Absorption of Surfactants by Membranes: Erythrocytes Versus Synthetic Vesicles

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ABSTRACT Three surfactants (chlorpromazine hydrochloride, thioridazine hydrochloride, and sodium deoxycholate) are found to absorb just as strongly into the protein-containing membranes of erythrocytes as into the phospholipid bilayers of synthetic vesicles. In the concentration region where hemolysis occurs and the Langmuir adsorption isotherm is no longer valid, one may use a phase partition model in which the erythrocyte membrane is one of the phases. The partition coefficients, expressed as the ratio of mole fraction surfactant in the membrane lipid phase to concentration of surfactant in the aqueous phase, have been calculated at the point of saturation in the erythrocyte membrane. These values are K_y = 430 M^{-1} (chlorpromazine, pH 5.9), 550 M^{-1} (deoxycholate, pH 7.6), and 640 M^{-1} (thioridazine, pH 5.9), in isotonic buffer at 27°C. Corresponding values for synthetic vesicles made from dimyristoylphosphatidylcholine are K_x = 230 M^{-1} (chlorpromazine, 0.12 M buffer/KCl pH 5.9), 440 M^{-1} (deoxycholate, 0.20 M buffer/NaCl pH 8.0) and 510 M^{-1} (thioridazine, 0.12 M buffer/KCl pH 5.9), at 27°C. It appears that the surfactants become an integral part of the bilayer in both vesicles and natural membranes and that the absorption is not of a peripheral nature. There is no evidence that the presence of proteins in the natural membrane inhibits the absorption of these surfactants in any way.

INTRODUCTION

Local anesthetic action by drugs and solubilization of membranes by surfactants are both preceded by the transfer of amphiphilic molecules from the aqueous phase to the oily phase of a cell membrane. Kwant and Seeman (1969) attempted to quantify this transfer by measuring the actual concentration of a strong sedative and tranquilizer, chlorpromazine (thorazine), in a natural membrane, erythrocyte ghost (EGM). This was accomplished by measuring the radioactivity of ^{35}S-labeled CPZ in the precipitate obtained by centrifugation of EGM. Using the Langmuir isotherm as a model, Kwant and Seeman observed ideal adsorption of the chlorpromazine (CPZ) up to a point of about 1.2–2.5% by volume of the wet membrane, corresponding to a "single set of binding sites." After these sites were occupied, the Langmuir isotherm was no longer followed and membrane concentration increased sharply as adsorption occurred at "lytic sites." These sites were not further characterized or identified. Roth and Seeman (1972) extended this study to include 44 anesthetics and for chlorpromazine reported a partition coefficient $K_{m/b}$ = 1600 membrane to buffer concentrations (mol CPZ per kg dried EGM/molarity of CPZ in the aqueous phase) at pH 7.

Conrad and Singer (1979, 1981) and Singer (1981) introduced a new filtration technique for separating EGM from the aqueous phase that they called "hygroscopic desorption." Using it they measured partition coefficients of CPZ between the volume fraction of erythrocyte ghost membrane and the aqueous phase. They found that K_p could not exceed 0.1 at pH 7.4, in contrast with the high value found by Seeman, which meant that the surfactant concentration was greater in the aqueous phase than it was in the membrane. Singer attributed this difference to the centrifugal method used by Seeman. He proposed that in the centrifugal method CPZ formed mixed micelles that were loosely associated with and became a part of the membrane centrifugate. Singer noted that the behavior of pure phospholipid vesicles was different. Even using the hygroscopic desorption method, synthetic vesicles were found to absorb CPZ strongly and not release it. For example, small (25 nm) vesicles composed of egg phosphatidylcholine in 10^{-5} M CPZ at pH 7.4 had a K_p value of 1350 M/M (Conrad and Singer, 1981). Singer (1981) suggested that the difference between his results with vesicles and with EGM was that the proteins in EGM produced an "internal pressure" that prevented integral absorption of CPZ.

Bondy and Remien (1981) tried to repeat the work of Conrad and Singer (1979) using a modified "hygroscopic desorption" method. However, they noted *distinct binding* of CPZ to EGM as well as to erythrocytes. Zachowski and Durand (1988) used a centrifugal method similar to that of Seeman on both EGM and soybean phosphatidylcholine vesicles. They found that the presence of proteins in EGM reduced the value of the partition coefficient, although it remained well above one. Other recent studies by Olivier et al. (1989) and by Davies and Jones (1992) have criticized the

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Abbreviations used: CMC, critical micelle concentration; DMPC, dimyristoylphosphatidylcholine; CPZ, chlorpromazine hydrochloride; TDZ, thioridazine hydrochloride; NaDC, sodium deoxycholate; EGM, erythrocyte ghost membrane; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MCHC, mean corpuscular hemoglobin concentration; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; Hct, hematocrit.

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"hygroscopic desorption" results and the significance of "internal pressure."

Binford and Wadsö (1984) determined K by measuring the heat of absorption of CPZ by dimyristoylphosphatidylcholine (DMPC) vesicles. Their value, $K = 1300$ M/M at pH 6.4 and 10^{-4} M CPZ, agreed well with that of Conrad and Singer (1981).

At concentrations of CPZ above 0.03 molal in EGM (Kwant and Seeman, 1969) or 2 mol% in synthetic membrane (Binford and Wadsö, 1984), the model for absorption begins to fail. The cause of this change is the increasing density of positive CPZ ions in the membrane, which increases the free energy for further binding. This effect should lead to smaller values of K expressed in terms of concentration as opposed to the true thermodynamic value of K in terms of activity.

Helenius and Simons (1975) described the steps in the solubilization of membranes by detergents as follows:

- Detergent is incorporated into the bilayer.
- The bilayer becomes saturated.
- The bilayer dissolves.

Binford et al. (1988) and Malloy and Binford (1990) observed a gradual increase in turbidity and particle size of DMPC vesicles (from 30 to 170 nm diameter) as they were mixed with CPZ. This was probably due to swelling of vesicles accompanied by aggregation and/or fusion. As more CPZ was added the turbidity decreased sharply and the particles became much smaller (15-nm diameter). Using light absorbance as a measure of turbidity a curve of absorbance versus surfactant concentration was plotted. Identifying the peak in the curve as the point where the membrane became saturated (e.g., 49 mol% CPZ), partition coefficients at saturation were calculated for a number of surfactants in DMPC vesicles. The advantage of this method was that it avoided any kind of intrusive separation of membranes from the aqueous phase either by filtration or by centrifugation. Kuroda and Kitamura (1984) established the position of CPZ molecules in pure phospholipid bilayers containing choline head groups. Using NMR, they located the phenothiazine ring protons next to the α-methylenes in the acyl groups of DMPC. This would place the positively charged polar end of CPZ near the phosphate group of DMPC. Thus, CPZ assumed an integral position in the synthetic membrane rather than peripheral. If the integrated proteins in natural membranes produced an internal pressure that profoundly reduced the absorption of amphiphiles like CPZ, that fact would render the vesicle of limited utility as a model system to study the properties of membranes (Conrad and Singer, 1981). That would have far reaching consequences because amphiphilic tags and tracers are widely used by membrane scientists to study the structure of lipid bilayers and their protein constituents.

In the present study, the hemoglobin released by erythrocytes is used as an indicator of saturation. Using the cationic surfactants CPZ and TDZ and the anionic surfactant NaDC, comparisons have been made between DMPC vesicles and whole erythrocytes. Hemolysis occurs very sharply as surfactant is added to erythrocytes. We have taken this point to be the saturation point of the surfactant.

We propose to show, by comparing partition coefficients under well defined thermodynamic conditions, that erythrocytes and synthetic vesicles absorb amphiphilic molecules in a very similar manner. Furthermore, the methods used do not require the physical separation of particles and solution.

MATERIALS AND METHODS

NaDC, chlorpromazine hydrochloride, and 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol (Bistris), were purchased from Sigma Chemical Co. (St. Louis, MO). Thioridazine hydrochloride was purchased from Aldrich Chemical Co. (Milwaukee, WI). 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), Baker grade, was purchased from J.T. Baker Inc. (Phillipsburg, NJ). All chemicals were used without being further purified. The wash solutions for erythrocytes consisted of isotonic 0.172 M Tris-HCl buffer (pH 7.61) for the NaDC trials and isotonic 0.50 M Bistris-HCl buffer (pH 5.85–5.90) in 0.1096 M NaCl for the phenothiazine trials. Human blood obtained from volunteer donors was the source of erythrocytes, which were washed to remove leukocytes and platelets in an IBM blood cell processor, Model 2991 (IBM Instruments, Inc., Armonk, NY). The washed erythrocytes were reconstituted to a hematocrit between 0.40 and 0.45 in the wash solution. The hematocrit was measured in an Adams Micro-Hematocrit II centrifuge (Becton Dickinson, Franklin Lakes, NJ). Blood was stored at 5°C and was used within 24–36 h.

Concentrations of titrants were 5.0 mM NaDC, 5.0 mM CPZ, and 2.5 mM TDZ. All titrations were carried out in a constant temperature room at 27°C.

A 30-ml sample of washed blood/buffer mixture was stirred with a magnetic stirrer for 15 min to allow it to reach ambient temperature. A predetermined amount of surfactant was added to the blood at 1-min intervals. After each addition, 1 ml of the suspension was removed and centrifuged at 2260 × g for 30 min. Spectrophotometric measurements at 540 nm were made on the supernatant liquid diluted with Tris or Bistris buffer as appropriate.

A preliminary run and a final run were carried out for each surfactant. The preliminary run was used to establish the approximate endpoint. In the final run, smaller increments of titrant were used for better precision as the endpoint was approached. The final run was carried out within 24 h of the preliminary run. Blood controls with no surfactant present were run simultaneously.

In the calculations that follow, the concentration of hemoglobin in the supernatant liquid is expressed as $A \times$ (dilution factor), where A is the absorbance at 540 nm. Assuming complete hemolysis of the cells, the mean corpuscular hemoglobin concentration, MCHC (Kjeldsberg, 1993), can be estimated from the Beer-Lambert law using the millimolar absorption coefficient $\epsilon = 14.6\ (\text{mM})^{-1}\ \text{cm}^{-1}$ for HbO_2 (mol. mass = 17,000 g mol^{-1} (Sunderman et al., 1953).

The experimental runs differed in the ratios of original blood volume to buffer volume added as follows: run I, 4:0; run II, 3:1; run III, 1:1; run IV, 1:3.

All concentrations are expressed in terms of the intercellular, or free, volume in the erythrocyte suspension. Calculations of the remaining [CPZ] take into account the number of micromoles CPZ removed for spectrophotometric analysis.

The vertical axis of the titration curve in Fig. 1 represents the amount of hemoglobin that has escaped from the erythrocytes in terms of the absorbance, A, at 540 nm. By correcting the original washed blood sample for dilution, the curves I–IV should all approach the same plateau after hemolysis. This fact aids in the identification of the endpoint.

As a test of the method, the MCHC can be calculated for a point on the plateau in Fig. 1. Given a cuvette having path length 1 cm, MCHC, the number of grams of hemoglobin per dl of packed red blood cells, is given by the equation

$$\text{MCHC} = \frac{\text{MW} \times \text{A} \times 0.0001 \times \text{dilution factor}}{\epsilon \times \text{Hct}}$$

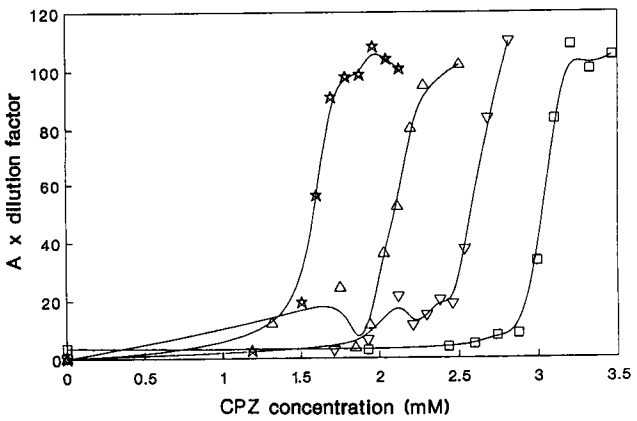

FIGURE 1 Titration curves of 30 ml total washed erythrocytes plus additional buffer vs. 5 mM chlorpromazine hydrochloride. Buffer is isotonic at pH 5.9. Absorbance measurements at 540 nm are made on the supernatant after centrifuging. Ratios of washed erythrocytes to additional buffer are (□) 4:0, (▽) 3:1, (△) 1:1, (☆) 1:3 by volume. Washed erythrocytes have *Hct* = 0.45 and contain 1.669 mM phospholipid based on total volume of the suspension.

where Hct is the volume ratio of packed red blood cells to volume of blood. The range of plateau MCHC levels observed in this study is 26–34 g/dl, which is a good approximation to the accepted value for MCHC in normal blood (Kjeldsberg, 1993).

The surfactant concentration at the inflection point of each curve in Fig. 1 is assumed to be an endpoint. The phospholipid concentration is determined by analysis for phosphorus. Total phosphorus in the washed blood sample is determined spectrophotometrically according to a modified procedure of Allen (Kates, 1972). The number of moles of phosphorus is assumed to equal the number of moles of membrane phospholipid. Other lipids are estimated from the ratio of total moles glycolipid and cholesterol to moles of phospholipid found in normal human erythrocytes, 1.02:1 (Telen, 1993).

The concentration of phospholipid at the endpoint is determined from the corresponding concentration of surfactant by interpolation of the tabulated values.

Several attempts were made to use the turbidimetric method on erythrocyte ghost membranes. This method was used earlier with synthetic vesicles (Binford et al., 1988; Malloy and Binford, 1990). Suspensions of EGM were prepared from fresh blood (Hanahan and Ekholm, 1974). Absorbance titrations at 630 nm were made on the cloudy white suspensions at a series of concentration levels ranging from 0.2 to 0.8 mM phosphorus. Using CPZ as the titrant at pH 6.3, the suspension became cloudier and did not clear up even at a final concentration level of 32 mM CPZ. A precipitate formed that was separated by centrifugation, washed, and dissolved in 1% SDS. A mixture of one part solution to three parts Coomassie blue concentrate (Bio Rad Laboratories, Hercules, CA) gave a blue-green color, indicating the presence of protein. Using NaDC as titrant at pH 8.0, the suspension of EGM gradually cleared, but there was no sharp decrease in absorbance that could be used as an endpoint. The final concentration of NaDC was 6 mM.

RESULTS AND DISCUSSION

In the region where the Langmuir isotherm model breaks down (Kwant and Seeman, 1969), the phase partition model should apply. The characteristic parameters of the phase partition model can be determined from a single unifying equation that is applicable at the saturation point of the membrane.

We determined the partition coefficient and the membrane composition at the point where the membrane became saturated. Because of the swelling in the membrane that occurred when large amounts of surfactant were added, concentration of surfactant in the membrane was expressed as mole fraction instead of molarity. In general, the partition coefficient for the distribution of surfactant S between the two phases is $K_x = x/[S]_{aq}$, where $x = n_S/(n_S + n_{PL})$ in terms of n_S mol of surfactant and n_{PL} mol of phospholipid in the membrane phase, and $[S]_{aq}$, the molar concentration of surfactant dissolved in the aqueous phase.

Linear relationships are observed in Figs. 2, 3, and 4 between total surfactant concentrations and phospholipid concentrations. Each graph is based on the linear regression of the endpoints found in four titrations, each using CPZ and NaDC as titrants and three titrations using TDZ as titrant. The linear regression parameters and their errors are given in Table 1.

It should be noted that [S]-intercept values for CPZ and TDZ are well above the blood serum levels for these surfactants when used as drugs in therapeutic doses.

The linear relationships just described are the experimental justification for choosing the phase partition model. Using free volume concentrations of the erythrocyte suspensions the total surfactant concentration $[S]_{tot}$, bound and unbound, is related to total phospholipid concentration [PL] in the equilibrium mixture by the following equation (Binford et al., 1988).

$$[S]_{tot} = \frac{x}{1 - x}[PL] + \frac{x}{K_x}$$

Note that $x/K_x = [S]_{aq} \cdot K_x$ would not be expected to remain constant at high concentrations of ionic surfactants because of the increasing charge density on the membrane. However, when the membrane is saturated some of the problems associated with the difference between concentration and thermodynamic activity are avoided. In all Runs I–IV using the same surfactant, the saturated membrane contains the same concentration of surfactant. Thus, the $[S]_{aq}$ value at the point of saturation in all Runs I–IV should be the same, based on

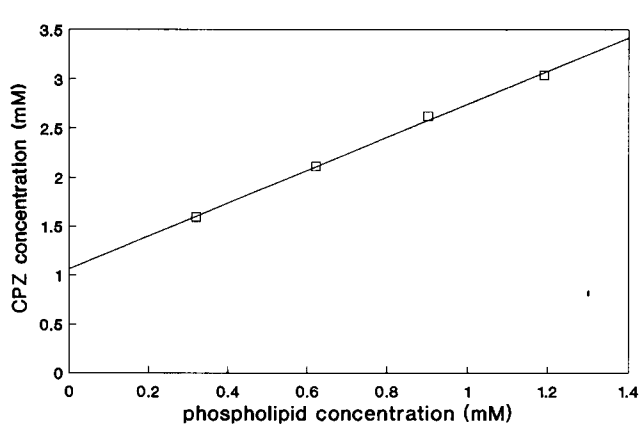

FIGURE 2 Endpoints of titrations of erythrocytes vs. CPZ in Fig. 1 and least-squares fit. Erythrocyte concentration is expressed in terms of its phospholipid content. CPZ includes both bound and unbound quantities. Concentrations are expressed in terms of intercellular volumes.

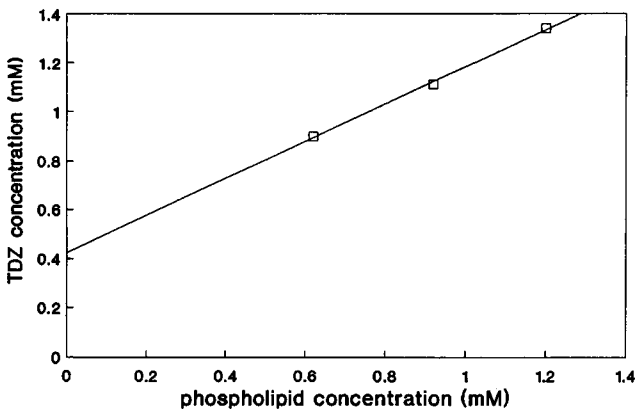

FIGURE 3 Endpoints of titrations of erythrocytes vs. TDZ in isotonic buffer at pH 5.9, and least-squares fit.

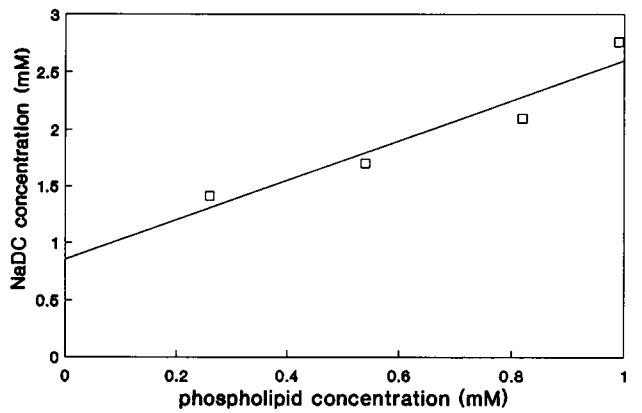

FIGURE 4 Endpoints of titrations of erythrocytes vs. NaDC in isotonic buffer at pH 7.61, and least-squares fit.

TABLE 1 Linear regression parameters of endpoint concentrations for absorbance titrations of erythrocytes

Titrant	R^2*	m‡	Error in m	b§	Error in b
		mM/mM		mM	mM
CPZ	0.998	1.683	0.054	1.064	0.035
CPZ‖	0.99994	1.803	0.010	1.041	0.0057
TDZ	0.998	0.758	0.035	0.424	0.014
NaDC	0.92	1.75	0.37	0.85	0.21

* Correlation coefficient.
† Slope.
§ [S]-intercept.
‖ Duplicate run using blood from another donor.

the phase partition model. This value of $[S]_{aq}$ is equal to x/K_x, which is the [S]-intercept. Because K_x and x are both constants at saturation, independent of [PL], the plot of endpoint values of $[S]_{tot}$ vs. [PL] should be linear.

Using slope, m, and intercept, b, from Table 1, we could calculate $x = m(m + 1)$ and $K_x = x/b$ as we did for phospholipid vesicles (Binford et al., 1988). However, the erythrocyte membrane contains significant amounts of cholesterol and glycolipid in addition to phospholipid. We did not include membrane proteins in our calculation because many of them are peripheral and all of them have such high molecular weights that their contribution to mole fraction of the membrane is small. To get the best comparison between erythrocyte membranes and vesicle bilayers (Binford et al., 1988; Malloy and Binford, 1990), we calculated the parameters y and K_y for erythrocytes in Table 2, where y is the mole fraction of surfactant based on total lipid.

Given that $K_y = y/[S]_{aq}$ and $y = n_S/(n_S + n_{TL})$, where n_{TL} is the total number of moles of membrane lipids, K_y can be calculated from the values of m and b (slope and intercept, respectively) from Table 2 and from the composition of normal blood. If n_C represents the number of moles of cholesterol and n_{GL} represents glycolipids, then the ratio $(n_C + n_{GL})/n_{PL}$ is given by 1.02 for normal blood (Telen, 1993).

A graph of $[S]_{tot}$ vs. [TL], the concentration of total lipid, has the same intercept as the graphs in Fig. 2, 3, or 4, thus $K_y = y/b$. However, the new graphs would have a different slope given by $m' = m/2.02$. Substituting this expression for m' into the equation $y = m'/(m' + 1)$ gives $y = m/(m + 2.02)$ and

$$K_y = \frac{m}{b(m + 2.02)}.$$

The results are summarized in Table 2. The data with vesicles are taken from Binford et al. (1988) and Malloy and Binford (1990).

It is apparent from Table 2 that the partition coefficient for a surfactant in erythrocyte membrane, K_y, is even greater than the partition coefficient for the same surfactant in synthetic phospholipid membrane. They are all three orders of magnitude greater than the values found by hygroscopic desorption (Conrad and Singer, 1979, 1981; Singer, 1981).

K_y: 430 (CPZ) < 550 (NaDC) < 640 (TDZ) M^{-1} in erythrocytes.
K_x: 230 (CPZ) < 440 (NaDC) < 510 (TDZ) M^{-1} in vesicles.

It is reasonable to assume that at these concentration levels, which are sufficient to saturate the membrane and disrupt it, the surfactant molecules are not just loosely bound to the surface but become an integral part of it. Furthermore, the [S]-intercepts in Table 1, which represent the calculated concentration of surfactant in the aqueous phase at saturation,

$[S]_{aq}$: 1.064 (CPZ) > 0.85 (NaDC) > 0.424 (TDZ) mM,

would not be expected to form micelles and rest on the pe-

TABLE 2 Partition coefficients and bilayer compositions of DMPC vesicles and erythrocytes saturated with surfactants*

Surfactant	With vesicles		With erythrocytes	
	K_x	x	K_y	y
	M^{-1}	mol/mol	M^{-1}	mol/mol
CPZ	230 ± 60	0.49 ± 0.09	430 ± 20	0.45 ± 0.02
CPZ‡			453 ± 4	0.472 ± 0.003
TDZ	510	0.43	640 ± 40	0.27 ± 0.01
NaDC	440 ± 40	0.22 ± 0.01	550 ± 20	0.46 ± 0.1

* Reported error is obtained from the linear regression parameters.
‡ Duplicate run is made using blood from another donor.

riphery of the erythrocyte because they are well below their critical micelle concentrations:

CMC: 3 (CPZ) > 1.7 (NaDC at 10°C) > 1.1 (TDZ) mM:

(Scholtan, 1955; Small, 1971; Binford et al., 1988, respectively). The CMC of NaDC changes very little with temperature.

Notice that the numerical values of CMC are in the reverse order of the partition coefficients. This is to be expected because the smaller the CMC value, the greater the tendency to escape from the aqueous phase by partitioning into the lipid bilayer. This is contrary to the view expressed by Singer (1981) that low CMC means that mixed micelles of surfactant and lipid from the bilayer are more likely to form and be carried away from the bilayer by hygroscopic desorption.

Finally, the contrast between partition coefficients for ideal absorption (~2% of wet EGM by volume) and absorption with strong ionic repulsion in the saturated erythrocyte membrane (~50 mol% surfactant in the lipid system) can be shown. In the former system, $K_{m/b}$ for CPZ is given as 1600 in units of mol kg^{-1} M^{-1} (Roth and Seeman, 1972), and in the latter system K_y for CPZ is given in Table 2 as 430 M^{-1}. Assuming that the dried EGM, which is the basis of membrane composition above, has the composition of normal human EGM (40% lipid, 52% protein, and 8% carbohydrate by weight (Telen, 1993)), 1 kg of dried membrane contains 400 g of lipid. Given also the fatty acid content of erythrocyte phospholipids, the average molar mass of the RCOO group is estimated to be 278 g mol^{-1}. Combining this value with the mole composition of cholesterol, glycolipid and the more common phospholipids in normal erythrocyte membrane (Telen, 1993), the average molecular mass of all lipids is found to be 573 g mol^{-1}. Thus, the conversion from molality to mole fraction in the membrane for $K_{m/b}$ is simply $1600 \times 573/400$ or 2300 M^{-1}. The value of 430 M^{-1} for saturated erythrocyte membrane represents a large deviation from ideality. However, it should be kept in mind that the charge density in the saturated membrane is an order of magnitude greater than at the concentration where it first begins to have an effect. Furthermore, it should be clear that the ratio of the mole fraction of surfactant in the membrane to its molarity in the aqueous phase, given by partition coefficients in Table 2, is only valid for saturated conditions. At other concentrations, the partition coefficient cannot be used to determine the composition.

We conclude that the type of absorption of the surfactants CPZ, NaDC, and TDZ in erythrocyte membrane is very similar to that in synthetic phospholipid vesicles in the concentration region where saturation occurs. Our comparisons of partition coefficients in the two membranes provide no evidence for the existence of "internal pressure" in protein-containing membranes.

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