Binding of two spin-labelled derivatives of chlorpromazine to human erythrocytes*

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The binding to human intact erythrocytes of two different spin-labelled derivatives of chlorpromazine has been studied. The influence of the positively charged side chain of the drug has been the focus of our attention. The positively charged amphiphilic compound (spin derivative I) is water-soluble up to 80 μM at pH values below 5.9. The apolar analogue (spin derivative II) aggregates in aqueous buffer from the lowest concentration tested. Both spin derivatives undergo a slow reduction inside the erythrocyte. The reduced nitroxides are readily reoxidized by adding a low, non-quenching, concentration of potassium ferricyanide to the intact erythrocytes. The fractions of spin label I and II bound to the erythrocyte membrane or to the erythrocyte-extracted lipids remain constant as a function of the temperature (3–42°C) and as a function of the concentration of the spin label up to 150 μM . E.s.r. spectra of both spin labels show a two-component lineshape when they are bound to intact erythrocytes. Below 35 °C for the positively charged spin probe, and below 32 °C for the apolar spin probe, the simulation of the lineshape shows that more than 50 % of the spectrum originates from a slow-motion component. This slow-motion component is also found in erythrocyte-extracted lipids probed by the positively charged spin label below 25 °C. In contrast, no slow-motion component is detected in the range 4–40 °C for the apolar spin label in erythrocyte-extracted lipids. In this environment the apolar probe experiences a single fast anisotropic motion with an exponential dependence on 1/temperature. Detailed lineshape simulations take into account the exchange frequency between binding sites where the probe experiences a fast motion and binding sites where it experiences a slow motion. The exchange frequency is strongly temperature-dependent. Characterization of the different motions experienced inside the different locations has been achieved and compared for whole erythrocytes and for the extracted lipids. The biochemical nature of the binding sites (membrane protein/acidic phospholipid) giving rise to the slow-motion component is discussed as a function of the polarity of the spin-labelled drug and as a function of the temperature controlling the fluidity of the lipid bulk and influencing the distribution of the drug inside the membrane.

INTRODUCTION

Numerous published studies claim that the mechanism of the therapeutic action for phenothiazine is initially a physicochemical alteration of the nerve-cell membrane. This concept is taken from local anaesthetics producing a conduction block (Boggs *et al.*, 1976). Experimental results favouring membrane action are numerous. Previous works emphasize the partitioning of the amphiphilic drug in the lipid bulk (Seeman, 1972). Modifications of the cell shape and of the osmotic resistance (Lovrien *et al.*, 1975; Araki & Rifkind, 1981; Isomaa *et al.*, 1987; Isomaa & Engblom, 1988), alteration of the fluidity (Neal *et al.*, 1976; Ogiso *et al.*, 1981) and lipid extraction by the drug (Maher & Singer, 1984) are non-specific phenomena which are reproduced with different amphiphiles. A biphasic behaviour has been recognized for phenothiazines as a function of the concentration. At low concentration the bilayer is preserved and the fluidity decreased in a cholesterol-poor membrane (Neal *et al.*, 1976). At medium concentration ($\approx 0.1 \text{ mM}$) the fluidity increases, and at high concentration, co-micellization of phenothiazine and membrane lipids disrupts the bilayer (Araki & Rifkind, 1981; Ogiso *et al.*, 1981; Forrest *et al.*, 1984).

The interaction of chlorpromazine with biomembranes has gained a lot of interest since a sharp discrepancy was observed between partition coefficients measured by various methods (Conrad & Singer, 1981). According to these data, the partition of chlorpromazine is reduced in biomembranes by an internal pressure expelling the molecule. Membrane proteins might be the driving force to develop this pressure, but it is also noticeable that cholesterol is able to reduce significantly the partition of the drug in pure lipid vesicles (Ahmed *et al.*, 1980; Forrest *et al.*, 1984). The interaction of chlorpromazine with lipids has recently been re-examined in detail (Luxnat & Galla, 1986; Müller *et al.*, 1986) and chlorpromazine has been found to partition in the biomembrane according to its cholesterol content. The partition has been also found to be constant up to the critical micellar concentration (between 3×10^{-5} and $5 \times 10^{-5} \text{ M}$), where extensive lipid extraction occurs from the membrane. As a function of the temperature, the partition is reduced 10-fold in the gel phase compared with the liquid-crystalline phase. The uptake of the drug can also be accounted for by the defects of the ripple phase $P_{\beta'}$.

In the search for a specific interaction of the phenothiazine with membrane proteins, several pieces of evi-

*This paper is dedicated to Dr. F. Leterrier, who, during the last 20 years, has initiated a number of e.s.r. studies devoted to phenothiazine.

dence have been obtained. Interactions of the drug have been observed with erythrocyte skeletal proteins (Minetti & Di Stasi, 1987) or with the δ -subunit of the acetylcholine receptor (Giraudat *et al.*, 1986; Heidmann & Changeux, 1986). On the basis of enzyme assay, interactions of phenothiazine are also expected with the Ca^{2+} pump (Rooney & Lee, 1983) and with the K^{+} extrusion pump (Eilam, 1984). Binding of phenothiazine can be assumed also to take place on membrane-bound calmodulin (Agre *et al.*, 1983). Remarkably, the specificity of chlorpromazine interaction with these various membrane proteins could be sustained by electrostatic binding of the side-chain terminal amino group, as has already been demonstrated for calmodulin (Prozialek, 1984; Olivier *et al.*, 1986).

In the present paper we use two different spin-labelled derivatives of chlorpromazine (Fig. 1) to probe the interaction with erythrocytes. It is noticeable that the side chain of the parent drug (*N*-dimethylaminopropyl) has been shortened in the spin-labelled analogues. Previous studies of spin-labelled phenothiazines bound to calmodulin (Olivier *et al.*, 1986) have shown that a short side chain (C_2 instead of C_3) hinders the motion of the reporter group and increases the resolution between freely rotating and bound spin probe. The terminal amino group of the side chain has been substituted by either a methyl (spin derivative I) or a cyanyl group (spin derivative II). Due to the presence of the cyanyl group, the terminal amino group of spin derivative II is uncharged over the range of pH studied here. The use of specific probes allows a direct monitoring by e.s.r. of the drug intercalated in the biomembrane. It minimizes the speculations in the interpretation of e.s.r. spectra especially in regard to the method using non-specific spin label (Yamaguchi *et al.*, 1985). In spite of this fact, it has been exceptionally used (Manian *et al.*, 1974). Using both positively charged and uncharged spin-labelled derivatives of chlorpromazine, we have been able to distinguish the influence of the electrostatic binding in addition to the lipophilic interaction of phenothiazine with membrane lipids.

MATERIALS AND METHODS

Erythrocytes were prepared from fresh human heparinized blood of healthy male volunteers. After centrifugation (2000 *g*, 14 min, 4 °C), the plasma and the buffy coat were removed. The erythrocytes were washed with 0.9% NaCl at 4 °C and centrifuged (2000 *g*, 14 min, 14 °C) three times. They were then suspended in PBS (10 mM-phosphate/140 mM-NaCl) and the haematocrit was adjusted to 20% or 50% respectively for bio-reduction monitoring or e.s.r. spectrum analysis. Lysate and ghosts are obtained after lysis of erythrocytes in 5 vol. of 10 mM-phosphate buffer and centrifugation for 15 min at 12500 *g*. Lipids were extracted from erythrocytes by the procedure described by Kates (1972) and, after careful evaporation of the solvent, they were suspended in PBS by extensive vortex mixing in the presence of the spin label. The suspension was stored overnight before e.s.r. measurements.

Spin derivatives I and II (Fig. 1) were synthesized as described by Kikelj *et al.* (1983), and the concentration of the solution was assayed by u.v. absorbance. Spin derivatives were added to erythrocytes as concentrated aliquots in dimethyl sulfoxide (< 0.5%, v/v).

Fig. 1. Structures of the spin-labelled derivatives (I and II) of chlorpromazine

E.s.r. spectra were recorded in the X band, with a spectrometer equipped with a variable-temperature unit. Temperature was measured by a thermocouple placed inside the e.s.r. cell, slightly above the cavity. Spectra were recorded from 42 to 3 °C. E.s.r. settings were: central field, 0.3262 T; modulation field, 0.2 mT or 0.16 mT; microwave (X band) power, 20 mW; sweep width, 10 mT. E.s.r. spectra were digitized as a 1000-points data block via a computer running the subtraction and integration program. The spectrum of bound spin derivative was obtained after subtraction of the narrow triplet corresponding to the spectrum of the free spin derivative I in aqueous buffer at pH 5.6. The end point of the subtraction is the reversion of the high-field resonance peak (Olivier *et al.*, 1986). The spectra considered thereafter were then devoid of resonance peaks from unbound probe.

The computer programs for simulating the e.s.r. spectra were run on an IBM 3090 computer. The single-component spectra, described for spin label II probing the membrane-extracted lipids, consist of three symmetric lines separated by 16 G. This is significant of a fast motional regime where the tumbling rate is significantly faster than the anisotropy of the hyperfine coupling tensor expressed in frequency unit, i.e. $\gamma_e \Delta A = \gamma_e [A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})] \approx 5.2 \times 10^8$ Hz for $A_{zz} = 3.5$ mT, $A_{xx} \approx A_{yy} \approx 0.64$ mT (Chachaty, 1984), γ_e being the electron gyromagnetic ratio and A_{zz} , A_{xx} and A_{yy} being the principal components of the hyperfine tensor (x being the direction of the N–O bond and z the $2p\pi$ orbital axis. This corresponds to an effective correlation time of about 2×10^{-9} s. For τ_{eff} (effective correlation time) $< 2 \times 10^{-9}$ s, the peak-to-peak linewidth (ΔH_{pp}) is related to the electron transverse relaxation time, T_{2e} , by an expression of the form (Nordio, 1976):

$$\Delta H_{\text{pp}} = 2/(\gamma_e T_{2e} \sqrt{3}) = a + bm + cm^2 \quad (1)$$

where a , b and c are constants dependent on the A and g tensors and on the motional correlation time $\tau_{\parallel} = (6D_{\parallel})^{-1}$ and $\tau_{\perp} = (6D_{\perp})^{-1}$, $D_{\parallel}$ and $D_{\perp}$ being the principal values of the rotational diffusion tensor and m the nuclear quantum number. Here the anisotropy of the motion results most likely from a fast reorientation of the tetramethylpiperidinyll N -oxyl group about the N -O direction (x -axis).

There are other contributions to the linewidth, independent of the nuclear quantum number, m , such as the modulation broadening, the unresolved hyperfine splitting and eventually the exchange among sites of different mobilities (see below).

In the absence of broad component, $\tau_{\parallel}$ and $\tau_{\perp}$ were obtained from the width and relative amplitude of these three nitroxide lines, by a minimization procedure (Chachaty, 1982). It was verified on theoretical spectra computed using the programs of Freed and co-workers (Freed, 1976; Moro & Freed, 1980, 1981) that this

procedure, while strictly valid below $\tau_{\perp} = 2 \times 10^{-9}$ s, holds within a few per cent up to an effective correlation time $\tau_{\text{eff.}} = (\tau_{\perp}\tau_{\parallel})^{1/2} \approx 3.10^{-9}$ s for $\tau_{\perp} \lesssim 5 \times 10^{-9}$ s. Above this limit it becomes apparent that the anisotropies of the A and g tensors are not completely averaged out and that the spectra correspond to the so-called 'slow-motional regime'. In this condition the spectra are computed by using either the program of Freed and co-workers (Freed, 1976; Moro & Freed, 1980, 1981) or the computation method of McCalley *et al.* (1972). Under our experimental conditions, the slow-motional spectra were always superimposed on the fast component, so that they cannot be accurately fitted. However, the shape of the slow component is consistent with an isotropic reorientation of the probe, the reorientation correlation time of which may be estimated from the total width of the spectrum, using a calibration curve derived from methods mentioned above. A more satisfactory approach was to simulate the entire spectrum including a exchange rate

Fig. 2. Influence of the exchange rate (ν_{ex}) on the shape of the nitroxide e.s.r. spectra

The spectra are computed for $\tau_s = 10^{-8}$ s, $\Delta H_{\parallel} = \Delta H_{\perp} = 0.5$ mT (slow component) $\tau_{\perp} = 2 \times 10^{-9}$ s, $\tau_{\parallel} = 2 \times 10^{-10}$ s (fast component) and a Gaussian broadening of 0.1 mT. The fraction of the fast component was 0.25. Values of ν_{ex} were as follows: (a) —, 10^5 s $^{-1}$; ----, 10^6 s $^{-1}$; -.-, 10^7 s $^{-1}$; (b) —, 10^8 s $^{-1}$; ----, 10^9 s $^{-1}$; -.-, 10^{10} s $^{-1}$.

between sites corresponding to the slow (S) and fast (F) motional regimes. This approach, suggested by various studies (Davoust & Devaux, 1982; Marsh, 1985) was supported by the fact that the linewidth of the fast component is nearly temperature-independent or even narrower in the low-temperature range ($< 20^\circ\text{C}$).

The lineshape was obtained from the imaginary part (v) of the complex magnetization

$$\hat{M} = U + iV = f\hat{M}_F + (1-f)\hat{M}_S$$

f being the fraction of fast component, and $\hat{M}_F$ and $\hat{M}_S$ being the magnetizations of the fast and slow components. Following the method of McCalley *et al.* (1972), the isotropic slow motion resulted in an exchange of magnetization, $\hat{M}_S$, among spherical zones corresponding to angles θ_j between the z axis of the magnetic tensors and the field H of the spectrometer and the adjacent zones $\theta_{j\pm 1}$. Under slow passage conditions and for a radiofrequency non-saturating field, H_1 , $\hat{M}$ was obtained by solving, for each value of H , a system of $N+1$ equations, N being the number of angular zones:

$$i\gamma_e H_1 M_{Sj}^m - \hat{M}_{Sj}^m [i\gamma_e (H - H_{Sj}^m) + T_{2Sj}^{-1} + k_j^- \tau_s^{-1} + K_{SjF}] + \tau_s^{-1} [k_{j-1}^+ \hat{M}_{Sj-1}^m + k_{j+1}^+ \hat{M}_{Sj+1}^m] + K_{FSj} \hat{M}_F^m \quad (2)$$

$$i\gamma_e H_1 M_F^m - \hat{M}_F^m \left[i\gamma_e (H - H_F^m) + (T_{2F}^m)^{-1} + \sum_{j=1}^N K_{FSj} \right] + \sum_{j=1}^N K_{SjF} \hat{M}_{Sj}^m = 0 \quad (3)$$

This calculation is successively performed for $m = 1, 0$ and -1 . In these expressions, H_{Sj}^m and H_F^m are the resonance fields, M_{Sj} and M_F are the longitudinal magnetization at equilibrium, for the slow and fast components respectively, with $M_F^m + \sum M_{Sj}^m = M_0/3$, M_0 being the total magnetization. τ_s is the slow-motional correlation time. The exchange rate from zone j to zones $j\pm 1$ is $k_j^- \tau_s^{-1}$. That from zones $j\pm 1$ to zone j is $k_{j\pm 1}^+ \tau_s^{-1}$. The superscript $(-)$ denotes the transfer of magnetization from zone j to $j-1$ and $j+1$, whereas the superscript $(+)$ denotes the transfer from zones $j-1$ and $j+1$ to j . The expression for k_j , k_{j-1} and k_{j+1} are taken from McCalley *et al.* (1972). For the slow component, $T_{2Sj} = \sqrt{3/2} \gamma_e (\Delta H_{\parallel}^2 \cos^2 \theta_j + \Delta H_{\perp}^2 \sin^2 \theta_j)^{1/2}$, where $\Delta H_{\parallel}$ and $\Delta H_{\perp}$ are the peak-to-peak width of the first derivative of the spectrum for $\theta = 0^\circ$ and $\theta = 90^\circ$ respectively. For the fast component, T_{2F} is given by eqn. (1).

The exchange rate between the sites F and S is defined as:

$$v_{\text{ex.}} = fK_{FS} = (1-f)K_{SF} \quad (4)$$

with

$$K_{SjF} = K_{SF}, K_{FSj} = K_{FS} P_j \quad (5)$$

P_j being the probability of the orientation θ_j , proportional to $\sin \theta_j$.

Some theoretical spectra, computed as a function of $v_{\text{ex.}}$, are given in Fig. 2. The line-broadening due to exchange becomes perceptible on the narrow component of the spectrum from $v_{\text{ex.}} = 10^6 \text{ s}^{-1}$. The collapse of the broad and the narrow component occurs for $10^7 \text{ s}^{-1} < v_{\text{ex.}} < 10^8 \text{ s}^{-1}$. The fast-exchange condition is achieved beyond $v_{\text{ex.}} = 10^9 \text{ s}^{-1}$. It is noticeable that, for fast components fraction $f > 0.5$, the parallel shoulders of the slow component become hardly perceptible beyond $v_{\text{ex.}} = 10^7 \text{ s}^{-1}$, but the contribution of this component is evidenced by an asymmetry of the low field line.

Fig. 3. Water-solubility of spin label I in 10 mM-PBS at 23°C (a) as a function of concentration [pH 5.6 (●) and 6.4 (○)] and (b) as a function of pH ([spin label I] = $50 \mu\text{M}$)

H^{+1} is the height of the low-field peak.

Fig. 4. Bioreduction of spin labels I (●) and II (■) by human erythrocytes at 23°C and of spin label I by ghosts (◆) and by lysate (◇)

Erythrocytes (haematocrit 20%) reduce the paramagnetism of spin labels I and II ([spin label] = $60 \mu\text{M}$). As a function of time, the percentage of initial signal is determined by double integration. After 1 h, potassium ferricyanide (0.5 mM) is added and the signal is extemporaneously requantified. For the purposes of comparison with erythrocytes, the lysate volume is adjusted.

Table 1. Lineshape simulation of e.s.r. spectra of spin-labelled chlorpromazine bound to erythrocytes

Temp. (°C)	Spin label I				Spin label II			
	Fast component ($\tau_{\text{eff.}}$ (ns))*	Slow component (τ_s)(ns)†	Exchange rate ($v_{\text{ex.}}$) (μs^{-1})	% slow component§	Fast component ($\tau_{\text{eff.}}$ (ns)	Slow component (τ_s) (ns)	Exchange rate ($v_{\text{ex.}}$) (μs^{-1})	% Slow component
42.5	—	—	—	—	0.45	5	50	20
40	0.8	6	30	30	—	—	—	—
38	—	—	—	—	0.55	5	20	30
36	0.8	6	30	40	—	—	—	—
32	0.8	6	30	50	0.55	5	15	50
27	0.8	6	20	50	0.63	7	5	75
22	0.77	7	15	60	0.63	6	5	75
17	1.5	10	7.5	80	0.63	6	5	75
14	1.5	10	5	80	—	—	—	—
12	—	—	—	—	0.63	7	2	85
7	1.5	10	0.1	87.5	0.63	7	2	87.5
4	1.5	10	0.1	90	—	—	—	—

* $\tau_{\text{eff.}} = [(\tau_{\parallel} \cdot \tau_{\perp})^{\frac{1}{2}}]$, effective correlation time of the fast motion.

† τ_s , correlation time of the slow isotropic motion.

‡ $v_{\text{ex.}}$, exchange rate between the slow-motion component and the fast-motion component.

§ '% slow component' means the percentage of slow-motion component used to fit the experimental e.s.r. spectrum.

Table 2. Lineshape simulation of e.s.r. spectra of spin-labelled chlorpromazine bound to extracted lipids

$\tau_{\text{eff.}}$, τ_s , $v_{\text{ex.}}$ and '% slow component' are defined in Table 1. (a) Two-components-spectra domain; (b) single-component-spectra domain.

Temp. (°C)	Spin label I				Spin label II
	Fast component ($\tau_{\text{eff.}}$ (ns)	Slow component (τ_s) (ns)	Exchange rate ($v_{\text{ex.}}$) (μs^{-1})	% Slow component	Fast component ($\tau_{\text{eff.}}$ (ns)
(a) 40	1.3	—	—	—	0.54
37	1.8	—	—	—	0.63
34	1.6	—	—	—	0.67
31	1.91	—	—	—	0.67
28	1.7	—	—	—	0.76
(b) 25	1.22	7.5	5	40	0.85
22	1.22	7.5	5	60	0.99
19	1.22	10	2	70	0.99
16	1.87	10	2	70	1.27
13	—	—	—	—	1.24
10	2.76	10	1	85	1.87
7	2.76	10	1	85	1.98
4	—	—	—	—	2.7

RESULTS

Spin derivative II is not soluble in aqueous buffer, even at the lowest concentration tested, and its e.s.r. spectrum is broadened by spin-spin interaction. Being amphiphilic and ionizable, the spin derivative I has a limited water-solubility (Fig. 3a) that varies with the pH (Fig. 3b). At acidic pH (pH < 5.6), the critical micellar concentration is about 80 μM . Above this value, the amplitude of resonance peaks is no longer linear with the concentration, because the spin derivative aggregates and the

spin-spin interaction broadens the resonance peaks. With spin derivative I, the process of aggregation in aqueous buffer is gradual, with no apparent sharp decrease of peak amplitude up to 150 μM . At pH 6.4, the critical micellar concentration is reduced 2-fold. The titration curve of spin derivative I (Fig. 3b) suggests, for the amino group, a pK of about 5.9. Deprotonation of the amino group induces aggregation of the spin label and collapse of the resonance triplet into a broad band above pH 7.0.

Spin derivative I and II complete binding to

erythrocytes in less than 2 min. Bioreduction of the nitroxide spin derivative to hydroxylamine by cells is a general phenomenon that is illustrated in Fig. 4. Spin derivatives I and II display the same rate of reduction by erythrocytes, in spite of their different solubility at pH 5.6. Bioreduction is related to a cytosolic agent, since ghosts are unable to reduce spin derivative I, but the lysate is able to. Assuming a first-order kinetic ($-d[N-O\cdot]/dt = k \times [N-O\cdot]$), one can estimate at room temperature the reduction rate (k) to be $2 \times 10^{-4} \text{ s}^{-1}$, a value in agreement with those in the literature (Bartosz, 1981) for Tempo (2,2,6,6-tetramethyl-1-piperidinyloxy) ($2.97 \times 10^{-4} \text{ s}^{-1}$) or for Tempol (4-hydroxy Tempo) ($1.05 \times 10^{-4} \text{ s}^{-1}$). The fact that both water-soluble and apolar spin derivatives display the same reduction rate and that lysate does not show a higher rate of reduction than the corresponding intact erythrocytes suggests that diffusion of the spin derivative through the membrane is not a limiting factor for bioreduction. This is also supported by the fast reoxidation of hydroxylamine to nitroxide by 0.5 mM-ferricyanide (Fig. 4). Non-permeant ferric ions added to the cell suspension reoxidize the reduced spin derivative in less than 1 min. Rapid recovery of the initial signal for spin derivative I or II suggests that the exchange rate between the intra- and extra-cellular volume is high. Thus the limiting step should be the reduction itself, or the access of the nitroxide substrate to the reducing system.

The absence of immobilization of the spin derivative added to lysate supernatant (results not shown) shows that the spin derivative is only bound to the membrane. The fraction of bound spin derivatives I and II is constant above 90% up to $150 \mu\text{M}$ at a haematocrit of 50% (results not shown). The fraction of bound spin derivatives is not influenced by temperature in the range 3–42°C if one quantifies the overall bound spectrum by double integration, and this holds for intact erythrocytes as well as for extracted lipids.

For intact erythrocytes probed by both spin labels (Table 1) the two-component spectra have been interpreted as a superimposition of spectra in the fast- and slow-tumbling regime. Systematic computer simulations of the slow component are consistent with a quasi-isotropic motion. A clearly different situation is encountered with the fast reorientation component, which displays an anisotropic motion. The slow component of the positively charged spin label is very similar to that of the apolar spin label when bound to the intact membrane, both qualitatively and quantitatively. By contrast, when the probes are bound to membrane-extracted lipids they differ considerably. For the apolar spin label, no slow-motion component has been detected down to 4°C, whereas for the positively charged spin label a slow component emerges rapidly below 25°C. The slow-motion component identified in erythrocyte-extracted lipids by spin label I resembles that observed in intact cells at the corresponding temperatures. In extracted

lipids, over the whole temperature range investigated by the apolar spin label, the spectra have been interpreted as an anisotropic reorientation of effective correlation time, varying according to an Arrhenius-law dependence on the temperature and with $D_{\perp}/D_{\parallel}$ about 5–8. By contrast, in intact erythrocytes the fast motion of spin labels I and II does not display the values predicted for τ_{eff} , by assuming an Arrhenius-law dependence. As suggested by Marsh (1985), this discrepancy is a consequence of the contribution to the linewidth of the exchange between the fast- and slow-motion resonance lines. As shown previously, the exchange alters significantly the lineshape, particularly in the low-field region of the spectrum, where the resonance peak is asymmetrically modified (Fig. 2). In Tables 1 and 2, the exchange rate is fitted, and the simulation shows a sharp temperature dependence of v_{ex} . From the physiological temperature down to 6–7°C, the exchange frequency is reduced by a factor of about 20–30. Owing to this exchange, the distribution of the probe between the various locations reaches different equilibrium states as a function of the temperature. When the temperature is decreased, the probes accumulate in the slow-motion binding sites of the membrane, which contribute over 85% to the spectra under 10°C. Interestingly, the variation of the motion rates inside of the binding sites with the temperature is very moderate compared with the variations of the exchange frequency and with the variation of the slow-component percentage. For this reason, these two parameters are considered determinant for lineshape analysis of the present spin labels. Appropriate fitting of the parameters displayed in Tables 1 and 2 release the simulated lineshapes presented in Fig. 5 and selected after visual matching.

DISCUSSION

In the present work we have obtained information on spin-labelled chlorpromazines probing biological membranes as a function of temperature. A different and complementary point of view was obtained previously on the influence of chlorpromazine on membranes probed with non-specific spin labels (Ogiso *et al.*, 1981; Yamaguchi *et al.*, 1985; Minetti & Di Stasi, 1987).

First, it must be established that the spin-labelled derivatives are representative analogues of the parent drug chlorpromazine. By using the activation by calmodulin of the myosin light-chain kinase, the efficiency of various spin-labelled chlorpromazines acting as anticalmodulin has been established (Olivier *et al.*, 1986). With this model, the conclusion reached is that anticalmodulin activity is sustained by a mixed hydrophobic/ionic interaction, given respectively by the phenothiazine ring and by the positively charged amino group.

Displacement of the spin-labelled derivative by the parent drug is usually proposed to establish their analogy. However, the non-feasibility of this test is obvious in

Fig. 5. Experimental (ripple line) and simulated (smooth line) spectra of spin labels I and II bound to erythrocytes (a) or to extracted lipids (b)

Experimental spectra were recorded in the presence of ferricyanide (0.5 mM) with erythrocytes (haematocrit 50%) suspended in PBS buffer, pH 5.6, and spin labels at a concentration of $50 \mu\text{M}$. Peaks of unbound spin probe have been subtracted from the total spectra. The parameters used for lineshape simulations are described in Table 1 (intact cells) and in Table 2 (extracted lipids).

high-capacity compartments such as the lipid bulk of the membrane (Holtzman, 1984). The limited water-solubility of chlorpromazine and its detergent property would dissolve the membrane before the probe is efficiently displaced. This situation impedes also competitive binding experiments of the parent drug versus the spin derivatives and does not allow estimation of their relative affinities.

The position of the drug in the membrane can be readily established. E.s.r. spectra of spin-labelled chlorpromazine are similarly altered in the presence of ghosts or intact erythrocytes. The resulting lineshape does not display the spectral distortion expected if spin-spin exchange occurs. This does not agree with mixed micelles or hemimicelles stuck on the membrane as assumed by Conrad & Singer (1981). The fact that addition of the quencher ferricyanide at high concentration (50 mM) completely suppresses the free component of spin-labelled chlorpromazine in the spectrum, and the absence of binding to the lysate, supports an exclusive location in the membrane.

The rate of diffusion of chlorpromazine through the membrane can be considered very high on the basis of the present reduction-reoxidation study. This agrees with the results of Ahmed *et al.* (1980), which have established a correlation between the partition coefficient in pure lipid vesicles and the efflux rate for different phenothiazines, chlorpromazine being in the middle of the range studied and cholesterol increasing the efflux to a considerable extent. Diffusion of a small ammonium spin probe through membrane has been found to be negligible for Tempo-choline (Ross & McConnell, 1975) or for Tempo-trimethylamine (C_4 derivatives of Tempo (Erickson *et al.*, 1986). In the present study, it is seen that the graft of a large hydrophobic planar ring on the small cationic reporter group (spin label I) very much enhances its partition in the biomembrane. The fast reoxidation by ferricyanide (0.5 mM) and the extensive binding to the membrane prompt us to assume a rapid flip-flop of the probe from one half to the other. The limiting step of the bioreduction of the spin-labelled derivatives of chlorpromazine is neither the intercalation in the membrane nor the flip-flop rate, but probably the reduction capability of the cell.

Spin-labelled fatty acids (Ogiso *et al.*, 1981; Yamaguchi *et al.* 1985; Minetti & Di Stasi, 1987) suggested a double location in erythrocyte membrane for chlorpromazine: in the lipid bulk or bound to membrane proteins. Boundary phospholipids around membrane enzyme (Ogiso *et al.*, 1981) or membrane proteins (Yamaguchi *et al.*, 1985) were recognized as spin-labelled-fatty-acid-binding sites when the lipid domain of erythrocyte membranes became packed with chlorpromazine. These observations have been obtained by using a higher concentration of drug than that used in the present study. Minetti & Di Stasi (1987) indicated the involvement of erythrocyte skeletal proteins as membrane target sites for active phenothiazines at relatively low concentration.

On the basis of data obtained with spin-labelled chlorpromazine, the simulation of the lineshape confirms a composite spectrum for the drug. The distribution of the drug between the slow-motion and the fast-motion binding sites is dependent on the temperature. The lipophilic nature of phenothiazine associated with a planar tricyclic ring favours the intercalation in the fluid

lipid matrix of the membrane. The fluid liquid-crystalline transition of the lipid matrix is known to influence positively the partition of the drug in the membrane (Luxnat & Galla, 1986). We observed in the present study that, at low concentrations of drug relative to membrane lipids, i.e. under conditions where more than 90% of the drug is inside the membrane, its location inside the membrane varies as a function of the temperature. The fluidity of the lipids favours the exchange of the drug between the possible binding sites. The exchange frequency increases and allows a thermodynamic equilibrium for the drug at increasing temperatures. The simulation of spin-labelled chlorpromazine spectra in membrane confirms an heterogeneous location, with two different environments influencing the drug mobility. Noticeably, the slow-motion component is favoured with the positively charged derivative in membrane or in extracted lipid at low temperature. For this reason the anionic binding sites offered by membrane proteins or by the acidic phospholipids of the inner half of the membrane are of particular concern. Apparently, these binding sites, where the probe experiences a considerable hindrance of motion, are not accessible to the apolar derivative in the membrane at physiological temperature or in extracted lipids. However, at low temperature the biological membrane offers to the apolar spin label a large array of slow-motion binding sites. We then considered the occurrence of two different binding sites of the slow-motion type: one selecting the ligand on the basis of the cationic group and the other on the basis of the phenothiazine ring. No assumption is possible on the difference between, or the identity of, these sites.

The low values of the effective correlation time for the fast component of both probes in biological membranes compared with those in extracted lipids are noticeable and may be related to the perturbing effect of membrane proteins on the lipid matrix. An internal pressure exerted by proteins (Conrad & Singer, 1981) may result in a superficial localization of the drug where the nitroxide group undergoes a faster reorientation than in the membrane lipid core.

Finally, if we correlate the pharmacological activity of phenothiazines, which requires the presence of a positively charged chain with the present binding study, we speculate that the relevant sites for neuroleptic drugs involve electrostatic interaction. This type of interaction, observed for spin label I in membrane-extracted lipids at low temperature, disappears at physiological temperature. For this reason, the role acted by membrane proteins at physiological temperature should be emphasized, whereas the role of acidic phospholipids should be considered cautiously.

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