INDEPENDENCE OF CENTRIOLE FORMATION AND DNA SYNTHESIS

J. B. RATTNER and STEPHANIE G. PHILLIPS

From the Departments of Biology and Anatomy, Washington University, St. Louis, Missouri 63130

ABSTRACT

The temporal relationship between cell cycle events and centriole duplication was investigated electron microscopically in L cells synchronized by mechanically selecting mitotic cells. The two mature centrioles which each cell received at telophase migrated together from the side of the telophase nucleus distal to the stem body around to a region of the cytoplasm near the stem body and then into a groovelike indention in the early G_1 nucleus, where they were found throughout interphase. Procentrioles appeared in association with each mature centriole at times varying from 4 to 12 h after mitosis. Since S phase was found to begin on the average about 9 h after mitotic selection, it appeared that cells generated procentrioles late in G_1 or early in S. During prophase, the two centriolar duplexes migrated to opposite sides of the nucleus and the daughter centrioles elongated to the mature length. To ascertain whether any aspect of centriolar duplication was contingent upon nuclear DNA synthesis, arabinosyl cytosine was added to mitotic cells at a concentration which inhibited cellular DNA synthesis by more than 99%. Though cells were thus prevented from entering S phase, the course of procentriole formation was not detectibly affected. However, cells were inhibited from proceeding to the next mitosis, and the centriolar elongation and migration normally associated with prophase did not occur.

INTRODUCTION

Independence of Centriole Formation and DNA Synthesis

In animal cells, a pair of centrioles normally occurs at each pole of a mitotic spindle. Thus, one pair of centrioles is regularly distributed to each daughter cell of a mitotic division. During interphase, precisely two new centrioles must be produced in order to repeat the allocation of one pair of centrioles per daughter cell at the next mitotic division. It is evident that the process of centriole production is under strict control; neither more nor fewer than two new centrioles are regularly formed during each intermitotic interphase.

The research to be described in this paper is concerned with the timing of centriolar formation and its coordination with other cell cycle events. It has been reported that in cultured mammalian cells, mature centrioles are first seen in association with newly forming "daughters" early in S phase (Robbins et al., 1968; Stubblefield, 1968; Brinkley and Stubblefield, 1970). It seemed possible that centriole formation might be coordinated in some way with nuclear DNA synthesis. Our experiments were designed to test whether new centrioles would form in the absence of DNA synthesis. We found that procentriole formation appeared to occur during G_1 in some cells. Elongation of the daughter centriole and migration of the centriolar duplexes to specific sites in the cytoplasm were found to be closely correlated with mitotic events. In cells inhibited from synthesizing DNA, pro-

centrioles appeared to form normally, but the centriolar events correlated with mitosis did not occur. In addition, our observations have provided some new information on the substructure of centrioles and their associated structures.

MATERIALS AND METHODS

Cell Maintenance and Synchronization

Experiments were performed on L929 cells (an established mouse cell line) which were initially obtained from the American Type Culture Collection. Cells were routinely grown as monolayer cultures in glass bottles and were subcultured by trypsinization every 4 days. They were maintained in Joklik-modified minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, N. Y.) with 10% fetal calf serum in the presence of 5% CO_2.

Experiments were performed on subconfluent cultures growing in roller bottles in 40 ml of medium. The technique for harvesting mitotic cells, modified from that of Terasima and Tolmach (1963), was to swirl the medium in the roller bottle so as to cause a shearing force over the cells. To get rid of debris, this was repeated twice at 1 h intervals before beginning an experiment. Thereafter, mitotic cells were harvested at intervals of 90 min. Mitotic indexes were ascertained for every sample at the time of harvest by fixing an aliquot of the cells with 3 parts methanol: 1 part acetic acid, air drying the cells on slides, and staining them with Giemsa. Mitotic indexes of cells obtained in this manner were routinely higher than 80%. Cultures with lower mitotic indexes were discarded. Immediately after harvest, mitotic cells were centrifuged into a pellet, resuspended by pipetting, and plated into 35 × 10 mm Falcon plastic Petri dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.). All operations were performed at 37°C.

For determination of the time until the onset of S phase, mitotic cells were plated onto glass cover slips inside of the plastic Petri dishes. Cover slip cultures were fixed with 3 parts methanol:1 part acetic acid at 2-h intervals. [^{3}H]thymidine (Schwarz Bio Research Inc., Orangeburg, N. J.), 3 Ci/mM, was added at a concentration of 4 μCi/ml for the last 15 min before fixation. Cover slips were extracted with 5% TCA at 4°C for 12 min, washed, and air dried. Mounted cover slips were autoradiographed using Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.), and the percent nuclei labeled was scored in 100 cells in each of three preparations for each time point.

Electron Microscope Analysis

Monolayer cultures growing on plastic were fixed *in situ* at intervals after mitosis with 3% glutaraldehyde in 0.1 M collidine buffer at pH 7.4. After 1 h the cultures were washed twice with collidine buffer and postfixed for 1–2 h in 1% osmium tetroxide solution buffered as above. The cultures were then dehydrated in a graded ethanol series and embedded in Epon 812 (Brinkley et al., 1967).

Epon disks obtained by the procedure described above were examined using a 100 × phase, oil immersion objective lens. Selected regions were scored with a diamond marker, cut from the Epon disk with a cork borer, and cemented on an Epon blank. Subsequently the blank was trimmed to approximately 0.5 × 0.5 mm. Serial sections in the silver range were cut on a LKB ultramicrotome III (LKB Instruments, Inc., Rockville, Md.) and collected on either copper-slotted grids coated with a 1% nitrocellulose in amylacetate solution or on 200-mesh copper grids. They were examined in a Siemens Elmiskop I microscope operated at 60 kV.

Synchronous cultures fixed at the following times after mitosis were examined by electron microscopy. Untreated controls: 4, 6, 8, 10, 12, 15, 18, 20, 25, and 40 h. Arabinosyl cytosine-treated cultures (described below): 4, 6, 10, 12, 15, 18, 20, 22, 25, and 40 h. The morphology of the centriolar complement and its position in the cell were evaluated in 20–40 cells at each time point.

Inhibition of DNA Synthesis

To inhibit DNA synthesis, arabinosyl cytosine (a gift of the Upjohn Co., Kalamazoo, Mich.) at a concentration of 10 μg/ml was added to mitotic cells at the time they were harvested and plated into Petri dishes. The drug remained in the cultures until they were fixed for electron microscopy. Experiments to demonstrate that arabinosyl cytosine was effective in inhibiting DNA synthesis in L929 cells were performed in the following manner: [^{3}H]thymidine (sp act 3 Ci/mM; 2 μCi/ml) was added to L929 cells freshly suspended into spinner cultures at a density of 5 × 10^5 cells/ml. Arabinosyl cytosine, 10 μg/ml, was added to half the cultures 30 min before the addition of isotope. 1 ml samples were removed at intervals and added to 9 ml of cold (0°C) Hanks' balanced salt solution. The cells were pelleted in the cold, extracted with 5% TCA at 4°C for 10 min, washed in 80% ethanol, and dissolved in 0.4 ml Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.). Replicate samples were incubated with DNase (Worthington Biochemical Corp., Freehold, N. J.), 0.2 mg/ml, for 2 h at 37°C before TCA extraction. Samples were counted in 10 ml scintillation fluid in a Packard Tri-Carb scintillation counter (Packard Instrument Co., Inc.).

RESULTS

The Cell Cycle

The doubling time of log phase L929 cells under routine culturing conditions in our labora-

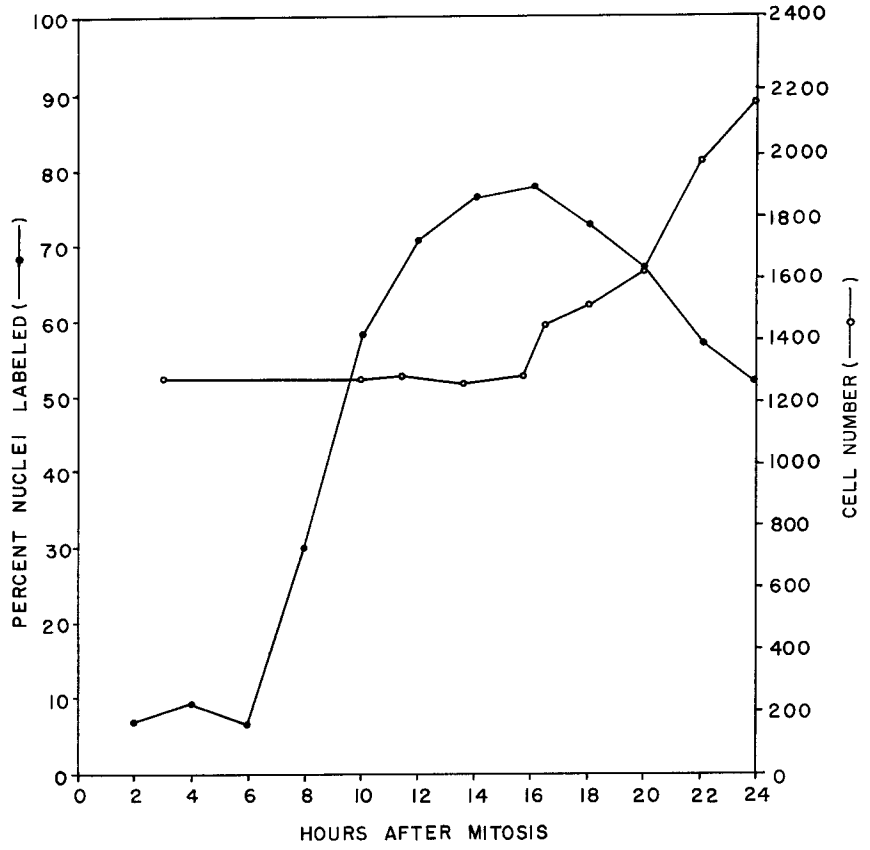


FIGURE 1 Cultures synchronized by mitotic selection were incubated with [^{3}H]thymidine for 15 min before fixation. Percent nuclei labeled (closed circles) was scored in autoradiographs of cultures fixed at indicated times after mitosis. 300 cells were scored for each time point. Cell number (open circles) was scored in similarly synchronized cell cultures by direct counts of living cells in ten preselected fields at various time points after mitosis.

tory is 17.5 h; however, growth was somewhat slower under the conditions of these experiments. That is, mitotic cells which had been agitated, centrifuged, and resuspended by pipetting, and plated out at low densities seemed to require slightly longer than expected to return to interphase. The doubling time of the population under experimental conditions, as determined by direct microscopic count of living cells, was about 19 h. The average time before onset of DNA synthesis, determined by scoring the percent of nuclei which incorporated [^{3}H]thymidine at various times after mitotic harvest, was about 9–10 h (Fig. 1).

The Centriole Cycle in L Cells

At prometaphase, a pair of centrioles was positioned at each spindle pole (Fig. 2). In prometaphase and metaphase cells, where sections transected both centrioles of a pair longitudinally, the daughter centriole appeared as long as the parent (0.55 μm). The daughter centriole was perpendicular to the parent and its tubules terminated 250 Å from the wall of the parent centriole.

Late in telophase, the pair of mature centrioles occupied a position within a cuplike concavity in the newly formed nucleus; this concavity occurred on the far side of the nucleus with reference to the midbody. In very early G_1 cells, the centriolar duplex was frequently found in the cytoplasm adjacent to the midbody (Fig. 3). The migration of the centriolar duplex across the cell occurred in early G_1 cells which were in transition from a rounded-up to a more elongate shape. Examples of early G_1 cells with centrioles in intermediate positions were found with low frequency. The

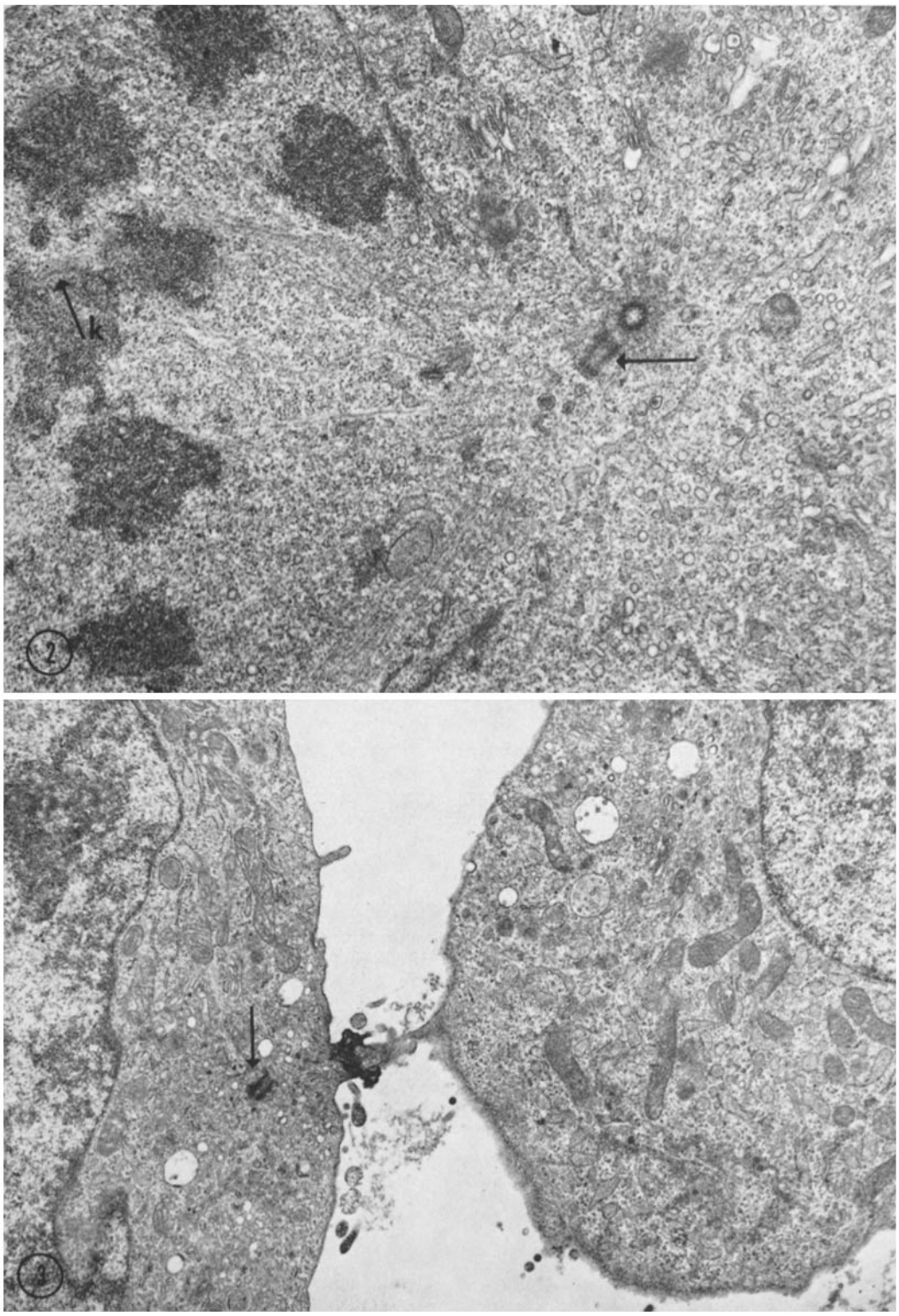


FIGURE 2 Late prometaphase cell. Mature daughter centriole (arrow) is 0.55 μm in length. (*k*) kinetochore. × 24,600.

FIGURE 3 Early G_1 cell 3 h after mitosis. The centrioles (arrow) have migrated from the telophase position at the far side of the nucleus into a region of cytoplasm near the stem body. × 12,300.

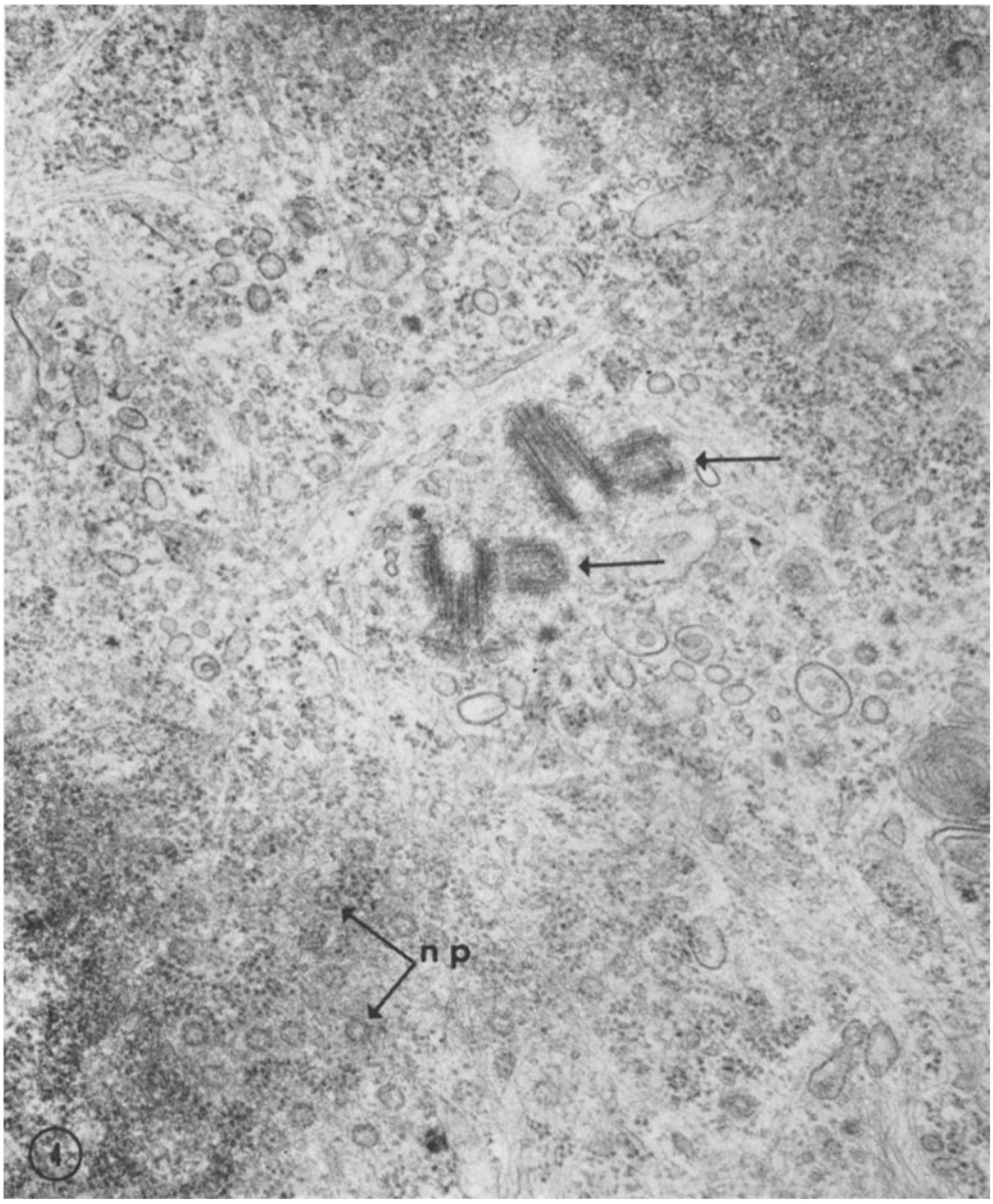


FIGURE 4 Cell fixed 4 h after mitosis. Two centriolar duplexes are located in an invagination on the nuclear surface. (*np*) nuclear pores. Each daughter centriole (arrow) is approximately 0.25 μm in length. × 41,200.

forces responsible for the centriolar migration from one side of the nucleus to the other were not obvious; the pericentriolar cytoplasm was relatively free of microtubules and microfilaments in these very early G_1 cells.

By 4 h after mitosis, centrioles were sometimes found in a depression which usually (in about 85% of cells examined) occurred in the top of the nucleus (Fig. 4), where the bottom of the cell was considered to be the surface attached to the plastic. At later times after mitosis, the frequency of cells with centrioles in the nuclear concavity increased; in cells fixed 10 h after mitosis, the centrioles were almost always found there. They

remained in this nuclear invagination until the onset of prophase (see Figs. 5, 7, 9). The cytoplasm adjacent to the nuclear invagination contained a network of microfilaments, microtubules, and small electron-opaque bodies (Figs. 4, 5). The electron-opaque bodies appeared as two morphological types: (*a*) 400–500 Å diameter fibrillar spheres (pericentriolar satellites) (as described by Robbins et al., 1968; and Brinkley and Stubblefield, 1970) which were found in the region of the centriole and were commonly associated with microtubules extending to the parent centriole and adjacent cytoplasm. (*b*) 150–200 Å irregularly shaped foci, less electron-opaque than the pericentriolar satellites, which were dispersed in the cytoplasm farther from the centrioles and were associated simultaneously with microtubules and microfibrils (Fig. 5). Both satellites and foci were observed in the centriolar region from late G_1 through G_2.

Before the centriolar duplex moved into the nuclear invagination in early G_1, the two centrioles occurred at right angles to one another. In cells in which the centrioles occurred in the invagination, the two centrioles were separated. When they were about 0.8 μm apart, four striated "arms" similar to those described in centrioles generating cilia (Sorokin, 1962) were seen extending radially from the outside wall of each centriole. The tips of these arms were associated with several microtubles (Fig. 6 *a*, *b*). The microtubules extended laterally several micra into the adjacent microtubular-microfibrillar network. This network was in evidence through S and G_2 (Fig. 7).

In cells fixed 4–6 h after mitosis, procentrioles were often seen at right angles to each mature centriole (Fig. 4). By 12 h most of the cells contained procentrioles. That is, in cultures fixed 4 h after mitosis, sections which passed through centrioles included procentrioles in four out of 39 cases. Procentrioles were found in 14 out of 65 cases at 6 h and in 33 out of 39 cases at 12 h after mitosis. The process of procentriole formation apparently occurred quite rapidly, for procentrioles less than 0.25 μm in length were infrequently observed. Thus, in L cells, procentrioles were most often formed 6–12 h after mitosis as structures similar to the parent centriole but about half its length. They remained this length throughout S phase and G_2 (Fig. 8).

At the initiation of prophase condensation, the two centriolar pairs lost their association with the nuclear invagination and began to migrate toward opposite sides of the nucleus. Centrioles of early prophase cells were devoid of the striated appendages seen on interphase centrioles and an organized microfilament network was not in evidence around the centriole at this stage (Figs. 9, 10). In cells in which the nuclear membrane had begun to break down (prometaphase), the centriole pairs were found on opposite sides of the nucleus and numerous microtubules were aligned between them (Figs. 10, 11).

The daughter centrioles of prophase cells were somewhat longer than in S and G_2 (0.35–0.37 μm). Elongation of the daughter centriole apparently began with the onset of prophase as it was seen in some cells in which the chromatin was condensed but the two centriole duplexes had not yet moved apart. Elongated daughters were not seen in cells in which chromatin condensation was not apparent. Centriolar elongation appeared to proceed slowly throughout prophase; daughter centrioles had achieved the mature length by prometaphase (Figs. 9, 10, 11). (The time-course of centriole elongation is summarized in Fig. 12.)

Inhibition of DNA Synthesis and the Centriole Cycle

Arabinosyl cytosine at 10 μg/ml was found to inhibit almost all incorporation of [^{3}H]thymidine into TCA-precipitable, DNase-removable material in L cells (Fig. 13). This result was confirmed in autoradiographs of monolayer cultures. Cell cycle experiments were performed in which mitotic cells were plated immediately into medium containing arabinosyl cytosine and were maintained in that medium until they were fixed. The cells thus could not initiate DNA synthesis. Cells were fixed for electron microscopy at various intervals up to 40 h after collection of mitotic cells. The sequence and timing of events in centriole movement into an invagination at the top of the nucleus, separation of the two mature centrioles, and daughter centriole formation were the same as in control cells. That is, in cultures treated with arabinosyl cytosine, by 4 h after mitosis, cells were seen (four out of 20 cases) in which the two mature centrioles were located in a depression on the top (the side facing away from the Petri dish) of the nucleus and had moved apart. By 8 h after mitosis, procentrioles were often observed. 18 h after mitosis, procentrioles were observed in almost all cells (20 out of 21) in which mature centrioles were observed. The group of four centrioles, two

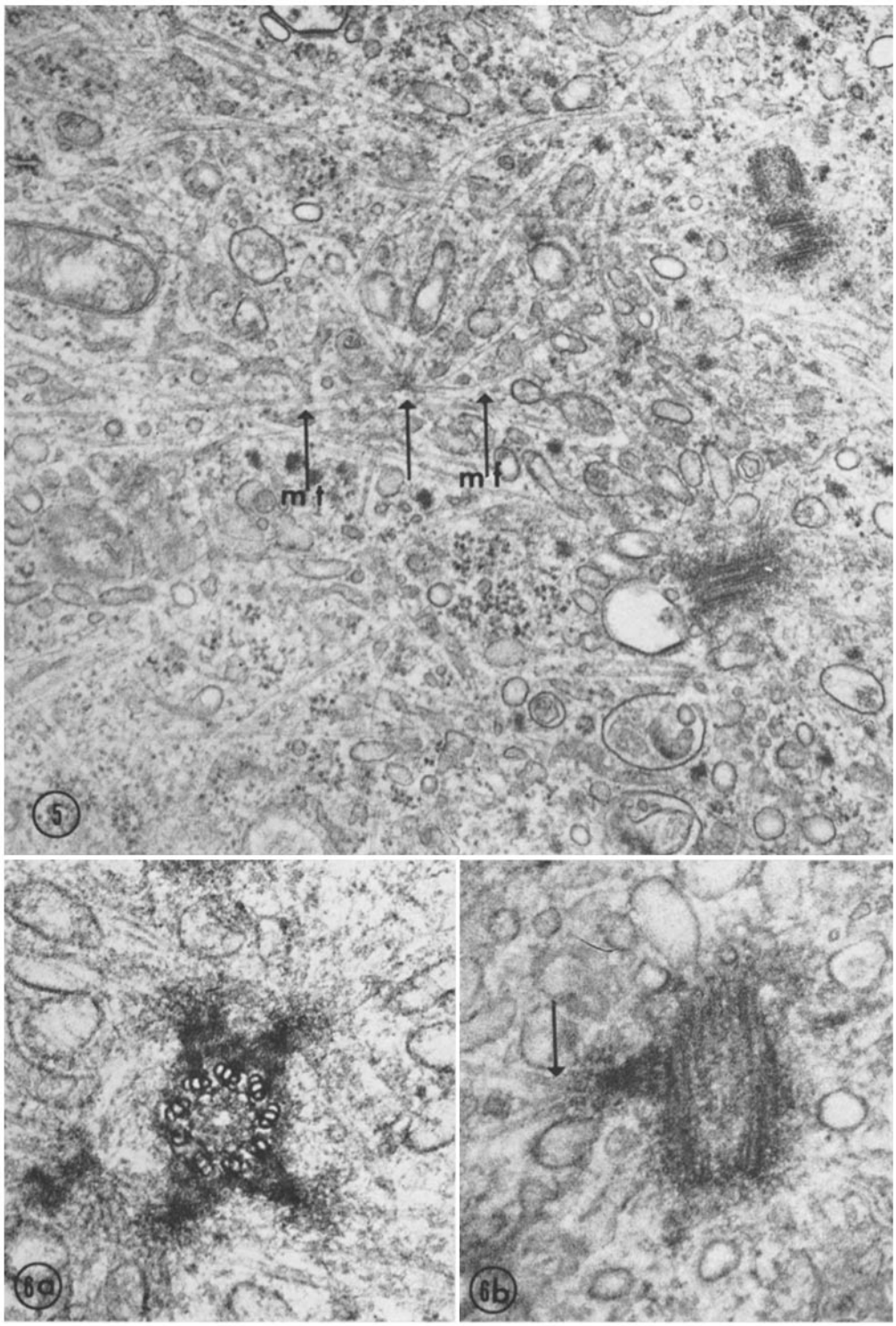
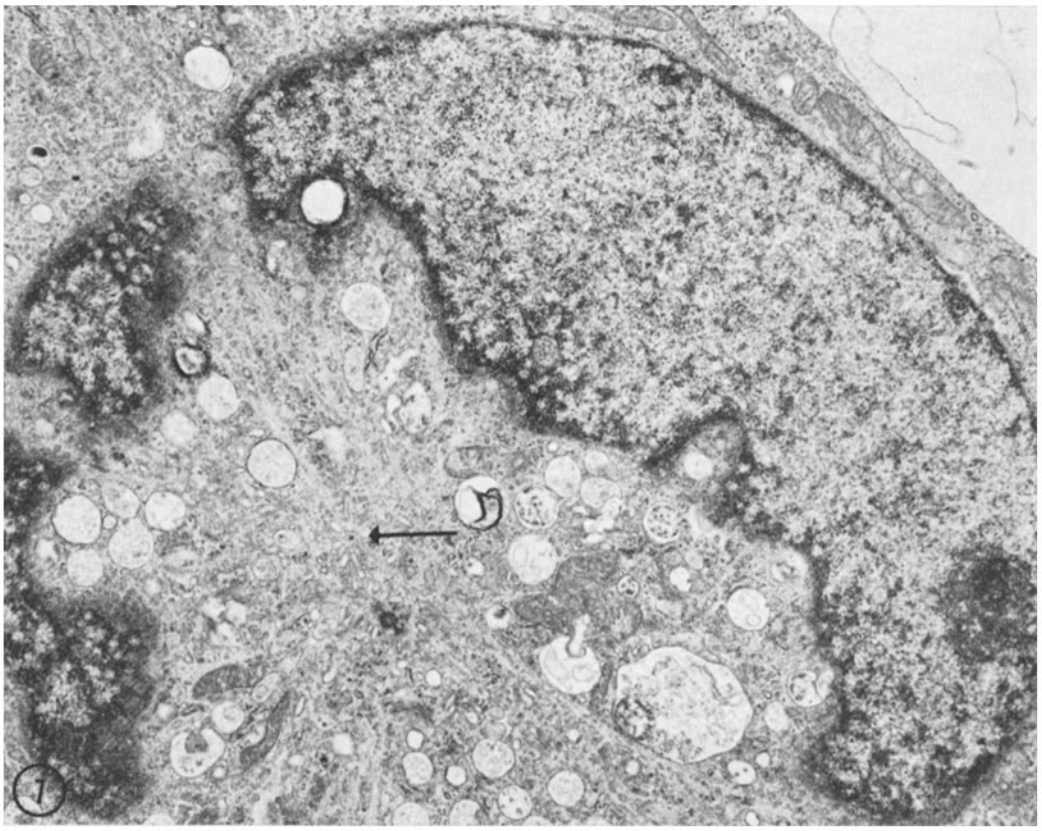


FIGURE 5 Cell fixed 12 h after mitosis. Arrows indicate cytoplasmic foci for microtubules (*mt*) and microfibrils (*mf*) which extend throughout the nuclear invagination in which the centrioles are positioned. × 47,350.

FIGURE 6 (*a*) Cross section through distal region of parent centriole of cell fixed 12 h after mitosis. Four wedge-shaped striated arms extend from the centriolar walls. × 92,250. (*b*) Longitudinal section through a parent centriole of cell fixed 12 h after mitosis. Several microtubules (arrow) impinge on the apex of the centriole appendage. × 91,250.



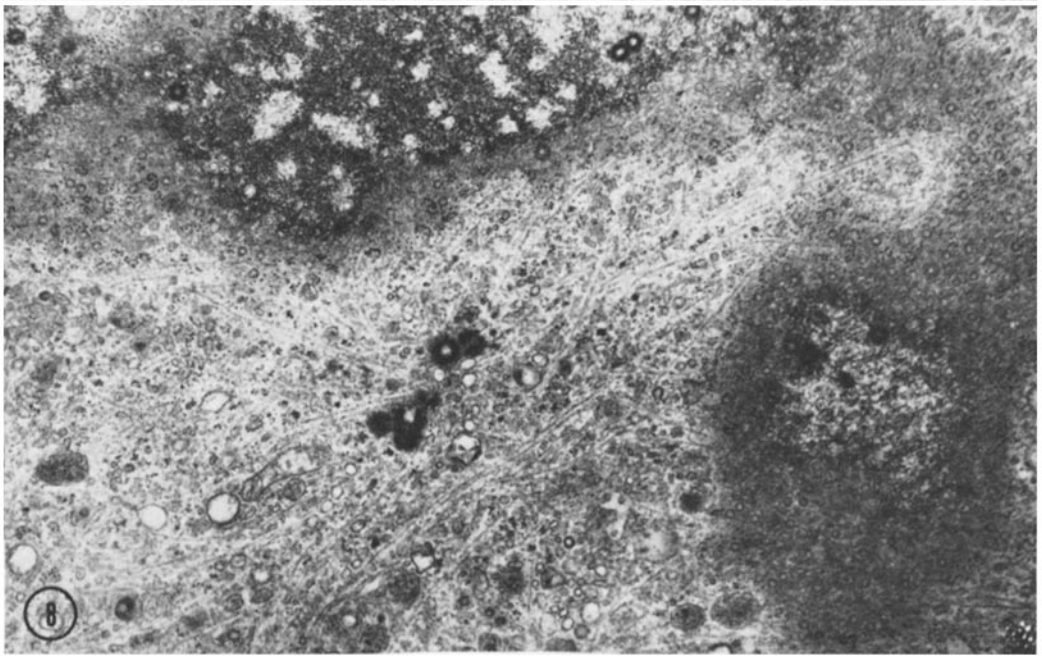


FIGURE 7 Section grazing top of nucleus of cell fixed 15 h after mitosis. The cytoplasm displays a meshwork of microfilaments radiating from the centriolar region and the cytoplasmic foci (arrow). × 7,400.

FIGURE 8 High magnification view of centriolar region of cell fixed 15 h after mitosis illustrating both centriolar duplexes. × 18,450.

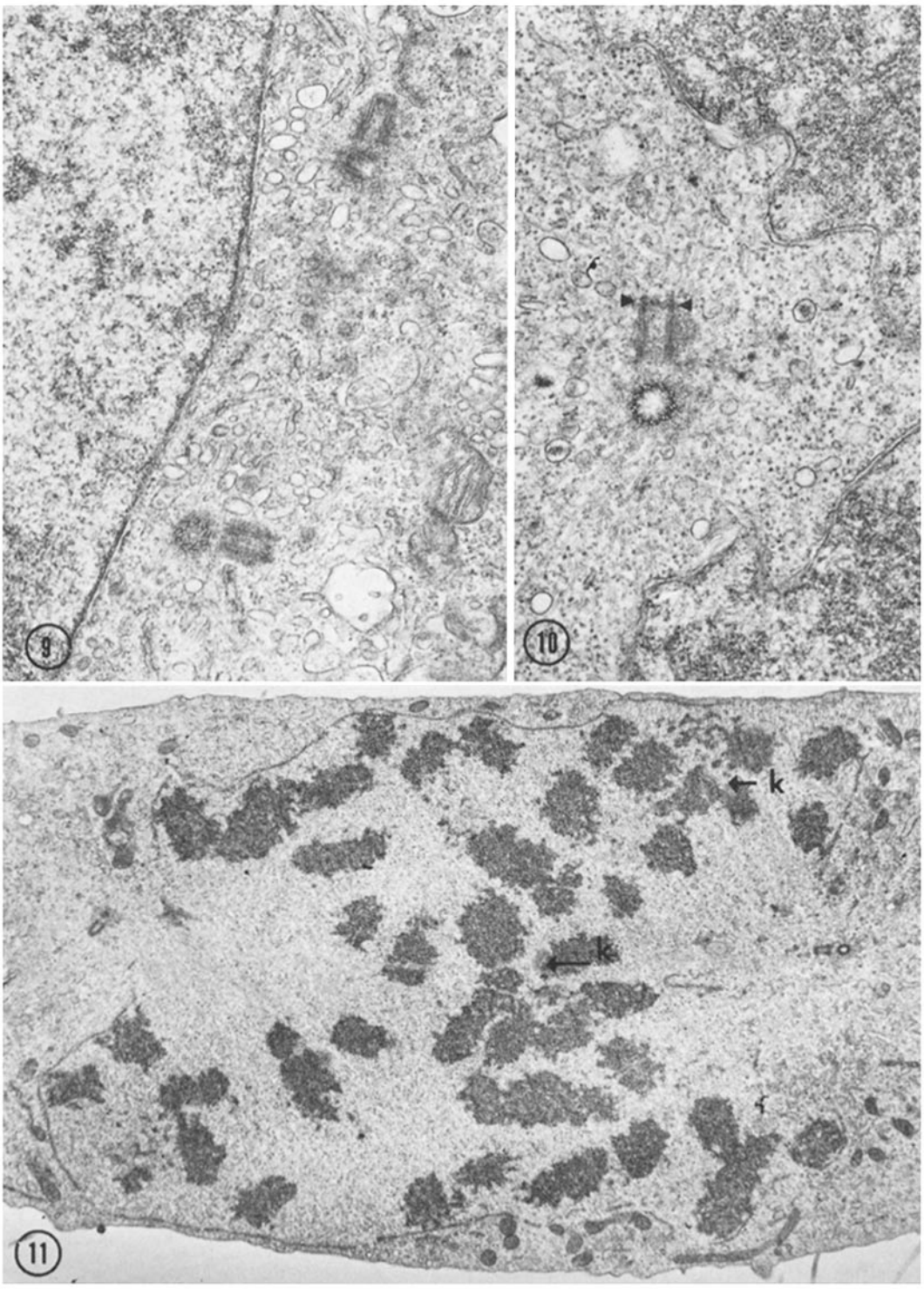


FIGURE 9 Early prophase (18 h after mitosis). The daughter centrioles have begun elongation. Note that the microfilament meshwork and nuclear invagination associations are no longer evident. × 30,750.

FIGURE 10 Prophase-prometaphase cell. The centriolar duplexes have moved to the presumptive poles. One such duplex illustrated here is associated with numerous microtubules. Note fuzzy dense bar at distal end of daughter centriole (arrowheads). × 45,000.

FIGURE 11 Prometaphase cell. The daughter centrioles of the two centriolar duplexes have attained the mature length of 0.55 μm. (*k*) kinetochores. × 8,200.

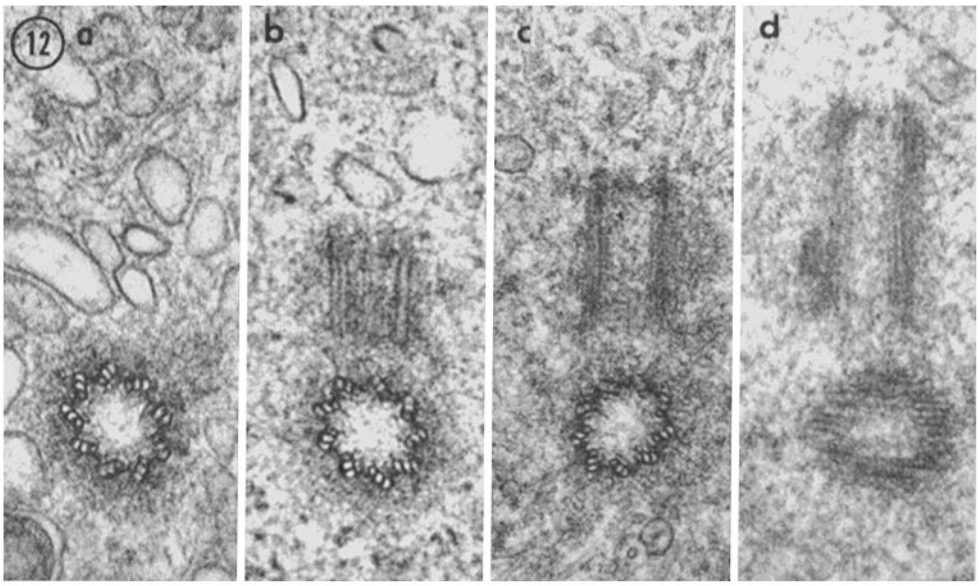


FIGURE 12 Summary of centriolar elongation during the cell cycle. (*a*) Early G_1. × 72,500. (*b*) S phase. × 73,000. (*c*) Prophase. × 73,000. (*d*) Metaphase. × 73,000.

parents and two daughters, occurred in close proximity to each other in the groove on top of the nucleus from 4 h after mitosis until the latest times observed, i.e., 40 h after mitosis. Migration of the two centriole pairs to opposite sides of the nucleus did not occur, and elongation of the daughter centriole to its mature length also did not occur (Figs. 14, 15). These two events are presumably linked with mitotic events which were, of course, inhibited because DNA synthesis had not taken place.

Centriolar Ultrastructure

The centriolar lumen appeared to contain a discrete, symmetrical substructure. In cross section, this structure appeared as a single dense line which ran along the inner margin of the nine triplet tubules. It was folded into nine ragged convexities, each semicircular convex region being associated with one set of triplet tubules at the junction between A and B tubules. Further, each semicircle had an arm which inserted on the outer margins of both the A and B tubules. The inner portion of the centriole lumen appeared to be filled with rather homogeneous, moderately electron-opaque material with a central electron-transparent region about 350–400 Å in diameter, and nine more peripheral electron-transparent circular areas partially outlined by the dense line structure (Fig. 16). These lumenal features were characteristic only of the distal two-thirds of the mature centriole (Fig. 16 *a–e*). No such structure was observed toward the end of mature centrioles where the daughter was associated (Fig. 12). The lumenal structures were not evident in daughter centrioles until early in G_1 just before parent-daughter centriole separation.

Though the immature daughter centriole did not possess the complex lumenal structure just described, it also had a characteristic feature in its morphology. From the time of its formation in the first half of the cell cycle until prometaphase, an electron-opaque amorphous material 100 Å in thickness was present at the end of the daughter farthest from the parent centricle. In longitudinal sections, this material appeared as a dense line extending across the centriolar lumen between opposing triplet blades (for examples see Figs. 4, 10, 15). This structure was not evident in mature, parent centrioles or mature daughters.

DISCUSSION

In two earlier studies on the centriole cycle in cultured cells, procentrioles were first seen in association with the mature parental centrioles early in S phase (Robbins et al., 1968; Stubblefield,

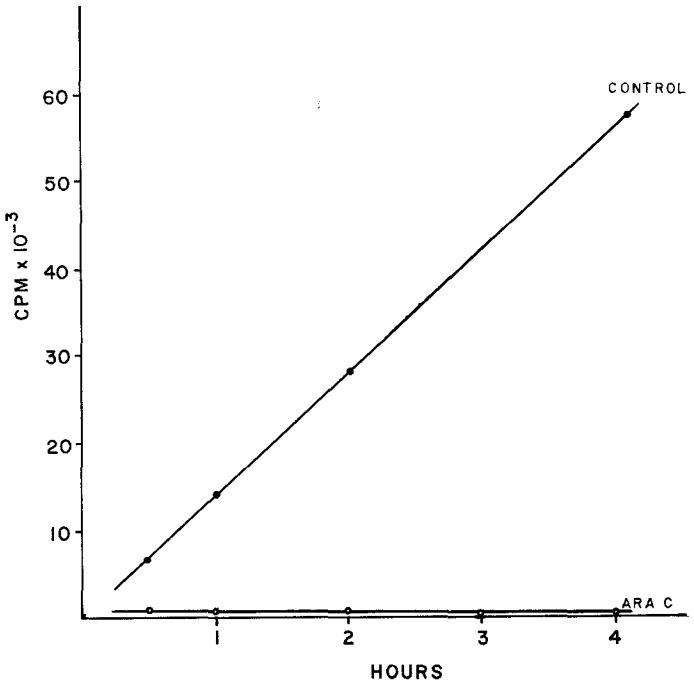


FIGURE 13 Incorporation of [^{3}H]thymidine into TCA-precipitable, DNase-removable material in L cells in suspension culture in the presence (open circles) or absence (closed circles) of arabinosyl cytosine (ARA C). Arabinosyl cytosine was added 30 min before time 0. [^{3}H]thymidine was added at time 0. Points represent the average of four replicate samples after subtracting the average count per minute remaining in four replicate DNase-treated samples.

1968; Brinkley and Stubblefield, 1970). It thus seemed possible that centriolar duplication was an event closely linked to cellular DNA synthesis. The results of our experiments argue against that hypothesis. In L929 cells, centriolar duplication may occur in some cells during G_1 rather than S. That is, procentrioles were seen in some cells as early as 4 h after mitosis. Thus, in the cells we have studied, since S phase usually starts about 9 h after mitotic selection, it appears possible that centriolar duplication may not necessarily be associated with the onset of nuclear DNA synthesis.

There are other reports in the literature which suggest that in certain systems, procentrioles may be generated at times other than S phase. By light microscopy, Pollister (1933) observed that in amphibian cells, two stainable dotlike structures (which presumably may be equated with centrioles or centriolar duplexes) occurred throughout interphase in amphibian cells. The two dots moved apart during prophase and each assumed a position at a spindle pole. In telophase cells, each dot appeared to have doubled (two dots were visualized at the spindle poles). In light of modern electron microscope observations, one might suppose that this "doubling" actually represented the separation of the two mature centrioles of the duplex, which occurs just before procentriole formation. This would suggest that procentrioles form in G_1 in amphibian cells. Mazia et al. (1960) used an ingenious approach to study the time of centriole duplication in echinoderm cleavage divisions. They found that when mercaptoethanol (which prevented cells from entering mitosis) was added to cells at anaphase of the first cleavage division and removed at a time after the next division would have normally occurred, each cell formed a spindle with two poles. However, when mercaptoethanol was added at telophase or G_1 of the first cleavage division and again removed at a time after the next division would have normally occurred, each cell subsequently formed a spindle with four poles. If one can equate number of pole organizing entities with number of centrioles, those findings would suggest that centriolar duplication occurs at about telophase

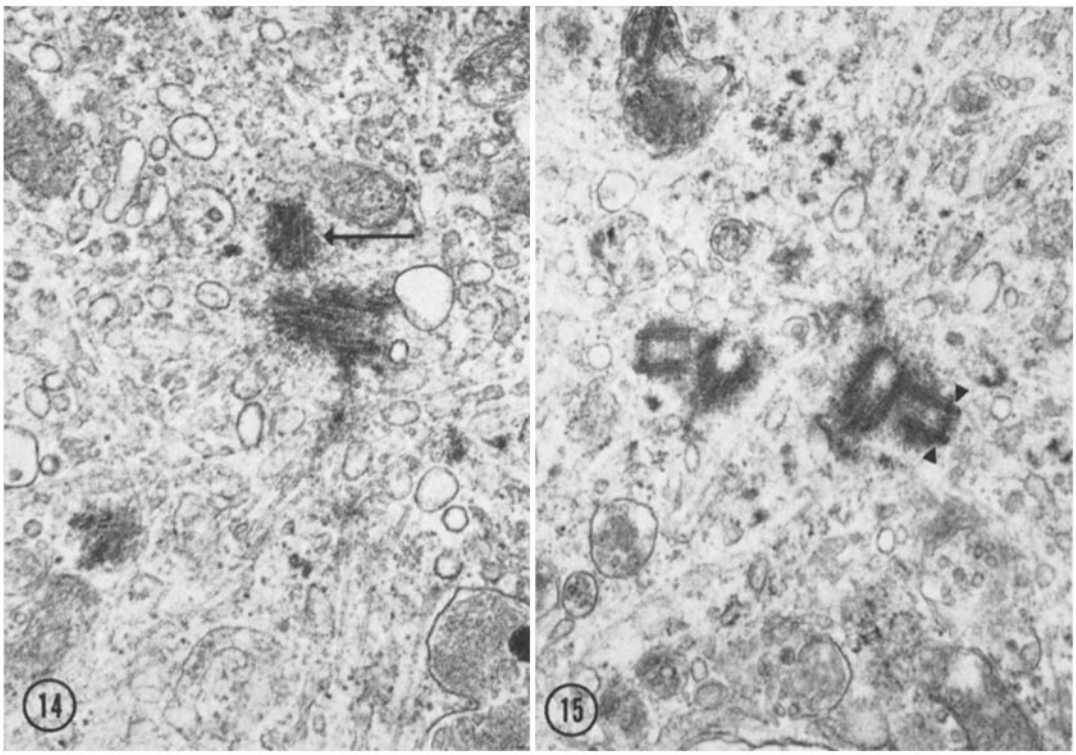


FIGURE 14 Cell from arabinosyl cytosine-treated culture fixed 4 h after mitosis. Arrow indicates the daughter centriole. × 47,350.

FIGURE 15 Cell fixed 24 h after mitotic harvest from culture maintained in the presence of arabinosyl cytosine from the time of harvest. Centriolar elongation and positioning has not taken place. Dense bar at the end of the daughter indicated by arrowheads. × 45,000.

in sea urchin and sand dollar eggs. Murray et al. (1965) reported that no more than two centrioles were seen in interphase thymic lymphocytes. They suggested that the time of procentriole formation in those cells might be around prophase. In male meiosis in a squirrel and a hyrax, procentriole formation was found to occur in prophase of meiosis I and again during interphase in secondary spermatocytes (Rattner, 1972). Though the time of centriolar duplication in prophase I coincides with the time of synthesis of a minor DNA component (Hotta and Stern, 1966), no DNA synthetic period is known to occur in secondary spermatocytes. The observations cited suggest that there is no necessary correlation between the time of centriolar formation and nuclear DNA synthesis, or, in fact, any other feature of the cell cycle. It would appear that the only constraint on the time of centriolar duplication is that it occur before cell division so that a complete centriolar duplex (i.e., one mother and one daughter centriole) occur at each spindle pole.

Our second observation bearing on this point is that procentriole formation occurred normally when DNA synthesis was inhibited by arabinosyl cytosine. The formation of ciliary basal bodies, structures analogous to centrioles, has also been found to be unaffected by DNA synthesis inhibitors such as X-irradiation (Sorokin and Adelstein, 1967), ethidium bromide, arabinosyl cytosine, hydroxyurea, and mitomycin C (Younger et al., 1972). These findings eliminate the possibility that centriolar duplication is linked to nuclear DNA synthesis. Our findings cannot, however, be taken as proof that centrioles are not semi-autonomous organelles (containing their own DNA). To disprove this controversial hypothesis (see Fulton, 1971, for review), one would have to be sure that arabinosyl cytosine was blocking *all* cellular DNA synthesis. Our data are only

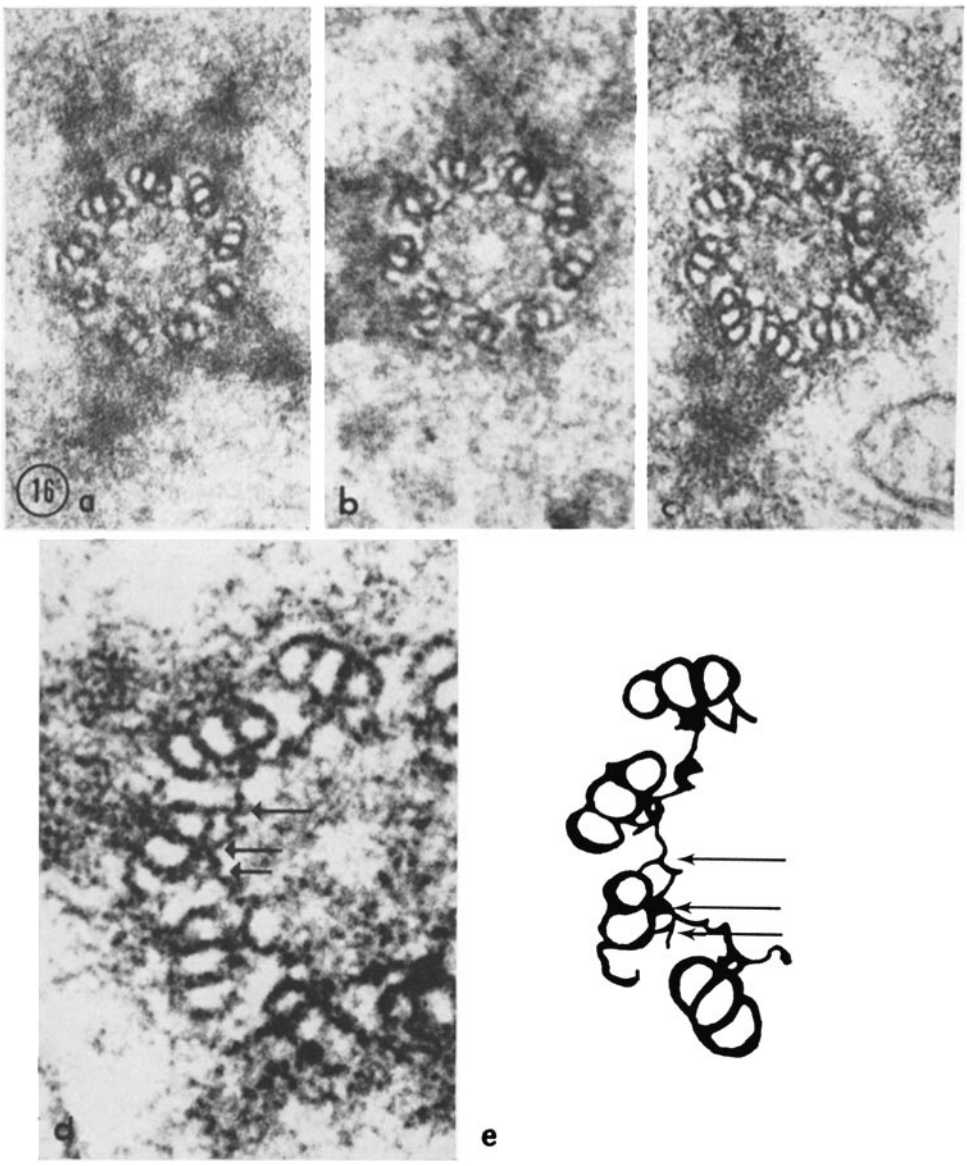


FIGURE 16 (*a–c*) Cross sections through the distal portion of three parent centrioles. In Fig. 16 *a* the linear structure can be seen in association with the centriolar blades. In Fig. 16 *b* the moderately electron-opaque lumenal material is apparent. In Fig. 16 *c* both structures are apparent. (*a*) × 135,000. (*b*) × 174,000. (*c*) × 174,000. (*d*) Higher magnification of Fig. 16 *c*. × 369,000. (*e*) Tracing of Fig. 16 *d* illustrating the association of the linear structure with the triplet blades. Note association (arrows) at edge of tubule A, A–B junction, edge of tubule B.

sufficient to conclude that arabinosyl cytosine blocked greater than 99% of DNA synthesis by 1 h after its addition. This degree of accuracy is not sufficient even to draw a conclusion about whether mitochondrial DNA synthesis (which constitutes about 0.15% of the total) (Nass, 1969) is blocked by the drug, and one would have to suppose that if centrioles have DNA it would account for an even smaller fraction of the cellular DNA than does mitochondrial DNA.

An interesting sidelight to this study was the observation that the positioning of the centrioles

in the cell is quite regular and is correlated with stage in the cell cycle. We found that in early G_1, centrioles moved to a region of cytoplasm near the midbody from a previous position on the far side of the nucleus. They then migrated into a groove in the nucleus before generating a procentriole. They stayed in this groove, which was associated with a meshwork of microtubules and microfilaments, until the onset of prophase. Then the two centriolar duplexes moved to opposite sides of the nucleus to begin the cycle anew.

In summary, we have found that new daughter centrioles in L929 cells could be generated in the presence of an inhibitor of cellular DNA synthesis. Thus, the formation of daughter centrioles apparently is not contingent upon cellular DNA synthesis. Elongation of the daughter centrioles to their mature length was an event correlated with the onset of mitosis in these cells.

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