End-to-end Joining of Taxol-stabilized GDP-containing Microtubules*

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By the use of the drug taxol, microtubules were assembled from tubulin that had GDP at its exchangeable nucleotide binding site. By means of dilution experiments and measurements of exchange of labeled subunits, it was determined that the rate of interchange of subunits between these microtubules and the solution is very slow: the upper limit of the dissociation rate constant was measured to be 0.2 subunit s\(^{-1}\) end\(^{-1}\). When they were broken into short pieces by gentle shearing, these microtubules were found to undergo a rapid subsequent spontaneous increase in length. This increase was attributed to end-to-end joining (also called annealing), because dynamic instability and other mechanisms involving either nucleotide hydrolysis or subunit interchange at the ends could be ruled out. To characterize the process, a diffusion-controlled joining mechanism was hypothesized, and a length-independent bimolecular rate constant, \(\Gamma\), was defined. Length distributions were measured at a series of times after the initial shearing. By means of a novel iterative calculation, the best-fitting value of \(\Gamma\) was determined from the time-dependent changes in the length distributions. Fitting was carried out at each of three concentrations of microtubules. The resulting values show that end-to-end joining of microtubules is remarkably efficient and that \(\Gamma\) is concentration-dependent.

Recent studies of the phenomenon of "dynamic instability" have drawn attention to spontaneous changes in the lengths of microtubules in solution (1–5). Although it is clear that growth and rapid collapse of microtubules can come about through this process of GTP-mediated subunit exchange at microtubule ends, other mechanisms may also contribute. By immunogold labeling of mixtures of short microtubules made from different tubulins, Rothwell et al. (6) demonstrated that end-to-end joining, or annealing, of microtubules can occur in vitro. They studied (7) the efficiency of this joining process by means of a detailed analysis of the density of immunogold label along annealed microtubules and concluded that annealing and subunit exchange were comparable in their contributions to the change in length of microtubules. Caplow et al. (5) measured the rate of elongation of microtubules grown from Tetrahymena axonemes in the presence of short pieces of microtubules and concluded that annealing was the predominant mechanism under those conditions. Kristoffersen et al. (3), however, in an analysis of dynamic instability that employed much longer microtubules prepared from distinguishable tubulins, concluded that annealing was probably not an important mode of length redistribution in those structures. In each of these prior studies, conditions were such that both annealing and subunit exchange throughout microtubules were occurring simultaneously, rendering quantitative assessment of either process somewhat uncertain. These studies also lack a framework for concrete analysis of the rate constant characterizing the joining process.

The present investigation was designed to measure the intrinsic rate of end-to-end joining by isolating that process under conditions where subunit exchange is suppressed and where all mechanisms of length redistribution involving GTP hydrolysis can be excluded. Its strategy makes use of the drug taxol, which is known to bring about the assembly of microtubules in the presence of GDP (8, 9) by binding to microtubules with an association constant near 1.1 \(\times\) 10\(^{10}\) M\(^{-1}\) (10), greatly reducing the critical concentration for assembly. Taxol has been found to reduce the rate of endwise subunit exchange to very small values (11, 12). By preparing taxol-stabilized microtubules from tubulin that has only GDP at its E-site, shearing them to produce short pieces, and studying quantitatively the kinetics of the subsequent spontaneous length redistribution, one can follow end-to-end joining in a system free of subunit exchange. By means of an appropriate mathematical model and data-fitting method, one can define an apparent bimolecular rate constant to describe the joining process and measure its value under different conditions. This paper describes a first application of this novel approach.

MATERIALS AND METHODS

Reagents and Protein Preparation—Taxol (Flow Laboratories) was a gift from the National Cancer Institute. Buffers were PM: 0.1 M Pipes, 1 mM MgSO\(_4\), 2 mM EGTA, 0.1 mM dithioerythritol, pH 6.9, or a Mg\(^{2+}\)-free buffer: 0.1 M Pipes, 2 mM EGTA, 0.1 mM dithioerythritol, pH 6.9. Centrifugal gel filtration columns of 5-ml volume were prepared and used as described by Penefsky (13). \([\text{H}]\)GDP (94 Ci/ mmol), obtained from Du Pont-New England Nuclear, was dried and redissolved in aqueous nonlabeled GDP (Sigma, Type II-S) to make a 50 mm stock (14). Phosphocellulose-purified (microtubule-associated protein-free) bovine tubulin was prepared as described in Williams and Lee (15), as modified (16). Protein concentrations were measured by the method of Bradford (17), with tubulin as standard (18).

Exchange of E-site Nucleotide—Methods for replacing GTP with GDP at tubulin’s E-site made use of an earlier study (19) of the relationship between Mg\(^{2+}\) concentration and affinity of tubulin for GTP and GDP. A solution of tubulin, 0.5 mM at approximately 5 mg/ml, was gel-filtered through a Penefsky column into 0.1 mM Pipes, 2 mM EGTA, pH 6.9, to remove free nucleotide. Sufficient \([\text{H}]\)GDP stock was then added to bring the solution to 1 mM. After 10 min at 4 °C, excess nucleotide was removed by a second centrifugal gel filtration into PM buffer. GDP was immediately added to most of this solution to bring it to 1 mM, but an aliquot was reserved. In each experiment the number of moles of nucleotide (GTP + GDP) bound

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per tubulin dimer was assayed in that aliquot by a spectrophotometric technique involving perchloric acid precipitation, and the relative amounts of GTP and GDP bound to tubulin were assayed by high performance liquid chromatography (19). The total number of moles of bound nucleotide (GDP + GTP) lay between 1.85 and 1.90 per tubulin dimer, and the fraction of GDP varied between 47.5 and 49.5. (The distribution of GDP, leading to the conclusion that 95-99% of the E-sites were filled with GDP.

**Microtubule Assembly, Shearing, Incubation, and Grid Preparation**—Microtubules were prepared by diluting a solution of GDP-tubulin to the desired concentration with PM + 1 mM GDP at 22 °C, adding taxol to 50 μM, and allowing polymerization to occur for 60 min. (The temperature was chosen to be close to room temperature for convenience in the shearing step described below, and to avoid the explosively rapid assembly that occurs at 37 °C in the presence of taxol.) Steady state, as measured by turbidity, was reached in about 15 min. The assembled structures appeared to be uniformly microtubular as judged by electron microscopy of negatively stained specimens. When microtubules were pelleted by centrifugation, the supernatant concentration was found to be in the range 0.8-1.0 μM tubulin.

For length redistribution studies, one aliquot of these microtubules was sheared by five gentle passages through a 26 gauge × 1/4 inch hypodermic needle. Another aliquot was left unshered. Samples of each aliquot were immediately withdrawn by means of a pipette with a wide-bore tip and fixed by the gentle addition of 1/6 volume of 8% glutaraldehyde (Polysciences, EM grade), followed by gentle stirring. After 10 min, the samples were diluted to 5 × 10^4 mg/ml, 40-μl aliquots were placed in the sample wells of an EM-90 Airfuge rotor (Beckman Instruments), and the microtubules were centrifuged onto freshly glow-discharge-treated 200-mesh, Formvar- and carbon-coated grids by spinning for 10 min at maximum speed (91,000 g). (The time of sedimentation was arrived at in preliminary experiments which showed that 90% of microtubules were pelleted at 5 min, and 98.9% were pelleted at 10 min.) The grids were negatively stained with 1% uranyl acetate. Incubation of the remaining solution was continued at 22 °C, and further aliquots were withdrawn at intervals for fixation and grid preparation.

**Measurement of Length Distributions**—Electron micrographs of randomly chosen grid squares were taken at ×1000. Lengths of all the microtubules in each micrograph were measured at a final magnification of ×2500 by means of a digitizing tablet and accompanying software (R&M Biometrics, Nashville, TN). More than 300 microtubules were measured in each sample. Microtubules that went off the micrograph were accommodated by multiplying their lengths by 2 (20), a procedure that maximizes the utilization of the micrographs at the expense of a slight broadening of the apparent distribution (21, 22).

**Rate of Dissociation of Assembled Microtubules**—Apparent rate constants for the dissociation of tubulin monomers from the ends of taxol-stabilized microtubules were measured by two methods (11, 12, 23). In the first method, dissociation was measured by the release of [3H]GDP from microtubules following dilution. Tubulin with [3H] GDP at its E-site was prepared as described. Microtubules which had been assembled in 50 μM taxol were separated into two 100-μl aliquots, one of which was sheared. The microtubules in both aliquots were pelleted (10 min at 122,000 × g in the A-100 rotor) to remove small amounts of inactive monomer, resuspended in PM + 1 mM GDP + 50 μM taxol, and then diluted (3-40-fold) with the same buffer to a final concentration of 0.5 μM tubulin. At succeeding 20-min intervals, aliquots were centrifuged (Airfuge, 10 min), and then the pellets and supernatants were separately counted. The apparent first-order rate constant for dissociation, k, (in units of subunits per s per end), was calculated from

\[ \frac{dF_{\text{free}}}{dt} = 2k_f (L) \]

where \( F_{\text{free}} \) is the fraction of the [3H] found in the supernatant and (L) is the measured or estimated mean length of the microtubules in the sample (in units of subunits). The value of k, will be a mean of the actual constants applicable at the two ends of the microtubule. Equation 1 invokes the simplifying assumption that the [3H] appearing in the supernatant corresponds to \([3H]\text{GDP} \) bound to tubulin subunits. It also incorporates the approximation that the number of microtubules in the incubation mixture remains effectively constant. Because the number of microtubules actually decreases with time due to end-to-end joining, only an upper limit on the value of k, (corresponding to the largest value of (L) measured in a given experiment) can be obtained.

In the second method ("exchange"), [3H]GDP-containing tubulin in PM buffer was allowed to polymerize for 60 min in 50 μM taxol and sheared. Unlabeled GDP (1 mM) was then added. At 2-4 intervals, aliquots were centrifuged (Airfuge, 10 min), and supernatants and pellets were counted. The value of k, (again an upper limit) was estimated (11, 24) from

\[ d(\text{free})/dt = \frac{4/(\sqrt{\pi})}{(1/(L)) \sqrt{2k_f}} \]

**Description of the Joining Process**—The data obtained from measurement of electron micrographs describe the temporal evolution of the distribution of microtubule lengths. This evolution is determined by the diffusional mobility of microtubules and by the likelihood that they will join when they make contact. The efficiency of joining can be described by an apparent bimolecular rate constant, the value of which can be obtained by fitting successive length distributions. We describe the method fully here because we believe it is novel and because it may be applicable to other instances of filament joining.

We assume that the joining of two microtubules is a bimolecular process. The rate, \( R_N \), of formation of a microtubule of length \( L_a \), from two shorter microtubules of lengths \( L_i \) and \( L_j \) is given by

\[ R_N = k_i(L_i L_j)/[L_i][L_j] \]

where \([L_i],[L_j]\), and \([L_a]\) are the concentrations of microtubules of lengths \( L_i \), \( L_j \), and \( L_a \), respectively, and \( k_i \) is the rate constant (applicable at the particular concentration) describing the joining of microtubules of those particular lengths. Note that \( k = i + j \). Microtubules of length \( L_a \) also disappear from solution by incorporation into longer microtubules at a rate \( R_a \),

\[ R_a = -[L_a] \sum_{n=1}^{M} k_n [L_n L_a] \]

where M is the index of the largest microtubule in solution. Change in \([L_a]\) can, in principle, also result from spontaneous fragmentation. At early times in the reaction, such fragmentation is probably negligible with respect to the active joining processes and is neglected here.) The net rate of change in \([L_a]\) is given by an appropriate sum of \( R_a \) and the many \( R_N \) values:

\[ d[L_a]/dt = 0.5 \sum_{n=1}^{M} k_n [L_n L_a - [L_a]] \sum_{n=1}^{M} k_n [L_n L_a] \]

In order to specify the manner in which \( k_i(L_i L_j) \) depends on \( L_i \) and \( L_j \), a "model" of the joining process is required. One that is simple enough to be tractable supposes that microtubules have the diffusional properties of long rods. The translational diffusion coefficient, \( D_s \), is then given to sufficient accuracy (25) by the Riseman-Kirkwood approximation (26):

\[ D_s = (kT/6\pi\eta L)^{1/2} \]

where \( L_\text{is} \) the length-to-diameter ratio of a microtubule of length \( L_a \) and \( \eta \) is the frictional coefficient of a sphere of the same diameter as a microtubule. The frequency of collision of microtubules i and j is governed by the geometry of the collision, as well as \( D_i \) and \( D_j \). Hill (27) has shown that the probability, \( p_{ij} \), that ends of two rods will approach within a distance \( \delta \) of each other, while their axes subvert an angle less than \( \theta \), is approximately

\[ p_{ij} = 4\delta^3/(2L_a + 3\delta)^3 \]

The values of \( \delta \) and \( \theta \) thus define the "collision," and the actual frequency of joining is then proportional to \( p_{ij} \) and to A, the probability that joining will result from a collision. Given this simple model,2

2 Microtubules are nonideal solutes. In principle, activities should be employed in place of concentrations. In practice, however, the activities are nearly proportional to concentrations throughout any particular end-to-end joining experiment. This fact, which is fully described under "Discussion," makes it reasonable to incorporate activity coefficients into the rate constant (which thus becomes concentration-dependent) and write concentrations explicitly.
one obtains an expression for the diffusion-controlled value of \( k_2(L_0) \):

\[
k_2(L_0) = A \cdot \left[ N_0(2kT\theta^2/1000d) \right]
\]

where \( d \) is the diameter of the microtubule, \( N_0 \) is Avogadro's number, and the units of \( k_2(L_0) \) are M\(^{-1}\) s\(^{-1}\). The term \((Z_i \ln Z_i + Z_j \ln Z_j)/Z_i Z_j (Z_i + Z_j)\) depends on the lengths of the two joining microtubules, while the term \( A \cdot [N_0(2kT\theta^2/1000d)] \) is length-independent and applies to the joining process as such. We define two convenient combinations of terms

\[
\Gamma = A \cdot [N_0(2kT\theta^2/1000d)]
\]

(9)

\[
\epsilon(L_0,L_d) = (Z_i \ln Z_i + Z_j \ln Z_j)/Z_i Z_j (Z_i + Z_j)
\]

(10)

**Fitting of Measured Length Distributions**—An iterative fitting process was devised to estimate the value of \( \Gamma \) from the data, according to the model just described. \(^*\) A length distribution measured at a particular time was used as the basis for calculation of the way in which that distribution would evolve.

(i) At a particular time, the length distribution is described as a set of \( M \) "bins." The \( k^{th} \) bin contains a number of microtubules, \( N_k(t) \), proportional to \( [L] \) through a constant, \( \alpha \). \( \alpha N_k(t) = [L] \). The measured length distribution at an early time, \( t_0 \), compared to its measured cognate at a later time, \( t \), and the root-mean-square difference between them, \( \chi^2 \), is computed.

\[
\chi^2 = \sum_{i=1}^{M} \left[ N_i(t_0) - N_i(t) \right]^2
\]

(11)

(ii) Beginning at \( t_0 \), the content of a bin a short time later is calculated by a small increment, \( \delta t' \), where \( t' = t + \delta t' \):

\[
N_k(t + \delta t) = N_k(t) + (\alpha/2) \sum_{i=1}^{k-1} (\delta t') \epsilon(L_0,L_d)N_i(t)N_k-i(t)
\]

\[
- \alpha N_k \sum_{i=1}^{M} (\delta t') \epsilon(L_0,L_d)N_i(t)
\]

(12)

The process is reiterated for each bin, to generate a complete set of incremented values.

(iii) The new set of values is compared with the second measured distribution, and a new value of \( \chi^2 \) is calculated.

(iv) Steps 2 and 3 are repeated until the value of \( \chi^2 \) begins to increase. The region of the minimum in \( \chi^2 \) is carefully mapped, and the number of iterations required to arrive at the minimum is designated \( n_{\text{opt}} \).

(v) If the model is applicable, then

\[
\Gamma = (\delta t')n_{\text{opt}}(t_2 - t_1)
\]

(13)

The method gave consistent results over a wide range of values of \( \delta t' \), with values of the bin-width ranging from 1 to 5 \( \mu \)M. The calculated mass was monitored and remained constant to within 1 \( \mu \)M. Estimates of experimental uncertainty in \( \Gamma \) were made by finding those values of \( \delta t' \) at which \( \chi^2 \) was 2\% greater than its minimum. This procedure takes into account the fact that small fluctuations in \( \chi^2 \) of 0.5–1\% of its value arose from the unevenness of the measured length histograms. Hence, the value of \( \pm 2\% \) represents a range of \( \chi^2 \) within which the true minimum was reasonably certain to lie.

**RESULTS**

In preliminary experiments, the rate of dissociation of subunits from microtubules assembled in the presence of 10 \( \mu \)M taxol was found to be comparatively large: 50\% of the incorporated \( [\text{H}] \)GDP was lost within 5 min after a 50-fold dilution (from 10 \( \mu \)M total tubulin to 0.2 \( \mu \)M). In addition, ribbon-like stretches interrupted the tubular profiles of many microtubules assembled from GDP-tubulin in the presence of 10 \( \mu \)M taxol. In contrast, microtubules prepared from 10 \( \mu \)M tubulin in the presence of 50 \( \mu \)M taxol appeared normal when viewed in negatively stained preparations. Profiles were uniform and ends were typically blunt, without extensive fraying. At short times (5–10 min) after the initiation of polymerization, some nonuniform profiles were seen, but they were no longer present at 30 min. Samples were allowed to polymerize for 60 min before being used in the present study.

**Upper Limit of Dissociation Rate Constant**—Table I lists values of the apparent first-order rate constant for release of nucleotides from freshly sheared GDP microtubules in the presence of 50 \( \mu \)M taxol. The rates of dissociation of nucleotide (and therefore of tubulin subunits) were very small, and the experimental uncertainties in the values of \( k_s \) were consequently nearly as large as \( k_s \) itself. No measurable difference was visible between the rate of release of nucleotide from sheared microtubules and unasheared microtubules; because the duration of these experiments was long, a significant fraction of the observed rate of release may have been due to denaturation of protein occurring during the experiment. Despite these limitations, an upper limit of 0.2 subunits end\(^{-1}\)

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\(^*\)This experiment was carried out with aliquots of the solutions employed to measure length changes (Fig. 3).

\(^*\)The values of \( \langle L \rangle \) are estimated from the experiment above. Duration of experiment was 2.5 h.

\(^*\)The value of \( \langle L \rangle \) is estimated. Duration of experiment was 5 h.
s$^{-1}$ may be placed on $k_r$ under the current experimental conditions. This value is so small that changes of microtubule length by processes involving subunit exchange must be inconsequential.

**Observed Length Redistribution**—Fig. 1 shows that taxol-stabilized GDP microtubules, broken into short pieces by shearing at apparent steady state and then incubated at 22 °C, gradually increased in mean length. The process continued for several hours. Qualitatively similar results were obtained at concentrations from 1.5 to 25 μM. This finding establishes the qualitative point that GTP is not necessary for end-to-end joining and shows that the system is amenable to quantitative analysis of changes in length distribution.

Fig. 2 illustrates the change in mean length with time after shearing of microtubules at three different concentrations. The mean length of the sheared microtubules increased rapidly, approximately doubling during the first hour. As the process proceeded, the rate of increase in length decreased. The mean length (near 20 μm) of the control microtubules remained essentially unchanged. Although the shape of the fitted distributions provides a more sensitive test of the model than the simple mean lengths, the means allow comparison of the behavior of the present system with reports in the literature.

A more detailed view of the process appears in Fig. 3, where the histogram bars drawn with broad lines represent (for data taken at one particular concentration) the length distribution of microtubules measured from electron micrographs at a series of times. The mean length increases, as noted, and the distribution broadens markedly during the 3 h of observation. These changes must reflect end-to-end joining of microtubules.

**Fitting of Data**—In order to express concisely the efficiency of the end-to-end joining process, fitting of the measured length distributions was carried out. The calculated temporal evolution of the length distribution in one experiment is shown by the broken histogram tops in Fig. 3. The measured initial distribution was taken as the basis for the calculation. As described under “Materials and Methods,” microtubules were assumed in the calculation to have the diffusional characteristics of long rods, moving freely in solution. Joining was assumed to result from end-to-end collisions. The good agreement between calculated and observed values provides support for the applicability of the model. Calculations carried out to fit data at other concentrations gave similarly close approximation of the calculated to the experimental distributions.

**Numerical Values of $\Gamma$**—Table II contains the values of the length-independent rate constant, $\Gamma$, obtained by fitting the data at different times. An important test of the validity of the novel fitting method employed here is whether it gives consistent results when applied to data obtained over different time intervals within the end-to-end joining process. Although the values of $\Gamma$ corresponding to different time intervals differ

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**Fig. 1.** Taxol-stabilized, GDP microtubules at three times after breakage by shearing. Times after shearing are: top, 1 min; middle, 15 min; bottom, 180 min. The tubulin concentration was 10 μM. This series of micrographs corresponds to the data shown in Fig. 3. The bar represents 5 μm.

**Fig. 2.** Mean lengths of taxol-stabilized GDP microtubules as a function of time after shearing. Three different concentrations are shown: 1.5 μM (○—○); 10 μM (△—△); 25 μM (■—■). The lines represent the trend of the data. The interruption of the abscissa represents the fact that the points ascribed to zero time were taken at a time uncertain by approximately ±1 min.
Annealing of Microtubules

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Suitability of Taxol-stabilized GDP-microtubules for Measuring Rates of End-to-end Joining—The values of $k_-$ in Table I confirm previous findings that taxol dramatically slows dissociation of microtubules. They agree substantially with the value of 0.34 subunits end$^{-1}$ s$^{-1}$ measured by Caplow and Zeeberg (11) under similar conditions (0.1 mM Mes, 0.5 mM MgCl$_2$, 1 mM EGTA, 50 mM taxol, pH 6.8, 37 °C), but for tubulin with GTP at its E-site. The substitution of GDP for GTP may slow the rate of subunit exchange somewhat. The small values of $k_-$ also agree qualitatively with the results of Wilson et al. (12), who found that dissociation of tubulin from microtubule-associated protein-containing microtubules is very slow at moderate concentrations (6.5 mM) of taxol.

When microtubule assembly is at steady state, essentially all subunits added to the ends of growing microtubules must arise from dissociation of existing microtubules. Hence, if $k_-$ is small, then all processes of shrinkage and growth which occur through subunit exchange at steady state must be slow. Because no nucleotide triphosphate is present to serve as a source of free energy for dynamic instability-like processes, the approximate theory of Oosawa (cf. Equation 23 of Ref. 28) must apply, with

$$T_{ax} = \frac{(i/2k_-)}{1/2k_+},$$

where $T_{ax}$ is the characteristic relaxation time of the length distribution toward its equilibrium value, and $(i)$ is the mean number of subunits per microtubule. The maximum value of 0.2 subunits end$^{-1}$ s$^{-1}$ corresponds to $T_{ax}$ on the order of months for microtubules longer than 1 μm. (Carlier and Pantaloni (9) observed length redistribution in taxol-stabilized microtubules similar to that reported here, but attributed it to subunit exchange at the ends of the microtubules. The conditions of polymerization employed in that work yielded many short microtubules (≈0.2 μm). Disappearance of these structures through total depolymerization was hypothesized on the basis of an estimated value of $k_-$ 2 orders of magnitude larger than that found in the present study. Such a mechanism is thus unlikely in the present context.) Hence, measurable redistribution of microtubule lengths through subunit exchange is not expected in these experiments. End-to-end annealing seems the only plausible mechanism. Simple measurements of length versus time therefore suffice to determine the joining rate.

Independence of GTP Hydrolysis—The results establish directly that end-to-end joining is not an "active" process, in that it does not require hydrolysis of GTP to occur.

Order and Molecularity of the Joining Reaction—The bimolecular reaction mechanism summarized in Equation 5 meets the primary test of correctness of a kinetic mechanism: it provides a good approximation of the observed process at each of three different concentrations (Fig. 3). End-to-end joining mechanisms of other molecularities (e.g. unimolecular, zero-molecular, termolecular) must be both more complicated and harder to imagine. Despite this fact, the actual reaction is clearly of less than second order: the observed rate of increase in the mean length of microtubules (Fig. 2) depends only slightly on the microtubule concentration. Similar behavior has been noted previously, in the case of actin (29) as well as microtubules (6, 7), and has been described as approx-

from each other by more than the (somewhat arbitrarily chosen) estimated uncertainty of measurement, they show no systematic trend with time. This fact, together with the excellent fit of the time-dependent broadening of the distribution (Fig. 3), argues strongly that the model describes the data well.

Mean values of $\Gamma$ for each concentration are shown in Table III, along with the standard deviations about the mean. Within the specified range of uncertainty, these values appear to describe the joining reaction under the conditions of the reaction. The data show that $\Gamma$, and hence the rate of end-to-end joining of microtubules of a given length, decreases as the concentration of the solution increases. This observation as well as the implications of the absolute magnitudes of $\Gamma$ are discussed below.

Microscopic Appearance of Microtubules—When observed at high magnification, a small fraction (less than 5%) of microtubules in the annealing preparation display short thickened regions for a short distance along their lengths. The frequency of occurrence of these regions is small, even at late times (e.g. 180 min after shearing). It is not known whether they represent microtubules in the process of joining. If they do, their rarity at late times argues that junctions between microtubules must "anneal" in this system to produce a smooth wall, since almost all of the long microtubules observed at late times have arisen by joining of multiple short ones but show no thickened regions.

**FIG. 3.** Length distributions of taxol-stabilized GDP-microtubules at five times after shearing. Experimental measurements are shown by the solid tops on the histogram bars that indicate experimental measurements. Interrupted tops on the histogram bars represent calculated values (see below). Arrows mark the experimentally determined mean length. The tubulin concentration was 10 μM. Note that the initial distribution is shown on a different vertical scale from the other graphs. Each histogram represents measurements of 250–350 microtubules. The interval (bin size) is 1 μm. Qualitatively similar curves are obtained with intervals of 2 or 5 μm. The calculated values represent successive times in the calculated temporal evolution of the experimentally determined initial distribution.
imulating zero-order kinetics. One might ask how these observations and the consequent concentration dependence of the measured value of $\Gamma$ (Table III) can be compatible with the bimolecular joining mechanism summarized in Equation 5.

Microtubule solutions are clearly nonideal. The way in which nonideality affects the kinetics of a bimolecular reaction can be well approximated in the usual transition-state model by the introduction of thermodynamic activity coefficients (30). If two microtubules, $A$ and $B$, join to form a longer one, $C$, then the rate constant $k$ for the reaction carried out at finite concentration, is given by

$$k = k_0 y_A y_B / y_{AB}$$  \hspace{1cm} (14)  

where $k_0$ is the (ideal) bimolecular rate constant pertaining to infinite dilution, and $y_A$, $y_B$, and $y_{AB}$ are the activity coefficients of the two reactants and of the rodlike transition-state complex. The concentration dependence of $k$ hinges on the concentration dependence of the activity coefficients and particularly on that of the ratio $(y_A y_B / y_{AB})$. At the ionic strength in the present experiments, excluded volume makes the primary contribution to the activity coefficients. Theoretical treatments of its magnitude in mixtures of cylindrical rods of different lengths have been given by Ogston (31), by Ogston and Winzor (32), and on an independent basis by Minton (33). The crucial point made by these authors is that the activity coefficient of a given rod-like solute in a mixture can be calculated from equations (y_A y_B / y_{AB}) and $y_{AB}$.

The constant $\Gamma$ is defined in Equation 9. When multiplied by the length-dependent term $\epsilon$ (defined in Equation 10), it gives the bimolecular rate constant for the joining of two microtubules of specified length. The experimental uncertainties are estimated from the steepness of the minimum of the curve of $\chi^2$ versus time, as described under "Materials and Methods."

### Table II

<table>
<thead>
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<th>Tubulin conc.</th>
<th>Initial time</th>
<th>Final time</th>
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<tr>
<td>$\mu M$</td>
<td>min</td>
<td>20 min</td>
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<tr>
<td>1.5</td>
<td>0</td>
<td>$1.9 \times 10^7$</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>($\pm 0.9 \times 10^5$)</td>
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<tr>
<td>40</td>
<td></td>
<td>$1.1 \times 10^8$</td>
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<tr>
<td>60</td>
<td></td>
<td>($\pm 2.2 \times 10^7$)</td>
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### Table III

Mean values of the length-independent rate constant, $\Gamma$ (means of the values shown in Table II)

<table>
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<th>Tubulin conc.</th>
<th>$\Gamma$</th>
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</thead>
<tbody>
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<td>$\mu M$</td>
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<td>$6.3 \times 10^8$ ($\pm 1.9 \times 10^9$)</td>
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<tr>
<td>25</td>
<td>$8.8 \times 10^8$ ($\pm 0.8 \times 10^9$)</td>
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</tbody>
</table>

In $y_i = \alpha_i m_i + \sum \alpha_{i,j} m_j$ + higher terms
undergo much larger differences between experiments. This fact implies that the activities of species \( L_1, L_2, \) and \( L_3 \) in Equation 5 are nearly proportional to their concentrations throughout the course of the reactions, and thus it explains why the bimolecular mechanism is both correct and successful in describing the observed length redistributions. The values of \( \alpha \) and \( \Gamma \) apply only at the concentrations at which they were measured.

Changes in the extent of nonideality almost certainly account for the less-than-second-order concentration dependence of the observed rates of length redistribution. The major differences in concentration between experiments, however, place severe demands on any attempt to understand quantitatively the overall concentration dependence of \( \Gamma \). Variations in factors other than the thermodynamic activity coefficients of Equation 14 are likely to be important determinants of the changes in rates. As discussed in this context by Caplow et al. (5), microtubules at the concentrations employed here experience restricted diffusional motion. Some of this restriction is mirrored in the thermodynamic activity coefficients and some of it is not, but an adequate theory is lacking. The large decrease in \( \Gamma \) observed between 1.5 and 10 \( \mu \text{M} \) tubulin may represent the onset of substantial diffusional nonideality in the solution. The reason for the lack of further significant change in \( \Gamma \) between 10 and 25 \( \mu \text{M} \) is not immediately obvious.

**Diffusion Control of the Joining Process**—Because short microtubules diffuse more rapidly than long ones, they participate in more frequent collisions and react more rapidly in a diffusion-controlled process. In a sense, each pair of microtubules, characterized by a given pair of lengths, constitutes a distinct set of reactants. The need always to consider the whole length distribution arises from the fact that each such pair has a different value of \( k_b(L_1,L_2) \). The constant \( \Gamma \) thus provides a length-independent measure of the tendency (which is assumed not to depend on length) of microtubules to join with each other, while \( \epsilon \) expresses the length-dependent part of \( k_b(L_1,L_2) \). A crucial aspect of the description is that the joining event occurs much more rapidly than the collision. The good fit of the calculated length distributions to those observed argues strongly that this description applies. The diffusion-controlled process assumed in the present analysis ought to be applicable to end-to-end joining reactions in a variety of contexts.

**Numerical Values of \( \Gamma \)—**The value of \( \Gamma \) under a particular set of conditions yields, through Equation 8, the values of the length-dependent rate constant, \( k_b(L_1,L_2) \), for any given pair of microtubules. For example, \( \Gamma = 6 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) (as seen with 10 \( \mu \text{M} \) tubulin) implies that for joining of a hypothetically pair of 1-\( \mu \text{M} \) microtubules, \( k_b(L_1,L_2) = 1.8 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \). For a pair of 10-\( \mu \text{M} \) microtubules, \( k_b(L_1,L_2) = 290 \text{ M}^{-1} \text{s}^{-1} \). For joining of a 1-\( \mu \text{M} \) to a 10-\( \mu \text{M} \) microtubule, \( k_b(L_1,L_2) = 1.9 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \). These rate constants, as one would expect from the size of the reacting species, are smaller by about 2 orders of magnitude than those found, for instance, in the association of subunits of common oligomeric proteins. These numbers also illustrate the marked decrease in the predicted annealing rate that occurs as microtubules become longer. Such an effect has been observed by Rothwell et al. (6) who noted that annealing rates decreased with increasing time, and that entire distributions of microtubules with large mean lengths annealed less rapidly than distributions with small mean lengths (7). Much of this decrease must occur because the diffusion coefficient decreases rapidly with increasing microtubule length, a fact reflected in the term \( (Z_1 \ln Z_i + Z_2 \ln Z_j)/Z_1 \) of Equation 10. The observed slowing of the increase in mean length with increasing time (Fig. 2) is also in qualitative accord with this aspect of the model.

**Comparison with a Calculated Diffusion-limited Value of \( \Gamma \)—**An illustrative value of \( \Gamma \), the probability that a collision yields a joining event, can be calculated from Equation 9, if appropriate guesses of the quantities \( \beta \) and \( \Theta \) are made. A range of reasonable assignments of \( \epsilon \) and \( \Theta \) gives widely varying predicted values of \( \Gamma \). Arbitrary but reasonable values \( \epsilon = 28 \text{ nm} \) and \( \Theta = 10^4 \text{ s}^{-1} \), for instance, yield \( \Gamma = (2.5 \times 10^5) \text{ A} \). Comparison of this value with the values shown in Table III yields a value of \( \Gamma \) within an order of magnitude of 1. Hence, the joining process, if it is regarded as resulting from endwise collisions, could be remarkably efficient. The geometry of the actual collisions remains unknown, of course, and with it the actual value of \( A \).

**Comparison with Values in the Literature**—Several pieces of evidence indicate that taxol-stabilized GDP-containing microtubules do not differ markedly in their annealing behavior from microtubules observed under more usual conditions. When allowances are made for differences in concentrations, temperatures and microtubule lengths, the rates of increase of mean length shown in Fig. 2 are comparable within an order of magnitude with those previously observed with unstabilized GTP-containing microtubules (Table IV). Rothwell et al. (Table I of Ref. 7) noted, in direct comparisons, that taxol produced only a small difference in annealing rate when length and concentration are taken into account. In a simple analysis that did not explicitly consider the effects of length, Rothwell et al. (6) inferred a value of \( k_b(L_1,L_2) \) of about \( 5 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) for annealing of microtubules of mean length about 1 \( \mu \text{M} \), giving a value of \( \Gamma = 1.7 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \), in fair agreement with the values observed here. Hence, end-to-end joining is not only possible in the absence of GTP, but it appears to proceed roughly as fast as it does in the presence of GTP. Unless taxol-stabilized GDP-containing microtubules anneal more rapidly than nonstabilized microtubules, these observations imply that annealing must make a contribution to in vitro length redistribution that is not negligible in comparison to the contribution of dynamic instability.

**Limitations of the Analysis**—The diffusion-controlled reaction mechanism presented here has the virtues of being

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**Table IV**

| Approximate values of rates of change of mean microtubule length |
|------------------|-----------------|-----------------|
| Experimental conditions | Observed change | Ref. |
| 50 \( \mu \text{M} \) tubulin, 37 °C | 10-70 \( \mu \text{M} \) in 60 min | 2 |
| 50 \( \mu \text{M} \) tubulin, 37 °C | 3.6-6.2 \( \mu \text{M} \) in 60 min | 7 |
| 10 \( \mu \text{M} \) tubulin, 37 °C | 12-20 \( \mu \text{M} \) in 60 min | 6, Fig. 10 |
| 10 \( \mu \text{M} \) tubulin, 37 °C | 1.1-3.3 \( \mu \text{M} \) in 30 min | 5 |
| 58 \( \mu \text{M} \) tubulin, 37 °C | 32.0-50.8 \( \mu \text{M} \) in 30 min | 3 |

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*Note:* The fact that a length-independent constant successfully characterizes the kinetics of joining of microtubules may not require the strict endwise collisions envisioned in Equation 7; other types of encounters between microtubules might also fit the model. Details are a matter for future work.
simple to understand, possible to apply to real systems, and reasonably accurate in its quantitative prediction of experimental results. It has several limitations, which might be overcome in a fuller treatment. 1) The fact that microtubules are actually somewhat flexible implies that Equation 6 may be slightly in error. 2) The neglect of the reverse reaction (i.e. fragmentation) in Equation 5 introduces an inaccuracy of unknown magnitude. It is likely to be most pronounced at late times in the course of the reaction. 3) In the fitting process, a plot of $x^2$ versus the number of iterations is somewhat "noisy" due to the limited precision of the data, rendering the estimates of the error in $\Gamma$ problematical. 4) Although $\Gamma$ is an experimentally defined number, its interpretation depends on a collisional model such as that represented by Equation 7. Endwise collisions may not be the only processes productive of joining: if microtubules are slightly "sticky," side-to-side collisions followed by rapid sliding of one microtubule over the other might also result in joining. A fuller treatment would consider the statistics of such processes.

The results, strictly construed, apply only to taxol-stabilized microtubules. Their extension to the general case requires the assumption that effects of taxol on the joining reactions are minimal. 6) Some inadvertent breakage of microtubules may occur during the fixation and grid-preparation process (21, 22). This work, in common with other studies, lacks a rigorous control for this possibility.

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