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Use of isothermal titration calorimetry to study the interaction of short-chain alcohols with lipid membranes[1]

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Abstract

The molecular mechanisms by which ethanol and other short-chain alcohols exert their effect in biological systems have been suggested to involve specific interactions with proteins and/or non-specific interactions with the lipid bilayer part of the cell membrane. To gain insight into the effect of short-chain alcohols on lipid bilayers, isothermal titration calorimetry (ITC) has been used to determine the energy involved in the association of the alcohols with lipid bilayers. Pure unilamellar DMPC liposomes and DMPC liposomes incorporated with different amounts of cholesterol, sphingomyelin and ganglioside (GM_1) were investigated at temperatures above, and below, the main phase-transition temperature of DMPC. The alcohols used were ethanol, 1-propanol, and 1-butanol. The calorimetric results reveal that the interaction of short-chain alcohols with the lipid bilayer is endothermic and strongly dependent on the lipid bilayer composition. In the presence of high concentrations of cholesterol, the binding enthalpy of ethanol is decreased, whereas the presence of ceramides enhances the enthalpic response of the lipid bilayer to ethanol. Isothermal titration calorimetry offers a new methodology of investigating molecular interactions and for determining partitioning coefficients for alcohols into lipid bilayers. We have estimated the partitioning coefficients for the three alcohols between the aqueous phase and the lipid bilayers of various lipid composition on the basis of calorimetric results. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Isothermal titration calorimetry; Lipid composition; Alcohol–liposome interaction; Temperature dependence; Partitioning coefficients

1. Background

Since Meyer and Overton [1,2], at the turn of the century, correlated the anesthetic potency of a molecule with the solubility in olive oil, a large number of models regarding the hydrophobic lipid-membrane core as the target for anesthetic drugs have been proposed. Aqueous suspensions of unilamellar liposomes of well-defined lipid compositions are convenient experimental model systems for the investigation of physical properties of relevance for the functional behavior of the lipid bilayer part of biomembranes [3,4].

The partitioning of alcohols into biological and model membranes at physiological and higher alcohol

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concentrations has been established [5–7]. Additionally, numerous investigations have indicated that alcohol-induced perturbations of the mechanical and structural properties of the cell membrane are important mechanisms underlying intoxication [8–10]. The amphiphilic nature of short-chain alcohols makes interfacial binding seem probable and detailed structural information on alcohol–membrane interactions has emerged from spectroscopy [11–14], revealing that ethanol resides predominantly in the lipid–water interface near the glycerol backbone. However, the location of alcohols in membranes may also be dependent on the lipid-membrane composition and it has been suggested that the penetration depth of water and ethanol into the membrane hydrophobic core can be modulated by lipid-chain unsaturation as well as lipid composition [9].

Isothermal titration calorimetry (ITC) has been applied to the study of a wide range of interactions in biological system; in particular, it has been used extensively for the study of saturable high-affinity binding of ligands to proteins [15]. However, the method was also used for the measurement of weak binding of co-solvents and denaturants to proteins [16] as well as for the measurement of non-saturable partitioning of small molecules into membranes [17]. In the present paper, a method to determine the enthalpy of transfer of molecules from an aqueous phase to a non-aqueous or lipid phase is presented.

2. Data treatment

It has previously been argued [18] that, since the partial excess enthalpy of a component j in solution, H_j^{E}, constitutes the enthalpic contribution of j to the total (integral) enthalpy of the system,

$$H_j^{\mathrm{E}} = (\partial H^{\mathrm{E}}/\partial n_j)_{T,p,n_i} \tag{1}$$

the sign of the derivative

$$H_{j-j}^{\mathrm{E}} = (\partial H_j^{\mathrm{E}}/\partial n_j)_{T,P,n_i} \tag{2}$$

may be used to elucidate intermolecular interactions. Since this is a purely thermodynamic approach, which makes no assumptions regarding the underlying molecular mechanism, derivatives similar to Eq. (2) may be applied to homogenous (*i-i* type) as well as heterogeneous (*i-j* type) interactions. Thus, the slope in a plot of H_j^{E} against concentration (molality; m_i) of another component i, m_i, indicates how incoming i-molecules enhance or decrease the average enthalpy of species j in the solution. Hence, the slope $\partial H_j^{\mathrm{E}}/\partial m_i$ (i.e. $\partial H_j^{\mathrm{E}}/\partial n_i$ in a system containing 1 kg of solvent water) is a measure of the enthalpy change generated from mutual *i-j* interactions. A situation where $\partial H_j^{\mathrm{E}}/\partial n_i > 0$ implies that additional i makes the contribution of j to the total enthalpy of the solution more positive; in other words, *i-j* interactions are associated with a positive enthalpy change. In the following, we will use such slopes, $\partial H_{\mathrm{lipid}}^{\mathrm{E}}/\partial n_{\mathrm{alcohol}}$, and $\partial H_{\mathrm{alcohol}}^{\mathrm{E}}/\partial n_{\mathrm{lipid}}$, to elucidate the enthalpic effects of membrane–alcohol interactions.

3. Materials and methods

Dimyristoyl phosphatidylcholine, DMPC (purity >99%), cholesterol (purity >98%) and sphingomyelin (purity >99%) were purchased from Avanti Polar Lipids (Birmingham, AL). Monosialoganglioside, GM_1 (bovine brain, purity > 99%) was supplied by Matreya (Pleasant Gap, PA). All lipids were used without further purification. Anhydrous ethanol (>99.9%), 1-propanol (>99.5%), and 1-butanol (>99.8%) were obtained from Merck (Darmstadt, Germany). The calorimetric results were obtained using concentrated (50–100 mM) suspensions of unilamellar liposomes composed of DMPC incorporated with cholesterol, sphingomyelin or ganglioside. Unilamellar liposomes were produced by standard extrusion techniques [19] (Lipex Biomembranes, Vancouver, Canada). Alcohol solutions were prepared from aqueous buffer (phosphate-buffered saline, pH = 7.4) by weighing. For a complete description of the sample preparation, see Trandum et al. [20].

3.1. Isothermal titration calorimetry

Numerical values for derivatives of the type described in the data analysis section can be obtained if the concentration dependence of the partial molar quantity is known with sufficient accuracy. In the present work, two different types of isothermal titration calorimetry (ITC) were conducted to determine the following:

(i) The excess partial enthalpy of liposomes in dilute alcohol–water mixtures, H^{E}_{lipid}.
(ii) The excess partial enthalpy of alcohol in solutions with liposomes of various lipid composition $H^{E}_{alcohol}$..

To measure the enthalpy change associated with transferring liposomes from water (the standard state) to an alcohol–water mixture (method (i)) Hess's law is used. The overall enthalpy change of a reaction is the sum of the enthalpies of the individual reactions into which the reaction may be divided. The following four-step procedure (A–D) was adopted to measure this enthalpy change as illustrated in Fig. 1. The measurements were carried out using a high-sensitivity ITC (MSC-ITC, MicroCal, Northamton, MA).

In the first step (A), liposomes are removed from the aqueous solution (where they are considered 'infinitely dilute') in to a small quantity of water. The enthalpy of this process is measured by titration of a concentrated liposome solution into water, i.e. the reverse process of A in Fig. 1. In the second step (B), the concentrated liposome solution from step (A) is titrated into the alcohol–water mixture. The third step (C) is to remove the same amount of water from the alcohol–liposome–water solution as was added in (B). The enthalpy of this step represents the heat of dilution and is determined as the reverse process (i.e. titration of the alcohol–liposome–water system with water). Finally, a small correction for the heat of adding water into water (D) is measured.

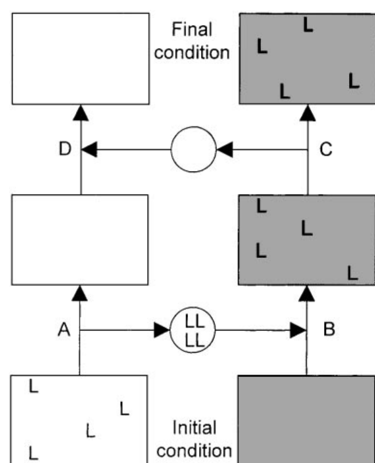

Fig. 1. Schematic illustration of the ITC procedure. Four separate measurements (A–D) were made to determine the transfer enthalpy of liposomes (L) at infinite dilution, from water (white) to an ethanol solution (shaded). The heats of process A (q_A) and B (q_B) are obtained by titrating the concentrated liposome solution into water and ethanol solutions, respectively. Similarly, q_C and q_D are measured by titration of water and the water–ethanol–liposome mixture with pure water.

The transfer enthalpy, Q_{trans}, is then calculated from the heats measured in each step (q_A, q_B, q_C, and q_D) as:

$$Q_{trans} = (M/\rho w_L)(q_A/V_A + q_B/V_B + v_W(q_C/V_C + q_D/V_D)) \quad (3)$$

where V_i is the volume titrated in step i (i: A, B, C, or D), M the molar mass of the lipids, ρ the density of the concentrated lipid solution removed in step A, and w_L and v_W are, respectively, the weight fraction of lipid and the volume fraction of water in the concentrated solution. The value of $\rho = 0.99$ g/ml was measured for DMPC (100 mmol/kg H_2O) by densitometry at 26°C (DMA 602, Anton Paar, Graz, Austria). We assume the density to be independent of the lipid composition. Fig. 2(A–D) shows examples of the raw titration data from the four-step procedure used to determine the transfer enthalpy, Q_{trans}, associated with transferring liposomes at 'infinite dilution' from water to an ethanol–water mixture. The example given is for an ethanol concentration of 300 mmol/kg H_2O. The contribution from steps A and D to the cycle are negligible. Fig. 2(B and C) show the excess enthalpy from introducing 8 µl aliquots of, respectively, liposomes and water into the ethanol solution. It is seen that the heats in steps B and C are of the opposite signs. Note that the introduction of liposomes into the water–ethanol solution is an endothermic process (heat is absorbed), whereas the dilution of the same water–ethanol solution by injection of water (buffer) is an exothermic process (heat is released). The difference between the two peaks represents the total amount of heat absorbed by transferring liposomes from aqueous solution to the ethanol–water mixture. As argued below, this is the excess partial enthalpy of the liposomes with respect to a standard state in which liposomes at infinite dilution are dispersed in pure water.

The excess partial enthalpy of alcohol in solutions with various liposomes contents (method (ii)) was measured by heat-conduction ITC (TAM 2277, Thermometric, Järfälla, Sweden). Small aliquots (3 µl) of neat alcohol were titrated into 3 ml solutions of a fixed liposome concentration (0, 50 and 100 mM). An

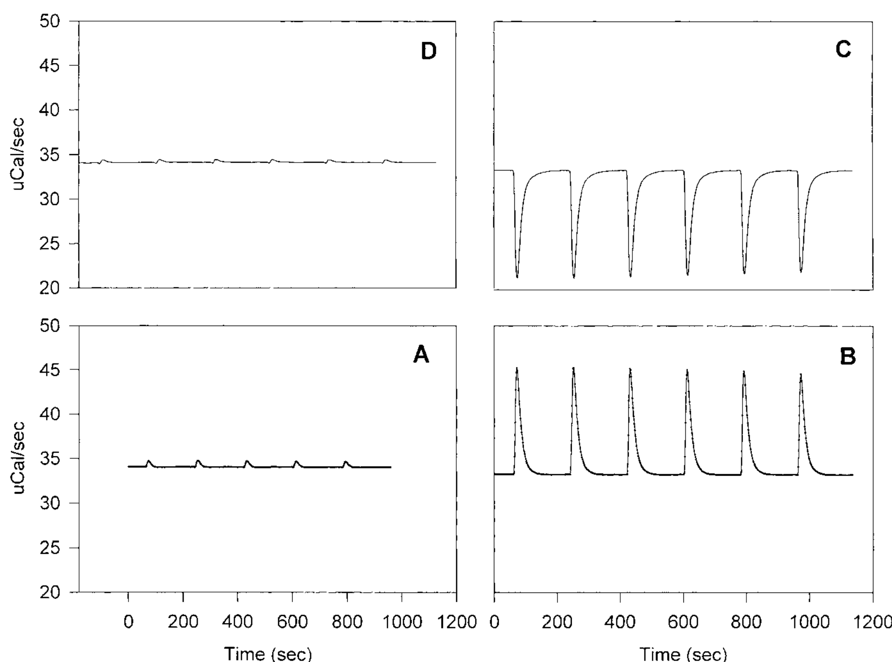

Fig. 2. Isothermal titration calorimetry profiles at 26°C. The figures A–D represent raw titration measurements for the four-step procedure (heat flow against time). (A) Titration of 8 μl aliquots of DMPC liposomes (100 mM) into 1.34 ml of water; (B) Titration of 8 μl aliquots of DMPC liposomes into an ethanol–water solution (300 mmol/kg H_2O); (C) 8 μl aliquots of water titrated into the liposome-ethanol-water solution (300 mmol/kg H_2O); and (D) titration of water into water.

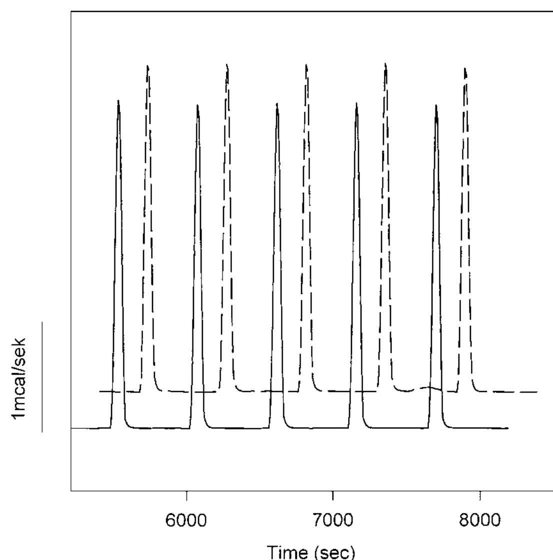

Fig. 3. An example of a thermogram for the data obtained using method (ii). Titration of 3 μl aliquots of ethanol into 3 ml (- - -) water and (———) 50 mM DMPC at 26°C.

example of a thermogram is shown in Fig. 3. This method provides direct information on liposome–alcohol interactions, but it is essentially complicated due to the large heats of dilution of alcohols. For the data in Fig. 3, for example, the presence of 50 mM DMPC reduces the peak size with ca. 3%. The method determines $H^{E}_{alcohol}$ with an uncertainty <0.5%.

The two types of isothermal titration calorimeters used in this study are fundamentally different. Comparison of Figs. 2 and 3 shows that, using method (i), it is possible to obtain very small heats of reaction (on the microcalorie scale), whereas method (ii) offers the possibility to measure large heats (millicalories).

4. Results and discussion

In Fig. 4, the excess partial molar enthalpy of liposomes, H^{E}_{lipid}, is plotted as a function of the ethanol concentration at 26°C. It appears that the slope for all types of liposomes (DMPC, DMPC/cholesterol, and

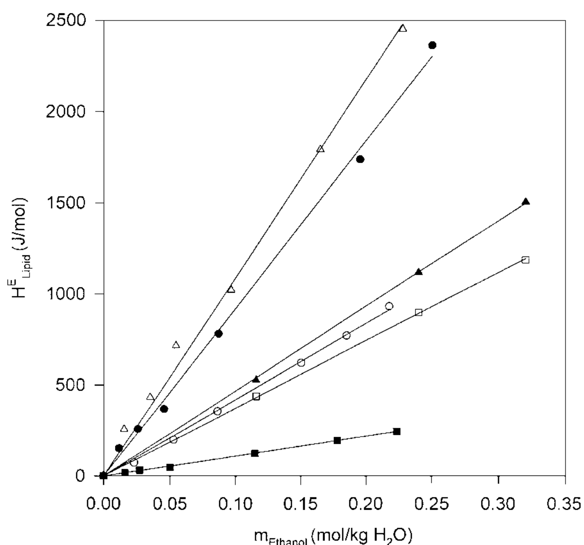

Fig. 4. Measured values of the excess partial enthalpy of liposomes of different lipid compositions. $H^{\mathrm{E}}_{\mathrm{Lipid}}$, in ethanol solutions calculated according to Eq. (3). DMPC (○); DMPC/sphingomyelin (●); DMPC/ganglioside (△); DMPC/cholesterol (10%) (▲); DMPC/cholesterol (20%) (□); and DMPC/Cholesterol (30%) (■).

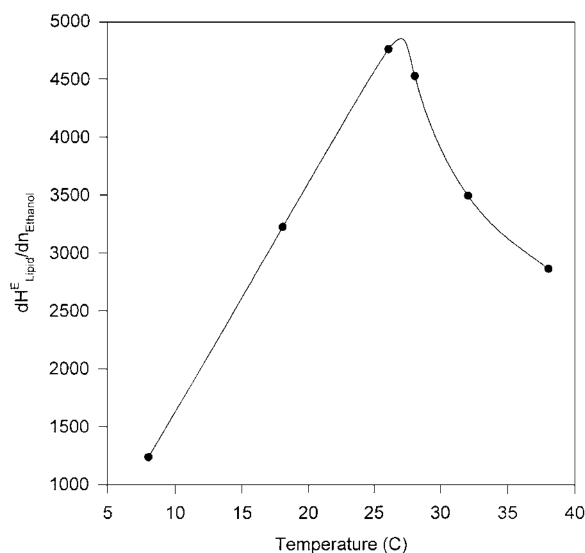

Fig. 5. The temperature dependence of the interaction parameter, $\partial H^{\mathrm{E}}_{\mathrm{lipid}}/\partial m_{\mathrm{ethanol}}$, for unilamellar DMPC liposomes.

DMPC/ceramide) is positive; i.e. $\partial H^{\mathrm{E}}_{\mathrm{lipid}}/\partial n_{\mathrm{ethanol}} > 0$. Hence, as pointed out in the data analysis section, the contribution of liposomes to the excess enthalpy of the entire solution is higher in dilute ethanol than in pure water, the bound complex is enthalpically unfavourable. Enthalpic effects for liposome-ethanol interactions are further elucidated in Fig. 5, which illustrates the temperature dependence of the interaction parameter, $\partial H^{\mathrm{E}}_{\mathrm{lipid}}/\partial n_{\mathrm{ethanol}}$, for pure DMPC liposomes above, and below, the main phase transition. For all temperatures, the transfer of liposomes from water into dilute ethanol solution is accompanied by an increase in the excess partial enthalpy of the liposomes. Consequently, the binding of ethanol to liposomes is entropy-driven in the studied range of temperature and composition. The effect of ethanol on the partial molar enthalpy of liposomes depends strongly on the temperature. In addition, a pronounced peak develops in the temperature range of the main transition. This unusual effect indicates a stronger affinity of ethanol to the bilayer in the transition region. Interestingly, computer simulation results of the interaction of small molecules with lipid bilayers have demonstrated a maximum in partitioning coefficients in the transition region due to the formation of heterogeneous lateral bilayers composed of fluctuating lipid domains and interfacial regions acting as adsorption sites [21].

Fig. 6 illustrates the excess partial molar enthalpy of ethanol, 1-propanol, and 1-butanol in buffer and 50 mM DMPC as a function of the respective alcohol concentrations at 26°C. It appears for all three alcohols that the large negative enthalpy of the alcohol becomes less negative in DMPC suspensions than in pure buffer ($\partial H^{\mathrm{E}}_{\mathrm{alcohol}}/\partial m_{\mathrm{lipid}} > 0$), implying endothermic alcohol–liposome interactions. A similar result is seen in Fig. 7 where the excess partial molar enthalpy of DMPC liposomes is plotted as a function of the concentration of the three different alcohols at 26°C. The slope for all three alcohols is positive, $\partial H^{\mathrm{E}}_{\mathrm{lipid}}/\partial m_{\mathrm{ethanol}} > 0$. The slopes of the curves in Fig. 7 may be considered as a measure of the average enthalpic effect of alcohol–liposome interactions. Thus, liposome–alcohol interactions are endothermic and become stronger with the increasing length of the acyl chain of the alcohol.

The data in Fig. 6 have been used to determine partitioning coefficients for the three alcohols between the aqueous phase and the lipid bilayer. In the case of ethanol, the partial molar enthalpy is reduced by ≈2.7% upon a change in the liposome concentration

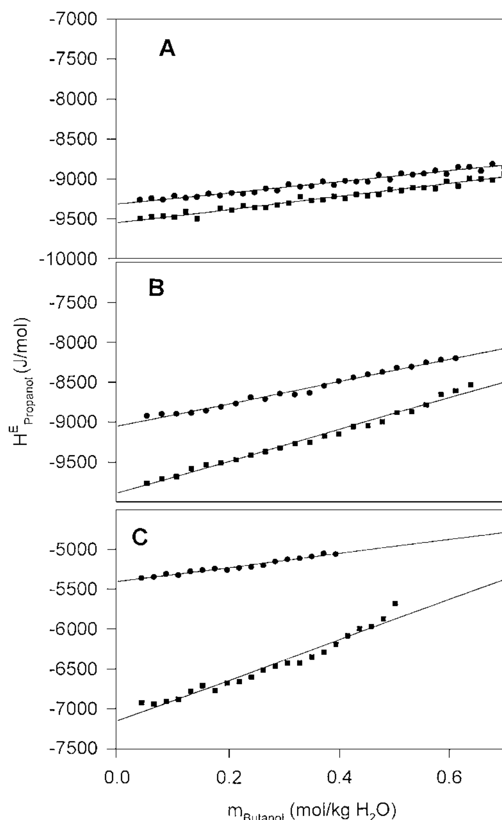

Fig. 6. Excess partial molar enthalpy of ethanol, $H^E_{Ethanol}$ propanol, $H^E_{Propanol}$, and butanol, $H^E_{Butanol}$, in solutions of buffer (■) and pure 50 mM DMPC liposomes (●) as a function of the alcohol molality, $m_{Alcohol}$. (a) Ethanol; (b) propanol; and (c) butanol.

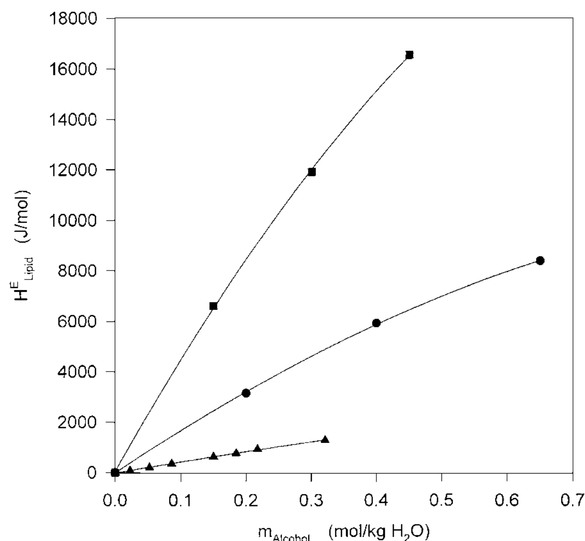

Fig. 7. Excess partial molar enthalpy of unilamellar DMPC liposomes, H^E_{Lipid}, in alcohol solutions as a function of the alcohol molality, $m_{Alcohol}$, at 26°C. Ethanol (▲); 1-propanol (●); and 1-butanol (■).

from 0 to 50 mM. Assuming that ethanol can be found in only two states, bound or aqueous, and that bound ethanol has the same enthalpy as pure liquid ethanol, we estimate partitioning coefficients from the formula $K_p = X_L/X_W$, where X_L is the mole fraction of ethanol in the lipid phase and X_W the mole fraction of ethanol in the solvent. The amount of ethanol bound to liposomes is then simply calculated as the relative reduction in the partial enthalpy of ethanol in solutions of liposomes. In the case of pure DMPC, $K_p = 28$. Sarasua et al. [7] found that the partition coefficient for ethanol between water and fluid lipid bilayers (albeit DPPC) was ca. 40. This accord between the solution enthalpy data and partitioning studies may suggest that the assumptions mentioned above are reasonable and, hence, dehydration of the alcohol upon association with the membrane is governing the energetics of the interaction. If this is the case, the observed partial enthalpy of ethanol would is reduced by 2.7% in 50 mM DMPC simply because that fraction is removed from the bulk. The same method has been applied to estimate partitioning coefficients for 1-propanol and 1-butanol between buffer and pure DMPC liposomes. The results are listed in Table 1.

The effects of cholesterol and ceramides at a physiologically relevant ethanol concentrations are illustrated in Fig. 4. It appears that the slope $\partial H^E_{lipid}/\partial m_{ethanol}$ is increasing at low cholesterol concentrations (<20 mol%), whereas the slope decreased by a factor of 2–3 in liposomes with 30 mol% cholesterol. This result concurrently suggests that the interaction

Table 1
Partitioning coefficients for short-chain alcohols into unilamellar DMPC liposomes at 26°C

	Bound alcohol(%) at 50 mM liposome	K_p
Ethanol	2.7	28
1-Propanol	8.2	106
1-Butanol	23.9	186

energy of ethanol with DMPC liposomes is significantly reduced by the addition of cholesterol to the bilayers. This observation might be particularly interesting in light of the in vivo observation that increased cholesterol content in biological membranes has been identified as an adaptive mechanism during chronic alcohol consumption [22]. Accordingly, Fig. 4 shows a 3–4-fold increase in the slope $\partial H^{E}_{\mathrm{ethanol}}/\partial m_{\mathrm{lipid}}$ upon addition of 10% sphingomyelin or ganglioside. This reduction (cholesterol) or increase (ceramides) could either be due to changes in the standard enthalpy of association or to a reduced (enhanced) affinity of the bilayers for ethanol. Further studies of these effects, particularly investigations of the Gibbs free energy of association, seems warranted.

Acknowledgements

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