ATP-induced Gelation–Contraction
of Microtubules Assembled In Vitro

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ABSTRACT   We report here an ATP-dependent formation and contraction, or syneresis, of a microtubule gel using microtubule proteins prepared from calf brains. Gel contraction is typically observable 15–30 min after ATP addition to microtubules assembled to steady state, and is complete after ~60 min, at which time the gel volume is reduced by as much as 75%. In contracted gels, microtubule bundles and aster-like structures are observable. Gelation–contraction requires only microtubule proteins present after purification by three cycles of assembly and disassembly.

Cytoplasmic microtubules are involved in various motile phenomena and in the control of cell shape, but little is known about the mechanism of microtubule function in the cell. In flagella, sliding of microtubules relative to one another is responsible for motility (25), but whether such a process is involved in cytoplasmic motility is not known (2, 30). Cytoplasmic microtubules are often organized into parallel arrays, or associated with microtubule-organizing centers, but the significance of such structures in either motility or cell shape is not understood. It obviously would be helpful to be able to form similar structures in vitro, and to induce in them some type of motile activity. Microtubules do undergo a type of internal motility in which subunits add at one end and gradually "treadmill" to the other end (15); however it is unclear if this phenomenon has any relationship to in vivo processes (11).

Cross-bridging of cytoplasmic microtubules in vitro has been obtained using ciliary dynein (7), a neuronal protein from squid (18), the enzyme glyceraldehyde 3-phosphate dehydrogenase (12), the spectrin-like protein "fodrin" (9), and the microtubule-stabilizing drug taxol (29), but these reactions have not been related to physiological processes and have not produced motile phenomena. Microtubules that have been cross-bridged with either dynein or glyceraldehyde 3-phosphate dehydrogenase or in the presence of taxol are sensitive to ATP, but the response to ATP in each case is the breaking of cross-bridges, not motility as such. We now report the ATP-dependent contraction, or syneresis, of microtubule gels formed in vitro using microtubule proteins prepared from calf brain by three cycles of assembly and disassembly. (Note that the terms gelation and contraction are used only to describe the observed phenomenon, and are not intended to imply any particular physiological or biochemical process.)

MATERIALS AND METHODS

Microtubule protein was isolated by slight modification of previously described methods (32), but we do not presently know if this procedure is essential to obtain a preparation competent to undergo gelation–contraction. Whole calf brains, processed within 2 h of slaughter, were homogenized in a Waring blender in 0.1 M 2-(N-morpholino)ethane sulfonic acid (MES)[1] buffer at pH 6.6 (this buffer and pH were used throughout) containing 0.1% mercaptoethanol, 0.1 mM GTP, and 25% glycerol, using two brains in 500 ml of medium. The homogenate was spun in a Beckman TI15 zonal (Beckman Instruments, Inc., Palo Alto, CA) rotor at 30,000 rpm for 60 min at 4°C. The supernatant was recovered and brought to 2 mM EGTA, 1 mM MgCl₂, and 0.5 mM GTP, and 33 ml of additional glycerol was added per 100 ml of supernatant. The supernatant was incubated at 37°C for 30 min to induce assembly, and the microtubules were collected in a Beckman TI14 zonal rotor at 45,000 rpm for 45 min at 25°C. The microtubule pellet was resuspended in 112 ml of MES buffer with 0.2 mM GTP and incubated at 0°C for 30 min, and glycerol was added to make a final volume of 150 ml (25% glycerol). The protein was stored overnight at −20°C and then centrifuged at 40,000 rpm for 20 min at 4°C in a Beckman TI60 rotor. The supernatant received 1 mM EGTA, 0.5 mM MgCl₂, and 0.2 mM GTP, and a second cycle of polymerization and centrifugation was performed (using centrifugations of 40,000 rpm for 30 min in a TI60 rotor). The microtubule protein was stored at −70°C in MES buffer, 0.1 mM GTP plus 25% glycerol. A third cycle of polymerization and centrifugation (using spins of 40,000 rpm for 30 min in a type-65 rotor) was performed to prepare the protein for each experiment. The third cycle pellet was resuspended in MES buffer and 1 mM dithiothreitol and then was spun through at least 20 vol of packed G-25 Sephadex (19) in MES buffer plus dithiothreitol, to remove most remaining traces of glycerol.

For each experiment, unless otherwise noted, the third-cycle protein was polymerized by incubation at 37°C for 30 min in 0.5 mM GTP, 1 mM EGTA, 0.5 mM MgCl₂. Protein concentrations were typically between 5 and 8 mg/ml. The solution was divided as needed, and the indicated additions were made. Experiments were generally performed in 1-ml polystyrene cuvettes to allow optimum visibility of the gels. Incubation was continued for at least 1 h after ATP addition, if gelation–contraction was observed, and for at least 3 h if gelation–contraction was not observed in any sample. The solutions were

[1] *Abbreviations used in this paper:* MES, 2-(N-morpholino)ethane sulfonic acid.

disturbed as little as possible during this time.

Protein concentration was determined by the Bradford method (1) using BSA standards. SDS acrylamide gel electrophoresis was done by the method of Laemmli (13). Chemicals were all reagent grade. Water was distilled and then passed through a Barnstead Mixed Bed deionizer (Barnstead Co., Boston, MA). Nucleotides and DNAse were all obtained from Sigma Chemical Co. (St. Louis, MO). Taxol was a gift from the National Institutes of Health.

RESULTS AND DISCUSSION

Approximately 30 min after ATP addition to steady-state microtubules, the turbid phase of the solution began to separate from the walls of the vessel and to either raise up from the bottom or separate from the meniscus (Fig. 1). A distinct gel was present at this time, as indicated by entrapment of bubbles and flow properties. The gel continued to decrease in volume for ~15–30 min after contraction, or syneresis, was first observable. The contracted gel often split, with half moving towards the meniscus and half settling to the bottom of the vessel (Fig. 1). Under less than optimum conditions, such as low protein concentration, a distinct gel may not form. Instead, the solution becomes flocculent and the turbid material settles slowly to the bottom of the vessel (not shown). The conditions for formation and the structure (e.g., the presence of aligned microtubules and aster-like structures) of the flocculent "precipitate" are similar to that of the contracting gel, and we consider them to reflect the same basic reaction.

The gels may contract to about ¼ the initial volume under optimum conditions. The volume and protein content of contracted gels were estimated by centrifugation in a graduated, narrow-bottom centrifuge tube in a table-top centrifuge. The amount of protein present in the gel ranged from 30 to 60% of the total protein present.

The contracted gels were temperature sensitive and were rapidly dispersed by incubation at 0°C. In most experiments a small amount (~10% of the initial volume) of insoluble material remained following cold incubation. Although the cold-stable material did not contain microtubules, the formation of the cold-stable aggregate appeared to correlate with gelation–contraction. Preliminary observations by timelapse video microscopy suggest that contraction involves the formation of microtubule focal centers which enlarge and move together, resulting in the formation of aster and spindle-like structures (visible in Fig. 2C). The cold-stable aggregate appear to be related to the microtubule focal centers.

Polarization microscopy of contracted gels indicated the presence of birefringent domains of various orientations (Fig. 2). No significant birefringence was observed before addition of ATP, at which time the solutions appeared uniformly grey (not shown). The pattern of birefringence was quite variable with respect to the size and anisotropy of birefringent domains. Although gel contraction was not detectable until ~30 min after ATP addition, birefringent domains were observable within 5 min. The birefringent structures formed early occupied the entire solution and eventually coalesced or contracted (Fig. 2C) at about the time that contraction was observed macroscopically.

Transmission electron microscopy by thin sectioning or negative staining with 0.5% uranyl acetate indicated that the gels contain microtubules aligned into roughly parallel arrays or bundles (Figs. 3 and 4). It is possible to observe apparent cross-bridges between microtubules, particularly in negatively stained preparations, but we do not know if these reflect real structures or are artifacts of the preparation (14). Considerable
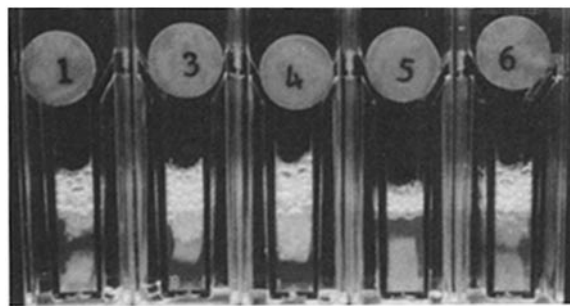

FIGURE 1 Examples of contracted microtubule gels. The samples, 0.4 ml each, are in plastic cuvettes. All samples contained 1 mM GTP, and the indicated additions and were incubated at 37°C for 30 min at which time 1 mM ATP was added to all the solutions. The photograph was taken ~1 h after ATP additions. Note that the movement of the gels may vary with essentially identical extents of contraction. The additions were: cell 1, 1 mM ATP only; cell 3, 1% DMSO; cell 4, 1% DMSO and 10 μM taxol; cell 5, 0.1 mM cAMP, cell 6, 0.1 mM cGMP.

variation has been observed in the degree of alignment and in the distance between microtubules. It is likely, however, that the wider spacing observed in thin sections reflects the true distribution of microtubules, while the negatively stained images have been distorted by flattening of the sample. Besides microtubules, dense aggregates of material are often observed. These aggregates frequently appear to be associated with microtubules, resulting in the formation of aster-like structures (Fig. 3C).

ATP was found to be effective above 0.5 mM in inducing gelation–contraction. At higher ATP concentrations contraction was observed sooner, and the extent of contraction increased (Fig. 5). GTP added to steady-state microtubules (GTP was always present at the start of incubation) was occasionally observed to induce gelation–contraction, but it was less effective than ATP (Fig. 6). When tested at 1 mM, ITP and CTP were unable to induce gelation–contraction (Fig. 6). No gelation–contraction was observed in 1 mM AMP-PNP (5' guanylimidodiphosphate) or AMP-PCP (β-γ methylene guanosine 5' triphosphate), nonhydrolyzable analogues of ATP (Fig. 7). AMP-PNP was also able to block contraction in ATP, indicating that it was effective as an analogue. In some experiments, but not in others (Fig. 6), 1 mM ADP was able to induce limited contraction, but it is not known if this was due to the action of ADP or to that of ATP generated by adenylate kinase activity or by phosphorylation from GTP.

To obtain optimum contraction it is helpful to add ATP to microtubules that have been allowed to assemble to steady state. This may be the result of ATPase activity in these preparations, which have an ATPase activity of ~10 nmol/ min per mg of protein, consistent with other reports (5, 27, 31). At this level, much of the ATP would have been hydrolyzed before microtubule assembly was complete.

In some experiments 0.1 mM cyclic AMP inhibited contraction, whereas in other experiments it had no observable effect. In preliminary experiments we examined the effect of 10 μM orthovanadate, an inhibitor of dynein and other enzymes (22), and observed no inhibition of the extent of contraction, although the rate of contraction was reduced. Stabilization of microtubules by 10 μM taxol (3) did not prevent gelation–contraction (Figs. 1 and 2D). To test the
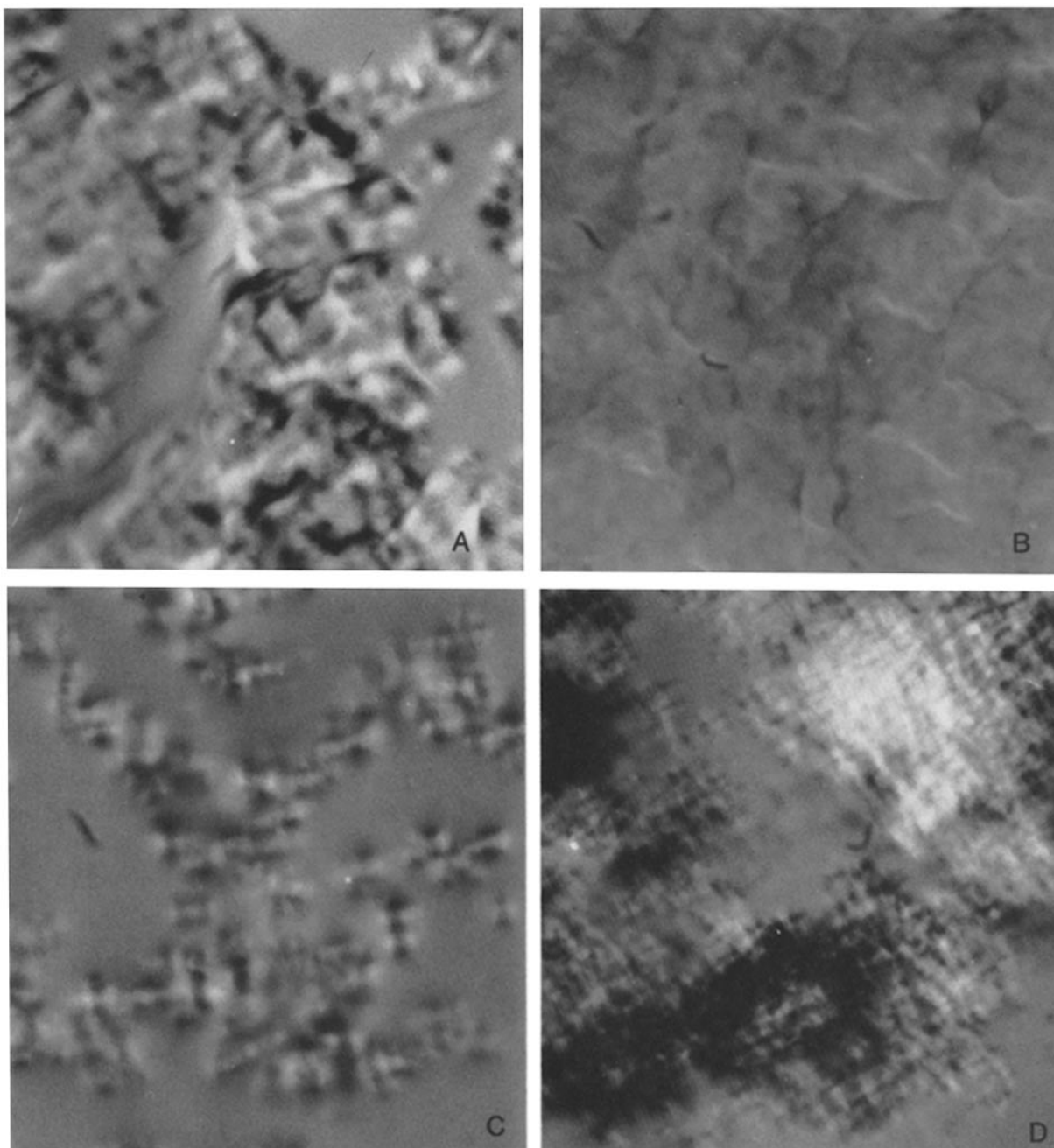
FIGURE 2   Polarization micrographs of contracted microtubule gels. (A) A well-contracted gel showing large, distinct birefringence domains. (B) A contracted gel showing a more homogeneous appearance with small domains. (C) Birefringent structures formed when gelation–contraction was induced in a thin solution on a microscope slide. (D) Gelation–contraction in the presence of 0.1 mM taxol; birefringence domains are larger than normal. (A–D) × 640.

possibility that actin may be involved in this reaction, we examined the effects of cytochalasin B (10 μg/ml) and DNAse I (1 mg/ml). These agents had no observable effect on microtubule gelation–contraction (not shown).

Preliminary analysis by SDS acrylamide electrophoresis of enriched gel proteins (obtained by low-speed centrifugation) and ungelled proteins revealed only minor differences. Tubulin comprised ~80% of the protein (Fig. 8), and the usual microtubule-associated proteins (23), as well as others, were present.

While much more remains to be learned about this phenomenon, several conclusion can be reached. First, the reaction is either the contraction or syneresis of a true microtubule-containing gel. Within the gel, microtubules are aligned to a significant degree. This is indicated by the birefringence of the gels and confirmed by electron microscopy. However, at this time we do not know whether microtubule alignment depends upon cross-bridges, is a result of physical interactions between rigid polymers (17, 26), or is related to the formation of apparent focal centers during contraction. Nor do we know
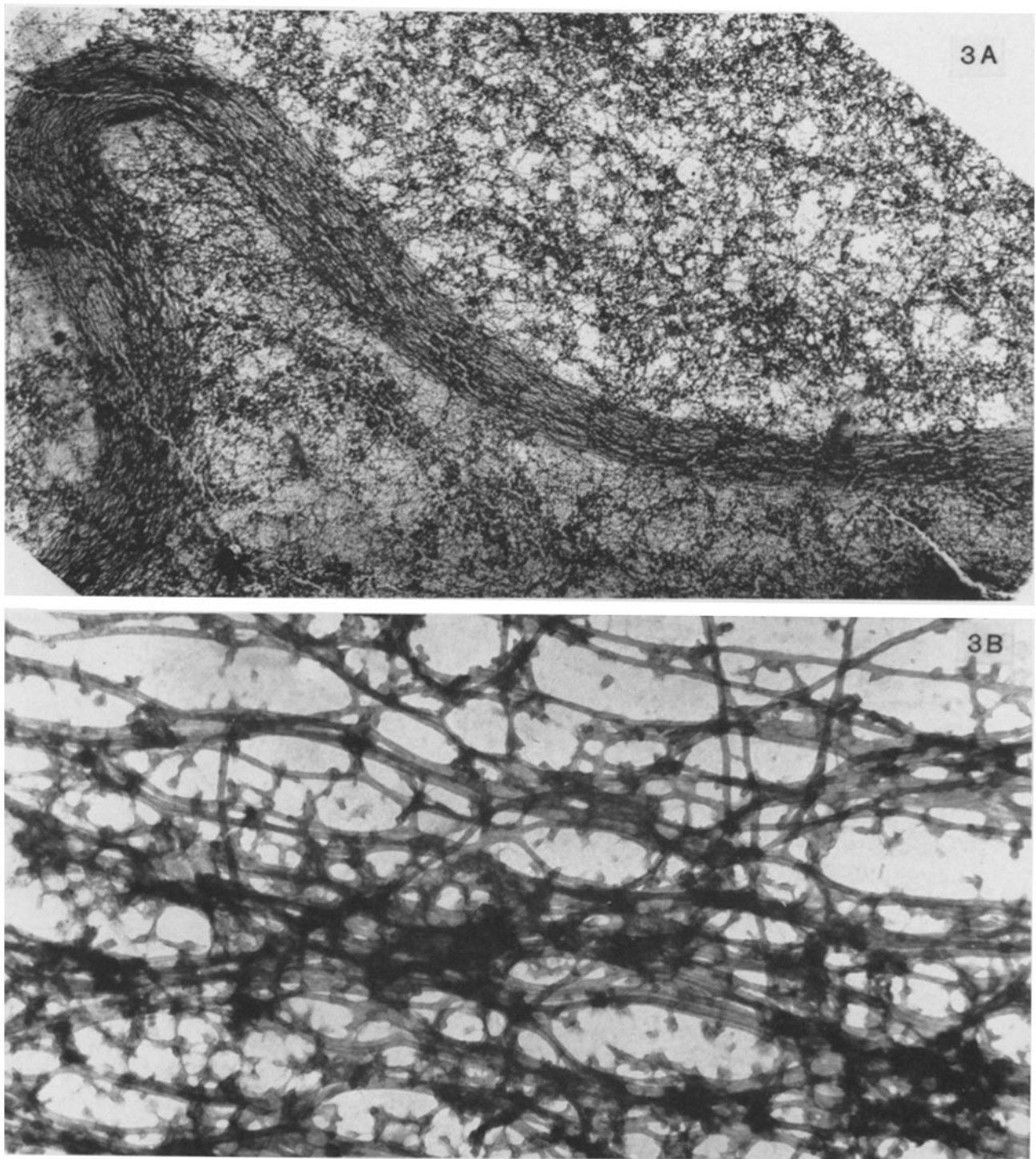
FIGURE 3 Negative stain electron micrographs of contracted microtubule gels, stained with 0.5% uranyl acetate on carbon-coated grids. To visualize gel structure it was helpful either to disperse the gels in a microtubule-stabilizing medium (1 M hexylene glycol at pH 6.2) or to fix the gels (with 2.5% glutaraldehyde) and then disperse in water. Ungelled microtubules showed no bundles when similarly treated. (A) A particularly long and uniform microtubule bundle. This bundle extended over two bars of a 200-mesh grid and had an estimated length of 0.25 mm. Dispersed in hexylene glycol. × 1,800. (B) A higher magnification view of a portion of the bundle in A. × 65,000. (C) An example of a particularly clear aster-like structure. Such structures are also observable with polarization microscopy (Fig. 2C). × 10,000 (D) Part of a bundle observed after fixation in glutaraldeyde and dispersal in water. Microtubule fine structure is much cleaner in these preparations. Possible cross-bridge structures are apparent, but they are heterogeneous in appearance and cannot be distinguished from structures (possibly protein aggregates) not associated with microtubules, × 150,000.

whether contraction of the microtubule gel is a result of microtubule alignment, the "zipping" of microtubule bundles (28), or the sliding of microtubules, or the result of an as yet unknown process. There is ATPase activity in these preparations, but its relationship to contraction is unknown. ATP may also act as a substrate for a protein kinase (10, 24) which
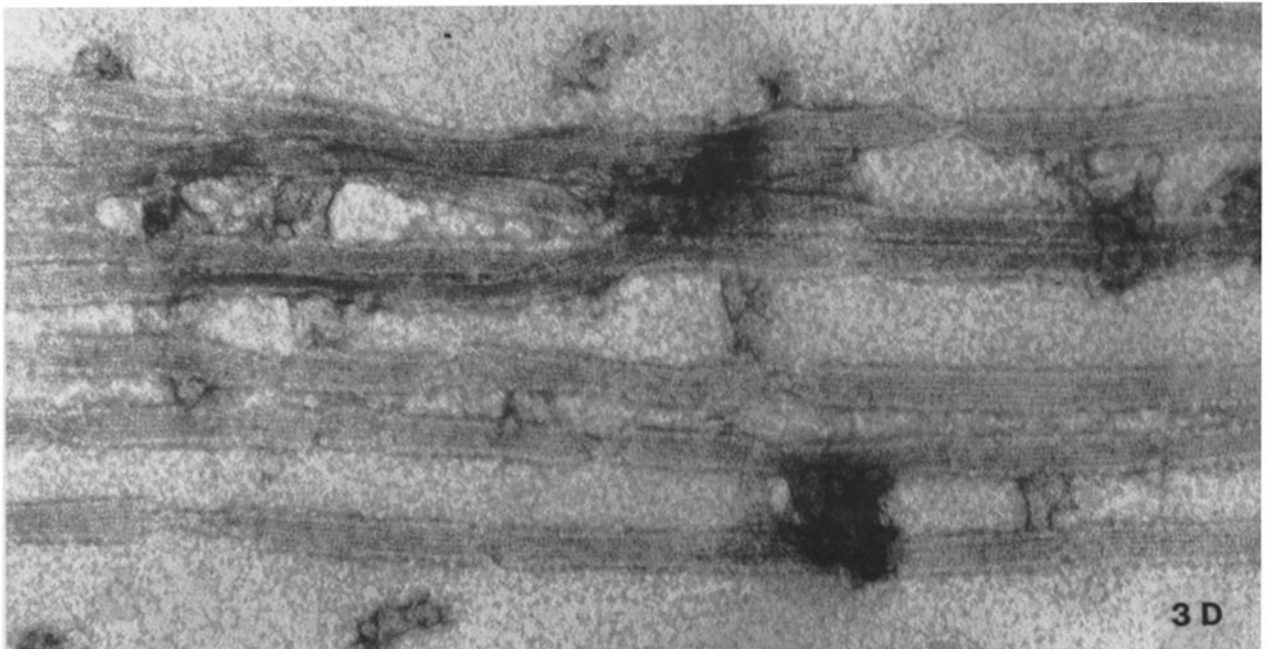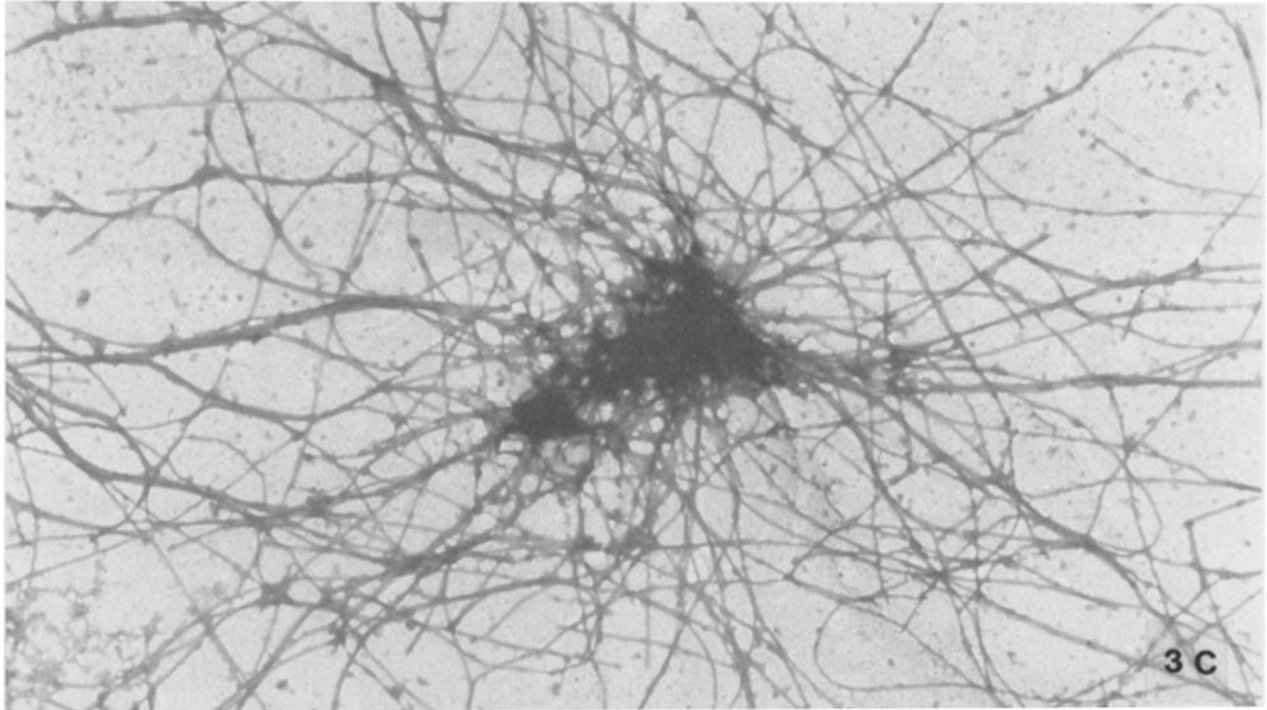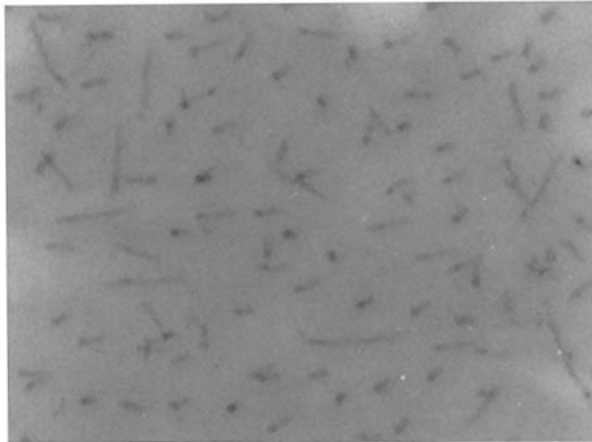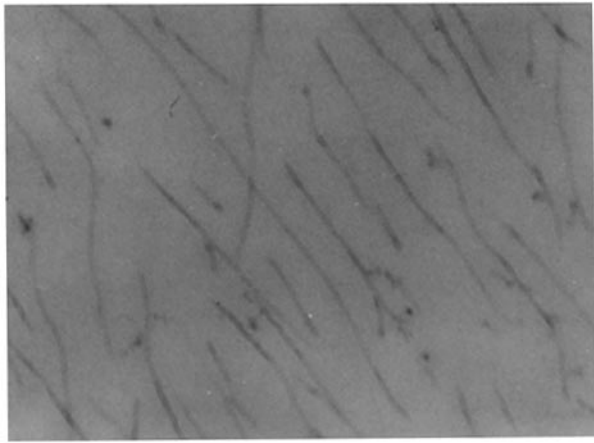
3 C

3 D

FIGURE 3—*Continued*

FIGURE 4 Thin-section electron micrographs of contracted gels. Gels were fixed in 2.5% glutaraldehyde (in the gelation–contraction medium), postfixed in 1% osmium tetroxide, and stained with uranyl acetate. As compared with negative stain preparations, the microtubules are more uniformly spaced. Cross-bridge structures are extremely rare. × 30,000.
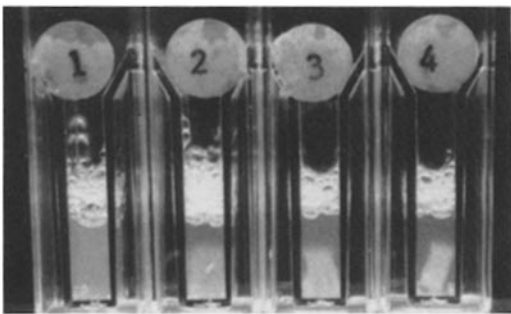

FIGURE 5 Effect of ATP concentration on gelation–contraction. Gels were formed under standard conditions with the indicated additions made 30 min after inducing polymerization, and the photograph was taken ~1.5 h later. Cell 1, 0.25 mM ATP, cell 2, 0.5 mM ATP; cell 3, 1.0 mM ATP; cell 4, 2.0 mM ATP.

stimulates gelation–contraction, but the fact that cAMP sometimes inhibits gelation–contraction argues against this. However, more than one reaction is required for gelation–contraction to occur. Microtubules must first be formed, and inhibition by cAMP may reflect inhibition of microtubule assembly (10). The rather long lag time (30 min) between the addition of ATP and the start of an observable contraction also suggests that more than a single process is occurring.

The relationship of the phenomenon reported here to events in the living cell is, of course, unknown. However the specificity for ATP is consistent with a physiological function for this reaction. Physiological significance is also indicated by the fact that ATP-dependent gelation–contraction requires
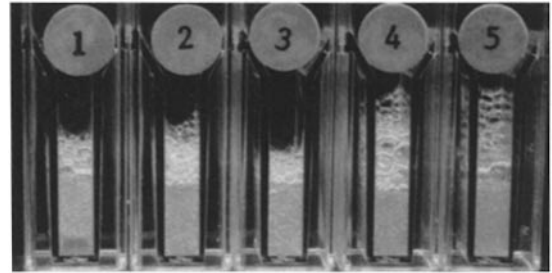

FIGURE 6 Effect of nucleotides on gelation–contraction. Microtubules were assembled under standard conditions and the indicated nucleotides, at 1 mM, were added at 30 min. Cell 1, ATP; cell 2, GTP; cell 3, ADP; cell 4, ITP; cell 5, CTP.
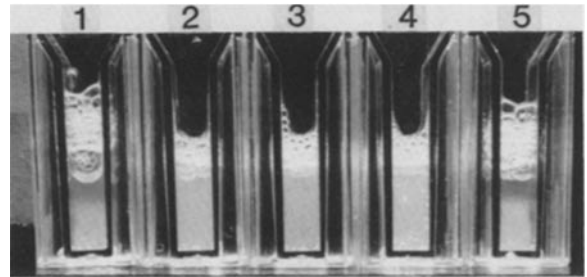

FIGURE 7 Effect of nonhydrolyzable analogues of ATP. Cell 1, 1 mM ATP control; cell 2, 1 mM AMP-PNP; cell 3, 1 mM AMP-PCP; cell 4, 1 mM ATP plus 1 mM AMP-PNP; cell 5, 1 mM ATP plus 1 mM AMP-PCP.

FIGURE 8 SDS acrylamide gel electrophoresis of third-cycle calf brain microtubule proteins. The acrylamide concentration of the gel was 5%, and the amount of protein loaded was 150 μg. Staining was with Coomassie Blue and the gel was scanned at 550 nm. Note that this gel is overloaded for tubulin and does not give an accurate indication of relative protein content. The band at the bottom of the gel is tracking dye.

only purified microtubule proteins, and as much as 60% of the total protein, and nearly all the microtubules, are incorporated into the contracted gel. Although the motility observed in vitro, contraction of a gel, has no obvious relationship to cellular motility as it occurs in nerve cells, this may reflect differences in the in vivo and in vitro environment of the microtubules (21). The reaction observed here may also reflect a developmental process, such as neurite extension. Results that indicate interactions between microtubules and actin (6), neurofilaments (8, 16, 20), and other microtubules (7, 9, 12, 18, 28) suggest the existence of complex interactions between cytoskeletal components in the living cell, consistent with ultrastructural observations (4, 8). The present observations may ultimately help resolve the role of such interactions.

Note Added in Proof: We have recently examined microtubule gelation-contraction by video-enhanced contrast microscopy in collaboration with Robert Day Allen (Dartmouth College) and Shinya Inoué (Marine Biological Laboratory, Woods Hole). We observed that gelation-contraction begins with the formation structures resembling mitotic spindle asters. These aster-like structures subsequently move towards one another at rates of 1–5 μm/min. In addition to the formation and movement of aster-like structures, we also observed linear particle movements. These are most often directed towards a single aster center, but are sometimes bi-directional, and particles may move back and forth between two connected asters. These results will be reported in a manuscript now in preparation.

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