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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC APPLICATION OF THE HUMMEL AND DREYER METHOD FOR THE DETERMINATION OF COLCHICINE-TUBULIN BINDING PARAMETERS*

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SUMMARY

An application of the Hummel and Dreyer gel chromatography procedure modified for high-performance liquid chromatography has been used to determine the dissociation constant for the colchicine-tubulin interaction at 25°C. The results obtained are compared with results of other equilibrium and non-equilibrium techniques and demonstrate that the initial interaction of colchicine with tubulin must be rapid and probably reversible. This rapid and sensitive technique, which does not require radioisotopes for measurement of the binding parameters, will be extremely useful for characterization of tubulin-ligand interactions.

INTRODUCTION

Non-equilibrium methods for the determination of the dissociation constants of ligands bound to tubulin include gel chromatography[1-3], filtration through DEAE-impregnated paper[4-8], and charcoal adsorption[6,7]. Equilibrium methods such as the Hummel and Dreyer procedure[8-10] and equilibrium dialysis[11,12] have rarely been used with tubulin. Recently an application of the Hummel and Dreyer[8] equilibrium gel chromatography method, adapted for high-performance liquid chromatography (HPLC) for studying ligand-macromolecular interactions was presented by Sebille *et al.*[13]. The results obtained were in close agreement with previously reported binding studies in which other techniques were used and showed the feasilility of using HPLC in evaluating macromolecular-ligand binding parameters.

This study reports preliminary results obtained with an HPLC-modified Hummel and Dreyer procedure in which size-exclusion columns are used for the determination of dissociation constants for the interaction between colchicine and renal tubulin. Previously, the apparent binding constant of colchicine to renal tubulin at 37°C has been determined by the filter assay on DEAE-impregnated paper disks[14]. Recently, the renal tubulin–colchicine dissociation constant at 5°C was determined using equilibrium dialysis[12]. Tubulin exhibits a decay of ligand binding capacity which is dependent on temperature, ligand type, and ligand concentration[6,15]. The slow rate of decay at 5°C permitted measurement of the tubulincolchicine interaction by equilibrium dialysis. However, the rate of decay at 37°C prevents equilibrium dialysis measurements, because several hours are required for equilibration. Therefore, the application of the equilibrium dialysis technique as a routine method for the measurement of binding constants is unfeasible at physiological temperatures. Other equilibrium and kinetic probes of the binding of colchicine to tubulin have met with limited success owing either to the tubulin decay phenomenon or to unexplained instability of the measurement. For example, the intrinsic fluorescence of colchicine has been employed as a kinetic probe of binding, but in addition to tubulin decay secondary fluorescent changes occur after an initial, rapid change, making reliable measurement of the binding constant difficult'[6,17].

Utilization of the HPLC-modified Hummel and Dreyer technique obviates the decay problem, since an analysis only requires *ca.* 15 min. This means that equilibrium ligand binding to tubulin can be examined over a wide range of temperature and concentration conditions.

EXPERIMENTAL

Materials

Colchicine was purchased from Aldrich (Milwaukee, WI, U.S.A.) and its purity was checked by thin-layer chromatography on silica gel G (Macherey, Nagel & Co., Duren, G.F.R.) in benzene-methanol (3:1). The purity was also determined by HPLC with an Ultrasphere™ C_{18} (Beckman Instruments, Irvine, CA, U.S.A.) reversed-phase column (1.8 ml/min; 3000 p.s.i.) using a modification of the acetonitrile-methanol-water (25:7:68) solvent system previously described[18]. Concentrations of colchicine solutions were calculated from values of absorbancy at 350 nm and the molar extinction coefficient of 16,600 $cm^{-1} M^{-1}$ (ref. 19). Sodium phosphate buffer (analytical grade) was dissolved in water that had been filtered through a Milli-Q® water purification system. All buffers were filtered through a 0.45-μm membrane filter and thoroughly degassed under vacuum prior to use. Sodium sulfate (reagent grade) was supplied by MCB (Los Angeles, CA, U.S.A.) and sodium dodecyl sulfate (sequanal grade) was obtained from Pierce (Rockford, IL, U.S.A.). All organic solvents used were chromatographic quality (Burdick & Jackson Labs., Muskegon, MI, U.S.A.).

Equipment

Apparatus, A Beckman Model 112 or a Model 110A solvent pump and a Gilford system 2600 microprocessor-controlled UV-VIS spectrophotometer equipped with an 8-μl quartz flow cell was used for all experiments.   An Altex Model 210 injector

equipped with a 250-μl loop was employed. Data were plotted on a Hewlett-Packard 7255A graphics plotter.

Size-exclusion columns. A TSK 3000 SW (10-μm particle size, $3 \cdot 10^5$ mol. wt.) protein column (60 cm × 7.5 mm I.D.) from Beckman Instruments, an I-125 (125-8, pore, 5–10-μm particle size) protein column (30 cm × 7.8 mm I.D.) from Waters Assoc. (Milford, MA, U.S.A.), and a Chromegapore MSE-100 (100-Å pore, 10-μm particle size, 10^5 mol. wt.) protein column (30 cm × 4.5 mm I.D.) from ES Industries, (Marlton, NJ, U.S.A) were employed for this study.

Experimental conditions

Preparation of tubulin. Tubulin was purified from bovine renal medulla by four cycles of in *vitro* assembly and disassembly of microtubules[14]. Renal medullary tubulin polymerized in *the* presence of dimethyl sulfoxide and glycerol does not contain microtubule-associated proteins. The renal medulla tubulin used in all binding experiments was at least 95% pure as determined by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate (SDS)[14]. Purified tubulin was dialyzed against 50-mM sodium phosphate buffer, pH 7.2, prior to the colchicine binding experiments by HPLC. Protein determinations were performed by the Lowry method, and bovine serum albumin was used as the standard[20].

Colchicine binding by the Hummel and Dreyer method. Three size-exclusion columns were investigated for their use in the colchicine binding study. The basic eluent for all columns was 50 mM sodium phosphate buffer, pH 7.2. All experiments were done at 25°C.

Column 1, TSK 3000 SW; eluents, 50 μM colchicine with and without 0.1% SDS or with 100 mM sodium sulfate in sodium buffer; samples, 95 μl of 50 mM sodium phosphate buffer, pH 7.2, and 95 μl of 75 μM colchicine solution; flow-rate, 1.0 ml/min; pressure, 400 p.s.i.

Column 2, Waters I-125; eluents, 50 μM colchicine with and without 100 mM sodium sulfate in sodium phosphate buffer; samples, 95 μl of 50 mM sodium phosphate buffer, pH 7.2, and 95 μl of 75 μM colchicine solution; flow-rate, 2.0 ml/min; pressure, 400 p.s.i.

Column 3, Chromegapore MSE-100; eluents, l-50 μM colchicine solutions in sodium phosphate buffer; samples, 100 μg of tubulin in l-50 μM colchicine solutions; flow-rate, 1.5 ml/min; pressure: 1100 psi.

Absorbances of free colchicine and the tubulincolchicine complex in column effluents were monitored at 350 nm.

Calculation of binding parameters. The Hummel and Dreyer technique[8] involves the equilibration of a size-exclusion column with a ligand solution. The macromolecule is dissolved in the ligand solution, applied to the column and then eluted with the same ligand solution. The concentrations of the ligand in the column eluent and the ligand in solution with the macromolecule must be different if binding occurs. The macromolecule is eluted from the column as the protein-ligand complex with a characteristic retention volume corresponding to its molecular weight. The ligand concentration in the column, depleted by the amount of ligand removed by binding to the protein, is observed as a **trough at** the included volume of the column. From the area of the trough, the amount of ligand bound may be determined. The area of the trough was determined electronically with the Gilford 2600 system, coupled to

the Hewlett-Packard plotter. A more precise measure of the extent of binding is obtained by plotting the area of the ligand trough (positive or negative) *versus* the excess moles of ligand, relative to that in the eluent, added to a constant amount of protein sample[21]. The binding ratio obtained by interpolating to zero trough area exactly equals the amount of ligand bound to the protein. Values of the binding ratio obtained at different concentrations of colchicine in the column eluent were analyzed as described by Scatchard[22]. Values of the dissociation constants and stoichiometries were calculated from the Scatchard plot[22].

Colchicine binding by non-equilibriumjltration. Binding of colchicine to tubulin was measured indirectly by competition with [³H]colchicine (Amersham Radiochemicafs). [³H]colchicine was incubated with tubulin (20 μg) in the absence and presence of unlabeled colchicine in 50 mM sodium phosphate, 1 mM magnesium sulfate, and 0.1 mM ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), pH 6.8, for 3 h at 37°C in a covered water-bath. The assay volume was 200 μl, and the concentration of [³H]colchicine was from 0.1-5.0 μM with a specific activity of 0.45 Ci/mmol. The [³H]colchicine–tubulin complex was isolated by retention on DEAE-cellulose paper disks and the radioactivity of the complex was measured as previously described by Barnes and Roberson[14]. Stock [³H]colchicine was stored at -20°C in ethanol and the solvent was evaporated under a stream of nitrogen prior to dissolving it for use in the assay. Concentrations of colchicine were calculated from absorbances at 350'nm. The binding of [³H]colchicine to renal tubulin was measured after 3 h of incubation under conditions of apparent equilibrium. Although the final apparent equilibrium is not reached until 6–8 h of binding, the time of 3 h was chosen so that the decay of colchicine-binding activity in the presence of colchicine was less than 13%[14]. Assays were performed in duplicate for each experiment and the competitive binding experiments were performed in quadruplicate.

RESULTS

The retention volume of colchicine was measured on each of the three size-exclusion columns. The values obtained were identical whether buffer containing colchicine or buffer without colchicine was applied the column. Identical retention volumes were observed for both positive and negative absorbances. As shown in Table I, colchicine was retained longest on the TSK 3000 SW column with a retention

TABLE I

RETENTION VOLUME OF COLCHICINE ON DIFFERENT SIZE-EXCLUSION COLUMNS

Column type	Column eluent	Retention volume (ml)
TSK 3000 SW	Buffer*	45
	+0.1% SDS	27
	+100 mM sodium sulfate	18
Waters I-125	Buffer*	26
	+100 mM sodium sulfate	32
Chromegapore MSE-100	Buffer*	15

* Eluent buffer: 50 mM sodium phosphate, pH 7.2, 50 μM colchicine.

volume of 45 ml. The observed retention volumes are related to column length, and the TSK 3000 SW column was twice as long (60 cm) as the other two columns (30 cm) examined. The use of shorter columns will reduce the elution time. The addition of 0.1% SDS and/or 100 mM sodium sulfate decreased the retention time by reducing the volume required for elution. In contrast, increasing the ionic strength had the opposite effect on the Waters I-125 column, which showed slightly longer retention volumes and considerable broadening of the colchicine peak. Since the retention volumes were long and peak broadening occurred, these columns were not studied further. The Chromegapore MSE-100 was the column of choice for the colchicine-tubulin binding measurements. The retention volume of colchicine on this column was 15 ml, the smallest retention volume exhibited by the columns tested. This lower
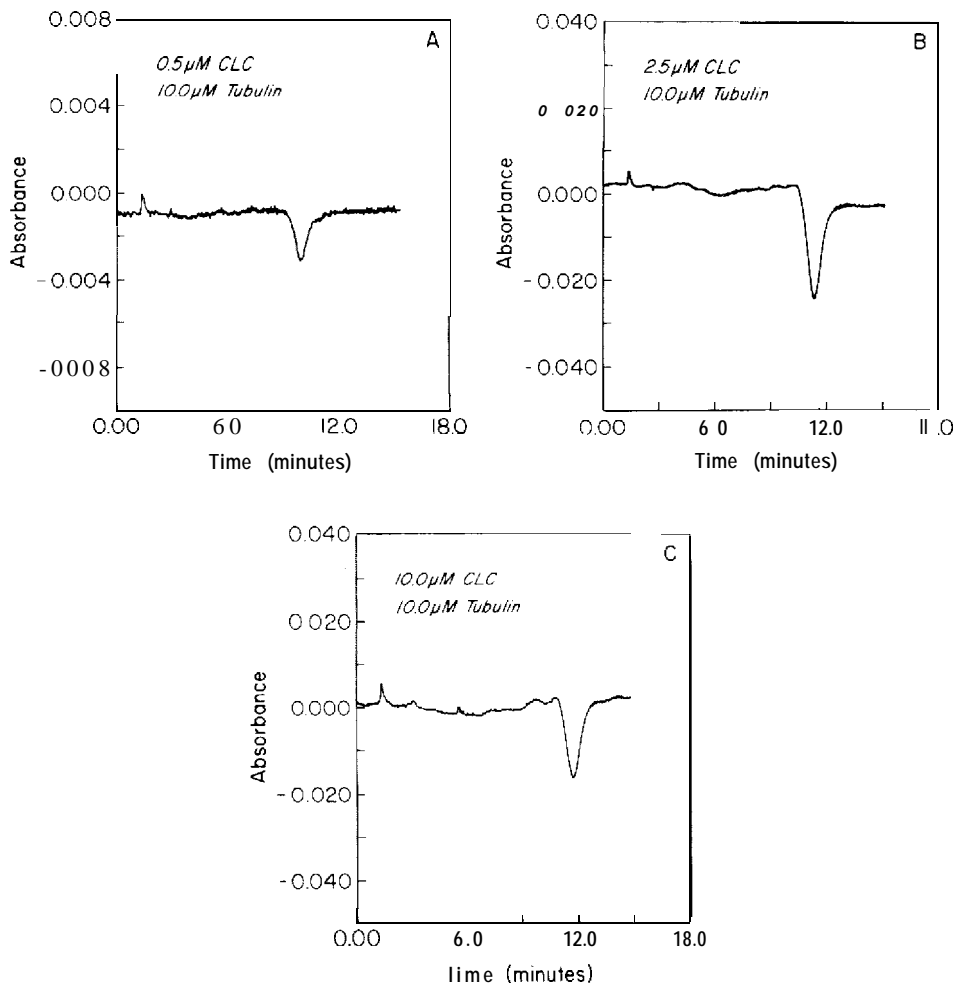

Fig. 1. Typical chromatograms of the Hummel and Dreyer procedure modified by HPLC on a Chromegapore MSE-100 column. Chromatographic conditions, 50 mM sodium phosphate, pH 7.2; flow-rate, 1.5 ml/min; pressure, 1100 p.s.i.; temperature, 25°C. Wavelength monitored, 350 nm. (A) 10 μM tubulin in 0.5 μM colchicine eluted with 2.5 μM colchicine. (B) μM tubulin in 2.5 μM colchicine eluted with 25 μM colchicine. (C) 10 μM tubulin in 10 μM colchicine eluted with 10 μM colchicine. CLC = colchicine.

retention volume resulted in sharper peaks, and the column shows excellent stability to repeated injections of tubulin. Although 100 mM sodium sulfate reduced the retention volume of the TSK 3000 SW column to close to that observed for the Chromegapore MSE-100 column, this effect of ionic strength upon tubulin binding parameters, as determined by HPLC, has not been established. Consequently, further study is required to validate the results obtained by using a TSK 3000 SW column.

A typical HPLC elution profile of the modified Hummel and Dreyer method using the Chromegapore MSE-100 column is presented in Fig. 1. A constant final
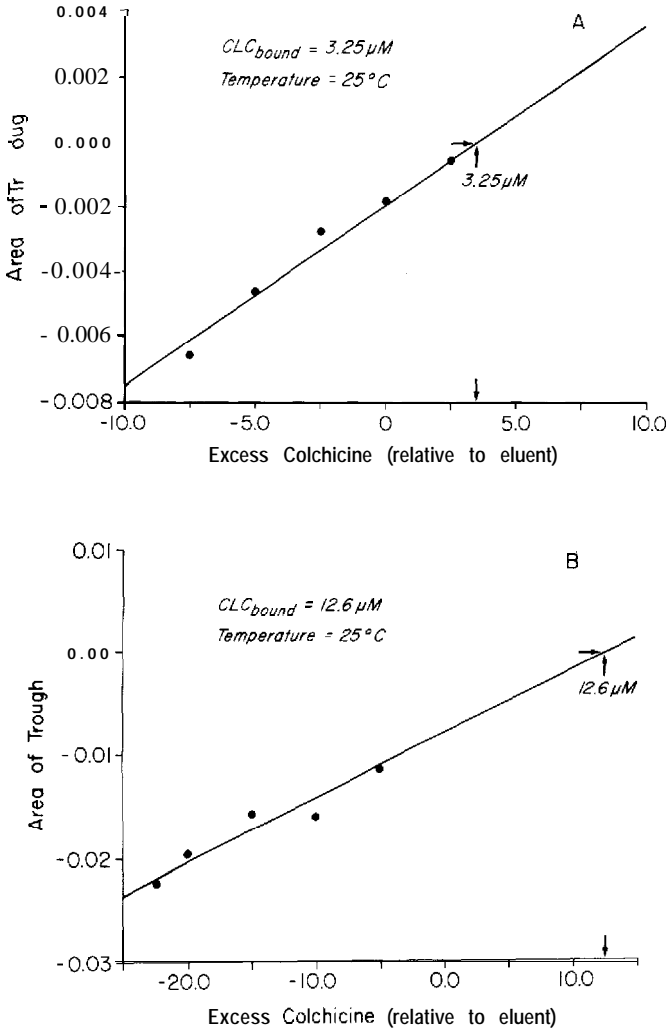

Fig. 2. Area of the colchicine trough as a function of excess of colchicine (CLC), relative to the eluent concentration. Areas of the troughs were determined from the HPLC chromatograms, as shown in Fig. 1. The abscissa intercept gives the concentration of CLC$_{bound}$. The chromatographic conditions are the same as in Fig. 1. (A) 10 μM tubulin injected with different concentrations of colchicine and eluted with 10 μM colchicine. (B) 10 μM tubulin injected with different concentrations of colchicine and eluted with 50 μM colchicine.

TABLE II

BINDING RATIO FOR THE COLCHICINE–TUBULIN INTERACTION

Colchicine eluent (μM)	Colchicine bound (μM)	Colchicine free (μM)	$\bar{r}$	$\bar{r}/Colchicine\ free$ $(l\ mol^{-1} \cdot 10^{-6})$
1.0	0.46	0.54	0.046	0.086
1.5	0.54	0.96	0.054	0.056
2.0	0.60	1.40	0.060	0.043
2.5	0.6	1.9	0.060	0.032
5.0	0.99	4.1	0.099	0.025
7.5	1.7	5.8	0.17	0.029
10.0	2.9	7.1	0.29	0.041
15.0	3.5	11.5	0.35	0.03
20.0	2.8	17.2	0.28	0.016
25.0	5.02	19.9	0.50	0.025
30.0	5.8	24.2	0.58	0.024
40.0	7.4	32.6	0.74	0.022
50.0	12.6	37.4	1.25	0.33

concentration of tubulin (10 μM) was mixed with various concentrations of colchicine and immediately applied to the column. The retention time of 1.36 min (2.04 ml) for the tubulin peak was constant for all injections. A minimum of five points were obtained for each determination of bound colchicine at each fixed concentration of colchicine in the eluent. Fig. 2 shows the data obtained by plotting the area of the ligand trough *versus* the concentration of excess colchicine bound to tubulin. The binding ratio, $\bar{r}$, of bound colchicine per mole of tubulin was calculated from the intercept of the abscissa. Table II summarizes the binding ratios determined from these plots. Examination of the data shows that the binding ratio, $\bar{r}$, varies with the colchicine concentration in a non-linear fashion. A Scatchard analysis of the resulting binding ratios obtained at various concentrations of colchicine in the HPLC eluent buffer is shown in Fig. 3. The binding constants were estimated as 0.3 μM and 65
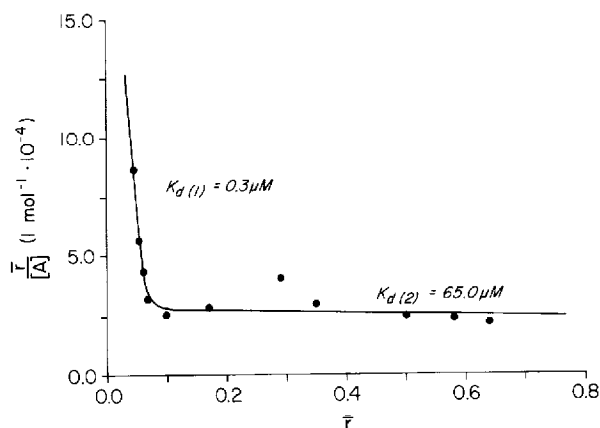

Fig. 3. Scatchard plot illustrating the binding of colchicine to renal tubulin at 25°C using the modified Hummel and Dreyer procedure on HPLC.
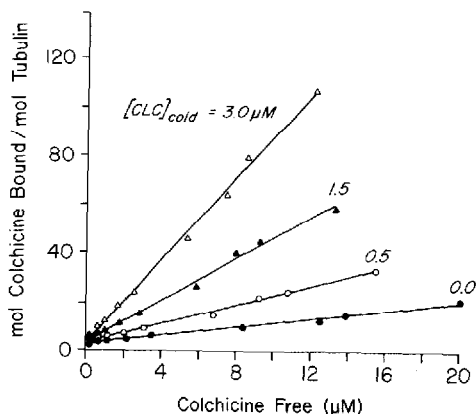
Fig. 4. Binding of [³H]colchicine to renal tubulin using the non-equilibrium filtration assay technique. Colchicine (unlabeled) was incubated with tubulin for 3 h at 37°C. Colchicine (CLC) = 0.0, 0.5, 1.5 and 3.0 μM.

μM with stoichiometries of 0.1 and 2.1, respectively. The binding of tubulin to colchicine by the non-equilibrium filtration method is shown in Fig. 4. The double reciprocal plot is typical of the standard colchicine binding assy[14]. Secondary plots of data (not shown), over a wide concentration range of colchicine show a non-linear dependence of binding upon colchicine concentration. This is consistent with the Scatchard plot shown in Fig. 3 and indicates that the colchicine–tubulin interaction is not a simple bimolecular process.

DISCUSSION

The method of Hummel and Dreyer adapted for use with HPLC, is a highly useful and rapid technique for the study of the tubulin–colchicine binding interaction. The method has several important advantages over other methods that have been used to examine tubulin–colchicine interactions: expensive radiolabeled compounds are not required since direct spectrophotometric monitoring of the column effluent allows sensitive detection of both the protein and drug. The method is very rapid, requiring only 15–20 min per binding measurement. Consequently, tubulin decay effects are minimized. This will allow a more complete temperature range to be examined and the thermodynamic binding parameters (enthalpy and entropy) to be obtained. Utilization of this procedure to measure the binding interactions between colchicine and tubulin can be easily extended to analogues of colchicine. Presently, the interaction of non-radiolabeled analogues with tubulin can only be measured by competitive inhibition between [³H]co'chicine and the analogue. Expansion of this procedure should greatly facilitate understanding of the complex tubulin–colchicine interaction.

By the present HPLC method, two classes of colchicine binding sites were detected on renal tubulin at 25°C. The high-affinity site, K_d (dissociation constant) = 0.3 μM, exhibited a low stoichiometry, while the low-affinity site, $K_d = 65 \mu M$, exhibited a stoichiometry of two moles of colchicine bound per mole of tubulin. In contrast, only a single class of colchicine-binding sites was detected by both the

non-equilibrium filter assay at 37°C[14] and the equilibrium dialysis assay at 5°C[12]. Table III compares the three techniques under different temperature conditions. The basis for the differences in binding parameters determined by the three different methods include the temperatures, duration of incubations, and possibly the methods themselves. The incubation periods for the filter assay[14] and the equilibrium dialysis assay[12] were several hours. The incubation time of tubulin and colchicine in the HPLC method was 15–20 min, the time required to elute the column. The incubation time prior to injection was negligible. Consequently, the HPLC method is not subject to the problems associated with the time-dependent decay of colchicine binding ability that tubulin displays. The inherent decay of tubulin is slower at 5°C, allowing the measurement of binding parameters by equilibrium dialysis at this temperature[12]. However, this technique cannot be employed at 37°C, owing to the large concentration corrections that would be required. Thus the HPLC method is the only available technique that can measure the equilibrium binding parameters of the colchicine–tubulin interaction.

The data obtained by the HPLC method are consistent with a rapid, reversible binding that occurs initially. The binding data obtained by the HPLC method are compatible with models proposed by Garland[16] and Lambeir and Engelborghs[17] that suggest a rapid initial binding, followed by a slower conformational change. Recent evidence from Andreu and Timasheff[10,23] also indicates that colchicine binds rapidly and induces a conformational change subsequent to the initial binding. Other data, suggesting that tubulin has more than one colchicine binding site[17,24,25], are also compatible with our observations. The low stoichiometry (0.1) of the high-affinity site is on the low side when compared with stoichiometries reported in the literature for colchicine binding to tubulin[15]. The reasons for observed stoichiometries considerably less than 1 are unexplained, but may be due to a domain interaction between tubulin molecules. If aggregation occurs, the binding ratio, $\bar{r}$, will be dependent on the tubulin concentration[26]. The stoichiometry (2) of the low-affinity site has not been observed by non-equilibrium binding assay procedures, and its significance is presently unclear. Evaluation of these considerations by HPLC is now feasible.

The effect of pressure on the binding of colchicine to tubulin is unknown; however, very high pressures are normally required to affect most chemical equilibria. All of the previously used colchicine-binding techniques have been performed at

TABLE III

COMPARISON OF BINDING PARAMETERS FOR THE TUBULIN–COLCHICINE INTERACTION

Technique	Temperature (°C)	Incubation time (h)	K_d (μM)	Stoichiometry
Filter assay	5*	3	32 ± 3.6	0.36 ± 0.03
	37	3	0.42 ± 0.04	0.46 ± 0.04
Equilibrium dialysis*	5	18	1.8 ± 0.4	0.50 ± 0.07
HPLC-modified Himmel-Dreyer	25	**	$k_{d(1)} = 0.3 \mu M$	0.1
			$k_{d(2)} = 65 \mu M$	2.1

* Data from ref. 12.
** Total analysis time is less than 20 min, with no preincubation of colchicine (CLC) and tubulin (TUB).

atmospheric pressure. Pressure does induce a depolymerization of microtubules[27-29]; consequently, the HPLC colchine-binding assay conditions should maintain tubulin in the monomeric form if there is any effect. Pfeffer *et al.*[29] demonstrated that outer doublet microtubules depolymerized at 16,000 psi, exhibiting typical values of K_a and stoichiometry for the binding of colchicine. The relatively low pressure (1100 p.s.i.) generated during the HPLC analysis probably does not alter colchicine binding parameters; nevertheless, its effect can and will be examined.

ACKNOWLEDGEMENTS

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