Polarity of Kinetochore Microtubules in Chinese Hamster Ovary Cells after Recovery from a Colcemid Block

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ABSTRACT The polarity of kinetochore microtubules was determined in a system for which kinetochore-initiated microtubule assembly has been demonstrated. Chinese hamster ovary cells were treated with 0.3 μg/ml colcemid for 8 h and then released from the block. Prior to recovery, microtubules were completely absent from the cells. The recovery was monitored using light and electron microscopy to establish that the cells progress through anaphase and that the kinetochore fibers are fully functional. Since early stages of recovery are characterized by short microtubule segments that terminate in the kinetochore fibrous corona rather than on the outer disk, microtubule polarity was determined at later stages of recovery when longer kinetochore bundles had formed, allowing us to establish unambiguously the spatial relationship between microtubules, kinetochores, and chromosomes. The cells were lysed in a detergent mixture containing bovine brain tubulin under conditions that allowed the formation of polarity-revealing hooks. 20 kinetochore bundles were assayed for microtubule polarity in either thick or thin serial sections. We found that 95% of the decorated kinetochore microtubules had the same polarity and that, according to the hook curvature, the plus ends of the microtubules were at the kinetochores. Hence, the polarity of kinetochore microtubules in Chinese hamster ovary cells recovering from a colcemid block is the same as in normal untreated cells. This result suggests that microtubule polarity is likely to be important for spindle function since kinetochore microtubules show the same polarity, regardless of the pattern of spindle formation.

The determination of the structural polarity of microtubules (MT) in situ has recently become possible through the development of two new techniques. These techniques reveal the intrinsic polarity of MT by decorating them with either dynein (16) or hook-like appendages of tubulin (17, 19). With these techniques, the structural polarity of MT has been analyzed in a number of different systems, and it has been shown that the polarity is remarkably uniform with respect to an organizing center (4, 11, 18). Following the convention of Borisy (2), the ends of an MT are designated plus or minus by comparison with reference MT, namely those of a flagellum. The end of a flagellar MT distal to the cell is designated plus, and the proximal end is designated minus. The polarity results indicate that > 90% of MT in interphase cells have their plus ends distal to their organizing centers. In the mitotic spindle the polarity is also uniform: Plus ends always face away from the pole (9, 10, 12, 19, 28).

This latter finding, which has been confirmed for three different cell types (PtK_1, surf clam eggs, and *Haemanthus* endosperm), raises several interesting questions. It suggests that the structural relationships between the MT and the two organizing structures of the mitotic spindle are different, namely that centrosomes are attached to MT at their minus ends and that kinetochores are attached to MT at their plus ends. This conflicts with earlier in vitro data from which, on the basis of MT growth rates, it was concluded that the kinetochore, like the centrosome, is attached to MT at their minus ends (1, 27).

In addition, it is at first sight difficult to relate the in situ findings to the apparent capacity of kinetochores to initiate or nucleate MT growth in vivo (6, 31). The in situ polarity findings could be explained simply if the mechanism by which kinetochores acquired MT was to attach to MT that formed at the poles. But then, what interpretation can be made of the evident ability of kinetochores to also nucleate MT in vivo? There seem to be two general possibilities. If kinetochores

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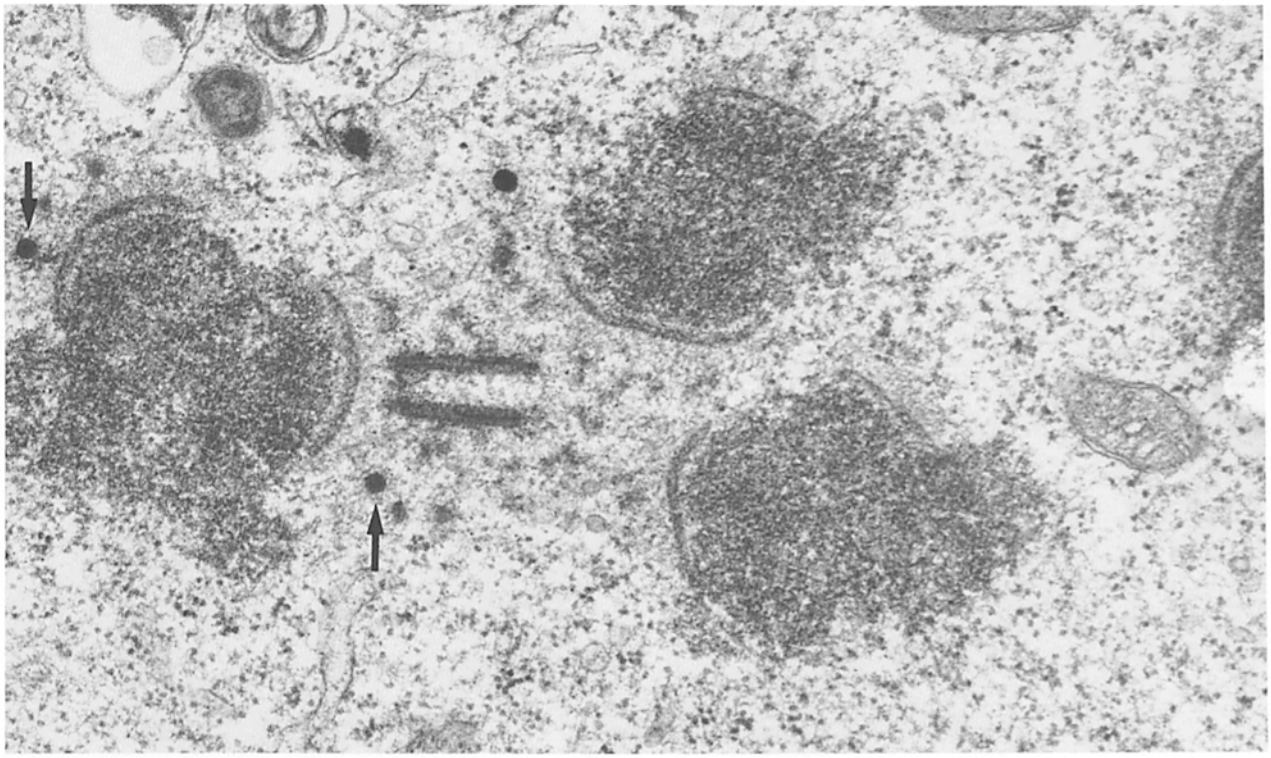

FIGURE 1 Thin section of aCHO cell blocked in mitosis after 8 h of colcemid treatment. The centriole is surrounded by a cloud of flocculent material. Some virus-like particles are present (arrows). Note the fibrous corona at all kinetochores. No MT are seen. × 37,000.

nucleate MT that have their plus ends at the kinetochores (as in untreated cells), then it has to be postulated either that MT grow at their minus (distal) ends or that new subunits are added at the kinetochores. On the other hand, if kinetochores nucleate MT that have their minus ends at the kinetochores (opposite to that in untreated cells), then it has to be concluded that the polarity of kinetochore MT is not intrinsic to the structure of the spindle, but depends on the kinetic path by which it is formed.

In the light of these considerations, we found it important to determine the polarity of kinetochore MT in cells where MT nucleation at the kinetochores had been demonstrated in vivo. This kinetochore-associated nucleation occurred during the recovery from treatment with the MT-depolymerizing drugs, colcemid and nocodazole. For practical reasons we chose the system used by Witt et al. (31) to study the polarity of MT nucleated at kinetochores during the recovery from colcemid because in this system, a large number of mitotic cells can be assayed easily in the electron microscope.

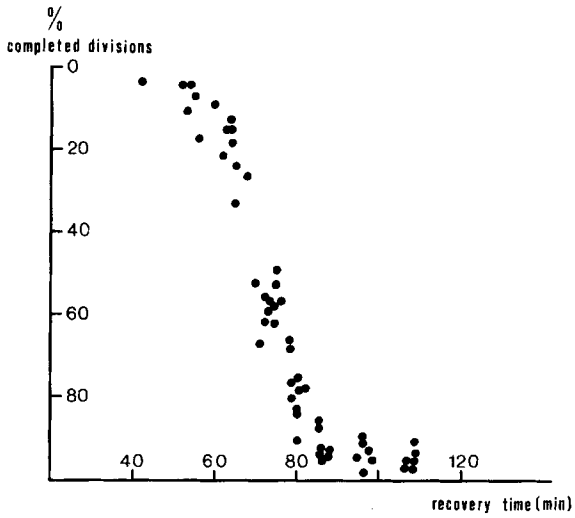

FIGURE 2 Time course of recovery for CHO cells after release from a colcemid block. The number of dividing cells was determined for various time points of recovery. Data from 10 sets of micrographs (three different experiments) were pooled for this plot. Most cells divided between 60 and 80 min after removal of the colcemid block.

MATERIALS AND METHODS

Cells: Chinese hamster ovary (CHO) cells, clone 13, were obtained from Dr. Smithies' laboratory, Department of Genetics, University of Wisconsin. They were grown in plastic tissue culture flasks in Ham's F-10 supplemented with 10% fetal calf serum (KC Biological, Inc., Lenexa, KS), 1% nonessential amino acids (Gibco Laboratories, Grand Island, NY), and a combination of antibiotics (600 U penicillin/ml; 10^6 U streptomycin/ml; Gibco Laboratories).

Tubulin Preparation: We prepared microtubule protein from bovine brain by modifying the method of Shelanski et al. (25). Twice-cycled protein was stored as a pellet at −70°C for subsequent use. To prepare the tubulin used to decorate cellular MT, the microtubule protein was cycled once in PMEG (0.5 M PIPES, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM GTP, pH 6.8) and then spun at 250,000 *g* for 2.5 h at 4°C. The resulting high-speed supernatant, which is called tubulin, was frozen in small aliquots and kept in liquid nitrogen.

Protein concentrations were determined according to the method of Bradford (3).

Experiments: All cells used in our experiments were treated with 0.3 μg/ml colcemid (Ciba-Geigy Corp., Pharmaceutical Div., Summit, NJ) for 8 h to disassemble all MT (31). After 1 h all rounded cells were discarded from the dish to remove the cells that were in mitosis when the drug was first added. After 8 h of colcemid treatment the blocked cells were collected by centrifugation at 400 *g* for 4 min and then either fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 30 min to serve as controls, or rinsed two times in fresh medium and then incubated at 37°C for the recovery.

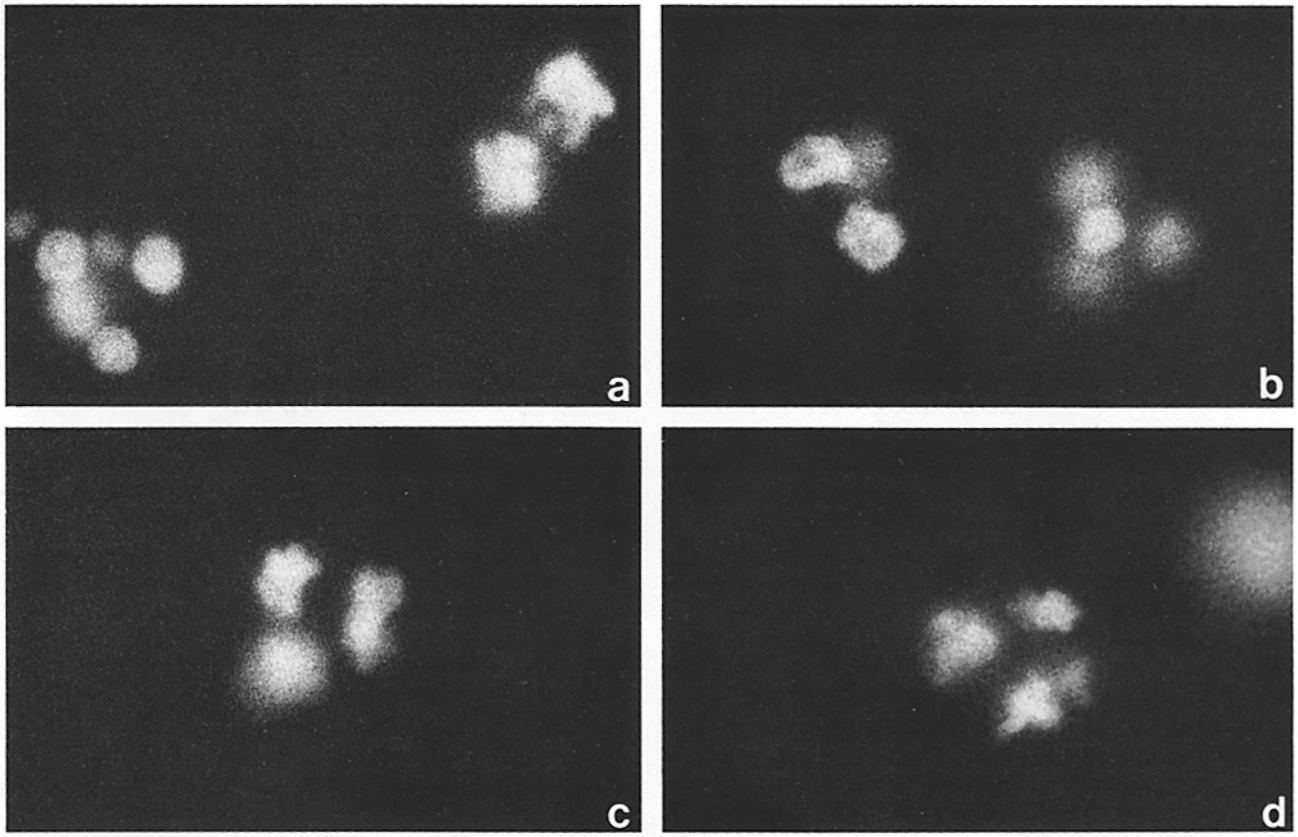

FIGURE 3 Localization of ethidium bromide-stained material in lysed CHO cells after ~ 80 min of recovery. The distribution of the ethidium bromide fluorescence indicates that the number of multipolar divisions is high. (a) Bipolar and multipolar division; (b) tripolar and tetrapolar division; (c) tripolar division; (d) tetrapolar division. × 1,250

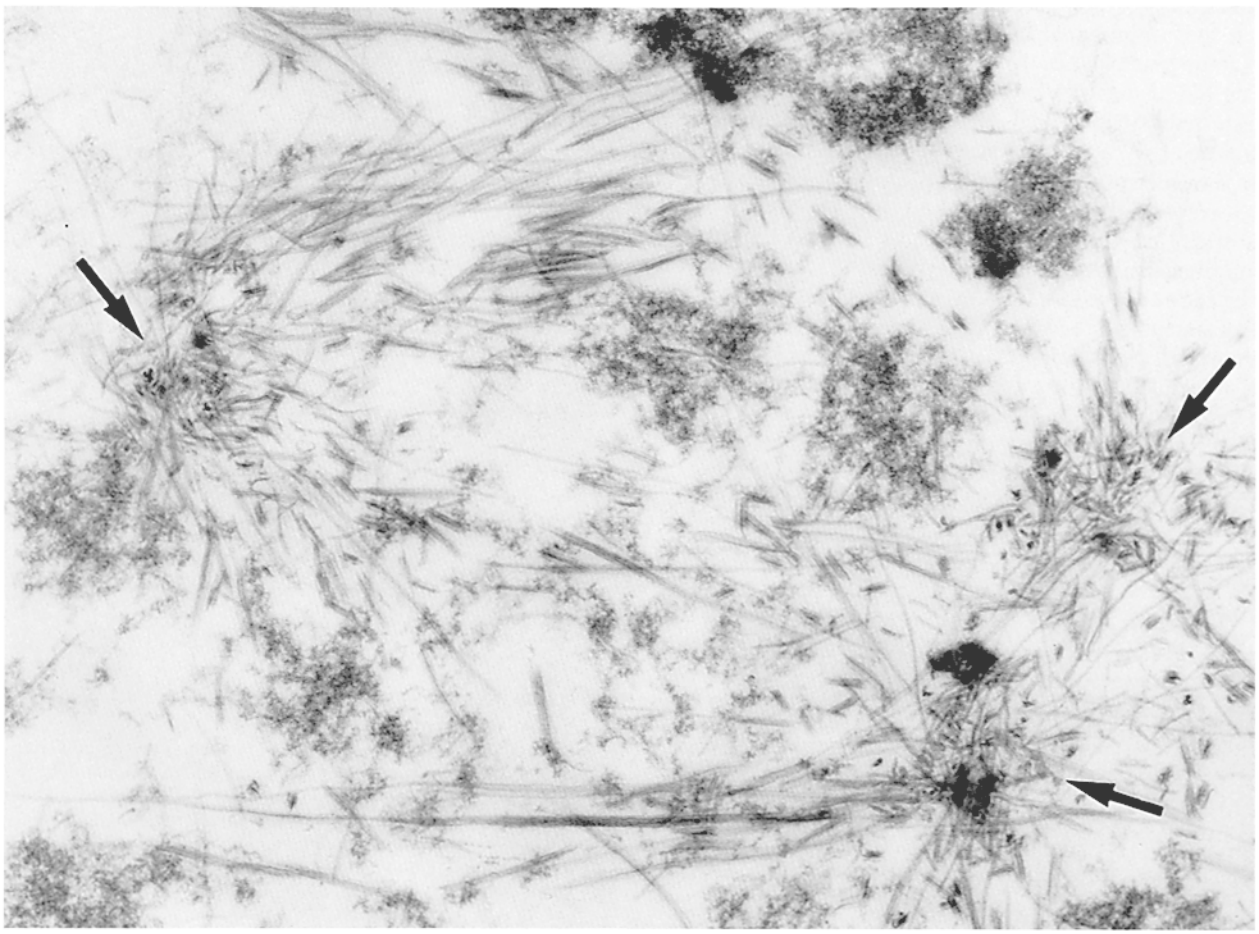

FIGURE 4 Thick section of a mitotic CHO cell that was lysed and treated with the hook-forming solution. Three polar areas can be distinguished in this plane (arrows); a total number of six poles was found in this cell. Note partial decondensation of the chromatin due to the treatment with 0.5 M PIPES. × 20,000.

The cells were monitored using phase contrast microscopy to determine the time course and frequency of recovery, and in some cases they were stained with ethidium bromide after lysis with Triton X-100 to assay chromatin distribution. For the latter experiment it was necessary to hold the round cells in place by means of a fibrin clot (13, 14). Some cells were prepared for electron microscopy after various times of recovery.

For the determination of MT polarity, cells were pelleted after 45 min of recovery, resuspended in 20 μl of phosphate buffer, and incubated with 200 μl of tubulin (1.2 mg/ml) in PMEG containing 1% Triton X-100, 0.5% deoxycholate, 0.02% SDS, and 2.5% dimethyl sulfoxide for 7 min at 28°C.

Cells were fixed by addition of 1 ml of 2% glutaraldehyde in 0.1 M PIPES, 1 mM $MgCl_2$, and 1 mM EGTA. Cells were postfixed and processed for electron microscopy as described earlier (8). All lysed cells were treated with 1% tannic acid after the glutaraldehyde fixation. Serial thick (0.25 μm) and thin (0.09 μm) sections were made on either a Sorvall MT2B (Dupont Co., Wilmington, DE) or a Reichert UM 3 ultramicrotome with a diamond knife. Thick sections were stained with 7.5% uranyl magnesium acetate at 60°C for 1 h followed by lead citrate for 15 min and subsequently carbon coated. They were observed and photographed at 1 MeV with the AEI-7 high voltage electron microscope at the Madison High Voltage Electron Microscope Laboratory. Thin sections were assayed with a Philips 300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) equipped with a tilting stage. Handedness of hooks on MT was determined at × 30,000–40,000.

RESULTS

We followed the protocol of Witt et al. (31) closely and repeated some of the controls because it seemed essential to verify the following prerequisites for our experiments: (*a*) No MT should be present in the cells at the end of the colcemid treatment. This was a necessary condition to observe subsequent nucleation of MT at the kinetochores. (*b*) The cells should recover from the colcemid block and progress through anaphase. This would indicate that the kinetochore fibers were fully functional.

We confirmed that CHO cells fixed after 8 h in 0.3 μg/ml colcemid did not contain any MT, as previously documented (31). In both thin (Fig. 1) and thick sections MT were absent. This was true for the vicinity of both centrosomes and kinetochores, regions where MT tend to be most resistant toward depolymerizing agents. The morphology of centrosomes and kinetochores corresponded to that described by Witt et al. (31). Kinetochores had a trilaminar substructure and a fibrous corona (Fig. 1). The centrioles were surrounded by a cloud of electron-dense material that often appeared flocculent. Centrosome-associated virus-like particles (30) were not always found in the immediate vicinity of the diplosomes. Some particles were regularly seen at some distance from the centrioles, occasionally near a kinetochore.

Within 45 min after removal of the drug, mitotic cells were characterized by the presence of abundant MT. To establish that the kinetochore MT were also functional, cells were placed in a petri dish for recovery and monitored by light microscopy. The cells divided 60–90 min after the mitotic inhibitor was removed (Fig. 2). About 90% of them underwent cytokinesis and began to spread on the substrate. As described earlier (31), an unusual abundance of multipolar divisions was noticed. To show that regular chromatin distribution did occur we used ethidium bromide to localize nucleic acids in the dividing cells. Fig. 3, *a–d* is an example of the patterns of chromatin distribution that were observed in recovering cells. The images suggest that all daughter cells contained chromatin. Proper chromatid segregation during anaphase was also confirmed at the fine-structural level (data not shown).

The inspection of lysed cells used for the polarity determination supported the observation that most spindles in our experiments contained more than two poles (Fig. 4). In the six cells studied in serial thick sections, either five or six poles were found. (For the purposes of this paper a pole is defined as a focal point for nonkinetochore MT and/or bundles of kinetochore MT.) Not all poles contained centrioles (20, 32).

All data on kinetochore MT polarity were obtained from cells that recovered for 45 min after the colcemid treatment. Since CHO cells round up during division it was not possible to select particular cells in a favorable orientation prior to sectioning. Further, due to the nature of the recovery process, kinetochore MT bundles were not restricted to two well-defined halfspindles, and screening of larger areas as in other studies of kinetochore MT polarity (9, 12, 28) was not feasible. Therefore, kinetochore MT bundles that could be tilted into cross-sectional view were identified close to a kinetochore and traced for ~0.7 μm. A tracing of kinetochore MT over longer distances was hindered because, as a result of cell lysis, MT did not remain very straight over the whole length of the bundle, and also because other spindle MT intermingled with the kinetochore MT.

The conditions used for hook formation (12, 19) affected chromosome and kinetochore organization, and kinetochore MT often elongated through the kinetochore and chromatid (Fig. 5). Therefore, it was necessary to determine unambiguously the spatial relationship between the chromosome, kinetochore, and MT bundle. Two different sequences of images were found while following a set of consecutive sections:

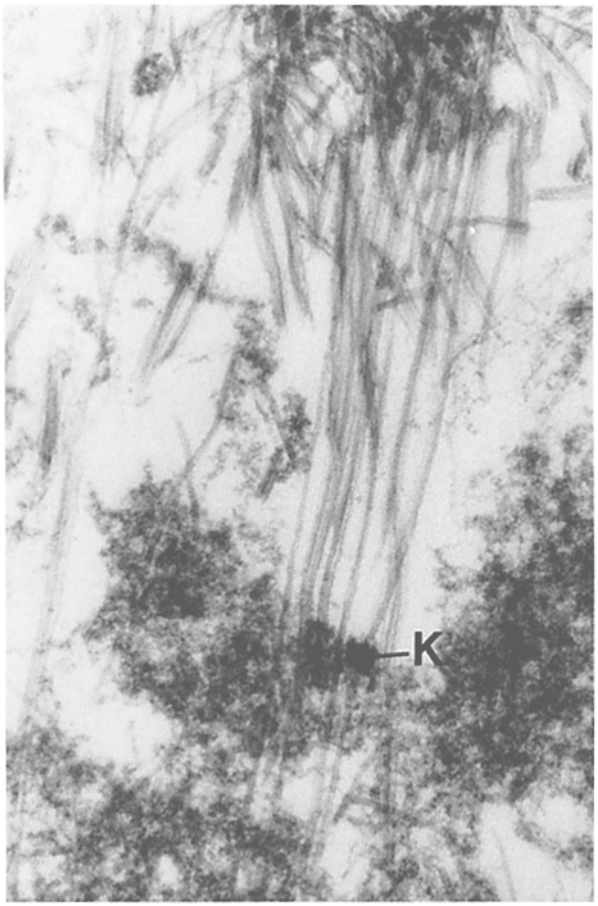

FIGURE 5 Thick section of a kinetochore fiber that was decorated with hooks. The kinetochore (*K*) is slightly more electron dense than the rest of the chromosome. Some kinetochore microtubules have elongated under the hook-forming conditions through the kinetochore and the adjacent chromatin. × 35,000.

kinetochore MT bundle, kinetochore MT at the kinetochore, and kinetochore MT within the chromatin (Fig. 6); or the reverse, kinetochore MT in the chromatin, kinetochore MT at the kinetochore, and kinetochore MT bundle (Fig. 7). If the first sequence of images is found, one is approaching a kinetochore, whereas the second sequence showed that one was moving away from it. We predominantly used thin sections for this part of the study because it was easier to identify the kinetochores unambiguously when viewed parallel to their surface. In our preparations kinetochores had a less fibrillar substructure than the surrounding chromatin and usually appeared slightly denser and darker (*k* in Figs. 6, *c* and *d* and 7 *d*).

We were able to identify 20 kinetochores with their attached MT and we analyzed the MT in the bundle for polarity. Table I summarizes these data. The number of MT per kinetochore varied but their average number, 11, was very similar to that found in untreated cells (14), cells recovering from colcemid block (12), and cells lysed after recovery from colcemid treatment (13; data from 32). The table shows that 67% of the kinetochore MT was decorated with hooks and that 94.6% of these had the same polarity with respect to the kinetochores, namely the plus ends were at the kinetochore.

We had wished to also analyze the polarity of kinetochore MT at earlier time points in the recovery since a participation of polar MT in the formation of the kinetochore fiber during later recovery stages cannot be totally ruled out based on the fine -structural data available. However, 15 or 30 min after release from the colcemid block most MT seen in the vicinity of a kinetochore were not attached to the kinetochore disk itself but were embedded in the fibrous corona and their orientation with respect to the kinetochore disk was not regular (31). It would therefore be very difficult to determine the polarity of these short MT. The number of MT per kinetochore was comparatively low and the extent of recovery at these time points showed greater variation from cell to cell than later in the recovery process (compare Fig. 8 with Fig. 11, in reference 31). Therefore, 45 min after release from the colcemid block was the earliest point in recovery where we could reasonably obtain data.

DISCUSSION

We have shown here that the polarity of kinetochore MT in CHO cells recovering from a colcemid block is the same as the polarity of kinetochore MT in untreated cells (9, 12, 28)—namely, in both systems the plus ends of the MT are at the kinetochores. For our study we used a system for which kinetochore-associated assembly was demonstrated previ-

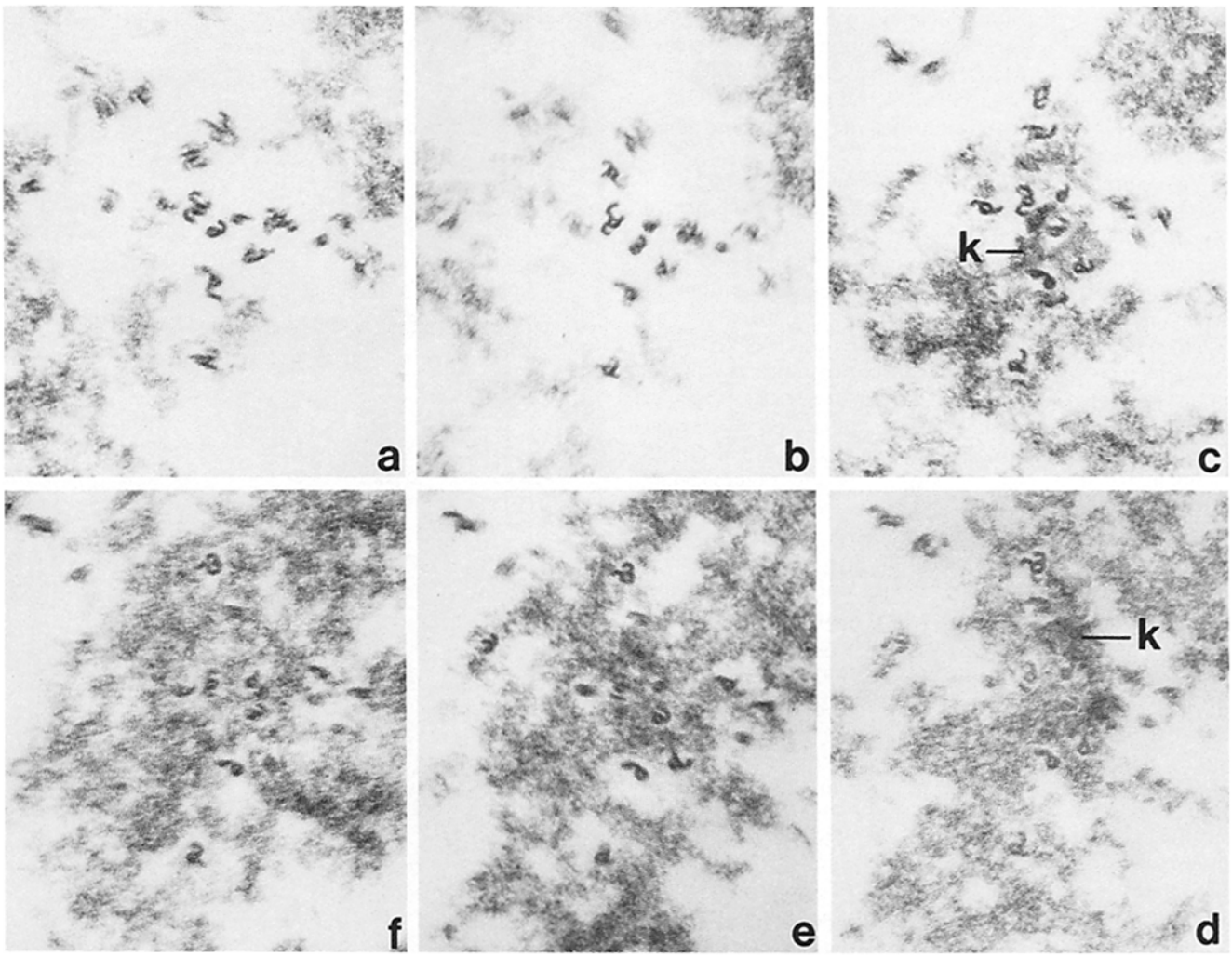

FIGURE 6 Series of thin sections through a kinetochore microtubule bundle presented in the sequence of sectioning. (*a* and *b*) Kinetochore bundle; (*c* and *d*) kinetochore microtubules at the kinetochore (*k*); (*c* and *f*) kinetochore microtubules in the chromatin. Most kinetochore microtubules are decorated with counterclockwise-curving hooks. × 65,000.

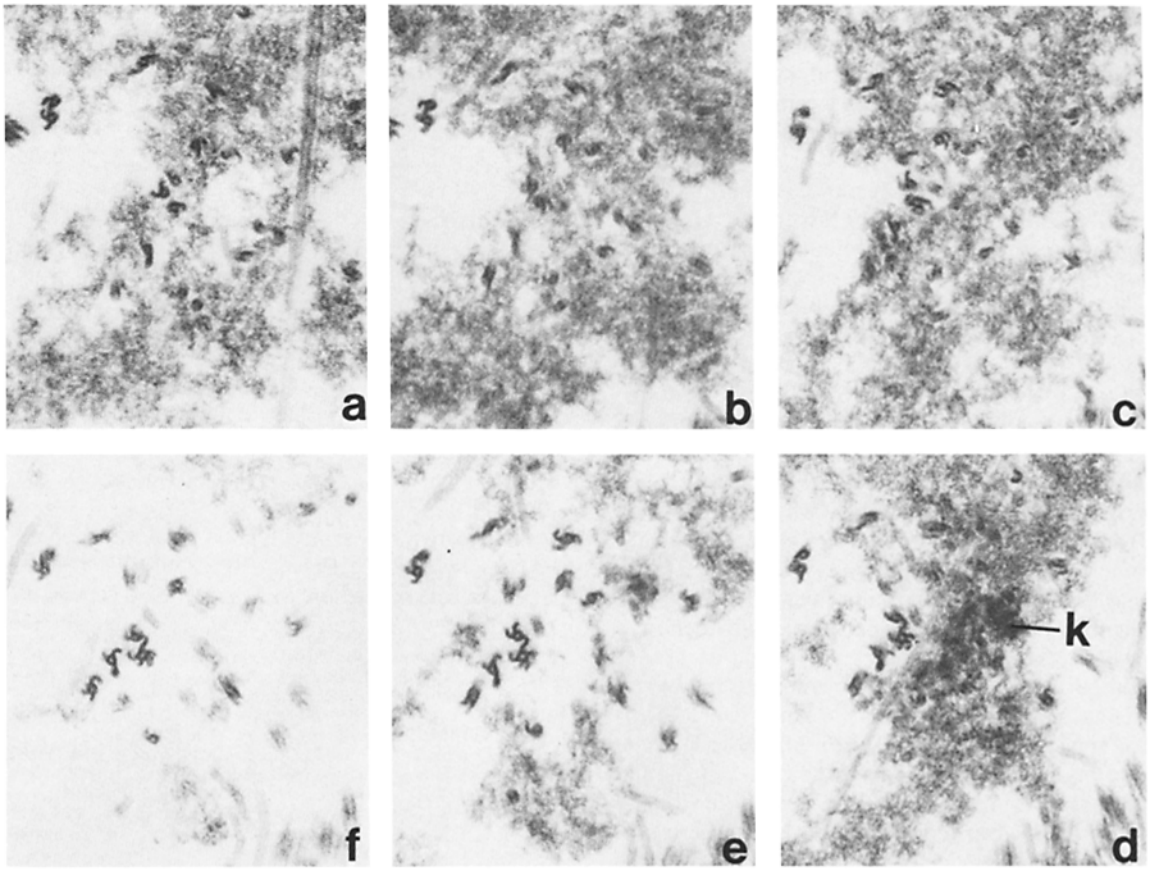

FIGURE 7 Series of thin sections through a kinetochore fiber approaching the kinetochore from the back (the chromatin side) (*a–c*) kinetochore microtubules in the chromosome; (*d*) kinetochore microtubules at the kinetochore (*k*); (*e* and *f*) kinetochore microtubule bundle. Most kinetochore microtubules bear clockwise-curving hooks. × 52,000.

ously (31). Electron microscopy was used to confirm that no MT were present in arrested cells after 8 h of colcemid treatment, and therefore that MT growth onto kinetochores did not occur by addition to pre-existing MT stubs. The recovery following the block was monitored using light and electron microscopy to prove that chromatids segregated during anaphase, thereby indicating that the kinetochore MT were functional and could be compared to kinetochore MT in untreated cells.

Although according to these criteria the recovery process looked normal, we observed an unusually high number of multipolar divisions. This effect, which has been described before (26), seems to depend on the duration of drug treatment more than on the colcemid concentration used. Based on the evidence available, there are two possible explanations for the increased number of poles found in most of the spindles: (*a*) drug-induced dispersion of pericentriolar material. If the centrosome-associated virus-like particles first described by Wheatley (30) indicate the presence of organizing centers, as suggested by Gould and Borisy (15), then cells recovering from colcemid treatment do indeed have an increased number of poles at the onset of recovery. Some cells contained as many as 10 different clusters of virus-like particles distributed throughout the cell. (*b*) Poles are randomly created by the convergence of MT bundles. Several bundles of elongating kinetochore MT (similar to Fig. 11 in reference 31) may be able to establish a pole area because they run into each other. Thus, pole formation would be correlated with

TABLE I

Polarity of Kinetochore Microtubules

Bundle	Total No. of MT	No. of MT with hooks	No. of MT with plus end at kinetochore	No. of MT with minus end of kinetochore	Ambiguous
a	12	9	8	—	1
b	14	10	9	1	—
c	12	10	8	1	1
d	10	9	8	1	—
e	9	6	6	—	—
f	10	5	5	—	—
g	15	13	13	—	—
h	11	6	6	—	—
i	12	8	8	—	—
j	16	8	8	—	—
k	13	8	8	—	—
l	11	6	5	—	1
m	12	8	7	—	1
n	10	10	10	—	—
o	9	5	5	—	—
p	8	7	7	—	—
q	11	8	7	—	1
r	10	7	6	—	1
s	11	10	10	—	—
t	10	5	5	—	—

The average number of MT in a kinetochore bundle was 11.3. 94.6% of the decorated MT had their plus end at the kinetochore.

kinetochore activity. Although it seems more straightforward to assume that a pole is in fact a specific entity or organelle with MT-initiating capacity, rather than the result of convergence of MT bundles by an as yet unspecified mechanism, the available evidence does not permit us to decide between these two possibilities.

It is not known how MT are organized into kinetochore bundles in cells recovering from drug treatment. However, any hypothesis concerning their formation has to explain two observations: (*a*) Early stages in the recovery process are characterized by short MT situated in the so-called corona and oriented more or less randomly with respect to the kinetochore disk (6, 23, 31). (*b*) The polarity of the kinetochore MT grown to a length where they are clearly identified as a bundle is identical to that of kinetochore MT in untreated cells.

So far, all MT whose polarity has been determined had their plus ends distal to the site endowed with the organizing capacity (4, 11, 18, 19). Based on this observation and additional morphological and experimental evidence (5, 21, 22, 24, 29), it has been proposed that kinetochore MT are polar MT that become attached to the kinetochore during prometaphase. According to this proposal, kinetochores do not, or only to a minor extent, participate in MT initiation. However, this hypothetical scheme of the origin of kinetochore MT is certainly not applicable to cells recovering from nocodazole (6, 7) or colcemid (31) where, independent of centrosomal activity, short MT form first in the fibrous corona of the kinetochore. Since the arrangement of these MT suggests that they are not initiated at the kinetochore disk directly, we think the easiest way to explain the results on kinetochore MT polarity is to postulate that kinetochore disks bind to the plus ends of MT preferentially. Hence, the short MT observed first in the fibrous corona would attach later in the "correct" orientation with their plus ends to the kinetochore disk.

The results presented here suggest that MT polarity might be an important factor for spindle function because kinetochore MT show the same polarity regardless of the pattern of spindle formation. In addition, the structural polarity of MT apparently nucleated at kinetochores contains implications as to how these MT grow. Either they grow at their minus (distal) ends or new subunits are added at the kinetochores.

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