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Thermodynamics of transfer of cholesterol from gel to fluid phases of phospholipid bilayers

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Abstract

The partitioning of cholesterol between gel and fluid phases of model membrane bilayers has been studied by differential scanning calorimetry. The partition coefficients and thermodynamics of transfer between phases of dimyristoyl-, dipalmitoyl-, distearoyl- and diarachidoylphosphatidylcholines were determined using a regular solution model for the partitioning process. The partition coefficient, which is the ratio of cholesterol in fluid to gel phase lipid, varies from 2.3 to 8.4 as the acyl chain length increases from the C_{14} to the C_{20} bilayer and determined at the phase transition temperature. The enthalpies of transfer increase from -46 to $+32$ kcal/mol as the chain length increases, and there is a compensating increase in the entropy of transfer. The results are interpreted in terms of a disruption of gel phase lipid by the cholesterol, which for the thinner bilayers is increased because the cholesterol molecule spans across the two leafs of the bilayer. At bilayer thicknesses between 18 and 19 carbons, the cholesterol can fit into one-half of the gel phase bilayer, and the enthalpy becomes positive, suggesting less disruption in the gel phase relative to the thinner bilayers. The data support the interpretation that cholesterol does mediate fluidity by acting to disrupt gel domains of lipid.

Keywords: DSC; Bilayer; Cholesterol; Partitioning; Phospholipid; Thermodynamics; Regular solution

1. Introduction

The partitioning behavior of membrane solutes between model bilayer gel and fluid phases has been examined for several purposes. First, there is some evidence that biological membranes under certain circumstances have coexisting fluid and gel domains [1–3]. Data on partitioning of membrane components between these phases are thus important, since the presence of coexisting lipid phases could influence the lateral distribution of membrane solutes during cell processes [4–7]. A second reason for obtaining data on gel-fluid partitioning is to evaluate interactions between membrane components, the partitioning data providing information on the steric and energetic requirements for stability in gel or fluid membrane environments [8–11]. For example, there is evidence that steric mismatch in the hydrophobic areas of model membrane bilayers relative to the solute hydrophobic regions determines the physical behavior and thus partitioning of membrane solutes between gel and fluid phases [10–15].

Differential scanning calorimetry (DSC) has been used to study partitioning behavior of bilayer solutes by a number of authors [16–19,11]. In a recent study we reported a comparison of partitioning data obtained by DSC with a fluorescence quenching method for indocarbocyanines of varying alkyl chain lengths in phospholipid bilayers [10,11]. An advantage of the DSC method for studying partitioning in model membranes is that the enthalpy and entropy of transfer of the membrane solute between gel and fluid phases as well as the free energy (partition coefficient) can be obtained [11], and thus more information on the energetics of partitioning can be obtained by this technique. The indocarbocyanine study showed that partitioning into the gel phase was most when there was an approximate matching of the alkyl chain length of the solute with the acyl chain length of the bilayer.

The gel-fluid partitioning behavior of cholesterol in model bilayers has not been determined. Several studies indicate that interactions between cholesterol and phospho-

Abbreviations: DSC, differential scanning calorimetry; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphorocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphorocholine; DAPC, 1,2-diarachidoyl-*sn*-glycero-3-phosphorocholine.

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lipids in the bilayer may be complex [14,20,21]. There is evidence that cholesterol at high concentration induces order in fluid bilayers by freezing out trans-conformers in the vicinity of the steroid ring if bilayer thickness is appropriate [14,20]. DSC studies of cholesterol in a series of saturated phosphatidylcholine bilayers showed that there is an optimum bilayer thickness to accommodate hydrophobic matching with cholesterol, and that this match occurs around 17 to 18 carbon atoms in the acyl chain of the bilayer [12]. At chain lengths shorter than 17 carbons in the bilayer it was suggested that the gel phase is stabilized by cholesterol, while at longer chain lengths the gel is destabilized. Thus, the partitioning of cholesterol between gel and fluid phases will depend on a rather complex pattern of interactions that depend on chain length in the phospholipid bilayer. A recent paper also shows the importance of the size of substituted cholesterol derivatives in relation to their interactions in phospholipid bilayers [13].

Since it has been shown that partitioning thermodynamics can be obtained by DSC measurements of dilute concentrations of solutes in phospholipid bilayers [11], this study was undertaken to determine the gel-fluid partitioning behavior of cholesterol in phospholipids of varying acyl chain lengths (C_{14}-C_{20}). The data are analyzed by the regular solution model and the enthalpy, entropy and free energy of transfer of cholesterol from gel to fluid phase are evaluated in mixtures of mole fractions of cholesterol between 0–0.04. The dependence of the transfer properties on chain length in the phospholipid is correlated with previous work on the physical behavior of cholesterol in model membranes.

2. Experimental procedures

2.1. Lipids and reagents

The phospholipids were synthetic saturated 1,2-diacyl-*sn*-glycero-3-phosphocholines, in which the diacyl chains were C_{14}-(DMPC), C_{16}-(DPPC), C_{18}-(DSPC), and C_{20}-(DAPC). All phospholipids were obtained from Avanti Polar Lipids, Alabaster, AL, and were used as received. The cholesterol was from Sigma, St. Louis, MO, and was recrystallized from methanol-ether mixtures. The buffer was a 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.01% azide. All organic solvents were HPLC grade.

2.2. Liposome preparation

Multilamellar vesicles of the cholesterol-phospholipid mixtures were prepared from thin films of the lipids co-deposited from chloroform solutions, the solvents removed by evaporation under argon and storing under vacuum overnight. For each phospholipid 8–10 mixtures were made with mol fractions of cholesterol in the range of 0–0.04. Buffer was added to obtain a concentration of thermally active lipid of 0.25 mM, and then the mixture heated above the phase transition temperature of the phospholipid followed by vortexing. The heating and vortexing were repeated three times to insure complete dispersal of the lipids.

2.3. DSC measurements and data analysis

DSC scans were made using a MicroCal MC-2 scanning calorimeter at a scan rate of 0.19 deg/min. Scans were repeated at least three times in order to insure that reproducible traces were obtained, and that the cholesterol was properly equilibrated in the lipid mixtures.

To analyze the data we have chosen the model developed by Inoue, et al. [22]. This method was chosen over other approaches to dealing with regular solutions because it provides a convenient way to evaluate ΔG_{tr} and K_p directly from the DSC data. In the Inoue method the temperature of half-melting of the gel-fluid phase transition of a phospholipid is related to the mol fraction of solute, X_s by the equation:

$$T_m = T_o + \frac{T_o}{\Delta H_o}\left((\Delta G_s^o)\frac{X_s}{1-X_s} + (\Delta U_s)X_s \right) \quad (1)$$

where T_m is the melting temperature, T_o is the transition temperature in the absence of solute, ΔH_o is the calorimetric enthalpy of the transition in the absence of solute, ΔG_s^o is the standard free energy difference of the solute in the gel and fluid phases at T_o, and ΔU_s is the excess interaction energy (difference in excess enthalpy of the solute between fluid and gel phase), which determines the nonideality of mixing in the two phases. This equation is valid at low mol fraction as long as the entropy of transfer of solute from gel to fluid phase is concentration independent in the range of compositions studied. The limiting slope of a plot of T_m vs. X_s is:

$$\left(\frac{dT_m}{dX_s}\right)_{lim} = \frac{T_o}{\Delta H_o}(\Delta G_s^o + \Delta U_s) \quad (2)$$

Thus, from the limiting slope one can calculate the free energy of transfer of the cholesterol from gel to fluid phase, $\Delta G_{tr} = \Delta G_s^o + \Delta U_s = -RT\ln K_p$, and also determine the partition coefficient, $K_p = X_s(fl)/X_s(g)$. This partition coefficient is based on a standard state according to Henry's law in which the dilute solution is considered to have constant properties in the range of concentrations considered, the 0–0.04 mol fraction region [11].

From the concentration dependence of the enthalpy of the phase transition of the phospholipid as solute concentration increases it is possible to obtain the enthalpy of transfer of the solute from gel to fluid phase [19].

$$\Delta H_{cal} = \Delta H_o + \Delta H_{tr}\left(\frac{X_s}{1-X_s}\right) \quad (3)$$

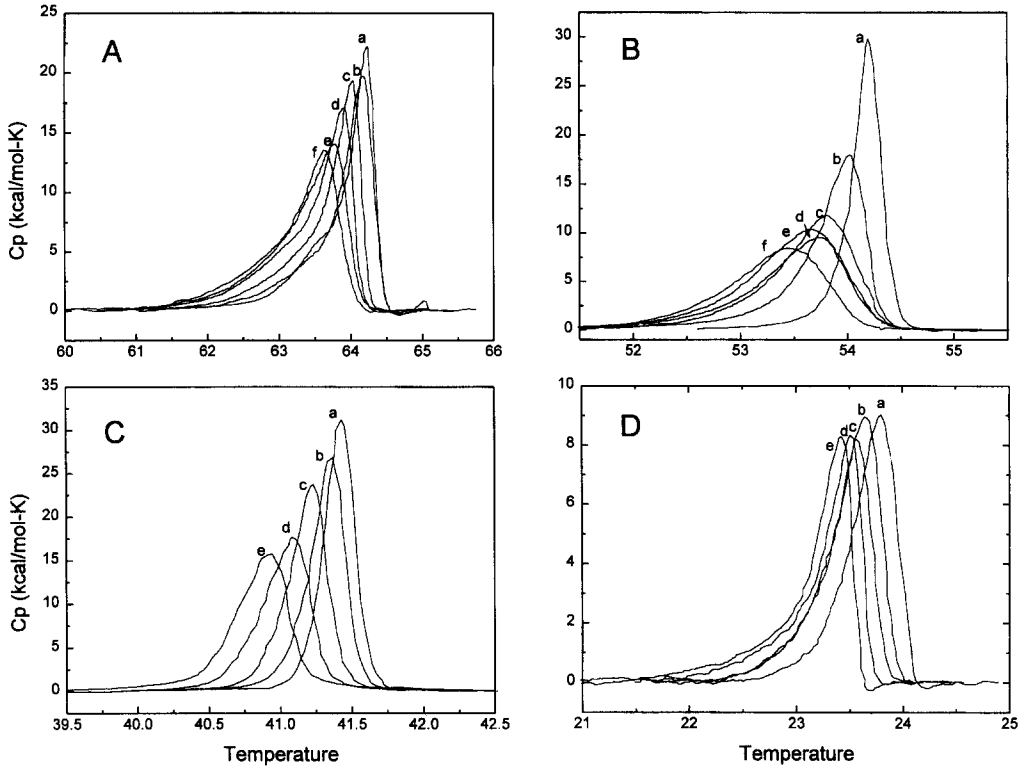

Fig. 1. Representative DSC transition curves for mixtures of cholesterol and A (DAPC), B (DSPC), C (DPPC), D (DMPC). Mol fractions of cholesterol are for A, a–f : 0, 0.01, 0.016, 0.027, 0.030, 0.037, resp.; B, a–f : 0, 0.01, 0.020, 0.025, 0.030, 0.035, resp.; C, a–e : 0, 0.005, 0.010, 0.020, 0.030, resp.; D, a–e : 0, 0.010, 0.017, 0.022, 0.026, resp.

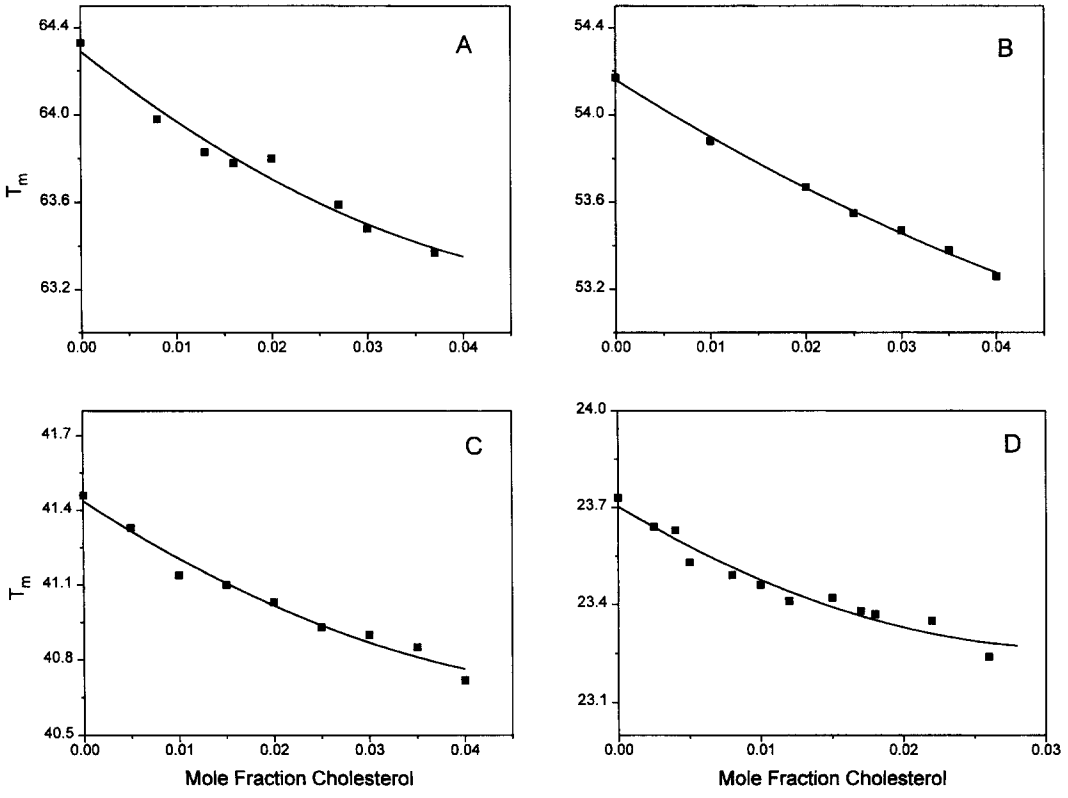

Fig. 2. Half-melting temperatures vs. mole fraction of cholesterol in mixtures with A (DAPC), B (DSPC), C (DPPC) and D (DMPC). The solid lines in the figures are calculated from the best fits to Eq. (1).

Therefore, from an analysis of the behavior of T_m and ΔH_{cal} as cholesterol is increased in dilute compositions, the free energy, enthalpy and entropy of transfer of the solute from gel to fluid phase can be obtained.

3. Results

Fig. 1 shows several typical DSC transition curves for mixtures of cholesterol with the various phospholipids. The curves all show the effects of the presence of a second component in the bilayer lipid, a broadening of the transition and lowering of the phase transition temperature with increased mol fraction of the cholesterol. The transitions are similar to those observed by others, except that because of the low mol fractions in this study, the second broad transition observed in phospholipid-cholesterol mixtures is not evident [12,23]. Also, since the pre-transition is not of interest in our work, the curves in Fig. 1 do not show the pre-transitions. The pre-transition peak areas decrease with increasing cholesterol, and are almost completely gone by about 5 mol%.

The midpoints (temperature of half-melting) were evaluated at the temperature for which the fractional area of the total transition was one-half. Values of T_m are plotted vs the mol fraction of cholesterol, X_s, in Fig. 2 for the four phospholipids studied. The T_m curves are nonlinear, the departure from linear behavior being greater at the higher mol fractions of cholesterol. This could be a result of the nonideality of interactions between cholesterol and the phospholipids as described by the regular solution model [22]. (See Eq. (1).) Another possible origin of the nonlinearity is the emergence of the second, broad component in the transition as cholesterol content is increased [12,23]. Interestingly, Harris, et al. [24] analyzed the nonlinearity for DPPC-cholesterol mixtures as due to transbilayer cholesterol dimer formation. This would seem unlikely at the lower concentrations used in our work. Because of the low concentrations used in our study, and since we are only interested in the limiting slope of these curves, the nonlinearity was not analyzed.

The free energies of transfer of the cholesterol from gel to fluid phase and thus the partition coefficients were evaluated from curve-fitting to Eq. (1). Table 1 shows the values of K_p and the transfer free energies obtained from the limiting slopes. The K_p values are all greater than 1, suggesting that partitioning is favored into the fluid phase in all cases at the transition temperature of each phospholipid bilayer. K_p values greater than 1 have been observed for most bilayer solutes that have been studied [8–11,25]. In general these results are consistent with the arguments that large bulky membrane solutes disrupt gel-phase packing, leading to a preference for partitioning into the fluid phase [8,9,25]. The only solutes which show preference for the gel phase have structures with hydrocarbon chains that are similar in length to the bilayer acyl chain lengths [10,11].

Table 1
Thermodynamic data for gel-fluid partitioning of cholesterol at the transition temperature

Bilayer	K_p (X_{fl}/X_g)	ΔG_{tr} (cal/mol)	ΔH_{tr} (kcal/mol)	ΔS_{tr} (cal/mol K)
DMPC	2.3 (0.3)	−500 (50)	−46 (13)	−153 (40)
DPPC	3.3 (0.3)	−700 (50)	−36 (8)	−112 (25)
DSPC	3.7 (0.3)	−900 (50)	−5 (13)	−13 (40)
DAPC	8.4 (0.4)	−1400 (40)	+32 (18)	+99 (50)

Numbers in parentheses are error estimates from fitting parameters and error propagation.

Estimates of the enthalpy of transfer from gel to fluid phase can be obtained from the concentration dependence of the calorimetric enthalpies for the transitions, as mentioned above. Fig. 3 shows the enthalpy data for the four bilayers in the low mol fraction range. At mol fractions higher than those used in this study the enthalpies for all of the transitions decrease, as has been observed by others [12,23]. While the enthalpies have fairly large uncertainties (repeatability of ΔH_c is about 0.4 kcal/mol), the data in Fig. 3 show trends in the behavior of the calorimetric enthalpy. The DMPC and DPPC mixtures with cholesterol show definite negative slopes, while DSPC has a slight negative and DAPC a positive slope. Using Eq. (3), it is possible to estimate the enthalpy of transfer of cholesterol from the gel phase to the fluid phase of the phospholipid bilayers. The solid lines in the figures are calculated by least squares fitting, and the ΔH_{tr} values are shown in Table 1. Fig. 4 summarizes the transfer thermodynamics for the process of moving cholesterol from gel to fluid phase at the phase transition temperature of each phospholipid bilayer. The results show a clear compensation be-

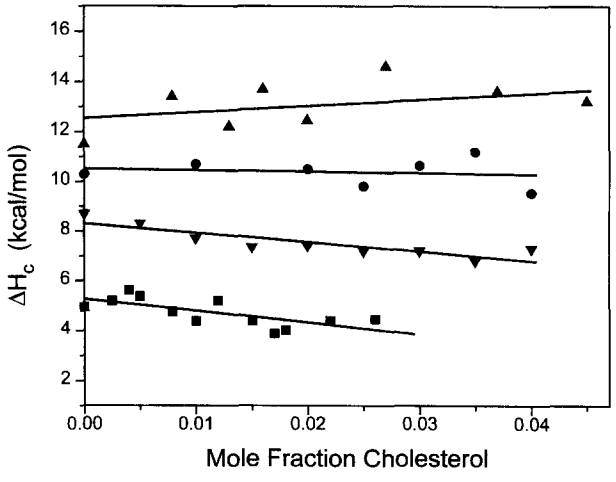

Fig. 3. Calorimetric enthalpies of gel-fluid phase transitions for phospholipid bilayers vs. mol fraction of cholesterol in the mixtures. DMPC (■); DPPC (▼); DSPC (●); DAPC (▲). Solid lines are best fits to Eq. (3).

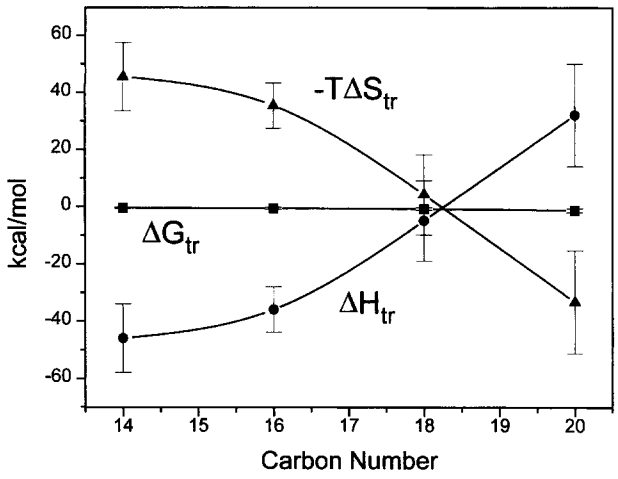

Fig. 4. Free energy, enthalpy and entropy of transfer of cholesterol from gel to fluid phase in bilayer vs. carbon number in the acyl chains of the bilayer.

tween the enthalpy and entropy effects for the transfer process. This behavior was also observed for the transfer of indocarbocyanines between gel and fluid phase lipids [11]. Thus, the preferences of cholesterol for the fluid phase, as indicated by the small negative free energies of transfer, result from a balance between rather large entropic and enthalpic effects.

4. Discussion

4.1. Thermodynamics of partitioning

The behavior of K_p for cholesterol when determined at the transition temperature for each phospholipid does not provide much insight into the characteristics of the partitioning process. The small variation from about 2 for the C_{14}-homolog to 8 for the C_{20}-phospholipid represents an only modest preference for the fluid phase that increases with acyl chain-length. (See Fig. 5.) Raman spectral data on these lipid bilayers show that there is more total order at the phase transition temperature for the longer chain homologs [28,29], and this observation is consistent with the trends in the enthalpies of melting of the bilayers [12]. Thus, one could explain the trend toward greater partitioning into the fluid phases of the longer chain bilayers to be a result of less disruption of the gel phase for DMPC because there is basically less gel to disrupt at the transition temperature, and since the order in the longer chain derivatives is greater, the cholesterol is more disruptive, and destabilizing of the packing in the gel phase lipid favoring partitioning into the fluid phase.

The partitioning behavior is somewhat more dramatic if the comparisons of K_p are made at a common reduced temperature, such as 10 degrees below the phase transition temperature in each case. Again the Raman data indicate substantially more order (more *trans* conformers) in the gel phases at temperatures below the phase transition temperature [28,29]. Since the ΔH_{tr} has been estimated, it is possible to use the van 't Hoff relationship to determine K_p at other temperatures. Fig. 5 presents results for K_p at the reduced temperature along with the values at the corresponding T_m for each phospholipid. This comparison shows that the acyl chain length of the phospholipid now becomes more important in determining the partitioning of cholesterol between the two phases. For DMPC the value of K_p increases dramatically to over 1000. On the other hand, K_p for DAPC decreases and shows a slight preference for gel phase partitioning at the reduced temperature.

That K_p changes so much in going from T_m to 10° below the transition temperature is a result of the differences in transfer enthalpies. The enthalpies of transfer of cholesterol from gel to fluid phase are quite negative for the DMPC and DPPC, and increase steadily to a positive value for DAPC as shown in Fig. 4. Because the free energy of transfer is small, the entropy of transfer is also negative and mirrors the trend in the enthalpy by decreasing with increased chain length in the phospholipid; that is, $-T\Delta S$ is positive and decreases with increasing bilayer thickness. This compensative behavior was also observed for partitioning of indocarbocyanines between gel and fluid phases of DPPC and DSPC [11]. In this case the indocarbocyanines which had markedly different chain lengths from those of the bilayer acyl chains showed large negative enthalpies and entropies of transfer. Negative enthalpies of transfer of solutes from gel to fluid phase can be interpreted as due to a destabilization of the gel phase relative to the fluid phase of the phospholipid [10,11]. A disruption of packing in the gel raises the enthalpy of the gel state, and if some ordering occurs in the fluid phase, negative enthalpies and entropies of transfer are expected. It has been argued by others that disruptive interactions

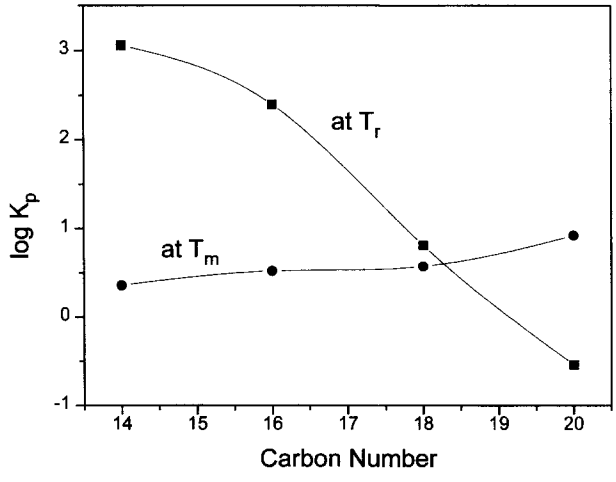

Fig. 5. Partition coefficient of cholesterol between gel and fluid phases at the melting temperature (T_m) of the bilayer and at reduced temperature (T_r, 10° below the phase transition temperature) vs. carbon number of the acyl chains in the bilayer.

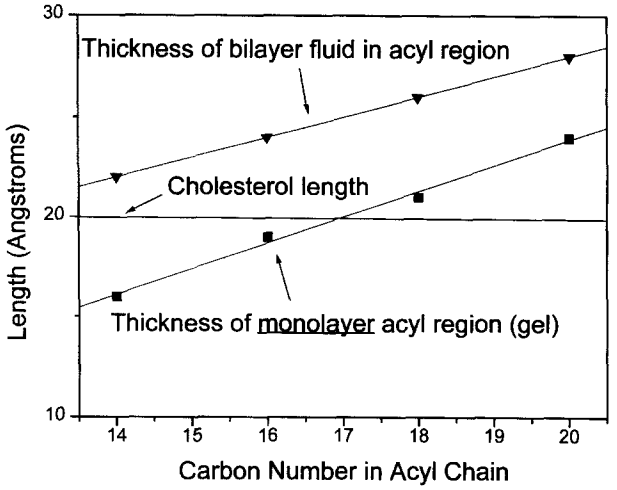

Fig. 6. Thickness of fluid bilayer region and thickness of gel monolayer (half of bilayer) region for phospholipids with varying carbon number in the acyl region. The horizontal line represents the length of an extended cholesterol molecule.

between membrane solutes and bilayer acyl chains in the gel phase are important in determining the overall partitioning of solutes between gel and fluid phases [9–11,26,27].

4.2. Partitioning and bilayer thickness

Several questions arise regarding the data presented in Figs. 4 and 5 for cholesterol partitioning. First, if we accept that the rather bulky steroid chain could be disruptive of the gel phase of a phospholipid bilayer, how does the bilayer thickness affect the partitioning behavior such that there is a cross-over of K_p to gel-phase preference at the reduced temperatures by about 19 carbons in the acyl region, and that the enthalpies of transfer are quite negative for the shorter chain bilayer, but increase as chain length of the acyl region increases. In order to analyze the trends in the partitioning, it is useful to examine estimates of the bilayer thicknesses that have been made for both fluid and gel bilayers. Sankaram and Thompson [14] have analyzed X-ray and NMR data on phospholipid bilayers, and report values for the effective acyl chain length in fluid bilayers for the 14, 16 and 18 carbon PC's, so it is possible to estimate the thickness of the acyl chain region in the fluid phase. The thickness for the C_{20}-PC can be obtained by extrapolation of these data. We present in Fig. 6 these data for the thickness of the fluid acyl region. Also shown in Fig. 6 are estimates of the length of acyl chains in the all *trans* conformation, which can be used to gauge the maximum possible half-thickness of the gel phase in the acyl region. The actual dimensions will be somewhat less because the gel phase is thought to have some 'melted' *gauche* conformers near the ends of the chains. Superimposed on the graph is a line representing the length of an extended cholesterol molecule, estimated from molecular bond lengths and atomic radii to be about 20 Å. Several issues arise from this graphical representation of the bilayer thickness data. First, although the total bilayer width of fluid lipid is 10–12 Å less than the corresponding gel bilayer, the total thickness of the acyl region for the fluid phases of DMPC through DAPC does not change very much. The variation is from about 22 to 28 Å in progressing from DMPC to DAPC. Note that if we assume that cholesterol positions itself within the acyl region with its OH-group oriented toward the carbonyl region of the acyl chains [14,20,21], it will be accommodated in the fluid phase with just enough room to cross the whole acyl chain region for DMPC and with 6–8 Å extra space in the acyl region for DAPC. Thus, one might expect little difference in the behavior of cholesterol in the various fluid phases considered here; that is, the thicknesses of the fluid bilayers are similar so that cholesterol would exist in a relatively similar environment in all of the fluid phases. From deuterium NMR data on the acyl chains in fluid phospholipids the order parameters for DMPC, DPPC and DSPC acyl chains are quite similar in the presence of cholesterol [14,20]. Thus, in dilute solutions of cholesterol in the phospholipids studied here, it is likely that there is relatively little difference, except that are related to the small thickness differences, in the thermodynamic parameters of the cholesterol in the four fluid phase bilayers studied.

The gel phases present a different situation, however. Note that in Fig. 6 the half-thickness of the gel phase phospholipids starts out less than the cholesterol length. This means that in DMPC cholesterol will extend across the bilayer into the opposite half of the acyl region. This condition will also prevail for the DPPC bilayer, although not so extensively. By the 18-carbon lipid the cholesterol will fit approximately into the half-bilayer acyl region, and the DAPC will have ample room. Thus, an explanation of the trends in the enthalpy of transfer is that for the thinner bilayers disruption across substantial regions of the gel acyl region occur, causing the partial molal enthalpy of the solute in the gel phase to be disproportionately high relative to the fluid phase, and thus produce large negative transfer enthalpies. If the disruption occurs only in one-half of the gel bilayer, as for the 20-carbon lipid, DAPC, then the enthalpy of the gel state cholesterol is lower, and the transfer enthalpy becomes, in fact, slightly positive. That the cross-over from net disruption to a slight ordering at between 18–19 carbon atoms in the acyl region occurs is consistent with the size of the cholesterol molecule just fitting within half of the gel phase bilayer at these distances. Because the enthalpy becomes positive at this cross-over point, the ordering effect of cholesterol in the fluid phase could be more than enough to compensate for the small disordering in the gel phase. This will lead to gel phase preference at lower temperatures, as is observed at the reduced temperatures for the DAPC case. It is clear that bilayer thickness is an important parameter in defining the behavior of cholesterol under these circumstances.

The results here suggest that for the shorter chain bilayers gel-phase disruption is a major factor in determining the partitioning behavior of the cholesterol in a two-phase mixture. One of the commonly defined roles for cholesterol in biological membranes is that it serves as a mediator of membrane fluidity by breaking up gel domains that would make the membrane too rigid. Our results show that except for the longer chain bilayers, major disruption of gel domains seems a very likely role for cholesterol in a gel membrane environment.

Acknowledgements

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