# UV Microbeam Irradiations of the Mitotic Spindle. II. Spindle Fiber Dynamics and Force Production

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*Abstract.* Metaphase and anaphase spindles in cultured newt and $PtK_1$ cells were irradiated with a UV microbeam (285 nM), creating areas of reduced birefringence (ARBs) in 3 s that selectively either severed a few fibers or cut across the half spindle. In either case, the birefringence at the polewards edge of the ARB rapidly faded polewards, while it remained fairly constant at the other, kinetochore edge. Shorter astral fibers, however, remained present in the enlarged ARB; presumably these had not been cut by the irradiation. After this enlargement of the ARB, metaphase spindles recovered rapidly as the detached pole moved back towards the chromosomes, reestablishing spindle fibers as the ARB closed; this happened when the ARB cut a few fibers or across the entire half spindle. We never detected elongation of the cut kinetochore fibers. Rather, astral fibers growing from the pole appeared to bridge and then close the ARB, just before the movement of the pole toward the chromosomes. When a second irradiation was directed into the closing ARB, the polewards movement again stopped before it restarted. In all metaphase cells, once the pole had reestablished connection with the chromosomes, the unirradiated half spindle then also shortened to create a smaller symmetrical spindle capable of normal anaphase later. Anaphase cells did not recover this way; the severed pole remained detached but the chromosomes continued a modified form of movement, clumping into a telophase-like group. The results are discussed in terms of controls operating on spindle microtubule stability and mechanisms of mitotic force generation.

THE UV microbeam offers a means by which structures containing microtubules (MTs)[1] can be experimentally manipulated by local disruption. This possibility exists because MTs are sensitive to irradiation of between 260–300 nM (20, 52). The technique has been used mostly for studying spindle structure and function (for example, see references 2, 12, 18–23, 26, 27), particularly by Forer and his colleagues (for example, see references 4, 7–9, 40, 41); on occasion, it has been used to probe other MT-based motility systems (for example, see references 25, 28; see also reference 49). A major problem with the technique is that no UV microbeam apparatus is commercially available. Standard glass lenses do not transmit UV light, and quartz objectives and condensers, when available, are not usually designed for polarization or DIC optics. Thus, all workers have had to design and develop their own instruments that have various technical and operational limitations. Recently, Zeiss offered a UV epifluorescence attachment for their IM35 inverted microscope. With relatively simple modifications, this attachment can provide UV microbeam capabilities (47).

Using our first UV microbeam apparatus and working with diatoms, our previous observations on spindle MT dynamics (26, 27) were significantly different from those reported by Forer in crane fly spermatocytes (7, 8; see Discussion). Specifically, Forer describes areas of reduced birefringence (ARBs) created by the irradiation, as moving polewards at metaphase and anaphase at about the rate of anaphase chromosome movement. This observation was widely interpreted as indicating the existence of a polewards flux of MT subunits in spindle fibers with the likelihood that the MTs were being assembled at the kinetochores during metaphase and disassembled at the poles. In our experiments on diatom central spindles, the two cut ends of MTs in the ARB behaved quite differently, with the polewards end of severed MTs disassembling polewards (increasing the size of the ARB) while the other end remained relatively stable; unlike the situation with Forer's ARBs, we never observed regrowth of the fibers from this edge. The buckling or collapse of the central spindle upon partial or complete severing also indicated the exis-

1. *Abbreviations used in this paper:* ARB, area of reduced birefringence; MT, microtubule.

tence of considerable compressive forces over the central spindle during metaphase.

One explanation of these differing results on MT dynamics is that we and Forer's group were using quite different spindle types. To clarify this possibility, we have now used our newly developed apparatus to create ARBs in more conventional spindle types and we follow the behavior of the cut edges. In addition, we wanted to further probe the largely uncharacterized force generating systems that move chromosomes and whether the creation of ARBs across metaphase spindles might lead to the collapse of the poles together as observed in diatoms.

Using PtK and newt lung spindles, we now show: (a) that the behavior of cut MT ends in ARBs behave initially as was observed in diatoms, but that during metaphase, there is rapid regrowth of the fibers from the poles to close the ARB; and (b) while there is no immediate collapse of the poles together upon severing the half spindle, there is always during metaphase, a movement of the severed pole back towards the chromosomes as the spindle fibers grow across the ARB; remarkably, the unirradiated half spindle soon also shortens till both are the same size and the reformed spindle is functional. This recovery does not, however, happen if the cell is in anaphase. We believe the results are significant in conceptualizing how the stability of MT systems in the spindle is controlled, confirming the importance of intrinsic MT polarity in this process. However, they do not suggest how the force generating system(s) might work and instead, pose further problems for current models of spindle force production.

## Materials and Methods

### Microscope and Microbeam Apparatus

The modified IM35 light microscope and the methods used in the microbeam irradiations, are described in a previous paper (47). The quartz objective used for observations and for focusing the microbeam was a Zeiss ultrafluar 100×/1.25.

Briefly, the cells were monitored with a Dage MTI SIT66 camera and the image processed with an Avia Image Sigma II digital image processor. Images were recorded on a Panasonic optical disc recorder (TQ 2025F). For some observations (e.g., real time observations of the formation of the ARBs), recording was at video rates in real time; for most observations, however, recording was in time-lapse mode with frames being taken at between 1 s and 1 min intervals, depending upon the rapidity of effects and the duration of the experiments. Timing data was recorded on each frame with a time/date generator; usually, timing was zeroed at the onset of irradiation. This information is displayed on many of the sequences reproduced here. An in-line video typewriter (VTW-100; FOR-A Co., Ltd., Chicago, IL) allowed us to record experimental details on blank frames before each experiment (e.g., Fig. 5 a); in addition, details such as irradiation wave length and duration were recorded directly on the recorded images during the irradiation. Distance measurements were obtained from comparison of distances measured on the TV screen with those from calibrated graticules recorded on video disc frames after the experiments.

Cells were monitored during the monochromatic irradiation since the UV light was invisible. Before each experiment, the size of the slit used was recorded on one frame (e.g., Fig. 5 a). In addition, the slit was adjusted to a specific position with respect to the microscope's removable photographic graticule, using visible light (Fig. 5 a). The monochrometer was then adjusted for the appropriate wavelength (285 nM) and before each irradiation the cell was positioned with respect to this graticule (e.g., Fig. 5 b; then the graticule was removed for the irradiation). Accurate placement of the invisible irradiation was thus facilitated.

### Cell Culture

Newts (*Taricha granulosa*) were collected from the wild near Eugene, OR,

were kept at room temperature in deionized water, and fed live brine shrimp weekly. Newt lung explants were placed on quartz coverslips (22 × 22 × 0.17 mm; Bond Optics Inc., Lebanon, NH) in a drop of filter-sterilized medium containing 60% L-15 medium (KC Biological, Inc., Lenexa, KS), 10% FCS (Flow Laboratories, Inc., McLean, VA), 0.11% BES ($N,N$-bis[2-hydroxyethyl]-2 aminoethane sulphonic acid), 0.15% Pipes (Sigma Chemical Co., St. Louis, MO), and 5% whole egg ultrafiltrate (Gibco, Grand Island, NY; a gift from Dr. C. Rieder, NY State Department of Health, Albany, NY) in deionized water and adjusted to a pH of 7.5. The explants were maintained in a 26°C incubator with fresh medium added on the third day. Cell divisions typically occurred 8–10 d after the initial cell plating.

PtK$_1$ cells (American Type Culture Collection, Rockville, Maryland) were maintained in culture in Ham's F-12 medium (Gibco) supplemented with 10% FCS and 0.03% ampicillin at 37°C in a 5% $CO_2$ incubator. They were subcultured on to quartz coverslips 48–72 h before use.

### Light Microscopy: Immunofluorescence

Cells were initially fixed after irradiations by immersion of the coverslip on which they were grown, in methanol, at −20°C for a minimum of 10 min. Later, the coverslips were set up in perfusion chambers; after irradiations, these cells were perfused with a chemical fixative consisting of 0.5% glutaraldehyde, 0.3% Triton-X in PBS (pH 6.9), for 30 min. The coverslips were removed into 1% glutaraldehyde in PBS for a further 2 h and then transferred into 25% methanol in PBS for a minimum of 2 h. Before processing further, these cells were placed into 0.5% sodium borohydride for 10 min in PBS.

All the coverslips were washed with PBS and incubated with mouse monoclonal antitubulin antibodies (M. Klymkowsky, University of Colorado, Boulder, CO) for 45 min at 37°C. The antitubulin solution was rinsed off with PBS and the coverslips were incubated with biotinylated, affinity-purified, horse anti-mouse IgG (H + L) immunoglobulin (anti-MIg-Bi) (Vector Laboratories, Inc., Burlingame, CA), again for 45 min at 37°C. This antibody was used diluted 1:100 in PBS. Fluorescein-labeled Avidin D (Vector Laboratories, Inc.) was conjugated with the biotin by incubation at 0.25 mg/ml in PBS for 45 min at 37°C. The stained specimens were washed in PBS followed by distilled water. The coverslips were mounted in 30% glycerol and 5% propylgallate (Sigma Chemical Co.) dissolved in PBS. Some irradiated cells were fixed and taken back to Oregon where they were stained with gold-labeled antitubulin, using the methods described by Mole-Bajer and Bajer (32).

The antibody-labeled cells were examined on a Zeiss IM35 microscope equipped for phase-contrast and epifluorescence optics using a 63× planapochromat phase objective. Photomicrographs were taken on XP1 400 film (Ilford Ltd., Paramus, NJ), developed according to manufacturers specifications, or images were recorded on the TV system described above.

## Results

Initial experiments were conducted using heterochromatic light for irradiations. Later, after fitting a monochrometer to the microscope, all irradiations were conducted at 285 nM with a bandwidth of 10 nM. As described previously (47), the creation of the ARBs (terminology of Forer [7]) and subsequent effects were indistinguishable whether monochromatic or polychromatic light was used, except that the irradiation time needed to form the ARB was slightly longer with the monochromatic light. The observations summarized here included over 500 irradiations recorded on video disc. Our efficiency at creating ARBs was close to 100%.

### Controls: General Effects of Irradiations

When irradiated on quartz coverslips with the UV microbeam, spindles lost birefringence. The immediate loss created the ARB, the effect of interest in these experiments. However, UV irradiation also has a more general, unspecific effect: the slower, more generalized loss of birefringence from the whole spindle (55; see Discussion). The latter loss was approximately proportional to the amount of irradiation the cell received; consequently, we kept both the duration of
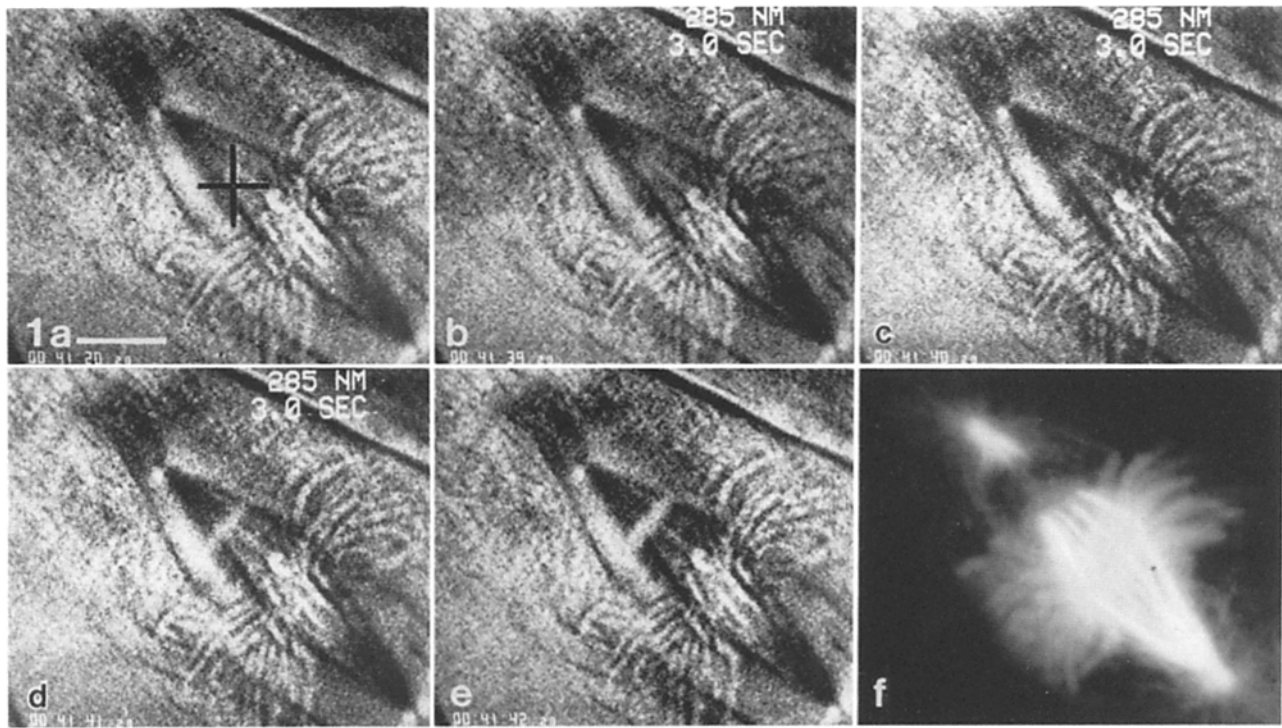
*Figure 1.* (*a–e*) Severing of spindle pole, metaphase newt spindle. (*a*) Cell positioned for irradiation. (*b–d*) Irradiation, starting at *t* = 41 min 38 s. (*e*) ARB immediately after irradiation. (*f*) Cell fixed 5 s after *E* and stained with antitubulin. ARB has expanded polewards. Bar, 10 μm.

irradiation and size of the irradiated area as small as possible (12).

When the cytoplasm outside the spindle area was irradiated close to the spindle, the latter would shorten as the poles congressed towards one another after ∼2 min in PtK₁ cells and 3 min in newt cells. When the beam was directed towards the outer regions of the cell, in most cases little or no effects on either spindle length or cell division were observed and anaphase was not affected.

### ARB Formation in Fixed Cells

Using an irradiation identical to that effective in live cells, an ARB was created in the spindle even with cells already fixed in glutaraldehyde. However, no subsequent changes in the ARB were seen.[2]

### Time Course of the Creation of an ARB

Since the UV irradiation in invisible (and therefore does not register on the sensitive TV camera), we could use the loss of birefringence to follow in real time the creation of the ARB during the irradiation (47). Typically, no effects were seen for about the first 1.7 s of irradiation, after which a rapidly cumulative effect created the ARB within the total period of irradiation of 3 s (e.g., Fig. 1).

### ARB Formation in Metaphase Cells

83 metaphase newt and PtK₁ spindles were irradiated and recorded for this part of the work. Once an ARB (typically

1.8 × 10 μM for newts) was created across the half spindle (Figs. 1, *a–d* and 2, *b–e*), the chromosomes in the metaphase plate withdrew slightly towards the unirradiated pole (Fig. 3). Invariably, birefringence at the cut edge proximal to the pole faded rapidly and evenly to that pole at a rate of 0.33 ± 0.01 μM/s over the next 10–25 s, depending on the length of the spindle (Figs. 2, *f–l*, 3, and 4). A small residual region of birefringence was left in the half spindle close to the pole, and the other astral fibers extending into the cytoplasm from the pole remained visible (Fig. 2, *f–l*). The fibers that remained in the half spindle appeared not to have extended into the cut region initially, although this could not be demonstrated definitively. Because of this residual astral birefringence, the position of the pole could easily be followed subsequently (see below). At metaphase, immediately after irradiation, the pole remained about in the same position as the ARB widened towards it; on a few occasions it moved a short distance away from the chromosomes. Later, as the ARB filled in, the pole always moved in a directed fashion back to the metaphase plate (see below) and most cells later continued into anaphase.

In contrast, the birefringence of the cut edge distal to the pole remained relatively stable (Figs. 1 and 2) although a brief, slight loss of birefringence was sometimes detectable. In general, this stability was marked although on occasion, when the slit size was large or the irradiations longer than ∼4 s, a noticeable loss of birefringence appeared to occur at this edge. Whether this was due to true loss or whether the fibrous elements (MTs) generating the birefringence were also being randomized, could not be ascertained.

Similar behavior of the cut edges were observed when only part of the spindle was irradiated, i.e., when one or more pe-
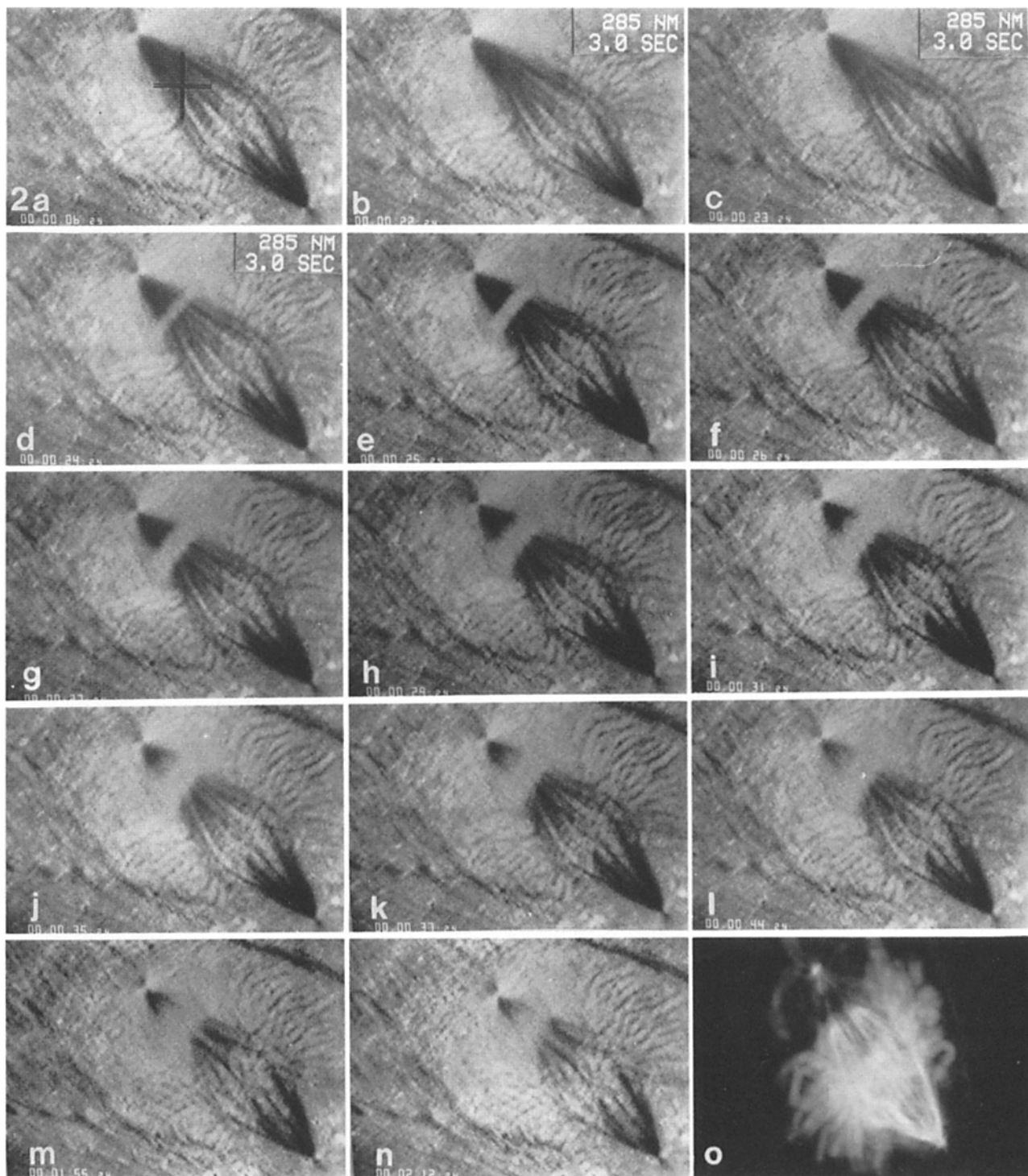
*Figure 2.* Formation of ARB, metaphase newt spindle. (*a*) Spindle positioned with respect to cross hairs for transverse cut. (*b–d*) Irradiation, initiated at $t = 21$ s. Course of irradiation ($t = 22–24$ s). (*e–l*) Disassembly of severed spindle fibers polewards (from $t = 25–44$ s) while kinetochore and adjacent stubs remain intact. Uncut astral fibers remain visible. (*m* and *n*) Initiation of movement of pole back to chromosomes as spindle shortens ($t = 1$ min 33 s, 1 min 50 s). (*o*) Cell fixed 5 s after *n*, and stained with antitubulin; reappearance of spindle fibers across the ARB.

ripheral kinetochore fibers were cut (Figs. 4, *b–f* and 5, *a–h*). In this event, the slight movement of chromosomes away from the pole only occurred with those spindles whose fibers were directly affected by the cut, and there was often some splaying outwards of the cut fiber.[2] Later, the pole moved in slightly and the whole spindle shortened as fibers were reestablished with the affected chromosomes (see below). The end result was that the ARB was no longer visible in the shortened spindle. These cells, too, usually went into anaphase subsequently (Fig. 5, *k* and *l*).
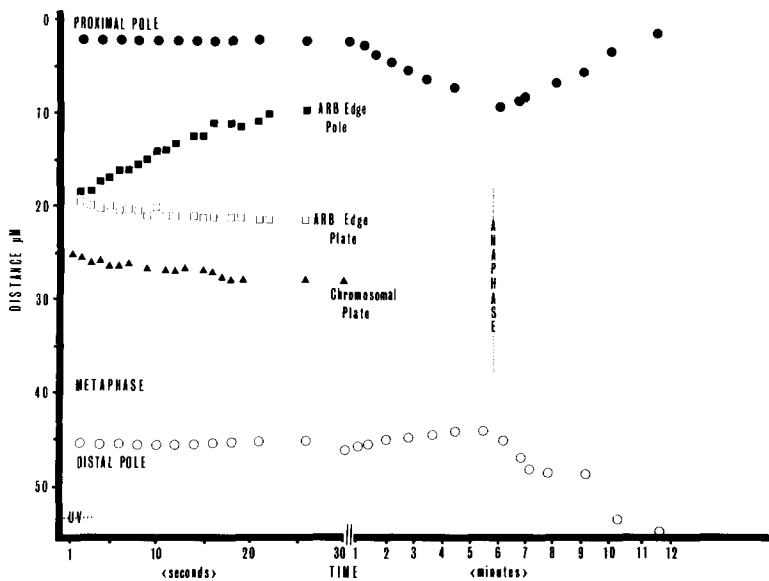
*Figure 3.* Graph showing relative positions of the poles, chromosomal plate, and edges of the ARB along a y-axis through both poles. The cell was irradiated at metaphase, with zero time immediately after the irradiation. The polewards edge of the ARB (*ARB Edge Pole*) rapidly moved polewards; the other edge (*ARB Edge Plate*) moved slightly away from that proximal pole as the metaphase chromosomal plate also retracted slightly. About 1 min later, the proximal pole started moving back towards the chromosomal plate, soon followed by the distal pole. The cell entered anaphase at ∿5.5 min.

In many experiments, we adjusted contrast settings during recording to visualize alternately the cut edge of the ARB and then individual spindle fibers. Thus, we could not attempt quantitative data on overall loss of birefringence, and these adjustments are partly responsible for some of the variations in total birefringence in certain sequences. For example, Fig. 2 *b* (before irradiation) resembles Fig. 2 *k* after irradiation; both are less dense than Fig. 2, *e* and *h* (after irradiation).

## Irradiation of Astral Fibers

While the behavior of irradiated astral fibers not associated with the chromosomes (i.e., away from the half spindles) was difficult to follow because of their weak birefringence, we could detect the appearance of an ARB and the polewards edge did show disassembly polewards as expected. This result was confirmed in cells irradiated thus and subsequently fixed for immunogold detection of MTs (Fig. 6).
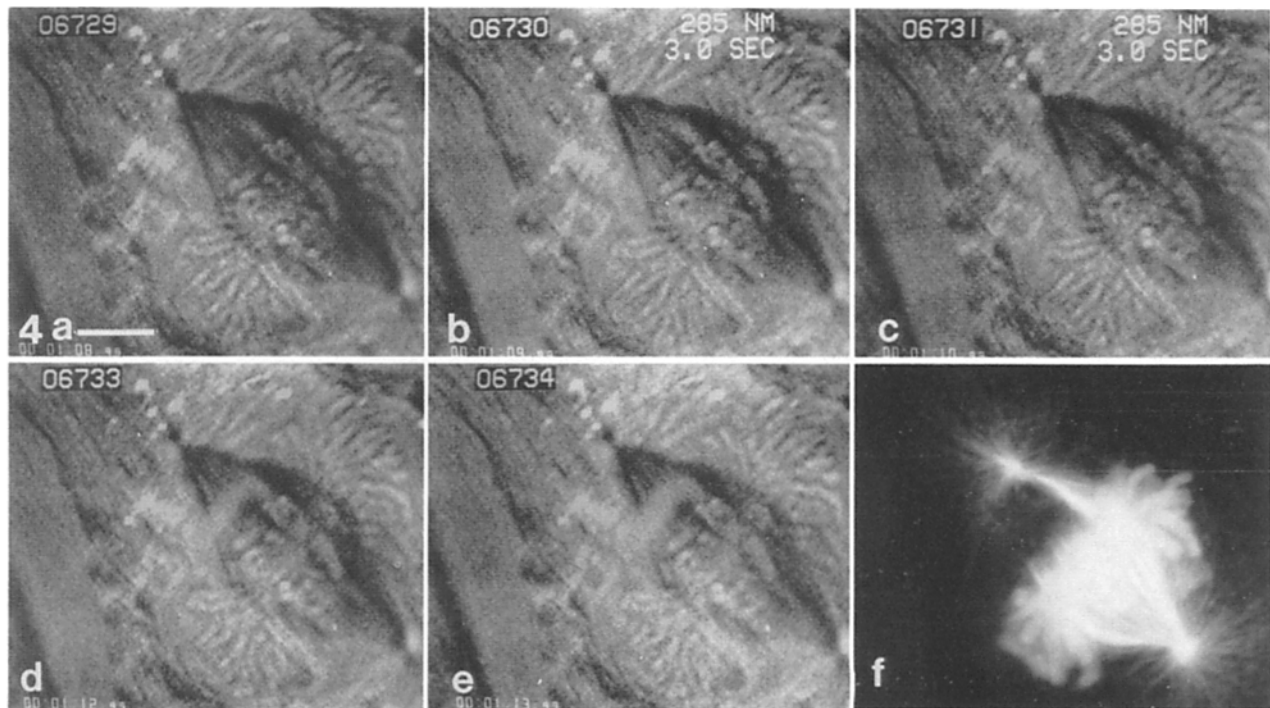


*Figure 4.* Irradiation of kinetochore and associated fibers, newt spindle. (*a*) Cell before irradiation. (*b–c*) Irradiation (starting at $t = 1$ min 8 s). (*d–e*) Initiation of polewards disassembly, 1 and 2 s after finish of irradiation. (*f*) Cell fixed ∿8 s after *e* and stained with antitubulin. Kinetochore stubs remain but ARB has grown polewards.
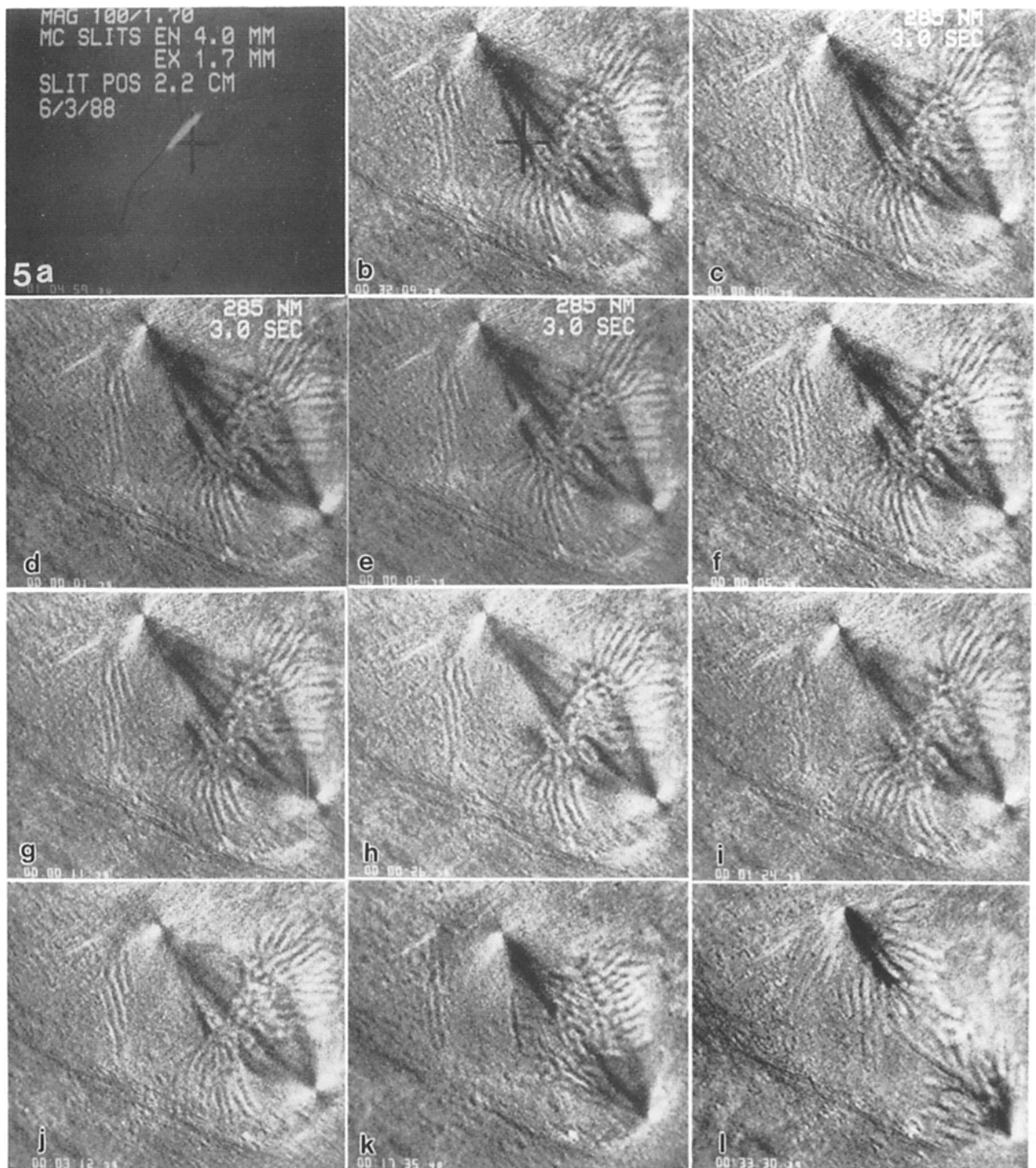
*Figure 5.* Cutting and reestablishment of kinetochore fiber, newt spindle. (*a*) Size and position of microbeam slit with respect to removable cross hairs. (*b*) Metaphase positioned for cut by cross hairs; initiation of irradiation (cross hairs removed). (*c–e*) Course of 3-s irradiation, timing zeroed so that $t = 0$ s in *c*. (*f–h*) Disassembly polewards of cut fiber ($t = 5$–26 s). (*i–j*) Reestablishment of cut fiber ($t = 1$ min 24 s, 3 min 12 s, respectively); spindle length decreased. (*k–l*) Normal anaphase ($t = 17$ min 35 s, 33 min 30 s, respectively).

### Subsequent Movement of the Pole Plateward and Shortening of the Whole Spindle at Metaphase

Once an ARB had been created across the half spindle in either newt or PtK₁ spindles, the severed pole and its array of fibers remained stationary for between 0.5–2.5 min. Then the pole moved steadily plateward (0.06 ± 0.02 μm/s; Table

I) and within a few minutes, the residual fibers appeared to have reestablished connection with the cut chromosomal fibers and associated chromosomes; this half spindle was now appreciably shorter than it was before the irradiation (Fig. 7) and shortening occurred when only some of the fibers were cut (Fig. 5). The time taken to initiate this move-
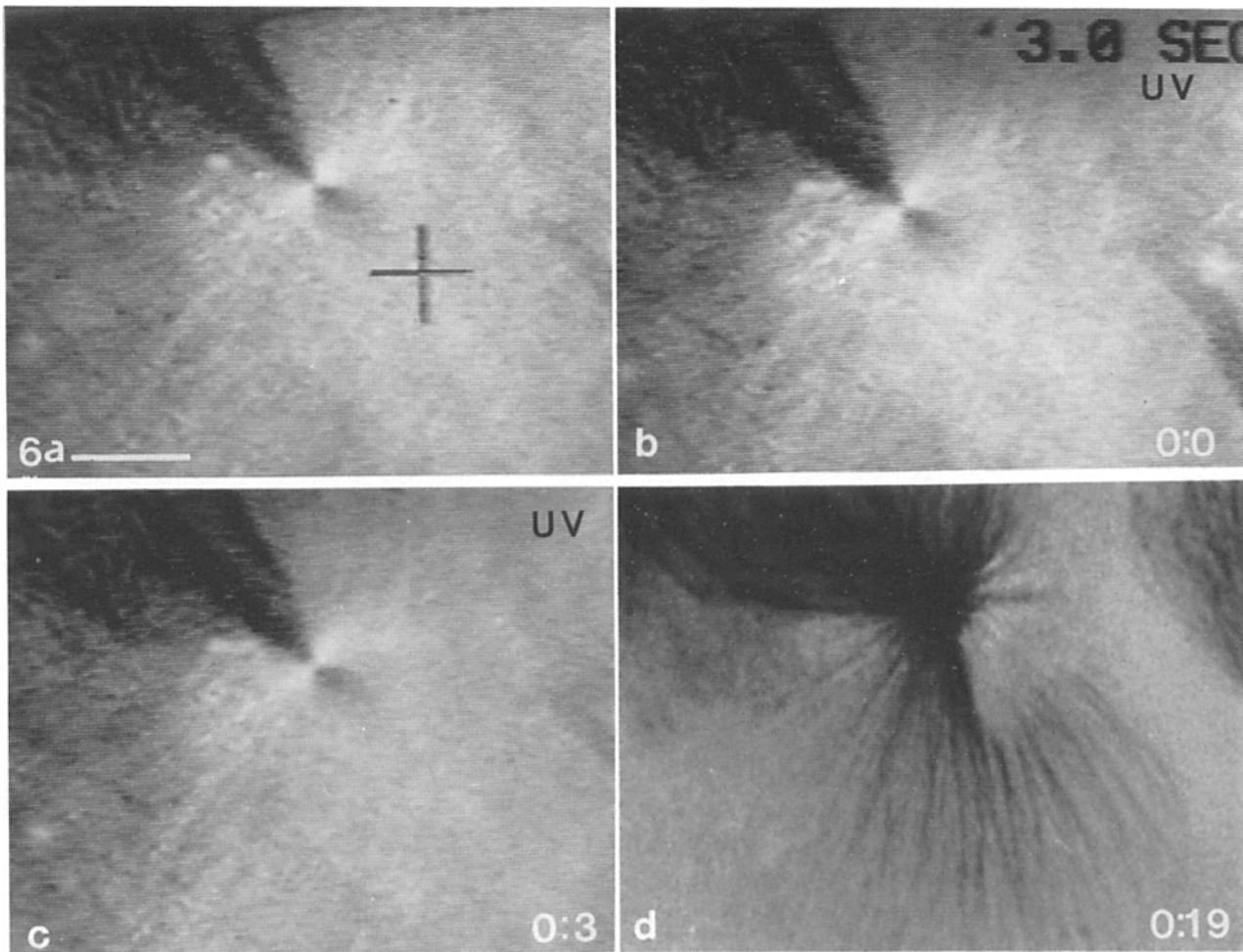
*Figure 6.* ARB in newt astral fibers. (*a*) Pole positioned for irradiation. (*b*) Irradiation; (*c*) a few seconds later. (*d*) Cell fixed 19 s after *c*, then stained with antitubulin. ARB clearly visible; MTs have disassembled polewards, but their distal segments still intact.

ment back to the plate after irradiation was somewhat dependent upon the position of the irradiation and enlarged ARB. If the irradiation was close to the plate so that the ARB was large, the movement started later (i.e., ~90 s) than if it were close to the pole (~45 s). In other words, the larger the ARB, the longer it took the pole to start this movement. Our images never suggested that this reestablishment of the connection between pole and chromosomes was the result of regrowth of the severed kinetochore fibers (e.g., Figs. 5 and 7). In some cells whose fibers were more clearly visible, not only did fibers appear to grow in all directions from the polar array, but those directed at the chromosomes appeared to thicken (presumably aggregating slightly) as they approached the kinetochore fiber stubs (Fig. 5, *i* and *j*). This subjective

impression was strong from viewing the video recordings but is difficult to document here.

Once the irradiated half spindle had shortened and reestablished a normal form, the other half spindle now also shortened steadily (0.03 ± 0.01 $\mu$m/s; Table I) until both were similar in length and birefringence. If the half spindles were initially slightly different in size (e.g., Fig. 5, *b* and *c*), they both shrank proportionally (Fig. 5, *i* and *j*). In cells where the spindle was nicked (i.e., those that had several kinetochore fibers severed), equivalent results were observed: the pole reestablished attachment to the severed ends of the fibers as it moved inwards a short distance, and the other half spindle then shortened a proportional amount (Fig. 5, *b–j*).

## Double Cut Experiments at Metaphase

To establish whether this inwards movement was due to reestablishment of spindle fibers, 12 newt cells were irradiated a second time in the previously formed ARB, as soon as the pole started coming in. In one cell, the pole then slowly wandered off into the cytoplasm; in the other 11 cells, the pole stopped moving inwards for a short time and then recommenced movement as before (results not illustrated here).

*Table I. Summary of Rates of Change in ARBs*

| Measurement | Number of cells | Rate $\mu$M/s |
|---|---|---|
| ARB polewards | 7 | 0.33 ± .01 |
| ARB platewards | 7 | 0.08 ± .03 |
| Proximal pole in | 5 | 0.06 ± .02 |
| Distal pole in | 5 | 0.03 ± .01 |

Summary of rates of change in position of the edges of ARBs, and subsequent movement of the two poles towards the chromosomal plate, after irradiation.
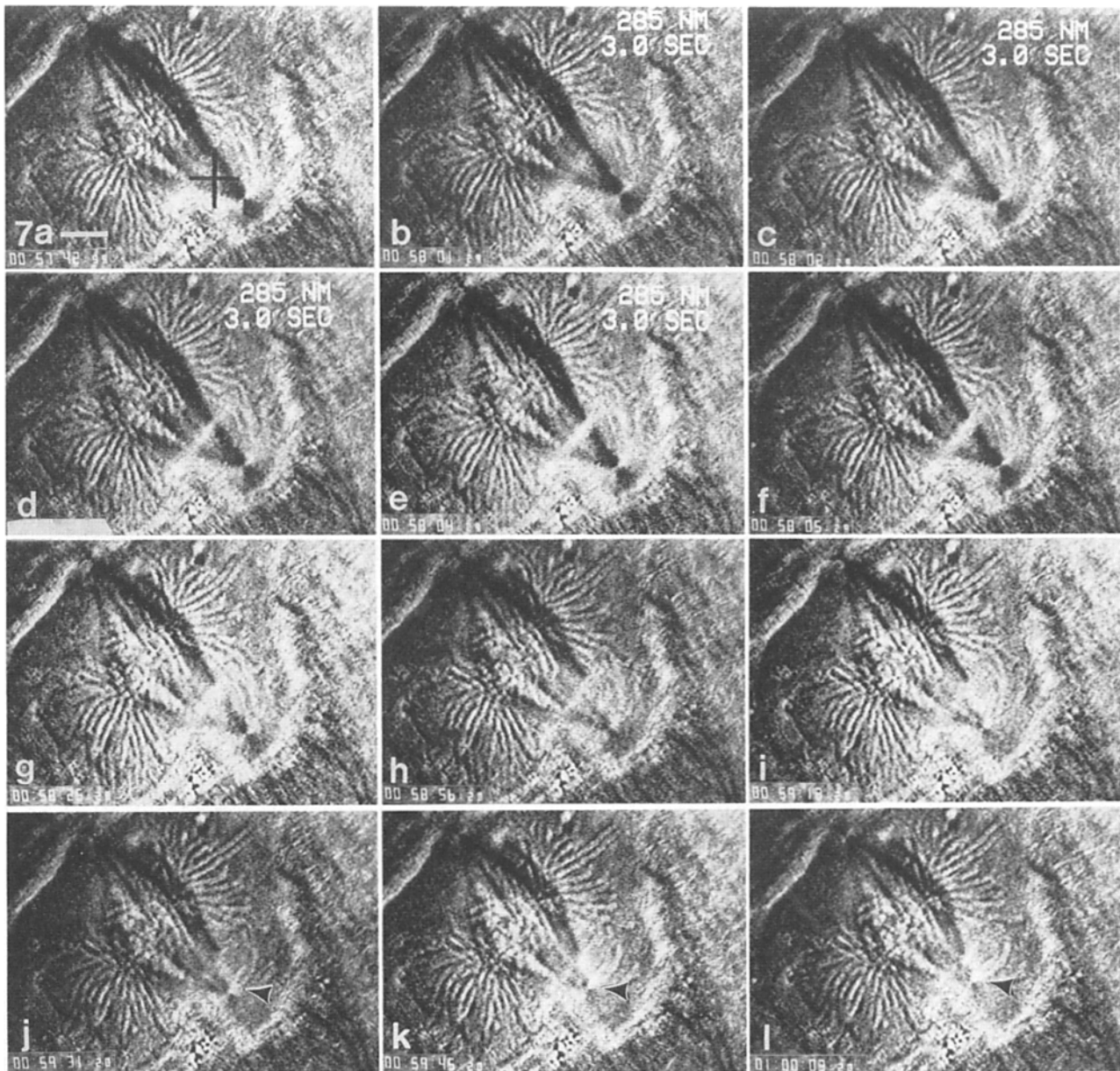
*Figure 7.* Shortening of metaphase newt spindle upon recovery from irradiation. (*a*) Cell positioned for irradiation. (*b–d*) Irradiation. (*e–i*) Growth of ARB polewards. (*j–l*) movement of pole back to reestablish connections to chromosomes; spindle shortened. One centrophilic chromosome is visible (*arrows*) accompanying the pole back towards the chromosomes.

### Anaphase in Cells Irradiated at Metaphase

Some of these irradiated cells were followed until they proceeded with a normal anaphase which occurred even though the spindle was short (as in Fig. 5, *j–l*). A few cells showed no sign of anaphase. In our experience, newt cells are particularly sensitive to experimentation and the illumination used for filming and TV recording. Thus, some such failed anaphases were considered inevitable regardless of whether or not the spindles had been irradiated with the UV microbeam.

### Behavior of Centrophilic Chromosomes and Particles in the Spindle

On a few occasions, newt spindles were irradiated that still had monopolar, oscillating chromosomes located at various positions on the half spindle. After irradiation, the ARB always grew as described above. If the oscillating chromosomes were located between the irradiation and the pole, as the ARB grew polewards, the fibers specifically associated with these chromosomes remained intact. If the chromosomes were between the metaphase plate and the irradiation, they remained attached to the fibers that were not disassembling. If the chromosomes were behind the pole, they remained tightly associated with that pole. In all cases, their oscillating activity continued throughout the experiment. As this pole later moved back towards the metaphase plate (see above), the monopolar chromosomes accompanied it (*arrows* in Fig. 7, *j–l*); subsequent behavior of the chromosomes was normal as they rejoined the full metaphase plate with the
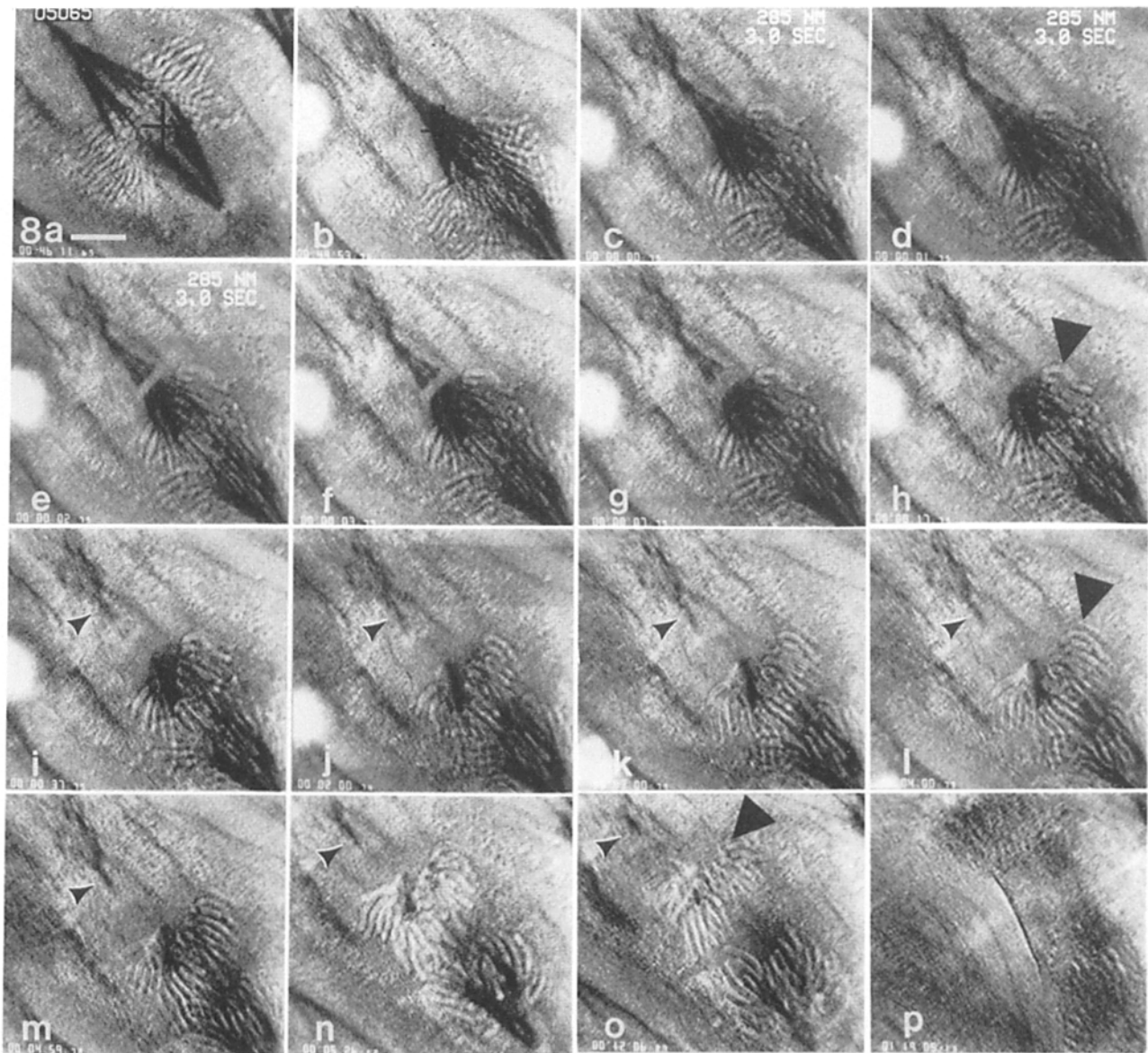
*Figure 8.* Severing of pole at anaphase, newt spindle. (*a*) Metaphase, whole spindle. (*b*) Spindle positioned with respect to cross hairs. (*c–e*) Irradiation at early anaphase, $t = 0$ s at *c*. (*f–h*) Loss of birefringence polewards ($t = 3$, 7, and 17 s); chromosome at top edge (*large arrows*) does not detach. (*i–o*) Anaphase continuing; upper group of chromosomes continuing to clump while detached from pole (*small arrows*): compare with the normal appearance of lower group of chromosomes. (*p*) Cleavage continuing.

smaller spindle. In addition, particle movement along the remaining spindle fibers appeared unaffected by the irradiation.

### Splaying of MTs On the Chromosomal Edge of the UV Lesion

PtK half spindles are typically hemispherical with the outer fibers appreciably curved. When the half spindle of such cells were severed by an ARB, the spindle fibers remaining attached to the chromosomes immediately splayed out until all were approximately parallel, perpendicular to the metaphase plate. These slowly curved back inwards as the pole moved in and reestablished the smaller but normal looking half spindle. This phenomenon is described and discussed more fully by Snyder et al.[2] Such splaying was less obvious

in the newt spindles that show little equivalent curvature in their spindle fibers.

### ARB Formation in Anaphase Cells

The half spindle of 20 early to mid-anaphase newt cells was severed with an ARB. In this event, there was invariably a loss of spindle birefringence polewards as described above (Figs. 8, *a–p*, 9, *a–l*). A significant difference, however, between metaphase and anaphase cells was that the apparently detached pole did not reattach to the remaining spindle; once severed, the pole as defined by the residual birefringence associated with it, moved irregularly around the cytoplasm (*arrows* in Figs. 8 and 9). On the two occasions when the pole did show some sign of moving back towards the chro-
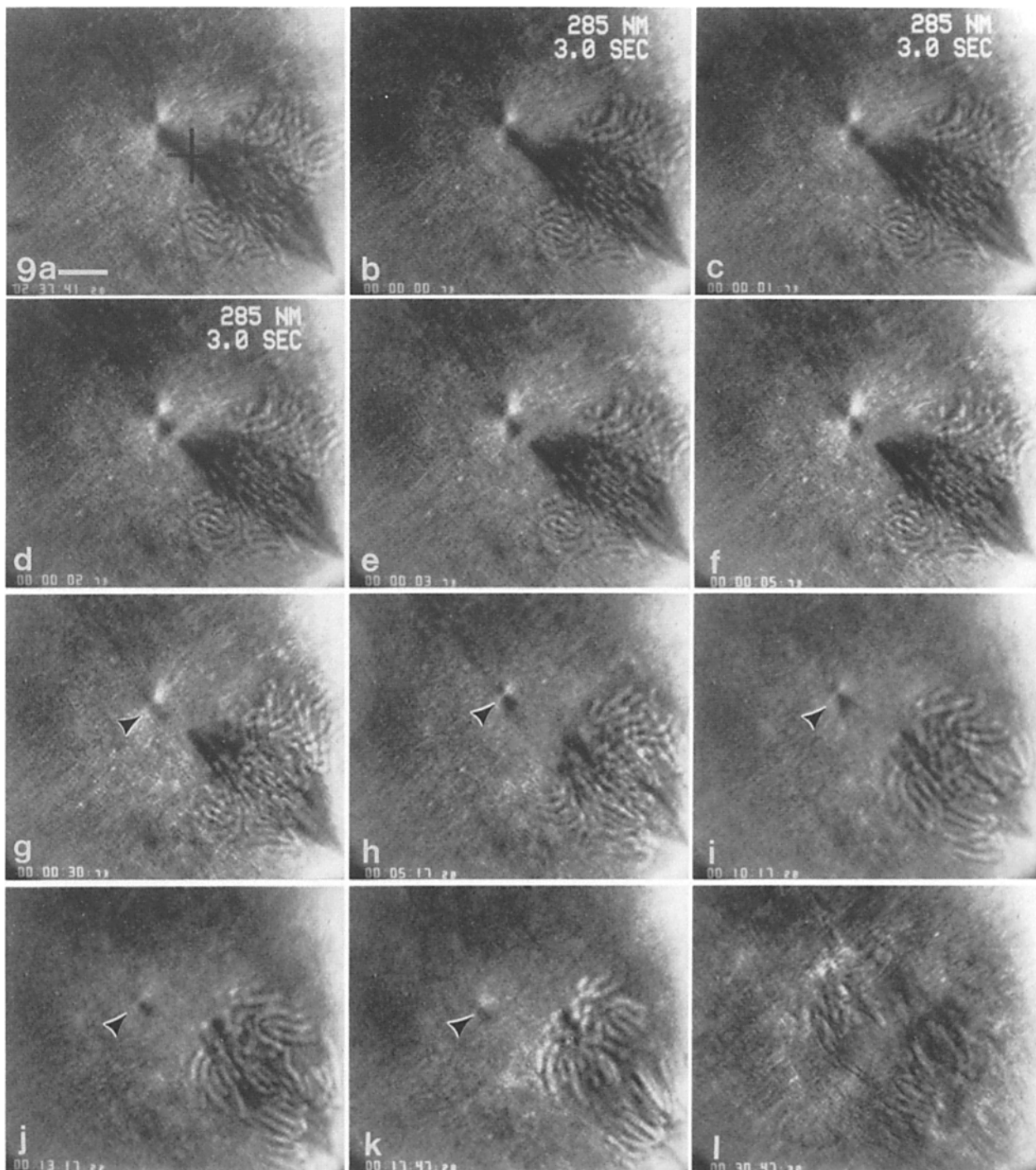
*Figure 9.* Severing pole from anaphase newt spindle. (*a*) Spindle positioned for irradiation. (*b–d*) Irradiation. (*e–g*) Lateral movement of pole (*arrows*) from spindle (*t* = 3, 5, and ∼30 s). (*h–l*) Continued movement of chromosomes into telophase-like clump; pole (*arrows*) clearly not associated with this movement (*t* = 5 min 17 s, 10 min 17 s, 13 min 17 s, 17 min 47 s, 30 min 47 s, respectively).

mosomes, careful inspection revealed that one fiber bundle remained unsevered across the ARB.

Immediately after the creation of the ARB, the mass of chromosomes appeared to spring back rapidly in the direction of the unirradiated pole. This movement was far more marked than the equivalent rebound effect observed at metaphase. Poleward chromosome movement in the irradiated half spindle was generally halted. The severed fibers be-

came aggregated at their splayed tips and then a form of chromosome movement appeared to restart as they clustered around a small region of cytoplasm, presumably a pseudopole (see reference 40) since the severed pole could be seen some distance away (Figs. 8, *i–n*, 9, *g–k*). The two half spindles looked different since the unirradiated spindle continued with normal anaphase A. It was difficult to decide the extent to which anaphase A and B activity resumed. The severed

kinetochore fibers were short and further shortening of kinetochore fibers was difficult to see.

### Effects of Irradiation on Anaphase B

If anaphase cells were irradiated in the interzone, the poles stopped moving apart. In many cases, anaphase appeared to cease completely. In favorable cells, the typical fading of bi-refringence polewards from the faint ARB (amongst the chromosome arms) could be detected.

## Discussion

### Dynamics of Severed Spindle Fibers: Polarity and Consequent Behavior of Spindle Microtubules

In another paper,[2] we demonstrate that the ARBs represent areas devoid of the MTs that constitute spindle fibers, as was shown for ARBs in diatom central spindles (26, 27) and crane fly spindles (53). The UV irradiation locally destroys the MTs of the spindle fiber (see also reference 17), thereby creating free ends of MTs.

The MTs in each half spindle are probably all of the one polarity (5, 6, 50) with the − or slow growing end (1, 48) located at the pole, and the + or fast growing end distal to the pole. The two edges of an ARB represent newly formed free + and − ends in the severed MTs. The − ends at the edge distal to the pole appear relatively stable since stubs of kinetochore and adjacent fibers usually remain intact for some while.[2] In contrast, the birefringence at the other edge of the ARB rapidly fades polewards. We conclude that when cut, their + ends rapidly disassemble polewards. All fibers, whether astral, kinetochore, or continuous fibers, be-have the same. The phragmoplast fibers in dividing cells of *Haemanthus*, whose MTs have the same polarity as the adja-cent spindle MTs (5), usually behave similarly although there is usually some detectable loss at first from the edge of the ARB proximal to the cell plate (results in preparation).

In diatoms, equivalent observations on ARBs in the cen-tral spindle (27) mirror its normal mode of unidirectional disassembly been observed in vitro when single MTs are cut by a UV microbeam (17, 20, 45); Walker et al. (52) report some slower growth from the − end in similar experiments.

### Dynamics of Severed Spindle Fibers: Capping and Rapid Disassembly

In the ARB, most or all fibers appear to be cut.[2] After the loss of birefringence from the + edge polewards, there still remained in the enlarged ARB, shorter fibers focused on the metaphase pole (see also reference 12, pages 16, 41). These fibers were apparently not cut since they initially did not ex-tend into the region of irradiation. The + end of free MTs growing from the pole are fairly stable, presumably because they are capped (14, 15, 24) and our results suggest that until severed, they remain capped and present after the irradia-tion. Continued growth of these or other, new fibers across the ARB is suggested to be involved in the reestablishment of continuity between the pole and chromosomes, a recovery evident from the movement of the pole towards the meta-phase plate (see below). The double cut experiments support this hypothesis; a second irradiation in the ARB as the pole started to move back immediately caused it to become dis-

engaged from the chromosomes for a further period until again starting its movement polewards. Gordon (12) also noted that soon after the cut fibers have disassembled pole-wards, ". . . the fibre birefringence just poleward of the irradi-ated region is increasing in an apparently separate process."

Once a free + end is created, the subsequent rate of MT disassembly from the + end is rapid. This observation is consistent with the catastrophic model of MT disassembly predicted theoretically (30) and observed in vivo (3, 39). Our rate of disassembly (19.8 $\mu$M/min; Table I) is close to the rate observed by Cassimeris et al. (17.3 $\mu$M/min; see ref-erence 3).

### Previous Reports on the Behavior of ARBs

In a classic early paper, Forer (7) created small ARBs in kinetochore fibers of crane fly spindles and described these as moving polewards at metaphase and anaphase at about the rate of anaphase chromosome movement, i.e., relatively slowly (see also reference 12). This widely cited result is reiterated in subsequent papers. Both the behavior of our ARBs and the speed of the effects differ significantly from those reported by Forer (7). If we nicked the metaphase spin-dle to create a small ARB like that described by Forer (7) and Gordon (12), we did get rapid reestablishment of the cut fibers, always accompanied by shortening of the irradiated half spindle. How this reestablishment occurred was not easy to determine; none of our images indicated a significant polewards growth of the chromosomal and other fibers at the cut edge of the ARB. The clearest visualizations were achieved by cutting across the spindle; the behavior of the resultant two long edges was consistent over many irradia-tions on PtK₁ and cultured newt cells (equivalent results were also recorded in mitotic diatoms). Electron micro-scopic analysis[2] supports our conclusion that at metaphase, the ARB fills as fibers grow back from the pole.

In attempting to reconcile these divergent results, a num-ber of points arise. They may reflect intrinsic differences in cell types. The timing factors involved are also crucial (and may reflect differences in cell types). Forer and his colleagues often used long irradiations (e.g., 3–375 s, 41) during which some effects we describe, including the reattachment of the pole to the metaphase chromosomes, are complete. This problem reappears when Wilson and Forer (52) described cells fixed "immediately" after irradiation. They estimate that cells were actually fixed within 2 min of irradiation (see page 458 of reference 52). In our cells, reattachment of the pole to metaphase chromosomes and shortening of the ir-radiated half spindle (effectively closing the ARB) had al-ready started well within this period. Gordon (12), although needing ∼1 s of heterochromatic irradiation to form ARBs, used a standardized 10-s exposure for his photographic re-cording, a protocol that would be unable to follow the more rapid of the changes we document with TV imaging.

The extent to which severed kinetochore and any intermin-gled fibers elongated polewards after irradiation is not clear-ly established in most reports. (This regrowth is essential if the ARB is to move polewards.) For example, in the cell in Fig. 4 of Inoué's review (19), the irradiated metaphase half spindle had not regained its former length by anaphase. Since the metaphase chromosomes were staggered, the extent to which the severed fibers actually recovered their length is

difficult to determine; by midanaphase, these kinetochore fibers remained shorter than before irradiation and shorter than those in the other half spindle. Moreover, most published micrographs show a reduction in the length of the irradiated half spindle (see below), further complicating the accurate determination of recovery in length of kinetochore and adjacent fibers. However, in other examples (particularly Fig. 1 in Wilson and Forer [53]), we agree that an elongation of a severed kinetochore fiber appears to have occurred. Inoué (18; cited in reference 19) also states that "the polar ends of spindle fibres, cut with a UV microbeam in dividing plant cells, rapidly disappear"; however, he adds, ". . . in a few minutes, they grow back from the severed fibre" (i.e., from the kinetochore stub). Izutsu (22) reported that an irradiated fiber disappeared completely; mechanical limitations did not allow him to follow the course of this disappearance.

Gordon's text (12) reveals many observations analogous to ours. The ARB in Gordon's irradiated kinetochore fibers enlarged polewards immediately after creation and "very rapidly" ($\sim$5 $\mu$M/min), 10 times faster than polewards chromosome movement at anaphase, i.e., the polewards edge disassembled as we have observed. This asymmetry in response of the two edges happened in all cells and furthermore, according to Gordon, (reference 12, page 16) ". . . In most cases, the arb behaviour (at metaphase) following the poleward enlargement is not clear"; he, like us, had trouble defining the behavior of the other cut edge of small ARBs. And Gordon later states (12) ". . . in three cases. . . the birefringence begins increasing in the poleward part of the enlarged arb restoring a microbeam sized arb (at least temporarily)." This result would be expected if MTs were growing back into the ARB from the pole as we suggest (see above). He notes that at anaphase, once this enlargement of the ARB has occurred, birefringence "makes no further appearance" in the ARB and there is no increase in the birefringence of the kinetochore fiber stub. Gordon also says that at anaphase "The kinetochoreward arb edge moves polewards at nearly the same rate as the arb kinetochore." These latter observations do not indicate growth of the severed anaphase kinetochore fiber and are equivalent to what we have seen.

In summary, our observations are similar to those of Gordon (12). The major discrepancy between our results and the previous reports concerns regrowth of the severed kinetochore fibers and therefore movement of the ARB towards the pole. We have never unequivocally observed this to happen. Thus, our observations do not support the inference from Forer's and Gordon's work that there is a flux of subunits through the kinetochore fiber to the pole at metaphase. However, Mitchison (29) and Mitchison et al. (31) have new evidence that supports such a scenario (see also reference 10). We cannot explain these differing results but our metaphase ARBs filled in quickly, within minutes, and slow growth of kinetochore fiber stubs, if it occurred, may have been masked by the speed of this recovery.

### Reestablishment of Chromosome-to-Pole Connections at Metaphase

An invariable response to irradiation was that the metaphase spindle lost birefringence; this was termed the "diffuse effect" by Gordon (12) and we agree that irradiated cells react as if treated with a small amount of anti–MT drug (56). In addi-

tion, after about 30-s recovery, invariably the pole began to move back towards the chromosomes until it had closed the ARB and created a shorter half spindle of normal appearance; this phenomenon occurs proportionally even when only a few fibers had been cut and the resultant shorter half spindle is also clear in many of Forer's (7) and Gordon's (12) micrographs. We believe that the movement follows growth of polar MTs across the ARB (44), a reconnection that was functional since most cells thereafter were capable of a normal anaphase. We cannot see a clear edge during this regrowth, so we cannot estimate the actual growth rate of the MTs; because the rate of movement of the pole back occurs by unknown mechanisms, it was not necessarily indicative of the growth rate of these MTs.

At anaphase, the poles may change their MT initiation properties.[2] Our results (44) are consistent with the possibility that in severed anaphase half spindles, fibers no longer grow from the pole or growth is much slower. These data partially explain why the poles did not reconnect to the chromosomes in a severed anaphase half spindle and if our interpretation is valid, the results give a rough estimate for the growth rate of MTs with free ends in vivo; at metaphase, they apparently bridge the ARB in $\sim$60–90 s, depending on its size. This explanation is oversimplified if other changes (e.g., in kinetochore properties) accompany the cell's entrance into anaphase. A second, not exclusive possibility, is that the severed anaphase pole cannot rejoin the spindle because of the permanent separation of forces which occurs with sister chromatid separation. Poles can never move plateward after the anaphase breakage of the kinetochore MT continuum since there is no opposing half spindle to serve as an anchor (for discussion see reference 43).

### Adjustments on Spindle Length

After the pole had reattached to the chromosomes, the irradiated metaphase half spindle was always smaller than it had been originally. Invariably, within a few minutes the other half spindle would shorten too until it matched in length the irradiated half spindle. Thus, these cells have a responsive mechanism for ensuring that the half spindles are normally the same size. The mechanism also functions when the half spindles had not achieved equality. Thus, the half spindles in Fig. 5 were unequal before irradiation; after irradiation and recovery, both were correspondingly smaller, but still slightly unequal. This response is a specific, direct consequence of irradiation at metaphase. The prometaphase spindle shrinks and becomes compact during progression through metaphase. However, this normal behavior occurs much more slowly than the readjustment we have observed although the two could be brought about by the same mechanisms. Irradiation may decrease the size of the tubulin pool and a smaller overall spindle would then be possible. However, if this were the only parameter responsible for the decrease in the unirradiated spindle size, we would expect both half spindles to respond concurrently. An alternate explanation has been proposed by the results of hyperosmotic shock treatment of PtK$_1$ cells (43). The population of nonkinetochore MTs is proposed to define spindle length, due to the compression they hold during the formation of the metaphase spindle. Severing any subpopulation of these MTs results in a decrease in spindle length (for discussion see reference 43 and footnote 2).

We cannot propose a simple explanation for these expressions of the cell's control over MT assembly/disassembly but spindle forces may be involved, with compression driving MT disassembly (16, 33, 37). The spindle behaves as if the force acting on a chromosome is proportional to the length of its fiber (13, 34, 54) and a balance of forces, resisted by the spindle structure, holds the metaphase chromosomes equidistant from each pole. If the size of one half spindle is decreased, then the overall compression in that half spindle is reduced because the kinetochore fibers are shorter. This matter is also addressed in a subsequent paper.[2]

## Generation of Spindle Forces

The mechanism by which the spindle generates polewards forces on kinetochores remains frustratingly obscure. We favor a model (35, 36) in which an undefined second component separate from MTs, generates and/or stores the polewards tension. We therefore expected that as the MTs are disassembled in the ARB, the pole would be drawn to the chromosomes (presuming this component was not concurrently destroyed by UV irradiation). This scenario does not happen as there is invariably a significant pause before the pole moves plateward, in response to the reformation of a MT continuum (43).[2] Other models fare no better in explaining our results. A model in which the kinetochore functions directly as the generator of the polewards force (11) does not suggest how such a kinetochore motor could draw the pole towards the metaphase plate without the kinetochore fibers concurrently shortening (see also 9, 38, 51).

The movement of the metaphase pole back towards the severed spindle fibers starts as the ARB is bridged by MTs;[2] thus, the generation of tension between the pole and chromosomes requires MTs, a well-documented observation from microbeam irradiations (for example, see references 12, 23, 33) and other observations. While the pole is usually the focal point of spindle fibers and anaphase movement, this correlation is coincidental rather than functional (see also reference 46). Anaphase chromosomes were still drawn into a tight cluster after the pole has been severed and demonstrably free from the chromosomes. The clustering around a focus requires the presence of residual kinetochore fibers; it cannot be attributed to a side effect of anaphase/telophase chromosome aggregation since the latter can be achieved, for example, without such movement in colchicine-treated telophases. This behavior raises a subtle and possibly central issue. When metaphase spindles are cut, particularly in PtK cells, the kinetochore fibers immediately splay outwards (footnote 2; and see, for example, Fig. 4 in reference 19). As these spindles recover and become reorganized, the severed kinetochore fibers become pointed and aggregated (12; the phenomenon is also visible in some of Forer's micrographs). This behavior is not seen with those fibers emanating from the pole and it is evident at anaphase as the chromosomes aggregate while the pole is severed. We suspect that this is a manifestation of the activity that generates polewards force, due to a kinetochore-, not pole-related component or activity that is functional along the length of the fiber.

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