Cholesterol–Phospholipid Interaction in Membranes. 2. Stoichiometry and Molecular Packing of Cholesterol-Rich Domains†

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ABSTRACT: A model for the molecular interaction between cholesterol and phospholipid in bilayer membranes is presented. We propose that cholesterol forms associations with phospholipids with stoichiometries of both 1:1 and 1:2. A hydrogen bond between the β-OH of cholesterol and the glycerol ester oxygen of a phospholipid is suggested as a likely mechanism for tight binding in a 1:1 complex. A second phospholipid molecule is loosely associated with the complex to form domains of 1:2 stoichiometry, which may coexist with pure phospholipid domains. Interfacial boundary phospholipid separates these two domains. Under conditions in which interfacial phospholipid is maximal, the perturbed phospholipid assumes a composition of 20 mol % cholesterol. To account for the phase behavior and surface properties of cholesterol–lipid membranes, we propose a molecular packing model for linear arrays within the cholesterol-rich domains. In this arrangement, two rows of 1:1 complex run antiparallel with loosely associated phospholipid intercalated between them. The loosely associated phospholipid can pack in the nearly hexagonal manner in which pure crystalline phospholipid is known to pack. The model provides maximal van der Waals contact in the hydrocarbon region of the bilayer and can maintain phospholipids as cholesterol's nearest neighbors at all concentrations up to 50 mol % cholesterol. The model is compatible with the diverse experimental observations compiled by many investigators over the past decade.

The most persistent and currently prevalent models for cholesterol–lipid interaction in biological membranes propose complex formation at 20, $33^1/_3$, or 50 mol % of the sterol. Such schemes have been based on calorimetry (Ladbrooke et al., 1968; Mabrey et al., 1978; Estep et al., 1978, 1979), X-ray diffraction (Engelman & Rothman, 1972), NMR[1] (Darke et al., 1972), fluorescence intensity and polarization (Lee, 1976; Rogers et al., 1979; Rubenstein et al., 1979), permeability (Blok et al., 1977; Tsong, 1975a,b), electron microscopy (Hui & Parsons, 1975; Copeland & McConnell, 1980), and ESR (Shimshick & McConnell, 1973; Shimoyama et al., 1978) studies. However, despite the wealth of data, theorizing, and speculation, there has been no conclusive interpretation of the behavior of cholesterol in lipid bilayer systems. The major reason that a concensus has not been reached is the difficulty in attaining a handle on these highly complex systems. Multicomponent bilayer membranes, which have relatively broad phase transitions, cannot be treated simply as macroscopic systems that satisfy the Gibbs phase rule, thus precluding rigorous construction of phase diagrams. Also, various experimental approaches necessarily highlight selected aspects of the structure, revealing information about one portion of the cholesterol molecule, one region of the lipid bilayer, or only one type of molecular interaction. All the data must be considered together in working out a cohesive picture of the behavior of these systems. This publication briefly reviews previously suggested explanations for cholesterol's behavior in lipid bilayers and then proposes a new, complete model for the mechanism of cholesterol–phospholipid interaction and the domain structure resulting from that interaction.

Cholesterol–Phospholipid Intermolecular Interaction. It has been suggested that hydrogen bonding between cholesterol's β-OH and the carbonyl groups of phospholipids is responsible for cholesterol's unique effects on lipid bilayer membranes (Brockerhoff, 1974). This idea was justified primarily by model building (Huang, 1976, 1977). A proton NMR investigation (Chatterjie & Brockerhoff, 1978), in which line widths of *sn*-3 and *sn*-1 dipalmitoylphosphatidylcholines responded differently to the addition of cholesterol, points to a stereospecific cholesterol–phospholipid interaction in the vicinity of the glycerol C-2. However, this study does not conclusively indicate an interaction with a carbonyl. In fact, recent permeability studies on diester, diether, and dialkyl lipids indicate that the carbonyl oxygens of phosphatidylcholines are not necessary for cholesterol interactions (Clejan et al., 1979). Also, infrared and Raman spectroscopic studies of the phosphatidylcholine carbonyl stretch bands (Bush et al., 1980) have shown that cholesterol does not hydrogen bond to the carbonyl groups of anhydrous DPPC. The possibility of hydrogen bonding between the sterol hydroxyl and the phosphate region of the lipid head group was excluded by ^{31}P NMR work (Yeagle et al., 1975). As a result of these recent studies, most investigators have discarded the idea that phospholipid participates in hydrogen bonding in complexing with cholesterol.

Another proposed molecular mechanism for the immobilization of phospholipids by cholesterol involves steric interaction of the planar α face of the sterol ring region with the phospholipid hydrocarbon chains (Rothman & Engelman, 1972; Yeagle et al., 1977). However, favorable van der Waals interactions alone are not sufficient to abolish the gel–liquid-crystal phase transition of phospholipids. This was demonstrated by our ESR studies with CSL in DPPC liposomes

† From the Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. *Received February 5, 1982.* This is Contribution No. 6466. F.T.P. was supported by a Fannie and John Hertz Foundation fellowship. This work was supported in part by Grant GM 22432 from the National Institute of General Medical Sciences, U.S. Public Health Service, and by BRSG Grant RR07003 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

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[1] Abbreviations: CSL, cholestane spin-label, 4′,4′-dimethylspiro[5α-cholestane-3,2′-oxazolidin]-3′-yloxy; DPPC, dipalmitoylphosphatidylcholine; ESR, electron spin resonance spectroscopy; NMR, nuclear magnetic resonance spectroscopy; T_m, gel–liquid-crystal transition temperature; CPK, Corey–Pauling–Koltun.

0006-2960/82/0421-3831$01.25/0

(Presti & Chan, 1982). Although this sterol spin probe has a nearly identical ring system with cholesterol's, it actually *induces* melting in neighboring phospholipid molecules. This behavioral difference must be due to the replacement of the β-OH "head group" with a nitroxide radical label. Thus, some head group interaction must also contribute to cholesterol's effects in bilayers. This is further substantiated by the fact that epicholesterol (α-OH) behaves very differently from cholesterol in monolayer and bilayer lipid systems (DeKruyff et al., 1973). Although androstane spin-label does have a β-OH group, it also, like CSL, induces local phospholipid melting (Presti & Chan, 1982). This most likely results from steric hindrance to some intimate sterol–phospholipid interaction, imposed by the bulky nitroxide group, even though it is embedded in the hydrocarbon region of the bilayer. Thus, a close fit in the hydrocarbon region may be essential to provide the proper geometry for cholesterol's specific head group interaction.

Cholesterol's Effect on Phospholipid Conformation. The disappearance of all phospholipid calorimetric transitions at 50 mol % cholesterol (Mabrey et al., 1978; Estep et al., 1978, 1979) suggests that each cholesterol molecule complexes with one lipid molecule, thereby excluding it from participation in the thermal phase transition. A clue to the nature of the interaction responsible for such complex formation was recently provided by Raman spectroscopic studies of the carbonyl stretch bands of DPPC (Bicknell-Brown & Brown, 1980). Anhydrous, crystalline DPPC exhibits a doublet in the C=O stretch frequency region, but hydration of the lipid reduces this to a single band in the gel phase. The investigators suggest that the doublet is due to the presence of two acyl linkage conformers in crystalline DPPC. When cholesterol is added to aqueous DPPC dispersions, the C=O stretch region again consists of two bands, located at the same frequencies as the crystalline DPPC Raman bands. Thus, the conformation adopted by the lipid carbonyl region in the presence of cholesterol appears to be very similar to that of anhydrous, crystalline phospholipid. This conclusion is supported by single-crystal X-ray diffraction data (Hitchcock et al., 1974), which show that the two hydrocarbon chains of a phosphatidylethanolamine lipid have different molecular configurations, consistent with the observation of a Raman doublet. Also, deuterium NMR studies have demonstrated that cholesterol induces motional nonequivalence in the two acyl chains of gel-state phosphatidylcholines (Oldfield et al., 1978). Since the conformation of the phospholipid carbonyl region may affect chain orientation and packing in the gel phase (Bicknell-Brown & Brown, 1980), it is likely that the entire hydrocarbon region of phospholipids (or at least the upper portion of the hydrocarbon chains) assumes the crystalline conformation in the presence of cholesterol. Indeed, deuterium NMR experiments (Jacobs & Oldfield, 1979) demonstrated that 50 mol % cholesterol induced an order and a rigidity in liquid-crystalline phospholipid that are comparable to those of the pure gel phase.

Bicknell-Brown & Brown (1980) also point out that the cholesterol OH group may be required for the splitting of the DPPC carbonyl stretch band, by recording only a single Raman band with cholesterol methyl ether. Thus, a specific interaction with cholesterol's β-OH may be responsible for the conformational change and rigidification of phospholipid molecules.

A New Look at Hydrogen Bonding. Careful inspection of CPK molecular models with DPPC in its pure crystalline configuration [deduced from Hitchcock et al. (1974)] shows

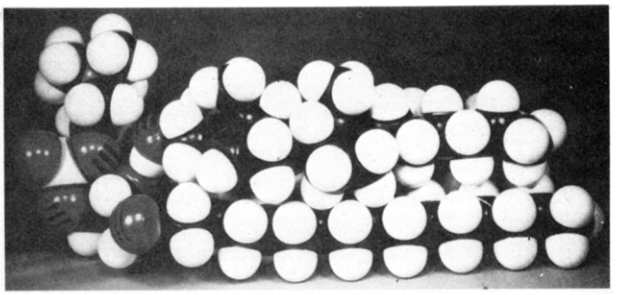

FIGURE 1: Space-filling CPK molecular model of cholesterol hydrogen bonded via its β-OH group to the glycerol oxygen that is esterified to the 2-position fatty acid chain of DPPC, which is in the all-trans rigid gel-state configuration.

that a collinear hydrogen bond between cholesterol's β-OH and the glycerol oxygen, which is esterified to the 2-position fatty acid chain of the phospholipid, is very favorable (see Figure 1). The carbonyl oxygens point upward in directions not particularly favorable for hydrogen-bond formation. However, the easily formed ester H bond permits close approach of the two molecules with intercalation of the protruding hydrogen atoms. The α-OH of epicholesterol does not have the proper geometry to form this interaction, consistent with the observations that this sterol does not exhibit the condensing effect or the reduction in lipid transition enthalpy, which are characteristic of cholesterol.

With the configuration depicted in Figure 1, the flat α face of cholesterol packs in tightly with the lipid hydrocarbon chains, maximizing the van der Waals interactions. The upper acyl chain region is constrained to be in the all-trans ordered state characteristic of pure gel-phase and crystalline phospholipids. This combination of strong van der Waals attraction and hydrogen bonding could account for the strong complex formation that has been suggested empirically. It is possible that the hydrogen bond provides the necessary extra binding energy to prevent the complexed phospholipid from participating in the normal gel–liquid-crystal phase transition.

Cholesterol as a "Filler". Less than 5 mol % cholesterol is sufficient to abolish the phosphatidylcholine pretransition (Estep et al., 1978), which is believed to be associated with a change in chain tilt and a structural transformation at the bilayer surface (Rand et al., 1975; Janiak et al., 1976). According to a model proposed by Nagle (1976) and McIntosh (1980), the phosphatidylcholine head group, in its position parallel to the bilayer plane (Seelig, 1978), has a larger excluded area than the hydrocarbon chains. Tilting enables the chains to fill in a potential void in the hydrocarbon region, permitting them to come in close contact, to maximize their van der Waals interactions. At the pretransition, a head group conformational change that reduces the excluded area of the head group allows the chains to straighten. Chain tilt is not necessary in phosphatidylethanolamines, which have a smaller head group, and indeed, no pretransition is observed in this group of phospholipids (Chapman et al., 1974). McIntosh (1980) has shown that long-chain alkanes such as tetradecane destroy the pretransition in phosphatidylcholine bilayers. In his filler model, *n*-alkanes partition into the hydrocarbon bilayer region, filling in the potential void, and make it energetically favorable for the chains to straighten. We are suggesting that, among its effects on phosphatidylcholine bilayers, cholesterol exhibits similar behavior as a filler and thus removes the pretransition.

Proposal for Cholesterol-Rich Domain Stoichiometry. The manner in which the cholesterol–phospholipid (1:1) complex units are distributed within the bilayer will greatly influence

the observed phase behavior. Macroscopic properties of bilayer membranes are dependent on composition, size, and distribution of cholesterol-rich domain regions, which are all determined by total cholesterol concentration within a particular phospholipid bilayer.

A phase domain region with a composition of $33^1/_3$ mol % cholesterol has been strongly suggested by X-ray diffraction (Engelman & Rothman, 1972), permeation (Tsong, 1975b; Blok et al., 1977), complement-fixing activity (Humphries & McConnell, 1975), surface pressure (Tajima & Gershfeld, 1978), and lateral diffusion (Rubenstein et al., 1979) studies. Bilayer systems with cholesterol-rich domains of this composition may also exhibit special effects around 20 mol % cholesterol, a point that will be clarified by the discussion below.

We propose that as cholesterol is added to phospholipid bilayers, the first cholesterol-rich regions to coalesce out are domains with a stoichiometry of one cholesterol to two phospholipid molecules. One of these phospholipids is complexed to the sterol via the hydrogen bond between cholesterol's β-OH and the glycerol ester oxygen of the lipid, as depicted in Figure 1. The second phospholipid molecule is loosely associated with the complex, probably simply by van der Waals interactions. These cholesterol-rich domains coexist with free phospholipid domains, which are responsible for the sharp calorimetric peak at the gel–liquid-crystal transition temperature. Phospholipid that is complexed to cholesterol does not undergo a thermal phase transition and does not contribute to the calorimetry curve, whereas interfacial boundary phospholipid and the phospholipid loosely associated with the complex contribute to the broad uncooperative transition observed slightly above the free bulk phospholipid melting temperature (Mabrey et al., 1978; Estep et al., 1978).

At about 20 mol % cholesterol content, free phospholipid domains disappear, although interfacial boundary phospholipids remain between the 1:2 cholesterol-rich domains. Hence, the main gel–liquid-crystal transition disappears at 20 mol % cholesterol, as has been observed by calorimetry (Mabrey et al., 1978; Estep et al., 1978), dilatometry (Melchior et al., 1980), and ESR (Presti & Chan, 1982) studies. Disappearance of pure phospholipid domains at 20 mol % cholesterol is also evident from a study that monitored lateral diffusion of a fluorescent lipid probe in phosphatidylcholine–cholesterol binary mixtures (Rubenstein et al., 1979). The sharp change in the diffusion coefficient seen in the pure phospholipid is present at the gel–liquid-crystal transition temperature up to 20 mol % cholesterol.

It is also at about 20 mol % cholesterol that there is a maximum enthalpy change in the broad calorimetry peak (Estep et al., 1978). This lends support to the theory that cholesterol-rich regions have a stoichiometry of 1:2, since it has been shown by Monte Carlo simulations (with interaction strengths deduced from calorimetry data) (Snyder & Freire, 1980) that, for domains of $33^1/_3$ mol % cholesterol, there is a maximum area of boundary phospholipid at about 20 mol % total cholesterol content. Molecular transport across bilayer membranes has also been shown to be maximal at about 17 mol % cholesterol, suggesting that the interfacial lipid regions mediate faster transmembrane permeation (Tsong, 1975b). The importance of phase-boundary regions in mediation of molecular transport is reinforced by kinetic data with pure phospholipid, which demonstrate that there is a maximum transport rate of amphiphilic molecules and ions at the temperature of the lipid gel–liquid-crystal phase transition (Tsong, 1975a; Marsh et al., 1976). In the cluster model of lipid phase transitions, there is a maximum number of solid and fluid clusters, and therefore of phase boundary regions, at the transition temperature (Marsh et al., 1976; Freire & Biltonen, 1978; Kanehisa & Tsong, 1978). The decrease in bilayer permeability both above and below the transition indicates that the enhanced permeability at T_m must arise from the maximal interfacial regions between solid and fluid phases and not from either phase domain.

The amount of interfacial boundary phospholipid decreases above 20 mol % cholesterol and, in our model, disappears completely at $33^1/_3$ mol %. This prediction is supported by the cessation of fast molecular transport at $33^1/_3$ mol % cholesterol (Tsong, 1975a). At this composition, only cholesterol-rich domain remains. The residual, very broad calorimetric transition that occurs at cholesterol concentrations between $33^1/_3$ and 50 mol % may be attributed to the highly uncooperative melting of the loosely associated phospholipid molecules still left around the 1:1 hydrogen-bonded complex pairs. These decrease in number as more 1:1 complex units are formed with the addition of more cholesterol. The last uncomplexed phospholipid molecule disappears at 50 mol % cholesterol, and further addition of the sterol results in separation of hydrated cholesterol crystals from the bilayer (Lecuyer & Dervichian, 1969).

Within the cholesterol-rich domains lateral diffusion should be comparable (within 1 order of magnitude) to that of fluid bilayers, because detailed crystalline interlocking between the all-trans hydrocarbon chains is lost (R. J. Pace and S. I. Chan, unpublished results). However, fast diffusion is limited to these cholesterol-rich regions if gel-state phospholipid is present. If the diffusion coefficient is monitored as a function of cholesterol content (Rubenstein et al., 1979), no change in the temperature dependence of this quantity is observed until the addition of around 20 mol %. At this point, the sigmoidal temperature-dependent curve becomes broader and shifts downward from T_m by a few degrees [Figure 4 (Rubenstein et al., 1979)]. As more cholesterol is added the curve becomes still broader and shallower and appears to be approaching a limit, not far above 30 mol % cholesterol (the highest sterol concentration used in that study) at which diffusion is comparable (fast) above and below T_m. These results are compatible with the existence of cholesterol-rich domains with a 1:2 stoichiometry. Although pure lipid domains disappear at 20 mol % cholesterol, rapid diffusion is still inhibited by gel-state boundary lipids, which melt increasingly uncooperatively as their area decreases with increasing cholesterol. Near $33^1/_3$ mol %, the concentration at which boundary lipid is expected to disappear, the diffusion coefficient becomes nearly temperature independent and approaches the level observed for fluid phospholipid bilayers.

Structure of Cholesterol-Rich Domains. There is some evidence that the cholesterol-rich domains consist of linear arrays of molecules, in which rows of sterol run parallel to rows of phospholipids. Fluorescence studies with sterol probes indicate that cholesterol molecules pack in phospholipid bilayers without close contact between the α faces, even with up to 50 mol % sterol present (Rogers et al., 1979). These investigators suggest that sterol molecules are organized linearly in an end-to-end fashion, with rows of phospholipid molecules separating these rows of sterol and preventing face-to-face overlap of sterols. It has also been suggested that phosphatidylcholesterol molecules may form linear arrays (Jain et al., 1980).

A linear packing arrangement can readily accommodate our model for cholesterol–phospholipid interaction. We propose that one row of phospholipid runs parallel to each row of 1:1

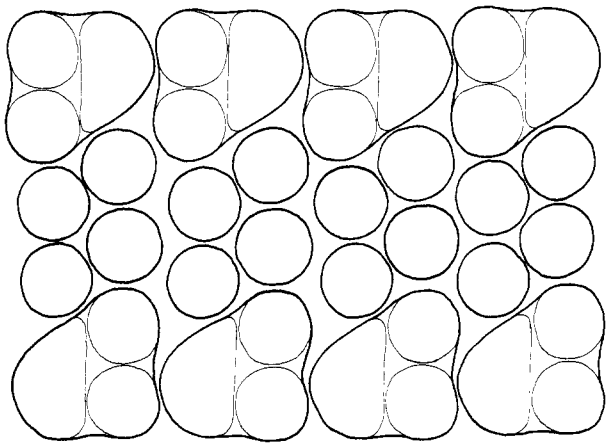

FIGURE 2: Model for domains of 1:2 cholesterol–phospholipid stoichiometry, as viewed from above the bilayer surface. A pair of circles represents the two hydrocarbon chains of a phospholipid molecule, while the slightly elongated shape denotes a cholesterol molecule. A line has been drawn around the 1:1 cholesterol–phospholipid complex pairs. One column the width of a single complex pair may be considered to be the unit cell of the packing structure.

sterol–lipid complex, consistent with the proposed 1:2 stoichiometry of cholesterol-rich domains. Of course, this would reduce to rows of complex alone as the cholesterol content is increased above $33^1/_3$ mol %.

In Figure 2 we present our model for packing of cholesterol-rich domains in a 1:2 stoichiometry. Two rows of 1:1 complex pairs run antiparallel with loosely associated phospholipid intercalating between them. In this model, the loosely associated phospholipid can pack in a quasi-hexagonal manner virtually identical with its pure lipid gel-state (L_β) packing (Elder et al., 1977). The suggested arrangement leaves very little intermolecular void and allows for maximal van der Waals contact in the hydrocarbon region of the bilayer. Since the orientation of the complex pairs in adjacent rows is alternated, cholesterol molecules do not contact each other, but have phospholipids for all their nearest neighbors. Removal of all loosely associated lipids at an overall bilayer composition of 50 mol % cholesterol leaves only 1:1 complex pairs, which are still packed in the antiparallel rows that provide cholesterol with only phospholipid nearest neighbors. Below 20 mol % cholesterol content, extra phospholipid molecules form pure lipid domains outside these linear regions of 1:2 plus boundary lipid.

The extended linear array model is consistent with freeze–fracture studies of the rippled structure of phospholipid bilayer surfaces. Above the phosphatidylcholine pretransition, but below T_m, the bilayer surface acquires a periodic undulation or ripple (Janiak et al., 1976; Luna & McConnell, 1977). If cholesterol is incorporated into the membranes, the ripple repeat distance increases until, at 20 mol % cholesterol, the rippling disappears completely (Copeland & McConnell, 1980). These investigators speculate that there might be phase separation in which thin strips of smooth 20 mol % cholesterol phase are separated by ripple lines of pure phosphatidylcholine. This 20 mol % phase would be the 1:2 cholesterol–phospholipid plus boundary lipid region in our model. The freeze–fracture data would not distinguish between the two models.

At the lowest cholesterol concentration utilized in these freeze–fracture studies, 4 mol %, there is a smooth interripple distance of 32.2 Å, which is about the width of one "unit cell" in our packing model. The most concentrated sample used that still exhibited the banding contained 16 mol % cholesterol, and this had an interripple distance equivalent to about 15 packing units. It seems likely that in order to induce the linear cholesterol-rich domain structure in phospholipid bilayers, a minimum cholesterol content, perhaps around 4 mol %, is required; below this concentration, 1:1 cholesterol–lipid complex pairs may be randomly distributed about the bilayer. Interestingly, 4 mol % is also about the minimum cholesterol concentration that abolishes the phosphatidylcholine pretransition (Estep et al., 1978). Thus, the cholesterol-rich linear domains may induce the same conformational state in the free phospholipid as occurs as a result of the thermal pretransition in pure phosphatidylcholine lipids (consistent with its role as a filler, as discussed above). If this is true, then 4 mol % cholesterol or more might induce rippling in phosphatidylcholine membranes at temperatures below the pretransition, where Luna & McConnell (1977) report no rippling in pure lipid bilayers. Also, with less than 4 mol % cholesterol present, one should observe no change in the ripple repeat distance from that measured for pure phospholipid bilayers above the pretransition, if this is the minimum cholesterol content required for these effects.

The macroscopic rippling of the entire membrane surface observed by freeze–fracture below T_m suggests the presence of long-range linear order over the whole bilayer surface. Above T_m, the long-range order is unlikely to be preserved, but molecules within cholesterol-rich domains may still be arranged quasi-linearly.

Conclusions. The molecular interaction and packing model presented here provides a unified interpretation for a wide range of seemingly contradictory published results obtained by various experimental techniques. Experiments that monitor dynamic properties of bilayers have shown significant changes around 20 mol % cholesterol, including lateral diffusion (Rubenstein et al., 1979), water permeation (Blok et al., 1977), lipid spin-label mobility (Rubenstein et al., 1980), and fluorescent dye transport (Tsong, 1975b). These phenomena are consistent with the calorimetry (Mabrey et al., 1978; Estep et al., 1978) and dilatometry (Melchior et al., 1980) studies that point to disappearance of a free bulk phospholipid phase at 20 mol % cholesterol. Other experiments that have shown evidence of special effects around $33^1/_3$ mol % cholesterol (Engelman & Rothman, 1972; Humphries & McConnell, 1975; Tsong, 1975b; Taylor et al., 1977; Rubenstein et al., 1980) lend support to our suggestion that boundary lipid disappears at this composition, leaving only cholesterol-rich domain. Finally, there is a multitude of experimental data supporting formation of a 1:1 cholesterol–lipid complex, including calorimetry (Ladbrook et al., 1968; Mabrey et al., 1978), X-ray diffraction (Bourgès et al., 1967; Lecuyer & Dervichian, 1969), nuclear magnetic resonance (Darke et al., 1972), fluorescence (Lee, 1975, 1976; Rogers et al., 1979), and electron diffraction (Hui & Parsons, 1975).

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