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Effect of Chlorpromazine on Proteins in Human Erythrocyte Membranes as Inferred from Spin Labeling and Biochemical Analyses

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SUMMARY

ESR spectra of erythrocyte membranes labeled with a maleimide spin label (MSL) show two types of label environment: a weakly immobilized component and a strongly immobilized component. Chlorpromazine (CPZ) markedly altered the spectra: at pH 8.0, 3 mM CPZ reduced the amplitude of the spectrum by 40%, and the weakly immobilized component was almost completely removed. In order to clarify the mechanisms of these spectral changes the protein release from erythrocyte membranes induced by CPZ has been followed. CPZ had a weak solubilizing effect on erythrocyte membranes: less than 1% of the membrane protein was released, mainly Band 6. By comparison with the protein release induced by low-salt treatment it was found that the "detergent-like" property of CPZ cannot explain the alterations in the ESR spectra. The nature of the spectral changes induced by CPZ was different from that of changes induced by lowering the pH to 4.5; correlated with other data this shows that changes in organization or conformation of membrane protein cannot explain the CPZ-induced alterations in the ESR spectra. These spectral changes appeared to be due to the reduction by CPZ of the nitroxide free radical. This was documented by the marked reduction of spin concentration of the labeled ghosts in the presence of CPZ resulting in a decrease in amplitude of the ESR spectrum of MSL-labeled erythrocyte ghosts induced by CPZ. The reduction by CPZ of the nitroxide free radical was compared with that induced by ascorbate. It was found that CPZ preferentially reduces the mobile component of the ESR spectrum of MSL-labeled ghosts. The action of CPZ in reducing free radicals may have consequences for patients receiving long-term treatment with phenothiazine derivatives.

INTRODUCTION

CPZ[2] and related phenothiazines are widely used in human therapeutics for various pharmacological actions. Their effects on biological membranes have been recorded by several authors (1-3). Studies of the interactions of CPZ with erythrocyte membranes are particularly challenging since the possibility has been considered that CPZ and its metabolites are transported to the brain by means of the erythrocytes and are possibly released there by a subsequent transfer mechanism to initiate pharmacological activity (4).

Various effects of phenothiazines on erythrocyte membranes have been reported: erythrocytes were lysed at high concentrations and stabilized at low concentrations of phenothiazines (5, 6), while the shape changed to stomatocytes and then to spherostomatocytes, depending on the drug concentration (7). Such effects have been attributed to molecular alterations of the membranes induced by CPZ, originating in the asymmetrical distribution of CPZ over the erythrocyte membrane or the increase in membrane fluidity induced by CPZ (7). However, the mechanism of the interaction of phenothiazines with the membrane has not been well elucidated (8).

Spin labeling has proved very useful in investigating molecular phenomena in erythrocyte membranes. Ogiso *et al.* (9) have reported that CPZ incorporation into the membrane makes the lipid environment more fluid than in the normal membrane. Changes in the ESR spectra from spin-labeled proteins in erythrocyte membranes induced by phenothiazine derivatives have been described by Piette and colleagues (10, 11) and by Leterrier *et al.* (12). These authors noticed that CPZ induced an immobilization of the protein-bound spin labels, and this was attributed to a drug-induced structural change in membrane proteins. However, the mechanism of the changes induced by CPZ has not been established.

Studies on spin-labeled synaptic membranes (2) have shown that CPZ induced changes in the ESR spectrum

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[2] The abbreviations used are: CPZ, chlorpromazine; MSL, 4-maleimido-2,2,6,6-tetramethylpiperidinoxyl; 5P8, 8 mM phosphate buffer (pH 8.0); SDS, sodium dodecyl sulfate.

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similar to those in erythrocyte membranes. These were attributed to preferential solubilization by CPZ of superficial synaptic membrane proteins. If CPZ is able to solubilize selectively proteins containing weakly immobilized sites in erythrocyte membranes, the apparent immobilization of the labels could be explained on the basis of such a "detergent-like" action.

Taking into account all of these data it was of interest to study the mechanism of the changes induced by CPZ in the ESR spectra of spin-labeled erythrocyte membranes, relating them to both possible changes in protein organization and/or conformation and to analysis of the "detergent-like" action of CPZ. In this paper the effects of CPZ on erythrocyte membranes were studied by spin labeling ESR and polyacrylamide gel electrophoresis under various conditions. We concluded that none of the two above-mentioned hypotheses regarding the action of CPZ on membranes can properly explain the ESR changes in erythrocytes. These appeared to be due to the reaction of CPZ with the spin label, resulting in a reduction of the nitroxide free radical.

MATERIALS

Chlorpromazine was obtained from EGYT (Budapest, Hungary). The spin label MSL was purchased from Syva Corporation (Palo Alto, Calif.). All reagents were analytical-grade.

METHODS

Preparation of Erythrocyte Ghosts

Fresh human blood from normal donors was used in all experiments. Several milliliters of blood were collected into heparinized tubes by venipuncture. The erythrocytes were pelleted by centrifugation at 3,000 × *g* for 5 min and the supernatant and buffy coat were carefully removed by aspiration. The cells were suspended in 150 mM NaCl/5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/0.1% glucose (pH 7.4) and washed three times under the same centrifugal conditions. The cells were lysed by mixing rapidly 1-ml portions into 20 ml of cold 5P8, and the ghosts were prepared as described by Dodge *et al.* (13) and Fairbanks *et al.* (14). After three or four washes in 5P8, followed each time by centrifugation at 20,000 × *g* for 20 min, the pellets were homogeneous and white.

Spin Labeling of Erythrocyte Ghosts

After washing, the ghost pellets were suspended in 5P8 to a density of about 3 mg of protein per milliliter. The protein concentration was estimated according to the method of Lowry *et al.* (15). Portions of a 4 mM solution of MSL in ethanol were evaporated to dryness under nitrogen; the ghost suspensions were then added in a 50:1 weight ratio to the spin label. The incubation was conducted for 1 hr at room temperature with gentle magnetic stirring followed by an overnight incubation at 4°. The spin-labeled ghosts were pelleted and the unreacted spin label was removed by washing the ghosts three or four times in the manner already described (until the samples gave a constant ESR signal).

Preparation of Membrane Samples of pH 4.5 and pH 8.0

The pelleted labeled ghosts were suspended to a protein concentration of 4 mg/ml in 5 mM sodium phosphate buffer (pH 4.5 or 8.0). The pH adjustment was made by adding a few drops of 100 mM H_3PO_4 or 100 mM NaOH, respectively. In case of incubation with CPZ a final pH adjustment with NaOH was performed. The samples were centrifuged at 20,000 × *g* for 20 min and the pelleted ghosts were used for ESR measurements.

Incubation with CPZ

Procedure A. Ghost suspensions (50 μl) of 4–8 mg of protein per milliliter were diluted with equal volumes of CPZ solutions (to obtain the final concentration of CPZ indicated in the figure legends) and incubated for 15 min at room temperature with occasional stirring. After dilution with 5P8 the samples were centrifuged at 20,000 × *g* for 20 min. The pelleted ghosts were used for polyacrylamide gel electrophoresis and ESR measurements. The supernatants were concentrated 40 times by ultrafiltration through a Diaflo membrane PM-10 using an Amicon ultrafiltration device (Lexington, Mass.). The concentrated supernatant was used for polyacrylamide gel electrophoresis and ESR measurements.

Procedure B. Ghost suspensions (50 μl) of 4 mg of protein per milliliter were mixed with 5 μl of a CPZ solution (to obtain 3 mM final concentration). The sample was then used for ESR measurements.

Selective Elution of Protein from Ghosts by Low-Salt Treatment (14)

Ghosts in 0.9 ml of 5P8 were diluted into 8.1 ml of 0.1 mM EDTA (pH 8) that had been warmed to 37°. The mixture was incubated at 37° for 20 min and then centrifuged at 100,000 × *g* for 30 min. The pellet was used for polyacrylamide gel electrophoresis and ESR measurements. The supernatant was concentrated 20 times by ultrafiltration and then was used for polyacrylamide gel electrophoresis and ESR measurements.

Polyacrylamide Gel Electrophoresis

One volume (usually 25 μl of ghosts containing 4 mg of protein per milliliter or 20 μl of concentrated supernatants) was added to 3 volumes of a solution containing 4% SDS, 30% sucrose, 2% β-mercaptoethanol, 4 mM sodium EDTA, 60 mM Tris-HCl (pH 8.8), and bromophenol blue (0.02 mg/ml). The mixture was heated for 3 min in a 100° bath. Membranes peptides were separated by using the discontinuous SDS polyacrylamide gel system designed by Laemmli (16). The slab gels used throughout this work consisted of a running gel of 10% acrylamide and 5% stacking gel. The acrylamide to bisacrylamide ratio was maintained at 36.5:1 in both the stacking and running gel. Samples of 25 μl (25 μg of protein) were applied, and the gels were run for 1 hr at 70 V and then 3 hr at 125 V (18–25°) in the running buffer (25 mM Tris/190 mM glycine/0.1% SDS). Following electrophoresis the slab gels were stained for 1 hr with a solution containing 0.075% Coomassie brilliant blue R-250, 45% methanol, and 10% acetic acid. Destaining was performed with a mixture of 5% methanol and 10% acetic acid.

Electron Spin Resonance Measurements

The pelleted ghosts or the concentrated supernatants were sealed in glass capillary tubes and supported vertically in a narrow tube. The temperature was controlled by placing the tube in a glass/quartz Dewar through which a stream of preheated N_2 gas was passed. An ART-6 ESR spectrometer (manufactured by the Institute of Physics and Nuclear Engineering, Bucharest-Măgurele, Roumania) equipped with a variable temperature control device was used for all measurements. The sample temperature was monitored by a digital readout device connected to a copper-constantan thermocouple placed immediately below the sample. The temperature was maintained at 20°. Instrumental parameters were optimized to avoid artifactual broadening.

The spin concentration was determined by dissolution of the labeled ghosts in 0.1 M NaOH to produce a narrow line ESR spectrum and comparing the intensity of this with that of MSL at known concentration (17).

RESULTS

The ESR spectrum of a ghost suspension labeled with MSL is shown in Fig. 1 (Spectrum *A*). The spin label is considered to be covalently bonded principally to sulfhydryl groups, although fewer than 5% of the labeled sites

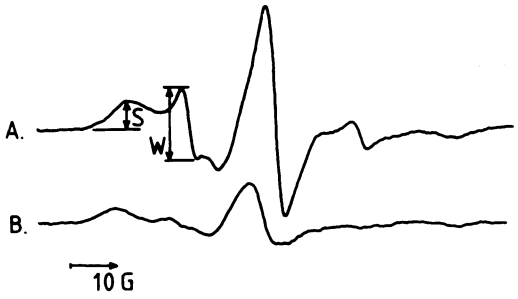

FIG. 1. *Typical ESR spectra of MSL-labeled erythrocytes at pH 8.0*
Spectrum *A*, without chlorpromazine; Spectrum *B*, with chlorpromazine. The amplitudes of low-field lines of the strongly immobilized component (S) and of the weakly immobilized component (W) are indicated.

may be amino groups (18). This type of spectrum has been described previously (10–12, 17–24) as a superposition of at least two spectra, reflecting at least two types of spin-label binding sites.

The amplitude ratio of the weakly and strongly immobilized signals (W/S) has often been used to monitor the relative amount of weakly and strongly immobilized label motions in the protein molecules in membranes under different conditions. As discussed by many authors (10–12, 17–24), the W/S ratio is a sensitive and convenient monitor of the physical state of membrane proteins. In particular, it has been shown (10–12) that CPZ provokes an "immobilization" of the protein-bound spin labels. These changes in ESR spectra have been considered to arise from drug-induced alterations of the protein conformation of one of the spin-labeled sites (10–12).

We have also noticed that CPZ markedly alters the ESR spectra of MSL in erythrocyte membrane. At a concentration of 3 mM (Spectrum *B* in Fig. 1), the amplitude of the spectrum is drastically reduced; more than that, the weakly immobilized spectrum is almost completely removed. The W/S ratio is consequently markedly decreased. By increasing progressively the concentration of CPZ it was found that the W/S ratio is gradually decreased (Fig. 2). The concentration of 3 mM CPZ

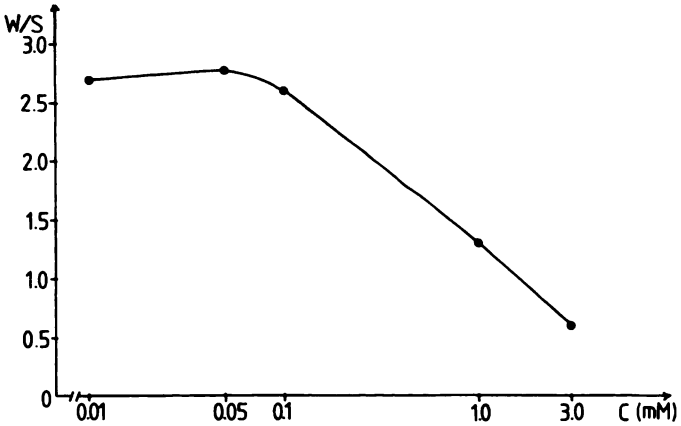

FIG. 2. *Variation of the W/S ratio as a function of CPZ concentration*

seemed to be the end-point of a titration, in agreement with previous reports (10–12).

We have tried to clarify the mechanisms of the changes of ESR spectra induced by CPZ. Thus far two possible mechanisms have been considered (8, 10–12). One mechanism is that of CPZ-induced changes in organization and/or conformation of membrane proteins resulting in a decreased binding of MSL at the weakly immobilized sites and an increased binding at the strongly immobilized site (17, 24). A second mechanism would be the CPZ-induced solubilization from erythrocyte membranes of some proteins containing most of the weakly immobilized sites. It has been suggested that CPZ acts preferentially on the superficial membrane protein layers (2). Therefore, if the proteins loosely bound to erythrocyte membranes are solubilized by CPZ and if such proteins contain most of the weakly immobilized label sites, the spectral changes induced by CPZ could be explained.

In order to discriminate between these two possible mechanisms we have studied CPZ-induced solubilization of proteins from the erythrocyte membrane in comparison with a standardized procedure for eluting loosely bound proteins (including spectrin) from erythrocyte membranes (14). On the other hand, the spectral changes induced by CPZ were compared with changes in the ESR spectra of MSL-labeled erythrocyte ghosts induced by decreasing pH to 4.5, a condition known to induce changes in the organization of proteins in the erythrocyte membrane.

The protein release from the erythrocyte membrane induced by CPZ has been followed by determination of protein concentration and by electrophoretic analyses of the pellet and the corresponding supernatant after membrane incubation in CPZ. A weak protein-solubilizing effect of CPZ has been noted: at a concentration of 3 mM after 15 min of incubation, less than 1% of the membrane protein is released in the supernatant. In contrast, low-salt treatment (14) releases as much as 28% of the membrane protein.

Electrophoretic comparisons of the proteins in erythrocyte ghosts and in the fractions obtained after CPZ incubation or by low-salt treatment are shown in Fig. 3, and the results of quantitative densitometry are listed in Table 1.

The labeling of erythrocyte ghosts with MSL induced only minor changes: Band 5′ was slightly increased and Band 8 was decreased (see notations in Fig. 3). After CPZ incubation (Fig. 3) some proteins were aggregated and did not enter the running gel. Some of these may have originated in spectrin, as Band 1 plus Band 2 (where spectrin is located) was markedly decreased in the pellet after CPZ incubation. A slight decrease in the percentage of Bands 4.5a and 6 could also be noticed. As shown in Fig. 3 and Table 1, the proteins released by CPZ were mainly Band 6 (around 60%) and Band 4.5a, as well as some polypeptides with molecular weights below 20,000 (which we called 8′). The low-salt treatment solubilized mainly Components 1, 2, and 5 (Fig. 3; Table 1), in agreement with Fairbanks *et al.* (14).

Butterfield *et al.* (22) suggested that spectrin contains many of the W sites. However, we found that ESR

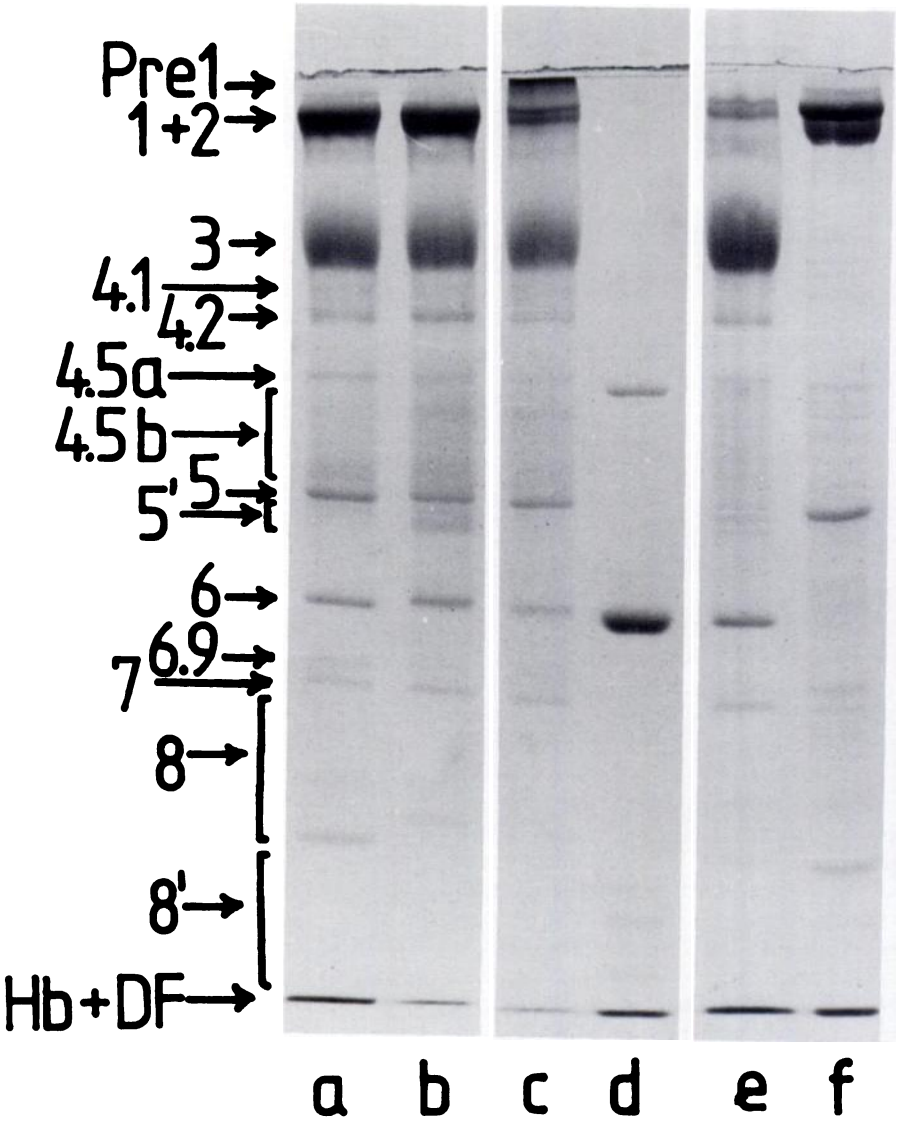

FIG. 3. *Electrophorograms*

a, Erythrocyte ghosts; *b*, ghosts labeled with MSL; *c*, pellet obtained after incubation of ghosts with chlorpromazine; *d*, polypeptides released in the supernatant after incubation with chlorpromazine; *e*, pellet obtained after low-salt treatment of ghosts; *f*, supernatant after low-salt treatment. Details of sample preparation are given under Methods. Hemoglobin and polypeptides are numbered following the system of Fairbanks *et al.* (14) with minor changes. *Hb*, Hemoglobin; DF, dye front.

spectra of the pellet and supernatant after EDTA treatment have both MSL binding sites. Therefore the W signal cannot be attributed to proteins loosely bound to erythrocyte membranes. On the other hand, the CPZ treatment of both pellet and supernatant (Fig. 4) after EDTA treatment showed a decrease in the amplitude of the ESR signal, with a marked reduction of the W/S ratio.

Taken together with the weak protein-solubilizing effect of CPZ and with the absence of any ESR signal in the supernatant obtained after CPZ incubation of MSL-labeled ghosts, these data show that the "detergent-like" property of CPZ (2) cannot explain the alterations in the ESR spectra of MSL-labeled erythrocyte membranes induced by this drug.

The possibility that changes in organization or conformation of membrane protein may explain the spectral changes induced by CPZ was explored by comparing the spectra of MSL-labeled ghosts at pH 4.5 and pH 8.0 in the presence and absence of CPZ. In agreement with previous reports (17, 24) we noted that lowering the pH to 4.5 in the absence of CPZ virtually eliminated the weakly immobilized component, presumably by conversion to the strongly immobilized component (Fig. 5). Values for the W/S ratio for membranes at pH 8.0 are 4.5 ± 0.5 and at pH 4.5 are 0.6 ± 0.2 (mean and standard error of the mean for five experiments). At pH 4.5 CPZ did not markedly change the spectra.

It is known from previous studies that a decrease in the W/S ratio may be an indicator of conformational changes in MSL-labeled proteins (25). However, in the presence of CPZ at pH 8.0 the weakly immobilized component was eliminated without conversion to the S component, but with a concomitant reduction in the amplitude of the S component. It seems therefore that the spectral changes induced by CPZ in MSL-labeled ghosts

TABLE 1

Distribution of protein in fractions of erythrocyte ghosts after various treatments (percentage of total membrane protein)

Band no.[a]	Control ghosts	MSL-labeled ghosts	CPZ treatment[b]		Low-salt treatment[c]	
			Sediment	Supernatant	Sediment	Supernatant
Pre 1	—	—	18.0	—	—	—
I and 2	31.9	34.7	26.1	—	15.8	47.4
3	27.8	27.9	30.0	—	50.7	7.8
4.1	2.2	2.2	1.9	—	1.9	—
4.2	3.5	4.6	3.1	—	4.0	—
4.5a	4.3	3.7	2.6	13.4	2.9	5.6
4.5b	7.0	8.6	4.6	—	5.5	3.7
5	4.9	4.0	3.6	—	2.4	8.6
5′	1.4	2.2	0.5	—	1.3	—
6	4.1	4.0	2.6	59.8	5.5	3.0
6.9	1.6	0.9	1.4	—	—	5.6
7	2.4	1.8	1.7	—	2.4	3.4
8	5.4	2.4	2.4	—	3.4	8.6
8′	—	—	—	11.9	—	—
Hb + DF	3.5	3.0	1.5	14.9	4.2	6.3

[a] According to the nomenclature of Fairbanks *et al.* (14) with minor modifications indicated in Fig. 3.
[b] Procedure *A* under Methods.
[c] After Fairbanks *et al.* (14) as described under Methods.

cannot be explained by conformational changes in membrane proteins.

When a sample of ghosts was first incubated with CPZ at pH 8.0 and then brought to pH 4.5, the weakly immobilized component was again eliminated; however, the amplitude of the spectrum was reduced (Spectrum *B* in Fig. 6 compared with Spectrum *B* in Fig. 5). This suggests again that a reduction of the nitroxide in MSL by CPZ is taking place.

The reduction of the label by CPZ was documented by determination of spin concentration after dissolution of the labeled ghosts in NaOH. In the absence of CPZ it was found that erythrocyte ghosts contained 39.8 nmoles of spin label per milligram of protein. After incubation with CPZ the spin concentration was reduced to 23.5 nmoles per milligram of protein. This means a 40% reduction by CPZ of the spin concentration.

The CPZ-induced decrease in amplitude of the ESR spectrum of MSL-labeled erythrocytes was followed under various conditions, taking for comparison the reduction of the ESR signal by ascorbate. In one series of experiments the incubation of ghosts with CPZ was performed according to Procedure A (see Methods); however, the period of incubation ranged from 0 to 15 min. As shown in Fig. 7, after 5 min of incubation the W component was markedly decreased and after 15 min it was completely reduced. The reduction of the S component was much slower: signal amplitude was 80% of the original after 5 min and 50% after 15 min. This shows that CPZ was reducing the weakly immobilized component much faster than the strongly immobilized one.

As depicted in Fig. 8, ascorbate (0.5 mM) reduced both components at the same rate. Since this reduction was followed as soon as ascorbate was added we tried to perform similar experiments with CPZ. Therefore Procedure B of incubation (see Methods) was followed. As shown in Fig. 9, under these conditions CPZ decreased the ESR signal amplitude more slowly as compared with incubation by Procedure A. However, there was still a difference in the manner of reduction of the two components of the ESR spectrum. During the first 3 hr of incubation the reduction of the W component was faster than that of the S component, the latter not being reduced at all for the first 20 min of incubation.

The reduction process did not seem to require oxygen, since it occurred (at a slightly higher rate) when both the MSL-labeled membranes and the CPZ solution were bubbled with nitrogen or argon. The nitroxide signal could not be restored in reduced membranes by bubbling oxygen. When oxygen was bubbled through a sample of partially reduced erythrocyte membranes, the reduction of the nitroxide was slowed considerably (Table 2).

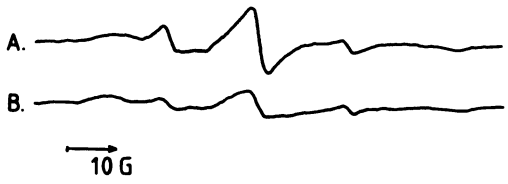

FIG. 4. *Effect of chlorpromazine on the ESR spectra of the supernatant obtained after low-salt treatment of erythrocyte ghosts*
Spectrum *A*, without CPZ; Spectrum *B*, with 3 mM CPZ.

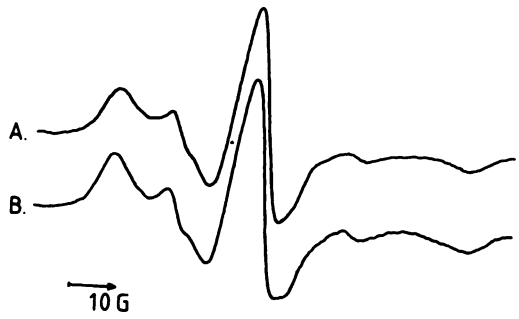

FIG. 5. *ESR spectra of MSL-labeled erythrocytes at pH 4.5*
Spectrum *A*, without CPZ; Spectrum *B*, with 3 mM CPZ.

FIG. 6. *Effect of chlorpromazine and pH on MSL-labeled erythrocyte membranes*

Spectrum *A*, no CPZ, pH 8.0; Spectrum *B*, after 15 min of incubation at pH 8.0 in 3 mM CPZ, the pH of the erythrocyte suspension was adjusted to 4.5.

We may therefore conclude that CPZ has a strong reduction activity toward the MSL nitroxide bound to erythrocyte membrane proteins. The differences in the manner of reduction under various conditions of incubation may be attributed to differences in the accessibility of CPZ to the nitroxide in Procedures A and B. It seems that Procedure A facilitates the contact of CPZ with MSL bound to W sites. The reduction of the ESR signal by CPZ can explain the spectral changes induced by this drug under all of the conditions mentioned above.

DISCUSSION

The typical ESR spectrum of erythrocyte membranes labeled with MSL at the sulfhydryl groups consists of two types of label environment: a weakly immobilized component and a strongly immobilized component. Comparison of one-half of the splitting of the outer hyperfine extrema of the strongly immobilized component to the T_{zz} principal values of nitroxides doped in appropriate host single crystals indicated that sulfhydryl groups in this environment are essentially completely immobilized (22). It was therefore considered that the strongly immobilized component may be associated with spin labels located within the membrane proteins in such a way that their motion is severely restricted (19).

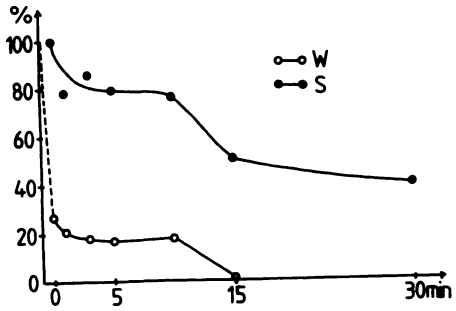

FIG. 7. *Decrease in the amplitude of the W and S components of MSL-labeled erythrocyte ghosts as a function of incubation with 3 mM chlorpromazine (Procedure A described under Methods)*

Portions (50 μl) from a ghost suspension containing 4 mg of protein per milliliter were labeled with MSL at pH 8.0 as described under Methods; they were then diluted with equal volumes of a 6 mM CPZ solution and incubated at room temperature for 0, 1, 5, and 15 min. After dilution with 5P8 the samples were centrifuged at 20,000 × *g* for 20 min and the pelleted ghosts were used for ESR measurements.

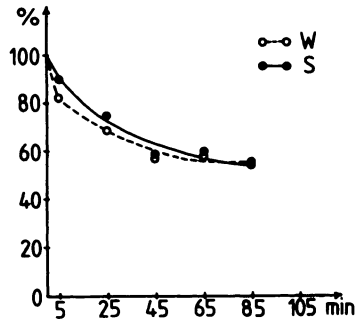

FIG. 8. *Decrease in amplitude of the W and S components of MSL-labeled erythrocyte ghosts as a function of incubation with 0.5 mM ascorbate*

A ghost suspension (50 μl) containing 4 mg of protein per milliliter was mixed with 5 μl of 5 mM ascorbate; the sample was then sealed in a glass capillary tube, and the ESR spectra were recorded after the time periods indicated.

For the weakly immobilized component, one-half of the splitting between the low- and high-field lines is a measure of A_N, the nitrogen isotropic hyperfine coupling constant. A comparison of A_N in the MSL ghost spectrum and in 5P8 and dodecane, respectively, indicated that the weakly immobilized sites are in a highly polar environment (22); these sites are therefore not likely to be in the lipid matrix of the membrane but rather exposed to the polar medium, i.e., bound on the membrane surface or in porelike structures within the membrane (19).

It is obvious (Figs. 1 and 2) that CPZ markedly alters the spectra of MSL in erythrocyte membranes. At pH 8.0 in the presence of 3 mM CPZ the weakly immobilized component is almost completely removed and the amplitude of the spectrum is drastically reduced.

Three possible mechanisms may be considered to explain the spectral changes induced by CPZ in MSL-labeled erythrocyte membranes: (a) selective solubilization by CPZ of some proteins which have an increased proportion of weakly immobilized sites over strongly immobilized sites, compared with the membrane; (b) changes in organization and/or conformation of membrane proteins; and (c) reduction of the nitroxide group of MSL by CPZ.

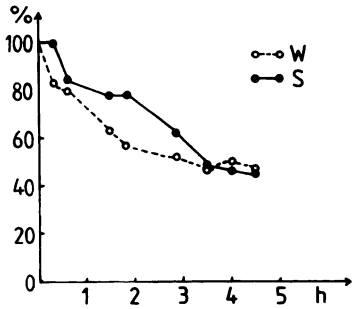

FIG. 9. *Decrease in the amplitude of the W and S components of MSL-labeled erythrocyte ghosts as a function of incubation with CPZ (Procedure B described under Methods)*

A ghost suspension (50 μl) containing 4 mg protein per milliliter was mixed with 5 μl of a CPZ solution (to obtain 3 mM final concentration); the sample was then sealed in a glass capillary tube, and the ESR spectra were recorded after the time periods indicated.

TABLE 2

Effect of oxygen on the spectral parameters of MSL-labeled erythrocyte membranes incubated with CPZ

Two identical samples of ghosts were incubated with 3 mM CPZ (Procedure *A* as described under Methods. One sample (I) was then sealed in a glass capillary tube. The other sample (II) was bubbled with oxygen for 3 min, then sealed in a glass capillary tube filled with oxygen.

Sample	Time	W/S	Amplitude of the components	
			S	W
	min		*% of control*	
I	0	2.08	1.00	24.6
	15	0.68	50	4.0
II (treated with oxygen)	0	2.05	100	24.6
	15	2.07	100	24.0

Selective solubilization of proteins cannot explain the spectral changes induced by CPZ. This mechanism has been proposed for maleimide spin-labeled synaptic membrane, where CPZ induced changes in the ESR spectra similar to those in MSL-labeled erythrocyte membranes (2). However, synaptic membrane protein solubilization by CPZ was much stronger than in erythrocytes. More than 25% of the synaptic membrane protein is released in the supernatant after incubation in 3 mM CPZ. The supernatant gave a spectrum composed of three sharp lines resembling free MSL (2). In the case of erythrocyte membranes, CPZ has a weak solubilizing effect: less than 1% of membrane protein was released by incubation in 3 mM CPZ. The polyacrylamide gel electrophoretic analysis showed that proteins released in the supernatant consist of Band 6, Band 4.5a, and some small molecular weight polypeptides.

No ESR signal could be noticed in the supernatant of MSL-labeled erythrocyte membranes after CPZ incubation. When loosely bound proteins were solubilized from such ghosts by low-salt treatment, both pellet and supernatant gave composite ESR spectra, containing the W and S label binding sites. It may be concluded that protein solubilization cannot explain the alterations in ESR spectra of MSL-labeled erythrocyte ghosts incubated in CPZ.

The pH dependence of the spectra (Fig. 5) reveals two things: (a) at pH 4.5 a part of the W component is converted to the S component, and (b) CPZ does not markedly alter the spectra. The first observation is in agreement with data of other authors (17, 24) and indicates a pH-induced change in the organization and/or conformation of the proteins in the erythrocyte membranes. It has been well documented from morphological studies that, upon decreasing pH, erythrocyte membranes shrink and eventually form aggregates at pH 4.5, the isoelectric point of spectrin/actin (26). It appears that the aggregation process is associated with a conversion of the MSL weakly immobilized binding sites to the strongly immobilized ones, so that a marked decrease in the W/S ratio can be noticed (Fig. 5). The lack of effect of CPZ on the ESR spectrum at pH 4.5 indicates that CPZ does not have access to the nitroxide groups in proteins aggregated at pH 4.5, and consequently little change occurs in the spectra.

It should be emphasized that the pattern of changes induced by CPZ at pH 8.0, even though the W/S ratio is decreased, is different from that induced by a pH value of 4.5. At pH 8.0 the W component is eliminated without conversion to the S component but with a concomitant reduction in the amplitude of the S component.

It may therefore be concluded that the ESR spectral changes induced by CPZ in MSL-labeled erythrocyte membranes cannot be explained by changes in conformation of the proteins because in such cases a decrease in the amplitude of one component is associated with an increase in amplitude of the second component. In case of conformational changes induced by *o*-phthaldehyde (27) the decrease in the W/S ratio is due to the conversion of weakly immobilized sites to strongly immobilized sites, whereas the reverse is true for the conformational changes induced by neutral salts in spin-labeled erythrocyte membranes (20, 21).

The common feature of the ESR changes induced by CPZ in MSL-labeled erythrocyte membrane preparations under various conditions is a reduction in the amplitude of the ESR signal. Accordingly, we interpret the ESR spectral changes to be a result of a reduction of nitroxide free radicals of MSL by CPZ. This interpretation is in agreement with the property of phenothiazine derivatives to yield free radicals; for example, this has been obtained by ultraviolet irradiation (28, 29). The free radicals generated by phenothiazine derivatives represent reactive species which destroy nitroxide free radicals, probably by a reduction reaction. As shown by the photochemical study of Leterrier and Kersanté (28) of the interaction of phenothiazine derivatives with spin-labeled fatty acids incorporated into lecithin multibilayers, the reduction reaction occurs only if the drug can reach the nitroxide group of the spin label. In the above-mentioned study (28), CPZ was found to be preferentially located in the polar part of the bilayer.

In another photochemical study, Leterrier *et al.* (29) noticed that under ultraviolet irradiation phenothiazine derivatives reduced the fatty acid spin labels included in erythrocyte ghosts and synaptic plasma membranes. Measurements of the reduction kinetic constants of two different types of spin labels gave information about the location of the drugs inside the membranes. The authors suggested that CPZ seems to localize at the contact area of the peripheral proteins with the polar head of phospholipids (29).

Several authors have found that CPZ is bound to proteins as well as to lipids of the erythrocyte membrane and is asymmetrically distributed over two halves of the membranes (see ref. 30 for a review), being preferentially bound to the inside face of the membrane. The greater number of binding sites on the inside face is associated with the presence of phosphatidylserine in that side of the membrane and possibly with the presence of more protein binding sites on the inner half as compared with the outer half of the membrane (30).

From the above-mentioned considerations one may assume that CPZ will preferentially reduce the nitroxide group of MSL which is in a polar environment, i.e., the W component. This proved to be the case (Figs. 6–9).

In conclusion we found that CPZ is quite powerful in reducing free radicals in membranes. If our interpretation is correct, this might have consequences for patients receiving long-term treatment with phenothiazine derivatives. However, more data with purified materials need to be obtained concerning this reaction outside of the membrane. Such studies are in progress in our laboratory.

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