Mechanism for Nucleotide Incorporation into Steady-State Microtubules†

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ABSTRACT: We have extended our previous theoretical analysis of the kinetics for radioactive GTP incorporation into steady-state microtubules [Zeeberg, B., Reid, R., & Caplow, M. (1980) *J. Biol. Chem. 255*, 9891–9899] to include the effects of a kinetic barrier for equilibration of labeled GTP with the tubulin E site. This binding has been found to be relatively slow; the half-time for GTP dissociation is approximately 25 s ($k = 0.028$ s^{-1}). The slow binding of radioactive GTP apparently accounts for the following observations: (a) more radioactive nucleotide is incorporated into steady-state microtubules in the first 20 s when tubulin–[^{3}H]GTP is used in a pulse than when [^{3}H]GTP is used; (b) when steady-state microtubules are pulsed for 20 s with tubulin–[^{3}H]GTP and then chased with excess nonradioactive GTP, radioactive nucleotide incorporation is not stopped immediately. Quantitative analysis of these results indicates that our steady-state microtubules do not contain significant amounts (>1%) of GDP or GTP which can exchange with added GTP. The principal route for labeled nucleotide incorporation appears to be from tubulin–[^{3}H]GTP subunit uptake, by diffusional and treadmilling processes.

The incorporation of radioactive guanine nucleotide into microtubules which are at steady state is believed to result from the treadmilling and diffusional uptake of tubulin–[^{3}H]GTP subunits. The treadmilling component of this process reflects the fact that as a result of GTP hydrolysis, the critical concentrations may be different at the two ends of a microtubule; this allows an excess of tubulin subunits to be added at one end, while an equivalent excess of subunits is lost at the opposite end. Diffusional subunit uptake results from the fact that while one fraction of the microtubule population is undergoing an excess of subunit additions, a different fraction will be undergoing an excess of subunit losses. Although it has been demonstrated that tubulin-bound GDP in the microtubule does not exchange with guanine nucleotide in solution (Jacobs et al., 1974; Kobayashi, 1975; Weisenberg et al., 1976), recent results from pulse–chase studies have been taken to indicate that GTP within subunits in the microtubule is able to freely exchange with nucleotide in solution (Carlier & Pantaloni, 1981). We wish to determine the significance of such exchange into steady-state microtubules, relative to the diffusional and treadmilling paths for label incorporation. Such an analysis has been found to be feasible, as a result of the fact that GTP in the E site of tubulin subunits dissociates relatively slowly. This has allowed a comparison of the reactivity of tubulin–[^{3}H]GTP and [^{3}H]GTP for label uptake in very brief pulse labeling experiments. Also, the slow dissociation of GTP has allowed analysis of tubulin–[^{3}H]GTP pulse/GTP chase experiments, in which the slow dissociation can influence the kinetics for the chase phase of the reaction.

We have previously described a quantitative analysis of labeled nucleotide incorporation into steady-state microtubules in which the early and later phases of the reaction were considered (Zeeberg et al., 1980). We now also consider the initial phase of the reaction, where the exchange of radioactive GTP into the tubulin subunit E site influences the rate; this analysis is provided in the supplementary material (see paragraph at end of paper regarding supplementary material).

Experimental Procedures

Materials. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (850 IU/mL) and yeast 3-phosphoglycerate kinase (6533 IU/mL) were obtained from Calbiochem. Ammonium sulfate was removed from the dehydrogenase by a column centrifugation procedure (Penefsky, 1977). In the experiment in which the phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase concentrations were 150 and 29 IU/mL, respectively, the enzymes were dialyzed at 4 °C for 1 h by using a Millipore VSW PO1300 membrane (Marusyk & Sergeant, 1980). Nucleotides and D(−)-3-phosphoglyceric acid were obtained from Sigma, and radioactive guanine nucleotides were purchased from New England Nuclear. Microtubular protein was prepared from pig brain, as described previously (Zeeberg, et al., 1980b). Microtubule-associated protein (MAP)[1]-free tubulin was obtained by chromatography of the microtubular protein on phosphocellulose (Witman et al., 1976).

Methods. Tubulin–[^{3}H]GTP used in the study of the E-site dissociation rate was prepared by a 60-min incubation at 0 °C of approximately 25 μM microtubular protein, 0.2 mM ATP, and a trace amount of [^{3}H]GDP. The transphosphorylase in microtubular protein (Jacobs, et al., 1974) is able to convert approximaately 70% of the E-site GDP to GTP under these conditions [see Figure 6 in Jameson & Caplow (1980)], and the low temperature prevents microtubule assembly. These precautions are not required with MAP-free tubulin, since this substance does not readily assemble when the E site contains GTP. In this case, MAP-free tubulin (approximately 30 μM) was simply incubated for 15 min at 0 °C with 30 μM [^{3}H]GTP. With both protein preparations, the tubulin–[^{3}H]GTP was isolated free of unbound nucleotides by a column centrifugation procedure (Penefsky, 1977) (at 4 °C) and then diluted with an equal volume of freshly prepared enzyme–cofactor solution containing 0.4 mM NADH, 12 mM 3-phosphoglycerate, and 10.4 units/mL glyceraldehyde-3-phosphate dehydrogenase; phosphoglycerate kinase was present at varying concentrations. Aliquots of the reaction mixture were quenched into 4% perchloric acid, and the nucleotides which were released were purified by thin-layer chromatography on PEI–cellulose (Brinkmann Polygram Cell 300 PEI) (Zeeberg & Caplow, 1978).

The flux of radioactive nucleotide into steady-state microtubules was determined as described previously (Caplow et al., 1982) with the following modifications. Aliquots (50 μL)

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[1] Abbreviations: MAP, microtubule-associated protein(s); PGK, phosphoglycerate kinase; PEI, poly(ethylenimine).

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of the reaction mixture were quenched into 7.0 mL of 32.3% glycerol in reassembly buffer [0.1 M 2-(*N*-morpholino)-ethanesulfonic acid, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 1 mM $MgCl_2$, pH 6.80] and then centrifuged for 20 min at 50 000 rpm at 37 °C in a Beckman Ti 50 rotor. The background was determined by quenching 50 μL of a solution of nonradioactive microtubules into 7 mL of glycerol–reassembly buffer containing an amount of [^{3}H]GTP which was identical with that used in the flux studies. In this case, the pelletted microtubules contained entrapped free radioactive nucleotide, as well as labeled nucleotide which became bound to the microtubule in the quenched reaction mixture. This background radioactivity was ordinarily about 225 cpm in reactions in which the microtubules which had been assembled in the presence of [^{3}H]GTP contained 40 000 cpm. Thus, in reactions in which uptake of label proceeds to as little as 3% of the maximum level (i.e., 1200 cpm), the background correction represented only about 20% of the measured radioactivity.

The very low background (225 out of 40 000 cpm) observed in these studies indicates that there was no nucleotide exchange into the microtubules in the glycerol quench solution. The possibility that the microtubules might disassemble in the glycerol quench solution was ruled out by the fact that when radioactive microtubules were diluted into the glycerol quench solution and pelletted over the course of several hours, the yield of radioactive microtubules did not change. Also, there was no new microtubule assembly in the glycerol quench solution, since only background levels of radioactivity were observed when 50 μL of an ice-cold solution of unpolymerized microtubular protein (30 μM), acetyl phosphate, acetate kinase, and 30 μM GTP was mixed with 7 mL of the glycerol quench solution (37 °C) containing a trace amount of [^{3}H]GTP.

Labeled guanine nucleotide uptake into steady-state microtubules was studied with both tubulin–[^{3}H]GTP and [^{3}H]GTP. The former material was generated by a 20-min incubation at 0 °C of 30 μM microtubular protein with a trace amount of [^{3}H]GTP, 15 mM acetyl phosphate, and acetate kinase (Sigma A2384, 0.075 unit/mL of reaction). This mixture was isolated free of unbound nucleotide by column centrifugation (Penefsky, 1977) and then diluted to approximately 5 μM. The protein-bound nucleotide, analyzed as described previously (Zeeberg & Caplow, 1978), was 75–95% [^{3}H]GTP and 5–25% [^{3}H]GDP. Reactions were initiated by adding 1-μL aliquots of either tubulin–[^{3}H]GTP or high specific activity [^{3}H]GTP to 50 μL of steady-state microtubules at 37 °C. This was immediately followed by 1 μL of either reassembly buffer or tubulin–GTP to make the reaction mixtures identical. To determine the amount of radioactive nucleotide incorporation which corresponds to 100% labeling of the microtubules, the radioactive solution ([^{3}H]GTP or tubulin–[^{3}H]GTP) was added to 50 μL of unpolymerized tubulin, and the mixture was assembled to steady state.

Uptake of label in the pulse phase of pulse–chase experiments utilized either tubulin–[^{3}H]GTP or [^{3}H]GTP. The former material was generated by adding a trace amount of [^{3}H]GTP to the supernatant obtained by centrifugation of steady-state microtubules, which were formed by using acetyl phosphate and acetate kinase, without excess added GTP. This material was incubated for 30 min on ice prior to its use. When the pulse was done with [^{3}H]GTP, a 1-μL aliquot of high specific activity [^{3}H]GTP was added to the microtubules.

Results

Rate for GTP Dissociation from the Tubulin E Site. Our quantitative analysis of pulse and pulse–chase studies (see below) requires a determination of the rate constant for GTP dissociation from the tubulin E site. The rate for GTP dissociation at 37 °C has been measured by following the conversion of tubulin–[^{3}H]GTP to [^{3}H]GDP in the presence of phosphoglycerate kinase and its substrates. The advantage of studying the rate by an assay which utilizes isotopes is that it allows use of low concentrations of tubulin–GTP. As a result, at relatively low concentrations of phosphoglycerate kinase, the rate for hydrolysis of GTP (i.e., k_2[PGK][GTP]) will be faster than that for rebinding of GTP by tubulin (k_{-1}[GTP][tubulin] in eq 1) so that k_1 can be determined (Brylawski & Caplow, 1983).

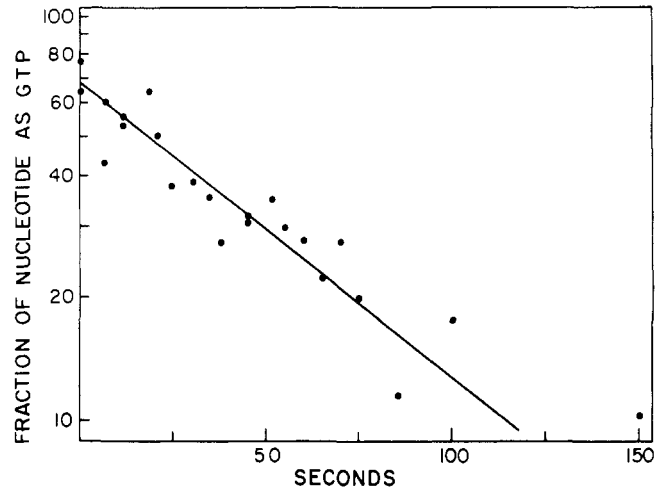

FIGURE 1: Rate of dissociation of GTP from the tubulin E site. In the reaction shown here, microtubular protein containing [^{3}H]GTP was reacted at 37 °C with 160 IU/mL PGK.

$$\text{tubulin–GTP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{tubulin} + \text{GTP} \xrightarrow[\text{PGK}]{k_2} \text{GDP} \quad (1)$$

In contrast, a spectrophotometric assay in which the release of GTP is coupled to NADH oxidation (Jacobs et al., 1974) requires high concentrations of tubulin–GTP; therefore, extremely high concentrations of PGK are required to make k_2[PGK] > k_{-1}[tubulin]; this relationship is required for the k_1 rate to be rate limiting so that the k_1 rate constant is correctly determined (Brylawski & Caplow, 1983).

Conversion of E-site-bound [^{3}H]GTP to [^{3}H]GDP has a half-time of approximately 60 and 30 s, respectively, when assayed with 16 or 160 IU/mL PGK. The time course for a typical reaction with the higher concentration of PGK is shown in Figure 1; in this experiment, the observed half-time is 31 s (k_1 = 0.022 s^{-1}); in a duplicate experiment, the measured rate constant was 0.028 s^{-1}. The rate constant determined under identical conditions with MAP-free tubulin was 0.034 s^{-1}. Since the rates are approximately the same with microtubular protein and with MAP-free tubulin, which does not contain oligomeric forms of tubulin (Witman et al., 1976), it is concluded that in our studies using microtubular protein we are measuring the rate for GTP dissociation from the tubulin dimer.

When we studied the GTP dissociation rate using the conditions previously employed in a spectrophotometric determination of the dissociation rate (1 IU of PGK/mL) (Terry & Purich, 1979), the half-time was found to be about 100 s; this is near the 132-s value previously reported. Since a faster rate is obtained with higher PGK concentrations, it is concluded that k_1 is not correctly measured with 1 IU of PGK/mL (Terry & Purich, 1979). The fact that the rate changes very little on increasing the PGK concentration from 16 to 160 IU/mL indicates that GTP which is released from the tubulin

E site is quantitatively trapped by the k_2[PGK] reactions at the highest PGK concentration. Under these conditions, the observed first-order rate constant is equal to k_1 in eq 1 [see eq 2 in Brylawski & Caplow (1983)]; on the basis of the results with the highest PGK concentrations, k_1 is equal to 0.028 ± 0.006 s^{-1}. Thus, dissociation of GTP from the E site occurs about 5 times more slowly than GDP dissociation (Brylawski & Caplow, 1983). The dissociation constant for GTP is 2.8-fold smaller than that for GDP (Zeeberg & Caplow, 1979), and the slower dissociation rate for GTP accounts for part of this difference. Results which are in general agreement with those reported here have been obtained (Engelborghs & Eccleston, 1982) by measurement of the kinetics for a change in fluorescence of (2-amino-6-mercapto-9β-ribofuranosyl)-purine 5′-triphosphate upon diplacement of GTP from the tubulin E site.

Dynamic Properties of the Microtubule Steady State. Radioactive nucleotide is rapidly incorporated into microtubules following the addition of [^{3}H]GTP to tubulin which has been assembled to steady state. This incorporation of label is not the result of an incomplete attainment of a steady state prior to addition of label, since the same amount of radioactive guanine nucleotide enters the microtubules during 100- and a 200-s incubations, when this is measured at any time between 1000 and 6000 s after the assembly is initiated (data not given). If the microtubules were not at steady state at 1000 s, then more radioactive nucleotide would have been incorporated during an incubation which is initiated at this time than at any later time. The dynamics of the microtubule steady state have been analyzed by using this procedure in pulse and pulse–chase studies.

Incorporation of [^{3}H]GTP and Tubulin–[^{3}H]GTP into Steady-State Microtubules. We had previously accounted for the initial phase for the incorporation of radioactive guanine nucleotide into steady-state microtubules as reflecting a diffusional exchange of tubulin–[^{3}H]GTP subunits for tubulin subunits at the microtubule ends (Caplow et al., 1982). We now evaluate the contributions to the rate of a reaction in which [^{3}H]GTP exchanges into tubulin subunits. In the former mechanism, the reactive species is tubulin–[^{3}H]GTP, while in the latter the reactive species is [^{3}H]GTP. The alternate mechanisms can be distinguished as follows.

If tubulin–[^{3}H]GTP is the predominant reactive species during the initial phase for label incorporation into steady-state microtubules, and if there is a significant kinetic barrier for incorporation of labeled nucleotide into the tubulin E site, then the initial rate for label incorporation into steady-state microtubules should be more rapid when tubulin–[^{3}H]GTP is used in a pulse than when the pulse is initiated with [^{3}H]GTP. The magnitude of the difference in rate of uptake of label when the pulse is done with tubulin–[^{3}H]GTP and with [^{3}H]GTP will depend upon two factors: (a) the duration of the incubation; (b) the relative magnitude of the rate constant for exchange of [^{3}H]GTP with the tubulin E-site GTP and that for addition of tubulin–GTP subunits to the microtubule. The dependence on the incubation time (i.e., factor a) is such that as this is increased, the kinetic advantage for a reaction initiated with tubulin–[^{3}H]GTP becomes progressively less significant. There are two reasons why this is the case. First, at longer incubation times, a significant fraction of the initially incorporated tubulin–[^{3}H]GTP subunits will be lost because of the diffusional nature of the process for labeled subunit uptake. A diffusional reaction is a random process in which labeled subunits are both gained *and* lost, so that the kinetic advantage for the tubulin–[^{3}H]GTP reaction is dissipated in prolonged incubations. The duration of the incubation (i.e., factor a) is also important because the fraction of the total label uptake, corresponding to label introduced before full isotopic equilibration of the tubulin E site, becomes progressively less significant as the reaction proceeds. For example, if the equilibration of the E site requires 20 s, then there is a more significant difference between the amounts of radioactivity incorporated when the reaction is initiated with tubulin–[^{3}H]GTP and with [^{3}H]GTP when this is measured after a 20-s pulse, as compared to a 200-s pulse. Factor b (see above) determines the advantage for a reaction which is initiated with tubulin–[^{3}H]GTP, since it reflects how much of a head start is provided by starting with the label bound and how much this advantage can be exploited before the E site becomes equilibrated with [^{3}H]GTP.

Table I: Percent Microtubule Labeled in a 20-s Pulse with Tubulin–[^{3}H]GTP and with [^{3}H]GTP[a]

[^{3}H]GTP pulse		tubulin–[^{3}H]GTP pulse	
% (±SD)	n[b]	% (±SD)	n[b]
2.54 (0.22)	8	11.02 (2.33)	9
3.91 (0.90)	10	14.05 (3.45)	8
3.84 (0.54)	6	11.29 (2.10)	6
2.88 (0.49)	8	22.1 (5.15)	8
1.97 (0.36)	8		
3.66 (0.15)	8	14.6 (5.17)[c]	
3.13 (0.79)[c]			

[a] 100% labeling corresponds to the amount of radioactivity incorporated when microtubules were assembled in the presence of either [^{3}H]GTP or tubulin–[^{3}H]GTP at concentrations identical with those used in the pulse experiments. 100% labeling corresponds to (40–50) × 10^3 cpm in typical reactions. [b] n indicates the number of experiments performed. [c] Mean value.

In fact, there is more radioactive nucleotide incorporated into steady-state microtubules in the first 20 s when tubulin–[^{3}H]GTP is used in a pulse than when [^{3}H]GTP is used (Table I). The average from these results shows that [^{3}H]-GTP is about 21% (i.e., 3.13/14.6) as effective as tubulin–[^{3}H]GTP for label incorporation in a 20-s pulse.[2] It should be noted that the opposite result is expected for a mechanism in which label uptake corresponds to [^{3}H]GTP exchange. This would be the case since the kinetic barrier for [^{3}H]GTP dissociation from tubulin–[^{3}H]GTP would reduce the average specific activity of the unbound [^{3}H]GTP during the incubation.

Pulse–Chase Kinetics for Label Incorporation and Loss with Steady-State Microtubules. In these experiments, the microtubules are assembled with approximately 30 μM microtubular protein, 1.0 μM added GTP,[3] and an acetyl phosphate–acetate kinase GTP-regenerating system. After a 20-s pulse, an excess of nonradioactive GTP is added, and uptake of label is followed. It was found that when a reaction which had undergone radioactive subunit flux for 20 s was made 200 μM in GTP (by addition of an aliquot of a concentrated solution of GTP dissolved in the supernatant derived by cen-

[2] We have not previously observed significant deviations from linearity in the earliest time points in studies of [^{3}H]GTP incorporation into steady-state microtubules [see Figures 4 and 6 in Caplow et al. (1982)]. The reason for this is that the effect of the [^{3}H]GTP binding step on the rate for label uptake is not easily detected, without multiple determinations.

[3] A relatively large excess of GTP (i.e., 30 μM) is not included in this reaction since we want rapid equilibration of the tubulin E site with [^{3}H]GTP [eq 4 in Brylawski & Caplow (1983) describes how and why the rate for attaining isotopic equilibrium depends upon the presence of excess nucleotide] and a high specific activity in the tubulin–[^{3}H]GTP which reacts in the pulse. Also, the chase is more effective when the unbound [^{3}H]GTP concentration is relatively low.

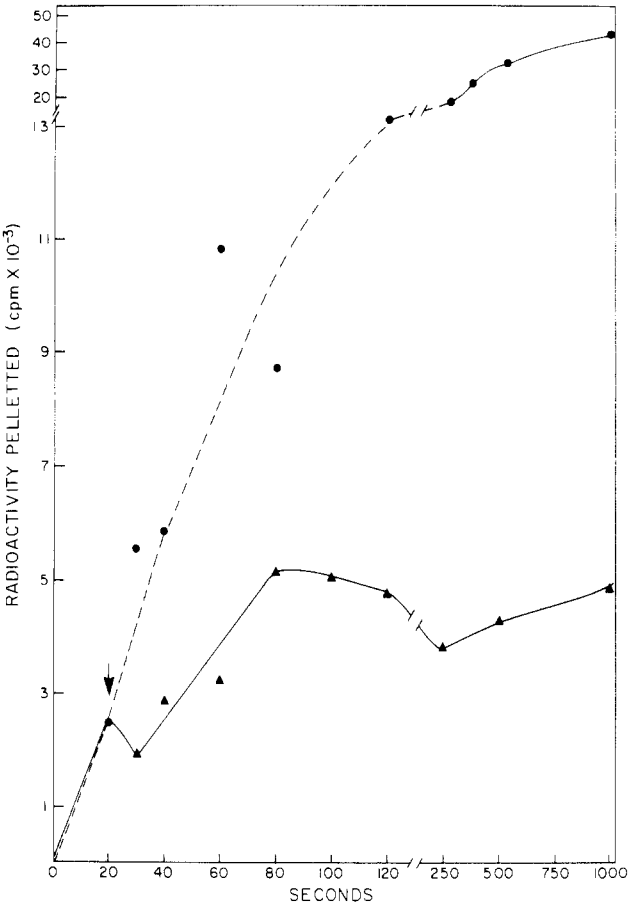

FIGURE 2: Radioactive nucleotide incorporation into steady-state microtubules. The reaction was initiated by addition of high specific activity [^{3}H]GTP (●); the reaction described by (▲) received 200 μM GTP at the time indicated by the arrow.

trifugation of steady-state microtubules), the incorporation of radioactive nucleotide did not stop immediately but continued at a relatively rapid rate for about 60 s (Figure 2). There is then a small loss of labeled nucleotide, which is complete in about 250 s, and then a progressive increase in label incorporation over the course of about 1500 s to a level equivalent to that which is seen when the microtubules are assembled in the presence of 200 μM [^{3}H]GTP. Although the added excess GTP does not immediately stop the pulse for incorporation of ^{3}H-nucleotide into the microtubules, it certainly does prevent label incorporation to the level seen in the absence of a chase (upper curve in Figure 2).

Another illustration of the ineffectiveness of a 200 μM GTP chase in preventing labeled nucleotide incorporation is seen in Figure 3, where the initial time course for uptake of label is shown for reactions which did and did not receive a 200 μM GTP chase at the 20-s point. The initial time course for label uptake is not significantly different in the presence or absence of added GTP. It should be noted that the 10-s time point following addition of excess nonradioactive GTP is lower than that which is expected from the observed rate for radioactive nucleotide uptake at earlier and later time points; this was observed in more than 20 experiments. This is also seen in the 10-s time point in the experiment shown in Figure 2. We believe that the rate for radioactive nucleotide incorporation during the first 10 s following the chase is influenced by one or both of the following: (a) There may be a small amount of net disassembly which results from a perturbation of the steady state by the added excess GTP. It has been found that very high concentrations of GTP cause disassembly of microtubules [see Figure 13 in Jameson & Caplow (1980)], and

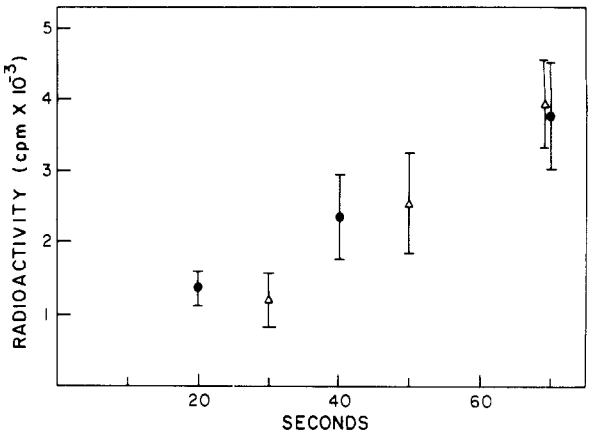

FIGURE 3: Kinetics of radioactive nucleotide incorporation into steady-state microtubules. At zero time, the reactions were pulsed with tubulin–[^{3}H]GTP, which was obtained by centrifugation of a reaction mixture containing microtubules at steady state (●). The reactions described by (△) received a chase with 200 μM GTP at 20 s.

this may occur to some small extent with 200 μM GTP. It was to avoid this effect that we assembled the microtubules using only a trace amount of excess GTP; if a significant excess of GTP had been used in the assembly reaction, it would have required a much higher GTP concentration in the chase. In the experiment shown in Figure 2, a loss of 500 cpm following the chase corresponds to a disassembly of about 1.0% of the microtubule mass (i.e., as indicated in Figure 2, the radioactivity incorporated in the reaction without the chase reaches about 50 000 cpm). Thus, in this experiment if 1.0% of the microtubule were to disassemble during the chase, then the uptake of label would be decreased by 500 cpm. (b) Approximately 1% of the nucleotide in steady-state microtubules may be able to rapidly exchange with added [^{3}H]GTP. Displacement of this label by the added excess GTP would appear as an interruption in the observed rate for label uptake from the residual tubulin–[^{3}H]GTP.

Overall, the results in Figure 2 are in accord with a mechanism in which uptake of label occurs as a result of a tubulin–[^{3}H]GTP reaction. It should be noted that different behavior is expected if label uptake came from a [^{3}H]GTP exchange. In this case, the majority of the label which had been incorporated in the pulse would be rapidly lost during the chase, and the rate for label uptake would have been immediately reduced by the chase.

Discussion

We have carried out pulse and pulse–chase kinetic studies to determine the relative contributions of tubulin–[^{3}H]GTP and [^{3}H]GTP for incorporation of labeled nucleotide into microtubules which are at steady state. The most direct method for making this evaluation would have involved simultaneous measurement of the rate of incorporation of labeled tubulin and radioactive GTP. We have used a less direct method because of the following reasons. First, we have not been able to obtain sufficient quantities of radioactive tubulin subunits with a high enough specific activity to allow measurement of the initial phase of the reaction. In three experiments in which an enormous quantity (2 mCi) of [^{35}S]-methionine was injected into the brains of a litter of 10–12-day-old rats, only very small quantities of tubulin dimer, with a specific activity of 32–65 mCi/μmol, could be obtained. Furthermore, as described previously in a study with in vivo labeled tubulin subunits (Cote & Borisy, 1981), upon centrifugation a small fraction of the labeled tubulin, either pu-

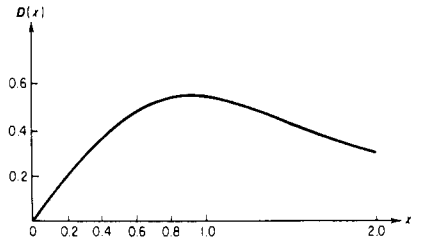

FIGURE 4: Probability integral of imaginary argument (Dawson's integral) in the form $D(x) = e^{-x^2}\int_0^x e^{y^2}\,dy$.

rified [^{3}H]ethyltubulin (Zeeberg et al., 1980b) of ^{35}S-tubulin (Zeeberg et al., 1980c), pellets independent of its incorporation into microtubules. The resulting high background reaction has relatively little effect when a significant fraction of the microtubules becomes labeled, but it obscures measurements of the initial phase of the reaction. It is necessary to analyze the initial rate, since a GTP exchange reaction is only expected to be significant during this phase of the reaction. Although we have not used labeled tubulin in our experiments, we have been able to estimate the contribution of a [^{3}H]GTP exchange reaction to the kinetics for labeled nucleotide incorporation.

Qualitative Analysis of the Pulse and Pulse–Chase Results. To determine the path for labeled nucleotide incorporation into steady-state microtubules, we have carried out studies of the loss of radioactivity during a GTP chase following a [^{3}H]GTP pulse and studies of the relative reactivities of [^{3}H]GTP and tubulin–[^{3}H]GTP for label incorporation. Our initial rationale for the pulse–chase studies was that if label uptake occurs by a first-order [^{3}H]GTP exchange, then the time course for label loss during a chase with excess GTP should be independent of the duration of the pulse. That is, for this mechanism, the rate for label loss is expected to be first order, with the duration of the pulse only influencing the amount of label which is lost in the chase. We were, however, surprised to find that added excess GTP did not immediately stop label uptake (see Figures 2 and 3). This observation required a change in our experimental protocol and resulted in a very different sort of analysis. Obviously, if the reactive species for label uptake is [^{3}H]GTP, then added excess GTP should immediately stop label uptake; however, if the label enters the microtubules from tubulin–[^{3}H]GTP *and* the equilibration of tubulin–[^{3}H]GTP with the added GTP is not very rapid (relative to that for subunit uptake), then the chase will not be immediately effective. We have established that there is a significant kinetic barrier for exchange of GTP with the tubulin E site; the half-time for this reaction is approximately 25 s (Figure 1). Thus, our observation that the GTP chase is not immediately effective can be accounted for, and it is established that tubulin–[^{3}H]GTP (rather than [^{3}H]GTP) is the reactive species for label incorporation. Confirmation of this point comes from our studies which show that the rate for labeled GTP uptake into steady-state microtubules is greater when the reaction is initiated with tubulin–[^{3}H]GTP, as compared with [^{3}H]GTP (Table I). The opposite result (i.e., that [^{3}H]GTP would react faster than tubulin–[^{3}H]GTP) is expected if the reaction path is an exchange of [^{3}H]GTP with microtubule-associated GTP.

Taken together, the results of our pulse–chase and pulse experiments indicate that the principal route for label incorporation into steady-statge microtubules involves tubulin–[^{3}H]GTP subunit incorporation by diffusional and treadmilling processes. It should be noted that our previous analysis of measurements of label uptake into steady-state microtubules (Caplow et al., 1982) was predicated upon the assumption that the uptake of radioactive nucleotide into microtubules corresponds to the uptake of tubulin–GTP subunits (rather than an exchange of [^{3}H]GTP into tubulin subunits which are in the microtubule). The results described here appear to justify this assumption.[4] Our results also suggest that our steady-state microtubules do not contain significant amounts (>1%) of guanine nucleotide which can exchange with added GTP (see below for a discussion of a related study).

Quantitative Analysis of the Results. A quantitative analysis of the pulse and pulse-chase experiments has been carried out, since it allows for a more rigorous discussion of the conclusions derived above and it constitutes an extension of our previous kinetic analysis of label uptake into steady-state microtubules (Zeeberg et al., 1980a; Caplow et al., 1982). We had not previously considered the kinetics for label uptake during the initial seconds of the reaction, when nucleotide exchange into monomeric subunits influences the observed rate. The new analysis necessitated a determination of the rate constant for dissociation of GTP from tubulin; this rate constant is equal to approximately 0.028 s^{-1}.

Pulse Experiments. We first discuss the relative effectiveness of tubulin–[^{3}H]GTP and [^{3}H]GTP in pulse experiments. Equation 2 describes the uptake of labeled tubulin subunits by a diffusional mechanism[5] when steady-state microtubules are pulsed in a reaction initiated with tubulin–[^{3}H]GTP:

$$\frac{L^*(t)}{L(t)} = f_1 - (f_1 - f_G)\left[1 - \frac{D(a_p)}{a_p}\right] \qquad (2)$$

The uptake of radiolabel per microtubule end at time t [$(L^*(t)$] depends upon f_1, the initial specific activity of the GTP bound to free tubulin, and f_G, the specific activity of the unbound [^{3}H]GTP. $L(t)$ is equal to the average number of "new" subunits which are introduced during the incubation and remain at time t. The a_p term is equal to $(k_1 t)^{1/2}$ where k_1 is the rate constant for isotopic equilibration of the tubulin E site with added [^{3}H]GTP. $D(a_p)$ is a "probability integral of imaginary argument" (Lebedev, 1965) which has a single local maximum of 0.54 when $(k_1 t)^{1/2}$ is equal to 0.92 (Figure 4). $D(a_p)/a_p$ varies from 1 to 0 as t varies from 0 to $+\infty$. The protocol for this experiment is outlined in Figure 5 (case 1).

Equation 3 describes the uptake of radiolabel for the case when the pulse is initiated with [^{3}H]GTP:

$$\frac{L^*(t)}{L(t)} = f_G\left[1 - \frac{D(a_p)}{a_p}\right] \qquad (3)$$

The symbols are defined above. The ratio of eq 3 and 2 is equal to

(label uptake in reaction initiated with [^{3}H]GTP)/(label uptake in reaction initiated with tubulin–[^{3}H]GTP) = $\{f_G[1 - D(a_p)/a_p]\}/\{f_1 - (f_1 - f_G)[1 - D(a_p)/a_p]\}$ (4)

[4] However, we believe that the rate constants calculated from these results (Caplow et al., 1982) are in error because we did not introduce a statistical correction to take into account the multistrand character of microtubules; this results in a large error in calculating the rate constants from the diffusional component for subunit incorporation. In an approximation of the statistical correction, we have assumed that the microtubule is composed of five independently reacting subhelices. From values of I_1, $t_{1/2}$, and S_1 equal to 400 subunits/helix, 35 s, and 0.82 subunit helix^{-1} s^{-1}, respectively [see Figures 4 and 6 and eq 3 and 4 in Caplow et al. (1982)], we calculate values for k_1, k_{-1}, k_2, and k_{-2} equal to 73.95, 73.13, 254.05, and 254.86 subunits helix^{-1} s^{-1}, respectively. These rate constants are considerably lower than those derived previously (Caplow et al., 1982).

[5] We only consider the diffusional mechanism for labeled subunit uptake, since this is a good approximation to the general case during short incubations. Equations are derived in the supplementary material.

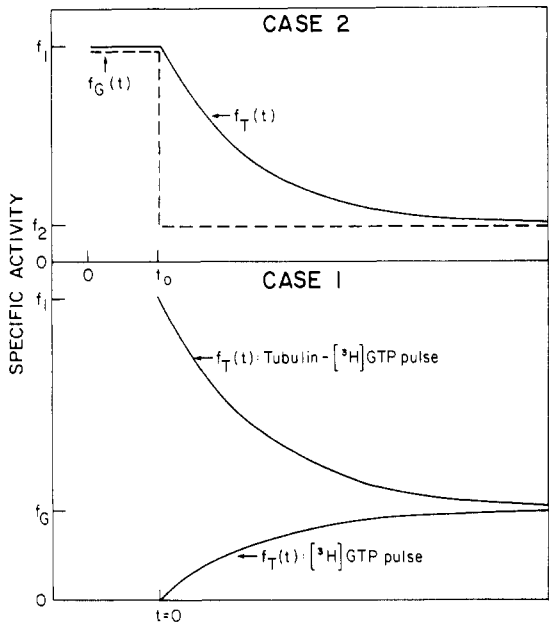

FIGURE 5: Schematic outline of the time course of the specific radioactivities of unbound GTP (f_G) and free tubulin E-site-bound GTP (f_T) in pulse (case 1) and pulse–chase (case 2) experiments. In case 1, $t = 0$ corresponds to the start of the pulse. The specific activity of added tubulin–[^{3}H]GTP falls (from f_1 to f_G) because the bound nucleotide is equilibrating with the added excess nucleotide. A pulse with [^{3}H]GTP results in a time-dependent increase in f_T. The magnitude in the change for this reaction (f_T goes from zero to f_G) is less than that for the reaction with tubulin–[^{3}H]GTP (f_T decreases from f_1 to f_G) since the pool of bound GTP is smaller than the pool of unbound GTP. In case 2, $t = 0$ corresponds to the start of the pulse. The tubulin–[^{3}H]GTP attains a high specific activity (i.e., $f_T = f_1$) almost immediately upon addition of [^{3}H]GTP, since the E-site equilibration is rapid in the absence of excess nucleotide;[3] this rapid increase is not shown in the figure. Also, in some cases. the pulse was done with tubulin–[^{3}H]GTP. The specific activity during the pulse remains approximately constant, since the pulse is done without significant added excess GTP. Following the chase (at t_0), f_T decreases exponentially. Our analysis of both cases is simplified by ignoring the effects of tubulin–GTP subunit addition to and dissociation from the microtubule ends on the specific activity of tubulin–[^{3}H]GTP subunits. For case 1, where the pulse is done with tubulin–[^{3}H]GTP, the specific activity of subunit tubulin–GTP will be decreased by dissociation of the microtubule's tubulin–GTP subunits which were present before $t = 0$ and will be increased by dissociation of tubulin–[^{3}H]GTP subunits which were introduced after $t = 0$. For case 1 where the pulse is done with [^{3}H]GTP, the subunit tubulin–[^{3}H]GTP specific activity will be decreased by dissociation from the microtubule of nonradioactive tubulin–GTP subunits which were in the microtubule before $t = 0$ and by nonradioactive subunits which were introduced after $t = 0$. For case 2, the effects in the pulse phase are equivalent to those described for case 1 where the pulse is done with tubulin–[^{3}H]GTP. During the chase, the specific activity of subunit tubulin–[^{3}H]GTP will be increased by loss from the microtubule of tubulin–[^{3}H]GTP subunits, which were introduced before t_0. Overall, the effect of ignoring changes in the subunit specific activity by microtubule-derived subunits is expected to be small in a brief (20-s) pulse. Also, subunit release and uptake will have an even less significant effect on the specific activity of unbound [^{3}H]GTP, so that our comparison with a model involving [^{3}H]GTP exchange remains valid.

To estimate the ratio in eq 4, we use a k_1 value equal to 0.0327 s^{-1},[6] so that with a 20-s incubation, a_p [$=(k_1 t)^{1/2}$] is equal to 0.809 [$D(a_p) = 0.533$]. The relative specific activities of the initially present tubulin-bound GTP (f_1) and unbound GTP (f_G) are estimated to be equal to 1 and 0.143, respectively. That is, the radioactivity in the added pulse of tubulin–[^{3}H]GTP is initially contained in the approximately 5 μM steady-state tubulin–GTP, and this radioactivity is eventually distributed in the 30 μM added excess GTP. On the basis of these values, the ratio calculated from eq 4 is equal to 0.09. That is, [^{3}H]GTP should be about 9% as effective as tubulin–[^{3}H]GTP for incorporation of radioactive guanine nucleotide into steady-state microtubules, when these are compared in a 20-s pulse. It should be noted that although eq 4 predicts that shorter incubations will result in a smaller ratio, the experimental error and background correction would constitute an excessive fraction of the total label uptake, for a reaction time of less than 20 s.

There is more radioactive nucleotide incorporated into steady-state microtubules in 20 s when tubulin–[^{3}H]GTP is used in a pulse, as compared to [^{3}H]GTP (Table I). The average from these results shows that [^{3}H]GTP is about 21% as effective as tubulin–[^{3}H]GTP for label incorporation in a 20-s pulse, which is in reasonable agreement with the ratio calculated from eq 4. The difference between the 0.21 ratio observed and the 0.09 calculated can be accounted for by either or both of the following: (a) The purity of the tubulin–[^{3}H]GTP varied from 70% to 95%, so that the observed label incorporation will underestimate the reactivity of this species. (b) Equation 3 is calculated from a simplified model which does not take into account the dilution of the subunit tubulin–[^{3}H]GTP by subunits which dissociate from the microtubule during the pulse (see legend to Figure 5). The conclusions derived from these studies will be given below.

Pulse–Chase Experiments. The pulse–chase protocol for the experiments described in Figures 1 and 2 is schematically outlined as case 2 in Figure 5. In an experiment in which the uptake of tubulin–[^{3}H]GTP subunits into steady-state microtubules is interrupted by an instantaneous decrease in the the specific activity of the unbound GTP (from f_1 to f_2; see Figure 5), the specific activity of the tubulin–[^{3}H]GTP decreases in an exponential reaction. Because of the slow decrease in the specific activity of the tubulin–[^{3}H]GTP, the uptake of label will continue for a period following the chase. The kinetics for this process have been quantitatively analyzed (see supplementary material), and the label uptake as a function of time [$L^*(t)$], as a fraction of the total new subunits which are taken up in the pulse time (i.e., t_0), is described by

$$\frac{L^*(t)}{L(t_0)} = \left\{ f_2\left(1 - \frac{t_0}{t}\right)^{1/2}\left[1 - \frac{D(a_c)}{a_c}\right] + f_1\left[1 - \left(1 - \frac{t_0}{t}\right)^{1/2}\left[1 - \frac{D(a_c)}{a_c}\right]\right]\right\}\left(\frac{t}{t_0}\right)^{1/2} \quad (5)$$

In this equation, $a_c = [k_1(t - t_0)]^{1/2}$, $D(a_c)$ is Dawson's integral, and the other symbols are as defined for eq 2 and in Figure 5. Equation 5 allows a calculation of the time course for label uptake and loss in the chase phase of a pulse–chase experiment. For example, in the experiments described in Figures 2 and 3, calculated values of $L^*(t)/L(t_0)$ are 1.12, 1.10, and 1.01, respectively, at the end of a 15-, 30-, and 50-s chase.[7] These values are calculated from the following: $f_1 = 1$ and $f_2 = 0.04$ (i.e., the addition of 200 mM GTP reduces the specific activity 25-fold); GTP exchanges into the E site with a half-time of 25 s ($k = 0.028$ s^{-1}, see above); the pulse time (t_0) is 20 s.

[6] Calculated from the relationship (Brylawski & Caplow, 1983) $k_{obsd} = (k_1[\text{tubulin–GTP}_{eq}] + [\text{GTP}_{eq}])/[\text{GTP}_{eq}]$ 0.028 s^{-1} ([5 μM + 30 μM]/[30 μM]). The 0.028-s^{-1} value is determined here, and the critical concentration for tubulin–GTP is approximated at 5 μM.

[7] An example of this calculation is pulse time (t_0) = 20 s, chase time = 15 s (t = 35 s), $[0.028(35 - 20)]^{1/2} = 0.648$, $D[0.028(35 - 20)]^{1/2} = 0.492$, and $L^*(t)/L(t_0) = [0.04(1 - 20/35)^{1/2}][1 - 0.492/0.648] + [1 - (1 - 20/35)^{1/2}(1 - 0.492/0.648)](35/20)^{1/2} = 1.12$.

Thus, following a chase, label uptake will continue for about 15 s, remain approximately constant for the next 15 s, and then decrease to the initial 20-s pulse level at about 50 s. Our results (Figures 2 and 3) are in general accord with this calculation, although the following deviations require consideration. First, the maximum label uptake in Figure 2 occurs at about 40–50 s instead of at 15 s. We believe that this discrepancy results from the fact that our analysis involves label uptake via a diffusional mechanism only (see supplementary material) and a more complex scheme (unpublished results) in which diffusional and treadmilling components contribute to the rate will make the chase process less effective so that maximum label uptake occurs at a later point. The results in Figure 2 are also not in perfect agreement with those predicted from eq 5 in that the extent of label uptake following the pulse exceeds that which we predict [the calculated values for $L^*(t)/L(t_0)$ are 1.12 and 1.10 at 15 and 30 s, respectively]. Aside from experimental error, we believe that the unexpectedly rapid label uptake following the GTP chase results from a contribution of treadmilling to the label uptake process. As noted above, our analysis only takes into account the diffusional component for labeled subunit uptake.

In summary, studies of the intial rate (20 s) for label uptake into steady-state microtubules reveal that [^{3}H]GTP is about 21% as reactive as is tubulin–[^{3}H]GTP. The reasonable agreement between this result and the value derived from our quantitative analysis (9%) suggests that label uptake into steady-state microtubules involves exchange of tubulin–[^{3}H]GTP, rather than an exchange of [^{3}H]GTP. The basis for the discrepancy between the calculated and observed reactivity is described above. Futhermore, the predictions derived from our quantitative analysis of label uptake and loss in a pulse–chase experiment are in reasonable agreement with a model in which it is assumed that label incorporation results from a reaction of tubulin–[^{3}H]GTP.

Exchangeability of Nucleotides in Microtubules. We have demonstrated that less than 1% of the nucleotide in steady-state microtubules is exchangeable.[8] Since microtubule-associated GDP is not exchangeable (Weisenberg et al., 1976), our observation indicates either that, if GTP in microtubules is exchangeable, less than 1% of the nucleotide in steady-state microtubules is GTP or that GTP in microtubules is not exchangeable. With regard to the first possibility, estimates of the size of tubulin–GTP caps with steady-state microtubules equal to 400 tubulin–GTP subunits/microtubule (Carlier, 1982) and 41 tubulin–GTP subunits/microtubule protofilament (Hill & Carlier, 1983) have been reported. (The latter value may be too low, since there was no statistical correction for the GTP hydrolysis rate constant.) The 400 tubulin–GTP subunits/microtubule was estimated to constitute about 5% of the microtubule mass, and an equivalent value would be indicated by the 41 subunits/protofilament estimate, if all of the microtubule's 10 protofilament ends are considered. These calculated values are in agreement with experimental determinations [see Table II in Caplow & Zeeberg (1980) and see Kirsch & Yarbrough (1981)], which indicates that 5–15% of the radioactivity in microtubules is in GTP, following an assembly reaction in the preesnce of [^{3}H]GTP. Thus, in light of the evidence that tubulin–GTP caps constitute more than 1% of the microtubule, our results can be taken to indicate that microtubule-associated GTP is nonexchangeable. Since this conclusion is not in accord with the interpretation of pulse–chase experiments, in which it was concluded that microtubule-associated GTP is readily exchangeable (Carlier & Pantaloni, 1981), we next describe an alternate interpretation of the earlier results in which we assume that microtubule-associated GTP is not exchangeable.

It was found [Figure 6 in Carlier & Pantaloni (1981)] that when microtubules were assembled with [γ-^{32}P]GTP and excess nonradioactive GTP was added at a point where the assembly was 90% complete, radioactive GTP within the microtubules was lost without hydrolysis. Also, when microtubules were assembled with [^{3}H]GTP and then chased with excess nonradioactive GTP at varying times before and after attainment of the steady state, there was a time-dependent decrease in the amount of ^{3}H-labeled nucleotide which could be lost in the chase [Figure 7 in Carlier & Pantaloni (1981)]. Both of these observations can be accounted for by the following mechanism, which is schematically outlined in Table II.

It is assummed that pre-steady-state microtubule assembly occurs at both ends of the microtubule and that the GTPase rate is lower at the steady-state net disassembly end. As a result, most and perhaps all of the subunits which are introduced at the steady-state net disassembly end will contain unhydrolyzed GTP. It has previously been pointed out (Carlier, 1982) that the steady-state net disassembly end may

Table II[a]

% of steady-state assembly completed	net assembly occurring at		microtubule composition	
	A end	D end	A end	D end
50	+	+	→DDDDTTTTTT←	
75	+	–	→DDDDDDDDDDTTTT→	
90	+	–	→DDDDDDDDDDDDDDDDDT→	

[a] To account for the results in Carlier & Pantaloni (1981), it is proposed that under the conditions of these experiments tubulin–GTP subunits are transiently introduced at the steady-state net disassembly end; these are progressively lost without hydrolysis as the reaction reaches the steady state. This table shows an extreme model in which GTP hydrolysis accompanies subunit addition at the steady-state net assembly end and there is no hydrolysis at the steady-state net disassembly end. A model in which there is a less significant difference in the hydrolysis rates at the two ends would lead to qualitatively similar conclusions. The time-dependent decrease in the amount of ^{3}H-labeled nucleotide displaced from the polymerized tubulin E site after a 2 mM unlabeled GTP chase [open circles in Figure 7 of Carlier & Pantaloni (1981)] is presumed to correspond to a decrease in the number of tubulin–GTP subunits at the D end which can be and are replaced by subunits added at the A end.

[8] The possibility that [^{3}H]GTP in a cap at the microtubule ends is lost during the centrifugation procedure is ruled out by considering the effect of this loss on the observed rate for the two routes for the incorporation of label:

reaction model	expected results for label uptake from pulse with [^{3}H]GTP	tubulin–[^{3}H]GTP
(A) tubulin–[^{3}H]GTP reacts and [^{3}H]GTP in a cap is not lost	slow	fast
(B) tubulin–[^{3}H]GTP reacts and [^{3}H]GTP in a cap is lost	slow + lag	lag
(C) [^{3}H]GTP reacts and [^{3}H]GTP in a cap is not lost	fast	slow
(D) [^{3}H]GTP reacts and [^{3}H]GTP in a cap is lost	lag	slow + lag

Models A and C are considered in the text. Models B and D will exhibit a lag for uptake of label, since it will be necessary for the label to be incorporated to a depth greater than the cap, before any radioactivity uptake is observed. Model A is believed to be correct, since the rate is faster with tubulin–[^{3}H]GTP than with [^{3}H]GTP (model C) and because no lag has been observed in the rate for uptake of label.

have a higher rate constant for tubulin subunit addition than the steady-state net assembly end. Therefore, if subunits introduced at this end have a reduced GTPase rate, the microtubule will contain very significant amounts of unhydrolyzed GTP at this end. When the assembly process starts to approach a steady state, the net disassembly end begins to undergo net disassembly [see Figure 5 in Zeeberg et al., (1980a)] so that tubulin–GTP subunits are lost from the microtubule; this occurs at a time when even more rapid net subunit addition is occurring at the steady-state net assembly end (required to account for the fact that net assembly is occurring). The loss of tubulin–GTP subunits from the net disassembly end during the terminal stages of the pre-steady-state reaction can account for the results in Figures 6 and 7 in Carlier & Pantaloni (1981). In conclusion, we are able to account for the previous results (Carlier & Pantaloni, 1981) by a reasonable and consistent mechanism which does not allow for free exchange of microtubule-associated GTP.[9] Our failure to observe [^{3}H]GTP exchange into microtubules is, therefore, not incongruous with these earlier results. There appears to be no conclusive evidence indicating that microtubule-associated GTP is exchangeable.

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Supplementary Material Available

Analysis of the kinetics for label uptake and loss in brief pulse and pulse–chase reactions of steady-state microtubules (9 pages). Ordering information is given on any current masthead page.

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[9] An alternate interpretation of the pulse–chase results (Carlier & Pantaloni, 1981) has the exchangeable GTP in "kinetic offshoot aggregates" (Mandelkow et al., 1983) rather than at the net disassembly end of the microtubule, as described in Table II.