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Contribution of trifluoperazine/lipid ratio and drug ionization to hemolysis

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Abstract

The interaction of the antipsychotic drug trifluoperazine (TFP) with membranes was investigated in terms of lipid phase perturbation. TFP partition coefficients (P) were measured by phase separation between octanol/water and model membranes/water. The profile of P values at pH 7.4 was: microsomes (7172 ± 1229) > liposomes (1916 ± 341) > erythrocyte ghosts (1380 ± 429) > octanol (452 ± 55). Hemolytic experiments showed a biphasic, protective (at lower concentrations) and hemolytic effect above the CMC (42 μM at pH 7.4) of the phenothiazine. By applying classical treatments for surface active compounds to the hemolytic curves, we could calculate P values in whole erythrocyte cells. The preferential binding of uncharged to charged TFP in the membrane was discussed, since it results in a ionization constant (pK_{app}) different from that observed in the aqueous phase (pK). The TFP ionization constant was decreased from 8.1 (in water) to 7.62 in the presence of membranes and almost the same ratio of charged/uncharged TFP species is present at physiologic pH. Taking into account the ΔpK, we calculated the average TFP partition coefficient between egg phosphatidylcholine liposomes and water, at pH 7.4 ($P_{average} = 1432$), which was well correlated with the measured one ($P^{lip} = 1916$). $P_{average}$ is highly influenced by the uncharged TFP species and the real base/acid ratio under physiologic conditions was discussed in terms of its possible role in the biological activity of TFP.

Keywords: Trifluoperazine; Erythrocyte; Hemolysis; Membrane solubilization; pK shift

1. Introduction

Phenothiazines are known as neuroleptics and antihistaminic agents [1]. Trifluoperazine (TFP), a phenothiazine derivative, is a widely used calmodulin antagonist and one of the most effective antipsychotic agents [2].

Abbreviations: CMC, critical micellar concentration; CPZ, chlorpromazine; C_{sat}, solute concentration for the onset of hemolysis; C_{sol}, solute concentration for total lysis; EPC, egg phosphatidylcholine; EPR, electron paramagnetic resonance; P, partition coefficient; PC, phosphatidyl choline; pK, ionization constant in water; pK_{app} ionization constant measured in water but in the presence of a membrane; R_e, solute/lipid molar ratio; TFP, trifluoperazine; TFP+, charged form; TFP:, neutral form

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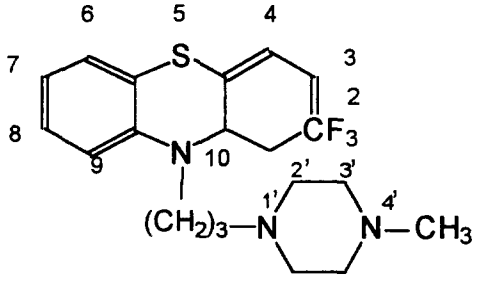

Trifluoperazine

The interaction between phenothiazines and erythrocyte membranes has been extensively studied. Seeman [3] described the biphasic effect of TFP on hemolysis: at micromolar concentration, it protects erythrocytes against hypotonic lysis, whereas at higher concentrations (>0.1 mM) it produces hemolysis. Another phenothiazine, chlorpromazine (CPZ), produces small holes in the membrane, suggesting a colloid–osmotic lytic mechanism [4]. A sublytic CPZ concentration also imposes an inward curvature on red cell membranes [5], an effect that could be attributed to the preferential partition of phenothiazines into the negatively charged cytoplasm leaflet of the bilayer [6,7]. Suda et al. [8], using electron paramagnetic resonance (EPR), observed that CPZ produces an increase in the rotational motion of stearic acid spin labels. In a similar way, previous results from our laboratory [9] employing EPR showed an increased erythrocyte membrane fluidity upon TFP addition, accompanied by changes in the lipid–protein interactions, as seen by fluorescence. EPR studies on erythrocyte ghosts revealed that CPZ binds preferentially to ghost proteins rather than to lipids [10] and phenothiazines have been reported to interact specifically with erythrocyte membrane proteins, such as cytoskeleton protein 4.1 [11], aminophospholipid flippase [12] and acetylcholinesterase [13].

We have previously shown that TFP, an amphiphilic molecule whose protonated species form micelles, is able to protect erythrocytes against mechanical hemolysis under isosmotic conditions [14] below its critical micellar concentration (CMC). Here we report further data about TFP-induced hemolysis. Incubation of erythrocytes with TFP (above its CMC) at pH 7.4 induces mixed micelle formation containing both TFP and erythrocyte phospholipids. The release of membrane lipids induces the collapse of the membrane (unpublished data). We quantitatively studied TFP and erythrocyte membrane interaction, to determine the effective TFP/lipid ratio for membrane activity (protection and lysis). Finally, considering that hydrophobic differences between charged and uncharged forms of TFP are determinant factors in TFP membrane interaction and that both forms are present at physiologic pH, we analyzed the contribution of each TFP species to isosmotic hemolysis.

2. Materials and methods

TFP hydrochloride (MW 480.4) and egg phosphatidylcholine (EPC) were obtained from Sigma, St Louis, MO.

2.1. Membrane preparation

Mouse liver microsomes were prepared as described previously [14]. EPC multilamellar vesicles were prepared by evaporating stock chloroform EPC solutions under a stream of wet nitrogen. The samples were left under vacuum for at least 2 h. Vesicles were obtained by the addition of phosphate-buffered saline, PBS (5 mM phosphate, pH 7.4, 150 mM NaCl), and vortexing for 5 min.

2.2. Erythrocytes

Freshly obtained mouse blood was collected into Alsiver's solution (27 mM sodium citrate/72 mM NaCl/114 mM glucose/2.6 mM citric acid) and washed three times in PBS. Erythrocyte ghost membranes were prepared as described by [15].

2.3. Protein determination

Total protein concentration was measured [16] using bovine serum albumin as a standard.

2.4. Phospholipid determination

Membrane phospholipid concentration was determined according to [17].

2.5. Partition coefficient (P) determination by phase separation

Membranes were prepared as described above and kept in PBS. A known amount of TFP was incubated with the membranes for 10 min at room temperature. The drug concentration remaining in the supernatant after centrifugation at 105 000×*g* for 1 h was optically detected at 256 nm ($\varepsilon_M = 23\,500$, [18]) against the respective control (membrane in PBS). For red blood cells, we used ghosts rather than whole erythrocytes because hemoglobin released during the experiment overlapped the TFP optical spectra, preventing the precise determination of TFP in the supernatant. The amount of drug bound to the lipid phase was obtained by subtracting the supernatant concentration from the total drug concentration measured before phase mixing. The partition coefficient, *P*, was determined according to Eq. 1 [19]:

$$P = \frac{n_m(s)/V_m}{n_w(s)/V_w} \quad (1)$$

where: *s* denotes the solute (TFP), *n* is the number of moles of solute, *V* = volume and the subscripts m and w refer to the membrane and aqueous phase, respectively. For erythrocytes, ghosts and microsomes, the apolar phase volume (V_m) was calculated assuming a lipid density of 1 g/ml [20,21]. For EPC multilamellar vesicles, *P* was determined after four freeze–thawing cycles, increasing the incubation time up to 30 min.

2.6. Octanol/water P determination

PBS and *n*-octanol solutions were pre-equilibrated overnight; after TFP addition the mixture was vortexed for 5 min and incubated for 10 min before centrifugation at 260×*g* for 5 min. *P* was optically determined as described for the phase separation experiments.

2.7. Hemolytic assay under hypotonic conditions

Erythrocytes (0.14% hematocrit) were incubated in hypotonic PBS (5 mM sodium phosphate, pH 7.4, 66 mM NaCl) that induced 50% hemolysis. TFP in the 1–125 μM range was added and the samples were incubated for 10, 30 and 60 min. After centrifugation at 260×*g* for 3 min, released hemoglobin was measured in the supernatant at 412 nm. Results are expressed on a relative absorbance (RA) scale ranging from <1 (protection against) to >1 (hemolysis). RA = 1 indicates 50% hemolysis obtained for the 66 mM saline control. The data were analyzed with the SAS statistical program [22]. PROC GLM programs were used for analysis of variance, with drug concentration and incubation time set as independent variables and RA as the variable answer. Means were compared by Duncan's multiple range test. Each RA value represents the mean of 15 independent experiments.

2.8. Isotonic hemolytic assay

TFP (1–200 μM) was prepared in isotonic PBS solution. Erythrocytes (hematocrits ranging from 0.04 to 0.14%) were added and the samples kept at room temperature (22–25°C) for 10 min. before centrifugation at 260×*g* for 3 min. Hemoglobin released into the supernatant was detected at 412 nm (for lower hematocrits) and 540 nm (0.14% hematocrit).

The hemolytic effect, measured as percent relative hemolysis (RH), was determined on the basis of released hemoglobin, according to the following formula:

$$RH = \frac{A_S - A_{c1}}{A_{c2} - A_{c1}} \quad (2)$$

where *A* is the absorbance, *S* the sample, c1 the mechanical hemolysis control (erythrocytes in PBS), and c2 the 100% hemolysis (erythrocytes in water) control.

2.9. C_{sat}, C_{sol} and TFP/lipid ratio (R_e) calculation

C_{sat} (solute concentration needed to start hemolysis) and C_{sol} (solute concentration for total lysis) [23–25] were graphically obtained for each hematocrit assayed. Plots of C_{sat} and C_{sol} as a function of lipid concentration allowed R_e (solute/lipid molar ratio) determination for initial (membrane saturation) and total hemolysis (solubilization), respectively. R_e was obtained from the slope of the resulting straight lines, as described in Eq. 3 [23–25]:

$$D_t = R_e[L + 1/K_b(R_e + 1)] \quad (3)$$

According to these authors, there is a linear relationship between the total solute concentration (D_t) producing the onset or completion of solubilization and the lipid concentration (L) in the system. The y intercept corresponds to the concentration of free solute, D_w, equivalent to its CMC [23,26]. K_b (M^{-1}) describes the solute binding to membranes in terms of equilibrium:

$$s + m \rightleftharpoons sm \quad K_b = \frac{[sm]}{[s][m]}$$

where s represents the solute and sm the membrane-associated species. Binding constants (K_b) and partition coefficients (P) are related through the partial molar volume, ∇, of the lipid phase [27,28].

$$K_b = P \cdot \nabla \quad (4)$$

For erythrocytes, ∇ was calculated as 0.658 l/mol, the average molecular weight of phospholipids, sphingolipids and cholesterol, according to the lipid composition of erythrocyte membranes described in [15].

2.10. Determination of CMC

CMC was determined with a K12 Krüss tensiometer. We measured the surface tension of TFP solutions ranging from 0.1 to 100 μM at room temperature using PBS buffer.

3. Results and discussion

Trifluoperazine, a rather soluble piperazine derivative (water solubility at pH 7.4 is 1 M according to [18]), has an ionizable amine group with a pK of 8.1 [18] so that, as discussed further in this paper, both charged (TFP+) and uncharged (TFP:) forms are present at physiologic pH. TFP+ has a CMC of 42 μM (experimental data determined in PBS, pH 7.4), indicating that both monomer and aggregate forms could be found in the concentration range employed here in the hemolytic experiments.

3.1. TFP partitioning between membranes and water

Table 1 shows TFP partition coefficients (P) between membrane (microsomes, erythrocyte ghosts, liposomes) and water and octanol/water systems. We can see that TFP partition into the membranes was higher than in the isotropic octanol phase and depended on membrane composition, so that: $P^{mic} > P^{lip} > P^{ghost}$.

The P^{oct} value obtained here is of the same order of magnitude as that found for TFP partition between hexane/water (PBS, pH 7.4) = 193 [29]. Absolute P values determined between organic phases (octanol) and water were lower than P values between membranes and water (Table 1) as also reported by other authors [29,30], revealing that P values between organic solvents and water are to be used carefully since they provide only poor estimates of the relative magnitude of the partition coefficients of the drugs in biomembranes [21,31–33].

The stronger binding of TFP to microsomes may reflect some TFP protein interaction, as the protein content of liver microsomes is higher (ca. 70% of the total membrane weight, [34]) than that of ghost membranes (55%, [35]). Besides, if we consider the fluidity of the ghost membranes we realize that the high cholesterol content (30%) of erythrocyte membranes [36] and the cytoskeleton [9] imposes a rigidity that could restrict TFP partition into them, explaining the rather small P^{ghost} value. In fact P^{ghost} was slightly lower than P^{lip}, an unexpected result since liposomes lack proteins. It seems that the reduced hydrocarbon chain dynamics [36] and increased bilayer bending [37] triggered by cholesterol, added to cytoskeleton rigidity, compensate for any TFP/protein interaction in ghosts.

Table 1
Partition coefficients[a] for TFP between microsomes (P^{mic}), erythrocyte ghosts (P^{ghost}), liposomes (P^{lip}) octanol (P^{oct}) and water (5 mM PBS, pH 7.4)

P^{mic}	P^{ghost}	P^{lip} [b]	P^{oct}
7172 ± 1229	1380 ± 429	1916 ± 341	452 ± 55

[a]Each P value represents the mean ± S.E. of nine experiments. Incubation time = 10 min at room temperature.
[b]For P^{lip} we used four freeze–thawing cycles, increasing the incubation time to nearly 30 min.

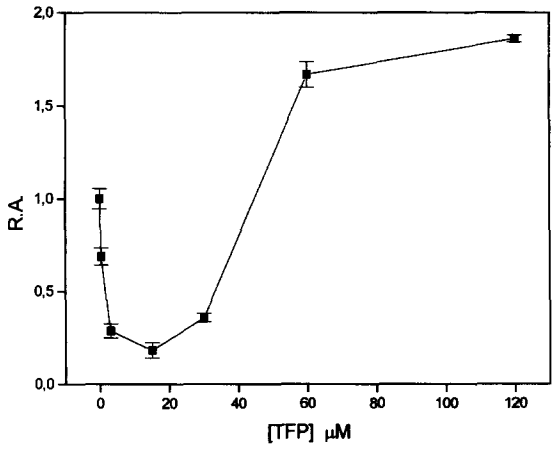

Fig. 1. TFP protection under hypotonic conditions. Hematocrit = 0.14% in hypotonic (66 mM NaCl) PBS, pH 7.4. Incubation time: 10 min at room temperature.

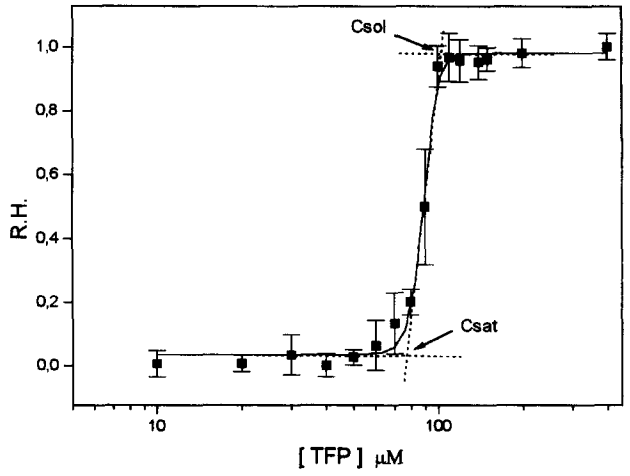

Fig. 2. TFP-induced isotonic hemolysis. Hematocrit = 0.14% in isotonic PBS. Incubation time: 10 min at room temperature. C_{sat} and C_{sol} (see text) determination are shown.

3.2. Studies on TFP induced hemolysis and TFP/lipid ratio determination

Phenothiazine stabilizes erythrocytes against hypotonic hemolysis at low concentration [3,37]. The biphasic TFP hemolytic curve under hypotonic conditions can be seen in Fig. 1, which shows that TFP at concentrations up to 17 μM protects erythrocytes ($H_t = 0.14\%$) against hypotonic lysis, while at higher concentrations its action on membranes is lytic (upward curve). The maximal protective concentration of TFP occurs in the 10–17 μM range, i.e. with TFP in its monomer form. The data obtained are in agreement with [3] who reported a maximal TFP protective concentration of 10 μM at pH 7.0, with a 0.2% hematocrit. Using the maximal protection average concentration we calculated the protective TFP/lipid ratio (R_e^{prot}) presented in Table 2.

Experiments like that illustrated in Fig. 1 (10 min incubation) were also performed with TFP pre-incubated for 30 and 60 min. The results obtained (data not shown) presented non-significant differences by the Duncan test, indicating that TFP equilibrium between the aqueous phase and the erythrocyte membrane was reached in less than 10 min.

Table 2
Effective drug/lipid molar ratios and related parameters in the protection and lysis of erythrocytes by trifluoperazine

R_e^{prot} [a]	0.16
R_e^{sat}	0.43
R_e^{sol}	1.45
D_w (μM)[b]	59
K_b (M^{-1})	4783

[a]Determined under hyposmotic conditions (Fig. 1).
[b]Taken from the saturation curve (Fig. 3).

Fig. 2 shows the hemolytic effect of TFP on erythrocytes under isotonic condition. No lytic effect was observed below 75 μM; up to this concentration the membrane incorporates TFP without losing its integrity. Beyond 75 μM, lysis increased quickly with TFP concentration due to the co-operative effect of the lipid bilayer. Membrane disruption occurs as lipids are released to the forming mixed-micelles.

We used hemolytic curves to obtain C_{sat} and C_{sol} values, i.e. TFP concentration for the onset and complete solubilization of erythrocyte membranes (Fig. 2). In Fig. 3, we plotted these values as a function of lipid concentration (experiments ranging from 0.15 to 1.5% hematocrit) to obtain the straight lines predicted by Eq. 3. The corresponding TFP to lipid molar ratio, R_e, (Table 2) was readily calculated from the saturation and solubilization lines in Fig. 3.

Theoretically [23], both straight lines in Fig. 3 should intercept the y axis at D_w, the free TFP concentration in water corresponding to the CMC of the amphiphilic molecule in the presence of membranes. The D_w values obtained (59 and 94 μM) closely resemble the CMC determined for TFP in water (42 μM at pH 7.4). Differences may be due to exper-

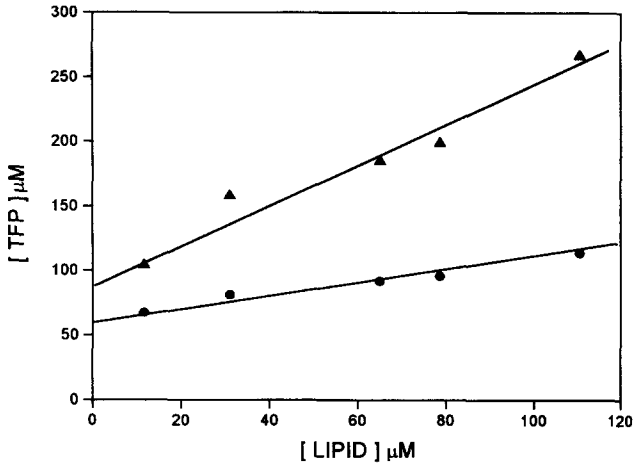

Fig. 3. Effective TFP/lipid molar ratio for membrane saturation and solubilization. C_{sat} (●) and C_{sol} (▲) are plotted as a function of erythrocyte lipid concentration. R_e values are taken from the slope of the straight lines.

imental errors, since the CMC value is very small, in the 10^{-5} M range.

This colloidal behavior of TFP to form micelles classifies it as a surface active compound [38]. Its CMC is lower than that of surfactants such as Triton X-100 (3.7×10^{-4} M), SDS (8.3×10^{-3} M) or CHAPS (3.5×10^{-3} M) [39,40], an indication of the high degree of intermolecular TFP hydrophobic interactions. In fact, TFP is among the most hydrophobic phenothiazines [29], as also shown in the present study by the very low concentration of TFP monomers in the water phase (small D_w) at pH 7.4.

Table 2 presents several parameters derived from the biphasic hemolytic experiments. At low TFP concentrations and under hyposmotic conditions a maximum protective effect was observed at 0.16:1 TFP/lipid molar ratio (R_e^{prot}, determined from Fig. 1). Under isotonic conditions, R_e^{sat} showed that a ratio of 0.43:1 TFP/lipid is necessary to reach membrane saturation. Higher ratios produced hemolysis, until complete membrane solubilization at ca. 1.5:1 TFP/lipid molar ratio (R_e^{sol}). Hemolytic phenomena is then a consequence of the erythrocyte membrane phospholipids migration to the hydrophobic micelle environment. Effective detergent/lipid ratios for phospholipid leakage calculated for many other surface active drugs are in agreement with the values observed for TFP. For example, egg phosphatidylcholine bilayers are able to incorporate Triton X-100 up to 0.71:1 detergent/lipid molar ratios; above 3:1 molar ratios all phospholipid is converted into mixed micelles [24,41]. In phosphatidylcholine vesicles, palmitoylcarnitine reaches membrane saturation and solubilization at 0.8:1 and 2:1 molar ratios, respectively [25].

Assuming ideal mixing of lipid and TFP in dilute aqueous media [26], the binding constant (K_b) for TFP distribution between bilayer and water could be obtained from the saturation straight line in Fig. 3, according to Eq. 5 [23,26]:

$$R_e^{sat} = K_b \cdot D_w/(1-K_b \cdot D_w) \quad (5)$$

The K_b value obtained for TFP between erythrocytes/water was 4.8×10^3 M^{-1} (Table 2) and, according to Eq. 4, it corresponds to a partition coefficient of 7268. Even considering the different methodologies employed and the well known variability in P values, this rather high partition, comparable to P^{mic} (Table 1), cannot be completely explained. It reflects, besides TFP hydrophobicity, some non-specific protein binding as, in fact, TFP specifically interacts with erythrocyte membrane proteins [11–13] and with hemoglobin [9], protecting the latter against H_2O_2-induced oxidation.

Using this partition coefficient, we can go back to R_e^{sat} and R_e^{sol} values in order to determine the real TFP/lipid molar ratio *inside the membrane*. For a $H_t = 0.15\%$ (7.9 μg/ml lipid) just 5.4% of the TFP would be partitioned in the bilayer (Eq. 1) and the molar ratio for saturation and solubilization would be 2:100 and 8:100 TFP/lipid, respectively. This rather small ratio indicates that hemolysis is not induced by real saturation of the membrane, but is a complex response for both TFP partition into the membrane (C_{sat}, C_{sol} increase according to membrane concentration, Fig. 3), and aggregation (micelle formation). In this case TFP micellar aggregation could play a synergistic effect on the drug–membrane interaction, explaining the high partition coefficient assigned from Fig. 3.

3.3. Further investigation of the real TFP charged/uncharged ratios for the hemolytic effect at physiological pH

In a previous study, we reviewed the effect of dif-

Table 3
Partition coefficients for charged and uncharged TFP species and ionization constant in water (pK); calculations of ionization constant in the presence of membrane (pK_{app}) and average partition coefficient at pH 7.4 ($P_{average}$)[a] according to pK_{app}

P^{lip}		pK[d]	pK_{app} [e]	$P_{average}$
TFP+[b]	TFP:[c]			
812	2463	8.1	7.62	1432

[a]Calculated by the Henderson-Hasselbalch equation, but using pK_{app} (real ionization constant under the assay condition) instead of pK (see text).
[b]Determined in 20 mM acetate buffer, pH 5.0.
[c]Determined with 20 mM carbonate buffer, at pH 10.5.
[d]According to [18].
[e]Calculated as in Eq. 6 for a hematocrit = 0.15%.

ferent partitioning of ionizable local anesthetics at their ionization constant [28]. It was shown that whenever the binding of charged/uncharged forms is different it will imply a pK shift of the partitioning compound. Tetracaine, for example, has its ionization constant down-shifted from 8.5 to 7.0 in the presence of egg PC multilamellar vesicles [42].

TFP shows quite different P values for its charged/uncharged forms (Table 3), the neutral form binding more strongly (P^{lip}: = 2463) than the protonated, less hydrophobic species (P^{lip}+ = 812). According to the formalism described by [43], we calculated pK_{app}, the apparent ionization constant in the presence of membranes, from:

$$pK_{app} = pK - \log[(P_{:} \cdot V_m + V_w)/(P_{+} \cdot V_m + V_w)] \quad (6)$$

pK_{app} was found to be 7.62 for TFP in the presence of 12 μM lipids (0.15% hematocrit, Table 3), revealing a down-shift of ca. 0.5 pH units in the presence of membranes. At higher phospholipid concentrations, calculated pK_{app} was the same.

Using pK_{app} in the Henderson–Hasselbalch equation (Eq. 7), one can see that the neutral (TFP:) to charged (TFP+) molar ratio at pH 7.4 is 1:1.5, instead of 1:4 as it would be expected if the pK shift was neglected. As a consequence, there is no predominant form of TFP in the hemolytic experiments described before (pH 7.4).

$$pH = pK + \log[\text{base}]/[\text{acid}] \quad (7)$$

An average partition coefficient can be calculated as shown by Eq. 8, taking into account the real TFP:/TFP+ ratio at pH 7.4 (using pK_{app} values). We call this $P_{average}$ (after Lee and Schreier [43], see below).

$$P_{average} = \frac{P_{+} + (P_{:} \cdot Z_{:})}{1 + Z} \quad (8)$$

where Z is the [base]/[acid] molar ratio at pH 7.4.

In fact, Lee and Schreier [43] described a similar equation for the calculation of $P_{average}$ that also consider the differences in pK and pK_{app}. We prefer to use Eq. 8 because it permits fast comparison of $P_{average}$, corrected or not, by changing the values of the [base]/[acid] ratio for pK_{app} and pK, respectively. The calculated values of $P_{average}$ obtained from the partition coefficients of the charged and uncharged TFP forms (measured at pH 5.0 and 10.0, respectively) between liposomes and water are listed in Table 3.

For TFP in multilamellar liposomes, the expected P value without considering ΔpK, would be 1086. $P_{average}$ is 1432, reflecting the contribution of the uncharged and charged forms of TFP to its partition at pH 7.4, and is in good correlation with the experimental value (P^{lip} = 1916, Table 1).

$P_{average}$ indicates that partitioning is higher than one would expect by ignoring the pK shift, so that hydrophobic membrane interaction is important for the hemolytic effect of trifluoperazine. These results explain the direct correlation between the hydrophobicity and biologic effects of phenothiazine compounds [3,7,29,44].

4. Conclusion

This work quantitatively describes the biphasic (protective/inductive) effect of TFP on mouse erythrocyte hemolysis. TFP protective effect seems to be related to its monomer insertion into the membrane, which occurs quickly at concentrations below those for membrane saturation (0.43:1, TFP/lipid molar ratios).

Above CMC, trifluoperazine solubilizes membranes. The results described should be of help in the understanding of the molecular mechanisms of hemolysis, because TFP acts as a surfactant on the

membrane system. Applying classical treatments for the interaction of surface active compounds with lipid vesicles [23] to the hemolytic curves it was possible to calculate TFP/lipid molar ratios for the onset of hemolysis (R_e^{sat}) and for complete membrane solubilization (R_e^{sol}). This approach seems to be quite adequate for the system as R_e values keep a good correlation with those determined for other surfactant molecules, and it also permitted the calculation of P values from the hemolytic curves, i.e. between whole erythrocytes and water.

Using the calculated partition coefficient, we determined rather small TFP/lipid molar ratios inside the membrane for saturation and solubilization what indicates that hemolysis is not induced by real membrane saturation, but also by TFP micelle formation that occurs in the concentration range used in the hemolytic experiments.

The amount of charged and uncharged TFP species in the hemolytic experiments was also analyzed taking into account the differences between TFP+ and TFP: binding to membranes and its effect on the real ionization constant, pK_{app} [28,42]. $P_{average}$, the mean partition coefficient at pH 7.4 was estimated and presented a good agreement with the data obtained. $P_{average}$ receives an important contribution of TFP:, the uncharged species that is present in almost the same proportion as TFP+ at pH 7.4, and which stronger binding is committed to the real membrane saturation.

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