ROLE OF SPINDLE MICROTUBULES IN THE CONTROL OF CELL CYCLE TIMING

GREENFIELD SLUDER

From the Program in Biophysical Cytology, Biology Department, University of Pennsylvania, Philadelphia, Pennsylvania 19104. Dr. Sluder's present address is the Zoology Department, University of California, Berkeley, California 94720.

ABSTRACT

Sea urchin eggs are used to investigate the involvement of spindle microtubules in the mechanisms that control the timing of cell cycle events. Eggs are treated for 4 min with Colcemid at prophase of the first mitosis. No microtubules are assembled for at least 3 h, and the eggs do not divide. These eggs show repeated cycles of nuclear envelope breakdown (NEB) and nuclear envelope reformation (NER). Mitosis (NEB to NER) is twice as long in Colcemid-treated eggs as in the untreated controls. Interphase (NER to NEB) is the same in both. Thus, each cycle is prolonged entirely in mitosis. The chromosomes of treated eggs condense and eventually split into separate chromatids which do not move apart. This "c-anaphase" splitting is substantially delayed relative to anaphase onset in the control eggs.

Treated eggs are irradiated after NEB with 366-nm light to inactivate the Colcemid. This allows the eggs to assemble normal spindles and divide. Up to 14 min after NEB, delays in the start of microtubule assembly give equal delays in anaphase onset, cleavage, and the events of the following cell cycle. Regardless of the delay, anaphase follows irradiation by the normal prometaphase duration.

The quantity of spindle microtubules also influences the timing of mitotic events. Short Colcemid treatments administered in prophase of second division cause eggs to assemble small spindles. One blastomere is irradiated after NEB to provide a control cell with a normal-sized spindle. Cells with diminished spindles always initiate anaphase later than their controls. Telophase events are correspondingly delayed. This work demonstrates that spindle microtubules are involved in the mechanisms that control the time when the cell will initiate anaphase, finish mitosis, and start the next cell cycle.

KEY WORDS cell cycle · Colcemid · irradiation · microtubules · mitosis · timing

In animal cells, the mitosis portion of the cell cycle consists of a series of nuclear and cytoplasmic events that precisely partition the chromosomes and cytoplasm into two functional daughter cells.

Spindle microtubules play an important role in the execution of these events. They are necessary for the establishment of spindle polarity (7, 37, 57, 78), chromosome attachment to the spindle (9, 58), prometaphase and anaphase movements of the chromosomes (7, 37, 46, 66), and for determining the location of the cleavage furrow

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/79/03/0674-18$1.00
Volume 80 March 1979 674-691

(32, 33, 63). They also give the spindle its characteristic birefringence (37, 67, 73).

In addition to being intimately involved in the execution of mitotic events, spindle microtubules may also be part of the mechanism that controls the cell's progress through the various stages of mitosis. Drugs and chemicals that inhibit microtubule assembly slow or stop the cell cycle in mitosis (10, 21, 23, 25, 30, 40). For many of these agents, the nature of the mitotic arrest is equivocal as a result of nonspecific side effects on metabolism or macromolecular synthesis. However, the action of colchicine and Colcemid, whose mode of action is specific and well characterized, shows that cells which cannot assemble spindle microtubules either are arrested in mitosis or stay significantly longer in mitosis than they normally would (16, 25, 29, 37, 54).

Inhibiting microtubule assembly may not completely stop the cell cycle. Some aspects of the cycle will continue, albeit delayed. The chromosomes of colchicine-treated cells condense, giving the familiar X-shaped figures characteristic of "c-metaphase" (25, 42, 43). Eventually, the sister chromatids may fall apart in "c-anaphase" and, later, nuclear envelopes will reform around individual chromosomes, giving a number of micronuclei (25, 35, 42, 43, 54). These same colchicine-treated cells may then enter mitosis once again with a greater number of chromosomes (20, 25, 42, 43). Fertilized sea urchin eggs can show cycles of cortical birefringence, nuclear envelope breakdown-reformation, and chromosome condensation-decondensation in the presence of colchicine (55, 76, 84).

Although these studies indicate that the assembly of spindle microtubules may influence the timing of the cell cycle, they were performed on cells of a variety of organisms with differing drugs and drug dosages. The timing of mitotic events was usually not precisely determined and compared to that of normal cells. Furthermore, these studies have not provided information on the timing of several other important mitotic events. The events listed below could proceed independent of microtubule assembly, but could not be detected in drug-treated cells. (*a*) Microtubule assembly: During mitosis the quantity of microtubules in the spindle changes in a stage-specific fashion in close coordination with nuclear events (26, 37, 73). Do the mechanisms that control microtubule assembly and disassembly still operate with normal timing when microtubule assembly is prevented? (*b*) Reproduction of mitotic centers: Sea urchin eggs normally replicate and split their mitotic centers (spindle poles) at the time of telophase (49, 51). If spindle microtubules are not assembled, do these events follow nuclear envelope breakdown at the normal time or are they delayed? (*c*) Cleavage: Does the egg cortex remain competent to form a furrow if mitosis is prolonged, or does the egg have to wait until the next cell cycle to cleave? (*d*) Progress through mitosis: When microtubule assembly is prevented, cells traverse the mitosis portion of the cell cycle more slowly. Does the cell cycle proceed at a constant but slower rate than normal? Possibly, it proceeds at a normal rate until some point and then stops. If so, this would lend credence to the saying that colchicine arrests cells at "metaphase."

The work described in this paper was designed to systematically investigate the role of spindle microtubules in the control of cell cycle timing. The eggs of the sea urchin *Lytechinus variegatus* were used for this work because they are hardy and rapidly traverse the cell cycle. First, I compared the cell cycle timing of eggs that are prevented from assembling microtubules to the timing of normal eggs. Second, I determined the extent to which the time at which the cell starts microtubule assembly influences the timing of mitotic events. Third, I tested to determine whether variations in the quantity of spindle microtubules affect the timing of the cell cycle.

For this work, the assembly of spindle microtubules was specifically inhibited by briefly treating fertilized eggs with 5×10^{-6} M Colcemid for a few minutes in prophase of first or second mitosis. Although constant immersion of the eggs in this concentration of drug is more than enough to prevent any microtubule assembly, the final dosage of the drug can be precisely determined by varying the duration of the treatment (68).

The results of this work show that spindle microtubules influence the timing of mitotic events and the overall duration of the cell cycle. That is, microtubules are not only necessary for the execution of mitotic events, but also are involved in the mechanisms that determine when the cell will decide to execute these events.

MATERIALS AND METHODS

Living Material

Lytechinus variegatus (Gulf Specimen Co. Inc., Panacea, Florida) were maintained at 22°C in Instant Ocean

aquaria (Aquarium Systems, Inc., Eastlake, Ohio) before use. Eggs were repeatedly obtained from individual females by intracoelomic injection of 0.5 M KCl (27). Sperm were taken "dry" from excised testes. Eggs were fertilized and allowed to develop at 22°C in artificial sea water. All experiments were performed at 21°–22.5°C.

Colcemid Treatment

To prevent the assembly of spindle microtubules, eggs were treated for 3.5–4 min with 5×10^{-6} M Colcemid (Ciba-Geigy Corp., Pharmaceutical Div., Summit, N. J.) as previously described (68). Since only the minimum dosage of Colcemid necessary to prevent assembly of spindle microtubules was used, treatment durations were adjusted to suit the eggs of particular females and the time during the spawning season. Treatments ranged from 3.25 min early in the season to 4.0 min later in the year. Treatments were terminated by centrifuging the eggs and resuspending them three times in fresh artificial sea water. This washes out the free intracellular Colcemid, leaving only the drug bound to the cells (14). *L. variegatus* eggs treated in this fashion recover very slowly from the drug treatment; even small spindles do not form for at least 3 h. Increasing the number of washes did not give early recovery from the drug. This is consistent with the finding that the colchicine bound to cells is only slowly lost when the cells are put into drug-free medium (77). The half-time for exchange of tritiated colchicine bound to in vitro preparations of tubulin is ~37 h (28). The amount of time for recovery from Colcemid varies with cell type (16, 37, 47, 77).

A stock solution of lumi-Colcemid was prepared by irradiating 1×10^{-4} M Colcemid/distilled water for more than 1 h, using unfiltered light from a 200W mercury arc bulb (Illumination Industries, Sunnyvale, Calif.) in a Zeiss lamphouse (Carl Zeiss, Inc., New York).

Microscopy and Observations

For observation, living cells were mounted as previously described (68). Control cells mounted in this way developed until at least the ciliated blastula stage.

Nuclear morphology in living and fixed cells was observed and photographed with Zeiss Nomarski differential interference contrast optics using a Plan 40X (NA 0.65) objective. Timing data were obtained by following individual cells and recording the times of nuclear envelope breakdown and nuclear envelope reformation.

Polarization microscopy was performed with a modified Nikon model S microscope body (Nikon Inc., Instrument Div., Garden City, N. Y.) (68). Timing data were obtained by following individual eggs and recording the times of nuclear envelope breakdown, irradiation, anaphase onset, and the following nuclear envelope breakdown. These events were used as timing markers since they are discrete occurrences that can be observed in vivo with the polarization microscope. Times were rounded off to the nearest half minute. Representative sequences were photographed with Kodak 35 mm Plus-X film developed in Kodak Microdol-X. Statistical methods were taken from Sokal and Rohlf (71).

Fixation, Cell Counting, and Staining

To determine the percentage of nuclear envelope breakdown, aliquots of eggs from Colcemid-treated and control cultures were fixed in ethanol-acetic acid (3:1). Eggs from each aliquot were then placed on a slide, gently flattened with a coverslip, and sealed with "Valap" (68). These slides were then scanned with a differential interference contrast microscope.

To observe chromosome morphology, eggs were fixed with ethanol-acetic acid (3:1) for several hours at room temperature. They were then transferred to 75% acetic acid. For observation, a drop of fixed eggs was placed on a slide and stained with a drop of acid-orcein (1% Orcein in 75% acetic acid). A coverslip was then placed on the eggs to gently flatten them, and the preparation was sealed. Photographs of chromosomes were taken with Zeiss phase contrast optics using a Neofluor 100X (NA 1.30) oil immersion objective.

Irradiation

To irradiate the eggs with 366-nm light, the polarizer was removed and the 546-nm filter was replaced with a Zeiss UG-1 filter (optical system described in references 68, 69). The image of the illuminator diaphragm (field stop) was used to limit irradiations to single cells or a portion of a cell. A single 15-s irradiation was sufficient to inactivate the doses of Colcemid used in this study.

RESULTS

Colcemid Control

To test for possible side effects of the Colcemid treatments used in this study, eggs were treated with lumi-Colcemid (photochemically inactivated Colcemid). Lumi-colchicine has some of the same toxic side effects as native colchicine, such as inhibition of nucleoside transport and binding to membranes (53, 72, 80), yet does not bind to tubulin and does not prevent microtubule assembly (3, 11, 68, 82, 83). Thus, the effect of lumi-Colcemid on the timing of the cell cycle should differentiate between the specific and nonspecific effects of Colcemid. Eggs were treated for 7 min with 5×10^{-6} M lumi-Colcemid or exposed continuously to 1×10^{-6} M lumi-Colcemid. Such treatments with native Colcemid are more than sufficient to prevent assembly of spindle microtubules. Neither treatment slowed the timing of first and second divisions in these eggs.

Entry into Mitosis

To determine whether microtubule assembly influences the time of nuclear envelope break-

down, as has been suggested (6), eggs from a single female were fertilized and then divided into three batches. The first served as the control and the second was treated for 3.5 min with 5×10^{-6} M Colcemid in early prophase of the first division. The third batch was treated for 7 min to test whether the drug had any side effects that would slow the entry of these eggs into mitosis. Aliquots from each batch were fixed at 3-min intervals starting before nuclear envelope breakdown. 100–180 eggs were scored for each aliquot to determine the percentage of nuclear envelope breakdown.

The results of one such experiment are shown in Fig. 1. The time of nuclear envelope breakdown in both treated batches is the same as that of the control. The spread in times of nuclear envelope breakdown reflects the asynchrony of the eggs used for this particular experiment. Identical results were obtained in three separate experiments.

Cyclical Disappearance-Reappearance of the Nuclear Envelope

Colcemid-treated eggs do not form a spindle or divide. For at least 3 h after treatment, there is no trace of spindle birefringence or of alignment of chromosomes and cytoplasmic granules which would indicate the presence of even a few microtubules.

During the next several hours, nuclear envelopes cyclically break down and later reform (Fig. 2). In each egg, the nuclear envelope breaks down, leaving an irregular, clear area about the same size as the original nucleus (Fig. 2*b*). This clear area persists for ~40 min and then tiny spherical karyomeres form in the nuclear area (Fig. 2*c*). With time, these karyomeres swell and a few may fuse (Fig. 2*d*). These events parallel those in normal nuclear reconstitution (Fig 3*d*, *e*, and *f*), except that in treated eggs not all kary-

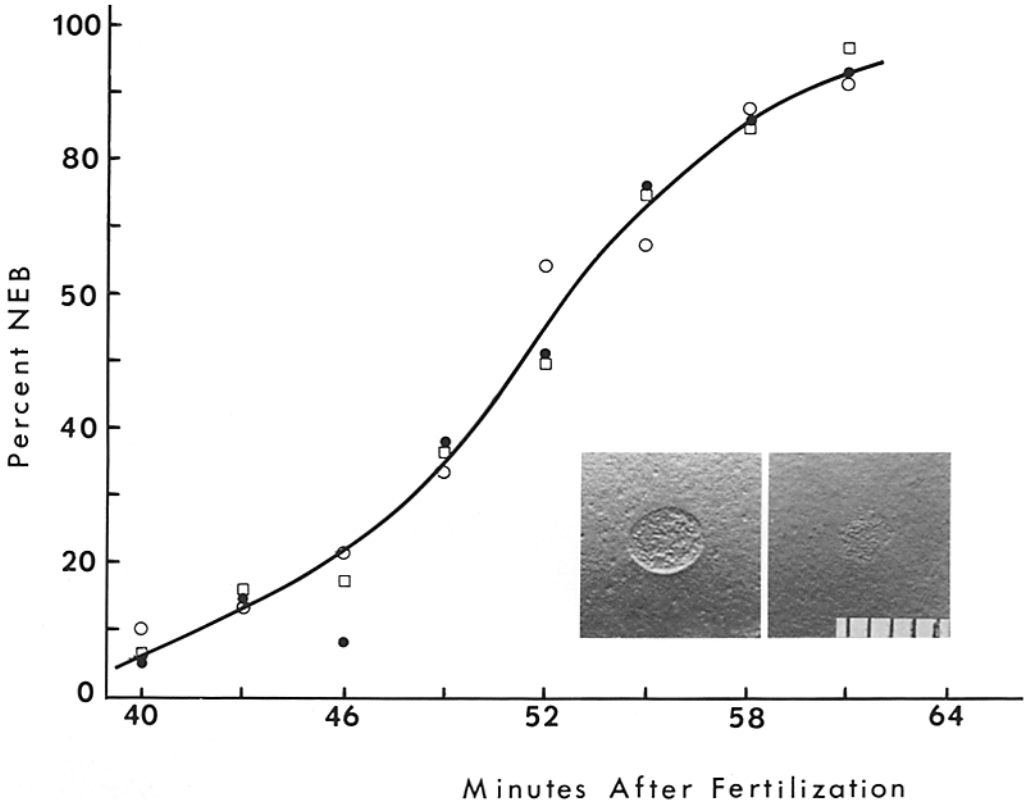

FIGURE 1 Percent nuclear envelope breakdown (NEB) as a function of time after fertilization. Closed circles: untreated control eggs. Open circles: eggs treated for 3.5 min with 5×10^{-6} M Colcemid. Open squares: eggs treated for 7 min with 5×10^{-6} M Colcemid. 100–180 eggs were scored for each data point. Inset: before and after nuclear envelope breakdown (fixed eggs). Nomarski differential interference contrast micrographs. Bar, 10 μm. × 275.

omeres fuse to give a single nucleus. There is no cleavage or sign of surface deformation. Later, the enlarged karyomeres of treated eggs synchronously break down, leaving an irregular clear area (Fig. 2*e*). This clear area persists for ~30 min and then small spherical karyomeres again reform (Fig. 2*f*). With time, these swell and a few may fuse before they all break down synchronously (Fig. 2*g* and *h*). With each pass through the nuclear cycle, a greater number of karyomeres are observed (Fig. 2*d* vs. *g*).

The times of nuclear envelope breakdown and nuclear envelope reformation (the first visible appearance of karyomeres) were recorded in treated and untreated eggs. Individual eggs were followed to precisely quantitate their timing and to avoid the complication of asynchrony in the population. Most of the population's asynchrony

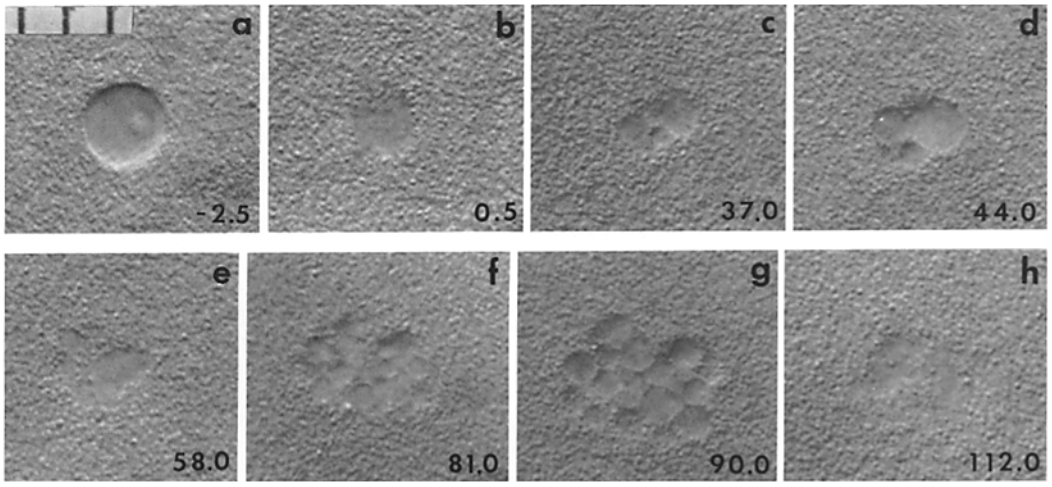

FIGURE 2 Disappearance-reappearance cycling of nuclear envelopes in an egg treated for 4.0 min with 5×10^{-6} M Colcemid. (*a*) before first nuclear envelope breakdown; (*b*) after nuclear envelope breakdown; (*c*) karyomeres forming; (*d*) karyomeres swell; (*e*) karyomeres synchronously break down; (*f*) karyomeres form for the second time; (*g*) these swell; (*h*) they break down. Minutes before and after first nuclear envelope breakdown are shown in the lower corner of each photograph. Nomarski differential interference contrast micrographs of the same living eggs. Bar, 10 μm × 600.

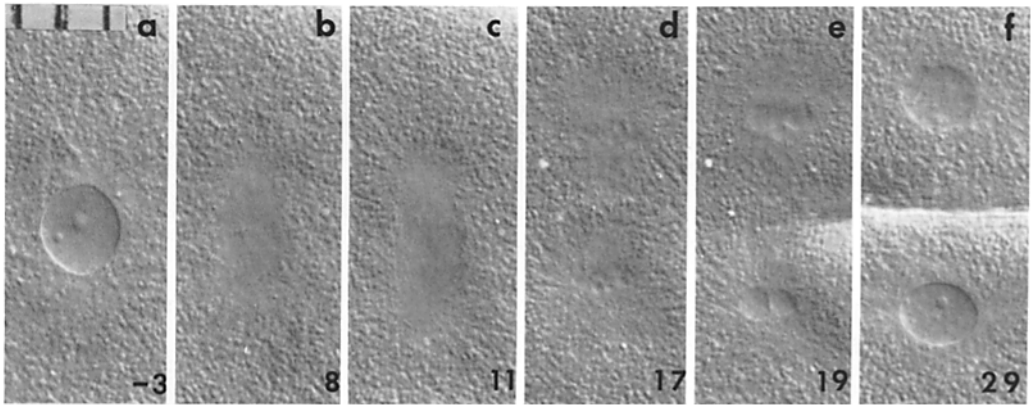

FIGURE 3 Mitosis and nuclear reformation in an untreated egg. (*a*) before nuclear envelope breakdown; (*b*) late prometaphase; (*c*) mid-anaphase; (*d*) nuclear envelope reformation in telophase; (*e*) karyomere fusion; (*f*) interphase, the line between the nuclei is the cleavage furrow. Minutes before and after first nuclear envelope breakdown are shown in the lower corner of each frame. Nomarski differential interference contrast micrographs of the same living egg. Bar, 10 μm × 600.

comes from a variability in the amount of time between fertilization and first nuclear envelope breakdown.

The timing of nuclear envelope breakdown and reformation is shown in Fig. 4. In the Colcemid-treated eggs, the interval from first nuclear envelope breakdown to first nuclear envelope reformation is about twice as long as that of the untreated eggs. The interval between the first nuclear envelope reformation and the second nuclear envelope breakdown is not significantly different in the treated and untreated eggs. After second nuclear envelope breakdown, the mitotic period is again almost exactly double in the Colcemid-treated eggs. The timing of subsequent cycles in treated and control eggs shows the same pattern.

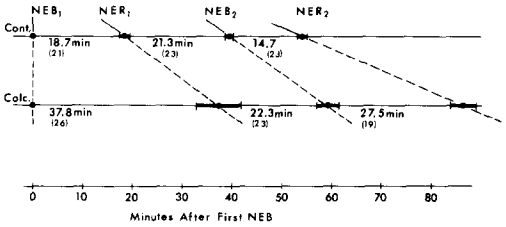

FIGURE 4 Timing of nuclear envelope breakdown (NEB) and nuclear envelope reformation (NER) in untreated (upper line) and treated eggs (lower line). Eggs were treated for 4 min with 5×10^{-6} M Colcemid ~20 min before first nuclear envelope breakdown. The horizontal lines represent time axes. First nuclear envelope breakdown is normalized to 0 min for all individual eggs. The mean times of nuclear envelope reformation and breakdown are shown by closed circles on the time axes. The heavy horizontal bars delimit the 95% confidence limits of the means. The larger numbers under each time axis show the mean duration of the various intervals. The small numbers in parentheses give the sample sizes.

Chromosome Morphology

The timing of the changes in chromosome morphology in treated and untreated eggs was determined by fixing and staining aliquots of eggs at 3-min intervals after first nuclear envelope breakdown. Treated and untreated eggs were taken from the same female and fertilized at the same time.

Fig. 5*A-J* shows typical examples of the changes in chromosome morphology of Colcemid-treated eggs. In late prophase, the chromosomes are relatively long and thin (Fig. 5*A*). After nuclear envelope breakdown, the chromosomes become progressively more condensed (Fig. 5*B-F*). This stage is equivalent to prometaphase in normal eggs. For comparison, the chromosomes

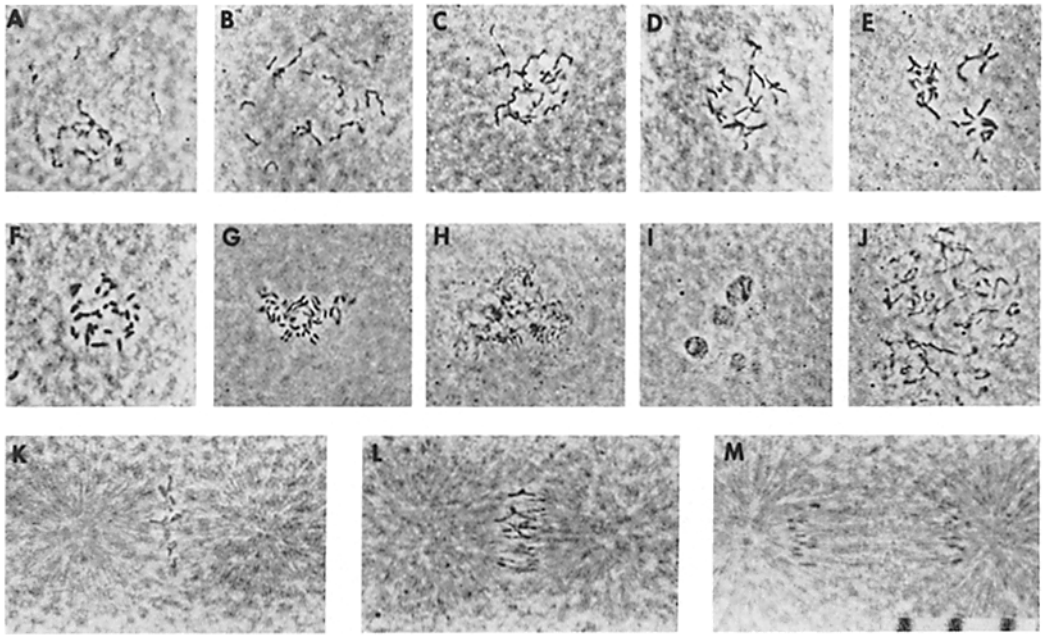

FIGURE 5 Chromosome morphology in Colcemid-treated (*A-J*) and untreated eggs (*K-M*). Phase contrast micrographs. Bar, 10 μm. × 700.

of an untreated egg at metaphase are shown in Fig. 5*K*. In treated eggs, the chromosomes remain randomly distributed over an area about the same size as the original nucleus. There is no alignment of the chromosomes that would indicate some spindle development.

After most of the control eggs have initiated anaphase (Fig. 5*L*), the chromosomes of the treated eggs continue to condense and may become even more condensed than normal metaphase chromosomes (Fig. 5*F*). Eventually, the chromosomes are observed to distinctly split (Fig. 5*G*). This splitting is synchronous since all chromosomes are single or split within any given egg. Although the sister chromatids do not move apart after splitting, this stage is analogous to anaphase in untreated eggs. The chromatids then decondense (Fig. 5*H*) and a number of separate karyomeres are formed (Fig. 5*I*). Subsequent cycles of chromosome condensation, splitting, and decondensation follow the same pattern, except that more and more chromosomes are observed with each cycle (Fig. 5*A* vs. 5*J*).

Chromosome splitting in treated eggs is delayed relative to anaphase onset in the untreated controls. Split figures are found only in aliquots of treated eggs that are fixed at least 15–20 min after most controls have entered anaphase. This delay in chromosome splitting accounts for the extra time that treated eggs spend in mitosis. The time from nuclear envelope breakdown to nuclear envelope reformation is 20 min longer in the treated eggs than in the controls (Fig. 4).

Delay of Spindle Assembly

Spindle assembly was experimentally delayed relative to the start of mitosis by individually irradiating Colcemid-treated eggs for 15 s with 366-nm light at various times after nuclear envelope breakdown. This photochemically inactivates the drug and allows the eggs to assemble microtubules if they are competent to do so (3, 11, 68). Their timing was compared to that of untreated eggs that were given the same irradiation shortly after nuclear envelope breakdown.

As a control, I tested for the effect of 366-nm light on the timing of the normal cell cycle. Fertilized eggs were allowed to divide once. Shortly after second nuclear envelope breakdown, one daughter was irradiated for the desired amount of time; the unirradiated daughter cell served as a control. Since second nuclear envelope breakdown occurred synchronously in the daughter cells, even 30-s differences in timing could easily be detected. Irradiations as long as 2 min, given anytime during prometaphase, do not influence the time of anaphase onset, cleavage, or the approximate time of nuclear envelope reformation. Such irradiations, however, delay the following nuclear envelope breakdown (in this case the third) in a dose-dependent fashion. The 15-s irradiations used throughout this study delay the following nuclear envelope breakdown by ~7 min. Irradiations given during telophase do not impede cleavage or nuclear envelope reformation, but produce similar delays in the following nuclear envelope breakdown. Thus, all eggs in the following studies were given equal doses of 366-nm light.

Colcemid-treated eggs enter mitosis but do not form a spindle (Fig. 6*a*). Upon irradiation, such eggs assemble functional spindles of normal appearance (Fig. 6*b–f*). 15–20 s after the irradiation, two small birefringent asters are seen on either side of the nuclear area. These asters move apart as the spindle forms. As in normal eggs, spindle size and retardation gradually increase, reaching a maximum at anaphase onset. In late anaphase, spindle birefringence rapidly fades, the egg cleaves, the mitotic centers split, and daughter nuclei reform (Fig. 6). The sequence and morphology of these telophase events are normal. Subsequent division cycles of these eggs appear normal. Thus, development proceeds normally after irradiation even though the start of microtubule assembly is substantially delayed. The egg shown in Fig. 6 was irradiated 8 min after nuclear envelope breakdown; an untreated egg at this time would be in metaphase. For comparison, mitosis in a normal egg, as seen with the polarization microscope, is shown in Fig. 7.

The quantitative results of these experiments show that delays of spindle assembly give equal delays in the time of anaphase onset and the time of second nuclear envelope breakdown (Figs. 8 and 9). Anaphase follows the irradiation by ~10 min regardless of the experimentally introduced delay of spindle assembly. This is the same amount of time that untreated eggs take to go from nuclear envelope breakdown to anaphase onset (see top line). The eggs shown on the bottom line of Fig. 8 (10- to 13-min delays) show normal spindle assembly and division even though they were irradiated when they should have been in mid-anaphase.

Delays in microtubule assembly correspond-

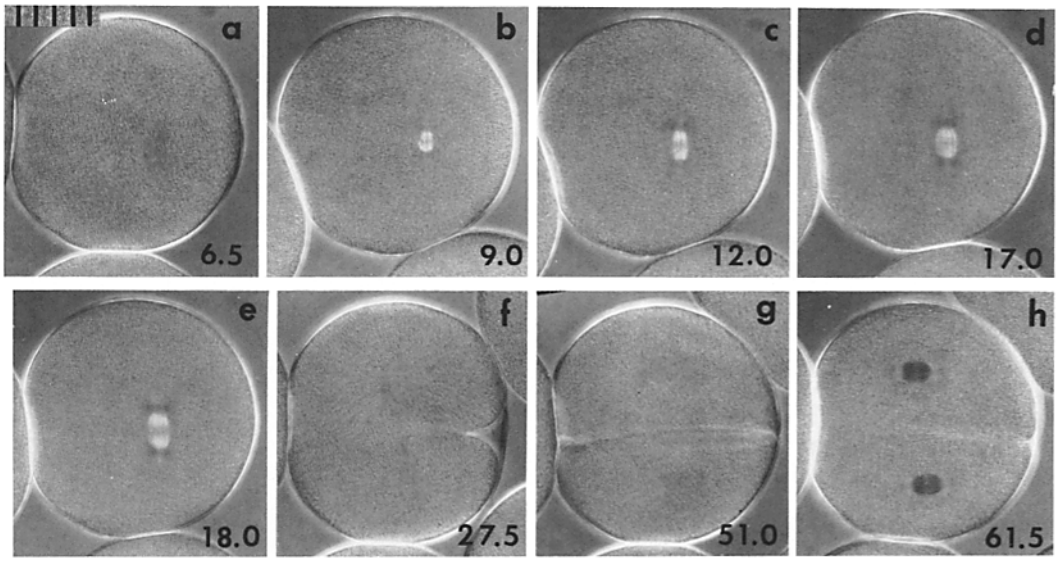

FIGURE 6 Mitosis in a Colcemid-treated egg after irradiation with 366-nm light. This egg was treated for 3.5 min with 5×10^{-6} M Colcemid in early prophase of the first division. 8 min after nuclear envelope breakdown, it was irradiated for 15 s with 366-nm light. (*a*) after nuclear envelope breakdown, but before irradiation; (*b–d*) recovery of the birefringent spindle after irradiation; (*e*) early anaphase; (*f*) cleavage; (*g*) nuclear envelopes have reformed; (*h*) second prometaphase. Minutes after first nuclear envelope breakdown are shown in the lower corner of each frame. Polarization micrographs; additive and subtractive compensation. Bar, 10 μm. × 240.

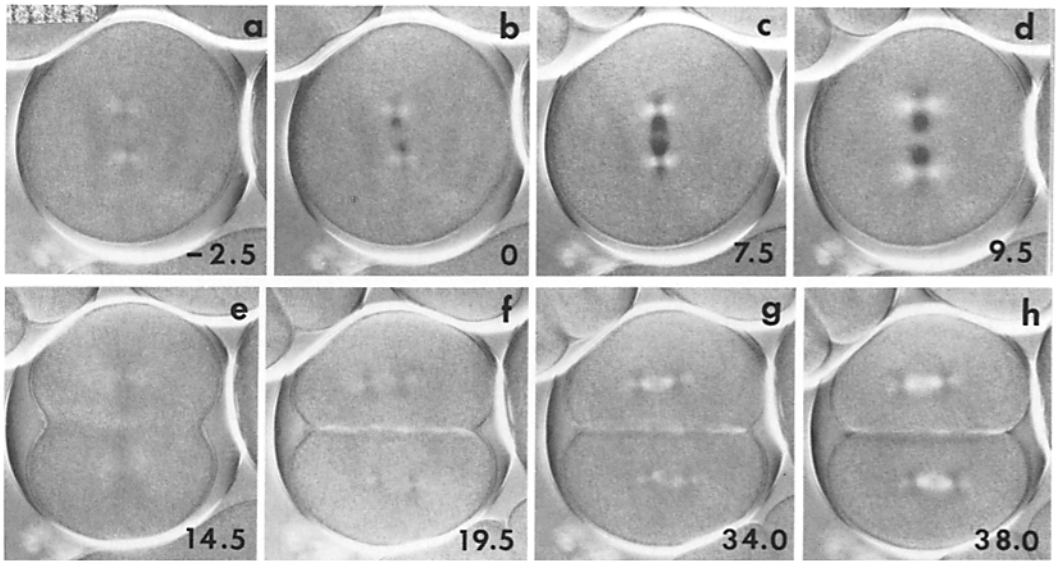

FIGURE 7 Mitosis in an untreated egg. (*a*) before first nuclear envelope breakdown; (*b*) nuclear envelope breakdown; (*c*) metaphase; (*d*) early anaphase; (*e*) telophase and cleavage; (*f*) prophase of second mitosis; (*g*) second nuclear envelope breakdown; (*h*) prometaphase of second mitosis. Minutes before and after first nuclear envelope breakdown are shown in the lower corner of each frame. Polarization micrographs; subtractive and additive compensation. Bar, 10 μm. × 225.

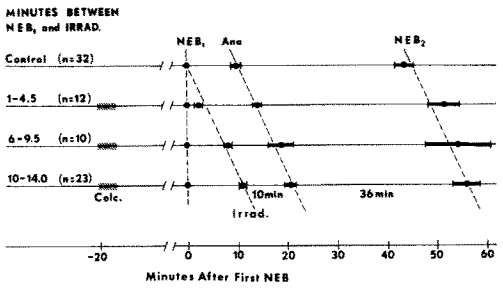

FIGURE 8 Results of Colcemid reversal experiments. Fertilized eggs were treated for 3.5 min with 5×10^{-6} M Colcemid in early prophase of the first division (cross hatching). They were irradiated for 15 s with 366-nm light at times ranging from 0.5 to 14 min after first nuclear envelope breakdown (NEB). The untreated control eggs (top line) were irradiated shortly after first nuclear envelope breakdown. Timing data from the Colcemid-treated eggs are collected into three classes based upon the number of minutes between nuclear envelope breakdown and irradiation. The light horizontal lines are the time axes. The time of first nuclear envelope breakdown is normalized to 0 min for all individual eggs. The mean times of irradiation, anaphase onset, and second nuclear envelope breakdown are shown as closed circles on the time axes. The heavy horizontal bars delimit the 95% confidence limits of the means. The parallel dotted lines are drawn through the irradiation, anaphase, and second nuclear envelope breakdown means to emphasize the constancy of the interval between irradiation and anaphase, as well as the interval between anaphase and second nuclear envelope breakdown. The numbers in parentheses give the sample sizes.

ingly delay following cell cycles. As shown in Fig. 8, second nuclear envelope breakdown follows anaphase onset by ~36 min in both control and experimentally delayed eggs. In Table I, the timing of subsequent cell cycles of treated eggs irradiated soon after first nuclear envelope breakdown ($\bar{x}$ = 2.6 min) is compared with that of treated eggs irradiated at a time when they should be entering anaphase ($\bar{x}$ = 10.4 min). This comparison shows that later cell cycles are not shorter to compensate for the experimentally introduced delay.

Identical results are obtained if the experiments are performed with eggs treated in prophase of the second divsion. A 15-s irradiation gives recovery of functional spindles of normal appearance in both daughter cells. As before, delays in spindle assembly give equal delays in the time of anaphase onset and division (Fig. 9).

To test the constancy of the interval between irradiation and anaphase onset, data obtained for first or second division eggs (shown in Fig. 9) can be plotted as: the amount of time between irradiation and anaphase onset (ordinate) as a function of the experimentally introduced delay (abscissa). The slopes and intercepts of lines drawn through the data were computed by the method of least squares (71). The slopes of the lines for first and second division eggs are not significantly different from zero: First division $t = -1.71844$, $p > 0.05$, Second division $t = 1.8950$, $p > 0.05$. This indicates that the interval from irradiation to anaphase onset is constant regardless of the delay in spindle assembly.

Also, the mean interval from irradiation to anaphase onset for all delayed first division eggs is not significantly different from the time of nuclear envelope breakdown to anaphase onset in untreated control eggs (Table I). For all delayed second division eggs, the mean time from irradiation to anaphase onset is 3 min longer than the nuclear envelope breakdown to anaphase interval for control eggs (Table I).

Spindle Size and the Time of Anaphase Onset

Eggs were treated for 1–3 min with 5×10^{-6} M Colcemid in second prophase. At second nuclear envelope breakdown, small barrel-shaped spindles were formed. Although reduced in length and birefringence, such small spindles are functional (68). One daughter cell was irradiated for 15 s with 366-nm light after second nuclear envelope breakdown. This led to the rapid recovery of an approximately normal-sized spindle which served as a control. The daughter cell with the small spindle was irradiated in telophase to equalize the doses of 366-nm light. Since both daughter cells underwent nuclear envelope breakdown synchronously, small differences in timing could easily be detected.

Photographs of a typical experiment are shown in Fig. 10. The upper daughter cell was irradiated after nuclear envelope breakdown and had an approximately normal-sized spindle. This spindle initiates anaphase 3 min sooner than does the small barrel-shaped spindle in the lower cell. This difference in the times of anaphase onset must be a result of the difference in quantities of microtubules since even 2-min irradiations per se do not influence the time of anaphase onset in untreated eggs. After anaphase, both cells cleave and reform

TABLE I

Cell Cycle Timing of Eggs Irradiated on the Average 2.6 min after Nuclear Envelope Breakdown vs. that of Eggs Irradiated on the Average 10.4 min after Nuclear Envelope Breakdown.

	Stage				
Delay	NEB_1 ····· Irrad ····· Ana_1 ····· NEB_2 ····· Ana_2 ····· NEB_3				
Short $\bar{x}$ (min)	2.6 ± 0.8 n = 18	11.2 ± 1.0 n = 18	35.5 ± 2.8 n = 18	9.8 ± 1.0 n = 15	37.2 ± 6.5 n = 5
Long $\bar{x}$ (min)	10.4 ± 0.6 n = 33	10.0 ± 1.0 n = 33	35.9 ± 1.9 n = 33	10.0 ± .9 n = 14	34.6 ± 5.2 n = 7
Corresponding values for untreated control cells (NEB to Ana)		9.6 ± 0.7 n = 35		7.1 ± 0.6 n = 34	

Mean value (in min), plus and minus the 95% confidence limits of the mean. Times shown are the duration of the intervals between the events shown at the top of the table. n is the sample size.

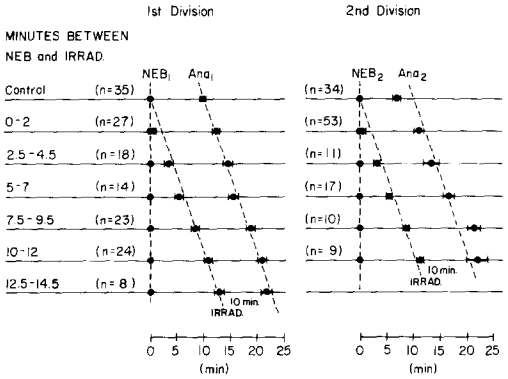

FIGURE 9 The relationship between the times of irradiation and anaphase onset in Colcemid-treated first and second division eggs. The timing of irradiated control eggs is shown on the top lines. Timing data from treated eggs are collected into classes based upon the number of minutes between nuclear envelope breakdown and irradiation. The parallel dotted lines are drawn through the irradiation and anaphase means to emphasize the constancy of this interval. The numbers in parentheses give the sample sizes. Data shown in Fig. 8 are included in this figure.

nuclei. The cell cycle in the cell with the small spindle remains delayed. Cleavage, nuclear envelope reformation, and third nuclear envelope breakdown occur at the same pace as in the control cell but are set back in time.

There is a qualitative correlation between spindle size and the delay in anaphase onset. Spindles that are 10% shorter than normal show little or no delay in anaphase onset. Those whose length and birefringence are reduced by 50% take as much as 7–8 min longer to reach anaphase than their full-sized counterparts. This represents almost a doubling of the normal interval between nuclear envelope breakdown and anaphase onset.

Fig. 11*a* and *b* show the quantitative results of a number of these experiments. The data from the cell pairs are artificially put into two classes (*a* and *b*) based upon the differences in time of anaphase onset within the cell pairs. Separation of the data into these two classes shows that third nuclear envelope breakdown follows anaphase onset by ~29 min regardless of the time of anaphase onset.

Abnormal Development from Long Delays in Spindle Assembly

Colcemid-treated eggs will eventually finish mitosis and start the next cell cycle without assembling microtubules. What then happens if treated eggs are irradiated later and later after nuclear envelope breakdown?

Although eggs will develop normally when spindle assembly is delayed by as much as 14 min, there is an increasing incidence of abnormal development when they are irradiated more than 11 min after nuclear envelope breakdown. Irradiation between 11 and 14 min after nuclear envelope breakdown leads in some cases to the recovery of spindles whose poles visibly split shortly before anaphase onset (Fig. 12*a* and *c*). These spindles are either tripolar or tetrapolar upon entering anaphase (Fig. 12*c*). Occasionally, one or both poles may be sufficiently split at first division so that after anaphase three or four small nuclei reform. Thus, the splitting of the mitotic

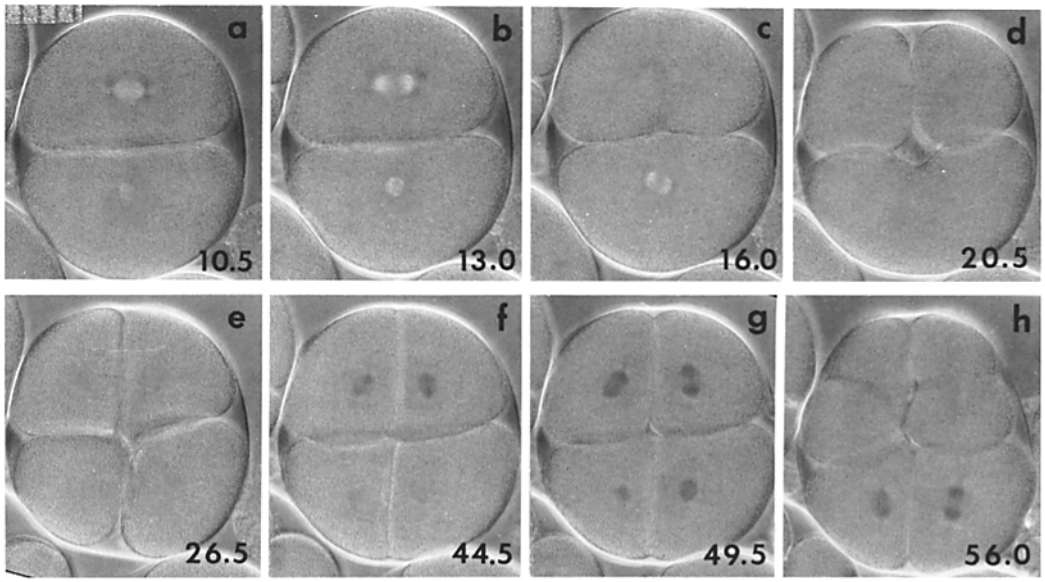

FIGURE 10 Mitosis of daughter cells with different-sized spindles. This zygote was treated for 2.5 min with 5×10^{-6} M Colcemid at first cleavage. Second nuclear envelope breakdown occurred synchronously in both daughter cells. Shortly thereafter, the upper blastomere was irradiated for 15 s with 366-nm light. The lower blastomere was irradiated for 15 s when it was in telophase to equalize the doses of 366-nm light. (*a*) prometaphase in irradiated and unirradiated blastomeres; (*b*) anaphase in the irradiated blastomere while lower blastomere is in metaphase; (*c*) telophase and the initiation of cleavage in irradiated cell while the unirradiated cell is in anaphase. The large spindle initiated anaphase 3 min earlier than the smaller one; (*d*) telophase and cleavage in lower blastomere; (*e*) nuclear envelope fully reformed in upper two cells but not in lower two; (*f*) prometaphase in upper cells while lower two are still in interphase; (*g*) anaphase in upper cells and prometaphase in lower cells; (*h*) cleavage in upper cells and anaphase in lower cells. Minutes after second nuclear envelope breakdown are shown in the lower corner of each frame. Polarization micrographs; additive and subtractive compensation. Bar 10 μm. × 243.

centers, which normally occurs in late anaphase or early telophase (49, 51), is out of phase with the rest of the mitotic events. These eggs cleave normally and reform daughter nuclei of equal size. At the next division, the poles of the daughters' spindles do not split before anaphase (Fig. 12*b* and *d*).

When irradiated 16–25 min after first nuclear envelope breakdown, treated eggs assemble spindles for a short but variable amount of time after the irradiation. These spindles then become diffuse and their birefringence fades completely before they are due to initiate anaphase. The poles of these weak or fading spindles may or may not be visibly split. Fig. 13 shows a typical case in which the egg was irradiated 23 min after nuclear envelope breakdown. Here, spindle birefringence disappears <6 min after the irradiation without a visible anaphase (Fig. 13*d*). Sometimes, eggs irradiated at this time assemble diffuse spindles with little birefringence.

Although anaphase may not be detectable in eggs delayed this long, chromosomes are moved, because two well separated daughter nuclei are formed. At the next mitosis, two bipolar spindles of normal appearance are assembled (Fig. 13*f*). Sometimes, the nuclei that reform are of unequal size, suggesting a breakdown in the mechanisms that ensure equal distribution of daughter chromosomes to opposite poles. These eggs sometimes initiate shallow furrows that later regress.

Irradiation between 25 and 35 min after nuclear envelope breakdown leads to the recovery of little spindle structure. Fig. 14 shows one such egg irradiated 29.5 min after nuclear envelope breakdown. Although there is no measurable spindle birefringence, cytoplasmic granules are aligned, indicating the presence of some astral microtubules (Fig. 14). With time, these spindles become larger, more diffuse, and may split one or both poles before nuclear envelope reformation (Fig. 14). There is no recognizable anaphase, yet chro-

mosomes must be moved since daughter nuclei reform away from the area occupied by the original first division nucleus. Either the egg reforms one large nucleus in association with a pair of daughter centers or reforms two smaller nuclei of unequal size. The egg shown in Fig. 12 reforms only one nucleus (Fig. 14*d*). There is usually no cleavage in these eggs. At the next division, cells with two nuclei form two spindles of unequal width that complete mitosis normally. Eggs that reform only one nucleus form one spindle, while the other two centers remain separate in the cytoplasm (Fig. 14*e*).

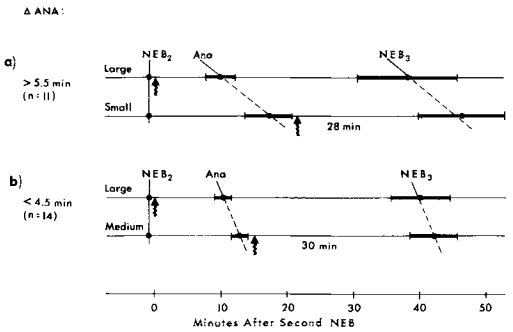

FIGURE 11 Timing data for sister blastomeres with different-sized spindles. Eggs were treated for 1–3 min with 5×10^{-6} M Colcemid during first cleavage. After second nuclear envelope breakdown, one of the two daughters was irradiated for 15 s with 366-nm light (arrow under top lines). The unirradiated blastomeres were irradiated in telophase to equalize the doses of 366-nm light (arrow under bottom lines). Second nuclear envelope breakdown was synchronous within all cell pairs and was normalized to zero time. Mean times of second anaphase and third nuclear envelope breakdown are shown as closed circles on the time axes; the heavy horizontal lines delimit the 95% confidence limits of the means. The data were artificially separated into two classes based upon the difference in times of anaphase onset within the cell pairs. This was done to emphasize that third nuclear envelope breakdown follows anaphase with a constant interval. Qualitatively, the smaller the diminished spindle, the greater the difference in anaphase times (large vs. small above, and large vs. medium below). Numbers in parentheses give the sample sizes.

If treated eggs are irradiated 35–40 min after nuclear envelope breakdown (about the time karyomeres become visible as clusters of small hyaline globules), no spindle is observed to form. When the karyomeres synchronously break down, a tetrapolar spindle forms. Identical tetrapolar spindles are immediately assembled if the eggs are irradiated after the karyomeres have broken down.

DISCUSSION

Use of Colcemid

A basic premise of the analysis presented here is that changes in cell cycle timing are only a result of the loss of microtubules and not of toxic side effects of Colcemid. Physiological processes that do not depend on microtubules should not be significantly affected. Even though Colcemid and its parent compound colchicine act by binding with good specificity to the tubulin dimer (12, 13, 14, 16, 37, 81), toxic side effects and nonspecific binding to cellular components are sometimes observed at drug concentrations which are greater than those needed to inhibit microtubule assembly. The extent of these side effects depends upon the drug's concentration and duration of the exposure.

To minimize possible side effects, I used Colcemid instead of colchicine since it is less toxic to animal cells and is effective at a lower external concentration (23, 31, 41, 65, 74, 85). The eggs were exposed to the drug for a discrete period and then washed with sea water. This not only removes the extracellular drug but also washes out

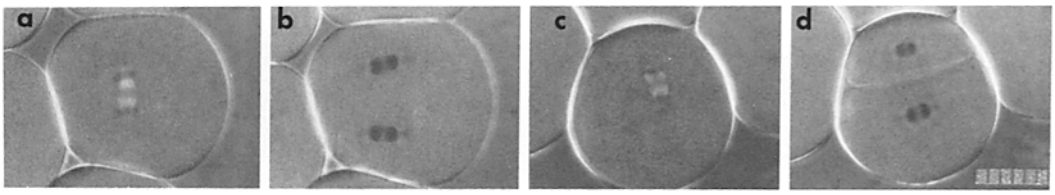

FIGURE 12 First and second division spindles of two Colcemid-treated eggs that were irradiated 11 min after first nuclear envelope breakdown (*a* and *b*), and 15 min after first nuclear envelope breakdown (*c* and *d*). (*a*) lower pole of this spindle is split at metaphase; (*b*) same egg in early anaphase of second mitosis; poles are not split; (*c*) both poles of this spindle are split at early anaphase; (*d*) same egg at second anaphase with poles that are not split. Polarization micrographs, additive and subtractive compensation. Bar, 10 μm. × 160.

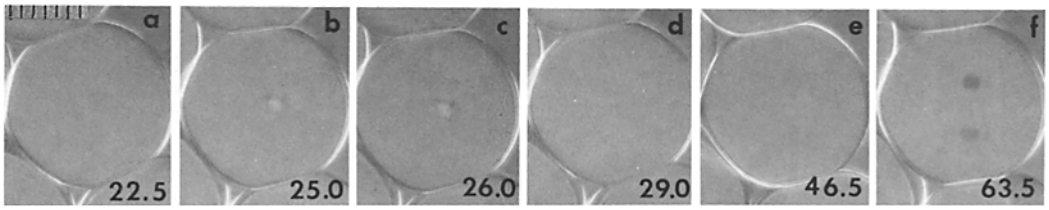

FIGURE 13 Mitosis in a Colcemid-treated egg irradiated 23 min after first nuclear envelope breakdown. (*a*) before irradiation; (*b* and *c*) spindle assembly after irradiation; (*d*) spindle birefringence fades 6 min after irradiation without a detectable anaphase; (*e*) nuclear envelope reformation; (*f*) second prometaphase. Minutes after first nuclear envelope breakdown are shown in the lower corner of each frame. Polarization micrographs, additive and subtractive compensation. Bar 10 μm. × 158.

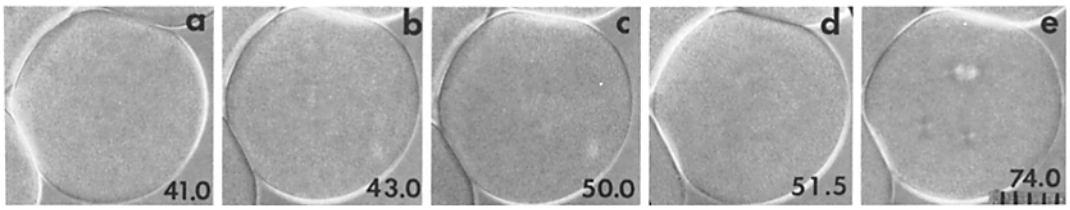

FIGURE 14 Mitosis in a Colcemid-treated egg that was irradiated 29.5 min after first nuclear envelope breakdown. (*a* and *b*) spindle assembly after irradiation, there is no measurable retardation, but some alignment of cytoplasmic granules can be seen; (*c* and *d*) the poles have split and a single nucleus is reforming; (*e*) second prometaphase, all of the chromosomes are associated with the upper two centers, while the lower centers are free in the cytoplasm. Minutes after first nuclear envelope breakdown are shown in the lower corner of each frame. Polarization micrographs, additive compensation. Bar, 10 μm. × 188.

the intracellular pool of unbound Colcemid (14). In this way, I could precisely control the dosage of Colcemid and be certain that I used only the minimum dosage that would prevent microtubule assembly.

Three observations from this study show that Colcemid, as used here, does not have detectable toxic side effects: (*a*) The rate at which a population of eggs goes through first nuclear envelope breakdown is not slowed by even twice the dosage of Colcemid sufficient to block assembly of spindle microtubules. (*b*) The interval between nuclear envelope reformation and subsequent nuclear envelope breakdown (interphase) is the same in treated and untreated eggs. (*c*) Treatments of lumi-Colcemid do not slow the cell cycle of these eggs.

Cell Cycle without Spindle Microtubule

When treated with Colcemid, the eggs of *L. variegatus* continue to go through the cell cycle without spindle microtubules. The sequence and morphology of the cell cycle events are normal, with several exceptions: daughter chromosomes are not moved apart after splitting; karyomeres tend not to fuse; and the cells do not cleave. The lack of chromosome movement and cleavage is understandable. Microtubules are necessary for chromosomes to be moved (7, 37, 46, 66) and two asters are normally required to trigger the egg cortex to initiate a furrow (63, 64). Incomplete karyomere fusion is probably not a result of alterations in the properties of the nuclear envelopes, because adjacent karyomeres are occasionally observed to fuse. Also, nuclei in Colcemid-treated eggs will fuse when brought into close apposition by centrifugation (2). This suggests that microtubules of the telophase aster, in normal cells, form a focus that is necessary to bring the karyomeres into close proximity so that they can fuse.

Although spindles do not form in Colcemid-treated eggs, daughter chromatids synchronously fall apart as they would at anaphase onset. The full-length gap between the daughter chromosomes indicates that both the chromosome arms and kinetochores have split. This splitting (often called "c-anaphase") has been reported for several other types of colchicine-treated cells (25, 34, 42, 43, 44, 45, 54). These observations are consistent

with the principle that anaphase onset consists of two distinct events: the splitting apart of the chromosomes and then the actual movement of the daughter chromosomes to the poles (49, 50).

The cell cycle of Colcemid-treated eggs is ~50% longer than normal. The increased duration of the cell cycle comes entirely from a doubling of the interval between nuclear envelope breakdown and nuclear envelope reformation. This is the portion of the cell cycle during which the spindle is normally assembled and the cell divides.

The timing of the changes in chromosome morphology indicates that primarily the prometaphase portion of mitosis is prolonged. Almost all the extra time that Colcemid-treated eggs spend in mitosis is a result of an increase in the interval between nuclear envelope breakdown and the c-anaphase splitting of the chromosomes. Once the chromosomes split, nuclear envelope reformation proceeds at an approximately normal pace. This observation is the same as that of Molè-Bajer on colchicine-treated *Haemanthus* cells (54). There, nuclear envelope reformation followed c-anaphase with the normal interval even though mitosis was substantially prolonged.

Delays of Spindle Assembly

To further define the interrelationship between microtubule assembly and the timing of mitotic events, Colcemid-treated eggs were irradiated for 15 s with 366-nm light at times after nuclear envelope breakdown. These experiments show that the assembly of spindle microtubules can be delayed relative to nuclear envelope breakdown and that the egg will still divide normally. After irradiation, spindle assembly, anaphase onset, chromosome movement, splitting of the spindle poles, cleavage, and nuclear envelope reformation proceed at a normal pace and remain properly coordinated with each other. This holds true whether spindle assembly starts right after nuclear envelope breakdown or at 14 min, a time when the egg would normally have been in late anaphase or early telophase.

The quantitative results of this work show that the time when the egg starts microtubule assembly determines when it will initiate anaphase, finish mitosis and enter the next cell cycle. For first or second division eggs, delays in microtubule assembly give equal delays in these events.

The timing of subsequent cell cycles shows that delays of microtubule assembly are not acting in a trivial sense by just delaying the expression of a few telophase events, leaving the basic mechanisms that drive the cell cycle untouched. Once the cycle is prolonged, following cell cycles do not resynchronize with the controls. Although delays of 14 min are small compared to the total cell cycle times of most cell types, delays of this length more than double the time from nuclear envelope breakdown to anaphase onset in these rapidly dividing eggs, and increase the total cell cycle duration by ~30%.

The conclusion that spindle microtubules are part of the mechanism that controls cell cycle timing is further strengthened by the observation that the quantity of microtubules in the spindle influences the timing of the cell cycle. Small spindles are still functional, but always take longer to reach anaphase than their normal counterparts. Significantly, there is a correlation between the size of the spindle and the time of anaphase onset. As before, the events that follow anaphase onset occur at a normal pace regardless of the actual time of anaphase onset.

Temporal Relationships

With respect to the onset of anaphase and subsequent cell cycles, Colcemid does not appear to arrest sea urchin eggs at "metaphase" as has been said for other types of cells (see references 16, 25, 30, 42, 43, 56, for examples). For at least 10–14 min after nuclear envelope breakdown, the temporal progression of mitosis does not begin until spindle microtubules start to assemble. Anaphase onset consistently follows the irradiation by 10.5 min, which is the normal interval between nuclear envelope breakdown and the start of anaphase for first division eggs. Also, the rate of microtubule assembly in the spindle upon irradiation is qualitatively normal regardless of the delay. For example, an egg irradiated when it should be at metaphase does not build a spindle any faster than a normal cell does after nuclear envelope breakdown.

However, chromosomes continue to condense after nuclear envelope breakdown even if microtubule assembly is prevented (this paper and references 5, 25, 49, 62). Normally, chromosome condensation may begin as early as the end of the S phase (59, 60, 62) and is observed to continue well into anaphase (5, 49). Thus, chromosome condensation does not determine the cell's prog-

ress through mitosis. Instead, it is an event that, once started, continues until nuclear envelope reformation.

The pattern of temporal relationships observed in this study shows that mitotic events are not rigidly linked to some timer mechanism. Instead, the portion of the cell cycle from nuclear envelope breakdown to anaphase onset is temporally flexible. Once anaphase has been initiated, however, events leading to and including the next nuclear envelope breakdown always go as a temporal linkage group. For delays up to 14 min, they remain properly coordinated and proceed at a normal pace. Thus, the cell is competent to execute and properly coordinate mitotic events well after the normal "clock" time.

These results argue against the possibility that the overall timing of the cell cycle in these eggs is governed solely by a continuous biochemical oscillator (38, 39, 52, 79), or by the linear readout of the genome (22, 24, 48). Microtubule assembly affects the duration of mitosis and this correspondingly changes the length of the cell cycle. Once the cell cycle is prolonged, it does not later return to its original schedule. The observation that the cell cycles of tissue culture cells can be synchronized by delaying the cells in mitosis with Colcemid and later washing out the drug (74, 75) indicates that this phenomenon is not unique to sea urchin eggs.

Also, not all the events of mitosis are part of a simple dependent sequence. Important mitotic events such as chromosome movements, cleavage, and karyomere fusion can be skipped, yet the cell cycle continues. The assembly of microtubules, as an event, is unusual in that it is not necessary for the cell cycle to continue, yet will influence its timing.

Prolonged Delays of Spindle Assembly

When Colcemid-treated eggs are irradiated more than 11 min after nuclear envelope breakdown, there is an increasing incidence of abnormal spindle development that indicates that the cells have spontaneously started to proceed through mitosis. This is evident in the cases where spindles develop split poles before anaphase and in cases where spindles start to assemble after irradiation, but then precociously fade away.

These cases are potentially interesting. Here are cases where the normal mitotic events of center splitting and spindle disassembly are experimentally put out of phase with other events of the cell cycle. However, these events do not remain out of phase in succeeding cell cycles; subsequent mitoses are of normal appearance and timing. Thus, the phasing of these mitotic events must be reset at each cell cycle.

When irradiated at the time karyomeres form, treated eggs recover little, if any, spindle structure. Only after karyomeres have broken down at the start of the next prometaphase will irradiation give immediate recovery of relatively large numbers of microtubules. These observations indicate that the cell's ability to assemble spindle microtubules continues to vary in proper coordination with the nuclear cycle even in the absence of microtubule assembly.

Force Production and the Time of Anaphase Onset

Several lines of reasoning indicate that the delays of anaphase onset observed in this study are a result of the role that microtubules have in the mechanisms that control timing, not the result of insufficient spindle microtubules to pull the chromosomes apart at the normal time. First, chromosomes do pop apart without any microtubules pulling on them (this report and references 42, 43, 54, 57). Secondly, unattached chromosomes or acentric fragments that lie outside the spindle are observed to split and separate slightly at the same time that the chromosomes in the spindle start their normal anaphase movements (4, 8, 9, 17, 18, 46, 49, 86). This suggests that a cell-wide influence, not force production by the spindle, determines when chromosomes can move apart in anaphase. Thirdly, relatively few microtubules are required to produce or transmit the force necessary for chromosome movement. Chromosomes or whole nuclei will move in the presence of only a few microtubules (1, 9, 15, 57, 66). Also, the tensile strength of a single microtubule should support the movement of a chromosome at normal rates (36). In the work presented here, the experimental eggs assembled what should have been more than enough microtubules to move chromosomes much earlier than they in fact did.

How Can Microtubule Assembly Influence Timing?

Conceivably, the cell could monitor how much of the cellular pool of tubulin has polymerized. This is unlikely since marine eggs use only a small

portion of the total cellular tubulin in the assembly of the first division spindle (19, 61, 77). A more likely possibility is that spindle microtubules provide the dividing cell with a structural framework that has the specific geometry necessary for the operation of some process of mitosis. If so, spindle microtubules would act as an essential structural cofactor that limits the rate at which this process can operate. For example, such a process could be the localization of substances within the dividing cell. Support for this hypothesis comes from micromanipulation experiments which show that the spatial arrangement of spindle microtubules influences the duration of mitosis (70).

Model

In summary, the observations of this study suggest a simple model for the way in which mitosis is integrated into the cell cycle: The cell has a fundamental rhythm that allows more time for mitosis than is actually used under normal circumstances. Starting with nuclear envelope breakdown, there is a waiting period that provides the cell with wide temporal tolerances to assemble the labile spindle structure and divide. Within this period, spindle microtubules are part of the mechanism that leads to a necessary event or physiological change that triggers the cell to execute the events that finish mitosis and start the next cell cycle. Once triggered, the cell cycle proceeds at a normal pace independent of microtubule assembly. At the next nuclear envelope breakdown, the timing of the cell cycle again becomes sensitive to the assembly of spindle microtubules. Thus, the cell cycle can be thought of as a stopwatch. Once the hand comes into the mitosis portion of the cycle, the watch can be reset to the start of the next cycle by a mechanism involving spindle microtubules. If these microtubules are not assembled, the watch will continue to cycle but does so at its fundamental rate.

The involvement of spindle microtubules in the timing of the cell cycle may be important to the cell. These microtubules are structural elements that are necessary for the precise alignment and separation of daughter chromosomes. Their role in the control of timing could be the way in which the cell ensures that spindle assembly and chromosome alignment are properly coordinated with anaphase onset, disassembly of the spindle, and the start of the next cell cycle.

It is a pleasure to express my gratitude to Drs. S. Inoué, H. Sato, and G. Ellis for their generous support with equipment and facilities. Their suggestions and criticisms have been valuable. The thoughtful help and encouragement of Drs. D. Begg, K. Fujiwara, E. Horn, D. Kiehart, L. Matthews, M. Mooseker, B. Nagle, and E. Salmon are gratefully acknowledged. Ralph and Bertha Woodward did a fantastic job caring for the sea urchins used in this study. I would especially like to express my appreciation for the invaluable technical assistance of S. T. Garland in the preparation of this manuscript. Finally, I am indebted to Dr. J. Aronson; his elegant work led me to think about the role of spindle microtubules in cell cycle timing.

Supported by National Science Foundation grant GB-31739 and National Institutes of Health (NIH) grant CA-10171 awarded to Dr. S. Inoué, and NIH ST01-GM00849-12 Predoctoral Traineeship to G. Sluder.

Submitted in partial fulfillment of the requirements for the Ph. D. degree at the University of Pennsylvania.

Received for publication 19 May 1978, and in revised form 20 November 1978.

REFERENCES

1. Aronson, J. 1971. Demonstration of a Colcemid-sensitive attractive force acting between the nucleus and a center. *J. Cell Biol.* **51**:579–583.
2. Aronson, J. F. 1973. Nuclear membrane fusion in fertilized *Lytechinus variegatus* eggs. *J. Cell Biol.* **58**:126–134.
3. Aronson, J., and S. Inoué. 1970. Reversal by light of the action of N-methyl N-desacetyl colchicine on mitosis. *J. Cell Biol.* **45**:470–477.
4. Bajer, A. 1958. Ciné-micrographic studies on chromosome movements in β-irradiated cells. *Chromosoma. (Berl.).* **9**:319–331.
5. Bajer, A. 1959. Change of length and volume of mitotic chromosomes in living cells. *Hereditas.* **45**:579–596.
6. Bajer, A., and J. Molè-Bajer. 1969. Formation of spindle fibers, kinetochore orientation, and behavior of the nuclear envelope during mitosis in endosperm, fine structural and *in vitro* studies. *Chromosoma (Berl.).* **27**:448–484.
7. Bajer, A., and J. Molè-Bajer. 1972. Spindle Dynamics and Chromosome Movements. Academic Press, Inc., New York.
8. Begg, D. A. 1975. Chromosome movement following detachment from the meiotic spindle by micromanipulation. *J. Cell Biol.* **67** (2, Pt. 2):25*a*. (Abstr.).
9. Begg, D. A. 1975. Micromanipulation studies of chromosome movement: the mechanical attachment of chromosomes to the spindle by birefringent chromosomal fibers and the behavior of chromosomes following detachment from the spindle. Ph. D. Thesis. University of Pennsylvania, Philadelphia, Pennsylvania.
10. Biesele, J. J. 1958. Mitotic poisons and the cancer problem. Elsevier Scientific Pub. Co., Amsterdam.
11. Borisy, G. G. 1971. Mechanism of U. V. reversal of colchicine inhibition of microtubule aggregation *in vitro*. Proceedings of the 11th Annual Meeting of the American Society for Cell Biology. **27**:35*a*. (Abstr.).
12. Borisy, G. G., J. B. Olmsted, and R. A. Klugman. 1972. *In vitro* aggregation of cytoplasmic microtubule subunits. *Proc. Natl. Acad. Sci. U. S. A.* **69**:2890–2894.
13. Borisy, G. G., J. B. Olmsted, J. M. Marcum, and C. Allen. 1974. Microtubule assembly *in vitro*. *Fed. Proc.* **33**:167–174.
14. Borisy, G. G., and E. Taylor. 1967. The mechanism of action of colchicine: binding of colchicine-^{3}H to cellular protein. *J. Cell Biol.* **34**: 525–533.
15. Brinkley, B. R., and R. B. Nicklas. 1968. Ultrastructure of the meiotic spindle of grasshopper spermatocytes after chromosome micromanipulation. *J. Cell Biol.* **39** (2, Pt. 2):16*a*. (Abstr.).
16. Brinkley, B. R., E. Stubblefield, and T. Hsu. 1967. The effects of Colcemid inhibition and reversal on the fine structure of the mitotic apparatus of Chinese hamster cells *in vitro*. *J. Ultrastruct. Res.* **19**:1–18.
17. Carlson, J. G. 1938. Mitotic behavior of induced chromosomal fragments in the neuroblasts of the grasshopper. *Proc. Natl. Acad. Sci. U. S. A.* **24**:500–507.

18. CARLSON, J. G. 1956. On the mitotic movements of chromosomes. *Science (Wash. D. C.).* **124:**203–206.
19. COHEN, W. D., and L. I. REBHUN. 1970. An estimate of the amount of microtubule protein in the isolated mitotic apparatus. *J. Cell Sci.* **6:** 159–276.
20. DAVIDSON, D., R. D. MACLEOD, and M. O'RIORDAN. 1966. Changes in mitotic index induced by colchicine. *Nature (Lond.).* **212:**1541–1542.
21. DEYSSON, G. 1968. Antimitotic substances. *Int. Rev. Cytol.* **24:**99–148.
22. DONACHIE, W. D., and M. MASTERS. 1969. Temporal control of gene expression in bacteria. *In* The Cell Cycle, Gene-Enzyme Interactions. G. M. Padilla, G. L. Whitson, and I. L. Cameron, editors. Academic Press Inc., New York. 37–76.
23. DUSTIN, P. 1963. New aspects of the pharmacology of antimitotic agents. *Pharmacol Rev.* **15:**449–480.
24. EHRET, C. F., and E. TRUCCO. 1967. Molecular models for the circadian clock. I. The chronon concept. *J. Theor. Biol.* **15:**240–262.
25. EIGSTI, O. J., and P. DUSTIN. 1955. Colchicine—in agriculture, medicine, biology and chemistry. Iowa State College Press, Ames, Iowa.
26. FUSELER, J. W. 1973. The effect of temperature on chromosome movement and the assembly-disassembly process of birefringent spindle fibers in actively dividing plant and animal cells. Ph. D. Thesis. University of Pennsylvania, Philadelphia, Pennsylvania.
27. FUSELER, J. W. 1973. Repetitive procurement of mature gametes from individual sea stars and sea urchins. *J. Cell Biol.* **57:**879–881.
28. GARLAND, D., and D. C. TELLER. 1975. A reexamination of the reaction between colchicine and tubulin. *Ann. N. Y. Acad. Sci.* **253:** 232–238.
29. GAULDEN, M. E., and J. C. CARLSON. 1951. Cytological effects of colchicine on the grasshopper neuroblast *in vitro* with special reference to the origin of the spindle. *Exp. Cell Res.* **2:**415–433.
30. GELFANT, S. 1963. Inhibition of cell division: a critical and experimental analysis. *Int. Rev. Cytol.* **14:**1–39.
31. HELL, E., and D. G. COX. 1963. Effects of colchicine and Colcemid on synthesis of deoxyribonucleic acid in the skin of guinea pig's ear *in vitro*. *Nature (Lond.).* **197:**287–288.
32. HIRAMOTO, Y. 1956. Cell division without mitotic apparatus in sea urchin eggs. *Exp. Cell Res.* **11:**630–636.
33. HIRAMOTO, Y. 1965. Further studies on cell division without mitotic apparatus in sea urchin eggs. *J. Cell Biol.* **25:**161–167.
34. HOLM, G., and A. BAJER. 1966. Ciné-micrographic studies on mitotic spiralization cycle. *Hereditas.* **54:**356–375.
35. HYYPIO, P. A., T. M. TSOU, and G. B. WILSON. 1955. Some notes on the "c-mitotic" action of colchicine and technical lindane. *Cytologia (Tokyo).* **20:**166–176.
36. INOUÉ, S., and H. RITTER. 1975. Dynamics of mitotic spindle organization and function. *In* Molecules and Cell Movement. S. Inoué and R. E. Stephens, editors. Raven Press, New York. 3–30.
37. INOUÉ, S., and H. SATO. 1967. Cell motility by labile association of molecules: the nature of mitotic spindle fibers and their role in chromosome movement. *J. Gen. Physiol.* (Suppl.) **50:**259–292.
38. KAUFFMAN, S. 1974. Measuring a mitotic oscillator: the arc discontinuity. *Bull. Math. Biol.* **36:**171–182.
39. KAUFFMAN, S., and J. J. WILLE. 1975. The mitotic oscillator in *Physarum polycephalum*. *J. Theor. Biol.* **55:**47–93.
40. KIHLMAN, B. A. 1966. Actions of chemicals on dividing cells. Prentice-Hall, Inc., Englewood Cliffs, N. J.
41. KLEINFELD, R. G., and J. E. SISKEN. 1966. Morphological and kinetic aspects of mitotic arrest by and recovery from Colcemid. *J. Cell Biol.* **31:**369–379.
42. LEVAN, A. 1938. The effect of colchicine on root mitoses in *Allium*. *Hereditas.* **24:**471–486.
43. LEVAN, A. 1954. Colchicine-induced c-mitosis in two mouse ascites tumors. *Hereditas.* **40:**1–64.
44. LIMA-DE-FARIA, A. 1955. The division cycle of the kinetochore. *Hereditas.* **41:**238–240.
45. LIMA-DE-FARIA, A., and S. BOSE. 1962. The role of telomeres at anaphase. *Chromosoma (Berl.).* **13:**315–327.
46. LUYKX, P. 1970. Cellular Mechanisms of Chromosome Distribution. Academic Press, Inc., New York.
47. MCGILL, M., and B. R. BRINKLEY. 1975. Human chromosomes and centrioles as nucleating sites for the *in vitro* assembly of microtubules from bovine brain tubulin. *J. Cell Biol.* **67:**189–199.
48. MASTERS, M., and A. B. PARDEE. 1965. Sequence of enzyme synthesis and gene replication during the cell cycle of *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U. S. A.* **54:**64–70.
49. MAZIA, D. 1961. Mitosis and the physiology of cell division. *In* The Cell, Biochemistry, Physiology, Morphology, vol. 3. J. Brachet and A. Mirsky, editors. Academic Press, Inc., New York.
50. MAZIA, D. 1974. Chromosome cycles turned on in unfertilized sea urchin eggs exposed to NH_4OH. *Proc. Natl. Acad. Sci. U. S. A.* **71:** 690–693.
51. MAZIA, D., P. J. HARRIS, and T. BIBRING. 1960. The multiplicity of the mitotic centers and the time course of their duplication and separation. *J. Biophys. Biochem. Cytol.* **7:**1–20.
52. MITCHISON, J. M. 1971. Biology of the cell cycle. Cambridge University Press, Cambridge, England.
53. MIZEL, S. B., and L. WILSON. 1972. Nucleoside transport in mammalian cells. Inhibition by colchicine. *Biochemistry* **11:**2573–2578.
54. MOLÈ-BAJER, J. 1958. Ciné-micrographic analysis of c-mitosis in endosperm. *Chromosoma (Berl.).* **9:**332–358.
55. MONROY, A., and G. MONTALENTI. 1947. Variations of the submicroscopic structure of the cortical layer of fertilized and parthenogenetic sea urchin eggs. *Biol. Bull.* **92:**151–161.
56. MUELLER, G. A., M. E. GAULDEN, and W. DRANE. 1971. The effects of varying concentrations of colchicine on the progression of grasshopper neuroblasts into metaphase. *J. Cell Biol.* **48:**253–268.
57. NICKLAS, R. B. 1971. Mitosis. *In* Advances in Cell Biology, Vol. 2. D. M. Prescott, L. Goldstein, and E. McConkey, editors. Appleton-Century-Croft, New York. 225–297.
58. NICKLAS, R. B., and C. A. KOCH. 1972. Chromosome micromanipulation. IV. Polarized motions within the spindle and models for mitosis. *Chromosoma (Berl.).* **39:**1–26.
59. PEDERSON, T. 1972. Chromatin structure and the cell cycle. *Proc. Natl. Acad. Sci. U. S. A.* **69:**2224–2228.
60. PEDERSON, T., and E. ROBBINS. 1972. Chromatin structure and the cell division cycle: actinomycin binding in the synchronized HeLa cells. *J. Cell Biol.* **55:**322–327.
61. RAFF, R. A., and J. F. KAUMEYER. 1973. Soluble microtubule proteins of the sea urchin embryo: partial characterization of the proteins and behavior of the pool in early development. *Dev. Biol.* **32:**309–320.
62. RAO, P. N., and R. T. JOHNSON. 1974. Induction of chromosome condensation in interphase cells. *Adv. Cell Mol. Biol.* **3:**136–190.
63. RAPPAPORT, R. 1971. Cytokinesis in animal cells. *Int. Rev. Cytol.* **31:** 169–213.
64. RAPPAPORT, R. 1975. Establishment and organization of the cleavage mechanism. *In* Molecules and Cell Movement. S. Inoué and R. Stephens, editors. Raven Press, New York. 287–304.
65. ROSENBAUM, J. L., and K. CARLSON. 1969. Cilia regeneration in *Tetrahymena* and its inhibition by colchicine. *J. Cell Biol.* **40:**415–425.
66. SALMON, E. D. 1975. Spindle microtubules: thermodynamics of *in vivo* assembly and role in chromosome movement. *Ann. N. Y. Acad. Sci.* **253:**383–406.
67. SATO, H., G. W. ELLIS, and S. INOUÉ. 1975. Microtubular origin of mitotic spindle form birefringence: demonstration of the applicability of Weiner's equation. *J. Cell Biol.* **67:**501–517.
68. SLUDER, G. 1976. Experimental manipulation of the amount of tubulin available for assembly into the spindle of dividing sea urchin eggs. *J. Cell Biol.* **70:**75–85.
69. SLUDER, G. 1976. Spindle microtubules and the cell cycle in the eggs of the sea urchin *Lytechinus variegatus*. Ph. D. Thesis. University of Pennsylvania, Philadelphia, Pennsylvania.
70. SLUDER, G., and D. A. BEGG. 1975. Influence of spindle geometry on the timing of morphological events in the mitotic cycle. *J. Cell Biol.* **67:** (2, Pt. 2):406*a*. (Abstr.).
71. SOKAL, R. R., and F. J. ROHLF. 1969. Biometry. The principles and practice of statistics in biological research. W. H. Freeman & Company Publishers. San Francisco.
72. STADLER, J., and W. W. FRANKE. 1974. Characterization of the colchicine binding of membrane fractions from rat and mouse liver. *J. Cell Biol.* **60:**297–303.
73. STEPHENS, R. E. 1972. Studies on the development of the sea urchin *Strongylocentrotus droebachiensis*. II. Regulation of mitotic spindle equilibrium by environmental temperature. *Biol. Bull.* **142:**145–159.
74. STUBBLEFIELD, E., and R. KLEVECZ. 1965. Synchronization of Chinese hamster cells by reversal of Colcemid inhibition. *Exp. Cell Res.* **40:** 660–664.
75. STUBBLEFIELD, E., R. KLEVECZ, and L. DEAVAN. 1967. Synchronized mammalian cell cultures. I. Cell replication cycle and macromolecular synthesis following brief Colcemid arrest of mitosis. *J. Cell. Physiol.* **69:**345–354.
76. SWANN, M., and J. M. MITCHISON. 1953. Cleavage of sea urchin eggs in colchicine. *J. Exp. Biol.* **30:**506–513.
77. TAYLOR, E. W. 1965. The mechanism of colchicine inhibition of mitosis. I. Kinetics of inhibition and the binding of H^3-colchicine. *J. Cell Biol.* **25:**145–160.
78. TILNEY, L. G. 1971. Origin and continuity of microtubules. *In* Origin and Continuity of Cell Organelles. J. Reinert and H. Ursprung, editors. Springer-Verlag, Berlin. 222–260.
79. TYSON, J., and S. KAUFFMAN. 1975. Control of mitosis by a continuous biochemical oscillation: synchronization; spatially inhomogeneous oscillations. *J. Math. Biol.* **1:**289–310.

80. WILSON, L. 1975. Microtubules as drug receptors: pharmacological properties of microtubule protein. *Ann. N. Y. Acad. Sci.* **253**:213–231.
81. WILSON, L., J. R. BAMBURG, S. B. MIZEL, L. M. GRISHAM, and K. M. CRESWELL. 1974. Interaction of drugs with microtubule proteins. *Fed. Proc.* **33**:158–166.
82. WILSON, L., and J. BRYAN. 1974. Biochemical and pharmacological properties of microtubules. *Adv. Cell Mol. Biol.* **3**:21–72.
83. WILSON, L., and M. FRIEDKIN. 1967. The biochemical events of mitosis. II. The *in vivo* and *in vitro* binding of colchicine in grasshopper embryos and its possible relation to inhibition of mitosis. *Biochemistry* **6**:3126–3135.
84. ZEUTHEN, E. 1951. Segmentation, nuclear growth and cytoplasmic storage in eggs of echinoderms and amphibia. *Pubbl. Sta. Zool. Napoli.* **23**:47–69.
85. ZIMMERMAN, A. M., and S. ZIMMERMAN. 1967. Action of Colcemid in sea urchin eggs. *J. Cell Biol.* **34**:483–488.
86. ZIRKLE, R. E. 1956. Cellular Aspects of Basic Mechanisms in Radiobiology. Nuclear Science Series Report, No. 8, Research Council, Washington, D. C., Publication No. 450, p. 1.