

# Stability of microtubule attachment to metaphase kinetochores in PtK<sub>1</sub> cells

LYNNE CASSIMERIS<sup>1,\*</sup>, CONLY L. RIEDER<sup>2,3</sup>, GERALD RUPP<sup>2,†</sup> and E. D. SALMON<sup>1</sup>

<sup>1</sup>Department of Biology, CB#3280, University of North Carolina, Chapel Hill, NC 27599-3280, USA

<sup>2</sup>Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201-0509, USA and

<sup>3</sup>School of Public Health, SUNY-Albany, Albany, NY 12222, USA

\* Author for correspondence at: Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA

† Present address: Department of Anatomy and Cell Biology, SUNY-Buffalo, Buffalo, NY 14214, USA

## Summary

Kinetochores are known to be differentially stable to a variety of microtubule depolymerization agents compared to the non-kinetochore polar microtubules, but the dynamics of microtubule attachment to the kinetochore is currently controversial. We have examined the stability of kinetochore microtubules in metaphase PtK<sub>1</sub> spindles at 23°C when microtubule assembly is abruptly blocked with the drug nocodazole. Metaphase cells were incubated in medium containing 34 µM nocodazole for various times before fixation and processing either for immunofluorescence light microscopy or serial-section electron microscopy. Microtubules not associated with kinetochore fibers disappeared completely in less than 1 min. Kinetochore fibers persisted and shortened, as the spindle poles moved close to the chromosomes over a 10–20 min interval. During this shortening process, the number of kin-

etochore microtubules decreased slowly. The mean number of kinetochore microtubules was 24±5 in control cells and zero in cells incubated with nocodazole for 20 min. The half-time of microtubule attachment to the kinetochore was ~7.5 min. These results show that when microtubule assembly is blocked, kinetochore microtubules shorten more slowly and persist about 10 times longer than the labile polar microtubules. If kinetochore microtubules shorten by tubulin dissociation at their plus-ends like the non-kinetochore polar microtubules, then the microtubule surface lattice must be able to translocate through the kinetochore attachment site without frequent detachment occurring.

Key words: metaphase, kinetochore, microtubule.

## Introduction

Chromosomes are segregated during mitosis by interactions between their kinetochores and the plus-ends of spindle microtubules (Nicklas, 1987; Mitchison, 1988; Salmon, 1989). Initially, a kinetochore on a chromosome attaches to polar microtubules from one spindle pole. When the opposing kinetochore attaches to microtubules from the opposite pole, the chromosome moves to the spindle equator, a position equidistant between the poles. This movement requires the concurrent lengthening of one kinetochore fiber and the shortening of the opposite fiber. At anaphase onset the sister chromatids separate and their kinetochore fibers shorten as the chromatids move poleward (reviewed by Mitchison, 1988; McIntosh and Koonce, 1989; Salmon, 1989).

Recent experiments have determined that labeled tubulin subunits add onto kinetochore microtubules at a site proximal to the kinetochore (Mitchison *et al.* 1986; Mitchison, 1988; Geuens *et al.* 1989). One interpretation of these results is that lengthening of a kinetochore fiber occurs by the growth and translocation of individual kinetochore microtubules at their attachment sites (Mitchison, 1988). Alternatively, the presence of kinetochore microtubules with labeled subunits localized proximal to the kinetochore could arise if the kinetochores rapidly released and rebound microtubules (free microtubule plus-ends would

elongate, incorporating labeled subunits and subsequent capture by the kinetochore would result in labeled subunits localized proximal to the kinetochore) (Cassimeris *et al.* 1988; Salmon, 1989).

In this paper we have investigated this issue by examining the stability of kinetochore microtubules at metaphase by blocking microtubule assembly. Microtubule elongation can be selectively inhibited in reconstituted preparations *in vitro* by simply diluting the tubulin concentration (Koshland *et al.* 1988). In living-cell experiments, selective dilution of the tubulin concentration has not been possible. However, a complex of tubulin and a colchicine-like drug can be used to block microtubule assembly rapidly *in vivo* (Salmon *et al.* 1984; Cassimeris *et al.* 1986). In the experiments reported here, we treated metaphase PtK<sub>1</sub> cells at room temperature with high concentrations (34 µM) of the tubulin-binding drug nocodazole and monitored the disappearance and shortening of kinetochore microtubules by immunofluorescence and serial-section electron microscopy. This concentration of nocodazole has been shown to induce rapid disappearance (half-life about 10 s at 37°C in PtK<sub>1</sub> cells (Salmon *et al.* 1984; Salmon, 1989)) of the non-kinetochore polar microtubules. Nocodazole rapidly penetrates the plasma membrane and has been shown to bind to the colchicine binding site on the tubulin dimer; this site is not exposed when the dimer is incorporated into the lattice wall of a microtubule

(Margolis and Wilson, 1977; Hoebeke *et al.* 1976; Lee *et al.* 1980). Our study was done at room temperature in order to correlate results with data on spindle microtubule dynamics we measured previously, using fluorescently labeled tubulin, and measurements of fluorescence redistribution after photobleaching (FRAP) (Cassimeris *et al.* 1988).

## Materials and methods

### Cell culture and nocodazole incubation

Stock cultures of PtK<sub>1</sub> cells were maintained at 37°C in Ham's F-10 medium supplemented with fetal bovine serum and antibiotics. Cells were plated onto polylysine-coated coverslips and used within 2 days after subculture. For experiments cells were transferred to 23°C HEPES-buffered culture medium and allowed to equilibrate at this temperature for 20–25 min. Coverslips were transferred to HEPES-buffered culture medium containing 10 µg ml<sup>-1</sup> (34 µM) nocodazole (Sigma) and fixed after various times in nocodazole.

At 23°C PtK<sub>1</sub> cells proceed through mitosis, but at a much slower rate than at 37°C (Rieder, 1981b). As a result of this extended time in mitosis several metaphase cells could be marked on each coverslip using a circular diamond objective scribe prior to incubation in nocodazole.

### Indirect immunofluorescence

Coverslips of control and nocodazole-treated cells were fixed and processed for anti-tubulin immunofluorescence as described previously (Cassimeris *et al.* 1986). After staining, cells were examined and photographed with a Nikon 100×/1.4 NA Plan Apochromat objective mounted on a Zeiss Universal microscope stand.

### Electron microscopy

Coverslips of control and nocodazole-treated cells were fixed for 30 min at 23°C in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.1). They were then washed in buffer, postfixed in 1% OsO<sub>4</sub> (in cacodylate buffer) for 15 min at 4°C, dehydrated in a graded series of ethanols, and flat embedded in Epon/Araldite. One to two cells from each time point, which were marked prior to nocodazole incubation, were relocated within each embedment, excised, and mounted on Epon pegs. Each cell was then serially thin sectioned with a diamond knife using a Sorvall Mt 6000 ultramicrotome. Ribbons of serial sections were mounted on Formvar-coated slot grids and stained with uranyl acetate and lead citrate (Rieder, 1981a). Sections were examined and photographed with either a Philips 301 or a Zeiss EM10C electron microscope operated at 80 kV.

### Data analysis

Electron microscopic negatives of serial sections were printed to a final magnification of ×20 000 to ×50 000. The number of microtubules terminating within the kinetochore (i.e. kinetochore microtubules) was then determined from the serial photographic prints. The kinetochore was defined on the basis of its trilaminar appearance in thin-section electron micrographs. Microtubules that clearly terminated within this recognizable structure were defined as kinetochore microtubules. In most cases, the entire kinetochore region was contained within six serial thin sections.

## Results

Kinetochore fibers are distinct in immunofluorescence micrographs of metaphase spindles fixed before nocodazole addition, but predominant in spindles fixed 10 s (data not shown) or longer after nocodazole addition (Fig. 1). Few, or no, non-kinetochore fiber microtubules were seen in cells fixed after ~30–60 s in nocodazole and no microtubules of any kind were seen in cells fixed ~20 min after incubation

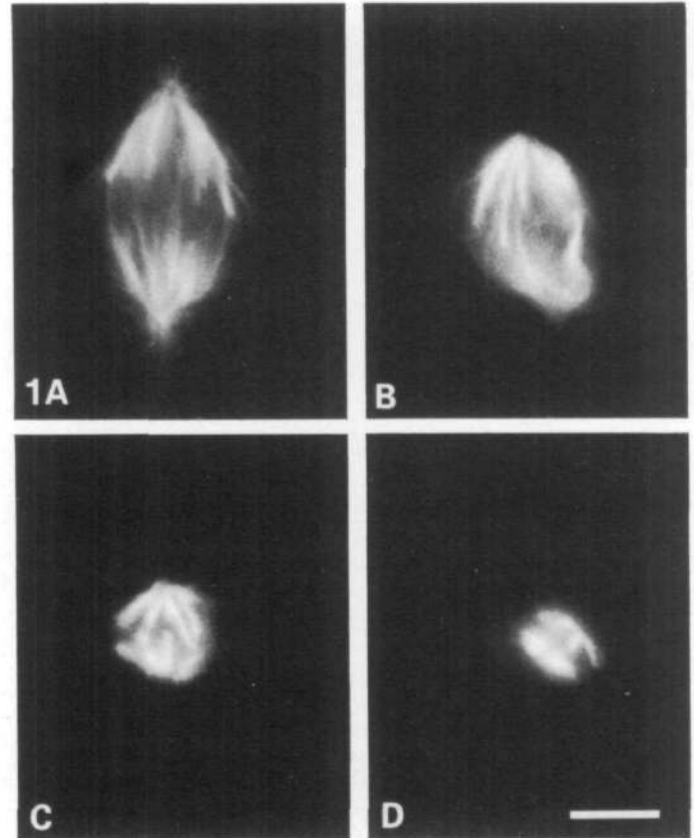
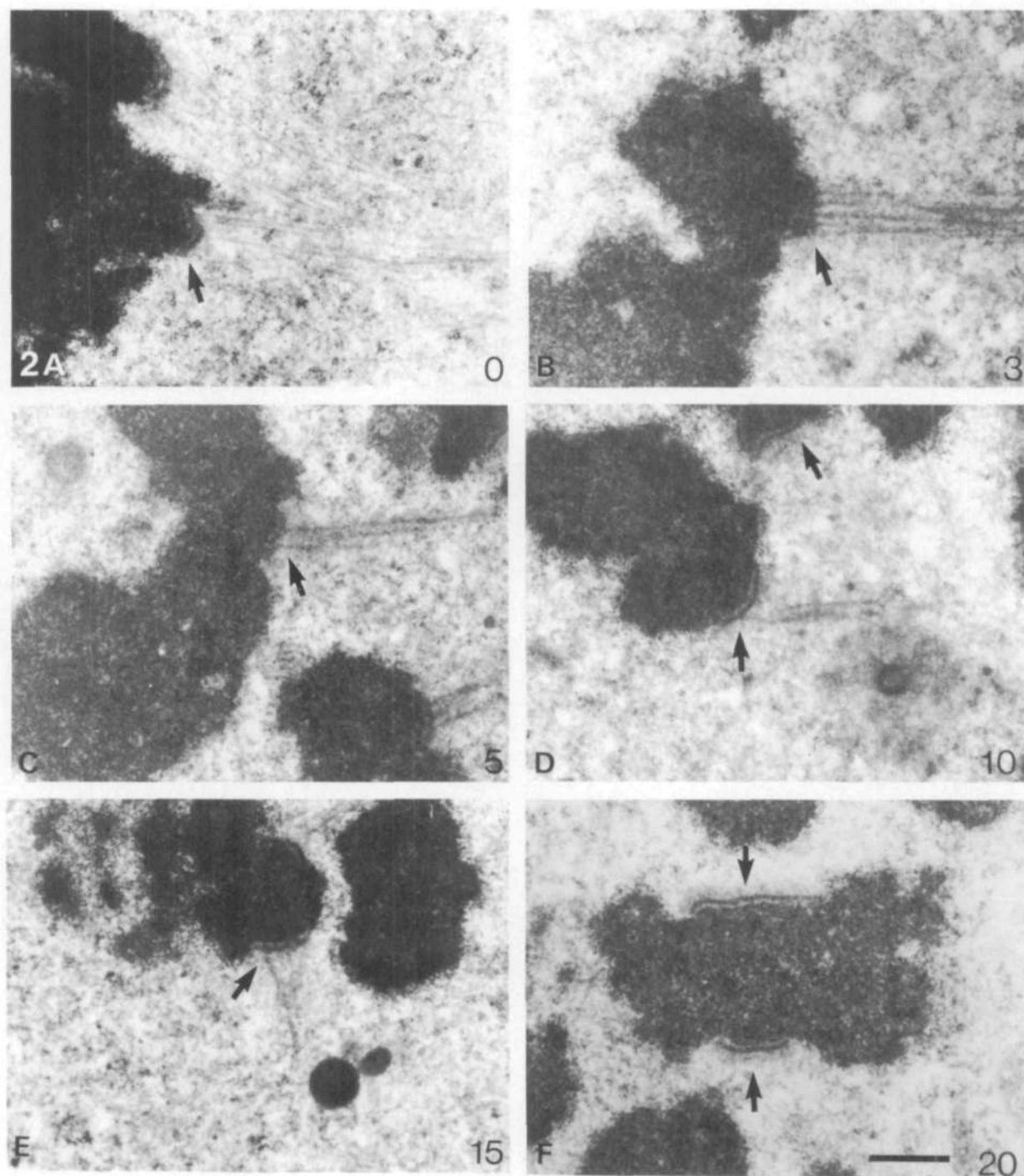


Fig. 1. Immunofluorescence micrographs of metaphase PtK<sub>1</sub> cells fixed after incubation in nocodazole. A, Control; B, 3 min; C, 5 min; and D, 10 min incubation in nocodazole. Bar, 5 µm.

in nocodazole. Bundles of kinetochore fiber microtubules were seen to span the distance between the kinetochores and the poles in electron micrographs of cells incubated with nocodazole for less than 20 min. In favorable sections, (those cut parallel to a kinetochore fiber), many microtubules could be followed continuously from the kinetochore to the pole (data not shown, similar to those of Rieder, 1981a and Witt *et al.* 1981).

The differentially stable kinetochore fibers shortened considerably when microtubule assembly was blocked and by 10 min in nocodazole they were only ~25–45% of their initial length. The poles moved inward to the chromosomes as the kinetochore fibers shortened. The kinetochore fibers always appeared to extend from the poles to the primary constrictions of the chromosomes; they never appeared to be detached from either the kinetochores or the poles in either fluorescent (Fig. 1) or electron micrographs (data not shown) of cells fixed during the shortening process. This result confirms previous observations of kinetochore fiber shortening after treatment of spindles with colchicine-like drugs, high hydrostatic pressure or cooling (reviewed by Inoue, 1981; Salmon, 1989).

The number of microtubules attached to a kinetochore decreased slowly with longer incubations in nocodazole, as seen in the electron micrographs in Figs 2 and 3. Representative sections through kinetochores at each time point are shown in Fig. 2. Fig. 3 shows serial sections through kinetochores fixed at 3 min, 5 min and 20 min after incubation in nocodazole. We determined the mean number of kinetochore microtubules and the average

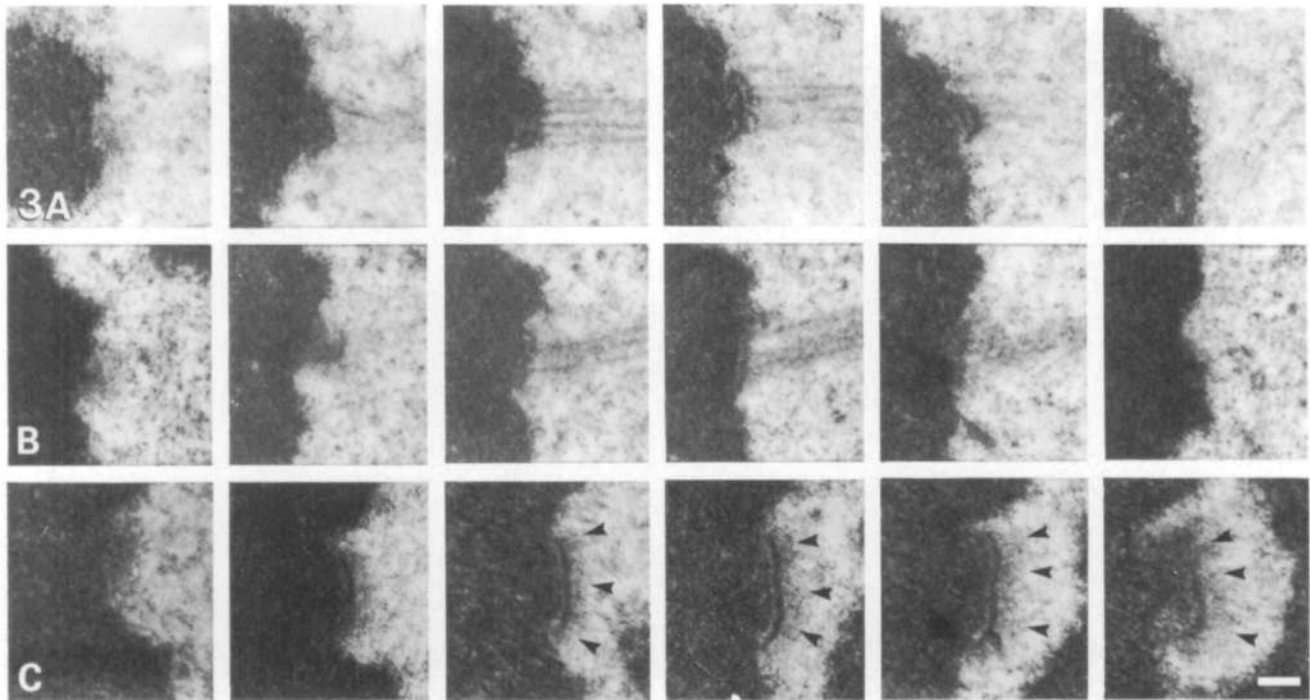


**Fig. 2.** Electron micrographs of representative sections through the kinetochore regions of cells fixed after incubation in nocodazole. The time of incubation (in min) in nocodazole is given in each frame. Kinetochores are denoted by arrows. Bar, 0.5  $\mu\text{m}$ .

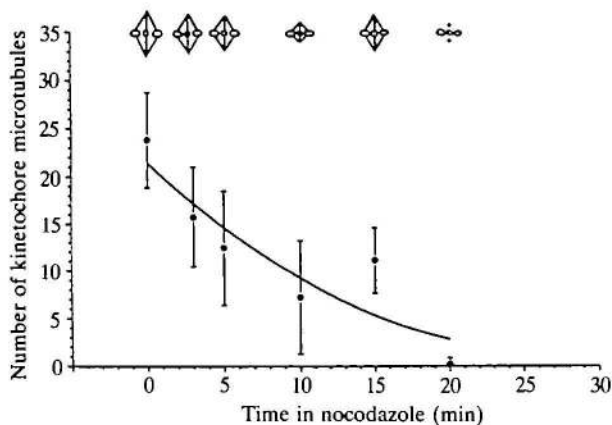
length of the kinetochore fibers from these and similar sets of serial sections through other cells fixed at different times after nocodazole treatment (Fig. 4). The average number of kinetochore microtubules was  $24 \pm 5$  in control cells. After nocodazole addition, the mean number of kinetochore microtubules decreased as a first-order reaction with a half-life of  $\sim 7.5$  min. The calculated half-life would be  $\sim 30$  s shorter without inclusion of the 15 min data point (Fig. 4), but since we have no reason to disregard the data from this seemingly anomalous cell, we

have included this point in the calculation of the half-life of microtubule attachment to the kinetochore.

We also looked for structural changes in the kinetochore correlated with decreased numbers of microtubules. The kinetochore corona material (reviewed by Rieder, 1982; Brinkley *et al.* 1989) became very distinct, and appeared to radiate away from the kinetochore plate as the number of attached microtubules decreased (Figs 2F, 3C). No other obvious structural changes in the trilaminar appearance of the kinetochore were observed.



**Fig. 3.** Serial sections through kinetochores in cells fixed 3 min (A), 5 min (B), and 20 min (C) after incubation in nocodazole. The corona material appears distinct and radiates away from the kinetochore after 20 min in nocodazole (arrowheads in C). Bar, 0.25  $\mu$ m.



**Fig. 4.** Kinetics of kinetochore microtubule number loss after incubation in nocodazole. The number of kinetochore microtubules was determined from electron micrographs of serial sections as described in Materials and methods. Each data point represents the mean number of kinetochore microtubules per kinetochore  $\pm$  standard deviation. The relative length of the kinetochore fibers is shown along the upper portion of the graph directly above the corresponding data point.

## Discussion

Our results showed that the average number of kinetochore microtubules in PtK<sub>1</sub> metaphase cells at 23°C was 24  $\pm$  5, approximately the same number as found previously for PtK kinetochores at 37°C (Brinkley and Cartwright, 1975; Roos, 1973) or 4°C (Rieder, 1981a). Because metaphase kinetochores of cells fixed at 37°C, 23°C and 4°C all have  $\sim$ 24 microtubules, this number is likely a measure of the average number of microtubule binding sites on PtK<sub>1</sub> kinetochores.

Our results also showed that nocodazole at 34  $\mu$ M initiates three events in metaphase PtK<sub>1</sub> cells: a rapid disappearance (<1 min) of the majority of non-kinetochore polar and astral spindle microtubules; a shortening of persistent kinetochore fibers; and a slow loss of kinetochore microtubules characterized by a half-life of  $\sim$ 7.5 min. It is clear from these results that kinetochore microtubules are much more stable than the non-kinetochore polar microtubules, a result found previously for other microtubule-depolymerizing agents such as cooling, high hydrostatic pressure, calcium and colchicine-like drugs (Inoue, 1952, 1976; Brinkley and Cartwright, 1975; Lambert and Bajer, 1977; Salmon, 1975; Salmon and Segall, 1980; Salmon and Begg, 1980; Rieder, 1981a; Salmon *et al.* 1984).

The  $\sim$ 7.5 min half-life for microtubule attachment to the kinetochore represents a minimum value for the stability of kinetochore microtubules, since two classes of mechanisms could generate the loss of kinetochore microtubules: (1) detachment induced by plus-end tubulin dissociation at the kinetochore without translocation to maintain microtubule attachment; or (2) detachment induced by tubulin dissociation at a site distal from the kinetochore (e.g. separation of minus-ends from the pole, with subsequent rapid minus-end depolymerization into the kinetochore). We cannot directly rule out either class of mechanism.

In addition to the mechanisms described above, it is also theoretically possible for the number of kinetochore microtubules to decrease by shortening at the kinetochore without any microtubule detachment. This decrease in the number of kinetochore microtubules could occur because not all kinetochore microtubules span the distance between the kinetochore and the pole (Rieder, 1981a). To determine whether this mechanism could account for the data, the theoretical numbers of kinetochore microtubules at positions closer to the pole (at positions approximating

the amount of spindle shortening, see Fig. 4) were determined from the published length distribution of the cold-stable kinetochore fiber (see Fig. 5, Rieder, 1981a). All microtubules were assumed to depolymerize from their plus-ends without detachment from the kinetochore. This mechanism could not account for our data, because it predicts very small changes in the number of microtubules attached to the kinetochore. For example, after 20 min in nocodazole this mechanism predicts that approximately 83% of the microtubules would remain attached to the kinetochore (based on Fig. 5 of Rieder, 1981a, the number of kinetochore microtubules would decrease from 29 to 24). In contrast, we found that the kinetochores were devoid of microtubules at this time point. Thus, the reduction in the number of kinetochore microtubules observed here cannot be accounted for solely by the shortening of kinetochore microtubules at the kinetochore without detachment of some microtubules during the shortening process.

These results have important implications for kinetochore regulation of microtubule turnover. According to the dynamic instability model of microtubule assembly dynamics (Mitchison and Kirschner, 1984; Kirschner and Mitchison, 1986), elongation of microtubules occurs by the addition of tubulin-GTP subunits to microtubule ends. Following dimer incorporation into an end, the GTP is hydrolyzed to GDP, producing a core of labile tubulin-GDP capped at the elongating end by newly added tubulin-GTP. When this 'tubulin-GTP cap' is lost, the microtubule end loses subunits rapidly and the microtubule rapidly shortens until it either disappears or is recapped. Since nocodazole-tubulin complex blocks microtubule elongation, both non-kinetochore and kinetochore microtubules are expected to lose their tubulin-GTP caps. The non-kinetochore microtubules rapidly disappear because their free plus-ends rapidly shorten back to the centrosome. The differential stability of the kinetochore microtubules is probably due to the attachment of their plus-ends to the kinetochore (Salmon *et al.* 1976; Mitchison and Kirschner, 1985), since other experiments have shown that kinetochore microtubules rapidly disappear upon detachment from the kinetochore (Nicklas and Kubai, 1985; Nicklas *et al.* 1982) and that microtubule plus-ends are stabilized *in vitro* by their attachment to kinetochores on isolated chromosomes (Mitchison and Kirschner, 1985). After nocodazole treatment, the rapid shortening expected for the tubulin-GDP ends of the microtubules attached to the kinetochores must be suppressed by the attachment sites within the kinetochore.

Although the kinetochore microtubules persist in nocodazole, they shorten as the poles move closer to the kinetochores at the metaphase plate. The site of tubulin subunit dissociation from kinetochore microtubules under these conditions (nocodazole-induced fiber shortening) has been determined by marking a position on the kinetochore fibers using fluorescence photobleaching. These experiments indicated that the majority of shortening occurred proximal to the kinetochore (Cassimeris and Salmon, 1988; Centonze and Borisy, 1988). Although some shortening may also occur proximal to the pole (Cassimeris and Salmon, 1988; Centonze and Borisy, 1988; see also, Mitchison, 1989), these results suggest that tubulin subunits dissociate predominately from a site proximal to the kinetochore as kinetochore microtubules shorten in nocodazole. During poleward movement of chromosomes in anaphase, tubulin subunits also dissociate from kinetochore microtubules from a site proximal to the kinetochore (Mitchison, 1986; Gorbsky *et al.* 1988). Microtubule de-

polymerization at the kinetochore implies that the microtubule attachment site of the kinetochore can translocate polewards along the surface of the microtubule as tubulin subunits dissociate from microtubule plus-ends (Mitchison, 1988).

The ~7.5 min half-life for microtubule attachment to kinetochores in nocodazole-treated cells is approximately twice as short as the 17 min half-time measured *in vitro* for the attachment of the plus-ends of taxol-stabilized microtubules to kinetochores on isolated chromosomes (Huitorel and Kirschner, 1988). This comparison suggests that the value of 7.5 min may represent the *in vivo* probability of microtubule depolymerization out of the microtubule attachment site under conditions where microtubule assembly is inhibited. Tubulin dissociation without concurrent poleward translocation of the kinetochore would produce detachment of plus-ends, which then could rapidly shorten and disappear like the free-ends of the polar, non-kinetochore microtubules. Conversely, under microtubule assembly conditions (i.e. conditions in an untreated cell), the life-time of a kinetochore microtubule could be longer than the ~7.5 min half-life measured here.

The relatively long life-time of a kinetochore microtubule determined here has important implications for the mechanism of tubulin subunit incorporation and dissociation from kinetochore microtubules as kinetochore fibers change lengths during prometaphase and metaphase. Kinetochore fibers change length during prometaphase as chromosomes congress to the metaphase plate. During metaphase, kinetochore fibers change length as chromosomes oscillate back and forth between the spindle poles over distances of several micrometers from the spindle equator for periods of 1–2 min in each direction (Salmon, 1989; D. Wise, L. Cassimeris, P. Wadsworth, C.L. Rieder and E.D. Salmon, unpublished data). Thus, the life-time of a kinetochore microtubule measured here is at least twice as long as the typical chromosome oscillation period observed during metaphase. This is consistent with the idea that tubulin subunits can incorporate into, and dissociate from, elongating and shortening kinetochore microtubules, respectively, without detachment occurring.

We previously examined microtubule turnover in prometaphase–metaphase PtK<sub>1</sub> spindles at 23°C using FRAP measurements (Cassimeris *et al.* 1988). The FRAP results indicated that 70% of the microtubules within the spindle at this temperature were dynamic and turned over with a half-time of ~77 s, while approximately 30% of the microtubules turned over at a much slower rate. In this preliminary report, we assumed that the majority of microtubules present at this temperature were kinetochore microtubules on the basis of the distinct appearance of kinetochore fibers in immunofluorescence micrographs such as those seen in Fig. 1A. Given this assumption, we suggested that the majority of kinetochore microtubules could be binding and releasing from the kinetochores at rates similar to the life-times of the polar non-kinetochore microtubules. This idea was initially proposed by McIntosh and Vigers (1987), based on FRAP measurements of PtK<sub>1</sub> spindles at 37°C. However, our subsequent ultrastructural analysis of the microtubule composition of PtK<sub>1</sub> spindles at 23°C revealed that kinetochore microtubules represented only 30% of the microtubules within the spindle (D. Wise, L. Cassimeris, P. Wadsworth, C.L. Rieder and E.D. Salmon, unpublished data). This percentage (30%) is identical to the percentage of microtubules within the spindle that turn over slowly as measured in our FRAP

studies. Thus, our initial suggestion was wrong. At 23°C, the life-time of kinetochore microtubules is much longer than the non-kinetochore polar microtubules as measured by the nocodazole studies reported here, the tubulin incorporation studies of Mitchison *et al.* (1986) as well as our FRAP experiments (Wadsworth and Salmon, 1986).

Recently, Gorbsky and Borisy (1989) examined kinetochore microtubule stability using FRAP techniques to mark kinetochore fiber microtubules in metaphase LLC-PK cells at 37°C. They estimated a half-time for turnover of microtubules within kinetochore fibers of about 70 s for 70% of the microtubules; 30% were relatively stable. A temperature-dependent turnover rate may account for the differences in microtubule stability observed by Gorbsky and Borisy (1989) and the results presented here. However, their FRAP numbers are strikingly similar to our previous FRAP results for PtK<sub>1</sub> cells at 23°C (Cassimeris *et al.* 1988). As discussed above, the 30% photobleached fluorescence that did not recover over several minutes may represent microtubules attached to the kinetochore, while the 70% fluorescence recovery during the first minute after bleaching may correspond to the dynamics of non-kinetochore microtubules that may have remained clustered with the kinetochore microtubules during the lysis procedures used in their experiments.

We also observed the appearance of fuzzy, filamentous corona material on the surface of the kinetochore regions previously occupied by kinetochore microtubules (Figs 2 and 3). The coronal filaments were not apparent in regions of the kinetochore with attached microtubules (Fig. 2). If the corona filaments were stretched out and tightly associated with the microtubule walls in this latter case, the corona filaments might not be apparent because their contrast could be lost in the contrast of the microtubule walls. Almost nothing is known about the corona filaments (Rieder, 1982; Brinkley *et al.* 1989), but recent observations indicate they could be involved in poleward kinetochore translocation along the walls of microtubules (Rieder and Alexander, 1990). If sites on the corona filaments were also responsible for the attachment of microtubules to the kinetochore, the corona filaments could provide the structural basis for the ability of the kinetochore attachment site to couple translocation to the growth and shortening of microtubules without detachment occurring.

In summary, the results reported here show that microtubule attachment to kinetochore persists during the shortening of kinetochore fibers induced by inhibiting microtubule assembly with nocodazole. Detachment occurs slowly in comparison to the dynamics of non-kinetochore microtubule assembly and the durations of chromosome movement during metaphase and anaphase. Although infrequent, detachment is probably important for the events of chromosome re-orientation that are involved in sister (mitosis) and homolog (meiosis) kinetochores becoming attached to polar microtubules from opposite poles. Attachment of a kinetochore to microtubules from both poles inhibits chromosome segregation and results in aneuploidy (Nicklas, 1985).

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