmented the birefringence (BR) of isolated *Chaetopterus* spindles. Tubulin was extracted from pig brain tissue in cold PEG buffer (0.1 M piperazine-*N*-*N'*-bis[2-ethane sulfonic acid], 1 mM ethylene bis-[oxyethylenenitrilo]tetraacetate, [EGTA], 2.5 mM guanosine triphosphate, [GTP], pH 6.94, at 25° C), and purified by two cycles of a reversible, temperature-dependent assembly-disassembly procedure. The spindle BR of the meiotic metaphase-arrested oocytes of *Chaetopterus* decreased linearly at a rate of 1.5 nm/min when perfused with PEG buffer without tubulin. In this hypotonic, calcium-chelating solution, the cell lysed within 1.5 min, and after a brief, transient rise, the BR disappeared in ca. 4 min from the time of buffer application. Cells perfused with tubulin in PEG buffer also showed BR decay at the same rate until cell lysis. Immediately upon cell lysis the spindle BR increased, initially at ca. 2.3 nm/min and then more slowly until the BR attained or exceeded intact cell values. Spindle and asters grew considerably larger than those in intact cells. From the kinetics of the transient BR increase after lysis, we infer that, initially, *Chaetopterus* cytoplasmic tubulin contributes to increased BR; further augmentation required added pig brain tubulin and most probably reflects the addition and incorporation of heterologous porcine tubulin into the spindle and asters. Isolated, augmented spindles depolymerized rapidly at 6° C. Upon return to 23° C, spindle BR returned slowly in tubulin-PEG. The BR of the isolates also decayed in solutions containing calcium ions 2.5 mM in excess of the EGTA. However, the isolates did not respond, or responded very slowly, to 1 mM colchicine or Colcemid and to dilution of tubulin with PEG solution. Microinjection into *Chaetopterus* oocytes of tubulin-PEG, but not PEG alone, enhanced spindle and aster BR which reversibly disappeared upon chilling the cell.

INTRODUCTION

Studies on living cells by polarized-light microscopy have led to the concept that the fibrous elements of the mitotic spindle are in dynamic equilibrium with subunits (1, 2). Fibers of the mitotic spindle lose their birefringence (BR) when living cells are exposed to cold, hydrostatic pres-

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sure, colchicine, and several other antimitotic alkaloids (see references 3 and 4 for reviews). These agents reversibly depolymerize microtubules of the spindle fibers in vivo and prevent chromosome movement upon the eradication of BR, indicating destruction of the tubules. Fiber BR, spindle organization, and chromosome movement recover after a brief delay when the spindle-disrupting agent is removed.

Earlier efforts to isolate the mitotic spindle have used stabilizing reagents such as ethanol (5) or glycols (6–9). In contrast to their lability in intact cells, spindles isolated by these procedures have generally been unresponsive to microtubule disruptive agents. Attempts to isolate the mitotic spindle in near physiological solutions have failed, presumably in part because in lysate of cells, the microtubule protein (tubulin) concentration is lowered and the equilibrium favors breakdown of microtubules into subunits.

With the development of conditions for the assembly of microtubules in vitro (10, 11) and for the purification of microtubule protein in a state competent to polymerize (12, 13), it became possible to reexamine the isolation of the mitotic spindle in aqueous solutions supplemented with tubulin.

In this paper[1] we describe the augmentation and growth with purified pig brain tubulin of the metaphase-arrested meiotic spindles of *Chaetopterus* oocytes under conditions which otherwise disintegrate the *Chaetopterus* spindle. The heterologous tubulin is applied to spindles by hypotonically lysing the cell in tubulin-containing solutions or by microinjecting the solution into intact oocytes. The augmented spindle is cold-labile and depolymerizes with elevated calcium concentrations. It is so far unresponsive to colchicine or Colcemid, or to tubulin dilution.

MATERIALS AND METHODS

Preparation of Tubulin

Tubulin was purified from porcine brain tissue[2] by two cycles of a reversible, temperature-dependent assembly procedure as described elsewhere (13). The purified material consisted of two components: 79% of the protein was tubulin and 17% was a high molecular weight species. The purified, polymerized microtubules were sedimented at 39,000 *g* for 0.5 h at 37°C to form a pellet, frozen in liquid nitrogen, and stored at −80°C. The frozen protein was stable at −80°C for at least a month. Frozen pellets were thawed before use and resuspended in PEG solution (0.1 M PIPES, 1 mM EGTA, 2.5 mM GTP, pH 6.9)[3] at 0°C to give protein concentrations between 5 and 12 mg/ml. Protein concentration was determined by refractometry and was checked by the procedure of Lowry et al. (15) using bovine serum albumin as a standard. Tubulin solutions were warmed to 22°C immediately before perfusion of the oocytes. In some experiments solutions containing 0.23 M PIPES (0.23 M PEG) or 0.5 M PIPES (0.5 M PEG) were used.

Preparation of Oocytes

Chaetopterus oocytes were obtained by clipping parapodia of mature females in MBL artificial seawater (Marine Biological Laboratory, Woods Hole, Mass.) (17). After two washings of the oocytes by suspension and sedimentation in artificial seawater, they were kept until use in finger bowls on a running seawater table. Germinal vesicles which persisted under this condition were induced to break down and form the first meiotic spindle by introducing the oocytes into filtered natural seawater approximately 30 min before use of the cells. Spindles were maintained in these metaphase-arrested oocytes for over 10 h and could be activated to continue their maturation divisions upon fertilization.

Perfusion Procedure

A perfusion chamber was constructed by placing a drop of cell suspension on a slide and applying a cover slip which rested at one end on two strips (2 × 10 mm) of 1-mil thick polyethylene film (Fig. 1). The edges of the cover slip parallel to the direction of flow were supported and sealed with Tackiwax (Cenco Corp., Chicago, Ill.). Perfusing solutions were applied between the polyethylene strips and fluid was drawn through the wedge chamber with a strip of filter paper. A line drawn across the top of the cover slip (with a wax pencil before assembly of the chamber) at the exit side prevented solution from creeping on to the top of the perfusion chamber. Slides and cover slips were previously cleaned by sonication in Alconox solution followed by successive rinses in tap water and distilled water. The wedge chamber provided a simple solution to the problem of attaining uniform perfusion. The wedge design also served to hold the oocytes in place, and to achieve a range

[1] A preliminary report (14) of this work was presented at the general meeting of the Marine Biological Laboratory, Woods Hole, Massachusetts. Experiments conceptually related to those reported here have also been carried out by Cande et al. using rat kangaroo cells (23) and by Rebhun et al. using *Spisula* oocytes (24, 25).

[2] Solutions and brain extracts were prepared as described in reference 16.

[3] *Abbreviations used in this paper:* ethylene bis-(oxyethylenenitrilo)tetraacetate, EGTA; guanosine triphosphate, GTP; piperazine-*N*-*N*'-bis(2-ethane sulfonic acid), PIPES.

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of flattening of the cells. Controlled flattening of the oocytes displaced the highly birefringent yolk granules and rendered the mitotic spindle clearly visible by polarized-light microscopy. Individual cells were followed throughout a perfusion experiment. After the experiment, the entire slide was scanned to check for uniformity of response.

Microscopy

Birefringence observations were made on a Leitz Ortholux microscope equipped with Nikon rectified lenses and a Glan-Thompson polarizer. Monochromatic, 546-nm illumination was provided by a high-intensity mercury-arc source equipped with an interference filter. Retardation was measured by extinguishing the central portion of the half-spindle with a Brace-Köhler compensator. The temperature of the specimen was controlled with a stress birefringence-free system described elsewhere (18).

OBSERVATIONS

Response of Oocytes to PEG Solution

The meiosis I spindle in the *Chaetopterus* oocyte is arrested at metaphase, and in seawater at 18–20°C its BR remains stable for several hours.

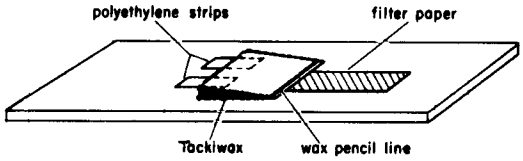

FIGURE 1 Diagram of wedge chamber used for perfusion experiments.

Oocytes were first perfused with a 19:1 mixture of sodium and potassium chlorides isotonic to sea water to reduce the concentration of divalent cations (19). In this solution, the size, shape, and BR of the spindle were not appreciably altered for several minutes. However, when oocytes were perfused with the hypotonic, calcium-chelating PEG solution, the oocyte swelled and the spindle BR decayed (Figs. 2, 4 *a–c*, 6 *a*, *b*). During BR decay, the fibers became less sharp, and the spindle swelled and became shorter by approximately 15%.

The BR decayed linearly with time at a rate of 1.5 nm/min for approximately 1.5 min until the cell lysed (Fig. 2). At the instant of lysis the BR ceased to decay and for a brief period often exhibited a transient rise during initial exposure of the cell interior to the PEG solution. At this stage the cytoplasm of the swollen cell, flattened by the cover glass, flowed out, often carrying with it the weakly birefringent spindle. Within a short period (<4 min) the spindle BR decayed further and disappeared altogether.

In solutions of higher tonicity the oocytes lysed after a longer period (in 0.23 M PEG) or did not lyse at all (0.5 M PEG). In these cases, the spindle BR did not decay appreciably and, in several instances, showed some gain. In PEG solution made approximately isotonic to seawater by the addition of 4 vol 0.85 M sucrose to 1 vol 0.5 M PEG, spindle BR also did not decay. Therefore, the decrease of spindle BR in the hypotonic PEG solution was probably associated with the influx of water. Although the patterns of behavior of oo-

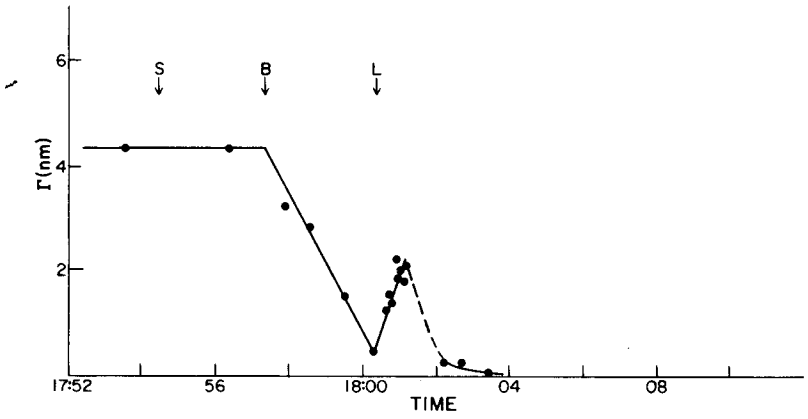

FIGURE 2 Change in spindle birefringence (BR) in a *Chaetopterus* oocyte upon exposure to PEG solution without tubulin. *S*, perfusion with 19:1 isotonic monovalent salt solution started; *B*, perfusion with PEG buffer started; *L*, time at which cell lysed. Ordinate, spindle retardation (Γ) in nm; abscissa, clock time in hours and minutes.

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cytes and spindles in these solutions are of interest in themselves, the effect of these solutions will not be discussed further since we are primarily concerned with the ability of tubulin to preserve and augment spindles which otherwise disintegrate spontaneously.

Spindle Response to Tubulin-PEG

When *Chaetopterus* oocytes were perfused with PEG solutions containing 5–12 mg/ml pig brain tubulin, they responded as to PEG without tubulin until the time of lysis. After lysis, however, the spindle increased in size and its BR continued to rise over the next several minutes (Figs. 3–7). The BR regained (Figs. 3, 7) and in several instances exceeded (Fig. 5) the values measured before exposure of the cells to PEG-tubulin solutions.

In overall shape and BR distribution, the augmented mitotic apparatus (see especially Fig. 8) more closely) resembled the first meiotic spindle in a grasshopper spermatocyte (reference 1, Fig. 15–18) than a typical *Chaetopterus* oocyte spindle (20). The half-spindle appeared more conical, with the fibers diverging radially from the spindle poles rather than curving gently towards the metaphase plate and exhibiting a characteristic roundish contour. In spite of this difference in overall morphology, all components of the spindle developed higher BR than at lysis. Together with increase in BR and diameter of the central spindle, the astral rays showed a striking increase in BR and length (Figs. 4, 6, and 8). The asters grew as long as or longer than the half-spindle. Chromosomal spindle fibers were clearly contrasted above the BR of the central spindle by adjusting the compensator orientation. The spindle itself increased in length relative to the untreated spindle by up to 20%, and by 35–40% relative to the spindle length at the instant of lysis.

Cold Lability of Tubulin-Augmented Spindles

The BR of the augmented spindle was reduced or abolished by chilling the preparation in a 6.5°C refrigerator for 6–8 min (Fig. 7). Although a 6-min treatment was just long enough to cool the slide to introduce a slight reduction in spindle BR, the BR of the tubulin wisps (probably bundles of free microtubules) surrounding the spindle was largely abolished by the treatment (cf. Fig. 6 *g* and *h*). Upon warming to room temperature, the BR of the augmented spindle and surrounding wisps recovered slowly.

The time-course of BR decay at 6.5°C and initiation of recovery at 22°C measured on a temperature control slide are illustrated in Fig. 10. The decay of BR was rapid and approximately linear, following a time-course similar to the BR decay of spindles accompanying similar cold treatment in intact *Chaetopterus* oocytes. Recovery, on the other hand, was slow, compared to that of the in vivo spindles whose rate of BR rise upon warming approximated the rate of BR decay after sudden chilling (21).

Fig. 9 shows the change in BR distribution upon chilling and rewarming the large augmented spindle whose retardation changes are plotted in Fig. 10. During early recovery, increase of BR at the two poles is especially prominent, although chromosomal spindle fibers and astral ray BR can readily be seen long distances from the poles.

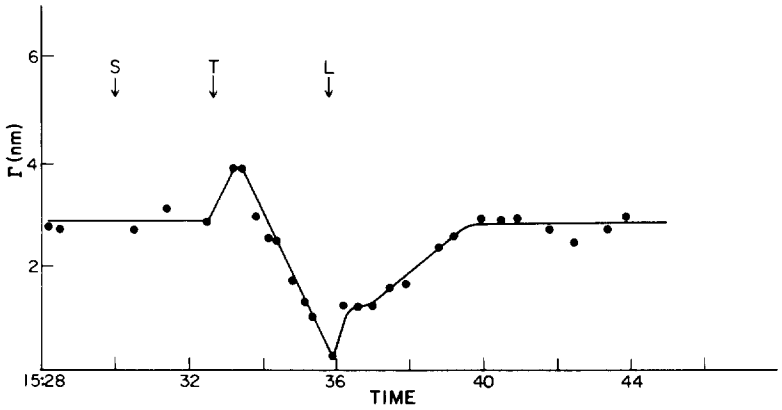

FIGURE 3 Change in spindle BR upon exposure of an oocyte to PEG containing 10 mg/ml tubulin. *T*, perfusion with PEG-tubulin started. Other details as in Fig. 2.

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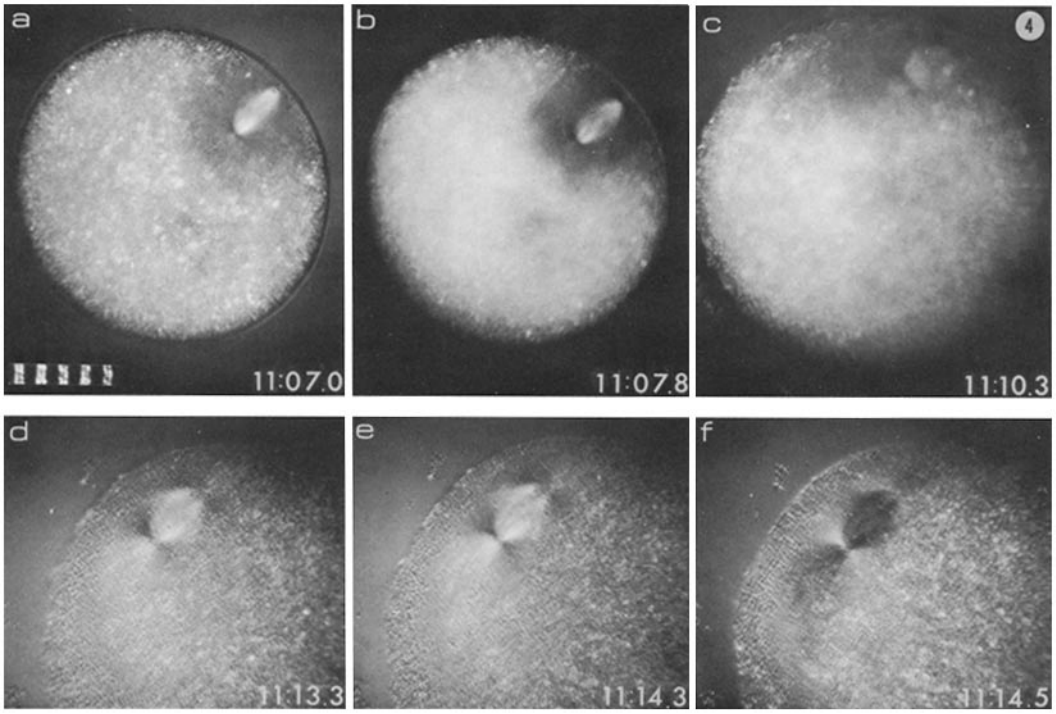

FIGURE 4 Response of *Chaetopterus* oocytes exposed to PEG solution containing 10 mg/ml tubulin. (*a*) In 19:1 NaCl-KCl solution. (*b*) Spindle BR dropping in tubulin-PEG. (*c*) Cell and spindle swollen and BR weak. (*d–f*) After lysis at 11:10.8, spindle and aster lengths and BR increasing. Crossed polarizers, compensator slow axis parallels spindle axis in (*a–e*), crossed in (*f*). Scale intervals (*a*) 10 μm; clock time in hours and minutes. × 300.

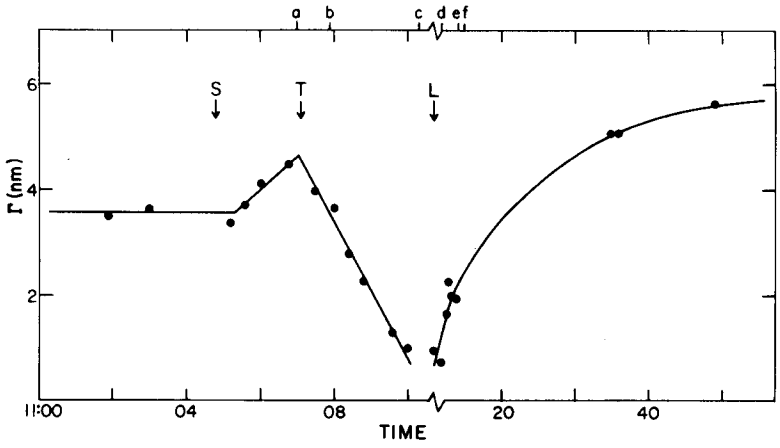

FIGURE 5 As in Fig. 3. Photographs (*a–f*) for this series shown in Fig. 4.

Sensitivity of the Augmented Spindle to Calcium Ions

Figs. 11 and 12 show the effect of increased calcium ion concentration on the augmented spindle. Perfusion with solutions containing calcium ions 2.5 mM above the EGTA concentration (1 mM) reduced the BR throughout the spindle. The BR decay followed a logarithmic time-course similar to that observed in intact cells during anaphase or as induced by colchicine at metaphase.

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Effect of Colchicine and Colcemid, and of Tubulin Dilution

Exposure of the augmented spindle to 1-mM and greater concentrations of colchicine or Colcemid did not appreciably reduce their BR for periods up to tens of minutes. By contrast, in intact *Chaetopterus* oocytes at 22°C the spindle BR was completely abolished in approximately 1 min by 5 mM Colcemid and in approximately 3 min by 0.5 mM colchicine (22). Similarly, the BR of the augmented spindle was not reduced upon dilution of the tubulin with PEG solution.

Augmentation of in vivo Spindle by Tubulin Microinjection

Chaetopterus oocytes injected with PEG solution alone showed a temporary reduction of spindle BR to about 75% of the original value. In

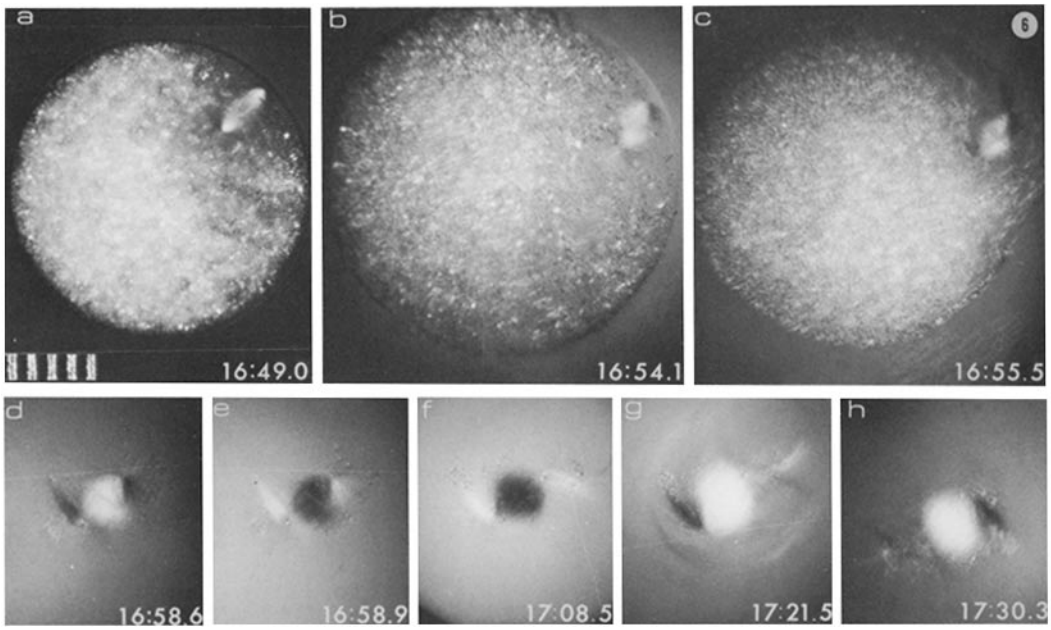

FIGURE 6 As in Fig. 4 but with mitotic apparatus well isolated from residual cytoplasm. × 265.

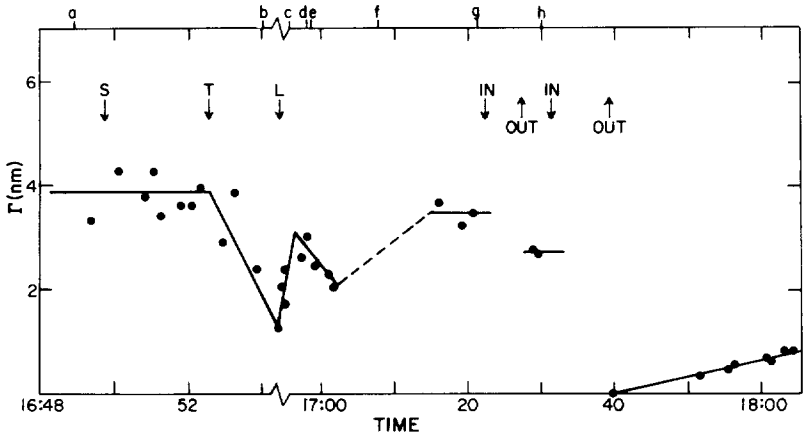

FIGURE 7 As in Fig. 3, 7 mg/ml tubulin. Preparation placed in and removed from 6.5°C refrigerator at times indicated by arrows. Photographs (*a–h*) for the series shown in Fig. 6.

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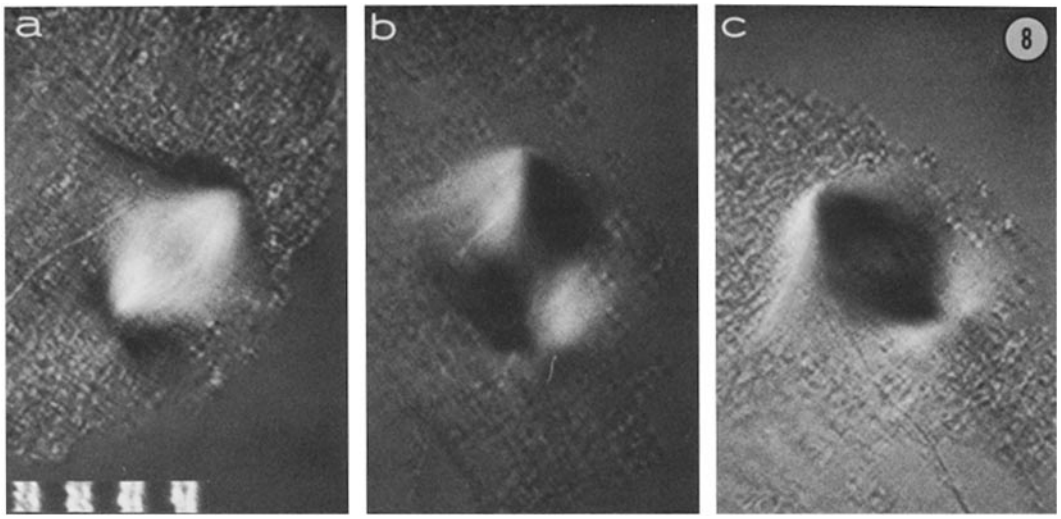

FIGURE 8 Structure and BR of isolated spindle augmented in 10 mg/ml tubulin. Spindle axis is oriented (*a*) parallel to slow axis of compensator; (*b*) parallel to polarizer transmission axis; (*c*) perpendicular to slow axis of compensator. Positive BR of chromosomal spindle fibers stand out in brighter or darker contrast against background spindle BR. Extensive astral rays at right-angles to spindle axis appear in reverse contrast. × 700.

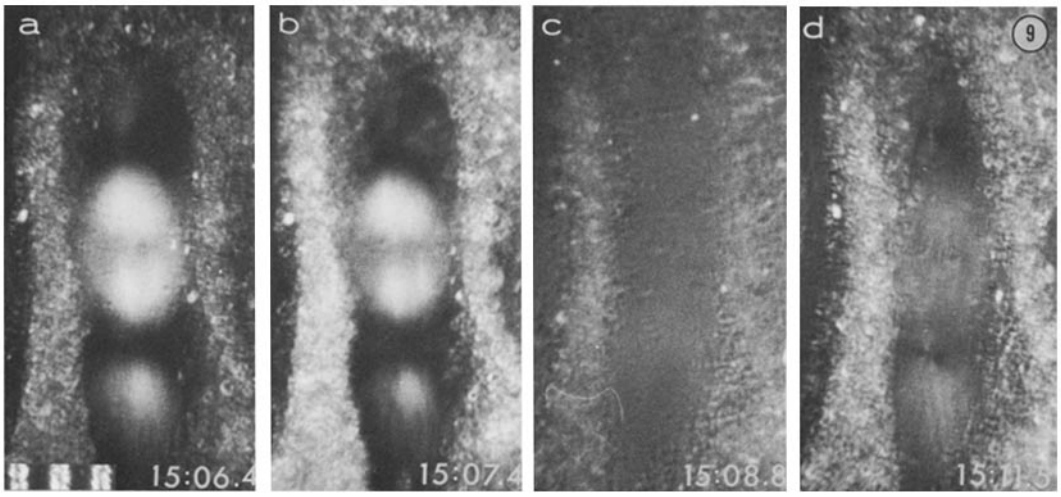

FIGURE 9 Cold depolymerization and repolymerization of isolated augmented spindle. A large spindle in PEG-tubulin (8 mg/ml) surrounded by expressed *Chaetopterus* cytoplasm. × 550.

approximately 10 min the spindle BR recovered to its original value.

When injected with PEG solution containing 10–12 mg/ml tubulin, the spindle and especially the asters, after the initial BR reduction, showed a striking increase of BR (Fig. 13 *a–e*). In these experiments the increased BR of the aster was enhanced further by chilling and rewarming the injected oocytes. In the cold (6.5°C), the spindle and aster BR disappeared as in the uninjected cells or the cells injected with buffer; upon raising the temperature, gigantic asters with very high BR were formed (Fig. 13 *f*). Cells injected with 8 mg/ml bovine serum albumin in PEG solution showed BR changes similar to those of cells injected with buffer alone.

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DISCUSSION

Comparison of oocytes lysed in PEG solution containing tubulin and in PEG solution alone led us to conclude that the presence of tubulin subunits prevents dissolution of the otherwise labile spindle.

In eggs exposed to PEG solution, spindle BR decayed rapidly until the moment of cell lysis. The cells swelled considerably in the hypotonic PEG solution and the initial BR decay may result, in part, from dilution of free cellular tubulin by influx of water, shifting the spindle equilibrium from the birefringent microtubules to the nonbirefringent tubulin subunits. However, unlike oocytes treated with colchicine or cold, in PEG-treated cells the spindles themselves showed considerable swelling, so that depolymerization of microtubules may not alone account for decrease of their BR.

During the transient BR rise after lysis the asters often increased in length beyond the value observed before lysis. We infer that this transient augmentation of spindle and aster BR in cells perfused with PEG solution alone reflects the polymerization of microtubules from homologous *Chaetopterus* tubulin. The initial increase in spindle and aster BR in cells perfused with PEG solution containing tubulin probably originates from the same cause.

In contrast to the transient rise, further augmentation of BR and growth of the spindle and asters occurred only in the presence of exogenous pig brain tubulin. Therefore, this second phase of spindle augmentation and growth most probably reflects the addition and incorporation of heterologous porcine tubulin into the spindle and asters.

In the augmented spindle, we observe growth and BR rise of the astral rays and there is indication of continuous and chromosomal fiber growth. The spindle grows in width and length, and chromosomal fiber BR remains clearly distinguishable above the generally increased BR of the central spindle region. Determination of the zone(s) of microtubule growth in these various spindle regions is of potential theoretical interest.

The augmented spindle retained its cold lability and sensitivity to concentrations of calcium ion which depolymerize microtubules in vitro (16). However, the spindle did not respond to colchicine or Colcemid, or to dilution of the tubulin by PEG solutions. Microtubules formed in vitro from purified tubulin are only partially broken down by

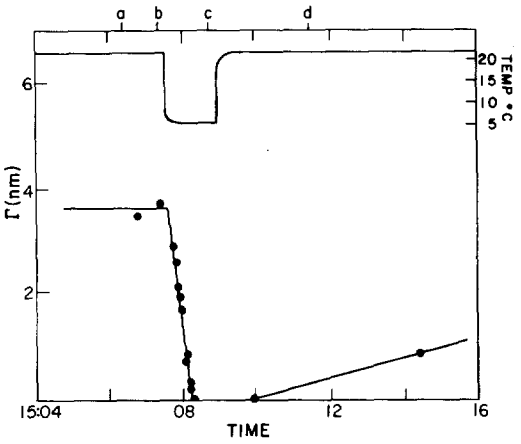

FIGURE 10 BR of augmented spindle followed on temperature control slide. Photographs (*a–d*) for this series shown in Fig. 9.

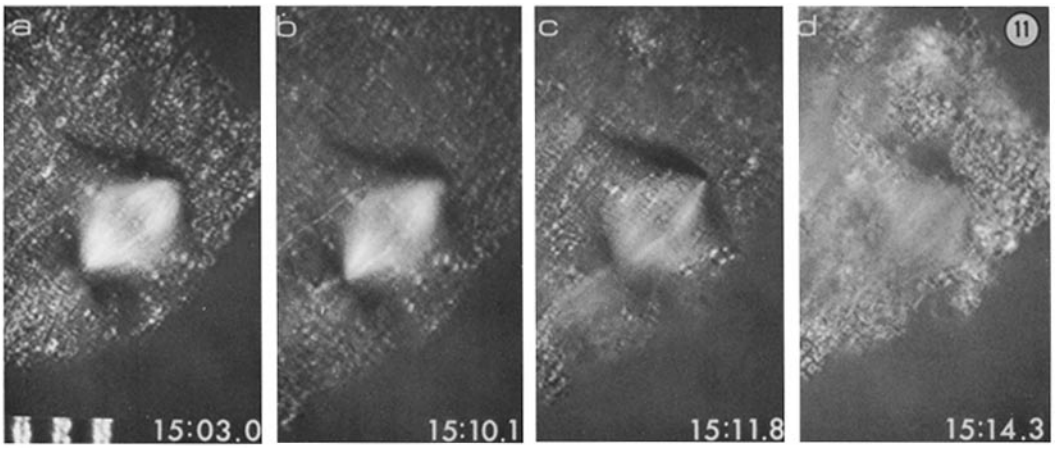

FIGURE 11 Dissolution by calcium ion of (10 mg/ml tubulin) augmented mitotic apparatus. Spindle was exposed at 15:11.0 to PEG solution containing 3.5 mM $CaCl_2$. × 525.

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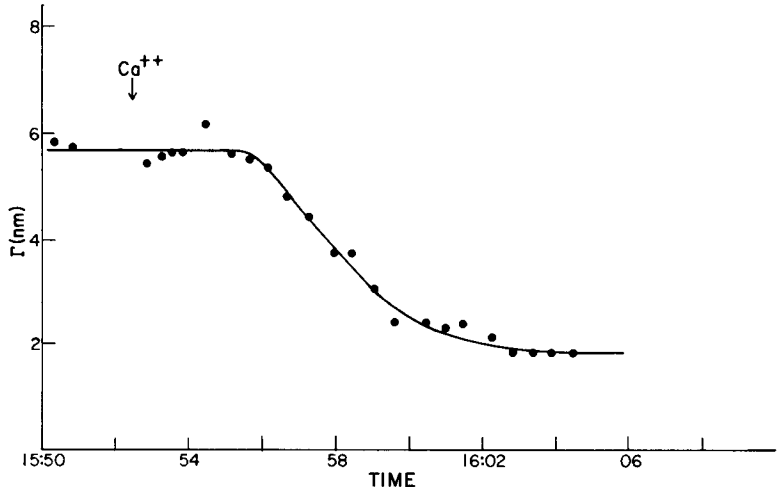

FIGURE 12 Reduction of augmented spindle BR by calcium ion. Spindle was exposed to PEG solution containing 3.5 mM $CaCl_2$ at time denoted by arrow.

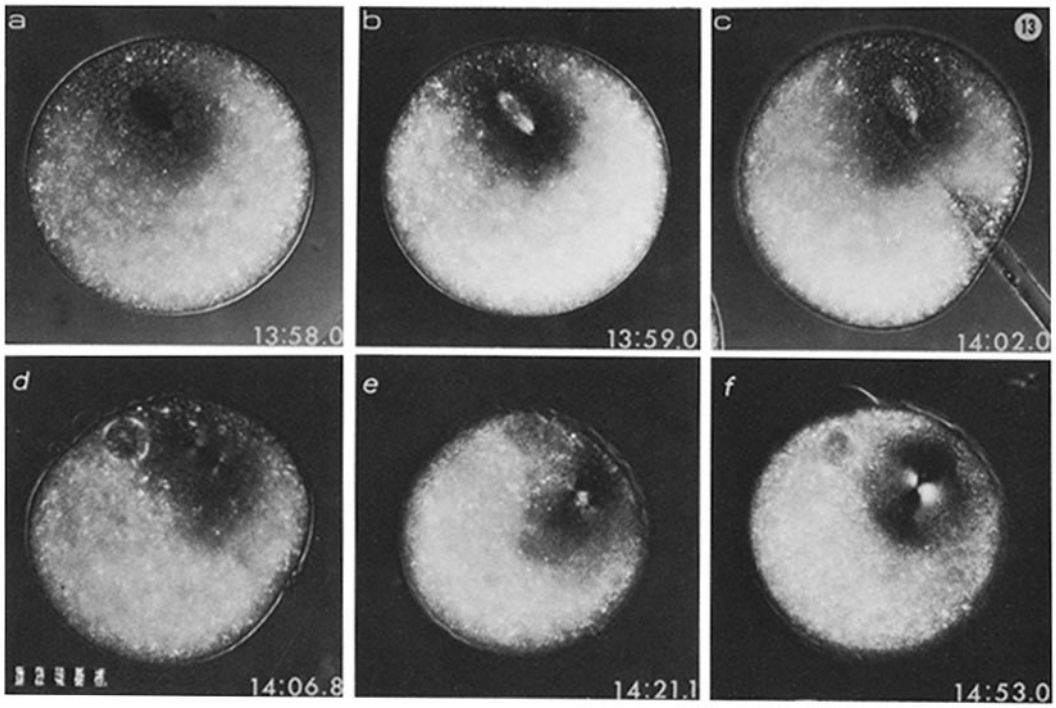

FIGURE 13 Microinjection of 12 mg/ml tubulin in PEG into *Chaetopterus* oocyte. (*a*, *b*) Oocyte before injection, spindle BR ca. 2.5-nm retardation; (*c*) oocyte impaled by micropipette containing tubulin and Wesson oil (Hunt-Wesson Foods, Inc., Fullerton, Calif.); (*d*) after injection completed at 14:02.6, oil drop near spindle demonstrates injection of material; (*e*) spindle and aster BR increased to 4.1 nm; (*f*) further increase in aster BR (max 7.0 nm) after chilling to 6.5°C at 14:24.8 and recovery at 22°C at 14:30.8. × 265.

colchicine (Olmsted and Borisy, unpublished observations); therefore, the insensitivity of the augmented spindle may in part reflect properties of the pig brain tubulin. Alternatively, the insensitivity to dilution and the very slow rate of BR recovery after cold treatment may indicate that we have not yet discovered sufficient conditions for obtaining native spindles in vitro. Further microinjection

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studies hopefully will aid in clarifying the latter point.

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