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Tubulin Polymerization in Dimethyl Sulfoxide*

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The self-assembly of tubulin devoid of microtubule-associated proteins (MAPs) has been studied using a MES buffer containing dimethyl sulfoxide (Me_2SO). Between 6% and 12% v/v Me_2SO, the tubulin forms polymers which resemble microtubules in their morphology and chemical properties. These Me_2SO microtubules, like normal microtubules, require GTP for assembly and are sensitive to cold, calcium ions, colchicine, and hydrostatic pressure. The polymerization shows a critical concentration which is dependent on the concentration of Me_2SO. 8% Me_2SO was found to be the optimum concentration for microtubule assembly. In these conditions, a linear Van t'Hoff plot is obtained, with $\Delta H^0/kJ \cdot mol^{-1} = 26.5$ over the range 10-35 °C, and $\Delta S^0/J \cdot K^{-1} \cdot mol^{-1} = 186$, in contrast to the assembly with MAPS or glycerol. The kinetics of polymerization shows that the apparent stoichiometry coefficient of nucleation has the value of 2. Ultracentrifugation analysis shows that there are no oligomers present at low temperatures in the absence of free nucleotide, while in identical conditions, tubulin with MAPs does form oligomers. Although the solvent conditions used supported propagation of assembly, nucleation was found to be very dependent on the transiently locally high Me_2SO concentrations formed when Me_2SO was added to initiate assembly. It is concluded that Me_2SO preferentially stabilizes the lateral interactions.

Microtubule protein isolated from brain by cycles of polymerization-depolymerization consists of 75 to 85% tubulin (1-3), the remainder being a mixture of microtubule-associated proteins. Removal of these MAPs[1] leaves the tubulin unable to form microtubules in the usual solution conditions, except at very high protein concentrations. The ability to polymerize at low protein concentrations is restored if the MAPs are added back, or if the solvent conditions are changed by the addition of Me_2SO or glycerol. The glycerol system was studied thoroughly by Lee and Timasheff (4). In glycerol and high Mg(II), however, a mixture of different types of polymer is formed (5). The Me_2SO system was studied previously by Himes (6, 7), but some interesting additional features are reported here. The Me_2SO system has also the advantage, for kinetic experiments, that it is of much lower viscosity than 3.4 M glycerol. The relation between mass concentration of polymer and turbidity is linear in Me_2SO, while it deviates strongly from linearity in glycerol due to nonideality (5).

We found that the characteristics of the microtubule assembly in Me_2SO depend largely on the transient high concentration of Me_2SO obtained immediately after its addition.

In the presence of MAPs, the Van t'Hoff plot of the propagation equilibrium is biphasic. This is attributed to the effect of the MAPs (8). In glycerol, the Van t'Hoff plot is curved, which is explained on the basis of changes in heat capacity, ΔC_p (4). In the presence of Me_2SO, however, the Van t'Hoff plot is linear.

Me_2SO is also known to stabilize microtubules formed in the presence of MAPs (9). These effects of Me_2SO are of interest as Me_2SO is sometimes used as a solvent for microtubule-active drugs (10-13). In this case, a distinction must be made between any effect of the Me_2SO and that of the drug itself.

MATERIALS AND METHODS

Microtubule protein was isolated from fresh porcine brains using the procedure of Shelanski *et al.* (14) modified as described previously (15). The standard buffer used for isolation and purification of the tubulin was 50 mM morpholinoethanesulfonic acid (Aldrich), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (Sigma), 0.5 mM $MgCl_2$, 70 mM KCl, 1 mM NaN_3, pH 6.4, I = 0.1, referred to as MES buffer.

Tubulin was purified by chromatography on a phosphocellulose (Whatman P-11) column (16), loaded with 3 to 4 mg of protein/ml of bed volume. The purified tubulin (PC-T) was frozen in liquid nitrogen immediately after elution, usually in 1 mM GTP. Its purity was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Free nucleotides were removed by gel filtration on Sephadex G-25 (Pharmacia).

Protein concentration was measured by the method of Lowry (17) using bovine serum albumin (Sigma) as a standard.

Polymerization of the PC-T into microtubules was followed by measuring the change in absorbance of the solution at 350 nm using a Cary 118 spectrophotometer, and using the hypothesis of Berne (18) in which turbidity is said to be proportional to the weight concentration of rod-like polymers, independent of their length. Temperature jumps were made using a thermostated cell with a half-time of 1.3 s for a jump between 35 and 4 °C (19), referred to as the fast T-jump cell, or in a cell with a half-time of 17 s (Hellma Cells, Jamaica, NY) referred to as the slow T-jump cell. For very slow or repeated assays, a GTP regeneration system was used (3 mM phosphoenolpyruvate 0.4 μM pyruvate kinase (Boehringer Mannheim GmbH)). The components of the regeneration system had been shown to have no effect on a single fast polymerization.

Me_2SO, analytical grade, was purchased from Merck. Addition of Me_2SO to MES buffer at the concentrations used did not affect the pH.

Samples for electron microscopy were prepared by fixation in 1.25% glutaraldehyde followed by staining with 1% uranyl acetate on collodion-coated grids.

Ultracentrifugation sedimentation analyses were carried out using

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[1] The abbreviations used are: MAPs, microtubule-associated proteins; Me_2SO, dimethyl sulfoxide; PC-T, tubulin purified by chromatography on phosphocellulose; MES, 4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

a Beckman-Spinco model E analytical ultracentrifuge equipped with Schlieren optics and an RTIC temperature control system.

RESULTS

Initiation of the polymerization of PC-T was attempted in three different ways: 1) prewarmed pure Me_2SO was added to a prewarmed solution of PC-T and GTP; 2) prewarmed GTP solution was added to a prewarmed solution of PC-T and Me_2SO; 3) a solution of PC-T, GTP, and pure Me_2SO was mixed in the cold and then warmed. In all three systems, polymers were formed. Only in the first, where Me_2SO is added to a prewarmed solution of PC-T in GTP, did the majority of the polymers resemble microtubules in their appearance and cold sensitivity. In the two other systems, microtubules were formed along with large amounts of sheets and amorphous aggregates. The first system was therefore used as the standard method of initiating polymerization. One reason for the difference between this system and the two others became apparent on studying the kinetics of polymerization.

A limited range of Me_2SO concentration allows the assembly of PC-T into microtubules. At 15% v/v Me_2SO, a significant proportion of sheets of various dimensions is formed, while at 4% Me_2SO, there are amorphous aggregates with the microtubules. Although the other solution constituents, for example Mg(II), are also changed by as much as 15%, this cannot account for the large differences seen in the polymerization. Assembly in 8 to 10% Me_2SO results in the almost exclusive formation of microtubules as judged by their appearance in the electron microscope.

These polymers resemble microtubules formed in the presence of MAPs in that polymerization is inhibited by 100 μM Ca(II) and by 10 μM colchicine, and that addition of these inhibitors to preformed microtubules results in their partial depolymerization. Similar inhibitory effects have been seen by Himes *et al.* (7).

The formation of PC-T microtubules in Me_2SO is temperature-dependent and is reversed on cooling. The final extent of polymerization represents a steady state which can be approached from a lower temperature by assembly, or from a higher temperature by disassembly. It is dependent on the total tubulin concentration, and shows a critical concentration below which no assembly occurs (Fig. 1). It should be noted that above the critical concentration, turbidity increases linearly, showing that the specific turbidity increase is constant, which is in contrast to the glycerol system (5). Since in these conditions only microtubules are formed, turbidity can be used as a measure of the weight concentration of the polymer.

The equilibrium constant for polymerization, K, is calculated from the critical concentration, C_c, by the relation $K = 1/C_c$. Calculation of K in 8% Me_2SO at different temperatures gives the Van t'Hoff plot shown in Fig. 2, from which it can be seen that $\Delta H^0/\text{kJ}\cdot\text{mol}^{-1} = 26.5$, and is constant over the range 10–35 °C, with $\Delta S^0/\text{J}\cdot K^{-1}\cdot\text{mol}^{-1} = 186$.

The polymerization follows an exponential approach to equilibrium, preceded in the slower polymerizations by a measurable lag time. This kinetics is characteristic of spontaneous nucleation, occurring during the lag time, followed by elongation of pseudo-first order kinetics which may be described by the equation

$$dc/dt = (k_+c_1 - k_-)M$$

where c_1 is the concentration of tubulin protomer, M is the product of the number of microtubule ends and the number of binding sites per end to which a protomer can add, and k_+ and k_- are the sum of the association and dissociation rate

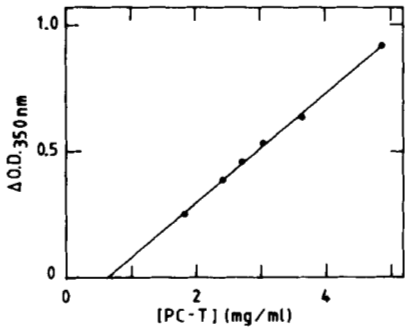

FIG. 1. **The change in turbidity on polymerization of PC-T in MES buffer containing 1 mM GTP and 8% Me_2SO at 35 °C.** The critical concentration, C_c, determined by extrapolation to zero turbidity change is 0.63 mg/ml.

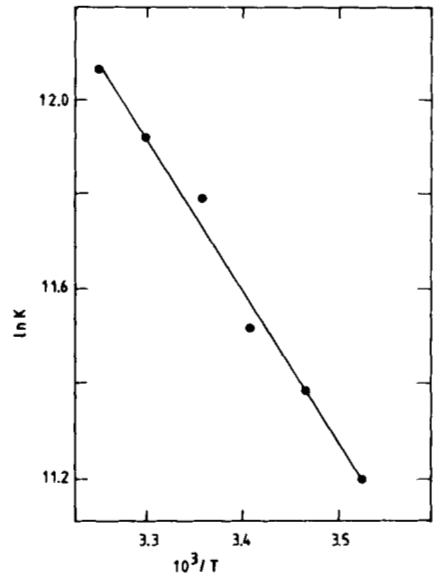

FIG. 2. **Van t'Hoff plot of the polymerization of PC-T in MES buffer, 1 mM GTP, and 8% ME_2SO.** $\Delta H^0/\text{kJ}\cdot\text{mol}^{-1} = 26.5$, and ΔS^0 $\text{J}\cdot K^{-1}\cdot\text{mol}^{-1} = 186$.

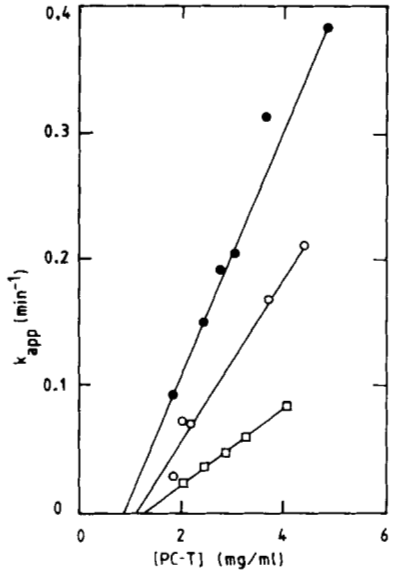

FIG. 3. **Dependence of the apparent rate constant of propagation in 8% Me_2SO on PC-T concentration: ●, at 35 °C; ○, at 30 °C; □, at 25 °C.** From the linear relationship it is calculated that the stoichiometry coefficient of nucleation, n, is 2.

constants of the protomer to and from both microtubule ends (20). During elongation, no further nucleation occurs so M remains constant. The apparent rate constant for elongation, $k_{app} = k_+M$. At lower temperatures, except where there is a high tubulin concentration, the polymerization is slow, resulting in the formation of temperature-insensitive aggregates in addition to microtubules. This can be avoided if the critical concentration, and hence K are determined by polymerization at 35 °C followed by depolymerization at the required temperature, and thus the apparent rate constant for elongation, k_{app}, could not be measured.

At higher temperature, k_{app} varies linearly with the total tubulin concentration, c_0 (Fig. 3). This permits calculation of the stoichiometry coefficient of nucleation, n, since k_{app} is proportional to $c_0^{n/2}$ (5, 21). Thus, for nucleation in these conditions, $n = 2$.

Since n may be influenced by the involvement of rings (15), their possible presence was investigated by analytical ultracentrifugation. However, unlike tubulin in the presence of MAPs, PC-T in 8% Me_2SO does not form 36 S or similar oligomers at low temperatures (Fig. 4).

The rate of polymerization is dependent on the concentration of the Me_2SO as it is added to initiate assembly. Instead of pure Me_2SO, solutions of different concentrations of Me_2SO in MES buffer, were used to initiate assembly. The variation of the apparent rate constant of the first order part of the polymerization, at a final Me_2SO concentration of 8%, is shown in Fig. 5 as a function of the Me_2SO concentration as it was added to initiate assembly. Instead of mixing immediately, as was the usual procedure on the addition of pure Me_2SO, there was a pause of 3 s before thorough mixing.

In the previous experiments, prewarmed Me_2SO was added to a prewarmed PC-T solution. If, however, Me_2SO is added at 0 °C (system 3), and polymerization is initiated by raising the temperature using the slow T-jump cell, the polymerization curve shows three components: a lag phase followed by a first order curve superimposed on an apparently linear increase. The change in absorbance of the solution due to the linear component is not reversed on lowering the temperature (Fig. 6), and some small sheets and aggregates are seen by electron microscopy. Subsequent repolymerizations show successively smaller amplitudes as more tubulin is consumed in the temperature-insensitive aggregates. The apparent rate constant of the reversible first order approach to the steady

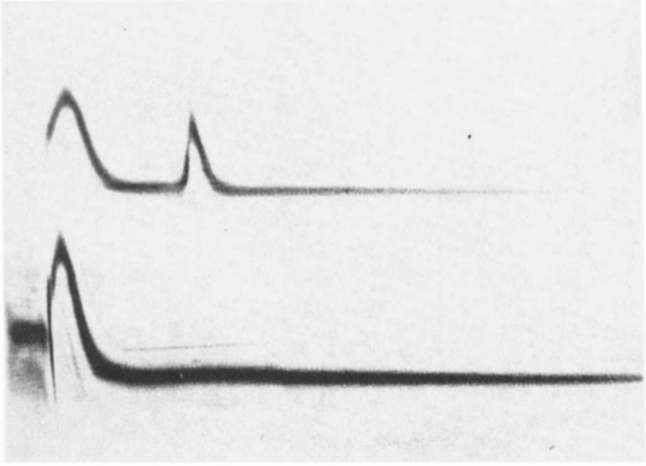

FIG. 4. **Comparison of the sedimentation patterns of tubulin with MAPs and tubulin in Me_2SO at 11 °C.** *Top,* tubulin and MAPs, as prepared by the modified Shelanski procedure described in the text, at 1.9 mg/ml in MES buffer, showing the oligomer peak; *bottom*, PC-T, 1.9 mg/ml in MES buffer and 8% Me_2SO.

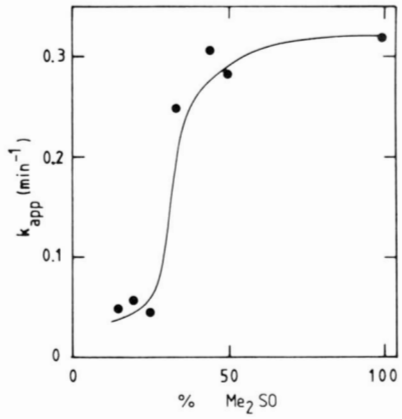

FIG. 5. **The dependence of the apparent first order rate constant, k_{app}, at 35 °C, on the concentration of Me_2SO as it was added to initiate polymerization.** Me_2SO was mixed with different volumes of MES buffer and warmed prior to its addition to the prewarmed PC-T solution of 3 mg/ml. The final Me_2SO concentration in each case was 8%.

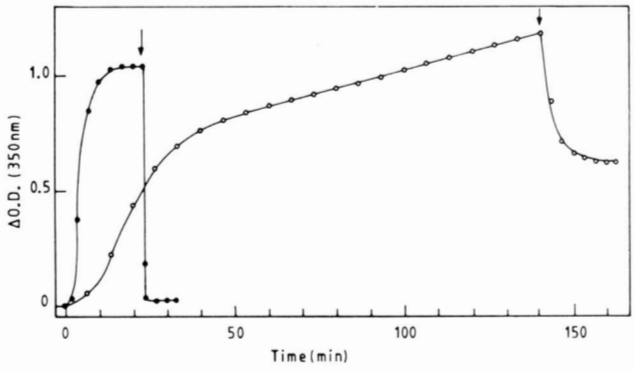

FIG. 6. **Dependence of polymerization on initiation conditions.** Polymerization was initiated by the addition of prewarmed Me_2SO to a solution of PC-T at 35 °C (●——●) or by raising the temperature from 3–35 °C of premixed PC-T and Me_2SO (○——○). In both cases, the final solution contained 5.4 mg/ml of PC-T, 8% Me_2SO, 1 mM GTP, 3 mM phosphoenolpyruvate, and 0.4 μM pyruvate kinase. The *arrows* indicate the time at which the temperature was decreased to 3 °C.

increase of turbidity is lower than that of the first order part of an identical polymerization in which the Me_2SO was added after raising the temperature. The polymerization is unaffected by the length of time, up to 3 h that the tubulin is stored in Me_2SO below 4 °C.

The kinetics of assembly during cycles of temperature jumps was studied for indications of the role of nucleation in high Me_2SO. If microtubules polymerized by system 1 at 35 °C are re-equilibrated at 25 °C, using the fast T-jump cell, and then subjected to a temperature jump back to 35 °C, the apparent rate constant of polymerization is the same for the two polymerizations. If, however, the microtubules are cooled from 35–0 °C, and are stored on ice for 30 min, the subsequent repolymerization on returning to 35 °C is very slow, and resembles that of system 3.

The polymerization requires GTP, and is inhibited by GDP. Addition of GDP before Me_2SO results in the total inhibition of microtubule formation, although cold-insensitive aggregates form slowly at high temperatures. Addition of GDP to polymerized microtubules results in a partial depolymerization. Subsequent polymerization-depolymerization cycles induced by temperature jumps of 35–25 °C in the fast T-jump cell show a reduced k_{app} (data not shown). If the GTP is added

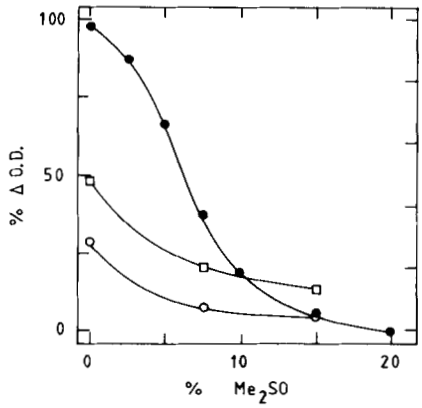

FIG. 7. **Effect of temperature and pressure on microtubules formed in the presence of MAPs and Me_2SO, as a function of the Me_2SO concentration.** The ordinate is the % *A* decrease at 350 nm as compared to the optical density change of the initial polymerization. The change is initiated by cooling from 35–0 °C (●), or by applying pressure: (○) 200 atm at 15 °C, (□) 400 atm at 15 °C.

after the Me_2SO (system 2), a very slow polymerization occurs with the formation of mainly temperature-insensitive aggregates.

Microtubules formed in the presence of both Me_2SO and MAPs are normal in appearance with the characteristic MAP side arms. The polymers are much more stable with respect to cold as reported previously (7, 9) and with respect to hydrostatic pressure (Fig. 7), than are the microtubules formed in either Me_2SO or MAPs.

DISCUSSION

This study shows that microtubules formed from pure tubulin in 8% Me_2SO have many chemical properties similar to those of microtubules assembled *in vitro* in the presence of MAPs, indicating that these properties are due to the nature of the tubulin itself. Pure tubulin from brain assembles in the absence of MAPs or a solvent such as Me_2SO or glycerol, but only with a very high critical concentration (4, 5). Tubulin from Ehrlich ascites tumor cells polymerizes with a C_c of 0.8 mg/ml at 37 °C in the absence of organic solvent and high molecular weight-associated proteins or τ (22). However, it is not clear whether another non-tubulin protein present serves the function of MAPs, or whether the tubulin itself is different. The action of glycerol is to increase the association constant by the preferential exclusion of glycerol on the formation of a tubulin-tubulin contact (4). As in the case of glycerol, Me_2SO must be present in high concentrations (>0.8 M) in order to be effective in promoting elongation, and even higher concentration for nucleation. This suggests that a nonspecific solvent interaction, similar to that of glycerol, may be involved. Circular dichroism studies (11) have shown that 12% Me_2SO does not induce significant structural changes in tubulin. Although the effect of MAPs is the same as that of Me_2SO and glycerol in lowering the C_c, the relatively low ratio of MAPs to tubulin indicates a different mechanism of action.

The thermodynamic parameters found for the polymerization of purified tubulin in 8% Me_2SO (ΔH^0/kJ·mol^{-1} = 26.5 and ΔS^0/J·K^{-1}·mol^{-1} = 186) are comparable with those of other microtubule assembly systems: in glycerol and 16 mM Mg(II), at 37 °C, ΔH^0/kJ·mol^{-1} = 9, ΔS^0/J·K^{-1}·mol^{-1} = 126, and at 23 °C, ΔH^0/kJ·mol^{-1} = 96, and ΔS^0/J·K^{-1}·mol^{-1} = 411 (4); in the presence of MAPs, between 20 and 37 °C, ΔH^0/kJ·mol^{-1} = 33, ΔS^0/J·K^{-1}·mol^{-1} = 209, while between 10 and 20 °C, ΔH^0/kJ·mol^{-1} = 192, and ΔS^0/J·K^{-1}·mol^{-1} = 753 (8). The linearity of the Van t'Hoff plot for assembly in Me_2SO is unusual: that for assembly with MAPs shows a sharp break near 20 °C. In a kinetic study, Johnson (8) showed that this was accompanied by a negative activation energy for dissociation. Such a phenomenon is due to the fact that the observed rate constant for dissociation is a product of a true rate constant and an equilibrium constant for a fast exothermic pre-equilibrium. The latter was assumed to be the binding equilibrium of the MAPs (8).

A study of the assembly in the absence of MAPs was therefore indicated. The system in glycerol is complicated by the strong curvature in the Van t'Hoff plot which was interpreted by Lee and Timasheff (4) as being due to a change in the heat capacity. There are two possible explanations for the linearity of the Van t'Hoff plot in our system: 1) the linearity is representative of the real intrinsic properties of tubulin and the ΔC_p values found in glycerol are due to the solvent contribution; 2) the linearity in Me_2SO is due to a compensation of the intrinsic protein and the Me_2SO solvent contributions such that the net ΔC_p is zero. Our results do not enable us to distinguish between the two possibilities.

The linear dependence of k_{app} on the total tubulin concentration leads to the calculation of the value of 2 for the stoichiometry coefficient for nucleation. *A priori* a much higher value for the cooperativity parameter was expected. Indeed, in the case of polymerization in glycerol and 5 mM Mg(II), a very high value of 10 to 12 was found (5), which is in sharp contrast to the value of 2 for polymerization in the presence of MAPs (15). The interpretation was that the difference is due to the existence of rings: when rings are present they contribute preferentially to nucleation (15). This has been confirmed by Pantaloni *et al.* (23) using radioactively labeled tubulin. As the association number of the rings is already quite high, a small number of rings, or even some dissociation products, is sufficient to form the nucleus probably by lateral association. When polymerization starts from protomers, it is clear that a much larger number of units is required, although association does not necessarily proceed via the formation of rings. Rings are assumed to be side products formed only when the main pathway of microtubule formation is blocked (21). However, we have shown that PC-T in 8% Me_2SO does not form rings.

The apparent anomaly of the low stoichiometry coefficient of nucleation and the absence of oligomers might be explained by the requirement for exposure of PC-T at a high temperature to a transient high Me_2SO concentration. Under these conditions, nucleating centers consisting of several protomers may be formed.

The same dependence of nucleation on high Me_2SO concentration explains the difference in the kinetics of growth when polymerization is initiated by addition of Me_2SO to a prewarmed tubulin solution or by raising the temperature of a premixed tubulin and Me_2SO solution. The conditions during the growth part of the polymerization are identical in both cases, so k_+ should remain constant. As k_{app} is very different, the number of active microtubule ends, *M*, must be different. Similarly, after a partial depolymerization induced by a small decrease in temperature, regrowth occurs by additions to existing microtubules. This occurs faster than repolymerization following the prolonged exposure of the microtubules to 0 °C, during which, presumably, the nucleating centers are destroyed.

Himes *et al.* (7), using 10% Me_2SO in a 20 mM MES buffer, obtained a partial depolymerization on cooling from approximately 37–5 °C. On rewarming, they found turbidity increased again. It is not possible to compare the kinetics of the first and second polymerizations since the temperature equilibration during rewarming was slow compared with the polymer-

ization itself. At 5 °C, turbidity did not decrease to its initial value, and repolymerization was relatively fast, which suggests that short exposure to 5 °C did not cause complete depolymerization. This was confirmed by electron microscopy. Unfortunately, it is not clear whether the first polymerization was initiated by an increase in temperature or by the addition of one of the reagents, which would have been interesting to compare with our results.

An interesting feature is the different Me_2SO requirements for nucleation and propagation: at final Me_2SO concentrations greater than 12%, many sheets of protofilaments, too wide to form microtubules, are formed while 8 to 10% appears to be the optimum concentration for microtubule formation. This indicates that Me_2SO stabilizes preferentially the lateral interactions. Maximal formation of nuclei, however, requires momentary exposure to at least 50% Me_2SO. Carlier and Pantaloni point to this competition between lateral and longitudinal interactions and the different polymer forms that result (5). In view of the dependence of nucleation on Me_2SO concentration, it is surprising that the addition of pure Me_2SO with immediate mixing leads to such uniform results in the apparent first order rate constant of propagation.

Our kinetic results emphasize the importance of considering transient as well as the final solution conditions, especially in the process of nucleation.

The dependence of the polymerization on GTP and the inhibition by GDP resemble those of microtubule polymerization in the presence of MAPs (24). The lower rate of polymerization when GTP is added after, instead of before, Me_2SO, is probably again due to the need for a short lived high Me_2SO concentration in assembly-competent PC-T (*i.e.* with bound GTP) to permit nucleation.

The increased stability of microtubules formed in the presence of both MAPs and Me_2SO with respect to cold and hydrostatic pressure suggests that the cold-sensitive interactions (supposed to be the lateral interactions (25)) are preferentially stabilized by Me_2SO. This seems to be apparent also in the conditions necessary for hook formation (26).

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