A COMPARISON OF THE DISTRIBUTION OF ACTIN AND TUBULIN IN THE MAMMALIAN MITOTIC SPINDLE AS SEEN BY INDIRECT IMMUNOFLUORESCENCE

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ABSTRACT

Rabbit antibodies against actin and tubulin were used in an indirect immunofluorescence study of the structure of the mitotic spindle of PtK_1 cells after lysis under conditions that preserve anaphase chromosome movement. During early prophase there is no antiactin staining associated with the mitotic centers, but by late prophase, as the spindle is beginning to form, a small ball of actin antigenicity is found beside the nucleus. After nuclear envelope breakdown, the actiactin stains the region around each mitotic center, and becomes organized into fibers that run between the chromosomes and the poles. Colchicine blocks this organization, but does not disrupt the staining at the poles. At metaphase the antiactin reveals a halo of ill-defined radius around each spindle pole and fibers that run from the poles to the metaphase plate. Antitubulin shows astral rays, fibers running from chromosomes to poles, and some fibers that run across the metaphase plate. At anaphase, there is a shortening of the antiactin-stained fibers, leaving a zone which is essentially free of actin-staining fluorescence between the separating chromosomes. Antitubulin stains the region between chromosomes and poles, but also reveals substantial fibers running through the zone between separating chromosomes. Cells fixed during cytokinesis show actin in the region of the cleavage furrow, while antitubulin reveals the fibrous spindle remnant that runs between daughter cells. These results suggest that actin is a component of the mammalian mitotic spindle, that the distribution of actin differs from that of tubulin and that the distributions of these two fibrous proteins change in different ways during anaphase.

The extent of our ignorance about molecular mechanisms for the cellular forces that move chromosomes is demonstrated by the number of different, successful models for mitotic movements (2, 10, 12, 23, 31, 34). In spite of this ignorance, several aspects of the process are reasonably well understood. There is general agreement that chromosomes are moved by forces acting on them at the kinetochore region (34). Since microtubules are the major fibrous element in the spindle, accounting for the majority (19) if not all of the spindle birefringence (42), and since microtubules

are the only fibers seen to be attached at the kinetochores, most models of force generation for anaphase movement of chromosomes assume that microtubules are somehow involved (2, 10, 22, 31, 34). This assumption is supported by experiments with colchicine, reduced temperature, or increased pressure, treatments which solubilize microtubules and which prevent normal chromosome movement (21, 22, 40).

Ultraviolet microbeam irradiation of spindle fibers has shown, however, that a zone of reduced birefringence can be made on a spindle fiber without slowing the chromosomes (11). Conversely, the chromosomes may be slowed without loss of birefringence. These results have been interpreted as suggesting that the system responsible for chromosome movement may not be congruent with the birefringent microtubule bundles of the spindle (11). Recent observations on the drug sensitivity and speed of prophase and prometaphase movement of chromosomes in insect spermatocytes also suggest that some chromosome movement may be due in part to a component other than microtubules (37). There are some reports of microfilaments in the spindle as determined by conventional methods of fixation and preparation for electron microscopy (3, 32, 33). After glycerination, spindle microfilaments are sometimes more distinct (18). After HMM decoration, a treatment that appears to be specific for actin filaments (24, 36), the thin filaments are more visible and more numerous (4, 13, 14, 17, 18, 20). Aronson (1) visualized the distribution of actin in isolated spindles using fluorescein-labeled HMM, and recently Sanger (41) found actin in spindles of PtK_2 cells using a similar technique. While the presence of actin in the spindle is no proof of its importance to chromosome motion, the evidence is sufficiently suggestive for several workers to have proposed models for chromosome motion based upon a muscle-like mechanochemistry (12, 18, 41).

The observations of spindle actin as determined by glycerination and heavy meromyosin (HMM) decoration can be criticized on two grounds: glycerination disrupts the organization of a cell and may displace existing cell components, and HMM is known to induce monomeric actin to polymerize (9, 13, 25, 50). It would therefore be useful to have an independent means to assess the actin content of a mitotic spindle, one which is less disruptive to cellular organization and which does not induce actin to polymerize. Biochemical dissection of isolated spindles has thus far not been a useful approach because isolated spindles contain many components and, as yet, will not move chromosomes. It is therefore not possible to determine which of the components in the isolate are important and which are not. A lysed cell system has been developed in which anaphase chromosome movement and spindle elongation will continue after lysis with a nonionic detergent (8, 30), but this cell model has not yet yielded new information about the identity of functionally significant spindle components.

We report here the use of antibodies against actin and of indirect immunofluorescence to determine the location of actin within dividing PtK_1 cells. To minimize fixation difficulties and contamination of the spindle with cytoplasmic actin, we have used cells fixed with formalin after lysis under conditions which preserve chromosome movement (8, 30). A fixed metaphase spindle from such a preparation shows antiactin fluorescence around the spindle poles and in discrete fibers running from chromosome to pole. During anaphase the fibers shorten as the chromosomes approach the poles, leaving the region between the chromosomes devoid of fluorescence. Under the same experimental conditions, but using antibodies against neurotubulin, kinetochore-attached fibers and fibers extending past the kinetochores into the "interzone" between sister chromosomes are seen in both metaphase and anaphase spindles. The presence of actin in fibers running from chromosome to pole but not in the interzone supports the suggestion that an actomyosin-like system is involved in the movement of the chromosomes to the spindle poles.

MATERIALS AND METHODS

Actin and Tubulin Purification

Chicken breast muscle actin was prepared from an acetone powder by the method of Spudich and Watt (45). When examined by sodium dodecyl sulfate (SDS) gel electrophoresis, overloaded gels contained no observable tropomyosin. Microtubule protein was prepared after the method of Weisenberg (50) with modifications by Borisy and Olmsted (5) or Shelanski et al. (43). Normally, two cycles of reversible temperature-dependent assembly were used to prepare polymerization-competent porcine brain tubulin to at least 80% purity (44).

Preparation of Antibodies

Antibodies against actin were prepared in rabbits. The antigen was either calf thymus actin that was purified by

column chromatography, denatured in SDS, and reduced with dithiothreitol before immunization, or mouse fibroblast actin purified by preparative SDS slab gel electrophoresis. The details of antibody preparation are described elsewhere (26, 28, 29).

The antibody against tubulin was prepared in rabbits using porcine neurotubulin, kindly provided by G. Borisy and D. Murphy from the University of Wisconsin. The tubulin appeared free of contaminating high molecular weight proteins when examined with SDS-acrylamide gel electrophoresis. Before immunization, the tubulin was further purified by preparative SDS-polyacrylamide gel electrophoresis (26). The antibody reacts with the mitotic apparatus of a number of mammalian cells grown in tissue culture and has been shown to react with intracellular microtubules of interphase cells (28).

Tissue Culture Cells and Lysis Conditions

PtK_1 cells were maintained in Falcon flasks in Ham's F-12 medium supplemented with 10% fetal calf serum, sodium ampicillin (200 μg/ml), and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer, pH 7.3. Cells were subcultured on glass cover slips in Falcon dishes (Falcon Plastics, Division of Bio-Quest, Oxnard, Calif.) for 48 h before use. For immunocytochemical experiments, cover slips were transferred to Falcon dishes maintained at 37°C and containing 4–8 mg/ml cycle II tubulin, 100 mM piperazine-*N*-*N'*-bis[2-ethane sulfonic acid] (PIPES), pH 6.9, 2 mM GTP, 2 mM ATP, 6 mM $MgSO_4$, and 0.04% Triton X-100 prewarmed for 5 min before use. Anaphase cells lysed in these conditions will continue chromosome separation for at least 20 min (8). For antibody experiments, cells were fixed within 10 min after lysis. Mitotic spindle structure was disrupted by adding Colcemid (Ciba Corp., Summit, N.J.) to cover slip preparations in conditioned Ham's F-12 medium at a final concentration of 1×10^{-5} M 20 min before lysis (44).

Fixation and Indirect Immunofluorescence

After lysis, the tubulin solutions were drained from the cover slips, and the lysed cells were fixed for 30 min at 37°C in 3.7% formalin (Fisher Scientific Co., Pittsburgh, Pa.), 50 mM *N*-Morpholenoethane sulfonate (MES, Sigma Chemical Co., St. Louis, Mo.) at pH 6.4, 10 mM $MgSO_4$, and 2% polyethylene glycol (Carbowax 20 M, Union Carbide Corp., Chemicals & Plastics, New York, N. Y.). In some experiments, cells were not lysed but were placed directly in the fixation medium for 20 min. Triton X-100 was then added to the fixation medium at a final concentration of 0.2% for the remainder of the fixation. In other experiments (tubulin antibody), cells were placed in 3.7% formalin (Fisher) in phosphate-buffered physiological saline containing 0.20 M sodium phosphate, pH 7.4 and 2 mM $MgCl_2$ (PBS) for 30 min; the cover slips were then passed through an acetone-water series (50% acetone, 100% acetone, 50% acetone) at room temperature over a 20-min period (27).

After fixation, cover slips were washed in PBS and incubated for 10 min at room temperature with nonimmune goat immunoglobulins (1–2 mg/ml, Colorado Serum Co., Denver, Colo.) to help reduce any subsequent nonspecific binding of the fluorescein-labeled goat immunoglobulins. The cover slips were then rinsed with PBS and incubated for several hours at 4°C or for 1 h at 37°C with 1–2 mg/ml rabbit antiactin- or antitubulin-containing immunoglobulins or non-immune rabbit immunoglobulins (NIR). The cover slips were rinsed with PBS, then incubated for 1 h at 37°C with fluorescein-labeled goat anti-rabbit immunoglobulins (GAR) prepared from commercial goat anti-rabbit serum (Grand Island Biological Co., Grand Island, N. Y.) in a 1:30 dilution with PBS. The GAR, NIR, antiactin, or antitubulin immunoglobulin fractions were prepared from serum by three 33% ammonium sulfate cuts and repeated dialysis against PBS. For microscopy, the cover slips were mounted in a 1:1 mixture of glycerol and PBS. For the preabsorption studies, ammonium sulfate-purified immunoglobulins against actin (250 μg in 100 λ PBS) were incubated for 12 h at 4°C with 250 μg of heat-denatured chick actin or 500 μg of hog brain tubulin that had been precipitated by the addition of 20 mM $MgCl_2$. The antitubulin immunoglobulins (250 μg in 100 λ PBS) were similarly incubated with 250 μg of chick actin or 250 μg of hog brain tubulin. At the end of the incubation period, the preparations were centrifuged at 10,000 rpm for 45 min in a RC-2 centrifuge and the supernates, properly diluted with PBS, were used as the absorbed antisera. The antiserum used in the spindle preabsorption control (Fig. 5) was prepared in a different manner; 100 μg of heat-denatured chick actin was added to 250 μg of immunoglobulin against actin. After 6 h, the antiserum was centrifuged as above, and the absorption step was repeated with an additional 100 μg of chick actin. The degree of interphase or spindle staining seen by using this antiserum was similar to that seen with antiserum prepared with one preabsorption with chick actin.

Microscopy

Specimens were viewed with a Zeiss universal microscope, using an HBO 200 mercury arc lamp, epifluorescence illumination, and a 100X Neofluar phase objective. Phase optics were available through the same lens, using a standard phase condenser, transmission illumination, and filters to approximate the epifluorescence image in color. Thus, no refocusing was necessary in passing from a phase to a fluorescence image; the light was simply redirected from transmission optics to epi-illumination.

Pictures were taken on Kodak Tri-X developed in Diafine to an ASA of 1600. All fluorescence pictures were taken, processed and printed under identical condi-

tions except as noted in the text. The exposure time for all antiactin and control preparations was 30 s, for antitubulin, 5 s.

Observations of spindle birefringence were made with Zeiss polarization optics, using a λ/30 Brace-Köhler compensator Carl Zeiss, Inc., New York, N.Y. for measurements of retardation.

Some cells were prepared for immunofluorescence as described above, and then fixed in 3% glutaraldehyde buffered in 100 mM PIPES, pH 6.9, osmicated, stained, dehydrated, and embedded for electron microscopy as described elsewhere (44). After sectioning, the specimens were stained with uranyl acetate and lead citrate and examined in a Philips 300 microscope.

RESULTS

Fixation

Glutaraldehyde is an excellent fixative for spindle microtubules, but, when it is used on PtK_1 cells in preparation for indirect immunofluorescence, subsequent treatment with fluorescein-labeled immunoglobulins results in nonspecific staining. Formaldehyde fixation, while less effective in preserving spindle microtubules, yields preparations that would react specifically with antibodies against actin and tubulin. The fixative described above was selected on the basis of its capacity to preserve spindle birefringence reasonably well and yet allow immunospecific staining. Figure 1*a–c* shows that spindle birefringence is preserved after fixation and repeated rinsings in PBS, although not at in vivo levels. The birefringence resulting from kinetochore-attached microtubules is well preserved by this fixation, but the birefringence contributed by the nonkinetochore microtubules is somewhat reduced.

To compare our experimental material after preparation for immunofluorescence with the same cell type after conventional preparation for fine structure study, we took cover slips of cells ready for fluorescence microscopy and fixed them in glutaraldehyde and processed them for electron microscopy. A comparison of Fig. 1*e–i* with published micrographs of PtK_1 cell spindles (6, 39, 44) shows that the spindles are severely extracted by our procedure. There are microtubules remaining, but the number of microtubules seen is substantially less than is found in a cell fixed by conventional methods. Nonkinetochore microtubules are observed in the spindle midzone of anaphase cells (Fig. 1*f*) and running past chromosomes in metaphase cells (Fig. 1*e*). A comparison of Fig. 1*h* and 1*i* shows that the antiactin-treated preparation contains more fuzzy, amorphous material associated with the kinetochore microtubules than in the NIR-treated cells. No obvious thin filaments are seen associated with this fuzz. The amorphous material may represent antibody binding to poorly preserved actin filaments.

Specificity of the Staining Reaction

The fluorescent pattern observed with tubulin antibody in interphase and mitotic PtK_1 cells after indirect immunofluorescence is similar to that described by Fuller et al. (16). In interphase cells (Fig. 2*a*), fluorescent fibers radiate out from a focus near the nuclear envelope or run as thin fibers lengthwise across the cell. The fibers are smaller than the antitubulin-staining bundles observed within the mitotic spindle (Figs. 22–29) and may represent just one or a few cytoplasmic microtubules. The specificity of the antibody is further documented by the preabsorption studies: antibody preabsorbed by chick actin still stains interphase fibers (Fig. 2*a*) and mitotic spindles (Fig. 26) but antibody preabsorbed with purified tubulin (Fig. 2*f*) does not stain these fibers (Fig. 2*b*) or the mitotic apparatus (Fig. 27).

Lazarides has already documented the specificity of the actin antibodies used in this study (26, 27). The antiactin yields a single detectable precipitation line against native, partially purified mouse fibroblast actin and native, highly purified chicken skeletal muscle actin by both double immunodiffusion and immunoelectrophoresis. Indirect immunofluorescent staining of chicken myofibrils shows a strong reaction with actin filaments (I band). When applied to interphase tissue culture cells, the antiactin binds to the phase-dense cytoplasmic fibers as judged by indirect immunofluorescent staining (Fig. 2*d*, *g*, and *h*) (26–29). In cells treated with actin antibody preabsorbed with tubulin (Fig. 2*d*), these fibers as well as the ruffling membrane around the cortex of the cell and around the nuclear envelope are stained. Preabsorption with chick action (Fig. 2*e*) blocks the staining of these fibers and the cell cortex (Fig. 2*c*), but the nucleolus and the area around the nuclear envelope still exhibit some fluorescence.

When applied to PtK_1 cells lysed in tubulin and fixed as described above, the antibodies against actin clearly stain the phase-dense fibers running the length of the interphase cell (Fig. 2*g* and *h*). The staining of the membranes at the cell cortex is removed by the Triton X-100 treatment, and staining of the nucleolus and the region around the

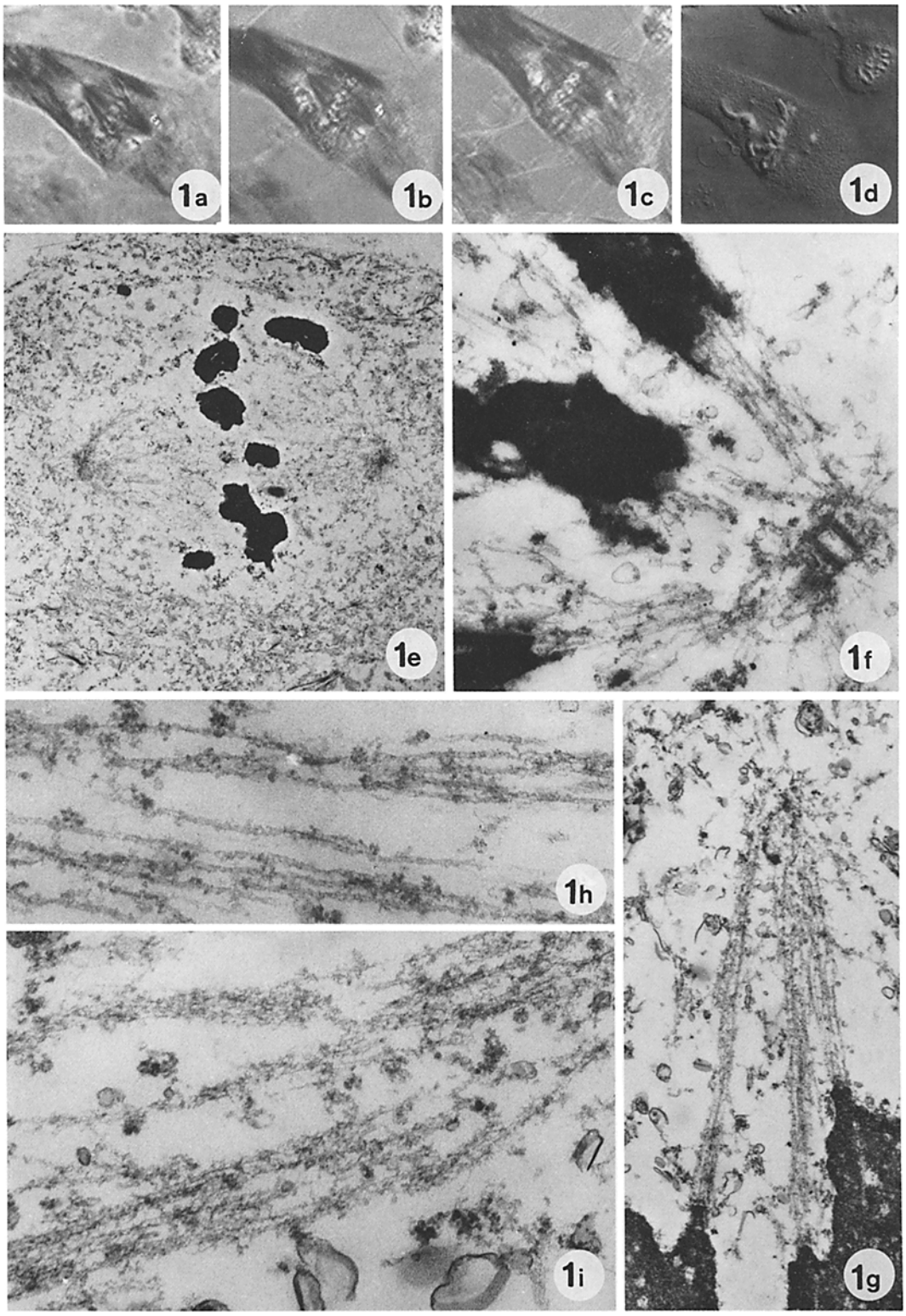


FIGURE 1 Effects of fixation on spindle birefringence. (*a*) polarization micrograph of the mitotic spindle after lysis in tubulin; (*b*) after fixation with formaldehyde-Carbowax; (*c*) after 6 h and repeated washes in PBS, and (*d*) Nomarski micrograph of same cell after 6 h in PBS. × 1,300. Electron micrographs of cells prepared for indirect immunofluorescence. (*e*) Nonimmune rabbit treated cell, × 5,000; (*f*) nonimmune rabbit treated anaphase cell, × 15,000; (*g*) antiactin-treated cell, × 15,000; (*h*) nonimmune rabbit treated cell, near a kinetochore, × 50,000; and (*i*) antiactin-treated cell near a kinetochore, × 50,000.

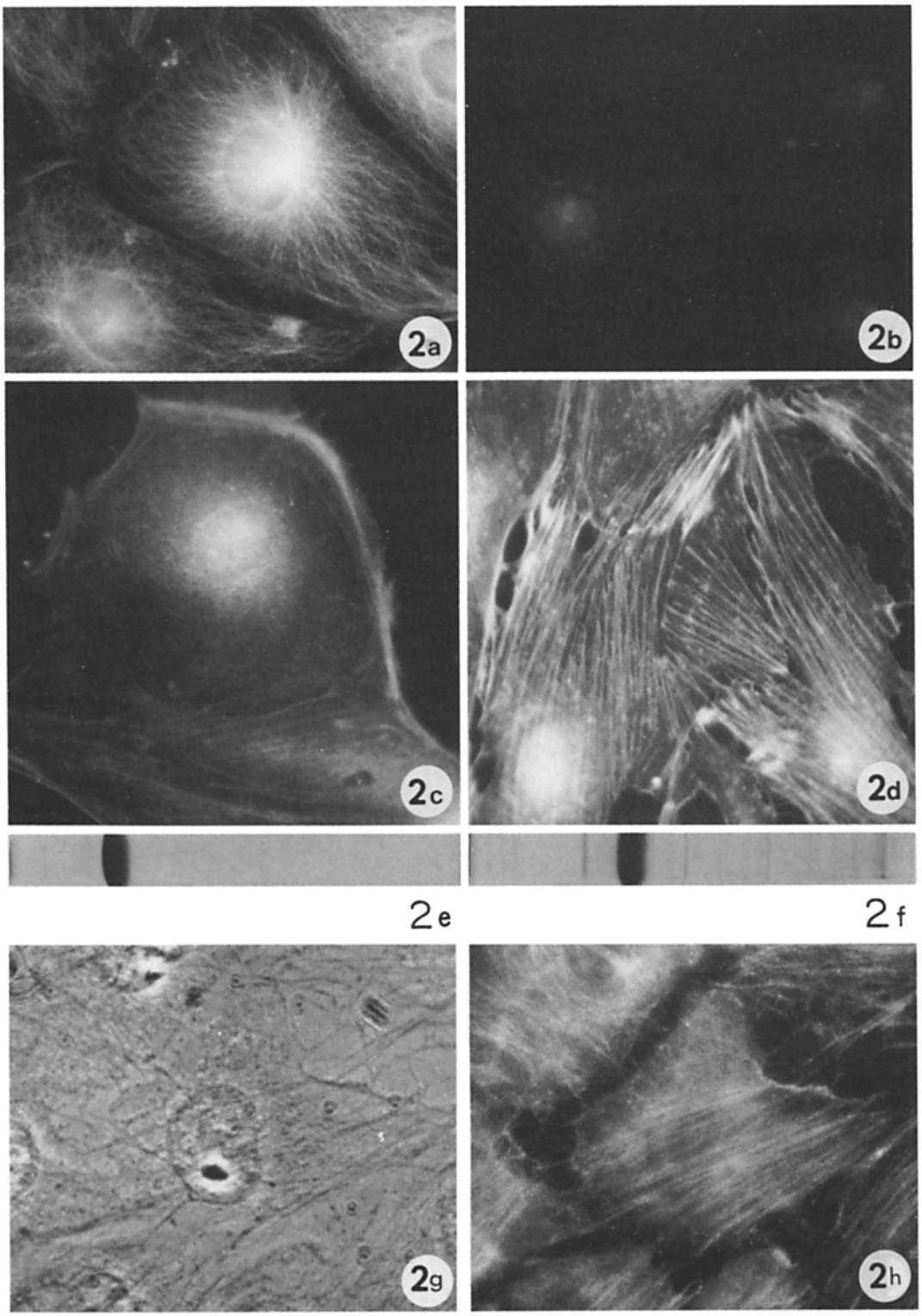


FIGURE 2 Indirect immunofluorescence of interphase PtK_1 cells with antibody preabsorbed with actin or tubulin: (*a*) antitubulin preabsorbed with actin; (*b*) antitubulin preabsorbed with tubulin; (*c*) antiactin preabsorbed with actin; and (*d*) antiactin preabsorbed with tubulin. Cells were fixed directly in formaldehyde-acetone. (*e*) Electrophoretic pattern of actin used in preabsorption; (*f*) electrophoretic pattern of tubulin used in preabsorption, (*g*); and (*h*) antiactin fluorescent and phase image of interphase PtK_1 cell lysed 7 min before fixation. All cells are × 1,600.

nuclear envelope is greatly reduced. Nonimmune rabbit treated cells lysed and fixed by several different procedures as described in Materials and Methods show a light granular staining pattern throughout the interphase or mitotic cell (Figs. 3 and 4). Cells that have not been pretreated with nonspecific goat immunoglobulins show an increase in nonspecific staining (data not shown). Mitotic cells treated with nonimmune rabbit immunoglobulins do not contain any fluorescent fibers within the spindle (Figs. 3 and 4). Mitotic cells treated with actin antibody preabsorbed with chick actin also do not reveal any fibers within the spindle, although these "preabsorption cells" do show some staining at the spindle poles (Fig. 5). This may represent nonspecific staining or an antibody component that cannot be preabsorbed by chick actin.

Actin Distribution in the Metaphase Cell

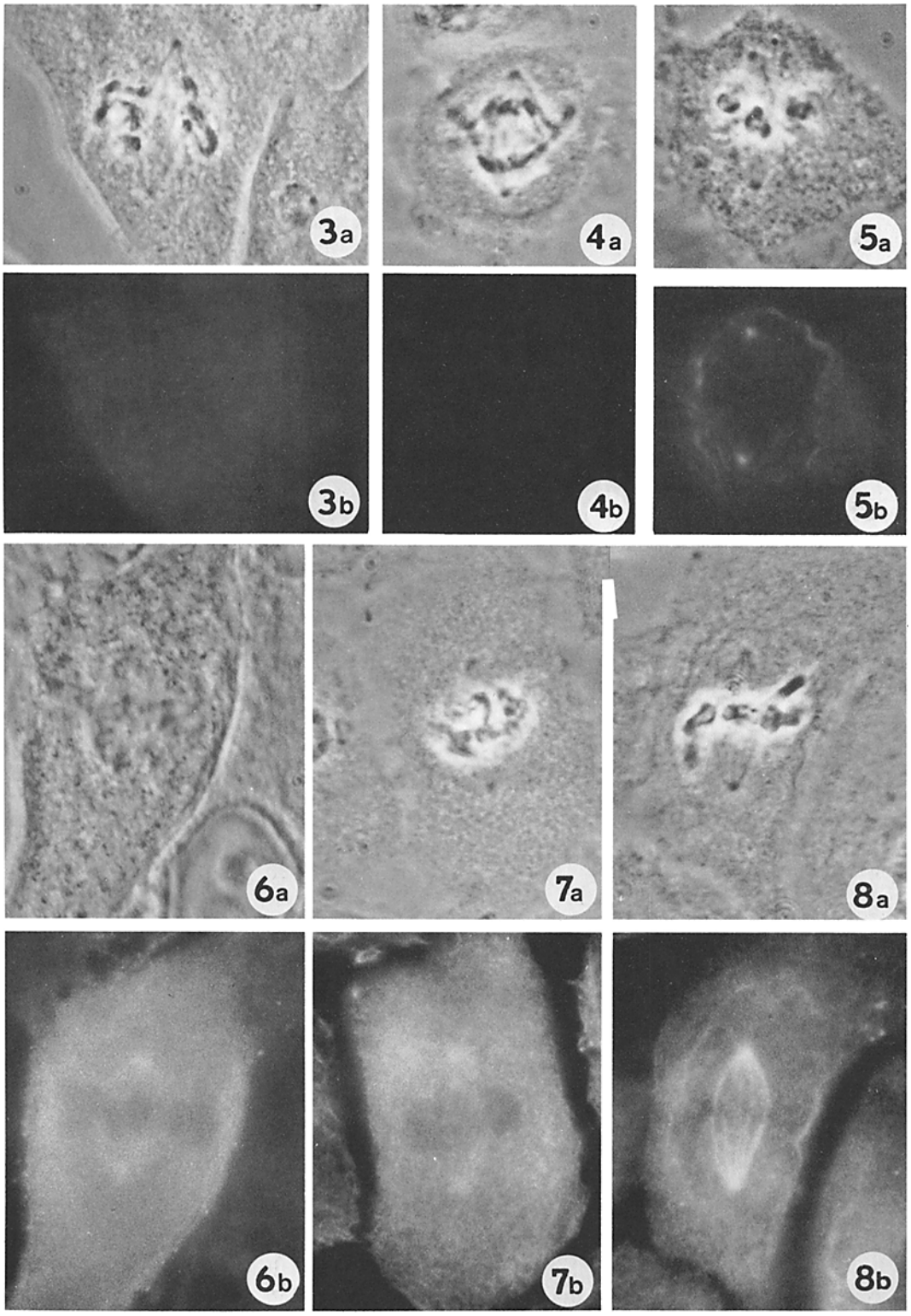
Metaphase PtK_1 cells fixed directly with formalin and then treated with detergent as described in Materials and Methods stain brightly for actin throughout the cytoplasm (Fig. 6). This staining is amorphous and is not distributed in long fibers as is seen in interphase cells. Optical sectioning shows that the fluorescence is not confined to the cell cortex. The spindle can be distinguished as a bright object against a bright background. To bring out the fluorescent spindle image, the negative for Fig. 6*b* was exposed for a shorter period of time in printing Fig. 6*b* than in printing the other figures in this paper. Lysis of the cells before fixation allows a progressive extraction of the amorphous cytoplasmic actin of the metaphase cell. Fig. 7 shows a cell lysed as described above and extracted for 3 min before fixation. Fig. 8 shows a cell extracted for 7 min before fixation. Since both preparation and photography of these two cells were otherwise identical, we infer from the reduction in general cellular staining that this amorphous actin is extracted by our lysis conditions. Since chromosome motion will continue for at least 10 min after lysis, although at slower rates (8, 30), we further infer that the loss of cytoplasmic actin does not interfere with spindle function. The observed variability in cytoplasmic fluorescence after lysis under the same conditions (Fig. 9–20) may reflect the variable loss of cytoplasmic components after lysis which has also been observed by electron microscopy (our unpublished data). Cells prepared with different lysis and fixation protocols, then treated with nonimmune rabbit immunoglobulins, may vary in the levels of nonspecific staining present (Figs. 3 and 4), but this variation cannot account for the differences in staining seen in Figs. 9–20. Mitotic cells from several cell lines handled with the conventional formalin-acetone fixative, then treated with antibodies against actin, also stain brightly for amorphous actin within the cytoplasm, thus obscuring the presence of actin in the spindle (Lazarides, E. Unpublished data).

The fluorescent staining pattern in the 3- and 7-min lysed cells is similar: actin is found as an amorphous halo at the spindle poles and as fibers running from chromosome to pole (Figs. 7, 8, 13, and 15). Because the fluorescent fibers are located at different focal planes, it has not been possible to determine whether there is an exact correspondence between fiber number and chromosome number. The six fibers seen in each half-spindle of Fig. 15 are the largest number observed in any one picture. PtK_1 contains 11 chromosomes per cell, so if fibers at all focal planes were recorded, there might be enough for one actin-containing fiber per chromosome. No fibers that cross the metaphase plate have been observed in the antiactin-treated cells. The fluorescent spindle is about 10% longer than the corresponding phase image in the four metaphase spindles shown here. The apparent difference in length is due to the increased size of the spindle pole as seen with fluorescence as opposed to phase optics. The staining at the poles appears to be distinct morphologically from the fiber fluorescence, with the pole fluorescence forming a symmetrical halo around the centrioles that extends into both the cytoplasm and the spindle.

The fluorescent fibers running from chromosome to pole at metaphase are curved rather than straight. This suggests that the actin fibers do not run directly from spindle pole to chromosome but rather are associated with the spindle microtubules which are known from electron microscopy to follow circular arcs (30).

Spindle Formation

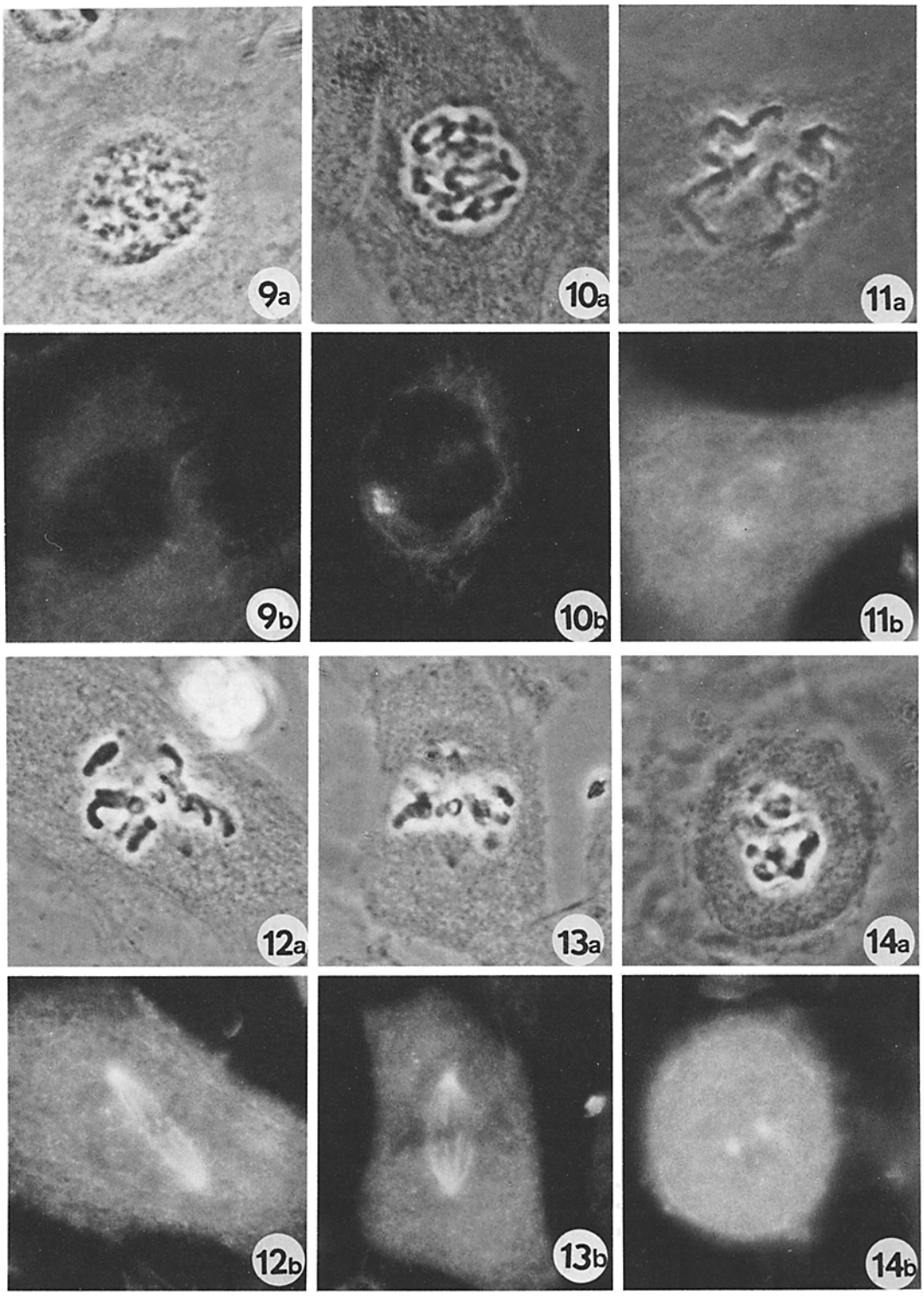
When prophase begins, the phase-dense cytoplasmic fibers that stain for antiactin disappear, and the level of amorphous staining in the cytoplasm increases (Fig. 9). Neither the condensing chromosomes nor the nucleolus in the prophase nucleus bind antiactin, but the region around the centrioles begins to stain intensely for actin at about the time of nuclear envelope breakdown (Fig. 10). During prometaphase, as the spindle



FIGURES 3–4 Indirect immunofluorescence with nonimmune rabbit immunoglobulin. Fig. 3, cell fixed directly in formaldehyde; Fig. 4, cell fixed 7 min after lysis. (*a*) Phase and (*b*) fluorescent micrograph of same cell. The magnification of this and all subsequent figures in the paper is × 2,000.

FIGURE 5 Indirect immunofluorescence with rabbit antibody preabsorbed with chick skeletal muscle actin. (*a*) Phase and (*b*) fluorescent micrograph of same cell.

FIGURES 6–8 Phase (*a*) and fluorescent micrograph (*b*) of same cell after antiactin treatment. Fig. 6, cell fixed directly in formaldenyde; Fig. 7, cell fixed 3 min after lysis; and Fig. 8, cell fixed 10 min after lysis in tubulin.



FIGURES 9–14 Phase (*a*) and fluorescent micrograph (*b*) of same cell after treatment with antibody against actin. All cells lysed for 7 min before fixation. Fig. 9, early prophase; Fig. 10, late prophase; Fig. 11, early prometaphase; Fig. 12, late prometaphase; Fig. 13, metaphase; and Fig. 14, mitotic cell pretreated with 10^{-5} M Colcemid before lysis.

microtubules assemble, the fluorescence around the poles increases (Fig. 11) and finally extends into fibers from the poles (Fig. 12). The distribution of actin in the late prometaphase spindle (Fig. 12) is similar to that of actin in the metaphase spindle (Fig. 13): staining shows a halo around the poles and fibers running between the chromosomes and poles. These fibers are of unequal length during prometaphase, consistent with the idea that they run from kinetochore to pole. Cells treated with Colcemid before lysis under conditions which prevent spindle formation (44) do not contain any distinct fluorescent fibers in the spindle region of the cell; the poles, however, still stain for actin (Fig. 14).

Anaphase and Telophase

During anaphase, as the chromosome-to-pole distance decreases, the length of the fluorescent fibers seen in the spindle correspondingly decreases and the amorphous halos of stain associated with the spindle poles move apart (Figs. 16–19). In late anaphase, the amorphous staining associated with the poles increases in area, but this may partly be due to the bunching of the short chromosome-to-pole fibers as the chromosomes approach and surround the poles (Figs. 18 and 19). No fluorescent fibers are observed in the spindle interzone during anaphase, although some wisps of fluorescence do dangle through the anaphase chromosome plates into the spindle interzone (Fig. 18). Late in anaphase (Fig. 19), a diffuse fluorescence is found in the spindle interzone and across the entire midregion of the cell where the cleavage furrow will form. Telophase cells (Fig. 20) stain brightly for actin in the cleavage furrow, but neither the poles, the decondensing chromosomes, nor the spindle midbody show any marked fluorescence.

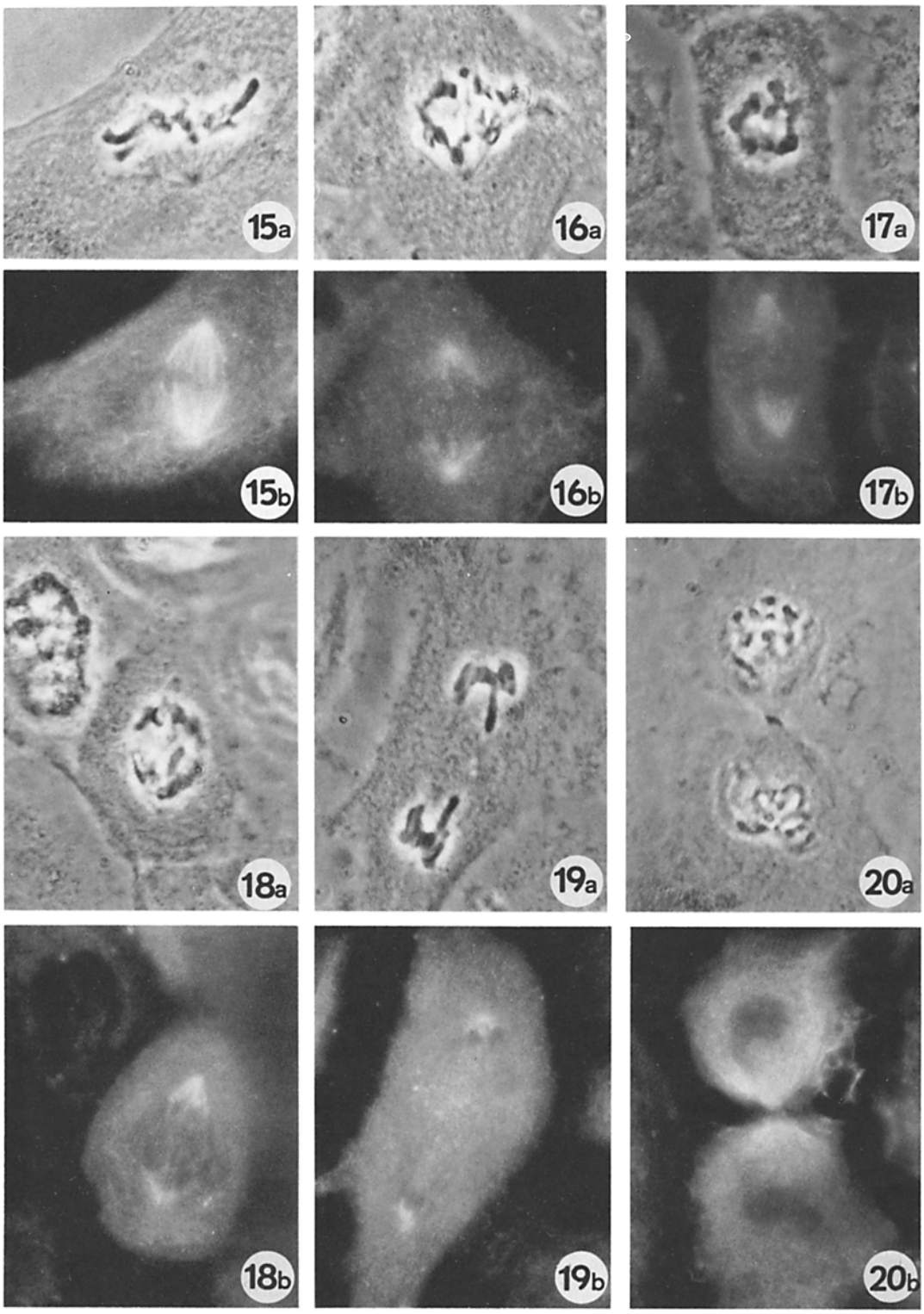
Distribution of Tubulin in the Spindles as Determined by Immunofluorescence

The distribution of tubulin in mitotic cells was studied in lysed cell preparations using the same formaldehyde-Carbowax fixation procedure employed in the study of actin distribution (Figs. 21–25). For comparison, we also include some pictures of unlysed cells fixed by the more conventional formaldehyde-acetone procedure (Figs. 26–30) and of cells treated with antibody preabsorbed with actin (Fig. 26) or tubulin (Fig. 27). Most of the fluorescent staining in the early prometaphase cells (Figs. 21 and 28) is concentrated around the poles, although some fluorescent fibers run between the poles or radiate out into the cytoplasm. Fluorescent fibers that end on chromosomes and fibers that cross the metaphase plate are readily observed in the metaphase spindles (Figs. 22 and 26). In the lysed preparation (Fig. 22), the chromosome fibers are distinct and regular. Other fluorescent fibers extend from the spindle poles as astral rays. A few slender filaments branch out from each half-spindle, intersecting at some distance from the spindle in the cytoplasm. A similar pattern is seen in the unlysed preparation except that the chromosome fibers are not so distinct in each half-spindle; the fluorescence blends together, and the fibers running through the spindle midzone are more prominent. In the anaphase cells (Figs. 23, 24, and 30), the poles stain intensely; microtubules are still observed radiating out into the cytoplasm from the poles. Some fluorescent fibers from each pole intersect in the peripheral cytoplasm where the cleavage furrow will later form. There are prominent fibers in the anaphase interzone; most of the fibers appear to run from pole to pole, but some fibers appear to overlap in the center of the spindle. In the telophase cell (Figs. 25 and 30), most of the stain is in the form of fibers, bunched together by the cleavage furrow, but also extending into the cytoplasm. The central region of this fiber bundle, the midbody, does not stain with antitubulin.

DISCUSSION

Our study shows that actin antigenicity is associated with the mammalian mitotic spindle, and that the spatial distribution of this antigenicity changes during spindle formation and function. The distribution of fluorescence suggests that actin is accumulated in the vicinity of mitotic centers at about the time that the spindle forms, and that actin in a fixable form is a component of the chromosomal spindle fibers.

Actin has been observed before in mitotic spindles by HMM as an actin-specific marker and glycerination of the cells as preparative treatment (1, 4, 13, 14, 17, 18, 20, 41). Recent work by Forer and Jackson (14) showing HMM decorated filaments in the spindles of endosperm tissue from the plant *Haemanthus* make it unlikely that cortical actin is the sole source of spindle actin, because these cells lack a defined, gelatinous cortex. All HMM studies, however, may be criticized on the



FIGURES 15–20 Phase (*a*) and fluorescent micrograph (*b*) of same cell after treatment with antibody against actin. All cells lysed for 7 min before fixation. Fig. 15, metaphase; Fig. 16, anaphase; Fig. 17, anaphase; Fig. 18, anaphase; and Fig. 19, late anaphase. Amorphous staining in spindle midzone is developing cleavage furrow; Fig. 20, telophase.

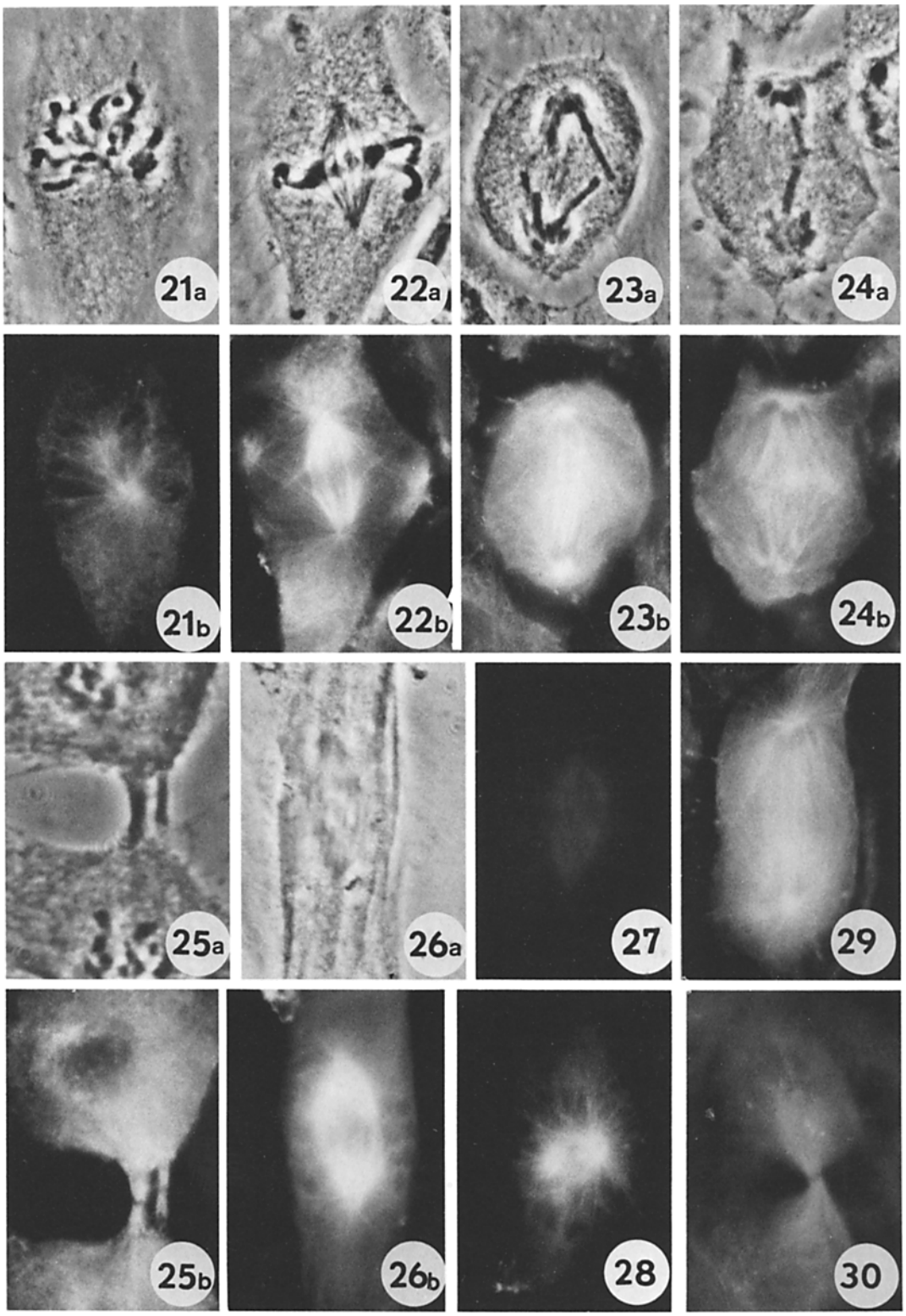
grounds that the actin filaments seen among the spindle tubules may have been formed during the HMM incubation (49, 51). While it is difficult to understand how the elegant images presented by Gawadi (18) of thin filaments running immediately beside spindle tubules either with or without HMM treatment could be preparative artifact, spindle tubules might align actin filaments as they form during preparation.

Our study supports the reality of spindle actin, because our methods of lysis and fixation as well as the indirect immunofluorescence techniques used to identify the distribution of actin are different from the methods previously employed. The formalin fixation and the antiactin treatment, unlike HMM treatment, are not likely to induce actin polymerization. We have reduced the possible artifacts to two classes: those characteristics of indirect immunofluorescence itself, for which we believe we have controlled, and those due to lysis and fixation. We cannot rule out the possibility that we are trapping cytoplasmic actin in the spindle. It may be that cells are filled with actin and that, by preserving the spindle as we extract the cytoplasm, we are maintaining in one region of the cell (the spindle) the high concentration of actin which is characteristic of the whole cell before lysis. The observation of a slight spindle-specific staining in cells lysed 20 min after formalin fixation, however, makes this explanation seem unlikely since the fixative should immobilize the cellular actin. The demonstration of both kinetochore- and nonkinetochore-attached spindle fibers with antitubulin staining after a treatment identical to the preparation for antiactin staining rules out the possibility that the actin localization is just a visualization of the microtubule distribution in the formalin-fixed spindle. Microtubules are found in the spindle interzone and in the midbody, but these regions of the spindle do not stain for actin. We cannot exclude the possibility that physiologically important actin is lost from the spindle after the fixation process, but functional spindle components must persist through the lysis step because the chromosomes continue to move after lysis.

The formaldehyde-acetone-treated cells and the cells lysed in tubulin, then formaldehyde fixed, give slightly different images after incubation with antitubulin. The kinetochore microtubule bundles are more distinct in the lysed preparations, probably because the fraction of staining due to nonkinetochore microtubules in these cells is slightly decreased. The differences between the two preparations are likely due to the extraction of some of the nonkinetochore tubules by the lysis-fixation-indirect immunofluorescent staining regime referred to above, although the acetone postfixation may cause bunching of fibers. The immunofluorescent images of mitotic cells after acetone fixation are similar to those described by Fuller et al. (16) who used antibody against tubulin and a similar fixation schedule.

Electron micrographs of the lysed, formalin-fixed cells stained for antiactin show that there are few recognizable filaments within the spindle. The fuzzy material associated with kinetochore microtubules in the antiactin-treated cells may represent antibodies decorating poorly preserved actin filaments. A similar decoration is observed on negatively stained plasmodium F-actin treated with plasmodium actin antibody (35). The difficulty in observing thin filaments in spindles after glutaraldehyde fixation, or in this case after formalin and glutaraldehyde fixation, may be due to general problems in the fixation of actin thin filaments for electron microscopy when the filaments are not associated with accessory proteins that copolymerize with actin. Szamer et al. (46) found that osmication after conventional glutaraldehyde fixation destroyed the filamentous nature of F-actin unless it was complexed with tropomyosin, and we have found that F-actin pellets, when prepared for EM by our methods, do not reveal thin filaments (our unpublished results). Immunological data (26, 29) suggest that nonmuscle cells contain some bundles of actin filaments that do not have tropomyosin associated with them. If spindle actin is simply F-actin without accessory proteins such as tropomyosin, which is consistent with the primitive position of the spindle among eucaryotic motile systems, then spindle microfilaments might be more difficult to fix for electron microscopy than other microfilament systems. Neither filaments nor fuzzy materials associated with microtubules are found in the NIR-treated cells.

It is gratifying that the distribution of actin within mammalian spindles as determined by indirect immunofluorescence and fluorescein-labeled HMM by Sanger (41) is similar. Sanger too has found fluorescein-labeled-HMM-decorated fibers running from chromosomes to pole. In his study, however, the fluorescence in these fibers decreases as one gets farther from the poles, and there is a discrete spot of stain on the chromosome at the fiber attachment point. In most cells we find no decrease in the staining intensity of the fibers as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they leave the pole region, and there is no discrete staining spot on the chromosome. Our preparations contain more amorphous staining than Sanger's, and while his methods stain the nucleolus, ours do not. These differences in staining between the two techniques may reflect differences in the preservation of the actin distribution after formaldehyde fixation as opposed to glycerination. By neither Sanger's methods nor our own is actin seen in appreciable amounts in the interzone fibers of anaphase cells. This result differs from the observations made with glycerination, HMM decoration, and electron microscopy where decorated filaments are found in the spindle midzone (4, 13, 14, 17, 18, 20). The cause of this discrepancy is not clear. Most likely, it reflects the difference in detection capacity between fluorescence microscopy and electron microscopy. Small amounts of actin may be present in the spindle interzone but in a form which cannot be observed by light microscopy.

The actin and microtubule distributions in interphase cells are clearly different: most of the actin is found in large bundles running the length of the cell or in cortical ruffles, while the tubulin is found in slender filaments running across the cell or radiating from a "cell center" near the nuclear envelope. During prophase, the mitotic centers begin to stain for actin at about the same time that they become competent to serve as spindle poles, that is, at about the time of nuclear envelope breakdown (44).

Actin staining appears to be associated with only the poles during the earliest stages of spindle formation; but as prometaphase progresses, chromosome to pole fibers develop. Two arguments suggest that the organization of the spindle actin depends upon the organization of spindle tubulin: although actin polymerization in vitro is insensitive to Colcemid, the drug blocks assembly of actin-containing spindle fibers in vivo (Fig. 14), and actin fibers in the spindle follow the curved trajectory of the chromosomal microtubules rather than run in a straight line from chromosome to pole. The close association of spindle tubules and actin filaments observed by Gawadi (18) indicates that there may be an association between these two proteins, perhaps by means of a third molecule serving as a bridge.

Until late anaphase-telophase, there is little actin-staining fluorescence in the spindle interzone. In contrast, the cells stained with antitubulin show that after the kinetochore microtubule bundles shorten, tubulin fibers are readily observable in the spindle interzone. At late anaphase and telophase there is a diffuse fluorescence in the antiactin-treated interzone, presumably due to the developing cleavage furrow. This fluorescence differs from that observed in the antitubulin cells in two ways: the antiactin fluorescence is not limited to the spindle but is also found in the adjacent cytoplasm at a similar intensity; and it is not organized into bundles. The presence of tubulin fibers which do not associate with actin and the fact that spindle tubules radiate in all directions from the poles while actin fibers are confined to the spindle suggest that there may be something special about the chemistry of microtubules that attach to a kinetochore. These tubules are known to be more stable than the rest of the metaphase spindle tubules (6, 7, 8, 30), but it is not yet known whether they are made from a different tubulin. Neither antitubulin nor antiactin stains the telophase spindle pole or the phase-dense midregion of the telophase midbody.

Fine structure studies of mammalian spindles at different stages of anaphase (30) and in vivo drug experiments (38) suggest that anaphase can be thought of as two distinct events: motions of the chromosomes to the poles on each half-spindle and separation of the two half-spindles by elongation of the interzone fibers. We suggest, on the

FIGURES 21–25 Phase (*a*) and fluorescent micrograph (*b*) of same cell after treatment with antibody against tubulin. Cells were fixed 7 min after lysis in tubulin. Fig. 21, prometaphase; Fig. 22, metaphase; Fig. 23, anaphase; Fig. 24, anaphase; and Fig. 25, telophase.

FIGURE 26 Phase (*a*) and fluorescent micrograph (*b*) of metaphase cell treated with antibody against tubulin preabsorbed with actin.

FIGURE 27 Metaphase cell treated with antitubulin preabsorbed with tubulin.

FIGURES 28–30 Cells treated with antibody against tubulin. Fig. 28, prometaphase; Fig. 29, anaphase; Fig. 30, telophase. Cells in Figs. 26–30 were fixed directly in formaldehyde-acetone.

basis of current evidence, that the forces for chromosome-to-pole motion are a result of mechanochemical events dependent in part upon actin. In support of this model, Fujiwara and Pollard report localization of myosin in mitotic spindles of HeLa cells using antibody against platelet myosin and direct immunofluorescence techniques (15). While actin-myosin interactions analogous to muscle are a possibility, the recent work of Tilney et al. (47) and Tilney (48) demonstrates that actin is capable of mechanical action, using other molecular mechanisms as well. Whatever the mechanism for force production, it is probable that the rate-limiting step in chromosome-to-pole motion is the disassembly of the kinetochore tubules which is required to allow each kinetochore to approach the pole toward which it points (22, 34). Given the paucity of actin seen in the spindle interzone, spindle elongation seems likely to be dependent upon microtubules but not actin. Perhaps there are two independent mechanisms for moving mammalian chromosomes that function in parallel to provide a fail-safe system for chromosome segregation, thereby reducing the chance of malfunction in this essential cellular process.

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