

Dynamics of Spindle Microtubule Organization: Kinetochore Fiber Microtubules of Plant Endosperm

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ABSTRACT Organization of kinetochore fiber microtubules (MTs) throughout mitosis in the endosperm of *Haemanthus katherinae* Bak. has been analysed using serial section reconstruction from electron micrographs. Accurate and complete studies have required careful analysis of individual MTs in precisely oriented serial sections through many (45) preselected cells. Kinetochore MTs (kMTs) and non-kinetochore MTs (nkMTs) intermingle within the fiber throughout division, undergoing characteristic, time-dependent, organizational changes. The number of kMTs increases progressively throughout the kinetochore during prometaphase-metaphase. Prometaphase chromosomes which were probably moving toward the pole at the time of fixation have unequally developed kinetochores associated with many nkMTs. The greatest numbers of kMTs (74–109/kinetochore), kinetochore cross-sectional area, and kMT central density all occur at metaphase. Throughout anaphase and telophase there is a decrease in the number of kMTs and, in the kinetochore cross-sectional area, an increased obliquity of kMTs and increased numbers of short MTs near the kinetochore. Delayed kinetochores possess more kMTs than do kinetochores near the poles, but fewer kMTs than chromosomes which have moved equivalent distances in other cells. The frequency of C-shaped proximal MT terminations within kinetochores is highest at early prometaphase and midtelophase, falling to zero at midanaphase.

Therefore, in *Haemanthus*, MTs are probably lost from the periphery of the kinetochore during anaphase in a manner which is related to both time and position of the chromosome along the spindle axis. The complex, time-dependent organization of MTs in the kinetochore region strongly suggests that chromosome movement is accompanied by continual MT rearrangement and/or assembly/disassembly.

Several current models for mitosis are based on the premise that spindle microtubules (MTs) are essential in the moving of chromosomes, producing or transmitting the motive force either directly—via assembly-disassembly and/or MT-MT interactions—or in conjunction with additional spindle components, such as actinlike filaments (see reviews in references 7, 22, 40, 51, 59). Therefore, a knowledge of the arrangement of individual MTs and, more importantly, of how their distribution normally varies throughout division and among various organisms, is central to understanding the mechanisms in mitotic movements.

We have considerable information derived from recent detailed serial-section analyses of the organization of MTs in the comparatively small and well-ordered spindles of certain fungi (reviewed in 26) and algae (39, 45, 53, 60, 61, 62). Nevertheless, our knowledge of the exact three-dimensional arrangement of individual spindle MTs throughout division in higher orga-

nisms is only fragmentary, mainly because of the technical problems in analysis of the much larger spindles of higher plants and animals, which at metaphase may consist of as many as 1,500–2,400 MTs in mammalian half-spindles (rat kangaroo PtK, 10, 42; HeLa, 44), 1,500–2,200 MTs in an insect meiotic half-spindle (crane fly spermatocyte, 20, 35), and 4,000 MTs in a higher plant half-spindle (*Haemanthus* endosperm, 30). Therefore, most studies of MT organization in such spindles have relied upon counts of MTs from selected thin sections cut transverse to the spindle axis (10, 17, 19, 20, 35, 42, 44) and upon descriptions of MT organization from thin sections (see reviews 7, 22, 40). Nevertheless, the complex three-dimensional organization of the mitotic spindles of higher organisms means that incomplete sampling of their morphology may lead to incorrect interpretations of their precise organization and function.

Recent graphic reconstructions of MTs from serial longitu-

dinal thin sections through a limited number of meiotic spindles of an insect (21, 23) and a plant (13) have shown that this method can provide useful information on spindle organization in higher plant and animal cells. I initiated these studies to provide extensive and accurate data on many mitotic spindles of a higher organism. The cells of *Haemanthus endosperm* were chosen for analysis not only because of their large size and clarity, which allowed precise orientation for sectioning and accurate localization of specific chromosomes, but also because additional information could be correlated with that obtained in studies with the light and electron microscopes (3, 4, 6, 7, 8, 18, 30, 31, 32, 36).

A previous paper (30) described the general arrangement of all spindle MTs in mitotic plant endosperm cells throughout division as determined by analysis of serial sections. Because the chromosomes are moved during division by forces acting on them at their kinetochore region (see reviews in references 1, 6, 11, 51), my study has attempted to obtain a more detailed picture of the continually changing arrangement, number, and length of MTs associated with this region. With serial thin-section reconstruction of individual MTs in spindles fixed at different stages of division and subsequently sectioned either parallel or perpendicular to the long axis of the spindle, I have been able to obtain a more accurate and detailed picture of the time-dependent changes in the organization of the kinetochore fiber and of its component MTs throughout division than can be obtained from either random thin sections or thick-section high-voltage electron microscopy. The inclusion of many cells (i.e., 45) also allowed examination of the organizational variability in different spindles at equivalent stages of division. Preliminary accounts of this study have appeared elsewhere (31, 32).

MATERIALS AND METHODS

Terminology

Kinetochore microtubules (kMTs) terminate at the kinetochore of a chromosome. Non-kinetochore microtubules (nkMTs) do not terminate at the kinetochore (30, 31, 32). This classification, also adopted by Fuge (21, 22, 23), is used in this paper. Therefore, "continuous," "free," or "interpolar" MTs lying between the poles, "chromosomal" MTs embedded in the chromatin in regions other than the kinetochore, and MTs which terminate outside the kinetochore are all referred to here as nkMTs. Non-kinetochore bundles are composed exclusively of MTs which do not terminate at a kinetochore. A kinetochore fiber (a term derived from light microscopy) consists of the kMTs attached to a single kinetochore, plus those nkMTs which are closely associated with them. The kinetochore fiber may be considered to connect the chromosome and the pole (see reference 60). It becomes progressively less distinct toward the pole because many nkMTs join the fiber along its length and intermingle with its constituent MTs (Fig. 4*a* and *d*; references 4 and 8). Nevertheless, kinetochore fibers are often distinguishable along their entire length in the light microscope (24), in longitudinal thin sections (Fig. 4*a*), and in composite tracings of serial thin sections (30).

Electron Microscopy

Endosperm cell preparations of *Haemanthus katherinae* Bak. (49) containing viable, dividing cells were fixed at 22°C for 15 min in 3% glutaraldehyde buffered to pH 6.9 with 0.1 M phosphate, postfixed for 15 min in 1% osmium tetroxide in the same buffer, dehydrated in graded ethanols, and flat-embedded in Epon-Araldite (50). 45 cells in stages from late prophase to late telophase were photographed as whole mounts with the light microscope and then serially thin-sectioned (70–90 nm thick) either parallel or perpendicular to the long axis of the spindle (hereafter referred to as the spindle axis) on a Porter-Blum MT2-B or LKB III ultramicrotome with a diamond knife. Ribbons of sections were placed with the aid of a "third hand" section lifter (58) on single-slot or 100-mesh parallel bar grids which had been coated with Formvar and stabilized with a thin layer of carbon. Sections were stained with 1% aqueous uranyl acetate and lead citrate (55). Photographs of serial sections through selected kinetochores were taken with a Philips EM 300 electron microscope at initial magnifications of

9,000 on 35-mm film or 35,000 on 3 × 4 in. sheet film. The negatives were printed with final magnifications of 72,000–90,000 on Ilford Ilfospeed paper or sheet film. Extreme care was taken not to alter the settings of the enlarger between prints so that all prints from an entire group of serial sections would be of exactly the same magnification, a critical prerequisite for accurately tracing MTs through serial sections.

To ensure as much uniformity as possible between spindles at different stages of mitosis, the 28 cells presented in Table II were taken from only two separate cell culture preparations which had been made at the same time and held at 22°C for 1 h before fixing. The information was supplemented and verified with seventeen cells from six other preparations.

Tracings from Sections Parallel with the Spindle Axis

Graphic reconstructions from sections parallel with the spindle axis (hereafter referred to as longitudinal sections) were made of 23 kinetochores from 6 different cells. Each kinetochore region analysed included all MTs immediately behind and surrounding the kinetochore, and up to 7 μm distal to the kinetochore, as well as any associated MTs from adjacent kinetochores (see Figs. 4*d*, 6, and 7*c*). The region analysed therefore included approximately one-fourth to one-half of the kinetochore fiber at metaphase (Fig. 4*b*). With sections approximately 70 nm thick, 10–24 serial longitudinal sections through each kinetochore region were required to include the entire region of interest in the reconstruction. Only complete sets of serial sections were used for longitudinal tracings. All MTs were traced from each photographic print onto a transparent plastic sheet, using a different colored pencil for each superimposed section in the sequence to produce a two-dimensional representation of the original three-dimensional organization. The MTs from two to ten sections, depending on the density of MTs, were traced onto a single plastic sheet. The tracing of one section was placed in exact superposition over the micrograph of the adjacent section in the sequence, and the point at which each MT left the plane of section on one photograph could be picked up on the photograph of the immediately adjacent section, allowing accurate alignment of successive sections. In this way, terminal ends of MTs could be distinguished from MTs leaving the plane of section. With this method all MTs in the kinetochore region, even those lying oblique to the plane of section, were traced.

In addition, the plastic sheets could be stacked, one on top of another, to visualize a graphic two-dimensional representation of the kinetochore. To visualize a three-dimensional representation of the MT arrangement in the kinetochore region, the tracings of individual serial sections were inserted between 6.3-mm thick sheets of transparent plastic (corresponding to a section thickness of 70 nm at a print magnification of 90,000), vertically aligned with respect to each other, and the entire stack was viewed through a light box (as described in references 13 and 18). In preparing the final graphic representations illustrated in this paper, I omitted some nkMTs of very dense arrays for the sake of clarity (Figs. 1*c*, 4*d*, and 5).

Tracking MTs through Sections Perpendicular to the Spindle Axis

The methods used to track MTs in sections perpendicular to the spindle axis (hereafter referred to as transverse sections) were similar to those described previously (25, 62). The MTs in 9 to 17 serial sections through the kinetochore and immediately distal to it were tracked in photographic prints, either by following them individually from one section to the next or by tracing all MTs onto transparent plastic sheets, numbering them, and noting their position in tracings of consecutive sections. Therefore, the position (or absence) of an MT in any one section was accurately determined by analysing the position occupied by each MT in the preceding and following sections. Each kMT was followed from its point of origin in the kinetochore to a point 1–1.5 μm distal to the kinetochore.

Counts of Kinetochore Microtubules

The number of kMTs was determined for each of 366 kinetochores from 34 cells in stages of division ranging from late prophase to late telophase. Whenever possible, at least five kinetochores from each cell were included. The data presented in Table II and Fig. 9 were derived from only those 201 kinetochores for which complete sets of serial sections through the kinetochores were available. Of the 201 kinetochores, 154 counts were derived from transverse sections and the remaining 47 from longitudinal sections. Kinetochores were selected at random. It was noted that those chosen included some from each half-spindle and from the center and the periphery of the spindle in metaphase, anaphase and telophase cells, and from throughout prometaphase cells.

The kMTs were identified accurately by following them through serial sections

as described above. MTs which terminated within the granular substance of the ball-shaped kinetochore region (see references 3, 6, and 7 for a discussion of kinetochore structure in *Haemanthus*) were classified as kMTs. Those which did not terminate within the kinetochore were, by definition, classified as nkMTs (also see Terminology).

Calculations of Cross-sectional Area of Kinetochore Microtubule Attachment

The cross-sectional area of kMT attachment was measured on photographic prints of the transversely sectioned kinetochores. Particular care was taken in selecting levels of section through different kinetochores, especially critical when comparing the prometaphase and metaphase kinetochores (in which the majority of MTs lie in parallel array) with kinetochores of anaphase and telophase (in which the peripheral kMTs are splaying out from the kinetochore). The level of section chosen was that nearest the equatorial region of the spindle but still containing all of the identified peripheral kMTs (as in Fig. 5e). A continuous curved line was drawn immediately outside the most peripheral kMTs. The resulting outlines were close to an ellipse in flattened cells, becoming almost circular in unflattened cells. The enclosed area was measured with a digitizing tablet (Hipad, Houston Instruments Div., Bausch & Lomb, Inc., Austin, Texas) coupled to a PDP-11 computer. The results were checked by treating each area as an ellipse, measuring major and minor axes (a and b) and calculating the area by the formula $A = 0.25(ab)\pi/m^2$, using the linear magnification (m) of the micrograph. The results agreed within the measuring error, those determined with the digitizing tablet being 0.03–1.5% less than when the kinetochore was treated as a true ellipse. The areas listed in Table II and Fig. 9 are those determined with the digitizing tablet.

Calculations of Microtubule Density

MTs were counted from photographic prints of kinetochores in a transverse section taken from the most proximal level at which all central MTs were present in the section (as in Fig. 5f). MTs were scored if at least half of the MT diameter lay within a circle with an area of $0.1 \mu\text{m}^2$ ($0.05 \mu\text{m}^2$ for the telophase kinetochores) whose center was the intersection of the major and minor axes of the kinetochore. The radius of each circle was determined by the formula, $r = m \times 10^{-4}(\sqrt{0.1/\pi})$ cm, using the linear magnification (m) of the micrograph.

The density of MTs within nonkinetochore bundles adjacent to the kinetochore was determined as described above for kMT density, using the same level of section. MTs were counted if they lay within a circle with an area of $0.1 \mu\text{m}^2$ at the center of the bundle.

RESULTS

Development of Spindle Microtubules in Early Stages of Division

My findings confirmed previous descriptions of the formation of the *Haemanthus* spindle during prophase and very early prometaphase (3, 6, 7, 36), showing that although kinetochore

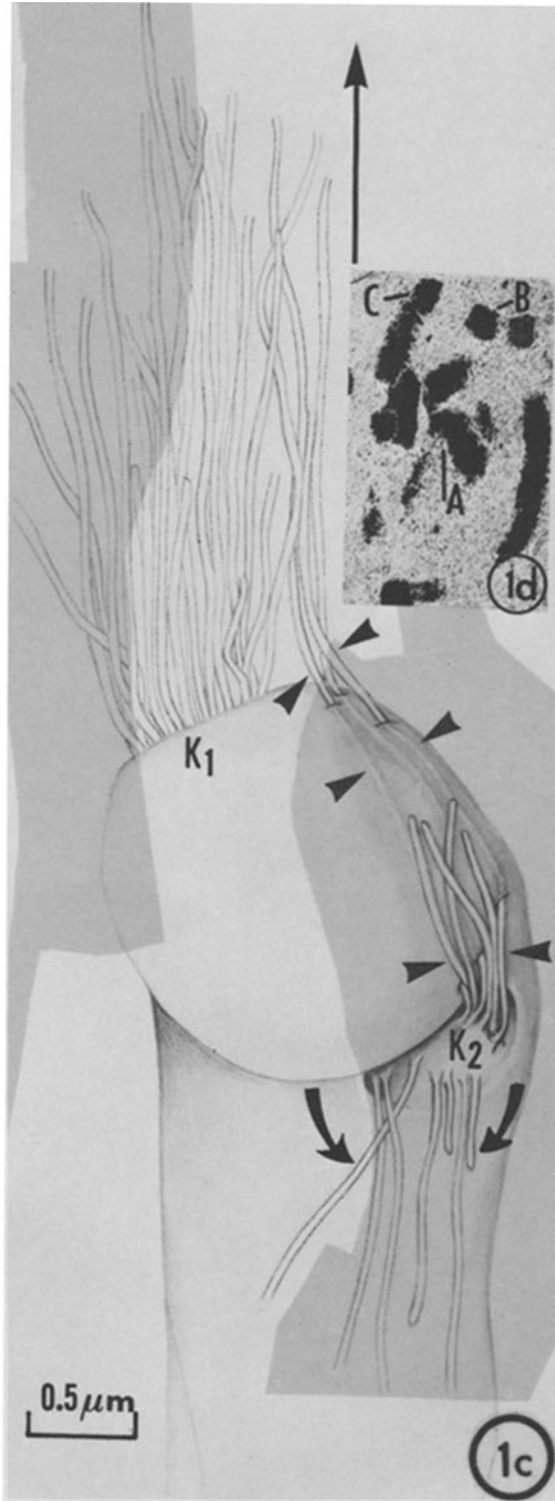
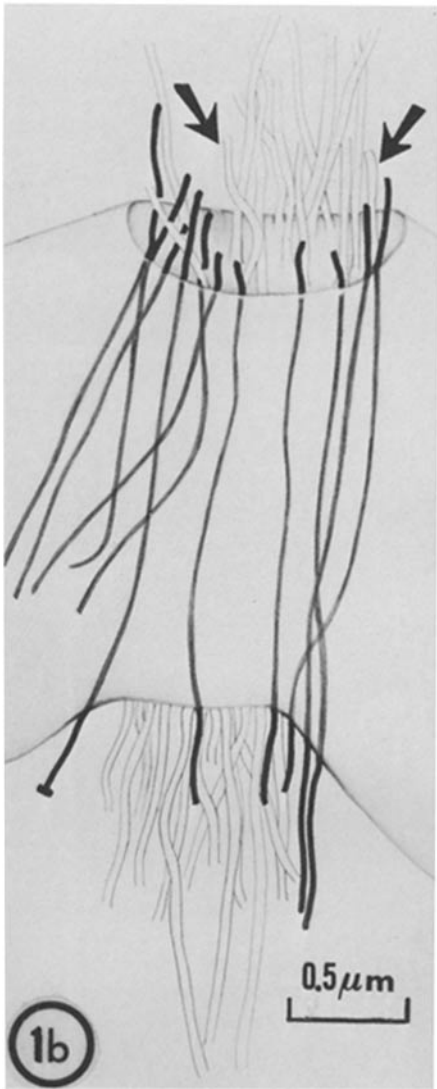
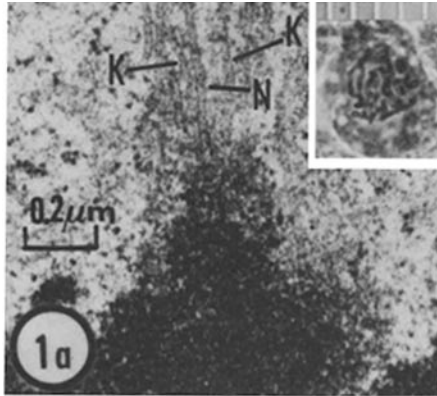
regions of chromosomes can be distinguished morphologically in very late prophase, no MTs are attached to them at that time. This study also confirmed that a clear zone immediately surrounding the nuclear envelope contains a network of MTs which progressively become aligned in parallel array as the nucleus assumes an elongate shape during late prophase, and that immediately after the nuclear envelope breaks, marking the onset of prometaphase, dense bundles and sheets of MTs penetrate from the clear zone into the nuclear area. Throughout prometaphase, some of the MTs become progressively more ordered into bundles lying parallel with the future long axis of the spindle.

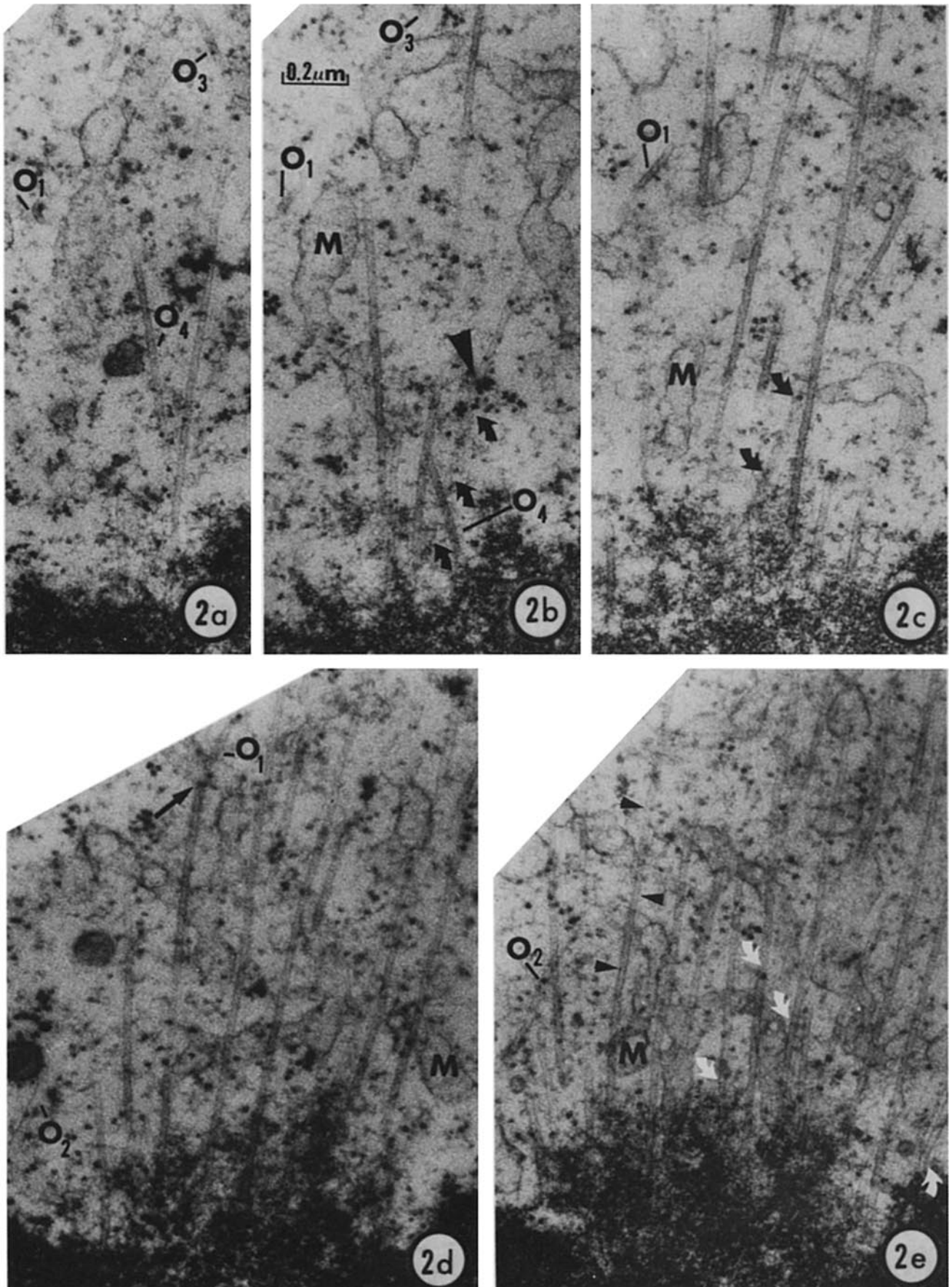
Organization of Kinetochore Fibers during Early Prometaphase

In the earliest prometaphase spindles studied (with chromosome arrangements corresponding to five to ten min after breakage of the nuclear envelope), all chromosomes examined possessed a well-developed kinetochore with the attached kMTs lying in almost parallel array (Fig. 1a and b). The shortest measured kMT was $1.5 \mu\text{m}$, but most kMTs were much longer. At this stage, one or two non-kinetochore bundles consisting of 8–10 MTs were closely associated with each kinetochore throughout the cell. In 60% of the kinetochores, these bundles passed through the chromatin in the same plane as the kinetochore and intermingled with the kMTs immediately distal to the kinetochore (as in Fig. 1b); in the remaining kinetochores, they passed through the chromatin to one or both sides of the kinetochore and joined the kinetochore fiber 0.3 – $1.5 \mu\text{m}$ distal to the kinetochore (not illustrated).

ANISOMETRIC CHROMOSOMES: In early prometaphase cells, similar numbers (i.e., 12–14) of kMTs were associated with each pair of sister kinetochores of most chromosomes (Fig. 1b). A striking exception was seen in those chromosomes which previously have been described as being associated with a single pole (misorientation, syntelic, or monopolar orientation; references 6, 7, 38, 57). Graphic reconstruction of serial sections through four chromosomes of this type, two close to the future metaphase plate and two close to a pole, showed clearly that the sister kinetochores of these chromosomes were organized very differently (Fig. 1c and d). Insofar as one kinetochore was more developed than the other, I have chosen to call these chromosomes “anisometric.” One kinetochore

FIGURE 1 Very early prometaphase, 5–10 min after breakage of the nuclear envelope. (a) Longitudinal section through a kinetochore of cell 1/72CC. kMTs (K) are embedded within the kinetochore. One nkMT (N) is clearly visible within the kinetochore fiber. In adjacent sections it was found to extend into the chromatin and intermingle also with the MTs of the sister kinetochore. $\times 51,000$. *Insert:* phase-contrast micrograph of the same cell in plastic; $10\text{-}\mu\text{m}$ intervals on scale. (b) Redrawn composite tracing from 15 serial thin sections through chromosome B in d. Fourteen MTs (shown in white) are attached to each of the sister kinetochores. nkMTs (shown in black) pass through the chromatin and intermingle with the kMTs of both kinetochores. A few MTs (arrows) were traceable to their ends distal to the kinetochore; all of the others are longer than indicated in the drawing. $\times 33,000$. (c) Redrawn composite tracing of kMTs in 24 sections through the kinetochore region of anisometric chromosome C shown in d. Long arrow indicates the direction of the pole toward which the chromosome is oriented. MTs which were traced to their termination are indicated by closed ends. The others are probably longer than indicated. Twenty-nine MTs are attached to the major kinetochore (K_1) facing the pole. Its sister, the minor kinetochore (K_2), has 14 MTs attached: one-half (between the arrowheads) are oriented toward the K_1 fiber, four of which are short, whereas the other three pass through the chromatin and intermingle with MTs of the K_1 fiber. The other seven MTs of the minor kinetochore (between the curved arrows) run toward the opposite pole. For the sake of clarity, nkMTs were not drawn. However, the grey-toned bands indicate the position of two large bundles of nkMTs associated with K_1 and K_2 . $\times 30,000$. (d) Thin section through cell 1/72CC showing the two chromosomes depicted in b (B) and c (C). The chromosome at A, cut through its surface in this section, also showed unipolar orientation and possessed an arrangement and number of kMTs similar to that of chromosome C (24 MTs attached to one kinetochore, 14 to the other). $\times 1,600$.





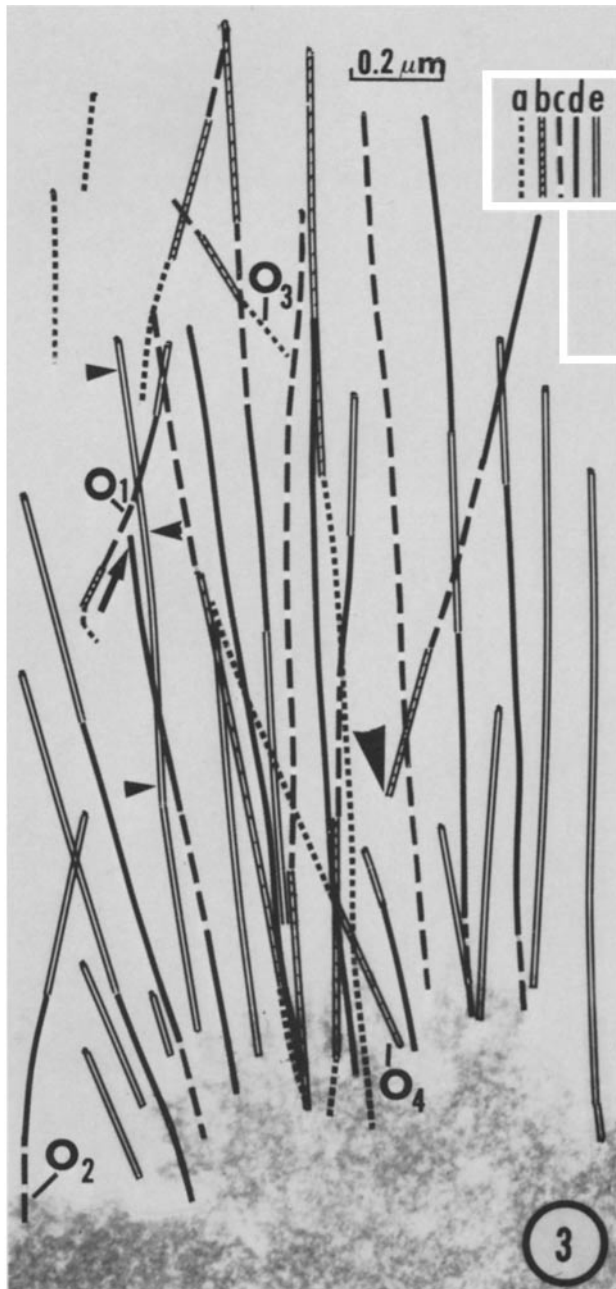


FIGURE 3 Late prometaphase-metaphase. Composite tracing of MTs from the five sections shown in Fig. 2. The legend labeled a–e designates the patterned lines used to depict MTs appearing in the sections shown in Fig. 2 a–e, respectively. For the sake of clarity, the MTs labeled with white arrows in Fig. 2 e have been omitted from the tracing. The outline of the kinetochore from Fig. 2 e is shown. Some oblique MTs (*O*) are present. One (*O*₂) terminates within the chromatin to the side of the kinetochore. One kMT (arrow) ends

(hereafter referred to as the major kinetochore—*K*₁ in Fig. 1 c) possessed a greater number of kMTs (i.e., 24, 25, 27, and 29) than the majority of kinetochores in the same cell. Its sister kinetochore (hereafter referred to as the minor kinetochore—*K*₂ in Fig. 1 c) possessed kMT numbers (i.e., 14) similar to that of most other kinetochores in early prometaphase cells. The minor kinetochore possessed connections to both poles. Approximately one-half of the MTs were retroflected and embedded in the chromatin; some of them were very long and intermingled with the kMTs of the major kinetochore. The remaining MTs attached to the minor kinetochore were very short, some being as short as 0.1 μm , and ran toward the opposite pole.

A consistent feature of anisometric chromosomes in prometaphase was the close association, with both kinetochores, of unusually large non-kinetochore bundles (position indicated by grey-toned bands in Fig. 1 c). Such bundles, containing 20–40 MTs, lay close to one side of the major kinetochore, intermingling with the peripheral kMTs immediately distal to the kinetochore. Another bundle of the same size passed directly through the region of the minor kinetochore, its constituent MTs intermingling with the kMTs.

Organization of Kinetochore Fibers during Late Prometaphase and Metaphase

In cells of progressively later stages of prometaphase the spindle MTs formed more discrete bundles, resulting in the definitive arrangement characteristic of late prometaphase and metaphase spindles (Figs. 2–5). In longitudinal thin sections of a cell in metaphase the kMTs tended to lie in almost parallel array or diverged only slightly, with a few kMTs lying oblique to the spindle axis (Figs. 2, 3, and 4 c and d). Graphic reconstructions of serial longitudinal sections revealed that large bundles of long nkMTs lay to one or both sides of the sister kinetochores of each chromosome, with some of their constituent MTs passing through the chromatin and intermingling with the kMTs at the edge of the kinetochore fiber immediately distal to both of the sister kinetochores (Fig. 4 d). Non-kinetochore bundles were often associated with the kinetochore fibers of adjacent nonsister kinetochores (Fig. 4 d, *K*₂ and *K*₃). Non-kinetochore bundles often lay oblique to the spindle axis along part of their length. In 40% of the kinetochores examined, a small bundle of 4–7 nkMTs terminated within the chromatin immediately adjacent to one side of the kinetochore and inter-

distal to the kinetochore. Other MTs (*O*₁ and small arrowheads) lie close to the termination. One MT (large arrowhead) does not appear to extend to the kinetochore. All other MTs are longer than depicted and could be traced into the adjacent sections. The kMTs for this kinetochore totalled 80, and for its sister kinetochore, 81. $\times 63,000$.

FIGURE 2 Late prometaphase-metaphase. A series of five consecutive longitudinal sections through a kinetochore from cell 2/72N-1, out of the total of 21 sections required to cut through the complete kinetochore region. MTs may be followed through adjacent sections. A composite tracing made from these sections is depicted in Fig. 3. The kMTs terminate within the granular material of the kinetochore, most of them lying in almost parallel array. A few oblique nkMTs (*O*₁, *O*₂, *O*₄) and one oblique kMT (*O*₄) are present. Membranous vesicles (*M*) are closely associated with the MTs. One MT (large arrowhead in b) does not appear to extend to the kinetochore, although there is a faint indication of its possible connection with the kinetochore (curved black arrows in b and c). One kMT (arrow in d) terminates at a membranous vesicle. The termination can be verified only by careful analysis of exactly superimposed serial sections, because two other MTs (*O*₁ in d and small arrowheads in e) lie close to the termination. All other kMTs in these five sections extend to the edge of the micrograph or could be traced into the section adjacent to e. White arrows in e indicate MTs not included in Fig. 3. Light micrograph of the cell is in Fig. 4b. All $\times 63,000$.

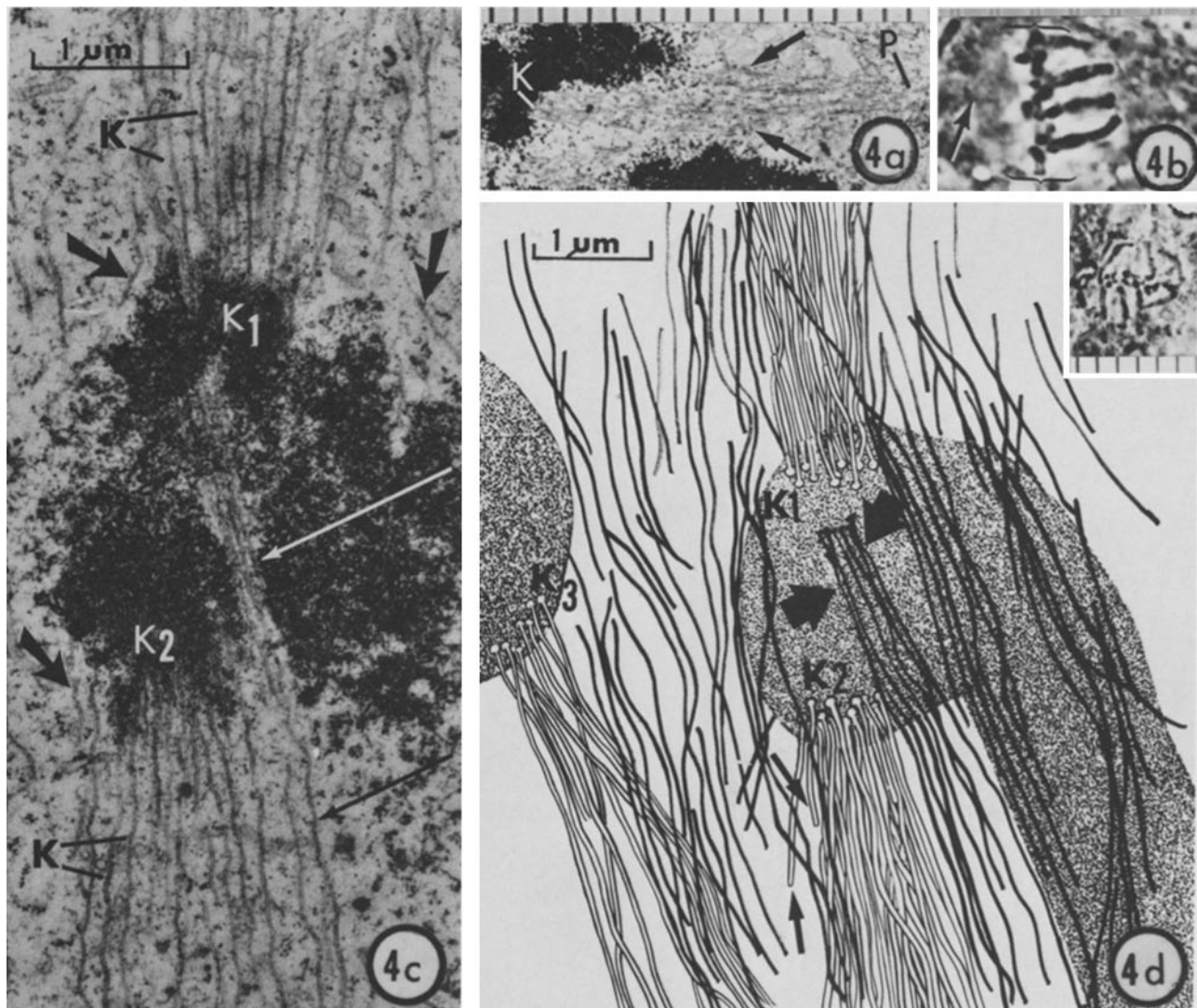


FIGURE 4 Late prometaphase-metaphase. (a) Longitudinal section through a metaphase cell. A distinct kinetochore fiber runs from a kinetochore (K) to the pole (P). It is composed of MTs associated with the kinetochore as well as additional MTs (arrows) which join the fiber distal to the kinetochore. 1- μ m intervals on scale. $\times 3,500$. (b) Phase-contrast micrograph of metaphase cell 2/72N-1. The brackets indicate the area which has been analysed in detail in each cell. This represents a region immediately behind and surrounding the kinetochore, and as much as 7 μ m distal to each kinetochore analysed, which is one-third to one-half the distance between the kinetochores and the pole (arrow) in this cell. 10- μ m intervals on scale. (c) One of the three sections from cell 2/72J used for the tracing in d. Two kinetochores (K_1 , K_2) possess attached kMTs (K) lying in almost parallel array. The waviness of the MTs attached to K_2 is probably a dehydration artifact associated with their lying next to a long chromosome arm (29). A bundle of nkMTs (long arrows) lies embedded within the chromatin and intermingles with the kMTs distal to the kinetochore. Other nkMTs (short arrows) are also closely associated with the kMTs distal to the kinetochore. Phase-contrast micrograph of cell is in b. $\times 23,000$. (d) Redrawn composite tracing of three sections through the center of the sister kinetochores (K_1 , K_2) of cell 2/72J, out of the total of 14 sections required to cut through the complete kinetochore region. The kMTs (some of them are shown here in white) for these two kinetochores totalled 76 (K_1) and 77 (K_2) and lie in almost parallel array. Several kMTs (thin arrows) can be traced to their ends distal to the kinetochore; the majority are much longer and their ends are not depicted. A bundle of seven nkMTs (shown in black at thick arrows) terminates within the chromatin and runs parallel with the kinetochore bundle in the vicinity of the kinetochore and distal to it. Additional nkMTs (also shown in black) lie singly and in bundles close to both kinetochores. A non-kinetochore bundle which is associated with K_1 and K_2 is also closely associated with the kMTs (shown in white) of an adjacent chromosome (K_3). For the sake of clarity, all nkMTs were not drawn. $\times 17,500$. Insert: phase-contrast micrograph of cell in plastic; 10- μ m intervals on scale.

mingled with the peripheral MTs of the kinetochore fiber (Fig. 4d, thick arrows).

In addition to the close association of some nkMTs with kMTs within the kinetochore and immediately distal to it, additional nkMTs merged with the kinetochore fiber 2-4 μ m distal to the kinetochore, increasing the size of the fiber and the number of constituent MTs (Fig. 4a). 5 μ m distal to the

kinetochore, the kinetochore fibers were composed of 130 to 170 MTs; therefore, about one-half of the MTs within the metaphase kinetochore fiber at this point are true kMTs.

In all stages from midprometaphase to telophase many irregularly shaped membranous vesicles resembling smooth endoplasmic reticulum were very closely associated with the MTs within the kinetochore fiber (Fig. 2). Particular care and

experience were required to follow MTs accurately in longitudinal sections where they lay above or beneath a membranous vesicle (see Fig. 2*d* and *e*).

Terminal Portions and Length of MTs in Late Prometaphase–Metaphase Cells

Within the granular material of the transversely sectioned late prometaphase and metaphase kinetochores the MTs tended to be spaced regularly (Figs. 5*a–f*). Most MTs were circular in cross-section and ~25 nm in diameter. The outer walls of some MTs were immediately surrounded by the dense material of the kinetochore, while others were surrounded by a less dense granular halo.

When specific MTs were located just distal to the kinetochore and followed through serial transverse sections to their insertion within the kinetochore, in most cases normal circular cross-sections disappeared between one section and the next, being replaced in the next successive section toward the kinetochore either by the dense granular material of the kinetochore (Fig. 5: MTs 5 and 55) or by an annular density (Fig. 5: MT 32). A few (4–9 per kinetochore at metaphase) circular MTs were, instead, replaced by C-shaped segments when traced to their proximal terminations within the kinetochore (Fig. 5: MTs 4, 30, and 46). The C-shaped segments could be followed through three to four consecutive sections toward the kinetochore before they disappeared completely. Occasional C-shaped segments which appeared in only one section, when followed to adjacent sections, were found to represent the proximal end of a circular MT which had been sectioned slightly obliquely (Fig. 5: MT 26). Proximal ends of MTs were located in various cross-sectional planes throughout the kinetochore, the peripheral MTs tending to be inserted most deeply into the kinetochore (Figs. 5*c* and *d*). A few MTs terminated right at the edge of the kinetochore (Fig. 5: MTs 4 and 26).

The majority of MTs traced in longitudinal sections through the kinetochore region terminated deep within the granular substance of the kinetochore (Fig. 2). However, occasional MTs (three to four per kinetochore), even when traced through adjacent sections, were not immediately surrounded by the granular substance of the kinetochore at their point of termination; instead, they terminated slightly distal to the kinetochore proper (Figs. 2*b* and *c*, large arrowhead). In some cases, there was a faint indication of their connection to the kinetochore (Figs. 2*b* and *c*, curved arrows).

Although some MTs could be tracked to their distal ends through serial transverse sections, the definitive length of kMTs was more easily and accurately determined by tracing them through adjacent longitudinal sections. Two or three short (as short as 0.6 μm) MTs were found attached to each metaphase kinetochore (Figs. 2*d*, 3, and 4*d* [thin arrows]), but the majority were much longer. 10% of the kMTs from metaphase cell 2/72N-1 could be followed in serial longitudinal sections as far as 7 μm distal to the kinetochore, slightly less than one-half the distance (~17 μm) between the kinetochore and the polar region of this cell. Some kMTs were probably longer than 7 μm but could not be followed farther because of the large numbers of nkMTs which had joined the kinetochore bundle between the kinetochore and this region (see Fig. 4*a*), many of them lying oblique to the spindle axis.

In transverse thin sections the kinetochores of late prometaphase and metaphase cells appeared either almost circular in unflattened cells (Fig. 5) or oval in flattened cells. Except for the shape of the kinetochore and a larger number of MTs

passing through the chromatin in flattened cells, none of the above features could be correlated exclusively with the degree of flattening of the cells, which were found to range from spherical (29- μm diameter in transverse section through the equatorial region of the cell) to extremely flat (4.8 μm \times 88 μm in transverse section through the equatorial region).

Organization of Kinetochore Fibers during Anaphase and Telophase

Throughout anaphase and telophase the kMTs, particularly those at the periphery of the kinetochore, appeared progressively more splayed out (Figs. 6 and 7*a* and *c*). In anaphase cells, many nkMTs, often in bundles of 10–30 parallel MTs which extended to the interzone (Fig. 6, long arrow), surrounded the kinetochore. The bundles themselves were sometimes parallel with the kinetochore bundle, and sometimes oblique. Bundles of MTs lay within the chromatin in the region of the kinetochore and intermingled with the kMTs distal to the kinetochore; additional bundles joined the kinetochore fiber 1 μm or more distal to the kinetochore, greatly increasing the number of MTs within the fiber. In addition, short MTs lay with various orientations adjacent to the kinetochore bundle and immediately behind it. Because of the nonparallel arrangement of the peripheral kMTs, and the bundles and short fragments of nkMTs lying oblique to the spindle axis, the MTs of the anaphase kinetochore fiber appeared less regularly arranged than in metaphase.

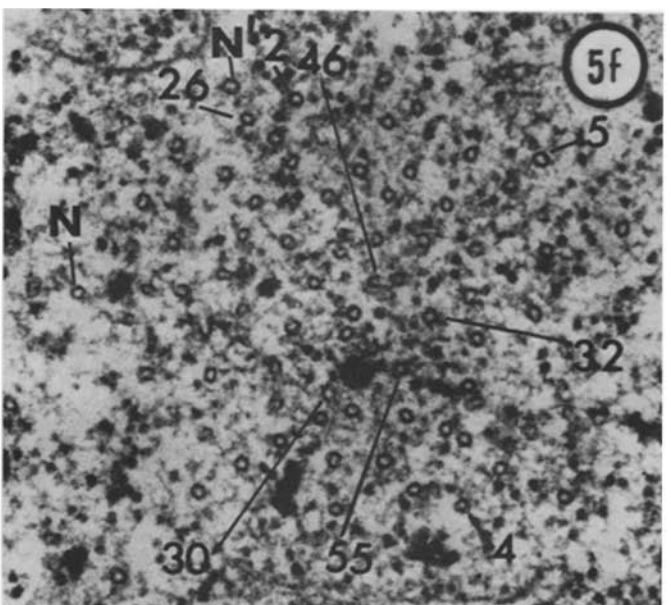
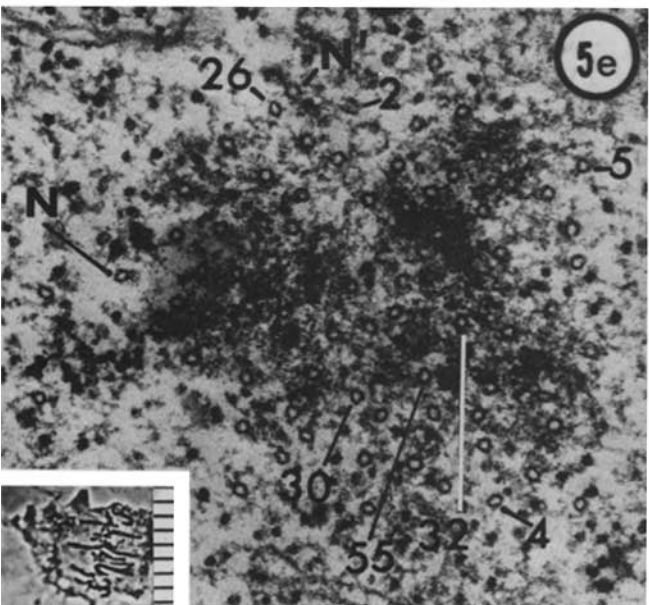
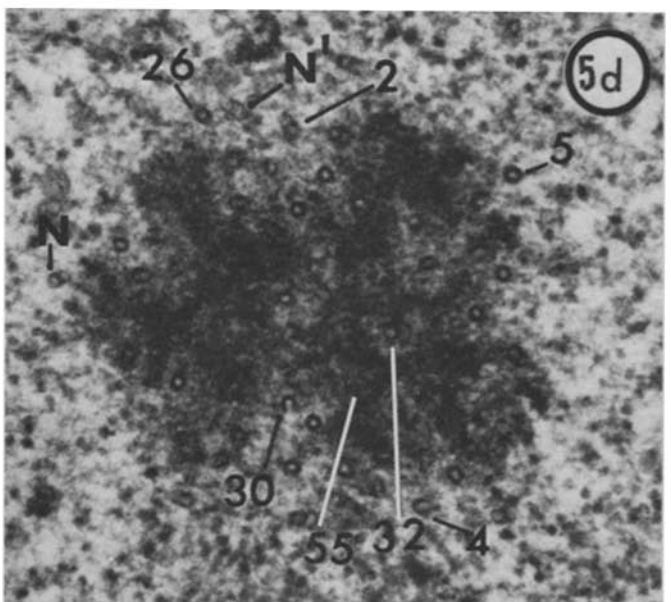
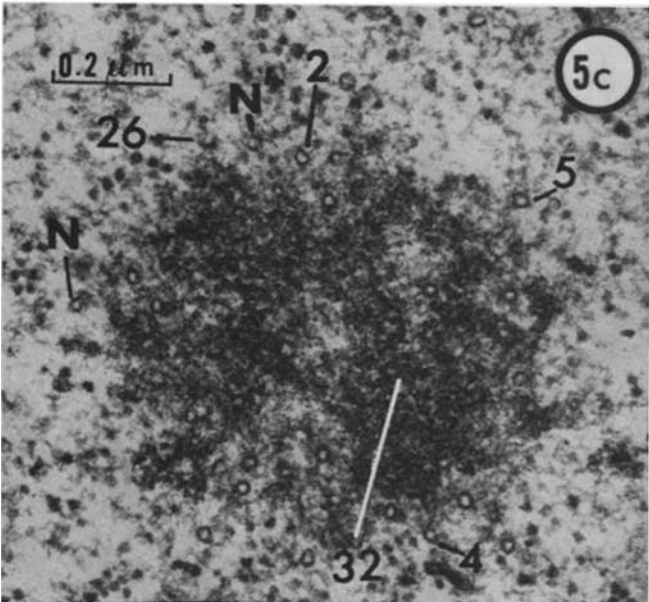
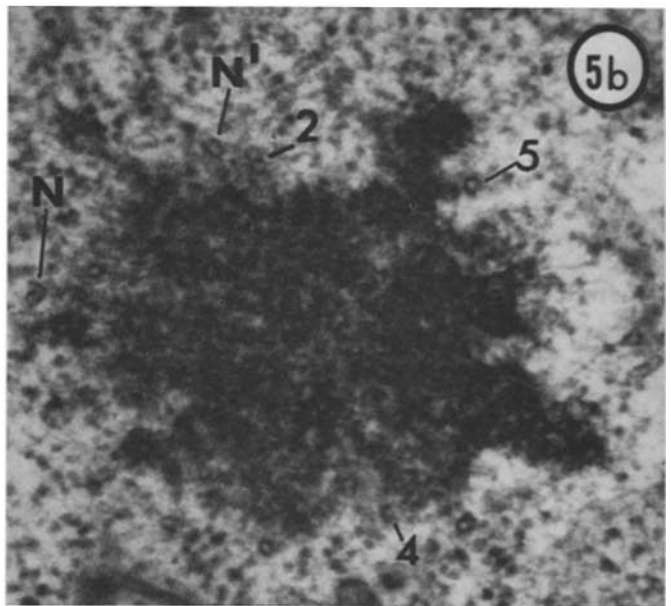
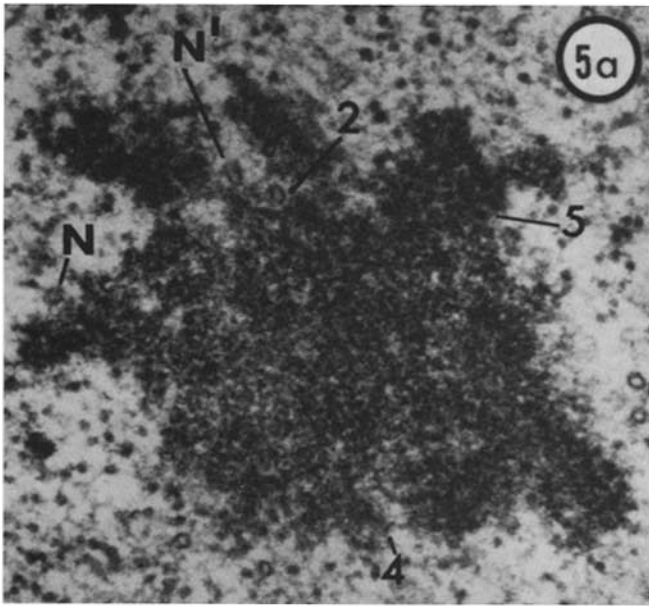
In telophase cells many short (<1 μm) nkMTs were present around the kinetochore and lay oblique to the spindle long axis (Fig. 7*c*). Fewer nkMTs intermingled with the kinetochore fiber than in cells of earlier stages (compare Figs. 1*b*, 4*d*, and 6 with 7*c*).

Divergence of kMTs throughout Division

The mean included angle between the most peripheral kMTs distal to each kinetochore, as measured from longitudinal tracings of kinetochore regions in moderately flattened cells, is detailed in Table I. The kMTs became progressively more splayed out throughout division, ranging from a mean included angle of 13.5° in prometaphase to 56° in telophase cells. The peripheral kMTs of the major kinetochore of anisometric chromosomes at prometaphase were more splayed out than other kMTs in the same cell (mean included angle of 34° vs. 13.5°, respectively), the included angle between peripheral kMTs being more similar to that for anaphase kinetochores. Delayed chromosomes possessed kMTs which were less splayed out than the kMTs of chromosomes closer to the pole (mean included angle of 19° vs. 36° in late anaphase and 25° vs. 56° in telophase).

Terminal Portions and Length of kMTs throughout Division

Throughout division, the terminal portions of MTs within the kinetochore resembled those described for the metaphase kinetochore. Fig. 8 illustrates the frequency of kMTs possessing a C-shaped segment at their proximal ends (the end embedded in the kinetochore). In early prometaphase cells, 20.6% of the kMTs possessed a C-shaped proximal segment. The frequency of C-shaped proximal terminations decreased progressively, reaching 8.9% in metaphase cells, 0.6% by early anaphase, and zero in mid- and late anaphase. It rose to 2.9% in very late anaphase cells and to 16.7% in telophase.



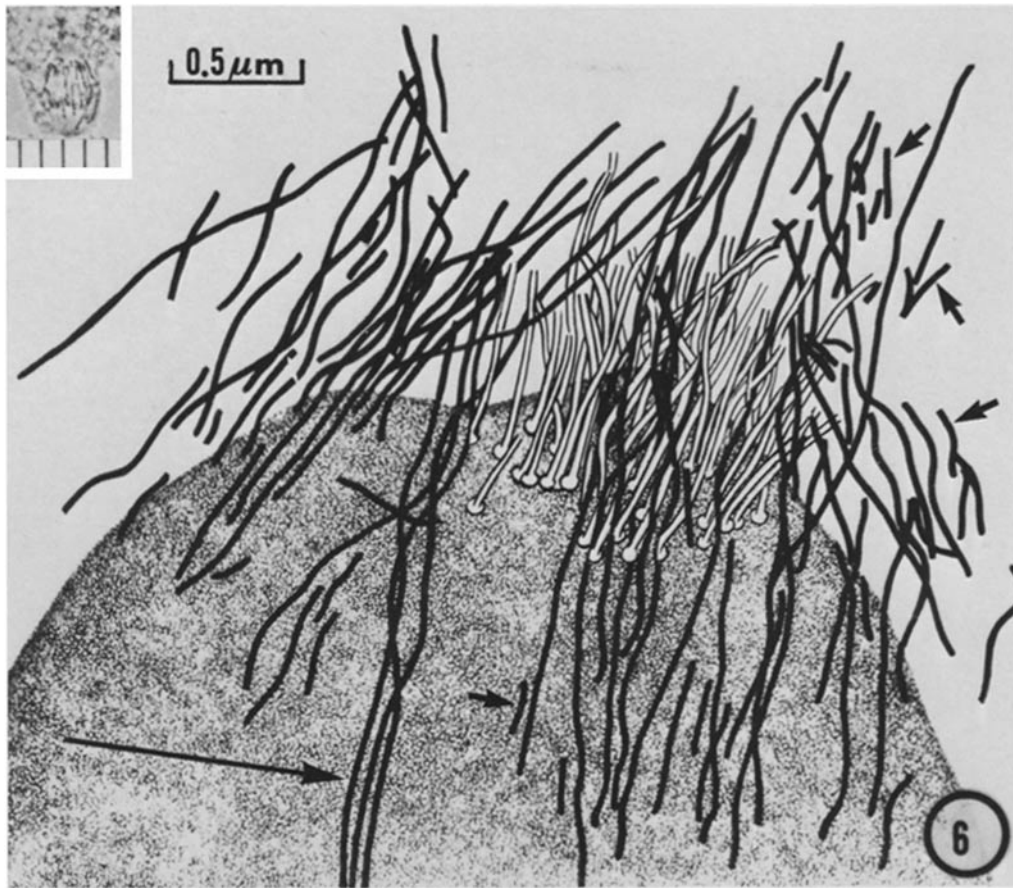


FIGURE 6 Anaphase. Redrawn composite tracing of 23 serial longitudinal sections through the kinetochore region from midanaphase cell 1/72DD. The 48 kMTs (shown in white) are all longer than depicted in the drawing and are more splayed than in metaphase. The kinetochore of the homologous chromosome was found to have 50 kMTs. Many nkMTs (shown in black) are closely associated with the kMTs, although for the sake of clarity all of those which were present are not depicted in the drawing. A bundle of nkMTs (long arrow) extends from the interzone through the chromatin to intermingle with the kMTs. Many short fragments of MTs (short arrows) lie next to the kinetochore and behind it. $\times 40,000$. Insert: phase-contrast micrograph of cell in plastic; 10- μm intervals on scale.

C-shaped terminations of nkMTs were also present in the kinetochore region, but their frequency differed from that of kMT C-shaped proximal terminations. In a 1- μm radius around the kinetochore, they averaged 3% of the nkMTs at early prometaphase, 6% in metaphase and early anaphase, 1.4% in mid-anaphase, 0.7% in late anaphase, and none in telophase cells. Although very few C-shaped nkMT terminations were located in the kinetochore region of late anaphase cells, many (mean = 24%) were seen in the interzone at this stage.

Except for some of the MTs attached to the minor kinetochores of anisometric chromosomes (Fig. 1c), no extremely short (<1 μm) kMTs were seen in early prometaphase cells. Two or three MTs <1 μm long were attached to each metaphase kinetochore; the majority were much longer, some being traced as far as 7 μm distal to the kinetochore. In contrast to metaphase cells, no short (<1 μm) kMTs were detected in anaphase cells,

most of them being at least 2 μm long and some probably being much longer (Fig. 6). However, in cells which had reached telophase, 80–85% of the kMTs were extremely short (0.1–0.8 μm ; Fig. 7c).

Kinetochore Microtubule Number and Density

NUMBER OF KINETOCHORE MICROTUBULES: The number of kMTs for 201 kinetochores in 28 cells belonging to two different cell preparations are detailed in Table II and diagrammed in Fig. 9. Wholly consistent with these data are counts of 113 other kinetochores from the same two cell preparations for which only a partial set of serial sections through the kinetochore was available, as well as 52 kinetochores from six other cell preparations. No kMTs were found attached to the kinetochores of prophase cells, before breakage

FIGURE 5 Late prometaphase-metaphase. (a–f) Serial sections Nos. 3, 4, 5, 6, 7, and 9 through a kinetochore of late prometaphase cell 1/72S. nkMTs (N) may be followed through adjacent sections. This kinetochore was determined to have 69 kMTs. No kMT distal ends are present in this series of sections. MTs 5 and 55 represent circular proximal terminations. MT 26 in c probably represents a circular-shaped proximal termination which has been sectioned obliquely. MTs 4, 30, and 46 represent C-shaped proximal terminations. MT 32 represents an annular density at its proximal termination. MTs 4 and 26 terminate at the edge of the kinetochore. MTs 2 and N' lie oblique to the plane of section in part of this series of sections. All $\times 81,000$. Insert e: phase-contrast micrograph of cell in plastic; 10- μm intervals on scale. $\times 200$.

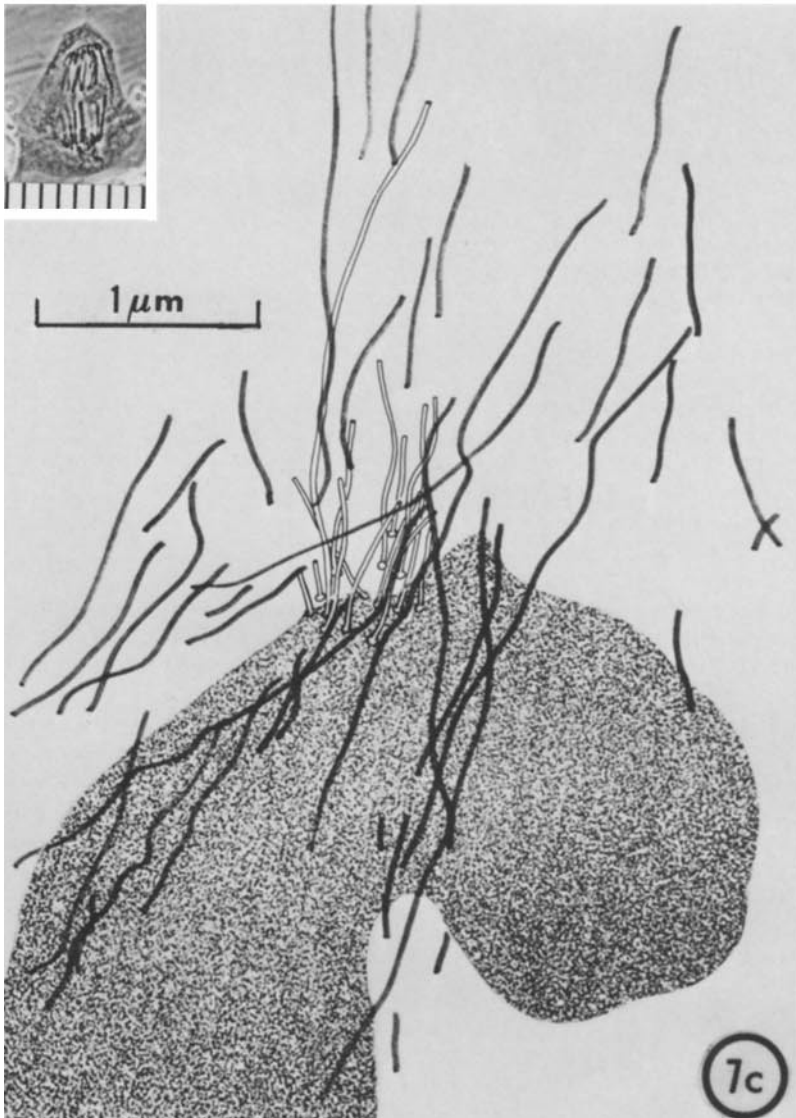
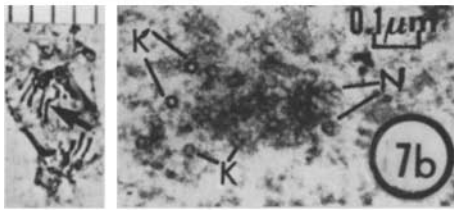
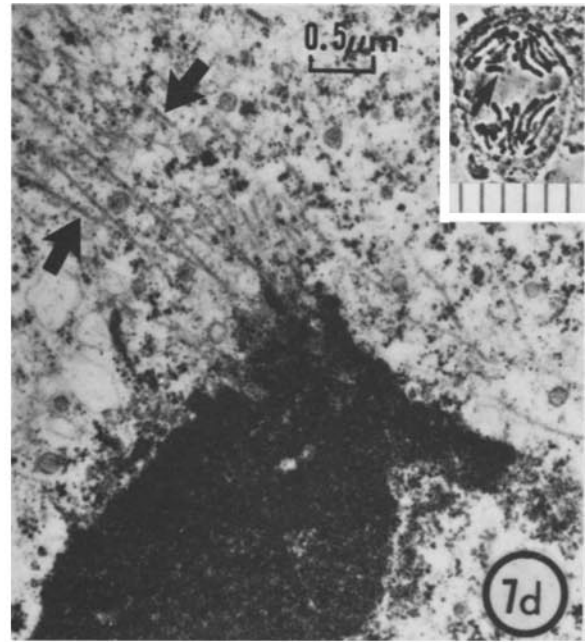
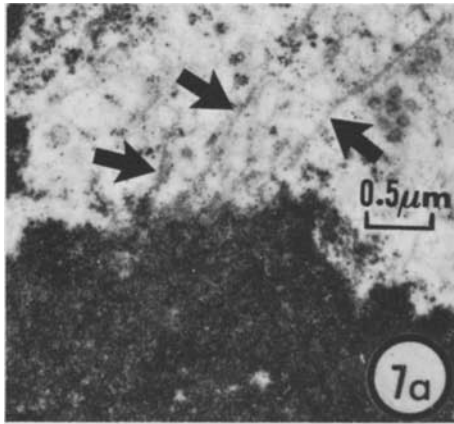


TABLE I
Included Angle between Peripheral Kinetochore Microtubules of a Kinetochore throughout Division

Stage of division	Normal chromosomes				Abnormal chromosomes		
	No. of kinetochores measured	Mean included angle		Distance of kinetochores from equator μm	No. of kinetochores measured	Mean included angle degrees	Distance of kinetochores from equator μm
		degrees	SD degrees				
Prometaphase	6	13.5	9.5	—	4*	34.3	—
Metaphase	31	17.5	5.1	—			
Midanaphase	3	28.0	—	13.5			
Mid-late Anaphase	18	30.2	8.0	17.0			
Late anaphase	8	36.3	8.2	21.0	1‡	19.0	7.5
Telophase	13	56.0	12.8	25.0	2§	25.0	10.0

* Anisometric chromosomes, major kinetochore.

‡ Lagging chromosome.

§ Bridge chromosomes.

of the nuclear envelope. Throughout prometaphase the mean number of MTs per kinetochore progressively increased, reaching a peak of 82.8 at metaphase. At the onset of anaphase, the mean dropped by 16% to 69.5, followed by a gradual linear decrease throughout anaphase which was correlated directly with the movement apart of the chromosomes. In late telophase cells no kMTs were discernible.

At all stages of division the number of MTs per kinetochore varied between chromosomes within the same cell as well as between cells, the range being greatest (74–109) at metaphase (Table II). However, there was little overlap between different stages of division. For example, the number of MTs per kinetochore in prometaphase cells was found to be 43–49; metaphase, 74–109; early anaphase, 63–76; midanaphase, 40–60; late anaphase, 29–39; and telophase, 8–14.

With the exception of those prometaphase chromosomes which were oriented to a single pole (Fig. 1c), in all 14 chromosomes where it was possible to obtain accurate counts of both sister kinetochores from graphic reconstructions of longitudinal sections, similar numbers of MTs (± 2 MTs) were found attached to sister kinetochores of prometaphase and metaphase chromatids as well as to homologous chromosomes separating during anaphase (see legends to Figs. 1b, 3, 4d, and 6). Also, in those cases where reliable measurements of chromosome length could be compared with kMT numbers, there was a positive correlation between the length of a metaphase chromosome and the number of kMTs attached to it (Fig. 10), ranging from the small chromosomes with 74 kMTs to the largest with 109 kMTs. This is also the range in number of MTs per kinetochore found for all metaphase cells.

AREA OF MICROTUBULE ATTACHMENT: The cross-sectional

area of MT attachment (as described in Materials and Methods) also increased during prometaphase and was greatest at metaphase and the onset of anaphase (Fig. 9 and Table I). It then decreased linearly during most of anaphase.

DENSITY OF MICROTUBULES: The density of kMTs within a central $0.1 \mu\text{m}^2$ circular cross-section of the kinetochore also increased throughout prometaphase, reaching the highest density of 197 MTs per μm^2 at metaphase (Table II). It decreased by 23% to 151 MTs per μm^2 at the onset of anaphase and remained relatively constant ($140\text{--}150 \text{ MTs}/\mu\text{m}^2$) throughout anaphase and telophase. In the latest stages of telophase at which kMTs were still discernible (cell 1/72M in Table II, and Fig. 7b), the density of MTs at the center of the kinetochore was found to have remained at $150 \text{ MTs}/\mu\text{m}^2$.

The overall density of MTs within early prometaphase kinetochores (based on a mean of 16.7 MTs per kinetochore and a mean kinetochore area of $0.23 \mu\text{m}^2$) was $73 \text{ MTs}/\mu\text{m}^2$. The overall density was greatest at late prometaphase, being $129 \text{ MTs}/\mu\text{m}^2$, dropping to $117 \text{ MTs}/\mu\text{m}^2$ in metaphase kinetochores and to $100 \text{ MTs}/\mu\text{m}^2$ within midanaphase kinetochores.

The density of MTs in the center of non-kinetochore bundles adjacent to the kinetochore was found to be $150 \text{ MTs}/\mu\text{m}^2$ during prometaphase, increasing to $170 \text{ MTs}/\mu\text{m}^2$ at metaphase, and decreasing during anaphase from $145 \text{ MTs}/\mu\text{m}^2$ at early anaphase to $120 \text{ MTs}/\mu\text{m}^2$ at midanaphase.

DELAYED KINETOCHORES: All delayed kinetochores which were examined (lagging and bridge chromosomes) in late anaphase–early telophase cells possessed more kMTs and a greater cross-sectional area of MT attachment than did kinetochores closer to the pole. For example, the delayed kinetochores of cells 2/72V and 1/72M possessed 30 and 36

FIGURE 7 Telophase. (a) Longitudinal section through a kinetochore near the pole of cell N27/68-1. Only a few short kMTs (arrows) remain and are more splayed than in earlier stages. $\times 19,200$. Phase-contrast micrograph of cell in Fig. 6d. (b) Cross section through kinetochore of cell 1/72M. Only a few kMTs (K) and nkMTs (N) are present and the kinetochore is greatly reduced in size compared with late prometaphase and metaphase kinetochores (compare with Fig. 5c). $\times 69,000$. Insert: phase-contrast micrograph of cell in plastic. Arrow, lagging chromosome; $10\text{-}\mu\text{m}$ intervals on scale. (c) Composite tracing from 22 sections through kinetochore region from cell 2/72-1. The 14 kMTs (shown in white) are very splayed and surrounded by nkMTs (shown in black) lying at various angles to the long axis of the spindle. Most MTs are very short. All MTs are drawn to their actual length. $\times 30,000$. Insert: cell in plastic; $10\text{-}\mu\text{m}$ intervals on scale. (d) Longitudinal section through kinetochore of bridge chromosome (indicated by arrow on the insert) of cell N27/68-1. The delayed chromosome possesses more kMTs (arrows) which are less splayed than the kMTs of a chromosome near the pole in the same cell (a). $\times 19,200$. Insert: phase-contrast micrograph of cell in plastic; $10\text{-}\mu\text{m}$ intervals on scale.

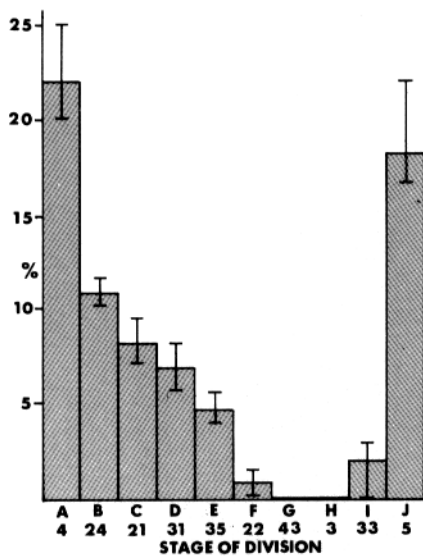


FIGURE 8 Frequency of kMTs which terminate at the kinetochore as a C-shape throughout division, expressed as the mean percent of the total number of MTs per kinetochore. The range for each stage is also indicated. Each C-MT was verified by tracking individual MTs through serial transverse sections. Stages of division: A, early prometaphase; B, mid-prometaphase; C, late prometaphase; D, metaphase; E, very early anaphase; F, early anaphase; G, midanaphase; H, late anaphase; I, very late anaphase; and J, midtelophase. Numbers beneath each bar indicate the number of kinetochores analysed. The samples for early prometaphase and midtelophase were smaller because in these stages the kinetochores lie with various orientations to the spindle axis; therefore, few of them were sectioned transversely.

kMTs, respectively, as compared with a mean of 12.5 and 9.2 MTs, respectively, attached to kinetochores close to the poles in the same cells (Table II; also compare Fig. 7a and d). However, the delayed kinetochores did not possess as many kMTs as did midanaphase chromosomes which had travelled an equivalent distance from the equator at the time of fixation. For example, the kinetochores of the midanaphase cell 2/72D which were 12 μ m from the equator had a mean of 52 MTs per kinetochore (range 47–59), while the kinetochore of the lagging chromosome in cell 1/72M which was also 12 μ m from the equator at the time of fixation had 36 attached kMTs (Table II).

DISCUSSION

I have described here the time-dependent changes in organization of kinetochore fibers in the *Haemaphysalis* spindle during mitosis. The studies represent probably the most detailed and extensive analysis so far undertaken of the kinetochore region from the mitotic spindles of a higher organism. Because the number and arrangement of MTs comprising the kinetochore fiber vary significantly throughout division and because the MTs invariably intermingle throughout the kinetochore fiber, detailed, valid quantitation of spindle structure is possible only on the basis of meticulous three-dimensional reconstructions based on both longitudinal and transverse serial sections through the spindles of many cells throughout all stages of division. Certain details of spindle structure and organization, including the three-dimensional arrangement of MTs in the vicinity of the kinetochore, the variation in numbers of MTs per kinetochore between chromosomes within the same cell and between different stages of division, the special features of

the kinetochores of lagging chromosomes, accurate identification of kMTs and nkMTs in the region of the kinetochore, and positive identification of C-shaped terminations of MTs could not have been ascertained in any way other than such a detailed analysis from serial thin sections.

Methodology

In analyses of spindle organization, the validity of the observations is dependent upon the reliability of the techniques used for preparation and analysis of the material. Therefore, in this study every effort has been made to ensure that the MT counts and reconstructions accurately reflect the total number and arrangement of each type of MT in each spindle in vivo. Nevertheless, the likelihood of errors must be recognized. Possible sources of error in studies of this type have been discussed previously (10, 44, 45, 53, 61, 62). These include: (a) variability between spindles either in vivo or as a result of processing, (b) loss and/or displacement of MTs during preparation, (c) failure to make correct linkages between corresponding MTs in adjacent serial sections, and (d) failure to identify all MTs present in the fixed material. Additional sources of error which could provide an inaccurate picture of spindle organization have been brought to light by the present study. These are: (a) variations in magnification or insufficient magnification of micrographs used for tracing MTs through serial sections, (b) inaccuracies in differentiating between kMTs and nkMTs in the region of the kinetochore, (c) failure to identify precisely the stage of division when making counts of kMTs, (d) variability in numbers of kMTs per kinetochore within a single cell and between different stages of division, and (e) errors in identifying C-shaped terminations of MTs. The studies have also shown the essential requirement of serial sections for accurately describing the kinetochores of prometaphase and lagging chromosomes, and the value of using longitudinal sections for certain aspects of the study.

VARIABILITY BETWEEN SPINDLES: To ensure a minimal variability between spindles at different stages of division, the numerical data were derived from cells belonging to only two separate cell preparations which had been prepared and fixed at the same time. The endosperm cells themselves, having originally been derived from a single triploid nucleus within the embryo sac through probably less than ten mitotic divisions, can therefore be considered a clonal population.

LOSS AND/OR DISPLACEMENT OF MTS DURING PREPARATION: There is no certainty that all MTs are preserved or that similar proportions are retained in all cells. Also, some MTs within bundles lying next to long chromosome arms may become bent and perhaps broken during dehydration (29). Waviness of MTs (as in Fig. 4c, MTs attached to K₂) is therefore probably a dehydration artifact. Nevertheless, the methods of preparation used are the most reliable known for *Haemaphysalis* (49). Since similar arrangements of MTs and ranges in numbers of kMTs were found in all cells of the same division stages from all preparations, the observations are valid to that extent.

FAILURE TO IDENTIFY MTS: In this study MTs lying oblique to the spindle axis, of which there are many in *Haemaphysalis* spindles, as well as some C-shaped MTs, were especially difficult to identify in single transverse or longitudinal thin sections. However, because in this study MTs were followed individually through adjacent serial sections, there was less likelihood that any were overlooked. Therefore, the counting error, as shown by comparison of counts of the same kinetochores obtained either by different observers or by myself

TABLE II
Summary of Kinetochores Microtubule Counts from 28 Cells

Cell	Stage of division	Plane of section*	No. of Counts	Mean no. MTs per			Mean K‡ areas§ μm ²	Mean central density	Distance of K‡ from equator μm ²
				K‡	Range	SD			
1/72EE	Late prophase	L	—	0	—	—	—	—	
1/72FF	Late prophase	L	—	0	—	—	—	—	
2/72-2	Very late prophase	L	—	0	—	—	—	—	
1/72CC	Very early prometaphase	L	10	13.7	12-14	0.89	0.16	—	
1/72F	Early prometaphase	T	3	16.7	15-18	1.53	0.23	10.0	
1/72A	Midprometaphase	T	12	46.5	43-49	4.50	0.42	14.3	
1/72S	Late prometaphase	T	17	65.9	48-78	9.16	0.51	19.0	
1/72E-1	Metaphase	T	2	—	—	—	—	—	
2/72E	Metaphase	T	11	—	—	—	—	—	
1/72E-2	Metaphase	T	5	82.8¶	74-109	4.69	0.70	19.7	
1/72C	Metaphase	T	9	—	—	—	—	—	
1/72N-1	Metaphase	L	5	—	—	—	—	—	
2/72J	Metaphase	L	5	—	—	—	—	—	
1/72J	Start anaphase	T	10	69.5	67-74	2.01	0.67	15.1	
1/72W	Very early anaphase	T	6	68.3¶	63-76	5.78	0.69	14.9	
2/72I	Very early anaphase	T	16	—	—	—	—	—	
1/72U	Early midanaphase	T	11	54.9	43-67	9.32	0.59	14.4	
2/72D	Midanaphase	T	5	52.0	47-59	4.47	0.49	14.4	
1/72DD	Midanaphase	L	6	49.0	48-50	1.41	0.51	—	
1/72I	Midanaphase	T	24	48.1	40-60	5.02	0.49	13.8	
2/72C-1	Late anaphase	T	6	39.0	38-39	—	0.41	14.0	
1/72O	Late anaphase	T	14	32.9	29-38	4.20	0.38	14.1	
2/72K-1	Very late anaphase	L	4	25.0	22-27	2.16	0.24	—	
2/72-1	Early telophase**	L	4	13.5	13-14	0.71	0.22	—	
2/72V	Midtelophase	L	8	12.5	10-14	—	0.21	—	
2/72V	Delayed chromosome	—	2	30.0	—	—	0.35	—	
1/72M	Midtelophase	L and T	5	9.2	8-11	1.30	0.13	15.0	
1/72M	Delayed chromosome	—	1	36.0	—	—	0.31	14.0	
1/72M-1	Late telophase	L	—	0	—	—	—	—	
2/72K-2	Late telophase	L	—	0	—	—	—	—	

* L, longitudinal; parallel with the spindle axis. T, transverse; perpendicular to the spindle axis.

‡ K, kinetochore.

§ Cross-sectional area of kMT attachment (see Materials and Methods).

|| Mean number of MTs within a 0.1 μm² central circle of the kinetochore.

¶ Data on all cells of this stage have been pooled.

** Stage at which chromosome arms have begun to contract.

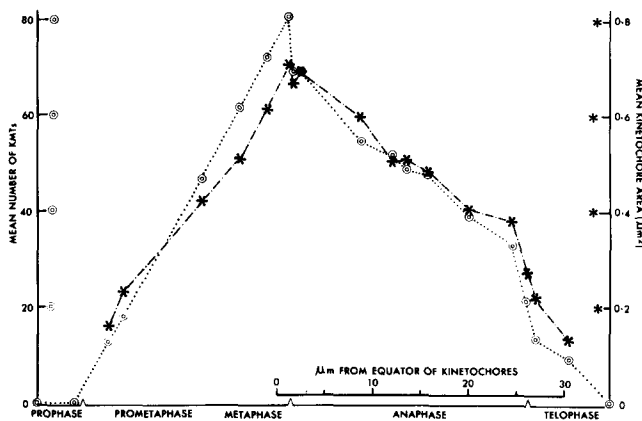


FIGURE 9 The mean number of kMTs (●) and mean area of kMT attachment (*) throughout division. Arbitrary distances on the abscissa have been chosen for all stages but anaphase. The onset of telophase is defined as the point at which chromosome arms have begun to contract.

on different occasions, was extremely low, being no greater than ±1 MT per kinetochore.

MAGNIFICATION OF PHOTOGRAPHS: This study high-

lighted the need for extremely precise enlargement of the photographs used for tracing MTs through serial sections, particularly longitudinal ones. Therefore, it was absolutely essential for a series of photographs to be enlarged to exactly the same degree and the print to be made on a surface with great dimensional stability such as glass, some films, or resin-coated photographic paper. Even slight variations in magnification in one direction on a series of prints (as can occur when putting photographic prints through the conventional drum dryer) can prevent accurate alignment of one section with the next, thereby producing mismatches between adjacent serial sections. When micrographs of a series were at exactly the same magnification, and great care was taken in aligning them, it was virtually impossible to mismatch adjacent sections, as with only one orientation would all MTs be lined up in perfect register from one section to the next (see Figs. 2 and 3). Also, following every MT with certainty required final print magnifications of at least 70,000.

DIFFERENTIATING BETWEEN kMTs AND nkMTs: Graphic reconstructions through the kinetochore region verify previous reports (4, 7, 8, 18, 27, 30) of the close association of nkMTs and kMTs throughout division in *Haemanthus*. To differentiate between kMTs and nkMTs in both transverse and

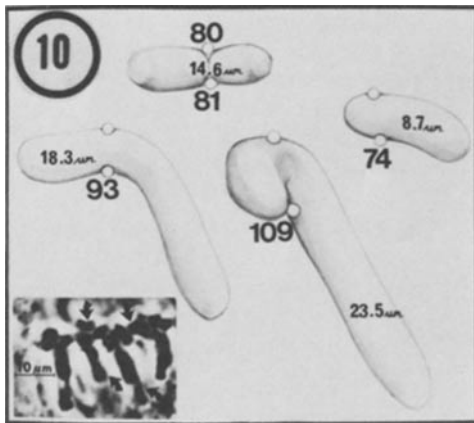


FIGURE 10 Correlation between chromosome size and number of associated kMTs in metaphase cell 2/72N-1. The four chromosomes marked with arrows in the phase-contrast micrograph insert are depicted graphically in the same topographical arrangement. The number of each chromosome represents its length as measured both on a phase-contrast micrograph of the cell in plastic and on electron micrographs of serial thin sections through the chromosomes. $23.5 \mu\text{m}$ = type A chromosome; $18.3 \mu\text{m}$ = type B; $14.6 \mu\text{m}$ = type C; and $8.7 \mu\text{m}$ = small chromosomes (2). The numbers at each kinetochore (circles) represent the number of kMTs attached.

longitudinal sections, it is essential to follow individual MTs, through serial sections, back to their insertion into the kinetochore. At metaphase the kinetochore fibers consist of up to 170 MTs $5 \mu\text{m}$ distal to the kinetochore; only about one-half of them are true kMTs. Rat kangaroo PtK mitotic spindles (56; C. Jensen, unpublished data) and insect spermatocyte spindles (21, 23, 34) also show a close relationship between kMTs and nkMTs in the region of the kinetochore, suggesting that such an arrangement may be characteristic of many of the cells of higher organisms rather than being unique to *Haemanthus* endosperm. Therefore, accurate data on the number of kMTs cannot be obtained by counting MTs in a single cross section through a kinetochore fiber distal to the kinetochore. For this reason, previous counts of the number of kMTs in various types of dividing cells (10, 44), including preliminary ones for *Haemanthus* (6, 7, 30, 31), have probably been too high.

STAGE OF DIVISION AND VARIABILITY IN NUMBER OF MTs: Because the number of kMTs increases throughout prometaphase and metaphase and decreases throughout anaphase and telophase (Fig. 9), it was essential to select cells in late metaphase for determinations of the definitive number of kMTs and the correlation between kMT number and chromosome size. The variability in numbers of MTs per kinetochore within a single cell and between different stages of division required that a large number of kinetochores be selected for MT counts.

The definition of onset of telophase has been unclear in the past. Here it is defined as the point at which the chromosome arms have begun to contract.

LONGITUDINAL VS. TRANSVERSE SECTIONS: Transverse sections have been used most successfully to track the almost parallel MTs of the small spindles in algae (39, 45, 60, 61, 62) and fungi (reviewed in reference 26), except for the polar regions where the MTs are not parallel in some of those spindles (61, 62). It would be difficult and probably impossible to obtain such precise and complete data in this manner for *Haemanthus* spindles. Obliquely sectioned MTs and large numbers of MTs are especially difficult to track through transverse sections (62). Therefore, because of the large numbers of MTs

in *Haemanthus* spindles, many of them directed oblique to the longitudinal axis of the spindle, the analysis of MT distribution from longitudinal sections was found to be an easier and more reliable way than analysis from transverse sections to follow every single MT in the kinetochore region for a reasonable distance. In particular, information on the association of kMTs and nkMTs at each stage of division (as in Figs. 1 b and c, 4 d, 6, and 7 c), the correlation between chromosome length and kMT number (as in Fig. 10), and the number of kMTs attached to early prometaphase (Fig. 1 b and c) and late telophase (Fig. 7 c) kinetochores, most of which were not oriented perpendicular to the long axis of the spindle, were most easily obtained through graphic reconstructions of longitudinal sections. Also, data on the length of MTs were obtained from analysis of longitudinal sections. In all cases, the data obtained from longitudinal sections were consistent with those obtained from analysis of transverse sections. Serial longitudinal sections have also been used successfully by others for the study of MT arrangements in the cells of various organisms (13, 18, 21, 23, 52, 53, 54).

LENGTH OF MTs: The lengths of the shortest MTs were determined by tracing them individually through serial longitudinal sections to their termination. In fact, many of the short MTs were contained within a single section. The only MTs whose length was recorded were those for which there was no ambiguity as to whether they actually terminated. Therefore, there may be more short MTs than actually depicted in the tracings. Also, because MTs coursing through longitudinal sections may appear shorter than they actually are, some of the measurements for short MTs may be slightly too low. It was felt that the use of transverse sections to determine the length of MTs in *Haemanthus* would be no more reliable (and much more time-consuming) than the use of longitudinal sections, since the section thickness, which is difficult to determine accurately, is critical for such measurements (44, 62).

No information could be provided on the maximum length of MTs. Because of the progressively increasing complexity of MT arrangement distal to the kinetochore (especially obvious in thick sections photographed with the high-voltage electron microscope; reference 8), the maximum distance which MTs could be traced in *Haemanthus* was $7 \mu\text{m}$, which represents one-half to one-third the distance from the kinetochore to the polar region at metaphase (37; Fig. 4 a and b). These distances were similar to or longer than those traceable in crane fly (21) and plant meiotic cells (13). Also, when longitudinal sections were used to trace MTs, because of the great depth of field of the electron microscope, several superimposed MTs could appear as a single one in longitudinal sections, thus making some MTs appear longer than they actually are. In practice it was found that, with careful analysis, only in very few cases was there any ambiguity as to whether or not a single MT was being traced.

Changing Organization of MTs in the Kinetochore Region throughout Division

SUMMARY OF TIME-DEPENDENT ALTERATIONS IN ORGANIZATION OF THE KINETOCHORE FIBER: The results of the current studies suggest that the functional unit of the spindle is the kinetochore fiber—a heterogeneous structure composed of kMTs which terminate at the kinetochore as well as of nkMTs which join the kinetochore fiber at the kinetochore itself or at different points along the fiber distal to the kinetochore. There is close association between the kMTs and nkMTs

in all stages of division, and a time-dependent alteration in their organization within the fiber. After breakage of the nuclear envelope, the number of MTs per kinetochore progressively increases during prometaphase and metaphase to >100 per kinetochore (Table II). At metaphase, when the kMT number is highest, the cross-sectional area of kMT attachment and the density of MTs within the central region of the kinetochore are also the highest of any stage during division. Once anaphase begins, certain progressive changes in the organization of the kinetochore fiber are initiated which last throughout anaphase and telophase. These changes include increasing obliquity of the arrangement of the kMTs (Table I), a decrease in number of kMTs, a decrease in the cross-sectional area of kMT attachment (Table II), and an increase in the number of short MTs to the side of anaphase kinetochores and behind them (Fig. 6). The frequency of C-shaped proximal terminations of kMTs is high at early prometaphase, drops progressively to zero during anaphase, and increases again during telophase (Fig. 8).

ORIGIN AND PROLIFERATION OF kMTs DURING PROMETAPHASE: By 5–10 min after breakage of the nuclear envelope the kinetochores are well developed and most MTs attached to them are relatively long (1 μm or longer). They may have been derived from the bundles of long MTs which penetrate the nuclear area during nuclear envelope breakage, as suggested by Bajer and Molè-Bajer (6). Possible ways in which kMTs may increase in number during prometaphase (as shown in Fig. 9) are: (a) binding by the kinetochore of already existing MTs (60); (b) assembly of MTs from subunits with the kinetochore acting as a nucleating site; or (c) a combination of the two processes (6). Addition of kMTs to the kinetochore during prometaphase occurs at its periphery as well as throughout the kinetochore, as shown by the observed progressive increase in cross-sectional area, in central density, and in overall density of MTs within the kinetochore during this time (Table II).

ANISOMETRIC CHROMOSOMES DURING PROMETAPHASE: The observations presented here on anisometric chromosomes in prometaphase (Fig. 1c) extend those of previous studies (6, 7, 38, 57). Bajer and Molè-Bajer (6, 7) have observed that >50% of the *Haemanthus* early prometaphase cells may possess chromosomes with kMTs oriented predominantly toward a single pole. Some or all of them may possess kinetochores organized like the anisometric chromosomes described in this study.

The distinct arrangement of the arms of the anisometric chromosomes corresponds to that of prometaphase chromosomes which have been seen to move abnormally (e.g., toward the polar region) in time-lapse films of living *Haemanthus* cells (6; C. Jensen, unpublished observations). Such chromosomes usually return to the equatorial region by metaphase. Therefore, at the time of fixation the anisometric chromosomes described here were probably in the process of moving toward the pole which the major kinetochore was facing. The unique features of the anisometric chromosomes (which are not characteristic of isometric chromosomes in the same cell) are the greater number of kMTs attached to the major kinetochore (24–29 vs. 12–14), the greater divergence of the kMTs (mean maximum angle of 35° vs. 13.5°), and the large bundle of nkMTs associated with both kinetochores. All of these are features held in common with mid-to-late anaphase kinetochores which are also moving toward the pole (see Figs. 1c and 6; and Tables I and II). Such features are most likely either a prerequisite for their poleward movement or a result of it,

and may therefore be important designators in considerations of force generation for chromosome movement. Because similar configurations were found throughout the spindle (close to the polar region as well as near the equatorial region of the spindle), it is unlikely that the original development of this type of chromosomal morphology is exclusively under the influence of the poles.

The minor kinetochores of anisometric chromosomes are also associated with a large bundle of nkMTs, a feature which may be important for their subsequent reorientation. These kinetochores may subsequently establish connections to a single pole by: (a) disassembly of kMTs, followed by reassembly in a new place; (b) rearrangement and elongation of existing kMTs; or (c) a combination of the above. The very short MTs attached to the minor kinetochore and oriented toward the opposite pole (Fig. 1c, curved arrows) may have been in the process of elongation in the direction of that pole at the time of fixation, before establishment of a chromosome with isometric kinetochores and bipolar orientation. The MTs attached to the minor kinetochore which face the major kinetochore (Fig. 1c, arrowheads) would have to undergo either rearrangement or disassembly before the standard metaphase arrangement of kMTs could be established. If MT rearrangement did not occur in these chromosomes to allow reorientation, non-disjunction might result.

It should be stressed that an anisometric configuration such as that shown in Fig. 1c can be visualized only through analysis of a complete set of longitudinal sections through both kinetochores (as discussed in 38). Because MTs attached to the minor kinetochore are often short or run in the direction of the major kinetochore, examining such chromosomes in single thin sections gives the false impression that few or no MTs are attached to the minor kinetochore.

ONSET OF ANAPHASE: Between metaphase and very early anaphase the mean number of MTs per kinetochore has dropped by 16% and the density of MTs within the center of the kinetochore has fallen by 23%, while the cross-sectional area of the kinetochore has remained almost unchanged (Table II). This suggests that MTs are lost from throughout the kinetochore at very late metaphase or the start of anaphase. The lost MTs may have been ones which were not “anchored” to the kinetochore and became transported toward the pole at this time (3). The C-shaped proximal terminations of metaphase kMTs (Fig. 8) may belong to this group. It is possible that some kMTs could be removed from the kinetochore in a similar manner during prometaphase-metaphase as well, but such a loss would not have been detected in this study because of the net increase in number of kMTs which occurs during those stages.

ANAPHASE-TELOPHASE: A striking feature of the anaphase and early telophase kinetochores is that the density of kMTs within the center of the kinetochore remains the same throughout this time. During these stages kMT numbers are decreasing steadily from a mean of 69 per kinetochore at the onset of anaphase to 12 per kinetochore at midtelophase, and MT attachment area decreases *pari passu* until late anaphase (Fig. 9). Throughout anaphase there is also an increase in splaying of the kMTs (Table I; also visible in *Haemanthus* with special light microscope techniques; 24), as well as an increase in the number of short nkMTs to the side of the kinetochore and behind it (as shown also by counts of MTs behind the kinetochore; 30). However, no extremely short kMTs are detectable. All these observations together strongly suggest that the peripheral kMTs are detached from the kinetochore and

left behind as the chromosomes move toward the poles during anaphase. The observations do not rule out the possibility that during anaphase the peripheral kMTs disassemble more rapidly than those in the center of the kinetochore, although disassembly would have to occur right up to the insertion of the MT into the kinetochore. Such a possibility is difficult to conceive unless the MTs were to become detached from the kinetochore first, since anchored spindle MTs are probably more stable than those MTs which are free at both ends (33).

The decrease in length of kinetochore fibers during anaphase and telophase (3) probably results from disassembly of MTs. Extremely short kMTs seen in longitudinal sections of prometaphase-metaphase (Figs. 1*b* and 4*d*) and telophase (Fig. 7*c*) cells may result from either of two processes: either they may represent MTs in the process of elongation or disassembly respectively at the time of fixation, or they may have been broken near the kinetochore.

On the basis of an analysis of three-dimensional spindle reconstructions, Fuge (23) also concluded that fragmentation of autosomal kMTs occurs during anaphase in crane fly spermatocytes, resulting in a progressive disorganization of the kinetochore fiber. Because there was no associated decrease in kMT number, he concluded that the MTs fragmented distal to the kinetochore. A progressive disorganization of the kinetochore fiber during anaphase has also been described in rat kangaroo PtK (56) and plant meiotic material (13). Such a disorganization, plus a decrease in number of kMTs, might be responsible for the progressive decrease in birefringence of the chromosomal fiber during anaphase described for a variety of cells (reviewed in reference 28). However, since other factors, such as variations in the density of MTs, as well as the presence of nonmicrotubule elements in the spindle and fixation artifacts, may influence spindle birefringence, no definite correlations should be attempted at present (see reference 46 for a recent discussion and references to earlier papers concerning the problems encountered in correlating spindle fiber birefringence with the number and arrangement of MTs). The changes in density of kMTs noted in this study may suggest a justified caution in interpreting results obtained with polarized light.

If kMTs are detached from the kinetochore during anaphase and left behind as the kinetochores move toward the poles, the same MTs which functioned originally as kMTs during metaphase and the first part of anaphase would become classified, as to both position and function, as nkMTs in later anaphase. Also, since similar numbers of kMTs are attached not only to sister kinetochores in metaphase but also to homologous chromosomes in anaphase, even though the kMT number has decreased from that found in metaphase cells, the loss of MTs from the kinetochore during anaphase appears to be a precisely regulated process.

There is little information regarding the number of MTs per kinetochore at different stages of division in other organisms. Although preliminary data suggest that there is no decrease in kMT number during the first half of anaphase in rat kangaroo PtK cells (41, 56; C. Jensen, unpublished data), further careful analysis is required to verify these observations. In crane fly spermatocytes the number of MTs per kinetochore remains the same for the autosomes during anaphase, whereas the number of kMTs attached to the late-segregating sex chromosomes decreases during their anaphase I movement (20). The autosomes move at the periphery of the spindle, whereas the sex chromosomes move in late anaphase within the central region of the spindle and are thus completely surrounded by nkMTs during their anaphase movement. Therefore, differences in the

spatial relationship between kMTs and nkMTs in various regions of the spindle as well as in the spindles of different organisms may be a factor in determining where, within the kinetochore fiber, MT rearrangement occurs. Thus, throughout anaphase, kMTs of *Haemanthus* may be detached from the kinetochore at their insertion, whereas they may become fragmented distal to the kinetochore in crane fly, as suggested by Fuge (20), as well as in PtK cells. Clearly, further analysis is necessary to explore this interesting possibility.

DELAYED KINETOCHORES: This study confirms preliminary observations (30) that lagging and bridge chromosomes possess more attached MTs and a greater area of MT attachment than do chromosomes closer to the poles in the same cell (Table II). This finding suggests that kMT number and area of MT attachment are related to the position of the chromosome with respect to the spindle long axis. However, as these chromosomes possess fewer kMTs than do anaphase chromosomes in other cells which have moved an equivalent distance, it is likely that loss of MTs from the kinetochore during anaphase occurs even when the chromosome is stationary or moving more slowly and, therefore, is related to time as well as position of the chromosome along the spindle axis.

C-SHAPED TERMINATIONS: In *Haemanthus*, the frequency of C-shaped terminations of kMTs within the kinetochore is highest in early prometaphase, progressively decreases to zero during anaphase, and increases again in telophase (Fig. 8). Because the number of C-shaped terminations consistently varies throughout division in *Haemanthus*, it is unlikely that they represent artifacts of fixation. These observations would not be inconsistent with previous suggestions (12, 22, 25, 30) that C-shaped MTs represent sites of assembly or disassembly; therefore, their frequency and distribution may reflect different rates of MT assembly and disassembly during the course of division, being highest in early prometaphase and midtelophase, respectively. Also, my results would suggest that kMTs may undergo assembly and disassembly at their proximal ends within the kinetochore, a concept not suggested by data derived from studies in vitro (9), but consistent with at least one model for mitosis (47) and with the observations on intrinsic polarity of kMTs in PtK cells examined with electron microscopy (16). The polarity of *Haemanthus* kMTs is probably the same as that of PtK cells (15, 16).

Chromosome Size and kMT Number

This study suggests that there may be a relationship between chromosome size and number of MTs attached to the kinetochore in metaphase cells of *Haemanthus* (Fig. 10). It is quite likely that the observed range in number of MTs per kinetochore at metaphase and anaphase is related to the range in chromosome size. Assuming that the counts presented in Table II were drawn randomly from the chromosome set, the proportion of higher to lower counts is the same as the proportion of larger to smaller chromosomes in the *Haemanthus* chromosome set of 27 (three of the longest chromosomes: Type A; three long chromosomes: Type B; three medium chromosomes: Type C; three medium chromosomes with subterminal kinetochore: Type D; and 15 small chromosomes: reference 2). It was not possible to obtain enough data for statistical analysis since the data presented in Fig. 10 were obtained by a time-consuming analysis of all kMTs in serial longitudinal sections through the kinetochore region of specific chromosomes whose length could be measured accurately from both light and electron micrographs. It would have been extremely difficult, and perhaps impossible, to obtain sufficient data from trans-

verse sections because of the large size of *Haemanthus* chromosomes (7 μm to $>20 \mu\text{m}$ long at metaphase) and the difficulty of locating both sister kinetochores of specific chromosomes cut in transverse section. Therefore, my results should be regarded as suggesting only that there may be a trend for the larger chromosomes to have more MTs attached.

Heath (26) and Fuge (22) have also suggested that there may be a correlation between chromosome sizes of different organisms and the number of MTs attached to the kinetochore. Moens (48) found that, although no strict proportionality exists between chromosome size and kMT number in insect mitotic and meiotic cells, there is a general correlation in that the larger chromosomes have more kMTs attached than do the smaller ones. He suggests that, in order to ensure flexibility, chromosomes have more attached MTs than the minimum needed to move the chromosome. His data also indicate that there is more likely to be a significant correlation in cells which were in late metaphase-very early anaphase than in earlier stages.

Clearly, more data on chromosome size and MT number are required to fully resolve the question. It is hoped that my results may serve as a stimulus to more of such analyses. Further studies could best be carried out with computer analysis using serial transverse sections of a cell with a small number of chromosomes with great differences in size, as done by Moens (see reference 48 for a further discussion of the implications of such studies). In attempting such analyses one must: (a) be absolutely sure of the stage of division, since the maximum number of kMTs may not be present on all chromosomes until late metaphase, and (b) be sure to differentiate between kMTs and nkMTs to obtain an accurate count of kMTs.

Possible Mechanisms of Chromosome Movement in *Haemanthus*

These studies indicate that movement of the chromosomes in *Haemanthus* must be accompanied by a pronounced rearrangement and assembly/disassembly of the constituent MTs. They also emphasize that all observed variations in MT arrangement which occur in various organisms (and how these change throughout mitosis) are features which must be considered in any postulated mechanism(s) of chromosome movement. Possible mitotic mechanisms which might be applicable to *Haemanthus* are: (a) sliding of MTs (43, 51); (b) specific patterns of MT assembly/disassembly, with or without concomitant MT sliding (28, 45, 47, 53); (c) lateral interaction ("zipping") of MTs (5, 8); and (d) non-MT spindle components acting upon a microtubular skeleton.

Sliding between MTs of opposite polarity during spindle elongation is a distinct possibility in the central spindle of diatoms (39, 45, 62). However, the arrangement of spindle MTs in *Haemanthus* does not resemble that of the almost parallel, highly cross-bridged MTs of the diatom spindle, nor does the distribution of nkMTs at anaphase in *Haemanthus* suggest a simple sliding in these cells (30).

If sliding between MTs does occur in *Haemanthus*, it would be more likely to be in concert with time-dependent patterns of assembly and disassembly of MTs in specific regions of the spindle, as suggested for several models of mitosis (45, 47, 53). The frequency of C-MTs at the proximal end of the kinetochore fiber at different stages of division in *Haemanthus* (Fig. 8) is consistent with the model of Margolis et al. (47), which predicts that assembly of MTs proximally within the kinetochore occurs during prometaphase and metaphase, and ceases at anaphase.

The recent demonstrations of MT polarity in *Haemanthus* and PtK₁ cells (15, 16) are also consistent with this theory.

My observations (including that of the intermingling of kMTs and nkMTs, the loss of MTs from the periphery of the kinetochore during anaphase, increased splaying of kMTs throughout anaphase-telophase, and increase in number of short MTs near the kinetochore during anaphase) are wholly consistent with the "zipper" hypothesis of spindle function (5, 8). This theory includes the premise that lateral interaction between kMTs and nkMTs angled to each other at the periphery of the kinetochore fiber results in movement of the chromosomes, accompanied by breakage and displacement of MTs.

The data for *Haemanthus* can not rule out the possibility that chromosome movement is accomplished by a non-MT spindle component, such as actin (as discussed in 59) or MT-associated protein, with the MTs playing a skeletal role. If so, the MT "skeleton" must be viewed as a structure which changes constantly and predictably throughout mitosis.

All the above models for mitosis incorporate the presence of linkages between MTs, to either bind MTs together or act as mechanochemical arms to bring about shearing between MTs. Intertubule cross-bridges have been clearly demonstrated using microdensitometer-computer correlation analysis (14). In *Haemanthus* they occur extensively between parallel MTs of the non-kinetochore bundles. They are also seen in limited regions both between kMTs within the kinetochore fiber, and between kMTs and nkMTs at the periphery of the fiber (C. Jensen, unpublished observations). These findings are entirely consistent with the zipper model.

I am deeply indebted to A. Bajer and J. Molé-Bajer for providing me with the embedded cell preparations and for many helpful discussions during the course of this research. I thank K. Speak and J. Fillery for technical assistance, R. Jameson, A. Ellis, and I. MacDonald for help in producing the illustrations, R. Baker for help with the statistical analysis, and J. Westbrooke for typing the manuscript. I am especially grateful to J. B. Carman for his extremely helpful comments during preparation of the manuscript.

The work was supported by a grant from the Medical Research Council of New Zealand, as well as National Science Foundation Grant Number GB-24 996X and National Institutes of Health grants GM 21741 and GM 26121 to A. Bajer.

Received for publication 23 December 1980, and in revised form 20 July 1981.

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