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Cell Division in Two Large Pennate Diatoms *Hantzschia* and *Nitzschia* III. A New Proposal for Kinetochore Function during Prometaphase

DAVID H. TIPPIT, JEREMY D. PICKETT-HEAPS, and ROGER LESLIE
Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309

ABSTRACT Prometaphase in two large species of diatoms is examined, using the following techniques: (*a*) time-lapse cinematography of chromosome movements in vivo; (*b*) electron microscopy of corresponding stages; (*c*) reconstruction of the microtubules (MTs) in the kinetochore fiber of chromosomes attached to the spindle. In vivo, the chromosomes independently commence oscillations back and forth to one pole. The kinetochore is usually at the leading edge of such chromosome movements; a variable time later both kinetochores undergo such oscillations but toward opposite poles and soon stretch poleward to establish stable bipolar attachment. Electron microscopy of early prometaphase shows that the kinetochores usually laterally associate with MTs that have one end attached to the spindle pole. At late prometaphase, most chromosomes are fully attached to the spindle, but the kinetochores on unattached chromosomes are bare of MTs. Reconstruction of the kinetochore fiber demonstrates that most of its MTs (96%) extend past the kinetochore and are thus apparently not nucleated there. At least one MT terminates at each kinetochore analyzed. Our interpretation is that the conventional view of kinetochore function cannot apply to diatoms. The kinetochore fiber in diatoms appears to be primarily composed of MTs from the poles, in contrast to the conventional view that many MTs of the kinetochore fiber are nucleated by the kinetochore. Similarly, chromosomes appear to initially orient their kinetochores to opposite poles by moving along MTs attached to the poles, instead of orientation effected by kinetochore MTs laterally associating with other MTs in the spindle. The function of the kinetochore in diatoms and other cell types is discussed.

Chromosomes attach to the mitotic apparatus during prometaphase. This remarkable process involves orienting chromosomes so that their kinetochores face toward opposite spindle poles, and then anchoring the chromosomes to the spindle by microtubule (MT) attachments which extend from each kinetochore to one or the other pole. The key structural and functional components of this process are the two kinetochores on each chromosome, because they are the sites on the chromosome which are oriented polewards, and they attach the chromosome to the spindle. However, the functioning of the kinetochore in vivo is still not well understood, although the overwhelming consensus presently is that it functions by nucleating MTs. Our work with diatom spindles unexpectedly suggests that the kinetochore in these organisms functions primarily, if not solely, by attaching to preexisting MTs in the spindle rather than by nucleating the MTs (39, 41, 56). We now present data relevant to this matter, examining prometaphase in detail with reference to chromosome movements in living cells in conjunction with corresponding electron microscope observations.

Diatoms offer unique advantages for studying prometaphase. Because the central spindle (a central bundle of MTs) is so clearly defined, both chromosomal movement around it and the subsequent attachment of chromosomes to it are easy to follow. In contrast, the chromosomes in most conventional spindles attach to a large and diffuse spindle structure. The

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first two papers of this series (42, 43) describe the course of mitosis and the structure of the spindle in the two diatoms *Hantzschia* and *Nitzschia* used in the present report.

MATERIALS AND METHODS

Culture of diatoms, preparation for electron microscopy, and time-lapse cinematographic techniques are described in the first two papers of this series (42, 43). Nomarski differential interference contrast micrographs (Figs. 2–6) were obtained as follows: time-lapse sequences were examined with a Photo-Optical Data Analyzer movie projector. Favorable sequences were carefully examined frame-by-frame. Selected frames were photographed directly using a 35-mm camera focused on the viewing screen; a pointer was included in most frames to unambiguously record the location of a particular chromosome or mitochondrion. This procedure causes some loss of image quality, but permits repeated forward and reverse viewing so that the identity of individual chromosomes cannot be confused even during periods of maximum movement.

Terminology

Polar MTs have one end at a pole and the other end free in the spindle. Free MTs have both ends free in the spindle (not attached to the poles). Kinetochore MTs (kMTs) have one end at a kinetochore. MTs with one end at a pole and the other at the kinetochore are by definition kMTs (26). The kinetochore fiber in these diatoms is operationally defined as the bundle of MTs and associated material that connects the chromosome to the pole. The kinetochore is a site of chromosome attachment to the spindle; some MTs may terminate at the kinetochore whereas others go past it. The central spindle is the essentially parallel set of MTs between the spindle poles (Figs. 2*a* and 18), which consists of two half-spindles that interdigitate in the middle overlap region. Metakinesis is movement of the chromosomes to the metaphase plate.

Serial Section Analysis

The kinetochore fiber at metaphase is reconstructed by tracking its MTs through transverse serial sections (see references 57 and 24 for details of MT tracking technique). Briefly, this is accomplished as follows:

An outline of each MT in the first micrograph of a series of serial sections (i.e. section 1) is traced onto acetate; the tracing is then laid over the next section (No. 2) and the best fit of each individual MT is determined. Similarly, MTs traced from section 2 are identified in section 3 and so on. A graph is then generated (Figs. 21–23) showing in one-dimensional form the endpoints of each MT, and an estimate of their relative length. Obliquely sectioned MTs, encountered near the kinetochore (Figs. 19 and 20), are tracked the same way as MTs which project circular profiles.

Fig. 1 shows which part of the kinetochore fiber is reconstructed. The first serial section of each reconstruction by definition begins just outside of the spindle pole (the region where MTs terminate which is usually nine sections in length). All MTs were tracked to within four to eight sections of the pole. The MTs were not tracked all the way to the pole because of the large number of other converging MTs. The kinetochore in transverse sections is defined as the densely staining region usually about five sections in length (Fig. 19) on the tip of the poleward-stretched chromosomes.

The following method was used to determine which of the many MTs around the chromosome are to be considered part of the kinetochore fiber. First, a series of serial sections starting at one pole and extending more than half way to the opposite pole is photographed using a JEOL 100 C electron microscope equipped with a goniometer stage. Each section is tilted and rotated until an optimum position is obtained such that most of the MTs of a particular kinetochore fiber project a circular profile. Each negative is enlarged to ×160,000. The print containing the kinetochore region is selected; an arbitrary sized circle, 0.3–0.5 μm in diameter, is drawn around the kinetochore and its associated MTs (Fig. 19). This circle is also traced onto each adjacent section towards the pole; the center of the circle is fixed by five or six centrally located reference MTs (Fig. 24). A similar circle is placed onto each section moving towards the overlap, but here the chromosome is used as the reference point to center the circle (Figs. 25, 26*a*, *b*). Collectively the circles in each section generate an imaginary cylinder extending from the pole through the kinetochore towards the overlap. Each MT inside this cylinder for more than two sections is tracked through the entire series of serial sections (i.e. usually ~40 sections) regardless of whether it stays in the cylinder or moves outside of it. Thus, an MT may be inside the cylinder near the pole but outside of it at the kinetochore, and vice versa. This positional change is documented in the reconstructions as follows. Each MT is designated by a line; the solid portion of the line denotes those sections in which this particular MT is inside the cylinder, while the dashed part of the line corresponds to the sections in which the MT is outside the cylinder. For example, the three MTs at the bottom of the reconstruction in Fig. 23 were in the cylinder in sections 10–14, and then moved outside it in sections 15–40. This is also illustrated in Figs. 24 and 26*b*.

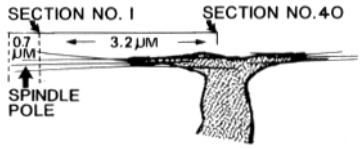


FIGURE 1 This drawing shows a typical chromosome and its kinetochore fiber in *Hantzschia* similar to those reconstructed in Figs. 21–23. These reconstructions contain ~40 sections; their position is shown in this drawing. The lines represent MTs and the chromosome is shaded.

RESULTS

In Vivo Observations of Prometaphase

CHROMOSOME MOVEMENT DURING METAKINESIS: We have presented a brief account of prometaphase in these cells as part of a description of the course of mitosis in vivo (42, 43). Here we examine metakinesis in detail, analyzing at least 50 cine sequences of this phase (a film of mitosis in these diatoms is in preparation).

It is difficult to document when the nuclear envelope breaks down in vivo. After a prolonged quiescent phase, the small birefringent prophase spindle suddenly initiates a phase of elongation; within 2 min it has tripled its length (42, 43). As the spindle starts elongating, the nuclear surface briefly appears to be indented. Suddenly, the chromosomes become agitated near the spindle and within 20 s the spindle clearly has entered the nucleus. Movement of chromosomes now rapidly becomes widespread; the first chromosomes occasionally are observed attached to the spindle within 10 s of the beginning of this generalized motion (Figs. 2*a* and *b*). Prometaphase attachment of chromosomes occupies ~15 min, although the majority of chromosomes independently attain their metaphase configuration within 3–5 min. Chromosome attachment has been observed up to a few minutes before the beginning of anaphase (Figs. 4*a–f*).

In every cell analyzed, prometaphase activity is characterized by rapid, irregular oscillations of the chromosomes either over the surface of the central spindle if they are close to it (Figs. 3*a–i*), or else along invisible tracks directed at the nearest edge of either pole (Figs. 4*a–f*; 5*a–f*). This chromosomal movement is strongly localized at one or two specific sites. Although kinetochores cannot be discerned under the light microscope, the correlation with electron microscopy (see section entitled electron microscopic . . .) is so precise, we can confidently identify these sites on the chromosomes as the kinetochores. The rest of each chromosome is relatively inactive, although they are visibly moved around by the motion inside the spindle.

The chromosomes rapidly and irregularly become drawn out to fine points at the kinetochores, displaying considerable pleomorphism. Repeatedly, they irregularly stretch at the kinetochore(s) and then relax as their interaction with the spindle proceeds (Figs. 5*a–f*). Initially, movement of kinetochores on any given chromosome is strongly towards one pole or the other, and the kinetochores of a chromosome may act independently, as they both oscillate along the same poleward directed track. The movements of an individual chromosome can thus be very complex. It will frequently move up to one pole, with minor stretching sometimes visible between kinetochores (Figs. 3*d* and 5*b*), and then move away from it. This

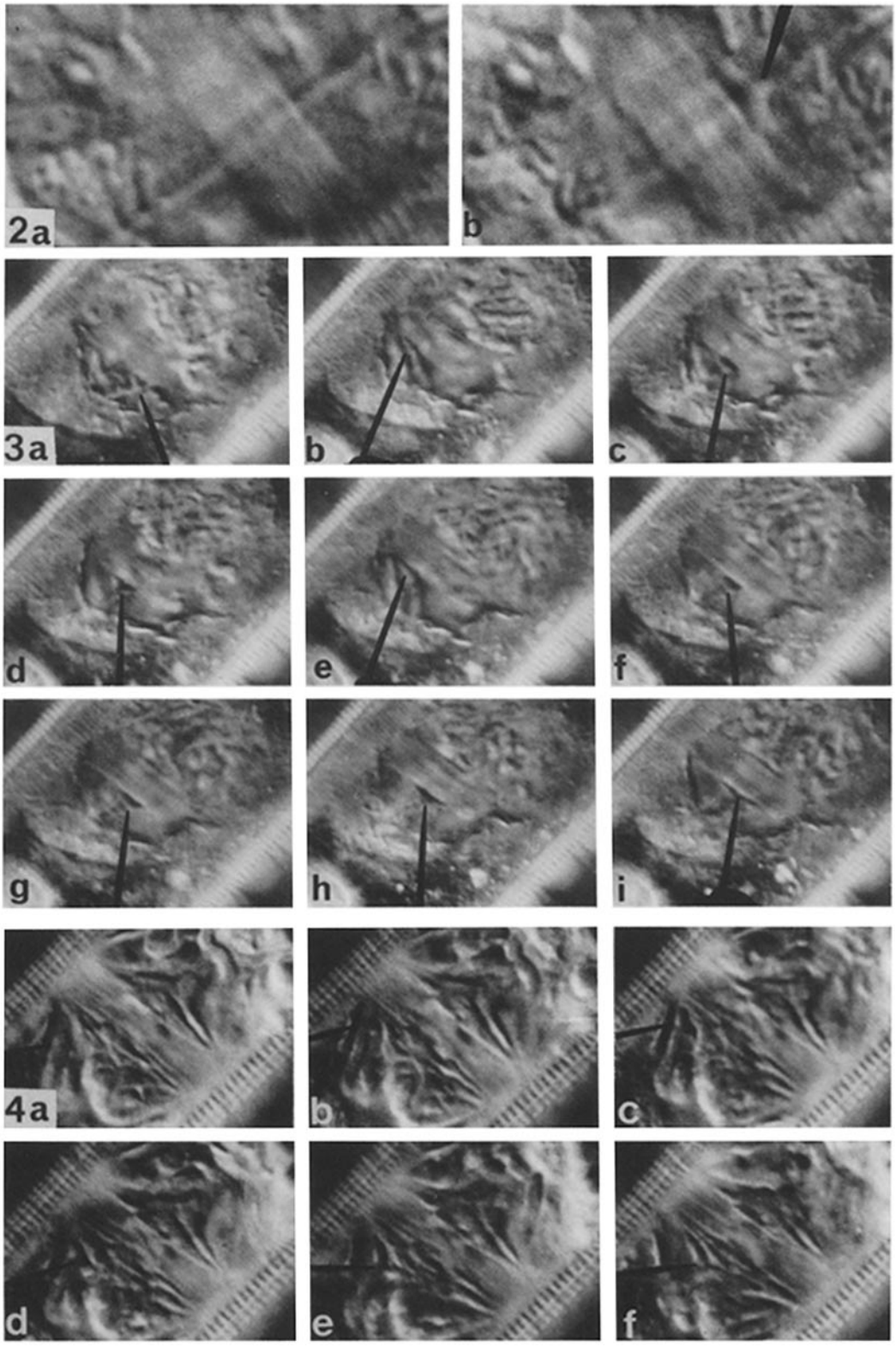


FIGURE 2 Very early chromosome attachment in *Hantzschia*. (*a*) Central spindle outside the nucleus (t = 0 s).(*b*) The central spindle has just entered the nucleus, and one chromosome is apparently attached to the spindle; see Fig. 15 (t = 6 s). × 2,650.

FIGURE 3 Early prometaphase in *Hantzschia*, showing chromosome oscillations and attachment. (*a*) Chromosomes are around the central spindle which is just out of the plane of focus; all are oscillating vigorously, and the one marked with the pointer will be followed subsequently. (*b*) The marked chromosome stretches to one pole. (*c*) It returns to the middle of the spindle. (*d*) It moves toward to the pole and the kinetochores have stretched apart. (*e*) It moves back to the original pole. (*f*) It returns to the center of the spindle again. (*g–i*) Final bipolar attachment is achieved as the kinetochores stretch apart (cf., with Figs 15 and 18). Timing: *a*, 0 s; *b*, 17 s; *c*, 21 s; *d*, 28 s; *e*, 40 s; *f*, 44 s; *g*, 45 s; *h*, 50 s; *i*, 57 s. × 1,250.

FIGURE 4 Late prometaphase chromosome attachment in *Hantzschia*. Many chromosomes are already attached to the spindle. (*a*) Chromosome slowly backing away from pole. (*b*) Tension between kinetochores briefly relaxes, and one kinetochore moves up to pole. (*c*) Tension reappears between kinetochores. (*d–f*) Tension increases, chromatids are partially separated over the central spindle as they achieve metaphase. Timing: *a*, 0 s; *b*, 12 s; *c*, 56 s; *d*, 74 s; *e*, 126 s; *f*, 184 s. × 1,150.

cycle may repeat several times and the overall motion is interspersed with numerous smaller oscillations. After several such major oscillations (a maximum of four to six is usual), the kinetochore furthest from the pole will suddenly start curving and then moving towards the opposite pole. Again, this new motion is at first irregular, and the initial connection (or stretched kinetochore) in several cases is apparently broken (cf. Figs. 5 *b–e*). Fairly soon however, connection to the second pole becomes firmly established. The chromosome immediately becomes more stable and less frenetic; within a few seconds there is a buildup of tension between the kinetochores as now they steadily stretch towards opposite poles (Figs. 3 *g–i*; 4 *c–f*; 5 *g–i*). Meanwhile, the inert chromosome arms are moved to the metaphase configuration where they extend perpendicular to the central spindle.

Time-lapse movies of metakinesis demonstrate a frenzied, disordered motion which very rapidly leads to the majority of chromosomes being attached. The first chromosomes attached seem to be stretched directly over the central spindle (Figs. 2 and 3). Succeeding chromosomes become stacked up on the first ones, as they are pulled in against the central spindle (Figs. 4*f* and 5 *i*), and thus by metaphase several layers of chromatin can be seen stretching over the central spindle. It is difficult to estimate the speed of these chromosome movements since they are so variable in duration, distance, and velocity. Chromosome movement at its fastest is clearly visible by direct observation and causes blurring of individual kinetochores with the half-second exposure per frame (maximum rate) used for ciné filming. We estimate the maximum velocity of kinetochore movement at around 2.5 μm/s; this rate varies from cell to cell and is temperature sensitive. Stretching of both chromosomes to the poles is slower, up to around 0.5 μm/s; this rate is difficult to estimate because of difficulty in identifying the leading edge of the chromosome. These movements were filmed at temperatures appreciably elevated (22°C) in comparison to growth temperature (4°–8°C) and are thus probably faster than in wild specimens.

MITOCHONDRIAL MOVEMENT IN THE SPINDLE: Mitochondria are particularly good nonchromosomal markers of spindle activity. They are long and thin, conspicuous with Nomarski optics, and highly pleomorphic; the changes in shape they undergo (e.g., stretching) demonstrate the forces to which they are being subjected. Their movement can be easily followed at metaphase, when their oscillations tend to remain in one plane of focus.

Mitochondria are usually lined up end to end and invariably move along invisible tracks (Figs. 6 *b, g, h*) directed at the closest edge of either pole (Figs. 6 *a–i*). They appear unexpectedly from the cytoplasm usually near the chromosome arms which laterally extend from the spindle. Their movement is very irregular and they will often move up to one pole (Figs. 6 *a, e, h*) before (e.g. ~30 s later) sliding with several oscillations back into the cytoplasm where they become invisible; during this movement they often go through cycles of stretching and relaxing. Sometimes they reappear and repeat this behavior (e.g., the top mitochondria in Figs. 6 *a–i*). The images of the mitochondria may change between frames (e.g. Figs. 6 *d–f*), making it difficult to determine their end points during movement. Their maximum velocity is estimated to be the same as that of the most active prometaphase kinetochores although they can maintain this speed over longer distances; for example, one pair of mitochondria moved from the equator of the spindle to one pole (9 μm) within ~4 s (Figs. 6 *c* and *d*). In rare cases, individual movie frames suggested the existence of a transitory fiber associated with such rapidly moving mitochondria (Figs. 6 *d* and *e*). However, it is likely that such an image is an artifact created by blurred movements of the organelles.

In Figs. 6 *a–i*, a mitochondrion moves to and from the upper pole, and then two others similarly move to the lower pole. Anaphase commences during the oscillations (at about frame *g*); as the chromosomes move polewards, the track these mitochondria follow is displaced laterally away from the central spindle by the chromosomal movement.

Electron Microscope Observations of Prometaphase

Kinetochores have not been identified in prophase nuclei, but can be found when the nuclear envelope starts to break down. Initially, the kinetochores are bare of MTs and have a distinctive morphology; they are often contained within one of three consecutive serial sections. Each kinetochore has a small basal lamella surmounted by a dense region of limited extent, which is often faintly fibrous (Figs. 7 *d* and 9).

As the nuclear envelope breaks down near the central spindle, MTs attached to the poles penetrate extensively into the nucleoplasm (Fig. 7). An association between kinetochores and MTs soon becomes widespread as prometaphase progresses. We have sectioned ~35–40 prometaphase cells (some serially sectioned), and have observed numerous examples of the following types of associations between kinetochores and MTs: (*a*) MTs directed toward and apparently attached to opposite poles (as observed in serial sections) are seen close to and often extending past a single kinetochore (Figs. 7 *a–c*). (*b*) Similarly, MTs attached to one pole run up to and often past both kinetochores on a chromosome (Fig. 12). (*c*) Most commonly, MTs attached to one pole are seen close to and often extending past one of the kinetochores on a chromosome, while the other kinetochore is bare; frequently, such chromosomes are very close to one pole (Figs. 9 and 10). Many times, both kinetochores are not in the plane of the section, but one kinetochore is observed laterally associated with MTs directed toward and apparently attached to the poles as shown by serial sections (Figs. 8, 11 *a, b*, 13). (*d*) On rare occasions, kinetochores were seen abutting the MTs of the central spindle, apparently caused by similar lateral interaction between the kinetochore and MTs of the central spindle (Fig. 14).

The manner by which the chromosomes orient so that the kinetochores face opposite poles is not discernible from electron microscopy alone. As prometaphase progresses, the chromosomes independently become arranged with their kinetochores clearly associated with MTs attached to opposite poles. After this orientation is achieved, the chromosomes stretch toward opposite poles and the kinetochores become unrecognizable (Figs. 15 and 18). During or after bipolar orientation, three other changes in the kinetochore are observed; (*a*) the number of MTs associated with each kinetochore increases, until a small bundle is formed which ensheathes each kinetochore and its attached strand of chromatin (Fig. 18, black arrow); (*b*) a faintly staining matrix often localized in the tip of the kinetochore is detectable permeating these bundles (Figs. 16 and 19); (*c*) the induction of tension is visible between the two kinetochores of each pair; as they increasingly separate, the chromatin develops a fine, fibrous, striated core stretched between them (Fig. 12 in reference 43). Sometimes, MTs are curved in association with such a stretched chromatin strand (Fig. 17).

At mid-prometaphase, some chromosomes are fully attached to the spindle while others on the edge of the nucleus contain



FIGURE 5 Repeated bipolar attachment in *Hantzschia*. (*a*) Blurred chromosome, not under tension, near one pole. (*b*) Tension between kinetochores. (*c*) Tension relaxed. (*d*) Tension reestablished. (*e*) Tension almost gone. (*f–i*) Increase in tension and establishment of bipolar attachment. Timing: *a*, 0 s; *b*, 38 s; *c*, 42 s; *d*, 48 s; *e*, 154 s; *f*, 172 s; *g*, 182 s; *h*, 198 s; *i*, 240 s. × 1,050.

FIGURE 6 Mitochondrial movement in the spindle of *Hantzschia*. (*a*) After several oscillations, one (possibly two) mitochondrion is near pole; note characteristic linear orientation along the invisible path it follows. (*b* and *c*) Movement to edge of spindle (one of several such oscillations). (*d*) Movement back to the pole; a faint fiber can be briefly detected alongside it. (*e*) As for *d*, but this adjacent frame shows the rapid change of shape it can undergo. (*f*) The mitochondrion moves back to the edge of the spindle. Anaphase commences at about this stage and the mitochondrion continues to move in this fashion. (*g*) A pair of mitochondria now appear, linearly oriented toward the other pole (pointer) note that the original mitochondrion has reappeared (white arrow) again, having undergone several oscillations not illustrated here. (*h*) Both mitochondria move rapidly to their respective poles. (*i*) Both mitochondria have moved away from the poles; as anaphase progresses, the tracks the mitochondria follow have become slightly bent. Timing: *a*, 0 s; *b*, 8 s; *c*, 22 s; *d*, 26 s; e, 28 s; *f*, 36 s; *g*, 56 s; *h*, 74 s; *i*, 84 s. × 1,050.

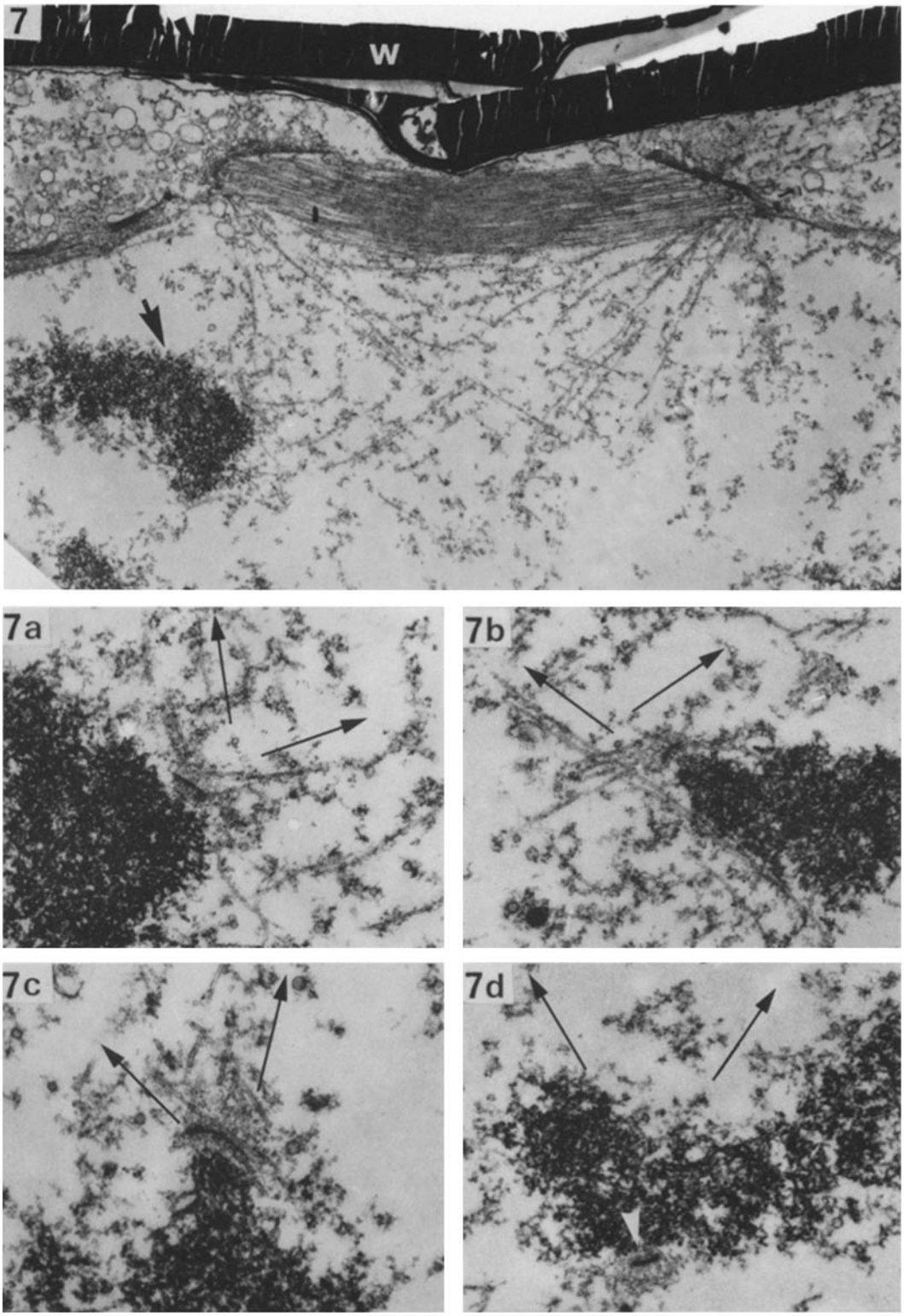
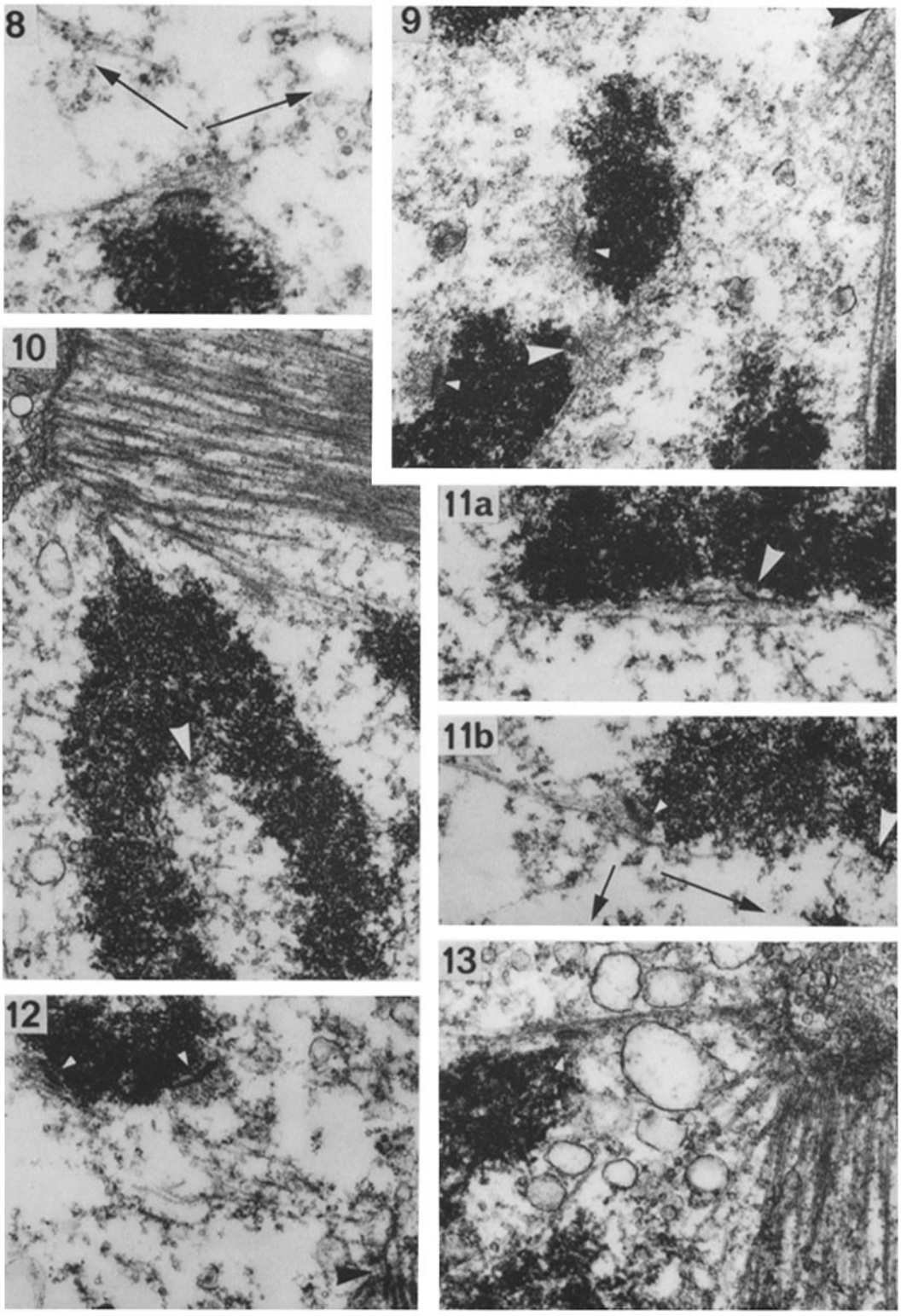


FIGURE 7 Early prometaphase in *Hantzschia*. The spindle forms near the siliceous wall (*w*) and starts sinking into the nucleus; numerous MTs attached to the poles invade the nucleoplasm and associate with the chromosomes (arrow). × 10,050. (*a–d*) These four micrographs show kinetochores from this spindle. The chromosome in *a* is a high magnification of the kinetochore in Fig. 7 (arrow), whereas other kinetochores are from different sections. The two arrows on each micrograph point toward the spindle poles; the arrows show the orientation of the chromosome in the spindle (cf. the orientation of the chromosome in Fig. 7 with the high magnification micrograph in *a*). (*a–c*) Kinetochores associated with MTs apparently attached to the poles; serial sections confirm most of these MTs extend to the pole. Occasionally, MTs from both poles are associated with a single kinetochore. (*d*) One of the many bare kinetochores (white arrow) in the spindle. × 24,900, × 23,300, × 25,700, × 23,000, respectively.


8
9
10
11a
11b
12
13

kinetochores which are devoid of MTs (Fig. 18); such late-attaching chromosomes are observed well into "metaphase".

Reconstruction of MTs Associated with the Chromosome

At metaphase, numerous MTs attach the chromosomes to the spindle poles. Serial longitudinal sections indicate that most of these MTs do not terminate at the tip of the stretched kinetochore; instead, they extend past it for a variable distance. To confirm this unexpected observation, we have reconstructed from transverse serial sections the MTs associated with single chromosomes already attached to the spindle. Five such reconstructions were completed from three different metaphase cells. Three representative samples are shown in Figs. 21–23; Figs. 19, 24–26 *a*, *b* are micrographs of the kinetochore fiber reconstructed in Fig. 23. Each kinetochore fiber is permeated by a densely staining matrix (Figs. 16, 19, 24) which extends from the kinetochore toward the pole.

Nearly 96% of the MTs from the five reconstructions do not terminate at the kinetochore; they extend from the pole past the kinetochore region (Figs. 19 and 20) towards the middle of the spindle. At least one MT per chromosome terminates in the region of the kinetochore (Figs. 21–23 and Table I). The MTs which extend past the kinetochore splay outwards away from the center of the chromosome after passing the kinetochore (Figs. 25 and 26 *a*).

Invariably, some of the MTs in these reconstructions are also central spindle MTs. For example, in the reconstruction shown in Fig. 23, the position of the same nine MTs is illustrated in a section near the pole (Fig. 24, section 12) and in a section half way through the reconstruction (Fig. 26 *b*, section 20: see figure legend). In Fig. 26 *b*, these MTs are part of the central spindle and are spatially removed from the kinetochore fiber which is encircled (see Materials and Methods); near the pole (Fig. 24) they intermingle with MTs associated with the chromosome. The reconstructions contain 9.8% free MTs (e.g. Fig. 23, the two MTs at the top of the reconstruction); every reconstruction contains at least two free MTs. In one reconstruction, a single MT was found to extend past one kinetochore and then curve over to an adjacent kinetochore on a nearby chromosome, thereby laterally associating with two separate kinetochores. (To track such curved MTs did not require further tilting of sections).

DISCUSSION

Prometaphase is a vital stage of mitosis; the chromosomes orient their pairs of kinetochores toward opposite poles of the spindle, and a fiber containing MTs is formed which connects each kinetochore to the pole it faces. There is variation in the morphology of the kinetochore fiber in different organisms. Certain fungi have a single kMT attached to each chromosome (13) but in other cells a more complicated fiber is observed which apparently contains some MTs that terminate at the kinetochore and other MTs which pass by it or do not reach it (11). Recently, it has become widely accepted that the MTs which terminate at the kinetochore are nucleated there. It follows therefore that prometaphase orientation of chromosomes is achieved by lateral interaction between these kMTs and the MTs from the pole (27, 49, 4, 26, 10, 22). MTs growing from the kinetochore are essential to this theory because without them there can be no lateral interaction to orient the chromosome and recruit MTs from the poles into the kinetochore fiber. Our observations on diatoms indicate that their chromosomes are not oriented by this mechanism (their chromosomes can apparently orient without MTs growing from the kinetochore), and that their kinetochore fiber is composed primarily, if not solely, of MTs originating from the poles.

Our earlier work on smaller diatoms indicated that most of their chromosomes attach to the surface of the central spindle, having been apparently directed there during metakinesis by MTs from the poles; between the attachment points of chromosomes and poles is a matrix, the "collar", permeating the outer MTs of the central spindle (56, 41). In contrast, these two larger diatoms have small bundles of MTs similar to conventional kinetochore fibers, ensheathing the stretched chromosomes and running to the poles. This difference in morphology is in part caused by the variation in the number and size of the chromosomes. A collar is not observed around the central spindle of these large diatoms; instead the collar may be represented by the dense material permeating the MTs of each kinetochore fiber.

Metakinesis in Diatoms

Our interpretation of how chromosomes orient to opposite poles in these diatoms at prometaphase, based upon a comparison of electron microscopy and time-lapse cinematography, is

FIGURE 8 Mid-prometaphase in *Hantzschia*. MTs graze the kinetochore of this chromosome. The two arrows point towards the spindle poles. × 28,200.

FIGURE 9 Three kinetochores are present in this prometaphase in *Hantzschia*; two are bare of MTs (small white arrows), but the third (large white arrow) associates with MTs attached to the corner of the pole (black arrow). The MTs of the central spindle are on the right. × 22,200.

FIGURE 10 Early prometaphase in *Hantzschia*. One of the kinetochores on this V-shaped chromosome is bare of MTs whereas the other is stretched slightly and associated with MTs attached to the poles. × 21,800.

FIGURE 11 These micrographs are serial sections of two kinetochores on two different chromosomes in *Hantzschia* which associate with the same MTs. In *a*, MTs extend past a kinetochore (large arrow); in *b* the same MTs laterally associate with another kinetochore (small white arrow; the large white arrow shows the position of the kinetochore in *a*) on a different chromosome. (These are two different chromosomes as indicated by the chromosome arms, not included, which are similar to those in Fig. 10). The black arrows point towards the poles. × 19,500.

FIGURE 12 The two kinetochores (small white arrows) of this chromosome in *Hantzschia* which is behind the spindle pole (black arrow), appear to be associated with MTs attached to that pole. In an adjacent section not included, the right kinetochore is more clearly associated with MTs. × 19,200.

FIGURE 13 Mid-prometaphase in *Nitzschia*. A kinetochore (arrow) on a chromosome near the pole is laterally associated with MTs attached to the pole. × 26,500.

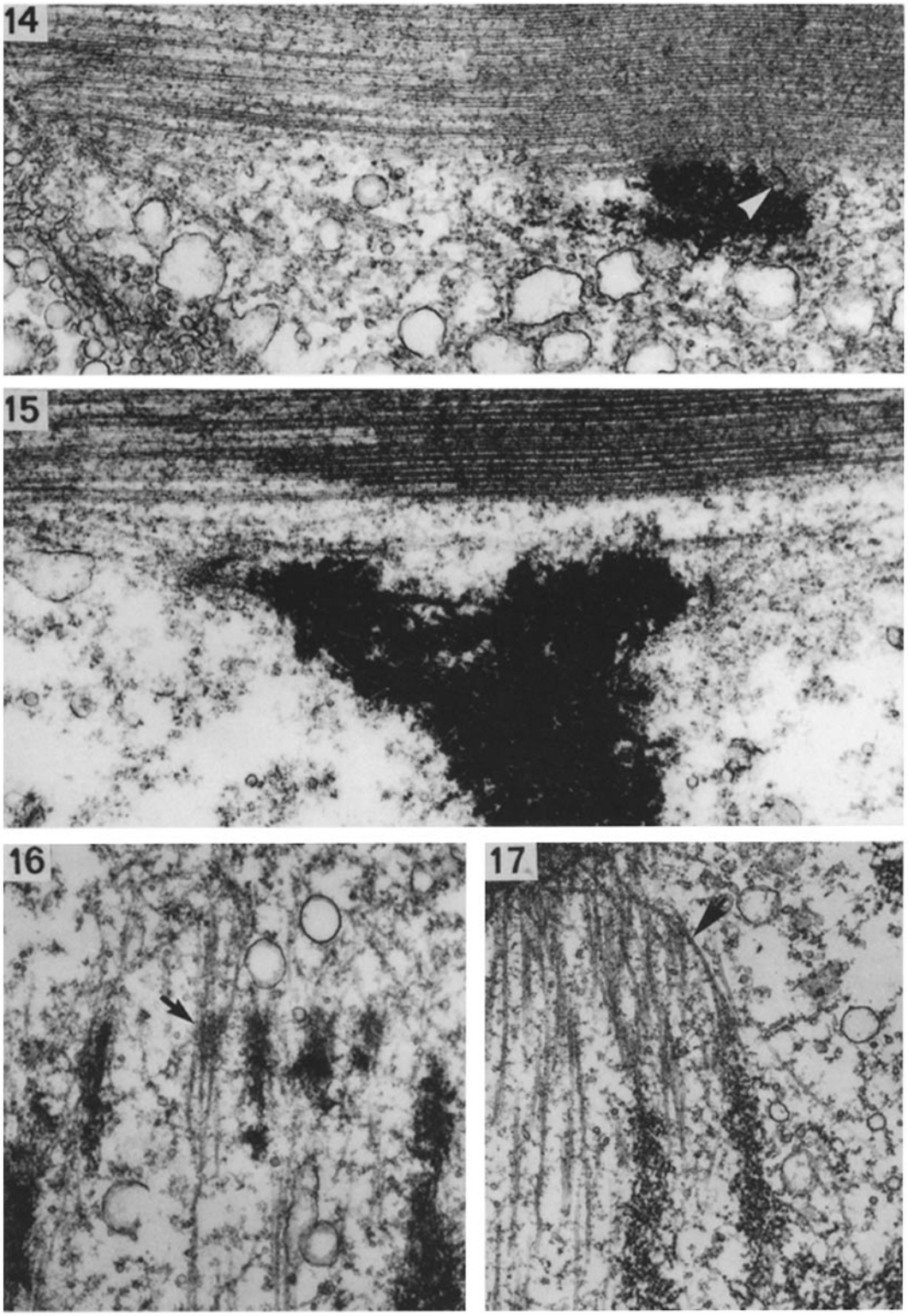
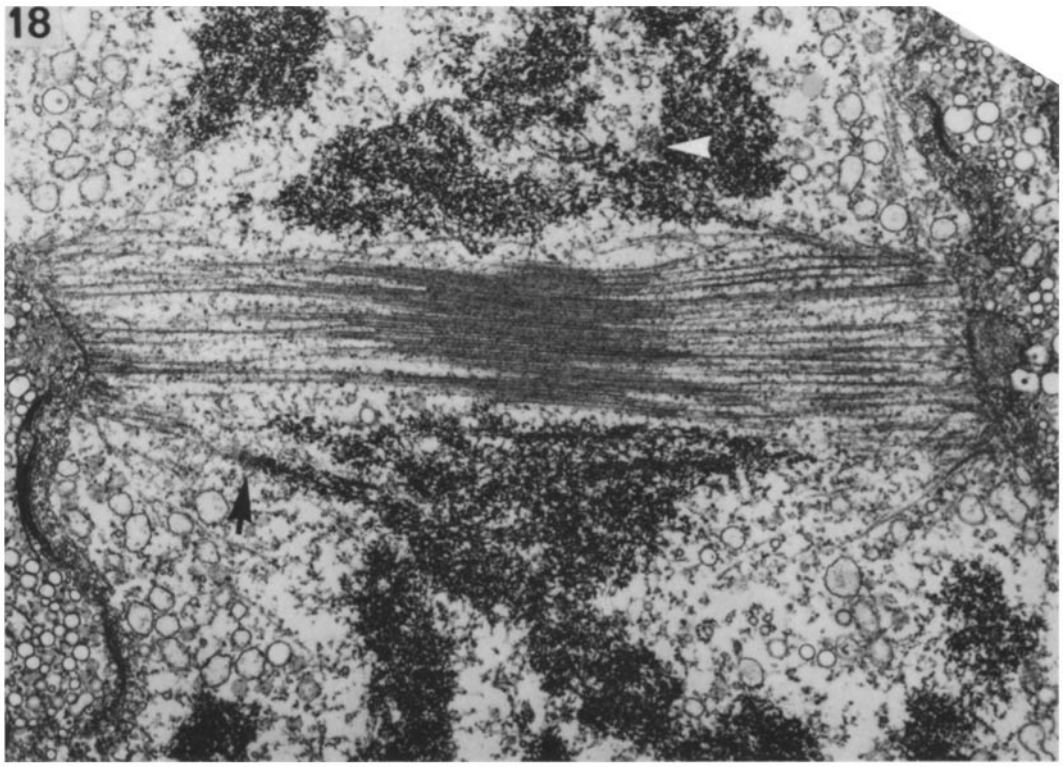


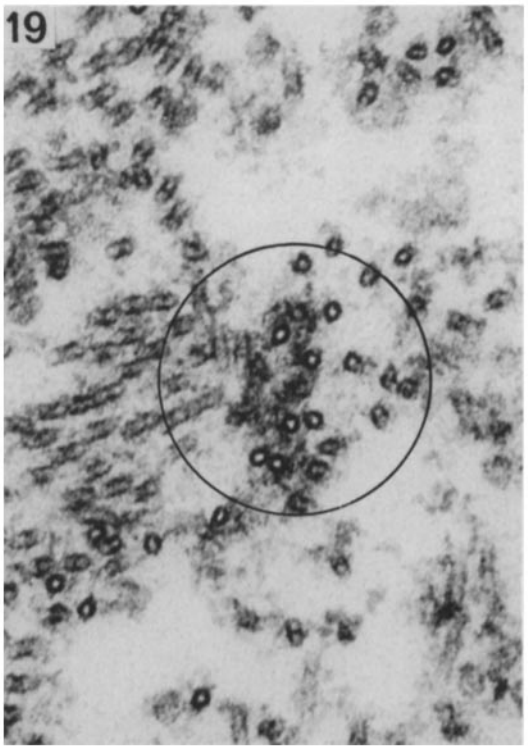
FIGURE 14 A kinetochore (arrow) of a chromosome at early prometaphase in *Hantzschia* is attached to the surface of the central spindle, possibly because of interaction between the kinetochore and these MTs. × 22,300.

FIGURE 15 This micrograph shows the first chromosome attaching to an early prometaphase spindle (cf with Fig. 2 *b*) in *Hantzschia*. The kinetochores, still recognizable, are beginning to stretch polewards and are associated with MTs. × 24,900.

FIGURE 16 This section grazes the tips of chromosomes in *Nitzschia*, which are already attached to a late prometaphase spindle. Densely staining material is associated with each kinetochore. × 19,900.

FIGURE 17 The MT marked with the arrow is conspicuously curved, possibly because of its attachment to a nearby chromosome in *Hantzschia*. × 22,200.





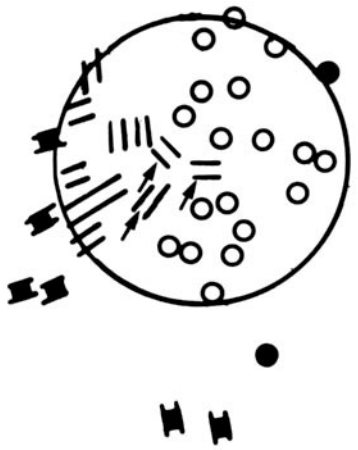
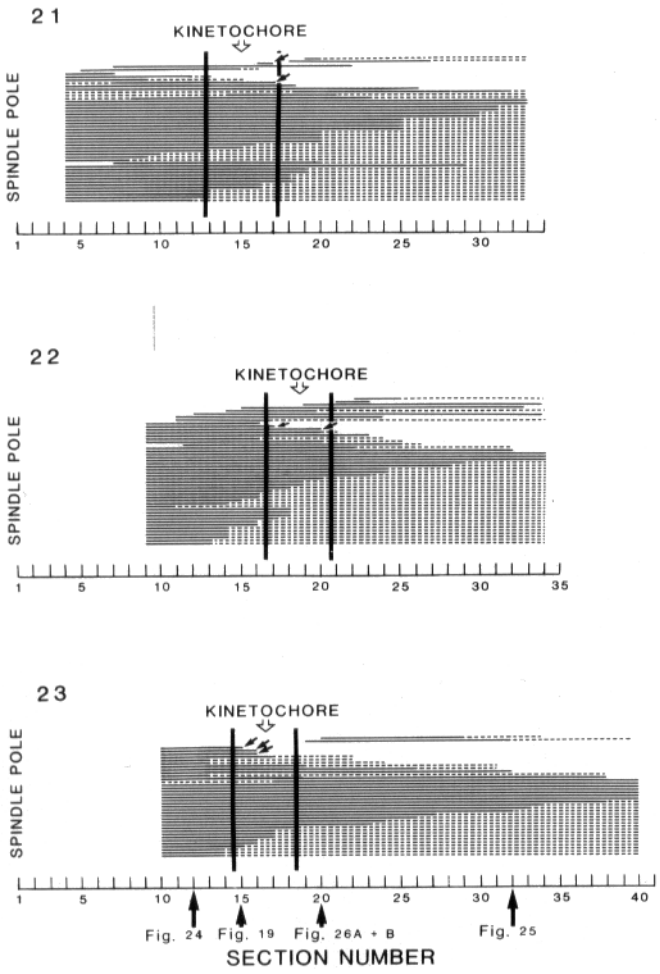


FIGURE 18 Mid-prometaphase in *Nitzschia*. At this stage, some chromosomes are fully attached to the spindle (black arrow), but other chromosomes still have no MTs associated with their kinetochores (white arrow). × 11,000.

FIGURES 19 and 20 Fig. 19 is a micrograph through the kinetochore region in *Hantzschia* (section 15) of the kinetochore fiber reconstructed in Fig. 23. Fig. 20 is a tracing of the MTs in Fig. 19; many of these MTs in the kinetochore region become skewed, especially those about to terminate (Fig. 20, the three MTs with arrows) in the next section. The skewed MTs are still trackable because there is very little background cytoplasm. The 28 MTs inside the large circle in Fig. 20 appear as solid lines in the reconstruction (section 15 in Fig. 23), and the eight shaded MTs are represented as dashed lines (see Materials and Methods). × 84,500.



FIGURES 21-23 These three diagrams are reconstructions of the MTs of three kinetochore fibers in *Hantzschia*. Each diagram is a reconstruction of the MTs associated with a single chromosome as shown in Fig. 1. Each line in these reconstructions represents a single MT; some lines are divided into a dashed portion and a solid portion, which denotes the spatial position of MTs (see Materials and Methods). Notes on reconstructions: arrows denote kMTs. The two MTs which terminate in section 31 in Fig. 23 were not tracked to their end point because they were too far displaced from the chromosome. MTs which are part of the central spindle (see Table I) are displayed at the bottom of each reconstruction. For example, the 13 central spindle MTs in Fig. 21 are displayed at the bottom of the reconstruction.

as follows: during spindle formation (43), each pole creates a set of MTs. A proportion of these MTs from each pole interact laterally to create a central spindle consisting of two interdigitated half-spindles. The remaining MTs (i.e., those not part of the central spindle) splay outwards from the poles throughout division. During prometaphase they invade the nucleoplasm, penetrate among the chromosomes, and create the structural basis for prometaphase chromosomal activity and subsequent attachment.

As the spindle enters the nucleus, chromosomes immediately start moving. This strongly localized movement is apparently caused by kinetochores moving along polar MTs. Usually, one kinetochore of a pair interacts with MTs first, leading to unstable linear oscillations, during which the chromosome may reorient. Two kinetochores of the same chromosome may interact with MTs from one pole, but soon they also reorient after unstable oscillations. When one kinetochore is moving thus, its accompanying sister kinetochore is able to encounter MTs from the opposite pole, whereupon it starts oscillating in that new direction. Thus, one kinetochore remains loosely attached to one pole until the other contacts MTs from the opposite pole, an elegantly simple method for ensuring that a chromosome will contact and then be able to attach to MTs from opposite poles. After bipolar contact has been established, both kinetochores stretch towards the opposite poles along the sets of MTs, and that chromosome thereafter maintains bipolar orientation and does not reorient. During these metakinetic movements, increasing numbers of MTs interact with a kinetochore until a discrete bundle is built up. During this process, individual MTs become bent and possibly broken because of their association with the kinetochore; some may thereby attach to and terminate at the kinetochore.

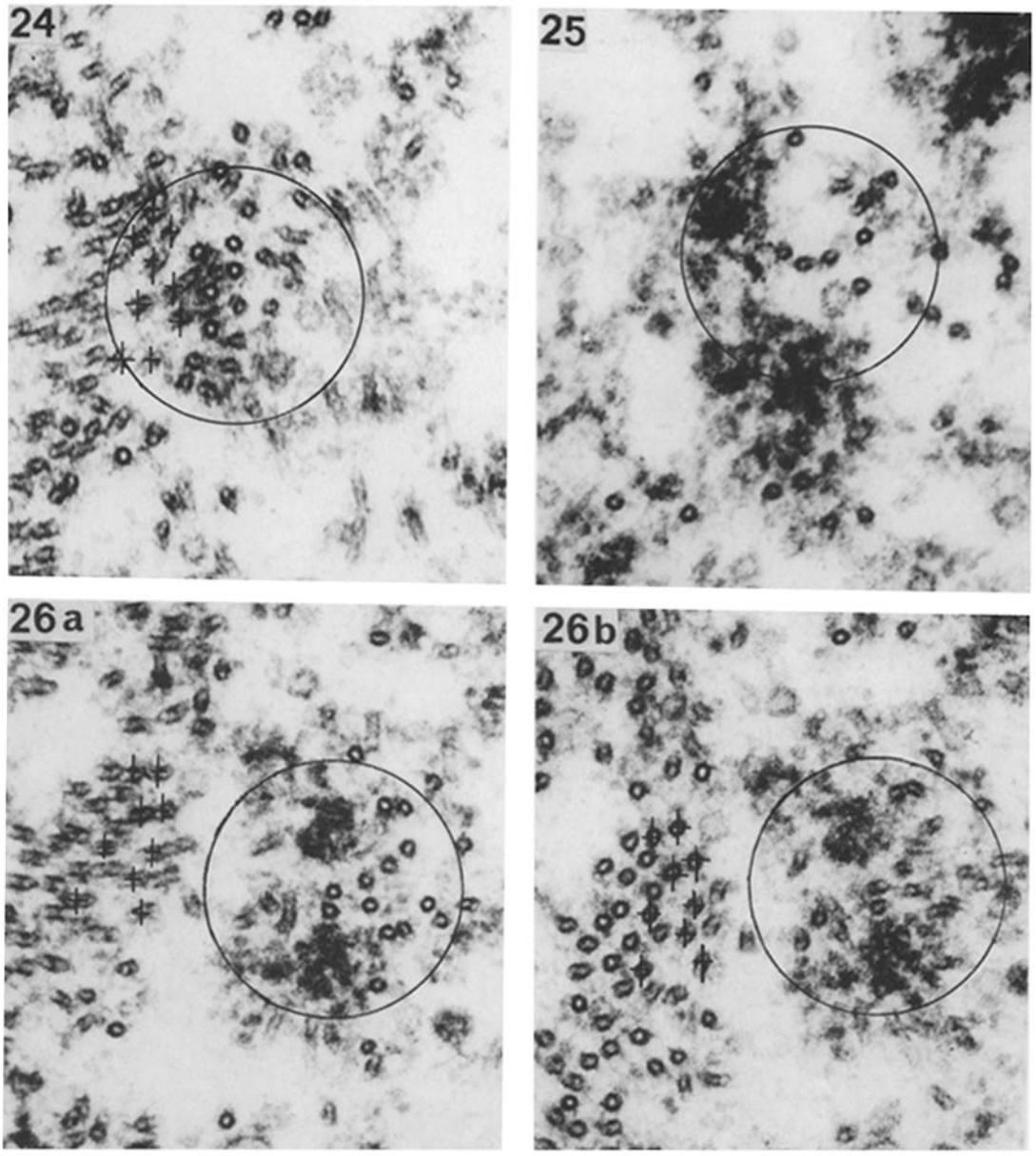
Thus, we postulate that metakinesis in these diatoms involves two distinct types of chromosome movements, during which kinetochores appear to be transported along preexisting polar MTs. First there are unstable oscillations of the chromosomes to one pole at a variable rate up to 2.5 μm/s; this is followed by the stretching of the sister kinetochores towards opposite poles at around 0.5 μm/s. Prometaphase oscillations are invariably strongly polewards directed, and the chromosomes often cluster unstably near either pole. It is possible that the intrinsic polarity of the MTs attached to the poles may be instrumental in the observed predominance of movements toward (instead of away from) the pole at early prometaphase.

Our interpretation is that tension (pull from opposite poles) induced by bipolar attachment, transforms the initial unstable, oscillatory motion into a strongly polewards-directed, stable bipolar orientation. This interpretation has been strongly influenced by the pioneering work of Nicklas and his colleagues (30, 33, 34, 14, 15) who demonstrated by direct experimental micromanipulation that tension on a chromosome confers upon it stability in the spindle. In particular, when a chromosome has both its kinetochores attached to one pole, they oscillate unstably until one connection is broken, whereupon that kinetochore attaches to the other pole to establish normal bipolar orientation. When a chromosome attached thus to one pole is restrained experimentally, the unstable oscillations immediately cease and the chromosome is now stable (33, 14). This experiment shows clearly that the kinetochores cannot intrinsically distinguish between which pole they are attached (34), and instead respond to tension that is normally induced between them. This confirms Dietz's (8) suggestion that bipolar orientation of chromosomes is stable, and that reorientation will occur repeatedly until this configuration is achieved.

TABLE I

Data from Reconstructed Kinetochore Fibers

Cell No.	No. of MTs that terminate at kinetochore	No. of MTs in reconstruction	No. of MTs part of central spindle	Diameter of tracking cylinder
				μm
1	1	32	7	0.402
2	1	32	8	0.378
3 (Fig. 21)	2	47	13	0.375
4 (Fig. 22)	2	47	12	0.430
5 (Fig. 23)	3	38	9	0.425



FIGURES 24–26 These are micrographs of sections 12, 32, and 20 respectively, from the kinetochore fiber reconstructed in Fig. 23. The large circle on each micrograph operationally defines the MTs to be included in the kinetochore fiber (see Materials and Methods). Figs. 26 *a* and *b* are two micrographs of the same section (i.e., section 20), but are photographed at a different tilt and rotation to maximize clarity of different MTs. Fig. 26 *a* shows the MTs which extend past the kinetochore toward the overlap, whereas Fig. 26 *b* shows MTs of the central spindle. Nine MTs are marked with crosses in Fig. 24; these same nine MTs are similarly marked in Figs. 26 *a* and *b*, but are more clearly viewed in Fig. 26 *b*. In Fig. 24 these nine MTs are within the circle (boundaries of the kinetochore fiber), but in Figs. 26 *a* and *b* they are part of the MTs that constitute the central spindle. × 84,500.

Metakinesis in Other Cell Types

The most widely accepted hypothesis to explain prometaphase orientation of chromosomes (27, 49) and indeed formerly endorsed by the second author (diagram 26 in reference 38) assumes that MTs arise concurrently from the two kinetochores on a chromosome; such MTs would be oriented at 180° to each other and are presumed to laterally interact with the parallel MTs from the poles and thereby orient the chromosomes. In spite of the widespread acceptance of this idea, we have yet to see one micrograph published which supports such a sequence of events (i.e., showing a bundle of MTs emanating from both kinetochores before the chromosome is oriented toward the poles). Often micrographs published of this reorientation stage show something quite different—a bundle of MTs from only one kinetochore, oriented to one pole (e.g., 3, 28, 49). Thus, the common explanation of prometaphase orientation is a theoretical concept since it has not been demonstrated that lateral interactions between kMTs and polar MTs can in fact orient chromosomes. Neither has the complexity of prometaphase oscillations been satisfactorily explained on this structural basis.

It may appear that the prometaphase chromosome activity of diatoms described here is unusual. However, we believe that there are important similarities between these metakinetic movements and those displayed by other cells. Ciné sequences and their descriptions (1, 48, 49) of mitosis in mammalian and

higher plant cells show some chromosomes moving at first irregularly polewards, often up to one pole before entering the metaphase configuration. Under favorable conditions, numerous such oscillations may be observed accompanied by "considerable stretching of kinetochores" (49; see also 29), but more frequently the oscillations are quickly damped. In *Barbulanympha*, kinetochores oscillate between the poles of the spindle on the surface of the intact nuclear envelope which undergoes pronounced deformation at the kinetochores (46, 17). The movements of centrophilic chromosomes at prometaphase in newt (28) are similar to those in diatoms. In *Haemanthus* (21) and spermatocytes of crickets (45), such movements are present but not so pronounced. McIntosh et al. (26) summarize numerous similar reports that metakinetic chromosomes "follow approximately circular arcs of different radii . . . defined by the elements of the polar spindle." This prometaphase activity is even more dramatic in abnormal unipolar spindles of newt, where chromosomes can oscillate up to 90 times, demonstrating extreme instability in the absence of bipolar attachment (A. J. Bajer, personal communication).

Rickards (45) has emphasized that oscillatory prophase and early prometaphase chromosome movement during meiosis in crickets is similar to the movement of particles in the aster. As the nuclear envelope disperses, these chromosome movements rapidly become localized at the kinetochore. The movements of particles, chromosomes and kinetochores are colcemid-sensitive. These prophase chromosome movements occur when there are apparently no MTs inside the nucleus (no electron microscopy of these cells is presented), suggesting that MTs from the aster influence such movements. We do not observe prophase chromosome movements in diatoms; however, recent work in preparation on the spindle of *Oedogonium* has revealed the existence of filaments in the nucleus at prophase which offer an explanation for these unusual chromosome movements.

In summary, oscillations and transport of small organelles (e.g., mitochondria) along MTs is a common feature of many nondividing eukaryotic cells, although molecular mechanisms to explain this movement are not yet known. Similar movement of organelles and particles within the spindle has been widely documented (2, 9, 44, present report). No one believes that such particles need to nucleate MTs to generate this movement; neither, in our opinion, do the kinetochores at this stage. We suggest that the transport properties associated with MTs may contribute to the unstable but vitally important prometaphase oscillations of chromosomes, and that the chromosomes (kinetochores) in certain cell types initially orient by moving along MTs already directed toward the poles.

Is the Kinetochore of Diatoms a Microtubule Organizing Center (MTOC)?

The diatoms offer advantages for analyzing structural aspects of mitosis; their spindle MTs are highly organized, and usually have one end terminating at the pole (57). In the absence of large numbers of confusing free MTs, the formation of the kinetochore fiber and the origin of its MTs can be followed. Let us now summarize the evidence that diatom kinetochores do not nucleate MTs:

(*a*) Reconstruction of several kinetochore fibers from transverse sections demonstrates that 96% of their MTs do not terminate at the kinetochore. This indicates these MTs were not nucleated at the kinetochore and implies that the kinetochore fiber is composed primarily of MTs from the poles. At least one MT of each fiber analyzed, terminates in the kinetochore region. Whether this single (occasionally two or three) kMT originated at the kinetochore or at the poles, cannot be determined.

(*b*) The first MTs associated with kinetochores during prometaphase are invariably revealed by serial longitudinal sections to be long, and to have one end attached to the pole. These MTs consistently run close to and extend past randomly oriented kinetochores in the early stages of prometaphase (Figs. 7–13); nucleation of MTs laterally by the kinetochore in such cells (Fig. 13) appears unlikely.

(*c*) The kinetochore fibers often do not develop simultaneously on a pair of kinetochores on the same chromosome. One kinetochore facing the pole very often has MTs associated with it, while its partner is bare, suggesting that the first has interacted with preexisting MTs (Figs. 9 and 10).

(*d*) MTs associated with the kinetochore are never observed facing any direction other than the pole. Intermediate stages of kinetochore fiber development (e.g. MTs extending from the kinetochore halfway to the pole) are not encountered.

(*e*) MTs attached to one pole may laterally associate with two different kinetochores on different chromosomes (Figs. 11 *a* and *b*). Nucleation of a MT from one kinetochore to another kinetochore appears unlikely (see comments in reference 25).

(*f*) The kinetochore appears to have an affinity for MTs as is demonstrated by its tendency to attach directly to the central spindle (Fig. 14), or else by conspicuously deforming MTs that pass nearby (Fig. 17).

(*g*) Time-lapse cinematography demonstrates repeatedly and unequivocally that the chromosomes make numerous fast oscillations back and forth to one pole, often up to it; it is not clear how this behavior could be effected by MT nucleation.

(*h*) At late prophase, MTs are not inside the nucleus. Within 1 min of the breakdown of the nuclear envelope, the first chromosomes are usually fully attached to the spindle. In Fig. 2, the first chromosome attaches in ~6 s after the beginning of generalized motion in the spindle which accompanies breakdown of the nuclear envelope. Thus, if kinetochores nucleate MTs, they must rapidly assemble these first kinetochore fibers (considerably faster than the MTs of the half spindle are assembled), while most of the other kinetochores are still bare of MTs.

(*i*) Bare kinetochores are ubiquitous at early prometaphase. Even by late metaphase, laggard chromosomes can be found (e.g., Fig. 18; also see plate IV*a*, in reference 40) which are unstretched and which have no MTs emanating from their kinetochores. Such laggard chromosomes are frequently seen in vivo to suddenly commence typical prometaphase movement, followed quickly by normal spindle attachment. If kinetochores nucleate MTs, such chromosomes would have to delay nucleating their kinetochore fiber until suddenly stimulated long after the rest have already attached; indeed, all chromosomes would have to commence kinetochore nucleation spontaneously, irregularly, and then be able to form the entire fiber very quickly.

In summary, we have analyzed over 50 cine sequences of prometaphase and many hundreds of prometaphase electron micrographs of various diatom genera (not just the two mentioned in this paper). We are convinced that the conventional view of kinetochore fiber formation cannot apply to diatoms (see first part of Discussion).

Is the Kinetochore an MTOC in Other Cell Types?

The recognition of the kinetochore by Metzner in 1894 (reviewed in reference 53) and of a fiber connecting it to the pole marked the beginning of an eighty-year debate on the origin of the chromosomal spindle fiber. By 1953 (53), there was evidence supporting two main theories. One hypothesis was that the fiber grew from the pole and connected to the kinetochore, the other that it originated at the kinetochore and grew to the pole. It seems that the former possibility has been almost entirely abandoned by most recent investigators; although a few (31, 36, 20, 23) still consider it an unresolved issue, virtually no one endorses this view. In the following discussion, we deliberately favor the interpretation that kinetochores during early prometaphase can bind to MTs, in order to emphasize that much of the evidence discussed can be interpreted in more than one way. Any recent review on mitosis presents the arguments which favor MT nucleation by the kinetochore.

The most convincing demonstration that MTs are nucleated by kinetochores, comes from in vitro studies (25, 54, 55, 12, 37, 6). These experiments, while often persuasive, are not without problems (see Discussion in reference 39); for example the kinetochore may renucleate MTs already bound to the kinetochore, but not removed by colcemid treatment, or the kinetochores may be "seeded" by binding to small MT fragments collected during chromosome preparation. All of these experiments use colcemid-treated chromosomes and it is not known whether these resemble in vivo prometaphase chromosomes. Kinetochores are far less vigorous in nucleating MTs when compared with the aster (54, 6), and Weisenberg and Rosenfeld (58) do not report any nucleation by kinetochores under conditions where asters are quickly formed. Thus, even if kinetochores nucleate new MTs in vitro, their nucleation properties are markedly different from those of the aster.

The light microscope does not reveal whether the kinetochore nucleates MTs in vivo, although the kinetochore obviously does organize the kinetochore fiber (16, 5). Electron microscope evidence from a wide variety of cells is usually interpreted to conform with the conclusion that MTs are nucleated by the kinetochore. Prometaphase is a poorly documented stage of mitosis, presumably because the spindle at this stage is disorganized and its structure difficult to evaluate, and open to different interpretations. In HeLa cells (35) and higher plants (50–52), it is suggested that the first MTs attached to the kinetochore could have come from the poles. Roos (47–49) and Bajer and Mole-Bajer (3) considered this possibility but they and most subsequent investigators have concluded that most or all kinetochore MTs arise from kinetochores. Some of their micrographs, and those of *Trichonympha* (18), do suggest that kinetochores may interact laterally with MTs as in diatoms (e.g., Figs. 17*b* and 18 in reference 3; Fig. 8 in reference 48; Fig. 6 in reference 49). Roos has shown that when chromosomes move toward one pole at early prometaphase, MTs are attached to the poleward-facing kinetochore whereas the other is bare (49). This has also been strikingly illustrated with the centrophilic chromosomes of newt (28). We have not encountered convincing reasons why the second kinetochore of such pairs, if it is an MTOC, should be bare. Lack of tubulin or the existence of hypothetical tubulin gradients (49) does not adequately explain this observation because the other kinetochores in such spindles are not affected and the spindle is permeated by MTs. A reinvestigation of prometaphase in *Oedogonium* (in preparation) indicates that the first MTs associated with kinetochores may be preexisting MTs from the poles. Two recent reports (20, 36) also suggest that kinetochores could function by attaching to preexisting MTs, but do not rule out nucleation. When chromosomes are removed from the spindle by micromanipulation and relocated near the edge of the cell, they move back towards the spindle (32); MTs attached to spindle pole run up to and laterally past the kinetochores of such chromosomes. The origin of these MTs is not addressed by the authors, but it is difficult to envisage the kinetochores nucleating MTs which extend laterally toward and away from the one pole.

In summary, the electron microscope evidence is quite equivocal in this issue, in spite of the consensus of opinion in its interpretation. It is difficult to ascertain the origin of kMTs because kinetochore nucleation is presumed to occur during prometaphase, precisely when MTs from the poles invade the nucleus (MTs are not observed in the prophase spindle although kinetochores are present [7, 10, 3]).

Function of Kinetochores in Conventional Spindles

We have been forced to reexamine the function of the kinetochore during mitosis, because the kinetochore fiber of the diatoms is not primarily composed of MTs nucleated by the kinetochore. Furthermore, the single kMT of each chromosome in these cells, even if nucleated by the kinetochore, could be unimportant during prometaphase because the kinetochore seems to be oriented and attached to the spindle primarily by MTs from the poles. At present, there is conflicting evidence generally as to whether kinetochores function in vivo entirely by nucleating MTs, or attaching to MTs and MT fragments which subsequently elongate. Neither of these possibilities alone presently appears to explain all of the observations on kinetochore fiber formation from a variety of cells. For example, attachment to preexisting MTs does not suggest why and how kMTs terminate at the kinetochores; how the number of kMTs increase after the chromosome is attached to the spindle; how certain fungi can attach to just one kMT per chromosome and never two. Alternatively, nucleation does not explain the diatom kinetochore fiber; the early prometaphase observations in numerous cell types which indicate that the first MTs that attach to kinetochores are from the poles; how kMTs grow precisely from the kinetochore to the pole and simultaneously orient the chromosome; prometaphase oscillations of chromosomes in vivo; or how the kinetochore can switch on nucleation on different chromosomes at different times (but usually just before attachment to the spindle and never during late prophase).

The main difference between metaphase in diatoms and that in other cells is that the kinetochores of diatoms remain stretched to the pole until anaphase commences. It seems likely that anaphase poleward movement is caused by the same mechanism as prometaphase poleward movements. Because these prometaphase movements occur without apparent sliding, zipping, or depolymerization of MTs (e.g., the chromosome in Fig. 13 has apparently moved close to the pole), we suggest that none of these mechanisms produce the force that moves chromosomes at anaphase in diatoms. Prometaphase stretching of chromatids is common in other cells (19, 21, 29, 48), but almost always the chromatids relax by metaphase. Perhaps, therefore, nucleation of MTs at the kinetochore (if it occurs) coincides with the relaxation of prometaphase tension, while

attachment to the poles is being maintained. Even if the kinetochore does nucleate MTs, this activity may be a relatively unimportant aspect of kinetochore function in some cell types, and perhaps has served over recent years to divert attention from other interesting possibilities.

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Note Added in Proof: A color film of mitosis in *Hantzschia* (including the sequences that gave Figs. 2-6) is available from J. Pickett-Heaps.

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