Evidence that Myosin Does Not Contribute to Force Production in Chromosome Movement

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ABSTRACT Antibody against cytoplasmic myosin, when microinjected into actively dividing cells, provides a physiological test for the role of actin and myosin in chromosome movement. Anti-*Asterias* egg myosin, characterized by Mabuchi and Okuno (1977, *J. Cell Biol.*, 74:251), completely and specifically inhibits the actin activated Mg^{++}-ATPase of myosin in vitro and, when microinjected, inhibits cytokinesis in vivo. Here, we demonstrate that microinjected antibody has no observable effect on the rate or extent of anaphase chromosome movements. Neither central spindle elongation nor chromosomal fiber shortening is affected by doses up to eightfold higher than those required to uniformly inhibit cytokinesis in all injected cells. We calculate that such doses are sufficient to completely inhibit myosin ATPase activity in these cells.

Cells injected with buffer alone, with myosin-absorbed antibody, or with nonimmune γ-globulin, proceed normally through both mitosis and cytokinesis. Control γ-globulin, labeled with fluorescein, diffuses to homogeneity throughout the cytoplasm in 2–4 min and remains uniformly distributed. Antibody is not excluded from the spindle region. Prometaphase chromosome movements, fertilization, pronuclear migration, and pronuclear fusion are also unaffected by microinjected antimyosin.

These experiments demonstrate that antimyosin blocks the actomyosin interaction thought to be responsible for force production in cytokinesis but has no effect on mitotic or meiotic chromosome motion. They provide direct physiological evidence that myosin is not involved in force production for chromosome movement.

Anaphase chromosome movement in eucaryotes is usually the result of two distinct motions: the chromosomal fibers shorten as the chromosomes move toward the spindle poles, and the central spindle elongates as the poles move apart. These motions, which together insure the appropriate segregation of the daughter chromosomes during cell division, are likely the result of different force-producing mechanisms (4, 5, 19, 44, 47). Because the spindle is labile, its ultrastructure is complex, and the actual force required to move the chromosomes is small (42, 54), a comprehensive catalog of the molecules responsible for force production in anaphase movement is not available. As a result, various theories that attempt to explain chromosomal fiber shortening and central spindle elongation have included virtually every known biological mechanochemical transducing system.

Three major theories or combinations of theories are currently in vogue (reviewed in reference 1, 19, 41, 52). Force production by the polymerization and depolymerization of biological polymers (18, 19, 22, 35, 36; and thermodynamically formalized by Hill, 16), remains the simplest model. While it is clear that polymerization and depolymerization of microtubules is a major feature of the structural changes in the mitotic spindle during mitosis, it is difficult to rule out force production by a parallel mechanochemical transducer that requires the integrity of the spindle fibers. Further, while force production as a result of polymerization of actin or tubulin (for pushing) is widely accepted (see reference 16), the depolymerization of polymers such as microtubules (for pulling) has not been readily embraced as a viable mechanochemical transducer.

Other current models incorporate the sliding filament sys-

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tems responsible for force production in muscle or flagella and cilia. They use the well-established mechanochemical transducers that are the ATPase activities associated with myosin or dynein (myosin: see below; dynein 4, 5, 7, 33, 40, 46, 49–51).

Actomyosin systems have been implicated in chromosome movement as a result of studies which report that actin and myosin are at least present and perhaps concentrated in the mitotic spindle. (actin: see reference 2 for a comprehensive bibliography, myosin: 10, 11, 43). These studies use fluorescent and ultrastructural localization techniques that, at best, reveal location, not function of contractile proteins in the spindle.

Mabuchi and Okuno (34) established that antibodies could be useful as probes for myosin function in living cells. They prepared and characterized a γ-globulin fraction that contained antibodies against cytoplasmic myosin isolated from starfish eggs. In vitro they found that this antimyosin inhibited the actin-activated ATPase of purified egg myosin. In vivo, they found that microinjected antimyosin could block cytokinesis, a process that almost surely requires an actomyosin system for force production (for review, see references 10, 53). They also found that doses of antimyosin sufficient to completely inhibit cytokinesis generally did not block nuclear division. However, in some antimyosin-injected cells they observed that mitosis did not occur and that in others daughter nuclei reformed abnormally close together. Their exciting observations suggested that perhaps antimyosin was inhibiting spindle assembly or one mode of chromosome movement, either chromosomal fiber shortening or central spindle elongation. Chloral hydrate (47), low doses of colchicine (44), and erythro-9-3-(2-hydroxynonyl) adenine (5), for example, can each block central spindle elongation without affecting chromosomal fiber shortening. Further evidence suggests that two distinct processes are responsible for anaphase chromosome movement in permeabilized cell models: central spindle elongation, but not chromosomal fiber shortening, depends on the presence of ATP (4).

We sought to examine directly the effects of antimyosin on chromosome movement. Our experiments demonstrate that chromosomal fiber shortening and central spindle elongation proceed normally even in cells injected with doses of antimyosin eight times that sufficient to completely inhibit cytokinesis. They provide strong evidence against force production by myosin in chromosome movement.

Abbreviated accounts of this work appear elsewhere (19–21, 28).

MATERIALS AND METHODS

Cells

Asterias forbesi were collected between May and July from waters near Woods Hole, MA. Sperm and oocytes were collected and prepared for use as described elsewhere (12). After spawning, oocytes spontaneously proceed through two meiotic maturation divisions, and could be fertilized after germinal vesicle breakdown. *Asterias forbesi* oocytes, eggs, and embryos could be injected at any stage after germinal vesicle breakdown and are particularly resistant to damage by microinjection (9).

Culture Medium

Eggs were immobilized in a chamber that allowed normal embryogenesis during high resolution observation before, during, and after microinjection (26, 27). The artificial sea water (8) used to fill the injection chamber was made up with 20% heavy water (D_2O) to improve visibility of the otherwise small and only weakly birefringent meiotic spindles (24). Cells in D_2O-containing sea water had larger, more birefringent spindles but were otherwise indistinguishable from cells in sea water that did not contain D_2O. Fertilized eggs in the injection chamber filled with 20% D_2O-sea water proceeded through meiosis, mitosis, cleavage, and embryogenesis somewhat more slowly than eggs allowed to develop under optimal conditions in H_2O-sea water (15, 25). However, no differences between swimming gastrulae which developed in the presence of 20% D_2O and those grown in its absence were observed.

Throughout these experiments cells were maintained at 15–18°C unless otherwise noted. All glassware, including glass slides, cover slips, and cover slip fragments, was detergent cleaned, and exhaustively rinsed (13).

Microscopy

(a) Before, during, and after injection, cells were routinely observed and photographed with a Nikon Model S microscope modified for polarized light microscopy (26). Micrographs were taken with a Leitz Micro Ibso camera on Kodak Plus X film.

(b) Certain specimens were examined alternately with polarized light and differential interference contrast microscopy in rapid succession to observe spindles, chromosomes, and nuclei. Specimens mounted on glass microscope slides were observed with a series of lenses, identical to those described in reference 48, mounted on a Leitz Ortholux microscope. For reviewing cells in the microinjection chamber, long working distance condensers were required. With the polarized light objectives a Nikon long (8 mm) working distance rectified condenser was used, and for the × 25 and × 40 Leitz Smith T objectives a Leitz UD 20 objective (working N.A. ~0.38) and a Leitz UMK 32 objective were used. For differential interference microscopy a Zeiss interference contrast Wollaston prism (Zeiss catalog number 47 4493), made for use for Zeiss Epiplan Pol 16/0.35 objective, was mounted beneath each long working distance condenser. This simple system, designed in conjunction with Dr. G. W. Ellis (Department of Biology, University of Pennsylvania, Philadelphia, PA), allowed high extinction, high resolution differential interference microscopy despite the long optical path lengths of cell preparations in the injection chamber. Illumination for this microscope was as described previously (48). A Leitz Orthomat camera was used to take 35-mm photographs on Kodak Plus-X or Panatomic-X film.

(c) Cells injected with fluorescein-conjugated γ-globulin were observed with a Zeiss Standard microscope equipped with a Zeiss IV F1 Epifluorescence system, a Leitz UMK 32 objective (working N.A., 0.40) and an HBO 50 mercury arc lamp. Standard Zeiss filters for epifluorescence observation of fluorescein were used. A 6-V incandescent lamp, in conjunction with a defocused Zeiss bright-field condenser (nominal N.A., 0.32), was used for bright-field observation. Photographs were taken with a Leitz Micro-Ibso system on Kodak Tri-X film.

Final magnification for all micrographs were calibrated by photographing a stage micrometer. All films were processed with Microdol X developer.

Spindle Stabilization

Because we were unable to see or photograph chromosomes routinely during meiosis or mitosis (see reference 14), we stabilized the spindles of individual injected cells in a new spindle isolation medium (100 mM K^+ ~EDTA pH 7.0, containing 1.0% Triton X-100). The isolation medium preserved spindle birefringence and morphology but lysed the cell membrane and removed light-scattering, cytoplasmic inclusions that had surrounded the spindle and obscured the chromosomes. To stabilize spindles, individual cells in mitosis were removed from the injection chamber with a large-bore micropipette. Next, while being observed with a dissecting microscope, each cell (in ~10 μl of artificial sea water) was rapidly plunged into 2–3 ml of isolation medium. In this medium a Triton-insoluble layer at the periphery of the egg remained to encapsulate the stabilized spindle and a variety of small particles that also remained after the Triton extraction. This layer proved useful in that the large cell "ghosts" were much easier to find and manipulate than individual isolated spindles. After the cell ghost had been in isolation medium for at least 1 min, it was transferred to a pool of fresh isolation medium on a clean glass microscope slide, a cover slip was applied, and the preparation was sealed. In stabilized spindles, chromosomes had sufficient contrast to be observed with both polarized light and differential interference contrast microscopy. At 19°C, stabilized spindles had a half-time of birefringence decay estimated to be 30–60 min.

γ-globulin Solutions

IMMUNE AND NONIMMUNE γ-GLOBULIN: The immune γ-globulin fraction we use in these experiments contains antibodies against purified *Asterias amurensis* egg myosin and is characterized elsewhere (34). Pertinent to our experiments, Mabuchi and Okuno demonstrated the following: *(a)* The immune γ-globulin formed a single precipitin line in Ouchterlony immunodiffusion tests when reacted against either purified egg myosin or crude egg homogenate; *(b)* it reacted with only the heavy and light chains of egg myosin in immunoelectrophoresis against purified egg myosin or crude egg fractions; *(c)* it blocked the actin activation of the egg myosin ATPase in vitro; and *(d)* the immune γ-globulin blocked cytokinesis in vivo when injected into starfish blastomeres. In contrast, preimmune γ-globulin displayed none of the above characteristics in vitro or in vivo. Here, we provide additional controls by evaluating the effects of microinjection of *(a)* a γ-globulin fraction isolated from a nonimmunized rabbit and *(b)* an immune γ-globulin fraction preabsorbed with purified starfish (*Asterias*

amurensis) egg myosin (34). In concert, these controls demonstrate that the only antigenic determinants in the cytoplasm of *Asterias* eggs with which the immune γ-globulin reacted to inhibit cytokinesis were found on egg myosin. We subsequently refer to this immune γ-globulin fraction as antimyosin. Nonimmune γ-globulin was prepared from a nonimmunized rabbit as described elsewhere (34).

Absorbed antimyosin was made by mixing a small aliquot of antimyosin (0.1 ml of 55 mg/ml) in PBS with 0.2 ml of 2 mg/ml egg myosin in high salt buffer (0.6 M KCl, 10 mM phosphate buffer, pH 7.0). In parallel, buffer without myosin was added to another aliquot of antimyosin to control for loss of antimyosin by other than specific absorption. Each mixture was incubated overnight during dialysis against 100 ml of high salt buffer (to keep the myosin in solution). Egg myosin and myosin-antibody complex were next precipitated by dialysis vs. low salt buffer (100 ml of 0.1 M KCl, 10 mM K^+~PO_4 pH 7.0) followed by pelleting at 20,000 *g* for 10 min. The supernatant was concentrated by ammonium sulfate precipitation and resolubilized by dialysis vs. high salt buffer. The absorption procedure was repeated, the final supernatant was reconcentrated, and finally the absorbed γ-globulin fraction was dialyzed into injection buffer (see below). Ouchterlony analysis verified the presence of antimyosin in the "buffer absorbed" sample and its absence in the myosin absorbed fraction. Microinjection experiments with absorbed antimyosin were compared directly with "buffer absorbed" antimyosin injection experiments performed in parallel.

FLUORESCEIN-LABELED NONIMMUNE γ-GLOBULIN: To evaluate the distribution of antibodies in injected cells we microinjected "nonimmune" antibodies (sheep anti-rabbit γ-globulin, Gibco, Grand Island Biological Co., Grand Island, NY) that had been fluorescein labeled. Fluorescein-labeled γ-globulin was dialyzed against >100 vol of injection buffer at 4°C for two changes at least 6 h apart. Fluorescein fluorescence migrated with the heavy (50,000-dalton) and light (25,000-dalton) chains of γ-globulin on 5% SDS polyacrylamide gels (with mercaptoethanol) using a Tris-glycine buffer system (3).

Microinjection

We microinjected γ-globulin and salt solutions into oocytes and eggs using the mercury pressure-volume transducer technique of Hiramoto (17) that is described in detail elsewhere (26, 27). Capillary reservoirs containing γ-globulin solutions were kept at 2–4°C except for brief periods during which micropipettes were loaded in preparation for microinjection. Along with each volume of aqueous solution, a small volume of a nontoxic vegetable oil (Wesson Oil, Hunt-Wesson Foods, Inc., Fullerton, CA), was injected into each target cell. In the micropipette the oil served as a "cap" that prevented the aqueous solution from mixing with the sea water that bathed the cells. Once injected, the small drop of oil served to distinguish injected cells from uninjected controls. Injected volume was determined as previously described and the dose was calculated as volume times protein concentration (26, 27).

All γ-globulin fractions were prepared for microinjection by dialysis vs. injection buffer (150 mM KCl, 10 mM K^+~PO_4 buffer, pH 7.2, containing 0.05% sodium azide) and were stored at 2–4°C until use.

Protein concentration was determined by optical density (10) or was estimated by the method of Lowry et al. (30) using bovine serum albumin as a standard. Protein concentrations of the various γ-globulin solutions used for microinjection were as follows: antimyosin 55 mg/ml; nonimmune γ-globulin 55 mg/ml, absorbed antimyosin 47 mg/ml, buffer-absorbed γ-globulin, 55 mg/ml; and fluorescein-labeled γ-globulin, 55 mg/ml.

RESULTS

Effects of Antimyosin on Cleavage and Nuclear Division

We confirm and extend Mabuchi and Okuno's (34) documentation of the dose (dose = volume × concentration) dependent effects of microinjected anti-starfish myosin on nuclear division and cytokinesis (Table I, Figs. 1 and 2). >0.6 ng of

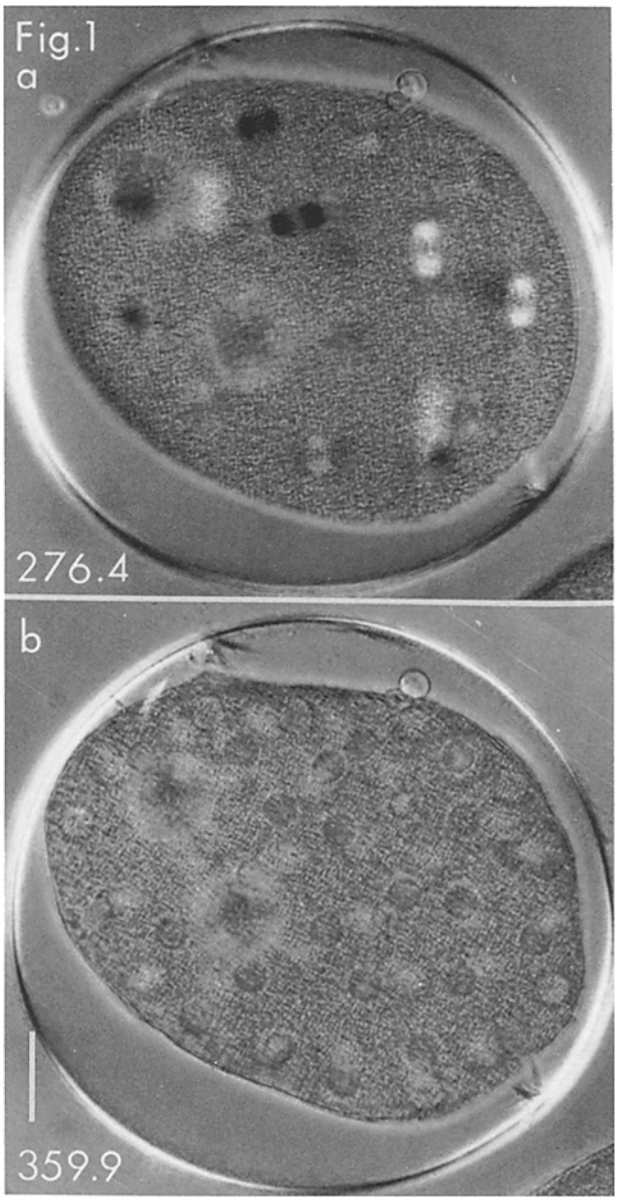

FIGURE 1 Microinjection of antimyosin inhibits cytokinesis but does not affect nuclear division. A fertilized egg was injected during late anaphase of second meiosis with antibody (1.1 ng immune γ-globulin) against egg myosin. (*a*) ~4.5 h after injection, the presence of numerous spindles in a common cytoplasm indicates that mitosis proceeds in the absence of cytokinesis. The large out-of-focus body to the upper left is the drop of Wesson Oil that was injected at the same time as the γ-globulin solution. (*b*) Later (6 h), the same cell in interphase contains numerous nuclei. Times are shown in minutes after injection. Bar, 30 μm.

TABLE I

The Dose-dependent Effect of Injected Antimyosin on Cleavage and Polar Body Function

	Amount of injected protein (ng)					
Effect	0–0.29	0.3–0.59	0.6–0.89	0.9–1.19	1.2–1.49	>1.5
no furrowing	—	—	5 + 5	11 + 2	10 + 4	11 + 21
furrowing, but no successful cytokinesis	—	1 + 0	—	—	—	—
cytokinesis	4 + 6	1 + 0	—	6 + 0 (control)	—	12 + 9 (control)

The numbers on the table indicate the numbers of cells (number of mitotic + number of meiotic) injected with each dose.
The control injections include injection of non-immune γ-globulin, fluorescein-labeled γ-globulin, absorbed immune γ-globulin, or buffer alone.

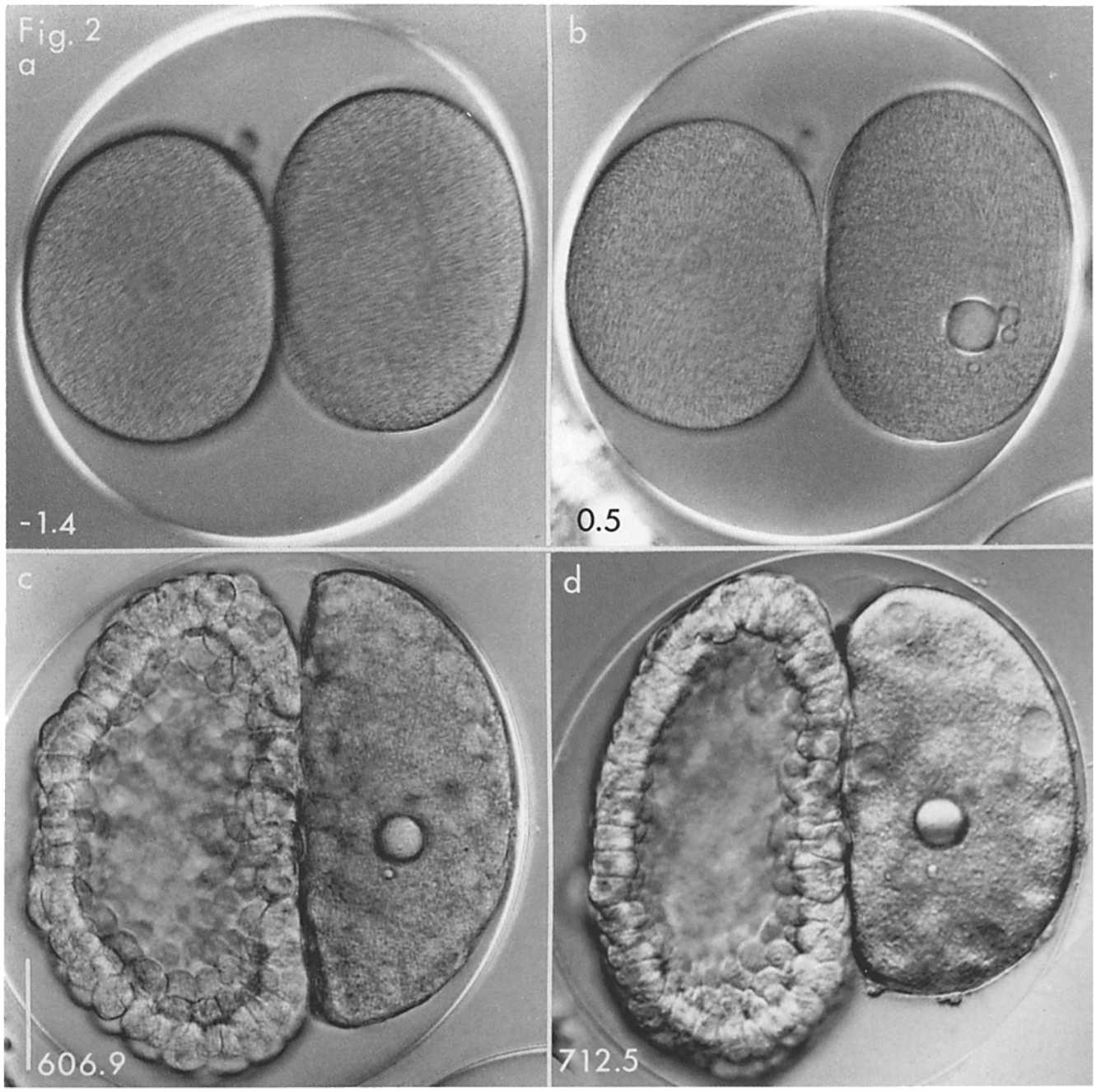

FIGURE 2 Injection of antimyosin at the two-cell stage of development. One blastomere (right, with oil droplet) was injected with antimyosin (0.8 ng immune γ-globulin), the other blastomere served as an uninjected control. (*a*) Cells before injection in early prophase of second mitosis. (*b*) Cell after injection. (*c*) After 10-h development, the control half of the embryo is normal. In the injected cell, cytokinesis is blocked but nuclear division proceeds normally, in approximate synchrony with the controls. (*d*) Nuclei in the injected cell are so numerous that they fill the cell and begin to fuse. Cilia form on both injected and control cells. Times are shown in minutes from time of injection. Bar, 40 μm.

antimyosin permanently blocks cytokinesis in all cells injected before anaphase. Most but not all cells injected with this dose of antimyosin during anaphase complete one cytokinesis, but subsequent cleavages are completely inhibited. We also find that the dose required to inhibit polar body formation (cytokinesis following meiosis) is the same as that required to block cleavage (cytokinesis following mitosis).

Nuclei of cells injected with antimyosin continue to divide (Figs. 1 and 2) in approximate synchrony with uninjected control cells. We confirm that numerous cycles of nuclear division occur in fertilized oocytes and eggs (48 different cells observed) in which furrowing had been completely inhibited by injected antimyosin (0.6–5.0 ng per egg).

Equivalent doses of control γ-globulin solutions or equivalent volumes of buffer alone do not inhibit cytokinesis (Table I, Figs. 3 and 4). All cells injected with <50 pl of control solution (equivalent to ~7% of the egg volume) divide normally. Most cells injected with larger volumes of control solutions (56–84 pl, 8–12% of egg volume) also divide normally, although at first more slowly than uninjected cells. In some eggs injected with larger volumes of control solution before first mitosis, a cleavage furrow is initiated but then regresses.

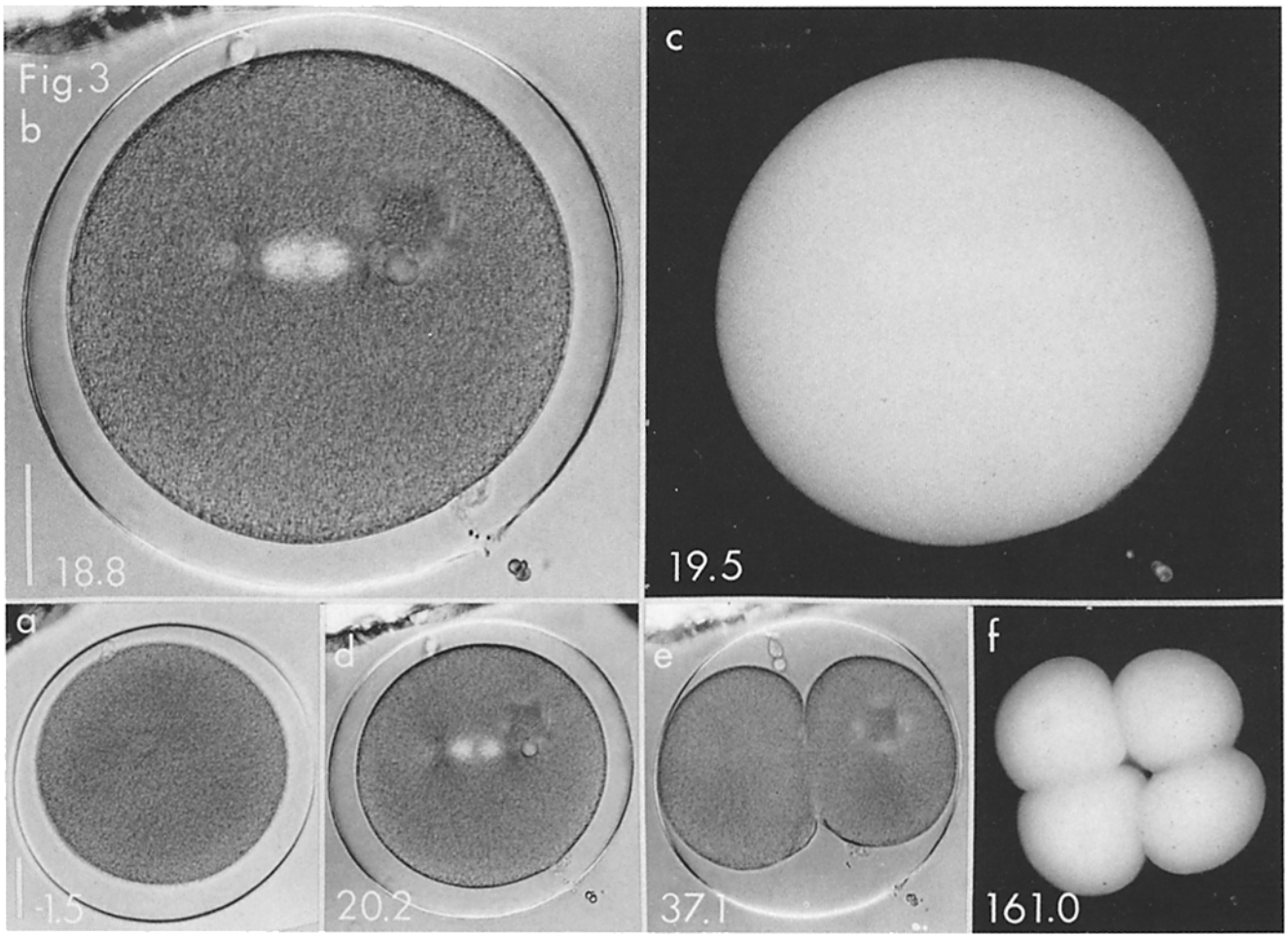

FIGURE 3 Injected fluorescein-labeled control γ-globulin infiltrates the spindle region and does not hinder karyokinesis or cytokinesis. 1.0 ng of fluorescein-labeled control γ-globulin was injected during first mitotic prophase. (*a*) Cell before injection. (*b*) Normal spindle in polarized light after injection (note oil droplet near right spindle pole). (*c*) Fluorescence microscopy of same cell shows a bright spindle region indicating penetration of labeled γ-globulin. (*d*, *e*, and *f*) Anaphase, cleavage, and subsequent development are normal. Note in (*f*) that fluorescein-labeled γ-globulin is excluded by the nuclei formed in its presence. Times are shown in minutes after injection. Bars, 40 μm.

Thereafter, two spindles form in a common cytoplasm and, after second mitosis, two furrows, oriented perpendicular to each other, cleave the single cell into four. All subsequent divisions in such cells are normal.

Spindle birefringence and size are often transiently affected by both injected immune and control γ-globulin solutions. Injected volumes equivalent to >1–2% of the cell's volume cause a transient reduction in spindle birefringence and size (see also reference 34). Both the magnitude and duration of these effects are roughly proportional to injected volume. Volumes of injected buffer or protein solutions >12–15% of the cell's volume (84–105 pl) cause considerable injury to the cell, and recovery does not always occur. Thus the maximum dose of antimyosin we could inject contained ~5 ng of γ-globulins.

Injections of fluorescein-labeled γ-globulin demonstrate that these proteins infiltrate the spindle region (Fig. 3). When injected during interphase, fluorescein-labeled γ-globulins diffuse to homogeneity throughout the cytoplasm within 2–4 min, but are excluded from the nucleus. Even nuclei which form after mitosis in the presence of labeled γ-globulin exclude fluorescence (Fig. 3*f*). In contrast, spindles formed before and after injection with labeled γ-globulin appear more fluorescent than the surrounding cytoplasm (cf. Fig. 3 *b* and *c*), probably as a result of the formed elements that the spindles exclude from their midst (21, 55).

Effects of Antimyosin on Chromosome Movement

The separation of chromosomes during nuclear division may be accomplished by either, or both, shortening of the chromosomal fibers (chromosomes move closer to poles) or elongation of the central spindle (poles move farther apart) (23). It is therefore possible that antimyosin could inhibit only one of these chromosome movement processes without inhibiting nuclear division. Indeed chloral hydrate, colchicine (in low doses), and EHNA cause just such effects (5, 44, 47). Consequently, it is essential to evaluate possible effects of antimyosin on each type of chromosome movement independently.

In healthy *Asterias* oocytes and eggs, chromosomes lack sufficient contrast to be visible in polarized light, phase contrast, or differential interference microscopy. Therefore, in cells injected with antimyosin (up to 8 times the dose required to block cleavage), we analyze chromosomal fiber shortening by (*a*) observing changes in the pattern of spindle birefringence during anaphase, (*b*) looking for formation of daughter nuclei

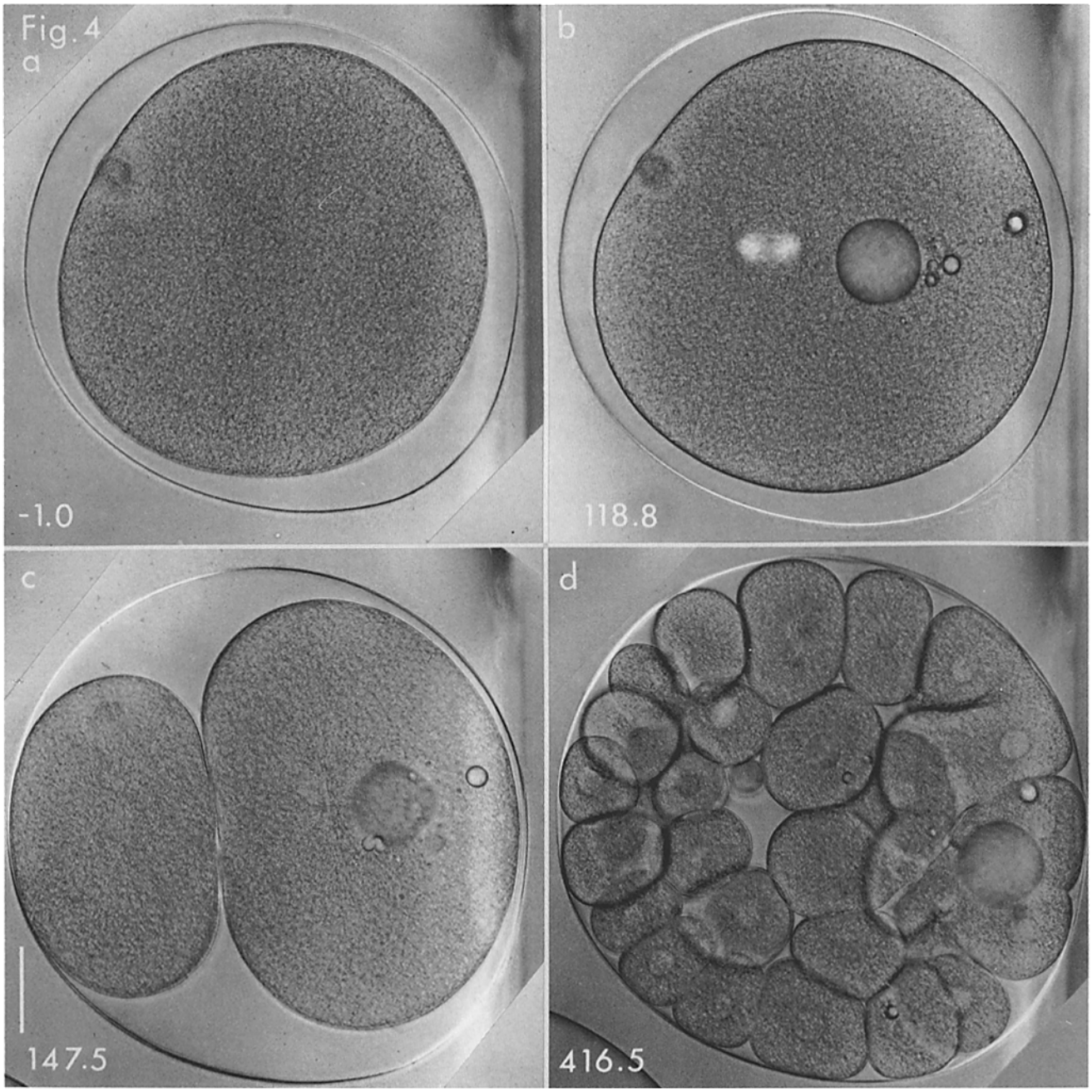

FIGURE 4 Neither cytokinesis nor nuclear division is inhibited by immune γ-globulin preabsorbed with egg myosin. This cell was injected before syngamy with 2.86 ng of absorbed immune γ-globulin. (*a*) Egg before injection. (*b*) A spindle formed in approximate synchrony with uninjected controls but was displaced by the large oil droplet. (*c*) First cleavage occurs normally but asymmetrically as a result of the position of the spindle. (*d*) 6.5 h later, numerous cleavages have occurred. Times are shown in minutes after injection. Bar, 40 μm.

after meiotic anaphase (there is no spindle elongation during normal meiosis in *Asterias forbesi* oocytes, see below), (*c*) documenting the position on the mitotic spindle where daughter nuclei form, and (*d*) observing directly the position of chromosomes in spindles stablized during late anaphase.

Chrosomal Fiber Shortening

SPINDLE BIREFRINGENCE CHANGES DURING ANAPHASE: Spindles in cells injected with up to 4.8 ng of immune γ-globulin undergo birefringence changes characteristic of anaphase in uninjected cells (Figs. 5 and 6). In meiotic anaphase, the birefringence next to the kinetochore and in much of the half-spindle (the region between the kinetochore and the pole) remains high. The birefringence in the interzone region (between separating chromosomes) is considerably lower. As anaphase progresses, both the length and the birefringent retardation of half-spindle decrease as the length of the interzone region of low birefringence increases (Fig. 5). Similarly, in both injected and uninjected mitotic cells the half-spindles are more strongly birefringent than in the interzonal region. As mitotic anaphase progresses the birefringence of the mitotic half-spindles decays and the gap between them grows as both the chromosomal fibers shorten and the spindle elon-

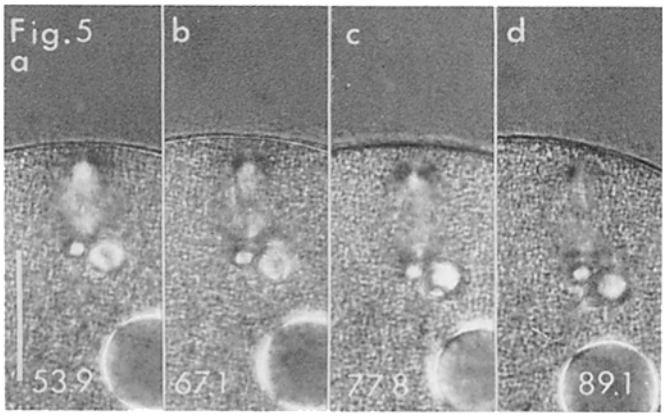

FIGURE 5 Normal meiotic changes of birefringence in an antimyosin-injected oocyte. An unfertilized oocyte, injected during first meiotic metaphase with antimyosin (4.8 ng immune γ-globulin), was observed with polarized light microscopy. (*a*) Metaphase to early anaphase. (*b*) Early anaphase (first meiosis). The birefringence spindle is oriented with one pole next to the cell surface. The large sphere below the spindle is an oil droplet injected at the same time as the antimyosin. The bright spindle fibers in each half-spindle contrast with the reduced birefringence in the interzonal region. (*c* and *d*) Mid and late anaphase, first meiosis. Normal anaphase birefringence changes suggest that chromosome-to-pole movement occurred as in uninjected controls. Meiotic cleavage was suppressed and no polar body formed in the presence of antimyosin. Times are shown in minutes after injection. Bar, 30 μm.

gates (Fig. 6).

FORMATION OF SPATIALLY SEPARATED SECOND MEIOTIC SPINDLES: In oocytes injected during prometaphase or metaphase of first meiosis with 0.8–4.8 ng of antimyosin, two spatially separated second meiotic spindles formed (19 cells observed, Fig. 7). To attribute this nuclear division to shortening of the chromosomal fibers alone, we verified that meiotic spindles to not elongate. In each of six oocytes the length of the spindle was measured from sets of photographs taken at different times throughout first meiotic anaphase. Very little change in the length of each spindle was observed. A least squares analysis was used to draw a regression line on a plot relating spindle length to time during anaphase for each cell. Using Student's *t* test we verified that the slope of each

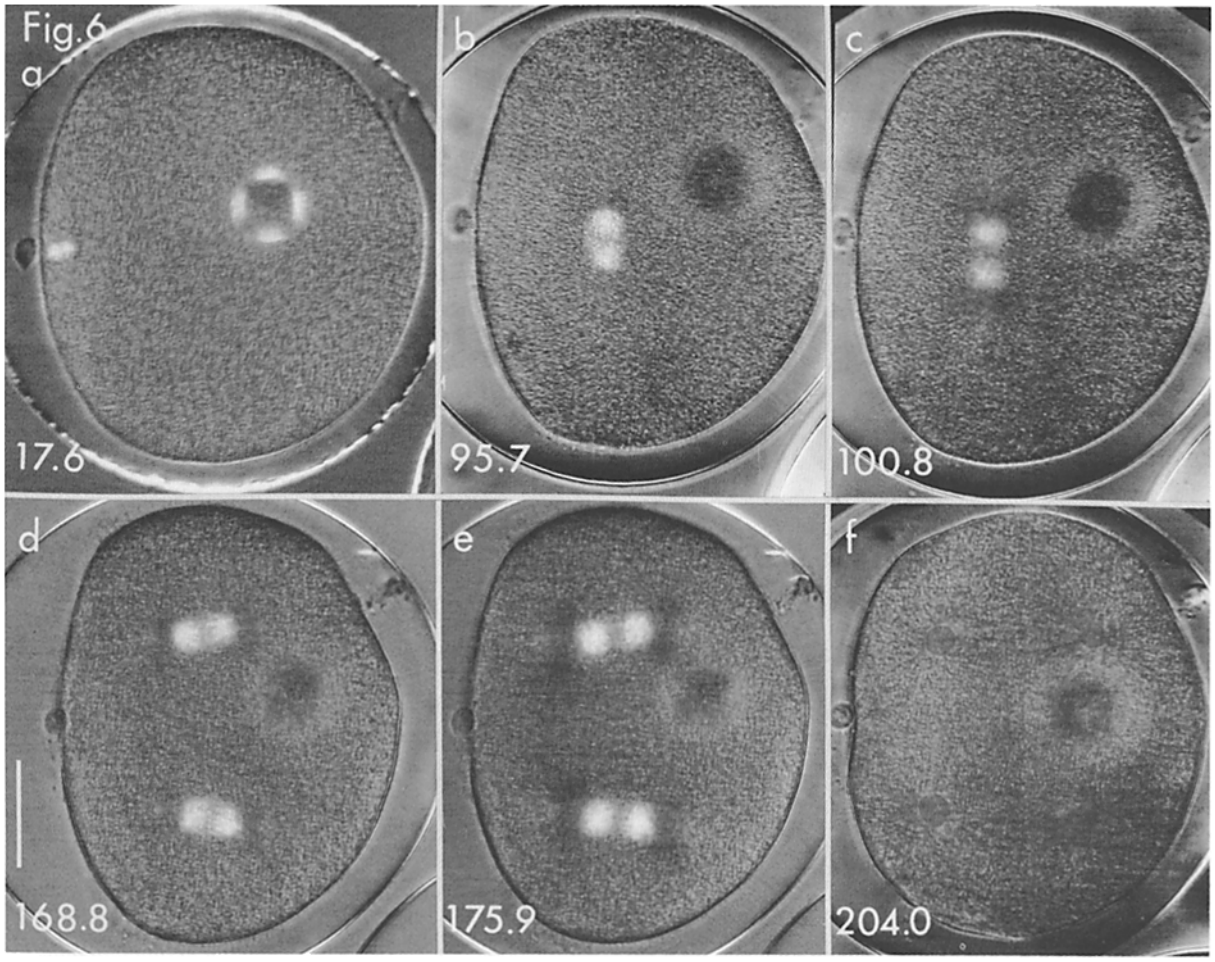

FIGURE 6 Central spindle elongation in the presence of antimyosin. An egg was injected during second meiosis with antimyosin (1.4 ng immune γ-globulin). (*a*) A small, birefringent second meiotic spindle can be seen at the surface of cell next to the first polar body. The large sphere to the right of center is an oil droplet injected at the same time as the antimyosin. (*b*) Later, the same cell in first mitotic metaphase. No second polar body formed. Pronuclear migration and fusion occur as in controls (not shown). (*c*) During anaphase of first mitosis, the interpolar distance increases, in the injected cell as in uninjected controls, by ~12.5%. (*d*) No cleavage occurs after first mitosis. Daughter nuclei move apart and second mitotic spindles form. (*e*) Central spindle elongation during anaphase again occurs normally. (*f*) Second cleavage is also blocked and four nuclei form in a common cytoplasm. Times are shown in minutes after injection. Bar, 40 μm.

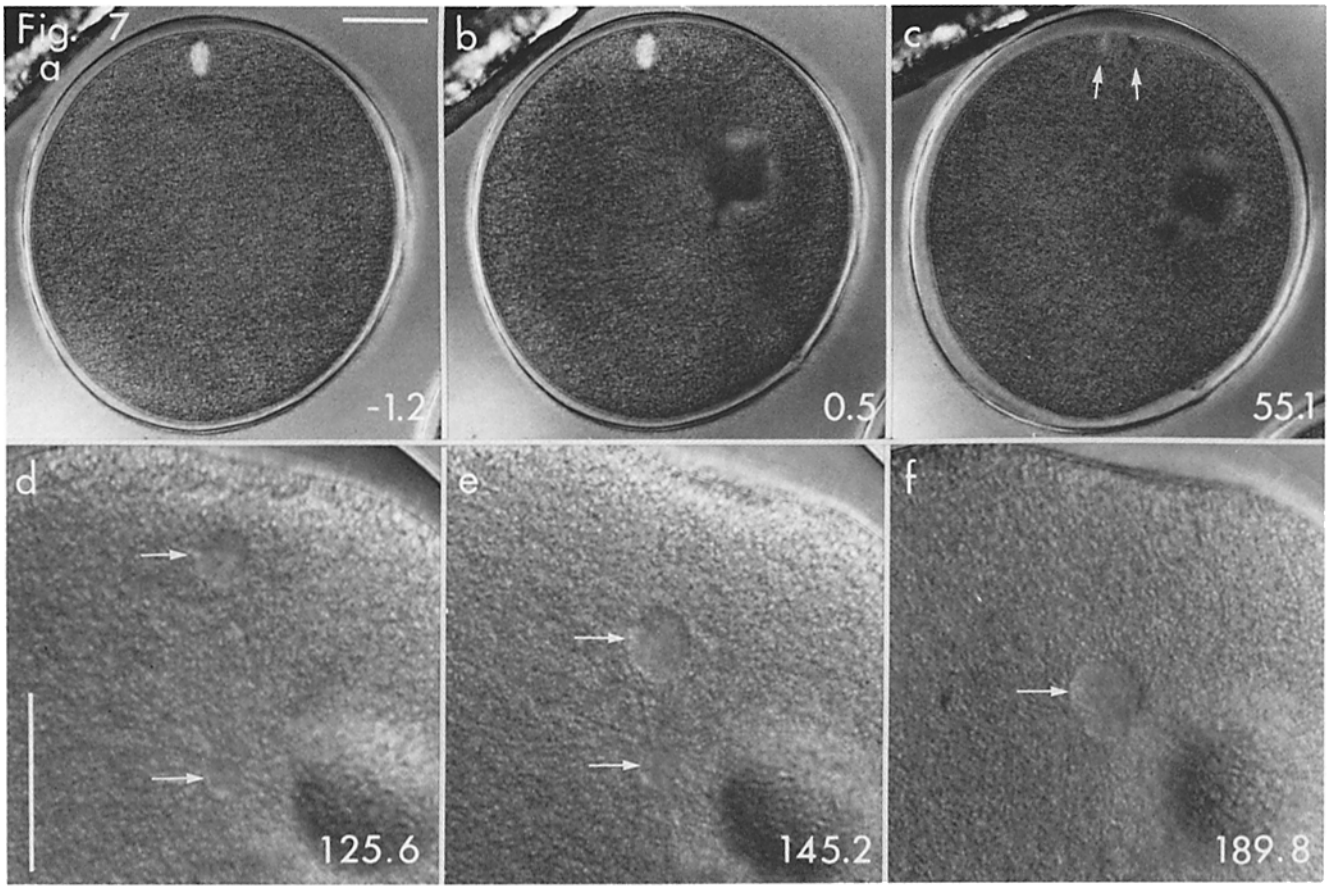

FIGURE 7 Poleward movement of chromosomes and pronuclear migration in an antimyosin-injected egg. This cell was injected during first meiotic metaphase with antimyosin (4.1 ng immune γ-globulin). Upper frames, polarized light microscopy. Lower frames, differential interference contrast microscopy. Temperature, 11°C. (*a*) Cell before injection. The birefringent meiotic spindle is next to the surface at the top of the cell. (*b*) An oil droplet, injected at same time as the antimyosin solution, marks the site of injection. (*c*) Two spatially separated second meiotic spindles are seen (arrows) in a common cytoplasm (no polar body formed). (*d*, *e*, and *f*) In the presence of enough antimyosin to block polar body formation, pronuclear migration and fusion occurs normally (arrows indicate pronuclei). Times are shown in minutes from time of injection. Bars, 40 μm.

regression line was not significantly different than zero within the 95% confidence interval.

DAUGHTER NUCLEI FORMATION ON MITOTIC SPINDLES: In oocytes each injected with 4.2 ng of antimyosin before or during first meiotic anaphase, we followed first mitosis, through the formation of daughter nuclei at the beginning of telophase (three cells observed). In differential interference contrast, chromosomes are not visible until early telophase, when they began to swell and fuse to form karyomeres (Fig. 8) at the poles of the spindles. Chromosomal fiber shortening must have occurred in these cells, otherwise karyomeres would have formed between the birefringent half-spindles and not at the spindle poles.

CHROMOSOME POSITION IN STABILIZED SPINDLES: We observed the chromosomes in stabilized spindles from antimyosin-injected cells to further verify that chromosomal fiber shortening had moved chromosomes poleward during anaphase. In stabilized spindles from late anaphase cells that had been previously injected with 1.7–3.0 ng immune γ-globulin, the chromosomes had all moved to the spindle poles (10 cells observed, Fig. 9). In spindles stabilized in mid-anaphase, chromosomes lay in two remarkably straight rows (Fig. 10) parallel to what had been the metaphase plate, indicating that in cells containing high concentrations of antimyosin the poleward movement of chromosomes had been orderly and synchronous.

Central Spindle Elongation

Central spindle elongation occurred normally in antimyosin-injected cells (Fig. 6). Based on Student's *t* test, the rate of central spindle elongation during mitotic anaphase in cells injected with up to seven times the dose of antimyosin required to block cleavage was indistinguishable from the rate of spindle elongation in uninjected controls (Fig. 11).

Effects of Antimyosin on Fertilization and Pronuclear Migration

In eggs injected with antimyosin before insemination, a fertilization membrane is elevated normally (six eggs observed). Syngamy and meiosis proceed normally, though cytokinesis (polar body formation) is inhibited (Fig. 12). After second meiosis in each egg, the female pronucleus moves toward the male pronucleus, which has already moved to a position near the center of the egg. The nuclei fuse normally (Fig. 7). Subsequently, each cell forms spindles and completes first mitosis even though cytokinesis never occurs. Thus antimyosin has no effect on fertilization or on pronuclear migration and fusion.

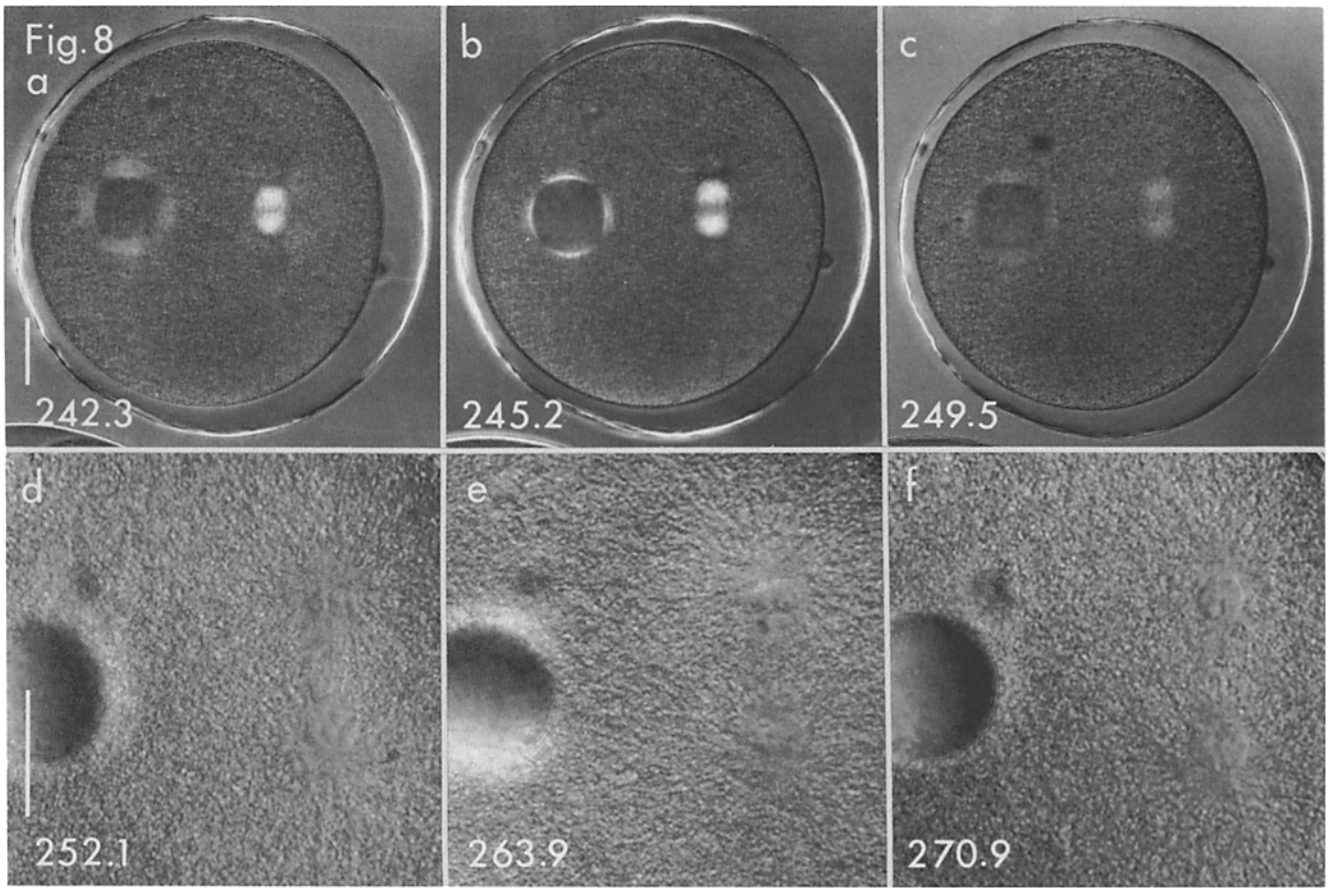

FIGURE 8 Chromosome movement by chromosomal fiber shortening and central spindle elongation in the presence of antimyosin. An oocyte was injected during first meiotic anaphase with antimyosin (4.1 ng immune γ-globulin). (*a–c*) Increased spindle length during first mitotic anaphase indicates that spindle elongation occurred in the presence of antimyosin. (Polarized light microscopy). (*d–f*) In early telophase, nuclear condensation at the spindle poles indicates that the chromosomal fibers must have shortened in the presence of antimyosin. (Differential interference contrast microscopy). Times are shown in minutes after injection. Bars, 30 μm.

Long-term Effects of Antimyosin on Embryogenesis

We intermittently observed cells that had been injected with 0.8–1.3 ng of immune γ-globulin for more than 14.5 h after injection (twelve cells followed). 12.5 h after we inject antimyosin, we estimate that there is in excess of 200 nuclei in each of these cells: as many as eight nuclear divisions have taken place in the absence of cytokinesis. Yet nuclei in these cells continue to divide. There are so many nuclei in each cell that nuclei in close proximity begin to fuse (Figs. 2 and 12). At this time uninjected control embryos start to form cilia. We also observed cilia on injected cells that had been completely inhibited from dividing. Later (14–15 h after injection), we observed changes in the shape of these cells. In each egg several widely separated furrows, positioned randomly around the cell periphery, form and progress toward the center of the cell (Fig. 13). The cells become highly polymorphous, each consisting of several large, rounded lobes connected by wide bridges of cytoplasm. When uninjected control embryos hatch and swim away, the injected polymorphous syncytium pinch into numerous, irregularly shaped "cells" and smaller "cytoplasmic droplets" that are 80 μm or less in diameter. Subsequently, these "cells" and "droplets" lyse, leaving cytoplasmic granules floating freely in the sea water.

DISCUSSION

Antibodies against starfish egg myosin did not block chromosomal fiber shortening in meiotic and mitotic starfish oocytes and eggs.

CHROMOSOMAL FIBER SHORTENING: No inhibition of chromosome-to-pole movement was observed even when as much as eight times the amount of antimyosin that is sufficient to completely inhibit furrowing was injected into these eggs. In injected cells, we observe: (*a*) normal anaphase changes in spindle birefringence, (*b*) the formation of spatially separated second meiotic spindles in oocytes (in which central spindle elongation does not conbtribute to anaphase movement), (*c*) karyomere, then nuclei formation at the poles of telophase mitotic spindles, and (*d*) chromosomes at the poles of stabilized anaphase spindles. These observations can be explained only if the chromosomes were moving poleward in the presence of antimyosin in amounts more than sufficient to completely inhibit cytokinesis.

CENTRAL SPINDLE ELONGATION: Antimyosin did not inhibit central spindle elongation. The rate of central spindle elongation, in the presence of up to seven times the dose of antimyosin required to inhibit cytokinesis, was not different than its rate in uninjected control eggs.

Mabuchi and Okuno (34) observed that daughter nuclei

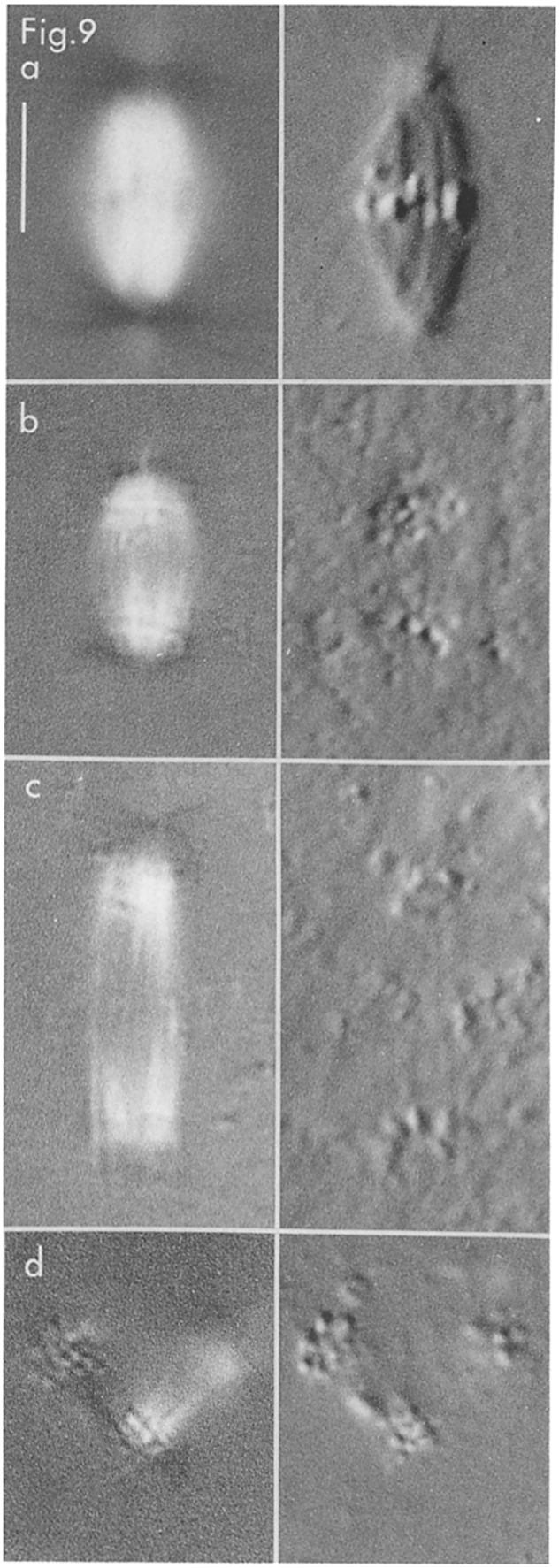

FIGURE 9 Chromosome distribution in stabilized anaphase spindles from oocytes injected with antimyosin. Spindles were stabilized during anaphase as described in the text. Left, polarized light; right, differential interference microscopy. (*a*) Stabilized spindle from a noninjected control cell shows normal metaphase birefringence pattern and chromosomes at the metaphase plate. (*b*) Stabilized cell in midanaphase, ~18 min after being injected with antimyosin (1.6 ng immune γ-globulin) during first meiotic metaphase. Chromosomes have moved approximately half the way to the poles. (*c*) Stabilized cell in late anaphase, ~18 min after being injected with antimyosin (2.0 ng immune γ-globulin) during first meiotic metaphase. Chromosomes have moved all the way to the poles. (*d*) Stabilized cell in second meiotic anaphase, ~83 min after being injected with antimyosin (0.8 ng immune γ-globulin). The cell completed first meiosis, but no polar body formed. Second meiotic spindles appeared and during anaphase the chromosomes moved all the way to the poles. (Only three of the four groups of chromosomes are in focus in these photographs.) Bar, 10 μm.

formed abnormally close together in antimyosin-injected cells. We attribute their observation to the transient reduction in spindle length that sometimes follows γ-globulin injection into oocytes and eggs.

While these experiments suggest that it is highly unlikely for myosin to play a role in either chromosomal fiber shortening or central spindle elongation, there are three principal objections that could be made against such a conclusion: one of access of antibody to myosin targets in the cell, one of the quantity of antibody of injected, and one of the immunological cross reactivity of injected myosin.

ACCESS: Perhaps injected antibody is prevented from interacting with putative "spindle" myosin, but not "cleavage furrow" myosin. This seems unlikely for two reasons. First, fluorescein-labeled control γ-globulin readily infiltrated even preformed spindles. Second, anaphase chromosome movement occurred normally even when spindles formed in the presence of injected antimyosin. This means that spindle myosin would have had to remain "protected" from interacting with antimyosin throughout the course of multiple cell cycles, even during interphase, when the mitotic apparatus is completely disassembled. The existence of such a stable, sterically unavailable myosin complex seems unlikely.

QUANTITY: A second argument is that although antimyosin blocks cleavage in injected cells, there is not enough antibody to inhibit all myosin function in those cells. Based on the amount of myosin (0.1 ng) in an *Asterias amurensis* egg (31, 32), the size of an *Asterias forbesi* egg (110 μm in diameter) compared to that of an *A. amurensis* egg (132 μm), and the assumption that each egg contains an amount of myosin proportional to its volume, we calculate that each *A. forbesi* egg contains ~0.058 ng of myosin. Mabuchi and Okuno (34) found that a weight-to-weight ratio of immune γ-globulin to myosin of ≃52:1 resulted in total inhibition of the actin-activated ATPase of egg myosin. The maximum dose of immune γ-globulin we injected was ~83 times the amount of myosin we estimate to be present in an *A. forbesi* egg. In vitro this amount of antimyosin would completely inhibit the actin-activated ATPase of myosin. It is unlikely that myosin is still functioning in motile processes in eggs we injected with such high doses of antimyosin.

CROSS REACTIVITY: A third argument is that putative "spindle" myosin is antigenically different from "cleavage furrow" myosin, and therefore is immunologically unreactive with our antimyosin probe for myosin function. This reasoning is fueled by reports of the existence of two myosins in single non-muscle cells (29, 37, 38, 45). While we cannot rule out this

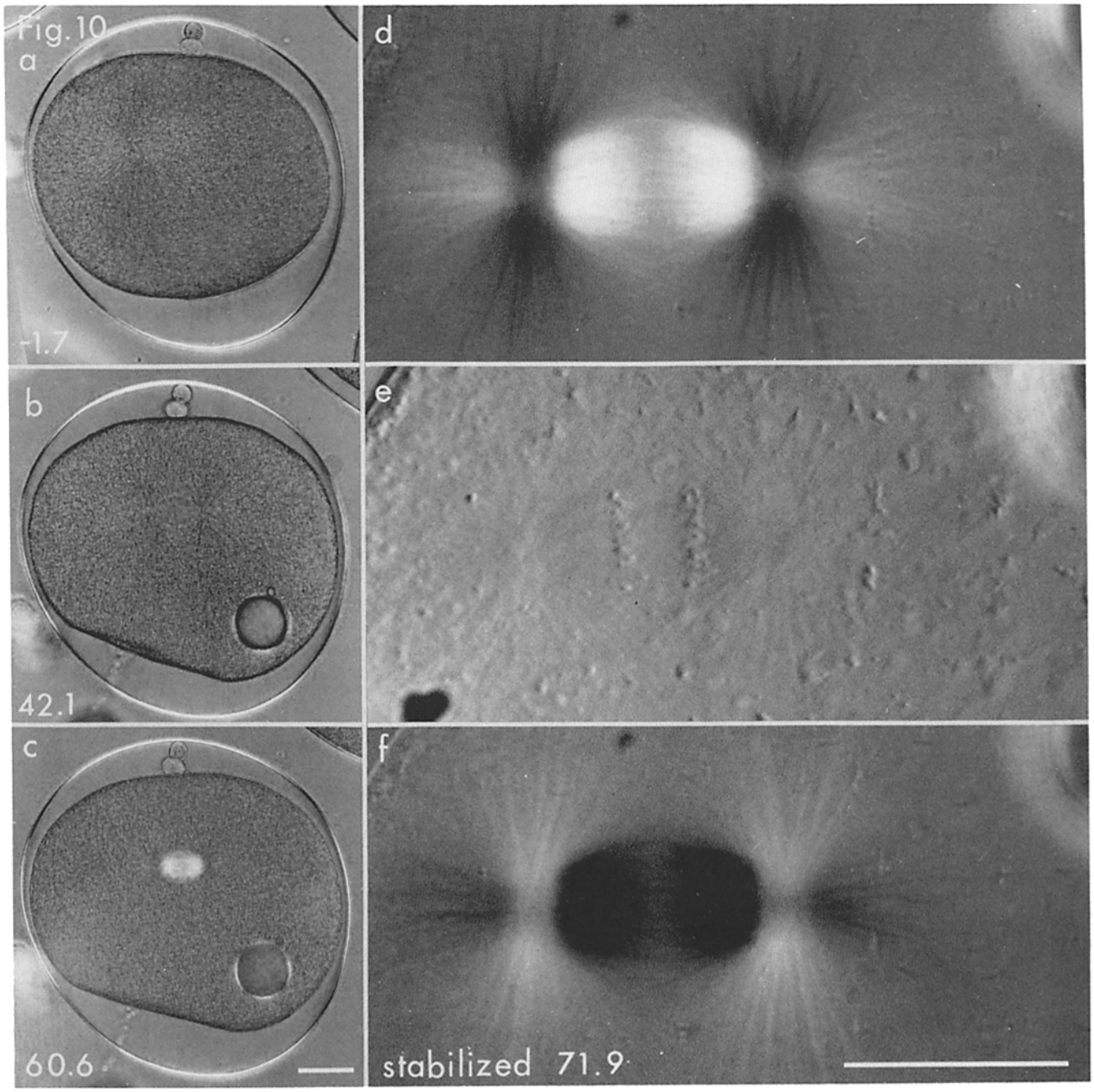

FIGURE 10 A spindle in a cell injected with antimyosin was stabilized to visualize the chromosomes. Cells were injected with antimyosin (1.8 ng γ-globulin) before pronuclear fusion. The spindle formed and anaphase ensued. The spindle was stabilized in mid-anaphase. (*a*) Target cell before pronuclear fusion. (*b* and *c*) Injected cell in prophase, then metaphase of first mitosis. (*d* and *f*) After spindle stabilization, polarized light, in bright and dark compensation, shows an anaphase spindle. Chromosomes are visible in *d*. (*e*) Differential interference microscopy more clearly shows the position and arrangement of the chromosomes. Times are shown in minutes after injection. Bar, 30 μm.

possibility, the role of either of these two myosins in motility has not been clarified and there is no evidence to suggest that two myosins, much less two antigenically dissimilar, and functionally active myosins, exist in starfish eggs.

Our data strongly suggest that myosin is not involved in chromosome movement. It is corroborated by the failure of *N*-ethylmaleimide-modified myosin subfragment 1, cytochalasin and antimyosin, agents reported to block actomyosin function, to inhibit chromosome movements in permeabilized cell models (6, 39, 51). Our data are consistent with the observation that ATP is not required for chromosomal fiber shortening in permeabilized cell models (4). Yet myosin and actin seem to be present in the mitotic spindle in background or even above background concentrations (2, 10, 11, 43). As techniques for the fixation and visualization of these proteins improve, their presence as relevant spindle components may or may not be confirmed. Ultimately, verification of their role in chromosome movement will require demonstration of their function during either chromosomal fiber shortening or central spindle elongation. We have provided strong evidence against a role for myosin function in chromosome movement.

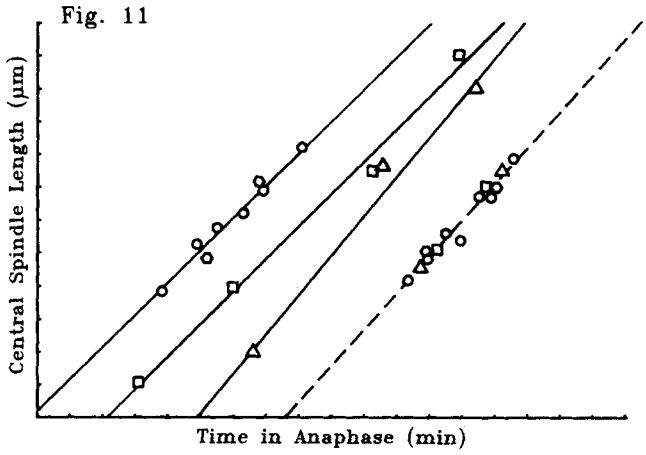

FIGURE 11 Rate of central spindle elongation in antimyosin-injected (solid lines) and uninjected control cells (broken line). Ordinate: Normalized spindle length in micrometers (the length of each spindle was adjusted by adding or subtracting a constant so that all spindles were of equal length midway through the data set). Abscissa: Normalized time in anaphase (time in minutes was adjusted so that the mid-point of each data set falls at 6 min). Neither manipulation affects the slope (rate of central spindle elongation) of spindle length vs. time. Different symbols indicated data points from different cells (four experimental, four control).

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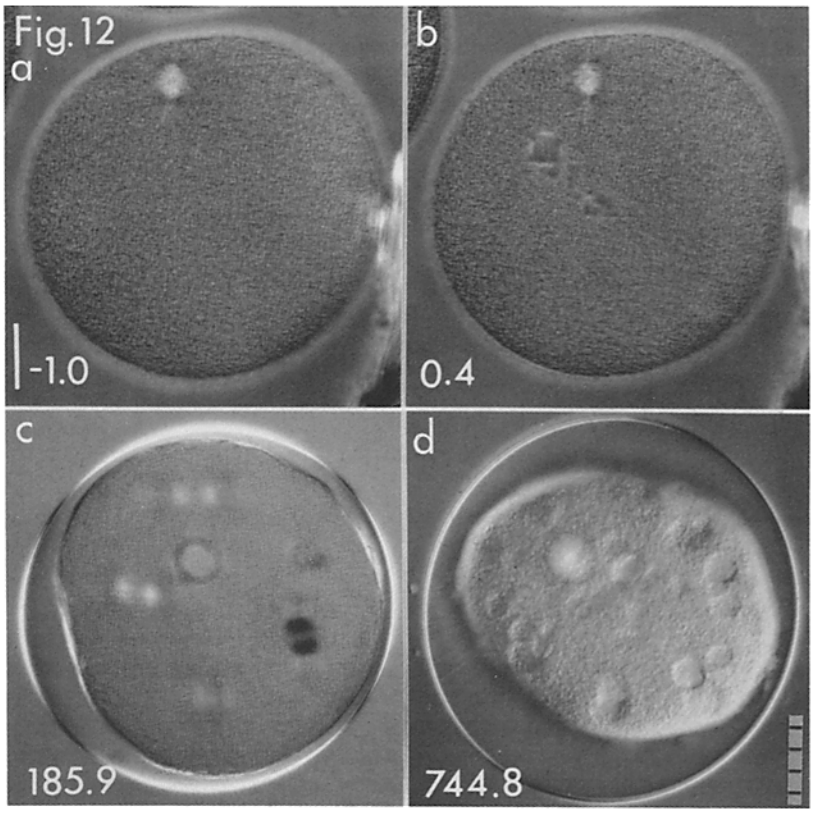

FIGURE 12 Antimyosin does not inhibit fertilization. (*a*) An unfertilized oocyte during first meiotic metaphase before injection. (*b*) The same oocyte after injection of antimyosin (1 ng immune γ-globulin). (*c*) ~30 min after injection, this oocyte was fertilized. 2.5 h later, several spindles in a common cytoplasm indicate that fertilization, syngamy, and nuclear division have occurred while cytokinesis was inhibited. (*d*) The same egg 12 h after injection viewed with differential interference microscopy. Numerous nuclei are visible. Times are shown in minutes from time of injection. Bar, 30 μm.

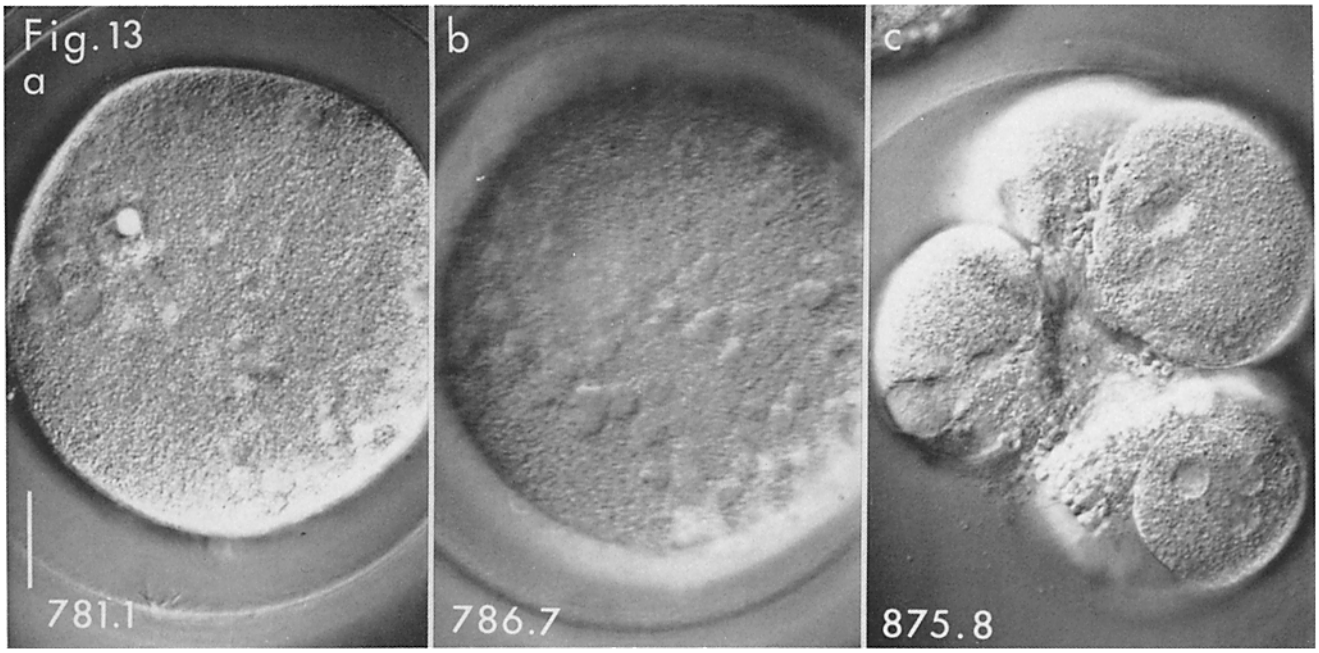

FIGURE 13 Fate of antimyosin-injected eggs. (*a*) ~13 h after injection of antimyosin (1.0 ng immune γ-globulin), a cell is full of nuclei. No cleavage furrows were observed up to this time. (Bright spot, upper left, is oil droplet injected with γ-globulin solution.) (*b*) Different focus, ~5 min later, shows numerous nuclei near the cell surface. (*c*) Later, when uninjected eggs have begun to ciliate, the injected cell fragmented. The randomly oriented "furrows" eventually pinched the egg into numerous irregular "cells" and "cytoplasmic droplets" which subsequently lysed. Photographs were taken with differential interference contrast microscopy. Times are shown in minutes after injection. Bar, 30 μm.

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