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A Functional Mitotic Spindle Prepared from Mammalian Cells in Culture

(microtubule assembly/motility/anaphase *in vitro*)

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ABSTRACT **Mitotic cells lysed into solutions of polymerizable microtubule protein contain a spindle which is similar to the living spindle in two respects: it will lose and gain birefringence when cooled and warmed, and it will move anaphase chromosomes to the opposite ends of the cell. Early anaphase cells lysed into buffers containing high molecular weight polyethylene glycol and nucleotide triphosphates will continue chromosome motion and spindle elongation in the absence of exogenous spindle subunits. These results suggest that while spindle growth requires microtubule polymerization, anaphase motions do not.**

Considerable effort has gone into the study of cell-free preparations of the mitotic apparatus (MA). Much has been learned about the structure of the isolated MA (1–4), and something is known of its chemistry (5–12), but two interesting questions have remained unanswered: what is the character of the equilibrium between the spindle and its subunits, and what is the nature of the motors that move the chromosomes?

Previous studies have isolated the MA by "stabilizing" it, i.e., making it reversibly less labile by lysing mitotic cells into buffers which are poor solvents for protein (9). In such buffers the MA will keep its birefringence and overall form for hours, but it will not shrink and regrow as a function of temperature the way the spindle does *in situ* (13). Since the work of Hoffman-Berling on chromosome motion in glycerol-extracted cells (14), no one has, to our knowledge, been able to study the physiology of anaphase-like processes *in vitro*.

In this paper we describe an experimental system for studying spindle functions *in vitro* and present some of our early results. We have taken advantage of the recent discovery of techniques for the reassembly of isolated microtubule protein (tubulin) (15, 16) to design a procedure based upon an equilibrium between assembled and disassembled subunits. Independent investigations based upon the same experimental design have been initiated by two groups at Woods Hole, Massachusetts. Preliminary reports of their findings have recently appeared in abstract form (17, 18). We lyse mitotic Pt K_1 cells with a nonionic detergent into buffers containing various concentrations of polymerizable tubulin, and we monitor the magnitude and longevity of spindle birefringence and spindle size with the light microscope. Our results show that the spindle is stable for more than 1 hr after lysis in solutions containing sufficient concentrations of tubulin. The stabilized spindles will lose birefringence when cooled and regain at least some of it when rewarmed. Cells deprived of spindle birefringence by cold treatment and then lysed into tubulin solutions will regrow birefringence after lysis when the preparation is warmed to 37°. Spindles prepared from cells lysed early in anaphase are able to move chromosomes in a fashion that is strikingly similar to normal anaphase.

Abbreviations: PIPES, Piperazine-*N*-*N'*-bis[2-ethane Sulfonic Acid]; EGTA, ethylene bis(oxyethylene-nitrilo) tetraacetic acid; GTP, guanosine triphosphate; GEP, GTP + EGTA + PIPES; ATP, adenosine triphosphate; Tx, Triton X-100; MA, mitotic apparatus.

MATERIALS AND METHODS

Microtubule protein was prepared from rat brain, following the method of Weisenberg (15) as modified by Borisy and Olmsted (19). Rat brains were homogenized at 0° in 0.1 M PIPES—KOH at pH 6.9 containing 1 mM EGTA and 2.5 mM GTP. We call this solution GEP (GTP, EGTA, and PIPES). The homogenate was spun at 40,000 × g for 30 min at 4°. The supernatant was decanted and incubated for 20 min at 37° and then spun at 40,000 × g for 30 min at 35°. The pellet was resuspended in a small volume of GEP at 37° and spun at 40,000 × g for 20 min at 35°. This pellet was resuspended in GEP at 0° and extracted for 30 min at 0°. After a 4° centrifugation as above, the supernatant was decanted. Tx was then added, and the preparation (Cycle I) was stored on ice until used. We sometimes took this material through one or two more cycles of warming and cooling with centrifugation to enhance purity. Total protein was estimated by the method of Lowry *et al.* (20). The purity of the tubulin preparations was assayed by acrylamide gel electrophoresis in 1% sodium laural sulfate (21). The capacity of our preparations to form microtubules *in vitro* was monitored in a qualitative fashion by negative staining and electron microscopy (22).

Pt K_1 cells (from the Rat Kangaroo) were maintained in Falcon flasks using Ham's F-12 medium supplemented with 10% fetal-calf serum and antibiotics. In preparation for light microscopy, cells were grown on glass coverslips, mounted on slides with coverslip fragments as spacers, and waxed down with a 1:1:1 mixture of vasoline, lanolin, and paraffin. Such a chamber filled with conditioned medium will support the initiation and completion of mitosis for about 4 hr. The preparations were maintained at 30° or 37° on the microscope stage with an air curtain incubator. Temperature jump experiments were performed by passing water of defined temperature through a thin perfusion chamber used as a supporting slide. Spindle structure and function were monitored with phase, differential interference contrast, and polarization optics.

When the medium bathing the cells was to be changed, the wax seal was broken on either side, and the new solution was flushed under the coverslip using filter paper to draw the old fluid out and the new one in. Photographs of changes in spindle

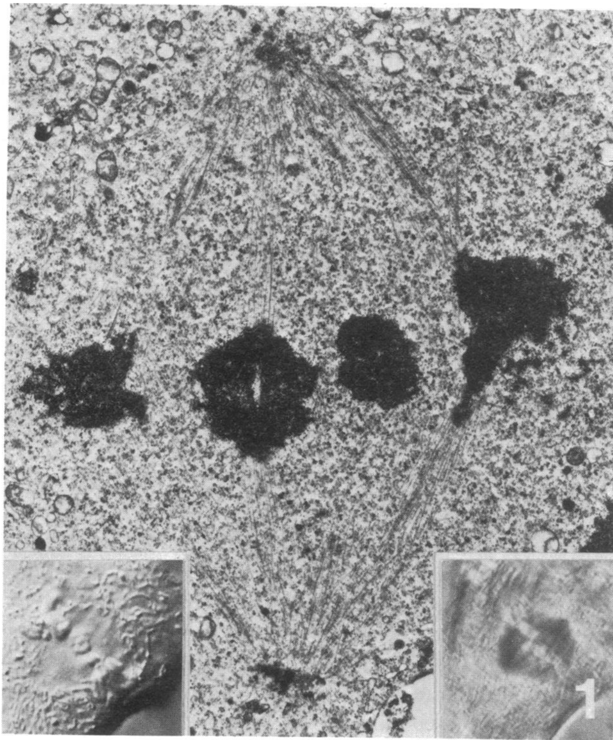

FIG. 1. Normal metaphase in Pt K_1. Electron micrograph is × 9000. *The left lower insert* is a differential interference contrast of a living cell; the *right lower insert* is a polarization optical light micrograph of the same cell. All light micrographs in paper are ×1000.

structure were made on Kodak TRI-X film developed in Diafine to a speed with an ASA rating of 1600.

For electron microscopy, cells were grown on coverslips or in Falcon dishes coated with Dri-slip (3-M Corp.) to facilitate removal of the hardened plastic. A thin layer of rat-tail collagen dried on top of the Dri-slip enhanced the sticking of the cells through fixation and dehydration. After experimental treatment, the preparations were fixed following the method of Brinkley *et al.* (23). Cells of interest were selected, excised, sectioned, picked up on slot grids, stained with uranyl acetate and lead citrate, and examined in a Philips 300 electron microscope.

RESULTS

1. Stability

Spindles vary considerably from one living cell to the next but during about 10 min at metaphase, the size, shape, and birefringence of a PT K_1 spindle are approximately constant and appear as shown in Fig. 1. When metaphase cells are treated at 37° with 0.03–0.2% Tx in GEP, the spindle shrinks in length and breadth, and the birefringence fades to insignificance (Fig. 2). The rate of spindle dissolution decreases as the concentration of detergent is increased, ranging from 1 min in 0.03% detergent to about 5 min in 0.2% detergent for total loss of birefringence and disappearance of essentially all microtubules. Probably the more rapid lysis at higher detergent concentrations allows quick entry of the buffer which supports tubule reassembly and thus promotes spindle stability, while slower lysis allows intermediate conditions to exist which are not so favorable. We have observed by serial section and electron microscopy that the bundles of microtubules that end on kinetochores (the chromosomes tubules) are slower to dissolve under conditions of rapid lysis than the tubules which do not end on a kinetochore (polar tubules).

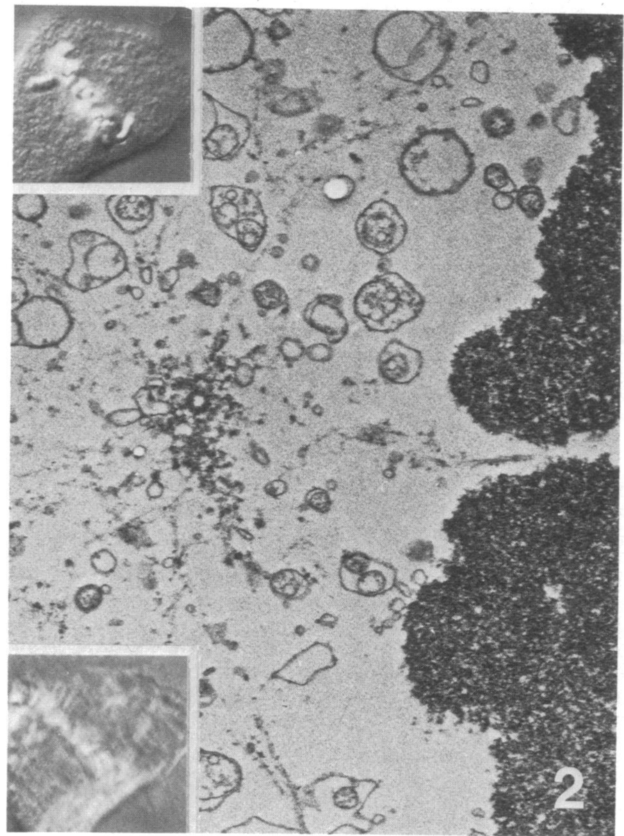

FIG. 2. Electron micrograph of cell lysed in GEP with 0.1% Tx at 37° 5 min prior to fixation (×16,000). *Inserts* show the same cell as the *inserts* in Fig. 1 about 3 min after addition of 0.05% Tx.

When cells are treated at 37° with 0.03–0.2% Tx in GEP which contains 3 mg/ml of the tubulin preparation characterized in Fig. 3, the interpolar distance does not decrease and the majority of the birefringence is stable for over an hour (Fig. 4). The stabilized spindles contain many microtubules but little matrix or granular material. When the matrix washes away during lysis, the spindle fibers can be discerned in the light microscope as well as the electron microscope (Fig. 4). As the fibers become visible, they appear to converge on a small, phase-dark body which is resolved in the electron microscope as two centrioles and the associated osmiophilic material of the spindle poles. We have come to use the first appearance of the poles in the light microscope as a criterion for cell lysis, although electron microscopy shows that in these preparations lysis is far advanced by the time the poles can be seen with differential interference contrast optics. Events which occur after the appearance of the poles are regarded as taking place in a lysed cell.

We have investigated the stability at 37° of the detergent-treated spindle as a function of tubulin concentration. In 1mg/ml of tubulin, the spindle is stable even though few microtubules are found in negative stained preparations made to test the ability of the tubulin to polymerize. At about 0.2 mg/ml, the spindle length decreases by about 1/4 in the first 4 min and is then approximately stable, while the birefringence

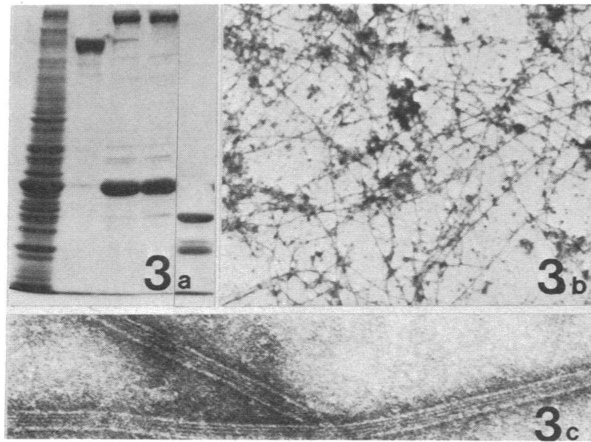

FIG. 3. Fig. 3*a* shows (*left to right*) the electrophoretic patterns of the supernatant from the first warm centrifugation, a myosin standard, Cycle I and Cycle II tubulin preparations, and an actin standard. Note the high molecular weight component that co-purifies with tubulin by this method. Quantitative scanning of the gels shows that the tubulin comprises approximately 60% of the Cycle I preparation and 75% of Cycle II. Fig. 3*b* shows tubules reassembled *in vitro* in GEP plus 0.1% Tx at 37° (×8,500). Fig. 3*c* shows the substructure of these tubules (×102,000).

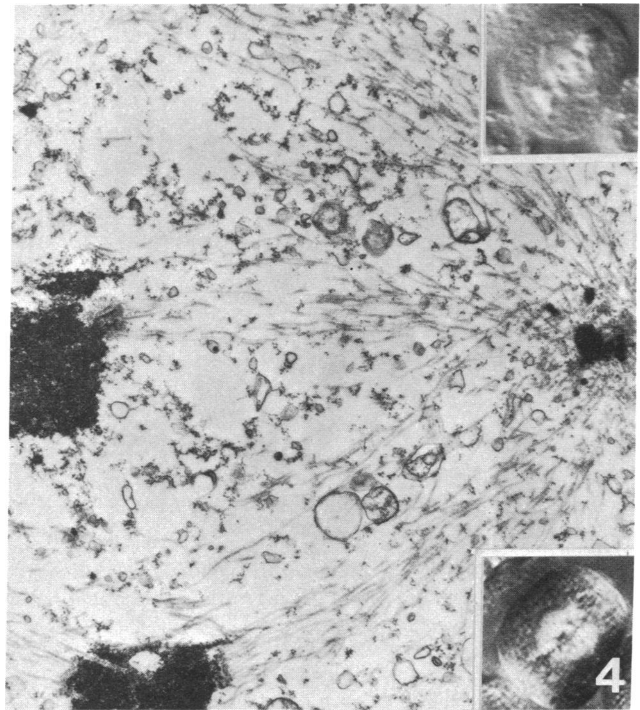

FIG. 4. Electron micrograph of a cell lysed in GEP with 0.1% Tx and 4 mg/ml of a Cycle II tubulin preparation at 37° 5 min prior to fixation (×13,600). Light micrographs show a cell which has been lysed for about 1 hr.

continues to fade; only a few chromosome fibers are left after 10 min. At 0.1 mg/ml the spindle shrinks both in length and in birefringence (Fig. 5). The rate of shrinking is less than that found with no tubulin at all. Below this concentration, the tubulin does not have any appreciable stabilizing effect.

We have asked whether the capacity of tubulin to preserve the mitotic spindle is blocked by the factors which interfere with microtubule assembly *in vitro.* If 5 mM $CaCl_2$ is added to a preparation known to preserve spindle length and birefringence, the spindle fades and shrinks as if there were no tubulin present. If a preparation known to stabilize the spindle is made 10^{-4} M in colcemid (the molarity of the tubulin is about 2.5×10^{-5} M), the spindle also shrinks as if there were no tubulin present. If tubulin is prepared without GTP by extracting washed tubules with GTP-free buffer at 0°, the preparation will confer limited stability to the spindle of a cell lysed at 37°: the birefringence fades over about 15 min. Tubulin preparations that are kept on ice for more than 8 hrs lose their ability to stabilize spindles. If a preparation of GEP with 0.1% Tx is made 5 mg/ml in bovine-serum albumin, the spindle fades as it does in GEP with Tx alone. In all these experiments the spindle birefringence behaves like an assembled, ordered form of tubulin that is in equilibrium with an unassembled, disordered form (13). The greater stability of the chromosome fibers suggests that either the equilibrium or the rate constant for the disassembly of these fibers is different from that of the polar spindle tubules.

2. Lability

Once a spindle has been stabilized by lysis into a sufficient concentration of repolymerizable tubulin, it is no longer so labile as it is *in vivo*. When the tubulin is washed away with GEP, the spindle is still stable over many minutes. It will disappear in about a minute, however, when washed with 0.1 M sodium phosphate buffer at pH 6.9. We conclude that GEP possesses some stabilizing properties in addition to those provided by the tubulin. If the stabilized spindles are cooled shortly after lysis, spindle birefringence will decrease significantly, but the spindles lose their cold lability with time.

3. Regrowth of birefringence

If a metaphase cell is lysed in GEP with tubulin and then cooled to make the spindle birefringence decrease, the birefringence will partially reappear when the preparation is warmed back up to 37° (Fig. 6). The length of the regrown spindle is less than that of the stabilized spindle prior to cold treatment, but the birefringence is essentially the same (Fig. 6*c*). We have also tried to regrow spindles which shrank and lost birefringence after detergent treatment in GEP with low concentrations of tubulin. Although the birefringence of a shrunken spindle will increase upon the addition of 2 mg/ml of tubulin at 37°, we have not been able to increase the spindle length (Fig. 7).

Fig. 8 follows a live cell as it is cooled to 0°, lysed at that temperature into GEP plus tubulin, and then rewarmed to 37°. There is a fully birefringent spindle with enlarged asters in the tubulin-containing sample, while a cell lysed into GEP without tubulin does not regrow birefringence (Fig. 8*g*). The birefringence of spindles isolated into tubulin-containing buffers thus seems to display a temperature dependence which is qualitatively the same as the birefringence of the spindle *in situ*.

4. Motility

Given the capacity of these spindles to respond to various treatments in a fashion resembling the living spindle, we have looked to see whether they can also move chromosomes.

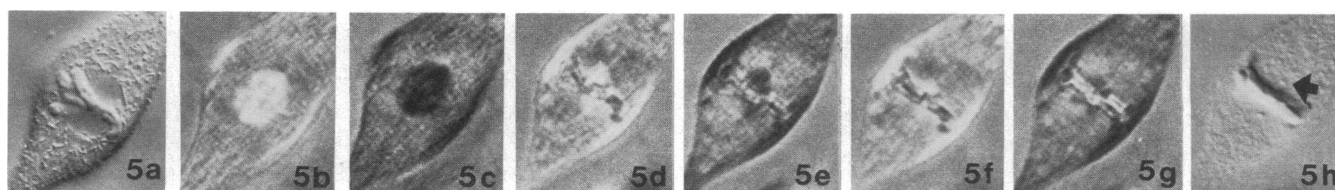

FIG. 5. Figs. 5*a*, *b*, and *c* show a cell before lysis; 5*d* and *e* are 100 sec after addition of 0.05% Tx in GEP with 0.1 mg/ml of tubulin at 37°; 5*f* and *g* are at 5 min. Fig. 5*h* is at 6 min. An *arrow* marks one presumptive pole.

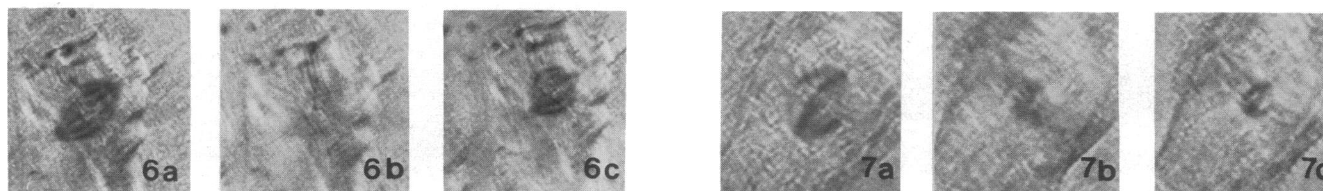

FIG. 6. Fig. 6*a* shows a spindle 3 min after lysis in GEP with 0.1% Tx and 0.6 mg/ml of tubulin at 37°; 6*b* is after 3 min at 0°; 6*c* is 2 min after the addition of 2.4 mg/ml of tubulin in GEP at 37° (18 min after lysis).

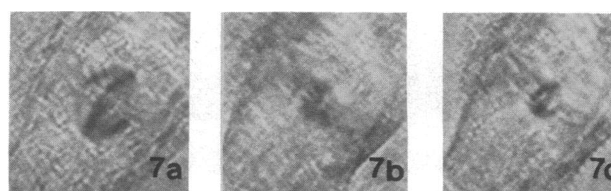

FIG. 7. Fig. 7*a* shows a spindle immediately after addition of 0.05% Tx in GEP with 0.1 mg/ml of tubulin at 37°; 7*b* is 3 min later; 7*c* is 5 min after addition of about 2 mg/ml of tubulin in GEP at 37°.

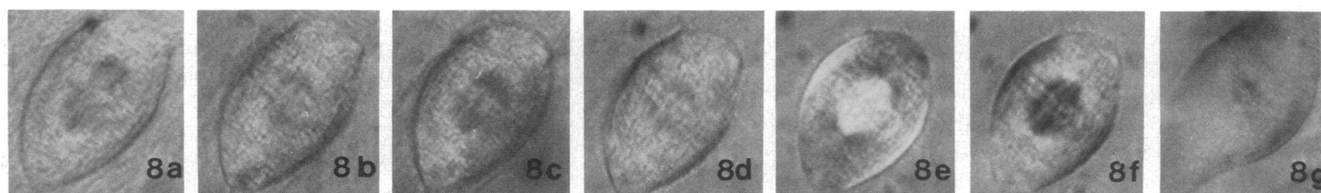

FIG. 8. A series showing regrowth after lysis at 0°. Fig. 8*a* is a normal cell; 8*b* has been cooled to 0° for 4 min; 8*c* has been rewarmed to 37°. Note that the spindle *in vivo* does not grow back to its normal length; 8*d* has been recooled to 0° and lysed with 0.1% Tx in GEP with 3 mg/ml of tubulin (the birefringence increases slightly upon lysis in this buffer); 8*e* and *f* are 4 min after a return to 37°; 8*g* shows a cell lysed at 0° into the same buffer but without tubulin. The picture was taken 4 min after warming to 37°.

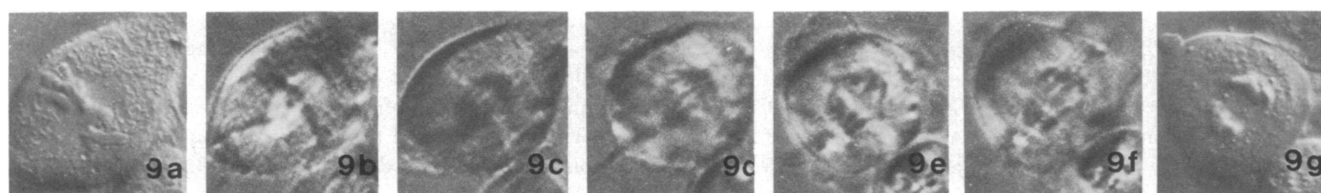

FIG. 9. A series showing anaphase-like motion of chromosomes after lysis. Figs. 9*a*, *b*, and *c* show a living metaphase cell; 9*d* is 3.5 min after addition of 0.05% Tx in GEP with about 2 mg/ml of tubulin at 37°; 9*e* is 9 min after, and *f* and *g* are about 20 min after addition of the detergent.

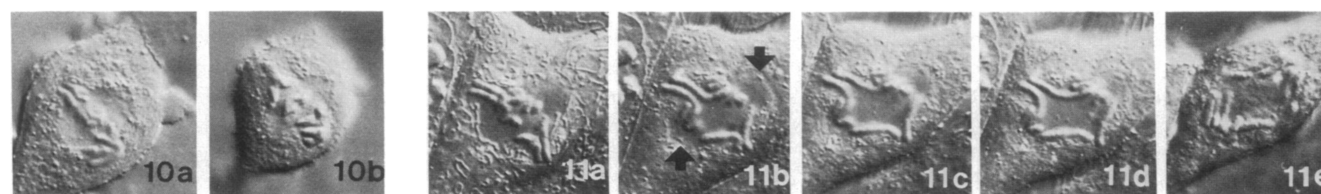

FIG. 10. The chromosomes stop after addition of 5% Carbowax in PIPES buffer with 1 mM EGTA and 0.1% Tx. Fig. 10*a* is 3 min after addition; 10*b* is 12 min after addition.

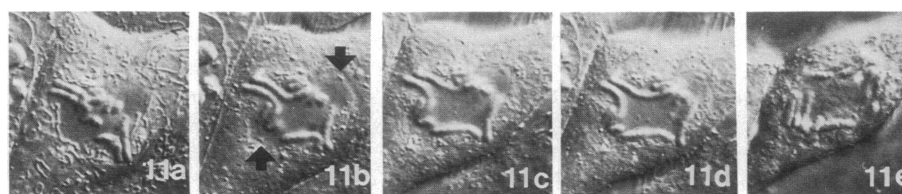

FIG. 11. A series showing anaphase-like motion of chromosomes after lysis in GEP with 0.1% Tx and 5% Carbowax at 37°. Fig. 11*a* is about 10 sec after addition; 11*b* is 2 min after. In 11*b* the mitochondria are no longer visible in the cytoplasm and the poles have appeared (*arrows*). Fig. 11*c* is after 4 min; 11*d* is after 5 min; and 11*e* is after 14 min.

Fig. 9 shows a cell which becomes distinguishable as an anaphase about 3.5 min after the addition of 0.05% Tx in GEP and tubulin. In half that time, a spindle in detergent and GEP alone would have dissolved, so we feel confident that the membrane is no longer a stringent permeability barrier. Nonetheless, the chromosomes separate as they would in normal anaphase, albeit approximately $^1/_5$ as fast.

Gibbons has found that high molecular weight polyethylene glycol (Carbowax 20M) improves the capacity of isolated sea urchin sperm tails to be reactivated (24). This observation prompted us to see if we might improve anaphase motility with the same ruse. We have found that 5% Carbowax not only improves the capacity of the tubulin-isolated spindles to function, it also supports spindle stability, chromosome motion, and spindle elongation in the complete absence of exogenous tubulin. Two percent Carbowax in PIPES buffer, pH 6.9 with 1 mM EGTA and 0.1% Tx will maintain spindle length for hours, although the birefringence gets somewhat weaker with time and the shape of the spindle is not faithfully preserved. The same solution added to an anaphase cell will stop the motion of the chromosomes within a few seconds of apparent cell lysis—that is, when the rod-shaped mitochondria disappear and the poles appear (Fig. 10). When 0.1–2.5 mM ATP or GTP is added to this solution and used to lyse an early anaphase cell, the chromosomes keep on moving in a remarkably normal fashion (Fig. 11).

The observed increase in spindle length during chromosome motion, in the absence of exogenous tubulin, implies that spindle fibers are repositioned during anaphase and that growth and dissolution of spindle tubules is not a sufficient hypothesis for the mechanism of anaphase motions. We cannot, however, exclude the possibility that our conditions of lysis in the presence of Carbowax are mild enough to retain an endogenous pool of tubule subunits which can contribute to the elongation of the spindle.

DISCUSSION

Spindles preserved by lysis into solutions of tubulin should be useful for analysis of at least two aspects of mitotic mechanism: the cellular control of spindle formation and the biochemistry of anaphase. For instance, a study of the differential stability of the chromosome and the polar spindle tubules originally described by Brinkley *et al.* (25) may reveal differences in the relevant cellular control systems. An understanding of why we cannot yet increase the length of a metaphase spindle *in vitro* may shed light on the cellular control of spindle growth in particular and microtubule assembly in general.

The capacity of the spindles in lysed cells to move chromosomes is perhaps the most convincing evidence that our preparations are an approximation to physiological normality. We hope to be able to use these preparations to study further the enzymes relevant for anaphase and thus to determine whether some known mechano-chemical protein such as myosin or dynein is an active component in chromosome motion.

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1. Kane, R. E. (1962) *J. Cell Biol.* **15**, 279–287.
2. Rebhun, L. I. & Sander, G. (1967) *J. Cell Biol.* **34**, 859–883.
3. Goldman, R. D. & Rebhun, L. I. (1969) *J. Cell Sci.* **4**, 179–209.
4. Cohen, W. D. & Gottlieb, T. (1971) *J. Cell Sci.* **9**, 603–619.
5. Borisy, G. G. & Taylor, E. W. (1967) *J. Cell Biol.* **34**, 535–548.
6. Bibring, T. & Baxandall, J. (1969) *J. Cell Biol.* **41**, 577–590.
7. Forer, A. & Behnke, O. (1972) *Chromosoma* **39**, 145–173.
8. Cohen, W. D. & Rebhun, L. I. (1970) *J. Cell Sci.* **6**, 159–176.
9. Kane, R. E. (1965) *J. Cell Biol.* **25**, **2**, 137–144.
10. Miki, T. (1963) *Exp. Cell Res.* **29**, 92–101.
11. Weisenberg, R. & Taylor, E. W. (1968) *Exp. Cell Res.* **53**, 372–384.
12. Mazia, D., Petzelt, C., Williams, R. O. & Meza, I. (1972) *Exp. Cell Res.* **70**, 325–332.
13. Inoué, S. & Sato, H. (1967) *J. Gen. Physiol.* **50**, 259–286.
14. Hoffman-Berling, H. (1954) *Biochim. Biophys. Acta* **14**, 182–194.
15. Weisenberg, R. (1972) *Science* **177**, 1104–1105.
16. Weisenberg, R. (1972) *J. Cell Biol.* **55**, 277 abstr.
17. Inoué, S., Borisy, G. G., & Kiehart, D. P. (1973) *Biol. Bull.* **145**, 441.
18. Rebhun, L., Lefebvre, P., & Rosenbaum, J. (1973) *Biol. Bull.* **145**, 451.
19. Borisy, G. G. & Olmsted, J. B. (1972) *Science* **177**, 1196–1197.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
21. Laemmli, U. K. (1970) *Nature* **227**, 680–685.
22. Huxley, H. E. (1963) *J. Mol. Biol.* **7**, 281–308.
23. Brinkley, B. R., Murphy, P., & Richardson, L. C. (1967) *J. Cell Biol.* **35**, 279–283.
24. Gibbons, B. H. & Gibbons, I. R. (1972) *J. Cell Biol.* **54**, 75–97.
25. Brinkley, B. R., Stubblefield, E. & Hsu, T. C. (1967) *J. Ultrastr. Res.* **19**, 1–17.