Acknowledgments

I thank Elżbieta Starzyńska for her skillful technical assistance.

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Exchange of Tubulin Dimer into Rings in Microtubule Assembly–Disassembly†

Barry Zeeberg,* Jeffrey Cheek, and Michael Caplow*

ABSTRACT: We have prepared native radioactive tubulin dimer from two species: [^{35}S]tubulin dimer, by in vivo labeling of rat brain, and porcine [^{3}H]ethyltubulin, as previously described [Zeeberg, B., Cheek, J., & Caplow, M. (1980) *Anal. Biochem. 104*, 321–327]. After microtubule assembly with radioactive tubulin dimer and nonradioactive dimer and rings, the tubulin in the rings and the dimer obtained upon disassembly have approximately equal specific activities. Therefore, during the reaction sequence

$$\text{dimer} + \text{rings} \xrightarrow{37\ ^\circ\text{C}} \text{microtubules} \xrightarrow{0\ ^\circ\text{C}} \text{dimer} + \text{rings}$$

the tubulin initially in rings becomes indistinguishable from tubulin initially in dimer. Under nonpolymerizing conditions (0 °C) radioactive tubulin dimer and radioactive guanine nucleotide are incorporated into rings at approximately equal rates. This indicates that there is a pathway for nucleotide incorporation into rings under nonpolymerizing conditions which involves the incorporation of dimer-bound nucleotide. We also report results on the lack of the mirror image equilibrium during the disassembly process, using porcine [^{3}H]-ethyltubulin dimer, rat [^{35}S]tubulin dimer, and a [^{3}H]-GDP·porcine tubulin dimer complex. In all three cases there is no significant disassembly-dependent incorporation of radioactivity into rings when microtubules are disassembled in the presence of radioactive dimer. These results demonstrate that, for rat and porcine tubulin, rings are formed during microtubule disassembly by direct cleavage of intact rings, without a tubulin dimer intermediate.

Microtubules have been postulated to be in a dynamic equilibrium with tubulin subunits in the cell (Inoue & Sato, 1967). The central role of microtubules in the mitotic apparatus and cytoskeleton has led investigators to examine the regulatory elements for in vivo microtubule properties and functioning. A number of recent studies have concentrated on the mechanism for microtubule assembly and disassembly (Margolis & Wilson, 1978, 1979; Karr & Purich, 1979; Sternlicht & Ringel, 1979; Bergen & Borisy, 1980).

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The product of cold-induced microtubule disassembly is a mixture of tubulin dimer (M_r 110000) and rings, which are composed of tubulin dimers and microtubule associated proteins. Recent studies revealed striking differences in the nucleotide binding properties of tubulin rings and tubulin dimer (Caplow & Zeeberg, 1980): the dimer rapidly binds 1 mol of added radioactive guanine nucleotide and the rings do not rapidly bind added guanine nucleotide. It was surprising, therefore, to find that microtubules formed in the presence of radioactive guanine nucleotide and a mixture of an approximately equal amount of ring and dimeric protein contain 1 mol of radioactive nucleotide. In addition, the rings derived from depolymerization of these microtubules were found to contain ~1 mol of radioactive nucleotide/mol of tubulin dimer within the rings. These observations led us to ask if there is a route for incorporation of dimeric tubulin into rings in a cycle of microtubule assembly and disassembly. The existence of this route could account for the incorporation of radioactive nucleotide into rings. Also, it led to the question whether there is a path for nucleotide incorporation into rings under nonpolymerizing conditions and whether this path involves incorporation of dimer-bound nucleotide or direct incorporation of nucleotide. In order to study these questions, we have used the approach of Weingarten et al. (1975) in which radioactivity in a [^{35}S]tubulin dimer was used to follow the path for dimer incorporation into microtubules.

We have found in these studies that the tubulin initially in rings and the tubulin in dimer are able to equilibrate during a cycle of assembly and disassembly. This result is in contrast to the results with chick tubulin (Weingarten et al., 1975) where the tubulin initially in rings remained in rings and the tubulin initially in dimer remained in dimer. The assembly and disassembly processes with chick tubulin are mirror images of each other, with respect to the lack of exchange of dimer and ring tubulins.

In order to determine whether the assembly and disassembly processes with rat and pig tubulins are mirror images of each other, we have studied whether there is equilibration of radioactive tubulin dimer into rings which are generated from disassembling microtubules. The answer to this question is used for determining the pathway for ring formation from disassembling microtubules: if tubulin dimer is an intermediate for ring formation, then radioactivity associated with tubulin dimer (added simultaneously to disassembly) will equilibrate into rings; a lack of equilibration would indicate that there is a direct cleavage of intact rings from microtubules.

Experimental Procedures

Preparation of Microtubular Protein from Pig Brain. Microtubular protein was prepared by a modification of the Shelanski assembly–disassembly procedure (Shelanski et al., 1973). Fresh pig brains were homogenized with an equal weight of ice-cold reassembly buffer (RB) [0.1 M 2-(*N*-morpholino)ethanesulfonic acid buffer, 0.5 mM $MgCl_2$, and 1.0 mM EGTA, pH 6.8] containing 0.1 mM ATP in a Waring Blendor for 30 s. The supernatant obtained from centrifugation (30000 rpm; 75 min; 4 °C) in a Beckman 30 rotor was diluted with an equal volume of glycerol–reassembly buffer (60.2 mL of glycerol–24 mL of reassembly buffer) containing 1 mM ATP and polymerized at 37 °C for 30 min. The assembled microtubules were then sedimented (Beckman 30 rotor; 30000 rpm; 75 min; 37 °C), and the pellet was stored at −40 °C under glycerol–reassembly buffer.

Immediately prior to an experiment, the protein was further purified by another cycle of polymerization to assure use of active protein. The pellets were resuspended in reassembly buffer (0.1 volume of the crude supernatant) by means of a Dounce homogenizer and depolymerized at 0 °C for 30 min. After centrifugation (50000 rpm; 10 min; 0 °C) in a Beckman Ti50 rotor, the supernatant was polymerized, as before. The microtubules were sedimented (50000 rpm in a Ti50 rotor; 30 min; 37 °C), the pellets were resuspended in reassembly buffer (0.025 volume of the crude supernatant), depolymerized (0 °C; 30 min), and centrifuged (50000 rpm; 10 min; 0 °C) to remove aggregates. The supernatant, containing depolymerized protein, was applied to a Sephadex G-25 column equilibrated with reassembly buffer to remove free nucleotides and glycerol. By the following criteria the proteins are identical when either ATP or GTP is used throughout the purification: microtubule yield in a subsequent GTP-induced assembly; rate of microtubule assembly in a subsequent ATP- or GTP-induced assembly; kinetics for cold-induced microtubule disassembly; kinetics for dilution-induced microtubule disassembly; average microtubule length following GTP-induced assembly, as measured by electron microscopy (B. Zeeberg and M. Caplow, unpublished results).

Protein concentrations were calculated from the absorbance at 278 nm and an extinction coefficient of 1.2 A/(mg/mL) (Jacobs et al., 1974). Fractionation of tubulin into ring and dimer was accomplished by chromatography, using Sepharose 6B at 4 °C. Electron microscopy of ring fractions, negatively stained with uranyl acetate on carbon-coated Formvar grids (kindly provided by Dr. E. D. Salmon), revealed that the only structure present is single rings. Electrophoresis of protein samples was done with 7.5% acrylamide, as described previously (Laemmli, 1970).

Preparation of Radioactive Tubulins. An aqueous solution containing 2 mCi of [^{35}S]methionine (100–400 Ci/mmol) was evaporated to dryness under a stream of air, dissolved in physiological saline, and then injected intracranially into six 11–12-day-old Long-Evans rats. The animals were decapitated after ~16 h, and the brains were quickly removed and homogenized with a Sorvall Omnimixer for 15 s at 0 °C in 6 mL of reassembly buffer containing 0.1 mM GTP. The supernatant obtained from centrifugation at 0 °C (Beckman Ti50 rotor; 50000 rpm) was mixed with 4.8 mL of glycerol–reassembly buffer (60.2 mL of glycerol–24 mL of reassembly buffer) and 3.25 mg of GTP. Microtubules formed by incubating this mixture at 37 °C for 20 min were harvested by centrifugation (30 min; Ti50 rotor; 50000 rpm). The pellet was homogenized in 0.75 mL of ice-cold reassembly buffer for 20 min by using a Pasteur pipet. This material was centrifuged at 0 °C (Ti50 rotor; 50000 rpm; 5 min), and the resultant pellet was discarded. The supernatant was mixed with 0.75 mL of glycerol–reassembly buffer and GTP (0.5 mM final concentration) for another polymerization cycle (37 °C; 20 min). Microtubules were harvested by centrifugation (37 °C; 30 min; Ti50 rotor; 50000 rpm) and then depolymerized for 20 min at 0 °C in 0.5 mL of reassembly buffer. Undepolymerized protein was removed by centrifugation at 0 °C (Ti50 rotor; 50000 rpm; 5 min). Nonradioactive rat brain tubulin was prepared simultaneously from littermates. For isolation of nonradioactive tubulin, the supernatant from the last centrifugation was passed through a column (1 × 12.5 cm) containing Sephadex G-25, which had been equilibrated with reassembly buffer at 4 °C. For isolation of pure [^{35}S]tubulin dimer free of rings and microtubule associated protein, the supernatant obtained from the last centrifugation was chromatographed on a Sepharose 6B column (1 × 12.5 cm) which had been equilibrated with reassembly buffer at 4 °C. The tubulin dimer peak, which was well resolved from the rings,

was collected and further purified by centrifugation at 0 °C (40 min; Ti50 rotor; 50000 rpm) to remove all contaminating tubulin rings. Rat tubulin was used on the day on which it was isolated. The [^{3}H]ethyltubulin dimer from pig brain was freshly prepared for each experiment, as described previously (Zeeberg et al., 1980).

Equilibration of Tubulin Dimer with Rings in Assembly–Disassembly. An aliquot (250 μL) of ^{35}S-labeled dimer (2.4 μM) was mixed with 400 μL of nonradioactive tubulin (25 μM), which had been prepared simultaneously with the ^{35}S-labeled tubulin, and polymerization was induced by adding 15 μL of 20 mM GTP and warming to 37 °C for 10 min. After the resultant microtubules had been depolymerized by cooling to 0 °C for 5 min, the entire reaction mixture was chromatographed at 4 °C on a Sepharose 6B column (1 × 12.5 cm) [10 min at 37 °C and 5 min at 0 °C are sufficient to ensure completion of polymerization and depolymerization, respectively (unpublished results)]. In a control experiment 400 μL of nonradioactive tubulin was assembled with 9 μL of 20 mM GTP at 37 °C for 10 min, and the resultant microtubules were disassembled at 0 °C for 5 min. This reaction was chromatographed immediately after addition of 6 μL of GTP and 250 μL of [^{35}S]tubulin dimer.

In experiments with porcine tubulin, 0.25 mL of 3.0 μM [^{3}H]ethyltubulin dimer was mixed with 0.35 mL of depolymerized porcine tubulin (25.0 μM). Polymerization was induced by addition of 30 μL of 20 mM GTP and warming to 37 °C for 10 min. The resultant microtubules were depolymerized by chilling for 5 min at 0 °C, and the entire reaction mixture was chromatographed at 4 °C on Sepharose 6B (1 × 12.5 cm). The control reaction lacked GTP and was maintained at 0 °C.

Pathway for Nucleotide Incorporation into Rings under Nonpolymerizing Conditions. An aliquot (400 μL) of nonradioactive rat tubulin (25 μM) was mixed with 250 μL of 2.4 μM [^{35}S]tubulin dimer and 10 μL of [^{3}H]GDP (1.0 μCi). After a 75-min incubation at 0 °C, the entire reaction mixture was chromatographed on a Sepharose 6B column (1 × 12.5 cm). In a control reaction the ^{35}S-labeled dimer and [^{3}H]GDP were added immediately before chromatography.

Reversibility of Nucleotide Binding and Dissociation with Porcine Tubulin Rings. The reversibility of nucleotide binding to rings in a slow reaction is demonstrated by incubation of 20 μM tubulin with 100 μM [^{3}H]GDP at 0 °C. Aliquots of the reaction were analyzed by column centrifugation (Penefsky, 1977) 200 s after addition of 757 μM nonradioactive GDP; in 200 s the nonradioactive GDP displaces only the rapidly exchangeable [^{3}H]GDP at the dimer binding site.

Kinetics for Nucleotide Dissociation and Binding with Porcine Tubulin. Kinetics were studied at 0 °C, with reaction mixtures maintained in an ice bath working in a 4 °C room. At different times 50-μL aliquots were removed and analyzed by column centrifugation (Penefsky, 1977) at 4 °C. The column centrifugation utilized tuberculin syringes, containing 1 cm^3 of packed Sephadex G-25, which had been equilibrated with the same buffer as had the protein. Approximately 0.9% of unbound guanine nucleotide is eluted from the column, as determined in control studies where the protein was not added. Protein and its associated bound E-site nucleotide are eluted from the column during centrifugation in ~35 s, although the centrifugation is allowed to proceed for 2 min as was originally described (Penefsky, 1977). There is no release of protein-bound nucleotide during column centrifugation, since identical binding was observed when the length and volume of the column were varied by a factor of 2.

Depolymerization Studies with [^{3}H]GDP and Porcine Tubulin. (A) Depolymerization Studies with Nonradioactive Microtubules. Microtubules, assembled from 2 mL of 35 μM tubulin by incubation at 37 °C with ATP (500 μM) for 10 min were isolated by centrifuging 1 mL of the reaction mixture through a 7-mL glycerol–reassembly buffer cushion (60.2 mL of glycerol–108 mL of reassembly buffer) at 37 °C in a SW50.1 rotor (30 min; 50000 rpm). The resultant pellet was depolymerized for 5 min at 0 °C by homogenization in 0.65 mL of 0.9 μM unpolymerized tubulin which had [^{3}H]GDP at the E site of the dimeric protein as a result of a 30-s preincubation with a trace amount of [^{3}H]GDP (~2 × 10^6 cpm; 10 mCi/μmol). The depolymerization product was resolved into rings and dimer by chromatography at 4 °C on a 1 × 12 cm Sepharose 6B column which had a 4-cm covering layer of Sephadex G-25. In a control experiment, the unpolymerized protein and [^{3}H]GDP were not present during depolymerization but were added after depolymerization had been completed. After an additional 5-min incubation, the control reaction was chromatographed.

(B) Depolymerization Studies with Microtubules Containing [^{3}H]GDP. Microtubules were assembled from 2 mL of 35 μM purified tubulin at 37 °C with ATP (500 μM) and 20 μCi of [^{3}H]GDP for 10 min. Approximately 1 mol of radioactive GDP is incorporated into microtubules by using this procedure (Caplow & Zeeberg, 1980). The microtubules were isolated by centrifuging 1 mL of the reaction mixture through a glycerol–reassembly buffer cushion, as in the preceding paragraph. The pellet was depolymerized at 0 °C in 0.6 mL of 100 μM GDP for 5 min [addition of higher concentrations of GDP is not compatible with the experiment because this results in dissociation of the rings (Penningroth & Kirschner, 1977)], and the resultant solution was chromatographed at 4 °C on a Sepharose 6B column (1 × 12 cm) with a 4-cm upper layer of Sephadex G-25. A control reaction was similarly treated except that the GDP was added 5 min after microtubule disassembly and an additional 5-min incubation was carried out before chromatography.

Depolymerization Studies with Porcine [^{3}H]Ethyltubulin Dimer and Rat [^{35}S]Tubulin Dimer. Nonradioactive porcine tubulin (18.9 μM, 1.1 mL), obtained by Sephadex G-25 chromatography, was incubated with GTP (20 mM, 50 μL) at 37 °C for 15 min. Two aliquots (500 μL) were layered on top of cushions (5 mL) composed of a mixture of glycerol–reassembly buffer/reassembly buffer (1:1 v/v) maintained at 37 °C. The microtubules were pelleted by spinning at 37 °C (20 min; 50000 rpm; Ti50 rotor), and the cushion was carefully removed from the pellets. One pellet was depolymerized at 0 °C for 10 min by using 500 μL of 2 μM porcine [^{3}H]-ethyltubulin dimer (60000 cpm/500 μL); the control pellet was depolymerized at 0 °C for 7 min with 500 μL of reassembly buffer and then incubated with 500 μL of [^{3}H]-ethyltubulin dimer at 0 °C for 10 min. Approximately 500 μL of each depolymerized pellet solution was chromatographed at 4 °C on a 1 × 12.5 cm Sepharose 6B column covered with 4 cm of Sephadex G-25. The eluted fractions (~0.5 mL) were assayed for radioactivity and protein.

Nonradioactive rat tubulin (400 μL of 25 μM) which had been purified by two cycles of assembly and disassembly and desalted by chromatography on Sephadex G-25 was assembled into microtubules by incubation with 450 μM GTP at 37 °C for 10 min. This mixture was added to 250 μL of a solution containing 2.4 μM purified rat [^{35}S]tubulin dimer (~88000 cpm total), with simultaneous cooling to 0 °C for 5 min. The solution was chromatographed at 4 °C on a 1 × 12 cm Se-

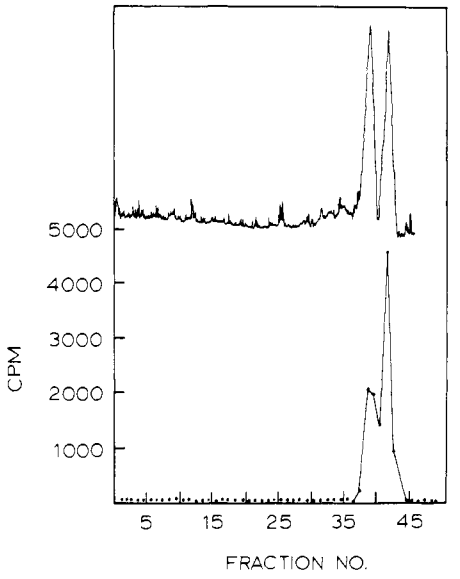

FIGURE 1: Protein and radioactivity profiles from $NaDodSO_4$ electrophoresis of [^{35}S]tubulin dimer. Radioactivity (lower curve) was determined by counting gel slices after Protosol digestion. Protein (upper curve) was determined from absorbance measurements at 583 nm of the Fast Green stained gel.

pharose 6B column. A control reaction differed only in that the rat [^{35}S]tubulin dimer was added after cooling the polymerization mixture to 0 °C for 5 min; this mixture was chromatographed after an additional 5-min incubation.

Results

Characterization of [^{35}S]*Tubulin.* Pure ^{35}S-labeled tubulin dimer (Figure 1) can be isolated from rats which have received an intracerebral injection of [^{35}S]methionine. Analysis of the purified tubulin dimer by isoelectric focusing under denaturing conditions (O'Farrell, 1975) revealed microheterogeneity in the α and β subunits. $NaDodSO_4$ slab gel electrophoresis of protein samples which had been initially fractionated by isoelectric focusing showed that the radioactive sulfur (measured by autoradiography) and the Coomassie-staining material had a mobility characteristic of tubulin. Isoelectric focusing studies also showed that the microheterogeneity of porcine brain tubulin dimer is different from that of rat.

Radioactivity in the ring fraction is present in both tubulin and nontubulin proteins (Figure 2); however, the majority of the radioactivity is associated with tubulin, with relatively low levels of radioactivity in the microtubule associated proteins. This pattern was found in three separate experiments.

Equilibration of Tubulin Dimer with Rings in Assembly–Disassembly. It is shown in these experiments with both rat and pig brain protein that tubulin dimer equilibrates fully with tubulin ring protein during microtubule formation. After rat ^{35}S-labeled dimer is copolymerized with nonradioactive rat tubulin, Sepharose 6B chromatography shows (Figure 3B) that the average for the ratio of specific activities of ring/dimer is equal to 0.73 (three separate experiments). Since rings are composed of ~32% nontubulin proteins and 68% tubulin (Figure 2), the observed ratio of specific activities of 0.73 indicates that the tubulin in dimer and rings had come to isotopic equilibrium.

Similar results were obtained in experiments with porcine [^{3}H]ethyltubulin dimer: Again, the polymerization effected an exchange of dimer into rings (Figure 4B); only minimal incorporation of radioactive dimer into rings was observed in the control (Figure 4A).

Characterization of Radioactive Nucleotide in Depolymerized Rat Brain Tubulin. Rat brain tubulin, which had been purified by two cycles of assembly and disassembly, was po-

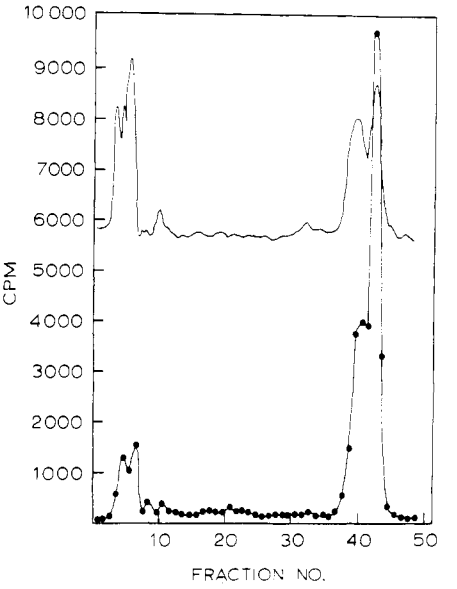

FIGURE 2: Protein and radioactivity profiles from $NaDodSO_4$ electrophoresis of [^{32}S]tubulin rings obtained from Sepharose 6B chromatography. The upper and lower curves represent protein and radioactivity, respectively.

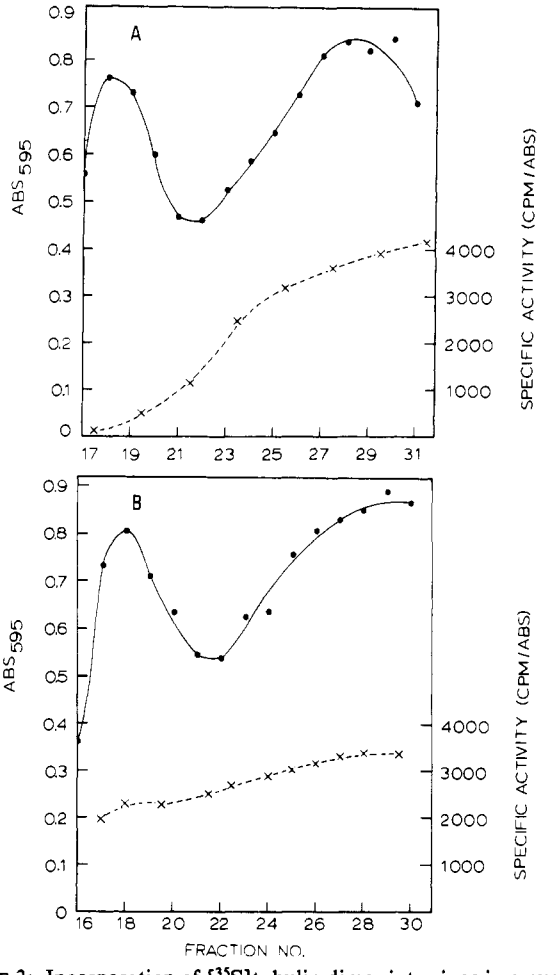

FIGURE 3: Incorporation of [^{35}S]tubulin dimer into rings in a cycle of microtubule assembly and disassembly. Reaction A: [^{35}S]tubulin dimer was added after microtubule assembly and disassembly. Reaction B: [^{35}S]tubulin dimer was present during microtubule assembly. Reaction mixtures were chromatographed at 4 °C on a 1 × 12.5 cm Sepharose 6B column, which had a 4-cm covering layer of Sephadex G-25.

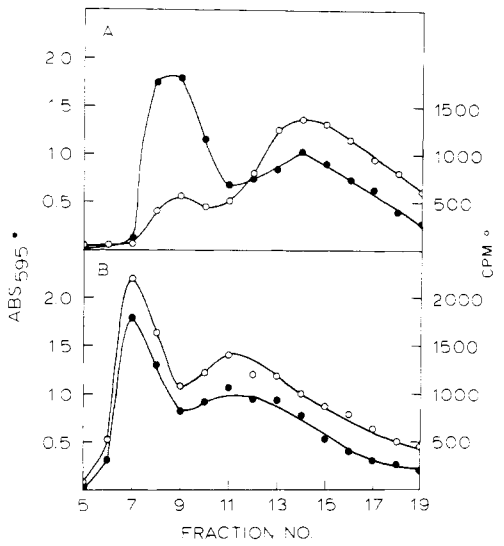

FIGURE 4: Incorporation of [^{3}H]ethyltubulin dimer into rings in a cycle of microtubule assembly and disassembly. Reaction A: [^{3}H]ethyltubulin dimer was added to unpolymerized nonradioactive tubulin (at 0 °C) 15 min before chromatography. Reaction B: [^{3}H]ethyltubulin dimer was present during microtubule assembly. The chromatography was as described for Figure 3.

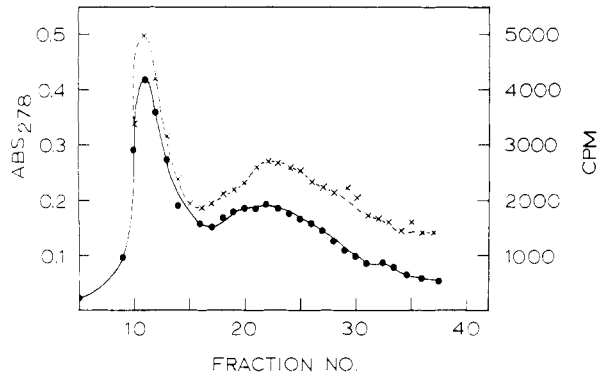

FIGURE 5: Chromatography of rat tubulin after polymerization with 488 μM [^{3}H]GTP (60 mCi/μmol). Protein (●) and radioactivity (×) were determined after chromatography, as described for Figure 3.

lymerized with [^{3}H]GTP. After cold-induced depolymerization, centrifugation to remove aggregates, and desalting on Sephadex G-25, the protein specific activity was 17 200 cpm A_{278}^{-1} (50 μL)$^{-1}$. Since the protein concentration was greater than 25 μM, tubulin-bound guanine nucleotide is not lost during this process (Zeeberg, 1980). Subsequent fractionation on a Sephadex G-25/Sepharose 6B column (Figure 5) showed that the specific activity of the rings was 13 900 cpm A_{278}^{-1} (50 μL)$^{-1}$ (fraction 11). The lower specific activity of the ring (13 900, fraction 11), as compared to the unfractionated protein (17 200), results from the fact that the ring is enriched in nontubulin proteins (Figure 2) which do not bind guanine nucleotide; rings do not rapidly equilibrate with added nucleotide (Caplow & Zeeberg, 1980) so that nucleotide dissociation from the rings during chromatography is insignificant.

Rings contain 1 mol of radioactive guanine nucleotide per 110 000 g of tubulin after an assembly reaction with radioactive GTP (Caplow & Zeeberg, 1980). Since ~68% of the protein in the rings is tubulin (Figure 2), the specific activity of the tubulin in the rings is approximately 13 900/0.68 = 20 400; this value is taken to be equal to the specific activity for dimeric tubulin also. The observed specific activity for the dimeric tubulin in the peak of the Sepharose 6B elution profile (fraction 24 in Figure 5) is 14 200. This is equal to (14 200/20 400) × 100% = 70%, so that the chromatography had resulted in the loss of 30% of the radioactive guanine nucleotide that had been bound to the dimer prior to chromatography. Therefore, in order to correct for the loss of nucleotide from dimer during chromatography, the experimentally observed specific activity of the eluted dimer must be divided by 0.7. Further evidence for the loss of radioactive nucleotide from the peak of the dimeric protein is the progressively higher specific activity in the trailing edge of the dimer peak; this value is 32 000 for fraction 37 in Figure 5. Depletion of guanine nucleotide from the tubulin dimer during gel chromatography has been previously observed and quantitatively analyzed (Zeeberg, 1980).

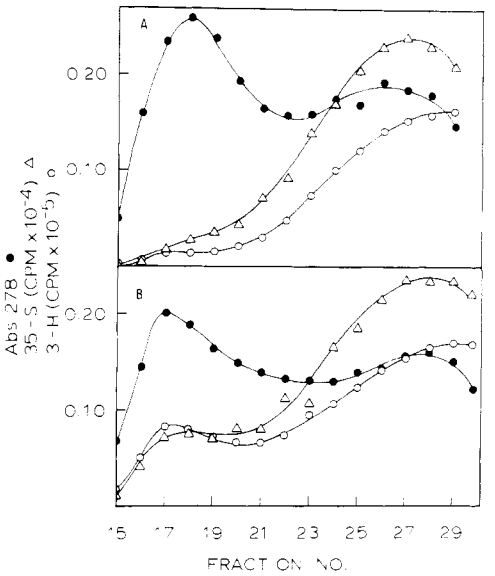

FIGURE 6: Incorporation of [^{35}S]tubulin dimer and radioactive guanine nucleotide into rings under nonpolymerizing conditions. Reaction A: chromatography of a zero-time reaction. Reaction B: chromatography after a 75-min incubation at 0 °C of nonradioactive tubulin, [^{35}S]tubulin dimer, and [^{3}H]GDP. The chromatography was as described for Figure 3 and the protein, ^{35}S, and ^{3}H profiles are shown.

Pathway for Nucleotide Incorporation into Rings under Nonpolymerizing Conditions. ^{35}S-Labeled dimer was used to determine whether the incorporation of added nucleotide into rings results from incorporation of dimer-bound radioactive nucleotide or direct incorporation of radioactive nucleotide. The analysis is predicated upon a comparison of the rates of incorporation of radioactive dimer and nucleotide into rings, which must be performed before either reaction is near completion, so that the results reflect the kinetic rather than the equilibrium behavior. We have found that at 0 °C, a 75-min incubation time is sufficient to permit significant incorporation without the reactions' being complete.

There is a time-dependent incorporation of ^{35}S-labeled dimer and radioactive nucleotide into rings, since the amount of radioactivity in rings is greater after a 75-min incubation (Figure 6A) than in a control reaction (Figure 6B) in which there was an incubation of ~10 min, corresponding to the time required for chromatographic resolution of the ring and dimer. In the reaction which had been incubated for 75 min, the average specific activity of the peak ring fractions (Figure 6A) is 3820 and 42 400 cpm/A for ^{35}S and ^{3}H, respectively. The specific activity of the dimer peak (fractions 25–27, Figure 6A) is 13 760 and 90 970 cpm/A for ^{35}S and ^{3}H, respectively. As described above, the dimeric protein has lost ~30% of the initially bound guanine nucleotide during chromatography, so that the specific activity of nucleotide in the dimer is equal to 90 970/0.7 = 130 000; no such correction is required for the [^{35}S]tubulin dimer specific activity. Therefore, the

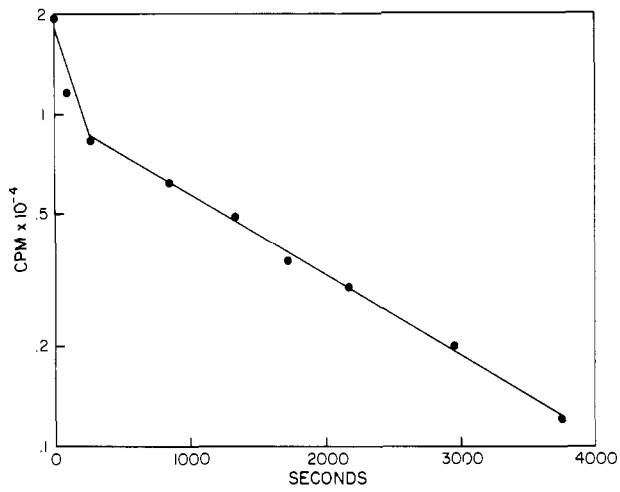

FIGURE 7: Semilogarithmic plot for the loss of [^{3}H]GDP from 20 μM tubulin in the presence of 109 μM GDP. An infinite-time value, equal to 10000 cpm, was subtracted from each time point. The theoretical infinite-time value is composed of two components: (a) there is a background equal to 0.9% of the unbound radioactive nucleotide which is eluted in column centrifugation (this is equal to 572 cpm); (b) 16% of the radioactive GDP remains bound to the tubulin E site since the added GDP only effects a 109/129 = 84% dilution of the specific activity of the initially bound radioactive nucleotide. The small difference between the theoretical infinite-time value (equal to 4900) and the observed value (10000 cpm) cannot be accounted for. This constitutes only 1/6 (4900/29400) of the total initially bound radioactive nucleotide. Dissociation of all of the radioactive nucleotide is seen on longer incubation with 7.0 mM GDP.

[^{3}H]/[^{35}S] ratio of specific activities in the dimer peak is 130000/13760 = 9.4, and the ratio in the ring peak is 42400/3820 = 11.1. The near equivalence of these ratios establishes that the rates for incorporation of nucleotide and dimeric tubulin into rings under nonpolymerizing conditions are nearly equal.

Kinetics for Nucleotide Binding and Dissociation with Porcine Tubulin. Radioactive nucleotide, which has been incorporated into microtubules as the result of polymerization in the presence of [^{3}H]GDP and ATP, dissociates at 0 °C from depolymerized tubulin in the presence of excess GDP, in a biphasic manner (Figure 7). The protein initially has associated 29400 cpm; after addition of GDP, this rapidly falls to 21700 cpm (Figure 7). In the slower phase, the bound radioactivity decreases to 10000 cpm. The loss of radioactivity in the slow phase of the reaction is first order (Figure 7) with a half-life equal to 1250 s ($k = 5.54 \times 10^{-4}$ s^{-1}). In the absence of excess GDP, the amount of bound tritiated nucleotide remains constant for at least 7200 s at 0 °C.

To determine whether it is the ring or dimer which loses radioactive nucleotide in the slow phase, protein containing [^{3}H]GDP was chromatographed on Sepharose 6B. The kinetics for radioactive nucleotide dissociation from the isolated tubulin ring fraction corresponded to the slow phase: at 23 °C (instead of 0 °C as in the study described above), the half-time is 700 s for tubulin rings in the presence of excess GDP. Radioactive nucleotide dissociation from the isolated tubulin dimer fraction in the presence of excess GDP was complete in less than 60 s.

The reversibility of nucleotide binding to rings in a slow reaction was demonstrated by the fact that the binding of [^{3}H]GDP (which is not displaced by excess GDP in 200 s) was time dependent, and the kinetics for binding are in agreement with the rate of ^{3}H-labeled nucleotide dissociation (and replacement with nonradioactive nucleotide) in the slow phase of the processes shown in Figure 7. This slow reaction was

Table I: Incorporation of Radioactive GDP into Tubulin Rings[a]

reaction	added GDP (μM)	incubn time (s)	protein-bound nucleotide sp act. (cpm/nmol)[b] ring	tubulin dimer
A	0	300	1450	14000
B	0	4500	16070	27270
C	100	150	872	6000
D	100	4500	4275	9000

[a] Tubulin (20 μM) was incubated at 0 °C with a trace amount of [^{3}H]GDP (1.76×10^5–3.43×10^6 cpm per 500-μL reaction) with either 0 or 100 μM nonradioactive GDP. A 500-μL aliquot was chromatographed on a 1 × 12 cm Sepharose 6B column at 4 °C, and the absorbance and radioactivity were measured in the eluted fractions. Ring protein is eluted in ~950 s, and the tubulin dimer peak is eluted in ~1700 s after starting the chromatography. The chromatographic results for reaction A are shown in Figure 8. [b] The variations in the specific activity of the proteins in different reactions result from the fact that a different amount of radioactive GDP was used in the reactions.

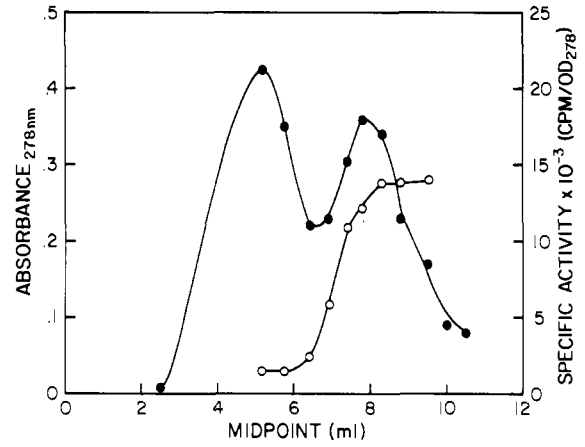

FIGURE 8: Sepharose 6B chromatography of reaction A in Table I. Column fractions were weighed and the midpoint weight between successive fractions is plotted. The absorbance (●) and the specific radioactivity (○) are shown.

shown to correspond to [^{3}H]GDP incorporation into rings, by Sepharose 6B chromatographic resolution of rings and tubulin dimer after incubation with [^{3}H]GDP (Table I). In reactions with high specific activity [^{3}H]GDP (A) or low specific activity [^{3}H]GDP (C), the rings have much lower specific activity nucleotide relative to that of dimer when chromatography follows a brief incubation. Results from a typical experiment are shown in Figure 8. On prolonged incubation the specific activity of the radioactive nucleotide in the rings approaches that of the dimer (reactions B and D, Table I).

Disassembly of Porcine Microtubules in the Presence of Tubulin–[^{3}H]GDP. When microtubules were depolymerized in the presence of the tubulin dimer–[^{3}H]GDP complex, the resulting solution had an absorbance at 278 nm equal to 2.22 and a specific activity equal to 159000 cpm (100 μL)$^{-1}$ A_{278}^{-1}. The depolymerization products were chromatographed on Sepharose 6B, the ring fractions had ~16% of the total radioactivity and a specific activity of 52000 cpm (100 μL)$^{-1}$ A_{278}^{-1}, the dimer fractions had ~50% of the total radioactivity and a specific activity of 160000 cpm (100 μL)$^{-1}$ A_{278}^{-1}, and ~34% of the total radioactivity was in the small molecule region. In a control experiment, [^{3}H]GDP and unpolymerized protein were added after depolymerization, and the ring fractions had ~10.5% of the total radioactivity and a specific activity of 31900 cpm (100 μL)$^{-1}$ A_{278}^{-1}, the dimer fraction had ~54% of the total radioactivity and a specific activity of 164000 cpm (100 μL)$^{-1}$ A_{278}^{-1}, and ~36% of the total ra-

dioactivity was in the small molecule region. The value for the dimeric protein must be increased by a factor of ~2 to reflect its specific activity before chromatography since there is significant loss of dimer-bound [^{3}H]GDP during column chromatography of the dilute tubulin solutions, as indicated by the nucleotide in the small molecule region; loss of tubulin-bound nucleotide during gel chromatography has been previously observed and quantitatively accounted for (Zeeberg, 1980). The depolymerization-independent incorporation of radioactive nucleotide into rings occurs during the 5-min incubation and during the initial stage of the chromatography; the result for the control experiment is in accord with that for a similar experiment with more concentrated tubulin (Table I, reaction A).

The depolymerization-dependent incorporation of [^{3}H]GDP into rings is equal to 52 000–41 000 = 11 000 cpm (100 μL)$^{-1}$ A_{278}^{-1}. If all of the added [^{3}H]GDP had entered the rings, then the ring-specific activity theoretically would have been 318 000 cpm (100 μL)$^{-1}$ A_{278}^{-1}, since the rings constitute approximately half of the protein (as estimated from the protein elution profile in numerous Sepharose 6B chromatography experiments) and the specific activity of the unfractionated protein was 159 000 cpm (100 μL)$^{-1}$ A_{278}^{-1}. Therefore, the depolymerization-dependent incorporation of [^{3}H]GDP into rings is equal to (11 000/318 000) × 100% = 3.5% of that possible if all of the radioactive nucleotide had been incorporated. Similar results were obtained in three experiments and the average depolymerization-dependent incorporation of [^{3}H]GDP into rings is 4.1% of that possible if all of the radioactive nucleotide had been incorporated.

Disassembly of Porcine Microtubules Containing [^{3}H]GDP in the Presence of GDP. Microtubules containing ~1 mol of [^{3}H]GDP/110 000 g of tubulin were depolymerized at 0 °C under three sets of conditions: (a) depolymerization in reassembly buffer was immediately followed by chromatography on Sepharose 6B; (b) after depolymerization in reassembly buffer, excess GDP was added and the mixture was chromatographed on Sepharose 6B; (c) after depolymerization in reassembly buffer containing excess GDP, the mixture was chromatographed on Sepharose 6B. The chromatographic separation of these reaction mixtures was equivalent to that described above for the reactions in which disassembly occurred in the presence of dimer containing [^{3}H]GDP. The specific activities (cpm (200 μL)$^{-1}$ A_{278}^{-1}) for ring and tubulin dimer were as follows: (reaction a) 81 000 and 126 000 (the lower specific activity for rings results from the fact that the ring fraction is enriched in microtubule-associated proteins); (reaction b) 63 000 and 42 500; (reaction c) 47 900 and 40 800. The decrease in the specific activity in the ring fraction in reaction b results from exchange of [^{3}H]GDP bound to the rings with the unbound low specific activity GDP during the column chromatography (it requires a few minutes for the rings to be separated from the small molecules). The specific activity for the rings in reaction c is similarly influenced; in addition, it is calculated that there is a [(63 000 − 47 000)/81 000] × 100 = 18.6% depolymerization-dependent decrease in the ring specific activity. Under these same conditions there is a [(126 000 − 40 800)/126 000] × 100 = 67% decrease in the dimer specific activity. In a duplicate experiment there was a 20% depolymerization-dependent decrease in the ring specific activity under conditions where the tubulin dimer specific activity decreased by 65% by excess GDP added simultaneously to microtubule disassembly.

Disassembly of Porcine Microtubules in the Presence of Porcine [^{3}H]Ethyltubulin and of Rat Microtubules in the Presence of Rat [^{35}S]Tubulin. There is little depolymerization-dependent incorporation of radioactive tubulin dimer into rings when microtubule pellets are depolymerized in the presence of [^{3}H]ethyltubulin (Figure 9) or [^{35}S]tubulin. With [^{3}H]ethyltubulin, the ratio of specific activities in the ring and dimer was 0.15, as compared to a ratio equal to 0.20 in the control; the values are identical, within experimental error. With rat [^{35}S]tubulin, the ratio of the specific activities of the ring and dimeric tubulin was 0.10 when the radioactive dimer was added simultaneously to microtubule disassembly, and 0.087 when the radioactive dimer was added after disassembly. In a duplicate experiment with rat tubulin, the comparable values were 0.089 and 0.10. These results indicate that there is no significant equilibration of tubulin dimer with tubulin in rings during rat or porcine brain microtubule disassembly.

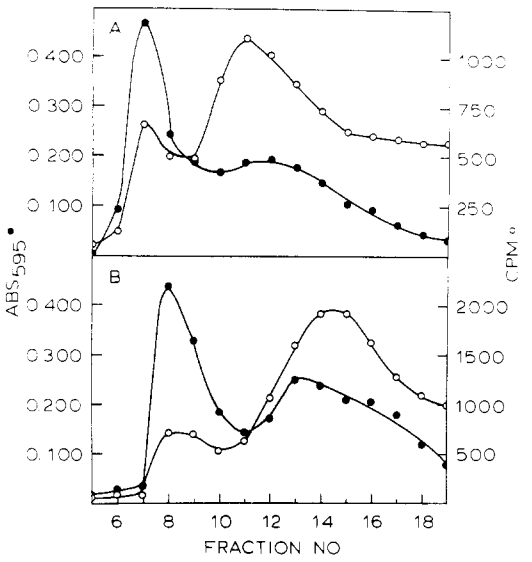

FIGURE 9: Depolymerization-dependent incorporation of porcine [^{3}H]ethyltubulin dimer into rings. Sepharose 6B chromatography of ring and dimeric tubulin which result from depolymerization of microtubules in the absence (A) or presence (B) of [^{3}H]ethyltubulin dimer.

Discussion

We have determined that porcine and rat tubulin dimers equilibrate with rings in a cycle of microtubule assembly and disassembly and that under nonpolymerizing conditions there is a pathway for nucleotide incorporation into rings which involves dimer-bound nucleotide. This has been accomplished by using two different native radioactive tubulin dimers: [^{35}S]tubulin dimer (prepared by in vivo labeling of rat brain) and porcine [^{3}H]ethyltubulin (Zeeberg et al., 1980).

There are two prior observations which suggested the approach leading to these conclusions. First, although rings do not readily bind added guanine nucleotide, the rings obtained from depolymerization of microtubules which had been formed in the presence of radioactive GTP contain 1 mol of radioactive guanine nucleotide/mol of tubulin (Caplow & Zeeberg, 1980). Second, the tubulin in rings is incorporated into microtubules, since the specific activity of the tubulin in microtubules is equal to that of the unpolymerized tubulin, when radioactive tubulin dimer is copolymerized with a mixture of nonradioactive dimeric and ring tubulin (Zeeberg et al., 1980); if the tubulin in rings had not polymerized, then the specific activity of the unpolymerized tubulin would have been very low. This led us to ask if there is a route for incorporation of dimeric tubulin into rings in a cycle of microtubule assembly and disassembly.

The existence of this route would result in the incorporation of dimer-bound radioactive nucleotide into rings. Also, we asked whether there is a path for incorporation of added radioactive nucleotide into rings under nonpolymerizing conditions and whether this path involves incorporation of dimer-bound radioactive nucleotide into rings or direct incorporation of nucleotide into rings.

When radioactive chick tubulin dimer is present during a cycle of microtubule assembly and disassembly, there is no incorporation of radioactivity into the rings which result from the disassembly step (Weingarten et al., 1975). The absence of equilibration throughout the entire cycle allows the conclusion to be drawn that there is no equilibration of tubulin rings with tubulin dimer during either assembly or disassembly; chick tubulin dimer remains distinct from tubulin in rings during microtubule assembly, in the microtubule and during disassembly. In contrast, when radioactive rat or porcine tubulin dimer is present during a cycle of assembly and disassembly with the corresponding nonradioactive tubulin dimer and rings, the rings subsequently obtained upon the disassembly step contain radioactivity. The equilibration seen during an assembly–disassembly cycle with rat and porcine tubulin indicates that tubulin in dimer becomes equivalent to tubulin initially in rings during assembly, during disassembly, or during both assembly and disassembly. For determination of the equilibration step, a complementary approach was required in order to study the properties of the discrete steps in an assembly and disassembly cycle. It is shown in these studies that there is no significant depolymerization-dependent equilibration of rat or porcine tubulin dimer and rings.

Incorporation of [^{3}H]GDP into Porcine Tubulin Rings during Disassembly. These studies depend on the fact that tubulin rings equilibrate with added guanine nucleotide very slowly, compared to the rate of equilibration of dimer with nucleotide: 1 mol of radioactive nucleotide is incorporated into microtubules when assembly is carried out with [^{3}H]GTP or with [^{3}H]GDP and ATP. After the microtubules are depolymerized in the cold, the resultant protein contains 1 mol of radioactive nucleotide/110 000 g of tubulin. The rings constitute ~45% of the depolymerized proteins, and both the ring and dimer contain ~1 mol of radioactive nucleotide/110 000 g of tubulin (Caplow & Zeeberg, 1980). The kinetics for nucleotide dissociation were determined from studies of the loss of bound radioactive nucleotide from the protein in the presence of an excess of added unlabelled nucleotide (Figure 7). The reversibility of this process was demonstrated by the incorporation of radioactive nucleotide into tubulin dimer and rings under conditions identical with those for studying radioactive nucleotide loss. Our results show that (a) the E site of the tubulin dimer equilibrates with added radioactive guanine nucleotide in ~60 s (Figure 7), (b) radioactive nucleotide in rings dissociates very slowly in the presence of added nonradioactive GDP (half-life of 1250 s with 100 μM GDP) (Figure 7), and (c) added radioactive nucleotide which enters rings in a slow reaction is not lost during chromatography on Sepharose 6B (Table I).

When microtubules are depolymerized in the presence of dimer-bound [^{3}H]GDP, there is only a 4.1% depolymerization-dependent incorporation of [^{3}H]GDP into rings; if dimer had equilibrated with rings during disassembly, then the dimer-bound [^{3}H]GDP would have been incorporated into rings. Therefore, there is insignificant equilibration of tubulin dimer with ring during microtubule disassembly. If such an equilibration were significant, then close to 100% of the added radioactivity would theoretically be incorporated into rings: the continuously supplied tubule-derived dimers would very efficiently extract the initially present trace amount of the [^{3}H]GDP–dimer complex en route to ring. A corollary is that the observed depolymerization-dependent incorporation of radioactivity into rings actually represents much less than a 4.1% contribution of a pathway involving tubulin dimer as an intermediate for ring formation during microtubule disassembly.

Weisenberg et al. (1976) have studied the incorporation of radioactive guanine nucleotide (added simultaneously to disassembly) into rings during the disassembly of bovine brain microtubules. However, the radioactive guanine nucleotide in the reaction was present at a different specific activity during disassembly and after disassembly, as a result of the addition of an excess of nonradioactive nucleotide in a chase. The ensuing calculation[1] of the stoichiometry for radioactive nucleotide incorporation is complicated by the variation in the specific activity. The experimental data and the treatment of them to derive the calculated values reported were not presented, which makes evaluation of this work difficult.

Disassembly of Porcine Microtubules Containing [^{3}H]GDP. When microtubules containing [^{3}H]GDP are disassembled in

[1] Results were reported which were taken as support for a mechanism for microtubule disassembly in which there is a facilitated pathway for incorporation of added guanine nucleotide into rings (Weisenberg et al., 1976). This pathway was presumed to involve the existence of a transiently available tubulin ring E site during depolymerization. It was stated that 0.64 mol of radioactive guanine nucleotide is bound to tubulin when depolymerization occurs in the presence of radioactive guanine nucleotide and 0.22 mol of radioactive nucleotide is bound when the radioactive nucleotide is added after depolymerization. That is, there was presumed to be 2.9 times (0.65/0.22) as much guanine nucleotide bound when the radioactive nucleotide was present during, as compared to after, the disassembly process. The basis of this calculated ratio for the relative number of moles of nucleotide bound equal to 2.9 is the observation that 2.9 times as much radioactivity was found associated with the protein. However, it is not correct to equate the observed ratio of counts bound (2.9) with the ratio of moles of nucleotide bound. As is calculated below, the reported values of 0.64 and 0.22 mol require a 22-fold difference in the observed radioactivities. Depolymerization was carried out in the presence of 0.1 mM radioactive guanine nucleotide, and the nucleotide concentration was adjusted to 1.1 mM after depolymerization. Under these conditions, the protein can bind radioactive nucleotide at two different specific activities, which differ by 11-fold. Since the nucleotide bound at the postulated transient E site during depolymerization does not equilibrate with nucleotide added after depolymerization, the following analysis applies:

$$\text{cpm bound} = (\text{nmol } 1)(\text{SA } 1) + (\text{nmol } 11)(\text{SA } 11) \quad (1)$$

SA 1 = lower specific activity, SA 11 = higher specific activity, nmol 1 = number of nanomoles bound per nanomole of tubulin at SA 1, nmol 11 = number of nanomoles bound per nanomole of tubulin at SA 11. In the experiment where the radioactive nucleotide is added at SA 1 after depolymerization is completed, eq 1 becomes

$$\text{cpm bound} = (\text{nmol } 1)(\text{SA } 1) \quad (2)$$

In the experiment where the radioactive nucleotide is added at SA 11 at the start of depolymerization and then the specific activity is adjusted to SA 1 at the completion of depolymerization, eq 1 becomes

$$\text{cpm bound} = (\text{nmol } 1)(\text{SA } 1) + (\text{nmol } 11)(11)(\text{SA } 1) \quad (3)$$

We now relate this analysis to the results reported previously. With nmol 1 = 0.22 and nmol 11 = 0.64 − 0.22 = 0.42, eq 2 becomes cpm bound = (0.22)(SA 1) and eq 3 becomes cpm bound = (SA 1)[0.22 + (11)(0.42)] = 4.84 SA 1. Therefore, the ratio of the cpm bound in the two experiments is expected to be 22. The observation that there is 2.9 times as much radioactive nucleotide associated with the protein in the reaction in which the 0.1 mM radioactive nucleotide was present during microtubule disassembly, as compared to the reaction in which radioactive nucleotide was added after microtubule disassembly, corresponds to a very low stoichiometry for nucleotide binding to a transiently available E site during microtubule disassembly. By use of eq 2 and 3, nmol 11 is calculated as equal to 0.037 mol, not 0.42 as incorrectly stated.

the presence of 100 μM added nonradioactive GDP, there is a 20% depolymerization-dependent decrease in the specific activity of the resultant rings. If rings had equilibrated with dimer during disassembly, then excess GDP would have been able to displace [^{3}H]GDP in the rings, and the resultant rings would have undergone approximately an 80% depolymerization-dependent decrease in specific activity. The success with which GDP would displace dimer E-site [^{3}H]GDP depends on the validity of the assumption that the lifetime of the dimer is sufficiently long to react with the GDP, so that this approach is subject to an ambiguity that is absent in the experiment described above. Also, the presence of 100 μM GDP causes the depolymerization-independent reaction to be higher than it was in the experiment described above without added GDP (unpublished results), so that there is a greater uncertainty in the calculations; therefore, this approach is not of the same degree of reliability as the above. Nonetheless, these results provide evidence for a lack of significant depolymerization-dependent equilibration of ring and dimer.

Disassembly of Microtubules in the Presence of Radioactive Tubulin Dimer. As described under Results, when porcine microtubules are disassembled in the presence of porcine [^{3}H]ethyltubulin dimer (Figure 9), or rat microtubules are disassembled in the presence of rat [^{35}S]tubulin dimer, there is no significant depolymerization-dependent incorporation of radioactivity into rings. These results provide evidence that the tubulin dimer is not an intermediate in disassembly of porcine and rat brain microtubules, since then the continuously supplied tubule-derived dimers would very efficiently extract the initially present [^{3}H]ethyltubulin or [^{35}S]tubulin dimers en route to ring. This would have resulted in very high depolymerization-dependent incorporation of radioactivity into rings.

Conclusion

With porcine and rat tubulin, the assembly and disassembly of microtubules are not mirror image processes: tubulin dimer equilibrates with the tubulin in rings in the microtubule assembly reaction but not in the disassembly reaction. The lack of equilibration of tubulin in dimer and rings during disassembly rules out a pathway for ring formation in which tubulin dimer is an intermediate. Intact rings are formed directly by a cleavage from the microtubule. Alternate mechanisms involving currently unknown tubulin species as intermediates for ring formation during microtubule disassembly cannot be ruled out.

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