Meiosis in *Drosophila melanogaster*. II. The Prometaphase-I Kinetochore Microtubule Bundle and Kinetochore Orientation in Males

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ABSTRACT Fourteen prometaphase kinetochore microtubule bundles have been examined in electron micrographs of serial sections. The majority (54%) of the microtubules extend from the polar region towards the kinetochore but do not end in the kinetochore proper. Rather, they stop short of the kinetochore (21%), graze the kinetochore (19%), or pass through the kinetochore (9%), displaying a free end distal to the pole. Other microtubules that make up the kinetochore bundle include: kinetochore-to-pole microtubules (24%), chromosome-to-pole microtubules (5%), pieces with two free ends (14%), and those microtubules with one end in the kinetochore and a free end distal to the kinetochore (9%). We conclude that the majority of the microtubules in the kinetochore bundle are most likely of polar origin rather than having been nucleated at the kinetochore.

Prometaphase-I kinetochores can display any one of four patterns of microtubule connections with the poles, but the pattern of microtubule connections is not always correlated with kinetochore position. For instance, a kinetochore directly facing one pole may have microtubule connections with both poles while a kinetochore positioned 90° to the spindle axis may have microtubules running towards one pole only.

Kinetochore orientation during prometaphase of meiosis can be very complex. The homologous kinetochores could theoretically participate in at least four orientation configurations: (*a*) unipolar orientation of homologous kinetochores; (*b*) bipolar orientation of homologous kinetochores; (*c*) unipolar orientation of one pair of sister kinetochores and bipolar orientation of the homologous pair; and (*d*) bipolar orientation of both pairs of sisters (1 cf. 8).

Bivalents, under normal conditions, usually achieve stable bipolar orientation before anaphase segregation. However, analyses of time-lapse ciné records of live cells in prometaphase demonstrate that brief unipolar orientation of homologous kinetochores is characteristic of some bivalents (1 cf. 18), as is unstable bipolar orientation (3 cf. 18). Mal-orientations are transient and reorientations of the kinetochores lead to stable bipolar orientation.

Although prometaphase kinetochore orientations have been frequently described in fixed cells by light microscopy and analyzed in live cells by ciné analyses, little is known about the structural basis for such orientations. That homologous kinetochores are mechanically connected to the same pole in bivalents showing unipolar orientation and to opposite poles in bivalents showing bipolar orientation was beautifully demonstrated in the classic micromanipulation experiments of Nicklas and co-workers (18, 19, 20, 21) and more recently confirmed by Begg and Ellis (2). Fragmentary ultrastructural analyses have shown that meiotic kinetochores may sometimes display "filaments" (14) or microtubules (31) that are directed towards both poles or at least towards both half-spindles (22). Although microtubule connections with both poles have been inferred for bipolar meiotic kinetochores, or with the same pole for unipolar kinetochores, documentation is lacking.

The spermatocytes of *Drosophila melanogaster* offer advantages in elucidating the fine structure of the meiotic spindle. The cells are relatively small and the microtubule number is low enough so that some aspects of the spindle can be quantitatively described (6, 12). Here we describe the orientation of meiotic prometaphase kinetochores as deduced from serial section analysis and examine the relationship of kinetochore position to microtubule disposition. We also analyze the microtubule composition of the kinetochore microtubule bundle at prometaphase.

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The major conclusions drawn from this analysis are: (*a*) at early prometaphase I, homologous kinetochores and sister kinetochores may show either unipolar or bipolar microtubule connections; (*b*) apparent kinetochore position and microtubule connections can be unrelated; (*c*) the majority of microtubules found in the vicinity of the kinetochore are most likely of polar origin; and (*d*) considerable microtubule pattern rearrangement occurs before metaphase of meiosis when virtually all bivalents have achieved bipolar orientation.

MATERIALS AND METHODS

Electron Microscopy

The flies used in this investigation were of the genotype In(1)sc^{4L}sc^{8R}/Y+; +/+; +/+; +/+. The wild-type Y chromosome and autosomes are from an Oregon R(OrR) stock (12). The inversion chromosome was placed in the OrR background by crossing In(1)sc^{4L}sc^{8R}/B^{s}Y males to females of the genotype yw:=/YOrR; OrR/OrR; OrR/OrR; OrR/OrR. Patroclinous male offspring were then back-crossed to females from the attached X strain for 10 generations. Flies were reared at room temperature on standard cornmeal molasses agar with propionic acid added to the medium.

Testes, dissected from prepupae, were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 2 to 4 h, washed three times in phosphate buffer (10 min each), three times with 0.1 M veronal acetate buffer (pH 7.2), and postfixed in 2% osmium tetroxide in 0.1 M veronal acetate buffer (pH 7.2) for 2 h. A prestain in 0.5% uranyl acetate (veronal acetate buffer) for 2 h was followed by a wash in distilled water and dehydration with ethanol (30%, 50%, 10 min each; 70%, 95%; 30 min each; 100%, three times, 10 min each). The testes were embedded in Epon (13) and, after sectioning, were poststained with bismuth subnitrate (24). Techniques for obtaining serial sections were the same as previously described (12).

Microtubule Analysis

Microtubules were tracked in electron micrographs of longitudinal, or slightly oblique from longitudinal, serial sections. A sheet of tracing paper was placed over the area of interest in the electron micrograph and all microtubule profiles were traced. The same paper was placed on the next section, the best alignment for all microtubules was achieved, and a second color was used to trace these microtubule profiles. Using a perfectly aligned second piece of paper, the new tracings were retraced (color coded) and this sheet was aligned on the next micrograph. This process was repeated through the entire series and resulted in a stack of papers each of which had the information from one section and its adjacent section. Composites were prepared from the tracings. Due to the relatively low number of microtubules associated with *D. melanogaster* kinetochores at prometaphase, it was possible to account for all microtubules in the kinetochore bundle. Stereo image computer reconstructions were obtained using the techniques and the computer facilities of Moens and Moens (17). The methods used for chromosome identification were those previously described (12).

RESULTS

General

The spermatocytes of *D. melanogaster* undergo intranuclear meiotic divisions. In fact, the nucleus of dividing cells is surrounded by multiple layers of double membranes in addition to the nuclear envelope (Fig. 1). For a detailed description of the origin and ultrastructure of this elaborate perinuclear membrane system in *Drosophila* the reader is referred to Tates (27) and Ito (9). At prometaphase, microtubules radiate from the extranuclear asters and are positioned in the spaces between the double membrane layers, forming a complete system of extranuclear microtubules extending the entire length of the nucleus (Fig. 2). The extranuclear microtubules are present at meiosis I and II although no quantitative estimates of their relative concentrations have yet been made.

At the polar regions (marked by asters) of primary spermatocytes in prometaphase, the perinuclear membranes are invaginated and microtubules appear to extend from the membrane folds into the nucleus (Fig. 1). These intranuclear microtubules often have one end positioned on the membrane vesicles near the poles or can be traced through the intermembrane space to the general area of the centriole pair.

Two major categories of intranuclear microtubules are observed at prometaphase. The first consists of relatively distinct bundles of microtubules that are characterized by the presence of an obvious fibrous matrix giving the bundle a grey appearance in low-magnification electron micrographs (Fig. 1). These bundles often, although not always, extend the entire length of the nucleus. We refer to them as bundles of intranuclear nonkinetochore microtubules. The second major category of microtubules are those observed in the vicinity of the kinetochores. This latter category will be described in detail in a later section. At this point it should be emphasized that the kinetochore bundles and the nonkinetochore bundles of microtubules are often spatially separated except where they converge at the poles, and that in the earliest prometaphase cells we have examined they represent the only microtubules present in the nucleus.

As prometaphase progresses, the distance which separates the two asters increases ($\bar{X} = 12.67 \pm 0.62$ μm, $n = 5$ cells at early prometaphase, $\bar{X} = 19.72 \pm 0.94$ μm, $n = 4$ at metaphase) and the number of intranuclear microtubules located in the region of the kinetochore also increases.

Classification of Microtubules Found in the Vicinity of the Kinetochore

Cells were judged to be in prometaphase if, in addition to an intact nucleolus, one or more pairs of homologous kinetochores did not show bipolar orientation or, if all kinetochores showed bipolar orientation, congression was not complete. Goldstein (6) described the ultrastructure of the *D. melanogaster* meiotic kinetochore. At prometaphase, the dyad kinetochore was observed to be a single dilaminar, hemispheric structure composed of an outer electron-dense layer and an inner layer of lesser electron density. As meiosis progresses, the kinetochore is transformed into a double disk structure. Our observations are in agreement with those of Goldstein (6), with two minor exceptions. In our preparations, the prometaphase kinetochore has a trilaminar appearance with a dense middle layer, a less dense inner layer, and a diffuse outer layer (see Figs. 3 and 7), and in rare cases a hint of doubleness can be observed in the prometaphase kinetochore. The boundary of the kinetochore is relatively well defined at least at the morphological level.

We attempted to track all microtubules that are found in the vicinity of the kinetochore. For early prometaphase cells, this is possible for virtually all kinetochores, since each is positioned far enough away from the nonkinetochore bundles to eliminate ambiguity. For late prometaphase cells, some kinetochores are so close to the intranuclear, nonkinetochore bundles that a completely unambiguous analysis is not possible. Thus our sample of prometaphase kinetochores is not random but is comprised of kinetochore bundles with the simplest microtubule composition. It should be emphasized that all bundles (both nonkinetochore bundles and kinetochore bundles) converge in the polar region of the nucleus and that microtubules that are recorded as reaching the pole could some times be tracked unambiguously to the membrane invagination but often times disappeared into the complex array of microtubules converging at the pole. A microtubule was defined as ending in the kinetochore if it could be tracked to and ended in any of the three layers. If a microtubule end was not physically associated with one of the three layers but was found free in

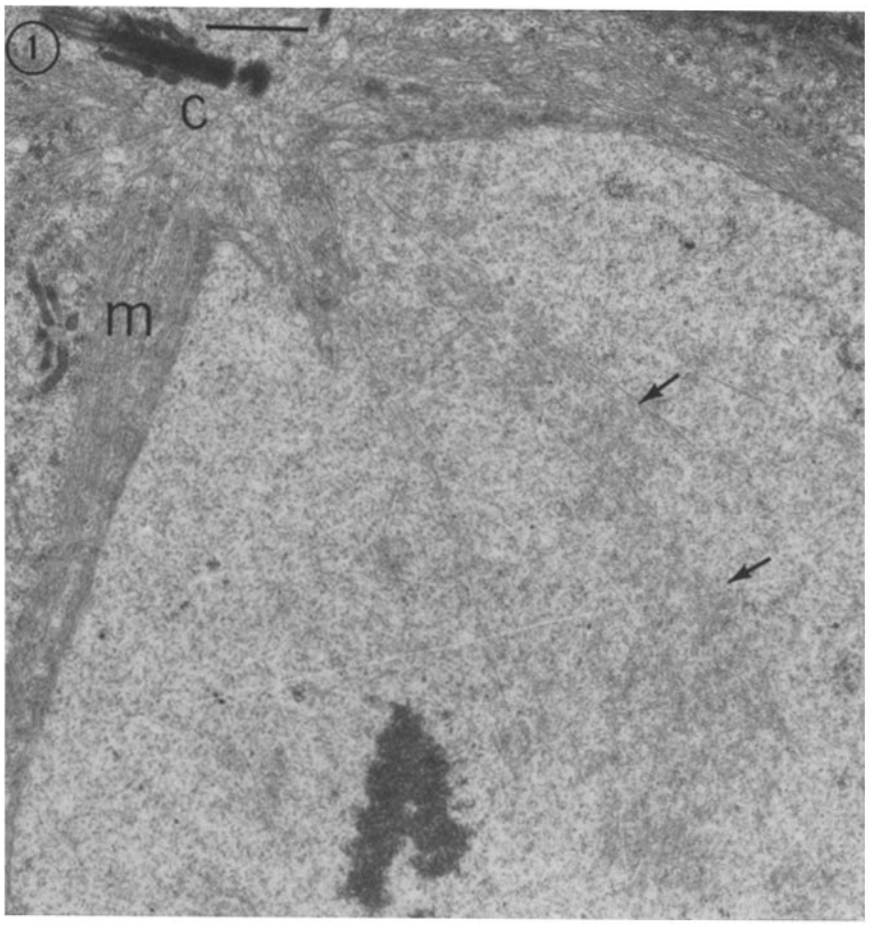


FIGURE 1 Primary spermatocyte in late prometaphase I of meiosis. Layers of perinuclear membranes (*m*) surround the nucleus. The poles are marked by paired centrioles (*c*) and the intranuclear interpolar microtubules are associated with a fibrous matrix (arrows) giving the bundles a grey appearance in the electron micrograph. Bar, 1.0 μm. × 13,000.

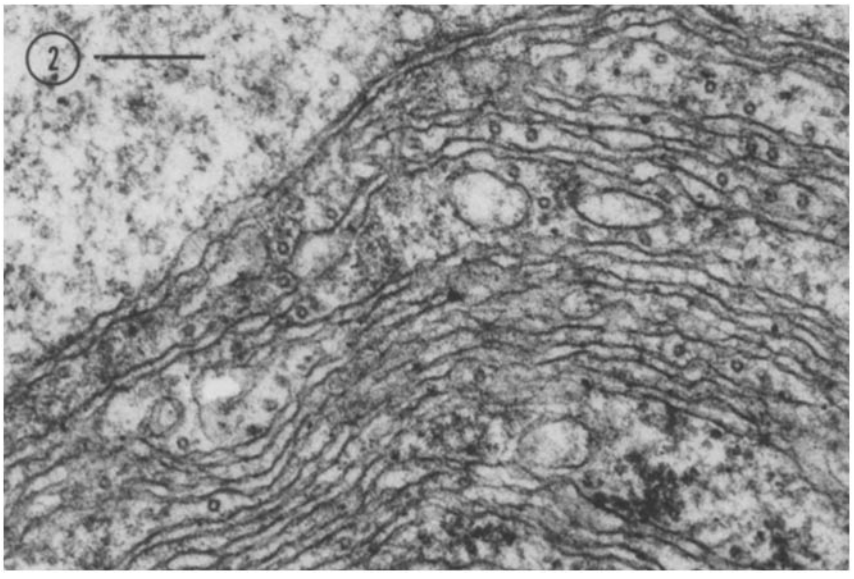
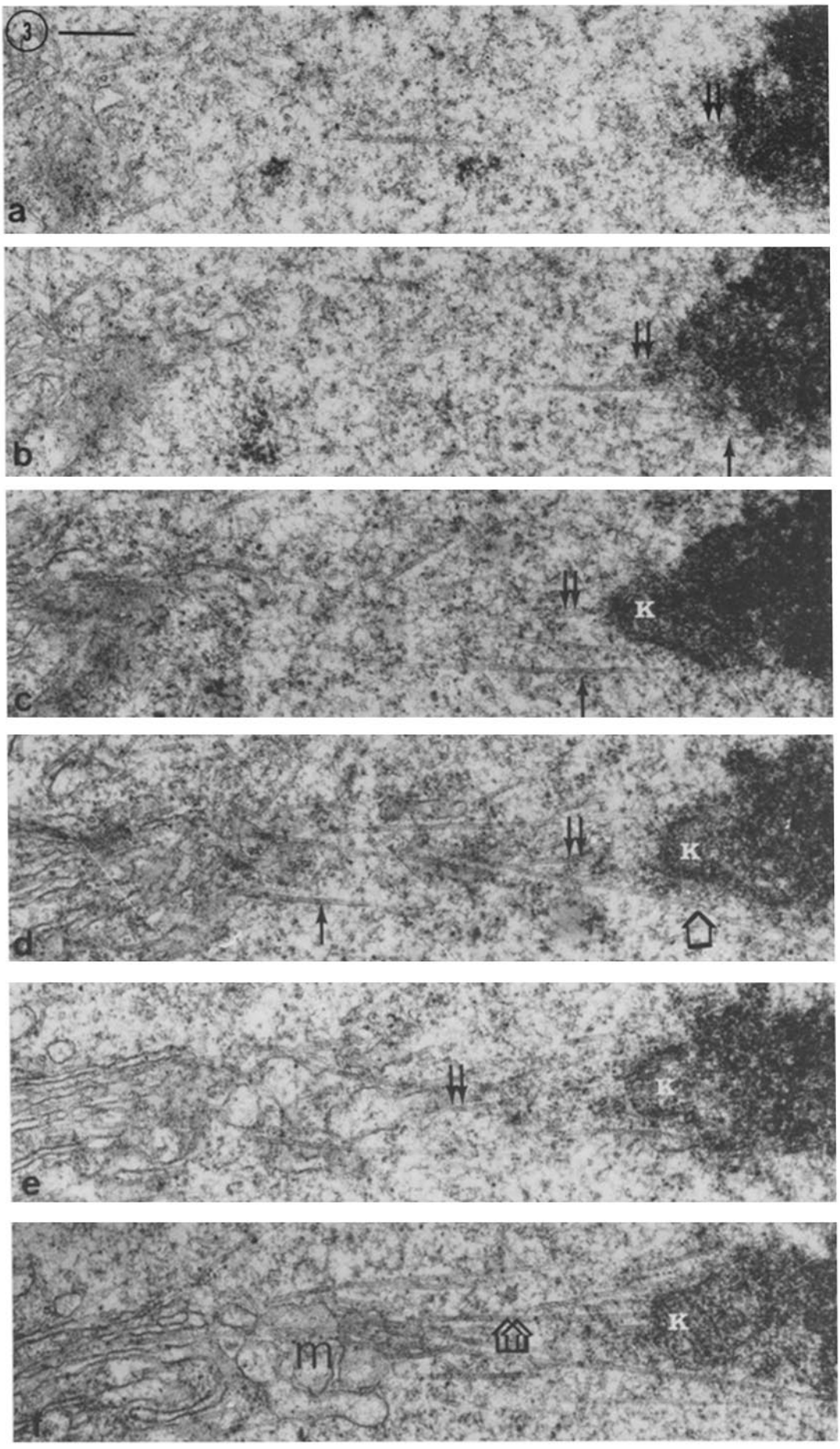


FIGURE 2 Cross-section of the perinuclear membranes taken near the midregion of a primary spermatocyte nucleus at late prometaphase I. Extranuclear microtubules are positioned between the double membrane layers. Bar, 0.25 μm. × 57,000.

the nucleoplasm or was embedded in the chromosome, it was recorded as such.

The following categories of microtubules were observed in the vicinity of the kinetochore: microtubules which extend from the polar region or nonkinetochore bundle toward the kinetochore and (*A*) end short of the kinetochore, (*B*) graze the outer layer of the kinetochore and end in the nucleoplasm past the kinetochore, (*C*) penetrate the kinetochore and exit with a free end in the nucleoplasm, (*D*) end in the kinetochore, or (*E*) end in the chromosome near to the kinetochore. Microtubules were also observed (*F*) with one end in the kinetochore and a free end extending toward the pole, and (*G*) with two free ends (i.e., pieces), Figs. 3 and 4 show a series through a kinetochore region and reconstructions of that region, respectively.

14 prometaphase-I kinetochores from five cells were examined, and all microtubules ($n = 150$) were tracked in their entirety. Table I shows the numbers and categories of micro-


3
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tubules found in the vicinity of each kinetochore and the overall frequency of each category. Inspection of the frequencies reveals that ~50% of the microtubules found in the vicinity of the kinetochore have one free end and that that end is distal from the pole (categories *A*, *B*, and *C* in Table I) and that 5% have one end at the pole and the other end embedded in the chromosome (category *E* in Table I). The simplest explanation for the occurrence of such microtubules is that they are of polar origin. The origin of the kinetochore-to-pole microtubules which comprise ~24% of the total (category *A*) cannot be determined. Roughly 9% of the microtubules extend from the kinetochore with a free end distal to the kinetochore (category *F*). Members of this latter category are candidates for origin by nucleation at the kinetochore although other origins cannot be ruled out.

Kinetochore Orientation and Microtubule Arrangement

One of the prometaphase-I cells was of particular interest since, judging from the number of microtubules present in the nucleus and the kinetochore positions, the cell was in an earlier stage than the other cells. Fig. 5 shows a reconstruction of low-magnification electron micrographs of this particular cell. Neither the X nor the Y kinetochore actually faces one or the other pole, but each is situated ~90° to the spindle axis (Fig. 5*a*). Both kinetochores have microtubule connections to both poles although the Y kinetochore displays more microtubules than the X (Figs. 6 and 7). Most of the microtubules extend from the polar regions or a nonkinetochore bundle and either penetrate the kinetochore or graze the kinetochore. Fig. 8*a* shows a reconstruction from serial sections through kinetochore 4*b* (see Fig. 5*b*). As with the X and Y kinetochores, bipolar microtubule attachments were observed. However, in this case,

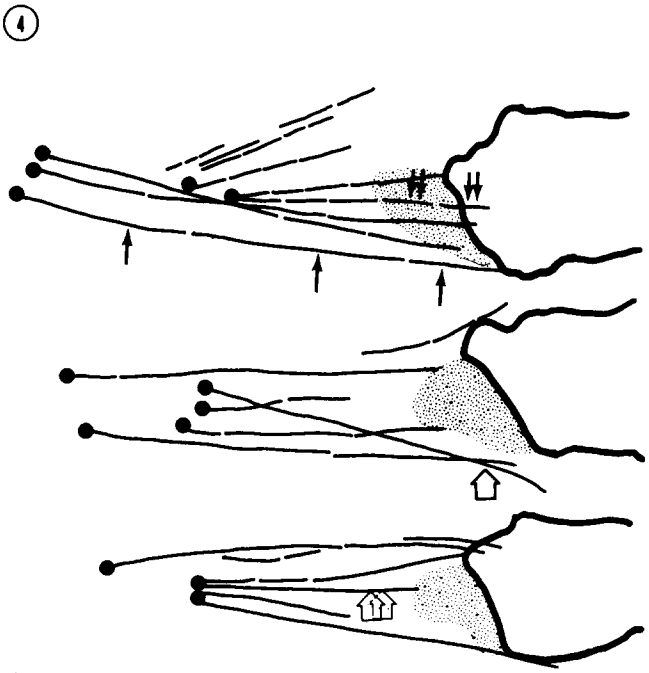

FIGURE 4 Reconstruction of 13 serial sections through a kinetochore region of a prometaphase-I spermatocyte. Arrows are keyed to arrows in Fig. 3. Circles indicate those microtubules that ended on a membrane vesicle.

TABLE I

Categories and Numbers of Microtubules in the Vicinity of the Kinetochore

Kinetochore number	Categories*							Total microtubules per kinetochore
	A	B	C	D	E	F	G	
1	3	2	1	4	4	0	6	20
2	0	5	2	8	0	4	2	21
3	2	0	1	2	0	1	0	6
4	4	2	0	4	0	0	4	14
5	3	4	0	1	1	1	0	10
6	1	3	1	3	0	1	2	11
7	3	0	1	1	0	1	1	7
8	3	1	2	3	1	1	0	11
9	2	3	1	4	0	2	2	14
10	1	2	2	1	0	0	0	6
11	3	1	1	2	0	0	3	10
12	2	2	1	1	0	0	0	6
13	3	1	0	1	1	2	0	8
14	1	2	1	1	0	0	1	6
Total	31	28	14	36	7	13	21	150
Frequency	0.21	0.19	0.09	0.24	0.05	0.09	0.14	1.00

* Microtubules which (*A*) end short of the kinetochore, (*B*) graze the kinetochore and end in nucleoplasm, (*C*) pass through the kinetochore and end in nucleoplasm, (*D*) end in the kinetochore, (*E*) end in the chromosome, (*F*) have one end in kinetochore and a free end distal to kinetochore, (*G*) have two free ends.

FIGURE 3 *(a-f)* Sections 3 through 8 of the kinetochore region which is reconstructed in Fig. 4. Examples of chromosome-to-pole microtubules (single arrows and double arrows), a glancing microtubule (open arrow), and a kinetochore (*K*)-to-pole microtubule (double open arrow) are illustrated. Each microtubule can be tracked to the membrane invagination (*m*) at the polar region. Bar, 0.25 μm. × 50,000.

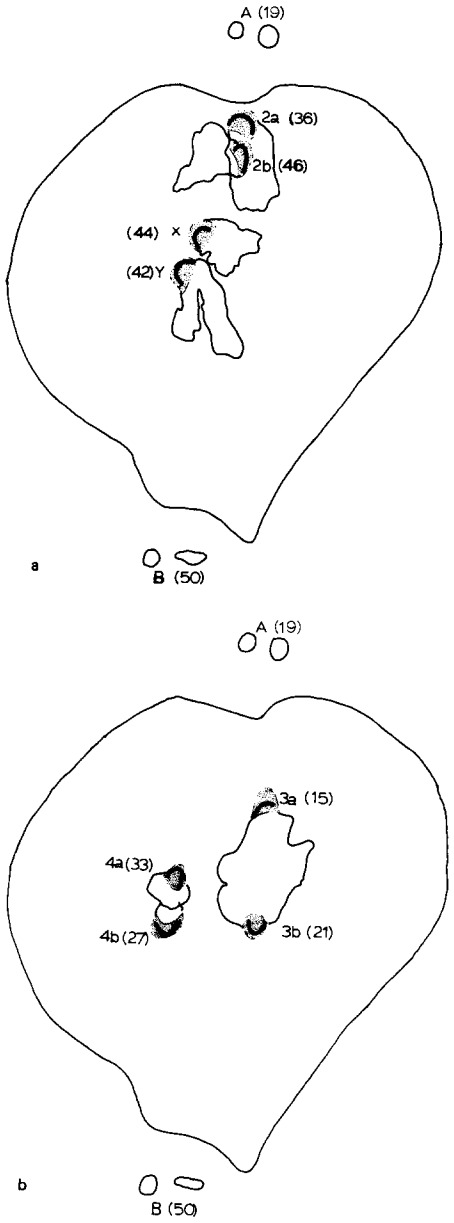

FIGURE 5 (*a* and *b*) Reconstruction from electron micrographs of serial sections of a nucleus in early prometaphase I. Numbers in parentheses indicate the section number midway through the kinetochore (stippled area) or the centriole pairs that mark the two poles (*A* and *B*). The *X* and *Y* kinetochores are oriented approximately 90° to the spindle axis. The *4a* and *4b* kinetochores appear to be oriented towards opposite poles as do the *3a* and *3b* kinetochores. The *2a* kinetochore faces pole A, and the *2b* kinetochore is oriented ~45° from pole *A*.

the kinetochore axis is perpendicular to the spindle axis (i.e., the kinetochore is clearly facing one pole). Nevertheless, two microtubules extend from the kinetochore, 180° in the opposite direction, and connect with the opposite pole and an additional microtubule extends from a nonkinetochore bundle to the kinetochore (Fig. 8*a*). The homologous kinetochore 4a (Fig. 5*b*) has microtubule connections only with the pole it faces (Fig. 8*b*). Thus, the kinetochores of the number 4 bivalent display both unipolar (4a) and bipolar (4b) microtubule connections. The observations on the X, Y, and 4b kinetochores demonstrate that microtubule connections with the poles at prometaphase are not necessarily related to the position of the kinetochore. These arrangements of microtubules might be expected if the initial interaction between a kinetochore and microtubules is unpolarized, so that the kinetochore can associate with microtubules at a variety of angles, and kinetochore movement in response to the prevailing distribution of microtubules does not occur immediately.

Bivalent number 2 is situated near one pole (Fig. 5*a*). Kinetochore 2a clearly faces pole *A* (Fig. 5*a*), and microtubule connections could be traced from the kinetochore to the pole. Kinetochore 2b, although located near pole *A*, is oriented at a 45° angle from pole *A*. However, it too has microtubule connections to pole *A*. In terms of microtubule connections, this is clearly a unipolar bivalent. Autosomal bivalent 3 has homologous kinetochores oriented towards opposite poles (Fig. 5*b*) and each kinetochore has microtubule connections with the pole it faces.

Another cell was judged to be in later prometaphase I by the increased number of microtubules found in the nucleus. All bivalents appeared to show bipolar kinetochore orientation but the bivalents had not yet completed congression. Upon examination of the higher-magnification electron micrographs, the Y kinetochore showed bipolar microtubule connections.

DISCUSSION

Prometaphase kinetochore behavior has been elegantly described from ciné analysis of live primary spermatocytes. Before the achievement of stable bipolar orientation, mal-oriented kinetochores may undergo repeated reorientations (8, 18, 20). Reorientation demands the presence of microtubules at the kinetochore (22) but for the most part the structural and molecular basis of kinetochore reorientation and bivalent congression is unknown. Our analysis emphasizes the complexity of the situation.

For example, there is clear evidence from ciné analysis that initial bipolar orientation is facilitated by the tendency for

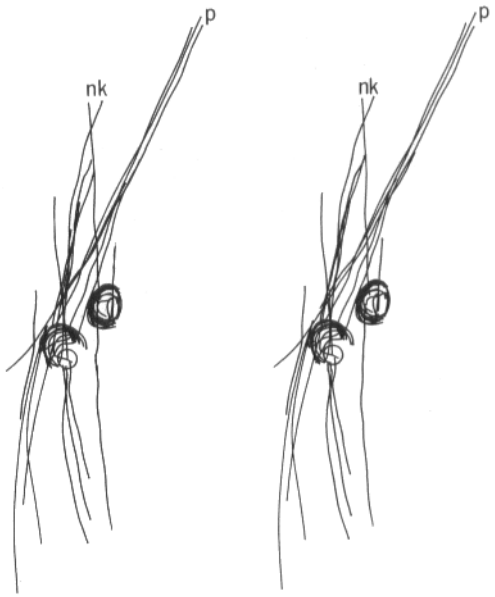

FIGURE 6 Stereo pair of the *X* and *Y* kinetochores (hemisphere lines) and associated microtubules. The *Y* kinetochore (on the left) has more microtubule associations than the *X* (on the right). However, both kinetochores have bipolar connections with poles. Some microtubules extend to a nonkinetochore bundle (*nk*) while others extend to the poles (*p*).

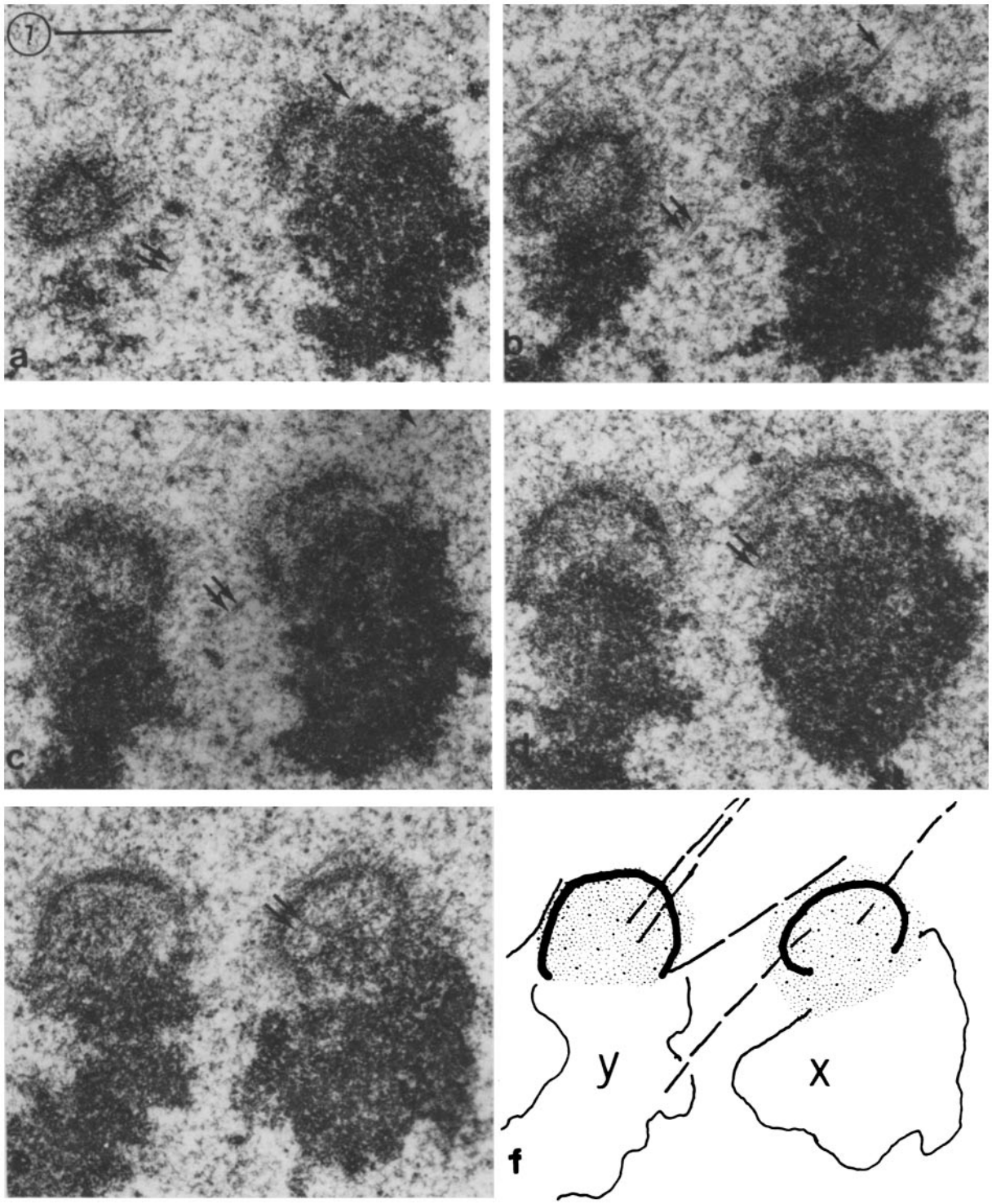

FIGURE 7 Sections 4–8 of the *X* and *Y* kinetochores (see Fig. 5 *a*) (14 sections were required to complete both kinetochores). Single and double arrows follow two microtubules that extend in opposite directions from the *X* kinetochore. Note the trilaminar appearance of the hemispheric kinetochore. Bar, 0.5 μm. × 37,000.

chromosome fibers to form to the pole to which a given half bivalent's kinetochore most directly points and to the intrinsic structure of the bivalent which ensures that homologous kinetochores face opposite directions (18, 22). Our observations indicate that although kinetochores may initially have the tendency to make microtubule connections to the pole to which they happen to be nearest (also see references 23 and 25), the actual polarity of the kinetochore may not be a determining factor. This situation is exemplified by the case of the number 4 kinetochore which directly faced one pole but displayed microtubule connections to both the facing pole and the opposite pole. We have also observed two additional examples of apparent bipolar positioning of homologous kinetochores; in each, one of the kinetochores has microtubule connections with both poles.

Our results suggest that, in terms of microtubule connections

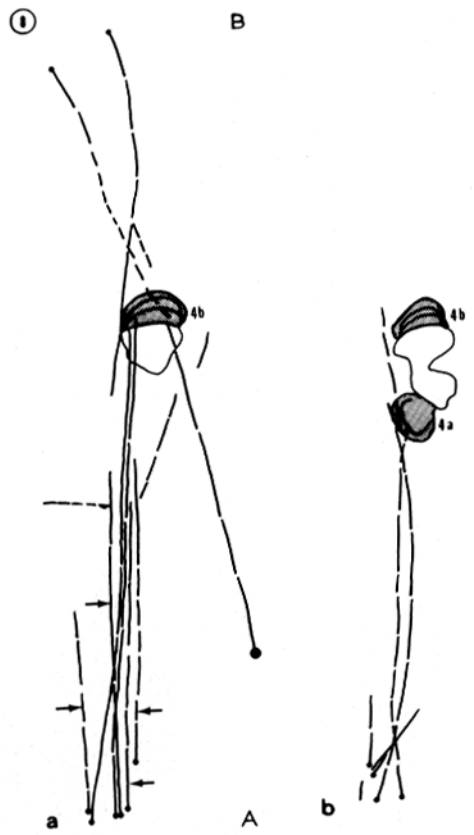

FIGURE 8 Higher-magnification reconstruction of the number 4 bivalent illustrated in Fig. 5 *b*. The *4b* kinetochore (*a*) although facing pole *B* (see Fig. 5 *b*) shows bipolar microtubule connections. Two microtubules (1 kinetochore-to-pole and 1 glancing) connect it to the pole it faces and 2 kinetochore-to-pole microtubules connect it to the opposite pole. Small circles indicate microtubules tracked to the membrane; large circle indicates a microtubule that extended from an interpolar bundle to the kinetochore. Arrows indicate four microtubules that extend from the polar region towards the kinetochore but do not connect with it. The homologous kinetochore *4a* (*b*) has microtubule connections to the pole it faces.

to the poles, prometaphase kinetochores can participate in all possible arrangements. However, we should emphasize that the bivalent, where homologous kinetochores each showed bipolar connections, is also a structural heterozygote (i.e., $Insc^{4L}sc^{8R}$). Whether this microtubule arrangement is caused by this particular structural rearrangement or can be a transient precursor to stable bipolar orientation in all bivalents is now being analyzed. It is interesting that the only other reported observations of this type of kinetochore orientation involved a bivalent with a structural rearrangement (32) and bivalents that were heat treated (7). It has been inferred that events leading to stable bipolar orientation of bivalents must involve considerable rearrangement or polymerization and depolymerization of microtubules. Our observations add documentation to these inferences. For example, to progress from a bivalent with unipolar microtubule connections to stable bipolar connections, one group of microtubules must either be eliminated or repositioned.

Until very recently, the most common view of prometaphase chromosome orientation was that it was achieved by the lateral interaction of microtubules originating from the kinetochores with interpolar microtubules of opposite polarity (5, 16, 25). Recently, Tippet et al. (30) have challenged this view of prometaphase kinetochore orientation. An analysis of cell division in two large pennate diatoms revealed that most microtubules at prometaphase do not terminate at the kinetochore but rather extend past the kinetochore, leading those authors to conclude that the kinetochore functions by attaching to or capturing preexisting microtubules, a possibility suggested earlier by Nicklas (19). Consistent with this hypothesis are observations by LaFountain (11), Kubai (10), Goldstein (6), Nicklas et al. (22), Rieder and Borisy (23), as well as the very recent observations that kinetochore microtubules in both metaphase and anaphase cells have the same polarity as the interpolar microtubules of the same half-spindle (4, 28). Tippit et al. (30) further proposed that invasion of polar microtubules during prometaphase forms the structural basis for prometaphase chromosome activity, i.e., motion is somehow caused by kinetochores interacting with and moving along polar microtubules.

Our observations are consistent with the hypothesis that kinetochores interact with polar microtubules. We have demonstrated that the majority of the microtubules found in the vicinity of the *Drosophila* prometaphase kinetochore most likely have not originated by nucleation at the kinetochore. Over 50% of the microtubules have one free end which is distal from the pole. All microtubules in this class either are directed towards a kinetochore, graze the kinetochore, or pass through the kinetochore. Furthermore, our observation that kinetochore microtubule orientation (i.e., parallel to the spindle axis) and kinetochore position can be unrelated in early cells is also consistent with a polar origin for the majority of the microtubules in a prometaphase kinetochore bundle. We suggest that in the case of meiotic prometaphase kinetochores, a given dyad kinetochore can initially interact with microtubules from one or both poles, and its homologue can do likewise. However, many of the initial interactions produce unstable microtubule configurations which may be correlated with characteristic prometaphase chromosome movements (i.e., oscillary poleward motion [24], kinetochore reorientation [20]). Stable microtubule configurations will eventually occur when, through trial and error, homologous kinetochores achieve bipolar microtubule connections and equal tension is directed towards opposite poles (20). It should be emphasized that our evidence most certainly does not rule out nucleation at the kinetochore as the origin of some kinetochore microtubules (15, 26, 29, 33) since we did observe microtubules with one end at the kinetochore and a free end distal to the kinetochore. Thus, we cannot rule out the possibility that lateral interactions between microtubules nucleated at the kinetochore and polar microtubules are involved in prometaphase chromosome movement (5, 16, 25). Finally, we do not know the relationships among the bundles of kinetochore microtubules, nonkinetochore microtubules and extranuclear microtubules in the complex region of the nucleus near the poles. The spacial proximity of the microtubules would most certainly allow lateral interactions among the different classes of microtubules in this region of the spindle apparatus.

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