

# Micromanipulated Bivalents Can Trigger Mini-Spindle Formation in *Drosophila melanogaster* Spermatocyte Cytoplasm

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**Abstract.** Single (individual) bivalents in cultured *Drosophila melanogaster* primary spermatocytes were detached from the spindle with a micromanipulation needle and placed in the cytoplasm. Such bivalents are prevented from rejoining the spindle by a natural membrane barrier that surrounds the spindle, but they quickly orient as if on a spindle of their own and the half-bivalents separate in anaphase. Serial section electron microscopy shows that a mini-spindle forms around the cytoplasmic bivalent, i.e., the microtubule density in the vicinity of the bivalent is much greater than in other cytoplasmic regions. This microtubule population cannot be accounted for solely by kine-

chore nucleation and/or capture of microtubules. Furthermore, the mini-spindles frequently form at odd angles to the main spindle, so that at least one pole has no relationship to the poles of the main spindle.

We conclude that a bivalent, or factors that become associated with the bivalent as a result of the manipulation, can either stabilize microtubules or promote their assembly. The bivalent activates latent microtubule organizing centers, or alternatively, polar organizing material has been passively transported from the main spindle to the cytoplasm by the micromanipulation procedure.

**M**OST investigations of mitotic spindle morphogenesis in animal cells have emphasized the role of microtubule organizing centers, the centrosome, and the kinetochore. The extent to which centrosomes and kinetochores determine when and where microtubules will be positioned in a cell is only beginning to be understood (7, 12, 13, 15). Although the importance of organizing centers is apparent, it is unlikely that all specificities for microtubule arrays are controlled by them. For example, while the mechanisms involved in microtubule length determination in vivo remain unknown, it is clear that microtubule length is controlled by something beyond tubulin concentration (1). Recent evidence suggests that chromosomes have an active role in spindle morphogenesis in addition to the role of their kinetochores in the origin of kinetochore microtubules. Chromosomes stabilize or promote the assembly of microtubules (7, 10, 17) and remarkably, the presence of chromosomes activates otherwise inactive centrosomes (7) and pericentriolar material (11). An understanding of how centrosomes, kinetochores, and chromosomes interact to organize a functional spindle apparatus will be essential if mechanisms of spindle morphogenesis and chromosome movement are to be resolved.

Cultured *Drosophila melanogaster* primary spermatocytes (6) are well-suited for micromanipulation experiments bearing on spindle formation. In contrast to most higher eucaryotes, several layers of membranes surround the spindle and separate it from the cytoplasm (24). In addition, the mem-

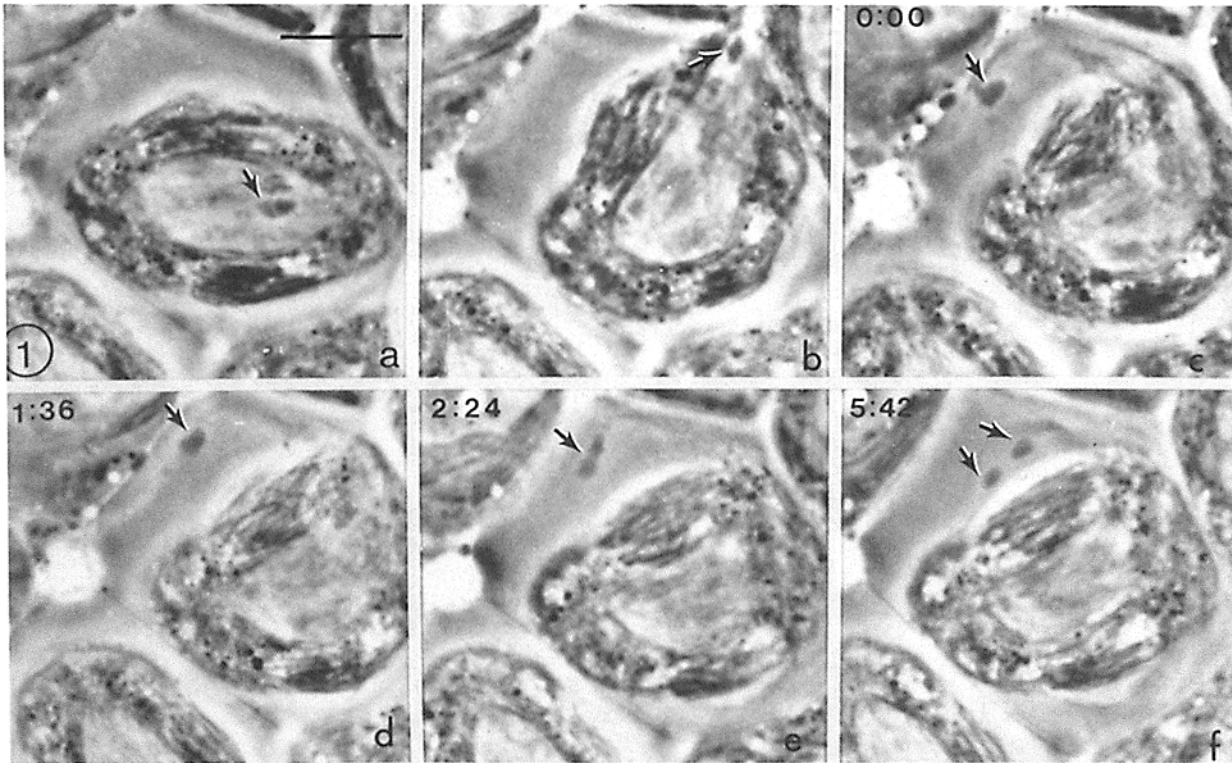
brane layers are circumscribed by a dense layer of mitochondria. Outside of this mitochondria-membrane-spindle complex, the cytoplasm is relatively free of large cytoplasmic organelles. Therefore, when bivalents are detached from the spindle and placed in the cytoplasm beyond the mitochondria, they are prevented from rejoining the spindle by the mitochondria-membrane barrier. We have investigated the fate of bivalents that have been placed in the cytoplasm by serial section electron microscopy. Our results suggest that the presence of a bivalent can trigger the formation of a functional mini-spindle in a place where a spindle does not normally occur. Furthermore, the poles of the mini-spindle often have no relationship to the poles of the main spindle. Preliminary results of this work have been reported elsewhere (19, 20).

## Materials and Methods

The primary spermatocytes used in this investigation were obtained from an *Oregon R*, wild type strain of *Drosophila melanogaster*. The flies were maintained at room temperature in half-pint milk bottles on standard cornmeal-molasses-agar medium supplemented with propionic acid as a mold inhibitor.

## Culturing and Micromanipulation

All experiments were performed at  $24 \pm 1.5^\circ\text{C}$ . Techniques for culturing *D. melanogaster* spermatocytes have been described (3). The instrumentation used for micromanipulation, light microscopy, and cinematography was described by Nicklas and Staehly (18). The karyotype of *D. melanogaster*



**Figure 1.** Prints from the cinematographic record of a micromanipulation experiment and documentary behavior of a bivalent (arrows) placed in the cytoplasm. Before the experiment, the bivalent is oriented normally on the spindle (a). The micromanipulation needle is inserted and the bivalent dragged poleward and through the membrane layers surrounding the spindle (b). The bivalent is moved to the cytoplasm beyond the mitochondria (c) and the needle removed (0 time). The bivalent stretches displaying half bivalents (d-e) and disjoins 5 min and 42 s after the operation (f). Bar, 10  $\mu$ m.

spermatocytes includes the sex chromosomal bivalent (X and Y), two large metacentric autosomal bivalents (2 and 3), and a small acrocentric autosomal bivalent (4). Manipulations involved bivalent 2 or 3 from cells in prometaphase or metaphase.

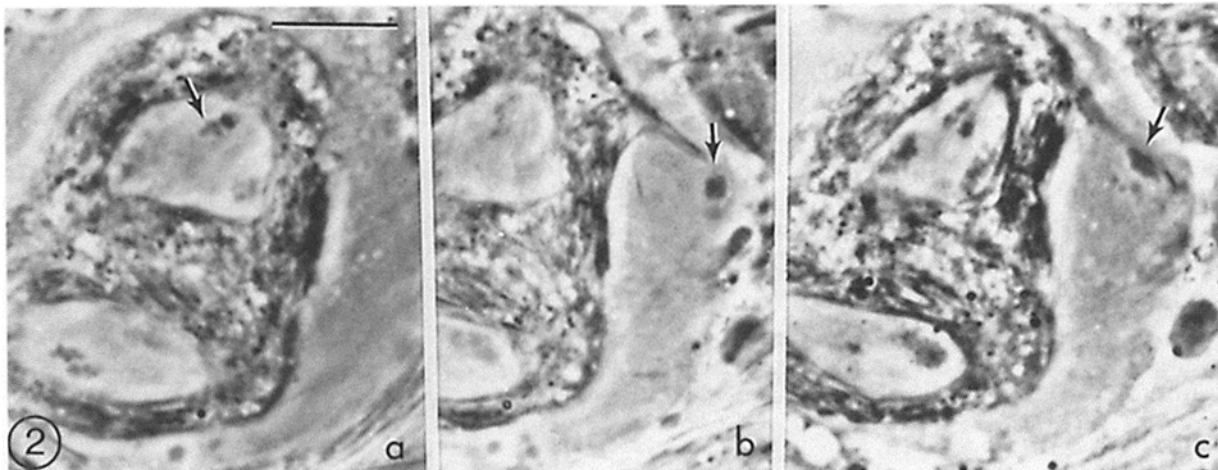
### Fixation and Electron Microscopy

Cells were fixed and prepared for electron microscopy as previously described (3). Methods for reconstruction of kinetochore microtubule bundles were as described in Church and Lin (2) and the computer programs of

Moens and Moens (16) were used to prepare representative two- and three-dimensional reconstructions from electronmicrographs of serial sections. Details of the procedure can be found in Nicklas et al. (21).

### Microtubule Length Measurements

Microtubule lengths were determined from approximately aligned acetate tracings of microtubule profiles from electronmicrographs ( $\times 18,000$ ) of serial sections. The summed lengths of the traced lines representing the microtubules were determined from each section using a digitizer (model



**Figure 2.** Prints from the cinematographic record showing a binucleate cell subjected to manipulation (arrow in a indicates the target bivalent). The bivalent is placed in the cytoplasm (b) and stretches 2.2 min after completion of the operation (c). At this time, the cell was fixed for electron microscopy.

1220; Numonics Corp., Lansdale, PA), a microcomputer (Kaypro 4, Kaypro Corp., Solano Beach, CA), and the computer programs originally developed by Moens and Moens (16). Details of the procedure and a discussion of the possible errors in the measurements can be found in reference 17. Volumes of cytoplasm sampled for microtubule length measurement were calculated from the formula

$$V = z \left( \sum_{i=1}^{i=n} a_i \right),$$

where  $z$  equals the section thickness and  $a$  equals the areas in section 1 through  $n$  of the region sampled. Proper conversions were applied to obtain the volume in  $\mu\text{m}^3$ .

## Results

### Manipulation of *Drosophila* Bivalents

Cells selected for manipulation are in prometaphase-metaphase and have either bivalent 2 or 3 positioned near the top surface of the spindle (Fig. 1 *a*). The needle is inserted, the bivalent detached, pulled poleward, and dragged through the membrane layers near a spindle pole (Fig. 1 *b*). It is placed in the cytoplasm as far from the spindle as possible (Fig. 1 *c*) and the needle is removed. Within a short period of time, the bivalent begins to move (Fig. 1 *d*); it stretches, clearly displaying both half bivalents (Fig. 1 *e*), and half-bivalent disjunction follows (Fig. 1 *f*). Disjunction always occurs in synchrony with anaphase in the main spindle. Under normal circumstances, *D. melanogaster* spermatocytes in culture require  $\sim 40$  min to complete prometaphase and metaphase (3). Timing of the events involving the manipulated bivalent depends on how near in time to anaphase the operation is performed. The minimum time after the operation that we have observed disjunction in the manipulated bivalent is  $\sim 5$  min.

### Mini-Spindles Are Formed around the Manipulated Bivalent

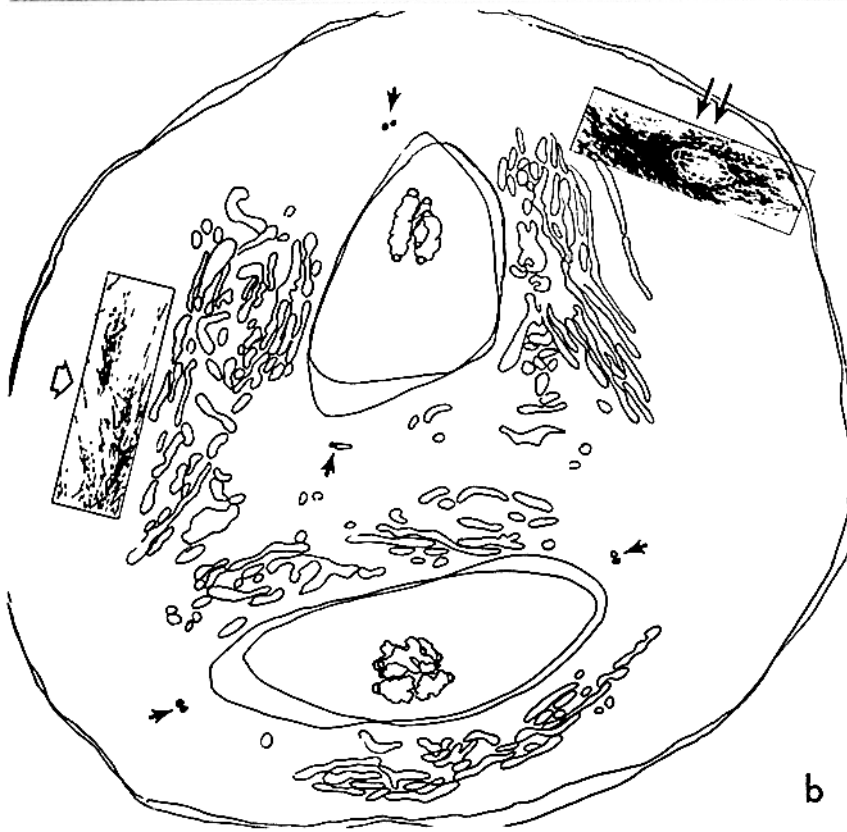
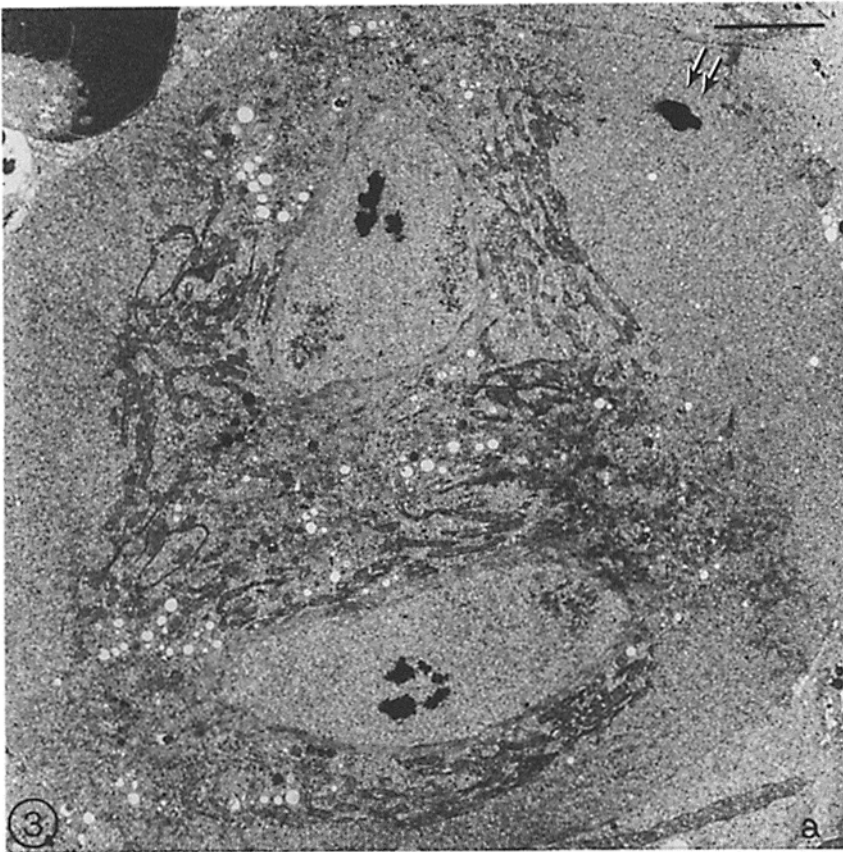
Examination with the electron microscope reveals that a mini-spindle rapidly forms around the manipulated bivalent. Fig. 2 shows a bivalent in the cytoplasm of a binucleate cell. Binucleate cells frequently occur under culture conditions and are often used in these experiments because they provide abundant cytoplasm in which to place the manipulated bivalent. The manipulated bivalent was followed in life (Fig. 2, *a-c*) until it stretched. It was then fixed for electron microscopy (Fig. 3 *a*). A two-dimensional reconstruction from electron micrographs of serial sections of the same cell is shown in Fig. 3 *b*. Inspection of the reconstruction indicates that the microtubule density surrounding the bivalent is much greater than in a comparable volume of cytoplasm. To quantify the increase in microtubule density in the vicinity of the manipulated bivalents, microtubule length measurements were made for five cells that were observed until the manipulated bivalent either stretched (two cells) or disjoined (three cells). After fixation and processing for electron microscopy, the cells were serially sectioned. Microtubule length measurements were made from acetate tracings of microtubule profiles from serial section electron micrographs. One rectangle was drawn to include the bivalent and some surrounding cytoplasm, and another of the same size was drawn in a chromosome-free area of the cytoplasm. To optimize the comparability of the two regions, the selected

chromosome-free region was located in a similar position relative to the centrosome, mitochondria, and nucleus, as was the manipulated bivalent (Fig. 3 *b*). All microtubules within the boundaries of the two rectangles were traced using every other section beginning with the section where the manipulated bivalent first appeared, and ending with the last section to include the bivalent. Tracings of the chromosome-containing and chromosome-free regions were aligned throughout the procedure. Microtubule profiles were digitized and their lengths calculated. Fig. 4 shows the detailed microtubule length data for a representative cell, and the data from all five cells are summarized in Table I (cells 1-5). The increased density of microtubules surrounding the bivalent is clear for all of these. In the most extreme cases (cells 1 and 2), the total length of microtubules in the vicinity of the manipulated bivalents is more than four times that found in the chromosome-free regions of the cytoplasm.

To test the possibility that micromanipulation transported a significant number of microtubules from the main spindle to the cytoplasm along with the manipulated bivalent, two cells were analyzed. These were treated as previously described except that they were fixed as soon as possible after the operation and before any apparent movement of the manipulated bivalent. The ratios [(length of microtubules in the vicinity of the bivalent)/(length in a chromosome-free region of the cytoplasm)] for those two cells were 1.3 and 1.1, respectively (Table I, cells 6 and 7). This suggests that microtubules may accompany the manipulated bivalent, but the length transported is not sufficient to account for the augmentation in total microtubule length occurring during the organization of the mini-spindle. We have not ruled out the possibility that short segments of microtubules brought from the main spindle during micromanipulation contribute to the microtubule length found in the mini-spindle. Such short segments may act as seeds for microtubule growth.

Undoubtedly, some of the increase in microtubule length associated with formation of the mini-spindle can be ascribed to formation of kinetochore fibers on the manipulated bivalent. To determine the proportion of mini-spindle microtubules that result from the kinetochore's capture of cytoplasmic microtubules, nucleation of new microtubules, and/or from growth of kinetochore microtubule seeds left from the manipulation procedure, three mini-spindle kinetochore microtubule bundles were reconstructed from high magnification electron micrographs. All microtubules that could have originated by kinetochore capture, nucleation, and/or kinetochore microtubule growth were traced (i.e., microtubules that had one end in the kinetochore, passed through the kinetochore, or grazed the kinetochore; see reference 2) and the total length of those microtubules was determined. The results show that the microtubules associated with a single kinetochore account for a maximum of 4% of the total increase in microtubule length in the vicinity of the bivalent.

We find the concentration of microtubules in regions of the cytoplasm far from the manipulated bivalent surprisingly high. For most cell types, microtubules are abundant in the spindle apparatus but relatively sparse in the cytoplasm during mitosis (25). It is possible that the abundant population of cytoplasmic microtubules in the *D. melanogaster* spermatocytes we studied is a result of the experimental procedure; i.e., the formation of cytoplasmic microtubules is stimulated by manipulation of the spindle and cytoplasm. To test



*Figure 3.* A survey electron micrograph (a) and two-dimensional reconstruction (b) of the cell shown in Fig. 2. The reconstruction was made from acetate tracings of every other section beginning with the first section including the bivalent and ending with the last section including the bivalent (a total of 37 sections). All microtubules in the vicinity of the bivalent (*double arrows*) were digitized as were microtubules in a comparable volume of chromosome-free cytoplasm (*open arrow*). Note that the density of microtubules surrounding the bivalent is greater than in the chromosome-free cytoplasmic region. The four centrioles that mark the poles of the two main spindles are indicated (*arrowheads*). Bar, 5  $\mu\text{m}$ .

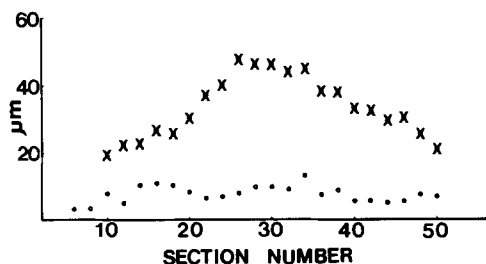


Figure 4. Microtubule length measurements from a cell where a bivalent is in the cytoplasm ( $x$ , microtubule length in the vicinity of the bivalent; solid circle, length in a cytoplasmic region that does not contain a bivalent). Note the augmentation in microtubule length in the regions surrounding the bivalent.

this, two cells in prometaphase were analyzed; one that was sham manipulated (the membrane layers disrupted by the manipulation needle without placing a bivalent in the cytoplasm) and one cell that was not manipulated. Those cells displayed 1.77 and 1.62  $\mu\text{m}$  of microtubules per unit volume of cytoplasm (Table I, cells 8 and 9). These values are not significantly different from each other or from values obtained for chromosome-free cytoplasmic regions in the micromanipulated cells (Table I).

### Odd Angle Mini-Spindles

The cytoplasmic mini-spindles often have one pole that has no relationship to either pole of the main spindle. Fig. 3 *b* shows a typical example. The mini-spindle is at an angle of about  $90^\circ$  to the axis of the nearer main spindle. The formation of mini-spindles after bivalent manipulation was recorded cinematographically for 15 spermatocytes. In 8 of the 15 cells, the mini-spindles formed at an odd angle to the main spindle. In the remainder, the mini-spindle was parallel to the main spindle axis. The mini-spindles formed at odd angles are completely functional. During anaphase, the bivalent disjoins at the odd angle (Fig. 5).

The formation of odd-angle mini-spindles obviously involves an additional new spindle pole. What is the source of such new spindle poles, e.g., the upper pole in the mini-spindle in Fig. 5? One possibility is that secondary, latent microtubule organizing centers within the primary spermatocyte cytoplasm are activated by the presence of a bivalent in their vicinity (11). We have not ruled out that possibility, but some observations make it unlikely. The angles at which the mini-spindles formed relative to the interpolar axis of the main spindle were measured from the cinematography records of the eight spermatocytes displaying odd-angle mini-spindles. Straight lines were drawn, one connecting the two centrosomes of the main spindle and another that connected both kinetochores of the odd angle bivalent. The angles at which these lines intercepted ranged from  $21^\circ$  to  $101^\circ$  (Table II), a result that suggests there is no favored position for a secondary pole to form. Angle measurements in two dimensions rather than three will be in error, but only slightly in such flattened cells. We conclude that if there are latent polar organizing centers within the cell, they must be very diffuse. A mini-spindle pole can form anywhere in the cytoplasm where a bivalent happens to be positioned.

We also considered the possibility that a new spindle pole

appears because we are bringing polar organizing material to the cytoplasm along with the manipulated bivalent. The bivalent is dragged from the spindle to the cytoplasm through the polar region (Fig. 1). Consequently, pericentriolar material might be picked up and carried along with the bivalent. We have looked for pericentriolar or other materials in two newly formed poles: the pole near the cell membrane in the cell shown in Fig. 3 *b*, and a second, similar example. In both cases, all kinetochore microtubules were followed in their entirety. The first kinetochore (Fig. 6) displayed a majority of short microtubules extending  $\sim 1.3 \mu\text{m}$  from the kinetochore proper with the remainder  $\sim 4.4 \mu\text{m}$  long. Neither the long nor short microtubules ended in any recognizable structure. There were membrane vesicles in the vicinity of both the kinetochore bundles (Fig. 6), and in one case, a long mitochondrion was positioned near the bundle. Most microtubules associated with the second kinetochore were longer, extending  $\sim 5 \mu\text{m}$  from the kinetochore (Fig. 7). Each ended in the cytoplasm with no recognizable structure at its end. In both cases, polar microtubules converged along with the kinetochore microtubules into diffuse foci that were devoid of any obvious structure (Fig. 7).

In the attempt to discover any polar organizing material that might be carried along with a manipulated bivalent, we also examined four bivalents that were fixed as soon as possible after completion of the operation. Of the eight kinetochores, two were completely devoid of microtubules. Others displayed short microtubules ranging in number from 2 to 20. In all cases masses of stacked membranes were found near the manipulated bivalent (Fig. 8). The poles of *D. melanogaster* spermatocytes contain a complex series of membranes. Bates (24) estimated that there are  $\sim 13$  membranes arranged as stacked umbrellas, with the center of the umbrellas containing the centrioles. Since stacked membrane masses are not normally found in the peripheral cytoplasm, the manipulation procedure has most likely resulted in the cytoplasmic location of the stacked membranes. Thus, if polar organizing material has accompanied the manipulated bivalent, a likely candidate is membrane material, or something associated with membrane material.

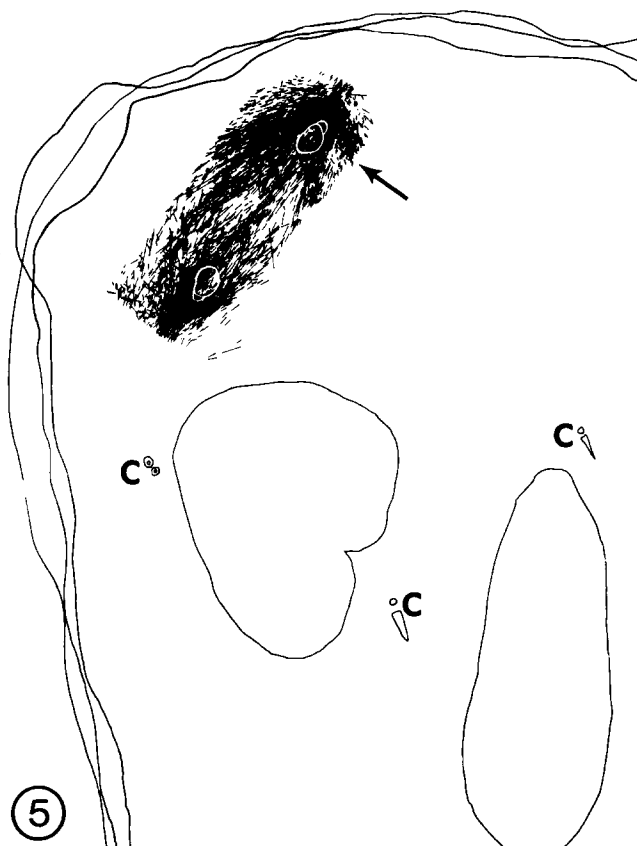
Table I. Microtubule Length Determinations on Serially Sectioned, Micromanipulated Cells

Cell No.	Time after operation*	Condition of bivalent	Microtubule length per unit volume <sup>‡</sup>		
			With chromosome	Without chromosome	Length ratio <sup>§</sup>
<i>min</i>					
1	2.2	Stretched	8.32	2.01	4.14
2	7.3	Stretched	10.58	2.38	4.45
3	8.3	Disjoining	7.29	1.95	3.74
4	23.0	Disjoining	6.04	2.47	2.45
5	21.2	Disjoining	4.44	1.76	2.52
6	1.0	Before movement	1.85	1.73	1.07
7	1.0	Before movement	2.36	1.80	1.33
8	17.2	Sham operated	—	1.77	—
9	—	No operation	—	1.62	—

\* Time interval between end of operation and fixation for electron microscopy.

<sup>‡</sup> Micrometers of microtubules per cubic micrometer in cytoplasmic regions of identical volume that did or did not contain a chromosome.

<sup>§</sup> Obtained by dividing numbers in column 4 by numbers in column 5.



**Figure 5.** Reconstruction of an odd angle mini-spindle with a pole (arrow) that has no relationship to the centrioles (c) of the main spindles. Only those microtubules found in the vicinity of the bivalent are displayed. The reconstruction was prepared from every other section of a series of 100 sections containing the half bivalents and demonstrates that the odd angle mini-spindles are functional in effecting disjunction of half bivalents.

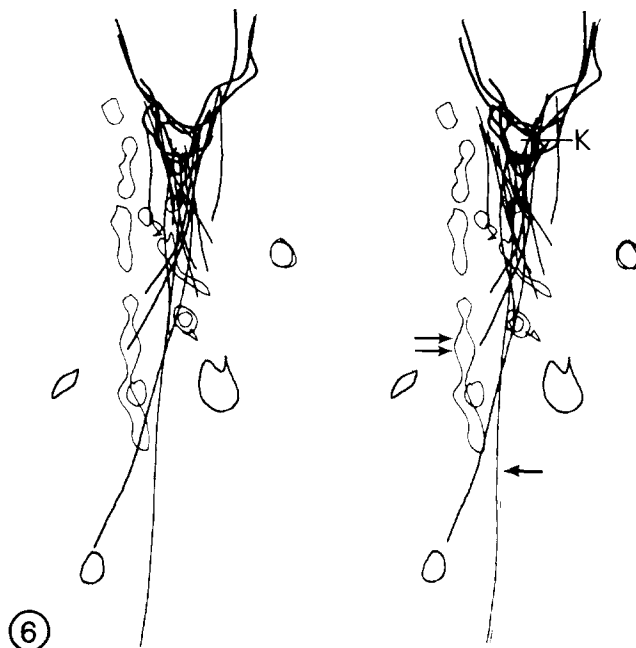
### Discussion

Recent evidence suggests that chromosomes have a role in spindle morphogenesis independent of the role of kinetochore as a microtubule organizing center (7, 10, 17). *Drosophila* mini-spindles may be another demonstration of the importance of chromosomes in the organization of a spindle.

**Table II. Degree to Which Odd-Angle Mini-Spindles Diverge from Being Parallel to the Main Spindle**

Cell number	Angle*
1	90°
2	92°
5	101°
11	53°
13	57°
15	45°
17	74°
18	21°

\* Determined by drawing one line that connects the centers of the two centrosomes of the main spindle and an intercepting line that connects both kinetochores of the stretched or disjoining bivalent in the odd-angle mini-spindle.



**Figure 6.** Stereopair of a reconstructed kinetochore (K) microtubule bundle. The kinetochore bundle is from the mini-spindle shown in Fig. 3 b, and is directed toward the cell periphery rather than toward a pole of the main spindle. All microtubules were followed until they ended. None ended in any recognizable structure. The longest microtubule (arrow) ends 0.8  $\mu\text{m}$  from the plasma membrane. Cytoplasmic membrane vesicles (double arrows) are found in the vicinity of the bundle.

In this case, not only is a microtubule array generated around the manipulated chromosome, but the array forms a functional spindle on which anaphase disjunction occurs. We do not know if the increase in microtubule length near the manipulated bivalent is the result of stabilization of a dynamically unstable population of cytoplasmic microtubules or the polymerization of new microtubules (5, 14). Whatever the mechanism, it occurs rapidly. Mini-spindles can form within 2 min after removal of the manipulation needle.

How do chromosomes enhance microtubule assembly or stability? One possibility is that chromosomes have enzymatic activities that increase the amount of assembly-competent tubulin in their vicinity (17). A group of mitosis-specific phosphoproteins has been shown to be associated with metaphase chromosomes (4). It remains to be seen if these or other chromosomal proteins affect microtubule assembly.

What we have observed in spermatocytes may be analogous to the situation in mouse oocytes. There, material detected with antibodies specific for pericentriolar material is located not only at the poles, but at discrete loci in the cytoplasm. Such loci can act as organizing centers if positioned near a chromosome (11). If polar organizing material is present at discrete locations in the cytoplasm of *Drosophila* spermatocytes, we might have expected to observe preferential locations of the mini-spindle in relation to the main spindle. Such was not the case. Rather, the observations suggest that if present, latent microtubule organizing capability is a diffuse property of the cell. A more likely explana-

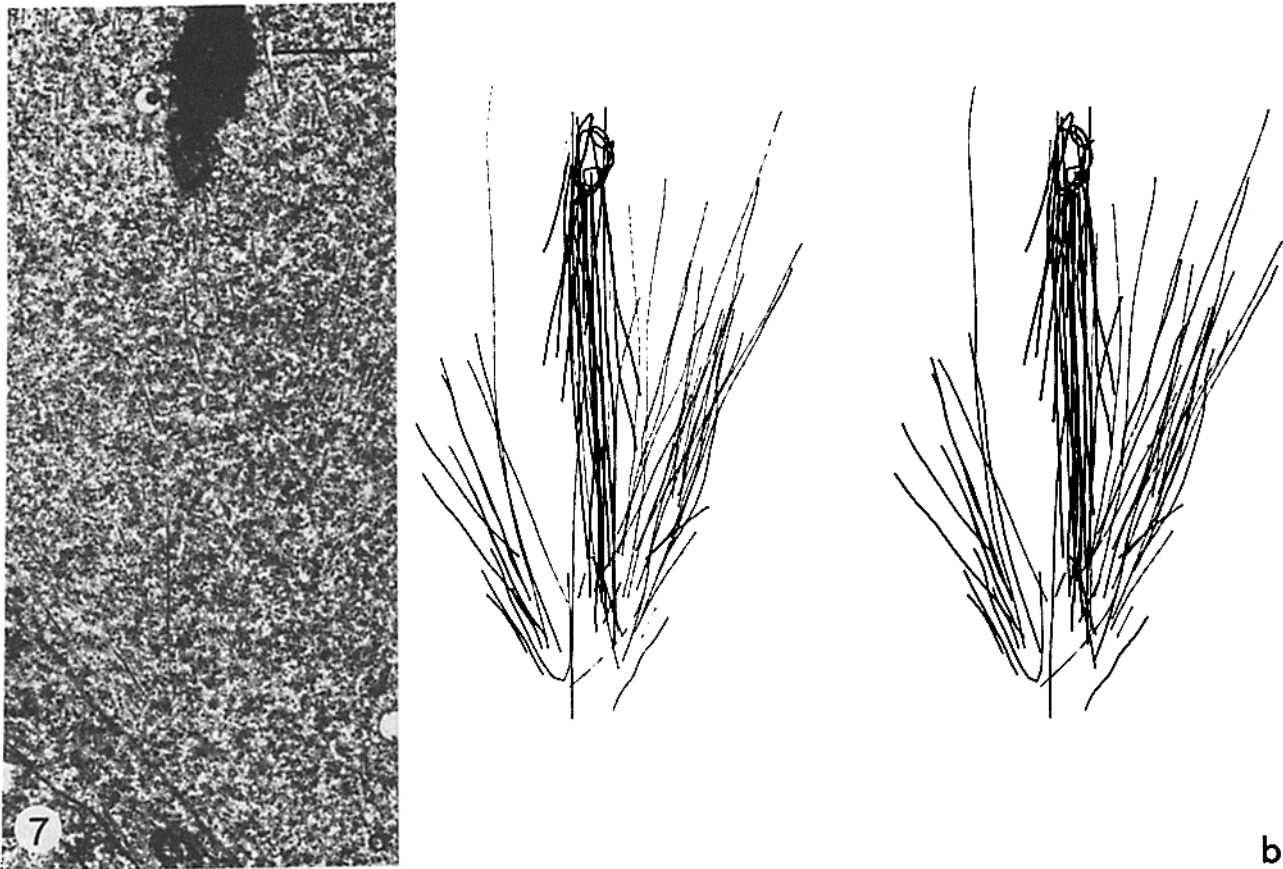


Figure 7. Electronmicrograph of kinetochore oriented toward the cell periphery rather than toward a recognizable pole (a). The cell was fixed 7.2 min after micromanipulation and an odd-angle minispindle had formed (Table I, cell 2). The kinetochore microtubule bundle and some of the "polar" microtubules were reconstructed (stereopair, b). They converge to foci that lack an obvious centrosome. Bar, 1  $\mu\text{m}$ .

tion, perhaps, is that factors associated with chromosomes or with extraneous materials brought along with the chromosome during manipulation, are involved in organizing the poles of the mini-spindle.

We have examined the ultrastructure of the poles of the mini-spindles and are unable to identify material resembling pericentriolar material. More definitive tests using antibodies to detect pericentriolar material are planned. It is possible that such material has hitchhiked on the bivalent during the manipulation. It is clear that membrane from the polar region usually accompanies the bivalent, but whether the membrane vesicles have a role in spindle organization is not known. Membrane vesicles can be found in the vicinity of the mini-spindle although not necessarily at the poles.

Karsenti et al. (7) noted that microtubules are assembled around metaphase chromatin injected into *Xenopus* eggs and suggested that anastral spindles were being formed in the absence of organizing centers. However, the preponderance of the evidence indicates that centers are necessary for bipolar spindle formation (12) and that chromosomes by themselves cannot organize functional bipolar spindles (23). Future work with the *Drosophila* system will focus on the source of the microtubules and the poles of the mini-spindles.

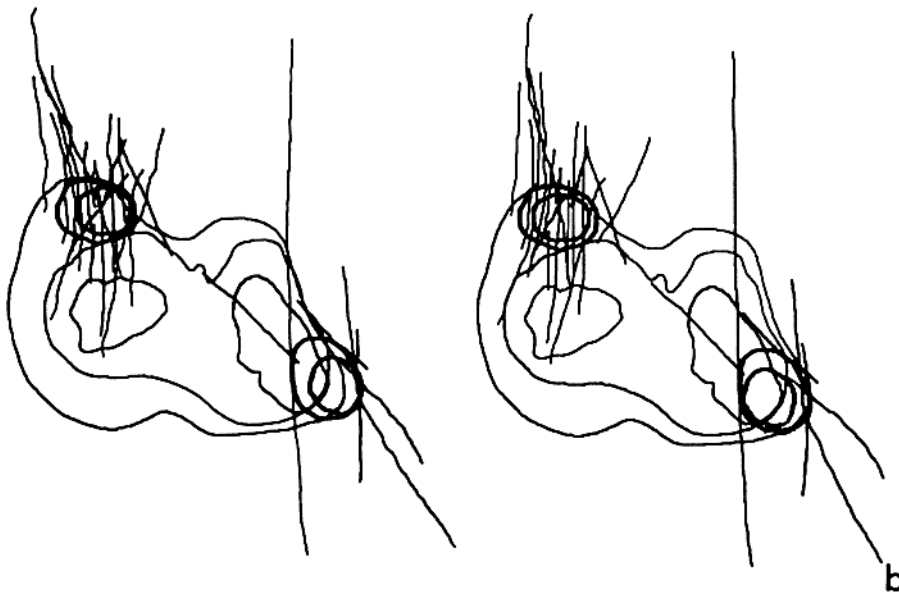
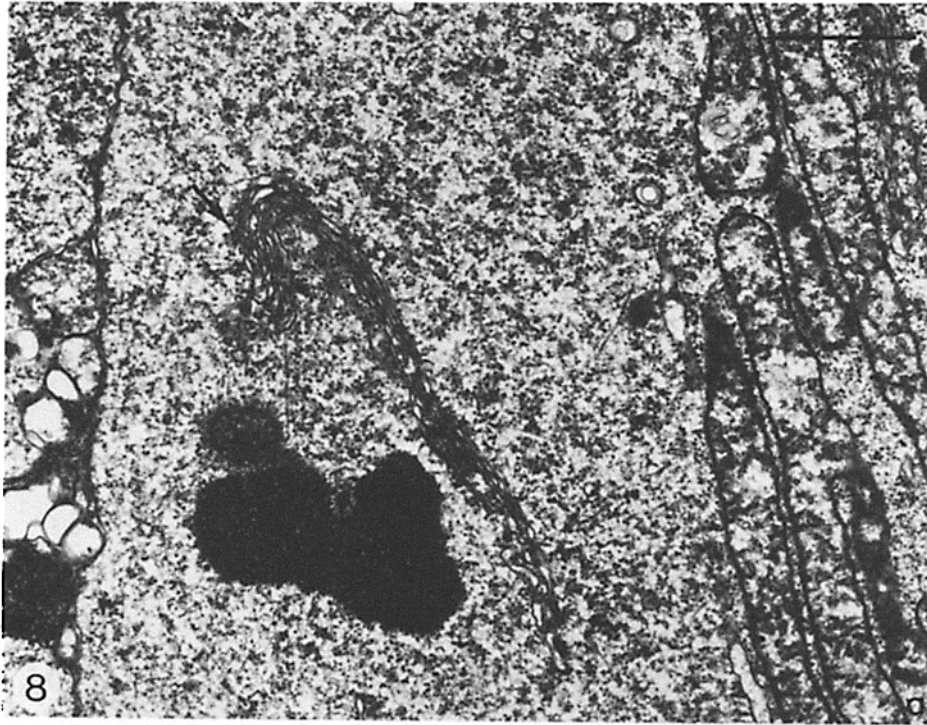
Before mitosis, interphase microtubule networks are disassembled, the mitotic spindle is constructed from the sub-

units, and virtually all cytoplasmic microtubules disappear (25). Consequently, it was surprising to observe that *Drosophila* spermatocytes displayed a significant number of cytoplasmic microtubules. However, if one considers that from each primary spermatocyte four sperm cells will eventually be produced, each possessing an axoneme 1.8 mm long (9), it might be expected that an abundance of tubulin would be present in the spermatocyte. Each primary spermatocyte must stockpile enough testes specific  $\beta$ 2-tubulin (8, 22) and  $\alpha$ -tubulin to produce roughly  $2 \times 10^5 \mu\text{m}$  of microtubules (4 sperm tails  $\times$  29 axoneme microtubules  $\times$  1.8 mm). Assuming that all tubulin involved in axoneme formation is synthesized in the primary spermatocytes, each cubic micrometer in the cell would have on average enough monomer and polymer to account for 25  $\mu\text{m}$  of microtubules (cell volume was determined from serial sections). Our observation of  $\sim 2 \mu\text{m}$  of microtubules per  $\mu\text{m}^3$  in the cytoplasm and 10  $\mu\text{m}/\mu\text{m}^3$  in the mini-spindle is certainly in the range of what might be expected.

We thank Dr. Donna Kubai for editorial assistance.

This work was supported by grants from the National Science Foundation (DCB 8207538) and the National Institutes of Health (GM 13745).

Received for publication 10 June 1986, and in revised form 27 August 1986.



**Figure 8.** Electron micrograph of a bivalent fixed 57 s after micromanipulation (a). Membrane (arrow) from the polar region of the main spindle has accompanied the manipulated bivalent to the cytoplasm. The kinetochores displayed numerous short microtubules (stereopair, b). Bar, 1  $\mu$ m.

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