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Interaction of Chlorpromazine with Brain Microtubule Subunit Protein

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SUMMARY

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Chlorpromazine at concentrations which approximate apparent physiological concentrations interacts reversibly with brain microtubule subunit protein *in vitro* and, in so doing, inhibits the rate of reassembly of microtubules and the binding of colchicine by the protein. It also causes dissassembly of microtubules formed in the absence of the drug. These results appear to provide a molecular explanation for inhibition by chlorpromazine of fast axonal transport of proteins *in vitro* in frog sciatic nerve, and provide a fresh clue as to the primary mechanism for the psychotropic effect of this drug.

INTRODUCTION

Since its introduction in 1954, the tranquilizing drug chlorpromazine [2-chloro-10-(3-dimethylaminopropyl)phenothiazine hydrochloride] has had a profound influence on medicine in general and on the treatment of mental illness in particular (1-3). Despite a vast literature the mechanism of action of this widely used drug is not understood, although it is well documented that phenothiazines produce changes in the surface properties of membranes (4-7). This communication reports the discovery of the interaction of CPZ[1] with microtubule subunit protein, tubulin.

Tubulin isolated from mammalian brain consists of two, nonidentical subunits (8) and has a molecular weight of about 120,000 (9). It specifically binds the antimitotic drug colchicine, whose mode of action *in vivo* is to depolymerize microtubules (10), and it repolymerizes into microtubules under pysiological conditions (11-13). The results described below demonstrate that CPZ interacts reversibly with tubulin and, in so doing, inhibits the binding of colchicine and the rate of repolymerization into microtubules.

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[1] The abbreviations used are: CPZ, chlorpromazine; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid.

MATERIALS AND METHODS

Microtubule reassembly. The conditions required for repolymerization of tubulin into microtubules *in vitro* have been reported (11-13). The reassembly process occurs maximally at 37° and pH 6.8, is inhibited by high concentrations of Ca^{++}, and is augmented by guanosine triphosphate and glycerol. In addition, reassembly may require the presence of "nucleating discs." Recently Olmsted and Borisy (14) have demonstrated that reassembly of microtubules in porcine brain extracts can be characterized by viscometry.

In our experiments viscometry was used to monitor reassembly of microtubules in a 30,000 × *g* supernatant fraction of mouse brain homogenate. The brains of adult mice (Texas Inbred, ICR) were homogenized in 1 volume of 10 mM imidazole HCl (pH 6.8), 1 mM EGTA (to chelate Ca^{++}), 0.5 mM $MgCl_2$, and 10% glycerol. Homogenization was carried out manually with a glass homogenizer fitted with a Teflon pestle, and the homogenate was centrifuged at 30,000 × *g* for 90 min at 0–4°. Supernatants prepared in this way will be referred to as 30,000 × *g* IMEG supernatants.

The IMEG supernatant contained 14.4–16.5 mg of protein per milliliter as determined by the method of Lowry *et al.* (15), using bovine serum albumin as a standard. Tubulin comprised about 14% of the total protein as estimated in the following manner. Samples of 30,000 × *g* and 100,000 × *g* IMEG supernatants from the same homogenate were analyzed for relative protein concentration and relative colchicine-binding capacity. The percentage of tubulin in the 100,000 × *g* supernatant was determined chromatographically by analysis of the protein elution profile from a DEAE-cellulose column (16), the tubulin having been tagged with [^{3}H]colchicine prior to application of the supernatant to the column. More than 93% of the protein applied to the column was recovered in the eluted fractions. Those fractions containing tubulin were identified by the label; the protein in these fractions was shown to be at least 95% tubulin by sodium dodecyl sulfate gel electrophoresis. The percentage of tubulin in the 30,000 × *g* supernatant (uncorrected for minor impurities in the eluted tubulin fractions and small over-all protein losses during chromatography) was calculated as the product of the percentage in the 100,000 × *g* supernatant and the relative colchicine-binding capacity, divided by the relative total protein concentration.

The reassembly process was initiated by the addition of 50 μl of 0.1 M GTP (Sigma, type II-S) to 1 ml of IMEG supernatant contained in a semimicro Cannon-Ubbelohde dilution viscometer (flow time for half-diluted IMEG buffer, about 100 sec) maintained at 37° in a constant-temperature water bath. In the inhibition experiments the aliquot of supernatant was incubated at 37° for 20–25 min with 10 μl of a freshly prepared solution of CPZ in water[2] prior to addition of GTP. Controls containing no CPZ were treated identically. (Incubation with CPZ for 90 min gave the same viscometric results within a small experimental error as incubation for 25 min, indicating that in these experiments the tubulin-CPZ binding reaction is complete and constant within the routine time of incubation.) Routinely, four viscometers were run simultaneously, one containing the control sample and the others containing test samples all derived from the same supernatant so as to be internally comparable. The increase in viscosity was followed as a function of time. The data are expressed as the fractional increase in viscosity,

$$F = \frac{\eta}{\eta^0} - 1$$

where η is the instantaneous viscosity and η^0 the viscosity immediately before addition of GTP, the value of η/η^0 being taken as the ratio of corresponding flow times through the viscometer capillary, neglecting any slight density change during reassembly.

As is evident from the work of Olmsted and Borisy (14), the kinetics of microtubule reassembly can be quite complex, depending upon experimental conditions. Under our conditions, however, a semilogarithmic plot of the quantity $1 - F$ against time t is approximately linear during the approach to maximum viscosity. Thus what is most certainly a complex sequence of bimolecular association reactions in the initiation and growth of microtubules is described empirically by a pseudo-first-order rate law. No doubt this description embodies hidden approximations and perhaps, experimental artifacts such as possible non-Newtonian flow and rupture of microtubules as a result

[2] These solutions contained no detectable (less than 1 μM) free radical form of CPZ as judged spectrophotometrically.

of shearing in the viscometer capillary. In order to gain some insight into the meaning of the empirical law, the kinetics of a model chain-polymerization reaction, represented by the following infinite sequence of stoichiometric equations, has been examined mathematically.

$$\begin{aligned} M_1 + M_1 &\xrightarrow{k_1} M_2 \\ M_1 + M_2 &\xrightarrow{k_2} M_3 \\ M_1 + M_3 &\xrightarrow{k_3} M_4 \\ &\vdots \\ M_1 + M_m &\xrightarrow{k_m} M_{m+1} \\ &\vdots \end{aligned} \qquad (1)$$

where M_1 designates a macromonomer such as a protein, M_m = m-mer, and k_m (milliliters per gram per second) = the specific rate constant for the nth equation. Given the assumption that all the k values are equal ($k_1 = k_2 = k_3 = \cdots = k$), it can be shown (17) that during the initial stages of the reaction, in which the concentrations of higher polymers are small compared to the concentrations of M_1, the rate law describing the time course of disappearance of monomer is

$$1 - \sum_{m=2}^{\infty} \phi_m = e^{-\lambda_0 t} \qquad (2)$$

where ϕ_m is the fraction of m-mer, $\lambda_0 = kc^0$ (sec^{-1}), and c^0 (grams per milliliter) is the initial concentration of monomer. While Eq. 2 is a pseudo-first-order rate law, we note that F is not equal to $\sum\phi_m$; i.e.,

$$F = \frac{\eta_w}{\eta^0} c^0 \bar{v} \sum_{m=2}^{\infty} (\nu_m - \nu_1)\phi_m \qquad (3)$$

where η_w is the viscosity of water, $\bar{v}$ = the partial specific volume of the protein, ν_m = the viscosity increment of the m-mer, and ν_1 = the viscosity increment of the monomer. Pursuing our analysis, we find that to the approximation that $\nu_m = m^2$ for rods (rigorously, $m^{1.81}$; see Eq. 20-8 and Fig. 19-8 in ref. 18, and let the ratio of length to diameter of M_1 equal 2:1 to 3:1),

$$\frac{d\ln(1 - F)}{dt} = -\lambda_0 G(\lambda_0 t) \qquad (4)$$

where $G(\lambda_0 t)$ is a monotonically increasing but relatively insensitive function whose value drifts less than 17% during 0.36 half-time of the reaction. When viewed in these terms alone, the empirical pseudo-first-order rate law is in part comprehensible.

If in our model system the rate of polymerization were to be inhibited by the binding of a small molecule to M_1, λ_0 in Eq. 4 would be replaced by

$$\lambda = \lambda_0(1 + KC^n)^{-1} \qquad (5)$$

where K [(moles per liter)$^{-n}$] is the equilibrium binding constant and C (moles per liter) is the equilbrium concentration of the small molecule. In Eq. 5 $n = 1$ for noncooperative binding to one or more identical sites on M_1, $n = N$ for highly cooperative binding to N sites, and $1 < n < N$ for moderately cooperative binding to N sites (19). Inhibition of the rate of polymerization can be quantitated in terms of the ratio λ/λ_0 and the relationship

$$\frac{1 - \lambda/\lambda_0}{\lambda/\lambda_0} = KC^n \qquad (6)$$

The value of n is given as the slop of the plot of $\ln[(1 - \lambda/\lambda_0)/\lambda/\lambda_0]$ against $\ln C$, which is a kinetic analogue of the Hill plot (19, 20).

With this as a guide, the inhibition of reassembly of microtubules by CPZ is characterized by the ratio k'/k'_0, where k' and k'_0 are the slopes of $\ln(1 - F)$ vs. t in the presence and absence of CPZ, respectively, and the quantity

$$X = \frac{1 - k'/k'_0}{k'/k'_0} \qquad (7)$$

Thus it becomes possible to construct a titration curve, k'/k'_0 vs. logarithm of the total concentration of added CPZ, [CPZ], and to determine a value of n as the slope of the plot of $\ln X$ vs. $\ln$ [CPZ]. Substitution of the total concentration of CPZ for its equilibrium concentration is justified by the fact that the concentration of tubulin in our experiments is of the order of 20 μM while the range of effective concentration of CPZ centers around a value of about 200 μM.

Assay for colchicine binding by tubulin.

The colchicine binding assay for tubulin was carried out essentially as described by Weisenberg *et al.* (9): 50-μl aliquots of 30,000 X*g* IMEG supernatant or a solution of purified tubulin (about 1 mg/ml) containing the desired concentration (routinely 24 μM) of [^{3}H]colchicine (New England Nuclear) were incubated at 37° for at least 90 min. The binding reaction was terminated with 2 ml of ice-cold 10 mM imidazole HCl, buffer, pH 6.8, containing 0.5 mM $MgCl_2$, in which the concentration of unlabeled colchicine was the same as in the incubated sample. The termination mixture was filtered by gravity through two Whatman DE-81 (2.5-cm) filter discs and washed under suction with 15 ml of the same buffer. The filters were homogenized in 2 ml of water and 10 ml of Triton-toluene-Permafluor (1:2:0.135) and counted at 27% efficiency. All assays were performed in duplicate.

Whereas most of the binding experiments were done as described above, in others the supernatants employed were prepared with either 10 mM imidazole HCl–1 mM EGTA–0.5 mM $MgCl_2$–10% glycerol (pH of supernatant, 7.35 ± 0.03) or 50 mM K_2HPO_4–1 mM EGTA–0.5 mM $MgCl_2$–10% glycerol (pH of supernatant, 7.49 ± 0.06) instead of the IMEG buffer. Such supernatants are referred to as 30,000 X*g* IMEGA and 30,000 X*g* PMEG supernatants, respectively.

Extraction of CPZ from reaction mixture. We have found that Parafilm M (American Can Company) has a high affinity for CPZ, thus providing a rather simple means of removing the drug from 30,000 X*g* supernatants. Routinely, 3 ml of supernatant containing CPZ were placed in a test tube packed with 50 cm^2 of Parafilm cut into small pieces. The tube was capped and gently rotated for 5 min in a 37° warm room, after which time the supernatant was transferred to a second tube containing fresh Parafilm for another 5 min of extraction. A total of six stages of extraction was employed. Control experiments on 0.5 mM CPZ in IMEG buffer showed that this was sufficient to remove more than 90% of the CPZ from solution.

Electron microscopy. We are grateful to Dr. Albert Vatter, Webb-Waring Institute and Department of Pathology, University of Colorado Medical Center, for his kindness in preparing electron micrographs. After addition of an equal volume of 2.5% glutaraldehyde to stabilize the specimen, the solution under examination was mixed with an equal volume of 1% aqueous uranyl acetate and applied to a collodion-coated grid, blotted, dried, and observed.

Other chemicals. CPZ was kindly supplied by Smith Kline & French Laboratories. Trimethylamine hydrochloride was obtained from Eastman. K & K Laboratories ethyldimethylamine was redistilled and its aqueous solution acidified with HCl.

RESULTS

Inhibition of microtubule reassembly by CPZ. Typically, addition of GTP to the 30,000 × *g* IMEG supernatant of mouse brain homogenate at 37° in the absence of CPZ causes a measurably slow 30–50% increase in viscosity (sometimes as high as 80% with young mice), which reaches its maximum value after about 45–90 min. The maximum level is stable for about 15 min, after which the viscosity slowly declines. The viscosity increase is almost completely inhibited by 0.5 mM CPZ, as is immediately apparent upon comparison of curves *a* and *b* in Fig. 1A. These experiments also show that colchicine causes a fairly rapid decline in the viscosity of both the control and the reaction mixture containing CPZ, indicating that the viscosity increase does in fact reflect reassembly of microtubules and that CPZ inhibits the reassembly process. Moreover, it has been found that addition of 0.3 mM CPZ to a control sample just prior to attainment of maximum viscosity causes a precipitous 60–70% drop in viscosity, which is indicative of disassembly of microtubules.

The possibility that CPZ might act somehow to labilize microtubules, making them more easily ruptured by shearing in the viscometer capillary, appears to be eliminated by the results of experiments in which a single viscosity measurement on both the control and the reaction mixture containing CPZ was made only after the

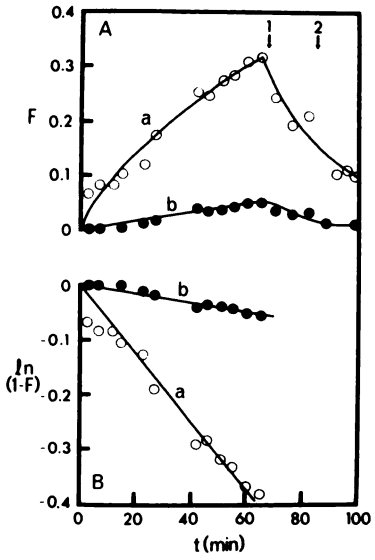

FIG. 1. *Inhibition of rate of reassembly of microtubules in 30,000 × g IMEG supernatant of mouse brain homogenate by CPZ as monitored by viscometry*

A. Plot of fractional increase in viscosity F initiated by addition of GTP at zero time, vs. time t. Curve *a*, control in the absence of CPZ; *b*, incubated with 0.5 mM CPZ for 15 min prior to initiation of reassembly with GTP. Point *1*, 190 μM colchicine added to both reaction mixtures; *2*, concentration of colchicine increased to 620 μM in both mixtures.

B. Semilogarithmic plot of $1 - F$ vs. t. Curve *a*, control in absence of CPZ; *b*, incubated with 0.5 mM CPZ prior to initiation of reassembly. The apparent induction period shown by curve *b* is not characteristic and was observed only once out of nine experiments with 0.5 mM CPZ and not at all at lower concentrations of the drug. Otherwise these results are reasonably representative.

viscosity of a second control had attained its maximum value. The inhibitory effect of CPZ was the same in these experiments as in those in which the viscosity increase was monitored continuously.[3]

Direct observations in the electron microscope support the conclusion drawn from viscosity measurements, that CPZ inhibits reassembly of microtubules. Thus examination of the 30,000 × *g* IMEG supernatant showed a complete absence of microtubules (Fig. 2a), while the supernatant incubated in the presence of GTP at 37° until reaching maximum viscosity showed an abundance of microtubules (Fig. 2b). In contrast, only an occasional microtubule was found in a supernatant which had been incubated for 30 min in the presence of 0.5 mM CPZ prior to incubation with GTP.

It is highly unlikely that the inhibitory effect of CPZ is due to a secondary effect on GTP, since the nucleotide concentration is 10 times higher than the drug concentration, which in turn is about 50 times lower than its critical micelle concentration in 50 mM NaCl (21). The remote possibility that inhibition might be due to release of Ca^{++} from membrane constituents or other components of the supernatant fraction was eliminated by increasing the concentration of EGTA in IMEG buffer from 1 to 10 mM without effect on the ability of CPZ to inhibit reassembly. A 30,000 × *g* supernatant prepared in the absence of any added Mg^{++} (homogenizing buffer, IMEG from which Mg^{++} was deleted) contains only 1.08 mM endogenous Mg^{++} (by atomic absorption spectroscopy, courtesy of Dr. Ernest B. Reeve, Department of Medicine, University of Colorado Medical Center). Thus the supernatant prepared with IMEG buffer has a maximum Mg^{++} concentration of 1.3 mM. Olmsted and Borisy (Fig. 10 in ref. 14) reported that exogenous addition of Mg^{++} to brain homogenate, which may lead to even higher levels of free Mg^{++} than our total Mg^{++} concentration, has only a minor inhibitory effect on the rate of repolymerization. It is highly improbable, therefore, that the effect of CPZ is due to release of Mg^{++}. Nor did the pH (6.8) drift during reassembly in the presence or absence of the drug. Furthermore, as will be seen shortly, CPZ also inhibits colchicine binding by the 30,000 × *g* supernatant and by purified tubulin, and does so in the absence of GTP. The conclusion seems justified, therefore, that the inhibitory ef-

[3] Although the ratio of maximum viscosity shown by the control to the viscosity of the sample containing CPZ was the same whether or not the viscosity increase was monitored continuously, the absolute viscosity values were about twice as large when only a single measurement was made. This result clearly implicates some rupture of microtubules as a result of shearing during repeated passage through the viscometer capillary.

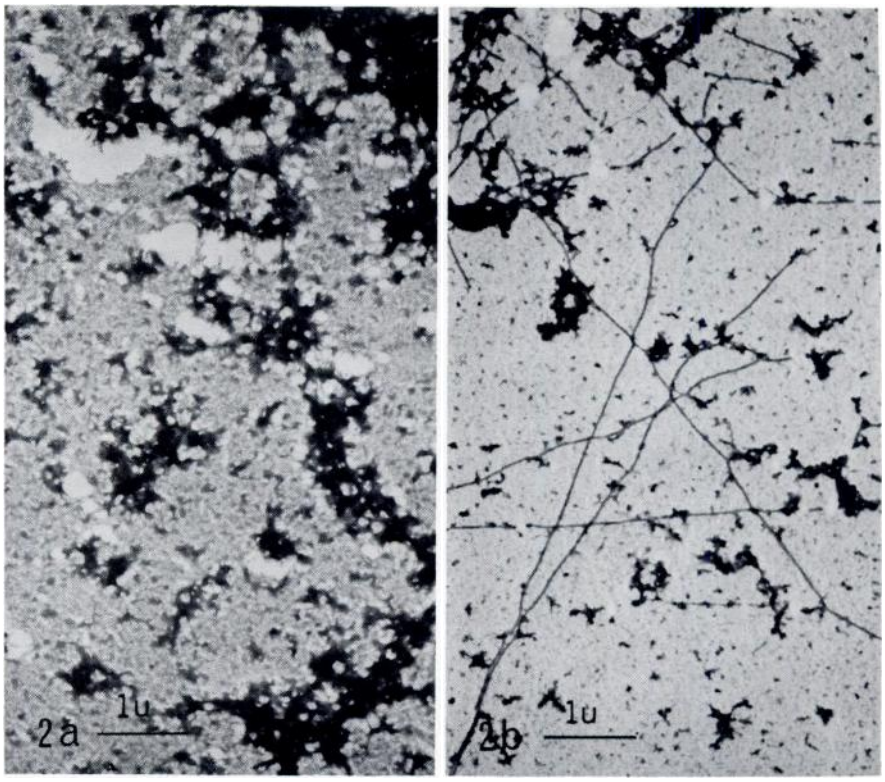

FIG. 2. *Electron micrographs of 30,000 × g IMEG supernatant of mouse brain homogenate (a) and 30,000 × g supernatant after incubation in the presence of GTP until reaching maximum viscosity (b), showing microtubules 240–300 A in diameter × 12,000.*

fect of CPZ on the reassembly of microtubules is due to direct interaction of the drug with microtubule subunit protein.

Moreover, the interaction is reversible, as demonstrated by the following experiment, made in triplicate. A 3-ml aliquot of 30,000 × *g* IMEG supernatant was incubated at 37° for 30 min with 0.5 mM CPZ, after which time the drug was extracted from the mixture with Parafilm. A control, containing no drug, was treated in an identical fashion. A second control, containing no drug, was incubated at 37° for 30 min but was not put through the extraction procedure. Upon subsequent initiation of the reassembly process with GTP, both the rate of increase and the maximum increase in viscosity were the same, within a small experimental error, for all three samples.

As for the specificity of the interaction, neither trimethylamine HCl nor ethyldimethylamine HCl at 0.5 mM had any significant effect on the reassembly process. Thus it can be concluded that the inhibitory action of CPZ specifically involves the phenothiazine moiety and is not due simply to the binding of a tertiary amine to tubulin.

In order to construct a titration curve for CPZ inhibition of the rate of reassembly, values of k'/k'_0 were derived from the slopes of semilogarithmic plots of $1\text{-}F$ vs. t, such as those illustrated in Fig. 1B. The titration curve, which is displayed in Fig. 3A as a plot of k'/k'_0 vs. the logarithm of CPZ concentration, extends over an approximately 30-fold range of CPZ concentration, 1–0.03 mM. Since preliminary experiments showed that a sufficiently high concentration of CPZ causes precipitation of protein from the 30,000 × *g* IMEG supernatant, all reaction mixtures used to establish the curve were carefully exam-

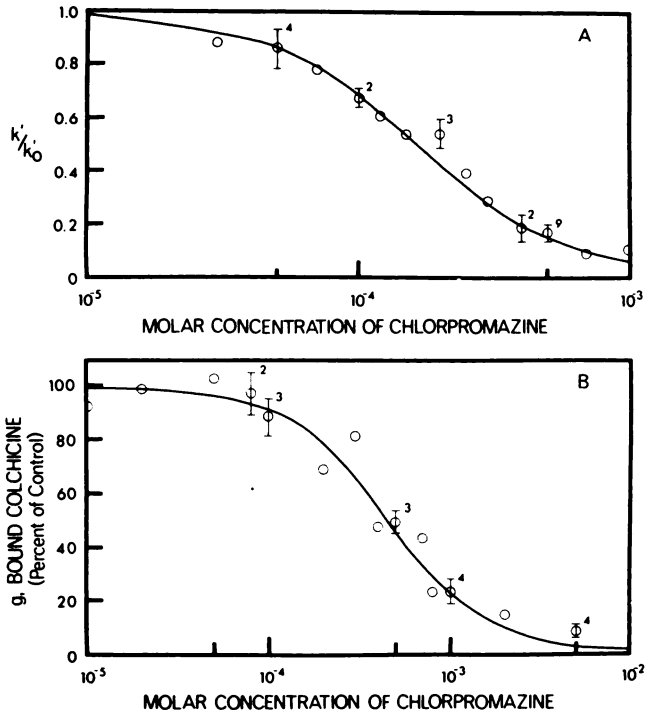

FIG. 3. *Titration curves for CPZ inhibition of rate of reassembly of microtubules in 30,000 × g IMEG supernatant of mouse brain homogenate (A) and colchicine binding by the same supernatant (B)*

Error bars denote mean deviations, and their superscripts, the number of observations averaged. The curves are theoretical ones computed from the Ising model as described in the text.

ined for incipient precipitation, which proved not to be a complication. Thus concentrations less than 0.5 mM did not produce the slightest Tyndall effect, 0.5 mM gave a barely perceptible Tyndall effect, and 1 mM, only a moderate one. The midpoint concentration of CPZ, at which the rate of reassembly is inhibited by 50% is 0.16 mM, and 0.5 mM causes about 85% inhibition. At the other extreme, as little as 30 μM CPZ still has a significant inhibitory effect.

The kinetic analogue of the Hill plot of these data is presented in Fig. 4A. The slope of the line, fitted by the method of least squares, is 1.36 ± 0.047. The fact that the slope is significantly greater than unity suggests cooperative binding to two sites. In order to test this idea, appeal was made to the statistical-mechanical Ising model of nearest-neighbor interactions, which has been applied with success to a number of biological systems, including the cooperative binding of oxygen to hemoglobin (20). For the case of cooperative binding of a small ligand molecule to two sites on a macromolecule the Ising model admits the following expression for the binding isotherm (Eqs. 3.8 and 3.9 in ref. 20 with the assignment $n = 2$),

$$f(\alpha) = \frac{N}{n} = \frac{\alpha(\alpha + e^{-4U}}{1 + \alpha(\alpha + 2e^{-4U})} \quad (8)$$

where N/n is the average fraction of sites occupied by ligand, α is the concentration of ligand in units such that half-saturation occurs at concentration unity, and U is the interaction parameter. The interaction is attractive when $U > 0$, as in the case of hemoglobin, and repulsive when $U < 0$. When $U = 0$ Eq. 8 reduces to the binding isotherm for independent binding to one or more identical sites (19). For inhibition of the rate of reassembly of microtubules by CPZ, the theoretical titration curve is a

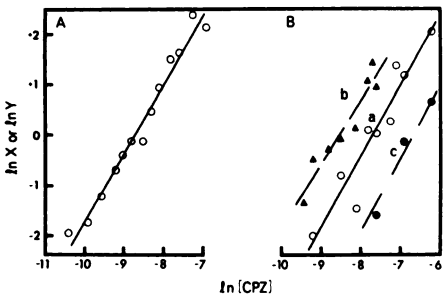

FIG. 4. *Hill plots of titration data*

A. CPZ inhibition of reassembly of microtubules in the 30,000 × *g* IMEG supernatant of mouse brain homogenate (n = 1.36 ± 0.047; midpoint concentration, $c_{1/2}$ = 1.6 × 10^{-4} M). B. CPZ inhibition of colchicine binding. Line *a*, 30,000 × *g* IMEG supernatant of mouse brain homogenate (n = 1.4 ± 0.12; $c_{1/2}$ = 4.6 × 10^{-4} M); *b* (- - -), purified tubulin prepared by DEAE-cellulose chromatography (16) in half-strength IMEG buffer, the buffer used to elute the tubulin from the chromatographic column having been exchanged for half-strength IMEG buffer on Sephadex G-25 (n = 1.3 ± 0.12; $c_{1/2} \cong 2 \times 10^{-4}$ M); *c* (- - -), purified tubulin prepared by affinity chromatography (22) in the elution buffer [0.01 M imidazole HCl (pH 6.8), 5 mM $MgCl_2$, 1 mM GTP, 0.1 M NaCl, and 10% glycerol], with duplicate measurements at each CPZ concentration ($n \cong 1.14$; $c_{1/2} \cong 1$ mM).

plot of $1 - f(\alpha)$ against the logarithm of drug concentration. The curve passing through the data points in Fig. 3A is the theoretical one calculated for moderately strong interaction, U = 0.35. In view of the goodness of fit, we favor interpretation of these data in terms of moderately cooperative binding of CPZ to two sites on the tubulin molecule.

Inhibition of colchicine binding by CPZ. In addition to inhibiting the reassembly of microtubules, CPZ also inhibits the binding of colchicine to microtubule subunit protein, and it does so over about the same range of drug concentration. Inhibition of colchicine binding likewise specifically involves the phenothiazine moiety, as witnessed by the fact that neither trimethylamine HCl nor ethyldimethylamine HCl inhibits colchicine binding when used at the same concentration, 0.5 mM, at which CPZ causes 50% inhibition.

Inhibition was demonstrated by colchicine binding assays on aliquots of 30,000 × *g* supernatants of mouse brain homogenates. Except for those experiments presented in Fig. 5, IMEG supernatants were used routinely. The supernatants were first incubated at 37° with various concentrations of CPZ. In the experiments described below the degree of inhibition increased with time of prior incubation until about 90 min, after which it remained essentially constant. Unless stated otherwise, prior incubation time was 90 min.[4] In order to correct for the natural decay of the colchicine-binding activity of the 30,000 × *g* supernatant with time (9), control samples containing no CPZ were also previously incubated for 90 min, and inhibition by CPZ is expressed relative to this control.

Although 24 μM colchicine was used routinely in the binding assays, it was found that the degree of CPZ inhibition was independent of colchicine concentration over the 500-fold range from 2.4 μM to 1 mM (Table 1). This finding indicates that inhibition is not an indirect effect, such as complex formation between CPZ and colchicine, but rather is due to direct interaction of CPZ with microtubule subunit protein, which in turn is not reversed by colchicine under our experimental conditions.

In contrast, it was found (Table 2) that CPZ causes partial reversal of the binding of colchicine by the subunit protein. This result provides additional evidence that the inhibitory effect of CPZ is due to direct interaction with tubulin.

These findings can be understood formally in terms of the reaction

$$T \cdot COL + CPZ \rightleftharpoons T \cdot CPZ + COL \quad (9)$$

where COL designates colchicine and T,

[4] On other occasions the rate of attainment of equilibrium with IMEG supernatants was about twice as slow. The reason for this is poorly understood, although we have eliminated impurities in our deionized water supply, variations between lots of imidazole, etc. We note, however, that the rate of reaction is highly pH-dependent: at pH 6.5 the reaction does not proceed at a measurable rate, and the rate increases by a factor of about 2 on changing the pH of the reaction mixture from 6.8 to 7.4. It may be that sometimes the buffering capacity of IMEG is insufficient to offset completely the strong buffering capacity of the protein solution at about pH 7.3.

TABLE 1

Noncompetitive nature of CPZ inhibition of colchicine binding

Aliquots of the 30,000 × *g* IMEG supernatant of mouse brain homogenate were incubated for 90 min with 0.5 mM CPZ and then assayed for colchicine binding. The assay mixtures contained varying concentrations of colchicine, and were incubated for 30 min at 37°. Controls were treated identically except for the absence of CPZ.

Colchicine concentration in assay mixture	Colchicine bound relative to control
μM	%
2.4	47 ± 8
24	52 ± 3
240	45 ± 2
1000	59 ± 7

TABLE 2

Release of tubulin-bound colchicine by CPZ

Duplicate samples of the 30,000 × *g* IMEG supernatant of mouse brain homogenate were incubated with 20 μM [^{3}H]colchicine for 30–115 min at 37° and then flooded with 200 μM unlabeled colchicine to prevent further significant binding of [^{3}H]colchicine, following which they were incubated with CPZ for 90 min at 37°. Controls were treated identically except for the absence of CPZ. The samples and controls were then assayed for bound [^{3}H]colchicine. The experiment was performed in triplicate. In one experiment the time course of release of colchicine was followed to establish that the reaction was complete within 90 min.

CPZ concentration	Bound [^{3}H]colchicine relative to control
mM	%
0.5	85 ± 4
0.9	70 ± 4

tubulin. The results presented in Table 2 give a provisional value of about 0.1 for the equilibrium constant of reaction 9, which indicates that the binding of colchicine by tubulin is an order of magnitude stronger than the binding of CPZ. In that case inhibition of colchicine binding by CPZ should be highly sensitive to the concentration of colchicine in the assay mixture; i.e., inhibition should be competitive. As shown in Table 1, however, inhibition is within experimental error independent of colchicine concentration. In other words, inhibition is actually noncompetitive. This means that reaction 9 is not in dynamic equilibrium, and that, for whatever reason, the reverse reaction does not take place.

The question immediately arises whether the interaction of CPZ with tubulin is reversible via mass action. That this is, in fact, the case is documented by Fig. 5A and its legend.

Together these results allow generation of a titration curve for CPZ inhibition of colchicine binding by plotting bound colchicine, expressed as a percentage of control, *g*, against the logarithm of the concentration of CPZ (Fig. 3B). The midpoint concentration of CPZ is 0.46 mM. Once again it should be emphasized that interpretation of the titration curve is free from complications due to precipitation of protein, which occurs only at a CPZ concentration of 5 mM or greater.

Hill plots of colchicine inhibition data, ln *Y* vs. ln [CPZ], where

$$Y = \frac{100 - g}{g} \qquad (10)$$

are presented in Fig. 4B, line *a*, for the IMEG supernatant and in Fig. 5B for the PMEG supernatant. The slope of the plot for the IMEG supernatant is 1.4 ± 0.12, which is the same, within experimental error, as the value obtained for inhibition of the reassembly of microtubules and is subject to the same interpretation. In fact, the curve passing through the data points in Fig. 3B is the theoretical titration curve [$1 - f(\alpha)$ vs. log [CPZ], Eq. 8] calculated from the Ising model for moderately cooperative binding of CPZ to two sites on the tubulin molecule, using the same value of the interaction parameter *U* employed to fit the data on inhibition of reassembly. Thus the only significant difference between the titration curves for inhibition of colchicine binding and for inhibition of microtubule reassembly is in the midpoint concentrations of CPZ, 0.46 mM and 0.16 mM, respectively. This difference is to a large extent explicable in terms of the critical concentration of tubulin which Olmsted and Borisy (14) found to be re-

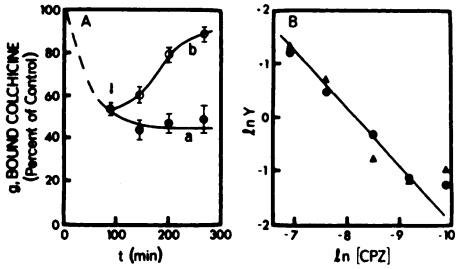

FIG. 5. *Inhibition of colchicine binding by 30,000 × g PMEG supernatant of mouse brain homogenate by CPZ*

A. Time course of inhibition by 0.5 mM CPZ (curve *a*) and of reversal of inhibition (curve *b*) after removal of CPZ by adsorption on Parafilm M at the time indicated by vertical arrow; average of three experiments. Reversal of inhibition was also demonstrated with IMEGA supernatants. In one experiment 80% of activity was recovered 250 min after removal of CPZ; in another 80% recovery was achieved in 390 min.

B. Hill plot of CPZ inhibition of colchicine binding by PMEG supernatant: ▲, after 150 min of incubation with CPZ; ●, after 210 min. These data are from one of three such sets of experiments, which together give $n = 0.98 \pm 0.058$ and a midpoint concentration of CPZ equal to $(3 \pm 1) \times 10^{-4}$ M.

quired for reassembly. Below the critical concentration c_c reassembly does not occur to any appreciable extent, and above it the maximum extent of reassembly is approximately linear in total tubulin concentration c_t. As a consequence it is to be expected that the titration curve for inhibition of reassembly would be shifted to lower CPZ concentrations relative to inhibition of colchicine binding. This is so because the fraction f of tubulin complexed with CPZ at the midpoint of the titration curve for reassembly is less than 0.5, depending upon the value of the ratio c_c/c_t; i.e., to a first approximation

$$f = 0.5 \left(1 - \frac{c_c}{c_t}\right) \qquad (11)$$

Reassembly experiments on diluted supernatant indicate that under our experimental conditions $c_c/c_t \cong 0.5$, so that $f \cong 0.25$. This value of f is conceptually equivalent to $g = 75$ in the case of inhibition of colchicine binding. Referring to Fig. 3B, the corresponding concentration of CPZ is about 0.23 mM, which is to be compared with the observed midpoint concentration for reassembly.

The slope of the Hill plot for PMEG supernatant is 0.98 ± 0.058. Clearly an unambiguous interpretation of the different values of n for IMEG and PMEG supernatants must await the results of direct binding experiments with purified tubulin, which are now in progress.

The results of other experiments with purified tubulin remove any doubt that CPZ inhibits colchicine binding by interacting directly with the microtubule subunit protein in the 30,000 × *g* supernatants. Measurements have been made in two different imidazole buffer systems whose ionic strengths were judged to encompass the ionic strength of the 30,000 × *g* supernatant. The results (broken lines *b* and *c* in Fig. 4B) show that CPZ does in fact inhibit colchicine binding by purified tubulin, the midpoint concentrations of CPZ bracketing the midpoint concentration of the 30,000 × *g* IMEG supernatant.

The experiments on purified tubulin are unencumbered by nonspecific proteins and other materials which might also bind CPZ. Still, the two Hill plots (Fig. 4B) together encompass about the same range of total CPZ concentration as in the case of the supernatant, which is a mixture. Moreover, their slopes are comparable in value to the slope shown by the supernatant. These results lend confidence to our analysis of the data obtained on the supernatant, which makes the assumption that the concentration of unbound CPZ can be approximated by the total concentration of CPZ.

DISCUSSION

It is apparent from the foregoing results that the effects of CPZ and colchicine on the microtubule system of brain *in vitro* have certain features in common. Both drugs inhibit reassembly of microtubules in brain extracts and cause their disassembly, most probably by direct interaction with microtubule subunit protein. In fact, both interact with purified tubulin. Interaction of CPZ with tubulin inhibits the binding of colchicine noncompetitively.

This observation allows construction of a titration curve for inhibition of colchicine binding by CPZ. The resulting curve strongly overlaps the titration curve for inhibition of reassembly of microtubules.

The inhibitory effect of CPZ on both the reassembly of microtubules and the binding of colchicine by tubulin is reversible. It is thus clear that CPZ does not act by increasing the rate of the natural decay of these two activities of tubulin (9, 14).

Consistent with our observations, it has been found that in tissue culture "CPZ in concentrations of 10^{-5} M to 5×10^{-6} M arrests cells in mitosis in a fashion similar to that of colcemide. This mitotic arrest is complicated by other toxic action which prevents cells from entering mitosis after approximately 2 hours. CPZ in the same concentration range also resembles the action of colcemide in disorganizing the organized microtubular structure produced by cyclic AMP. The cell-stretching produced by the latter compound tends to be reversed and the characteristic knobs, which develop at the cell surface when colcemide or other agents disrupt the microtubular organization produced by cyclic AMP, are produced by the addition of CPZ."[5]

The importance of microtubules in brain function is becoming increasingly apparent, particularly their role in the maintenance or establishment of cell structure and their involvement in axoplasmic transport (23). It is of considerable interest that a recent study (24) demonstrated that CPZ inhibits the fast axonal transport of labeled proteins *in vitro* in frog sciatic nerve. Transport is inhibited 50% by 0.1 mM and almost 100% by 0.5 mM CPZ. Inhibition by 0.1 mM CPZ is reversible. Furthermore, the number of microtubules in the cell is generally decreased and the number of neurofilaments increased at these drug concentrations, which have negligible or small effects on protein synthesis and oxidative phosphorylation. We believe that our results may provide a molecular explanation of these observations. It is particularly striking that in our experiments CPZ caused 50% reversible inhibition of the reassembly of microtubules at a concentration of 0.16 mM (33% at 0.1 mM) and about 85% inhibition at 0.5 mM (Fig. 3A).

Although the concentrations at which CPZ exerts its effects on the properties of tubulin *in vitro* approximate apparent physiological concentrations based on extracellular space and average therapeutic dosages, the crucial consideration is the tissue localization of the drug. While there is consensus that CPZ accumulates in adrenals, kidney, liver, and lung, discrepancies exist in the literature concerning localization in the brain. Some papers report high levels in brain; others report brain to plasma ratios close to 1.0. [For a review and literature citations bearing on this problem, see Gordon (25), De Jaramillo and Guth (26), and Mahju and Maickel (27).] According to the latter two groups of investigators, these discrepancies are probably explicable in terms of the diversity of techniques, experimental design, and species used. In particular, sampling times are evidently critical; Mahju and Maickel (27) reported that CPZ localizes in the brain shortly after administration of the drug and then decays with a half-time of about 7 hr. Their studies of brain and plasma levels after a single dose to rats (intraperitoneal administration of dosages selected on the basis of similarity to clinical dose and lack of toxicity) show clear localization in brain, with a brain to plasma ratio of 6.15, 1 hr after dosage, and a brain level of 15.50 ± 1.30 μg/g (50 μmoles/kg). Moreover, after administration of five doses at 12-hr intervals the drug continued to accumulate in brain with each successive dose, with the brain to plasma ratio reaching 10.6 and the brain level, 24.01 ± 2.01 μg/g (80 μmoles/kg). In their kinetic studies on the uptake of CPZ by various anatomical regions of the brain of the dog, De Jaramillo and Guth (26) found transient levels as high as 210 ± 42 μg/g (700 μmoles/kg) in some regions. These brain concentrations are comparable to the concentrations in our experiments, but it must be borne in mind that *in vivo* the drug is undoubtedly partitioned into membranes (7) and other cellular compo-

[5] T. T. Puck, personal communication.

nents (25), and by and large is not free as such.

With due cognizance of these physiological considerations, our findings, together with the biological experiments *in vitro* discussed above, provide a fresh clue to the primary mechanism of the psychotropic action of CPZ. Without implying that CPZ might cause a collapse of the microtubule system *in vivo*, it is conceivable that the drug could, through direct interaction, play a modulating role in tubulin action, such as the dynamics of assembly and maintenance of the functional integrity of microtubules that occurs continually in the neurons as well as other cells. Such a mechanism would be consistent with the observation that CPZ inhibits fast axonal transport in the hypothalamo-neurohypophysial system of the rat without changing the number and distribution of microtubules (28).

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