it seems that the presence of a high correlation between parameter estimates may not necessarily indicate that the statistical properties of these final estimates are meaningless.

In conclusion, meaningful statistics of the final parameter estimates of E_a and t_{90} can be obtained by the nonlinear approach described in this paper. This approach is applicable over a wide range of different theoretical values of E_a and t_{90}, different orders of reaction, different levels of noise in data, and different types of data structure. The advantage of this nonlinear approach is that it uses data of drug content, time, and temperature to provide a direct estimation of shelf-life with relevant statistics. This method may be potentially useful for the realistic prediction of drug stability of pharmaceutical products.

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Behavior of ATP Toward Phenothiazine Drugs

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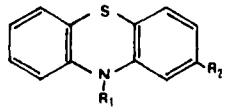
Abstract □ The tricyclic amines promazine, promethazine, chlorpromazine, triflupromazine, and trifluperazine form solid ion pairs with ATP in a 1:2 molar ratio. There is a good correlation between the measured K_{sp} and the apparent diffusion constants of the ion pairs with the critical micelle concentration (CMC) of the corresponding phenothiazines. Solid ion pairs are solubilized by phenothiazine micelles; the binding constants of ATP to drug micelles are calculated from solubility data at 25°C and can be related to the CMC of the phenothiazines.

Keyphrases □ ATP—behavior toward phenothiazine drugs, binding, micellar solubilization □ Binding—behavior of ATP toward phenothiazine drugs, micellar solubilization □ Phenothiazine drugs—behavior of ATP, binding, micellar solubilization □ Micellar solubilization—behavior of ATP toward phenothiazine drugs, binding

The mechanism of action of phenothiazine drugs is difficult to explain because of their great variety of biochemical and physiological effects. Membrane interactions seem to be important particularly in the case of chlorpromazine (1–4). Phenothiazines, like many tricyclic amines, have amphiphatic properties and are surface-active drugs (4–7). Their micelles are able to solubilize *in vitro* various high molecular weight drugs such as pteridine and porphyrin derivatives (8, 9); the binding constants of solubilized compounds with micelles are rather high. Previously, Blei (10) studied the decrease of chlorpromazine surface tension in the presence of ATP, while Moriguchi *et al.* (11) observed the formation of a 1:1 complex between the neuroleptic agent and ATP.

The aim of the present work is to investigate the behavior of five phenothiazines with different pharmacological activity



Promethazine	$R_1 = CH_2CH(CH_3)N(CH_3)_2$	$R_2 = H$
Promazine	$R_1 = CH_2CH_2CH_2N(CH_3)_2$	$R_2 = H$
Chlorpromazine	$R_1 = CH_2CH_2CH_2N(CH_3)_2$	$R_2 = Cl$
Triflupromazine	$R_1 = CH_2CH_2CH_2N(CH_3)_2$	$R_2 = CF_3$
Trifluperazine	$R_1 = CH_2CH_2CH_2$—N N—CH_3	$R_2 = CF_3$

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toward ATP, paying attention particularly to variations in the lipophilicity of ATP in the presence of the drugs and to its possible solubilization by phenothiazine micelles.

EXPERIMENTAL

Materials—Promethazine hydrochloride[1], promazine hydrochloride[1], chlorpromazine hydrochloride[1], trifluperazine hydrochloride[1], and triflupromazine hydrochloride[1], ATP[2], non-reinforced dimethylpolysiloxane[3] sheeting in a labeled thickness of 5 mil [12.5×10^{-3} cm, thoroughly rinsed and treated as previously described (12)]; 0.25-μm filter disks[4], Sephadex G-25 medium[5], and glass microfiber papers[6] were commercial samples. A pH meter[7], a spectrophotometer[8], a plate tensiometer[9], a conductimeter[10], and an automatic fraction collector[11] were also used.

Preparation of Solid Complexes—The solid complexes were obtained in aqueous media by mixing 50 mL of ATP (8.0×10^{-3} M) with 50 mL of tricyclic amine ($16.0\text{-}32 \times 10^{-3}$ M). The suspensions were pH 3.5-4.0. All the phenothiazines formed a precipitate, which was separated and analyzed. The same molar ratios of ATP-phenothiazine were employed throughout. The pH was fixed at 5.5 and 6.0 by adding 0.1 M NaOH and 0.1 M 2-morpholineethansulfonate buffer, respectively. This buffer was selected because permeability measurements have shown (12) that it does not form ion pairs with phenothiazines.

Solutions at fixed concentrations of ATP (4.0×10^{-3} M) and chlorpromazine (8.0×10^{-3} M) and increasing concentrations of calcium chloride (2.0×10^{-3}, 4.0×10^{-3}, and 8.0×10^{-3} M) were prepared. No solid complex was obtained from the solution with 8.0×10^{-3} M calcium chloride.

Analysis of Solid Complexes—Solid ion pairs (8-15 mg) were treated with 10 mL of 2 M HCl, and the solution was heated for 15 min in a water bath to promote ATP hydrolysis, then diluted to 25 mL with 2 M HCl. ATP was determined by the malachite green method (13) to evaluate the phosphate obtained by hydrolysis.

The concentration of phenothiazine was determined spectrophotometrically at 310 nm (chlorpromazine, log ϵ = 3.62; promazine, log ϵ = 3.56; promethazine, log ϵ = 3.56; triflupromazine, log ϵ = 3.43; and trifluperazine, log ϵ = 3.60). ATP does not interfere at this wavelength.

The analysis data are reported in Table I. Each molar ratio is the average of three determinations. All the phenothiazines studied form a 1:2 solid complex with ATP. A 1:2 molar ratio was also obtained for the solid ATP-chlorpromazine complex formed at different molar ratios of reactants and in the presence of calcium chloride. In Table II the yields of the solid complex obtained in different media are listed.

Determination of Solubility Products—*Spectrophotometry at 25°C*—Glass-stoppered cylinders containing an excess of solid complex in 6 mL of water were shaken in a water bath kept at 25 ± 0.5°C for 24 h. The samples were then filtered through 0.25-μm filter disks. The ATP and phenothiazine concentrations were determined by the malachite green method and absorbance measurements, respectively, on the filtrate as previously described. The results reported in Table III confirm an ATP-tricyclic amine molar ratio of 1:2.

Conductimetry—Twenty-five milliliters of the drug solution was placed in the titrimeter cell kept at 25 ± 0.5°C. A concentrated solution of ATP was then added; the rate of addition was generally between 0.1 and 1 mL/min, with titration usually complete within 5-10 min. Complexation between the two ions was taken to be the point on the conductimetric titration trace where a marked change in the gradient is observed (Fig. 1a).

Several titration curves were determined using different concentrations of phenothiazines. Double-logarithmic plots were then constructed, reporting ATP concentrations at the break point against drug concentrations. All the studied systems exhibited a straight-line relationship between cation and anion concentration (Fig. 1b), showing that, over the concentration ranges studied, the areas of ion self-association have been avoided (14, 15). Analysis of these double-logarithmic plots gave slopes of ~0.5; the intercept was at log $K_{sp}/2$. By substituting the values:

$$\log [\text{drug}] = \frac{\log K_{sp}}{2} - \frac{\log [\text{ATP}]}{2} \qquad \text{(Eq. 1)}$$

[1] Rhône-Poulenc.
[2] Merck.
[3] Silastic.
[4] Millipore.
[5] Pharmacia.
[6] Whatman.
[7] Orion-701/A.
[8] Perkin-Elmer EPS-3T.
[9] Dognon-Abribat.
[10] Amel Model 123.
[11] LKB-Redirec 2112.

Table I—Experimental Molar Ratios [a] for ATP-Phenothiazine Complexes

Complex	Amount, g	ATP, mol $\times 10^{-5}$ [b]	Phenothiazine, mol $\times 10^{-5}$ [b]
ATP-chlorpromazine	0.0150	1.2	2.5
ATP-promazine	0.0080	0.65	1.4
ATP-promethazine	0.0100	1.0	2.1
ATP-triflupromazine	0.0150	1.0	2.2
ATP-trifluperazine	0.0130	1.0	1.9

[a] Determined spectrophotometrically. [b] Standard deviation of each value is within 5-10%; *e.g.*, for the ATP-chlorpromazine complex, the molar ratio, averaged over three separate measurements, is 2.08 ± 0.11. If the molar ratio is averaged over all the measurements concerning all the complexes (assuming that the ratio is the same for all the phenothiazines) a value of 2.09 ± 0.08 is obtained.

Table II—Solid ATP-Phenothiazine Complexes (1:2) Formed in Solutions of ATP [a] and Phenothiazine Drugs [b] in Water and in the Presence of Increasing Concentrations of $CaCl_2$

	Yield, %					
				In the Presence of $CaCl_2$		
				2.0×10^{-3}	4.0×10^{-3}	8.0×10^{-3}
Drug	H_2O [c]	pH 5.5	pH 6.0	M	M	M
Promethazine	4.2	1.8	7.5	—	—	—
Promazine	19.1	7.4	31.9	—	—	—
Chlorpromazine	26.8	35.0	44.2	18.1	13.8	0
Triflupromazine	30.0	25.5	42.3	—	—	—
Trifluperazine	30.0	42.5	44.9	—	—	—

[a] 4.0×10^{-3} M. [b] 8.0×10^{-3} M. [c] pH 3.5-4.0.

Table III—Solubility Products of Phenothiazine-ATP Ion Pairs

	K_{sp}, $(\text{mol/L})^3$		
	25°C		37°C
Drug	Spectrophotometry	Conductimetry	Conductimetry
Promethazine	6.1×10^{-8}	8.3×10^{-8}	9.3×10^{-8}
Promazine	4.6×10^{-8}	4.7×10^{-8}	5.3×10^{-8}
Chlorpromazine	1.4×10^{-8}	1.6×10^{-8}	1.5×10^{-8}
Triflupromazine	9.2×10^{-9}	9.2×10^{-9}	1.2×10^{-8}
Trifluperazine	1.0×10^{-8}	8.7×10^{-9}	1.1×10^{-8}

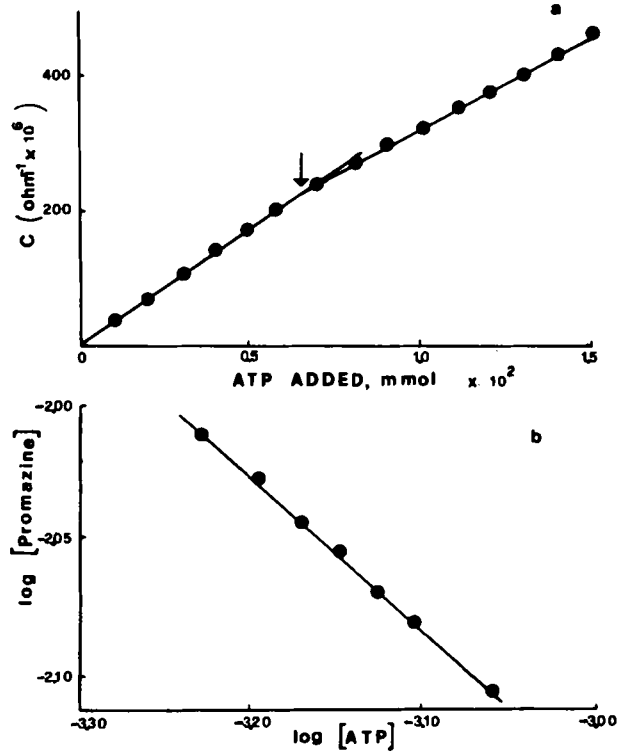

Figure 1—*(a) Conductimetric titration for ATP* versus *promazine at 25°C, original promazine concentration: 9.0×10^{-3} M. (b) Double logarithm plot for the promazine-ATP system of the concentration of each interacting ion at the K_{sp} point: slope 0.47.*

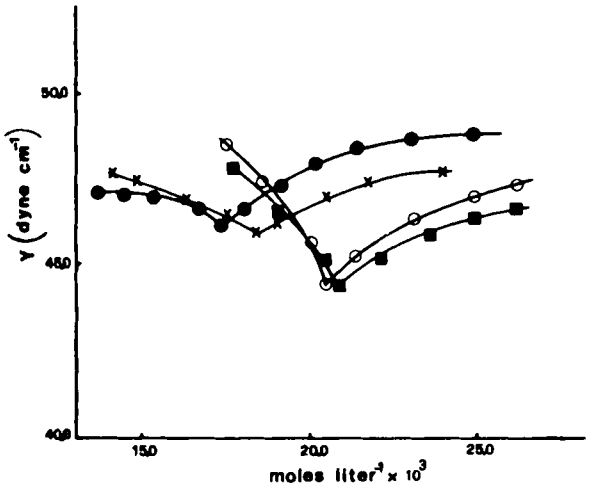

Figure 2—*Surface tension* versus *the molar concentration of chlorpromazine at 25°C. Key: (○) chlorpromazine alone; (●) chlorpromazine in the presence of 0.8 × 10⁻⁴ M ATP; (×) chlorpromazine in the presence of 0.8 × 10⁻⁴ M ATP and 0.6 × 10⁻⁴ M $CaCl_2$; (■) chlorpromazine in the presence of 0.8 × 10⁻⁴ M ATP and 0.8 × 10⁻⁴ M $CaCl_2$.*

a 1:2 interaction between the ATP and the drug was inferred.

Determination of the Critical Micelle Concentration—The critical micelle concentrations (CMC) of phenothiazines were determined by measurements of surface tension using a plate tensiometer at 25 ± 0.5°C in 0.9% NaCl at pH 6.0. The CMC of chlorpromazine was also determined in the presence of ATP and of calcium ions ([ATP] = 0.8 × 10⁻⁴ M; [Ca^{2+}] = 0.6 × 10⁻⁴ M ÷ 0.8 × 10⁻⁴ M) (Fig. 2).

Determination of the Apparent Diffusion Constant—The diffusion cell was of the type used by Nakano and co-workers (16, 17). The glass cell consists of donor and receptor compartments (volume of each, 20 mL) and a membrane (available area, 3.80 cm^2). Twenty milliliters of 0.01 M HCl were added to one arm, and an equal volume of the test solution was placed in the other arm. The concentration of the diffusible phenothiazine drug in the desorbing hydrochloric acid solution was kept at zero, maintaining diffusing phenothiazine in a dissociated form. All solutions were brought to 37°C before being placed in the cell, which was kept at 37°C. The contents of each compartment were stirred by a magnet attached to an electric motor turning at 300 rpm. At scheduled times an aliquot (0.5 mL) of the receptor solution was removed for the absorbance measurements, and the same volume of 0.01 M HCl was added.

The equation derived by Garrett and Chemburkar (18) for steady-state diffusion was used to obtain the apparent diffusion constants, $D = C_1XV/tC_2S$ where C_1 is the concentration of diffusate in the desorbing solution, X is the thickness of the membrane, S is the available area of membrane, V is the volume of the desorbing solution, and C_2 is the concentration of the diffusate. The diffusing solution in 0.9% NaCl at pH 6.0 at 37°C contained 0.8–1.0 × 10⁻³ M tricyclic amine and an ATP concentration eight times the molarity of the drug. D values are reported in Table IV.

Determination of the Binding Constants of ATP to Phenothiazine Micelles by a Solubility Method—Increasing concentrations of tricyclic amines (0.5–40.0 × 10⁻³ M) were added to the solutions of ATP (1.0 × 10⁻³ M) in glass-stoppered cylinders at pH 6.0 in 0.9% NaCl. The cylinders were placed in a water bath and rotated at 25 ± 0.5°C until equilibrium was reached. Agitation for 24 h was sufficient. The ionic strength of the solutions was brought to 0.155 with 0.9% NaCl; therefore, the small amounts of sodium hydroxide used for pH adjustment did not appreciably influence the ionic strength. After 24 h an appropriate volume (4 mL) was filtered through a 0.25-μm filter disk. The concentration of ATP was determined by the malachite green method; each value is an average of three determinations. Comparable results were obtained using glass microfiber papers instead of filter disks. The same procedure was employed to study the solubility of the ATP–chlorpromazine complex at increasing concentrations of chlorpromazine in the presence of a fixed concentration of calcium chloride (7.5 × 10⁻³ M).

The solubility measurements for all the studied phenothiazines were also performed in 0.1 M 2-morpholineethansulfonate buffer (pH 6.0). The results are in agreement with previous data. Variation in the solubility of the ATP–phenothiazine complex was quite similar to the variation of the solubilization of slightly soluble hydrophobic solutes by aggregating micelle-forming surfactants (19, 20). At first, ATP concentration decreases because of solid complex formation; at higher concentrations of the drug, ATP concentration increases since the solid ion pair is solubilized by surfactant micelles. From the solubilization curves the apparent binding constants of ATP with phenothiazine micelles were calculated using (21):

$$\frac{S_{M,W}}{S_W} = 1 + K_bC \qquad \text{(Eq. 2)}$$

where S_W is the concentration of ATP at the CMC, $S_{M,W}$ is the concentration in the presence of micelles of the drug, and C is the surfactant concentration (molarity), which exceeds the CMC value (Table V).

Determination of the Binding Constant of ATP with Chlorpromazine Micelles by Gel Filtration—The binding constant (K_b) of ATP with chlorpromazine micelles was determined by gel filtration according to Herries *et al.* and Yatsimirskii *et al.* (21, 22). The chromatography of Sephadex G-25 was carried out on a 2-cm diameter column with a total volume of 72 mL. Using blue dextran 2000, the void volume V_0 of the packed column was determined to be 29.5 mL. The imbibed volume of the gel V_i, calculated from the water regain and wet density data, was 34.0. Prior to each run the column was first equilibrated with 0.9% NaCl at pH 6.0 and then with the appropriate concentration of chlorpromazine (in 0.9% NaCl at pH 6.0). The run was initiated by addition of 0.5 mL of ATP solution (2 mg/mL) to the column. All runs were done at 25°C. The elution volume V_e was calculated by ATP determined according to the method previously described. The K_b value was determined using (22, 23):

$$\frac{V_i}{V_e - V_0} = \frac{\overline{V}(K_p - 1)}{k'K_d}C + \frac{1}{k'K_d} \qquad \text{(Eq. 3)}$$

where V_i, V_e, and V_0 are the imbibed (stationary), elution, and void volumes, $\overline{V}$ is the molar volume of chlorpromazine in the micelle, K_d is the molecular sieving constant, k' is the proportionality constant between the solute absorbed per unit volume of gel matrix and the equilibrium concentration of monomer solute in liquid, C is the concentration of chlorpromazine, and K_p is the micelle–water partition coefficient of ATP. A plot of the left-hand side of Eq. 3 ($V_i/V_e - V_0$) *versus* chlorpromazine concentration (C) is shown in Fig. 3. At low drug concentrations the values of $V_i/V_e - V_0$ are independent of the concentration of the surfactant. Above the CMC, the values of $V_i/V_e - V_0$ increase linearly with the micelle concentrations.

The product $\overline{V}(K_p - 1)$ can be determined from the slope of the straight line according to Yatsimirskii *et al.* (21). The binding constant is then calculated as $K_b = \overline{V}(K_p - 1)$. Intersection of the lines in Fig. 3 define the CMC of chlorpromazine hydrochloride in the presence of the solute and of Sephadex G-25. The CMC is about twice as great as that determined by the surface tension method. According to other authors (23, 24), such a discrepancy is probably due to strong interaction between the Sephadex dextran and chlorpromazine. In Table V the K_b value obtained by gel filtration is reported.

Table IV—Apparent Constants of Diffusion Through Dimethylpolysiloxane Membrane at 37°C of Phenothiazines[a] Alone and in the Presence of ATP

Drug	D, L/s·cm: Alone	D, L/s·cm: In the Presence of ATP[b]
Promethazine	1.3 × 10⁻¹⁰	4.6 × 10⁻¹⁰
Promazine	1.4 × 10⁻¹⁰	5.3 × 10⁻¹⁰
Chlorpromazine	1.7 × 10⁻¹⁰	16.1 × 10⁻¹⁰
Triflupromazine	4.7 × 10⁻¹⁰	14.8 × 10⁻¹⁰
Trifluperazine	2.7 × 10⁻¹⁰	21.4 × 10⁻¹⁰

[a] In 0.9% NaCl, pH 6.0. [b] Eight times the concentration of the tricyclic amines.

Table V—Binding Constants of ATP to Phenothiazine Micelles[a]

Drug	K_b: Solubility Method, In the Absence of $CaCl_2$, micelles/L	K_b: Solubility Method, In the Absence of $CaCl_2$, mol/L	K_b: Solubility Method, In the Presence of $CaCl_2$, micelles/L	K_b: Solubility Method, In the Presence of $CaCl_2$, mol/L	K_b: Gel Filtration Method, mol/L
Promethazine	1215	45	—	—	—
Promazine	1665	45	—	—	—
Chlorpromazine	3360	80	1974	47	79
Triflupromazine	8000	200	—	—	—
Trifluperazine	6000	120	—	—	—

[a] In 0.9% NaCl, pH 6.0, at 25°C.

RESULTS

ATP interacts with phenothiazine drugs to form water-insoluble complexes. A 1:2 ATP–drug complex was obtained (Table I) in the presence and absence

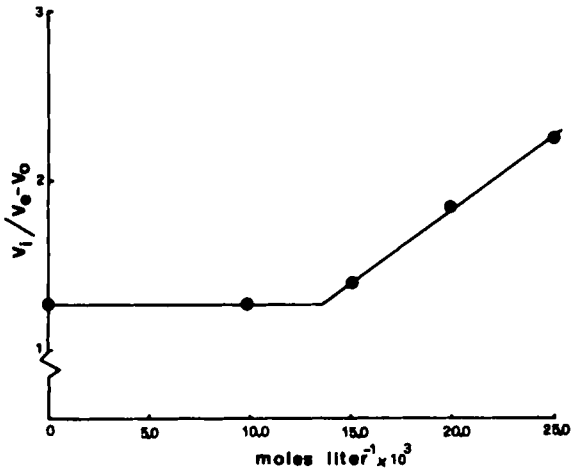

Figure 3—*Plots of* $V_i/V_e - V_0$ versus *chlorpromazine molar concentration.*

of calcium ions. Trifluperazine with two basic sites on the piperazine ring shows a similar behavior. The yields change with the different drugs examined and are pH dependent; all the phenothiazines show a maximum at pH 6.0, where ATP dissociation is highest (Table II). The formation of the ion pairs at pH > 6.0 was not studied because of the partial coprecipitation of some phenothiazine bases.

The CMC of chlorpromazine (Fig. 2) varies in the presence of ATP and calcium ions; by adding only ATP at low concentration, the CMC decreases, probably due to the greater hydrophobicity of the ion pairs. If calcium ions are then also added to the solution, the CMC rises and when the molarity of calcium and ATP are equal, the initial CMC is obtained. This behavior could indicate that a high calcium ion concentration interferes with ion pair formation.

The permeation rate of different phenothiazines through a dimethylpolysiloxane membrane increases in the presence of ATP (Table V). The effect of ATP on the apparent diffusion constant (D) was studied examining solutions containing ATP at concentrations only eight times the molarity of the drug, to avoid the precipitation of the solid complex. Phenothiazine micelles solubilize ATP, giving high apparent binding constants (K_b). The concentration of the drug was indicated in two ways: as molar concentration (C) and as micellar concentration (C_m), where $C_m = (C - \text{CMC})/N$ (N is the aggregation number obtained from the literature (6, 25) assuming that the solubilized compound does not change the size of the micelles). The binding constant of ATP with chlorpromazine micelles decreases in the presence of calcium ions in aqueous solution. The K_b of ATP with chlorpromazine was verified by two methods, obtaining comparable values within the limits of experimental error.

DISCUSSION

The solubility products of the 1:2 solid complexes, obtained by two methods (Table IV), are in good agreement with the yields obtained at pH 6.0. The sequence of solubility products is consistent with that of the surface tensions of the phenothiazine drugs in Ringer's solution at pH 6.0 (26).

A good correlation was also established between the logarithm of the solubility product and the logarithm of the CMC of the phenothiazine involved in the interaction (Fig. 4a, b). A similar dependence was previously observed (15). These results suggest that the increased hydrophobic nature of the interacting amines raises the ion pair stability. Further support to this hypothesis can be noted from Fig. 5, where log D *versus* CMC of phenothiazine is reported. The CMC describes the lipophilicity of the amphiphilic substances; the relation between log D and log CMC shows a linear trend and could be an indirect measure of the lipophilicity of the ion pair. As a consequence, in the presence of phenothiazines at concentrations lower than the CMC, the lipophilicity of ATP greatly increases.

Baur (27) noted that for tricyclic amines the tranquilizing potency rises exponentially with increasing lysis index, a measure of the relative hemolytic potency. Using the lysis index of chlorpromazine (*i.e.*, 1) (27) for comparison, we note a relationship between log (lysis index) and log D of the phenothiazines in the presence of ATP (Fig. 6); permeability data, in this case, can be related to data obtained *in vivo*. On the contrary, no relationship can be found between permeability constants of the ion pairs and partition coefficients (27, 28) or pK_a values (29) of the phenothiazines.

It was shown that certain drugs can be absorbed in their undissociated state,

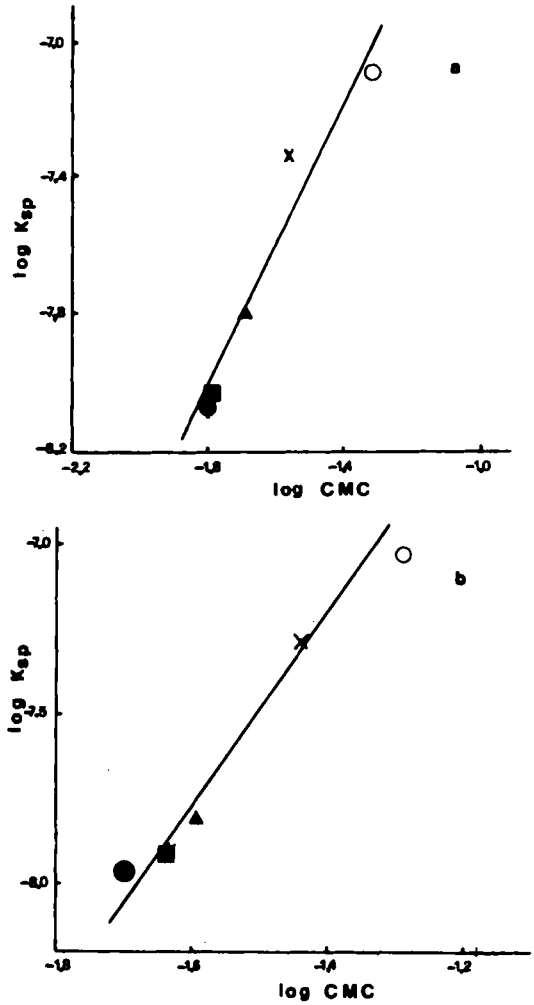

Figure 4—*Relationship between solubility products (log* K_{sp}*) of the ion pairs determined by conductimetry and log CMC of the phenothiazines at 25°C (a) and at 37°C (b). Key: (○) promethazine; (×) promazine; (▲) chlorpromazine; (■) triflupromazine; (●) trifluperazine.*

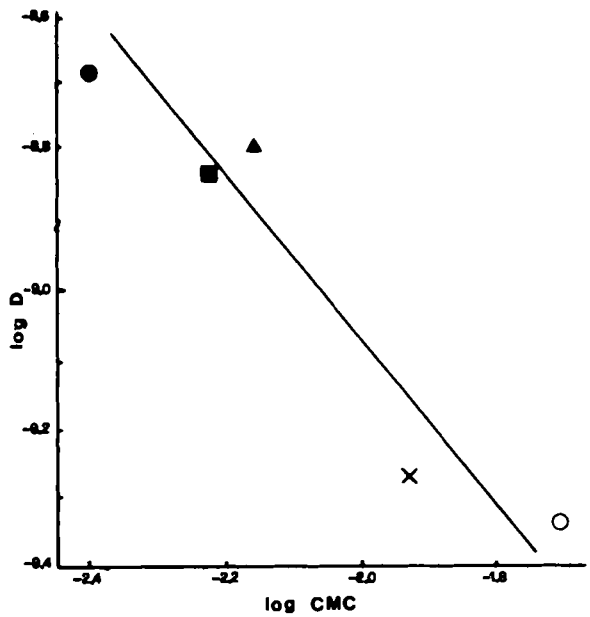

Figure 5—*Relationship between apparent diffusion constants* D *of phenothiazines in the presence of ATP and log CMC (0.9% NaCl, pH 6.0, at 37°C). Key: (○) promethazine; (×) promazine; (▲) chlorpromazine; (■) triflupromazine; (●) trifluperazine.*

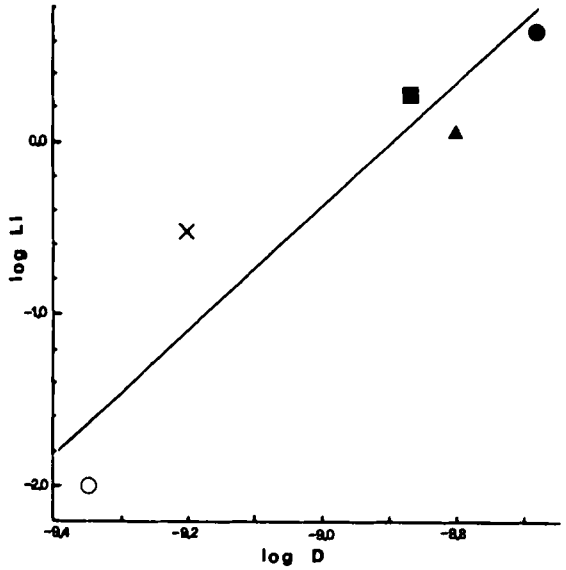

Figure 6—*Relationship between lysis index (LI) and apparent diffusion constants* D *of phenothiazines in the presence of ATP (0.9% NaCl, pH 6.0, at 37°C). Key: (○) promethazine; (×) promazine; (▲) chlorpromazine; (■) triflupromazine; (●) trifluperazine.*

either directly or by ion pair or complex formation[12]; but ion pairs can also be formed in biological fluids, *e.g.*, between basic drugs and biogenic acids. The role of ATP in the uptake and in the release of biogenic amines is complex and not clearly understood. The behavior of chlorpromazine in the release and uptake of serotonin and biogenic amines from storage sites has not yet been explained (30). In particular, the existence of serotonin–ATP aggregates (up to 12,000–15,000 mol. wt.) was shown by Berneis *et al.* (31) in the storage organelles of blood platelets. Chlorpromazine may interfere with ATP–serotonin aggregates in storage organelles. It is possible that the hydrophobicity of the depressant at first promotes its transport through biological membranes. Subsequently, chlorpromazine might form an ion pair with ATP, partially removing the nucleotide from its aggregates with serotonin. The consequence might be an interference in the uptake mechanism of the monoamine.

Basically, we can regard the micelles as dispersed oil droplets in water (32); the peculiar solvent property of micelle-forming surfactants is closely related to the peculiar nature of the self-association responsible for the formation of the micelles themselves. Micellar solubilization can provide considerable insight into the nature of the interaction of molecules with other lipid assemblies, such as biological membranes, which are responsible for the binding or uptake of molecules and their transport (33).

Figure 7 shows clear trends that suggest a linear correlation, between log CMC and log K_b; probably, lipophilicity of phenothiazines could determine the binding stability between ATP and the micelles. Further support for this

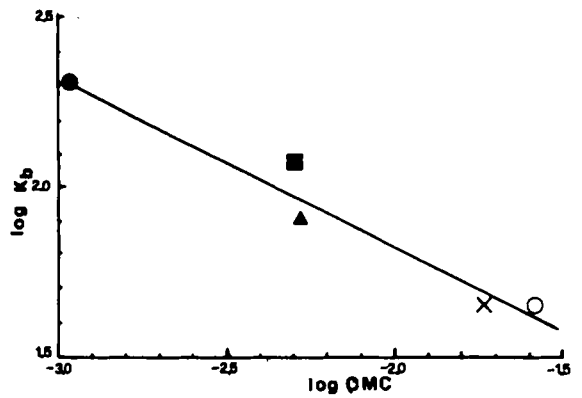

Figure 7—*Relationship between binding constants of ATP to phenothiazine micelles and log CMC of the drugs (0.9% NaCl, pH 6.0, at 25°C). Key: (○) promethazine; (×) promazine; (▲) chlorpromazine; (■) triflupromazine; (●) trifluperazine.*

[12] Unpublished results.

hypothesis arises from the increase of the binding constants (Table V) going from promethazine, an antihistaminic drug, to neuroleptics. The location and distribution of ATP inside micelles could be of interest in elucidating the role of its molecular structure in the solubilization process and in understanding the solubilization capacity of micelles. Phenothiazines are ionic surfactants and their micelles (34) have a core formed from their tricyclic moiety with their cationic head-group outside.

Previously (8, 9), we noted that phenothiazine micelles can solubilize polar molecules; their solubilization occurs, in all cases examined, only when the formation of an ion pair is noted. The solubilization of ATP by phenothiazine micelles can be explained in several ways. Solubilization of ATP might occur as the poorly dissociated ion pair; the lipophilicity of the ion pair is greater than that of ATP, as permeability constants show, for the presence of two phenothiazine hydrophobic molecules. Furthermore, solubilization of the ion pair could be facilitated because the phenothiazine molecules forming the complex are the same as those forming the micelles.

It is possible that *in vivo* bioactive molecules can be solubilized into micelles formed by a drug alone, into mixed micelles of drug and phospholipid, or into membranes, by an ion pair mechanism. Such an interaction might be responsible in part for the activity of drugs and endogenous substances.

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High-Performance Liquid Chromatographic Determination of Tracazolate and Its Major Metabolite in Plasma

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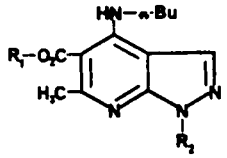
Abstract □ A high-performance liquid chromatographic (HPLC) method is reported that provides for either separate or simultaneous plasma determination of tracazolate and its major metabolite, the free carboxylic acid. Tracazolate is extracted from plasma with hexane and is quantitated by reverse-phase HPLC using an internal standard. The metabolite is extracted in an additional step, methylated, and either quantitated using the same HPLC conditions in a separate determination or combined with the hexane extract for the simultaneous HPLC determination of drug and metabolite. The plasma concentrations of metabolite were as much as 60 times greater than free drug in some animal species, therefore requiring the use of separate determinations for the two compounds. The analyses have limits of reproducible quantitation of 20 ng/mL for tracazolate and 50 ng/mL for the metabolite in plasma. The simultaneous analysis for tracazolate and the metabolite was used to evaluate plasma levels of both compounds *versus* time and dose. Data generated in conjunction with a toxicology study are reported to demonstrate the applicability of the procedure.

Keyphrases □ Tracazolate—free carboxylic acid metabolite, individual and simultaneous assay in plasma, HPLC □ HPLC—tracazolate and its free carboxylic acid metabolite, individual and simultaneous assay in plasma

Tracazolate (I) (ICI 136,753; 4-(butylamino)-1-ethyl-6-methyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxylic acid ethyl ester), a nonbenzodiazepine agent with potential clinical utility as a nonsedative anxiolytic drug (1, 2), is extensively metabolized by a first-pass effect in the rat and dog. The major metabolite (II) is the acid resulting from deesterification of I (3). Although II is inactive in pharmacological screening tests, it is of interest since the circulating levels can be 3- to 60-fold greater than I depending on the species and dosage. Metabolite (II) may be the only drug-related compound in sufficient concentration to measure in plasma, therefore, the quantitation of II may provide an indication of absorption of I at low dosages should plasma levels of I fall below the limits of detection.

The analytical procedures described herein allow for either the separate determination of I and II or for the simultaneous determination of both in plasma. The internal standard used was the 1-butyl analogue of tracazolate (III). Compound IV is the methyl ester of II.



I: $R_1 = R_2 = -CH_2CH_3$ (mol. wt. 304)
II: $R_1 = H$; $R_2 = -CH_2CH_3$ (mol. wt. 276)
III: $R_1 = -CH_2CH_3$; $R_2 = -(CH_2)_3CH_3$ (mol. wt. 332)
IV: $R_1 = -CH_3$; $R_2 = -CH_2CH_3$ (mol. wt. 290)

EXPERIMENTAL

Chemicals and Reagents—All organic solvents used were HPLC grade[1]. The water was purified by ion exchange, activated charcoal filtration, and distillation. Ammonium hydroxide was high purity[2], and 10% aqueous trichloroacetic acid was made from reagent-grade trichloroacetic acid[3]. The methylation of II was carried out with diazomethane[4] (4).

Instrumentation—A liquid chromatograph fitted with an automatic sampler[5] was used with a variable-wavelength UV detector[6] set at 240 nm. The columns used were: a 30 cm × 3.9 mm i.d. stainless steel precolumn (prior to the injector) hand packed with 30–38-μm pellicular packing[7], a 3-cm guard cartridge (between the injector and the analytical column) packed with 5-μm octadecylsilane-bonded packing (ODS)[8], and a 25 cm × 4.6-mm i.d. stainless-steel analytical column packed with 7-μm spherical ODS packing[9]. The mobile phase of methanol–water–20% NH_3OH (90:10:0.05 v/v/v) was pumped at ~2 mL/min and produced ~3000 psi back-pressure. A strip-chart recorder[10] provided a real time record of the chromatography; however, the quantitation was done by computer[11]. The detector signal for the computer was tapped prior to attenuation and had an output of 1 V/AU.

Standard Solutions—Stock solutions of I, II, IV, and the internal standard (III) (1.0 mg/mL) were prepared in methanol. Aliquots of the above stock solutions were further diluted in methanol as spiking solutions. Standard solutions of I, III, and IV were brought to volume with mobile phase solution and were chromatographed directly. Ester IV was previously synthesized in bulk and was used directly to make a stock solution. Matrix standards were prepared with control plasma, 200 μL in a 15-mL centrifuge tube, and spiked with methanol solutions of I and III, for the analysis of I only; II only for the separate analysis of II; or I, II, and III for the dual analysis of I and II. Matrix blanks were not spiked. The internal standard (III) was added to samples obtained from dosed animals for either the single or dual analysis where I was being determined. A 20-μL aliquot of a 1.0-μg/mL internal standard solution in methanol was added to the 200-μL plasma aliquots. The resulting concentration of internal standard was 110 ng/mL.

Sample Preparation—For the extraction of I, 2.5 mL of *n*-hexane was added to each plasma sample in a 15-mL centrifuge tube, vortexed for 30 s, and centrifuged. The hexane layer containing I and III was removed with a Pasteur pipet, and if I was to be measured it was transferred into a labeled test tube. If II was to be extracted, a 10% trichloroacetic acid solution (0.04 mL) and 2.5 mL of methyl *tert*-butyl ether were added to the aqueous residues, which were then vortexed and centrifuged. The methyl *tert*-butyl ether layer containing II was removed with a Pasteur pipet and transferred into a test tube. If both I and II were to be determined simultaneously the methyl *tert*-butyl ether layer was added to the *n*-hexane layer from the previous extraction. The

[1] Burdick and Jackson; methanol, *n*-hexane, methyl *tert*-butyl ether, and ether; USI Chemicals: 200 proof ethanol.
[2] Baker Chemicals; "Ultrex" grade 20% ammonium hydroxide.
[3] Baker Chemicals; trichloroacetic acid.
[4] Aldrich Chemical Co., diazald (*N*-methyl-*N*-nitroso-*p*-toluene); Baker Chemical Co., glacial acetic acid (diluted 25% in water); Fisher Chemical Co., potassium hydroxide (0.7 M in 10% v/v water–ethanol).
[5] Model 1081B HPLC; Hewlett-Packard, Palo Alto, Calif.
[6] Spectromonitor III (model 1204); Laboratory Data Control, Riviera Beach, Fla.
[7] 30–38-μm HC Pellosil; Whatman, Clifton N.J.
[8] Guard Holder and 5 μm C18 cartridges; Brownlee, Santa Clara, Calif.
[9] Zorbax ODS packing and columns; DuPont, Wilmington, Del.
[10] Model 391 recorder; Linear Instruments, Irvine, Calif.
[11] Model 3356 Laboratory Automation System; Hewlett-Packard, Palo Alto, Calif.

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