The Influence of Cholesterol on Nearest-Neighbor Recognition in Saturated Phospholipid Membranes[1]

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Abstract: The thermodynamic preference for one phospholipid to become a covalently attached nearest neighbor of another in the bilayer state provides unique insight into the supramolecular structure of that membrane [Krisovitch, S. M.; Regen, S. L. *J. Am. Chem. Soc.* **1992**, *114*, 9828]. In this work, such "nearest-neighbor recognition" measurements have been used to study the effects of cholesterol on phospholipid mixing in the physiologically relevant fluid phase and in the gel–fluid coexistence region. Specific disulfide-based dimers that have been employed were derived from 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine. When the difference in alkyl chain length between the equilibrating monomers is small (i.e., two methylene groups per chain), the presence of cholesterol (10, 30, and 40 mol%) has no effect on the dimer distribution; in each case, a statistical mixture is observed. This finding establishes that these monomeric units are randomly arranged in the bilayer at the molecular as well as the supramolecular level. When the difference in chain length is increased to four methylene units, high concentrations of cholesterol (≥30 mol %) significantly enhance homodimer formation in the fluid phase but not in the gel–fluid region. It has also been found that increasing concentrations of cholesterol result in increased rates of dimer equilibration in the fluid and gel–fluid states. The biological implications of these nearest-neighbor recognition studies are briefly discussed.

Introduction

One of the most significant challenges presently facing chemists and biologists is to define the supramolecular structure of biological membranes. In particular, the time-averaged, lateral distribution of the lipids and proteins that make up these biological enclosures remains to be clarified.[3] We have recently devised a chemical method for probing the supramolecular structure of simple model systems.[4,5] Unlike most other approaches that have previously been employed, ours is directly applicable to the physiologically relevant fluid phase. The essence of this technique may be summarized as follows: a 1:1 molar mixture of two phospholipid homodimers (AA and BB) is equilibrated *via* a thiolate–disulfide interchange reaction.[6] In order to ensure that an equilibrium state has been reached, a similar equilibration reaction is carried out starting with the corresponding heterodimer, AB. The extent to which the ratio of AA/AB/BB deviates from a molar ratio of 1/2/1 (a random distribution) reflects the thermodynamic preference for one phospholipid to become a covalently attached, nearest-neighbor of another; i.e., it defines the ability of an equilibrating phospholipid monomer to "recognize" a nearest-neighbor. When dimer distributions are found to be purely statistical, this fact, in and of itself, proves that the lipid components are randomly distributed throughout the membrane at the molecular as well as the supramolecular level. It establishes that there is no thermodynamic driving force for nearest-neighbor recognition and domain formation. For those cases in which nearest-neighbor recognition is observed, the existence of domains is inferred. This inference rests on the assumption that the packing forces that govern nearest-neighbor recognition are the same as (or very similar to) those that govern domain formation. Although these lipid dimers bear a resemblance toward cardiolipin, we explicitly point out that biological membranes are largely comprised of single phospholipid molecules, not dimers. Thus, it is important to realize that these chemically-equilibrating dimer-based bilayers must be considered as membrane mimetic in character.

In this paper, we report the influence that cholesterol has on nearest-neighbor recognition within two such membrane-mimetic systems.[7] The reason we have chosen to examine cholesterol as a perturbing agent was based on two important considerations. First, cholesterol represents a major component of mammalian cell membranes. In human red blood cells, for example, it accounts for almost 50% of the total lipid content.[3] Second, the precise role that cholesterol plays in determining the supramolecular structure of biological membranes is not well understood. In particular, its influence on the time-averaged lateral distribution of other biomembrane components remains to be defined. In view of the strong condensing and ordering effects that cholesterol is known to have on fluid phospholipid membranes,[8] we hypothesized that its presence could promote "organization" within the phospholipid component due to an enhancement of van der Waals forces. It is noteworthy that although cholesterol–phospholipid interactions have been the subject of numerous reports,[9–28] the

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(2) Unilever Graduate Fellow, 1989–1992.
(3) For an excellent review regarding the detection of lipid domains in biological membranes, see: Tocanne, J. F. *Comm. Mol. Cell Biophys.* **1992**, *8*, 53.
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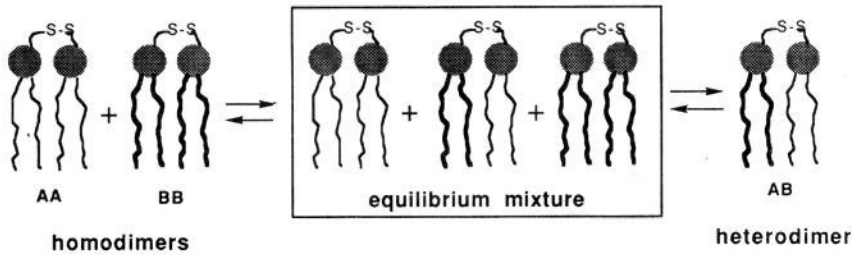
Scheme I



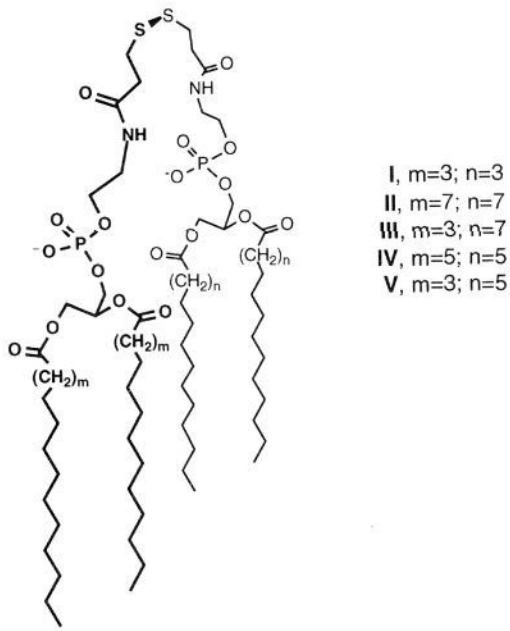


Figure 1. Structure of lipid dimers.

question of whether or not cholesterol can alter the mixing behavior of phospholipids in the physiologically relevant fluid phase has not (to the best of our knowledge) previously been considered. One caveat with our use of nearest-neighbor recognition in the present study is that it does not provide insight into the supramolecular structure of components other than the phospholipid dimers, themselves. Thus, the lateral distribution of cholesterol within the specific membranes described herein cannot be assessed by this technique; only its influence on the phospholipid portion of the bilayer can be measured.

Results

Choice of the Phospholipid Dimers Used in This Study. The specific phospholipid dimers that have been selected for this work were those that we have previously reported (Figure 1).[5] Lipids **I, II,** and **IV** may be viewed as dimeric analogs of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), on the basis of their fatty acid content and their similarity in melting behavior.[5] Lipid dimers **III** and **V** represent the corresponding heterodimers. Because the differences in chain length among the monomeric components are small, compared with the range of fatty acids that are commonly found in naturally occurring phospholipids (i.e., 12–22 carbon atoms in length),[29] we felt that any cholesterol effects seen here would represent a lower limit, in terms of what is likely to exist in biological membranes.

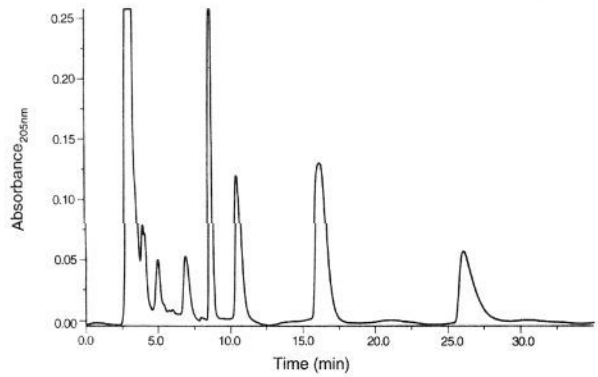


Figure 2. High-pressure liquid chromatogram of an equilibrated dimer mixture formed from a 1:1 molar mixture of **I** and **IV** plus 40 mol % cholesterol, after 2 h at 53 °C. Retention times for **I, V,** and **IV** are 10.5, 16.2, and 26.0 min, respectively. Retention time for cholesterol is 8.6 min. Thiol lipid monomers have retention times of 4.9 (14-carbon alkyl chain) and 6.8 min (16-carbon alkyl chain).

Protocol for Dimer Equilibration. The protocol that we have used for carrying out dimer equilibration reactions was similar to that previously described.[5] Large unilamellar vesicles (1000 Å) were first prepared via extrusion methods.[30] Chemical equilibration was then carried out by partial reduction to the corresponding thiol phospholipid monomers, followed by intramembrane thiolate–disulfide interchange.[6] The course of the equilibration process was monitored by HPLC (Figure 2).

Nearest-Neighbor Recognition in the Fluid Phase. From our previous studies, the gel to liquid–crystalline phase transition temperatures (T_m) for **I–V** were found to be 22.7, 55.4, 33.9, 41.9, and 31.2 °C, respectively.[4,5] In order to probe nearest-neighbor recognition in the fluid phase, we chose reaction temperatures that were higher than the T_m of the higher melting homodimer within each series.

In Figure 3, we show the equilibration of lipid membranes derived from **I–III** in the presence of varying concentrations of cholesterol. The equilibrium ratio of **III/I** (ratio of heterodimer to the "shorter"-homodimer) that was observed with 10 mol % cholesterol was the same as that found in its absence; both mixtures were statistical (10 mol % cholesterol, 1.98 ± 0.06/1; 0 mol %

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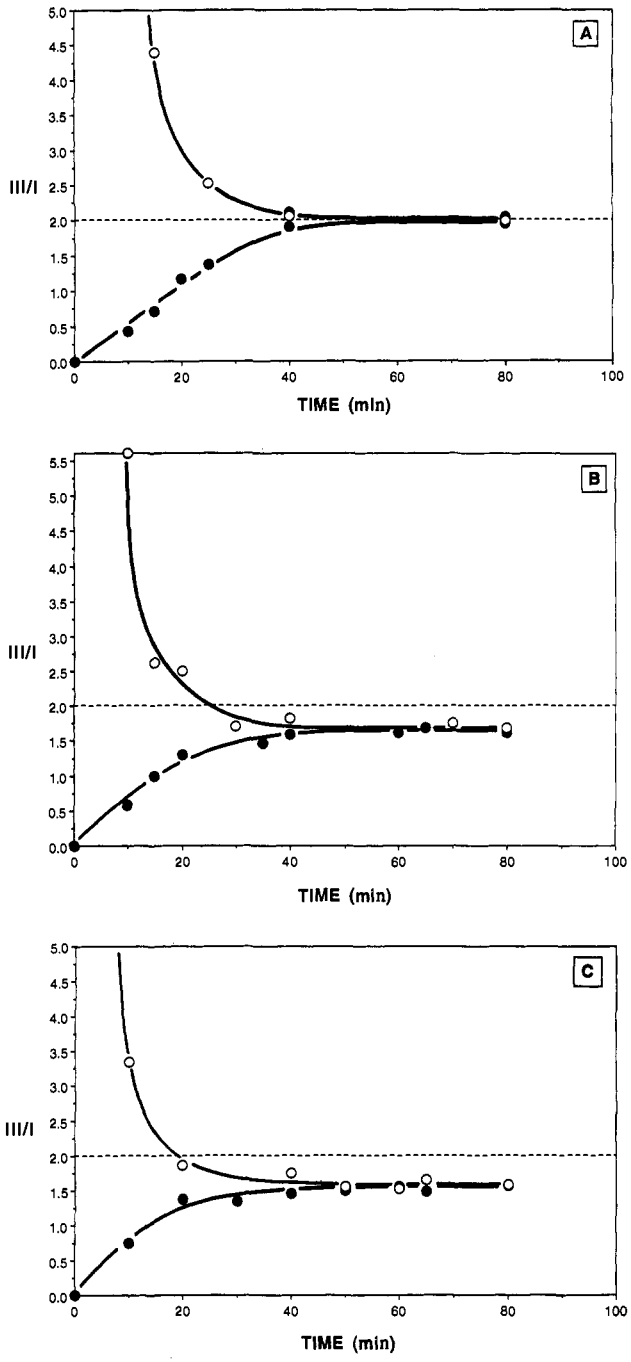

Figure 3. Plots of the molar ratio of **III**/**I** plus (A) 0 mol % cholesterol, (B) 30 mol % cholesterol, and (C) 40 mol % cholesterol, as a function of equilibration time at 60 ± 1 °C. Vesicles were prepared from **III** plus various cholesterol concentrations (○) and also from a 1/1 molar mixture of **I**/**II** plus various cholesterol concentrations (●). In all cases, equal molar ratios of symmetrical dimers were produced (±5%).

cholesterol, 2.01 ± 0.06/1). When the cholesterol content was increased to 30 mol %, however, the dimer ratio was 1.68 ± 0.07/1. This level of nearest-neighbor recognition corresponds to a thermodynamic preference for homodimer formation of ΔG = 0.12 ± 0.03 kcal/mol. Analogous membranes that contained 40 mol % cholesterol yielded a ratio of 1.55 ± 0.04/1 (ΔG = 0.17 ± 0.02 kcal/mol). The error values that are indicated here and throughout this paper represent two standard deviations from the mean, using a minimum of five independent experiments.

Similar equilibration reactions that were carried out using bilayers derived from **I**, **IV**, and **V** gave a dimer distribution that was found to be statistical over the entire range of cholesterol that was employed: 0% cholesterol, 2.01 ± 0.06/1; 10% cholesterol

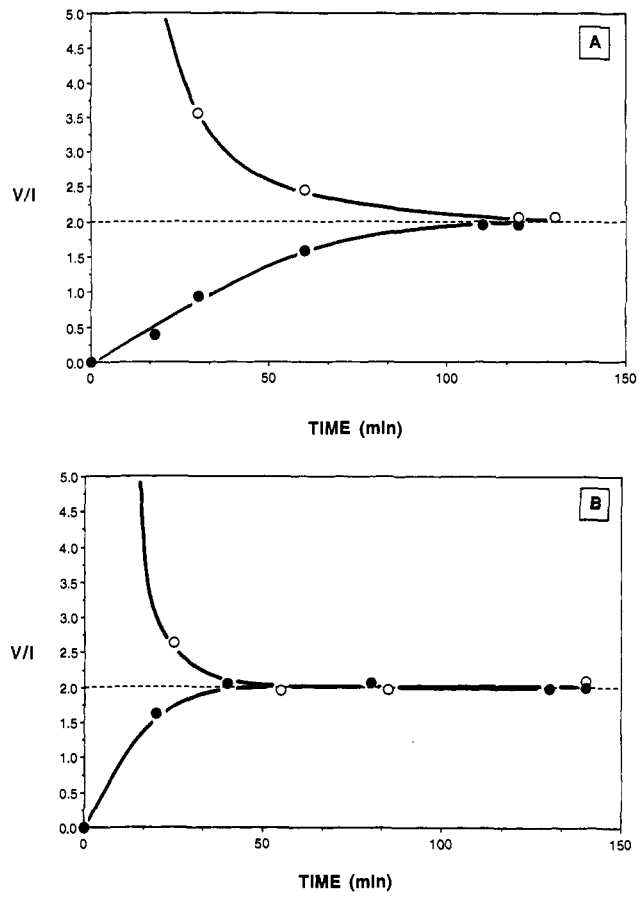

Figure 4. Plots of the molar ratio of **V**/**I** plus (A) 0 mol % cholesterol and (B) 40 mol % cholesterol, as a function of equilibration time at 53 ± 1 °C. Vesicles were prepared from **V** plus various cholesterol concentrations (○) and also from a 1/1 molar mixture of **I**/**IV** plus various cholesterol concentrations (●). In all cases, equal molar ratios of symmetrical dimers were produced (±5%).

2.05 ± 0.06/1; 30% cholesterol, 2.06 ± 0.06/1; 40% cholesterol, 2.02 ± 0.04/1 (Figure 4).

Nearest-Neighbor Recognition in the Gel–Fluid Coexistence Region. Equilibrium compositions of **I**–**III** that were obtained in the presence of varying concentrations of cholesterol at 33 °C are summarized in Figure 5. Within experimental error, the inclusion of 10 and 35 mol % of the sterol did not significantly alter the extent of nearest-neighbor recognition; i.e., the ratios of **III**/**I** for 0, 10, and 35 mol % cholesterol were 0.76 ± 0.06/1, 0.69 ± 0.06/1 amd 0.69 ± 0.06/1, respectively.[31]

In Figure 6, we show the influence that cholesterol has on the melting behavior of a membrane made from **I**–**III**. The specific dimer ratio that was used in these experiments corresponds to the equilibrium ratio within the gel–fluid region (33 °C), in the absence of cholesterol. It should be noted that these high sensitivity differential scanning calorimetry (*hs*-DSC) thermograms were recorded under nonexchanging conditions. In the absence of cholesterol, two broadened endotherms were clearly visible; one was centered at 23 °C and the other at 48 °C.[5] Incremental addition of cholesterol resulted in *a continuous shift of both endotherms toward lower temperatures*. In addition, the transition enthalpy for each endotherm decreased with increasing

(31) In order to rule out the possibility that the presence of DTT influences the intrinsic structure of the membrane, we have carried out analogous equilibrium exchange reactions for gel–fluid bilayers made from **I**–**III** (33 °C) that contain a 1/1 mixture of the corresponding thiol monomers (13 mol %). In this case, the thiolate–disulfide interchange is initiated simply by pH adjustment; i.e., *DTT is completely absent from the system.* The fact that we observe the same dimer distribution as that obtained when DTT is used to generate thiol monomer, proves that DTT has no significant affect on nearest-neighbor recognition.

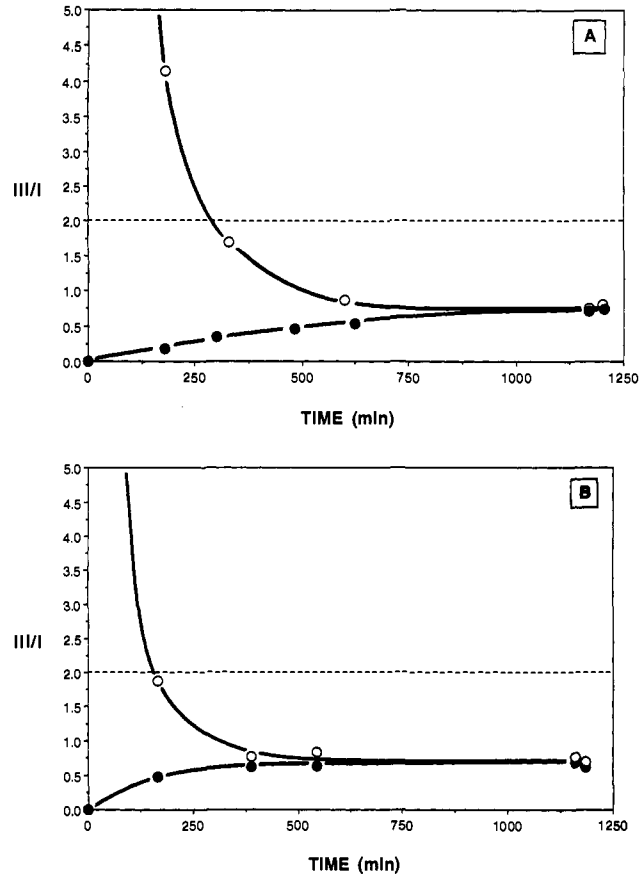

Figure 5. Plots of the molar ratio of **III/I** plus (A) 0 mol % cholesterol and (B) 35 mol % cholesterol, as a function of equilibration time at 33 ± 1 °C. Vesicles were prepared from **III** plus various cholesterol concentrations (○) and also from a 1/1 molar mixture of **I/II** plus various cholesterol concentrations (●). In all cases, equal molar ratios of symmetrical dimers were produced (±5%).

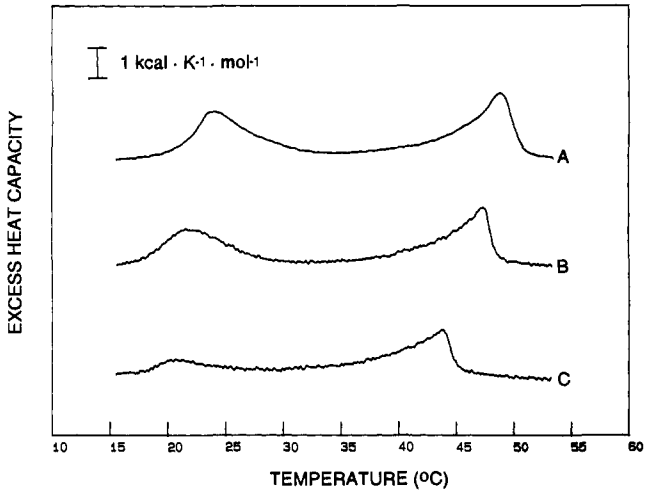

Figure 6. High-sensitivity excess heat capacity profile of **I/III/II** in a 1/0.7/1 molar ratio plus (A) 0 mol % cholesterol, (B) 10 mol % cholesterol, and (C) 35 mol % cholesterol.

cholesterol concentrations; i.e., on going from 0–35 mol % cholesterol, the transition enthalpy of the lower endotherm decreased by ca. 75% and the higher endotherm by ca. 30% (Figure 7).

Figure 8 shows the equilibrium compositions of gel–fluid bilayers that were derived from **I**, **IV**, and **V** in the presence of varying amounts of cholesterol. For these experiments, it was found necessary to slightly lower the equilibration temperature in order to maintain the gel–fluid state, vide infra: 0% cholesterol

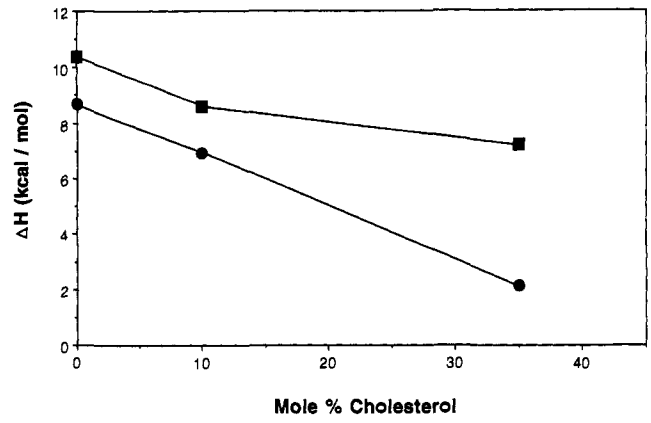

Figure 7. Plot of enthalpy as a function of cholesterol concentration for 1/0.7/1 molar mixtures of **I/III/II**; enthalpy of the lower endotherm (●); enthalpy of the upper endotherm (■).

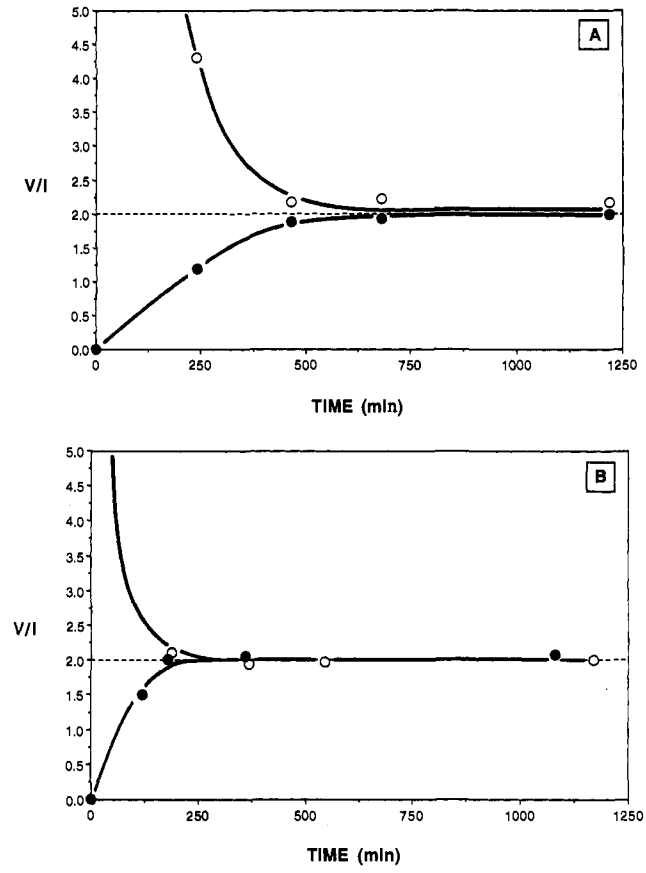

Figure 8. Plots of the molar ratio of **V/I** plus (A) 0 mol % cholesterol and (B) 40 mol % cholesterol, as a function of equilibration time. Equilibration temperature was 33 ± 1 °C in (A) and 28 ± 1 °C in (B). Vesicles were prepared from **V** plus various cholesterol concentrations (○) and also from a 1/1 molar mixtures of **I/IV** plus various cholesterol concentrations (●). In all cases, equal molar ratios of symmetrical dimers were produced (±5%).

(33 °C), 10 and 30% cholesterol (30 °C), and 40% cholesterol (28 °C). In each case, the ratio of **V/I** was found to be statistical; i.e., the ratios were 2.03 ± 0.05/1, 2.03 ± 0.08/1, 2.00 ± 0.06/1, and 2.01 ± 0.04/1 for 0, 10, 30, and 40 mol % cholesterol, respectively.

In the absence of cholesterol, a 1/2/1 mixture of **I/V/IV** displayed a single broadened endotherm having a T_m of 33 °C (Figure 9).[5] Introduction of the sterol into the membrane resulted in a continuous lowering of T_m and a decrease in the transition enthalpy (Figure 10). One striking feature in this set of

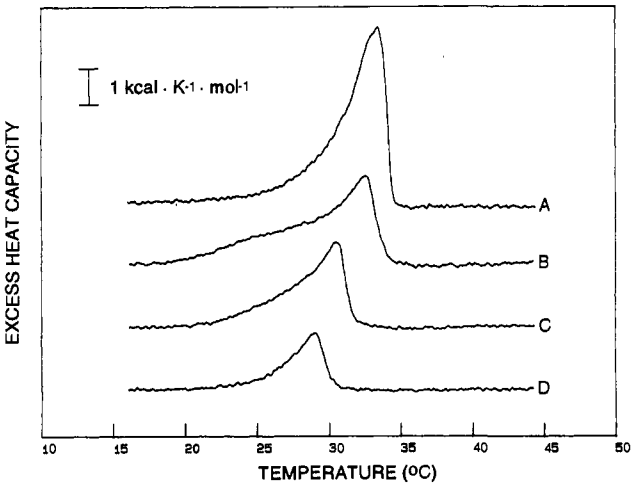

Figure 9. High-sensitivity excess heat capacity profile of I/V/IV in a 1/2/1 molar ratio plus (A) 0 mol % cholesterol, (B) 10 mol % cholesterol, (C) 30 mol % cholesterol, and (D) 40 mol % cholesterol.

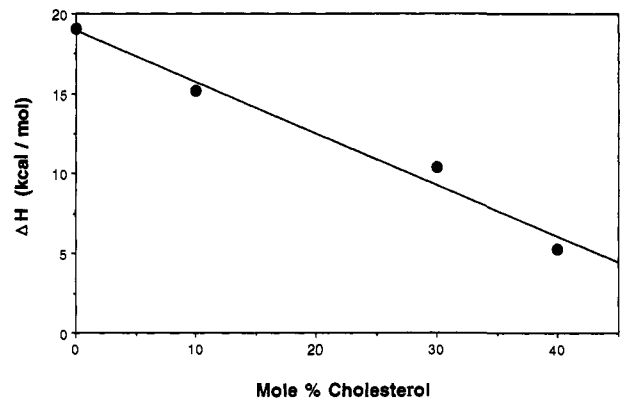

Figure 10. Plot of enthalpy as a function of cholesterol concentration for 1/2/1 molar mixtures of I/V/IV.

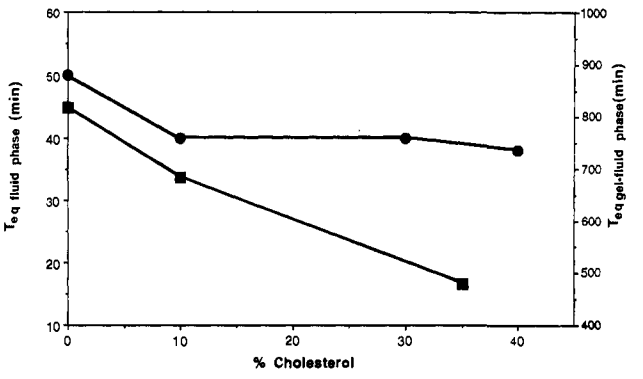

Figure 11. Plot of the time required to reach equilibrium (T_{eq}) as a function of percent cholesterol for equilibration experiments using lipid dimers I, II, and III in the fluid phase (●) and the gel–fluid coexistence region (■).

thermograms is the pronounced broadening and asymmetry that is associated with the membrane that contains 10 mol % cholesterol.

Influence of Cholesterol on the Time Required to Reach Equilibrium. Although the primary aim of the present study has been to define equilibrium product mixtures, as a function of cholesterol content, we note one intriguing aspect of the exchange kinetics. Specifically, the rate of dimer equilibration was found to increase with increasing concentrations of cholesterol in both the fluid and gel–fluid regions (Figures 11 and 12).

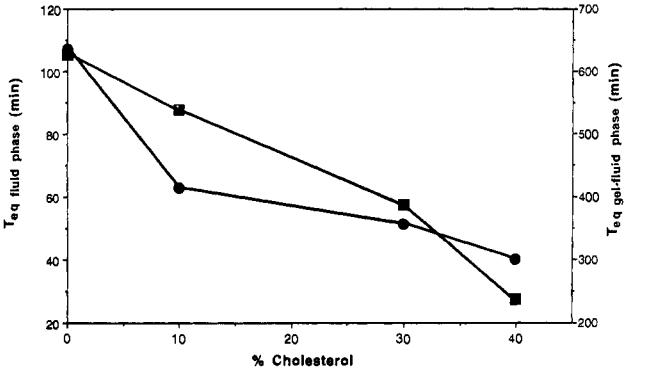

Figure 12. Plot of the time required to reach equilibrium (T_{eq}) as a function of percent cholesterol for equilibration experiments using lipid dimers I, IV, and V in the fluid phase (●) and the gel–fluid coexistence region (■).

Discussion

Differences in Alkyl Chain Length of Two Carbons Lead to Random Mixtures of Phospholipids in the Fluid Phase and in the Gel–Fluid Coexistence Region Regardless of Cholesterol Content. The equilibrium dimer distributions that were measured for fluid and gel–fluid bilayers made from I, IV, V, and cholesterol show that this sterol has no influence on the lateral arrangement of these phospholipids. In every case, a statistical mixture of dimers was observed. This finding establishes that *the monomeric units are randomly arranged throughout the bilayer at the molecular and supramolecular level.*

The unusual broadening and asymmetry that is evident in the *hs*-DSC thermogram of a bilayer composed of a 1/2/1 molar ratio of I/V/IV plus 10 mol % cholesterol is noteworthy (Figure 9). This behavior indicates that all of the lipid components are not randomly distributed within the gel–fluid phase. Since our nearest-neighbor recognition results establish that *the phospholipids, themselves, are randomly arranged*, this melting behavior implies that it is the cholesterol portion of the membrane that is nonrandom. The lateral arrangement of cholesterol in the other membranes of this series is less certain.

One general feature of the dimeric phospholipid systems that have been used in this study deserves comment; i.e., the persistence of phase transition behavior in the presence of high concentrations of cholesterol. This type of behavior is normally not observed for bilayers composed of single phospholipid molecules; instead, extreme broadening is usually observed.[12,14,21] The persistence of phase transition behavior in our dimer-based phospholipid membranes is a likely consequence of an "enforced cooperativity" in the melting process that results from the covalent coupling of nearest neighbors.

Cholesterol Induces Nearest-Neighbor Recognition in the Fluid Phase When the Difference in Alkyl Chain Length Is Four Carbons. The fact that high concentrations of cholesterol (≥30 mol %) can enhance homodimer formation in fluid bilayers made from I–III, but not in membranes made from I, IV, and V, indicates that *the sterol's effect on nearest-neighbor recognition is a sensitive function of the difference in chain length among the equilibrating monomers.* It is noteworthy that a similar chain-length dependency on nearest-neighbor recognition is also evident in the gel–fluid coexistence region, in the absence of cholesterol; i.e., membranes that are made from I–III (but not I, IV, and V) favor homodimer formation in the gel–fluid region. We presently believe that these two recognition phenomena are, in fact, closely related. Specifically, we hypothesize that by increasing the compactness of the bilayer, cholesterol "moves" the liquid-crystalline phase toward a gel–fluid-like state (*condensing effect*), where the extent of van der Waals forces are greater and where nearest-neighbors can be recognized when the difference in chain length between the equilibrating monomers is sufficient.

Cholesterol Effects on Nearest-Neighbor Recognition in the Gel–Fluid Coexistence Region Have Not Been Found. Our finding that cholesterol lowers the melting temperatures and the transition enthalpies for bilayers made from I–III and I, IV, and V indicates that this sterol creates a less tightly packed gel phase. In principle, such a *fluidizing effect* should reduce the degree of nearest-neighbor recognition by lowering the extent of van der Waals interactions in the *gel portion* of the gel–fluid coexistence region. At the same time, the presence of cholesterol in the fluid region should have the opposite effect; i.e., it should increase the degree of recognition by condensing the fluid phase, *vide ante*. Moreover, the likely preference for cholesterol to reside in the fluid portion of gel–fluid bilayers of I–III (as is strongly suggested by the larger reduction in transition enthalpy for the lower melting endotherm) should amplify this effect. Apparently, the net difference in cholesterol's fluidizing and condensing effects is simply too small to be detected by these nearest-neighbor recognition measurements.

Cholesterol Increases the Rate of Dimer Equilibration. Our observation that the rate of equilibration increases with increasing concentrations of cholesterol is intriguing. Although we have not attempted to separate the kinetics of dimer reduction and equilibration, the fact that a near-maximum in thiol monomer content is reached at the *first* time period in which the membrane composition is analyzed (ca. 15–20% of reduced dimer in all cases) implies that the latter is rate-limiting.

In principle, the kinetics of the intramembrane displacement could be controlled by diffusion or by chemical reaction. If the displacement were reaction-controlled, then one plausible explanation for this phenomena is that cholesterol increases the effective concentration of the reactants by reducing their vertical fluctuations in the bilayer.[32] In essence, the thiolate and disulfide moieties would become localized on the periphery of the vesicle's interior and exterior. Alternatively, if the displacement were diffusion-controlled, then our findings suggest that cholesterol increases the rate of lateral diffusion in the fluid and gel–fluid regions. Previous reports that have described the effects of cholesterol on the lateral diffusion of saturated phosphatidylcholines, at temperatures in excess of T_m, appear to be contradictory. While fluorescent recovery after photobleaching (FRAP) measurements indicate that cholesterol tends to decrease lateral diffusion,[33-34] more recent pulse gradient NMR and ELDOR experiments indicate that cholesterol can increase as well as decrease lateral diffusion, depending upon the specific concentration of sterol that is present in the bilayer.[31,35] These previous FRAP studies further indicate that cholesterol increases lateral diffusion below T_m. Definitive conclusions concerning the effects of cholesterol on the rates of dimer equilibration cannot be made at the present time. Detailed kinetic studies, which are aimed at exploring this question further, are now under investigation.

Conclusion

In this paper we have shown how cholesterol influences the equilibrium distribution of a homologous series of phospholipid dimers in the physiologically relevant fluid phase and also in the gel–fluid coexistence region. These equilibration reactions were made possible via thiolate–disulfide interchange between these phospholipid dimers and the corresponding thiol monomers. When the difference in chain length between the equilibrating monomers was small (two methylenes per chain), cholesterol was found to have no significant effect on the equilibrium distribution; a statistical mixture was observed in every case. This fact, in and of itself, *establishes that these phospholipids are randomly dispersed throughout the membrane at the molecular as well as the supramolecular level*. When the difference in chain length was greater (four methylenes per chain), cholesterol enhanced homodimer formation but only in the fluid phase. For gel–fluid membranes that were composed of an equilibrium mixture of I/V/IV (molar ratio of 1/2/1) plus 10 mol% of cholesterol, a significantly broadened and asymmetric endotherm was observed, reflecting a nonhomogeneous mixture of lipids. Since the phospholipids, themselves are randomly dispersed in this membrane, we conclude that the cholesterol that is present is nonrandom. The fact that cholesterol significantly *increases* the rate of dimer equilibration has been accounted for in terms of higher effective concentrations of reactants within the bilayer and/or increased rates of lateral diffusion.

In a broader context, the cholesterol–induced nearest-neighbor recognition that we have observed for fluid bilayers derived from I–III *suggests the presence of phospholipid domains*. We believe that this inferential evidence does, in fact, represent the strongest indication to date that phospholipid mixtures *can* become "organized" in the physiologically relevant fluid phase. The fact that mammalian cells are known to be rich in cholesterol leads us to hypothesize that this sterol plays an analogous role in "organizing" the supramolecular structure of these life-sustaining membranes. In principle, the technique of nearest-neighbor recognition should be applicable to even more complex model systems, provided that additional membrane components do not interfere with the thiolate–disulfide interchange reaction and that the analytical chemistry remains tractable. Efforts aimed at exploring such possibilities are in progress.

Experimental Section

General Methods. Phospholipids I–V were synthesized using procedures similar to those that were previously reported.[5] Cholesterol was obtained from Sigma (99+%) and was recrystallized twice from ethanol prior to use. Hexane and ethanol (denatured) that were used for high-pressure liquid chromatography were HPLC grade and were filtered through 0.45 μm nylon filters prior to use. Choline chloride (99+%) was dissolved in ethanol (3.7 g/L) and then filtered through a 0.45 μm nylon filter prior to use in HPLC. House-deionized water that was used for HPLC and vesicle formation was purified by use of a Milli-Q system consisting of one carbon, two ion exchange and one Organex-Q stages.

Preparation of Large Unilamellar Vesicles by Extrusion. Vesicle dispersions composed of phospholipid and cholesterol were prepared by mixing appropriate aliquots of stock chloroform solutions and removing the solvent under a stream of nitrogen. The test tube containing the thin film of lipid was fitted with a No-air rubber septum and repeatedly degassed by evacuating the test tube under reduced pressure (50 mmHg 1 min) and replenishing the test tube with nitrogen (four times in succession). The test tube was then placed under reduced pressure for at least 12 h at 0.4 mmHg (23 °C) in order to remove residual chloroform. Typically, ca. 1 mg of phospholipid was used per sample. Cholesterol was incorporated in ratios of 10–40 mol % based on the total amount of lipid *monomer* present. Briefly, liposomes were prepared by the following method:[5] 2 mL of a 10 mM borate buffer solution (140 mM NaCl and 2 mM NaN_3, pH 7.4) was added to the dried lipid film, and the tube was placed in a water bath for 30 min at 60 °C. The lipid was dispersed in the buffer *via* vortex mixing. In order to prepare a more homogeneous liposomal dispersion, the solution was frozen (77 K), thawed in a water bath (60 °C), and vortex mixed. This procedure was repeated four times. The resulting liposomal dispersion was sequentially extruded through 0.4, 0.2, and 0.1 μm polycarbonate filters (Nuclepore) in a Lipex extrusion apparatus (Vancouver, British Columbia). The Lipex Biomembrane chamber was maintained at a temperature at least 5 degrees above the gel to liquid–crystalline phase transition temperature of the lipid (or the higher melting lipid of a mixture) *via* an external water bath. In all cases, the mean diameter of the vesicles (ca. 1000 Å) was confirmed by dynamic light scattering using a Nicomp 270 submicrometer particle size analyzer.

Thiolate–Disulfide Interchange Reaction. In a typical experiment, large unilamellar vesicles (1000-Å diameter) composed of III plus 10 mol % cholesterol were prepared *via* extrusion. A separate dispersion was

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prepared from a 1:1 molar mixture of **I** and **II** plus 10 mol % cholesterol. Each of these dispersions were prepared from 0.70 μmol phospholipid and 0.14 μmol cholesterol in a 2.2 mL borate buffer (pH 7.4) and were treated identically throughout the entire experiment. The procedure that was used to promote the thiolate–disulfide interchange reaction had been previously reported.[5] Briefly, each dispersion was degassed *via* argon using a Firestone Valve, and the pH was then raised to 8.5. Each dispersion was divided into 0.3-mL samples and placed into separate test tubes, which were subsequently placed into an oil bath maintained at the desired temperature (33 ± 1, 53 ± 1, 60 ± 1 °C). An aqueous solution of dithiothreitol (1 equiv relative to phospholipid) was then injected into each tube in order to initiate the thiolate–disulfide interchange reaction. After a specified time, ranging from 10 to 1200 min, the reaction was quenched by lowering the pH to 5.0. Each aliquot was vortex mixed and immediately freeze dried.

Analysis of Dimer Distributions by High-Pressure Liquid Chromatography. Phospholipid–cholesterol mixtures were analyzed by high-pressure liquid chromatography using a 4.6 × 250 mm Altex Ultrasphere C18 reverse-phase column (5 μm particle size, Beckman Instruments), with 20 mM choline chloride in ethanol/water/hexane (78/13/9, v/v/v).[36] The flow rate was 0.8 mL/min, and detection was made by UV at 205 nm.

Freeze-dried vesicle samples were prepared for HPLC analysis by dissolving the lipid portion in 2 mL of chloroform, removing the chloroform solution from the undissolved buffer components, and filtering through glass wool. The chloroform was then evaporated, and the lipid was redissolved in 50 μL of eluent and injected onto the HPLC column. The retention times for lipids **I**, **II**, **III**, **IV**, and **V** were 11, 71, 26, 28, and 17 min, respectively. Cholesterol had a retention time of 9 min. Chromatography was performed at room temperature for the **I**/**V**/**IV** system and at 31.2 °C for the **I**/**III**/**II** system. Based on the quantity of lipid that was used to prepare the initial multilamellar dispersions, the mass balance of lipids that was recovered and analyzed by HPLC was typically ca. 90%. In all cases, the ratio of cholesterol to phospholipid analyzed by HPLC agreed (±2%) with the original ratio used to form the vesicle dispersion.

Differential Scanning Calorimetry (DSC). Calorimetry was performed on multilamellar vesicle dispersions that were prepared by mixing appropriate aliquots of chloroform solutions containing the phospholipids and cholesterol. The chloroform was removed by evaporation under a stream of nitrogen, and the test tube containing the thin lipid film was then placed under reduced pressure for at least 12 h. A multilamellar dispersion was formed by dispersing the lipid film (1–2 mg) in 1.6 mL of 10 mM borate buffer (140 mM NaCl and 2 mM NaN_3, pH 7.4) at 60 °C. Calorimetry was performed using a Microcal MC-2 calorimeter with DA-2 data acquisition and analysis software. In all cases, the scan rate was $0.5°\cdot min^{-1}$, and heating scans were recorded between 10 and 60 °C. The melting behavior of the multilamellar dispersion was measured, using the same buffer solution as a reference. Three DSC runs were performed for each sample; no difference was observed among the scans. A borate buffer baseline was also collected and subtracted from each thermogram. The calorimetric data were analyzed to yield phospholipid excess heat capacities as a function of temperature, and the transition enthalpies were calculated by employing software supplied by Microcal. Thermograms were normalized from mcal/min to mcal/deg by dividing by the scan rate. The heat capacity in the units of kcal/deg-mol was determined by dividing by the number of moles of phospholipid present in the vesicle dispersion.