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The Attachment of Kinetochores to the Pro-Metaphase Spindle in PtK_1 Cells

Recovery from Low Temperature Treatment

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Abstract. When late prophase PtK_1 cells are chilled to 6° C the nuclear envelope (NE) breaks down as in normal cells but the spindle is inhibited from forming. When these cells are subsequently warmed to 18° C the spindle slowly forms and pro-metaphase congression ensues. Using this approach we have been able to experimentally eliminate the influence of asynchronous NE breakdown on the formation and development of the spindle, and also to slow down (and thus increase the temporal separation of) the subsequent events which occur during the initial stages of spindle formation. Correlative light and high voltage electron microscopic studies on these cells, fixed after various times of recovery, reveal the following results: 1) the centrosomes generate microtubules (MTs) well before MTs are seen to be associated with the kinetochores; 2) as in untreated PtK_1 cells (Roos, 1973a, 1976) the order in which chromosomes attach to the forming spindle is influenced by their proximity to a centrosome–kinetochores closest to a centrosome appear stretched towards the centrosome at a time during recovery when other kinetochores, more distal to the centrosome appear unstretched and unoriented; 3) as in untreated cells (Heneen, 1970; Roos, 1976) the predominant behavior during recovery is for a chromosome to initially mono-orient and associate with the near centrosome and only later to develop a bipolar association; and 4) MTs associated with early pro-metaphase kinetochores are almost always oriented towards a centrosome. – From our results we conclude that the proximity effect and the tendency of pro-metaphase chromosomes in PtK_1 to initially mono-orient and associate with the near centrosome cannot be ascribed, as suggested by Roos (1976), to influences arising during the asynchronous breakdown of the NE. Rather, our data clearly demonstrate that a kinetochore-centrosome interaction occurs during spindle formation which cannot be attributed to transient influences. The proximity effect and the predominant tendency of PtK_1 pro-metaphase chromosomes to mono-orient to the near pole are taken to signify the existance of a centrosomal influence on the attachment and orientation of chromosomes.

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Two possible mechanisms for this influence, both involving a structural interaction between the centrosome and the kinetochore, are outlined.

Introduction

In higher plant and animal cells the disintegration of the nuclear envelope (NE) during late prophase initiates and influences the subsequent formation and development of the pro-metaphase spindle (see review of Bajer and Mole-Bajer, 1972; Mole-Bajer, 1975; Roos, 1976). The rat kangaroo cell lines (PtK_1 and PtK_2) have proven to be especially suitable material for correlative studies on this phenemenon at the light and electron microscopic level. In these cells the relative timing of centrosome migration to the presumptive spindle poles is variable and may occur before, during or after the breakdown of the NE (Roos, 1973a; Rattner and Berns, 1976b; McIntosh et al., 1975; Aubin et al., 1980). The separation is accompanied by a rearrangement in the distribution of the centrosomal microtubules (Rattner and Berns, 1976a). Nuclear envelope breakdown occurs asynchronously over its surface and is initiated in the region of the centrosomes (Heneen, 1970; Roos, 1973a, 1976). The early pro-metaphase spindle then invades into the nucleus through gaps in the polar region of the NE (Roos, 1973a). Further growth of the early pro-metaphase spindle is a rapid process and is thought to occur between the centrosomes by elongation of microtubules (MTs) nucleated primarily in the polar regions (Roos, 1973a; Rattner and Berns, 1976a; DeBrabander et al., 1979a, b). Chromosomes attach to the forming spindle as soon as the NE begins to rupture and proximity to a centrosome influences the order of attachment; those chromosomes closest to the centrosomes are the first to attach while those more removed attach later, after further rupture of the NE (Roos, 1973a, 1976).

The tendency in this material is for chromosomes to initially mono-orient and associate with the nearer pole and only later to develop bipolar associations. Roos (1976) attempted to account for the proximity effect and for the initial mono-orientation by suggesting that kinetochore MTs would form first in regions of high tubulin concentration and that gradients of decreasing tubulin concentration would develop from the polar areas during NE breakdown. The suggestion of a tubulin gradient is plausible since the available evidence does indicate that tubulin subunits are absent from the interphase nucleus in higher eukaryotes (DeRobertis et al., 1978) and NE breakdown does begin in the polar areas. However, the existence of a tubulin gradient does not necessarily mean that it is the cause of the observed centrosomal influence (i.e. the proximity effect) or of chromosome mono-orientation. To test this idea a method which would allow the NE to breakdown well before the formation of the pro-metaphase spindle would be highly desirable. In this manner the influence of asynchronous NE breakdown on the initial stages of spindle formation and chromosome orientation would be eliminated. This in turn should allow for a clearer assessment of the role of the NE and other possible factors involved in the attachment of kinetochores to the forming spindle.

We have found that when late prophase PtK_1 cells are cooled to 6° C, the NE disintegrates without the subsequent formation of the MT component

of the spindle (i.e., kinetochore, astral and free MTs are inhibited from forming). Upon rewarming, the cells recover and complete division. We have taken advantage of the fact that the rate at which the pro-metaphase spindle is allowed to form can be manipulated in these cells by simply varying the temperature of the cell. Thus when PtK_1 cells are prepared in the above manner, and then subsequently warmed to 18° C, the spindle slowly forms (relative to the rate at 37° C) and the congression of pro-metaphase chromosomes ensues. Using this approach we have been able to eliminate the influence of asynchronous NE breakdown on the formation and development of the pro-metaphase spindle, and also to slow down (and thus increase the temporal separation of) the subsequent events which occur during the initial stages of spindle formation.

Materials and Methods

1. Culture Methods. Cells from the rat kangaroo (*Potorous tridactylus*) line PtK_1 were grown at 37° C on 22 mm sq. glass coverslips in L-15 medium buffered to pH 7.2 with 10 mM N,N-Bis(2-Hydroxyethyl)-2-aminoethane sulfonic acid (BES) and supplemented with 10% fetal calf serum and antibiotics. L-15 medium is buffered with free amino acids instead of bicarbonate. Consequently cells were grown in free gas exchange with the atmosphere (see Leibovitz, 1963). This permitted cultures to be manipulated in the air, for extensive periods of time, without inducing rapid fluctuations in the pH of the culture. The addition of 10 mm BES to L-15 was found to further increase the buffering capacity of the medium without any loss in the rate of PtK_1 proliferation (see Jensen, et al., 1979).

2. Temperature Shifts. All experiments which required either the chilling or the chilling and subsequent rewarming of cells were conducted in a cold room held at 6 ± 1° C. The cold room contained a Zeiss phase contrast microscope equipped with a 35 mm Zeiss Ikon camera and a Lauda K-2R incubator (Brinkman Instruments). For these experiments coverslips containing actively growing PtK_1 cultures were mounted in conditioned L-15 medium on the cell chamber of a temperature control apparatus.

The characteristics of the temperature control apparatus permitted the continuous observation of selected cells during temperature shifts and fixation. It has been described in detail elsewhere (Lambert and Bajer, 1977; Rieder and Bajer, 1977). Briefly, it consists of a cell chamber which is in direct contact with a temperature control (TC) slide. The cell chamber (onto which the cell cultures are mounted) is indirectly heated (or cooled) by passing an H_2O-Ethanol mixture, warmed to the desired temperature by the Lauda incubator, through the TC slide. Temperature changes within the slide and cell chamber were determined by monitoring two thermistors, one attached to the outflow of the TC slide, the other attached to the cell chamber (see Rieder and Bajer, 1977).

Chilling of PtK_1 cells was achieved by placing the TC apparatus (which contained an attached cell culture) in the cold room and allowing it to equilibrate to 6° C. After approximately 30 min (i.e., after equilibration) the TC apparatus was mounted on the microscope stage and 3–6 late prophase cells, all of which possessed intact nuclear envelopes, were located within the culture and photographed on Plus-X film using heat filtered (Zeiss KG 1 filter) green (Baird Atomic filter, 546 nm) light. Each cell was subsequently photographed at approximately 1 h intervals until the nuclear envelope had broken down in most or all of the cells selected for inidividual examination (i.e. between 2–12 h). Cells in which the NE had broken down at 6° C were then either: 1) fixed after various times in the cold room with 6° C glutaraldehyde and processed for electron microscopy as described below, or 2) gradually warmed to 18° C and fixed at various times during recovery by perfusing the cell chamber with isothermal glutaraldehyde. Gradual warming to 18° C was achieved by initially pumping 6° C H_2O-ethanol through the TC slide and then by rapidly changing the incubator thermostat from 6° C to 20° C. The deleterious influence of the objective lens (i.e., it acts as a heat sink–see Rieder and Bajer, 1977), on the temperature of the cell culture during warming, was obviated by equipping the objective lens with a collar of coiled copper tubing.

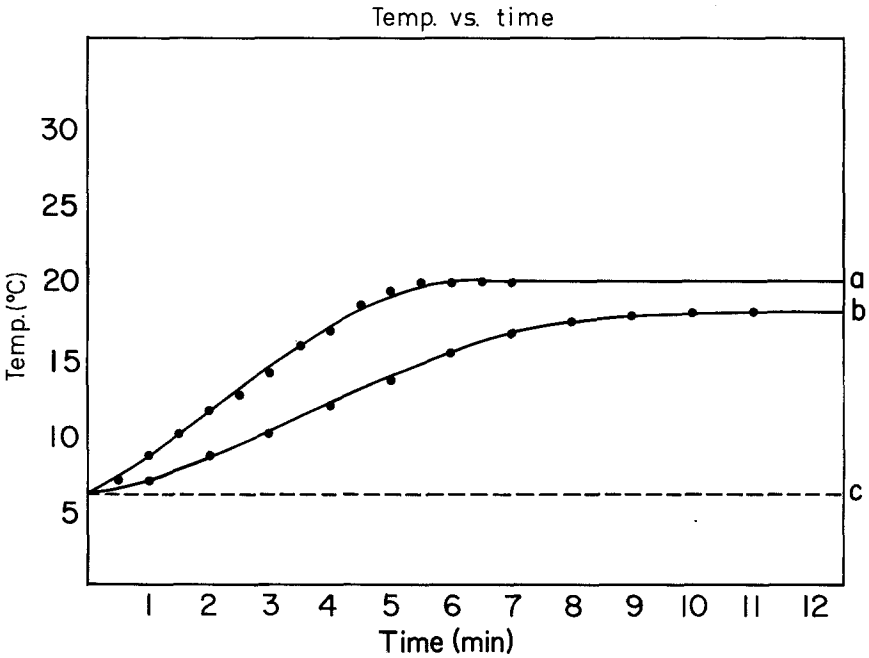


Fig. 1. Recovery of cells from cold treatment at 6° C is initiated by pumping a 6° C water-ethanol mixture, contained in a Lauda incubator, simultaneously through the temperature control (*TC*) slide and objective collar (see text for details). The incubator thermostat is then rapidly changed from 6° C to 20° C. The water-ethanol mixture in the incubator (*curve a*) reaches 20° C about 6 min later. However, the warming of the cell chamber is a more gradual process (*curve b*) and it reaches a stable 18° C about 10 min after the initiation of recovery. The 2° C temperature difference between the incubator and cell chamber arises from heat loss to the 6° C cold room (*line c*) as the incubator fluid is pumped via tygon tubing to the TC slide. Under the above conditions the warming characteristics of the cell chamber remained constant and reproducible as long as a constant flow rate was maintained through the TC slide

Cotton was then packed around the TC apparatus and objective lens to create a dead air space between the glass coverslip growth substrate and the objective lens. Under these conditions simultaneously pumping the warming H_2O-ethanol mixture through both the TC slide and objective collar gave a constant and reproducible rate of warming as long as a constant flow rate was maintained (see Fig. 1).

3. Electron Microscopy. Control and experimental cells were initially fixed by perfusing isothermal glutaraldehyde (3.1% in 0.1 M PO_4 buffer, pH 7.1) through the cell chamber and fixed for an additional 20 min in room temperature glutaraldehyde. They were then postfixed for 1 h in 2% OsO_4 (in 0.1 M PO_4 buffer), washed in buffer, dehydrated in a graded series of ethanol, and flat embedded in Epon-Araldite. During dehydration, the majority of the cultures were stained for 1–2 h "en block" with 2% uranyl acetate in 70% ethanol.

The glass coverslip growth substrate was removed from the embedded cultures by placing them in cold (4° C) hydrofluoric acid (see Moore, 1975). The experimental cells were then relocated, circled with a diamond objective marker, excised from the cultures and mounted on Epon pegs for trimming and serial sectioning. Serial sections 0.25 μm thick were collected on slot grids by the method of Behnke and Rostgaard (1964) and subsequently stained with uranyl acetate (5%; 60° C for 120–150 min) and lead citrate (23° C for 25–30 min).

All sections were examined and photographed with the University of Wisconsin (Madison)

1 MeV AEI-EM7 High Voltage Electron Microscope (HVEM) operated at 900 KV, using an objective aperture of 35 μm. A total of 15 room temperature control and 5 chilled control cells (which were fixed prior to NE breakdown at 6° C) were examined by HVEM. An additional 28 experimental cells, which were fixed either after NE breakdown at 6° C or at various times during recovery, were examined by HVEM. Many of these cells were completely serially sectioned.

Results

1. The Ultrastructure of Prophase Cells at 37° C

The ultrastructural features of prophase PtK_1 cells have been previously described in detail (Roos, 1973a; Heneen, 1975; Rattner and Berns, 1976a) and only those points relevant to the present study will be reviewed here. Centrosome separation in PtK_1 may occur at any time during late prophase or early prometaphase. During this time numerous microtubules (MTs) are associated with and terminate in the dense cloud of pericentriolar material surrounding the centriole pair of each centrosome. The condensing chromosomes lie near the periphery of the nucleus and are often attached to the nuclear envelope (see Roos, 1973a; Rieder, 1980). Kinetochores are recognized, during the later stages of chromosome condensation, as discrete globular packets of a finely fibrillar material, approximately 0.5 μm in diameter, which lie on opposite sides of the primary constriction. They appear slightly less electron opaque than the rest of the chromosome and are associated with the chromosome in a "ball and cup" fashion (Roos, 1973b; Heneen, 1975; Rieder, 1980). MTs are not found within the prophase nucleus.

2. The Effect of Chilling Prophase PtK_1 Cells to 6° C

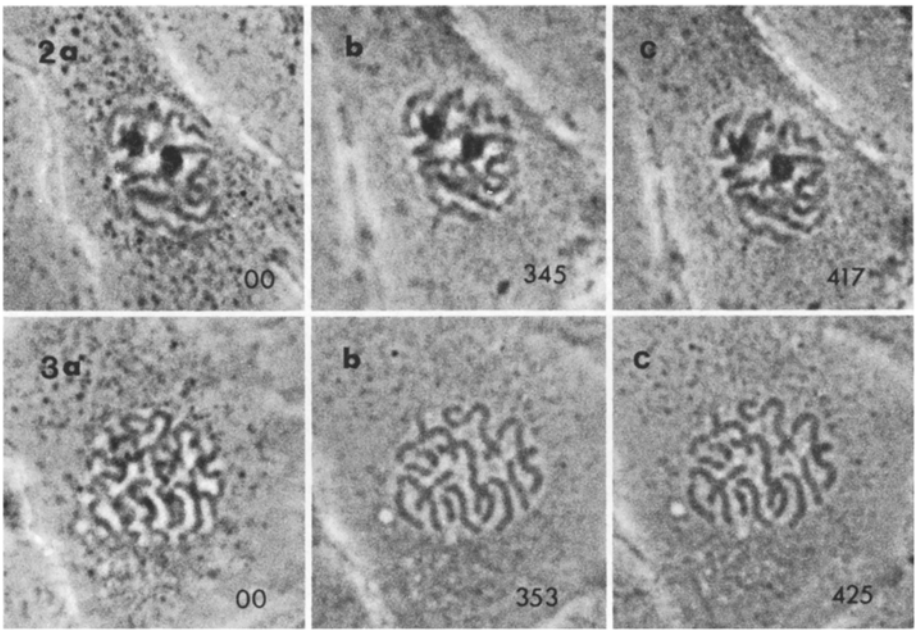
Chilling to 6° C arrested and reversed chromosome condensation in early prophase PtK_1 cells (data not shown). However, cells that were subjected to chilling at a later stage of prophase, when the chromosomes were well condensed and distinct from each other, did not revert directly back to interphase. Rather, at some time during the cold treatment the nuclear envelope (NE) broke down and the chromosomes became scattered throughout the area of the former nucleus where they subsequently lay motionless. After a prolonged period (greater than 36 h) at 6° C the chromosomes in these cells coalesced and the division was aborted.

With phase contrast microscopy the presence of the NE in chilled prophase cells could be directly ascertained by slowly focusing through the nucleus. It was rarely possible, however, to unambiguously document on film the presence of the NE in these cells. Since the intent of the present investigation was to chill the prophase cells prior to NE breakdown, we had to develop a more reliable assay for the presence or absence of the NE in these cells.

During the early stages of this investigation we noted that the chromosomes in chilled prophase cells often underwent a dramatic and abrupt change in their position. Other observations indicated that aside from this abrupt change in position, chromosome motion within the prophase nucleus was frozen at 6° C. Since the arms of the condensing chromosomes are intimately associated

with the NE in prophase cells (see above), it was reasoned that this abrupt change in the position of the prophase chromosomes was indicative of and probably induced by the breakdown of the NE. This inference was subsequently confirmed by electron microscopy. Figure 2 shows a prophase cell which was chilled to 6° C for 7 h prior to fixation. Photographs of this cell taken at various times during the treatment reveal very little change in the position of the chromosomes throughout the duration of the shock. The corresponding ultrastructural study of this cell revealed that the NE was intact. Alternatively, Figure 3 depicts a prophase cell, subjected to the same experimental conditions, in which the chromosomes underwent an abrupt change in their position at 6° C (cf Fig. 3a and b) and then appeared motionless (cf Fig. 3b and c). Electron microscopy showed that the NE had broken in this cell.

Two criteria were therefore used to determine that the NE had broken down in prophase cells held at 6° C: 1) during the early stages of the cold treatment the NE had to appear to be intact upon focusing through the nucleus, and 2) at some time, well after the initiation of the cold treatment, the chromosomes had to undergo an abrupt change in their position within the cell.



Figs. 2 and 3[1] Prophase cells. **Fig. 2a–c.** Fixed after 7 h at 6° C. Note that there is very little change in the position of the chromosomes throughout the duration of the cold treatment. Subsequent electron microscopy of this cell revealed that the NE was intact. **Fig. 3a–c.** A cell which was subjected to the same experimental conditions as in **Fig. 2.** The abrupt change in the position of the chromosomes between the photographs in **a** and **b** was correlated at the ultrastructural level with the disintegration of the NE (cf. **Fig. 2**; see text for details)

[1] In these and all subsequent light micrographs the first photograph of the series (i.e., **a** of the figures) is the initial picture of the cell taken after equilibration to 6° C and is labelled 00. In the subsequent photographs of a series the time in min relative to the initial photograph is printed in the lower right hand corner of each picture. All light micrographs are 2,000 ×

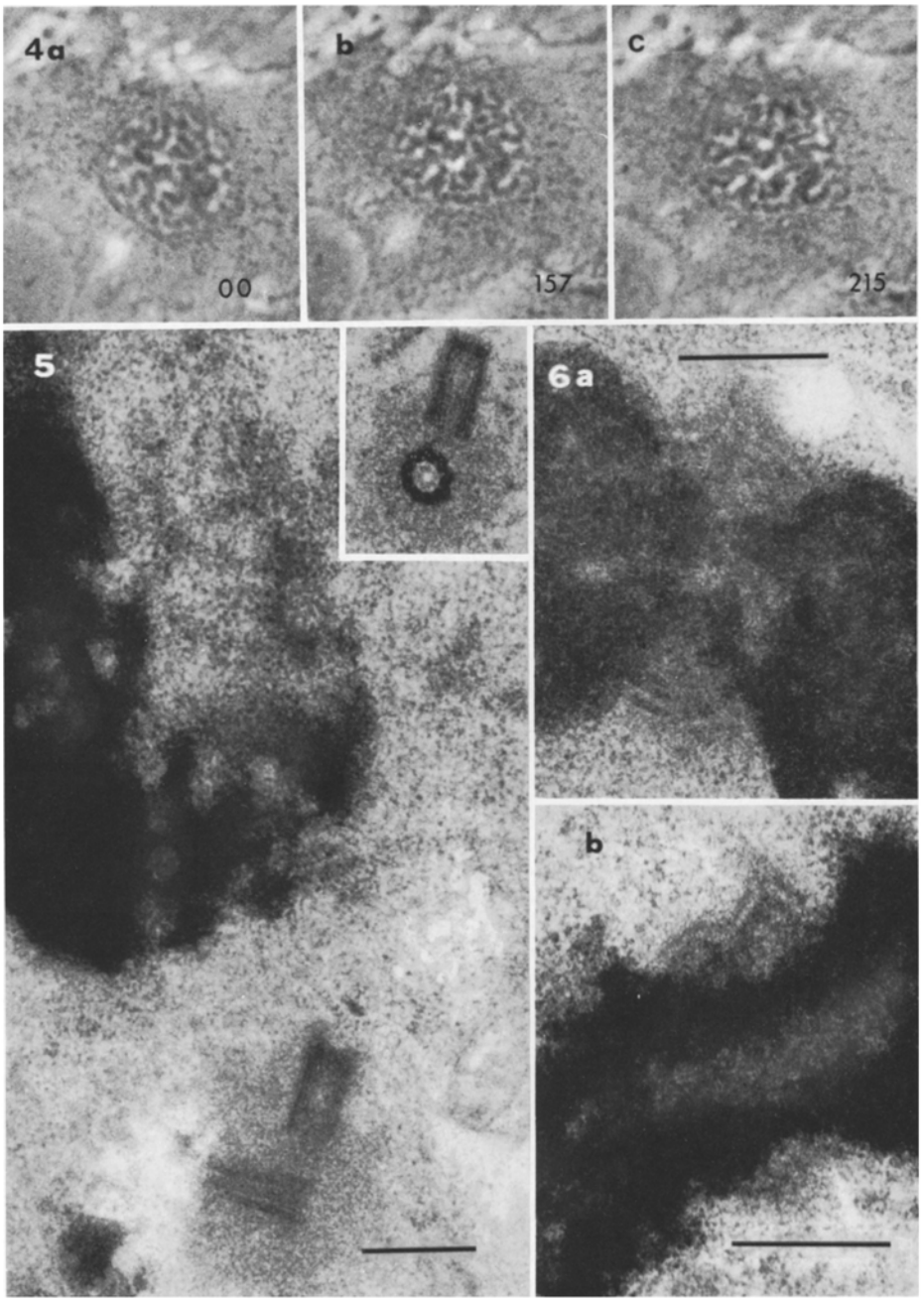
3. The Structure of Prophase Cells Held at 6° C

a) Cells in Which the NE Remained Intact. Late prophase cells in which the NE remained intact during the cold shock and prior to fixation possessed kinetochores which appeared similar to the "ball and cup" kinetochores of control cells. The chromosomes in cells kept at 6° C appeared mottled, a condition which presumably reflects an alteration in the normal prophase process of chromosome coiling and condensation. As in control cells, the centrosomes were most often well separated and positioned close to the nucleus. The centrioles remained structurally intact and were surrounded by an electron opaque cloud of pericentriolar material. There was however, a complete absence of centrosomal MTs in prophase cells kept at 6° C for 2 h or more.

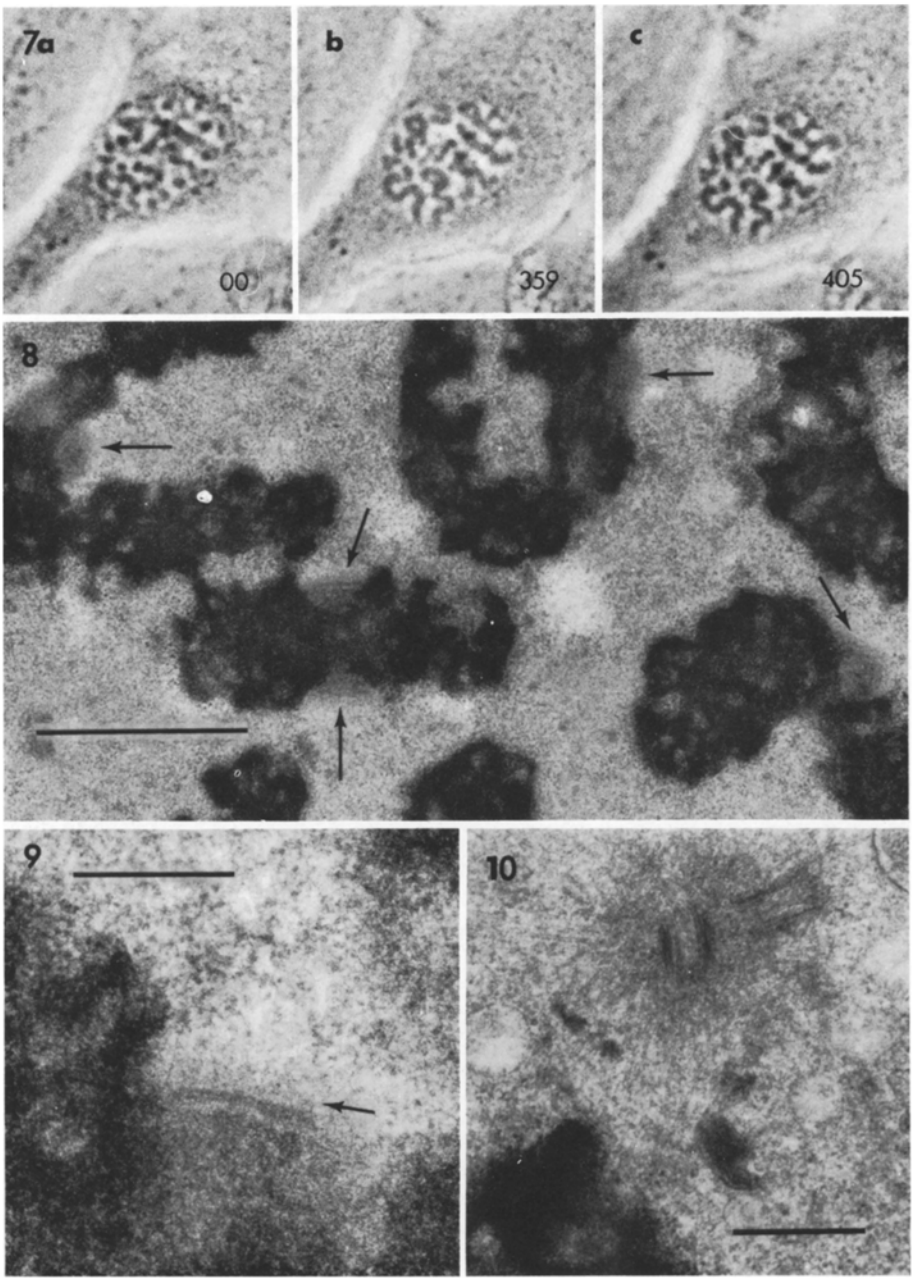
b) Cells in Which the NE had Broken Down During the Cold Treatment. After breakdown of the NE in prophase cells kept at 6° C the chromosomes remained motionless and were positioned throughout the area of the former nucleus (Figs. 3, 4). Due to limitations inherent in our methodology we were unable to examine the fine structure of cells during the initial stages of NE breakdown at 6° C. However, cells fixed 2–12 h after disruption of the NE appeared structurally similar to each other. The typical fine structural features of these cells are exemplified by the cell shown in Figures 4–6. In this cell, which was fixed 3.5 h after equilibration to 6° C and approximately 2 h after the NE had broken down, disruption of the NE was complete and we were unable to distinguish NE remnants from membranes of the golgi or endoplasmic reticulum. The centrosomes were well separated and positioned at the periphery of the centrally located mass of chromosomes. This observation suggests that the centrosomes in this cell were in the process of separating as the cold treatment was initiated. The centrioles were structurally intact and each pair was surrounded by a distinct cloud of granular pericentriolar material (Fig. 5). The kinetochores were easily recognizable and stained less electron opaque than the chromosomes. In many sections they appeared as disc structures (Fig. 6). The centromeric chromatin subjacent to the kinetochores (see Rattner et al., 1975; Roos, 1977) was notable in that it appeared much less electron opaque than the surrounding chromosomes and varied in thickness. A conspicuous feature of these cells was the complete absence of all classes of spindle MTs (i.e., kinetochore, astral and free).

4. The Structure of Recovering Cells in which the NE had First Broken Down in the Cold

a) Approximately 5 min after the Initiation of Recovery. A comparison of light micrographs taken of cells before the initiation of recovery and just prior to their fixation 5 min later revealed that the chromosomes remained motionless during this period (see example in Fig. 7). By inspecting Figure 1 it can be deduced that the temperature of these cells was approximately 14° C at the time of fixation. Their ultrastructure was similar to that of non-recovering cells with the exception that in all cases numerous MTs of undetermined length were associated with the centrosomes (Fig. 10). The chromosomes remained mottled and were frequently seen bridged to neighbors (Fig. 8). An analysis of numerous serial sections taken from these cells failed to reveal any MTs



Figs. 4–6. Fig. 4a–c. A prophase cell fixed after approximately 3.6 h at 6° C. A comparison of **a** and **b** indicates that the NE in this cell disintegrated within the first 2.6 h of the shock. **Fig. 5.** An electron micrograph showing one of the centrosome regions of the cell pictured in **Fig. 4.** Note the absence of both the NE and centrosomal MTs. The centriole pair of the centrosome is surrounded by an electron opaque cloud of pericentriolar material. The inset, in the upper right hand corner, is of a centrosomal region from a similarly treated cell. Both micrographs are 31,000×; Bar=0.5 μm. **Fig. 6a and b.** Kinetochores (**a, b**) from the cell pictured in **Fig. 5.** Note their layered organization (see text for details). 39,000×; Bar=0.5 μm



Figs. 7–10. Fig. 7a–c. A cell, exposed to 6° C for approximately 7 h before its fixation 6 min after the initiation of recovery. A comparison of **a** and **b** indicates that the NE in this cell broke in the cold. Recovery was initiated after 399 min at 6° C. A comparison of micrographs of this cell taken before the initiation of recovery (**b**) and just prior to fixation (**c**) reveals no movement of the chromosomes. **Fig. 8.** An electron micrograph of a section through the chromosomes of the cell pictured in **Fig. 7.** The chromosomes appear mottled and are frequently bridged to each other. The kinetochores (*arrows*) appear more electron translucent than the chromosomes and are free of MTs. The electron translucent areas within the section are due to holes in the formvar support film. 14,000×; Bar=2.0 μm. **Fig. 9.** A high magnification micrograph of a kinetochore from the cell pictured in **Fig. 7.** The kinetochore plate and its associated light staining corona material (*arrow*) is clearly revealed in this section. That part of the chromosome which is subjacent to the kinetochore (i.e., the centromeric heterochromatin) appears more electron translucent than the remainder of the chromosome (cf. **Fig. 8**). 43,000×; Bar=0.5 μm. **Fig. 10.** A photomicrograph of a section through one of the centrosomes of the cell pictured in **Fig. 7.** Note that numerous MTs are associated with the pericentriolar material (cf. Fig. 5). 35,000×; Bar=0.5 μm

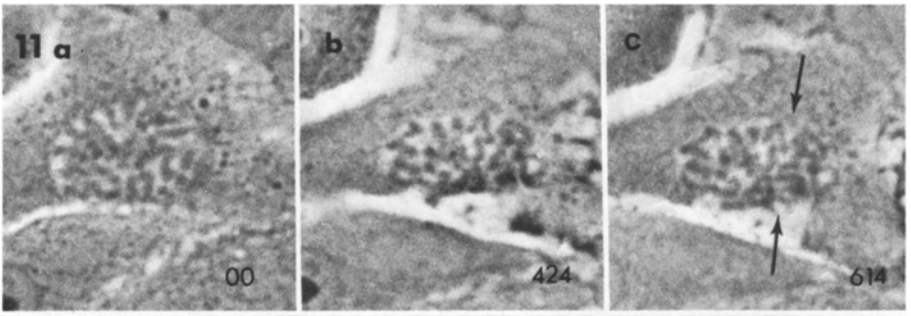
on the kinetochores. In general the kinetochore appeared to be better organized than in non-recovering cells (cf Figs. 9 and 6). However, as in non-recovering cells the chromatin subjacent to the kinetochore appeared more electron translucent than the rest of the chromosome, and varied in thickness (Figs. 8, 9). A filamentous corona (see Jokelainen, 1967), 50–100 nm thick, was often associated with the outer kinetochore disk in those sections which contained good kinetochore profiles (see arrow in Fig. 9). This material was also seen associated with outer disks of those unoriented kinetochores in cells fixed 10–15 min after the initiation of recovery (see Section b; Fig. 15).

b) 10–15 min after the Initiation of Recovery. A comparison of photomicrographs taken of cells before recovery and immediately prior to fixation, 15 min after the initiation of recovery, revealed no gross movement of the chromosomes. The corresponding ultrastructural studies on these cells revealed numerous MTs associated with each of the centrosomes which were invariably well separated. However, the area in the middle of the separated centrosomes was, in general, poorly organized and contained only a few individual MTs of an undetermined length.

Although the chromosomes in these cells had appeared to remain motionless, some of them had their kinetochores stretched poleward. We take this poleward stretching to be indicative of a force applied to the kinetochore and to signify that it is attached to the forming spindle. Invariably, the kinetochores displaying this stretching were those located closest to the centrosomes. MTs were found to be associated with these stretched kinetochores, but due to extensive MT nucleation by the centrosomes and to the thickness of the sections, it was not possible to conclude that the MTs unequivocally terminated at the kinetochore. Instead, we prefer to use a criterion similar to that of Witt et al. (1980), in a related study on CHO cells, for defining kinetochore associated MTs. Since our sections are 0.25 μm thick we consider MTs to be associated with a kinetochore if they are within 0.25 μm of the kinetochore.

In these early recovering cells those chromosomes further from the centrosomes invariably possessed unstretched kinetochores completely free of associated

Figs. 11–13. Fig. 11a–c. This cell was held at 6° C for 10 h, allowed to recover by rewarming for 15 min and then fixed. A comparison of **a** and **b** indicates that NE breakdown occurred within the first 7 h of the cold treatment. Recovery was initiated after 600 min at low temperature. A comparison of photographs taken prior to the initiation of recovery (**b**) and immediately before fixation (**c**) reveals no gross movement of the chromosomes. **Fig. 12.** An electron micrograph of a section through the middle of the spindle from the cell pictured in **Fig. 12.** The three chromosomes (i.e., 1–3) that show attachment to the spindle in this cell are located nearest the lower pole. Additional kinetochores (7, 9, 10; *short arrows*), which were farther from the polar areas, are free of associated MTs and show no attachment to the spindle (see Figs. 13 and 14). Very few MTs can be found in the region between the two centrosomes. The asterisks note the position of electron translucent membrane inclusions which are often found in these cells. 8,000×; Bar= 3.0 μm. **Fig. 13.** Seven serial sections were used to reconstruct the position of 14 kinetochores from the cell pictured in **Fig. 11.** Kinetochores 1–3 were attached and oriented towards the lower pole (cf. Fig. 12); the kinetochores (4) near the upper pole were apparently unattached although numerous MTs were found in their vicinity; MTs were not attached to nor found in the vicinity of the remaining kinetochores. The kinetochores on chromosomes 10 and 9 are pictured in Figure 15a and b respectively. Bar=0.5 μm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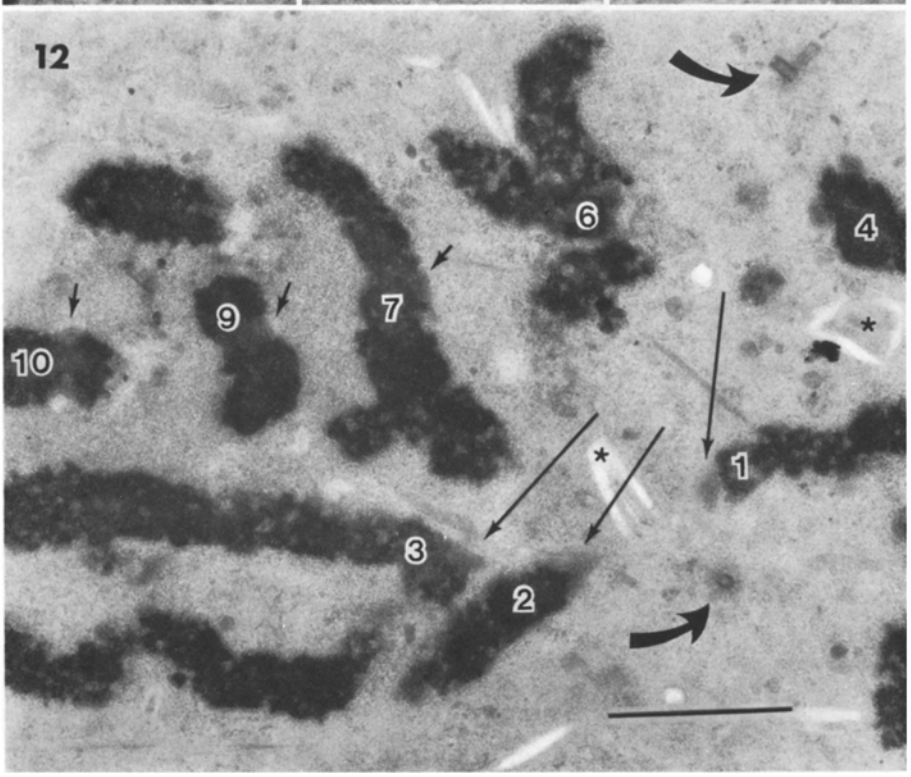
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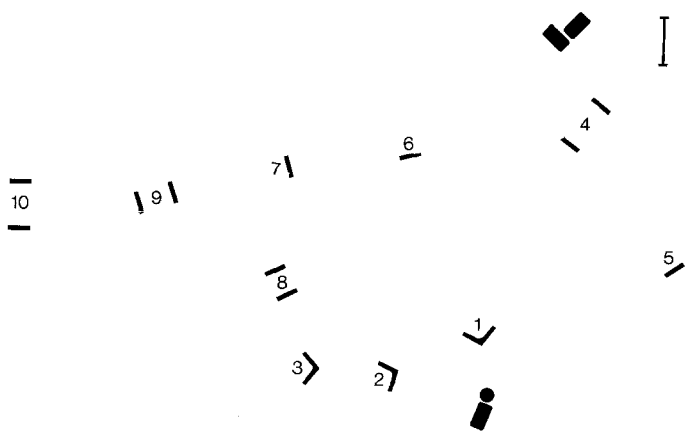
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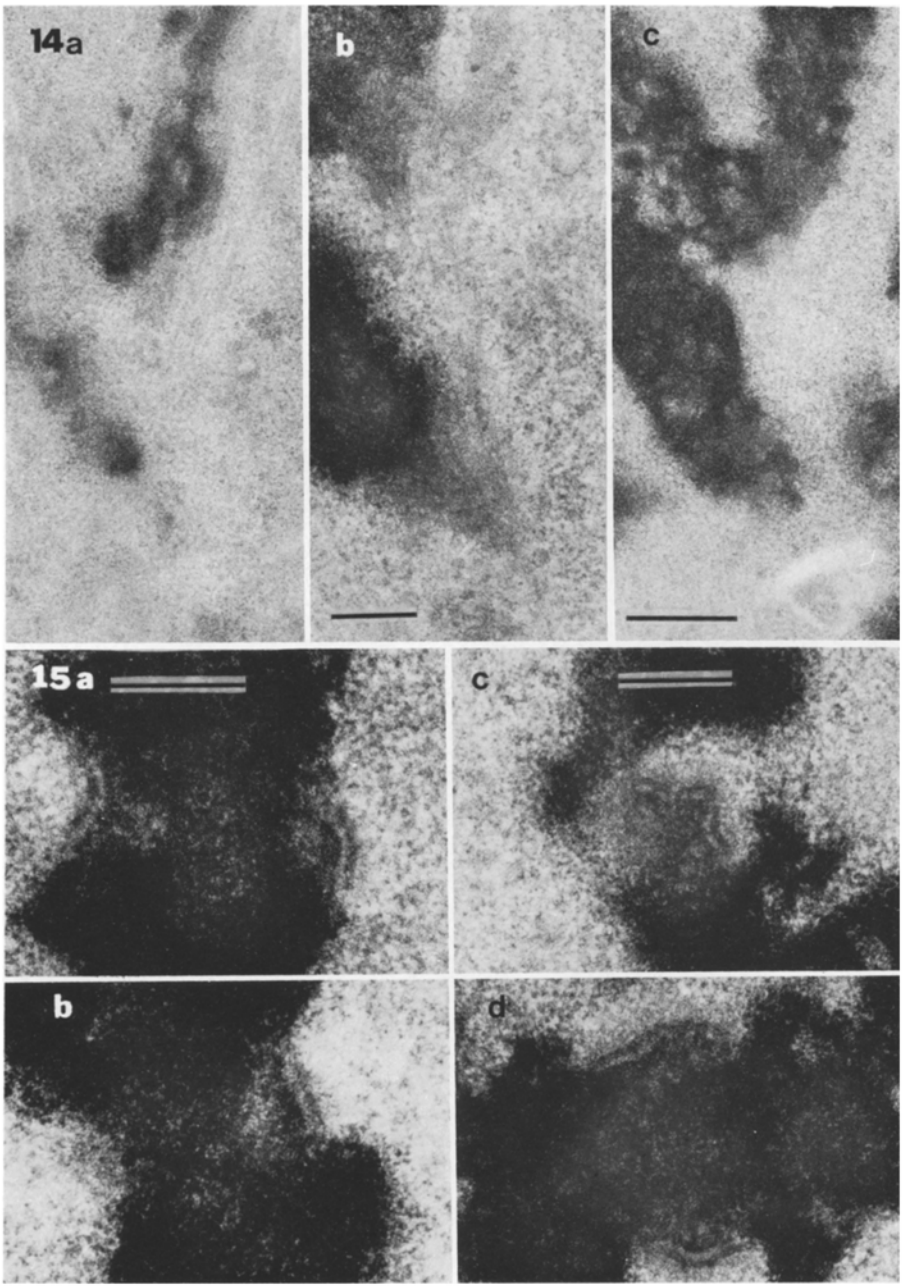


Figs. 14 and 15. Fig. 14a–c. Three serial sections of two of the attached kinetochores (i.e., 2 and 3) noted by the long arrows in Figure 12. These micrographs are oriented so that the centrosomal area would be positioned at the bottom right hand corner of the plate. Both of these kinetochores are found only in section B and they are stretched towards the centrosome. **b** Slightly more magnified than **a** and **c** to reveal associated MTs. **a, c** 15,000×, **b** 55,0000×; Bar in **b** 0.25 μm, 1.0 μm. **Fig. 15a–d. a and b** Electron micrographs of two of the unattached kinetochores (i.e., 10 and 9 in Figs. 12 and 13) from the cell pictured in **Figure 11.** MTs are not associated with these kinetochores and they remain unstretched. The kinetochores in **c** and **d** are from a cell fixed under similar conditions to that in Figure 11. Note that a filamentous corona appears to be associated with the outer disk of each kinetochore (cf. Fig. 9). **a, b** 38,000×, **c, d** 30,000×; Bars in **a** and **c** 0.50 μm

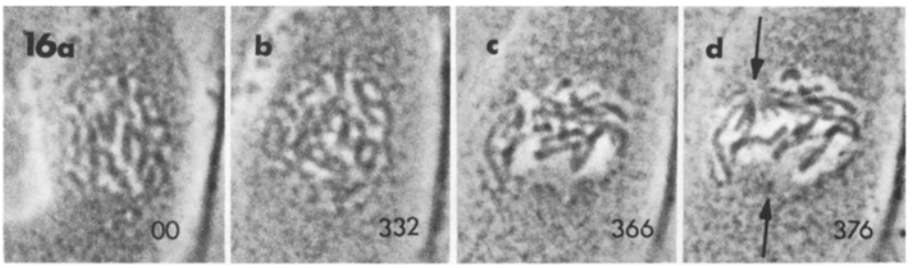
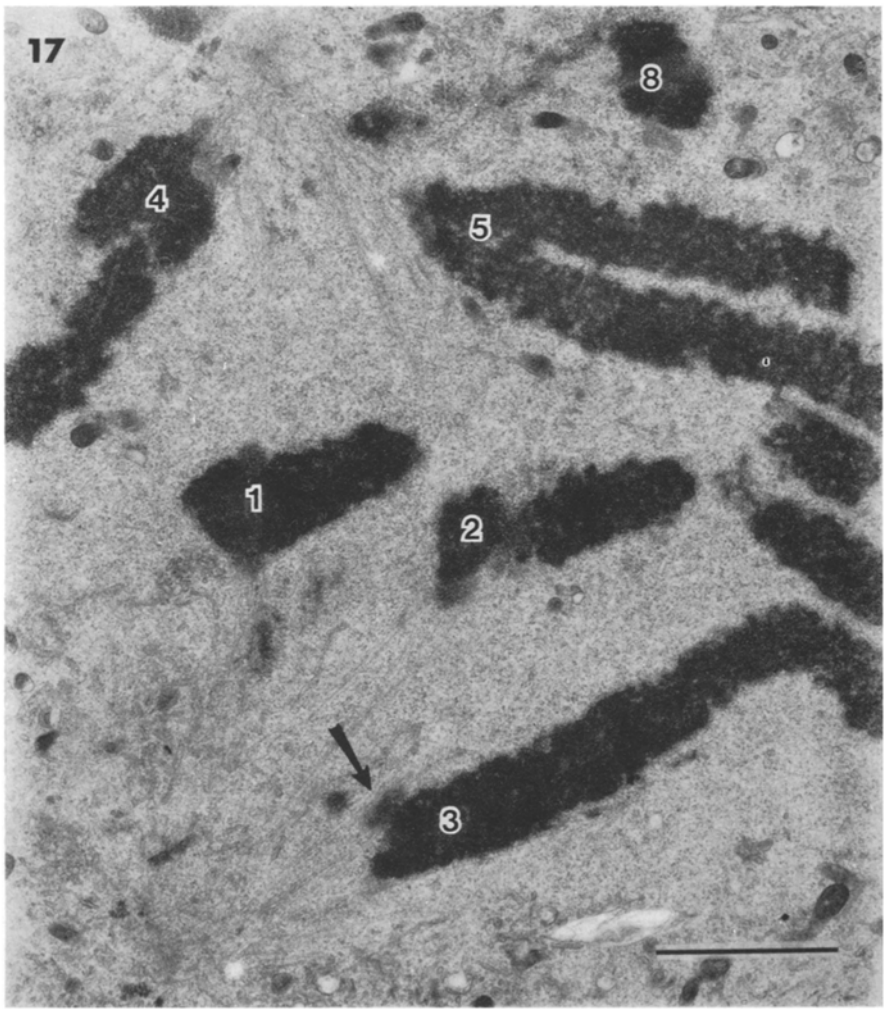


Fig. 16a–d. A prophase cell exposed to 6° C for 346 min and then fixed 30 min after the initiation of recovery. A comparison of **a** and **b** indicates that the NE broke down in the cold. **c** and **d** Pictures of the cell 20 and 30 min, respectively, after the initiation of recovery. The cell was fixed immediately after taking the photograph in **d**. Thin arrows in **d** mark the position of the centrosomes

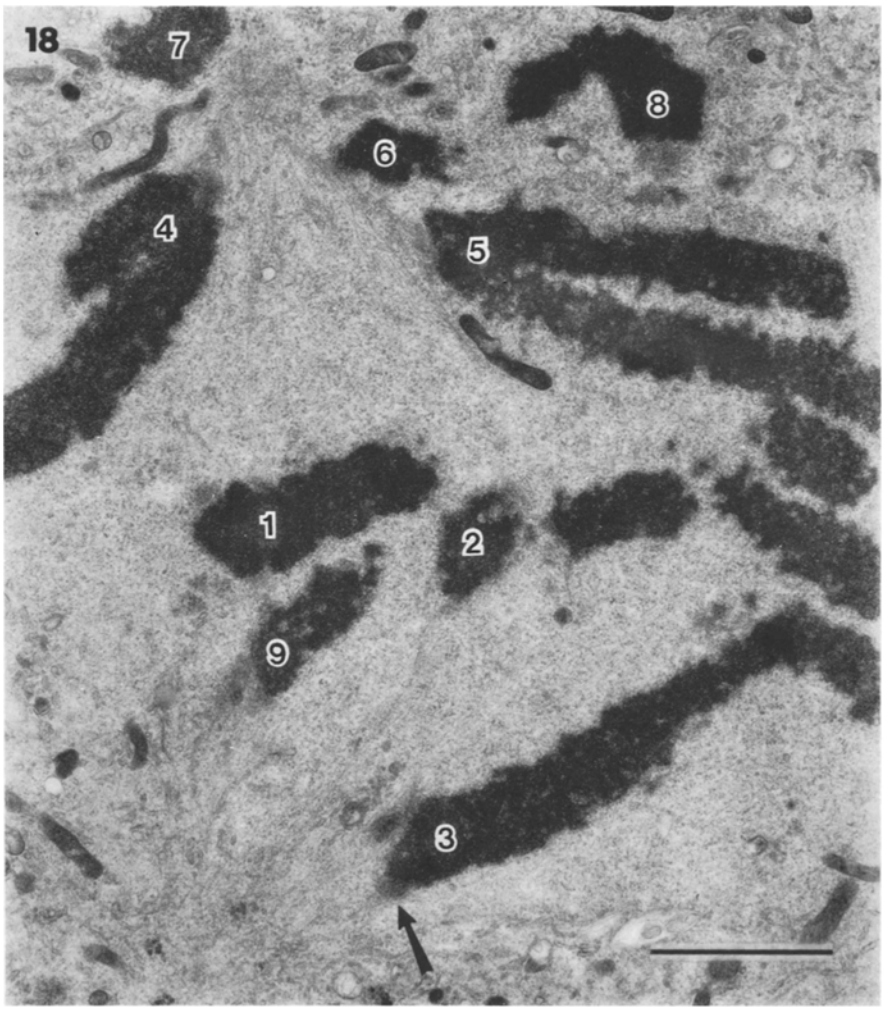
MTs. These unattached kinetochores appeared similar in structure to those found in cells fixed 5 min after initiating recovery, the only notable exception being that the chromatin subjacent to these kinetochores now appeared more electron opaque than those in cells fixed earlier in recovery (cf. Figs. 8, 9 and 12, 15).

The above observations are clearly illustrated by Figures 11–15. The series of photomicrographs in Figure 11 depicts a prophase cell that was kept at 6° C for 10 h and allowed to recover for 15 min. The abrupt change in the position of the chromosomes (cf. Fig. 11a and b) indicated that the NE had broken down within the first 7 h of the cold treatment. A comparison of photographs taken before recovery (Fig. 11b) and immediately prior to fixation (Fig. 11c) reveals very little if any movement of the chromosomes. However, an analysis of serial sections from this cell showed that 3 of the 11 chromosomes were, by the criterion of poleward stretching, attached to the spindle and all of these plus both poles were captured in a single fortuitous thick section (Fig. 12). A reconstruction of kinetochore position and status within this cell, based on 7 serial 0.25 μm sections and showing 14 of the 22 kinetochores, is shown in Figure 13. The chromosomes which showed attachment to the spindle (1–3; long arrows in Fig. 12; see also Fig. 14) were those that were located closest to the lower centrosome. Figure 14 is a set of three serial sections through two (i.e., chromosomes 2 and 3) of the attached kinetochores pictured in Figure 12, showing that the stretched appearance is not an artifactual result of a grazing section. By the criterion of closeness (see above) MTs were considered to be associated with the stretched kinetochores on these chromosomes (Fig. 14b). As previously mentioned the attachment of these kinetochores to the forming spindle occurred without an obvious movement of the chromosomes. An analysis of serial sections revealed that those kinetochores that were further from the centrosomes in this cell (short arrows in Fig. 12) were unstretched and completely free of MTs (Fig. 15a and b). Although numerous MTs were associated with both of the well separated centrosomes, the spindle between the centrosomes in this cell was poorly organized (see Fig. 12). This suggests that centrosome separation had already occurred prior to the initiation of the cold treatment.



Figs. 17 and 18. Two serial sections taken from near the middle of the spindle from the cell pictured in **Figure 16.** Nine chromosomes can be identified in these two sections. An analysis of such serial sections revealed that chromosomes 1 and 2 were amphi-oriented near the middle of the spindle, 3 and 9 were mono-oriented to the lower pole while the rest were mono-oriented to the upper pole. Note that both kinetochores of chromosome 3 are found in these two sections; one is attached and oriented to the lower pole (*arrow* in **Fig. 18.**) while its sister (*arrow* in **Fig. 17**) is unattached and shows no orientation. Both micrographs are 8,500×; Bar = 3.0 μm

c) 30 min after the Initiation of Recovery. With few exceptions, the chromosomes in cells fixed 30 min after the initiation of recovery showed movement and were therefore, by this light microscopic criterion, considered attached to the spindle. An analysis of serial sections of these chromosomes revealed that their kinetochores were stretched poleward and that MTs terminated at the kinetochores. The majority of these chromosomes were closely associated with one or the other centrosome (i.e., they were mono-oriented) and only a few showed



connections to both poles (i.e., amphi-orientation). Those chromosomes which were amphi-oriented were invariably positioned equidistant between the separated centrosomes. In most instances the configuration of the kinetochores on mono-oriented chromosomes could not be unambiguously established. However, when the kinetochore status on these mono-oriented chromosomes could be clearly defined, the majority were found to possess a unitelic configuration (i.e., the kinetochore facing the centrosome possessed MTs and was stretched while its sister, facing in the opposite direction was unstretched and free of MTs) and only a few had the syntelic configuration (in which both kinetochores on a replicated chromosome were oriented to the same pole). The salient point for this paper is, however, that irrespective of whether the kinetochores on these chromosomes were unitelic or syntelically oriented, both reflect mono-

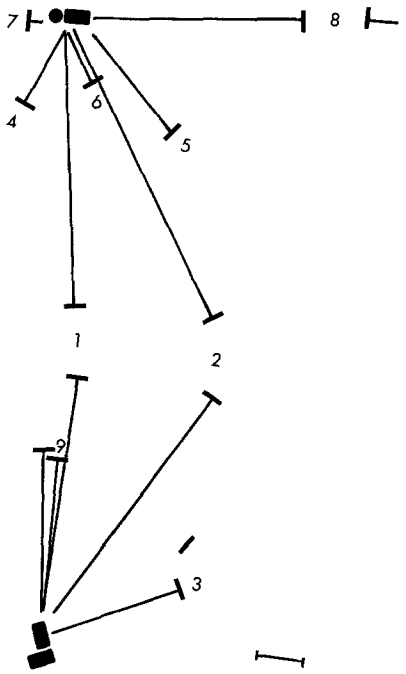


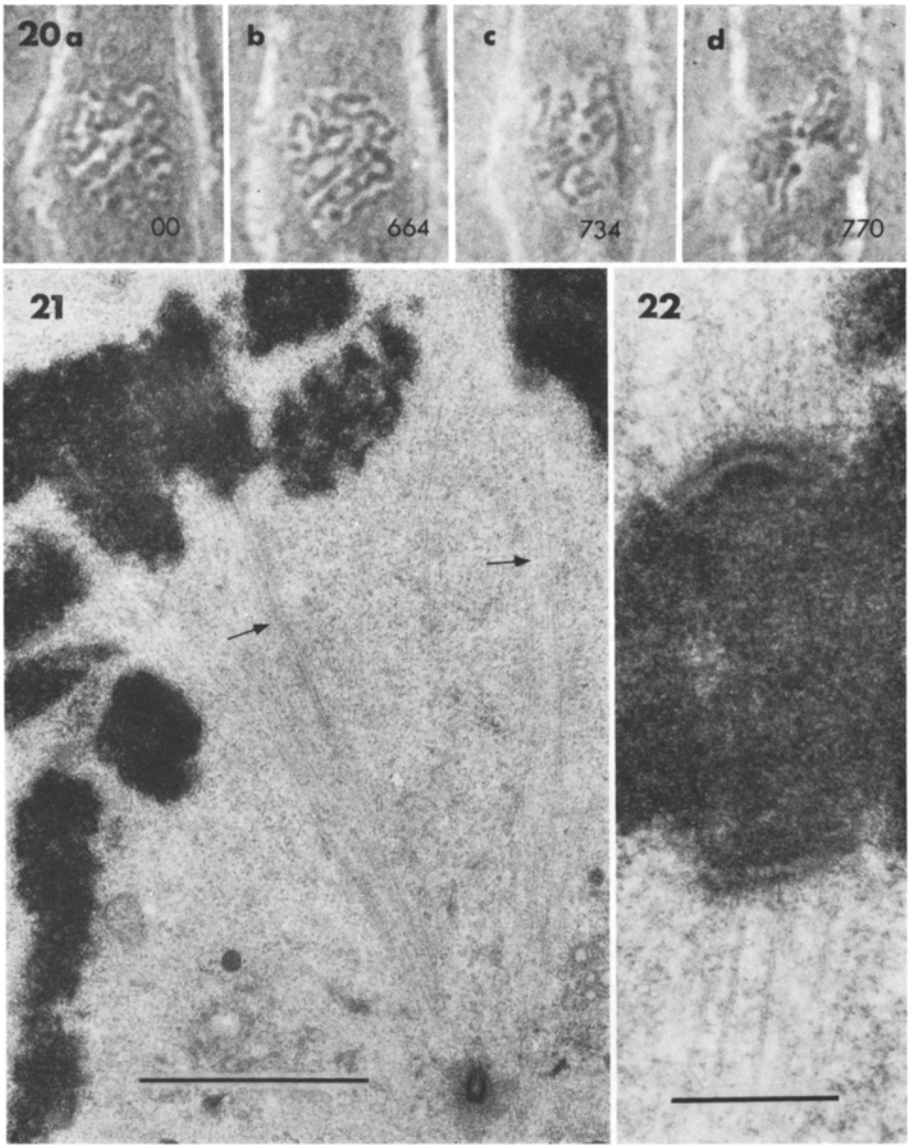
Fig. 19. Six serial sections were used to reconstruct the position of kinetochores in the cell pictured in Figure 16. Fourteen kinetochores were recognizable in the sections used to make this reconstruction. Only two (i.e., 1, 2) of the 9 chromosomes included within the reconstruction were amphi-oriented, the rest were mono-oriented to one or the other centrosomes (cf. Figs. 17 and 18). The kinetochore distal to the pole on chromosome 8 was the only example found of a kinetochore which possessed MTs without being oriented towards a centrosome. Bar = 0.5 μm

orientation and may be taken as an expression of a centrosomal influence on kinetochore attachment to the spindle.

These observations are illustrated by the cell pictured in Figure 16. After 5.75 h at 6° C this cell was warmed to 18° C and fixed 30 min after the initiation of recovery (inspection of Fig. 1 indicates that it was fixed about 20 min after equilibration at 18° C). A comparison of Figure 16a and b indicates that the NE in this cell broke down in the cold. Figure 16c and d are photographs of the same cell taken 20 and 30 min, respectively, after the initiation of recovery.

The ultrastructural features of this particular cell are presented in Figures 17–19. The cell was sectioned in such a way that both poles were captured in a single section. This greatly facilitated the subsequent reconstruction of the position of each kinetochore on the spindle. An analysis of six serial sections (two are shown in Figs. 17–18) revealed the orientation of nine of the eleven chromosomes (Fig. 19). Two of the chromosomes near the middle of the spindle were amphi-oriented (Chromosome 1 and 2 on Figs. 17–18), two were mono-oriented to the lower pole (Chromosomes 3 and 9) and the rest were positioned around and mono-oriented to the upper pole. Thus, 7/9 of the identified chromosomes in this cell could be documented at the EM level to be mono-oriented and attached by microtubules to a single pole.

d) 90 min after the Initiation of Recovery. Eight cells were fixed for electron microscopy 90 min after initiating recovery from an extended (8–12 h) cold treatment. All 8 cells were either in pro-metaphase or at metaphase. Four of



Figs. 20–22. Fig. 20a–d. In this prophase cell recovery was initiated after 690 min at 6° C and the cell was near metaphase when it was subsequently fixed 90 min later. **Fig. 21.** A low power micrograph of a section through the middle of the lower half spindle from the cell pictured in **Fig. 20.** Prominent bundles of kinetochore MTs (*arrows*) can be seen between the chromosomes and the polar region (see text for details). 10,000×; Bar=3.0 μm. **Fig. 22.** Two kinetochores of a chromosome from the cell pictured in Fig. 20. Numerous MTs can be seen to terminate in the outer disk of the trilaminar structure. 43,000×; Bar=0.5 μm

these cells were subsequently serially sectioned. In all cases the chromosome ultrastructure in these cells was indistinguishable from that of a normal metaphase cell at room temperature (compare Figs. 21–22 of the present study with Figs. 1 and 4 of Rieder, 1979).

The ultrastructure of the spindle in these cells was typified by the cell pictured in Figure 20. This cell was fixed 90 min into recovery following a cold treatment of 12 h. An analysis of serial sections revealed that all the chromosomes in this late pro-metaphase cell were amphi-oriented on the spindle. The majority of spindle MTs appeared to be associated into prominent bundles (Arrows in Fig. 21). These MT bundles were in all cases traced to the outer disk of the kinetochore where they terminated (Fig. 22). Many of the individual MTs were followed for up to 3–4 μm within a section before they were lost from view. However, we were unable to trace any single kinetochore MT from its kinetochore into the corresponding centrosome. Numerous astral Mts were seen associated with each centrosome and these appeared similar in structure to those published for controls. Although our impression is that the majority of MTs between the centrosomes in these spindles were kinetochore MTs, in most sections we were able to find MTs near the metaphase plate which could not be traced to a kinetochore. We consider it likely that these represent non-kinetochore MTs since many could either be followed between half spindles or past a metaphase kinetochore. The structure of the kinetochore in these cells was similar to that previously published for room temperature or 37° C cells (compare Fig. 22 of this study with Figs. 1 and 4 of Rieder, 1979).

Discussion

1. Some General Remarks

Many cells divide over a broad range of temperatures and over this range the rate at which a particular cell proceeds through division is exponentially related to the temperature of the cell (see Mazia, 1961). Cells at lower temperatures complete the various stages of mitosis as a slower rate than those at higher temperatures.

Although there are a few exceptions (see Stephens, 1972; Lambert, 1980), spindle formation is inhibited at low temperatures in most types of dividing cells (see Ris, 1949). A lengthy and continual exposure of dividing cells to cold generally results in the formation of restitution nuclei with a corresponding increase in ploidy number (see Wilson, 1925; Barber and Callan, 1942). However, it is well documented that cells in which chromosome movement has been arrested at low temperatures for shorter periods of time complete mitosis when subsequently returned to a temperature at which the cell will divide normally (Ris, 1949; Inoué, 1952, 1964; Roth, 1967; Goode, 1973; Lambert and Bajer, 1977). In fact, Inoué (1964) notes that the cold induced disruption of the spindle "can be performed on many types of cells over and over again during a single division... without impeding the ability of the spindle fibers to become reorganized again".

We found that when late prophase PtK_1 cells were chilled to 6° C the

centrosomal MTs disassemble and the NE breaks down without the subsequent formation of the spindle. Thus, although the disruption of the NE is influenced by and correlates with the growth of the astral MTs, its breakdown is not strictly dependent on the presence of these MTs. This cold induced inhibition of spindle formation was, as expected, reversible: pro-metaphase chromosome congression ensued when these cells were subsequently warmed to 18° C. The recovery temperature of 18° C was low enough to slow down the early events which occur during spindle formation yet high enough to insure recovery.

The results we obtained for cells recovering at 18° C are remarkably similar to those obtained earlier by Roos (1976) for the initial behavior of pro-metaphase chromosomes in untreated PtK_1 cells at 37° C. Since the predominant pathway, mono-orientation followed by amphitely and congression, was observed in both treated and untreated cells, we consider the mitotic process in these cold treated cells to be fundamentally normal.

2. The Proximity Effect and the Mono-Orientation of Chromosomes

During the pro-metaphase of astral divisions the first chromosomes to attach to the forming spindle are those located nearest the polar areas at the time of NE breakdown (Paweletz, 1974; Molè-Bajer, 1975; Roos, 1973b, 1976; Izutsu et al., 1977). Roos (1976, Fig. 13a) attributed this phenemenon (and that of mono-orientation) to influences resulting from the asynchronous breakdown of the NE. He proposed that MT subunits are concentrated in the astral region during late prophase and that the initial breakdown of the NE in the polar areas allows for the diffusion of MT subunits into the nucleus "so that two gradients of decreasing concentration of subunits initially exist from the polar areas to the center of the nucleus". Those chromosomes closest to the poles during NE breakdown would be the first to attach to the spindle since they would see an environment containing a high concentration of MT subunits which would favor fiber formation. As a result these chromosomes would initially mono-orient and move towards the pole due to the formation of a functional fiber only on the kinetochore nearer the pole.

Although the mechanism outlined by Roos may contribute to the path of spindle formation in normal cells, our results demonstrate that the differential breakdown of a barrier separating regions of high and low tubulin concentration is not the cause of the observed "proximity effect" or necessary for the mono-orientation of chromosomes. In the cold-treated cells, the NE broke down long before recovery was initiated and it is likely that gradients of diffusable substances formed at the onset of breakdown would have been transient and long since ceased to exist.

The initial mono-orientation of pro-metaphase chromosomes in PtK_1 cells is similar to that which occurs in a variety of untreated cells which possess well developed asters (see references in Mazia, 1961; Molè-Bajer et al., 1975; Roos, 1976). This phenomenon correlates most frequently with the initial formation of a functional fiber only on that kinetochore of a chromosome which is closest to (and facing) a pole; the sister kinetochore, which sits less than

0.5 μm away and faces in the opposite direction, invariably possesses no MTs (Roos, 1973b, 1976; Molè-Bajer, 1975; Rattner and Berns, 1976a; Figs. 17–18 of this study). However, mono-orientation may also arise when both kinetochores on a chromosome form a connection to the same pole (i.e., syntelic mono-orientation – see Roos, 1973b, 1976; Molè-Bajer, 1975). In either case the tendency to mono-orient correlates with chromosome position at the time of NE breakdown; in general those chromosomes near a polar area during NE breakdown mono-orient to that pole while those chromosomes near the middle of the spindle quickly achieve amphi-orientation (Roos, 1976). We have shown, however, that influences arising from the breakdown of the NE are not the cause of mono-orientation. Therefore, the explanation for these phenomena must lie elsewhere.

We take the mono-orientation of pro-metaphase chromosomes, and the related proximity effect, to be manifestations of a centrosomal influence on the attachment and orientation of chromosomes. From our results, and those of others, we suggest that an interaction occurs between the centrosomes and kinetochores during spindle formation which results in the formation of a fiber first on those kinetochores closest to and facing the centrosomes.

3. The Nature of the Centrosome–Kinetochore Interaction

The nature of the interaction between the centrosome and the kinetochore is currently unknown. However, we observed that those kinetochores which acquire MTs during spindle formation were, with only one exception (which could be interpreted as a re-orienting chromosome), oriented towards a polar area. Similar observations have been reported by DeBrabander et al. (1979a, b, 1980a, b – see also Nicklas et al., 1979) who found that kinetochore fiber formation in PtK_1 occurs mainly along the kinetochore to pole axis. Indeed, it appears that this phenemenon is a rule in astral divisions since a clear case of fiber formation on an unoriented kinetochore has yet to be documented in the ultrastructural literature of normally dividing *untreated* pro-metaphase cells (the initial formation of a fiber on an unoriented kinetochore has long been actively searched for since it would support the notion that kinetochores nucleate MTs in vivo, that this nucleation significantly contributes to the formation of a kinetochore fiber, and that orientation occurs subsequent to the initiation of fiber formation). The recent data therefore indicates that kinetochore fiber formation is favored only when it can be directed towards a polar region. This is significant since it suggests that kinetochore fiber formation and the poleward orientation of the kinetochore are closely coupled events which either occur simultaneously or in near synchrony. Therefore, to be consistent with the data, any mechanism of kinetochore fiber formation must explain the nature of the kinetochore to pole interaction which results in the formation of kinetochore fibers first on those chromosomes closest to the centrosome, and also in the immediate poleward orientation of the kinetochore.

Our results exclude transient gradients (Roos, 1976) as a cause of these observations. However, a related possibility is that gradient formation is an active expression of a property of the poles. If we assume that most pro-metaphase kinetochores in these cells are similarly physiologically competent

to acquire MTs[1] then any hypothesis attempting to explain the proximity effect must deal with the fact that on a unitelic mono-oriented chromosome, the kinetochore facing away from the pole possesses no MTs at a time when another chromosome, more distant from the pole, may possess MTs on both kinetochores and the amphi-oriented. This observation is not consistent with the thesis that the proximity effect (and the related mono-orientation) arises from the asynchronous nucleation and poleward growth of kinetochore fibers in response to a tubulin gradient actively maintained by the polar regions.

The observation that the kinetochores facing away from the near centrosome on a unitelic mono-oriented chromosome is free of MTs suggests that a centrosome and kinetochore interact to form a fiber via a structural rather than a diffusable factor. The most logical candidate for this structural interaction is the centrosomal MT. The proximity effect and the related mono-orientation of chromosomes are consistent with the concept that early pro-metaphase kinetochores initially associate with (and are subsequently transported along) Mts nucleated from the centrosome (as suggested by Tippit et al., 1980 for diatom spindles). Such an initial attachment mechanism predicts that kinetochore fiber formation and poleward orientation would be closely coupled events which occur in near synchrony. In addition it offers a straightforward explanation as to how the centrosome influences kinetochore orientation and attachment, how the initial mono-polar orientation of pro-metaphase chromosomes arises and why it is most pronounced in cells with well developed centrosomes.

However, Witt et al. (1980) have recently shown that when mitotic CHO cells are allowed to recover from a prolonged arrest in colcemid, kinetochores acquire MTs without facing a centrosome at a time when the MT nucleating capacity of the centrosome is supressed. One can conclude from this result that kinetochores can nucleate MTs in vivo. Furthermore, in this high voltage EM study the lack of kinetochore orientation correlates with the absence of centrosomal MTs suggesting that centrosomal MTs are involved in kinetochore orientation. Similar conclusions were reached by DeBrabander et al. (1979a, 1980a, b) who used light and electron microscopic methods to study kinetochore fiber formation in PtK_1 cells recovering from a nocodazole block. These investigators initially found short MTs nucleated in the vicinity of the kinetochores. These MTs failed to elongate, however, unless the elongation could be directed

1 The suggestion that pro-metaphase mono-orientation results from the asynchronous differentiation of sister kinetochores (Jokelainen, 1965) is unlikely. There is no reason to believe that the kinetochore lacking Mts on a unitelic mono-oriented chromosome would not acquire MTs if it were the one located nearer to (and facing) the pole at the time of NE breakdown. Indeed, the unoriented kinetochore on these chromosomes is similar in structure to the oriented kinetochore with the notable exception that it lacks MTs. Roos (1973b; see also Molè-Bajer et al., 1975) found kinetochores attached to MTs immediately after NE breakdown and concludes that late prophase kinetochores are "physiologically competent' to acquire MTs. Roos (1976) also notes that those chromosomes located near the spindle equator as the NE breaks down directly congress, a phenemenon which would require a similar differentiation level of both sister kinetochores. Finally, when prophase cells are treated with low concentrations of colcemid the chromosomes mono-orient around the unseparated centrosomes in a unitelic fashion during the subsequent pro-metaphase (Brinkley et al. 1967). When these "chromosomal spheres" are lysed into a reassembly buffer containing tubulin the kinetochores facing away from the poles acquire MTs (McGill and Brinkley, 1975). Thus, at least in this system, both kinetochores possess the capacity to acquire MTs in vitro but only those facing the pole do so in vivo

towards a polar area. They suggest that centrosome nucleated MTs interact with the short kinetochore nucleated MTs to orient, stabilize and promote the poleward growth of the forming kinetochore fiber. Such an interaction would be expected to occur very rapidly (i.e., as soon as short MTs are organized in the vicinity of the kinetochore) due to the abundant supply of centrosome nucleated MTs which make up the bulk of the untreated pro-metaphase spindle (Rattner and Berns, 1976a). In this mechanism the orienting influence of the centrosome on kinetochore fiber formation is therefore attributed to an interaction between centrosome nucleated and kinetochore nucleated MTs. The proximity effect would arise due to the fact that those kinetochores closest to the centrosomes at the time of NE breakdown would initially see the highest density of centrosomal MTs which would, as a result, facilitate their attachment. The initial position of sister kinetochores on a chromosome, relative to the proximal centrosome, would then determine whether the subsequent orientation would be syntelic or unitelic. A prediction of this hypothesis is that the kinetochore fiber forms as a hybrid structure consisting of both centrosome and kinetochore nucleated MTs and that not all of these MTs need terminate in the kinetochore.

Thus, we propose that kinetochore fibers form from centrosomal MTs which interact either directly with the kinetochore or with short MTs nucleated by the kinetochore. The same proposal has been made to explain the reattachment to the spindle of chromosomes detached by micromanipulation (Nicklas et al., 1979).

It should be noted that the short MTs which are initially seen to be associated with the kinetochores recovering from mitotic inhibitors are oriented randomly around the corona material of the kinetochore (i.e., they are not necessarily organized perpendicular to the long axis of the outer kinetochore plate nor do they terminate in this plate – see Witt et al., 1980; DeBrabander et al., 1979a, 1980a, b). This "loose" association with the kinetochore may facilitate a lateral interaction between these MTs and centrosome nucleated MTs. However, it may also confer a greater lability to these MTs. It is possible that these short kinetochore associated MTs may be disassembled during the 30 seconds it takes to fix a cell using our perfusion method (see Molè-Bajer and Bajer, 1968; Jensen and Bajer, 1969). Such an event would be reflected as a lack of MTs on those unoriented kinetochores in PtK_1 cells recovering from the cold treatment.

4. Conclusion

We have shown that an interaction occurs during spindle formation between the kinetochores and the centrosomes which is manifested by the attachment first of those kinetochores closest to the centrosome. We argue that this proximity effect does not arise from kinetochore fiber formation in response to influences related to the asynchronous breakdown of the NE, from an actively maintained polar gradient, or from asynchronous sister kinetochore differentiation. The proximity effect and the observation that kinetochore fiber formation occurs predominantly towards a polar region are most simply explained by the hypothesis that centrosome nucleated MTs interact either directly with kinetochores or with kinetochore nucleated MTs.

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