EXPERIMENTAL MANIPULATION OF THE AMOUNT OF TUBULIN AVAILABLE FOR ASSEMBLY INTO THE SPINDLE OF DIVIDING SEA URCHIN EGGS

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ABSTRACT

Spindle assembly is studied in the eggs of the sea urchin *Lytechinus variegatus* by experimentally varying the amount of polymerizable tubulin within the egg. Aliquots of fertilized eggs from the same female are individually pulsed for 1-6 min with 1 × 10⁻⁶ M Colcemid at least 20 min before first nuclear envelope breakdown. This treatment inactivates a portion of the cellular tubulin before the spindle is formed. Upon entering mitosis, treated eggs form functional spindles that are reduced in length and birefringent retardation but not width. With increased exposure to Colcemid, the length and retardation of the metaphase spindles are progressively reduced. Similar results are obtained by pulsing the eggs with Colcemid before fertilization, which demonstrates that the tubulin found in unfertilized sea urchin eggs is later used in spindle formation. Spindles, once assembled, are responsive to increases in the amount of polymerizable tubulin within the cell. Rapid increases in the amount of polymerizable tubulin within a Colcemid-treated cell can be experimentally effected by irradiating the cells with 366-nm light. This treatment photochemically inactivates the Colcemid, thereby freeing the tubulin to polymerize. Upon irradiation, the small prometaphase spindles of Colcemid-treated cells immediately increase in length and retardation. In these irradiated cells, spindle length and retardation increase as much as four times faster than they do during prometaphase for normal spindles. This suggests that the rate of the normal prometaphase increase in retardation and spindle size may be determined by factors other than the maximum rate of tubulin polymerization in the cell.

Assembly of the spindle is a key event in cell division. Aligned microtubules are important structural elements of the spindle and give the spindle its characteristic birefringence (14, 22, 29, 35, 38). Inoué's observations that the spindle birefringence could be reversibly abolished with cold (18) or colchicine (17) led him to propose that spindle fibers are labile and exist in a "dynamic" equilibrium with a pre-existing pool of unoriented subunits (18, 19, 20, 21). According to this model, the quantity of aligned microtubules in the spindle is determined by the equilibrium constant of the assembly reaction and by the size of the tubulin pool. Evidence for this model has been provided by a number of studies using temperature (8, 9, 19, 22, 34) or high hydrostatic pressure.
the cellular tubulin is separated in time from the important advantages: (a) the time at which a portion of the cellular tubulin is inactivated is discrete and known. (b) Since the inactivation of Colcemid is only slowly lost when the cells are in drug-free medium (40). This approach has several advantages: (a) the time at which a portion of the cellular tubulin is inactivated is discrete and known. (b) Since the inactivation of the cellular tubulin is separated in time from the assembly of the spindle, the spindle microtubules are built in relation to a fixed but reduced pool of subunits. This eliminates the complication of continued inactivation of the cellular tubulin while the spindle is being assembled, as would be the case if the eggs were continuously immersed in Colcemid. (c) When the Colcemid in treated cells is photochemically inactivated by 366-nm light as described by Aronson and Inoue (1), the cellular tubulin cannot be bound again by external drug. Thus, irradiations with 366-nm light can be used to give rapid, stable increases in the quantity of polymerizable tubulin within the living cell.

In this paper, I present evidence that the size and retardation of the spindle can be reduced by inactivating a portion of the cellular tubulin before mitosis. These reduced spindles are functional and are immediately responsive to increases in the amount of polymerizable tubulin within the cell.

MATERIALS AND METHODS

*Lytechinus variegatus* (Gulf Specimen Co., Inc., Panama, Fla.) were maintained in modified Instant Ocean seawater before use. For each series of experiments, eggs were repeatedly obtained from a single female by intracoelomic injection of 0.5 M KCl as described by Fuseler (12). Sperm were taken “dry” from excised testes. Eggs were fertilized and allowed to develop in artificial seawater (10). All experiments were performed at 21–22.5°C.

Eggs were treated (“pulsed”) with Colcemid (Ciba Corp., Summit, N.J.) by gently centrifuging them in a 15-ml conical centrifuge tube and then resuspending them with 10-4 M Colcemid in artificial seawater for the desired time. The treatment was terminated by centrifuging the eggs and resuspending them three times in fresh artificial seawater. This should wash out the free intracellular Colcemid, leaving only the drug bound to the cells (4). *Lytechinus variegatus* eggs treated in this fashion recover very slowly from the drug treatment; they show slight, if any, recovery for at least 1 h from a Colcemid treatment given 20 min before the first nuclear envelope breakdown. Increasing the number of washes did not alter the observed results. This is consistent with the findings that colchicine bound to cells is slowly lost when the cells are placed in drug-free medium (40). The amount of time of recovery from Colcemid varies with cell type (see 5, 22, 25, 40).

For observation, a drop of egg suspension was placed on a clean 18 x 18 mm cover slip which was then inverted and put on a 22 x 40 mm cover slip bearing a drop of FC-47 fluorocarbon oil (3M Co., St. Paul, Minn.). A square of Teflon tape (about 0.0035 inch thick) with the center cut out served to contain the oil and act as a spacer. This gently flattens the eggs between the 18 x 18 mm cover slip and the nontoxic oil. This preparation was sealed with a mixture of Vaseline, lano-
directly from 35-ram negatives. Since the exact position
tor. determined from the rotational angle of the compensa-
tion were consistently about 1 nm higher than those
cal density of the egg cytoplasm next to the spindle
tometer (Heath Co., Benton Harbor, Mich.). The opti-
values were determined by a Heathkit “Colorval” pho-
microscopy. An Olympus PO 40 × (NA 0.65) was used
objective was used as the condenser. A Brase-Kohler
compensator (E. Leitz, Inc., Rockleigh, N.J.) was used
to measure the birefringent retardation of the spindle. A
200-W mercury arc bulb (Illumination Industries, Inc.,
Sunnyvale, Calif.) in a Zeiss lamphousing was used as
the light source. For observations, two Corning no. 4602
heat-absorbing filters (Corning Glass Works, Science
Products Div., Corning, N.Y.) enclosing a 3.8-cm column
of 5% CuCO₃ in water were used in conjunction
with a 546-nm interference filter (Baird Atomic, Inc.,
Bedford, Mass.). To irradiate the cells with 366-nm
light, the polarizer (Polaroid unlaminated HN-22, Pola-
roid Corp., Cambridge, Mass.) was removed, and the
546-nm filter was replaced with a Zeiss UG-1 filter (Carl
Zeiss, Inc., N.Y., N.Y.). The intensity of the 366-nm
light at the specimen was estimated to be 1.8 × 10⁴
quanta/s/µm² with an Eppley thermopile (Eppley Labo-
ratory, Inc., Newport, R.I.)

For a given treatment, between 5 and 10 cells were
photographed at metaphase. For each cell, the retar-
dation of the spindle was determined as the mean of several
individual measurements of the region midway between
the pole and the metaphase plate. Spindle retardation
and size, taken together, provide a good measure of the
total amount of microtubules in the spindle (14, 22, 31,
35, 38). The retardation values for the metaphase spin-
dles were determined directly from the rotational angle
of the compensator (15, 35). In the case of the 366-nm
irradiated cell shown in Figs. 6 and 7, the spindle retar-
dation was obtained from densitometric analysis of pho-
tographs on Kodak Plus-X film developed in Kodak
Microdol X (Eastman Kodak Co., Rochester, N.Y.). At
a fixed value of positive compensation, the density of the
spindle image is a function of the retardation of the
spindle plus scattered light. Retardation values were
obtained from the film by subtracting the optical density
of the background from the optical density of the spindle
and comparing this remainder to optical densities of the
film for known values of compensation. Optical density
values were determined by a Heathkit “Colorval” pho-
tometer (Heath Co., Benton Harbor, Mich.). The opti-
cal density of the egg cytoplasm next to the spindle
showed that the retardation values obtained in this fash-
ion were consistently about 1 nm higher than those
determined from the rotational angle of the compensa-
tor.

Spindle length and width measurements were taken
directly from 35-mm negatives. Since the exact position
of astral foci was difficult to determine accurately in the
Colcemid-treated cells, spindle length was measured as
the length of the birefringent central spindle instead of the
full aster-to-aster distance.

RESULTS

Colcemid Treatment 20 Min before First
Nuclear Envelope Breakdown

Fertilized eggs were treated for 1–6 min with 1 × 10⁻⁶ M Colcemid 30 min after fertilization
(early prophase of first mitosis). About 20 min later, first nuclear envelope breakdown occurred,
and the cells formed functional spindles that were significantly reduced in length and retardation but not width
(Fig. 1). During mitosis, these diminished spindles showed the same pattern of retardation changes as the
control spindles. Spindle retardation increased during prometaphase, reached a peak at anaphase
onset, and then rapidly decayed during late anaphase. Although anaphase onset was slightly de-
layed in these diminished spindles, cleavage fur-
rows were initiated and separate daughter nuclei were formed. The extent of the delay in anaphase
onset depended upon the size of the spindle; the
very small spindles produced by longer Colcemid
treatments were sometimes delayed by as much as
5–7 min (36). Cleavage was generally complete in
eggs treated with Colcemid for 2 min or less. In
eggs treated for longer times, the smaller the spind-
le, the less the cleavage furrow constricted the
egg before regressing. With the smallest spindles
(5- to 6-min Colcemid treatment), no furrowing
was observed. Thus spindle size appears to deter-
mine the “strength” of the cleavage furrow in
these eggs.

To quantitate the relationship between the du-
ration of the drug treatment and the amount of
microtubular material in the metaphase spindle,
eggs were repeatedly obtained from the same fe-
male and pulsed for different durations with 1 × 10⁻⁶ M Colcemid about 20 min before the first
nuclear envelope breakdown. One batch of ferti-
ilized eggs served as the untreated controls, and
each of the other batches was pulsed for 1–6 min.
Individual cells were followed from mid-prometa-
phase to early anaphase. Due to possible changes
in the time of anaphase onset, only the photo-
tographs and retardation measurements taken 2 min
before the cell was observed to start anaphase
were used for analysis. This ensured that the spin-
dles were measured at equivalent points in mito-
sis. The results were not dependent upon a partic-
Figure 1 Mitosis in the fertilized egg of *Lytechinus variegatus* as seen by polarization microscopy. The upper sequence shows the spindle of a normal cell. The lower sequence shows the diminished spindle of a cell immersed for 2.5 min in $1 \times 10^{-4}$ M Colcemid 20 min before nuclear envelope breakdown. Minutes after nuclear envelope breakdown are indicated in the lower corner of each frame. 10 μm per scale division, additive compensation. × 960.

ular Colcemid concentration, i.e. identical results were obtained by using higher Colcemid concentrations for shorter times. They also were not unique to first division eggs; the same treatments produced similar results in second division cells.

The appearance of typical metaphase spindles for each of the different Colcemid treatments is shown in Fig. 2. With increasing treatment duration, there is a progressive reduction in metaphase spindle length with little change in spindle width. This gives spindles the appearance of becoming more and more “clipped off” to the extent that 6-min treatments give spindles that are often wider than long. The size and retardation of the asters also decrease progressively with lengthening drug treatments to a point where the asters are barely detectable with the optical system used here. Furthermore, the asters do not maintain a normal separation but instead get closer to the metaphase plate as the central spindles get shorter. The chromosomal fibers appear more distinct than in the controls; with longer treatments, distinct regions of low retardation surround these chromosomal fibers (see Fig. 2 d, e, and Fig. 7). This may be the result of a preferential loss of nonchromosomal spindle fibers. The average length, width, and retardation of these spindles are shown as a function of pulse duration in Fig. 3. The length and retardation of the metaphase spindle decrease with longer drug treatments while spindle width changes little over the entire range. Some drug-treated eggs have spindles that are even wider than those of the controls. The reasons for this are not clear but may result from a loosening of the overall spindle structure due to the reduction in numbers of astral and nonchromosomal fibers.

Eggs treated longer than 6 min with Colcemid do not form spindles at division. If treated for 7–8
min, only a small hyaline zone forms at the time of nuclear envelope breakdown which then becomes diffusely birefringent over the next 5–10 min. Short rods or “fibers” with a retardation of about 1 nm are often seen within this weakly birefringent area. Longer drug treatments prevent the formation of any birefringent structure after nuclear envelope breakdown.

**Colcemid Treatment before Fertilization**

To determine the earliest time at which the tubulin pool used in spindle assembly can be inactivated, aliquots of eggs from the same female were treated for 3–7 min with $1 \times 10^{-6}$ M Colcemid before fertilization. 5 min after treatment, the eggs were fertilized and allowed to develop in drug-free artificial seawater.

The appearance of typical metaphase spindles from control and drug-treated cells is shown in Fig. 4. The average length, width and birefringent retardation of these spindles are shown in Fig. 5. Spindle length and retardation are again observed to decrease with increasing Colcemid pulse duration while spindle width remains constant or may even increase slightly. There is a practical limit to the extent that the metaphase spindle can be diminished by treating the egg before fertilization. Treatments longer than 7 min block pronuclear fusion, thereby preventing normal development.

**Photochemical Inactivation of the Colcemid**

Fertilized eggs were treated for 1 min with $3 \times 10^{-6}$ M Colcemid approx. 20 min before first nuclear envelope breakdown. These cells formed spindles of reduced length and retardation. Several minutes after nuclear envelope breakdown, the cells were individually irradiated for 15 s with 366-nm light ($1.8 \times 10^{14}$ quanta/s/µm²). Upon irradiation, the spindles of such prometaphase cells increase rapidly in length and retardation with only a slight increase in width (Figs. 6 and 7). The rates of length and retardation increases for this spindle are approx. 4.0 µm/min and 2.6 nm/min, respectively. The drop in spindle retardation after the initial rapid rise as seen in Fig. 6 is often observed in such irradiated cells. Fig. 8 shows two other typical examples of this phenomenon. Here the retardation values were obtained from the rotational angle of the compensator in the same way as those of spindles represented in Figs. 3 and 5. Comparison of these figures shows that, within a minute of irradiation, spindle retardation goes

**Figure 2** Metaphase spindles of eggs treated for various durations with $1 \times 10^{-6}$ M Colcemid. The eggs were pulsed with Colcemid 20 min before first nuclear envelope breakdown for (a) 0 min, (b) 1 min, (c) 2 min, (d) 3 min, (e) 4 min, (f) 5 min, and (g) 6 min. These eggs were all taken from the same female. Polarization micrographs, additive compensation, 10 µm per scale division. × 790.

**Figure 3** Average length, width, and birefringence (BR) of metaphase spindles as a function of Colcemid pulse duration. The eggs were pulsed with $1 \times 10^{-6}$ M Colcemid 20 min before first nuclear envelope breakdown. Each point represents the mean of values from at least five spindles. All eggs were from the same female.
FIGURE 4 Metaphase spindles of eggs treated before fertilization for various durations with $1 \times 10^{-6}$ M Colcemid. Pulse durations were for (a) 0 min, (b) 3 min, (c) 4 min, (d) 5 min, and (e) 7 min. All eggs were from the same female, except the cell pulsed for 4 min (frame c). Polarization micrographs, additive compensation 10 $\mu$m per scale division. $\times$ 970.

FIGURE 5 Average length, width, and birefringence of metaphase spindles as a function of Colcemid pulse duration. The eggs were treated with $1 \times 10^{-6}$ M Colcemid before fertilization. Each point represents the mean of values from at least five spindles. All eggs were from the same female.

even higher than the metaphase retardation of untreated cells. Spindle retardation may then drop 15–35% from peak values over a period of 2–3 min. This does not necessarily reflect a loss in the total quantity of oriented microtubules; spindle length is increasing during this period, probably leading to a redistribution of existing microtubules.

DISCUSSION

In these experiments the amount of polymerizable tubulin in the cell is directly manipulated by means of brief Colcemid treatments and irradiations with 366-nm light. Inactivation of a portion of the cellular tubulin before mitosis reduces the length and retardation of spindles that are later formed. With increased exposure to Colcemid the length and retardation of the metaphase spindles are progressively reduced. Although diminished, such spindles are functional and the cells complete mitosis. Irradiation of treated cells with 366-nm light photochemically inactivates the bound Colcemid, thereby quickly increasing the amount of polymerizable tubulin in the living cell (1). Such irradiations produce an immediate and rapid increase in the length and retardation of the spindles in drug-treated cells. These results support Inoué’s initial assumption that spindle fibers are in a “dynamic” equilibrium with a pool of unpolymerized subunits (18, 19, 22).

Experimental manipulations of this subunit pool need not be done at the time of mitosis in order to affect the spindle. Colcemid treatments administered even before fertilization reduce the amount of microtubular material in spindles that are formed later. This indicates that the tubulin found in unfertilized sea urchin eggs by biochemical techniques (26, 27, 28) does, in fact, participate in spindle formation. This does not rule out a contribution to spindle assembly by postfertilization synthesis of tubulin as some have suggested (24, 27), but shows that this synthesis is not the primary means by which the pool of polymerizable subunits is formed for the first divisions. Interestingly, longer Colcemid treatments are needed before fertilization than at 20 min before nuclear envelope breakdown to produce equivalent reductions in the amount of spindle microtubules. This could be due to either a contribution to the tubulin pool by protein synthesis, a partial recovery from the drug, or a reduced ability of the tubulin in unfertilized eggs to bind Colcemid.

Although the results of these experiments confirm Inoué’s assumption that the subunit pool ex-
ists before spindle assembly (18, 19), they should be put in context with present knowledge of the control of spindle assembly. In these eggs, the amount of microtubules in the spindle varies in a stage-specific fashion during the cell cycle. The mechanisms that determine the time-course and extent of their polymerization are still poorly understood. The results of this study indicate that the normal prometaphase increase in the amount of oriented microtubules must be determined by factors other than the maximum rate of tubulin polymerization in the cell. For the irradiated spindle shown in Fig. 7, the length and retardation increase at approx. 2.6 nm/min and 4.0 μm/min, respectively. This is four times faster than the normal prometaphase increases in retardation and length of first division spindles (approx. 0.7 nm/min and 1 μm/min, respectively). The overshoot in spindle retardation after irradiation suggests that the polymerizable tubulin is rapidly incorporated into microtubules as soon as it is available and that spindle length reaches its equilibrium more slowly. These results are similar to the observation of Salmon that changes in spindle length lag changes in spindle retardation after rapid shifts in hydrostatic pressure (33).

There is evidence that dividing marine eggs control the amount of cellular tubulin which is competent to polymerize. Even before fertilization, sea urchin eggs contain at least three to five times the amount of tubulin that is polymerized into the microtubules of the first division metaphase spindle (11, 28). Also, during mitosis the number of microtubules in the spindle can be dramatically increased by immersing the cells in seawater containing D₂O (22, 39) or glycols (30). This augmentation must come from the utilization of previously un polymerized tubulin since the effects are rapid, reversible, and occur in the virtual absence of protein synthesis (22, 30).

An elegant demonstration that sea urchin eggs can control the amount of tubulin used in spindle

![Figure 6](image-url)

**Figure 6** Typical spindle growth in a Colcemid-treated egg upon irradiation with 366-nm light. Spindle length, width, and birefringence are shown as a function of time before and after irradiation. The egg was treated for 1 min with 3 × 10⁻⁸ M Colcemid 20 min before first nuclear envelope breakdown. During prometaphase, the cell was irradiated for 15 s with 366-nm light (stippled area). Birefringence values were obtained from densitometric analysis of 35-mm negatives. Photographs of this spindle are shown in Fig. 7.
FIGURE 7 Polarization micrographs of the spindle described in Fig. 6. The first frame shows the prometaphase spindle before irradiation with 366-nm light. Subsequent frames are taken at 20-s intervals starting 10 s after the end of the irradiation. This spindle reached metaphase about 5 min after the irradiation. Seconds before and after the end of the irradiation are shown in the lower corner of each frame. 10 μm per scale division, additive compensation. × 970.

FIGURE 8 Typical changes in spindle birefringence after 15-s irradiation with 366-nm light (stippled area). Open and filled circles represent birefringence measurements of two prometaphase spindles from eggs treated for 2.5 min with 5 × 10^{-4} M Colcemid 20 min before first nuclear envelope breakdown. Birefringence values were calculated from the rotational angle of the compensator. Curves were drawn through the points by eye. One cell (filled circles) initiated anaphase 6.5 min after irradiation, and the other (open circles) initiated anaphase 8 min after irradiation.

assembly comes from the work of Stephens with the sea urchin Strongylocentrotus drobachiensis (38, 39). He induced a natural variation in the size of the subunit pool by altering the growth temperature during prometaphase. Cells raised at 8°C had larger, more birefringent spindles at a given observational temperature than cells raised at 0°C. Since the spindles assembled at different temperatures had similar thermodynamic parameters, these differences in spindle size and birefringence were due to different sizes of the subunit pool. Interestingly, treating these eggs with D_{2}O seawater irreversibly "erased" the temperature-induced variation in pool size (39). This suggests that D_{2}O may bypass the mechanisms that normally determine how much tubulin is available for assembly.

Although the way in which the cell controls the amount of polymerizable tubulin is still unknown, a number of interesting possibilities have been suggested. Weisenberg found in the eggs of Spisula solidissima a nonmicrotubule particulate form of tubulin, which disappears as the spindle is being assembled (43). This may be a storage form for tubulin, and perhaps the shift out of this form at division is the partitioning event that determines how much tubulin is available for spindle assembly. Bryan et al. report that polyanionic substances such as RNA found in homogenates of sea urchin eggs can limit microtubule polymerization (7). Synthetic polynucleotides or natural RNAs bind to a basic, heat-stable protein identical to the tau factor described by Weingarten (42) which may be necessary in stoichiometric quantities for microtubule assembly (7). In proper concentrations, such polyanions prevent spontaneous nucleation of microtubule assembly while allowing elongation of existing microtubules. In the living cell, this would allow polymerization on only specific nucleating sites such as kinetochores (41) and centrosomes (45) as well as control the extent of polymerization.

Modulating the pool of polymerizable tubulin is not the only way the cell can control the number of microtubules in the spindle. The cell could vary the equilibrium constant of the assembly reaction. Temperature and pressure both change the quan-
tity of spindle microtubules (8, 9, 18, 19, 21, 33, 34, 38, 39). Under constant environmental conditions, however, intracellular changes in the concentration of Ca**+ (6, 32) or nucleotides (23, 44) could determine the extent of microtubule polymerization.

The cell could also regulate microtubule polymerization by modulating the nucleating ability of the kinetochores and centrosomes. In eggs of *Sula solidissima*, microtubule polymerization may be limited by the state of the microtubule-organizing centers. Homogenates of unactivated eggs will not polymerize microtubules until organizing centers of activated eggs are added (45). Similarly, in unfertilized *Xenopus* eggs, cellular tubulin can polymerize but will not do so until competent organizing centers are introduced. Microinjection of isolated basal bodies from *Chlamydomonas* and *Tetrahymena* give aster formation in unfertilized eggs (16). The ability of centrioles in lysed PtK cells to initiate microtubule assembly from exogenous tubulin depends upon stage in the cell cycle. Centrioles from cells lysed in late prometaphase nucleate more microtubules than centrioles from cells lysed earlier in mitosis (37). Even so, nucleating centers may not be the sole factor limiting the extent of microtubule polymerization during mitosis. Spindles of marine eggs can be made to incorporate more microtubules than they do normally, both in vivo by D2O (22) or glycols (30) and in vitro by addition of exogenous tubulin (20, 31).

In principle, control of spindle assembly could be effected through any one of these three possible mechanisms alone. However, in the living cell a subtle interplay between the pool of polymerizable tubulin and the other factors may operate to regulate spindle assembly, disassembly; one or the other would be limiting at different times during the cell cycle. Although there is a great deal yet to be learned about this subject, the work presented here demonstrates that the microtubules of active spindles are assembled from a pool of subunits which can be "titrated" with Colcemid long before the spindle forms and that these spindles respond immediately to changes in the amount of the polymerizable tubulin.

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