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Protein Kinase Associated with Tubulin: Affinity Chromatography and Properties†

I. V. Sandoval‡ and P. Cuatrecasas*,§

ABSTRACT: Rat brain tubulin purified by colchicine–agarose affinity chromatography contains protein kinase activity. The kinase activity can be separated *completely* from tubulin by chromatography on casein columns and is not subsequently retained by colchicine affinity columns. Protein kinase activity associated with purified tubulin does not correlate with the total content of protein kinase activity in brain homogenates, since microtubules isolated from 48 000*g* fetal brain supernatants contain twice as much protein kinase activity than adult microtubules, although the total protein kinase activity is twice as high in the 48 000*g* adult supernatant. The protein kinase of tubulin preparations, while corresponding to a different molecule than tubulin, is probably not simply the result of contamination. These observations are interpreted in terms of specific associations between protein kinase and tubulin complexes. The protein kinase–tubulin association may be an important determinant in the regulation of tubulin function. Fetal tubulin polymerizes twice as well as adult tubulin in the absence of glycerol at the same tubulin concentration. The preferred substrate for the protein kinase either in vivo or in vitro (pH 7.4, 37 °C) is a specific high-molecular-weight protein, distinct from tubulin, which copurifies with tubulin through different kinds of isolation procedures (i.e., colchicine affinity chromatography and ammonium sulfate precipitation followed by diethylaminoethyl-cellulose chromatography). The tubulin-associated protein kinase is completely dependent on cyclic adenosine monophosphate ($K_m = 10^{-7}$ M), as demonstrated by the complete suppression of activity upon addition of the protein kinase modulator, a well-known specific inhibitor of cAMP-dependent protein kinases.

Microtubules are highly organized structures that are ubiquitously present in eukariotic cells and that result from the polymerization of the asymmetric dimeric protein, tubulin (Taylor, 1965; Renaud et al., 1968; Weisenberg, 1972). The formation and dissolution of microtubules are fast processes that occur in cells in response to changing physiological conditions. Mechanisms must therefore exist for regulating the tubulin–microtubule equilibrium. Recent experimental evidence suggests that proteins present in purified tubulin preparations may be fundamental to the processes that regulate microtubule formation (Kuriyama, 1975; Weingarten et al., 1975; Sandoval and Cuatrecasas, 1976). In addition, the multiplicity and diversity of microtubular functions suggest the existence of additional regulatory mechanisms controlling the functional role of the microtubule. Specialized microtubule functions can be the result of different types of tubulin emerging from diverse posttranslational modifications of the protein (Ratt et al., 1971). Alternatively, specialized organizational centers, whose activity may vary according to the physiological conditions of the cell, can functionally diversify an initially homogeneous population of tubulin (Gibbons et al., 1969; Inoue, 1964; Weisenberg et al., 1972; Witman, 1973).

Phosphorylation of tubulin has been proposed as a possible device for regulating tubulin function. Protein kinase activity is present constantly in tubulin prepared from different sources, e.g., *Tetrahymena axonemes* (Muro Fushi, 1973; Kaji, 1973), rat brain (Goodman et al., 1970), and by different procedures (Goodman et al., 1970; Soifer, 1975; Shigekawa et al., 1975). One of the subunits of the tubulin dimer appears to be in a phosphorylated state in vivo (Eipper, 1974a). cAMP,[1] a well-known effector in protein kinase reactions (Brostrom et al., 1970), is able to promote microtubule formation (Prasad et al., 1971) or dissolution (Bitensky et al., 1965) in the intact cell. Despite these findings, there is no conclusive evidence for the existence of a protein kinase specific for tubulin phosphorylation, and a variation in its state of phosphorylation has not been rigorously correlated with any change in function.

The present studies report that: (a) rat brain tubulin purified by affinity chromatography copurifies with a specific protein kinase that can subsequently be separated in an active state by chromatography on casein–agarose columns, (b) brain protein kinase content does not correlate with microtubule protein

† From the Department of Pharmacology and Experimental Therapeutics and Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and the Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709. *Received December 23, 1975.* This work was supported by grants from the National Institutes of Health (AM 14956), the American Cancer Society, and the Kroc Foundation.

‡ Recipient of a postdoctoral fellowship from the Program of Cultural Cooperation Spain-USA.
§ Recipient of a USPHS Research Development Award (AM 31464).

[1] Abbreviations used are: DAC, deacetylcolchicine; IDAC, isodeacetylcolchicine; (I)DAC, isodeacetylcolchicine–deacetylcolchicine; TMCA, trimethylcolchicinic acid; cAMP, adenosine 3′,5′-cyclic monophosphate; GTP, ATP, guanosine and adenosine 5′-triphosphates; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; OD, optical density; Mes, 4-morpholineethanesulfonic acid.

kinase activity, (c) a correlation exists between the protein kinase content of tubulin and microtubule formation, and (d) the enzyme, whose activity is completely dependent on the presence of cAMP, *preferentially* phosphorylates an endogenous, high-molecular-weight protein which is closely associated with tubulin rather than tubulin itself.

Materials and Methods

Affinity Chromatography Columns

Preparation of Colchicine Derivatives. The conversion of colchicine to the amino-reactive species, deacetylcolchicine (DAC) and isodeacetylcolchicine (I-DAC), was carried out according to the procedure of Wilson and Friedkin (1966). Methylation of trimethylcolchicinic acid (TMCA), the acidic hydrolytic product of colchicine, to DAC and IDAC by treatment with diazomethane, in methylene chloride, was always incomplete as revealed by methanol chromatography on silica gel 60F-254 plates (TMCA, R_f 0; DAC, R_f 0.4; IDAC, R_f 0.3). Prolonged (e.g., 1 week, 4 °C) incubation resulted in nearly complete disappearance of TMCA with the appearance of two new unidentified spots in the chromatogram with R_f values close to those of DAC and IDAC. The presence in the affinity column of TMCA as a third ligand does not interfere with the purification of tubulin, since its behavior as an affinity ligand is identical to that of DAC and its isomer (to be published).

Coupling of the Ligands to Agarose. Coupling of either the colchicine derivatives or casein to Sepharose-4B was carried out according to March et al., (1974). Two-hundred milligrams of the colchicine derivative was reacted (4 °C, pH 9.5) overnight with 7.5 ml of packed, activated Sepharose-4B. The first agarose batch was discarded and the uncoupled material was reacted again with 7.5 ml of activated agarose; 80 nmol of ligand was coupled per ml of agarose. For the preparation of casein–agarose, 60 mg of casein was reacted (4 °C, pH 9.5) overnight with 20 ml of packed, activated Sepharose-4B. Since the efficiency of the coupling was 95%, the final protein concentration was 34 nmol/ml of agarose.

Utilization of the Affinity Columns. The bed volume of the (I)DAC columns utilized in these experiments was 2 ml, with a total ligand content of 160 nmol. The 100 000*g* supernatant of a 10-g rat brain, homogenized (4 °C) in 10 ml of 10 mM sodium phosphate, pH 6.75, containing 5 mM $MgCl_2$ plus 1 mM GTP (buffer A), constituted the sample. This sample contained about 2.7 μmol of tubulin assuming a protein content of 15 mg/100 mg of tissue and an average concentration of tubulin of 20 mg/100 mg of protein. Since the stoichiometry of the tubulin–colchicine reaction is 1 to 1, the quantity of tubulin was about 15 times more than the total amount of colchicine ligand in the column. Given that the protein with the greatest avidity for colchicine is tubulin, such conditions were considered optimal for obtaining tubulin preparations of high purity (Figure 1). After passing the brain homogenate sample through the column (4–8 °C), extensive washing (50 ml of buffer A) was carried out. Tubulin was first eluted (4 °C) by increasing the ionic strength of buffer A to 110 mM with NaCl (Hinman and Morgan, 1973). To calculate the total amount of tubulin retained by the column, the column was subsequently eluted with 7 M guanidine-HCl, pH 7.2.

Casein columns, with a bed volume of 10 ml and a total protein content of 230 nmol, were used to dissociate the 0.1 M NaCl eluate of (I)DAC–Sepharose columns into tubulin and protein kinase activity. These affinity columns were preequilibrated (4 °C) with 10 mM sodium phosphate, pH 6.75, containing 5 mM $MgCl_2$, 1 mM GTP, and 5×10^{-6} M cAMP (buffer B). The protein kinase activity retained by the column (4 °C) was eluted by increasing the ionic strength of buffer B to 310 mM with NaCl. The 0.1 M NaCl protein eluted from the (I)DAC–Sepharose columns was diluted with buffer A to a final ionic strength of 50 mM and incubated (4 °C) with 5×10^{-5} M cAMP for 30 min before applying to the casein–Sepharose column. When required, the protein kinase activity eluted with 0.2 M NaCl from the casein column was desalted on a 30-ml Sephadex G-25 coarse column before application on a second (I)DAC–Sepharose column.

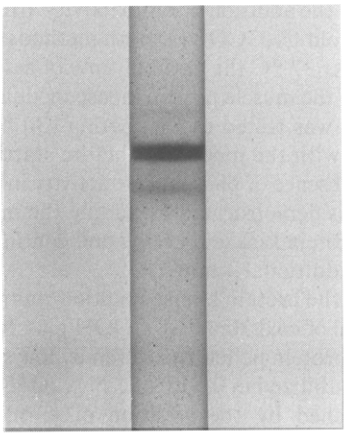

FIGURE 1: Tubulin purification by (I)DAC affinity chromatography. 10 g of rat brain were homogenized (4 °C) in 10 ml of sodium phosphate buffer, pH 6.75, containing 5 mM $MgCl_2$ and 1 mM GTP (buffer A). The 100 000*g* supernatant of this homogenate was sampled (4 °C) on a 2-ml (bed volume) (I)DAC–Sepharose affinity column containing 160 nmol of active ligand. The column was washed with 50 ml of buffer A and the entire protein adsorbed was eluted with 7 M guanidine-HCl, pH 7.2. Guanidine was removed by dialysis (4 °C, overnight), against 4 l. of water and the desalted eluate was lyophilized. The lyophilized, dried residue was resuspended in 300 μl of 15 mM Tris-HCl buffer, pH 7.5, containing 8 M urea, 0.1% sodium dodecyl sulfate, and 30 mM dithiothreitol, and boiled for 5 min. 75 μg of protein were sampled on 1 × 7 cm 0.1% sodium dodecyl sulfate, 8 M urea, 7.5% polyacrylamide, pH 8.7, gels. Electrophoresis was at 2.5 mA/gel until the tracking dye, Bromophenol blue, reached the bottom of the gels. Described are band H_1 (high-molecular-weight protein), bands T (tubulin dimer bands), and band L (low-molecular-weight protein).

Protein Kinase Assay

[γ-^{32}P]ATP was synthesized by the Glynn and Chappell (1964) procedure. The reaction mixture of the protein kinase assay contained, in a final volume of 0.2 ml, 10 μmol of sodium phosphate buffer, pH 7.5, 1 μmol of $MgCl_2$, 100 μg of histone (Sigma, fraction II-A) or α-casein, 0.5 nmol of cAMP (when required), 0.4 μmol of theophylline, 2 nmol of unlabeled ATP (as carrier), and 2 (exogenous phosphorylation) or 4×10^6 cpm (endogenous phosphorylation) of [γ-^{32}P]ATP (95% ATP). Protein kinase activity in column eluates was assayed by adding 100-μl aliquots of each column fraction to the reaction mixture. The quantity of protein kinase activity not eluted from the (I)DAC–Sepharose column by 0.1 M NaCl was determined by assaying 50 μl of the agarose beads diluted with 50 μl of buffer A. The protein kinase activity associated with microtubules was studied in microtubule pellets corresponding to a second polymerization cycle obtained as described by Shelanski et al. (1973) but in the absence of glycerol. Endogenous phosphorylation was assayed by omitting exogenous substrate (histone or α-casein). The reaction was always begun by addition of the labeled phosphate donor. Blanks were determined either by the inclusion in the assay mixture of 2 μmol

of EDTA or by the addition of radioactivity after stopping the reaction with cold Cl_3CCOOH. Both methods gave identical blank values. At 37 °C the optimal time of assay was 5 min.

The effect of the muscle protein kinase modulator on protein kinase activity was tested by incubating (30 °C, 4 min) the assay mixture with the modulator before starting the kinase reaction. The absence of phosphatase activity in the modulator preparation was demonstrated by adding the modulator after 5 min of the protein kinase reaction and continuing the incubation for an additional 4 min.

In all cases, the protein kinase reaction was stopped by the addition of 3 ml of cold 20% Cl_3CCOOH plus 300 μg of serum albumin. The protein pellet, recovered by low speed centrifugation, was solubilized in 0.5 ml of 1 N NaOH and reprecipitated and washed by the addition of 4 ml of cold 20% Cl_3CCOOH. After suspending the pellet in 0.5 ml of 1 N NaOH, the ^{32}P incorporated into protein was counted in 10 ml of Bray's solution.

Colchicine-Binding Assay

Two-hundred-microliter samples of the fractions collected from the columns, containing 200 nmol of GTP, 1 μmol of MgCl, and 2 μmol of sodium phosphate buffer, pH 6.75, were incubated with 4.3 nmol of [^3H]colchicine (5.5×10^5 cpm) (New England Nuclear Corp.). The reaction was allowed to proceed at 30 °C for 75 min and stopped with 1 ml of cold 10 mM sodium phosphate, pH 6.75, containing 1 mM $MgCl_2$, and sampled on 1 ml DEAE 52-cellulose disposable columns. After being washed with 12 ml of cold 10 mM sodium phosphate buffer, pH 6.75, the cellulose was placed in counting vials, shaken for 30 min in 1 ml of 10% sodium dodecyl sulfate and counted for ^3H. The presence of 0.2 M sodium chloride in the assay did not interfere with the binding of colchicine by tubulin.

Microtubule Polymerization Assay

Brains were homogenized in 1 volume of 0.1 M Mes, 1 mM $MgCl_2$, 1 mM GTP, 1 mM β-mercaptoethanol, 0.5 mM EDTA, 1.5 mM EGTA, pH 6.75, and centrifuged (4 °C) at 48 000*g* for 1 h. The tubulin content of the supernatant was determined by the colchicine binding assay, as described earlier, and protein by the Lowry method. Aliquots of 1 ml from the 48 000*g* supernatants were supplemented with 1 mM GTP, incubated 30 min at 37 °C, centrifuged at 100 000*g* (30 min, 35 °C), and the tubulin content of the supernatants was determined by colchicine binding. Microtubule polymerization was calculated from the difference in colchicine binding capacity between the 48 000*g* and 100 000*g* supernatants. Since the tubulin content of foetal brains (15–20 days of pregnancy) is higher than that of adult brains and tubulin polymerization is directly proportional to its concentration, the above mentioned differences in colchicine binding were corrected in a calibrated curve in which tubulin concentration was plotted against polymerized tubulin.

Other Procedures

Protein was determined (Lowry et al., 1951) utilizing serum albumin as standard. All initial absorbance measurements (at OD_{750}) were corrected for the presence of GTP by using appropriate GTP standard curves.

The method of Walsh et al., (1971) for purification of the muscle protein kinase modulator was followed except that the last step (Sephadex G-25 column) was omitted because, as reported, it did not increase the specific activity of the inhibitor.

TABLE I: Distribution of Tubulin and Protein Kinase Activity on Colchicine Affinity Columns.[a]

	Vol. (ml)	Protein Total (mg)	Protein %	Protein Kinase Act. %
0.1 M NaCl eluate	10.4	6.2	35	42
7 M guanidine-HCl	8	11.1	65	
Bed column (beads)				58

[a] Elution profile of a 2 ml (I)DAC-Sepharose column, sampled with the 100 000*g* supernatant of four rat brains homogenized in 1 volume of 10 mM sodium phosphate buffer, pH 6.75, containing 5 mM $MgCl_2$ and 1 mM GTP. The column was washed with 25 bed volumes of buffer A before elution (see Methods).

Neither protein kinase nor phosphatase activity contaminated the inhibitor preparation.

The cAMP-dependent lobster tail protein kinase was purified according to the procedure described by Kuo et al., (1970), while the rat liver kinase was purified according to Gilman (1970).

Polyacrylamide disc gel electrophoresis was performed by using sodium dodecyl sulfate (0.1%)–urea (8 M)–polyacrylamide (7.5%), pH 8.7, essentially according to Eipper (1974b) with the exceptions that $K_3Fe(CN)_6$ was not included in the gel mixture and β-mercaptoethanol was replaced by boiling the sample mixture in 3 mM dithiothreitol. The samples were boiled for 5 min before being placed on the gels. The intensity of the current applied was 2.5 mA/gel. The gels were run until the tracking dye, bromophenol blue, reached the bottom of the gel.

Tubulin and associated proteins were labeled in vivo by the following procedure. Male rats weighing 150 g were anesthetized and injected intracranially through the ear with 1.5 mCi of carrier-free ^{32}P. After 12 h the animals were killed and tubulin was purified (Weisenberg et al., 1968). Identification of the in vivo ^{32}P-labeled proteins was done by fractionation of the purified tubulin on Sephadex G-150, and analysis of the proteins contained in the breakthrough and included volumes of the column was carried out by sodium dodecyl sulfate–urea disc gel electrophoresis.

Iodination of the protein kinase purified by casein–Sepharose chromatography, and thus separated from tubulin, was performed with carrier-free $Na^{125}I$ by the chloramine T procedure (Cuatrecasas, 1971; Hunter and Greenwood, 1962).

Results

Purification of Tubulin Protein Kinase Activity by (I) DAC-Sepharose Chromatography. Both tubulin and protein kinase activity are eluted from (I)DAC–Sepharose columns when the ionic strength of the washing buffer A is increased to 0.1 M with NaCl. Under these conditions, where the affinity ligand is saturated with tubulin (see Methods), the 0.1 M NaCl elution yields tubulin that is 90–95% pure by disc gel electrophoresis (Figure 1).

A very large part (65%) of the total tubulin originally adsorbed to the column remains attached to the column after elution with 0.1 M NaCl. Complete elution of the retained protein is achieved by the addition of 7 M guanidine-HCl, pH 7.2. Protein kinase activity is also found in this tightly bound fraction of tubulin, as manifested by the ability of the 0.1 M NaCl-washed bed matrix to catalyze incorporation of ^{32}P from [γ-^{32}P]ATP into either the protein retained on the adsorbent

TABLE II: Activity of Fractionated Protein Kinase Towards Different Substrates and cAMP Dependency.

Tubulin Preparation	Substrate	Protein Kinase Act.[a] Basal	cAMP (2.5 μM)	cAMP (2.5 μM) + Modulator[b]
(I)DAC-Sepharose 0.1 M NaCl	Histone	8.6	14	0
	Casein	3.2	3.5	0
	Endogenous	1.6	2	0
(I)DAC-Sepharose (beads)	Histone	9.7	10	0
	Casein	1.9	2	0
	Endogenous	1.9	1.7	0
Tubulin purified according to Weisenberg (36S fraction)	Histone	20	33	0
Tubulin polymerized by two cycles of polymerization	Histone	2.7	11.5	0
	Endogenous	3	8	0

[a] pmol of ^{32}P incorporated (5 min) into the substrate (endogenous) present in 100 μg of purified tubulin and, where indicated, 100 μg of histone or casein (pH 7.5, 37 °C). [b] Activity (zero) was not detectable. When purified cAMP-dependent liver protein kinase was preincubated with muscle inhibitor, the enzymatic activity was 0–10% of that measured in the absence of the inhibitor.

(endogenous substrate) or into histone or α-casein (exogenous substrate). Furthermore, the distribution of protein kinase activity through the column is symmetric with respect to the distribution of tubulin (Table I).[2]

The identity of the protein kinase activity found in the 0.1 M NaCl eluate with that which is most tightly retained by the column is further suggested by the fact that both enzymes phosphorylate histone better than endogenous protein, and the latter similarly to α-casein (Table II).

Separation of Tubulin from Protein Kinase Activity. After incubating (30 min, 4 °C) the fraction that is eluted with 0.1 M NaCl from (I)DAC-Sepharose columns with 5×10^{-6} cAMP, chromatography on a casein-Sepharose column yields two reproducible peaks of tubulin, A and B, which contain protein kinase activity (Figure 2). These peaks are clearly separated and detected in the early, less retarded fractions. The low protein content of peak A, which is below the range of sensitivity of the Lowry method for protein determination, did not permit calculation of specific activities for colchicine binding or protein kinase activities. However, both of these activities are clearly much higher than those observed with peak B. Notably, the protein kinase activity retained by this first casein-Sepharose column, and that is subsequently eluted with 0.3 M NaCl (peak C, Figure 2), does not exhibit any colchicine binding activity. No colchicine-binding activity is found in the absence of protein kinase activity throughout the elution profile.

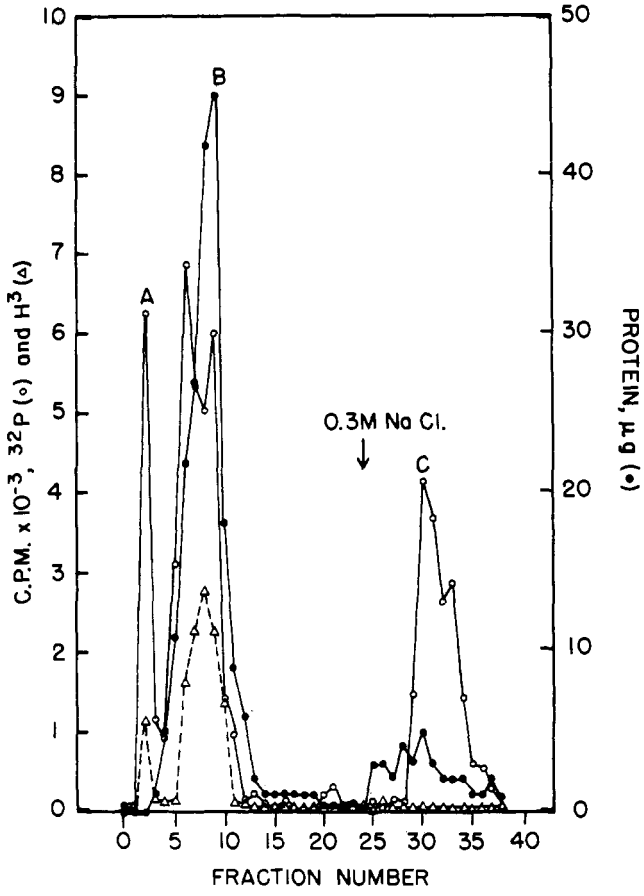

FIGURE 2: Chromatography of (I)DAC-purified tubulin on casein-Sepharose affinity columns. Tubulin eluted from (I)DAC affinity columns with 0.1 M NaCl was incubated for 30 min (4 °C) in 1 volume of buffer A containing 5×10^{-5} M cAMP. Sampling was carried out on a 10-ml (bed volume) casein-Sepharose column containing a total of 230 nmol of casein and equilibrated with 10 mM sodium phosphate, pH 6.75, containing 5 mM $MgCl_2$, 1 mM GTP, and 5×10^{-6} M cAMP (buffer B). The protein retained by casein affinity column was eluted with buffer B containing 0.3 M NaCl. (●) protein, (○) protein kinase activity (^{32}P, cpm), (△) colchicine binding (^{3}H, cpm).

The incomplete resolution of protein kinase activity from

[2] The "uneven" distribution of tubulin on (I)DAC-Sepharose columns has the following characteristics. Samples of crude brain homogenates or of highly purified tubulin give the same type of tubulin distribution on these columns. After initial application of tubulin to the affinity column, 35% of the tubulin adsorbed can be removed with 0.1 M NaCl, while the remaining 65% is bound more tightly and requires 7 M guanidine-HCl for elution. This pattern is independent of the column-bound ligand concentration but is dependent on the length of time that the tubulin remains adsorbed on the column. After remaining on the column for 5 h, the proportion of tubulin tightly bound to the column increases up to 90%. These patterns of distribution are not due to the existence of two separate ligand binding sites in the tubulin molecule, as inferred by the dependence on time. Similarly, the distribution is not explained by the existence in the (I)DAC-Sepharose column of different ligands with different properties, since (I)DAC columns with and without any TMCA both show the same pattern of tubulin distribution. The behavior of the columns is poorly understood. From binding studies of free colchicine to tubulin, binding does not occur at 4 °C and high concentrations of NaCl have no effect (0.1 M) or increase the rate of binding (1 M) (Sandoval and Cuatrecasas, unpublished results).

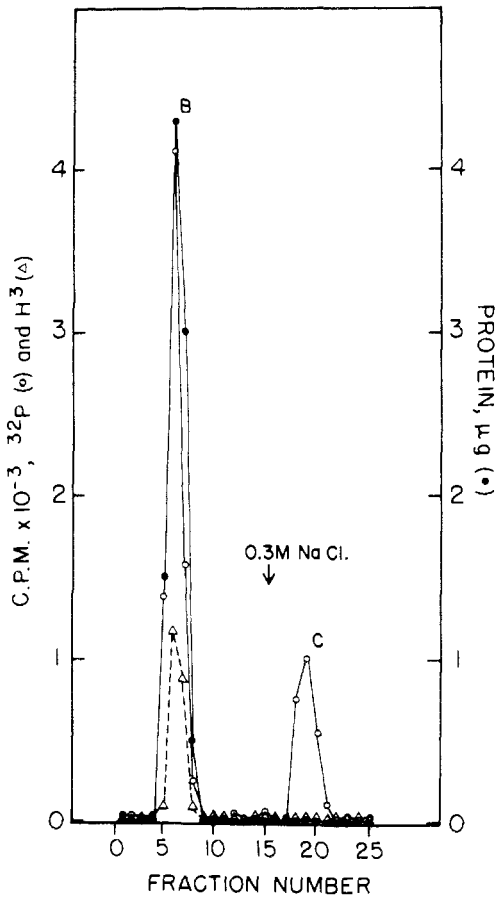

FIGURE 3: Chromatography of the breakthrough material (peaks A and B) of a casein–Sepharose column (Figure 2) on a second casein–Sepharose affinity column. Conditions were as described in Figure 2. (●) Protein, (○) protein kinase activity (^{32}P, cpm), (△) colchicine binding (^{3}H cpm).

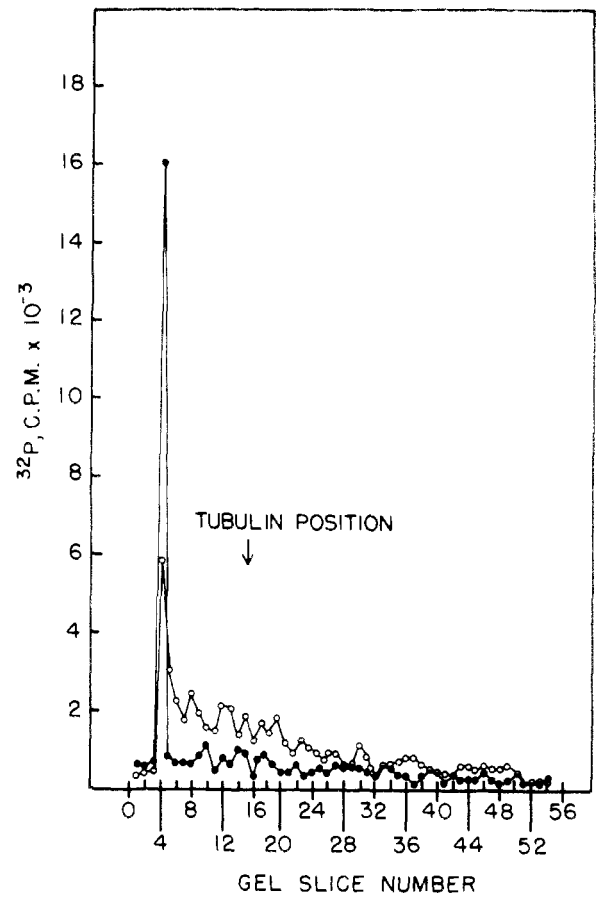

FIGURE 4: Disc gel electrophoresis of (I)DAC-purified tubulin phosphorylated in vitro. Phosphorylation of tubulin in vitro, in the presence (●) and absence (○) of 2.5×10^{-5} M cAMP, was carried out as described under Materials and Methods (see protein kinase assay) except that the enzymatic reaction was stopped by adding 10 µl of 10% sodium dodecyl sulfate. After overnight dialysis (4 °C) against 4 l. of water, the reaction mixture was processed and subjected to gel electrophoresis in parallel with 75 µg of pure tubulin, as described in Figure 1. The profile of protein phosphorylation was studied on the Coomassie blue-stained gels that were cut in 1-mm slices and counted for ^{32}P.

TABLE III: Patterns of Tubulin, Protein Kinase Free of Tubulin, and α-Casein Corresponding to Sodium Dodecyl Sulfate (0.1%)–Urea (7 M)–Acrylamide (7.5%) Gel Electrophoresis.

Protein Samples		R_f	Total Protein[a] %
Tubulin:	High-molecular-weight band	0.25	1
	Tubulin dimer, β band	0.32	95
	α band	0.35	
	Low-molecular-weight band	0.42	4
Protein kinase free of tubulin			
	Band 1	0.037	97
	Band 2	0.084	3
α-Casein:	Band 1	0.1	8
	Band 2	0.26	92

[a] Densitometric tracings of gels stained with Coomassie blue yielded a quantitative evaluation of the proteins entering the gels.

tubulin after the first casein column (peaks A and B) suggests either the existence of two kinds of protein kinase, including the possibility that tubulin is itself a protein kinase, or the presence of a complex in which both tubulin and protein kinase are closely and tightly integrated. In the latter case the complex may be partially separated and resolved by the presence of high concentrations of the exogenous protein kinase substrate, casein. The progressive resolution of peak B into protein kinase activity free of colchicine-binding activity after a second (Figure 3) and a third passage (not shown) through identical casein columns strongly supports the existence of a unique protein kinase activity separate from the tubulin protein itself but that forms part of the tubulin complex.

It has been reported (Eipper, 1974a) that tubulin fractionated on Bio-Gel A 0.5-m columns shows protein kinase activity free of colchicine-binding capacity in the breakthrough fractions. However, despite the absence of colchicine-binding activity, these fractions contain more than 80% tubulin when examined by sodium dodecyl sulfate–urea–polyacrylamide gel electrophoresis. Therefore, the inability to bind colchicine is alone not sufficient to exclude the presence of tubulin. In the case of the protein kinase fractions retained by the second and third casein columns of the present experiments, the absence of even traces of tubulin can be demonstrated by sodium dodecyl sulfate–urea polyacrylamide gels of these fractions (Table III). Instead, two high-molecular-weight bands appear in a position distinct from that of casein (Table III). Because of the tendency of the protein containing kinase activity to aggregate, even after boiling in the gel sample mixture (see Methods), its molecular weight cannot be gauged with confidence from its position on the gel.

Protein Kinase Associated with Tubulin Is Not Retained by Colchicine Columns in the Absence of Tubulin. The protein

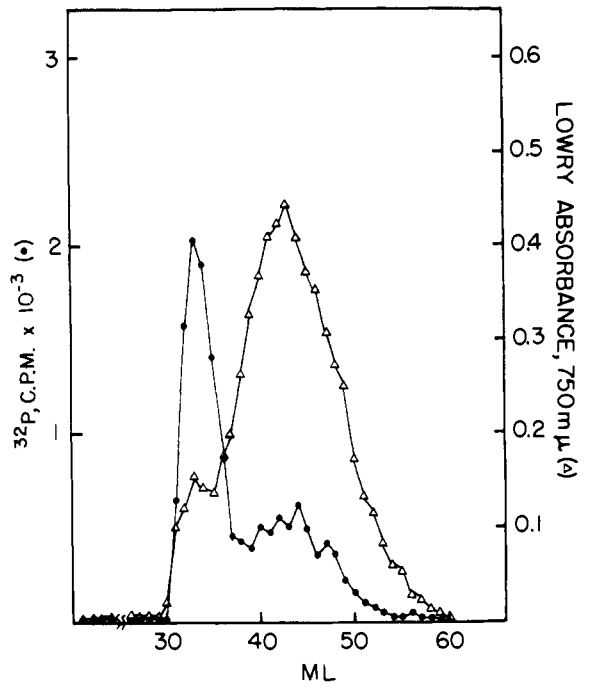

FIGURE 5: Phosphorylation of tubulin and associated proteins in vivo. The rat brain proteins were labeled in vivo with ^{32}P by individual intracranial injection of 1.5 mCi of ^{32}P to six adult male rats. Twelve hours after injection the rats were sacrificed and brain tubulin was purified according to Weisenberg et al. (1968). Purified tubulin was further fractionated, according to its state of aggregation, on a 30 × 2 cm Sephadex G-150 column. The protein (OD 750 mμ/100 μl, △) and ^{32}P (cpm/100 μl, ○) contents of the aggregated (V_0) and nonaggregated (V_i) tubulin fractions were determined.

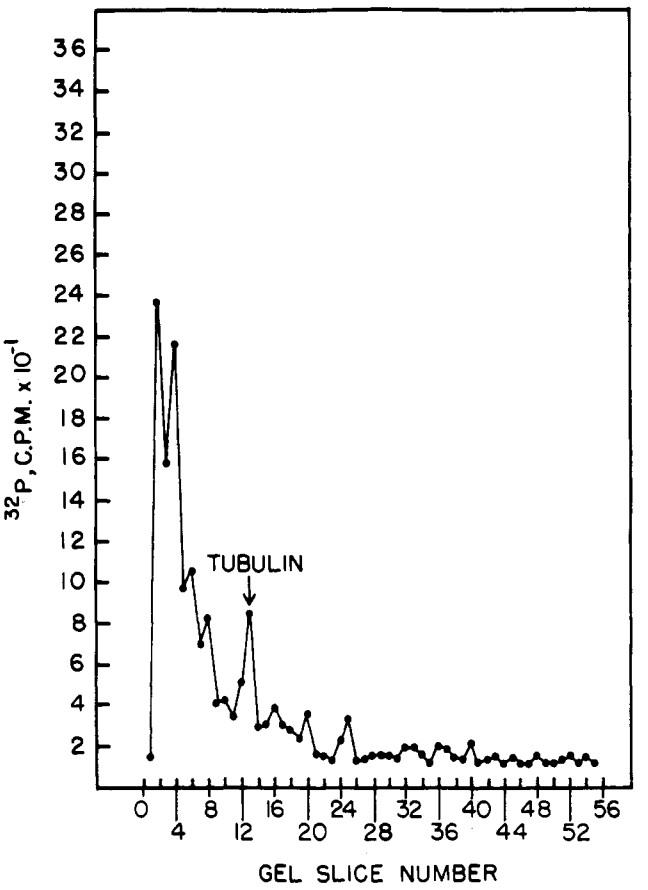

FIGURE 6: Disc gel electrophoresis of tubulin aggregates (36S) phosphorylated in vivo. One-hundred microliters of the V_0 fraction (described in Figure 5), having the highest ^{32}P specific activity, was electrophorized under the conditions described in Figure 1. The profile of protein phosphorylation was studied on gels that were cut in 1-mm slices and counted for ^{32}P.

kinase activity coeluting with tubulin from (I)DAC-Sepharose columns is retained by these columns in proportion to its tubulin content. For example, very little protein kinase activity is retained by (I)DAC columns with samples eluted from initial casein-Sepharose columns, in agreement with the trace amounts of tubulin that can be detected by disc gel electrophoresis. Furthermore, ^{125}I protein kinase from second and third passages on casein columns is not retained by (I)DAC-Sepharose columns, in harmony with the complete absence of detectable tubulin on sodium dodecyl sulfate-urea disc gels (Table III). In addition, cAMP-dependent protein kinase purified from lobster tail, which is completely free of tubulin, is not retained by (I)DAC-Sepharose columns (data not shown).

Attempts to reconstitute the tubulin-protein kinase complex failed as ^{125}I protein kinase free of tubulin was not retained by tubulin partially freed from protein kinase attached to an (I)DAC column (4 °C). This failure suggests that the initial copurification of the protein kinase with tubulin is not the result of an easy unspecific interaction.

In Vitro and in Vivo Endogenous Protein Kinase Substrates. In vitro the protein kinase activity associated with tubulin catalyzes preferentially the phosphorylation of a high-molecular-weight protein that copurifies with tubulin on (I)DAC affinity columns (Figure 4). At the pH (7.5) used, very minimal or no phosphorylation of tubulin itself is detected (37 °C).

Rat brain tubulin labeled in vivo with ^{32}P and purified by the procedure of Weisenberg et al. (1968), when fractionated on Sephadex G-150, contained 60% of its radioactivity in the excluded volume of the column (V_0) despite the fact that this fraction contained only 13% of the total protein (Figure 5). The remainder of the radioactivity (40%) migrated with the major (87%) portion of the total protein, which was included in the column (V_i). Sodium dodecyl sulfate-urea disc gel electrophoresis of both fractions reveals that phosphorylation in the V_0 fraction (Figure 6) is accounted predominantly by two high-molecular-weight proteins. One of these ^{32}P bands is found on the very top of the gel, while the other clearly enters the gel. The presence of these proteins may account for the tenfold higher specific activity (^{32}P, cpm/mg of protein) of tubulin aggregates (V_0) compared to the dimer (V_i) which appears phosphorylated in both fractions. The preferential phosphorylation of these high-molecular-weight proteins is consistent with the endogenous in vitro (pH 7.4, 37 °C) phosphorylation data described above.

Protein Kinase Associated with Tubulin Is Completely cAMP Dependent. A highly variable percent of the protein kinase activity of tubulin purified by (I)DAC-Sepharose affinity chromatography is cAMP independent in both 0.1 M NaCl and 7 M guanidine eluates. The protein kinase that copurifies with tubulin when this is purified by ammonium sulfate precipitation followed by DEAE chromatography also shows a variable percent of cAMP-independent activity. From experiment to experiment the most constant cAMP-dependent activity is that found in tubulin purified by two cycles of polymerization-depolymerization (Table II).

The artifactual nature of this independence on cAMP is manifest when protein kinase modulator is added to the assay mixture (Table II). This modulator protein is known to spe-

TABLE IV: Levels of Protein Kinase Activity in Foetal and Adult Brain Supernatants (48 000*g*) and in Their Corresponding Microtubules.[a]

	Protein kinase act.[b]			
	48 000*g* supernatant		Microtubule pellet	
	Fetus	Adult	Fetus	Adult
Experiment 1	3.1	8	8	1.21
Experiment 2	4.2	9.4	5.4	2.3

[a] Brain microtubules were obtained by two cycles of polymerization–depolymerization from 48 000*g* brain supernatants (foetal and adult) in the absence of glycerol. Protein kinase activity was measured in both the 48 000*g* supernatant and microtubules in the presence of cAMP (2.5×10^{-6} M) and with histone (Sigma fraction IIA) as substrate. Two different experiments are reported. Foetuses in the 20th day of development were used. [b] pmol of ^{32}P incorporated in 5 min into 0.1 mg of histone/100 μg of microtubule protein (pH 7.5, 37 °C).

TABLE V: Comparison of Polymerization Capacity of Tubulin from Adult and Foetal Brains.[a]

Brain Preparation	nmol of Tubulin/g of Wet Brain	nmol of Tubulin Polymerized in Vitro	% of Tubulin Polymerization
Adult	135	47	34
Newborn	190	74	38
Fetus, 19-day pregnancy	165	130	79
Fetus, 14-day pregnancy	60	35	56

[a] nmol of tubulin in adult brain were calculated from the protein content (15%) of wet brain, of which 20% is tubulin. The tubulin content of brains at different ages of development was calculated by referring the colchicine binding of the respective 40 000*g* supernatants to the colchicine binding of adult brains, which corresponds to 136 nmol of tubulin/g of tissue. Tubulin polymerization was calculated from the difference in colchicine binding between the 48 000*g* supernatants (0 °C, before polymerization) and the 100 000*g* supernatant (37 °C, after polymerization). As it is known that tubulin polymerization correlates directly with the initial tubulin concentration, over a C_0 (critical concentration), initial polymerization values were corrected on calibrated curves in which the percent of tubulin polymerized was plotted against its concentration in the 48 000*g* supernatant. Addition of 1 M NaCl to the binding assay (to disrupt the 36S tubulin structures) did not affect the steady state binding of colchicine but increased the rate (Sandoval and Cuatrecasas, unpublished results).

cifically inhibit only cAMP-dependent protein kinases (Walsh et al., 1971), to stimulate the cGMP-dependent kinases (Kuo, 1974), and not to affect cyclic nucleotide-independent kinases (Appleman et al., 1966; Bingham and Farrell, 1974). In the absence of any detectable phosphatase activity, addition of the modulator to the protein kinase assay mixture causes total inhibition of the enzymatic activity associated with tubulin when assayed with either endogenous or exogenous substrate. These results argue against the existence of two kinds of protein kinase differing in their cAMP dependence (Table II), but they do not exclude the possible existence of a cAMP-independent protein kinase that is completely dependent on a cAMP-dependent kinase for activation, as occurs in the glycogen phosphorylase system.

The K_a of the protein kinase for cAMP is about 10^{-7} M, which is in the same range of values reported for other protein kinases.

Correlation of Protein Kinase in Brain Homogenates and Purified Tubulin. The 48 000*g* supernatants from adult brains contain twice as much protein kinase activity than equivalent supernatants from fetal brains (Table IV). Nevertheless, microtubules isolated from such supernatants (two cycles of polymerization) show a much higher (2–6 times) content of kinase activity in foetal than in the adult preparation (Table IV). This pattern of distribution is contrary to that expected from simple contamination of tubulin preparations by protein kinases.

Differences in Polymerization between Foetal and Adult Tubulins. Tubulin from rat brain foetus (14–19 days of development) has a higher capacity to polymerize than the corresponding adult brain tubulin (Table V). When the pattern of both tubulin and associated proteins is studied on disc gel electrophoresis, no substantial difference can be detected in the high molecular weight proteins corresponding to unfractionated tubulins or in their 36 and 6S fractions. The only difference so far detected is the higher content of protein kinase activity associated with the foetal microtubules (Table IV).

Discussion

Rat brain tubulin purified by (I)DAC–Sepharose columns contains protein kinase activity. This enzymatic activity has been detected in tubulin prepared by a variety of methods including ammonium sulfate–DEAE cellulose chromatography (Goodman et al., 1970), vinblastine precipitation (Soifer, 1975), successive cycles of polymerization–depolymerization (Shigekawa and Olsen, 1975), and colchicine affinity chromatography (this report). Furthermore, when tubulin is fractionated by sieve gel electrophoresis in its aggregated and dimeric states, protein kinase activity is found in both fractions (Shigekawa and Olsen, 1975; Eipper, 1974; Letterrier et al., 1974).[3] The constant presence of protein kinase activity in tubulin preparations has prompted the proposal that the activity is an intrinsic property of tubulin (Lagnado et al., 1972; Soifer, 1975). Proposals negating such an identity are based on the lack of coincidence, during chromatography, of the maximal colchicine-binding and protein kinase activities (Shigekawa and Olsen, 1975; Letterrier et al., 1974). However, the extensive overlapping and lack of coincidence could also result from the presence of more than one protein kinase or from differences in the expression of kinase activity depending on the state of aggregation of tubulin.

The present studies show that casein–Sepharose columns can effectively resolve protein kinase activity free of colchi-

[3] Eipper (1974a) has reported that the specific activity of the kinase in the aggregated tubulin (V_0) is ~80 times higher than that of the dimer. This result differs from that of Shigekawa et al., (1975), who reported a similar distribution of protein kinase activity between tubulin aggregates and tubulin dimers purified by two cycles of polymerization–depolymerization, and also from the studies of Letterrier et al., (1974), who reported substantial protein kinase activity in the tubulin dimer prepared by ammonium sulfate precipitation–DEAE cellulose chromatography. The differences between these studies may possibly be explained by differences in the stability of the protein kinase or the tubulin dimer that may vary with the purification procedure. In addition, the Eipper (1974) preparations contain sodium pyrophosphate, which is a potent inhibitor of the protein kinase and which could therefore reduce substantially the detection of enzymatic activity in the fractions containing lower amounts of protein kinase.

cine-binding capacity, starting with preparations that contain 90–95% pure tubulin and that also contain substantial quantities of protein kinase activity (Figures 2 and 3). However, since the in vitro colchicine-binding capacity is inversely correlated with the state of aggregation of tubulin (Eipper, 1974a), the main criterion in support of the complete tubulin-protein kinase separation is the gel electrophoretic analysis of the protein kinase fractions that are free of colchicine-binding activity. Peak C from the first casein–Sepharose columns (Figure 2) frequently contains some tubulin accompanying a major high-molecular-weight protein, but Peak C from the second and third casein columns shows the latter without any detectable tubulin (Table III).

Several major findings in this report support the existence in vivo of tubulin–protein kinase complexes. The protein kinase activity appears to reside in a macromolecule distinct from tubulin, although there is no evidence that it corresponds to one of the proteins described in Table III. (I)DAC–Sepharose columns retain the same proportion of protein kinase activity under conditions in which the column affinity ligand is saturated with tubulin. The affinity of the protein kinase for the (I)DAC ligand parallels that of tubulin. The separation of protein kinase from tubulin is a slow, progressive process which requires the presence of casein (possibly acting as a competitor of the natural substrate). (I)DAC–Sepharose columns retain the protein kinase of tubulin previously purified from such columns only when tubulin is present, but not after it has been removed by casein–Sepharose columns. Colchicine-binding activity is always accompanied by protein kinase activity during chromatography on (I)DAC- or casein–Sepharose columns. The amount of protein kinase activity copurifying with tubulin does not correlate with the total protein kinase content of the tissue (brain) homogenate from which tubulin is purified. The physical association of protein kinases with their substrates in vivo may prove to be a general mechanism for determining the specificity of these enzymes (which in vitro exhibit characteristically broad specificity). Eipper's principal conclusion (1974a) that the protein kinase activity copurifying with tubulin results from nonspecific adsorption was based on the differences in the patterns of tubulin phosphorylation observed in vivo and in vitro, but no studies were performed on the proteins that copurify with tubulin and that may be functionally related. In addition, the fact that tubulin itself appears to be phosphorylated in vivo in a highly specific way (Eipper, 1972; this report) was not considered (Eipper, 1974a).

The nature of the possible components of the protein kinase–tubulin structural complex remains to be determined. Recent evidence suggests that high-molecular-weight proteins are decisive in the process of tubulin polymerization into microtubules (Kuriyama, 1975; Weingarten et al., 1975; Sandoval and Cuatrecasas, 1976). Protein kinase and its endogenous substrate (Sloboda et al., 1975, and this report), both of which are high-molecular-weight proteins which copurify with tubulin, may be among these important high-molecular-weight proteins.

The formation of microtubules has been formulated as 6S (tubulin dimer) $\rightleftarrows$ 36S (coiled protofilaments) $\rightleftarrows$ microtubules, while 6S tubulin dimers can be incorporated directly to incomplete microtubules and complete their elongation (Weingarten et al., 1974). The above mentioned high-molecular-weight proteins are reportedly required in the 6S to 36S transition (Weingarten et al., 1975), which is also dependent on the concentration of 6S dimer and Mg^{2+} (Weisenberg and Timasheff, 1970; Frigon and Timasheff, 1975) but independent of temperature and unaffected by the presence of Ca^{2+} and nucleoside triphosphates. It is reasonable to postulate that the protein factors whose incorporation into the 36S protofilaments may be required for displacing the 6S $\rightleftarrows$ 36S equilibrium towards the 36S form (Weingarten et al., 1975) might also be responsible for the further incorporation of 36S protofilaments into microtubules. Their structural modification, affecting in turn the general ring structure of the 36S species, could for specific functional requirements facilitate the incorporation of the latter into microtubules. Conceivably, such structural modification could be initiated by high-molecular-weight protein and/or tubulin phosphorylation, which could explain the temperature, nucleoside triphosphate, and Ca^{2+} (a potent inhibitor of the protein kinase reaction) dependence of the 36S $\rightleftarrows$ microtubule step. Alternatively, the activation (e.g., by phosphorylation) of independent microtubule initiation centers could stabilize the assembled protofilaments (36S) into microtubules in a way similar to that which occurs with the basal bodies of Chlamydomonas (Snell et al. 1974). With the exception of tubulin synthesis in response to nerve growth factor (Kolber et al., 1974), tubulin polymerization in vivo and in vitro has been demonstrated to be independent of de novo protein synthesis and it is a very fast process (Weisenberg, 1972; Kuriyama, 1975). Moreover, cAMP or agents that increase its cellular concentration have been described to affect microtubule formation (Prasad et al., 1971; Bitensky et al., 1965) and to enhance (Willems et al., 1970; Williams and Wolf, 1970; Bdolah and Schramm, 1965; Ridderstap and Bonting, 1969; Schofield, 1967; Fleischer et al., 1969) or inhibit (Bitensky and Burstein, 1965; Lichtenstein and De Bernardo, 1971; Goldstein et al., 1973; Loeffler et al., 1971) cell functions dependent on microtubules. It is pertinent that cAMP-dependent protein kinases, like the one described in this report, are commonly admitted to be one of the first and faster expressions of changing nucleotide levels (Brostrom et al., 1970).

It should also be considered that phosphorylation of high-molecular-weight proteins, or of tubulin itself, may play a role in the transition from monomeric to dimeric state of tubulin, in the determination of the rate of turnover of tubulin or in the distribution of tubulin among different cellular pools related to different functional requirements.

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