Biochemical Pharmacology, Vol. 25, pp. 2469–2474. Pergamon Press, 1976. Printed in Great Britain.

INTERACTION OF CHLORPROMAZINE WITH BIOLOGICAL MEMBRANES. A PHOTOCHEMICAL STUDY USING SPIN LABELS

FRANÇOIS LETERRIER, ALAIN MENDYK and JACQUES VIRET

Division de Biophysique du Centre de Recherches du Service de Santé des Armées, 1 bis rue du Lieutenant Raoul Batany, 92140-Clamart, France

(*Received* 22 *January* 1976; *accepted* 26 *May* 1976)

Abstract—Fatty acid spin labels have been included into erythrocyte ghosts and synaptic plasma membranes in order to study the interaction of phenothiazine derivatives (particularly chlorpromazine—CPZ) with these membranes. The following results have been obtained. (1) Weak modifications of the spin label spectroscopic response are observed only on the label of the polar part of the membrane and with CPZ concentrations higher than 5×10^{-4} M. (2) Under ultraviolet irradiation ($\lambda = 310$ nm) phenothiazine derivatives reduce fatty acids spin labels. Measurements of the reduction kinetic constants of two different types of spin labels give information about the location of the drugs inside the membranes. The photochemical interaction is influenced by the membrane proteins. These results suggest that, in the pharmacologically active concentration range, chlorpromazine seems to localize at the interface between the phospholipids and the proteins of the membranes.

In a recent communication [1] we have reported a photochemical method for studying the interaction of phenothiazine derivatives with lecithin multibilayers. The principal thought underlying this method is that under u.v. irradiation, phenothiazine derivatives are oxidized with the formation of free radicals [2] which reduce nitroxide derivatives. When these nitroxide free radicals are incorporated as spin labels inside lipidic structures such as lecithin layers or natural membranes, the measurement of their paramagnetic signal decay kinetics under irradiation in the presence of phenothiazines gives information about phenothiazine location inside the membranes.

In this report, we present the first results obtained with this method in the study of the interaction of phenothiazine derivatives with natural membranes (erythrocytes ghosts and rat brain synaptic membranes). These photochemical results, together with the direct spectroscopic responses given by the spin labels, seems to indicate a preferential location of chlorpromazine at the interface between the phospholipids and the proteins of the membranes.

MATERIALS AND METHODS

(1) *Membrane preparation*

(a) *Erythrocyte ghosts.* Recently out-dated human blood was used for the ghost preparation. After washing the red blood cells with 0.17 M NaCl, they were hemolyzed in 5 mM phosphate buffer (pH 7.5–1 mM EDTA) according to Dodge *et al.* [3]. These membranes will be called, "PO_4 ghosts". Other red blood cells were hemolyzed in 10 mM Tris–HCl (pH 7.4, 1 mM $MgCl_2$) according to Radda and Smith [4]. These ghosts will be called "Tris Mg^{2+} ghosts". Membrane protein concentration [5] was 5 mg/ml.

(b) *Synaptic membranes.* Rat brain synaptic plasma membranes were prepared by the method of Morgan *et al.* [6]. They are free of myelin and of glial cell membranes [7]. The preparation was controlled by measurement of acetylcholinesterase [8] and lactic dehydrogenase [9] activities and by electron microscopy.

(2) *Spin labelling of membranes*

Nitroxide fatty acids were purchased from SYVA Corp., Palo Alto and were used without further purification. Spin label I was 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl and spin label II was 2-(10-carboxydecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl. Formulas of these compounds are given in Fig. 2. Stock solutions of the spin labels were prepared in ethanol (2×10^{-3} M). These solutions were added to the membrane preparation in order to obtain a final concentration of $2–4 \times 10^{-5}$ M (1–2% in ethanol).

No free spin label was observable on the spectra recorded with erythrocyte ghosts. In the case of synaptic plasma membranes, excess spin label was removed by centrifugation (100,000 *g* 1 hr), and resuspension of the pellet in 100 μl of incubation medium.

(3) *Irradiation of the samples and recording of the spectra*

E.S.R. spectra were recorded with a Varian E 3 spectrometer, in aqueous quartz cells, at room temperature. Irradiation of the samples was performed inside the spectrometer cavity, as previously described [1] (HBO 200 W high-pressure mercury lamp, interferential filter centered at 310 nm).

Spin labelled membranes were incubated for 15 min at room temperature in phenothiazine solutions of concentrations ranging from 3×10^{-7} to 10^{-4} M. Membranes were centrifuged (18,000 *g*, 20 min) and the pellet was introduced into the quartz cell. After recording the spectrum, the decay of the central line was recorded during u.v. irradiation. Kinetic constants (k:min^{-1}) were calculated by measuring the slopes of the lines obtained by plotting log I/I_0 as a function of time, where I is the signal intensity at

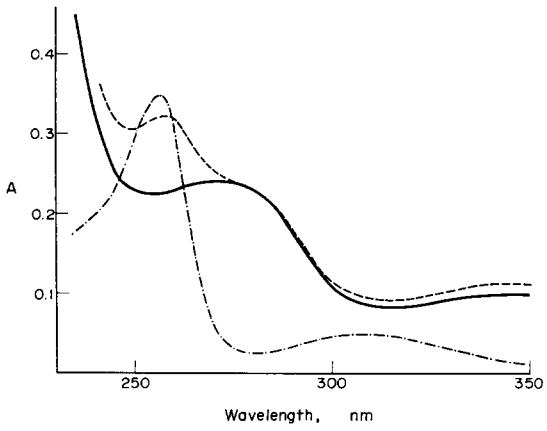

Fig. 1. Absorption spectra of the supernatants obtained after membrane centrifugation. ——: "Tris Mg^{2+}" ghosts without CPZ. – – –: "Tris Mg^{2+}" ghosts after incubation with 3×10^{-5} M CPZ. —·—·—: Spectrum of a 10^{-5} M CPZ solution in Tris Mg^{2+} buffer.

time t and I_0 the signal intensity before irradiation. These plots gave straight lines showing first-order kinetics.

(4) *Measurement of the membrane-bound concentrations of phenothiazines*

After incubation with membranes and centrifugation, free phenothiazine concentrations in the supernatant were measured spectrophotometrically (Aminco DW 2). These spectra show the presence of proteins and traces of hemoglobin (277 and 345 nm). The chlorpromazine (CPZ) absorption spectrum shows maxima at 258 and 308 nm and a trough at 277 nm with a very low extinction coefficient (Fig. 1). CPZ concentration in the presence of proteins was evaluated as follows: the absorption ratio between 257 and 277 nm was measured for supernatant solutions of samples incubated without CPZ. This ratio was used for calculation of the protein absorbance at 257 nm in the presence of CPZ. The difference between the observed and calculated values was taken as due to the presence of CPZ. Its concentration was obtained using molar extinction coefficients determined in parallel experiments with pure CPZ in phosphate ($\epsilon = 33{,}000\,l\,mole^{-1}\,cm^{-1}$) and Tris–HCl ($\epsilon = 31{,}500\,l\,mole^{-1}\,cm^{-1}$) buffers.

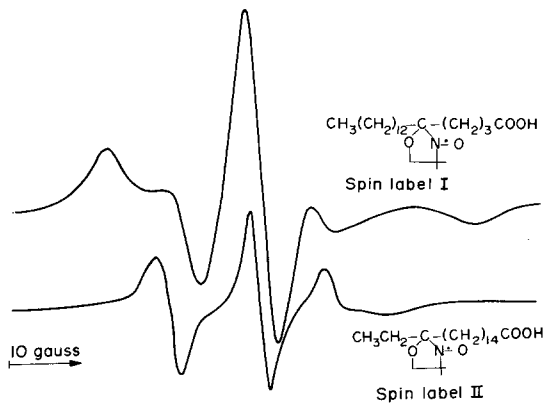

Fig. 2. E.S.R. spectra of synaptic plasma membranes labelled with nitroxide derivatives of stearic acids I and II (modulation amplitude 1 g, incident power 10 mW, time constant 1 sec, scanning rate 12.5 g/min).

RESULTS

I. *Effect of phenothiazines on spin-labeled membranes, without irradiation*

Figure 2 shows the spectra obtained with spin-labels I and II in synaptic plasma membranes. Qualitatively analogous spectra were obtained with erythrocyte ghosts, and they are identical with those previously published [10, 11].

We have measured the order paramater according to Seelig [12] and McConnell [13]. We have found $S = 0.75$ and 0.71 for erythrocyte ghosts and synaptic membranes respectively. These values are not modified after incubation in the presence of CPZ or promethazine (PMZ) in the range of concentrations studied (10^{-4} M maximum). A slight but significant increase was observed with 5×10^{-4} M CPZ in synaptic membranes at 35°, however, ($S = 0.580 \pm 0.15$ without CPZ and $S = 0.620 \pm 0.20$ in the presence of 5×10^{-4} M CPZ) [14].

Rotational correlation times of spin label II, evaluated according to Henry and Keith [15], were 2.25 and 1.9 nsec for erythrocyte ghosts and synaptic membranes at 22°C. These values were not modified by the presence of phenothiazine derivatives, even at high concentrations.

II. *Spin label signal decay kinetics under u.v. irradiation*

(1) *Photosensitivity of the spin labels in solution.* (a) *Photosensitivity of the spin labels alone.* Spin labels I and II were dissolved at 2×10^{-5} M in methanol–water (40/50 v/v). Under irradiation at 310 nm, no variation of their signal amplitude was observed for 8 min. When using a filter absorbing wavelengths below 260 nm in place of the interferential filter, a slow photoreduction was observed: $k = 0.11\,min^{-1}$ for both labels.

(b) *Photosensitivity of the labels in the presence of chlorpromazine.* Experiments were performed in the same solvent as above but with CPZ added at 5×10^{-5} M. Under irradiation (310 nm) we observe a decay of the spin label signals. The kinetic constants ($0.09\,min^{-1}$) were identical for both labels. With promethazine, the kinetic constants were also identical for both labels, but they were higher ($0.25\,min^{-1}$) than with CPZ.

(c) *Photosensitivity of the labels in the presence of tryptophan.* In the presence of 5×10^{-5} M tryptophan in place of CPZ, the spin labels are rapidly destroyed by u.v. irradiation ($\lambda > 260$ nm) ($k_{TRY} = 3.1\,min^{-1}$).

(2) *Photosensitivity of the spin labels included inside membranes.* (a) *In the absence of phenothiazine derivatives.* During irradiation at 310 nm, the amplitude of the signal of spin labels included in membranes decreases slowly. The kinetic constants depend on the spin label and on the mode of preparation of the membrane. The results are summarized in Table 1.

In the case of "PO_4" ghosts, the photoreduction kinetic parameter of spin label I decreases rapidly as a function of the number of washes in phosphate buffer. The spin label II decay parameter is always

Table 1. Variation of the kinetic constants of spin label reduction, in absence of CPZ, as a function of the type and the mode of preparation of membranes

Type of membrane	Erythrocyte ghosts						Synaptic membranes
Mode of preparation	Low ionic strength PO_4 EDTA				Tris Mg^{2+}	Tris Mg^{2+} washed in PO_4	PO_4 EDTA
No. of washing	1	2	3	5	5	5	
Decay kinetic constants of (min^{-1})							
Spin label I	0.200	0.120	0.050	0.045	0.130	0.072	0.035
Spin label II	0.065	0.032	0.034	0.034	0.084	0.034	0.025

lower and is less sensitive to the effects of washing. In the case of "Tris Mg^{2+}" ghosts, the kinetic constants of both labels are higher than with "PO_4" ghosts. Washing of Tris Mg^{2+} membrane with low ionic strength phosphate buffer diminishes the speed of reduction of both labels.

(b) *In the presence of phenothiazine derivatives.* The results vary with the type of membrane and with their mode of preparation.

"Tris Mg^{2+}" ghosts show important difference between the kinetics of both labels. With spin label I, and at a low CPZ concentration, we observe a moderate but significant decrease of the kinetics. For CPZ concentrations higher than 6×10^{-6} M, the kinetic constants increase markedly and reach 1.4 min^{-1} at 10^{-4} M. With spin label II, the kinetic constants are not modified for concentrations lower than 3×10^{-5} M, but they increase abruptly and reach the value obtained with spin label I at 10^{-4} M CPZ. In the presence of promethazine, the variations of the kinetic constants are identical for both labels.

These results are shown in Fig. 3. We have plotted the ratio k/k_0 as a function of phenothiazine concentration, where k is the kinetic constant in the presence of drug and k_0 is the kinetic constant in its absence. This mode of representation permits a better comparison of the results obtained with both kinds of labels since the kinetic values of the controls are not the same.

With "PO_4" ghosts, no significative difference are observed between the decay kinetics of both labels. However, the measured values vary strongly as a

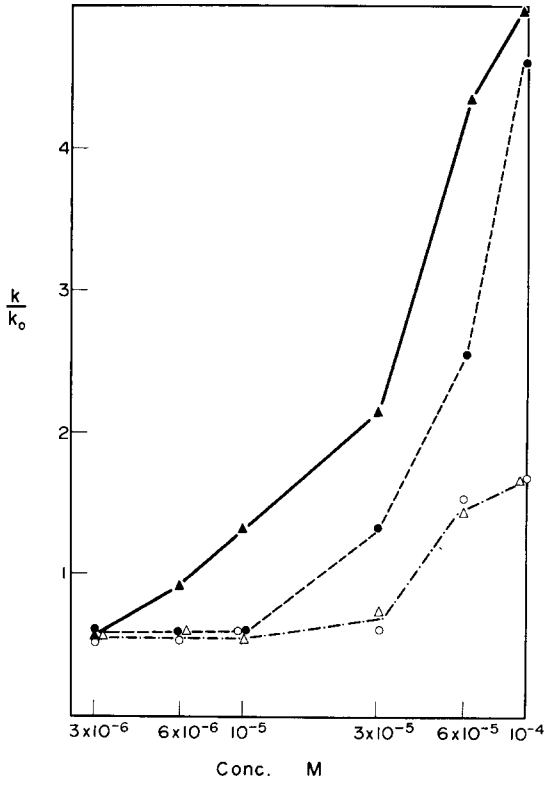

Fig. 3. Relative variation of the kinetic constants for spin label reduction as a function of chlorpromazine or promethazine concentration in the case of "Tris Mg^{2+}" erythrocyte ghosts. For an explanation of k/k_0, see text. ▲ CPZ, spin label I, ● CPZ, spin label II, △ PMZ, spin label I, ○ PMZ, spin label II.

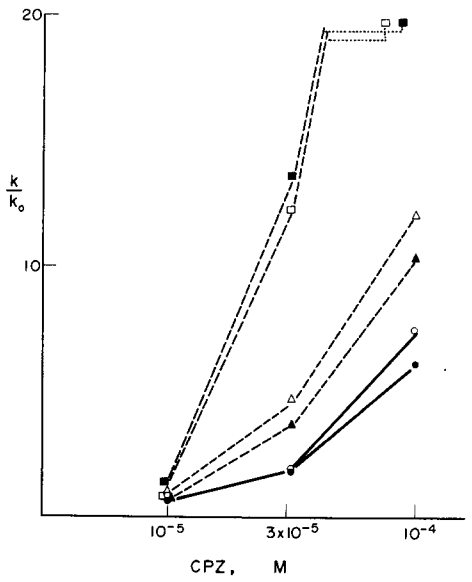

Fig. 4. Relative variation of the kinetic constants of spin label reduction, in the case of "PO_4" ghosts, as a function of the number of washes and of CPZ concentration. After the third wash, in the presence of 10^{-4} M CPZ, the measured kinetic constants are very high. The k/k_0 values are respectively 38 with spin label I, and 40 with spin label II.

Number of washes	Spin label I	Spin label II
1	●	○
2	▲	△
3	■	□

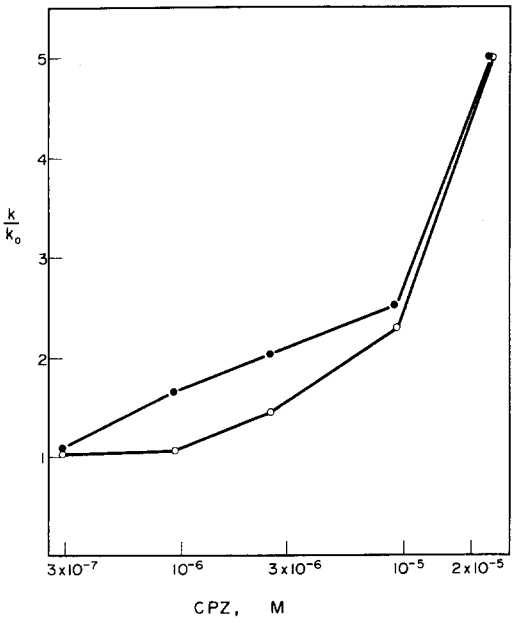

Fig. 5. Relative variation of the reduction kinetic constants of lipidic spin label, as a function of CPZ concentration, in the case of synaptic plasma membranes. ●: spin label I, ○: spin label II.

function of drug concentration and of the number of washes. Results are shown in Fig. 4. With spin label I, we also observe a decrease of the reduction kinetic parameter at low CPZ concentrations with membranes washed one or two times. With thrice-washed membranes, the kinetic constant is strongly increased and reaches 2.3 min^{-1}. With label II, the parameter is not modified at 10^{-5} M, but is identical with those of spin label I at higher concentrations.

The low yield in the preparation of synaptic membranes did not permit us to perform as many experiments as with erythrocytes ghosts. The results are shown in Fig. 5. The effects are observed at lower concentrations than with erythrocyte ghosts since an increase in the reduction kinetics is observed in the presence of 10^{-6} M CPZ with spin label I. At low CPZ concentrations, spin label I is more rapidly reduced than spin label II. The kinetic constants are identical at 2×10^{-5} M and higher concentrations.

III. *Affinity of the membrane for the drug*

The spectroscopic determination of CPZ in the supernatant of centrifuged spin-labelled membranes permitted us to evaluate the number of CPZ molecules bound per mg of membrane protein and the apparent CPZ dissociation constant. This determination was performed by the classical Scatchard's method. Results are given in Table 2. "PO_4" ghosts have a high number of binding sites with moderate affinity. With "Tris Mg^{2+}", two types of sites are observed. The first are not abundant but show a high affinity, whereas the second are ten times more numerous and have affinity for CPZ fifty times lower. Two types of sites are also observed with synaptic plasma membranes.

The binding of CPZ decreases with the number of washes of "PO_4" ghosts, as can be seen in Table 3 when the CPZ concentrations measured in the supernatant are given as a function of the number of washes.

DISCUSSION

I. *Effect of CPZ on the spin label spectra*

The location of nitroxide stearic acid spin labels inside the lipidic structure seems to be well established by numerous works [16]. Spin label I explores the polar part of the lipidic structure, whereas spin label II is sensitive to modifications in the environment of the membrane hydrophobic core. The e.s.r. spectra obtained in this study confirm previous results which show that the lipidic polar zone is rigid and relatively highly ordered, whereas the hydrophobic core is fluid. Small differences are observed between erythrocyte ghosts and synaptic membranes. These latter are more fluid than the former, as can be seen by the lower values of the order parameters and rotational correlation times [17].

Phenothiazine derivatives, in the range of concentration from 10^{-6} to 10^{-4} M, do not significantly modify the spectra. A moderate increase in the order parameter is observed with CPZ, only with spin label

Table 2. Affinity parameters of CPZ for erythrocyte ghosts and synaptic membranes

Type of membrane	Moles of CPZ bound per mg protein	K_D (mole·l^{-1})
"PO_4" erythrocyte ghosts five times washed	4.5×10^{-7}	3×10^{-6}
"Tris Mg^{2+}" erythrocyte ghosts	1: 1.5×10^{-8}	2.8×10^{-7}
	2: 1.5×10^{-7}	1.2×10^{-5}
Synaptic membranes	1: 4.6×10^{-7}	5.7×10^{-6}
	2: 1.3×10^{-6}	1.1×10^{-4}

Table 3. Variation of the CPZ concentration measured in the supernatant of centrifugation as a function of the number of washes of PO_4 erythrocyte ghosts. The experiment was performed as follows: ghosts were washed in PO_4 buffer without CPZ, as many times as indicated, then the pellet was incubated in the presence of CPZ, and the concentration of CPZ in the supernatant measured spectrophotometrically

Number of washes	1	2	3	5
CPZ concentration in the supernatant (mole/l)	4.7×10^{-7}	1.7×10^{-6}	2.2×10^{-6}	2.9×10^{-6}

I, and for concentrations higher than 5×10^{-4} M in the case of synaptic membranes studied at 35°C [14]. At these concentrations we have observed very substantial modifications of the ESR spectra of spin-labelled membrane proteins [18, 19]. This observation is a first argument in favour of a preferential interaction of chlorpromazine with the proteins rather than with the lipids of the membrane. This interpretation seems to be confirmed by the results obtained with the photochemical method.

II. *Photosensitizing action of the membrane proteins*

Under u.v. irradiation of the membranes, the spin labels are slowly destroyed, even in the absence of drugs. The chromophores responsible for this effect are probably the proteic aromatic amino-acids, particularly tryptophan. In membranes, the reduction kinetic parameter is considerably increased when the irradiation is performed around the tryptophan absorption maximum, rather than at 310 nm ($k_{280}/k_{310} = 75$). Furthermore, in solution, tryptophan is an efficient photosensitizer. The measured speed of reduction in the presence of 5×10^{-5} M TRY is approximately the same as in the presence of membranes. Since tryptophan absorbs slightly at 310 nm and since our interferential filter has about 1% transmission at 295 nm, this aromatic amino acid is irradiated even in the presence of the filter and its photoproducts can react with the spin labels. In fact, with PO_4 ghosts, one observes an important decrease of the kinetics parameter of spin label I, as the number of washes increases, whereas the kinetic constants of spin label II decrease only slowly. This result can be explained by a progressive extraction of the less tightly bound proteins [20, 21], which are superficially disposed in contact with the polar part of the phospholipid structure, i.e. in the proximity of the nitroxide group of spin label I. After a sufficient number of washes only the structural proteins are present; they are strongly anchored in the lipidic structure, and even extend across both faces of the membrane. They are then in contact with both types of spin labels.

This interpretation is strengthened by the fact that in "Tris Mg^{2+}" ghosts, even after five incubation–centrifugation cycles, one observes a marked difference in the decay kinetics of both labels. This difference is decreased by washing in low ionic strength phosphate buffer. It has been shown that the presence of divalent ions inhibits the solubilization of peripheral proteins [4, 21].

III. *Photochemical actions of phenothiazines on spin labels*

(1) *In solution.* Although the photosensitization by PMZ is more efficient than by CPZ, no difference is observed between the reactivity of spin label I and II. Thus the difference observed when the spin labels are included inside membranes can be related to local variations of the phenothiazine concentration. It seems noteworthy to us that the kinetic constants measured in solution with 5×10^{-5} M CPZ ($0.09\ min^{-1}$) are lower than those observed in the presence of membranes at the same concentration ($0.3–1\ min^{-1}$). This observation is an indication of the inclusion of the drugs inside the membrane.

(2) *Location of phenothiazine inside membranes.* It is somewhat surprising to observe a decrease of the spin label reduction kinetics at low phenothiazine concentration. Since this reduction, in the absence of the drugs, is due to proteins, it is probable that this result is due to interactions between highly reactive photochemical products of tryptophan and phenothiazines, as for consequence a decrease in the availability of reducing species for the nitroxide-free radicals. In order to verifty this hypothesis, it will be necessary to experiment with an irradiation device using a monochromator in place of the interferential filter, in order to avoid simultaneous irradiation of tryptophan and of CPZ. Such an irradiation device is now in construction in this laboratory.

When the phenothiazine concentration is increased, the reaction between the drug and the nitroxides become predominant, and the results depend on the nature of the mode of preparation of membranes.

(a) "*Tris* Mg^{2+}" *ghosts.* At CPZ concentrations higher than 6×10^{-6} M, the reduction kinetics is higher with spin label I than with spin label II. This result indicates that CPZ is more concentrated at the level of the polar part of the lipidic structure. At these low concentrations, the ghosts have a high affinity for the drug and a relatively low number of binding sites ("specific" sites) (Table 1). As these sites disappear by low ionic strength phosphate buffer treatment, they are probably of proteic nature. It seems therefore that CPZ is located at the contact area of the peripheral proteins with the polar heads of phospholipids.

When the drug concentration increases, these "specific" sites are saturated and CPZ invades the whole membrane structure: the decay kinetics tend to become identical for both labels.

With PMZ, the photochemical reaction is observable at higher drug concentration, and is identical for both labels. As the photosensitizing action of this drug in solution is higher than that of CPZ, this result show that PMZ has a very lower affinity for the membrane and that its localization is not specific.

(b) "PO_4" *ghosts.* No significant differences are observed between the decay kinetics of the two labels studied. As shown in Table 2, these membranes have lost their "specific" binding sites for CPZ. At low concentration of the drug ($\leqslant 10^{-5}$ M), the interaction is thus very weak, and only the direct reaction between CPZ and protein is observable ($k/k_0 < 1$). As the concentration increases, the drug penetrates inside the lipidic part of the membrane and this penetration is favoured by the disparition of the proteins under the influence of successive washes.

(c) *Synaptic membranes.* As in the case of "Tris Mg^{2+}" ghosts these membranes possess two types of binding sites for CPZ, and the decay kinetic constant of spin label I is higher than that of spin label II in the presence of low CPZ concentrations (10^{-6}–10^{-5} M). The drug is thus probably bound at the polar lipidic interface.

This result may be compared with those obtained by the protein spin labelling method previously reported [19]. In this case we observed modifications of the e.s.r. spectra which correlated with the degree of solubilization of membrane proteins. This effect was observed for CPZ concentrations higher than

10^{-4} M. At this concentration in the present study, the decay kinetics are very high but they are the same for both labels.

A considerable amount of work has been done to understand the mechanism of action of neuroleptic phenothiazines. Their impact on membrane structure is well established [22] but their precise mode of membrane interaction is unclear. In the light of the present results and of those previously established by the spin label [8, 19, 23] and fluorescence method [24–27] it seems that the interaction of chlorpromazine with biological membranes depends strongly on the proteic structure of these membranes. This compound does not penetrate in the hydrophobic core of the lipid bilayer in the range of concentrations which are of pharmacological interest. It is thus possible to suggest that its fundamental pharmacological properties [28–31] are mediated by a topological modification of the membrane protein arrangement. One indication for the validity of this hypothesis is given by the present results obtained with promethazine which is a weak antipsychotic agent: its location in the membrane is not the same as that of chlorpromazine.

Acknowledgements—This work was supported by Direction des Recherches et Moyens d'Essais (Contract No. 74/1088 and 75/1071). The useful suggestions and critical reading of the manuscript by Pr. E. S. Copeland are gratefully acknowledged.

REFERENCES

1. F. Leterrier and R. Kersante, *Biochem. biophys. Res. Comm.* **63,** 515 (1975).
2. A. Felmeister and C. A. Discher, *J. pharm. Sci.* **53,** 756 (1964).
3. J. T. Dodge, C. Mitchell and D. J. Hanahan, *Archs Biochem. Biophys.* **100,** 119 (1963).
4. G. K. Radda and D. S. Smith, *Biochim. biophys. Acta* **318,** 197 (1973).
5. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
6. I. G. Morgan, L. S. Wolfe, P. Mandel and G. Gombos, *Biochim. biophys. Acta* **241,** 737 (1971).
7. I. G. Morgan, G. Vincendon and G. Gombos, *Biochim. biophys. Acta* **320,** 671 (1973).
8. G. L. Ellmann, K. D. Courtney, Jr., V. Andros and R. M. Featherstone, *Biochem. Pharmac.* **7,** 88 (1961).
9. M. K. Johnson, *Biochem. J.* **77,** 610 (1960).
10. H. M. McConnell and B. G. McFarland, *Qu. Rev. Biophys.* **3,** 91 (1970).
11. J. L. Rigaud, C. M. Gary-Bobo and C. Taupin, *Biochim. biophys. Acta* **373,** 211 (1974).
12. J. Seelig, *J. Am. chem. Soc.* **92,** 3881 (1970).
13. W. L. Hubbel and H. M. McConnell, *J. Am. chem. Soc.* **93,** 314 (1971).
14. F. Leterrier and J. Breton, *Archs Biochem. Biophys.* submitted to publication.
15. S. Henry and A. Keith, *Chem. Phys. Lipids* **7,** 245
16. I. C. P. Smith, *The spin label method, in biological applications of electron spin resonance* (Eds. H. M. Swartz, J. R. Bolton and D. C. Borg) pp. 483–539. Wiley Interscience (1972).
17. J. Viret and F. Leterrier, *Biochim. biophys. Acta* **436,** 811 (1976).
18. F. Leterrier, F. Rieger and J. F. Mariaud, *J. Pharmac. exp. Ther.* **186,** 609 (1973).
19. F. Leterrier, F. Rieger and J. F. Mariaud, *Biochem. Pharmac.* **23,** 103 (1974).
20. S. L. Marchesi, E. Steers, V. T. Marchesi and T. W. Tillack, *Biochemistry* **9,** 50 (1970).
21. G. Fairbanks, T. L. Steck and D. F. H. Wallach, *Biochemistry* **10,** 2606 (1971).
22. F. Seeman, *Pharmac. Rev.* **24,** 583 (1972).
23. D. E. Holmes and L. H. Piette, *J. Pharmac. exp. Ther.* **173,** 78 (1970).
24. F. Leterrier, J. Canva, J. F. Mariaud and J. M. Calloud, *C. r. Acad. Sci.* **274,** 2094, Série D. (1972).
25. F. Leterrier and C. Thiery, in *The phenothiazines and structurally related drugs* (Eds. I. S. Forrest, C. J. Carr and E. Usdin) p. 77. Raven Press, New York (1974).
26. V. M. C. Madeira and M. C. Antunes-Madiera, *Biochem. biophys. Acta* **323,** 396 (1973).
27. S. T. Christian, *Int. J. Neurosci.* **6,** 57 (1973).
28. P. Seeman and T. Lee, *Science* **188,** 1217 (1975).
29. J. W. Kebabian, G. L. Petzold and P. Greengard, *Proc. natn. Acad. Sci. U.S.A.* **69,** 2145 (1972).
30. G. C. Palmer, G. A. Robinson and F. Silser, *Biochem. Pharmac.* **20,** 236 (1971).
31. G. C. Palmer, G. A. Robinson, A. A. Manian and F. Silser, *Psychopharmacologia* **23,** 201 (1972).