A Thermodynamic Study of the Effects of Cholesterol on the Interaction between Liposomes and Ethanol

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ABSTRACT The association of ethanol with unilamellar dimyristoyl phosphatidylcholine (DMPC) liposomes of varying cholesterol content has been investigated by isothermal titration calorimetry over a wide temperature range (8–45°C). The calorimetric data show that the interaction of ethanol with the lipid membranes is endothermic and strongly dependent on the phase behavior of the mixed lipid bilayer, specifically whether the lipid bilayer is in the solid ordered (so), liquid disordered (ld), or liquid ordered (lo) phase. In the low concentration regime (<10 mol%), cholesterol enhances the affinity of ethanol for the lipid bilayer compared to pure DMPC bilayers, whereas higher levels of cholesterol (>10 mol%) reduce affinity of ethanol for the lipid bilayer. Moreover, the experimental data reveal that the affinity of ethanol for the DMPC bilayers containing small amounts of cholesterol is enhanced in the region around the main phase transition. The results suggest the existence of a close relationship between the physical structure of the lipid bilayer and the association of ethanol with the bilayer. In particular, the existence of dynamically coexisting domains of gel and fluid lipids in the transition temperature region may play an important role for association of ethanol with the lipid bilayers. Finally, the relation between cholesterol content and the affinity of ethanol for the lipid bilayer provides some support for the in vivo observation that cholesterol acts as a natural antagonist against alcohol intoxication.

INTRODUCTION

Cholesterol is an important component of eukaryotic plasma membranes and during the past decades, cholesterol–phospholipid interactions have been a topic of substantial interest (for reviews see Finegold, 1993; Mouritsen and Jørgensen, 1994; McMullen and McElhaney, 1996). A substantial part of the experimental work has focussed on a quantitative evaluation of the thermodynamic effects of various amounts of cholesterol on the pure lipid bilayer main phase transition, leading to the widely accepted phospholipid–cholesterol phase diagram illustrated in Fig. 1 (Thewalt and Bloom, 1992). According to this phase diagram, which is considered to be relatively independent of the precise chemical structure of the PC molecule, cholesterol in large amounts ($X_{chol} > \sim 25\%$) promotes the formation of a liquid-ordered phase, which is a liquid (fluid) from the point of view of lateral disorder and diffusion, and hence of importance for the mobility of membrane-incorporated compounds, but, at the same time, the lipid acyl-chains are characterized by a high degree of conformational order. In other words, at high concentrations, cholesterol decouples the positional and conformational degrees of freedom of the phospholipid molecules (Nielsen et al., 1999). At low cholesterol concentrations ($X_{chol} < \sim 10\%$) the influence of cholesterol on the lipid bilayer is quite different. As it appears from the phase diagram, only a marginal reduction in the transition temperature of the main phase transition occurs. The interpretation of this phenomenon is that, at these low concentrations, cholesterol is neither able to fully break the crystalline order in the solid-ordered phase nor able to fully induce order of the acyl chains in the fluid phase. The result is that cholesterol prefers both lipid phases equally and becomes an interfacially active molecule, which promotes lipid-domain formation and, consequently, enhances the dynamic membrane heterogeneity (Cruzeiro-Hansson et al., 1989; Mouritsen and Jørgensen, 1994).

The content of cholesterol in eukaryotic plasma membranes is rather high, e.g., 20 wt% in human erythrocytes, whereas much lower cholesterol concentrations are found in the intercellular membranes, e.g., the Golgi apparatus has about 8 wt%, the endoplasmic reticulum has 6 wt%, and the mitochondria only about 3 wt% (Jamieson and Robinson, 1977). It is generally accepted that the high levels of cholesterol in the plasma membrane are important for imparting the membrane with mechanical coherence, resistance to mechanical fatigue, and for stabilizing a high permeability barrier (Yeagle, 1988; Needham and Nunn, 1990; Bloom et al., 1991; Finegold, 1993), while the membrane, at the same time, maintains the fluid character. However, the physiological reason for the lower concentrations of cholesterol in the internal membranes are, as yet, unclear, although it has been suggested that the cholesterol concentration gradient found from the endoplasmic reticulum and Golgi toward the plasma membrane is instrumental for the sorting of proteins

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Abbreviations used: DMPC, dimyristoyl phosphatidylcholine (14:0); ITC, isothermal titration calorimetry

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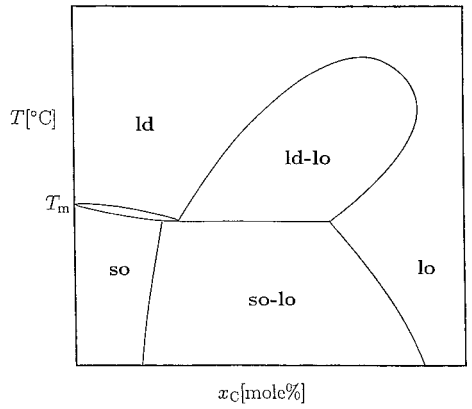

FIGURE 1 Schematic phase diagram for mixtures of DMPC and cholesterol (From Ipsen et al. 1987). T_m is the main transition temperature of pure DMPC. The different phases are labeled: so, solid-ordered, the gel phase; ld, liquid-disordered, the fluid phase; and lo, liquid-ordered.

in the secretory pathway (Brescher and Munro, 1993; Pelham and Munro, 1993). Only very few quantitative biophysical studies on the effect of small levels of cholesterol on the physical properties of lipid bilayers have been reported (de Gier et al., 1979; Blume and Hillmann, 1986; Genz et al., 1986; Michels et al., 1989; Corvera et al., 1992; Lemmich et al., 1997; Richter et al., 1999; Raffy and Teissié, 1999). Taken together, these studies demonstrate that cholesterol in the low-concentration regime leads to an enhancement of the lipid-domain formation in the region close to the main phase transition, softening of the bilayer, and reduction of the thermal bending rigidity, and, as a particular consequence, that the passive bilayer permeability to small ions is increased.

Although the mechanisms of acute and chronic effects of alcohol intoxication are not known, it is generally accepted that physiological changes are facilitated, at least in part, by altering the structure and dynamics of the lipid bilayer of the cell membrane (Chin and Goldstein, 1977; Barry and Gawrisch, 1995), in particular, the degree of order and heterogeneity (Mouritsen and Jørgensen, 1994). A macroscopic manifestation of the effect of many general anesthetics on membrane properties is a depression of the transition temperature and a smearing of thermodynamic response functions, e.g., the heat capacity (Rowe, 1983). A microscopic picture of the interactions of anesthetics with lipid membranes has emerged from computer simulation studies. These calculations show that the formation of dynamically coexisting domains of gel and fluid lipids is strongly influenced by the presence of small organic molecules (Jørgensen et al., 1993). The calculations also revealed that solute molecules have a high affinity for the interfaces formed between coexisting gel and fluid domains close to the main phase transition in pure lipid bilayers, and might thereby explain the enhanced affinity of ethanol for pure DMPC bilayers in this temperature region as previously reported (Trandum et al., 1999a).

In a recent work (Trandum et al., 1999c), we examined the bilayer response to ethanol for liposomes of various compositions of the lipids most commonly found in the neural cell membranes, i.e., sphingomyelin, ganglioside, and cholesterol. We found that, in the fluid phase at temperatures above the main phase transition of DMPC, the presence of ceramides enhances the partitioning of ethanol, whereas DMPC liposomes incorporated with large amounts of cholesterol (30 mol%) significantly reduce the partitioning coefficient of ethanol compared to pure DMPC bilayers.

In the present paper, we report a systematic study of the effect of cholesterol concentration on the association of ethanol with unilamellar lipid bilayers. Partial molar enthalpies obtained by ITC will be used to evaluate molecular interactions and to determine partitioning coefficients for the partitioning of ethanol between water and unilamellar DMPC liposomes incorporated with various amounts of cholesterol (0–40 mol%) over a wide temperature range (8–45°C).

MATERIALS AND METHODS

DMPC (purity > 99%) and cholesterol (purity > 98%) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). The lipids were used without further purification. Anhydrous ethanol (purity > 99.9%) was obtained from Merck (Darmstadt, Germany).

Preparation of unilamellar liposomes

For each series of isothermal calorimetric measurements, concentrated (50–70 mg/ml) suspensions of unilamellar liposomes were prepared. The mixtures studied were as follows: pure DMPC, and DMPC with 0.5, 1, 4, 5, 10, 15, 20, 25, 30, and 40 mol% cholesterol. After weighing, the dry lipids were cosolubilized in a chloroform/methanol 2:1 mixture (or hydrated immediately in the case of pure DMPC). Chloroform/methanol was driven off by a stream of nitrogen, and the samples were stored under low pressure for at least 72 h. The resulting dry lipid films were then dispersed in a 50 mM saline buffer (10 mM phosphate, 137 mM NaCl, 2 mM KCl; pH 7.4). The temperature was kept at 40°C for 1 h, during which the suspensions were shaken vigorously several times. Unilamellar vesicles were produced by standard extrusion techniques using twelve repeated extrusions through two stacked polycarbonate filters (Nucleopore, 0.1 μm pore size) and a hydrostatic pressure of 25 atm at 40°C (Mayer et al., 1986). The final concentration of lipid was determined gravimetrically by freeze drying 100-μl aliquots of the suspensions. Ethanol solutions were prepared from aqueous buffer by weighing. After mixing, the apparent pH of the ethanol solutions were was measured and, if necessary, readjusted to pH = 7.4.

Isothermal Titration Calorimetry

Numerical values for derivatives of the type described in the data analysis section below can be obtained if the concentration dependence of the partial molar quantity is known with sufficient accuracy. In the present work, excess partial molar enthalpies of liposomes (100 mM) in dilute ethanol–water mixtures have been measured by high sensitivity ITC (MSC-ITC, MicroCal, Northamton, MA) using a four-step procedure described in detail elsewhere (Trandum et al., 1999a).

Partitioning coefficients for ethanol into pure DMPC bilayers is determined using the method of Zhang and Rowe (1992). The method is based

on the determination of the free solute concentration in a solute–lipid–water suspension. Aliquots from the syringe, containing a solute–lipid–water suspension, are added to the reaction cell, which contains various concentrations of solute dissolved in water. Heat is absorbed if the free solute concentration in the cell is higher than that in the lipid suspension, whereas heat is released if the solute concentration in the cell is lower than in the lipid suspension. When the solute concentration in the cell matches the free solute concentration in the syringe, no heat is generated upon mixing. The amount of ethanol partitioned into the DMPC bilayer is determined by subtraction of the free concentration from the total concentration, and the partitioning coefficient is calculated as described in Data Analysis.

DATA ANALYSIS

The contribution of a species *a* in solution toward the total enthalpy of the system is signified by its excess partial molar enthalpy, H_a^E. In the following, the composition dependence of partial molar enthalpies will be used to elucidate the enthalpic effects of intermolecular interactions in aqueous solution. Hence, we define an "interaction function" H_{a-b},

$$H_{a-b} = dH_a^E/dm_b, \quad (1)$$

which reflects the influence of additional *b* molecules on the enthalpy of *a* (Koga, 1996). m_b is the molal concentration of component *b*. If H_{a-b} is positive (i.e., if additional *b* molecules in the system act to increase the enthalpy of *a,* the enthalpy change generated from mutual *a–b* interactions is positive. In such a case, *a–b* interactions are said to be unfavorable in terms of enthalpy, or endothermic. Conversely, negative values of H_{a-b} indicate exothermic interactions, and *a–b* interactions are said to be favorable in terms of enthalpy (Trandum et al., 1999a,b).

The simplest and most commonly used approach to determine alcohol partitioning coefficients considers alcohol to be distributed between the lipid bilayer and aqueous phases, described by the reaction,

$$A(aq) \leftrightarrow A(lipid) \quad (2)$$

where A(aq) and A(lipid) indicate alcohol in aqueous solution and lipid bilayer phase, respectively. The partitioning coefficient is the given as

$$K_p = \frac{X_{ethanol}^{(lipid)}}{X_{ethanol}^{(aq)}} \quad (3)$$

where $X_{ethanol}^{(lipid)}$ is the mole fraction of ethanol in the lipid phase and $X_{ethanol}^{(aq)}$ is the mole fraction of ethanol in the solvent.

RESULTS AND DISCUSSION

Lipid bilayers undergo a number of different phase transitions (Kinnunen and Laggner, 1991) among which the main phase transition is considered important for biological systems. The main phase transition takes the lipid bilayer from a gel phase (solid-ordered phase) to a fluid phase (liquid-disordered phase) (Mouritsen, 1991). The terminology reflects the fact that two different degrees of freedom are involved in the main transition, the translational variable and the acyl-chain intermolecular variable (Mouritsen and Jørgensen, 1994; Nielsen et al., 1999). In the main transition of a single-component phospholipid bilayer, the two sets of degrees of freedom are coupled, and the bilayer orders in terms of them simultaneously (Mouritsen, 1991). As mentioned in the Introduction, cholesterol can be used to modulate this order–disorder phenomenon; at high concentrations, cholesterol is even able to decouple these two sets of variables completely (Nielsen et al., 1999). The main objective of the present study is to use the interaction parameter, $H_{lipid-ethanol}$, to discuss the effect of cholesterol concentration and temperature on the association of ethanol with lipid bilayer membranes, in particular, in relation to the phase equilibria. This might provide further insights into the possible role of lipid composition in the functional dynamics of membranes.

Figure 2 shows the excess partial specific enthalpy of DMPC/cholesterol liposomes of various lipid composition, H_{lipid}^E as a function of the alcohol concentration at 28°C. For all lipid compositions, transfer of the liposomes from water into the dilute ethanol solution is accompanied by an increase in the excess partial enthalpy of the liposomes; i.e., $\partial H_{lipid}^E/\partial m_{ethanol} > 0$. Thus, the well-documented association of ethanol with lipid membranes is endothermic (the bound complex is energetically less stable than the dissociated components) (Trandum et al., 1999b). The slopes of the curves in Fig. 2 are measures of the average enthalpic effect of ethanol–liposome interactions as defined in Eq. 1, and they signify the enthalpic interaction parameter. Hence, liposome–ethanol interactions are endothermic but strongly dependent on the cholesterol content in the bilayer.

This is further illustrated in Fig. 3, where the interaction parameter, $H_{lipid-ethanol}$, as a function of cholesterol concentration at 28°C, is shown. For liposomes containing small amounts of cholesterol, the enthalpic effect of ethanol–

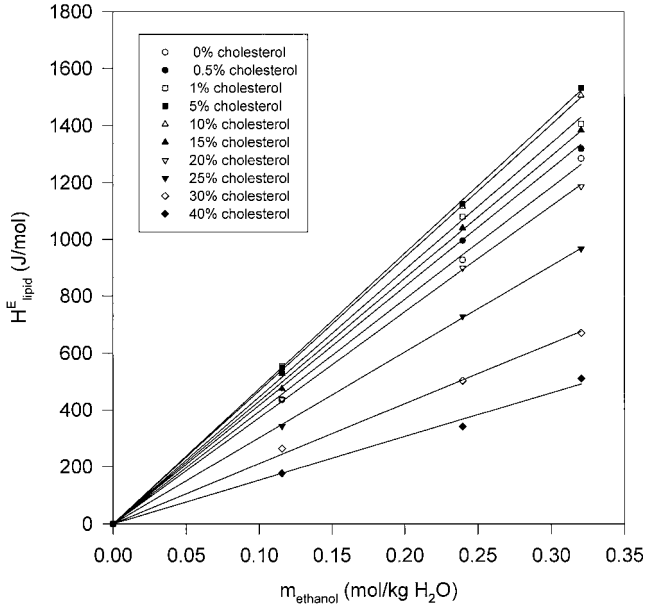

FIGURE 2 Measured values of the partial enthalpy, H_{lipid}^E, of DMPC liposomes of different cholesterol content in ethanol solutions calculated according to Eq. 1 and plotted as a function of the ethanol molality, $m_{ethanol}$ at 28°C. ○, 0 mol%; ●, 0.5%; □, 1%; ■, 5%; △, 10%; ▲, 15%; ▽, 20%; ▼, 25%; ◇, 30%; and ◆, 40% mol% cholesterol.

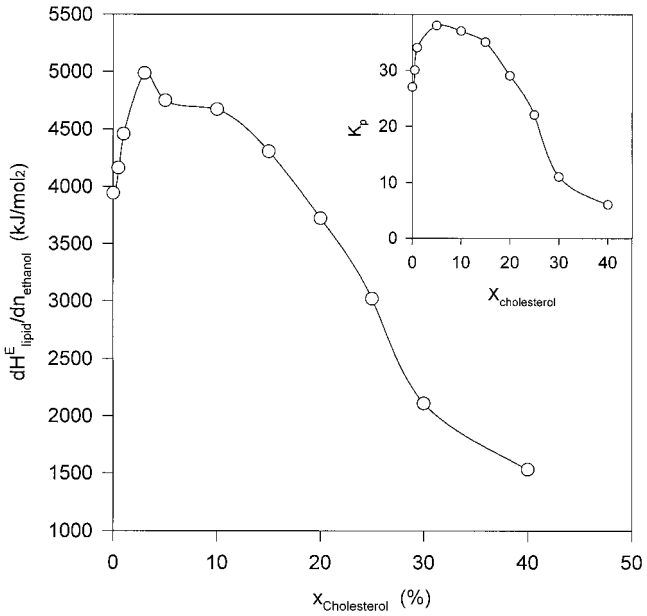

FIGURE 3 The interaction parameter, $H_{\text{lipid–alcohol}}$, as a function of cholesterol concentration at 28°C. The interaction parameter is taken directly as the slope of the curves in Fig. 2. The partitioning coefficient of ethanol (mole fraction scale) is plotted as a function of the cholesterol concentration in the inset.

liposome interactions is enhanced, with a maximum at approximately $X_{\text{cholesterol}} = 4\%$. Conversely, incorporation of large amounts of cholesterol in the liposomes reduces the enthalpic response of liposomes to ethanol by a factor of 2–3 compared to pure DMPC liposomes. This result is in qualitatively accordance with H^2-NMR results obtained by Barry and Gawrisch (1995), who found that the effect of ethanol on the order in DMPC bilayers depends on the cholesterol concentration in a complex manner, with a maximum in the order parameter at about 12 mol% cholesterol, and a response similar to that of no cholesterol at roughly 25 mol%.

To gain further insight into the effect of cholesterol content on the association of ethanol with lipid bilayers, partitioning coefficients of ethanol between the aqueous phase and the lipid bilayer phase are estimated. In a recent study (Trandum et al., 1999b), we reported that the standard enthalpy of transferring ethanol from water into pure DMPC bilayers is of the same magnitude but opposite sign as the enthalpy of transferring ethanol from the pure liquid into water, and we provided evidence for proportionality between the interaction parameter and the partitioning coefficient for DMPC liposomes incorporated with various amounts of ceramides (Trandum et al., 1999c). These results allow for estimation of the amount of ethanol partitioned into cholesterol containing liposomes from the interaction parameter. The results are plotted in the inset in Fig. 3. The figure shows that the partitioning of ethanol into lipid bilayers of low cholesterol concentration is enhanced compared to pure DMPC bilayers, whereas cholesterol in large amounts reduces the partitioning of ethanol significantly. The observation of decreasing partitioning coefficients for high cholesterol concentrations is in agreement with results obtained by Rowe et al. (1998).

The strong dependence of the partitioning coefficient on cholesterol content might be inferred from the phase diagram for DMPC/cholesterol, as shown schematically in Fig. 1. At low cholesterol concentrations, there is a very narrow phase separation region, which has been suggested to be accompanied by dynamically coexisting gel and fluid lipid domains (Cruzeiro-Hansson et al., 1989). Furthermore, computer simulation calculations have revealed that small organic molecules such as ethanol have an enhanced affinity for the interfaces between coexisting gel and fluid domains in pure lipid bilayers (Jørgensen et al., 1993). These results, together with the data presented in this paper, suggest that the partitioning of ethanol increases until the phase boundary of the low concentration phase is reached. Between the low concentration region and the liquid ordered phase at high cholesterol concentrations, static phase separation occurs. In this area the partitioning should decrease with cholesterol concentration until the phase boundary for the high concentration phase is reached. After crossing the phase boundary to the liquid-ordered phase the decrease in partitioning should then continue with increasing cholesterol concentration. This is indeed what the data in the insert in Fig. 3 shows.

The temperature dependence of the interaction of ethanol with pure DMPC bilayers is evaluated in Fig. 4 *A*, which shows the enthalpic interaction parameter as a function of temperature at temperatures below and above the main phase transition of pure DMPC. Each data point is determined as the slope of graphs similar to those in Fig. 2. To evaluate the experimental reproducibility, three separate sets of H^E_{lipid} data for pure DMPC were measured. The reproducibility was found to within 3% (S.D.). Several observations are evident from the figure. The interaction of ethanol with pure DMPC liposomes is endothermic in the entire temperature range investigated, implying unfavorable interactions in terms of enthalpy. Consequently, the association of ethanol with lipid bilayers must be entropy driven in the concentration and temperature range studied. In addition, a pronounced peak develops in the temperature range close to the main phase transition. This unusual effect could either be due to changes in the standard enthalpy of association or to an enhanced affinity of the liposomes for ethanol. To resolve the interaction parameter into these two terms, we have adopted the solvent-null method of Zhang and Rowe (1992). This technique allows for direct determination of partitioning coefficients from estimates of the free (bulk) ethanol concentration in a liposome suspension. The results for ethanol partitioning into pure DMPC liposomes at temperatures between 14 and 36°C are illustrated in Fig. 4 *B* (*circles*). The data show that partitioning of ethanol into

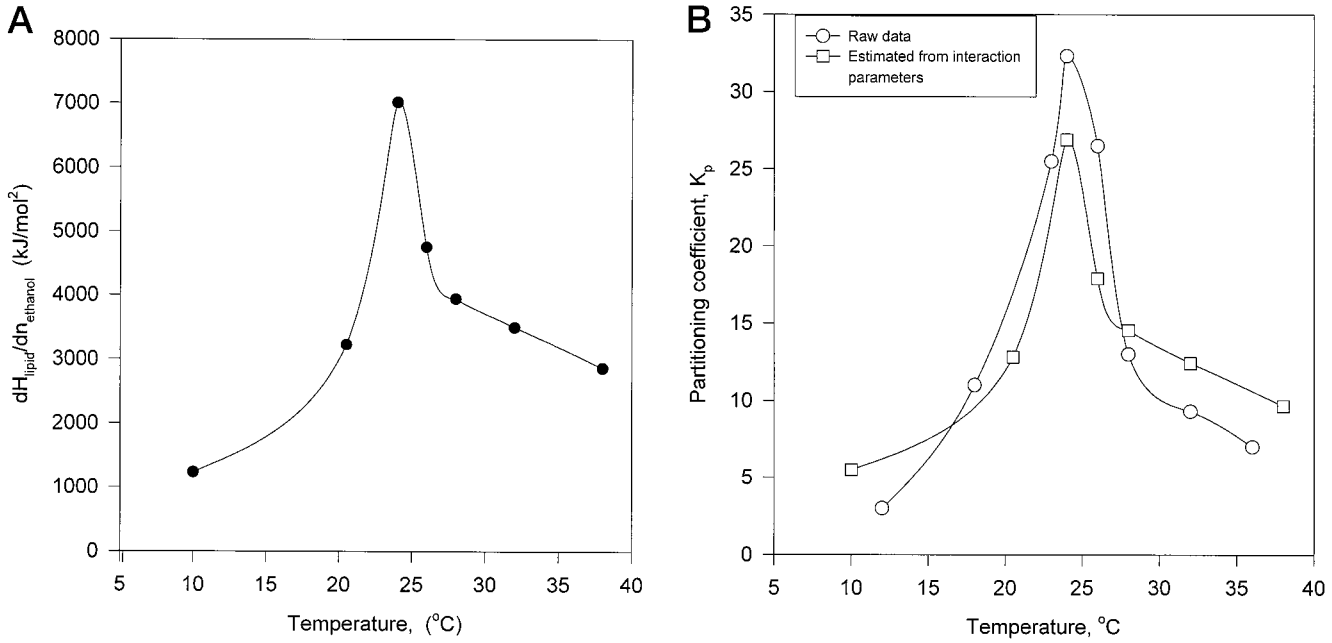

FIGURE 4 (*A*) The temperature dependence of the interaction parameter, $H_{\text{lipid–alcohol}}$. (*B*) The temperature dependence of (○) partitioning coefficients of ethanol into pure DMPC bilayers and (□) partitioning coefficients determined from the interaction parameter, $H_{\text{lipid–alcohol}}$. Applying a standard partitioning model, where ethanol is considered distributed between the membrane and the aqueous phase, partitioning coefficients (in molal units) can be estimated from the interaction parameters using the equation $K_m = H^E_{\text{lipid–ethanol}}/\Delta H^\circ M_{\text{lipid}}$ (Trandum et al., 1999b).

pure DMPC liposomes is proportional to $H_{\text{lipid–ethanol}}$ in the investigated temperature range. This implies that the standard enthalpy of partitioning is independent of the physical state of the bilayer, or in other words, the peak in the interaction parameter is due to an enhanced number of partitioned ethanol molecules rather than change in the standard enthalpy of partitioning. For a more direct comparison of the two data sets, partitioning coefficients estimated from the enthalpic interaction parameter are plotted in the same figure (*squares*). This is done by application of the standard partitioning model mentioned above and use of the equation $K_m = H^E_{\text{lipid–ethanol}}/\Delta H^\circ M_{\text{lipid}}$ (Trandum et al., 1999b), where $\Delta H^\circ = 16$ kJ/mol. K_m is here in molal units. To estimate K_m at temperatures other than 26°C, where ΔH° is known, the standard enthalpy of partitioning is determined using $\Delta H^\circ(T) = \Delta H^\circ(26^\circ\text{C}) + (T - 26^\circ\text{C})C_{p,\text{ethanol}}$. The molar heat capacity, $C_{p,\text{ ethanol,}}$ is found to be 136 J/mol K (Westh and Trandum, 1999). The reproducibility of the partitioning coefficient data in Fig. 4 *B* is only within ~30%, but the general trend, i.e., the development of a peak close to T_m, is the same. Due to the difference in uncertainty on the two different experimental methods, we have based the discussion of ethanol–liposome interactions for cholesterol containing liposomes on interaction parameters rather than partitioning coefficients.

The strong temperature dependence of liposome–ethanol interactions is further elucidated in Fig. 5. The figure illustrates the temperature dependence of the interaction parameter, $H_{\text{lipid–ethanol}}$, of five different lipid compositions above and below the main phase transition of pure DMPC. The DMPC liposomes contained 0, 4, 15, 25, or 35 mol% cholesterol. For pure DMPC bilayers and bilayers containing 4 mol% cholesterol, a pronounced peak develops close

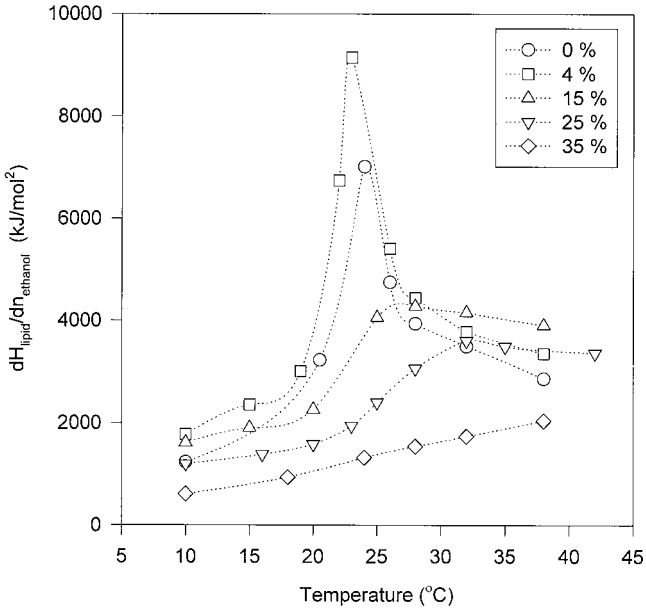

FIGURE 5 The temperature dependence of the interaction parameter, $H_{\text{lipid–alcohol}}$, at various cholesterol concentrations. ○, Pure DMPC; □, 4; △, 15; ▽, 25; and ◇, 35 mol%.

to the main phase transition. Assuming that the standard enthalpy of partitioning is not affected by changes in the bilayer state as Fig. 4 provides evidence for, the result implies that there is an increase in ethanol partitioning into lipid membranes containing low concentrations of cholesterol close to T_m. Furthermore, Fig. 5 shows that the peak in the interaction parameter (or partitioning coefficient) decreases with increasing cholesterol concentration, until eventually the interaction parameter becomes linearly dependent on temperature at cholesterol concentrations larger than 25%. At this cholesterol content, the lipid membrane is in the fluid-ordered phase in the entire temperature region studied (see Fig. 1). The overall variation of the interaction parameter across the lipid–cholesterol phase diagram shown in Fig. 5 fits into a more general picture of how cholesterol influences various physiochemical and functional properties of lipid bilayer membranes. The enhanced affinity of ethanol at low cholesterol concentrations parallels the finding of increased ion permeability (de Gier et al., 1979; Corvera et al., 1992), increased membrane fluctuations (Blume and Hillmann 1986; Genz et al., 1986; Michels et al., 1989; Raffy and Teissié, 1999), increased bilayer softness (Lemmich et. al., 1997; Richter et al., 1999), and an increase in the susceptibility of the bilayer to enzymatic degradation (Liu and Chong, 1999). Similarly, the suppressed binding of ethanol at high cholesterol concentrations parallels the reversed trend also found in the properties listed above. Interestingly, the overall appearance of the curves in Fig. 4 parallels qualitatively closely that observed for the specific heat profiles (Almeida et al., 1992; Vist and Davis, 1990 [DPPC/cholesterol]).

Finally, if the interaction parameter studied in this paper indeed reflects the partitioning coefficient, the linear increase in partitioning coefficient with temperature (for the DMPC liposomes with high cholesterol concentration) might be particularly interesting in the light of the enhanced alcohol sensitivity at higher temperatures observed both in vivo and for isolated cell membranes (Alkana et al. 1985; Bejanian et al. 1991). This implies that enhanced alcohol sensitivity simply could be due to an increased amount of alcohol in the lipid bilayer compared to the aqueous phase.

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