

Aster-free Spindle Poles in Insect Spermatocytes: Evidence for Chromosome-induced Spindle Formation?

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Abstract. Tipulid spermatocytes form normally functioning bipolar spindles after one of the centrosomes is experimentally dislocated from the nucleus in late diakinesis (Dietz, R., 1959, *Z. Naturforsch.*, 14b:749–752; Dietz, R., 1963, *Zool. Anz. Suppl.*, 23:131–138; Dietz, R., 1966, *Heredity*, 19:161–166). The possibility that dissociated pericentriolar material (PCM) is nevertheless responsible for the formation of the spindle in these cells cannot be ruled out based on live observation. In studying serial sections of complete cells and of lysed cells, it was found that centrosome-free spindle poles in the crane fly show neither peri-

centriolar-like material nor aster microtubules, whereas the displaced centrosomes appear complete, i.e., consist of a centriole pair, aster microtubules, and PCM. Exposure to a lysis buffer containing tubulin resulted in an increase of centrosomal asters due to aster microtubule polymerization. Aster-free spindle poles did not show any reaction, also indicating the absence of PCM at these poles. The results favor the hypothesis of chromosome-induced spindle pole formation at the onset of prometaphase and the dispensability of PCM in *Pales*.

CENTROSOMES (4) function as polar foci in a great number of organisms both in mitosis and meiosis. It was and still is a widely accepted hypothesis that centrosomes are compulsory for spindle formation (2, 19, 20), most probably by nucleating microtubules (18, 22, 28, 31) that become associated with the kinetochores of the chromosomes during early prometaphase (26). On the other hand, many cell types perform nuclear division in the absence of centrosomes, for example, the cells of higher plants and fungi (25), or mammalian oocytes (6, 29). Electron microscopical investigations show that normal centrosomes are compound structures composed of a centriole pair, a cloud of darkly staining pericentriolar material (PCM),¹ and microtubules radiating from it ("aster" microtubules). It became evident that the capacity of the centrosome to nucleate microtubules is due to the PCM (15, 30), and the PCM is regarded as the important component for spindle pole formation, while the centrioles do not seem to play a critical role during this process (3, 5, 6, 17). The presence of PCM could recently even be verified at the spindle poles in plant cells, a well known example of acentric spindle formation (8, 32).

In several light microscopical studies Dietz showed that in primary spermatocytes of the crane fly fully functional spindles may develop in prometaphase around the chromosomes, even if one centrosome does not participate in this process (9–11). In these studies one centrosome had experimentally

been separated from the nucleus in late diakinesis by flattening of the cells. Dietz showed that despite the absence of one pole-determinating centrosome, the formation of a bipolar spindle, the regular distribution of the chromosomes to the daughter cells, and cytokinesis proceeded without irregularities in these cells. Dietz took the findings as evidence that centrosomes in the crane fly may be dispensable during nuclear division. In an attempt to explain these results, which are contradictory to the widely accepted opinion about the importance of centrosomes, Peterson and Berns argued (25) that in Dietz' experiments the flattening of the spermatocytes displaced the centrioles from the nuclei but could have left the PCM behind. Dissociation of the PCM from the centrosome would, however, not be recognizable in the light microscope by live observation. Keryer et al. (17) concluded from their experiments with Chinese hamster ovary cells that the PCM may even "have the capacity to self-aggregate and serve as poles in the absence of centrioles." If the PCM in crane fly cells displayed such autonomy, it could indeed perform its regular role in determining the spindle poles. To investigate this possibility we carried out the present study of combined light and electron microscopy.

Materials and Methods

Intact Cells

Testes from fourth instar larvae of *Pales ferruginea* (Nematocera, Tipulidae) were disrupted under a drop of liquid paraffin (A. G. Merck, Darmstadt, FRG)

1. Abbreviation used in this paper: PCM, pericentriolar material.

on a polylysine-coated cover slip. This freed spermatocytes suspended in the testis fluid. In the drop of fluid that adhered to the glass surface the cells were dispersed by moving the testis sheath with a needle across the coverslip. This procedure resulted in a certain flattening of the cells. Cell preparations were observed with a Diavert microscope (Leitz, Wetzlar, FRG) equipped with phase contrast optics. For fixation the technique described by Nicklas (23) was slightly modified. The fixative (6% glutaraldehyde [Serva, Heidelberg, FRG] in 0.1 M Pipes buffer, pH 6.8) was applied under the paraffin oil using a fine glass capillary $\sim 10 \mu\text{m}$ in diameter. A microsyringe connected to the capillary by a silicon tube controlled the flow of the fixative. After 2–5 min the paraffin oil was removed by slowly immersing the coverslip in 2% glutaraldehyde in the same buffer. The cells sticking to the coverslip were fixed for 30 min. After being rinsed in buffer (three times for 15 min) the cells were treated for 1 h with 2% tannic acid in distilled water, adjusted to pH 7.0 with KOH. After a 2-h rinse in 0.05 M cacodylate buffer, the cells were postfixed for 8 min in 1% OsO_4 and treated overnight in 2% uranyl acetate. After dehydration in an acetone series cells were flat-embedded in the polyester resin Mikropal (Ferah, Berlin), and serial sectioning was carried out with an Ultratome III (LKB Instruments, Inc., Gaithersburg, MD) at a feed of 500 Å. Sections were routinely double-stained with uranyl acetate and lead citrate, and complete series through the centrosome and polar regions were examined with a Zeiss EM 10A electron microscope. We reconstructed a chromosome fiber according to the methods Fuge described (13, 14).

Lysed Cells

Testes smears were prepared under liquid paraffin on a polylysine-coated coverslip. The coverslip was placed upside-down on a microscope slide to which two strips of coverslip glass had been glued, thus forming a cell chamber for live observation ("hanging drop" preparation). For electron microscopy the lysis solution (modified after Capco and Penman, reference 7) containing 10 mM Pipes (pH 6.8), 100 mM NaCl, 3 mM MgCl_2 , 300 mM sucrose, 0.5% Triton X-100, and 10 $\mu\text{g}/\text{ml}$ taxol for microtubule stabilization (kindly provided by Dr. M. Sufness, National Cancer Institute, Bethesda, MD) was applied from the side under the coverslip. After 5 min the lysis medium was replaced by a fixative (2% glutaraldehyde in 0.1 M Pipes, pH 6.8, containing 1 mM MgCl_2 and 1 mM EGTA). After 5 min the coverslip was removed from the slide by gently immersing the whole slide in the fixative. The lysed cells sticking to the coverslip were prepared as described above. For light microscopical preparation a lysis buffer was used that had different concentrations of Pipes (50 mM) and sucrose (100 mM). This change in concentration had no effect on the morphology of spindles at the light microscopical level. For light microscopy fixed cells were transferred to 20% ethanol (10 min), stained with Coomassie Blue (100 mg/50 ml 30% ethanol) for 15 min, dehydrated in an ethanol series, and embedded in Euparal (Chroma, Stuttgart, FRG).

Microtubule polymerization studies were carried out in a medium containing 100 mM Pipes (pH 6.8), 1 mM EGTA, 1 mM MgCl_2 , 100 mM fructose, 1.3 mM guanosine-5'-triphosphate, 1% Triton X-100, and 4 mg/ml pig brain tubulin purified by four polymerization-depolymerization steps (the tubulin was kindly provided by Dr. David Russell, Max-Planck-Institut für Biologie, Tübingen). After several pilot tests with differently composed buffers this polymerization buffer gave optimal results regarding nucleation of microtubules at centrosomal sites, and with respect to the visibility of the spindle after lysis in the light microscope. The medium was applied from the side under the coverslip as described above, and the cells were examined with Zeiss phase contrast optics. Controls were run with bovine serum albumin (BSA) instead of tubulin.

Results

The flattening of the spermatocytes can cause different positions of centrosomes in late diakinesis. Both centrosomes can remain together at one side of the nucleus, i.e., the normal movement of one centrosome around the nucleus to the opposite position before spindle formation is prevented. One centrosome may also become separated from the nucleus. The centrosome remains separate only if it lies far enough away from the forming spindle. Then it is no longer involved in spindle formation. In both cases a bipolar spindle is formed in early prometaphase with one pole lacking a centrosome. In another case one separated centrosome can remain in a position closer to the nucleus, and contact and fuse with the forming spindle from the side. This results in the formation

of a tripolar spindle. Displaced centrosomes are no longer properly distributed to the daughter cells, and thus secondary spermatocytes occasionally arise which lack any pole-determining organelles. Nevertheless, a bipolar mitotic spindle is formed in these cells, and chromosome distribution and cytokinesis proceed (11).

In this study a total of 35 cells with dislocated centrosomes was studied by live observation. Three of the cells were fixed during live observation, embedded, and serially sectioned for electron microscopical examination. These cells are characterized as follows.

Cell I. Observation began when the cell was in late diakinesis. The separated centrosome was several micrometers away from the nucleus. After breakdown of the nuclear envelope, a normal bipolar spindle was formed. The displaced centrosome did not contact the spindle. The cell was fixed 70 min after the onset of prometaphase.

Cell II. Observation began in early prometaphase. The separated centrosome was far away from the spindle and remained there until the cell was fixed (~ 80 min after the breakdown of the nuclear envelope).

Cell III. This cell was observed from early prometaphase up to metaphase. The separated centrosome was initially near the spindle equator. At the time of fixation at metaphase (~ 90 min after nuclear envelope breakdown) the centrosome had not changed its position and had fused with the spindle to produce a tripolar configuration. The true tripolar nature of the spindle, however, was only detected with the electron microscope.

Examination of complete section series through cells I and II reveals the spindle to be conventionally, i.e., approximately biconically shaped. Fig. 1, *a* and *b*, shows two consecutive sections through the centrosome at one spindle pole of cell II. Fig. 1, *c–f*, are four consecutive sections through the dislocated centrosome of the same cell lying at the cell periphery. Both centrosomes possess a pair of centrioles. One centriole is caught in all the sections shown. The centrioles are surrounded by darkly staining PCM filling an area of $\sim 1–2 \mu\text{m}$ in diameter. The PCM is interspersed with irregularly arranged aster microtubules caught in longitudinal and cross section. The dark mass of PCM-like material is best outlined against the surrounding cytoplasm in Fig. 1, *e* and *f*. The ultrastructure of the displaced centrosome thus does not seem to differ from the centrosome at the spindle pole. The same was found in cell I.

The apex of the half-spindle lacking a centrosome in cell II reaches to the cell membrane. Fig. 2 shows six consecutive sections of a complete series through the centrosome- and aster-free spindle pole of this cell. Centrioles are, of course, absent. There are also no indications of microtubules arranged in an aster-like fashion nor of electron-opaque masses resembling the PCM of centrosomes. Centrosome-free spindle poles in cells I and III revealed these same features.

The orientation of microtubules within the half-spindle lacking polar centrosomes is the same as in the opposite half-spindles or in normal spindles with a complete set of polar centrosomes. To document this, one centrosome fiber from cell II pointing in the direction of the centrosome-free pole was reconstructed (Fig. 3). Kinetochore microtubules extend up to $4 \mu\text{m}$ into the half spindle. They are associated with hundreds of nonkinetochore microtubules, many of which

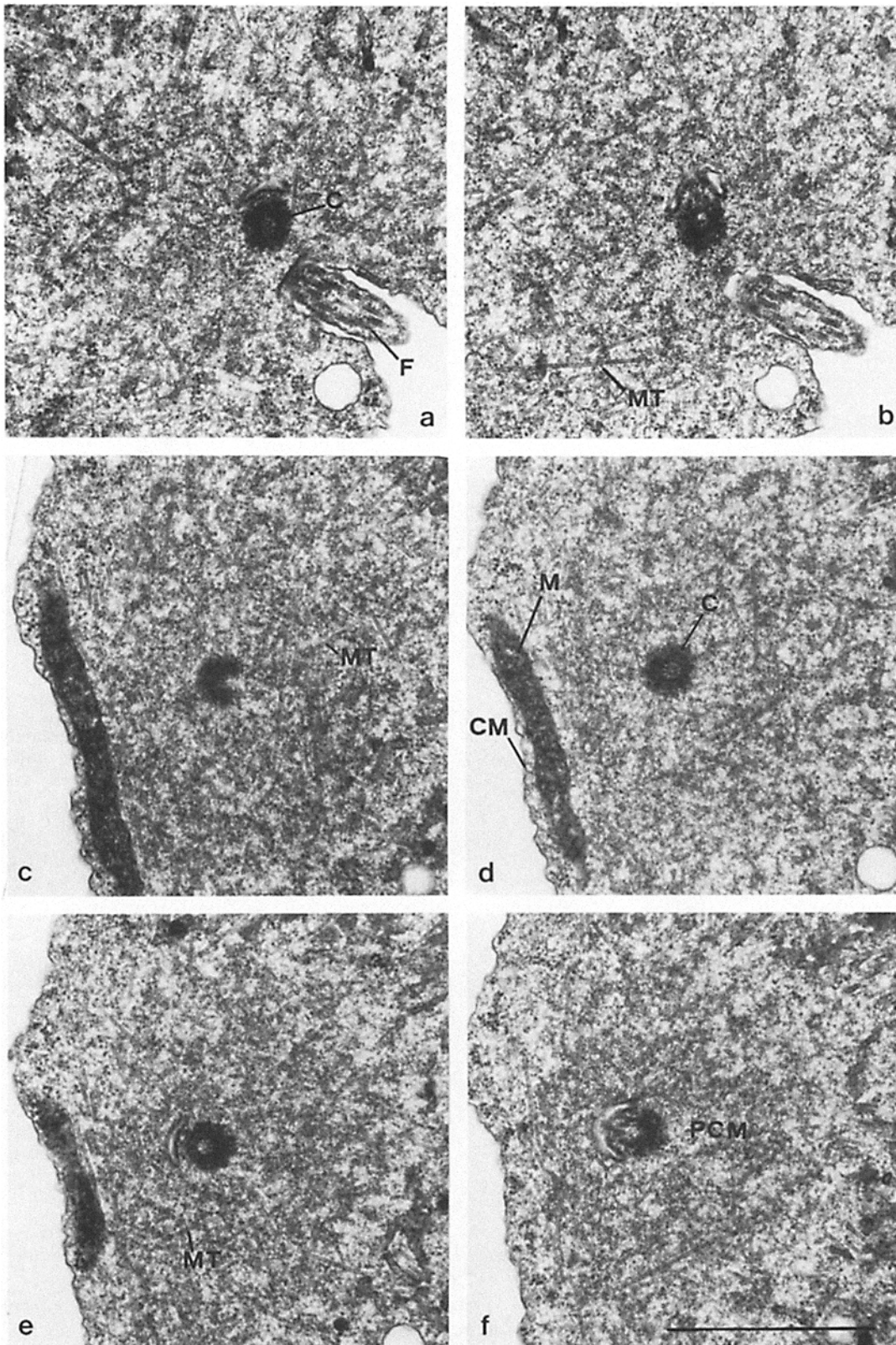


Figure 1. Cell II. (a and b) Two consecutive sections through the centrosome lying at one pole. (c-f) Four consecutive sections through the dislocated centrosome lying at the cell periphery. Aster microtubules (MT) and diffuse PCM surrounding the centrioles (C) are seen in all sections. CM, cell membrane; F, flagellum; M, mitochondrion. Bar, 1 μ m.

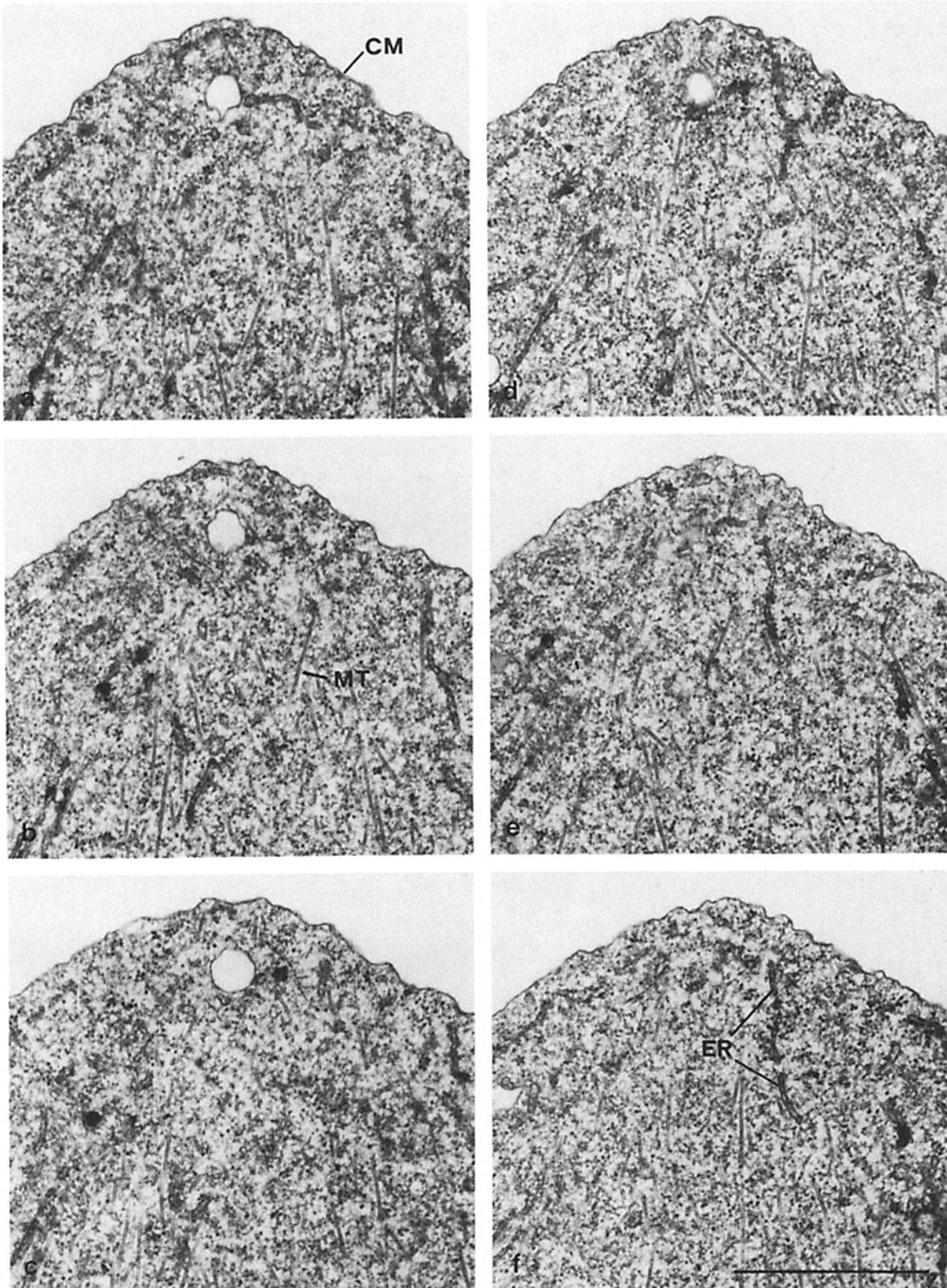


Figure 2. Cell II. (a-f) Six consecutive sections through the centrosome- and aster-free spindle pole. *CM*, cell membrane; *ER*, endoplasmatic reticulum; *MT*, spindle microtubules. Bar, 1 μ m.

run obliquely to the chromosome-pole axis (13, 14).

The spindle of cell III had a protrusion pointing in the direction of the dislocated centrosome, thus establishing a

tripolar configuration. Microtubules in this protrusion oriented toward this centrosome (no micrograph shown). Kinetochore microtubules of the chromosomes were, however,

oriented toward the centrosome-free spindle pole and toward the pole occupied by the nondislocated centrosome, but not toward the dislocated centrosome which had fused with the spindle. Hence, the displaced centrosome influenced the orientation of the nonkinetochore microtubules but not of the kinetochore microtubules (for microtubule orientation in tri-

polar spindles see the isolated spindle in Fig. 5).

Various investigators have shown that PCM is still present and functionally active in mammalian cells after cells were lysed in buffers containing 0.25–0.5% Triton X-100 (15, 18, 30). To obtain images of centrosomes and polar regions that are more clearly outlined than in intact cells, spermatocyte preparations were subjected to different lysis media in another set of experiments. A good preservation of spindles and centrosomes with a minimum of spindle matrix material adhering to the microtubules was achieved with a Triton X-100 and taxol-containing buffer. Larger than the sex univalents, and as dark as chromosomes, the centrosomes presented themselves as most conspicuous and unmistakable components of the persisting cytoskeletons. If the spermatocytes were not flattened before lysis, the two centrosomes occupied positions at the spindle poles. After lysis of flattened spermatocytes one centrosome was frequently found at various positions off the spindle pole. Fig. 4 shows light micrographs of isolated tripolar spindles stained with Coomassie Blue. Bundles of aster microtubules are partly visible. Half-spindles lacking centrosomes are cone-shaped without any polar differentiations.

Two isolated spindles, each with one centrosome-free pole, were investigated with the electron microscope. Fig. 5 shows different sections from a complete series through an isolated late anaphase spindle with one dislocated centrosome. The spindle is tripolar. One set of autosomal half-bivalents moves toward the pole occupied by an aster and the other set toward the pole lacking an aster. Sex univalents X and Y still lag in the equator region at this stage of anaphase segregation, as is usual in tipulids (12). The third pole of the spindle is formed by the dislocated centrosome. Centrosomes each consist of a pair of centrioles and a dense mass of PCM with aster microtubules radiating from it (details in Fig. 6). Centrioles already bear short axonemes of future sperm flagella (Fig. 5). The aster-free spindle pole not only is devoid of radiating aster microtubules, but also does not seem to possess PCM-like material.

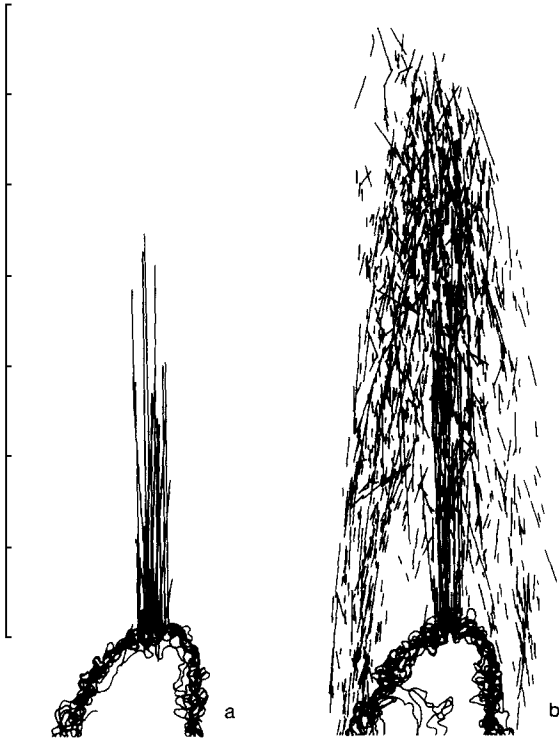


Figure 3. A chromosome fiber from cell II pointing in the direction of the centrosome-free pole. (a) Reconstruction of kinetochore microtubules achieved by tracing the tubules through the section series. (b) Reconstruction of the whole fiber achieved by superimposing the microtubule fragments as seen in the sections. Scale unit, 1 μm .

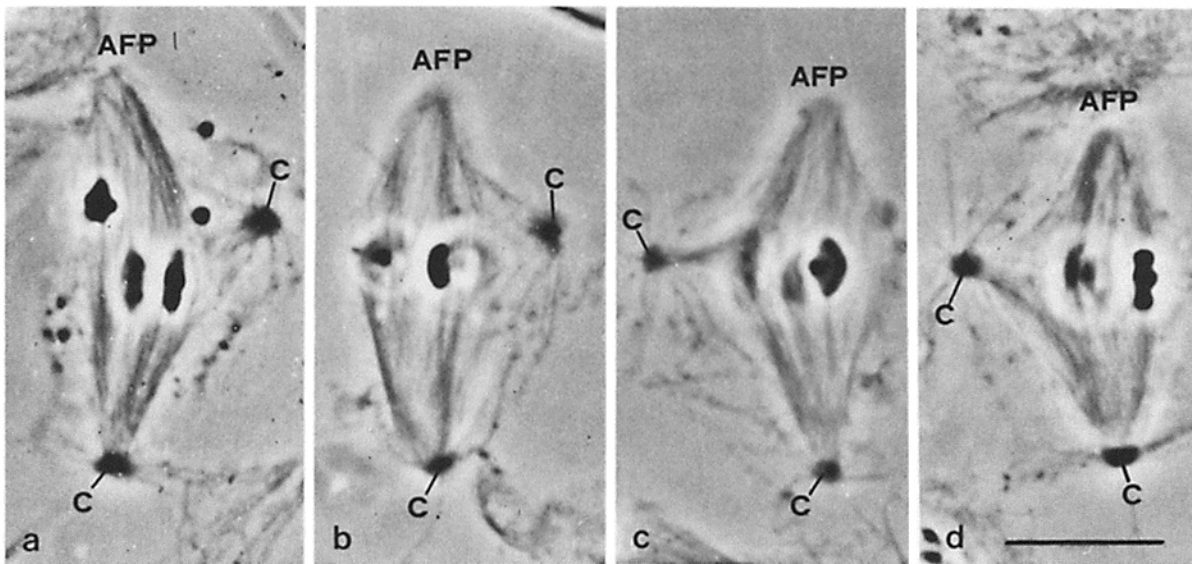


Figure 4. (a–d) Spindles isolated by cell lysis in a Triton X-100-containing buffer, stained with Coomassie Blue. (a) Late prometaphase. (b–d) Metaphase. Centrosomes (C) are darkly stained. All spindles are tripolar with one aster-free pole (AFP). The dislocated centrosome forms the third pole. Chromosome fibers are only oriented toward the aster-free pole and the nondislocated centrosome. Phase-contrast optics. Bar, 10 μm .

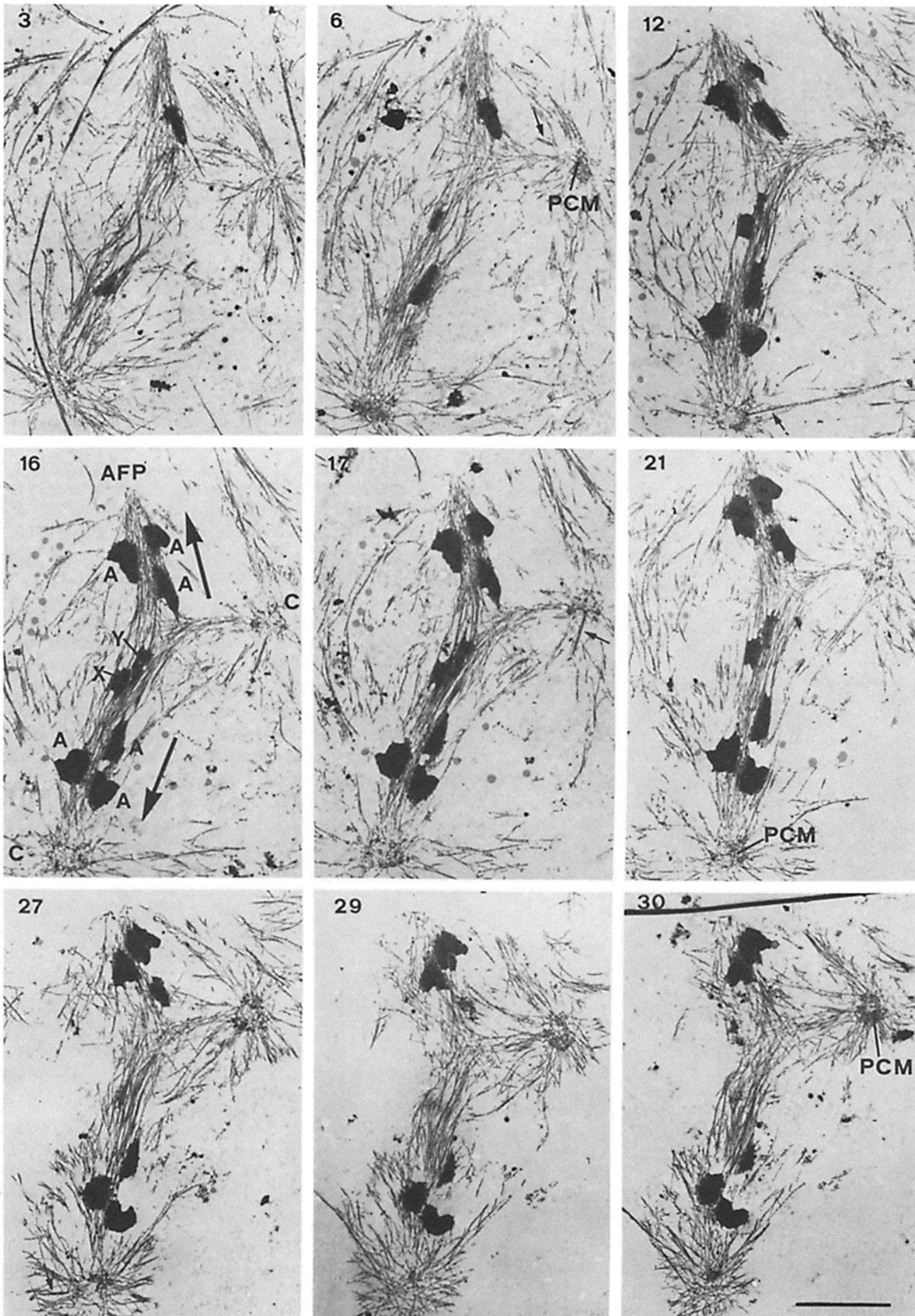


Figure 5. Nine sections from a series through an isolated anaphase spindle apparatus (section number indicated). The spindle is tripolar with one aster-free pole (AFP). Centrosomes (C) with PCM and radiating aster microtubules. The dislocated centrosome at the right side of the

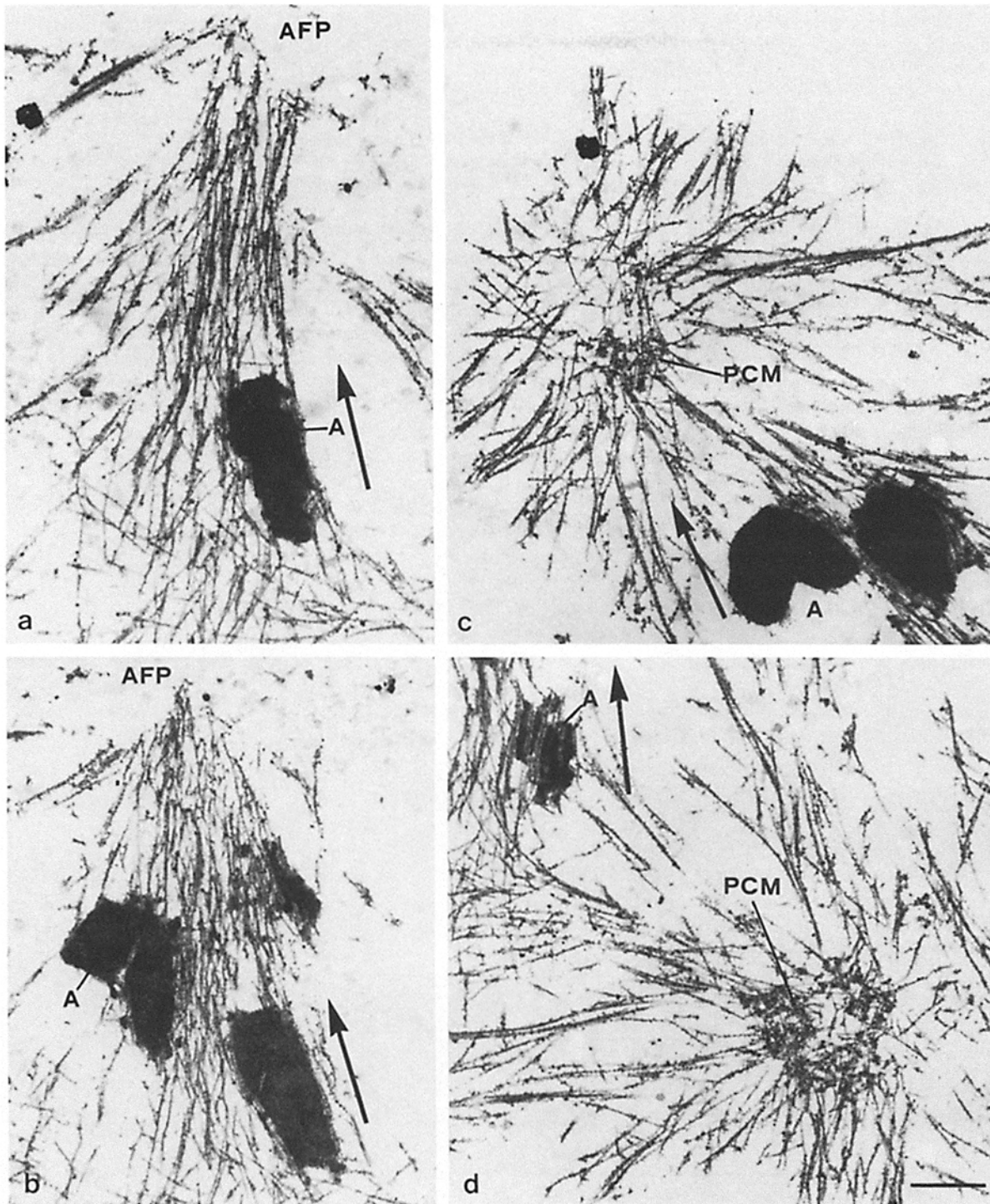


Figure 6. (a and b) Aster-free spindle pole (AFP) at higher magnification (sections 5 and 9 from the series shown in Fig. 5). (c) Aster of the nondislocated centrosome (from section 28). (d) Aster of the dislocated centrosome (from section 28). A, autosomal half-bivalents; arrows indicate direction of chromosome movement. Bar, 1 μ m.

spindle forms the third pole. Position of the four centrioles is indicated by four axonemes (small arrows in sections 6, 12, 17, 27). Autosomal half-bivalents (A) move toward the AFP and the nondislocated centrosome (arrows in 16). Sex univalents (X, Y) lag at the equator (reference 12). Bar, 5 μ m.

To detect traces of PCM by their ability to nucleate microtubules, cells were lysed using a polymerization buffer containing 4 mg/ml tubulin and 1% Triton X-100. A total of 20 cells possessing one aster-free spindle pole was studied both live and after lysis with the phase contrast microscope. In all cells the centrosomal asters began to increase in diameter shortly after lysis. Aster-free spindle poles did not show any changes, even after the slide had been warmed for several minutes (Fig. 7). In 18 cells the aster-free poles did not reveal any polar differentiations, i.e., they looked like the poles described above (Figs. 4-6). Two cells revealed a dark spot at the aster-free pole immediately after lysis, but this spot did not change in size also after prolonged incubation (Fig. 8). The nature of this density zone is not clear. In controls, where tubulin was replaced by BSA, centrosomes did not grow. We conclude that the growing of centrosomal asters is due to additional nucleation of microtubules by the PCM. The failure to detect any additional polymerization at the centrosome-

and aster-free poles also seems to indicate a lack of PCM at these sites.

Discussion

Radiating aster microtubules and PCM could not be detected at centrosome-free spindle poles, either in serial sections of intact cells or in isolated spindles. In the same cells the displaced centrosomes contain amounts of aster microtubules and PCM which do not seem to be different in magnitude from the amounts in centrosomes not displaced from the nuclei. Furthermore, an exposure to pig brain tubulin in a polymerization buffer did not reveal additional nucleation of microtubules at centrosome-free poles, whereas in the same cells, the PCM-containing centrosomes increased due to microtubule polymerization. These observations suggest that, during cell flattening, the centrosomes in the investigated cells were displaced as intact organelles, i.e., the assumption that

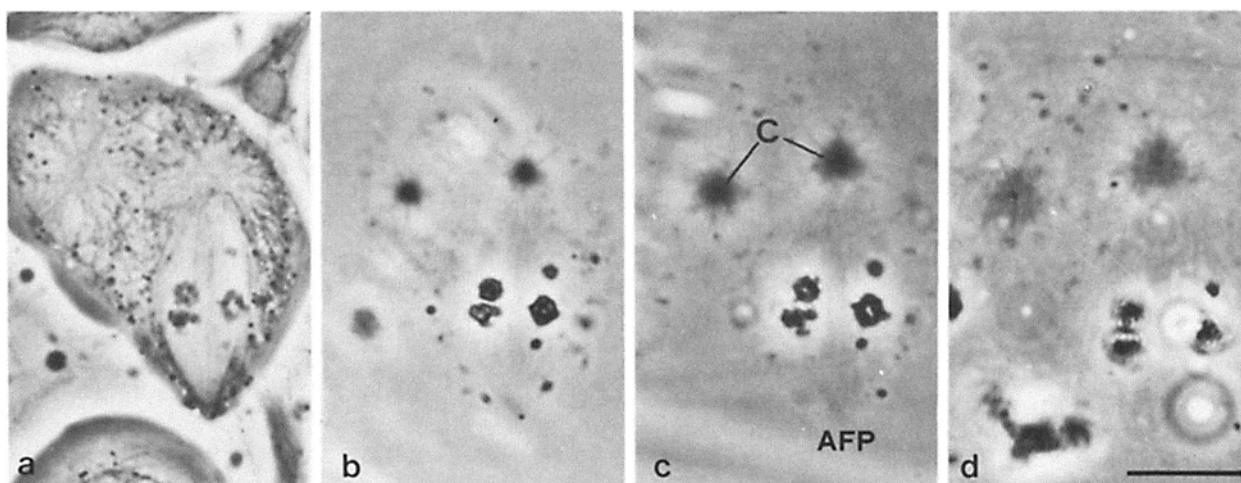


Figure 7. Lysis of a prometaphase cell in polymerization buffer containing 4 mg/ml tubulin. (a) Living cell 2 min before lysis. (b) Immediately after lysis. (c) 2 min after lysis. (d) 8 min after lysis with the last 5 min at 37°C. C, centrosome; AFP, aster-free spindle pole. Note the increase in diameter of the centrosomes (b-d). Phase-contrast optics. Bar, 10 μ m.

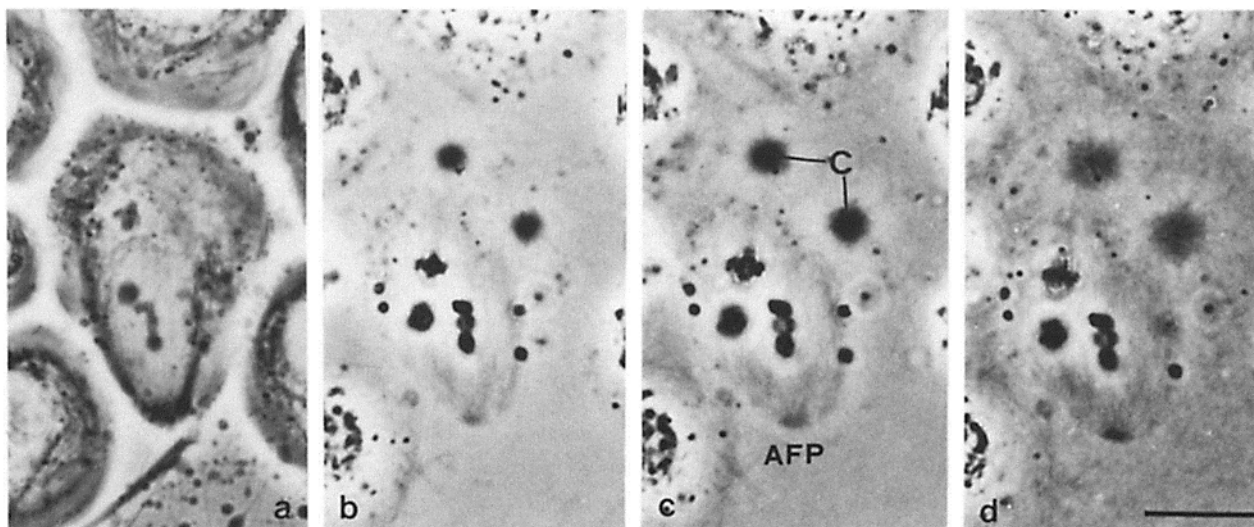


Figure 8. Lysis of a prometaphase cell in polymerization buffer containing 4 mg/ml tubulin. (a) Living cell 3 min before lysis. (b) Immediately after lysis. (c) 2 min after lysis. (d) 13 min after lysis with the last 10 min at 35°C. C, centrosome; AFP, aster-free spindle pole. Note the increase in diameter of the centrosome (b-d). Phase-contrast optics. Bar, 10 μ m.

PCM might have dissociated from the centrosome during this procedure (25) does not seem to be correct. These conclusions are corroborated by experiments using a PCM-specific antibody (manuscript in preparation). The finding of Keryer et al. (17) that spindle poles lacking centrioles in Chinese hamster ovary cells nevertheless are characterized by PCM could not be confirmed in crane fly spermatocytes. Their notion that the PCM may self-aggregate to serve as a pole-determining center does not seem to apply to Pales.

If PCM and asters are not found in polar positions, what was functioning as organizing centers during the formation of the centrosome-free poles? Generalizing our electron microscopical findings and applying them to the earlier studies of living cells, a bipolar mitotic spindle can even be developed when both poles lack centrioles and PCM (some secondary spermatocytes observed by Dietz, reference 11). Dietz suggested that the chromosomes or their kinetochores can function as organizing centers during formation of the spindle. However, observation in other organisms must be interpreted differently. In sea urchin eggs, chromosomes do not seem to be able to form a spindle (21, 27). The same seems to be true for newt lung chromosomes (1). On the other hand, it could be shown by experiments that kinetochores of Chinese hamster ovary cell chromosomes can form fibers of kinetochore microtubules; this activity is independent of centrosomes (16, 33). The question remains how all the nonkinetochore microtubules of the spindle are nucleated in Pales. Nicklas and Gordon recently showed in grasshopper spermatocytes (24) that the total length of nonkinetochore microtubules in a half-spindle is dependent on the chromosome number, which implies that kinetochores in some way can also determine the amount of nonkinetochore microtubules. This finding agrees with the observation that the number of kinetochore microtubules is positively correlated with the total length of all microtubules constituting the chromosome fiber in meta- and anaphase of Pales spermatocytes (14). Although we cannot conclusively answer how nonkinetochore microtubules originate in the absence of polar PCM, our observations indicate that a chromosome-induced formation of a half-spindle is realized in the crane fly, as was previously suggested. The relative unimportance of centrosome constituents for the division process in Pales is most clearly documented in the tripolar spindles (cell III and Fig. 4, 5). Although the displaced centrosomes are parts of the spindles, the chromosome fibers do not focus on these centrosomes but converge to form "their own" centrosome-free pole and move toward this pole.

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