Concerning the Efficiency of the Treadmilling Phenomenon with Microtubules*

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Radioactive tubulin subunit incorporation into porcine and dogfish shark brain microtubules which are at steady state has been found to result primarily from a diffusional reaction, in which subunits are incorporated although there are an equal number of tubulin subunit additions to and losses from each of the two microtubule ends in a unit of time. Treadmilling is very inefficient, and the Wegner s-values are equal to 0.0065-0.001.

At steady state there are approximately 5000 (pig brain) or 2500 (dogfish brain) tubulin subunits lost from the two microtubule ends/s; an equivalent number of subunit addition reactions maintains a constant microtubule mass. The rate constants for subunit loss and addition with porcine brain microtubules, determined from analysis of the steady state rate for radioactive subunit incorporation, are much larger than those measured previously, when the rate constants were determined from the disassembly rate following perturbation of the steady state by dilution (Zeeberg, B., Reid, R., and Caplow, M. (1980) J. Biol. Chem. 255, 9881-9899). To account for this discrepancy it is suggested that at steady state the microtubule is capped by a short finite length of tubulin-GTP subunits, which undergo extremely facile association and dissociation (2500-5000 subunits/microtubule/s). This cap would be rapidly lost following dilution so that the observed rate only measures the relatively slow loss of tubulin-GDP subunits (129 subunits/microtubule/s) which had been in the interior of the microtubule; this is not equal to the rate constant for subunit loss (and addition) from the ends at steady state. Because of this, previous estimates of the Wegner s-value for treadmilling which utilized dilution for determining the steady state molecular rate constants for subunit loss are believed to be too high.

The efficiency of treadmilling may be quantitatively expressed in terms of the number of tubulin subunit addition reactions (or subunit losses) required for the net incorporation (or loss) of a tubulin subunit into a microtubule at steady state. From studies of the rate of the pre-steady state elongation of the distinguishable ends of flagellar seeds by brain tubulin subunits, and the rate following dilution for loss of microtubule mass from the two ends, a Wegner s-value equal to 0.07 has been derived (11). This means that it requires 14 (i.e. 0.07^-1) tubulin subunit additions (and losses) to the two microtubule ends for a net addition (or loss) of a tubulin subunit to one end of a microtubule. Bergen and Borisy (11) constrained this relatively low efficiency with the s-value at 1.0, implicit in an earlier study (1). This discrepancy was subsequently resolved (12).

We have previously determined the efficiency for subunit treadmilling with porcine brain microtubules by analysis of the rate for radioactive subunit incorporation at steady state, and the rate for dilution-induced disassembly (12). The Wegner s-value calculated from these results was 0.015. More recently, we have studied the rate for radioactive subunit incorporation into steady state microtubules formed with MAP-free tubulin in the presence of taxol (13). This reaction was found to be sufficiently slow so that the diffusional component for radioactive subunit incorporation (12) could be clearly observed. Diffusional subunit incorporation results from the fact that even when there are equal numbers of additions to and losses from each of the two microtubule ends in a unit of time, because of the statistical nature of this process there is net incorporation of subunits into a portion of the ensemble of microtubule ends. When the results obtained

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in the presence of taxol were quantitatively analyzed using a rate equation which describes subunit incorporation resulting from a diffusional reaction (Equation 13 in Ref. 12), it was possible to determine the molecular rate constants for steady state subunit addition and loss. In contrast to this approach where the rate constants are obtained without altering the steady state, in earlier studies of porcine brain (12), bovine brain (14), and flagellar outer doublet (14) microtubules, as well as the heterologous microtubules formed on flagellar seeds (11), the steady state molecular rate constant for tubulin subunit dissociation was determined from study of microtubule disassembly following perturbation of the steady state by dilution. It is, therefore, of interest to make a comparison of the steady state molecular rate constants derived from studies without perturbation of the steady state, to those obtained previously (12). This has been found to be feasible using a quench procedure, which allows determination of the initial phase for radioactive tubulin subunit incorporation, during which time the diffusional component for the reaction is the predominant path. This method has also been used for analysis of the kinetic properties of steady state microtubules formed from dogfish shark brain, which contains an altered MAP composition (15, 16). We also describe here an extension of our previously published theoretical analysis of steady state subunit incorporation (12), which allows a determination of the four rate constants for tubulin subunit addition to and loss from the two microtubule ends.

**EXPERIMENTAL PROCEDURES**

**Tubulin Purification**—Dogfish shark brain tubulin was purified through two cycles of assembly-disassembly as described previously (15). The pelleted microtubules, which had been stored frozen at −70 °C, were dissolved in ice-cold reassembly buffer (100 mM 2-(N-morpholino)ethanesulphonic acid, 1 mM EGTA, 0.5 mM MgCl₂, pH 6.9) and centrifuged at 4 °C for 30 min at 35,000 rpm in a Beckman 40 rotor. Contaminating glycerol and excess nucleotide were removed by chromatography on Sephadex G-25. Porcine brain tubulin was prepared as described previously (17). Porcine brain MAPs were purified by phosphocellulose chromatography (18), and the NaCl used for elution was removed by chromatography on Sephadex G-25. Proteins were analyzed by sodium dodecyl sulfate-gel electrophoresis (19).

**Materials**—A stock solution of acetyl kinase (obtained from Sigma) was prepared fresh for each experiment by dissolving a small quantity of the crystals in reassembly buffer; the ammonium sulfate was removed by column centrifugation (20). Ethanol and contaminating tritium oxide were removed from [3H]GTP (obtained from New England Nuclear) by evaporation of an aliquot of the stock solution at 37 °C.

**Microtubule Length Determination**—Aliquots of steady state microtubules which had been diluted into glycerol-reassembly buffer (36 volume % glycerol) were applied to formvar-carbon coated grids for 20 s and then stained with 2% uranyl acetate. The lengths of approximately 500 microtubules were determined, as described previously (12). It is necessary to know the average microtubule length in order to determine the specific activity of a tubulin dimer in a microtubule which has been formed in the presence of [3H]GTP (see below). It is also necessary to measure the distribution of microtubule lengths so that it is possible to correct for the fraction of microtubules which become fully labelled during flux experiments. That is, a microtubule no longer contributes to the rate of subunit flux after all of the tubulin subunits within this microtubule have fully exchanged with radioactive tubulin dimers in solution. The fraction of microtubules which are not fully labeled at different stages during subunit flux is given in the inset for Fig. 1; these values were calculated from the histogram representing the frequency of microtubule lengths in the population of microtubules (Fig. 1). For example, the histogram indicates that 9.7% of the microtubules had a length of less than 1.0 micron. Since there are 1639 dimers/micron of microtubule length (21), the fraction of microtubules which remain to be labeled after 1639 radioactive dimers have been incorporated in the microtubules is equal to 1.0 − 0.097 = 0.903. Thus, the observed rate can be corrected for the progressively smaller fraction of microtubules which contribute to the reaction. This was a relatively small correction since the reaction was followed during a time course in which an average of only about 3000 radioactive subunits were incorporated per average microtubule.

**Incorporation of Radioactivity into Microtubules at Steady State**—Dogfish shark brain tubulin (25.2 μm) was assembled to steady state at 37 °C in the presence of 10 mM acetyl phosphate and 30 μM added GTP, using acetyl kinase (approximately 0.1 units/ml) to regenerate GTP. Porcine brain tubulin was reacted under similar conditions, using 1.0 mM ATP instead of acetyl kinase and acetyl phosphate. Spectrophotometric measurements of the solution's turbidity indicated that a steady state was attained within 10 min and the mass of microtubules formed remained constant for the duration of the kinetic measurements. Following the addition of a small volume containing a trace amount of [3H]GTP (7.5 Ci/mmol), 10-μl aliquots were removed after varying time intervals and diluted into 200 μl of glycerol-reassembly buffer (32 volume % glycerol). The microtubules in these diluted aliquots were subsequently pelleted by centrifugation through a 7-ml cushion of glycerol-reassembly buffer (36 volume % glycerol) using a Beckman Ti 50 rotor (35 °C, 30 min, 50,000 rpm). Radioactive nucleotide in the pellet was extracted by a 20-min incubation with 5% (w/v) HCIO₄ and measured in a scintillation counter. In order to determine how much radioactivity incorporated would correspond to 100% labeling of the microtubule, an identical microtubule assembly reaction was carried out in which the [3H]GTP was present when assembly was initiated. Based upon the fact that the amount of radioactive nucleotide found associated with the microtubule which had been coassembled with [3H]GTP remained constant for 1.5 h following a 21-fold dilution into 32 volume % glycerol-reassembly buffer, it is concluded that radioactive subunit dissociation does not occur after dilution. It is also concluded that radioactive subunit association and dissociation from the microtubule cease after a 21-fold dilution of steady state microtubules into 32 volume % glycerol-reassembly buffer, since the amount of radioactivity in the microtubule pellets did not depend upon the duration of contact with the glycerol-reassembly buffer. For example, in the experiment shown in Fig. 2, every alternating time point was obtained with microtubules which had been in contact with glycerol-reassembly buffer for either 15 or 60 min, respectively.

In a typical experiment (Fig. 2) the specific activity for the reaction in which the [3H]GTP was present when microtubule assembly was initiated was 1.132 (cpm/dimer) (microtubule/pellet). This was calculated as follows. The pelleted microtubules contained 9230 cpm and their average length was 4.97 microns (Fig. 1). Since there are 1639 dimers/micron of microtubule length (21) the specific activity is 9230 cpm/(4.97 microns × 1639 dimers/micron) = 1.132 (cpm/dimer) (microtubule/pellet). This is not, however, the same as the specific activity of a dimer when subunit flux is initiated, since when flux starts the initially added [3H]GTP is not diluted by the guanine nucleotide which will exchange out of the microtubule when subunits are incorporated. Dilution of the specific activity...
progressively occurs as nonradioactive dimers which are in the microtubule exchange with radioactive dimers in solution; we next describe how the specific activity for radioactive dimers was calculated. [3H]GTP added to the steady state microtubules is diluted by: (a) excess GTP which is added prior to the microtubule assembly; (b) guanine nucleotide at the E-site of tubulin dimers which had not been incorporated into the microtubule; there is no contribution from the E-site of tubulin dimers which are released from the microtubule as a result of subunit flux. In order to minimize contributions by (b) and (c) the amount of added excess GTP (a) was relatively high (28.7 μM).

It is not possible to work at a significantly higher added GTP concentration without excessively reducing the specific activity of the added [3H]GTP and thereby making measurements of subunit incorporation less sensitive. To determine the contribution of (b), two experimental results are required. First, the amount of E-site guanine nucleotide in the polymerized and unpolymerized tubulin was determined by spectrophotometric measurement (256 nm) of the deproteinized solution following perchloric acid treatment (2 min at 100°C with 2.5% HClO4). It was assumed for dogfish tubulin, based upon earlier results with porcine brain tubulin (22), that half of this nucleotide is N-site and half is E-site. The so-calculated total E-site guanine nucleotide is present in the polymerized and unpolymerized tubulin was determined by spectrophotometric measurement (256 nm) of the deproteinized solution following perchloric acid treatment (2 min at 100°C with 2.5% HClO4).

It has been previously found that dogfish shark brain microtubular protein preparations are primarily composed of tubulin, with relatively low amounts of a single high molecular weight protein (see gel scan 2a in Ref. 15). The absorbance profile of a fast green-stained sodium dodecyl sulfate gel of the protein used in the kinetic studies described here is shown in Fig. 3. A single high molecular weight band, which has a shoulder derived from a lower molecular weight band, is observed; several other protein bands of intermediate and low molecular weight are present in lesser amounts. When the dogfish tubulin is assembled into microtubules in the presence of exogenously added purified porcine MAPs, the isolated microtubules contain significant amounts of both MAP 1 and MAP 2 (Fig. 3B). This result shows that the gel system is capable of resolving MAP 1 from MAP 2, and that microtubules formed with dogfish shark microtubular protein are able to coassemble with both MAP species.

Subunit Incorporation—In order to analyze the dynamic properties of microtubules at steady state, the rate of incorporation of radioactive subunits was studied. The procedure used in these experiments is to assemble the tubulin to steady state (which requires about 10 min), at which time a trace quantity of [3H]GTP is added. Aliquots taken from this mixture at sequential time points are diluted into a microtubule-stabilizing medium and the labeled microtubules are isolated free from unpolymerized radioactive subunits by centrifugation through a glycerol cushion. In order to determine how much radioactivity would correspond to 100% labeling of the microtubule, an aliquot of unpolymerized tubulin is coassembled with an aliquot of [3H]GTP identical with that used in the rate measurements. These microtubules are similarly diluted in the microtubule-stabilizing medium prior to isolation, by centrifugation through a glycerol cushion. The time course for radioactive subunit incorporation in a representative experiment with dogfish shark brain tubulin is shown in Fig. 2; equivalent results were obtained in four separate experiments. Results with porcine brain tubulin are described in Fig. 4. In both reactions the rate of incorporation of radioactivity is very rapid immediately after the addition of [3H]GTP and this rate falls off with time.
FIG. 4. Incorporation of radioactive guanine nucleotide into porcine brain steady state microtubules at 37 °C. The triplicate zero time determinations were done with tubulin which had been maintained at 0 °C (without added ATP) prior to the glycerol quench; identical values are obtained when nonradioactive steady state microtubules are diluted into a glycerol quench mixture containing [3H]GTP. The inset shows the time course for [3H]ATP hydrolysis.

The calculated total subunits incorporated (column 8 in Table I) is plotted versus \( \left( \frac{2}{\pi} \right)^{1/2} (s)^{1/2} \) in Fig. 5. Fig. 5 also shows the

TABLE I

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>( \left( \frac{2}{\pi} \right)^{1/2} )</th>
<th>Specific activity* (cpm/potassium dimer/particle)</th>
<th>Counts incorporated/µm²</th>
<th>Dimer incorporation/µm²</th>
<th>Frac- tion microtubules reacting</th>
<th>Corrected increment</th>
<th>Total subunits incorporated/µm²</th>
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<tr>
<td>25</td>
<td>4</td>
<td>1.66</td>
<td>1.57</td>
<td>5.72</td>
<td>2.20</td>
<td>0.975</td>
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<tr>
<td>56</td>
<td>6</td>
<td>1.61</td>
<td>1.57</td>
<td>4.35</td>
<td>1.97</td>
<td>0.964</td>
<td>1.97 [256, 256]</td>
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<tr>
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<td>8</td>
<td>1.59</td>
<td>1.57</td>
<td>4.70</td>
<td>1.96</td>
<td>0.952</td>
<td>1.96 [256, 256]</td>
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<td>1.57</td>
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<td>1.71</td>
<td>0.930</td>
<td>1.71 [256, 250]</td>
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<td>2.80</td>
<td>1.71</td>
<td>0.918</td>
<td>1.71 [256, 250]</td>
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<td>2.80</td>
<td>1.71</td>
<td>0.908</td>
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<td>2.80</td>
<td>1.71</td>
<td>0.900</td>
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<td>1.57</td>
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<td>1.71</td>
<td>0.895</td>
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<td>1.50</td>
<td>1.57</td>
<td>2.80</td>
<td>1.71</td>
<td>0.898</td>
<td>1.71 [256, 250]</td>
</tr>
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<td>1.49</td>
<td>1.57</td>
<td>2.80</td>
<td>1.71</td>
<td>0.898</td>
<td>1.71 [256, 250]</td>
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<td>1.57</td>
<td>2.80</td>
<td>1.71</td>
<td>0.898</td>
<td>1.71 [256, 250]</td>
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<td>1.46</td>
<td>1.57</td>
<td>2.80</td>
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<td>0.898</td>
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<td>1.57</td>
<td>2.80</td>
<td>1.71</td>
<td>0.898</td>
<td>1.71 [256, 250]</td>
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<td>1.57</td>
<td>2.80</td>
<td>1.71</td>
<td>0.898</td>
<td>1.71 [256, 250]</td>
</tr>
<tr>
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<td>1.57</td>
<td>2.80</td>
<td>1.71</td>
<td>0.898</td>
<td>1.71 [256, 250]</td>
</tr>
<tr>
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<td>1.41</td>
<td>1.57</td>
<td>2.80</td>
<td>1.71</td>
<td>0.898</td>
<td>1.71 [256, 250]</td>
</tr>
</tbody>
</table>

* The specific activity was derived from the line given in Fig. 2.

The radioactivity incorporated during an increment in time was obtained from the line given in Fig. 2. For example, the last entry (330 cpm at 2025 s) was obtained by subtracting the counts/min at the point on the line at 1806 s from the counts/min at 2025 s.

Calculated by dividing counts/min incorporated pellet by the specific activity.

Interpolated values, derived from the curve given in Fig. 2.

Subunits incorporated/(fraction microtubules reacting); i.e. column 5/column 6.

The curve describing the time course for radioactive subunit incorporation for the experiment shown in Fig. 2 has been corrected for changes in specific activity, and variation in the fraction of the microtubules which participate in the reaction, in Table I, columns 3 and 6, respectively. The values for the rate as a function of time (Table I, column 4) were derived from a smooth curve drawn through the experimental points.

FIG. 5. Radioactive subunit incorporation into dogfish brain steady state microtubules. The subunits incorporated, corrected for changes in the [3H]GTP specific activity, and fraction of the microtubules reacting (described in Table I) (solid line) and the uncorrected results from Fig. 2 (open circles) are shown. The curved upward sloping line is a theoretical curve calculated for contributions of diffusional and directional components, with an s-value equal to 0.00078.
rate for subunit incorporation where the number of subunits was calculated from the initial specific activity only, and there is no correction for the change in the fraction of the microtubules which participate in the reaction. This curve is included so as to show that the corrections (Table I) are relatively insignificant. It is seen that both the corrected and uncorrected subunit incorporation (● and □, respectively, in Fig. 5) show an approximately linear dependence on \( \frac{2}{n} \) and in neither case is there a positive deviation from linearity at longer time intervals. Results with porcine brain tubulin were similarly analyzed (Fig. 6) and in this case there are significant positive deviations from linearity in the plot of radioactive subunits incorporated versus \( \frac{2}{n} \). 

It is important to establish that the E-site GTP concentration in unpolymerized tubulin remains constant during the entire time course of these experiments. The sufficiency of the acetate kinase/acyetyl phosphate GTP generating system was demonstrated by thin layer chromatographic analysis of the nucleotide composition of reaction mixtures in which the \([\text{H}] GTP\) was added at the start of microtubule assembly. Under conditions where the GTP regenerating system is operating effectively so that the E-site is 100% GTP, approximately 69% of the total radioactive nucleotide will theoretically be \([\text{H}] GTP\), since the nucleotide in the microtubule is \([\text{H}] GDP\) (22), and this constitutes 31% of the total nucleotide (fraction tubulin assembled) (E-site nucleotide)/(total guanine nucleotide) = (0.67(25.2))/(25.2 + 28.7); see “Experimental Procedures”. Chromatographic analysis showed that 65% of the radioactive nucleotide was \([\text{H}] GTP\) during the entire period during which the rate was measured.

NTPase Reaction—It is of interest to compare the steady state nucleotide concentration constant for tubulin subunit addition and loss with the rate constant for ATP hydrolysis. With dogfish shark brain tubulin, following the attainment of a steady state GTP hydrolysis continues (Fig. 7). In the experiment shown here, the hydrolysis of the 2.84 mM GTP occurs at a rate equal to approximately 0.012%/s; similar rates were observed with 1.0 and 2.0 mM GTP concentrations, although in the former case, much of the GTP had hydrolyzed by the time that a steady state was attained. It is not possible to determine precisely the rate constant for GTP hydrolysis at the microtubule ends, since there is probably significant hydrolysis by other components of the microtubular protein mixture. For calculation of an upper limit for this rate constant we assume that all of the tubulin is incorporated into microtubules. Based upon an average microtubule length found to be equal to 2.9 microns for the experiment shown in Fig. 7 and the fact that there are 1639 tubulin dimers/micron of microtubule length, with the tubulin concentration equal to 31.3 μM, the microtubule concentration would be equal to 6.6 × 10⁻⁹ M. The approximately 0.012%/s disappearance of the 2.84 mM GTP therefore corresponds to a rate constant equal to 52 mol of GTP hydrolyzed/microtubule/s.

With porcine brain tubulin, the hydrolysis of 0.96 mM ATP in the presence of tubulin is approximately linear with time, with a rate equal to 4.2 × 10⁻⁷ M/s (Fig. 4). Microtubule assembly was complete after 500 s, so that the observed rate reflects the ATP hydrolysis rate at steady state. The ATPase rate is expected to be approximately equal to the tubulin GTPase rate, since the microtubular protein contains a high nucleotide diphosphate kinase activity which equilibrates the terminal phosphate moiety of GTP and ATP. Again, it is not possible to determine precisely the rate constant for ATP hydrolysis at the microtubule ends, since there is probably significant ATP hydrolysis by other components of the microtubular protein mixture (23). For calculation of an approximate value for this rate constant, we divide the observed rate by the microtubule concentration. This concentration is approximately equal to the tubulin concentration (28.2 μM) divided by the average length of the microtubules (equal to 3.94 microns in this experiment) × 1639 tubulin dimers/micron of microtubule length (21). The calculated rate is 105 ATP/microtubule/s.

**Effect of Podophyllotoxin and Microtubule Number on Subunit Incorporation**—We have recently found that porcine brain tubulin-podophyllotoxin subunits are able to cap the two ends of the microtubule, and thereby prevent radioactive subunit flux at steady state (24). The occurrence of a similar reaction with dogfish shark microtubules is indicated by our observation that the rate for radioactive subunit incorporation is dramatically reduced by 6 μM tubulin-podophyllotoxin: less than 200 radioactive subunits were found to be incorporated at any point during a 600-s incubation; simultaneously run
control reaction in which podophyllotoxin was omitted gave results which resembled those shown in Fig. 2. In another control reaction in which 6 \( \mu \)m tubulin-podophyllotoxin was added to steady state microtubules which had been assembled in the presence of \(^{3}H\)GTP, there was no detectable (less than 5%) loss of radioactive subunits (average of 5 measurements) during a 600-s incubation. It was previously observed (25) that addition of tubulin-podophyllotoxin results in a decreased loss of microtubule mass, as compared to that produced by the addition of a similar concentration of podophyllotoxin, apparently because the added tubulin-podophyllotoxin is able to cap the microtubule before significant disassembly occurs. Idetical results were obtained in studies of the effect of added tubulin-podophyllotoxin on radioactive subunit incorporation using porcine brain tubulin.

The fact that tubulin-podophyllotoxin effectively blocks subunit flux indicates that steady state subunit incorporation occurs from the microtubule ends. If the mechanism had involved an exchange of \(^3\)H-labeled nucleotide or \(^{3}H\)GTP-tubulin dimer along the length of the microtubule wall we would expect no effect of added tubulin-podophyllotoxin, since the concentrations of the two species which could participate in such a reaction, \(^{3}H\)-labeled nucleotide which is not in the microtubule, and \(^{3}H\)GTP tubulin dimer, are unchanged by this addition. It has been shown (Fig. 2 in Ref. 26) that tubulin-colchicine, (and presumably also tubulin-podophyllotoxin, since colchicine and podophyllotoxin appear to bind to the same site (27)) binds to steady state microtubules at a single class of sites, which is presumably the microtubule ends.

To determine the effect of the number of microtubule ends on the rate for steady state subunit incorporation, the microtubules were sheared. It was found in three experiments with dogfish brain microtubules, with an initial mean length of 1.5, 2.9, and 4.1 microns, that the mean length was unchanged by 7, 14, or 22 passages, respectively, through a Hamilton 22S needle. There was also no change in the subunit flux rate induced by this treatment. With pig brain microtubules, 20 passes through a 22S needle reduced the mean microtubule length from 4.35 to 2.6 microns and 5 vigorous passages through a 27 needle reduced the length from 6.57 to 0.64 microns. In two kinetic studies with sheared porcine brain microtubules (Fig. 8 shows results from one experiment) the reaction mixtures were assayed at 13 time points during the first 60 s of reaction and it was found that in 8 of the reactions the radioactivity incorporated was greater in the sheared as compared to the control microtubules. The rate is extremely fast during the first 60 s and the experimental error is relatively large because of difficulty in determining the precise time when the reactions were initiated and quenched.\(^9\) During the 60–700-s period the reaction mixtures were assayed at 20 time points (10 in each of the two experiments). In 19 of these determinations the amount of radioactivity incorporated was greater in the sheared microtubules (see Fig. 8). The experimental scatter in these results precludes a quantitative analysis; however, these results are in qualitative agreement with the conclusion derived from the study of the effect of podophyllotoxin, that subunit incorporation occurs from the microtubule ends.

**DISCUSSION**

**Theoretical Analysis of Radioactive Tubulin Subunit Incorporation into Microtubules at Steady State—Steady state microtubule assembly-disassembly is schematically outlined in Equation 1:**

\[
\text{Tubulin} \overset{k_1}{\rightleftharpoons} \text{End 1} \overset{k_{-1}}{\rightarrow} \text{MICROTUBULE} \overset{k_2}{\rightarrow} \text{End 2} \overset{k_{-2}}{\rightarrow} \text{tubulin}
\]

where \( k_1 \) and \( k_{-1} \) are the rate constants for subunit addition and removal, and \( k_2 \) and \( k_{-2} \) are those for subunit removal and addition, respectively. The rate of subunit addition is \( k_1 [\text{Tubulin}] \) and the rate of subunit removal is \( k_{-2} [\text{End 2}] \). The net rate of subunit addition is \( k_1 [\text{Tubulin}] - k_{-2} [\text{End 2}] \).

\(^9\) The rate constants for subunit addition \( (k_1 \text{ and } k_{-1}) \) are pseudo-first order rate constants which have been corrected for the critical tubulin concentration.
which end has a more rapid diffusional incorporation of subunits; the extent of incorporation by a treadmilling mechanism at End 1 is proportional to time (i.e., it is equal to \((k_1 - k_{-1})t\)) while the diffusional incorporation at End 2 is proportional to \(\sqrt{t}\), so that the relative extent of subunit incorporation by treadmilling at End 1 and diffusion at End 2 varies as a function of time (see Equations 16 and 17 in Ref. 12).

There are four rate constants defining the scheme given in Equation 1 and it is theoretically possible to determine the value for each of these from an analysis of the rate for radioactive subunit incorporation at steady state. Equation 2, which is related to Equation 18 in Ref. 12 by making the substitutions \(p_1 = k_1/(k_1 + k_{-1}); p_2 = k_2/(k_2 + k_{-2}); q_1 = 1 - p_1; p_2 = k_2/(k_2 + k_{-2}); q_2 = 1 - p_2\),
describes the rate for subunit incorporation at the two microtubule ends:

Label incorporated at both microtubule ends

\[
\frac{k_1 + k_2}{k_1 - k_{-1}} + \frac{k_1 - k_{-1}}{k_1 - k_{-2}} = (k_1 - k_{-1})t
\]

Equation 2 indicates that a plot of radioactive subunit incorporation versus time yields the reciprocal of the Wegner s-value as an intercept, and the treadmilling rate constant from the slope. Except for the special cases where the treadmilling efficiency is 0% \((k_1 = k_{-1})\) or 100% \((k_1 = k_{-1} = 0)\), the rate for subunit incorporation will be biphasic, with an initial rapid phase resulting from the diffusional subunit incorporation and a later linear phase which reflects the treadmilling process. At early time points the diffusional mechanism is the predominant route for radioactive subunit incorporation, if treadmilling efficiency is low.

The individual values for \(k_1, k_{-1}, k_2,\) and \(k_{-2}\) may be determined (see “Appendix”) from Equations 3 and 4:

\[
k_{+1} = 0.5 S_1 \left( \frac{(S_1)_{l(t)}}{0.16 \text{ dimers}^{-1}} \right)
\]

\[
k_{-1} = 0.5 S_1 \left( \frac{(S_1)_{l(t)}}{0.16 \text{ dimers}^{-1}} \right)
\]

where \(S_1\) is equal to the slope of the linear phase of the plot of label incorporated at both microtubule ends versus time (Equation 2), \(I_1\) is the intercept, and \(t_{1/2}\) is the half-time for reaching the linear phase of the reaction. In essence, the feasibility of determining all four of the rate constants shown in the scheme given in Equation 1 depends on the fact that it is theoretically possible to make three measurements in a study of the kinetics for radioactive subunit incorporation. These are: 1) the linear rate when the diffusionless route for subunit incorporation is insignificant \((S_1)\); 2) the intercept of the plot of subunit incorporation as a function of time \((I_1)\); 3) the rate of the reaction when the diffusionless component for subunit incorporation is the predominant path, as reflected by the half-time for reaching a linear rate for subunit incorporation \((t_{1/2})\). In addition to these three experimental measurements, the special relationship which defines the steady state \(i.e., k_1 + k_2 = k_{-1} + k_{-2}\) provides the requisite fourth equation necessary to unravel all four rate constants.

It is not always possible to determine experimentally the four rate constants for the scheme given in Equation 1. For example, in our study of radioactive subunit incorporation into microtubule which are at steady state in the presence of taxol (13), the rate constants for subunit dissociation \((k_{-1} + k_{-2})\) are dramatically reduced relative to that in the absence of taxol so that even after 16 h the diffusionless component for subunit incorporation is the predominant observable route for incorporation of radioactivity. Therefore, the possible contribution by a treadmilling mechanism \(i.e., S_1\) in Equations 3 and 4 was not measurable. On the other hand, one may be unable to measure the diffusionless component for subunit incorporation \(i.e., t_{1/2}\) in Equations 3 and 4) if: 1) the rate constants \(k_{-1} + k_{-2} + k_1 + k_2\) are so large that the diffusionless reaction is virtually complete before measurements can be made; or 2) the directional incorporation is so efficient that the intercept on the ordinate in the plot of radioactive subunit incorporation as a function of time is close to the origin. In this case the very small contribution of the diffusionless process even at the earliest time points precludes an accurate determination of \(t_{1/2}\) and \(I\).

Experimental Analysis of Radioactive Tubulin Subunit Incorporation—When the calculated number of subunits which are incorporated is plotted as a function of time (Figs. 2 and 6A), the reaction is biphasic. With porcine brain tubulin, the intercept of the slow linear phase \((I_1)\) is in the range 1000–2000 tubulin subunits, from which a Wegner s-value equal to 0.0005–0.001 may be calculated (Equation 2). The estimated values for \(S_1\) and \(t_{1/2}\) (see the discussion of Equations 3 and 4) are 44.2 s^{-1} and 35 s, respectively, with \(I_1\) equal to 2000; for \(I_1\) equal to 1000 \((i.e., \text{the lower estimate for the intercept})\), \(S_1\) and \(t_{1/2}\) are 6.3 s^{-1} and 12 s, respectively. Using Equations 3 and 4, the calculated rate constants (subunits/microtubule/s) are: \(k_1 = 1901.5\) (1515.3), \(k_{-1} = 1927.3\) (1509), \(k_2 = 6742.5\) (4534.5), \(k_{-2} = 6488.5\) (4511) \((\text{values in parentheses were calculated using} I_1 = 1000 \text{ and} t_{1/2} = 12 \text{ s}; \text{ the number of significant figures is meaningful only for comparing the corresponding} k \text{ and} k_{-1}\) \(\text{values})\). A theoretical curve calculated from these rate constants (Fig. 6A) shows a good correlation with the experimental results.

The 0.0005–0.001 Wegner s-value derived from the results shown in Fig. 6A indicates a very low efficiency for the treadmilling. The relatively small contribution of treadmilling to subunit incorporation is most clearly indicated by the near linearity of the plot of subunits incorporated versus \(\left(\frac{2}{\pi}\right)^{1/2} \sqrt{t}\) (Fig. 6B). This plot will be linear (with a slope equal to \(2^{1/2} k_{-1}\), when \(k_{-1} = k_{-2}\); see Equations 7 and 13 in Ref. 12) if there is only diffusional subunit incorporation. The slope of the line drawn in Fig. 6B gives \(k_{-1} = k_{-2} = 2500 \text{ subunits/microtubule end/s}; \text{ the observed small positive deviations from linearity at longer time points are indicative of a small contribution of treadmilling to the reaction.}\)

The results with dogfish shark brain microtubules (Fig. 2) are in qualitative agreement with those obtained with porcine brain microtubules. We have not analyzed these results using Equation 2, since the plot given in Fig. 5 indicates that there is no significant treadmilling. In order to estimate an upper limit for the Wegner s-value for this reaction we have calculated the time course for subunit incorporation with varying degrees of directional incorporation superimposed upon the diffusional rate. This calculation uses Equations 7, 16, 17, and that given for \(G(G)\) in the legend to Fig. 7 in Ref. 12. The radioactive subunit incorporation, calculated for a hypothetical case when \(k_1 = k_{-2} = 1250.9375\), \(k_{-1} = k_2 = 1249.0625\), and a Wegner s-value \(= (k_{-1} - k_{-2})/(k_1 + k_{-2}) = 0.00075\), is given in the upward curving line in Fig. 5. We conclude that this is an upper limit for the Wegner s-value, since the calculated values at later time points are all considerably above the experimental curve.

Analysis of Earlier Results—Although the rate constant reported here for treadmilling with steady state porcine brain microtubules \((4.2–6.3 \text{ subunits/microtubule/s})\) is in reasonable agreement with the 1.8 subunits/microtubule/s previously reported \((12)\), the s-value of 0.0005–0.001 is much less than

\* There is a typographic error in the equation for \(G(G)\) in the legend to Fig 7 in Ref 12. Every occurrence of a small c should be changed to a capital C.
the 0.015 previously calculated (12). We believe that the earlier result is incorrect, because it was derived using the dilution-induced disassembly rate for estimating the steady state rate constants for subunit dissociation from the microtubule ends. We now recognize that for the situation where the microtubule is capped by short segments which have unusually high subunit association and dissociation rate constants, the rate which can be observed following dilution does not correspond to that which exists at the microtubule ends at steady state. Instead, since the caps would be rapidly lost following dilution, the observed rate would reflect the dynamic properties of a portion of the microtubule which is not generally exposed for reaction at steady state; i.e., the interior portion of the microtubule containing tubulin-GDP. A similar factor may have complicated studies of the rate for dilution-induced disassembly of microtubule fragments which had been formed at the ends of flagellar seeds (11), and the disassembly of radiolabeled bovine brain and flagellar outer doublet microtubules (14). In these cases the calculated Wegner s-values would be incorrect.

Although there have been a number of reports which suggest that treadmilling efficiency may be relatively high, in some cases this parameter may not have been correctly measured. Also, the difference between the very low s-values reported here and the larger values reported elsewhere may be related to some as yet unknown variable in the protein preparations used. A number of earlier studies are summarized as follows.

1) Results from a pulse-chase experiment have been taken to indicate that the Wegner s-value is about 1.0 (1). We have, however, calculated that these results are also consistent with s-values in the range 0.02-1.0 (see Table I in Ref. 12).

2) The Wegner s-value has been found to inexplicably vary, from 0.8 at a low GTP concentration, to about 0.1 at high GTP concentrations (29). In this study the s-values were derived from determinations of podophyllotoxin-induced microtubule disassembly rates, in the presence of either GDP or GTP. This calculation is predicated upon an assumption that the tubulin-GTP-podophyllotoxin complex only caps the assembly end of microtubules, so that the disassembly rate in the presence of podophyllotoxin and GTP measures the rate constant for subunit loss at the disassembly end of the microtubule. However, we have demonstrated (24) that tubulin-GTP-podophyllotoxin caps both microtubule ends, so that the calculated s-values (29) are incorrect.

3) Terry and Purich (7) have measured a treadmilling rate equal to 0.3 s⁻¹; using the dilution-induced disassembly rate (114 s⁻¹, Ref. 30) for estimating the rate for the steady state assembly process, an s-value equal to 0.0026 is calculated. It is, however, puzzling that there is no indication of the expected diffusional component to subunit incorporation in the time course for this treadmilling (Fig. 3 in Ref. 7). From Equation 2, it is expected that the zero-time extrapolate should be at approximately 0.5 μM (1/0.0026 = 380 subunits/microtubule); a much higher extrapolate is expected if the dilution-induced disassembly rate underestimates the rate for subunit addition at steady state.

4) Radiolabeled subunit incorporation into microtubules has been found to occur at a rate equal to 27 s⁻¹, under steady state conditions where it is calculated that the microtubules undergo subunit gain and loss of 102 subunits/s; s equals 0.26 (31). The rate for the latter reaction was calculated by adjusting the second order rate constant for presteady state microtubule assembly (1.7 × 10⁻⁵ m⁻¹ s⁻¹) for the critical tubulin concentration (6.7 × 10⁻⁹ M). The assumption here is that the pre-steady state assembly process is a simple, single step reaction so that the second order rate constant correctly measures the rate for subunit addition. In contrast, if tubulin subunit addition is a two step process involving a kinetically significant unimolecular reaction of the addition complex composed of the microtubule and added tubulin subunit (such as a protein conformation change end or GTP hydrolysis), then the measured second order rate constant only provides a lower limit for the rate of subunit addition. A There is considerable evidence which suggests that the assembly reaction is, in fact, a multi-step process: (a) the rate for microtubule assembly has a hyperbolic dependence on the tubulin concentration (Fig. 6 in Ref. 11, Fig. 4 in Ref. 32, Fig. 3 in Ref. 33, Fig. 2 in Ref. 34, Fig. 11 in Ref. 35); (b) It has been shown that in microtubule assembly the GTP hydrolysis occurs in a discrete rate-limiting step which follows the subunit addition reaction (36, 37); (c) The negative activation enthalpy for microtubule disassembly has been accounted for in terms of a two step reaction involving a rate-limiting conformation change (38). Thus, the s-values calculated from the pre-steady state assembly rate (31) may not be correct, because their calculation is based upon an incorrect assumption that the subunit addition reaction is a single step process.

A Proposed Mechanism for Pre-steady State Microtubule Assembly and Rapidly Reversing Subunit Dissociation at Steady State—We have previously estimated the steady state molecular rate constant for porcine brain microtubule disassembly from analysis of the disassembly rate following dilution (12); the rate constant obtained for this reaction is 120 subunits/microtubule/s. This rate constant is very much smaller than the approximately 5000 subunits/microtubule/s value obtained here, using a method which does not involve perturbation of the steady state. The nonequivalence of the steady state molecular rate constant and the rate constant for disassembly induced by dilution requires that there be an inhomogeneity in the microtubule such that the subunits at the ends are not equivalent to the bulk of subunits in the microtubule. We propose that the inhomogeneity at the ends of steady state microtubules consists of a cap containing tubulin-GTP subunits, which undergo extremely rapid (about 5000 subunits/microtubule/s) dissociation and association.

The basis for our assumption that the inhomogeneity consists of tubulin-GTP subunits is our observation that at steady state the upper limit to the rate for GTP hydrolysis at microtubule ends (105/microtubule/s, Figs. 4 and 7) is less than the molecular rate constants for tubulin-GTP subunit addition and loss. Therefore, the microtubule ends will contain tubulin subunits which have not yet hydrolyzed GTP. The proposed mechanism is also supported by kinetic studies of pre-steady state microtubule assembly which indicate that the elongating microtubule contains GTP (34), and the observations that 4.7% (8) to 10% (22) of the guanine nucleotide which is contained in steady state microtubules is GTP (22). The existence of tubulin-GTP caps at the ends of steady state microtubules has been proposed in a preliminary report (39).
It is required that the putative tubulin-GTP caps are of limited length. This conclusion is based upon the fact that if the steady state rate constants determined here described the rate for tubulin subunit loss from the entire microtubule, then the rate for dilution-induced microtubule disassembly would be too fast to measure. However, if the GTP-tubulin caps constitute only a small fraction of the microtubule, then only a portion of the microtubule would be lost prior to the first measurement in studies of the dilution-induced disassembly (12, 39). An estimate of the length of the putative cap can be made from the zero time extrapolate of the plot of tubulin subunit incorporation as a function of time (Fig. 6A). This extrapolate, equal to 1000-2000 subunits, constitutes the fraction of the microtubule which participates in rapid diffusional subunit incorporation. Since rapid diffusional subunit incorporation may also contribute to subunit incorporation into the main body of the microtubule, the extrapolate provides an upper limit to the length of the tubulin-GTP caps.

According to the proposed mechanism, the dilution-induced disassembly rate (120 subunits/microtubule/s) reflects the dissociation rate of tubulin-GDP subunits from the interior of the microtubule. As a result, the rate constant determined by dilution does not measure the steady state molecular rate constant for tubulin subunit addition and loss. It has previously been suggested (40) that: (a) dilution studies only yield the molecular rate constant for loss of tubulin-GDP subunits from the interior of the microtubule; (b) that the molecular rate constant measured by dilution is very much greater than that for subunit loss from steady state microtubules because the microtubule end contains tubulin-GTP, which is lost very slowly. We are in agreement with (a) but have the opposite conclusion concerning (b); we suggest that tubulin-GTP is lost more rapidly than is tubulin-GDP.

The tendency for the tubulin-GTP cap to have a finite length may represent negative cooperativity; i.e., the equilibrium constant for tubulin-GTP addition is less favorable at positions distal to the tubulin-GDP microtubule. The less favorable equilibrium constant would be manifested in a lower second order rate constant for tubulin-GTP subunit addition, and/or a higher first order rate constant for tubulin subunit dissociation. As described next, the kinetic consequences of negative cooperativity can account for: (a) a discrepancy between the observed rate for pre-steady state assembly and the rate calculated from the steady state molecular rate constants derived here; (b) the observation (11, 32-35) that the initial rate for microtubule elongation shows a decreasing dependence on the tubulin concentration, at high concentrations.

(a) The pre-steady state rate for microtubule assembly according to the scheme given in Equation 1, can be calculated from Equation 5 (12):

\[
N_r = \frac{N_{in}(k_1 + k_2) - (k_1 + k_2)e^{-1}}{k_1 + k_2}
\]

where \(N_r\) and \(N_{in}\) are the “concentration” of tubulin dimers in the microtubule, and the total tubulin concentration, respectively. Using the rate constants derived from the results in Fig. 6A, and assuming a nucleating “seed” concentration equal to 10^-8 M (i.e., a concentration which would allow growth to about 10,000 subunits/microtubule in a reaction with 10^9 M tubulin), it can be calculated that the pre-steady state assembly would be complete in about 1 s, if there was no restriction to the addition of tubulin-GTP subunits to the growing microtubule. Our mechanism includes such a restriction; it assumes that a tubulin-GTP cap will tend to be of finite length. That is, the rate constants for subunit addition and loss which reflect the postulated negative cooperativity, rather than the steady state molecular rate constants, determine the rate for pre-steady state microtubule elongation.

(b) It has been found (11, 32-35) that the initial rate for microtubule elongation shows a decreasing dependence on the tubulin concentration, at high concentrations. This behavior, which has not previously been dealt with, can be accounted for if it is assumed that the degree of negative cooperativity progressively increases as the tubulin-GTP cap is elongated. Generation of a limiting rate at high tubulin concentrations would result from a balance between the tendency for an increasing tubulin concentration to increase the length of a pre-steady state cap, and a progressive increase in the difficulty for net subunit addition as the cap is elongated.

### SUMMARY

1. There is a parallel between the mechanism proposed here and those which have been previously advanced to account for the properties of steady state microtubules. Subunit addition to form a cap of finite length at the ends of steady state microtubules occurs with tubulin-GTP (this study), tubulin-GDP (41), tubulin-podophyllotoxin (24), and tubulin-colchicine (26). As was previously pointed out (41), the fact that the addition of tubulin-GDP subunits to a microtubule is limited to an isoenergetic exchange means that the microtubule contains two kinds of tubulin-GDP subunits, those introduced in a reaction accompanied by GTP hydrolysis and those added without GTP hydrolysis. It is only when the former type of tubulin-GDP subunits are at the end of a microtubule that tubulin-GDP in solution can undergo net addition (41). The mechanism proposed here is equivalent in that it is suggested that at steady state in the presence of GTP the microtubule can contain two types of tubulin subunits, tubulin-GDP subunits which are introduced in a reaction which is accompanied by GTP hydrolysis, and tubulin-GTP subunits. Addition of tubulin-GTP from solution occurs more readily to an end containing the tubulin-GDP subunits, as compared to an end with tubulin-GTP subunits; i.e., tubulin-GTP subunits at a microtubule end are equivalent to tubulin-GDP subunits which are introduced without GTP hydrolysis. Thus, as suggested by Carlier and Pantaloni (34) and by Weisenberg (10), GTP hydrolysis is not required for tubulin-GTP subunit addition. Rather, GTP hydrolysis in an incorporated subunit “ratchets” the subunit into place so that additional subunit addition can occur. That hydrolysis of the GTP locks the subunit into the microtubule is indicated by the fact that the rate constant for subunit dissociation is apparently reduced from about 5000 to 120/s, as a result of GTP cleavage. A similar conclusion has been derived elsewhere (37).

2. The predominance of diffusional as compared to direct microtubin incorporation seen here with porcine brain microtubules, has been previously observed with MAP-free porcine brain tubulin in the presence of taxol (13). Results from other studies (7, 11, 14) are also in accord with a low efficiency for treadmilling. It will be of interest to determine how this efficiency may be increased.

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### APPENDIX

The individual rate constants for the scheme given in Equation 1 can be calculated from Equation 6,
L(t) = (k_1 - k_-)t + \frac{U_1}{2 k_1 - k_-} (k_1 + k_-)G(C_1) + (k_2 - k_-)G(C_2)

which is derived from Equation 18 in Ref. 12, by substitution of the values for \( L_s(t) \) and \( L_0(t) \) from Equations 16 and 17 (Ref. 12), respectively; the rate constants defining \( p_1 \) and \( q_1 \) have also been substituted in deriving Eq. 6.

In Equation 6 \( L(t) \) is the average amount of label incorporated for microtubules during time \( t \), \( U_1 \) equals 1 tubulin dimer, and \( C_1 = [(k_1 - k_-)/(\sqrt{k_1} + k_-)]U_2/2 \), where \( U_2 = 1/\sqrt{1} \) tubulin dimer). The term \( G(C) \), which measures the degree to which diffusional subunit incorporation (as opposed to directional incorporation) has taken place, is defined in the legend to Fig. 7 in Ref. 12.

Analysis of Equation 6 is accomplished by plotting the radioactive tubulin subunits incorporated as a function of time. In this plot we define \( S_t = \text{slope} = \lim (dL/dt) = k_1 - k_- \), and \( I_1 = \text{intercept} = (k_1 + k_2)/(k_1 - k_-)U_1 t/2 \). There is a time point during the initial phase of the reaction, defined as \( t/2 \), which corresponds to the time where \( C_1 = 0.403 \); from the definition of \( G(C) \) given in the legend to Fig. 7 in Ref. 12, it can be calculated that this corresponds to the point when \( G(C) \) equals 0.5, so that the diffusional component has reached half of its limiting value. In Equation 7, this value for \( C_1 \) is substituted into the definition of \( G \), (see above):

\[
0.40 = (\sqrt{k_1} + k_-)\sqrt{t}/2 \text{ dimer}
\]

\[
0.40 = (\sqrt{S_t} \sqrt{2})/(\sqrt{k_1} + k_-) \sqrt{t} \text{ dimer}
\]

\[
0.16 = S_t/(t/2)(k_1 + k_-) (1 \text{ dimer})
\]

\[
k_1 = \frac{1}{2} S_t (S_t/2) 0.16 \text{ dimers} \pm 1
\]

The equation for \( k_2 \) (Equation 4) is derived by substituting the value for \( k_1 \) (Equation 10) into the relationship defining \( I_1; k_2 \) is derived from the relationship: \( k_1 - k_- = k_2 \).

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