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CAN REGULAR SOLUTION THEORY BE APPLIED TO LIPID BILAYER MEMBRANES?

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Summary

Direct measurement of the partition coefficient of *n*-hexane into phosphatidylcholine and phosphatidylcholine-cholesterol bilayers showed that (a) isotropic liquids are not good models for lipid bilayers and (b), Regular Solution Theory cannot, in general, be applied to lipid bilayer membranes at temperatures above their phase transition. Theoretical and experimental evidence is given.

Introduction

The solubility and mixing properties of molecules with lipid bilayers and biological membranes is a subject that recently has evoked much interest [1—6]. This is because the interaction of well characterized molecules with solvents two molecules thick, such as lipid bilayers, present problems that are of interest to both physical chemists and physiologists, as many of these well characterized molecules are drugs. In this context it is of interest to ascertain whether theories of mixing that were found to be predictive in many other systems can be applied to lipid bilayers (or biological membranes).

In this paper we will examine the applicability of Regular Solution Theory to lipid bilayer membranes. Regular Solution Theory has been found most useful for predicting the mixing properties of gases and liquids (usually nonpolar molecules) with isotropic liquids, polymers and even monolayers [7]. This theory has been found to be applicable beyond its original assumptions [8]. This is perhaps most evident where Regular Solution Theory has been successfully applied to asymmetrical amphiphilic molecules that are arranged

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as a monolayer at an air-water interface [9]. The applicability of Regular Solution Theory to bilayers and biological membranes has previously been suggested in regard to mechanisms of anesthesia [10]. However, the assumptions regarding its use to these anisotropic materials has not yet been justified in any rigorous manner. The lipid bilayer by virtue of its extreme thinness and geometrical arrangement has been found to possess many solvent properties that are not found generally in other solvent systems [6,34]. We find that the inherent anisotropic nature of the lipid bilayer, combined with its extreme thinness, make it impossible to specify a solubility parameter to it that will be applicable to all normal solutes as is the case for isotropic liquids.

Theory

In 1970 Davis [11] derived an equation that relates the partition coefficient of a solute between two immiscible liquids (one of them being water) to the solubility parameters of the solute (i) and solvent (j) phases. The equations were derived from the famous Scatchard-Hildebrand equation that gives an expression for the excess free energy, $\overline{F}^{E}$, of a two component regular solution at constant temperature

$$\overline{F}^{E} = (X_1 V_1 + X_2 V_2)(\delta_1 - \delta_2)^2 \phi_1 \phi_2 \tag{1}$$

where X_i, V_i, ϕ_i are the mol fraction, molar volume and volume fraction of component i, respectively. δ_1 is the solubility parameter of component 1 and $\delta_1 = (-E_1^v/V_1)^{1/2} \approx (\Delta H_1^v - RT/V_1)^{1/2}$ where $-E^v/V$ is the cohesive energy per unit volume of the liquid and ΔH^v is the heat of vaporization of liquid. The solubility parameter is a semi-empirical constant that characterizes the liquid [8]. The molal free energy of a solute (i) in a solvent (1) in the limit of infinite dilution, $\phi_1 \to 1$, is simply

$$RT \ln \gamma_{i\infty} = V_i(\delta_1 - \delta_i)^2 \tag{2}$$

Thus the partition coefficient between two liquids, one of which is water, can be written (in concentration units) as

$$\ln K = \frac{V_i}{RT}[(\delta_a - \delta_i)^2 - (\delta_o - \delta_i)^2] + \ln \frac{V_a}{V_o} \tag{3}$$

where V_o represents the volume of the 'oil' phase, δ_a the solubility parameter of the aqueous phase and V_i the volume of solute i.

Recently, however, Srebrenik and Cohen [12] refined the above analysis so that a more accurate prediction of K could be obtained. They did this by allowing for the change in partial molal volume that occurs when a solute is dissolved in a solvent. This eliminated the need to use the solubility parameter of water which is ambiguous [11]. The equation they derived is written

$$\ln K = \frac{V_i}{RT}[(\delta_1 - \delta_i)^2 - (\delta_2 - \delta_i)^2] + (\overline{V}_{i1} - V_i)\left(\frac{\delta_1^2}{RT} - \frac{1}{V_1}\right)$$
$$- (\overline{V}_{i2} - V_i)\left(\frac{\delta_2^2}{RT} - \frac{1}{V_2}\right) - V_i\left(\frac{1}{V_1} - \frac{1}{V_2}\right) \tag{4}$$

$\overline{V}_{ij}$ is the partial molal volume of solute i in solvent j. $j = 1,2$.

The above authors have shown that the correction terms can account for about 10%. The above equation can be put in a more tractable form (as the partial molal volumes of almost all molecules have not been measured in bilayer systems) by using the equation derived by Hildebrand et al. [7] that relates the difference in molar volume to the partial molal volume:

$$\overline{V}_{ij} - V_i = V_i n_j \beta_j (\delta_j - \delta_i)^2 \tag{5}$$

where β_j is the isothermal compressibility of liquid j and $n_j = (\partial E_j/\partial V_j)_T/(\Delta E_j/V_j)$ which experimentally results at approximately unity for most liquids [7].

Eqn. 5 shows that there is an increase in volume for systems that obey regular solution theory as all the terms on the right hand side are positive. Substituting Eqn. 5 into Eqn. 4 with the unnecessary, but physically reasonable, assumptions (especially for bilayer) [13] that $V_1 = V_2 = V$, $\beta_1 = \beta_2 = \beta$, $n_1 = n_2 = n$ we obtain

$$\ln K = \frac{V_i}{RT}\left[\left(1 + \frac{nV_n\beta\delta_1^2}{V_i}\right)(\delta_1 - \delta_i)^2 - \left(1 + \frac{nV_n\beta\delta_2^2}{V_i}\right)^2 (\delta_2 - \delta_i)^2\right] \tag{6}$$

If $n \approx 1$ then

$$\ln K = \frac{V_i}{RT}\left[\left(1 + \frac{V\beta\delta_1^2}{V_i}\right)(\delta_1 - \delta_i)^2 - \left(1 + \frac{V\beta\delta_2^2}{V_1}\right)(\delta_2 - \delta_i)^2\right] \tag{7}$$

where it is easy to show that for most liquids where $\delta^2 = 3 \cdot 10^3$ atm [8], $\beta = 5 \cdot 10^{-5}$ atm^{-1} [14] the ratio $V\beta\delta_2^2/V_1$ is approx. 0.1 or about a 10% difference between Eqn. 7 and Eqn. 3. This form is useful in that the compressibility for some bilayer membranes has been measured [15].

What we did, therefore, was to measure the partition coefficient of n-hexane into well characterized bilayers of various compositions in the presence and absence of cholesterol and show that the differences in δ values obtained do not reflect the polarity of the solvent, but instead, primarily reflect the reduced entropy of the acyl chains of the lipid.

Furthermore, we set out to show that the predictive value of Eqn. 1, which states that the more similar the two liquids are to each other the more ideally they will mix, is unreliable in bilayer systems of a given acyl chain length where it is found that the closer the acyl chain length is to the alkane chain length, the less ideal the mixture to the extent that phase separation takes place [25,26]. Thus, there is not a single δ value that can characterize a bimolecular membrane.

Finally we show that when using usual methods for calculating δ values from thermodynamic parameters (i.e. $\delta \approx (T\alpha/\beta)^{1/2}$); the δ values for bilayers come out extremely low to preclude physical meaning or comparison with the apparent solubility parameters obtained from partition coefficient measurements of isotropic liquids.

Materials and Methods

Soybean phosphatidylcholine was purchased from Avanti Biochemicals (Alabama, U.S.A.) and used as purchased. Dimyristoyl phosphatidylcholine was

purchased from Supelco Inc. (Bellfonte, Pa.). Both lipids were checked for purity with thin layer chromatography where they migrated as a single spot and microelectrophoresis [16], where the drift was considerably greater than the lateral movement in the presence of a field of 10 V/cm. Cholesterol was obtained from the Applied Science Laboratory and was used as purchased. Water used was doubly distilled in an all quartz still. Sodium chloride was roasted at 600°C for 24 h to remove organic impurities. The *n*-[1-^{14}C]hexane was purchased from New England Nuclear. The radioactive hexane was diluted with ultrapure hexane and its specific activity determined.

The lipids were stored in chloroform under argon at −20°C until ready for use. Vesicles were prepared by evaporating the chloroform under reduced pressure and then adding 0.1 M NaCl to give a concentration of approx. 10 mg/ml. The dispersion was then gently vortexed until uniform composition was observed. When the lipids were mixed with cholesterol, both components were initially dissolved in chloroform and the procedure above was repeated.

The partition coefficient of *n*-hexane between 0.1 M NaCl (hereafter referred to as water) was measured in a gas solubility cells as described by Wishnia and Pinder [17]. The description and sampling procedure are described elsewhere [6,18].

In these experiments, reproducible results were obtained upon heating and cooling once a fixed amount of *n*-hexane was injected into the gaseous phase. In all experiments, unless otherwise specified, we attempted to keep the amount of radioactivity in the gas phase constant. However, as pressure of hexane in the gas phase was not strictly fixed, the activity coefficients of hexane in the water and bilayer are not known. In this paper, they were assumed to be unity following the procedure outlined in refs. 17 and 32. We have determined here and elsewhere [6] that the ratio of the activity coefficients are constant at a given temperature.

Results

The partition coefficients (mol fraction units) as a function of temperature between aqueous dispersions of soybean-phosphatidylcholine and soybean-phosphatidylcholine-cholesterol (2 : 1 and 1 : 1) are shown in Fig. 1. In all three disperions the partition coefficient was found to be independent of concentration of *n*-hexane in the aqueous phase until saturation of *n*-hexane was reached. This implies that the activity coefficient is a constant and that we are operating in the Henry's Law region [19]. Note also that the temperature dependence of the partition decreases as the temperature increases in all three dispersions.

If the molal volume of hexane and the hydrocarbon region is equal to the molar volume of each of these components then at saturation of hexane in the aqueous phase we may calculate the volume fraction occupied of *n*-hexane in the bilayer assuming that the volume of the acyl region of soybean-phosphatidylcholine is the same as that of egg phosphatidylcholine of 640 cm^3/mol [13]. Under these circumstances the volume fraction of hexane in the bilayer is about 0.04. Thus, 4% of the membranes volume could be occupied by *n*-hexane under these circumstances. The temperature dependence of the partition coeffi-

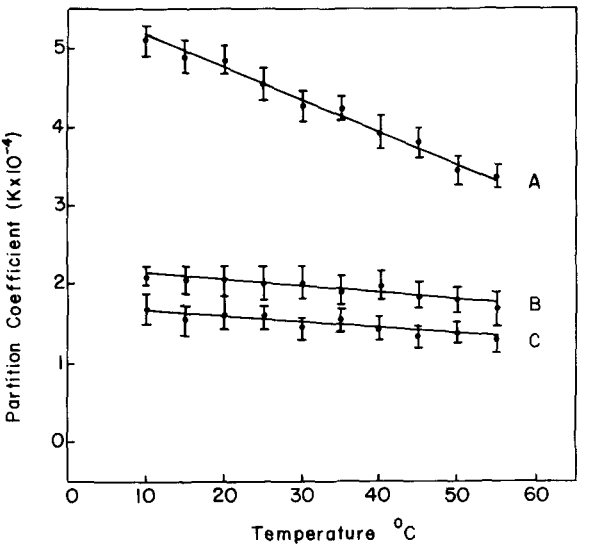

Fig. 1. The partition coefficient K, as a function of temperature of n-hexane from water to dispersions of soybean phosphatidylcholine (A), soybean phosphatidylcholine-cholesterol (2 : 1) (B), soybean phosphatidylcholine-cholesterol (1 : 1) (C). The solid lines refer to Eqns. 8, 9 and 10 in the text.

cient in the range 10—55° C is given by the following equations.

Soybean phosphatidylcholine

$$K = 5.62 \cdot 10^4 - 424\,T \quad r^2 = 0.98 \tag{8}$$

Soybean phosphatidylcholine-cholesterol (2 : 1)

$$K = 2.22 \cdot 10^4 - 79\,T \quad r^2 = 0.97 \tag{9}$$

Soybean phosphatidylcholine-cholesterol (1 : 1)

$$K = 1.72 \cdot 10^4 - 66.6\,T \quad r^2 = 0.97 \tag{10}$$

where the temperature is given in degrees Celsius.

The partial molal energy $\Delta\overline{G}$, enthalpy $\Delta\overline{H}$, and entropy $\Delta\overline{S}$, of transfer of n-hexane from water into these various bilayers may be obtained in a straightforward manner by using the equation

$$\Delta\overline{G} = -RT \ln K = \Delta\overline{H} - T\Delta\overline{S} \tag{11}$$

along with the Gibbs-Helmholtz equation.

The values of these numbers are presented in Table I along with other thermodynamic data for the transfer of n-hexane into other liquids.

The membrane water partition coefficient of n-hexane from water into dimyristoyl phosphatidylcholine is shown in Fig. 2. The abrupt change seen in the partition coefficient at about 20°C is the well known phase transition that occurs in this saturated lipid at 23°C [3]. The experiments done with dimyristoyl phosphatidylcholine were generally performed with very dilute

TABLE I

THERMODYNAMIC TRANSFER PARAMETERS [a] OF *n*-HEXANE FROM WATER [b] TO VARIOUS SOLVENTS AT 25°C

Solvent	$\overline{\Delta G}$ (kcal/mol)	$\overline{\Delta H}$ (kcal/mol)	$\overline{\Delta S}$ (cal/mol per degree)	References
Soybean phosphatidylcholine	−6.35 ± 0.03	−1.6	15.9	This work
Soybean phosphatidylcholinecholesterol (2 : 1)	−5.87 ± 0.03	−0.7	17.3	This work
Soybean phosphatidylcholinecholesterol (1 : 1)	−5.71 ± 0.03	−0.75	16.6	This work
Dimyristoyl phosphatidylcholine	−6.33 ± 0.03	−2.2	13.9	This work
Dioleoyl phosphatidylcholine	−6.18 ± 0.04	−1.7	15.0	[6]
Egg phosphatidylcholine	−6.65 ± 0.04	−2.2	14.8	[6]
Sodium dodecyl sulfate	−6.56 ± 0.04	−2.1	15.0	[6]
n-Hexane	−7.74	0.0	26.0	[32,33]
Octanol	−5.42			[31]

[a] Unitary free energy units.
[b] By water we refer to 0.1 M NaCl.

n-hexane solutions in the aqueous phase to minimize the freezing-point depression effect that occurs when hexane is mixed with saturated lipids [20]. The change in the partition coefficient with temperature from 24 to 40°C is best described by the equation

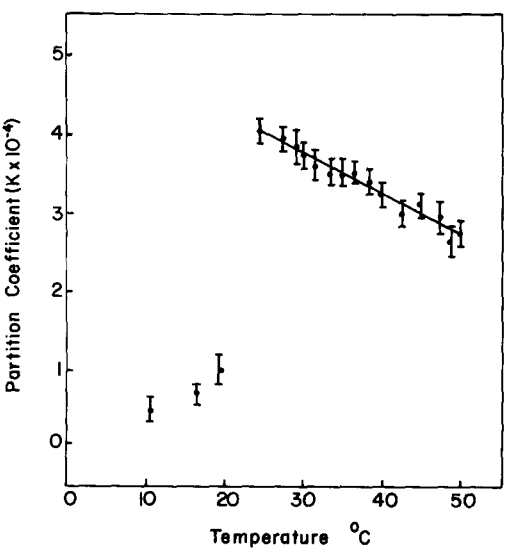

Fig. 2. The partition coefficient K, as a function of temperature of *n*-hexane into dispersions of dimyristoyl phosphatidylcholine. The solid line refers to Eqn. 12 in the text.

Dimyristoyl phosphatidylcholine

$$K = 5.27 \cdot 10^4 - 507\,T \quad r^2 = 0.97 \tag{12}$$

The values of the partial molal free energies enthalpy and entropy of transfer of *n*-hexane dimyristoyl phosphatidylcholine at 25°C are given in Table I. It is noteworthy that the partition coefficient of *n*-hexane was found within experimental error to be independent of the aqueous concentration of *n*-hexane at 30°C.

A comparison of the partition coefficients of *n*-hexane for soybean-phosphatidylcholine-cholesterol dispersions with that of dimyristoyl phosphatidylcholine at 20°C show that these two systems have similar partial molal free energies of transfer. The incorporation of cholesterol into soybean-phosphatidylcholine liposomes reduces the *n*-hexane partition coefficient by a factor of 2.7 for 2 : 1 mol ratios and about 3.5 for a 1 : 1 mol ratio. The effect of cholesterol on reducing the partition coefficient is reduced as the temperature is increased. For example at 50°C cholesterol reduces the partition coefficient by 2.5 at 2 : 1 mol ratios and 3.2 at 1 : 1 mol ratios.

Discussion

From the data in Table I it is evident that the thermodynamic transfer parameters of *n*-hexane going from water into various liquids are significantly different if the liquids are isotropic organic, lipid bilayers with or without cholesterol or micelles. The reasons for this difference have been explained elsewhere [6] but basically bilayers are different than isotropic liquids in that they significantly decrease the entropy of transfer. From the enthalpy of transfer differences it was argued that on the average the *n*-hexane was aligned parallel to the acyl chains.

The approach we are using is to find the apparent solubility parameter of a membrane and, if it exists, to determine its physical significance. From Table I we may calculate the differences in free energy of *n*-hexane in going from *n*-hexane to any of the other systems. As a check for the essential correctness of the analysis we shall use the value of the differences in free energy of transfer of *n*-hexane from *n*-hexane to octanol to calculate the value of δ octanol and then compare it to the literature value [8] of $\delta = 10.3\ (\text{cal/cm}^3)^{1/2}$.

Under these conditions Eqn. 7 may be written

$$\Delta\overline{G} = RT \ln K = V_{\text{hexane}} \left[\left(1 + \frac{V_{\text{octanol}}\beta_{\text{octanol}}\delta^2_{\text{octanol}}}{V_{\text{hexane}}}\right) \times \right.$$

$$\left. \times\ (\delta_{\text{octanol}} - \delta_{\text{hexane}})^2\right] \tag{13}$$

Inserting the appropriate values of the constants in the above equation $\delta_{\text{hexane}} = 7.3\ (\text{cal/cm}^3)^{1/2}$, $V_{\text{hexane}} = 132\ \text{cm}^3/\text{mol}$, $V_{\text{octanol}} = 157\ \text{cm}^3/\text{mol}$ and $\beta_{\text{octanol}} = 7.05 \cdot 10^{-5}\ \text{atm}^{-1}$. We find from the above that $\delta_{\text{octanol}} = 10.62\ (\text{cal/cm}^3)^{1/2}$ in comparison to the literature value of 10.3. This amounts to a 3% error. In a similar manner we may calculate the value of δ_{membrane} if some addi-

tional assumptions are made. (1) For physical reasons we will assume that the hydrocarbon is the region into which the *n*-hexane partitions and that it has a volume of 640 cm^3/mol (the same as egg phosphatidylcholine) [13] and (2) that the isothermal compressibility, as measured for dipalmitoyl phosphatidylcholine above its transition temperature, is $13.6 \cdot 10^{-5}$ atm^{-1}, which is the same for single component bilayers above their transition temperature [15]. Under these conditions the value of δ for soybean phosphatidylcholine from Eqn. 13 is 9.4 $(cal/cm^3)^{1/2}$, which is also similar to the value obtained for dimyristoyl phosphatidylcholine above its transition temperature.

In the case of the soybean-phosphatidylcholine-cholesterol dispersions we assume the bilayer compressibility to be $1 \cdot 10^{-5}$ atm^{-1}. Although no data are available, numerous studies have shown that cholesterol makes bilayers above their transition temperature less compressible and we feel a factor of 10 is not unreasonable [21]. For the soybean-phosphatidylcholine-cholesterol 2 : 1 dispersion, a bilayer volume of 586 cm^3/mol is calculated from which a δ value for this dispersion is found to be 11.3 $(cal/cm^3)^{1/2}$. This value is very close to the solubility parameter of *n*-butanol [19] (δ = 11.4 $(cal/cm^3)^{1/2}$). The value for δ of a 1 : 1 egg phosphatidylcholine-cholesterol dispersion is 14.1 $(cal/cm^3)^{1/2}$, which is similar to the δ value of methanol (14.6 $(cal/cm^3)^{1/2}$) [8].

If we now examine the predicted solubility behavior of *n*-alkanes with bilayers free of cholesterol ($\delta \approx 9.3$ $(cal/cm^3)^{1/2}$) it is predicted that the solubility of the longer chain alkanes should be greater than those of the shorter chain alkanes. This should occur for two reasons. (1) The enthalphy of mixing term should become smaller, (2) the entropy of mixing should become more or less ideal as the chain lengths approach each other [8]. In fact, experiments that measure the solubility of alkanes in planar bilayers [25,34] (above their transition temperature) clearly demonstrate that as the acyl chain lenght of the alkane approaches that of the amphiphile, these two components become less miscible with each other to the extent that complete immiscibility between hydrocarbon and amphiphile was observed when squalene was mixed with bilayers [26].

The reason for this apparent contradiction is seen in Table I. Here it is clear that the reason for this effect is the great reduction in entropy of transfer that a molecule like hexane experiences in going from water to an isotropic liquid as compared to a lipid bilayer. This effect gets larger the larger the acyl chain length of the alkane. When cholesterol is added to the lipid dispersion, the apparent solubility parameter of the mixture increases, not because the bilayer interior becomes significantly more polar (as seen from electron density profiles [27]) but rather that the enthalpy and entropy of transfer combine to reduce the free energy of transfer.

In this regard it is not correct to say that the membrane interior behaves like *n*-butanol or other isotropic liquids [22]. When molecules such as small non-electrolytes [23] or gases [24] of a volume smaller than *n*-hexane partition into a pure bilayer it is found, in some cases, that the partition coefficient may be very similar to that of an isotropic liquid. The reason for this is fortuitous as there is a cancellation of entropic and enthalpic contributions to the free energy of transfer [22].

Hildebrand has shown how it is possible to estimate the solubility parameter

of a liquid from the internal pressure, $(\partial E/\partial V)_T$. The analysis show that

$$\delta \approx \left[T\left(\frac{\partial P}{\partial T}\right)_V\right]^{1/2} = (T\alpha/\beta)^{1/2} \tag{14}$$

where α, the thermal expansion coefficient, $= 1/V_o\ (\partial V/\partial T)_P$

and $\beta = 1/V_o\ (\partial V/\partial P)_T$

The values of α for bilayers above their transition temperature as measured by dilatometry is $4 \cdot 10^{-4}\ °C^{-1}$ [29]. Using the value of β of $1.36 \cdot 10^{-4}$ atm^{-1} [15], at 298 K a value of δ for a cholesterol free bilayer above the transition temperature is 4.6 $(cal/cm^3)^{1/2}$. The reason that this appears to be such a low number is that the bilayer is inherently anisotropic so that for a bilayer, with a fixed number of molecules, any changes in area will be compensated by a change in thickness [30]. Thus, if the area increases with temperature the thickness decreases by the same amount, which is an order of magnitude larger than the change in volume with temperature.

It has been experimentally found that

$$\frac{1}{A_0}\left(\frac{\partial A}{\partial T}\right)_{\overline{T}} = -\frac{1}{L_0}\left(\frac{\partial L}{\partial T}\right)_{\overline{T}} \approx -2 \cdot 10^{-3}\ °C^{-1}$$

where A is the bilayer area, L the bilayer length and $\overline{T}$ the bilayer tension.

Thus, there is an internal inconsistency in attempting to calculate the solubility parameter of a bilayer of a single component (in addition to water of course) via the prescribed method that is used for isotropic liquids.

Next we would like to point out that the differences in free energy of a particular group (i.e. CH_2) is not a constant in bilayers but depends on the types of molecules being considered. For example Stone [18] measured the free energy of transfer between *n*-hexane and *n*-octane and found $\Delta\overline{G}(CH_2) = -1000$ cal/mol. Similarly Katz and Diamond [22] measured the free energy of transfer between methanol and ethanol and found $\Delta\overline{G}(CH_2) = -450$ cal/mol.

The reason for the difference is that the alkanes partition into a different region of the bilayer than the small alcohols. The former partition 'deep' in the interior whereas the latter are closer to the aqueous phase. In this context, molar attractive constants [11] or Hansch π values [31] that have been so useful in predicting the partition efficients in isotropic liquids must be used with caution in lipid bilayers.

The differences in partial molal free energy of transfer of *n*-hexane from water to cholesterol-free dispersion of soybean-phosphatidylcholine-egg phosphatidylcholine, sodium dodecyl sulfate (SDS), dioleoyl phosphatidylcholine are not very great and such that the free energy of transfer of *n*-hexane is essentially independent of the length and charge of the amphiphile whether it be in an SDS micelle (C_{12}) or a dioleoyl phosphatidylcholine bilayer (C_{18}). These results, however, are significantly different than the free energy of transfer of *n*-hexane going from water to *n*-hexane or octanol. As stated elsewhere, this discrepancy occurs because *n*-hexane is more immobilized in these bilayer systems than in an isotropic bulk hydrocarbon liquids [6]. What is somewhat surprising is that the entropy of transfer of *n*-hexane from water to cholesterol-containing bilayers is greater than in cholesterol-free bilayers. The

enthalpy of transfer is also reduced. This could be explained, in part, if the hexane principally partitioned in the region between the end of the steroid nucleus (8 Å from the carbonyl moiety) to the geometric center of the bilayer [27]. This region, as shown by spectroscopic techniques, is considerably more 'fluid' than the regions closer to the aqueous interface and may indeed be more representative of an isotropic liquid [32].

In this regard it is interesting that the soybean-phosphatidylcholinecholesterol 2 : 1 and 1 : 1 bilayers have the same enthalpy of transfer but different free energies of transfer suggesting, as Gershfield [35] pointed out, that above a 2 : 1 mol ratio cholesterol may exist in the bilayer as a separate phase that is unavailable for partition of *n*-hexane.

In summary we suggest that Regular Solution Theory is not, in general, a useful starting point for describing the miscibility of non-polar molecules with lipid bilayers (or biological membranes). This is because a particular value of the membrane solubility parameter cannot be used to describe the solubility of even the simple molecules like gases and alkanes. The addition of a second component to the bilayer such as cholesterol causes these deviations to be even more pronounced.

Acknowledgements

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