

THE ROLE OF THE CENTRIOLAR REGION IN ANIMAL CELL MITOSIS

A Laser Microbeam Study

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ABSTRACT

An argon ion laser microbeam (488 and 514 nm) was used to selectively irradiate one of the two centriolar regions of rat kangaroo *Potorous tridactylis* (PtK₂) prophase cells in vitro. The cells were sensitized to the laser radiation by treatment with acridine orange (0.1–0.2 µg/ml). Ultrastructural examination of the irradiated centriolar regions demonstrated that the primary site of damage was the pericentriolar material. This result suggests that nucleic acid is present in the pericentriolar material. Behavioral and ultrastructural analysis demonstrated that cells with one damaged pericentriolar zone could undergo (a) nuclear membrane breakdown, (b) chromosome condensation, (c) metaphase plate formation, and (d) cytokinesis. However, the chromosomes neither separated nor exhibited any anaphase movements. Detailed ultrastructural analysis revealed the presence of kinetochore microtubules on both sides of the chromosome mass and a lack of microtubules in the cytokinesis constriction. These results indicate that the pericentriolar material is important in spindle organization and essential for the formation of the interpolar microtubules.

The centriole is a controversial organelle. There are diverse opinions in the literature with respect to its molecular organization and its function in the cell division process. About the only aspect of centriole structure generally agreed upon is that the centriole contains triplets of microtubules that are usually arranged in the typical 9 + 0 configuration (11). The triplets are arranged in a pinwheel-like configuration, and connections are often detected either between the triplets or extending from the triplets into the center of the centrioles where they may connect with other electron-dense elements. However, there is considerable variability

among cells, and it is possible that this variation is due to differences in fixation, staining, angle of sectioning, or even cell function (Figs. 1, 2).

Also associated with the centriole is an electron-dense "amorphous cloud" (20), generally referred to as pericentriolar material. This material is often found surrounding the centriole proper. It varies considerably in appearance, and, quite frequently, smaller electron-dense spheres (referred to as "pericentriolar satellites") are observed in this region (11). In this manuscript, we define the "centriolar region" as containing two major components: (a) the centriole proper and (b) the peri-

centriolar cloud, which may or may not contain pericentriolar satellites.

In addition to the above variations in centriolar region organization, there is considerable question with respect to the presence of nucleic acid. Early studies employing RNase digestion suggested that RNA was present in the connections between the triplet blades (26) and in the pericentriolar satellites (11).

Other studies on two centriolar structures, the pellicle of *Paramecium* (24) and the basal body of *Tetrahymena* (21), have suggested the presence of DNA. However, there still seems to be considerable question with respect to the presence or absence of nucleic acids and their precise location within the centriolar region.

The role of the centriole in the organization and function of the cell division apparatus is by no means well understood. Despite the fact that most of the textbooks imply that centrioles determine the spindle poles and are intimately involved in assembly and organization of spindle microtubules, there is considerable diversity of opinion on this subject. The minority view espoused by Pickett-Heaps (19) is that the centrioles in plants and animals "are an appendage attached to the spindle rather than a vital component of it. . . ." The argument is based on several points: (a) spindles are formed and function in higher plants without centrioles; (b) microtubules are broken down and repolymerized in different regions of the cell without centrioles; (c) there are basic chemical differences between flagellar fibers that are associated with the centriole tubules and other cytoplasmic microtubules, and (d) in certain plant cells, centrioles can appear *de novo* and become associated with the spindle poles (i.e., at one point in the life cycle, centrioles are associated with the mitotic apparatus, and, at other times, they are absent from the mitotic spindle).

In addition to the arguments made by Pickett-Heaps, Dietz (14) demonstrated that dislocation of the centriole from the meiotic spindle apparatus of grasshopper spermatocytes did not interfere with spindle function. Furthermore, it has been shown that no centrioles at all are present in mouse oocyte meiotic spindles (27), and the centrioles that are present in the meiotic cells of the sea lettuce *Ulva* are located in a position removed from the spindle poles (10). The same observations were made on the mitotic vegetative cells of *Ulva* (17).

Despite the previous observations, the major

view is that centrioles are directly involved in spindle organization and function. Evidence attesting to these roles are: (a) colcemid block experiments demonstrating that inhibition of centriole separation prevents the formation of a bipolar spindle, (b) the formation of continuous microtubules as the centrioles separate following recovery from colcemid, and (c) the formation of multipolar spindles in cells with more than the normal number of centrioles (20). In addition, a series of recent studies employing lysed tissue culture cells have shown that centrioles can act as microtubule organizing centers (12, 18, 25).

It is evident that there is considerable disagreement with respect to the centriole. It is not our intention to resolve it at this time. However, the combination of experimental manipulation by microbeam irradiation, behavioral analysis after the manipulation, and concomitant ultrastructural analysis could be a fruitful approach to studying this ubiquitous organelle. Indeed, ultraviolet microbeam irradiation has already been employed in the study of mitosis in plant (1, 2) and animal cells (15, 16). These experiments have produced mixed results, in part, because of considerable secondary effects due to general ultraviolet light absorption, inadequate or no ultrastructural analysis, and the technical problems associated with ultraviolet microscopy and dosimetry.

The use of visible laser light either alone or in combination with selectively binding vital dyes (4) has proven to be very useful in the study of numerous cell organelles. The absorption of laser energy is very localized (down to 0.25 μm on a single chromosome region), and the damage is confined to only the precise region irradiated (22). In addition, the total exposure time is between 10^{-5} and 10^{-9} s; thus, the cell is perturbed by the irradiation for an extremely short period of time. (This compares to times of 5–20-s exposure for the UV studies.) The use of visible laser light does not require the expensive and difficult-to-handle quartz or reflecting objectives and the necessity for growing the cells on a quartz coverglass.

In this manuscript, we will describe studies aimed toward elucidating the function and organization of the centriolar region in *Potorous tridactylis* (PtK₂) mitotic cells in vitro.

MATERIALS AND METHODS

Cells

The cells employed in these studies were taken from several *Potorous tridactylis* PtK₂ established lines. Most

of the experiments were performed on a clonal subline, PtK₂W (9, 23), that was derived originally as a tetraploid line from the normal near-diploid PtK₂ line. These cells have reverted to the near diploid state. In addition, the cells have unusually clear centriolar regions. The centriolar duplexes are visible in living cells as small phase dark dots in a clear region. This permits easy recognition followed by selective microirradiation.

Stock cultures were grown in T30 plastic flasks in Eagle's MEM (GIBCO F15, Grand Island Biological Co., Grand Island, N.Y.) fortified with 10% fetal calf serum. No antibiotics were used, and the cells were subcultured once per week. 2-3 days before an experiment, the cells were enzymatically removed from the plastic flasks and injected into Rose multipurpose culture chambers (8). In the experiments reported in this manuscript, the cells were exposed to a solution of acridine orange (0.1-0.2 $\mu\text{g}/\text{ml}$ of culture medium) for 5 min before laser microirradiation. As indicated in earlier studies (5, 7), the acridine orange binds to nucleic acid, selectively sensitizing specific regions of the cell to visible laser light. Control irradiation experiments were conducted on both acridine orange-treated cells and cells that had not been exposed to acridine orange.

Laser Irradiation of Experimental and Control Cells

The laser microbeam system employs an argon ion laser with primary wavelengths at 488 and 514 nm. The energy used in these experiments was ~ 50 - $100 \mu\text{J}$ in a 0.25 - 0.5 - μm^2 focused spot. The entire system is identical to the one described earlier (3). A cell with a clear centriolar region was moved under a cross-hair on the monitor, and the laser was fired. The cells were maintained at 37°C by an air curtain incubator. After irradiation, the cells were either observed behaviorally and/or fixed for electron microscopy. In all the experiments reported here, only one of the two centriolar duplex regions was irradiated in each cell. The other duplex served as a control. All cells reported here were irradiated in early prophase before nuclear membrane breakdown began. Control irradiations involved (a) irradiation of centriolar region without acridine orange treatment, (b) irradiation of a noncentriolar juxtannuclear region in cells treated with acridine orange and (3) irradiation of partially condensed prophase chromosomes in acridine orange treated cells. Between 20 and 25 cells were irradiated in each control and in the experimental series. At least five of each group were examined ultrastructurally.

Electron Microscopy

Single cell electron microscopy was performed according to the procedures described earlier (22, 23). The cell was photographed after irradiation and fixed in 3% glutaraldehyde either within 1 min of irradiation or after

the observation of a particular behavior pattern. The cell was photographed again under low power after fixation, and a circle was drawn around the cell on the outer surface of the Rose chamber glass coverglass with a wax pencil. The chamber was opened, and the cells were run through standard postfixation with osmium tetroxide, dehydration, and flat-embedding procedures (22). The Epon disk containing the cells was separated from the coverglass by treatment with liquid nitrogen, trimmed, and mounted on a blank Epon block. Serial thin sections in the silver to gray range were made with a diamond knife. Sections were placed on copper single-slotted grids coated with 0.5% Formvar and carbon and examined under a Siemens Elmiskop 1A at 60 kV.

RESULTS

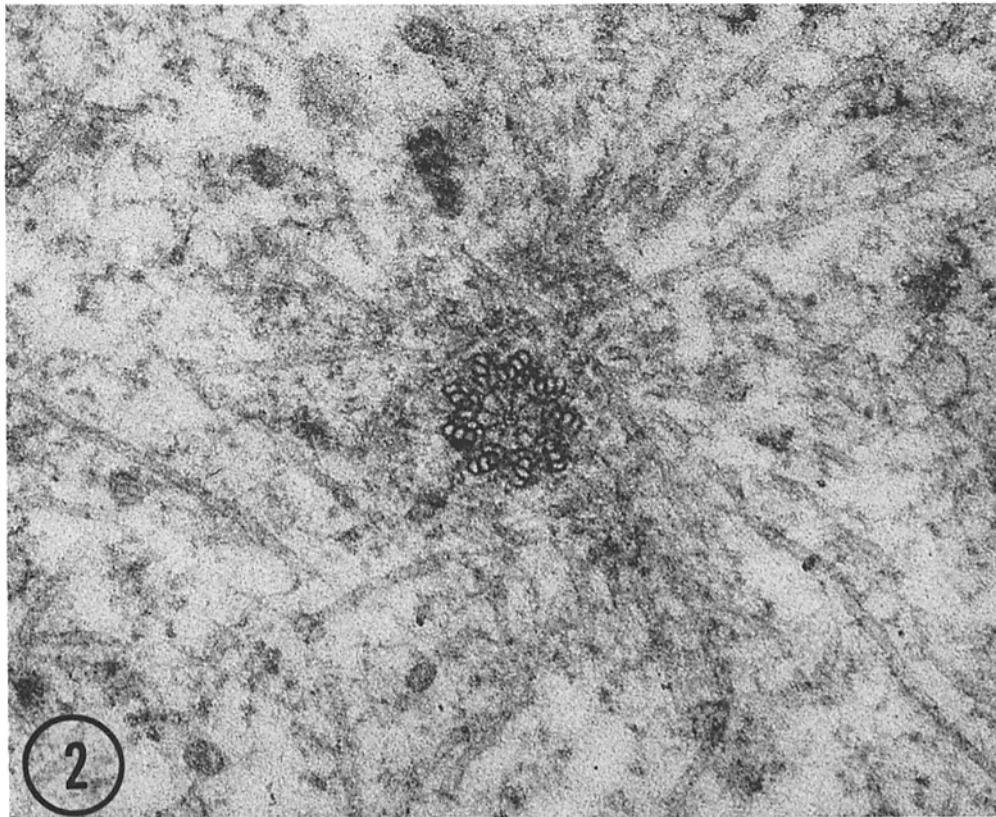
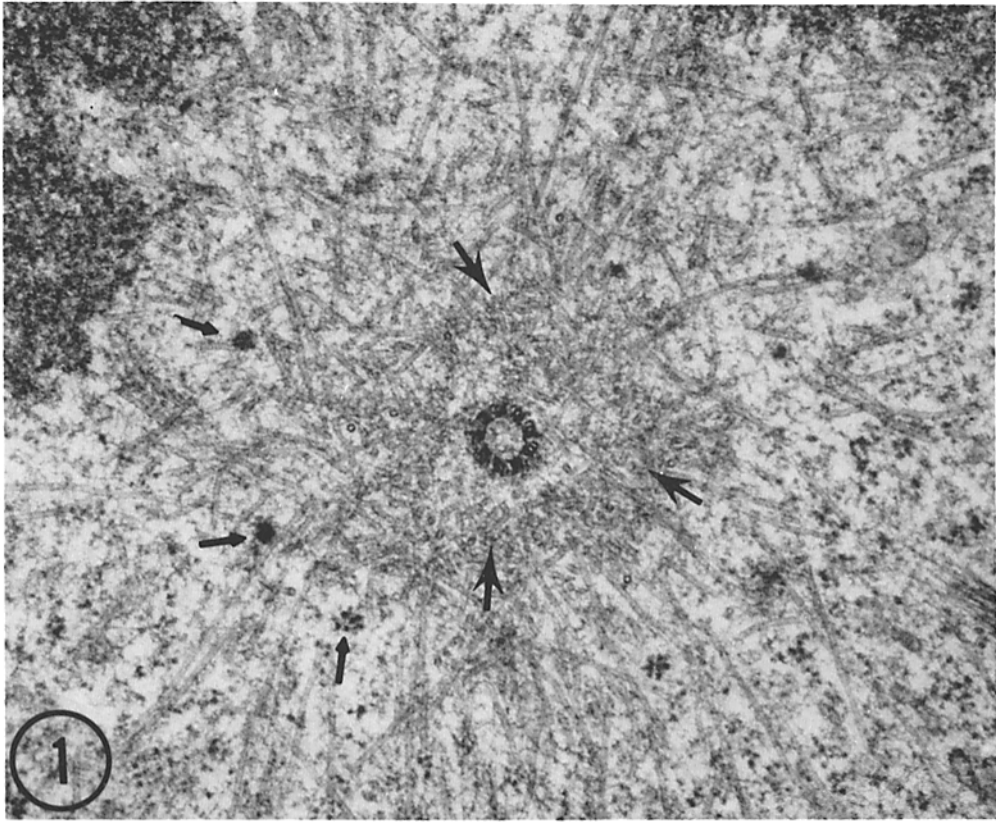
Control Irradiation

Laser microirradiation of the centriolar region in cells that were not treated with acridine orange did not result in any ultrastructural alterations of the centriole or pericentriolar material. In addition, the cells continued through a normal mitosis. Fig. 1 illustrates the fine structure of an irradiated centriolar region. The centriole proper, the pericentriolar cloud (larger arrows), and some pericentriolar satellites (smaller arrows) are all clearly visible. Also note the abundance of microtubules entering (or leaving?) the pericentriolar cloud. Another control irradiation cell depicted in Fig. 2 illustrates near perfect centriolar morphology. Note the internal structure of the centriole and the clear definition of the triplet blades. In comparison to the previous cell, there is less pericentriolar cloud material and fewer pericentriolar satellites. These differences reflect normal variations seen between centriolar regions of different cells. The other centrioles of the duplexes are located in different serial sections.

Control irradiation of acridine orange-treated cells involved irradiation of prophase chromosomes and noncentriolar cytoplasm near the nucleus. The ultrastructure of the centrioles in these cells was normal and very similar to that of the cells depicted in Figs. 1 and 2. Behaviorally, these cells underwent a normal mitosis (Figs. 3-5).

Experimental Irradiation

Centriolar zones were irradiated initially with different laser energies, followed by treatment with different acridine orange concentrations. The purpose of these experiments was to determine the best combination of parameters for producing



a consistent structural and behavioral effect. One of the first and most consistent observations made was that it was extremely difficult to destroy the centriole proper but that there was a high degree of selective damage produced in the pericentriolar zone. This observation was amplified in cells where the higher acridine orange concentration and/or higher laser energies were used (Figs. 6, 7). Note that the damage in the pericentriolar cytoplasm is distinct, yet it is still very localized to the region within 1–2 μm of the centriole (smaller arrows indicate margins of lesion area). With this degree of damage, partial disruption of the centriole proper was repeatedly observed in numerous cells and was clearly not an artifact of sectioning angles (larger arrows indicate that partially disrupted centriole). Numerous microtubules are evident because irradiation took place in mid-prophase and fixation occurred within 2 min of irradiation. In the actual experiments described in this manuscript, the damage was one degree lower than depicted in Figs. 6 and 7, and consisted of visible damage essentially only in the pericentriolar material. (See experimental cells in Figs. 15, 18, and 20.)

The main group of experimental cells involved irradiation of one of the two centriolar regions in prophase. The cells were then allowed to continue through mitosis. The following mitotic events were observed in these cells with the light microscope (Figs. 8–11): (a) nuclear membrane breakdown, (b) chromosome condensation, (c) formation of a metaphase-like plate, (d) a cytokinesis constriction, (e) reformation of the nuclear membrane. The striking feature about these cells was that the chromosomes did not separate and undergo any anaphase movements. The cytokinetic constriction either constricted the “metaphase-like” mass of chromosomes (Figs. 12–14) or constricted to one side of the chromosome mass, resulting in the entire mass being pushed into one cell (Fig. 11)

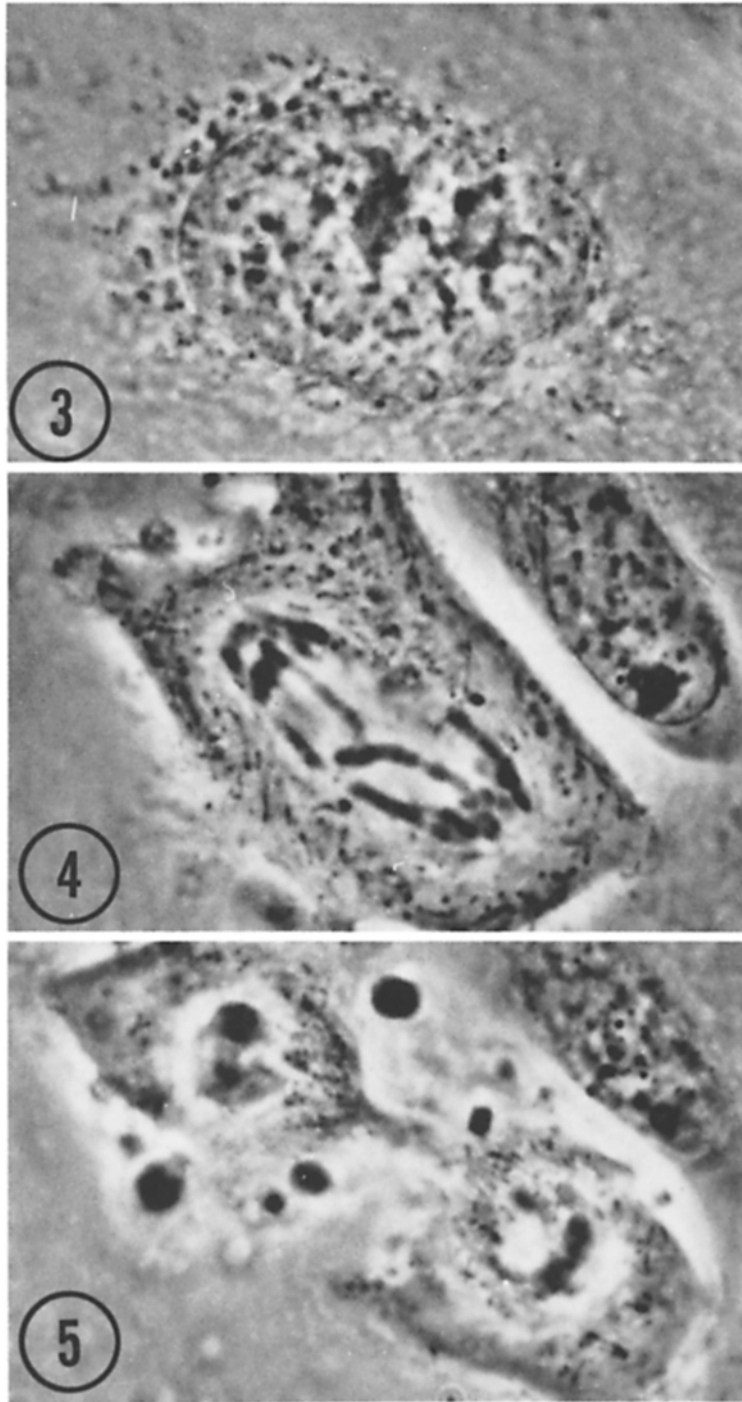
Ultrastructural Analysis of Irradiated Cells

Ultrastructural analysis was performed on cells that were fixed well into the cytokinesis phase. Data are presented on three cells designated 485 (Figs. 15, 16), 546 (Figs. 17 and 18), and 550 (Figs. 19–22). Cell 485 is the same cell illustrated in the light micrographs in Figs. 8–11, and cell 550 is the same cell illustrated in Fig. 12. For ease of presentation, the electron micrographs from each of the cells are grouped together. However, in the following presentation of the data, various micrographs from these three cells will be discussed together.

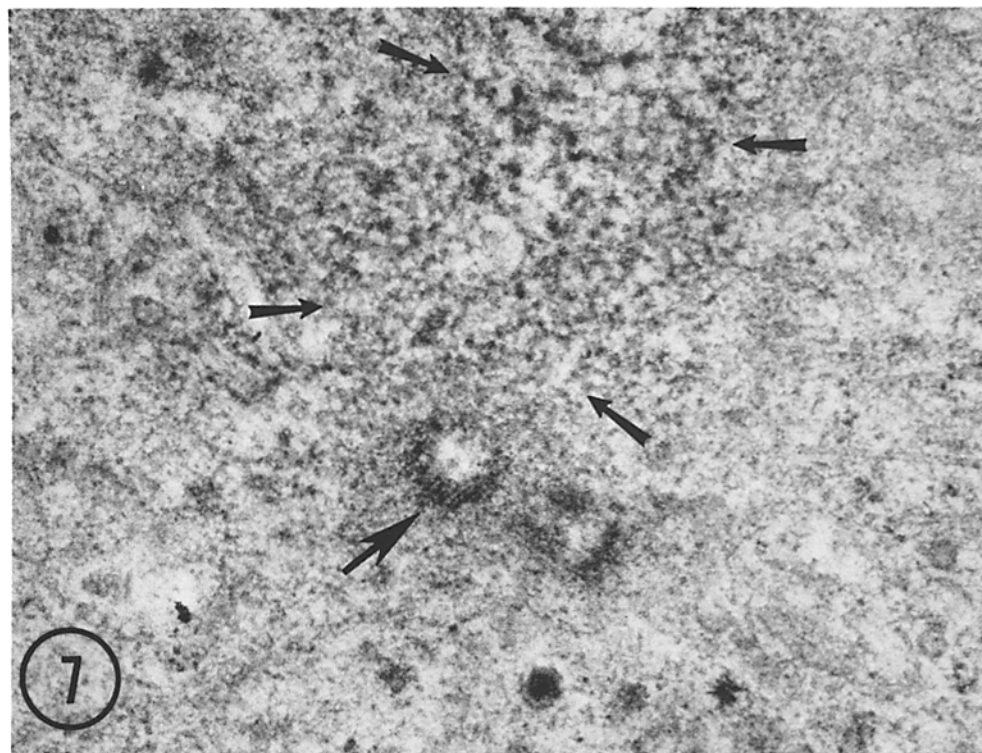
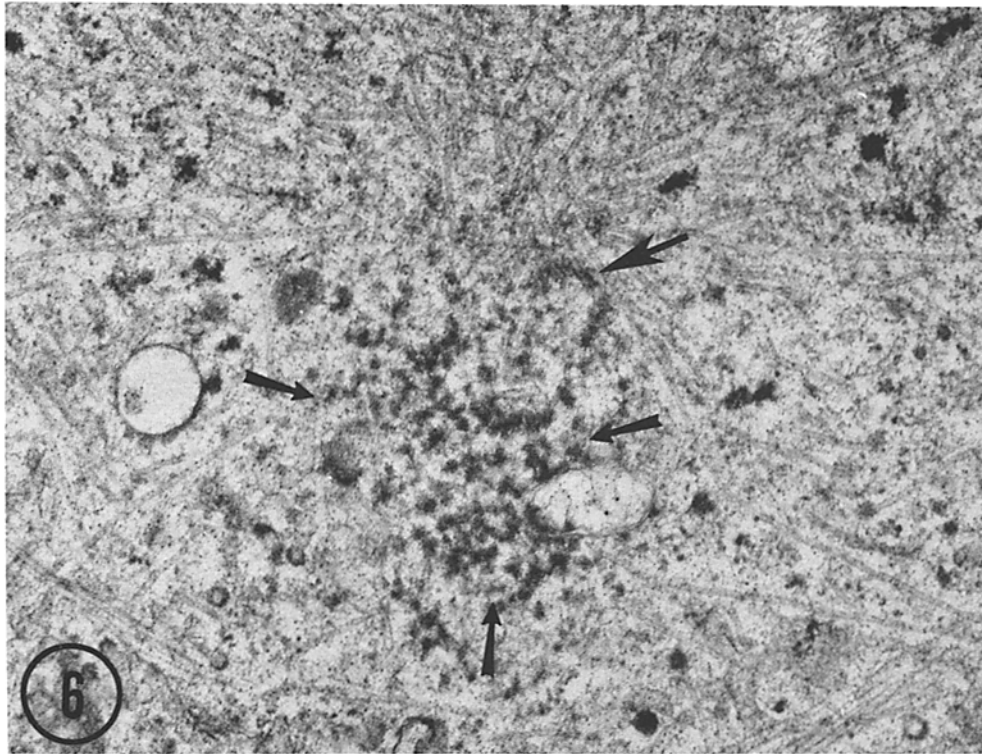
Several consistent patterns were evident. First, it was clear that the centriole duplexes had separated and were on opposite sides of the chromosome material. However, the position of the irradiated centrioles was quite variable with respect to distance from the chromosomes (see Fig. 17 for cell 546). Second, the control unirradiated centriolar region appeared structurally similar to non-irradiated centrioles in hundreds of cells examined in our laboratory and had many microtubular profiles associated with it (see Fig. 18 for cell 546 and Fig. 16, inset, for cell 485). Third, the irradiated centriolar region contained normal-appearing centrioles, but the pericentriolar material appeared to be absent. In particular, in the irradiated centrioles in Figs. 17 and 21, the triple-blade microtubular structure can be discerned even though the angle of sectioning was oblique to both the longitudinal and cross-sectional axes. In addition, there was almost a complete lack of pericentriolar microtubules (see Figs. 19 and 21, cell 550; Fig. 17, inset, cell 546; Fig. 15, inset, cell 485). Occasionally, individual microtubule elements, or microtubule fragments, were detected near the irradiated centriole. In addition, in several sections from different cells, there appeared to be electron-dense lesion material between the irradiated cen-

FIGURE 1 Electron micrograph of control centriole that was irradiated with the laser microbeam. The cell was not sensitized with acridine orange. Note the pericentriolar zone (large arrows), the pericentriolar satellites (small arrows), and the numerous microtubules. $\times 40,000$.

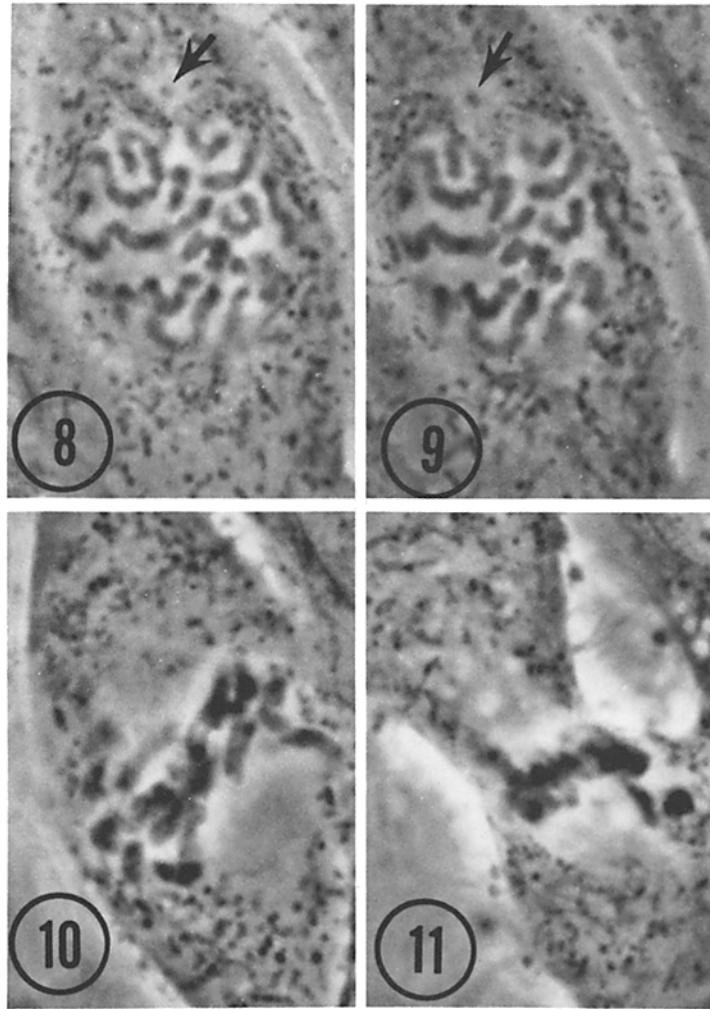
FIGURE 2 Control cell treated the same way as the above cell (Fig. 1). Note the clarity of the centriole triplets and the electron-dense internal structure. In comparison to the preceding cell, note the reduction in pericentriolar material and satellites. The centrioles depicted in both Figs. 1 and 2 demonstrate the normal range of variability between cells. $\times 80,000$.



FIGURES 3-5 Light micrograph series of control cell treated with acridine orange that was laser-irradiated adjacent to the nucleus in a noncentriolar region. Fig. 3 is prophase just after irradiation; Fig. 4 is midanaphase; and Fig. 5 is the phase of cytokinesis. $\times 1,500$.



FIGURES 6 and 7 Electron micrograph of the centriolar regions in cells that were irradiated with moderate energy levels after acridine orange treatment. Note that the primary damage (outlined with small arrows) is in the region adjacent to the centriole proper. The centrioles (large arrows) appear damaged in the region closest to the area of primary damage. The total area of damage is about 1 μm . These cells were fixed within 2 min of irradiation. Fig. 6, $\times 35,000$; Fig. 7, $\times 50,000$.



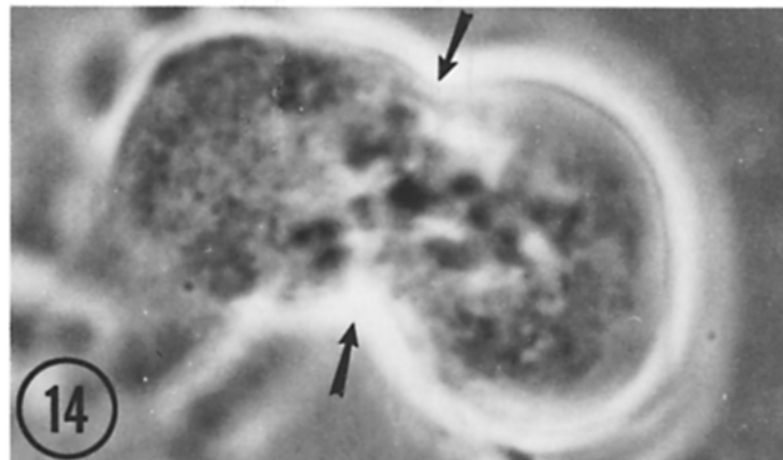
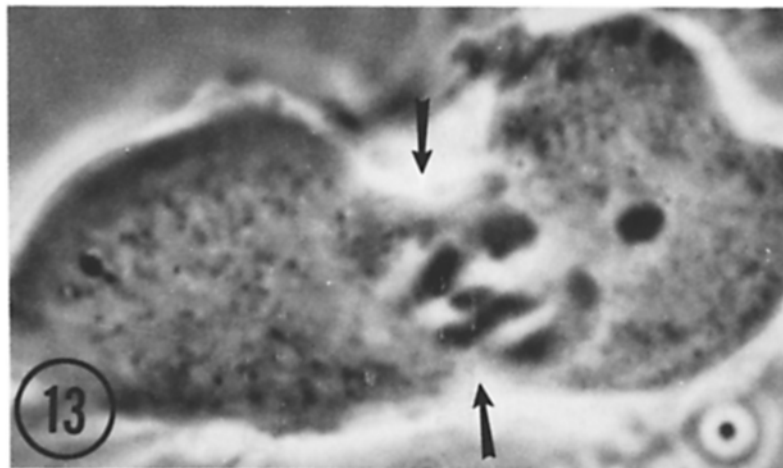
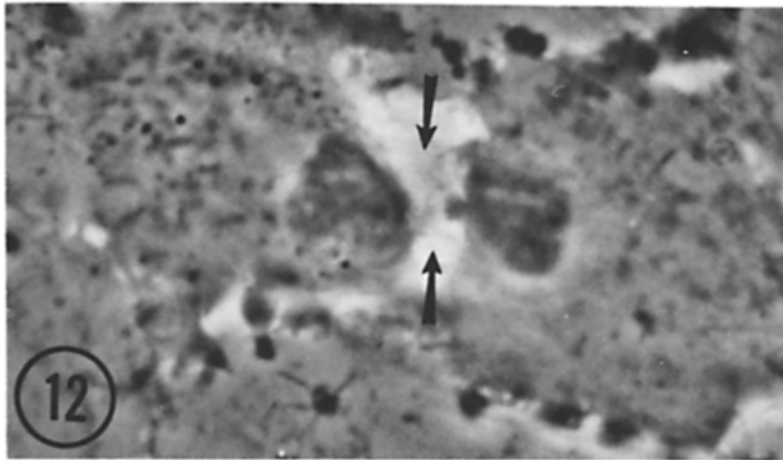
FIGURES 8-11 A series of light micrographs of cell 485 after irradiation of one centriolar region. Fig. 8 is preirradiation, and the centriolar region is evident as a small dot in a clear zone (arrows). Immediately postirradiation (Fig. 9), the centriolar region appears slightly darker. Approximately 15 min postirradiation (Fig. 10), the chromosomes have condensed and become aligned in a metaphase-like configuration. By 30 min postirradiation (Fig. 11), cytokinesis has begun. The chromosomes have not separated, and they remain on one side of the constriction. Figs. 15 and 16 are the electron micrographs of this cell. $\times 1,000$.

triole and the chromosome material (Fig. 19, cell 550; Fig. 15, cell 485, arrows). Under higher magnification, some microtubule profiles were observed in association with this material (Fig. 20). It is suggested that this material is damaged pericentriolar material that has separated from the irradiated centriole.

A fourth set of observations involves the cytokinesis constriction. In order to provide a frame of reference, we have presented a normal cytokinesis constriction in Fig. 23. Note the abundance of

microtubules converging into the constriction region and the electron-dense material in the region of greatest constriction. The cytokinesis constriction regions of all three cells were virtually devoid of microtubules and electron-dense material (Figs. 15, 17, and 22). Occasionally, some microtubule fragments were detected, and in cell 546 (Fig. 17) considerable microfilaments were detected (open arrows).

A fifth and final set of observations involves kinetochore microtubules. Cell 485 was fixed be-



FIGURES 12-14 Cells after irradiation of one centriolar region in which the cytokinesis constriction constricted the chromosome material. The arrows indicate the points of the constriction furrow. $\times 2,000$.

fore nuclear membrane reformation. Numerous microtubules were seen emanating directly from the chromosomes facing both the unirradiated and irradiated centriolar regions (Fig. 16). However, those microtubules facing the irradiated centriolar region did not extend through the cytokinesis constriction to the centrioles. The microtubules emanating from the chromosomes facing the unirradiated control centrioles did extend to the pole.

DISCUSSION

The initial experiments to determine the best combination of laser energy and acridine orange treatment indicate that it is much easier to damage the pericentriolar material than the centriole proper. In fact, in none of our acridine orange-sensitized irradiation experiments (which were more than 25) was there any gross destruction of the centriole. It appeared that the centriole could be affected only indirectly, with the primary effect occurring in the pericentriolar zone. Since acridine orange is a standard fluorochrome in fluorescence microscopy for DNA and RNA (13), our results indicate that nucleic acids or nucleotides are located in the pericentriolar material.

Earlier experiments employing acridine orange as a laser-sensitizing agent for chromosomes (6) indicated that very low amounts of the dye could be detected by laser microirradiation. By assaying chromosome "paling" (a change in refractive index of the irradiated chromosome region), we found it possible to demonstrate the presence of chromosome-bound acridine orange even though there was not enough dye present to produce detectable fluorescence. Further centriolar region irradiation studies employing DNA-specific light-sensitive compounds are under way to determine whether DNA and/or RNA is present in the pericentriolar material.

The behavior of the prophase cells after irradiation of one centriolar region indicates that several mitotic events can occur independently of the immediate function of one centriolar region. The breakdown of the nuclear membrane, further condensation of the chromosomes, and the congression of chromosomes at the center of the cell to form a metaphase-like configuration all occur within 15 min of irradiation of one centriolar region. In addition, in all the cells examined by electron microscopy, the two centriolar regions

FIGURE 15 Electron micrograph of cell 485 that had one centriolar region irradiated after acridine orange treatment. Note the irradiated centriole (large arrow) and the damaged material that is most likely pericentriolar material (small arrows). The irradiated centriole proper appears normal; however, there is a conspicuous lack of microtubules associated with it (see inset). Note the lack of microtubules in the constriction zone and compare it with a control constriction in Fig. 23. Large micrograph, $\times 10,536$; inset, $\times 19,363$.

FIGURE 16 Electron micrograph of the cell 485 as in Fig. 15. Note the chromosome material with kinetochore microtubules emanating from both sides (arrows). Inset shows the unirradiated centriolar region and demonstrates the presence of numerous microtubules. Large micrograph, $\times 18,200$; inset, $\times 49,680$.

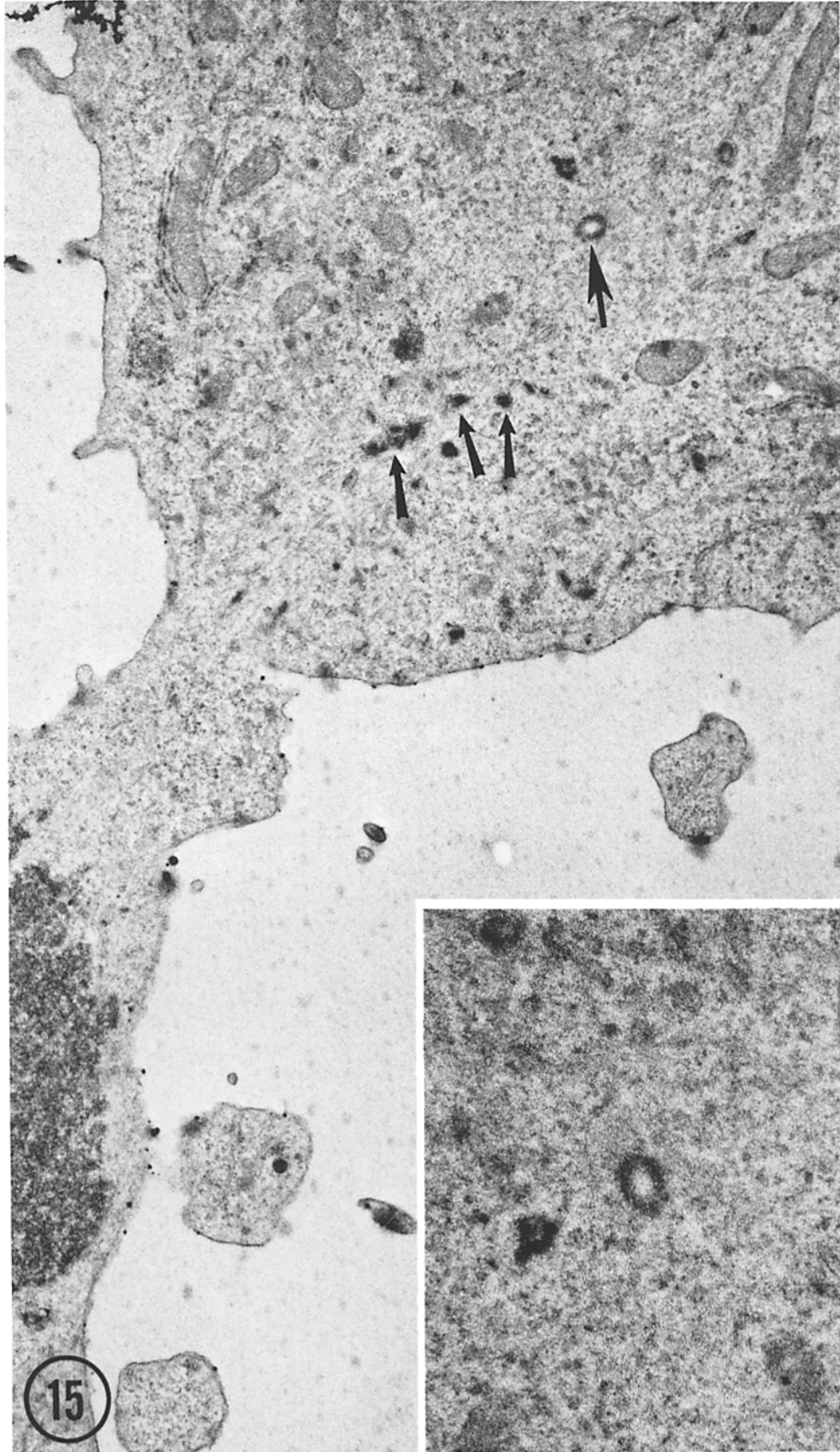
FIGURE 17 Cell 546 with an irradiated centriole caught in the cytokinesis constriction (solid arrow). Virtually no microtubules or pericentriolar material are associated with this centriole (see inset) even though the centriole proper is normal appearing. Note the absence of microtubules in the cytokinesis constriction and the presence of numerous microfilaments (open arrow). See Fig. 18 for the unirradiated centriolar region from the same cell. Large micrograph, $\times 23,000$; inset, $\times 61,600$.

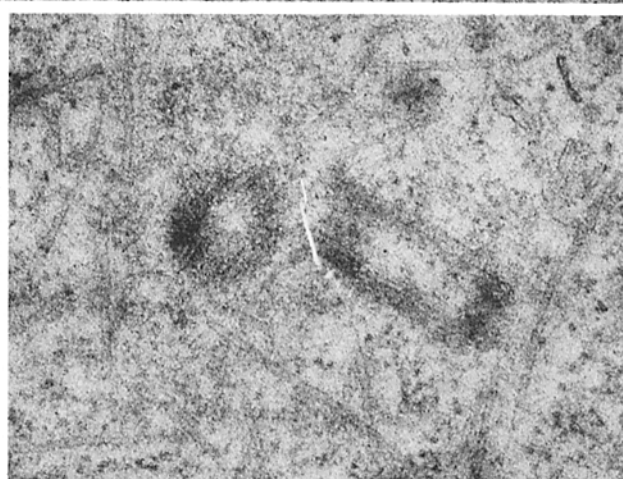
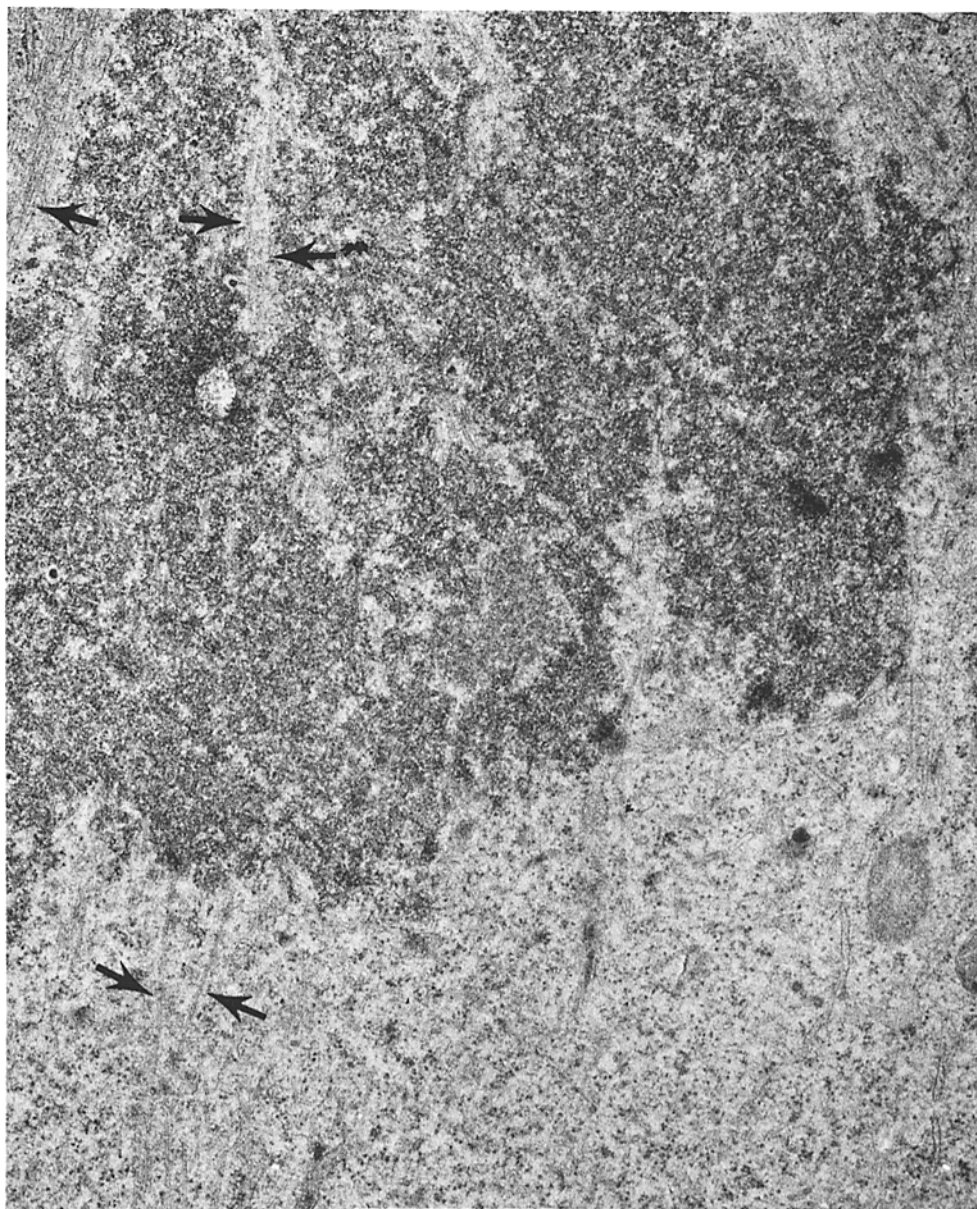
FIGURE 18 Unirradiated centriolar region in the same cell as above (Fig. 17). Note the microtubules emanating from the pericentriolar zone. $\times 28,800$.

FIGURE 19 Cell 550 that has one irradiated centriolar region. The centriole proper (open arrow) appears normal. Note the general absence of pericentriolar material and microtubules from the vicinity of the centriole. There appears to be some lesion material between the chromatin and the centriole proper (solid arrow). $\times 25,600$.

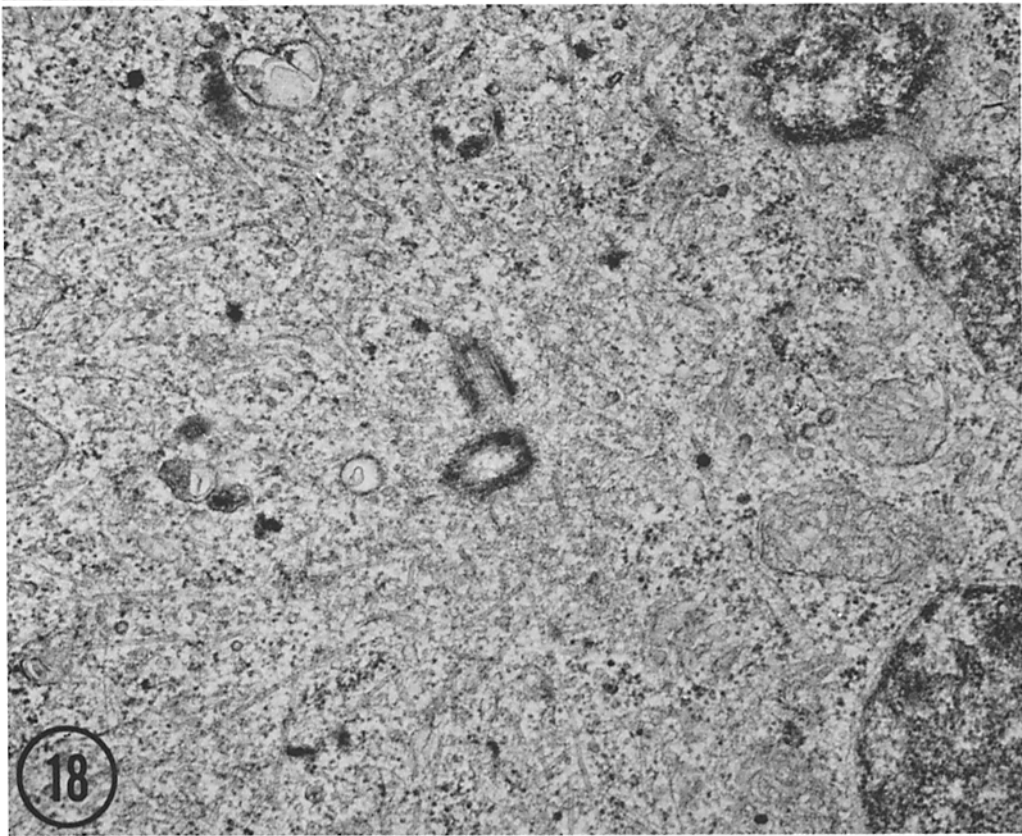
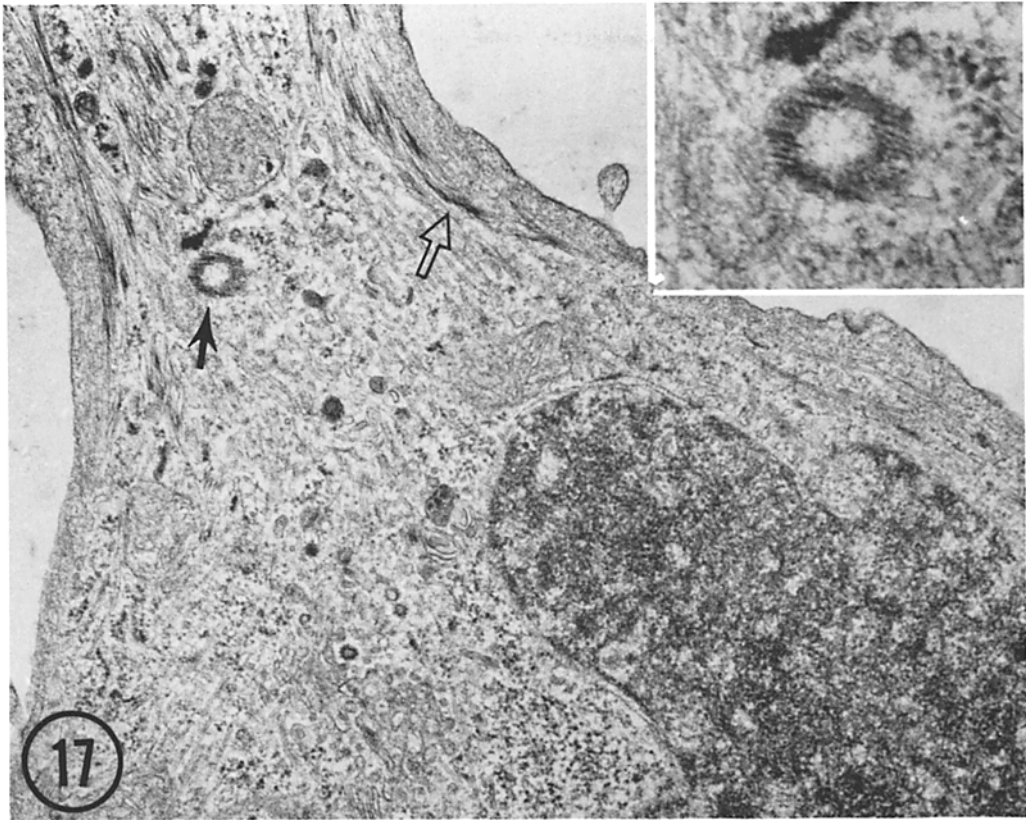
FIGURE 20 High magnification of lesion material from cell 550. Note the microtubular elements associated with this material (arrows). $\times 89,900$.

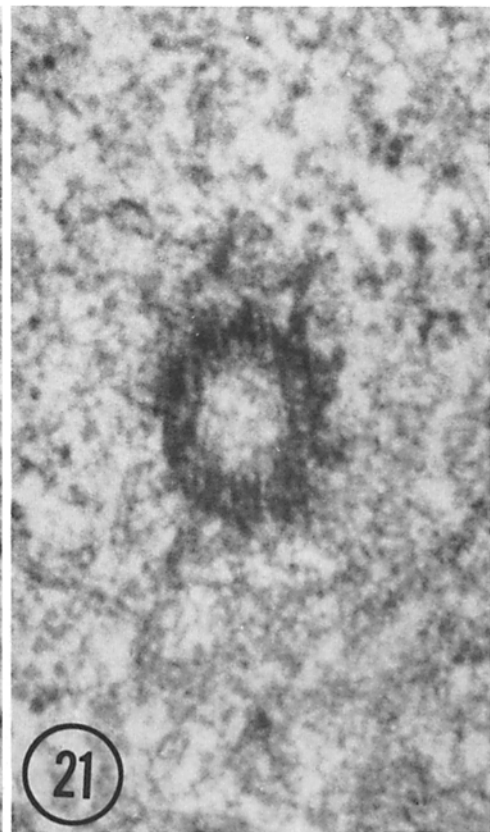
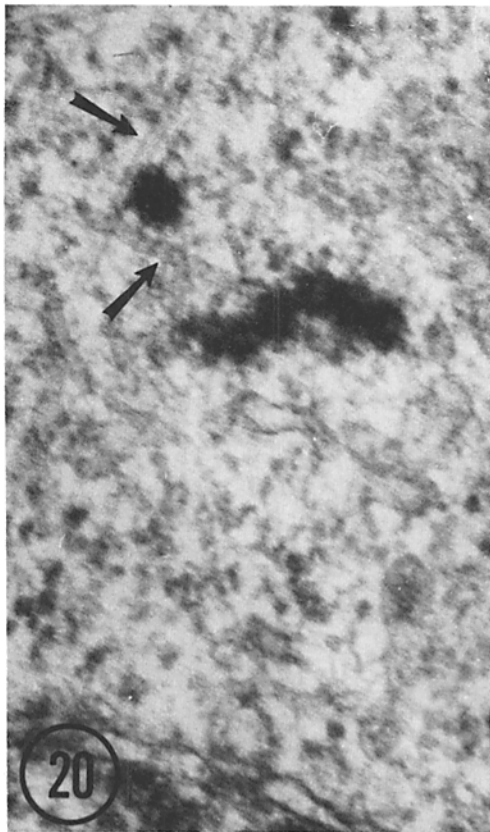
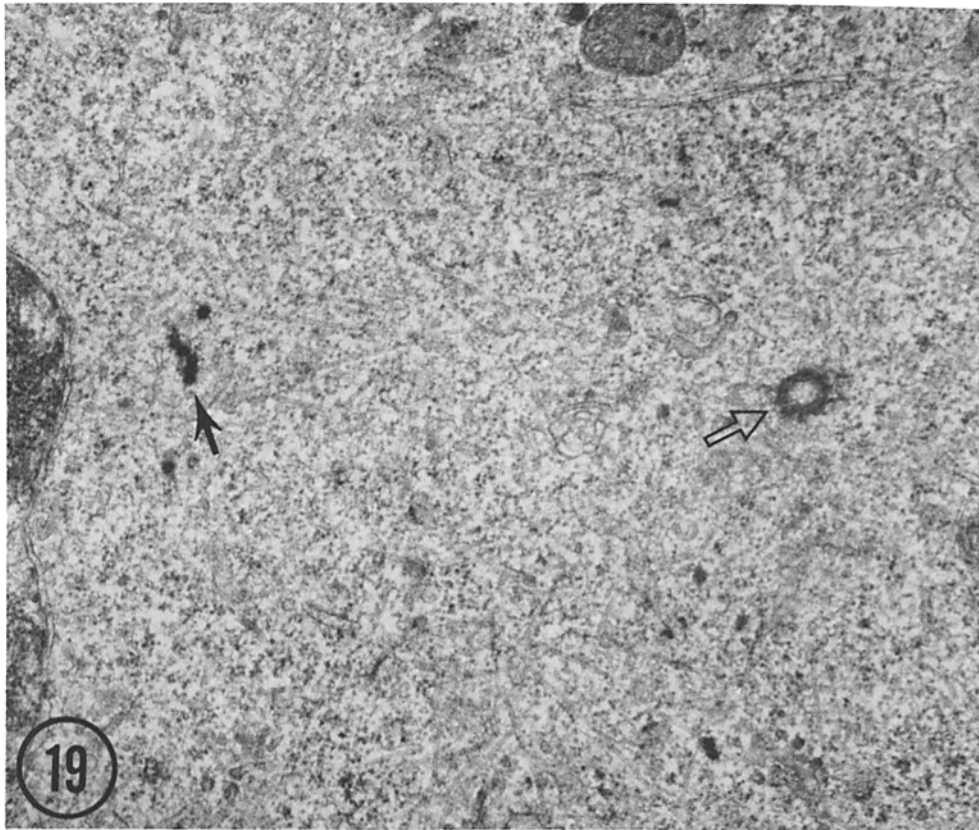
FIGURE 21 High magnification of irradiated centriole from cell 550. $\times 104,400$.





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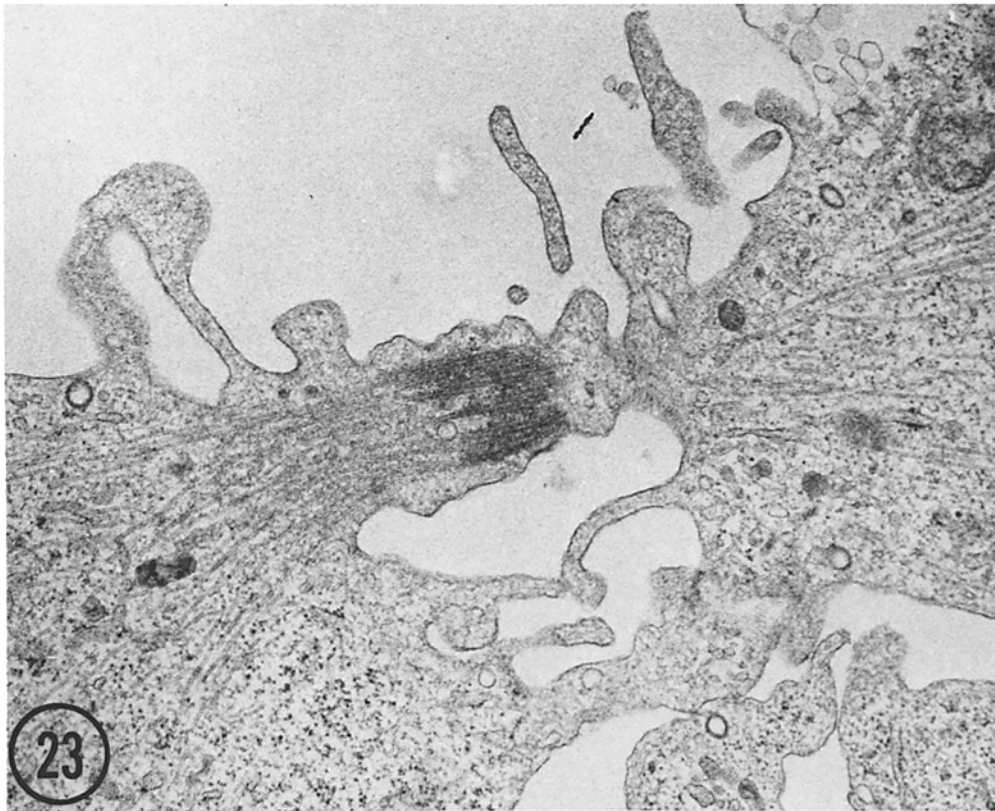
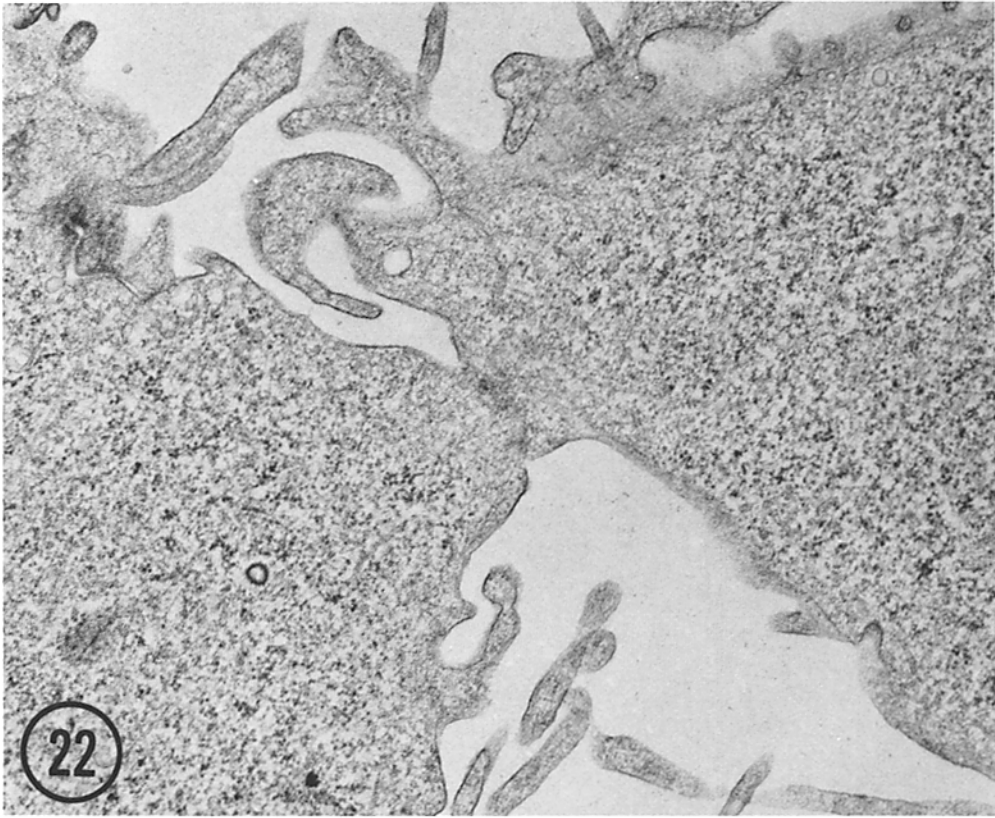


FIGURE 22 Cytokinesis constriction from cell 550 that has been irradiated in one centriolar region. Note complete lack of microtubular elements; compare to the control cell in Fig. 23. $\times 23,200$.

FIGURE 23 Control unirradiated cell illustrating a normal cytokinesis constriction with numerous microtubule elements. $\times 23,200$.

appear to have migrated apart and taken up positions on opposite sides of the chromosomes. However, the position of the irradiated centriolar region seemed to be quite variable and certainly not normal. It is recognized that any or all of these events may be dependent on centriolar participation. It is possible that the centriolar participation involves some kind of control or synthetic process that occurs some period of time before the actual morphological event (such as the production of some compound necessary for the assembly of microtubules). Inactivation of the centriolar region would not be detected in terms of behavior or spindle morphology until some period of time after irradiation.

On the basis of our results, it would appear that chromosome separation and anaphase movements toward the poles are inhibited by irradiation of one centriolar region. Furthermore, no movement of chromosomes was detected toward the unirradiated centriolar region even though there were numerous microtubules associated with the centriolar region, and kinetochore microtubules extended out from the chromosomes (see cell 485, Fig. 16). In effect, even though there was a half spindle, it did not function. These results would suggest that any poleward movements of chromosomes require two functional centriolar regions.

A consistent finding was that the centrioles within the irradiated centriolar regions appeared structurally similar to unirradiated centrioles found in numerous control unirradiated cells. As indicated in Results, it was possible to discern microtubular structure in the irradiated centrioles even though the sections were considerably oblique. The unirradiated centrioles in the irradiated cells also appeared similar to typical centrioles found in unirradiated cells, in terms of both structure and orientation with respect to each other. However, since we are dealing with recovery and electron microscopy of individual cells, it must be recognized that interpretation is often subjective and relies upon a limited number of experiments. The pericentriolar material was either absent or dislocated away from the centrioles towards the chromosomes. Furthermore, the dislocated pericentriolar material appeared damaged (Figs. 15, 20) and did have some fragments of microtubules associated with it. These observations strongly implicate the pericentriolar material in microtubule organization, as suggested by Pickett-Heaps in 1968. The possibility remains, however, that the association of the pericentriolar material with the centriole is essential for normal

spindle organization. To rule out this possibility, we would have to show that a normal spindle can be organized and can function in the absence of the centriole proper and in the presence of the pericentriolar material. Experiments of this nature are described in the following paper.

Another consistent finding was the occurrence of a cytokinesis constriction. It appears that the process of cytokinesis occurs independently of centriolar region function and chromosome movements. In addition, the almost complete absence of longitudinal microtubule profiles in the constriction region demonstrates that they are not necessary for cytokinesis. What is perhaps more interesting about these observations is that the absence of these microtubules would indicate that the centriolar regions are responsible for the organization of the microtubules often referred to as continuous, interzonal, or interpolar. These are the microtubules that apparently do not attach to the chromosomes. Since there are numerous kinetochore microtubules present on both sides of the chromosomes, the absence of the interpolar microtubules from the cytokinesis constriction may partially explain the lack of anaphase movements. We feel that this experimental result indicates that both the kinetochore microtubules and nonkinetochore microtubules are necessary for anaphase chromosome movements.

In this paper, we have presented data that we hope have clarified some aspects of the role of the centriolar region in cell division in PtK₂ cells *in vitro*. We have purposely avoided interpreting our experiments in terms of any of the currently proposed models for cell division. We feel that aspects of our results may be viewed as both consistent and inconsistent with essential components of each of the models. Much more extensive work combining laser microirradiation with electron microscopy, analysis of cell behavior, and biochemical analysis employing immunofluorescent procedures is necessary before any real definitive conclusions can be made with respect to "models".

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