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A Circular Dichroism Study of the Interaction of Chlorpromazine with Mouse Brain Tubulin†

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ABSTRACT: A circular dichroism study with subsidiary ultracentrifuge measurements on the interaction of chlorpromazine with mouse brain tubulin establishes the previous inference from binding studies that the drug induces a change in the structure of the protein. Binding of the first mole of chlorpromazine causes an alteration in secondary structure, which is reversible with respect to drug concentration, without detected change in tertiary structure or significant change in the state of association of the protein. The conformationally altered tubulin binds additional chlorpromazine molecules without further change in secondary structure.

The tranquilizing drug chlorpromazine hydrochloride [2-chloro-10-(*N,N*-dimethylaminopropyl)phenothiazine hydrochloride] interacts reversibly in vitro with mouse brain microtubule subunit protein, tubulin, as revealed by inhibition of the rate of reassembly of microtubules and the binding of colchicine (Cann and Hinman, 1975), and by direct binding measurements (Hinman and Cann, 1976). Chlorpromazine binds reversibly to tubulin via two well-resolved processes: One CPZ[1] molecule binds strongly compared to eight to nine molecules which bind weakly and with moderately weak cooperativity (Hill constant of 2.8). This behavior implies a macromolecular structural change, which is substantiated in the study described below.

Materials and Methods

Preparation of Tubulin. Purified tubulin was prepared by DEAE-cellulose chromatography (Eipper, 1972). The excised brains of 15–18 adult mice (Texas Inbred, ICR) were homogenized manually in 2.5 volumes of PMS buffer. The homogenate was centrifuged at 100 000*g* for 60 min, and the supernatant was applied to a column of DEAE-cellulose (bed volume, 1.5 × 26 cm) which had been equilibrated with PMS buffer plus 0.15 M NaCl. After eluting the column with PMS buffer plus 0.15 M NaCl to remove extraneous proteins, the tubulin was eluted with PMS buffer plus 0.3 M NaCl. The center fractions from the tubulin peak were pooled, and solid sucrose was dissolved in the sample to give a final concentration of 1 M when required. All of these operations were carried out at 0–4 °C, and the resulting stock solution of tubulin was maintained in an ice bath for the rest of the day's experimentation.

The purity of the tubulin thus prepared was established by

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[1] Abbreviations used are: CPZ, chlorpromazine; DEAE, diethylaminoethyl; PMS buffer, 0.05 M sodium pyrophosphate, 2.5 × 10^{-3} M $MgCl_2$, 7.3 × 10^{-3} M sucrose, adjusted to pH 6.8 with HCl; PMHS buffer, same as PMS buffer except that the sucrose concentration is 1 M; $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism.

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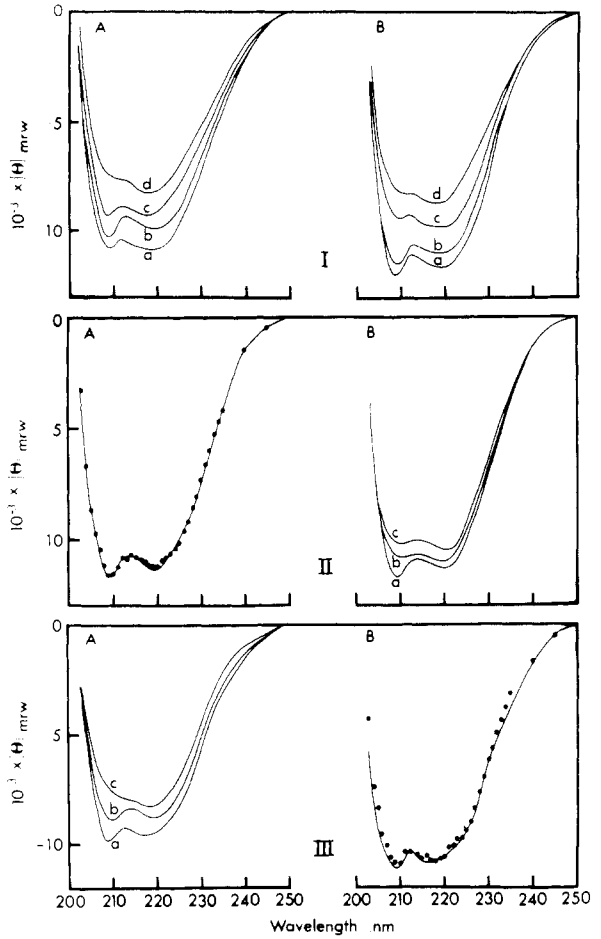

FIGURE 1: Far-ultraviolet CD spectra of tubulin. I: Spectra recorded at 27 °C showing the stabilizing effect of 1 M sucrose and pilot observations on the interaction of CPZ with tubulin. (A) Low sucrose concentration (PMS buffer plus 0.075 M NaCl): curve a, spectrum obtained immediately upon preparation; curve b, sample preincubated at 27 °C prior to measurement; curve c, preincubation at 37 °C; curve d, preincubation at 37 °C in the presence of 2×10^{-4} M CPZ. Preincubation at 27 °C with 2×10^{-4} M CPZ gave a spectrum intermediate between curves c and d. The preincubation lasted 1 h in these and subsequent experiments. (B) 1 M sucrose (PMHS buffer plus 0.075 M NaCl): curve a, spectrum obtained either immediately upon preparation or after preincubation at 27 °C; curve b, preincubation at 37 °C; curve c, preincubation at 37 °C in the presence of 2×10^{-4} M CPZ; curve d, preincubation at 50 °C. Preincubation at 43 °C gave a spectrum falling midway between curves b and c. II: Spectra recorded at 27 °C showing the effect of preincubation with CPZ at that temperature. (A) (—) Control containing no CPZ; (●) 2×10^{-5} M CPZ. (B) Curve a, control; curve b, 5×10^{-5} M CPZ; curve c, 1×10^{-4} M CPZ. III: (A) Spectra recorded at 37 °C showing the effect of preincubation with CPZ at that temperature: curve a, control; curve b, 5×10^{-5} M CPZ; curve c, 1×10^{-4} M CPZ. (B) Reversal of the effect of 2×10^{-4} M CPZ at 37 °C by removal of the drug (see text for details): (●) CD at 27 °C after removal of the drug; (—) parallel control.

colchicine binding, electrophoresis, and ultracentrifugation. The preparations in 1 M sucrose bind 0.5 mol of colchicine per 110 000 g of protein, as measured by the standard filter assay of Weisenberg et al. (1968); this value compares favorably with literature values (Weisenberg et al., 1968; Wilson and Meza, 1972; Hinman et al., 1973). The $NaDodSO_4$ discontinuous acrylamide gel electrophoretic patterns show two bands corresponding to the α and β subunits of the tubulin heterodimer. Densitometry scanning indicated that the two bands represent about 98% of the protein. The velocity sedimentation patterns of preparations concentrated by ultrafiltration through a Diaflo membrane in the absence of 1 M sucrose (with a change of solvent to PMS buffer plus 0.075 M NaCl) show a major 6S component with about 10% of a more rapidly sedimenting peak, 26S. As might be expected (Lee et al., 1975), addition of 1 M sucrose before concentrating (final solvent PMHS plus 0.075 M NaCl) prevents formation of the 26S aggregates. Finally, the preparations have an A_{280}/A_{260} ratio of 1.3–1.4 (corresponding to about 2 mol of bound guanine nucleotides) and an $E_{280}^{0.1\%} = 1.15$, which are comparable to the values of Eipper (1974) for rat brain tubulin; $E_{274}^{0.1\%} = 1.15$ in 6 M guanidine hydrochloride in agreement with Lee et al. (1978) for calf brain tubulin.

Other Materials. CPZ was kindly supplied by Smith Kline and French Laboratories. [*ring C, methoxy*-^{3}H]Colchicine (99% radiochemically pure) was obtained from New England Nuclear. DEAE-cellulose (DE52) was a product of Whatman Biochemicals, Ltd., Sephadex G-25 was from Pharmacia Fine Chemicals, and UltraPure sucrose was from Schwarz/Mann. All other chemicals were of reagent grade.

Physical Methods. Protein concentrations were measured spectrophotometrically using the aforementioned extinction coefficient, which was determined with tubulin solutions whose concentrations had been estimated by the Lowry method (Lowry et al., 1951). Electrophoresis was carried out in $NaDodSO_4$ according to the discontinuous procedures of Ludueña and Woodward (1975), with 30–100 μg of protein loaded on each gel. 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 °C.

CD spectra were recorded on a Cary Model 60 spectropolarimeter with a Model 6001 circular dichroism attachment, fitted with a thermostable cell holder calibrated with a thermistor probe. The instrument had been modified in the field to eliminate possible artifactual signals on passing through intense absorption bands. Slits were programmed to yield a 15-Å bandwidth at each wavelength. For the far-ultraviolet spectral region, the optimum path length of 0.05 cm was dictated by various practical considerations, including the concentration and volume of stock tubulin solution and the strong absorbance of CPZ; 2×10^{-4} M CPZ is the upper limit for this path length. Aliquots of stock tubulin were diluted fourfold with either PMS or PMHS buffer to give a protein concentration of 0.2–0.3 mg/mL and a NaCl concentration of 0.075 M. For the near-ultraviolet, a path length of 1 cm (upper limit of CPZ, 1×10^{-4} M) was necessary because of the weak CD of tubulin; the NaCl concentration of the tubulin solution was adjusted to 0.075 M by concentrating the stock (in PMHS buffer + 0.3 M NaCl) fourfold by ultrafiltration followed by fourfold dilution with PMHS buffer, final protein concentration of 1 mg/mL. Mean residue ellipticities $[\theta]_{mrw}$ (deg cm^2)/dmol, were calculated in the usual fashion using a value of 115 for the mean residue weight.

Finally, a few words with respect to reproducibility are in order. Each CD spectrum reported in this study is the average of at least two and in the vast majority of cases three (sometimes four to five) determinations made in matched pairs with the control in order to average out biological variations as well as experimental error (Frigon and Timasheff, 1975). The mean deviation of the amplitude in the far-ultraviolet is less than ±5% for measurements made at 27 °C and ±5–10% for 37 °C, and the several effects reported were qualitatively the same and of the same magnitude for each matched pair of experiments, irrespective of the particular preparation. The same applies to the near-ultraviolet, except for a surprisingly large variation in intensity of the positive 265-nm band from one preparation to another.

Results

Stabilization of the CD of Mouse Brain Tubulin. The far-

ultraviolet CD spectra displayed in Figure 1 (IA, curves a–c) illustrate the thermal lability of mouse brain tubulin under conventional conditions of solvent composition. Thus, the amplitude of the CD at 27 °C decreases quite significantly during 1-h preincubation at that temperature and changes markedly during preincubation at 37 °C. Moreover, if the CD of samples preincubated at 37 °C is also measured at 37 °C, the resulting spectrum is virtually the same as when the measurements are made at 27 °C; i.e., the CD does not relax significantly upon lowering the temperature. Previously, we found (Hinman and Cann, 1976) that the CPZ-binding equilibrium of mouse brain tubulin is stabilized by 1 M sucrose (Frigon and Lee, 1972). This agent also has a stabilizing effect on the CD [Figure 1 (IB)]. Not only is the amplitude of the CD at 27 °C greater in 1 M sucrose [compare curves a in Figure 1 (IA and IB)], but 1-h preincubation at 27 °C has no significant effect on the spectrum. Although 1 M sucrose does not protect significantly against a change in CD on preincubation at 37 °C as measured at that temperature [look forward to curve a in Figure 1(IIIA)], it does allow the spectrum to relax almost completely upon lowering the temperature to 27 °C [curve b in Figure 1 (IB)]. In contrast, it does not prevent the essentially irreversible change in CD brought about by higher temperatures [curve d in Figure 1 (IB)], nor does it give complete protection against the changes occasioned by freezing and thawing of stored material. (It is for this reason that a fresh stock of tubulin was prepared for each day's experimentation.)

Far-Ultraviolet CD of the CPZ-Tubulin System. Spectrum d in Figure 1 (IA) and c in Figure 1 (IB) reveal that preincubation of tubulin at 37 °C in the presence of 2×10^{-4} M CPZ has a major effect on the far-ultraviolet CD as measured at 27 °C. These pilot observations establish that the binding of CPZ causes a substantial change in the secondary structure of the protein, irrespective of whether or not the incubation mixture contains 1 M sucrose, and the change is of the same magnitude under both conditions when comparison is made with the corresponding control. Subsequent experiments were done in 1 M sucrose solution because, in addition to the above considerations, we wished to make the measurements under conditions known to stabilize both the CPZ-binding equilibrium and the colchicine-binding activity of mouse tubulin (Hinman and Cann, 1976). Also, except where noted, the CD was recorded at the same temperature as used for preincubation of the protein with CPZ. The effect of varying CPZ concentration at 27 °C is shown in Figure 1 (II). A concentration of 2×10^{-5} M has virtually no effect on the CD, but increasing the concentration to 1×10^{-4} M decreases the amplitude significantly, while a further increase to 2×10^{-4} M causes essentially no further change. The same picture is seen at 37 °C, except that the effect is accentuated [Figure 1 (IIIA)]. Although the time of preincubation in these experiments was 1 h, the change in CD is more rapid than that, 15-min preincubation sufficing.

The effect of CPZ upon the CD of tubulin is reversed upon removal of the drug. In these experiments, the protein was incubated for 0.5 h at 37 °C with 2×10^{-4} M CPZ, after which time the mixture was returned to room temperature and the CPZ removed (as confirmed spectrophotometrically) by passage through a column of Sephadex G-25 equilibrated with PMHS plus 0.075 M NaCl. The CD at 27 °C of material so treated is compared with the parallel control in Figure 1 (IIIB); these spectra are to be compared, in turn, with curve c in Figure 1 (IIIA) and curve b in Figure 1 (IB). In contrast to this result, the magnitude of the effect of CPZ is only partially relaxed when the temperature of the incubated CPZ–tubulin mixture is lowered from 37 to 27 °C, even after 1.5 h at the lower temperature. [Compare spectra c in Figure 1 (IB, IIB, and IIIA) bearing in mind that 1×10^{-4} and 2×10^{-4} M CPZ give virtually the same spectrum, at either temperature.) The hysteresis is more pronounced when the system is incubated at 43 °C and brought back to 27 °C. These results preclude determination of thermodynamic parameters, and further measurements in the far-ultraviolet were not deemed essential for the conclusions drawn from the following analysis.

TABLE I: Analysis of Far-Ultraviolet CD Spectra in Terms of Secondary Structure.[a]

sample	ν (mol of CPZ bound/ mol of tubulin heterodimer)	% of structure α	β	R
control[b]	0	15	41	44
2×10^{-5} M CPZ[c]	0.34	13	39	48
5×10^{-5} M	0.66	12	42	46
1×10^{-4} M	1.3	8	45	47
2×10^{-4} M[d]	4.2	9	43	48
		9	46	45

[a] At 37 °C in a medium containing the tubulin stabilizer, sucrose; i.e., PMHS buffer plus 0.075 M NaCl. [b] Of four spectra (each an average of two to three measurements) determined over a period of 9 months, three gave 15% and one 14% α helix. [c] Each of three matched-paired experiments showed no significant difference between the spectrum of the CPZ–tubulin mixture and the control. It is the average of the three controls which analyzed 14% α helix. [d] Determination of the two spectra was spaced by 4 months to check reproducibility.

The spectra were analyzed in terms of the secondary structure of the protein according to the procedure of Greenfield and Fasman (1969). In the absence of CPZ, the spectrum at 27 °C analyzed 22% α helix, 37% β pleated sheet, and 41% random (three spectra determined over a period of 1 y gave this same result): in the presence of 1×10^{-4} M CPZ, 19% α helix, 38% β-pleated sheet, and 43% random. Analyses of the spectra at 37 °C are presented in Table I in which a comparison is also made between the change in structure and the number of moles of CPZ bound per mole of tubulin heterodimer, ν, calculated from the binding data of Hinman and Cann (1976). The conclusions drawn are: (a) The effect of CPZ binding can be formally ascribed to a decrease in the apparent α-helical content of the protein (about a 40% decrease at 37 °C for $1\text{–}2 \times 10^{-4}$ M CPZ) with a concomitant increase in β-pleated sheet and possibly random structure. (b) This change in secondary structure is induced by the binding of the first mole of CPZ. (c) The transition evidently does not set in until $\nu > 0.3$, suggesting that more than one thing may be happening to the protein. (d) There is no further change in secondary structure, within experimental error, on going from $\nu = 1.3$ to 4.2, which is well into the cooperative phase of CPZ binding [See Figure 3 of Hinman and Cann (1976)]. These conclusions are in accord with a superficial reading of the CD spectra.

Near-Ultraviolet CD. In contrast to its far-ultraviolet CD, the near-ultraviolet CD of tubulin at 37 °C is not significantly affected by 1×10^{-4} M CPZ (Figure 2). For the constituent concentrations of tubulin and CPZ used in these experiments, $\nu = 1$.

Ultracentrifugation. The results of subsidiary velocity sedimentation experiments on tubulin and CPZ–tubulin mixtures in PMS buffer plus 0.075 M NaCl at 27 °C can be summarized as follows: (a) Tubulin concentration, 2.6 mg/mL; in the absence of CPZ, uncorrected sedimentation coefficient, 5.9 S; 5×10^{-4} M CPZ, 6.0 S. (b) Tubulin concentration, 5.0 mg/mL; absence of CPZ, 6.1 ± 0.15 S; 5×10^{-4} M CPZ, 6.3

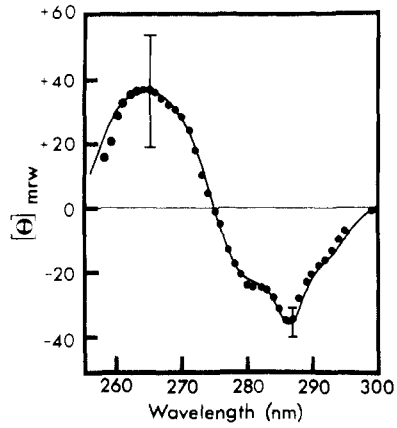

FIGURE 2: Near-ultraviolet CD spectrum of tubulin at 37 °C: (●) 1×10^{-4} M CPZ; (—) control.

± 0.10 S. (c) The amount of 26S aggregate is the same (~10%) in the presence as in the absence of CPZ. Clearly, the well-known tendency of tubulin to self-associate does not play a significant role in the mechanism of its interaction with CPZ.

Discussion

The foregoing results validate the inference drawn previously (Hinman and Cann, 1976) from an empirical analysis of the CPZ-binding isotherm that the drug induces a change in the structure of mouse brain tubulin. Thus, the binding of the first mole of CPZ causes a change in the secondary structure of the protein, which is reversible with respect to drug concentration, without a detected change in the tertiary structure or a significant change in its state of association. An alteration in secondary structure, as revealed by the far-ultraviolet CD, is formally characterized by a reduction in the apparent content of α helices and an increase in β structure. The insensitivity of the near-ultraviolet CD to CPZ speaks against extensive change in tertiary structure, or at least against significant perturbations of the asymmetric environment of those aromatic residues which contribute to the near-ultraviolet CD. This result points to a localized change in secondary structure, suggesting that tubulin has more than one structural domain. Lee et al. (1978) have suggested that in calf brain tubulin the disulfide bond maintains a highly stable structural domain containing aromatic chromophores which, along with the disulfide bond, give rise to the near-ultraviolet CD bands. Apparently, it is not known whether mouse brain tubulin contains disulfide bonds; however, rat brain tubulin does not contain disulfide bonds (Eipper, 1974). A less likely explanation of our observations is that the change in secondary structure is accompanied by a significant change in tertiary structure around the chromophores responsible for the near-ultraviolet CD but that the CD differences associated with them compensate. In any case, it follows from comparison of the CD results with the CPZ-binding behavior that the conformationally altered tubulin binds additional CPZ molecules with moderately weak cooperativity but without further change in secondary structure. Ligand-induced macromolecular association (Cann and Hinman, 1976) has been eliminated as the source of the cooperativity. The source might be subtle changes in conformation or conceivably to stacking of bound CPZ molecules at a single site in much the same way as in micelle formation (Florence and Parfitt, 1971; Attwood et al., 1974). Although the critical micelle concentration of CPZ is an order to magnitude greater than the highest concentration used in these studies, the protein molecule might present a hydrophobic microenvironment conducive to such an interaction.

The CPZ–tubulin system is unconventional in several aspects: (a) Its initial state shows an unusual dependence upon temperature; i.e., the secondary structure of tubulin in the absence of CPZ is temperature sensitive,[2] and, rigorously speaking, the effect of temperature is not completely reversible even on going from 37 to 27 °C. (b) The change in secondary structure of the protein induced by CPZ shows temperature-dependent saturation; i.e., the final state of the interaction is temperature dependent in the unusual sense that the change in secondary structure of the protein induced by CPZ at 27 °C cannot be driven to the final structure induced at 37 °C by increasing the concentration of drug. (c) The system is only partially reversible with respect to temperature. These observations indicate that the effects of CPZ and temperature are different and that the CPZ–tubulin complex has a metastable conformational state(s).

It is thus of interest to examine more closely the comparative effects of CPZ and temperature upon the CD. The analyses according to the procedure of Greenfield and Fasman (1969), which gives the best least-squares fit of the observed spectrum to a linear combination of polylysine reference spectra, would suggest a similarity between the two effects; i.e., loss of α helix and gain in β structure. On the other hand, it is evident from a visual inspection of the spectra that the effects are actually qualitatively different. This was substantiated by comparison of difference spectra obtained by arithmetic substraction of the data points. The shape of the difference spectrum of tubulin at 27 °C referred to 37 °C is close to that of an α helix. In contrast, when the temperature is held constant at either 27 or 37 °C, the shape of the difference spectrum of tubulin referred to an admixture of tubulin with 1×10^{-4} M CPZ is more like one might expect from a loss of α helix and gain in β structure. The discrepancy between least-squares analysis and empirical reading of the spectra is attributable to the oversimplifying assumptions of the Greenfield–Fasman procedure[3] (Adler et al., 1973; Lee et al., 1978).

As for the biological significance of the interaction of CPZ with tubulin, in tissue culture CPZ resembles the action of colcemide in causing mitotic arrest and in disorganizing the organized microtubule structure produced by cyclic AMP [see private communication from T. T. Puck quoted by Cann and Hinman (1975)]. The mechanisms of the antimitotic action of these two drugs most likely differ in detail, however, since, unlike CPZ, colchicine does not effect the CD of tubulin (Ventilla et al., 1972). We believe that the interaction of CPZ with tubulin provides a fresh clue as to the primary molecular mechanism of its psychotropic action and may also have a bearing on its side effects. A test of this idea is to compare the interaction with tubulin of various phenothiazine tranquilizing drugs with different clinical potencies. Toward this end, we have initiated a study of the tubulin–trifluoperazine interaction. The results of pilot experiments show that concentrationwise trifluoperazine, which is about an order of magnitude more potent clinically than CPZ, is at least five times as effective as CPZ in causing changes in the secondary structure of tubulin, as monitored by far-ultraviolet CD. While it is evident that phenothiazine drugs block dopamine receptor sites in the central nervous system (Seeman et al., 1978), it can also

[2] The temperature dependence of the CD of mouse brain tubulin stands in contrast to the insensitivity of calf brain tubulin (Lee et al., 1978) but is less marked than with porcine brain tubulin (Ventilla et al., 1972).

[3] The reference spectra derived by Chen et al. (1972) from the CD of proteins of known three-dimensional crystallographic structure gave unacceptable fits to all of our spectra.

be said that microtubules apparently play an important role in determining the mobility and topography of receptor sites on cell membranes (Yahara and Edelman, 1975; Nicholson, 1976).

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Platelet 5-Hydroxytryptamine Transport, an Electroneutral Mechanism Coupled to Potassium†

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ABSTRACT: Transport of 5-hydroxytryptamine into plasma membrane vesicles isolated from porcine blood platelets is stimulated when a potassium gradient (in > out) is imposed across the vesicle membrane. This stimulation occurs in the absence of measurable electrical potential across the membrane. Addition of valinomycin induces a membrane potential of approximately 50 mV (interior negative) as estimated by uptake of the lipophilic cation triphenylmethylphosphonium, but has surprisingly little effect on 5-hydroxytryptamine transport. Addition of 2,4-dinitrophenol dissipates the valinomycin-induced membrane potential. In the absence of valinomycin, 2,4-dinitrophenol has no effect on 5-hydroxytryptamine transport but valinomycin and 2,4-dinitrophenol together inhibit transport, probably by dissipation of the K^+ gradient. These results are consistent with an electroneutral mechanism in which 5-hydroxytryptamine influx is directly coupled to potassium ion efflux and argue against an electrogenic mechanism in which there is a net influx of positive charge with 5-hydroxytryptamine.

Transport of 5-hydroxytryptamine (5-HT)[1] is an energy-dependent, carrier-mediated process with an absolute requirement for Na^+ and Cl^- in the external medium (Sneddon, 1969; Lingjaerde, 1971; Rudnick, 1977). Similarities between 5-HT transport into platelets and synaptosomes and brain slices have led to the proposal that the same transport system is present in both tissues (Sneddon, 1973; Paasonen, 1968). A previous report from this laboratory demonstrated that plasma membrane vesicles isolated from human blood platelets accumulate 5-HT to concentrations approximately 100 times greater than in the external medium (Rudnick, 1977). Accumulation is stimulated by imposition of a potassium ion gradient (in > out) across the vesicle membrane. This observation can be interpreted in one of two ways: (1) K^+ efflux stimulates transport by creating an electrical potential (interior negative) across the membrane, or (2) K^+ interacts directly with the 5-HT carrier, which couples K^+ efflux to 5-HT uptake. The

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[1] Abbreviations used: 5-HT, 5-hydroxytryptamine; $TPMP^+$, triphenylmethylphosphonium.

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