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Synchronous oscillations in microtubule polymerization

(phase transition/GTP cap/nonlinear kinetics/GTP hydrolysis)

M. F. CARLIER†, R. MELKI†, D. PANTALONI†, T. L. HILL‡, AND Y. CHEN‡

†Laboratoire d'Enzymologie, Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France; and ‡Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **Under conditions where microtubule nucleation and growth are fast (i.e., high magnesium ion and tubulin concentrations and absence of glycerol), microtubule assembly *in vitro* exhibits an oscillatory regime preceding the establishment of steady state. The amplitude of the oscillations can represent >50% of the maximum turbidity change and oscillations persist for up to 20 periods of 80 s each. Oscillations are accompanied by extensive length redistribution of microtubules. Preliminary work suggests that the oscillatory kinetics can be simulated using a model in which many microtubules undergo synchronous transitions between growing and rapidly depolymerizing phases, complicated by the kinetically limiting rate of nucleotide exchange on free tubulin.**

Conditions have been found such that tubulin polymerization into microtubules *in vitro* exhibits rather persistent oscillations of large amplitude that dampen in the approach to steady state. These oscillations are due to periodic depolymerization and repolymerization processes of the bulk population of microtubules, acting at least partially in synchrony. The oscillations can be conveniently monitored turbidimetrically. This behavior differs strikingly from the classical monotonic growth up to steady state. This phenomenon is consistent with the known nonlinearity in microtubule growth (1, 2) and can be accounted for by synchronous transitions between the GTP-capped and uncapped phases of microtubule ends (3–5). It is worth noting that modest damped oscillations have already been reported, in the approach to steady state, in Monte Carlo simulations of the time course of microtubule growth (5). The present results document another aspect of the "dynamic instability" behavior of microtubules, now widely acknowledged in a variety of *in vitro* and cellular systems (6–12).

The possible biological importance of large periodic changes in the concentrations of both microtubules and free tubulin in the cell is discussed.

MATERIALS AND METHODS

Tubulin was purified through three assembly–disassembly cycles (13) followed by phosphocellulose chromatography (14), and kept at −80°C in 0.05 M Mes, pH 6.8/0.25 mM $MgCl_2$/0.5 mM EGTA/3.4 M glycerol/200 μM GTP at a concentration of 3–8 mg/ml. Before each experiment, tubulin was polymerized for 30 min at 37°C in the presence of 0.4 mM GTP/6 mM $MgCl_2$. Microtubules were pelleted and then suspended in ice-cold buffer P, which routinely consisted of 0.1 M Mes, pH 6.8/1 mM EGTA/12 mM $MgCl_2$/2 mM GTP. After 20 min of depolymerization at 0°C, the solution was clarified by centrifugation at 25,000 × *g*, 4°C, for 20 min and used as such for polymerization. At this stage, tubulin could also be rapidly frozen in liquid nitrogen and used without any loss in polymerization properties for up to at least 3 weeks. Polymerization was carried out at 37°C in a 120-μl thermostated cuvette (optical path, 0.5 cm) and monitored turbidimetrically at 350 nm. The reaction was started by switching the temperature from 8°C to 37°C (rising time, 15 s). Quantitative negative-staining electron microscopy was performed using an EM90 rotor in an Airfuge.

RESULTS

A typical series of polymerization curves in buffer P, at different tubulin concentrations, is shown in Fig. 1. At low tubulin concentration, e.g., 25–30 μM, a classical time course was observed; i.e., a lag time preceded the development of turbidity up to a plateau, following a simple sigmoidal curve. As the tubulin concentration was increased, nucleation was faster and an overshoot appeared in the time course, preceding the establishment of steady state. At higher tubulin concentrations (>50 μM), nucleation was achieved within <30 s, and polymerization was fast, reaching a maximum within 1–2 min that was followed by an oscillatory regime in which the turbidity decreased and increased periodically and substantially. While the amplitude of the oscillations gradually damped until a stable nonoscillatory steady state was reached, the period of the oscillations itself remained at a constant value of 70–80 s that also did not change much with tubulin concentration. Up to 20 oscillations could be observed easily in the best cases, when the tubulin concentration was above 100 μM (10 mg/ml). The phenomenon was perfectly reproducible under standard conditions; however, the amplitude and persistence (damping) of the oscillations varied somewhat with the rate of nucleation.

Electron microscopy of negatively stained samples at different times of the oscillatory process showed the presence of closed microtubules exclusively. These microtubules are identical to the ones that form at a lower concentration of tubulin when oscillations do not occur. Sheets, ribbons, and other known large structures of polymerized tubulin were never observed.

Changes in buffer conditions were made to determine the parameters crucial to the oscillations. Details of these experiments will be reported elsewhere. The main conclusions are the following. Oscillations were observed as well in Mes or Pipes or even phosphate buffer, although to a lesser extent. Polymerization was low and did not show oscillations in imidazole buffer, which is known to complex metal ions. Increasing the ionic strength by addition of either sodium chloride (optimum 40 mM) or potassium acetate, sulfate, or phosphate decreased the damping and increased the amplitude of the oscillations (note that these conditions are closer to physiological). The optimum pH range was 6.5–6.9. The dependence of oscillations on Mg^{2+} concentration in buffer P was studied at 100 μM tubulin: at low (5–6 mM) Mg^{2+} concentration, an overshoot was noticed in the polymerization time course; as the concentration of Mg^{2+} was increased, oscillations appeared (8–9 mM Mg^{2+}) and were

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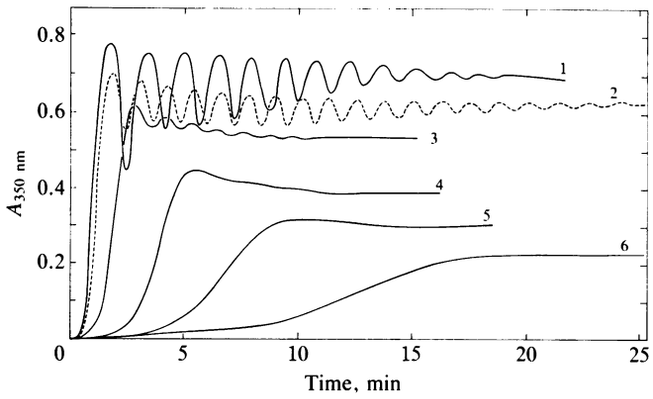


FIG. 1. Tubulin-concentration dependence of the oscillatory kinetics for microtubule assembly. Tubulin was polymerized in buffer P at various concentrations: curve 1, 150 μM; curve 2, 100 μM (dashed); curve 3, 75 μM; curve 4, 50 μM; curve 5, 37.5 μM; curve 6, 30 μM. Turbidity at 350 nm was recorded (light path, 0.5 cm).

observed in the presence of up to 20 mM Mg^{2+}, but the formation of amorphous aggregates increased noticeably above 12 mM Mg^{2+}. No oscillations were observed when 3.4 M glycerol was added to buffer P. Finally, oscillations were also observed in the presence of microtubule-associated proteins (MAPs), either on three times cycled whole microtubule protein in buffer P, or when boiled MAPs (0.2 mg/ml) were added to pure tubulin in buffer P.

The geometry and volume of the cuvette did not affect the oscillations. Oscillations could also conveniently be monitored by light scattering at 90° using a Spex Fluorolog 2 (Spex Industries, Edison, NJ) spectrofluorimeter (370 nm). The extent of light scattering periodic changes represented only 5% of the signal, most of the scattered light being absorbed due to the concomitant increase in turbidity of the solution. Stirring the solution did not affect the oscillations. Therefore we believe that the temporal phenomenon is not associated with some spatial periodic organization of microtubules in the solution.

Role of GTP and GTP Hydrolysis. The GTP concentration dependence of the oscillations is shown in Fig. 2. Tubulin (100 μM) was prepared in buffer P containing 2 mM GTP, then treated with Dowex-1X8-100 in batches to eliminate most of the unbound nucleotide. The resulting solution contained 130 μM free GTP. Various amounts of GTP up to 2 mM were added to several samples of this solution, and each sample was polymerized. In the presence of 130 μM free GTP, only the first maximum in turbidity was reached, then the microtubules depolymerized totally in a monotonic curve. (When 2 mM GTP was added during the depolymerization, repolymerization followed by oscillations was observed.) As the GTP concentration was increased, the depolymerization slowed and eventually the system began to oscillate. However, these oscillations were superimposed onto a slow depolymerization process; no stable plateau was reached until the GTP concentration was high enough to maintain sustained oscillations and a stable steady state for at least 15 min. The fact that, in the presence of a low concentration of GTP, the depolymerization of microtubules causes a turbidity decrease that can be superimposed onto the first turbidity decrease observed in the presence of 2 mM GTP, when oscillations occur, indicates that the oscillations correspond to actual polymerization and depolymerization processes of microtubules. Finally, sustained oscillations were observed in the presence of only 0.2 mM GTP, provided that a GTP-regenerating system was present (2 mM acetyl phosphate plus acetate kinase at 1 unit/ml).

The time course of GTP hydrolysis accompanying the oscillations is shown in Fig. 3. Stepwise waves of GTP hydrolysis were associated with the polymerization phases; no GTP hydrolysis occurred during the depolymerization phases.

The above experiments indicate that GTP is hydrolyzed during the polymerization processes and GDP-tubulin is liberated during the depolymerization processes; tubulin is unable to repolymerize if GTP is not present in sufficient amount to convert GDP-tubulin into GTP-tubulin.

Effect of Breakage of Microtubules on the Oscillations. The data shown in Fig. 1 suggest that oscillations in microtubule polymerization are initiated when a large number of growing microtubules switch to the depolymerization phase. To verify this possibility, microtubules were sheared at the second turbidity maximum and at steady state. Fig. 4 shows that, at a high tubulin concentration, producing many GDP-ends by shearing resulted in much more pronounced oscillations (larger amplitude) than in the unsheared control. Shearing at the plateau also generated a new set of oscillations, although they were more damped than the former ones. In contrast, at a low tubulin concentration, when no spontaneous oscillations were observed, shearing the microtubule solution at the approach to the plateau, although increasing the number of microtubules, did not generate oscillations. These data sug-

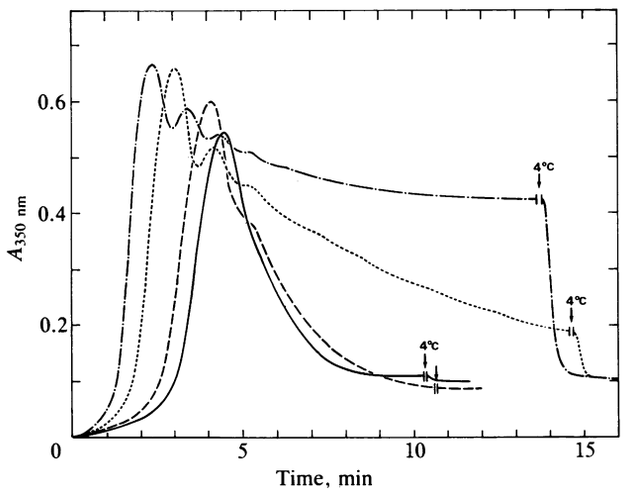


FIG. 2. Role of GTP in the oscillations. Tubulin-GTP (102 μM) was polymerized in buffer P containing various concentrations of free GTP: —, 130 μM; – – –, 230 μM; - - -, 530 μM; – · –, 1730 μM.

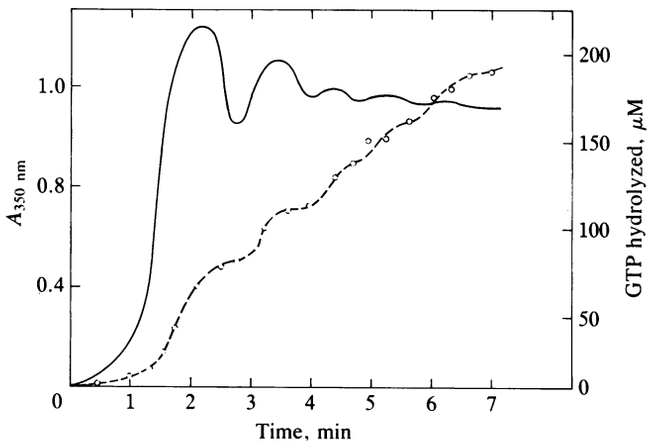


FIG. 3. GTP hydrolysis accompanying the oscillations in tubulin polymerization. Tubulin (120 μM) was polymerized in buffer P containing 1 mM [γ-^{32}P]GTP. —, Turbidity recording (light path, 1 cm); ○—○, GTP hydrolysis, monitored by extraction of ^{32}P-labeled phosphomolybdate complex.

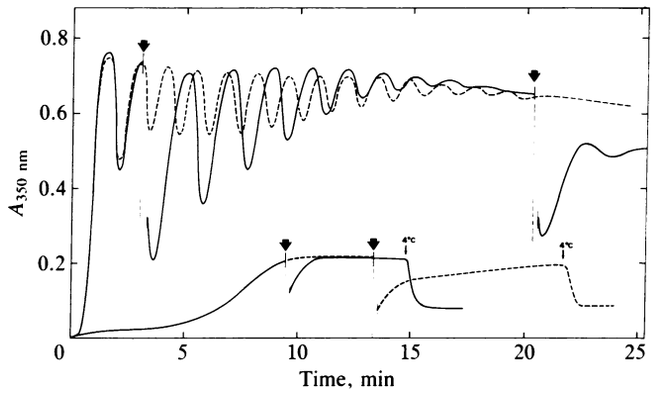


FIG. 4. Effect of breakage of microtubules by shearing on the oscillations. Tubulin was polymerized at 138 μM (top curves) and 27.6 μM (bottom curves). Microtubules were sheared by two passages in a 25-gauge needle, at the times indicated by the arrows. —, Sheared; – – –, unsheared control.

gest that a large number of microtubules may not be the sole factor responsible for the occurrence of an oscillatory regime. Probably a high-enough free tubulin concentration is also an important parameter.

Change in Length Distribution During the Oscillations. The mass amount of polymerized tubulin can be determined by turbidity measurements. To investigate how the number of microtubules and the shape of the length distribution change during the oscillations, histograms of length distribution were derived from electron micrographs of samples taken from a polymerizing 101.5 μM tubulin solution at different times (Fig. 5): at the first maximum in turbidity, the mean length of the microtubules was 13–17 μm (average of several experiments); at the first minimum, it was 7–8 μm; at the second maximum, and at time 9 min, when the oscillations had damped, it was at least 20 μm. These data show that microtubules shorten in the depolymerization process and increase in average length during the oscillations. Comparison of the turbidity and average lengths at first maximum and steady state also indicates that many microtubules disappear in the depolymerization phases and that extensive redistribution to fewer but longer microtubules accompanies the oscillations.

Nature of Free Tubulin During the Oscillations. When the oscillations had damped and steady state was reached, the microtubules were rapidly sedimented at 170,000 × *g* for 3 min at 30°C in an Airfuge. Irrespective of the total tubulin concentration, the amount of tubulin polymerized in microtubules at steady state represented only 35–40% of total tubulin. Tubulin in the supernatant, cooled on ice for a few minutes, repolymerized spontaneously when brought to 37°C, provided that its concentration was above 25–30 μM, and showed marked oscillations above 60 μM. These results demonstrate that free tubulin coexists with microtubules at steady state at a concentration well above the critical concentration. This tubulin is not inactive; once separated from microtubules it behaves like native tubulin. The simplest scheme accounting for these results is that in the dynamic monomer–polymer exchange reactions that are accompanied by GTP hydrolysis, GDP-tubulin is liberated in large amounts in the fast depolymerization processes, and the rate of nucleotide exchange on dimeric tubulin may not be very fast as compared to the rate of other reactions involved. Therefore, a steady high concentration of free GDP-tubulin may be maintained by dynamic instability at steady state. An identical situation has been encountered in the case of actin (15, 16). Rapid sedimentation of microtubules "freezes" free tubulin but, once separated from the polymers and in the presence of excess GTP, GDP-tubulin converts into GTP-tubulin, which is able to polymerize in a second cycle.

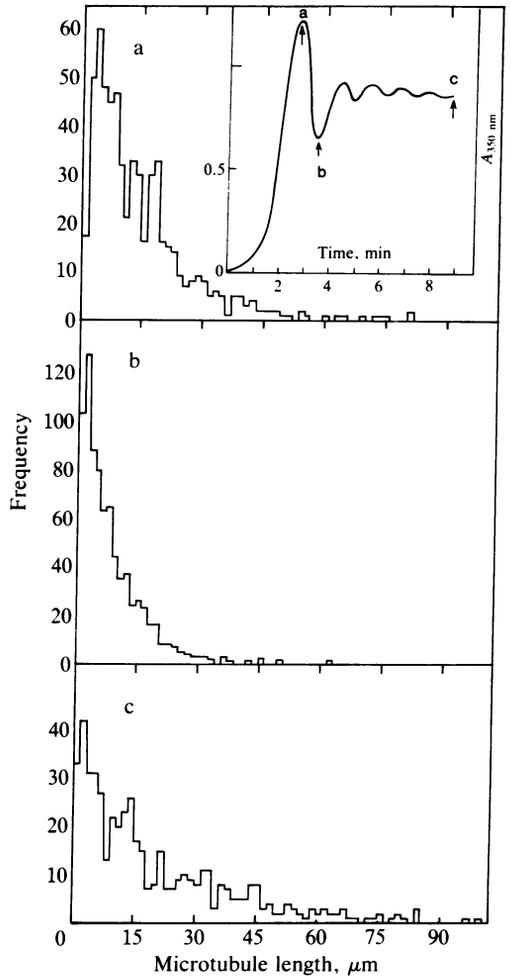


FIG. 5. Changes in microtubule length distribution during the oscillations. Tubulin (101.5 μM) was polymerized in buffer P. Aliquots (30 μl) were taken from the cuvette at various times (*Inset*, arrows a, b, and c), immediately fixed for 1 min by addition of 3 μl of 1% glutaraldehyde at 37°C, and diluted 1:10^5 in buffer P containing 0.1% glutaraldehyde at 37°C. Aliquots (40 μl) of the diluted solution were spun over 200-mesh grids in an EM90 rotor of an Airfuge at 30°C, 170,000 × *g* for 3 min, and these were stained with 1% uranyl acetate.

MODEL AND DISCUSSION

A minimal model able to account for the results presented above includes the following features.

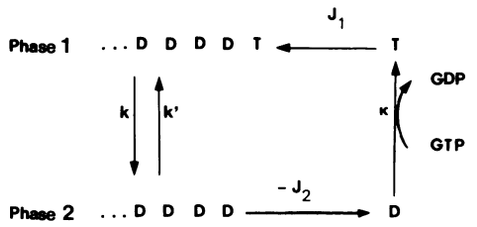
(*i*) At high tubulin concentrations, nucleation is fast and a large number of microtubules are nucleated within a very short time. Because of the high GTP-tubulin concentration, these microtubules grow rapidly and in synchrony. GTP hydrolysis follows with a slight delay (17, 18). When the concentration of free dimeric GTP-tubulin gets low enough, the GTP cap at the ends of microtubules will be very small and will be lost, for an appreciable number of microtubules, leading to the uncapped state and depolymerization. This catastrophic event occurs for a large number of microtubules at about the same time, because all microtubules are in the presence of the same pool of free GTP-tubulin (see *k* in Eq. 5 below).

(*ii*) Microtubule depolymerization leads to the appearance of a large amount of GDP-tubulin that is only slowly con-

verted into polymerizable GTP-tubulin by nucleotide exchange. Thus GDP-tubulin regulates the concentration of GTP-tubulin.

(*iii*) As soon as the concentration of GTP-tubulin is high enough to rebuild a cap of terminal GTP-subunits, many microtubules change phase again and start to repolymerize (see k' in Eq. **5**).

The three steps just described can be repeated over and over, leading to a regime of oscillations between the two phases 1 and 2, as described by the following scheme:



We have made some preliminary attempts to calculate polymerization curves theoretically. Our object at this early stage is simply to establish qualitatively that damped periodic behavior in c_p^* (the concentration of polymerized subunits) can indeed be obtained from the model and theory. Our main approach so far has been to use mean-value differential equations (solved numerically), as described below, although a few Monte Carlo simulations (5) have also been made. The differential equation formulation ignores the possible disappearance of polymers (microtubules) by shortening. In fact, such disappearance (5) requires the use of the alternative stochastic Monte Carlo method. Because we feel that polymer disappearance is indeed important in this system, our future calculations will emphasize the Monte Carlo method.

At first we assumed, as is conventional, that free GDP-tubulin becomes GTP-tubulin very rapidly by exchange. With this assumption, though, we were unable to obtain any periodic solutions that did not dampen very rapidly. The introduction of an exchange step with a finite (one-way) rate constant κ eased this difficulty to some extent.

We assume in this treatment that only one polymer end is active and that the end can exist in two different phases (phase 1, growing, presumably with a GTP cap; phase 2, shortening, presumably with no cap). The differential equations are

$$dc_p/dt = KJ_1c^n, \qquad dp_1/dt = k'(1 - p_1) - kp_1, \quad [1]$$

$$dc/dt = -J_1p_1c_p + \kappa c_D, \quad [2]$$

$$dc_D/dt = -J_2(1 - p_1)c_p - \kappa c_D, \quad [3]$$

where c_p is the polymer concentration, p_1 is the fraction of active ends in phase 1 with growth rate J_1, J_2 (negative) is the growth rate in phase 2, c is the free GTP-tubulin concentration, c_D is the free GDP-tubulin concentration, k is the rate constant for phase 1 → phase 2 at a polymer end, k' is the rate constant for 2 → 1, and K is the equilibrium constant for fast-equilibrium formation of nuclei of size n (we take $n = 7$). The first equation above is based on the approach of Wegner and Savko (19) and of Voter and Erickson (20). The system is closed, with a fixed volume and with total tubulin concentration c_t. The concentration of subunits in polymers is then

$$c_p^* = \bar{N}\, c_p = c_t - c - c_D, \quad [4]$$

where $\bar{N}$ is the mean number of subunits per polymer. At $t = 0$: $c = c_t$, $c_p = 0$, $\bar{N} = 0$, $p_1 = 1$, $c_D = 0$, and $c_p^* = 0$. An example of $c_p^*(t)$, from Eqs. **1–4**, with

$$k = 0.12\, e^{2-c}, \quad k' = 0.12\, e^{c-8}, \quad c_t = 40 \text{ or } 100,$$
$$J_1 = 3c, \quad J_2 = -400, \quad \kappa = 0.025, \quad K = 3.6 \times 10^{-19} \quad [5]$$

is shown in Fig. 6. The units here are μM for concentrations and s^{-1} for rate constants. Steady-state properties ($t \to \infty$) for $c_t = 100$ are

$$c = 6.51, \quad c_D = 22.34, \quad p_1 = 0.9534, \quad c_p = 0.03,$$
$$c_p^* = 71.15, \quad \bar{N} = 2372, \quad \text{oscillation period} = 79.3 \text{ s.} \quad [6]$$

Fig. 6 resembles some experimental curves (e.g., Fig. 1) qualitatively, but the example is not meant to be realistic. At early times, while $p_1 = 1$ and $c_D = 0$, there is a simple relation between c_p and c:

$$c_p = K^{1/2}(c_t^8 - c^8)^{1/2}/2. \quad [7]$$

Unless c_t is small, the steady-state value of c_p is $K^{1/2}c_t^4/2$. This assumes, however (see above), no polymer disappearance by shortening.

A negligible term (here), $(1 - p_1)d \ln c_p/dt$, has been omitted from the equation for dp_1/dt (Eq. **1**).

CONCLUSION

The role of oscillating dynamics in cellular functions, especially in the formation of defined patterns of spatial organization, has been studied extensively (for review, see refs. 21 and 22).

In this paper, we have shown that, under conditions of fast nucleation and growth, tubulin polymerization exhibits oscillations of large amplitude during which microtubules can alternatively shorten and lengthen up to 10 μm with a period of 70–80 s. Such a behavior is consistent with microtubule phase transitions in the region of the critical concentration (1, 2). The results point to the importance of GTP and GTP hydrolysis in the establishment and persistence of oscillations. The oscillatory pattern can be reproduced theoretically using the phase-transition macroscopic model (5), with the addition of the rate of nucleotide exchange on dimeric tubulin. Whether this additional ingredient is sufficient to ensure the buffering of free tubulin necessary to obtain the large amplitudes actually observed remains to be investigat-

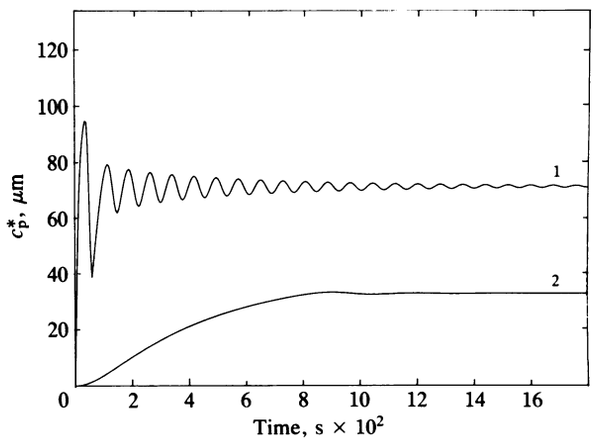


FIG. 6. Simulation of oscillatory polymerization pattern of tubulin. The time course of polymerization, $c_p^*(t)$, is calculated by integration of Eqs. **1–4**, using the parameter values given in Eq. **5**. Curve 1, $c_t = 100$ μM; curve 2 $c_t = 40$ μM.

ed. Note that the formalism will be the same whether κ represents nucleotide exchange on free tubulin or any other rate-determining step preceding GTP-tubulin polymerization—e.g., dissociation of an oligomeric species into dimers or isomerization of GTP-tubulin (25).

The extensive length-redistribution process that accompanies the oscillations has not been considered thus far in the simplified analytical modeling; Monte Carlo calculations are necessary to take this feature into account. However, there does appear to be a qualitative conflict between the model in its present form and the length distributions in Fig. 5. It is probably necessary to include heterogeneous nucleation (20, 26) in the model.

The present experiments allow one to visualize dynamic instability on a bulk population of microtubules that are made partially synchronous by conditions favoring fast nucleation and growth. Whether similar situations exist *in vivo* is an open question. Actually, oscillations of chromosomes have been reported (23) at all stages of mitosis, with amplitudes and periods comparable to the values found here *in vitro*. Also the rates of depolymerization and growth we have observed in the oscillations *in vitro* are similar to those found for the highly dynamic microtubules of the mitotic spindle (24). Therefore we believe that oscillations may have important biological implications in the functional properties of microtubules. In particular, the observation of large periodic changes in the concentration of dimeric tubulin contrasts with the established notion of a steady-state tubulin concentration. If such large changes in the concentration of free tubulin also occur in the cell, one can easily imagine that, once combined with other cellular complexities, they might generate a lively sequence of connected events.

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