Structural Polarity of Kinetochore Microtubules in PtK_1 Cells

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ABSTRACT The polarity of kinetochore microtubules (MTs) has been studied in lysed PtK_1 cells by polymerizing hook-shaped sheets of neurotubulin onto the walls of preexisting cellular MTs in a fashion that reveals their structural polarity. Three different approaches are presented here: (a) we have screened the polarity of all MTs in a given spindle cross section taken from the region between the kinetochores and the poles, (b) we have determined the polarity of kinetochore MTs selectively in cold-treated spindles; this approach takes advantage of the fact that kinetochore MTs are more stable to cold treatment than other spindle MTs; and (c) we have tracked bundles of kinetochore MTs from the vicinity of the pole to the outer layer of the kinetochore in cold-treated cells. In an anaphase cell, 90–95% of all MTs in an area between the kinetochores and the poles are of uniform polarity with their plus ends (i.e., fast growing ends) distal to the pole. In cold-treated cells, all bundles of kinetochore MTs show the same polarity; the plus ends of the MTs are located at the kinetochores. We therefore conclude that kinetochore MTs in both metaphase and anaphase cells have the same polarity as the aster MTs in each half-spindle.

These results can be interpreted in two ways: (*a*) virtually all MTs are initiated at the spindle poles and some of them are "captured" by matured kinetochores using an as yet unknown mechanism to bind the plus ends of existing MTs; (*b*) the growth of kinetochore MTs is initiated at the kinetochore in such a way that the fast growing MT end is proximal to the kinetochore.

Our data are inconsistent with previous kinetochore MT polarity determinations based on growth rate measurements in vitro. These studies used drug-treated cells from which chromosomes were isolated to serve as seeds for initiation of neurotubule polymerization. It is possible that under these conditions kinetochores will initiate MTs with a polarity opposite to the one described here.

The majority of the recent models for mitosis propose mechanisms for chromosome and pole movement that are based upon microtubules (MTs) (3, 12, 22, 23, 27, 29, 34). Several of these models assume that the polarity of different spindle MTs is an important factor in force generation (23, 27, 29, 34). Attempts have been made, therefore, to develop methods to determine the polarity of spindle MTs to see whether the real polarities conform to the predictions of any of the models. The polarity of an MT is due to its construction out of asymmetric subunits (for review, see reference 2). Polarity is reflected in several MT properties, e.g., the different rates at which the two ends of a MT add new subunits (1, 6, 11). On the basis of these rates the ends are denoted "fast-growing" (the plus end) and "slow-growing" (the minus end), respectively (4, 7). MT polarity has thus far been determined by growth kinetics in cilia, asters, and kinetochore fibers (1, 5, 35). The results from these studies indicate that in all cases the plus end is distal to the relevant organizing center. However, the method used involves a more or less severe disruption of the *in situ* morphology and might be displaying a misleading result. Two more recently discovered methods have the potential of revealing MT polarity under conditions that leave the overall architecture of the spindle relatively unaffected. One is the attachment of isolated dynein to nonciliary MTs. This method, introduced by Haimo et al. (17), takes advantage of the asymmetries of the dynein molecule. The fine-structural image of dynein associated with

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MTs permits the determination of MT polarity in both longitudinal and cross sections, but this method is not yet easily applicable to all systems of interest. The other method is the decoration of MTs with curved sheets of tubulin protofilaments, called hooks. This technique, first described by Heidemann and McIntosh (19), allows one to detect MT polarity in lysed cells with comparative ease. The method has already been successfully applied to the MTs of a variety of structures, including the asters of mammalian spindles (19), cilia, heliozoan axopodia, and fish melanophores (15), mammalian spindle midbodies, and plant phragmoplasts (14).

The polarity of kinetochore MTs is at present a controversial issue, because the polarity studies using growth rates were carried out with neurotubules grown on drug-treated, isolated chromosomes. We report here on the use of the hook decoration method to study the polarity of kinetochore MTs in metaphase and anaphase spindles from PtK_1 cells. In contrast to the data obtained from MT growth rates (5, 35), our results suggest that the plus ends of the kinetochore MTs are located at the kinetochores, i.e., distal to the spindle poles.

MATERIALS AND METHODS

Tubulin Preparation

Microtubule protein (MTP) was prepared from bovine brain by a modification of the method of Shelanski et al. (33). A high-speed supernate of depolymerized cycle two MTP in 0.5 M piperazine-N-N'-*bis*(ethane sulfonate) (PIPES), pH 6.9, 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM GTP (henceforth called 0.5 PMEG) was used in all experiments to decorate spindle MTs with hooklike appendages, as previously described (14).

Cells

PtK_1 cells were grown on teflon- and polylysine-coated microscope slides (14). Four different sets of experiments were performed. (*i*) After a brief wash in 0.5 PMEG, the cells were treated for 5–7 min with a mixture containing 1% Triton X-165, 0.5% deoxycholate, 0.02% sodium dodecyl sulfate, and 2.5% dimethyl sulfoxide (DMSO) (20) in 0.5 PMEG at 22°C containing 1.5 mg/ml tubulin. (*ii*) The cells were transferred from a 37°C incubator into cold culture medium and kept at 4°C for 1 h. (*iii*) The cells were first cold treated (as in *ii*). After a brief wash in 0.5 PMEG, they were lysed for 20 s with the detergent mixture in 0.5 PMEG at 22°C (as in *i*), but free of added tubulin. (*iv*) After the cold treatment (as in *ii*), cells were washed briefly with 0.5 PMEG and then lysed and incubated for 5–7 min as in *i*.

Electron Microscopy

After one of the above treatments, cells were rinsed in 0.1 M PIPES, pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA (0.1 PME), and then fixed with 2% glutaraldehyde in 0.1 PME for 30 min, washed in 0.1 M cacodylate buffer, pH 7.4, and then postfixed in 1% OsO_4 in cacodylate buffer. In most cases, 1% tannic acid was added to the glutaraldehyde fixative. Dehydration, including en bloc staining with 1% phosphotungstic acid and 0.5% uranyl acetate, and embedding were carried out according to standard procedures (13). Some of the cold-treated cells were sectioned parallel to the substrate, but most cells were remounted for transverse sectioning.

Serial sections were cut on a Sorvall MT2 microtome (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) and observed in a Jeol 100 C or a Philips 300 electron microscope. To include a representative number of MTs from the sections cut between chromosomes and poles, tilting of the specimen in the microscope was usually required. In some cases, up to eight pictures at different tilt angles were taken from a single section. MT polarity was determined by assessing the direction of curvature of the hooks attached to the spindle MTs at a final magnification of ×30,000–40,000. Those few MTs showing an equal number of hooks of either polarity were excluded from our counts. The handedness of hook curvature is directly related to MT polarity (14, 15, 19): a clockwise-curving hook generally means that one is looking from the plus end toward the minus end of the MT, whereas a counterclockwise-curving hook indicates that one is looking along the MT in the opposite direction.

RESULTS

The high molarity of PIPES buffer necessary for the growth of hooks promotes a decondensation of the chromatin, so the conditions used in previous studies of MT polarity had to be modified in this investigation. We have found that a combination of a comparatively high tubulin concentration (1.5 mg/ml) with a short incubation time (5–7 min) and a moderate incubation temperature (22°C) adequately preserves chromatin structure and at the same time gives sufficient hook decoration on preexisting spindle MTs to reveal their polarity (Fig. 1). Approximately 90% of all spindle MTs became decorated with hooks under these conditions. Previous studies indicate that the detergent mixture and buffer used for the decoration of cellular MTs with hooks preserve most if not all cytoplasmic and spindle MTs in PtK_1 and HeLa cells for many minutes, even in the absence of added tubulin (14, 20). Cells lysed under these conditions should thus contain a number and distribution of MTs that reflect the state before lysis. The kinetochore MTs should therefore be a defined subset of the observed spindle MTs. To determine the polarity of kinetochore MTs in PtK_1 cells we chose three approaches: (*a*) screen all spindle MTs present in the region between the kinetochores and the poles; (*b*) look selectively at the kinetochore MTs by removing the other MTs with cold; and (*c*) identify kinetochore MTs by tracking them to the kinetochores in cold-treated spindles.

Cells Not Treated with Cold

We looked at cross sections taken from the spindles of three cells treated as described in method *i*: a metaphase, an early anaphase, and a mid-anaphase cell. One to several sections in each half-spindle from a region halfway between the kinetochores and the poles were micrographed at several tilt angles and screened for MT polarity according to hook curvature (Fig. 1). The data are summarized in Table I. They reveal that in all three stages of mitosis the majority of the MTs are oriented with the plus end distal to the poles, as would be expected for aster MTs. MTs with hooks of opposite hand (5–20%, Table I) seem to be randomly distributed throughout the spindle in all sections examined (Fig. 1). The fraction of MTs

TABLE I

Polarity of Spindle MTs in Cells Not Cold Treated

Mitotic stages		MTs with plus ends distal to pole	MTs with plus ends proximal to pole	Number of MTs screened	Position of section
		%	%		
$Metaphase_1$	h_1	80.4	19.6	495	Near chromosomes
	h_2	81.7	18.3	447	
	h_1	90.6	9.4	974	Near poles
	h_2	83.6	16.4	1,393	
$Anaphase_1$	h_1	91.5	8.5	505	Near chromosomes
	h_2	88.8	11.2	537	
	h_1	93.9	6.1	491	Near poles
	h_2	91.2	8.8	375	
$Anaphase_2$	h_1	94.7	5.3	189	Near chromosomes
	h_2	95.5	4.5	425	

The polarity of MTs present in the two half-spindles (h_1, h_2) of three cells was determined according to hook curvature. Only sections from the area between the kinetochores and the poles were screened.

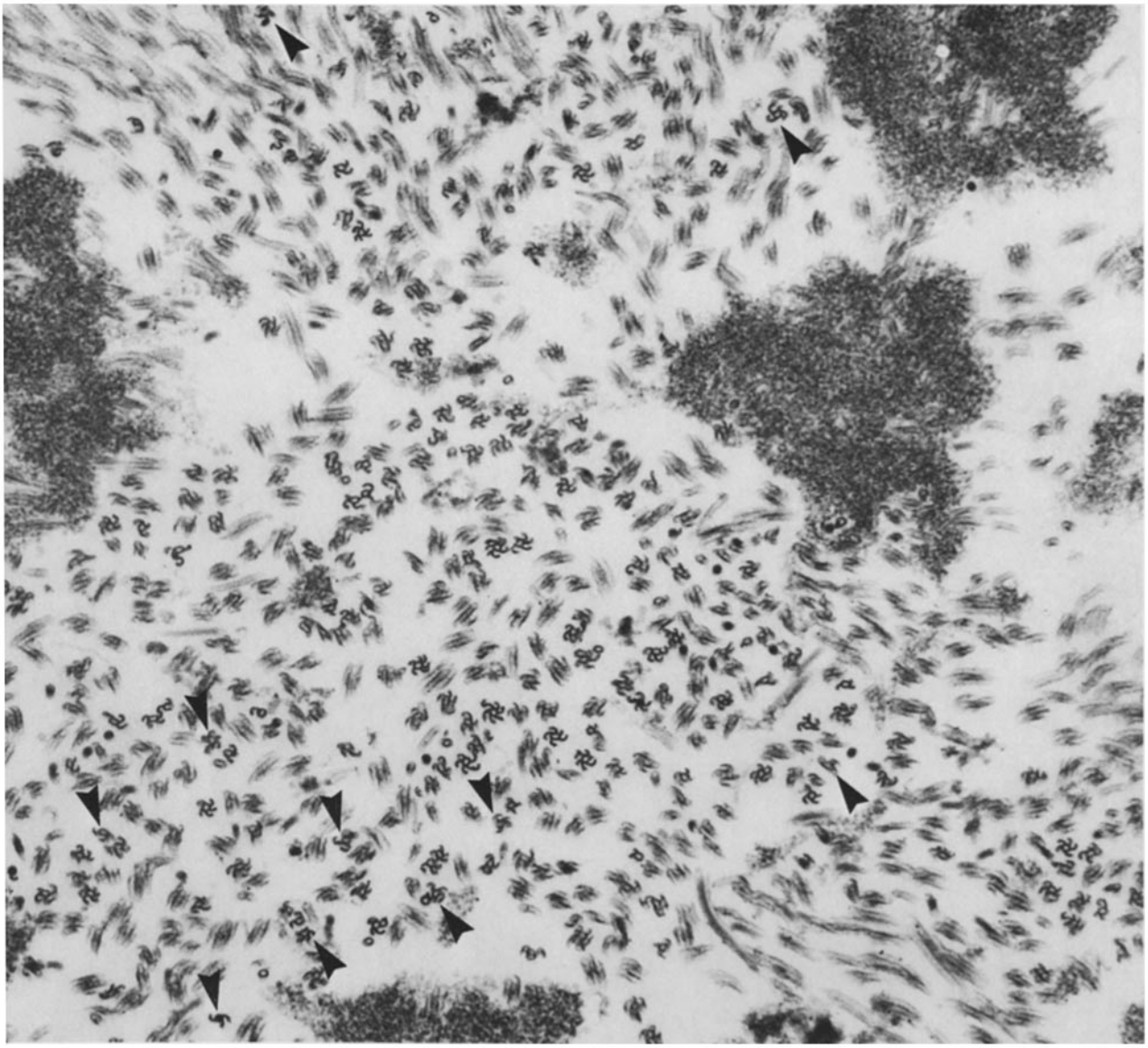


FIGURE 1 Treatment *i*. Cross section through an early anaphase cell cut close to the kinetochores in the area between the kinetochores and the poles. Most of the MTs seen in transverse view are decorated with hooks; the predominant hook curvature is counterclockwise (91.5%). All MTs with opposite polarity that could be identified in this micrograph are marked with arrowheads. They are scattered over most of the spindle cross section. × 48,000.

with hooks of opposite curvature decreases as one looks at sections closer to the poles. The fraction is also smaller midway between chromosomes and poles in the cells fixed during anaphase. These observations indicate that the fraction of MTs with the same polarity in a given spindle cross section increases with increasing distance from the equatorial region.

Cold-treated Cells

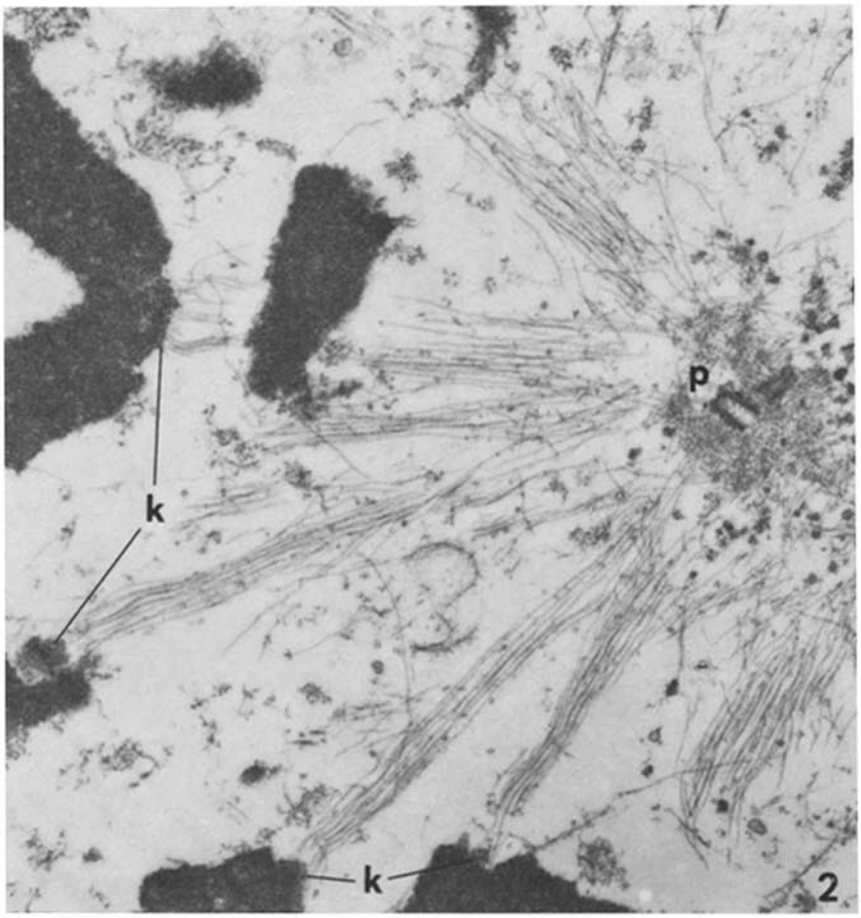
It has previously been shown that the different classes of spindle MTs differ in their sensitivity to cold treatment (9). Aster MTs are sensitive in all stages of mitosis, whereas interpolar MTs appear to be sensitive only before mid or late anaphase; kinetochore MTs seem to be fairly stable throughout mitosis. Cold treatment should therefore enable us selectively to look at kinetochore MTs in cross sections taken from the area between the kinetochores and the poles in both metaphase and anaphase.

In spindles fixed after our cold treatments (method *ii* and *iii*), astral MTs and MTs between the poles, as well as MT fragments, have by and large disappeared. We confirm the

FIGURE 2 Treatment *iii*. Longitudinal section through a former metaphase spindle. Mainly kinetochore MTs are left after 1 h at 4°C. Around the pole an accumulation of very short MT fragments is observed. Kinetochores = *k*, pole = *p*. × 12,000.

FIGURE 3 Treatment *ii* (*a*) and *iii* (*b*). Cross sections through bundles of kinetochore MTs from cold-treated cells. MTs can be identified more easily in the lysed spindle (*b*) than in the unlysed (*a*). × 72,000.

FIGURE 4 Treatment *iv*. Cross section through a metaphase cell in the region between the kinetochores and the pole. Several bundles of kinetochore MTs can be identified (arrowheads), all of which show the same polarity. The hooks curve clockwise in this view looking toward the pole. × 46,000.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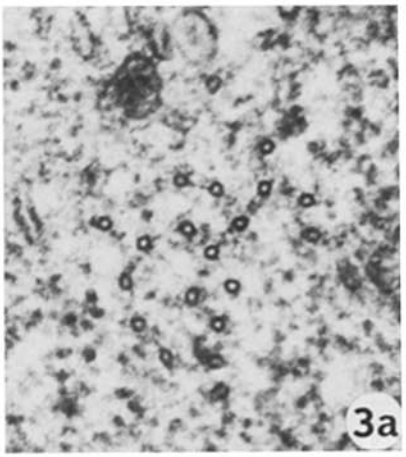
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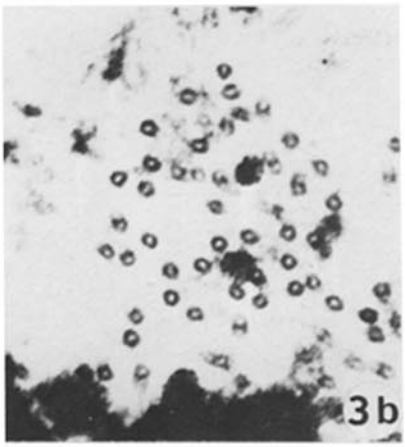
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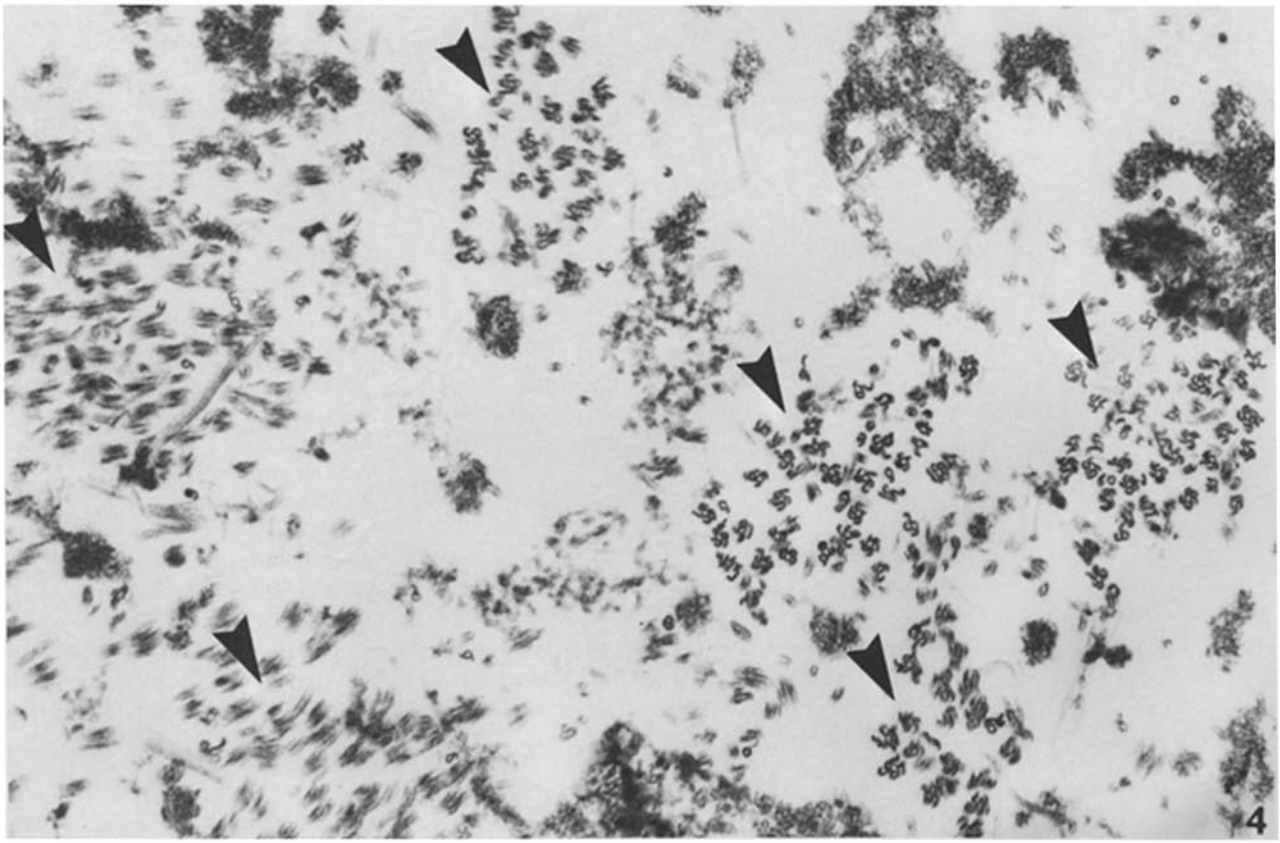
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TABLE II

Number of MTs per Kinetochore Bundle

Treatment	Mean number of MTs	Standard deviation of mean	Number of kinetochore bundles counted
ii	20.5	4.5	29
iii	33.1	7.3	11
iv	31.4	4.8	17

The treatments (*ii*, *iii*, and *iv*) are specified in Materials and Methods.

TABLE III

Polarity of Spindle MTs in Cold-treated Cells

		MTs with plus ends distal to pole	MTs with plus ends proximal to pole	Number of MTs screened
		%	%	
Metaphase$_1$	h_1	99.3	0.7	147
	h_2	97.7	2.3	341
Metaphase$_2$	h_1	98.4	1.6	187
	h_2	94.6	5.4	186
Anaphase$_1$	h_1	96.3	3.7	352
	h_2	90.3	9.7	154
Anaphase$_2$	h_1	96.5	3.5	397
	h_2	97.3	2.7	372
Anaphase$_3$	h_1	95.6	4.4	183
	h_2	95.8	4.2	662
Anaphase$_4$	h_1	97.3	2.7	150
	h_2	97.6	2.4	369
Anaphase$_5$	h_1	94.7	5.3	394
	h_2	94.5	5.5	271

Polarity data from the half-spindles (h_1, h_2) of seven cold-treated cells. Only sections from the area between the kinetochores and the poles were screened.

previously published finding (9) that mainly kinetochore MTs are left (Fig. 2). In addition, a large cloud of very short fragments, probably representing former aster and polar MTs, is found associated with the poles. Cross sections of cold-treated spindles show the kinetochore fibers as clusters of relatively tightly spaced MTs (Fig. 3). We were concerned, however, that cell lysis under hook-forming conditions might promote initiation of new MTs from the kinetochores, confusing our results. We therefore counted the number of kinetochore MTs in spindles treated with cold under various conditions (*ii*, *iii*, and *iv*). The results (Table II) show that there is no apparent difference between the number of kinetochore MTs in cells lysed in the presence of tubulin vs. those lysed in its absence. We conclude that no significant MT nucleation takes place at the kinetochores in our experimental conditions. Further, Roos (32) and McIntosh et al. (24) have reported 25–40 MTs per kinetochore in PtK$_1$ cells, so it seems that few, if any, kinetochore MTs are lost under our conditions of cold treatment and lysis. The reduced number of MTs seen per kinetochore in cold-treated, unlysed cells is probably an underestimate because the high density of the cytoplasm, regularly observed in fixed, cold-treated cells, may prevent the identification of some MTs in our sections.

Two metaphase and five anaphase cells were serially sectioned to obtain data on the polarity of kinetochore MTs in cold-treated spindles. In sections taken about halfway between the kinetochores and the poles, one finds parts of chromosomes and bundles of kinetochore MTs gradually converging toward the poles (Fig. 4). On the average, the polarity of about half of the MTs present in a section could be determined by the obvious handedness of the associated hooks. The data we obtained are shown in Table III. All cold-treated spindles contained MTs of predominantly one polarity: 95% have their plus ends located distal to the pole. In one of the two metaphase cells, a section from the region between the kinetochores and the poles was tilted so that all kinetochore MT bundles could be seen in transverse view. In all of these bundles the MT polarity was the same. Of the 187 MTs whose polarity could be determined in that section, only 3 bore hooks of the opposite curvature.

Careful inspection of the cold-treated metaphase cells revealed that in cross sections close to the metaphase plate several bundles of MTs with opposite polarity were present (Fig. 5). Longitudinal sections of cells treated by method *iv* showed that kinetochore MTs will elongate past the kinetochore and through the adjacent chromatin (Fig. 6). The conditions used here for the decoration of spindle MTs, although more protective of chromosome morphology than those used in earlier experiments, seem to affect the structure of the kinetochores, so that MTs can elongate beyond the kinetochore itself. Their polarity is, however, unaltered—their plus ends are distal to the respective pole. Fortunately, they do not grow more than 1–2 μm during the incubation time used, so bundles of MTs with opposite polarity are not found in anaphase cells in the area between the kinetochores and the poles. By this stage of mitosis, the two sets of chromatids have separated far enough that the elongating kinetochore MTs do not reach the region where the second set of kinetochores is located. In our sections of metaphase cells taken half way between the kinetochores and the poles, again only bundles of one polarity are present, suggesting that even if kinetochore MTs in these cells do elongate past the kinetochore, they do not reach this region of the spindle.

To obtain additional evidence concerning the origin of the MT bundles remaining after cold treatment and lysis, four kinetochore bundles were tracked through complete serial transverse sections. Several others were traced less rigorously in the electron microscope without taking micrographs. Fig. 7 shows an example of a tracking analysis. In this particular case, about 25 MTs of the bundle could be followed into the outer layer of the kinetochore. One can easily identify several MTs throughout the section series according to their individual hook decoration patterns, which may be seen even within the electron-dense outer layer of the kinetochore. In all cases the data so obtained were in perfect agreement with the results obtained from polarity determinations in whole-spindle cross sections.

DISCUSSION

We have applied a method for revealing the structural polarity of MTs to mitotic spindles in cells lysed from physiological conditions and cells lysed after a cold treatment designed specifically to reveal the kinetochore MTs. Our results suggest that essentially all the MTs in the region between the chromosomes and the poles, including kinetochore MTs, have the same polarity: their plus ends are distal to the pole.

At metaphase in cells lysed without cold treatment, sections near the metaphase plate contain some MTs with opposite polarity, a number that is close to the number of kinetochore MTs present in a normal, unlysed PtK$_1$ cell spindle (25). The distribution of these MTs does not, however, support the idea

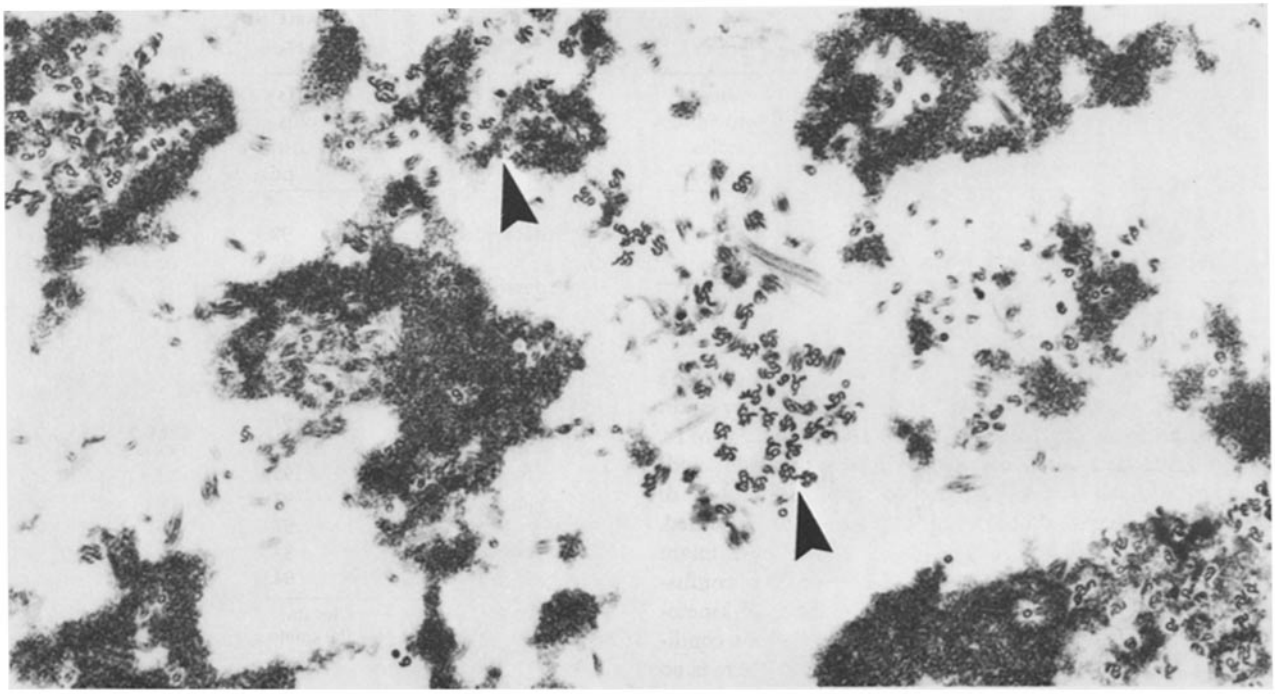

FIGURE 5 Treatment *iv*. Cross section through a metaphase cell between the pole and the chromosomes near the equatorial region. The view is toward the kinetochores. Parts of chromosomes and bundles of kinetochore MTs are present. In this area some bundles of opposite polarity can be found (arrows) although the same cell reveals predominantly MTs of one polarity closer to a pole (see Fig. 5). × 38,000.

that kinetochore MTs are oriented with their plus ends distal to the kinetochore. The MTs of opposite polarity are distributed uniformly throughout the spindle cross section (Fig. 1), whereas kinetochore MTs are normally arranged in bundles. Our interpretation of the MTs with opposite polarity in the equatorial region of metaphase cells is that nonkinetochore MTs from the half-spindle across the metaphase plate interdigitate with MTs of the half-spindle under examination. There is fine-structural evidence from PtK_1 and other cell types (8, 24, 25, 28) supporting an MT distribution similar to the one schematically presented in Fig. 8. The observations that the number of MTs with opposite polarity decreases with increasing distance from the equatorial region and that the number is low in anaphase half-spindles are also consistent with this picture. Our data from untreated metaphase and anaphase spindles can therefore be explained if we assume that the MTs with opposite polarity present during metaphase close to the equatorial region (about 20%) represent polar MTs coming from the far pole. We cannot with current data, however, assess the contribution of MT elongation during lysis to this interdigitation.

To analyze the polarity of kinetochore MTs with minimum interference from polar MTs, we subjected unlysed metaphase and anaphase spindles to a 1-h cold treatment at 4°C to depolymerize aster and nonkinetochore MTs (9). We think that our data (Table II) exclude the possibility that the number of kinetochore MTs either decreases significantly during cold treatment and lysis or increases markedly upon addition of tubulin under hook-forming conditions. We conclude that our polarity data apply to essentially the entire MT bundle that ends on a kinetochore in vivo.

All the data obtained from normal and cold-treated cells in either metaphase or anaphase indicate that kinetochore MTs are oriented with their plus ends distal to the pole. It is not clear, however, whether our observations also apply to cell

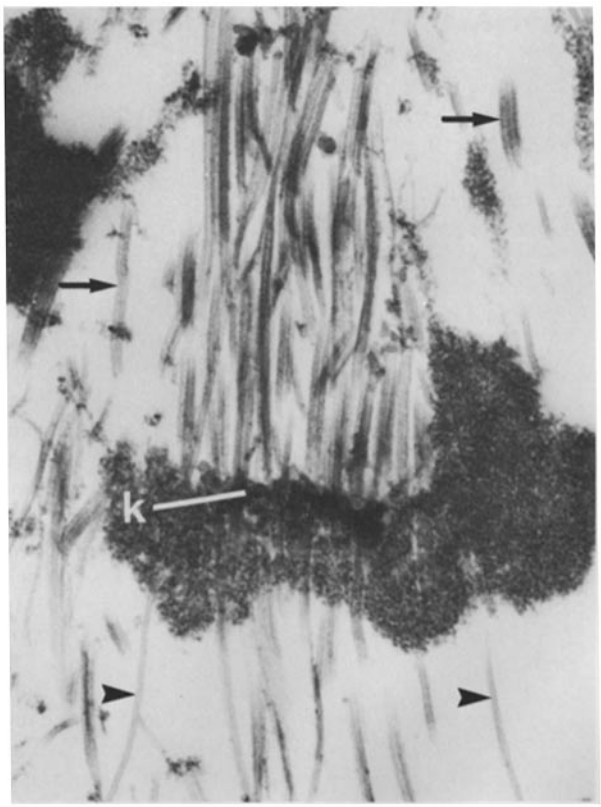

FIGURE 6 Treatment *iv*. Kinetochore of a metaphase chromosome. Because the spindle was cold treated before processing for hook decoration, a distinct bundle of kinetochore MTs is seen. MTs do not end at the kinetochore itself (*k*) but rather extend through it and the adjacent chromatin. The decoration of MTs along their long axis is obvious (compare decorated [arrows] and undecorated [arrowheads] MTs). × 34,000.

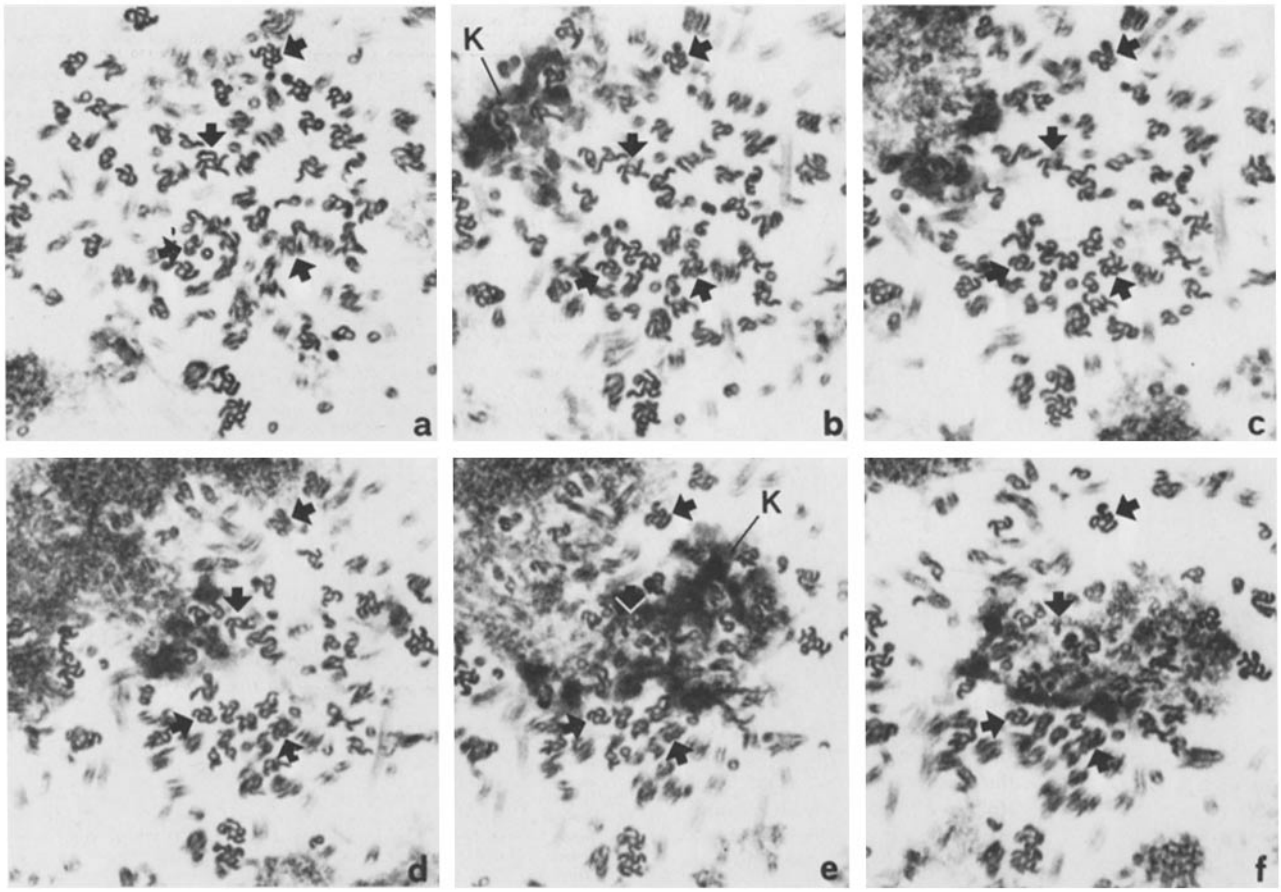

FIGURE 7 Treatment *iv*. Series of cross sections through a bundle of kinetochore MTs. The view is looking toward a kinetochore (*K*); most hooks attached to the MTs curve counterclockwise. Six sections from the series are shown; *b–f* are consecutive. Two sections between *a* and *b* are not shown because they resemble *a* so closely. Several MTs in the bundle can be easily followed through the series as their hook pattern facilitates their identification (arrows). × 56,000.

types other than PtK_1. Some indirect evidence obtained from HeLa cells (18) and preliminary results from *Haemanthus* endosperm cells in anaphase (26) suggest that a similar situation exists in these cells. It is tempting to speculate, therefore, that kinetochore MTs in most cell types possess the polarity described here for PtK_1 cells. If this is the case, then those models for mitosis that require an antiparallel arrangement of polar and kinetochore MTs for chromosome movement must be incorrect. The only published model for mitosis that assumes or predicts MT polarities in agreement with all our polarity data is that of Margolis et al. (23).

Our observations on the polarity of kinetochore MTs *in situ* are at odds with in vitro experiments suggesting the opposite polarity for neurotubules nucleated from isolated chromosomes. Summers and Kirschner (35) and Bergen et al. (5) reported that the fast growing or plus ends of neurotubules initiated from kinetochores are located distal to the kinetochore. Because kinetochores in a living cell can also, under certain experimental conditions, initiate the growth of MTs (10, 37), the in vitro growth rate polarity has, reasonably enough, been taken as suggestive evidence for a kinetochore MT polarity opposite to that of aster MTs. It is noteworthy, however, that all the experiments, both in vivo and in vitro, demonstrating nucleation of MTs at kinetochores have involved a previous treatment with a mitotic inhibitor. This treatment could have induced some change in the kinetochore that caused it to behave abnormally.

The present observations on the polarity of kinetochore MTs can be interpreted in two ways: (*a*) kinetochores initiate the growth of MTs with their minus ends distal to the site of initiation; or (*b*) kinetochores "capture" by some unknown mechanism MTs initiated by the spindle poles. Several lines of circumstantial evidence support the latter idea as the major mechanism for normal spindle formation. If cold treatment is used instead of antimitotic drugs to block the normal formation of spindle MTs, kinetochores do not show initial nucleation capacity in the subsequent recovery period (30). Careful investigations of prometaphase events in a hypermastigote flagellate (31) and in several algal spindles (36) likewise suggest a "capturing" mechanism. There can be little doubt from the in vitro work that kinetochores have the potential of nucleating MT assembly, but this potential could be masked during the normal course of mitosis. It must be noted, however, that the stated possibilities are not mutually exclusive. If the surface of the kinetochore binds the plus ends of MTs to capture them, it might also bind the plus ends of tubulin dimers, initiating MTs upside down. Such MTs would probably grow slowly and might constitute the few short kinetochore MTs sometimes found (16).

If kinetochores bind the plus ends of preexisting MTs to organize a bundle, they must still be regarded as microtubule organizing centers (MTOCs), but they are clearly distinct from MT initiating sites, such as the centrosomes. Likewise, the equator of the phragmoplast (at the margin of the cell plate), which has long been regarded as an MTOC (21), is the location of the plus ends of the phragmoplast MTs (14). We propose

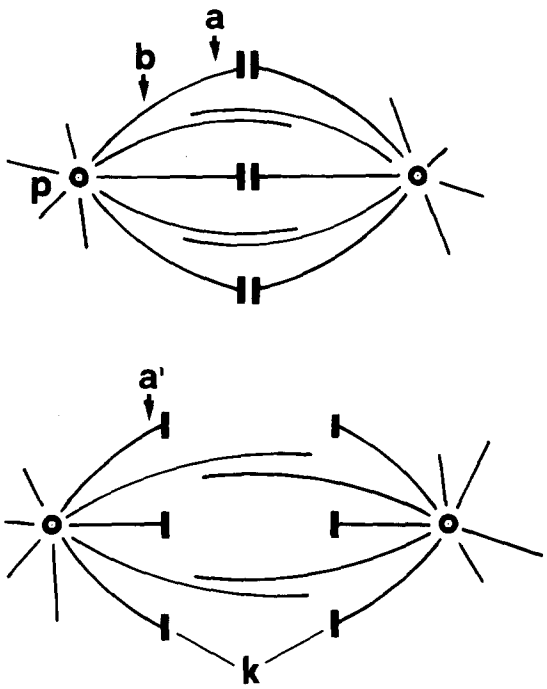

FIGURE 8 Schematic representation of the MT distribution in a normal metaphase and anaphase spindle. According to this arrangement, MTs associated with the opposite pole are found in the area between the kinetochores and the poles in a metaphase cell (plane *a*) but not in an anaphase cell (plane *a'*). However, only a few of these oppositely directed MTs would be expected in a metaphase cell in sections closer to the poles (plane *b*). Poles = *p*, kinetochores = *k*.

that the concept of an MTOC should be broken into two parts: MT initiating sites and MT positioning sites. The positioning sites, like the kinetochores and phragmoplast equator, might initiate some MTs, but they seem to exert most of their effect by interaction with MTs initiated elsewhere. This distinction may help us to develop a more detailed understanding of the ways in which living cells regulate the polymerization and distribution of their MTs.

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