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THE MEMBRANE CONCENTRATION OF A LOCAL ANESTHETIC (CHLORPROMAZINE)

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SUMMARY

1. The classical partition theory of anesthesia proposed by MEYER[1], BAUM[2], OVERTON[3] and MEYER AND HEMMI[4] predicted that the concentration of anesthetic in the membrane should be of the order of 0.03–0.06 mole anesthetic per l of membrane lipid. In order to measure the membrane concentration of a local anesthetic directly, the adsorption of chlorpromazine to hemoglobin-free erythrocyte ghost membranes was measured at concentrations known to be membrane stabilizing for both nerve fibers and erythrocytes.

2. In the chlorpromazine concentration region for membrane stabilization and anesthesia, namely $2 \cdot 10^{-6}$–$2 \cdot 10^{-5}$ M, the adsorption of chlorpromazine by erythrocyte ghost membranes was associated with a single set of binding sites. When these sites were 50% occupied (50% membrane stabilization) the membrane concentration of the anesthetic was about 0.03 mole drug per l wet membrane. When the sites were fully occupied (at around $2 \cdot 10^{-5}$ M) the membrane concentration was 0.066 mole anesthetic per l wet membrane. The range of 0.03–0.066 M approximately agrees with the values predicted by the partition theory.

3. At chlorpromazine concentrations higher than $3 \cdot 10^{-5}$ M, which corresponds to lytic concentrations of this surface-active anesthetic, the membrane concentrations increased very steeply. The membrane concentration of these "lytic sites" is much higher than 0.066 mole/l wet membrane.

4. The maximum membrane concentration of 0.066 M for the "stabilization sites" did not vary with the temperature.

5. The affinity constant for the adsorption of chlorpromazine was 165000 M^{-1} at 22°. The affinity constant varies inversely with the temperature.

6. The effect of pH on the membrane concentration of chlorpromazine was also examined. At the extracellular concentration of $2.5 \cdot 10^{-6}$ M the membrane/buffer partition coefficient was 4300 at pH 6.8 and 3500 at pH 6.2.

7. The free energy of adsorption was —9400 cal/mole. The enthalpy of adsorption was negative at 14° (—500 cal/mole), and became more negative (—3000 cal/mole) at higher temperature. These findings, according to the theory of NEMETHY AND SCHERAGA[52,53], suggest that the chlorpromazine–membrane interaction is hydrophobic in nature.

8. The bulk volume of 0.066 mole chlorpromazine in 1 l wet membrane would occupy between 12 and 25 cm^3 or 1.2–2.5 % of the wet membrane volume. The bulk

volume of the molecules cannot completely account for the 4.5 % expansion of the erythrocyte membrane area that is known to occur in the presence of $2 \cdot 10^{-5}$ M chlorpromazine.

INTRODUCTION

The classical partition theory of anesthesia formulated by MEYER[1], BAUM[2], OVERTON[3] and MEYER AND HEMMI[4] (see ref. 5 for a review) predicts that the concentration of anesthetic in the membrane-lipid phase is of the order of 0.03–0.06 mole of anesthetic per l of membrane lipid when animals are anesthetized.

As ARIENS *et al.*[6] have recently summarized, this value is obtained for a wide variety of anesthetics and organisms, and is calculated by multiplying the extracellular aqueous concentration of the anesthetic by the olive oil/water solubility coefficient of the anesthetic. For example, the average concentration for a variety of anesthetics in the membrane lipid is calculated as 0.033 mole/l of lipid under conditions of inhibition of the reflex response of tadpoles[4,6]. For anesthesia of mice a higher average value of 0.065 mole anesthetic per l membrane lipid has been calculated (ref. 5, p. 190).

Since membranes contain 40–50 % protein[7] as well as a lipid composition very different from that of olive oil[7], the choice of olive oil as a membrane model for determining membrane anesthetic concentrations is not suitable. It would be more desirable to examine the narcotic concentration in the membrane directly. This task is difficult if not impossible for the plasma membrane of the neuron because the neurolemma is intimately surrounded by Schwann cells. There are as yet no methods available for obtaining large yields of pure neuron plasmalemmata, free of glial membranes. This is unlike the situation for erythrocyte plasma membranes which can be obtained in abundant amount and in a very clean state[8].

For the following reasons the erythrocyte membrane has been used as a quantitative model for studying the molecular mechanism of the nerve membrane stabilization which occurs in anesthesia[9–21].

(1) It is known that anesthetics have an anti-hemolytic and membrane-expanding effect on erythrocyte membranes[9–19,22–25].

These effects occur at anesthetic concentrations which are almost identical to those which anesthetize nerve fibers[9]. The anesthetic potencies of the alcohols correlate with the erythrocyte stabilizing abilities over a 10000000-fold concentration range.

(2) All compounds which protect or stabilize erythrocytes against hemolysis can also act as local anesthetics[13].

(3) Both the neutral and cationic forms of local anesthetics which are tertiary amines are known to be active in anesthetizing the nerve cell membrane from the interior and exterior aspects[18,26–31]. This is also true for the erythrocyte membrane[18].

Both SHANES[32] and SKOU[33] have predicted an expansion of biomembranes in the presence of anesthetics, on the basis that such a drug-induced expansion does occur in a lipid monolayer at the air–water interface[34–37].

Recent work[16–18] shows that at the $E.D._{50}$, the anesthetic concentration which inhibits hypotonic hemolysis by 50 %, the membrane area of the intact erythrocyte expands by around 3 %. At the $E.D._{50}$ the erythrocyte ghost area expands by

1.3–1.6 % for the alcohol anesthetics and by 1.5–2.7 % for chlorpromazine. At higher but sublytic concentrations of these drugs, the maximum amount of membrane area expansion observed was around 5 %.

As already discussed by SEEMAN *et al.*[17], anesthetics might expand the membrane by: (1) dissolving right into the membrane and occupying bulk space[9,10,38,39] without thickening the membrane[12]; (2) an induced secondary extension of the membrane following the adsorption of the anesthetic, the "adsorption–extension" hypothesis of SCHNEIDER[40]; (3) displacing membrane-bound components which normally keep the membrane condensed, such as Ca^{2+} (see ref. 16 for references); (4) inducing conformational changes in the membrane proteins[41].

The studies reported in this paper were carried out in order to test directly and quantitatively the first possibility mentioned above. The main findings are as follows.

(1) The erythrocyte ghost membrane concentration of local anesthetic, chlorpromazine·HCl, under conditions of 50 % membrane stabilization is around 0.033 mole anesthetic per l wet membrane. This compares with the value of 0.03 mole/l of membrane lipid predicted by the classical partition theory for anesthesia of 50 % of the animals.

(2) The bulk volume of the anesthetic molecules can only account for at most approximately a third of the observed membrane area expansion. To explain the drug-induced membrane expansion completely, therefore, other mechanisms, such as those mentioned above, must be considered.

METHODS

Preparation of erythrocyte ghost membranes

Human blood, stored in acid–citrate–dextrose medium, was obtained from the Red Cross blood transfusion service of Toronto. The blood was centrifuged at 3000 × *g* for 5 min and the plasma and buffy coat were removed. The remaining erythrocytes were washed 3 times with 154 mM NaCl in 6.25 mM sodium phosphate buffer (pH 7.4). 1 mM EDTA was present in all solutions. Ghosts were then prepared by the method of DODGE *et al.*[8]. The hemolyzing solutions contained 1 mM EDTA (ethylene diamine tetraacetate, sodium salt) and were buffered at pH 7.4 with 6.25 mM sodium phosphate buffer; the total ideal osmolarity was 20 mosM. The resulting ghosts were washed 3 times with 10 mM sodium phosphate buffer (pH 7.0). In these ghosts hemoglobin constitutes approx. 1.4 % of the dry weight[8]. The membranes were pooled and concentrated by centrifugation at 36900 × *g* for 40 min.

The dry weight of membranes present in the ghost suspension was determined by drying at 90° for at least 24 h and by correcting for the dry weight of the buffer salts. As determined by the dry weight, the concentration of ghosts in the stock ghost suspension was usually between 1 and 2 % (g dry membrane per 100 ml of ghost suspension).

Determination of the membrane concentration of chlorpromazine

For the adsorption experiments 0.2 ml of $2 \cdot 10^{-4}$ M ^{35}S-labeled chlorpromazine (specific activity, 4.7 mC/mmole; Amersham, Great Britain) was mixed with 0.5-ml aliquots of ghost in 10 mm × 75 mm polycarbonate tubes. The final drug concen-

tration was adjusted by addition of 0.2 ml containing varying concentrations of the nonradioactive compound. After 30 min incubation the ghosts were spun down at 36900 × *g* for 20 min in a temperature-controlled centrifuge. The radioactivity in 0.1-ml samples of the supernatant was determined in a liquid scintillation counter with the liquid scintillator described by BRAY[42].

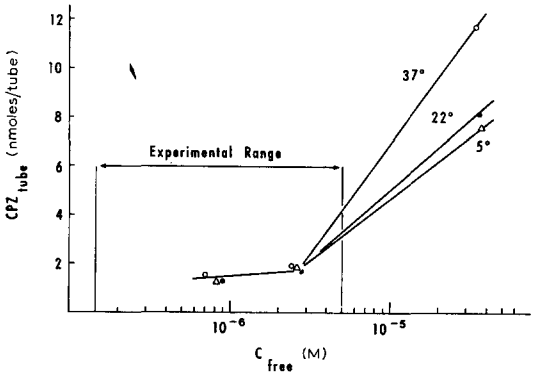

Fig. 1. The adsorption of chlorpromazine·HCl to the walls of polycarbonate tubes. CPZ_{tube} represents the amount of chlorpromazine in nmoles which has been adsorbed per tube. Each tube contained 0.2 ml (with 188 nC) of [^{35}S]chlorpromazine·HCl, 0.5 ml of 10 mM sodium phosphate buffer, and 0.2 ml of nonradioactive chlorpromazine solution. c_{free} is the concentration in the final mixture after 30 min incubation and centrifugation at different temperatures.

In the absence of membranes a low but finite amount of drug adsorbed to the walls of the tube. The amount of chlorpromazine adsorption to the polycarbonate tubes was measured at different drug concentrations and at different temperatures. The results are shown in Fig. 1 where the amount of moles bound per tube, CPZ_{tube}, is plotted against the free chlorpromazine concentration. From the results shown in Fig. 1 it can be seen that the absolute amount of chlorpromazine bound per tube is small and is also rather insensitive to the temperature in the free concentration range of experimental interest. The membrane concentration of chlorpromazine, c_m, in moles drug per l of wet membrane was obtained as follows:

$$c_m = \frac{CPZ_m \times 1000 \times 1.17}{1.00/0.70} \quad (1)$$

where CPZ_m is the amount of drug bound to 1 g of dry membrane, where the amount of water in the hydrated membrane is taken as 30 % of the total weight[43], and where the density of the wet membrane is taken to be 1.17 (refs. 44 and 45).

CPZ_m (moles/g dry membrane) was obtained as follows:

$$CPZ_m = \frac{(c_{total} - c_{free})0.0009 - CPZ_{tube}}{0.5 \times D.W.} \quad (2)$$

where c_{total} and c_{free} are the total and free chlorpromazine concentrations (M) in the tube, where CPZ_{tube} is the amount of drug adsorbed to each tube (moles/tube) at the c_{free} which is present (Fig. 1), and D.W. is the percent dry weight of the added ghost suspension in g dry solids per ml ghost suspension. The final volume in each tube was 0.9 ml, 0.5 ml of which had been added from the ghost suspension. The

free drug concentration present after adsorption to the ghost membranes could be calculated from:

$$c_{free} = \frac{\text{disint./min (sup)} \times c_{total}}{\text{disint./min (control)}} \quad (3)$$

where disint./min (sup) are the radioactive disint./min found per ml of supernatant, and disint./min (control) represents the disint./min per ml found in control tubes in which 0.5 ml of the ghost suspension had been replaced by 0.5 ml of buffer. Disint./min (control) was first corrected for the adsorption of chlorpromazine to the test tube.

The membrane/buffer partition coefficient, P, for chlorpromazine was readily obtained:

$$P = c_m/c_{free} \quad (4)$$

Unless otherwise stated, the experiments were carried out in 10 mM sodium phosphate buffer (pH 7.0) at 22°.

Effect of temperature and pH on the membrane concentration of chlorpromazine

For the temperature experiments incubation and centrifugation were temperature controlled. An adsorption isotherm at 22°, using the same ghost suspension, was always determined on the same day.

Measurements of the pH in the final mixture of ghosts and chlorpromazine, as well as in the supernatant after centrifugation were made with a Beckman combination electrode (5 mm diameter). At the temperatures used, the pH of the complete mixture was 0.1 pH unit lower than the pH of the supernatant. This is probably due to a somewhat smaller pH in the direct vicinity of the membranes[46,47].

To study the effect of pH on adsorption small aliquots of 1 M HCl were added to the solutions. Chlorpromazine adsorption to the membranes was determined with and without HCl being present.

Calculation of the number of binding sites and the affinity constant of the membrane for chlorpromazine

The number of sites present in the membranes and their affinity for the drug were determined by plotting the reciprocals of the drug concentration in the medium and in the membrane according to the equation:

$$\frac{1}{c_m} = \left(\frac{1}{c_m^{max}} \cdot \frac{1}{K}\right) \frac{1}{c_f} + \frac{1}{c_m^{max}} \quad (5)$$

where c_m is the drug concentration in the membrane expressed per l of wet membrane, c_m^{max} is the drug concentration at complete saturation of a set of sites, c_{free} is the free concentration of chlorpromazine, and K is the equilibrium constant for the binding between drug and membrane (see ref. 48 for further references on this equation).

Any linear portion in this plot corresponds to a set of sites with the same affinity for the drug. The equilibrium constant for such a site and chlorpromazine and also the amount of drug present at complete saturation of this site can be calculated directly from Eqn. 5.

Calculation of the free energy, enthalpy and entropy of chlorpromazine adsorption to the membrane

The free energy change when 1 mole of drug binds to a certain site can be calculated from its equilibrium constant:

$$\Delta F = -RT \ln K \tag{6}$$

where ΔF is the free energy change, R is the gas constant and T is the absolute temperature. K has to be multiplied by 55.5, the number of moles of water per l, in order to change its units from l/mole to (mole fraction)$^{-1}$ (see ref. 48).

The value of K at different temperatures allows a calculation of the enthalpy change occurring during the reaction. Integration of the Van 't Hoff equation:

$$\frac{\mathrm{d} \ln K}{\mathrm{d}T} = \frac{\Delta E}{RT^2} \tag{7}$$

gives:

$$\ln K = -\frac{\Delta E}{RT} + \text{constant} \tag{8}$$

where ΔE is the total energy change in the reaction under consideration[49]. Since energy changes due to volume changes are usually very small, no error is introduced by taking ΔE to be equal to the enthalpy difference, ΔH. When at a temperature T_1 the equilibrium constant is found to be K_1 and when at T_2 a value of K_2 is determined, then for this temperature interval the enthalpy change is:

$$\Delta H = \frac{R \cdot \ln K_1 \cdot K_2}{\frac{1}{T_2} - \frac{1}{T_1}} \tag{9}$$

To obtain an estimate of the entropy change, ΔS, as a function of temperature, ΔF and ΔH for two temperatures were obtained. If at T_1 and T_2 the free energy change was ΔF_1 and ΔF_2, respectively, and if the enthalpy change calculated for this interval was ΔH, then at the temperature $(T_1 + T_2)/2$ the free energy change was assumed to be $(\Delta F_1 + \Delta F_2)/2$ and the enthalpy change ΔH. The entropy change ΔS was determined from the equation:

$$\Delta F = \Delta H - T\Delta S \tag{10}$$

RESULTS

The membrane concentration of chlorpromazine at different values of c_{free}

The concentration of chlorpromazine in the erythrocyte ghost membrane was determined for a wide range of extracellular chlorpromazine concentrations. The results of a representative experiment are shown in Fig. 2. The curve can be divided into two parts:

(1) *Reciprocal linear region.* At low extracellular chlorpromazine concentrations (c_{free}), the membrane concentration, c_m, depends on c_{free} in a hyperbolic fashion; the reciprocal of c_m varies directly with the reciprocal of c_{free}.

(2) *Nonlinear region.* At high extracellular chlorpromazine concentrations the c_m increased markedly in a nonlinear fashion.

The change occurs at a value of $1/c_{free}$ smaller than $0.06 \cdot 10^6$ M^{-1}, corresponding to a c_{free} value larger than $1.6 \cdot 10^{-5}$ M.

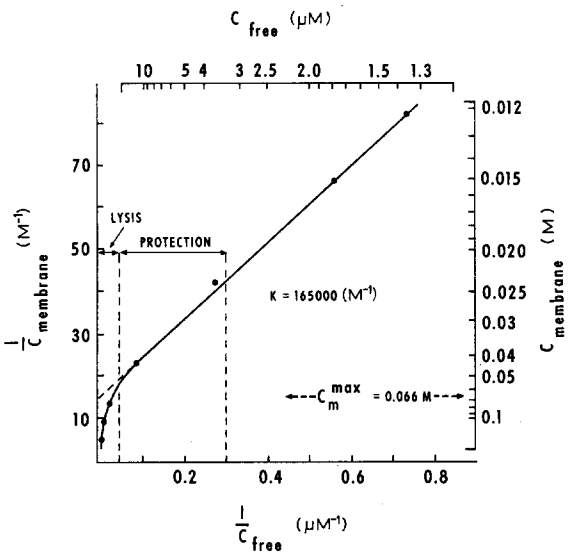

Fig. 2. The erythrocyte membrane concentration of chlorpromazine at different values of c_{free}. Each tube contained 0.2 ml [^{35}S]chlorpromazine (with 188 nC), 0.5 ml of erythrocyte ghosts and 0.2 ml of a chlorpromazine solution. After incubation and centrifugation at 22°, the chlorpromazine concentration in the supernatant (c_{free}) was determined. The membrane concentration ($c_{membrane}$), expressed as moles of drug bound per l of hydrated or wet membrane, was calculated according to the formulae in the text, and taking the membrane water content as 30% (ref. 43) and the density as 1.17 (refs. 44, 45). In the region of chlorpromazine which stabilizes membranes, namely $2 \cdot 10^{-6}$–$2 \cdot 10^{-5}$ M, the uptake of chlorpromazine into the membranes is associated with a set of sites having an affinity constant of 165000 M^{-1} and a maximum concentration of 0.066 mole drug per l of wet membrane.

Using Eqn. 5 and the data in Fig. 2, the maximum membrane concentration for chlorpromazine (c_m^{max}) extrapolates to 0.066 mole drug per l wet membrane; the affinity constant for the linear region works out to be 165000 M^{-1}. In different experiments the value of K varied from 100000 to 200000 M^{-1} causing a ΔF variation from —9100 to —9500 cal/mole. The reason for this variability is not known. Possibly age of the ghosts or individual variations between the donors are of importance.

Effect of temperature on the membrane concentration of chlorpromazine

To examine the effect of temperature on the membrane concentration of chlorpromazine, adsorption isotherms were obtained at 5, 22 and 37° (see Fig. 3).

It was observed that: (1) the extrapolated maximum of the membrane concentration, c_m^{max}, was independent of the temperature, and (2) the affinity constant, K, varied inversely with the temperature (see Fig. 4).

The average value for c_m^{max} was 0.066 M (for all temperatures), and the average value for K at 22° was 165000 M^{-1}. Despite the fact that c_m^{max} was not affected by

temperature (Fig. 3), the c_m^{max} of some preparations were higher or lower than this average (as mentioned in the previous section). This is the case for the 5° experiment shown in Fig. 3A. For the sake of comparison and to study the effect of temperature on the affinity constants, it was necessary to normalize the temperature results using an adsorption isotherm obtained at 22° on the same day and on the same ghost preparation. The results in Fig. 3A are uncorrected in any way; the data in Fig. 3B are those of Fig. 3A where the adsorption isotherms at 22° have been replaced by the average isotherm at 22° (*i.e.* with c_m^{max} of 0.066 M and a K of 165000 M^{-1}), and where the membrane concentrations of chlorpromazine at 37 and 5° have been

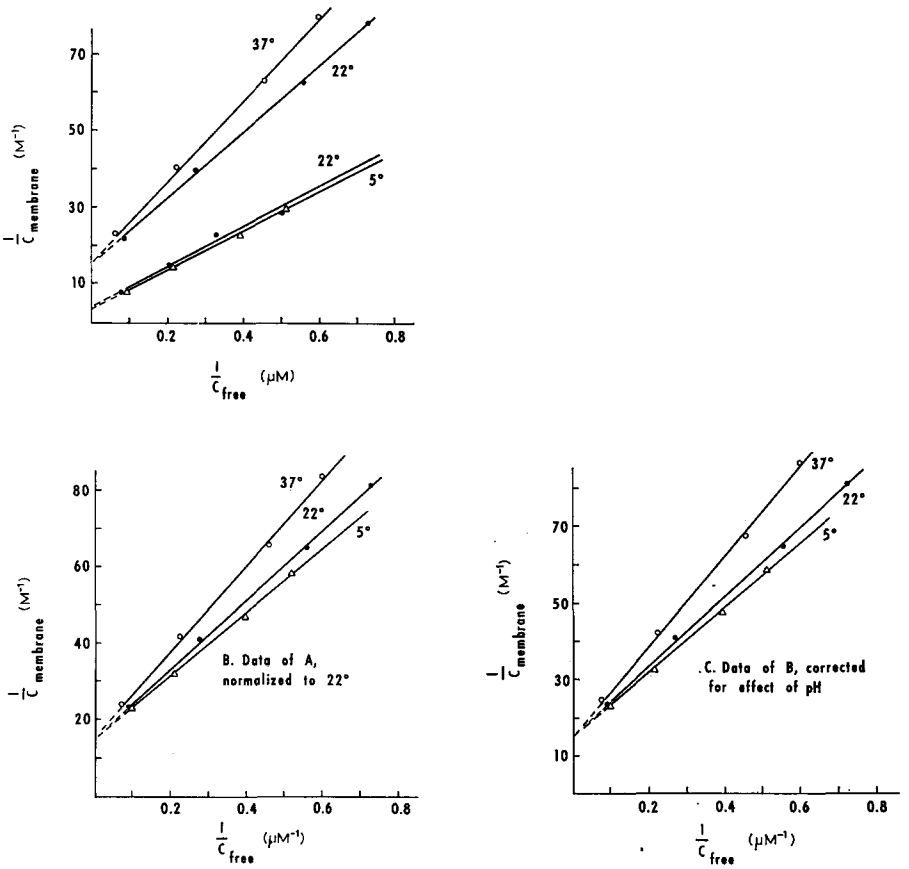

Fig. 3. The effect of temperature and pH on the membrane concentration of chlorpromazine. The procedure was as described for Fig. 2. (A) The membrane concentrations at different values for c_{free} at 5, 22 and 37°. As explained in the text, the membrane uptake varied for different ghost preparations. It was necessary, therefore, to carry out an adsorption experiment at 22° on the same day with the same ghosts for each variation in temperature. (B) Here the adsorption isotherms at 22° of A have been replaced by the average isotherm at 22° (*i.e.* with c_m^{max} of 0.066 M and a K of 165000 M^{-1}). The membrane concentrations of chlorpromazine at 37 and 5° have been corrected to the same extent as was necessary to alter the isotherm of 22° of each day's experiment to the average isotherm of 22°. (C) The data here are the same as those of B after the latter c_m values have been slightly corrected for the change in pH with temperature, as explained in the text.

corrected to the same extent as was necessary to alter the isotherm of 22° of each day's experiment to the average isotherm of 22°.

Effect of pH on the membrane/buffer partition coefficient for chlorpromazine

Since temperature changes are generally accompanied by changes in pH, the pH values of the suspensions were measured at the different temperatures. At 5, 22 and 37° the pH values of the ghost–drug suspensions were 6.65, 6.8 and 6.9, respectively.

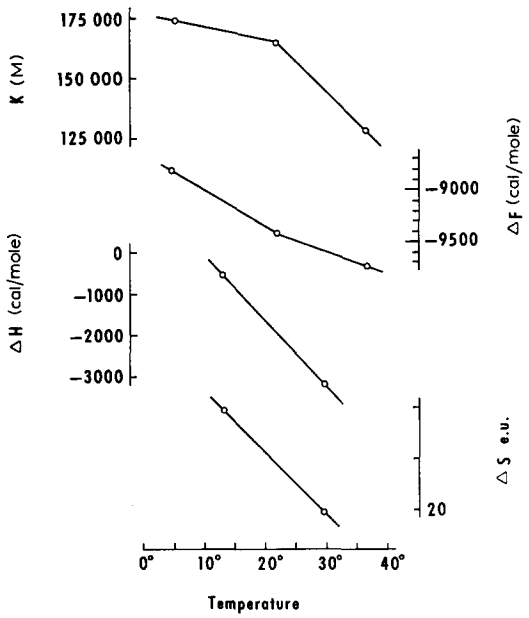

Fig. 4. From the data in Figs. 2 and 3, the affinity constant, the free energy, enthalpy and entropy of chlorpromazine adsorption to the membrane was derived. The fact that ΔF is about −9 kcal/mole, and that ΔH becomes more negative with temperature suggests that the chlorpromazine–erythrocyte membrane interaction is hydrophobic in nature.

The effect of pH on the membrane/buffer partition coefficient for chlorpromazine was examined, therefore, at constant temperature. At an extracellular concentration of chlorpromazine of $2.5 \cdot 10^{-6}$ M, the membrane/buffer partition coefficient was 4300 at pH 6.8, and 3500 at pH 6.2. Assuming linearity of the membrane/buffer partition coefficient within the small pH interval between 6.8 and 6.2, it was possible to correct the data of Fig. 3B for the slight differences in pH. The corrections, in fact, were almost negligible. The data in Fig. 3C are those of Fig. 3B after correction for the dependence of the partition coefficient on pH; the graphs are virtually identical.

Free energy, enthalpy and entropy of chlorpromazine adsorption to membrane

From the data of Figs. 2 and 3 the affinity constant, K, was 165000 M^{-1}. The free energy of chlorpromazine adsorption associated with this value of the affinity constant is given by Eqn. 6 and is −9400 cal/mole. The data in Figs. 3 and 4 show

that the affinity constant and the free energy of adsorption fell with increased temperature.

Figs. 3 and 4 show that the enthalpy of adsorption was negative and became more negative as the temperature was increased. The entropy of adsorption decreased at higher temperature (Fig. 4).

DISCUSSION

The membrane concentration of chlorpromazine at different values of c_{free}

The adsorption studies (Figs. 2 and 3) indicate the following:

(1) In the region of chlorpromazine concentrations which stabilize membranes[11,17,18,25] namely, $2 \cdot 10^{-6}$–$2 \cdot 10^{-5}$ M, the uptake of chlorpromazine into erythrocyte ghost membranes is associated with a single set of adsorption sites. These sites have a maximum membrane concentration of 0.066 M wet membrane, and an average affinity constant of 165 000 M^{-1}.

(2) In the region of high chlorpromazine concentrations where membrane lysis occurs[11,17,18,25], namely above $2 \cdot 10^{-5}$–$3 \cdot 10^{-5}$ M, other sites are involved in the adsorption of chlorpromazine. The membrane concentration of these lytic sites is much higher than 0.066 M wet membrane; the affinity constant is lower than that for the membrane stabilizing sites.

This distinction of at least two sets of adsorption sites from the adsorption isotherm is similar to that made by SCHNEIDER *et al.*[48] (on the adsorption of acids and alcohols to polystyrene) and by RAY *et al.*[50] (on the adsorption of sodium dodecyl sulfate to bovine serum albumin).

The results of Fig. 2 indicate that at the chlorpromazine concentration of around $2 \cdot 10^{-5}$ M, most of the first set of sites are occupied while few of the second set of sites are occupied by drug molecules. This agrees with the observation by KWANT AND VAN STEVENINCK[25] that $2 \cdot 10^{-5}$ M chlorpromazine gives almost maximum (or optimal) protection of intact erythrocytes against osmotic hemolysis. It has also been observed by SEEMAN *et al.*[17] and by SEEMAN AND KWANT[18] that this concentration of $2 \cdot 10^{-5}$–$3 \cdot 10^{-5}$ M is associated with maximum membrane expansion of the erythrocyte ghost.

The membrane concentration of chlorpromazine at the extracellular anesthetic concentration which is 50% effective

The data of Figs. 2 and 3 indicate that at an extracellular chlorpromazine concentration of $5 \cdot 10^{-6}$ M the membrane concentration is 0.033 mole chlorpromazine per l wet membrane. This concentration is known to cause nerve anesthesia (see ref. 10 for references) and also represents the erythrocyte $E.D._{50}$, or concentration at which there is 50% erythrocyte membrane protection against osmotic hemolysis[11,17,18,25].

This finding of 0.033–0.066 M in the chlorpromazine concentration in the cell membrane (for 50–100% membrane stabilization, respectively) is of the same order of magnitude as that predicted by the classical partition theory of anesthesia. As mentioned in the INTRODUCTION, this theory predicts that the concentration of anesthetic in the membrane-lipid phase is of the order of 0.03–0.06 mole anesthetic per l of membrane lipid, depending on the depth and type of anesthesia.

The energy, nature and thermodynamic parameters of the membrane–anesthetic bond

Some insight into the nature of the membrane–anesthetic bond can be obtained by calculating the free energy of chlorpromazine adsorption, and by considering the variation in enthalpy with temperature.

The free energy of adsorption is —9400 cal/mole, using our Eqn. 6 and expressing concentrations in terms of mole fraction (see ref. 48). This value for the free energy of the membrane–anesthetic interaction is intermediate in strength between the ionic bond and the Van der Waals bond[51]. The value of 9–10 kcal/mole is compatible with a hydrophobic bond[48,52].

NEMETHY AND SCHERAGA[52,53] have predicted that the enthalpy for a hydrophobic bond diminishes with increased temperature. In the present experiments the enthalpy of chlorpromazine adsorption was found to be negative and diminished (became more negative) with temperature. This is shown in Figs. 3 and 4. A small reduction in the affinity constant was found on increasing the temperature from 5 to 22°; this indicates that the enthalpy, ΔH, was close to zero, around —500 cal/mole. This finding of a very small change in the affinity constant between 5 and 22° is virtually identical to the result of BALZER *et al.*[54]. They found no difference in the amount of chlorpromazine bound to sarcoplasmic microsomes between 0 and 22°.

At higher temperature, however, the affinity constant for chlorpromazine adsorption became more reduced, and the enthalpy involved became more negative, around —3000 cal/mole (Figs. 3 and 4).

This variation of the enthalpy with temperature, therefore, fits the criterion for a hydrophobic bond as explained by NEMETHY AND SCHERAGA[52,53]. As would be expected for the hydrophobic bond, the entropy gain becomes smaller at increasing temperatures (Fig. 4). This change is due to decreasing amounts of ice-like water around the molecules in solution. In order for the drug to bind with the membrane less hydrogen bonds have to be broken, allowing ΔH to become more negative. Moreover, the net gain in "bulk" water will be less, causing ΔS to become less positive.

In conclusion, therefore, the absolute value of ΔF as well as the variation of ΔH and ΔS with temperature indicate that the interaction between chlorpromazine and the erythrocyte membrane is hydrophobic in nature.

Effect of pH on the membrane/buffer partition coefficient for chlorpromazine

Changing the pH from 6.8 to 6.2 resulted in a smaller membrane/buffer partition coefficient. This finding agrees qualitatively with others. Studies on binding of chlorpromazine to red blood cells[55] and of promazine to bovine serum albumin[56] showed a higher uptake at higher pH. Without more systematic studies, conclusions about the contribution of the ionic group in the binding cannot be drawn from this general finding, however, since the sites for both species may differ.

The membrane–aqueous interface as a possible site for chlorpromazine adsorption

It is possible that the adsorption of chlorpromazine may occur at the junctions of the membrane with the aqueous regions rather than the drug dissolving completely into a hydrophobic space. This possibility is raised by the following considerations.

The adsorption of carboxylic acids and *n*-alkanols to an interface is accompanied by a negative enthalpy change. This has been shown for the polystyrene–aqueous interface[48] and for the air–aqueous interface[47,57]. It is also known that the critical

micelle concentration of chlorpromazine increases as the temperature goes up[58], indicating that the transfer of a molecule from the aqueous solution to the micellar "interface" is an exothermic process as well.

On the other hand, transfer of alcohols from an aqueous phase to the bulk region of a nonpolar solvent is associated with a positive ΔH (ref. 53).

The observation that the binding of chlorpromazine to the red cell membrane is an exothermic reaction may indicate, therefore, that the site of chlorpromazine adsorption is at the immediate interface of the membrane–aqueous region.

Membrane area-expansion cannot be completely accounted for by the bulk volume of the adsorbed chlorpromazine molecules

In a previous paper[17] it was shown that at the 50% effective concentration for stabilizing erythrocytes by alcohols there was an associated membrane area expansion of between 1.35 and 1.62 %. By means of adsorption studies it was also found that the membrane concentration of alcohol (at the 50% effective concentration) was 0.048 mole drug per l wet membrane. Taking 30% as the amount of membrane water and 1.17 as the density of the wet membrane (see INTRODUCTION), the membrane concentration of alcohol became 0.04 mole drug per l wet membrane. It was calculated that the bulk volume of the drug molecules at this concentration in the membrane could only account for about 0.5–0.6% membrane area expansion. It was concluded, therefore, that the bulk volume of the alcohol drug molecules was not enough to account for the entire membrane area expansion observed.

The results of the present chlorpromazine binding studies also indicate that the volume of the chlorpromazine molecules in the membrane is not enough to account for the observed membrane area expansion induced by chlorpromazine. This is shown as follows. At complete saturation of the sites associated with protection, 1 l of wet membrane adsorbs approx. 0.066 mole of chlorpromazine. These molecules correspond to a bulk volume of 12.4 cm^3, using a Van der Waals molecular volume of 186 cm^3/mole for chlorpromazine[59]. Using the molar volume or parachor for chlorpromazine (see ref. 9 for references), the bulk volume of the chlorpromazine molecules occupies about 25 cm^3/l of wet membrane.

These 12–25 cm^3 represent 1.2–2.5% of the volume of 1 l of membrane. If it is assumed that the membrane does not become thicker[12], this maximum uptake of chlorpromazine might account for a membrane area increase of 1.2–2.5%. It has been shown[17], however, that at chlorpromazine concentrations close to complete occupation of the protection sites the membrane area is expanded by about 4.5 %.

Since it is not possible for the volume of the chlorpromazine molecules to account completely for the drug-induced expansion of the membrane, it is likely that the adsorption of chlorpromazine indirectly leads to membrane expansion. As mentioned in the INTRODUCTION, the drug may cause the following processes to occur, and these are being tested in current experiments: (1) "drug adsorption + membrane extension"[40]; (2) displacement of membrane Ca^{2+} (ref. 16); (3) induced conformational change in membrane proteins[41].

The biochemical nature of the chlorpromazine receptor site

Both lipids[1–6] and proteins[41] of the membrane have been proposed as the possible receptor for anesthetic molecules. It is interesting, therefore, to compare the

number of anesthetic molecules taken up by the membrane with the amount of membrane phospholipid and protein.

The phospholipid content of the erythrocyte membrane is $3.2 \cdot 10^{-7}$ mole/mg (ref. 7). The amount of chlorpromazine taken up at complete saturation of the protective sites is $6.6 \cdot 10^{-8}$ mole/mg. This represents, therefore, an uptake in the membrane equal to 20% of its phospholipid content. A similar figure is obtained from the results of BALZER *et al.*[54] for the adsorption of chlorpromazine to sarcoplasmic microsomes.

It has been suggested[60–62] that local anesthetics compete with Ca^{2+} for the negative sites on the phospholipid molecules of the membrane. It is interesting to note, therefore, that the maximum uptake of chlorpromazine, 0.066 M, is of the same order of magnitude as the maximum uptake of Ca^{2+} by the membrane, 0.048 M (recalculated from ref. 63, taking 1.17 as the membrane density and 30% as the amount of membrane water).

It is also possible that the membrane proteins may act as the receptor sites for the local anesthetics. This can be shown as follows. It is known that detergents and surface-active drugs, including sodium dodecyl sulfate, act as local anesthetics and also can protect erythrocytes against osmotic hemolysis[10,15]. It is also known that long chain alkyl sulfates and sulfonates can bind to proteins[50,64]. Bovine serum albumin has about 6–8 binding sites for these detergents per molecule of albumin. This would correspond to a concentration of $9 \cdot 10^{-5}$–$12 \cdot 10^{-5}$ moles detergent per g albumin. Accepting the value of 50% as the amount of erythrocyte membrane protein[7], the maximum membrane concentration of chlorpromazine, expressed on a membrane protein basis, is $15 \cdot 10^{-5}$ mole chlorpromazine per g of membrane protein. It is conceivable, therefore, that membrane protein may also be the chlorpromazine receptor.

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REFERENCES

1 H. H. MEYER, *Arch. Exptl. Pathol. Pharmakol.*, 42 (1899) 109.
2 F. BAUM, *Arch. Exptl. Pathol. Pharmakol.*, 42 (1899) 119.
3 E. OVERTON, *Studien über die Narkose*, Jena, 1901. Quoted by V. E. HENDERSON, *Physiol. Rev.*, 10 (1930) 171.
4 K. H. MEYER AND H. HEMMI, *Biochem. Z.*, 277 (1935) 39.
5 V. E. HENDERSON, *Physiol. Rev.*, 10 (1930) 171.
6 E. J. ARIENS, A. M. SIMONIS AND J. M. VAN ROSSUM, in E. J. ARIENS, *Molecular Pharmacology: The Mode of Action of Biologically Active Compounds*, Academic Press, New York, 1964, p. 287.
7 L. L. M. VAN DEENEN, in R. T. HOLMAN, *Progress in the Chemistry of Fats and other Lipids, Phospholipids and Biomembranes*, Vol. 8, Part 1, Pergamon Press, Oxford, 1965, p. 4.
8 J. T. DODGE, C. MITCHELL AND D. J. HANAHAN, *Arch. Biochem. Biophys.*, 100 (1963) 119.
9 P. SEEMAN, *Biochem. Pharmacol.*, 15 (1966) 1632.

10 P. SEEMAN, *Intern. Rev. Neurobiol.*, 9 (1966) 145.
11 P. SEEMAN AND J. WEINSTEIN, *Biochem. Pharmacol.*, 15 (1966) 1737.
12 P. SEEMAN, *Biochem. Pharmacol.*, 15 (1966) 1753.
13 P. SEEMAN, *Biochem. Pharmacol.*, 15 (1966) 1767.
14 J. METCALFE, P. SEEMAN AND A. S. V. BURGEN, *Mol. Pharmacol.*, 4 (1968) 87.
15 P. SEEMAN, T. SAUKS, W. ARGENT AND W. O. KWANT, *Biochim. Biophys. Acta*, 183 (1969) 512.
16 P. SEEMAN, W. O. KWANT, T. SAUKS AND W. ARGENT, *Biochim. Biophys. Acta*, 183 (1969) 520.
17 P. SEEMAN, W. O. KWANT AND T. SAUKS, *Biochim. Biophys. Acta*, 183 (1969) 476.
18 P. SEEMAN AND W. O. KWANT, *Biochim. Biophys. Acta*, 183 (1969) 490.
19 P. SEEMAN, *Biochim. Biophys. Acta*, 183 (1969) 499.
20 H. SCHNEIDER, *Biochim. Biophys. Acta*, 163 (1968) 451.
21 J. METCALFE AND A. S. V. BURGEN, *Nature*, 220 (1968) 587.
22 E. PONDER, *J. Gen. Physiol.*, 31 (1947) 325.
23 M. BARAC-NIETO, B. OSPINA, A. DUENAS, I. MARTINEZ-PINTO, C. MEJIA, E. RODRIGUEZ AND F. R. HUNTER, *J. Cellular Comp. Physiol.*, 61 (1963) 223.
24 J. VAN STEVENINCK, W. K. GJÖSUND AND H. L. BOOIJ, *Biochem. Pharmacol.*, 16 (1967) 837.
25 W. O. KWANT AND J. VAN STEVENINCK, *Biochem. Pharmacol.*, 17 (1968) 2215.
26 W. D. DETTBARN, *Biochim. Biophys. Acta*, 57 (1962) 73.
27 J. M. RITCHIE AND P. GREENGARD, *J. Pharmacol. Exptl. Therap.*, 133 (1961) 241.
28 J. M. RITCHIE AND B. R. RITCHIE, *Science*, 162 (1968) 1394.
29 J. M. RITCHIE, B. R. RITCHIE AND P. GREENGARD, *J. Pharmacol. Exptl. Therap.*, 150 (1965) 160.
30 J. M. RITCHIE AND P. GREENGARD, *Ann. Rev. Pharmacol.*, 6 (1966) 405.
31 T. NARAHASHI AND D. T. FRAZIER, *Federation Proc.*, 27 (1968) 408.
32 A. M. SHANES, *Proc. 22nd Intern. Congr. Physiol.*, 1 (1962) 93.
33 J. C. SKOU, *J. Pharm. Pharmacol.*, 13 (1961) 204.
34 R. A. DEMEL AND L. L. M. VAN DEENEN, *Chem. Phys. Lipids*, 1 (1966) 68.
35 A. D. BANGHAM, M. M. STANDISH AND N. MILLER, *Nature*, 208 (1965) 1295.
36 G. ZOGRAFI AND D. E. AUSLANDER, *J. Pharm. Sci.*, 54 (1965) 1313.
37 J. A. CLEMENTS AND K. M. WILSON, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 1008.
38 L. J. MULLINS, *Chem. Rev.*, 54 (1954) 289.
39 D. F. SEARS AND E. L. FULLER, *Respiration Physiol.*, 5 (1968) 175.
40 H. SCHNEIDER, *Federation Proc.*, 27 (1968) 912.
41 D. BALASUBRAMANIAN AND D. B. WETLAUFER, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 762.
42 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
43 J. CLIFFORD, B. A. PETHICA AND E. G. SMITH, in L. BOLIS AND B. A. PETHICA, *Membrane Models and the Formation of Biological Membranes*, North Holland, Amsterdam, 1968, p. 19.
44 C. D. MITCHELL AND D. J. HANAHAN, *Biochemistry*, 5 (1966) 51.
45 M. BARCLAY, R. K. BARCLAY, E. S. ESSNER, V. P. SKIPSKI AND O. TEREBUS-KEKISH, *Science*, 156 (1967) 665.
46 M. B. FEINSTEIN, *J. Gen. Physiol.*, 48 (1964) 357.
47 J. T. DAVIES AND E. K. RIDEAL, *Interfacial Phenomena*, Academic Press, New York, 1961, p. 94.
48 H. SCHNEIDER, G. C. KRESHECK AND H. A. SCHERAGA, *J. Phys. Chem.*, 69 (1965) 1310.
49 S. GLASSTONE, *Textbook of Physical Chemistry*, Van Nostrand, New York, 2nd ed., p. 1088.
50 A. RAY, J. A. REYNOLDS, H. POLET AND J. STEINHARDT, *Biochemistry*, 5 (1966) 2606.
51 W. DROST-HANSEN, *Intern. Sci. Technol.*, 58 (1966) 86.
52 G. NEMETHY AND H. A. SCHERAGA, *J. Phys. Chem.*, 66 (1962) 1773.
53 G. NEMETHY, I. Z. STEINBERG AND H. A. SCHERAGA, *Biopolymers*, 1 (1963) 43.
54 H. BALZER, M. MAKINOSE, W. FIEHN AND W. HASSELBACH, *Arch. Exptl. Pathol. Pharmakol.*, 260 (1968) 456.
55 M. A. SPIRTES, *Agressologie*, 9 (1968) 2.
56 J. KRIEGLSTEIN AND G. KUSCHINSKY, *Arch. Exptl. Pathol. Pharmakol.*, 260 (1968) 160.
57 J. H. CLINT, J. M. CORKILL, J. F. GOODMAN AND J. R. TATE, *J. Colloid Interface Sci.*, 28 (1968) 522.
58 W. SCHOLTAN, *Kolloid Z.*, 142 (1955) 84.
59 A. BONDI, *J. Phys. Chem.*, 68 (1964) 441.
60 M. P. BLAUSTEIN AND D. E. GOLDMAN, *Science*, 153 (1966) 429.
61 M. B. FEINSTEIN, M. PAINRE AND M. LEE, *Transl. N.Y. Acad. Sci.*, 30 (1968) 1073.
62 H. HAUSER AND R. M. C. DAWSON, *Biochem. J.*, 109 (1968) 909.
63 W. L. G. GHENT, J. R. TROUNCE AND M. WALSER, *Arch. Biochem. Biophys.*, 105 (1964) 582.
64 J. A. REYNOLDS, S. HERBERT, H. POLET AND J. STEINHARDT, *Biochemistry*, 6 (1967) 937.