MODIFICATION OF TUBULIN BY TYROSYLATION IN CELLS AND EXTRACTS AND ITS EFFECT ON ASSEMBLY IN VITRO

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ABSTRACT

A post-translational modification of tubulin with potential regulatory significance has been revealed by the discovery of an enzyme (tubulin-tyrosine ligase) in brain extracts which can add a tyrosine residue to the α chain, apparently through peptide bond linkage to a C-terminal glutamate. We have investigated whether this modification also occurs in vivo, and whether it alters the extent to which tubulin can assemble in vitro. Cytoplasmic tubulin purified from bovine brain by cycles of assembly was shown to be partially tyrosylated. Carboxypeptidase A digestion of isolated α chains liberated about 0.3 equivalent of tyrosine. Brief digestion of native tubulin increased the proportion of α chains which could be tyrosylated by ligase, from 25 to 45%. The tubulin assembled to the same extent before and after carboxypeptidase treatment. When tubulin was purified after introducing labeled tyrosine with ligase, the labeled species assembled in the same proportion as unlabeled. Thus tubulin can be incorporated into microtubules in vitro with or without C-terminal tyrosine. An apparent resolution of α chain into two components by hydroxylapatite chromatography was shown not to be due to the presence or absence of C-terminal tyrosine. Tubulin-tyrosine ligase was found in extracts of every rat tissue examined, but was not detected in sea urchin eggs before or after fertilization, in *Tetrahymena* cells or cilia, or in yeast. Cultured neuroblastoma cells fixed tyrosine into tubulin α chains under conditions where protein synthesis was inhibited; this in vivo fixation appeared to be into an insoluble moiety of tubulin. Incidental to these studies, a new assay utilizing an enamine substrate for carboxypeptidase was investigated.

Since microtubules may form and disappear rapidly at times when the total cell content of tubulin appears to be constant, it seems unlikely that transcriptional and translational controls are sufficient to regulate microtubule assembly. Post-translational modifications may therefore be involved in modulating the assembly (or function) of microtubules, either by directly affecting the competence of tubulin to polymerize, or by influencing the partition of tubulin among different cell compartments (31). One example of tubulin modification is the binding, and certain transformations, of guanine nucleotides (15, 18, 28, 44). Another is the phosphorylation of serine residues in tubulin (10) as well as in some proteins that coassemble with tubulin (37), and a third is redox changes in

tubulin sulfhydryl groups (34). However, none of these has so far been implicated in controlling assembly in vivo, nor has any report appeared of enzymes that specifically reverse the phosphorylations or sulfhydryl oxidations.

A fourth kind of modification has been revealed by work in Caputto's laboratory which showed that brain extracts could incorporate tyrosine into a protein very similar to tubulin (4), in the absence of tRNA or other components required for protein synthesis. The reaction was reversible in extracts, and the tyrosine appeared to be introduced through a peptide bond to C-terminal glutamate or glutamine (2).

We have previously reported (30, 31) that the tyrosine is indeed fixed in the α chain of tubulin. A tyrosylating enzyme, called tubulin-tyrosine ligase pending more insight into the nature of the reaction, was partially purified from bovine brain; the reactions involving addition of tyrosine in the presence of ATP, and its release in the presence of ADP + P_i, appear to be catalyzed by the same enzyme, since the two activities increased in parallel during purification (33). The apparent size of the ligase was 150,000 daltons in extracts, but 35,000 after anion exchange chromatography. Addition of purified tubulin converted the latter back to the larger species (33), and a preliminary titration of the amount of tubulin required to form a stoichiometric complex with 35,000-dalton enzyme suggests that brain extracts may contain about 1 mol of enzyme/150 mol of tubulin. The enzyme was very specific for tubulin; 6*s* tubulin dimer was a substrate and axonemal outer doublet microtubules appeared not to be. Tyrosylation did not affect the partition of tubulin between dimers and 36*s* rings, nor did it block incorporation of tubulin into microtubules in vitro (30); it was unclear whether tubulin could assemble without C-terminal tyrosine.

We report here evidence that tubulin can also assemble in vitro without C-terminal tyrosine. Some studies will be described of the cellular and subcellular distribution of the enzyme, as well as the results of preliminary experiments on the species of tubulin that can accept tyrosine in vivo in neuroblastoma cells. Evidence will be presented that the fraction of cytoplasmic brain tubulin isolated by cycles of assembly is partially tyrosylated.

MATERIALS AND METHODS

We have described elsewhere (33) the procedure for preparing tubulin from bovine brain by three cycles of assembly, and the assays for tubulin-tyrosine ligase and for detyrosylation of tubulin by the ligase.

Analytical Procedures

A small-scale sedimentation was used as a quantitative assay for the ability of tubulin to assemble into microtubules (16), since much smaller amounts of tubulin were needed than for light scattering or viscometry. Maximal assembly was obtained with tubulin concentrations ≧1 mg/ml. Incubations were carried out for 30 min at 37°C in a volume of 250 μl of reassembly buffer (100 mM Na^+2-(*N*-morpholino)ethane sulfonic acid (MES), pH 6.6, 0.5 mM $MgSO_4$, 1 mM GTP) in stoppered 6 × 50 mm Sorvall centrifuge tubes (DuPont Instruments, Sorvall Operations, Newtown, Conn.). From 250 to 500 μg of tubulin was incubated with and without 0.1 mM colchicine. The tubes were centrifuged for 20 min at 44,000 *g* at 25°-30°C. After the supernatants were decanted and the liquid was removed from the walls of the tubes with absorbent paper, the pellets were dissolved in 200 μl of 2% Na_2CO_3 in 0.1 N NaOH, with a Vortex mixer (Vortex Corp., Cincinnati, Ohio), and aliquots of both fractions were assayed for protein. With satisfactory preparations of purified tubulin, 70–80% of the protein pelleted, and 10–15% pelleted in the controls with colchicine; the amount of the latter denatured protein was usually less if glycerol had not been used in the preparation. Microtubule assembly was also qualitatively confirmed by darkfield microscopy (39) and occasionally by electron microscopy.

Colchicine-binding capacity of tubulin was measured by the filter assay of Weisenberg et al. (43). Protein was incubated for 90 min at 37°C with 6×10^{-6} M [^{3}H]colchicine (100 mCi/mmol) in 0.5 ml of P-Mg buffer (10 mM potassium phosphate, 5 mM $MgCl_2$, pH 6.9) supplemented with 0.1 mM GTP and 240 mM sucrose. Reactions were stopped by adding an equal volume of cold 0.1 mM colchicine in P-Mg, and the mixtures were filtered through 3-cm DE-81 disks, premoistened with 0.1 mM colchicine in P-Mg, as described by Weisenberg et al. (43). The wet disks were placed in vials containing 1 ml of water and 10 ml of Hydromix, (Yorktown Research Inc., S. Hackensack, N. J.) and counted after the colchicine was allowed to elute off the paper overnight. When colchicine binding of ^{14}C-tyrosylated samples was assayed, the paper disks were kept overnight in 12 ml of 0.4% 2,4-diphenyloxazole in toluene. This eluted all of the [^{3}H]colchicine but none of the ^{14}C in either free or fixed tyrosine; the solution was counted after removing the filters. The maximum binding that we observed for tubulin purified by three cycles of assembly was 0.3 nmol/110 μg (1 nmol) of protein. This value would be low even if corrected for denaturation during the 90-min incubation, but it was quite reproducible.

Protein was determined by the procedure of Lowry et al. (19), standardized with bovine serum albumin. In experiments carried out to determine the amino acids released by carboxypeptidase from α chain purified by

polyacrylamide gel electrophoresis, the amount of α chain was determined by amino acid analysis after 24-h hydrolysis in constant boiling HCl at 110°C.

Preparations: Cells and Extracts

For the assay of ligase in various rat tissues, organs were obtained from adult male Osborn-Mendel rats immediately after decapitation. The chilled tissues were minced or teased with a scalpel, and homogenized with 5–10 strokes of a motor-driven Teflon-in-glass homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.) in: 10 mM K^+MES, pH 6.6, 250 mM sucrose, and 1 mM each EDTA and α-mercaptoethanol. The volume of buffer was chosen to give a final soluble protein concentration of 10–15 mg/ml. The supernates, after centrifugation for 1 h at 4°C at 102,000 *g*, were assayed for ligase.

N18TG2, a thioguanine-resistant mutant of the N18 clone of the C1300 mouse neuroblastoma isolated in the laboratory of Dr. M. Nirenberg, was cultured for us by Dr. Bruce Schrier. Cells were grown to confluence in 75 cm^2 Falcon flasks in Dulbecco's modification (high glucose) of Eagle's medium, from Grand Island Biological Co. (Grand Island, N. Y.), containing 10% (vol/vol) fetal calf serum. The undifferentiated round cells were dislodged by gentle agitation with Ca^{2+}- and Mg^{2+}-free growth medium, and pelleted by centrifugation for 5 min at 25°C at 600 *g*. A portion of the cells was extracted for ligase assays by brief sonication in cold buffer: 25 mM K^+MES, pH 6.6, 100 mM KCl, and 1 mM each EDTA and mercaptoethanol; assays were done on the supernate after centrifugation for 1 h at 4°C at 102,000 *g*. For the study of tubulin tyrosylation in vivo, the remainder of the cells were suspended at 25°C in medium containing 1% glucose and enough (about 50% vol/vol) of the following (amounts in milligrams per liter) to constitute 330 mosmol/liter: *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (7,130), NaCl (6,700), KCl (400), KH_2PO_4 (60), Na_2HPO_4 (53), $MgSO_4 \cdot 7H_2O$ (100), $MgCl_2 \cdot 6H_2O$ (100), $CaCl_2$ (140); adjusted to pH 7.25 with NaOH. Cell suspension was incubated with [^{14}C]tyrosine as described in the Results section. The complete amino acid mixture was that as described by Eagle (9), with tyrosine omitted.

Eggs and sperm from the sea urchin *Lytechinus variegatus* (Gulf Specimens, Panacea, Florida) were obtained by injecting 0.6 M KCl, and collected in filtered artificial seawater (Instant Ocean, Aquarium Systems Inc., Eastlake, Ohio) at 25°C. The eggs were pelleted by centrifugation for 3 min at 800 rpm in a 269 head of a PR2 International centrifuge (International Equipment Co., Needham Heights, Mass.), resuspended in seawater, and filtered through cheesecloth, then washed twice more by decantation; during this process the jelly coats separated. Washed eggs from four animals were suspended in 400 ml of seawater at 15°C with slow stirring, and an excess of sperm was added. Within 5 min, 92% of the eggs had elevated fertilization membranes. Before, and 1/2, 1 1/2 and 18 h after fertilization, aliquots were centrifuged for 6 min at 1,500 rpm in the 269 rotor. The pelleted eggs were homogenized for ligase assays by brief sonication in the buffer used (above) for neuroblastoma. After centrifugation for 1 h at 2°C at 102,000 *g*, both pellet and supernate were assayed.

Log phase cells of two strains of *Tetrahymena* (ATCC nos. 30006 and 10542, provided by Drs. Paulette Royt and Yoko Nagata) were sonicated with four 15-s pulses in the buffer used (above) for neuroblastoma. Frozen cells of *Saccharomyces cerevisiae* (ATCC 9255, provided by Dr. Maria Mazon) were homogenized in the same buffer by two passages through a French pressure cell. The homogenates were centrifuged for 1 h at 4°C at 27,000 *g*, and precipitate and supernatant fractions were assayed for ligase.

Carboxypeptidase Digestion of Tubulin

Carboxypeptidase A was washed just before use by suspending 1 mg in 1 ml of cold deionized water, centrifuging for 5 min at 3,000 *g*, and discarding the supernate. The pellet was either resuspended in water or dissolved in cold 0.5 M $NaHCO_3$ adjusted to pH 9 with NaOH. Carboxypeptidase was assayed by measuring the phenylalanine liberated from hippurylphenylalanine with ninhydrin (1, 26). Various reports that sodium dodecyl sulfate (SDS) does not inhibit carboxypeptidase must reflect the use of large amounts of enzyme in very short, effective incubations, since we found the enzyme to be 90% inhibited after it had been exposed to 0.1 or 0.5% SDS for 5 min.

We take the occasion to mention a potentially much more convenient assay, analogous to one used previously for an aminopeptidase (11). In this assay, a peptidase reaction product decomposes spontaneously to an α-ketoacid which is measured continuously *in situ* with DPNH and lactic dehydrogenase. We were thus able to assay carboxypeptidase A with *N*-chloroacetyl-α-aminocrotonate (11) as substrate. The usefulness was limited by the fact that the K_m was 20 mM and the V_{max} under the conditions used was only 0.2% of that with hippurylphenylalanine. We have not searched for more favorable conditions or a more reactive enamine substrate.

Tubulin and reference proteins, cytochrome *c* and β-lactoglobulin, were digested in the native state, or after denaturation by reduction and alkylation, or by performic oxidation (10). Qualitative analyses of amino acids released from tubulin were done by thin-layer chromatography of dansyl derivatives, using a mixture of tubulin $\alpha + \beta$ chains purified by elution from a column of Celex-P (Bio-Rad Laboratories, Richmond, Calif.) after three cycles of assembly. The details will not be described, since these experiments confirmed, but did not add to, the quantitative results obtained with the amino acid analyzer.

Isolation of Tubulin α Chain by Preparative Polyacrylamide Gel Electrophoresis

8 mg of reduced and alkylated tubulin was applied to a

0.3 × 8 × 14 cm polyacrylamide slab, and electrophoresed in the Luduena and Woodward (22) system with a current of 65 mA. At the end of the run, the unstained slab was placed in Saran Wrap (Dow Corning Corp., Midland, Mich.) over a plastic fluorescent sheet (Eastman chromogram 6063 [Eastman Organic Chemicals, Rochester, N. Y.]). With a short-wave ultraviolet lamp (Mineralight UVS-54 [Dow Corning Corp.]) the well-separated heavy bands of α and β tubulin could easily be visualized as areas quenching the fluorescence (weaker protein bands could not be detected this way). The location of the protein bands was confirmed several times by staining guide strips cut from the slab. The appropriate areas were excised, and the gel was minced by forcing it through a syringe. The minced gel was added to 24 ml of freshly prepared running gel, and the slurry was allowed to solidify in a 22-mm diameter Canalco Prep Disc column (Ames Co., Div. of Miles Lab., Inc., Elkhart, Ind.) The gel was restrained with nylon mesh and a cellophane bag was secured around the bottom of the column, with a rubber band above the lower buffer level. Protein was eluted with the same electrophoresis buffer used to develop the gel, with a current of 10 mA for 16 h. To remove SDS, the eluate was lyophilized, dissolved in 1 ml of 0.1 M $NaHCO_3$ (adjusted to pH 9 with NaOH), 6 M urea, and applied to a 0.5 × 8 cm column of Dowex 1-X2 (Dow Corning Corp. [200–400 mesh]) equilibrated with the same buffer (41). For amino acid analysis after carboxypeptidase digestion, the protein which eluted in the void volume was dialyzed for 3 days at +2°C against several changes of 0.05 M ethylmorpholine acetate, pH 8.5. The recovery of tubulin α chain from the Dowex 1 column was poor. For radioautography, slab gels were vacuum-dried on a sheet of Whatman 1 paper, wrapped in Saran Wrap or similar plastic, and exposed to Kodak single-coated blue sensitive film. ^{14}C-Tyrosylated α chain could also be electrophoretically eluted after the whole gel slab had been dried and radioautographed, and the radioactive band cut out and rehydrated. Procedures for analytical gel electrophoresis were those described previously (33).

Materials

Carboxypeptidase A (treated with diisopropylphosphofluoridate [DFP]) was obtained from Worthington Biochemical Corp. (Freehold, N. J.). DEAE-Sephadex and Sephadex G-50 were obtained from Pharmacia Inc. (Piscataway, N. J.); hydroxylapatite (Biogel HTP) and Coomassie Brilliant Blue from Bio-Rad Laboratories. β-Lactoglobulin, cytochrome *c*, hippuryl-L-phenylalanine, and GTP were purchased from Sigma Chemical Co. (St. Louis, Mo.). Amersham/Searle Corp. (Arlington Heights, Ill.) was the source of L-[U-^{14}C]tyrosine and [^{3}H]colchicine. Colchicine and L-tyrosine were obtained from Calbiochem (San Diego, Calif.); ATP (ultrapure) from Schwarz/Mann (Orangeburg, N. Y.); and *N*-ethylmorpholine (sequanal grade) from Pierce Chemical Co. (Rockford, Ill.).

RESULTS

Tubulin-Tyrosine Ligase in Various Cells and Tissues

The highest level of ligase was found in extracts of cultured, undifferentiated neuroblastoma cells (Table I); the pellet after high-speed centrifugation also had 1/4 of the total U, sp act 0.03. Appreciable activity was found in the supernatant fraction from every rat tissue examined (Table I), when the fraction was incubated with purified brain tubulin. In agreement with Barra et al. (5), only fresh extracts of brain had significant activity when not supplemented with additional tubulin. The difference can be attributed to the lower tubulin content of the other tissues (36) plus the rapid denaturation of tubulin in the buffer used to extract ligase (30), but we cannot exclude the possibility that only the tubulin from brain serves as a substrate.

Fertilization of sea urchin eggs results in synchronous assembly of spindle tubules from a preexisting tubulin pool (29, 38). Cell systems of this kind provide a means of testing the role of tyrosylation in controlling assembly in vivo. As a preliminary to such experiments, we assayed ligase in the supernatant and insoluble fractions of extracts of *Lytechinus variegatus* eggs, before and at intervals (Materials and Methods section) after fertilization. The results were equivocal; if present at all, the ligase specific activity was not more than

TABLE I

*Tubulin-Tyrosine Ligase Activity in Extracts of Various Tissues and Cells**

Enzyme source	Ligase sp act
	nmol min^{-1} mg^{-1}
Neuroblastoma N18TG2	0.12
Bovine brain	0.050
Porcine brain	0.050
Rat brain	0.055
Rat liver	0.016
Rat lung	0.012
Rat large intestine	0.011
Rat kidney	0.006
Rat spleen	0.005

* Assays were done on supernates obtained from centrifuging for 1 h at 4°C at 100,000 *g* after extracting as described in Materials and Methods section. No measurable activity (sp act ≦ 0.0005) was found in extracts of sperm of the sea urchin *Lytechinus variegatus*, or of eggs before or after fertilization; of *Tetrahymena*, or *Saccharomyces*. These extracts did not inhibit tyrosylation of brain tubulin by brain ligase.

2% of that in brain extract. Since tubulin is present in all eukaryotes, we also assayed supernatant and insoluble fractions of extracts of yeast and *Tetrahymena* (Table I); no activity was detected. Negative results were also obtained with preparations from *Tetrahymena* cilia (30).

Liver ligase appears to be in the soluble subcellular fraction (Table II). A portion of the activity from brain and neuroblastoma was not solubilized, though this could be due to incomplete homogenization. More careful isolation of intact organelles (8) will be needed to confirm this localization. The distribution of some organelle enzymes (27) between our two fractions is shown in Table IV. Glucose-6-phosphatase assays suggest that ligase is not present in liver microsomes. The results were inconclusive with regard to lysosomes (acid phosphatase) and mitochondria (succinic dehydrogenase remains insoluble after mitochondrial lysis).

We undertook to study tubulin tyrosylation in vivo in cultured neuroblastoma cells because of the possibility that the natural substrate might not be tyrosine itself but something related, possibly even a protein with N-terminal tyrosine, or that tyrosine might be further modified, in vivo, after reacting with tubulin. This aim has not yet been realized, but preliminary experiments yielded the interesting observation that tyrosine was fixed mostly into an insoluble moiety of tubulin (Table III). Sonication solubilizes 90% of the colchicine-binding protein of these cells (23), but only 10% of the [^{14}C]tyrosine fixed was solubilized. When the pellet from the sonicate of the incubation mixture containing cycloheximide and colchicine (Table III) was dissolved in hot SDS and analyzed by polyacrylamide gel electrophoresis, all the ^{14}C appeared to be in tubulin α chain. Much less tyrosine was fixed under protein synthesis conditions (Fig. 1); this may have been due simply to inhibition of the ligase by phenylalanine. An extract of these cells would have fixed tyrosine > 100 times faster (for comparison with Fig. 1, 1 mg cell protein = 0.25 mg extract protein would fix 0.03 nmol in 1 min). A small amount of cell lysis could have accounted for the observed fixation, were it not that the reaction product appeared to

TABLE III

*In Vivo Fixation of [^{14}C]Tyrosine into an Insoluble Moiety of Tubulin by Neuroblastoma Cells**

Additions to reaction mixture			Solubilized by sonication	
Amino acid mixture	Cycloheximide	Colchicine	Protein	Fixed [^{14}C]tyrosine
	μg/ml	*mM*	%	
+			23	10
	120		21	5
	120	0.12	27	14

* Freshly harvested neuroblastoma cells were suspended in 2 vol of medium (Materials and Methods section) to give a total protein concentration of 25 mg/ml. Aliquots, supplemented as indicated and with 0.1 μmol (50 μCi) of [^{14}C]tyrosine per ml, were incubated for 2 h at 37°C. After freezing and thawing, the mixtures were exposed in a salt-ice bath, to four 30-s bursts at 60 watts with a Branson model W185 sonifier (Branson Sonic Power Co., Danbury, Conn.) equipped with a microtip. The sonicates were centrifuged for 30 min at 0°C at 43,000 *g*, the pellets were suspended in water, and aliquots were assayed for protein and fixed [^{14}C]tyrosine.

TABLE II

*Partition of Protein, Tubulin-Tyrosine Ligase, and Some Organelle Marker Enzymes between Soluble and Particulate Fractions of Rat Brain and Liver Homogenates**

Protein or enzyme activity	Brain		Liver	
	In supernate + wash	In washed pellet	In supernate + wash	In washed pellet
	%			
Protein	33	67	51	49
Tubulin-tyrosine ligase	88	12	100	0
Glucose-6-phosphatase			0	100
Succinic dehydrogenase	0	100	0	100
Acid phosphatase	16	84	53	47

* Tissues were homogenized (in 250 mM sucrose, 10 mM K$^+$MES, and 1 mM each EDTA and mercaptoethanol, pH 6.6) and the homogenates centrifuged for 1 h at 100,000 *g*, as described in the Materials and Methods section. The pellets were gently resuspended in the same buffer and centrifuged again. The two supernates were combined and the final pellets resuspended in buffer. Marker enzyme assays were done according to Swanson (40) for glucose-6-phosphatase, Hendler and Burgess (13) for succinic dehydrogenase, and Kersters and DeLey (17) for acid phosphatase.

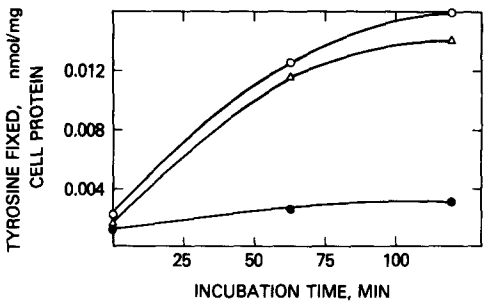

FIGURE 1 Time-course of [^{14}C]tyrosine fixation by neuroblastoma cells. The reaction mixtures, described in Table III, contained: complete amino acid mixture, ●; cycloheximide, △; and cycloheximide + colchicine, ○. At intervals, 5-μl aliquots were spotted on paper disks, and fixed tyrosine was measured as in the standard assay for ligase.

be an insoluble form of tubulin. Very similar results were observed in another experiment with a mince of adult rat brain.

Assembly of Tubulin In Vitro with or without C-Terminal Tyrosine

By incubating relatively little tubulin with crude ligase for 2 h, we had been able to tyrosylate up to 25% of the α chains (30). However, tubulin was denatured in such long incubations, as documented by colchicine-binding assays. With 20-fold purified ligase, we have been able to rule out denaturation as the reason for the apparent limit to the capacity of tubulin, as isolated after three cycles of assembly, to accept tyrosine. In 15-min incubations of 170 μg of tubulin with from 0.03 to 0.24 U of ligase, the result in every case was that 0.25 mol of tyrosine was fixed per mol of tubulin.[1] The short incubation time and invariance over 8-fold range of enzyme shows that this value is maximal.

To study the effect of tyrosylation on assembly, we prepared maximally tyrosylated tubulin. A purified fraction of ligase (7.4 U) eluted from DEAE-cellulose (33) was incubated with 36 mg of tubulin (prepared without glycerol) and 0.48 μmol (15 μCi) of [^{14}C]tyrosine for 40 min at 37°C in 5 ml of 10 mM K^+MES, pH 6.6, containing: 40 mM KCl, 2.5 mM ATP, 0.2 mM GTP, 10 mM $MgCl_2$, 0.4 mM β-mercaptoethanol, and 2 mM $CaCl_2$ (to ensure inhibition of assembly). After chilling to 0°C and adding ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetate (EGTA) to 4 mM to chelate the Ca^{2+}, the mixture was applied at once to a 3 × 34 cm Sephadex G-50 (medium) column, pre-equilibrated with reassembly buffer which was 1 mM in EGTA and 0.1 mM in GTP. 4-ml fractions were collected in the cold, and aliquots quickly counted in Hydromix. The column separated ^{14}C-tyrosylated protein from free [^{14}C]tyrosine. The first peak (16 ml) was made 0.4 mM in GTP, and the tubulin was repurified by three cycles of assembly, as shown in Table IV, part A. Each warm and cold supernate was assayed for protein, [^{14}C]tyrosine content, and colchicine-binding capacity. With our procedure pure tubulin binds 0.3 mol colchicine/mol; thus by this criterion tubulin was almost pure after the first cycle (Table IV). However, the continuing release of free tyrosine during each warm period indicates incomplete removal of ligase. In a previous purification (30) of 5% tyrosylated tubulin, where the ligase to tubulin ratio was 1/16 of that used here, the percent of free tyrosine released was relatively lower. In the latter preparation, tubulin assembled poorly only in the first cycle (the third cycle results are reproduced at the bottom of Table IV, part B), as it did from crude extracts. In the maximally tyrosylated tubulin the yield of microtubules continued to be low through all three cycles. This is not due to the high percent of C-terminal tyrosine; we found the same result after incubating large amounts of purified ligase with tubulin under detyrosylating conditions (ADP + P_i in the absence of tyrosine) in attempts to prepare completely detyrosylated tubulin. When diluted with carrier tubulin, for experiments described below, the [^{14}C]tubulin of Table IV assembled in proportion with the carrier.

Clearly, tyrosylation does not block incorporation of tubulin into microtubules in vitro, although there appears to be a slight enrichment of ^{14}C in the nonpolymerizing fraction of tubulin (the warm supernate; last column, Table IV). We do not know exactly what proportion of the α chains in tubulin as isolated already have C-terminal tyrosine, i.e., whether the 75% that will not accept tyrosine already have it. To test whether tubulin which lacks C-terminal tyrosine can be incorporated into microtubules. we looked for conditions that could remove all the labeled tyrosine that we

[1] Although it has not been rigorously proven, it is extremely likely that tubulin is a heterodimer of one α and one β subunit. Then the extent to which it is tyrosylated can be expressed either as the percent of α chains having C-terminal tyrosine, or as moles of C-terminal tyrosine present per mole of tubulin dimer.

TABLE IV

Purification of Tyrosylated Tubulin by Cycles of Assembly

Fraction	Protein	Colchicine-binding capacity			Fixed [^{14}C]tyrosine			Free [^{14}C]tyrosine	Ratio of tyrosine fixed to colchicine bound
		Total	Yield	mol/mol Tubulin*	Total	Yield	mol/mol Tubulin*		
	mg	*nmol*	*%*		*nmol*	*%*		*% of fixed*	*mol/mol*
A. Maximally tyrosylated tubulin									
Tyrosylation reaction mixture	56	89.4		0.21					
G-50 Sephadex eluate	35.2	60.1	(100)	0.22	57.0	(100)	0.21		0.95
First cycle									
Warm supernate	21.0	26.8	45	0.17	25.4	45	0.16	8	0.95
Cold supernate	12.4	31.1	52	0.32	24.2	42	0.25	6	0.78
	33.4	57.9	97		49.6	87			
Second cycle									
Warm supernate		9.7	16		8.5	15		15	0.88
Cold supernate	5.4	15.4	26	0.37	10.8	19	0.26	1	0.70
		25.1	42		19.3	34			
Third cycle									
Warm supernate	1.8	3.6	6	0.26	3.3	6	0.24	8	0.89
Cold supernate	2.8	6.4	11	0.30	4.8	8	0.22	0	0.75
	3.6	10.0	17		8.1	14			
B. Submaximally tyrosylated tubulin									
Third cycle									
Warm supernate	1.3	2.0	6	0.20	0.58	4	0.058	2.5	0.29
Cold supernate	11.0	20.0	56	0.24	4.21	29	0.042	0	0.21
	12.3	22.0	62						

* We assume that 85% of the protein at each step is tubulin. This is the usual tubulin content after three cycles of assembly. The assumption is essentially correct after the first warm supernate when, as indicated by the colchicine binding, protein introduced with the ligase fraction has been removed.

had put on, assuming that this would remove prior unlabeled tyrosine equally well. The reverse reaction catalyzed by the ligase was not satisfactory for this purpose since we were not able to drive it to completion (33). Carboxypeptidase had been shown to release the C-terminal tyrosine from tubulin (3). Although this enzyme might have blocked assembly by also digesting the β chain or the protein(s) which coassemble and seem required for assembly, we were encouraged by finding that [^{14}C]tyrosine release was unusually sensitive to carboxypeptidase, going to completion in a few minutes with a weight ratio (enzyme: substrate) of 1:100,000.

Table V (top 2 lines) documents that the [^{14}C]tubulin of Table IV, when mixed with carrier, assembled in proportion to the carrier and did not pellet in the presence of Ca^{2+}. Our assay for assembly always showed that 70–75% of the protein pelleted under assembly conditions, and 10–15% in presence of colchicine or calcium; the latter fraction contained no microtubules when examined by darkfield light microscopy (39). When EGTA was added after Ca^{2+}, the [^{14}C]tubulin again coassembled with carrier (Table V). When the tubulin was preincubated for 10 min with just enough carboxypeptidase to release all the [^{14}C]tyrosine, and then allowed to assemble for 20 min, the percent of protein which assembled was unchanged from the control preincubated without carboxypeptidase (some tyrosine was also released during the latter incubation, presumably due to residual ligase). The experiment was repeated with Ca^{2+} present during the carboxypeptidase digestion (Table V), since we do not know whether the tyrosine is as accessible to the enzyme in microtubules as it is in dimeric tubulin. All the results suggest that tubulin which lacks C-terminal tyrosine can be incorporated into microtubules in vitro.

What Proportion of α Chains Have C-Terminal Tyrosine in Brain Tubulin as Isolated?

We have done two kinds of experiments which indicate that brain tubulin as isolated by three cycles of assembly is partially tyrosylated. In one experiment, tubulin containing trace amounts of [^{14}C]tyrosine was digested with enough carboxypeptidase to release the latter, carboxypeptidase was separated away, and the maximal capacity of the tubulin to accept more [^{14}C]tyrosine was compared with that of undigested tubulin. To remove carboxypeptidase the tubulin was eluted from DEAE-Sephadex (43). Fig. 2 shows the elution

TABLE V

*Tubulin Assembly after Treatment with Carboxypeptidase to Remove C-Terminal Tyrosine from the α Chain**

Added during 10 min prior incubation			Assays after 2nd 20 min incubation		
				[^{14}C]Tyrosine	
Ca^{2+}	Carboxypeptidase	EGTA added after 10 min prior incubation	Protein in pelleted microtubules	Free	In pelleted microtubules
mM	*μg*	*mM*	%	%	%
0	0		71	16	61
2	0		14	14	11
2	0	3	69	10	62
0	0.01		69	100	5
2	0.01		12	99	3
2	0.01	3	69	88	11

* Untreated tubulin, purified by three cycles of assembly, was mixed with 1/50 amount of the tyrosylated tubulin of Table IV, in which 25% of the α chains contained newly introduced C-terminal [^{14}C]tyrosine. In 250 μl of reassembly buffer, 370 μg of the tubulin was incubated for 10 min at 37°C with the indicated additions. After adding EGTA where indicated, incubations were continued another 20 min, and the mixtures were centrifuged as in the standard assembly assay. Supernates and pellets were assayed for protein, fixed ^{14}C, and total ^{14}C. Carboxypeptidase was added as a water suspension; however, at this high dilution significant activity was not sedimentable.

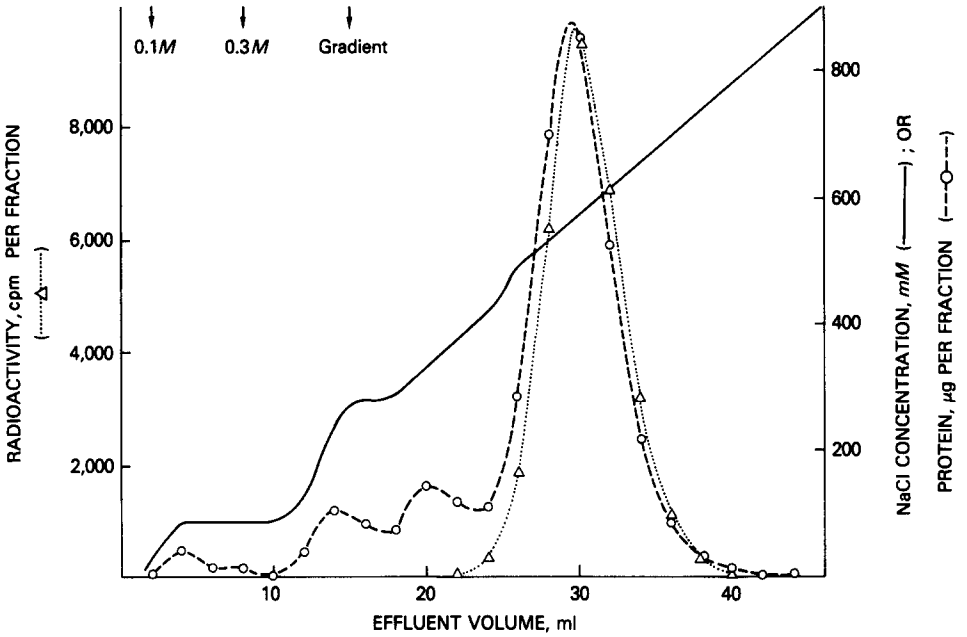

FIGURE 2 Further purification of ^{14}C-tyrosylated tubulin by elution from DEAE-Sephadex after three cycles of assembly. Tubulin (5 mg = 30,000 cpm in 0.5 ml) of Table V was applied to a 1 × 7 cm column of DEAE-Sephadex A-50 equilibrated with reassembly buffer, with GTP decreased to 0.1 mM and containing 100 mM NaCl. 2-ml fractions were collected at 0.2 ml/min while eluting with 100 or 300 mM NaCl, as indicated, then with a gradient formed from 15 ml each of 300 and 1,000 mM NaCl in the reassembly buffer.

pattern for a sample of ^{14}C-tyrosylated tubulin (which had not been digested). This eluted tubulin did not shed any free tyrosine when warmed, in contrast to that shown in Tables IV and V. DEAE-Sephadex columns would be expected to remove all residual ligase from tubulin since the enzyme is eluted at much lower salt concentration (33). Comparison of protein and ^{14}C peaks in Fig. 2 indicates that the leading edge of the tubulin peak is still slightly contaminated with other pro-

teins, but DEAE chromatography clearly does not itself select out a population of detyrosylated tubulin which might have a higher capacity to accept tyrosine. Table VI shows that carboxypeptidase digestion did increase the capacity of the tubulin to accept tyrosine from 0.25 to 0.37 mol/mol. With the use of colchicine binding to correct for denaturation of the digested tubulin, i.e., during chromatography and concentration, it appears that the acceptor capacity was increased by 80% (Table VI). This indicates that, in removing the tyrosine that we had put on, carboxypeptidase also took off some more that was already there, or removed something else that was blocking tyrosylation.

A second approach was to isolate α chains by slab gel SDS electrophoresis and monitor the products released by carboxypeptidase with an amino acid analyzer. In an earlier experiment with a mixture of performic oxidized $\alpha + \beta$ chains, we had not observed any amino acids released (32). In the experiment of Table VII, we included β-lactoglobulin in one incubation mixture, in order to be sure that carboxypeptidase was still active in the presence of the tubulin preparation. β-Lactoglobulin, which has two identical chains with C-terminal Ile and penultimate His, had been denatured with 4% SDS (2 min at 100°C), and then freed of SDS with Dowex-1 by the same procedure used for tubulin α chains (Materials and Methods section). The tubulin also contained trace amounts of [^{14}C]tyrosine. Although carboxypeptidase rapidly releases all of the latter from native tubulin, we have found that a portion becomes resistant after denaturation either by performic oxidation or by alkylation and SDS electrophoresis. In this case, 30% of the ^{14}C was not released even by large amounts of carboxypeptidase. This preparation also contained 10–20% of β chain; the preparative electrophoresis system gave pure β, but the α band, though well separated (see Fig. 3), contained some β. As shown in Table VII the control mixtures had appreciable amounts of amino acids compared with the reaction mixtures, and in both cases the amounts were near the limit of resolution of even the Durrum analyzer (Durrum Instrument Corp., Sunnyvale, Calif.). Only small amounts of α chain were available because of the losses during removal of SDS (Materials and Methods section). Other experiments showed that the control amino acids were a background present at zero time in the tubulin. Table VII shows that distinctly more tyrosine was liberated than anything else, the amount being 0.3 mol/mol α chain, or 0.45 if one corrects for the resistant fraction of [^{14}C]tyrosine. Although the amount is uncertain it appears that, in this preparation of brain tubulin, some α chains do have C-terminal tyrosine.

The α chains lacking tyrosine probably have C-terminal Glu or Gln (2), neither of which was released in significant amounts (Table VII). We have not been able to remove any Glu from other

TABLE VI

*Extent to which Tubulin can Accept Tyrosine before and after Treatment with Minimal Amount of Carboxypeptidase**

Tubulin preparation	Colchicine bound	Tyrosylation conditions		[^{14}C]Tyrosine fixed	Ratio of tyrosine fixed to colchicine bound
		Tubulin	Ligase		
	mol/mol tubulin	*nmol*	*U*	*mol/mol tubulin*	
Untreated	0.28	0.41	0.020	0.26	0.89
			0.037	0.23	
			0.074	0.26	
Treated	0.23	0.43	0.020	0.36	1.61
			0.037	0.36	
			0.074	0.37	

* 10 mg of tubulin containing trace amounts of C-terminal [^{14}C]tyrosine (Table V) was incubated with 0.05 μg of carboxypeptidase until 99% of the ^{14}C was released (50 min). To remove the carboxypeptidase, the chilled mixture was applied to a DEAE-Sephadex column and eluted as in Fig. 2. The peak fractions containing tubulin were concentrated at 0°C from 12 to 1 ml by ultrafiltration through an S and S collodion bag no. 100 (Schleicher & Schuell, Inc., Keene, N. H.) with simultaneous dialysis against reassembly buffer. Recovery was 3.3 mg protein. The tubulin was then tyrosylated in 100 μl vol for 40 min with the indicated amounts of a DEAE fraction of ligase (sp act 0.3). The indicated nanomoles of tubulin are calculated on the assumption that the untreated is 85% pure, and the treated, i.e., after DEAE, is 95% pure.

TABLE VII
*Amino Acids Released from α Chain of Tubulin by Carboxypeptidase**

Amino Acid	α Chain (2 nmol)		α Chain (2 nmol) + β-lactoglobin (2 neq)	
	Amount recovered less control	Increase over amount in control	Amount less control	Increase over amount in control
	nmol	%	*nmol*	%
Ala	0.26	28	0.19	20
Asp	0.11	11	0	0
Glu	0.18	67	0.14	51
Gly	0.19	9	0.11	5
His	0.07	13	1.80	450
Ile	0.13	43	2.31	770
Leu	0.40	84	0.32	67
Lys	0.04	15	0.03	11
Ser + Asn + Gln	0.36	11	0.18	6
Thr	0.18	27	0.06	9
Tyr	0.64	250	0.56	220

* 2 nmol of tubulin α chain (110 μg) ± 2 neq of β-lactoglobulin (36 μg) were incubated for 30 min at 37°C in 250 μl of 0.05 M *N*-ethylmorpholine acetate buffer, pH 8.5, containing 2 nmol of norleucine and 5 μg of a suspension of carboxypeptidase. Reactions were stopped by adding 10 μl of concentrated acetic acid. Controls were incubated without carboxypeptidase, and 5 μg of the latter (which had also been separately incubated 30 min at 37°) was added after acidification. The residues after lyophylization were suspended in 50 μl of analyzer buffer, and 30 μl of the supernate after centrifugation was assayed with a Durrum amino acid analyzer. The results shown are the differences between experimental and control mixtures, based on amounts present in the total 50 μl and normalized to a recovery of 2 nmol of norleucine. Amino acids not shown corresponded to peaks insufficiently resolved from background to be integrated by the analyzer.

proteins with carboxypeptidase A, i.e., from cytochrome *c*, at pH 6 or 9, or 5.3 after performic oxidation. Since Gln is easily attacked, the terminal residue to which tyrosine is linked is presumably Glu.

A convenient way to determine the amount of C-terminal tyrosine would be provided if one could physically separate the tyrosylated α chains. Several authors have described apparent resolution of α chains into multiple components. Lu and Elzinga (20) reported that hydroxylapatite chromatography in SDS (24) fractionated α tubulin into two peaks. We repeated their experiment with ^{14}C-tyrosylated tubulin, but found comparable amounts of ^{14}C in both fractions (Fig. 3).

DISCUSSION

It appears that brain tubulin could be mostly tyrosylated in vivo, insofar as enzyme is in excess over tubulin in extracts (33) and insofar as the concentration of free tyrosine in brain exceeds the K_m concentration for the enzyme (35). Our results indicate that a portion of α chains do contain C-terminal tyrosine in vivo, at least in that moiety of soluble tubulin that is isolated after three cycles of

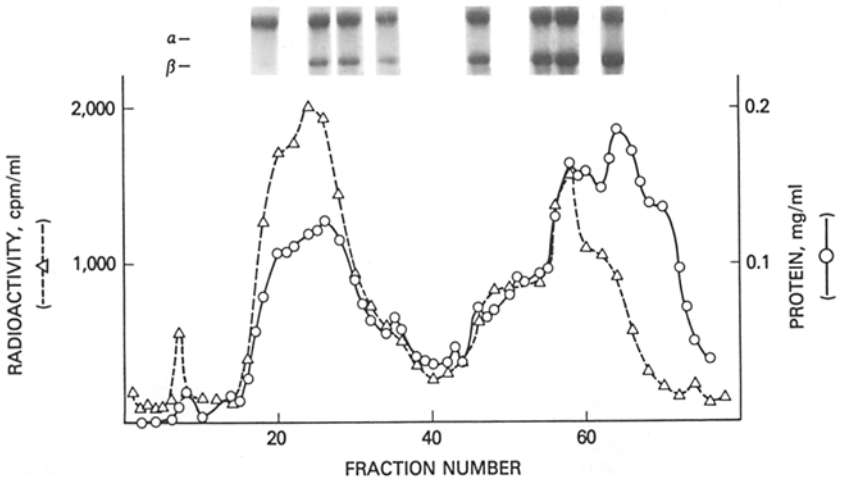

FIGURE 3 SDS-hydroxylapatite column chromatography of ^{14}C-tyrosylated tubulin. A mixture of tubulin (11 mg) and ^{14}C-tyrosylated tubulin (10^5 cpm), both purified by three cycles of assembly as in Table IV, was reduced and alkylated, heated in 1% SDS (22), and dialyzed against 10 mM sodium phosphate, pH 6.4, containing 0.1% (wt/vol) SDS and 0.1% (vol/vol) mercaptoethanol. The sample was applied to a 0.9 × 11.5 cm hydroxylapatite column equilibrated with the same buffer, and eluted with a linear gradient prepared from 100 ml each of 100 and 500 mM sodium phosphate containing the above components (Lu and Elzinga, (20) and personal communication). Fractions of 96 drops were collected. Towards the end of the elution a precipitate formed in the 500 mM sodium phosphate solution. Aliquots of each fraction were counted in Hydromix, and assayed for protein after oxidation of the mercaptoethanol with I_2. Some fractions were analyzed by SDS gel electrophoresis (22). Photographs of the portions of the stained gels containing the α and β chains of tubulin are shown above the corresponding fractions.

assembly. The amino acid analysis of products released from α chain by carboxypeptidase needs to be repeated with more substrate to obtain a higher ratio of tyrosine to background. The observation that some tyrosine (0.2–0.5 mol/mol α chain) was released is supported by the experiment showing that carboxypeptidase digestion increased the capacity of tubulin to accept tyrosine, from 0.25 to about 0.45 mol/mol α chain, but the capacity should have been increased more if 75% of the isolated α chains originally had C-terminal tyrosine. The apparent limited increase may be due to equilibrium or exchange processes, or to some of the α chains being blocked in some other way or with something other than tyrosine.

Recently, Lu and Elzinga (21) have reported the complete sequence of a 25-residue cyanogen bromide C-terminal α chain peptide. They reported it to have C-terminal glutamate, suggesting that we are looking at the same C-terminus, but found no evidence for a fractional population having an additional tyrosine in this position. They now find (personal communication), however, that 30–50% of the peptide molecules do have a C-terminal tyrosine. It is significant that they isolated α chain without at any time warming the brain extract (21), since in our case the C-terminal tyrosine might conceivably not have been present in vivo but rather might have been added by the ligase during the first warm assembly step. The C-terminal peptide is extremely acidic (21). That it occupies an exposed position in native tubulin dimers is indicated by its unusual accessibility to carboxypeptidase A, as well as to tubulin-tyrosine ligase. The serine which is phosphorylated in β chains appears to reside in a similarly acidic C-terminal region (10, 21).

Tubulin-tyrosine ligase is widely distributed in rat tissues (7, 32). There is no reason to doubt that cytoplasmic tubulin of these tissues serves as substrate, although so far only brain tubulin has been shown to do so. If the ligase is confined to mammalian tissues it cannot have a universal role in microtubule assembly or function, but we have not tested enough invertebrates or unicellular eukaryotes to show this (Table I), and perhaps we do not know enough about the enzyme to prove its absence. It is conceivable but quite unlikely that the C-terminal tyrosine is a coded residue, and that the initial post-translational modification is its removal by the reversible reaction catalyzed by the ligase. There are precedents for a hydrolytic digestion being requisite to the formation of $\alpha\beta$ dimer, but not for such a hydrolase that can add back the residue with the expenditure of ATP.

Our results show that tyrosylation does not block incorporation of tubulin into microtubules in vitro, and suggest that detyrosylated tubulin can also be incorporated, because tubulin assembled normally after digestion with carboxypeptidase (Table V). In these experiments we have measured only the final extent of assembly. It remains to be seen whether tyrosylation will affect the kinetics of assembly or disassembly, or the ability of microtubule-associated proteins to coassemble. Besides catalyzing a reaction, the ligase apparently exists in brain in the form of a tight complex with tubulin (33). The fact that tubulin assembled poorly after relatively large amounts of purified ligase were added to it (Table IV), might suggest that assembly could be aborted by incorporation of this complex. However, the ratio of enzyme units to milligrams of tubulin is actually higher (0.5) in crude extracts than it was in the experiment of Table IV. In any case, the requirements for in vivo assembly, where the many critical conditions of pH, ionic strength, Mg^{2+} concentration, etc., may be only marginally satisfied, may be more stringent. The incompletely characterized microtubule-associated proteins (25, 42), which may be essential for assembly in vivo, can be replaced in vitro with various unnatural polycations (14) or with high concentrations of glycerol and Mg^{2+} (12), suggesting that in vitro assembly need not be a valid model of intracellular phenomena. Perhaps many factors will prove to be significant in regulating assembly. We have begun to look for changing ligase activity in cells (neuroblastoma, sea urchin eggs) which can be induced to synchronously assemble microtubules from preexisting pools of tubulin. Since the ligase reaction is reversible, this is not sufficient to test whether tyrosylation controls assembly in vivo, and we are currently seeking (T. Martensen, unpublished results) more precise methods to determine the actual extent to which tubulin is modified before and after assembly.

Tyrosylation might also control the partition of tubulin between different cell compartments. In a preliminary experiment, neuroblastoma cells fixed tyrosine almost exclusively into an insoluble fraction of tubulin (Table III). Experiments are in progress (J. Nath, unpublished observations) to determine the amount of C-terminal tyrosine in the nonmicrotubule, membrane-bound fraction of brain tubulin (6), and to see whether this tubulin is

a substrate for ligase or can convert the 35,000 dalton enzyme (33) to the larger form.

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