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Reversible Binding of Chlorpromazine to Brain Tubulin

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SUMMARY

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Chlorpromazine binds reversibly to brain tubulin via two well-resolved processes. One chlorpromazine molecule binds strongly compared with 8-9 molecules that bind weakly. Binding to the higher-affinity site is chiefly entropy-driven, implicating hydrophobic forces. The two binding processes are compared with the biological activities of chlorpromazine *in vitro*.

INTRODUCTION

Recently we reported (1) that the tranquilizing drug chlorpromazine [2-chloro-10-(3-dimethylaminopropyl)phenothiazine hydrochloride] interacts reversibly *in vitro* with microtubule subunit protein, tubulin, as revealed by inhibition of the rate of reassembly of microtubules in the 30,000 × *g* supernatant fraction of mouse brain homogenate and inhibition of the binding of colchicine by both the supernatant fraction and purified tubulin. CPZ[2] also causes disassembly of microtubules formed in its absence. McGuire *et al.* (2) found that a sufficiently high concentration of CPZ selectively precipitates tubulin from fractions of guinea pig brain homogenate. They too found that the drug inhibits reassembly of microtubules, in their case from purified tubulin. The present paper describes direct measurements on the reversible binding of CPZ to purified mouse brain tubulin.

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[2] The abbreviations used are: CPZ, chlorpromazine; PMS buffer, 0.05 M sodium pyrophosphate, 2.5 mM $MgCl_2$, and 2.5% sucrose, pH 6.8; PMSS buffer, 0.025 M sodium pyrophosphate, 1.25 mM $MgCl_2$, 0.125 M NaCl, and 1 M sucrose, pH 6.8; PM buffer, 0.025 M sodium pyrophosphate, 1.25 mM $MgCl_2$, and 0.125 M NaCl, pH 6.8; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Preparation of tubulin. Purified tubulin was prepared by DEAE-cellulose chromatography (3). The brains of 10 adult mice (Texas Inbred, ICR) were excised and homogenized in 2.5 volumes of PMS buffer. Homogenization was carried out manually with a glass homogenizer fitted with a Teflon pestle, and the homogenate was centrifuged at 100,000 × *g* for 60 min at 1-4°. The supernatant will be referred to as PMS supernatant. After centrifugation, 9 ml of PMS supernatant were applied to a column of DEAE-cellulose (bed volume, 1.5 cm in diameter × 17 cm) which had been equilibrated with PMS buffer. The column was then eluted with a gradient of NaCl in PMS buffer; 1- or 2-ml fractions were col-

lected, and aliquots were analyzed for protein by the method of Lowry *et al.* (4). More than 50% of the protein did not bind to the column and emerged as a large, single peak in early fractions. The remainder emerged as three peaks, the last peak being eluted at a NaCl concentration of about 0.25 M as determined conductometrically. A pilot experiment, in which 1 ml of PMS supernatant was first incubated with [^{3}H]colchicine and then mixed with 8 ml of unlabeled supernatant prior to application to the chromatographic column, showed that the last peak contained the tubulin. All chromatography was carried out at 4°.

The center fractions from the tubulin-containing peak were pooled to give a final volume of about 2 ml. Solid sucrose was then dissolved in the sample to give a final concentration of approximately 1 M and a protein concentration of about 2 mg/ml. Aliquots (0.3 ml) of the sample were frozen at −20°.

The purity of the tubulin prepared by this procedure was established by electrophoresis, ultracentrifugation, and colchicine binding. The SDS discontinuous acrylamide gel electrophoretic patterns showed two major bands, corresponding to the α and β subunits of the tubulin heterodimer. Densitometry scanning indicated that the two bands represented 90–95% of the protein. Ultracentrifugal analysis of a sample of tubulin immediately after removal from the chromatographic column and before addition of 1 M sucrose showed that the material sedimented essentially as a single component, with $s_{20,w}$ = 6.4 S, and contained only a trace of more rapidly sedimenting material ($s_{20,w} \cong$ 20 S). Using the standard filter assay of Weisenberg *et al.* (5), the fresh preparation in 1 M sucrose bound 0.6 mole of colchicine per 110,000 g of protein at the saturating concentration of 20 μM colchicine, which compares favorably with literature values for purified tubulin using this assay (5–7). The colchicine binding activity did not decline during frozen storage for 4 days and was not followed further because other investigators had already shown that 1 M sucrose stabilizes frozen calf brain tubulin for at least 2 weeks (8, 9) and that 30% sucrose stabilizes frozen rat brain tubulin for 90 days (10).

Seven preparations of tubulin were used for measurement of CPZ binding once our technique had been developed. More than half the measurements were made within 1 week of preparation of the protein, and the remainder within 2.5 weeks. There was no significant change (±8%) in the stoichiometry of CPZ binding to the protein during frozen storage for this period of time.

Assay for CPZ binding to tubulin. The binding of CPZ to tubulin was quantitated using a modification of the method of Hummel and Dreyer (11). A jacketed column of Sephadex G-25 (0.5 cm in diameter × 19 cm) was equilibrated with PMSS buffer containing the desired constituent concentration of CPZ (concentration of the drug in all its forms) mixed with a trace amount (less than 10 nM) of [^{3}H]CPZ. The temperature of the column was maintained by circulating bath water and was checked with a thermistor probe. Except where indicated, all experiments were performed at 37°.

Tubulin solution (200 μl) containing on the average about 1.8 mg of protein per milliliter and the same concentration of CPZ as in the equilibrated column was applied to the column. The tubulin solution was prepared by incubating 225 μl of tubulin from a frozen aliquot (prepared as described above) with 25 μl of PMSS buffer containing 10 times the concentration of CPZ-[^{3}H]CPZ used to equilibrate the column. Incubation was generally carried out for 5 min at the temperature of the column. The column was eluted with the same CPZ-containing buffer that was used to equilibrate it. Accurately determined fractions of about 0.1 ml were collected in glass liquid scintillation vials. (Glass vials were used because plastic vials adsorb CPZ from solution; it is the experience of this laboratory that, in general, contact between solutions of CPZ and plastics other than Teflon must be avoided.) In each case a 50-μl aliquot was transferred to another vial. Then 10 ml of Triton–toluene–Permafluor (1:2:0.135) were added to each vial, and the samples were counted.

In this way it was possible to construct an elution profile of concentration of total CPZ vs. fraction number, and to calculate the volume of each fraction and the total amount of CPZ in each fraction. In more than half the experiments a 10-μl aliquot was also removed from each fraction for protein analysis (4). It was found that the concentrations of CPZ used in these experiments did not interfere with the protein analysis. The protein emerged in the void volume fractions of the column, and material balance was good to within 4%. The average number of moles of CPZ bound per mole of tubulin (ν) was calculated using (a) the total amount of protein in the void volume fractions and (b) the net amount of CPZ in the void volume fractions, calculated from the total CPZ reckoned from volume and CPZ concentration and the concentration of CPZ in the fractions preceding the void volume fractions. This value of ν corresponds to the equilibrium between tubulin and its CPZ complexes at the concentration of unbound CPZ in the equilibrated column.

In order to determine the concentration of unbound CPZ in the equilibrated column, it was necessary to determine the extent of complex formation between sucrose and CPZ. These experiments were done as described above, except that the buffer system used to equilibrate and elute the column contained no sucrose, and the 200-μl sample applied to the column was an aliquot of 225 μl of PMSS plus 25 μl of the buffer system used to equilibrate the column but containing 10 times more CPZ. The sucrose-CPZ complex emerged from the column in fractions centered about a volume equal to $^9/_{10}$ of the totally included volume of the column.

The equilibrated column was calibrated in the usual fashion with blue dextran and NaCl. The totally included volume was twice the void volume. Another type of calibration experiment, in which a trace amount of [^{3}H]CPZ was passed through an equilibrated column containing only unlabeled CPZ, showed that CPZ was retarded on the column and emerged in fractions centered about a volume equal to about 2.5 times the totally included volume as determined with NaCl. This was confirmed by passing unlabeled CPZ through an equilibrated column containing unlabeled CPZ mixed with a trace amount of [^{3}H]CPZ.

All measurements were made at constituent concentrations of CPZ at least an order of magnitude less than its critical micelle concentration,[3] and a fresh Hummel-Dreyer column was prepared for each measurement.

Other analytical methods. Electrophoresis was carried out in SDS according to the discontinuous procedure of Davis (13). The trailing ion was glycine; the leading ion was Cl^-, and Tris was the buffer counterion. The separating gel was 10% acrylamide containing 0.1% SDS, and the pH in the separating gel was 8.8. Tubulin was prepared in 1% SDS and 0.1% 2-mercaptoethanol, and 40 μg of protein were applied to each gel. Gels were stained with Amido black and scanned at 650 nm.

Ultracentrifugal analysis of tubulin was performed in a Spinco model E ultracentrifuge operating at 48,000 rpm. Velocity sedimentation was carried out in a 30-mm double-sector cell at 8°, using about 0.2% protein and, as solvent, the buffer solution which eluted tubulin from the DEAE-cellulose column.

Fractions from the Hummel-Dreyer column were analyzed for sucrose by the resorcinol method of Roe *et al.* (14).

The identities of commercial [^{3}H]CPZ and [^{3}H]colchicine were confirmed by thin-layer chromatography: [^{3}H]CPZ on polyethyleneimine cellulose developed with 1 M acetic acid; [^{3}H]colchicine, on silica gel, using chloroform–methanol (95:5, v/v).

Sources of chemicals. Nonradioactive CPZ was kindly supplied by Smith Kline & French Laboratories. [^{3}H]CPZ ([*methyl*-^{3}H]chlorpromazine, 99% radiochemically pure) was obtained from Schwarz/Mann. [*ring C, methoxy*-^{3}H]colchicine (99% radiochemically pure) was obtained from New

[3] Florence and Parfitt (12) reported values of 4.5 and 5.5 mM for the critical micelle concentration of CPZ in 0.15 M NaCl at 20° and 34°, respectively. Using their pH titration method, we determined the critical micelle concentration in 0.125 M NaCl plus 1 M sucrose to be 4 mM at room temperature (approximately 24°).

England Nuclear. DEAE-cellulose was a product of Whatman Biochemicals, Ltd.; Sephadex G-25, Pharmacia Fine Chemicals; and ultrapure sucrose, Schwarz/Mann. All other chemicals were of reagent-grade or equivalent quality.

RESULTS

Nature of Hummel-Dreyer elution profile for CPZ-tubulin. The two types of Hummel-Dreyer elution profiles encountered with the CPZ-tubulin system are illustrated in Fig. 1. In both cases the tubulin and its complexes with CPZ emerged from the column in the void volume fractions, but neither profile shows a trough at the total volume of the column, because CPZ was retarded on the column. If a sufficiently large number of fractions had been collected, the trough would have emerged from the column at about fraction 90. The striking difference between the two types of elution profiles is the artifactual peak of CPZ devoid of protein centered at about fraction 30 in Fig. 1B but absent from Fig. 1A. This peak was observed only when the Hummel-Dreyer column was equilibrated with CPZ of relatively high specific activity (expressed as counts per minute per mole of constituent CPZ). Its nature was established by three different experiments, taking care to maintain the CPZ specific activity at a level where the artifactual peak was seen. (a) When a composite of the central void volume fractions from a Hummel-Dreyer column was applied to a second Hummel-Dreyer column, the resulting elution profile did not show the artifactual peak, even when the concentration of protein applied to the second column was the same as routinely used in single-column experiments. This fractionation experiment shows that the artifactual peak did not arise from some kind of protein interaction, e.g., reversible ligand-mediated macromolecular association (see total ligand pattern in Fig. 3B of ref. 15). (b) When the stock tubulin solution was equilibrated with respect to sucrose by passage through a Sephadex G-25 column equilibrated with PMSS buffer just prior to a Hummel-Dreyer experiment, the Hummel-Dreyer elution profile did not show the artifactual peak. This result indicates that the artifactual peak did not represent a tubulin-bound contaminant released by CPZ. (c) A modified Hummel-Dreyer experiment was performed, in which the column was equilibrated with sucrose-free PM buffer containing CPZ rather than with PMSS buffer containing the drug. The resulting elution profile was of the type shown in Fig. 1B, but the artifactual peak was much larger. Qualitative analysis of the fractions showed that this large artifactual peak contained a peak of sucrose. We conclude that sucrose complexes with CPZ, and that the artifactual peak in Fig. 1B corresponds to a small excess of sucrose-CPZ complex in the tubulin solution applied to the column as compared with the solution used to equilibrate the column. That is, the stock tubulin solution contained a slightly higher concentration of sucrose than PMSS buffer.

The sucrose was originally added to the purified tubulin to stabilize it while frozen during storage (8–10), and was retained to

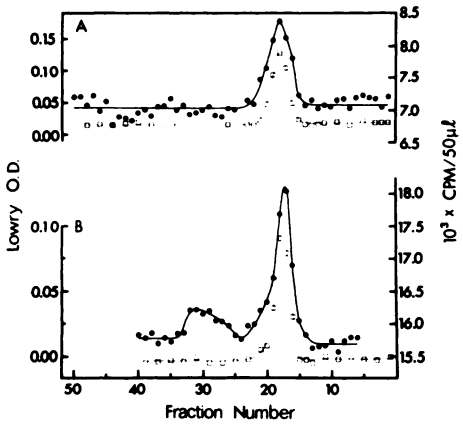

FIG. 1. *Representative Hummel-Dreyer elution profiles for CPZ-tubulin system*

A. Profile for $\dot{C}$ = 300 µM CPZ (C = 200 µM) with a specific activity of 4.7×10^{11} cpm/mole of constituent CPZ. B. Profile for $\dot{C}$ = 25 µM CPZ (C = 16.7 µM) with a specific activity of 1.3×10^{13} cpm/mole of constituent CPZ. ●——●, constituent concentration of CPZ, expressed as counts per minute per 50 µl, vs. fraction number; □····□, protein concentration, denoted as "Lowry O.D.," where 0.1 absorbance unit corresponds to 0.95 mg of protein per milliliter of fraction relative to bovine serum albumin as the standard.

prevent loss of biological activity during the 1-hr exposure to 37° required for measurement. In the absence of sucrose or other stabilizing agents the half-time for the loss of colchicine binding activity is about 2.5 hr at pH 6.8 and 37° (16). Circular dichroism measurements showed that at this temperature the protein undergoes a slow conformational change which increases its lability to irreversible denaturation accompanied by aggregation (17). Actually, a set of measurements was made on sucrose-free Hummel-Dreyer columns equilibrated with PM buffer containing CPZ. It was expected that most of the sucrose would be removed from the tubulin sample on such a column without interfering with the concomitant binding reaction. Experiments were done at two different CPZ concentrations. In each case the value of ν obtained was twice as large as the value in the presence of 1 M sucrose for the same concentration of unbound CPZ in the equilibrated column (see below); more disturbingly, the system was not at equilibrium. Thus, when a composite of central void volume fractions was passed through a second sucrose-free column, the value of ν increased by another 20% at each of the two concentrations. Since interpretation of these results is complicated, we did not attempt to determine a binding isotherm in the absence of sucrose. In any case, we wished to make the CPZ binding measurements under conditions known to stabilize the biological activities of tubulin.

Frigon and Lee (8) have shown that 1 M sucrose stabilizes tubulin in unfrozen solution without interfering with colchicine binding, and Garland and Teller (18) took advantage of this finding in their kinetic analysis of the colchicine-tubulin interaction. Glycerol (usually about 1 M) is widely used to stabilize tubulin with respect to both colchicine binding and reassembly into microtubules, and apparently does not interfere with GTP binding (19). Both 1 M sucrose and 4 M glycerol promote the slow assembly of microtubules (half-times of 5 and 3.7 hr, respectively) from tubulin in reassembly buffer without the addition of GTP (20). This phenomenon did not complicate our CPZ binding measurements, however, since our buffer did not contain the Ca^{++}-chelating agent, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid, required for reassembly. These several observations suggest that glycerol could replace sucrose in CPZ binding experiments, keeping in mind the possibility that this agent might also complex with CPZ.

Because sucrose complexes weakly with CPZ, it was necessary to correct for its presence in PMSS buffer. The correction was made as follows. The average number of moles of CPZ bound per mole of sucrose (ν') was determined as a function of the equilibrium concentration of CPZ, using the Hummel-Dreyer method (Fig. 2A). Since the sucrose-CPZ complex evidently contains a single mole of sucrose,[4] ν' can be considered as being independent of sucrose concentration. It follows that these data permit determination of the concentration of unbound CPZ (C) in a Hummel-Dreyer column equilibrated with a given constituent concentration of the drug ($\bar{C}$) in PMSS buffer. Since PMSS buffer contains 1 M sucrose, the relationship $\bar{C} = C + \nu'$ can be used to construct a plot of C vs. $\bar{C}$ for the buffer, using the data in Fig. 2A. This construction is displayed in Fig. 2B. Knowing $\bar{C}$ for a given CPZ-tubulin experiment, we determined the value of C from Fig. 2B by interpolation.

Controls. As described above, values of ν for the CPZ-tubulin system were calculated from the composition of the void volume fractions in the Hummel-Dreyer elution profiles. In order to ensure that these values could be interpreted in terms of the number of binding sites on the tubulin molecule and their binding constants, it was necessary to demonstrate that binding

[4] Pilot experiments done in this laboratory by Mr. Gary A. Ludi show that at pH 6.8 and 27° the spectrum of CPZ is red-shifted by sucrose from λ_{max} = 253.5 nm in water to 255 nm in 1.5 M sucrose, and that the spectra show an isosbestic point at 252.8 nm. A double-reciprocal plot of the change in absorbance at 260 nm vs. sucrose concentration is linear within a very small experimental error over the range of sucrose concentrations from 0.2 to 1.5 M. These results indicate formation of a single type of sucrose-CPZ complex containing 1 mole of sucrose.

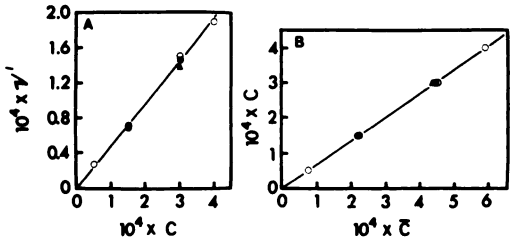

FIG. 2. *Complex formation between sucrose and CPZ*

A. Plot of average number of moles of CPZ bound per mole of sucrose, ν', vs. equilibrium concentration of CPZ, C: ●, 27°; ○, 37°; ▲, 42°. B. Plot of concentration of unbound CPZ, C, vs. constituent concentration of CPZ, $\bar{C}$, in PMSS buffer.

was reversible and that the system was at equilibrium. Reversibility was demonstrated by experiments in which a composite of the central void volume fractions from a Hummel-Dreyer column (C = 100 μM, where ν = 2.4 ± 0.26) was passed through a Sephadex G-25 column equilibrated with PMSS buffer alone. The tubulin was stripped of CPZ on the CPZ-free column so that it emerged at the void volume uncomplexed with the drug. The CPZ emerged in a peak centered about a volume equal to 2.5 times the totally included volume of the column, and there was no protein in the CPZ peak. The same results were obtained in a variation of this experiment, in which a tubulin-CPZ mixture was simply incubated for 50 min before passage through a CPZ-free column.

That the system was at equilibrium was self-evident when the elution profile was of the type shown in Fig. 1A, but when the profile showed two peaks, as in Fig. 1B, auxiliary experiments became necessary. In these experiments a composite of the central void volume fractions from one Hummel-Dreyer column was passed through a second Hummel-Dreyer column, an aliquot of the composite having been analyzed for protein. The value of ν calculated from the second elution profile agreed within experimental error with the value from the first profile: C = 48 μM; ν = 0.96 from the first profile and 0.97 ± 0.02 from the second one. It can be concluded, therefore, that equilibrium had already been established on the first column.

Isotherm for CPZ binding to tubulin. The binding of CPZ to tubulin at 37° was examined over a 100-fold range of CPZ concentration, C = 4.5–400 μM. Qualitative reading of the binding isotherm displayed in Fig. 3 indicates that the tubulin molecule can bind 8 or more CPZ molecules, but that binding is not of the classical type characterized by a hyperbolic isotherm. At least two binding processes are discernible, since the isotherm shows an inflection point[5] at $C \cong 35$ μM. Below the inflection point CPZ appears to bind to a single site on the tubulin molecule, while at higher concentrations relatively weak cooperative binding to several sites apparently dominates the interaction. These qualitative deductions are supported quantitatively by the following analysis of data.

The double-reciprocal plot of the binding data (Fig. 4) has an unusual shape (21, 22), showing two distinct segments: (a) an approximately linear segment which extrapolates to $1/\nu \cong 1$, indicative of a single binding site, and whose reciprocal slope gives a binding constant k of $(4 \pm 1) \times 10^4$ M^{-1}, and (b) at smaller reciprocal concentrations a curvilinear segment which is concave toward the abscissa, as is characteristic of cooperative binding (22), and which extrapolates to $1/\nu \cong 0.1$, indicative of a total number of about 10 binding sites on the tubulin molecule. As for the total number of sites, a plot of ν vs. $1/C$ is fortuitously linear[6] for ν = 2.4–7.7 and extrapolates to ν = 9.4. Since this extrapolation appears to be more precise than extrapolation of the curvilinear segment of the double-reciprocal plot, the value of 9.4 is assigned for computational purposes to the total number of sites, n.

A Scatchard plot of the binding data (23) is shown in Fig. 5A. Its shape is consistent with the foregoing interpretation of the data in terms of two binding processes.

[5] This reading of Fig. 3 is unbiased by the calculated curve through the data points, since the adjusted fit shows an even more pronounced inflection.

[6] In general a plot of ν vs. $1/C$ is theoretically linear only in the limit $1/C \rightarrow 0$, where the last binding site is being titrated, and is predicted to extrapolate to the total number of sites (21).

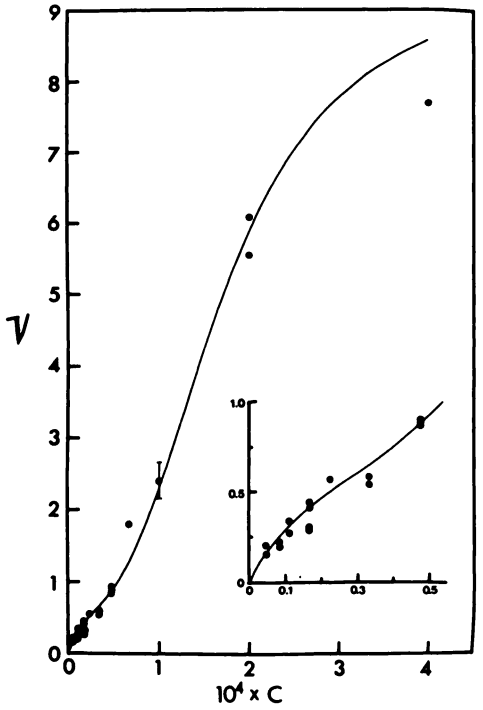

FIG. 3. *Isotherm for CPZ binding to tubulin at 37°*

The average number of moles of CPZ bound per mole of tubulin, ν, is plotted against equilibrium concentration of CPZ, C. The data point with an error bar designating mean deviation is the average of seven determinations on four different preparations of tubulin; all other data points are single determinations. The curve through the data points was calculated as described in the text.

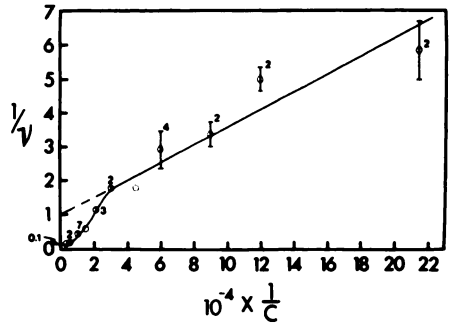

FIG. 4. *Double-reciprocal plot of ν vs. C for binding of CPZ to tubulin at 37°*

Error bars designate mean deviations, and their superscripts or subscripts, the number of determinations averaged. The intercept on the ordinate is indicated by an arrow.

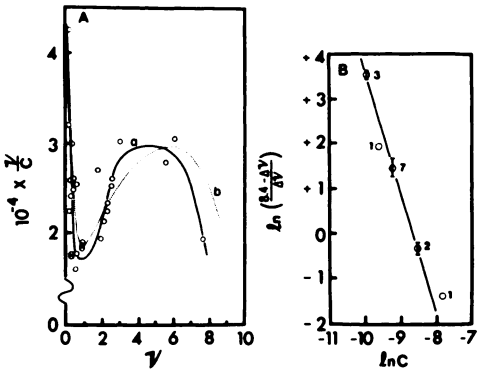

FIG. 5. *Binding of CPZ to tubulin at 37°*

A. Scatchard plot of binding data, ν/C vs. ν: ——, best curve through experimental points; · · · ·, curve calculated as described in the text. B. Hill plot, ln $[(8.4 - \Delta\nu)/\Delta\nu]$ vs. ln C, constructed as described in the text. Error bars designate mean deviations for the indicated number of determinations averaged.

Thus the initial branch of the plot indicates that at low concentrations CPZ binds to one site on the tubulin molecule with a binding constant of 4–5 × 10^4 M^{-1}, while the open-downward portion of the plot suggests that at higher concentrations an additional 8 or 9 CPZ molecules bind to the protein in a cooperative fashion (24).

Given this interpretation, it should be possible to fit the binding data to the two-term equation

$$\nu = \frac{kC}{1 + kC} + \frac{(n - 1)KC^m}{1 + KC^m} \qquad (1)$$

in which the first term on the right-hand side represents noncooperative binding of CPZ to a single site, while the second term is the Hill expression for cooperative binding (21) to $(n - 1) = 8.4$ sites. The Hill constant m is a measure of the degree of cooperativity, and K is an apparent binding constant. Empirical values of m and K were obtained from a Hill plot constructed in accordance with the relationship

$$\ln \frac{(n - 1) - \Delta\nu}{\Delta\nu} = -\ln K - m \ln C \qquad (2)$$

In this equation $\Delta\nu$ is the difference be-

tween the experimentally observed value of ν for given C and the number of moles of CPZ bound to the single site as computed from the first term in Eq. 1, using $k = 3.9 \times 10^4$ M^{-1}. The Hill plot is shown in Fig. 5B. Within experimental error the data points may be fitted to a straight line. From the slope of the line, $m = 2.75$, and from the slope and the midpoint value of C, $K = 2.20 \times 10^{10}$ M$^{-2.75}$. The value of m indicates moderately weak cooperativity. The value of K suggests that on the average the binding affinity for the cooperative process is about an order of magnitude less than for binding to the single site (compare $K^{1/2.75} = 5.8 \times 10^3$ M^{-1} with $k = 3.9 \times 10^4$ M^{-1}). The indicated values of k, $(n - 1)$, m, and K were substituted into Eq. 1, and a binding isotherm was calculated. As shown in Figs. 3 and 5A, the calculated isotherm is a satisfactory representation of the experimental data, which lends confidence in our interpretation.

The above experiments were performed at 37° because tubulin is most active with respect to reassembly of microtubules *in vitro*, etc., at this temperature, but it would be mechanistically desirable to know the effect of temperature on CPZ binding, particularly the binding of the first mole of drug. With this in mind, binding data were obtained for $C = 16.7$ μM over a 10° temperature range centered at 37°. The results presented in Table 1 show that the binding of the first mole of CPZ is quite insensitive to temperature in this range. Values of k were calculated using the approximate relationship

$$\nu \cong \frac{kC}{1 + kC} \tag{3}$$

which neglects the small contribution to ν made by the more weakly binding CPZ molecules at this low concentration (approximately 4% contribution at 37° as judged from Eq. 1, which is about the same as the error in ν; see Table 1). There is no statistically significant difference between the values of k estimated in this way at the three temperatures; the van't Hoff $\Delta H° = 0 \pm 2$ kcal/mole. Evidently the binding reaction is chiefly entropy-driven: $\Delta G° = -RT \ln k = -6.5 \pm 0.3$ kcal/mole, and $\Delta S° = (\Delta H° - \Delta G°)/T = 21 \pm 6$ e.u. at 37°.

TABLE 1

Binding of CPZ to tubulin at different temperatures

Duplicate determinations of ν at $C = 16.7$ μM CPZ were made at three different temperatures on aliquots of the same preparation of tubulin to ensure internal consistency with respect to protein concentration.

Temperature	ν
	mole CPZ bound/mole tubulin
32°	0.27 ± 0.017
37°	0.29 ± 0.010
42°	0.29 ± 0.012

DISCUSSION

The foregoing results show that CPZ binds reversibly to tubulin. Under our experimental conditions a total of 9–10 moles of CPZ can be bound via two processes with sufficiently different binding constants to permit their resolution. One CPZ molecule binds to the protein strongly, compared to the 8–9 molecules which bind cooperatively.

Binding to the site of relatively high affinity is evidently entropy-driven, and the values of the thermodynamic functions are strikingly similar to those for micelle formation of CPZ (25), which involves hydrophobic bonds. It seems likely that hydrophobic forces play an important role in the binding of CPZ to tubulin, as in the case of binding to serum albumin (26, 27).

The weaker cooperative binding of the other CPZ molecules implies a macromolecular structural change, but as yet there are no biophysical measurements to support this idea. Another testable idea is that the cooperatively binding CPZ molecules actually stack vertically at a single site, in much the same way as in micelle formation (12, 25). Although the concentration of CPZ is an order of magnitude less than its critical micelle concentration, it is conceivable that the protein molecule might present a hydrophobic microenvironment conducive to such an interaction.

It is interesting that the activities of CPZ *in vitro* also fall into two groups, which correlate with the two binding processes. Thus the action of 1–50 μM CPZ in causing mitotic arrest and in disorganizing the organized microtubular structure produced in cells by cyclic AMP[7] can be placed in formal correspondence with the binding of CPZ to the high-affinity site. In contrast, inhibition of assembly of microtubules and of colchicine binding by tubulin (1) and inhibition of fast axonal transport in sciatic nerve (28) have midpoint concentrations in the range 0.1–1 mM CPZ, which corresponds to the region of the isotherm where the drug binds cooperatively to tubulin. Moreover, it was concluded previously that inhibition of reassembly and colchicine binding are cooperative with respect to CPZ (1).[8]

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REFERENCES

1. Cann, J. R. & Hinman, N. D. (1975) *Mol. Pharmacol.*, **11**, 256–267.
2. McGuire, J., Quinn, P. & Knutton, S. (1974) *J. Cell Biol.*, **63**, 217a.
3. Eipper, B. A. (1972) *Proc. Natl. Acad. Sci. U. S. A.*, **69**, 2283–2287.
4. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
5. Weisenberg, R. C., Borisy, G. G. & Taylor, E. W. (1968) *Biochemistry*, **7**, 4466–4478.
6. Wilson, L. & Meza, I. (1972) *J. Cell Biol.*, **55**, 285a.
7. Hinman, N. D., Morgan, J. L., Seeds, N. W. & Cann, J. R. (1973) *Biochem. Biophys. Res. Commun.*, **52**, 752–758.
8. Frigon, R. P. & Lee, J. C. (1972) *Arch. Biochem. Biophys.*, **153**, 587–589.
9. Lee, J. C., Frigon, R. P. & Timasehff, S. N. (1975) *Ann. N. Y. Acad. Sci.*, **253**, 284–291.
10. Eipper, B. A. (1974) *J. Biol. Chem.*, **249**, 1398–1406.
11. Hummel, J. P. & Dreyer, W. J. (1962) *Biochim. Biophys. Acta*, **63**, 530–532.
12. Florence, A. T. & Parfitt, R. T. (1971) *J. Phys. Chem.*, **75**, 3554–3560.
13. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.*, **121**, 404–427.
14. Roe, J. H., Epstein, J. H. & Goldstein, N. P. (1949) *J. Biol. Chem.*, **178**, 839–845.
15. Cann, J. R. & Goad, W. B. (1970) *Science*, **170**, 441–445.
16. Wilson, L. (1970) *Biochemistry*, **9**, 4999–5007.
17. Ventilla, M., Cantor, C. R. & Shelanski, M. (1972) *Biochemistry*, **11**, 1554–1561.
18. Garland, D. & Teller, D. C. (1975) *Ann. N. Y. Acad. Sci.*, **253**, 232–238.
19. Levi, A., Cimino, M., Mercanti, D. & Calissano, P. (1974) *Biochim. Biophys. Acta*, **365**, 450–453.
20. Shelanski, M. L., Gaskin, F. & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U. S. A.*, **70**, 765–768.
21. Van Holde, K. E. (1971) *Physical Biochemistry*, Ch. 3, Prentice-Hall, Englewood Cliffs, N. J.
22. Nichol, L. W. & Winzor, D. J. (1972) *Migration of Interacting Systems*, Ch. 1, Clarendon Press, Oxford.
23. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.*, **51**, 660–672.
24. Changeux, J.-P. & Rubin, M. M. (1968) *Biochemistry*, **7**, 553–561.
25. Attwood, D., Florence, A. T. & Gillan, J. M. N. (1974) *J. Pharm. Sci.*, **63**, 988–993.
26. Jähnchen, E., Krieglstein, J. & Kuschinsky, G. (1969) *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.*, **263**, 375–386.
27. Krieglstein, J., Meiler, W. & Staab, J. (1972) *Biochem. Pharmacol.*, **21**, 985–997.
28. Edström, A., Hansson, H.-A. & Norström, A. (1973) *Z. Zellforsch. Mikrosk. Anat.*, **143**, 53–70.

[7] T. T. Puck, personal communication quoted in ref. 1.

[8] *Corrigendum*. Change the last line of Fig. 4 in Cann and Hinman (1) to read: concentration ($n \cong 1.4$; $C_{1/2} \cong 1$ mM).