# Thermodynamics of Membrane Partitioning for a Series of *n*-Alcohols Determined by Titration Calorimetry: Role of Hydrophobic Effects[†]

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*Received August 29, 1997; Revised Manuscript Received December 18, 1997*

ABSTRACT: Recent studies have shown that the traditional paradigm relying on hydrophobic effects is not adequate to describe membrane partitioning of amphiphilic solutes. To elucidate the thermodynamics and determine the role of the hydrophobic effect in the partitioning of small amphiphilic molecules into lipid bilayers, we have used titration calorimetry to directly measure the enthalpy, partition coefficients, and heat capacity change for the partitioning of a series of *n*-alcohols into lipid bilayers of several lipid compositions. The incremental thermodynamic quantities have been compared with model compound data for partitioning into bulk hydrocarbon solvents. We have found that there is a large negative heat capacity change upon partitioning, indicating a major contribution from the dehydration of nonpolar solute moieties; however, these hydrophobic effects also involve changes in lipid interactions with water in the interfacial region of the bilayer. In addition, we have found that the enthalpy effects are large, indicating that the partitioning process is accompanied by significant changes in the intralipid interactions within the bilayer. Cholesterol has a large effect on partitioning thermodynamics, making both the enthalpy and entropy contributions significantly more positive, resulting in a relatively small net decrease in the negative free energy of partitioning. These results demonstrate that while hydrophobic interactions play a major role in partitioning, the process is considerably more complex than the partitioning of model compounds between water and bulk hydrocarbons, with major contributions coming from changes in the structure and thermodynamic state of the bilayer, including the interfacial region. The results are discussed in terms of both the thermodynamics of partitioning and the role of lipid properties in membrane function. Our results support a paradigm for membrane structure and function in which the thermodynamic state, which is a function of lipid composition, temperature, and dissolved solutes, is a critical membrane property.

The biological role of the physical properties of specific membrane lipids has not been clearly established. However, the wide diversity of lipid physical states and dynamic properties combined with the tight regulation of membrane lipid compositions suggests that these properties are essential to the structure and function of biological membranes. Possible roles include the establishment and maintenance of the lateral and vertical segregation of membrane components into various regions or domains and the creation and/or regulation of the microenvironment of particular membrane proteins. It has been proposed by Gruner that the lipid properties are controlled, by means of the content of non-bilayer-forming lipids, to maintain a certain energy property termed the "intrinsic curvature", which is itself a critical membrane property (*1*). In a study by Seelig and co-workers of the effect of vesicle size on partitioning thermodynamics, it was suggested that the membrane possesses an "entropy-producing potential" which is important to function (*2*). This proposal is related to Gruner's hypothesis by its reliance on a critical role for lipid packing to determine a functionally critical internal thermodynamic status. Thus, while the evidence is strong that lipid properties are critical for membrane structure and function, a consensus has not yet appeared.

It is well established that many small molecules partition into lipid bilayers with considerable effects on the bilayer physical properties. These small molecules include natural metabolites and peptides as well as hormones, drugs, alcohols, anesthetics, and environmental pollutants. These interactions are of great importance in the mechanism of actions of drugs, drug delivery, peptide translocation, toxicity of environmental pollutants, and other important biological phenomena. To understand the function of biological membranes, it is very important to elucidate the means by which membranes interact with small molecules and the effects of these interactions on membranes.

Bilayer:water partition coefficients or lipid solubilities for many small molecules have been measured over many years, and in general they behave similarly to partition coefficients or solubilities in hydrocarbon solvents. The correlation of these solubilities with anesthetic potency led to the lipid hypothesis of general anesthesia of Meyer and Overton in 1901 (*3*−*5*). However, some systematic differences between the solubilities and membrane:buffer partition coefficients

[†] This work was supported by the Medical Research Service of the Department of Veterans Affairs.

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S0006-2960(97)02160-0 CCC: $15.00 
Published on Web 02/04/1998

in hydrocarbon solvents and lipid bilayers, as well as variations in partition coefficients for lipids as a function of lipid composition and temperature, have been noted in a number of studies (e.g., refs *6*−*14*).

Traditionally, because of these longstanding correlations, the partitioning of small organic molecules such as general anesthetics into membranes has been considered as being primarily driven by the hydrophobic effect, i.e. as being thermodynamically well modeled by the partitioning of model compounds between aqueous solution and bulk hydrocarbon. The primary characteristic of hydrophobic interactions is an anomalous heat capacity change which gives rise to temperature-dependent enthalpy changes, as discussed for example by Dill (*15*), Tanford (*16*), and Baldwin (*17*). The molecular interpretation of the hydrophobic effect focuses on the effect on water structure of the presence of nonpolar moieties in which the entropy is decreased and the heat capacity is increased. The removal of such nonpolar entities from the water and into the hydrocarbon phase then increases the overall entropy of the system and decreases the heat capacity. In this paradigm any thermodynamic changes within the bulk hydrocarbon phase are negligible compared to the large changes due to dehydration of hydrophobic solute moieties.

In recent years, as the structure of membranes has become understood in more detail, partitioning phenomena have been more closely examined thermodynamically, and some deviations from strictly hydrophobic behavior have been reported. In one example showing the inadequacy of the analogy of bilayer partitioning with partitioning into bulk hydrocarbons, DeYoung and Dill showed that the partition coefficients of benzene and hexane in lipid bilayers were a function of the surface density of the bilayer, a property characteristic of interfacial phases as opposed to bulk phases (*18*, *19*). In a recent study using measurements of the partition coefficients as a function of temperature, Wimley and White compared the partitioning of several indoles into lipid bilayers and into cyclohexane and found that the heat capacity change was lower and the enthalpy more negative for the lipid bilayer compared to cyclohexane (*20*). Several studies by Seelig and co-workers using titration calorimetry have found that the enthalpies are sometimes considerably larger than expected for purely hydrophobic partitioning, leading to the suggestion of the existence of "nonclassical" hydrophobic partitioning (*2*, *21*, *22*).

The partitioning of drugs and alcohols into lipid bilayers has been measured by a variety of means. Many measurements have been made of the partition coefficients, giving a measure of the free energies, but until recently the other thermodynamic quantities could only be determined by measurement of the temperature dependence of the partition coefficients. With the development of titration calorimetry, it is now possible to measure directly both the enthalpy and the partition coefficient; carrying out these measurements as a function of temperature provides the heat capacity change. The use of titration calorimetry thus now makes it possible to obtain all of the thermodynamic properties of partitioning with a precision not previously available.

In our laboratory we have adapted the titration calorimetry method to the study of membrane partitioning of alcohols. We previously reported the partition coefficients and thermodynamics of butanol partitioning into DPPC[1] as a function of its phase state (*23*). In the present study, we have used titration calorimetry to measure directly for the first time the enthalpy, partition coefficients, and heat capacity change for the partitioning of a series of *n*-alcohols into lipid bilayers of several lipid compositions. By measuring a homologous series of *n*-alcohols, we have determined the incremental thermodynamic quantities which can be compared with model compound data for partitioning into bulk hydrocarbon solvents. The results which we report demonstrate that while hydrophobic interactions play a major role in partitioning, the process is considerably more complex, with major contributions coming from changes in the structure and thermodynamic state of the bilayer. The inadequacy of the paradigm of membrane partitioning as being analogous to partitioning into bulk hydrocarbons is particularly demonstrated by the profound effect of cholesterol on the partitioning thermodynamics. The results are discussed in terms of both the thermodynamics of partitioning and the role of lipid properties in membrane function. A preliminary part of this work was presented at the International Workshop on Anesthetic Mechanisms, in Takamatsu, Japan (*24*).

## MATERIALS AND METHODS

*Materials*. The lipids were purchased from Avanti Polar Lipids and used without further purification. Alcohols were obtained from Aldrich Chemical Co. (Milwaukee, WI) or Supelco (Bellefonte, PA).

*Sample Preparation*. Large unilamellar extrusion vesicles were used for all titration experiments. Bangham's method was used to prepare the DPPC multilamellar vesicles (*25*). Chloroform stock solutions of DPPC were dried under a steady stream of nitrogen gas and then kept overnight on a vacuum pump to remove all residual chloroform. The stock suspensions were hydrated with distilled water at 50 °C for at least 2 h with occasional vortexing. Extruded vesicles were prepared by extrusion of multilamellar vesicles through two stacked polycarbonate filters of 400 nm pore size at 50 °C, using the thermostated Extruder from Lipex (Vancouver, BC, Canada) (*26*). The extrusion was repeated 10 times. The extruded vesicles for each experiment were characterized by the NICOMP Model 370 Submicron Particle Sizer (Pacific Scientific Co., Silver Spring, MD). The distributions were unimodal, centering around values between 300 and 425 nm. Samples removed from the titrator were occasionally rechecked, and it was found that no changes in size occurred during the titration experiments. Lipid concentrations were determined by phosphorus assay by the method of Bartlett (*27*). To minimize oxidation of unsaturated lipids, they were kept under nitrogen gas at room temperature. Butylated hydroxytoluene (1 mol %) was added to SAPC samples.

*Titration Calorimetry*. Titration calorimetry experiments were carried out using the OMEGA titration calorimeter from Microcal, Inc. (Northampton, MA) (*28*), as described previously (*23*). The lipid suspension was placed in the syringe and the alcohol solution in the cell; this arrangement minimized the contributions from the heat of dilution of the

[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DLPE, dilaurylphosphatidylethanolamine; DOPG, dioleoylphosphatidylglycerol; SAPC, 1-steroyl-2-arachidonoylphosphatidylcholine; DPH, diphenylhexatriene.

alcohol. Since the partitioning of alcohols into lipids involves nonsaturable low-affinity partitioning, as opposed to the high-affinity saturable specific-site model which is appropriate for protein−ligand binding, we developed a routine to fit the data to the partitioning model. In this procedure, the equation for mole fraction partition coefficients was fitted to the accumulated heat curve, with the two fitting parameters being $\Delta H$ and $K$. The mole fraction partition coefficient is expressed by the relation

$$K_X = X_{a,b}/X_{a,w}$$

where $X_{a,b}$ is the mole fraction of alcohol in the bilayer and $X_{a,w}$ is the mole fraction of alcohol in the solution.

*Partition Coefficient Units*. The units used in this study were the traditional mole fraction units. Because it has been suggested that volume fraction units and Flory−Huggins corrected volume fraction units may be more appropriate, we have also calculated these for purposes of comparison (*19*, *20*, *29*−*31*). The volume fraction partition coefficient is expressed by the relation

$$K_V = \phi_{a,b}/\phi_{a,w}$$

where $\phi_{a,b}$ is the volume fraction of alcohol in the bilayer and $\phi_{a,w}$ is the volume fraction of alcohol in the solution. Refitting the data with this quadratic equation for selected experiments gave results which differed from the mole fraction values by a constant factor, equal to the ratio of the molar volumes of lipid and water, as expected for dilute solutions (*19*). Therefore the volume fraction partition coefficients shown in Figure 3 were calculated by applying this factor to the fitted mole fraction values. The molar volume used for the lipid was 0.6 L/mol, as suggested by Wimley and White (*20*); this value is lower than the molar volume of the total phospholipid molecule, because it can be assumed that the polar head groups are not part of the lipid "solvent". They showed that the results are not very sensitive to the exact value used between 0.4 and 0.8 (*20*). The alcohol molar volumes were calculated from their densities. The Flory−Huggins corrected volume fraction partition coefficient is calculated by the relation

$$\ln K_{VFH} = \ln K_V + V_s((1/V_b) - (1/V_w))$$

where $V_s$, $V_b$, and $V_w$ are the molar volumes of solute, bilayer, and water, respectively(*19*).

*Determination of Partition Coefficients Using Optical Density*. The effect of each alcohol on the gel to liquid crystal phase transition temperature of DPPC was determined by following the absorbance at 400 nm using the Cary 3 spectrophotometer. Transition temperatures were measured for both heating and cooling scans. Transition temperatures were determined for a minimum of six alcohol concentrations for each alcohol, the data were fitted linearly, and the partition coefficient was calculated from ideal-solution theory as described by Rowe (*32*).

## RESULTS

*The Method: Octanol Partitioning into DPPC*. Titration calorimetry experiments were performed by adding aliquots of the suspension of lipid vesicles from the syringe into the reaction cell containing the alcohol solution. Figure 1A shows an example of a typical experiment for octanol with a sequence of 25 injections. The area under the peaks is integrated to give the amount of heat released or taken up. Figure 1B shows the accumulated heat of injections from Figure 1A. The enthalpy $\Delta H$ (in kcal/mol of alcohol bound) and mole fraction partition coefficient $K_X$ are determined by the fitting procedure described above; the line in Figure 1B is the fitted curve. The other thermodynamic parameters are calculated from the relation

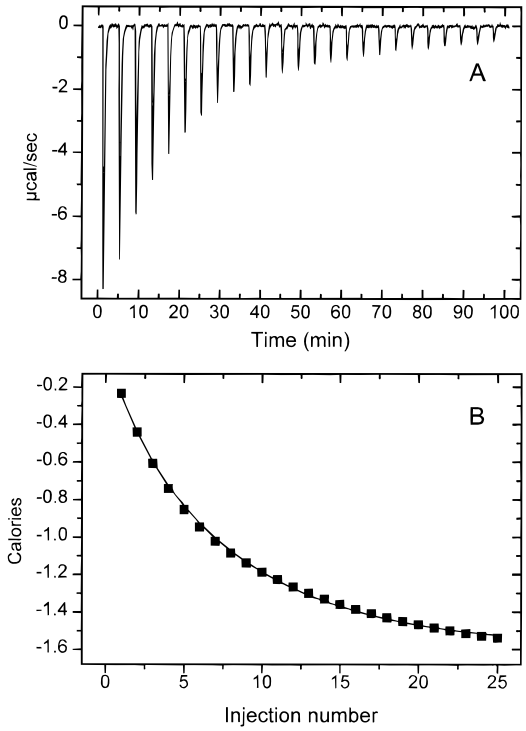


FIGURE 1: (A) Titration calorimetry data for octanol−DPPC binding. The cell contained 0.614 mM octanol; 10 $\mu$L injections of 47.4 mM lipid vesicles at 4 min intervals; temperature 47.5 °C. (B) Accumulated heat as a function of injection number for the data of part A. The line represents the best fit, determined as described in the text.

$$\Delta G = -\mathrm{RT} \ln K = \Delta H - T\Delta S$$

Each experiment was performed at a constant temperature ranging from 45 to 60 °C, which is above the main transition temperature of each of the lipids and mixtures studied. The heat capacity change for the partitioning reaction is calculated from the slope of the plot of enthalpy against temperature.

Figure 2 shows the enthalpies and partition coefficients for octanol partitioning into DPPC as a function of temperature. The least-squares fit of the enthalpy as a function of temperature gives the heat capacity change for the interaction of $-140 \pm 44$ cal/(deg mol).

*Partition Coefficient Units*. The choice of partition coefficient units for membrane-partitioning phenomena has been discussed in the literature recently, and because of the large size disparity between the two "solvents", water and lipid bilayer, it has been suggested that the Flory−Huggins corrected volume fraction partition coefficients are more appropriate than mole fraction units for characterizing the partitioning of small molecules into lipid bilayers (*19*, *20*, *29*−*31*). However, this is still controversial, and Holtzer (*33*) has made a convincing argument that the mole fraction

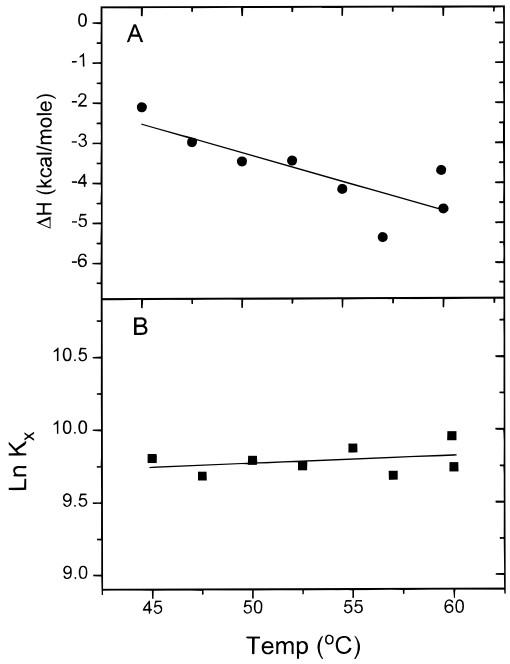


FIGURE 2: (A) Enthalpy of partitioning for octanol in DPPC as a function of temperature; expressed as enthalpy per mole of bound alcohol. (B) Mole fraction partition coefficient for octanol−DPPC partitioning.

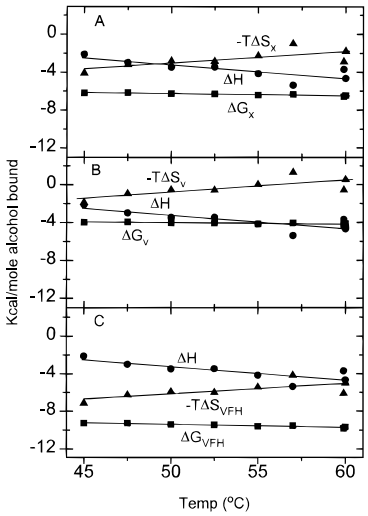


FIGURE 3: Thermodynamic quantities for octanol−DPPC partitioning, determined using different partition coefficient units for free energy calculation: (A) mole fraction units; (B) volume fraction units; (C) Flory−Huggins corrected volume fraction units.

treatment is still valid and may still be the most appropriate for these systems. As pointed out by Holtzer (*33*) and discussed by DeYoung and Dill (*19*), the Flory−Huggins treatment was derived for much larger differences in molecular size than is considered here, namely systems involving large polymer molecules with ideal behavior (*31*). There are additional problems in choosing the appropriate molecular volume for the bilayer molecules, since portions of the bilayer molecules (i.e., the charged head groups) are not part of the "solvent", as discussed by Wimley and White (*20*).

To compare the units and determine their significance, we have calculated the thermodynamic data for octanol−DPPC partitioning using mole fraction, volume fraction, and Flory−Huggins corrected volume fraction units to determine the free energy. The enthalpy is the same for each since it is independent of units; all of the effects of changing units for the free energy appear in the entropy term. Figure 3 shows the free energies, along with the contributions from enthalpy and from the entropy, expressed in terms of $-T\Delta S$, plotted as a function of temperature for each choice of units. In Figure 3A, in terms of mole fraction units, the entropy and enthalpy contributions to the free energy are both negative and are of similar magnitude, with the entropy contribution decreasing and the enthalpy contribution increasing with increasing temperature. In Figure 3B, where the free energy is calculated using volume fraction units for the partition coefficients, the entropy contribution is less positive and the reaction is driven primarily by the enthalpy. In Figure 3C, where the volume fraction partition coefficients have been corrected according to the Flory−Huggins theory, the entropy contribution becomes dominant.

The comparison of the thermodynamic results summarized in Figure 3 shows graphically that the choice of partition coefficient units alters the free energy by altering the entropy contribution to the free energy, since the enthalpy is not affected by units. The major benefit of using the volume fraction and Flory−Huggins corrected values is in comparisons of systems with large molecular size differentials. From the point of view of our study, the differences in size among our solutes (four alcohols) and our solvents (lipids) are not sufficient to change any conclusions in the comparisons among them. For simplicity, and to make use of the thermodynamic quantities in the literature which are generally reported in mole fraction units, we report our results using the traditional mole fraction units.

*Thermodynamics of Octanol Partitioning in DPPC.* Thermodynamic quantities for the partitioning of octanol into DPPC from 45 to 60 °C based on mole fraction units are summarized in Figure 3A; the values at 45 °C are shown in Table 1. The entropy at 45 °C is given in Table 1; in Figure 3A the entropy contribution is presented as $-T\Delta S$, to show its contribution to the free energy. The enthalpy is temperature dependent, giving a heat capacity change of $-140 \pm 44$ cal/(deg mol) for the partitioning process. The relative contributions of enthalpy and entropy depend upon the choice of units for the partition coefficient as discussed above. However, considering the quantities derived using the traditional mole fraction units, the values are consistent with an interaction which is at least partially driven by hydrophobic forces, i.e. the dehydration of the hydrophobic acyl chains of octanol. For example, although the enthalpy is

Table 1: Thermodynamic Parameters for DPPC−Alcohol Interactions at 45 °C

| alcohol chain length | $K$ | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $T\Delta S$ (kcal/mol) | $\Delta S$ (cal/(mol K)) | $\Delta C_p$ (cal/(mol K)) |
|---|---|---|---|---|---|---|
| 6 | 839 | $-4.3 \pm 0.1$ | $1.6 \pm 0.2$ | $5.8 \pm 0.3$ | $18 \pm 1$ | $-210 \pm 16$ |
| 7 | 2150 | $-4.9 \pm 0.1$ | $-1.2 \pm 0.6$ | $3.6 \pm 0.7$ | $11 \pm 2$ | $-120 \pm 54$ |
| 8 | $1.81 \times 10^4$ | $-6.2 \pm 0.1$ | $-2.1 \pm 0.6$ | $4.1 \pm 0.7$ | $13 \pm 2$ | $-140 \pm 44$ |
| 9 | $5.55 \times 10^4$ | $-6.9 \pm 0.1$ | $-4.0 \pm 0.2$ | $2.9 \pm 0.3$ | $9 \pm 1$ | $-77 \pm 11$ |

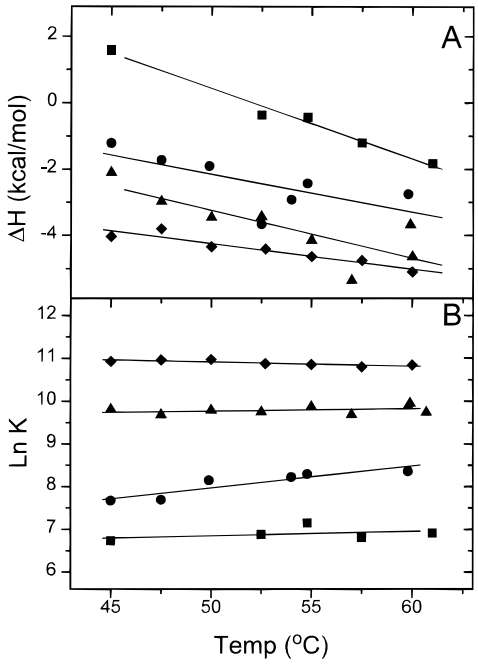


FIGURE 4: Enthalpy and mole fraction partition coefficients for partitioning into DPPC for four alcohols as a function of temperature: squares, hexanol; circles, heptanol; triangles, octanol; diamonds, nonanol.

negative at 45 °C and makes a significant contribution to the free energy, the enthalpy extrapolates to zero at 27.5 °C, near room temperature.

The most significant factor for identification of hydrophobic interactions is the change in heat capacity. As shown in Figures 2 and 3 and Table 1, the heat capacity change is large and negative, consistent with a strong hydrophobic contribution to the interactions.

*Variation of Thermodynamic Parameters with Alcohol Chain Length*. The titrations of DPPC with the series of alcohols from hexanol to nonanol were carried out as a function of temperature as described above. Figure 4A shows the enthalpies for each of these alcohols as a function of temperature. The heat capacity change is calculated from the slope of these plots. Figure 4B shows the mole fraction partition coefficients of each of these alcohols as a function of temperature.

To obtain independent confirmation of our fitting procedures, the partition coefficients for several of these alcohols into DPPC were also determined by the transition temperature depression method (*32*). This method only allows measurement at one temperature, the temperature of the phase transition. The free energies calculated from mole fraction partition coefficients of the series of *n*-alcohols at 41 °C calculated by the temperature depression method are shown in Figure 5, along with the 45 degree values from the calorimetry measurements. This plot shows that similar results were obtained with both methods. This agreement is particularly important because the values from the transition temperature depression method are determined at the limit of low alcohol concentration, whereas the calorimetry data are obtained over a range of alcohol concentrations. These partition coefficients are also in good agreement with published values (*34*, *35*).

Table 1 summarizes the thermodynamic results obtained in this study at 45 °C, using mole fraction units. The values

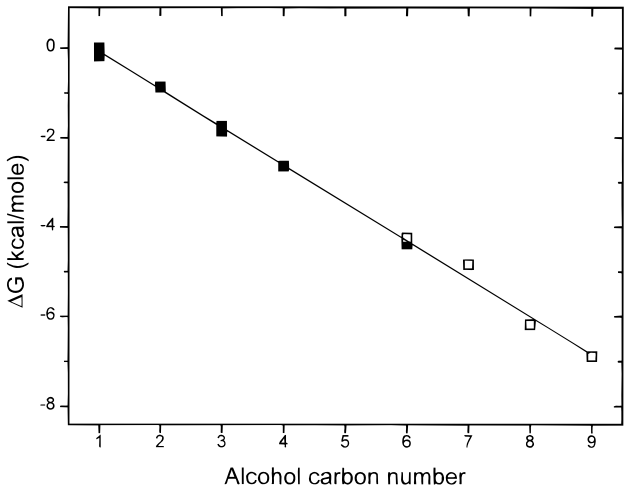


FIGURE 5: Free energy of partitioning of alcohols as a function of alcohol carbon number at either 45 or 41 °C (mole fraction untis): solid squares, partition coefficients determined by melting temperature depression; open squares, partition coefficients determined by titration calorimetry.

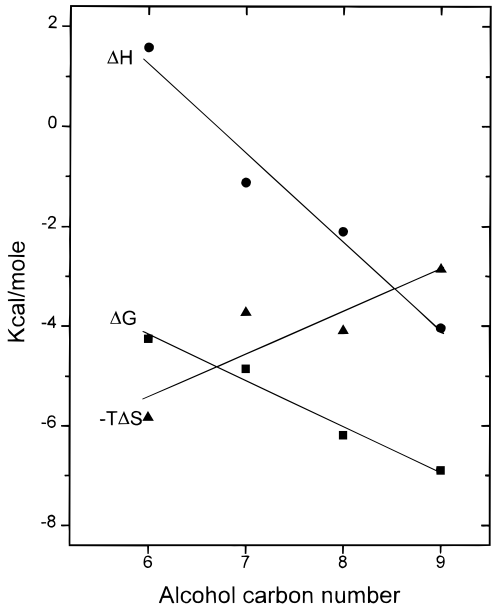


FIGURE 6: Thermodynamic parameters for alcohol partitioning into DPPC at 45 °C as a function of alcohol carbon number (mole fraction units): squares, free energy; circles, enthalpy; triangles, entropy contribution to free energy expressed as $-T\Delta S$.

at 45 °C of the enthalpy, free energy, and the entropy term $-T\Delta S$ are plotted against the number of carbons (C) in Figure 6. The slopes of these plots give a measure of the incremental value with each methylene group. These are useful for comparison with values obtained from the data for the partitioning of model compounds between aqueous and pure hydrocarbon solvents, which represent classical hydrophobic partitioning.

The dependence of the free energy on carbon number (C) from Figure 6 is −0.929 kcal/(mol C). For the data from $n = 1$ to 9 in Figure 5, the slope is −0.847 kcal/(mol C). This compares well with the values of −0.884, −0.884, and −0.860 summarized by Tanford for the dependence on the number of carbons of the free energy of transfer of alkanes, alkenes, and dienes, respectively, from water to hydrocarbon (*16*).

Table 2: Partitioning of Octanol at 45 °C

| lipid | $K$ | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $T\Delta S$ (kcal/mol) | $\Delta S$ (cal/(mol K)) | $\Delta C_p$ (cal/(mol K)) |
|---|---|---|---|---|---|---|
| DPPC | $1.81 \times 10^4$ | $-6.2 \pm 0.1$ | $-2.1 \pm 0.6$ | $4.1 \pm 0.7$ | $13 \pm 2$ | $-144 \pm 44$ |
| DOPC | $1.98 \times 10^4$ | $-6.3 \pm 0.1$ | $-1.9 \pm 0.1$ | $4.4 \pm 0.2$ | $14 \pm 1$ | $-99 \pm 6$ |
| DLPE | $1.19 \times 10^4$ | $-5.9 \pm 0.1$ | $-1.2 \pm 0.1$ | $4.8 \pm 0.1$ | $15 \pm 1$ | $-149 \pm 10$ |
| DOPG | $1.29 \times 10^4$ | $-6.0 \pm 0.1$ | $-1.6 \pm 0.2$ | $4.4 \pm 0.3$ | $14 \pm 1$ | $-63 \pm 15$ |
| SAPC | $2.12 \times 10^4$ | $-6.3 \pm 0.1$ | $-1.3 \pm 0.1$ | $5.1 \pm 0.2$ | $16 \pm 1$ | $-83 \pm 17$ |

A similar analysis of the dependence on n of the enthalpy of the interactions for alcohol partitioning into DPPC at 45 °C gives a slope of −1.784 kcal/(mol C). This is very different from the value obtained for the heats of transfer of *n*-alcohols from water into the pure alcohols. As summarized in Tanford (*16*), the variation of enthalpy with chain length for the transfer of *n*-alcohols was quite small, e.g. −0.185 kcal/(mol C). The strong dependence of the enthalpy of partitioning of alcohols into lipid bilayers on the number of carbons is not consistent with an interaction which is purely hydrophobic, i.e. based on the dehydration of nonpolar moieties by removal from water. It suggests that the interactions of these alcohols with bilayers involve some changes in specific interactions among the lipid molecules or between the alcohols and the lipid moieties. For example, the introduction of the alcohol into the lipid bilayer may involve significant changes in the bilayer structure and dynamics. It may be noted that the sign of the enthalpy changes from positive to negative with increasing chain length, suggesting that the smaller alcohols disrupt interactions in the bilayer while the longer alcohols enhance, and participate in, such interactions. Because of the large heat capacity change, the crossover in sign of the enthalpy occurs at different temperatures for each chain length.

The change of the entropy with additional carbons is negative, as seen by inspection of Table 1. The entropy of the interaction is positive for each alcohol, as expected for hydrophobic interactions; however, the magnitude of the positive entropy decreases with increasing chain length. This is in contrast to the results for the transfer of alcohols from water to pure alcohol reviewed by Tanford (*16*), in which the entropy contribution increases with increasing alcohol chain length. The classical interpretation is that the entropy change upon partitioning arises from the decrease in entropy in the water phase when the hydrocarbon moiety is removed from it; this should be proportional to the size of the hydrocarbon moiety. In the case of the alcohols partitioning into lipid bilayers, the negative increment for additional methylene groups suggests that changes in addition to the removal of the alcohol from water are occurring. One possibility is that there are compensating changes in exposure of hydrophobic moieties of the lipid to water in the interfacial region. Another possibility is that the configurational entropy of the bilayer phase is altered by the introduction of the alcohol.

The most significant factor for identification of classical hydrophobic effects is the anomalous heat capacity change which has been observed in model compound studies of partitioning of small molecules between water and hydrocarbon solvents (*15*−*17*). According to the classical interpretation, the change in heat capacity is due to the larger heat capacity of the water when it is forced to accommodate moieties which cannot form hydrogen bonds. The heat capacity then decreases when the hydrocarbon moiety is removed from the water. The magnitude of this effect is dependent on the molecular surface area of the hydrophobic moiety. The data summarized in Table 1 show that, as expected, there is a large negative heat capacity change associated with partitioning of each of these alcohols into lipid bilayers. However, as is evident by inspection of Figure 4A and Table 1, the magnitude of the negative heat capacity is not increasing with alcohol chain length. In fact, a plot of these data against carbon number (C) gives a slope of 37 ± 17 cal/(mol K C), indicating a trend opposite to the expected direction, although nearly zero when the error is taken into account. The absence of an increase in the magnitude of the heat capacity change with alcohol chain length suggests that changes are also occurring in the lipid upon partitioning, which compensate for the changes in the water phase caused by the removal of the alcohol hydrophobic moieties. The change is most likely a compensating exposure of hydrophobic lipid moieties to the aqueous phase in the interfacial region of the bilayer.

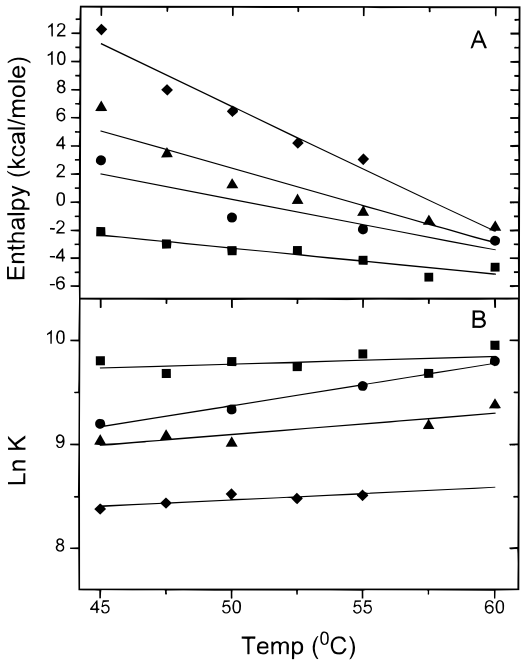


FIGURE 7: Effect of cholesterol on enthalpy and partition coefficients for octanol partitioning into DPPC as a function of temperature (mole fraction units). Cholesterol concentrations: squares, 0%; circles, 16 mol %; triangles, 19 mol %; diamonds, 31 mol %.

*Effects of Head Groups and Chain Unsaturations*. To survey the role of the lipid head groups and acyl chain unsaturations in the partitioning of alcohols, the partitioning of octanol into several additional lipids was measured. Table 2 summarizes the thermodynamic data from results obtained at 45 °C for DPPC, DOPC, DLPE, DOPG, and SAPC. The other temperatures up to 60 °C were also measured and the results used to calculate the heat capacity change (data not shown). The variations among these lipids of the free energy

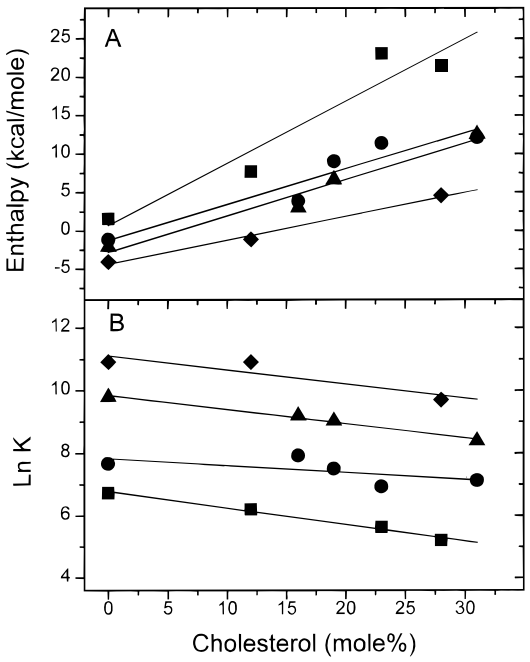


FIGURE 8: Effect of cholesterol on enthalpy and partition coefficients for alcohol partitioning into DPPC at 45 °C for series of alcohols (mole fraction units): squares, hexanol; circles, heptanol; triangles, octanol; diamonds, nonanol.

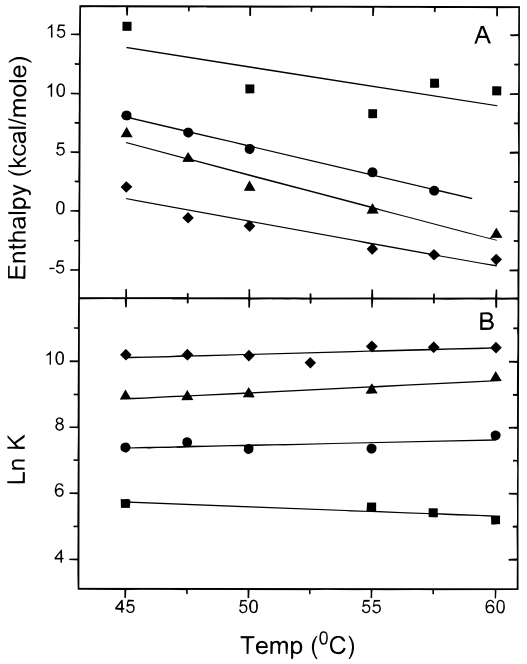


FIGURE 9: Enthalpy and partition coefficients for alcohols partitioning into DPPC with 20 mol % cholesterol as a function of temperature (mole fraction units). 20 mol % values for each temperature and alcohol were determined by interpolation as described in text: squares, hexanol; circles, heptanol; triangles, octanol; diamonds, nonanol.

of octanol partitioning is nearly within the experimental error. The enthalpy variations of octanol partitioning are greater, ranging from −1.2 kcal/mol for the short-chain DLPE to −2.1 kcal/mol for the longer-chain saturated DPPC. However, these variations are small and near the experimental error; further studies are needed to determine if they are significant. The largest variation is in the heat capacity change; the two saturated lipids have heat capacity changes which appear to be significantly different from those of the three unsaturated lipids. These data suggest that the lipid acyl chain unsaturation may affect alcohol partitioning thermodynamics; however, more studies are needed to make a conclusive determination of this effect. Further detailed systematic investigations of the effects of acyl chain unsaturation and head group variations on alcohol partitioning are in progress.

*Effect of Cholesterol on Partitioning*. The effect of cholesterol on octanol partitioning into DPPC is shown in Figure 7, where the enthalpy and partition coefficients are shown as a function of temperature for four cholesterol compositions. The partition coefficient decreases with increasing cholesterol content, as shown in Figure 7B. Figure 7A shows that the enthalpy becomes increasingly positive with increasing cholesterol content. It is also clear from Figure 7A that the heat capacity change for partitioning (slopes of enthalpy curves) becomes increasingly negative with increasing cholesterol content.

The effect of cholesterol on partitioning was also determined for nonanol, heptanol, and hexanol. For each alcohol, data analogous to that in Figure 7 for octanol were collected at four cholesterol contents as a function of temperature (data not shown). The 45 °C data for each are summarized in Figure 8 where the enthalpy and partition coefficients at 45 °C are plotted against the cholesterol concentration. The data for the other alcohols were similar to those for octanol; cholesterol reduced the partition coefficient and increased the enthalpy as a function of cholesterol concentration.

For the further discussion and comparison of cholesterol data for the four alcohols, we have interpolated these data to obtain the partition coefficients and enthalpies for 20 mol % cholesterol for each alcohol as a function of temperature. For each alcohol, the enthalpy and partition coefficients for each temperature have been fitted by linear regression and the 20 mol % value has been determined. The enthalpy and partition coefficients for 20 mol % cholesterol for each alcohol are plotted in Figure 9 as functions of temperature. The heat capacity change for each alcohol is determined from the slopes of the enthalpy plots. The thermodynamic quantities at 45 °C for each alcohol partitioning into DPPC with 20 mol % cholesterol are summarized in Table 3.

The dependence on the alcohol carbon number of the thermodynamic contributions to the free energy of partitioning are shown in Figure 10 for DPPC with 20 mol % cholesterol at 45 °C. The corresponding data for the absence of cholesterol from Figure 6 are also shown for comparison. Figure 10 demonstrates graphically that the effect of cholesterol in reducing the free energy of alcohol partitioning for each of these alcohols is the result of much larger effects on the enthalpy and entropy contributions, which compensate for each other. In addition to the effects of cholesterol on each alcohol, there is also a large effect of cholesterol on the chain length dependence of the enthalpy and the entropy terms, which again compensate for each other, leaving the chain length effect on the free energy unaffected by cholesterol.

The data presented in Figures 7−10 and Table 3 show that the most profound effect of cholesterol on the partitioning thermodynamics is on the enthalpy of the interaction, with a compensating effect on the entropy. The effect of cholesterol on the enthalpy is to make it increasingly positive

Table 3: Thermodynamic Parameters for Alcohol Partitioning in DPPC Containing 20 mol % Cholesterol at 45 °C

| alcohol chain length | $K$ | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $T\Delta S$ (kcal/mol) | $\Delta S$ (cal/(mol K)) | $\Delta C_p$ (cal/(mol K)) |
|---|---|---|---|---|---|---|
| 6 | 296 | $-3.6 \pm 0.1$ | $16 \pm 2$ | $20 \pm 2$ | $60 \pm 6$ | $-324 \pm 184$ |
| 7 | 1615 | $-4.7 \pm 0.1$ | $8.1 \pm 0.2$ | $12.8 \pm 0.3$ | $40 \pm 1$ | $-492 \pm 20$ |
| 8 | 7657 | $-5.6 \pm 0.1$ | $6.6 \pm 0.8$ | $12.2 \pm 0.9$ | $38 \pm 3$ | $-550 \pm 67$ |
| 9 | $2.7 \times 10^4$ | $-6.4 \pm 0.1$ | $2.0 \pm 0.7$ | $8.5 \pm 0.8$ | $27 \pm 2$ | $-378 \pm 54$ |

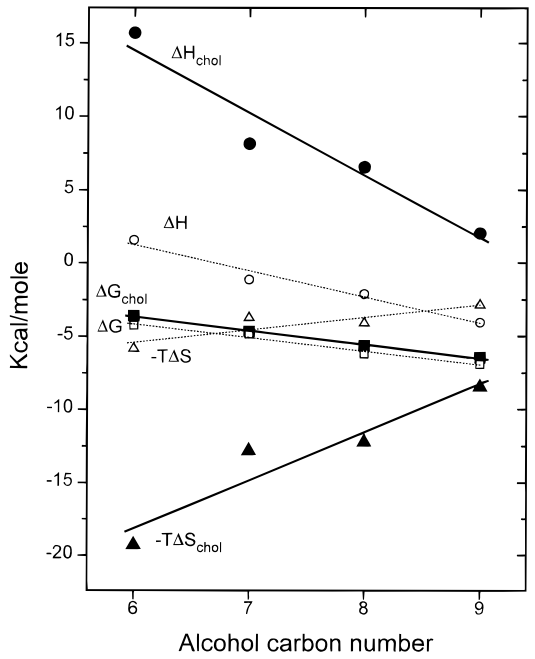


FIGURE 10: Thermodynamic parameters for alcohol partitioning into DPPC containing 20 mol % cholesterol at 45 °C as a function of alcohol carbon number, compared with data of Figure 3 for no cholesterol (mole fraction units): squares, free energy; circles, enthalpy; triangles, entropy contribution to free energy expressed as $-T\Delta S$; solid symbols and lines, 20 mol % cholesterol; open symbols and dotted lines, data from Figure 3 for no cholesterol.

with increasing cholesterol content. The positive enthalpy of partitioning suggests that the alcohols are disrupting the packing in the bilayer, reducing the energy of the interactions among the lipid components. This is occurring to a much greater extent in the presence of 20 mol % cholesterol than without cholesterol. As discussed above, in the absence of cholesterol, the chain length dependence of the enthalpy of partitioning indicates that there is an important contribution to partitioning from the lipid−lipid interactions, in which for the shorter alcohols the change is positive and for the longer ones it is negative. The effect of cholesterol is to shift all of the alcohols to positive enthalpy, indicating a greater disruption of bilayer packing for the cholesterol-containing bilayer.

The corresponding greater positive entropy contribution of partitioning for the cholesterol-containing bilayer is probably due both to a greater increase in the configurational entropy of the bilayer and to larger changes in the degree of exposure to water of the hydrophobic moieties of the lipid interfacial region. As discussed below, the heat capacity changes support the latter as making a significant contribution.

The dependence of the enthalpy on the carbon number, shown in Figure 10, is even greater than in the absence of cholesterol, giving a slope of −4.26 kcal/(mol C). Following the discussion above, the positive enthalpy contributions indicate that the alcohol is reducing the energy of the interactions of cholesterol and lipid in the bilayer. The effect of alcohol in reducing the van der Waals packing is greatest for the smallest alcohols and becomes less with increasing chain length, leading to the very large negative slope of the enthalpy plot of Figure 10. The greater heat capacity change for this interaction in the presence of cholesterol means that the difference between cholesterol and no cholesterol decreases with increasing temperature.

The negative heat capacity change upon partitioning is also significantly increased with increasing cholesterol, as is evident by inspection of Figure 7A and comparison of Figures 4A and 9A and by comparison of Tables 1 and 3. For example, the heat capacity change for octanol partitioning into DPPC is −140 cal/(deg mol) without cholesterol, and −550 cal/(deg mol) in the presence of 20 mol % cholesterol. The larger magnitude of the heat capacity change in the presence compared to the absence of cholesterol must arise from the different structure of the cholesterol−lipid bilayer compared to that with no cholesterol. The most likely possibility for such a large change in heat capacity lies in the interaction between hydrophobic moieties and water; this large change would indicate that the overall exposure of hydrophobic moieties to water is reduced by the partitioning process to a greater extent in the presence than the absence of cholesterol. Since the dehydration of the alcohols themselves is not affected by cholesterol, alcohol partitioning must cause a larger decrease in exposure of water to hydrophobic moieties in the interfacial region in cholesterol-containing lipids than in the absence of cholesterol. The introduction of the alcohol may expel some water from hydrophobic sites in the interfacial region by increasing the mobility and allowing the alcohol to shield some lipid moieties from water. Thus the partitioning process protects not only the alcohol hydrocarbons but also lipid moieties from exposure to water.

The heat capacity change, in contrast to the enthalpy and entropy, shows no dependence on the alcohol chain length, as is evident by inspection of Figure 9A and Table 3. A plot of these data against carbon number gives a slope of $-22 \pm 54$ cal/(mol K C), clearly within experimental error of zero. As in the case of no cholesterol, this again suggests that although the overall change in exposure of hydrophobic moieties to water is large for partitioning, and is even larger in the presence than in the absence of cholesterol as noted above, it is similar for each alcohol. This again suggests that there are changes in exposure to water of membrane moieties upon partitioning that are dependent on the alcohol size and which compensate for the known dependence on molecular size of water exposure to the alcohol in the aqueous phase.

## DISCUSSION

We have reported the measurement of the partitioning of a series of *n*-alcohols into several lipids using titration

calorimetry. This method has allowed us to measure directly for the first time the enthalpy and the partition coefficients for these alcohols and thus to obtain all the thermodynamic quantities with a precision that has not been possible using other methods which measure only the partition coefficient as a function of temperature. The results that have been obtained are not as expected on the basis of the classical hydrophobic paradigm of membrane partitioning. These results provide new insight into not only the partitioning thermodynamics but also the structure and dynamics of lipid bilayers.

*Partitioning Thermodynamics*. Our results demonstrate that while hydrophobic effects play a major role in the partitioning process, the process is considerably more complex than the simple classical hydrophobic model based on the partitioning of model compounds between water and bulk hydrocarbon solvents. We have found that there is a large heat capacity change upon alcohol partitioning into bilayers, which is recognized as the critical property of hydrophobic processes (*15*−*17*). However, there are several deviations from the classical hydrophobic effect. Like others, we have found a large negative enthalpy for some compounds. However, since we have the additional information of the alcohol chain length dependence we have also found a large dependence of the enthalpy on the alcohol chain length, which cannot be explained by the hydrophobic effect, so that the enthalpy is large and negative for the longer alcohols at high temperatures, but positive for smaller alcohols at lower temperatures. Also inconsistent with a purely hydrophobic partitioning is the finding that the heat capacity change and the entropy change are not dependent on the size of the alcohol. Finally, we have observed the reduction of partitioning free energy caused by the addition of cholesterol into the bilayer which has been observed by others (*19*). However, by doing direct independent measurements of the enthalpy of binding we have found that the change in free energy due to the presence of cholesterol is the resultant of much larger changes in the contributions of enthalpy and entropy to the partitioning free energy. There is no ready interpretation of these observations within the paradigm of bilayer partitioning as being modeled by partitioning between water and bulk hydrocarbon solvent.

The large negative enthalpy increment of increasing chain length, as well as the large positive changes in the enthalpy of partitioning in the presence of cholesterol suggests that van der Waals interactions play a major role in lipid interactions with amphiphiles, as originally suggested by Seelig and Ganz (*21*). However, our results emphasize that it is the lipid−lipid interactions which probably contribute more to these changes than the solute−lipid interactions. The large contribution of enthalpy changes to partitioning thermodynamics indicates that the packing of the lipids is an important membrane property which is perturbed by partitioning of alcohols. The shorter-chain alcohols have positive enthalpy of partitioning, indicating that the total van der Waals interaction energy is reduced by partitioning, while the longer-chain alcohols have a negative enthalpy of partitioning, indicating that they enhance the lipid−lipid interactions and participate in them.

Cholesterol changes the thermodynamics of partitioning by an increase in both the positive enthalpy and positive entropy contributions. This is consistent with the known effects of cholesterol on lipid packing of bilayers, in making them more rigid and increasing the surface density (*18*, *19*). In this system, the alcohols disrupt the packing as indicated by the positive enthalpy contribution, reducing the total van der Waals energy to a greater extent than in the absence of cholesterol. The greater positive entropy change suggests a greater increase in configurational entropy, as well as a greater decrease in exposure of nonpolar moieties to water, compared to the system in the absence of cholesterol. Thus, the total cholesterol effect is a relatively small reduction in partitioning free energy which is the resultant of much larger changes in enthalpy and entropy.

The thermodynamics of partitioning of the series of alcohols is also characterized by a large negative change in heat capacity; this is the primary characteristic of hydrophobic processes (*15*−*17*). It indicates that partitioning is accompanied by the dehydration of nonpolar moieties. The presence of cholesterol causes the change in heat capacity for partitioning to be much larger than that without cholesterol. Both with and without cholesterol, the heat capacity change does not increase with the size of the alcohol. These observations together indicate that there is significant contribution of changes in water exposure of lipid moieties involved in the partitioning. The much larger heat capacity change for alcohol partitioning in the presence of cholesterol is consistent with the larger membrane area per phospholipid head group and the consequent greater amount of water exposure of lipid moieties in the presence of cholesterol (*36*−*38*). The greater effect of alcohol binding to reduce this exposure is probably due to some direct shielding of hydrophobic moieties as well as to the general change in lipid−lipid interactions in the bilayer.

Our results indicate that the lipid packing in the bilayer has a profound effect on the thermodynamics of alcohol partitioning. The finding by Beschiaschvili and Seelig (*2*) that the size of vesicles can also make a large change in the thermodynamics of partitioning is in some ways analogous to the effect of cholesterol. They found that the partitioning of a cyclic peptide has a large negative enthalpy for partitioning into sonicated vesicles but a nearly zero enthalpy for the larger vesicles. In contrast, the free energy of partitioning was almost independent of vesicle size. It is well-known that the lipid packing in the limiting-size small vesicles formed by sonication is different from that in relaxed larger vesicles. The difference in packing and surface curvature can also be expected to lead to a difference in the water structure in the interfacial region in these bilayers, contributing to the large differences in the enthalpy−entropy balance of partitioning observed by Beschiaschvili and Seelig (*2*). This would be expected to be reflected in large heat capacity changes; however, they did not measure the change in heat capacity in their system.

*Implications for Membrane Structure*. The results obtained in the present study demonstrate the limitations of the paradigm of the lipid bilayer as being well modeled by bulk hydrocarbon solvents and of partitioning of small molecules as being well modeled by the partitioning of model compounds between water and bulk hydrocarbon solvents. As pointed out by DeYoung and Dill (*18*−*19*) in their studies of the effects of surface density on partitioning, the interfacial region has a whole host of unique properties which are not analogous to those of either aqueous solution or bulk

hydrocarbon. Moreover, that portion of the bilayer which may be somewhat similar to a bulk hydrocarbon phase is not isotropic; it clearly has orientational properties which vary with distance from the surface, lipid composition, temperature, and the presence of dissolved solutes.

The conclusion that the structure and energy of the lipid bilayer are complex is supported by the recent studies of rhodopsin by Litman and his colleagues (*38*−*41*), who have extensively investigated the relationships between the physical properties of the lipids and the functional properties of rhodopsin in the natural lipids and in reconstituted systems. They have developed a lipid structural parameter $f_v$ based on multiparameter analysis of time-resolved DPH fluorescence, which is a measure of the "free volume" within the lipid bilayer. They have measured this for a variety of lipid compositions, including cholesterol, and correlated this property with the MI to MII conformational transition of the membrane receptor rhodopsin, which is involved in its mechanism of action. This work shows that there are significant variations in the lipid properties as seen by DPH and reflected in the rhodopsin conformational transition as a function of lipid composition and cholesterol content.

Our results indicate that there are significant changes within the lipid bilayer upon partitioning of alcohols. Litman and co-workers have studied the effect of *n*-alcohols from ethanol to decanol in their system (*42*, *43*). They found that ethanol increases the $f_v$ parameter significantly, correlated with a shift in the MI−MII equilibrium of rhodopsin in favor of the larger-volume MII conformation. There is a progressive change in the effects of the alcohols with chain length, and they found that as the alcohols increase in chain length, the effect on the $f_v$ parameter, and the MI−MII equilibrium, decreases. For hexanol and above, the effect is opposite to that of the shorter-chain alcohols; the longer-chain alcohols decrease the MI−MII equilibrium and decrease the $f_v$ parameter. These results demonstrate that increasing the alcohol chain length has more effect than merely increasing the nonspecific interactions which would be relevant in the bulk hydrocarbon model of the membrane. We have found that the enthalpy of partitioning is strongly dependent on the alcohol chain length, with the shorter-chain alcohols having a positive enthalpy of binding, suggesting a disruption of membrane interactions, whereas the longer ones have a negative enthalpy, suggesting an increase in membrane interactions with participation by the alcohols in those interactions. Our results are consistent with those of Litman and co-workers. However, direct comparison of the alcohol chain length at which the crossover in the sign of the enthalpy is observed with that at which the effect on the MI−MII equilibrium reverses is not possible because of the different temperatures and lipid compositions used in the two studies.

Our results demonstrating the effects of alcohol partitioning on the bilayer are also consistent with NMR studies of ethanol interactions with PC's showing that ethanol decreases the order of the lipid in the hydrophobic core, even though its direct interactions are predominantly in the interfacial region (*44*). The same approach showed that cholesterol compositions below 22% enhanced the disordering effect of ethanol (*45*). Our conclusion that there are changes in water-exposed lipid moieties in the interfacial region is supported by the recent report that the water content in the interfacial region of DOPC bilayers increases with binding of alcohols, proportional to the size of the alcohol (*46*).

*Conclusion*. The results we have reported are consistent with a paradigm of membrane structure in which the lipid packing and the interfacial water structure have a major role in the regulation and control of the membrane properties. Our results demonstrate the large effects of changing the thermodynamic state of the bilayer by the introduction of cholesterol on the thermodynamics of partitioning of small molecules.

The thermodynamics of lipid bilayers have been discussed previously by Gruner and colleagues in terms of the intrinsic curvature of bilayers (*1*). They have suggested that there is an inherent energy stored in the bilayer due to the presence of non-bilayer-forming lipids and proposed that it is a critical regulated biological property. Seelig and Beschiaschvili have also been concerned with the thermodynamic state of the bilayer in their discussion of the observation that the enthalpy of binding of a cyclic peptide to lipid vesicles was dependent on the vesicle size, with compensating effects on the entropy and little effect on the free energy (*2*). They have shown from thermodynamic principles that the plasticity of the bilayer with respect to changes in bilayer area (with compensating changes in thickness) lead to compensating changes in the entropy and enthalpy of bilayers; they have suggested that the bilayer is a reservoir of "entropy potential" (*2*). Both of these proposals involve the concept that the inherent thermodynamic state of the bilayer is an important biological property.

Our measurements of alcohol partitioning reveal large effects on the partitioning thermodynamics caused by cholesterol, also suggesting the importance of the bilayer thermodynamic state. Litman and co-workers' studies of rhodopsin demonstrate the capacity of changes in these thermodynamic states, for example due to partitioning of alcohols, to affect biological function. If this inherent thermodynamic status of lipid bilayers is a critical regulated property, our results suggest that the regulation of bilayer properties, and thus of biological function, is a property not only of the lipid composition but also of the milieu of small molecules, both natural and environmental, with which they come into contact.

From a practical point of view, our studies suggest that direct thermodynamic measurements using titration calorimetry of partitioning can be used to probe the thermodynamic state of the bilayer as well as the effects of various solutes on this state. In particular, it will be very interesting to determine if there is a systematic effect of non-bilayer-forming lipids on partitioning thermodynamics. These studies are in progress in our laboratory.

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BI9721602