Centriole Number and the Reproductive Capacity of Spindle Poles

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ABSTRACT The reproduction of spindle poles is a key event in the cell's preparation for mitosis. To gain further insight into how this process is controlled, we systematically characterized the ultrastructure of spindle poles whose reproductive capacity had been experimentally altered. In particular, we wanted to determine if the ability of a pole to reproduce before the next division is related to the number of centrioles it contains. We used mercaptoethanol to indirectly induce the formation of monopolar spindles in sea urchin eggs. We followed individually treated eggs in vivo with a polarizing microscope during the induction and development of monopolar spindles. We then fixed each egg at one of three predetermined key stages and serially semithick sectioned it for observation in a high-voltage electron microscope. We thus know the history of each egg before fixation and, from earlier studies, what that cell would have done had it not been fixed. We found that spindle poles that would have given rise to monopolar spindles at the next mitosis have only one centriole whereas spindle poles that would have formed bipolar spindles at the next division have two centrioles. By serially sectioning each egg, we were able to count all centrioles present. In the twelve cells examined, we found no cases of acentriolar spindle poles or centriole reduplication. Thus, the reproductive capacity of a spindle pole is linked to the number of centrioles it contains. Our experimental results also show, contrary to existing reports, that the daughter centriole of a centrosome can acquire pericentriolar material without first becoming a parent. Furthermore, our results demonstrate that the splitting apart of mother and daughter centrioles is an event that is distinct from, and not dependent on, centriole duplication.

At the end of mitosis, each new daughter cell receives a single spindle pole from the parent cell and by the start of the next mitosis, each of these cells has two, and only two, spindle poles. This reproduction of spindle poles must be tightly controlled by the cell since the wrong number of poles would inevitably lead to aneuploidy and a consequent loss of viability for the progeny of that cell. The mechanisms that are involved in the precise doubling of the spindle poles before each mitosis are poorly understood.
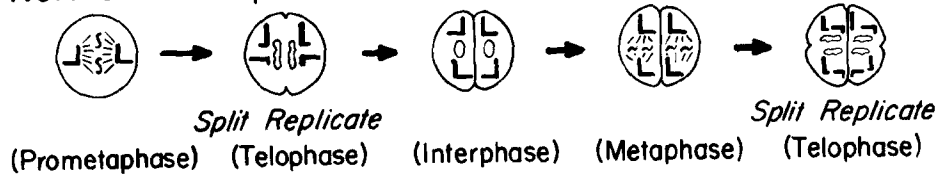
A number of studies have shown that the reproduction of spindle poles can be experimentally manipulated in sea urchin eggs (15, 28, 29a). When the first mitosis of the fertilized egg is prolonged by any of several techniques (mercaptoethanol, colcemid, or micromanipulation), the two existing spindle poles are observed to split and separate to form a tetrapolar spindle (29a). Although these four poles appear normal, the

subsequent development of the daughter cells shows that these poles have only half the reproductive capacity of normal poles. Each of the four daughters that result from the tetrapolar division form only a monopolar spindle at the next mitosis.
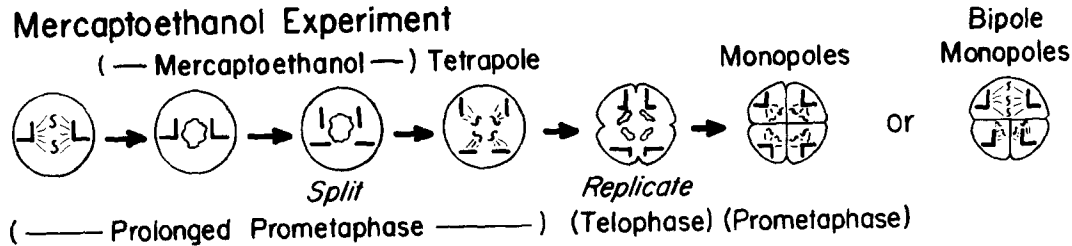
These monopolar spindles are truly half of a spindle because two of them can come together to give a bipolar spindle of normal appearance and function (15, 29). Subsequent divisions of the progeny of such cells are normal. Thus, monopolar spindles have one pole with full reproductive capacity.

When a monopolar spindle is formed individually in its own cell, that cell often spends significantly more time in mitosis than normal (29). In such cases, the single pole is observed to split to form two poles that separate and transform the monopolar spindle into a bipolar spindle of normal appearance and function (28, 29a). Although the two new poles appear normal, they have only half the normal reproductive

A. Normal Development

Split Replicate
(Prometaphase) (Telophase)   (Interphase) (Metaphase) (Telophase)

B. Mercaptoethanol Experiment

(— Mercaptoethanol —) Tetrapole   Monopoles   Bipole Monopoles

Split   Replicate
(—— Prolonged Prometaphase ——) (Telophase) (Prometaphase)

C. Development of Monopolar Spindles (scale enlarged)

Split
(Prolonged Prometaphase)   (Telophase) (Interphase) (Prometaphase)

—→ Normal Development

Split Replicate
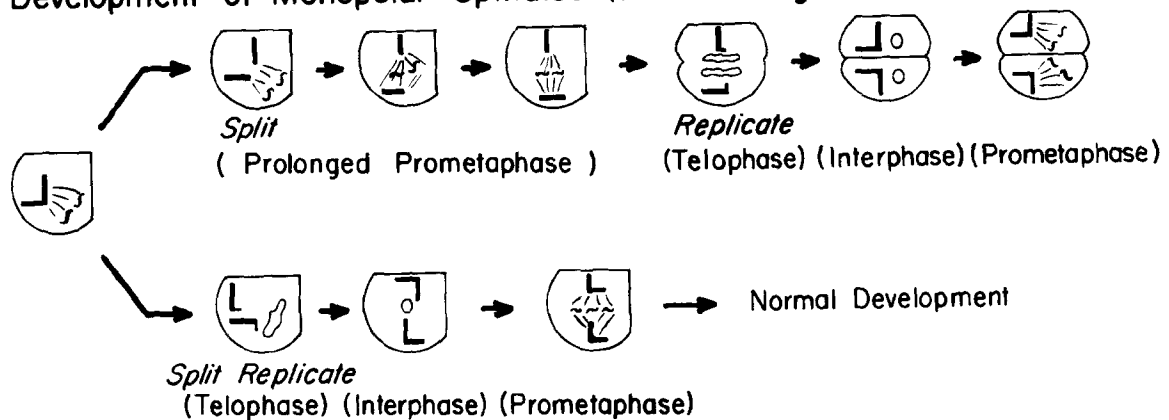(Telophase) (Interphase) (Prometaphase)

FIGURE 1   Pattern of the splitting and duplication of centriole pairs in normal or mercaptoethanol-treated eggs.

capacity since the daughter cells resulting from this division each form only monopolar spindles at the next division (28, 29a).

The observations outlined above have provided important information on the functional properties of the reproductive mechanisms for spindle poles. However, the structural and molecular bases of these phenomena have remained a mystery. It has been suggested (15, 28) that the splitting and duplication of centriole pairs (diagrammed in Fig. 1) could explain the phenomena described above. However, no evidence has been obtained to support this possibility because the large size of the sea urchin egg has precluded a conventional ultrastructural analysis of this system (13).

Methods using semithick serial sections have recently been developed that allow for the systematic ultrastructural reconstruction of selected areas from large cells or groups of cells (24, 25). We reasoned that these same methods could be used to systematically characterize the ultrastructure of sea urchin egg spindle poles at key stages during the induction and development of monopolar spindles.

In the study described here, we followed individual sea urchin eggs in vivo with a polarization microscope after release from mercaptoethanol. These same individual eggs were then fixed on the coverslip at one of three key stages, embedded, and serially semithick sectioned for observation with a high-voltage electron microscope. Our major goal was to determine if the reproductive capacity of a spindle pole is

related to the number of centrioles it contains. In effect, this experimental system provided a unique opportunity to test whether the centriole would play any role in controlling the reproduction of spindle poles. If we could find cases of acentriolar spindle poles or of spindle poles containing more than two centrioles, then we would have convincing evidence that the reproduction of spindle poles could be uncoupled from the reproduction of centrioles.

Several features of our correlative light microscope and high-voltage electron microscope approach are worth noting. By following each experimental egg in vivo, we knew its prior history. This information and the existing characterization of this experimental system allowed us to know how each of these eggs would have developed had it not been fixed. Our high-voltage electron microscope approach then enabled us to serially reconstruct, within a reasonable period of time, all of the spindle poles in each experimental cell. Finally, we know that the ultrastructure we observed must reflect the true properties of the eggs and not some toxic side-effect of the mercaptoethanol treatment, because identical monopolar spindles can be directly induced by other unrelated methods (29a).

MATERIALS AND METHODS

and fertilized in natural sea water. The fertilization envelopes were subsequently removed by first adding 3 vol of Ca^{++}-free sea water to the egg suspension and then by passing the eggs through a 102-μm mesh "Nitex" screen (Tetko, Inc., Elmsford, NY). The fertilized, demembranated eggs were then cultured in Ca^{++}-free sea water at 20°C.

Mercaptoethanol Treatments: Fertilized eggs were immersed, at first prometaphase, in 0.1 M mercaptoethanol (Sigma Chemical Co., St. Louis, MO) in Ca^{++}-free sea water and left in this solution until a separate control culture reached second metaphase. The mercaptoethanol was then washed out by several changes of sea water. After 5-10 min, the eggs were spread on a cleaned coverslip that had been treated with 3-5% protamine sulfate (Sigma Chemical Co.) in distilled water. This anchored the cells to the coverslip. The coverslip was then inverted, placed on a drop of FC47 fluorocarbon oil (3M, Inc., St. Paul, Minn.), and sealed as previously described (27).

Light Microscopy, Fixation, and Embedding: Individual eggs were observed with a Zeiss ACM microscope (Carl Zeiss, Inc., Thornwood, NY) modified for polarization microscopy. Selected eggs were photographed on Kodak Plus X film and these eggs were marked by scratching a circle onto the top of the coverslip with a diamond scribe. For fixation, the coverslip was removed from the preparation and immediately immersed in a dish of 20°C 1% OsO$_4$ buffered to pH 6.1 with 0.4 M Na acetate (8). After 5 min, the fixative was cooled to 0°C for 90 min. The fixed eggs, still attached to the coverslip, were washed in 0.4 M Na acetate buffer (pH 6.1), dehydrated in a graded series of ethanol, and flat embedded in Epon-Araldite.

Electron Microscopy: Eggs previously followed in vivo were relocated within the embedded cultures with the aid of the reference scribe marks and low-power reference photomicrographs. Each was again circled with a diamond scribe on the Epon Araldite side of the coverslip. This allowed the selected eggs to be relocated after the coverslip was removed with a brief cold (4°C) hydrofluoric acid treatment (22). Each egg of interest was then excised from the embedded culture, mounted on an Epon peg, and serially sectioned with a diamond knife to a thickness of 0.25 μm. The ribbons of sections were mounted on Formvar (0.5%) coated slot grids (21) and subsequently stained in uranyl acetate (3.5%; 60°C for 90 min) and lead citrate (23°C for 30 min).

Slot grids containing serial sections from a given egg were then placed section side up on a clean microscope slide. This allowed each ribbon of sections to be screened for content with a 40× phase contrast objective (23). Those grids containing serial sections through the spindle and centrosomes (each of which was clearly visible under the 40× objective) were then examined and photographed at 800-1,000 kV with the New York State Department of Health 1.2 MeV AEI-EM7 high-voltage electron microscope employing a 30-μm objective aperture.

The 0.25-μm sections used in this study proved advantageous in several respects. First, an egg containing a tetrapolar spindle could be completely sectioned in 275-325 sections whereas those blastomeres containing monopolar spindles or bipolarized-monopoles required only 150-190 sections. A similar ultra-thin (silver-gold) section study would require at least 1,000 sections through each tetrapolar egg and 500 sections through each blastomere. Thus, three eggs could be serially sectioned in the time it would normally take to serially thin-section one egg. Second, ribbons of 0.25-μm-thick (stained) sections could be quickly prescreened for centrosomes and chromosomes by phase contrast light microscopy (23). This eliminated the need to examine nonuseful sections with the electron microscope.

RESULTS

Tetrapolar Spindles

A typical egg with a mercaptoethanol-induced tetrapolar spindle is shown in the inset of Fig. 2. The photograph was taken a few minutes before this cell was fixed for electron microscopy. The low-power survey micrograph shows the ultrastructure of the spindle region of this same cell. This section includes three of the four centrosomes (i.e., *I-III*);

centrosome *IV* was found ~1.0 μm below this section. It is not uncommon for the four poles of a tetrapole to try to establish an orthogonal arrangement relative to each other. The spindle region appears in sections as an area clear of large yolk granules and mitochondria. The centrosomes are visible as distinctive electron-opaque regions, ~2-2.5 μm in diameter, against a background of microtubules, membranous elements, and ribosomes. By the time this egg was fixed, it was well into anaphase as evidenced by the fact that chromosomes surrounded by nuclear envelopes (karyomeres) were seen in the vicinity of all centrosomes (e.g., arrows in Fig. 2).

Serial sections through each of the four centrosomal regions in this egg are shown in Fig. 3, *I-IV*. Each centrosome was found to consist of a single centriole surrounded by an irregular cloud of pericentriolar material ~2-3 μm in diameter. These centrioles were ~0.2-0.25 μm in diameter, 0.25-0.35 μm in length, and consisted of the usual nine triplet microtubule blades embedded in an amorphous electron matrix. Numerous microtubules radiated from the extensive cloud of pericentriolar material which surrounded each centriole. This pericentriolar material consisted of a moderately electron-opaque diffuse fibro-granular material that contained numerous small but discrete (50-100 nm) electron-opaque aggregates (Fig. 3, *I-IV*). On the electron microscope screen it appeared that centrosomal microtubules terminated in or arose from these discrete aggregates (in this respect, see reference 17). However, attempts to convincingly validate this impression, by reconstructing the centrosome from serial thin sections, have as yet proven unsuccessful.

We serially sectioned portions of mercaptoethanol-induced tetrapolar spindles in five eggs from two females and reconstructed 15 centrosomes. For two cells we serially sectioned through all four poles. In all cases, only one centriole was found in each centrosomal region.

Monopolar Spindles

Individual eggs were followed with the polarization microscope as they recovered from the mercaptoethanol treatment. They invariably assembled tetrapolar spindles, completed anaphase, and then cleaved directly into three or four blastomeres. At the next nuclear envelope breakdown, the resultant blastomeres formed monopolar spindles. Functional studies (15, 28, 29a) show that these monopolar spindles behave as half of a spindle; that is, two monopoles in the same cytoplasm can come together to form a functional bipolar spindle of normal appearance.

Fig. 4 shows two cells from the same embryo, each of which contains a monopolar spindle. The living cells are shown, a few minutes before fixation, in Fig. 4a. These same cells are also pictured in plastic (Fig. 4b) and in a 0.25-μm section (Fig. 4c). Serial sections through the sizable centrosomal region in the left-hand cell are shown in Fig. 5L, *a-h* while those through the sizable centrosome in the right-hand cell

Figures 2 and 3 Low power electron micrograph of a section through the late anaphase spindle from the tetrapolar cell photographed before fixation (inset). The four centrosomal regions (*I-IV*) of this cell are reconstructed in Fig. 3, *I-IV*. Note that three (*I-III*) centrosomal regions are visible within this one section. Arrows point to chromosomes surrounded by nuclear envelope. 10 μm per scale division for inset (× 200). Bar, 4.0 μm for electron micrograph. × 9,900. Fig. 3 shows consecutive serial sections through the four centrosomes of the cell shown in Fig. 2. I, II, III, and IV sections through centrosomes I, II, III, and IV, respectively. Each centrosome contains only one centriole which is shown by arrowheads in those sections that contain portions of the centriole. Bars, 1.0 μm. × 22,000.
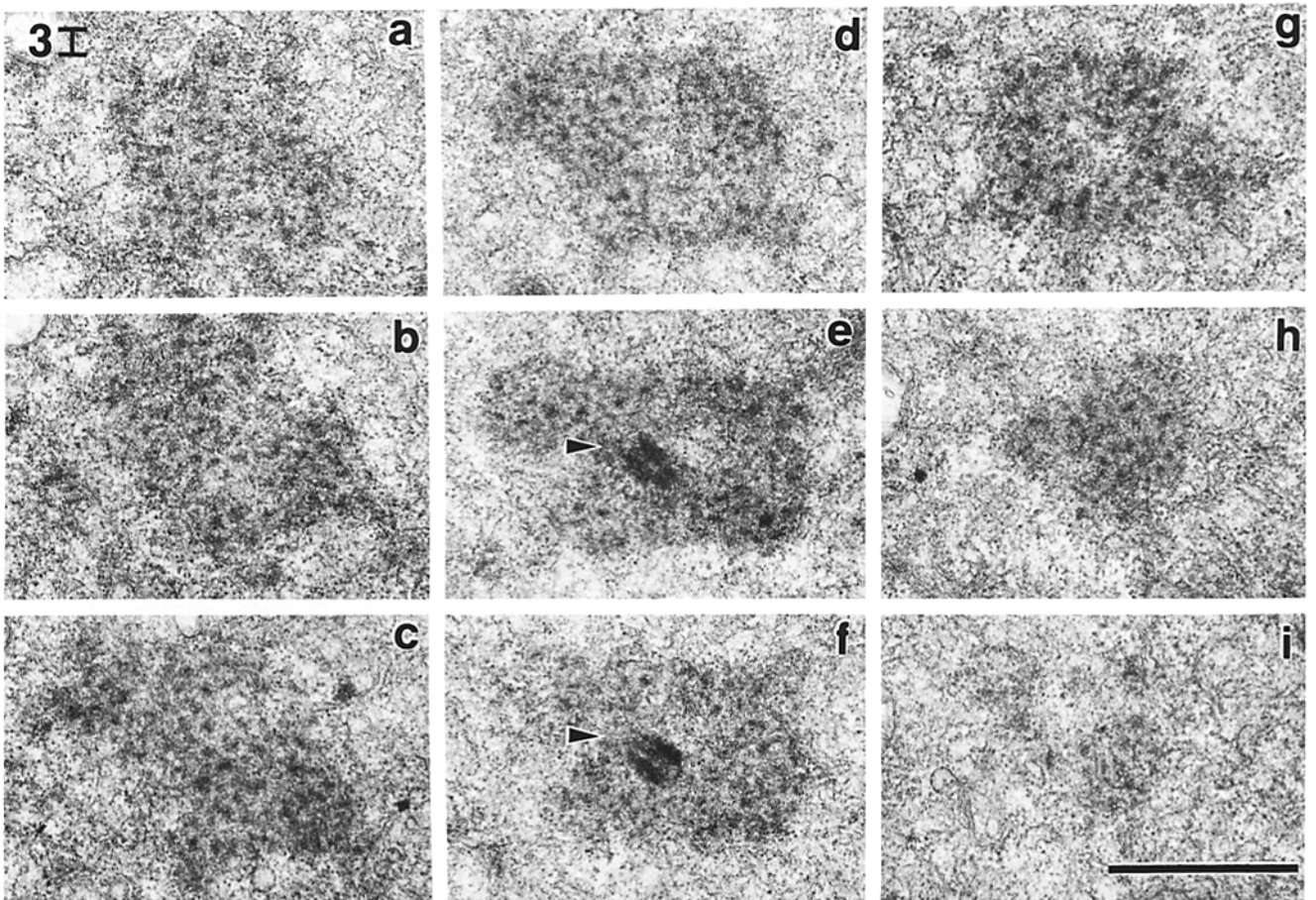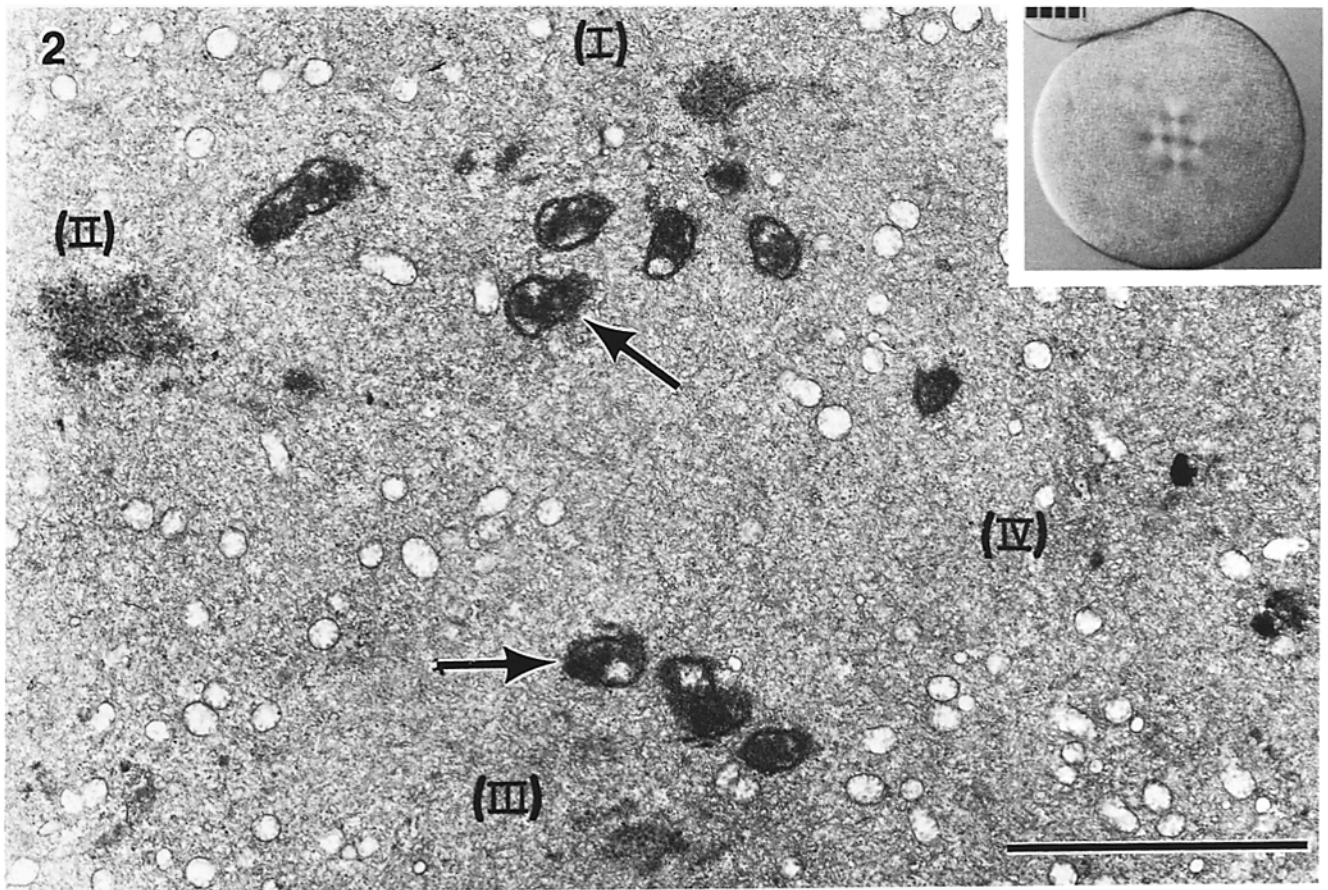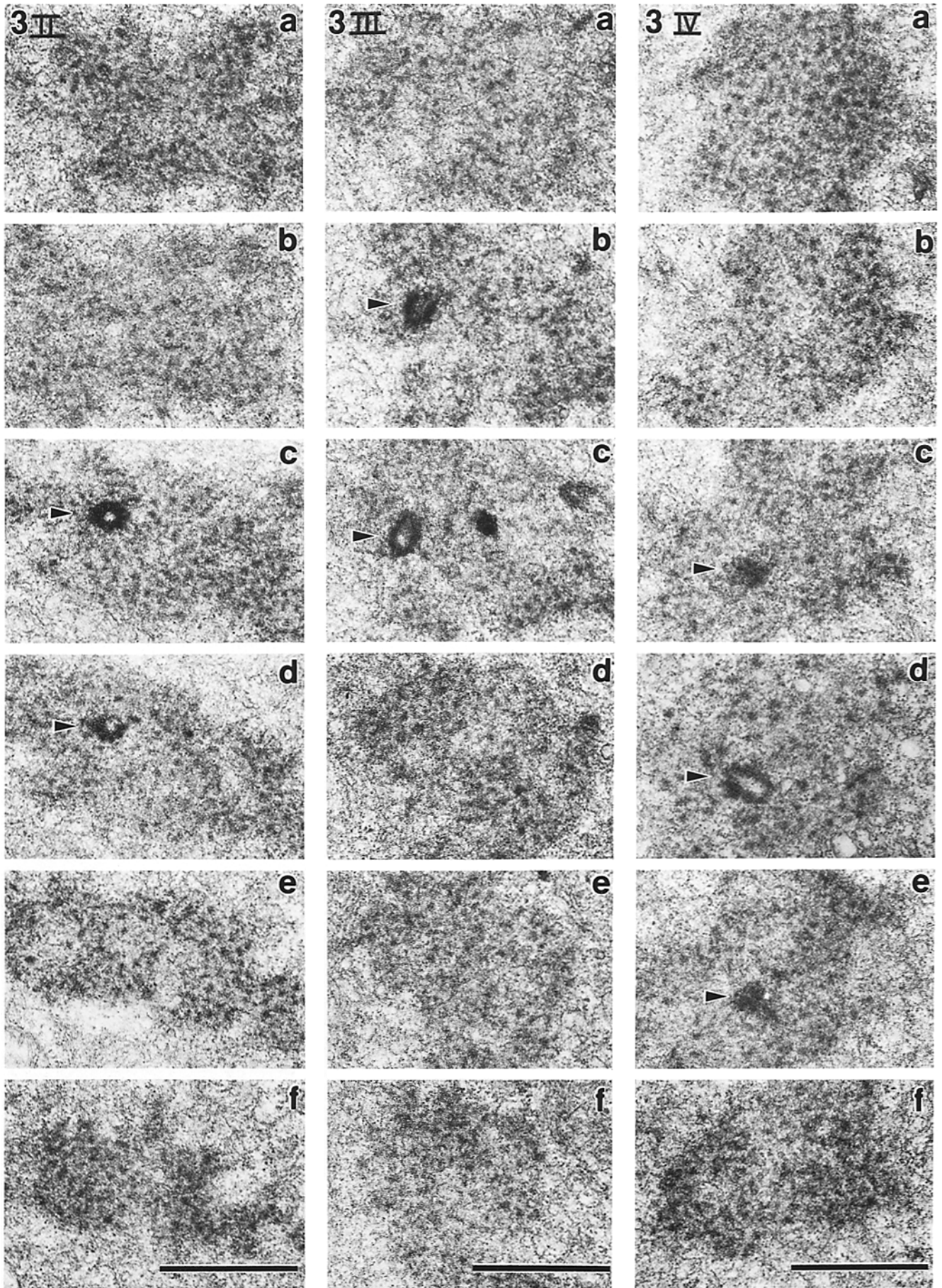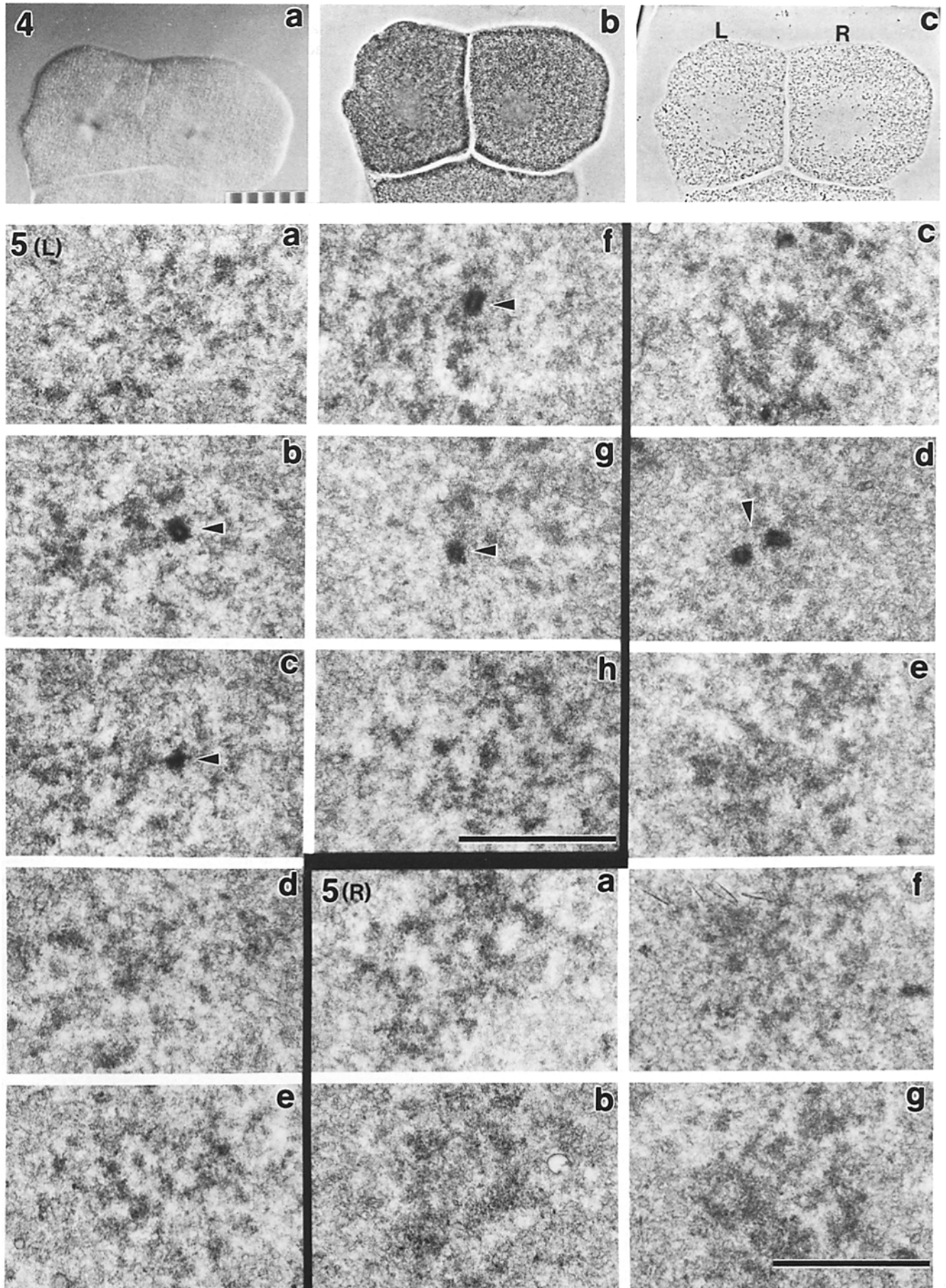
are shown in Fig. 5R, *a–g*. Each centrosome is observed to contain two centrioles (arrows in Fig. 5). Sections above and below those presented in Fig. 5 contained no additional centrioles. The irregular cloud of pericentriolar material around the centriole pair was ~2–3 μm in diameter.

For this portion of the study, we serially sectioned four cells with monopolar spindles from two embryos. In all cases, two centrioles were found in each centrosomal region.

Bipolarized-Monopolar Spindles

If a blastomere with a monopolar spindle (such as those shown in Fig. 4) stays in mitosis long enough, the aster is observed to split and the two resulting asters move apart to give a bipolar spindle of normal appearance and function. Functional studies indicate that these two poles have only half the normal reproductive capacity since monopolar spindles are always formed at the next division (28, 29a).

We photographed the development of individual blastomeres that clearly had monopolar spindles. Such cells were followed throughout the splitting and bipolarization of the monopolar spindle. At the appropriate moment, selected cells were circled, fixed, and embedded for electron microscopy. Thus, we were certain of the prior history of each cell that we examined with the electron microscope.

The development of one such cell is shown in Fig. 6, *a–d*. Serial sections through both of the polar areas of this cell are shown in Fig. 7I, *a–d* and 7II, *a–d*. In both cases, there is only one centriole in each centrosome (arrows). (Note: Sections prior to the first one shown in Fig. 7II *a* were inspected in the electron microscope and contained no additional centrioles. Unfortunately they were lost when the Formvar film broke during photography.)

Additional documentation of the bipolarization of a monopole is shown in Fig. 8, *a–c*, and serial sections of one centrosome from this cell are shown in Fig. 9, *a–d*. This centrosome, and the one not pictured, both contained only one centriole. Fig. 8 *d* is a survey phase-contrast photomicrograph of a slot grid containing ribbons of stained sections through a bipolarized monopole. This micrograph demonstrates the good visibility of the spindle region, even at this low magnification, in 0.25-μm thick sections.

For this portion of the study, we serially sectioned three cells and examined all six centrosomes. In two cells, which were at metaphase, we found only one centriole in each centrosome. The size of the cloud of pericentriolar material at each pole of these cells (1–2 μm) was about half that found in tetrapolar and monopolar spindles. The third cell was in early telophase and both centrosomes contained one full-sized centriole and a small daughter centriole (data not shown). By fixing this cell late in mitosis, we could see centriole duplication. This is consistent with the observation that the polar organizers in fertilized sea urchin eggs normally duplicate during telophase (15).

DISCUSSION

Previous functional studies on this experimental system (15, 16, 29a) have made the important distinction between the spindle pole, as it is seen in the microscope, and the entity that organizes the pole. This entity, which we call the polar organizer, is the essential determinant around which the cell elaborates structures of the centrosome. The polar organizer is not the pole itself but the kernel of macromolecules that determine where a pole will form and how it reproduces. The number of active polar organizers in a cell thus determines the number of spindle poles that a cell will have. Thus far, the polar organizer has been defined only in operational terms as an organizing activity whose reproduction can be indirectly altered by a number of techniques (15, 16, 20, 28).

In the present study, we experimentally manipulated the reproduction of spindle poles and sought to determine if any structure in the spindle pole showed the same pattern of reproduction as the polar organizer. We found that the number of centrioles at a pole coincides with the reproductive capacity of that pole. Cells that will form bipolar spindles at the next division receive a pole with two centrioles from the preceding division. Cells that form monopolar spindles at the next mitosis receive a pole with only one centriole. In the 12 cells examined, we found no cases of reduplication of the centrioles during the mercaptoethanol treatment, nor did we find cases of acentriolar spindle poles.

Our present data, and the results of preceding functional studies (15, 28, 29a), support the model shown in Fig. 1 for centriole reproduction and distribution during the induction and development of monopolar spindles. In normal sea urchin egg mitosis (Fig. 1*A*), the mother and daughter centrioles split and duplicate concurrently in telophase. This corresponds to the time when the polar organizer splits and duplicates as determined by functional criteria (15). When the eggs are treated with mercaptoethanol (Fig. 1*B*), the prometaphase-metaphase portion of mitosis is prolonged and the mother and daughter centrioles in each of the two centrosomes split apart but do not duplicate. Upon release from the drug, the four single centrioles separate with the four poles. When the egg cleaves into four, each daughter receives a spindle pole containing only one centriole. This centriole then duplicates at telophase, producing a single diplosome identical to that found at a single normal spindle pole. Thus, at the next nuclear envelope breakdown, all four daughter cells form only a monopolar spindle. Given this information, it is not surprising that two monopolar spindles can come together to give a functional bipolar spindle whose poles reproduce normally in all subsequent divisions (15, 28, 29a).

Once formed, a monopolar spindle can proceed down one of two lines of further development (Fig. 1*C*). If a cell with a monopolar spindle stays in mitosis long enough, the single diplosome splits and one centriole separates with each of the two new poles, giving a bipolar spindle with a single centriole

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FIGURES 4 and 5   (4*a*) Two cells of the same embryo, both of which have assembled a monopolar spindle. Photograph was taken of the live cells just before fixation. (*b*) The same cells photographed with phase-contrast optics after embedding. (*c*) The same cells seen in section. 10 μm per scale division. × 300. Fig. 5 shows consecutive serial sections through the centrosomal region of the cells shown in Fig. 4. (5L, *a–h*) Micrographs of the centrosome in the left-hand cell. (5R, *a–g*) Micrographs of the centrosome in the right-hand cell. The centrosome region of each cell contains a pair of centrioles shown by arrowheads in the appropriate sections. Bar, 2.0 μm. × 14,500.
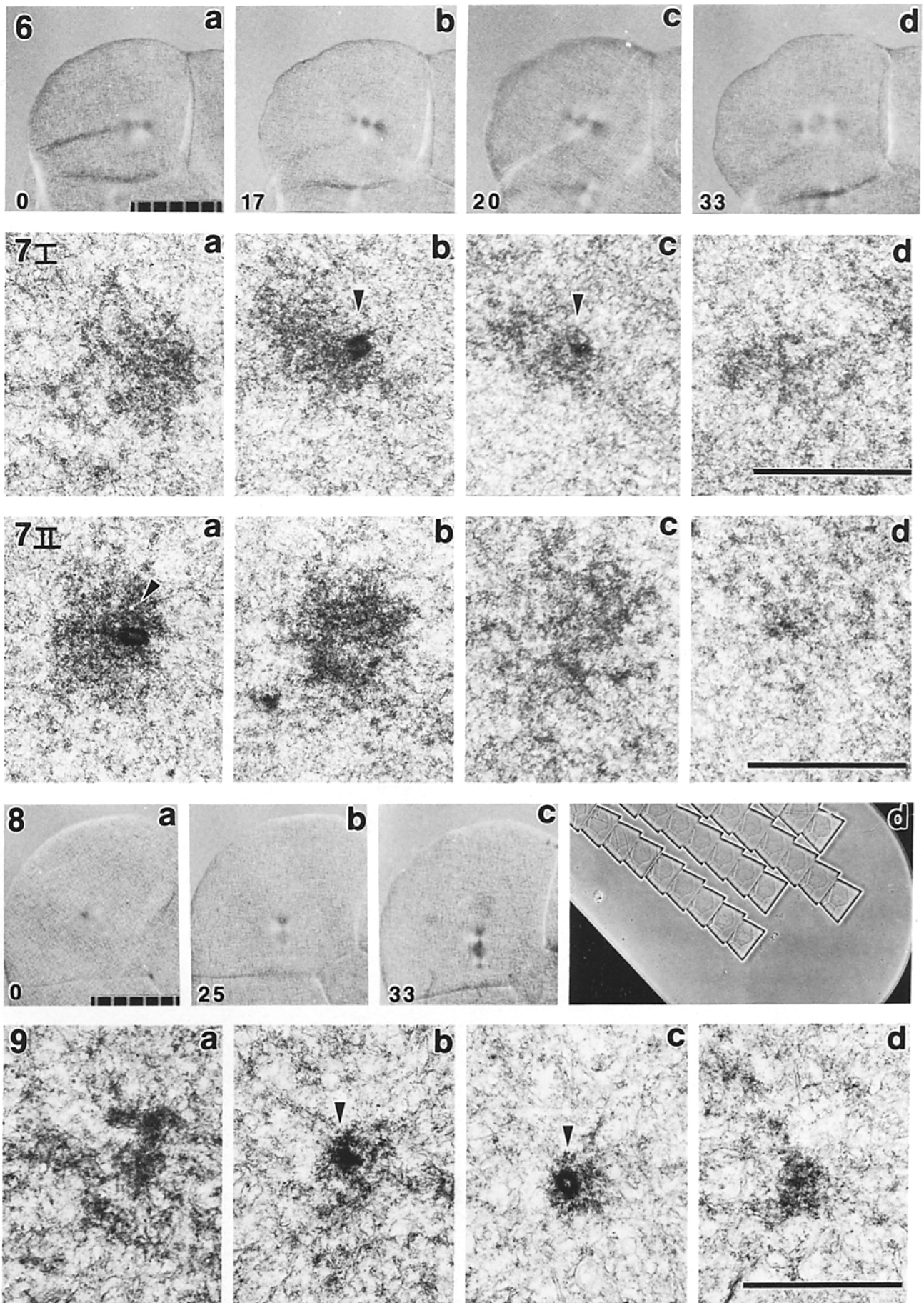
at each pole (Fig. 1C; upper). When the cell cleaves, each daughter receives only one centriole, which duplicates before the next mitosis. The poles of the two daughter cells are now the same as that of the original parent; each has one diplosome and forms only one spindle pole at the next mitosis. This sequence may then be repeated. Alternatively, a cell with a monopolar spindle may enter telophase before the diplosome splits (Fig. 1C; lower). Since the spindle never bipolarizes, the cell does not cleave and a single nucleus reforms. Concurrently, the two centrioles split apart and duplicate, forming two diplosomes associated with one nucleus. At the next mitosis, a normal bipolar spindle forms.

Role of the Centriole in Spindle Pole Formation

Perhaps the most interesting question raised by our work concerns the possible role of the centriole in controlling the formation and reproduction of the spindle poles. The hypothesis that the centriole could play a causal role in the formation and reproduction of the spindle pole is supported by three lines of evidence. First, the reproduction of poles is temporally coincident with the reproduction of centrioles (15, 26). Second, the results of our present study show that the reproductive capacity of a spindle pole is coincident with the number of centrioles it contains. Third, isolated basal bodies or partially purified centrioles from sperm will induce asters in a roughly dose-dependent fashion when microinjected into amphibian or sea urchin eggs (7, 9, 10, 11, 14).

These latter studies show that this aster-inducing activity must be tightly associated with the isolates because it survives the rigors of both isolation and injection. Importantly, the isolated basal bodies do not form asters when put into tubulin-containing solutions—they simply show biased growth of microtubules off of the existing nine triplet structures (10, 30). Also, the basal bodies or centrioles are not associated with asters before isolation; they are serving as basal bodies of flagellae in nondividing cells. The observation that their aster-organizing activity is RNAase sensitive (10, 11) raises the intriguing possibility that the RNAase sensitive fibers found inside basal bodies (4) and centrioles (1, 20) might play a role in organizing the spindle pole.

Thus, whatever organizes the spindle pole seems to be spatially, mechanically, and functionally associated with the centriole when it is present. Does this show that the centriole is the polar organizer? The answer to this question may depend upon how one chooses to define the centriole. If it is simply the nine triplet microtubules only (32), then the centriole clearly does not organize the spindle pole, and the polar organizer is only associated with the nine triplet microtubules.

There is ample evidence to show that the centriole, by this definition, is not needed for pole formation, function, or reproduction. For example, cells of all higher plants and some animals divide without recognizable centrioles (reviewed in 13, 19, 20, 23). Even a mutant cell line from Drosophila has been described that divides without visible centrioles (3).

On the other hand, if one defines the centriole as the complex of structures bounded by the triplet array, then some centriolar component, other than the nine triplet microtubules, might be responsible for pole formation and reproduction. This would most easily explain why isolated basal bodies and centrioles can induce supernumerary asters upon injection. Thus, the centriole, by this definition, should play a key role in mitosis. In this respect, the absence of visible centrioles in the poles of certain cell types would only mean that the nine triplet microtubule portion of the centriole is missing. Other centriolar components such as the core structures could be present and functional; they would simply not be visible in thin section without something to signal their location.

In fact, the notion that cells can have polar organizers in a cryptic form is not without precedent. When treated with hypertonic sea water, sea urchin eggs can assemble large numbers of cytasters by the time of first mitosis. These cytasters contain centrioles and can act as spindle poles (5, 12, 17, 18). In addition, Fulton and Dingle (6) have demonstrated that the ameboid form of Naegeleria can stably propagate indefinitely without visible centrioles, even at mitosis. Yet, under appropriate conditions, this organism transforms into a flagellated form and assembles only two typical basal bodies per cell, presumably from cryptic precursors.

CENTRIOLE REPLICATION AND SPLITTING: Under normal circumstances, the splitting of parent and daughter centrioles is approximately coincident with procentriole formation (22, 26, 31). This temporal coincidence could be interpreted to indicate that the molecular events occurring at the inception of the procentriole are the same ones that split the mother from the daughter. However, our present work demonstrates that the splitting of the centriole pair is an event that is distinct and separable from centriole replication; the two events can be put permanently out of phase with each other. Centriole splitting and separation can occur while the cell is in the prolonged prometaphase/metaphase portion of mitosis; procentriole formation is never observed until telophase. Thus, centriole replication, as seen by procentriole formation, is not a prerequisite for the splitting of the parent from daughter centrioles. Thus, the centriole cycle, under these experimental conditions, shows identical properties to the reproductive cycle of polar organizers.

FIGURES 6–9 Fig. 6 shows polarized light micrographs of a monopolar spindle splitting and bipolarizing. Minutes after the first photograph are shown in the lower corner of each frame. This cell was fixed immediately after the last photograph was taken. Sections of both centrosomes are shown in Fig. 7. 10 μm per scale division. × 290. Fig. 7, I and II shows consecutive serial sections through both centrosomes of living cells shown in Fig. 6. Each centrosome contains only one centriole (arrowheads). In Fig. 7II, the sections before the first (a) were inspected visually but were not photographed. These sections contained no centriolar profiles. See text for details. Bar, 2.0 μm. × 14,500. Fig. 8, a–c shows polarized light micrographs of a monopolar spindle undergoing a transition into a bipolar spindle. Minutes after the first photograph are shown in the lower corner of each frame. the cell was fixed immediately after the last photograph was taken and one of its centrosomal regions is shown in Fig. 9. Fig. 8d is a survey phase-contrast micrograph of four ribbons of serial sections on a slot grid. These sections, cut from a cell that underwent a monopolar-bipolar transition, have been stained with uranyl and lead. 10 μm per scale division. × 290. Fig. 9 shows consecutive serial sections through one of the centrosomes from the cell pictured in Fig. 8. Note the presence of a single centriole (arrowheads in b and c). The second centrosome from this cell also contained a single centriole (data not shown). See text for details. Bar, 2.0 μm. × 14,500.

CENTRIOLE MATURATION: The interrelationship between the mother and daughter centriole, as well as the time course of maturation for the daughter centriole, have been matters of uncertainty. Recent ultrastructural analyses of the centriole cycle in cultured animal cells have shown that daughter centrioles do not seem to become associated with visible amounts of pericentriolar material until they become parents themselves (2, 22, 31). These observations suggest that daughter centrioles are immature, and may not be a functional part of the spindle pole until they split from their mothers and replicate a full cell cycle later. In this respect our present results, as well as those of another recent study (13), show that daughter centrioles can acquire functional pericentriolar material in the same cell cycle as they are "born" in, provided that the mitotic cycle is prolonged experimentally. Thus, the appearance of pericentriolar material in association with the daughter centriole is not linked to centriole replication, as has been suggested, but appears instead to be dependent on the splitting of mother and daughter from each other.

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