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ATP-dependent formation and motility of aster-like structures with isolated calf brain microtubule proteins

(mitosis/tubulin/transport)

RICHARD C. WEISENBERG*, ROBERT DAY ALLEN†, AND SHINYA INOUÉ‡

*Department of Biology, Temple University, Philadelphia, PA 19122; †Department of Biological Sciences, Dartmouth College, Hanover, NH 03755; and ‡The Marine Biological Laboratory, Woods Hole, MA 02543

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ABSTRACT **Microtubule proteins isolated from calf brain will undergo gelation–contraction in the presence of ATP. We have now examined this process by video-enhanced contrast microscopy. After ATP addition to steady-state microtubules, slow (1–5 μm/min), linear movements of particles and microtubules toward aggregation centers occur. The resulting structures resemble mitotic spindle asters. During the time when gel contraction occurs, asters move (at 1–5 μm/min) toward other nearby asters. This is accompanied by the apparent shortening of the microtubules running between the asters. This is the first example of isolated microtubules undergoing a process that has similarities to half-spindle shortening during anaphase A. Formation of aster-like structures without preformed microtubule organizing centers raises the possibility that a similar process may contribute to microtubule organization *in vivo*.**

Cytoplasmic microtubules have been implicated in the movement of organelles in many cells (for review, see ref. 1), including nerve cells, where their role in fast axonal transport of vesicles has been demonstrated (2, 3). Very little is known about the mechanism of action of cytoplasmic microtubules. A requirement for ATP has been demonstrated for anaphase B (spindle elongation) (4) and for particle movements along neuronal microtubules (2, 3, 5), but the mechanism of action of ATP in these processes remains unknown.

It has been found recently that microtubules isolated from calf brain by three cycles of assembly and disassembly will undergo "gelation–contraction" in the presence of ATP (6). We have now examined this process by computer-assisted, video-enhanced contrast microscopy (7–9). These observations reveal the formation and motility of structures with a striking resemblance to mitotic asters and spindles. The motility observed differs significantly from that reported previously by isolated microtubules but displays some similarities to half-spindle shortening during anaphase A.

METHODS

For each experiment, microtubule proteins were isolated as described (6) except that the third-cycle microtubule protein was frozen by dripping the solution into liquid nitrogen. The protein was stored at liquid-nitrogen or dry-ice temperature until needed, at which time the frozen protein pellets were rapidly thawed and then polymerized at 37°C in 1 mM GTP/1 mM EGTA/0.5 mM $MgCl_2$/0.1 M 4-morpholeneethanesulfonic acid (Mes), pH 6.6. The protein was used either undiluted, at about 5 mg/ml, or was diluted 1:1 to 1:2 with buffer before ATP addition to make individual microtubules more visible. Incubation was carried out for 30 min to assemble microtubules to a steady-state array, at which time 2 mM ATP was added. Slides were immediately prepared by using a single drop of solution, and the coverslip was sealed with VALAP [a 1:1:1 (wt/wt) mixture of Vaseline, lanolin, and paraffin) or clear nail polish. The sample was maintained at approximately 37°C either by placing the microscope in a heated room (when using a ×100 oil immersion objective) or by heating the stage with an air curtain incubator (when using a dry objective). The air curtain incubator was positioned to operate continuously while holding the slide at the desired temperature to eliminate artifacts caused by cyclic temperature changes.

Observations were performed by using either phase-contrast, differential-interference-contrast (DIC), or polarization optics. Details and operation of the image-enhancement systems have been described elsewhere (7–9). Measurements were performed by using either an *x-y* video signal generator (Colorado Video, model 321) or by analysis of still photographs of the video image.

RESULTS AND DISCUSSION

A survey view of the process of microtubule gelation–contraction as observed using DIC optics is shown in Fig. 1 *a–e*. Fig. 1*a* was taken 7 min after addition of ATP to the sample (the time required to prepare the slide and to perform the image-enhancement operations). At this time the solution appears to be almost uniformly "grainy," with particulate matter dispersed throughout the solution (the nature of the particulate matter is discussed below). Although microtubules are not clearly visible in this figure, direct observation of the video image and negative-stain electron microscopy reveal the presence of microtubules and particles throughout the time period investigated.

The first visible change in the solution, which occurs by 10 min after ATP addition, is the formation of particulate "islands" (Fig. 1*b*). The formation of the particulate aggregation centers appear to occur, at least in part, by directed motility of particles. Particle movements follow linear tracks at rates that vary from about 1 to 5 μm/min and are primarily directed towards the aggregation centers (Fig. 2 and 3). Particle movements are discussed in more detail below.

The particulate aggregations gradually become more defined, as they accumulate more material and increase in density. Microtubules are observed to radiate out from the particulate aggregation centers, forming structures resembling mitotic spindle asters (6). The precise mechanism by which microtubules and particulates become organized into the aster-like structures is not yet clear, but motile processes are clearly involved. Microtubules present initially (Fig. 4*a*) appear to interconnect small particles, and it is likely that directed movements of microtubules and particles result in aster formation. Comigration of particulates and microtubules towards a growing aster is visible in Figs. 2 and 4;

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Abbreviations: DIC, differential-interference contrast; MTOCs, microtubule-organizing centers.

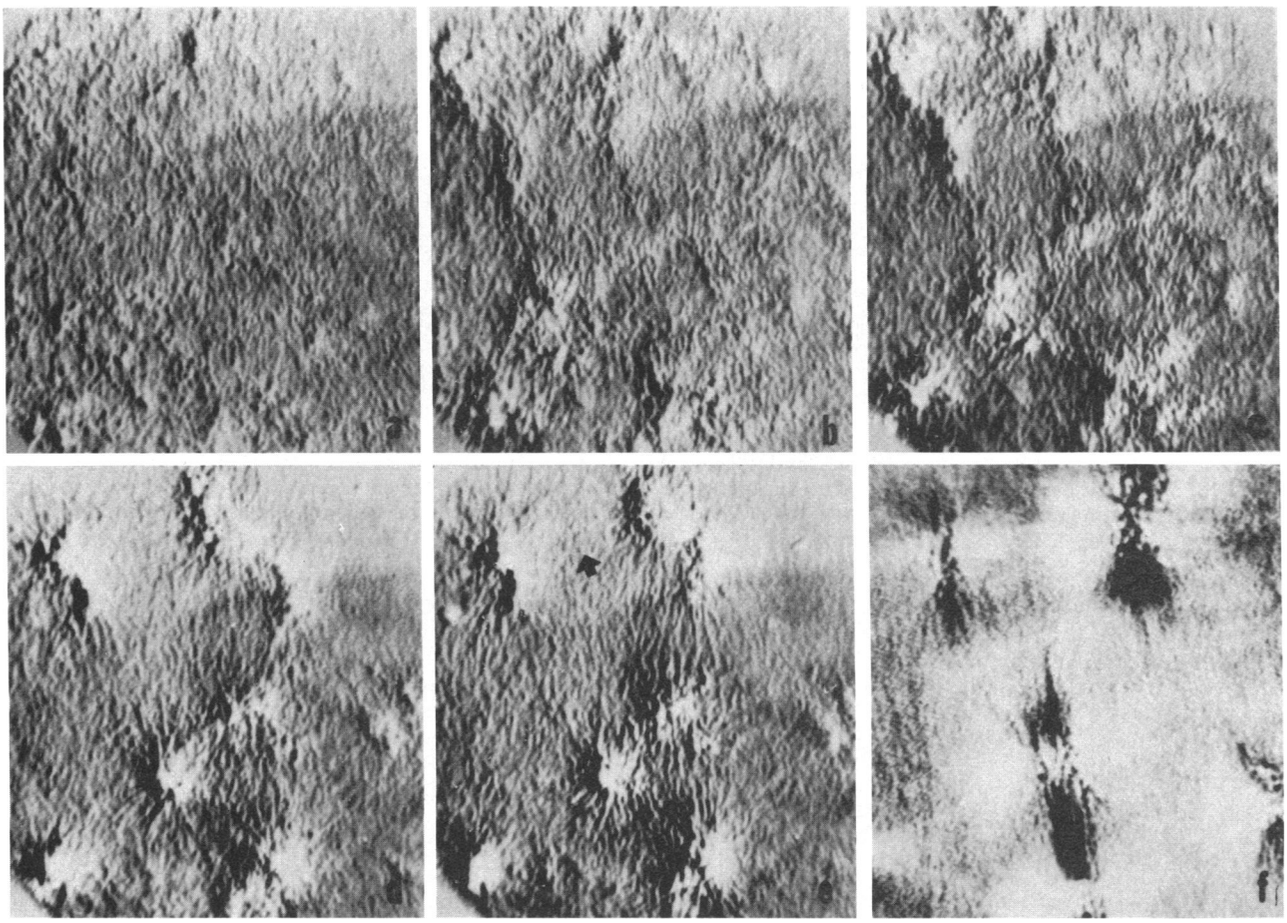


FIG. 1. Microtubule gelation–contraction viewed by DIC and polarization optics. This experiment was performed with a rectified microscope (8) with a ×40 n.a. 0.95 lens. Micrographs were taken at specified times after addition of 2 mM ATP to microtubules assembled to a steady-state array under standard conditions: 7 min (*a*), 12 min (*b*), 17 min (*c*), 22 min (*d*), 27 min (*e*), and 60 min (*f*). *f* was taken with polarization optics after slight rotation of the sample. The arrow indicates the location of a particle that underwent bidirectional movement. (×1200.)

however, the actual behavior of the microtubules has not been possible to follow in detail. Significant motility was not observed at low microtubule densities, and, at high microtubule and particulate concentrations, visualization of individual microtubule behavior is difficult. No examples of microtubule "gliding" motions (2, 3) have been observed. [Note that individual microtubules cannot be distinguished from small bundles of microtubules and that the smaller particles observed may be below the limit of resolution of the microscope (7–9) and cannot be assigned a size.]

Asters that are close together, such that their microtubule arrays overlap, appear structurally similar to animal cell

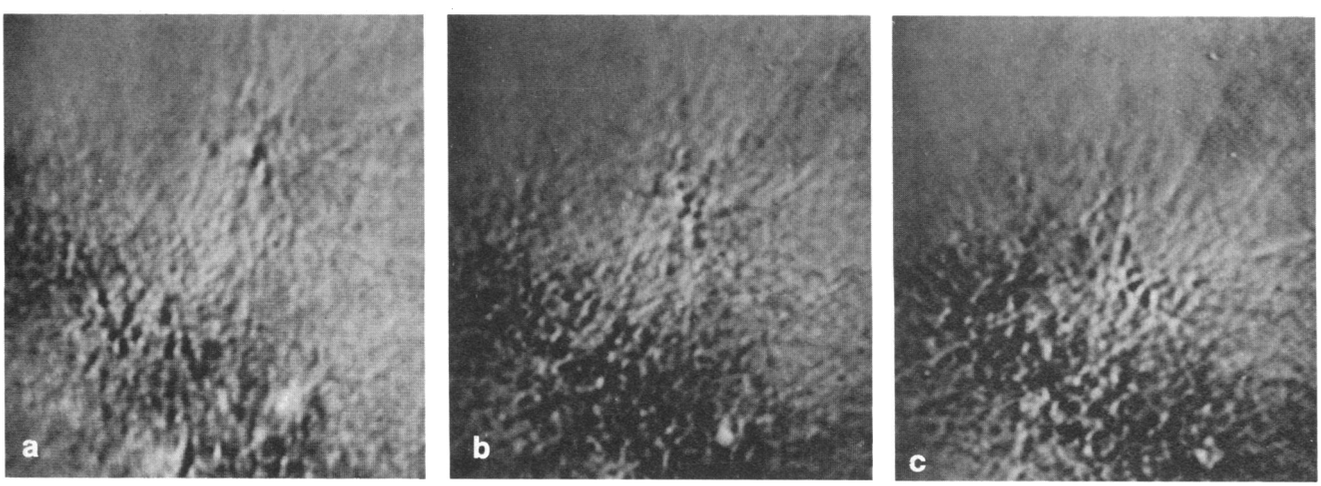


FIG. 2. Movement of a group of particles toward a microtubule focal center, observed with DIC optics. (*a*) Micrograph taken approximately 20 min after ATP addition. (*b* and *c*) Subsequent micrographs taken at 2-min intervals. Note that both particulates and microtubules are transported toward the growing aster center. Individual particles within a group appear to move independently of other, nearby particles. This phenomenon is shown more clearly in Fig. 3. (×2200.)

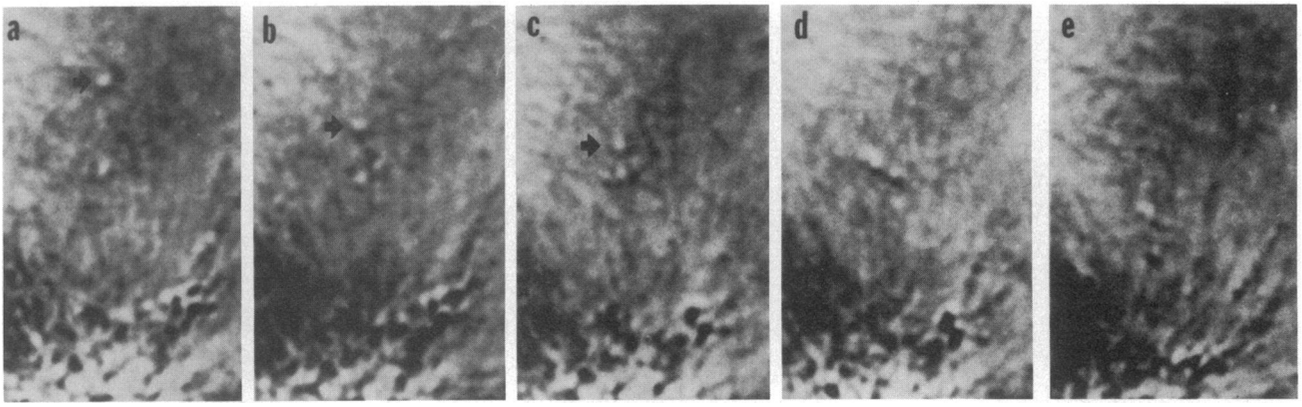
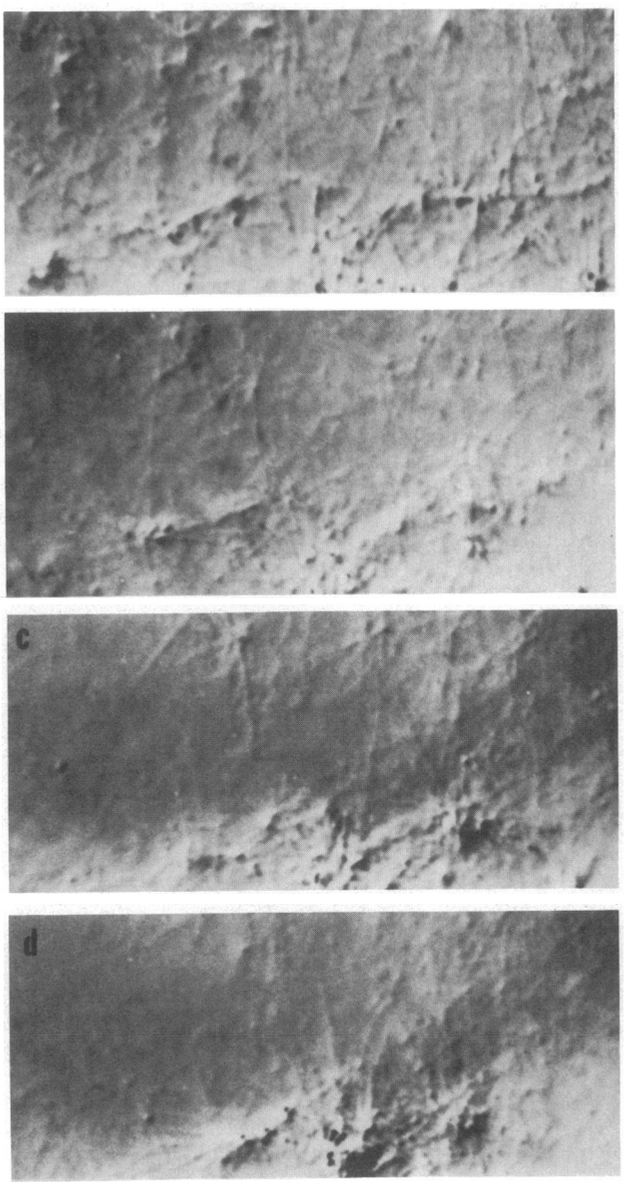


FIG. 3. Independent movement of particles. The particle indicated by the arrow in *a* was initially stationary and then began to move in a straight line at a uniform velocity. A group of particles (just above the center of the field) remained stationary, although they were directly in line with the moving particle. After "collision" of the moving and stationary particles (*d*), movement appeared to stop briefly. Movement then continued, but it was not possible to still identify the original particle. Micrographs *a–d* were taken at 30-sec intervals, and *e* was taken 1.5 min after frame *d*. (×2800.)

mitotic spindles. Such spindle-like structures frequently shorten, indicating the existence of physical interaction between the asters. This phenomenon is visible in Fig. 1 and is shown more clearly in Fig. 5, in which two large aster-like structures are seen to move closer together. Such movements may continue until the aster centers can no longer be resolved as separate structures, and a single larger aster-like structure is formed. The rates of these movements (as measured by the decrease in distance between the aster centers) is 1–5 μm/min, about the same as that of individual particle motions, and are independent of the size of the asters. Much faster rates of movement (relative to a stationary reference) have been observed, but this appears to be the result of the summation of the velocities of several asters connected in series. Microtubule focal centers also have been observed to move farther apart (e.g., Fig. 1 *Upper*), but these movements may be due to forces generated from other centers out of the field of view. Frequently three or more centers may be interconnected and all will move toward a central location.



The fate of the microtubules that exist between asters that are moving together is not known. One possibility is that the microtubules shorten. Apparent shortening of microtubules is seen in Fig. 6, in which particulate material appears to be connected to a focal center by visible fibers, presumably microtubules, which appear to shorten as the particles move toward the center. Note in Fig. 6 that the distances between the growing focal center (at the lower right corner of Fig. 6) and each of the two groups of attached particles decrease, as does the distance between the two groups of particles. Although fibers may appear to shorten, as shown in Fig. 6, the fate of the microtubules involved has not been determined, and we presently have no reason to believe that individual microtubules shorten. It also is possible that they pass into and through the approaching aster centers.

The movement of particles is overwhelmingly toward microtubule focal centers (only a few examples of bidirectional movements along a single track have been observed). Particles may move independently of other nearby particles (Fig. 3). The indicated particle in Fig. 3 moved in a uniform velocity along a straight tract until it encountered a group of stationary particles. Particle movements continued along the original track, although it is not possible to identify the original moving particle. Even where several particles appear

FIG. 4. Formation of aster-like structures at high resolution. The microtubule protein solution was diluted (to approximately 2 mg/ml) and observed with DIC optics. Note that microtubules and particulates are nearly randomly distributed in *a* (taken about 10 min after addition of ATP) but that they form an interlocking "network." The fibers connect particulates that subsequently move together to form astral centers. (×4100.)

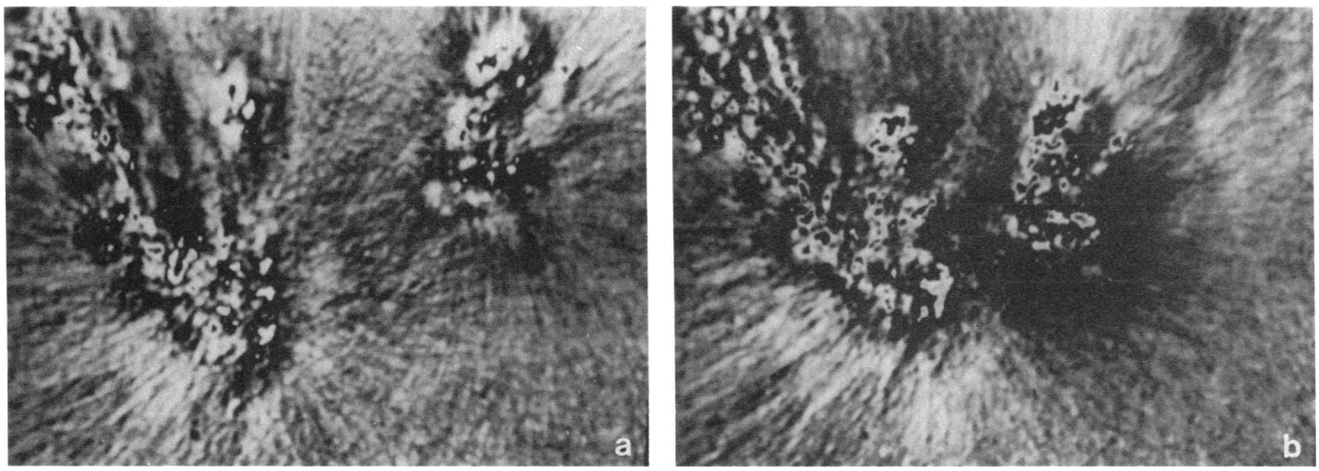


FIG. 5. Movement of asters toward one another. The bright areas in the centers of the asters are an artifact of contrast enhancement. The fate of microtubules that are between the focal centers at the onset of contraction cannot be determined from this image. The time interval between *a* and *b* was 8 min. (×3300.)

to move as a group (Fig. 2), close examination of the time-lapse tapes indicates that individual particles within each group are moving independently.

The nature of the particulate material has not been determined. A cold- and calcium-stable aggregate is present in these preparations (6), and it probably accounts for most of the particulate matter observed. Preliminary results into the ultrastructure and biochemistry of the cold-stable aggregate have not revealed any consistent differences between the protein composition of the cold-stable aggregate and the bulk microtubule preparation. There is no indication of membrane-limited vesicles in our preparations. The cold-stable aggregate is heterogeneous in appearance, cannot yet be distinguished from aggregates of "denatured" protein frequently observed in microtubule protein preparations, and may consist of such aggregates. It should be noted, however, that a cold- and calcium-stable aggregate of tubulin is a major component of nerve cells (10, 11) and nonmicrotubular aggregates of tubulin may exist in mammalian (12) and molluscan cells (13).

It is not clear how the present observations relate to the behavior of microtubules *in vivo*. While microtubules have been shown to support vesicle transport both *in vivo* and *in vitro* (2, 3), the rate of particle movement typically observed here (1–5 μm/min) is substantially slower than that of particles moving by fast axonal transport (100–300 μm/min). Furthermore, the particles present in our preparations are protein aggregates, not membrane-bound vesicles, which are the major component of fast axonal transport. The rates observed here are most similar to that reported for anterograde slow axonal transport of actin and soluble proteins (14) and retrograde axonal transport of a 68,000-dalton protein (15). It is not yet known, however, if the conditions we are using yield optimum velocities or if cytoplasmic vesicles will undergo motility in our system.

Another difference between the present observations and those previously reported for microtubules *in vitro* is the reorganization of microtubules and particulates into aster-like structures. The organization of microtubules in cells is under the control of microtubule-organizing centers (MTOCs), which are believed to act by nucleating the assembly of microtubules (16), but they may also function as microtubule attachment sites (17). The relationship of the microtubule focal centers formed *in vitro* to cellular MTOCs is unknown. The formation of foci by a contractile system is not unexpected (18), and the similarities of the structure formed *in vitro* to cellular structures may be accidental. The present observations may be related to the process by which MTOCs undergo maturation (19, 20) and redistribution in

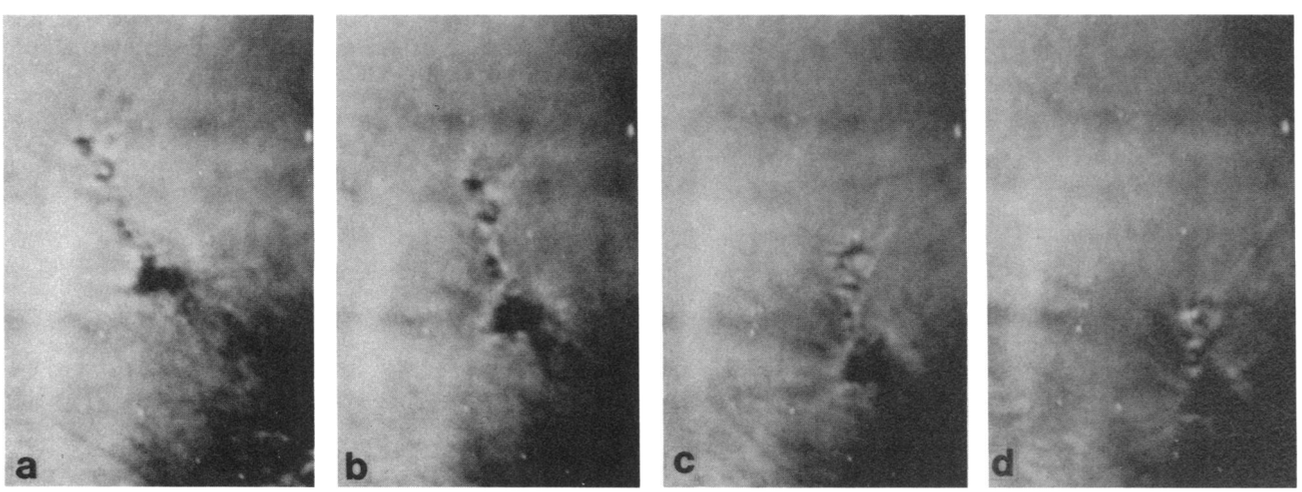


FIG. 6. Movement of connected particulate structures. A growing center (just off the lower right corner of the figure) is connected to a large object which is connected to a set of smaller particulates. Note that the upper particles move toward the lower one, while both move toward the growing center. (×2700.)

cells, such as the change from multiple to single MTOCs in developing neurites (21).

We have seen no indication that microtubules grow from the particulate centers, and since microtubules are present continuously, nucleated assembly from the centers appears unlikely. The possibility exists that some microtubules may undergo complete disassembly, and the released subunits could then grow new microtubules from the centers (16). However, we have not observed any indication of such a phenomenon. Most likely, microtubules move along with attached particles and become assembled into aster-like structures by such movements.

Another unique aspect of our observations is the movement of asters toward one another. This movement appears most like the shortening of the half spindle during anaphase in that it involves the apparent shortening of a microtubule array and occurs at approximately the same rate. It has been reported that half-spindle shortening does not require the presence of ATP (4), which would appear to distinguish it from the present results. However, it should be noted that movement of asters in our system does not occur until sometime after the addition of ATP, and it is not yet known if ATP is still required during this period.

A characteristic feature of microtubule gelation–contraction is a delay of about 30 min that occurs between the time of ATP addition and the time when contraction of the gel is first visible (6). From the present observations, this delay appears to reflect the time required to assemble an interconnected network of asters. Subsequent movements of the asters towards one another result in contraction of the gel. Gelation–contraction of microtubules *in vitro* does not appear directly related to cellular phenomena usually associated with microtubules; however, extending neurites cut with a microneedle undergo an energy-dependent "collapse," or contraction. This process may be dependent upon microtubule function and has been proposed to reflect the normal mechanisms of neuronal growth (22).

The mechanism of the movements observed here is unknown. ATP can induce sliding of flagella microtubules (23) and microtubule depolymerization (24) and modulates the cross-bridging of microtubules to other microtubules (25, 26) and to other cytoskeletal elements (27, 28). Microtubule protein prepared by cycles of assembly contains both ATPase (29) and protein kinase activity (30). Many mechanisms are thus possible for ATP-dependent motility by microtubules as reported here. Furthermore, a single mechanism of microtubule-based motility appears unlikely to explain the numerous functions of microtubules *in vivo*. The significant differences observed between fast particle transport by microtubules (2, 3) and microtubule gelation–contraction as reported here indicate the operation of distinct mechanisms for these processes.

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