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BINDING OF CHLORPROMAZINE AND THIOPROPERAZINE *IN VITRO*—I.

RESULTS OF CENTRIFUGATION METHODS WITH TISSUE AND MITOCHONDRIA FROM RAT LIVER AND HUMAN LEUKOCYTES

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Abstract—Binding of two phenothiazine neuroleptics, chlorpromazine (CPZ) and thioproperazine (TPZ) was studied with rat liver and human leukocytes as sources of tissue and mitochondria. After 10 min of incubation at 24° in 15 or 30 μg CPZ/ml Hank's solution, rat liver bound 66–88 per cent, whereas human leukocytes bound 27–29 per cent of the drug. Eight sequential centrifugal washings of the rat liver tissue released 30 per cent of the CPZ, but under the same conditions human leukocytes released 84–100 per cent of the bound drug. In contrast, we observed no difference in mitochondrial binding due to difference between tissues. Liver and leukocyte mitochondria were centrifuged at 8° through linear sucrose density gradients that contained a constant concentration of 15 or 30 μg phenothiazine/ml. After 5 min of centrifugation through a sucrose gradient containing 15 μg CPZ/ml, 3·7 μg CPZ/ml was bound/mg mitochondrial protein; after 15 min, 5·3 μg drug/ml was bound/mg protein. With 30 μg CPZ/ml in the gradient, 2·2 and 9·9 μg of drug/ml were bound/mg protein after 5 and 15 min, respectively. If TPZ, at either 15 *or* 30 μg/ml, was present in the gradient, 3·0 and 4·4 μg of drug/ml were bound/mg protein after 5 and 15 min. Cooperative effects on binding by the mitochondria and the drugs were observed as a function of duration of exposure at 8°. Significant quantities of each drug, measured spectrophotofluorimetrically, were removed from solution by the mitochondrial suspensions.

THIS paper reports results obtained by centrifugation methods for the binding of chlorpromazine (CPZ) and thioproperazine (TPZ) to rat liver and human leukocytes. These tissues are easily obtained, readily characterized, and would normally be exposed to high levels of unmetabolized phenothiazine after medication *in vivo*. The two tissues were chosen because leukocytic motility, and liver function tests related to mitochondrial activity, showed marked changes during treatment *in vivo* with these and other neuroleptic drugs.[1, 2] The effects of phenothiazine derivatives on cell membranes,[3, 4] on intracellular incorporation of other compounds,[5–7] and particularly on mitochondrial physiology[8–11] suggested that measurement of the binding of these two drugs to mitochondria would be of particular interest. There are at present few direct, systematic studies[8, 12] of binding of these two neuroleptics to mitochondria.

Variations in reported metabolic turnover, tissue incorporation, and anatomical localization of these drugs have been described to 'species' and binding differences. Mitochondrial binding[8] has been reported to vary depending upon the species and

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tissue.[12, 13] In the present study, there was a definite difference in the binding of CPZ by rat liver tissue and by human leukocytes at 23°, but mitochondria from each tissue bound similar amounts of CPZ at 8°. In addition, the amount of CPZ or TPZ bound by mitochondria *in vitro* changed with the duration of exposure and this markedly affected the concentration and distribution of both drugs in the suspending medium.

METHODS

Preparation of tissues, cells, and mitochondria

Preparation of liver tissue. Rat livers were obtained from Wistar or Sherman males fasted for 18–24 hr, and weighing 150–250 *g*. They were killed by cervical dislocation and the livers were perfused with saline containing 2–4 units of heparin/ml. The organs were blotted, forced through a syringe, and the fine mince was suspended in 0·25 M sucrose. After agitation in a double cheesecloth bag in Hanks' solution, pH 7·2, without phenol red indicator dye,[14] the suspended liver particles were centrifuged at 300 *g* for 5 min. The precipitate was washed by centrifugation in Hanks' solution, at the same speed, until the supernatant was clear. The washed precipitate was suspended in Hanks' solution for use in drug-binding or enzyme assays.

Preparation of leukocyte tissue. Small quantities of human leukocytes were obtained by centrifugation in special tubes (Fig. 1). These 'buffy coat' preparations contained

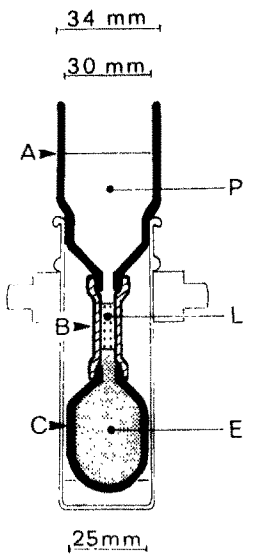

FIG. 1. Centrifuge tube apparatus for leukocyte tissue isolation from whole blood. (A, C) Pyrex glass; (B) polyvinyl chloride tubing 1/8 in. i.d., 1/4 in o.d.; (P) plasma; (L) leukocytes and platelets, 'Buffy coat'; (E) packed red cells. Carrier: No. 320 test tube shield, No. 325 trunnion and rubber cushion, International Equipment Co. The transparent flexible plastic tubing is cut with a razor blade to remove the 'buffy coat'.

6–30 red cells/leukocyte. When the 'buffy coat' was suspended in 10 ml 0·3 M sucrose, containing 1 unit heparin/ml, the red cells clumped and settled at 6°. Larger quantities of leukocytes were prepared by the methods of Christlieb, Sbarra and Bardawil.[15] None of the blood donors received any medication for at least 6 weeks before the blood was

collected. Cell counts and smears were made of leukocytes suspended in 1% human albumin in 0·9% NaCl.

Preparation of liver mitochondria. The rat liver tissue that remained in the cheese cloth bag (described above) was homogenized for 120 sec at 800–1000 rpm (with a Teflon-glass Potter–Elvehjem homogenizer) in 40 ml of 0·25 M sucrose containing 0·01 M phosphate buffer, pH 7·2. Mitochondria were prepared by the method of Schneider,[16] and the dark, heavy sediments were washed three times.

Preparation of leukocyte mitochondria. Leukocytes for this purpose were obtained through a macromodification of the procedure of Baron and Roberts,[17] since large volumes of blood were required. Recovery of leukocytes from the original blood was 37 per cent.

Methods used for the study of tissue and cell fraction binding of phenothiazines

Tissue binding. Leukocyte and rat liver preparations were incubated at 23–24° in 15 or 30 μg/ml of the drugs in a total volume of 40 ml of Hanks' solution. After 10 min the suspensions were divided in four parts. The *first* aliquot was centrifuged at 30,000 *g* for 30 min at 8° and the supernatant was aspirated. The precipitate was gently suspended in 1·0 ml of Hanks' solution, incubated for 10 min, and centrifuged again. The individual supernatant solutions were analyzed for drug and protein. This process[18] continued until no drug was detected in the supernatant.

A *second* aliquot of the original tissue suspension was dialyzed against a ten-fold larger volume of Hanks' solution at 8° with constant agitation. The diffusate was analyzed for phenothiazine content and was changed at 30-min intervals. This was repeated until no drug was detected in the diffusate. A *third* aliquot of the original tissue–drug incubation mixture was filtered through double Whatman 40-H preweighed paper circles and the filtrate was analyzed for drug. The top filter paper was dried with the tissue at 100° for 18 hr and was then reweighed. The *fourth* aliquot was used for dialysis and protein determinations. Dialysis and filtration procedures were not satisfactorily accurate methods for determining phenothiazine binding. However, these two procedures established the approximate range of ligand concentrations for the centrifugation techniques.

Binding of drug to rat liver and human leukocyte mitochondria. Concentrated mitochondrial suspensions were obtained by rapid vibration of the heavy pellet in the polypropylene conical centrifuge tube after the third preparative wash. Four uniform siliconized capillary tubes (20-μl Drummond Microcaps) were filled with the suspension and sealed with soft wax (1 part Lubriseal:2 parts paraffin). They were centrifugated in a hematocrit centrifuge for 12 min at 16,000 *g*. The ratio of supernatant to precipitate in the original volume was measured. The tubes were cut carefully 1 mm above the sediment layer and the surface supernatant absorbed on to paper. The sediment was pushed out of the tube and weighed. The average density was found to be 1·19 ± 0·02 g/ml. The rat liver mitochondrial sediments, containing 417 μg protein/mg wet weight ± 31 μg (standard deviation, N = 71) in 0·25 M sucrose, pH 7·2, 0·01 M phosphate buffer, were resuspended and layered over sucrose gradients in 5-ml vol. (0·5 × 2 in) nitrocellulose tubes. The gradients were linear from 0·25 to 1·1 M sucrose and contained a constant amount of 0·01 M phosphate and phenothiazine drug at all levels.

Liver and leukocyte mitochondrial suspensions were dispensed from calibrated constriction pipettes. The pipettes were rinsed with additional volumes of 0·25 M sucrose to fill the 5-ml SW-39 rotor tubes. The filled tube contained 0·1–0·25 ml mitochondrial suspension (2–20 mg wet weight mitochondria) layered over a 4·5 ml density gradient.

After centrifugation in a Spinco model L ultracentrifuge at 30,000 *g* for 20 min, the tubes were punctured and single drops were collected for measurement of drug and protein content. The protein and drug fluorescence were plotted for tube fractions to present a profile of the gradient contents after centrifugation. One control tube was centrifuged with each experimental pair of tubes. The control tubes contained: (a) no mitochondria; (b) particles saturated with phenothiazine, after incubation for 60 min with a level of drug the same as, or higher than that contained throughout the gradient; or (c) gradients without drug. Only 8 experiments were performed with leukocyte mitochondria because of the small amount of material obtained.

Protein was determined by the method of Lowry *et al.*[19] Phenothiazines were determined by fluorescence. Spectra are shown in Fig. 2.

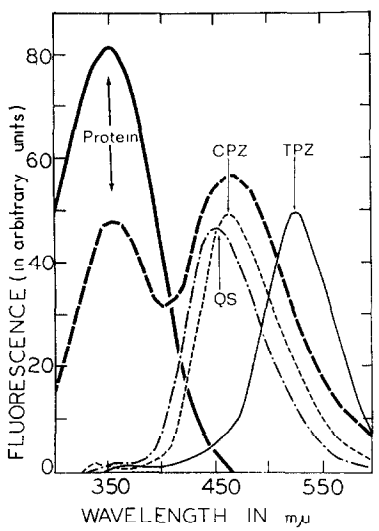

FIG. 2. Fluorescence intensity spectra of CPZ, TPZ, and protein in comparison with a quinine sulfate (QS) standard. Solutions of TPZ and CPZ, 0·2 mM in pH 7·0, 0·01 M Tris-HCl containing 0·002 M EDTA were activated at 360 and 340 mμ, respectively, in 1-cm² cells at 25°. Human crystalline albumin, 100 μg/ml in 2 ml of 0·9% NaCl was activated at 290 mμ; then 160 μg CPZ was added and the fluorescence spectrum was repeated. The fluorescence of 1·0 μg QS/ml 0·1 N H_2SO_4 activated at 355 mμ is shown for comparison with the two phenothiazines under the same instrumental conditions. Key: albumin alone, heavy solid line; albumin plus CPZ, heavy broken line. CPZ, thin broken line; TPZ thin solid line; QS, dash-dot line.

Hanks' solution was modified from Hanks and Wallace.[14] Tris-HCl buffers were made from "121" primary standard Tris (hydroxymethyl) aminomethane, Sigma Chemical Co.

Phenothiazines. CPZ,* chlorpromazine HCl, mol. wt. 355·33; recrystallized from methyl alcohol–water. Thin-layer chromatography on silica gel (10% methanol: ethyl acetate) indicated only one component under u.v. light.

* Thorazine®. Smith Kline and French Laboratories.

TPZ* thioproperazine dimethane sulfonate, mol. wt. 638·84; purity 99·71%. Alternate nomenclature: 2-dimethylsulfonamido-3[(methyl-4-piperazino)-3 propyl]-10 phenothiazine, *bis* methane sulfonate; 70·2 g active product/100 g TPZ.

RESULTS

Binding of CPZ to leukocytes and rat liver

The amount of CPZ in the first supernatant, obtained after centrifugation of the 10-ml tissue–drug incubation suspension, was depleted by the tissue suspension as shown in Table 1. However, 29–30 per cent of the CPZ originally removed from the incubation supernatant by the tissue pellet reappeared in the 1-ml 'wash' supernatants (Fig. 3). Protein loss from the tissue pellet after the centrifugation of the tissue–drug incubation medium was greatest with leukocytes incubated with 30 μg CPZ/ml, and least with rat liver incubated with 15 μg CPZ/ml (Table 1). Additional protein released into the supernatants during eight 'wash' procedures averaged 4 per cent of the original 2 mg of tissue protein in the incubations.

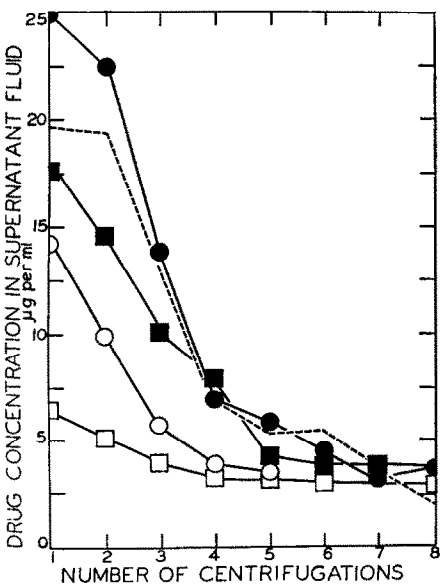

FIG. 3. Release of CPZ and protein during repeated centrifugal washing of tissue previously removed from drug incubation medium. Key: (●) human leukocytes originally incubated in 30 μg CPZ/ml; (○) human leukocytes originally incubated in 15 μg CPZ/ml; (■) rat liver particles originally incubated in 30 μg CPZ/ml; (□) rat liver mince particles originally incubated in 15 μg CPZ/ml, (– – –) protein (μg/ml) averages for the four sets of determinations. (See legend for Table 1.)

Binding of CPZ and TPZ to mitochondria (density gradient studies)

Some results after gradient centrifugation of rat liver mitochondria with the two phenothiazines are shown in Fig. 4. Fractions 1–20 were from the centrifugal base of the tube; fractions 65–70 were from the centripetal, air–liquid interface.

Controls. Fig. 4 (curves **a–c**) shows the results obtained from two of the experimental controls. The linearity of CPZ throughout the density gradient after centrifugation without mitochondria is shown in curve **a**, with the exception of some

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TABLE 1. RECOVERY OF CPZ AND PROTEIN FROM SUPERNATANTS OF RAT LIVER AND HUMAN LEUKOCYTE TISSUE AFTER INCUBATION AND CENTRIFUGAL 'WASHING' PROCEDURES AT 23–24°*

Tissue	Original CPZ concentration	Total of original ligand recovered in supernatants:								CPZ bound after 1 centrifugation		Total of bound CPZ released in 8 wash steps
		After first incubation				After 8 wash steps						
		protein		CPZ		protein		CPZ				
	(μg/ml)	(μg)	(%)	(μg)	(%)	(μg)	(%)	(μg)	(%)	(μg)	(%)	(%)
Rat liver	30	286 ± 9·7†	14·3	96 ± 3·4‡	32	82 ± 3·1†	4·1	61·3 ± 2·1‡	20·4	204	68	30
	15	202 ± 6·1	10·1	51 ± 3·6	34	63 ± 2·7	3·2	29·5 ± 3·2	19·7	99	66	29·8
Human leukocytes	30	514 ± 17	25·7	218 ± 7	72·7	136 ± 8·1	6·8	84 ± 4·1	28	82	27·3	102
	15	335 ± 10	16·8	107 ± 11	71·3	38 ± 4·8	1·9	36 ± 2·1	24	43	28·7	83·7

* After 10 min of incubation and centrifugation at 30,000 $g \times 10$ min, the tissue pellets were "washed" by resuspension in 1 ml Hanks' solution minus drug. They were centrifuged again at 30,000 $g \times 10$ min for the first of 8 successive 'wash' procedures.

† Mean ± estimated standard error, duplicate analyses at 2 dilutions of four replicates. All initial protein concentrations were 2 mg in 10 ml Hanks solution.

‡ Mean ± estimated standard error, duplicate anzlysis of four replicates. Initial CPZ concentrations were 150 or 300 μg/10 ml. The total amount of CPZ bound after one centrifugation was obtained by subtracting the amount of drug recovered in the supernatant after the first incubation from the 150 or 300 μg originally present. The percentage of the bound CPZ released in the washing procedures (last column) was obtained by dividing the total μg present in the 8 wash supernatants by the total μg bound after one centrifugation, and by converting this to a percentage for purposes of comparison with the other data. The figure of 102 per cent recovery of CPZ bound by leukocytes at 30 μg/ml is due to the large protein loss from the tissue pellet during the wash procedures.

increased drug fluorescence at the air-fluid interface. The fluorescence of another tube's fractions is shown in curves **b** and **c**. At the bottom of this second tube, fractions 1–20 showed flourescence of 20 units, equivalent to 8 μg CPZ/ml. Below fraction 18 there was no detectable protein. The protein above this tube level (broken line curve **c**) spread over all the fractions obtained, with a peak at fraction 30–33, the peak

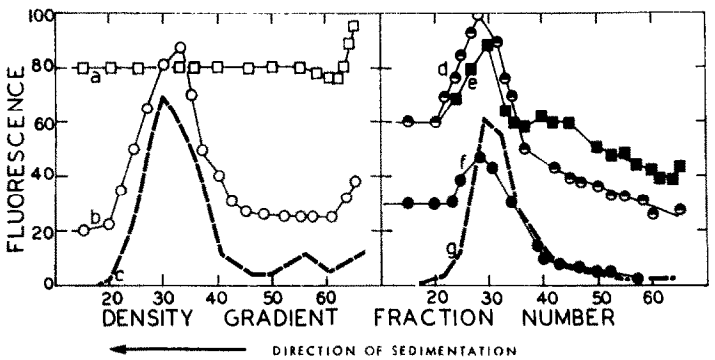

FIG. 4. Fluorescence analysis profiles of sucrose density gradients originally containing a constant level of phenothiazine after centrifugation at 30,000 *g* for 20 min with and without mitochondria; 1 mg wet weight mitochondria = 417 ± 31 (S.D.) μg protein. Curve **a** (□), a control tube; no mitochondria were layered over the CPZ at 30 μg/ml in gradient. Fluorescence was activated at 340 mμ and measured at 465 mμ. Curve **b** (○), another control tube; 20 mg rat liver mitochondria pretreated in 30 μg CPZ/ml for 1 hr, followed by centrifugation through 8 μg CPZ/ml in sucrose density gradient. Curve **c** (– – –), protein distribution (light scattering at 520 mμ, fluoresecnce at 350 mμ activated at 285 mμ) from curve **b**. Curve **d** (◓) 30 μg TPZ/ml in gradient plus 12 mg rat liver mitochondria; fluorescence activated at 360 mμ, measured at 525 mμ. Curve **e** (■), 30 μg TPZ/ml plus 8 mg rat liver mitochondria. Curve **f** (●), 15 μg TPZ/ml plus 12 mg rat liver mitochondria. Curve **g** (– – –), protein distribution of curve **f**.

of drug fluorescence (curve **b**). Protein and drug fluorescence above fraction 40 indicate that both CPZ and protein were left behind in the gradient levels through which the particles sedimented. We observed that a more prolonged treatment of the particles with > 10 μg drug/ml/mg protein (for instance for 20 min at 23°), before centrifugation through the gradient, disturbed the constant phenothiazine level in the tube and caused spreading of the mitochondrial protein peak.

Experimental. The 'sweeping' (binding activity of mitochondria passing through the gradient levels) was adjusted by changing the sedimentation time and force. This altered the duration of exposure of the particles to the phenothiazine. Forces between 10 and 20 $\times$ 10^3 *g* moved the mitochondrial band through the gradient levels slowly. The drug was almost completely removed from the upper tube levels. When the centrifugation stopped, the mitochondria were no longer 'sweeping' the drug.

Some results with TPZ are shown in Fig. 4, curves **d**–**f**. The relative fluorescence of the drug in the profile of the gradient has as its own control fluorescence in fractions below 20. As the particles sedimented, phenothiazine was removed from fractions above 35 and was concentrated into the peak shown for fractions 20–35. The sweeping of the phenothiazine above fraction 35 is shown by the line connecting the points that represent the fluorescence intensity of TPZ remaining in fractions 40–65 (Fig. 4,

curves **d** and **f**). In contrast, curve **e** shows results of centrifuging only 8 mg mitochondria through the same phenothiazine concentration used in Fig. 4, curve **d**. At fraction 45, the sweeping stopped. After the 8 mg of mitochondria passed this point during the centrifugation, it no longer bound or swept additional TPZ. When the concentration of TPZ was only 15μg/ml in the gradient, nearly all the drug was removed in the upper tube fractions by 12 mg of mitochondria (Fig. 4, curve **f**). Note that the band of protein (curve **g**), fractions 21–39, coincides with the peak of TPZ fluorescence (curve **f**), fractions 22–40.

Some examples of results from the centrifugation of rat liver mitochondria through CPZ are shown in Fig. 5. Curve A shows the bimodal CPZ fluorescence peak observed

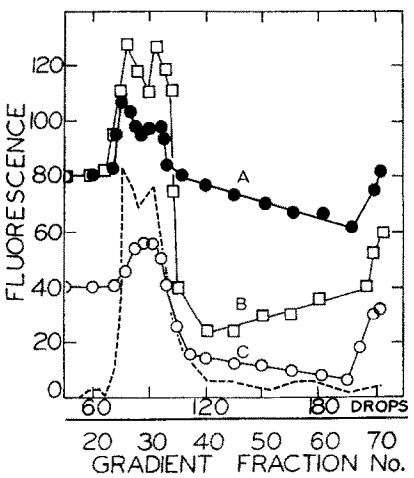

FIG. 5. Fluorescence analysis profiles of sucrose density gradients originally containing a constant CPZ concentration after centrifuging rat liver mitochondria through the gradient; 1 mg wet weight mitochondria = 417 ± 31 (S.D.) μg protein. Curve A (●), 30 μg CPZ/ml originally in gradient, plus 8·5 mg mitochondria; Curve B (□), 30 μg CPZ/ml plus 12 mg mitochondria; Curve C (○), 15 μg CPZ/ml plus 8·5 mg mitochondria; (– – –) Protein (fluorescence activated at 285 mμ, measured at 350 mμ) from experiment A. Drops were collected from fine polyethylene tubing attached to the hypodermic needle outlet of the tube puncturing device.

with a bimodal protein band (broken line) in fractions 22–35. With some concentrations of mitochondria, the removal of drug from the gradient increased as the particles sedimented (Fig. 5, curve B). Thus, with 30 μg CPZ/ml and 12 mg mitochondria, after the mitochondria moved a short distance through the gradient, they removed progressively greater amounts of CPZ from the gradient.

The results from gradient centrifugation experiments are summarized in Fig. 6 and Table 2. After 5 min of exposure (after particles passed through fractions 60–70, circles Fig. 6) to TPZ at 15 or 30 μg/ml, rat liver mitochondria removed this phenothiazine from the gradient in linear proportion to the mass of particles present. After 15 min of exposure (fractions 40–50, squares, Fig. 6), the amount of TPZ removed/ml of tube fractions was less than at 5 min, but increased more with mitochondrial increments than at the beginning of centrifugation. With 20 mg of mitrochondria, the amount of TPZ bound (in μg/ml removed from solution) was the same at 15 or 30 μg/ml, at the beginning and at the end of the centrifugation.

An apparent rate of binding was determined for two intervals during centrifugation. The first interval at 5 min after centrifugation started occurred when the particles sedimented through fractions 60–70 (circles, Fig. 6) at the top of the gradient. The second interval at fractions 40–50 (squares, Fig. 6) occurred at 15 min of exposure to

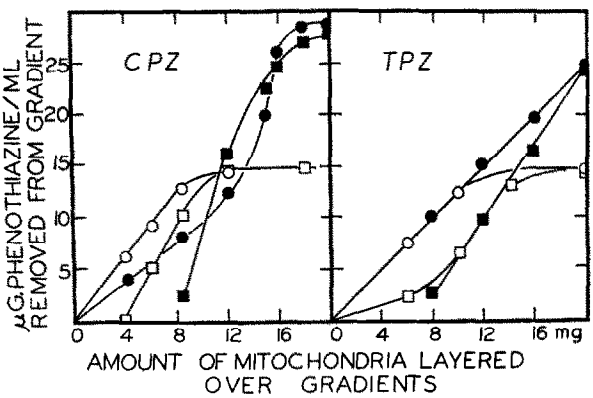

FIG. 6. Results of centrifuging rat liver mitochondria through TPZ and CPZ at 15 or 30 μg/ml after 5 and 15 min of exposure to the drug in the gradient: (○) gradient fractions 60–70, 5 min exposure to 15 μg drug/ml; (□) gradient fractions 40–50, 15 min exposure to 15 μg drug/ml; (●) gradient fractions 60–70, 5 min exposure to 30 μg drug/ml; (■) gradient fractions 40–50, 15 min exposure to 30 μg drug/ml. Experimental points are averages of duplicates centrifuged simultaneously. Mitochondrial concentration is in mg (wet weight in 0·25 M sucrose)/tube; 1 mg = 417 ± 31 μg (S.D.) protein. Centrifugation temperature 8°. The amount of phenothiazine removed from the gradient fractions was obtained by averaging the fluorescence of fractions 40–50 and 60–70 and subtracting it from fluorescence of control fractions below the protein peak (below fraction 20). Because the fluorescence of both phenothiazines is linearly proportional to their concentration between 2 and 60 μg/ml, at pH 7·2, the ordinate values are the difference in drug concentration between control and swept fractions (e,g, 12 mg mitochondria, centrifuged through a gradient containing 30 μg/ml TPZ at all levels, after 5 min removed an amount of TPZ equal to 15 μg/ml in fractions 60–70 and after 15 min removed 9·8 μg/ml fractions in 40–50; see also Fig. 4, curve d).

the drugs. The apparent binding rate equals the amount (μg) of drug removed/ml (of the fractions collected)/mg (of mitochondria in the gradient)/fraction interval (time). This rate increased with increasing mitochondrial concentrations. This is indicated by the steeper slopes of the lines connecting square symbols in Fig. 6, e.g. TPZ binding, at 15 or 30 μg/ml original gradient drug concentration during the second interval, after > 10 min of exposure to the drug in the gradient. The rate also increased with duration of exposure to CPZ at 15 or 30 μg/ml and with > 12 mg of mitochondria in the gradient.

The experimental binding rates that were calculated from straight-line portions of curves shown in Fig. 6 are shown in Table 2 after a 5- and a 15-min exposure of mitochondria at 8° to both drugs while the particles were sedimenting through the gradient. Near the end of the centrifugation, after > 10 min of exposure to the drug and while the particles sedimented past fractions 40–50, the amount of drug removed by the organelles was less than that removed initially, until 16–20 mg of mitochondria was present in either TPZ concentration. In comparison, as the concentration of CPZ was doubled, the intial (5-min) binding rate decreased 40 per cent, but the final (15-min)

rate and the minimal amount of mitochondria required to continue drug removal from the gradient after 15 min were approximately doubled. Thus, the amount and initial rate of CPZ (at 15 μg/ml) binding to < 12 mg mitochondria were greater than the amounts and initial rates of binding at 30 μg CPZ/ml. However, after 15 min of exposure to either 15 or 30 μg CPZ/ml, the rate of binding was greatly increased

TABLE 2. APPARENT BINDING OF CPZ AND TPZ BY RAT LIVER MITOCHONDRIA IN SUCROSE DENSITY GRADIENTS AFTER 5 AND 15 MIN OF CENTRIFUGATION THROUGH THE DRUGS AT 8°*

Drug	Concentration in gradient (μg/ml)	Drug removed from gradient (μg/ml/mg mitochondrial protein)	
		At 5 min (Fractions 60–70)	At 15 min (Fractions 40–50)
CPZ	15	3·7	5·3†
CPZ	30	2·2	9·9‡
TPZ	15 or 30	3·0	4·4§

* The amount of phenothiazine removed was calculated by subtracting the drug fluorescence of the fractions above the protein peak from that of control fractions below the protein peak (below fraction 20) as described for Fig. 6. Binding data for the two time intervals are the slopes of straight-line portions of curves shown in Fig. 6 multiplied by 2·398 (mg mitochondria/mg protein)

† When $>$ 4 mg mitochondria/tube was used.

‡ When $>$ 8 mg mitochondria/tube was used.

§ When $>$ 10 mg mitochondria/tube was used.

compared to the binding rate at 5 min. For example, after 15 min of exposure, 12 mg of mitochondria removed more CPZ from the gradient in fractions 40–50 than in fractions 60–70 at the beginning of centrifugation, as illustrated in Fig. 5, curve B

The results of a limited series of binding determinations with leukocyte mitochondria are shown in Table 3 for comparison to rat liver mitochondria under similar conditions. The leukocyte mitochondria contained 12 per cent less protein than the rat liver mitochondria per mg wet weight, but bound amounts of the two phenothiazines similarly to the rat liver mitochondria. To compare the binding by the small amounts of leukocyte mitochondria to binding by small amounts of rat liver mitochondria, some figures in Table 3 were extrapolated from curves shown in Fig. 6. For instance, after 15 min of exposure to 30 μg CPZ/ml there was no further binding by 4 mg of rat liver mitochondria and 'sweeping' of the phenothiazine from the gradient ceased before sedimentation stopped (Fig. 4, curve **e**). In comparison, 20 mg of mitochondria bound nearly all of the original 30 μg CPZ/ml in the upper 2·4 ml of the gradient, which increased the concentration of CPZ from $8{\cdot}4 \times 10^{-5}$ to $3{\cdot}2 \times 10^{-4}$ M in the gradient fractions that contained the particles.

DISCUSSION

Tissue studies

With tissue suspensions containing 0·2 mg protein/ml, there was no difference between the amounts of CPZ bound when the drug concentration was either 15 or 30

μg/ml. There was a difference in the total binding and release of CPZ by the two tissues. Rat liver bound 2·4-fold more CPZ/μg protein than leukocytes. Leukocytes released nearly all of the bound drug during the centrifugal 'wash' procedures, whereas rat liver released only 30 per cent of the CPZ initially bound. However, the amounts of the bound drug that were released/'wash' were similar for both tissues and were in proportion to the initial binding. The approximately linear decrease in the amount of CPZ released from the tissue pellets, between each of the first 3 to 4 'wash' steps, suggests that the mechanism involved may not be due to simple passive diffusion.

TABLE 3. PHENOTHIAZINE CONCENTRATION REMOVED FROM DENSITY GRADIENT AT TWO DIFFERENT TUBE LEVELS BY RAT LIVER AND HUMAN LEUKOCYTE MITOCHONDRIA*

Mitochondria	Initial drug concentration in density gradient							
	CPZ (15 μg/ml) (μg drug removed/ml at gradient fraction numbers)		TPZ (15 μg/ml)		CPZ (30 μg/ml)		TPZ (30 μg/ml)	
(mg wet wt./tube)	60–70†	40–50‡	60–70	40–50	60–70	40–50	60–70	40–50
Leukocyte, 4	5·8	0·7	4·7	0·6	3·2	0·8	5·2	0·7
Rat liver, 4	6·2	0·5	4·9§		3·8		4·9§	
Leukocyte, 12	13·2	13	13·2	8·7	9·7	15·2	15·4	7·9
Rat liver, 12	13·5	13·5	13·5	9	11·2	15	15	8·5

* Leukocyte mitochondria, 143·4 mg packed wet weight containing 367 $\pm$ 21 μg (S.D.) protein/mg were obtained after homogenizing 90 ml ($26·6 \times 10^9$) packed leukocytes from 12 litres of human whole blood. The mitochondria were suspended in 0·25 M sucrose–0·01 M phosphate, pH 7·2. Differential centrifugation removed granules, chromatin, platelet, and erythrocyte residues. Other conditions were as for Fig. 5.

† Less than 5 min of exposure to drug in gradient.

‡ More than 10 min of exposure to drug in gradient.

§ Calculated from curves shown in Fig. 6, since the smallest weight of liver mitochondria centrifuged through TPZ-containing gradients was 6 mg/tube.

Further interpretation of the data is complicated by concurrent protein release into supernatants by centrifugation methods that pack tissue into a pellet. Most of the protein loss occurred when the tissue was initially centrifuged from the incubation medium. This loss was greater with leukocytes than with rat liver and greater with 30 than with 15 μg CPZ/ml.

Dialysis was unsatisfactory for measurement of phenothiazine binding because the tubing absorbed varying amounts of the drug. Leukocytes in dialysis tubing sacs disintegrated after 2 hr of incubation at 8°. There was continuous leakage of amino nitrogen into the external medium, which was significant when less than 25 mg (dry weight) of leukocytes was used.

Use of fluorescence characteristics of the drugs provided very sensitive, rapid, convenient measurements of their concentration in the gradients. The fluorescence emission and activation points of the two drugs are separate from those of protein (Fig. 2). Measurements of drug fluorescence in protein suspensions are facilitated by polarizers in the spectrophotofluorometer. We did not observe appreciable quenching of drug fluorescence by protein at the wavelengths, concentrations, and pH conditions used in this study. Preliminary reports have been presented of additional studies

concerning soluble and particulate protein–drug complexes investigated by fluorescence methods.[20, 21] These are still in progress.

Mitochondrial studies

The fluorescence profile of fractions obtained after the centrifugation of these particles through the sucrose density gradient, which initially contained a constant phenothiazine concentration, provided a 'negative' image of mitochondrial binding activity and a 'positive' image of protein lost at each gradient level. The 'negative' was the disappearance or decrease of drug fluorescence in the fractions compared to the control fractions at the base of each gradient. The 'positive' was the appearance of protein fluorescence in fractions other than those showing light scattering and in the characteristic (20–40) position for mitochondria in the gradient. The experimental procedure chosen for the mitochondrial experiments obviated resuspension, agitation, and physical compaction of the particles after they came into contact with non-limiting external phenothiazine concentration. (The mechanical factors probably were responsible for the protein loss observed in the tissue studies.) Each gradient contained at the bottom a phenothiazine solution, which was not contaminated or depleted by mitochondria, as a control of the linearity of drug concentration and fluorescence for each tube. In addition, the particles did not become compacted at the tube end as they do in ordinary centrifugation procedures. Thus, puncturing the tube allowed reproducible collection of the upper gradient levels after the mitochondria and denser gradient levels drained out.

In a previous study, Spirtes and Guth[8] indicated a small increase in 10^{-5} M CPZ binding to rat liver mitochondria between 10 and 20 min after the particles were first mixed with the drug. The usual separation of mitochondria from supernatant by complete sedimentation requires at least 15 min of exposure to the drug solution. No measurement of binding is possible from the time the cell particles and drug are first mixed in the centrifuge tube until after the supernatant is separated. By centrifuging the particles into the gradient containing the drug, binding was measured after only 5 min of exposure when the mitochondria sedimented past the upper gradient layers. Any possible disadvantages of the effect of high osmolar sucrose on mitochondrial structure and enzymatic function limit the comparison of binding data obtained by the gradient centrifugation method with data obtained by other methods. However, sucrose gradients are routinely used for the preparation of brain mitochondria. In those procedures the particles are exposed to 1·2 M sucrose for >1 hr. The low temperature of 8° was employed because mitochondria incubated with the phenothiazines at room temperature release protein into solution[8] (Fig. 4).

Changes in biological and biochemical activities of enzymes, mitochondria, etc., as a function of duration of exposure to phenothiazines have been reported.[11, 22, 23] Increased exposure to the drugs usually increased the inhibition of enzymes, and this was correlated with the production of phenothiazine free radicals.[24, 25] The marked decrease in amount of drug bound after 15 min exposure at 8° to the two phenothiazines by low mitochondrial concentrations (below 8 mg/tube) suggests several possibilities. Mitochondrial binding sites may be saturated. This has been suggested by results from other methods.[20] The concept of saturation is also supported by data from experiments where there was no further CPZ binding by small amounts of mitochondria (after 15 min of exposure) beyond that of 6·2 and 4·0 μg CPZ, as

shown in Fig. 6. Perhaps increasing the relative concentration of mitochondria/drug may have prevented mitochondrial agglutination by the phenothiazines,[8, 11] because when the particles aggregate drug binding might be expected to decrease. The marked nonlinear changes in binding, evident at 30 μg/ml CPZ and with >8 mg mitochondria/tube, therefore may be due to changes in aggregation of the particles as they sedimented.

The TPZ binding rate increased after 15 min of exposure to the drug. Our results suggest that similar cooperative effects of CPZ are greater than those of TPZ on mitochondrial binding after 5 min of exposure (see Fig. 5, curve B). This may be due to particle decomposition because of surface activity by the drugs.[26] The latter is suggested by the surface concentration and the former by the bimodal protein and drug band observed in the CPZ centrifugation data shown in Fig. 5. Increased mitocondrial binding of the phenothiazines after long exposure would explain, for instance, the perinuclear fluorescent halo effect seen by Murray and Peterson[27] in tissue cultures treated with CPZ.

There is controversy concerning the validity of extrapolating results from experiments *in vitro* (using more than 10^{-5} M drug) to possible explanation of effects *in vivo*. This occurs because the extent and frequency of subcellular drug localization is often unknown. After brief incubation at 8° there is marked drug binding to mitochondria, which may increase further with time. We suggest that localized intracellular concentrations of drugs much above 10^{-4} M may also be found after relatively brief chemotherapy. These high concentrations would be due not only to drug distribution between tissues[13, 28] or 'pools', but also to more intense binding by subcellular particles.

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