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# Partition of Amphiphilic Molecules into Phospholipid Vesicles and Human Erythrocyte Ghosts: Measurements by Ultraviolet Difference Spectroscopy[†]

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ABSTRACT: Molar partition coefficients for chlorpromazine and methochlorpromazine between phospholipid vesicles or human erythrocyte ghosts and buffer are determined by ultraviolet difference spectroscopy. The partition coefficients between small unilamellar egg phosphatidylcholine vesicles and buffer at pH 7.4 are $4.4 \times 10^5$ for chlorpromazine and $0.8 \times 10^5$ for methochlorpromazine, determined with 10 $\mu$M amphiphile. An increase in the partition of chlorpromazine into vesicles is seen as the pH is increased to the p$K_a$ of chlorpromazine at 9.2. Chlorpromazine also partitions preferentially into fluid-phase phospholipid compared to solid-phase phospholipid. Molar partition coefficients between unsealed human erythrocyte ghosts and buffer at pH 8.0 with 10 $\mu$M amphiphile are determined to be $6.5 \times 10^5$ for chlorpromazine and $2.5 \times 10^5$ for methochlorpromazine. Difference spectroscopy is an equilibrium technique that does not require separation of bound from free amphiphile, as do many other methods of determining membrane-buffer partition coefficients. This method is useful for any amphiphile that has an appreciable absorbance below its critical micelle concentration and whose absorbance is sensitive to environment.

Chlorpromazine and methochlorpromazine are amphiphilic amines that are clinically useful as tranquilizers. For many years the anesthetic properties of these amines and other amphiphilic compounds have been correlated with their membrane solubility. This correlation has recently been reviewed (Janoff et al., 1981). The shape changes produced in erythrocytes by chlorpromazine and methochlorpromazine have also been attributed to their membrane solubility (Deuticke, 1968; Sheetz & Singer, 1974). The current assessment of the molecular basis of chlorpromazine-lipid bilayer interaction derives from nuclear magnetic resonance spectroscopy (Frenzel et al., 1978; Kuroda & Kitamura, 1984) and suggests that the phenothiazine ring is located near the $\alpha$-methylenes of fatty acyl chains and that the positively charged alkylamine group is in the proximity of the phosphate of the phospholipid polar head group. It is less clear how other effects of these drugs on membranes and membrane-related processes are related to the intercalation of amphiphile into the membrane lipid bilayer. For example, chlorpromazine affects the activity of a variety of phospholipases (Kunze et al., 1976; Vanderhoek & Feinstein, 1979), as well as phospholipid biosynthetic enzymes (Sturton & Brindley, 1977; Zborowski & Brindley, 1983). Recent work from this laboratory describes the inhibition of phosphatidylinositol transfer protein from bovine brain by both chlorpromazine and methochlorpromazine (Mullikin & Helmkamp, 1984).

A high solubility of chlorpromazine in red cell ghosts was first demonstrated directly by Roth & Seeman (1972). These studies involved mixing erythrocyte ghosts with amphiphiles, then centrifuging the ghosts, and determining the amount of ghost-associated amphiphile. However, later measurements of the partition of these compounds into cell membranes and phospholipid vesicles led to the conclusion that there was practically no cell membrane associated chlorpromazine or methochlorpromazine (Conrad & Singer, 1979, 1981). The latter method involved the separation of vesicles or membranes from the free amphiphile in buffer by a series of filters.

In order to resolve this discrepancy and to assess what factors affect the binding of chlorpromazine and methochlorpromazine to membranes, we undertook the present study. The vesicle-buffer and ghost-buffer partition properties of these compounds were investigated by ultraviolet difference spectroscopy. This method takes advantage of a shift in the absorption spectra of these drugs upon going from an aqueous to a hydrophobic environment. Furthermore, it is an equilibrium method that does not require the separation of bound and free amphiphile. We demonstrate that chlorpromazine and methochlorpromazine partition strongly into phospholipid vesicles and erythrocyte ghosts. Our results on ghosts support and extend those obtained by Bondy & Remien (1981) using

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the filtration technique. We also examined partition of these amphiphiles into vesicles as a function of amphiphile and lipid concentration, pH, and lipid bilayer phase.

## Experimental Procedures

*Materials.* Chlorpromazine hydrochloride was purchased from Sigma Chemical Co., St. Louis, MO. Methochlorpromazine was synthesized from chlorpromazine and methyl iodide and recrystallized from acetone-diethyl ether (Huang at al., 1970). *cis*-Parinaric acid was from Molecular Probes, Inc., Junction City, OR. Buffers used for vesicles were 50 mM NaCl-10 mM Hepes[1] (pH 6.8-8.0) and 50 mM NaCl-10 mM glycine (pH 9.2). For ghosts, 5 mM sodium phosphate (pH 8.0) was used. Egg PC was purified by silica gel column chromatography from a crude PC fraction from fresh frozen egg yolk obtained from Sigma. PCs of defined molecular species were synthesized by the method of Gupta et al. (1977).

*Vesicle Preparation.* Solvent was removed from the lipids by using a rotary evaporator and then a vacuum pump. Buffer was added, and the contents were vortexed to produce multilamellar vesicles. To make small, unilamellar vesicles, the suspension was sonicated with a probe sonicator until opalescent. These vesicles were centrifuged at 100000*g* for 1 h. The material above the level of the pellet represented a uniform population of small, single bilayer vesicles (Barenholz et al., 1977). The concentration of all vesicle stocks was determined by phosphate assay (Rouser et al., 1970). In general, the stock concentration was about 25 mM phospholipid. Vesicles were stored at 2 °C and used within 2-3 days of preparation.

*Erythrocyte Ghost Preparation.* Outdated packed human red blood cells were obtained from the Community Blood Center, Kansas City, MO. The cells were washed 3 times with isotonic, phosphate-buffered saline (pH 7.5), and each time, the buffy coat was removed after low-speed centrifugation. Unsealed, right-side out ghosts were prepared by the method of Steck (1974). Ghost protein was determined by the method of Peterson (1977). Lipid concentration of the ghosts was calculated by assuming 0.50 μmol of phospholipid plus 0.45 μmol of sterol/mg of protein. Other minor lipid components were ignored.

*Spectroscopy.* A Varian Cary 219 dual-beam UV-visible spectrophotometer was used for all measurements. "Regular" spectra were obtained in 1-cm quartz cuvettes with the appropriate reference of solvent, buffer, or lipid vesicles. Difference spectral titrations were done in tandem cuvettes with buffer in one 0.45-cm compartment and a solution of amphiphile in buffer in the other 0.45-cm compartment of each cuvette. The "automatic base-line correction" mode was used for all spectral recordings.

Initially, no lipid was present in either tandem cuvette containing amphiphile and buffer, and thus, no difference spectrum was observed. A titration was performed by adding concentrated lipid vesicles or erythrocyte ghosts to the amphiphile solution in the sample cuvette and to the buffer in the reference cuvette. The solution was mixed by pipetting. Thus, absorbance changes due to scattering by the vesicles were eliminated from the observed spectra. Equal volumes of buffer were added to those cuvette compartments not containing membranes. Amphiphile concentrations stated under Results are initial concentrations. These concentrations were reduced by less than 10% by the addition of vesicles or ghosts; the absorbances were corrected for the volume changes. After addition of vesicles or ghosts, the cuvettes were allowed to equilibrate for 3 min at 37 °C (or another stated temperature) before the spectrum was recorded. There were no significant differences between spectra recorded at 3 min and spectra recorded after 1 h at 37 °C. Thus, a rapid equilibrium was attained. No settling of vesicles or ghosts was detectable during the measurements. The magnitude of the difference spectrum, $\Delta A$, was measured from the minimum (248 nm for chlorpromazine and methochlorpromazine, 319.5 nm for *cis*-parinaric acid) to the maximum (262.5 nm for chlorpromazine and methochlorpromazine, 327 nm for *cis*-parinaric acid).

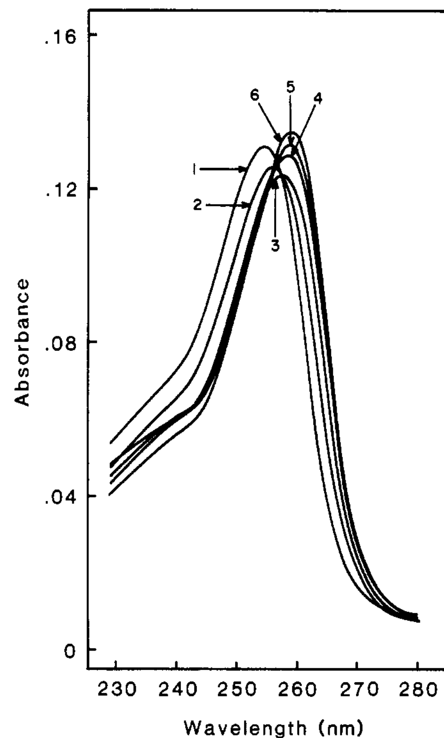


FIGURE 1: Absorption spectra of 5 μM chlorpromazine at 25 °C. The buffer was 50 mM NaCl-10 mM Hepes (pH 7.4). The concentration of egg PC vesicles was 0 (curve 1), 59 (curve 2), 174 (curve 3), 345 (curve 4), 620 (curve 5), and 1139 μM (curve 6). The total chlorpromazine concentration was kept constant during these vesicle additions.

## Results

*Solvent Studies.* A red shift in the absorption spectrum was observed for chlorpromazine and methochlorpromazine in solvents of increasing polarizability. The major peak in the ultraviolet spectrum of chlorpromazine had a maximum at 254.5 nm in water, 256.7 nm in ethanol, 257.5 nm in chloroform, and 259.7 nm in carbon tetrachloride. Similar wavelength maxima were observed with methochlorpromazine. For the amphiphilic amines, no significant changes in the extinction coefficient were found as a function of solvent. A similar red shift had previously been noted for *cis*-parinaric acid, another amphiphilic molecule (Sklar et al., 1977a). The absorbance of *cis*-parinaric acid that we examined had a maximum between 318 and 325 nm, depending on solvent polarizability (Sklar et al., 1977a).

*Adsorption Spectral Changes Induced by Lipid.* When egg PC vesicles are added to a solution of chlorpromazine in buffer, some of the chlorpromazine partitions into the lipid. This produces a spectrum that includes both the absorbance of the chlorpromazine in water (with a maximum at 254.5 nm) and the absorbance of chlorpromazine in the lipid phase (with a

[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; SEPC, 1-stearoyl-2-elaidoylphosphatidylcholine; PDPC, 1-palmitoyl-2-docosahexaenoylphosphatidylcholine.

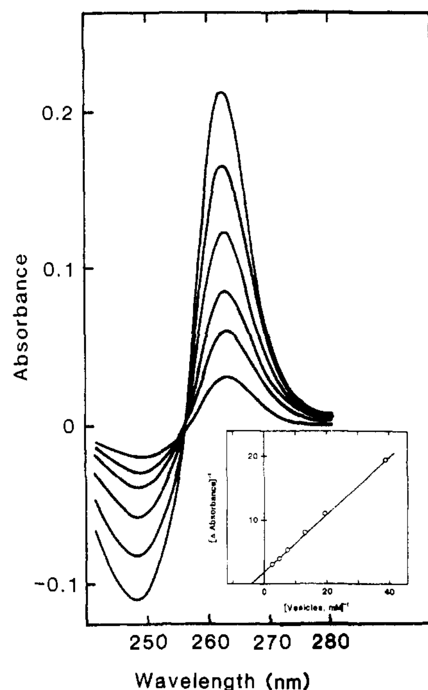


FIGURE 2: Difference spectral titration of 50 $\mu$M chlorpromazine with egg PC vesicles in 50 mM NaCl–10 mM Hepes (pH 7.4) at 37 °C. Increasing-size curves correspond to egg PC concentrations of 26, 51, 77, 128, 204, and 330 $\mu$M. (Inset) Double-reciprocal plot of these data, measured and corrected as described under Experimental Procedures.

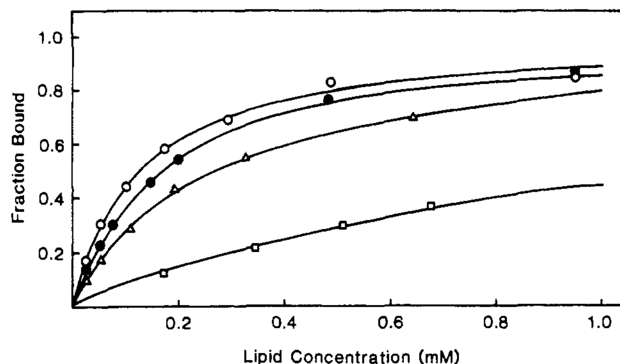


FIGURE 3: Fraction of lipid-associated amphiphile as a function of lipid concentration. The buffer was 50 mM NaCl–10 mM Hepes (pH 7.4) at 37 °C. Fraction bound = $\Delta A/\Delta A_{max}$. Amphiphiles investigated were 10 $\mu$M chlorpromazine (○), 25 $\mu$M chlorpromazine (●), 100 $\mu$M chlorpromazine (△), and 10 $\mu$M methochlorpromazine (□). The hyperbolic curves have been derived from least-squares best fit double-reciprocal plots.

maximum at a higher wavelength). Such spectra are shown in Figure 1. The difference in the wavelength maxima for chlorpromazine in lipid and water is too small for the two broad peaks to be resolved. Instead, the two peaks appear as one with an increasing wavelength maximum on lipid addition. Again, the extinction coefficient of chlorpromazine is largely independent of environment. There appears to be an isosbestic point at about 256 nm, which indicates that there are only two spectrally distinct forms of chlorpromazine. We assume that these are a free form and a lipid-associated form.

*Difference Spectroscopy and Data Analysis.* In order to determine the free and bound amphiphile concentration accurately, difference spectra were recorded, and the overall change in absorbance, $\Delta A$, was measured. This method is applicable when the extinction coefficient of the amphiphile remains essentially constant, as for chlorpromazine and methochlorpromazine, or is significantly different in lipid compared to water, as for *cis*-parinaric acid. As shown in Figure 2, the magnitude of $\Delta A$ increases with increasing lipid additions. $\Delta A$ is proportional to the amount of lipid-associated amphiphile. At high lipid concentrations, $\Delta A$ approaches $\Delta A_{max}$, the value corresponding to 100% amphiphile bound. $\Delta A/\Delta A_{max}$ (at any lipid concentration) is then the fraction of the total amphiphile associated with lipid. Plots of $\Delta A$ vs. lipid concentration yield hyperbolic curves. The data can, therefore, be plotted on a double-reciprocal plot as shown in the inset of Figure 2 by using the data from the experiment shown in this figure. We define the molar partition coefficient of amphiphile between buffer and (water) and lipid as

$$K_P = \frac{\text{mol of amphiphile in lipid/mol of lipid}}{\text{mol of amphiphile in water/mol of water}} \quad (1)$$

This is equivalent to

$$K_P = \frac{\text{fraction of amphiphile in lipid/[lipid]}}{\text{fraction of amphiphile in water/[water]}} \quad (2)$$

where [lipid] and [water] are expressed as molar concentrations. Since the fraction of amphiphile in lipid + the fraction of amphiphile in water = 1

$$K_P = \frac{(\Delta A/\Delta A_{max})/[\text{lipid}]}{(1 - \Delta A/\Delta A_{max})/[\text{water}]} \quad (3)$$

This can be rearranged to give

$$1/\Delta A = \frac{[\text{water}]}{K_P \Delta A_{max}} \frac{1}{[\text{lipid}]} + \frac{1}{\Delta A_{max}} \quad (4)$$

Equation 4 [which is modified from Sklar (1980)] can be used to obtain $\Delta A_{max}$ and, thus, $K_P$ from the double-reciprocal plot.

*Comparison of Difference Spectral Data with Previous Spectroscopic Data.* In order to evaluate the difference spectral method, we analyzed the partition properties of *cis*-parinaric acid. We employed a concentration of 3 $\mu$M in our studies; the saturating concentration of *cis*-parinaric acid is about 4 $\mu$M (Sklar, 1980). Applying the same difference spectral techniques used with chlorpromazine and methochlorpromazine, we determined the $K_P$ of *cis*-parinaric acid between large, multilamellar, fluid-phase PDPC vesicles and water at 25 °C to be $(3.4 \pm 1.5) \times 10^6$. By examining the spectral shifts of *cis*-parinaric acid directly, Sklar et al. (1979) determined a $K_P$ of approximately $1 \times 10^6$ into the same multilamellar vesicles at 22 °C. These investigators also observed that their spectral method for parinaric acid gave similar results to a centrifugation technique (Sklar et al., 1977b). A $K_P$ for *cis*-parinaric acid into the surface lipid of low-density lipoprotein has been determined to be about $3 \times 10^6$ by a fluorescence titration assay (Sklar, 1980). Thus, our method produces results in excellent agreement with those previously reported for *cis*-parinaric acid.

*Partition Coefficient into Egg PC Vesicles.* Binding of chlorpromazine and methochlorpromazine to egg PC vesicles is shown in Figure 3, and partition coefficients calculated by fitting the data with a hyperbolic curve are shown in Table I. The molar partition coefficients for methochlorpromazine were about 5 times lower than those for chlorpromazine. It can also be noted that $K_P$ exhibits a dependence on the amphiphile concentration.

*pH Dependence of Partition Coefficients.* Chlorpromazine, a tertiary amine, has a p$K_a$ of 9.2 or 9.3 (Murthy & Zographi, 1970; Chatten & Henri, 1962). This p$K_a$ corresponds to a single ionizable amine proton and thus the loss of a positive charge at higher pH. Methochlorpromazine, on the other hand, remains positively charged throughout the pH range of

Table I: Dependence of Molar Partition Coefficients on Amphiphile Concentration[a]

| amphiphile concn ($\mu$M) | partition coefficient, $K_p$ ($\times 10^{-5}$) | |
|---|---|---|
| | chlorpromazine | methochlorpromazine |
| 5 | 6.7 | |
| 10 | 4.4 ± 0.1 (2) | 0.8 ± 0.1 (3) |
| 25 | 3.5 ± 0.4 (2) | 0.5 ± 0.1 (2) |
| 50 | 2.1 | |
| 100 | 1.9 ± 0.2 (2) | 0.4 ± 0.2 (2) |

[a] Partition coefficients between small, unilamellar egg PC vesicles and buffer (50 mM NaCl, 10 mM Hepes, pH 7.4) were determined by difference spectroscopy at 37 °C and calculated, in all cases, from the least-squares, best fit line in double-reciprocal and [lipid] vs. [lipid]/ΔA plots. For each titration experiment, difference spectra were recorded at 5–14 lipid concentrations. The values represent the mean ± SD. The number of titration experiments is given in parentheses.

Table II: pH Dependence of Molar Partition Coefficient with 10 $\mu$M Amphiphile between Egg PC Vesicles and Buffer at 37 °C

| pH | partition coefficient, $K_p$ ($\times 10^{-5}$) | |
|---|---|---|
| | chlorpromazine | methochlorpromazine |
| 6.8 | 4.4 | 0.6 |
| 7.4 | 4.4 | 0.8 |
| 8.0 | 5.5 | 0.4 |
| 9.2 | 21.3 | 0.4 |

Table III: Lipid-Phase Dependence of Molar Partition Coefficient for 10 $\mu$M Chlorpromazine[a]

| lipid | temp (°C) | phase[b] | partition coefficient, $K_p$ ($\times 10^{-5}$) |
|---|---|---|---|
| egg PC | 56 | fluid | 2.4 |
| | 37 | fluid | 4.4 |
| | 15 | fluid | 4.4 |
| DPPC | 56 | fluid | 4.7 |
| | 15 | solid | 2.0 |
| SEPC | 45 | fluid | 2.8 |
| | 15 | solid | 0.8 |

[a] Partition coefficients were determined between small, unilamellar vesicles and buffer (50 mM NaCl, 10 mM Hepes, pH 7.4). [b] The gel to liquid-crystalline transition occurs at 41 °C for DPPC and at 33 °C for SEPC (Ladbrooke & Chapman, 1969). The transition temperature of egg PC is −5 to −15 °C (Ladbrooke et al., 1968).

6.8–9.2, due to its quaternary amine function. As seen in Table II, there is no significant change in the $K_P$ of methochlorpromazine in this pH range, while the $K_P$ of chlorpromazine is about 5 times higher at pH 9.2 than at pH 6.8 or 7.4. A simple interpretation of these data is that the uncharged form of chlorpromazine partitions more strongly into lipid than either its charged form or methochlorpromazine.

*Lipid-Phase Effect on Partition Coefficient.* The dependence of $K_P$ on both temperature and lipid phase was examined. These results are summarized in Table III. For egg PC, which is fluid at all temperatures used in the experiment, $K_P$ of chlorpromazine increases slightly as the temperature is decreased. The $K_P$ values of chlorpromazine into DPPC and SEPC, when these lipids are in the fluid phase, are similar to those observed for egg PC. However, at temperatures below the phase transitions of DPPC and SEPC, the $K_P$ values are clearly lower than those calculated above the phase transitions. These differences are in contrast to what was observed for egg PC with decreasing temperature. These data suggest that chlorpromazine partitions less strongly into solid-phase phospholipid vesicles than fluid-phase bilayer membranes.

*Partition Coefficients into Human Erythrocyte Ghosts.* Binding of chlorpromazine and methochlorpromazine to erythrocyte ghosts is shown in Figure 4, and the calculated $K_P$ values are summarized in Table IV. As was the case for partition into phospholipid vesicles (Figure 3), the binding curve is hyperbolic; thus, no significant change in $K_P$ is found as a function of membrane concentration. Again, $K_P$ exhibits a significant dependence on amphiphile concentration. This magnitude of $K_P$ for chlorpromazine and methochlorpromazine calculated for erythrocyte ghosts is similar to that calculated for phospholipid vesicles.

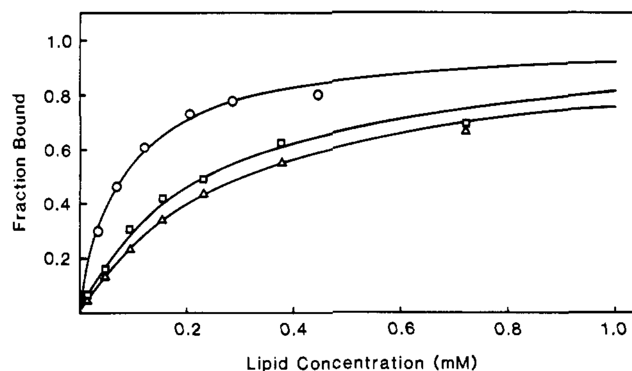


FIGURE 4: Fraction of erythrocyte membrane-associated amphiphile as a function of membrane concentration. The buffer was 5 mM sodium phosphate (pH 8.0) at 37 °C. Fraction bound = $\Delta A/\Delta A_{max}$. Amphiphiles used were 10 $\mu$M chlorpromazine (○), 100 $\mu$M chlorpromazine (△), and 10 $\mu$M methochlorpromazine (□).

Table IV: Molar Partition Coefficients of Amphiphiles between Human Erythrocyte Ghosts and Buffer at 37 °C

| amphiphile concn ($\mu$M) | partition coefficient, $K_p$ ($\times 10^{-5}$)[a] | |
|---|---|---|
| | chlorpromazine | methochlorpromazine |
| 10 | 6.5 ± 0.4 (2) | 2.5 ± 0.4 (2) |
| 100 | 1.6 ± 0.2 (2) | 0.6 ± 0.2 (2) |

[a] The number of titration experiments is given in parentheses.

## Discussion

We have determined values of $K_P$ for chlorpromazine and methochlorpromazine into phospholipid vesicles and erythrocyte ghosts by using ultraviolet difference spectroscopy. This technique takes advantage of the shift in the absorbance spectra of these compounds upon movement from an aqueous to a membrane or membranelike environment. The method is applicable because chlorpromazine and methochlorpromazine have sufficient absorbance at concentrations below their critical micelle concentrations to produce easily measurable difference spectra upon titration with lipid vesicles or membranes. The amphiphile concentrations used are well below the critical micelle concentrations of chlorpromazine, determined to be 2 mM[2] to 4 mM (Scholtan, 1955), and methochlorpromazine, determined to be 30 mM (Conrad & Singer, 1981). Furthermore, the concentrations are similar to those used in previous studies using centrifugation (8.1 $\mu$M chlorpromazine) (Roth & Seeman, 1972) and filtration techniques (6–600 $\mu$M chlorpromazine and methochlorpromazine) (Conrad & Singer, 1981).

As previously observed by Roth & Seeman (1972) for positively charged local anesthetics in erythrocyte membranes, we observe a dependence of $K_P$ on the concentration of chlorpromazine and methochlorpromazine. This dependence is similar for partition into both vesicles and ghosts. $K_P$ is, however, independent of the concentration of vesicles or ghosts, as indicated by the good fit of the data to hyperbolic curves.

[2] Determined by optical scattering measurements at pH 7.0; L. J. Mullikin and G. M. Helmkamp, Jr., unpublished results.

This suggests that the observed dependence of $K_P$ on amphiphile concentration is due not to saturation of the lipid by amphiphile but rather to a form of aggregation of the amphiphile in aqueous solution that occurs well below the critical micelle concentration.

In order to compare our data on partition properties of chlorpromazine and methochlorpromazine with those of Roth & Seeman (1972) and Conrad & Singer (1979, 1981), it is necessary to convert their partition coefficients, which are equal to the amphiphile concentration in the membrane divided by the amphiphile concentration in the water and based on membrane weight (in kilograms) or volume (in liters, inulin-exclusion method) and buffer volume (liters), to units of moles of lipid and water. For small, unilamellar phospholipid vesicles, 1 L of lipid is assumed to be equal to 1 kg, which is about 1.33 mol of phospholipid. Thus, $K_P$(molar) = $42K_P$ (vol) for small, unilamellar vesicles. If the inulin-excluded volume of erythrocyte ghosts containing 0.457 mol of lipid (phospholipid and cholesterol) is 1 L (Gaffney et al., 1983), then $K_P$(molar) = $120K_P$(vol) for ghosts. From use of these conversions, the $K_P$ for either chlorpromazine or methochlorpromazine into small, unilamellar egg PC vesicles determined by filtration by Conrad & Singer (1979, 1981) is about $0.6 \times 10^5$. This compares well with the data presented in this paper for methochlorpromazine partition, as well as with a value of $0.3 \times 10^5$ determined by equilibrium gel-filtration chromatography (Mullikin & Helmkamp, 1984). However, we find that chlorpromazine partitions more strongly than methochlorpromazine into both vesicles and red cell ghosts. This difference in partition might have been predicted from the difference in the critical micelle concentration of the two amphiphiles, which suggests that chlorpromazine has more tendency toward hydrophobic associations than methochlorpromazine. Also, the concentrations of these compounds that afford normal human erythrocytes 50% protection against hypotonic lysis differ, being 60 $\mu$M for chlorpromazine and 300 $\mu$M for methochlorpromazine (Conrad & Singer, 1981). Assuming that this protection is due to intercalation of an equal amount of either amphiphile into the erythrocyte membrane, this implies that chlorpromazine must partition more strongly than methochlorpromazine. The assay conditions used by Conrad & Singer are $2.7 \times 10^7$ cells/mL (Seeman et al., 1969; Seeman & Kwant, 1969), which is equivalent to about 15 $\mu$g of ghost protein/mL or about 15 $\mu$M lipid (Steck, 1974). Thus, one can calculate that if the $K_P$ for 60 $\mu$M chlorpromazine is 2.0 $\times 10^5$; the $K_P$ for 300 $\mu$M methochlorpromazine would be 0.4 $\times 10^5$. This is similar to the difference we observe between chlorpromazine and methochlorpromazine.

To compare our value for chlorpromazine partition into erythrocyte ghosts with that derived by the centrifugal method (Roth & Seeman, 1972), we convert their value to a molar partition coefficient of $1.9 \times 10^5$. Bondy & Remien (1981) obtained a value equivalent to $4.0 \times 10^5$ for ghosts by using the filtration method. These values are in relatively good agreement with our data. The major qualitative difference between our work and that of Conrad & Singer (1979, 1981), however, is in the data on amphiphile binding to erythrocyte ghosts. We observe partition coefficients of the same magnitude as observed with phospholipid vesicles, while Conrad & Singer, using the filtration technique, observed virtually no binding. Thus, their result is also in contrast to that of Roth & Seeman and Bondy & Remien.

An advantage of difference spectroscopy over filtration or centrifugation is that it is a purely equilibrium technique. Other equilibrium techniques, such as electron paramagnetic resonance and fluorescence spectroscopy, have derived equivalent values for partition of various amphiphiles into vesicles and membranes (Gains & Dawson, 1982; Gaffney et al., 1983; Pjura et al., 1984). Conrad & Singer (1981), however, originally suggested that the apparent high partition coefficients of amphiphiles determined by spectroscopic techniques are due to the formation of micelles of amphiphiles and membrane-derived material rather than intercalation of amphiphile into the membrane. While our data do not address this question, two recent studies do. In the study of partition of spin-labeled amphiphiles into membranes by Gaffney et al. (1983), they argue that if micelles were present containing 20% or more of their amphiphile, a spectrum derived from these micelles would have been generated. Such spectra were not observed. Maher & Singer (1984) measured the material extracted from erythrocyte membrane by amphiphile directly. With the filtration technique, they found that about 11% of the lipid from red cell ghosts was dissociated from the ghosts as small vesicles in the presence of 60 $\mu$M chlorpromazine. Thus, the filtration technique induces the extraction of some ghost material, with which the amphiphle is presumably associated. As Maher & Singer (1984) imply, one possible resolution of the data of Conrad & Singer (1981) with our data and with that of others is that the amphiphile intercalates exclusively into specialized cell membrane regions that are extracted only by filtration under their conditions.

Acknowledgments

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**Registry No.** PDPC, 37449-27-7; DPPC, 2644-64-6; SEPC, 20375-96-6; chlorpromazine, 50-53-3; methochlorpromazine, 19077-31-7; ethanol, 64-17-5; chloroform, 67-66-3; carbon tetrachloride, 56-23-5; *cis*-parinaric acid, 593-38-4.

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# Biochemical Characterization of Complex Formation by Human Erythrocyte Spectrin, Protein 4.1, and Actin[†]

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ABSTRACT: Ternary complex formation between the major human erythrocyte membrane skeletal proteins spectrin, protein 4.1, and actin was quantified by measuring cosedimentation of spectrin and band 4.1 with F-actin. Complex formation was dependent upon the concentration of spectrin and band 4.1, each of which promoted the binding of the other to F-actin. Simultaneous measurement of the concentrations of spectrin and band 4.1 in the sedimentable complex showed that a single molecule of band 4.1 was sufficient to promote the binding of a spectrin dimer to F-actin. However, the molar ratio of band 4.1/spectrin in the complex was not fixed, ranging from approximately 0.6 to 2.2 as the relative concentration of added spectrin to band 4.1 was decreased. A mole ratio of 0.6 band 4.1/spectrin suggests that a single molecule of band 4.1 can promote the binding of more than one spectrin dimer to an actin filament. Saturation binding studies showed that in the presence of band 4.1 every actin monomer in a filament could bind at least one molecule of spectrin, yielding ternary complexes with spectrin/actin mole ratios as high as 1.4. Electron microscopy of such complexes showed them to consist of actin filaments heavily decorated with spectrin dimers. Ternary complex formation was not affected by alteration in $Mg^{2+}$ or $Ca^{2+}$ concentration but was markedly inhibited by KCl above 100 mM and nearly abolished by 10 mM 2,3-diphosphoglycerate or 10 mM adenosine 5′-triphosphate. Our data are used to refine the molecular model of the red cell membrane skeleton.

The membrane skeleton of the human red cell consists of an extensively cross-linked, self-associated network of proteins which coats the cytoplasmic surface of the cell membrane. It is commonly held that the membrane skeleton is responsible for the remarkable flexibility and resiliency of the red cell membrane and that it plays a large part in maintaining the shape of the cell and its isolated plasma membrane. The major proteins of the membrane skeleton, spectrin, actin, and band 4.1, as well as other components such as band 4.9, have all been purified and at least partially characterized [reviewed in Cohen (1983)], and studies of the association of these proteins in solution have led to the development of molecular models of the red cell membrane skeleton which share certain common features. Nearly all models show that the membrane skeletal network contains short actin filaments cross-linked by tetramers (or higher oligomers) of spectrin [see, for example, Cohen (1983), Palek & Lux (1983), Goodman & Shiffer (1983), and Gratzer (1983)]. Band 4.1 plays the important role of promoting or strengthening the attachment of spectrin to actin, and the entire complex along with such accessory proteins as band 4.9 is bound to the membrane by the protein ankyrin, which fastens the membrane skeleton to the integral membrane protein band 3.

The development of this model of the membrane skeleton was based largely on studies of binary associations such as spectrin binding to ankyrin, spectrin binding to band 4.1, and spectrin binding to actin in the presence or absence of band 4.1. However, extrapolation from studies of binary interactions may not provide a complete or accurate picture of membrane skeletal organization. The present work provides a preliminary characterization of the ternary spectrin–actin–band 4.1 complex in vitro. This complex is of interest also because defects in its formation have been implicated in the inherited blood disorder hereditary spherocytosis and may also be involved with other red cell diseases as well [reviewed in Palek & Lux

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