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THE ABSENCE OF CENTRIOLES FROM SPINDLE POLES  
OF RAT KANGAROO (PtK<sub>2</sub>) CELLS  
UNDERGOING MEIOTIC-LIKE REDUCTION DIVISION IN VITRO

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ABSTRACT

Light and electron microscopy were used to study somatic cell reduction division occurring spontaneously in tetraploid populations of rat kangaroo *Potorous tridactylis* (PtK<sub>2</sub>) cells in vitro. Light microscopy coupled with time-lapse photography documented the pattern of reduction division which includes an anaphase-like movement of double chromatid chromosomes to opposite spindle poles followed by the organization of two separate metaphase plates and synchronous anaphase division to form four poles and four daughter nuclei. The resulting daughter cells were isolated and cloned, showing their viability, and karyotyped to determine their ploidy. Ultrastructural analysis of cells undergoing reduction consistently revealed two duplexes of centrioles (one at each of two spindle poles) and two spindle poles in each cell that lacked centrioles but with microtubules terminating in a pericentriolar-like cloud of material. These results suggest that the centriole is not essential for spindle pole formation and division and implicate the cloud region as a necessary component of the spindle apparatus.

The two-step reduction division process of meiosis is generally confined to the germ line in animals and to those cells giving rise to pollen or ova in plant tissue. Notwithstanding, there are numerous examples in plants and animals of apparent reduction in chromosome number through unusual cell division events. For example, in certain insects and ascarids (24, 28, 31), there is a selective elimination of specific chromosomes during meiosis. In some cases, there is evidence for the segregation of parental chromosome sets (30), and, in the mosquito gut, there is a reduction to lower ploidy levels from the polyploid condition (9). The formation of multiple mitotic spindle poles is also a frequently described mechanism for

the reduction of whole or partial chromosome complements (17, 18). Similarly, in plants, numerous authors discuss "meiotic-like reduction division" in somatic tissue (10, 11). The present work documents a two-step reduction division in vertebrate tissue culture cells where the cells have been followed through the two divisions, cloned, and the subsequent ploidy has been determined.

In order for a cell to undergo a two-step reduction division, certain events must occur which are different from those of normal somatic cell mitosis. First, there must be established a total of three somewhat complete spindle apparatuses: one for the initial reduction division and one more for each of the two subsequent mitotic-like divisions.

Relative to a normal interphase, this process must be accomplished in a very short period of time. Microtubules involved in spindle formation quite possibly exist in subunit form as a "pool" in the cell (13) and thus might be available for association into new spindle fibers in the second phase of reduction division. Second, the number and distribution of centrioles in reduction division cells versus normal mitotic cells will be shown to be different.

In addition to spindle fibers, there are two different additional noteworthy materials associated with centrioles. One type, referred to as the pericentriolar satellites, is sometimes present as small, round, electron-opaque spheres scattered radially outward from the centriole. The other type of material is an electron-dense, amorphous cloud directly surrounding the centriole.

Whether the centriole actually plays a role in the function of the mitotic apparatus is a highly controversial question. One opinion has implicated the centriole as the microtubule organizing center of the spindle (6, 12). Much experimental evidence has been accumulated in support of this idea, including electron micrographs which appear to show connections between spindle microtubules and centrioles (6, 14), experiments showing prevention of spindle formation by colcemid block of centriole separation (7), and studies which have shown centrioles acting as microtubule organizing centers in lysed cell preparations (16).

The other view (19, 20) implicates the pericentriolar cloud rather than the centriole itself with the role of spindle organization. Evidence for this theory includes the infrequency of photographs showing microtubule-centriole connections (26), the observation that the pericentriolar cloud region seems to undergo cyclical changes with respect to spindle formation (25), and the occurrence of divisions with no centrioles in higher plants and mouse oocyte meiotic spindles (29). In addition, it has been shown that meiotic spindle function in crane fly spermatocytes is not disrupted by dislocation of the centriole from the spindle (8), and in both mitotic and meiotic spindles of *Ulva* the centrioles are naturally located at points in the cell other than spindle poles (5, 15).

The somatic reduction division phenomenon which we will describe in this paper is of interest because it not only documents a meiotic-like ploidy-control mechanism *in vitro* but also clearly demonstrates that the spindle pole can be organized without centrioles.

## MATERIALS AND METHODS

### *Cell Culture*

Rat kangaroo kidney cells (PtK<sub>2</sub>) originally were obtained from the American Type Culture Collection and were grown as monolayer cultures in a modified Eagle's medium containing 0.85 gm NaHCO<sub>3</sub>/liter, supplemented with 10–20% fetal calf serum. Cultures were subdivided once a week into 25 cm<sup>2</sup> plastic Falcon T-flasks, using 0.125% trypsin or Viokase solutions. Cells harvested from stock cultures were seeded into Rose chambers for observation, fixation for electron microscopy, or cloning. All observations reported in this paper were made on a stable tetraploid cell line which was established by cloning a single cell with two nucleoli, twice the normal diploid nucleolar number (4). This line has been subcultured for over 2 years, and the tetraploid level has been maintained at greater than 90%.

### *Time Lapse Photography*

Time lapse photographs were made with a Sage series 500 microphotographic apparatus (Sage Instruments, Div. of Orion Research, Cambridge, Mass.) using Kodak Plus-X reversal film. The rate of cinephotography ranged from 2 to 20 frames per min with a .5 s exposure. One or more heat filters were employed to protect the cells from the infrared wavelengths of the microscope light. Cells were exposed to the light as briefly as possible when a series of still photos was being taken. The Rose chambers were maintained at 37°C by using a Sage air curtain incubator or by keeping the cells in a 37°C hot room.

### *Cloning*

The cloning procedure employed was a modified version of the method described earlier (1, 2). Both the initial reduction division and the two succeeding normal divisions were recorded by time lapse photography. Invading cells were removed by placing the chamber under a laser microbeam system and exposing the cells to multiple pulses of laser light through either a Neofluar × 40 or × 100 oil immersion objective. When the clonal population reached 64 cells, the chamber was opened, and the clone was further isolated by micromanipulation. After cloning, the chamber was covered with a fresh coverslip, the medium was changed, and the chamber was inverted and returned to a 37°C incubator. The pH was maintained at 7.2 by changing the medium frequently and placing the chamber in an airtight box containing 5% CO<sub>2</sub> in air. When the clone contained 100–300 cells, it was transferred to a separate culture flask following the procedures described earlier (2).

### *Karyotyping*

The karyotype procedure used was that of Prescott and Bender (21). Cells were exposed to .06 μg/ml of colcemid for 2.5–4 h and treated with cold Carnoy's

fixative (3:1). The fixed cells were placed on chilled glass slides and stained with 1% aceto-orcein.

### *Electron Microscopy*

Single cell electron microscopy was performed according to procedures described earlier (22, 23). The cell was photographed undergoing reduction division and fixed at the desired stage in 3% glutaraldehyde. It was then photographed again under low power, and a circle was drawn around the cell on the outer surface of the Rose chamber coverglass with a wax pencil. The chamber was opened, and the cells were run through a standard osmium tetroxide postfixation, ethanol dehydration, and flat embedding. The Epon disk containing the embedded cells was separated from the coverglass with liquid nitrogen and mounted on a blank Epon block. From 70 to 100 (depending on cell thickness) thin serial sections were cut with a diamond knife and placed on copper single slotted grids coated with .5% Formvar and carbon. Each section was examined and photographed with a Siemens Elmiskop 1A at 60 kV or a Zeiss model 9S microscope.

### RESULTS

Cells likely to undergo a two-step reduction division were first identified when the two chromosome masses were observed moving apart towards apparent spindle poles (Figs. 1 and 2). This corresponds to anaphase of the first meiotic-like division. Note that the chromosomes are composed of two chromatids and that the clear zone originally between the chromosome masses became filled with granules. Unlike meiosis, however, cytokinesis associated with this phase of the somatic process was at times incomplete and marked only by a temporary narrowing of the cytoplasm between the two groups of chromosomes (Fig. 3, lower inset). Electron microscopy of this area at different points in the division process generally revealed numerous microfilaments. However, unlike normal mitosis, there was a complete lack of microtubules in the stem body or mid-body (Fig. 3). In some cases, this narrowing resulted in the

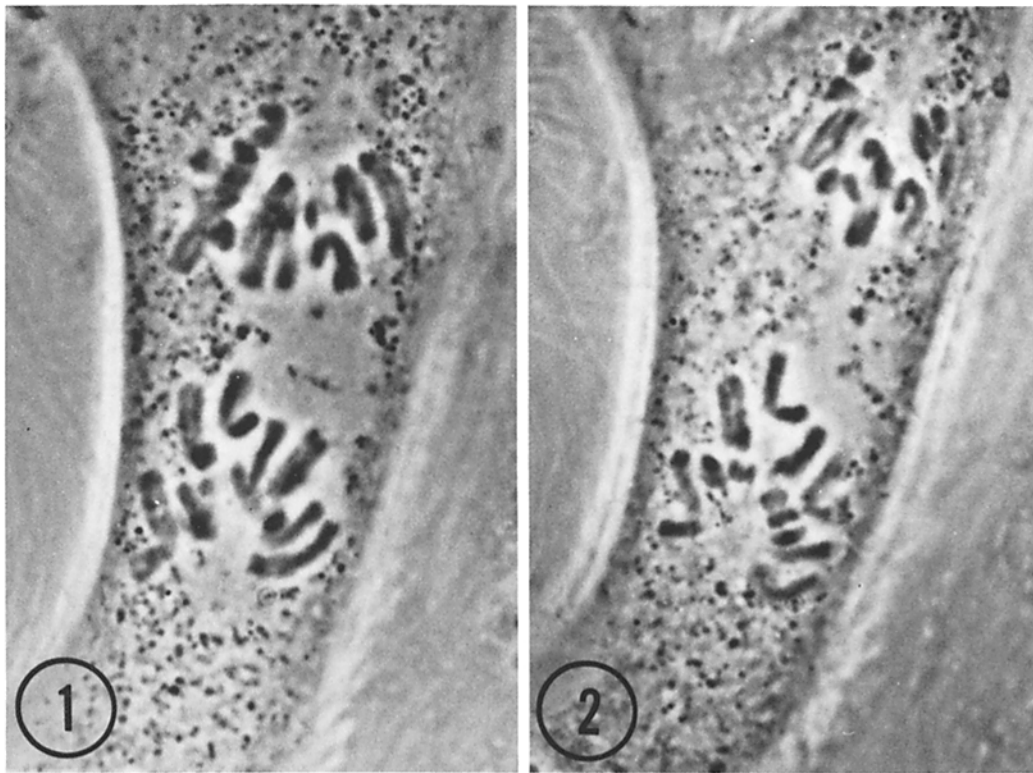


FIGURE 1 Light micrograph of a cell undergoing a two-step division. The two groups of double chromatid chromosomes are moving apart towards opposite poles. Note the clear area between the chromosome masses.  $\times 2,200$ .

FIGURE 2 The same cell after about five minutes. The clear area between the chromosome groups has become granular, and the pole to pole distance has increased.  $\times 2,200$ .

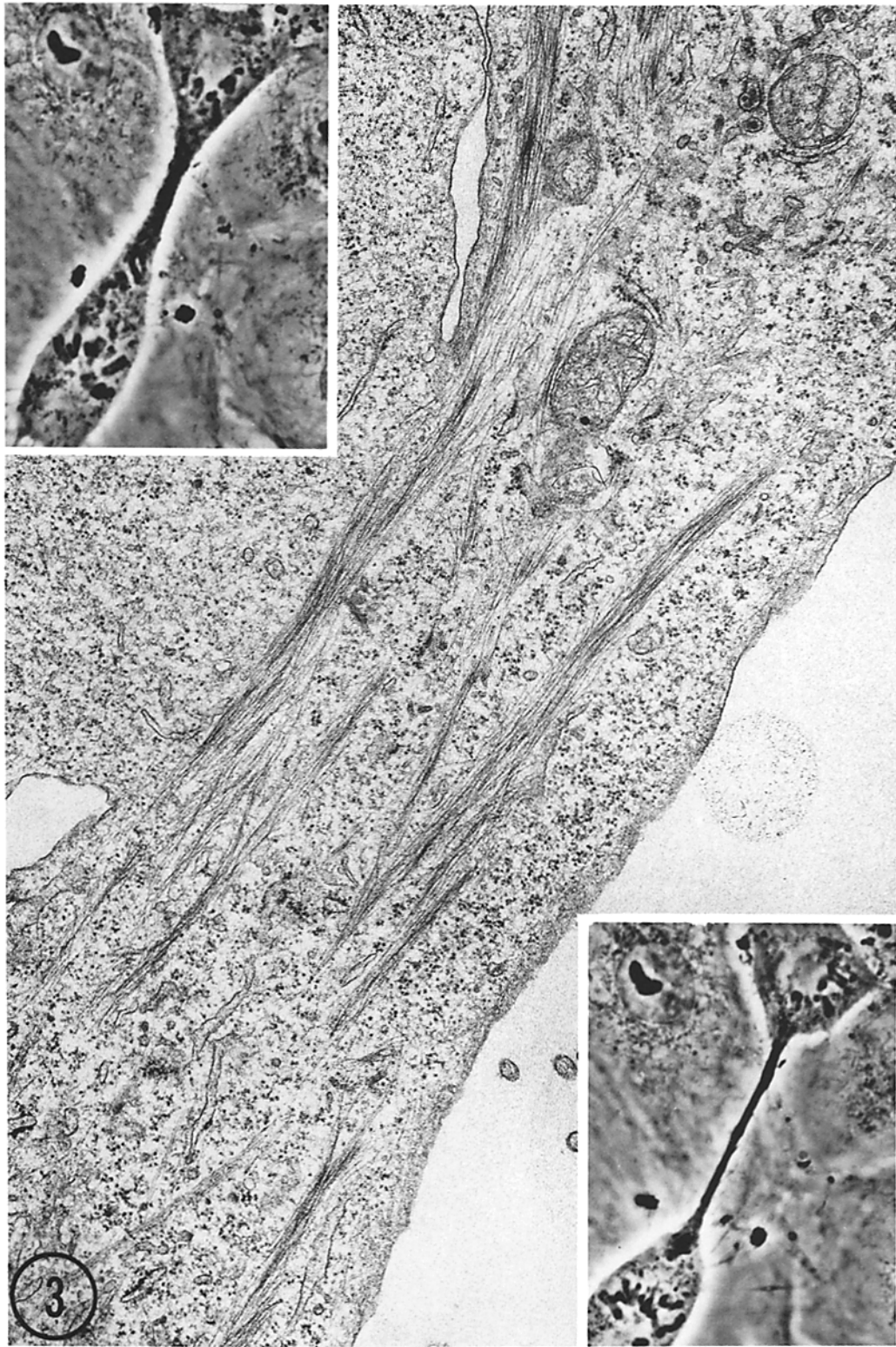


FIGURE 3 Electron micrograph of the constricted area of cytoplasm between the two chromosome masses showing numerous microfilaments and the lack of microtubules. Insets: light micrographs of the constricted area showing the long, thin bridge joining the two parts of the cell. Large micrograph,  $\times 22,500$ .

complete separation of the chromosome masses into two separate cells. Intermediate conditions also were observed in which the two cells remained connected by a very attenuated cytoplasmic strand (see Fig. 3, upper inset).

The second phase of reduction division began within 15 min. The chromosomes in each of the groups formed a metaphase plate and divided as in a normal mitosis, resulting in four spindle poles (Figs. 4–8) and eventually four nuclei. Though the formation of the metaphase plate was not always synchronous in the two chromosome groups (Fig. 6), there was a remarkably high degree of synchrony of anaphase movements. This was particularly true when a vast cytoplasmic connection remained between the groups of chromosomes (Figs. 5–8). As has been noted in conventional multipolar mitosis (17, 18), the number of nuclei formed in these divisions was usually equal to the number of poles. In all of the two-step divisions followed, four nuclei resulted. The nuclei came to reside in three or four cells depending upon whether the cytokinesis associated with the first step was partial or complete. If even a narrow cytoplasmic bridge persisted, the two middle nuclei would form a binucleate cell.

Due to the low frequency of these two-step divisions (18 cases have been clearly documented) and the inability to predict in which cells they were likely to occur before chromosome separation, it is not known what events preceded them. Light microscope observations of some cells indicated that the two masses of chromosomes did not appear to contain the same number of chromosomes; in other cells, the masses appeared to be of the same size. In order to determine whether these masses of chromosomes were composed of normal or abnormal chromosome sets and could participate in the formation of viable cells, we established a clone from the four daughter cells that resulted from a two-step division.

All four cells looked healthy and normal. Each contained the two nucleoli typical of stable tetraploid PtK<sub>2</sub> cells (4); presumably, the parent cell contained the 8N number of chromosomes. These cells were followed by time lapse photography for the first 96 h after the reduction division, so that the subsequent pattern of cell division could be documented. 26 h after the original division, about the normal cell cycle time of tetraploid PtK<sub>2</sub> cells, the first of the four cells entered into a normal, bipolar mitosis. Within a few hours, the other three cells also divided normally, indicating

that all four cells contained chromosome complements capable of supporting division. The cells produced a normal binucleolate population which was subsequently karyotyped. The karyotype of this clone, designated WAR, was indistinguishable from that of other stable tetraploid clones studied. It contained 14 long autosomes, seven short autosomes, two X chromosomes, one Y<sub>1</sub>, and two Y<sub>2</sub> chromosomes. 92% of the karyotypes contained between 22 and 28 chromosomes (Fig. 9). The distribution had no hint of bimodality, suggesting that either all four of the original cells contained the same number of chromosomes or that a selective pressure in culture had favored cells with chromosome numbers in this range. Ultrastructural studies of the WAR cells are now in progress.

A total of seven reduction division cells at various stages of division were fixed for electron microscopy and serial sectioned. Each cell was fixed when its chromosome masses were greatly separated by granular cytoplasm so as to avoid fixation of a normal, prometaphase cell. In general, the cells appeared normal with the usual-looking chromosomes, tri-layered kinetochore structure, and attached kinetochore microtubules. The most outstanding result of electron microscope examination was the consistent finding of only two complete centriolar duplexes in each cell, independent of the stage at which the cell was fixed. In two of the cells fixed at the "second anaphase" of reduction division, the two duplexes were positioned with one at each end of the cell (Fig. 10, insets). The two central poles had no centrioles. The same arrangement of centrioles was also found in a cell fixed before the double metaphase stage. No centrioles were found in the center region of any of these cells, though two or four were expected since two distinct poles were organized in that region. In two other cells also fixed at the second anaphase, one duplex was found at one of the outer spindle poles, and one in the central region. Two poles still lacked centrioles in these cells. Close examination of the centriole-lacking pole regions revealed slightly electron-dense areas at which kinetochore microtubules and other microtubules terminated (Figs. 11 and 12, arrows). This region resembled what is referred to as the pericentriolar cloud (including satellites) which is normally found around a centriolar duplex and in the sections above and below a normal duplex (Fig. 13). The cloud region has already been implicated as an important part of spindle organization (3,

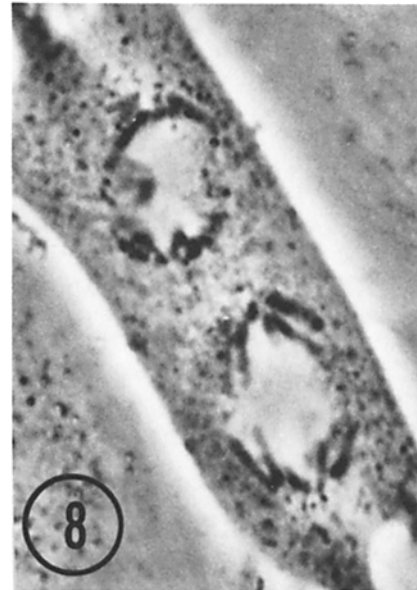
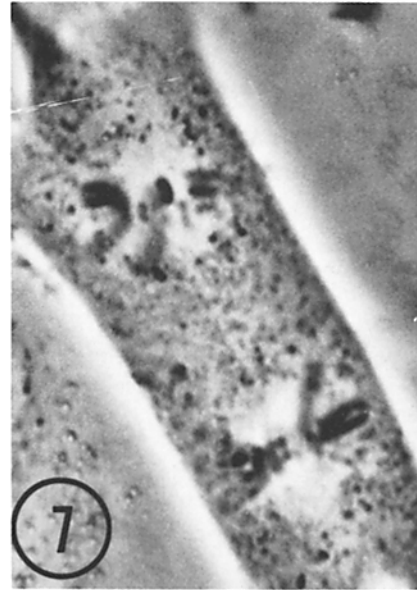
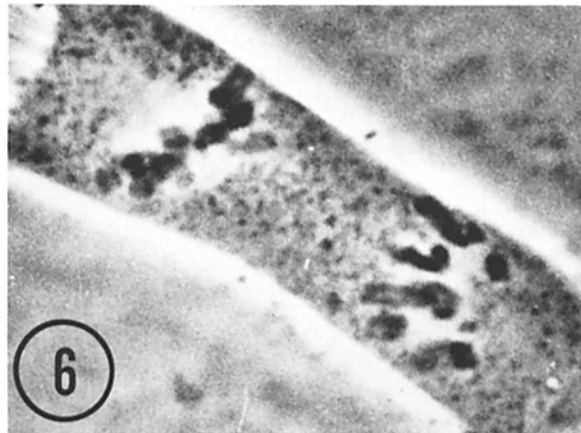
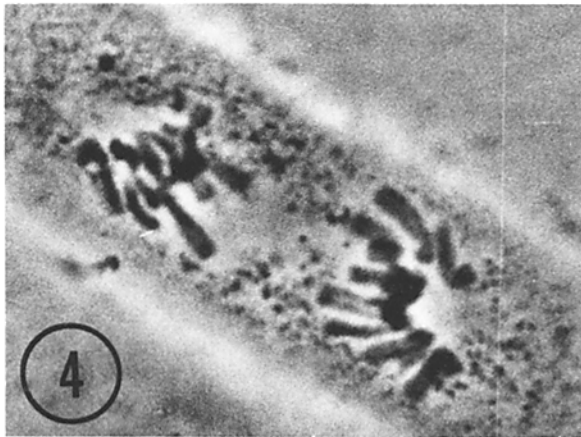


FIGURE 4 Light micrograph of a cell beginning a two-step division. The clear area between chromosome masses is still visible.  $\times 1,280$ .

FIGURE 5 The distance between chromosome masses has increased, and the clear area has disappeared.  $\times 1,280$ .

FIGURE 6 Asynchronous formation of the first of two metaphase plates.  $\times 1,280$ .

FIGURE 7 Cell with both metaphase plates observable.  $\times 1,280$ .

FIGURE 8 Synchronous anaphase of a second step of division. In this case, there was a very wide cytoplasmic bridge between the two anaphase spindles. This bridge did contain numerous microfilaments when examined with the electron microscope.  $\times 1,280$ .

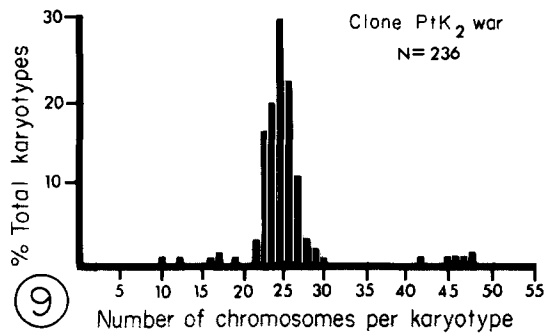


FIGURE 9 Percentage of karyotypes of WAR cells containing a given number of chromosomes. 92% of the karyotypes contain between 22 and 28 chromosomes.

19). Work is now in progress involving isolation of reduction division daughter cells and possible establishment of a clone lacking centrioles.

In order to eliminate the possibility that these two-step divisions originate from binucleate cells, we followed more than 40 binucleate cells into and through mitosis. None of them divided in the manner just described. In all binucleate cells we followed, the chromosomes from both nuclei intermingled during prometaphase, formed a single metaphase plate, and divided in a single, multipolar mitosis (Figs. 14–16). No case was found in which each nucleus formed an independent mitotic spindle. Electron microscopy of binucleate cells in early prophase has consistently shown the presence of four or more duplexes (8 or more centrioles) (Figs. 17–19). Therefore, it seems highly unlikely that reduction division cells (always containing two duplexes) represent nuclear division in binucleate cells.

## DISCUSSION

The meiotic-like reduction division in these vertebrate tissue culture cells has been shown to involve two cell divisions. The first division is a reduction division (possibly involving homologues) in which chromosomes with two chromatids segregate into two different daughter cells. The second division employs chromatid separation in a mitotic-like division yielding a total of four separate nuclei. Both divisions occur within one hour, and the resulting daughter cells are viable. Karyotype data from the clonal population of reduction division daughter cells indicate that the chromosome number after reduction division is stable and appears identical to the chromosome number and distribution in the populations from which the original reduction cell

arose. This suggests that reduction division may play a role in the determination and maintenance of ploidy levels in cultured cells.

One of the main arguments against the reduction division phenomenon described in this paper is that we are merely seeing binucleate cells in which each nucleus sets up its own independent spindle. We feel that this is not the case because (a) over 40 binucleate cells have been followed through mitosis, and no divisions resembling the two-step reduction process have been observed, and (b) the number of centrioles associated with binucleate cells has been shown to be consistently higher than in the reduction division cells.

Electron microscope observation of these cells has shed further light on the ultrastructural events which have occurred and affords us an opportunity to at least speculate on possible causes and mechanisms of this interesting phenomenon. The absence of microtubules in the first division constriction seems to imply that this first reduction spindle is lacking in continuous spindle microtubules. Whether or not this is a cause of the reduction steps is at this point unknown. It is interesting to compare this step to a normal prometaphase step frequently occurring in PtK<sub>2</sub> cells. This prometaphase movement has been observed in our own laboratory and has been reported by Roos (27). It involves the anaphase-like movement of two groups of chromosomes with two chromatids to opposite poles of the cell and their subsequent return to the center of the cell to form a normal metaphase plate. EM studies of this movement reveal a nonsynchronous “hook-up” of kinetochore microtubules from the two chromatids of each chromosome to the opposite poles as possibly responsible for this initial false anaphase movement. Perhaps it is a failure of the second kinetochore hook-up which could allow for a reduction division.

The absence of centrioles at organized and functional spindle poles and the presence of pericentriolar cloud-like material at which microtubules terminate lead to further questions about spindle mechanics. Since centrioles themselves appear to be unnecessary for the organization and function of a spindle pole, our results suggest that it is the pericentriolar cloud region which is actually responsible for pole organization. This is evidenced both by the EM studies presented here and by studies also done in our laboratory involving preferential destruction or damage of the pericentriolar material with laser microbeam irradiation (3).



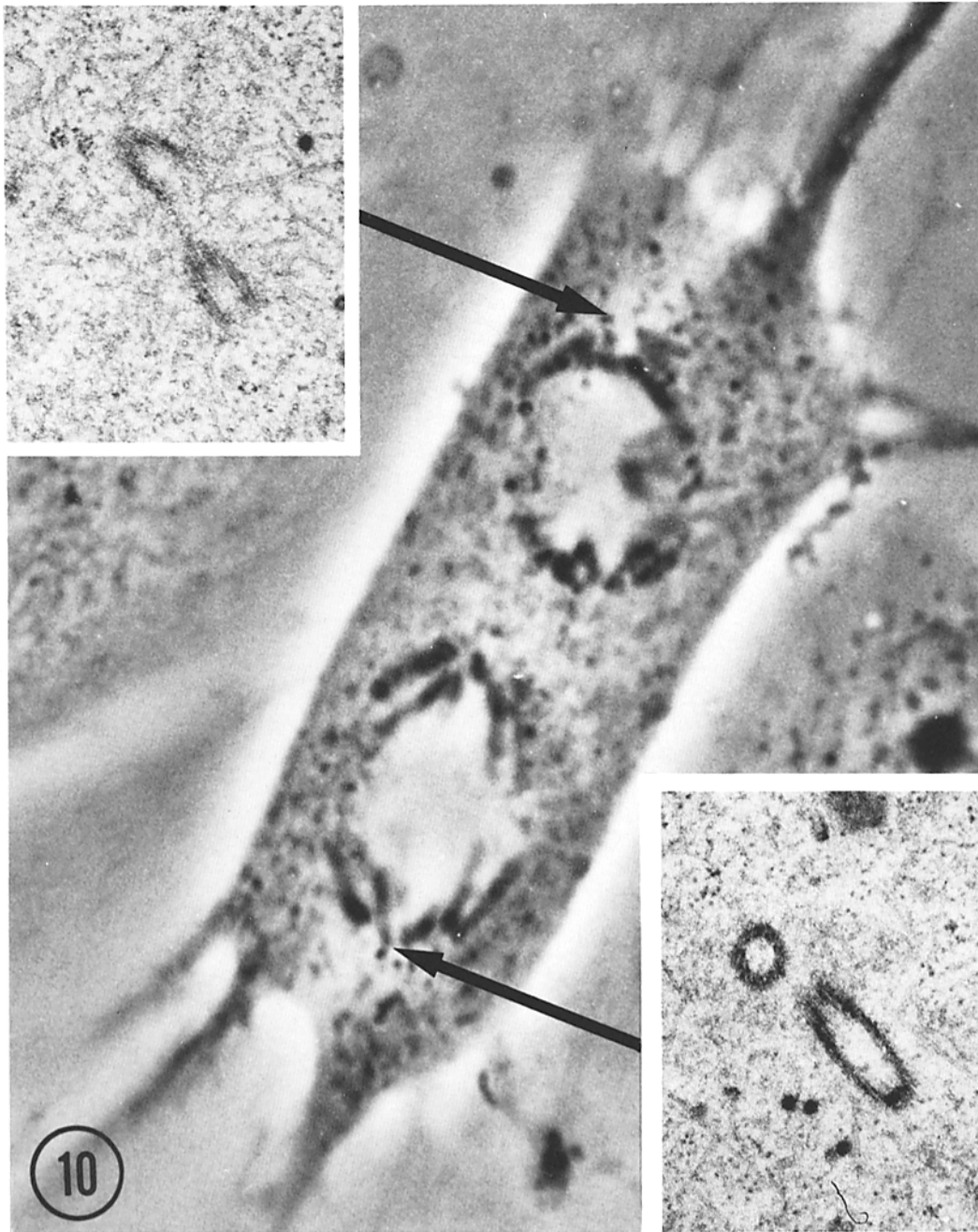


FIGURE 10 Enlargement of light micrograph of a fixed reduction division cell at the second anaphase stage. Arrows indicate the points at which the two duplexes of centrioles were found. Insets: the two duplexes found in the electron microscope examination of fixed cell,  $\times 33,600$ . Large micrograph,  $\times 2,240$ .

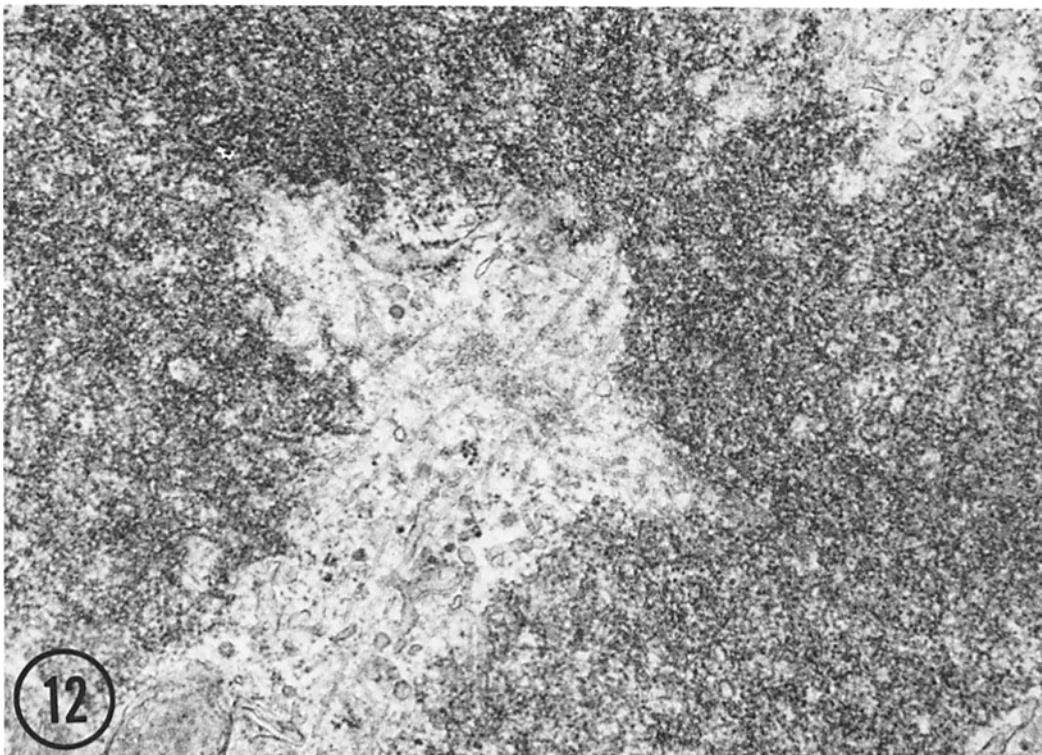
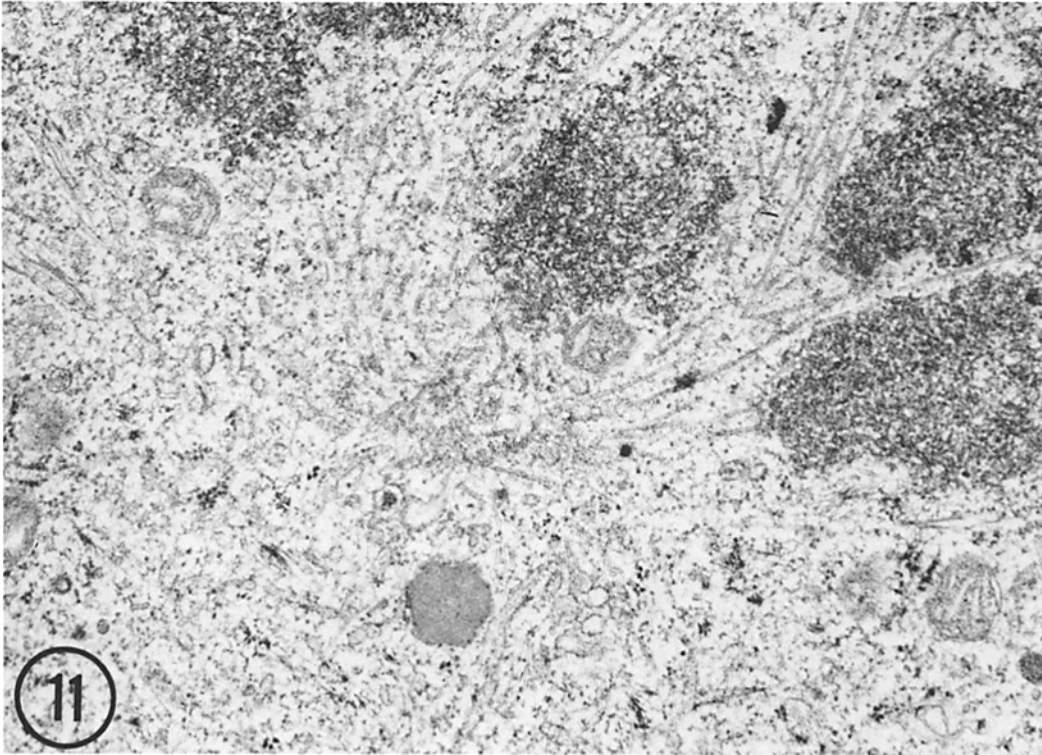


FIGURE 11 Electron micrograph of centriole-lacking pole of a reduction division cell. Arrows point to the electron-dense region which serves as a termination point for continuous and kinetochore microtubules. Also note the presence of a pericentriolar satellite. (Other satellites are seen in greater numbers in other serial sections of the same cell.) This region resembles the pericentriolar cloud area around a normal centriolar duplex.  $\times 25,600$ .

FIGURE 12 A second cell also showing the cloud-like material at a pole without centrioles.  $\times 25,000$ .

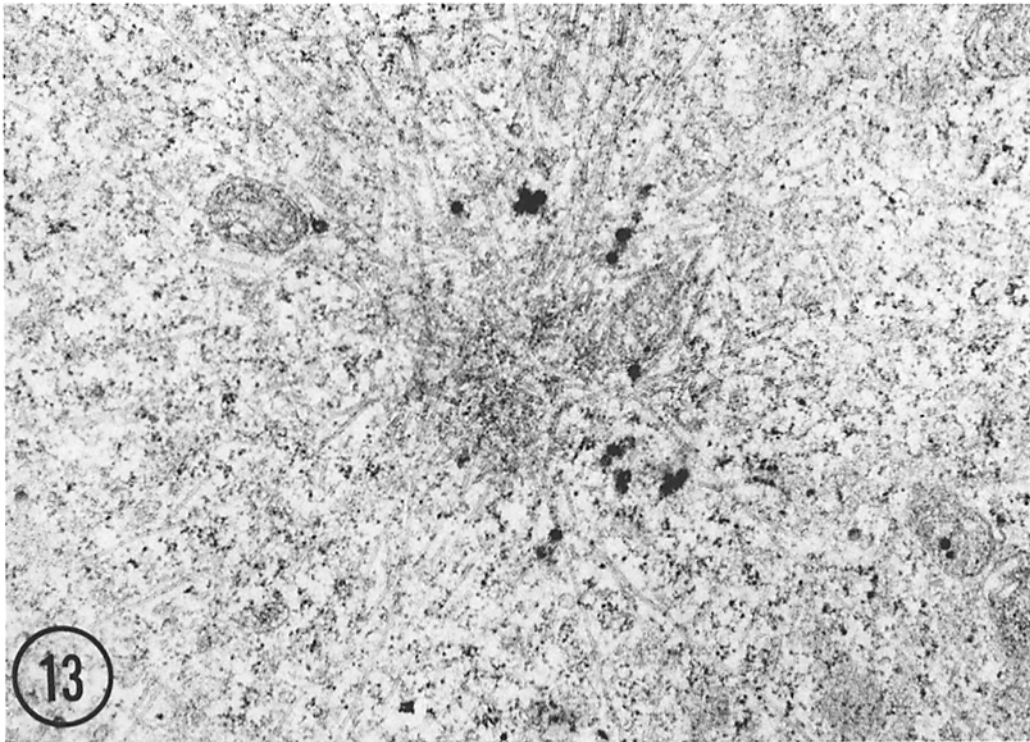


FIGURE 13 A section through a control cell of the area above a normal duplex. Note the similarity between this photograph and the previous two.  $\times 25,600$ .

These irradiation studies showed that cells which were damaged in the pericentriolar region of one pole but left with ultrastructurally normal centrioles could undergo cytokinesis at metaphase, but both chromatid separation and anaphase movements were arrested. A combination of these two results, i.e., functional poles without centrioles but with pericentriolar material and partially nonfunctional poles with centrioles but with damaged pericentriolar material, implicate the pericentriolar material as an essential component for normal mitosis.

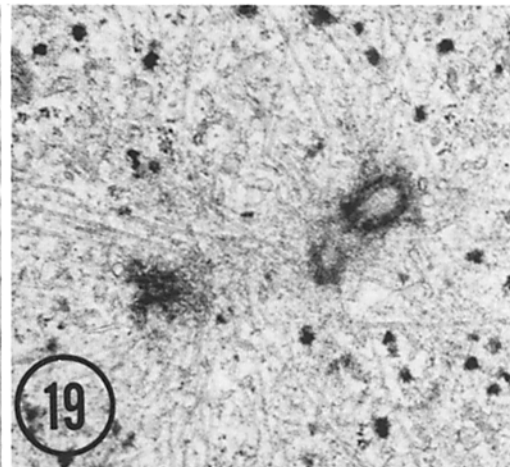
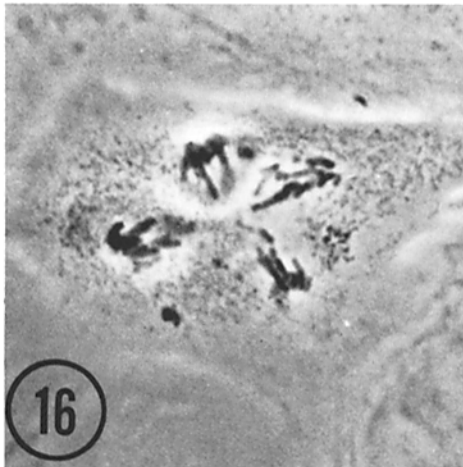
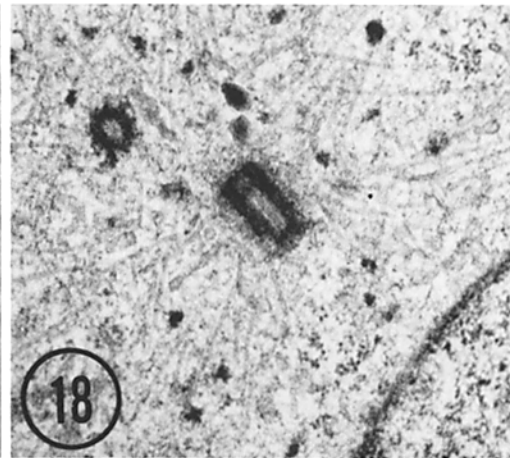
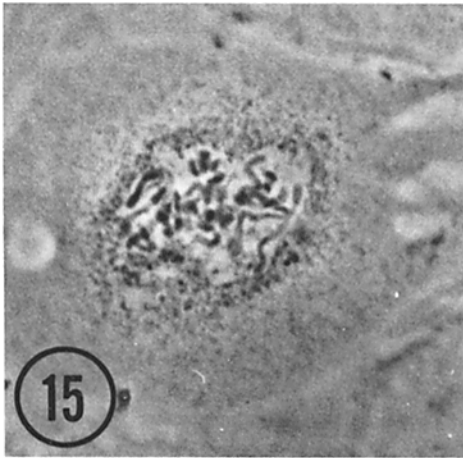
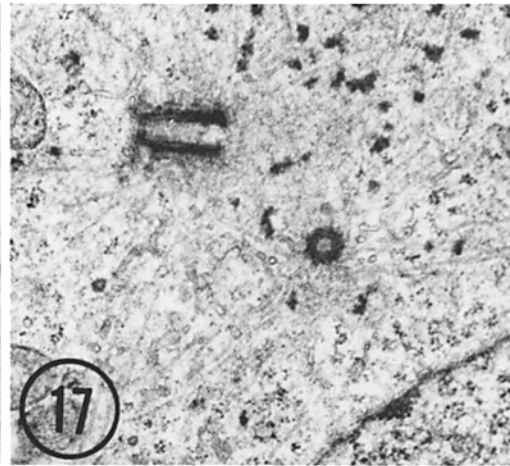
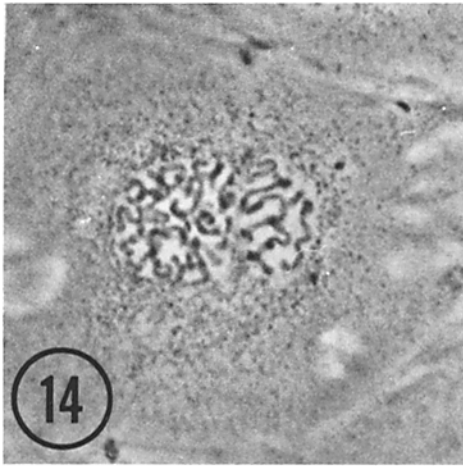
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FIGURE 14 Binucleate cell at early prophase. Nuclear membranes are still intact. × 880.

FIGURE 15 After nuclear membrane breakdown, the chromosomes from both nuclei mingle. × 880.

FIGURE 16 Tetrapolar anaphase. × 880.

FIGURE 17 Two of the eight centrioles found in a binucleate prophase cell. These centrioles are not a duplex but rather each forms a duplex with a second centriole sitting in a plane below it, as pictured in Fig. 18. × 20,000.

FIGURE 18 Two other centrioles found in a plane below that of those in Fig. 17. These four make up two duplexes. The same nuclear membrane is shown for orientation. × 20,000.

FIGURE 19 Three of another group of four centrioles found on the other side of the binucleate cell nuclei. (The fourth is found in another plane not pictured.) × 25,600.