Computation of Mixed Phosphatidylcholine-Cholesterol Bilayer Structures by Energy Minimization

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ABSTRACT The energetically preferred structures of dimyristoylphosphatidylcholine (DMPC)-cholesterol bilayers were determined at a 1:1 mole ratio. Crystallographic symmetry operations were used to generate planar bilayers of cholesterol and DMPC. Energy minimization was carried out with respect to bond rotations, rigid body motions, and the two-dimensional lattice constants. The lowest energy structures had a hydrogen bond between the cholesterol hydroxyl and the carbonyl oxygen of the *sn*-2 acyl chain, but the largest contribution to the intermolecular energy was from the nonbonded interactions between the flat α surface of cholesterol and the acyl chains of DMPC. Two modes of packing in the bilayer were found; in structure A (the global minimum), unlike molecules are nearest neighbors, whereas in structure B (second lowest energy) like-like intermolecular interactions predominate. Crystallographic close packing of the molecules in the bilayer was achieved, as judged from the molecular areas and the bilayer thickness. These energy-minimized structures are consistent with the available experimental data on mixed bilayers of lecithin and cholesterol, and may be used as starting points for molecular dynamics or other calculations on bilayers.

INTRODUCTION

Computational model building is used in this work to determine the energy-minimized structures of bilayers of cholesterol and dimyristoylphosphatidylcholine (DMPC) at a 1:1 mole ratio. The assumption is made that local order is present and that this local order can be generated by crystallographic symmetry operations. The asymmetric unit was assumed initially to be a heterodimer of DMPC and cholesterol, but in the final cycle of calculations an asymmetric unit of twice this size was introduced. The energy-minimized bilayer structures so obtained may be used as starting points for molecular dynamics calculations or for studies of membrane electrostatics (Zheng and Vanderkooi, 1992).

Background

The role of cholesterol in biological membranes has been a problem of longstanding interest. Although cholesterol does not form bilayer membranes by itself, it readily interacts with phospholipids, especially sphingomyelin and phosphatidylcholine (Demel et al., 1977; van Dijck et al., 1976), to form stable mixed bilayers. The structure of the cholesterol molecule is remarkably well suited for interaction with these phospholipids; alteration of the steroid structure in any of several ways has been shown to diminish or abolish its ability to give the characteristic properties of cholesterol-phospholipid membranes (Demel et al., 1972a, b).

The solubility of cholesterol in phosphatidylcholine bilayers has been determined by equilibrium mixing experiments to be 1.0 ± 0.1 mol/mol; at higher mixing ratios cholesterol tends to separate out as microcrystals of cholesterol monohydrate (Collins and Phillips, 1982). Other techniques, including x-ray diffraction (Engelman and Rothman, 1972; Finean, 1989), calorimetry (Mabrey et al., 1978; McMullen et al., 1993), and dilatometry (Melchior et al., 1980) have given evidence that bilayers having cholesterol to phospholipid mole ratios of 1:2 and 1:4 also have special properties that may correspond to structurally identifiable phases. These data have been extensively reviewed (Martin and Yeagle, 1978; Presti et al., 1982; Yeagle, 1985; Hui, 1988; Finean, 1990; McMullin et al., 1993), and numerous attempts have been made to explain the properties of these phases in structural terms. The strongest evidence for local order applies to bilayers having a 1:1 mole ratio (Finean, 1990). At this mole ratio there is clearly a tight packing of the components and restricted motional freedom, but yet the diffuse nature of the wide angle x-ray diffraction pattern and the lack of a thermal phase transition implies that there is no long-range crystallinity (Finean, 1990; McMullen et al., 1993). Finean (1990) has suggested that these somewhat paradoxical characteristics may be explained in terms of microcrystalline order rather than molecular disorder, with local order being retained.

Surface pressure measurements on monolayers of mixed films have given much information on the molecular structural requirements for a favorable interaction between phospholipids and cholesterol. Mixing of lecithin with cholesterol in monolayers gives a "condensing effect," meaning that there are large negative deviations from additivity of the molecular areas of the unlike molecules in the film. This nonideal behavior is evidence for attractive interactions between the unlike molecules. Steroids other than cholesterol give a weakened condensing effect, showing that this effect is structure-dependent. Through comparative studies involving a series of steroids, Demel et al. (1972a) concluded that a β-OH group, a planar steroid nucleus, and an intact side

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chain at C-17 are necessary for a maximal condensing effect. Of particular interest is that epicholesterol, which has an α-OH group, gives a much diminished condensing effect as compared to cholesterol. These authors also showed that the steroid structural requirements for decreasing the permeability of egg lecithin liposomes to glucose and other substances parallels the requirements for the condensing effect on monolayers (Demel et al., 1972b).

De Kruyff et al. (1973) investigated the phospholipid structural requirements for the condensing effect and found that, although phosphatidylcholine analogs that lack the ester group at *sn*-2 still give condensation with cholesterol, the strongest effect is given by phosphatidylcholine per se. In another study, Cadenhead and Muller-Landau (1979) compared the condensing effect of 3-doxyl cholestane and 3-doxyl-17-hydroandrostane with that of cholesterol. They found little or no condensation with the androstane derivative, but appreciable condensation with 3-doxyl cholestane, which lacks the β-OH group (although less than with cholesterol). They concluded that hydrophobic interactions involving the nonpolar parts of the steroid molecule are of primary importance for the condensing effect, and that polar interactions involving the β-OH group are not a requirement for condensation. When all of the monolayer studies are considered together, however, it is clear that the β-OH group does contribute to the condensation, as do the hydrophobic interactions involving the nonpolar parts of the molecules. The latter interactions are dependent upon the three-dimensional shape of the steroid.

Information on the location of cholesterol molecules in phosphatidylcholine bilayers has been obtained from x-ray diffraction studies of oriented multibilayers (McIntosh, 1978). Comparison of the one-dimensional electron density maps of phosphatidylcholine and cholesterol-phosphatidylcholine at a 1:2 mole ratio indicated that the long axis of cholesterol is aligned parallel to the alkyl chains of the phospholipid, and the cholesterol hydroxyl group appeared to be at the level of the lipid carbonyl groups. It was observed further that the addition of cholesterol caused no detectable change in the layer parallel orientation of the phosphocholine head groups.

Spectroscopic measurements have been used to identify specific interactions that may occur between lecithin and cholesterol in heterodimer formation. The possibility of hydrogen bonding between the cholesterol hydroxyl and the phosphate group was ruled out by ^{31}P-NMR, because cholesterol had no effect on the NOE, T_1, or chemical shift of the phosphorous resonance (Yeagle et al., 1975). A small shift was observed in the ^{13}C-NMR carbonyl resonance upon the addition of cholesterol, however, which was deemed to be consistent with the formation of a hydrogen bond between the cholesterol hydroxyl and a lecithin carbonyl group (Yeagle and Martin, 1976). Other ^{13}C-NMR measurements by the same group confirmed the conclusion, previously inferred from surface pressure measurements (Demel et al., 1972a), that a flat α-surface of the steroid ring system is required for tight interaction with lecithin (Yeagle et al., 1977).

Clear evidence has been obtained from FT-IR measurements for the formation of a hydrogen bond between the β-OH of cholesterol and the *sn*-2 carbonyl group of phosphatidylcholine in the anhydrous state (Wong et al., 1989). Separate peaks were identified in the IR spectrum of phosphatidylcholine as being due to the *sn*-1 and *sn*-2 carbonyl groups. Addition of cholesterol caused a shift in the *sn*-2 carbonyl peak but not the *sn*-1 peak, from which it was inferred that the cholesterol specifically bonded to the *sn*-2 group. A shift in the phosphate bands in the IR spectrum was also noted, which indicated the existence of hydrogen bonding (in the anhydrous state) between cholesterol and the phosphate group. Comparison of the magnitude of the effects of water on the carbonyl and phosphate IR spectral bands (Wong and Mantsch, 1988) with the effects of cholesterol showed that the cholesterol-carbonyl hydrogen bond is stronger than a water-carbonyl bond, but that, conversely, a water-phosphate bond is stronger than the cholesterol-phosphate bond (Wong et al., 1989). Hence, they conclude that only the cholesterol-carbonyl bond will persist in aqueous media. The results of Wong et al. (1989) disagree with the earlier work of Bush et al. (1980), who found no evidence for hydrogen bonding between cholesterol and phosphatidylcholine. The latter workers used a racemic synthetic phosphatidylcholine rather than the naturally occuring L isomer, and this may have a bearing on the difference in the results obtained by the two groups.

Model building

Molecular model building has been used in several attempts to account for the interactions that occur between cholesterol and phospholipids. The earliest of these was evidently that of Finean (1953), who proposed that specific interactions exist between phospholipids and cholesterol on the basis of the low angle x-ray diffraction pattern of myelin. Subsequent workers proposed heterodimer models that included phosphate-hydroxyl hydogen bonding (Darke et al., 1971; Verma and Wallach, 1973); but Brockerhoff (1974) argued in favor of a carbonyl-hydroxyl bond on structural and energetic grounds. Yeagle et al. (1975) also favored a carbonyl-hydroxyl bond, because their ^{31}P-NMR work showed that cholesterol does not interact with the phosphate group. Huang (1977a, b) developed a detailed model for the interaction of cholesterol with a lecithin molecule having an unsaturated alkyl chain at *sn*-2. He proposed, on the basis of molecular model building, that the cholesterol hydroxyl makes a hydrogen bond with the carbonyl at *sn*-1.

Presti et al. (1982) used CPK space-filling models to construct a lecithin-cholesterol dimer structure. This model included a hydrogen bond from the cholesterol hydroxyl to the *sn*-2 glycerol ester oxygen rather than to the carbonyl oxygen. This type of hydrogen bonding was used to maximize

nonbonded interactions, as judged by examination of the model, with the observation being made that the carbonyl group was angled in an unfavorable direction for hydrogen bond formation. They quoted the IR results of Bush et al. (1980), which found no evidence for hydrogen bonding involving the carbonyl group; but, as already mentioned, the more recent FT-IR measurements of Wong et al. (1989) do support the presence of a carbonyl hydrogen bond. Finean (1990) also published photos of a CPK model of a lecithin-cholesterol dimer that appears to be identical to the model of Presti et al. (1982); his model likewise includes a hydrogen bond to the *sn*-2 glycerol oxygen. No experimental evidence has been adduced by either of these groups in support of this type of hydrogen bond.

The experimental results quoted above are consistent with dimer formation of lecithin and cholesterol in which there is an *sn*-2 carbonyl-hydroxyl hydrogen bond. The flat α face of the cholesterol apparently makes close contact with the lipid alkyl chains, because cholesterol derivatives that have protrusions on the α surface do not interact well with lecithin (Yeagle et al., 1977). In addition, it is consistent with the x-ray data (McIntosh, 1978) to assume that the long axis of cholesterol lies essentially parallel to the long axis of the phospholipid molecule, normal to the bilayer plane. Any acceptable model should be able to account for these observations.

MATERIALS AND METHODS

Atomic coordinates

Initial coordinates for DMPC were taken from the results of energy minimization of the DMPC:2H_2O crystal structure (Pearson and Pascher, 1979: Hauser et al., 1981; Vanderkooi, 1991). Six trial sets of atomic coordiates were used for DMPC, corresponding to molecules 1 and 2 in the energy-minimized structures I, II, and III described previously (Vanderkooi, 1991). Coordinates for cholesterol were obtained from the low temperature (123K) crystal structure of cholesteryl acetate (Sawzik and Craven, 1979a), by replacing the acetate group of molecule A with a hydroxyl. The C-H bond lengths in cholesterol were also adjusted to standard values. The C-17 aliphatic chain in this structure is in the extended (*trans*) conformation.

Energy calculations

The computational methods, energy functions, and parameters employed are the same as those previously described (Vanderkooi 1990a, 1991). Briefly, the empirical energy of a crystal is computed as a sum of intramolecular and intermolecular contributions, and includes nonbonded, electrostatic, hydrogen-bonded, and torsional energy terms. Intermolecular energies are computed between the molecules within the crystallographic asymmetric unit and between the reference asymmetric unit and its surrounding environment, which is generated by symmetry. A nominal cutoff distance of 12 Å was used, with care being taken not to segment any charge clusters by application of the cutoff distance. As before (Vanderkooi, 1990a, 1991), the dielectric constant was set at 2.0. Energy minimization was carried out with respect to all intramolecular bond rotations, rigid body motions, and lattice constants whose values are not dictated by the symmetry of the space group. The derivative-based DUMING unconstrained minimization program of the IMSL Math Library (Version 2.0, 1991) was used to direct the course of the minimization. All calculations were carried out on the Amdahl 5890 computer at Northern Illinois University.

Outline of the computational approach

The major steps in the calculation were: (1) determination of trial DMPC-cholesterol dimer structures; (2) generation of monolayers from the dimer structures by the application of two-dimensional symmetry operations; and (3) formation of bilayers by the action of a twofold rotation or screw axis operating on the energetically favorable monolayers obtained in step 2.

In step 1, an isolated DMPC molecule was placed with its long axis parallel to the *z* axis of a Cartesian coordinate system. (The *z* axis will become the bilayer normal, with the *x* and *y* axes defining the monolayer or bilayer plane.) A cholesterol molecule was placed at several locations and orientations around the DMPC molecule, with its long axis parallel to the *z* axis and the hydroxyl group being in the vicinity of the DMPC polar head group. Multiple energy minimizations were carried out from starting points generated in this manner, with the result that 13 dimer structures of relatively low energy were found. One or more of these structures included a hydrogen bond from cholesterol to the *sn*-1 carbonyl oxygen (O32), to the *sn*-2 carbonyl oxygen (O22), and to each of the nonesterified phosphate oxygens (O13 and O14). No structures were found with hydrogen bonding to the glycerol ester oxygens or to the esterified phosphate oxygens. No effort was made to determine the "best" dimer structure, because the totality of the intermolecular interactions in a monolayer or bilayer may be expected to overshadow the limited number of interactions that occur within an isolated dimer. All 13 low energy dimer structures, therefore, were carried along for use in the next step.

In step 2, monolayers were generated by the application of two-dimensional symmetry operations to the dimer structures obtained in step 1 using the dimers as the crystallographic asymmetric unit. Many complete energy minimizations were carried out on the monolayers, using as starting points each of the 13 dimeric structures, taken in combination with all permissible two-dimensional space groups.

The four two-dimensional space groups employed are listed in Table 1, together with the rigid body parameters and lattice constants which are variables in each case. The rigid body parameters that are used for specifying the positions and orientations of a pair of molecules on a surface are defined in Fig. 1. Table 1 also lists the three-dimensional space groups that can be derived from each of the two-dimensional space groups by the addition of a twofold rotation or screw axis. In principle, the two-dimensional c1- and c2-centered space groups would be redundant with the p1 and p2 primitive space groups if a nonorthogonal lattice angle were permitted, but in this work a lattice angle of 90° was maintained in the monolayer calculations

TABLE 1 Two and 3-dimensional space groups employed in the generation of monolayers and bilayers*

p1 Monolayer, Primitive $Z = 1, \Gamma = 90°$ variables: a, b, τ_2	c1 Monolayer, Centered $Z = 2, \Gamma = 90°$ variables: a, b, τ_2
Derived bilayers (monoclinic): P2, $P2_1$ $Z = 2, \alpha = \Gamma = 90°, \beta \neq 90°$ *b* diad or screw axis variables: a, b, c, τ_2	Derived bilayers (monoclinic): C2 $Z = 4, \alpha = \Gamma = 90°, \beta \neq 90°$ *b* diad axis variables: a, b, c, τ_2
p2 Monolayer, Primitive $Z = 2, \Gamma = 90°$ *c* diad axis variables: a, b, τ_1, τ_2, r	c2 Monolayer, Centered $Z = 4, \Gamma = 90°$ *c* diad axis variables: a, b, τ_1, τ_2, r
Derived bilayers (orthorhombic): P222, $P2_122$, $P22_12$, $P2_12_12$ $Z = 4, \alpha = \beta = \Gamma = 90°$ *c* diad axis *a*, *b* diad or screw axis variables: $a, b, c, \tau_1, \tau_2, r$	Derived bilayers (orthorhombic): C222, $C22_12$ $Z = 8, \alpha = \beta = \Gamma = 90°$ *c* diad axis *a*, *b* diad or screw axis variables: $a, b, c, \tau_1, \tau_2, r$

* The rigid body variables are defined in Fig. 1. The variables d, ϕ_1, and ϕ_2 shown in Fig. 1 define the heterodimer structure and are used in addition to the variables listed here. *Z* is the number of asymmetric units per unit cell, and *a*, *b*, *c*, α, β, Γ are the lattice constants, with the *a* and *b* axes lying in the bilayer plane.

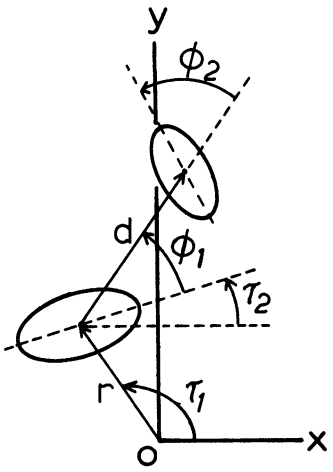

FIGURE 1 Definition of rigid body variables used in specifying the positions of DMPC and cholesterol relative to each other and to the fixed coordinate system, in two dimensions.

in order to permit bilayer formation by the addition of a twofold axis lying in the bilayer plane. (Note that there is no physical reason requiring that the two halves of a bilayer be related by a twofold axis; this is a simplifying assumption. Reflection and inversion operations are not permitted, however, because of the chiral nature of the molecules involved.)

Monolayer minimizations were started at 15–30° intervals with respect to rotational variables, and translational variables were set initially at values large enough to prevent atomic overlaps but small enough so there would be an attractive interaction between the molecules involved.

In step 3, bilayers were generated from the low energy monolayers found in step 2, with all of the three-dimensional space groups listed in Table 1 being tested to find out which would give the lowest energy in each case. Only monolayers of relatively low energy were carried forward to step 3 because the major part of the energy of a bilayer comes from interactions within the two monolayers, and a relatively small contribution comes from midplane interactions between the monolayers (Vanderkooi, 1990b). Hence, a low-energy monolayer is a prerequisite for obtaining a good low-energy bilayer.

The present calculations were carried out for the anhydrous state. Addition of water would not be expected to have an appreciable effect on the geometrical structures of the energy-minimized bilayers because these are largely determined by packing considerations, but the relative energies of the different low energy structures may be dependent upon the degree of hydration.

RESULTS

Two basic questions are to be answered. First, what is the structure of the asymmetric unit? Second, how do these asymmetric units pack in a bilayer array? Obviously these questions are interrelated because the crystal-packing energies will affect the structure of the asymmetric unit, and conversely. The low-energy structures found for isolated DMPC-cholesterol dimers are of little interest in and of themselves because the dimer structure may be expected to change upon incorporation into a monolayer or bilayer. All 13 low energy isolated dimer structures found in step 1 of the calculations (as described in Materials and Methods) were used, therefore, as starting points for monolayer and bilayer formation. From these calculations, both the energetically preferred asymmetric unit structure and the most probable space groups were determined.

Hydrogen bonding

The dimer structures are categorized on the basis of the intermolecular hydrogen bond type that is present. Monolayers and bilayers were generated that included each of four types of hydrogen bond, and the lowest bilayer energy found for each hydrogen bond type is listed in Table 2. This table also gives the bilayer space group and the energy of a monolayer having the same structure as found in the corresponding energy-minimized bilayer. In the global minimum bilayer structure, hydrogen bonding from cholesterol to DMPC involved the *sn*-2 carbonyl oxygen (O22). The hydrogen bond type that gave the second lowest energy was to one of the phosphoryl oxygens (O14). Structures having either of the other two types of hydrogen bond (i.e., involving the *sn*-1 carbonyl or the O13 phosphoryl oxygen) gave significantly higher energies and were excluded from further consideration.

In energetic terms, the structure listed in Table 2 as having hydrogen bonding to O14 should be considered as acceptable because its bilayer energy differs by only 1.7 kcal/mol from that of the lowest energy structure. It appears unlikely, however, that this structure will exist to any significant degree in an aqueous environment. The present calculations were done for the anhydrous state. Water will compete with cholesterol for hydrogen bonding to the DMPC phosphoryl oxygens that are exposed to the aqueous environment, but will probably not compete significantly for hydrogen bonding to the carbonyl oxygens, which are more deeply located in the bilayer. Hence, the presence of water would effectively disfavor structures in which cholesterol hydrogen bonding is to the phosphoryl oxygens and would favor those in which bonding is to the carbonyl oxygens. This reasoning is in agreement with the conclusions arrived at by Wong et al. (1989) on the basis of FT-IR measurements. Their evidence indicated that cholesterol will preferentially hydrogen bond to the *sn*-2 carbonyl oxygen when in an aqueous environment, but they also gave evidence that hydrogen bonding may occur to the phosphoryl oxygens in the anhydrous state. The remainder of this

TABLE 2 Lowest energy values found for DMPC:cholesterol bilayer structures with each type of hydrogen bonding

Hydrogen Bond	Space Group	Energy (kcal/mol)*	
		Monolayer	Bilayer
O—H···O22 = C	C222	−104.59	−107.16
O—H···O14 = P	$P22_12$	−97.32	−105.48
O—H···O13 = P	$P2_12_12$	−96.49	−98.70
O—H···O32 = C	C222	−92.10	−93.56

* The total energy is given, including the intramolecular and intermolecular contributions. The energy is expressed per mole of DMPC-cholesterol dimers. The monolayer energy is calculated at the structure of the bilayer minimum, so that the difference between the monolayer and bilayer energies equals the energy of interaction between the monolayers.

paper will be devoted to describing structures having hydrogen bonding to the *sn*-2 carbonyl group.

Space group

The packing of the asymmetric units in a monolayer is governed by the choice of space group. There are four two-dimensional space groups from which bilayers can be generated by the action of a twofold rotational or screw axis lying in the bilayer plane, as listed in Table 1. After the three-step procedure outlined in Material and Methods, energy-minimized monolayers were first formed using each of these two-dimensional space groups, after which bilayers were made from the monolayers. Bilayers were formed using each of the three-dimensional space groups listed in Table 1 that are derivable from the respective two-dimensional groups. The lowest energies for bilayers derived from each of the two-dimensional groups are given in Table 3, together with the designation of the three-dimensional space group that gave this energy, and the energy of a monolayer having the same structure as the energy-minimized bilayer. The energies of the intermediate energy-minimized monolayers are not reported. All structures referred to in Table 3 had hydrogen bonding to the *sn*-2 carbonyl group.

The two-dimensional space groups having a twofold rotational axis normal to the bilayer plane (p2 and c2) consistently gave lower energies than p1 and c1, which lack this symmetry element. The difference in energy between these types of space groups is mainly due to the electrostatic term. The intermolecular electrostatic energy in the minimized structures having p2 and c2 symmetry is −12.3 to −12.6 kcal/mol, whereas with p1 or c1 symmetries this energy term is in the vicinity of −9.0 kcal/mol. This result is plausible because the twofold axis generates pairs of dimers with the P-N head group dipoles pointing in opposite directions in the bilayer plane, which is electrostatically favored over the case in which all dipoles are pointing in the same direction.

TABLE 3 Lowest energies found for DMPC:cholesterol bilayer structures derived from each 2-dimensional space group

2-d Space group	3-d Space group	Z‡	Energy (kcal/mol)* Monolayer	Bilayer
Dimeric asymmetric unit				
p1	$P2_1$	2	−92.43	−102.22
c1	C2	4	−94.73	−100.69
p2	P222	4	−101.57	−105.11§
c2	C222	8	−104.59	−107.16¶
Tetrameric asymmetric unit				
c1	C2	4	−104.66	−109.59‖

* The total energy is given, being the sum of intramolecular and intermolecular terms. The energy is expressed per mole of DMPC-cholesterol dimers. The monolayer energy is calculated at the structure of the bilayer minimum.

‡ Z is the number of asymmetric units per unit cell in the bilayer, with the asymmetric unit containing either a dimer or tetramer of DMPC and cholesterol.

§ Structure B.

¶ Structure A, dimeric asymmetric unit.

‖ Structure A, tetrameric asymmetric unit, but with the average energy per dimer being given.

Contents of the asymmetric unit

Initially, the assumptions were made that the crystallographic asymmetric unit contains one molecule each of cholesterol and DMPC, and that the two halves of the bilayer are related by a twofold symmetry axis. Visual examination of the lowest energy-bilayer structure so obtained (C222 symmetry, see Table 3) showed that the fit between the two monolayers at the bilayer midplane was rather poor. (This fact was also reflected in a relatively small monolayer-monolayer interaction energy of −2.57 kcal/mol). It was necessary to enlarge the contents of the asymmetric unit and to drop one element of symmetry to improve the fit between the monolayers. Either of the twofold axes, parallel or perpendicular to the bilayer plane, could be dropped with equivalent results; the latter option was selected. The enlarged asymmetric unit was tetrameric, containing two dimers of DMPC and cholesterol that were related by a local twofold axis before reminimization. Complete minimization of the bilayer followed, with the result that the two halves of the bilayer shifted relative to each other by a sufficient amount to give a good steric fit at the bilayer midplane. The two dimers in the asymmetric unit became nonequivalent, but remained related by an approximate pseudo-twofold axis normal to the bilayer plane. The average total energy per dimer decreased from −107.16 to −109.59 kcal/mol. This change in energy was due almost entirely to the improved fit at the bilayer midplane, with the midplane energy decreasing from −2.57 to −4.93 kcal/mol.

A tetrameric asymmetric unit was also tested for the second lowest energy structure listed in Table 3 (P222 symmetry), but this resulted in a negligible change in both the structure and the energy. Visual examination of this structure showed that a good fit already existed between the two halves of the bilayer.

Detailed information will be given for the two lowest energy structures listed in Table 3. These will be referred to as structure A (space group C222 or C2, with a dimeric or tetrameric asymmetric unit, respectively, as indicated) and structure B (having space group P222 and a dimeric asymmetric unit).

Molecular conformation

The conformation of DMPC in structure A is similar to molecule 1 in the DMPC:$2H_2O$ crystal structure, whereas in structure B it is similar to molecule 2 of the crystal structure (Pearson and Pascher, 1979). The dihedral angles θ_1, θ_3, and β_1 around the C2 glycerol carbon define the phosphatide structural class (Vanderkooi, 1973); these have the values 70, 176, and 94° in structure A; and 184, 168, and 145° in structure B, respectively.

The cholesterol side chain is all *trans* in the energy-minimized structures. The flat α surface of the cholesterol

ring is in contact with the DMPC molecule to which it is hydrogen-bonded. Cholesterol makes van der Waals contacts with both of the acyl chains of DMPC in structure A, but predominantly with the β chain in structure B.

A lateral view of the DMPC-cholesterol dimer found in structure A is shown in Fig. 2. It can be seen that when cholesterol is hydrogen-bonded to the *sn*-2 carbonyl, the terminal methyl groups of cholesterol extend to a position that is intermediate between that of the terminal methyls of the 2 myristoyl chains of DMPC.

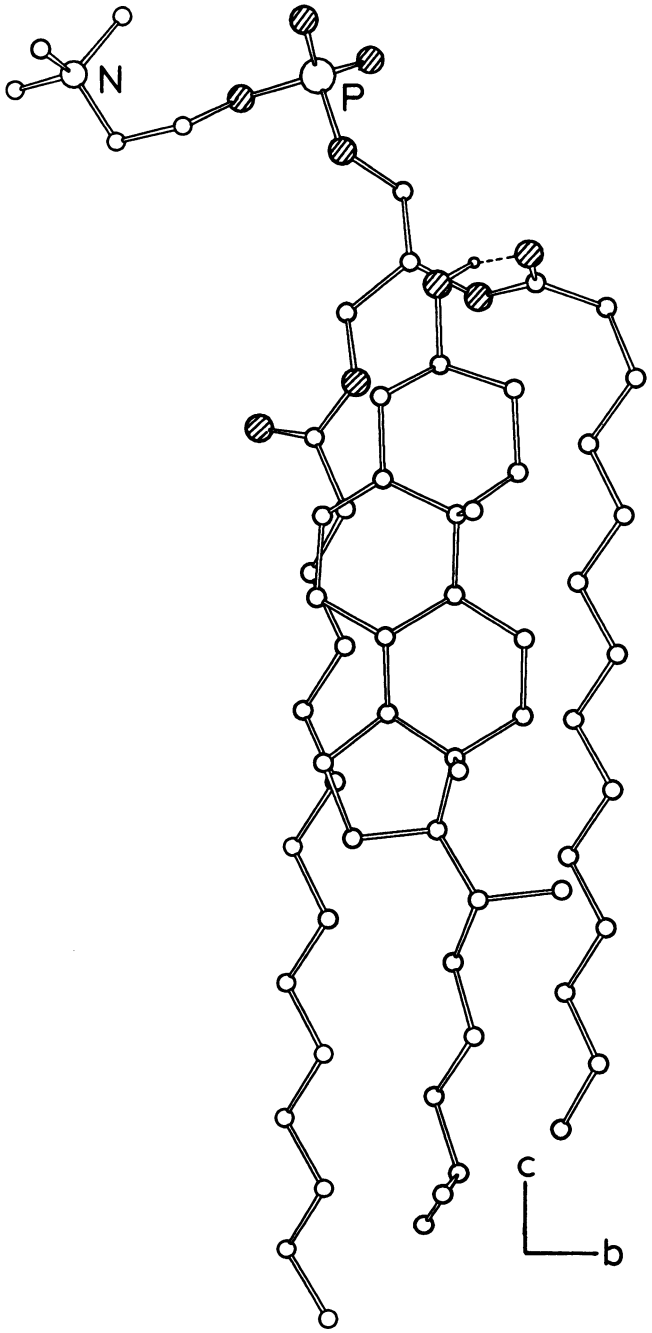

FIGURE 2 Conformation of the DMPC-cholesterol dimer found in structure A (dimeric asymmetric unit). The projection is on the *bc* plane. Oxygen atoms are hatched and the hydrogen bond is indicated by a dashed line.

Monolayer and bilayer packing

The molecular packing of DMPC and cholesterol in struc tures A (dimeric asymmetric unit) and B are illustrated i Figs. 3 and 4, in projections onto the *ab* planes. These figure are divided into three panels: the entire molecules are show in the central panels, whereas in the left panels only th phosphocholine head groups are shown, and in the right pan els the diacylglycerol esters and the cholesterol molecules ar shown.

The spacial organization of cholesterol and the acyl chai portions of DMPC differs markedly between the structure A and B, as can be seen from the right panels in Figs. 3 an 4. In structure A (Fig. 3), cholesterol molecules alternate wit the diacylglycerol groups along both axes, so that neares neighbors are always unlike molecules. In structure B, on th other hand, there are bands of diacylglycerol groups tw molecules thick running parallel to the *a* crystal axis sepa rated by bands of cholesterol two molecules thick. Thus like-like interactions predominate in structure B.

In both structures, the P-N vectors of the polar head group are aligned approximately parallel to the *b* crystal axis. Thes vectors alternately point in opposite directions, as require by the twofold symmetry that relates pairs of dimers. Th head groups form rows or bands parallel to the a axis. I structure A, the choline quaternary ammonium group of on DMPC lies above the cholesterol molecule of another dime to which it is related by a twofold axis. In structure B, on th other hand, the quaternary ammonium group of one DMP is above another DMPC molecule, and the cholesterol mol ecules are exposed to the external space.

The junction between the two halves of the bilayer in struc ture A with a tetrameric asymmetric unit is illustrated in th lateral view shown in Fig. 5. It can be seen that the long axe of both DMPC and cholesterol are aligned essentially per pendicularly to the bilayer plane. There is a good fit betwee the two halves of the bilayer, with the midplane having zigzag profile.

Structural analysis

Table 4 gives geometrical data for the DMPC-cholesterc bilayer structures. Included for comparison purposes are th corresponding data for the energy-minimized crystal struc ture of DMPC:2H_2O (Vanderkooi, 1991).

The cross sectional area of cholesterol in the crystal struc ture of cholesterol monohydrate is 36.2 Å^2 (Craven, 1976) and the area per molecule of DMPC in the DMPC:2H_2O crystal structure is 38.9 Å^2 (Pearson and Pascher, 1979). Th sum of these areas is 75.1 Å^2. In structure A the surface are per dimer is 76.1 Å^2, differing by only 1 Å^2 from the sum o the experimental areas. The nonpolar region thickness is als less in the mixed bilayer than it is in the DMPC crystal struc ture (Table 4). These comparisons indicate that crystallo graphic close packing was achieved in the mixed bilaye structure as a result of energy minimization.

The orientation of the P-N dipole more closely approache

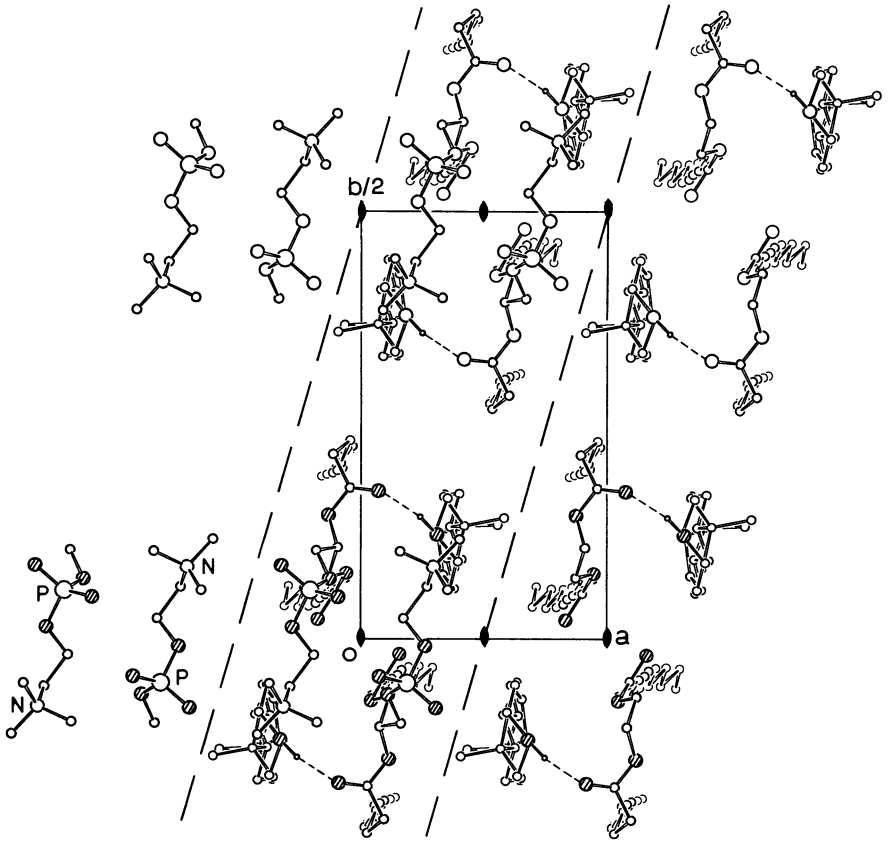

FIGURE 3 Molecular packing of DMPC and cholesterol in structure A (dimeric asymmetric unit). A monolayer is shown in projection down the *c* axis, onto the monolayer plane. The space group is C222; one-half of a unit cell is indicated by the rectangular box. Only the DMPC polar head groups are shown in the left section, and the cholesterol and diacylglycerol esters are shown on the right, with the entire molecules being shown in the middle panel. Oxygen atoms are indicated with cross hatching, and the hydrogen bonds are shown as dashed lines.

in the pure DMPC bilayer, as can be seen from the vector angles given in Table 4. In pure DMPC the bilayer parallel orientation is prevented by the bulkiness of the polar head groups, but in the mixed bilayer the cholesterol molecules act as spacers so that the combined cross sectional area of the nonpolar region is greater than that of the head group. This permits the head groups to adopt orientations that optimize the electrostatic energy, without the necessity of satisfying the steric constraints that are present in pure DMPC. For the same reason, there is no need for acyl chain tilt in the mixed bilayer. Chain tilt occurs in pure DMPC to increase the effective cross sectional area of the acyl chain region in order to match the tail area with the head group area, but because in the mixed bilayer the nonpolar region already has a greater area than the head group, the driving force favoring chain tilt is no longer present.

Energetic analysis

The total energy (intramolecular plus intermolecular) for structures A and B is given in Table 3, from which it can be seen that structure A (with a tetrameric asymmetric unit) has the lowest total energy. The intramolecular energy for A and B was essentially the same; the difference in total energy was mainly due to the intermolecular energy terms.

A further breakdown on the intermolecular energy is given in Table 5. The intermolecular energies of interaction of DMPC and cholesterol with their respective environments were summed separately and are listed in this table. (The sum of the DMPC and cholesterol interaction energies equals the total intermolecular energy.) The DMPC interaction energy is the same in the two mixed bilayer structures (−60.3 kcal/mol), and the values of each of the components of the DMPC energy (electrostatic, nonbonded, and hydrogen-bonded) are also the same in the two structures. The cholesterol interaction energy differs considerably between the two structures, however; the nonbonded component of the cholesterol energy is primarily responsible for the difference in total energy between the two packing arrangements.

The DMPC interaction energy in the mixed bilayers is close to the interaction energy of DMPC calculated for a pure anhydrous DMPC bilayer (−61.4 kcal/mol; see Vanderkooi, 1991), although the partitioning of the energy between the electrostatic and nonbonded terms differs. This implies that a DMPC molecule should be able to exchange in a virtually isoenergetic manner between a domain of pure gel state bilayer and a contiguous domain of 1:1 DMPC-cholesterol bilayer.

Table 6 gives a breakdown of the intermolecular energy in terms of like-like and like-unlike interactions. This table shows quantitatively what is qualitatively evident from Figs. 3 and 4, namely that in structure A like-unlike interactions predominate, but in structure B the like-like interactions play a greater role. For both structures, a large part of the like-unlike energy comes from the interaction between the molecules of the hydrogen-bonded dimer; this amounts to −20.45 kcal/mol in structure A (out of a total of −51.81 kcal/mol) and −17.52 kcal/mol in structure B (out of a total

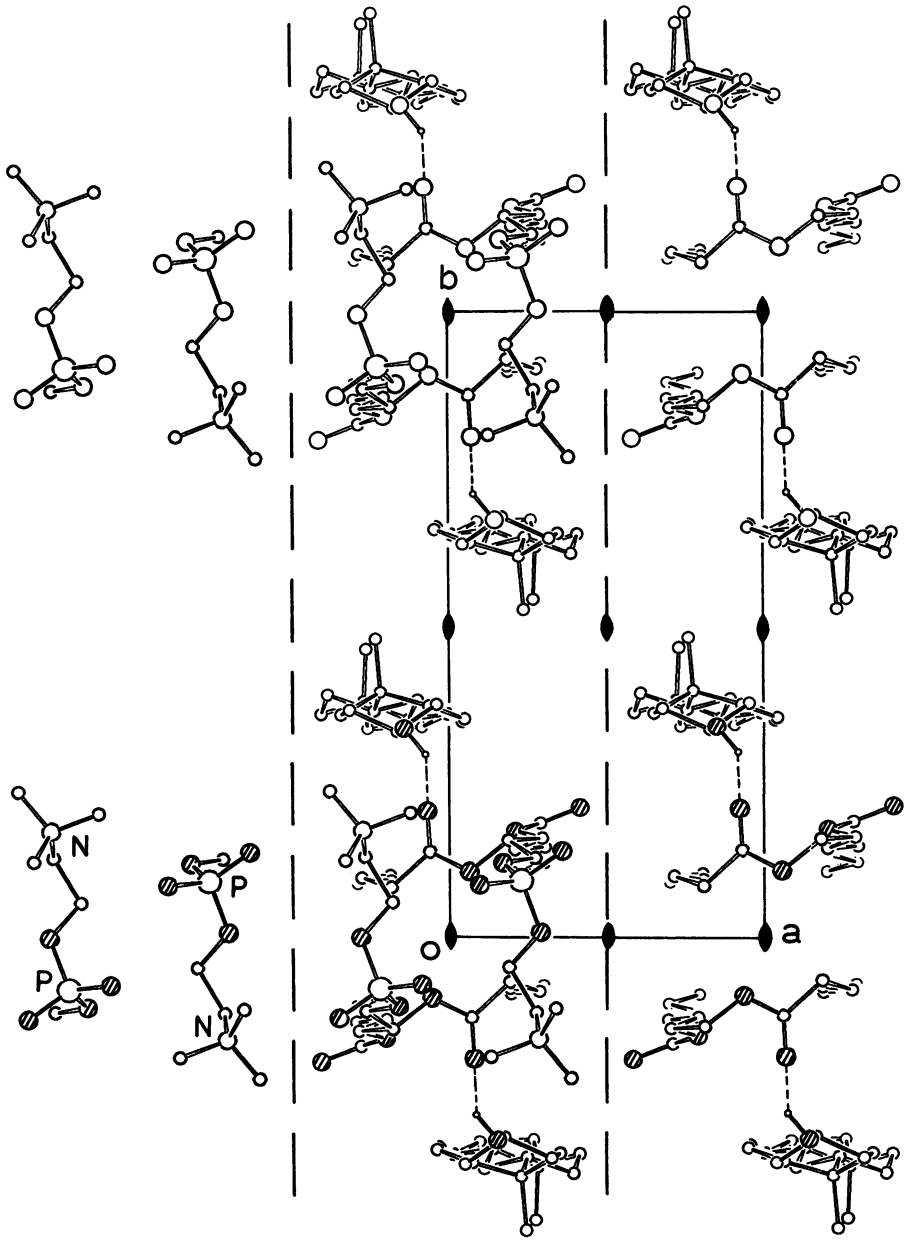

FIGURE 4 Molecular packing of DMPC and cholesterol in a monolayer of structure B, shown in projection down the *c* axis. The space group is P222; the rectangular box includes one unit cell. See the legend of Fig. 3 for other details.

of −28.90 kcal/mol). Only a small part of the interaction energy of the hydrogen bonded dimer is due to the hydrogen-bonded interaction per se; most of the energy of interaction comes from favorable nonbonded contacts between cholesterol and the acyl chains of DMPC.

Acyl chain interaction energy

The nonbonded energy per CH_2 or CH_3 group is plotted in Fig. 6 as a function of chain position for structure A and for a pure DMPC bilayer. The average energy per CH_2 group is −1.18 kcal/mol for structure A, and is −1.44 kcal/mol for pure DMPC. The latter value is similar to the CH_2 interaction energies previously calculated for other gel state bilayer lipids (Vanderkooi, 1990b). The fact that the CH_2 interaction energy is less in the mixed bilayer than in the pure bilayer is not surprising, considering the disparate nature of the cholesterol and DMPC molecules involved. It is remarkable that the difference is not greater than was found.

DISCUSSION

The results obtained by these calculations provide a structural basis for understanding some of the experimental observations described in the Introduction. For example, the seemingly inconsistent data on the requirements for the condensation effect in monolayers can now be clarified. Early results were interpreted to mean that a β-OH group on cholesterol was essential for the condensing effect (Demel et al., 1972a), but later work showed this was not so because 3-doxyl cholestane also gave condensation (Cadenhead and Muller-Landau, 1979). The requirement of a particular

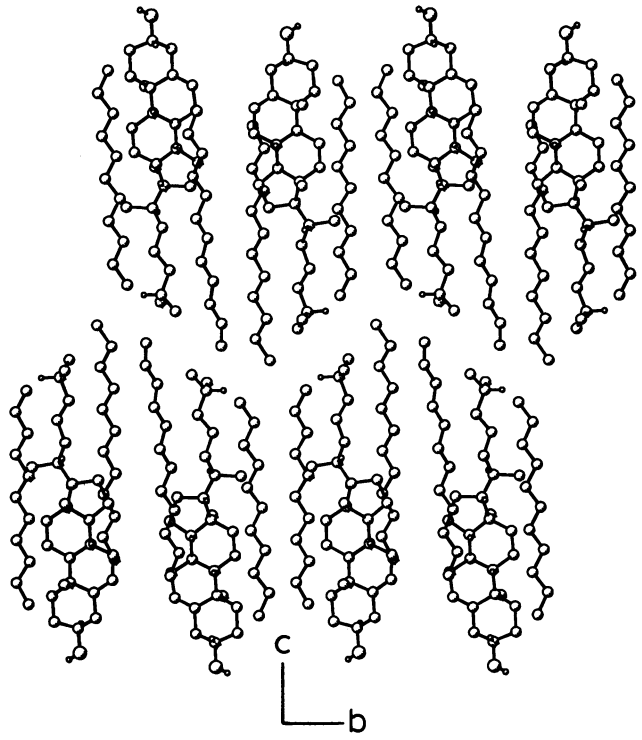

FIGURE 5 Lateral view of structure A (tetrameric asymmetric unit), showing the packing arrangement at the bilayer midplane. The projection is down the *a* axis. Only the cholesterol molecules and the acyl chains are shown, with the polar parts of the DMPC molecules being omitted for clarity.

TABLE 4 Geometrical parameters for DMPC:cholesterol bilayer structures, as compared wtih the structure of a pure DMPC bilayer

Parameter	DMPC:cholesterol 1:1		DMPC‡
	Structure A*	Structure B	
Space group	C2	P222	$P2_1$
Mol. per asym. unit	4	2	2
Asym. units per unit cell	4	4	2
2-d lattice constants:			
a (Å)	9.317	8.829	8.573
b (Å)	32.664	17.799	9.000
Surface area per dimer§ (Å²)	76.1	78.6	77.2
Nonpolar region thickness¶ (Å)	38.0	41.2	38.6
P-N vector angle (deg)‖	12.3°, −5.8°	2.1°	9°, 25°

* Data are for structure A with a tetrameric asymmetric unit.
‡ The DMPC bilayer parameters are for structure II of the energy minimized DMPC bilayer described by Vanderkooi (1991).
§ The surface area is given for a heterodimer of DMPC and cholesterol or for a homodimer of DMPC.
¶ The nonpolar region thickness is measured as the mean distance between the planes of the glycerol C2 atoms in the two halves of the bilayer.
‖ The angle is measured between the P-N vector and the bilayer plane.

phospholipid structure for condensation was also made uncertain by the work of De Kruyff et al. (1973), who found that similar condensation effects were given regardless of whether there was an ester, ether, or alkane linkage at the 2 position of glycerol.

These experimental results are explainable in terms of the structural and energetic information given above. The energy values given in Table 6 show that the largest contribution to the DMPC-cholesterol interaction energy is the nonbonded energy, with only a relatively small part of it being due to the hydrogen bond. Thus, it is understandable that the cholesterol OH group can be replaced with a doxyl group as in the work of Cadenhead and Muller-Landau (1979), with the consequent loss of the hydrogen bond but still giving the condensing effect. Likewise, replacement of the *sn*-2 ester linkage with an alkane or ether linkage will eliminate the hydrogen bond, but will not disrupt the nonbonded interactions between cholesterol and the acyl chains; and condensation can still occur (De Kruyff et al., 1973).

In epicholesterol, the H and OH groups on C3 are interchanged relative to their positions on cholesterol. The axial H atom on C3 in cholesterol points toward the DMPC molecule and is in van der Waals contact with it in the energy-minimized structures. Hence, if this H is interchanged with the much larger hydroxyl group, as is the case in epicholesterol, not only will the geometry of the hydroxyl be incorrect for hydrogen bonding, but more importantly the cholesterol and DMPC molecules will be forced apart by the axial hydroxyl, thereby severely weakening the whole array of nonbonded interactions between the molecules. A similar effect would be given by any other group protruding on the α surface of the cholesterol ring system. This confirms and emphasizes the need for a flat α surface on cholesterol as a requirement for condensation, as previously inferred from surface pressure data by Demel et al. (1972a).

Although hydrogen bonding is not an essential requirement for the favorable interaction between cholesterol and DMPC, the fact that this single pairwise interaction contributes 2.0 kcal/mol to the dimer interaction energy (Table 6) ensures that there will be a high probability for hydrogen bond formation if hydrogen bonding is structurally possible. The energy calculations show that hydrogen bonding will occur primarily between the cholesterol OH and the carbonyl oxygen of the *sn*-2 ester group, which is in agreement with the FT-IR results of Wong et al. (1989).

Presti et al. (1982) and Finean (1990) built space-filling models of lecithin and cholesterol and, on this basis, proposed hydrogen bonding to the *sn*-2 glycerol ester oxygen rather than to the carbonyl oxygen. A special effort was made to reproduce their model by computer to obtain an energy-minimized dimer that included hydrogen bonding to the glycerol oxygen, but no low energy structures of that type could be found.

It is well known that mixed bilayers readily form that contain lesser amounts of cholesterol than the 1:1 mole ratio studied here. It may be expected that dimers of lecithin and cholesterol will still be found in such bilayers, considering the goodness of fit that occurs between these two kinds of molecules. In structure B there are alternating bands of cholesterol and DMPC, with the DMPC bands being two molecules thick (see Fig. 4). Increasing the DMPC to cholesterol

TABLE 5 Intermolecular energies of interaction of DMPC and cholesterol with their environments

Energy term* (kcal/mol)	DMPC		Cholesterol		Total	
	Str. A‡	Str. B	Str. A‡	Str. B	Str. A‡	Str. B
Electrostatic	−12.25	−12.09	−0.33	−0.22	−12.58	−12.31
Nonbonded	−47.07	−47.23	−28.96	−23.62	−76.03	−70.85
Hydrogen bonded	−1.00	−1.01	−1.00	−1.01	−2.00	−2.02
Total	−60.32	−60.33	−30.29	−24.85	−90.61	−85.18

* The energies are expressed per mole of molecules of each type. Averages are given of the energies of the 2 nonidentical DMPC and cholesterol molecules in the asymmetric unit of structure A.

‡ Data are for structure A with a tetrameric asymmetric unit.

TABLE 6 Energy of interaction between like and unlike molecules in DMPC:cholesterol bilayers

Energy term (kcal/mol)	Type of interaction*					
	DMPC-DMPC		chol-chol		DMPC-chol	
	Str. A	Str. B	Str. A	Str. B	Str. A	Str. B
Electrostatic	−11.88	−11.81	+0.04	+0.06	−0.74	−0.56
Nonbonded	−22.54	−34.07	−4.42	−10.45	−49.07	−26.33
Hydrogen bonded	0.0	0.0	0.0	0.0	−2.00	−2.02
Total	−34.42	−45.88	−4.38	−10.39	−51.81	−28.91

* The DMPC-DMPC energy equals one-half the sum of all interactions between a reference DMPC molecule and all other DMPC molecules in the bilayer, out to the specified cutoff distance, and the cholesterol-cholesterol and DMPC-cholesterol energies are similarly defined.

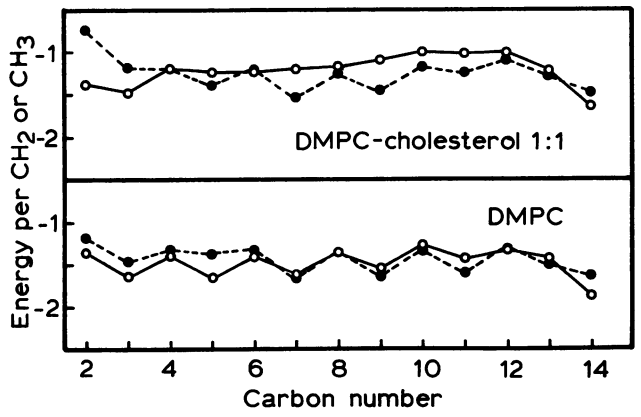

FIGURE 6 The intermolecular energy (kcal/mol) per CH_2 or CH_3 group in the acyl chains of DMPC is plotted as a function of chain position. The upper panel gives the interaction energy in the mixed DMPC-cholesterol bilayer (structure A, tetrameric asymmetric unit), and the lower panel gives the corresponding energy values in a pure DMPC bilayer (structure II of Vanderkooi, 1991). In each case the plotted value is the average for the two DMPC molecules in the asymmetric unit. The β chains are indicated by ● and - - - -, and the Γ chains are shown with ○ and ——.

ratio by the addition of DMPC to this type of structure might simply add to the already existing DMPC bands, making them thicker, without disrupting the DMPC-cholesterol interactions at the band boundries. Bulk DMPC not in direct contact with cholesterol may be expected to undergo the usual DMPC thermal phase transition.

The interesting question remains as to why 1:1 DMPC-cholesterol mixed bilayers do not show a thermal phase transition (Mabrey et al., 1978; McMullen et al., 1993). This is evidently a problem of gel state bilayer energetics, and some insight may be gained regarding it from the energy calculations. It was shown in Table 4 that cholesterol and DMPC each occupy approximately the same surface area, but the energy of interaction of cholesterol with its neighbors given in Table 5 is only half that of the interaction energy of DMPC. (The interaction energy of DMPC in the mixed bilayer is about the same as it is in pure gel state DMPC.) Thus, the average intermolecular energy per molecule and the intermolecular energy per unit area are considerably weaker in the mixed bilayer than in a pure DMPC bilayer. The interaction energy in the mixed bilayer evidently is not of sufficient strength to hold the bilayer together in an ordered array over extended distances, as required for a cooperative gel to liquid crystalline phase transition. Finean (1990) has suggested that short range or microcrystalline order may be present in lecithin-cholesterol mixed bilayers, although long-range order is not present as demonstrated by the diffuse x-ray diffraction pattern.

Cholesteryl esters have been studied extensively by x-ray crystallography (Craven, 1986), and it has been suggested that the packing arrangements found in these crystals may provide some understanding of the cholesterol-phospholipid interactions that occur in mixed lipid bilayers and membranes (Dahlen, 1979; Sawzik and Craven, 1979b). Cholesteryl laurate crystals contain two nonsymmetry-related molecules (A and B) in the asymmetric unit; the steroid rings of the B molecules are almost entirely surrounded by aliphatic chains of neighboring molecules. This observation, taken together with packing density considerations, led Dahlen (1979) to conclude that "Cholesterol and hydrocarbon chains can thus pack together just as effectively as when they are arranged in separate regions in a crystal structure."

The results of the present calculations are in agreement with this, considering that in the lowest energy packing arrangement (structure A) the nearest neighbors of cholesterol are all DMPC molecules, with ring-chain interactions predominating in the hydrophobic domain.

Cholesteryl myristate is structurally homologous with cholesteryl laurate but crystallizes in an entirely different packing mode (Craven and DeTitta, 1976; Craven, 1986). Cholesteryl myristate forms stacked bilayers with alternating layers of steroid rings and interdigitated aliphatic chains. Ring-ring and chain-chain interactions are present, but the ring-chain interactions prevalent in the laurate ester are absent. The lateral packing of the steroid rings in this structure is remarkably similar to the arrangement in the cholesterol bands of structure B shown Fig. 4 (compare with Fig. 3 of Craven and DeTitta, 1976, or Figs. 6–25 of Craven, 1986). The cholesterol rings are parallel and are back to back, with the protruding CH_3 groups on the β surfaces being staggered. Like-like interactions predominate in structure B just as in the cholesteryl myristate crystals. Hence, crystallographic precedents exist for the essential types of nonpolar interactions found in both structures A and B. There is evidently no strong driving force favoring either like-like or like-unlike interactions between the steroid rings and the aliphatic chains. These observations provide empirical support for the findings of the present calculations, in which it was shown that two quite different packing arrangements have similar energies, with like-like interactions being mainly present in the one, but like-unlike interactions in the other.

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