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Microtubule-Associated Proteins Connect Microtubules and Neurofilaments in Vitro†

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

ABSTRACT: Neuronal intermediate filaments (neurofilaments) prepared from brain form a viscous, sedimentable complex with microtubules under suitable conditions [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]. Under the same conditions, neurofilaments prepared from spinal cord did not form such a complex. Brain neurofilaments were shown to differ from spinal cord neurofilaments in part by having proteins that resemble microtubule-associated proteins (MAPs) attached to them. MAPs became bound to spinal cord neurofilaments when the two structures were incubated together. The resulting MAP-decorated neurofilaments formed a viscous complex with microtubules, showing that some component of the MAPs mediated the association between the two filamentous organelles. By means of gel filtration, the MAPs were separated into two major fractions. The large Stokes radius fraction was active in producing neurofilament–microtubule mixtures of high viscosity, while the small Stokes radius fraction was not. The dependence of the viscosity of neurofilament–microtubule mixtures upon the concentration of MAPs was found to possess a maximum. This result suggests that the MAPs serve as cross-bridges between the two structures. Neurofilaments, with and without bound MAPs, were allowed to adhere to electron microscope grids. The grids were then exposed to microtubules, fixed, and stained. The grids prepared with MAP-decorated neurofilaments bound numerous microtubules, each in apparent contact with one or more neurofilaments. The grids prepared with untreated neurofilaments lacked microtubules. These results show that one or more of the MAPs mediates association between microtubules and neurofilaments.

The cytoplasm of eukaryotic cells contains at least three types of filamentous organelles: microtubules, intermediate filaments, and microfilaments, connected together to form the cytoskeleton. The axons of vertebrate neurons have provided a convenient system in which to study the interaction of these cytoskeletal elements and their involvement in growth and transport. Within the axon, the chief cytoskeletal elements appear to be microtubules and the intermediate filaments of neurons, the neurofilaments (Peters et al., 1976). The neurofilaments and microtubules are aligned roughly parallel to the long axis of the axon and appear to be linked to each other and to membranous organelles through thin cross-bridges (Wuerker & Palay, 1969; Bertolini et al., 1970; Wuerker, 1970; Smith, 1971; Smith et al., 1977; Ellisman & Porter, 1980; Hodge & Adelman, 1980; Rice et al., 1980; Metuzals et al., 1981; Hirokawa, 1982; Schnapp & Reese, 1982). At least some of these bridges must break and re-form as the cell transports material and moves. The cross-bridges seen in axons resemble the cross-connecting filaments of 20–40-Å diameter seen in numerous cell types (Wolosewick & Porter, 1976; Porter et al., 1979; Heuser & Kirschner, 1980).

Apparently as a result of this cross-bridging, neurofilaments and microtubules move together in the slowest component of

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axonal transport (Hoffman & Lasek, 1975; Mori et al., 1979; Lasek & Brady, 1981). The normal attachment of microtubules to neurofilaments is disrupted when the compound β,β′-iminobis(propionitrile) is administered (Griffin et al., 1978). Transport of neurofilaments ceases, and neurofilaments move to the periphery of the axon while the microtubules and membrane-bounded organelles move to the center (Papasozomenos et al., 1981, 1982), rearranging the normal interspersed array of these structures.

Bloom & Vallee (1983) found that in primary cultures of rat brain cells, filamentous microtubule-like structures are stained by anti-tubulin, by anti-MAP_2, and by anti-vimentin, showing that these proteins are present in the same regions of the cell. After treatments that induce the disassembly of microtubules, the vimentin filaments form perinuclear cables, and the anti-MAP_2 and anti-vimentin both stained these cables. The authors suggested that MAP_2 connects the microtubules to the intermediate filaments in vivo.

Evidence for attractive interaction in vitro between microtubules and neurofilaments has also been obtained. Berkowitz et al. (1977) found that neurofilaments are a tenacious contaminant of microtubules prepared from brain by multiple cycles of assembly and disassembly. They inferred that neurofilaments might adhere to assembled microtubules and be pelleted with them during the centrifugation of microtubules. Runge et al. (1981b) and Runge & Williams (1981) carried these findings a step further and showed that a viscous and sedimentable complex can form in vitro between microtubules and neurofilaments from bovine brain. This result has been confirmed for components of porcine brain by Minami et al. (1982). Leterrier et al. (1982) showed that neurofilaments can inhibit the assembly of microtubule protein that contains microtubule-associated proteins (MAPs)[1] by sequestering the MAPs. The MAPs, both MAP_1 and MAP_2, were recovered in the pellet when neurofilaments were centrifuged out of a solution of unassembled microtubule protein. The binding of ^{32}P-labeled MAPs to neurofilaments was measured by these workers. It appeared to be saturable, although measurements were not made at a high enough concentration of MAPs to establish this point firmly. Although it remains to be determined whether, or how, the complex observed in vitro is related to the cross-linked structures seen in situ, further investigation of the mechanism of formation of the observed complex which forms in vitro is warranted.

This paper reports a study of the proteins that must be present in the mixtures of microtubules and neurofilaments for a viscous complex to form. It shows that neurofilaments prepared from spinal cord lack many of the proteins associated with neurofilaments prepared from brain. In particular, the spinal cord neurofilament preparation, unlike the brain neurofilament preparation, contains only minute amounts of proteins that comigrate with high molecular weight microtubule-associated proteins (HMW-MAPs). The spinal cord neurofilaments will not spontaneously form a viscous complex with microtubules. When MAPs are added to these neurofilaments, however, some of the HMW-MAP proteins become bound to the neurofilaments, and the resulting MAP-containing neurofilaments form a complex with microtubules. These findings are taken as evidence that the association between microtubules and neurofilaments is mediated by one or more of the HMW-MAPs.

Materials and Methods

Materials. Bio-Gel A-15m was purchased from Bio-Rad Laboratories, PIPES was purchased from BDH Biochemicals, and nucleotides, EGTA, dithioerythritol, PMSF, and polyglutamic acid were purchased from Sigma. ATP was bought from P-L Biochemicals, Inc. Phosphocellulose P-11 was obtained from Whatman and prepared according to the manufacturer's instructions. Other chemicals were reagent grade. PM buffer contained 0.1 M PIPES, 1.0 mM EGTA, 0.5 mM $MgSO_4$, and 2.0 mM dithioerythritol at pH 6.9.

Preparation of Microtubule Proteins. Microtubule proteins were prepared from bovine brain by the assembly–disassembly method of Shelanski et al. (1973), as modified by Berkowitz et al. (1977), and modified further as follows. The supernatant from the first high-speed centrifugation was made 2.5 mM in ATP by the addition of 100 mM ATP and was incubated at 35 °C for 30 min to induce microtubule assembly. Assembled microtubules were collected by centrifugation in a Beckman T35 rotor at 34000 rpm for 75 min at 30 °C. After the supernatant was decanted, the microtubule pellets were cooled on ice and resuspended at 0 °C by means of a glass Dounce homogenizer in a volume of PM buffer equal to one-fifth of the supernatant volume. After a 30-min incubation on ice, the depolymerized tubulin was centrifuged at 34000 rpm for 60 min in a T35 rotor at 4 °C. The supernatant was diluted 1:1 with PM buffer containing 8 M glycerol and 1.0 mM GTP. The microtubule protein was taken through two more such cycles of polymerization and depolymerization. After the final warm centrifugation, the volume of the supernatant was measured, and the microtubule pellets were resuspended in a volume of PM buffer equal to one-tenth of the supernatant volume. The microtubule protein was incubated on ice for 30 min and centrifuged for 30 min at 34000 rpm in a T35 rotor at 4 °C. The resulting supernatant is referred to as 3× microtubule protein.

Separation of Tubulin and MAPs. Phosphocellulose-purified tubulin was prepared from freshly isolated 3 times cycled microtubule protein by the method of Weingarten et al. (1975) as modified by Detrich & Williams (1978). Phosphocellulose-purified tubulin is approximately 96% pure (Detrich & Williams, 1978). The purified tubulin was frozen dropwise into liquid nitrogen and stored under liquid nitrogen. Before each experiment, an aliquot of tubulin was thawed and centrifuged at approximately 10000*g* for 15 min at 4 °C to remove any aggregated protein, and the buffer was exchanged with fresh PM buffer, containing 0.1 mM GTP, by gel filtration over Sephadex G-25.

The MAPs were eluted from the phosphocellulose by application of PM buffer containing 0.75 M NaCl. The eluent was centrifuged at 34000 rpm in a T35 rotor at 4 °C to remove neurofilaments and aggregated proteins. Ammonium sulfate was added to the supernatant at 55% of saturation. The precipitated MAPs were collected by centrifugation, resuspended to a concentration of about 12 mg/mL in PM buffer containing 0.1 mM PMSF, and then dialyzed against 250 mL of this buffer for 12 h with three changes of buffer. This preparation is referred to as whole MAPs. Electrophoretic analysis of the proteins present in whole MAPs is shown in Figure 1 (lanes A and D). These MAPs were frozen dropwise in liquid nitrogen and stored at −80 °C.

Whole MAPs were fractionated by gel filtration on Bio-Gel A-15m. An aliquot of 1.0 mL of whole MAPs at 12.6 mg/mL was thawed and centrifuged at approximately 10000*g* for 15

[1] Abbreviations: MAPs, microtubule-associated proteins (Sloboda et al., 1975); HMW-MAPs, high molecular weight microtubule-associated proteins; LMW-MAPs, low molecular weight microtubule-associated proteins; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; PIPES, piperazine-*N,N′*-bis(2-ethanesulfonic acid).

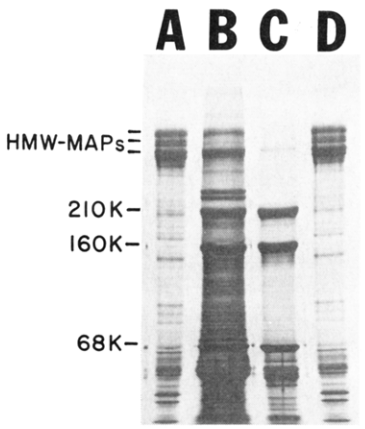

FIGURE 1: SDS–polyacrylamide–urea gradient gel of whole MAPs (lanes A and D), brain neurofilaments (lane B), and spinal cord neurofilaments (lane C) stained with Coomassie blue. Proteins that comigrate with HMW-MAPs are present in amounts easily detectable by this method in the brain neurofilament preparation, but not in the spinal cord neurofilament preparation. The amounts of protein applied to lanes B and C were adjusted so that the neurofilament proteins (68K, 160K, and 210K) were present in approximately the same amount in each of the two neurofilament preparations. Lanes A and D, 10 μg of whole MAPs; lane B, 100 μg of brain neurofilaments; lane C, 35 μg of spinal cord neurofilaments.

min. The supernatant was gel filtered on a 72 cm by 1.5 cm diameter column of Bio-Gel A-15m equilibrated with PM buffer containing 0.1 mM PMSF, and 1.0-mL fractions were collected. The protein concentration of each fraction was measured, and samples from across the column profile were electrophoresed on SDS-polyacrylamide gels. The fractions were combined into three pools according to their position in the column profile and their protein composition.

Purified MAP_2 was prepared by the method of Kim et al. (1979) as modified by Vallee (1980). The MAP_2 obtained was of comparable purity to that shown by Vallee (1980, Figure 1) when assayed by SDS–polyacrylamide gel electrophoresis.

Isolation of Neurofilaments. Neurofilaments were prepared from fresh bovine spinal cord by the method of Delacourte et al. (1980) as modified in Jones & Williams (1982). The 6000*g* centrifugation was done in a Sorval GSA rotor, and the 95000*g* centrifugations were done in a Beckman T35 rotor. The second-cycle neurofilaments were frozen dropwise in liquid nitrogen and stored under liquid nitrogen. Before these neurofilaments were used, they were centrifuged at 10000*g* for 15 min at 4 °C to remove aggregated proteins. Figure 1 (lane C) shows the proteins present in this preparation.

Neurofilaments were also prepared from bovine brain by the method of Runge et al. (1981a). Figure 1 (lane B) shows the protein present in this preparation.

Falling Ball Viscometry. Falling ball viscometry was done by means of 100-μL capillary micropipets and 0.25-mm stainless-steel balls as described by MacLean-Fletcher & Pollard (1980). The protein solutions were mixed on ice, the ice-cold solutions were drawn into the viscometers at 4 °C, and the experiments were started by immersing the viscometers in a Tamson water bath controlled to ±0.01 °C at 37 °C. The apparent viscosity was determined from the velocity of the ball as it dropped through the solution. All of the measurements were done with the viscometers at an angle of 80° to the horizontal. A PDP-8E computer equipped with a clock was used to collect and store the data and to calculate times and viscosities. The apparent viscosity was calculated by comparison to a standard curve obtained by measurement of glycerol solutions of known viscosities. The adjective "apparent" reflects the fact that these mixtures display distinctly non-Newtonian behavior. The apparent viscosities reported are true only for this type of viscometer at an angle of 80° to the horizontal. Because the apparent viscosity of a solution was often changing over the course of a measurement, the measured apparent viscosity is the average viscosity of the solution over the time required to make the measurement. [This time is given in seconds by t = viscosity (in centipoise) × 0.786.] Therefore, the reported time of measurement is the time halfway through the measurement, rather than that time at which the measurement was begun. Because the solutions were thixotropic, each viscometer was used for only one measurement.

Affinity Electron Microscope Grids. Neurofilament affinity electron microscope grids were prepared in a manner similar to the immunoaffinity electron microscope grids described by Willard et al. (1980). An aliquot of 1.0 mL of neurofilaments at a concentration of 2.0 mg/mL and whole MAPs at 3.0 mg/mL was incubated for 1.0 h in PM buffer at 35 °C. At the same time, controls of neurofilaments at 2.0 mg/mL without added MAPs and of MAPs at 3.0 mg/mL without neurofilaments were run. The neurofilaments were separated from the free MAPs by centrifugation in a Beckman T40 rotor at 30000 rpm for 20 min at 4 °C. The neurofilament pellets were rinsed once with fresh buffer and then resuspended in 3.7 mL of PM buffer by the use of a 7.0-mL glass Dounce homogenizer. The protein concentration of the neurofilament- and MAP-containing sample was 0.12 mg/mL. Drops of the resuspended mixtures were placed on 400-mesh copper electron microscope grids freshly coated with a film of formvar followed by a thin coat of evaporated carbon. After 10 s, the neurofilament solution was removed from the grids by aspiration, and the grids were floated face down on drops of 1% polyglutamic acid in PM buffer for 1.0 h at room temperature. The grids were then transferred to drops of purified tubulin at 3.5 mg/mL in PM buffer plus 0.5 mM GTP at 4 °C. The tubulin was warmed to 35 °C to induce microtubule assembly. Each of the drops became turbid and highly viscous, confirming that microtubules had formed. After 20 min, the grids were briefly transferred to drops of 1% glutaraldehyde in PM buffer at room temperature to fix the microtubules. The grids were then floated face down on drops of filtered 0.1 M ammonium acetate. These operations were carried out without allowing the grids to dry. The grids were then transferred to drops of filtered 1% uranyl acetate in double-distilled water. After 10 s, all but a thin coat of the stain was removed by aspiration. The grids were photographed in a JEOL Model 180 electron microscope at 80 kV at magnifications ranging from 3000× to 30000×. The fields shown were randomly chosen by making use of the field position indicator to allow a broad sampling of the squares on the grid.

Protein Determination. Protein concentrations were determined by the method of Bradford (1976) calibrated for tubulin by Detrich & Williams (1978), and calibrated for neurofilaments prepared by the method of Delacourte et al. (1981) by Jones & Williams (1982). This assay was also calibrated for bovine serum albumin. Concentrations of proteins other than tubulin or neurofilament protein prepared by the method of Delacourte are based on the bovine serum albumin calibration.

Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was done on polyacrylamide–urea exponential gradient gels of 4–16% polyacrylamide and 1.0–8.0 M urea (Kim et al., 1979). Gradient gels provide better resolution of HMW-MAPs than do gels of uniform composition.

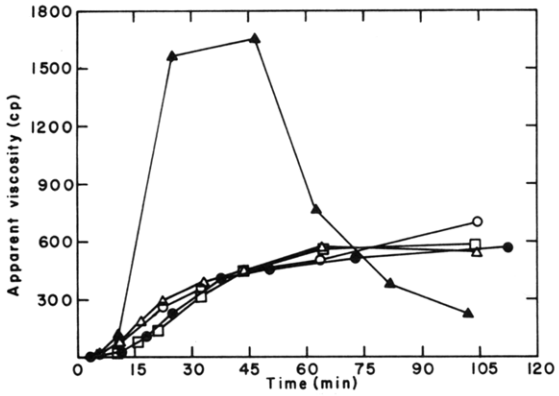

FIGURE 2: Apparent viscosity of tubulin and of neurofilament–tubulin mixtures as a function of time. Tubulin was polymerized to make microtubules, in mixtures containing spinal cord neurofilaments, GTP, and ATP, by warming to 37 °C. Zero time represents the time at which the mixtures were immersed into a 37 °C water bath. (▲) Tubulin at 3.5 mg/mL, brain neurofilaments at 0.7 mg/mL, ATP at 1.0 mM, and GTP at 0.5 mM; (○) tubulin at 3.5 mg/mL, spinal cord neurofilaments at 0.7 mg/mL, ATP at 1.0 mM, and GTP at 0.5 mM; (△) tubulin at 3.5 mg/mL, spinal cord neurofilaments at 0.7 mg/mL, and GTP at 0.5 mM; (□) tubulin at 3.5 mg/mL, ATP at 1.0 mM, and GTP at 0.5 mM; (●) tubulin at 3.5 mg/mL and GTP at 0.5 mM.

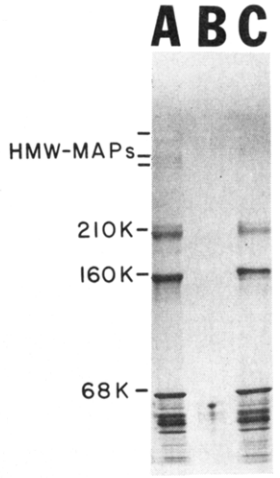

FIGURE 3: MAP binding to spinal cord neurofilaments. SDS–polyacrylamide–urea gradient gel showing spinal cord neurofilaments that were first incubated at a protein concentration of 1.0 mg/mL with whole MAPs at a protein concentration of 0.1 mg/mL for 1 h at 35 °C and then separated from the free MAPs by centrifugation through 20% sucrose. (A) Neurofilaments and MAPs. (B) Whole MAP control experiment. Whole MAPs at 0.1 mg/mL were subjected to the procedure described above. (C) Neurofilament control experiment. Neurofilaments at 1.0 mg/mL were taken through the procedure described above.

Results

Differences in Complex Formation between Spinal Cord Neurofilaments and Brain Neurofilaments. Tubulin was mixed with neurofilaments prepared from bovine brain, at 0 °C, in the presence of ATP and GTP. Aliquots of the mixture were introduced into falling ball viscometers, which were then placed into a water bath maintained at 37 °C. As shown in Figure 2 (closed triangles), the apparent viscosity of this material increased rapidly to a peak near 1600 cP and then decreased. In contrast, when tubulin was mixed, under the same conditions, with neurofilaments prepared from bovine spinal cord, the apparent viscosity increased to a plateau near 600 cP (open circles). This rate and extent of viscosity formation resembled the behavior of tubulin in the presence of GTP (closed circles) or of tubulin in the presence of GTP and ATP (open squares), as well as the behavior of a mixture of tubulin and spinal cord neurofilaments in the presence of GTP (open triangles). Spinal cord neurofilaments at 0.7 mg/mL, alone or in the presence of GTP, had viscosities of less than 4 cP throughout the time of the experiment. The large apparent viscosity attained by the mixture containing brain neurofilaments confirms the results of Runge et al. (1981b), obtained with similar materials. It can be taken as evidence that, after the tubulin has assembled into microtubules as a consequence of warming of the solution, a complex forms between microtubules and neurofilaments. However, the apparent viscosity of a similar mixture containing spinal cord neurofilaments in the place of brain neurofilaments did not rise above that of a solution of tubulin alone. Spinal cord neurofilaments evidently differ from brain neurofilaments in that they do not, by themselves, form a complex with the microtubules. Electron microscopy was used to confirm that microtubules were formed in these mixtures.

The decrease in apparent viscosity exhibited at later times by the mixture of brain neurofilaments and tubulin was unexpected. The experiments described in the following paper (Aamodt & Williams, 1984) show that it resulted from hydrolysis of the GTP in the solution.

Differences in Protein Composition between Spinal Cord Neurofilaments and Brain Neurofilaments. Figure 1 shows the electrophoretic patterns of spinal cord neurofilaments (lane C), of brain neurofilaments (lane B), and of whole MAPs (lanes A and D). The amount of brain neurofilaments applied to the gel was chosen to contain amounts of the three major neurofilament proteins (M_r 68 000, 160 000 and 210 000) approximately equal to the amounts present in the sample of spinal cord neurofilaments. This figure shows that four proteins of high molecular weight (approximately 250 000–350 000), as well as numerous other polypeptides, are present in appreciable amounts in the brain filament preparation but are nearly absent in the spinal cord preparation. These polypeptides comigrate with the slowest migrating and the third slowest migrating HMW-MAPs, and with the two polypeptides of MAP_2 (Sloboda et al., 1975). In the HMW-MAP region, the spinal cord neurofilament preparation contained only small amounts of two proteins that migrate more slowly than MAP_2.

Binding of HMW-MAPs to Spinal Cord Neurofilaments. Spinal cord neurofilaments at a concentration of 1.0 mg/mL were incubated with whole MAPs at a concentration of 0.1 mg/mL at 35 °C for 1 h. The mixture was layered on top of 20% sucrose in PM buffer and centrifuged in a Beckman T40 rotor at 35 000 rpm for 1 h. The resulting pellets, which contained neurofilaments and any MAPs that were bound to the neurofilaments, were resuspended in sample buffer and electrophoresed on polyacrylamide–urea gradient gels. Neurofilaments alone, at a concentration of 1.0 mg/mL, and whole MAPs alone, at a concentration of 0.1 mg/mL, were subjected to the same treatment. The results are shown in Figure 3. Here, one can see that small amounts of five of the MAPs have become bound to the spinal cord neurofilaments: the slowest migrating HMW-MAP, the two MAP_2 proteins, and two LMW-MAPs. The identification of these polypeptides as MAPs is, of course, based on their origin in the whole MAP fraction.

Effect of MAPs on the Association of Microtubules and Neurofilaments in Vitro. Spinal cord neurofilaments at a concentration of 3.3 mg/mL were incubated with whole MAPs at 0.33 mg/mL in PM buffer for 1 h at 35 °C. The mixture was cooled to 0 °C and added to a solution of tubulin and GTP. (The final concentrations of the components of the

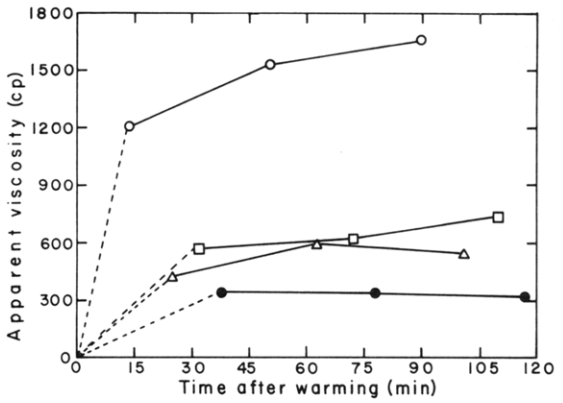

FIGURE 4: Apparent viscosity of mixtures of tubulin with spinal cord neurofilaments, with and without MAPs. This experiment was performed as described in the text. The final concentrations were the following: (○) tubulin at 3.5 mg/mL, neurofilaments at 1.0 mg/mL, MAPs at 0.1 mg/mL, GTP at 0.5 mM; (●) tubulin at 3.5 mg/mL, whole MAPs at 0.1 mg/mL, and GTP at 0.5 mM; (□) tubulin at 3.5 mg/mL, neurofilaments at 1.0 mg/mL, and GTP at 0.5 mM; (△) tubulin at 3.5 mg/mL and GTP at 0.5 mM. The dashed lines indicate that at zero time the apparent viscosities of these mixtures were less than 4 cP.

mixture are given in the legend to Figure 4). The experiment was started by warming the viscometers to 37 °C. The apparent viscosity of the mixture was then measured, together with the viscosities of three control solutions: spinal cord neurofilaments, tubulin, and GTP; tubulin, whole MAPs, and GTP; and tubulin and GTP. Results are shown in Figure 4. The mixture containing tubulin, neurofilaments, and whole MAPs became more than twice as viscous as any of the controls. Evidently, the presence of the MAPs brought about this difference, a result which suggests that the spinal cord neurofilaments could form a cross-connected network after the neurofilaments had bound MAPs.

Mixtures of spinal cord neurofilaments, whole MAPs, tubulin, and GTP were made at the same concentrations used in Figure 4 with the addition of β,β'-iminobis(propionitrile) at concentrations ranging from 0.0 to 10.0 mM. The mixtures were warmed to 37 °C, and after 40 min the apparent viscosities were measured. The viscosities of these mixtures were not lowered by the presence of the β,β'-iminobis(propionitrile). This showed that the in vitro association of microtubules and neurofilaments is not affected by this drug. Therefore, the apparent disruption of microtubule–neurofilament associations in vivo by β,β'-iminobis(propionitrile) may not result from a direct action of this drug on the cross-connections.

Mediation of Viscosity Increase by Subfractions of Whole MAPs. Figure 5A shows the chromatogram of whole MAPs eluted from an A-15m column. The first peak (the excluded MAPs) included tubulin, neurofilament proteins, all of the HMW-MAPs, and some of the low molecular weight MAPs. This first peak probably represented aggregated proteins and neurofilaments that were excluded from the A-15m, and it was set aside. The leading and middle portions of the second peak (fraction A) contained as predominant proteins each of the HMW-MAPs and two LMW-MAPs, but only small amounts of protein in the region of the τ MAPs (Cleveland et al., 1978). The trailing portion of the second peak (fraction B) contained the τ MAPs as well as some HMW-MAPs. Figure 5B shows a SDS-polyacrylamide gradient gel of the whole MAPs that were applied to the column, as well as fraction A and fraction B.

The fraction A MAPs and the fraction B MAPs were tested for their ability to mediate the association of microtubules and neurofilaments. Figure 6 shows the results of these experiments and controls. A viscous gel rapidly formed in a mixture of tubulin and spinal cord neurofilaments that had been incubated with fraction A MAPs (Figure 6, open circles). Smaller viscosities were observed in the mixtures of tubulin

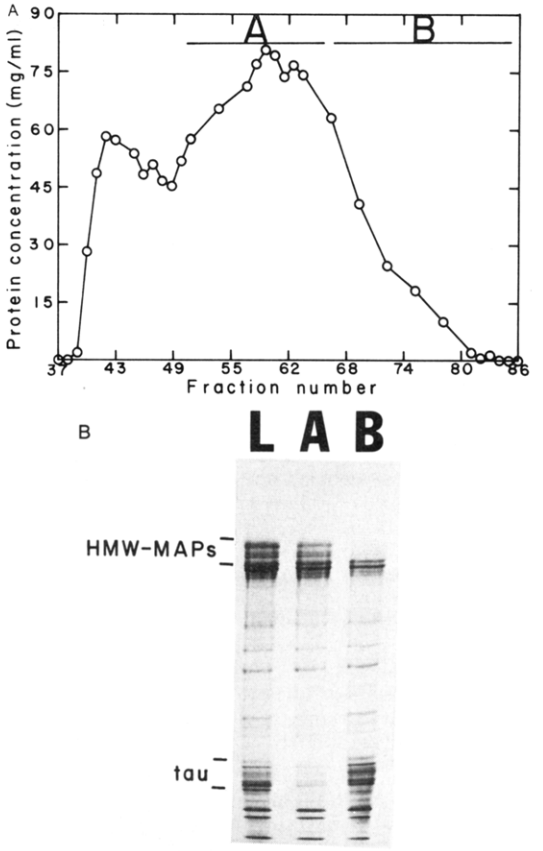

FIGURE 5: Separation of whole MAPs by gel filtration on Bio-Gel A-15m. (A) Protein concentration profile of the fractions from the column. (B) Polyacrylamide–urea gradient SDS gel of the loaded material (lane L) and of the pooled fractions. Ten micrograms of protein was applied to each track. The fractions were pooled into a HMW-MAP-containing fraction (lane A), and a τ-MAP-containing fraction (lane B). The pooled fractions were used in the experiments shown in Figures 6–8.

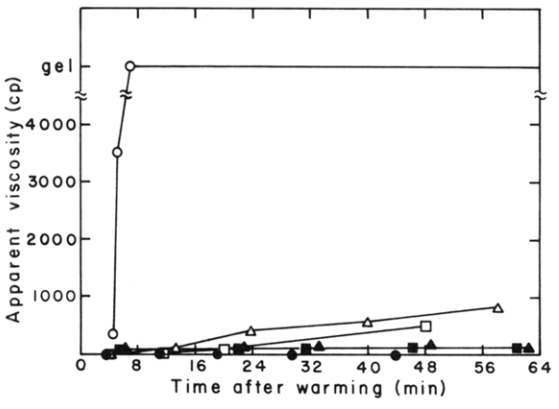

FIGURE 6: Apparent viscosity of mixtures of tubulin and neurofilaments with subfractions of MAPs. These experiments were done as described in Figure 4. (○) Tubulin at 3.5 mg/mL, neurofilaments at 1.0 mg/mL, fraction A MAPs at 0.1 mg/mL, and GTP at 0.5 mM; (△) tubulin at 3.5 mg/mL, fraction A MAPs at 0.1 mg/mL, and GTP at 0.5 mM; (□) tubulin at 3.5 mg/mL and GTP at 0.5 mM; (●) neurofilaments at 1.0 mg/mL, fraction A MAPs at 0.1 mg/mL, and GTP at 0.5 mM; (▲) tubulin at 3.5 mg/mL, neurofilaments at 1.0 mg/mL, fraction B MAPs at 0.1 mg/mL, and GTP at 0.5 mM; (■) tubulin at 3.5 mg/mL, fraction B MAPs at 0.1 mg/mL, and GTP at 0.5 mM. The break in the vertical axis represents the approximation that a solution with apparent viscosity greater than 12000 cP was taken to be a gel.

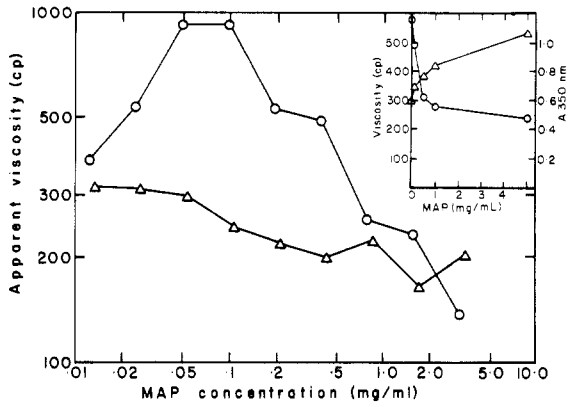

FIGURE 7: Apparent viscosity of mixtures of neurofilaments, microtubules, and MAPs as a function of MAP concentration. (○) Neurofilaments were incubated with fraction A MAPs at 35 °C for 1 h, cooled to 0 °C, and combined with MAP-free tubulin and GTP. The final concentrations were 3.5 mg/mL tubulin, 0.1 mg/mL neurofilaments, and 0.5 mM GTP. The MAP concentration varied as shown on the abscissa. The solutions were loaded into falling ball viscometers and warmed to 37 °C for 40 min before the viscosities were measured. (△) MAP control. Fraction A MAPs were taken through the procedure described and added to a solution of tubulin without neurofilaments. Note that both ordinate and the abscissa are logarithmic scales. The inset shows how the apparent viscosity (circles) and turbidity (triangles) of tubulin solutions polymerized to a steady-state level of assembly vary with the MAP concentration.

with fraction A MAPs (Figure 6, open triangles), neurofilaments with fraction A MAPs (Figure 6, closed circles), and tubulin (Figure 6, open squares).

Fraction B MAPs, in contrast, reduced the apparent viscosity of a mixture of tubulin and spinal cord neurofilaments (Figure 6, closed triangles) below the values that were attained by a solution of tubulin alone (Figure 4, open triangles). Addition of fraction B MAPs also decreased the apparent viscosity developed by a tubulin solution upon incubation at 37 °C (Figure 6, closed squares) below the level that was observed in the absence of MAPs. These results clearly show that MAPs enhance the viscosity of mixtures of microtubules and spinal cord neurofilaments and that the viscosity-enhancing property of the MAPs lies in the fraction of MAPs with a large Stokes radius, the fraction A MAPs. Furthermore, since fraction B MAPs lowered the apparent viscosity of a mixture of tubulin and spinal cord neurofilaments, their presence in the unfractionated whole MAPs would explain why whole MAPs appear to be less effective (per gram) than fraction A MAPs in mediating the interaction of microtubules and neurofilaments.

To test whether MAP_2 mediates the complex, purified MAP_2 at 0.058, 0.116, 0.232, and 0.465 mg/mL was incubated with spinal cord neurofilaments at 2.33 mg/mL for 1 h at 35 °C. The mixtures were cooled and then mixed with tubulin and GTP at 0 °C. The final concentrations were the following: tubulin, 3.5 mg/mL; GTP, 0.5 mM; neurofilaments, 1.0 mg/mL; and MAP_2, 0.025, 0.05, 0.1, and 0.2 mg/mL. Controls of MAP_2 and tubulin, neurofilaments and tubulin, and tubulin alone were run at the same time. The mixtures were warmed to 37 °C for 40 min, and the apparent viscosities were then measured. The viscosities of the MAP_2-containing samples decreased with increased MAP_2 concentration in a manner similar to the decrease in the viscosity of microtubules with increased whole MAP concentration (inset of Figure 7). The viscosities of the neurofilament-, MAP_2-, and tubulin-containing mixtures were slightly lower than those of the MAP_2- and tubulin-containing mixtures, and all of the mixtures that contained MAP_2 had lower viscosities than tubulin alone. It can be concluded that MAP_2 prepared by heat treatment of 3 times cycled microtubule protein (Kim et al., 1979) does not mediate complex formation. It is not possible to tell from this result whether MAP_2 that has not been exposed to high temperature will mediate complex formation.

Dependence of Apparent Viscosity on Concentration of Fraction A MAPs. Aliquots of neurofilaments were incubated with a series of concentrations of fraction A MAPs at 35 °C for 1 h, cooled to 0 °C, and mixed with tubulin and GTP. The final concentrations of tubulin, neurofilaments, and GTP were fixed, but the concentration of MAPs in the initial incubation and in the final mixture (recorded as the abscissa in Figure 7) was varied. The cold solutions were introduced into viscometers and incubated at 37 °C for 40 min before apparent viscosities were measured. Results are shown in Figure 7. The apparent viscosity of the tubulin–neurofilament mixture increased with increasing MAP concentration up to approximately 0.1 mg/mL. At higher MAP concentrations, the apparent viscosity decreased. A control experiment in which the neurofilaments were replaced by an equal volume of buffer is shown by the triangles. The apparent viscosity of the microtubules decreased with increasing MAP concentration.

The insert in Figure 7 shows an experiment in which tubulin at a concentration of 3.7 mg/mL was mixed with GTP at 0.5 mM and whole MAPs at the concentrations shown. A portion of each mixture was transferred into a falling ball viscometer and the remaining portion into a spectrophotometer cuvette prewarmed to 37 °C. The viscometers were also warmed to 37 °C, and after 40 min, the turbidities and the apparent viscosities of these mixtures were measured. The turbidity increased with increased MAP concentration, and the apparent viscosity decreased with increased MAP concentration. The increase in turbidity occurs because the MAPs lower the critical concentration for assembly of the tubulin, causing more of the tubulin to polymerize into microtubules. The apparent viscosity decreased with increasing MAP concentration probably because of an increase in the number of nuclei for formation of microtubules. Such an increase would result in more, shorter, microtubules at the higher MAP concentrations, and therefore, the apparent viscosity would decrease. This effect explains both the MAP-dependent decrease in viscosity of microtubules shown in Figure 7 (triangles) and the fact that the apparent viscosity of the MAP-containing tubulin sample in Figure 6 is lower than that of the tubulin control in Figure 4.

If HMW-MAPs mediate the association of microtubules and neurofilaments in vitro by cross-connecting these structures, then the apparent viscosity of a solution of microtubules and neurofilaments should increase as the MAP concentration is increased, since more cross-connections will be made, up to an optimal MAP concentration. However, if the MAP concentration is increased above the optimal concentration, the apparent viscosity would be expected to decrease since free MAPs will bind to the MAP binding sites both on the microtubules and on the neurofilaments and thereby prevent cross-connections from forming. (The situation is analogous to the classical immunological precipitin curve.) The fact that the observed mixtures behave in this way is consistent with the hypothesis that such cross-connections are indeed formed, though other interpretations are also possible.

Electron Microscopy of Microtubules on Neurofilament-Coated "Affinity Grids". Neurofilament affinity electron microscope grids were made as described under Materials and Methods. As shown in Figure 8, many microtubules became bound to those grids that had been coated with neurofilaments

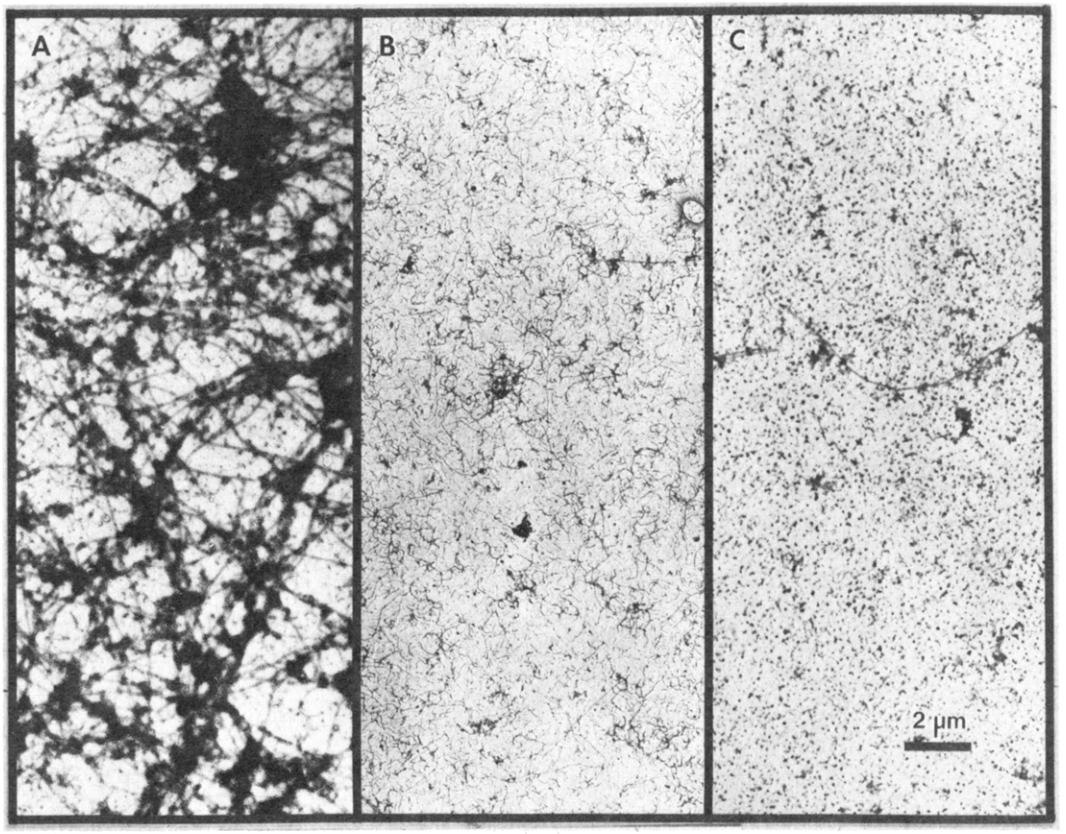

FIGURE 8: Affinity grid electron micrographs. (A) Neurofilaments were incubated with fraction A MAPs for 1 h to bind the MAPs to the neurofilaments and then prepared for electron microscopy as described under Materials and Methods. (B) and (C) are control experiments. In (B), neurofilaments were taken through the same procedure without MAPs being present. In (C), MAPs were subjected to the same procedure without neurofilaments being present.

that had first been incubated with whole MAPs and centrifugally separated from unbound MAPs. In contrast, few microtubules became bound to the grids coated with neurofilaments which had been taken through the same procedure without MAPs, or to the grids coated with the small amount of free MAPs which came through the same procedure without neurofilaments. This result supports the hypothesis that MAPs mediate the association of microtubules and neurofilaments by connecting these two structures.

Discussion

When neurofilaments isolated from brain are mixed with tubulin in a buffer that promotes assembly of tubulin, and the solution is warmed to polymerize the tubulin into microtubules, the neurofilaments and microtubules become connected to form a viscous network (Runge et al., 1981b; Minami et al., 1982). When neurofilaments prepared from spinal cord were used in a similar experiment, no such connected network was formed. The brain neurofilament preparation contains small amounts of proteins resembling HMW-MAPs, but the spinal cord neurofilament preparation does not. When the spinal cord neurofilaments were incubated with whole MAPs, they bound a small amount of several of the MAPs. Upon binding of the MAPs, the neurofilaments formed a highly viscous mixture when incubated with microtubules. A subfraction of the MAPs contains most of the viscosity-promoting activity and most of the HMW-MAPs. It appears from these results that a large protein, or group of proteins, in the fraction A MAPs mediates the association of microtubules and neurofilaments.

Protein Composition of Neurofilaments from Brain and Spinal Cord. It can be inferred from previous studies (Lasek & Hoffman, 1976; Liem et al., 1978; Schlaepfer & Freeman, 1978; Delacourte et al., 1980; Jones & Williams, 1982) that neurofilaments prepared from spinal cord have associated with them smaller amounts of proteins that comigrate on SDS gels with HMW-MAPs than do neurofilaments prepared from brain. The reasons for the presence or absence of these proteins are not clear, although distribution studies (Matus et al., 1981; Vallee, 1982; Binder et al., 1982) suggest that MAP_2 is present only in small amounts in axons. Since the bulk of the neuronal material in spinal cord is axonal, one would expect little MAP_2 to be present. The adventitious proteins, including MAPs, that are present in the brain neurofilament preparation are tightly bound (Runge et al., 1981a) to fast-sedimenting structures, probably the neurofilaments themselves. This observation suggests that brain neurofilaments possess MAP binding sites.

Binding of MAPs to Neurofilaments. The observation of binding of HMW-MAPs to previously MAP-free neurofilaments is in accord with the findings of Leterrier et al. (1982). Our results show that at least five MAP polypeptides bind to spinal cord neurofilaments: the slowest migrating MAP (probably MAP_1) (Kim et al., 1979), the two components of MAP_2, and two LMW-MAPs (Berkowitz et al., 1977). These proteins may bind to separate sites on the neurofilaments, or they may compose a complex of proteins that together bind to a single site.

Mediation of Microtubule–Neurofilament Association by Bound HMW-MAPs. Brain neurofilaments, which possess bound MAPs, have been shown to participate in the formation of viscous complexes with microtubules (Runge et al., 1981b; Runge & Williams, 1981; Minami et al., 1982). The present results demonstrate that one or more of the MAPs plays an essential part in the process of complex formation. Runge et al. (1981b) had previously observed that the viscous sedimentable microtubule–neurofilament complex could form in mixtures of brain neurofilaments and tubulin without the

addition of exogenous MAPs. They mistakenly interpreted these results to mean that "MAPs may not be required for formation of the complex", although they noted that the possible participation of these proteins in complex formation could not be ruled out. Those observations can now be understood on the basis of the present results, since in the earlier study the MAP binding sites on the brain neurofilaments are likely to have been occupied before exogenous MAPs were added. Minami et al. (1982) found that at high concentrations (greater than 0.5 mg/mL) MAPs inhibit the formation of a complex of microtubules and neurofilaments. They interpreted these data to mean that MAPs are not involved in the formation of the complex. However, as discussed below, these data can be taken as evidence that MAPs are involved in the formation of microtubule–neurofilament connections, and at high concentrations, the MAPs prevent connections from forming by blocking the MAP binding sites on the microtubules. The preparation of brain neurofilaments employed in complex formation by Minami et al. (1982) also appeared to contain MAPs. Hence, in all cases in which a microtubule–neurofilament complex has been observed, HMW-MAPs have been present. It appears that one or more of these proteins mediates formation of the complex.

Fractionation of MAPs. The question of which of the MAPs mediates complex formation is partially answered by the gel filtration experiments. The A fraction, composed of materials of large effective Stokes radius, apparently contains the mediating molecule (or molecules). Although it is not possible to specify with certainty which of the several polypeptides is the mediator, it seems likely that one or more of the HMW-MAPs may be. MAP_2 purified by boiling and gel filtration on Bio-Gel A-15m did not cause an increase in the viscosity of microtubule–neurofilament mixtures. This result means either that MAP_2 is not, by itself, the mediating molecule or that the boiling step destroyed its cross-linking activity.

Do MAPs Form Cross-Bridges? The fact that HMW-MAPs form projections from the microtubule wall (Amos, 1977; Kim et al., 1979; Vallee & Borisy, 1978), together with their observed binding to neurofilaments, is compatible with a mechanism of complex formation in which the relatively large HMW-MAPs form bridges between microtubules and neurofilaments. Leterrier et al. (1982), on the basis of measurements of binding of HMW-MAPs to neurofilaments, speculated that this might be the case. Participation of HMW-MAPs in connecting microtubules and secretory granules has been demonstrated by Suprenant & Dentler (1982), and a similar geometry may apply in the present case. The existence of an optimum concentration of MAPs for viscosity development is strong evidence that the added MAPs, or a complex of added MAPs, are forming a double-ended bridge. This phenomenon is well-known in the precipitation of a multivalent antigen by a bivalent antibody [cf. Bellanti (1978)]. In analogy to that reaction, at low MAP concentration, each added molecule would have a high probability of finding a vacant site on a neurofilament and on a microtubule. As MAP concentration increases, the extent of cross-bridging would increase, as would the viscosity. At high MAP concentrations, however, the probability would become great that each site is occupied by a single MAP, and consequently, both cross-bridging and viscosity would be expected to decrease.

Affinity Grid Electron Microscopy. The affinity grid electron micrographs shown in Figure 8 demonstrate by a second method that spinal cord neurofilaments which have bound MAPs will then bind microtubules, while spinal cord neurofilaments without bound MAPs will not bind microtubules.

The use of affinity electron microscopy to analyze associative interactions may be applicable in other studies of the binding of relatively large structures to either large or small ligands. For instance, the binding of vesicles to microtubules or actin filaments, the binding of MAPs to microtubules, or the binding of actin binding proteins to actin filaments could be studied by this technique.

The question of whether the MAPs form bridges or mediate association through some other mechanism must still be considered open until firm structural evidence can be obtained. Likewise, the question of whether the complex formed in vitro resembles the axonal cytoskeleton in its essential interactions must remain unanswered for the present.

Acknowledgments

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

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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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