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Thermodynamics of alcohol–lipid bilayer interactions: application of a binding model

Peter Westh [a,b,*], Christa Trandum [b]

[a] *Department of Life Sciences and Chemistry, Roskilde University, P.O. Box 260, DK-4000 Roskilde, Denmark*

[b] *Department of Chemistry, Technical University of Denmark, DK-2800 Lyngby, Denmark*

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Abstract

Several recent reports have provided evidence that interactions of small alcohols with lipid bilayer membranes are dominated by adsorption to the membrane–water interface. This mode of interaction is better modeled by binding models than solution theories. In the present study, alcohol–membrane interactions are examined by applying the 'solvent exchange model' [J.A. Schellmann, Biophys. Chem. 37 (1990) 121] to calorimetric measurements. Binding constants (in mole fraction units) for small alcohols to unilamellar liposomes of dimyristoyl phosphatidylcholine were found to be close to unity, and in contrast to partitioning coefficients they decrease through the sequence ethanol, 1-propanol, 1-butanol. Thus, the direct (intrinsic) affinity of the bilayer for these alcohols is lower the longer the acyl chain. A distinction between binding and partitioning is discussed, and it is demonstrated that a high concentration of solute in the bilayer (large partitioning coefficients) can be obtained even in cases of weak binding. Other results from the model suggest that the number of binding sites on the lipid bilayer interface is 1–3 times the number of lipid molecules and that the binding is endothermic with an enthalpy change of 10–15 kJ/mol. Close to the main phase transition of the lipid bilayer the results suggest the presence of two distinct classes of binding sites: 'normal' sites similar to those observed at higher temperatures, and a lower number of high-affinity sites with binding constants larger by one or two orders of magnitude. The occurrence of high-affinity sites is discussed with respect to fluctuating gel and fluid domains in bilayer membranes close to the main phase transition.

Keywords: Binding constant; Partitioning coefficient; Surface adsorption; Hydrophobic drive; Direct affinity; Solvent exchange

1. Introduction

The influence of alcohols on the properties of lipid bilayers has been extensively investigated. The principal motivation for this work has been the potential of lipid bilayer membranes to serve as model systems for studies of various biological processes including permeability of the plasma membrane and molecular mechanisms of anesthesia. While it has been well established that monohydric alcohols bind or partition into lipid membranes, the location of the partitioned molecules remains to be fully elucidated. The analogy with oil–water partitioning has inferred that alcohols may be dissolved in the non-polar interior of the membrane, but an increasing number of results have suggested a mode of interaction in which small amphiphilic solutes compete with water for sites in the membrane–solvent interface [1–6]. Recently, important direct support for an interfacial

* Corresponding author.

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location of partitioned ethanol has been provided through the NMR studies of Gawrisch and coworkers, who concluded that ethanol was predominantly bound to the region near the glycerol backbone of the phospholipid [7,8]. This information is clearly of most immediate importance for the structural understanding of ethanol-membrane complexes, but it also raises interesting questions concerning the thermodynamics of the interaction.

Traditionally the outset for a thermodynamic treatment of alcohol-membrane interactions involves enumeration of the bound or partitioned molecules at a given bulk concentration of the alcohol. It should be recognized, however, that the division of solute molecules into 'bound' and 'free' sub-populations is extra-thermodynamic, and that the arbitrariness of this distinction becomes of significant practical importance in cases of weak binding [9,10]. A weakly bound complex is defined operationally through some experimental observable, and the thermodynamic description of complex formation relies on the model or 'reaction' chosen to describe the process. In the simplest and most commonly used approach, this reaction is the equilibrium between a solute (A) in the free and partitioned state

$$\mathrm{A(aq)} \leftrightarrows \mathrm{A(lip)}, \tag{1}$$

where (aq) and (lip) indicate respectively 'aqueous solution' and 'partitioned in the lipid bilayer'. The partitioning coefficient, K_p, is the ratio, at equilibrium, of the solute concentration in the two phases. The access to a thermodynamic treatment of the process is the expression for $\Delta G°$ noted in Eq. 2. The underlying assumptions for this equation are that A molecules are *dissolved* in the two phases and that their chemical potential can be expressed as $\mu_A^i = \mu_A^{\circ i} + RT\ln x_A^i$ where μ_A° is the standard chemical potential of the alcohol and x_A is the mole of alcohol defined as the number of moles of alcohol molecules divided by the total number of moles in the system. Superscript i identifies the solvent (water or lipid). At equilibrium:

$$\mu_A^{\circ lip} - \mu_A^{\circ aq} = \Delta G^\circ = -RT\ln(x_A^{lip}/x_A^{aq}) = -RT\ln K_p \tag{2}$$

Eq. 2 has been extensively utilized to discuss hydrophobic effects based on oil–water partitioning experiments [11], but it may have shortcomings in membrane partitioning studies [12]. For example, it ignores the size difference of the two types of solvent molecules (water and phospholipid). Attempts to account for this through the Flory–Huggins theory has recently been discussed [13] and incorporated into some experimental works [6,14]. In the light of the improved structural understanding of alcohol–membrane complexes it seems there are other fundamental limitations in the use of Eq. 2 as the starting point for a thermodynamic analysis. Hence, if A molecules are indeed arranged in a rather well defined way in the membrane–solvent interface, their entropy will not be appropriately accounted for – as is assumed in Eq. 2 – by the cratic entropy of an ideal solution of A in the total volume of the membrane. The translational freedom of an A molecule bound or adsorbed to the interface will be smaller than that of a dissolved A which is free to occupy any spatial location throughout the volume of the lipid phase. As a result, molecular interpretations of thermodynamic functions based on Eq. 2 will be ambiguous.

In an attempt to find a thermodynamic approach, which is in better accordance with the notion of 'nonspecific surface binding' [7,8] we examine here the application of a surface binding model as an alternative to Eq. 1.

2. Materials and methods

Large unilamellar vesicles were prepared from pure (99%) dimyristoylphosphatidylcholine (DMPC) purchased as powder from Avanti Polar Lipids (Birmingham, AL, USA). Unilamellar liposomes (0.1 μm) were produced by standard extrusion techniques [15]. Briefly, lipid samples, hydrated in saline buffer (pH 7.4, 10 mM phosphate, 137 mM NaCl and 2 mM KCl) and kept above the main transition temperature for more than 1 h, were extruded through two stacked polycarbonate filters. Ethanol (99.9% Merck, Darmstadt, Germany), 1-propanol (99.8%, Merck) and 1-butanol (99.9%, Merck) were used without further purification.

The interaction of alcohols with DMPC liposomes was investigated by isothermal titration calorimetry (ITC) according to procedures discussed in general elsewhere [16,17]. In the present work, a 2.5 ml sam-

ple (either pure buffer or a 50 mM liposome suspension), loaded into the calorimetric cell, was titrated with 30–50 small aliquots (3–5 μl) of pure alcohol. Since the amount of alcohol per aliquot, n_A, was small compared to the content of the calorimetric cell (ca. 3×10^{-5} moles alcohol vs. ca. 10^{-1} moles sample) the measured enthalpy change for each injection, ΔH_{inj}, signifies the partial enthalpy of the alcohol[1], H_A, in a given solution ($\Delta H_{inj}/n_A \approx dH/dn_A = H_A$, where H is the total enthalpy of the system). The enthalpy of liposome-alcohol interaction is reflected in the difference between the partial enthalpy of the alcohol in the two component (buffer+alcohol) system, ^2H_A, and that in the three component (buffer+alcohol+liposomes) system, ^3H_A. Thus, if the presence of liposomes induce an increase in H_A, i.e. $^3H_A > {}^2H_A$ (as is indeed the case) the liposome–alcohol interaction is endothermic.

Titration experiments were carried out at 26, 32 and 40°C and alcohol concentrations up to a mole fraction of about 0.015 (ca. 0.8 molal). The equipment used was a TAM 2270 calorimeter (Thermo-Metric, Järfälla, Sweden) equipped with a titration cell (type 2250) and a LUND precision injection system. This setup allows determination of the rather large mixing enthalpies (typically 100–500 mJ for each injection) with a precision of about 0.3%.

To determine activity coefficients of the three alcohols in aqueous solution the total vapor pressure of alcohol–water mixtures was measured at 25°C and 10–20 different compositions. The differential equipment used in this study provides a precision of 0.01 torr on the total pressure measurement and has been described in some detail earlier [18,19]. Partial pressures were derived from the total pressures by numerical procedures based on the Gibbs–Duhem equation [20,21].

[1] H_A is the excess partial molar enthalpy of the alcohol with respect to the pure liquid state. For convenience we will refer to H_A as the 'partial enthalpy of A' throughout.

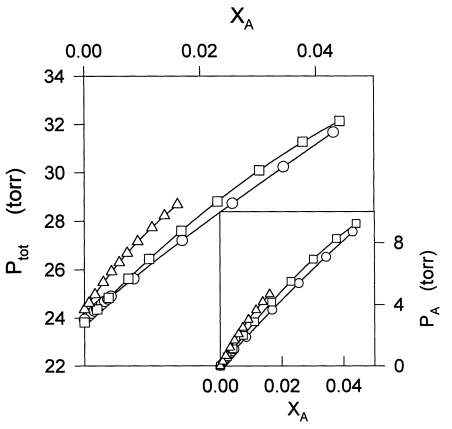

Fig. 1. Composition dependence of the total vapor pressures, P_{tot}, of aqueous mixtures of ethanol at 25.2°C (circles), 1-propanol at 25.2°C (squares) and 1-butanol at 25.4°C (triangles). Partial pressures of the alcohols are plotted as a function of the alcohol mole fraction, x_A, in the inset (symbols as in main figure).

3. Results

3.1. Activity coefficients

The measured total pressures of aqueous solutions of ethanol (EtOH), 1-propanol (PrOH) and 1-butanol (BuOH) are illustrated in Fig. 1; calculated partial pressures of the alcohols are plotted in the inset. The experimental temperatures were 25.2 ± 0.003°C for EtOH and PrOH and 25.4 ± 0.003°C for BuOH. Activity coefficients of the alcohols, γ_A, at 25°C are determined as $\gamma_A = p_A/x_A p_A^*$, where p_A and x_A are vapor pressure and (liquid) mole fraction of the alcohol and the asterisk indicates pure liquid (vapor pressures of the pure alcohols were measured separately). To estimate γ_A at other temperatures, the partial molar heat capacity, $C_{p,A}$, of each alcohol was estimated. This was done by plotting data from Figs. 3–5 (discussed below) to show ^2H_A as a function of temperature at selected alcohol concentrations. The slope of the resulting lines (not shown) is the partial heat capacity of the solutes, $C_{p,A} = d(^2H_A)/dT$. The obtained values of $C_{p,A}$ depend slightly on the composition; extrapolation to infinite dilution yielded 136, 211 and 259 J/mol K for EtOH, PrOH and BuOH. These values (in saline buffer) are consistently 12–14% less that those reported in pure water at 25°C [11]. The partial enthalpy of alcohol at a given temperature and concentra-

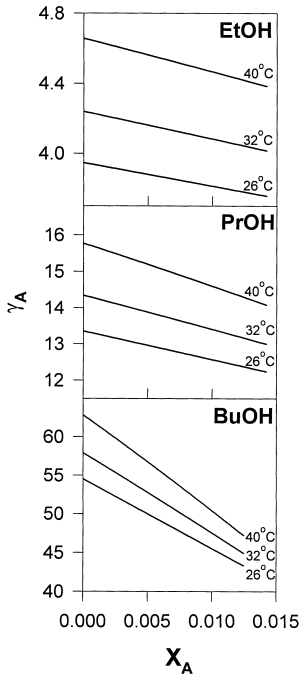

Fig. 2. Activity coefficients of ethanol (EtOH), 1-propanol (PrOH) and 1-butanol (BuOH) at the three experimental temperatures as a function of the alcohol mole fraction, x_A.

tion, ${}^2H_A(x_A,T) = {}^2H_A(x_A, 26°C)+(T-26°C)C_{p,A}(x_A)$, was then calculated and used to estimate γ_A in the x_A, T-field

$$\left(\frac{\partial \ln \gamma_A(x_A,T)}{\partial T}\right) = -\frac{H_A(x_a,T)}{RT^2}$$

Numeric solution of this equation yields the values of γ_A shown in Fig. 2.

3.2. Calorimetry

The partial enthalpies of the alcohols in respectively buffer (${}^2H_A$) and 50 mM DMPC solutions (${}^3H_A$) are shown in Figs. 3–5. As mentioned previously, the increased values of H_A observed in liposome solutions (${}^3H_A > {}^2H_A$) are indicative of endothermic alcohol-lipid interactions. It appears from Figs. 3–5 that this increase is dependent on the alcohol concentration. In the case of PrOH at 26°C, for example, the presence of 50 mM DMPC increases H_A (makes it less negative) by about 900 J/mol in the most dilute samples, but only by 450 J/mol when the mole fraction of alcohol is 0.015. For BuOH this effect is even more pronounced. This behavior is in accordance with a mode of interaction where adsorption sites are gradually saturated as the alcohol concentration increases.

A fundamental assumption for the further discussion of the data in Figs. 3–5 is that the enthalpy of the partitioning process is independent of the alcohol concentration. This implies that the difference (${}^3H_A - {}^2H_A$) between a set of curves is proportional to the number of alcohol molecules partitioned as the result of the addition of one alcohol aliquot. Under this assumption the calorimetric data in Figs. 3–5 in essence 'counts' the extent of alcohol uptake upon increasing concentration.

3.3. Model

The aim of modeling the calorimetric data is to extract intrinsic (microscopic) binding constants for the association of an alcohol molecule with a contact point or 'binding site' on the membrane surface. As will become apparent below these association constants are small ($k < 10$ on the mole fraction scale, i.e. < 0.2 in molal units). The thermodynamics of low-affinity binding has been discussed extensively by Schellman [22–25]. One of the essential results of this work is the introduction of the 'solvent exchange model' in which the traditional concept of ligand attachment has been replaced by a one-to-one exchange of water and ligand at the binding site. This model was shown to give meaningful results in cases where the interaction at the site was weakly attractive, or even repulsive. Applied to the present system, this approach assumes that a liposome (L) has n_{max} potential sites for alcohol association. In buffer each of these sites are occupied by one water molecule. During the titration trial, these water molecules are sequentially replaced one-for-one by alcohol. The first step of this sequence can be written:

$$L(H_2O)_{n_{max}} + A \leftrightarrow LA(H_2O)_{n_{max}-1} + H_2O,$$

$$k_1 = \frac{a_W a_{L(H_2O)_{n_{max}-1}}}{a_A a_{L(H_2O)_{n_{max}}}} \quad (3)$$

A full description of the association requires n_{max} equilibria like Eq. 3, each with a separate binding constant, k_i.

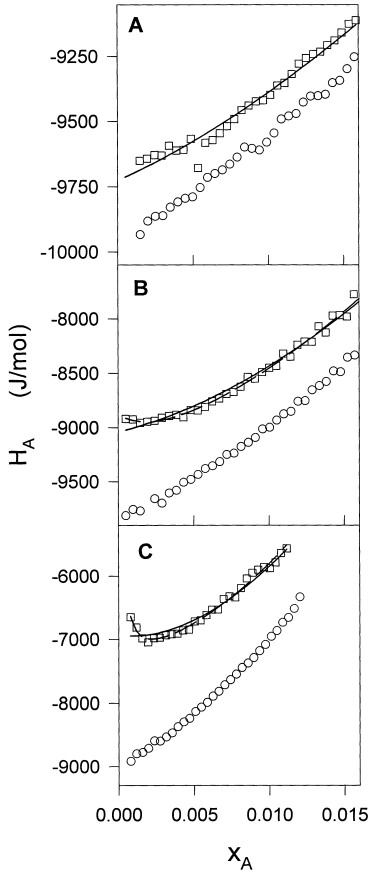

Fig. 3. Selected data from the calorimetric measurements at 26°C for ethanol (A), 1-propanol (B) and 1-butanol (C). The partial molar enthalpy of the alcohol, H_A, in buffer (circles) and a 50 mM suspension of DMPC liposomes (squares) is plotted as a function of the alcohol mole fraction, x_A. Solid curves are the results of non-linear regression of Eq. 6 (i.e. a model assuming similar, independent sites). The dashed lines represent fitting of a model expression based on a mode of interaction with two distinct classes of binding sites.

The degree of mean occupancy at the ith site, θ_i, can be written [23]

$$\theta_i = \frac{k_i a_A}{a_w + k_i a_A} \quad (4)$$

where a_A and a_w are the activities of alcohol and water respectively. If all n_{max} binding sites are assumed to be similar and independent, all equilibria can be characterized by the same constant, k ($k_1 = k_2 = ...k_{n_{max}} = k$). Although this assumption is certainly an oversimplification, the rather homogeneous surface of a liposome may lend some support to its validity. The average number, n, of alcohol molecules occupying a site can now be expressed simply as $n = n_{max}\theta = n_{max}ka_A/(a_w + ka_A)$. We will assume that the small alcohols under study readily permeate unilamellar DMPC liposomes and thus that both sides of the membrane (i.e. the total number of lipid molecules) are available for interaction. Also, it should be noted that n_{max} signifies the total number of sites available for the alcohol–water exchange, and that this number is expected to be much smaller than the ca. 20 water molecules involved in full hydration of fluid phase phospholipid membranes (see [26] and references therein). As discussed below the experimental data indeed suggest that n_{max} is about 2, and thus that only a limited fraction of the hydration water can be exchanged.

The model is most easily interpreted when alcohol and water are treated on a symmetric concentration scale, and we will hence use mole fractions units throughout. The standard states that define the (mole fraction) activities are the pure liquids for

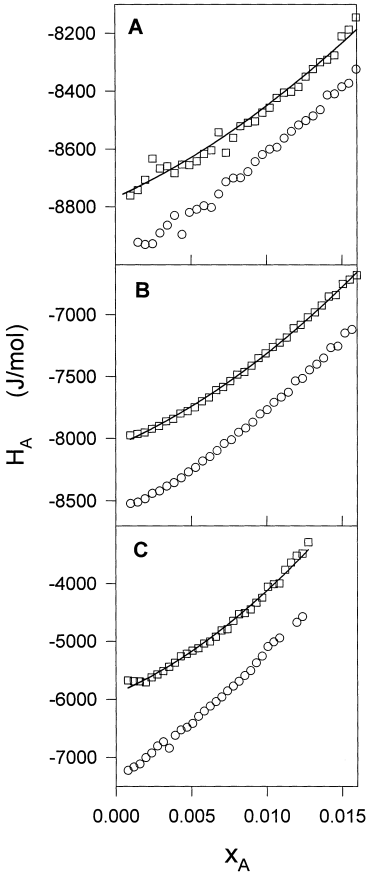

Fig. 4. Selected data from the calorimetric measurements at 32°C (symbols and lines as in Fig. 3).

water and alcohols and infinitely dilute aqueous solution for liposomes. From the vapor pressures presented in Fig. 2, the activity of water is found to lie in the range of 0.99–1 for all the experimental conditions used in this study (while the range of a_A is much broader, e.g. 0–0.7 for 1-butanol). Due to the limited variation of a_w, Eq. 4 can be approximated $\theta = ka_A/(1+ka_A)$. We note that this expression is equivalent to the degree of saturation for a standard binding model (L+A $\rightleftharpoons$ LA, see e.g. [27]), but we will discuss results with respect to Eq. 3 due to its conceptual compatibility with small binding constants.

To proceed we need to express the observed enthalpy effects in terms of θ. To do so we utilize that the data in Figs. 3–5 reflect the enthalpic response of the system upon addition of a small amount of alcohol, n_A. We assume that this perturbation displaces the n_{max} equilibria of Eq. 3 slightly towards the right-hand side, and that this displacement is the origin of the difference between a set of curves (buffer vs. 50 mM liposome) in Fig. 3. If the displacement is expressed as the change in θ we can write

$$\left(\frac{\partial\theta}{\partial n_A}\right)_{T,P,n_L} = \frac{1}{N}\frac{d(\gamma_A\theta)}{da_A} = \frac{1}{N}\frac{k\left(\gamma_A + x_A\left(\frac{d\gamma_A}{dx_A}\right)\right)}{(1+ka_A)^2} \quad (5)$$

The change of variable in the first equation of Eq. 5 is based on the definition of the alcohol mole fraction, $x_A = n_A/N$, where N is the total number of moles in the system, and the expression for the activity coefficient of the alcohol, $\gamma_A = a_A/x_A$. Hence, $dn_A = (N/\gamma_A)da_A$. The extensive quantity N in Eq. 5 occurs because the concentration of lipid has not yet been introduced.

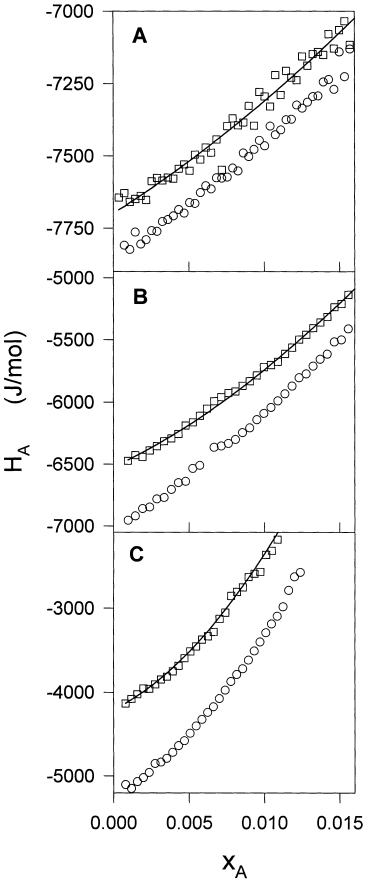

Fig. 5. Selected data from the calorimetric measurements at 40°C (symbols and lines as in Fig. 3).

To simplify Eq. 5 for further numeric analysis the composition dependence of the activity coefficient is ignored (i.e. $d\gamma_A/dx_A \approx 0$); evaluation of the data in

Table 1
Parameters of the non-linear regression of Eq. 6 to the experimental data

Alcohol	26°C		32°C		40°C	
	k	$x_{max}\Delta h$	k	$x_{max}\Delta h$	k	$x_{max}\Delta h$
Ethanol	5	(13)	3	(18)	2	(20)
1-Propanol	1.6	40	0.8	55	1.0	30
	13, 0.4	*3, 100*				
1-Butanol	0.8	50	0.5	50	0.3	50
	45, 0.3	*2, 80*				

The parameters in italics (for propanol and butanol at 26°C) were obtained by an extended model allowing two separate, independent classes of sites (see text for details). The $x_{max}\Delta h$ parameter for ethanol listed in parentheses can only be determined with high uncertainty; one S.D. confidence intervals for the other parameters are ±10–35% (see text).

Fig. 2 and Table 1 indicates that the maximal error on $d\theta_A/dn_A$ from this assumption is 5–15%.

The molar binding enthalpy for the reactions in Eq. 3 is Δh (see Fig. 6). Hence, the partial enthalpy of alcohol in the three component system, ${}^3H_A$, can be expressed as the sum of the partial enthalpy in the two component (liposome free) system, ${}^2H_A$, and the enthalpy associated with displacement of the equilibria. The latter quantity is the product of the total binding enthalpy (occupation of all sites: $n_{max}\Delta h$) and the change in the mean occupancy expressed in Eq. 5. Thus, using the simplified version ($d\gamma_A/dx_A \approx 0$) of Eq. 5, the partial enthalpy of alcohol in the three component system can be expressed

$$ {}^3H_A = {}^2H_A + n_{max}\,\Delta h\,\frac{\gamma_A}{N}\,\frac{k}{(1+ka_A)^2} \qquad (6) $$

Eq. 6 was incorporated into a non-linear, least square optimization routine and fitted to the experimental data in Figs. 3–5. The results of the regressions are illustrated by solid curves in the same figures. As mentioned above Δh is assumed to be independent of the alcohol concentration. The effect on the binding constant of this approximation can be evaluated from the composition dependence of ${}^3H_A$. Hence, a modified version of Eq. 6 in which the alcohol uptake was defined by the relative decrease in enthalpy, i.e. $({}^3H_A - {}^2H_A)/{}^3H_A$, was tested against the experimental data. It was found that this approach gave rise to somewhat smaller k values than Eq. 6; the effect was strongest for BuOH where up to 30% discrepancy was observed. Based on the level of precision required for the discussion of this work and the general disparity of equilibrium constants determined by other methods [28–30] it was deemed inexpedient to introduce a more complicated version of Eq. 6.

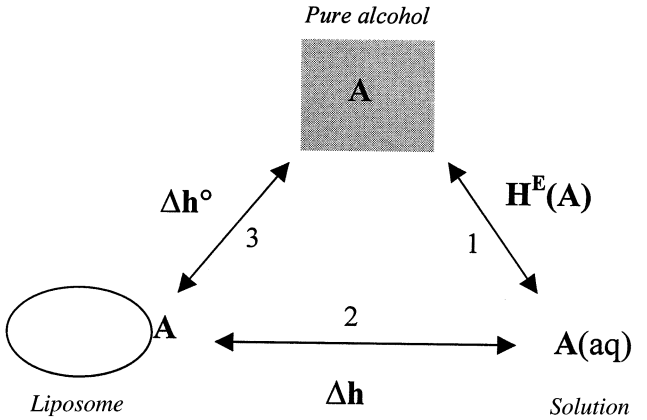

Fig. 6. Illustration of the enthalpy functions used in the model. Addition of (a small amount of) pure alcohol to the buffer (process 1) gives rise to an enthalpy change equal to the partial molar enthalpy, H_A, of the alcohol in solution. Exchange of an aqueous alcohol molecule with a water molecule on a site on the liposome surface (process 2) is associated with the molar enthalpy change Δh. Exchange of alcohol from the pure (standard) state is characterized by $\Delta h°$ (process 3).

The two parameters directly obtained from the regression are k and the term ($n_{max}\Delta H\gamma_A/N$). These are presented in Table 1 as k and $x_{max}\Delta h$. To calculate the latter, we used known values of γ_A (Fig. 2), and introduced the 'mole fraction of binding sites' in the solution, $x_{max} = n_{max}/N$, a quantity proportional to the liposome concentration.

The accuracy of the obtained model parameters was evaluated through the experimental reproducibility as well as a mathematical analysis of the variance of the fitting procedure. For propanol at 26°C, four separate sets of ${}^3H_A$ and ${}^2H_A$ data were measured and the reproducibility was found to be about 15% (S.D.) on both parameters. Similar reproducibility was found in double trials performed at other solute/temperature conditions. To further evaluate the quality of the model parameters, particularly their mutual dependence, a 'grid search' [31,32] was conducted. In this procedure a series of fixed values of one of the parameters is inserted in Eq. 6. Subsequently, the maximum likelihood values of the other parameter along with the variance of the fit are determined (cf. [33]). For PrOH and BuOH the variances showed clear minima at the values in Table 1, supporting the assumption that the listed parameters are maximum likelihood values. If a 67% confidence interval is defined by a 14% increase in the variance of the fit [34] we find that the k values for BuOH and PrOH fall within a rather symmetric one S.D. confidence interval of ±20–25% of the values in Table 1. A similar evaluation of the $x_{max}\Delta h$ parameter shows an asymmetric confidence interval of −10% to +30% of the listed values. As a consequence of the comparably lower degrees of mean occupancy for EtOH the model parameters for this solute are less accurate. For example, the analysis predicts that the binding constant $k(26°C) = 5$ falls in a one S.D. confidence interval of 2–10. Also, as a result of parameter covariance the $x_{max}\Delta h$ parameter for EtOH is uncertain

with a highly asymmetric confidence interval from −40% to a maximum value of several hundred percent. Hence, this parameter, noted in parentheses in Table 1, is not used in the further discussion.

4. Discussion

Our main objective is to discuss the applicability of the solvent exchange model (Eq. 3) for the interaction of small alcohols and lipid bilayer membranes. Before we turn to this, however, it is useful to discuss briefly some general consequences of the introduction of activity coefficients defined by symmetric standard states (pure liquid for both water and alcohol). This will be done in the framework of Eqs. 1 and 2, i.e. using solution theory for both bulk phase and partitioned alcohol.

4.1. Solution theory

Solutes in partitioning studies are most often highly dilute and activity coefficients are typically defined with respect to an infinitely dilute solution. This offers the convenience that the activity coefficient is close to unity under the experimental conditions (cf. Eq. 2). Deviations from unity at higher concentrations primarily reflect effects of solute–solute interactions. Consequences of solute-solvent effects in dilute solution are manifested in the standard chemical potential. If, on the other hand, the activity coefficient is defined by the pure liquid standard state, its value at high dilution (free of effects due to mutual solute-solute interactions) constitutes a measure of net solute-solvent effects. In Fig. 2 we find activity coefficients at infinite dilution and 26°C of 3.9, 14.4 and 54.5 for ethanol, 1-propanol and 1-butanol respectively. These values, which translate into excess chemical potentials of 3.4, 6.5 and 9.9 kJ/mol, are measures of the propensity of water to expel the alcohols, i.e. the 'hydrophobic push' on the alcohols towards another phase. If membrane partitioned alcohols formed ideal mixtures with the lipid molecules (as is assumed in Eq. 2) the partitioning coefficient, K_p, would be determined solely by this 'push' and it would attain a value equal to the aqueous activity coefficient, γ_A^{aq}. This follows from writing Eq. 2 for real solutions. At equilibrium

$$\mu_A^{aq} = \mu_A^{lip} =$$

$$\mu_A^* + RT \ln\{\gamma_A^{aq} x_A^{aq}\} = \mu_A^* + RT \ln\{\gamma_A^{lip} x_A^{lip}\}$$

$$K_p = x_A^{lip}/x_A^{aq} = \gamma_A^{aq}/\gamma_A^{lip} \quad (7)$$

Hence, if the activity coefficient of a partitioned alcohol, γ_A^{lip}, is unity, $K_p = \gamma_A^{aq}$. The simple relationship of Eq. 7 follows from choosing the same standard state for both aqueous and partitioned alcohol. Since K_p and γ_A^{aq} are experimentally assessable the activity coefficient of alcohol in the lipid phase, γ_A^{lip}, can be calculated. This quantity gives direct information on deviations from ideality of the partitioned alcohol molecules. If, for example, $\gamma_A^{lip} < 1$ (i.e. $K_p > \gamma_A^{aq}$) the chemical potential of partitioned alcohol is lower than predicted by ideal solution theory and favorable (direct) interactions between the bilayer and the alcohol contribute (together with the hydrophobic 'push') to the partitioning process. Conversely, $\gamma_A^{lip} > 1$ ($K_p < \gamma_A^{aq}$) indicates that bilayer–alcohol interactions contribute negatively to partitioning.

The partitioning coefficients of EtOH, PrOH and BuOH (in mole fraction units) into DMPC bilayers at 25°C are about 16, 48 and 120 respectively [27]. These values are 2–4 times larger activity the coefficients in Fig. 2. According to Eq. 7 this suggests that γ_A^{lip} is well below unity and thus that partitioning is driven by a combination of unfavorable interactions in the aqueous phase and attraction arising from direct bilayer–alcohol effects. For the long chain alcohols decanol and dodecanol this picture is different. The mole fraction partitioning coefficients for these solutes into fluid dipalmitoyl phosphatidylcholine (DPPC) bilayers are respectively 2.2×10^5 and 3.4×10^6 [35], which is rather close to the aqueous activity coefficients, $\gamma_{decanol}^{aq} = 2.8 \times 10^5$ and $\gamma_{dodecanol}^{aq} = 2.4 \times 10^6$, estimated from the aqueous solubility of the solutes [36]. In a solution theory approach, the coincidence between K_p and γ_A^{aq} suggests that net effects of interactions of partitioned alcohols in fluid DPPC are similar to those in the neat alcohol. Hence, accumulation in DPPC bilayers is almost fully driven by unfavorable interactions in the aqueous phase for some long chain alcohols. For small alcohols, on the other hand, favorable direct interac-

tions between solute and bilayer contribute significantly to partitioning, the more so the shorter the alcohol acyl chain.

4.2. Exchange model

As discussed in Section 1, both direct and indirect results have indicated that the association of small alcohols and other amphiphilic solutes with lipid bilayers involves predominantly adsorption to the lipid–water interface. Binding models may thus be more appropriate than solution theory for an analysis of the energetics of the association process. Table 1 presents data from such an approach (Eq. 3) applied to calorimetric measurements of lipid–alcohol interactions. It appears that at 26°C, for example, the microscopic equilibrium constant for the exchange of a water molecule with an alcohol molecule at a site on the lipid bilayer surface is respectively 5, 1.7, and 0.8 for EtOH, PrOH and BuOH. As discussed above, these values signify the affinity of the bilayer for the alcohols, devoid of contributions from unfavorable (hydrophobic) interactions in the aqueous phase (cf. Fig. 6). The affinity of the DMPC bilayer for EtOH and PrOH is slightly higher than that for water (the standard free energy, $\Delta g° = -RT\ln(k)$, for the exchange reaction of Eq. 3 is negative). For BuOH k is less than unity indicating that the affinity of the bilayer for water is (slightly) higher than that for the alcohol. At all three temperatures we find that the affinity of alcohol–bilayer association decreases with increasing chain length of the alcohol. This means that the well established increase in K_p with increasing acyl chain length (see e.g. [28]) relies on the enhanced magnitude of the hydrophobic effect while direct alcohol–bilayer interactions become less favorable the larger the alcohol, and hence increasingly counteract alcohol-bilayer complexation. This is the main experimental result of the work. It conveys fundamental information on the mode of (direct) interaction of alcohols with lipid membranes. Hence, an obvious interpretation of the chain length dependence illustrated in Table 1 would be that alcohol–membrane association is promoted by polar interactions between the DMPC head group and the –OH group of the alcohol. Effects due to the insertion of the acyl chain of the solute into the bilayer, on the other hand, contribute unfavorably to the binding. As a result, the affinity for *n*-alcohols, which all have the same potential for the polar interaction, decreases with increasing length of the acyl chain.

The data in Table 1 consistently show decreasing k values with increasing temperature. This may suggest that $\Delta h°$ (see Fig. 6) is negative and that its value can be derived from a van 't Hoff treatment of the $k(T)$ data. However, previous work [17,37,38] has indicated that solutes show an anomalous propensity for membrane partitioning at temperatures close to the main transition temperature (24° for DMPC). If this is the case, the mode of interaction depends on temperature and neither van 't Hoff analysis nor qualitative arguments based on Le Chatelier's principle can be applied to the temperature dependence of k. Instead the model must be modified to reflect effects of changes in temperature. This will be discussed in more detail below.

As discussed in detail by Schellman [23] weak complexation ($k < 10^2$) requires distinction between binding and site occupancy. At high concentration (activity) some ligand molecules will occupy binding sites 'by chance', i.e. even in the absence of specific ligand–receptor attraction. Thus, (weak) binding defined as the concentration of ligand close to the macromolecule is not meaningful in a thermodynamic sense [9,22]. A thermodynamically sound treatment of weak binding can be obtained through so-called selective interactions which are defined as the excess of ligand with respect to random distribution (bulk vs. binding site). For the present system it can be expressed as the number of alcohol molecules required to restore the alcohol's chemical potential when one lipid molecule is added to a solution [23]. It can attain both positive and negative values. The binding constants in Table 1 may be interpreted in terms of selective binding. If we define random distribution to be a situation where the mean site occupancy (θ in Eq. 4) is equal to the bulk activity of the alcohol, we find that for EtOH the interfacial concentration is above random distribution while for BuOH it is below. Following this approach, partitioning coefficients are measures of site occupancy – not binding – and it would be relevant to compare site occupancy calculated from the present model with published K_p values. However, since the model parameter $x_{site}\Delta h$ (Table 1) cannot be resolved into

its term, this calculation is not readily done. If we assume, as a first approximation, that saturation of the bilayer surface requires one alcohol molecule for each lipid molecule (i.e. $n_{lipid} = n_{max}$) we can estimate the occupancy at the experimental conditions. At 26°C and a bulk alcohol mole fraction of 0.005, for example, the mean degree of site occupancy (Eq. 4) is 9.6%, 13% and 28% for EtOH, PrOH and BuOH respectively. Under the assumption that $n_{lipid} = n_{max}$, these values are equivalent to partitioning coefficients of 16, 23 and 45. While these coefficients are, of course, dependent on the assumed value of n_{max}, they serve to demonstrate that the high occupancies observed in partitioning studies can be pertinent to cases of very weak binding (or even to 'repulsion' as defined by $k < 1$). If the preceding estimation is reversed and published K_p values [28] are used as measures for site occupancy we find that the number of binding sites per lipid is 2–3 for BuOH and PrOH. From these values of x_{max} it follows that Δh (see Fig. 6) is 10–15 kJ/mole for the investigated alcohols. This is close to what has been directly measured in other calorimetric studies [17,39].

An interesting consequence of a surface adsorption mechanism is the enhanced local concentration of alcohol. The combined volume of the glycerol backbone and the phosphatidylcholine group in DMPC is about 344 Å^3 [40] while the volume of the whole molecule at 26°C is 0.975 cm^3/g [40] or 1100 Å^3/molecule. Hence, if the alcohols are confined to the polar zone of the membrane their local volume fraction will be more than three times larger than the average membrane concentration. If again $x_A = 0.005$ is used as an example, the volume fraction of alcohol in the membrane–water interface is estimated to be 1.5%, 4% and 10% for respectively EtOH, PrOH and BuOH, under the assumption that the volume of partitioned alcohol is similar to that of the neat organic liquid [41,42].

Inspection of Fig. 3 shows that at 26°C a small but systematic deviation between the model and the measurements occurs at the most dilute concentrations ($x_{Alc} < 0.0015$ or 80 mM). This is particularly apparent for BuOH but four separate titration experiments with PrOH (not shown) consistently showed a 100 J/mol difference between model and experiment in the most dilute range. This trend is strongly reduced at 32°C (Fig. 4) and not observed

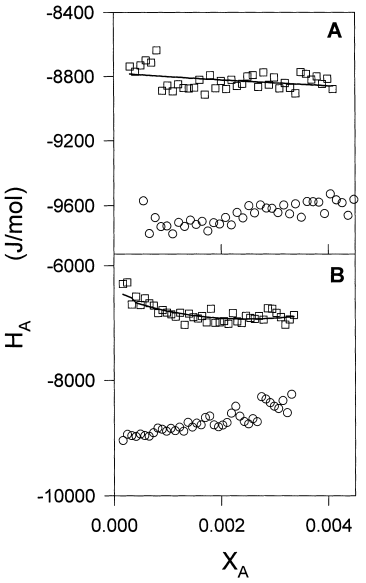

Fig. 7. Partial enthalpy, H_A, of 1-propanol (A) and 1-butanol (B) at 26°C measured in small increments of alcohol mole fraction. Symbols and lines have the same meaning as in Fig. 3.

at all at 40°C (Fig. 5). The high curvature of the experimental data at 26°C and low concentration suggest that a moderate number of sites with higher alcohol affinity exist at this temperature. To investigate this further we made a set of experiments for PrOH and BuOH at 26°C with very small (1 μl) injection aliquots. Although the scatter of this procedure is higher, it has the advantage of a better resolution in the most dilute concentration range (Fig. 7). Applying Eq. 6 to these data ($x_{Alc} < 0.0035$) gave binding constants of 4 and 2 for PrOH and BuOH, i.e. approximately three times the values averaged over a broader concentration interval (Table 1). The $x_{site}\Delta h$ parameter, which is proportional to the number of surface sites, is about 20 for both alcohols in Fig. 7. This is half the value in Table 1, and these results thus support the suggestion that a moderate number of stronger binding sites dominate at 26°C and high dilution.

In a theoretical study Jørgensen et al. [38] discussed the anomalous increase in the partitioning of non-polar molecules into single component lipid bilayer close to the liquid to gel transition temperature (T_m). They concluded that enhanced values of K_p were related to a dynamic heterogeneity manifested in the formation of coexisting domains of gel phase and fluid lipids. In the interfacial regions of

such domains, the packing properties of the lipid molecules gives rise to high affinity sites for the binding of non-polar solutes [38]. It is interesting to note that results of this computer simulation study are in good accordance with the interpretation of the present model. For BuOH at 26°C (2°C above T_m for DMPC), we find a binding constant of 2.0 for $x_{BuOH} < 0.0035$ (Fig. 7). Separate modeling of the data in Fig. 3 at higher concentrations ($0.004 < x_{BuOH} < 0.012$) shows weaker binding characterized by a constant of about 0.5. This behavior may reflect the existence of two classes of sites (high and low affinity) at 26°C. For comparison the data for BuOH at 40°C (16°C above T_m of DMPC) were modeled in the same two concentration regions ($x_{BuOH} < 0.0035$ and $0.004 < x_{BuOH} < 0.012$). In this case we obtain k values for the two concentration ranges which are similar (0.3 and 0.4) and close to the low affinity sites found at 26°C. Hence, there is no indication of a dual mode of interaction at 40°C.

The relative binding strength of the two classes of sites is reflected in the ratio of the binding constants obtained by modeling the two concentration regions separately. However, overlapping binding effects, which tend to average out the difference between the two constants, hamper the validity of this procedure. As an alternative we extended Eq. 6 to allow for two independent classes of sites (high affinity/low affinity). This was done simply by adding a second 'displacement term' analogous to the last term of Eq. 6. The resulting (four parameter) equation was applied to the data for BuOH and PrOH at 26°C. The model showed good accordance with the experimental data (Fig. 3) with the parameters noted in italics in Table 1. It is interesting to note that the low affinity constants are close to the k values found at higher temperatures, where fluctuating domains are practically absent [43]. The high affinity constants are larger by about two orders of magnitude. In the light of these observations and the results of Jørgensen et al. [38] we tentatively suggest that the high affinity/low affinity constants can be assigned to strongly binding interfacial and 'normal' fluid phase binding sites.

4.3. Solution or adsorption

The application of both solution theory and the solvent exchange model led to the conclusion that the hydrophobic interaction was the dominant driving force for the partitioning process. However, some important differences between the two approaches emerge. For example, the contribution to the driving force due to direct alcohol–bilayer effects appears weaker when experimental data are treated according to the binding model. This may reflect differences in the interpretation of the entropy of partitioned alcohols discussed in the Introduction. Further experimental work is needed to establish which approach provides the most realistic outset for a thermodynamic treatment. To this end, direct ('model free') data on the free energy of the interaction (e.g. measurements of preferential interactions) may prove particularly useful. An important study by Rowe at al. [14] recently contributed to this discussion. In this work a calorimetric technique was used to determine partitioning coefficients and enthalpies over a broad temperature range. This comprehensive data set allows some evaluation of the applicability of the solution theory approach since the directly measured (model free) enthalpies of partitioning can be compared with (model dependent) van 't Hoff enthalpies derived from the temperature dependence of the partitioning coefficient. If solution theory (i.e. Eqs. 1 and 2) gave a realistic picture of the partitioning process the van 't Hoff treatment would be valid and the two ways of obtaining the enthalpy would bring about similar values. However, attempts to extract $\Delta H°_{van\ 't\ Hoff}$ from the figures in reference [14] showed poor accordance with the measured enthalpies in the same work; in some cases (e.g. octanol) $\Delta H°_{van\ 't\ Hoff}$ and $\Delta H°_{measured}$ did not even carry the same sign. Although the work of Rowe et al. addressed larger alcohols (C6–C9) than the present study, the discrepancy of $\Delta H°_{measured}$ and $\Delta H°_{van\ 't\ Hoff}$ calls into question the general validity of solution theory for alcohol-membrane partitioning.

4.4. Closing remarks

Partitioning coefficients signify the presence of solute molecules in or close to the membrane, and this concept has proven extremely useful in numerous aspects of membrane studies. These include some physical properties of the bilayer and discussions of

thermodynamic functions such as enthalpy and volume, which are governed solely by the concentration of solute in the bilayer membrane. A general thermodynamic description of solute-membrane interactions, however, requires determination of selective binding rather that 'site occupancy' as expressed by K_p. Thus, the chemical potential of lipid, μ_L, in a mixed solvent can be expressed $\mu_L = \mu^\circ_L + RT \ln [L] + \mu^{ex}$, where [L] is the total concentration of lipid and μ^{ex} is the excess free energy generated from membrane–solute interactions [25]. The latter quantity relates to the excess preferential binding – not to the number density of solute in the membrane.

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