

# FUNCTIONAL IMPLICATIONS OF COLD-STABLE MICROTUBULES IN KINETOCHORE FIBERS OF INSECT SPERMATOCYTES DURING ANAPHASE

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## ABSTRACT

In normal anaphase of crane fly spermatocytes, the autosomes traverse most of the distance to the poles at a constant, temperature-dependent velocity. Concurrently, the birefringent kinetochore fibers shorten while retaining a constant birefringent retardation (BR) and width over most of the fiber length as the autosomes approach the centrosome region. To test the dynamic equilibrium model of chromosome poleward movement, we abruptly cooled or heated primary spermatocytes of the crane fly *Nephrotoma ferruginea* (and the grasshopper *Trimerotropis maritima*) during early anaphase. According to this model, abrupt cooling should induce transient depolymerization of the kinetochore fiber microtubules, thus producing a transient acceleration in the poleward movement of the autosomal chromosomes, provided the poles remain separated. Abrupt changes in temperature from 22°C to as low as 4°C or as high as 31°C in fact produced immediate changes in chromosome velocity to new constant velocities. No transient changes in velocity were observed. At 4°C (10°C for grasshopper cells), chromosome movement ceased. Although no nonkinetochore fiber BR remained at these low temperatures, kinetochore fiber BR had changed very little. The cold stability of the kinetochore fiber microtubules, the constant velocity character of chromosome movement, and the observed Arrhenius relationship between temperature and chromosome velocity indicate that a rate-limiting catalyzed process is involved in the normal anaphase depolymerization of the spindle fiber microtubules. On the basis of our birefringence observations, the kinetochore fiber microtubules appear to exist in a steady-state balance between comparatively irreversible, and probably different, physiological pathways of polymerization and depolymerization.

A major proposition in the "dynamic equilibrium theory" of mitotic spindle assembly and function (23-26, 51) is that pushing and pulling forces for moving chromosomes are generated by assembly

and disassembly of the microtubules that make up the spindle fibers. In his dynamic equilibrium theory, Inoué (23, 25, 26) proposes that the spindle fiber microtubules are in a continuous state of flux

that he sees as the consequence of a simple, labile, physiochemical equilibrium between the polymerized microtubules and a cellular pool of tubulin subunits. According to Inoué's view, changes in the equilibrium constant or in the concentration of activated tubulin subunits initiate microtubule assembly or disassembly, thus generating forces for mitotic movements.

The major support for the dynamic equilibrium theory came from experiments with metaphase-arrested oocytes of *Chaetopterus* in which the spindle microtubules were induced to disassemble by cooling (23, 25, 26), pressurization (50–52, 56), or the addition of colchicine (22). As the microtubules depolymerized, the spindle shortened and the chromosomes were moved from their metaphase-plate position toward the pole that anchored the spindle to the cell surface. When the depolymerizing conditions were reversed, the spindle regrew, moving the chromosomes away from the anchored pole. We felt that more convincing evidence for the dynamic equilibrium theory would be provided if the same microtubule-depolymerizing agents, when applied during anaphase, would cause the chromosomes to accelerate as they moved poleward.

More specifically, we predicted on the basis of the dynamic equilibrium theory that abruptly cooling or pressurizing a cell during anaphase would cause the spindle fiber microtubules to depolymerize and shorten rapidly until they reached a new equilibrium state. During this transitional period of rapid microtubule depolymerization, we expected to observe an increase in the chromosomes' poleward velocity, provided the spindle poles remained separated. Then, as a new equilibrium became established, we expected chromosome velocity to decrease until it reached a new steady-state rate. Steady-state chromosome velocity increases exponentially with temperature, with a  $Q_{10}$  of 2.6–4.3 depending on cell type (18, 59). As illustrated in Fig. 1 for a hypothetical example, abrupt cooling should induce transient acceleration of the chromosomes (region *i*) followed by a steady-state velocity slower than originally observed (region *ii*).

For our studies we used primary spermatocytes from two insects, primarily the crane fly *Nephrotoma ferruginea* and, secondarily, the grasshopper *Trimerotropis maritima*. These cells were chosen because they contain large spindles with relatively few chromosomes and because they develop distinct chromosomal fibers during anaphase (13, 14,

30–32, 46, 47). The crane fly spermatocytes appeared to be highly suitable for this study because (a) they have only three autosomes and two sex chromosomes, (b) both spindle poles are well anchored at the cell surface (33), (c) the autosomes move poleward at a nearly constant velocity (13, 14), and (d) the interpolar spindle does not usually elongate (called "Anaphase B" by Inoué and Ritter [25]) until the autosomes have nearly completed their movement to the poles ("Anaphase A") (13, 14). Consequently, for living crane fly spermatocytes, observation of changes in chromosome velocity could be related directly to changes in the assembly and length of the birefringent kinetochore fibers by using sensitive polarization microscopy methods. Measurements of birefringent retardation (BR) have been shown to be good measures of the number and distribution of spindle fiber microtubules in crane fly spermatocytes (32, 33) and other cell types (58).

To our surprise, unlike the results reported earlier for marine eggs and plant cells (18, 23, 27, 51, 61), extensive cooling of these spermatocytes did not cause appreciable reduction in the birefringence of the kinetochore fibers, nor did it induce a transient acceleration of the chromosomes. In this paper we discuss the temperature dependence of the poleward velocity of the chromosomes and examine the implications of the striking differential stability that kinetochore and nonkinetochore fiber microtubules have to cooling. Results of similar experiments using hydrostatic pressure to depolymerize the microtubules will be reported in a subsequent paper. A preliminary report of our work has been presented previously (53).

## MATERIALS AND METHODS

Primary spermatocytes of the crane fly *Nephrotoma ferruginea* Fabricius and the grasshopper *Trimerotropis maritima* Harris were used for these studies. Crane flies were reared in the laboratory according to the method described by Begg (4) and grasshoppers were periodically collected during August through October from a wild population near Falmouth, Massachusetts. Spermatocyte smears were prepared as described previously (5, 6). Glass coverslips containing a spermatocyte smear were sealed to a temperature-controlled slide with valap (a 1:1:1 mixture of Vasoline, paraffin, and lanolin).

### Temperature Control

The temperature of the spermatocyte preparation was regulated and monitored by a water-flow controlled-temperature slide, similar in construction and operation to that described by Stephens (61) (Fig. 2). Cell temperature was taken to be the mean of the influx and efflux water temperatures, as measured during the experiment by thermistor probes (61). The water flow

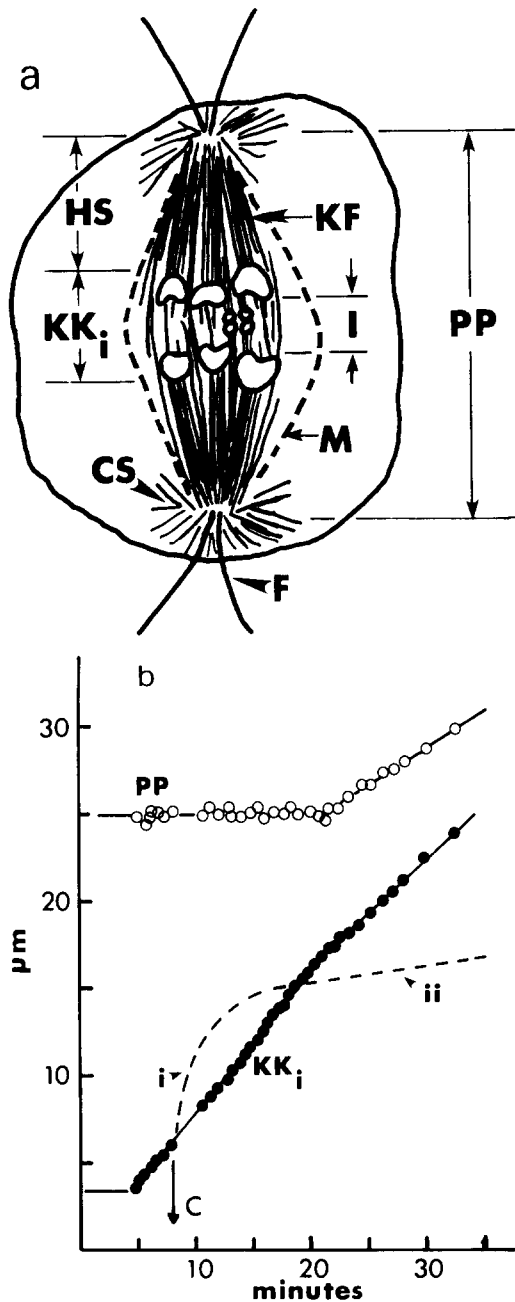


FIGURE 1 (a) Schematic drawing of a crane fly spermatocyte during early anaphase of meiosis I, magnified  $\times 2,000$ . *KF*, birefringent kinetochore fiber; *I*, interzone; *M*, mitochondrial sheath surrounding the spindle; *F*, flagella; *HS*, half-spindle region; and *CS*, centrosome-pole region.  $KK_i$ , the separation distance between kinetochores of sister half-bivalents from which chromosome poleward velocities were calculated;  $PP$ , interpolar distance (constant for most of anaphase A). (b) Typical

was driven by gravity and the slide temperature was controlled by switching between two 5-gallon insulated reservoirs, one maintained near  $22^\circ\text{C}$  and the other at the desired experimental temperature. Fogging of the slide at low temperatures was prevented by blowing dry air over both windows of the slide.

### Microscopy

Phase contrast observations were made using a Nikon model S microscope equipped with a Zeiss 40x (NA 0.65) phase objective and a Zeiss model IS long-working-distance phase condenser (NA 0.7; working distance, 11 mm). Spindle fiber birefringence was observed with the high extinction, inverted polarization microscope of Inoué (23) (crane fly experiments) or a Zeiss WL stand (grasshopper experiments) equipped with a Nikon rectified 40x (NA 0.65) objective and a Nikon 16-mm long-working-distance rectified condenser (NA 0.52) (Fig. 2). The illuminating beam for both microscopes was filtered with a glass heat-cut filter and a 546-nm interference filter (Baird Atomic, Inc., Bedford, Mass.).

### Photography and Data Analysis

Photographic records were made with a Nikon Microflex AFM camera system, using a Zeiss 10x ocular and Kodak Plux X 35-mm film. The films were developed in Kodak Microdol X Developer at  $75^\circ\text{F}$ .

Brief exposure times were required to record changes in BR and kinetochore position after temperature shifts, because changes in temperature induced small deformations in the slide causing the transient displacement of cells. The high intensity illumination and sensitivity provided by the Inoué polarization microscope allowed exposure times of 3–5 s, which were sufficient to record BR changes in the cooling experiments. Exposure times for phase contrast microscopy were  $\sim 1$  s.

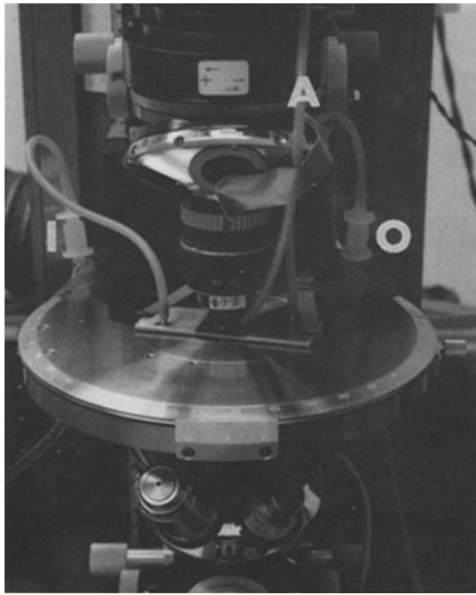
Kinetochore positions and interpolar distance were measured from photographs printed at a final magnification of  $\times 1,500$ . Magnification was calibrated by photographing a stage micrometer scale.

### Measurement of Spindle BR

Spindle fiber BR was usually measured visually with a Zeiss Brace-Koehler  $\lambda/30$  rotating compensator ( $\Gamma = 18.45$  nm), using the device described by Salmon and Ellis (54) for displaying extinction angles.

We have also used a method similar to the procedures described by Swann and Mitchison (63) and Forer (16) to measure the changes in BR along single anaphase kinetochore fibers by densitometric analysis of time-lapse 35-mm negatives. Spindles in flattened crane fly spermatocytes with clearly defined peripheral kinetochore fibers were photographed on the polarization microscope at 1-min intervals, using a constant exposure interval

kinetics of chromosome movement (●) and interpolar elongation (○) during anaphase at  $22^\circ\text{C}$  in the meiosis I spindle of crane fly spermatocytes.  $KK_i$  and  $PP$ , as defined in Fig. 1a, were measured on phase contrast micrographs similar to those in Fig. 3. Time = 0 at the onset of anaphase. The dashed line represents the expected changes in chromosome movements resulting from sudden cooling (C) in early anaphase, based on the dynamic equilibrium theory (26). The two regions of the predicted curve (i) and (ii) are explained in the text.



**FIGURE 2** The temperature-regulated specimen slide mounted on the stage of S. Inoué's inverted optical-bench polarization microscope (23). Teflon strips 0.5-mm thick insulate the slide from the metallic microscope stage. For observation with the inverted microscope arrangement, the spermatocyte preparation was placed on the bottom coverslip of the temperature-controlled slide. (J) Inlet water connector, (O) outlet water connector, and (A) a dry-air jet directed at one optical surface of the temperature-controlled slide. A similar dry-air jet (not visible) is mounted beneath the stage and directed upwards.

and a constant compensator setting of  $5^\circ$  off background-light extinction. Film density was correlated with BR by taking a series of photographs of a blank field adjacent to the cell at  $1^\circ$  increments in the compensator vernier dial away from the position of extinction of the background light. Changes in image intensity along single kinetochore fibers were measured by projecting the 35-mm negative in an Omega D2 enlarger (Simmons Bros., Inc., New York) equipped with a 250-W #213 GE bulb and 50-mm lens at  $f/4.5$ . The densitometer probe was translated beneath the focused image using a Craftsman milling table (Sears, Roebuck and Co., #9GT2495L). The densitometer was a Mitchell Fotoval 9 (Heathkit model PM-14, Benton Harbor, Mich.) that had the scale calibrated to display BR from the calibration curve. A piece of aluminum foil with a circular aperture 0.3 mm in diameter covered the photocell on the densitometer probe. The area around the pinhole was covered with white tape to permit accurate focusing and visual tracking of individual kinetochore fibers. A magnet anchored the probe to the upper surface of the milling table. An image of a stage micrometer, photographed in the microscope under the same conditions as the spermatocyte spindles, was used to calibrate distances in the projected image of the spindle. Total magnification of the projected image was  $\times 2,000$  and the equivalent densitometer probe aperture was  $0.4 \mu\text{m}$ . BR was recorded at the equivalent of  $0.3\text{-}\mu\text{m}$  intervals and

plotted, and smooth curves were drawn through the data points. Hand movement of the densitometer probe beneath the spindle image permitted rapid assessment of the magnitude of BR throughout the spindle region.

### Experimental Protocol

The temperature regulator slide containing a spermatocyte smear was mounted on the microscope stage and connected to the temperature control apparatus. The temperature was adjusted to  $\sim 22^\circ\text{C}$ . In the temperature-jump experiments, the autosomes were allowed to move approximately one-quarter to one-third of the distance toward the spindle poles before the temperature was shifted to the new experimental value. The temperature was generally held at this value for 10–30 min. In a few experiments, the temperature was returned to  $22^\circ\text{C}$  before the chromosomes reached the spindle poles.

Most of the chromosome movement data during anaphase was obtained with phase contrast microscopy. During the experiment, one pair of autosomal half-bivalents was kept in sharp focus, but frequently, in well-flattened cells, a second pair was in sufficiently sharp focus to permit identification of the kinetochore positions. Velocity was measured from cells photographed at 15–30 s intervals from metaphase through the onset of cytokinesis.

The effects of changes in temperature upon the BR of the spindle fibers were followed by polarization microscopy. The BR of individual kinetochore fibers was measured by visual compensation at a distance of about one-third the length of the fiber away from the kinetochore. Fibers at the periphery of the spindle were usually chosen because they were the most distinct.

## RESULTS

### Crane Fly Spermatocytes

**NORMAL ANAPHASE:** In crane fly spermatocytes, the autosomes traversed the distance to the poles (anaphase A) almost completely before elongation of the spindle (anaphase B) separated them further (Fig. 3). The autosomes moved at a nearly constant velocity over approximately three-quarters of the distance to the spindle poles. Velocity then decreased progressively as the autosomes moved the remaining distance to the poles. Although sister half-bivalents always moved at the same velocity, velocities often differed substantially between different pairs of half-bivalents within the same cell, or in different cells at the same culture temperature (cf. references 13, 14, and 59). For example, in our experiments at  $22^\circ\text{C}$ , initial autosome velocities ranged from  $0.3\text{--}0.8 \mu\text{m}/\text{min}$ .

As the autosomes moved poleward, their birefringent kinetochore fibers shortened at a corresponding rate, while the fiber diameter ( $0.7\text{--}1.1 \mu\text{m}$ ) remained constant (Fig. 4) (see also La-Fountain, 30, 31). Densitometric measurements of individual kinetochore fibers demonstrated that their BR remained nearly constant ( $\cong 2 \text{ nm}$  at

22°C) along most of their length, with a gradient of decreasing BR near the spindle poles (Figs. 4, 5, and 8). The region of decreasing BR appears to correspond to the area of the centrosome. The profile of BR along the kinetochore fibers did not change markedly as they shortened (Fig. 5) (see also reference 15 for similar results). The region of constant BR became progressively shorter until

the kinetochores reached the centrosome region (Fig. 4, 8 min; Fig. 5). As the autosomes moved the remaining distance to the poles, the BR of their kinetochore fibers decreased (Fig. 4, 13.4 min). This period of decay in kinetochore fiber BR coincided with the time of deceleration in autosome velocity.

In crane fly spermatocytes, the univalent sex

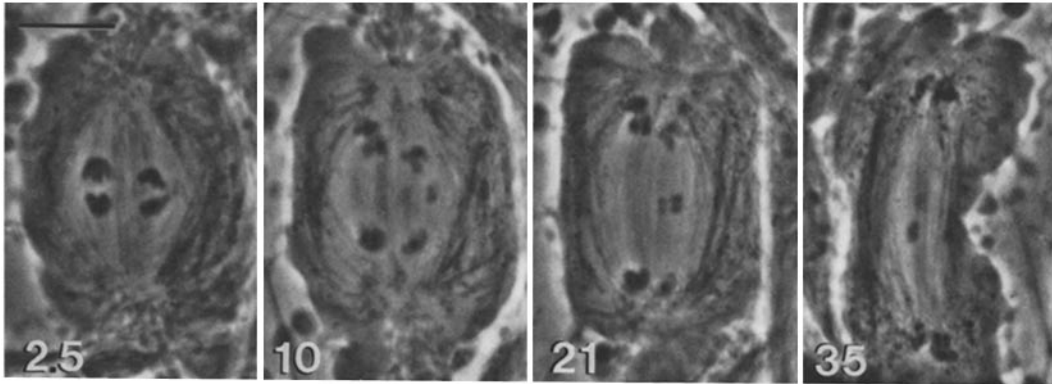


FIGURE 3 Normal anaphase movements of the chromosomes and changes in the spindle shape during meiosis I in a living spermatocyte of the crane fly *Nephrotoma ferruginea*, observed with phase contrast microscopy. The number of minutes after the onset of anaphase is indicated on each frame. Two pairs of separating autosomal chromosomes are in focus throughout anaphase. The third autosome pair is beneath the plane of focus. The two sex chromosomes remain near the metaphase plate as the autosomes move to the poles (21 min). They then segregate to the poles during interpolar elongation and cytokinesis (35 min). Bar, 10  $\mu$ m. The spindle region is demarcated by a sheath of elongated mitochondria that surrounds the spindle. The fibrous structures of the spindle are only weakly visible with phase contrast microscopy in comparison to polarization microscopy (see Fig. 4 for comparison).

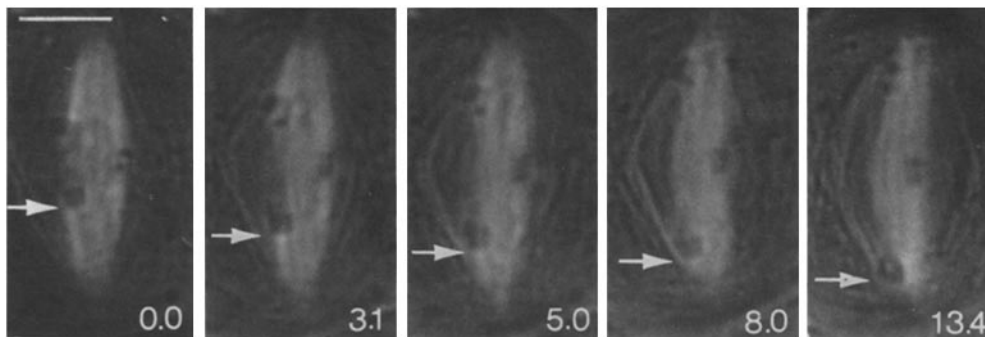


FIGURE 4 The distribution of birefringent spindle fibers during the poleward movement of autosomes in anaphase of meiosis I crane fly spermatocytes, viewed with polarization microscopy. The kinetochore fiber (arrows) of the lower lefthand autosome, which remains in the plane of focus throughout the movement of the autosome to the lower pole, is seen to shorten with little change in diameter or birefringence retardation until the autosome is nearly at the pole. The interzone contains the birefringent kinetochore fibers of the two sex chromosomes, plus other nonkinetochore fibers and diffuse birefringence. Time on each frame is in minutes. Bar, 10  $\mu$ m. The interpolar axis of the spindle is oriented at 45° to the analyzer-polarizer directions. The compensator is set for 3-nm BR from the background light extinction. The astral fibers perpendicular to the spindle interpolar axis appear dark in contrast and are not distinctly visible in these micrographs.

chromosomes did not migrate to a pole until after the autosomes arrived at the poles (15; Figs. 3, 4, and especially 8). In the interzone, as the auto-

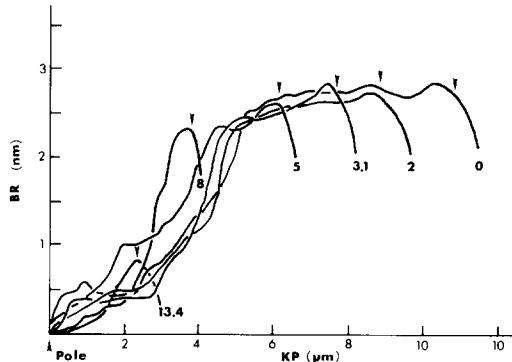


FIGURE 5 Profiles of the birefringent retardation ( $BR$ ) along a single kinetochore fiber at intervals during the movement of an autosome in the crane fly spermatocyte shown in Fig. 4. The number of minutes after the onset of anaphase is indicated on each profile. The arrows indicate the position of the kinetochore determined by the leading edge of the chromosome. The pole position was taken to be the position along the scan where the density along the fiber became equal to the density of the medium outside the cell. The densitometer probe (relative aperture,  $0.4 \mu\text{m}$ ) was scanned in a straight line from kinetochore to pole following approximately the central axis of the projected  $1.0\text{-}\mu\text{m}$ -Diam kinetochore fiber.

somes moved poleward, we could still see the birefringent kinetochore fibers that connected each univalent sex chromosome to both poles (Fig. 8, 17.8 min). Also, in the interzone there was a substantial amount of nonkinetochore fiber  $BR$  ( $1.0\text{--}1.5 \text{ nm}$  at  $22^\circ\text{C}$ ) as measured by scanning the densitometer probe.

**RESPONSES TO ABRUPT CHANGES IN TEMPERATURE:** Abrupt increases or decreases in temperature during anaphase produced rapid changes in autosome velocity to new constant velocities characteristic for the temperature (Figs. 6 and 7), but autosomes were never observed to undergo a transient acceleration after the temperature had been lowered. Autosome velocities ranged from nearly 0 at  $4^\circ\text{C}$  to  $1.9 \mu\text{m}/\text{min}$  at  $31^\circ\text{C}$  (Fig. 6*a* and *c*). The interpolar distance appeared unaffected by temperature changes (Figs. 6 and 8).

The relationship between temperature and anaphase autosome velocity is illustrated in Fig. 7. Because of the variation in velocity between different pairs of sister half-bivalents, autosome velocities after a temperature change were divided by their initial velocity at  $22^\circ\text{C}$  to normalize the experimental values. Excluding data at  $4^\circ\text{C}$ , where autosome movement became unmeasurably slow (Fig. 6*a*), the normalized velocities closely approximate an exponential curve corresponding to a  $Q_{10}$

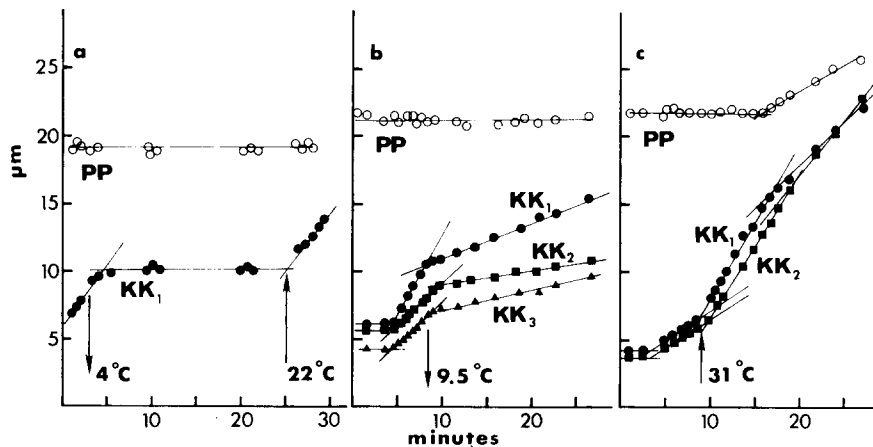


FIGURE 6 Changes in the poleward movement of autosomes induced by sudden temperature changes during first meiotic anaphase in crane fly spermatocytes. Temperature changes were from  $22^\circ$  to (a)  $4^\circ$ , (b)  $9.5^\circ$ , and (c)  $31^\circ\text{C}$ ;  $\sim 1$  min was required to complete 95% of the temperature change to a new equilibrium value. In *b*,  $KK$  separation distance (as defined in Fig. 1*a*) is plotted for all three autosomes in the cell to illustrate the different velocities that can occur within the same cell at constant temperature. Cooling induced no transient velocity increases, only a change in autosome velocity to new constant magnitudes. At  $4^\circ\text{C}$ , chromosome movement ceased 3–5 min after cooling, but the autosomes almost immediately resumed normal velocity after warming to  $22^\circ\text{C}$ .

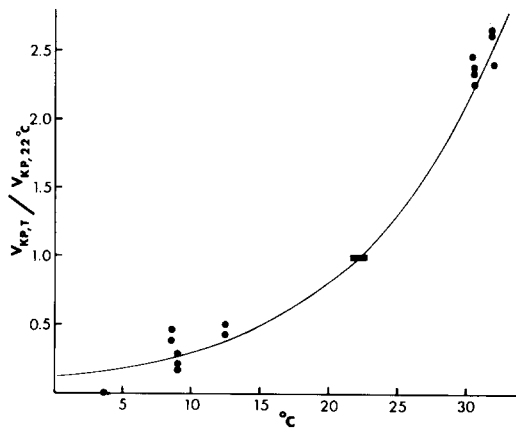


FIGURE 7 The temperature-dependence of autosome poleward velocity ( $V_{KP}$ ) after abrupt cooling or warming of crane fly spermatocytes during early anaphase of meiosis I.  $V_{KP} = \frac{1}{2}(V_{KK} - V_{PP})$  (see Fig. 1a).  $V_{KK}$  was taken to be the slope of a straight line fitted by eye to the data points of kinetic curves such as in Fig. 6.  $V_{PP} = 0$  for crane fly spermatocytes during most of anaphase A. Because  $V_{KP}$  varied significantly from one pair of separating sister bivalents to another, the values of  $V_{KP}$  after temperature changes were normalized (divided by the autosome's initial velocity at  $\sim 22^\circ\text{C}$ ). The curve was calculated from a best-fit straight line drawn through the data points in an Arrhenius plot [ $\ln(V_{KP,T}/V_{KP,22^\circ\text{C}})$  vs.  $(1/T)$ ] for temperature ( $T$ ) from  $8.5^\circ\text{C}$ – $31.5^\circ\text{C}$ . The slope of the Arrhenius plot yielded a value for the energy of activation,  $E_{\text{act}} = 16.6$  kcal/mol, according to the formula: slope =  $-E_{\text{act}}/R = d \ln V/d(1/T)$ . The solid rectangle represents the normalized initial velocities at  $\sim 22^\circ\text{C}$ .

= 2.7 and an activation energy  $E_{\text{act}} = 16.6$  kcal/mol, as determined from an Arrhenius plot of the data between  $8^\circ$  and  $31^\circ\text{C}$  (solid curve in Fig. 7).

In several experiments, the temperature was returned to near  $22^\circ\text{C}$  before the kinetochores had reached the centrosome region. In all cases, the autosomes rapidly returned to approximately their original velocities (Fig. 6a) without pronounced delays (within 1 min).

In the range  $31^\circ$ – $4^\circ\text{C}$ , the BR of the kinetochore fibers showed very little dependence on temperature. In contrast, the BR of the nonkinetochore fibers was a sensitive function of temperature. In the cell shown in Fig. 8b, a decrease in temperature from  $22^\circ$  to  $4^\circ\text{C}$  caused only a 25% reduction in kinetochore fiber BR (from 2.1 to 1.6 nm). At the same time, almost all nonkinetochore fiber BR was eradicated, except for a few strongly birefringent bundles in the interzone (Fig. 8b, 17.8 min). Nonkinetochore fiber BR re-formed within 1 min

of rewarming to  $22^\circ\text{C}$  (Fig. 8b, 23.8 min). Individual kinetochore fibers became progressively more distinct with cooling as a result of the increased contrast produced by the reduction in nonkinetochore fiber BR. At  $4^\circ\text{C}$ , for example, kinetochore fibers can be seen clearly as distinct white rods of constant diameter extending from the kinetochores to the centrospheres (see the sex-chromosomal fibers in Fig. 8b).

A similar differential temperature stability of kinetochore and nonkinetochore fiber BR was also observed in metaphase spindles (data not shown). Similarly, metaphase spindles did not shorten when cooled.

### Grasshopper Spermatocytes

In spermatocytes of the grasshopper *Trimerotropis maritima*, the poleward movement of autosomes and spindle elongation frequently occurred simultaneously, making the analysis of anaphase chromosome movement more complex than in crane fly spermatocytes. The observation of individual kinetochore fibers was also more difficult because grasshopper spermatocytes have more than twice as many chromosomes as crane fly spermatocytes (11 vs. 5). However, the spindle poles could be clearly distinguished with phase contrast microscopy, which facilitated the measurement of kinetochore-to-pole and pole-to-pole distances. As in crane fly spermatocytes, the majority of spindle BR in anaphase was localized in the region of the kinetochore fibers (Fig. 9). However, inter-polar fiber BR in the grasshopper spermatocytes was significantly lower than in the crane fly, reaching a maximum value of 0.5 nm in the interzone at  $22^\circ\text{C}$  (for example, see 47). In addition, there were no strongly birefringent bundles of nonkinetochore, interzonal fibers such as occur in the crane fly spermatocyte.

In spite of these differences, the response of grasshopper spermatocytes to abrupt changes in temperature was similar in most respects to that observed in crane fly spermatocytes. Drops in temperature rapidly produced corresponding reduction in autosome velocity to new constant rates (Figs. 10 and 11). Chromosome movement stopped entirely at  $10^\circ\text{C}$  (Fig. 11) without a substantial loss of kinetochore fiber BR. Kinetochore fibers were more temperature stable than nonkinetochore fibers. At  $10^\circ\text{C}$ , interzonal BR was undetectable, whereas the kinetochore fibers were more distinct and maintained at least 70% of their original BR (Fig. 11).

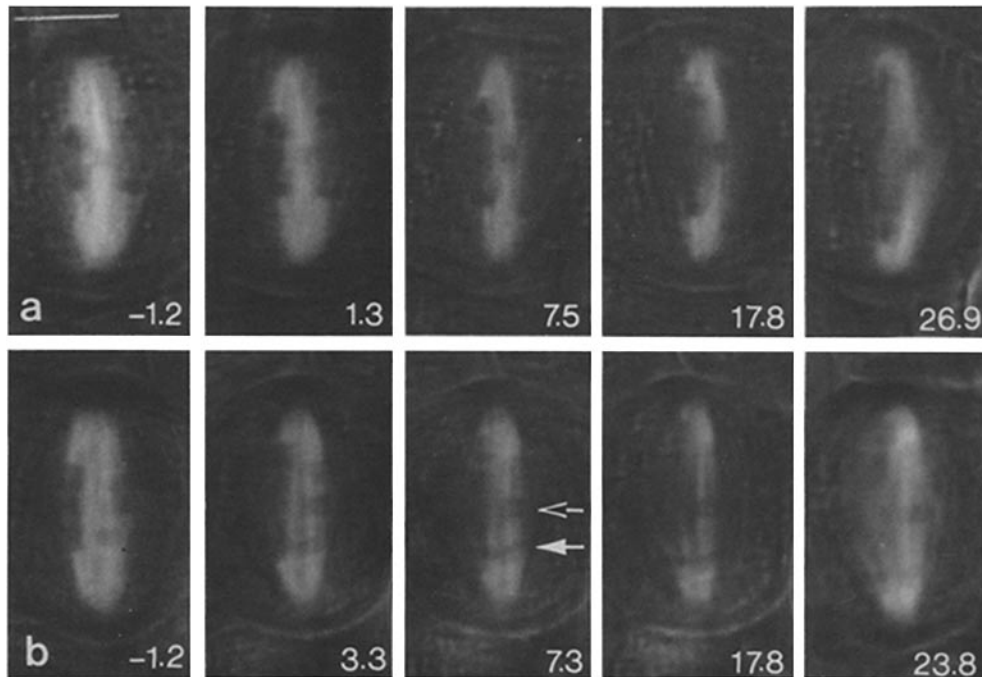


FIGURE 8 Changes in the pattern of BR in spindles and crane fly spermatocytes during early anaphase of meiosis I after abrupt cooling from 22° to (a) 8.5° and (b) 4°C. Time is in minutes from the time of cooling. It required ~1 min to complete 95% of the temperature change to a new equilibrium value. Temperature was returned to 22°C after ~20 min. Note the persistence and distinctiveness of the birefringent kinetochore fibers, particularly at 4°C after 7.3 min (autosomes, white arrow; sex chromosomes, white/black arrow). The diffuse nonkinetochore fiber BR was rapidly abolished in the interzone and half-spindle region, leaving only the distinct kinetochore fibers and a few interzonal nonkinetochore fibers. The BR of the latter was seen to dissolve slowly from the center of the interzone poleward. By 7.3 min after cooling, chromosome movement had halted at 4°C. The diffuse nonkinetochore BR in the interzone and half-spindle rapidly returned after warming, and chromosome poleward movement and kinetochore fiber shortening returned to precooling velocities. These cells were not flattened as much as the cell in Fig. 4. Bar, 10  $\mu$ m.

The rate of elongation in grasshopper spindles was also a direct function of temperature (Figs. 10 and 11). Below 14°C, elongation ceased (Fig. 11), and below 10°C, interpolar distance even shortened in one cell (Fig. 10*b*). (See Nicklas, reference 47, for effects of long-term cooling on grasshopper spermatocyte spindles.) When the temperature was returned to 22°C, the magnitude and distribution of spindle fiber BR returned to normal, and the normal rates of poleward movement and of spindle elongation resumed (Fig. 10). However, in contrast to the effect on crane flies, there was a 1.5–2.0 min delay in the resumption of autosome movement upon rewarming to 22° after cooling to 10°C or below (Fig. 10*b*).

#### DISCUSSION

Abrupt cooling of crane fly and grasshopper sper-

matocytes did not produce the predicted transient acceleration of the autosomes moving poleward during anaphase. In contrast to earlier studies on metaphase spindles of marine oocytes (22, 23, 24, 27, 50–52, 56), cooling these spermatocytes to as low as 4°C also did not cause the metaphase spindle to shorten. This absence of a transient shortening of the chromosomal spindle fibers at either metaphase or anaphase resulted from an unexpected cold stability of the kinetochore fiber microtubules. The birefringence profiles of crane fly kinetochore fibers at 4°C showed little change from 22°C, thus indicating little change in the number and length of kinetochore fiber microtubules. In contrast, nonkinetochore fiber BR was gone at 4°C. Consequently, we can make no conclusion regarding the relationship of experimentally induced kinetochore fiber disassembly to the



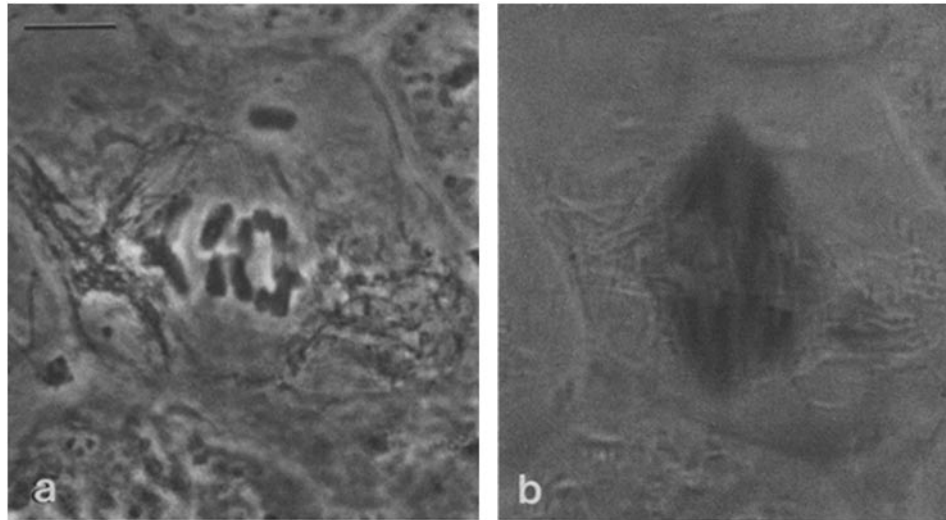


FIGURE 9 Mid-anaphase of meiosis I in living spermatocytes of the grasshopper *Trimerotropis maritima*, viewed with (a) phase contrast and (b) polarization microscopy in dark contrast. As in crane fly spermatocytes (Fig. 3), elongating mitochondria outline the spindle region. The birefringent half-spindle fibers shorten as the chromosomes move poleward, leaving behind an interzone of weak and diffuse birefringence. The interzone shows no fibrous structures as compared to the crane fly spermatocytes (Figs. 4 and 8). The sex chromosome (upper right near pole) in grasshopper spermatocytes segregates to one pole before or during autosomal poleward movement. Bar, 10  $\mu$ m.

generation of forces for chromosome movement as we originally had intended.

The extreme stability of kinetochore fibers to extensive cooling that we observed, compared to nonkinetochore fibers, has caused us to reconsider a more basic postulate of Inoué's dynamic equilibrium theory (23, 25, 26), that the spindle microtubules are in a simple monomer-polymer equilibrium with a cellular pool of subunits. At the very least, our data demonstrate there must be two equilibria—one for the kinetochore fiber microtubules and one for the nonkinetochore fiber microtubules. The behavior of astral and interpolar microtubules in response to cooling appeared consistent with predictions for a reversible physicochemical equilibrium (27, 29, 34, 50, 51). In contrast, we feel the data suggest that the kinetochore fibers are not in a simple, bidirectional monomer-polymer equilibrium, but in an active steady-state assembly based on distinctly different, and perhaps spatially separated, chemical pathways of polymerization and depolymerization (36–38, 64).

Differential stability of spindle fibers in crane fly and grasshopper spermatocytes is not related to any anomalies in anaphase autosome movement and, indeed, is not even peculiar to these cells.

Investigators have noted differential stability of the kinetochore and nonkinetochore fiber microtubules in several cell types to various depolymerizing conditions, including cooling (8, 34, 47), pressurization (49, 51, 52, 56), anti-mitotic drugs (9, 43, 44), dilution of the tubulin pool (10), and increased calcium concentration (55). Rarely, however, have the differences in stability been as great as we observed in the crane fly and grasshopper spermatocytes. Even the slight decrease in kinetochore fiber BR that we did measure may have resulted from the disassembly of nonkinetochore microtubules that normally form a sheath around the kinetochore fibers (17, 32, 33).

The basis for the differential stability of the morphologically similar spindle fiber microtubules is currently a matter of speculation. The differences could be attributable to differences in tubulin composition, such as have been found between ciliary axonemal microtubule structures (62). Two types of  $\alpha$ -tubulins have been found in mitotic apparatus isolated from sea urchins (3), but their structural origins are unknown. Another possibility is that factors similar to microtubule-associated proteins (MAP) that stabilize purified brain microtubules in vitro (29, 45) could be associated selectively with the kinetochore fiber mi-

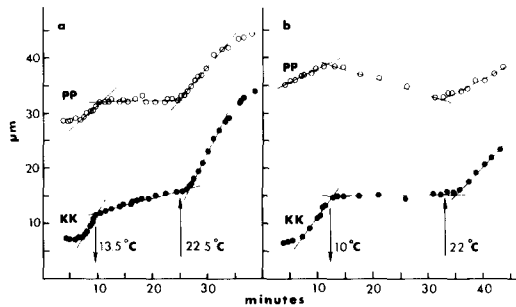


FIGURE 10 Changes in autosome movement (*KK* distance as defined in Fig. 1*a*) and interpolar elongation (*PP* distance) induced by sudden temperature changes during anaphase of meiosis I in grasshopper (*Trimerotropis*) spermatocytes. In the examples shown, temperature was dropped from  $\sim 22^\circ$  to (a)  $13.5^\circ$  and (b)  $10^\circ\text{C}$ . In both examples, *KK* separation was measured from one pair of separating autosomes that were in focus, but the results are typical for all 11 autosomes in both cells. It required  $\sim 1$  min to complete 95% of the temperature change to a new equilibrium value. Note the rapid shifts to new constant velocities, the stoppage of *KK* separation at  $10^\circ\text{C}$ , and the short delay (1–1.5 min) in the resumption of *KK* separation after rewarming from  $10^\circ\text{C}$ . The example in (b) was chosen to illustrate the slow shortening of *PP* distance that occurred in this cell. *PP* distance did not shorten in the three other cells cooled to  $10^\circ\text{C}$  nor in two cells cooled to  $7.5^\circ\text{C}$ ;  $V_{\text{KP}}$  and  $V_{\text{PP}}$  in these cells were zero.

cro-tubules or could be cross-bridging them (12, 15, 60). In addition, studies using immunofluorescent actin antibodies or myosin labeling (11, 21) suggest that actin filaments may also be associated with kinetochore fibers. Differential stability could be attributable to differences in the sites of origin and/or insertion of the microtubule ends. To explain the differential stability of *Chaetopterus* and HeLa cell spindle microtubules to increased hydrostatic pressure, Salmon (49, 51, 52, 56) proposed that microtubules anchored at both ends (kinetochore and interpolar microtubules) are more stable than those with one or both ends free (nonkinetochore and astral microtubules). Microtubule bundling is correlated with stability (34) (e.g., see Fig. 8*b*), which indicates that kinetochore microtubule stability is probably a consequence of post-assembly modifications, addition of accessory proteins, cross-bridging, or engagement of ends, after initiation of microtubule growth off the kinetochore and/or centrosome complexes (7, 23, 35). An examination of the differential stability of kinetochore and nonkinetochore fiber microtu-

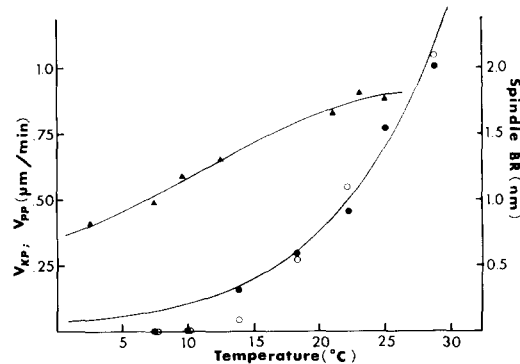


FIGURE 11 Comparison of average values of  $V_{\text{KP}}$  (●),  $V_{\text{PP}}$  (○), and BR (▲) of the half-spindle region after sudden temperature changes during early anaphase in meiosis I of grasshopper (*Trimerotropis*) spermatocytes. In each cell,  $V_{\text{KK}}$  and  $V_{\text{PP}}$  were determined from the slopes of lines drawn through the data points in graphs such as those in Fig. 10.  $V_{\text{KP}}$  was calculated from:  $V_{\text{KP}} = \frac{1}{2}(V_{\text{KK}} - V_{\text{PP}})$ . In a separate set of experiments, half-spindle BR was measured in the region halfway between the kinetochores and the poles of the spindle, 7–10 min after the temperature was changed. The BR values are typical of both late metaphase and mid-anaphase spindles. The  $V_{\text{KP}}$ ,  $V_{\text{PP}}$ , and BR data were pooled and averaged at each temperature, except that we excluded the one cell shown in Fig. 10*b* in which the interpolar distance shortened slowly after cooling to  $10^\circ\text{C}$ . The  $V_{\text{KP}}$  and  $V_{\text{PP}}$  data represent the average of two cells at  $7.5^\circ\text{C}$ , three cells at  $10^\circ\text{C}$ , and four or five cells at each of the other temperatures. The SD of the  $V_{\text{PP}}$  and  $V_{\text{KP}}$  data was  $\sim 20\%$ . The BR measurements varied within  $\pm 0.15$  nm at each experimental temperature. The solid curve passing through the  $V_{\text{KP}}$  data of  $14^\circ\text{C}$  and above was derived from an Arrhenius reciprocal-temperature graph of the  $\ln V_{\text{KP}}$  data, the slope of which yielded  $E_{\text{act}} \cong 21$  kcal/mol.

bules during early prometaphase would help clarify this issue, as distinct birefringent bundles are not pronounced until metaphase.

An important aspect of the differential stability, aside from its source, is that the relative persistence of kinetochore and nonkinetochore fiber microtubules during experimental changes in equilibrium conditions is not the same as their relative persistence during normal anaphase. Kinetochore fibers persist longer than nonkinetochore fibers when equilibrium conditions are experimentally shifted, whereas, during anaphase, the nonkinetochore fibers persist and may even lengthen while the kinetochore fibers continuously disassemble and shorten. Consequently, the changes in spindle assembly during mitosis cannot be explained by

changes in a single equilibrium constant or by inactivation of the tubulin pool alone: these changes should lead to the early disappearance of nonkinetochore fibers instead of kinetochore fibers.

The cold stability of kinetochore fiber microtubules does not mean, however, that they are static structures like ciliary axonemes. Instead, they seem to exist in a constant state of flux, which we judged to be steady-state equilibrium between distinctly different processes of polymerization and depolymerization. Evidence for tubulin flux in crane fly kinetochore fibers at metaphase is provided by experiments in which  $10^{-6}$  M colchicine abolished metaphase kinetochore fiber BR and, concurrently, caused the spindle to shorten (6). Both effects were reversed by removing or inactivating the colchicine. The current interpretation is that colchicine, or its derivative Colcemid, blocks microtubule polymerization by forming a complex with the tubulin dimer, preventing further incorporation of tubulin subunits into a microtubule (37, 64). If this hypothesis is correct, then the spindle fibers disassemble in the presence of colchicine because polymerization is blocked, while depolymerization actively continues.

Additional evidence for an active depolymerization mechanism and a continuous flux of tubulin through the kinetochore fiber microtubules comes from experiments in which Forer (13) irradiated kinetochore fibers in crane fly spermatocytes at metaphase with a UV microbeam, thus creating a small area of reduced BR in the fibers. While the autosomes remained at the metaphase plate, the area of reduced BR moved poleward at a velocity similar to the poleward velocity of autosomes during anaphase. The poleward movement of areas of reduced BR in Forer's UV microbeam experiments has been cited as evidence that the polymerization and depolymerization processes occur at opposite ends of the microtubules (13, 25, 26, 36, 38), creating a unidirectional flow of tubulin poleward in the kinetochore fiber. This is an intriguing idea, but methods for directly measuring tubulin flux within the spindle fibers need to be developed to clarify this critical issue.

Our results demonstrate that the depolymerization of the kinetochore fibers during anaphase is closely coupled to the poleward movement of the chromosomes. The relatively constant profile of kinetochore BR that we observed as the chromosomes moved poleward indicates that the number of microtubules in each kinetochore fiber remains

constant throughout the constant velocity phase of anaphase A in agreement with the earlier electron microscopy observations of LaFountain (33). Thus our birefringence measurements equate chromosome velocity directly with the rate of kinetochore fiber microtubule depolymerization and shortening.

The rate of depolymerization of the kinetochore fiber microtubules and chromosome velocity appear to be characteristic of a catalyzed process. At constant temperature, kinetochore microtubule shortening and autosome movement proceed at a constant rate for most of anaphase A. Abrupt changes in temperature produce rapid changes to new constant rates over a wide temperature range. The Arrhenius relationship we observed between autosome poleward velocity and temperature, after the abrupt temperature shifts, was similar to the relationship between chromosome velocity and temperature in other cells held at different constant temperatures throughout anaphase (18, 59).

It is important to note that in both crane fly and grasshopper spermatocytes there were critical temperatures not predicted from the Arrhenius plots (Fig. 7) below which the kinetochore fibers stopped shortening and the chromosomes stopped moving ( $4^{\circ}\text{C}$  for crane fly,  $10^{\circ}\text{C}$  for grasshopper). It may be that these cold temperatures deactivate the depolymerization process so that the "force generator" cannot effect chromosome movement. Alternatively, as proposed recently by several investigators (15, 36, 38, 40–42, 57), the force generator, in moving the chromosomes poleward, may actually be triggering the microtubule depolymerization process as well. If so, inactivating the force generator would halt depolymerization and kinetochore fiber shortening. In addition, because nonkinetochore fiber BR disappeared completely at these critical cold temperatures, it may be that the force-generating mechanism for anaphase chromosome movement requires some minimum number of nonkinetochore fiber microtubules to operate (e.g., see references 1, 2, 36, 38–40, 46, 48). The time required for grasshopper-spermatocyte nonkinetochore fiber microtubules to repolymerize after rewarming may account for the noticeable delay we observed before normal chromosome velocity resumed.

Our results indicate that a major component of the mitotic apparatus is a mechanism that can actively and selectively depolymerize microtubules, independent of the microtubules' thermal stability. Recent evidence suggests that elevation

of calcium ion concentration within the spindle microenvironment can produce spindle microtubule depolymerization. Micromolar concentrations of calcium ions have been shown to depolymerize spindle microtubules in living cells (28) and in isolated spindles whose microtubules are cold stable (55). Perhaps the membranous reticulum of the mitotic apparatus, which has been seen concentrated in the centrosome region of several spindle types (19, 20), is a major structural component of the mitotic apparatus and locally sequesters and releases calcium ions as the sarcolemmal reticulum does in muscle cells.

In any case, the depolymerization process is intimately coupled to the movement of chromosomes poleward during anaphase. This active mechanism of depolymerization appears to operate in addition to the labile equilibrium assembly-disassembly processes described earlier by Inoué and others. Existence of an active mechanism was revealed by the distinct cold stability of the kinetochore fibers in the insect spermatocytes examined here. Both isolated kinetochore and pole complexes have been shown to serve as sites for reversible nucleated condensation polymerization of cold-labile microtubules *in vitro* (7). Thus additional factors are required to explain the differential cold stability of microtubules and their mechanism of depolymerization.

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