Evidence that kinetochore microtubules in crane-fly spermatoocytes disassemble during anaphase primarily at the poleward end

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SUMMARY

Anaphase chromosome motion involves the disassembly of kinetochore microtubules. We wished to determine the site of kinetochore microtubule disassembly during anaphase in crane-fly spermatoocytes.

In crane-fly spermatoocyte spindles, monoclonal antibody 6-11B-1 to acetylated α-tubulin labels kinetochore microtubules almost exclusively, with an area immediately adjacent to the kinetochore being weakly or not labelled. This ‘gap’ in acetylation at the kinetochore serves as a natural marker of kinetochore microtubules in the kinetochore fibre. We measured the length of the gap on kinetochore fibres in metaphase and anaphase in order to deduce the fate of the gap during anaphase; we used this information to determine where kinetochore microtubules disassemble in anaphase. Gap lengths were measured from confocal microscope images of fixed spermatoocytes dual labelled with 6-11B-1 to acetylated α-tubulin and YL1/2 to tyrosinated α-tubulin, the latter being used to determine the positions of kinetochores.

In metaphase the average gap length was 1.7 µm. In anaphase, the gap appeared to decrease in length abruptly by about 0.4 µm, after which it decreased in length by about 0.2 µm for every 1 µm that the chromosome moved poleward. PacMan models of chromosome movement predict that this ‘gap’ in staining should disappear in anaphase at a rate equal to that of chromosome movement. Thus, our results do not support theories of chromosome motion that require disassembly solely at the kinetochore; rather, in crane-fly spermatoocytes kinetochore microtubule disassembly in anaphase seems to take place primarily at the poles.

Key words: mitosis, meiosis, microtubule, anaphase chromosome motion, spindle fibre disassembly, acetylated tubulin

INTRODUCTION

Anaphase kinetochore microtubule (kMT) disassembly forms a cornerstone of many models of chromosome motion. Various studies have attempted to determine the site of kMT disassembly in anaphase by marking the kMTs and observing how the mark behaves. Marks that do not move but disappear as the chromosomes move into and through the marked zone suggest that disassembly occurs at the kinetochore, as predicted by various models including the ‘PacMan model’ (e.g. see Casimeris et al., 1987; Gruzdev, 1972; Pickett-Heaps et al., 1982). Marks that move poleward with the chromosomes suggest that disassembly takes place primarily at the pole, as a continuation of the metaphase ‘flux’, where subunits add at the kinetochore and come off at the pole (see Forer, 1965; Margolis et al., 1978; Margolis and Wilson, 1981; Mitchison, 1989; Mitchison and Sawin, 1990; Mitchison et al., 1986; Sawin and Mitchison, 1991). Marks that disappear without poleward movement suggest that microtubule turnover occurs throughout the fibre, as originally postulated by Inoué and colleagues (Inoué and Ritter, 1975; Inoué and Sato, 1967). All three results have been reported and thus experimental data concerning kMT disassembly during anaphase do not present a consistent view.

In one of the first marking studies (Forer, 1965), a microbeam of ultraviolet light was used to create areas of reduced birefringence (ARBs) on kinetochore fibres. The ARBs moved poleward in both metaphase and anaphase, suggesting that kMTs disassemble at the pole in both metaphase and anaphase. However, electron microscopic studies revealed that the ARBs are areas in which microtubules are destroyed (Wilson and Forer, 1989b).

In a different approach, Mitchison et al. (1986) allowed injected biotinylated tubulin to incorporate into metaphase BSC1 cell spindles, then fixed the cells and determined the site of incorporation using immunogold electron microscopic techniques. The labelled tubulin was incorporated at the kinetochore in metaphase but was lost at anaphase, suggesting that the metaphase ‘flux’ ceases in anaphase. However, using a similar technique but injecting anaphase cells, Wadsworth et al. (1989) found that biotin-tubulin was incorporated at the kinetochore during early- and mid-anaphase.

A third method of marking kMTs is to photobleach local regions of spindles that have incorporated fluorescently labelled tubulin. In LLC-PK cells, no movement of the photobleached area was detected in anaphase (Gorbsky and Borisy, 1989; Gorbsky et al., 1987, 1988), suggesting that disassembly during...
anaphase occurs primarily at the kinetochore. In contrast, in sand dollar eggs the photobleached area both moved poleward and filled in (Hamaguchi et al., 1987), though it is unclear whether the latter results were due to kMTs or other spindle microtubules. The interpretations of photobleaching experiments also are complicated by evidence that under certain conditions irradiation of fluorophores can alter cytoskeletal components such as actin and myosin (Knight and Parsons, 1991), and can affect the dynamic behaviour and integrity of the labelled microtubules (Leslie et al., 1984; Vigers et al., 1988). In PC12 neurites different photobleaching protocols can alter dramatically the behaviour of the photobleached zones (Keith and Farner, 1993). Thus different photobleaching experiments have yielded different results, with the technique itself possibly contributing to this variation.

In the most recent approach to marking kMTs, tubulin tagged with a non-fluorescent fluorescein derivative that becomes fluorescent when photoactivated is allowed to incorporate into spindles; then a small region of the spindle is photoactivated, marking the microtubules. With this technique there is unambiguous poleward motion of the photoactivated area in anaphase A in newt lung cells, though at a rate slower than chromosome motion (Mitchison and Salmon, 1992). The results suggest that in newt cells the kMTs disassemble at both the kinetochore and pole ends: early in anaphase the relative contributions to kinetochore fibre shortening made by the kinetochore and the pole are 75% and 25%, respectively, shifting to 63% and 37%, respectively, later in anaphase. Using a similar technique in LLC-PK cells, Zhai et al. (1993) estimate that anaphase chromosome motion is 84% due to disassembly at the kinetochore and 16% due to disassembly at the pole.

It is difficult to extrapolate any of the above results into other cell systems, since different techniques (and different cell types) yield different outcomes. An added concern is evidence that different microtubule structures may contain different arrays of tubulin isotypes (Fackenthal et al., 1993; Oka et al., 1990), that isotype specificity may be crucial to microtubule organization and functioning (Fackenthal et al., 1993), and that microtubule behaviour can be altered by altering the ratios of tubulin isotypes (e.g. see Lu and Ludueña, 1993, 1994; Matthews et al., 1993; reviewed by Ludueña, 1993). These data confound interpretations of experiments using microinjected tubulin, since one does not know if subtle effects on spindle dynamics and behaviour might be caused by the introduction of various brain tubulin isotypes. We therefore have used a different approach in studying chromosome motion in crane-fly spermatocytes, which, in any event, are not amenable to microinjection (Czaban et al., 1993).

In this study we have marked kMTs using antibodies to different forms of α-tubulin. We previously showed that antibodies to acetylated α-tubulin label crane-fly spermatocyte kMTs to the general exclusion of other spindle microtubules (Wilson and Forer, 1989a), and that a region close to the kinetochore binds antibody poorly, resulting in a ‘gap’ in fluorescence in front of the kinetochore. This gap is a natural marker of kMTs, visible without prior microinjection or disruption of the cell. In anaphase, if disassembly occurs at the kinetochore, then the gap should disappear at the same rate as chromosome motion to the pole; if disassembly occurs at the pole, then the gap should move poleward with the chromosome.

In the experiments described below we measured the ‘gap’ in metaphase and anaphase cells. The results suggest that during anaphase in crane-fly spermatocytes the kMTs disassemble initially at the kinetochore and then primarily at the pole.

### MATERIALS AND METHODS

#### Cell preparations

Crane-fly (Nephrotoma suturalis (Loew)) spermatocytes were prepared as described previously (Czaban and Forer, 1985a; Wilson and Forer, 1988). Briefly, spermatocytes from four-week-old larvae were attached to coverslips with a fibrin clot (thrombin from Sigma Chemical Co., St Louis, MO; fibrinogen from CalBiochem., La Jolla, CA) under low-viscosity oil (Series 7 halocarbon oil from Halocarbon Products Corp., Hackensack, NJ). Before lysis and fixation the oil was replaced with insect Ringer’s solution (0.13 M NaCl, 0.005 M KCl, 0.002 M CaCl2 and 0.01 M Sorensen’s phosphate buffer, final pH 6.8).

#### Immunofluorescence procedures

Cells were lysed by replacing the Ringer’s solution with lysis medium (100 mM PIPES, 10 mM EGTA, 5 mM MgSO4, 5% DMSO, 0.1% NP40; final pH 6.9). After 10-20 minutes in lysis medium the cells were fixed for 10 minutes in 0.5% glutaraldehyde in phosphate buffered saline (PBS: 0.13 M NaCl, 6 mM phosphate buffer, final pH 6.9), followed by 20 minutes in NaBH4 (1 mg/ml in PBS). Coverslips were rinsed well in PBS and immunostained as described below, or stored in PBS/glycerol (1:1, v/v) until use.

For immunostaining, stored coverslips were first rinsed in PBS. All steps were carried out at room temperature and all antibodies were diluted in PBS or in PBS with 0.1% bovine serum albumin (Sigma) and 0.02% NaNO3. Coverslips were incubated for 30 minutes with monoclonal antibody 6-11B-1 against acetylated α-tubulin (α-AC) (Piperno and Fuller, 1985; a generous gift from Dr G. Piperno) diluted 1:10. Following the rinse steps (2× 5 minutes in PBS and 1× 5 minutes in PBS with 0.1% Triton X-100) they were incubated for 30 minutes with rat-absorbed FITC-conjugated goat anti-mouse IgG (Caltag Laboratories, San Francisco, CA) diluted 1:10, then rinsed as above, incubated for 30 minutes with rat monoclonal antibody YLI/2 against tyrosinated α-tubulin (α-TYR) (Serotec Limited, Oxford, England) diluted 1:100,000, rinsed, and incubated for 30 minutes with mouse-absorbed Texas Red-conjugated goat anti-rat IgG (Caltag Laboratories, San Francisco, CA) under low-viscosity oil (Series 7 halocarbon oil from Halocarbon Products Corp., Hackensack, NJ). These data inferred that the appropriate conditions irradiation of fluorophores can alter cytoskeletal components also are complicated by evidence that under certain conditions the photobleached zones (Keith and Farner, 1993). Thus different photobleaching experiments have yielded different results, with the technique itself possibly contributing to this variation.

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#### Confocal microscopy

Cells were observed with a Bio-Rad MRC-600 confocal microscope equipped with a krypton-argon laser, attached to a Nikon Optiphot microscope, using a ×60 objective lens (NA = 1.4). Simultaneous dual channel imaging was used to detect α-AC (fluorescein) and α-TYR (Texas Red) in single optical sections: each channel occupied half the video screen (split-screen imaging). For each cell we recorded a series of optical sections spaced 0.5-1.0 μm apart (a ‘Z-series’) and adjusted the background and sensitivity values so that all grey-scale values
were from 1 to 255 (none saturated the detector). Collected images were Kalman averaged but otherwise had no image enhancement.

### Intensity analysis and gap length determinations

We determined the length of the gap in acetylation by comparing kMT fluorescence intensity profiles in the two channels. The intensity profile of α-TYR labelling was used to determine the position of the kinetochore and the intensity profile of α-AC labelling was used to determine the initial position of maximum acetylation. The gap was the distance between these two points. This analysis required that we obtain intensity profiles along lines that were identically placed in both the fluorescein and Texas Red channels. Software with this capability was developed by Mr James Kelley. Briefly, images recorded on 3.5 inch optical disks were analysed on a DOS personal computer that contained an Oculus 300 frame-grabber (Coreco, Montreal, Quebec). One of the two split-screen images was displayed on the video monitor and a mouse-driven cursor was used to mark the two ends of a line along a kinetochore fibre. Then we offset the image by 384 pixels (the width of a single split-screen image) in the X-direction to put the line in exactly the same position in the other image. We visually determined whether the two lines were accurately placed along the kinetochore fibre with the aid of pre-viewed intensity plots. Once the lines were satisfactorily placed the computer extracted the pixel intensity at each point along each line. We stored the data on our hard disk and made working prints of each image (with superposed lines) using a Mitsubishi video printer (model P40U).

A separate programme converted inter-pixel distances into μm to yield arrays of distance versus intensity for each line. Data were graphed using Lotus 123 and SlideWrite.

Sample intensity profiles for a kinetochore fibre are shown in Fig. 1. Each profile began on the equator side of the kinetochore and ended past the initial point of maximum intensity in acetylated tubulin (as judged by eye). As shown in Fig. 1, the intensity of α-TYR labelling increased rather abruptly at the kinetochore region, reaching maximum values at distances <1 μm from the low intensity plateaus. (These scans are similar to intensity profiles across interference microscope images of molecularly sharp edges of myoglobin crystals of 0.8 μm thickness in the Z direction (Huxley and Hanson, 1957).)

The intensity of α-AC labelling, on the other hand, increased with a less steep slope: in Fig. 1 it reaches a maximum intensity about 2 μm from the low-intensity plateau. We determined the kinetochore position from the α-TYR profile as the intersection of a line through the points representing the upwards slope of the curve and a horizontal line through the points of peak intensity (point A in Fig. 1). We estimated the position of the peak intensity at the end of the gap in the same manner from α-AC profiles (point B in Fig. 1). The length of the gap is the distance between points A and B.

Intensities were extracted from the raw data recorded on the hard disk (e.g. Figs 1, 2H), or after passing the images through a low-pass filter (e.g. Fig. 2E). The low-pass filter smoothed out the curves (by reducing large jumps in intensities) but did not alter the positions at which we chose the kinetochore or maximum intensities.

We discuss in the Appendix certain difficulties encountered in our analyses of the data.

### Accuracy of gap measurements

#### Magnification

Images were obtained over a period of 3 years using different cell preparations and microscopes. At various ‘zoom’ settings for each microscope we recorded images of a stage micrometer (lines spaced 10 μm apart) to determine the magnifications of each microscope. On our personal computer we measured each scale image 4-10 times to determine average number of pixels per μm for each microscope. The individual values for a microscope never ranged outside 2% from the average, generally less than 1%. For the microscope at York University, on which most of the data were taken, we recorded images of the scale lines at five different times; with the exception of one very hot day, the magnifications varied by less than 1% from the mean, and the magnification on that unusual day was 8% from the average of the other four. Thus we are confident that the magnifications we used were accurate to within 2%, with an upper limit of 8%.

#### Reproducibility of gap measurements

Many fibres were analysed independently by both P.W. and A.F. (one example is illustrated in Fig. 2). Each person also analysed certain fibres up to 3 times. For inter-person comparisons, when A.F.’s values were subtracted from P.W.’s values, the average difference between the two gap measurements for 112 fibres was 0.2±0.4 (s.d.) μm, which is not statistically different from zero using the Student’s t-test (P>0.5), indicating that neither of us had a consistent bias in measurements. The average absolute difference between our measurements was 0.3±0.3 μm; the data presented herein are averages of the different analyses and should therefore be accurate to within about half that, or 0.2 μm.

Our overall accuracy can be estimated another way. Since we measured the position of the kinetochore independently from the position of the maximum intensity in α-AC labelling, and since in determining ‘gap lengths’ we always subtracted the position of the kinetochore from the position of the maximum intensity, the fact that averaged negative values for gap lengths were all within 0.1 μm from zero (Fig. 6) suggests that the gap lengths were accurate to within 0.1-0.2 μm.

### Photography

Colour prints were made from slides photographed from pseudocolour images on the Bio-Rad monitor (using a Nikon F3 camera and Fujichrome film, ASA 50). Prints were made with the Ilfochrome Classic colour print system (Ilford, Mobberley-Cheshire). Black and white prints are from the Mitsubishi video printer.

### RESULTS

#### Description of cells

Cranefly spermatocytes in meiosis-I have three autosomal bivalents and two univalent sex chromosomes. The sex chromosomes, which are amphitelic (i.e. they have kinetochore
fibres to both poles), remain at the equator in anaphase as the autosomal half-bivalents move poleward. The sex chromosomes enter anaphase once the autosomes have reached the poles; as they move to opposite poles each maintains kinetochore fibres to each pole. The spindles do not elongate until the sex chromosomes segregate in anaphase (Forer, 1964, 1980). ‘Precocious’ sperm tail flagella emanate from the centriole at each pole: two flagella per pole in meiosis-I and one per pole in meiosis-II.

We analyzed cells in metaphase-I and anaphase-I. At the time of data acquisition we determined the division stage of each cell by phase-contrast microscopy. However, since mistakes are sometimes made, prior to analysis we reviewed the fluorescence images of each cell to confirm the stage it was in. We distinguished meiosis-I from meiosis-II based on cell size, number of spindle fibres and number of flagella at the poles. For cells in meiosis-I, we distinguished prometaphase from metaphase by the following criteria: during metaphase chromosomes are at the equator, the kinetochore fibres are thicker and more prominent and spindles label well for acetylated tubulin. It was difficult to distinguish between genuine metaphase and late prometaphase, though we tried to be ruthless in eliminating all cells that might be in late prometaphase. We distinguished metaphase from anaphase on the basis of chromosome position and kinetochore fibre length. However, since we could not always see the chromosomes clearly in the fluorescent images, we may have made some mistakes in distinguishing metaphase from very early anaphase.

In cells dual labelled with α-TYR and α-AC, labelling with α-TYR was similar to that described earlier in cells labelled with polyclonal antibodies to tubulin (Wilson and Forer, 1989a), revealing kMTs, non-kinetochore microtubules and astral microtubules (Fig. 2). In contrast, α-AC labelling was present only in the kinetochore fibres and the flagella at the poles (Fig. 2).

In kinetochore fibres at metaphase, α-TYR labelling was of more or less constant intensity from the kinetochore to the pole (Fig. 2). α-AC labelling, however, was less intense closer to the kinetochore, leaving a gap in labelling (Fig. 2; Wilson and Forer, 1989a). In the fluorescent images, the autosomal kinetochore fibres were about 0.9-1 μm in diameter; in the α-TYR channel the kMT bundles were of more or less constant diameter but in the α-AC channel the portions closest to the kinetochores sometimes seemed to taper or decrease in diameter (Fig. 2). The kinetochore fibres associated with the sex chromosomes appeared to have diameters of about 0.5-0.6 μm, noticeably smaller than those associated with the autosomes. (Sex chromosome fibres are more difficult to detect in the photographs presented here because they fluoresce less intensely and tend to be lost when exposures are set to optimize autosomal fibres.) The autosomal fibres often seemed to be composed of two sub-fibres, apparently one from each of the two chromatids associated with a spindle pole (data not shown). Intensity scans of α-AC labelling showed that flagella generally labelled with very similar intensity to kMT bundles (data not shown).

We measured the gaps in α-AC labelling in metaphase and anaphase cells and we used these data to deduce the fate of the gap in anaphase.

### At metaphase-I, the average length of the gap in α-AC labelling is 1.7 μm and is independent of kinetochore fibre length

We measured gaps in 74 kinetochore fibres from 16 metaphase cells, one of which is illustrated in Fig. 2. The average gap length was 1.7±0.7 μm (s.d.). We also normalized all lengths to correspond to the average pole-to-pole length of 21.5 μm, in case the gap lengths were influenced by spindle size. This analysis did not reveal any new trends but did narrow the ranges of kinetochore-to-pole distances (kinetochore fibre lengths), as listed in Table 1. To be more certain of our conclusions, we analyzed both normalized and non-normalized data.

Gap sizes varied considerably but were independent of the kinetochore fibre length, judging by the nearly horizontal best-fit line for the data when plotting gap length versus kinetochore fibre length (Fig. 3A). Data from Fig. 3A also were grouped according to kinetochore fibre length within 1 μm ranges (e.g. 6.0-6.9 μm, 7.0-7.9 μm) and the average gap length for each fibre length category was calculated (Fig. 3B). For kinetochore fibre lengths between 7 and 11 μm, the average gap lengths are not statistically different using ANOVA analysis (P>0.25), again suggesting that the gap length is independent of kinetochore fibre length. Similar results were obtained with non-normalized data (data not shown). The slight positive slope in Fig. 3A is due to slightly lower values in the 6-8 μm range, which may be due to the small sample size in this range, or to our mistaking early anaphase for metaphase.

In conclusion, the average gap length is 1.7 μm, near that estimated crudely by Wilson and Forer (1989a), and, with the possible exception of small kinetochore fibre lengths, the length of the gap is independent of kinetochore fibre length in metaphase.

### In anaphase-I, the gap length decreases at a rate of approximately 0.2 μm per 1 μm of kMT shortening

We measured gaps in 116 kinetochore fibres from 23 anaphase cells at various stages of anaphase, two of which are illustrated in Figs 4 (mid-anaphase) and 5 (late anaphase). We plotted anaphase gap lengths versus kinetochore fibre lengths, both as normalized and non-normalized data. Forer, 1989a). In the fluorescent images, the autosomal kMTs, non-kinetochore microtubules and astral microtubules (Fig. 2). In contrast, α-AC labelling was present only in the kinetochore fibres and the flagella at the poles (Fig. 2).

In conclusion, the average gap length is 1.7 μm, near that estimated crudely by Wilson and Forer (1989a), and, with the possible exception of small kinetochore fibre lengths, the length of the gap is independent of kinetochore fibre length in metaphase.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Averages±s.d. (μm)</th>
<th>Range (μm)</th>
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<tbody>
<tr>
<td>Gap</td>
<td>1.7±0.7</td>
<td>1.7±0.8</td>
</tr>
<tr>
<td>Pole-to-pole</td>
<td>21.5±2.9</td>
<td>21.5</td>
</tr>
<tr>
<td>KT-to-pole</td>
<td>9.0±1.6</td>
<td>9.0±0.7</td>
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The gap represents the distance between the kinetochore and the initial point of maximum intensity in labelling with α-AC. Values are for spindle lengths that have been normalized for the average pole-to-pole distance of 21.5 μm (Norm.) and for lengths that have not been normalized (Not norm.).
Fig. 2. A metaphase spindle dual labelled with α-TYR and α-AC as visualized by dual channel imaging with the confocal microscope. (A,C,F) Texas Red channel (α-TYR). (B,D,G) Fluorescein channel (α-AC). Only the kinetochore fibres and the flagella label strongly with α-AC. (A,B) Ten optical sections taken through the cell were superposed to form an image of the complete spindle (a Z-series). (C,D) Single optical sections of the cell in A,B showing the line along the kinetochore fibre from which the intensity profile (E) was derived. (E) Intensity of pixels along the lines in C (▲) and D (▲), beginning at the end labelled 0. The image was smoothed using a low-pass filter prior to extracting pixel intensities. (F-H) A second, independent scan of the same fibre, by a different person, without using the low-pass filter. Bars, 5 μm.
that the kinetochore fibre shortens. Assuming that the gap is a
value. For example, consider a point in Fig. 3 in which the
to shorten quickly by about 0.4 µm. During the rest of anaphase
m increase in interkinetochore distance,
mosomes move poleward at about one-fifth the speed of the
a rate of 0.2 µm. Similar values were obtained with
not-normalized data (data not shown). If depolymerization
were occurring solely at the kinetochore, we would expect the
to be unity, i.e. the gap would disappear at the same rate
kinetochore-to-pole shortening (Fig. 6A). Clearly, then, the
gap decreases in size during anaphase, but not at the rate
expected if disassembly were solely at the kinetochore. To be
sure that this conclusion is valid, we analysed the data in two
other ways.

First, we plotted gap lengths versus interkinetochore
distances (the distances between the two kinetochores of the
separating half-bivalents). In anaphase the gap decreases by
~0.1 µm for every 1 µm increase in interkinetochore distance,
as seen in both the scatter diagram (Fig. 7A) and the grouped
data (Fig. 7B). Since the interkinetochore distance increases 2
µm for every 1 µm that each kinetochore fibre shortens, the
results are exactly the same as from the analysis with respect
to kinetochore fibre length.

Second, we used the data on gap length in metaphase (Fig.
3) to predict the kinetochore fibre length at which each gap
would disappear if the shortening was exclusively at the kine-
tochore; we then compared the predicted with the actual
values. For example, consider a point in Fig. 3 in which the
gap length is 1.9 µm and the kinetochore fibre length is 8.6
µm. If in anaphase this fibre shortened only from the kineto-
chore end, the gap would disappear at a fibre length of (8.6
µm-1.9 µm) = 6.7 µm. We calculated such predicted values for
each point in Fig. 3A and plotted the results as a cumulative
percentage of fibre lengths at which the fibres have zero gaps
(Fig. 8). By the time the kinetochore fibres shorten to 6.4 µm,
75% of the anaphase kinetochore fibres should have zero gap
lengths if disassembly is solely at the kinetochore (broken line
in Fig. 8), yet the actual measurements (Fig. 6) show that the
gaps are only beginning to be zero at kinetochore fibre lengths
of 6.4 µm. Thus, this alternative approach also suggests that
most of the disassembly of KMTs during anaphase is not at the
kinetochore end of the fibre.

Evidence for a short burst of disassembly at the
kinetochore at anaphase onset
We extrapolated the best-fit line for the anaphase graph of gap
length versus kinetochore fibre length (Fig. 6A) back to the
average metaphase kinetochore fibre length of 9.0 µm. The
expected metaphase gap length from the extrapolated line is
1.3 µm, which is shorter by 0.4 µm than the measured
metaphase value of 1.7 µm (Table 1). Similarly, when the best-
fit line for gap length versus interkinetochore distance in
anaphase is extrapolated to the average metaphase interkine-

tochore length of 4.2 µm, the gap length is shorter by 0.4 µm
than the measured metaphase gap length of 1.7 µm. These
statements also are true for the grouped data (Figs 6B and 7B),
and for the best-fit lines through the scatter data omitting kine-
tochore lengths ≤4.5 (see legend of Fig. 6). Though the dif-
fERENCE of 0.4 µm is small, it suggests to us that there may be
a rapid loss of gap length at the start of anaphase.

We conclude, then, that the gap may decrease quickly by
about 0.4 µm at the onset of anaphase, but it then decreases at
a rate of 0.2 µm per 1 µm of kinetochore fibre shortening.

Gap lengths on amphitelic sex-chromosome fibres
During sex-chromosome anaphase, the amphitelic sex chro-
mosomes move poleward at about one-fifth the speed of the
autosomes (Schaap and Forer, 1979). As each moves, one kine-
tochore fibre shortens and the other kinetochore fibre elongates
(Forer, 1980). In our earlier study (Wilson and Forer, 1989a)
we noted that the gap on the elongating fibre appeared to
increase in length while that on the shortening fibre decreased
in length. Measurements of gaps on sex-chromosome fibres
during anaphase (Fig. 9) confirm these initial qualitative obser-
VATIONS. Thus as the sex chromosomes separate in anaphase the
gap length decreases on the shortening fibre and increases on the
elongating fibre.

DISCUSSION
We have used quantitative confocal microscopy of both
metaphase and anaphase cells to measure the length of the
kinetochore fibre in front of the kinetochore that does not label
strongly with α-AC. At the start of anaphase the ‘gap’ seems
to shorten quickly by about 0.4 µm; during the rest of anaphase
the gap decreases in length at a rate of 0.2 µm for every 1 µm
that the kinetochore fibre shortens. Assuming that the gap is a

![Fig. 3. Gap length vs kinetochore fibre length in metaphase. (A) For
each fibre, the length of the gap in acetylation was plotted against the
kinetochore-to-pole length. Each point represents data from a single
kinetochore fibre. The best-fit line for the data points is shown.
(B) Values from A were grouped according to kinetochore-to-pole
length within 1 µm ranges, and for each group an average gap value
was calculated and plotted. The group labelled 6.5 represents data for
kinetochore-to-pole lengths of 6.0-6.9 µm, etc. Error bars = s.d.
Numbers above the error bars represent the number of points in that
group. All values are normalized to spindle lengths of 21.5 µm.]
marker of kMTs in anaphase, these results suggest that during anaphase the kMTs initially disassemble at the kinetochore but during the remainder of anaphase at least 80% of kMT shortening is due to disassembly at the pole, in agreement with the hypothesis that during anaphase both the kinetochore and the pole can act as sites of microtubule disassembly (Forer and Wilson, 1994).

The above interpretation rests heavily on certain assumptions regarding the nature of the gap; for this reason further discussion of the gap is warranted.

We explain the absence of acetylated kMTs near the metaphase kinetochore as follows: kMTs constantly polymerize (add subunits) at the kinetochore, the subunits move (‘flux’) towards the pole where they are removed, and the ‘gap’ represents the time lag between polymerization and acetylation.

We favour this hypothesis for the following reasons (also see Wilson and Forer, 1989a).

With respect to incorporation of tubulin into kMTs, there now is ample evidence that tubulin incorporates at the kinetochore during metaphase. This evidence arises from different experimental approaches, such as preventing disassembly by colcemid using UV light (Czaban and Forer, 1985b), electron microscopy after microinjection of biotin-labelled tubulin (Mitchison et al., 1986), fluorescence microscopy after microinjection of DTAF-tubulin (Wise et al., 1991), studying fluorescence recovery after photobleaching (Gorbsky and Borisy, 1989), and photoactivation of ‘caged’ tubulin (Mitchison, 1989; Mitchison and Salmon, 1992).

With respect to acetylation, the evidence from the literature supports the view that acetylation occurs after polymerization. In *Chlamydomonas reinhardtii* acetylation of tubulin is closely
tied to flagellar growth and hence to microtubule polymerization (Brunke et al., 1982; L’Herneault and Rosenbaum, 1983). Only assembled tubulin in the flagellum is acetylated (Greer and Rosenbaum, 1989), and while tubulin dimers can be acetylated, the polymer is a better substrate than the dimer (Maruta et al., 1986). In tissue culture cells, acetylated tubulin is found only in the pellet fraction containing microtubules (Piperno et al., 1987). For newly polymerized microtubules in cells, acetylation lags behind microtubule assembly by 3-15 minutes (dePennart et al., 1988; Piperno et al., 1987; Wilson and Forer, 1989a). Lastly, cytoplasmic microtubules stabilized by taxol become increasingly acetylated over time (Piperno et al., 1987), suggesting that acetylation is a post-polymerization event.

In summary, previous work supports the view that in metaphase tubulin adds at the kinetochore and acetylation takes place some time after polymerization. That the gap gradually increases in fluorescence as one follows the kMTs from the kinetochore towards the pole also is consistent with an increase in acetylation with time as the subunits flux towards the pole.

To extend our explanation to anaphase, we assume that polymerization at the kinetochore ceases in anaphase. Although some recent evidence suggests that tubulin can add to kMTs during anaphase (Shelden and Wadsworth, 1992; Wadsworth et al., 1989), and we have argued that the growth of UV-severed kMTs during anaphase is due to addition of subunits at the kinetochore (Forer and Wilson, 1994), these examples appear to be a result of the experimental treatment itself and not something that occurs under normal conditions (e.g. see Shelden and Wadsworth, 1992). Given that polymerization at the kinetochore ceases in anaphase, the fact that the gap persists once the chromosomes have moved the 1.7 µm length

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**Fig. 5** The gap in late anaphase. (A,B) Confocal Z-series of a cell in late anaphase dual-labelled with α-TYR (A) and α-AC (B). (C,D) Single optical sections from the Z-series shown in A,B, showing the lines from which fluorescence intensity profiles were taken. (E) The fluorescence intensity profiles from the lines depicted in C,D for labelling with α-TYR (C, ▲) and α-AC (D, △). In this cell a gap is absent. Bars, 5 µm.
Anaphase microtubule depolymerization

of the gap (Fig. 6) strongly suggests that disassembly does not occur primarily at the kinetochore. A straightforward interpretation of the loss of gap by 0.2 µm for every 1 µm shortening of kMTs is that 80% of the shortening is due to disassembly at the poles and 20% to disassembly at the kinetochores.

There is an alternative interpretation for our results, however: it is possible that the gap in metaphase is due to deacetylating enzymes emanating from (or activated by) the kinetochore. Similarly, in anaphase the persisting gap could represent a ‘wave’ of deacetylation in front of the kinetochore. We outlined previously reasons why we think that this is unlikely (Wilson and Forer, 1989a). Briefly, we know that tubulin can add at the kinetochore and that there likely is a lag between polymerization and acetylation, so our explanation fits known data. We know of no precedent for expecting newly polymerized tubulin to be deacetylated, on the other hand. Nor is there any precedent we know of for microtubules being deacetylated without first being disassembled. Secondly, we do not see a reasonable explanation for why the gap gradually would decrease in length during anaphase when the chromosomes move poleward if the gap is due to a zone of deacetylation. Thirdly, the gap associated with sex-chromosomal fibres in metaphase is smaller than that associated with autosomal fibres in metaphase, 1.1 µm±0.4 µm (Fig. 9) compared with 1.7 µm±0.7 µm (Table 1), a difference that is statistically significant (using Student’s t-test) at a level of P<0.001. Whereas this might be understandable in terms of our assumptions if this is related to the slower speeds with which the two kinds of chromosomes move (and therefore slower rates of incorporation), we see no reason why deacetylation levels should be different in the two cases. Finally, when sex chromosomes move poleward in anaphase, the elongating fibre...
of the fiber” (Mitchison and Salmon, 1992).

If one accepts that the gap in metaphase kMTs arises because of a lag between polymerization and acetylation, then one can be deacetylated. The activity was detected only in the cell body, whereas disassembly of acetylated microtubules in flagella appears to take place within the flagellum (Greer and Rosenbaum, 1989), suggesting that deacetylation occurs after disassembly. In addition, the acetylation state of tubulin does not affect assembly/disassembly kinetics, and therefore there is no need to deacetylate tubulin prior to disassembly (Maruta et al., 1986). These data are consistent with the suggestion that deacetylation occurs after disassembly, but they do not provide unequivocal evidence that this is the only way in which tubulin can be deacetylated.

In conclusion, we feel that the existing data favour the hypothesis that in metaphase the gap arises because of a lag between polymerization and acetylation of microtubules, with the corollary that in anaphase the gap persists for the same reason.

Is gap loss due to disassembly or to acetylation?

If one accepts that the gap in metaphase kMTs arises because of a time lag between polymerization and acetylation, then one must ask how the gap disappears during anaphase. Two possible mechanisms can account for it: (a) disassembly at the kinetochore, or (b) continued acetylation of the polymerized microtubules once polymerization ceases and the chromosome begins to move poleward. While we cannot distinguish unequivocally between the two possibilities, acetylation could account for the entire loss of the gap, in the following way.

The metaphase gap is 1.7 µm long, and the gap shortens at a rate of 0.2 µm per 1 µm of shortening of kMTs; thus the gap would disappear on average after the kinetochore fibre shortens by about 8.5 µm (1.7 µm/0.2 µm). N. suturealis chromosomes move poleward at about 0.7-1 µm per minute (Marzec, 1993; Shaap and Forer, 1979); shortening by 8.5 µm corresponds, then, to 8.5-12 minutes. Experimentally, microtubules that repolymerize become acetylated within 3-15 minutes after reappearing (de Pennart et al. 1988; Piperno et al., 1987; Wilson and Forer, 1989a). Thus, it is conceivable that during anaphase the loss of the gap occurs by acetylation, and that there is no disassembly at the kinetochore.

Our overall interpretation, then, is that there is an initial, brief disassembly of kMTs at the kinetochore at the start of anaphase, but that the remainder of anaphase occurs by disassembly at the pole. We might speculate that this represents the general rule, that earlier anaphase is predominantly due to disassembly at the kinetochore whereas later anaphase is due to disassembly at the pole, since this also seems to occur in at least some newt cells: early in anaphase there was primarily disassembly at the kinetochore but later in anaphase, “... in some cells the kinetochores and the marks (on the kMTs) tended to move together, indicating that disassembly was now mainly at the polewards end of the fiber” (Mitchison and Salmon, 1992).

**Fig. 8.** Predicting the anaphase outcome for metaphase cells if disassembly of kMTs is entirely at the kinetochore. If disassembly occurs entirely at the kinetochore, then the predicted fibre length for which the gap would be 0, once metaphase cells entered anaphase, would be equal to the metaphase kinetochore-to-pole length minus the gap length for that fibre. This value was calculated for each of the fibres represented in Fig. 3 and plotted above against the cumulative percentage of the total number of fibres in Fig. 3. The vertical broken line represents the point at which 75% of the fibres should have no gap, if disassembly occurs solely at the kinetochore.

Has an increased gap length while the shortening fibre has a decreased gap length (Wilson and Forer, 1989a; Fig. 9): this is understandable from our working hypothesis, since the elongating fibre must have an increased rate of incorporation in order to elongate, but is not understandable if one assumes that the gap is due to a region of deacetylation. All these ‘indirect’ arguments are reasons why we think that the deacetylation explanation is unlikely.

We could decide directly if deacetylation is a possible explanation if we knew the characteristics of the deacetylation enzymes, and whether they can cause deacetylation of polymerized tubulin. Unfortunately there is very little relevant information available. To our knowledge, the only study that has approached this topic directly is that of Maruta et al. (1986), who detected tubulin deacetylase activity in *Chlamydomonas reinhardtii*, presumably due to the presence of the deacetylating enzyme. The activity was detected only in the cell body, whereas disassembly of acetylated microtubules in flagella appears to take place within the flagellum (Greer and Rosenbaum, 1989), suggesting that deacetylation occurs after disassembly. In addition, the acetylation state of tubulin does not affect assembly/disassembly kinetics, and therefore there is no need to deacetylate tubulin prior to disassembly (Maruta et al., 1986). These data are consistent with the suggestion that deacetylation occurs after disassembly, but they do not provide unequivocal evidence that this is the only way in which tubulin can be deacetylated.

**Fig. 9.** Gaps on sex chromosome fibres during sex chromosome anaphase. As sex chromosomes move poleward, they maintain kinetochore fibres to both poles, so that one fibre shortens and the other elongates. Gaps in acetylation measured for sex chromosomes that were still at the equator (●) and for both elongating (△) and shortening (crosses) sex chromosome fibres in anaphase were plotted against the distance between the two separating sex chromosomes. The continuous line represents the best-fit linear curve for the values from shortening fibres (slope = 0.1 µm/µm) and the broken line represents the best-fit linear curve for the values from the elongating fibres (slope = 0.2 µm/µm). The four data points for chromosome separation of 5.4 µm are all from the same cell and therefore may not be representative.
Comparing our results with other studies

Like previous studies using caged fluorescence, we find evidence for disassembly both at the pole and at the kinetochore (Mitchison and Salmon, 1992; Zhai et al., 1993). In addition, like Mitchison and Salmon (1992), we find a greater contribution due to disassembly at the kinetochore early in anaphase.

The key difference between our results and those of other marking experiments is the relative contribution made by the pole during disassembly. Our results suggest that, after the start of anaphase, at least 80% of the kinetochore fibre shortening is due to disassembly at the poleward end of the kMTs. This contrasts dramatically with the range of 0 to 37% seen by others (Gorbsky and Borisy, 1989; Gorbsky et al., 1987, 1988, Salmon and Mitchison, 1992; Zhai et al., 1993). While this difference could be due to different experimental approaches, we think that both disassembly at the kinetochore and disassembly at the pole take place during anaphase, to different degrees in different cell types and different circumstances. We have suggested elsewhere (Forer and Wilson, 1994) that the kMTs may be pushed poleward by motor molecules in the spindle matrix, and that, as a consequence, in different cells (or circumstances) one would expect disassembly predominantly at the kinetochore (as in newt cell early anaphase), or predominantly at the pole (as in most of crane-fly spermatocyte anaphase), or mixtures of the two (as in some newt and tissue culture cells). Thus, the fact that different cells have varying degrees of one form of anaphase disassembly or the other does not necessarily mean that the basic mechanisms for movement are different.

How many of the kMTs are acetylated?

Without analysis by immuneelectron microscopy, one cannot tell how many of the kMTs in crane-fly spermatocytes are acetylated. Nonetheless, rough estimates can be made based on our observation that the kMT bundles in one optical section have about the same level of acetylation as the flagella at the spindle poles in the same cells, and that flagellar microtubules are highly acetylated (Brunke et al., 1982; Glyn and Gull, 1990; L’Herneault and Rosenbaum, 1983; Sasse et al., 1987). Given the diameter and microtubule density in a flagellum (Behnke and Forer, 1967), a kinetochore fibre diameter of 0.9 µm (see results), an estimated depth of field of 0.7 µm (Fricker and White, 1992), and a kMT density of 125 to 160 kMTs per µm² (based on data from LaFountain, 1976; Janicke and LaFountain, 1984; Scarcella et al., 1986), we estimate that either 70% to 90% of the kMTs in the bundle are acetylated to the same degree as the flagellar microtubules, or that all of the kMTs are acetylated but only to 70% to 90% of the level of acetylation of the flagellar microtubules. We realize that these estimates are very crude, but they indicate that in studying the acetylated kMTs in crane-fly spermatocytes we have not studied a trivially minor portion of the kMT bundle, but rather a sizeable fraction of the kMTs.

In conclusion, we have marked kMTs using a novel, non-invasive approach; namely, the differential staining pattern seen on kMTs labelled with antibodies to acetylated α-tubulin. We have argued that the gap in acetylation represents the time lag between polymerization of tubulin and acetylation; that from measurements of gap sizes in anaphase the initial stage of anaphase is due to disassembly at the kinetochore and the remainder of anaphase is due to disassembly at the pole; and that the acetylated kMTs make up a sizable fraction of the kMT bundle.

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APPENDIX

Problems and controls

Choosing the best optical section

The purpose of using confocal microscopy is to resolve a single kinetochore fibre without interference from fluorescence above and below it. We encountered some experimental difficulties, however. One concerned focus. Unless the focus steps are very small (and hence the number of sections unreasonably large) the images recorded may not be in optimal focus for some of the fibres in the cell. Consequently, some kinetochore fibres were in focus in more than one optical section. For these fibres, and for kinetochore fibres that were at an angle to the plane of focus (e.g. see Figs 2, 5), we chose the section in which the region near the kinetochore was best visualized. When we could not do this satisfactorily, the fibres (or cells) were not used in our analysis.

Focus shifts

Dual images were recorded simultaneously for each single optical section, but sometimes there were vertical and horizontal phase shifts between the Texas-Red and the FITC images. By vertical shift we mean that a kinetochore fibre in one bundle would appear best focussed in one optical section, but in the other channel the same fibre would appear best focussed in an adjacent optical section. To study how the vertical shift arose, we labelled interphase cells with one anti-tubulin antibody and two different secondary antibodies (fluorescein-labelled and Texas Red-labelled). In imaging single microtubules we did not see shifts in focus. Thus we attribute the vertical shifts in focus with the kMT bundles to the fact that the bundles were of the order of 1 µm in diameter and that because of the way the series of pictures were taken, the level of focus might have been slightly off from the best (optimal) focus. As a consequence, the chromatic aberration in the lenses, estimated theoretically (Martin, 1966) and experimentally (Fricker and White, 1992) to be of the order of half the depth of field, was enough to cause the observed shift in focus. In support of this interpretation we note that in general only some of the fibres in a given cell were shifted in focus. Regardless of the correctness of our interpretation, each individual fibre was considered separately and the best optical section for each channel was chosen.

Lateral shifts in focus also occurred, but generally were not a problem, in that we usually could obtain a line that accurately reflected both the kinetochore position (α-TYR labelling) and the intensity of acetylation along the kMTs (α-AC labelling). On a very few occasions we had to shift one of the two images
by 1 or 2 pixels (corresponding to ~0.1-0.2 μm) in order to bring the two lines along the kMTs into closer register.

Overlapping fibres

Out of focus fibres sometimes interfered with our analysis. P.W. and A.F. both separately determined gap lengths for many spindle fibres, locating the position of the kinetochore independently of the end of the gap. We then compared curves and lengths of the gaps. When the gap lengths differed by more than 0.5 μm P.W. and A.F. each re-analysed the same fibres. In the re-analysis we eventually realized that the major cause of disagreements was vertical overlap of kinetochore fibres.

Vertical overlap occurred when fibres that were superposed in the X and Y direction but separated vertically at the kinetochores (i.e. were in the same position in the X and Y directions but were in different sections in the Z-series) came together (‘merged’) closer to the poles. The overlapping fibre contributed to the intensities that we measured along what we thought were individual kinetochore fibres and consequently we sometimes saw more than one peak in intensity along a kinetochore fibre with α-AC labelling, with the second peak higher in intensity and closer to the pole. (In our original analysis (Wilson and Forer, 1993) we mistakenly chose the second peak.) This difficulty did not occur regularly, but was not infrequent; it arose primarily when the image was not in the best plane of focus for the fibre in question. We eliminated this difficulty by analysing the intensities in the fluorescein channel (α-AC) in the optical section further removed by one from the offending kinetochore fibre or by choosing the first intensity peak. If neither were possible, we eliminated the fibres from the analysis.

Lateral overlap, from kinetochore fibres that were spatially separated laterally at the kinetochores but that merged as the fibres came closer to the pole, also occurred but easily was recognized and avoided.

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