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FLUORESCENCE STUDIES OF MEMBRANE INTERACTIONS OF CHLORPROMAZINE AND CHLORIMIPRAMINE

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Abstract—The location of chlorpromazine and chlorimipramine, when these drugs are bound by the erythrocyte membrane, is discussed. The binding of these drugs to ghosts, to liposomes of erythrocyte ghost lipid and to isolated ghost proteins suggest that both the lipid and the protein phase are binding domains. The average distance between drug and membrane tryptophan was determined by fluorescence spectroscopy. These results and experiments with the fluorescent probe 1-anilinonaphtalene-8-sulfonate (ANS) confirmed the indicated location.

The interaction of anesthetics with membranes has been the subject of many investigations because the anesthetic effect is the main action or side action of many clinically important compounds [1]. Though the mechanism of action of anesthetics is far from clear, it seems evident that they influence the properties of the cell membrane. The erythrocyte membrane has proved to be a suitable model system for the studies of molecular events involved in the membrane action of anesthetics [1]. All lipid-soluble anesthetics protect erythrocytes against hypotonic lysis and here a clue has been sought to an understanding of the molecular basis of anesthetic action.

The effects of anesthetics on lipid model membranes in some respects resemble their effects on biological membranes. This suggests a predominant interaction of such compounds with lipid components of the membrane. In recent years however, increasing attention has been paid to membrane proteins and the results of some investigations suggest that anesthetics influence the conformation of membrane proteins [2–12].

Fluorescence spectroscopy can be used to study anesthetic–membrane interactions. Firstly the membrane itself has an intrinsic fluorescence, due to aromatic amino acids in the protein part, especially tryptophan. The presence of certain drugs may cause radiationless transfer of energy from tryptophan to the drug molecule, depending on spectroscopic properties and the location of the drug in the membrane. Secondly, the fluorescence of probes like 1-anilinonaphtalene-8-sulfonate (ANS) depends on the micro-environment of the probe. The interaction of a drug with the membrane can influence the fluorescence of membrane-bound ANS by affecting its micro-environment.

In this study the location of some anesthetics with regard to the protein and lipid phase will be considered. Because of suitable spectroscopic properties the tranquillizer chlorpromazine and the antidepressant chlorimipramine were chosen. Their partition over the protein and lipid phase was studied at a concentration (2×10^{-5}) when they provide a strong protection against hypotonic lysis of erythrocytes [1, 13].

MATERIALS AND METHODS

Pig erythrocyte ghost membranes were prepared according to the procedure of Dodge [14]. The concentrated ghost suspension was diluted in order to obtain a stock suspension with 600 μg protein/ml. Experiments were carried out with a final protein concentration of 60 μg per ml in 20 mOsm phosphate buffer (pH 7.4). Protein was estimated by the Lowry method [15], using bovine serum albumin as a standard. A ghost protein fraction was obtained by removal of the lipids from the ghosts by the butanol-extraction method of Rega [16], followed by extensive dialysis to remove the butanol. This method was chosen because it leads to minimal configurational changes, while over 80 per cent of the membrane protein is recovered. The lipids of the membrane were isolated according to the extraction procedure of De Gier and Van Deenen [17]. The lipids were converted into liposomes by sonicating them for 15 min, under a nitrogen atmosphere and in ice, in 15 mM phosphate buffer (pH 7.4).

Chlorpromazine HCl was obtained from SPECIA and ANS from K & K Laboratories. Chlorimipramine was a gift from CIBA-Geigy B.V. All other chemicals were analytical grade reagents. Unless otherwise indicated ANS concentration was 10^{-5} M; the drug concentration was 2×10^{-5} M.

The binding of chlorpromazine and chlorimipramine added to ghosts was determined by measuring the absorption at 260 nm of the supernatant after centrifugation. The binding to ghost protein and to liposomes was determined by equilibrium dialysis with a Dianorm apparatus, measuring the absorption in the dialysate. The values were corrected for contamination with other 260 nm absorbing material.

Fluorescence measurements were carried out with an Aminco Bowman Spectrofluorimeter. The excitation wavelength for tryptophan emission was

Table 1. Percentage of chlorpromazine and chlorimipramine bound to ghosts, liposomes and ghost proteins at a drug concentration of 2×10^{-5} M*, as compared to total drug quantity added

	Chlorpromazine	Chlorimipramine
Ghosts	41 ± 3†	36 ± 3
Liposomes of ghost lipid	48 ± 4	39 ± 5
Isolated ghost protein	47 ± 4	37 ± 7

* Concentration for ghosts and isolated protein: 60 μg protein/ml; for liposomes: 60 μg lipid/ml.

† Reproducibility was hard to obtain between different batches of erythrocytes. Determinations were carried out in 5-fold with ghosts and ghost components from the same batch of erythrocytes. Here the S.D. was small, ranging from 1–2%. The values given in this Table represent the mean of the results of three different batches; here the deviation is considerable.

290 nm. The distance R between membrane tryptophan and the drug molecules was estimated according to the theory of Förster concerning energy transfer [18]. Förster has shown that when there is an overlap between the fluorescence emission spectrum of a donor molecule (e.g. tryptophan) and the absorption spectrum of an acceptor molecule, resonance transfer of energy from the donor to the acceptor will occur. The critical distance R_0, at which transfer of energy is 50 per cent efficient is given by:

$$(R_0)^6 = \frac{9 \times 10^6 (\ln 10)^2 \kappa^2 c \tau_s}{16 \pi^4 n^2 N^2 \bar{\nu}_0^2} \cdot J_\nu$$

where κ is an orientation factor of the dipole pair, τ_s is the donor lifetime in the absence of an acceptor, n is the refractive index of the medium, $\bar{\nu}_0$ is the wavenumber of the 0,0 transition of the donor and J_ν is the integral of the product of all wavenumbers of donor emission intensity and acceptor absorption [19, 20]. A random orientation of drug molecules in the membrane has been assumed, in which case κ^2 amounts to 2/3. The refractive index was taken as 1.6. The factors τ_s and $\bar{\nu}_0$ refer to the properties of membrane tryptophan and have been taken from a paper of Brocklehurst *et al.* J_ν was calculated from the corrected emission spectrum of membrane tryptophan and the absorption spectrum of the drugs. The distance R was calculated from R_0 and the efficiency of energy transfer T from the relationship

$$T/(1 - T) = (R_0/R)^6 \qquad (21)$$

The T value was determined from the quenching of membrane tryptophan fluorescence after correction for the inner filter effect using solutions of free tryptophan and the drug in appropriate concentrations.

The excitation wavelength for ANS was 370 nm, the emission was measured at 475 nm. The data were represented as relative fluorescence intensities. A double reciprocal plot of ANS fluorescence versus membrane concentration was used to obtain a measure for the quantum yield. The intercept with the ordinate will be indicated with F_m.

RESULTS

The binding of chlorpromazine and chlorimipramine to ghosts has been compared with the binding to its isolated lipid and protein components. For this purpose the isolated ghost lipids were converted into liposomes, because these structures are considered as an appropriate model system for the bilayer structure in the membrane. The binding in a 2×10^{-5} M drug solution is represented in Table 1.

The value for the overlap integral J_ν for chlorpromazine and chlorimipramine with tryptophan were determined from the corrected emission spectrum of tryptophan and the absorption spectra of the drugs. The J_ν value was found to be 2.3×10^{10} cm^3 mM^{-2} for tryptophan to chlorpromazine and 2.2×10^9 cm^3 mM^{-2} for tryptophan to chlorimipramine. The latter value is small because there is only a small overlap of the absorption spectrum of chlorimipramine with the emission spectrum of tryptophan. Thus the error in R_0 is here more significant than for chlorpromazine. This may be in part responsible for the difference between the R-values for chlorpromazine and chlorimipramine. The corresponding R_0-values could be calculated and were 18Å for chlorpromazine to tryptophan and 12Å for chlorimipramine to tryptophan.

Table 2. Values for transfer efficiency T* and drug—membrane tryptophan distance (R) in ghosts and isolated ghost protein

	Ghosts			Isolated ghost protein		
	$T(2 \times 10^{-5}$ M)	T_{max}	R	$T(2 \times 10^{-5}$ M)	T_{max}	R
Chlorpromazine to tryptophan	0.34 ± 0.02	0.41 ± 0.02	19_5 Å 19 Å	0.58_5 ± 0.03_5	0.71_5 ± 0.04	17 Å 15_5 Å
Chlorimipramine to tryptophan	0.12 ± 0.012	0.14 ± 0.014	17 Å 16_5 Å	0.32 ± 0.03	0.40 ± 0.04	14 Å 13 Å

* The T-values are the average of five experiments.

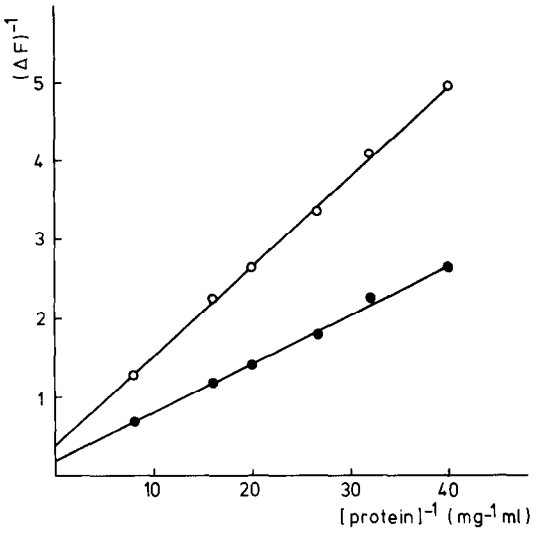

Fig. 1. Double reciprocal plot of ANS fluorescence versus ghost protein concentration. Effect of chlorimipramine on ANS fluorescence. ANS concentration: 10^{-5} M in 20 mOsm phosphate buffer, pH 7.4. Fluorescence intensities are given in arbitrary units; F is the observed fluorescence, corrected for fluorescence of ANS in the absence of membranes and background fluorescence. Each point is the mean of three measurements. The intercept of the line, plotted by the method of least squares, on the ordinate is a measure for quantum yield. The whole experiment was repeated 8 times. Taking $1/F_m$ for ghosts + ANS as unity, then $1/F_m$ for ghosts + ANS + chlorimipramine was 0.60 ± 0.14. (——○——) ghosts without drug; (——●——) ghosts with 2×10^{-5} M chlorimipramine.

Assuming that energy transfer is the only reason of quenching, the efficiency of energy transfer T can be estimated according to the relationship

$$T = 1 - Q_T/Q_0,$$

where Q_T is the quantum yield in the presence of drug and Q_0 the quantum yield in the absence of drug. The ratio Q_T/Q_0 was obtained from the emission spectra of membrane tryptophan, with and without drug, after correction for the inner filter effect. The T-value for maximal energy transfer, T_{max}, was obtained from a plot of the reciprocal fluorescence increment, corrected for the inner filter effect, and the reciprocal drug concentration. The T-values for a 2×10^{-5} M drug concentration, T_{max}, and the corresponding distances drug-to-tryptophan are given in Table 2.

The transfer efficiency in the isolated ghost protein was much higher than in intact ghosts (Table 2); the distance R between the drug molecules and tryptophan is accordingly smaller in the isolated protein than in ghosts.

The addition of ghosts to an ANS-containing solution results in a strong increase of ANS fluorescence (Fig. 1). This is due to an increase in quantum yield of the ANS bound to the membrane. The presence of chlorimipramine (2×10^{-5} M) in the ANS-containing ghost suspension results in a further fluorescence increase, as can be seen in Fig. 1. This change in fluorescence may have two causes: (a) a change in quantum yield, due to a change in the micro-environment of the ANS molecule; (b) a change in the affinity or binding capacity of the membrane for ANS.

To decide whether a change in quantum yield is responsible for the observed increase of ANS fluorescence, plots were made of reciprocal fluorescence change versus reciprocal protein concentration. The intercept on the ordinate, F_m, is a measure for the quantum yield of bound ANS. In our case the protein concentration can be considered as a measure of membrane concentration. As can be seen in Fig. 1, the quantum yield of the ANS-bound protein is increased considerably in the presence of chlorimipramine.

Chlorpromazine presents certain difficulties in fluorescence studies with ANS. When excited at 370 nm there is a considerable emission at 475 nm which overlaps with ANS fluorescence. The fluorescence depends on the membrane concentration because the fluorescence of chlorpromazine depends on its micro-environment. Kramer and Li [22] have described chlorpromazine action in connection with 2-*p*-toluidinyl-6-sulfonate (TNS) fluorescence and erythrocyte membranes; here a decrease in quantum yield was found. We found no significant difference between chlorpromazine and chlorimipramine; both gave an enhancement of ANS quantum yield. The different results obtained by Kramer and Li may be ascribed to the different structure of TNS. Tasaki *et al.* [23] found that the behaviour of ANS was very different from that of TNS, due to a complete different geometry of the molecules.

Chlorimipramine influences the fluorescence of ANS bound to liposomes in the same way as the fluorescence of ANS bound to ghosts, corresponding to a roughly similar quantum yield. The liposomes of ghost lipid (Fig. 2) were compared with liposomes of lecithin, liposomes of lecithin with 20% phosphatidylserine and liposomes of lecithin, phosphatidylserine and cholesterol (Fig. 3). The fluorescence—and quantum yield—of ANS in lecithin liposomes was much higher than in ghost lipid liposomes. The addition of phosphatidylserine causes a decrease of fluorescence, but here too the fluorescence was higher than

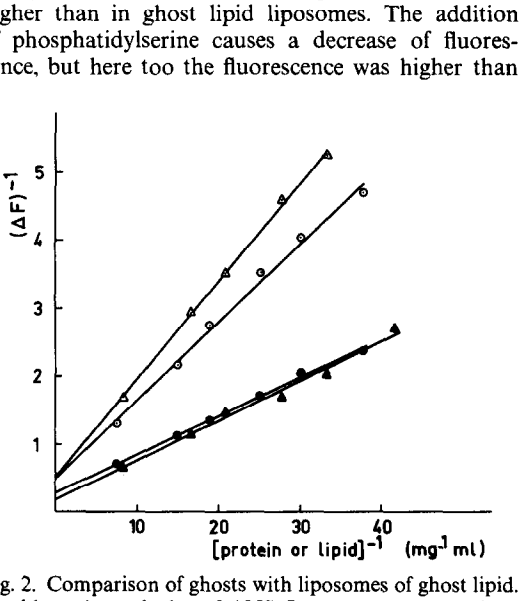

Fig. 2. Comparison of ghosts with liposomes of ghost lipid. Double reciprocal plot of ANS fluorescence versus ghost protein concentration or ghost lipid concentration. Conditions as in Fig. 1. (——○——) ghosts; (——△——) liposomes; (——●——) ghosts with 2×10^{-5} M chlorimipramine; (——▲——) liposomes with 2×10^{-5} M chlorimipramine.

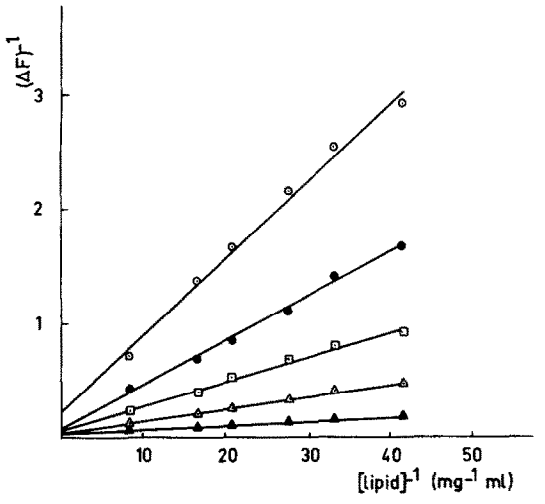

Fig. 3. Comparison of liposomes with different composition. Double reciprocal plot of ANS fluorescence versus lipid concentration. Liposomes were made of pure lecithin, lecithin with 20% phosphatidylserine and lecithin with phosphatidylserine and cholesterol (wt ratios 100:25:40). Conditions as in Fig. 1. (——△——) liposomes of lecithin; (——▲——) liposomes of lecithin with 2×10^{-5} M chlorimipramine; (——□——) liposomes of lecithin with 20% phosphatidylserine; (——○——) liposomes of lecithin, phosphatidylserine and cholesterol; (——●——) liposomes of lecithin, phosphatidylserine and cholesterol, with 2×10^{-5} M chlorimipramine.

with liposomes of ghost lipid. The presence of cholesterol causes a further decrease of fluorescence. The fluorescence pattern of ANS in the latter type of liposomes with and without chlorimipramine resembles that of ghost lipid liposomes.

The behaviour of isolated ghost proteins was completely different from that of ghost lipid liposomes or ghosts (Fig. 4). The increase of ANS fluorescence as well as F_m is considerably higher with ghost proteins as compared with ghosts. In the presence of

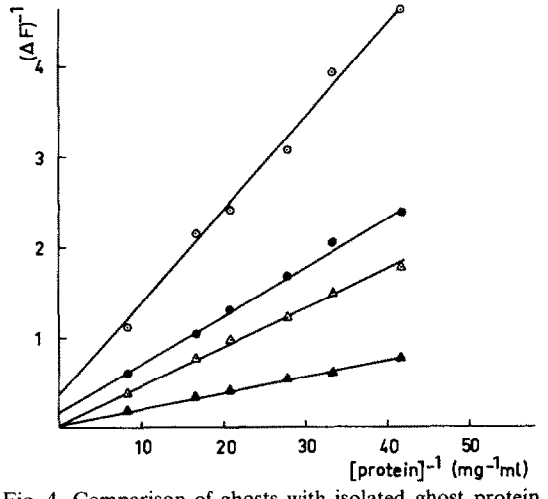

Fig. 4. Comparison of ghosts with isolated ghost protein. Double reciprocal plot of ANS fluorescence vs protein concentration. Conditions as in Fig. 1. (——○——) ghosts; (——●——) ghosts with 2×10^{-5} M chlorimipramine; (——△——) isolated ghost protein; (——▲——) isolated ghost protein with 2×10^{-5} M chlorimipramine.

chlorimipramine the fluorescence was further enhanced but because of the low value of $1/F_m$ a difference in quantum yield could not be measured accurately.

Some anesthetics give highly fluorescent precipitates with ANS [24]. With ANS and chlorimipramine in the concentration used in this investigation no increase of fluorescence was observed. However, with higher concentrations of ANS and chlorimipramine a strong increase of fluorescence was observed. The increase was dependent on the concentration of both reagents. At 10^{-5} M ANS the increase started at a drug concentration of 4×10^{-4} M. With 10^{-4} M ANS and 10^{-4} M chlorimipramine also a strong increase of fluorescence occurs.

DISCUSSION

Several authors have postulated that the action of anesthetics is due to their interaction with the lipid components of membranes, while others maintain that an interaction with the protein part of the membrane is responsible [2–12]. The question is obviously where to locate the anesthetic in the membrane.

Comparison of the binding of the drugs to ghosts with the binding to its isolated components shows that the drugs are bound to each of the components to an important degree. It should be remembered, however, that liposomes are a model system and that the isolation for proteins may produce a change in the binding capacity by disruption of protein–lipid interactions or by denaturation of protein. The fluorescence experiments were designed to evaluate the results of the binding experiment.

The study of energy transfer in fluorescence may be a useful tool in membrane studies. This method has been used to calculate the distance between dyes or drugs and tryptophan in membranes and proteins [25–28]. The method yields average values for the distance of for instance tryptophan to drug molecules. The orientation of drug molecules with regard to the tryptophan molecules is unknown. The results can therefore only be used for an approximate location of the drugs in the membrane by considering the extremes of drug location: the presence of nearly all drug molecules in either the protein or the lipid phase. The distances found seem too large for a nearly exclusive location of the drugs in the protein phase, considering the number of drug molecules bound and the dimensions of the membrane. Our results indicate that the binding of the drug reflects the situation in the intact membrane: both the lipid and the protein phase are important binding domains in the intact membrane. Only in that situation the average distance of drug to tryptophan can be explained.

The drug–tryptophan distances found for isolated ghost protein are considerably smaller than for intact ghosts. The protein isolation procedure probably induces a change in the binding pattern for the drugs. The isolation procedure implies the removal of the lipid phase. According to Rega [16] the structure of the protein is largely preserved. However, a certain degree of denaturation is not excluded. If we assume that changes in protein structure due to denaturation can be neglected, then we may conclude that in the intact membrane the presence of the lipid bilayer has

an important influence on the binding pattern of the drug molecules in the protein phase.

Because of the resemblance between ANS fluorescence in lipid model systems and membranes, and the change in ANS fluorescence in membranes treated with protease or phospholipase C, many authors point to the lipid phase as the main binding domain for ANS [28–35]. Studies of Gulick-Krzywicki *et al.* [36] and Leslauer [34, 35] strongly suggest that the interface, i.e. the region of polar head groups of the lipid bilayer, is the site of ANS binding. In this place ANS is near enough to the protein phase to fulfill the requirement that the distance of ANS to membrane tryptophan is 20–25 Å, as has been calculated by Brocklehurst *et al* [20]. There are also some investigators who suggest that the protein phase is the main binding domain for ANS [37–39].

Our experiments support the idea that ANS is located in the lipid phase of the membrane. The fluorescence of ANS in liposomes corresponds to that of ANS in ghost membranes, with and without chlorimipramine. A much higher fluorescence and quantum yield is given by ANS in the presence of isolated membrane proteins. Perhaps in the intact membrane the lipids prevent ANS binding to proteins; hence the fluorescence properties of the membrane coincide with those of the lipids. An increased hydrophobicity in the isolated protein, leading to an enhanced quantum yield, is unlikely. We found that the tryptophan fluorescence emission maximum of isolated protein (343 nm) is higher than that of tryptophan in ghosts (338 nm), which indicated more polar surroundings for the tryptophan in the isolated protein [40].

The most striking effect of chlorimipramine is the enhancement of the quantum yield of ANS bound to ghost membranes. The nature of this effect differs from that obtained with cations. Relatively high concentrations of cations induce an enhancement of ANS fluorescence, due exclusively to an increased binding of ANS as a consequence of shielding of membrane charges, leaving quantum yield unaltered [24, 29].

A reasonable explanation of this increased quantum yield is that complex formation, comparable to that of high concentrations of chlorimipramine with ANS in solution, occurs in the membrane. The membrane apparently behaves as a matrix where the drug and dye molecules are concentrated. This suggests that part of the drug appears to be in the same phase as ANS, i.e. in the lipid phase, which is in agreement with the hypothesis mentioned before.

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