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The Interaction of Colchicine and Some Related Alkaloids with Rat Brain Tubulin

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SUMMARY

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A study of the interaction of colchicine and purified rat brain microtubule protein is presented. Experimental data agree with a theory derived to describe the system. Protein stabilized with 0.1 mM vinblastine and 1 mM GTP lost the ability to bind colchicine in a first-order reaction, with a rate constant of $1.1 \pm 0.1 \times 10^{-5}$ sec $^{-1}$. The colchicine-microtubule protein complex appeared to be stable. The rate constant for the second-order association of colchicine and microtubule protein is 128.7 ± 6.5 M^{-1} sec^{-1}, while the rate constant for dissociation is $8.1 \pm 0.8 \times 10^{-6}$ sec^{-1}. The dissociation constant calculated from these two numbers is 6.3×10^{-8} M. By using other ligands to perturb the interaction between colchicine and microtubule protein, the affinities of these ligands for microtubule protein could be measured. The methods developed are applicable in general for ligands of microtubule protein. Podophyllotoxin binds firmly to microtubule protein ($K_d = 0.28 \pm 0.06$ μM), while picropodophyllin and lumicolchicine bind much more weakly than their respective isomeric partners. All three ligands appear to compete with colchicine for a binding site on microtubule protein. The diastereoisomeric pair of podophyllotoxin and picropodophyllin should be a useful tool with which to study the involvement of microtubules in physiological processes.

INTRODUCTION

Although we do not yet know the range of physiological activities in which microtubules play some role, these structures have been strongly associated with such processes as cell elongation, mitosis, and intracellular movement. In almost all cases the involvement of microtubules in physiological processes has been inferred from an inhibitory action by the alkaloids which bind to tubulin, the protein subunit of microtubules. A number of recent papers challenge this association. Trifaró *et al.* (1) found that the inhibition by colchicine of release of catecholamines from the adrenal is due not to an action on microtubules, but rather to a direct antagonism of the acetylcholine released from the innervating nerves. In a similar fashion, the same authors showed that the action of colchicine in blocking ganglionic conduction does not involve an effect upon microtubules, but is due to direct postsynaptic antagonism of acetylcholine. Colchicine inhibits the uptake of nucleosides into HeLa cells (2). This action may be unre-

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lated to an action upon microtubules, since lumicolchicine, a related molecule which binds poorly to tubulin, is an active inhibitor of nucleoside transport. Colchicine can also exhibit a direct action on systems which appear unrelated to microtubules. For example, the drug inhibits isolated aldose reductase (3).

Despite the shortcomings of the approach, the use of inhibitors which interact with microtubule protein is a rapid way in which to ask whether a given physiological process involves microtubules. In the study of systems which are either rare or physically small in size, this is the approach which is most feasible. If a pharmacological approach is to be effective in detecting those functions dependent upon microtubules, the qualitative approach often used in the past must be replaced by quantitation both of the effect of the inhibitor upon the process in question and of the interaction of the same inhibitor with tubulin. To be most effective, a variety of inhibitors must be found, and their actions should be compared. Such an approach has been used to study the role of microtubules in thyroid function (4). In this paper the interaction of four alkaloids with tubulin is described. Podophyllotoxin and picropodophyllin, two alkaloids used sparingly up to this time, are proposed as stereospecific probes of microtubule-related functions.

METHODS

Preparation of microtubule protein. The following three buffers were employed routinely. Reassembly buffer (5) contained 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Sigma), 0.1 mM ethylene glycol bis(β-amino ethyl ether)-*N,N'*-tetraacetic acid (Sigma), 1 mM GTP (grade II-S, Sigma), and 0.5 mM $MgCl_2$, adjusted to pH 6.7 with 10 N NaOH. Reassembly buffer with vinblastine contained all these components plus 0.1 mM vinblastine sulfate (Lilly). For the third buffer, glycerol and RB[3] were mixed (59:41, v/v; final concentration of glycerol, 8 M) and adjusted to 1 mM GTP.

Microtubule protein (tubulin) was prepared by a slight modification of the method of Shelanski *et al.* (6). Rat brains, removed immediately after decapitation, were washed at 4° in RB before being homogenized in the same buffer (1 ml/g) in a motor-driven glass-Teflon homogenizer. Homogenates were centrifuged at 30,000 × *g* for 10 min, and the resulting supernatants were further centrifuged at 10,000 × *g* for 1 hr at 4°. The supernatant at this stage (S_1) was used directly in studies concerned with stabilizing the protein. For all other experiments, further purification was carried out. To S_1, an equal volume of RBG was added. The resulting solution was incubated at 37° for 30 min to polymerize microtubules, and was then centrifuged at 100,000 × *g* for 1 hr at 25°. The supernatant was discarded, and the pellet, containing polymerized microtubules, was homogenized with fresh RB at 4°. The solution was incubated in an ice bath for 30 min to depolymerize the microtubules, after which undissociated material was removed by centrifugation at 100,000 × *g* for 1 hr at 4°. For further purification, the procedure of polymerization and depolymerization was repeated. After two cycles of the purification scheme, RB with 1.0 mM vinblastine was added to give a final concentration of 0.1 mM vinblastine. This preparation was kept at 4° and was used within 3 days. A slight turbidity was observed in the solution of microtubule protein stored in the presence of vinblastine. This turbidity was probably due to vinblastine-induced aggregates of the protein (7). Over a period of 3–4 days, however, the suspension remained homogeneous and no loss of the colchicine binding capacity of the protein was observed. Analysis for purity was carried out on sodium dodecyl sulfate–polyacrylamide gels stained with Coomassie blue (8). The microtubule protein was approximately 85–90% pure. No single contaminant binding more than 1–2% of the total adsorbed stain was detected.

Colchicine binding assays. Colchicine binding to microtubule protein was measured using the DEAE-cellulose filter disc assay of Borisy (9). [^{3}H]Colchicine (New England Nuclear) was obtained in a solution of benzene–ethanol (9:1). After re-

[3] The abbreviations used are: RB, reassembly buffer; RBG and RBV, reassembly buffer with glycerol and vinblastine, respectively.

moval of the solvent under vacuum, sterile 10 mM phosphate buffer (pH 6.8) was added to give a stock solution with a final concentration of 5 μM. Final determination of the concentration was carried out spectrophotometrically, using an extinction coefficient of 1.71×10^4 M^{-1} cm^{-1} at 350 nm (10 mM phosphate buffer, pH 6.8). Routine binding assays were carried out as follows: 10 μl of 5 μM [^{3}H]colchicine were added to 120 μl of RBV which contained 3–6 μg of microtubule protein and enough unlabeled colchicine to give the desired final concentration. These assay mixtures were incubated at 37° for 1.5 hr and then cooled at 4° for 5 min. Each of two 50-μl aliquots from an assay mixture was applied to a double thickness of DEAE-cellulose filters, 1 in. in diameter, which had been moistened with 10 mM phosphate buffer (pH 6.8). After 10 min each filter stack was washed four times with 10 ml of 10 mM phosphate buffer (pH 6.8). A multiple-unit filter apparatus allowed several assays to be conducted simultaneously. Under these conditions microtubule protein, and any associated colchicine, binds quantitatively to the filters while colchicine not bound to microtubule protein is washed free. After removal of excess moisture by suction, the filters were placed in scintillation vials and counted in a Beckman LS-230 liquid scintillation spectrometer 24 hr after the addition of scintillation mixture (10). Counting efficiencies were 21–25%. In all the experiments considered in this paper, the total [^{3}H]colchicine bound was always less than 10%, and typically less than 5%, of the total colchicine included in the assay mixtures.

Several of the agents used in this study inhibit the binding of colchicine to microtubule protein. To measure the effects of these compounds, the assay described above was modified. Before colchicine was added to the assay mixture, microtubule protein and the compound of interest were incubated in 110 μl of RBV for 1.0–1.5 hr at 37°. Then 10 μl of 5.0 μM [^{3}H]colchicine and 10 μl of a solution containing the desired amount of unlabeled colchicine were added. The sample was incubated for an additional 1.5 hr at 37°, cooled to 4°, and assessed for bound [^{3}H]colchicine as described above.

The kinetics of colchicine binding, both alone and in the presence of inhibitors, was also examined. These assays were modified from those described above by changing the times of incubation of colchicine and/or the inhibitor with microtubule protein.

During the course of the binding studies it was found that both lumicolchicine and picropodophyllin could inhibit binding of colchicine only at concentrations close to their limits of solubility. Concentrated solutions of lumicolchicine were prepared by dissolving approximately 2 mg in 50 μl of 95% ethanol and diluting with RB to a final concentration of 5 mM. Solutions of picropodophyllin were prepared by dissolving about 1 mg in 200 μl of 95% ethanol by warming in a 60° water bath, then diluting with RB, also at 60°, to a final concentration of 0.4 mM. When used, further dilutions of this stock solution were prepared immediately with RB at 60°. Podophyllotoxin solutions were prepared in a manner similar to those of picropodophyllin, but at room temperature. All lumicolchicine and picropodophyllin solutions were placed in a 37° water bath soon after preparation and used in the binding studies within several hours.

Preparation of lumicolchicine. Colchicine was isomerized by irradiation at 350 nm (11) to a mixture of three isomers (α, β, and γ). Colchicine (60 mg) was dissolved in 150 ml of 95% aqueous ethanol, placed in a round-bottomed quartz flask, and irradiated in a Srinivasan-Griffin photochemical reactor with sixteen 350-nm lamps. The first-order loss of colchicine could be followed by monitoring the absorbance at 350 nm. After reaching a constant absorbance at 350 nm, the reaction was allowed to proceed for eight additional half-lives. Comparison of the ultraviolet absorption spectra of the preparation with those of Grewe and Wolfe (12) showed that the main products were the β and γ isomers, in agreement with the finding of Wilson and Friedkin (11). This mixture of isomers is referred to as lumicolchicine.

Analysis of the lumicolchicine prepara-

tion by thin-layer chromatography, using developing solvents of methanol and ether in ratios of 100:0, 50:50, 10:90 and 0:100, indicated the presence of two major and two minor products. The weak competition of lumicolchicine for the binding of colchicine to microtubule protein demonstrated that there could be no more than 0.1% residual contamination with colchicine (see RESULTS).

Materials. Female rats (225–250 g) of the NLR strain were procured from National Laboratories, St. Louis. Podophyllotoxin was obtained from Delta Chemical Works, New York, and colchicine, from Sigma Chemical Company. Picropodophyllin was a gift from Professor E. Smissman, and vinblastine sulfate was provided by Eli Lilly and Company. All other chemicals were of reagent grade unless otherwise specified.

RESULTS

Stabilization of colchicine binding in crude preparations of microtubule protein. Microtubule protein (mol wt 115,000) binds 2 moles of GTP, 1 mole of vinblastine (13), and 1 mole of colchicine (13, 14). Any of these compounds can partially stabilize the protein. To determine an optimum stabilization buffer, 0.5 ml of buffer (20 mM phosphate and 100 mM glutamate, pH 6.7) containing the compounds of interest was mixed with 0.5 ml of a crude preparation of microtubule protein (4 mg of protein per milliter, final concentration). After incubation at 4° for various times, a 0.1-ml aliquot was withdrawn from each solution, mixed with a solution of [^{3}H]colchicine, and incubated at 37° for 1.5 hr. The aliquots were subsequently assessed for bound [^{3}H]colchicine using the DEAE-cellulose filter disc procedure. The loss of colchicine binding activity for each experiment was followed for 40–50 hr. Since the loss of binding activity followed first-order kinetics, these experiments can be compared by using as a measure of stabilization the time of incubation required to lose one-half the colchicine binding activity (Table 1). To determine whether the stabilizing effects of GTP and vinblastine were additive, incubation mixtures containing both compounds were examined. Mixtures containing both GTP and vinblastine were more effective in stabilizing colchicine binding activity than either of the components tested separately (Table 1). For subsequent work with microtubule protein a stock buffer containing 1 mM GTP and 0.1 mM vinblastine was used. Neither of these two ligands has any marked influence upon the binding of colchicine to tubulin (13–16).

TABLE 1

Stabilization of colchicine binding activity of rat brain microtubule protein by vinblastine and GTP

Incubations were carried out at a colchicine concentration of 18 μM, pH 6.7, at 37° for 1.5 hr. See the text for details.

Vinblastine	GTP	$MgCl_2$	Binding activity half-life
mM	*mM*	*mM*	*hr*
0	0	0	7.5
0	0.1	0	9.5
0	1.0	0	47.5
0	1.0	4.0	47.5
0.01	0	1.0	24.5
0.10	0	1.0	33.0
1.00	0	1.0	45.5
0.01	1.0	1.0	70
0.10	1.0	1.0	190
1.00	1.0	1.0	630

Stoichiometry of colchicine - tubulin complex. The binding stoichiometry between colchicine and tubulin preparations used in this study was assessed immediately after purification of the tubulin. Microtubule protein in RBV was incubated in the presence of 5–10 μM [^{3}H]colchicine for 1.5–2 hr at 37°. Under these conditions more than 95% of the colchicine binding sites are occupied (see below). Bound [^{3}H]colchicine was determined after removal of free [^{3}H]colchicine on a small column of Sephadex G-50 (14). Calculated stoichiometries ranged from 0.51 to 0.65 mole of colchicine per 115,000 g of protein. Possible loss of colchicine binding activity during the 12-hr purification procedure was not investigated.

Interaction of tubulin and colchicine. A time course of binding of 1 μM [^{3}H]-colchicine to microtubule protein is shown

in Fig. 1 (circles). Maximum binding was seen at about 10 hr, followed by a slow decrease in binding at longer times. It is not clear from these data whether equilibrium was established. The shape of the curve suggests, in fact, that the binding observed as equilibrium was approached was offset, at least in part, by loss of binding due to denaturation of the protein. Similar observations have previously been made using crude tubulin (17).

The loss of colchicine binding activity by microtubule protein may be described by

$$P \xrightarrow{k_3} P' \tag{1}$$

and

$$C \cdot P \xrightarrow{k_3'} C + P' \tag{2}$$

where P' represents inactivated microtubule protein, and k_3 and k_3' are the first-order rate constants for decay of the free and colchicine-bound protein, respectively. An estimate of k_3 can be readily obtained. Aliquots, removed at appropriate time intervals from a solution of purified microtubule protein incubated at 37°, were incubated for an additional 90 min with 1 μM [^{3}H]colchicine. Bound [^{3}H]colchicine was then assessed as described in METHODS. The loss of colchicine binding activity with time was exponential (Fig. 1, triangles). The apparent first-order rate constant for the decay equals k_3, the rate constant for the loss of colchicine binding activity of the free tubulin. The value of this constant is 1.1×10^{-5} sec^{-1}, which corresponds to a half-time for denaturation of about 18 hr (Table 2).

The possibility that the protein could become denatured while bound to colchicine (k_3') was also examined. Solutions of tubulin and colchicine were mixed to produce concentrations of colchicine of 5–10 μM. Under these conditions binding reached a plateau value in approximately 5 hr (not shown). Calculations made using Eq. 4, with the constants collected in Table 2, indicate that at this time over 97% of the available sites on the tubulin were saturated with colchicine. Any decrease seen after this time in the amount of radioactivity bound in the complex must have been due to denaturation of the protein while actually bound in the colchicine-protein adduct. Over an additional 660 min, following the attainment of maximal binding at 300 min, no measurable loss of binding was seen. With an allowable error of ±5%, the maximal value which could be assigned to the first-order rate constant describing the denaturation of the colchicine-protein complex is about 1.0×10^{-6} sec^{-1}. This value is negligible in comparison with competing reactions, and the associated reaction has therefore been excluded from consideration in the formal treatment given in the APPENDIX.

The rate of dissociation of the protein-colchicine complex was also measured. After incubation of microtubule protein with 1 μM [^{3}H]colchicine for 2 hr at 37°, a 500-fold excess of nonradioactive colchicine was added. Because of the large excess of unlabeled colchicine, no further binding of [^{3}H]colchicine was observed. Loss of bound [^{3}H]colchicine from the previously formed complex was then followed by assaying aliquots of the incubation mixture as described in METHODS. The loss of binding activity was exponential (Fig. 1, squares), with an apparent first-order rate constant (k_{-2}) of 8.1×10^{-6} sec^{-1} (Table 2). The observed value of k_{-2} corresponds to a half-time for dissociation of about 24 hr.

With values of k_{-2} and k_3 available, the measurement of k_2 could be undertaken. It was clear from a consideration of initial data that the final values of the dissociation constants for ligands, K_1, would depend more strongly upon k_2 than upon any of the other constants. We therefore considered the evaluation of this constant in some detail. Two methods are available by which k_2 can be measured.

In the first method, the variation of binding of colchicine to tubulin was measured at various times. Solutions of colchicine and tubulin were mixed, and aliquots were withdrawn at intervals and assessed for binding of [^{3}H]colchicine. Under these conditions Eq. 8 (APPENDIX) may be abbreviated to

$$\ln (\mathrm{cpm}_{max} - \mathrm{cpm}) = \ln (\mathrm{cpm}_{max}) - k_{obs}t \tag{3}$$

where cpm is the observed counts per min-

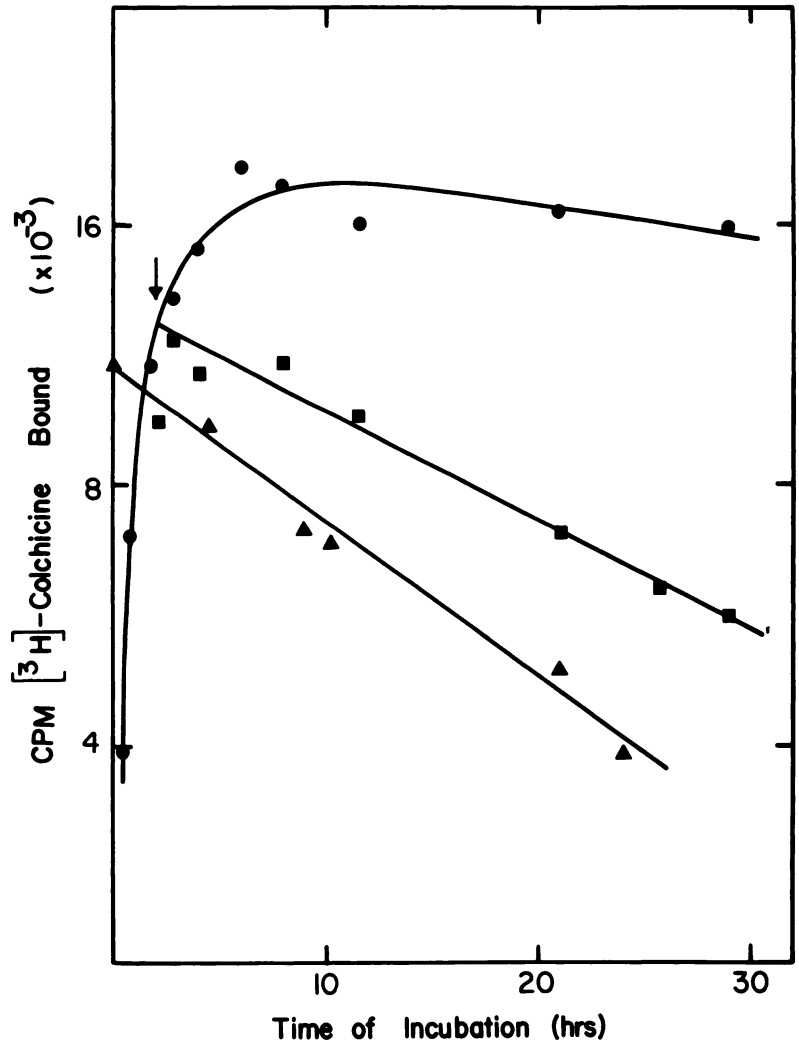

FIG. 1. *Time course of binding of colchicine to tubulin* (●), *dissociation of colchicine-tubulin complex* (■), *and denaturation of free tubulin* (▲)

To measure the binding of colchicine to tubulin, solutions containing tubulin were incubated with 1 μM [^{3}H]colchicine. At various times the amount of bound colchicine was measured. To measure the dissociation of the tubulin-colchicine complex, the reaction mixture was made to 500 μM in colchicine (arrow) by adding unlabeled colchicine, and the loss of bound radioactivity was followed. To measure the denaturation of free tubulin, solutions of purified protein were incubated in the absence of colchicine for various times, after which the amount of radioactivity bound during a 90-min incubation with 1 μM [^{3}H]colchicine was measured. The concentration of protein was 50 μg/ml in RBV, at a temperature of 37°. Each point represents the mean of duplicate measurements. See the text for further details and discussion.

ute bound at time t and

$$\text{cpm}_{\max} = \frac{\sigma k_2 C_0 P_0}{[\alpha^2 - 4k_{-2}k_3]^{1/2}} \qquad (4)$$

in which σ is the specific activity of the colchicine, and the remaining symbols are defined following Eq. 8. Both $\text{cpm}_{\max}$ and k_{obs} may be considered parameters to be obtained from data relating cpm and t. The parameter $\text{cpm}_{\max}$ may be evaluated by a least-squares procedure which minimizes the curvature in a plot of ln ($\text{cpm}_{\max}$ –

TABLE 2

Collected values of constants for interaction of tubulin and selected ligands

Ligand	Constant	Value	n^a
Colchicine	k_2	128.7 ± 6.5 M^{-1} sec^{-1}	5
Colchicine	k_{-2}	8.1 ± 0.8 × 10^{-6} sec^{-1}	1
Colchicine	k_3	1.1 ± 0.1 × 10^{-5} sec^{-1}	1
Colchicine	K_1[b]	6.3 ± 0.7 × 10^{-8} M[c]	
Lumicolchicine	K_1[d]	1.57 ± 0.24 × 10^{-3} M	1
Podophyllotoxin	K_1[d]	2.79 ± 0.058 × 10^{-7} M	9
Picropodophyllin	K_1[d]	3.78 ± 0.80 × 10^{-5} M	5

[a] n = number of experiments. In those cases where n = 1, the standard deviation is that of the least-squares line predicted by Eq. 12.

[b] Calculated from k_{-2}/k_2.

[c] Standard deviation calculated from the root mean square coefficient of variation of k_2 and k_{-2}.

[d] Evaluated from Eq. 12 and minimized data. See the text for details.

cpm) vs. t, after which k_{obs} may be calculated from the slope of the straight line (Fig. 2). The value of k_{obs} may be related to k_2 by

$$k_2 = \left[\frac{k_{obs} - k_3}{C_0}\right]\left[1 - \frac{k_{-2}}{k_{obs}}\right] \qquad (5)$$

from which k_2 may be calculated after k_{-2} and k_3 are known.

Values of k_2 were evaluated by this method, using concentrations of colchicine ranging from 0.60 to 5.60 μM (Table 2). No systematic variation was seen within this range.

The second method with which k_2 can be evaluated involves experiments in which the time of incubation is maintained constant, and the amount of colchicine bound is measured using varying concentrations of colchicine. Solutions of tubulin were mixed with solutions of colchicine of various concentrations. After an incubation of 90 min, aliquots of these solutions were removed and the amount of tubulin-bound colchicine was determined. As the concentration of colchicine was increased, the amounts of the complex also rose (Fig. 3A). Although the data in this case should be described by Eq. 8, no simple linear transform could be utilized. It was possible, however, to program a minicomputer to calculate a criterion of the fit between experimental and computed points, after which values of the desired parameters could be determined in order to minimize the chosen criterion. We used as a criterion the usual sum of the squares of the differences between experimental and computed values of the bound radioactivity. At saturating concentrations, the bound radioactivity will be σP_0, where σ is the specific activity of the colchicine and P_0 is the concentration of active binding sites on tubulin at the beginning of the experiment. Since P_0 is not known with certainty, we treated the product σP_0 as a second parameter. Values of k_2 and σP_0 were chosen to minimize the least-squares criterion. To test for the over-all quality of the fit, the theoretical curve predicted using these values was compared with experiment (Fig. 3A, solid line). No deviations

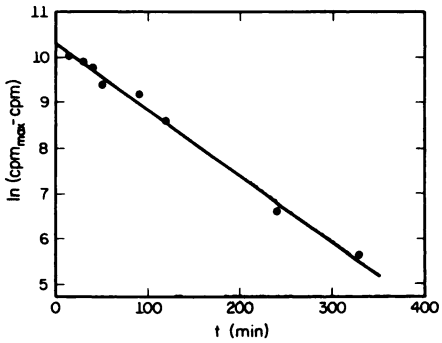

FIG. 2. *Binding of [^3H]colchicine to purified microtubule protein*

The data of an experiment similar to that of Fig. 1 are presented in the form of Eq. 3. cpm, bound radioactivity at time t; cpm_{max}, maximal binding predicted as $t \to \infty$ (see Eq. 4); ln, natural logarithm. The concentration of colchicine was 1 μM, at 37°, in RBV. See the text for further details.

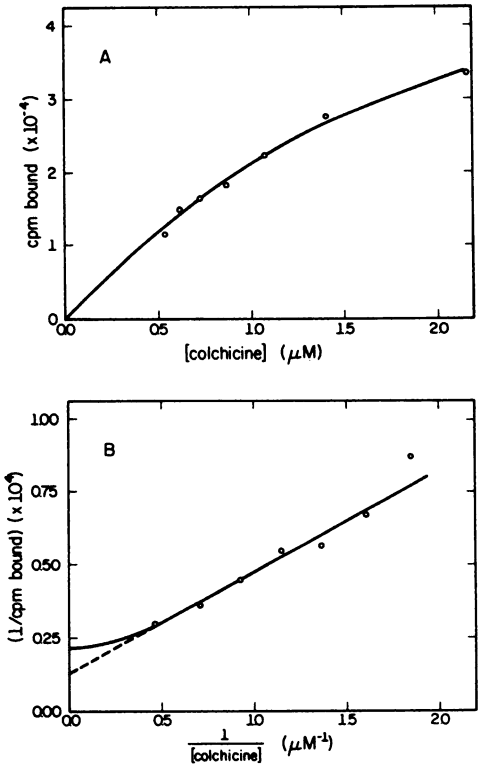

FIG. 3. *Variation with colchicine concentration of binding of colchicine to microtubule protein*

A. solutions of [^{3}H]colchicine and purified tubulin (45 μg/ml in RBV) were incubated for 90 min at 37° before measuring the amount of bound radioactivity. The points are the means of duplicate measurements. The line was calculated from Eq. 8, using the constants of Table 2.

B. Double-reciprocal presentation of the data of Fig. 3A. The dashed line is a linear extrapolation of the line through the points, while the solid line was calculated from Eq. 8, using the constants of Table 2.

other than those expected from experimental error were detected. The value of k_2 calculated by this means was the same as that calculated by varying the time of the experiment (Table 2). The fact that equal values of k_2 were measured in two quite different experiments strongly supports the use of Eq. 8 as a satisfactory theoretical model for the binding of colchicine to microtubule protein.

Despite the fact that none of these reactions reached maximal binding, the data obtained in this experiment mimic a normal equilibrium titration of tubulin with colchicine. When the data were plotted in double-reciprocal fashion, for example, adequate straight lines were obtained (Fig. 3B). The curve predicted by Eq. 8 (Fig. 3B, solid line) is also linear within a wide range of concentrations of colchicine, but flattens out as $1/C_0$ approaches zero.

Interaction of tubulin with ligands other than colchicine. In conjunction with studies involving the role of microtubules in axoplasmic transport, it was desired to study the binding of other ligands to tubulin by measuring their effect upon the interaction of colchicine with the protein. The diastereoisomers podophyllotoxin (13, 15, 16, 18–23) and picropodophyllotoxin (18, 22) are inhibitors of the interaction of colchicine with tubulin. Moreover, podophyllotoxin is thought to bind to the same site on the microtubule subunit as colchicine (13). Lumicolchicine, an isomer produced by ultraviolet irradiation of colchicine, binds much less effectively to tubulin than does colchicine (13, 18). Podophyllotoxin, picropodophyllin, and lumicolchicine were examined further as inhibitors of the binding of colchicine to purified rat brain microtubule protein.

Several experiments on the interaction of tubulin with podophyllotoxin and picropodophyllin suggested that equilibrium was established within 1 hr. Since results with podophyllotoxin and picropodophyllin were essentially identical, only the binding of podophyllotoxin is considered here in detail. Solutions of tubulin and podophyllotoxin were prepared and incubated at 37° for 1 hr. At the end of this time a second solution was added, and the incubation was continued for an additional period of 1, 2, or 3 hr. The second solution always contained [^{3}H]colchicine to produce a final concentration of 1 μM, and was constituted in such a way that the concentration of podophyllotoxin was either increased or decreased. Control experiments in which concentrations of 0.9 μM podophyllotoxin were present during the second period of incubation displayed a depression of binding to about 20% of values obtained in the absence of podophyllotoxin (Table 3). Concentrations of 0.3 μM in the

TABLE 3

Reversibility of binding of podophyllotoxin to microtubule protein

All solutions were made up in RBV and maintained at 37°. The concentration of colchicine, which was present only during the second incubation, was 1 μM. Tubulin was used at a concentration of 6 μg in the 110-μl assay volume during the second incubation, and at 3 times this concentration during the first incubation. Results are presented with respect to a zero-time control which lacked podophyllotoxin. There is no statistically significant difference between experiments 1 and 2, or between 3 and 4.

Expt.	Concentration of podophyllotoxin in		Time of second incubation	[^{3}H]Colchicine
	First incubation (1 hr)	Second incubation		
	μM	*μM*	*hr*	*% control*
1	0.9	0.3	1.25	40
			2.0	38
2	0.0	0.3	1	38
			2	40
			3	43
3	0.0	0.9	1	18
			2	22
			3	21
4	0.3	0.9	0.75	19
			1.5	17
			3.0	16

second incubation depressed binding only to about 40%. Even when the first incubation was carried out at 0.3 μM, which should result in 40% binding, a concentration of 0.9 μM in the second incubation resulted in a depression of binding to 20%. Similarly, dilution of an initial concentration of 0.9 μM to a final concentration of 0.3 μM produced the same inhibition of binding as seen in a second incubation carried out entirely at 0.3 μM (Table 3). These data indicate strongly that the interaction of podophyllotoxin and tubulin approaches equilibrium in less than 60 min.

Inhibition of the binding of colchicine to tubulin was investigated by evaluating dose-response curves for podophyllotoxin, picropodophyllin, and lumicolchicine. Solutions of the ligand and tubulin were mixed, using various concentrations of the ligand, and incubated for 90 min (time period 1). At the end of this time [^{3}H]colchicine was added, and the incubation was continued for an additional 90 min (time period 2). At the end of time period 2, each sample was assessed for colchicine bound to tubulin (Fig. 4). All three drugs depressed the binding of colchicine to tubulin. Podophyllotoxin was the most active, followed by picropodophyllin and lumicolchicine.

Two problems were encountered in carrying out the experiments presented in Fig. 4. First, the most concentrated test solutions of lumicolchicine and picropodophyllin contained up to 4% ethanol, used to dissolve the compound. To determine the effect of this solvent on the binding of [^{3}H]colchicine and microtubule protein, various concentrations of ethanol were added to solutions of microtubule protein and incubated with 1 μM [^{3}H]colchicine for 90 min at 37°. A 15–20% stimulation of colchicine binding was observed at concentrations of ethanol between 1.5% and 7.5%. To correct for this effect, the observed binding in all solutions containing over 1.5% ethanol was compared with that of identical solutions containing no ligand. Second, the inhibition of colchicine binding by picropodophyllin came to a constant level of inhibition (approximately 70%) at concentrations greater than 100 μM. Solutions of 100 μM picropodophyllin, prepared as described in METHODS, began to form visible precipitates within 3–4 hr. Precipitates appeared earlier in more concentrated solutions. As the total period of incubation was 3.0 hr, it is possible that the plateau level represents inhibition by a saturated solution of picropodophyllin.

Equation 8 predicts that the inhibitory activity of ligands will vary when measured using different concentrations of colchicine. To examine this point, a comparison of the inhibition of colchicine binding by podophyllotoxin at two concentrations of colchicine was carried out (Fig. 5). When lower concentrations of colchicine were employed as a "test" agent, podophyllotoxin was more effective in reducing the binding of colchicine.

In order to determine the equilibrium constant (K_1), data such as those of Fig. 4 or 5 may be analyzed in several ways (see

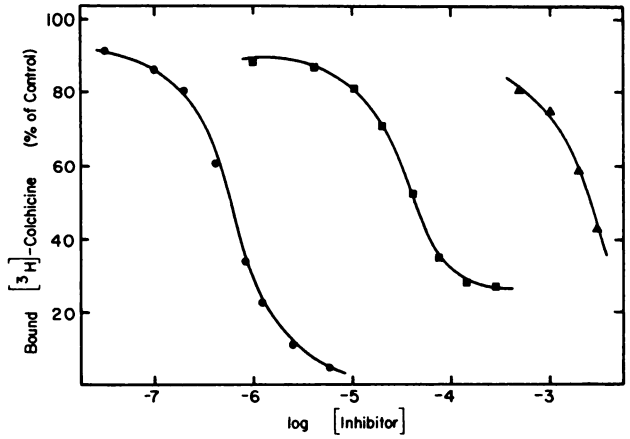

FIG. 4. *Inhibition of binding of colchicine to microtubule protein by podophyllotoxin (●), picropodophyllin (■), and lumicolchicine (▲)*

Microtubule protein was incubated with an inhibitor for 90 min before a second incubation of 90 min with 1 μM [^{3}H]colchicine. Binding is expressed with respect to controls in which the inhibitors were absent. The concentrations of inhibitors are expressed as the common logarithm of their molar concentration. Each point is the mean of duplicate determinations. For other details, see the text.

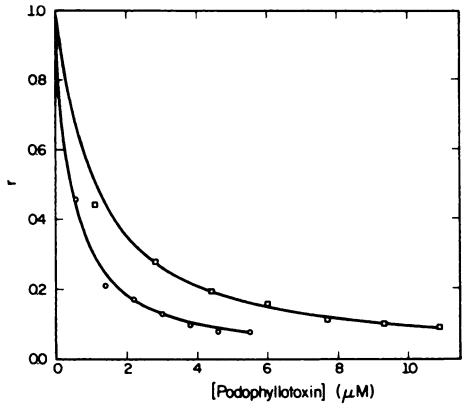

FIG. 5. *Comparison of inhibition of colchicine binding by podophyllotoxin at two concentrations of colchicine*

Binding experiments were performed as described in Fig. 4, using podophyllotoxin as an inhibitor of the binding of either 4.2 μM (□) or 1.1 μM (○) colchicine to tubulin. Data are presented as the ratio (r) of counts per minute of [^{3}H]colchicine bound in the absence of podophyllotoxin to counts per minute bound in the presence of podophyllotoxin (see Eq. 10). The solid lines were calculated from Eq. 8, using the collected constants presented in Table 2.

APPENDIX). In particular, the ratio r of binding in the presence and absence of ligand can be transformed through the use of Eq. 11 into a new variable, β. According to Eq. 12, $1/\beta$ should be a linear function of L_0, with a slope and intercept which are independent of the concentration of colchicine. The data satisfy these predictions (Fig. 6). From the slope of the line of Fig. 6, a value may be calculated for the dissociation constant of the podophyllotoxin-tubulin complex (Table 2).

Two other arguments also suggest that Eq. 8 provides an adequate description of this system. First, there are two other methods by which K_1 may be extracted from data such as those presented in Figs. 4 and 5 (see APPENDIX). When tested, both methods yield values of K_1 which are equal to the values calculated from the slopes of plots such as those of Fig. 6. Second, K_1 may be evaluated in a very different type of experiment. If podophyllotoxin is present initially when colchicine and tubulin are mixed together, the entire time course of binding should be altered. Experiments were carried out in which the time course of binding of colchicine to tubulin was followed in the presence of podophyllotoxin (data not shown). A least-squares minimization was then employed to allow selection of the value of K_1 which produced the best fit of Eq. 8 to the experimental data, using known values of the other constants. Values of K_1 evaluated in this way were

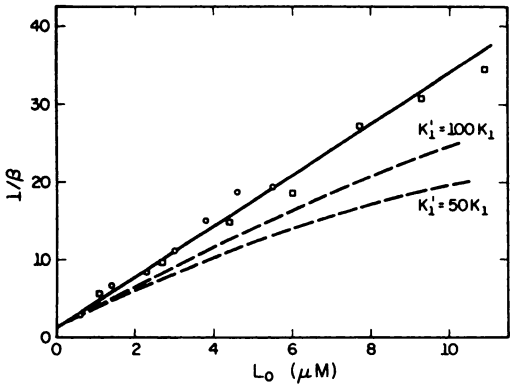

FIG. 6. *Variation of $1/\beta$ with concentration of podophyllotoxin (L_0)*

The data of Fig. 5 were transformed using Eq. 11 and the constants of Table 2, and replotted according to Eq. 12. Concentration of colchicine: ○, 1.1 μM; □, 4.2 μM. The straight line was calculated by the method of least squares. For comparison with theoretical functions describing a noncompetitive interaction between colchicine and podophyllotoxin, the dashed lines were computed for two cases: $K_1' = 100\ K_1$ and $K_1' = 50\ K_1$. See the text for details.

not different from those measured as described above. Since the various methods weight the data in quite different ways, the agreement between them suggests that Eq. 8 correctly describes the system. The average value of K_1 for the podophyllotoxin-tubulin complex, using data from all the methods presented above, is 0.28 ± 0.06 μM (Table 2). To demonstrate the agreement between Eq. 8 and the experimental data, theoretical lines were computed for the data of Fig. 5 (solid lines), using the constants collected in Table 2.

The same techniques described above for podophyllotoxin were employed to measure the binding of picropodophyllin to tubulin. Data transformed to the coordinates of Eq. 12 yield a straight line, with a slope independent of the concentration of colchicine (1.0–5.2 μM range). The dissociation constant for the picropodophyllin-tubulin complex is 38 μM (Table 2), about 130 times higher than that measured for the binding of podophyllotoxin to tubulin.

Lumicolchicine can depress the binding of colchicine to tubulin, but only incompletely, even at the highest concentrations allowed by the solubility of the compound. Within the range of inhibition which could be achieved, data plotted according to Eq. 12 gave a straight line from which a dissociation constant for the lumicolchicine-tubulin complex could be evaluated (Table 2). Because of the narrow range of values of r which could be considered (0.5–1.0), this value can only be considered tentative. Because of the low level of inhibition, the binding of lumicolchicine to tubulin was not studied in more detail.

DISCUSSION

The ligands considered in this paper have previously been examined as inhibitors of the process of axoplasmic transport. It was felt that a comparison of the concentrations of drugs necessary to inhibit axoplasmic transport with the concentrations necessary to bind to microtubule protein would provide evidence either to support or to refute the hypothesis that microtubules are involved in axoplasmic transport. Accordingly, studies necessary to measure the binding of ligands to tubulin were begun. Because of the slow binding of colchicine to tubulin (13, 15, 16, 22, 23) and the decay of the binding activity of the free protein (17), it was not practical to carry out experiments under equilibrium conditions. Therefore, in order to use colchicine as a probe of the binding of other ligands, the interaction of tubulin and colchicine was considered as a kinetic system.

The equations developed in the APPENDIX predict that, even though strongly time-dependent, the data obtained in a "titration" of tubulin with colchicine will yield a straight line when plotted in double-reciprocal fashion (Fig. 3). As the time of incubation is lengthened, the line in a double-reciprocal plot remains straight and continues to strike nearly the same intercept on the ordinate, but the slope is reduced. Behavior of this type has been demonstrated previously by Wolff and Williams (4). Since the ordinate intercept is usually taken as the reciprocal of the maximum amount of binding possible at a saturating concentration of ligand, it is tempting to calculate the stoichiometry of binding of colchicine from this point. Modeling studies carried out with Eq. 8 suggest that such a conclusion is invalid.

In fact, theoretical curves are linear at values of $(1/C_0)$ away from the ordinate, but bend sharply to much lower slopes as $1/C_0$ approaches zero. As a result, extrapolated values of the maximal number of counts per minute which may be bound will be too large. An analysis of Eq. 8 shows that the error introduced by a linear extrapolation of a double-reciprocal plot is a maximum of exactly 2-fold; i.e., the true stoichiometry may be as little as 50% of that calculated from the linear extrapolation. In practice, data presented in this paper (Fig. 3B) would overestimate the stoichiometry by 1.6–1.7 times, indicating that the true binding is only about 60% of that predicted by extrapolation. These calculations raise a problem when considered in connection with the published stoichiometries of binding of colchicine to tubulin. The conclusion that stoichiometries in the literature may be too high, however, is in agreement with observations of Kirschner *et al.* (24), who found by fractionation of purified tubulin that about 40% of the protein is incapable of binding colchicine.

Since the colchicine-tubulin system is not at equilibrium, it is not possible to evaluate a dissociation constant from the intercept on the abscissa of a double-reciprocal plot. The affinity of colchicine for tubulin may, however, be obtained from the rate constants for formation (k_2) and dissociation (k_{-2}) of the complex. In this manner a dissociation constant of 0.063 μM may be computed. This value indicates binding over 40 times tighter than that represented by the apparent "dissociation constant" of 2.7 μM, which could be calculated from the linear extrapolation of Fig. 3B.

Once described, the colchicine-tubulin system could be used as a means with which to measure the binding of other ligands to tubulin. Despite the complexity of the theoretical treatment, a method such as this is useful because tritiated colchicine is commercially available, and a rapid and effective method exists with which its binding to tubulin can be measured (9). The only general requirements for the use of analyses such as those described in the APPENDIX are that the ligand being examined must in some way modify the interaction of colchicine and tubulin, and must itself be in equilibrium with the protein. By the use of this system, an analysis was made of three ligands: podophyllotoxin, picropodophyllin, and lumicolchicine. The analysis given in RESULTS fits well with a simple competitive interaction between any of these ligands and colchicine; i.e., either the ligand or colchicine can bind to tubulin, but simultaneous binding of both small molecules may not occur. On the evidence of various forms of double-reciprocal plots, the statement has been made that podophyllotoxin competes with colchicine for a site on tubulin (13, 16, 19). In view of the limited utility of such plots in the colchicine-tubulin system, however, the question of competitive vs. noncompetitive interaction must be viewed with caution. In the case of the three ligands studied here, the analysis given in the APPENDIX suggests that the interaction is, in fact, competitive. If a second set of reactions is allowed in which either of the two binary complexes can bind a second ligand to form a ternary complex, a noncompetitive system will result. Solution of this system for the amount of total bound colchicine yields Eq. 15, which, when modeled, predicts that the presentation of Fig. 6 will not be linear. As shown by the dashed lines in Fig. 6, a noticeable curvature would be observed if the binding of the ligand to the colchicine-tubulin complex occurred with an affinity of as little as 1–2% of that describing the binding of the ligand to the free protein. Since no such curvature is seen, we conclude that colchicine and these three ligands either bind at the same site, in the usual model for competitive interaction, or bind at different sites which interact in such a way that only one may be occupied at any time.

In the case of colchicine, the reaction with tubulin is quite slow. Under the conditions used here, periods of several hours were necessary to achieve maximal binding to tubulin. Although tubulin isolated from different sources may vary from the protein from rat brain, and physiological systems studied *in vivo* may vary in other

ways, it seems likely that the rate of interaction of colchicine with tubulin in tissues is no faster than the rates we have seen, and may be slower. The effects of colchicine upon secretion from the thyroid require several hours (4), as does the inhibition of axoplasmic transport (20, 25, 26). While it is possible to consider the slow rate as a potential aid in diagnosing the involvement of microtubules in these processes, so many other factors also slow the action of drugs that such a criterion is of only limited utility. Furthermore, colchicine acts quickly in some systems which certainly involve microtubules. For example, spindle fibers of living oocytes from *Pectinaria* are dissociated in minutes by colchicine (27).

Although colchicine and lumicolchicine have been, and will certainly continue to be, useful agents in the study of physiological processes involving microtubules, other compounds which could also act as pharmacological probes of these systems would be very welcome. Pairs of compounds which possess similar solubility properties but greatly different anti-microtubule activity would be particularly valuable. Since such molecules should be distributed in biological systems in a similar manner, a differential inhibitory action of the two could with even greater confidence by associated with microtubules. It would appear that the diastereoisomers podophyllotoxin and picropodophyllin should be useful as such a pair of molecules. These two alkaloids differ in their affinity for tubulin by over 100-fold, which should be sufficient to serve as a useful diagnostic aid in most physiological systems. For example, axoplasmic transport through several types of neurons is inhibited by podophyllotoxin, but is unaffected by much higher concentrations of picropodophyllin (20, 28). Similarly, mitosis is strongly inhibited by podophyllotoxin. To achieve equal inhibition with picropodophyllin requires concentrations 15–200 times greater (29, 30). Both compounds bind rapidly to tubulin and interact with the protein in a simple manner. Since they appear to compete with colchicine for a binding site, it should be possible to use podophyllotoxin in experiments in which colchicine was previously employed. Finally, data from our laboratory suggest that podophyllotoxin penetrates even the elaborate myelin sheath around axons, for the concentration of this drug needed to inhibit axoplasmic transport through myelinated fibers is in good agreement with the value of K_1 listed in Table 2 (20, 28). If podophyllotoxin is found to penetrate all membranes readily, it would be even more useful as a probe of microtubule function in cellular systems.

APPENDIX

The purpose of this study was to examine the binding of several agents to tubulin by utilizing their ability to inhibit the binding of colchicine to microtubule protein. During the course of the experiments, however, it became clear that useful information concerning the binding of the inhibitors to tubulin could be obtained only after the binding of colchicine to tubulin was more clearly defined. Some aspects of the binding of colchicine to microtubule protein have previously been described by Borisy and Taylor (17), who developed in brief form a theoretical treatment which is similar to that given here. Several problems appear to be common to the study of the binding of colchicine to microtubule protein. Colchicine binding activity in crude extracts is labile, with times for loss of half the activity as short as 4 hr at 4° (14). In addition, the forward rate constant for the formation of the colchicine–microtubule protein complex is slow, with measured rates of 30–130 M^{-1} sec^{-1} (17, 31).

A mechanism which describes the studies presented in RESULTS follows. In an initial time period, tubulin (P) is allowed to interact with a nonradioactive ligand (L). It is assumed that the reaction comes to equilibrium during the time of this first incubation (see RESULTS) and remains at equilibrium during all following studies. At the end of the first incubation, radioactive colchicine is added to the system and allowed to interact during a second time period. The reaction of colchicine with tubulin does not come to equilibrium in the 90-min period usually considered in experiments involving ligands. Therefore this

interaction must be considered from a kinetic standpoint. It is further assumed that during both time periods free tubulin suffers a progressive loss of its ability to bind colchicine. Since we shall never use the absolute value of the tubulin concentration, however, any tubulin which denatures during time period 1 will simply disappear from consideration, without altering the ligand-tubulin equilibrium. As a result, it is necessary formally to consider only the denaturation of free tubulin during time period 2. The system may be formalized in the following equations.

Time period 1:

$$L + P \overset{K_1}{\rightleftharpoons} L \cdot P \tag{6}$$

Time period 2:

$$L + P \overset{K_1}{\rightleftharpoons} L \cdot P \tag{6}$$

$$C + P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C \cdot P \tag{7}$$

$$P \overset{k_3}{\rightarrow} P' \tag{1}$$

where $L \cdot P$ and $C \cdot P$ refer to the complexes of ligand and colchicine, respectively, with tubulin, and P' is a denatured form of tubulin which is no longer capable of binding colchicine.

The mechanism given above assumes competition between the ligand and colchicine, i.e., that no ternary complex of tubulin, ligand, and colchicine can exist. The system can be solved with the aid of two further assumptions: $L_0 \gg P_0$, and $C_0 \gg P_0$, where L_0, C_0, and P_0 refer to the initial concentrations of the three components. The colchicine-tubulin complex, $C \cdot P$, is the only species which contains protein-bound radioactivity, and which will therefore be detected by the filter disc assay. The concentration of this species is given by

$$[C \cdot P] = \frac{k_2 C_0 P_0 \beta}{[\alpha^2 - 4k_{-2}k_3\beta]^{1/2}} \cdot [\exp(-m_1 t_2/2) - \exp(-m_1 t_2/2)] \tag{8}$$

where t_2 is the time elapsed during time period 2, $\beta = K_1/(K_1 + L_0)$, $\alpha = k_{-2} + (k_3 + k_2C_0)\beta$, $m_1 = (\alpha - [\alpha^2 - 4k_{-2}k_3\beta]^{1/2})$, and $m_2 = (\alpha + [\alpha^2 - 4k_{-2}k_3\beta]^{1/2})$.

In the studies described below we wish to evaluate K_1, the equilibrium constant for the dissociation of the ligand-tubulin complex. Using Eq. 8, we have been unable to find any way in which K_1 can be evaluated without first knowing values for the rate constants k_2, k_{-2}, and k_3, which describe the system in the absence of added ligand.

The constants k_{-2} and k_3 may be obtained from relatively simple experiments, which are described in RESULTS. In particular, it is true that the loss of binding activity of the protein (k_3) can be measured easily. If no ligand is present, $\beta = 1$; if, in addition, the second time period (t_2) is maintained constant while the first time period (t_1) is varied, a simple first-order loss of binding activity will be observed in which the observed first-order rate constant will be k_3. Experimental values for k_2 may also be obtained, but the analysis (see RESULTS) is more complex in this case.

With values available for k_2, k_{-2}, and k_3, the evaluation of K_1 may be considered. Since Eq. 8 is transcendental in β, and therefore in K_1, indirect means of solution must be sought. Three methods are available. (a) A least-squares criterion may be defined for the fit of Eq. 8 to a set of data, using K_1 as an adjustable parameter. A value of K_1 can then be chosen which minimizes the criterion. In practice, this method has proven rapid, and was routinely used to evaluate values of K_1. The method suffers from the criticism that a minimal value of the criterion must exist, even though the data may deviate from Eq. 8 in a systematic way. (b) K_1 and L_0 occur in Eq. 8 in such a way that the effect of any ligand upon $[C \cdot P]$ is related only to the ratio L_0/K_1. It follows immediately that the concentration of ligand (L_0) necessary to depress binding of colchicine by a given amount (e.g., 50%) will be directly proportional to K_1, and the proportionality will not be a function of the particular ligand involved. Although any extent to which binding is depressed could be employed, 50% gives the greatest sensitivity, since $d[C \cdot P]/dL_0$ is maximal at or near this value. This method also suffers from the criticism that a comparison is made

only at a single point, and deviations from theory at higher and lower values of L_0 would go unnoticed. (c) A final method involves an approximation to Eq. 8. In the case of colchicine and tubulin, $(k_3 + k_2C_0)\beta > k_{-2}$ if $\beta \gtrsim 0.05$. From this it follows also that $\alpha^2 \gg 4k_{-2}k_3\beta$, and $\exp(-m_1t_2/2) \cong 1$, an approximation which is true in this paper to never worse than 1%. Expanding Eq. 8 and introducing these assumptions, we obtain

$$[C\cdot P] = \frac{k_2C_0P_0}{(k_3+k_2C_0)} \cdot[1 - \exp(-(k_3 + k_2C_0)\beta t_2)] \quad (9)$$

Define the function r, which can be measured experimentally:

$$r = \frac{[C\cdot P] \text{ in the presence of ligand}}{[C\cdot P] \text{ in the absence of ligand}} \quad (10)$$

Equations 9 and 10 yield a solution for $\beta(r)$:

$$\beta \cong \left[\frac{1}{(k_3 + k_2C_0)t_2}\right] \cdot \ln\left[\frac{1}{1 - r(1 - \exp(-(k_3 + k_2C_0)t_2))}\right] \quad (11)$$

from which numerical values of β may be calculated from experimentally determined values of r. Since $\beta = K_1/(K_1 + L_0)$, evaluation of K_1 is straightforward. Of the several means by which this may be accomplished, we have found the following rearrangement most useful:

$$\frac{1}{\beta} = 1 + \frac{1}{K_1}(L_0) \quad (12)$$

which predicts that a plot of $1/\beta$ vs. L_0 should be a straight line of slope $(1/K_1)$. This function tests the theory directly over a wide range of values of L_0, and has been used extensively by us. It may be the most useful method when using this general technique to study ligands other than those considered here.

Noncompetitive interaction. It is also possible to consider a reaction system analogous to the preceding, but which involves noncompetitive interaction between the ligand and colchicine, i.e., in which the two small molecules are bound to different sites on tubulin, and a ternary complex can exist. The following equations describe this system:

Time period 1:

$$L + P \overset{K_1}{\rightleftharpoons} L\cdot P \quad (6)$$

Time period 2:

$$L + P \overset{K_1}{\rightleftharpoons} L\cdot P \quad (6)$$

$$C + P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P\cdot C \quad (7)$$

$$P \overset{k_3}{\rightarrow} P' \quad (1)$$

$$C + L\cdot P \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} L\cdot P\cdot C \quad (13)$$

$$L + P\cdot C \overset{K_1'}{\rightleftharpoons} L\cdot P\cdot C \quad (14)$$

The system may be solved if it is assumed that both Eqs. 6 and 14 are in equilibrium that $L_0 \gg P_0$, and that $C_0 \gg P_0$. It is assumed that both Eqs. 7 and 13 are controlled by kinetic factors. Under these conditions the sum of the concentrations of the colchicine-containing (radioactive) complexes is given by

$$[P\cdot C] + [L\cdot P\cdot C] = \frac{k_2C_0P_0}{[\alpha'^2 - 4k_{-2}k_3\beta]^{1/2}} \cdot[\exp(-m_1't_2/2) - \exp(-m_2't_2/2)]\left[\frac{\beta}{\beta'}\right] \quad (15)$$

where t_1, t_2, and β are defined following Eq. 8, $\beta' = K_1'/(K_1' + L_0)$, $\alpha' = k_{-2} + (k_3 + k_2C_0/\beta')\beta$, $m_1' = (\alpha' - [\alpha'^2 - 4k_{-2}k_3\beta])^{1/2}$, and $m_2' = (\alpha' + [\alpha'^2 - 4k_{-2}k_3\beta])^{1/2}$. Again, $\exp(-m_1't_2/2) \cong 1$. For a comparison of noncompetitive and competitive behavior, binding curves were computed directly from Eq. 15 with assumed values of K_1', and are presented in RESULTS.

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