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Association of Microtubules and Neurofilaments in Vitro Is Not Mediated by ATP†

Eric J. Aamodt and Robley C. Williams, Jr.*

ABSTRACT: Runge et al. [Runge, M. S., Laue, T. M., Yphantis, D. A., Lifsics, M. R., Saito, A., Altin, M., Reinke, K., & Williams, R. C., Jr. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1431–1435] found that mixtures of microtubules and neurofilaments formed a viscous, sedimentable complex when incubated at 37 °C for 20 min in the presence of ATP. They did not observe the high viscosities associated with the complex when the incubation was carried out in the absence of ATP. This paper reports an investigation of the roles of time and ATP in the formation of the complex. Microtubules assembled in a mixture containing GTP and neurofilaments prepared from bovine brain remained assembled for a shorter period of time than they did in similar solutions containing no neurofilaments. Adding ATP to the neurofilament-containing solutions, or doubling their GTP concentration, extended the time during which the microtubules remained assembled. These mixtures then became highly viscous. These phenomena resulted from the action of at least two enzymes present in the neurofilament preparation. A GTPase raised the GDP/GTP ratio, in the mixtures in which ATP was absent, to levels sufficient to cause disassembly of the microtubules. When ATP was present, a nucleotide diphosphokinase catalyzed regeneration of GTP from GDP while converting ATP to ADP. This process kept the GDP/GTP ratio low and delayed the disassembly of the microtubules. These results show that the apparent ATP dependence of formation of the microtubule–neurofilament complex observed by Runge et al. is attributable to a GDP-induced disassembly of microtubules rather than to a disruption of microtubule–neurofilament contacts. Those contacts can form in the absence of ATP.

Runge et al. (1981b) showed that neurofilaments prepared from bovine brain can cross-connect microtubule-associated protein (MAP)[1] -free microtubules in vitro and form a viscous sedimentable network. Because this in vitro network may

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[1] Abbreviations: MAP, microtubule-associated protein; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); MT, microtubule; NF, neurofilament.

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result from the same kind of cross-connections that link microtubules and neurofilaments in vivo [e.g., see Wuerker & Palay (1969), Hoffman & Lasek (1975), Papasozomenos et al. (1981), and Hirokawa (1982)], the identity of the proteins and cofactors that are required for its formation is of interest. Runge et al. (1981b) observed that the network formed only when ATP was added to the solutions of microtubules and neurofilaments. They concluded, therefore, that ATP induced the association of microtubules with neurofilaments. Minami et al. (1982), however, repeating these studies with porcine brain neurofilaments and porcine MAP-free microtubules, found that a network of microtubules and neurofilaments formed even when ATP was not added. The preceding paper confirmed and extended these findings (Aamodt & Williams, 1984) and showed that a network of bovine microtubules and bovine spinal cord neurofilaments will form without the addition of ATP. A study of the role of ATP in the formation of the complex of neurofilaments and MAP-free microtubules is reported in this paper.

Materials and Methods

Materials. Bio-Gel A-150m was purchased from Bio-Rad Laboratories; ATP was bought from P-L Biochemicals Inc., and [γ-^{32}P]ATP at a specific activity greater than 5500 Ci/mmol was from New England Nuclear. PM buffer was 0.1 M PIPES, 1.0 mM EGTA, 0.5 mM $MgSO_4$, and 2.0 mM dithioerythritol at pH 6.9. Other materials are described in the preceding paper (Aamodt & Williams, 1984).

Preparation of Neurofilaments. Neurofilaments were prepared from bovine brain by the method of Runge et al. (1981a). A fresh brain was homogenized in PM buffer; the homogenate was centrifuged first at 8000 rpm for 15 min in a Sorval GSA rotor at 4 °C and then at 34 000 rpm for 60 min in a Beckman T35 rotor at 4 °C. The supernatant was chromatographed on Bio-Gel A-150m, and neurofilaments were collected from the excluded material by centrifugation. The neurofilaments were resuspended in a small volume of PM buffer by means of a Dounce homogenizer and recentrifuged through 20% sucrose in PM buffer to remove lipids. The pellet was resuspended again in PM buffer with a Dounce homogenizer. When analyzed by electron microscopy, the resulting material was seen to contain numerous filaments of approximately 100-Å diameter, as well as some smaller filaments and amorphous clumps. The preparations were frozen dropwise in liquid nitrogen and stored under liquid nitrogen. Before each experiment, an aliquot was thawed and centrifuged at approximately 10000*g* for 15 min at 4 °C to remove any aggregated proteins. A SDS–polyacrylamide gel of this material appears as Figure 1 in the preceding paper (Aamodt & Williams, 1984).

Thin-Layer Chromatography. Thin-layer chromatography was done on 20 × 20 cm poly(ethylene imine)-impregnated cellulose MN300 sheets containing a fluorescent indicator (Brinkmann Instruments Inc.). The chromatograms were developed either with 1.0 M formic acid containing 0.25 M LiCl or with 0.85 M KH_2PO_4 at pH 3.4 as indicated. The developed sheets were photographed under ultraviolet illumination. Autoradiography of the chromatograms was done with the use of Kodak X-Omat RP X-ray film and an intensifying screen for 14 h at −80 °C.

GTP Hydrolysis. The inorganic phosphate released during GTP hydrolysis was measured by the method of Lanzetta et al. (1979). Duplicate samples of either 50, 10, or 5 μL depending on the concentration of free phosphate in the solutions, were assayed at the times indicated. Color reagent was added immediately, the samples were stirred on a vortex mixer, and

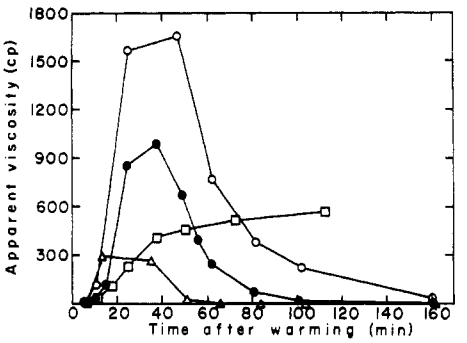

FIGURE 1: Apparent viscosity of mixtures of brain neurofilaments and tubulin as a function of time of incubation at 37 °C. (△) Tubulin at a concentration of 3.5 mg/mL, brain neurofilaments at a concentration of 0.7 mg/mL, and GTP at 0.5 mM; (○) tubulin at 3.5 mg/mL, brain neurofilaments at 0.7 mg/mL, GTP at 0.5 mM, and ATP at 1.0 mM; (□) tubulin at 3.5 mg/mL and GTP at 0.5 mM; (●) tubulin at 3.5 mg/mL, brain neurofilaments at 0.7 mg/mL, and GTP at 1.0 mM.

1 min later 0.1 mL of 34% (w/v) sodium citrate was added. After at least 20 min, the solution was placed in a 1.0-mL cuvette, and its absorbance at 660 nm was measured.

Other Methods. Protein determination, falling ball viscometry, polyacrylamide gel electrophoresis, and the preparation of purified tubulin were done as described in the previous paper (Aamodt & Williams, 1984).

Results

Time Course of Network Formation. The dependence of the apparent viscosity of a mixture of brain neurofilaments, microtubules, GTP, and ATP on time is shown in Figure 1 (open circles). The apparent viscosity of this material increased rapidly to a peak near 1600 cP and then decreased to below 40 cP. When ATP was not present in the mixture and a similar experiment was performed (Figure 1, triangles), the apparent viscosity rose rapidly at first but began to decrease sooner. Within 35 min the apparent viscosity was smaller than that of a control mixture containing tubulin and GTP (Figure 1, squares). These experiments suggest that the microtubule–neurofilament interactions were interrupted by some time-dependent process that disrupts the structure of the microtubules.

At 20 min after the initiation of the reaction, the observed viscosities were comparable to those reported at the same elapsed time by Runge et al. (1981b), who did not study the variation of apparent viscosity with time. The solution of tubulin and GTP (squares) did not show a decrease in its viscosity within the time of measurement.

To examine the effect of GTP concentration on the stability of the viscosity of the tubulin–neurofilament mixtures, tubulin at a concentration of 3.5 mg/mL and neurofilaments at a concentration of 0.7 mg/mL were mixed in PM buffer. These protein concentrations are the same as those used in the experiment shown in Figure 1 (triangles), but the GTP concentration was increased from 0.5 to 1.0 mM. As shown in Figure 1 (closed circles), the apparent viscosities of aliquots of this mixture were measured at a series of times after warming. The higher GTP concentration caused a delay in the onset of the decrease in viscosity and allowed the apparent viscosity to rise to a maximum of approximately 1000 cP, a value substantially higher than that observed with tubulin and GTP alone. Thus, the association of MAP-free microtubules and bovine brain neurofilaments can occur even when ATP is not added to the solution.

GTPase Activity in Brain Neurofilaments. The GTPase activity of the neurofilament preparation was determined by

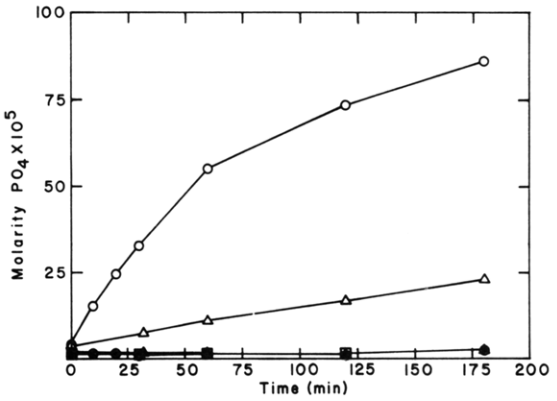

FIGURE 2: PO_4 released into solution from mixtures of neurofilaments and GTP incubated together at 35 °C as a function of time. PO_4 was rapidly released from GTP when bovine brain neurofilaments at a concentration of 0.7 mg/mL and GTP at a concentration of 0.5 mM were were incubated together (○), but little PO_4 was released into solution in the control mixtures of brain neurofilaments alone (□) or GTP alone (●). PO_4 was released more slowly in mixtures of spinal cord neurofilaments at a concentration of 0.7 mg/mL and 0.5 mM GTP (△). A control mixture of spinal cord neurofilaments alone released little PO_4 (▲).

measuring the inorganic phosphate released by GTP incubated with brain neurofilaments. Brain neurofilaments at a concentration of 0.7 mg/mL were mixed with GTP at a concentration of 0.5 mM in PM buffer at 0 °C. Control solutions of 0.5 mM GTP in PM buffer and of neurofilaments at a concentration of 0.7 mg/mL in PM buffer were observed at the same time. To obtain the 0-min time point, duplicate aliquots of 50 μL were removed from each of the freshly mixed cold solutions, and the free phosphate initially present in each solution was measured. The solutions were then placed into a 35 °C water bath, and duplicate measurements of the free phosphate in each solution were made at the times indicated in Figure 2.

Free phosphate was rapidly released when GTP was incubated with the brain neurofilament preparation (Figure 2, open circles). Within 52 min, 1 mol of phosphate had been released for each mol of GTP in the mixture of GTP and brain neurofilaments, and at later times, more than 1 mol of phosphate had been released for each mol of GTP. This result indicates the presence of GDP-hydrolyzing enzymes as well as the GTPase. Very little free phosphate was released from the control solutions of brain neurofilaments in PM buffer (Figure 2, open squares) and GTP in PM buffer (Figure 2, closed circles).

Figure 2 also shows (open triangles) the free phosphate released when neurofilaments prepared from bovine spin cord by the method of Delacourte et al. (1980) were incubated with 0.5 mM GTP in PM buffer. The spinal cord neurofilament preparation evidently had less GTPase activity than the brain neurofilament preparation. This finding is consistent with the fact that the complex of spinal cord neurofilaments and microtubules is stable for times of a few hours (Aamodt & Williams, 1984). Little free phosphate was released into a control solution of spinal cord neurofilaments incubated without GTP (Figure 2, closed triangles).

Thin-layer chromatography was used to determine the products of brain neurofilament-induced GTP hydrolysis. Neurofilaments at a concentration of 1.0 mg/mL were mixed with GTP at a concentration of 3.0 mM in PM buffer, and the mixture was incubated at 35 °C. A control solution of neurofilaments at a concentration of 1.0 mg/mL in PM buffer and one of 3.0 mM GTP in PM buffer were run at the same time. At times from 0 to 80 min, 2.0-μL samples were applied to thin-layer sheets. The chromatograms are shown in Figure 3.

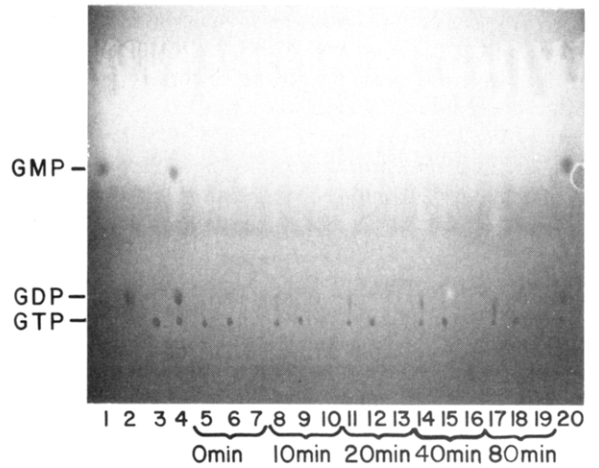

FIGURE 3: Thin-layer chromatogram of the products produced when brain neurofilaments were incubated with GTP. A solution containing neurofilaments at a concentration of 1.0 mg/mL and 3.0 mM GTP (lanes 5, 8, 11, 14, and 17), one containing GTP at 3.0 mM (lanes 6, 9, 12, 15, and 18), and one containing neurofilaments at a concentration of 1.0 mg/mL (lanes 7, 10, 13, 16, and 19) were prepared on ice and then incubated at 35 °C. At the times indicated, 2-μL samples were removed from each of the mixtures and spotted onto the thin-layer sheets. The chromatograms were developed with 1.0 M formic acid containing 0.25 M LiCl. A spot which comigrates with GDP was present in the mixture of neurofilaments and GTP after 10 min. The bright area between the front and the GMP spots is due to a diffuse band of the fluor moving with the solvent front.

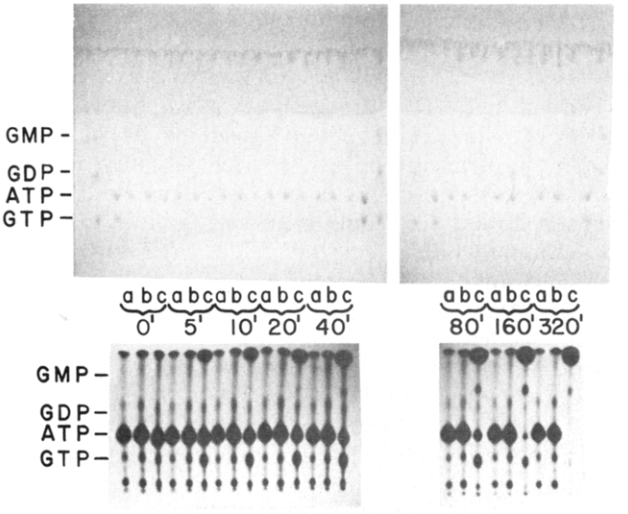

FIGURE 4: Transfer of ^{32}P from [γ-^{32}P]ATP to GDP. Chromatogram (upper panels) and corresponding autoradiogram (lower panels) of [^{32}P]ATP incubated with GDP and brain neurofilaments. Mixtures containing ATP (lanes a), ATP and neurofilaments (lanes b), and ATP, GDP, and neurofilaments (lanes c) were incubated at 35 °C. At the times indicated, 2-μL samples were applied to the thin-layer sheets. The sheets were developed with 0.85 M KH_2PO_4 at pH 3.4, and autoradiographs were made. The spots at the top of the autoradiogram correspond to inorganic phosphate; those immediately above the mark for GDP and at the mark for GTP correspond to a small amount of [^{32}P]ADP and [^{32}P]GTP present as contaminants in the [^{32}P]ATP preparation.

In the aliquot withdrawn immediately after the neurofilaments and GTP were mixed, only a single spot that migrated with GTP was present. By 10 min, two spots that migrated with GTP and GDP were visible on the chromatogram of the solution of both neurofilaments and GTP. In other experiments, including that shown in Figure 4, the GTP was clearly hydrolyzed further to GMP at later times. The control solution

of GTP showed only a single spot that migrated with GTP throughout this experiment, while the control solution of neurofilaments did not show any spots corresponding to nucleotide phosphates at any of the times.

Nucleotide Diphosphokinase Activity in Brain Neurofilaments. Thin-layer chromatography was used to test for a nucleotide diphosphokinase activity in the brain neurofilament preparation. [γ-^{32}P]ATP at a concentration of 2.0 mM and a specific activity of 4.0 mCi/mmol, GDP at a concentration of 1.0 mM, and neurofilaments at a concentration of 0.5 mg/mL were incubated in PM buffer at 35 °C. Control solutions of [γ-^{32}P]ATP at a concentration of 2.0 mM in PM buffer, of [γ-^{32}P]ATP at a concentration of 2.0 mM, and of GDP at a concentration of 1.0 mM were also treated in an identical manner at the same time. At the times indicated in Figure 4, samples were removed from each solution and spotted onto thin-layer chromatography sheets. The chromatograms were developed, and autoradiographs were made from them. Figure 4 shows both. In the solution containing [γ-^{32}P]ATP, GDP, and neurofilaments, [^{32}P]GTP is already visible on the autoradiogram at 5 min after mixing and warming. At progressively later times, one can see a gradual increase in the amount of [^{32}P]GTP, followed by a diminution. The amount of ^{32}P increases monotonically with time. This result was observed with two different chromatographic systems. Evidently, the [^{32}P]GTP initially formed was subsequently broken down by the GTPase activity in the neurofilaments so that by 320 min nearly all the [^{32}P]ATP and [^{32}P]GTP were gone from this solution. The control solutions were unchanged over the time of the experiment.

GDP-Induced Disassembly of Microtubules. As a test of whether a high GDP/GTP ratio will cause the disassembly of microtubules formed under the conditions used in the viscometry experiments, GDP was added to a solution of microtubules, and the disassembly of the microtubules was measured over time. Three aliquots of a solution of tubulin at a concentration of 3.5 mg/mL in PM buffer containing 0.2 mM GTP were placed in prewarmed (37 °C), matched cuvettes in the automatic sample changer of the Cary 118 spectrophotometer. When the change in turbidity that accompanies microtubule assembly was complete, 0.04 mL of 50 mM GTP was added to one cuvette, 0.04 mL of 50 mM GDP was added to a second cuvette, and 0.04 mL of water was added to the third cuvette, and the samples were mixed by pipetting 3 times. As shown in Figure 5, the turbidity of the microtubule solution to which GTP was added and that to which water was added changed transiently, but quickly recovered to near their plateau values, while the turbidity of the microtubule solution to which GDP was added decreased rapidly to a value near the turbidity of the unpolymerized tubulin. At the time indicated by the second arrowhead, all the solutions were cooled to 4 °C. The turbidity of the solutions to which GTP or water was added decreased after cooling to near the turbidity of the solution to which GDP was added. The solution to which GDP was added showed no further turbidity decrease, indicating that the addition of GDP had caused essentially complete depolymerization of the microtubules in this solution.

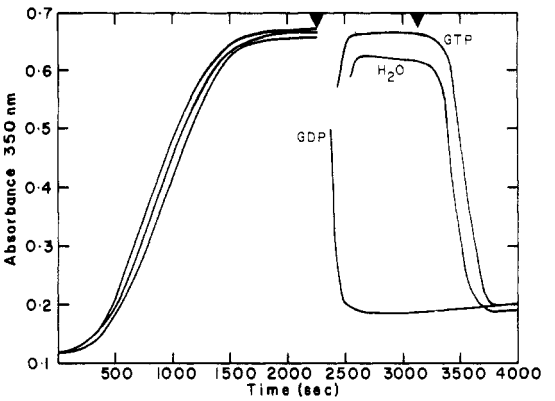

FIGURE 5: GDP-induced disassembly of microtubules. The assembly of tubulin into microtubules in three solutions that contained tubulin at a concentration of 3.5 mg/mL and 0.2 mM GTP was monitored by measurement of their turbidity at 350 nm. Ice-cold tubulin was added to the 37 °C cuvettes at time zero. After the turbidities had reached an asymptote (first arrowhead), one mixture was made 2.0 mM in GDP by the addition of 0.04 mL of 50 mM GDP. A second mixture was made 2.2 mM in GTP by the addition of 0.04 mL of 50 mM GTP. To the third mixture was added 0.04 mL of water. At the times indicated by the second arrowhead, the cuvettes were cooled to 4 °C.

Discussion

The presence of the brain neurofilament preparation was found to have three effects on the apparent viscosity of a solution of tubulin under conditions where microtubules are formed. It raised the rate at which the apparent viscosity increased; it caused the apparent viscosity to decrease soon after it had started to rise; in the presence of ATP or high concentrations of GTP, it caused a large increase in the apparent viscosity above that attained by microtubules alone. The presence of ATP or high concentrations of GTP appeared to extend the time during which the microtubules were formed. This allowed the observation of the high apparent viscosities.

In view of previous findings (Runge et al., 1981; Minami et al., 1982; Aamodt & Williams, 1984), the high viscosity in mixtures of microtubules, neurofilaments, GTP, and ATP probably results from the formation of an associated complex between the microtubules and the neurofilaments. Because ATP apparently serves to prolong the existence of this complex, experiments were performed to help elucidate the mechanism by which the prolongation of viscosity development was brought about.

The results shown in Figure 2 clearly demonstrate the presence of a GTPase activity in the neurofilament preparation. From the measured rate of hydrolysis of GTP, one can calculate that after 20 min, under the conditions of the viscometry experiments, approximately half of the GTP in a solution of 0.5 mM GTP would have been hydrolyzed.

Although the role of guanine nucleotides in microtubule assembly and disassembly is not well understood, it has been generally accepted that tubulin dimers have two GTP binding sites. One of these sites is exchangeable, and the other is nonexchangeable (Weisenberg et al., 1968). Assembly of tubulin dimers into microtubules is followed, with some delay, by the hydrolysis of the GTP at the exchangeable site (Carlier & Pantaloni, 1981). GDP can bind to the exchangeable site on the free dimer with an affinity not greatly different from that of GTP (Zeeberg & Caplow, 1979). The tubulin dimer with GDP bound (T–GDP) cannot be assembled into microtubules (Zackroff et al., 1980; Jamison & Caplow, 1980; Carlier & Pantaloni, 1982); therefore, only the concentration of tubulin dimers with GTP bound to the exchangeable site ([T–GTP]) determines the critical concentration for microtubule assembly. According to Carlier & Pantaloni (1982), in the case $K_T \ll [GTP]$ and $K_D \ll [GDP]$, where K_T is the dissociation constant of GTP from T–GTP ($=6.1 \times 10^{-8}$ M) and K_D is the dissociation constant of GDP from T-GDP ($=5.5 \times 10^{-8}$ M)

$$[T\text{–}GTP] = C_s/(1 + \alpha[GDP]/[GTP])$$

and

$$[T\text{–}GDP] = (C_s\alpha[GDP]/[GTP])/(1 + \alpha[GDP]/[GTP])$$

where C_s = [T–GTP] + [T–GDP] = the concentration of tubulin dimers and $\alpha = K_T/K_D$. From these equations, it is apparent that [T–GTP] is dependent only on the ratio [GDP]/[GTP]. Thus, if this ratio increases, the concentration of tubulin dimers that are competent to assemble into microtubules, [T–GTP], will decrease. When [T–GTP] falls below the critical concentration, either due to the addition of GDP, as in the experiment shown in Figure 5, or due to the hydrolysis of the GTP present in these solutions to GDP, the microtubules will disassemble. This occurred in the experiment shown in Figure 5. The rapid disappearance of high viscosity shown in Figure 1 can, therefore, be attributed to the action of the GTPase raising the GDP/GTP ratio in these solutions.

The presence in the neurofilament preparation of a nucleotide diphosphokinase as well as the GTPase is clearly shown in Figure 4. The effect of this enzyme in tubulin–neurofilament mixtures would be to replenish the GTP as it is hydrolyzed by the GTPase, but only when ATP is present to act as a donor of γ-phosphate groups. Hence, the disassembly of microtubules brought about by the increase in GDP/GTP ratio would be expected to be delayed by the addition of ATP, as observed. A similar delay can be brought about by increasing the concentration of GTP directly.

Because the brain neurofilament preparation is not pure, there is no reason to believe that the enzyme activities reported here are associated with neurofilaments in the brain.

Runge et al. (1981b) observed a high viscosity in mixtures of tubulin and neurofilaments incubated at 37 °C for 20 min, in the presence of ATP but not in its absence. They did not explore the time dependence of the viscosities of the mixtures. The results described here strongly suggest that in the study of Runge et al. the GDP/GTP ratio was small at the time of measurement in the ATP-containing mixtures, but it was large in the ATP-free mixtures. In that study, the concentration of neurofilaments was 4 times as great as the concentration in the experiment shown in Figure 2. The initial rate of hydrolysis of GTP would, therefore, have been approximately 4 times as great. Thus, the apparent ATP dependence of complex formation observed by those workers very likely arose from an ATP-mediated delay in the disassembly of microtubules, rather than arising from an ATP-mediated formation of connections between microtubules and neurofilaments.

Acknowledgments

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Registry No. ATP, 56-65-5; GTP, 86-01-1; GDP, 146-91-8; GTPase, 9059-32-9; nucleotide diphosphokinase, 9026-51-1.

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