Microtubule-associated Proteins–dependent Colchicine Stability of Acetylated Cold-Labile Brain Microtubules from the Atlantic Cod, *Gadus morhua*

Martin Billger, Elisabeth Strömberg, and Margareta Wallin

Department of Zoophysiology, Comparative Neuroscience Unit, University of Göteborg, S-400 31 Göteborg, Sweden

Abstract. Assembly of brain microtubule proteins isolated from the Atlantic cod, *Gadus morhua*, was found to be much less sensitive to colchicine than assembly of bovine brain microtubules, which was completely inhibited by low colchicine concentrations (10 μM). The degree of disassembly by colchicine was also less for cod microtubules. The lack of colchicine effect was not caused by a lower affinity of colchicine to cod tubulin, as colchicine bound to cod tubulin with a dissociation constant, K_d, and a binding ratio close to that of bovine tubulin.

Cod brain tubulin was highly acetylated and mainly detyrosinated, as opposed to bovine tubulin. When cod tubulin, purified by means of phosphocellulose chromatography, was assembled by addition of DMSO in the absence of microtubule-associated proteins (MAPs), the microtubules became sensitive to low concentrations of colchicine. They were, however, slightly more stable to disassembly, indicating that posttranslational modifications induce a somewhat increased stability to colchicine. The stability was mainly MAPs dependent, as it increased markedly in the presence of MAPs. The stability was not caused by an extremely large amount of cod MAPs, since there were slightly less MAPs in cod than in bovine microtubules. When "hybrid" microtubules were assembled from cod tubulin and bovine MAPs, these microtubules became less sensitive to colchicine. This was not a general effect of MAPs, since bovine MAPs did not induce a colchicine stability of microtubules assembled from bovine tubulin. We can therefore conclude that MAPs can induce colchicine stability of colchicine labile acetylated tubulin.

MICROTUBULES constitute a dynamic part of the cytoskeleton in eukaryotic cells. They can be isolated for studies in vitro, and such studies show that microtubules are built of tubulin and several microtubule-associated proteins (MAPs)[1]. Microtubule proteins in vitro reflect the dynamics in vivo, in that they can be brought through cycles of assembly and disassembly. Furthermore, they may be disassembled by several microtubule-disrupting agents, such as cold, Ca^{2+} or colchicine.

Previous studies have shown that an isolated fraction of cold-labile microtubules, from brains of the Atlantic cod (*Gadus morhua*) exhibit atypical features, i.e., they are not broken down by calcium, and they are associated with an unusual set of MAPs (Strömberg et al., 1989). In addition, neurophysiological studies on cod autonomic innervation, where colchicine was employed to increase the levels of neuropeptides in autonomic ganglia cells, failed, possibly due to an ineffectiveness of colchicine to disrupt cod axonal transport (Drs. S. Holmgren and A.-C. Jönsson, Department of Zoophysiology, Comparative Neuroscience Unit, University of Göteborg, Sweden, personal communication). This observation might indicate that cod microtubules also are colchicine stable.

Tubulin is known to be subject to different kinds of posttranslational modification. After synthesis, tubulin may be cyclically detyrosinated (Hallak et al., 1977; Argaraña et al., 1980) and tyrosinated (Barra et al., 1973), as well as acetylated (L'Hernault and Rosenbaum, 1983, 1985). The study of the interconversion of these tubulin states has been greatly enhanced by the development of monospecific antibodies against tyrosinated, detyrosinated, and acetylated α-tubulin (Gundersen et al., 1984; Kilmartin et al., 1982; Piperno and Fuller, 1985; Wehland and Weber, 1987).

In recent years, several studies have shown that many cell types contain a stable subset of microtubules. The reason(s) for microtubule stability are not known, but posttranslational modifications of tubulin, such as acetylation (Black and Keyser, 1987) and detyrosination (Kreis, 1987; Webster

Part of these results have been presented in preliminary form at the 4th Meeting of the European Cytoskeletal Club, held in Lyon, France, in 1988 (Billger, M., E. Strömberg, and M. Wallin. 1988. Difference in colchicine sensitivity between microtubules from lower and higher vertebrates. *In* Structure and Functions of the Cytoskeleton. B. A. F. Rousset, editor. INSERM/John Libbey Eurotext, Lyon, France. 171:393–398.).

1. *Abbreviation used in this paper*: MAP, microtubule-associated protein.

et al., 1987) have been proposed, since these modifications often correlate with stable microtubules. Other studies of microtubule stability indicate that posttranslational modification of tubulin per se is not the reason for microtubule stability. Other factors, possibly including specific MAPs, seem necessary to commit microtubules for stability (Schulze et al., 1987). An extensive review of these matters is given by Greer and Rosenbaum (1989).

In the present study, we show that isolated cod brain microtubules have a much lower colchicine sensitivity than mammalian brain microtubules, but that the tubulin is competent of colchicine binding in a manner similar to that of bovine brain tubulin. Cod tubulin was found to be highly acetylated and predominately detyrosinated. Furthermore, assembly of acetylated and detyrosinated tubulin in the absence of MAPs, was inhibited by low concentrations of colchicine. In addition, reconstitution of cod tubulin with bovine MAPs resulted in colchicine-stable "hybrid" microtubules, although cod MAPs seemed to be more effective in generating colchicine stability in cod microtubules. Thus, our results suggest that microtubule stability is not a function of posttranslational modifications of tubulin per se, but of the presence of MAPs.

Materials and Methods

Chemicals

GTP (type II-S) was purchased from Sigma Chemical Co. (St. Louis, MO) and [^{3}H]colchicine was obtained from Amersham International (Amersham, UK). Unless otherwise stated, all other chemicals were of reagent grade.

EM

Negative staining of microtubules was achieved by applying 5 μl of microtubule-containing solution, usually diluted twentyfold in PEMG (100 mM Pipes, 1 mM EGTA, 1 mM GTP, and 0.5 mM $MgSO_4$) with 50% (wt/vol) sucrose, to a Formvar-coated copper grid. The grid was dried with a piece of filter paper and subsequently stained with 1% (wt/vol) uranyl acetate.

Preparation of Microtubule Proteins

Bovine microtubule proteins were isolated by two cycles of assembly–disassembly in PEMG buffer as described in Wallin et al. (1986). Cold-labile cod brain microtubules were isolated by one cycle of a temperature-dependent assembly–disassembly method as described by Strömberg et al. (1986). Microtubule assembly of bovine and cod microtubules was induced by the addition of 1 mM GTP and raising the temperature to +37°C and +30°C, respectively. Phosphocellulose-purified tubulin was prepared as described by Weingarten et al. (1975) in the presence of 0.5 mM Mg^{2+} (Williams and Detrich, 1979). Several preparations of cod brain microtubules were pooled, corresponding to 600 animals (~90 mg microtubule proteins), and applied without prior concentration to a phosphocellulose column (2 mg protein per ml wet packed phosphocellulose). All tubulin-containing fractions were pooled, concentrated by means of pressure filtration, and the solution was reconstituted to the original assembly buffer by adding a concentrated stock, GTP was added to 1 mM, and centrifuged at 200,000 *g*, for 30 min at +4°C. The purified tubulin was checked for purity by means of SDS-PAGE and for assembly competence by raising the temperature in the presence of 1 mM GTP and 8% (vol/vol) DMSO. Bovine brain tubulin was prepared using the same protocol. MAPs were eluted by adding 0.6 M NaCl, and the eluate was desalted and concentrated.

Assembly/Disassembly

Assembly of microtubules was determined in a temperature-controlled spectrophotometer by measuring the change in light scattering at 350 or 450 nm. The longer wavelength was used for experiments with colchicine to avoid the absorption of this compound at shorter wavelengths. It was established that the amplitude of light scattering was lower at 450 nm but still proportional to the 350-nm reading (see also Gaskin et al., 1974).

Colchicine Binding to Tubulin

The binding of colchicine to tubulin was determined by employing the cellulose filter absorption method (Borisy, 1972). Isolated microtubule proteins were incubated at concentrations corresponding to 10 μM tubulin at +30°C in PEMG buffer. The specific activity of [^{3}H]-colchicine was kept in the range of 5–100 TBq/mol colchicine. After 1 h, the incubation was interrupted by chilling the solutions on ice, and aliquots were diluted and filtered through a stack of three filter disks (model DE81; Whatman Inc., Clifton, NJ). The tubulin-to-filter absorption time was adjusted to 1 min by adjusting the water flow of the vacuum pump. The filters were then placed in scintillation vials and 10 ml of Optiscint HiSafe (LKB Instruments Inc., Bromma, Sweden) was added. After allowing for 24 h desorption by gentle shaking in darkness, the radioactivity was determined in an LKB Wallac liquid scintillation counter programmed with an appropriate quench correction curve. Values from samples without proteins were subtracted from protein-containing samples.

Gel Electrophoresis

Proteins were separated by SDS-PAGE on a 1.5-mm-thick discontinuous vertical slab gel (LKB Instruments Inc.) using 5–12% linear gradient polyacrylamide (1:37 crosslinker) according to Laemmli (1970). The gels were stained in 0.25% (wt/vol) Coomassie brilliant blue R250 (Sigma Chemical Co.) in methanol/acetic acid/water (5:1:5, vol/vol) and destained in 7% (vol/vol) acetic acid and 5% (vol/vol) methanol. Alternatively, gel electrophoresis was performed on a PhastSystem™, using precast 12.5% PhastGels™ (Pharmacia, Uppsala, Sweden).

Relative Amounts of MAPs

Relative amounts of MAPs in microtubule preparations were determined by laser densitometry of Coomassie brilliant blue G-250–stained SDS-PAGE gels using an Ultroscan XL laser densitometer (LKB Instruments Inc.), using manual integration. The amount of MAPs was calculated as the relative amount of nontubulin proteins.

Immuno-electroblotting and Related Procedures

Semi-dry electrophoretic transfer was performed using PhastTransfer™ (Pharmacia LKB). After SDS-PAGE in 12.5% polyacrylamide homogenous SDS-gels (Pharmacia PhastGel™), the gel was laid on a nitrocellulose membrane soaked in transfer buffer (25 mM Tris [hydroxymethyl]-aminomethan, 192 mM glycine, and 20% [vol/vol] methanol) and three presoaked filter papers (as described by Pharmacia LKB) were placed on each side of the stack. The stack was placed between graphite electrodes, and the transfer was performed at 1.0 mA/cm^2 (25 mA per gel) for 15 min at +15°C. This method allows for the rapid and effective transfer of microtubule proteins.

After transfer, the membrane was washed in TBS (20 mM Tris [hydroxymethyl]-aminomethan, 500 mM NaCl, pH 7.5) for 10 min and soaked in blocking solution (TBS with 5% wt/wt fat-free milkpowder [Semper AB, Stockholm, Sweden]) with very gentle agitation for 30 min. The membrane was then incubated with the primary antibody in the same buffer for another 90 min. After one wash in distilled water and two 10-min washes in TBS with 0.05% vol/vol Tween-20, the membrane was incubated with the secondary antibody for 1 h in blocking solution and washed in water. Subsequently, the membrane was developed in darkness in a solution freshly made of 0.5% (wt/vol) 4-chloro-1-naphthol (Bio-Rad Laboratories, Cambridge, MA), 17% (vol/vol) methanol, and 0.015% (vol/vol) H_2O_2 in TBS for 15–30 min. The development of the membrane was arrested by soaking the membrane in distilled water.

As a control of the electrophoretic transfer, symmetric halves of the membranes were stained with 0.1% (wt/vol) Amido black in 45% (vol/vol) methanol and 10% (vol/vol) acetic acid, destained in the same solution, and documented.

Antibodies

The mouse monoclonal 6-11B-1 against acetylated α-tubulin was kindly supported by Dr. G. Piperno (Rockefeller University, New York). mAbs against tyrosinated and detyrosinated α-tubulin, respectively, were a generous gift

from Drs. C. Bulinski and G. Gundersen (University of California). Secondary antibodies were purchased from Cappel (Göteborg, Sweden) (HRP-conjugated goat anti-mouse IgG) or BioRad (Stockholm, Sweden) (HRP-conjugated goat anti-rabbit IgG).

Miscellaneous Procedures

Protein concentrations were determined according to the method of Lowry et al. (1951) by using BSA as a standard. Colchicine binding data were represented as polynomial regression plots of moles colchicine bound per mole tubulin versus the logarithm of free colchicine concentration, to assess the approach to saturation (Thompson and Klotz, 1971). Free colchicine concentration was calculated as the difference between total (added) and bound (measured) colchicine. Scatchard plots were performed by plotting mole colchicine bound per mole tubulin per free colchicine concentration versus mole colchicine bound per mole tubulin, according to Scatchard (1949). Assuming that tubulin has one binding site for colchicine and a molecular mass of 100,000 kD, the dissociation constant, K_d, was calculated as -1/slope for the linear regression line, and the stoichiometry was determined from the abscissa intercept. Statistical treatment of data was performed with a Student's *t* test.

Results

Assembly of Cod Brain Microtubules Is Much Less Sensitive to Colchicine than That of Bovine Brain Microtubules

The assembly of isolated cod brain microtubules was only scarcely affected by the presence of 10 μM colchicine (Fig. 1 *A*), as opposed to bovine brain microtubules, where assembly was completely inhibited (Fig. 1 *B*). The assembly of cod microtubules was not completely inhibited even at concentrations as high as 1 mM. It was also seen that colchicine added to preformed microtubules, was much less efficient in inducing disassembly of cod microtubules. Instead, cod microtubules disassembled and reached a new steady-state level slightly below the original value (Fig. 2). In contrast, bovine microtubules showed a progressive disassembly throughout the experiment. Colchicine did not induce any aberrant forms, since cod brain microtubules assembled in the presence or absence of colchicine had the same morphology as bovine brain microtubules, as judged from negative staining (not shown).

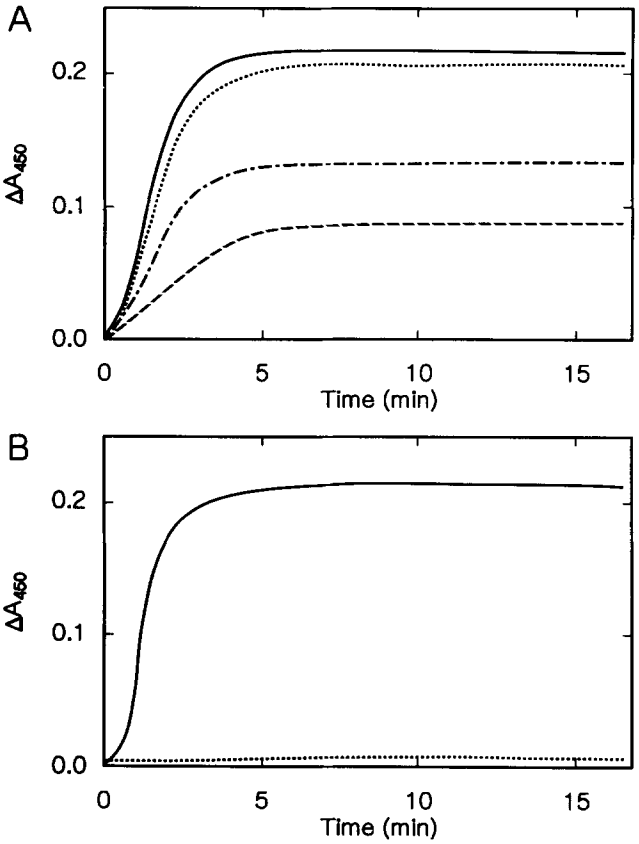

Figure 1. The assembly of microtubule proteins (2 mg/ml) was followed spectrophotometrically in the absence (control) or presence of colchicine and plotted. Control (———); 10 μM (· · ·); 100 μM (— · — · —); 1,000 μM (— — —). (*A*) Cod brain microtubules; (*B*) bovine brain microtubules.

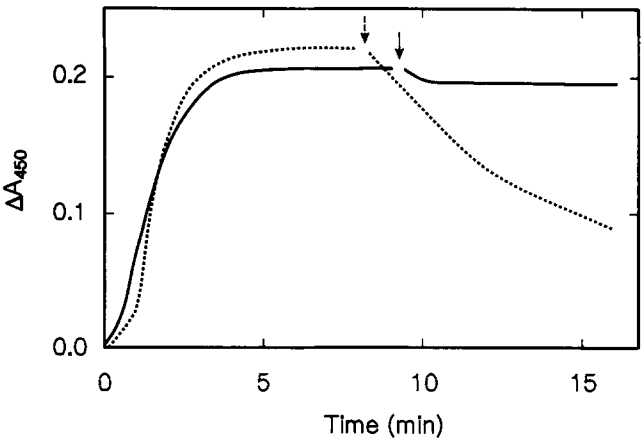

Figure 2. Microtubule proteins from cod (———) and bovine (· · ·) brain were assembled as in the legend to Fig. 1, and at steady state, 1 mM colchicine was added (*arrow*).

Cod and Bovine Brain Tubulin Have the Same Affinity to Colchicine

Lack of colchicine effects on cod brain microtubules would be predicted if cod tubulin either is unable to bind colchicine or binds colchicine with a low affinity. To exclude such possibilities, a colchicine-binding analysis on isolated microtubule proteins was performed. A semi-logarithmic plot of the bound colchicine fraction versus free colchicine, showed a sigmoidal pattern, as would be expected for a single class binding site reaction approaching equilibrium (Fig. 3 *A*). A Scatchard plot of the binding data showed that the dissociation constant, K_d, was in the same range for both cod and bovine brain tubulin (8.5 and 7.7 μM, respectively) (Fig. 3 *B*). The stoichiometry of colchicine binding to tubulin was calculated as 0.4 mol colchicine bound per mol of cod and bovine tubulin, respectively.

Composition and Relative Abundance of MAPs

The main MAPs of bovine microtubules are MAP1, MAP2, and the *tau* proteins. Cod MAPs consist of MAP2, but not MAP1. In addition, there are two further MAPs, ~400-kD MAP and another with a molecular mass below MAP2 (Fig. 4).

Semiquantitative densitometric analysis of microtubule proteins separated on SDS polyacrylamide gels showed that bovine brain microtubule proteins consisted of ~70% tubulin and cod brain microtubules contained ~80% tubulin,

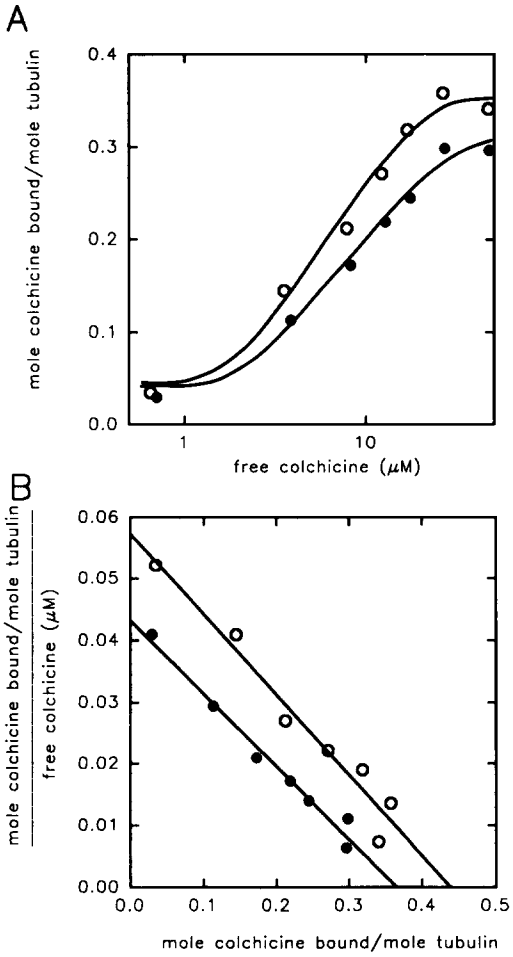

Figure 3. (*A*) Microtubule proteins from bovine (○) and cod (●) brains were incubated corresponding to 10 μM tubulin in the presence of radiolabeled colchicine at the indicated concentrations. After incubation at +30°C for 60 min, the amount of bound colchicine was determined as described in Materials and Methods, and the data were fitted to a third-order polynomial regression line. (*B*) Scatchard plot of colchicine binding to bovine (○) and cod (●) tubulin. Radiolabeled colchicine-liganded tubulin was treated as described in Materials and Methods and the results were fitted to a straight line. The dissociation constants, K_d, were calculated as −1/(slope) of the regression lines and the number of binding sites was estimated from the abscissa intercepts. Polynomial and linear regression correlation coefficients for the plots were always better than 0.91.

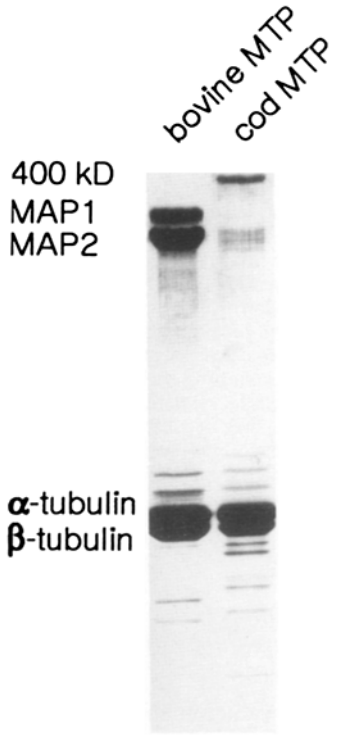

Figure 4. Analysis of protein contents in preparations of microtubule proteins from cod and bovine brains. Microtubule proteins isolated from bovine (*left lane*) and cod (*right lane*) brains were separated under identical conditions on 5–12% SDS-polyacrylamide vertical slab gels and stained with Coomassie blue. Identified proteins were denoted as indicated. Each lane was loaded with 50 μg microtubule proteins.

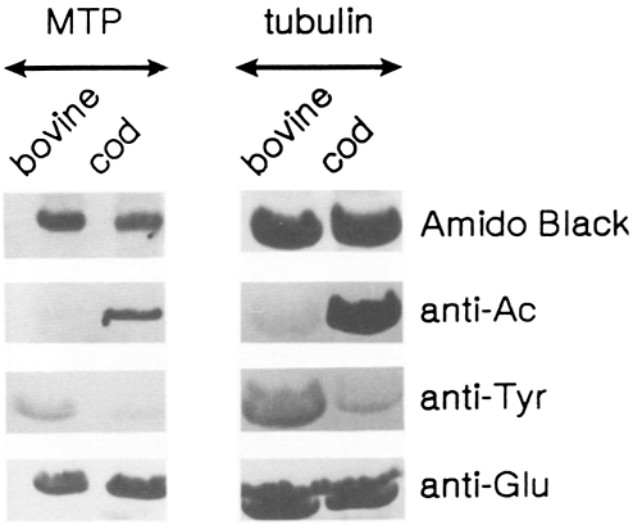

Figure 5. Immunoblotting of microtubule proteins. Microtubule proteins, consisting of either tubulin and MAPs (MTP) (*left lanes*) or phosphocellulose-purified tubulin (*right lanes*) from bovine and cod brain, were electrophoresed on 12% PhastGels™, transferred to nitrocellulose membranes, and immunoblotted with the 6-11B-1 antibody against acetylated α-tubulin (*anti-Ac*) and antibodies against tyrosinated (*anti-Tyr*) and detyrosinated (*anti-Glu*) α-tubulin, respectively, as indicated. A control membrane was stained with Amido black to detect tubulin, as indicated. Each lane was loaded with 8 μg microtubule proteins.

when separated under identical conditions on the same gel (data not shown). These figures were obtained from summing the integrated areas for all identified microtubule proteins. These figures are only semiquantitative, since the background staining of the gel supposedly varied with the polyacrylamide gradient (i.e., the baseline established by the densitometer is based on the lowest absorption in the gel lane, whereas the true baseline coincides with the polyacrylamide gradient).

Isolated Cod Brain Microtubules Consist of Acetylated Tubulin, and a Mixture of Tyrosinated and Detyrosinated Tubulin

Having established that cod brain microtubules are colchicine stable, we sought a reason for this. As posttranslational modifications of tubulin often are associated with microtubule stability, we asked whether this is also the case for cod microtubules. Immunoblots of brain microtubule proteins showed that cod tubulin is highly acetylated, whereas bovine

brain tubulin was only slightly acetylated. Cod tubulin contained a mixture of tyrosinated and detyrosinated tubulin, although the detyrosinated tubulin appeared to dominate. In addition, bovine tubulin consisted of both tyrosinated and detyrosinated tubulin, although the relative amounts were much less obvious (Fig. 5).

Cod Tubulin in the Absence of MAPs Is Sensitive to Low Concentrations of Colchicine

In an attempt to clarify whether the observed colchicine stability of cod brain microtubules is dependent on the intrinsic properties of tubulin, or conferred by the presence of MAPs, we studied the effects of colchicine on the DMSO-induced assembly of tubulin in the absence of MAPs. When tubulin was purified by phosphocellulose chromatography, the distribution of acetylation, tyrosination, and detyrosination persisted (Fig. 5). Assembly of these microtubules was completely inhibited by 10 μM colchicine initially (Fig. 6 *A*). This result was congruent with the inhibited assembly of phosphocellulose-purified bovine brain tubulin (Fig. 6 *B*). When 1 mM colchicine was added to preformed MAPs-free microtubules, cod microtubules disassembled by ~50%, whereas bovine microtubules disassembled almost completely (Fig. 6). To examine whether the remaining microtubules were enriched in acetylated tubulin, they were centrifuged at 200,000 *g* at +30°C for 30 min. The pellet and the supernatant were electrophoresed, blotted, and examined for acetylated and detyrosinated α-tubulin. As judged from these immunoblottings, there was a slight enrichment of acetylated, but no obvious change in detyrosinated α-tubulin, after treatment with colchicine (Fig. 7).

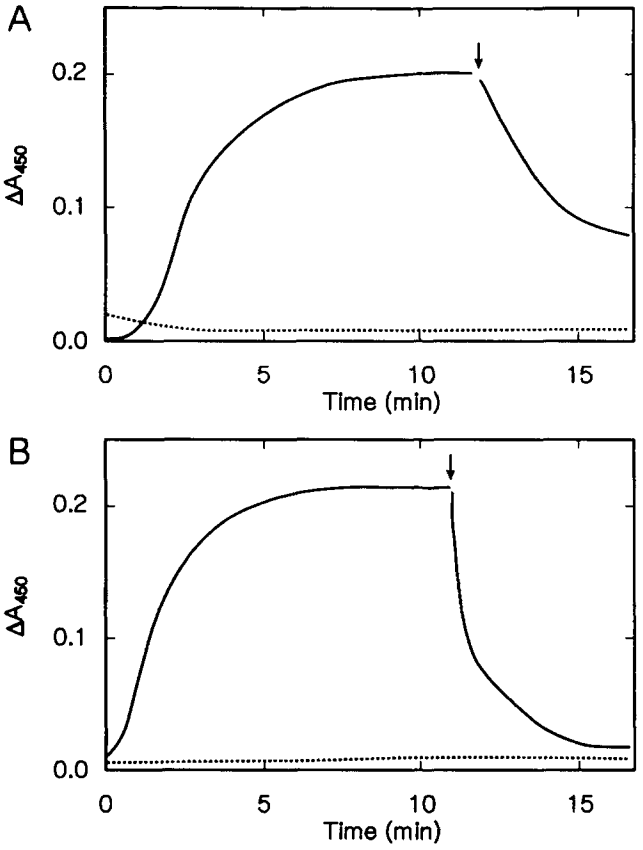

Figure 6. Phosphocellulose-purified tubulin at 2 mg/ml was assembled by the addition of 1 mM GTP and 8% DMSO and raising the temperature to +30°C. Control (———), 1 mM colchicine added at steady state of assembly (*arrow*); 10 μM colchicine added initially (· · ·). (*A*) Cod brain tubulin; (*B*) bovine brain tubulin.

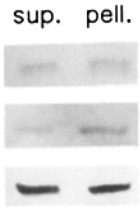

Figure 7. Immunoblotting of microtubule proteins. Cod tubulin at 2 mg/ml was assembled in 180 μl at +30°C by the addition of 8% (vol/vol) DMSO and 1 mM GTP. At steady state of assembly, 1 mM colchicine was added. After 20 min incubation, the suspension was centrifuged at 200,000 *g* at +30°C for 30 min. The pellet and the supernatant were electrophoresed on 12% PhastGels™ and electroblotted. The transferred proteins were stained as indicated. Each lane was loaded with the same amount of tubulin.

Reconstitution of "Hybrid" Microtubules

Purified cod MAPs were unable to initiate the assembly of phosphocellulose-purified cod or bovine tubulin, at a MAP concentration similar or higher than that in the samples of unseparated microtubule proteins (not shown). In contrast, cod tubulin and bovine MAPs coassembled quite well (Fig. 8). These "hybrid" microtubules were less sensitive to colchicine than bovine microtubules. In the presence of 10 μM colchicine, assembly was ~50% of control, indicating that bovine MAPs can confer colchicine stability to cod tubulin. The induced stability was not as high as for that of cod brain microtubule proteins, where an approximate inhibition of only 10% was found (compare Figs. 8 and 1 *A*).

Cod tubulin assembled readily at a concentration of 4 mg/ml in the absence of MAPs. Reconstitution of hybrid microtubules from cod tubulin and bovine MAPs showed an increasing extent of assembly at increasing bovine MAPs concentration (Fig. 9 *A*). The correlation was linear at least up to 1 mg/ml bovine MAPs. Colchicine at 10 μM inhibited the assembly of pure cod tubulin completely. When bovine MAPs were included in the samples, this inhibition was reversed. The amount of assembly in the presence of 10 μM colchicine

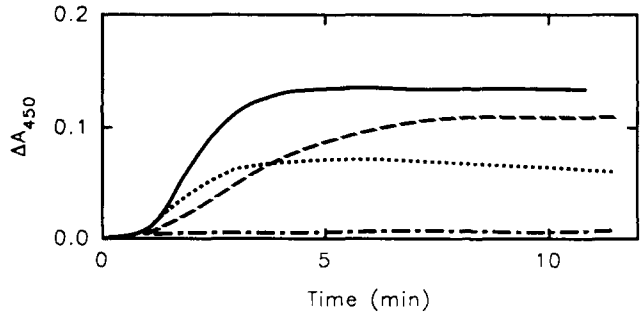

Figure 8. Assembly of "hybrid" microtubules. Cod tubulin at 4 mg/ml was assembled in the presence or absence of bovine MAPs at 1 mg/ml and in the presence or absence of 10 μM colchicine. (———) Cod tubulin and bovine MAPs; (· · ·) cod tubulin and bovine MAPs, 10 μM colchicine initially; (— — —) cod tubulin; (— · — · —) cod tubulin, 10 μM colchicine initially.

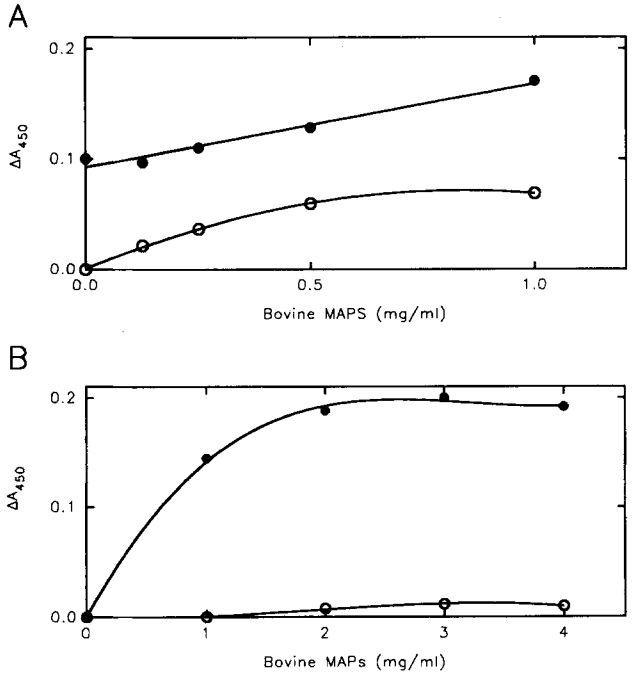

Figure 9. Concentration dependency of bovine MAPs for the assembly of cod tubulin in the presence or absence of colchicine. (*A*) Cod tubulin at 4 mg/ml in the absence of DMSO was brought to assembly in the presence of varying concentrations of bovine MAPs and in the presence (○; second order regression) or absence (●; linear regression) of 10 μM colchicine. At steady state of assembly, the amount of assembly, expressed as ΔA_{450}, was estimated and plotted against bovine MAPs concentration. (*B*) Bovine tubulin at 4 mg/ml in the absence (●) or presence (○) of 10 μM colchicine was brought to assembly at varying bovine MAPs concentration as in *A*.

increased with an increasing amount of bovine MAPs. The results showed, however, a diverting curve, indicating a saturation of assembly stimulation effect. There was no complete reversibility, bovine MAPs at 1 mg/ml could only induce an assembly of ~50% of the control. The results therefore suggest that bovine MAPs can induce a partial colchicine stability of cod microtubules, but not as extensive as for the cod MAPs. The induction of colchicine stability is, however, not an intrinsic property of MAPs, since even though bovine MAPs stimulated the assembly of bovine tubulin, they did not induce any colchicine stability at a similar tubulin/MAPs ratio as cod tubulin and bovine MAPs (Fig. 9 *B*). The conclusion is, therefore, that both cod and bovine MAPs can induce stability of cod brain microtubules, most probably because cod tubulin is posttranslationally modified.

Discussion

In the present study cod microtubules were found to be highly insensitive to colchicine. In general, substoichiometric binding of colchicine causes disassembly and inhibits assembly of microtubules isolated from various sources. In cod preparations a concentration as excessive as 1 mM failed to completely inhibit the assembly of cold-labile brain microtubules. However, the dissociation constants, K_d, for colchicine binding were similar for both cod and bovine brain tubulin, thus, excluding the possibility that cod tubulin do not bind colchicine. Tubulin is known to have one high affinity site for colchicine (Weisenberg et al., 1968), but as discussed by Hains et al. (1978), binding data reported in the literature varies considerably. We found that both cod and bovine tubulin bound ~0.4 mol of colchicine per mol of tubulin. This falls in the same range as that found for renal tubulin, which is close to 0.6 (McClure and Paulson, 1977; Nunez et al., 1979) and 0.5 for bovine renal tubulin (Barnes et al., 1983). One cannot of course exclude the possibility that a low stoichiometry might reflect methodological differences or a partial denaturation of the proteins during preparation, but both bovine and cod tubulins are fully assembly competent and the morphology of the assembled microtubules appears normal. In the present study bovine brain microtubules have been included to serve as an internal reference.

Tubulin belongs to a multigene family and most microtubules are formed from a mixture of these tubulins (for a review, see Sullivan, 1988). In spite of the fact that most isotypes of tubulin seem to be randomly dispersed in microtubules, one finds microtubules with different characteristics in the cell. In addition to complex and drug-insensitive microtubules in the centrioles (Behnke and Forer, 1967), a small fraction of drug-insensitive and cold-labile cytoplasmic microtubules are usually posttranslationally modified by acetylation of a lysine on α-tubulin and detyrosination of the carboxyl terminal on α-tubulin (for a comprehensive review, see Greer and Rosenbaum, 1989). These modifications have therefore been suggested to confer stability to microtubules. Isolated cod brain microtubules were found to contain highly acetylated tubulin and a mixture of tyrosinated and detyrosinated tubulin, where detyrosination seemed to dominate. It is not known whether drug-insensitive acetylated cytoplasmic microtubules in vivo are able to bind to colchicine, but our in vitro results indicate that colchicine can bind to isolated acetylated tubulin without affecting the assembly in the presence of MAPs.

Acetylation of tubulin has been shown to occur preferentially on preformed microtubules, rather than on free tubulin dimers (Maruta et al., 1986; Black et al., 1989). The site of acetylation then must be accessible on the surface of microtubules. Similar to acetylation, detyrosination occurs on the assembled microtubule (Wehland and Weber, 1987; Bré et al., 1987). There are some indications that posttranslational modification of tubulin per se is not the reason for microtubule stability (Schulze et al., 1987). Instead, these modifications would serve as markers for stable microtubules. One likely candidate which has been suggested to be involved in the induction of drug stability of acetylated microtubules is MAPs (see Greer and Rosenbaum, 1989). It is known that MAPs are important for assembly as well as for microtubule function. When MAPs were removed by phosphocellulose chromatography, the assembly of cod brain tubulin became as sensitive to low concentrations of colchicine added initially, as bovine brain microtubules. They were however still less sensitive to 1 mM colchicine added at steady state than assembled bovine tubulin. The remaining microtubules seemed to be slightly enriched in acetylated tubulin. This indicates that acetylated tubulin in itself has a slightly increased stability to colchicine. However, one cannot exclude the possibility that the nondisassembled micro-

tubules might contain isoform differences other than those associated with acetylation and detyrosination. In spite of this, our experiments have clearly shown that the enhanced stability against colchicine for cod microtubules is MAP dependent, since microtubules composed of cod tubulin and MAPs had a high stability to colchicine. This effect was, although, not specific for cod MAPs. Hybrid microtubules made of cod tubulin and bovine MAPs produced microtubules with enhanced stability against colchicine, but to a somewhat lesser degree. Thus, it seems that cod microtubules have a MAP-dependent colchicine stability, although these MAPs need not be of cod origin.

Greer and Rosenbaum (1989) have discussed that the amount of MAPs relative to the amount of tubulin may interfere with the stability of acetylated microtubules. Semiquantitative analysis of cod microtubule proteins showed however that this is not true as judged from densitometry of SDS-gels. The amount of MAPs is similar or even slightly lower in cod brain microtubules compared to bovine brain microtubules. The effect of added MAPs seemed to reach saturation since increased amounts of bovine MAPs increased the amount of assembly without a concomitant increase in stability. Nunez et al. (1979) have previously shown that MAPs competitively inhibit colchicine binding to tubulin. MAPs usually comprise ~20% of isolated microtubule proteins. The finding that MAPs competitively inhibit colchicine binding, would predict that assembly inhibition would be inhibited by raising the MAPs-to-tubulin ratio. However, in the present study even when bovine MAPs were added to bovine tubulin at an equal amount, no, or only a very slight, increase in stability to colchicine was found. Our results suggest, therefore, that MAPs only increase the colchicine stability of acetylated cod tubulin.

In light of the results discussed above, it would seem that qualitative differences between MAPs are not the reason for colchicine stability. Isolated cold-labile cod brain microtubules have a composition of MAPs that is different from bovine brain microtubules. A heat-labile, MAP2-like protein is present in cod brain microtubules as identified by anti-MAP2 antibodies, but MAP1-like proteins are missing. In addition, a 400-kD MAP and a heat-stable MAP with a molecular mass slightly lower than that of MAP2 is present (Strömberg et al., 1989). Further characterization of the 400-kD MAP is in progress. It is not yet known whether only one, or several MAPs are involved in the colchicine stability.

STOPs (stable tubule only polypeptide) have previously been found to induce stability in microtubules. These microtubules are stable to low temperature, to millimolar calcium, and to podophyllotoxin (Job et al., 1982; Pirollet et al., 1983). The extent of cold stability was demonstrated by chilling the microtubules and measuring the amount of residual microtubules (Margolis et al., 1986). It was found that in the absence of STOPs, the measured amount of assembly always returned to zero. The colchicine stability of cold-labile cod microtubules is most probably not caused by STOPs. Cod microtubules disassemble completely when chilled on ice (Strömberg et al., 1986). Furthermore, calcium does not disassemble cod microtubules but induces the formation of spirals (Strömberg et al., 1989). Preliminary results have also shown that the microtubules do not disassemble when both calcium and calmodulin are present (Strömberg, E., M. Billger, and M. Wallin, manuscript in preparation).

There is, however, probably more than one route leading to the stabilization of microtubules. In lower eukaryotes, e.g., the myxamoebae of *Physarum polycephalum*, the colchicine stability seems to be intrinsic with respect to tubulin. Tubulin isolated from *Physarum* is assembly competent in vivo as well as in vitro in the presence of colchicine (Quinlan et al., 1981), and in contrast to cod tubulin, has a very low affinity for colchicine (Roobol et al., 1980). Furthermore, when isolated *Physarum* tubulin was microinjected to PtK_2-cells, the recipient cytoskeleton incorporates the injected tubulin and becomes resistent to colchicine-induced disassembly of microtubules (Prescott et al., 1989). It will be most interesting to determine whether microinjection of cod tubulin and/or MAPs will transfer colchicine stability to microtubules in PtK_2-cells.

In conclusion, we have investigated the mechanism of colchicine stability of microtubules isolated from the brain of Atlantic cod, *Gadus morhua*. We have found that cod tubulin is highly acetylated and consists of a mixture of tyrosinated and detyrosinated α-tubulin. We suggest that these posttranslational modifications are not the main cause for microtubule stability, since cod microtubules assembled in the absence of MAPs are much more sensitive to colchicine than in their presence. The ability of the MAPs to induce stability is, however, not an intrinsic property of cod MAPs. Bovine MAPs can also induce stability to cod but not to bovine tubulin, indicating that this is a more general property of MAPs when they bind to posttranslationally modified tubulin.

We thank Mrs. Ann-Britt Källström, Mrs. Inger Holmqvist, and Mrs. Birgitta Wallander for excellent technical assistance; Mr. Tony Roysson for supplying the fish; and Dr. Michael C. Thorndyke for careful reading of the manuscript. We are indebted to the staff at the Kristineberg Marine Biological Station, Sweden, at which part of this study was conducted.

This study was supported by grants from the Swedish Natural Science Research Council (to M. Wallin), Anna Ahrenbergs Fond, Hierta-Retzius fond för Vetenskaplig Forskning, Hjalmar Theels Fond för Vetenskaplig Forskning and A. F. Regnells Zoologiska Gåvomedel (to M. Billger), Kungliga och Hvitfeldtska Överskottsfonden, Längmanska Kulturfonden, Magnus Bergvalls Stiftelse, and Wilhelm och Martina Lundgrens Vetenskapsfond.

Received for publication 25 September 1990 and in revised form 2 January 1991.

References

Argaraña, C. E., H. S. Barra, and R. Caputto. 1980. Tubulinyl-tyrosine carboxypeptidase from chicken brain: properties and partial purification. *J. Neurochem.* 31:114–118.

Barnes, L. D., A. K. Robinson, R. F. Willimas, and P. M. Horowitz. 1983. Binding of colchicine to renal tubulin at 5°C. *Biochem. Biophys. Res. Commun.* 116(3):866–872.

Barra, H. S., J. A. Rodriguez, C. A. Arce, and R. Caputto. 1973. A soluble preparation from rat brain that incorporates into its own proteins [14C]arginine by a ribonuclease-sensitive system and [14C]tyrosine by a ribonuclease-insensitive system. *J. Neurochem.* 20:97–108.

Behnke, O., and A. Forer. 1967. Evidence for four classes of microtubules in individual cells. *J. Cell Sci.* 2:169–192.

Black, M. M., and P. Keyser. 1987. Acetylation of α-tubulin in cultured neurons and the induction of α-tubulin acetylation in PC12 cells by treatment with nerve growth factor. *J. Neurosci.* 7:1833–1842.

Black, M. M., P. W. Baas, and S. Humphries. 1989. Dynamics of α-tubulin deacetylation in intact neurons. *J. Neurosci.* 9:358–368.

Borisy, G. G. 1972. A rapid method for quantitative determination of microtubule protein using DEAE-cellulose filters. *Anal. Biochem.* 50:373–385.

Bré, M.-H., T. E. Kreis, and E. Karsenti. 1987. Control of microtubule nucleation and stability in Madin-Darby canine kidney cells: the occurrence of noncentrosomal, stable detyrosinated microtubules. *J. Cell Biol.* 105:1283–1296.

Gaskin, F., C. R. Cantor, and M. L. Shelanski. 1974. Turbidimetric studies

of the *in vitro* assembly and disassembly of porcine neurotubules. *J. Mol. Biol.* 89:737–758.

Greer, K., and J. L. Rosenbaum. 1989. Post-translational modifications of tubulin. *In* Cell Movement. Kinesin, Dynein and Microtubule Dynamics. Vol. 2. F. D. Warner and J. R. MacIntosh, editors. Alan R. Liss, Inc., New York. 47–66.

Gundersen, G. G., M. H. Kalnoski, and J. C. Bulinski. 1984. Distinct populations of microtubules; tyrosinated and nontyrosinated alpha tubulin are distributed differently *in vivo*. *Cell.* 38:779–789.

Hains, F. O., R. M. Dickerson, L. Wilson, and R. J. Owellen. 1978. Differences in the binding properties of *Vinca* alkaloids and colchicine to tubulin by varying protein sources and methodology. *Biochem. Pharmacol.* 27: 71–76.

Hallak, M. E., J. A. Rodriguez, H. S. Barra, and R. Caputto. 1977. Release of tyrosine from tyrosinated tubulin. Some common factors that affect this process and the assembly of tubulin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 73:147–150.

Job, D., C. T. Rauch, E. H. Fischer, and R. L. Margolis. 1982. Recycling of cold-stable microtubules: evidence that cold stability is due to substoichiometric polymer blocks. *Biochemistry.* 21:509–515.

Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576–582.

Kreis, T. E. 1987. Microtubules containing detyrosinated tubulin are less dynamic. *EMBO (Eur. Mol. Biol. Organ) J.* 6:2597–2606.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

L'Hernault, S. W., and J. L. Rosenbaum. 1983. *Chlamydomonas* α-tubulin is post-translationally modified in the flagella during flagellar assembly. *J. Cell Biol.* 97:258–263.

L'Hernault, S. W., and J. L. Rosenbaum. 1985. *Chlamydomonas* α-tubulin is post-translationally modified by acetylation on the ε-amino group of a lysine. *Biochemistry.* 24:473–478.

Lockwood, A. H. 1979. Molecules in mammalian brain that interact with the colchicine site on tubulin. *Proc. Natl. Acad. Sci. USA.* 76:1184–1188.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Margolis, R. L., C. T. Rauch, and D. Job. 1986. Purification and assay of a 145-kDa protein ($STOP_{145}$) with microtubule-stabilizing the motility behavior. *Proc. Natl. Acad. Sci. USA.* 83:639–643.

Maruta, H., K. Greer, and J. L. Rosenbaum. 1986. The acetylation of α-tubulin and its relationship to assembly and disassembly of microtubules. *J. Cell Biol.* 103:571–579.

McClure, W. O., and J. C. Paulson. 1977. The interaction of colchicine and some related alkaloids with rat brain tubulin. *Mol. Pharmacol.* 13:560–575.

Nunez, J., A. Fellous, J. Francon, and A. M. Lennon. 1979. Competitive inhibition of colchicine binding to tubulin by microtubule-associated proteins. *Proc. Natl. Acad. Sci. USA.* 76:86–90.

Piperno, G., and M. T. Fuller. 1985. Monoclonal antibodies specific for an acetylated form of α-tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J. Cell Biol.* 101:2085–2094.

Pirollet, F., D. Job, E. H. Fischer, and R. L. Margolis. 1983. Purification and characterization of sheep brain cold-stable microtubules. *Proc. Natl. Acad. Sci. USA.* 80:1560–1564.

Prescott, A. R., K. E. Foster, R. M. Warn, and K. Gull. 1989. Incorporation of tubulin from an evolutionarily diverse source, Physarum polycephalum, into the microtubules of a mammalian cell. *J. Cell. Sci.* 92:595–605.

Quinlan, R. A., A. Roobol, C. I. Pogson, and K. Gull. 1981. A correlation between *in vivo* and *in vitro* effects of the microtubule inhibitors colchicine, parbendazole and nocodazole on myxamoebae of *Physarum polycephalum. J. Gen. Microbiol.* 122:1–6.

Roobol, A., C. I. Pogson, and K. Gull. 1980. *In vitro* assembly of microtubule proteins from myxamoebae of *Physarum polycephalum. Exp. Cell Res.* 130:203–215.

Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. NY Acad Sci.* 51:660–672.

Schulze, E., D. J. Asai, J. C. Bulinski, and M. Kirschner. 1987. Posttranslational modification and microtubule stability. *J. Cell Biol.* 105:2167–2177.

Strömberg, E., A.-C. Jönsson, and M. Wallin. 1986. Are microtubules cold-stable in the Atlantic cod, *Gadus morhua? FEBS (Fed. Eur. Biochem. Soc.) Lett.* 204:111–116.

Strömberg, E., L. Serrano, J. Avila, and M. Wallin. 1989. Unusual properties of a cold-labile fraction of cod (*Gadus morhua*) brain microtubules. *Biochem. Cell Biol.* 67:791–800.

Sullivan, K. F. 1988. Structure and utilization of tubulin isotypes. *Annu. Rev. Cell Biol.* 4:687–716.

Thompson, C. J., and I. M. Klotz. 1971. Macromolecule-small molecule interactions: analytical and graphical reexamination. *Arch. Biochem. Biophys.* 147:178–185.

Wallin, M., J. Nordh, and J. Deinum. 1986. Changes in the hydrodynamic properties of microtubules induced by taxol. *Biochim. Biophys. Acta.* 880: 189–196.

Webster, D. R., G. G. Gundersen, J. C. Bulinski, and G. G. Borisy. 1987. Differential turnover of tyrosinated and detyrosinated microtubules. *Proc. Natl. Acad. Sci. USA.* 84:9040–9044.

Wehland, J., and K. Weber. 1987. Turnover of the carboxy-terminal tyrosine of α-tubulin and means of reaching elevated levels of detyrosination in living cells. *J. Cell Sci.* 88:185–203.

Weingarten, M. D., A. H. Lockwood, S.-Y. Hwo, and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA.* 72:1858–1862.

Weisenberg, R. C., G. G. Borisy, and E. W. Taylor. 1968. The colchicine binding protein of mammalian brain and its relation to microtubules. *Biochemistry.* 7:4466–4478.

Williams, Jr., R. C., and H. W. Detrich, III. 1979. Separation of tubulin from microtubule-associated proteins on phosphocellulose. Accompanying alterations in concentrations of buffer components. *Biochemistry.* 18:2499–2503.