PRESENT WORK
ROLE OF B-RING OF COLCHICINE IN ITS BINDING TO TUBULIN: PART IA AND IB
Several properties of the colchicine tubulin interaction such as association rate, reversibility and the promotion of drug fluorescence have been related to the B-ring of colchicine. The B-ring itself retards the binding rate and substitution at C-7 leads to further decreases which appear to be related to both substituent bulk and the presence of a N-acyl group. Thus, the decreasing order of binding rates is 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropolone (AC compound) > desacetamido-colchicine > deacetylcolchicine > colcemid > colchicine > N-benzoyldeacetylcolchicine etc. On the other hand, the apparent irreversibility of the binding seems clearly more closely related to the presence of an N-acyl group than the bulk of the substituent at C-7. Substitution at C-7 also affects the tropolone fluoreophore. Thus, amines (deacetylcolchicine, colcemid or N-methylcolcemid) fluoresce poorly in the presence of tubulin, whereas substitution of the amino group with an acyl group enhances fluorescence. When these compounds fluoresce in glycerol and n-octanol the yields are very different: N-methylcolcemid > methiodide > deacetyl colchicine > colcemid > N-methylcolcemid. There is little correlation between the ability of analogs at C-7 to fluoresce in viscous solvents and the quantum yields of the complex. The presence of an N-acyl group at C-7 is essential for enhanced fluorescence. We conclude that in addition to A- and the C-ring portion of the molecule, the B-ring of colchicine is a third determinant recognized by the binding site on tubulin.
Introduction

Structure function relationships in colchicine (Fig. 1) and its congeners can be conveniently described in terms of the three rings of the molecule: the A ring or trimethoxybenzene moiety; the 7-membered B ring; and the methoxy tropone moiety or C ring. Substantial evidence has accumulated that the colchicine binding site of tubulin, through which most of the desired drug effect must, presumably, operate, contains a domain that recognizes the A ring and a second domain that recognizes the C ring (1-3). This was first concluded from the finding that podophyllotoxin competed for colchicine presumably via the mutual trimethoxybenzene moiety (4). On the other hand, tropolone and methoxypriopone block colchicine but not podophyllotoxin binding (5). It was thus proposed that colchicine has at least two attachment points to its binding site on tubulin, one for the A ring and one for the C ring (4,5). Subsequently, it was shown (6) that these domains can be independently occupied by single ring analogues such as mescaline (A ring) or methoxypriopone (C ring). However, affinities for these single rings are low and marked enhancement of binding affinity is attained when these are linked as in colchicine, etc. The sum of the individual binding energies (plus a correction for the cratic entropy resulting from incorporation of these rings into a single molecule) was similar to that of colchicine.
Fig. 1. Structure of colchicine and its analogs.

R = H, Desacetamidocolchicine (DAC);
R = NH₂, Deacetylcolchicine
R = NH·CH₂, Colcemid; R = NH·CO·CH₃, Colchicine.

2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone (AC compound).
Because of the finding that an analog containing the A and C rings but lacking the B ring [2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone] referred to hereafter as the AC compound, has high biological and tubulin binding activity (8), this compound has been widely used as a simple and flexible model for colchicine. It binds rapidly, possibly due to the facile rotation about the biaryl bond, and with an activation energy lower than colchicine (9,10). Like colchicine, the AC compound exhibits marked enhancement of fluorescence upon binding and free energies of binding consistent with the A and C ring contributions (7,9,10).

The contributions of the B ring to the binding of colchicine congeners to tubulin has been considered to be of a minor nature. Thus, using AC as a model compound, a bifunctional ligand model has been proposed for colchicine in which the bulk of the interactions with the tubulin site, including the conformational changes in tubulin, are accomplished by the A and C rings (7,10). Since colchicine binding to tubulin is a two step process (11,12), it has been proposed that the tropolone moiety binds before the remainder of the molecule and promotes the conformational change in the protein that now facilitates binding of the A ring.

When present, however, B ring substituents at position C-7 have major effects on the association and dissociation rates of the drugs and on the temperature dependence of binding as exemplified by the difference between colchicine,
coli cremid and deacetamidocolchicine (9,13). Moreover, the unnatural enantiomer, (+)colchicine, shows no tubulin binding activity (not shown). The present study was undertaken to elucidate the contributions of the B ring and substitutions at C-7 to the kinetics, binding constants and fluorescence properties of colchicine analogs.

Results

Equilibrium Constants

Binding constants for colchicinoids with different substitutions at C-7 of the B ring analogues obtained by displacement of 1 μM colchicine [3H] are listed in Table I. It is apparent that substitutions on the nitrogen at C-7 had an effect on the binding constants obtained but that, despite large variations in the bulk of the substituents, all constants were within -1 order of magnitude of each other. It is of interest that an even bulkier group (fluorescein isothiocyanate) has been reported to bind with an affinity -1/10th that of colchicine (20), and an azidoaromatic photo-affinity label at C-7 (21), and a spinlabel at C-7 of allo-colchicine (22), have good specificity for the site. It is apparent, therefore, that this portion of the B ring has relatively modest effects on this binding parameter although the introduction of a positive charge through formation of a quartenary nitrogen causes loss of binding activity. Another conclusion permitted by the present results is that hydrogen
### Table I

**Binding Parameters of E-ring Analogs of Colchicine**

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>$K_C$</th>
<th>$K_1$</th>
<th>Association Rate Constant</th>
<th>Reversibility$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>7-desacetamidocolchicine (H)</td>
<td>5</td>
<td>6.0</td>
<td>$10^6$</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>5,6-dehydro-7-desacetamido-</td>
<td>-</td>
<td>5.8</td>
<td>$10^6$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>colchicine [H, (5,6-CH=CH-)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>N-deacetylcolchicine (NH$_2$)</td>
<td>12</td>
<td>1.8</td>
<td>$10^6$</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Colcemid (Demecolcine) (NHCH$_3$)</td>
<td>60</td>
<td>1.9$^b$</td>
<td>$10^6$</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>N-methylcolcemid [N(CH$_3$)$_2$]</td>
<td>30</td>
<td>-</td>
<td>$10^6$</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>N-dimethylcolcemid (iodide)</td>
<td>Inactive</td>
<td>-</td>
<td>$10^6$</td>
<td>-</td>
</tr>
</tbody>
</table>

Contd.......

$^a$ Reversibility means whether the binding is reversible or not.

$^b$ 1.9$^b$ indicates a value slightly higher than 1.8.
<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>$K_i$ (µM)</th>
<th>Association Rate Constant $(\text{M}^{-1}\text{h}^{-1} \times 10^6)$</th>
<th>Reversibility $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.</td>
<td>Colchicine</td>
<td>9.4</td>
<td>0.34</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(NHCOC$_3$H$_3$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>N-methylcolchicine</td>
<td>30</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$[\text{N}($CH$_3$)$_2$COCH$_2$]$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>N-deacetyl(trifluoracetyl) colchicine</td>
<td>10</td>
<td>0.30</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(NHCOC$_3$F$_3$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>N-(trifluoracetyl)colcemid</td>
<td>12</td>
<td>0.18</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$[\text{N}($CH$_3$)$_2$COCF$_3$]$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>N-deacetyl-N-benzoyl-colchicine</td>
<td>24</td>
<td>0.09</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(NHCOC$_6$H$_5$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>N-deacetyl-N-trimethoxy-benzoyl colchicine</td>
<td>54</td>
<td>0.07</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$[\text{N}($HOC$_6$H$_2$$(OOC$_3$H$_3$)$_3$]$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Contd........
<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>$K_i$ (µM)</th>
<th>Association Rate Constant $(M^{-1}h^{-1}x10^6)$</th>
<th>Reversibility$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.</td>
<td>N-deacetyl-N-pivaloyl-colchicine [NHCOC(CH$_3$)$_3$]</td>
<td>70</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>14.</td>
<td>N-deacetyl-N-retinoyl-colchicine (NH-Retinoyl)</td>
<td>50</td>
<td>0.01</td>
<td>-</td>
</tr>
<tr>
<td>15.</td>
<td>2-methoxy-5-(2,3,4-trimethoxyphenyl)tropone (AC compound)</td>
<td>-</td>
<td>120</td>
<td>+</td>
</tr>
</tbody>
</table>

$^a$With 100 times excess podophyllotoxin for 60 min at 37°C. + indicates exchange of podophyllotoxin (reversibility) and - indicates unexchange of podophyllotoxin (irreversibility).

$^b$Taken from Ref. 29.
bonding between the NH group and the tropolone carbonyl group is not essential (23) since the disubstituted analogs N-methylcolchicine, N-methylcolcemid and N-trifluoroacetyldemecolcine showed good binding activity. Comparison of these results with the one point assays performed previously (2,19) shows some discrepancies in the rank order of affinities particularly with deacetylcolchicine, N-trifluoracetyldemecolcine and N-acetyldemecolcine and demecolcine. Part of these differences can be ascribed to the fact that highly active analogs are compared on the nonlinear portion of the dose response curve in a one point assay. It should be noted that the total absence of the B-ring, as in the AC compound, yields an equilibrium binding constant of the same order as a number of the analog containing the intact B-ring as reported by other (7,10).

**Binding Rates**

Association rate constants were measured by enhancement of fluorescence of the analogs that occurs upon binding to tubulin (23). At the concentrations used, there was only negligible fluorescence of these compounds in buffer but in the absence of tubulin. Quantum yields differed markedly (see below) and all rates are expressed as a suction of the maximal attainable fluorescence for that particular analog in the presence of 8-10 fold excesses of tubulin where all of the compound may be assumed to be bound (see below).
Although colchicine binding has long been known to exhibit very slow kinetics, there is a very wide spread in the binding rates of the colchicine analogs. This is demonstrated for B ring analogs at C-7 in Table I. Association rate constants vary by nearly four orders of magnitude as we move from no B ring at all, as exemplified by the AC compound to the N-retinoyl derivative at the 7 position. The bare, unsubstituted B ring of desacetamidocolchicine, causes a twenty fold reduction in the association rate constant. Substitution at C-7 in the B ring further lowers the association rate constant and this occurs as a rough function of the level of the substituent. It seems possible to propose, therefore, that the seven membered B ring itself retards binding and that substitution at C-7 leads to further rate losses that appear to be functions both of the bulk and the presence of a N-acyl group.

Fluorescence

The very slow dissociation of the tubulin-colchicine complex has proved a boon in binding studies and has been explained by an activation energy barrier for dissociation that is substantially larger than for the reversible analog, AC (10). The binding of colchicine to tubulin is accompanied by a marked enhancement of fluorescence from the tropolone moiety with characteristics of $\pi^* \rightarrow \pi$ transition (24) and a lifetime, $\tau$, of 1.14 nsec (12). A substantial fraction of this
fluorescence is caused by the immobilization of this drug in the binding site on tubulin, rather than any hydrophobic environment that the site may provide (25). It has also been suggested that stabilization of the drug in a conformation that makes the A and C rings more nearly coplanar contributes to fluorescence (10).

B ring substitution at C-7 has a profound effect on the fluorescence properties of the methoxy tropone fluorophore of colchicinoids. This was first suggested by the poor fluorescence of coleemid (13). The quantum yields of analogs of colchicine are listed in Table II for assays carried out with an 8-10 fold molar excess of tubulin for 60-90 min (to attain plateau values), followed by separation of the complex and checking for unbound drug.

The unsubstituted B ring or desacetamido-colchicine gave a fluorescence yield about 2/3 that of colchicine. When a double bond was introduced as in 5,6-dehydro, 7-desacetamido-colchicine the expected red shift occurred in the absorption spectrum (λ<sub>max</sub> = 358 nm) but there was a reduction in the quantum yield to $\Phi = 0.013$. Dreyding models suggest that this may be due to the fact that neither atropimer can attain the near coplanar biaryl angle that is possible with the saturated B ring. This is consistent with the postulate of Bane et al. (10). Addition of the free amino or alkyl amine groups at position 7 yields analogs that fluoresce very poorly or not
### Table II

Fluorescent Properties of B-ring Analogs of Colchicine

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Quantum Yield of Tubulin Complex</th>
<th>Fluorescence in Glycerol</th>
<th>Fluorescence in Octanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>7-desacetamidocolchicine (H)</td>
<td>0.023</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>5,6-dehydro-7-desacetamidocolchicine [H, (5,6-CH=CH-)]</td>
<td>0.013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>N-deacetylcolchicine (NH&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>0.0057</td>
<td>108</td>
<td>331</td>
</tr>
<tr>
<td>4.</td>
<td>Golcemid (Demecolcine) (NHCH&lt;sub&gt;3&lt;/sub&gt;)</td>
<td>0</td>
<td>102</td>
<td>111</td>
</tr>
<tr>
<td>5.</td>
<td>N-methylcolcemid (N(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>0</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>6.</td>
<td>N-dimethylcolcemid (iodide) [N&lt;sup&gt;+&lt;/sup&gt;(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;I&lt;sup&gt;-&lt;/sup&gt;]</td>
<td>0</td>
<td>150</td>
<td>222</td>
</tr>
</tbody>
</table>

Contd......
<table>
<thead>
<tr>
<th>No.</th>
<th>Compound R&lt;sub&gt;0.7&lt;/sub&gt;</th>
<th>Quantum Yield of Tubulin Complex</th>
<th>Fluorescence in Glycerol&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fluorescence in Octanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.</td>
<td>Speciosin [N-(\text{CH}_2)\text{CH}_2\text{C}_6\text{H}_4\text{OH}]</td>
<td>0</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>8.</td>
<td>Colchicine (\text{NHOCH}_3) (\text{NHOCH}_3)</td>
<td>0.030</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>9.</td>
<td>\text{N-methylcolchicine} [N(\text{CH}_2)\text{COCH}_3]</td>
<td>0.032</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10.</td>
<td>\text{N-deacetyl}(trifluoracetyl) \text{colchicine} (\text{NHOOCF}_3)</td>
<td>0.026</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11.</td>
<td>\text{N-(trifluoracetyl)colcemid} [N(\text{CH}_2)\text{COOCF}_3]</td>
<td>0.028</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12.</td>
<td>\text{N-deacetyl-N-benzoyl-colchicine} (\text{NHOOC}_6\text{H}_5)</td>
<td>0.036</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>No.</td>
<td>Compound</td>
<td>Quantum Yield of Tubulin Complex</td>
<td>Fluorescence in Glycerol</td>
<td>Fluorescence in Octanol</td>
</tr>
<tr>
<td>-----</td>
<td>-----------------------------------------------</td>
<td>----------------------------------</td>
<td>--------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>13.</td>
<td>N-deacetyl-N-trimethoxybenzoyl colchicine</td>
<td>0.029</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(\text{[NHCOO} \cdot \text{H}_2(\text{OCH}_3)_3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>N-deacetyl-N-pivaloyl colchicine</td>
<td>0.0175</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(\text{[NHCOO(CH}_3)_3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>N-deacetyl-N-retinoylcolchicine (NH-Retinoyl)</td>
<td>0.018</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone</td>
<td>0.021</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(AC compound)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Compared to colchicine = 100.
al all. By contrast, the N-acyl containing derivatives exhibited quantum yields of the order of colchicine and fluorescence was not particularly sensitive to the bulk of the substituent. Dreyding models show that, in one conformation of colchicine, the N-acyl group could approach the electrons over the A ring. To test whether this could enhance fluorescence by supplying extra electrons to the A ring and thus to the tropolone ring by extended conjugation, we tested N-deacetylsuccinylcolchicine (prepared (-)-deacetylcolchicine and succinic anhydride -m.p. 255°C, M^+ 1 = 468). However, this compound had slightly lower fluorescence than colchicine under identical conditions (data not shown).

Attempts were made to determine whether the differences in quantum yield between N-alkyl and N-acyl derivatives of colchicine were intrinsic properties of the fluorophore or were the result of the binding interaction with tubulin. To this end we employed solvents of high viscosity such as glycerol or n-octanol which are known to promote colchicine fluorescence by immobilization, albeit less efficiently than tubulin (25). As seen in Figs. 2 and 3, there is little correlation between the ability of the C-7 substituted analogs to fluoresce in viscous solvents and the quantum yields of the tubulin complex (Table II).
FIG. 2
Both tertiary amine compounds, methylcolchicine and speciosine \((\text{N(CH}_3\text{)}(\text{CH}_2\text{O}_6\text{H}_4\text{OH}))\), failed to fluoresce in either solvent (or on tubulin), although both can displace \(^{3}\text{H}\)-colchicine from tubulin. The reason for this is not clear. Both colcemid and the quarternary analog fluoresce in the solvents but the quarternary analog does not fluoresce in the presence of tubulin, presumably because it does not bind. The high fluorescence intensity of deacetylcolchicine in the solvents contrasts sharply with the low quantum yield in the tubulin complex. Such differences suggest that factors other than simple immobilization may contribute to the fluorescence of the analog-tubulin complex. Whether or not this results from reduction of the biaryl angle that occurs on the protein but not in the solvent \((10)\), remains to be established but provides a promising hypothesis. Despite these discrepancies, it seems clear that some B ring dependent interactions with tubulin, substitution at C-7, and especially an N-acyl group, are required in the fluorescence response of colchicinoids to binding to tubulin.

**Discussion**

One of the peculiar properties of colchicine binding to tubulin is the slow equilibration, which often takes 60-90 minutes. Garland first proposed a two step model in which rapid, reversible binding to tubulin is followed by slow
conformational changes that convert the initial complex to a more stable, less easily reversible state (11). This model, with some modifications, is the currently most used one and it has been shown (26) that the promotion of fluorescence best coincides with the formation of the second complex. The conformational changes leading to the more stable state have been postulated to be due to changes in the tubulin molecule and the colchicine molecule (27,28), or both. It is not clear whether these mutual conformational changes are concerted or sequential with selection of the proper fit. The assumption made is that initial binding occurs with the skewed conformation whereas binding to the second state occurs with the more planar conformer (10).

Is it possible that the B ring or substitutions at C-7 contributes to these conformational changes? Certainly the B ring provides constraints on the spatial relationship of the A and G rings to each other. This ring contributes three properties to the drug: a) bulk (both the three carbon chain of the ring and substituents at C-7), b) the N-acyl group, and c) atropisomerism. The present studies clearly show that bulk at C-7 impedes the binding process and this effect is greater on the association rate than on the equilibrium constant (Table I). On the other hand, the apparent irreversibility of the binding seems more closely
related to the presence of a N-acyl group, although the nature of its interaction with the binding site is not clear at present. Since the presence of an N-acyl group is also associated with higher quantum yields of the colchicine-tubulin complex (Table II), the question of the relation of the irreversibility to the quantum yield may well be raised. The lower quantum yield (0.021) for the AC compound (Table II) is consistent with a connection between the two phenomena.

The role of the B ring itself has been more difficult to rationalize. The drug loses its negative circular dichroic band at 340 nm and it was postulated that binding stabilizes rotation about the biaryl bond from an angle of 53° to 19°, thereby facilitating extended conjugation and hence fluorescence (10,27). Such a mechanism would explain the 20 fold greater association constant of the AC compound compared to deacetamidocolchicine (Table I), provided it is assumed that the more planar conformation forms the stable complex. Additional impediments to approaching complanarity appear to occur in 5,6-dehydro, 7-deacetamidocolchicine and the quantum yield is reduced (Table II). To what extent these considerations pertain to the excited state remains to be determined. Whether this effect, or the reduction of vibrational energy dissipation
from the excited state, is the chief factor promoting fluorescence is difficult to determine, since both occur as a consequence of binding to tubulin. Other points for speculation are (1) whether the binding of the B ring causes additional immobilization and thus enhanced fluorescence; and (2), whether the thermodynamic differences between binding of colchicine and the AC compound can, in part, be accounted for by the third interaction site rather than the cratic component.

In any case, it seems likely that the B ring of colchicine with the acetamido group at C-7 is a third determinant recognized by the binding site. The domain plays a major role in the kinetics of binding, reversibility, and the induction of fluorescence.
PART - IB : Role of Bering Side Chain of Colchicine on Its Binding to Tubulin

Abstract

The chemical specificity of colchicine binding site of tubulin is less stringent for the presence of the B-ring than the A- and the 0-rings of colchicine. But when present, the B-ring itself retards the binding rate to tubulin. Here we examined the effect of the substituents present at the 7 position of the B-ring, as exemplified by desacetamido-colchicine (referred to hereafter as DAC), on binding to tubulin. We observed that DAC binds to tubulin 12-50 times faster than colchicine with an association rate constant $6 \times 10^6 \text{M}^{-1}\text{h}^{-1}$. DAC binding is about 20-30 times slower than 2-methoxy-5-(2',3',4-trimethoxyphenyl)tropone (henceforth termed AC because it lacks the B-ring of colchicine) but 3-4 times faster than colcemid binding to tubulin. Thus a series can be represented as $\text{AC} \succ \text{DAC} \succ \text{colcemid} \succ \text{colchicine}$. However, the value determined for the activation energy for DAC tubulin interaction (12 kcal/mol) was similar to that for colcemid and AC binding to tubulin but about 10 kcal/mol less than that for colchicine. Apparent thermodynamic parameters determined for DAC binding to tubulin were: standard enthalpy $\Delta H^0 = 0.5 \text{ kcal/mol}$, standard free energy $\Delta G^0 = -7 \text{ kcal/mol}$ and $\Delta S^0 = 24.1 \text{ e.u.}$.

Like AC and colcemid, DAC binding to tubulin was observed to be reversible. We conclude that while the rate of drug tubulin association is inversely related to the size of the substituent present at the 7 position of the B-ring, no such correlation could be drawn for the reversibility of binding. However, it appears that irreversibility of binding is related to the presence of the carbonyl group on the side chain of the B-ring.
Introduction

It was first reported from this laboratory that the slow association rate of colchicine is a phenomenon of the B-ring of colchicine molecule (13). Thus, colchicine analogs with smaller (colcemid) or no substituents in the B-ring (desacetylcolchicine) bind tubulin remarkably faster than colchicine. A compound without the B-ring (AC compound) binds tubulin instantaneously at 37°C. These findings were confirmed recently by others (7,10). Bane et al. found that the activation energy of AC binding to tubulin is 7-11 kcal/mol less than that for colchicine binding to tubulin (10).

The activation energy of the colchicine tubulin reaction is sum of the energy required for the colchicine to achieve the binding conformation plus the energy required for the tubulin conformation change. Thus the high activation energy for colchicine binding compared to that of AC has been accounted due to the B-ring imposed rigidity on colchicine molecule. In order to distinguish the effect of B-ring itself on the binding reaction from that of the side chain present at C-7 position of the B-ring, it is appropriate to choose a congener which has the B-ring imposed rigidity such as colchicine but no side chain on the B-ring.

In the present study, the binding parameters of DAC (having no side chain on the B-ring have examined and
compared those with other colchicine analogs like colcemid and deacetylcolchicine both having the side chain (Fig. 1) or AC which lacks the B-ring (Fig. 1). It is observed that DAC binding to tubulin is about 20 times slower than that of AC indicating that the B-ring alone indeed affect the rate of binding (Part IA, Table I). Nevertheless, DAC binding rate to tubulin is 18 times faster (Part IA, Table I) compared to that of colchicine suggesting that the side chain on the B-ring contributes a unfavourable environment addition to the B-ring alone in the association of colchicine congeners to tubulin.

To get a better insight about the binding reaction, the activation energies of several analogs with different side chains (Fig. 1) have determined. It is observed that the activation energy of the reaction is not affected by the allicyclic ring itself or even by the presence of NH₂ or NH.CH₃ at 7 position of the B-ring. It is the carbonyl group whose presence significantly contributes to the activation and also the irreversibility of the colchicine tubulin interaction.

Results and Discussion

Fluorescence of DAC-Tubulin Complex

The fluorescence excitation and emission spectra of DAC in the presence of tubulin are shown in Figure 4A.
While free colchicine has almost no fluorescence, this analog has a little fluorescence in a buffer containing MES-MgCl$_2$-EGTA under identical condition (data not shown). The fluorescence is enhanced at least 270 fold when DAC binds to tubulin. Uncorrected excitation and emission maxima of the complex are at 362 nm and 424 nm respectively. The quantum yield of this drug-tubulin complex is 0.023. Tryptophan fluorescence of tubulin has an emission peak at 335 nm when excited at 280 nm and this fluorescence has been found to be quenched by about 20-25% when tubulin is observed between the tryptophan and the bound DAC molecule (Fig. 4B, Curve 2-4). Figure 5 (inset) shows the time dependence of fluorescence of DAC-tubulin complex. This experiment is carried out under pseudo first order conditions, with DAC present in at least 50 fold molar excess. Under these experimental conditions, where a single exponential increase of fluorescence is expected, the logarithmic plot clearly shows presence of two phases (Fig. 5). Similar two phases are also observed in the case of colchicine binding to tubulin (11,26). Recently Bane et al. (10) also observed similar two phases in AO binding to tubulin. In this study, we did not analyze further these two phases which are expected to give similar results as reported to its other analogs viz. colchicine (11,26) and AO (10).
FIG. 4

A

Excitation spectra

Emission spectra

B

FLUORESCENCE AT ARBITRARY UNITS

WAVELENGTH (nm)

FIG. 4
FIG. 5

$\ln(F_{\text{max}} - F_t)$ vs. Time (min)

Fluorescence 430 nm

Time (min)
Association Rate Constant and Activation Energy of Binding

The bimolecular association rate constants have been described in Part IA: Table I. The mean value of the second order rate constant ($k_1$) for DAC tubulin binding has been found to be $6 \times 10^6 \text{ M}^{-1}\text{h}^{-1}$. This value is about 12-50 times higher than that for the colchicine tubulin interaction (31) reflecting the effect of removal of the B-ring side chain ($R = \text{NH}_2\text{CO.CH}_3$, Fig. 1). However, this value of rate constant for DAC tubulin association is about 30 times slower than that reported for AC tubulin interaction (10). The difference between association rate constants for AC and DAC clearly indicates that B-ring alone (without side chain) significantly influences the rate of association. This can be explained to be the result of restriction provided by the B-ring on the spatial relationship between the A- and the C-rings to each other as suggested by Bane et al. (10). Nevertheless, the observation that DAC binds 12-50 times faster than colchicine, suggests that a more substantial constrains is contributed by the side chain present at the 7 position of the B-ring. The rate constant for association, it is reported, for colcemid tubulin interaction is $1.88 \times 10^6 \text{ M}^{-1}\text{h}^{-1}$ (29) which is again 3-4 times slower than DAC tubulin interaction, but about 7-10 times faster than colchicine tubulin interaction. Thus the higher value for association rate constant for
Colcemid compared to colchicine results from depletion of carbonyl group from colchicine. It is not clear, whether this is a steric effect or the carbonyl group is involved in any type of interaction with the protein. Nevertheless, bulk or size of the B-ring side chain appears to play a significant role. Thus smaller the size of the B-ring side chain higher is the value of association rate constant (Table III).

Table III
Comparison of the Association Rate Constants ($k_1$) and Activation Energies ($E_a$) of Colchicine Analogs Binding to Tubulin

<table>
<thead>
<tr>
<th>Colchicine congeners</th>
<th>Substituent at 7 position of the B-ring (R in Fig. 1)</th>
<th>Association rate constant ($M^{-1}h^{-1}$)</th>
<th>Activation energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colchicine</td>
<td>-NH₂. CO. CH₃</td>
<td>0.12 - 0.48 x 10⁶⁺</td>
<td>20 - 24⁺</td>
</tr>
<tr>
<td>Colcemid</td>
<td>-NH₂. CH₃</td>
<td>1.88 x 10⁶⁻</td>
<td>12</td>
</tr>
<tr>
<td>Deacetyl colchicine</td>
<td>-NH₂</td>
<td>1.80 x 10⁶</td>
<td>13.80</td>
</tr>
<tr>
<td>Desacetamido colchicine (DAC)</td>
<td>-H</td>
<td>6 x 10⁶⁻</td>
<td>13</td>
</tr>
<tr>
<td>2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone (AC).</td>
<td>No B-ring</td>
<td>1.87 x 10⁸⁻</td>
<td>13⁻</td>
</tr>
</tbody>
</table>

⁺The values were obtained from Cortese et al. (5), Ray et al. (29) and Bane et al. (10).
Interestingly enough the activation energies of DAC and colcemid binding to tubulin (Fig. 6) have been found to be 13 kcal/mol and 12 kcal/mol respectively. Similar value for activation energy has been reported for AC binding to tubulin (10). These values are about 8-12 kcals less than that for the colchicine tubulin interaction i.e. 20-24 kcal/mol (5). Thus it appears from these studies that the rate of binding is controlled at several points of the B-ring, that is, allicylic ring itself, carbonyl group and the overall size of the side chain at the 7 position. However, the activation energy of the reaction is not affected by the allicylic ring itself or even by the NH-CH$_3$ group present at the 7 position of the B-ring but the carbonyl group significantly contributes to the high activation energy (Table III).

**Binding Parameters at Equilibrium**

Striking difference between colchicine and AC in the thermodynamics of their binding to tubulin is that colchicine reaction is entropy driven whereas AC reaction is enthalphy driven and has small positive entropy (10). Andreu et al. (7), however, reported high entropy value for the same reaction using different buffer composition. In order to evaluate the thermodynamic parameters, the temperature dependence of the binding reaction was examined. Tubulin induced fluorescence of DAC was used to determine the number
Fig. 6. Temperature dependence of the association rate constants. Rate constants at each temperature was calculated as described in Materials and Methods.
of DAC binding site per dimer of tubulin. The number of binding site was determined from the Scatchard plot. The number of DAC binding sites per tubulin dimer was found to be 0.8. Similar value was also obtained for colchicine binding with the same protein preparation. The binding of DAC to tubulin was found to be weakly dependent upon temperature as shown by the Van't Hoff plot (Fig. 7). Thus, the value for standard enthalpy of binding (ΔH°) calculated from the slope of the curve (Fig. 7) was 0.5 kcal/mol. The standard free energy (ΔG°) of binding at 37°C was equal to -7 kcal/mol and ΔS° = 24.19 cal/deg/mol. Similar ΔG° value has been reported for AC binding to tubulin. However, in contrast to AC, the affinity for the DAC tubulin interaction is not lowered at high temperature. A little increase in affinity is observed and hence the Van't Hoff plot gives negative slope (Fig. 7).

**Reversibility**

The binding of colchicine to tubulin can be considered to be practically irreversible. The rate constant of dissociation is indeed extremely small (31). Colcemid and AC binding to tubulin are reversible (13,30). In Part IA, Table I, reversibility is tested qualitatively for several B-ring analogs. It is found that in addition to colcemid and AC, DAC and deacetyl colchicine also reversibly bind to tubulin. Here the reversibility is measured quantitatively. The reversibility of
Fig. 7. Van't Hoff plot of DAC binding to tubulin. Affinity constants were calculated from the Scatchard plot at each temperature.
FIG. 7

\[ \ln(KA) \] vs. \( \left( \frac{1}{T} \right) \times 10^3 \)

[Graph showing a linear relationship between \( \ln(KA) \) and \( \left( \frac{1}{T} \right) \times 10^3 \).]
drug tubulin binding is tested by adding 100 fold excess of podophyllotoxin (a competitive inhibitor of colchicine analogs) at the steady state level of drug tubulin interaction (Fig. 8). It is observed that addition of podophyllotoxin at the steady state decreases the fluorescence of DAC-tubulin complex with time. In 60 minutes about 32\% decrease of maximum fluorescence is observed. Under identical condition fluorescence of (a) AC tubulin is decreased by 80\% (b) deacetyl colchicine tubulin is decreased by 40\% and (c) colchicine tubulin remains practically unchanged. Reversibility of colcemid binding was examined by chasing ^3H-colcemid-tubulin complex (which does not fluoresce) with unlabelled colcemid and 65\% reversibility was observed. So, the series of reversibility of colchicine analog and tubulin binding can be written as AC (80\%) \succ colcemid (65\%) \succ deacetyl colchicine (40\%) \succ DAC (32\%) \succ colchicine (0\%).

Thus no correlation is apparent between reversibility of the drug tubulin binding and the size of the B-ring substituent at 7 position of the colchicine congeners. However, the presence of carbonyl group in B-ring side chain contributes to irreversibility of colchicine. This is also supported by activation energy data.
Fig. 8. Reversibility of drug-tubulin complexes. Time course of $5 \times 10^{-6} \text{M}$ colchicine analogs binding to $2.5 \times 10^{-6} \text{M}$ tubulin at $37^\circ \text{C}$. Podophyllotoxin ($1 \times 10^{-4} \text{M}$) was added at the time indicated by the first (from left) and the second arrow indicates the amount of analogs remain bound to tubulin after incubation with podophyllotoxin at $37^\circ \text{C}$ for 60 min.
FIG. 8

Desacetoxy Colchicine
A-C Compound
Colcemid
Colchicine
Deacetyl Colchicine

TIME (MIN)

FLUORESCENCE 430 nm

100
80
60
40
20
0
0
10
15
20
30
40
50
60
70
80
90
100

(4--------4) 3H-Colcemid (CPM x 10^2)
Conclusion

Activation energy of the drug tubulin reaction is the sum of the energy required for the drug to achieve the binding conformation plus the energy required for the tubulin conformational change. Since the activation energy required for AC to achieve the binding conformation is expected to be quite low due to free rotation about the biaryl bond, the difference in the activation energies between AC binding and colchicine binding to tubulin reflects the increased energy required for the colchicine conformational change. Since the restriction on the free rotation between the A and C-ring is provided by the allicyclic B-ring, it is expected that the activation energy for the DAC binding to tubulin should be higher compared to that of AC. However, our results show that values are very similar, although the binding conditions for AC is different than that of DAC reported here. Our results indicate that the side-chain on the B-ring significantly contributes to the activation energy only when carbonyl group is present. There are several ways carbonyl group may create unfavorable environment and hence higher activation energy for the binding reaction. Thus, it is possible that the carbonyl group is involved in any type of interaction with tubulin and thus protein undergoes more stringent conformational change compared to that needed when it binds analog without carbonyl group. Secondly, the
presence of carbonyl group imposed a restriction on the free rotation of the side chain as experienced from the Dreiding model. Thus it is possible that the colchicine having a carbonyl group at the side chain will need different amount of activation energy than that of DAO and colcemid (having no carbonyl group) in order to achieve the binding conformation. In Part IA, it is emphasized the role of carbonyl group in the colchicine fluorescence. In absence of carbonyl group from the C-7 substituent, there was a sharp reduction of quantum yield of tubulin drug fluorescence.

**Materials and Methods**

Both $^3$H-colchicine and $^3$H-colcemid having specific activity of 9.3 Ci/mmol and 27.8 Ci/mmol respectively were the products of New England Nuclear Corporation. GTP (Grade IIS) and colchicine were products of Sigma. Colcemid was from K and K. DE-81 and GF/C were obtained from Whatman. Des-acetamidocolchicine and AC compound [2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone] were gifts of Dr. T.J. Fitzgerald, Florida A and M University. The quarternary amine was generously provided by Dr. M. Röser (NIH postdoctoral fellow). It was prepared from colcemid by reaction with methyl iodide (m.p. 213-215°C, $M^+ + 1 = 385$). Colchicinoids with different substituents at C-7 were prepared as described (17-19). All other chemicals used were reagent grade.
Preparation of Tubulin

Tubulin was prepared by phosphocellulose purification of either goat or rat brain microtubule protein, prepared by two cycles of temperature dependent polymerization (14). Protein was stored at liquid nitrogen in tubulin assembly buffer (0.1 M MES, 0.5 mM MgCl₂, 1 mM EGTA, pH 7.0). Same assembly buffer without GTP was used for all radioactive binding experiments and fluorescence measurements to prevent polymerization. Protein was estimated according to the method of Lowry et al. using BSA as a standard (32).

Fluorescence Measurements

Fluorescence measurements were carried out in thermostated cuvettes at 37°C in a Perkin-Elmer MFP 3L instrument with an excitation wavelength of 353 nm and emission at 430 nm except when stated. Corrected emission spectra were measured with a modified Bowman Aminco Spectrofluorometer. The quantum yields of colchicine analog-tubulin complexes were calculated by comparison with quinine sulfate in 0.1 M H₂SO₄, whose quantum yield was taken as 0.546 at 25°C (15).

Measurements of Colchicine Analogs Binding

The binding of colchicine analogs to tubulin was determined by competition for 1 μM [³H]-labeled colchicine
binding. 250 μl aliquots (0.25-0.5 mg/ml) of rat brain tubulin solution containing 100 mM MES buffer (pH 7.0), 1 mM MgCl₂ and 1 mM EGTA and 2 μM ³H-colchicine and analogs (added at the beginning of the incubation to concentration ranges from 10⁻⁶ to 10⁻⁴ M), were incubated for 60 min at 37°C. The extent of binding was measured by the DE-81 filter disc method (16). Apparent Kᵣ values, the amount of colchicine analog required to inhibit the ³H-colchicine binding to tubulin by 50%, were then determined from the semilog plot of colchicine binding activity against the concentration of analog.

**Colcemid Binding Assay**

Colcemid binding activity was measured by using radioactive colcemid as this drug gives no fluorescence as a result of binding to tubulin. Binding was assayed by the GF/C filter disc method as described by Ray et al. (15). The radioactive discs were counted in Beckman LS 1800 Liquid Scintillation System using 5 ml of toluene based fluor containing 0.5% PPO and 0.005% POPOP.

**Association Rate Constant**

The bimolecular association rate constant (k₁) can be represented as:

\[ k₁ = \frac{d(CT)}{dt} \cdot \frac{1}{[O][T]} \]
where $\frac{\Delta [CT]}{\Delta t}$ is the rate of formation of the complex CT (colchicine analog-tubulin) and $[C]$ and $[T]$ are the concentrations of free colchicine analog and unbound tubulin, respectively. Conditions were adjusted such that <10% of the reactants were consumed during the reaction and progress curves were linear. We have thus assumed that $[C] = [C]_0$ and $[T] = [T]_0$ where $[C]_0$ and $[T]_0$ are the initial concentrations of colchicine analog and tubulin, respectively. The amount of analog bound protein was determined by fluorescence as follows: $[CT] = (F_c/F_0)x C_0$ where $[CT]$ is the amount of complex, $F_c$ is the fluorescence of a given solution of analog-tubulin complex, and $F_0$ is the fluorescence of an equal concentration of analog in excess tubulin, such that all the analog is bound and $C_0$ is the total analog concentration. Fluorescence of the analogs in water was negligible at the concentrations used. Each value of the rate constant is an average of three determinations. In general, analog concentration was held constant (3-5 µM) and linear curves were produced over a concentration range of 3-8 µM of tubulin.

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ROLE OF B-RING ON (i) ABSORPTION SPECTRA AND (ii) PHOTO-INDUCED REARRANGEMENT OF C-RING OF COLCHICINE
Visible absorption spectra (between 300 nm and 400 nm) of colchicine have been found to be related with the B-ring part of colchicine. Absence of B-ring causes a significant blue shift in the absorption band. Thus 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone has absorption maxima at 341 nm whereas that of desacetamidocolchicine is at 352 nm. A significant red shift in the visible absorption band of colchicine, desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone also occur when they are immobilized in the binding site to tubulin or in pure glycerol.

We also observed that B-ring substituent of colchicine has no role in the ultraviolet induced rearrangement of colchicine into lumicolchicine. However, in absence of B-ring such as in the case of 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone, the rearrangement reaction slowed down significantly.
Introduction

Several properties of colchicine tubulin interaction such as association rate, reversibility and the promotion of drug fluorescence have been found to be related to the B-ring of colchicine. The B-ring itself retards the binding rate and substitution at C-7 leads to further decreases which appear to be related to both substituent bulk and the presence of N-acyl group. Thus, the decreasing order of binding rates is 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone (AC compound) > desacetamidocolchicine > desacetyl colchicine > colcemid > colchicine. On the other hand, the apparent irreversibility of the binding seems more clearly related to the presence of an N-acyl group rather than the bulk of the substituent at C-7. Substitution at C-7 also effects the tropolone fluorophore. Thus amines (deacetyl colchicine, colcemid or N-methyl colcemid) do not fluoresce in the presence of tubulin, whereas the substitution of the amino group with an acyl group enhances fluorescence. All these observations are described in both Part IA and Part IB.

In the present study, we show some other properties of colchicine such as its spectral (visible) properties and its photoconversion into lumicolchicine in the presence of ultra violet light which are also related to the B-ring of the drug.
Results and Discussion

Absorption spectra of colchicine can be divided into two parts: one ranging from 200 to 300 nm which is composed of the absorption bands with extinction coefficient values of $10^4 - 10^5$ and the other ranging from 300 to 400 nm which is a little weaker in intensity ($\sim 1.5 - 1.8 \times 10^4$). Colchicine and all its analogs we are discussing in this study have $\text{C} = 0$ (at C-ring) and $\text{C} = 0$ conjugated with each other. Such compounds show two kinds of electronic bands: one due to $\text{N} \rightarrow \text{Vn}$ type transitions and the other due to the $\text{N} \rightarrow \text{En}$ type transition. The bands of the first kind are related to the $\pi$ electrons extending along the entire system of conjugated bonds and are strong in intensity ($\xi \sim 10^4$), while the bands of the second kind are related to the electrons in the nonbonding molecular orbital, largely localized on the oxygen atom are weak in intensity ($\xi \sim 10^1$).

Two kinds of bands observed in the case of colchicine (Fig. 1A) belongs to $\text{N} \rightarrow \text{Vn}$ transition. Figure 1B shows the visible spectra of colchicine, desacetamidocolchicine and 2-methoxy-5-$(2',3',4'$-trimethoxyphenyl)tropone in water. Values of extinction coefficients are very similar for all of them. Colchicine and desacetamidocolchicine have absorption maxima at 352 nm where as in the case of 2-methoxy-5-$(2',3',4'$-trimethoxyphenyl) tropone, it is at 341 nm. Compare to 2-methoxy-5-$(2',3',4'$-trimethoxyphenyl)tropone, this significant red shift
Fig. 1A. Ultra violet absorption spectra of colchicine (2.27 x 10^{-6} M) in water or in assembly buffer containing 100 mM MES (pH 7.0), 1 mM Mg^{2+} and 1 mM EGTA Vs. water or assembly buffer without drug.

Absorption maxima: \( \lambda_{\text{max}_1} = 247 \text{ nm} \) and \( \lambda_{\text{max}_2} = 352 \text{ nm} \).

Fig. 1B. Absorption spectra (visible region, \( \lambda_{\text{max}_2} \)) of colchicine (2.7 x 10^{-6} M), DAC (2.8 x 10^{-6} M) and AC compound (3 x 10^{-6} M) in assembly buffer Vs. assembly buffer.

1 - Colchicine, \( \lambda_{\text{max}} = 352 \text{ nm} \)
2 - DAC, \( \lambda_{\text{max}} = 352 \text{ nm} \)
3 - AC compound, \( \lambda_{\text{max}} = 341 \text{ nm} \).
(341 nm to 353 nm) in the absorption spectra is due to the presence of allycyclic B-ring into it. It is known that intensities and positions of peaks depend on the length of the conjugated system: the longer such a system, the longer the wavelength of the absorption and larger the extinction coefficient. When the \( \pi \) electron system is prevented from achieving coplanarity, the degree of overlap of the \( \pi \) electron system will be diminished, resulted a marked effect on ultra violet spectrum (blue shift). Thus, the presence of B-ring might have done two things: it makes A- and C-ring more planar in the case of desacetamidocolchicine compared to 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone; other possibility is that A- and the C-ring which can rotate freely along C\(_{15}\) - C\(_{16}\) bond in 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone become immobilized when they are anchored by the B-ring as in the case of desacetamidocolchicine. Recently it was shown that colchicine which fluoresce when binds tubulin is mainly due to the immobilization of the drug in the binding site and this response to immobilization of colchicine depends, in part on the partially flexible nature of the drug (5). Thus conclusion of the colchicine fluorescence was based upon the findings that colchicine shows a remarkable fluorescence in pure glycerol and this fluorescence decreases with decreasing viscosity of the medium. Figure 2A shows the visible absorption spectra of colchicine when immobilized to
Absorption spectra (visible region, \(\lambda_{\text{max}}\)) of colchicine, DAC and AC compound in assembly buffer (1), in 100% glycerol (2) and in presence of tubulin (3). In case of (1) and (2), the absorption spectra are taken by adjusting the background of assembly buffer and 100% glycerol respectively. In case of (3), the drugs are incubated with 5 times excess of tubulin (15 \(\mu\)M) in assembly buffer without GTP at 37°C for 30 mins and the absorption spectra are taken by adjusting the background of tubulin in assembly buffer.

A - Colchicine (2.7 \(\times\) 10^{-6}M)  
\(\lambda_{\text{max}}\) : (1) 352, (2) 358, (3) 354,

B - DAC (2.8 \(\times\) 10^{-6}M)  
\(\lambda_{\text{max}}\) : (1) 352, (2) 355, (3) 356 and

C - AC compound (3 \(\times\) 10^{-6}M)  
\(\lambda_{\text{max}}\) : (1) 341, (2) 348, (3) 346.
its binding site at tubulin or in pure glycerol. Both cases a significant red shift of the absorption maxima is observed. Similar red shifts in $\lambda_{\text{max}}$ are observed in the case of desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone when they are bound to tubulin or present in pure glycerol (Fig. 2B and Fig. 2C).

Ultra violet irradiation causes the conversion of colchicine to $\beta$- and $\gamma$-lumicolchicine (1-3). This photon induced rearrangement occurs at the C-ring part of colchicine (Fig. 3). It has been reported that position of the groups present at C-ring affect the rate of rearrangement reaction (4). The marked effect is observed when the position of oxygen and the methoxy group in ring C are interchanged. Thus isocolchicine shows remarkable stability in presence of ultra violet light (4). The effect of B-ring substituent at C-7 position has been found to be less significant. In fact when the structure of colchicine and lumicolchicine is compared (Fig. 3), no direct involvement of B-ring is noticed. However, in this study we observed that the absence of B-ring from colchicine make a compound which is very stable towards ultra violet irradiation. Thus the conversion of 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone into its lumicolchicine derivative is much less than that of colchicine (Fig. 4A). Since the conversion of colchicine or its analogs into their corresponding lumicolchicine derivative take place with the
Fig. 3. Conversion of colchicine to $\beta$- and $\gamma$-lumi colchicine.
loss of chromophorore C-ring (which absorbs light with the absorption maxima at 350 nm) this photochemical conversion can be followed easily by measuring the decrease of absorbancy at 350 nm. Figure 5 shows a typical irradiation experiment with colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone. It can be seen that 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone has much slower break down than colchicine and 30 minutes irradiation caused 33% and 80% decrease of O.D. (at their corresponding λ_max, ) respectively. Isobestic point which is a proof for the authenticity of a compound has found to be at 303 nm and 292 nm for colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone respectively. Desacetamidocolchicine, a compound having the B-ring but no substitution at C-7 position has been found to be equally susceptible like colchicine (Fig. 4A). All these irradiation experiments were done by dissolving drug in 95% ethanol.

Figure 4B shows how colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone are effected by irradiation when they are bound to tubulin. Irradiation of the colchicine-tubulin complexes with ultra violet light caused lowering of fluorescence at 430 nm which is characteristic property of the tubulin-colchicine complex and after 10 min irradiation, 33% of the fluorescence was lost, whereas under identical conditions tubulin-AC complexes remained unaffected.
Fig. 4A. Time kinetics of ultra violet induced rearrangement of colchicine, DAC and AC compound in 95% ethanol. The ultra violet irradiation experiment has been done as described in Materials and Methods.

Colchicine (Δ—Δ), DAC (○—○) and AC compound (○—○).

Fig. 4B. Time course of release of drug from drug-tubulin complex (measured by decrease in fluorescence at 430 nm) during irradiation with ultra violet light.

2.5 μM tubulin in 100 mM PIPES (pH 6.9) - 0.5 mM Mg²⁺ - 1 mM EGTA was incubated with 5 μM drug at 37°C for 30 mins. The resultant drug-tubulin complex was irradiated for the indicated times and fluorescence at 430 nm was measured.

Colchicine-tubulin complex (○—○) AC-tubulin complex (Δ—Δ).

Fig. 5. Absorption spectra of colchicine and AC compound at different time intervals during irradiation with ultra violet light. The irradiation experiment has been done as described in Materials and Methods. Absorption spectra are taken by adjusting the background of 95% ethanol.

A - Colchicine, isobestic point - 303 nm
B - AC compound, isobestic point- 292 nm.
FIG. 4

FLUORESCENCE, at 430 nm

ABSORBANCE, at 350 nm

TIME (min)
Materials and Methods

Materials are used as described in Part I.
Tubulin was prepared as described in Part I.

Absorption spectra were obtained on a Cary Model 219
recording spectrophotometer, using 1 cm light path.

For the ultra violet irradiation of free drug,
each compound is diluted in 95% ethanol and then placed in
a 1 ml quartz cuvette (1 cm light path) exactly 2.5 cm from
a short wave uv lamp Model uv 4-11 (ultra violet products,
Inc., San Gabriel, California, USA) in a dark room. After
irradiation the cuvette was transferred to the spectrophotome-
ter for an absorbancy measurement at 350 nm and then
returned to further irradiation.

In case of bound drug, colchicine and AC was
preincubated with tubulin in assembly buffer for 30 min
at 37°C and then irradiated for different time intervals.
Fluorescence measurements were done with the Perkin-Elmer
MPF 44B Spectrofluorometer.

References

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pH EFFECT ON TUBULIN POLYMERIZATION
IN THE PRESENCE OF DIFFERENT B-RING
ANALOGS OF COLCHICINE
Abstract

Assembly of DEAE purified tubulin is significantly affected by alteration of pH. At pH 6.4, the polymers formed are ribbon shaped or sheet structure whereas at 6.94, microtubules are formed. In contrast, cycle-purified tubulin makes microtubule at both pH. Tubulins prepared by both methods show biphasic kinetics at pH 6.4 and single phase at pH 6.94. At pH 6.4, DEAE purified tubulin is unable to assemble in presence of suprastoichiometric concentration colchicine, desacetamidocolchicine and 2-methoxy-5-(2',3',4'- trimethoxyphenyl)tropone. Although, all these drugs substoichiometrically inhibit the assembly reaction at this pH. However, cycle-purified tubulin can assemble at pH 6.4 in the presence of suprastoichiometric concentration of colchicine, desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone. Tubulins prepared by any one of these methods can not polymerize at 6.94 in the presence of suprastoichiometric concentration of these drugs.
Introduction

The discovery by Weisenberg (1) of conditions favouring microtubule formation in vitro led to purification methods, based on repeated cycles of assembly and disassembly. Shelanski et al. (2) described a purification method which used 4 M glycerol in the assembly buffer. Glycerol free assembly disassembly procedures have also been developed by Borisy et al. (3). Other differences between these two methods are that MES buffer of pH 6.4-6.5 has been used by Shelanski et al. whereas PIPES buffer of pH 6.94 has been used by Borisy et al. Recently a significant variation of the properties of microtubules protein as a result of changing solution conditions have been reported (4). Barton and Riazi (5) presented kinetic evidence indicated the existence of two consecutive steps in the growth phase of microtubule. Their work has been supported by others (6,7). Johnson and Borisy (8) performed a similar analysis and their data yielded only one first order process. Although in both cases PIPES buffer has been used, pH was 6.5 and 6.94 of Barton and Riazi (5) and Johnson and Borisy (8) respectively. Recently, Martin et al. (9) showed that three components of microtubule protein 6S dimer and two digomers species containing tubulin and MAPs (18S oligomer and 30S ring species) and particularly are markedly sensitive to protein concentration, ionic composition and pH. They showed both
oligomers 10S and 30S species being much more stable at pH 6.4 than at 6.9. They also showed the near ultra violet circular dichroic properties of microtubule protein as a result of changing in pH, ionic composition and protein concentration etc. In this section, it is shown the effect of pH in the assembly of tubulin isolated by two different methods. Three aspects of assembly at different pH are analysed in this chapter: (i) morphology of the polymers, (ii) kinetics of the assembly reactions and (iii) effect of colchicine and its B-ring analogs in the assembly reactions.

Results

Figure 1 shows the assembly of DEAE purified tubulin at pH 6.4 and 6.9 where identical amount of tubulin (2.4 mg/ml) and PIPES buffer (100 mM) have been used. Although the rate of polymerization and the amount of turbidity at the plateau level is much higher at pH 6.4, polymers formed at both pH are highly cold sensitive. The ratio of polymer mass to its turbidity values at these pH are shown in following Table. Since the steady state turbidity indicates the polymer mass, it is expected that the critical concentrations for polymerization will be different at two pH. Surprisingly, it has been observed that the critical concentrations at these two pH are almost identical (Fig. 2).
Fig. 1. Assembly of DEAE purified tubulin at pH 6.4 and 6.9.

DEAE tubulin (2.4 mg/ml) in 100 mM PIPES - 0.5 mM Mg²⁺ - 1 mM GTP - 4 M glycerol was incubated at 37°C and polymerization was monitored spectrophotometrically at 400 nm. After steady state was reached, the cuvette was placed in ice for 2-3 mins (indicating by arrow).

Fig. 2. Critical protein concentration of DEAE purified tubulin assembly at pH 6.4 and 6.9.

Assembly condition as in legend to Fig. 1. Tubulin at different concentration was polymerized and steady state turbidity at 400 nm was measured spectrophotometrically.
FIG. 1

O.D. 400 nm

TIME (min)

pH = 6.4

Cold

pH = 6.94

FIG. 1
Values of Steady State Turbidity and Sedimented Polymer Mass of the Assembled Polymer at pH 6.4 and pH 6.94.

<table>
<thead>
<tr>
<th>pH</th>
<th>Steady State Turbidity</th>
<th>Sedimentation Measurements&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Specific Turbidity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O.D. at 400 nm from Figure 1</td>
<td>Pellet Protein mg/ml</td>
<td>Supernatent Protein mg/ml</td>
</tr>
<tr>
<td>6.4</td>
<td>0.544</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>6.94</td>
<td>0.184</td>
<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

<sup>+</sup> Assembled mixtures (500 µl each) at two different pH as described in Fig. 1, are centrifuged at 100,000 x g for 30 min. The pellets are suspended in equal volume (500 µl).

Similar values for critical concentrations are obtained by sedimentation assay method. These results indicate that polymers formed at these two conditions will have different morphology. Electron microscopic observation of the assembled polymers formed at these two pH show different structures (Fig. 3). Microtubules are observed only at pH 6.9 whereas either sheet or ribbon structure are observed at pH 6.4. These structural variations of polymers at two pH explained the differences in mass to turbidity ratio of polymers at these pH. Elongation reaction of tubulin assembly follows
Fig. 3. Electron micrographs of assembled polymers of DEAE tubulin at pH 6.4 and 6.9.

Assembly condition and protein concentration as in legend to Fig. 1. Grids were prepared as described in Materials and Methods.

a and b - pH 6.4; c - pH 6.9.
Magnification: a - 60,000 x 2;
             b - 60,000 x 4;
             c - 60,000 x 2.
the pseudo first order kinetics (10). Here it is found that the elongation reaction has two growth steps at pH 6.4 and the reaction has single growth step at pH 6.9 (Fig. 4).

Identical experiments were done with two cycled purified microtubule protein (MTP). Figure 5 shows the polymerization of microtubule protein at pH 6.4 and 6.9. There are no significant differences either in the rate or in the extent of assembly at two conditions. Like DEAE tubulin assembly at pH 6.9 has more lag than at pH 6.4 and the pseudo first order kinetic analysis shows two step growth process at pH 6.4 and one step at 6.9. Electron microscopic analysis shows microtubular structures at both pH (Fig. 6). These results indicate that pH dependency of the assembly reaction is dependent upon the method by which tubulin has been prepared. In the case of cycle prepared tubulin, there is no significant change either in the rate or in the extent of assembly reaction. However, a significant differences in the rate, extent as well as in the morphology of polymers occurs when tubulin is prepared either by DEAE or by phosphocellulose method.

Although the assembly of MTP remains unaffected between pH 6.4-6.9, a significant pH dependency is noticed when this MTP is bound to colchicine or to its B-ring analogs.
Fig. 4. First order kinetic analysis of DEAE tubulin assembly at pH 6.4 and 6.9.

\[ \ln(A_\alpha - A_t) \text{ Vs. time plot.} \ A_\alpha \text{ indicates optical density at steady state and } A_t \text{ is the optical density at different time interval. Assembly condition and protein concentration as described in legend to Fig. 1.} \]

Fig. 5. Assembly of cycle purified microtubule protein at pH 6.4 and 6.9.

Second cycle microtubule protein (1.5 mg/ml) in 100 mM PIPES - 0.5 mM Mg\(^{2+}\) - 1 mM GTP was incubated at 37°C and polymerization was monitored spectrophotometrically at 400 nm. After steady state was reached, the cuvette was placed in ice for 2-3 mins (indicated by arrow).

Inset: First order kinetic analysis of microtubule protein assembly.

Polymerization: a - pH 6.4 and b - pH 6.9.

Fig. 6. Electron micrographs of assembled polymers of microtubule protein at pH 6.4 and 6.9.

Assembly condition and protein concentration as described in legend to Fig. 5.

a - pH 6.4, Magnification - 60,000 x 2.
b - pH 6.9, Magnification - 60,000 x 2.
FIG. 4

$\ln(A - A_t)$ vs. TIME (min)

- pH-6.94
- pH-6.4
Figure 7 shows the polymerization of MTP liganded to colchicine or to its B-ring analogs. At acidic pH, colchicine-tubulin, desacetamidocolchicine-tubulin and AC-tubulin can polymerize and the rate of polymerization drastically decreases at higher pH and no polymerization is detected at pH 6.9. The rate and the extent of assembly is highest in the case of AC-tubulin followed by DAC-tubulin and colchicine-tubulin complexes indicating the role of B-ring of colchicine. This assembly of drug-tubulin complexes also dependent upon the method by which tubulin was prepared. Instead of MTP when DEAE purified tubulin was used, colchicine-tubulin complex does not polymerize even at pH 6.4, whereas there is a significant amount of polymerization of AC-tubulin complex and of desacetamidocolchicine-tubulin complexes (Fig. 8). Reversibility of the drug-tubulin complex probably does not play any role in this observation since colcemid-tubulin complex which is highly reversible also does not polymerize. Figure 9 shows the effect of drug concentration on the polymerization of drug-tubulin complexes. At substoichiometric concentrations colchicine and its B-ring analogs such as desacetamidocolchicine and AC inhibit assembly whereas at the suprastoichiometric drug concentration assembly occurs in the case of DAC and AC. To polymerize colchicine-tubulin complex, we noticed that addition of MAPs in the reaction mixture is essential. To find role of MAPs in
Fig. 7. Assembly of microtubule protein at different pH in presence of colchicine and its B-ring analogs.

Microtubule protein (1.87 mg/ml) in 100 mM Pipes – 0.5 mM Mg\(^{2+}\) – 1 mM GTP was incubated at 37°C in presence of drugs and polymerization was monitored spectrophotometrically. Protein was isolated in 100 mM Pipes (pH 6.9) up to second cycle. The second cycle pellet was dissolved in 100 mM Pipes buffer of different pH. Rate of assembly denotes the maximum elongation rate during assembly reaction.

(1) No drug; (2) 100 µM colchicine; (3) 100 µM DAC and (4) 100 µM AC compound.

Fig. 8. Assembly of DEAE purified at pH 6.4 and 6.9 in presence of colchicine and its B-ring analogs.

DEAE tubulin (2.4 mg/ml) in 100 mM Pipes – 0.5 mM Mg\(^{2+}\) – 1 mM GTP – 4 M glycerol was incubated at 37°C in presence of drugs and polymerization was measured spectrophotometrically.

No drug – Curve 1 – pH 6.4 and Curve 1 – pH 6.9; 100 µM Colchicine – Curve 2 – pH 6.4 and pH 6.9; 100 µM DAC – Curve 3 – pH 6.4 and Curve 2 – pH 6.9 and 100 µM AC compound – Curve 4 – pH 6.4 and Curve 2 – pH 6.9, 100 µM Colcemid – Curve 2 – pH 6.4 and pH 6.9.
FIG. 8

O.D. 400 nm

TIME (min)

0 5 10 15 20 25

0.0 0.2 0.4 0.6 0.8 1.0

1 1' 3 4

FIG. 8
Fig. 9. Assembly of DEAE purified tubulin in presence of different drug concentrations.

DEAE tubulin (1.71 mg/ml) in 100 mM PIPES (pH 6.4) - 0.5 mM Mg^{2+} - 1 mM GTP - 4 M glycerol was incubated at 37°C in presence of drugs and polymerization was monitored spectrophotometrically. Elongation rate denotes the maximum elongation rate at different drug concentration at 400 nm and at 37°C.
Elongation Rate (O.D. 400/Sec x 10^3)

- Control
- Colchicine
- DAC
- Ac compound

[Drug], M

FIG. 9
drug-tubulin complexes assembly, microtubule protein is separated into two fractions by high speed ultracentrifugation. Pellet fractions called oligomer which is rich in MAPs and the supernatent called dimer containing very low quantity of MAPs. MAPs content is measured in both fractions by polyacrylamide gel electrophoresis. At pH 6.4 both fractions can assemble but the rate and extent of assembly of oligomer is higher than dimer. This is due to presence of high quantity MAPs. Microtubule protein mixtures containing different MAPs/tubulin ratio can be prepared by mixing the oligomer and dimer at different ratio. Microtubule protein mixtures are assembled in the presence of suprastoichiometric concentration of colchicine or AC compound (Fig. 10). When Figure 10B and 10C is compared, it is observed that at a particular MAPs/tubulin ratio, the polymerization of AC-tubulin complex is always much higher than that of colchicine-tubulin complex. Figure 11 shows the electron microscopic observation of polymers formed from AC-tubulin complexes. No clear structural features is observed.

Discussion

Microtubule assembly is a pH dependent process and takes place between pH 6.0 to 8.0 (11,12). Burton and Himes (13) reported earlier that at acidic pH phosphocellulose purified tubulin (DMSO induced) forms ribbon or sheet structure
Fig. 10. Assembly of tubulin and drug-tubulin complexes at different MAPs/tubulin ratio.

Second cycle microtubule protein is centrifugally separated into two fractions (i) oligomer (30S) and (ii) dimer (6S). MAPs/tubulin ratio in two fractions were determined by scanning of polyacrylamide gel analysis. Assembly experiment were performed at different oligomer/dimer ratio with a fixed protein concentration (oligomer + dimer = 1.1 mg/ml). Assembly buffer contains 100 mM PIPES (pH 6.4) - 0.5 mM Mg$^{2+}$ - 1 mM GTP. Drug-tubulin complexes are preformed by incubating oligomer and dimer with 100 µM drug at 37°C for 30 min. Polymerization was monitored spectrophotometrically at 37°C.

A - Tubulin; B - Colchicine-tubulin complex and C - AC-tubulin complex.

MAPs/tubulin ratio: (1) 0.874; (2) 0.619; (3) 0.491; (4) 0.364.

Fig. 11. Electron micrographs of the assembled polymer of AC-tubulin complex.

DEAE tubulin (2.4 mg/ml) in 100 mM PIPES - 0.5 mM Mg$^{2+}$ - 1 mM GTP - 4 M glycerol was polymerized in presence of 100 µM AC compound at 37°C. Grid was prepared as described in Materials and Methods.

Magnification - 1,00,000 x 2.
FIG. 10

(A) [Graph with curves labeled 1, 2, 3, 4]

(B) [Graph with curves labeled 1, 2, 3, 4]

(C) [Graph with curves labeled 1, 2, 3, 4]

O.D. 400 nm

TIME (min)

0 2 4 6 8 10

FIG. 10
instead of microtubules. At pH 6.4, we also observed similar ribbon and sheet structure in our DEAE purified tubulin preparation which contains a little amount of microtubule associated proteins. However at pH 6.9 microtubules are observed. When two cycled microtubule proteins are used, instead of DEAE tubulin then even at pH 6.4 we observed microtubules. In our preparation we do not find any composite structures at acidic pH as reported by Mandelkow and Mandelkow (14). This differences in observation might be due to the differences in the assembly conditions which in our case do not contain any glycerol.

Although the assembly of MTP remains unaffected between pH 6.4-6.9, a significant pH dependency is noticed when MTP is bound to colchicine or to its B-ring analogs. We observed that the structure of the drug (specifically the side chain present at C7 carbon atom of the B-ring) determines the polymerization efficiency. Presence of B-ring substituent of colchicine makes the drug-tubulin complex less competent to assemble at acidic pH. Thus it can be suggested that the B-ring substituent produces extra conformational change of tubulin besides the other part of the drug.

In addition to morphology of the polymers and drug inhibition property, the kinetics of the polymerization reaction has been found to be different at different pH. Such
phenomena is not affected by the MAPs. Barton and Riazi (5) reported two phase kinetics of tubulin assembly at acidic pH(6.4). At neutral pH (6.94), Johnson and Borisy (8) found one phase kinetics in the assembly of tubulin. The reaction mixture pH is 6.5 in case of former group. But in case of latter group, it is neutral (pH 6.94). Our results also shows that such two phase kinetics occurs at acidic pH. Barton and Riazi explained that the fast phase of the reaction is due to the incorporation of the oligomer (MAPs + tubulin) in the polymeric structure and the slow phase is due to the incorporation of dimer (free tubulin) in the polymer. Martin et al. (9) reported that at pH 6.4, oligomer is more stable than at pH 6.9. According to their explanation, it can be suggested that at neutral pH, the incorporation of oligomer does not take place during assembly.

MAPs have been considered basic proteins on the basis of their behavior on ion exchange resins, electrophoretic mobility (15-17), the resemblance of their effect on tubulin polymerization to those produced by polycations (18,19) and the apparent competition for MAPs by polycations such as RNA and DNA (20,21). The MAPs and pH sensitivity of the polymerization reaction suggests the possibility of charge-charge interaction in the polymerization process. When the amino acid sequence of α and β tubulin was solved, it immediately became apparent that the carboxyl terminal
regions of both subunits were highly acidic and contained 17/24 anionic groups of the protein in their last 20 amino acid residues (22,23). At the pH range (6.4-6.9) customarily used for polymerization reaction, the Glu- and the Asp-carboxyls would be dissociated, forming random coils presumably extending into the solvent. They would also tend to repel neighbouring carboxyl termini in the polymer and might be expected to impede the polymerization process. Recent proteolytic digestion studies suggest that this acidic domains interacting with MAPs (22). It has been predicted that the purpose of MAPs is to bind at the carboxyl termini to neutralize the excess anionic charges (23,24) we are at present not sure whether these amino acids responsible for the pH dependency are at carboxyl termini or somewhere else in the protein.

Materials and Methods

Materials are used as described in Part I.

Isolation of Microtubule Protein

Microtubule protein was prepared from goat brain by two cycles of an in vitro assembly procedure according to the method of Borisy et al. (5) with some modifications. Glycerol was used at 4 M concentration in first polymerization step to increase the yield. All steps of purifications of this
protein were performed in solution containing 100 mM PIPES (pH 6.94 at 25°C with NaOH) 1 mM EGTA, 0.5 mM MgCl₂ and 1 mM GTP. Microtubule protein was stored as 2nd cycle hot pellet in liquid nitrogen. Before use, the pellet was thawed and suspended in experimental buffer and centrifuged at 10,000 rpm in SS-34 rotor for 20 min at 4°C and the supernatant is known as 2nd cycle cold supernatent or C₂S of microtubule protein.

Microtubule protein was separated into 30S and 65 fraction by high speed centrifugation at 250,000 x g for 90 min at 4°C in L8-70 ultracentrifuge according to the method of Johnson and Borisy (25).

Isolation of DEAE-Purified Tubulin

Tubulin was purified from goat brain in Pi/MgCl₂/GTP buffer (10 mM K-PO₄, pH 7.0, 10 mM MgCl₂ and 0.1 mM GTP) by the procedure of Weisenberg et al. (26) except that DEAB-cellulose was used instead of DEAE-sephadex. The active fractions as judged from a colchicine binding assay were pooled, concentrated by overnight dialysis at 4°C against 8 M Glycerol in different assembly buffer and stored in liquid nitrogen.

Protein concentrations were determined as described by Lowry et al. (27) using bovine serum albumin as a standard.
Purity of tubulin and the proportion of microtubule associated protein present was determined by polyacrylamide gel electrophoresis according to the method of Laemmli (28). The stained gel was quantitatively scanned by 2202 Ultroscan Laser Densitometer and the ratio of MAP(s)/tubulin in a microtubule preparation was determined.

Microtubule polymerization was measured spectrophotometrically at 400 nm and at 37°C using the turbidimetric method as described by Gaskin et al. (11). Shimadzu Double-Beam Spectrophotometer UV-210A was used and it contained water-jacketted cuvette chamber. Temperature was controlled by B. Braun thermomix 1441. Absorbance was measured using 1 cm path length cuvette.

Electron microscopy was done as described by Banerjee et al. (29). A 10 μl microtubule sample was placed for 2 min on a carbon/formvar-coated copper grid (300 mesh) and displaced successively with 4 drops of each of distilled water and 1% uranyl acetate. The excess stain was removed with a filter paper. Grids were examined with a JEOL 200 CX electron microscope at an operating voltage of 60 Kv

References


