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Cytochalasin induces abnormal anaphase in crane-fly spermatocytes and causes altered distribution of actin and centromeric antigens

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Abstract. Inhibition of cytokinesis by cytochalasins without an effect on karyokinesis has been demonstrated in several types of cells. We report here that treating crane-fly spermatocytes with cytochalasins at concentrations (10 μM CE, 100 μM CD, and 200 CB) in excess of that needed to inhibit cell division induces one or more half-bivalents to lag at anaphase during the first meiotic division. The behavior of the laggards is similar to that of maloriented half-bivalents. Following treatment at these concentrations, probing with rhodamine-phalloidin or bodipy-phallacidin reveals loss of filamentous actin from the poles and its appearance in the spindle, predominantly in regions where centromeres and kinetochores are normally found. When either N350 anti-actin monoclonal antibody or rhodamine DNase I was used to probe for actin in cytochalasin-treated cells, a similar redistribution of actin was observed. CD and CE treatments alter the pattern of fluorescence at centromere/kinetochore regions after staining with scleroderma CREST serum: CREST-positive structures become broader, with spikes extending from them toward the pole; in addition, some strands of CREST fluorescence appear that are apparently extraneous, and not associated with chromosomes. Probes for actin yield staining patterns in centromere/kinetochore regions that match closely the cytochalasin-altered pattern of CREST staining. Our finding of actin in the vicinity of kinetochores under conditions that result in abnormal chromosome behavior raises numerous questions about the possible role(s) of actin in meiosis, particularly in chromosome orientation.

Introduction

Evidence for actin as a component of the spindle apparatus in mitotic and meiotic cells has been obtained with a number of approaches, including heavy meromyosin (HMM) decoration after glycerination (Gawadi 1971), immunofluorescence after cell permeabilization (Cande et al. 1977; Seagull et al. 1987), and microinjection of fluorescently conjugated actin into living cells (Sanger et al. 1989). The postulate that actin may participate with myosin in the mechanism of chromosome movement has been challenged by results of experiments that attempted to perturb the function of myosin through either inhibition with anti-myosin antibodies (Kiehart et al. 1982) or use of recombinant DNA technology that disrupted myosin heavy chain gene expression (DeLozanne and Spudich 1987), although the possibility remains that myosin I is involved at some stage.

Evidence against a functional role of actin in the spindle has come from studies using cytochalasins, a group of fungal metabolites that have been used to test the role of actin in numerous motility-related phenomena (Tanenbaum 1978). Specific interactions have been shown to occur between cytochalasins and both isolated actin monomers and F-actin in vitro (Howard and Lin 1979; Cooper 1987), but limited data are available concerning mechanisms underlying cellular responses to cytochalasin treatment. Previous studies on dividing animal cells have documented cytochalasin treatments that can inhibit cytokinesis but have no ostensible effect on karyokinesis (Carter 1967).

Prominent in the literature covering the possible role(s) of actin in chromosome segregation are crane-fly spermatocytes. These meiotic cells were used in the initial studies of Forer and Behnke (1972) that revealed decorated filaments in the spindle after probing glycerinated spermatocytes with HMM. Evidence interpreted in agreement with an actin-myosin model of chromosome movement has also been obtained by Forer from UV microbeam experiments on crane-fly spermatocytes (Forer 1985, 1988). Ultrastructural studies done on this material in our laboratory (LaFountain 1975) did not reveal actin microfilaments in spindles of non-glycerinated cells but did provide evidence for actin filaments in the contractile ring. We have also found evidence for

Abbreviations: CREST, calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia

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cortical actin in centrifugation-sheared spermatocytes (Strauch and LaFountain 1982). Using biochemical approaches, we have isolated spermatocyte actin and determined that it is distributed between sedimentable (supramolecular) and non-sedimentable (monomeric) forms (Strauch et al. 1980).

In this paper, we report on studies in which cytochalasins B, D and E (CB, CD, CE) were used to perturb actin in spermatocytes of the crane fly *Nephrotoma suturalis*. In general agreement with previous work, we have found that over a wide range of concentrations and durations of exposure, chromosome movements do not cease in spermatocytes in which subsequent cytokinesis is inhibited. This is not to say, however, that chromosome behavior is totally unaffected by cytochalasin treatment. At the highest concentrations that were used to block cytokinesis (20 μM CE, 100 μM CD, and 200 μM CB), the pattern of chromosome movement at anaphase was not normal. Here we report on the inhibition of cytokinesis and on the induction of anaphase lag in crane-fly spermatocytes as a result of cytochalasin treatment. We also describe the effects of cytochalasin treatment on (a) the distribution of actin as revealed by fluorescence after staining with rhodamine-conjugated phalloidin and (b) the distribution of fluorescence in the vicinity of kinetochores following immunofluorescence with scleroderma CREST serum.

Materials and methods

Crane-fly spermatocytes. Larvae used in these experiments were obtained from a laboratory colony of *N. suturalis*. Testes containing spermatocytes in desired stages of division were removed from larvae into Tricine insect buffer (Begg and Ellis 1979). These testes are rich in spermatocytes in meiotic stages from prophase I through telophase II and also contain some spermatids.

All cytochalasin and control treatments described below were performed on spermatocytes contained within isolated testes. Rupturing of testes to release spermatocytes was done either (a) under oil to make living cell preparations, (b) on glass slides after which cells were air dried, fixed, and Giemsa stained, (c) after fixation and permeabilization of cells that were to be stained for fluorescence microscopy, or (d) on pieces of coverslip before transfer of cells into collection tubes prior to SDS denaturation and gel electrophoresis.

Cytochalasin treatment. CB, CD, and CE (Sigma Chemical Co., St. Louis, Mo.) were prepared in stock solutions at concentrations of 1 mg/ml (approximately 2 mM) or 10 mg/ml (approximately 20 mM) in dimethyl sulfoxide (DMSO) and stored in the dark. Stock solutions were diluted in Tricine insect buffer with constant stirring. For all of the dilutions used, the final concentration of DMSO did not exceed 1% (v/v). Cytochalasin treatments and control experiments on spermatocytes within testes were done by immersing 2 to 4 testes in a 0.3–0.5 ml drop of diluted cytochalasin or DMSO-containing Tricine insect buffer. During incubation at room temperature, samples were kept in the dark.

To test the reversibility of cytochalasin effects, testes were transferred after cytochalasin treatment into drops of cytochalasin-free buffer containing 1% DMSO for the first 30 min of recovery, then, if longer periods were required, into Tricine insect buffer for recovery periods up to 4 h.

Living cells. Preparations of living spermatocytes were made in halocarbon oil as described elsewhere (LaFountain 1982). Each testis incubated either in cytochalasin with 1% DMSO or in 1% DMSO (controls) was dipped in Tricine insect buffer at the conclusion of the incubation period for 5 s or less. Omission of this rinse resulted in reduced viability of cells. Testes were then submersed in oil, where buffer and adhering fat tissue were separated from them.

Giemsa-stained cells. For analysis of cytokinesis inhibition and induction of anaphase anomalies in populations of cells, spermatocytes were smeared on glass slides, fixed, and processed for Giemsa staining as described previously (LaFountain 1985). Representative smear preparations from single testes were scanned and every primary spermatocyte meeting pre-established criteria (described in Fig. 1) was scored. For analysis of chromosome segregation, we scored cells from mid-anaphase (Fig. 1 a) to late anaphase (Fig. 1 d) as containing autosomal laggards if one or more autosomal half-bivalents had failed to reach a position more than half-way between the equator and the segregating half-bivalents (Fig. 1 b and c). As illustrated, the sex univalents normally lag at anaphase in these cells. The behavior of sex chromosomes, although often observed to be anomalous after cytochalasin treatment, was not analyzed. For analysis of cytokinesis inhibition, telophase cells (Fig. 1 e) were scored. Cytokinesis was judged to be occurring in these cells if the cleavage furrow had narrowed by at least half the diameter of the rest of the cell.

Each smear from a cytochalasin-treated testis was compared with a control – the partner testis of the same larva exposed only to the vehicle solution containing 1% DMSO.

Cells prepared for fluorescence microscopy. Among the numerous protocols (including use of cold methanol) that we tested, the following method of fixation with formaldehyde proved most successful; it preserved cell morphology well with minimal cell loss and permitted staining with fluorescent antibodies, phalloidin, HMM, or DNase I. Intact isolated testes were fixed for 25 min using a solution containing 2% formaldehyde (prepared fresh before use from paraformaldehyde), 0.05 M PIPES buffer, 1 mM EGTA, 1 mM $MgCl_2$ and 0.1% (v/v) Triton X-100, the mixture adjusted to pH 6.9 (modified from Bastmeyer et al. 1986). For phalloidin staining, Triton X-100 was omitted in some preparations without affecting the stainability of cells; however, detergent was a necessary ingredient if immunofluorescence was intended. Fixation and all subsequent steps were done at room temperature.

All fixations were followed by rinsing testes twice (15 min each rinse) with a permeabilization buffer (perm buffer) (Bastmeyer et al. 1986) that included 1 mM EGTA, 1 mM $MgCl_2$, 0.1% Triton X-100 (v/v) and 10 mg/ml bovine serum albumin (Sigma) in PBS, pH 7.2. (PBS was prepared as a 10 × stock solution with 0.5 g KCl, 0.5 g KH_2PO_4, 20 g NaCl, and 5.4 g $Na_2HPO_4 \cdot 7H_2O$ in 250 ml of distilled water.) Then, testes were ruptured in < 5 μl perm buffer on pre-cleaned (Janicke and LaFountain 1989) coverglasses that had been attached with VALAP (1 part each of vaseline, lanolin, and paraffin) to a microscope slide through which a 3/4 in. hole had been cut to make a well slide, and the contents of testes were smeared over the coverglass. This preparation was covered and held in a humid chamber for 15 to 20 min to permit attachment of cells to the glass, after which they were air dried and either stained immediately or stored at 4° C for subsequent staining. Air-dried preparations were used up to 7 days after drying without any adverse effects. Longer storage periods were not attempted. Rehydration of cells and all subsequent incubation and wash steps were done by applying and withdrawing solutions from well preparations with a pipet, such that cells were maintained in a hydrated state after rehydration.

To test the extent to which results were influenced by air drying, testes were fixed (both with and without Triton) as above and ruptured in 10–12 μl of perm buffer containing rhodamine-labeled phalloidin at a 1/15 dilution and, to label the chromosomes, 1 μg/ml Hoechst 33258 (Sigma). These cells were incubated in a humid chamber for 30 min and then covered with oil for observation. Phalloidin staining in these preparations was similar to that of

preparations that were air dried. Because air-dried cells stuck to the glass, facilitating the multiple wash steps required for immunofluorescence staining, the air-drying protocol was used routinely, and, unless otherwise stated, all micrographs presented in Results are of cells prepared with the air-drying step.

Two types of immunofluorescence studies were done. The first utilized anti-actin monoclonal antibody (code N. 350 from Amersham, Arlington Heights, Ill.) as the primary antibody (diluted 1/200) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (μ-chain specific) (Sigma Chemical Co, St. Louis Mo.) as the secondary antibody (diluted 1/100). The second utilized CREST serum from patient EK, diluted 1/30 with PBS, as the primary antibody and fluorescently labeled anti-human (rhodamine-labeled goat anti-human IgG from Jackson Immunoresearch, West Grove, Pa., or fluorescein-conjugated goat anti-human IgG from Organon Teknika, West Chester, Pa.), diluted 1/100, as the secondary antibody. For both, cells were rehydrated for 10 min in PBS and incubated with primary antibody for 40 min, followed by washing with several changes of PBS over the course of 30 min. Incubation with secondary antibody was for 35 min, followed by washing for 25 min with PBS. This was followed by incubation in Hoechst at 0.33 μg/ml for 10 min and a final 20 min wash in PBS before mounting in Elvanol (Dupont). Elvanol was prepared by dissolving 0.35 g in 3 ml of distilled water that had been heated in a test tube; then 1.5 ml of glycerol was added.

For each well slide to be stained with phalloidin, 7.5 μl of rhodamine-phalloidin or bodipy-phallacidin (Molecular Probes, Eugene, Ore.) was transferred to a small test tube and left in a fume hood until the methanol had evaporated. Phalloidin was then dissolved in 150 μl of PBS as a 1/20 dilution and used within 2 h. After rehydration for 10 min in PBS, cells were stained with 150 μl of phalloidin for 40 min. In most cases, 5 μl of 10 μg/ml Hoechst was added for the last 10 min of phalloidin incubation. After two very brief, incomplete washes with PBS, coverslips were mounted on the preparations using Elvanol.

To double stain with antibody and phalloidin, the procedure for immunofluorescence staining was followed through incubation with rhodamine-labeled secondary antibody. Then, after a 5 min wash in PBS, slides were incubated with bodipy-phallacidin for 40 min and processed subsequently as for phalloidin staining.

For staining with DNAse I, preparations were rehydrated in PBS then stained for 40 min in tetramethylrhodamine-conjugated DNase I (Molecular Probes) diluted 1/50 to 1/200 in PBS. This was followed by a 15 min wash in PBS, 10 min in 0.33 μg/ml Hoechst, 5 min washing in PBS, and mounting.

For HMM staining, a stock solution (a gift from Joseph Sanger) of 8 mg/ml lissamine-rhodamine-labeled HMM in 50% glycerol was diluted in PBS. Cell preparations were rehydrated 10 min in PBS, then incubated for 40 min with labeled HMM at concentrations of 20, 200, and 1,000 μg/ml or for 5 h with 1,000 to 2,000 μg/ml. After washing for 15–30 min in PBS, preparations were incubated in Hoechst at 0.33 μg/ml for 10 min, washed 5 min in PBS, and mounted.

Immunoblotting. For immunoblotting of spermatocyte proteins, homogenates were prepared for denaturation in SDS as described by Strauch et al. (1980): contents of treated or untreated testes were ruptured into Tricine buffer on pieces of broken coverglasses, where testicular contents were separated from the testicular sheath and adhering fat tissue. Cells were collected in 1 ml conical test tubes and were concentrated by centrifugation in a clinical centrifuge at high speed (ca. 1,500 rpm) at 4° C for 10 min. Pellets were dispersed in sample buffer consisting of 40 mM Tris-HCl, pH 7.4, 2% (w/v) SDS, 0.5% bromphenol blue, and 5% (v/v) beta-mercaptoethanol on ice for 15 min, and final denaturation was done by immersing tubes in a boiling water bath for 10 min.

Protein blot analysis was performed essentially as described previously (Balczon and Brinkley 1987, 1989). Spermatocyte proteins were separated on 12.5% SDS-polyacrylamide gels using the procedures published by Laemmli (1970) and the proteins were transferred to nitrocellulose using the methods reported by Towbin et al. (1979). After being blocked with 3% milk in PBS for 1 h, the nitrocellulose blots were probed for 3 h at room temperature with primary antibody diluted to working concentration in the 3% milk solution. After three rinses for 5 min each, the blots were incubated with the appropriate peroxidase-labeled antispecies IgG antibody for 2 h (diluted 1:500–1:1,000 in 3% milk). After three rinses with PBS, the blots were developed with 3,3′-diaminobenzidine and H_2O_2.

Microscopy. Giemsa-stained and living-cell preparations were viewed with a Zeiss universal microscope, using primarily a 40×/0.75 N.A. Neofluar phase-contrast objective; a narrow-band green (546 nm) interference and a heat-absorbing filter were used. Fluorescently stained preparations were viewed with a Zeiss photomicroscope III equipped with epifluorescence optics using a 100×/1.3 N.A. oil-immersion Neofluar objective. Photographs were taken on Tri-X (Eastman Kodak, Rochester N.Y.), T-Max 400 (Kodak) or Technical Pan 2415 (Kodak) film and processed in diafine (Acufine, Chicago Ill) or HC-110 (Kodak) developer. Photography was done within 1–2 days of staining.

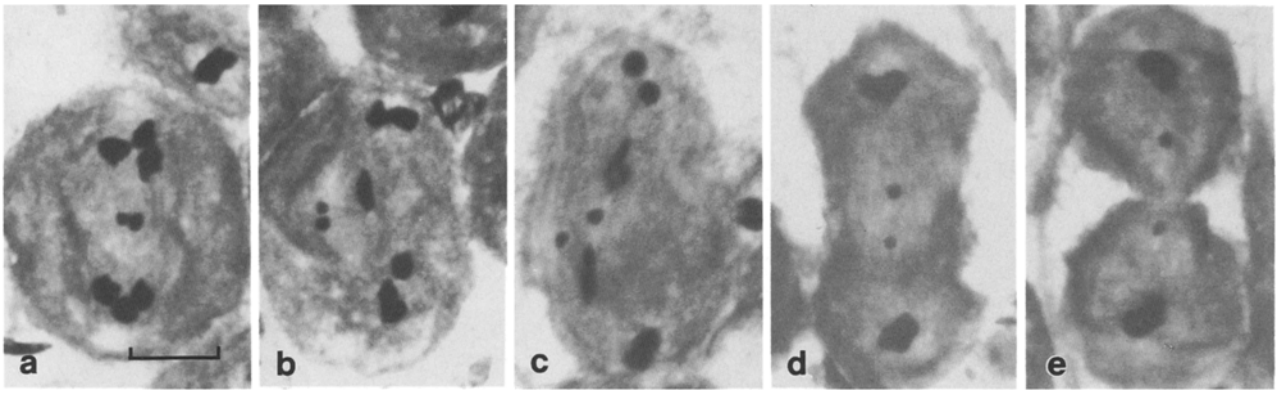


Fig. 1a–e. Giemsa-stained spermatocytes of *Nephrotoma suturalis* illustrating details of criteria used in analysis of cytokinesis and chromosome segregation. **a**, **d**, and **e** Controls treated with 1% DMSO for 3.5 h. **a** Normal chromosome segregation at mid-anaphase. Autosomes are at least half-way but not yet all the way to the poles. The sex univalents lag at the equator. **b** Cell treated with 200 μM CB for 3.5 h; one autosomal laggard. **c** Cell treated with 100 μM CD for 3 h; two autosomal laggards. **d** Normal segregation at late anaphase. Autosomes are at the poles and are still partially distinguishable from one another. Sex chromosome segregation and cytokinesis are beginning. **e** Normal telophase/cytokinesis. Autosomes are fused together at the poles, but sex univalents are still distinguishable. Cytokinesis has reduced the cell diameter in the mid-zone to less than half the diameter of the rest of the cell. Bar represents 10 μm

Results

Cytokinesis and anaphase lag in cytochalasin-treated spermatocytes

Anaphase in spermatocytes from the crane fly *N. suturalis* is often abnormal after exposure to concentrations of cytochalasins (CB, CD, and CE) in excess of the minimum doses that inhibit cytokinesis. Following these treatments, many anaphase cells had one or two autosomal half-bivalents lagging near the spindle equator as other half-bivalents moved poleward (Fig. 1). Some cells had three or more lagging half-bivalents. In control testes (exposed only to the DMSO vehicle), cytokinesis was present in many cells (although cytokinesis fails in some untreated as well as some control cells), and the percentage of mid-to-late anaphase cells with autosomal laggards (7.6 ± 3.9) was not statistically different (at $\alpha = 0.01$) from that in untreated cells (6.4 ± 5.9) (Janicke and LaFountain 1982; LaFountain 1985; Ladrach and LaFountain 1986).

With each of the three cytochalasins studied, 2 μM cytochalasin was sufficient to prevent cytokinesis. For CE, significant (at $\alpha = 0.01$) lagging was induced at 2 μM after 3.5 h; for CD and CB, induction of anaphase lag was not apparent until higher concentrations were used (Table 1); the percentage of cells in which anaphase lag was induced tended to increase with increasing concentrations of cytochalasin and with longer exposures, with CE being the most potent inducer and CB the least. Lagging induction was not seen with an exposure shorter than 1 h in duration.

Inspection of the smear preparations used in this analysis provided no evidence that cytochalasins cause homologs to fail to pair or to separate prematurely from one another and exist as univalents at prometaphase or metaphase.

Table 1. Effects of cytochalasins D, B and E on crane-fly spermatocytes

Drug (μM)		Duration of exposure (h)	Cytokinesis[a]	Percentage anaphase lag[b]
CD	2	3.5	−	7
	10	2.5	−	9
	20	2.5	−	5
	20	3.5	−	20
	20	3.5	−	39
	60	3.5	−	59
	60	3.5	−	46
	80	3.5	−	84
	100	0.5	+	10
	100	1	−	39
	100	3	−	58
	100	3.5	−	63
	100	3.5	−	61
CB	2	2.5	−	2
	20	2.5	−	6
	20	3.5	−	7
	100	3.5	−	10
	140	3.5	−	43
	200	1	−	9
	200	3.5	−	55
	200	3.5	−	85
CE	2	2.5	−	14
	2	3.5	−	17
	2	3.5	−	18
	20	2.5	−	46
	20	4	−	48

[a] Observed (+), inhibited (−)
[b] Percentage of mid-to-late anaphase primary spermatocytes having one or more autosomal laggards

Living-cell observations

The effects of cytochalasins were further evidenced in living cells (Fig. 2). The following results emerged regarding cells treated with 20 μM CE, 100 μM CD, or 200 μM CB.

1. The laggard autosomes and other anomalously segregating autosomes in cytochalasin-treated anaphase and telophase cells derive from bivalents that are resolved into half-bivalents at anaphase onset. The induced anaphase laggards did not exist as univalents during prometaphase.

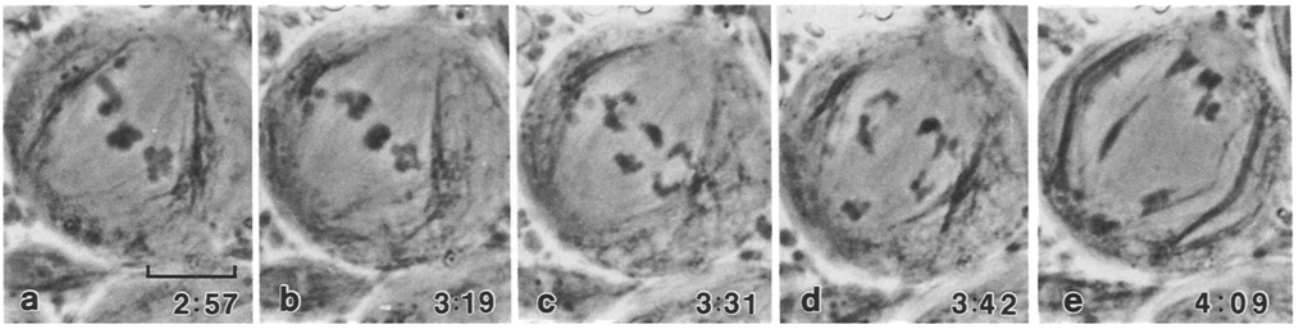


Fig. 2a–e. Series of phase-contrast micrographs in a living spermatocyte after incubation in 100 μM CD. Times are indicated in hours and minutes (h:min) after start of cytochalasin treatment. The middle bivalent appears tilted in prometaphase but reorients by metaphase and yields no laggards. The bivalent on the right has its lower centromere directed toward the spindle periphery rather than toward the lower pole at prometaphase; this appears to have begun to reorient but is still tilted when anaphase begins; the lower half-bivalent lags while the upper one segregates. The bivalent on the left also has an unusual configuration at prometaphase and metaphase, and it yields one laggard and one non-laggard. Bar represents 10 μm

2. Many metaphase bivalents appear unusually tilted with respect to the spindle axis in configurations that we have previously shown (Janicke and LaFountain 1986) to be indicators of bipolar malorientation in one or both half-bivalents, i.e., a single half-bivalent having kinetochore microtubules extending toward both poles (amphitelic or merotelic orientation) instead of toward only one pole (syntelic orientation).
3. Bivalent chromosomes and sex univalents may acquire an unorthodox "metaphase" position prior to anaphase onset. A bivalent may be located near one of the spindle poles when anaphase commences, in which case both half-bivalents remain associated with that pole during anaphase. Bivalents positioned closer to, but still off, the equator at anaphase onset often yield at least one half-bivalent that fails to reach a pole.
4. Chromosomes at metaphase in cytochalasin-treated cells tend to be more peripherally situated than they are in untreated cells. In untreated cells, metaphase bivalents are rarely as close to the mitochondria that outline the spindle as they are in Fig. 2.
5. Spindle poles are generally broader in cytochalasin-treated cells than in control and untreated cells. This effect may result from changes in the organization of actin at the spindle poles (described later).
6. During anaphase, spindle elongation occurs (to a greater extent in CE-treated cells than in CB- and CD-treated cells); this is followed by collapsing of the elongated structure such that the previously segregated chromosomes are repositioned closer to one another within the uncleaved cytoplasm. A spherical interkinesis cell results, and the chromosome events of the second division occur in that cell, usually with a tri- or tetrapolar spindle.
7. In all cases, the duration of time between nuclear membrane breakdown and the onset of anaphase among cytochalasin-treated cells (and in control cells exposed to 1% DMSO) ranged between 65 and 90 min, similar to the duration seen in untreated cells.

Cytokinesis and anaphase lag during recovery

To investigate the reversibility of cytochalasin effects, testes previously treated with cytochalasins were transferred to cytochalasin-free buffer. Following short recovery periods (for example, 30 min), anaphase lagging was still widespread and cytokinesis was still inhibited. Thus, laggards did not "catch up" with non-laggards during recovery, nor did cells that had failed at cytokinesis form cleavage furrows.

To test whether cells that had been in early stages (prophase, prometaphase) during cytochalasin treatment were able to undergo normal anaphase and cytokinesis during recovery, longer recovery experiments were performed. Since the duration of time between prometaphase onset and late anaphase is normally about 2 h, and since that schedule is comparable in cytochalasin-treated cells, cells in anaphase or telophase after more than 2 h of recovery would have been in prometaphase or prophase at the end of cytochalasin exposure. Table 2 shows that frequencies of anaphase lag were restored to normal with long recovery periods (3–4 h) following CD or CB exposure. The effects of CE on chromosome segregation were not reversible, even after 4 h of recovery. Cytokinesis was restored to the cell population with long periods of washing following CD and some CB treatments, but again, not following washing periods of up to 4 h following CE exposure. Difficulty in reversal of CE effects has also been reported by others (Mookerjee and Jung 1984) and interpreted to result from the strong binding affinity of CE for actin. Evidence for the viability of CE-treated cells comes from our observation in CE-treated preparations of many cells that appeared to be undergoing the second meiotic division (often with a multipolar spindle) after having failed to cleave at the first meiotic division.

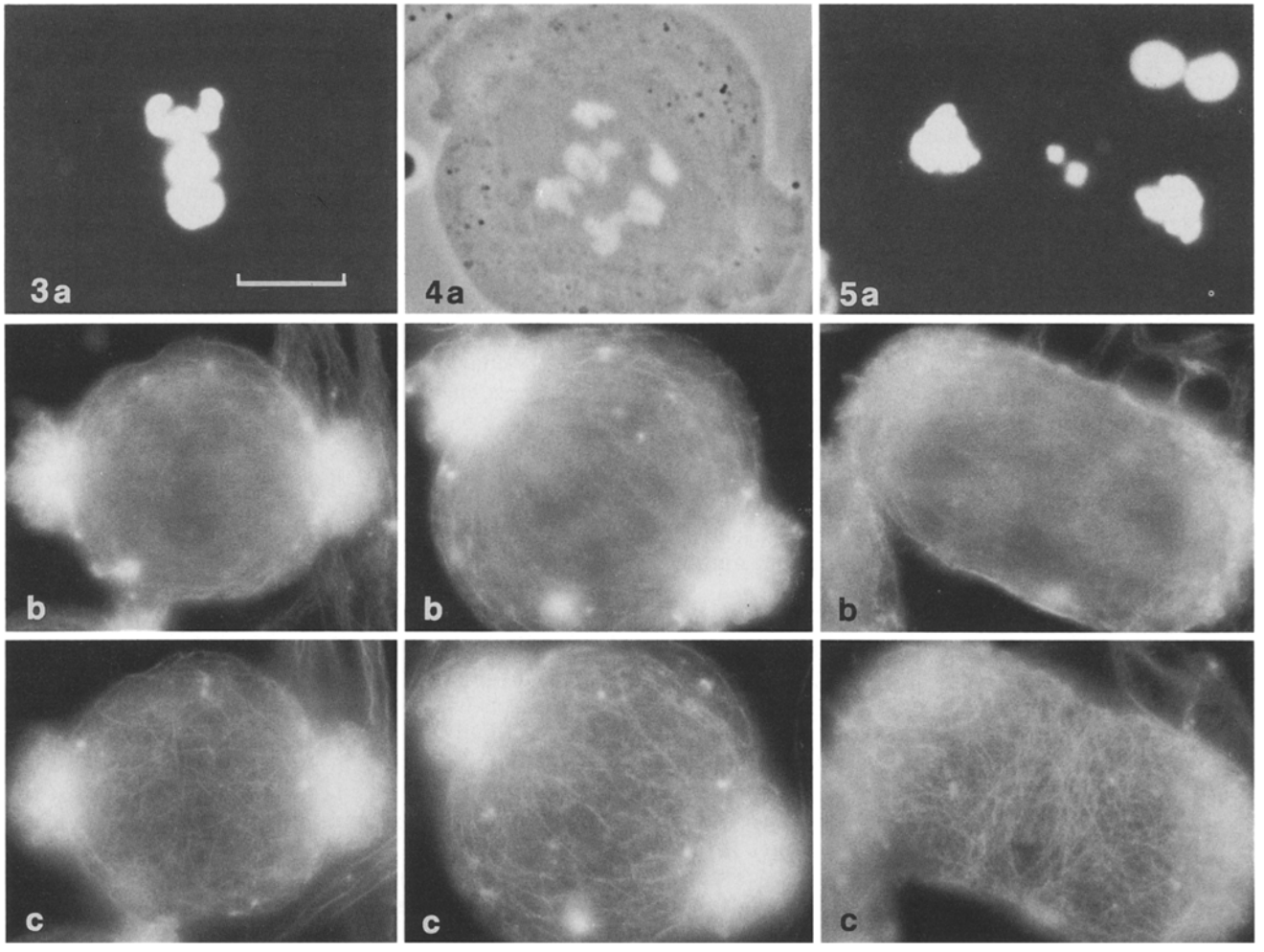
Fluorescence distribution of labeled actin probes

Untreated cells. To assess the distribution of actin filaments in untreated cells for comparison with that of cytochalasin-treated cells, we stained cells with fluorescently labeled phalloidin, which binds to F-actin polymers but not to monomeric G-actin (Wieland 1977; Barak et al. 1981). Results on the distribution of phalloidin fluorescence within different focal planes were best achieved either with cells that were fixed, prior to drying

Table 2. Recovery of crane-fly spermatocytes from cytochalasin treatment

Drug (μM)	Duration of exposure (h)	Cytokinesis[a]		Percentage anaphase lag[b]	
		Cytochalasin treated	After washout (h)	Cytochalasin treated	After washout (h)
CD 100	1	−[c]	+(2)	39[c]	19 (2)
100	2		+(2)		47 (2)
100	2.5	−	+(4)	57	5 (4)
CB 200	1	−[c]	+(2.5)	9[c]	3 (2.5)
200	3.5	−	−(3)	79	9 (3)
CE 20	2.5	−	−(4)	29	94 (4)

[a] Observed (+), inhibited (−)
[b] Percentage of mid-to-late anaphase primary spermatocytes having one or more autosomal laggards
[c] Testis not from the same larva as recovering testis



Figs. 3–5. Untreated spermatocytes fixed in formaldehyde without Triton X-100, stained in perm buffer to avoid air drying, and observed in an oil preparation; cell flattening is minimized and different focal planes are resolved

Fig. 3. a Hoechst staining in the focal plane of the chromosomes seen in **b** with phalloidin fluorescence; **c** cortical meshworks in focus

Fig. 4. a Hoechst staining with transmitted phase contrast of the focal plane of the chromosomes seen in **b** with phalloidin fluorescence; **c** cell cortex in focus

Fig. 5. a Hoechst staining of the focal plane seen in **b**; **c** cell cortex. Bar represents 10 μm

down, in solutions that did not contain detergent or with cells that were not dried down during preparation (Figs. 3–5). Cells prepared in these ways were not as flat as cells that were detergent treated and air dried, and thus it was easier to differentiate between focal planes through the cortex and spindle. Fluorescence staining of untreated spermatocytes probed with either rhodamine-phalloidin or bodipy-phallicidin revealed three major results, irrespective of which probe was used.

1. Numerous filaments are labeled within a cortical meshwork. Filaments are deployed without any apparent pattern of alignment up to metaphase (Fig. 3). In a number of early anaphase cells, filaments oriented parallel to the spindle axis predominate (Fig. 4), then at telophase, a group of filaments perpendicular to the spindle axis forms a contractile ring subjacent to the cleavage furrow (Fig. 5).
2. At metaphase and anaphase, an additional structure, resembling a pompon, is formed at each spindle pole. Pompons are not detectable at diakinesis and they become dispersed and ultimately disappear at telophase.
3. Phalloidin fluorescence at the focal planes of the spindle and chromosomes is generally amorphous with uniform intensity. The only exception observed (not shown) is that, in non-dried cells, very faintly fluorescent filaments were occasionally seen oriented parallel to the mitochondria that ensheath the spindle. So far, in untreated cells, we have not found evidence for concentrated fluorescence that might correspond to either spindle fibers or kinetochores.
4. When rhodamine-phalloidin or biodipy-phallicidin was used subsequent to pre-incubation of cells with unlabeled phalloidin, no phalloidin fluorescence was seen.

In addition to phalloidin, we employed three other actin probes to confirm our results (data not shown): (1) fluorescently labeled HMM, which stains F-actin, as demonstrated by Sanger (1975); (2) DNase I, which binds to both G-actin and F-actin (Hitchcock et al.

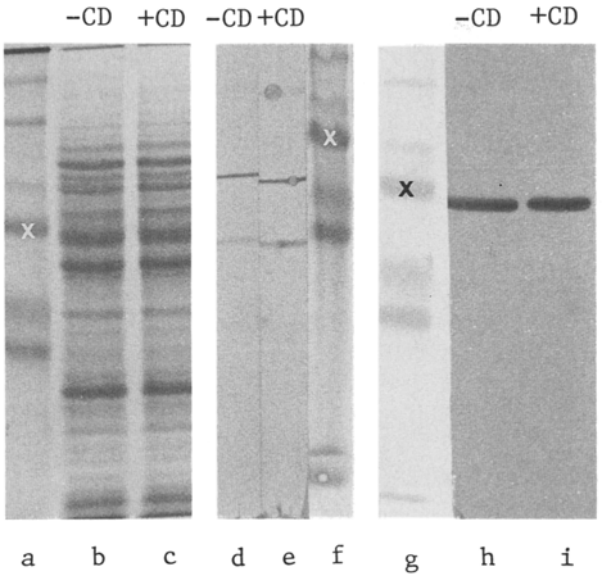


Fig. 6. Protein blot analysis of crane-fly spermatocyte homogenates (untreated cells and cells treated with 100 μM CD for 2 h). *a–c* Coomassie-stained gels: *a* molecular weight standards for *b* and *c*: 26, 36, 48, 58, 84, 116, and 180 kDa; the *letter x* indicates the 48 kDa standard; the 84 kDa band is very faint; *b* homogenates from untreated crane-fly testes, and *c* homogenates from CD-treated crane-fly testes. *d* and *e* Blots of crane-fly spermatocyte homogenates probed with anti-actin antibody N. 350: *d* untreated cells, *e* CD-treated cells. The apparent difference in mobility of the actin bands in *lanes d* and *e* is due only to a tilting of the bands. The distance migrated on the left-hand side of *lane d* corresponds to that on the left-hand side of the band in *lane e*, as do the right sides of the bands in each case. *f* Molecular weight standards for *d* and *e*: same standards as in *a*; 48 kDa band marked with an *x*. *g* Molecular weight standards for *h* and *i*: same standards as in *a*; 48 kDa band marked with an *x*. *h* and *i* Blots of crane-fly spermatocyte homogenate probed with anti-tubulin: *h* untreated cells, *i* CD-treated cells

1976); and (3) Amersham monoclonal anti-actin antibody N. 350, which has been used by a number of investigators as a probe for actin in aldehyde-fixed cells (Trass 1987; Tang et al. 1989). N. 350 antibody detected three bands on protein blots (Fig. 6) of crane-fly spermatocyte homogenates (Fig. 6d): (1) a band at about Mr 42,000, presumed to be actin; (2) a band at about Mr 23,000; and (3) another at about Mr 64,000. Protein blots probed using monoclonal antibody to alpha-tubulin (Sigma product T-9026) and the same secondary antibody used with N. 350 revealed only one band (Fig. 6h), indicating that additional bands detected with N. 350 were not due to non-specific staining by the secondary antibody. It is not uncommon for N. 350 to detect additional bands at higher and lower molecular weights than actin in protein blots (reviewed by Tang et al. 1989). The identity of the additional bands is not known.

In agreement with results obtained with phalloidin, these other actin probes applied to untreated cells (data not shown) revealed (1) evidence for fluorescence intensity at poles resembling polar pompons and (2) in cells in advanced stages of cytokinesis, a cortical ring having increased fluorescence over the background staining of the cytoplasm. Again, no evidence was obtained for concentrated fluorescence within the spindle, even with long incubations and high concentrations of these actin probes (DNase at a 1/50 dilution for 40 min, HMM at 2,000 μg/ml for 5 h).

The results with these probes differed from those obtained with phalloidin in that the discrete cortical filaments seen with phalloidin could not be detected. This difference may be explained by the relatively intense staining of other forms of actin within the cytoplasm by N. 350 anti-actin, DNase I, and HMM. Double labeling with both anti-actin and phalloidin or both DNase I and phalloidin permitted resolution of cortical filaments with phalloidin, even though those structures were not resolved in the same cell with N. 350 anti-actin or with DNase I.

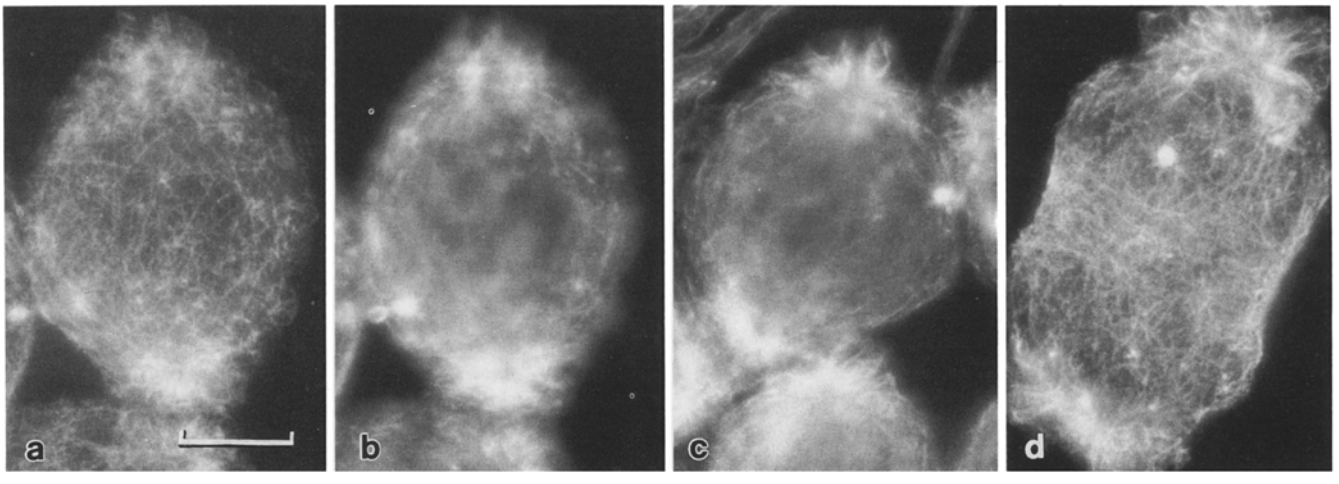


Fig. 7a–d. Spermatocytes that were incubated in 1% DMSO in Tricine buffer (**a–c**) or in Tricine buffer alone (**d**) for 2.5 h prior to fixation and staining with rhodamine-phalloidin. **a** and **b** A metaphase cell at two focal planes: **a** cortical meshwork in focus, **b** focal plane of chromosomes. Compare these frames with Fig. 3b and c, the same planes of an untreated metaphase cell. **c** An anaphase cell at focal plane of chromosomes; **d** late anaphase at focal plane of cortical filaments. Bar represents 10 μm

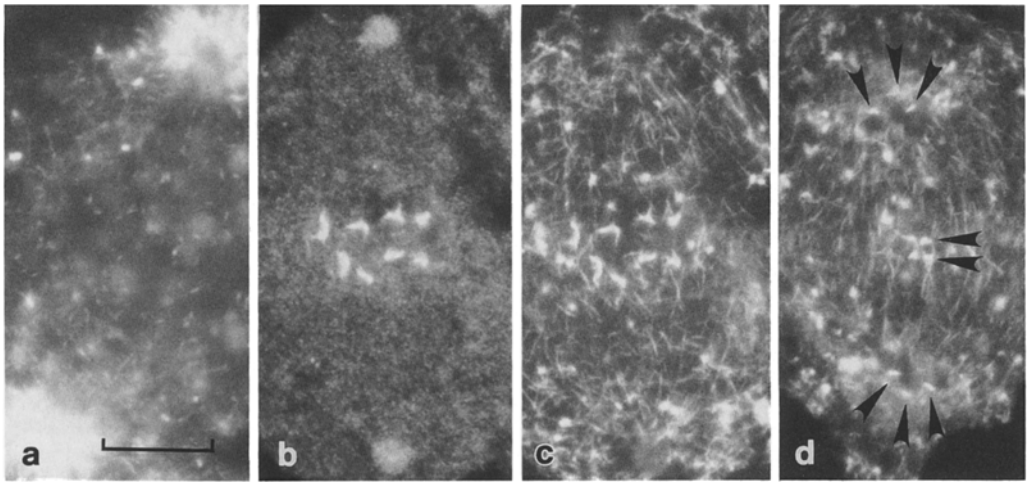


Fig. 8a–d. Double-stained cells showing phalloidin and CREST fluorescence following CD exposure. **a** After 90 s of exposure to 100 μM CD, focal plane of chromosomes has phalloidin-staining specks and filaments in the region of the spindle. **b** CREST image of the same cell as **c**. **c** and **d** Phalloidin images after 5 min in 100 μM CD at **c** metaphase (focal plane of chromosomes) and **d** late anaphase (chromosomes). In **d**, *arrowheads* indicate centromere/kinetochore regions (CKR) revealed by CREST staining (not shown). Bar represent 10 μm

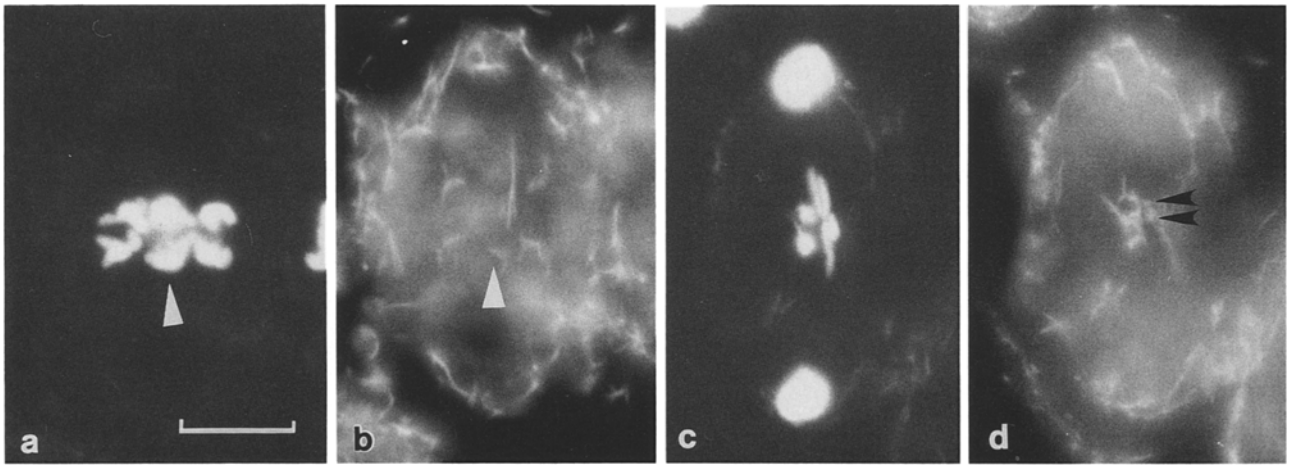


Fig. 9a–d. Early and late anaphase cells after exposure to 100 μM CD for 2 h. Cells were stained with rhodamine-phalloidin in perm buffer and not air dried before covering with oil. **a** Hoechst staining of the focal plane seen in **b**; **b** kinetochore regions contain broad, spiked fluorescence and strands of fluorescence are in the spindle region. *Arrowheads* locate corresponding points in the two images. **c** Hoechst and **d** phalloidin staining; *arrowheads* locate fluorescence at CKRs of the lagging autosome. Bar represents 10 μm

DMSO control cells. Results from cells incubated in 1% DMSO (the vehicle used to dissolve cytochalasins in the following experiments) prior to phalloidin staining were similar to those obtained from untreated cells except that, in some cases, polar pompons in DMSO-treated cells did not appear to be as tightly organized around their central core as in untreated cells at the same stage (Fig. 7).

CD-treated cells. The following results were obtained from cells exposed to 100 μM CD, then either air dried (Fig. 8) or stained without air drying (Fig. 9).

a. Within 1–3 min, phalloidin is concentrated in fluorescent aggregates located in the vicinities of the centromeres and/or kinetochores (Fig. 8c; for comparison of a cell treated for a longer time with an untreated cell after the same fixation, see Figs. 9b and 4b). Since this fluorescence has not yet been localized ultrastructurally to a particular structure but rather to a region of the spindle, we hence forth use the acronym *CKR* when referring to results on fluorescence within this *centromere/kinetochore region.* The initial pattern of phalloidin fluorescence at CKRs resembles that which we have seen for kinetochores in untreated cells by electron microscopy. After just 5 min of exposure, these actin-containing structures are broader than kinetochores normally are and they are often spiked (Fig. 8c). However, as will be described later, this pattern of fluorescence is closely matched by that of CREST immunofluorescence after CD treatment (Fig. 8b).

CD-induced staining of CKRs by phalloidin was observed in cells from prometaphase through telophase,

including cells that must have begun anaphase prior to CD treatment based on our observation of living cells (Fig. 8d), and in all of the chromosomes of the complement – segregating half-bivalents as well as laggards (Fig. 9d). Intensity and extent of phalloidin fluorescence at CKRs appeared to diminish during anaphase (Fig. 8d), although in some instances, accurate assessment of phalloidin changes during anaphase was complicated by the presence of additional actin-containing structures in either the spindle and/or cortical regions of CD-treated cells.

b. Phalloidin staining of polar pompons disperses within 5 min of exposure to 100 μM CD (Fig. 8c; also, compare Figs. 9b and 4b).

c. After 5 min in 100 μM CD, the spindle contains many short, thin, phalloidin-stained filaments aligned parallel to the spindle axis, with some in contact with fluorescence at CKRs (Fig. 8c and d). With exposures of 1–3 h (Fig. 9), spindle regions contain phalloidin fluorescence in the form of longer and thicker strands, some of which appear to be attached to CKRs.

d. The cortical meshwork of filaments begins to change within 5 min of exposure. The contractile ring does not appear in cells that progress into telophase during CD treatment, and in cells that presumably had a contractile ring when CD treatment began, contractile ring filaments begin to become disordered. With longer exposures, cells at stages when cytokinesis should be occurring contain several thick fibers oriented parallel to the plane of cell elongation. In the place of the cortical meshwork found before CD treatment are fewer, thicker fibers and star-shaped structures composed of short filaments radiating from particulate foci.

To confirm that these CD-induced changes in phalloidin staining reflected changes in the distribution of actin, anti-actin monoclonal antibody N. 350 and DNase I were also used to probe CD-treated cells (data not shown). In CD-treated cells, these probes had patterns of distribution similar to those seen after phalloidin staining, with the exception that with DNase I, the region of the centrosome sometimes appeared slightly fluorescent. Background cytoplasmic staining, which was pronounced with anti-actin and DNase I in untreated cells, was substantially reduced following CD treatment. Blots of proteins derived from CD-treated cells and probed with monoclonal anti-actin antibody N. 350 gave results qualitatively similar to those obtained from untreated material (Fig. 6).

Loss of the effect of CD on phalloidin fluorescence requires at least 1 h. When cells were exposed to 100 μM CD for 2 h then washed with CD-free medium, fluorescence at CKRs was evident after 30 min of washing, but at 60 min, it was either absent or too faint to be documented. Instead of large cortical filaments seen after 15 min of recovery, the cortex at 30 min contained numerous short filaments and particulate fluorescence; filamentous meshworks resembling those in controls had formed by 60 min. Structures resembling polar pompons had also formed by 60 min of recovery, and cells after 2–4 h of washing appeared indistinguishable from untreated controls.

Immunofluorescence with CREST serum

Untreated cells. Others (e.g., Brenner et al. 1981) have shown that CREST serum stains the centromeric region of chromosomes in many mammalian cell types by immunofluorescence. Following immunofluorescence with CREST serum (from patient EK) on untreated crane-fly spermatocytes, the cytoplasm appeared weakly fluorescent, the centrosomal regions at the two spindle poles had fluorescence intensity greater than background, and intense fluorescence was seen in the regions of centromeres/kinetochores (Fig. 10). As discussed for phalloidin staining, we have not localized CREST staining ultrastructurally to a specific structure at the centromere/kinetochore region, so we refer again to this simply as staining at the CKR.

In contrast to mammalian cells, crane-fly spermatocyte CKRs were also stained by normal human sera, although not as intensely as by EK serum. Secondary antibody alone did not stain the cells. Blotting of spermatocyte proteins (Fig. 11) revealed several protein bands that reacted with CREST (EK) serum. The bands at about Mr 56,000, 53,000 and 23,000 remained prominent in blots using a 1:800 dilution of EK. We attempted to affinity purify EK antibodies from these bands to be used for immunofluorescence but were unable to detect any fluorescence with eluted antibodies. In interpreting these immunoblots, one should bear in mind that our material is not well suited for these biochemical approaches from two standpoints. First, the quantities of protein available for electrophoresis are limited: each manually dissected testis contains 3 μg or less of total protein. Second, the content of each testis is variable, containing spermatocytes in various stages of meiosis, plus a variable proportion of spermatids.

Based on the above results, we cannot state which one (or more) of the bands in immunoblots binds antibodies that localize at CKRs and which contributes to centrosomal or background fluorescence following immunofluorescence with EK serum. Nor can we state whether any of the CREST-reactive proteins in crane-fly spermatocytes correspond to the antigens stained by this serum in mammalian cells (Earnshaw and Rothfield 1985; Kingwell and Rattner 1987). Nevertheless, the intensity of staining at CKRs by EK serum makes the serum a useful probe.

In numerous cases, CREST immunofluorescence of autosomes at metaphase and anaphase resolved two fluorescent masses (each measuring about 0.5 μm across by about 0.2 μm thick) at the poleward edge of each half-bivalent of a bivalent, in the vicinities of the two sister centromeres (and the two sister kinetochores). In other cases, this doubleness in CREST staining at the region of sister centromeres/kinetochores was not resolved, probably because they overlapped along the optical axis.

The morphology of CREST-positive structures at CKRs of autosomes was irregular at prometaphase (Fig. 10b), when the CREST-positive structures were often elongated toward a pole. CREST staining of sex chromosomes revealed a fluorescent band alongside each

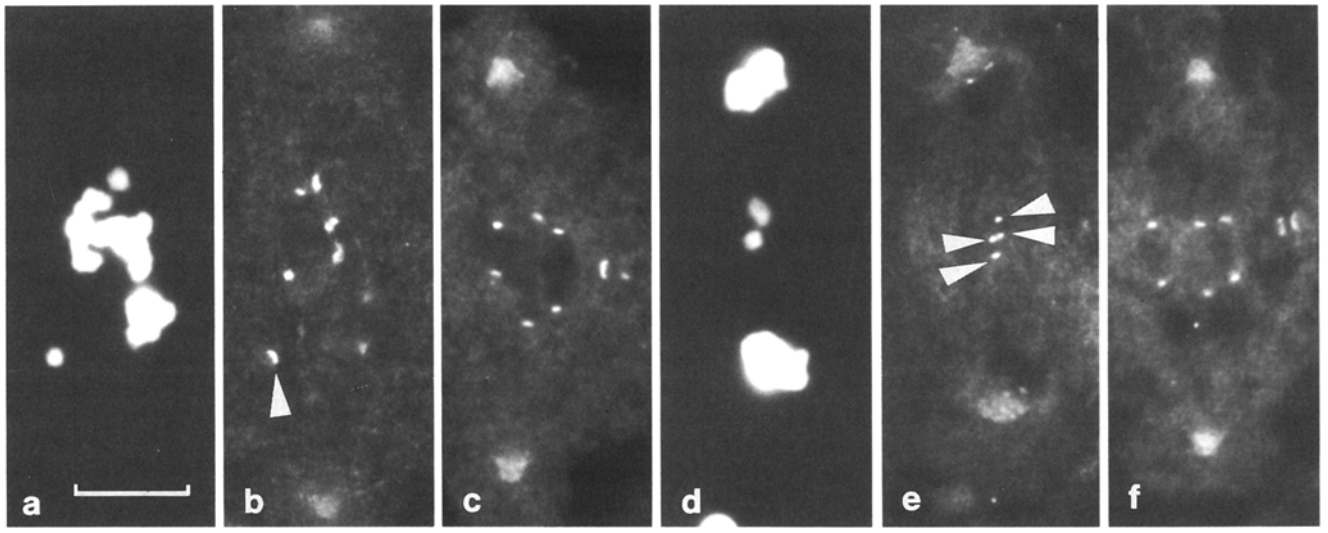


Fig. 10a–f. CREST immunofluorescence of untreated crane-fly spermatocytes: **a** Hoechst staining and **b** CREST staining of a prometaphase cell. *Arrowhead* locates the kinetochore band of a sex univalent. **c** CREST staining of a late prometaphase cell; **d** Hoechst and **e** CREST staining of a late anaphase cell; *left-pointing arrowheads* locate the two distinctly resolvable sister kinetochores of a sex univalent; *right-pointing arrowheads* locate the sister kinetochores of the other univalent; **f** CREST staining of a DMSO-treated control cell at metaphase. Bar represents 10 μm

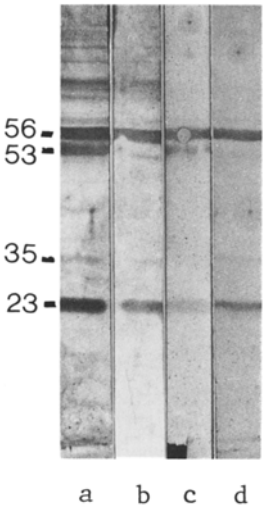


Fig. 11. Protein blot analysis of crane-fly spermatocyte homogenates probed with CREST (EK) serum: *a*, *b*, *c* blots of untreated cells (-CD) using EK serum at dilutions of 1/200, 1/500, and 1/800 respectively; *d* blot of cells treated with 100 μM CD for 2 h using EK serum at a dilution of 1/800. Bands at 56, 53, 35, and 23 kDa are labeled

univalent at prometaphase (Fig. 10b) that differentiated by early anaphase into two distinctly resolvable structures oriented toward opposite poles. These patterns of CREST staining at CKRs are consistent with those seen ultrastructurally for kinetochores in crane-fly spermatocytes (Müller 1972; Fuge 1974), both in the case of autosomes, which become oriented syntelically, and in the case of sex chromosomes, which become oriented amphitelically by anaphase onset.

CREST fluorescence intensity at CKRs of autosomes diminished during autosomal anaphase, while the CKRs of the sex univalents (which normally lag at anaphase in these cells) retained a high level of fluorescence in the interzone (Fig. 10e); the latter diminished as the sex chromosomes segregated during cytokinesis.

DMSO control cells. Results from cells incubated in 1% DMSO prior to CREST immunofluorescence (Fig. 10f) were similar to those obtained from untreated cells.

CD-treated cells. After 5 min incubation of cells with 100 μM CD, CREST fluorescence at CKRs appears somewhat broader than normal and sometimes has spikes extending toward the poles (Fig. 8b). With longer exposures (30 min to 1 h), the CREST-stained region at the vicinity of kinetochores may be 3–4 times broader than normal (Figs. 12, 13a), with obvious spikes extending toward the poles. Sex univalents often appear to be encased in CREST-positive material (Fig. 13a). After such exposures, numerous strands of CREST-positive material are also observed extending considerable distances from kinetochores (Figs. 12, 13c and d), some possibly being unattached to chromosomes.

Double labeling with CREST serum and phalloidin (Figs. 8b and c, 13) shows that once phalloidin appears at CKRs during CD treatment, the pattern of CREST fluorescence there closely matches the pattern of phalloidin staining. Regarding the strands of material extending considerable distances from CKRs or unassociated with them, some label with both phalloidin and CREST (including some strands that appear unattached to kinetochores, for example, the strand indicated with double arrowheads in Fig. 13c, d), while others label with phalloidin, but not with CREST (for example, the strand indicated with a single arrowhead in Fig. 13d).

CD-induced changes in CREST staining at CKRs are found in laggards (Fig. 12b) as well as non-laggards.

As anaphase progresses, the intensely fluorescent, broad structures seen at CKRs at metaphase and early anaphase become less broad, less spiked, and less fluorescent (Fig. 13c), suggesting that material contributing to their structure at early anaphase is lost during anaphase or becomes less accessible to antibody.

CD treatment had no ostensible effect on the diffuse CREST fluorescence of cytoplasm seen in untreated cells or on the presence of CREST-positive material at the centrosomal regions, although subtle alterations are possible.

CD-induced effects on CREST fluorescence disappear from the cell population upon incubation in CD-free buffer. Following treatment for 2.5 h in 100 μM CD and 20 min in CD-free medium, strands disappear and the broadened, spiked fluorescence at kinetochore regions is less evident. With 30 min of recovery, CREST fluorescence appears similar to that in untreated cells at comparable stages. For lagging autosomes, the two CREST-staining regions on each half-bivalent are separated and directed toward opposite poles, similar to the CREST-staining regions on amphitelically oriented lagging sex univalents (Fig. 14).

Figure 11d shows an EK serum immunoblot of spermatocyte homogenate prepared from cells that had been treated with 100 μM CD for 2 h before SDS denaturation. The two bands at Mr 56,000 and 23,000 detected in untreated material with 1/800 EK are prominent, and faint bands are detectable at MR 53,000 and at about 35,000, where bands were also resolved with the 1/500

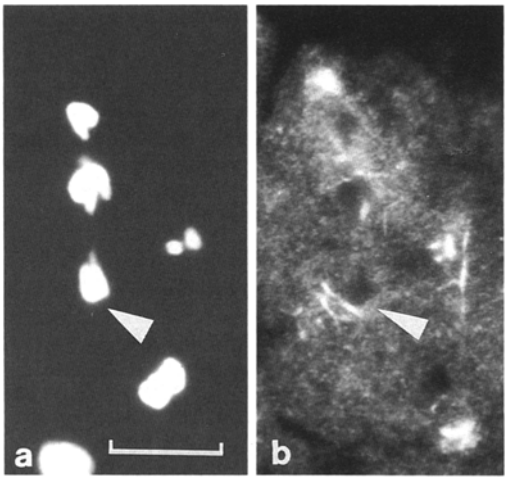


Fig. 12a, b. CREST immunofluorescence of crane-fly spermatocytes after exposure to CD for 2.5 h. *Arrowheads* locate an autosomal laggard shown stained with Hoechst in **a** and a CREST-staining strand associated with it in **b**. Bar represents 10 μm

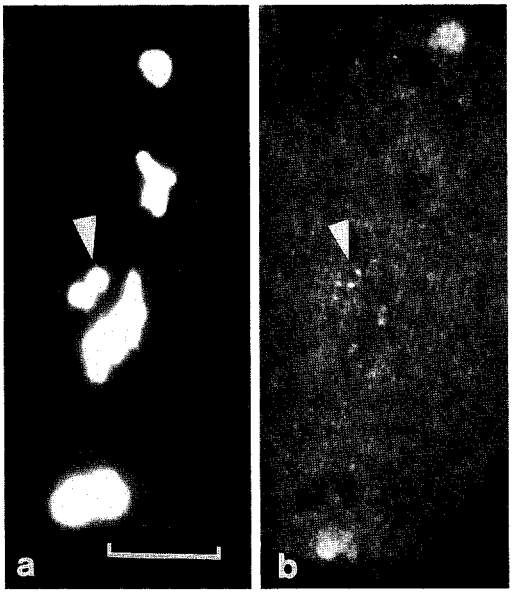


Fig. 14a, b. Recovery of normal CREST fluorescence following CD reversal. Testes were exposed to 100 μM CD for 2.5 h then washed for 30 min. *Arrowheads* locate corresponding points in the Hoechst (**a**) and CREST (**b**) images. Bar represents 10 μm

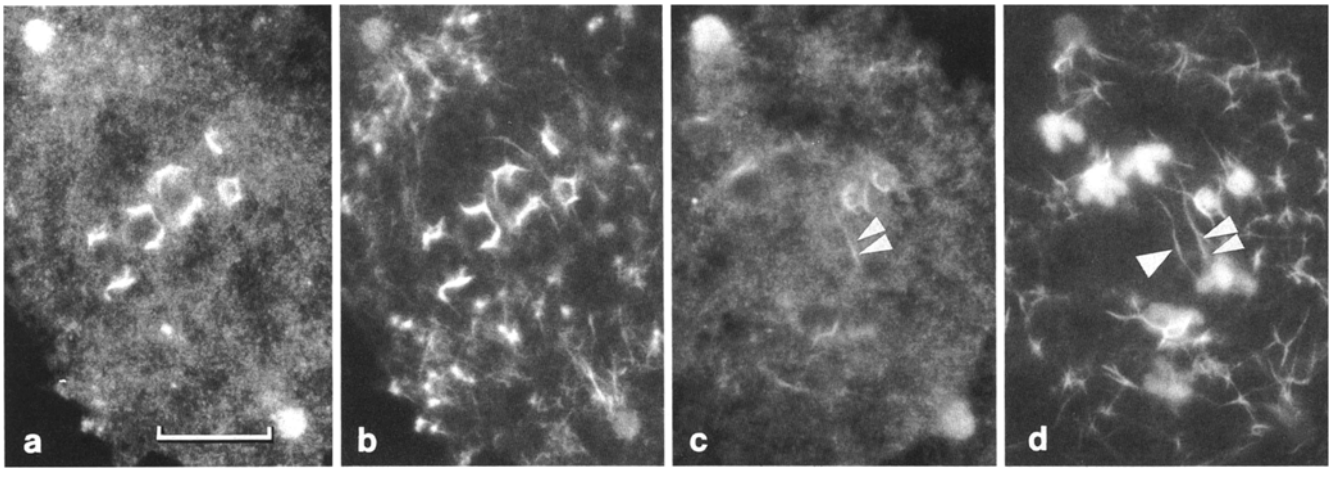


Fig. 13a–d. Double labeling of CD-treated cells with rhodamine-labeled CREST serum (**a** and **c**) and bodipy-labeled phallacidin (**b** and **d**); **a**, **b** 30 min CD exposure; **c**, **d** 1 h CD exposure. Chromosomes were labeled with Hoechst, the fluorescence of which is sometimes visible with the filter system used for bodipy fluorescence. *Double arrowheads* in **d** locate an actin strand that is CREST positive in **c**. *Single arrowhead* in **d** locates a strand that is not CREST positive in **c**. Bar represents 10 μm

dilution of EK applied to untreated material. Thus, it appears that changes in CREST staining of kinetochores do not involve the addition of new CREST-reactive antigens to the protein composition of CD-treated spermatocytes.

Phalloidin and CREST staining of CB-treated cells

After incubation of spermatocytes in 200 μM CB for 2–2.5 h, anomalous segregation of chromosomes is common, but that treatment has no detectable effect on the distribution of CREST fluorescence. Broadening and spiking of CREST staining at kinetochore regions were not seen after treatment for 2.5 h in 200 μM CB or after even 4.5 h exposures to 200 μM CB (Fig. 15). Background fluorescence of the cytoplasm and centrosomal fluorescence were observed after CB treatment, as they were in untreated cells.

After a 2 h exposure to 200 μM CB, most of the phalloidin fluorescence is contained within structures located in subpolar regions (just equatorial of the poles) (Fig. 16). Polar pompons are no longer present. The limited fluorescence in other regions includes both filaments and particles, neither of which is comparable in size or intensity of fluorescence to those seen in CD-treated cells. Phalloidin fluorescence is detectable at CKRs when chromosomes are near the equator (metaphase, early anaphase, lagging chromosomes) (Fig. 16). Clear evidence for phalloidin at CKRs of half-bivalents that have reached the pole is lacking, probably in part because it becomes obscured by fluorescent structures near the poles; perhaps there is also a diminution in phalloidin fluorescence at CKRs as anaphase progresses. As was found with CD, phalloidin fluorescence is found at both laggard and non-laggard kinetochore regions. Phalloidin fluorescence at CKRs of laggards (Fig. 16d) is in two discrete spots separated along the pole-to-pole axis, as would be expected for kinetochores of chromosomes with amphitelic orientation (Janicke and LaFountain 1984).

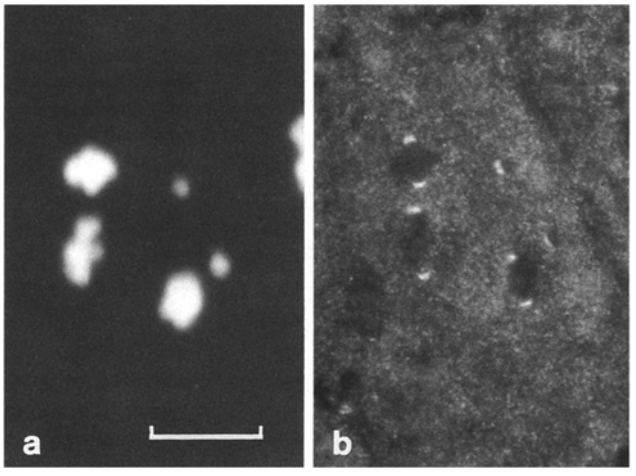


Fig. 15a, b. Prometaphase in a cell from a testis exposed to 200 μM CB for 4.5 h: **a** Hoechst, **b** CREST staining. Bar represents 10 μm

In recovery experiments, we found that the effects of CB at 200 μM are not as rapidly lost as the effects of CD at 100 μM, in that polar pompons had not yet appeared after 2 h following transfer to CB-free buffer. Cortical meshworks recovered within 1.5 to 2 h. Up until about 2 h following transfer, the fluorescence of short filaments and particles in focal planes of chromosomes confounded the unambiguous resolution of fluorescence at CKRs, at which time no concentrated fluorescence at CKRs was seen.

Phalloidin and CREST staining of CE-treated cells

Treatment with 20 μM CE for 2 h caused redistribution of actin as detected by phalloidin staining (Fig. 17) comparable to that found only with higher concentrations of CD (100 μM for 2 h). Polar pompons were absent, star-shaped structures with particulate foci were found instead of a cortical meshwork, and broad and some-

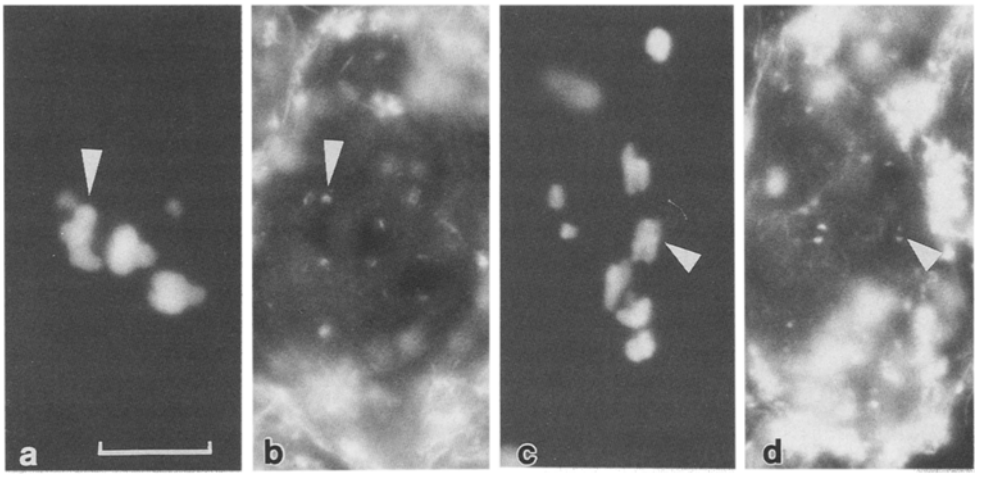


Fig. 16a–d. Hoechst (**a**) and phalloidin (**b**) fluorescence of a prometaphase cell after 2 h in 200 μM CB. Hoechst (**c**) and phalloidin fluorescence (**d**) of an anaphase cell after 4.5 h in 200 μM CB. *Arrowheads* locate corresponding points in the pairs of images. Bar represents 10 μm

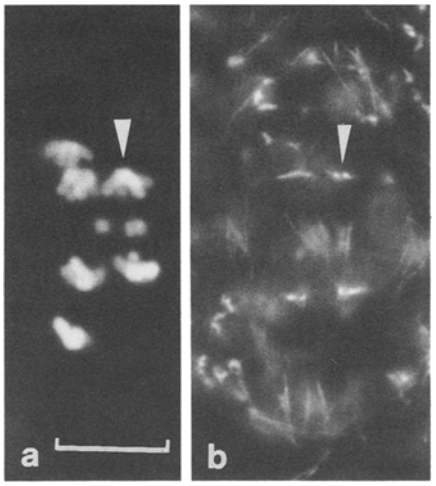


Fig. 17a, b. Hoechst (**a**) and phalloidin (**b**) staining at early anaphase after 2 h treatment in 20 μM CE. *Arrowheads* locate corresponding points in the pair of images. Bar represents 10 μm

times spiked phalloidin-staining and CREST-staining aggregates (data not shown) were detected at CKRs. Extraneous strands and specks of CREST-staining material not associated with chromosomes were also found in the spindle. Background CREST fluorescence of cytoplasm and of centrosomal regions was found in CE-treated cells, as in untreated cells.

After recovery periods as long as 3 h, the effects of 20 μM CE (1–2 h) on actin distribution and CREST fluorescence were irreversible (data not shown). This parallels the irreversibility of CE inhibition of cytokinesis and CE induction of anaphase lag and attests to the high potency of CE in causing cytochalasin effects.

Phalloidin staining after low-dose cytochalasin treatment

We examined the dose-response relationship between CD and effects on phalloidin staining. After 3.5 h in 20 μM CD (the lowest dose found to induce anaphase lag), phalloidin stainability of CKRs had been induced, as had loss of phalloidin-stained pompons from the poles. Filaments were present in the spindle, some extending from kinetochores in the direction of poles. After 3.5 h in 2 μM CD (which does not induce anaphase lag), phalloidin staining was detected at only a few kinetochores, and that staining was faint; phalloidin at the poles was altered: many of the filaments normally present there in untreated cells had been lost and in their place numerous phalloidin-stained particles were found. Cells treated with 0.2 μM CD appeared as untreated cells: polar pompons were present and only diffuse background staining was found in the spindle region.

Thus, for 3.5 h exposures, changes in phalloidin staining at the poles and CKRs were detected in concentrations of CD that were lower than those required to induce anaphase lag. Consistent with this result, we found that both CB and CE caused disruption of phalloidin-staining pompons at the poles and the appearance of phalloidin-staining filaments in the spindle at concentrations lower than that required to induce anaphase lag.

Discussion

Three major findings are described in this paper. (1) CB, CD, and CE at concentrations in excess of that required to inhibit cytokinesis induce anaphase lag in crane-fly spermatocytes. (2) These treatments result in changes in actin-containing structures, including the loss of actin filaments from the poles and their appearance in non-polar regions of the spindle and in CKRs, as revealed by three actin probes. (3) The treatments with CD and CE also induce alterations of CREST staining at CKRs

Incubation of spermatocytes for 3.5 h in 2 μM CD or CB, or for 2.5 h in 2 μM CE, produced results similar to those obtained by others on dividing cells: inhibition of cytokinesis with no apparent effect on karyokinesis (Carter 1967). Induction of abnormal anaphase with 3.5 h of exposure was seen with 2 μM CE, 20 μM CD and 70 μM CB. The greater potency of CE and lesser potency of CB compared with the potency of CD in inducing anaphase lag is in accord with the known heirarchical order of cytochalasin effects on cellular actin (Lin and Lin 1979; Howard et al. 1981).

The finding that induction of segregation anomalies requires higher concentrations of cytochalasins than does inhibition of cytokinesis may be related to the different effects exerted by cytochalasins on cellular actin at these concentrations. For 3.5 h exposures, we found that between 2 μM CD, the concentration at which cytokinesis is inhibited but anaphase lag is not induced, and 20 μM CD, polar pompons change from slightly disrupted to highly disrupted, specks in the spindle are replaced by filaments in the spindle, and phalloidin staining of CKRs changes from faint and occasional to pronounced and consistent. Staining of CKRs, loss of polar pompons, and the appearance of actin filaments in the spindle are changes that were always seen after treatments that induced anaphase lag and that began to be observed at lower concentrations and shorter exposure times than induction of anaphase lag, consistent with a cause and effect relationship.

The mechanism underlying the change in actin distribution induced by cytochalasins is not clear. One possibility is that kinetochores or structures near the kinetochores that bind CREST serum also bind cytochalasins, which in turn bind actin. If so, the CREST-binding antigens at the centrosome would have to be different from CREST-binding antigens at the kinetochores, since CREST-positive centrosomes do not stain with phalloidin following CD treatment.

Another possibility is that the actin-containing structures induced at kinetochore regions and in non-polar regions of the spindle by cytochalasin treatment result from transport of actin filaments from the poles into the region of spindle fibers and ultimately to the kinetochores. In a recent report of CD effects on root cells of *Allium*, Palevitz (1988) described the CD-induced for-

mation of actin-containing specks (foci) at stages when rhodamine-phalloidin staining normally appeared amorphous. In cells with spindles, specks accumulated at the spindle poles, with spindle microtubules (MTs) appearing to guide and/or propel actin toward the poles. Polar clustering of specks was shown to be energy dependent. Palevitz's results are consistent with known transport properties of the spindle: acentric bodies (Bajer and Molé-Bajer 1972; Nicklas and Koch 1972) have been shown to migrate parallel to spindle MTs from equator to pole. In accord with Palevitz's results and those of others who have studied CD effects (Weber et al. 1976; Schliwa 1982), the CD-induced structures that we observed in spermatocytes included specks (both particles and foci of star-shaped structures) as well as short filaments and long strands. These were found both in the cortex and in the spindle. In contrast to results from *Allium*, structures seen at poles in untreated crane-fly spermatocytes (polar pompons) disappear during CD treatment, and we see accumulation of CD-induced structures at CKRs. The possibility exists that the small filaments that we have seen in the spindle region after treatment for 5 to 10 min in 100 μM CD could be actin filaments that originate at the poles and, subsequent to their fragmentation, are transported through the spindle to kinetochores, where they form broad, spiked aggregates. This interpretation is based on evidence that actin filaments may be fragmented by CD (reviewed by Cooper 1987) as well as on the assumption that the alignment of filaments and strands parallel to spindle MTs is indicative of possible interaction between actin and MTs (Griffith and Pollard 1978). Thus, our results may be providing evidence for transport within the spindle following a pathway from pole (MT minus ends) to kinetochore (MT plus ends). We do not know why transport of actin in crane-fly spermatocytes treated with CD might occur with a directionality opposite to that of actin in *Allium*. Nor do we know the relationship between this postulated mode of actin redistribution and the polar exclusion forces that have been observed in other cells (Rieder et al. 1986; see also Bajer and Molé-Bajer 1972).

On the other hand, actin may normally be a component of the kinetochores and/or nonpolar regions of the spindle. In the absence of cytochalasins, non-polar spindle regions and kinetochores may normally contain actin but (1) it may not be sufficiently concentrated for our methods of detection, (2) it may not be preserved in filamentous form by our methods of fixation [the deleterious effects of aldehyde fixation on actin have been documented (Lehrer 1981)], (3) it may in some way be obscured or inaccessible to actin probes, or (4) it may be present and functional in a non-filamentous or oligomeric state. With anti-actin antibody N. 350 and with DNase I, probes that recognize G-actin as well as F-actin, background fluorescence in non-polar regions of the spindle region was intense in untreated cells but diminished substantially as actin-containing structures appeared in these regions following cytochalasin treatment. This is consistent with the possibility that high doses of cytochalasins may render actin filaments more readily visible through promotion of the aggregation of fine filaments into larger ones and/or through the promotion of G-actin assembly, evidence for which has also been found in vitro (Löw and Dancker 1976; Brown and Spudich 1979).

Evidence for actin in isolated mammalian kinetochores has been presented previously (Valdivia and Brinkley 1984), and there is also in situ evidence for the concentration of actin at kinetochores in other animal cells (Sanger 1975). Reports of actin concentrated within untreated spindles of plant and animal cells have come from several laboratories (Seagull et al. 1987; Traas et al. 1987; Sanger et al. 1989). These previous results have been open to two criticisms: (1) actin detected in the spindle or at kinetochores could have resulted from cytoplasmic contamination during preparative procedures, and (2) the presence of actin in the spindle, even if real, may not be significant, based on experimental evidence against the involvement of myosin in chromosome transport (Kiehart et al. 1982; DeLozanne and Spudich 1987).

Our finding that cytochalasin-induced redistribution of actin correlates with abnormal anaphase raises numerous questions about the possible involvement of actin in producing the observed anaphase anomalies, as well as the involvement of actin in chromosome segregation in untreated cells. If actin is not normally located at the kinetochores and/or in non-polar regions of the spindle, its appearance there may be cytochalasin dependent and result in segregation problems. Induction of lagging by cytochalasin does not seem to be due to interference by actin in anaphase movement of kinetochores. Non-laggards as well as laggards have actin at CKRs after cytochalasin treatment. Moreover, if induction of lagging were due simply to an interference by actin in anaphase movement, one would expect to see laggards within minutes after exposure to cytochalasin, since actin is seen at CKRs of anaphase cells within 3 min of exposure to CD. In actuality, laggards are not seen until after about 1 h of cytochalasin exposure, which is substantially longer than the 20 min required for autosomes to segregate to the poles during anaphase in these cells. The possibility remains that the observed lagging of chromosomes at anaphase results from an accumulation of actin at kinetochores affecting a key process at an earlier stage, such as prometaphase.

Alternatively, the induction of segregation anomalies by cytochalasins may occur through perturbation of the state of the actin that normally participates in the mechanism of chromosome segregation. If indeed actin does play a role in normal chromosome segregation, perhaps it functions properly only when it is in a dynamic, non-aggregated state at the kinetochore and/or in its native state at the poles. Our treatments do not cause all chromosome movements to cease, but rather, cause lagging of one or more autosomal half-bivalents at anaphase. Thus, actin may be functioning not in the motor for chromosome movement but in the control of chromosome segregation. Whether or not this role depends on myosin awaits further investigation.

We have previously shown induction of anaphase lag

by cold (Janicke and LaFountain 1984) and MT inhibitor (LaFountain 1985; Ladrach and LaFountain 1986) treatments that induce bipolar malorientation of bivalents: sister kinetochores having kinetochore MTs extending toward both poles instead of to the same pole during the first meiotic division. Bivalents with bipolar malorientations in those studies displayed "tilted" configurations (Janicke and LaFountain 1986) similar to those seen in numerous CD-treated cells in this study. Although we have initiated ultrastructural analysis of kinetochore-MT associations in CD-treated cells, an understanding of the extent of amphitely (sisters oriented to opposite poles) and/or merotely (individual kinetochores oriented to both poles) within cytochalasin-treated bivalents will not be achieved quickly, since the structure of kinetochore regions after CD treatment is more complicated than after either cold or Colcemid induction of malorientation. Nevertheless, we do have preliminary evidence for sister kinetochores of laggards connected to MT bundles extending toward opposite poles. Further evidence that malorientation exists in laggards is our observation of CREST-stained, CD-recovering cells having laggard half-bivalents with two CREST-positive structures separated along the pole-to-pole axis (as if sister kinetochores were subject to forces directed toward opposite poles).

In untreated cells, centromere maturation occurs to separate kinetochores of sister chromatids in preparation for the second meiotic division, during which they should orient to opposite poles (Janicke and LaFountain 1989). During previous treatments (cold and Colcemid) that induced bipolar malorientation, the schedule of meiosis was delayed such that sister centromeres in bivalents matured from a first meiotic division arrangement (sister centromeres together) to a second meiotic division arrangement (sister centromeres separated) before bivalents had achieved their metaphase I orientation. Based on our observations of living cells, the duration from end of prophase to onset of anaphase in CD-treated cells is similar to that seen in untreated cells. Thus, if malorientation is the cause of anaphase lag induced by CD, its induction does not appear to involve delays in the schedule of meiosis.

Normally, proper orientation is achieved by metaphase. Thus, if lagging is due to inability to achieve proper orientation, we would have predicted that, as observed, cells that had already reached metaphase when exposed to cytochalasin would have displayed normal segregation at anaphase. When CD is washed out, phalloidin staining at kinetochores is lost after perhaps 60 min, yet it takes at least an additional 1.5 h for frequencies of anaphase lag to begin to return to normal. Thus, prometaphase centromeres that become maloriented during cytochalasin exposure may remain malooriented through anaphase, even if cytochalasin is removed.

Anomalies associated with spindle poles have appeared under the experimental conditions (Colcemid and cold) that we have used previously to induce bipolar malorientation (LaFountain 1985; Janicke and LaFountain 1986). With CD, there is a striking alteration in the organization of actin at the spindle poles. Polar pompons are dispersed, and in living cells, the region of the polar centrosome appears broader than normal. The achievement of proper orientation may depend directly or indirectly on the organization of actin within the polar pompons, The extent to which the structure of polar regions of spermatocytes is associated with either the induction of anaphase lag or the mechanism of chromosome orientation is a question that remains open to investigation.

After treatment with 100 μM CD, CREST staining revealed fluorescent structures at CKRs that were broader than normal and had spikes extending into the spindle. In addition, strands staining with CREST serum were found after CD treatment, both associated with chromosomes and in the spindle region, apparently not associated with chromosomes. Assuming sufficient resolution capacity of our immunoblots, we can conclude that these differences in distribution of material staining with CREST serum did not result from addition of a new CD-induced protein to these cells. Other possible models that could explain these changes are: (1) CD may induce dispersion into the spindle area of antigens normally concentrated in centromeres and/or kinetochores, or (2) antigens reacting with CREST serum may normally be located not only in the CKR but also at other sites in the cell, and cytochalasin treatment could cause the condensation of the dispersed antigens onto CKRs and in the spindle fiber region.

Changes in kinetochore morphology are a common feature of prometaphase in untreated cells (Müller 1972; reviewed by Rieder 1982). Altered kinetochore structure has also been induced experimentally (Rieder 1982; Snyder 1985). The possibility that cytochalasin-induced changes in the distribution of CREST staining at CKRs have nothing to do with the structure of kinetochores has not been ruled out. However, the data also do not eliminate the possibility that changes in CREST staining at kinetochore regions reflect cytochalasin-induced changes in kinetochore morphology. If the latter is found to be true, these changes would be particularly intriguing because they occur not only in lagging chromosomes, but also in kinetochores that are actively moving. The finding that the morphology of the CREST-staining material at CKRs is altered within 10 min of CD treatment and restored to normal after 30 min of recovery would be compatible with the concept that kinetochores may be both structurally (reviewed by Rieder 1982) and functionally (Gorbsky et al. 1987; Nicklas 1989) dynamic.

Whereas during cytochalasin treatment, phalloidin staining of CKRs precedes alterations in the distribution of CREST staining, recovery of normal CREST morphology (within 30 min) is accomplished before loss of the phalloidin staining in the vicinities of kinetochores (longer than 30 min). Thus, morphological change in CREST-staining material appears to be a more extreme effect of cytochalasins than is the appearance of actin at CKRs. This is further supported by our observations that (1) phalloidin fluorescence at kinetochore regions is induced by 20 μM CD, but altered CREST fluores-

cence is not, and (2) while all three cytochalasins (CB, CD, and CE) induce stainability of CKRs by phalloidin, altered CREST fluorescence was observed only with CD and CE after the incubation times used in this study and was not observed with CB after incubations as long as 6 h.

Since all three cytochalasins induced anaphase lagging but only CD and CE altered the pattern of CREST staining, the latter clearly was not essential for induction of anaphase lag. Rather, the loss of actin from the poles and appearance at CKRs correlates with induction of anaphase lag. When actin was found at CKRs of cytochalasin-treated cells, a striking correspondence was maintained between its distribution and that of CREST-positive material there, even in cases where the distribution of CREST-staining material at CKRs was altered. The pattern of CREST-positive extraneous strands in the spindle induced by cytochalasin treatment was also matched by fluorescence patterns achieved with actin probes. Since not all actin was CREST positive, and since CREST-positive material at the centrosomes did not stain with phalloidin or N. 350 anti-actin antibody in cytochalasin-treated cells, we conclude that CREST serum was not simply staining actin. Instead, our results point to an intimate association of actin with CREST-positive material at or near the kinetochores. The rapid appearance (within 5 min) of actin at CKRs following cytochalasin treatment could also be interpreted as reflecting specificity in kinetochore-actin interaction.

We conclude that the close association maintained between CKRs and actin during CD treatment, together with the finding that alterations of spindle actin induce anaphase lag, is consistent with the hypothesis that actin has a function in chromosome segregation. An involvement of actin in motive force generation during chromosome movement has been debated for a number of years. Results reported here are consistent with an involvement of actin in chromosome orientation, a new concept that deserves further study.

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