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COMPARATIVE STUDIES OF SYNAPTIC MEMBRANE PROTEIN SOLUBILIZATION BY CHLORPROMAZINE AND SODIUM DODECYLSULFATE

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Abstract—Chlorpromazine (CPZ) and sodium dodecylsulfate (SDS) induce qualitatively similar changes in the spectra of protein spin-labelled rat brain symaptic membranes. Comparative study of the yield of protein and spin label solubilization as a function of drug concentration indicates that the effect of CPZ is limited to the proteins which bind spin-labels, whereas SDS acts on the whole depth of the membrane structure. This is confirmed by polyacrylamide gel electrophoresis of the solubilized proteins and by sucrose gradient centrifugation of the various forms of solubilized acetylcholinesterase. The mild detergent-like action of CPZ is tentitatively correlated with some biochemical properties of phenothiazine drugs.

CHLORPROMAZINE (CPZ) and related compounds, are widely used in human therapeutics for their various pharmacological actions. They possess interesting biochemical and biophysical properties. They modify the activities of numerous isolated or membrane-bound enzymes[1–7] and induce, in some cases, protein transconformations.[8] Their actions on biological membranes have been recorded by several authors.[9–12] Most of them think that these compounds interact with the lipidic part of the membrane, but the possibility of protein modifications cannot be ruled out.[13]

Spin labels can be used to study this problem. Depending on the chemical structure of the paramagnetic molecule, they can be attached to the proteins or included in the membrane lipids, and thus they give specific responses on the structural modifications induced in their environment.[14–15] Piette *et al.* on the erythrocyte ghost[16–17] and ourselves on various fractions of brain membranes[18] have shown that compounds like CPZ provoke an "immobilization" of the protein bound spin labels.

The mechanism of this effect has not been established. It could be due to a modification of the membrane protein structure,[19] but the spin label responses which were observed with relatively high concentrations of CPZ, were only partially reversible. Thus the possibility of partial protein solubilization cannot be excluded. Since CPZ possess high tensio-active properties,[20–22] we have tested this hypothesis by comparing the effects of CPZ and of a "true" detergent such as sodium dodecylsulfate (SDS) on the spin labelled rat brain synaptic membrane.

MATERIALS AND METHODS

Isolation of membranes. Brain cortex, from 250 g male Wistar rats, was fractionated by the method of Whittaker.[23] Only the synaptic fractions F and G according to Whittaker's nomenclature (synaptosomes ghosts) were collected for this study.

Spin labelling of membranes. The spin label used was the 3 maleimide 22′, 55′-tetramethyl-pyrrolidine *N* oxyl (maleimide spin label: MSL) purchased from Synvar. It binds covalently on the membrane protein SH groups.[24] The labelling procedure has been previously described in detail.[18] Labelled membranes were suspended in a Ringer solution composed of 0·2 M Tris–HCl pH 6·65 buffer, 160 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 10 mM $MgCl_2$.

Incubation with drugs. Aliquots of membrane pellets (about 2 mg protein) were suspended in 3 ml of drug-Ringer solution, and allowed to stand for 30 min at 0°. Membranes were collected by centrifugation (20,000 *g* for 30 min) and gently homogenized in 150 μl of the drug solution.

Recording of the ESR spectra. Homogenized pellets or aliquots of the supernatants were introduced into a Varian V 4548 quartz flat cell. The same cell was used throughout the experiment; great care was taken to place the cell always in the same position in the ESR cavity. Spectra were recorded on a Varian E3 spectrometer at room temperature.

Modifications of the spectra were evaluated by measuring the mobility index *M* according to Schneider.[25] This index is the ratio of the intensities of the lines *I* and *A* (Fig. 2). A low value of *M* indicates an increase in the proportion of labels with low rotational freedom in the membranes.

The number of membrane bound MSL molecules and the MSL concentrations in the supernatants were evaluated by double integration of the spectra and by standardization with known concentrations of free nitroxide in 80% glycerol.

Protein concentration measurements. The protein concentration of pellets and supernatants was determined by the Lowry method.[26] CPZ reacts with the Folin–Ciocalteu reagent, coloration due to CPZ being additive with that due to protein. Thus, in order to determine exactly the protein concentration in presence of this drug, we made a reference curve for each CPZ concentration used. CPZ was measured by the method of Meunier *et al.*[27]

Disk polyacrylamide gel electrophoresis. Proteins solubilized either by CPZ or by SDS were qualitatively distinguished by disk polyacrylamide gel electrophoresis. The classical method of Davis was used with an Acrylophor apparatus.[28]

In pH 9 glycine buffer, CPZ precipitates and protein migration was perturbated. The solutions were therefore dialysed with a Diaflo membrane UM 10 (filtration limit mol. wt 15,000), against Ringer solution. Control and "SDS" supernatants were dialysed under the same conditions. Gels were loaded with 0·2 ml of dialysed supernatant containing about 0·15 mg/ml protein. Gels were stained first with naphtylacetate which revealed all the forms of cholinesterases,[29] and then with 1% Coomassie blue in 7% acetic acid in the presence of BioRad AG IX 8, for the other proteins.

Acetylcholinesterase sedimentation in sucrose gradient. Supernatants of synaptic membranes incubated with CPZ (3×10^{-3} M), SDS (5×10^{-4} M) or Triton X-100 (1%) were centrifuged in sucrose gradients under experimental conditions previously described.[30] Acetylcholinesterase activities were measured by hydrolysis of acetylcholine in the presence of bromothymol blue. No butyrylcholinesterase activity was

found. This method gave an estimation of the sedimentation constants of the acetylcholinesterase forms, by reference to the marker enzymes β-galactosidase (16 S) and alcohol dehydrogenase (7·4 S).

Depth of membrane labelling. In order to determine the depth of spin label penetration in the membrane structure, we labelled the synaptic membranes with maleimide nitroxides of increasing length.[31] Formulas of these spin labels are given on Table 3.

All chemicals were analytical grade reagents (Sigma, Merck). CPZ was a gift from Rhône-Poulenc. Solutions were prepared just before use, in order to avoid photooxidation.

RESULTS

Effect of a series of compounds acting on the membrane structure

In the first series of experiences we tested various compounds known to denature membranes, in order to determine if any of them possess qualitative effects similar to those previously observed with phenothiazines.[16–18] The results are shown in Table 1. Trypsin and urea increased the spin label mobility. Deoxycholate (DOC) had a drastic effect: at 0·01 %, no modification was observed, whereas at 0·1 % the collected pellet was so small that it was impossible to record the spectrum. With Triton X-100, the *M* index decreased slightly, but the total intensity of the spectrum was reduced about twice. The variation due to SDS was in the same range as with CPZ. Thus we decided to compare the membrane action of these latter compounds.

TABLE 1. EFFECT OF VARIOUS COMPOUNDS ON THE *M* INDEX OF SPIN LABELLED SYNAPTIC MEMBRANES

Compound	*M* index	Observations
Control (Ringer solution pH 6·7)	8·4	
Chlorpromazine 10^{-3}M	2·5	
Chlorpromazine 3×10^{-3} M	0·8	
Trypsin (1 mg/ml)	10·5	(Incubation 30 min at 4°)
Urea 6 M	∞	Only three sharp lines
Triton X-100 0·01 %	6·8	
Triton X-100 0·1 %	6·5	The intensity of the spectrum is twice reduced
DOC 0·01 %	8·5	
DOC 0·1 %	—	Centrifugation pellets are very small
SDS 0·01 % ($3·5 \times 10^{-4}$ M)	8·0	The intensity of the spectrum is only 40 per cent
SDS 0·1 % ($3·5 \times 10^{-3}$ M)	4·4	of the control

Effects of SDS and CPZ on the ESR spectra

Variation of the M index as a function of drug concentration. Figure 1 shows the relative variation of the *M* index after incubation of synaptic membranes in increasing concentrations of drugs. Spectral modifications induced by SDS were measurable at a lower concentration than in the case of CPZ, but the maximum effect was stronger with this drug.

Spectra of the spin labelled membranes, reversibility of the action. Figure 2 shows the spectra obtained on membranes with 3×10^{-3} M CPZ and 10^{-3} M SDS, concentrations for which the maximum effect is observed with both drugs. In order to test the reversibility of this effect, membranes were washed free from the drug by dilution

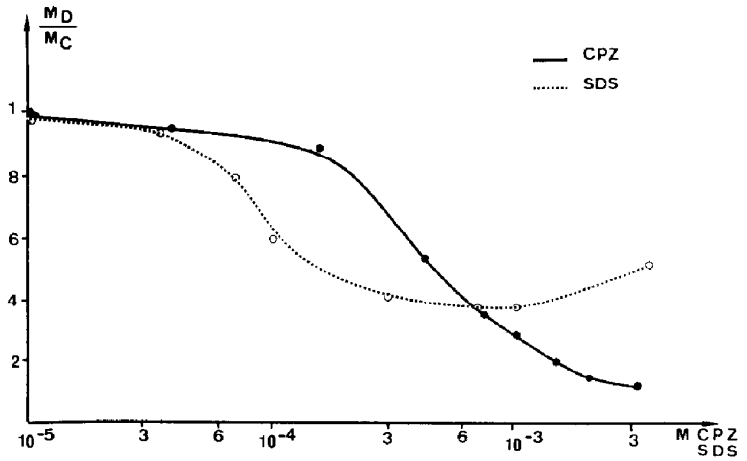


FIG. 1. Relative variation of the mobility index as a function of CPZ or SDS concentration.

$$\frac{Md}{Mc} = \frac{M \text{ index of spin labelled synaptic membrane in presence of drug}}{M \text{ index of spin labelled synaptic membrane without drug.}}$$

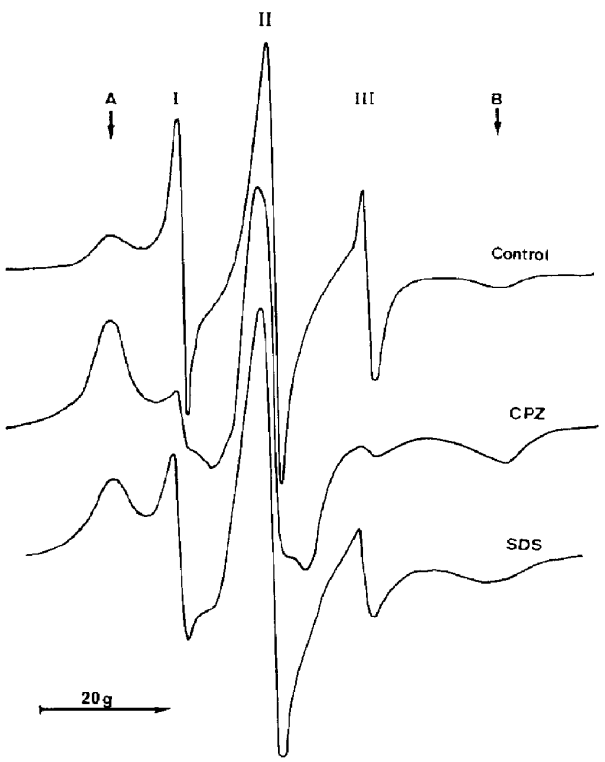


FIG. 2. ESR spectra of the spin labelled synaptic membrane. Lines I and III are due to weakly immobilized spin labels. Lines A and B to nitroxides with low rotational freedom. $M = I/A$ is the mobility index. Control: synaptic membrane in Ringer solution. CPZ: 3×10^{-3} M, SDS: 10^{-3} M.

in 10 ml of Ringer solution and spectra were measured again after centrifugation. No significant change was observed with either CPZ or SDS. At 7×10^{-4} M CPZ, 50% reversibility was measured.

If membranes were first incubated in CPZ (3×10^{-3} M), then in SDS (10^{-3} M), the spectrum did not differ from that recorded with CPZ alone. In the reverse situation, an increase in MSL immobilization was observed.

Spectra of the supernatants. The supernatants obtained after centrifugation of drug membrane suspensions contained spin labels, spectra of which are shown in Fig. 3.

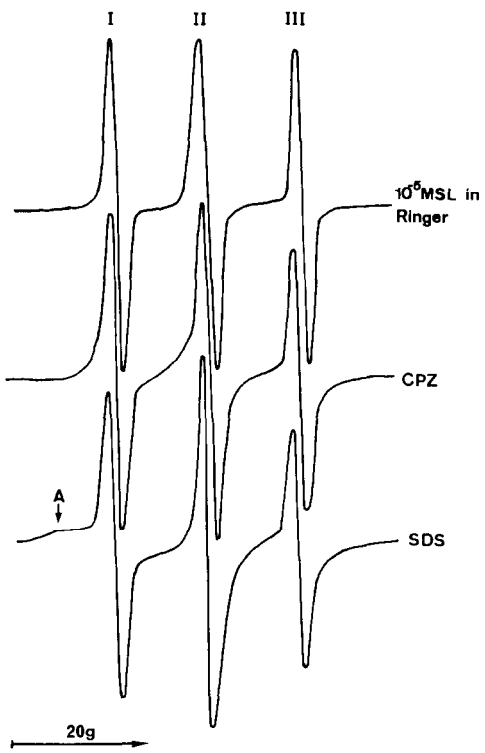


FIG. 3. ESR spectra of the supernatant obtained after centrifugation of drug incubated synaptic membranes (CPZ 3×10^{-3} M, SDS 10^{-3} M). Control is the spectrum of a 10^{-5} M MSL solution in Ringer. Note the presence of a weak *A* line in the "SDS" spectrum.

With CPZ, we observed three sharp lines looking like free MSL in solution. With SDS, the spectrum showed partially immobilized spin labels with an *M* index of 28. In the absence of drug, no spectrum could be recorded in the supernatant fluid.

Variations in the amount of protein and of nitroxides in the membrane and in the supernatants

Simultaneous determinations of protein and MSL concentrations in the pellets and in the corresponding supernatants were made after synaptic membrane incubation in increasing concentrations of CPZ and SDS. Results are given on Table 2. We observed a stronger protein solubilizing effect with SDS than with CPZ. With SDS, the protein and spin label solubilization levels were identical. On the other hand,

TABLE 2. AMOUNTS OF PROTEINS AND OF MSL RECOVERED IN THE PELLETS AND IN THE SUPERNATANT AFTER CENTRIFUGATION OF SPIN LABELLED MEMBRANES IN INCREASING CPZ OR SDS CONCENTRATIONS

		mg of protein				nmoles of MSL				nmoles MSL/mg protein	
Drug	Concn	Pellet	Supernatant	Total	% Solubilization	Pellet	Supernatant	Total	% Solubilization	Pellet	Supernatant
	Control	1·77	0	1·77	0	4·53	0	4·53	0	2·56	
	7×10^{-4}	1·63	0·7	1·70	4	3·65	1·0	4·65	21	2·24	14·5
CPZ	$1·5 \times 10^{-3}$	1·45	0·17	1·62	10·4	3·16	1·41	4·57	31	2·18	8·3
	2×10^{-3}	1·36	0·32	1·68	19	2·63	2·10	4·73	44	1·93	6·6
	3×10^{-3}	1·19	0·41	1·60	25·6	2·30	2·09	4·39	47	1·93	5·1
	Control	1·85	0	1·85	0	4·50	0	4·50	0	2·43	
	$5·2 \times 10^{-4}$	1·38	0·42	1·80	23·3	3·11	1·33	4·44	30	2·25	3·2
SDS	7×10^{-4}	0·90	0·82	1·72	47·5	2·45	1·91	4·36	44	2·72	2·3
	10^{-3}	0·75	1·0	1·75	57	2·13	2·22	4·35	50	2·84	2·2
	$3·5 \times 10^{-3}$	0·57	1·33	1·90	70	1·2	3·27	4·47	73	2·10	2·3

All incubations were performed in 3 ml. Amount of MSL were measured by double integration of the Spectra and comparison with known concentration of the spin label.

with CPZ the solubilization yield was higher for spin labels than for proteins. The MSL/protein ratio (Table 2, last column) decreased when CPZ concentration increased, but it was approximately constant in the case of SDS.

We have verified that this difference is not due to the presence in the SDS supernatants of membrane fragments which would not have been precipitated by the 20,000 *g* centrifugation. Aliquots of supernatants were centrifuged for 3 hr at 300,000 *g*, and protein concentrations were found to be identical after low or high speed centrifugation within the limit of the experimental error (5%).

Disk polyacrylamide gel electrophoresis

Synaptic membranes were incubated in 5×10^{-4} M SDS or 3×10^{-3} M CPZ, in order to obtain identical concentrations of solubilized proteins. Both supernatants were submitted to disk polyacrylamide gel electrophoresis. Important differences in the repartition of protein bands can be seen in Fig. 4. SDS solubilized much more

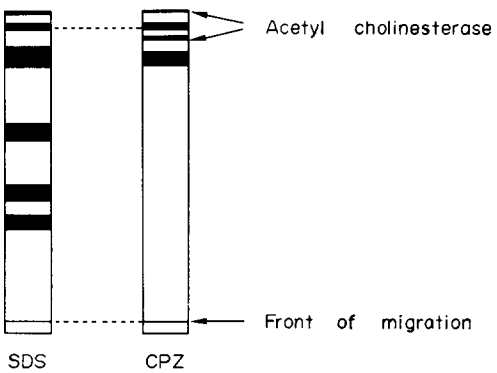


FIG. 4. Drawing of the bands observed after disk polyacrylamide gel electrophoresis of SDS or CPZ supernatants.

protein or polypeptides than CPZ. The solubilization of various forms of acetylcholinesterases seems to be more pronounced with CPZ than with SDS, for the intensity of the corresponding band was higher after incubation with the phenothiazine drug. These results are not due to a modification of the membrane sensitivity to the action of the drugs as a consequence of spin labelling, since we observed the same effects with unlabelled membranes.

Characterization of the solubilized forms of acetylcholinesterase

The various forms of solubilized acetylcholinesterases were separated by sucrose gradient centrifugation as shown in Fig. 5.

With Triton X-100, acetylcholinesterase activity was recorded principally between 12 and 17 S. With CPZ, in spite of a low resolution due to the low concentration of solubilized proteins, the peaks were also observed between 12 and 17 S. With SDS, the centrifugation pattern was entirely different. The main part of the activity was found between 25 and 35 S. This fact agrees with the polyacrylamide gel electrophoresis experiment where we observed that the main part of acetylcholinesterase did not enter the gell.

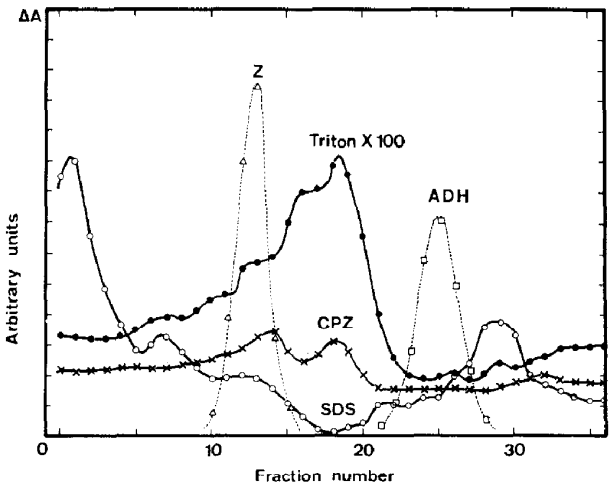


FIG. 5. Sucrose gradient centrifugation patterns of the supernatant obtained after synaptic membrane incubation in Triton X-100 (1%), SDS (5×10^{-4} M) or CPZ (3×10^{-3} M). Conditions of centrifugation: 10^{-2} M Tris–HCl pH 7 buffer, NaCl 1 M, $MgCl_2$ 0·05 M. Sucrose gradient 5/20%. Rotor: Spinco SW 56; 16 hr 37,000 rev/min. Markers; Z: *E. Coli* β galactosidase. ADH: yeast alcohol dehydrogenase.

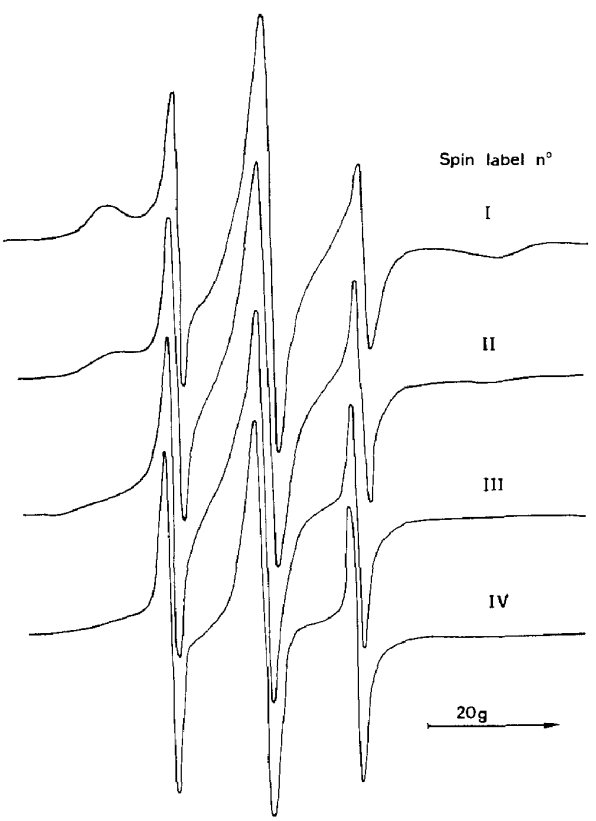


FIG. 6. Spectra of synaptic membranes labelled with nitroxides of increasing length. Spin label formulas are given in Table 3.

Depth of bonding of the spin label

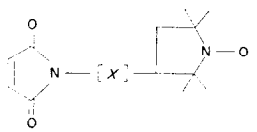
Figure 6 shows the variation of the ESR spectra as a function of the carbon chain length between the maleimide and the nitroxide parts of the labels. Corresponding variations in the *M* index are given in Table 3. We observed a regular increase in this index which reached an infinite value for the longest molecule, the length of which is about 15 Å, indicating that the depth at which the labels were bound on the membrane did not exceed this value.

TABLE 3. VARIATION OF THE *M* INDEX OF SPIN LABELLED SYNAPTIC MEMBRANES AS A FUNCTION OF SPIN LABEL LENGTH

No. of spin label	I	II	III	IV	V
M index	8·1	11	15	25	∞

Formulas of the spin labels:

General structure



No. I: direct bonding between A and B.
No. II: $X = -CH_2-$
No. III: $X = -CO-NH-CH_2-CH_2-$
No. IV: $X = -CO-NH-CH_2-CH_2-CH_2-$
No. V: $X = -CO-NH-CH_2-CH_2-O-CH_2-CH_2-$

DISCUSSION

The membrane effects of detergents have been discussed in numerous studies. Schneider and Smith[25] have shown that 0·2% SDS (7×10^{-3} M) solubilizes 98% of the proteins from MSL labelled human erythrocyte ghosts. They found an *M* value of 22 in the solubilized fraction; our value of 28 is thus in good agreement with this experiment. Fiszer and de Robertis[32] obtained 20% protein solubilization with 0·1% Triton X-100 on rat synaptic vesicles, and 70% acetylcholinesterase solubilization.

DOC is the most used detergent in solubilization studies of membranes proteins. Its drastic effect has been observed by Ernster *et al.*[33] This effect was found also on the spin labelled synaptic membranes (Table 1).

Our results show that the SDS solubilizing action can be observed with concentrations much lower than that generally used. As CPZ and SDS provoked very similar changes in the spin labelled membrane spectra, one may think that the CPZ action is only a trivial detergent-like effect, which can be correlated with the strong tensioactive properties of phenothiazines drugs.[20–22]

However, analysis of the results revealed significant differences in the properties of both compounds. These differences were characterized by the spectra of the supernatants (Fig. 3), by the variations of MSL/protein ratios as a function of drug concentration (Table 2), by polyacrylamide gel electrophoresis (Fig. 4) and by acetylcholinesterase centrifugation gradient (Fig. 5).

The sites of action are thus not the same for both drugs. As the MSL penetrating depth does not exceed 15 Å (Fig. 6), the high amount of spin label per mg of solubilized protein measured after CPZ incubation indicates that this compound acts preferentially on the superficial membrane protein layers, which are the most strongly labelled.

On the other hand, SDS acts on the whole depth of the membrane structure: it solubilizes both labelled and unlabelled proteins, thus giving a less marked modification of the spectrum and a higher M index for the solubilized fraction. This fact was confirmed by the successive drug incubation experiment. When membranes were incubated first in SDS, the "superficial" proteins were not entirely solubilized and CPZ could further modify the spectrum. In the reverse case, SDS did not reinforce the action of CPZ because the detergent acted at a depth where the proteins were not labelled.

At higher concentrations, SDS provoked a complete disorganization of the membrane, the M index increased and the solubilization yield reached 100 per cent.

The spectral change observed on spin labelled membranes under CPZ or SDS action is thus due to the decrease in the number of mobile spin label. Since the ESR lines of this latter type of site were sharp, the spectrum was very sensitive, even to a low yield of solubilization.

The CPZ solubilizing effect was observed only with relatively high concentrations of drug. It can thus be considered as the terminal step of a progressive modification of the membrane structure which begins with lower CPZ concentrations. These modifications are evidenced by other techniques. The work of Seeman *et al.*[11–13] on erythrocytes demonstrates that phenothiazines and related compounds possess a biphasic effect. At low concentration they protect against hypotonic hemolysis. At high concentration they are hemolytic agents. Hemolysis could be related to protein solubilization. Furthermore, experiments with the fluorescent probe ANS have revealed membrane modifications induced by CPZ in a range of concentrations ten to a hundred times lower than in the present study.[34]

Protein solublization by CPZ indicates that this drug interacts strongly with the proteic part of the membrane. However, it cannot be excluded that this solubilization could be an indirect consequence of lipid modification. Preliminary work with spin labels reveals no important spectral change even with high concentration of CPZ.[35]

The detergent-like property of CPZ and probably of many other phenothiazine drugs seems to be original and could be used in the characterization of the superficial enzymes and protein of various membranes. In spite of the low solubilization yield obtained with CPZ in comparison with true detergents such as Triton X-100 or SDS, it will be interesting to use this drug to study acetylcholinesterase, which seems to be localized at the periphery of the neural membrane.[30,36]

Activation of microsomal NADH cytochrome *c* reductase has been observed with CPZ and other phenothiazine derivatives.[6] This enzyme is not solubilized by CPZ, but it is possible that the drug solubilizes, or even only "displaces", a non-enzymic protein fraction that hinders partially the substrate accessibility to the active center of the enzyme. This hypothesis is being investigated.

It is difficult to correlate this CPZ property with its numerous pharmacological actions. However, a good correlation has been established in a previous study[18] between the modification of the spin labelled synaptic membrane spectra and the known neuroleptic activities of a series of phenothiazine drugs.

It is thus possible that these compounds, at pharmacologically active concentrations, could modify the interaction between the various proteic fractions thus inducing perturbations in the membrane permeability and in the kinetics properties of membrane enzymes.

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