Copolymerization of Tubulin–Colchicine Complex and Unliganded Tubulin in a Nonmicrotubular Polymer†

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ABSTRACT: We have shown previously [Saltarelli, D., & Pantaloni, D. (1982) *Biochemistry 21*, 2996–3006] that the tubulin–colchicine complex is able to polymerize in vitro into peculiar "curly" polymers, under the solution conditions permitting polymerization of unliganded tubulin into microtubules. Here it is further demonstrated that unliganded tubulin can be incorporated into these "curly" polymers. The partial critical concentration of tubulin–colchicine is decreased upon incorporation of unliganded tubulin into the copolymer. GTP hydrolysis occurs on unliganded tubulin upon incorporation in the copolymer. Tubulin–podophyllotoxin does not copolymerize with tubulin–colchicine to form a large polymer but interacts with it, preventing tubulin–colchicine polymerization. The data have been analyzed within a model of random copolymerization of unliganded tubulin and tubulin–colchicine into "curly" polymers. A corollary is that unliganded tubulin is virtually able to self-assemble into curly polymers with a critical concentration 10-fold higher than the critical concentration found for microtubule assembly. Consequently, these peculiar tubulin homopolymers cannot be observed except as transients at high concentrations, or when microtubule assembly is inhibited. Kinetic measurements of the T–TC copolymerization process and associated GTP hydrolysis at different T/TC ratios provide supplementary information about some privileged interactions between tubulin and tubulin–colchicine molecules. A comprehensive phase diagram of the various possible polymers formed in the presence of tubulin and tubulin–colchicine is presented.

It has been discovered early (Taylor, 1965) that microtubule functions in vivo were blocked by colchicine at concentrations estimated small relative to the tubulin concentration in the cell. The same finding has been obtained later in vitro (Olmsted & Borisy, 1973), and this amazing property of colchicine instigated extensive studies of its binding to tubulin and of the characteristics of its inhibition of microtubule assembly, with the challenging aim to elucidate the mechanism of tubulin polymerization. From these studies a picture emerges: Colchicine binds almost irreversibly to tubulin to form a 1/1 complex (Owellen et al., 1972; Wilson et al., 1974; Bhattacharyya & Wolff, 1976), through a complex two-step reaction (Garland, 1978; Lambeir & Engelborghs, 1981) in which conformational changes both of the colchicine (Detrich et al., 1981) and of the tubulin itself (Detrich et al., 1982) are involved. The tubulin–colchicine complex is able to bind actively to microtubule ends (Margolis & Wilson, 1977), in a reversible manner and with a binding constant of the same order as the equilibrium binding constant of unliganded tubulin (Lambeir & Engelborghs, 1980), thus inhibiting further microtubule elongation. A few tubulin–colchicine molecules can, however, be incorporated into the microtubule pending considerable destabilization of the polymer (Sternlicht & Ringel, 1979; Farrell & Wilson, 1980), which suggests the possibility of interactions between tubulin and tubulin–colchicine molecules. The binding of colchicine to tubulin induces a GTPase activity on tubulin (David-Pfeuty et al., 1979) which has been found to be generated by interactions between tubulin–colchicine, or tubulin and tubulin–colchicine molecules (Heusèle & Carlier, 1981). It is now established that colchicine binds to the ring oligomer of tubulin (Weisenberg & Timasheff, 1970; Penningroth, 1980; Lambeir & Engelborghs, 1981), in which the longitudinal interactions of the microtubule protofilament are involved (Mandelkow et al., 1982). Polymerized ribbon structures have been observed with tubulin–colchicine in the presence of the GTP analogue guanyl-5′-yl methylenediphosphate (Sandoval & Weber, 1979). More recently we have shown evidence for the polymerization of pure tubulin–colchicine complex into a nonmicrotubular three-dimensional aggregate, under the same solution conditions under which pure unliganded tubulin polymerizes into microtubules, and the physicochemical properties of this peculiar tubulin–colchicine polymer have been extensively studied (Saltarelli & Pantaloni, 1982) and corroborated by Andreu & Timasheff (1982).

The present paper presents an extension of our previous work (Saltarelli & Pantaloni, 1982) and demonstrates that unliganded tubulin can be incorporated into the tubulin–colchicine polymer. The questions addressed here concern the general problem of the nature of the different homologous and heterologous protein–protein interactions that take place in solutions containing tubulin and tubulin–colchicine. Specific points approached are the following: Does unliganded tubulin incorporation stabilize the copolymer? Is the copolymer formed by a random distribution of T and TC, or does it exhibit a particular order in which some specific interactions between T and TC would be privileged? How are the tubulin properties modified upon interaction with tubulin–colchicine, in particular its GTPase activity?

Materials and Methods

Chemicals. Unlabeled colchicine was bought from Prolabo; *C*-methoxy[^{3}H]colchicine (5 Ci/mol) and [γ-^{32}P]GTP (10 Ci/mol) came from Amersham; GTP was from Boehringer. All other chemicals used in buffers were analytical grade.

Tubulin Preparation. Tubulin was purified from pig brain by three cycles of assembly–disassembly according to Shelanski et al. (1973), followed by phosphocellulose chromatography according to Weingarten et al. (1975), then processed, and stored at −70 °C as previously described (Saltarelli & Pantaloni, 1982).

Biochemical Assays. All assays performed in this work have been done under the same conditions as described previously (Saltarelli & Pantaloni, 1982). Unless otherwise indicated,

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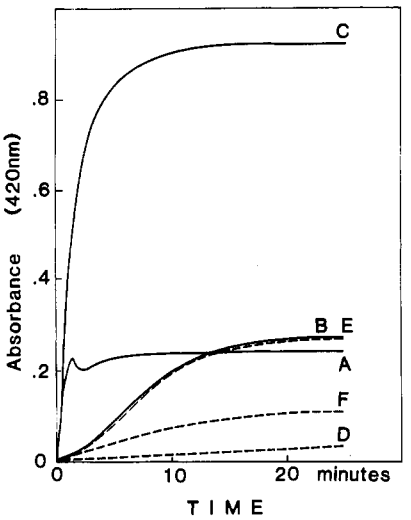

FIGURE 1: Polymerization of tubulin and tubulin–colchicine. The polymerization process was monitored turbidimetrically at 420 nm under the following conditions: 0.05 M Mes buffer, pH 6.6, 270 μM GTP, 8 mM Mg^{2+}, 30% glycerol (w/v), 37 °C. A, unliganded tubulin (24 μM) (microtubule formation); B, tubulin–colchicine (15 μM); C, mixture of tubulin (24 μM) and tubulin–colchicine (15 μM). D, E, and F samples (dashed lines) are the same as A, B, and C, respectively, except that 200 μM podophyllotoxin was present.

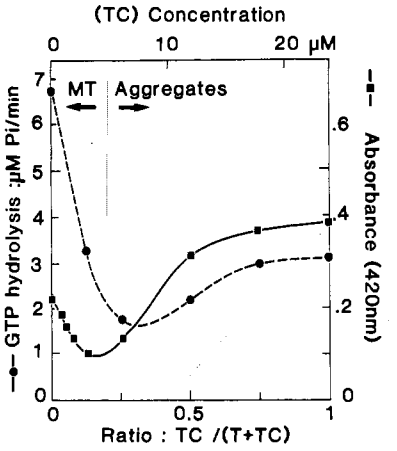

FIGURE 2: Inhibition of microtubule assembly by tubulin–colchicine and copolymerization of unliganded tubulin and tubulin–colchicine. The experimental conditions are the same as Figure 1. The sum of unliganded tubulin and tubulin–colchicine concentrations was kept constant (24 μM), while the ratio TC/(T + TC) was varied from 0 to 1. (●) Initial rate of GTP hydrolysis; (■) absorbance change due to microtubule assembly and copolymerization of T and TC; (···) absorbance change due to the formation of tubulin–colchicine homopolymer expected theoretically in the absence of unliganded tubulin. The two domains, microtubules and aggregates, are featured on the figure.

the buffer in all experiments consisted in 50 mM Mes,[1] pH 6.6, containing 20% glycerol, 7 mM Mg^{2+}, 0.5 mM EGTA, and 0.25 mM GTP (P buffer). The colchicine binding assay was performed essentially according to Sherline et al. (1974). Tubulin–colchicine complex was isolated deprived of unbound colchicine as previously described (Saltarelli & Pantaloni, 1982); GTP hydrolysis was measured according to Nishizuka et al. (1968); polymerization at 37 °C was followed both by turbidimetric methods at 420 nm and by sedimentation at 100000*g* for 20 min in a Beckman Spinco L50 centrifuge or at 160000*g* for 5 min in a Beckman Airfuge; protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard and the correction for tubulin of David-Pfeuty et al. (1977). All experiments were repeated a large number of times, and the same data were quantitatively obtained on different tubulin preparations. The experimental errors are 2% in the tubulin concentration determinations, 2% in turbidity measurements, 5% in the rates of GTP hydrolysis, and 10% in measurements of the lag times.

Results

Evidence for the Copolymerization of Tubulin–Colchicine Complex with Unliganded Tubulin. We have previously shown that the tubulin–colchicine complex at a concentration higher than 10 μM was able to polymerize into a particular type of aggregate when brought to 37 °C in P buffer (Saltarelli & Pantaloni, 1982). Polymerization can be monitored turbidimetrically at 420 nm. Figure 1 shows that when pure (phosphocellulose chromatographed) unliganded tubulin (24 μM) is added to tubulin–colchicine (15 μM) and when the temperature of the solution is raised to 37 °C, a much faster rate of polymerization is observed, and a 3-fold larger amount of polymer is formed than in the control sample B containing tubulin–colchicine alone. This result was assessed both by the turbidity measurements shown in Figure 1 and by sedimentation assays. Under these conditions of tubulin and tubulin–colchicine concentrations, no microtubules can be formed. This was further checked by electron microscopic examination of negatively stained samples, which indicated that the preparation was homogeneous and contained one single type of aggregate morphogically similar to the tubulin–colchicine "curly" polymer previously described (Saltarelli & Pantaloni, 1982). When 200 μM podophyllotoxin was added to the two samples, no modification of the polymerization of the tubulin–colchicine complex alone was observed, in good agreement with the fact that podophyllotoxin does not bind to tubulin–colchicine (Wilson et al., 1974). On the other hand, a strong inhibition of polymerization was observed upon addition of podophyllotoxin to the sample containing both tubulin and tubulin–colchicine. The extent of polymerization obtained in sample F was even lower than in the control sample B (TC homopolymer). This result indicates not only that the binding of podophyllotoxin to tubulin prevents the formation of the copolymer but also that moreover interactions between tubulin–podophyllotoxin and tubulin–colchicine do exist and prevent the formation of the TC homopolymer.

The requirements for the copolymer formation were the same as for the tubulin–colchicine homopolymer, which have been extensively studied (Saltarelli & Pantaloni, 1982) and will not be developed here. They mainly include the presence of Mg^{2+} ions at a concentration larger than 5 mM, the presence of GTP, and a temperature above 30 °C.

Figure 2 shows the development of the two types of tubulin polymers, microtubules and TC-type curly polymers, at a constant concentration of total (T + TC) tubulin, upon varying the partial concentration of TC. In the absence of TC, microtubules are present as well as S-sheet polymers which are known to have the same structure as microtubules (Carlier & Pantaloni, 1978; Mandelkow & Mandelkow, 1979). When TC partial concentration is increased, an initial inhibition of microtubule formation, in the TC/T = 10^{-2} to 10^{-1} range, is followed by the formation of the (T–TC) copolymer, as shown by the increase in turbidity beyond the level observed for the TC homopolymer formed at the same concentrations of tubulin–colchicine (dotted line). In the region where microtubule polymerization was inhibited by tubulin–colchicine, electron

[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

Table I: Copolymerization of Tubulin-[^{3}H]Colchicine (TC) and Unliganded Tubulin (T)[a]

total tubulin concn			protein concn in supernatant			concn of polymerized tubulin		
TC + T	TC	T	T + TC	TC	T	T + TC	TC	T
1	1	0	0.9	0.8	0	0.2	0.2	0
1.68	1	0.68	1.04	0.59	0.45	0.64	0.41	0.23
2.25	1	1.25	1.20	0.51	0.69	1.05	0.49	0.56
2.83	1	1.83	1.40	0.47	0.93	1.43	0.53	0.90
3.30	1	2.30	1.50	0.43	1.07	1.80	0.57	1.23
3.88	1	2.88	1.50	0.36	1.14	2.38	0.64	1.74
4.43	1	3.43	1.58	0.32	1.26	2.85	0.68	2.17
5.50	1	4.50	1.60	0.24	1.36	3.90	0.76	3.14

[a] Increasing amounts of T were added to a constant amount (1 mg/mL) of TC. Protein concentrations are expressed in milligrams per milliliter. Partial concentration of tubulin-[^{3}H]colchicine in supernatant is derived from radioactivity measurement, knowing the specific radioactivity of [^{3}H]colchicine. The amount of polymerized tubulin is calculated as the difference between total and supernatant concentrations.

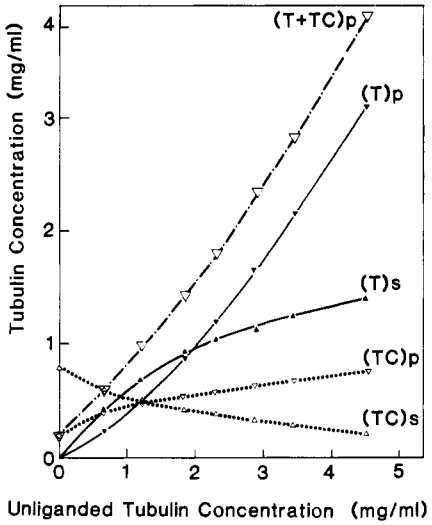

FIGURE 3: Copolymerization of unliganded tubulin and tubulin–colchicine. Plot of the weight amount of unliganded tubulin $(T)_p$ and $(T)_s$ and tubulin colchicine $(TC)_p$ and $(TC)_s$ in the polymer (subscript p) and in the supernatant (subscript s), respectively, vs. the concentration of unliganded tubulin added to a constant amount (10 μM, i.e., 1 mg/mL) of tubulin–[^{3}H]colchicine. All concentrations were derived from protein concentration determinations and radioactivity measurements of [^{3}H]colchicine bound (see text). The experimental conditions are the same as in Figure 1, except that 10 mM Mg^{2+} and 20% glycerol are present.

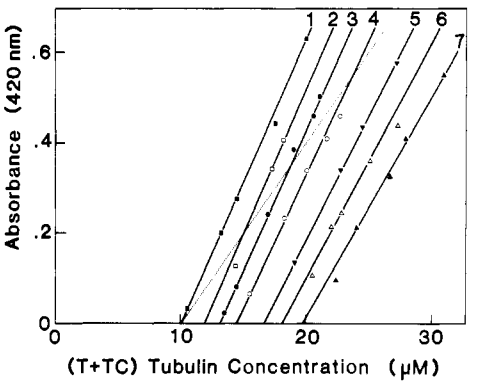

FIGURE 4: Stability of the (T–TC) copolymers as a function of their relative composition in T and TC. The data are plotted as the amount of polymer formed (from turbidity measurements) vs. the total (T + TC) tubulin concentration. Each straight line is experimentally obtained with mixtures of T and TC in which the percent amounts of TC are (from 1 to 7) 100, 69, 56, 44, 35, 30 and 18. The polymerization buffer contained 10 mM Mg^{2+}, 20% glycerol, and 1 mM GTP. The dotted line represents the absorbance of polymer formed from solutions containing 10 μM TC and variable concentrations of T.

microscopic examination of negatively stained samples revealed that microtubules were shorter than in the absence of tubulin–colchicine.

The (T–TC) copolymer could be sedimented in the Airfuge under the same conditions as the (TC) homopolymer. Upon addition of increasing amounts of unliganded tubulin to a constant concentration of TC, increasing amounts of polymer were sedimented in parallel with an increase in the development of turbidity. The change in specific turbidity accompanying the formation of the copolymer, $\Delta\theta_{420nm}$, was the same as for the TC polymer and took the value of 1.1 cm^2 mg^{-1} as long as the ratio T/TC in the copolymer varied between 0 and 2. The constancy of $\Delta\theta_{420nm}$ indicated that the morphologies of the TC and T–TC polymers were similar in this range of stoichiometry of T and TC. The value of $\Delta\theta_{420nm}$ was 5.5-fold larger for the T–TC polymer than for microtubules.

By use of [^{3}H]colchicine-bound tubulin, the relative amounts of T and labeled TC* in the polymer and in the nonsedimentable material could be examined after centrifugation (Table I). Figure 3 shows that the addition of increasing amounts of unliganded tubulin, which does not polymerize by itself, to tubulin–colchicine kept at a constant concentration of 10 μM leads to an increased polymerization with a concomitant decrease of nonsedimentable TC and increase of both T and TC incorporation into the sedimentable material. The evolutions of polymerized T and TC, respectively, are described by intersecting curves (Figure 3). This means that the stoichiometry $(T/TC)_{polymer}$ is lower than 1 at low concentrations of unliganded tubulin and becomes higher than 1 at high concentrations above the crossing point.

In another experiment, a series of stock solutions of mixed tubulin and tubulin–[^{3}H]colchicine containing different partial concentrations of TC* were prepared. Each stock solution was diluted to different total tubulin concentrations, the ratio TC*/(T + TC*) being thus maintained constant, and the resulting solutions were polymerized. Figure 4 shows that the plots of the amount of polymer formed vs. the total tubulin concentration were parallel straight lines, each corresponding to a given TC*/(T + TC*) value. Each plot extrapolated on the abscissa to a corresponding apparent critical concentration C_c which was the sum of the partial critical concentrations $(TC)_c$ and $(T)_c$. It can be observed that the apparent critical concentration C_c increases upon decreasing the ratio TC*/(T + TC*). Measurements of the relative amounts of TC* and T in the supernatant after the polymers were sedimented gave the values of the partial critical concentrations $(TC)_c$ and $(T)_c$ at different concentrations of T and TC. It was observed that upon copolymerization with increasing amounts of unliganded tubulin, the partial critical concentration of TC decreased and the measured values $(TC)_c$ and $(T)_c$ obeyed a linear relationship (Figure 5). The existence of such a linear relationship

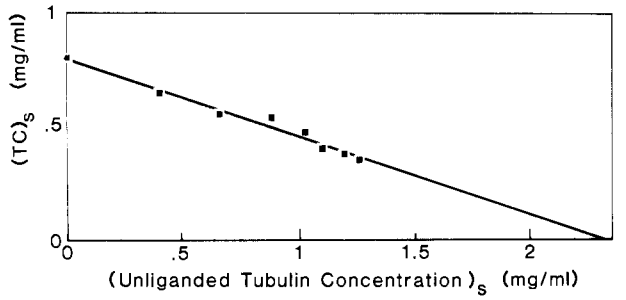

FIGURE 5: Random copolymerization of tubulin–colchicine and unliganded tubulin is demonstrated by the linear relationship between the partial critical concentrations T_c and TC_c in equilibrium with the copolymer. The experiment is performed with labeled tubulin–[^{3}H]colchicine so that the partial T_c and TC_c concentrations in the supernatant can be directly determined after the polymer is sedimented.

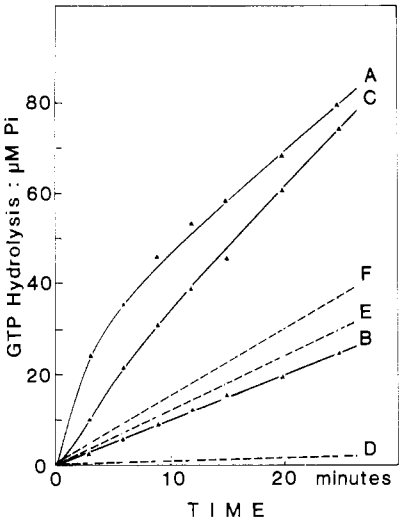

FIGURE 6: GTP hydrolysis associated with polymer formation: microtubules (A), tubulin–colchicine homopolymer (B), (T–TC) nonmicrotubular copolymer (C). D, E, and F (dashed lines) are the controls corresponding to A, B, and C, respectively, with 200 μM podophyllotoxin present to prevent polymerization. Samples A–F are the same as in Figure 1 in which the corresponding polymerization processes are shown.

is characteristic of copolymer formation as described by Oosawa & Asakura (1975): $(TC)_cK_{TC} + (T)_cK_T = 1$, where $(TC)_c$ and $(T)_c$ are the partial critical concentrations measured for TC and T, and K_{TC} and K_T are the intrinsic equilibrium association constants of TC and T for this particular nonmicrotubular polymer. Figures 5 and 11 show that two different techniques, sedimentation and turbidimetry, yielded the values $K_{TC} = 9 \pm 1$ μM and $K_T = 23 \pm 2$ μM. That is, unliganded tubulin can polymerize not only into microtubules with a critical concentration of 3 μM but also into nonmicrotubular polymers having the structure of the TC polymer with a critical concentration of 23 μM. Since the latter value is much higher than that of the critical concentration for microtubule formation, only microtubules can be observed in the absence of tubulin–colchicine.

GTP Hydrolysis Associated to the Copolymerization of Tubulin–Colchicine and Unliganded Tubulin. We had previously shown that GTP hydrolysis catalyzed by tubulin–colchicine was independent of assembly (Saltarelli & Pantaloni, 1982). When unliganded tubulin was added to tubulin–colchicine under conditions of copolymerization, an increased rate of GTP hydrolysis was observed as compared to tubulin–colchicine alone. Figure 6 shows the time courses of GTP hydrolysis in samples in which the overall reaction

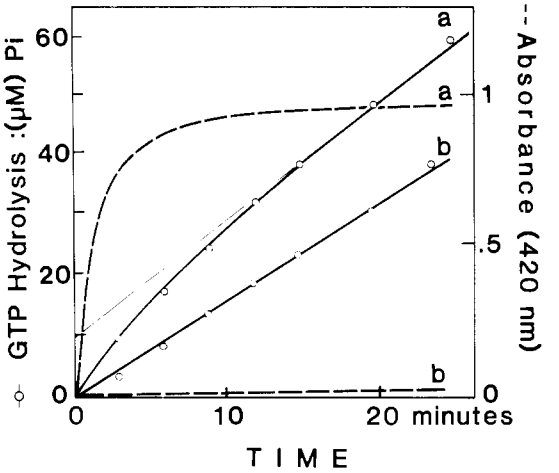

FIGURE 7: Evidence for a pre-steady-state GTP hydrolysis associated to unliganded tubulin incorporation in the (T–TC) copolymer. Tubulin (19 μM) and tubulin–colchicine (10 μM) are mixed in the presence of 10 mM Mg^{2+} (a) or 1 mM Mg^{2+} (b). (---) Time courses of turbidity development upon bringing the solution to 37 °C; (O) plots of the GTP hydrolyzed concomitantly. A burst of GTP hydrolyzed in (a) is concomitant with the copolymerization process.

consisted of an initial burst of liberated P_i and a subsequent steady state of GTP hydrolysis (sample C). At steady state, GTP was hydrolyzed at a higher rate by the copolymer than by the homopolymer TC (sample B). In addition the value of the burst of P_i, obtained by extrapolation of the steady-state straight line, was 10 μM. In the same experiment, the phase diagram shown in Figure 11 indicates that 9 μM tubulin–colchicine and 15 μM unliganded tubulin were incorporated in the polymer. A control sample carried out in the presence of podophyllotoxin shows that inhibition of copolymer formation was accompanied by the disappearance of the burst phase and a decrease in the steady-state rate of GTP hydrolysis. These data suggest that the increase in GTP hydrolysis is due to the incorporation of T in the copolymer, but they do not tell unambiguously whether GTP is hydrolyzed on T or TC in the copolymer. In control F, the rate of GTP hydrolysis was higher than in control E which did not contain unliganded tubulin. This result is in agreement with the reported interaction between tubulin–colchicine and tubulin–podophyllotoxin leading to a faster GTP hydrolysis reaction (Heusèle & Carlier, 1981). It should be emphasized that GTP hydrolysis accompanying copolymer T–TC formation exhibited the same kinetic characteristics as microtubule formation from pure tubulin under the same medium conditions, i.e., was assembly dependent but kinetically uncoupled from polymerization (Carlier & Pantaloni, 1981).

Figure 7 further emphasizes the dependence of enhanced GTP hydrolysis on the copolymerization process. The time course of GTP hydrolysis by a mixture of tubulin and tubulin–colchicine was followed, and the extent of copolymerization was controlled by varying Mg^{2+} ion concentration. Again the existence of a burst phase and increased steady-state rate of GTP hydrolysis were characteristic of copolymer formation. Figure 8 documents the quantitative correlation between the increase in the amount of polymer formed at equilibrium and the amplitude of the burst of GTP hydrolyzed, when the Mg^{2+} ion concentration was varied between 0.5 and 10 mM.

In Figure 9 are summarized the parallel evolutions of initial rate of GTP hydrolysis, absorbance change, and polymer weight at steady state when increasing amounts of unliganded tubulin were added to a constant amount (12.5 μM) of tubulin–colchicine under copolymerization conditions. The data show an increase in the values of all three parameters in the

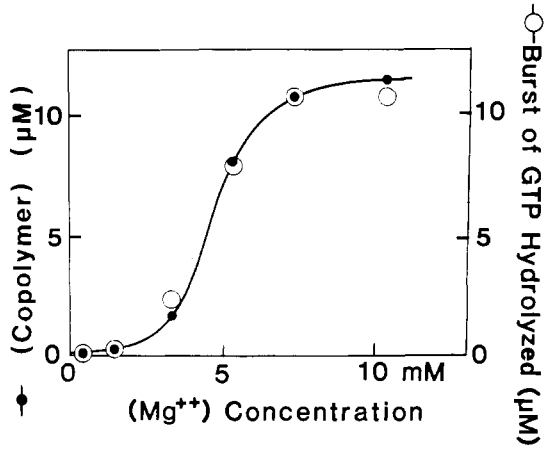

FIGURE 8: Correlation between the amounts of copolymer formed and of GTP hydrolyzed in the burst phase. Copolymerization of 19 μM tubulin and 10 μM tubulin–colchicine is promoted by increasing Mg^{2+} ion concentration. No polymer was formed from tubulin–colchicine alone at 10 μM at all Mg^{2+} concentrations. (●) Extent of copolymer formed, derived from the measurement of turbidity change at 420 nm; (○) amount of P_i liberated in the pre-steady-state step, measured by extrapolation to zero time of the steady-state linear time course of P_i liberation. At the plateau, 12 μM tubulin was polymerized. This amount consisted of 8 μM unliganded tubulin and 4 μM tubulin–colchicine (from protein and radioactivity measurements).

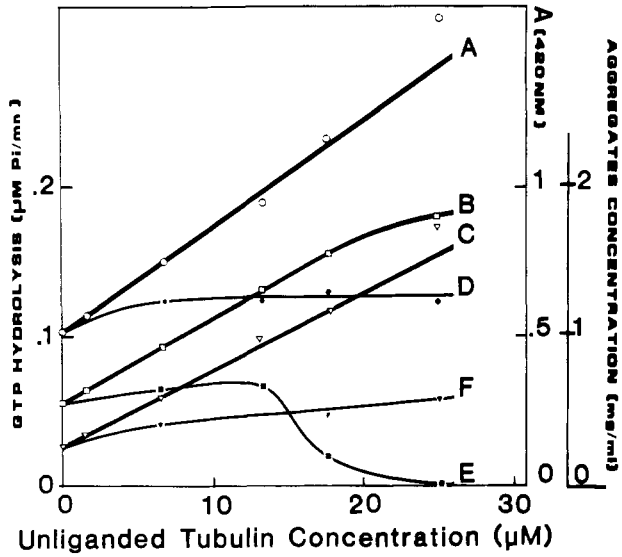

FIGURE 9: Correlated changes in initial rate of GTP hydrolysis (A), turbidity reached (B), and weight of polymer sedimented at equilibrium (C). Increasing amounts of unliganded tubulin were copolymerized with 12.5 μM tubulin–colchicine. Other buffer conditions were 8 mM $MgCl_2$, 30% glycerol, and 0.3 mM GTP. D, E, and F curves represent the controls of A, B, and C, respectively (same full symbols), in the presence of podophyllotoxin (200 μM).

range of tubulin concentrations used. This increase was prevented by podophyllotoxin. Again it can be seen (as in Figure 1) that high tubulin–podophyllotoxin concentrations (T–podophyllotoxin/TC > 1) impair the formation of the tubulin–colchicine homopolymer itself, as indicated by turbidity measurements (curve E, Figure 9). However, the weight amount, if not the morphology of sedimented material, was not changed, suggesting that an interaction between TC and T–podophyllotoxin could take place through the formation of a polymer unable to assemble into large aggregates but large enough to be at least partially sedimented at 160000g for 5 min. The sigmoidal shape of curve E emphasizes the cooperative nature of the inhibition of TC polymerization by tubulin–podophyllotoxin and is a further support to the interaction between tubulin–colchicine and tubulin–podophyllotoxin.

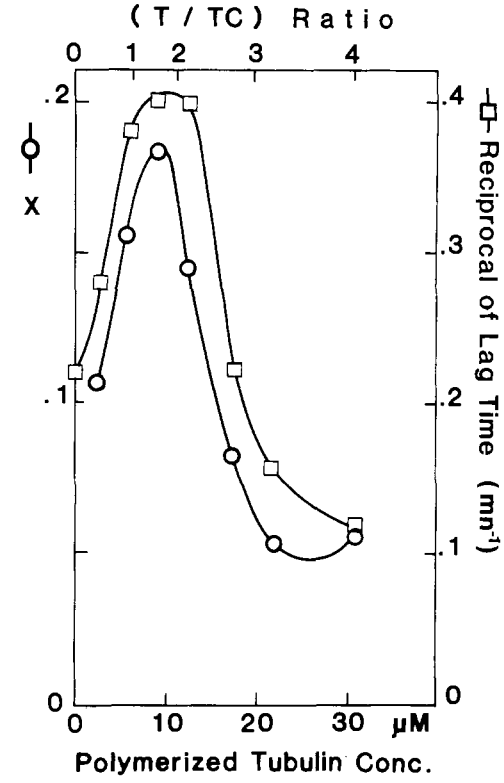

FIGURE 10: Evolution of the kinetics of copolymerization and of the initial rate of GTP hydrolysis as a function of the stoichiometries of T and TC in the copolymer. Increasing amounts of unliganded tubulin were copolymerized with a constant concentration of tubulin–colchicine (10 μM). (□) Reciprocal of lag time τ preceding polymerization; (○) x, increase in the initial rate of GTP hydrolysis upon incorporation of unliganded tubulin into the polymer divided by the amount of unliganded tubulin polymerized.

Stoichiometry of Tubulin–Colchicine and Unliganded Tubulin in the Copolymer. As shown in Figure 3, high proportions of unliganded tubulin in the copolymer can be obtained and ratios of (T/TC) in the copolymer as high as 4 have been reached experimentally. Moreover, upon addition of increasing amounts of unliganded tubulin, the stoichiometry $(T/TC)_{polymer}$ seems to increase in a monotonous fashion. However, the question can be raised whether all the properties of the copolymer vary monotonously with the ratio $(T/TC)_{polymer}$ or exhibit peculiar features for some defined stoichiometries. To answer this question, we examined the behavior of two parameters that give an insight into the mechanism of formation of the aggregates, namely, the lag time τ preceding the turbidity development (which reflects the nucleation reaction) and the increase x in the initial rate of GTP hydrolysis divided by the amount of T incorporated into the copolymer aggregates. Figure 10 shows that these two parameters indeed exhibited a nonmonotonous variation when studied as a function of the amount of polymerized unliganded tubulin. When the incorporation of T into the copolymer was increased, both $1/\tau$ and x first increased, reached a maximum at $(T/TC)_{polymer} = 1$, and then decreased abruptly when the stoichiometry $(T/TC)_{polymer}$ became higher than 2. Concomitantly with this decrease in $1/\tau$ and x, the extent of turbidity reached at equilibrium was smaller and smaller upon increasing the ratio $(T/TC)_{polymer}$ above 2 (Figure 9, curve B). However, no discontinuity appeared in the increase in the weight amount of polymer sedimentable at 160000g (Figure 9, curve C). A preliminary analysis of these results indicates

that the homologous tubulin–tubulin interactions which increasingly predominate in the copolymer at high ratios of T/TC yield polymers which can be sedimented at 100000g for 20 min but have a lower tendency to self-associate into large amorphous aggregates. Consequently a lower turbidity is observed.

Discussion

It has been well established that in buffers containing GTP, 3.4 M glycerol, and 5–10 mM Mg^{2+} ions, pure tubulin polymerizes into microtubules and sheets (Lee & Timasheff, 1977; Carlier & Pantaloni, 1978) with a critical concentration of 0.3 mg/mL in Mes buffer and a specific turbidity of 0.35 $cm^2\ mg^{-1}$ at 350 nm. It has also been shown that under the same medium conditions, pure tubulin–colchicine polymerizes into curly filaments, packing together into large aggregates with a critical concentration of 1 mg/mL and a specific turbidity of 1.1 $cm^2\ mg^{-1}$ at 420 nm (Saltarelli & Pantaloni, 1982). Here we report in addition that mixtures of tubulin–colchicine and unliganded tubulin can copolymerize either into microtubules or into polymers of the tubulin–colchicine type, depending on the respective concentrations of the two protomers. Sternlicht et al. (1980) first provided experimental evidence for the incorporation of tubulin–colchicine into microtubules, but the proportion of TC in microtubules was never greater than 1%. In contrast, in the alternate nonmicrotubular type of copolymer studied here, any proportion of T and TC can be obtained. The thermodynamics of copolymerization equilibria have been well studied by Oosawa & Asakura (1975) for a variety of cases. These authors demonstrated that in a solution containing two kinds of monomers α and β able to copolymerize, the critical concentration of one kind of monomer is related to the critical concentration of the other kind of monomer and that under polymerization conditions the following relation takes place:

$$C_{c\alpha}K_\alpha + C_{c\beta}K_\beta = 1$$

in which $C_{c\alpha}$ and $C_{c\beta}$ are the measured partial critical concentrations and K_α and K_β are the intrinsic equilibrium association constants of α and β monomers, respectively, for polymer formation.

Figure 11 shows a phase diagram derived from the data shown in Figure 4 (presented here as filled circles) and in which the data of Sternlicht & Ringel (1979), (Figure 6C) have been included (filled squares on line 1). The state of the solution is determined by the total concentrations of monomers $(T)_t$ and $(TC)_t$ listed on the abscissa and ordinate, respectively. Several regions can be distinguished: (1) *In the A region* no polymer is formed, and both the T and TC monomers coexist in solution. (2) *In the B region* (above line L) a T–TC copolymer is formed and is in equilibrium with TC and T monomers at partial concentrations $(TC)_c$ and $(T)_c$ which obey the following equation:

$$(TC)_cK_{TC} + (T)_cK_T = 1 \qquad (1)$$

This equation describes line L; K_{TC} and K_T are the intrinsic association constants of TC and T, respectively, for the corresponding homopolymers (TC) and (T) and are defined as the reciprocals of the intercepts of L with the ordinate and abscissa axes, respectively. The points on line L have been obtained from the data on the partial critical concentrations $(T)_c$ and $(TC)_c$ shown in Figure 4. Any solution composition in the B region is defined by a single point, with abscissa $(T)_{total}$ and ordinate $(TC)_{total}$ corresponding to an empty square from which the partial critical concentrations $(TC)_c$ and $(T)_c$ have been calculated from the extrapolations of lines 1–7 on the abscissa in Figure 4. The amounts of TC and T in the polymer can then easily be calculated:

$$(TC)_{polymer} = (TC)_{total} - (TC)_c \qquad (2)$$

$$(T)_{polymer} = (T)_{total} - (T)_c \qquad (3)$$

Alternatively these values can be measured directly by sedimentation as shown in Figure 5, which yielded a straight line quite comparable to the present line L. These data support the model of a random copolymerization of two types of monomers, T and TC, at least in a first approximation. (3) *In the C region* (below line 1, i.e., at high T/TC ratios), T and TC copolymerize into microtubules in which a very low amount of TC has been found incorporated by Sternlicht & Ringel (1979). (4) *In the D region*, both types of copolymers, i.e., microtubules and curly polymers of the TC type, coexist.

It should be observed that while line L has a negative slope, indicating that the partial TC critical concentration decreases upon incorporation of T into the copolymer, line 1 has a positive slope which means that the partial critical concentration of T for the formation of microtubules increases upon incorporation of TC into the microtubule and the extrapolated

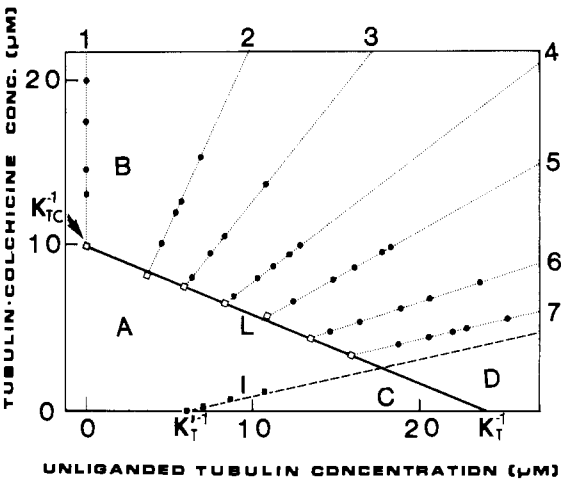

FIGURE 11: Phase diagram featuring the domains of existence of tubulin and tubulin–colchicine copolymers. Total unliganded tubulin and tubulin–colchicine concentrations are represented on the abscissa and ordinate, respectively. The intrinsic critical concentrations for tubulin and tubulin–colchicine polymerization into curly polymers are K_T^{-1} = 24 μM and K_{TC}^{-1} = 10 μM, respectively. In region A, only T and TC monomers coexist. In region B, curly polymers are present in equilibrium with T and TC monomers. In region C, microtubules are present. Region D is the overlap of B and C, where microtubules and curly polymers are (see text) observed by electron microscopy (data not shown). Filled circles are derived from the data presented in Figure 4, and the dotted lines are numbered 1–7 in the order of decreasing ratios R = TC/(T + TC), according to the legend of Figure 4. The abscissa and ordinate of each point are equal to the partial total concentrations of T and TC, respectively [$(TC) = R(T)_{total}$ and $(T) = (1 - R)(T)_{total}$]. Empty squares are defined by the partial critical concentrations TC_c and T_c calculated from the apparent total critical concentration extrapolated on the plots of Figure 4. These empty squares determine line L which is the limit below which curly polymers cannot be found in significant amounts under the defined buffer conditions. The intercepts of L with the abscissa and ordinate axes represent the intrinsic critical concentrations of T and TC, respectively, for the formation of the curly homopolymer: K_T^{-1} = 24 μM and K_{TC}^{-1} = 10 μM. The data obtained by Sternlicht & Ringel (1979) (Figure 6C) define line 1 (dashed) under which are formed microtubules in which TC can be incorporated. The intrinsic critical concentration for TC polymerization into microtubules is defined by the intercept of line 1 with the ordinate axis which gives a negative value. This point is discussed in the text. K'^{-1}_T is the intrinsic critical concentration for microtubule formation from unliganded tubulin.

intrinsic TC critical concentration for microtubule formation has a negative value. In other words, incorporation of TC decreases the stability of microtubules. This is indeed a paradoxic finding since, while it is conceivable in a copolymerization scheme that one of the monomers has a weak association equilibrium constant for the polymer, on the other hand the occurrence of a negative association constant is physically meaningless. A possible explanation of this paradoxic observation is that the microtubular T–TC copolymer is not in equilibrium with TC monomers but that it is a structure formed under nonequilibrium conditions in which TC has been trapped during the course of assembly and the resulting distortions of the lattice decrease the stability of microtubules. In other words, tubulin–colchicine can be incorporated in two kinds of polymers: (1) to a very small extent, in microtubules in which the structural strains tend to prevent the interaction between tubulin–colchicine and tubulin; (2) to a larger extent, in curly polymers in which the structural strains are looser and make possible an interaction between tubulin–colchicine and tubulin.

This diagram further shows that in the absence of TC, unliganded tubulin itself can polymerize into two types of homopolymers with two different critical concentrations: a high critical concentration, $K_T = 24\ \mu M$, defined above (eq 1) for the formation of the curly TC-type polymer; a low critical concentration, $K_T = 3\ \mu M$, for the formation of microtubules. Obviously at tubulin concentrations above 24 μM, only the more stable polymers, i.e., microtubules, will be formed, and curly polymers will be obtained only under conditions of inhibition of microtubule assembly. "Curly" polymers of tubulin could be formed, however, as transients in the time course of microtubule assembly at high tubulin concentrations, provided that their activation energy was lower than that of microtubule formation. In relation to this point, the turbidity overshoot, which has been observed currently in the early phase of the time course of microtubule assembly at high tubulin concentration (Figure 1), could support the existence of such polymeric intermediates characterized by a high-specific turbidity. These unstable transient polymers would dissociate rapidly, while the more stable microtubule species of lower specific turbidity was being formed.

In conclusion, sedimentation measurements and studies of the stoichiometry of T and TC in the "curly" polymers are in agreement with a model of random copolymerization of the two monomers, T and TC. However, kinetic studies of the polymerization and associated GTP hydrolysis showed that upon increasing the ratio $(T/TC)_{polymer}$, a dramatic change in the evolution of these parameters was observed in the region of $1 < (T/TC)_{polymer} < 2$: (1) The GTP hydrolysis induced upon incorporation of unliganded tubulin into the copolymer increased upon increasing $(T/TC)_{polymer}$ (Figures 6 and 10), reached a maximum for $(T/TC)_{polymer} = 1$, and then decreased abruptly at $(T/TC)_{polymer} > 2$.

(2) Concomitantly, while no change was observed in the amount of sedimented material upon incorporation of more and more T into the copolymer, the specific turbidity decreased for $(T/TC)_{pol} > 2$ (curve B, Figure 9). Analysis of the time course of polymerization showed that incorporation of small amounts of T into the copolymer $[(T/TC)_{pol} < 1]$ facilitated the polymerization process as indicated by a large increase in the reciprocal lag time (Figure 10). The value of $1/\tau$, however, reached a maximum and decreased again abruptly for $(T/TC)_{pol} > 2$.

These results can be interpreted within a scheme in which one-to-one interactions between two adjacent tubulin and tubulin–colchicine molecules in the polymer induce GTP hydrolysis in a single turnover slow reaction on the incorporated tubulin molecule, so that a burst of 1 mol of GTP hydrolyzed per mol of incorporated tubulin is observed as long as $(T/TC)_{pol} < 1$.

The dramatic changes in lag time and initial rate of GTP hydrolysis upon varying the $(T/TC)_{polymer}$ stoichiometry are in apparent contradiction with a random distribution of T and TC in the copolymer demonstrated by equilibrium measurements. Indeed, random distribution means that a change of position of the two kinds of monomers in the copolymer does not result in a change in interaction energy (Oosawa & Asakura, 1975). A very small interaction energy change, however, could result in an appreciable change in enzymatic activity, and even more in the rate of the nucleation reaction which results from n successive addition steps making an n-fold amplification of the energy change. As an illustration, if we assume that the alternating configuration TC–T–TC–T–TC... is necessary to observe an increased GTPase activity, the maximum activity will be obtained at a stoichiometry $(T/TC)_{polymer} = 1$. A more extensive study would be necessary, however, to propose a more exact model of the possible interactions in the copolymer.

The fact that GTP hydrolysis on polymerized tubulin takes place as a burst phase, as is observed for microtubule assembly (David-Pfeuty et al., 1977; Carlier & Pantaloni, 1981), suggests that, in the curly polymer, too, GTP hydrolysis associated with unliganded tubulin incorporation could be limited by the rate of exchange of GTP for GDP on the polymerized tubulin. It is known that GDP is nonexchangeable on microtubules except at the ends, but the exchangeability has not been measured on the curly polymers. The appearance of a burst contrasts with the linear catalytic GTP hydrolysis of tubulin–colchicine which is independent of polymerization (Saltarelli & Pantaloni, 1982).

The steady-state rate of GTP hydrolysis is also increased upon incorporation of tubulin into the copolymer. We cannot assess here whether the GTP is hydrolyzed on tubulin at the steady state in parallel correlation with nucleotide exchange as in the case of microtubules or if the turnover of GTP on tubulin–colchicine itself is increased upon interactions with tubulin, as has been reported to occur under conditions under which no polymer was formed (Heusèle & Carlier, 1981). Further experiments are needed to elucidate this problem.

It may be worthwhile to point out that although the podophyllotoxin binding site on tubulin overlaps the colchicine site to a large extent (Cortese et al., 1977), tubulin–podophyllotoxin by itself does not polymerize into the same "curly" polymers as TC and cannot be incorporated into the tubulin–colchicine polymer as is unliganded tubulin. The formation of this type of polymer thus seems to depend on the peculiar conformation of tubulin induced by colchicine binding and not by podophyllotoxin (Detrich et al., 1982). However, some other type of interaction between tubulin–colchicine and tubulin–podophyllotoxin takes place, as demonstrated by the inhibition of tubulin–colchicine polymerization into large aggregates (Figures 1 and 9), the formation of a smaller sedimentable polymer, and an increase in the rate of GTP hydrolysis (Figure 6; Heusèle & Carlier, 1981) upon addition of tubulin–podophyllotoxin to tubulin–colchicine. These data emphasize the multiplicity of tubulin–tubulin interactions and correlated effects on polymer morphology and the GTP hydrolysis reaction.

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