MOLECULAR PHARMACOLOGY, 19: 295–301

A Comparative Study of the Interaction of Chlorpromazine, Trifluoperazine, and Promethazine with Mouse Brain Tubulin

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Received August 6, 1980; Accepted November 3, 1980

SUMMARY

APPU RAO, A. G., AND J. R. CANN. A comparative study of the interaction of chlorpromazine, trifluoperazine, and promethazine with mouse brain tubulin. *Mol. Pharmacol.* **19**:295–301 (1981).

The comparative interaction of chlorpromazine, trifluoperazine, and promethazine with mouse brain tubulin has been examined via their effects on the assembly of tubulin *in vitro*, the circular dichroism of tubulin under nonpolymerizing conditions, and the fluorescence intensity of the protein. Of the two tranquilizing drugs, the more potent trifluoperazine inhibits assembly more strongly than does chlorpromazine, whereas the nontranquilizing drug promethazine does not inhibit but instead enhances assembly somewhat. Likewise, trifluoperazine has a stronger effect on the far-ultraviolet circular dichroic spectrum than does chlorpromazine, whereas promethazine is without effect. Thus, there are two separate correlations between biophysical measurements and the clinical potencies of these three phenothiazines. The fluorometric measurements, in turn, correlate in a provocative way with the assembly and circular dichroism results. The possible biological significance of these findings is discussed.

INTRODUCTION

The tranquilizing CPZ[1] interacts reversibly *in vitro* with the microtubule subunit protein tubulin. The interaction has been demonstrated and partially characterized by inhibition of the binding of colchicine and the reassembly of microtubules (1, 2), direct binding measurements (3), and changes in the secondary structure of the protein as probed by CD (4).

In tissue culture, CPZ arrests cells in mitosis and disorganizes the organized microtubule structure produced by cyclic AMP.[2] It causes a reduction in the number of microtubules in spinal ganglion cells (5, 6) and neuroblastoma cells (7) *in vitro*, and interferes with axoplasmic transport *in vivo* (8) as well as *in vitro* (5), a process requiring an organized microtubular structure (9, 10).

This work was supported in part by Research Grant 5 R01 HL 13909-29 from the National Heart, Lung and Blood Institute, National Institutes of Health, United States Public Health Service. This publication is No. 734 from the Department of Biophysics and Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262.

[1] The abbreviations used are: CPZ, chlorpromazine hydrochloride, 2-chloro-10-(3-dimethylaminopropyl)-phenothiazine hydrochloride; TFP, trifluoperazine dihydrochloride, 2-trifluoromethyl-10-[3′-(1-methyl-4-piperazinyl)propyl]-phenothiazine dihydrochloride; PMZ, promethazine hydrochloride, 10-(2-dimethylaminopropyl)-phenothiazine hydrochloride; MES, 2(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

[2] T. T. Puck, personal communication as quoted by Cann and Hinman (1).

These several observations lead us to believe that the interaction of CPZ with tubulin provides a clue to the primary molecular mechanism of its psychotropic action and may also have a bearing on its side effects. To test the plausibility of this idea we have compared the interaction with tubulin of three phenothiazines with different clinical potencies: CPZ; TFP, which is approximately 1 order of magnitude more potent clinically as a tranquilizing drug than is CPZ (11); and PMZ, which is not a tranquilizer but an antihistamine (11, 12). The interaction was monitored by assembly of tubulin *in vitro*, CD, and fluorescence quenching, along with subsidiary velocity sedimentation experiments.

MATERIALS AND METHODS

Preparation and determination of tubulin. Mouse brain tubulin was prepared by a modification of the DEAE-cellulose chromatographic procedure of Eipper (13). The protocol and characterization of the purified tubulin are the same as those described in detail previously (4). Suffice it to repeat that tubulin represents approximately 98% of the protein in the preparations by the criterion of sodium dodecyl sulfate-discontinuous acrylamide gel electrophoresis, which showed no evidence of microtubule-associated proteins even in overloaded gels. A fresh stock of tubulin was prepared for each day's experimentation; tubulin concentrations were determined spectrophotometrically using $E_{280}^{0.1\%} = 1.15$.

Other materials. CPZ and TFP were kindly supplied

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by Smith Kline & French Laboratories, Philadelphia, Pa., and PMZ by Wyeth Laboratories, Inc., Philadelphia, Pa. DEAE-cellulose (DE52) was a product of Whatman Biochemicals, Ltd., London, England; Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Ultrapure sucrose was obtained from Schwarz/Mann, Orangeburg, N. Y.; and MES, GTP grade II sodium salt, EGTA, and crystalline tetrasodium pyrophosphate were obtained from Sigma Chemical Company, St. Louis, Mo. All other chemicals were of reagent grade.

Assembly of tubulin. Assembly of tubulin *in vitro* in sucrose medium (14) was followed by the turbidimetric method of Gaskin *et al.* (15). Turbidity measurements were made at 350 nm on a Beckman Model 25 recording spectrophotometer fitted with a temperature control accessory, employing 1-cm path length semimicro cells.

Approximately 20 ml of freshly prepared tubulin solution were brought to 5 mM EGTA, 0.2 mM GTP, and 1 M sucrose by the addition of solids and then concentrated to approximately 5 mg of protein per milliliter by ultrafiltration through a Diaflow P-30 membrane. About 2 ml of the concentrated solution were passed through a Sephadex G-25 column equilibrated with 0.06 M MES buffer, pH 6.8, plus 1 M sucrose in order to exchange the supporting electrolytes. The effluent protein solution was reconcentrated to 4.5 ± 0.1 mg/ml, and the sucrose concentration was adjusted to 1.5 M by the addition of solid. All of these operations were carried out at 4°, and the tubulin solution was stored in an ice bath.

An aliquot of the tubulin solution was incubated with phenothiazine at 37° for 5 min (same protein concentration without drug for the control), after which appropriately small volumes of solutions of $MgCl_2$, EGTA, and GTP were added to give the final composition 0.055 M MES, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM GTP, 1.25 M sucrose (confirmed by refractometry), and protein, 3 mg/ml. The sample was rapidly transferred to the spectrophotometer cell and the absorbance at 37° ± 0.1° was recorded as a function of time (less than 1 min required for temperature equilibration; blank, 0.055 M MES containing 1.25 M sucrose and drug at the concentration being treated; chart speed 0.5 inch/min). The increase in absorbance reached a plateau within 15–20 min. The reversibility of assembly was checked in the usual fashion by cooling the solution at the end of 26 min to 0° for 0.5–2 hr and recording the absorbance at ambient temperature. The percentage of reversibility was calculated as described by Gaskin *et al.* (15), and the percentage of polymerization activity of the sample was calculated as 100 times the product of the ratio of the change in absorbance of the sample after 26 min to that of the control by the ratio of reversibility of the sample to the control.

Assembly was conducted in 1.25 M sucrose rather than the usual concentration of 1 M (14, 16) in order to shorten the lag period without sacrificing reversibility; 3.4 M glycerol was not used because a pilot experiment indicated that in this medium CPZ can cause extensive irreversible, presumably nonspecific, aggregation of tubulin. Because of the use of sucrose, the tubulin fibers formed in our experiments may not be true microtubules as suggested by Timasheff *et al.* (16). Accordingly, we adopt the notation "assembly of tubulin" and use "assembly" and "polymerization" interchangeably.

Physical methods. Velocity sedimentation was carried out with a 12-mm double-sector cell in a Spinco Model E ultracentrifuge operating at 60,000 rpm at 27°. CD spectra were recorded on a Cary Model 60 spectropolarimeter with a Model 6001 CD attachment, fitted with a thermostatable cell holder calibrated with a Thermistor probe. Slits were programmed to yield a 15 A bandwidth at each wavelength. Mean residue ellipticities $[\theta]_{mrw}$ (deg cm^2)/dmole, were calculated in the usual fashion using a value of 115 for the mean residue weight. The sole departure from our previous protocols (4) is a slight difference in buffer composition, the concentrations of buffer components and NaCl being 10% less than before in order to accommodate the relatively limited solubility of TFP as compared with that of CPZ at high salt concentrations. Control experiments showed that this difference has no significant effect on the CD and causes an increase of less than 2% in the uncorrected sedimentation coefficient.

Fluorescence measurements were made on a Farrand Mark 1 spectrofluorometer. Early measurements were at ambient temperature, 27° ± 0.5° as determined by a Thermistor probe inserted into the cuvette immediately after reading the fluorescence intensity. Later on, the temperature was regulated at 27° or 10° using a constant-temperature cell holder connected to a constant-temperature (±0.02°) circulating water bath. The experimental protocol was as follows: Tubulin, concentrated to 5–6 mg/ml in 0.2 mM GTP and 1 M sucrose by ultrafiltration, was passed through a Sephadex G-25 column equilibrated with 0.05 M MES buffer, pH 6.8, followed by centrifugation at 100,000 × *g* for 1 hr to remove any turbidity. Except as noted below, measurements of protein fluorescence were made on 0.2 mg of tubulin per milliliter (1.8 μM) in the absence and presence of phenothiazine at an excitation wavelength of 290 nm and an emission wavelength of 342 nm.[3] The percentage quenching of the fluorescence intensity of the protein by drug was corrected empirically for internal absorption and filtration by subtracting the percentage quenching by the same concentration of drug of the fluorescence of a tryptophan solution, equivalent in absorption to tubulin at 290 nm. The fluorescence intensity of the tubulin controls in the absence of drug did not change during the course of the experiment.

Quenching as a function of drug concentration was analyzed in terms of binding of the drug by tubulin using established procedures (17–19). Thus, if it is assumed that the binding of each drug molecule causes the same degree of quenching and that binding is statistical (see Appendix), the intrinsic drug-binding constant, K, is given by the equation (19)

$$K = \frac{\beta}{1-\beta}\cdot\frac{1}{C_f} \qquad (1)$$

where $\beta = Q/Q_{max}$ and $C_f = C - n\beta\bar{T}$, in which Q is the

[3] The fluorescence maximum of tubulin is at 332 nm. The first absorption band of CPZ, TFP, and PMZ overlaps the emission band of the protein, the wavelengths of maximal absorbance being 306, 308, and 298 nm, respectively. All three drugs show a weak emission spectrum with maxima at 455, 476, and 442 nm, respectively.

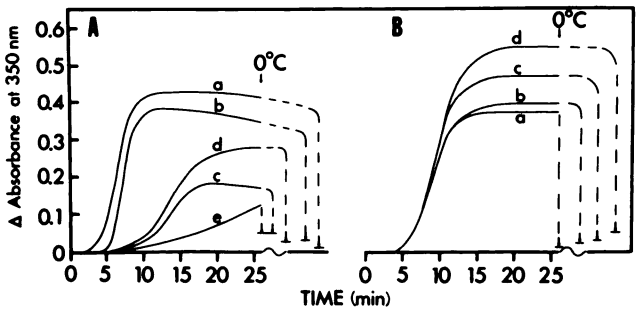

FIG. 1. *Effect of CPZ, TFP, and PMZ on the time-course and extent of assembly of tubulin at 37° as measured turbidimetrically (——)*

The polymerization mixtures were cooled to 0° at the end of 26 min as indicated; - - -, Reversal of assembly (no kinetic significance) to the indicated Δ absorbance levels. Data points were read off the continuous recording of absorbance at 1- to 2-min intervals. Initial concentration of tubulin, 3 mg ml^{-1}; critical concentration, 1 ± 0.2 mg ml^{-1}.

A. Effect of CPZ and TFP: curve *a*, control containing no drug; *b*, concentration of CPZ in polymerization mixture, 100 μM; *c*, 300 μM CPZ; *d*, 80 μM TFP; *e*, 120 μM TFP.

B. Effect of PMZ: *a*, control; *b*, 100 μM PMZ; *c*, 300 μM; *d*, 500 μM.

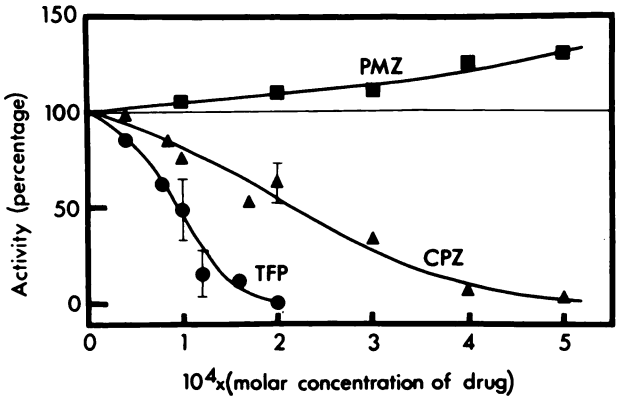

FIG. 2. *Effect of phenothiazines on the assembly of tubulin: plot of polymerization activity against the concentration of drug in the polymerization mixture*

All but a few of the data points are average values of two to six determinations, the error bars indicating ± mean deviation; where not shown, the error bars are the size of the symbols or smaller.

corrected percentage quenching; Q_{max}, the maximal quenching; C_f, the molar equilibrium concentration of unbound drug; C, the molar constituent concentration of drug; T, the molar constituent concentration of tubulin; and n, the binding stoichiometry. The value of K is given by the slope of a plot of $\beta/1-\beta$ against C_f. Q_{max} was determined by extrapolation of a plot of $1/Q$ against $1/C$ to $1/C = 0$ (18). In both cases, the data were fitted to a straight line by the method of least-squares. The value of n for TFP was estimated by Job's method of continuous variation (19, 20).[4] The sum of the concentrations of tubulin and TFP was held constant at 10 μM, their relative concentrations being varied; Q was plotted against the mole fraction of TFP; and the linear portions of the curve were extrapolated, the molar ratio of tubulin and drug in the complex being taken from the point of intersection (see ref. 23 for critique). This method was not applied to CPZ and PMZ for technical reasons, particularly the relatively low Q with correspondingly larger errors at low C. The value of n for these two drugs was assumed to be the same as that for TFP. This was justified by the heavy excess of drug in the reaction mixtures, a pilot calculation showing that the value of K increases by only 2% in going from $n = 1$ to $n = 5$.

RESULTS

Assembly of tubulin. The effects of CPZ, TFP, or PMZ on the time-course and extent of assembly of tubulin are compared in Fig. 1.[5] CPZ and TFP inhibited assembly, TFP inhibiting much more than CPZ (Fig. 1A). Although the kinetics is complex (15, 24), both the initiation and propagation steps appeared to be inhibited. In marked contrast, PMZ enhanced assembly (Fig. 1B), evidently exerting its effect only on the propagation step.

Titration of the polymerization activity of tubulin with these drugs is shown in Fig. 2. The concentration-effect curve for CPZ was similar to the one obtained previously by viscometry (1) using the 30,000 × *g* supernatant fraction of mouse brain homogenate stabilized with 1 M glycerol: the midpoint concentrations were 220 μM and 160 μM, respectively, and the interaction was obviously cooperative with an estimated Hill constant of approximately 2 as compared with the previously found value of 1.4. In the case of TFP, the midpoint concentration was 94 μM, and the estimated Hill constant was 3–4. The concentration-effect curve for PMZ underscores the qualitative difference between the effect of this drug and the two tranquilizers on the assembly process.

CD. CD measurements were made both with and without the tubulin stabilizer, 1 M sucrose, present. The spectra displayed in Fig. 3A, which were obtained with-

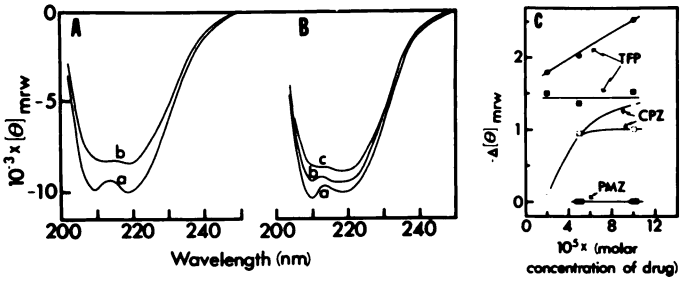

FIG. 3. *Effect of phenothiazines on the far-ultraviolet CD of tubulin*

A. CD spectra in Buffer A,[6] incubated at 37° for 45 min prior to measurement at 27°: *a*, control containing no drug; *b*, 20 μM TFP.

B. CD spectra at 37° in a medium containing the tubulin stabilizer, 1 M sucrose, i.e., Buffer B[6]: *a*, control; *b*, 20 μM TFP; *c*, 100 μM TFP.

C. Comparison of the effect of CPZ, TFP, and PMZ on the ellipticity of tubulin at 209 nm (● and ○) and 219 nm (■, □) under the same conditions as in A.

[4] Job's method is said to assume a single complex (20), but this may not always be a limitation since we note that the method has been successfully applied to the binding of two molecules of vinblastine by tubulin (19). This system must contain at least three complexes, since vinblastine induces the self-association of tubulin (19, 21, 22).

[5] CPZ, TFP, and PMZ complex weakly with sucrose, but this does not affect the comparison of their interactions with tubulin. Pilot experiments, similar to those done on CPZ by G. A. Ludi and reported by Hinman and Cann (3), show that the association constants are approximately the same, 0.6 ± 0.1 M^{-1}, for all three drugs.

[6] Buffer A: 0.045 M sodium pyrophosphate, 2.3 mM $MgCl_2$, 6.6 mM sucrose, 0.068 M NaCl, adjusted to pH 6.8 with HCl; Buffer B: Buffer A plus 1 M sucrose.

out stabilizer, show that TFP had a major effect on the far-ultraviolet CD of tubulin. These spectra were analyzed in terms of the secondary structure of the protein according to the procedure of Greenfield and Fasman (25). In the absence of drug the spectrum analyzed 17% α-helix, 37% β-pleated sheet, and 46% random; in the presence of 20 μM TFP, 11% α-helix, 41% β-pleated sheet, and 48% random. Thus, as in the case of CPZ (4), the alteration in secondary structure produced by TFP can be formally characterized by a reduction in the apparent content of α-helices and an increase in β-structure. However, with regard to concentration, TFP was more than 5 times as effective as CPZ in bringing about these changes. This conclusion follows from the comparisons drawn in Fig. 3C. Whereas 20 μM CPZ caused no significant change in the CD, the same concentration of TFP was more effective than 100 μM CPZ; at 100 μM, TFP was 1.5–2 times as effective as CPZ. In contrast to TFP and CPZ, PMZ had no significant effect on the CD at concentrations up to 100 μM.

Similar relationships were shown in media containing 1 M sucrose. At both 27° and 37°, 20 μM CPZ had no significant effect on the CD whereas the same concentration of TFP was at least as effective as 50 μM CPZ [compare our Fig. 3B with fig. 1 (IIIA) in Appu Rao *et al.* (4)]. Here too, PMZ was without effect at concentrations up to 100 μM].

As was the case with CPZ in sucrose-containing media at 37°, (a) the change in the far-ultraviolet CD of tubulin produced by 100 μM TFP was reversed upon removal of the drug by filtration through Sephadex G-25 and (b) the near-ultraviolet CD was not significantly affected by 100 μM TFP.

Fluorescence quenching. All three drugs quenched the fluorescence intensity of tubulin, the order of effectiveness being PMZ < CPZ < TFP as shown by the quenching curves of Q versus C presented in Fig. 4A. The corresponding double-reciprocal plots are presented in Fig. 4B, from which two conclusions can be drawn. First, within experimental error, CPZ and TFP had the same maximal quench (Q_{max} = 36 ± 1.5 and 33 ± 4.1, respectively) indicating that these drugs bound at the same sites on the tubulin molecule and with the same geometry. Second, PMZ had a contrastingly lower Q_{max} (21.7 ± 0.85), which superficially would suggest different binding sites for this drug. Actually, however, it evidently binds at the same sites as CPZ and TFP since, as is shown in Table 1, PMZ and TFP bound competitively. These observations are subject to at least two interpretations. We favor the view that PMZ has a different binding geometry than do CPZ and TFP; i.e., the drug is

TABLE 1

Competitive binding of PMZ and TFP to tubulin

The experimentally determined quenching of the fluorescence intensity of tubulin by mixures of PMZ and TFP at 27° is compared with theoretically expected quenching values for binding of the two drugs either (a) to two totally independent sets of sites or (b) competitively to the same set of sites. The experimental results are in accord with the predictions for competitive binding.

Concentration of drug		Theoretical quenching values		Experimental quenching values
PMZ	TFP	Independent sites[a]	Same sites[b]	
μM	μM	%	%	%
30	30	36.0	23.9	24.1 ± 0.9[c]
40	20	34.8	21.7	21.4 ± 0.7[d]
50	10	30.4	18.9	14.4 ± 0.5[d]

[a] $Q = Q_1 + Q_2$, where Q_1 and Q_2 are experimental values for the tabulated concentrations of PMZ and TFP taken alone.

[b] $Q = (K_1C_1Q_{max,1} + K_2C_2Q_{max,2})/(1 + K_1C_1 + K_2C_2)$, where subscripts 1 and 2 refer to PMZ and TFP; $K_1 = 3.8 \times 10^4$ M^{-1}, $K_2 = 9.6 \times 10^4$ M^{-1}, $Q_{max,1} = 21.7$ and $Q_{max,2} = 33$. See text for determination of these parameters.

[c] Average of three determinations.

[d] Average of two determinations.

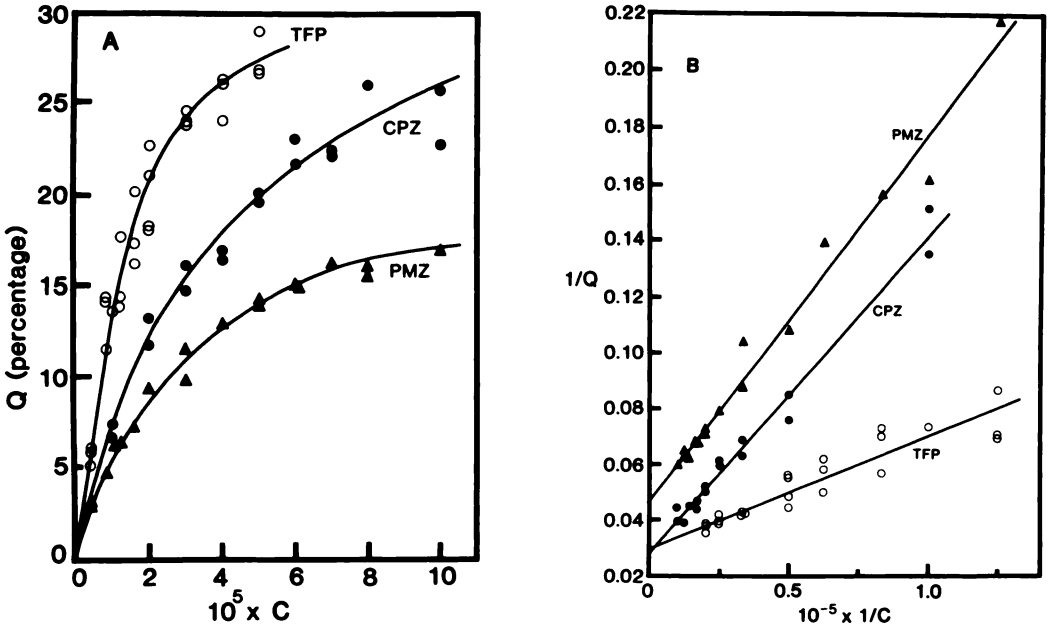

FIG. 4. *Quenching of the fluorescence intensity of tubulin by phenothiazines at 27°*
A. Quenching curves of Q against C.
B. Corresponding double reciprocal plots.

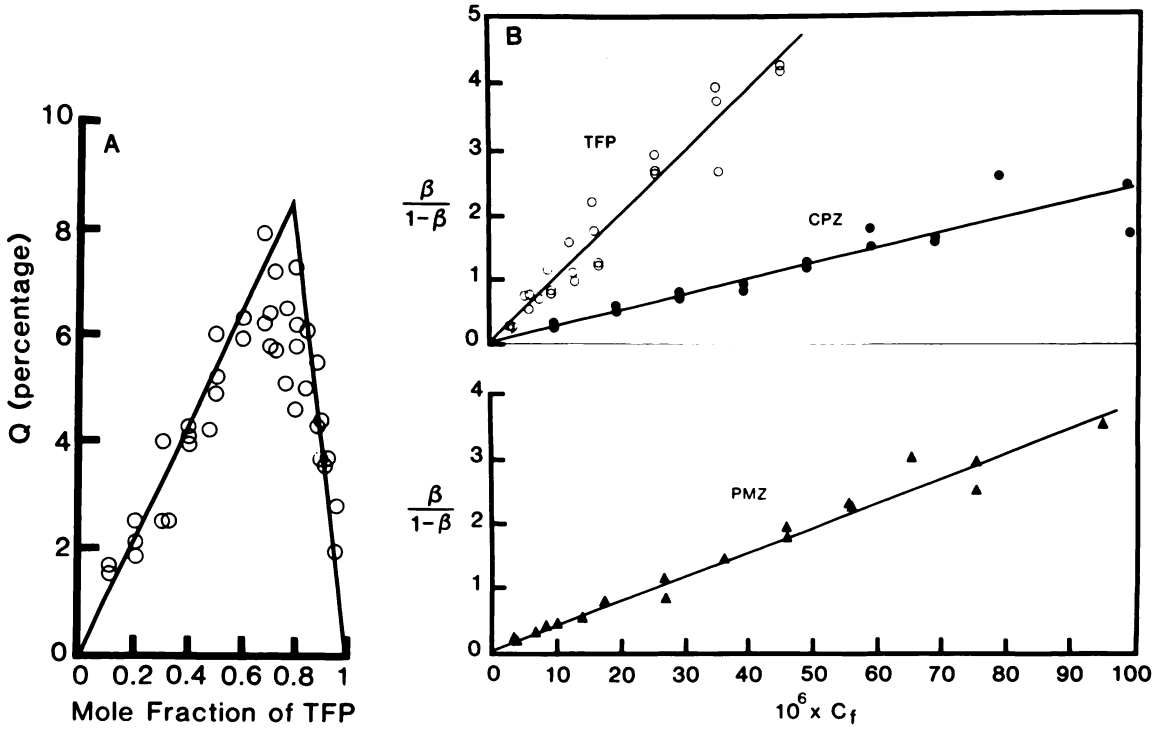

FIG. 5. *Quenching of tubulin fluorescence by phenothiazines at 27°*
A. Job's plot for TFP.
B. Mass-action plots of $\beta/1-\beta$ against C_f for determination of drug-binding constants in accordance with Eq. 1, K being given by the slope of the plot.

oriented differently in the sites so that it quenches less efficiently. A less likely possibility is that it binds to the same but fewer sites with the same binding geometry.

The Job's plot in Fig. 5A yields a value of $n = 3.5$ for TFP, while the mass-action plots in Fig. 5B give the following values for the drug-binding constants at 27°: PMZ, 3.8×10^4 M^{-1}; CPZ, 2.4×10^4 M^{-1}; and TFP, 9.6×10^4 M^{-1}.

As in the case of CPZ (3), the binding of PMZ and TFP was insensitive to temperature. Thus, values of Q determined at 10° for TFP fell within the scatter of the 27° values presented in Fig. 4A. Although the Q values for PMZ at 10° were smaller than those at 27°, the value of Q_{max} was compensatingly smaller so that the value 4.2×10^4 M^{-1} determined for the binding constant was the same, within experimental error (≃10%), as that at 27°. Thus, the van't Hoff $\Delta H^0 \simeq 0$ kcal mole^{-1}, and the binding reactions were entropy-driven: $\Delta G^0 = -RT\ln K = -6.3$, -6.0, and -6.8 kcal mole^{-1} for PMZ, CPZ, and TFP, respectively, and $\Delta S^0 = (\Delta H^0 - \Delta G^0)/T = 21$, 20, and 23 eu at 27°. The values of the thermodynamic functions are quite similar to those for formation of micelles by these drugs (26), which processes involve hydrophobic bonding. Evidently, such forces also play an important role in their binding to tubulin.

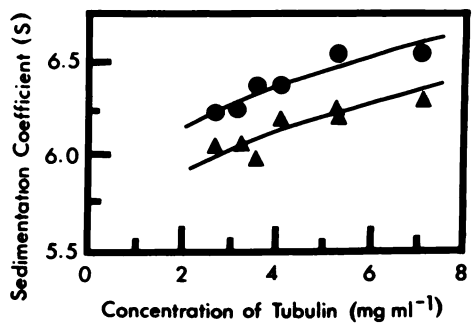

FIG. 6. *Plot of uncorrected sedimentation coefficient of tubulin against its concentration*
Buffer A[6]: ▲, in the absence of TFP; ■, in the presence of 100 μM TFP.

Ultracentrifugation. The results of subsidiary velocity sedimentation experiments on tubulin and TFP-tubulin mixture are presented in Fig. 6. TFP at a level of 100 μM increased the sedimentation coefficient of tubulin in Buffer A[6] by approximately 4% as compared with 2–3% for 500 μM CPZ (4).[7] The small amount (≃10%) of 26 S aggregate, which forms while concentrating the protein by ultrafiltration in the absence of 1 M sucrose (4), was the same in the presence as in the absence of either drug. It is concluded that the well-known tendency of tubulin to self-associate does not play an important role, if any, in the mechanism of its interaction with either of these drugs.

DISCUSSION

The results of the biophysical measurements described above show two separate correlations with the clinical potencies of the three phenothiazine drugs examined. Thus, both of the tranquilizers, CPZ and TFP, inhibit the assembly of tubulin, the more potent drug, TFP, being the stronger inhibitor. [Previously it was shown by

[7] A higher concentration of TFP was not used because incipient precipitation of tubulin occurs at 200 μM TFP as compared with >500 μM CPZ.

electron microscopy that CPZ inhibits the reassembly of microtubules (1).] In contrast, PMZ, which is not a tranquilizer, does not inhibit. In fact, it has the opposite effect of enhancing assembly somewhat. Likewise, CPZ and TFP act similarly in causing a change in the secondary structure of tubulin under nonpolymerizing conditions as monitored by far-ultraviolet CD, TFP being more effective with regard to concentration, whereas PMZ is without effect. The fluorometric measurements, in turn, correlate in a provocative way with the assembly and CD results. The quenching data are interpreted conventionally to mean that CPZ and TFP bind to the same sites on the tubulin molecule and with the same binding geometry, TFP binding the more strongly. PMZ also binds to these sites, in fact more strongly than CPZ, but the binding geometry is evidently different. This subtle difference in binding mechanism between PMZ and the other two drugs somehow translates into a profound difference in their effects on the secondary structure and the assembly propensity of tubulin.

These several correlations lend support to our suggestion that the primary molecular mechanism of the psychotropic action of CPZ may involve interaction with tubulin, with the reservation that this could be one of a set of orchestrated mechanisms. At the cellular level, such an interaction could secondarily result in the CPZ-induced modifications of the Golgi complex of nerve cells *in vitro*, as suggested by Thyberg *et al.* (6), and in other manifestations such as the inhibition of dopamine receptor sites in the central nervous system (27–29). With respect to the latter, it should be noted that microtubules apparently play an important role in determining the mobility and topography of receptor sites on cell membranes (30). It has also been demonstrated that microtubules are associated with membranes of the postsynaptic junctions in the rat and that isolated synaptosomal membranes from the brain of man and swine contain a tubulin-like protein (30). Finally, it warrants repeating that the concentrations at which CPZ exerts its effects on the properties of tubulin *in vitro* approximate apparent therapeutic concentrations. As reviewed earlier (1), rat and dog brain concentrations in the range of 50–700 μmoles kg^{-1} have been reported.

ACKNOWLEDGMENTS

The authors thank Mr. Robert O. Coombs for technical assistance, Dr. Jerry L. Brown for use of his spectrofluorometer for early measurements, and Dr. Philip G. Archer for mathematical consultation.

APPENDIX

The theoretical analysis of the fluorescence-quenching data makes the assumptions that binding of the drug, L, to tubulin, T, is statistical and that each molecule of drug that binds causes the same degree of quenching. This appears to be the only set of assumptions that predict a linear relationship between $\beta/1-\beta$ and C_f. Consider the most general case of nonstatistical binding to n sites on the tubulin molecule with no restrictions as to the degrees of quenching. Under these assumptions the mass-action expression is

$$\frac{1}{C_f}\frac{\beta}{1-\beta}=\frac{\sum_{j=1}^{n}\lambda_{j,n}\,C_f^{j-1}\prod_{l=1}^{j}K_l}{1+\sum_{j=1}^{n-1}(1-\lambda_{j,n})\,C_f^{j}\prod_{l=1}^{j}K_l} \tag{2a}$$

$$\lambda_{j,n}=(1-f_j)/1-f_n) \tag{2b}$$

in which K_l is the association constant for the reaction $TL_{l-1}+L \rightleftarrows TL_l$, and $f_j = F_j/F_T$ is the relative fluorescence intensity of complex TL_j where F_j and F_T are the fluorescence intensities of TL_j and T, respectively. These equations predict a nonlinear plot of $\beta/1$-β versus C_f with limiting slopes equal to $K_1\,\lambda_{1,n}$ for $C_f \rightarrow o$ and $K_n\,(1-\lambda_{n-1,n})^{-1}$ for $C_f \rightarrow \infty$. For the special case in which each molecule of bound drug produces the same degree of quenching (i.e., $(1-f_j)/j$ same for all j), $\lambda_{j,n}=j/n$ and the predicted plot is still nonlinear, limiting slopes K_1/n and nK_n.

For statistical binding with no restriction on the degree of quenching produced by each molecule of bound drug

$$\frac{1}{C_f}\frac{\beta}{1-\beta}=K\left\{\frac{(1+KC_f)^{n-1}+\sum_{j=o}^{n-2}\alpha_{j,n}\,(KC_f)^{j}}{(1+KC_f)^{n-1}-\sum_{j=o}^{n-2}\alpha_{j,n}\,(KC_f)^{j+1}}\right\} \tag{3a}$$

$$\alpha_{j,n}=\frac{\binom{n-1}{j+1}(1-f_{j+1})+\binom{n-1}{j}(f_n-f_{j+1})}{(1-f_n)} \tag{3b}$$

where K is the intrinsic binding constant. Here too, the theoretical plot of $\beta/1-\beta$ versus C_f is nonlinear, limiting slopes $K(1+\alpha_{o,n})$ and $K(1-\alpha_{n-2,n})^{-1}$; but for the particular case of equal degree of quenching $\alpha_{j,n}=0$ and Eq. 3a reduces to the linear relationship given by Eq. 1.

Thus, the fact that the experimental plots of $\beta/1-\beta$ versus C_f are linear and give K values approximately equal to the reciprocal of the midpoint concentration of drug in the Q-C plots is pragmatic justification for the assumptions made in our analysis of the data. A few more words concerning the assumption of statistical binding are in order, since we previously reported (3) nonstatistical binding of CPZ to tubulin. However, those direct measurements were made in a solvent of considerably different composition (0.025 M sodium pyrophosphate, 1.25 mM $MgCl_2$, 0.125 M NaCl, and 1 M sucrose, pH 6.8) from the 0.05 M MES used in the fluorescence-quenching experiments. The $MgCl_2$ in particular might affect the binding characteristics of the drug, since Mg^{2+} binds weakly to tubulin (16).

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