The Solubility of Amphipathic Molecules in Biological Membranes and Lipid Bilayers and Its Implications for Membrane Structure†

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ABSTRACT: The equilibrium binding of several small amphipathic molecules to a variety of biological membranes has been determined by a new filtration method called hygroscopic desorption [Conrad, M. J., & Singer, S. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5202-5206]. The binding was found to be so small as to be undetectable under the conditions used, indicating that the equilibrium partition coefficients, K_p, for these compounds into the membranes could be no larger than about 0.1. On the other hand, by the same method, the binding of the same compounds to phospholipid vesicles was very large ($K_p \sim 10^3$). In this paper, the method of hygroscopic desorption is described in detail, and an intensive comparative study of the apparent binding of chlorpromazine and its quaternary amine analogue methochlorpromazine to several biological membranes and phospholipid vesicles, as measured both by hygroscopic desorption and by the conventional centrifugal method, is presented. The results show that the two compounds bind identically and additively to large unilamellar phospholipid vesicles made from soybean phosphatidylcholine, with K_p = 1490 at 37 °C, with both methods giving identical results. With erythrocyte or lymphoma cell membranes, or sarcoplasmic reticulum vesicles, however, the hygroscopic desorption method revealed no detectable binding of either compound. By the centrifugal method, apparent binding of the two compounds to the membranes was observed, as had been previously reported for chlorpromazine and erythrocyte membranes [Roth, S., & Seeman, P. (1972) *Biochim. Biophys. Acta 255*, 207-219]. However, by several criteria, this apparent binding was irreconcilable with the proposal that these compounds dissolve in the lipid bilayer portions of the membranes. Instead, the apparent binding of the two compounds to the membranes was correlated with their capacities to form micelles in aqueous solutions. We conclude that the true solubility of amphipathic molecules in a wide range of biological membranes is at least 10^3-fold lower than in phospholipid vesicles and that the lipid in the membranes is subject to some kind of large "internal pressure" that excludes the amphipaths from the membranes.

In the course of studying the solubility properties of small amphipathic molecules in membranes, we have obtained results which have led us to postulate the existence of a large "internal pressure"[1] of the lipids in biological membranes that does not exist in unilamellar phospholipid bilayer vesicles (Conrad & Singer, 1979). Amphipathic molecules, which include many of our important drugs and metabolites, have two distinct domains, one hydrophobic and the other hydrophilic. It is generally believed at present that such compounds are quite soluble in biological membranes (Seeman, 1972). The quantitation of such solubility is expressed by an equilibrium partition coefficient, K_p,[2] which is the ratio of the concentration of the compound dissolved in the membrane to that in the surrounding aqueous phase at equilibrium. Most determinations of K_p for amphipaths and membranes have been made by a centrifugal method (Seeman, 1972) in which cells or isolated membranes suspended in a solution of the amphipath are sedimented from suspension and the concentration of unbound amphipath in the supernatant is measured. Values of $K_p \gg 1$ have usually been obtained by the centrifugal method; an example of interest for the studies reported in this paper is the value K_p = 1600 for chlorpromazine in human erythrocyte membranes (Roth & Seeman, 1972). Because such values generally correspond to those for the partition of the same compounds between olive oil and water (Seeman, 1972), they have seemed reasonable. The presumption has been that the amphipathic molecules dissolved in the membranes by intercalation of their hydrophobic domains into the hydrophobic interior of the membrane, while their hydrophilic domains were positioned at the membrane–aqueous interface.

We have recently, however, developed a new method for measuring the binding of amphipaths by cells and membranes with which we have obtained different results from those of the centrifugal method. This new method is referred to as hygroscopic desorption and involves a filtration procedure described below. With three amphipathic compounds, chlorpromazine (positively charged at neutral pH), 2,4-dinitrophenol (negatively charged), and 1-decanol (uncharged), and four different types of cell membranes, it was found that at equilibrium the binding to the membranes was not detectable above controls, signifying that K_p was substantially less than 1.0 in all cases. On the other hand, with phospholipid bilayer vesicles, the same compounds were found to exhibit large values of K_p ($\gg$1) by hygroscopic desorption.

These results carry the important implication already mentioned that many biological membranes are characterized by a profound structural alteration of their lipids ("internal pressure") that does not exist in synthetic phospholipid bilayers and that serves to exclude the amphipaths from the membranes (Conrad & Singer, 1979). Such a pressure is not generally recognized as a characteristic feature of membranes at the present time. In order for such a concept to be credible, however, the hygroscopic desorption method has to be validated, and the wide discrepancy between the binding results obtained by the centrifugal and hygroscopic desorption methods must be satisfactorily explained. We have found that

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[1] The term "internal pressure" is meant to be taken figuratively rather than literally. It does not correspond to the usage of the term in the theory of liquids. We have therefore enclosed it in quotation marks throughout the text.

[2] Abbreviations used: Con A, concanavalin A; K_p, equilibrium partition coefficient.

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an intensive comparative study of the membrane binding properties of chlorpromazine and its closely related analogue methochlorpromazine (Figure 1) has been especially illuminating in these regards, and the results of this study are presented in this paper, together with the first detailed description of the procedures used in the hygroscopic desorption method. This comparison provides strong evidence that the hygroscopic desorption method does give valid data on the membrane binding of amphipaths and that the apparent membrane binding of amphipaths as measured by the centrifugal method does not represent true partition into the membrane phase. Instead, it is most likely attributable to some hitherto unsuspected type of micelle formation by these compounds. The true equilibrium solubility of these amphipaths in the biological membranes studied is therefore indeed very small.

Materials and Methods

Materials. L-α-Phosphatidylcholine from soybeans (Sigma: lecithin, type II-s) was extracted as described (Kagawa & Racker, 1971). When redissolved in $CHCl_3/CH_3OH$ (9:1), this lipid comigrated as a single spot with phosphatidylcholine standards (Supelco) during two-dimensional thin-layer chromatography on silica gel H (Brinkman, 20 × 20 × 0.2 cm plates). By this criterion, and by cholesterol oxidase assay (Heider & Boyett, 1978), the final phosphatidylcholine preparation was free of cholesterol. Egg phosphatidylcholine (Supelco) was used without further purification. Chlorpromazine hydrochloride was purchased from Sigma and its purity confirmed by both thin-layer chromatography and by high-pressure liquid chromatography (Waters Associates, μBondapak C_{18}) in acetonitrile–water. Methochlorpromazine was synthesized in this laboratory from chlorpromazine and methyl iodide (Aldrich) as reported (Huang et al., 1970). After five recrystallizations from acetone, the methochlorpromazine migrated as a single distinguishable band during both thin-layer chromatography and high-pressure liquid chromatography. Stock solutions of each compound were prepared at 6.0×10^{-2} M in 150 mM NaCl, pH 6.8, and were stored in the dark at 4 °C until used for preparing membrane suspensions. Concanavalin A (Boehringer) was purified (Agrawal & Goldstein, 1967) and stored as the lyophilized powder at −20 °C. Inulin (Sigma: dahlia tubers) was used as supplied to prepare sterile 5×10^{-3} M solutions for later incorporation inside liposomes.

Radiochemicals. Adenosine [^{32}P]triphosphate, [^{14}C]sucrose, [^{14}C]dipalmitoylphosphatidylcholine, [^{3}H]methyl iodide, and [^{3}H]inulin were purchased from New England Nuclear and were cold-diluted and used without further purification. [^{3}H]Chlorpromazine hydrochloride (Commissariat a l'Energie Atomique: 39 Ci/mmol) was assayed for purity as described above and used without further treatment, while [^{3}H]methochlorpromazine (82 mCi/mmol) was prepared in this laboratory by the same procedures used for the preparation of the nonradioactive compound described above. [^{125}I]Concanavalin A (86 Ci/mmol) was prepared from affinity-purified concanavalin A by the chloramine T method (Hunter, 1973).

Preparation of Cells, Membranes, and Liposomes. Human erythrocytes were obtained from blood which had been freshly drawn into anticoagulant buffer (0.07 M sodium citrate, 0.04 M citric acid, and 0.14 M D-glucose). The murine lymphoma cells were of two types, T cells (S49·ITB·2·3) and B cells (S194/5·XX0·BU·1), which were obtained from Dr. R. Hyman of the Salk Institute for Biological Studies. The unsealed membranes of the erythrocytes and lymphoma cells were prepared by hypotonic lysis on the day of an experiment as previously reported (Conrad & Singer, 1979). The erythrocyte "ghosts" were, of course, entirely plasma membrane; the lymphoma cell "ghosts", however, also contained intracellular and nuclear membranes which were free of nucleic acids.

Highly purified sarcoplasmic reticulum vesicles were isolated from rabbit muscle (Meissner & Fleischer, 1971), quick-frozen in liquid nitrogen, and stored at −70 °C for use within 1 month of preparation.

Large unilamellar phospholipid vesicles were prepared from soybean phosphatidylcholine by a modification of the method of Szoka & Papahadjopoulos (1978). A 25 mM solution of phosphatidylcholine in diethyl ether was sonicated briefly (MSE 150-W ultrasonic disintegrator; 5 min, 0 °C) at low power (probe tip amplitude, 8 μm, $^1/_8$-in. diameter exponential probe) with a one-third volume of 150 mM saline + 5 mM inulin. In some experiments, 2×10^{-4} mCi/mL [^{3}H]inulin or 2.5×10^{-4} mCi/mL [^{3}H]glucose was included in this aqueous phase to determine the internal volume of the vesicles. The organic solvent was then removed by using a rotary evaporator, under reduced pressure, at 25 °C. The final aqueous suspension was washed 3 times (Sorvall SS-34 rotor, 8000 rpm) in 30 volumes each of 150 mM NaCl, and the pellet was resuspended in 10% Dextran T70 (Pharmacia) in the same buffer. This material was separated by centrifugation in a 1%–10% Dextran T70 gradient (Sorvall HB-4 rotor, 7000 rpm at 4 °C for 16 h) into three discrete bands. The upper and lower layers consisted of small diameter vesicles (25–100 nm) and large oligolamellar liposomes, respectively, and were discarded. The liposomes used in this study formed the middle band with an apparent bouyant density of 1.10. These have been shown to be unilamellar by methods which will be described elsewhere. After these vesicles were washed 3 times by centrifugation (8000 rpm, 30 volumes each), the final pellet was assayed for the volume fraction of vesicles and the P concentration (see below). The pellets were then stored at 4 °C for use within 2 days. A portion of these vesicles, after fixation with 1% osmium tetroxide, was found by scanning electron microscopy to be regular spheres whose diameters ranged from 450 to 550 nm. For our present purposes, their average diameter was taken to be 500 nm.

A few experiments were also carried out with small diameter vesicles made from egg phosphatidylcholine prepared by a sonication procedure as previously reported (Conrad & Singer, 1979). These small unilamellar vesicles, which averaged 25 nm in diameter, were prepared at 4 °C and used within 3 h.

In the hygroscopic desorption experiments, it was essential to determine that each type of membrane or vesicle was completely retained on the top filter (see below). In separate experiments for this purpose, membranes and vesicles were prepared in labeled form. Red cell ghosts were labeled with [^{125}I]Con A as follows. Intact erythrocytes were reacted with [^{125}I]Con A under the set of conditions specified by Schekman & Singer (1976), and the ghosts were then prepared by the usual hypotonic lysis procedure with less than 1% loss of label. The ghosts were labeled at an activity of 7.6×10^{-3} mCi/mL of membrane. Intact lymphoma cells were also labeled with [^{125}I]Con A under the same conditions and then lysed. (This extent of [^{125}I]Con A labeling did not cause an aggregation of the cells.) In the case of sarcoplasmic reticulum vesicles, labeling with [^{32}P]-ATP was carried out as described (Meissner & Fleischer, 1971) and the product used within 4 h. The activity of the vesicles used was 3×10^{-3} mCi of ^{32}P/μL of membrane. In order to prepare labeled unilamellar phospholipid vesicles separate preparations were made, starting with either soybean phosphatidylcholine or egg phosphatidylcholine

to which was added a small amount of [^{14}C]dipalmitoylphosphatidylcholine. The deposition of solutions containing known amounts of labeled membranes on the top filter in the hygroscopic desorption experiments described below allowed measurements to be made of the degree of retention of the membranes on the filter.

Hygroscopic Desorption. A simple apparatus consisting of an assembly of three filters was devised to isolate cells or membranes free from most of the surrounding fluid in a few seconds. The top filter, which served to retain the cells or membranes, was made from a solvent-cast polycarbonate film which was either 2 μm thick (Mobay Chemical Corp.; Makrofol KG) or 5 μm thick (Nuclepore Corp.: 113602). These were obtained as 8 × 10 in. sheets which had been extensively irradiated with a ^{235}Cf source (Nuclepore Corp.). The radiation-damaged regions of the films were dissolved in 6 N NaOH for periods of $^1/_4$ h (0.03-μm maximum pore diameter) to 2 h (0.6-μm maximum pore diameter) to produce pores through the polycarbonate sheets at a nominal density of 6 × 10^8 pores/cm^2. It was through these pores that the surrounding fluid was withdrawn from cells or membranes during desorption. In order to obtain water permeability through these pores, the polycarbonate films had to be treated with a surfactant [e.g., poly(vinylpyrrolidone)] prior to their use as the upper filters for desorption. The porous sheets were washed in water, neutralized in 15% acetic acid, and cut into squares 47 mm on a side. Treatment of these squares by vacuum filtration with ethanol–water solutions (1:6 v/v) which contained from 0.01% to 1% poly(vinylpyrrolidone) resulted in filters for which the rates of water flow under standard conditions (20 °C, 10 psi) ranged from 1 to 100 mL/(min per cm^2). Thus, for filters of a given pore diameter and density, the rate of hygroscopic desorption could be routinely prescribed by pretreatment with a solution containing an appropriate concentration of the poly(vinylpyrrolidone). (Over this range of flow rates, the background uptake of amphipath by the polycarbonate filters during hygroscopic desorption was reduced to negligible levels.) After final washes in 15% 2-propanol and dilute acetic acid (5%–20% v/v), the sheets were dried and stored between glassine papers. The middle filter was an inert glass fiber spacer of 100-μm thickness and 47-mm diameter (Schleicher & Schuell, grade 30 glass) whose function was to keep the underside of the top filter from being wetted by the contents of the bottom filter. The bottom filter was a pad of absorbent white cellulose of 1.0–1.5-mm thickness and 37-mm diameter (Millipore, AP1003700). The three filters were then assembled, placed upon a Teflon-coated, stainless steel screen, and mounted in a filtration unit (Sartorius SM 16-3-16) which had been modified to allow collection of a filtrate under vacuum, if required.

When a small volume (0.2–1.5 mL) of a suspension of cells or membranes was placed on a central area of the top filter of the assembly, the bulk of the extracellular fluid was absorbed within a few seconds, without suction, into the bottom filter. The cells or membranes could be completely retained on the appropriate top filter, together with very little of the extracellular fluid (see below). The top filter was then removed and analyzed for any of several components, depending on the experiment, by dissolving the entire filter and its contents in Soluene 350, as described below.

In a typical series of hygroscopic desorption experiments to determine quantitatively the binding of an amphipathic compound to unlabeled membranes or vesicles, the membranes were suspended in a solution containing either (a) [^{3}H]inulin or (b) the radioactive amphipath. After equilibration, the desorption of the inulin-containing suspensions was carried out. The ^{3}H counts remaining on the top filter, when corrected for the ^{3}H counts left after desorption of a solution without membranes, allowed the calculation of the residual external solution left with the membranes on the top filter. Repeated experiments under standardized conditions showed the results to be quite reproducible. After desorption of the parallel *amphipath*-containing suspensions, the ^{3}H counts remaining on the top filter, when corrected for the ^{3}H counts left after desorption of an amphipath solution without membranes and also corrected for the ^{3}H counts to be expected from the residual amount of external fluid, yielded the total moles of amphipath bound to the membranes on the filter. This information, together with the measured volume of membrane suspension deposited on the filter and the volume fraction, V_m, of membranes in the suspension (see below), allowed the calculation of a value of K_p for the amphipath–membrane systems involving unsealed erythroctye ghosts and lymphocyte membranes.

In the case of the unlabeled sarcoplasmic reticulum vesicles or the soybean phosphatidylcholine vesicles, which were sealed, the vesicles were retained along with their internal fluids on the top filter after hygroscopic desorption. Following desorption, however, these vesicles could be quantitatively lysed on the top filter by the application of a measured vacuum to the filter assembly, with full retention of the lysed membranes on the top filter and up to 99% removal of the cytoplasmic fluid contents into the bottom filter. When such quantitative lysis was used, the equilibrium concentration of an amphipath in the internal fluid volume of the vesicles was measured. An additional correction for the amount of amphipath in the cytoplasm was then made in order to calculate a value of K_p for the membranes of intact sarcoplasmic reticulum vesicles. In the case of the soybean phospholipid vesicles and the small egg phosphatidylcholine vesicles, this correction was negligible.

The Centrifugal Method. An extensive body of results (Seeman, 1972) has been obtained on the apparent binding of amphipaths by membranes by this method, using the experimental procedure described by Seeman et al. (1971). The procedures we have used are similar, but all measurements were made at 37 °C. In a typical centrifugal experiment, membranes or vesicles suspended in a solution containing a known concentration of a radioactive amphipath were brought to equilibrium at 37 °C. A 1.4-mL sample of each suspension was centrifuged for 1.5 min at 37 °C in a Beckman microfuge, after which the supernatants were sampled in triplicate by using calibrated micropipettes and then counted. The pellet was assayed for radiolabel and total P to determine V_m, as described below. (Parallel hygroscopic desorption experiments and V_m determinations were carried out on the remainder of the suspension.)

Determination of the Volume Fraction of Membrane V_m. In order to calculate partition coefficients, K_p, for both the hygroscopic desorption and the centrifugal methods, the concentration of membranes or vesicles in a suspension containing an amphipath was required. The concentration is expressed throughout this paper as the volume fraction V_m of the membrane in the suspension. This was routinely determined from analysis for the concentration of total P in the stock suspensions of membranes or vesicles that were used in the uptake experiments. For each type of membrane or vesicle preparation, separate experiments were carried out to determine the relationship between the concentration of total P and V_m. For unsealed erythrocyte and lymphoma cell membranes, this was measured as the inulin-impermeable volume in the

stock solution, as follows. To a series of tubes which contained various volumes of a stock suspension containing membranes at a volume fraction of V_m in buffer was added a fixed amount of [^{3}H]inulin, and then each tube was brought to a fixed final volume, V_t, with buffer and thoroughly mixed. The membranes were then sedimented out of the suspensions, and measured fixed volumes of each supernatant were counted. The membrane volume fraction, V_m, of such suspensions is given by $(1 - c'/c)$ where c'/c is the ratio of the final concentration of ^{3}H in a sample without membrane to the concentration in a sample with $V_m > 0$. (These values agreed well with V_m obtained independently from dry weight determinations on the stock solutions.) Total phosphorous analysis of the pellets in the tubes allowed calculation of the desired relation between P concentration and V_m for erythrocyte ghosts and lymphoma cell membranes.

For sarcoplasmic reticulum vesicles, soybean phosphatidylcholine vesicles, and egg phosphatidylcholine vesicles, a similar procedure was used to determine the inulin-impermeable volume in the stock solution of vesicles, except that, in addition, a correction had to be made for the internal volume of trapped fluid inside the vesicles. This was done in separate parallel experiments by incorporating [^{14}C]glucose passively into the sarcoplasmic reticulum vesicles (which were permeable to glucose but not to inulin) or into the soybean phosphatidylcholine or egg phosphatidylcholine vesicles during their preparation. (Loss of labeled glucose from the soybean lipid vesicles was less than 1% per day.) Total P analyses of the stock solutions then gave the relation between P concentration and V_m for these three types of vesicle preparations.

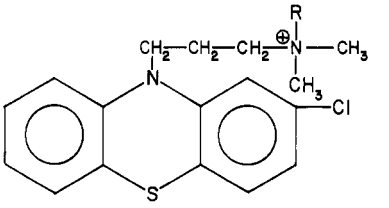
Chemical and Radioactive Assays. Colorimetric assay for total phosphorus (Applebury et al., 1974) was used to measure the phospholipid concentration in suspensions of membranes and liposomes.

For radioactive counting, samples containing ^{3}H, ^{14}C, and ^{32}P were dissolved in 1 mL of Soluene 350 (Packard), which was then diluted in 10 mL of Dimilume 30 (Packard) and assayed in a Beckman LS-200 scintillation counter. Quench factors were determined with external ^{3}H, ^{14}C, or ^{32}P standards (New England Nuclear). Samples containing ^{125}I were assayed for radioactivity, without further treatment, in a Nuclear-Chicago γ counter.

Viscometry. Viscosities of the amphipath solutions, in the absence of membranes, were measured by using Cannon–Manning semimicrocapillary viscometers (CM SMU, Size 5, 0.8–4 cS). Sample temperatures were maintained (±0.1 °C) in a thermostated glass water bath and measured with a Markson digital thermometer equipped with an immersible platinum probe (Y51-724).

Results

The Retention of Intact Cells and Membranes on the Top Filter upon Hygroscopic Desorption. The results presented below depend on the demonstration that membranes and vesicles are essentially completely retained in intact form on the polycarbonate top filter after hygroscopic desorption. For each type of preparation, experiments were first performed to determine the appropriate maximum pore size in the top filter required for such complete retention under the conditions used for the amphipath-binding measurements. For the erythrocyte ghost membranes and the lysed lymphoma cells, more than 98% of both the membrane protein (as measured by [^{125}I]Con A label) and 97% of the phospholipid (as measured by total P) were retained on top filters, with a maximum pore diameter of 0.45 μm or less, in the presence or absence of 6×10^{-5} M chlorpromazine. Similarly, more than 98% of both the [^{32}P]ATP-labeled protein and total phosphorus of the sarcoplasmic reticulum vesicles were retained on filters of maximum pore diameter 0.2 μm of less. When the large diameter phospholipid vesicles of soybean phosphatidylcholine were made to include some [^{14}C]dipalmitoylphosphatidylcholine for analytical purposes, it could be shown that greater than 99% retention was achieved on top filters of maximum pore diameter 0.2 μm or less. The small vesicles made of egg phosphatidylcholine were similarly retained by filters of 0.03 μm maximum pore diameter.



R = H chlorpromazine
R = CH_3 methochlorpromazine

FIGURE 1: Structures of chlorpromazine and methochlorpromazine.

Table I: Retention of Labeled Compounds on the Top Filter in Hygroscopic Desorption[a]

sample	dpm/mL added	dpm left on top filter	percent retention of labeled compound
(1) [^{3}H]inulin alone	2.2×10^6	75 ± 5	<0.01
(2) [^{3}H]inulin + erythrocyte ghosts	2.2×10^6	165 ± 6	0.01
(3) [^{3}H]inulin + phospholipid vesicles	2.2×10^6	142 ± 4	0.01
(4) [^{3}H]chlorpromazine alone	1.9×10^6	285 ± 7	0.016
(5) [^{3}H]chlorpromazine + erythrocyte ghosts	1.9×10^6	330 ± 8	0.018
(6) [^{3}H]chlorpromazine + phospholipid vesicles	1.9×10^6	5.9×10^5	42

[a] In each individual experiment, 0.75 mL of an equilibrated sample in isotonic Tris-NaCl buffer, pH 7.4, at 37 °C was applied to the top filter, which had a nominal density of 6×10^8 pores/cm^2 and a maximum pore diameter of 0.4 μm. Desorption occurred within 15 s. In experiments 2, 3, and 5, the volume of the membranes applied was 4×10^{-3} mL, and in experiment 6, it was 3.6×10^{-4} mL. The phospholipid vesicles in experiments 3 and 6 were large soybean phosphatidylcholine vesicles (450–550-nm diameter).

It should also be recalled that when intact erythrocytes are subjected to hygroscopic desorption they can be retained intact on the top filter, with no change in cell shape and no loss of cytoplasmic contents (Figure 1, Conrad & Singer, 1979). This further attests to the maintenance of the integrity of the membrane after hygroscopic desorption.

An Example of the Data Obtained by Hygroscopic Desorption Experiments. Most of the binding results presented in this and the preceding paper (Conrad & Singer, 1979) are given as final calculated values of K_p. It is important, however, in order to assess these results, to present at least some of the raw data for these experiments. Therefore, some typical experimental results, along with controls, are given in Table I for the retention of [^{3}H]inulin or [^{3}H]chlorpromazine in hygroscopic desorption experiments with erythrocyte ghosts or large unilamellar vesicles (450–550-nm diameter) made with soybean phosphatidylcholine. These representative data illustrate several points: (a) The retention of [^{3}H]inulin on the appropriately conditioned top filter after hygroscopic desorption of erythrocyte ghosts or vesicles was very small and

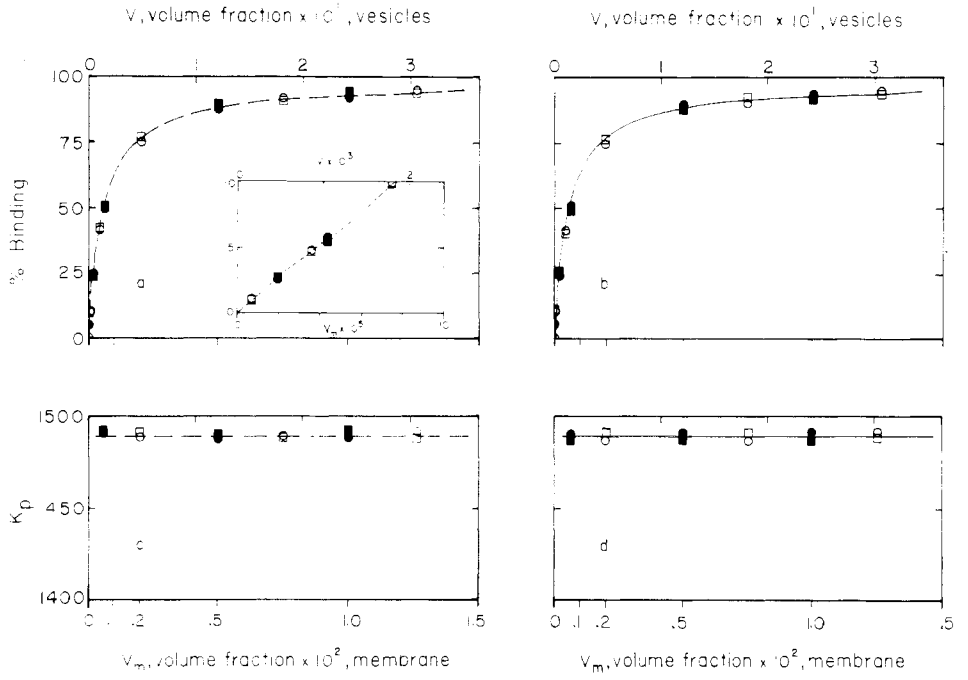


FIGURE 2: The binding of chlorpromazine or methochlorpromazine or their mixtures to vesicles of soybean phosphatidylcholine, as measured by hygroscopic desorption (open symbols) and the centrifugal method (filled symbols) at 37 °C in isotonic Tris-NaCl buffer, pH 7.4. Although data were obtained by both methods at each volume concentration of vesicles, for clarity, the data for the two methods have been plotted at alternate volume concentrations. (a) A constant concentration of [^{3}H]chlorpromazine, 6×10^{-5} M (squares), or of [^{3}H]methochlorpromazine, 3×10^{-4} M (circles), was studied as a function of the volume concentration of vesicles in the suspension. The volume concentrations are given in two ways. The lower abscissa of each figure, V_m, is the actual volume fraction of vesicle membrane in milliliter per milliliter of suspension, while the upper abscissa, V, is the volume fraction occupied by the entire intact vesicles in milliliter per milliliter of suspension. The region of small V is expanded in the inset. (b) Binding of chlorpromazine or methochlorpromazine from mixtures of the two compounds in which only one was radiolabeled. Aliquots of a mixture containing 6×10^{-5} M [^{3}H]chlorpromazine plus 4.3×10^{-5} unlabeled methochlorpromazine (mole ratio 1.4) (squares) or of a mixture containing the same concentrations of unlabeled chlropromazine and [^{3}H]methochlorpromazine (circles) were equilibrated with different volume concentrations of the large phospholipid vesicles, and binding of the radioactive compound was measured by hygroscopic desorption (open symbols) or the centrifugal method (filled symbols). The line drawn through the data in (a) is redrawn as a solid line. The binding results obtained with each compound in the mixture are therefore indistinguishable from those obtained with each compound alone. (c, d) Equilibrium partition coefficients, K_p, calculated from the data in (a) and (b), respectively.

indicated that less than 0.01% of the original external buffer remained with the cells on the top filter. (b) The background retention of [^{3}H]chlorpromazine on the top filter was very small. (c) The retention of [^{3}H]chlorpromazine with the erythrocyte ghosts was not detectable above background. This low value was uneffected by times of prior incubation of the drug and the ghosts between 10 and 80 min, and therefore it represents an equilibrium value. (d) By contrast, the binding of [^{3}H]chlorpromazine to the large diameter phospholipid vesicles was over 3 orders of magnitude greater than background controls. This great difference in [^{3}H]chlorpromazine retention by the erythrocyte ghosts and the soybean phospholipid vesicles represents the crux of our observations and is so large as to be entirely outside any experimental uncertainties of the method.

From such results with the phospholipid vesicles, including (a) the measured amounts of phospholipid (hence, of V_m), of external residual solution, and of internal solution retained on the top filter, (b) the concentration of amphipath in the original membrane suspension, and (c) the amount of amphipath retained with the membranes on the top filter (corrected for the small amounts of amphipath in the external and internal solutions and amphipath nonspecifically retained by the top filter), values of K_p could be calculated. Such K_p values are included in Figure 2c. On the other hand, since the erythrocyte ghost and other membrane preparations showed no detectable binding of the amphipath over background controls, only a maximum possible value of K_p could be calculated (Table II) as discussed below.

Binding of the Two Amphipaths by Phospholipid Vesicles Measured Both by Hygroscopic Desorption and by the Centrifugal Method. Results are shown in Figure 2a for the binding of [^{3}H]chlorpromazine or [^{3}H]methochlorpromazine by the large soybean phospholipid vesicles at 37 °C, as a function of the volume concentration of vesicles and as measured independently by both the hygroscopic desorption and centrifugal methods. In these and all remaining binding ex-

Table II: Maximal Values for K_p, $K_{p(max)}$, of Chlorpromazine and Methochlorpromazine in Several Membranes As Measured by Hygroscopic Desorption[a]

amphipath	erythrocyte ghosts	B lymphoma cell membranes	T lymphoma cell membranes	sarcoplasmic reticulum vesicles
chlorpromazine	0.1	0.03	0.07	0.09
methochlorpromazine	0.1	0.09	0.07	0.09

[a] Suspensions of membranes or vesicles, in isotonic Tris-NaCl, pH 7.4, were prepared so that the volume fraction of membranes was 0.01. The concentrations of the ^{3}H compounds were 6×10^{-5} M for chlorpromazine and 3×10^{-4} M for methochlorpromazine, and results were not changed by longer incubations of the cell suspensions with the amphipaths prior to the desorption. Hygroscopic desorption was measured at 37 °C by using top filters of 0.4-μm maximum pore diameter for the erythrocyte ghosts and lymphoma cell membranes and 0.2-μm diameter for the sarcoplasmic reticulum vesicles. Retention of the cells, membranes, or vesicles was quantitative in each case.

periments, unless otherwise specified, a constant concentration of each of the amphipaths was employed, selected as the one that afforded intact normal human erythrocytes 50% protection against hypotonic lysis, as measured under controlled conditions (Conrad & Singer, 1979; Deuticke, 1968; Seeman et al., 1967). In our experiments, this concentration was 6×10^{-5} M for chlorpromazine and 3×10^{-4} M for methochlorpromazine. The values of K_p calculated from the data are presented in Figure 2c.

A number of important points are demonstrated by these results with the phospholipid vesicles. First, K_p is a true constant. It is independent of the concentration of the vesicles at a constant concentration of amphipath or of a 100-fold change in the concentration (6×10^{-6} to 6×10^{-4} M for chlorpromazine and 1×10^{-5} to 1×10^{-3} M for methochlorpromazine) of amphipath at a constant concentration of vesicles (not shown). Second, the same value of K_p is obtained for chlorpromazine as for methochlorpromazine (1490 ± 12) with these vesicles. This identity is a point that is worth emphasizing. It is, in fact, to be expected if binding of these compounds to the vesicles occurs by an intercalation of their identical hydrophobic domains (Figure 1) into the interior of a homogeneous phase of lipid bilayer, and the small difference in the polar head groups of the two compounds has no effect. Third, the two quite different methods of measuring binding give identical results with these large phospholipid vesicles.

Closely similar results were obtained if binding to the small egg phosphatidylcholine vesicles (~25-nm diameter) was measured by both hygroscopic desorption and centrifugal methods, except that the value of K_p for both chlorpromazine and methochlorpromazine was 1350 ± 10, slightly smaller than the value for the large vesicles.

In addition to these binding experiments with the two amphipaths separately, mixtures of the two (one radioactive and the other unlabeled) were studied with the large phospholipid vesicles. The data in Figure 2b,d show that the presence of neither methochlorpromazine nor chlorpromazine affected the binding and K_p value of the other, as measured either by hygroscopic desorption or the centrifugal method. These results demonstrate that at the concentrations of the amphipaths used the two compounds bound to the synthetic vesicles independently and additively.

The results described in this section are therefore exactly those expected from the solubilization of chlorpromazine and methochlorpromazine into a homogeneous phase by intercalation into the bilayer of the phospholipid vesicles. In addition, they show that the method of hygroscopic desorption yields reliable binding data when such solubilization occurs.

Binding of the Amphipaths by Several Biological Membranes Measured by Hygroscopic Desorption. When the binding of the two amphipaths by several biological membranes was studied by hygroscopic desorption, very different results were obtained from those with the phospholipid vesicles. The membranes were those of lysed normal human erythrocytes, lysed T and B mouse lymphoma cells, and rabbit muscle sarcoplasmic reticulum. In every case, as in the example of [^{3}H]chlorpromazine and human erythrocyte ghosts shown in Table I, the binding of the amphipaths to the membranes was not detectable above background and controls. This means that K_p could not have exceeded certain maximal values. If we make the conservative estimate that less than twice the background levels of binding could not have been reliably detected, values of $K_{p(max)}$ can be calculated. These are listed in Table II. Similar results were obtained over a 100-fold range of concentration for both chlorpromazine (6×10^{-6} to 6×10^{-4} M) and methochlorpromazine (1×10^{-5} to 1×10^{-3} M). The values of $K_{p(max)}$ are generally less than 0.1; i.e., *these amphipaths are less than one-tenth as soluble in the biological membranes as in water and less than 10^{-4} times as soluble in the membranes as in the phospholipid vesicles.*

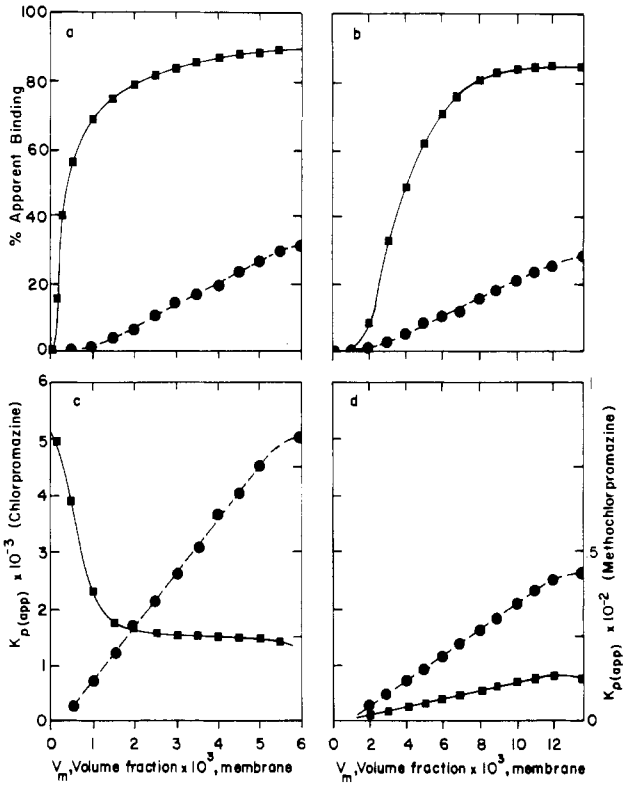


FIGURE 3: Apparent binding of chlorpromazine or methochlorpromazine to lysed human erythrocyte membranes (a,c) and to lysed T lymphoma cell membranes (b,d), as measured by the centrifugal method. (a,b) A constant concentration of 6×10^{-5} M [^{3}H]chlorpromazine (squares) or 3×10^{-4} M [^{3}H]methochlorpromazine (circles) was studied as a function of the volume fraction of membrane in the suspension. Other details are as in Figure 2a. The results with the B lymphoma cell membranes were indistinguishable from those shown for the membranes of the T lymphoma cells, but are omitted for the sake of clarity. (c,d) Apparent partition coefficients, K_p, calculated from the data in (a) and (b), respectively. The K_p ordinate scale on the left of each figure is for chlorpromazine and on the right for methochlorpromazine. Note the difference in the two scales.

Apparent Binding of the Amphipaths by Membranes Measured by the Centrifugal Method. When the apparent binding of the two amphipaths by lysed normal human erythrocytes or by lysed mouse B or T lymphoma cells was studied by the centrifugal method, in which the volume concentration of membranes was varied at a fixed concentration of the amphipath, there was under most conditions a substantial depletion of the amphipaths in the supernatants of the pelleted samples. This is shown in Figure 3a,b. Such depletion had been observed before in centrifugal experiments in the particular case of chlorpromazine and human erythrocyte membranes (Roth & Seeman, 1972) and was taken to reflect the solubilization of the amphipath in the pelleted membranes. From their results, an apparent value of K_p = 1600 could be calculated for this system. From Figure 3c, it is observed that our results also lead to an apparent $K_p \simeq 1600$ at $V_m \simeq 3 \times 10^{-3}$. However, when examined in detail, the results in Figure 3a–d are strongly anomalous in several respects. First, chlorpromazine and methochlorpromazine were markedly discriminated by both the erythrocyte ghosts and the lymphoma cell membranes in the centrifugal uptake experiments, whereas by the same technique, the two compounds

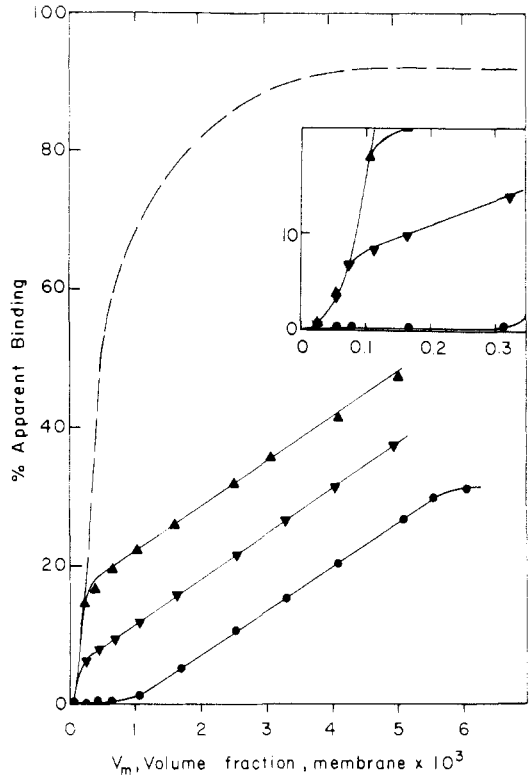


FIGURE 4: Apparent binding to lysed human erythrocytes of [^{3}H]-methochlorpromazine from mixtures containing different fixed amounts of unlabeled chlorpromazine, as measured by the centrifugal method. (▲) 12.0 × 10^{-5} M chlorpromazine plus 8.5 × 10^{-5} M [^{3}H]methochlorpromazine; (▼) 6.0 × 10^{-5} M chlorpromazine plus 8.5 × 10^{-5} M [^{3}H]methochlorpromazine; (●) 8.5 × 10^{-5} M [^{3}H]-methochlorpromazine alone. The dashed curve is the apparent binding curve for 6 × 10^{-5} M [^{3}H]chlorpromazine alone (from Figure 3a). It is not altered by the addition of unlabeled methochlorpromazine in mole ratios of 0.5–1.5 to the [^{3}H]chlorpromazine. The inset shows the region of small membrane volume fractions amplified.

showed indistinguishable binding to the phospholipid vesicles (Figure 2a). Second, the individual binding curves are peculiar. They are not characteristic of single-valued K_p curves, as would be expected if solubilization were occurring in a homogeneous phase. The large variation in the apparent K_p value as a function of the concentration of the membrane is shown in Figure 3c,d. In the case of chlorpromazine, as the concentration of the erythocyte membranes decreased toward zero, the apparent K_p markedly increased to close to 5000, whereas on the contrary, at low concentrations of the lymphoma cell membranes, the apparent K_p for chlorpromazine became vanishingly small. The apparent K_p for methochlorpromazine also varied strongly with the membrane concentration and was always more than an order of magnitude smaller than the corresponding values for chlorpromazine. At volume fractions of erythrocyte membrane smaller than 0.001, there was an enormous difference in the apparent K_p of chlorpromazine compared to methochlorpromazine, whereas at similar concentrations of either type of lymphoma cell membrane, the apparent K_p of both compounds became very small. The centrifugal uptake results for the two compounds by intact erythrocytes and lymphoma cells (not shown) were closely similar to those for their respective lysed membranes; that is, the marked disparity between the apparent binding of chlorpromazine and methochlorpromazine was observed for the intact cells as well as for their isolated membranes. These data are therefore entirely inconsistent with the notion that the apparent binding of the amphipaths to the membranes as measured by the centrifugal method represents a solubilization

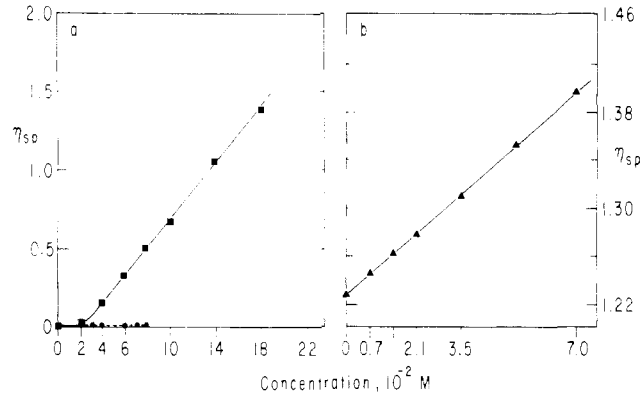


FIGURE 5: Specific viscosities of aqueous solutions of chlorpromazine, methochlorpromazine, and their mixtures in isotonic Tris-NaCl buffer, pH 7.4, at 37 °C. (a) Specific viscosity as a function of concentration for chlorpromazine (solid line) or methochlorpromazine (dashed line). (b) Specific viscosities of solutions containing a fixed concentration of 0.15 M chlorpromazine and increasing concentrations of methochlorpromazine.

of these compounds in the lipid bilayer of the membranes.

Further strong confirmation of the anomalous nature of the binding results obtained by the centrifugal method with membranes was provided by studying the apparent binding in mixtures of the two amphipaths. Mixtures containing a constant concentration of the radioactive form of one compound together with different fixed concentrations of the unlabeled form of the other were investigated for their apparent binding of the radioactive compound by lysed erythrocyte membranes. An example of these experiments is shown in Figure 4. The addition of unlabeled chlorpromazine to a constant concentration of [^{3}H]methochlorpromazine in mole ratios between 0.5 and 1.5 caused marked increases in the apparent uptake of the methochlorpromazine. For example, at low concentrations of erythrocyte membranes (inset, Figure 4) where methochlorpromazine itself showed very little apparent binding, the addition of the chlorpromazine caused about a 100-fold increase in the apparent binding of methochlorpromazine so that its binding was indistinguishable from that of chlorpromazine itself. If simple intercalation of these amphipathic compounds into the membrane bilayer were occurring, such synergistic effects on binding would not be expected (and were not observed in binding to the phospholipid vesicles, Figure 2b).

Viscosity of Aqueous Solutions of the Amphipaths. Amphipathic molecules of the appropriate structure tend to form micellar aggregates in aqueous solutions. This is a well-known fact in the case of our more common detergents, but it is not as well appreciated that amphipaths like chlorpromazine can also form such micellar aggregates (Scholtan, 1955). The difference between a detergent such as sodium dodecyl sulfate and chlorpromazine as measured by their respective critical micelle concentrations is not very great: 1.33 mM compared to 4.0 mM at 0.1 ionic strength (Helenius et al., 1979; Scholtan, 1955). At concentrations exceeding its critical micelle concentration, chlorpromazine can form micelles containing >10^2 molecules/micelle (Scholtan, 1955), much as in the case of sodium dodecyl sulfate, and these micelles can be detected by a variety of physical chemical measurements such as analytical ultracentrifugation, surface tension, and viscosity.

In order to illustrate some consequences of these detergent-like properties, comparative viscosity measurements were carried out on isotonic aqueous solutions of chlorpromazine, methochlorpromazine, and their mixtures. Some representative

results are shown in Figure 5. In the concentration range indicated (Figure 5a), chlorpromazine solutions exhibited a large specific viscosity which increased with increasing concentration, most likely due to the formation of increasingly large and asymmetric micelles with increasing concentration (Scholtan, 1955). Under the specified conditions, however, methochlorpromazine solutions showed the small specific viscosity characteristic of molecularly dispersed solutes (i.e., little or no micelle formation occurred). From measurements of the surface tensions of isotonic aqueous solutions of chlorpromazine (Scholtan, 1955) and methochlorpromazine (M. J. Conrad and S. J. Singer, unpublished results), we have estimated that the critical micelle concentration of methochlorpromazine is about 3×10^{-2} M under these conditions—substantially larger than the value of 4×10^{-3} M for chlorpromazine.

Experiments with mixtures of the two compounds showed that they interact with one another in aqueous solution. For example, as shown in Figure 5b, the addition of increasing concentrations of methochlorpromazine to solutions at a fixed concentration of chlorpromazine produced a regular increase in specific viscosity (although in this concentration range methochlorpromazine itself caused no significant viscosity increment, Figure 5a). These results very likely mean that the size and/or asymmetry of the micelles formed by chlorpromazine alone was increased by the incorporation of increasing amounts of methochlopromazine into them to form mixed micelles (Shinoda et al., 1963) containing the two compounds. Furthermore, the maximum amount of methochlorpromazine that could be solubilized at equilibrium was markedly increased in the presence of chlorpromazine.

These observations can be rationalized as follows. The packing of methochlorpromazine compared to chlorpromazine molecules into one-component micelles must be relatively impeded by the bulk of the extra methyl group at the hydrophilic head of the methochlorpromazine molecule. Such steric effects on critical micelle concentrations have previously been observed with other phenothiazine derivatives (Scholtan, 1955). On the other hand, in *mixed* micelles in which methochlorpromazine is incorporated with excess chlorpromazine, such steric effects are diluted out.

Discussion

Significance of the Binding Results Obtained by Hygroscopic Desorption. The direct binding of chlorpromazine and methochlorpromazine as well as of the amphipaths 2,4-dinitrophenol and 1-decanol previously studied (Conrad & Singer, 1979) to several different biological membranes cannot be detected at the level of sensitivity of the hygroscopic desorption method as we currently employ it. If these results are correct, they signify that the K_p values for all of these systems cannot be larger than about 0.1. Because these values are surprisingly small, we have sought for possible artifacts in the hygroscopic desorption measurements, but have been unable to find any. These are equilibrium values unaffected by the length of time during which the membranes are suspended in the amphipath solution up to 3 h. One possibility which we considered was that the amphipaths did indeed intercalate and solubilize in the membranes but were ejected during hygroscopic desorption, perhaps through physical changes in the membrane produced in the last stages of removal of the external fluid from the intact cells or membranes. However, no evidence for such an ejection at later stages in the desorption process could be obtained (Conrad & Singer, 1979). Furthermore, we showed that despite the low solubility in the membrane chlorpromazine had equilibrated across the membrane of the intact erythrocyte and was present in the cytoplasm at the same concentration as in the external fluid. If an ejection of chlorpromazine from the membrane bilayer had indeed occurred upon hygroscopic desorption of intact cells, the chlorpromazine dissolved in the inner half of the bilayer should have been ejected into the cytoplasm and have been readily detected there in the intact cells. Such an increase in concentration of chlorpromazine in the cytoplasm was not observed (Conrad & Singer, 1979). A related possibility is that in the last stages of removal of the external fluid from the cells or membranes during hygroscopic desorption the equilibrium between membrane and external fluid is somehow perturbed and the chlorpromazine or other amphipaths leave the membrane to enter the aqueous phase. In the short time involved in the last stages of hygroscopic desorption (at most a few seconds), however, any chlorpromazine dissolved in the inner half of the membrane bilayer of the intact cell would remain in equilibrium with the chlorpromazine in the cytoplasm and should therefore have been left in the membrane and have been detected. It was not.

Finally, in the present paper, we have shown that with amphipaths and phospholipid vesicles, the method of hygroscopic desorption yields large K_p values that are single valued, are the same for chlorpromazine and methochlorpromazine, and are indistinguishable from the values obtained by the centrifugal method. This demonstrates that where true solubilization occurs in the bilayer the method of hygroscopic desorption can measure it without artifact. We therefore conclude from this discussion that the low solubility of amphipaths in the membranes studied is a meaningful result.

Significance of the Binding Results Obtained by the Centrifugal Method. The large apparent binding and K_p values obtained with the amphipaths and biological membranes by the centrifugal method are in clear contradiction to the small values obtained for the same systems by hygroscopic desorption measurements. We have therefore devoted considerable attention to this wide discrepancy. Our results demonstrate that the apparent binding obtained by the centrifugal method cannot be attributed to the solubility of the amphipaths in the membrane. The apparent K_p values are highly variable in a given system, and they are widely different for the two membranes studied. They are also widely different for the two compounds despite the fact that chlorpromazine and methochlorpromazine are identical in their hydrophobic moieties and indeed show the same large K_p values for binding to the phospholipid vesicles. It should be emphasized that these were equilibrium values, unchanged by longer incubations of the membranes with the amphipaths prior to centrifugation. While the apparent binding results with membranes, therefore, cannot be explained by solution in the membrane, they show a correlation with the micelle-forming capacities of chlorpromazine and methochlorpromazine in aqueous solutions. The larger apparent membrane uptake of the former compound than that of the latter correlates with the lower critical micelle concentration of the former in aqueous solution. Furthermore, and most strikingly, the synergistic rather than simply additive membrane binding observed in mixtures of the two amphipaths by the centrifugal method correlates with the capacity of the two to form mixed micelles with one another in aqueous solutions. The apparent binding to the membranes obtained by the centrifugal method therefore seems to involve some kind of aqueous micelle formation by the amphipaths in contrast to their solubilization in the phospholipid vesicles. We do not, at present, have a completely satisfactory molecular explanation for this apparent binding to the membranes nor, for

our present purposes, (which are primarily to validate the low binding results obtained by the hygroscopic desorption method) do we require one. Nevertheless, it is an interesting problem, for which several clues exist that are worth noting.

In previous sections, we have provided evidence that aqueous micelle formation may somehow be involved in the apparent binding. On the other hand, the concentrations of chlorpromazine and methochlorpromazine used in the membrane binding studies were some 2 orders of magnitude smaller than their respective critical micelle concentrations in membrane-free aqueous solutions. Furthermore, certain amphipaths such as decanol [which also gives large values of apparent binding by the centrifugal method (Seeman, 1972) but no detectable binding by hygroscopic desorption (Conrad & Singer, 1979)] do not form their own micelles in aqueous solutions. When aqueous solutions of decanol exceed a critical equilibrium concentration, the decanol forms a separate liquid phase rather than micelles (Tanford, 1973). Thus, simple, one-component micelle formation in the aqueous phase of the membrane suspensions cannot be the explanation of the apparent binding of amphipaths. On the other hand, the formation of mixed micelles (Shinoda et al., 1963) incorporating the amphipath, in which the other components of the mixed micelles were derived from the membranes, could rationalize many of the results. Such mixed micelles could exist at concentrations of amphipaths much lower than the critical micelle concentrations of the amphipaths themselves and could include amphipaths like decanol which do not form micelles themselves. The components derived from the membrane could differ for different amphipaths and different membranes. If such mixed micelles were loosely associated with (and perhaps partially stabilized by) the membrane surface, then they might sediment with the membranes in the highly hydrated pellet in the centrifugal method but be stripped from the membrane surfaces during hygroscopic desorption. Under such circumstances, incidentally, equilibrium dialysis methods might also be expected to exhibit the apparent large binding of amphipaths to membranes that are found by the centrifugal method.

It is also possible that amphipaths bind to the surfaces of membranes by way of "hemimicelle" formation (Fuerstenau, 1956; Somasundaran et al., 1964), as occurs when amphipaths are adsorbed at solid–water interfaces formed between aqueous solutions and quartz, alumina, and similar solids. In these cases, the individual molecules of an amphipath adsorb by their hydrophilic ends to groups at the solid surface. At high enough concentrations, however, they associate with each other laterally to form a two-dimensional half- or hemimicelle. The critical concentration for hemimicelle formation can be lower than for bulk-phase micelle formation. Mixed hemimicelles can also form.

These suggestions are made to provide a basis for a consideration of mechanisms of appparent amphipath binding to membranes that do not involve solubilization into the interior of the lipid bilayer of the membrane. Further consideration of these suggestions does not seem warranted here, however, and will be deferred until more direct experimental information bearing on the problem is acquired.

Whatever may be the correct explanation of the apparent binding of the amphipaths to membranes that is obtained by the centrifugal method, the conclusion that it does not represent a true solubilization in the membrane bilayer has many implications, a few of which should at least be mentioned here. In the past, many studies have been carried out by using amphipathic fluorescent or spin-labeled molecules as probes for the lipid environment within biological membranes in which solutions of such amphipaths have been added directly to the membrane suspensions [as early examples, see Freedman & Radda (1969) and Rubalcava et al. (1969)]. These amphipathic probes then were found to exhibit spectroscopic characteristics that showed that they were dispersed in a hydrophobic environment; this environment was naturally presumed to be the interior of the membrane bilayer. In view of the results and conclusions presented in this paper, however, it is likely that in at least some of these instances the amphipathic molecules were not solubilized in the membrane interior. They might instead, for example, have been present in mixed micelles or hemimicelles distinct from the membranes themselves, as discussed above, within which they would also be expected to show the spectroscopic characteristics due to an hydrophobic environment. In order to affirm that the amphipathic molecules were indeed appreciably dissolved in the membranes, hygroscopic desorption or equivalent binding measurements would have to be carried out. This has not been done in the past.

These suggested artifacts might not apply, however, in all such cases. For example, where an amphipathic fluorescent or spin-labeled compound was added along with bovine serum albumin to a membrane suspension (Hubbell & McConnell, 1969), if the amphipath were initially bound to the albumin, an *exchange* of the amphipath with some membrane component (such as a free fatty acid) could occur, and a true solubilization of the amphipath in the membrane might result. This could also be checked by hygroscopic desorption measurements.

Other implications of this proposal have to do with the well-known effects of amphipaths on the shapes of erythrocytes and their resistance to hypotonic lysis and, at high enough concentrations, in causing their isotonic lysis (Deuticke, 1968; Seeman et al., 1967). Previously, the direct intercalation of the amphipaths into the lipid bilayer of the membrane (Seeman, 1972; Sheetz & Singer, 1974) was suggested to be the determining factor in these effects. However, in the light of our present results, the fact that methchlorpromazine causes the same equilibrium erythrocyte membrane changes as chlorpromazine but *only at higher concentrations* (Sheetz & Singer, 1974) correlates with the micelle-forming capabilities of the two compounds. An explanation of the amphipath-induced membrane changes that takes these considerations into account is therefore required.

Significance of the Low Solubility of Amphipaths in Biological Membranes. In these and our previous studies (Conrad & Singer, 1979), we have examined four widely different amphipaths with four different membrane preparations by the method of hygroscopic desorption and have found in all cases that the solubility of the compounds in the membranes is low. The membranes have involved not only those of erythrocytes but also of more typical eukaryotic cells, such as lymphoma cells, and have included an intracellular type of membrane (sarcoplasmic reticulum) as well as the plasmalemma. In addition, the lysed lymphoma cells retained much of their intercellular membranes along with their plasma membranes, and yet the entire preparation exhibited low amphipath binding. Thus, these findings appear to be fairly general. On the other hand, it is possible that with certain amphipaths or specific membranes significant exceptions to these findings will be observed. Prokaryotic cell membranes, thylakoid, and mitochondrial membranes should be among those examined in extending the present studies, as well as the membranes of excitable cells. In this connection, the results of Easton et al. (1978) are of considerable interest. They found that the

amphipathic dye merocyanine 540, which fluoresces in a hydrophobic environment but not an aqueous one, showed a strong fluorescence enhancement when added to cells with excitable membranes (such as nerve and muscle cells) but no enhancement when added to any of a wide range of cells that were not excitable (including erythrocytes, cultured fibroblasts, epithelial cells, and blood leukocytes). The latter results are consistent with our present findings that amphipathic compounds are only slightly soluble in such nonexcitable membranes but suggest that our hygroscopic desorption measurements should be extended to excitable membranes. However, in what follows, the low solubility of amphipaths in membranes is taken to be sufficiently general as to warrant an examination of its significance.

It should first be pointed out that the low equilibrium solubility is not incompatible with the permeability of the amphipaths through the membranes. In the case of intact erythrocytes (Conrad & Singer, 1979), it was shown directly by hygroscopic desorption that [^{3}H]chlorpromazine rapidly equilibrated between the intracellular and extracellular fluids. The low solubility of the amphipath should affect the rate of permeation but not the ultimate attainment of equilibrium across the membrane. The low solubility, furthermore, does not rule out the possibility that, although undetectable by hygroscopic desorption, the small amount of amphipath that dissolved in the membrane interior may have important consequences, particularly if it is concentrated in the boundary regions where the lipid fatty acyl chains abut the hydrophobic surfaces of the integral proteins (Bieri & Wallach, 1975).

We have suggested that the reason that amphipathic compounds are excluded from biological membranes but are quite soluble in phospholipid vesicle bilayers is that there is a large "internal pressure"[1] in the lipid bilayer portions of membranes that does not exist in the simple bilayers (Conrad & Singer, 1979). This difference could be related to compositional differences between the real membranes and simple bilayers, the former generally containing large concentrations of integral proteins and in some cases neutral lipids such as cholesterol, as well as attached peripheral proteins, along with the phospholipids. Although a wide variety of studies by many investigators has demonstrated that integral proteins and cholesterol can affect the structural and thermodynamic properties of phospholipids [for reviews, see Gennis & Jonas (1977), Shinitzky & Inbar (1976), and Chapman et al. (1979)], there has been no agreement about the range or magnitude of these effects. We have embarked on a program of systematic reconstitution of integral proteins, cholesterol, and other components into phospholipid vesicle membranes, examining the resultant preparations for changes in their capacity to bind amphipaths as measured by hygroscopic desorption. Our preliminary studies have indicated that integral membrane proteins or cholesterol incorporated into the large soybean phospholipid vesicle membranes do produce significant reductions in the K_p for chlorpromazine binding. These studies and their relation to protein–lipid and lipid–lipid interactions in biological membranes will be presented and discussed elsewhere.

The existence of a large "internal pressure" in the lipids of membranes, whatever its precise origins, could have profound effects on membrane functions. It might greatly increase the tensile strength of the membranes. By increasing the effective microviscosity of the membrane, it could reduce the rates of lateral diffusion of integral proteins and lipids in the membrane and affect the activities of membrane enzymes and transport proteins. A large "internal pressure" could also greatly reduce the rate with which cell membranes fuse with one another or with phospholipid bilayer vesicles. Furthermore, such large structural differences between biological membranes and phospholipid bilayer vesicles render the vesicles of limited utility as model systems to study the properties of membranes and could affect the significance of many such model studies that have been made in the past. Phenomena may be observed in phospholipid vesicle systems that do not have their counterparts in real membranes because of the large "internal pressure" in the membranes.

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High-Resolution Nuclear Magnetic Resonance Studies of the *Lac* Repressor. 1. Assignments of Tyrosine Resonances in the N-Terminal Headpiece†

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ABSTRACT: The DNA binding site of the *lac* repressor protein has been implicated to lie within the N-terminal 51 amino acid fragment termed headpiece (HP-51 or LR-51). High-resolution NMR suggests that isolated HP-51 retains most of the secondary and tertiary structure which it has in the whole repressor. Four of the eight tyrosines of repressor are in HP-51. ^{1}H NMR spectra (360 MHz) over the aromatic region of native HP-51 show that the four tyrosines are nonequivalent with an unusual distribution of chemical shifts. Denaturation leads to loss of these chemical shift differences. Homonuclear decoupling and a two-dimensional autocorrelated spectrum allow unequivocal pairing of resonances from Tyr A at 6.99 and 6.79 ppm, Tyr B at 6.98 and 6.39 ppm, Tyr C at 6.70 and 6.54 ppm, and Tyr D at 6.39 and 6.33 ppm. The 2,6 protons are low field of the 3,5 protons for each Tyr residue. Selective chemical modification with nitration reagents allows assignments of Tyr A to Tyr-47, Tyr B to Tyr-7, Tyr C to Tyr-12, and Tyr D to Tyr-17 in HP-51. All four tyrosines are essential for maintaining the structure of the isolated headpiece, and Tyr-7, -12, and -17 appear to be stacked.

The *lac* repressor (LR) is a regulatory protein of *Escherichia coli* originally isolated by Gilbert & Muller-Hill (1966). Its function in the intact bacterium is to form a complex with the operator region of the *lac* operon, blocking transcription and hence the production of lactose metabolizing enzymes and transport proteins. Allolactose and various galactosides serve as inducers of protein synthesis by binding to the repressor and cause dissociation of the DNA-repressor complex (Jobe & Bourgeois, 1972). Intact repressor has a molecular weight of 154 500 and is a tetramer of four identical subunits, each with a known sequence of 360 amino acids (Beyreuther, 1978; Farabaugh, 1978). The three-dimensional structure of this protein has not been determined. Given the primary sequence, NMR may allow some insight into the structure, and the present studies were undertaken on this basis.

Proteolytic cleavage of the repressor (Geisler & Weber, 1977) permits the preparation of two fragments: the tetrameric "tryptic core" (TC- or LR-60-360), which contains the inducer binding site, and the N-terminal headpiece (HP- or LR-51 or 59), which is monomeric and contains the major portion of the operator (DNA) binding site (Pfahl et al., 1974; Miller et al., 1975, 1977; Barkley et al., 1975). ^{1}H NMR spectroscopy has given the surprising result that the spectra of HP and TC are simply additive, yielding a spectrum identical with that of the whole repressor (Wade-Jardetzky et al., 1979; Buck et al., 1978). This result suggests that the headpiece is a separately mobile domain of the repressor molecule and is consistent with recent low-angle X-ray scattering experiments (Pilz et al., 1980) which demonstrate that HP lies at the ends of a cigar-shaped tetrameric core.

HP contains four of the eight tyrosines and one of the six histidines in the repressor. The tyrosine resonances are nonequivalent with an unusual distribution of chemical shifts. It is the purpose of this paper to report an analysis of the aromatic region of the HP ^{1}H NMR spectrum and the assignment of the tyrosine resonances. In subsequent reports, we will discuss other features of the structure and dynamics of the *lac* repressor and its structured fragments HP and TC, which can be inferred from high-resolution NMR spectroscopy.

Materials and Methods

Lac repressor protein was prepared from cultures of the SQ mutant of *E. coli* as previously described (Matthews et al., 1977). The tryptic core (TC) and headpiece (HP) fragments of LR were prepared according to the procedure of Geisler & Weber (1977). LR, at a protein concentration of 15 mg/mL in 1 M Tris-HCl buffer (pH 7.6), 30% glycerol, and 3×10^{-4} M dithiothreitol, was digested with 1.5% of its weight of DCC-treated trypsin (Sigma Chemical Co.) for 2 h at 20 °C. The trypsin was then inactivated by a 3-fold excess of soybean trypsin inhibitor (Sigma Chemical Co.). Under these conditions, peptide bond hydrolysis at lysyl residue 59 goes to completion, while a partial cleavage occurs at arginyl residue 51. The monomeric HP fragments (residues 1-51 and 1-59) and the tetrameric TC (residues 60-360) were separated by gel filtration at 5 °C on a column of Sephadex G-150 eluted with 0.2 M ammonium bicarbonate and 3×10^{-4} M dithiothreitol. HP was further purified by gel filtration on a column of Sephadex G-50 with the bicarbonate buffer, and HP-51 and HP-59 were then separated by ion-exchange chromatography on a column of CM-Sephadex C-25 and eluted with a gradient

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