A QUANTITATIVE ANALYSIS OF MICROTUBULE ELONGATION

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ABSTRACT

Methods have been developed for differentially inhibiting microtubule nucleation and elongation in vitro. By use of polyanions, assembly-competent tubulin solutions of several milligrams/milliliter can be prepared which do not exhibit appreciable spontaneous assembly during the time-course of an experiment. Microtubule elongation can be initiated by the addition of known numbers of microtubule fragments. A detailed analysis of the resulting process demonstrates that: (a) rings are not obligatory intermediates in the nucleation sequence, and neither rings nor protofilament sheets are obligatory intermediates in the elongation reaction. (b) The end of an elongating microtubule often has a short region of open protofilament sheet or “C-microtubule” similar to that observed in vivo. (c) The development of turbidity follows a simple exponential approach to an equilibrium value. (d) The final equilibrium values are independent of the number of added nucleating fragments, while the initial growth rates and half-times to reach equilibrium are dependent on the number of added nuclei. (e) The final lengths of the microtubules at equilibrium are inversely proportional to the number of added fragments. (f) The equilibrium constants are independent of microtubule length. (g) The number of assembly and disassembly sites per microtubule is not a function of microtubule length. (h) The forward rate constants, the final polymer concentrations, and growth rates of microtubules are dependent upon the concentration of polyanion present. These results are strongly supportive of the idea that microtubule assembly is a “condensation-polymerization” and provide basic information on the kinetics and length distributions of the elongation in vitro.

We have demonstrated previously that several nonneural cells contain inhibitors which will block spontaneous-microtubule assembly when mixed with brain tubulin (6, 7, 8, 9). These “inhibited” brain tubulin solutions contain all the components required for assembly and will support elongation of added sonicated microtubule fragments (7, 9). In sea urchin eggs, the major inhibitors appear to be nonspecific nucleic acids (4, 8, 9). These “natural” inhibitors or added synthetic polyanions appear to sequester an essential heat-stable nontubulin protein(s) present in cycled brain tubulin which is required for both nucleation and elongation under the assay conditions used (8, 9). Here, we support this notion by demonstrating that the morphological effects of adding large polyanions to assembly-competent tubulin are equivalent to removing or “stripping” tubulin solutions of their promoter proteins (8, 9) or “tau” factors (39). We then examine the nucleation sequence after addition of excess promoter protein to either polyanion-inhibited tubulin or tubulin previously
isolated from promoter protein. Finally, we examine the elongation sequence observed after addition of known numbers of sonicated microtubule fragments to tubulin-polyanion mixtures in which spontaneous nucleation is greatly retarded. The results of these analyses support the previous arguments (8, 9), question the role of rings in assembly, and allow a detailed study of the kinetics of elongation. We have determined the forward rate constant for the elongation reaction, calculated and measured the rate of elongation, looked at the influence of polyanion concentration on these parameters and demonstrated that the final length of microtubules is a simple function of the number of nuclei used to initiate the reaction. These results strongly support the idea that microtubule assembly is a condensation-polymerization (21, 31, 32, 33) and further suggest that cellular polyanions may act nonspecifically to block undirected spontaneous microtubule assembly in vivo (34).

MATERIALS AND METHODS

**Tubulin Preparation**

Rabbit brain tubulin was purified by two cycles of assembly using modifications (7) of the procedures described by Shelanski et al. (37). The actual percentage of tubulin α- and β-subunits in each preparation was determined by densiometry of SDS-polyacrylamide slab gels (24). The preparations, after two cycles, routinely contain 83-85% α + β tubulins. Protein concentrations were determined by the procedure of Lowry et al. (26), using bovine serum albumin as a standard.

**Preparation of Inhibited Tubulin Solutions**

For convenience, the polyanion used routinely was polyadenylic acid (Poly A; Miles Laboratories, Inc., Elk hart, Ind.). It is relatively inexpensive and easily quantitated. using the OD at 257 nm and an extinction coefficient of 10.4 mM⁻¹. Inhibited tubulin solutions were prepared by running an inhibition curve on each preparation (8) and using sufficient added Poly A to block the turbidity increase for at least 45-60 min after warming to 30°C. (See text for individual concentrations.)

**Isolation of Nontubulin Factors and Preparation of Stripped Tubulin**

The nontubulin factors were isolated as described previously (8) following the methods of Weingarten et al. (39) using phosphocellulose. When phosphocellulose (PC) was used, a preincubation of the tubulin with PC at 37°C for 15 min and chromatography at room temperature improved the efficiency of the stripping procedure.

**Preparation of Fragments and Calculation of Numbers and Lengths**

To control the solution conditions accurately, sonicated fragments were prepared by a two-step process. Initially, a 1-ml solution of inhibited tubulin was seeded with a small volume (50-100 μl) of sonicated fragments obtained from a spontaneously assembled preparation. These seeded tubules were allowed to grow and to reach equilibrium length; then the OD increase at 350 nm was recorded. The preparation was then sonicated for 20 s with a Branson Sonifier (Branson Instruments, Inc., Stamford, Conn.) at setting 2. Samples were immediately taken for negative staining, and the average lengths of 100 or more fragments were subsequently measured directly from low-power electron micrographs. These fragment solutions were used within 2-3 min after sonication. The number of fragments was determined as follows. The OD₃₅₀ measurement was converted to milligrams/milliliter of polymer using the calibration procedure described previously (7). The values of milligrams of protein per OD₃₅₀ vary somewhat between preparations but typically equal 5.1-5.3 (mg/OD₃₅₀). This is normalized to milligrams of tubulin per OD₃₅₀ by taking into account the proportion of α + β tubulins present in the microtubule pellet (as determined by gel electrophoresis). The conversion factor for the total length of microtubule present is approx. 3.4 × 10⁶ μm of microtubule/mg of tubulin dimer. The average number of fragments is then equal to the total length divided by the average fragment length. In common terms, a 1-ml cuvette with 1 mg of tubulin in polymer contains approx. 3.4 × 10⁶ meters of microtubule. If this is sonicated into fragments of average length = 0.5 μm, the average number of fragments is 3.4 × 10⁶ μm divided by 0.5 μm = 6.8 × 10⁴ fragments (or approx. 1.13 × 10⁻⁸ M fragments).

**Negative Staining, Counting, and Length Determinations**

Carbon-coated, Formvar-covered copper grids were used. Approx. 20 μl of solution were transferred to the grid and allowed to sit for 20 s, then 8-10 drops of 1% uranyl acetate were applied. The excess stain was removed by touching the edge of the grid to a filter paper, and the grids were then air dried. Grids were examined in a Philips EM 300 operated at 60 kV. For counting, several fields were photographed at a suitable magnification; at least 100 tubules were then measured either with calipers (short lengths) or with a map-reading device (longer lengths). The data given are the mean values; the bars are the standard errors of the mean values.

**Curve Fitting**

The data from fragment addition experiments were analyzed using a program which computes the least squares fit of n pairs of data points (xᵢ, yᵢ) for an expo-
nential function of the form $y = ae^{bx}$. The equation is linearized into $\ln y = \ln a + bx$. The coefficients $a$ and $b$ are computed from:

$$a = \exp \left[ \frac{\sum \ln y_i}{n} - b \frac{\sum x_i}{n} \right]$$

and

$$b = \frac{\sum x_i \ln y_i - \frac{1}{n} (\sum x_i)(\sum \ln y_i)}{\sum x_i^2 - \frac{1}{n} (\sum x_i)^2}$$

For straight line data a similar linear regression program was used.

RESULTS

Rings

The rings or doughnuts present in partially purified tubulin preparations have been described by others (5, 13, 14, 22, 30). In these particular preparations after storage in 4 M glycerol at −20°C, there are both closed single and double ring structures (Fig. 1). When sufficient Poly A to block spontaneous assembly is added to a cold solution of tubulin, there appears to be a reduction in the number of rings, although a good quantitative study has not yet been done. The rings which remain are predominately single walled (Fig. 2a). When the inhibited solutions are warmed to 30°C for 2–3 min, no rings can be found (Fig. 2b). Recooling does not result in ring formation (Fig. 2c). This behavior parallels our experience with an insoluble Poly A coupled to agarose, where incubations at higher temperatures were more effective in removing the promoting factors (8, 9). The morphological results are also similar to those observed when tubulin is treated with Poly A agarose or PC (39); the “stripped” tubulin has no rings (Fig. 3a) while reconstitution with the nontubulin factor containing salt wash at low temperature (0–4°C) results in ring formation.
FIGURE 2 The effect of adding a soluble polyanion in the form of Poly A to an assembly-competent tubulin preparation. (a) Addition of a concentration of Poly A which will completely block assembly to a cold (0°C) tubulin solution appears to reduce the number of rings present. The rings which remain appear to be predominately single-walled. This micrograph should be compared with the one shown in Fig. 1. (b) Warming the solution shown in a to 37°C for 3-5 min completely eliminates the remaining rings. The equivalent control micrograph would be Fig. 5. (c) Cooling of the inhibited solutions to 0°C does not regenerate rings. The tubulin concentration is 2.35 mg/ml containing 0.21 mM Poly A as phosphate. × 90,000.

(Fig. 3b). Essentially the same results can be obtained by adding excess salt wash at low temperatures to preparations of tubulin inhibited with Poly A. These results indicate that the rings are probably a cold-stable aggregate of tubulin and the nontubulin factors. Lowering the activity of these factors by complexing with added polyanion reduces the number of rings; adding excess factor reconstitutes the rings.

**Normal Assembly Sequence**

In order to have a background for comparison, the normal assembly sequence of tubules was studied. The results, given in Figs. 4, 5, 6, and 7, show the most common intermediates observed. The results are very similar to those described by Erickson (13, 14) and by Kirschner et al. (23). Within 30-45 s after transfer of the tubulin solution to 30°C, short bundles of some three to five protofilaments are produced which are usually associated with rings (Fig. 4a and b). Within 1 min (Fig. 4c), longer sheets of protofilaments (5-7) are observed which often show the hook described by others (13, 14, 23). At 3 min a wide variety of structures (Fig. 5) are present including sheets running to 11 protofilaments, spiral structures (usually < eight protofilaments) and an occasional closed tube (Fig. 6). Tube closure appears to occur in these preparations by addition of the final one or two protofilaments or subunits which make up these protofilaments (Fig. 7a). The basis for this statement is twofold. First, we never see open sheets with more than 11 protofilaments, and second, if we examine the regions immediately adjacent to the region of closure, we do not find evidence for more than 12 protofilaments. It
seems reasonable that the protofilament sheets are actually "C"-shaped in solution and that the closure event is the addition of the final subunit or subunits, which close the circle. The protofilament sheets represent the C's which flatten during staining. The regions of closed tube which would be thermodynamically more stable and resistant to flattening would produce the images in Fig. 7a and b. A schematic representation of the assembly sequence is given in Fig. 8.

**Assembly after Addition of the Nontubulin Factors**

The mixture of precooled solutions of PC-treated tubulin (PC-tubulin) or Poly A-inhibited tubulin with excess nontubulin factor(s) produces rings (Fig. 3a and b); if these solutions are subsequently warmed, the observed assembly sequence is identical to that described under the normal sequence. The more interesting experiment is to mix prewarmed solutions, rapidly sample and look for intermediates. The first intermediates (within 30-45 s) observed were short bundles of protofilaments, usually three-five in number (Fig. 9). These were never seen, however, in association with rings or ringlike structures. Under these mixing conditions, rings were never observed. Using lower concentrations of the factor, the time necessary for the formation of the protofilament bundles could be increased to several minutes, but no rings or ringlike structures were found. It seems reasonable to conclude that the rings are not an **obligatory** intermediate in this nucleation sequence.

The further assembly sequence is essentially identical with that described under normal assembly (Fig. 8), except that no "hooks" are observed associated with the growing protofilament sheets. Similar closure figures were observed, however, and, after allowing polymerization to proceed in these solutions for 30-45 min, large numbers of microtubules were present. If calcium (2 mM) was
Normal assembly sequence: (a) 30 s after transfer to 30°C. The first detectable intermediates seem to be a ring or perhaps several rings connected to a short sheet of three or more protofilaments. Occasionally rings are seen at both ends of the protofilament sheet (b). (c) By 45-60 s after transfer to 30°C, longer protofilament sheets are found which often have the "hook" described by Erickson (13, 14). At this time, few free rings remain. (a and c) × 188,000; (b) × 250,000.

Absence of Elongation of Microtubule Fragments Added to Factor-Depleted Tubulin

In order to see whether the factors are required for elongation, the following type of experiment was done. Tubulin, as free of factors as possible, was prepared by treatment with PC, O-(diethylaminoethyl)cellulose (DEAE-cellulose), or a sequential treatment first with PC then with DEAE-cellulose. The preparations would not assemble spontaneously at the concentrations and buffer conditions used. To test for elongation, 1 ml of a factor-free preparation at 4 mg/ml with 1 mM GTP was incubated at 30°C for 5 min. This solution was then "seeded" with 10 μl of a sonicated
After 2-3 min at 30°C, no rings are visible but large numbers of open protofilament sheets and "spirals" of these sheets are observed. Both the length and number of protofilaments in a sheet are highly variable. The maximum number seldom exceeds 11 protofilaments in the large open sheets. Single protofilaments are only very rarely observed. x 100,000.

A mixture of 2 vol of stripped tubulin (4 mg/ml) with 1 vol of sonicated fragments (4.5 mg/ml) resulted in a six-fold length increase (0.35-2 μm). We strongly suspect that this elongation, which is only a fraction of that expected at this tubulin concentration, is due to the free factor present in the added fragment solutions. The tubulin itself is able to assemble since the addition of a small amount of crude nontubulin factor to these preparations resulted in a few extremely long tubules, while a mixture of equal amounts of the proteins produced a large number of short tubules.

Elongation of Microtubule Fragments Added to Polyanion-Inhibited Tubulin

The important consideration in this series of experiments was to rule out the possibility that the addition of fragments produced spontaneous microtubule formation in addition to elongation and growth of the added nuclei. The morphological data (Fig. 10) are given for three times after addition of fragments to prewarmed Poly-A-inhibited tubulin solutions. The times correspond reasonably to those shown in the control sequence. Fig. 10a is immediately after addition of fragments (X = 0.35 μm); Fig. 10b is at 40-60 s after addition (X = 0.85 μm) and should be compared with Fig. 4; Fig. 10c is 4 min after addition (X = 2.5 μm) and should be compared with Fig. 5. The data very clearly indicate three things: (a) The average...
length measurements ($\bar{X}$) indicate that the added fragments grow. (b) No free intermediates including rings, protofilaments, or sheets of protofilaments are ever observed, indicating that none of these structures are obligatory intermediates in the elongation reaction. We interpret the absence of these intermediates as evidence that no spontaneous assembly is promoted by fragment addition. (c) Frequently, the end of the elongating tubule exhibits a natural polarity in the form of a short region of protofilament sheet. This could be due to an unraveling during staining; this seems unlikely, however, since the sonicated fragments do not exhibit a similar region. Quantitatively, tubules with a single differentiated end predominate. In 88 cases in which the ends had a region of sheet and were well visualized, 70 of the tubules had a single differentiated end. In the remaining 18 tubules, both ends were splayed out.

The morphological observations are consistent with the idea that the added nuclei are growing by addition of subunits primarily at one end (1, 11), while the failure to find free intermediates argues that fragment addition does not promote spontaneous assembly. In this case, we establish the important point that the number of growing tubules will be equal to and determined by the number of added nuclei or fragments.

**Quantitative Aspects of Microtubule Elongation**

**THE END-ON-ADDITION MODEL***: In order to quantitate the elongation reaction, it is assumed (see references 32 and 33 for discussion) that the assembly of a microtubule occurs in two distinct steps, nucleation and elongation, and that these can be separated using polyanions. A third process called "redistribution" is also presumed to occur (31). This process involves a change in polymer size and leads to an equilibrium length distribution where the number concentration of polymers containing $i$ subunits decreases exponentially with $i$. This redistribution of polymer size is a slow process in comparison with the elongation or nucleation reactions, and for the sake of simplicity we
have ignored it for the present analysis. This introduces one complexity. When the lengths of microtubules are measured or computed, the values given are not final, equilibrium values, but represent the operational values obtained in the absence of redistribution effects.

Under conditions where nucleation is suppressed and redistribution effects are ignored, Oosawa's equations can be rewritten to include terms as follows:

\[
D + aF + T_n \xrightarrow{k_+^*} T_{n+1},
\]

where \( D \) is the tubulin dimer concentration, \( F \) is the nontubulin factor concentration, and \( T_n \) and \( T_{n+1} \) are microtubules with \( n \) and \( n + 1 \) subunits. \( k_+^* \) and \( k_-^* \) are the rate constants for assembly and disassembly, while \( a \) is a coefficient describing the stoichiometry. The conversion of dimer to polymer is given by:

\[
\frac{dD}{dt} = -k_+^*(D)(F)^a \left( \sum_{n=0}^{j-1} T_n \right)
\]

\[
+ k_-^* \left( \sum_{n=0}^{j-1} T_{n+1} \right),
\]

where \( j \) is the number of dimers in a tubule of maximum length and \( i_o \) is the average number of subunits in an added fragment. With end-on-addition and suppressed nucleation, the two
summations are equal. The number of active polymer ends, defined by the summations, are then equal to \( m \), the number of added fragments of average size \( i_o \); this is a constant for a given experiment. This was confirmed by morphological data which ruled out spontaneous assembly after fragment addition.

Since the nontubulin factors appear to complex with the added polyanions (8, 9), the concentration of the factors is reduced and buffered below the original concentration. We make, as a first order approximation, the assumption that the concentration of \( F \) is a constant under the conditions used. With these two constraints, \( (F) = \) constant and

\[
m = \sum_{n=0}^{i_o} T_n = \sum_{n=1}^{i_o} T_{n+1},
\]

eq. 2 can be integrated with appropriate boundary conditions to give:

\[
D(t) - D(\infty) = [D(o) - D(\infty)] e^{-k_e m}.
\]

\( D(o), D(\infty), \) and \( D(t) \) are the concentrations of free, unpolymerized dimer at zero time, at equilibrium, and as a function of time \( t \), respectively, and \( k_e = k_e^* (F)^e \) is a pseudo rate constant which will depend on the buffered concentration of factor and hence on the concentration of added polyanion.

Eq. 3 can be rewritten in terms of the number of moles of dimer in polymer, using the conservation relations:

\[
D(o) = D(t) + C_p(t)
\]

\[
C_p(\infty) = D(o) - D(\infty),
\]

where \( C_p(t) \) and \( C_p(\infty) \) are defined as the number of moles of dimer in polymer at time \( t \) or at equilibrium. Substituting eq. 4 and 5 into eq. 3 and rearranging gives:

\[
C_p(t) = C_p(\infty) (1 - e^{-k_e m})
\]

This equation predicts, as we have previously noted (7, 9), that there will be a simple exponent-
Some examples of the first detectable intermediates present after addition of prewarmed factors to prewarmed stripped tubulin. Rings are never observed. After the formation of the short protofilament sheets, the sequence is identical with that described under the normal assembly sequence. PC tubulin at 1.1 mg/ml and factors at 0.5 mg/ml, 30 s after mixing at 37°C. × 165,000.

The data from one experiment are given in Fig. 11. The results were analyzed using the curve-fitting procedures outlined in the Methods section. The lines are the best fit curves to the experimentally measured values of $C_p(t)$ and $C_p(\infty)$ which are determined using the turbidity assay. The fitting program gives us the product $k+m$. If the average concentration of nuclei is given in moles/liter and the time in minutes, then $k_+$ has units of M$^{-1}$ minutes$^{-1}$.

To see to what extent the reaction is well behaved and reasonably approximated by the equations, we have looked at four different areas: (a) the dependence of $k_+m$ on $m$; (b) the dependence of $k_+$ on the concentration of poly-anion; (c) the velocity of elongation and the relationship between the final polymer concentration, the calculated velocities and poly-anion concentrations; and (d) the dependence of polymer length ($X$) on $m$, the number of nuclei added.

**The dependence of $k_+m$ on $m$:** Fig. 12 demonstrates that there is a reasonably linear relationship between $k_+m$ and $m$ as would be expected if $k_+$ is constant. This observation is critical since it provides strong support for the assumption that the nontubulin factors are buffered by added poly-anions and that the free factor level remains approximately constant under the conditions of our experiment. Two types of data are given in Fig. 12: the $k_+m$ values obtained using the fitting program and the $1/t^{1/2}$ values calculated by measuring the time ($t^{1/2}$) for the turbidity to develop to a half maximal value. Both sets of data are linear with small volume additions. With volume additions greater than 8–10%, the data become increasingly nonlinear although it is not completely clear...
FI6vaE 10 Nucleated assembly sequence. (a) Sonicated microtubule fragments were added to a solution of Poly A-inhibited tubulin, and a sample was removed immediately and negatively stained with uranyl acetate. In these solutions, there are no rings or sheets of protofilaments present. The average size of the nuclei was 0.35 μm × 80,000. (b) Appearance of the growing tubules after 30–45 s at 30°C in polyanion-inhibited tubulin. No free intermediates are present in the form of rings or protofilament sheets. One end of the growing tubule does, however, often exhibit a short region of protofilament sheet. The average length of the microtubules is 0.8 μm × 85,000. This micrograph should be compared with the picture obtained for spontaneous assembly after transfer to 30°C (see Fig. 4). (c) The appearance of the growing tubules approx. 4 min after transfer to a polyanion-inhibited tubulin solution at 30°C. The absence of free intermediates is apparent, and, again, one end of the tubule has a short length of open sheet present. The average length at this time is 2.5 μm × 40,000. This micrograph should be compared with Figs. 5 and 6 in the control assembly sequence. The tubulin concentration is 2.35 mg/ml with Poly A added to 0.21 mM (as phosphate).

The dependence of $K^+$ on the concentration of polyanions

In principle, we would like to know the functional dependence of $K_+$ on free-factor concentration. However, at the moment, there is no independent method for measuring the free-factor concentration. We have therefore pursued the idea that the factors are buffered by added polyanions, in which case increasing the polyanion concentration should lower the nontubulin factor concentration and, in turn, lower the apparent $K_+$ since $K_+ = K_+^*(F)^\alpha$. The dependence of $K_+$ on added Poly-A shown in Fig. 13 follows the general prediction and argues against the added polyanion simply blocking nu-
Figure 11  The kinetics of microtubule assembly in a polyanion-inhibited tubulin preparation after addition of microtubule fragments. The inhibited solution was incubated at 30°C for 20 min before addition of nuclei. The dots are experimental points taken from the spectophotometer trace; the lines are the least-squares computer fit values obtained using eq. 6. The tubulin concentration was 2.35 mg/ml in reassembly buffer with 2 M glycerol, 0.5 mM GTP, and 0.21 mM Poly A (as phosphate). The number of nuclei added were calculated as described in the Materials and Methods section. Curve 1 = 6 x 10¹⁰ nuclei; curve 2 = 18 x 10¹⁰ nuclei, curve 3 = 30 x 10¹⁰ nuclei, and curve 4 = 60 x 10¹⁰ nuclei. The measured average length of the added nuclei was X = 0.62 μm. The maximum volume addition was in cuvette 4 with 0.8 ml of tubulin and 0.1 ml of added nuclei.

Calculation. An extrapolated value of the apparent rate constant was obtained by plotting 1/kₐ versus polyanion concentration. The extrapolated value compares favorably with that published by Binder et al. (3).

The velocity of elongation and polyanion concentrations: After we have measured kₐ for the growth reaction under a given set of conditions, the method for calculating the initial velocity of tubule growth is straightforward. The first derivative of eq. 6 evaluated at t = 0 is simply:

$$\frac{d}{dt}[C_\rho(t)] = C_\rho(\infty) k_\alpha m.$$  (7)

This relationship can then be applied to calculate the initial rates of total polymer formation. The procedure used was to first (a) calculate the initial rate of total polymer formation in terms of molar quantities of dimer assembling into polymer per minute, then (b) convert this to meters of microtubule formed per milliliter per minute using the values given in the Materials and Methods section, and (c) finally convert to the velocity of elongation of an average individual microtubule by dividing the value obtained under (b) by the number concentration of elongating tubules which is determined by the number of added nucleating sites. The calculated values for various polyanion concentrations are given in Fig. 15.

A calculated value was compared with an actual value by a direct determination at one polyanion concentration of the average rate of microtubule growth. The data are given in Fig. 14 as a plot of the average tubule length in micrometers as a function of time after fragment addition. The calculated growth rate from the slope of the line was 0.46 μm/min (±0.025 μm/min) which is in excellent agreement with the value obtained using eq. 7, which was 0.45 μm/min (see Fig. 15, first closed circle). This agreement is important since it demonstrates that the measured rates of turbidity increase are a simple product of the average number of elongating tubules and the rate of elongation of an individual tubule.

One further point deserves to be made using the velocity data. It has been proposed by several workers that microtubules can or must depolymerize along their length in order to move chromosomes (12, 18, 19, 35, 38). This argument implies that the number of sites of subunit insertion will be a function of tubule length provided the simplest assumption, that a disassembly site is also an assembly site, holds. If this were the case, we would expect to see nonlinear be-
concentration of dimers in polymer and if $m$ is the molar concentration of added nuclei, then $\bar{X}$ is the degree of polymerization or number of subunits per polymer. The mean length can be calculated using 8.0 nm per 13 dimers. This form of equation is somewhat cumbersome for expressing the actual data; therefore, we have used an equivalent but more useful form:

$$\bar{X} = \frac{1}{m} C_p(x) + \bar{X}_i. \quad (8)$$

where $\bar{X}_i$ is the average length of the initial nuclei. The data for one experiment are shown in Fig. 16. Fragments were added, allowed to grow to equilibrium, then prepared for negative staining, and the length determinations were done.

Figure 12: Plot of the time constants ($k_+, m$) vs. the concentration of added nuclei ($m$) obtained from the experimental data presented in Fig. 11 (solid circles) and from measurements of the half-time to reach equilibrium (open circles). The molar concentration of nuclei was calculated using Avogadro's number and the number of nuclei per milliliter. The data are reasonably linear for volume additions below 10%, but become increasingly nonlinear with larger additions. The slope of the line gives $k_+$, the pseudo-rate constant.

Behavior in Fig. 14 with an upward curving line, in effect an acceleration as more and more insertion sites are generated. The available in vitro kinetic data (see also reference 3), however, strongly argue against a mechanism in which the number of sites of assembly and disassembly are a function of the length of the microtubule.

The Dependence of Equilibrium Length on the Number of Added Nuclei: A final area that has been looked at involves the relationship between the number of nuclei or fragments which have been added and the final lengths of the polymerized microtubules. Since there is no spontaneous assembly, the total number of tubules at equilibrium (but before "redistribution" has occurred) is equal to $m$, the number added. In this case, the total amount of polymer is given by the following relationship:

$$C_p(x) = m\bar{X},$$

where $\bar{X}$ is the mean final length of the polymers formed. If $C_p(x)$ is given in terms of the molar concentration of dimers in polymer and if $m$ is the molar concentration of added nuclei, then $\bar{X}$ is the degree of polymerization or number of subunits per polymer. The mean length can be calculated using 8.0 nm per 13 dimers. This form of equation is somewhat cumbersome for expressing the actual data; therefore, we have used an equivalent but more useful form:

$$\bar{X} = \frac{1}{m} C_p(x) + \bar{X}_i. \quad (8)$$

where $\bar{X}_i$ is the average length of the initial nuclei. The data for one experiment are shown in Fig. 16. Fragments were added, allowed to grow to equilibrium, then prepared for negative staining, and the length determinations were done.

Figure 13: Effect of increasing polyanion concentration upon the pseudo-rate constant ($k_+$). The solid circles represent measured values of $k_+$ at the indicated concentrations of Poly A (plotted as mM nucleic acid phosphate). The dashed line and open circle are the extrapolated values in the absence of Poly A, which were estimated using the plot of the same data in a somewhat different form as shown in the inset. The tubulin concentration was 2.35 mg/ml (0.8 ml), and $2.4 \times 10^{11}$ nuclei of average length of 0.34 μm were added in a vol of 30 μl.
Calculated Growth Rate
$= 0.46 \pm 0.025 \mu m/min$

![Diagram](image)

**Figure 14** Data for the determination of the average rate of growth of individual microtubule fragments. Tubulin, at 2.35 mg/ml, was inhibited with 0.21 mM Poly A (as phosphate) for 15 min at 30°C, then seeded with 1.2-μm long fragments. Samples were removed as indicated and prepared for electron microscopy. The average length ($X$) was determined by measuring the lengths of approx. 100 tubules at each time-point. The bars indicate the standard error of the mean values. The slope of the line is the average growth rate $= 0.46 \mu m/minute$. The calculated growth rate, using equation 7, was 0.45 μm/min.

The data demonstrate two things: a linear relationship, as eq. 8 predicts, between $\bar{X}$ and $\frac{1}{m}$ and, in addition, an excellent correlation between the $C_p(\infty)$ value determined from the slope of the line ($= 5.5 \pm 0.6 \mu M$ dimer in polymer) and the value calculated from the spectrophotometric data ($= 5.9 \mu M$). In short, the data tell us what we expect intuitively: if there is a constant total amount of dimer in polymer, then the solution which has the fewest microtubules must have the longest microtubules.

**DISCUSSION**

**Morphological Observations**

Rings have been demonstrated in partially purified tubulin preparations by several groups (5, 13, 14, 22, 23, 30). The data presented here demonstrate that the addition of polyanions to a solution of cycled tubulin promotes the breakdown of the rings or 36 S aggregates. The results with PC-stripped tubulin confirm those presented by Weingarten et al. (39) that a nontubulin protein is essential for ring formation (at reduced temperature) and subsequent microtubule assembly at higher temperatures. These results, with the previous demonstration that Poly A-agarose-treated tubulin will not assemble unless these nontubulin proteins are added, strongly suggest that the added soluble polyanions inhibit assembly by complexing or sequestering these promoter proteins and reducing their activity (8, 9). This reduction of factor activity could serve in vivo as a "negative control" to suppress unwanted and unoriented microtubule assembly (see Discussion in references 6 and 34).

The data do not really answer or attempt to answer questions on the nature or specificity of the promoter proteins (cf. references 15, 29, 39) or tell us whether one can circumvent the need for these molecules with, for example, very high Mg$^{++}$ levels (16). Using the present solution conditions, it is clear, however, that this protein or proteins are required for both nucleation and elongation and that the nucleation steps are more...
sensitive to lower promoter activity than the elongation step.

The failure to observe rings in either the nucleated reactions or those experiments involving mixture of prewarmed stripped tubulin with prewarmed promoters indicates that the ring structures normally seen in negatively stained preparations are not obligatory intermediates in either the nucleation reactions or the elongation process. A similar result holds for other free intermediates in the elongation step; none seem to be required. These results argue that the intermediates described here and investigated in a great deal more detail by others (13, 14, 22, 23, 30) represent parts of the nucleation pathway which lead to the formation of a closed tubule.

We would like to extrapolate the present results and argue that free ring intermediates (as defined by negative staining) should not be stable in vivo both because they are thermostable in the presence of nucleotides and because other cellular polyanions should restrict the promoter activity required for their formation. However, it is reasonable to suppose that rings could form in vivo if there are areas of the cytoplasm rich in the factors, for example, in the centrosome region.

There is evidence, on the other hand, for structures which can be interpreted as nucleation intermediates. These are the partial microtubules or C-microtubules routinely observed in the sectioned mitotic apparatus. (For a brief review of this literature, see references 20 and 25)

It is not clear whether these structures are due to fixation problems (28) or whether these partial tubules could be identified with the small region of open protofilament sheet that we observe in our negatively stained preparations. The significance of this region is not yet clear, beyond the fact that it demonstrates a natural differentiation of one end. It seems reasonable to speculate that this may be the growing end, as we have indicated in Fig. 8, and that the short region of open protofilament sheet represents multiple sites of subunit insertion. In this view, the elongation process becomes more complex than a simple single subunit helical addition reaction. A complete description would have to take into account subunit insertion at an undefined number of sites around the protofilament sheet followed by final tube closure as the 13th subunit is inserted. Fortunately, for our present purposes, the optical density assay is dominated by the tubule; therefore, the rate-limiting step will be the addition of the closing subunit.

Other workers (2) have argued that C-microtubules represent microtubules in a state of either assembly or disassembly, and Manton et al. (27) and Cohen and Gottlieb (10) have suggested that C-microtubules are in fact the growing end of a microtubule. The available results are consistent then with the idea that, in vivo, the growing end of a microtubule consists of a short region of "unclosed" tubule or C-microtubule which can be demonstrated under in vitro conditions when the concentrations of promoter proteins are regulated at a low level.
Analysis of the Elongation Reaction

The observations that added polyanions inhibit tubulin assembly and that the nucleation and elongation reactions are differentially sensitive to this addition provided a method for the isolation of the elongation reaction. Despite the complexities discussed above for the morphology of the growing end, the experimental results have been quantified and interpreted using a simple end-on-addition model. The detailed analysis stems from the theoretical work of Oosawa (31), Oosawa and Higasi (32), and Oosawa and Kasai (33) with an extension to take into account the essential factors, the velocity of elongation, the length distributions, and polyanion concentration problems. Other workers have used the end-on-addition model to interpret tubule disassembly induced by cooling (21, see also reference 17) and to interpret the kinetics of tubule growth in vivo (36).

The equations describe the addition of subunits to a fixed number of sites per polymer when the number concentration of polymers is held constant. The analysis makes several simple predictions: (a) The time constants (as $k_m$ or $1/t_2$) should be a linear function of the number of added nucleating sites. (b) The equilibrium polymer concentration $[C_p(\infty)]$ should be independent of the number of added nucleating sites, which is a reflection of the fact that the equilibrium constant is independent of $m$ and tubule length. (c) The final polymer lengths, in the absence of redistribution, should be a function of the number of added nucleating sites. (d) The polymer concentration as a function of time should show an exponential form. (e) The measured initial velocity of elongation should be approximately constant and should agree with the calculated values.

Each of these predictions has been tested and found to hold within experimental limits. It seems reasonable to conclude that the Oosawa model provides a valid, although somewhat incomplete, description of microtubule assembly in vitro.

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