THE UNIVERSITY OF MANITOBA

SPIN LABEL STUDIES OF CHOLESTEROL-PHOSPHOLIPID INTERACTIONS IN ORIENTED THIN FILMS

by

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TO MY PARENTS

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ABSTRACT

The spin label technique has been used to investigate the interaction of cholesterol with phospholipids in oriented thin lipid films. Two model systems were investigated: (a) egg phosphatidylcholine, and (b) bovine brain sphingomyelin. In hydrated egg phosphatidylcholine films, the hydrocarbon regions are in a liquid state at room temperature. Addition of cholesterol reduces the random motion of the fatty acid residues. Cholesterol increases the fluidity of the hydrocarbon regions of sphingomyelin films at room temperature but makes them more rigid at higher temperatures. The fluidity of the polar regions of the sphingomyelin films show a marked temperature dependence ascribed to intermolecular interactions involving the polar moieties of the sphingomyelin molecules. A comparison of the ordering effects of cholesterol and several structural derivatives in both model systems demonstrate that the nature of cholesterol-phospholipid interactions is dependent on the chemical structure of the phospholipid. To be an effective ordering agent in egg phosphatidylcholine films, a steroid with a planar nucleus requires a hydroxyl group at C_3 in the β configuration, and an eight carbon fully saturated side chain attached at C_{17}. In sphingomyelin films, the C_3 substituent need not be a hydroxyl group and more latitude is permitted in the structure of the C_{17} substituent.

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CHAPTER I

INTRODUCTION

1) Membranes and Lipids

The study of cellular organization has revealed that the structural integrity of cells is maintained by a complex system of membranes. The external boundary of a cell is provided by the plasma membrane. Inside the cell, many highly specialized organells are bounded by membranes. All membranes possess the properties of highly selective active and passive transport of molecules and ions. The plasma membrane also controls cell-to-cell association and recognition. In specialized cells, the plasma membrane provides the matrix for certain enzymatic processes. Detailed discussions of the structure and function of cell membranes are given in several monographs.[1,2,3]

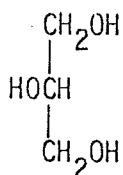
Membranes are composed mainly of proteins and lipids. In general, there are three types of lipids: phospholipids, glycolipids, (with one or more carbohydrate moieties), and sterols. Thought to be the most important class of lipids in membrane structure are the phospholipids. In animal membranes, cholesterol is the most predominant sterol. There have been many models suggested for the lipid - protein arrangement in membranes,[4-7] but at this time the elucidation of the structure of biological membranes at the molecular level is a very complex problem due to the great number of chemical species present and our lack of understanding of the manner in which these molecules are integrated in the overall structure. A critical discussion of membrane models is found in a recent review.[8] A simplified approach has been to study the physical and chemical properties of model membranes consisting only of lipids with the expectation that knowledge of these systems will yield some insight into biological

membrane structure and function.

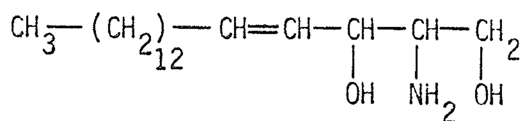
In the following sections, the properties of phospholipids and cholesterol will be discussed.

2) Phospholipids

Phospholipids are derivatives of either glycerol or sphingosine.

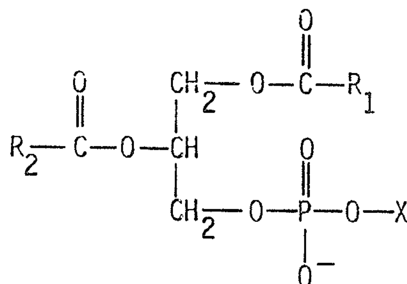


glycerol



sphingosine

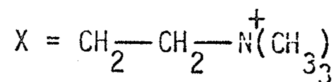
The phosphoglycerides have the general structure



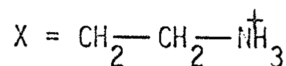
where R_1 and R_2 are long chain fatty acid residues. Some of the phosphoglycerides are:

phosphatidic acid

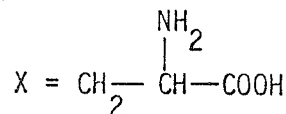
phosphatidylcholine



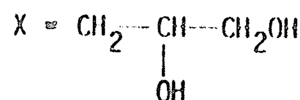
phosphatidylethanolamine



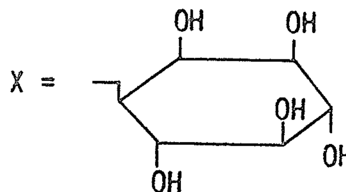
phosphatidylserine



phosphatidylglycerol

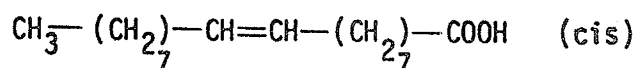


phosphatidylinositol

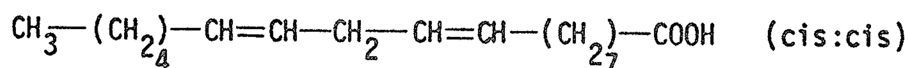


The most predominant phosphoglyceride found in mammalian cells is phosphatidylcholine which has the trivial name lecithin. Phosphatidylcholine is a waxy solid with no definite melting point. It is a zwitterion over a wide pH range.[9] Phosphatidylcholine isolated from a natural source generally has a heterogeneous fatty acid composition. Stearic ($C_{17}H_{35}COOH$) and palmitic acid ($C_{15}H_{31}COOH$) are the major saturated fatty acid constituents of mammalian phospholipids. Unsaturated fatty acids which have been found to be associated with phospholipids include:

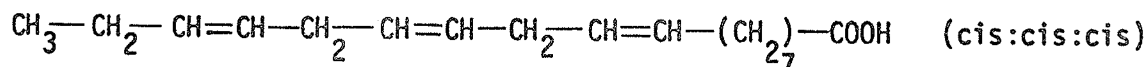
oleic acid:



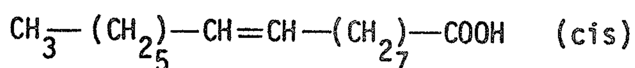
linoleic acid:



linolenic acid:



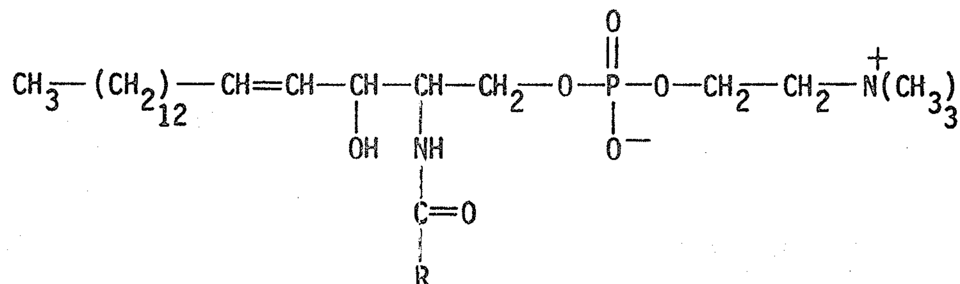
palmitoleic acid:



In egg phosphatidylcholine, unsaturated fatty acids are preferentially

located at the 2-position.[10] At the other end of the phosphatidylcholine molecule is the so called polar head group consisting of several functional groups: the ester linkages, the negatively charged phosphate group, and the positively charged choline group.

The best characterized phosphosphingoside is sphingomyelin. Sphingomyelin is a white crystalline solid with a melting point around 200°C. It is found in many membranes, notably in nervous tissue.



sphingomyelin

Depending on the source, R is usually palmitic, stearic, lignoceric ($C_{23}H_{47}COOH$) or nervonic ($C_{23}H_{45}COOH$) acid.[6] Thus, sphingomyelin tends to have a higher proportion of saturated fatty acid residues than phosphatidylcholine. It is interesting to note that the basic structure of phosphatidylcholine and sphingomyelin are similar, each having two hydrocarbon chains and a phosphorylcholine moiety.

3) Physical Properties of Phospholipids

The chemical structure of a phospholipid is such that two different regions in the molecule can be distinguished:

(a) the hydrophobic region, consisting of the long chain fatty acid residues,

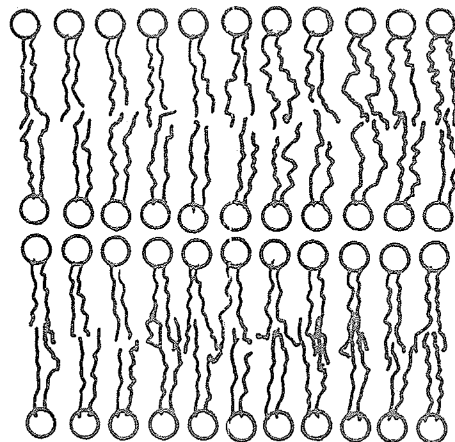
(b) the hydrophilic region, consisting of the polar head group which has a permanent dipole moment.

These structural features give rise to the unique physical properties of phospholipids. The physics and chemistry of phospholipids are discussed in great detail in several comprehensive review articles and monographs[6,11-16a] and only a brief account will be given here.

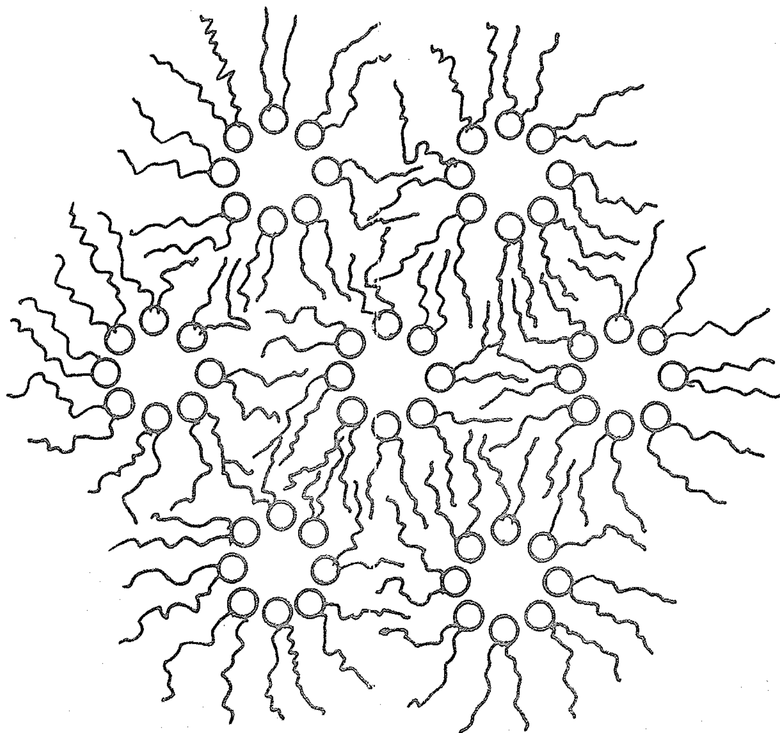
Provided the temperature is low enough, all dry phospholipids exist in a crystalline state. On heating a crystalline phospholipid, there is a transition from the crystalline to liquid crystalline state. The temperature at which this transition occurs depends on the chemical structure of the phospholipid, particularly on the composition of the hydrocarbon chains. For phospholipids having the same polar head group, the longer and more saturated the fatty acid residues are, the higher the transition temperature will be. In the liquid crystalline state, the hydrocarbon chains are melted and exhibit liquid-like behavior. The polar head groups hold the molecules together maintaining some degree of long range order.

X-ray diffraction studies have shown that anhydrous phospholipids exist in only two liquid crystalline phases:

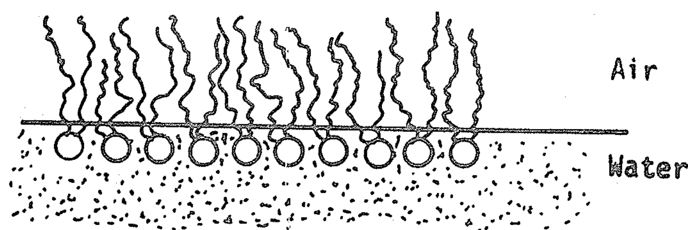
(a) the lamellar phase, periodic in one dimension,



(b) the hexagonal phase, periodic in two dimensions.



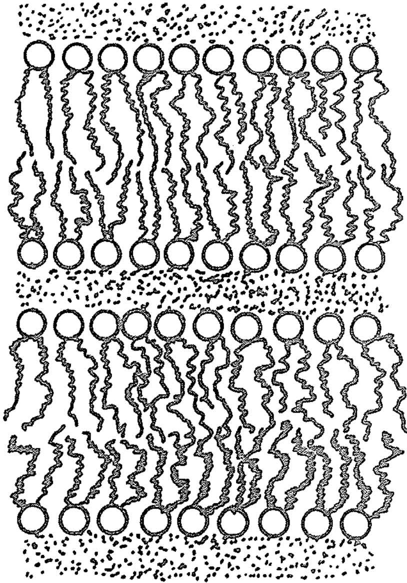
A knowledge of the physical properties of phospholipids in the presence of water is necessary for understanding membrane structure. Since there are two chemically different entities in a phospholipid molecule, the solubility of both regions in a solvent will be different. Molecules displaying this bimodal character are called amphiphilic molecules. Phospholipid molecules which are spread on a water surface by means of a volatile carrier solvent are spontaneously oriented with the polar head groups in the water and the paraffin chains above the surface. Thus, phospholipids are able to form monolayer films at an air-water interface.

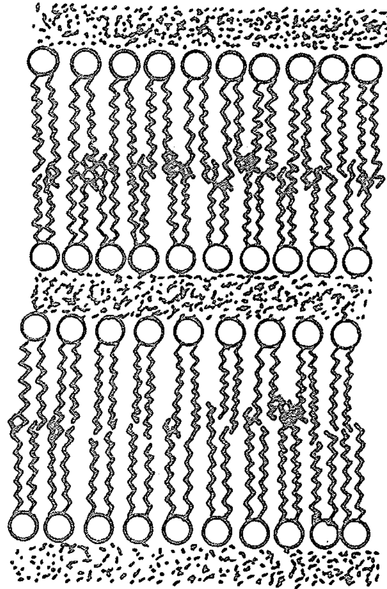


Monolayers are formed regardless of whether the phospholipid is in the crystalline or liquid crystalline state. The concepts of molecular orientation of amphiphilic molecules at air-water interfaces are primarily due to Langmuir.[17] There are other structures that phospholipids form in water which are shown in Figure 1:1. The most common ones are: (a) lamellar, (b) gel, (c) hexagonal I, (d) hexagonal II. The lamellar phase consists of bimolecular lipid leaflets, or bilayers of phospholipids in the liquid crystalline state, alternating between layers of water. The bilayer is thought to be the most important structural arrangement

Figure 1.1

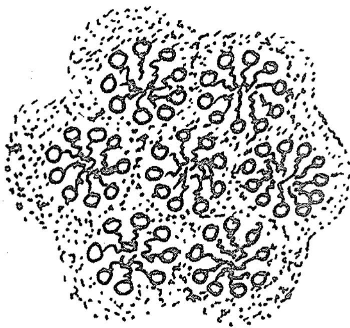
Some phases formed by phospholipids and water: (a) lamellar, (b) gel, (c) hexagonal I, (d) hexagonal II.

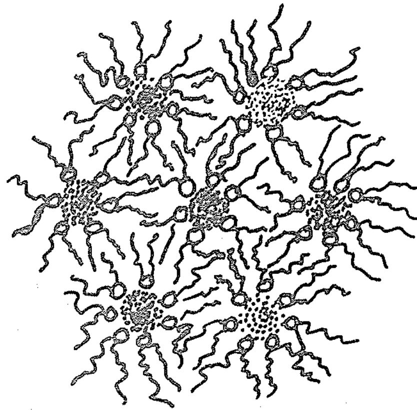




a

b





c

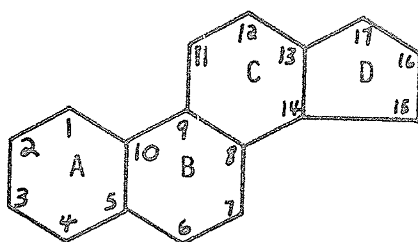
d

of phospholipids in biological membranes. The gel phase is formed when the lamellae are cooled to a temperature where the hydrocarbon chains become crystalline. The presence of water lowers the crystalline to liquid crystalline transition temperature of phospholipids. The transition temperature of dry dipalmitoylphosphatidylcholine is approximately 98 °C.[18] In the presence of excess water, the transition temperature is 41 °C.[18]

The two phospholipids which have been used in this research are egg yolk phosphatidylcholine and bovine brain sphingomyelin. A phase diagram of the egg yolk phosphatidylcholine - water system has been determined by X-ray diffraction.[19] At 25 °C, egg phosphatidylcholine is in a liquid crystalline state and forms a lamellar phase with water. The lamellar phase can accomodate up to 45% water by weight. Increasing the water concentration above 45%, results in the formation of two phases: the lamellar phase and an aqueous phase. The crystalline to liquid crystalline transition for egg phosphatidylcholine in the presence of water has been determined calorimeterically to occur over the range -15 °C to -7 °C.[16] The broadness of the transition is ascribed to the heterogeneous fatty acid composition of the phosphatidylcholine molecules. An X-ray diffraction study[20] of bovine brain sphingomyelin in the presence of water showed that at 25 °C, the paraffin chains were crystalline. At 40 °C, a lamellar phase exists which can incorporate up to 40% water before the formation of a separate aqueous phase.

4) Cholesterol

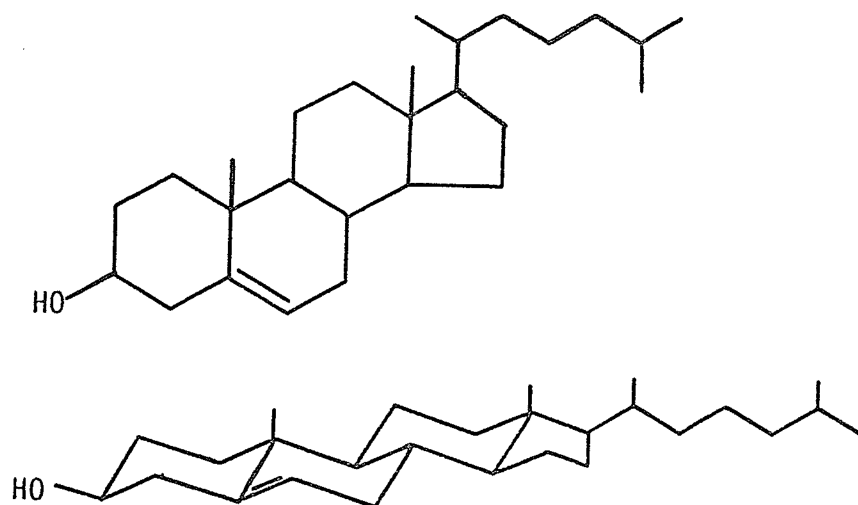
One aspect of membrane composition and structure which has intrigued scientists for many years is the association of cholesterol with phospholipids in many cell membranes. Cholesterol is a white crystalline solid with a melting point of 148.5 °C. It is a member of the class of compounds called steroids. The basic building block of the steroids is perhydrocyclopentanophenanthrene which consists of four fused carbon rings. Substituents on the tetracyclic ring system can



perhydrocyclopentanophenanthrene

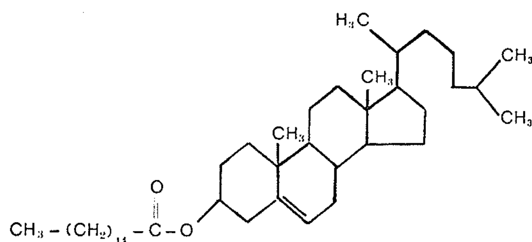
have two possible configurations. Those that project above the general plane of the steroid nucleus, that is above the plane of the paper, are considered to be in the β configuration and the bond is represented by a solid line. Substituents which are α oriented project below the plane of the paper and are denoted by a broken line bond. The A/B, B/C, and C/D ring fusions in the cholesterol molecule are trans. There is a hydroxyl group attached at C_3, a methyl group at C_{10} and C_{13}, an eight carbon fully saturated chain at C_{17}, and a double bond between C_5 and C_6. All the substituents are β oriented. The 2 and 3 dimensional structural formulas of cholesterol are shown on the following page. Since cholesterol has a hydroxyl group, it is a member of the special

subclass of steroids called sterols. Its correct chemical name is Δ^5 - cholesten - 3β - ol. Cholesterol is present in all vertebrates



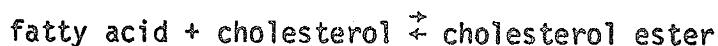
Cholesterol

and in some invertebrates, and is found mainly in plasma membranes. It is the major sterol found in man. All cells appear to be able to synthesize cholesterol but the major formation is thought to be in the liver and the adrenals. Cholesterol serves as the precursor for bile salts and hormones.[21] In addition to free cholesterol, cholesterol esters are found in animals, notable in the liver, adrenals, and plasma. These molecules consist of long chain fatty acids attached to the cholesterol molecule through an ester linkage at C_3.



Cholesteryl palmitate

In vitro studies of cholesterol esterases [22-24] have demonstrated substrate specificity of the enzyme. The 3-hydroxyl group must be in the β configuration; a double bond between C_5 and C_6 is not essential; saturation at C_7 is necessary, and the A/B ring fusion must be trans, for esterification. Unsaturated fatty acids were most readily esterified. It also appears that one enzyme[22,23] possesses the esterifying and hydrolytic properties of the reaction



fatty acid + cholesterol $\rightleftarrows$ cholesterol ester

although the presence of two enzymes could not be ruled out. In plasma, cholesterol esters are associated with complexes of proteins and other lipids. These complexes are called lipoproteins and are thought to play a role in the transport of lipids to cells or to be the precursors of membranes.

5) Cholesterol and Membranes

Since many membranes contain large amounts of cholesterol, it would appear that the physiological role of this molecule is intimately connected with the nature and extent of its interaction with other membranous components. To familiarize the reader with the current trends of opinion regarding the role of cholesterol in membranes, a brief review of the literature will be presented in the following subsections.

(a) Monolayers

Almost fifty years ago, Leathes[25] demonstrated that there is an interaction between cholesterol and phospholipids. The addition of cholesterol, which is assumed to have an invarient cross sectional area, to a monolayer film of lecithin resulted in a decreased average area occupied by the phospholipid molecules. The effects of cholesterol on monolayer phospholipid films have been studied extensively up to the present time using natural and synthetic phospholipids. A monolayer study of egg phosphatidylcholine by De Barnard[26] demonstrated the condensing effect of cholesterol and indicated the possible formation of molecular complexes at cholesterol to phospholipid ratios of 1:3 and 3:1. Demel *et al*[27] also found that cholesterol reduced the molecular area of films made from purified phospholipids from natural sources. However, their experiments showed no evidence of a phospholipid - cholesterol complex at 1:3 or 3:1 mole ratios for human erythrocyte phosphatidylcholine, egg phosphatidylcholine or human plasma sphingomyelin. Although the condensing effect of cholesterol depends on the nature of the fatty acid constituents of the phospholipid, there do not seem to be any straightforward conclusions

regarding cholesterol effects that can be reached from monolayer studies using purified synthetic phospholipids.[28] The most important factor appears to be the physical state of the phospholipid film, with the chemical composition of the hydrocarbon chains being of secondary importance. The ability of cholesterol to condense phospholipid films which are already in a very expanded or condensed state is very small. Shah and Schulman[29] have argued that there is no cholesterol condensing effect and that cholesterol acts as a fluidizing agent in membranes. They interpret the condensing effect of cholesterol in egg phosphatidylcholine to arise from the presence of molecular cavities. At low surface pressures, the thermal motion of the fatty acid chains of a phospholipid is thought to sweep out an effective volume in the shape of a cone. Therefore, a vacancy or molecular cavity will exist between adjacent phospholipid molecules. These authors state that the cholesterol molecules occupy these cavities and therefore cause no proportional increase in the average area per molecule in mixed monolayers thus giving rise to the apparent condensing effect. Their results based on surface potential measurements indicated that there was no interaction between cholesterol and either egg phosphatidylcholine or dipalmitoyl phosphatidylcholine. They also concluded that cholesterol decreased the cohesive forces between phospholipid molecules due to its rigid planar asymmetrical shape and increased the fluidity of the fatty acid chains in a phosphatidylcholine monolayer.

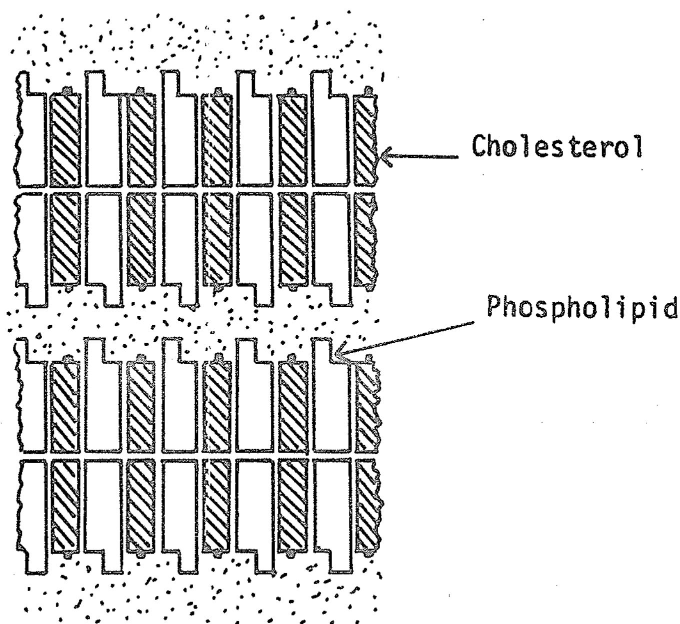
(b) X-ray Studies

The phase diagram for the 3 component system, water-egg yolk phosphatidylcholine - cholesterol was determined by Bourgès, Small and

Dervichian[30] using polarized light microscopy. Cholesterol was found to be insoluble in water. The phosphatidylcholine formed a lamellar, liquid crystalline phase which could incorporate up to 45% water. Cholesterol was soluble in the lamellar phase up to approximately a 1:1 mole ratio of cholesterol to phospholipid. Lecuyer and Dervichian[31] have carried out a systematic X-ray analysis of this system. They found that for egg phosphatidylcholine alone, the molecular area occupied by each phospholipid molecule increased with increased water content. The molecular area was 58 $Å^2$ with 15% water, 61 $Å^2$ with 25% water, and 64 $Å^2$ with 35% water. Because the molecular area is a function of the motion of the fatty acid chains, it was concluded the motional freedom of the hydrocarbon chains increased with increasing hydration of the phospholipids. The addition of cholesterol increased the thickness of the bilayers and decreased the mean molecular area occupied by the phospholipids. With 25 mole % water, the mean molecular area decreased from 61 $Å^2$ with no cholesterol to 48 $Å^2$ with 50 mole % cholesterol and there was a corresponding increase in the thickness of the bilayers from 32.5 Å to 37.5 Å. The presence of cholesterol also decreased the thickness of the water layers in the lamellar phase. The authors ascribe this to the increase in the bilayer thickness and to an increase in the volume available to the water molecules. Since the aqueous layers in the lamellar phase contain the phospholipid polar groups, increasing the cholesterol concentration results in an effective decrease in the number of bulky polar groups in an aqueous layer. Thus, there should be more space available to the water molecules resulting in a decrease in the thickness of the aqueous layers.

The presence of a 4.5 Å diffraction band corresponding to the interchain distance of the phospholipid molecules, and the relatively compact area occupied by these molecules led these authors to emphasize the ordering of the hydrophobic regions of the bilayers despite the fact the chains were in a fluid condition.

Rand and Luzzatti[32] have determined the phase diagram for an erythrocyte lipid-water system. The erythrocyte lipid extract contains a number of different phospholipids and about 26% cholesterol. They found the bilayers of the lamellar phase were condensed compared to similar systems containing no cholesterol. Rand and Luzzatti[32] and Lecuyer and Dervichian[31] have postulated the palisade arrangement for cholesterol - phospholipid bilayers. In this structure, the cholesterol molecules are interdigitated between the phospholipid molecules with the hydroxyl groups of the sterol molecules at the bilayer - water interface.



Ladbrooke *et al*[33] used X-ray diffraction to study the effect of cholesterol on aqueous dispersions of dipalmitoylphosphatidylcholine at 25 °C. The phospholipids formed a lamellar phase in water and cholesterol was soluble up to 50 mole %. In the absence of cholesterol, a sharp 4.2 Å diffraction corresponding to the phospholipid interchain distance was observed. This diffraction is very sharp since the chains are in a crystalline state. With 50 mole % cholesterol present, this spacing increased to 4.45 Å and became very diffuse. This change was interpreted as arising from an increase in fluidity of the chains due to a reduction in the cohesive forces caused by the cholesterol molecules. This fluidization effect did not occur with less than 30% water. In this study, they also examined the effect of cholesterol on the long spacings which are related to the thickness of a bilayer plus the thickness of one aqueous layer. They interpreted the changes in the long spacings with addition of cholesterol as evidence that cholesterol actually decreased the thickness of the dipalmitoylphosphatidylcholine bilayers due to its fluidizing effect. However, in the light of recent spin label experiments,[34,35] (to be discussed subsequently), a large decrease in the order of the paraffin chains in the presence of cholesterol is highly doubtful.

Levine and Wilkins[36] have recently reported an X-ray diffraction study of oriented egg phosphatidylcholine multibilayers. The multilayers were formed by evaporating a chloroform - methanol solution of lipids on a flat aluminum surface. The lipid film consisted of stacked phospholipid bilayers, the planes of the bilayers being essentially

parallel to the plane of the film supporting surface. Their results indicated that at low hydration, 14% water, the hydrocarbon chains in the phospholipid bilayers exist in an extended configuration perpendicular to the bilayer plane. An electron density distribution map showed that a large number of the terminal methyl groups of the hydrocarbon chains were well localized in the center of the bilayer at 14% water content. Increasing the water concentration to 21%, the positions of the terminal methyl groups became less definite, consistent with other results[31] that suggest increasing hydration increases the thermal motion of the hydrocarbon chains. The electron density map for egg phosphatidylcholine bilayers containing 50 mole % cholesterol showed a very marked localization of the terminal methyl groups at the bilayer center at 9% water content. There was very little change in the motion of the chains at 17% water content.

All the X-ray data are consistent with the idea that cholesterol has an ordering effect on the phospholipid bilayers. In other words, cholesterol inhibits the random thermal motion of the paraffin chains in the liquid crystalline state, resulting in a more extended configuration of the chains normal to the plane of the bilayers than would exist in its absence. The X-ray measurements do not support the arguments put forward by Shah and Shulman for molecular cavities or that cholesterol increases the random motion of the paraffin chains.

(c) Nuclear Magnetic Resonance Studies

A high resolution nuclear magnetic resonance (NMR) experiment concerning the effect of cholesterol on egg phosphatidylcholine has

been reported by Chapman and Penkett.[37] If egg phosphatidylcholine is dispersed in excess water by means of ultrasonic radiation, a high resolution NMR spectrum can be observed. At 33.5 °C, the peak assigned to the methylene protons of the fatty acid chains of the phospholipids was fairly sharp. In a sonicated aqueous dispersion of egg phosphatidylcholine containing 50 mole % cholesterol, the methylene proton peak was considerably broadened and almost not observable. This line broadening effect was interpreted as arising from an increase in the correlation time of the hydrocarbon chains due to the presence of cholesterol.

Oldfield and Chapman[38] have used deuteron magnetic resonance (DMR) to study the effect of cholesterol on phospholipid chain mobility. They used di(perdeuterio) myristoylphosphatidylcholine in which 95% of the protons of the fatty acid chains were replaced by deuterons. The gel to liquid crystalline transition temperature in excess water was 23 °C for both the perdeuterated and unmodified phospholipid. At 10 °C, the phospholipid is in the gel phase but the DMR spectra indicated there was some motion of the chains occuring. At 30 °C, the phospholipid is in the liquid crystalline state and the spectra showed an increase in the motional freedom of the hydrocarbon chains. At 30 °C, the spectra of phospholipid dispersions containing 50 mole % cholesterol indicated that the motion of the chains was more anisotropic than in the pure phospholipid dispersion. Cooling the sample to 10 °C caused little change in the spectrum. These results suggested that cholesterol does inhibit the motion of phospholipid chains and extends the temperature range of the liquid crystalline state.

(d) Differential Scanning Calorimetery

Experiments by Ladbrooke *et al*[33] have shown that cholesterol increases the motional freedom of the fatty acid chains of saturated phospholipids below their gel to liquid crystalline transition temperature. In the gel state, the hydrocarbon chains are in a frozen crystalline condition. Above the gel to liquid crystalline transition temperature, the chains are melted and possess some degree of liquid-like behavior. Dipalmitoylphosphatidylcholine containing 50% water is in the gel phase up to 41 °C. Ladbrooke *et al*[33] found that adding increasing amounts of cholesterol to this phospholipid dispersion reduced the heat absorbed in the endothermic transition from a gel to a liquid crystalline phase. With 50 mole % cholesterol present, the transition was not observable. These results provided evidence that one effect of cholesterol is to keep phospholipids, which are at a temperature below their gel to liquid crystalline transition temperature, in a fluid state. These authors suggested a possible role for cholesterol in membranes is to control the fluidity of the hydrocarbon chains of the phospholipids giving a coherent structure stable over a wide temperature range and permitting some latitude in the fatty acid content of the lipids. Recent experiments have shown that cholesterol does indeed increase the fluidity of saturated hydrocarbon chains but this motion remains restricted. Therefore, to say that cholesterol liquifies saturated phospholipids without emphasizing its ordering effects tends to be misleading.

(e) Laser Raman Spectroscopy

A study of the effect of cholesterol on dipalmitoylphosphatidylcholine has recently been reported by Lippert and Petocolas.[39] Using laser Raman spectroscopy, they were able to monitor the structure of the paraffin chains. Their results indicated that there was a sharp transition of the hydrocarbon chains of dipalmitoylphosphatidylcholine dispersed in excess water from an all trans to a fluid configuration at 38 - 39 °C. For a sample containing a 1:1 mole ratio of dipalmitoylphosphatidylcholine and cholesterol, this transition was very broad extending over approximately 0 - 70 °C. These authors interpret the results as suggesting that cholesterol changes the gel - liquid crystal transition from a co-operative to a non-co-operative event by decreasing the interactions between adjacent paraffin chains in the bilayers. Thus, in the presence of cholesterol, the phospholipid will show properties more fluid than the pure lipid below the transition temperature, while above this temperature, the system will exhibit more rigidity. The authors suggest that the biological role of cholesterol may be to fluidize membranes making them less sensitive to temperature variations, but they do not discount the possibility of a chain ordering role of cholesterol for phospholipids which are in the liquid crystalline state.

(f) Sterols and *Mycoplasmas*

There are two major groups of *Mycoplasmas* or pleuropneumonia-

like organisms that are distinguished by their growth requirements. One group, the *Mycoplasma laidlawii*, *Mycolplasma granularum*, and *Mycoplasma* S-410, and S-743 strains do not require cholesterol in their growth medium while all other known strains require exogeneous sterol for growth.[40] The sterol requiring strains only grow when supplied with steroids of a highly specific structure.[41] A growth supporting steroid must have a OH group at C_3 in an equatorial configuration with respect to the plane of the A ring, and a hydrocarbon chain at C_{17}. The presence or position of double bonds in the B ring appears to be of little importance and it does not matter if the A/B ring fusion is cis or trans. Thus, cholesterol, 5α - cholestane - 3β - ol, β - sitosterol, ergosterol, and epicoprostanol support growth while Δ^4 - cholesten - 3 - one, 5α - cholestane, testosterone, epicholesterol and coprostanol do not. Both groups can absorb steroids from the growth medium. The absorbed steroids are associated with the lipids of the cell membrane. Steroids possessing a side chain are irreversibly absorbed while those lacking a hydrocarbon tail are not. The 3-OH is not necessary for this process of absorption by the cells. The necessity of having a hydrocarbon chain at C_{17} for irreversible absorption points out the importance of attractive hydrophobic forces between absorbed steroid and other membrane lipid components.

In the absence of sterol, the only nonsaponifiable lipids synthesized by non - sterol requiring strains, (except for S-410 and S-743), are carotenoid pigments.[42] Two components have been detected: neurosporene in minor quantities, and neurosporol in greater quantity.

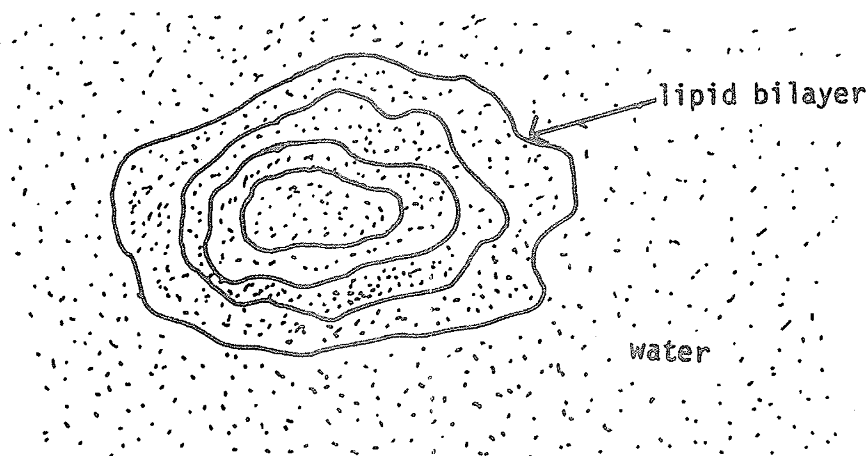
It has been found[41] that glucose fermenting, sterol-requiring strains grown with exogeneous cholesterol contain free and esterified cholesterol and cholesteryl β-D-glucoside. Glucose fermenting non-sterol-requiring strains contain neurosporene, free and esterified neurosporol, and a neurosporyl glucoside. Since these compounds can be isolated from cells, it has been suggested[41] that at least in the glucose fermenting strains, the cholesterol or carotenol is involved in the active transport of glucose into the cell and the transport of the degradative product, acetic acid, out of the cell. However, due to the large amounts of cholesterol incorporated into the cell membranes of sterol-requiring strains, it is generally thought that the primary function of sterol is structural.

(g) Permeability Studies

There are several studies which deal with the effects of cholesterol on the permeability of phospholipid liposomes to ions, and non-electrolytes such as glycerol and glycol.[43-46] Liposomes consist of many spherically concentric lamellae. The liposomes form a closed system so the aqueous layers between bilayers are isolated from the bulk aqueous phase.

In all these experiments, it was found that the presence of cholesterol led to a reduction in the permeability of liposomes made from phospholipids having unsaturated fatty acid chains. It has also been demonstrated that the removal of cholesterol from human erythrocytes causes an increase in their glycerol permeability.[47] A decrease

in the permeability of phospholipid bilayers containing cholesterol is consistent with the view that cholesterol orders the hydrocarbon chains, causing an increase in thickness of the bilayers making it more difficult for molecules to traverse them.



cross sectional view of a liposome

The permeability characteristics of liposomes made with fully saturated phosphatidylcholines have been studied by De Gier *et al.*[48] They found that below a certain temperature - approximately 20 °C for dimyristoylphosphatidylcholine, 36 °C for dipalmitoylphosphatidylcholine, and 44 °C for disteroylphosphatidylcholine - the presence of 30 mole % cholesterol enhanced the rate of glycerol and glycol penetration of the liposomes, but above these temperatures the reverse effect was found. These authors state that below a certain temperature cholesterol does prevent an ordering of the chains but above the temperatures mentioned above, this sterol reduces the random thermal motion of the saturated phospholipids. They emphasized the fact that most of the phospholipids of biological

membranes have at least one unsaturated fatty acid chain so that the ordering effects of cholesterol would appear to be most important. However, assuming a non-random distribution of phospholipids in a membrane, they did not rule out the possibility of a localized fluidizing effect by cholesterol involving saturated phospholipids.

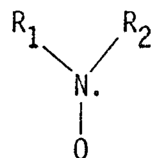
A study of the glycerol permeability of *Mycoplasma laidlawii* B cells has recently been reported by McElhaney *et al.*[49] These authors have grown *Mycoplasma laidlawii* cells in the presence and absence of exogeneous cholesterol. No cholesterol could be detected in the membrane lipids of cells grown in the absence of cholesterol while cholesterol accounted for 12% by weight of the total membrane lipids of cells grown in the presence of cholesterol. It was found that the permeability of intact cells and liposomes made from the cell membrane lipids depended on their cholesterol content. The permeation by glycerol was faster in cells and liposomes which contained no cholesterol. These results indicate that cholesterol affects the structural integrity of the lipid components of biological membranes since the permeability of intact cells and derived liposomes show the same general trends.

6) Spin Label Studies

(a) Spin Labels

The spin label technique was developed by McConnell and his associates to study conformational changes in proteins.[50,51] Since the technique was first applied to the investigation of membrane structure,[52-55] it has proven to be an invaluable addition to the methods available for the study of model and biological membrane systems. The theory of electron spin resonance, (ESR), is covered in many comprehensive monographs[56-59] and the theory and applications of spin labelling are described in several excellent review articles;[60-64] no attempt will be made here to provide the reader with a rigorous and complete discussion of the spin labelling technique.

The compounds which have found the most use as spin labels are the nitroxide free radicals having the general formula

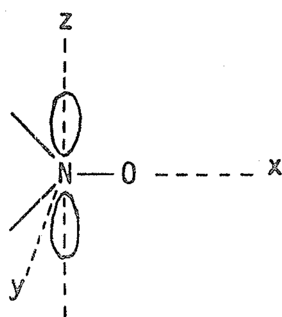


The approximate spin Hamiltonian for a nitroxide is given by

$$\mathcal{H} = \beta \underline{H} \cdot \underline{\underline{g}} \cdot \underline{\hat{S}} + \underline{\hat{S}} \cdot \underline{\underline{T}} \cdot \underline{\hat{I}} \qquad (1)$$

where β is the electron Bohr magneton, $\underline{H}$ is the laboratory magnetic field vector, $\underline{\hat{S}}$ is the electron spin angular momentum operator, $\underline{\hat{I}}$ is the nuclear spin angular momentum operator and $\underline{\underline{g}}$ and $\underline{\underline{T}}$ are the electronic g factor and hyperfine coupling tensors. The first term, the electron

Zeeman term, represents the interaction of the unpaired electron with the applied magnetic field. The second term represents the interaction of the unpaired electron with the nitrogen nucleus. The nitrogen atom has a nuclear spin of 1 and therefore there are three quantized nuclear spin states. Since X-band electron spin resonance spectrometers operate at relatively low magnetic field strengths - approximately 3400 gauss - there is essentially equal probability of the electron interacting with all three nuclear spin states. There are two mechanisms of interaction of the unpaired electron with the nitrogen nucleus: (a) the Fermi contact interaction which is isotropic and requires the electron molecular orbital to have some s character, (b) the anisotropic dipolar interaction, which results from a coupling of the magnetic moments of the electron and the nucleus. From electron spin resonance studies of single host crystals doped with small amounts of a nitroxide, it has been deduced that the unpaired electron occupies a 2pπ atomic orbital on the nitrogen atom with a spin density of approximately 0.8 - 0.9.[60]



It is found that if the principle axes of the nitroxide are

chosen with the x axis along the N-O bond, the z axis parallel to the $p\pi$ orbital and the y axis perpendicular to both the z and x axes, the g and T tensors are diagonalized and the Hamiltonian can be written

$$\mathcal{H} = \beta \underline{H} \cdot \begin{vmatrix} g_{xx} & 0 & 0 \\ 0 & g_{yy} & 0 \\ 0 & 0 & g_{zz} \end{vmatrix} \cdot \hat{\underline{S}} + \hat{\underline{S}} \cdot \begin{vmatrix} T_{xx} & 0 & 0 \\ 0 & T_{yy} & 0 \\ 0 & 0 & T_{zz} \end{vmatrix} \cdot \hat{\underline{I}} \qquad (2)$$

The g and T tensor components of all nitroxides determined thus far by single crystal measurements have approximately the same values with $g_{xx} > g_{yy} > g_{zz}$ and $T_{zz} > T_{xx} \simeq T_{yy}$. As an example, the tensor components of 3-spiro- {2'-(N-oxyl- 4', 4' - dimethyloxazolidine)}-cholestane in a single crystal of cholesteryl chloride have been determined by Hubbell;[65]

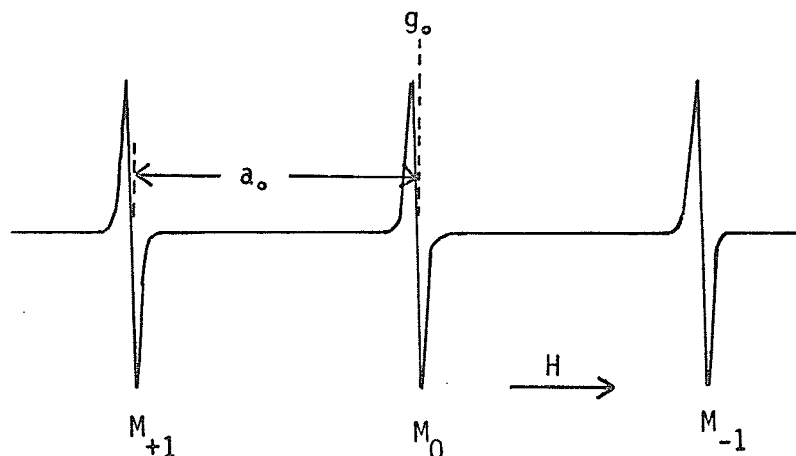
g_{xx} = 2.0089 ± 0.001 T_{xx} = 5.8 gauss ± 0.5 gauss

g_{yy} = 2.0058 T_{yy} = 5.8 gauss

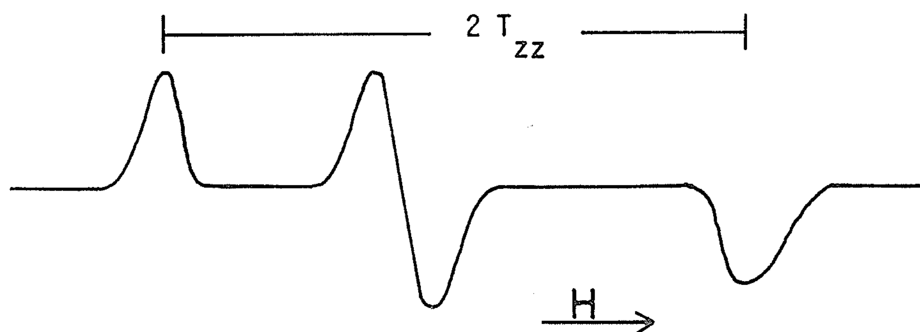
g_{zz} = 2.0021 T_{zz} = 30.8 gauss

When a nitroxide is dissolved in a suitable non-viscous solvent, it undergoes isotropic motion. If the nitroxide tumbles isotropically at a frequency very much greater than the maximum difference between the components of the g tensor, $(g_{xx}-g_{zz})\beta H h^{-1} \simeq 3.2 \times 10^7$ sec^{-1}, and the hyperfine tensor, $T_{zz}-T_{xx} \simeq 7.5 \times 10^7$ sec^{-1} a spectrum of 3 lines of equal intensity is observed with a g value, g_o, and a hyperfine splitting constant a_o, where $g_o = \frac{1}{3}(g_{xx} + g_{yy} + g_{zz})$ and $a_o = \frac{1}{3}(T_{xx} + T_{yy} + T_{zz})$. a_o is the magnitude of the Fermi contact interaction since the dipolar interac-

tions average to zero when the nitroxide undergoes rapid isotropic motion.



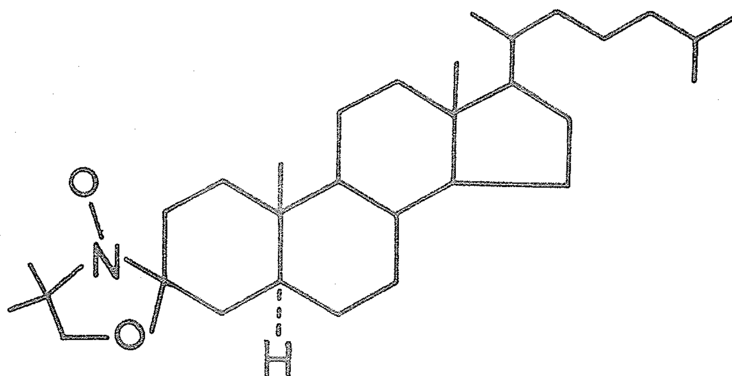
If the isotropic tumbling rate of the nitroxide is decreased by increasing the viscosity of the solvent, asymmetric line broadening occurs with the high field line being broadened first, then the low field line, and finally the center line. This broadening results from an incomplete averaging of the g and T tensor components. The rotational correlation times of isotropically tumbling nitroxides can be estimated from Stokes law,[66] $\tau = \frac{4\pi\eta r^3}{3kT}$, ($\tau$ is the correlation time, η is the viscosity, r is the effective radius of the molecule, k is the Boltzman constant and T is the absolute temperature), or from the observed spectral parameters.[51] If the viscosity of the solvent is increased to the point where molecular motion has "ceased", an ensemble of randomly oriented nitroxides results, and the spectrum one obtains is the so called powder spectrum.



The powder spectrum comprises an envelope of spectra for nitroxides in all possible orientation with respect to the magnetic field vector. The maximum hyperfine splitting corresponds to $2T_{zz}$ and is approximately 64 gauss. The width of the center line is approximately $2T_{xx}$. The position of the center peak is unsymmetric with respect to the outer peaks because of g anisotropy.

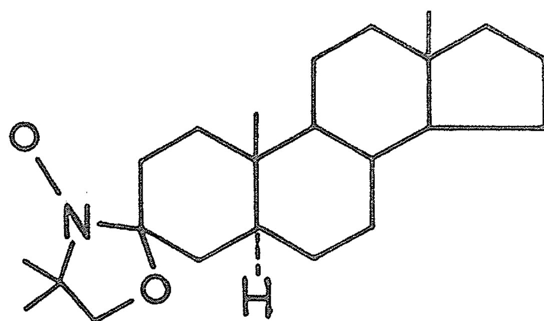
A consequence of the ionic character of the N-O bond is a sensitivity of the hyperfine and g tensor values to the polarity of the nitroxide's environment. It is found experimentally that isotropic hyperfine splitting constants increase and g values decrease with increasing solvent polarity.[67]

A spin label is a molecular probe. In order to minimize the perturbation effects of introducing such a molecule into a system, it is necessary that the probe resemble as closely as possible a component of the system under examination. Spin label studies of membrane structure have utilized spin labelled lipids, particularly the nitroxide derivatives of steroids and fatty acids. Some examples of spin labelled steroids are shown below.



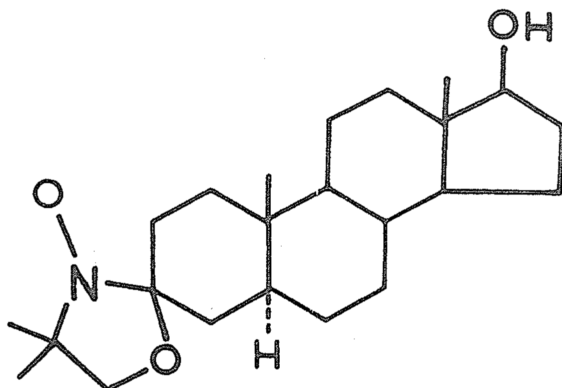
I

3-spiro- {2'-(N-oxyl- 4', 4' - dimethyloxazolidine)} - cholestane



II

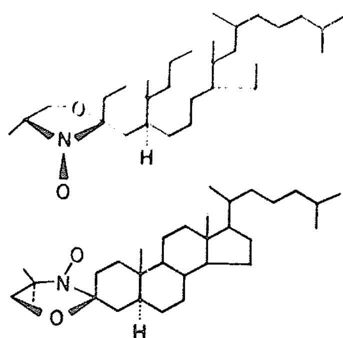
3-spiro- {2'-(N-oxyl- 4', 4'- dimethyloxazolidine)} - androstane



III

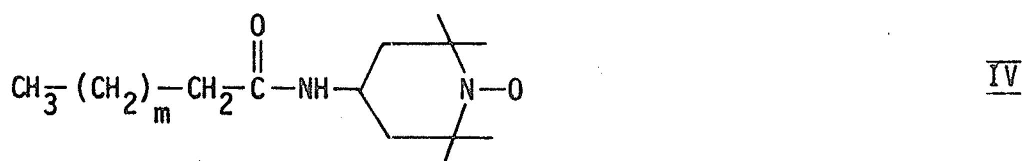
3-spiro- {2'-(N-oxyl- 4', 4'- dimethyloxazolidine)} - androstane - 17β - ol

The important point to note about the steroid spin labels is that the oxazolidine ring is rigidly attached to the A ring. Therefore, the motion of the nitroxide reflects the motion of the steroid nucleus. It is not known which epimer is formed in the synthesis of these spin labels but that makes little difference since in both cases the principle axes of the nitroxide have the same geometrical relationship to the long axis of the steroid nucleus. The *y* axis of the nitroxide is

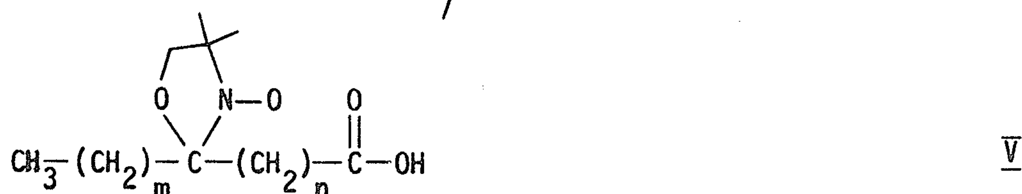


essentially parallel to the steroid long axis and the x and z axes are essentially perpendicular to the steroid long axis.

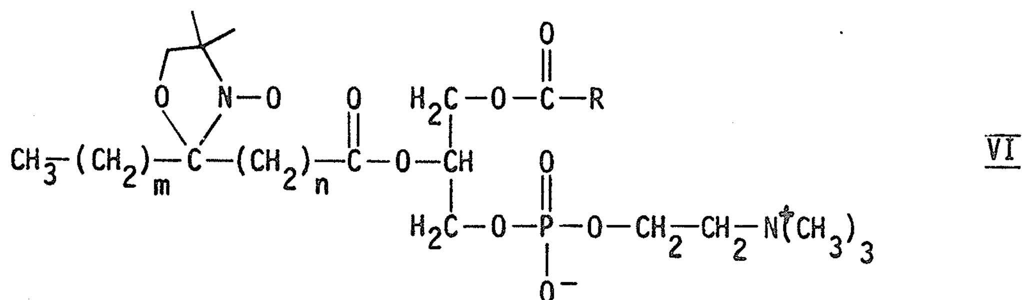
Some fatty acid spin labels either free or incorporated into phospholipid molecules are shown below.



IV



V



VI

The piperidinyl ring of the fatty acid amide spin labels can move independently of the rest of the molecule so a quantitative analysis of the motion of these spin labels is extremely complicated.

However, if the molecule is in the all trans position, it can be seen that the z and y axes of the nitroxide are perpendicular to, and the x axis is parallel to, the long axis of the molecule. The fatty acid spin labels in which an oxazolidine ring is rigidly attached to a carbon atom in the fatty acid chain offer some improvement in interpreting the motion of the molecule. The carboxyl group of a fatty acid spin label or the polar head group of a phospholipid spin label anchors the molecule at the membrane-water interface with the fatty acid portion of the molecule dissolving in the hydrophobic region of the membrane. If a fatty acid spin label is in the all trans configuration, then the z axis of the nitroxide is parallel to the long axis of the molecule while the x and y axes are perpendicular to this axis. The motion of the nitroxide reflects the motion of the carbon atom to which the oxazolidine ring is attached but due to the flexibility of the fatty acid chain, it does not necessarily depend on the motion of the rest of the molecule. Information concerning the overall motion of the hydrocarbon chain can be obtained by synthesizing a series of fatty acid spin labels with the oxazolidine ring attached to different carbon atoms in the chain.

We shall now consider a description of the motion of a spin label in oriented multibilayers using the approach of Seelig.[68] Oriented multilayer arrays possess a unique rotational symmetry axis, the normal to the plane of the bilayers. We denote by x', y', and z' the axes of a cartesian co-ordinate system related to the oriented multilayers where z' coincides with the rotational symmetry axis of the system.

The $\underline{g}$ and $\underline{T}$ tensors of equation 2 which are molecular fixed can be transformed into the x', y', z' system to give

$$\underline{g}' = \begin{vmatrix} g_\perp & 0 & 0 \\ 0 & g_\perp & 0 \\ 0 & 0 & g_{//} \end{vmatrix} \qquad \underline{T}' = \begin{vmatrix} T_\perp & 0 & 0 \\ 0 & T_\perp & 0 \\ 0 & 0 & T_{//} \end{vmatrix} \tag{3}$$

where the components of $\underline{g}'$ and $\underline{T}'$ are suitable time averages of the components of $\underline{g}$ and $\underline{T}$. The components of $\underline{T}'$ in equation 3 are related to $\underline{T}$ by the following equations:

$$T_\perp = l^2_{x'x}T_{xx} + l^2_{x'y}T_{yy} + l^2_{x'z}T_{zz} \tag{4}$$

$$T_\perp = l^2_{y'x}T_{xx} + l^2_{y'y}T_{yy} + l^2_{y'z}T_{zz} \tag{5}$$

$$T_{//} = l^2_{z'x}T_{xx} + l^2_{z'y}T_{yy} + l^2_{z'z}T_{zz} \tag{6}$$

where l^2_{ij} is the time average of the square of the direction cosine between the i and j axes, $< \cos^2\Theta_{ij} >$. For a nitroxide $T_{xx} \simeq T_{yy}$ so equations 4 and 5 can be combined and simplified using the standard relationship for the squares of direction cosines to give

$$T_\perp = \tfrac{1}{2}(1 + l^2_{z'z})T_{zz} + \tfrac{1}{2}(1 - l^2_{z'z})T_{zz} \tag{7}$$

Similarly, for equation 6, where $l^2_{z'x} + l^2_{z'y} + l^2_{z'z} = 1$,

$$T_{//} = (1 - l^2_{z'z})T_{xx} + l^2_{z'z}T_{zz} \tag{8}$$

Let Θ_1, Θ_2 and Θ_3 be the angles between the symmetry axis z' and the x, y and z axes of the nitroxide. The order parameter[69,70] S_3 is

defined as

$$S_3 = \tfrac{1}{2}(3 < \cos^2\theta_3 > - 1) \qquad (9)$$

Order parameters range between values of 1 and $-\frac{1}{2}$. If $S_3 = 1$, then the axis of the nitroxide is aligned parallel to the symmetry axis; $S_3 = -\frac{1}{2}$ means that the z axis is perpendicular to the symmetry axis. When $S_3 = 0$, there is a random orientation of the z axis corresponding to isotropic motion of the nitroxide. It follows from the definition of the order parameter that

$$S_1 + S_2 + S_3 = 0 \qquad (10)$$

Combining equations 7 and 8

$$S_3 = \frac{T_{/\!/} - T_{\perp}}{T_{zz} - T_{xx}} \qquad (11)$$

Rewriting equation 11

$$< \theta_3 > = \text{arc cos}\left[\frac{2}{3}\left(\frac{T_{/\!/} - T_{\perp}}{T_{zz} - T_{xx}} + \frac{1}{2}\right)\right]^{\frac{1}{2}} \qquad (12)$$

The magnitude of the tensor components T_{zz} and T_{xx} depend on the polarity of the nitroxide's environment. Since the trace of a tensor is invarient under rotation

$$2T_{\perp} + T_{/\!/} = 2T_{xx} + T_{zz} \qquad (13)$$

From single crystal studies of nitroxides, it is found that the ratio of T_{xx} to T_{zz} is almost constant[68] with

$$\frac{T_{xx}}{T_{zz}} = 0.188 \qquad (14)$$

Using equations 13 and 14, it is possible to calculate reasonably accurate values of T_{xx} and T_{zz} for equation 11.

In a similar manner, an expression for S_2 can be derived from the tensor components of $\underline{g}'$. Thus, it is possible to obtain all three order parameters from equation 10. In the special case of the steroid spin labels when there is rapid rotational motion about the molecular long axis, it has been shown that the average angle between the symmetry axis and both the x and z molecular axes is the same, so

$$S_2 = - 2S_3 \tag{15}$$

Thus, using this equation, the average angle between the symmetry axis and the long axis of a steroid spin label, $< \Theta_2 >$, can be calculated without a knowledge of the components of $\underline{g}'$. The general equation for the observed hyperfine splitting is,

$$T_{obser.} = (T_{//}^{2} \sin^{2}\phi + T_{\perp}^{2} \cos^{2}\phi)^{\frac{1}{2}} \tag{16}$$

where ϕ is the angle between the magnetic field vector and the plane of oriented multilayers.

There are some limitations of this description of the motion that must be kept in mind. Firstly, one cannot account for observed spectral line shapes using this simple model since it has been assumed that all the spin labels have the same orientation, and time dependant fluctuations in the Hamiltonian have been neglected. Also, this description of the motion breaks down when the motion of the spin label ceases to be sufficiently anisotropic.

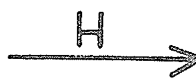
Three methods of preparing oriented multibilayers of phospholipids containing trace amounts of a spin label have been reported. The first method consists of drying an aqueous dispersion of phospholipid and spin label on a flat glass slide.[71] The second technique makes use of a standard ESR aqueous cell which consists of two flat parallel quartz plates having the approximate dimensions 5.5 cm. X 1.0 cm. separated by a distance of approximately 0.050 cm. A chloroform-methanol solution of phospholipid and spin label is placed in the cell and a lipid film is deposited on the flat quartz surfaces by evaporating the solvents with vacuum[72] or a stream of nitrogen gas.[73] The third method involves pressing a sample of phosphatidylcholine containing spin label between two flat quartz plates to obtain orientation of the bilayers.[74] In all these methods, the planes of the bilayers are essentially parallel to the plane of the glass surface. Figure 1.2 illustrates the orientation of a lipid film, prepared in a quartz ESR aqueous cell, in the laboratory magnetic field for the measurement of $T_{//}$ and $T_{\perp}$. Seelig[68] has found that the lamellae of the system sodium decanoate-decanol-water (28 wt. %: 42.1 wt. %: 29.9 wt. %) orient in a quartz aqueous cell with the planes of the bilayers parallel to the plane of the quartz surfaces.

Libertini *et al*[71] have shown that spectral line shapes of spin labels in oriented multilayers can be accounted for by assuming a gaussian distribution of orientations of nitroxides. They found that the long axes of both the cholestane, I, and 12-stearic acid, V, (m,n = 5,10), spin labels are aligned essentially perpendicular to the plane of the multibilayers of egg phosphatidylcholine, but that all the spin labels do not have the same orientation with respect to the plane of the glass

Figure 1.2

Orientation of a quartz aqueous cell in the magnetic field for the measurement of: (a) $T_{\parallel}$, (b) $T_{\perp}$.

a



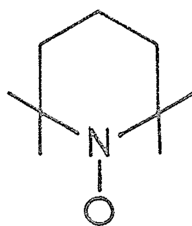
b

slide due to imperfections in the ordering of the bilayers, and also probably because of the fatty acid heterogeneity of the phospholipids. Recently, Jost *et al*[75] reported a study of oriented egg phosphatidylcholine multibilayers using a series of fatty acid spin labels. Using a restricted random walk model for the motion of a nitroxide, they found good agreement between experimental and calculated hyperfine splittings even when the motion of the nitroxide approached an isotropic limit. Combining this model with a gaussian distribution of orientations, they were able to obtain a good fit of simulated and experimental spectra. McFarland and McConnell[74] have analysed the spectra of a series of phospholipid spin labels, VI, in oriented phosphatidylcholine multilayers containing approximately 25% water. Treating time dependant fluctuations in the Hamiltonian by Block-Wangness and Redfield theory, they have found that near the polar headgroups the chains are bent at an angle of 30° from the normal of the bilayer surface but near the terminal methyl groups, the chains are on the average parallel to the normal.

The reviews by McConnell and McFarland,[62] Smith,[63] and Mehlhorn and Keith[64] survey the spin label studies of biological and model membranes up to and including part of 1971. The present discussion will be limited to a resumé of spin label studies of cholesterol effects in liposomes and oriented multibilayers.

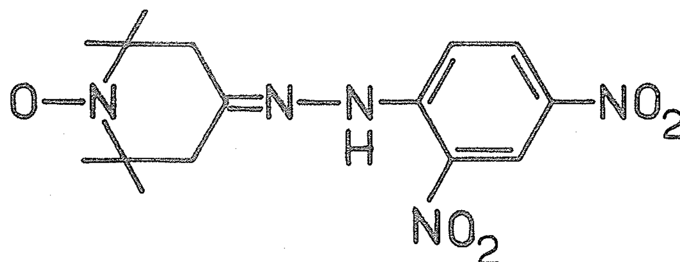
(b) Cholesterol Effects

Hubbell and McConnell[53] found that the solubility of N-oxyl 2,2,6,6 tetramethylpiperidine in phospholipid liposomes was decreased by the presence of cholesterol. They attributed this solubility effect



N-oxyl 2,2,6,6 tetramethylpiperidine

to a decrease in the volume of the hydrophobic regions available to the spin label with the addition of cholesterol to the liposomes. Barrett *et al*[76] also found that the solubility of the dinitrophenyl spin label in dioleoylphosphatidylcholine was decreased when cholesterol was added to the liposomes. In addition, the mobility of the



dinitrophenyl spin label

spin label in the bilayers was decreased in the presence of cholesterol.

Waggoner *et al*[77] were the first to examine the effect of cholesterol in egg phosphatidylcholine liposomes using a lipid spin label, the 12-stearic acid spin label, V, (m,n = 5,10). They found that the viscosity of the local environment of the nitroxide increased with the addition of cholesterol consistant with the idea that cholesterol reduces the motion of the fatty acid chains.

Hsia *et al*[78] investigated the effect of cholesterol in egg phosphatidylcholine liposomes using the cholestane and stearamide, IV,

(m = 15), spin labels. No anisotropic motion of the cholestane spin label was evident from the spectra but a broadening of the lines in the presence of cholesterol was evidence that cholesterol decreased the mobility of the label in the liposomes. The spectra of the stearamide spin label did not indicate any anisotropic motion of the label in liposomes with and without cholesterol. There was an increase in the width of the peaks of the spectrum with liposomes containing cholesterol again indicating decreased mobility of the label in the presence of cholesterol. Another effect was also observed with this label. In the absence of cholesterol, the line width of the low field (M_{+1}) line was less than that of the center (M_0) line. With addition of cholesterol this relationship was reversed with the peak to peak line width of the center line being smaller than that of the low field line. The authors attribute this relative line width change to a change in the spatial position of the nitroxide when cholesterol is added to the liposomes. Hubbell and McConnell[79] have examined the motion of a series of fatty acid, V, and phosphatidylcholine, VI, spin labels in egg phosphatidylcholine liposomes containing 33% cholesterol. They found that as the distance between the oxazolidine ring and the carboxyl group or the glycerol linkage increased, the degree of anisotropic motion of the nitroxide decreased. Thus, near the phospholipid polar groups, the fatty acid chains appear to be tightly packed. At increasing distances from the polar interface, the motion of the hydrocarbon chains becomes more fluid. This fluidity gradient in bilayers has been studied using a series of spin labelled fatty acids in both biological membranes,[79,80]

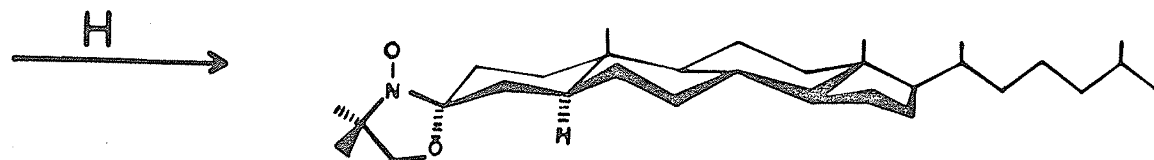
and oriented multilayers[68,75] which contained no cholesterol.

Oldfield and Chapman[81] have used the 12-methyl stearate spin label to investigate the effect of cholesterol in aqueous dispersions of egg phosphatidylcholine, dipalmitoylphosphatidylcholine, and bovine brain sphingomyelin. They found that the motion of the nitroxide in aqueous dispersions of dipalmitoylphosphatidylcholine and sphingomyelin at 20°C was very slow giving rise to characteristic strongly immobilized spectra. The restricted motion of the spin label is presumably due to the crystalline state of the hydrocarbon chains of these two phospholipids at 20°C. However, in dispersions of these phospholipids containing 50 mole % cholesterol, the spectra of the spin label very strongly resembled the spectrum of the spin label in egg phosphatidylcholine liposomes containing 50 mole % cholesterol at 20°C. Since the hydrocarbon chains of egg phosphatidylcholine are in a liquid crystalline state at 20°C, the authors suggest that the biological role of cholesterol may involve keeping the saturated hydrocarbon chains in a fluid condition.

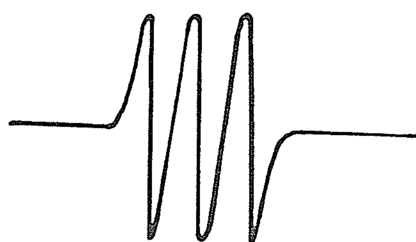
Hsia _et al_ have used the cholestane spin label to investigate cholesterol effects in oriented phospholipid multibilayer films.[34,72] Figure 1.3 illustrates the theoretical spectra for the cholestane spin label oriented in the magnetic field for the two cases where the axial rotational frequency of the spin label is $<< 7.5 \times 10^7$ sec^{-1} and $>> 7.5 \times 10^7$ sec^{-1}. In a dry dipalmitoylphosphatidylcholine film containing 50 mole % cholesterol, $T_{//}$ was 6.0 ± 0.5 gauss and $T_{\perp} = 32.0 \pm 1$ gauss.[34] Since the spectrum obtained when the magnetic field was parallel to the plane of the film supporting surface was a powder spectrum, the authors concluded that the long axis of the spin label was perpendicular to the bilayer

Figure 1.3

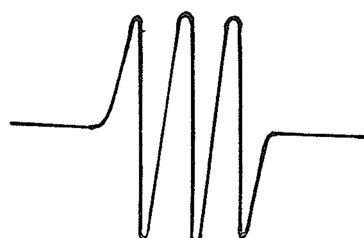
Spectra of the cholestane spin label oriented in the magnetic field with an axial rotational frequency $<< 7.5 \times 10^7 sec^{-1}$ and $>> 7.5 \times 10^7 sec^{-1}$.



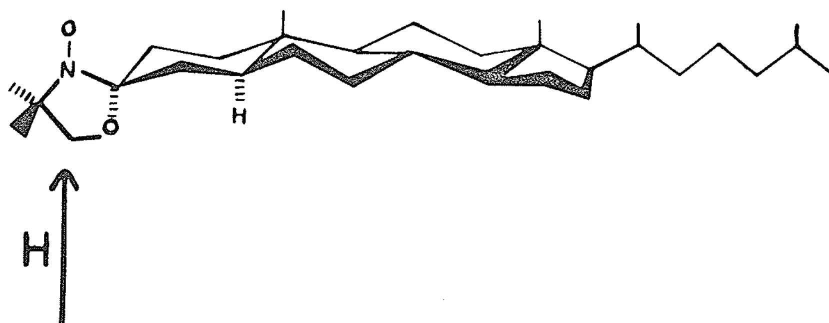
10G

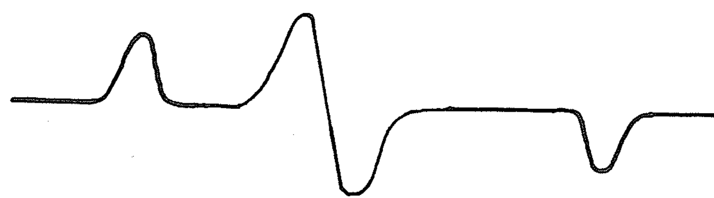


$\ll 7.5 \times 10^{7}\ \mathrm{sec}^{-1}$

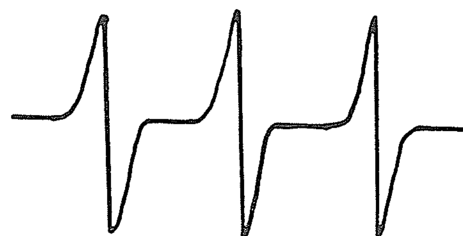


$\gg 7.5 \times 10^{7}\ \mathrm{sec}^{-1}$





$\ll 7.5 \times 10^{7}\ \mathrm{sec}^{-1}$



$\gg 7.5 \times 10^{7}\ \mathrm{sec}^{-1}$

plane and there was a random distribution of orientations of T_{zz} and T_{xx} indicating the rotational frequency about the molecular long axis was << 7.5 x 10^7 sec^{-1}. Upon hydration of the film, there was little change in $T_{//}$ but $T_{\perp}$ decreased to 19.0 gauss. Therefore, there was no change in the alignment of the spin label but the axial rotational frequency was great enough (>> 7.5 x 10^7 sec^{-1}) to average the T_{zz} and T_{xx} components. If the spin labels are perfectly aligned normal to the plane of the bilayers, then

$$T_{obser.} = \left[\left| \frac{T_{zz} + T_{xx}}{2} \right| \cos^2\phi + T_{yy}{}^2 \sin^2\phi \right]^{\frac{1}{2}} \qquad (17)$$

where ϕ is the angle between the plane of the bilayers and the magnetic field vector. (The original notation used by the authors[34,72] has been altered to be consistant with the notation used in this discussion). Assuming T_{zz} = 32.0 gauss and T_{xx} = T_{yy} = 6.0 gauss, the observed hyperfine splittings were in good agreement with the calculated values from equation 17. These authors found that the orientation of the spin label in egg phosphatidylcholine - cholesterol films was not as good as in corresponding dipalmitoylphosphatidylcholine - cholesterol bilayers,[34,72] presumably due to the fatty acid chain heterogeneity. In hydrated films with no cholesterol $T_{//}$ = 9.5 gauss and $T_{\perp}$ = 16.5 gauss. For films containing 25 - 50 mole % cholesterol, $T_{//}$ = 6.5 gauss and $T_{\perp}$ = 19.0 gauss.

The authors described the motion of the spin label in terms of rapid anisotropic motion using equation 18 where θ_2 is the deviation angle, the average angle between the steroid long axis and the normal of the bilayer plane.

$$T_{obser.} = \left[\left(\frac{T_{zz} + T_{xx}}{2}\right)^2 \left(\frac{\cos^2(\phi + \Theta_2) + \cos^2(\phi - \Theta_2)}{2}\right) + T_{yy}^2\left(\frac{\sin^2(\phi + \Theta_2) + \sin^2(\phi - \Theta_2)}{2}\right)\right]^{\frac{1}{2}} \qquad (18)$$

When $\phi = 90^\circ$, equation 18 simplifies to

$$T_{//} = \left[\left(\frac{T_{zz} + T_{xx}}{2}\right)^2 \sin^2\Theta_2 + T_{yy}^2\cos^2\Theta_2\right]^{\frac{1}{2}} \qquad (19)$$

It was found that there was a difference of approximately 5° in the deviation angle calculated from $T_{//}$ or $T_{\perp}$ which the authors attributed to the simplicity of equation 18. However, they emphasized that while the values of calculated deviation angles could not be considered as absolute, this in no way detracted from their use in making comparative measurements. At room temperature Θ_2 was calculated to be $28^\circ \pm 5^\circ$ in hydrated egg phosphatidylcholine bilayers. Upon addition of cholesterol, there was a linear decrease in Θ_2 up to 25 mole % cholesterol. For 25 mole % cholesterol $\Theta_2 = 10^\circ \pm 3^\circ$. There was no change in Θ_2 from 25 mole % to 50 mole % cholesterol. It has also been determined that the average deviation angle of the cholestane spin label decreases from approximately 18.5° ($T_{//}$ = 8.3 gauss, $T_{\perp}$ = 18.9 gauss) in hydrated dipalmitoylphosphatidylcholine multilayers, to 6.2° ($T_{//}$ = 6.3 gauss, $T_{\perp}$ = 20.7 gauss) in hydrated films of this phospholipid containing 50 mole % cholesterol.[35] Since the orientation of the intercalated cholestane spin label depends on the orientation of the surrounding phospholipid molecules, it follows from the decrease in Θ_2 with addition of cholesterol, that this molecule has an ordering effect on the phospholipids

tending to align the phospholipid long axes perpendicular to the plane of the bilayer plane. It is important to note that cholesterol has an ordering effect on the dipalmitoylphosphatidylcholine films even though it does increase the fluidity of the chains at room temperature. A decrease in the deviation angle of the phospholipids with addition of cholesterol leads to an increase in bilayer thickness, consistent with X-ray results.[31,36]

Butler *et al*[82] have used the cholestane spin label to study the ordering effect of cholesterol in two multilayer systems: (a) the lipids of fraction I of the white matter of bovine brain, and (b) the polar lipids of human erythrocyte ghosts. Therefore, instead of having only one phospholipid component, the multilayer films used by these authors contained a complex mixture of phospholipids including phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. Addition of cholesterol to these films resulted in increased anisotropic motion of the cholestane spin label indicative of the ordering effect of this sterol on the multilayers. They also examined the ordering effects of many cholesterol derivatives in the multilayer films and concluded that to be an effective ordering agent, a steroid must have a single hydroxyl group in the 3β configuration and a planar steroid nucleus. A hydrocarbon chain at C_{17} was not crucial but increased the ordering effect. They also pointed out the correlation between the structure of steroids which induce ordering in the oriented multibilayer films and the structure of steroids which support the growth of sterol-requiring *Mycoplasmas*. Thus, Butler *et al*[82] and Hsia *et al*[34]

have proposed that the biological function of cholesterol is to control the orientation of membrane phospholipids and some of the membrane dimensional parameters.

7) Nature of the Problem

A great deal of information is now available concerning the effects of cholesterol on the mobility and orientation of phospholipids. If mechanisms proposed for the biological activity of cholesterol and related steroids are to have a plausible structural basis, detailed knowledge of the nature of the forces involved in the cholesterol-phospholipid interaction is essential. Finean[83] and Vandenheuvel[84] have both proposed models for the interaction of cholesterol with phospholipids which take into account polar forces between the hydroxyl group and the N terminal group of the phospholipid and apolar forces between the cholesterol molecule and the paraffinic chains of the phospholipids. The geometrical arrangements of the molecules in the cholesterol-phospholipid complex proposed by both authors have been critized.[84a]

Cholesterol has three structural features: (a) C_3 β-OH group, (b) steroid nucleus, and (c) C_{17} sidechain. A comparative study of the effects of cholesterol and structural derivatives on membrane structure would provide information concerning the forces involved in cholesterol-phospholipid interactions. Hsia *et al*[34,72] have demonstrated that the oriented multibilayer technique is an excellent method for examining the ordering effect of cholesterol in bilayers. Therefore, a comparative study of the ordering effects of systematically varied structural derivatives of cholesterol in oriented phospholipid films was carried out in the hope of elucidating the nature of the cholesterol-phospholipid interaction.

The structural derivatives of cholesterol used in the experi-

ments are:

(1) C_3 derivatives

Δ^5-cholesten-3β-methoxy	cholesteryl methyl ether
Δ^5-cholesten-3β-chloride	cholesteryl chloride
Δ^5-cholesten-3β-thiol	thiocholesterol
Δ^5-cholesten-3α-ol	epicholesterol

(2) B-ring derivatives

5α-cholestane-3β-ol	
Δ^7-cholesten-3β-ol	
$\Delta^{5,7}$-cholestadien-3β-ol	7-dehydrocholesterol

(3) C_{17} derivatives

Δ^5-cholesten-3β-ol-24 -ethyl	β-sitosterol
$\Delta^{5,7,22}$-cholestatrien-3β-ol-24β-methyl	ergosterol
5α-androstane-3β-ol	
5α-androstane-3β-ol-17-one	

The structural formulas of the steroids are shown in Figure 1.4.

The effects of cholesterol and related steroids were examined in two model systems:

(1) egg phosphatidylcholine

(2) bovine brain sphingomyelin

These two systems were used since both phospholipids are membrane components. In addition, it should be possible to determine whether or not the properties of each system and the interaction with cholesterol are governed by the chemical nature of the phospholipid.

The effects of intercalated steroids in oriented thin films

Figure 1.4

Structural formulas of the cholesterol derivatives:

(a) cholesteryl methyl ether

(b) cholesteryl chloride

(c) thiocholesterol

(d) epicholesterol

(e) 5α-cholestane-3β-ol

(f) Δ^7-cholesten-3β-ol

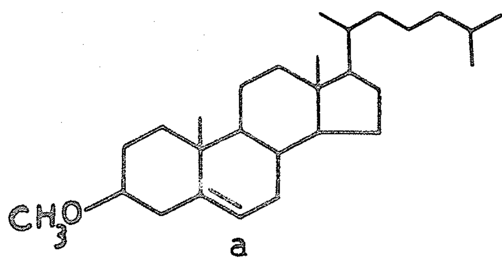
(g) 7-dehydrocholesterol

(h) ergosterol

(i) β-sitosterol

(j) 5α-androstane-3β-ol

(k) 5α-androstane-3β-ol-17-one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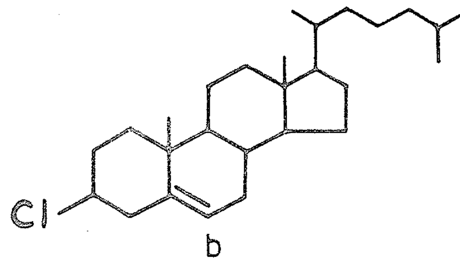
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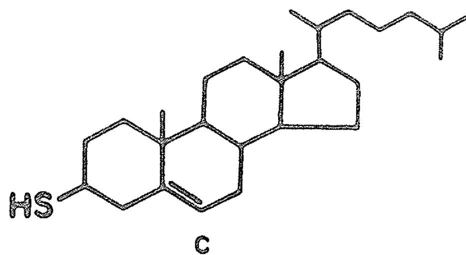
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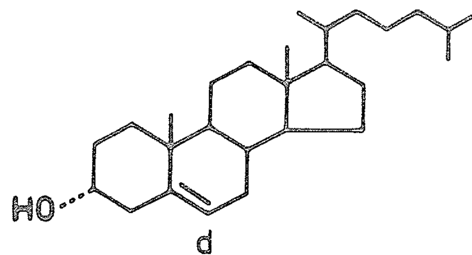
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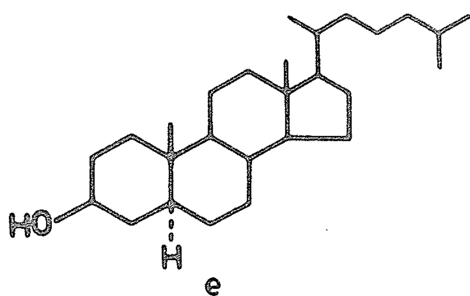
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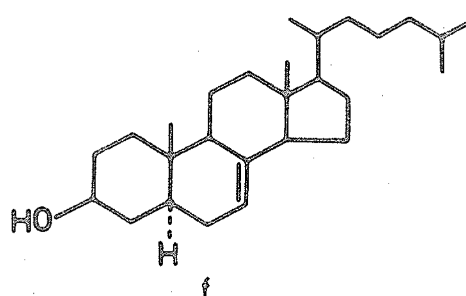
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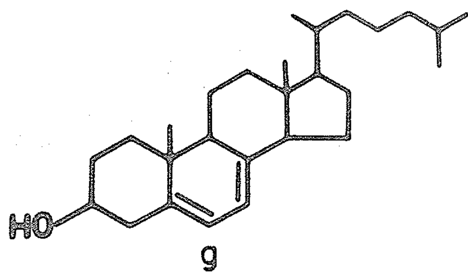
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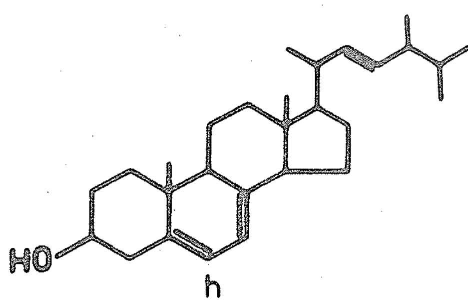
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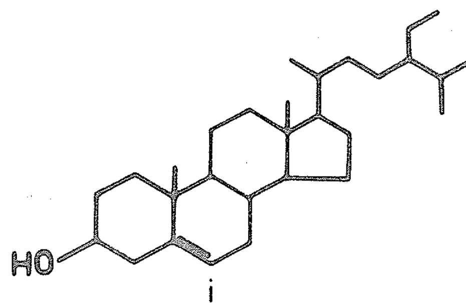
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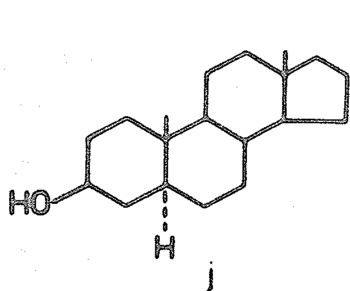
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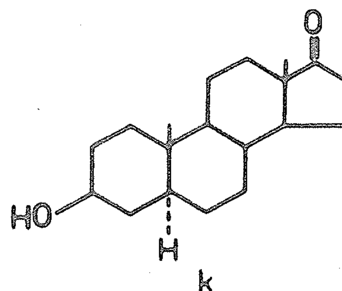
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were monitored with three spin labelled lipids:

(a) the cholestane spin label, I,

(b) the stearamide spin label, IV (n = 15),

(c) the 12-stearic acid spin label, V (m, n = 5, 10).

The first two labels provide information regarding the orientation and motion of the phospholipids near their polar head groups. The third label is useful for examining the motion and orientation of the hydrocarbon chains of the phospholipids.

CHAPTER II

EXPERIMENTAL

1) Materials

The spin label, 3-spiro-[2'-(N-oxyl-4', 4'-dimethyloxazolidine)]-cholestane was prepared by the method of Keana *et al.*[85] 12-Spiro-[2'-(N-oxyl-4', 4'-dimethyloxazolidine)]-stearic acid was prepared according to Waggoner *et al.*[77] N-(1-oxyl-2,2,6,6 tetramethyl-4-piperidinyl) stearamide was prepared by the method of Hsia *et al.*[86] These spin labels were generously supplied by Dr. J. C. Hsia. A 10^{-4} molar stock solution of each spin label in chloroform was prepared and stored at -20 °C.

Chromatographically pure egg yolk phosphatidylcholine was a gift from Dr. J. C. Hsia. The phosphatidylcholine was extracted from *Gallus domesticus* eggs and purified by aluminum oxide and silicic acid chromatography following the method of Papahodjopoulos and Miller.[87] Bovine brain sphingomyelin, (melting point 210 - 215 °C), was purchased from Mann Research Laboratories (New York, N. Y.) and was used without further purification. Chromatograms of the phosphatidylcholine and sphingomyelin developed on silica gel F-254 (E. Merck AG Darmstadt Germany) with chloroform-methanol-water, (65:35:10 by volume) and stained with either iodine vapours or 0.0012% aqueous Rhodamine 6G showed only a single spot under ultraviolet light. A stock solution of each phospholipid (15×10^{-3} molar in chloroform-methanol 1:1 by volume) was adjusted to 15×10^{-3} molar, using the colourimeteric semi-micro-phosphorous analysis method described by Marinette.[88] Concentrations determined in this manner are estimated to be accurate to within ±5%.[88] A Unicam SP 600 Series 2 spectrophotometer was used for absorbance measurements.

Thiocholesterol was obtained from Eastman Organic Chemicals, (Rochester, N.Y.). Ergosterol was obtained from the Sigma Chemical Company, (St. Louis, Mo.). All the other steroids were purchased from Mann Research Laboratories (New York, N.Y.). All the steroids except epicholesterol, Δ^7-cholesten-3β-ol, 7-dehydrocholesterol and ergosterol were recrystallized from methanol until sharp melting points were obtained. Melting points, (uncorrected), were determined on a Fisher-Johns melting point apparatus, (Table 2:1). Insufficient quantities of epicholesterol and Δ^7-cholesten-3β-ol did not permit further purification of these compounds by recrystallization. Due to their air and light sensitivity, 7-dehydrocholesterol and ergosterol were not further purified and were stored in the crystalline state in a desiccator at -20 °C until used. All the steroids except 7-dehydrocholesterol and ergosterol were stored as 15 X 10^{-3} molar chloroform solutions at -15 °C. 7-Dehydrocholesterol and ergosterol chloroform solutions of the same concentration, (15 X 10^{-3} molar), were made up immediately before use to minimize the formation of any decomposition products.

Table 2.1

Melting Points of Steroids

Steroid	Source	Melting Point (°C) Found	Melting Point (°C) Reported[a]
cholesterol	Mann Research Laboratories	148.5	148.5
cholesteryl chloride	Mann Research Laboratories	96-98	96
cholesteryl methyl ether	Mann Research Laboratories	85.5-86.5	84.5
epicholesterol	Mann Research Laboratories	139-140	141.5
thiocholesterol	Eastman Organic Chemicals	94-96	96-97[b]
5α-cholestane-3β-ol	Mann Research Laboratories	146-147	146-147
Δ^7-cholesten-3β-ol	Mann Research Laboratories	113-114	122-123
7-dehydrocholesterol	Mann Research Laboratories	133-137	142-143
5α-androstane-3β-ol	Mann Research Laboratories	146-147	147.5-148
5α-androstane-3β-ol-17-one	Mann Research Laboratories	173-174	175-176
β-sitosterol	Mann Research Laboratories	137-138	136-137
ergosterol	Sigma Chemical Co.	154-155	163

(a) Dictionary of Organic Compounds, 4th edition, edited by J. R. A. Pollock and R. Stevens, Eyre and Spottiswood Ltd., London, (1965).

(b) O'Conner, G. L., and H. R. Nace: *J. Amer. Chem. Soc.* 75, 2118, (1953).

2) Method

(a) Preparation of Lipid Films

Thin lipid films were prepared on the two interior parallel surfaces of a standard quartz ESR aqueous cell. The aqueous cells used in this work were purchased from J. F. Scanlon Co., Whittier, California and had the approximate cell dimensions: 5 cm., (length), X 1.0 cm., (width), X 0.05 cm., (separation between the two parallel surfaces). Prior to use, a cell was soaked in dilute nitric acid, rinsed with distilled water, rinsed with chloroform-methanol (1:1, by volume) and then evacuated for approximately five minutes. A solution of spin label, phospholipid and the appropriate steroid was made up by volume from the stock solutions. The spin label-to-phospholipid mole ratio was always 1:150. Approximately 50 μl. of this solution was placed in the cell and the solvents were slowly evaporated by subjecting the cell to partial vacuum produced by a rotary vacuum pump. During evaporation of the organic solvents, the lipids were deposited on the flat walls of the cell. This procedure was repeated three or four times so that each film preparation contained approximately 1 - 2 mg. of material. After the last addition of lipid solution, the cell was evacuated for about 10 minutes to insure the removal of all organic solvents from the lipid film.

(b) Electron Spin Resonance Measurements

All resonance spectra were recorded at ambient temperature (20 °C ± 2 °C), unless otherwise stated, on a Varian E-3 X-band electron

spin resonance spectrometer. The following instrument settings were used in all the experiments:

magnetic field scan	-	100 gauss
microwave power	-	5 milliwatts
amplitude modulation	-	4 gauss
scan time	-	8 minutes
time constant	-	0.3 seconds

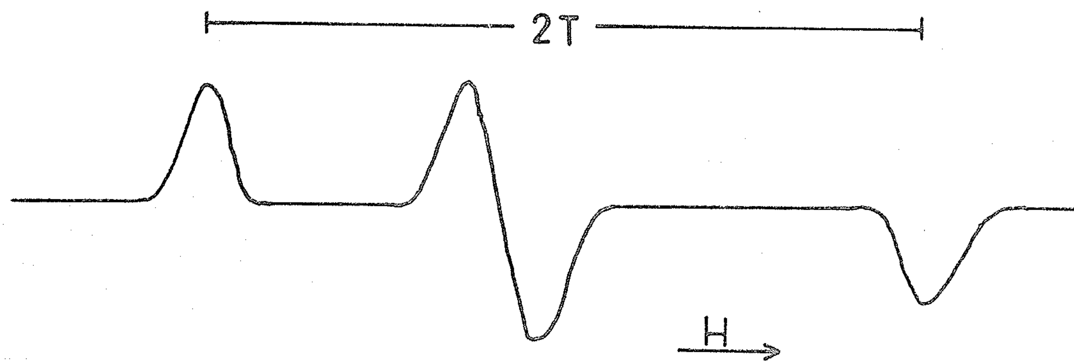
The magnetic field scan of the spectrometer was calibrated regularly with Fremys' salt, $K_2NO(SO_3)_2$, dissolved in aqueous 0.1 molar K_2CO_3. The peroxylamine disulfonate ion, $NO(SO_3)_2^{--}$, has an isotropic hyperfine splitting constant of 13.091 gauss.[89]

The dry film spectra were recorded with the cell under vacuum to prevent the phospholipids from absorbing water from the atmosphere. The spectra in a dry film were recorded with the film-supporting surface parallel and perpendicular to the magnetic field vector. After obtaining the dry film spectra, the cell was opened to the air and filled with a 0.15 molar NaCl aqueous solution, made with double-distilled water. To minimize the dielectric loss in the cavity, the excess aqueous phase was drained, but approximately 50 μl. of the saline solution always remained in the cell. The angle between the plane of the cell and the magnetic field direction, ϕ, was determined with a one cycle goniometer. The spectra of a spin label in a particular film for $\phi = 0^\circ$ and 90° are displayed as overlapping spectra where the position and amplitude of the two spectra are arbitrary. The solid and broken line represent the spectrum obtained when $\phi = 0^\circ$ and 90°,

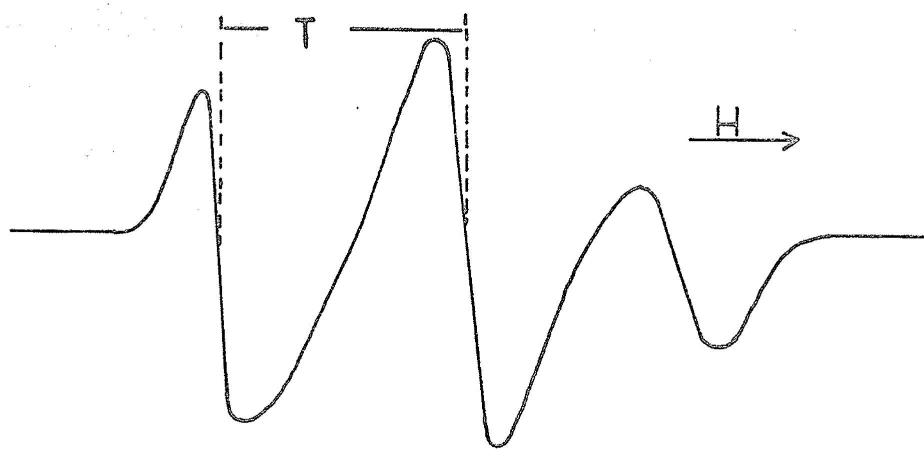
respectively. The temperature of the cell was varied by passing pre-heated nitrogen gas through the cavity of the spectrometer. The temperature was stabilized using a thermistor sensor solid-state temperature controller.[90] A chromel-alumel thermocouple was used to determine the temperature in the cavity of the spectrometer. The maximum drift in the temperature at any setting was found to be ±0.1 °C.

(c) Spectral Analysis

The hyperfine splitting constants of the cholestane spin label were measured from the resonance spectra in the following manner. For a powder spectrum, the hyperfine splitting constant was calculated as one half the separation, in gauss, between the low and high field peaks. In some cases, the broadness of the high field peak did not allow a measurement of the hyperfine splitting.



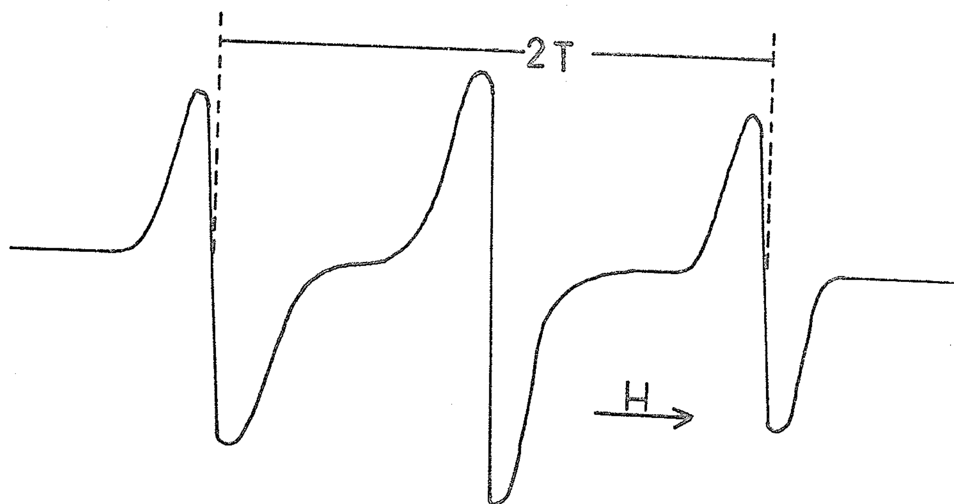
For triplet spectra, the hyperfine splitting constant was measured as the separation, in gauss, between the mid-points of the low field and center lines of the spectrum. $< \Theta_2 >$, the average angle between the steroid spin label long axis and the normal to the plane



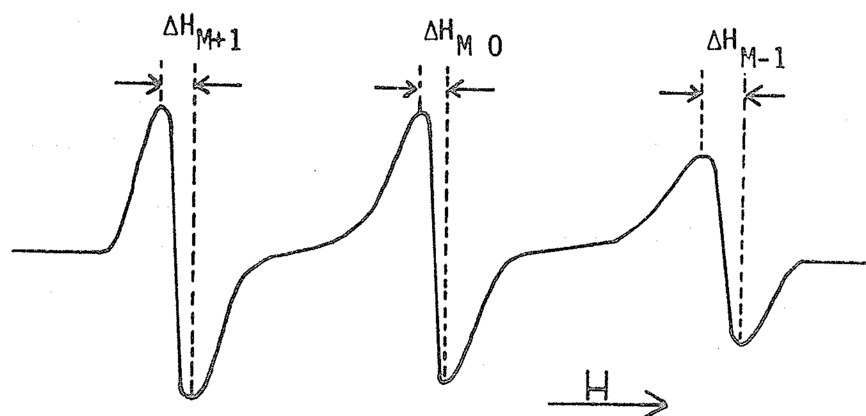
of the hydrated bilayers was calculated using equation 15 and equation 19.

The resonance spectra of the cholestane spin label in different thin film preparations having the same composition were reproducible in both the dry and hydrated states. The maximum deviation in hyperfine splitting constants measured from powder spectra was ±0.5 gauss. The maximum deviation of $T_{//}$ and $T_{\perp}$ values in hydrated films having the same composition was ±0.2 gauss.

The lineshapes of the spectra of the stearic acid spin label in dry films were very complex due to the slow anisotropic motion of the spin label, and it was not possible to extract accurate values of $T_{//}$ and $T_{\perp}$ from these spectra. Therefore, dry film spectra were used primarily for qualitative comparisons between dry films of different composition. In hydrated films, the hyperfine splitting constant of a spectrum was measured as one half the distance in gauss between the mid-points of the low and high field lines. The order parameter, S_3, of the label in hydrated films was calculated from equation 11. The maximum deviation of $T_{//}$ and $T_{\perp}$ values of the label in hydrated films having the same composition was ±0.2 gauss.



Stearamide spin label spectra were analysed in the following manner. The hyperfine splitting of powder spectra were determined as for the cholestane spin label. In hydrated films, the hyperfine splitting was measured as one half the splitting between the mid-points of the low and high field lines. The peak-to-peak linewidths of a spectrum designated ΔH_{M+1}, ΔH_{M0}, and ΔH_{M-1} were measured as shown below.



The order parameter of the spin label, S_3, in hydrated films was calculated from equation 11. $T_{\parallel}$ and $T_{\perp}$ values were reproducible to within ±0.2 gauss in hydrated films having the same composition.

CHAPTER III

RESULTS AND DISCUSSION

1) Egg Phosphatidylcholine Thin Films

(a) Cholestane Spin Label Results

The results of the experiments are summarized in Table 3:1. The spectra of the spin label in dry phosphatidylcholine multilayers (Figure 3:1A) resemble those obtained by Libertini *et al.*[71] Since $T_{//}$ = 6.8 gauss and $T_{\perp}$ = 32 gauss, the spin labels are aligned with their long axes essentially perpendicular to the plane of the multilayers with a rotational frequency << 7.5 x 10^7 sec^{-1}. The broadness of the triplet obtained for the perpendicular orientation arises from a gaussian distribution of label orientations.[71] Hydration of the film yields spectra similar to those found by Hsia *et al.*[72] An increase in $T_{//}$ and decrease in $T_{\perp}$ compared with the dry film spectra indicate a decrease in the orientation of the spin labels with respect to the symmetry axis, in conjunction with an increased axial rotational frequency, >> 7.5 x 10^7 sec^{-1}. These results are compatible with the fact that there is an increase in the fluidity of the phospholipids upon hydration. The average deviation angle of the spin label, $< \theta_2 >$, in hydrated phosphatidylcholine was calculated to be 24° using equation 19 and 30.7° using equation 15. The assumption is made that any change in the average deviation angle of the intercalated spin label upon addition of a steroid to the film can be discussed in terms of specific phospholipid-steroid interactions.

The presence of 50 mole % cholesterol, (1:1 mole ratio of phospholipid : cholesterol), does not alter significantly the distribution of orientations or motion of the spin labelled steroid molecules in

Table 3.1

Ordering Effects of Cholesterol and Structural Derivatives in Egg Phosphatidylcholine Multilayers

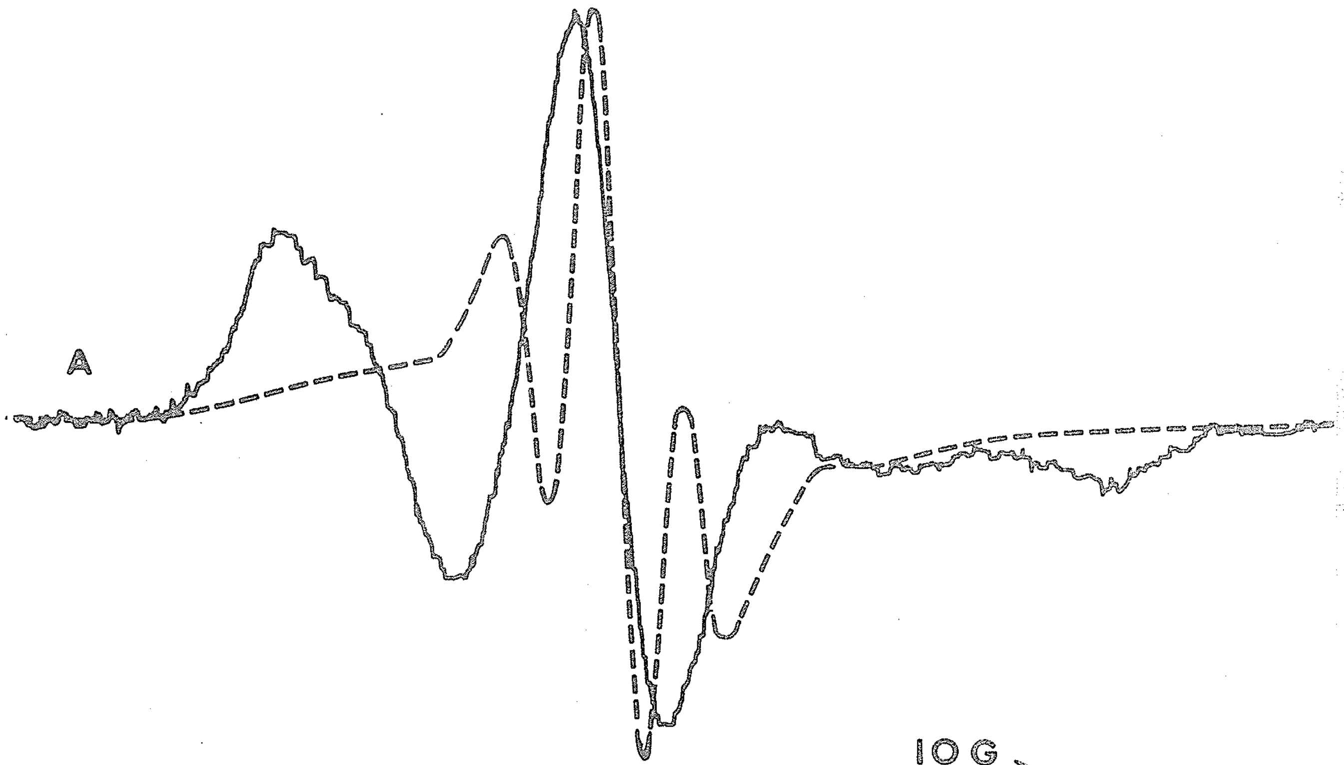
Steroid	Mole %	Dry Film		Hydrated Film			
		$T_{//}$	$T_{\perp}$	$T_{//}$	$T_{\perp}$	$<\Theta_2>^{\dagger}$	$<\Theta_2>^{\S}$
cholesterol	0	6.8	33.0	9.5	17.5	24.0	30.7
	50	6.6	32.5	6.6	19.9	8.7	8.1
cholesteryl methyl ether }	25	6.9	31.5	8.6	17.8	20.0	26.3
cholesteryl chloride }	50	30.0	31.3	8.6	17.6	20.0	26.8
thiocholesterol	9	---	---	---	---	---	---
epicholesterol	50	---	---	7.8	18.7	16.0	20.5
5α-cholestane-3β-ol	50	6.6	33.1	6.6	19.5	8.7	8.5
Δ^7-cholesten-3β-ol	50	6.9	33.0	6.6	20.2	8.7	6.5
7-dehydrocholesterol	50	6.7	33.0	6.7	20.7	9.5	9.1
ergosterol	50	6.7	32.6	7.4	18.9	14.0	17.7
β-sitosterol	50	6.7	32.0	7.4	18.8	14.0	18.0
5α-androstane-3β-ol	50	29.9	31.3	7.9	18.8	16.5	20.7
5α-androstane-3β-ol-17-one	10	---	---	9.3	17.8	23.2	29.2
	50	---	---	10.2	17.3	27.2	33.7

† calculated from equation 19

§ calculated from equation 15

Figure 3.1

ESR spectra of the cholestane spin label in an egg phosphatidylcholine film. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.


A
10 G

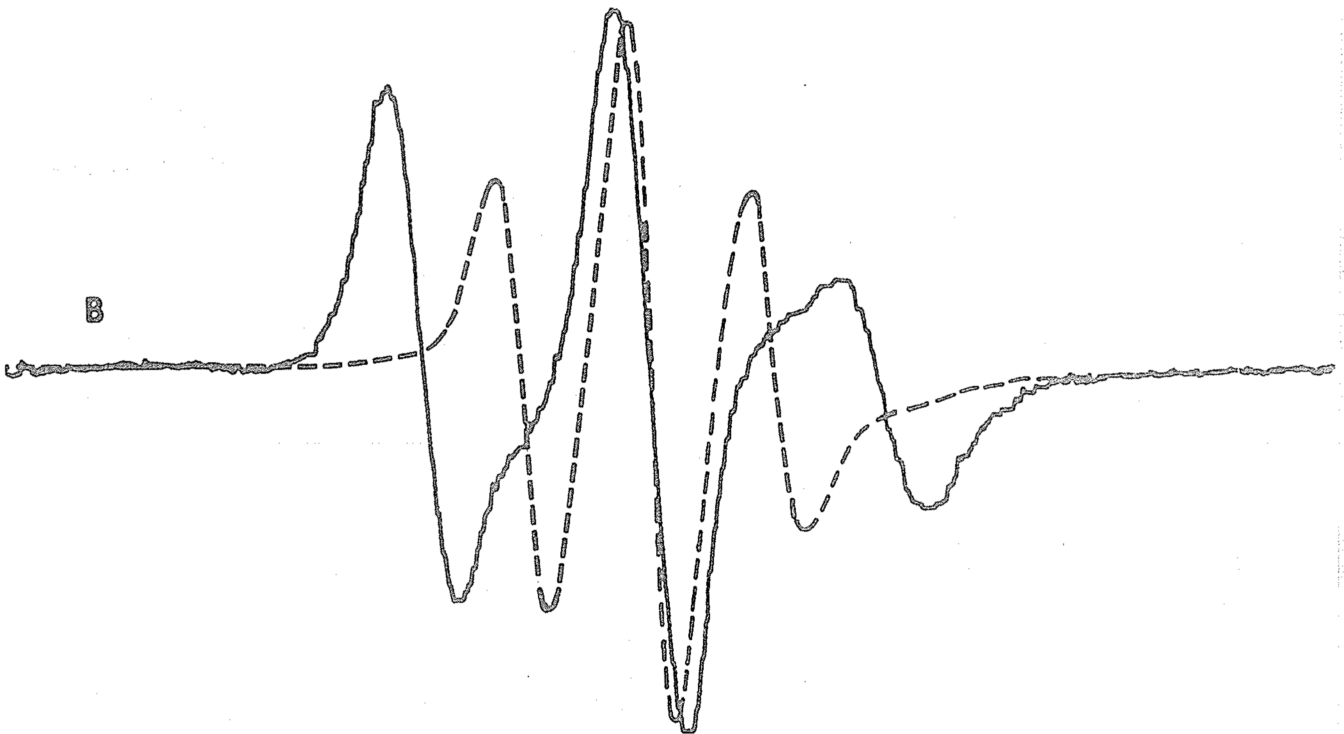
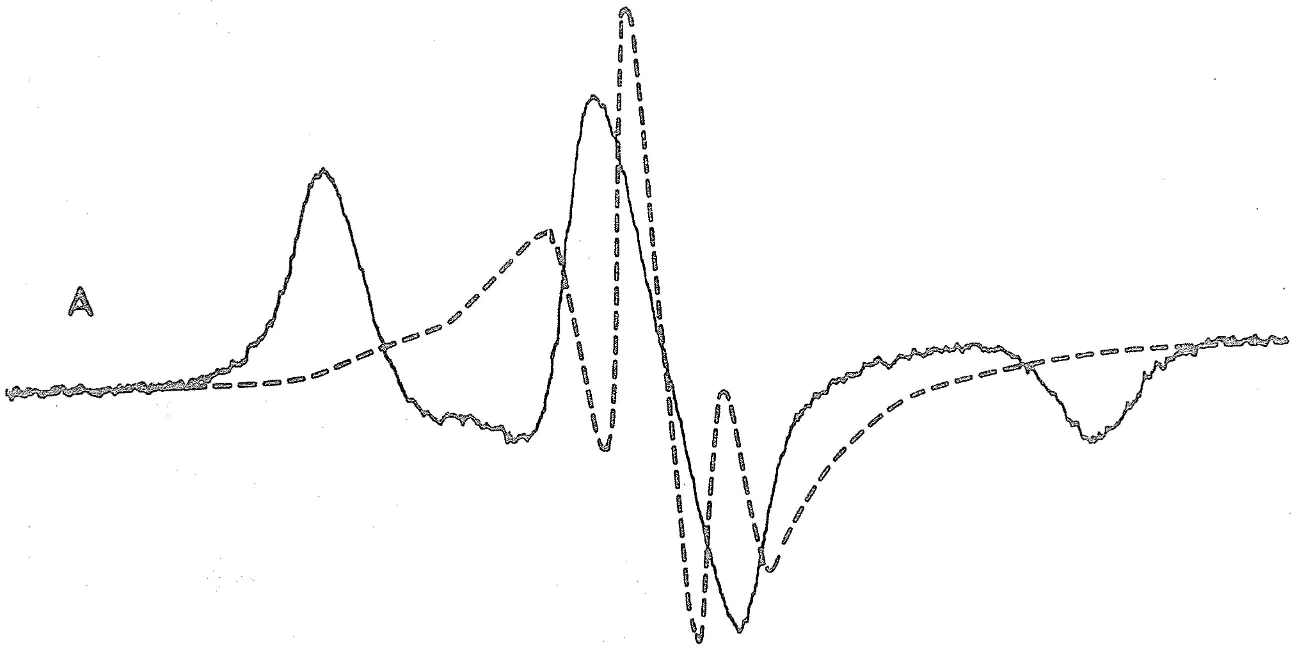

B

Figure 3.2

ESR spectra of the cholestane spin label in an egg phosphatidylcholine film containing 50 mole % cholesterol. The spectra were recorded with the plane of the film parallel, (——), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.



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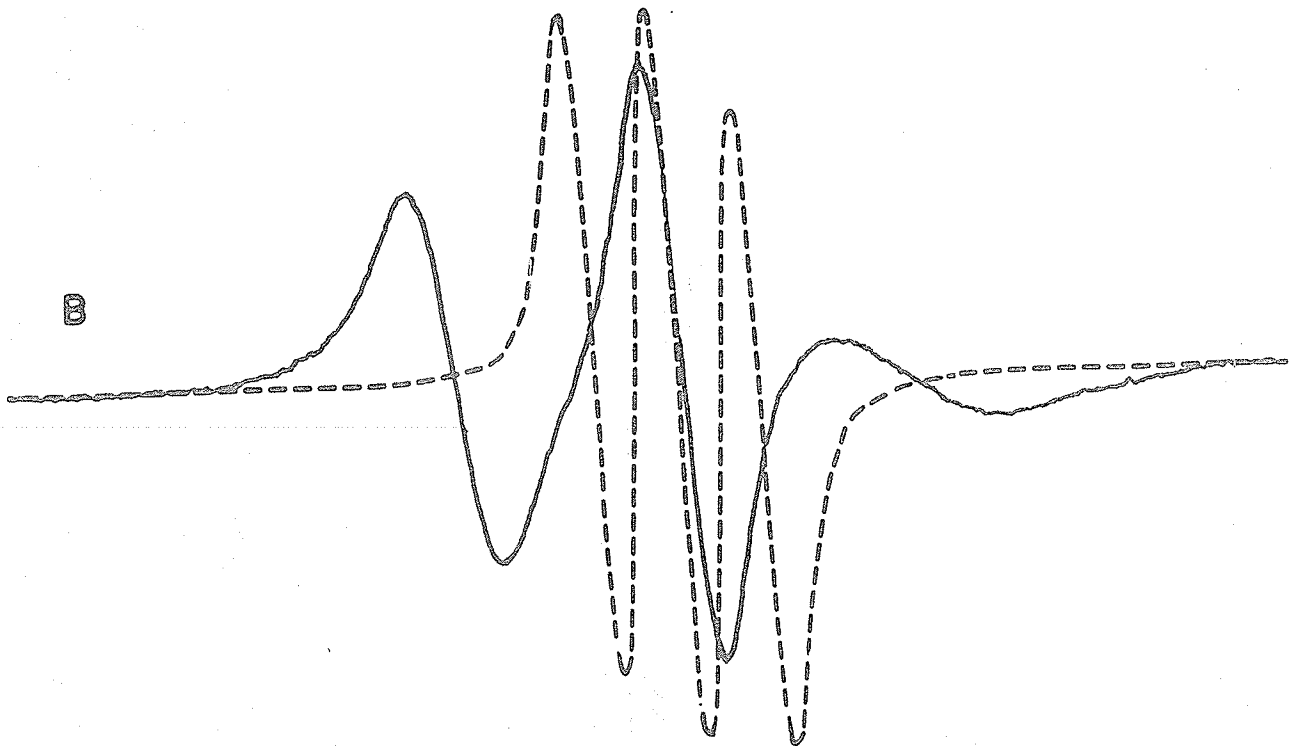


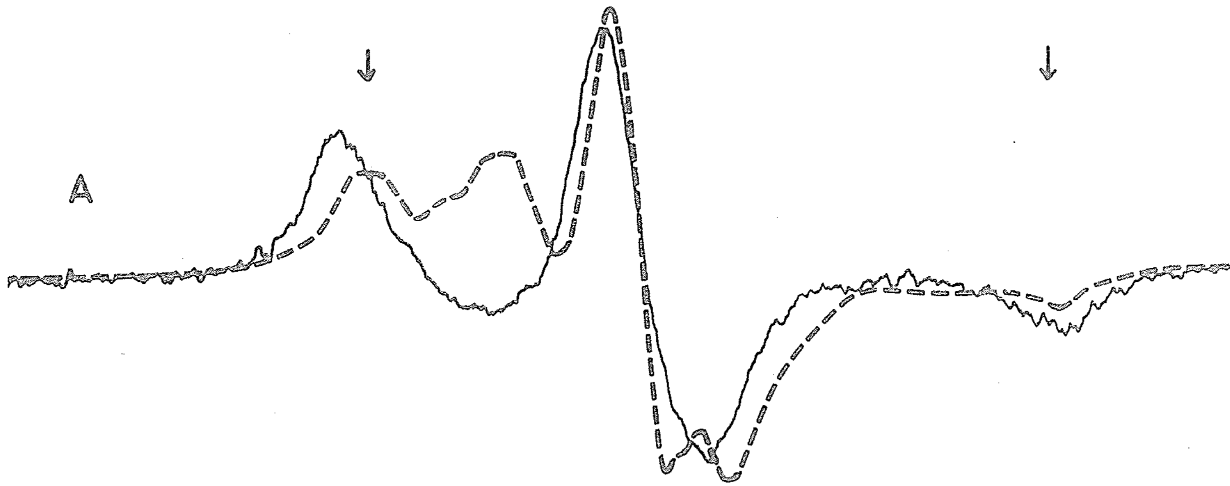
the dry lamellae since the spectra obtained, (Figure 3.2A), are similar to those of the label in dry phosphatidylcholine alone. Hydration of the cholesterol-phosphatidylcholine film allows rapid rotational motion about the long axes of the spin labels with a frequency >> $7.5 \times 10^7 sec^{-1}$, (Figure 3.2B), deduced from the decrease in $T_{\perp}$ from 32.5 gauss to 19.9 gauss. The sharpening of the lines of the spectrum of the perpendicular orientation, ($\phi = 90^\circ$), upon hydration, (Figure 3.2B), indicates a much narrower distribution of orientations of spin labels in the hydrated lamellae. The average deviation angle of the lipids in hydrated lamellae containing 50 mole % cholesterol is estimated to be 8.1°- 8.7°. Thus, there is a decrease of approximately 15° in $< \Theta_2>$ upon addition of cholesterol to the bilayers, clearly demonstrating the ordering effect of this sterol on the phospholipid molecules.

C_3 Derivatives

Cholesteryl methyl ether and cholesteryl chloride have no apparent effect on the dry lamellae up to concentrations of 25 mole %. In hydrated films containing 25 mole % of either steroid, the average deviation angle is much greater than in a film containing an equal concentration of cholesterol. This would suggest that the ordering effect of cholesterol involves polar interactions between the 3-OH group and the phospholipids. Increasing the concentration of these steroids above 25 mole %, results in the appearance of powder spectra which overlap the spectra of spin labels in the lamellar phase. The powder spectra can be rationalized as arising from spin labels intercalated in a polycrystalline steroid matrix. Figure 3.3 shows the

Figure 3.3

ESR spectra of the cholestane spin label in an egg phosphatidylcholine film containing 50 mole % cholesteryl methyl ether. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.



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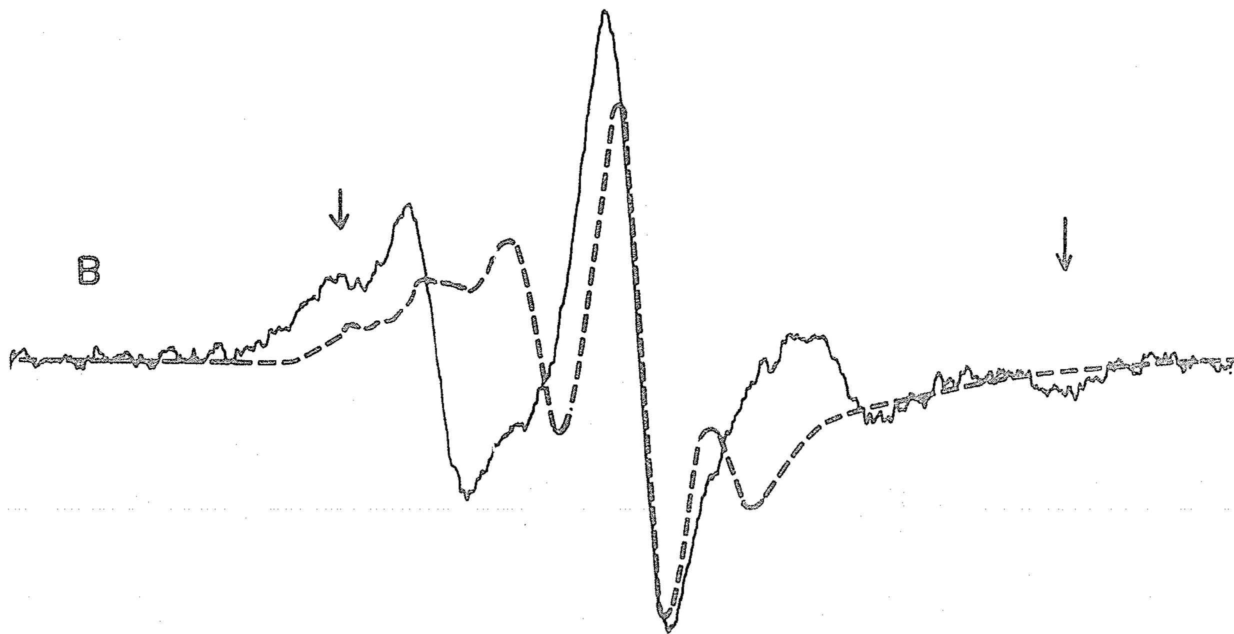
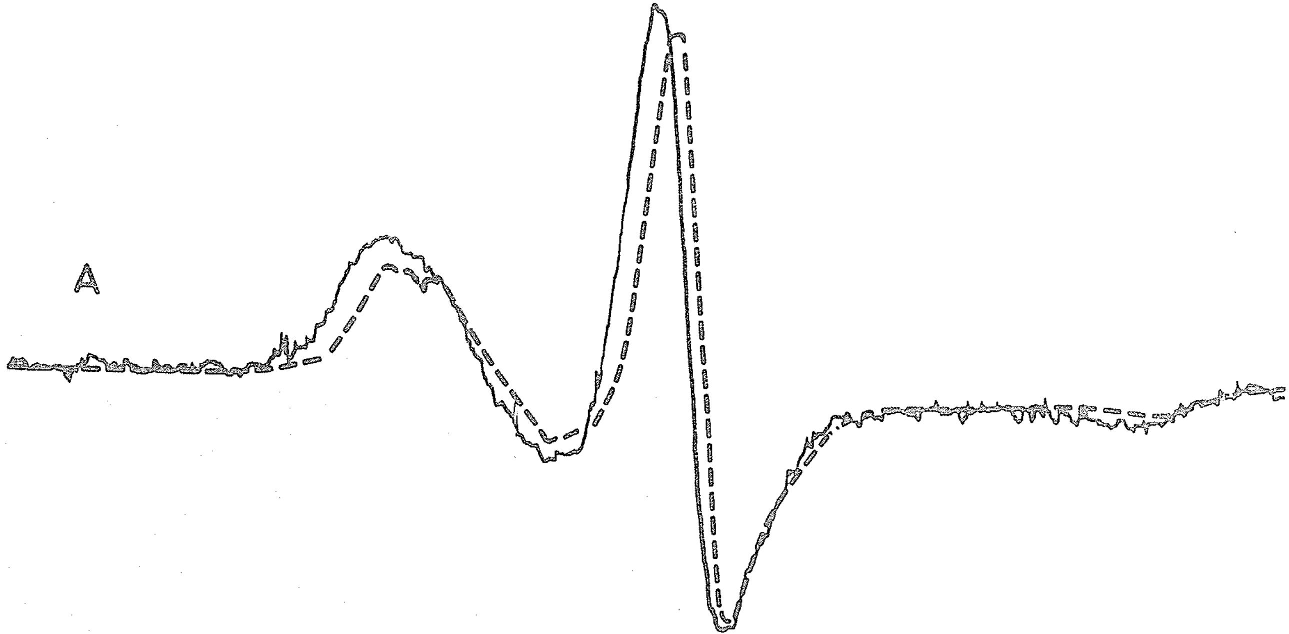


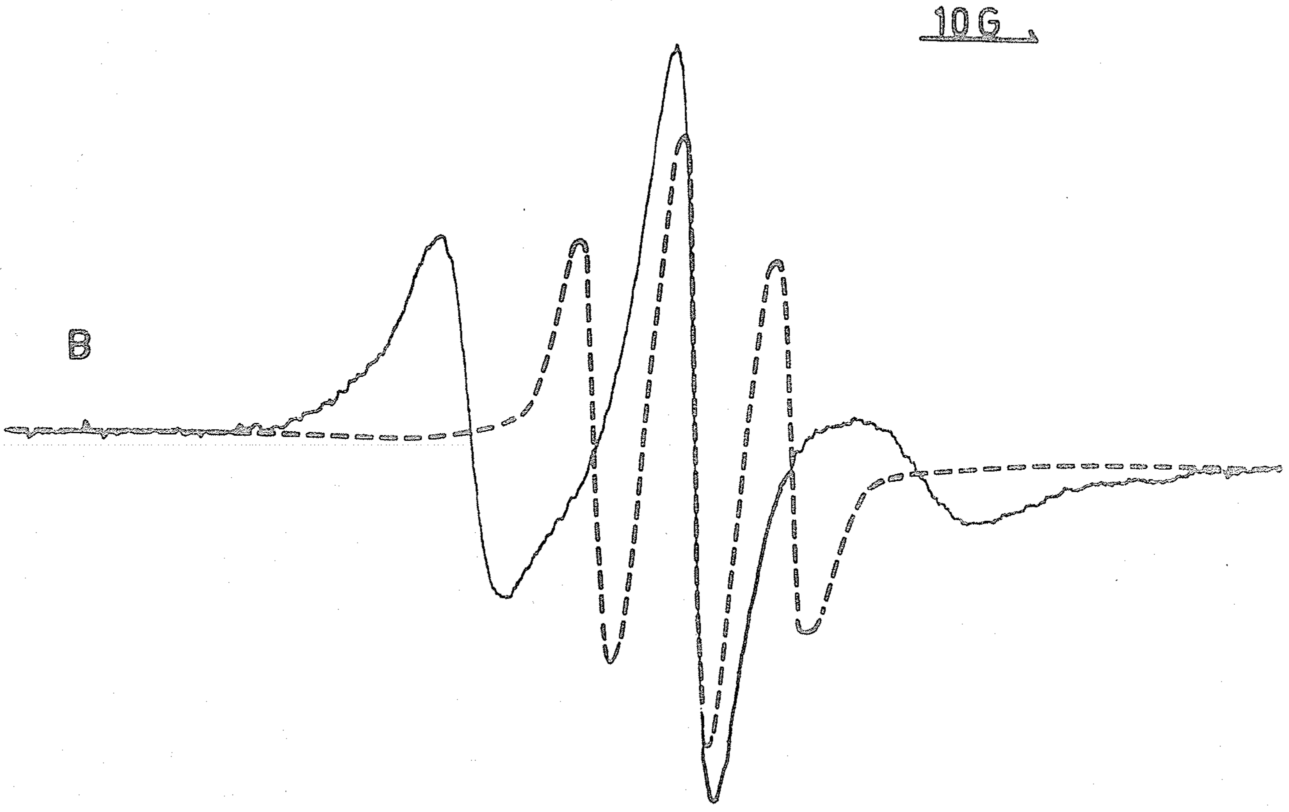
spectra obtained from phosphatidylcholine films containing 50 mole % cholesteryl methyl ether. The high and low field peaks of the powder spectra are indicated by arrows (↓). Thus, in both the dry and hydrated films, there is a distribution of spin labels between the lamellar phase and the separate crystalline steroid phase. The limited solubility of cholesteryl methyl ether and cholesteryl chloride in egg phosphatidylcholine liposomes has been observed using phase contrast microscopy.[81] If one takes the view that cholesterol is incorporated in the phosphatidylcholine bilayers due to its hydrophobic nature, then removal of the hydroxyl group should have little effect on its solubility in the lamellar phase. Since incorporation of the cholestene nucleus into both dry and hydrated lamellae shows a dependance on the presence of the OH group, this is more evidence for the existance of specific electrostatic interactions involving this group and the polar moieties of the phospholipid molecules.

Epicholesterol, the 3α-OH isomer of cholesterol had a profound effect on the dry film spectra. The presence of 50 mole % epicholesterol in the dry phospholipid film resulted in angular independent spectra of the spin label, (Figure 3.4A). This denotes a complete disruption of the oriented lamellar structure in the dry film. Presumably, a 3-OH group in the α configuration is not favourably oriented for polar interactions with the phospholipid head groups, and the epicholesterol molecules cannot be accomodated without disturbing the structure of the lamellae. In the presence of water (Figure 3.4B), the spectra become angular dependent indicating a reformation of oriented lamellae.

Figure 3.4

ESR spectra of the cholestane spin label in an egg phosphatidylcholine film containing 50 mole % epicholesterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.


A


10 G
B

However, epicholesterol is less effective than cholesterol in reducing the average deviation angle of the lipids. Consequently, a hydroxyl group in the β configuration is necessary for the maximum ordering effect which suggests that the interaction of the 3-OH group with the phospholipids is stereospecific.

Huang *et al*[91] have shown that thiocholesterol can be incorporated into phosphatidylcholine vesicles up to one mole of thiocholesterol per ten moles of phospholipid. Titration and spin labelling experiments demonstrated that the thiol group is exposed to the aqueous phase.[91] The preparation of dry films containing 9 mole % thiocholesterol resulted in a rapid decrease in the signal intensity of the cholestane spin label resonance spectrum. Shortly after formation of the film, no resonance spectrum of the label could be detected. It is well known that nitroxides react with hydrogen donors, such as sulfhydryl groups, forming $\rangle$N–OH which results in the destruction of the paramagnetic center. Since the nitroxide of the cholestane spin label must be in very close proximity to the sulfhydryl groups of thiocholesterol in the dry phospholipid lamellae, it can be concluded that the cholestane spin label has the same spatial orientation as thiocholesterol with the nitoxide being in the vicinity of the polar headgroup region. Unfortunately, no deviation angles could be determined from the thiocholesterol-phospholipid films due to rapid destruction of the spin labels.

B - Ring Derivatives

From Table 3.1, it can be seen that 5α-cholestane-3β-ol, Δ^7-cholesten-3β-ol and 7-dehydrocholesterol have the same ordering effect

as cholesterol in the dry and hydrated films. Therefore, it appears that any hydrophobic phospholipid-sterol interactions involving the B ring do not significantly depend on its degree of unsaturation or the position of double bonds.

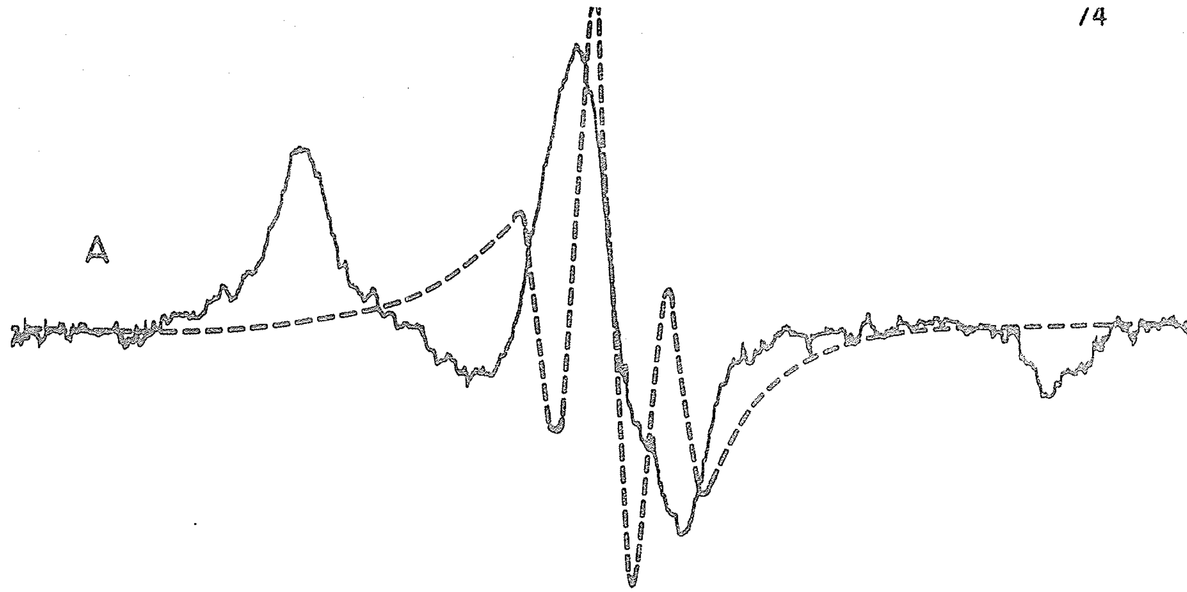
C_{17} Derivatives

β-Sitosterol and ergosterol, two common plant sterols, represent C_{17} derivatives in which the hydrophobic nature of the hydrocarbon chain is only slightly different from that of cholesterol. Resonance spectra of the spin label in dry films containing either 50 mole % β-sitosterol or ergosterol are essentially the same as spectra obtained with an equivalent amount of cholesterol. However, in the hydrated multilayers, these sterols are less effective than cholesterol in reducing the average deviation angle of the phospholipids. A possible explanation for the larger deviation angles produced by two sterols in comparison with cholesterol, is that the fatty acid composition of the phospholipids is not suitable for maximum hydrophobic interactions between the sterol side chains and the phospholipid molecules.

A rapid decrease in the angular dependance of the spectra of the spin label in films containing 50 mole % ergosterol was observed when the films were hydrated, (Figure 3.5). This process was equally rapid in the presence or absence of light. It is well known that ergosterol irradiated with ultraviolet light isomerises to vitamin D_2.[92] Butler *et al*[82] have reported that vitamin D_2 is ineffective in reducing the average deviation angle in phospholipid films. The decomposition

Figure 3.5

ESR spectra of the cholestane spin label in an egg phosphatidylcholine film containing 50 mole % ergosterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film, (C) spectra obtained approximately 45 minutes after hydration of the film.



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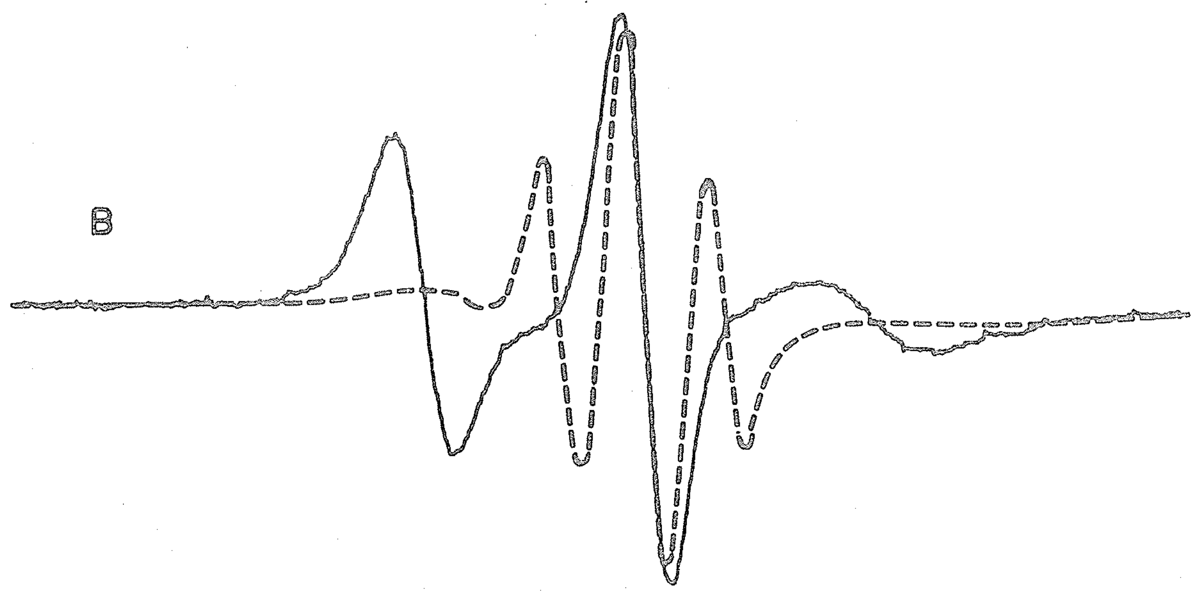
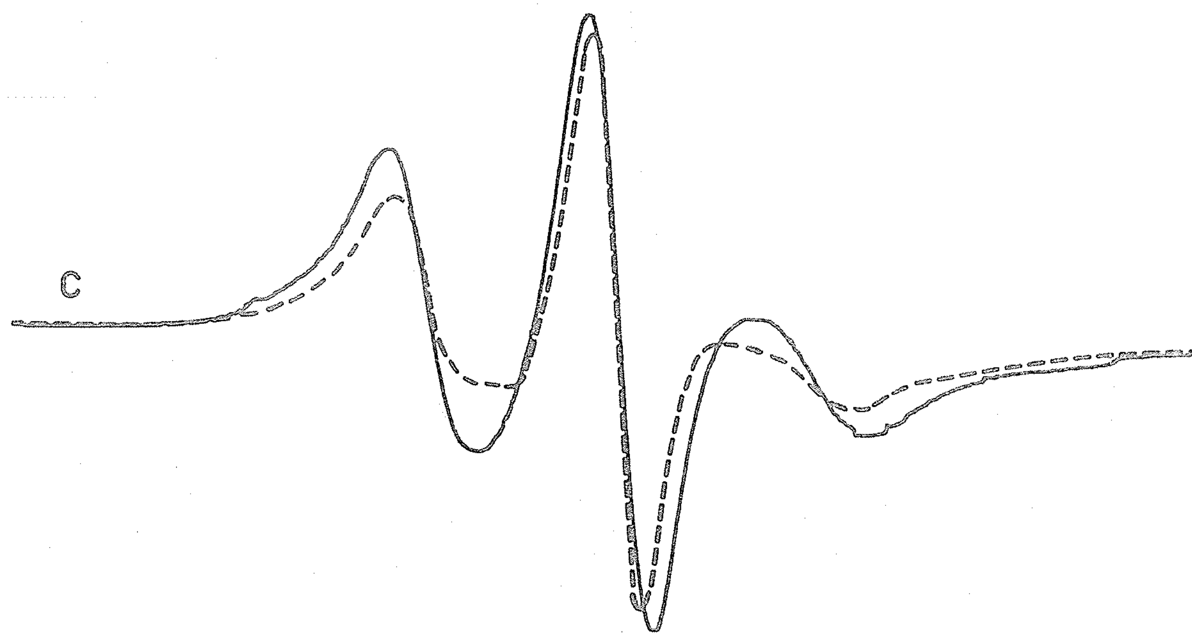
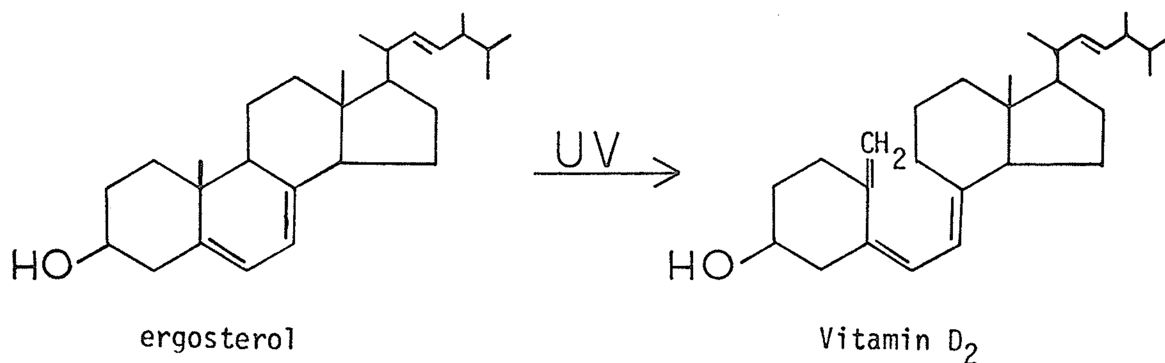






of ergosterol to vitamin D_2 might be used as an explanation for the decrease in the orientation of the spin label except that the disordering of the label occurs equally fast in the dark. Also, films containing 7-dehydrocholesterol, which rearranges to form vitamin D_3 in the same manner as ergosterol are very stable in the presence of water and light. Therefore, a clear statement regarding this phenomenum is not possible with the present information and further investigations are required.

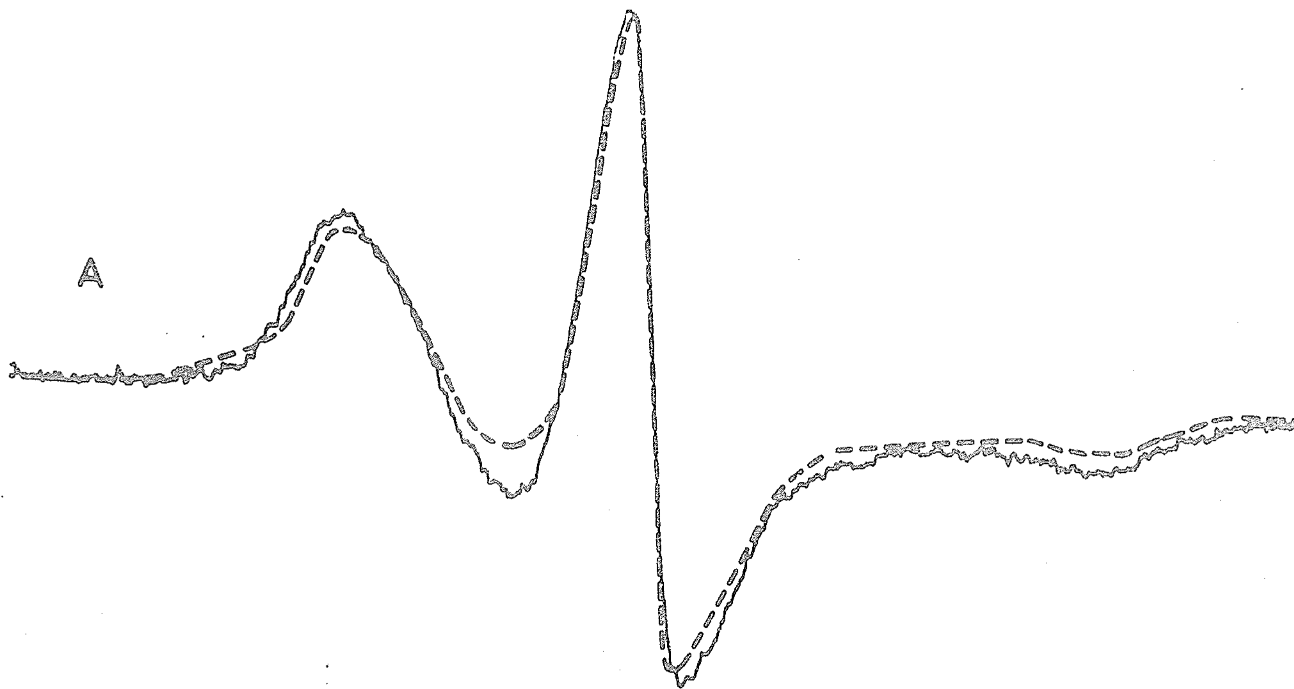
5α-Androstane-3β-ol and 5α-androstane-3β-ol-17-one are derivatives of 5α-cholestane-3β-ol where the nature of the C_{17} substituent has been drastically altered. No orientation dependence of the spectra of the spin label was found in dry films containing 50 mole % of either sterol, (Figure 3.6A and Figure 3.7A). Thus, these two sterols which lack a hydrocarbon side chain must have a disruptive effect on the dry lamellae. It would appear that for a sterol to be incorporated into the dry phospholipid lattice, a hydrocarbon side chain is necessary for anchoring the molecule in the hydrophobic interior of the bilayer. Hydrophobic forces between the side chain and the phospholipid fatty

acid residues would provide a counterbalance for the polar interactions between the sterol and the phospholipids and assist in maintaining the long axis of the sterol molecule roughly parallel to the long axes of the phospholipids.

When films containing 5α-androstane-3β-ol or 5α-androstane-3β-ol-17-one are hydrated, the spectra of the spin label become angular dependent, indicating a reorganization of the lipids into oriented lamellae in the presence of water but both sterols are less effective than 5α-cholestane-3β-ol or cholesterol in reducing the average deviation angle of the lipids. Therefore, an interaction between the hydrocarbon side chain of the cholesterol molecule and the phospholipid fatty acid chains is necessary for a maximum reduction in the random thermal motion of the phospholipids. For a hydrated film containing 50 mole % 5α-androstane-3β-ol, an extra low field peak indicated by an arrow, (↓), in Figure 3.6B can be seen when the plane of the film is perpendicular to the magnetic field. This extra spectral component probably arises from spin labels in regions of local disorder in the film. 5α-Androstane-3β-ol-17-one which has the typical structure of a steroid hormone, having polar substituents at C_3 and C_{17}, has a much more disruptive effect on the hydrated phosphatidylcholine films than 5α-androstane-3β-ol. It can be seen from Figure 3.8B and Figure 3.7B which depict spectra of films containing 10 mole % and 50 mole % 5α-androstane-3β-ol-17-one, respectively, that the intensity of the isotropic spectral component, (↓), increases with steroid concentration. In addition, the average deviation angle of the lipids in the film

Figure 3.6

ESR spectra of the cholestane spin label in an egg phosphatidylcholine film containing 50 mole % 5α-androstane-3β-ol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.


A

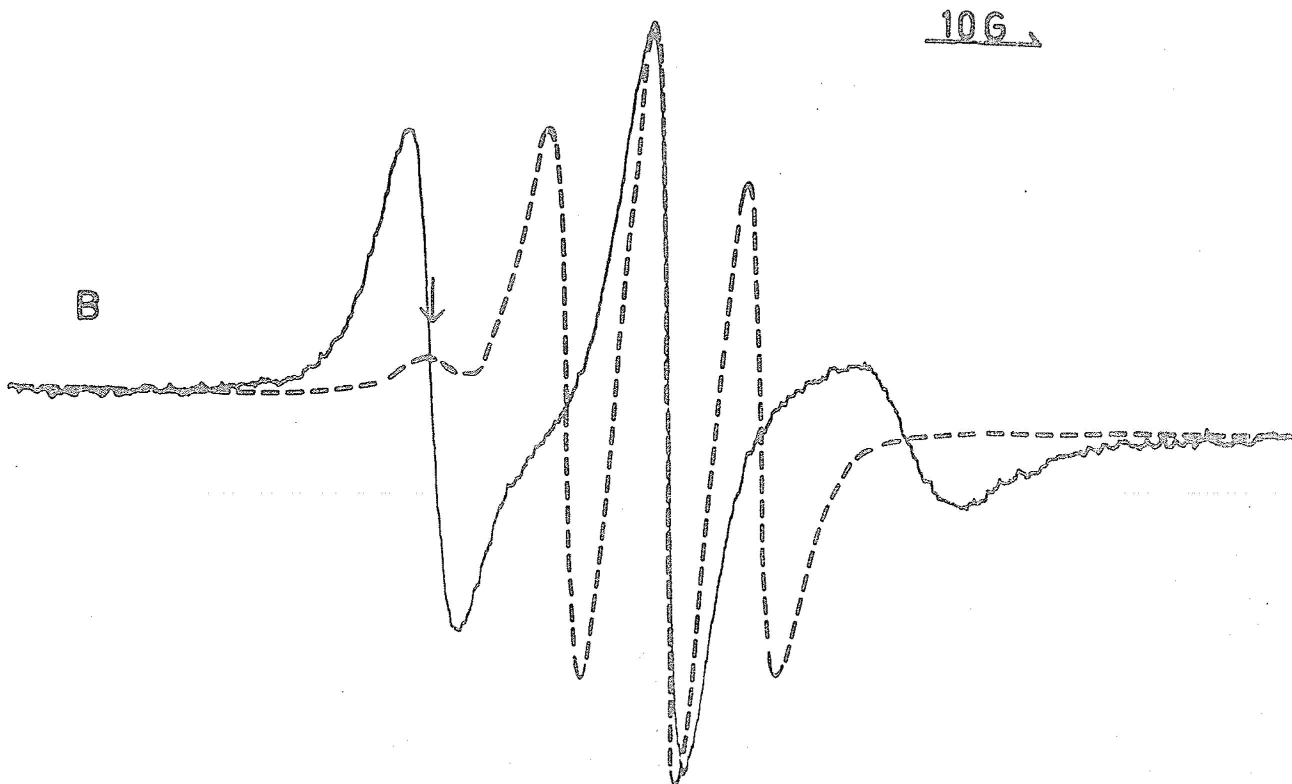
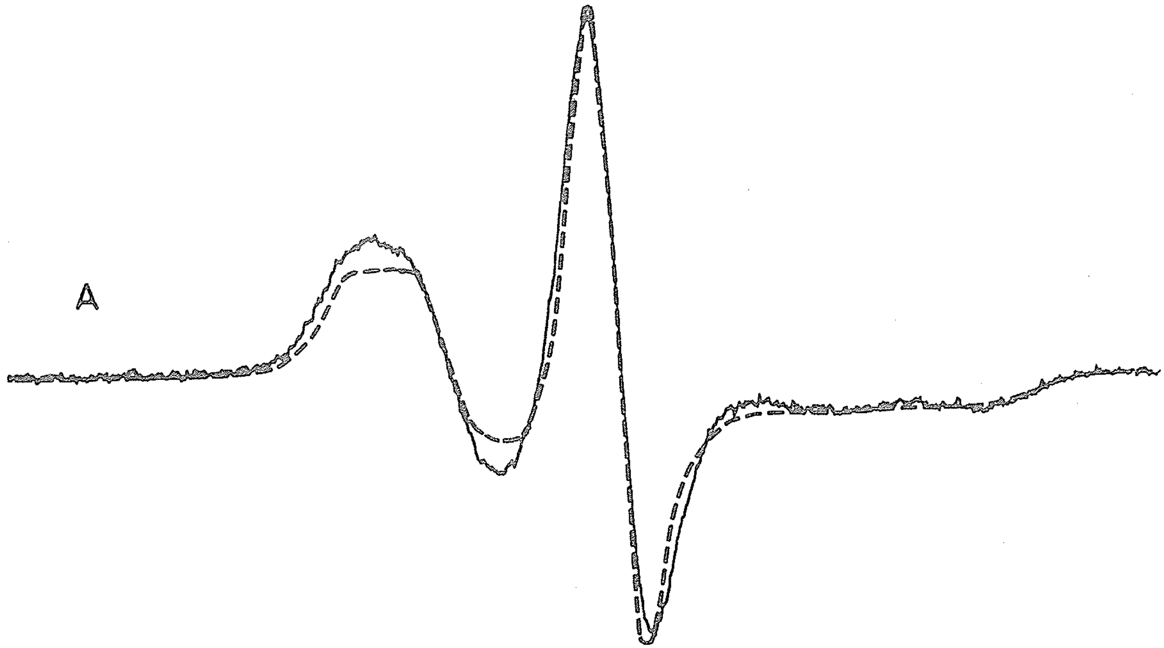

B
10G

Figure 3.7

ESR spectra of the cholestane spin label in an egg phosphatidylcholine film containing 50 mole % 5α-androstane-3β-ol-17-one. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.


A

10G

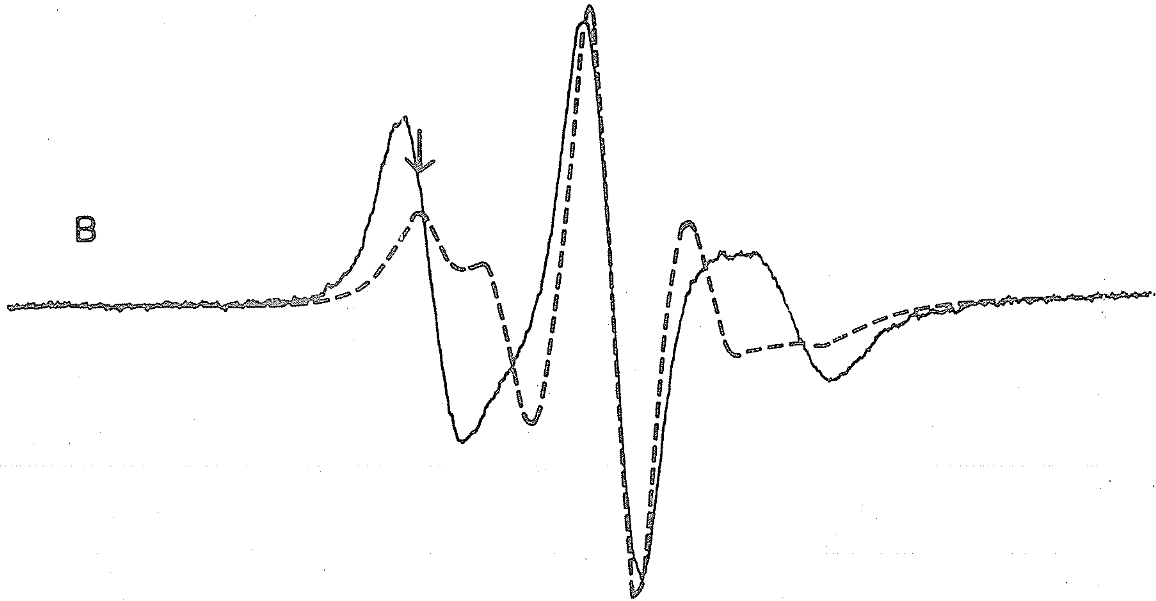
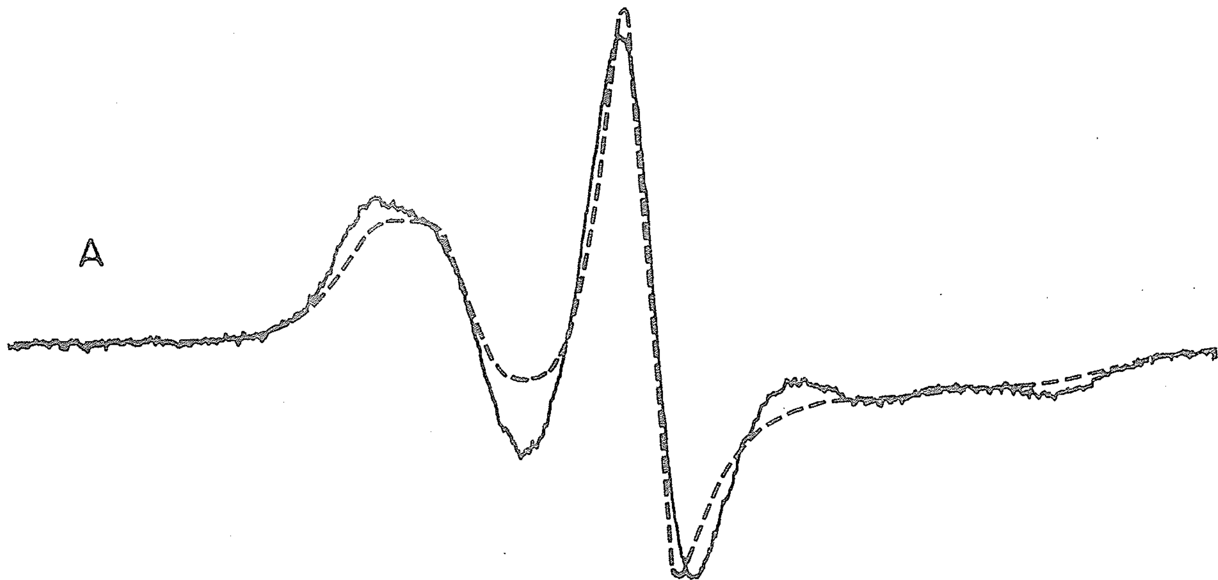
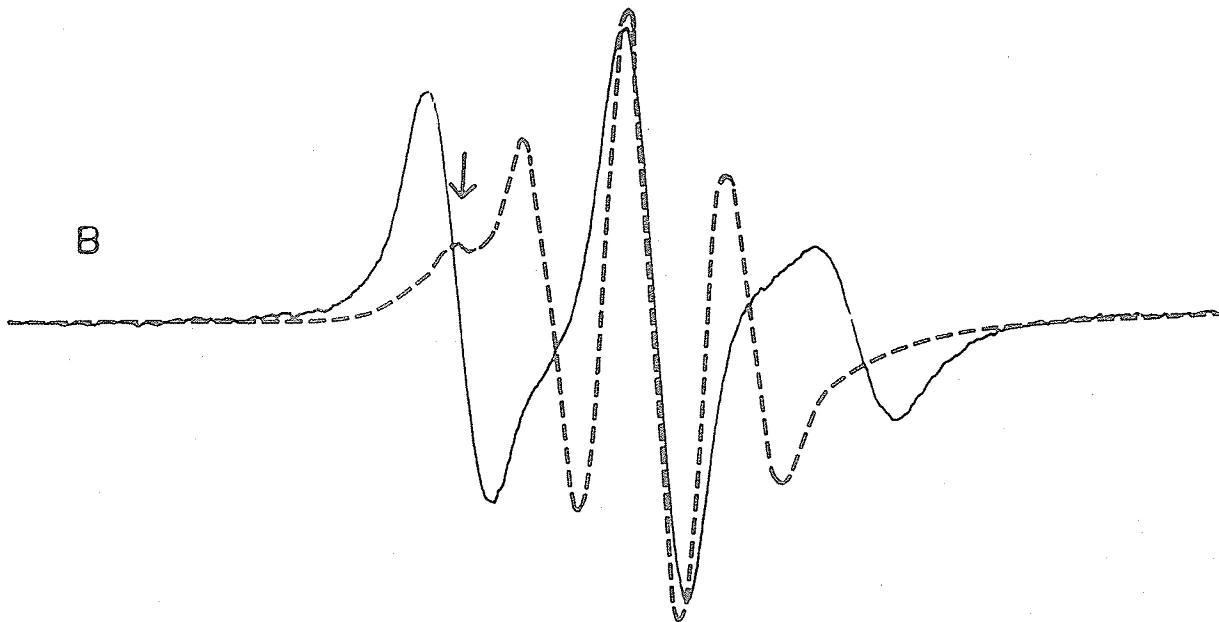

B

Figure 3.8

ESR spectra of the cholestane spin label in an egg phosphatidylcholine film containing 10 mole % 5α-androstane-3β-ol-17-one. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.



10G



containing 50 mole % 5α-androstane-3β-ol-17-one is actually greater than the deviation angle in phosphatidylcholine alone. Thus, it is necessary for the C_{17} substituent to be of a hydrophobic nature to permit co-operative interactions between the steroid and the phospholipids.

It would be advantageous at this point to summarize the major conclusions obtained from the cholestane spin label experiments. The results of this study are consistent with a model of the phosphatidylcholine-cholesterol-water system in which the linear cholesterol molecule is interdigitated between the phospholipid molecules in the bimolecular lipid leaflets and so reduces the random thermal motion of the paraffin chains.[31,36] The 3β-OH group anchors the cholesterol molecule at the membrane water interface by interacting stereospecifically with the phosphatidylcholine head groups. The most apparent form of interaction would be a hydrogen bond, involving the hydroxyl group and an oxygen atom of the head group. A likely consequence of this interaction is a reduction in the mobility of the polar head groups. The C_{17} hydrocarbon side chain extends towards the center of the lipid bilayer and interacts with the phospholipid fatty acid chains through attractive London dispersion forces.

The binding of the cholesterol molecule at both hydrophilic and hydrophobic ends of the phospholipid molecule tends to keep the phospholipid long axis parallel to the long axis of the sterol, accounting for a reduction in the thermal motion of the phospholipids. The steroid nucleus provides the rigid long axis for the cholesterol molecule and allows for a correct degree of separation between the hydroxyl group and the hydrocarbon side chain for maximum interaction

with the phospholipid molecules. It appears that any hydrophobic forces involving the tetracyclic nucleus and the phospholipids are not dependent on the presence, or position of double bonds in the B-ring.

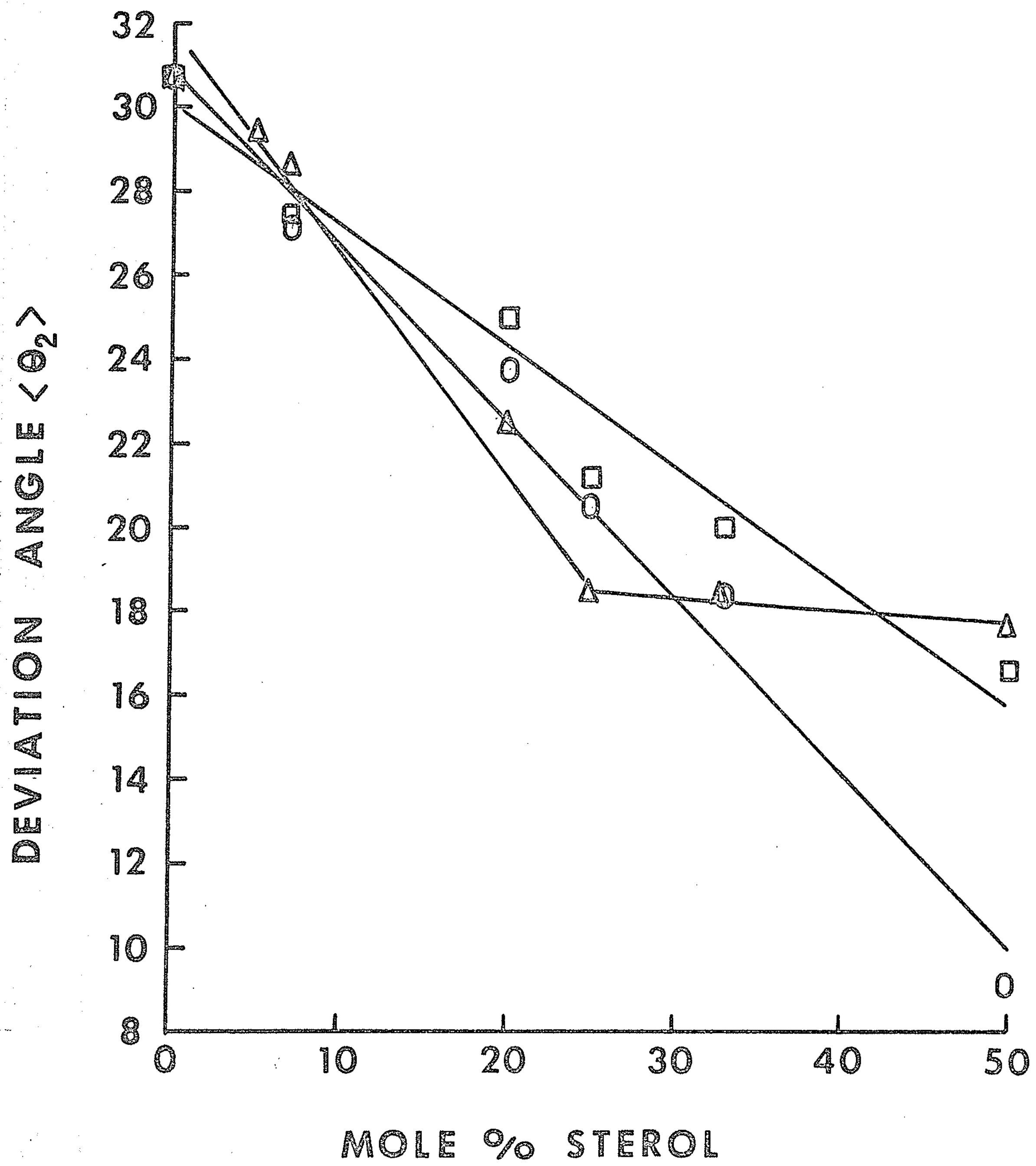
It has been reported[47] that the solubilities of 7-dehydrocholesterol, ergosterol, and β-sitosterol in sonicated egg phosphatidylcholine liposomes are 7 mole %, 7 mole % and 33 mole % respectively. The results of a concentration study of these three sterols in hydrated egg phosphatidylcholine films using the cholestane spin label are presented in Figure 3.8a. The deviation angle, $\langle\theta_2\rangle$, of the spin label was calculated using equations 13, 14 and 15. If the solubility limits noted above were applicable to the oriented lamellae system there should be no change in $\langle\theta_2\rangle$, after the sterol solubility was exceeded. In fact, the presence of a separate polycrystalline steroid phase should give rise to powder spectra as observed with the experiments involving cholesteryl methyl ether and cholesteryl chloride.

The deviation angle, $\langle\theta_2\rangle$, decreases continually over the range 0-50 mole % sterol for 7-dehydrocholesterol and β-sitosterol. In ergosterol containing films there is little change in $\langle\theta_2\rangle$ between 25-50 mole % sterol. This is probably due to the instability of hydrated films containing ergosterol noted previously. Powder spectra were not detected in any of the film preparations.

These results indicate that these sterols are soluble up to 50 mole % in the oriented phosphatidylcholine multibilayers and that a comparison of the ordering effects of each of these sterols with cholesterol at a concentration of 50 mole % is probably valid.

Figure 3.8a

A plot of $<\theta_2>$ versus sterol concentration for the cholestane spin label in hydrated egg phosphatidylcholine films: O 7-dehydrocholesterol, □ β-sitosterol, Δ ergosterol.



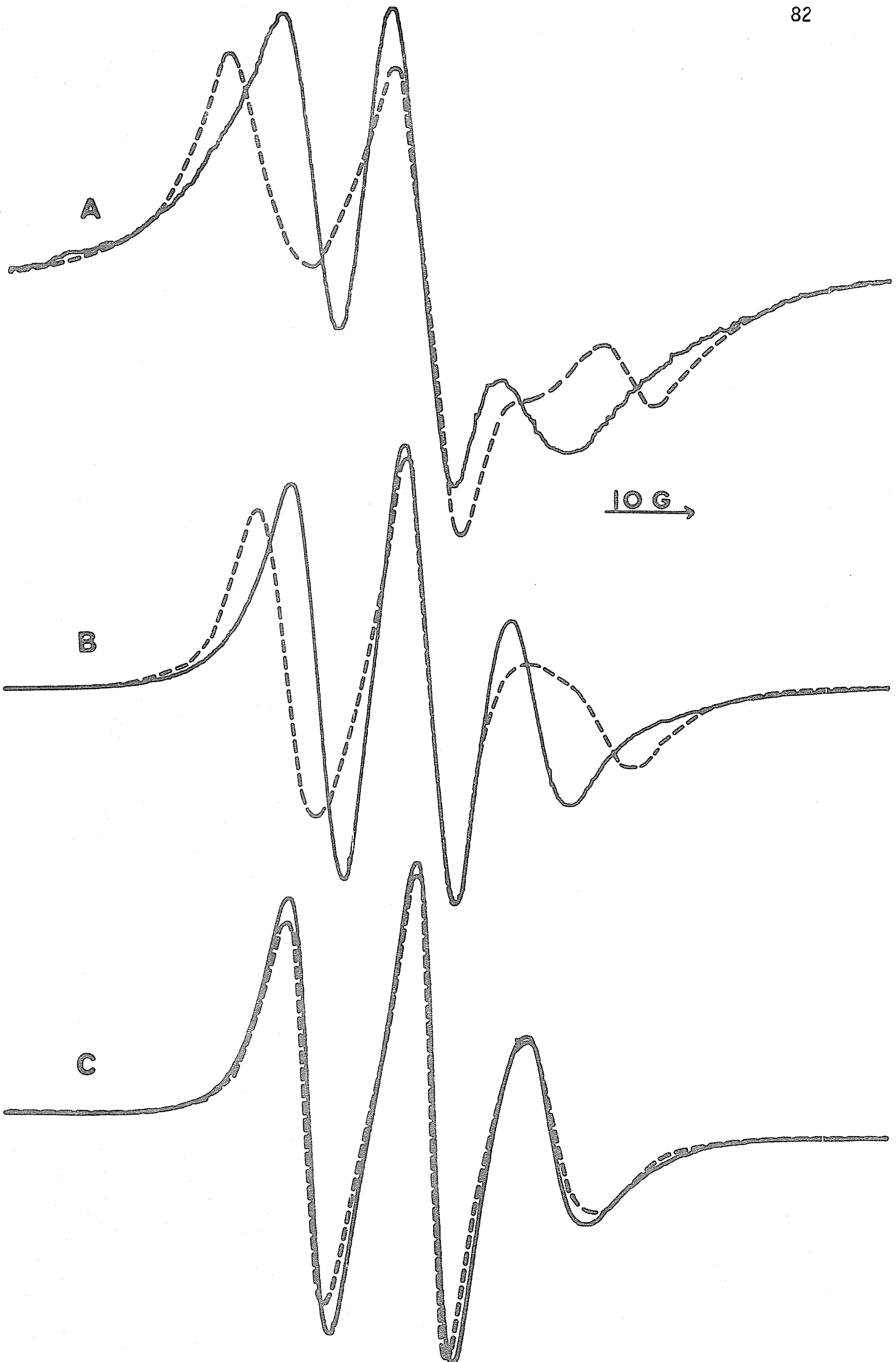
DEVIATION ANGLE ⟨θ_2⟩
32
30
28
26
24
22
20
18
16
14
12
10
8
0
10
20
30
40
50
MOLE % STEROL

(b) 12-Stearic Acid Spin Label Results

In a dry egg phosphatidylcholine film, the spectra of the stearic acid spin label are angular dependent, (Figure 3.9A). The largest hyperfine splitting is $T_{//}$ and is approximately equal to 21.4 gauss, measured as one half of the separation between low and high field peaks. $T_{\perp}$ meanwhile is equal to 11.8 gauss. Therefore, the angular dependence of the hyperfine splittings indicate that the long axis of the stearic acid molecule is preferentially aligned perpendicular to the plane of the bilayers, consistent with the results of Libertini *et al*.[71] Thus, the spin label accurately reflects the orientation of the phospholipids since X-ray data[36] have shown that the fatty acid chains of dry phospholipid multilayers exist in an extended configuration with their long axes perpendicular to the bilayer plane. It can also be seen from Figure 3.9A that the spectral lines, especially the high field lines, are very broad. These broad lines indicate that the anisotropic motion of the spin label in the dry bilayers is very slow, presumably a consequence of the low degree of motion of the phospholipid fatty acid chains in the absence of water. Figure 3.9B shows the spectra of the spin label after 1 µl. of water was introduced in to the cell. The lines are much sharper for both the parallel and perpendicular orientations of the film indicating an increase in fluidity of the hydrocarbon chains. $T_{//}$ is still greater than $T_{\perp}$ which means that the increased motion of

Figure 3.9

ESR spectra of the 12-stearic acid spin label in an egg phosphatidylcholine film. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) film in the presence of 1 μl. of water, (C) hydrated film.


A
10 G
B
C

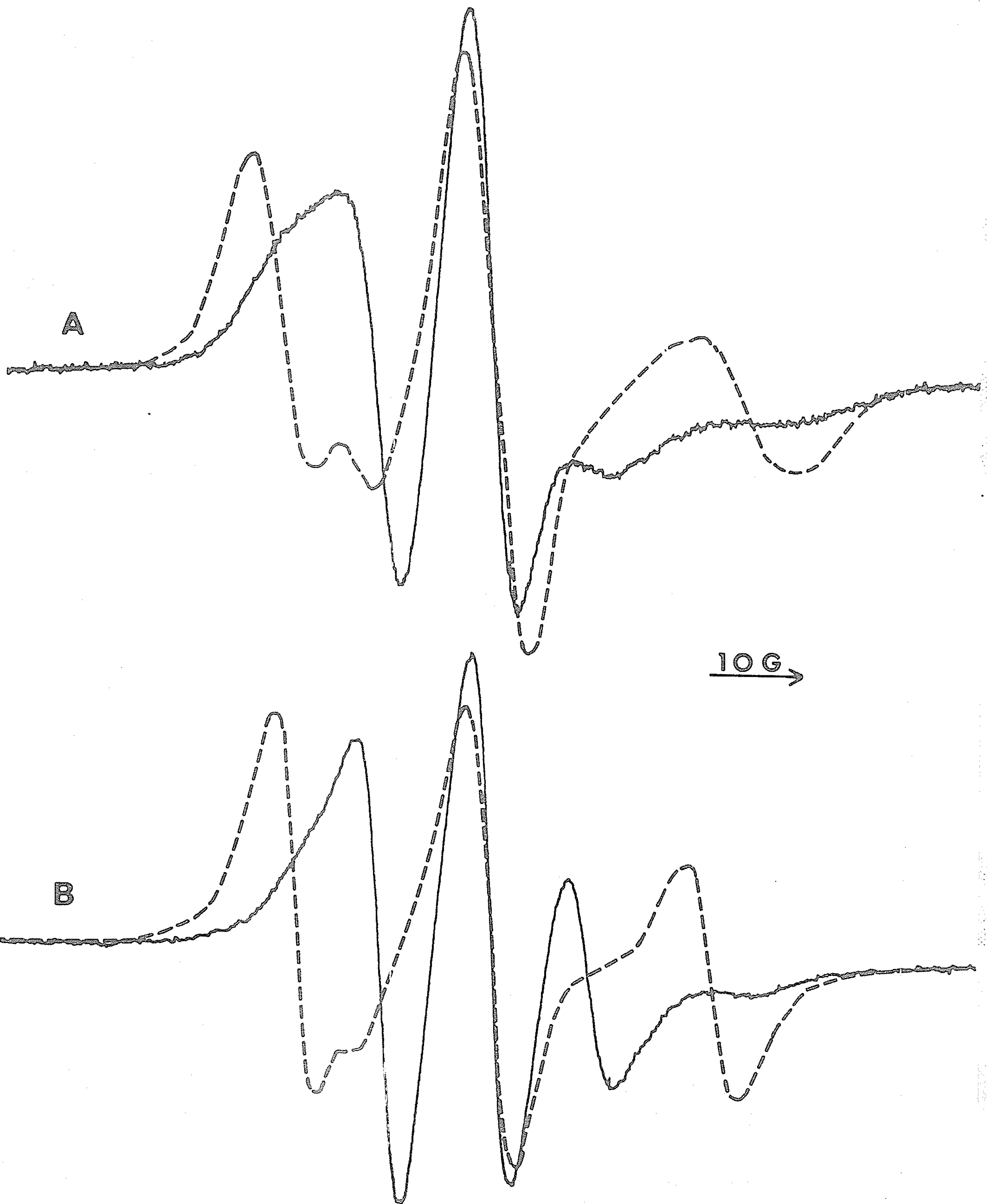
chains still results in anisotropic motion of the spin label about the symmetry axis of the bilayers. Figure 3.9C represents the spectra of the spin label after the film has been exposed to an excess of water. Now the motion of the spin label is essentially isotropic with no preferential alignment of the nitroxide. Therefore, in the presence of excess water, the hydrocarbon chains of egg phosphatidylcholine bilayers are very fluid and are in a state of disorder at least in the vicinity of the nitroxide. The increased molecular motion of the stearic acid spin label with increasing hydration of the phospholipids is consistent with X-ray results[31,36] which have shown that the motion of the phospholipid hydrocarbon chains increases with increasing hydration. Jost *et al*[75] have carried out a systematic study of the motion of this spin label in oriented phosphatidylcholine multilayers at different levels of hydration, and also find that the motion of the label increases with increasing hydration.

In a dry equimolar phosphatidylcholine-cholesterol film, the spectra of the spin label are angular dependent, (Figure 3.10A). The largest hyperfine splitting $T_{\parallel}$ is 23.8 gauss, measured as one half the distance between the low and high field lines. In the parallel orientation, the hyperfine splitting $T_{\perp}$ is estimated to be 10.0 gauss, measured as one half the distance between the mid-points of the low field and center lines. Thus, there is little difference in the alignment of the stearic acid spin label in dry phosphatidylcholine bilayers with or without added cholesterol.

Hydration of the equimolar phosphatidylcholine-cholesterol film causes a sharpening of the resonance spectra, (Figure 3.10B), indicative

Figure 3.10

ESR spectra of the 12-stearic acid spin label in an egg phosphatidylcholine film containing 50 mole % cholesterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.


A
B
10 G

of increased fluidity in the hydrophobic regions of the bilayers. However, the spectra of the spin label remain angular dependant after hydration of the film with $T_{\parallel}$ = 22.0 gauss and $T_{\perp}$ = 10.6 gauss. Figure 3.11 depicts the resonance line positions of the spectra of the stearic acid spin label in hydrated films of phosphatidylcholine, and 50 mole % phosphatidylcholine-cholesterol, with respect to the center line of the Fremys' salt resonance spectrum. The order parameter of the nitroxide, S_3 = 0.02 in hydrated phosphatidylcholine alone, but increases to 0.44 with the addition of 50 mole % cholesterol. The increased anisotropic motion of the spin label upon addition of cholesterol is in agreement with the results of many other physical studies[26,27,31,32,34,36,37,49,77] which indicate that cholesterol reduces the thermal motion of the phospholipid fatty acid chains increasing their alignment perpendicular to the bilayer plane. If the long axis of the stearic acid spin label were perfectly aligned perpendicular to the plane of the bilayers, the order parameter would equal 1. Since S_3 = 0.44, we can conclude that with the presence of cholesterol the hydrocarbon chains possess some degree of fluidity. Figure 3.12 is a plot of the order parameter versus the cholesterol concentration of hydrated phosphatidylcholine multilayers. There is a rapid rise in S_3 up to 25 mole % cholesterol followed by a slower increase up to a constant value at approximately 40 mole % cholesterol. Using the cholestane spin label, Hsia *et al*[34] have found that the maximum ordering effect in egg phosphatidylcholine bilayers occurs at 25 mole % cholesterol. The fact that the maximum ordering effect determined with the stearic acid spin label occurs at a higher sterol concentration, points out the greater sensitivity of

Figure 3.11

Resonance line positions of the spectrum of the 12-stearic acid spin label with respect to the center line of the Fremys' salt spectrum, as a function of the angle between the plane of the film and the direction of the external magnetic field: (-----) hydrated egg phosphatidylcholine film, (———) hydrated equimolar phosphatidylcholine-cholesterol film.

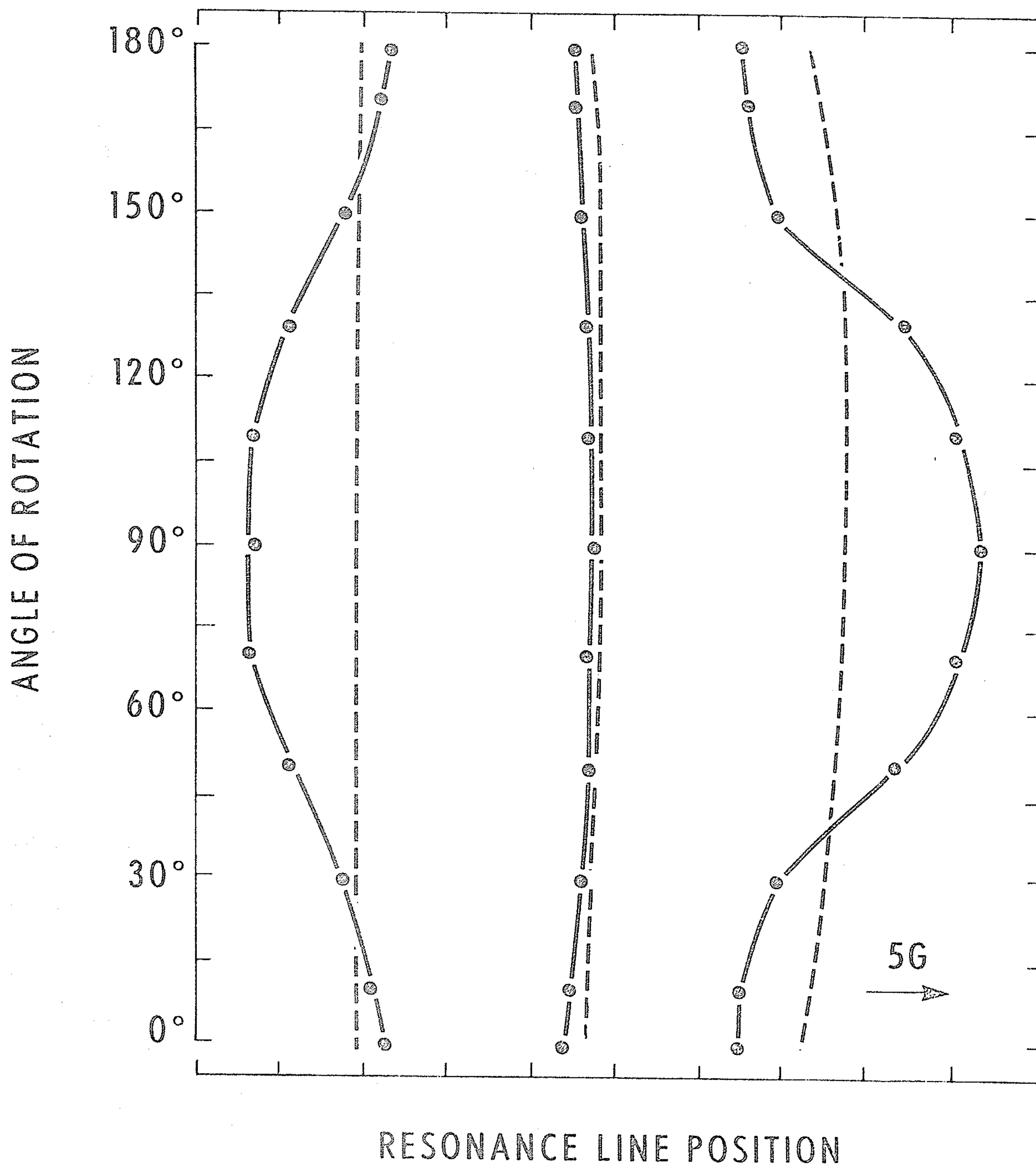
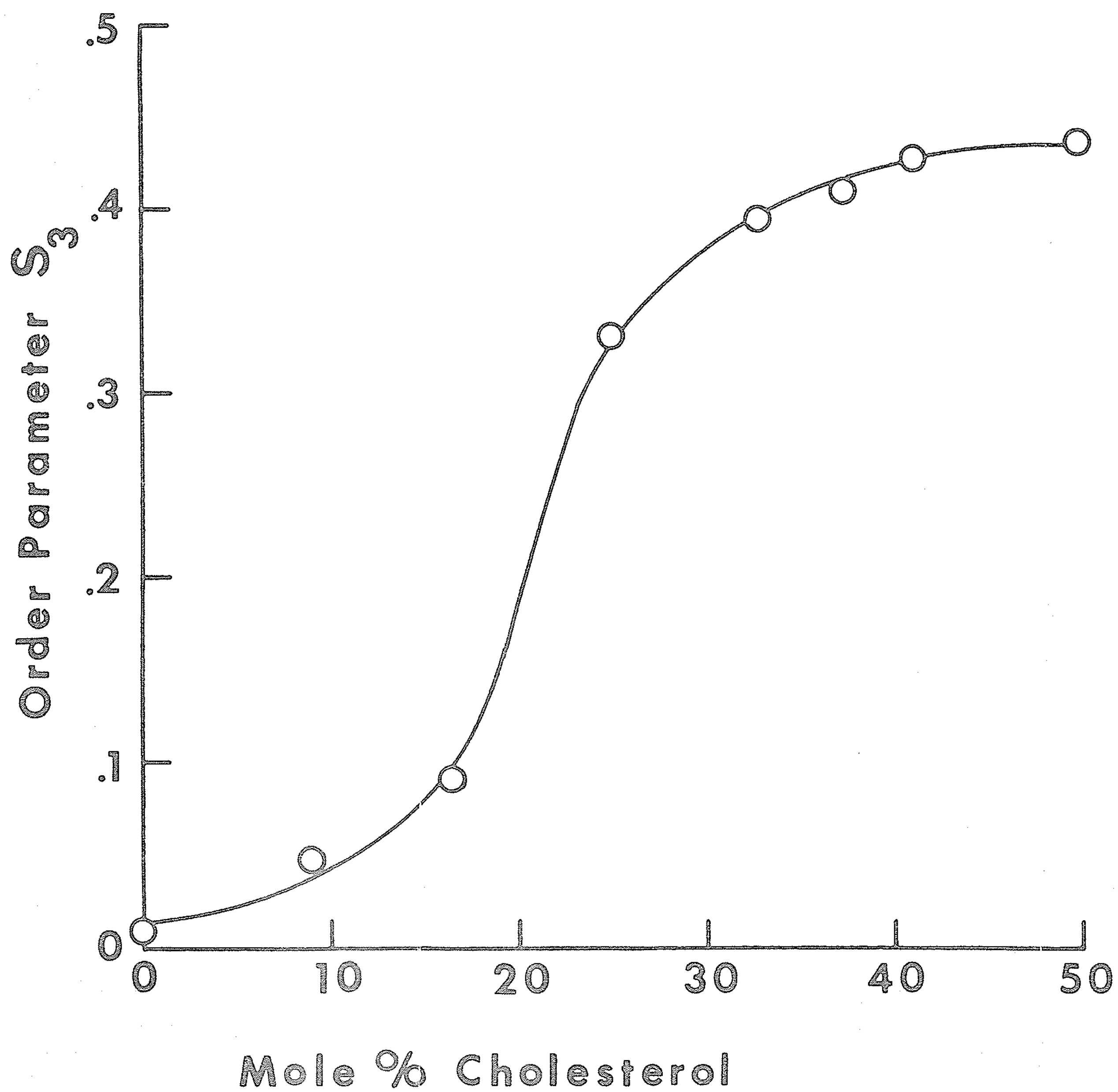

180°
150°
120°
90°
60°
30°
0°
ANGLE OF ROTATION
5G
RESONANCE LINE POSITION

Figure 3.12

The order parameter S_3 of the 12-stearic acid spin label in hydrated egg phosphatidylcholine films as a function of cholesterol concentration.



Order Parameter S_3
.5
.4
.3
.2
.1
0
0
10
20
30
40
50
Mole % Cholesterol

this label to changes in the motion of the phospholipid molecules.

Table 3.2 lists the order parameter of the stearic acid spin label in hydrated egg phosphatidylcholine multilayers containing several structural derivatives of cholesterol.

C_3 Derivatives

The spectra of the stearic acid spin label in a film containing 25 mole % cholesteryl methyl ether are shown in Figure 3.13. The dry state spectra are essentially the same as the spectra of the label in dry phosphatidylcholine multilayers alone indicating that there is no perturbing effect on the film with incorporation of this steroid. In the hydrated state, the calculated order parameter, ($S_3 = 0.01$), is indicative of almost isotropic motion of the spin label. Compared with the order parameter of the label in a hydrated film containing an equal amount of cholesterol, ($S_3 = 0.33$), one can conclude that the presence of the hydroxyl group is necessary for reducing the motion of the phospholipid chains.

From the cholestane spin label results, it is known that cholesteryl methyl ether is not soluble in egg phosphatidylcholine above a concentration of 25 mole %. Figure 3.14 represents the spectra of the stearic acid label in a 50 mole % cholesteryl methyl ether-phospholipid film. In the dry film, the spectra of the label are angular independant but there is no indication from the spectra of the existance of a separate polycrystalline steroid phase. The fact that there is no longer preferential alignment of the label in the film points out the disruptive effects of the separate steroid phase on the orientation of the multibilayers, but the fatty acid character of the spin label

Table 3.2

Order Parameter of the 12-Stearic Acid Spin Label in Hydrated Phosphatidylcholine Films

Steroid	Mole %	$T_{//}$	$T_{\perp}$	T_{zz}	T_{xx}	S_3
cholesterol	0	14.0	13.5	29.7	5.6	0.02
	25	20.2	11.7	31.6	5.9	0.33
	50	22.0	10.6	31.3	5.6	0.44
cholesteryl methyl ether	25	14.4	14.0	30.8	5.6	0.01
	50	14.0	13.4	29.6	5.5	0.02
cholesteryl chloride	50	14.0	13.7	30.0	5.6	0.01
epicholesterol	50	14.2	13.3	29.6	5.5	0.03
thiocholesterol	9	14.5	13.3	29.8	5.6	0.04
5α-cholestane-3β-ol	50	21.7	10.6	31.1	5.8	0.43
Δ^7-cholesten-3β-ol	50	22.3	11.6	32.4	6.1	0.42
7-dehydrocholesterol	50	22.6	10.7	31.9	6.0	0.45
ergosterol	50	14.0	13.1	29.2	5.4	0.03
β-sitosterol	50	15.1	12.9	29.7	5.5	0.09
5α-androstane-3β-ol	50	13.7	13.5	29.5	5.5	0.01
5α-androstane-3β-ol-17-one	50	13.8	13.2	29.2	5.4	0.02

Figure 3.13

ESR spectra of the 12-stearic acid spin label in an egg phosphatidylcholine film containing 25 mole % cholesteryl methyl ether. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.

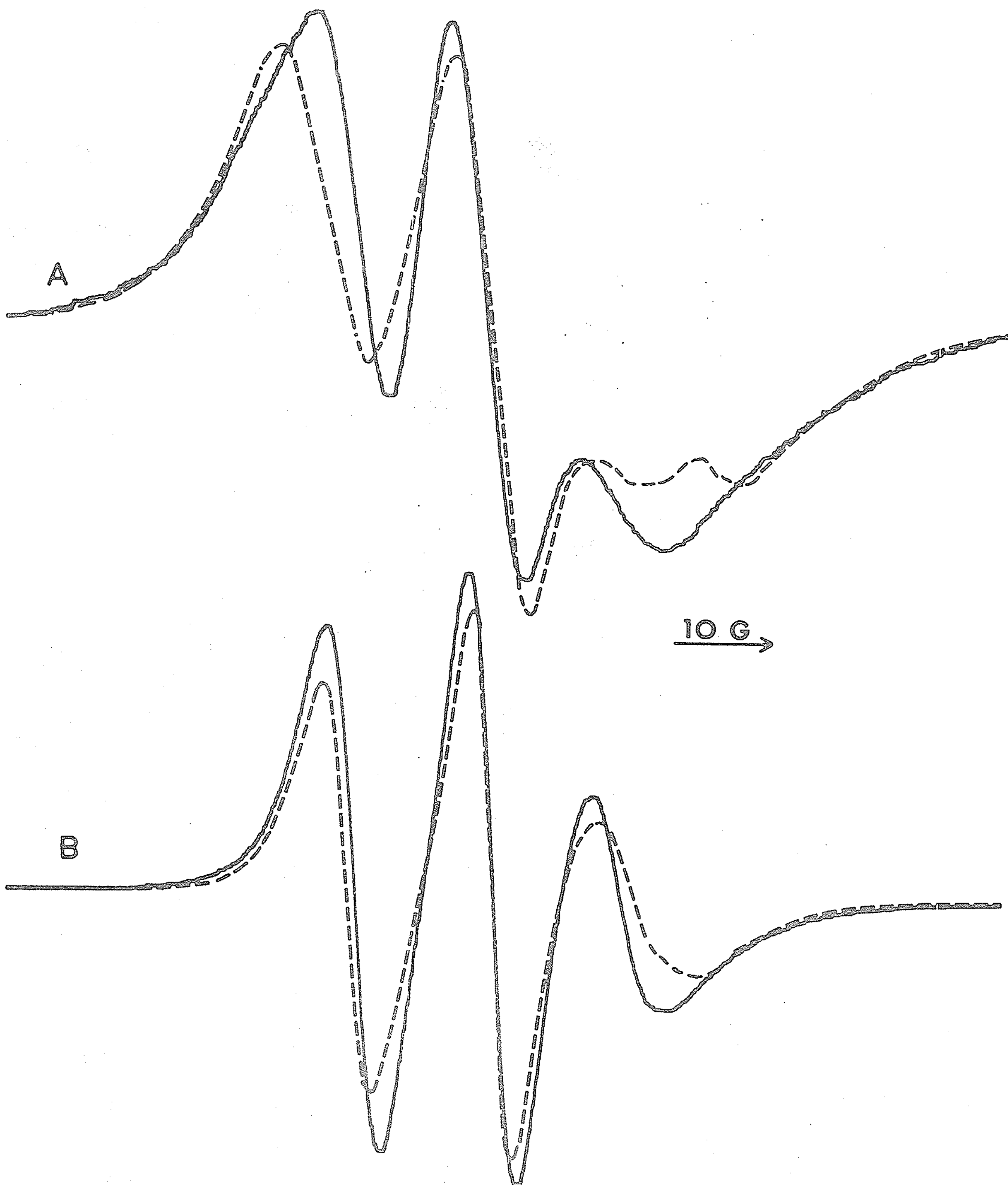
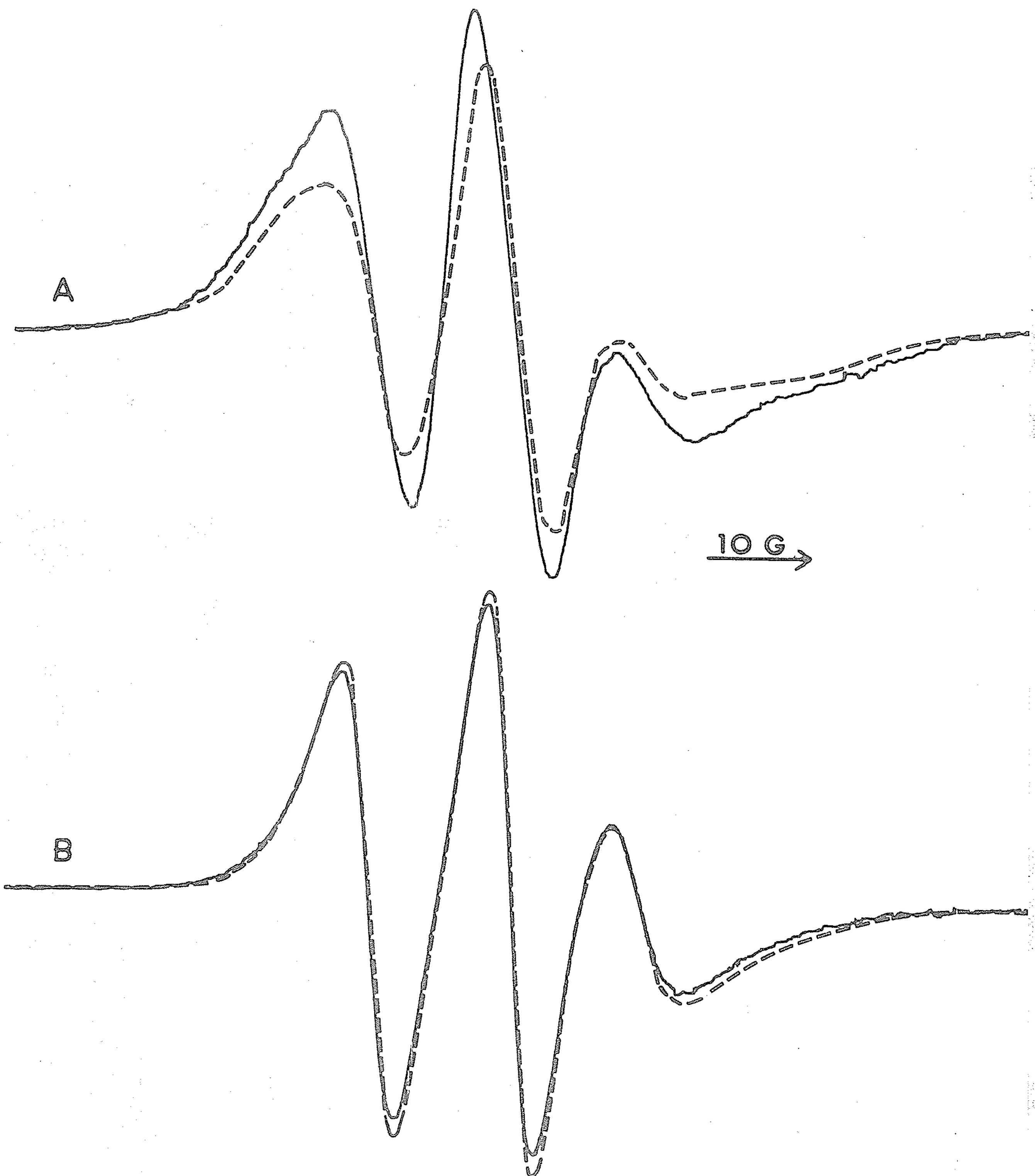

A
B
10 G

Figure 3.14

ESR spectra of the 12-stearic acid spin label in an egg phosphatidylcholine film containing 50 mole % cholesteryl methyl ether. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.


A
10 G
B

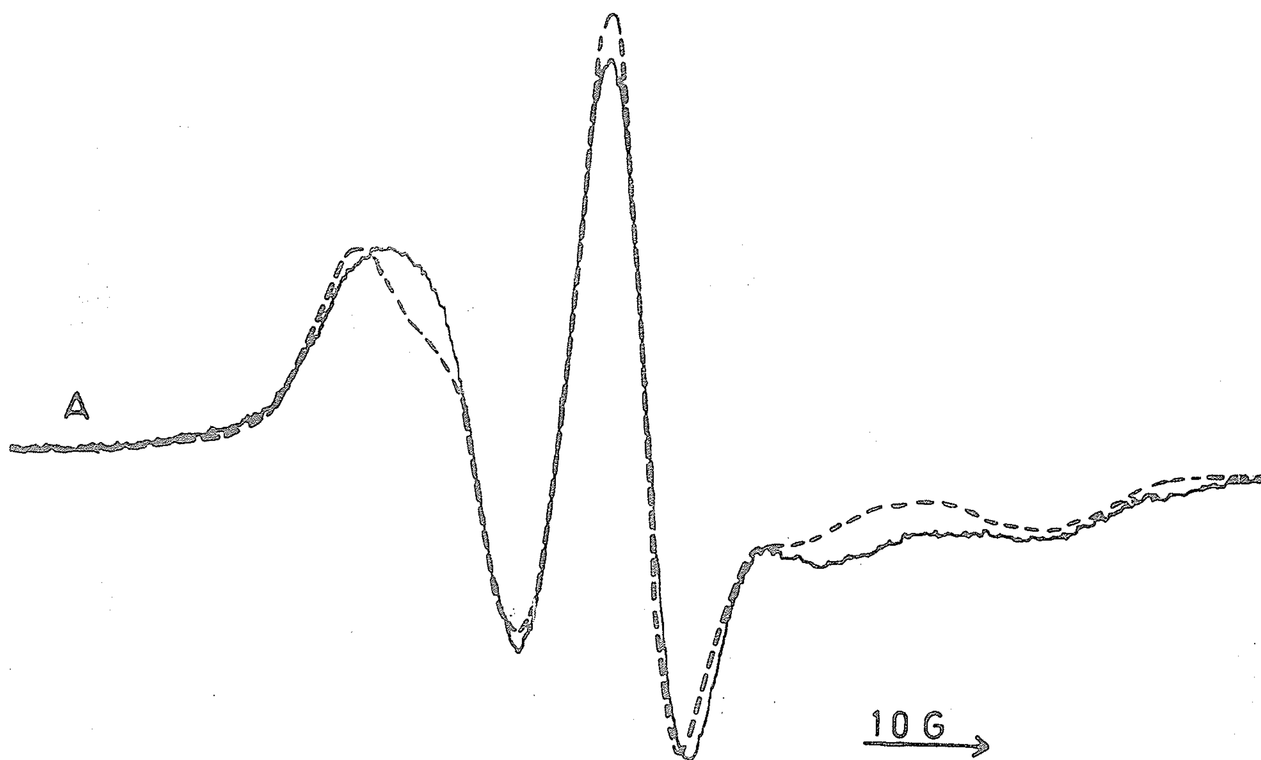
appears to preclude its incorporation into the separate steroid phase in the presence of phospholipid. In the hydrated state, the spin label remains associated with the lamellar phase and the motion of the nitroxide is almost isotropic, (S_3 = 0.02). The same results were obtained when cholesteryl chloride was substituted for the methoxy derivative.

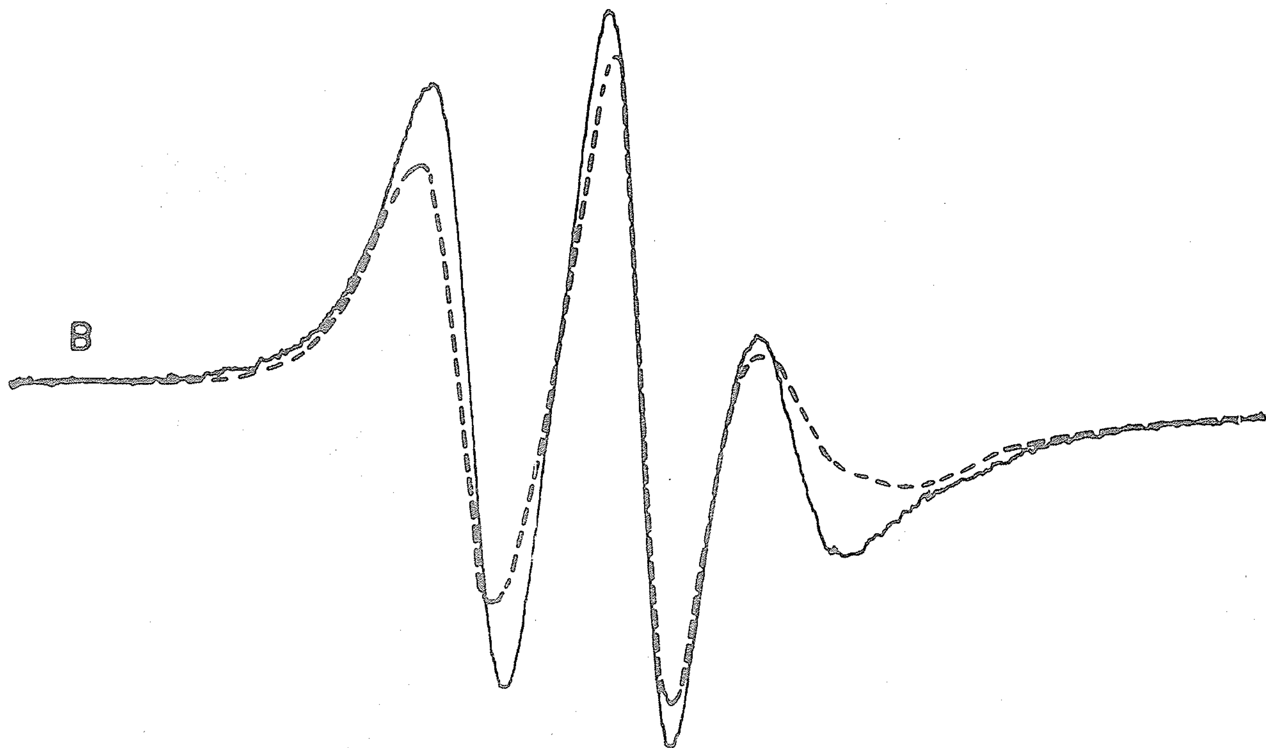
Spectra of the label in films containing 9 mole % thiocholesterol are similar to those obtained in films containing 25 mole % cholesteryl methyl ether. Since 9 mole % is the maximum solubility limit of thio-cholesterol in egg phosphatidylcholine,[91] it is difficult to evaluate the ordering effect of this steroid. It is interesting to note, that unlike the cholestane spin label, the nitroxide of the stearic acid label is not destroyed by the incorporation of thiocholesterol into the lipid film presumably due to location of the oxazolidine rings in the interior of the bilayers.

The spectra of the label in dry films containing 50 mole % epi-cholesterol, (Figure 3.15A), are angular independant demonstrating the inability of the phospholipids to form oriented multilayers containing this steroid. These results are in good agreement with the cholestane spin label spectra which also indicated disruption of the dry multibi-layers containing this steroid. Upon hydration of this film, the order parameter of the label is not much greater than that in pure phospha-tidylcholine. Thus, to be effective in ordering the paraffin chains, the 3-OH group of the steroid must be in the β configuration, pointing to the existance of stereospecific interactions between the cholesterol molecule and the phospholipids.

Figure 3.15

ESR spectra of the 12-stearic acid spin label in an egg phosphatidylcholine film containing 50 mole % epicholesterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.


A
10 G


B

B-Ring Derivatives

The spectra of the stearic acid spin label in dry and hydrated phosphatidylcholine films containing either 50 mole % 5α-cholestane-3β-ol, Δ^7-cholesten-3β-ol or 7-dehydrocholesterol are essentially the same as the spectra of the label in films containing 50 mole % cholesterol. The order parameters of the lipid chains in the presence of these three sterols are equal to the order parameter induced by cholesterol. Therefore, the presence or position of double bonds in the B-ring of the steroid nucleus appear to be unimportant for the membrane condensing effect.

C_{17} Derivatives

The dry state spectra of the label in films containing 50 mole % ergosterol or β-sitosterol, (Figure 3.16A and Figure 3.17A), indicate that these two sterols do not have any perturbing effect on the alignment of the stearic acid molecule in the dry multilayers. From the very small values of S_3 in hydrated films containing these two sterols, one may conclude that these molecules are very ineffective in ordering the phospholipid chains. This would indicate that the structural differences in the C_{17} side chains of these sterols make them incompatible for maximum hydrophobic interactions with the phosphatidylcholine chains. The low S_3 value for 50 mole % ergosterol-phosphatidylcholine films may in part be due to the disordering effect of this sterol in the presence of water, noted with the cholestane spin label.

The spectra of the spin label in dry films containing 50 mole % 5α-androstane-3β-ol, (Figure 3.18),and 50 mole % 5α-androstane-3β-ol-17-one,

Figure 3.16

ESR spectra of the 12-stearic acid spin label in an egg phosphatidylcholine film containing 50 mole % β-sitosterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.

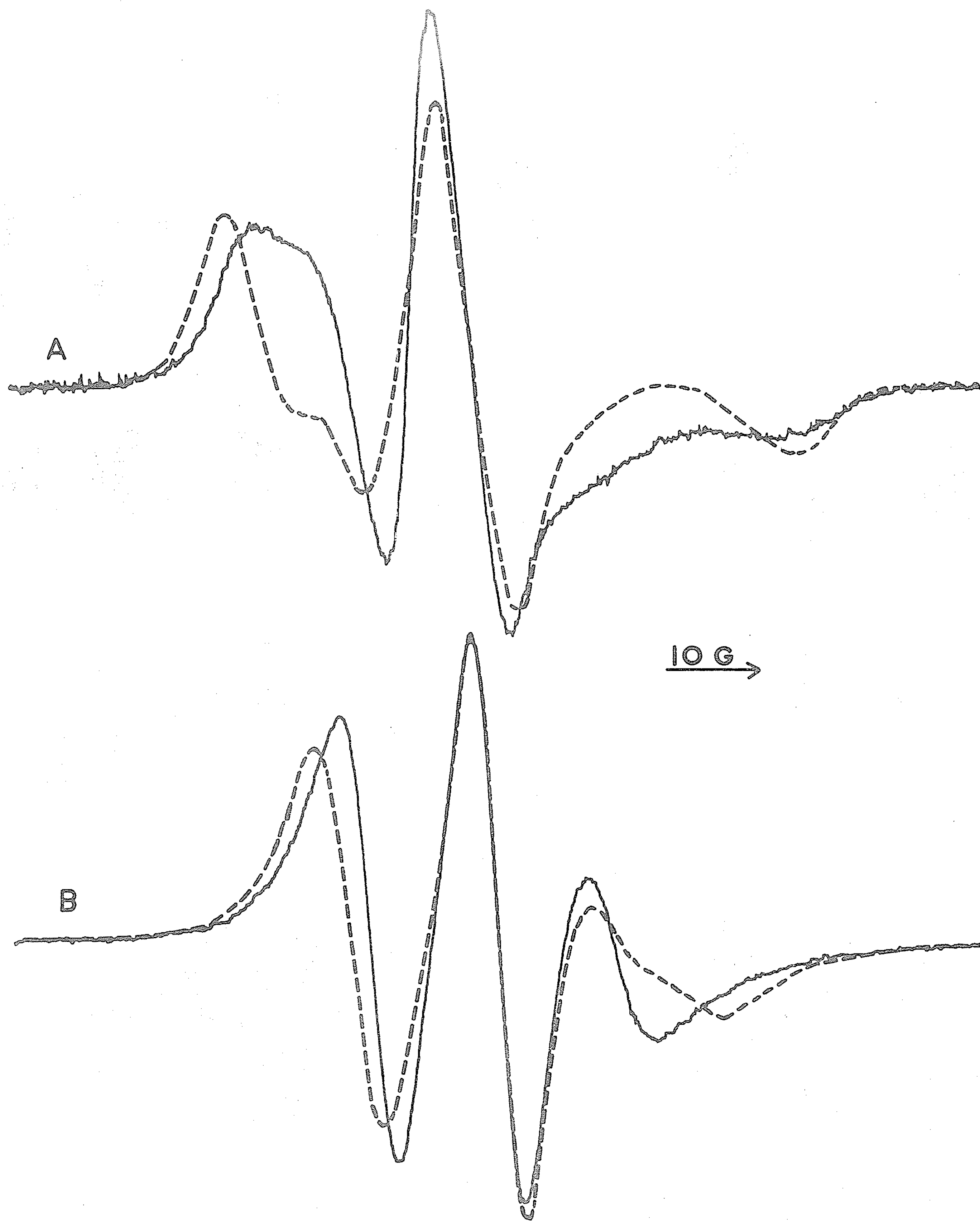
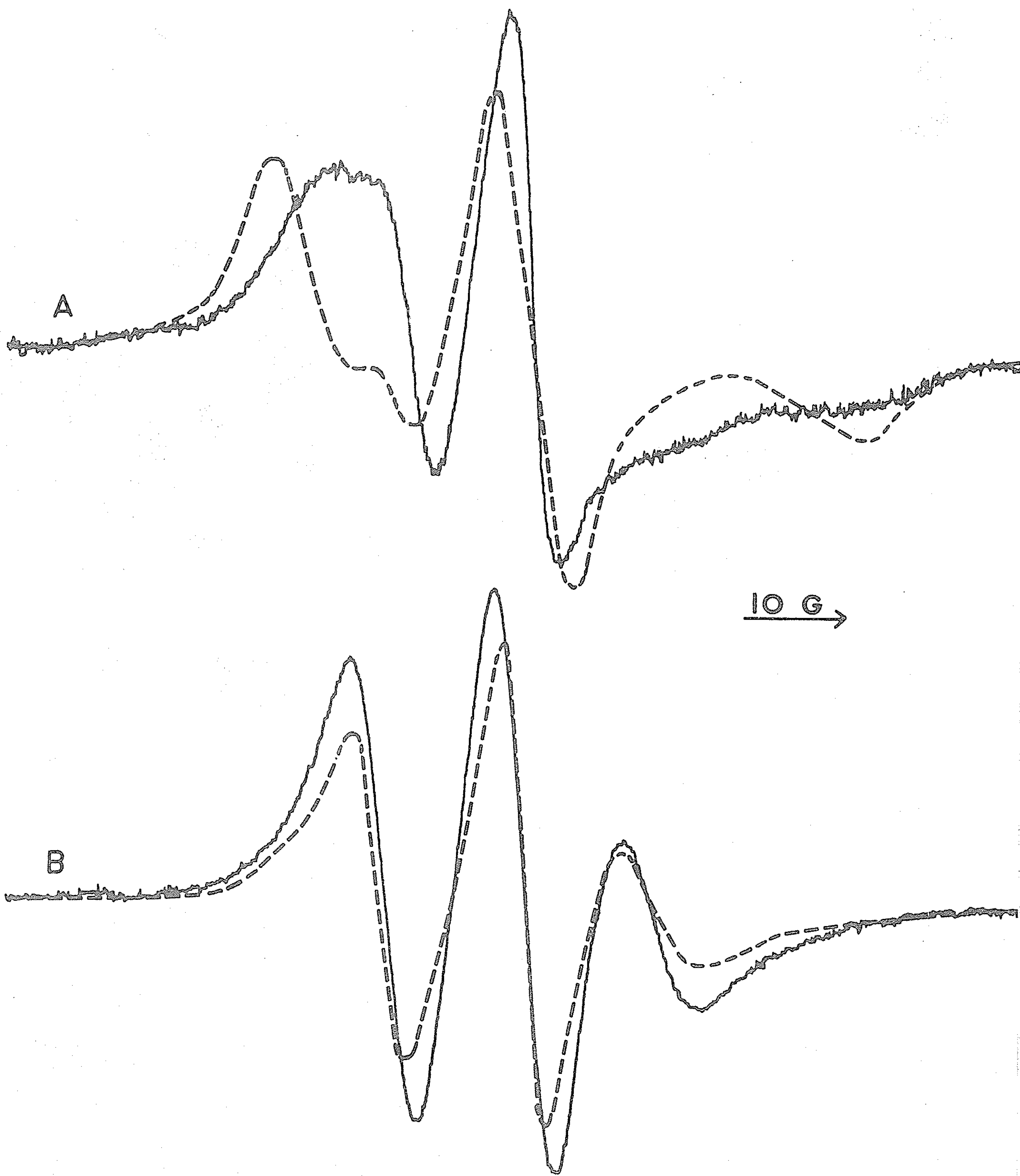

A
10 G
B

Figure 3.17

ESR spectra of the 12-stearic acid spin label in an egg phosphatidylcholine film containing 50 mole % ergosterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.


A
B
10 G

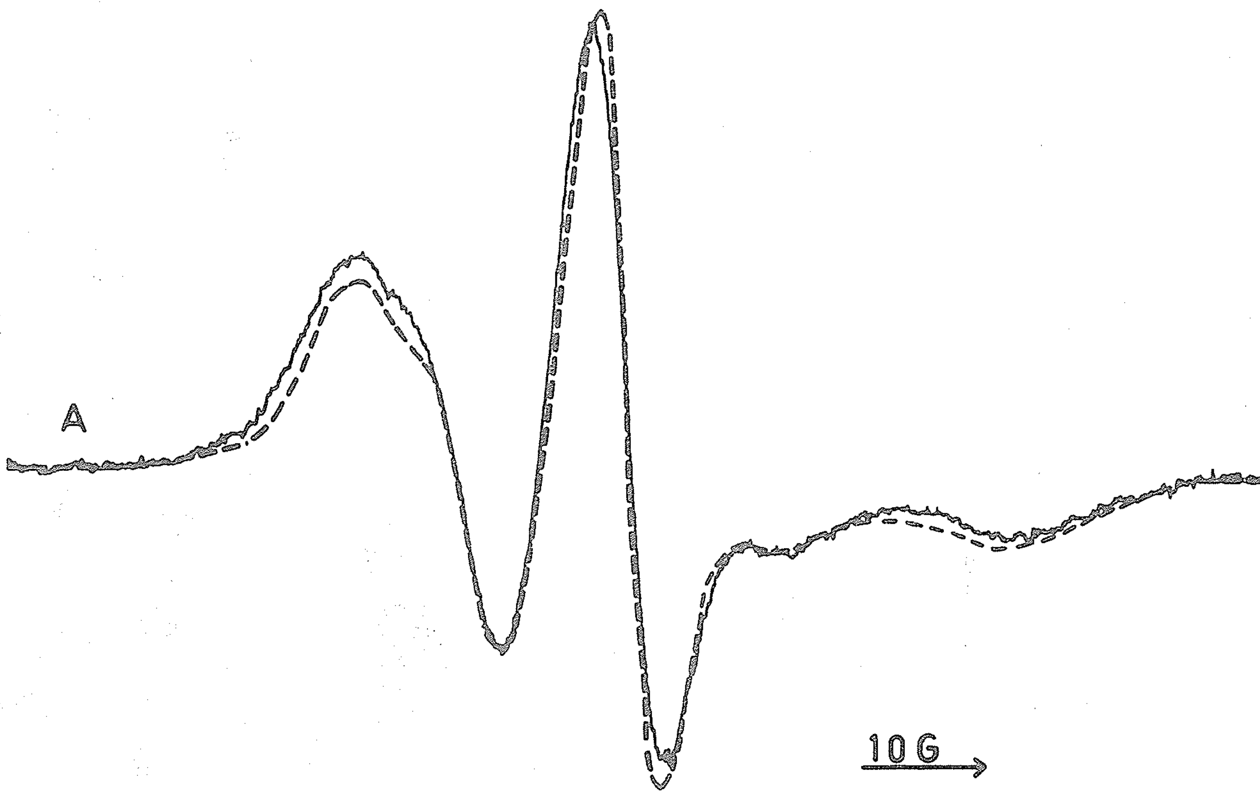
(Figure 3.19), are angular independent, and demonstrate the disruptive effects that a steroid lacking a hydrocarbon side chain has on dry multilayer structure. Therefore, hydrophobic interactions between the steroid and phospholipid involving the C_{17} side chain appear necessary to maintain the structural integrity of dry oriented multilayer films.

From the S_3 values of hydrated films containing either 50 mole % 5α-androstane-3β-ol or 5α-androstane-3β-ol-17-one, it can be seen that these two steroids are almost totally ineffective in ordering the phospholipid chains in the interior of the hydrated bilayers. Therefore, the C_{17} side chain of cholesterol plays a crucial role in the ordering effects. The local disordering effects of these two sterols, detected by the cholestane spin label are not evident from the stearic acid spin label spectra since the motion of the label, even in the unperturbed areas of the multilayers, is nearly isotropic.

The increased anisotropic motion of the stearic acid spin label in hydrated phospholipid films with addition of cholesterol, is further verification of other studies, namely that cholesterol increased the orientation of bilayer phospholipids. It has been assumed that any changes in the motional characteristics of a spin label in phospholipid multibilayers, upon intercalation of a steroid, are primarily due to specific phospholipid-steroid interactions. However, the possibility exists that perturbing effects on the motion of the spin label are due to steroid-spin label interactions. Since the cholestane and stearic acid labels have different chemical structures, it is unlikely that both spin labels would be involved in similar steroid-spin label inter-

Figure 3.18

ESR spectra of the 12-stearic acid spin label in an egg phosphatidylcholine film containing 50 mole % 5α-androstane-3β-ol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.


A
10 G

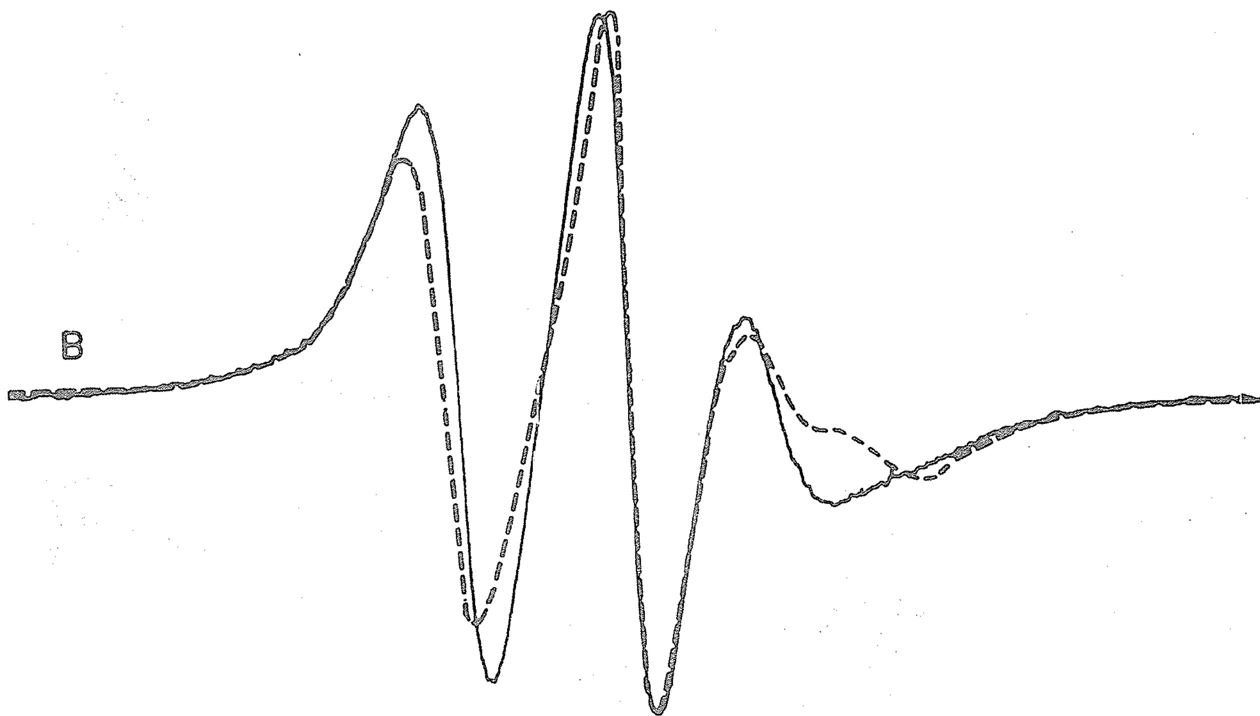
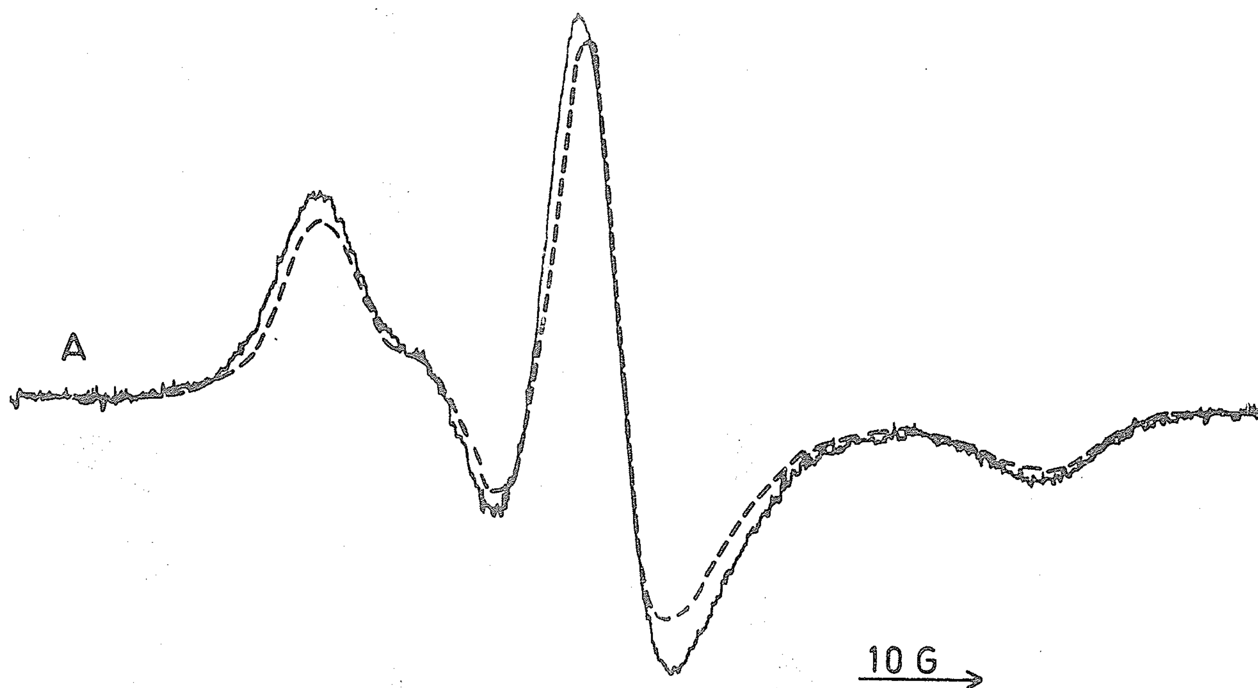
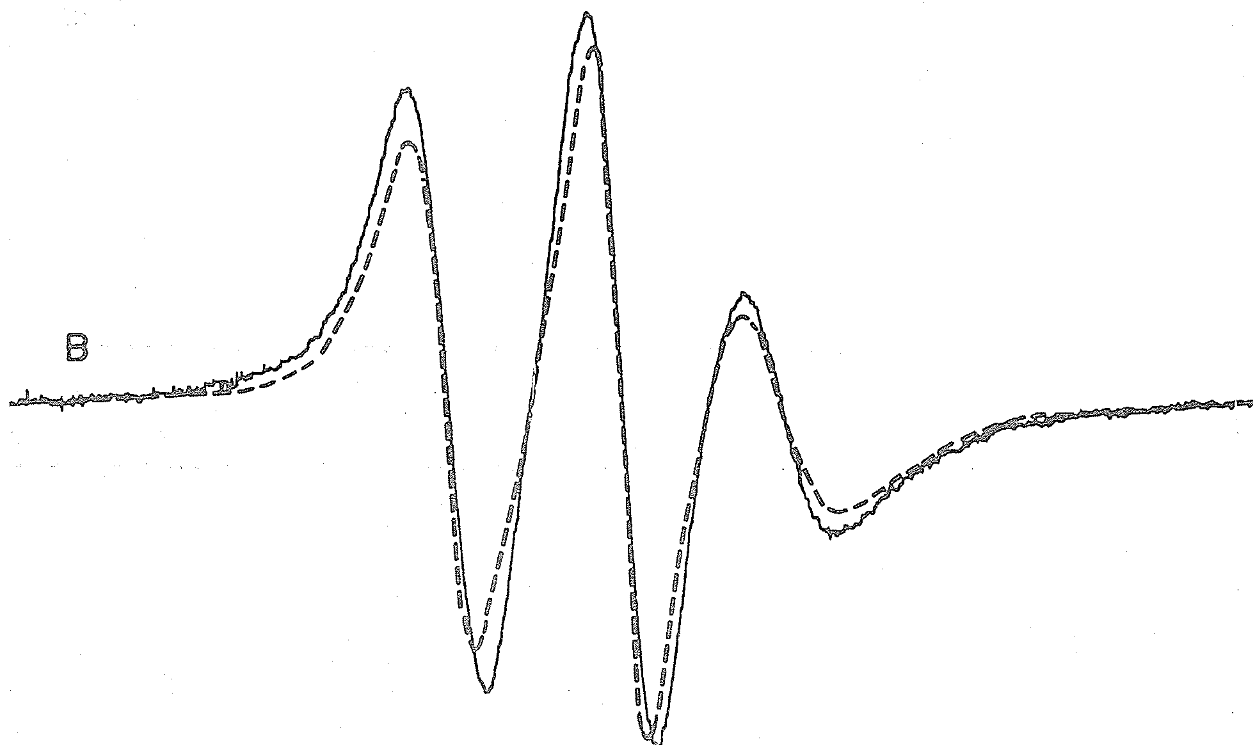

B

Figure 3.19

ESR spectra of the 12-stearic acid spin label in an egg phosphatidylcholine film containing 50 mole % 5α-androstane-3β-ol-17-one. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.


A
10 G


B

actions. Clearly, the self-consistency of the ordering effects reported by both labels in Table 3.1 and Table 3.2 indicate that the motional freedom of these labels depend mainly on phospholipid-steroid interactions. A greater sensitivity of the stearic acid spin label to hydrophobic interactions between the C_{17} side chain of cholesterol and the phosphatidylcholine apolar chains has indicated that the sterol's fully saturated eight carbon chain is absolutely necessary for increasing the orientation of the phospholipid fatty acid chains.

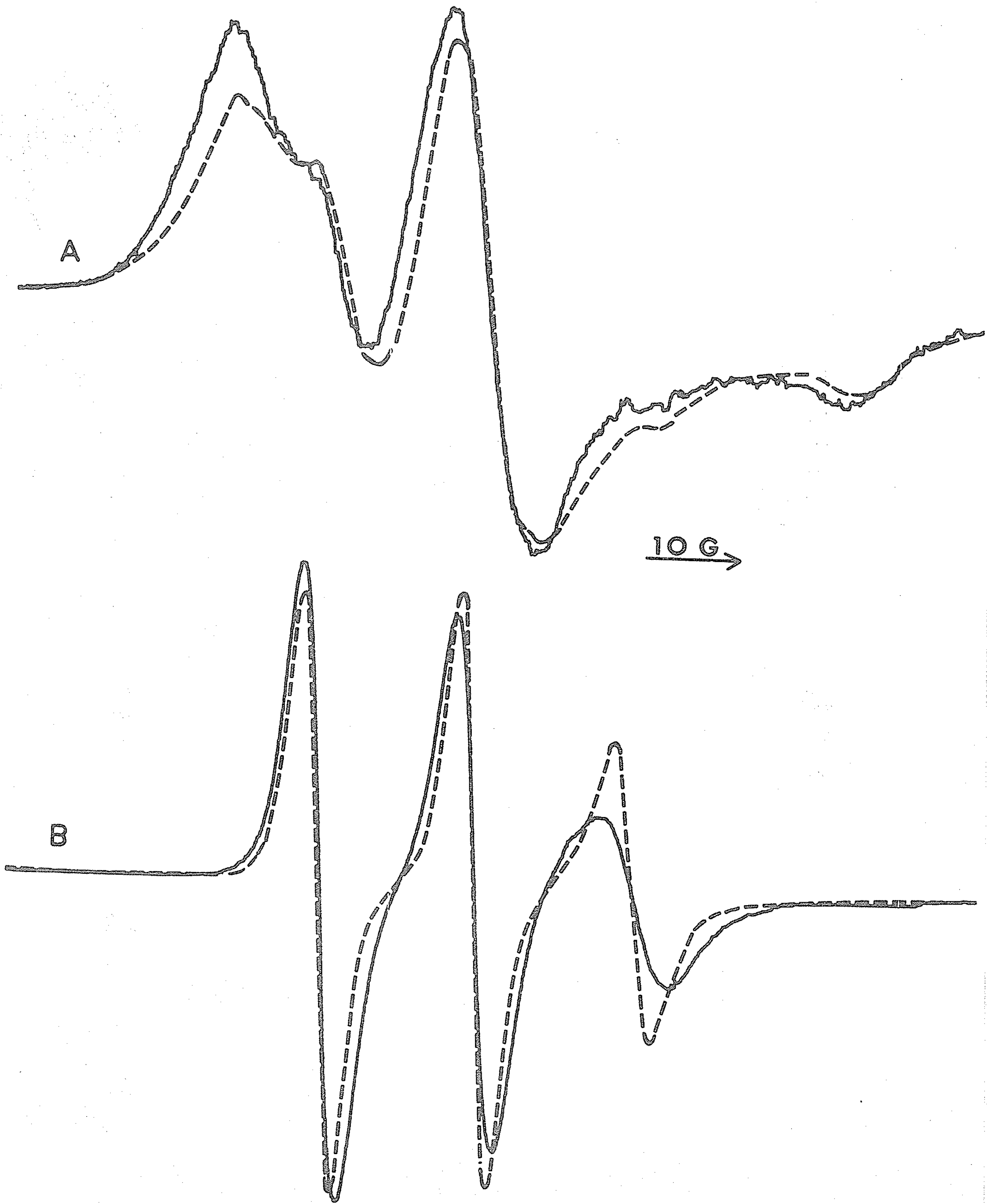
The same mechanism of the formation of the cholesterol-phosphatidylcholine complex can be proposed from the above results as was suggested previously, involving polar and apolar forces. The cholesterol molecule interacts with the phospholipid head group through a stereospecific hydrogen bond involving the 3β-OH group and an oxygen atom of the head group. The C_{17} hydrocarbon side chain interacts with the phospholipid paraffin chains through attractive London dispersion forces. The linear steroid nucleus provides rigidity to the complex thus reducing the thermal motion of the phospholipids and increasing their orientation.

(c) Stearamide Spin Label Results

The spectra of the stearamide spin label in an egg phosphatidylcholine film are shown in Figure 3.20. In the dry film, the spectra are identical for both the parallel and perpendicular orientations, ($T_{\parallel} = T_{\perp} = 33.3$ gauss). Therefore, there is no preferential alignment of the nitroxide in the dry lamellae. The spectra have the characteristic

Figure 3.20

ESR spectra of the stearamide spin label in an egg phosphatidylcholine film. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.


A
10 G
B

shape of strongly immobilized nitroxides indicating the piperidinyl rings are in a region of high viscosity in the dry multilayers.

Upon hydration of the dry film, there is a dramatic increase in motion of the nitroxides indicated by the spectra of Figure 3.20B. The spectral parameters are summarized in Table 3.3. The average hyperfine splitting constant for both the parallel and perpendicular orientations is the same, ($T_{//} = T_{\perp} = 16.6$ gauss). Therefore, the nitroxide

Table 3.3

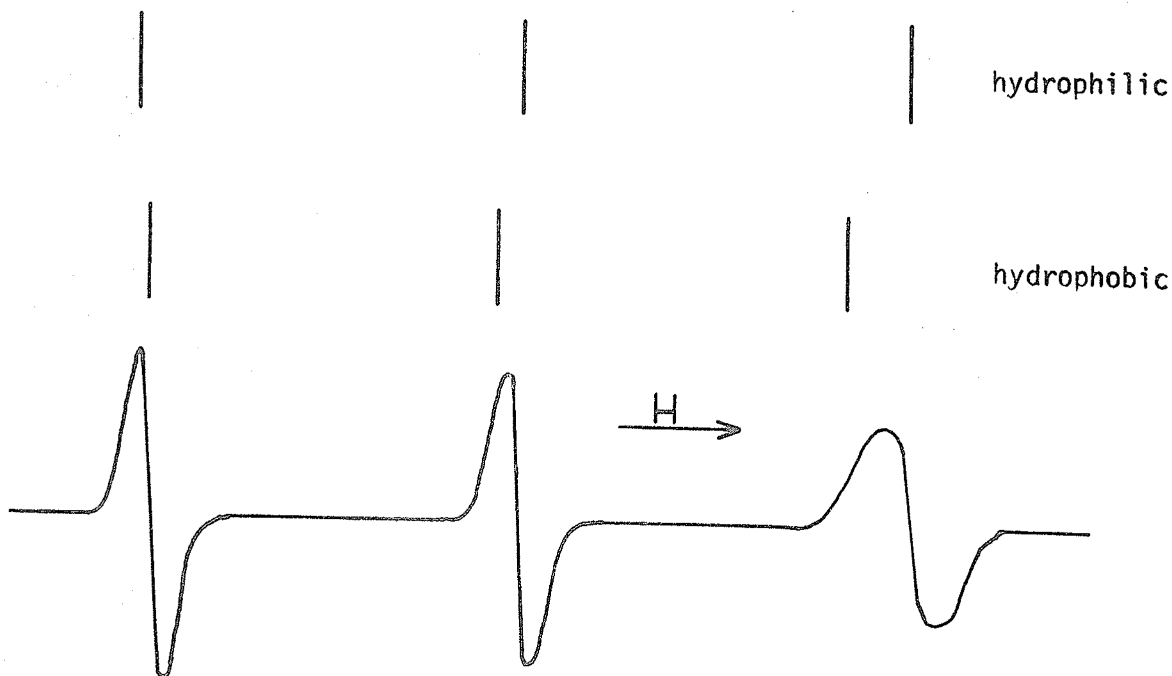
Spectral Parameters of the Stearamide Spin Label In Hydrated Phosphatidylcholine Films

Mole % Cholesterol	$T_{\perp}$	ΔH_{M+1}	$\Delta H_{M\,0}$	ΔH_{M-1}	$T_{//}$	ΔH_{M+1}	$\Delta H_{M\,0}$	ΔH_{M-1}	S_3
0	16.6	4.5	5.2	8.3	16.6	3.8	3.5	4.5	0.0
50	18.4	5.4	5.2	10.7	14.7	4.5	4.7	5.4	-0.12

groups are undergoing rapid isotropic motion in the hydrated multilayers, ($S_3 = 0.0$). The increase in the motion of the labels with hydration of the film is consistent with an increase in fluidity of the phospholipids.

Table 3.3 lists the peak-to-peak line widths, (ΔH), of the three lines of the nitroxide spectrum in the hydrated multilayers. It is evident from this table that the relative line width ratio of the spectrum is asymmetric In the parallel orientation ($\phi = 0°$), $\Delta H_{M-1} > \Delta H_{M\,0} > \Delta H_{M+1}$ while in the perpendicular orientation $\Delta H_{M-1} > \Delta H_{M+1} > \Delta H_{M\,0}$. The relative line width ratio of the spectrum of nitroxides undergoing isotropic tumbling in a viscous environment of uniform polarity is always

$\Delta H_{M-1} > \Delta H_{M+1} > \Delta H_{M\,0}$. It is well known that the isotropic g and hyperfine splitting values are sensitive to the polarity of a nitroxide's environment. As the polarity of the medium increases, the isotropic hyperfine splitting value increases and g_o decreases. The hydrated phospholipid multilayers have regions of hydrophobic and hydrophilic character. Therefore, a distribution of nitroxides in regions of different polarity will result in two slightly different resonance spectra. The hyperfine splitting of the spectrum of nitroxides in the hydrophilic environment will be slightly greater, and the g_o value slightly smaller, than the corresponding spectrum of the hydrophobic nitroxides. A simple stick diagram representing the two spectra resulting from the two spin label environments is shown below.



Resultant spectrum for nitroxides having correlation times of the order of 10^{-9} seconds in environments of different polarity.

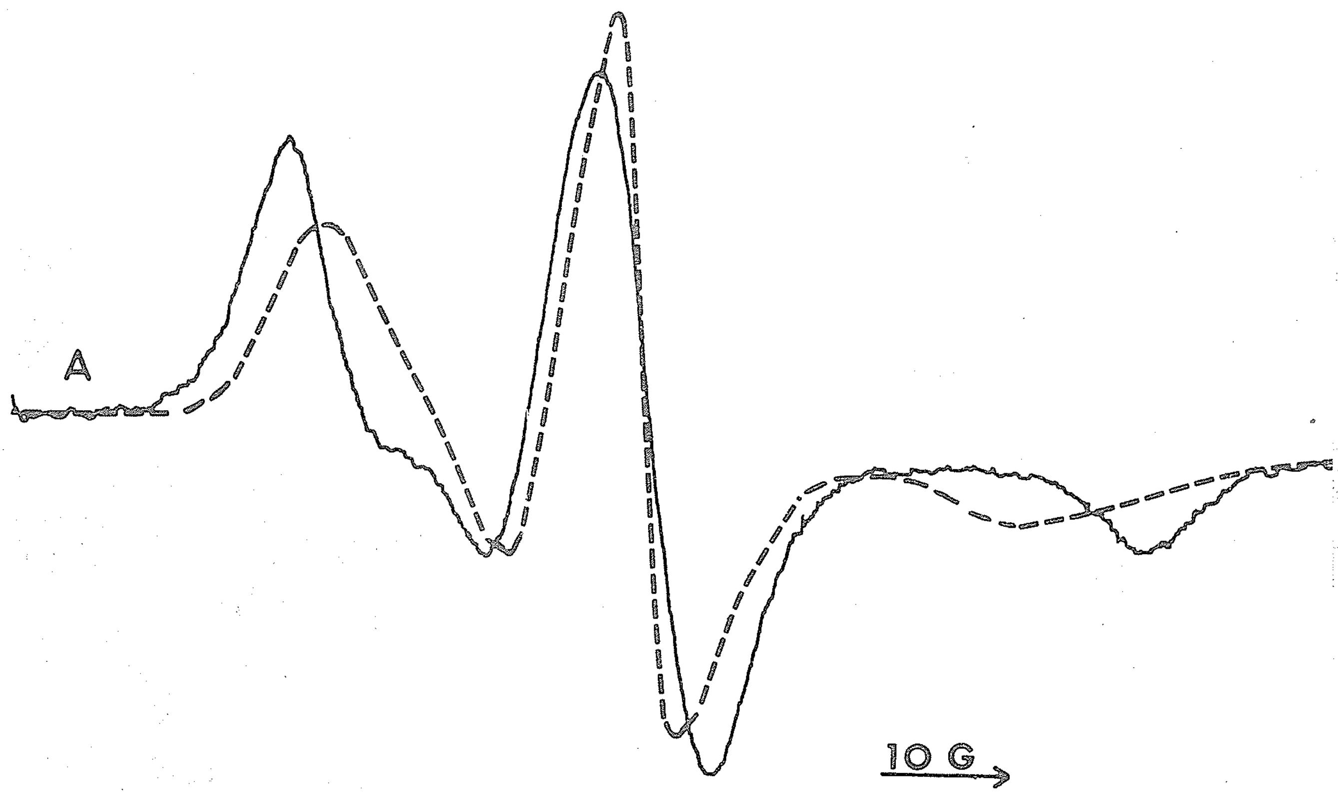
With a fixed microwave frequency, the larger the g_o value, the smaller the magnetic field strength needed for the resonance condition to be met. Therefore, the center point of the hydrophobic label spectrum is displaced to the low field side of the center of the hydrophilic label spectrum. It can be seen from the stick diagram that for the resultant spectrum made up of both the hydrophilic and hydrophobic spectra, the high field line will be broadened the most, then the center line and finally the low field line. Consequently, a spectrum of a system in which there are spin labels in two environments of different polarity will have the line width ratio $\Delta H_{M-1} > \Delta H_{M\,0} > \Delta H_{M+1}$.

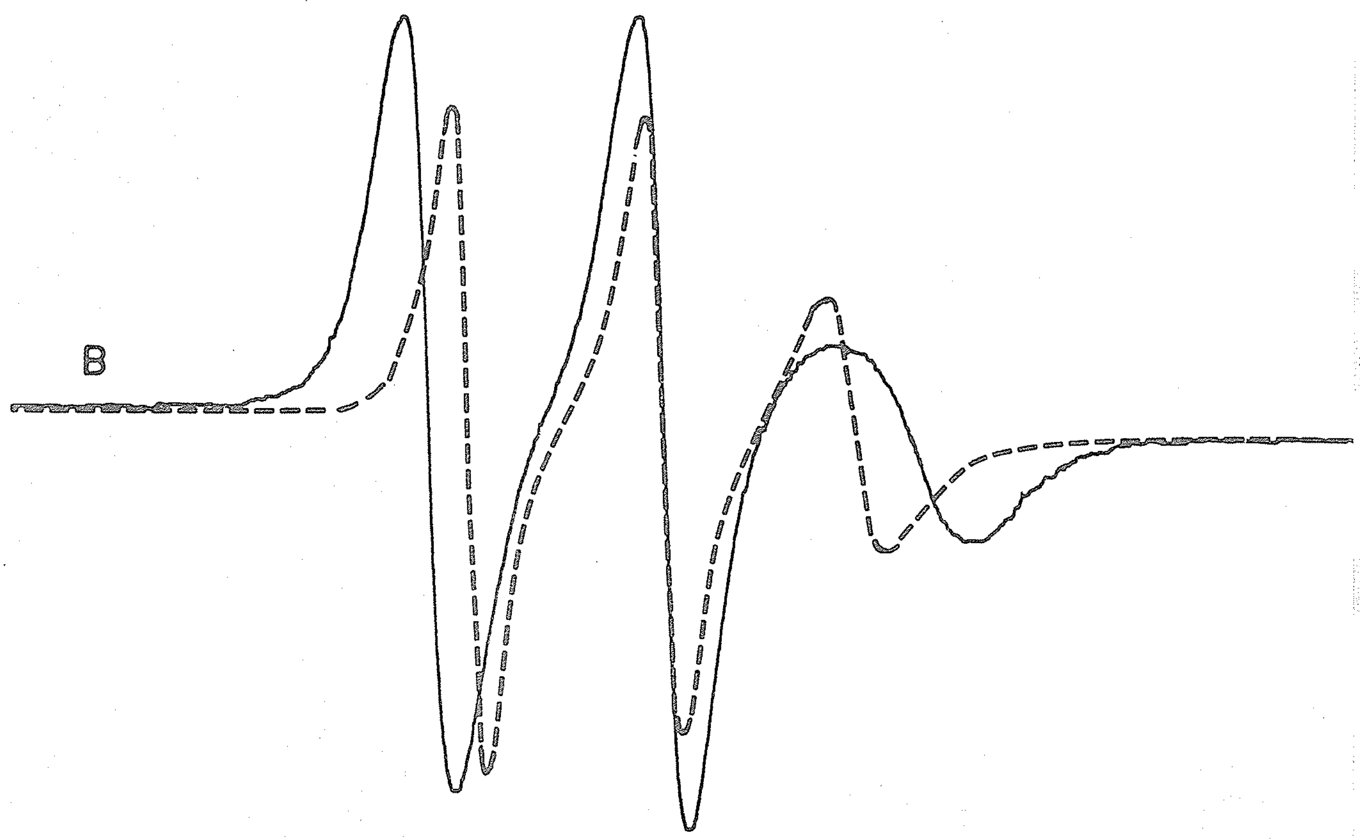
From the spectra of Figure 3.20B, we can conclude that piperidinyl rings of the stearamide spin labels are in two regions of different polarity in the hydrated phosphatidylcholine bilayers. It is not apparent at the present time why the line width ratio of the spectrum is dependent on the angle between the magnetic field direction and the plane of the phospholipid multilayers.

The spectra of the stearamide spin label in a film containing 50 mole % cholesterol are shown in Figure 3.21. In the dry state, the mobility of the nitroxides are highly restricted but there appears to be some improvement in the alignment of the labels compared to a film containing no cholesterol, since the spectra are orientation dependent with $T_{\parallel} = 26.6$ gauss and $T_{\perp} = 33.3$ gauss. Hydration of the film allows for a rapid increase in the motion of the spin label but the hyperfine splittings of the spectra remain angular dependent. Therefore, the presence of cholesterol results in an increase in anisotropic motion

Figure 3.21

ESR spectra of the stearamide spin label in an egg phosphatidylcholine film containing 50 mole % cholesterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, (B) hydrated film.


A
10 G


B

of the label. Since $T_{\perp} > T_{\parallel}$, we can conclude that there is orientation of the nitroxide with the direction of the N-O bond preferentially aligned parallel to the symmetry axis of the bilayers, (S_3 = -0.12). The anisotropic motion of the piperidinyl ring in the cholesterol-containing film is undoubtedly a consequence of a reduction in the thermal motion of the phospholipids by the sterol. The relative line width ratios of the spectrum were angular dependent in the cholesterol containing films as in the pure phospholipid multilayers but the angular dependence was reversed. Thus, for $\phi = 90^\circ$, $\Delta H_{M-1} > \Delta H_{M\ 0} > \Delta H_{M+1}$, and for $\phi = 0^\circ$, $\Delta H_{M-1} > \Delta H_{M+1} > \Delta H_{M\ 0}$. Therefore, in the hydrated bilayers containing 50 mole % cholesterol, the spin labels still experience two environments of different polarity.

It is interesting to compare these results with the study by Hsia *et al*,[78] of cholesterol effects in egg phosphatidylcholine liposomes using the same spin label. A liposome model system does not have a unique symmetry axis and therefore, there is an isotropic distribution of spin labels in all possible orientations. The relative line width ratio of the spectrum of the label in liposomes containing no cholesterol is the same as that of the spectrum of the label in the oriented multilayers in the parallel orientation, with $\Delta H_{M-1} > \Delta H_{M\ 0} > \Delta H_{M+1}$. The hyperfine splitting of the label in oriented bilayers is approximately one gauss larger than the splitting obtained in liposomes. The relative line width ratio of the spectrum of the stearamide spin label in liposomes containing 20 mole % cholesterol is the same as that of the spectrum of the label in oriented multilayers in the parallel orientation, with $\Delta H_{M-1} > \Delta H_{M+1} > \Delta H_{M\ 0}$. No anisotropic motion of the

label in the liposomes containing cholesterol was detectable. The authors attributed the changes in the relative line widths of the label upon addition of cholesterol to the liposomes as arising from a change in the position of the label. The oriented film results do not indicate that the addition of cholesterol influences the positions of the labels in the bilayers, but that it does reverse the angular dependence of the relative line width ratio of the spectrum.

2) Bovine Brain Sphingomylin Thin Films

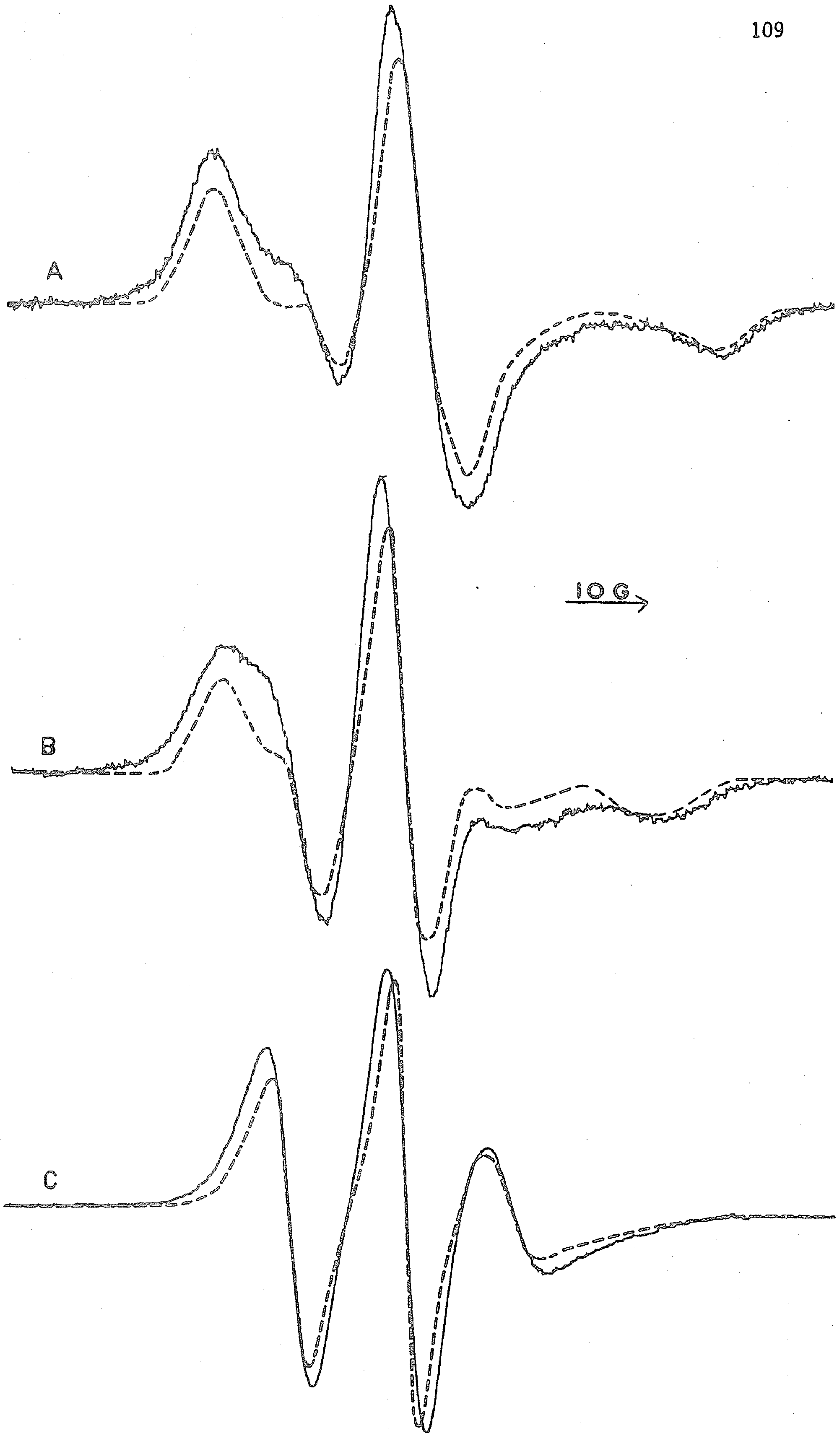
(a) 12-Stearic Acid Spin Label Results

The stearic acid label with the oxazolidine ring attached at C_{12} is a useful probe for the hydrophobic regions of sphingomyelin thin films. In a dry sphingomyelin film at 20 °C, the spectra of the label are angular independent and have the line shapes typical of the spectrum for strongly immobilized nitroxides, (Figure 3.22A). The high viscosity of the environment, indicated by the slow motion of the nitroxide groups, ($T_{\parallel} \simeq T_{\perp}$ = 31.6 gauss), is presumably a consequence of the crystalline state of the sphingomyelin chains at this temperature.[20] There is little change in the motion or orientation spin labels upon hydration of the film, ($T_{\parallel}$ = 26.1 gauss and $T_{\perp}$ = 27.2 gauss). As the temperature of the hydrated sphingomyelin film is increased, the motion of the label increases. At 37 °C, (Figure 3.22C), the spectra indicate the spin labels undergo rapid isotropic tumbling in the hydrated film, ($T_{\parallel}$ = 14.0 gauss, $T_{\perp}$ = 13.9 gauss). The rapid isotropic motion of the label in the hydrated film at 37 °C would indicate the sphingomyelin molecules are in a liquid crystalline state at this temperature.

Figure 3.23 shows the spectra of the spin label in a sphingomyelin film containing 50 mole % cholesterol. The dry state spectra, ($T_{\parallel}$ = 23.8 gauss, $T_{\perp}$ = 12.3 gauss), are similar to those obtained for equimolar cholesterol-egg phosphatidylcholine dry multilayers, and suggest a preferential alignment of the stearic acid long molecular axis perpendicular to the plane of the film. The similarity of results in-

Figure 3.22

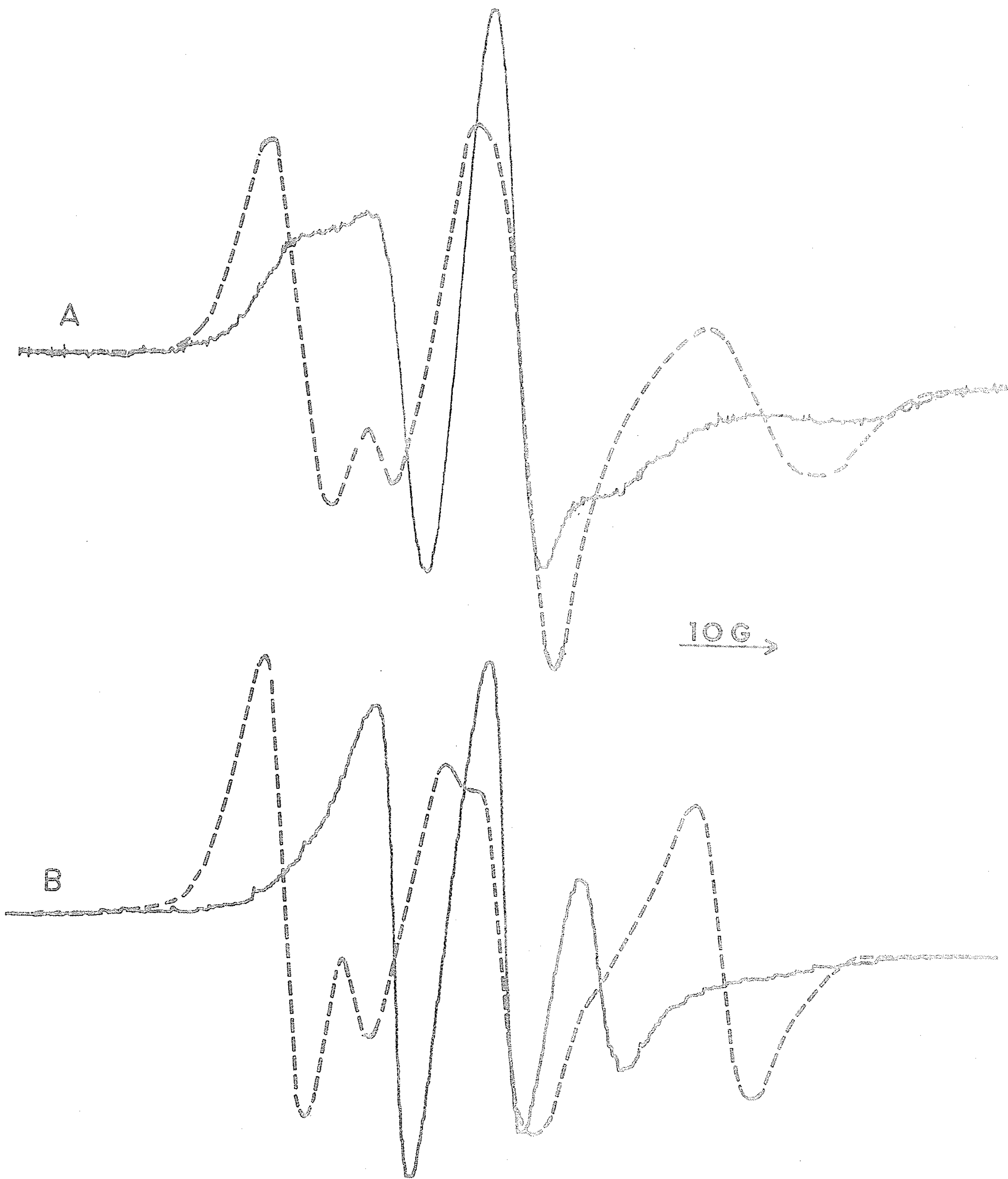
ESR spectra of the 12-stearic acid spin label in a sphingomyelin film. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film 20 °C, (B) hydrated film 20 °C, (C) hydrated film 37 °C.


A
B
C
10 G

dicate that the sphingomyelin-cholesterol film probably consists of oriented multibilayers with the planes of the bilayers being parallel to the film-supporting surface. It is interesting to note that the $T_{\parallel}$ and $T_{\perp}$ values demonstrate that the hydrophobic regions of the dry sphingomyelin-cholesterol film are more fluid than the corresponding regions of dry sphingomyelin alone. Upon hydration, (Figure 3.23B), there is a general sharpening of the spectral lines but the hyperfine splitting constants remain angular dependant. The $T_{\parallel}$ and $T_{\perp}$ values of the label in this film are practically identical to the values obtained in 50 mole % cholesterol egg phosphatidylcholine multilayers. Thus, the label has the same degree of anisotropic motion in both cholesterol containing sphingomyelin and phosphatidylcholine films at room temperature. Therefore, the fluidity of the hydrophobic regions of both sphingomyelin and phosphatidylcholine must be approximately the same. Since hydrated sphingomyelin is in a crystalline state at 20 °C, we can conclude that cholesterol induces an apparent gel to liquid crystalline transition in the sphingomyelin film at this temperature. This is an example of the general fluidizing effect of cholesterol on saturated phospholipids studied by a variety of physical techniques. The increase in liquidity of the phospholipid chains in aqueous dispersions of sphingomyelin upon addition of cholesterol has been observed by Oldfield and Chapman using the methyl ester of the 12-stearic acid label as a probe.[81] Even though the thermal motion of the chains at 20 °C increases in the presence of cholesterol, it must be emphasized that the spectra of the label indicate some remaining degree of chain

Figure 3.23

ESR spectra of the 12-stearic acid spin label in a sphingo-myelin film containing 50 mole % cholesterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular to the direction of the external magnetic field: (A) dry film 20 °C, (B) hydrated film 20 °C.


A
10 G
B

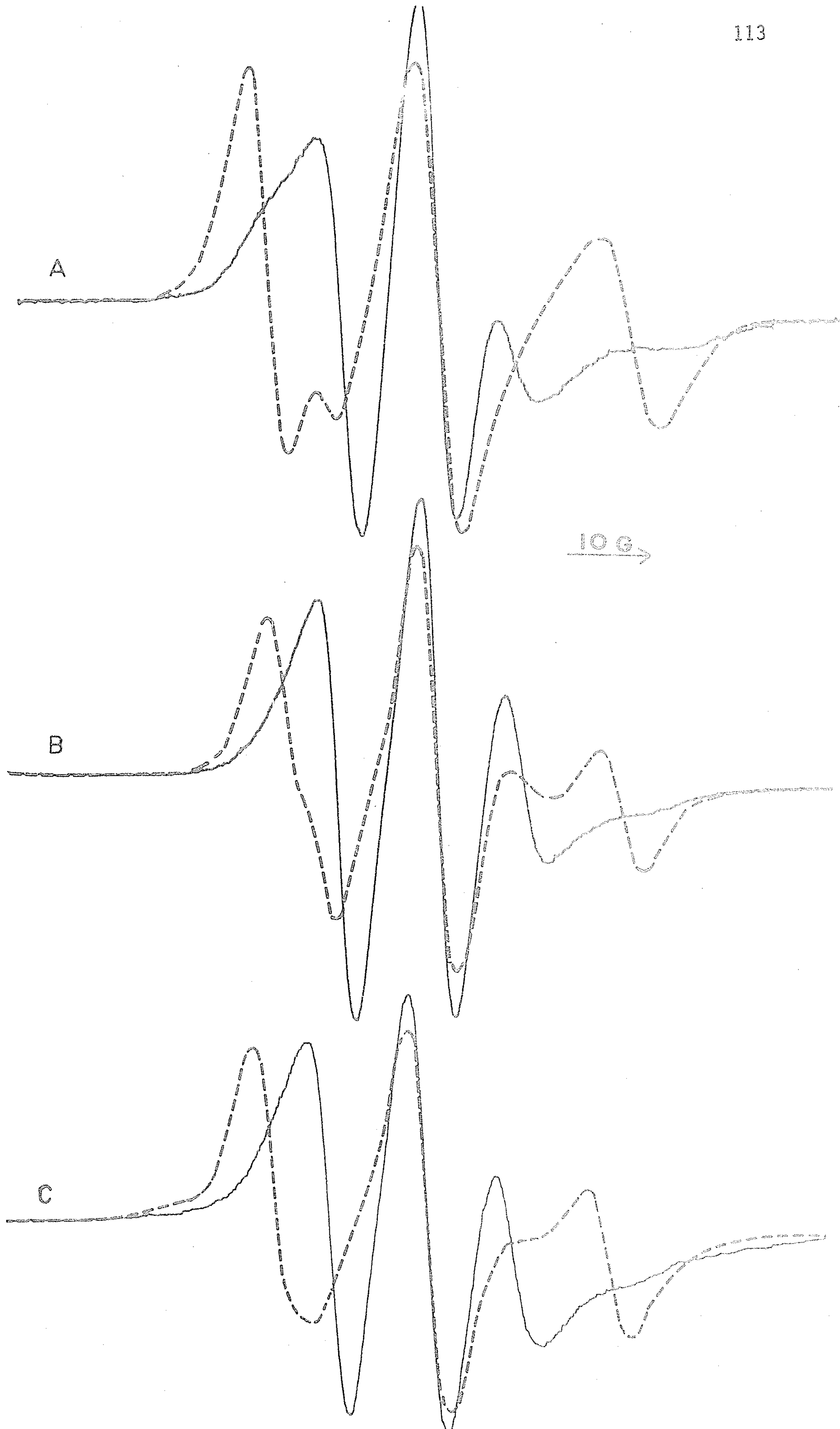
ordering comparable with that in equimolar egg phosphatidylcholine-cholesterol multibilayers. From Figure 3.23B, it can be seen that the center line of the spectrum for the perpendicular orientation is very asymmetric. Lowering the cholesterol concentration in the film to 33 mole %, drastically decreases the asymmetry of perpendicular orientation spectrum in the hydrated film at room temperature, (Figure 3.24A). Thus, it would appear that at a cholesterol concentration of 50 mole %, some spin labels are forced out of the bilayers resulting in the appearance of a spectrum of randomly tumbling labels. The reason for the exclusion of stearic acid spin labels from the bilayers is not apparent at the present time. Increasing the temperature of the hydrated sphingomyelin multilayers containing 33 mole % cholesterol to 37 °C, results in little change in the spectra of the spin label, (Figure 3.24B) which are similar to those of the label in an equimolar cholesterol-egg phosphatidylcholine film at the same temperature, (Figure 3.24C).

At 37 °C, the degree of order in the sphingomyelin bilayers containing cholesterol indicated by the anisotropic motion of the spin labels, compared with the random liquid state of the fatty acid chains in hydrated sphingomyelin alone, demonstrates that the influence of cholesterol is to decrease the random motion of the paraffin chains. Thus, it may be erroneous to conclude that the apparent gel to liquid crystalline transition of the sphingomyelin chains induced by the presence of cholesterol at room temperature has any biological significance, based on the results obtained at physiological temperature.

Only the effects of two derivatives, (cholesteryl methyl ether, and cholesteryl chloride), on sphingomyelin membrane structure were

Figure 3.24

ESR spectra of the 12-stearic acid spin label in phospholipid films. The spectra were recorded, with the plane of the films parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) hydrated sphingomyelin film containing 33 mole % cholesterol at 20 °C, (B) same film at 37 °C, (C) hydrated egg phosphatidylcholine film containing 50 mole % cholesterol at 37 °C.


A
B
C
10 G

investigated. Figure 3.25 shows the spectra of the label in a sphingomyelin thin film containing 50 mole % cholesteryl methyl ether. The

Table 3.4

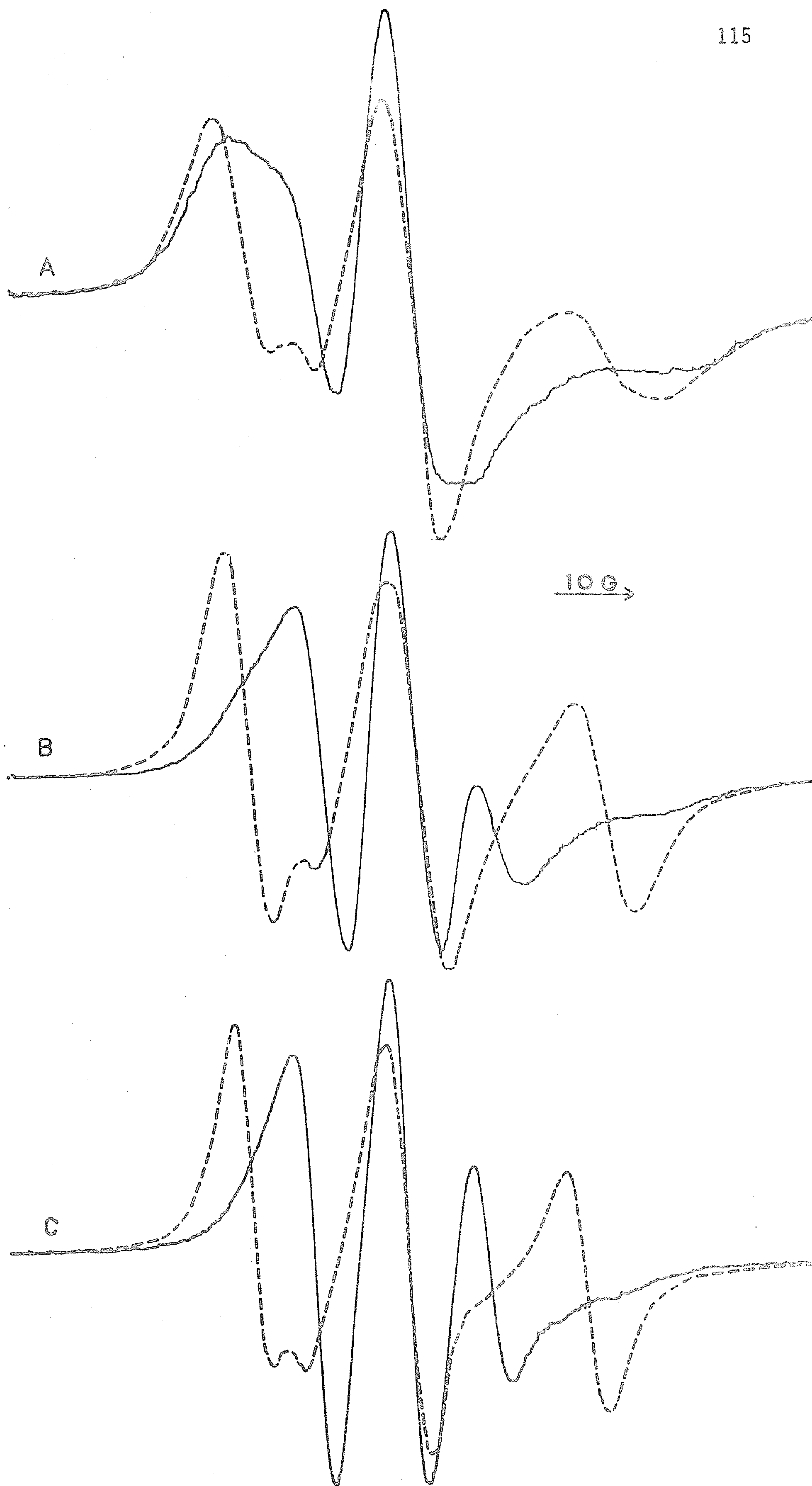
Hyperfine Splittings of the Stearic Acid Spin Label in Hydrated Sphingomyelin Films

Steroid	Mole %	20 °C		37 °C	
		$T_{\parallel}$	$T_{\perp}$	$T_{\parallel}$	$T_{\perp}$
Cholesterol	0	26.1	27.2	14.0	13.9
	33	22.6	10.7	20.2	11.6
	50	21.2	10.5	----	----
Cholesteryl methyl ether	50	22.5	10.8	21.3	11.1
Cholesteryl chloride	50	22.2	11.6	19.9	11.9

spectra and the results of Table 3.4 indicate that the methoxy derivative is equally effective as cholesterol in ordering the lipids of the dry and hydrated films. There was no alignment of the label in dry films containing 50 mole % cholesteryl chloride but results from the hydrated film spectra demonstrated that the chloro derivative is essentially equal to cholesterol in its effect on the motion of the sphingolipids at 20 °C and 37 °C. These results are in marked contrast to the influence of these derivatives in phosphatidylcholine multibilayers. The methoxy and chloro derivatives were only soluable in phosphatidylcholine up to 25 mole % and had no ordering effect on the phospholipids. The presence of a separate steroid phase resulting from

Figure 3.25

ESR spectra of the 12-stearic acid spin label in a sphingomyelin film containing 50 mole % cholesteryl methyl ether. The spectra were recorded with the plane of the film parallel, (——), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, 20 °C, (B) hydrated film, 37 °C.


A
B
C
10 G

insolubility in the lipid film can only be detected with the cholestane spin label. However, the present results strongly suggest that the interaction of sphingomyelin and cholesterol is not dependant on forces involving the 3β-OH group of the sterol.

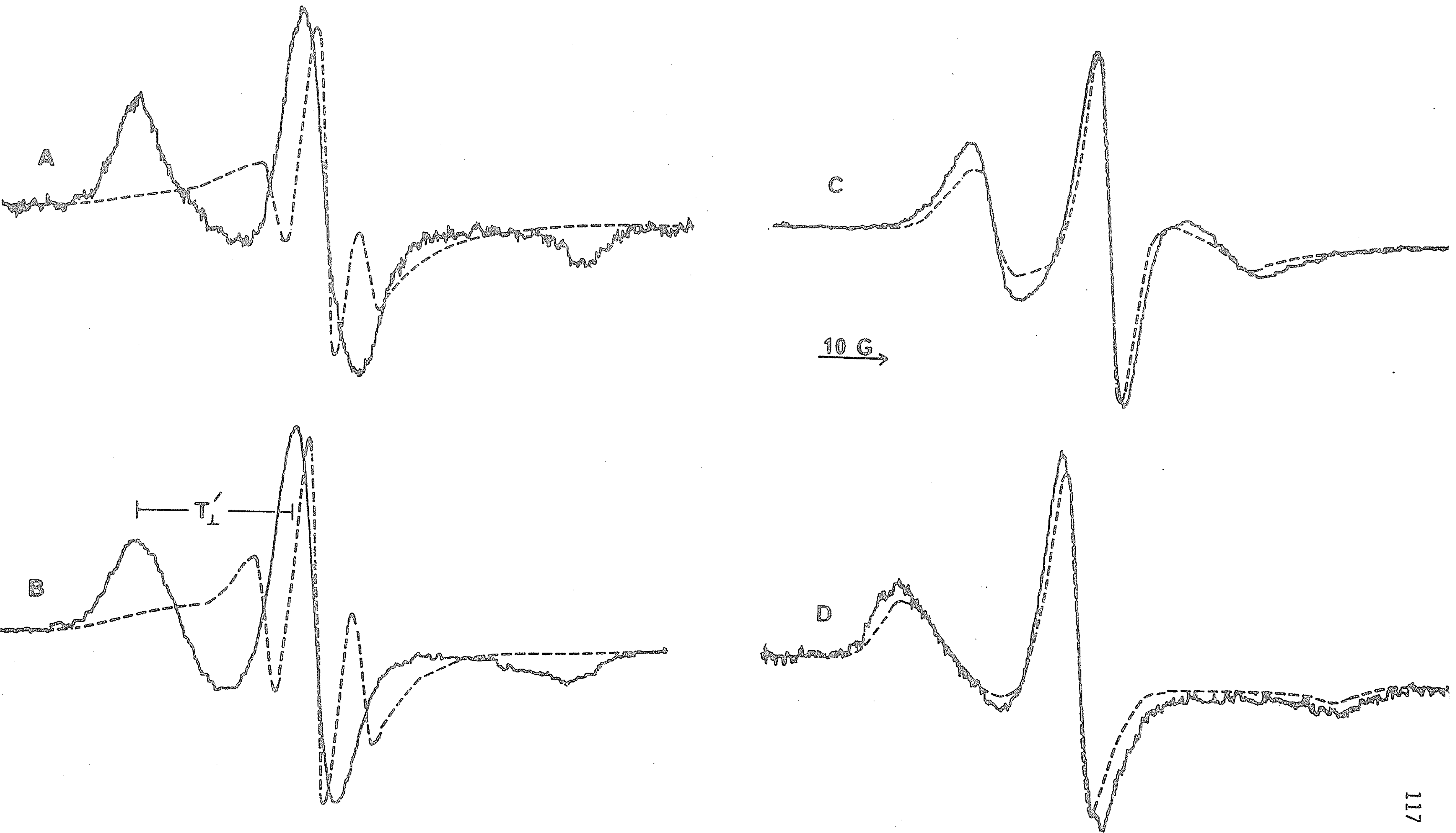
(b) Cholestane Spin Label Results

Attempts to obtain preferential orientation of the labelled steroid in dry sphingomyelin films using the method described in Chapter II were unsuccessful. The same problem is encountered in the formation of dipalmitoylphosphatidylcholine films.[34] The difficulty in forming oriented multilayers of these phospholipids is undoubtedly due to the crystalline state of the hydrocarbon chains of both phospholipids at room temperature. This difficulty can be overcome in the case of dipalmitoylphosphatidylcholine by heating the dry film *in vacuo* to approximately 100 °C.[35] At this temperature, the paraffin chains melt and oriented multilayers are formed. On cooling the film to room temperature, the film retains the multibilayer structure as indicated by the spectra of the spin label but, of course, the chains become crystalline again. By warming dry sphingomyelin films *in vacuo* to approximately 180 °C, it was found that upon cooling the film to room temperature, preferential alignment of the cholestane spin label was obtained.

The spectra of the spin label in a sphingomyelin film are shown in Figure 3.26. The magnitude and angular dependence of the hyperfine splitting constants of the label in the dry film at 20 °C, ($T_{//}$ = 7.3 gauss, $T_{\perp}$ = 33.0 gauss), indicate that the cholestane spin label is

Figure 3.26

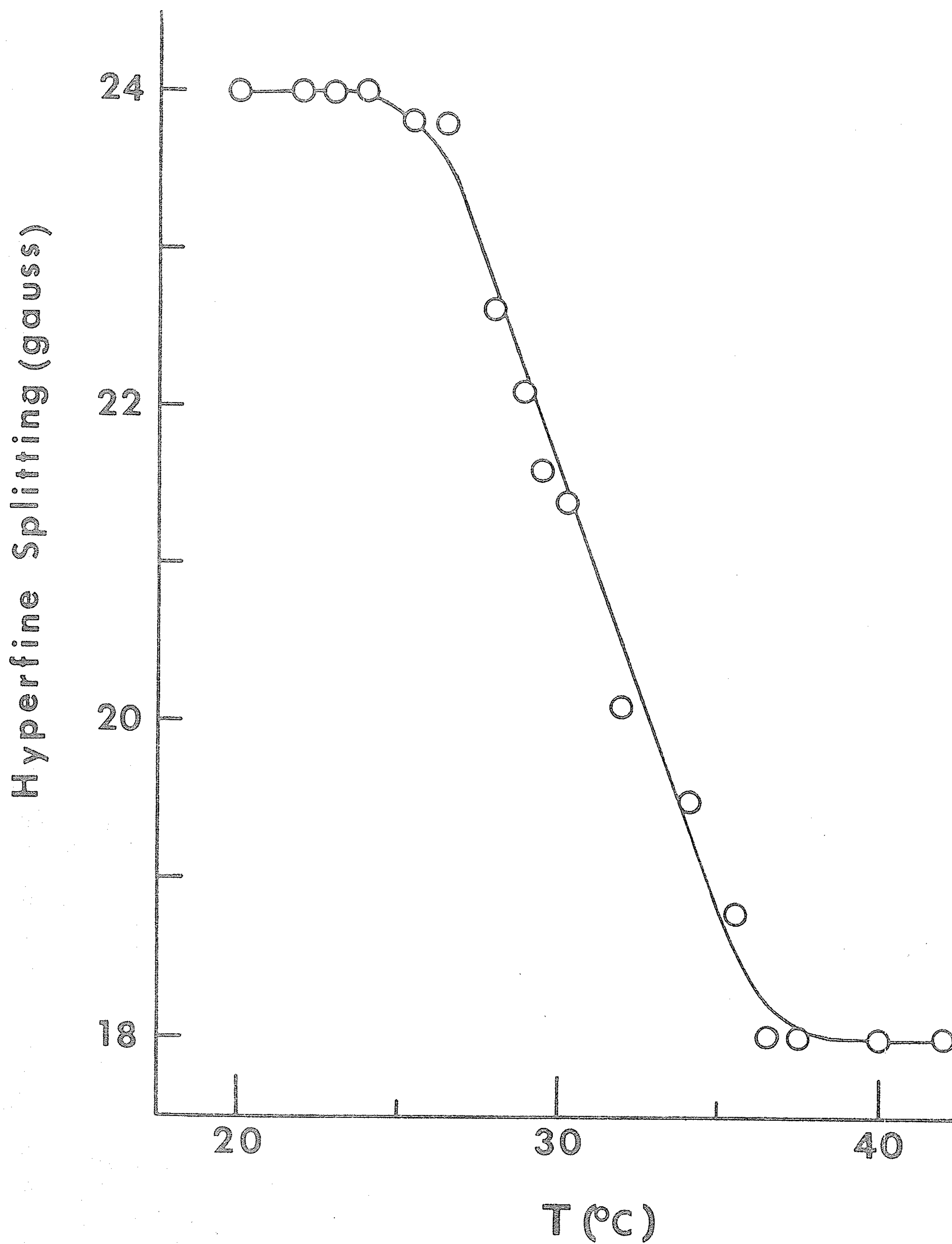
ESR spectra of the cholestane spin label in a sphingomyelin film. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, 20 °C, (B) hydrated film, 20 °C, (C) hydrated film, 37 °C, (D) hydrated film returned to 20 °C.


A
C
10 G
T′⊥
B
D

aligned with its long axis preferentially perpendicular to the plane of the film-supporting surface with an axial rotational frequency << 7.5 x 10^7 sec^{-1}. The similarity of these spectra to those obtained in dry egg and dipalmitoylphosphatidylcholine films which are known to form multilayer arrays,[36,93] strongly suggests the same structural arrangement of the sphingomyelin film. Hydration of the film at 20°C, results in effectively no change in the spectra of the label, ($T_{//}$ = 7.3 gauss, $T_{\perp}$ = 31.8 gauss). This is in contrast to the substantial effect of hydrating the dipalmitoyl and egg phosphatidylcholine films at room temperature which results in rapid anisotropic motion of the labels with axial rotational frequencies >> 7.5 x 10^7 sec^{-1}. It does not appear that the restricted rotational motion of the label is a consequence of the crystalline state of the sphingolipids since the label undergoes rapid rotational motion in hydrated dipalmitoylphosphatidylcholine. Thus, the restricted motion of the label must arise from the sphingolipids being much more tightly packed in the multilayers compared to the phosphatidylcholines. As an approximate measurement of the rotational freedom of the label in the hydrated sphingomyelin film, a spectral parameter designated $T_{\perp}'$ was used. $T_{\perp}'$ is the peak-to-peak separation of the low field and center line of the spectrum for ϕ = 0°. Figure 3.27 is a plot of $T_{\perp}'$ versus temperature obtained from the spectrum of the cholestane spin label in a hydrated sphingomyelin film. There is no change in $T_{\perp}'$ from 20 - 25°C. Above 25°C, there is a linear decrease in $T_{\perp}'$ reaching a minimum value at approximately 37°C. There is little change in $T_{//}$ or the line shape of the spectrum for ϕ = 90° up to approx-

Figure 3.27

A plot of $T_{\perp}'$ for the cholestane spin label in a hydrated sphingomyelin film as a function of temperature.



Hyperfine Splitting (gauss)
24
22
20
18
20
30
40
T (°C)

imately 30°C. Between 30 - 36°C, $T_{//}$ changes from 8.0 gauss to 16.8 gauss. At 37°C, (Figure 3.26C), the spectra of the label are identical for both parallel and perpendicular orientations of the film, ($T_{//} = T_{\perp} = 16.8$ gauss). The values of $T_{//}$ and $T_{\perp}$ at this temperature indicate an isotropic distribution of label orientations with respect to the plane of the film-supporting surface and the axial rotational frequency of the spin labels is >> 7.5×10^{7} sec^{-1}. On cooling the film to 20°C, powder spectra are observed for both orientations, ($T_{//} \approx T_{\perp} = 32.1$ gauss). Reheating the film to 37°C, results in spectra as shown in Figure 3.26C. The results indicate that the sphingomyelin molecules are arranged in oriented planar lamellae on initial formation of the film. Warming the hydrated film above 30°C, results in an irreversible structural rearrangement of the lamellae to an isotropic structure, probably liposomes. The rotational freedom of the spin labels in both the oriented multibilayers and liposomes is restricted below ≈ 37°C indicative of the tight packing of the sphingolipid molecules in the bilayers.

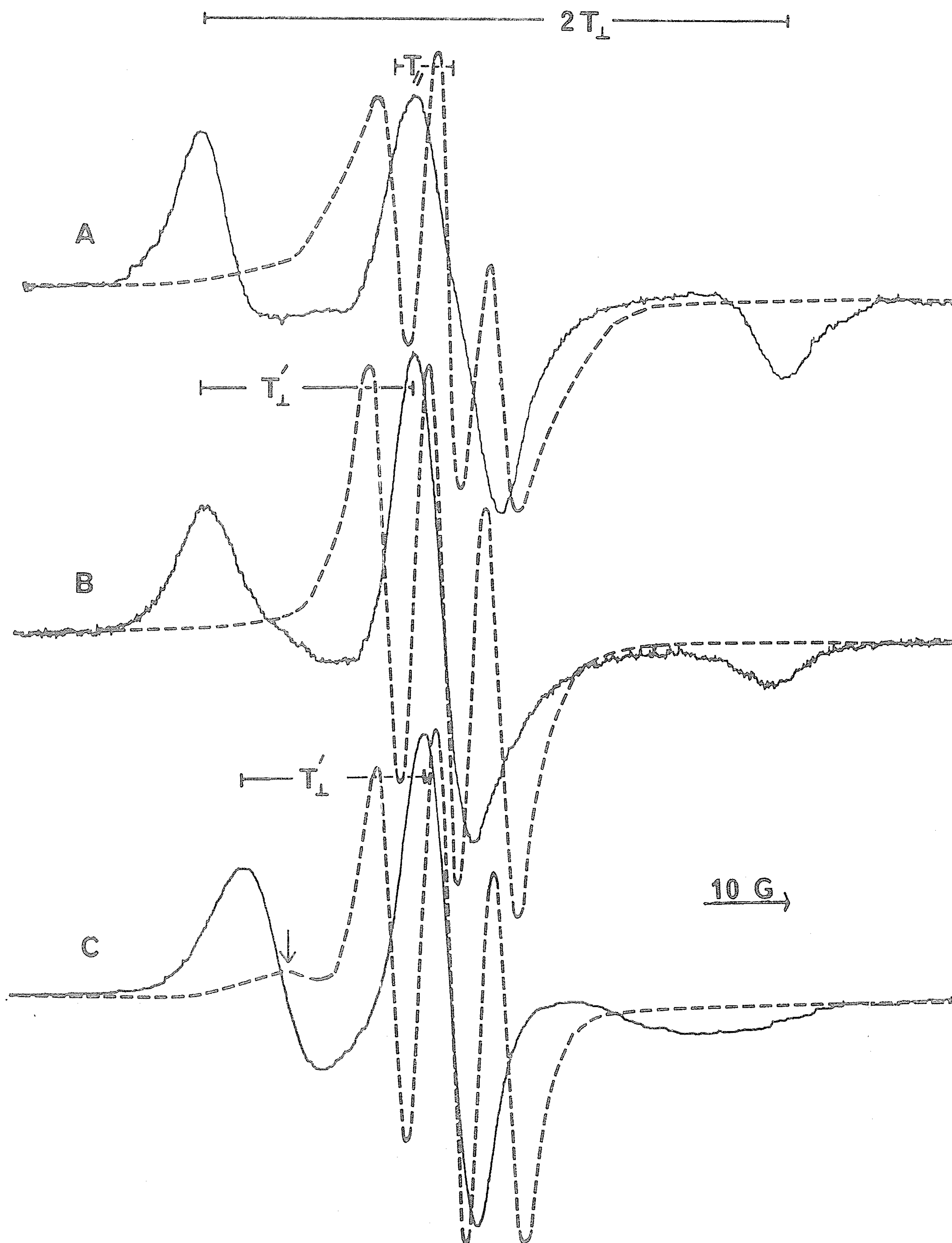
Sphingomyelin films incorporating 25 - 50 mole % cholesterol give a preferred alignment of the cholestane spin label without heating the dry film. Figure 3.28 shows the spectra of the label in a 50 mole % cholesterol-sphingomyelin film. In the dry state at 20°C, a broad triplet, ($T_{//} = 6.4$ gauss), is found in the perpendicular orientation and powder spectrum, ($T_{\perp} = 33.0$ gauss), is observed in the parallel orientation of the film. These spectra are essentially the same as those of the label in cholesterol containing egg and dipalmitoylphosphatidylcholine multilayers. Therefore, it is reasonable to conclude

that dry sphingomyelin films containing cholesterol consist of stacked multibilayers with the long axes of the cholestane spin labels aligned perpendicular to the plane of the bilayers with axial rotational frequencies $<< 7.5 \times 10^7$ sec^{-1}. The ease with which oriented dry multilayers of sphingomyelin can be prepared with added cholesterol is undoubtedly related to the increase in liquidity of the hydrocarbon chains in these films, as indicated by the stearic acid label results. The spectra of the label in the hydrated equimolar sphingomyelin cholesterol film are shown in Figure 3.28B. The outer lines of the spectrum for the perpendicular orientation are much sharper than in the dry state denoting a decrease in the width of the distribution of orientations of the labels, ($T_{//}$ = 6.3 gauss). Thus, it appears that wobbling motion of the cholestane spin label is permitted at 20°C in the hydrated sphingomyelin film containing cholesterol but not in a film consisting of sphingomyelin alone. In the parallel orientation, a powder spectrum was still observed, ($T_{\perp}$ = 32.0 gauss). Therefore, even in the hydrated film at 20°C, the axial rotational frequency of the labels is $<< 7.5 \times 10^7$ sec^{-1}. This is contrary to results obtained with egg or dipalmitoylphosphatidylcholine multilayers containing 50 mole % cholesterol, where hydration of these lipid films permits rapid rotational motion, ($>> 7.5 \times 10^7$ sec^{-1}) of the spin labelled cholestane molecules at room temperature.

Raising the temperature of the film causes a change in the spectra of the label. At 37°C, (Figure 3.28C), the hyperfine splittings of the spectra are $T_{\perp}$ = 19.7 gauss and $T_{//}$ = 6.4 gauss. Thus, at this temperature, the labels are still well aligned, $< \theta_2 > = 5.9°$,

Figure 3.28

ESR spectra of the cholestane spin label in a sphingomyelin film containing 50 mole % cholesterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, 20 °C, (B) hydrated film, 20 °C, (C) hydrated film, 37 °C.



2 T⊥
T∥
A
T⊥'
B
T⊥'
C
10 G

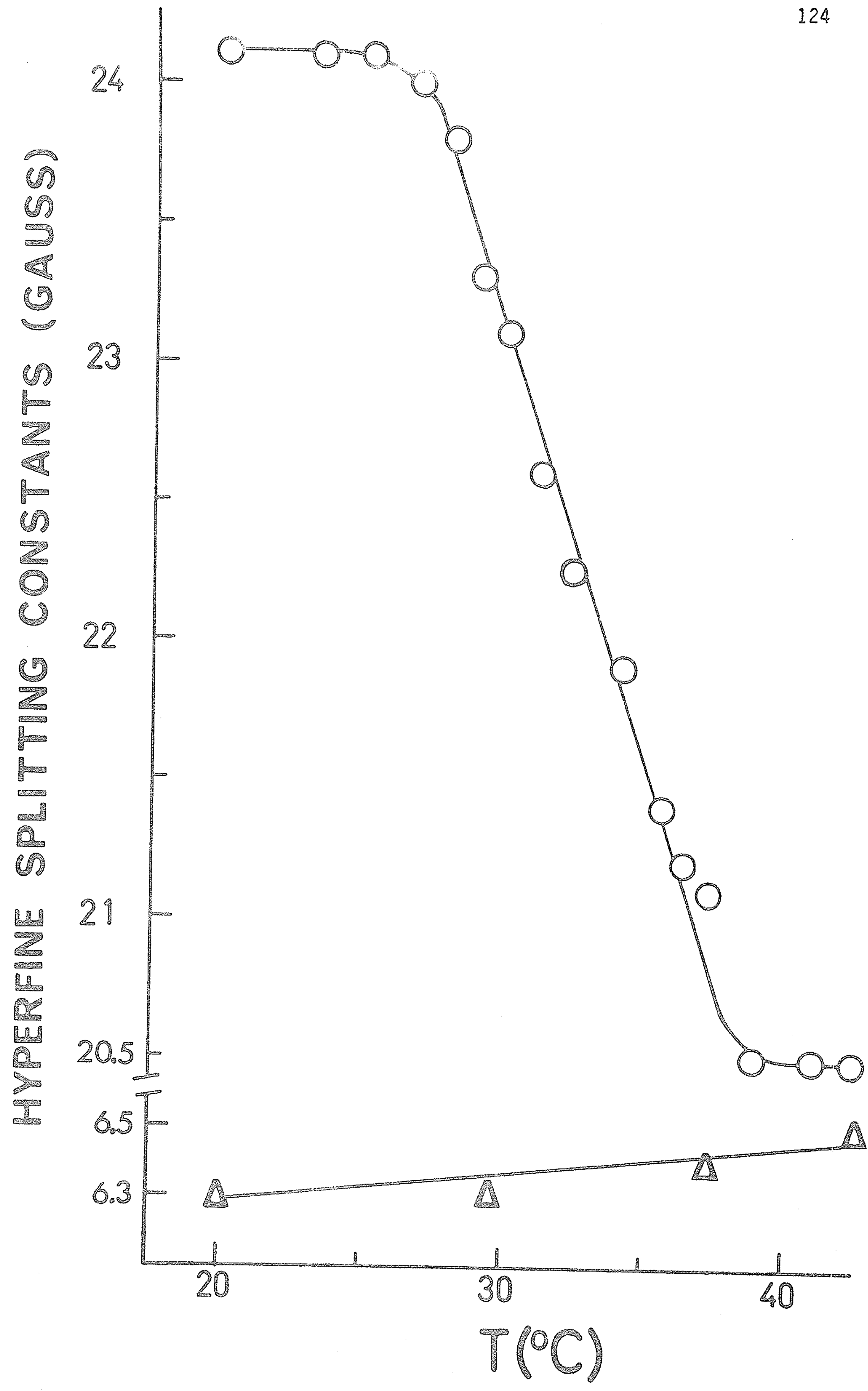
but the axial rotational frequency of the labels is >> $7.5 \times 10^7 sec.^{-1}$. Therefore, to account for the increased rotational freedom of the label at 37 °C, we must assume that the lipids occupy greater surface areas in the bilayers at this temperature. The parameter $T_{\perp}'$, defined previously, was used as a measure of the rotational freedom of the cholestane spin label in the equimolar sphingomyelin-cholesterol film. Figure 3.29 shows that $T_{\perp}'$ is constant up to 25 °C with a linear decrease occurring over the range 26 - 38 °C. Comparing Figure 3.28 to Figure 3.26, it can be seen that the $T_{\perp}'$ values of the cholestane spin label in films with and without cholesterol exhibit the same general temperature dependance although in the case of the cholesterol free film, the distribution of spin label orientations becomes irreversibly isotropic at higher temperatures. Figure 3.29 also includes a plot of $T_{||}$ versus temperature. Since the change in $T_{||}$ over the range 20 - 42 °C is approximately 0.2 gauss, we may conclude that the lattice rigidity of the bilayers can be altered without a significant change in the alignment of the spin labels. This is undoubtedly due to the presence of cholesterol which appears to maintain the planar multibilayer structure of the film above 30 °C. The broad line appearing at elevated temperatures in the spectrum indicated by an arrow in Figure 3.28C denoting a decrease in the alignment of some spin labels may arise from the fatty acid heterogeneity of the sphingomyelin, or imperfections in the bilayers. This line disappears when the film is returned to 20 °C. The temperature effects in the film are fully reversible.

The temperature dependance of the rotational freedom of the

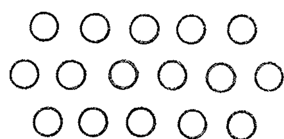
Figure 3.29

A plot of $T_{\perp}'$ and $T_{//}$ as a function of temperature for the cholestane spin label in a hydrated sphingomyelin film containing 50 mole % cholesterol.

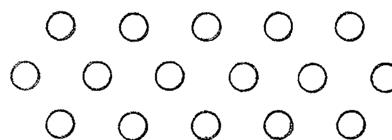
(O) $T_{\perp}'$, (Δ) $T_{//}$


HYPERFINE SPLITTING CONSTANTS (GAUSS)
24
23
22
21
20.5
6.5
6.3
20
30
40
T(°C)

cholestane spin label in sphingomyelin film with and without added cholesterol suggest that a thermal phase transition occurs over the range ≈ 25 - 38 °C resulting in an increase in the average surface area per sphingomyelin molecule. Below 25 °C, the sphingolipids are more tightly packed than phosphatidylcholine molecules presumably the result of strong intermolecular interactions. Raising the temperature will increase the thermal motion of the molecules decreasing the effectiveness of intermolecular forces and resulting in a greater intermolecular separation in the bilayers. We can represent the phase transition by a schematic diagram where the circles represent phospholipids viewed along the normal to the plane of a bilayer.



20°C



37°C

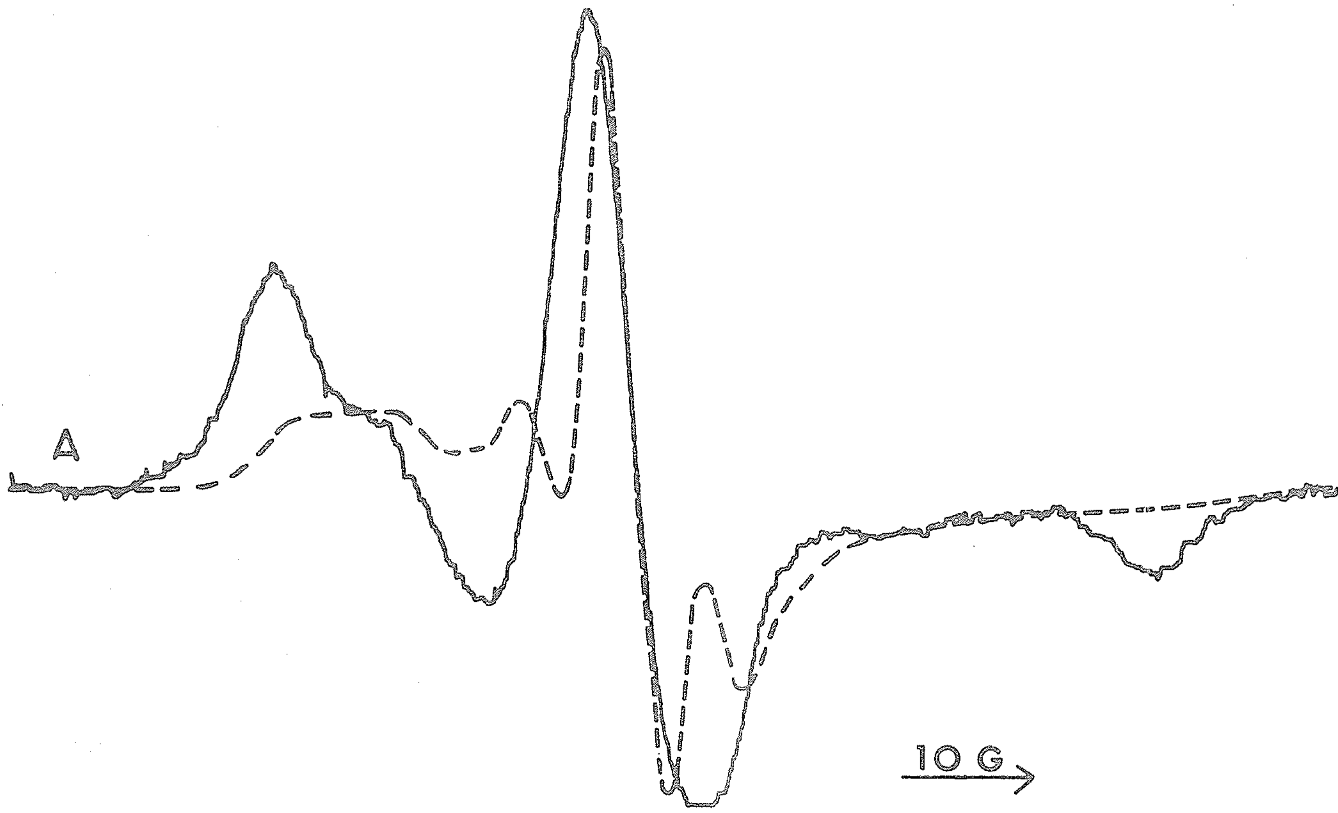
Since the 12-stearic acid spin label results suggest that the hydrophobic regions of equimolar sphingomyelin-cholesterol and phosphatidylcholine-cholesterol bilayers have approximately the same degree of fluidity at room temperature, the strong intermolecular interactions between sphingomyelin molecules below 37 °C must involve the polar head groups of these molecules. The importance of polar intermolecular forces can be demonstrated by disrupting the homogeneity of the head group

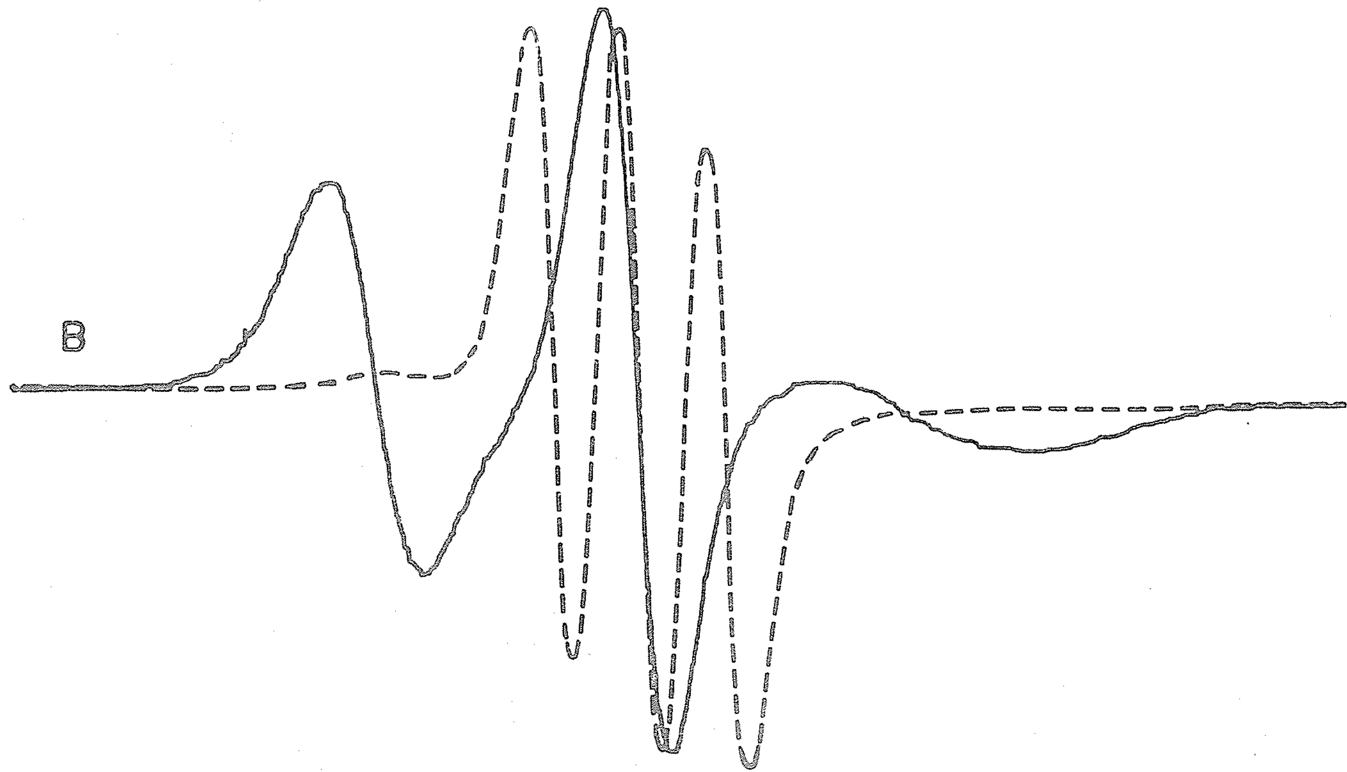
regions of the sphingomyelin films. Figure 3.30 shows the spectra of the label in a film containing a 1:1:2 mole ratio of egg phosphatidylcholine : sphingomyelin : cholesterol. Due to the method of film preparation, we may reasonably expect a random distribution of lipids in the bilayers. In the dry state, the orientation of the labels is not as good as obtained in equimolar phospholipid-cholesterol films of phosphatidylcholine or sphingomyelin alone. Upon hydration at room temperature, there is an increase in the orientation of the label indicated by the sharpness of the triplet, ($T_{//}$ = 6.4 gauss), observed for $\phi = 90^\circ$. In the parallel orientation, $T_{\perp}$ = 19.9 gauss and there is no evidence from the spectrum of labels having axial rotational frequencies << 75 MHz. Thus, it would appear that the presence of phosphatidylcholine head groups precludes the formation of a rigid sphingomyelin lattice by preventing the necessary cross-linking interactions. With the present information, it is not possible to pinpoint the exact nature or mechanism of intermolecular interactions in the sphingomyelin films but either or both of the chemical entities, the hydroxyl group and the amide group, which are not present in phosphatidylcholine are probably involved. This would suggest that the dominant mechanism of interaction is hydrogen bonding.

Shah and Schulman[94,95] have investigated some physical properties of dipalmitoylphosphatidylcholine and sphingomyelin monolayers. Since monolayers of both phospholipids had the same limiting area, (42 - 44Å^2 per molecule), they concluded that the intermolecular distance was the same in each system. Surface potential measurements indicated the ionic properties of the two phospholipids were different

Figure 3.30

ESR spectra of the cholestane spin label in a film containing a 1:1:2 mole ratio of egg phosphatidylcholine: sphingomyelin : cholesterol at room temperature: (A) dry film, (B) hydrated film.


A
10 G


B

and that the sphingomyelin monolayer possessed a net positive surface charge. Micro-electrophoresis experiments showed that the particles in aqueous dispersions of sphingomyelin also possessed a positive surface charge. To explain their observations, the authors proposed the existance of an intramolecular ion-dipole association between the hydroxyl group and the negatively charged oxygen atom of the phosphate group, decreasing the negative charge of the phosphate group leaving the trimethylammonium group with a net positive charge. The spin label results suggest that the properties of the phosphatidylcholine and sphingomyelin head groups differ but not that the intermolecular distance of each species is the same.

Cholesterol Derivatives

In dry films which were not preheated, some degree of preferential alignment of the cholestane spin label was observed only in those films incorporating cholesteryl methyl ether, β-sitosterol, or epicholesterol. In all other dry films, $T_{\parallel}$ and $T_{\perp}$ were approximately equal to 32.0 gauss. In hydrated films containing each one of the cholesterol derivatives, the spectra indicated there were always some spin labels showing a preferred alignment. Hyperfine splittings were measured at 20 °C and 37 °C. The reversible thermal phase transition was observed in films containing each of the derivatives. The ratio of the low field and center lines separated by $T_{\parallel}$, designated A/B, was used as a measurement of the distribution of labels in ordered and isotropic regions of a film. The average deviation angle of the label in a hydrated film

was not calculated when A/B was less than 0.6 since for those cases, the majority of spin labels were randomly oriented with respect to the film supporting surface. The results are summarized in Table 3.5.

$\underline{C_3}$ Derivatives

Figure 3.31 shows the spectra of the cholestane spin label in a sphingomyelin film containing 50 mole % cholesteryl methyl ether. In the dry state, the spectra are essentially identical to those obtained in a film containing an equal amount of cholesterol. There is no evidence from the spectra of a separate steroid phase as is the case for egg phosphatidylcholine films containing 50 mole % cholesteryl methyl ether. Upon hydration at 20 °C, the spectra indicate good alignment of the spin label with an axial rotational frequency $<< 7.5 \times 10^7 sec^{-1}$. At 37 °C, the axial rotational frequency of the spin label is $>> 7.5 \times 10^7 sec^{-1}$, but the labels are still aligned with an average deviation angle of approximately 11°. The absence of any powder spectra at 37 °C suggests that all the cholesteryl methyl ether is solubilized in the sphingomyelin multibilayers. No preferred alignment of the label in films with 50 mole % cholesteryl chloride was observed but the spectra in hydrated films were almost the same as those shown in Figure 3.31. These results are in good agreement with those obtained with the stearic acid spin label.

The spectra of the label in a sphingomyelin film containing 50 mole % thiocholesterol are shown in Figure 3.32. In the dry state, there is no preferred alignment of the labels. In dry phosphatidylcholine films containing thiocholesterol, there was a rapid destruction of

Table 3.5

Ordering Effects of Cholesterol and Structural Derivatives in Hydrated Sphingomyelin Films

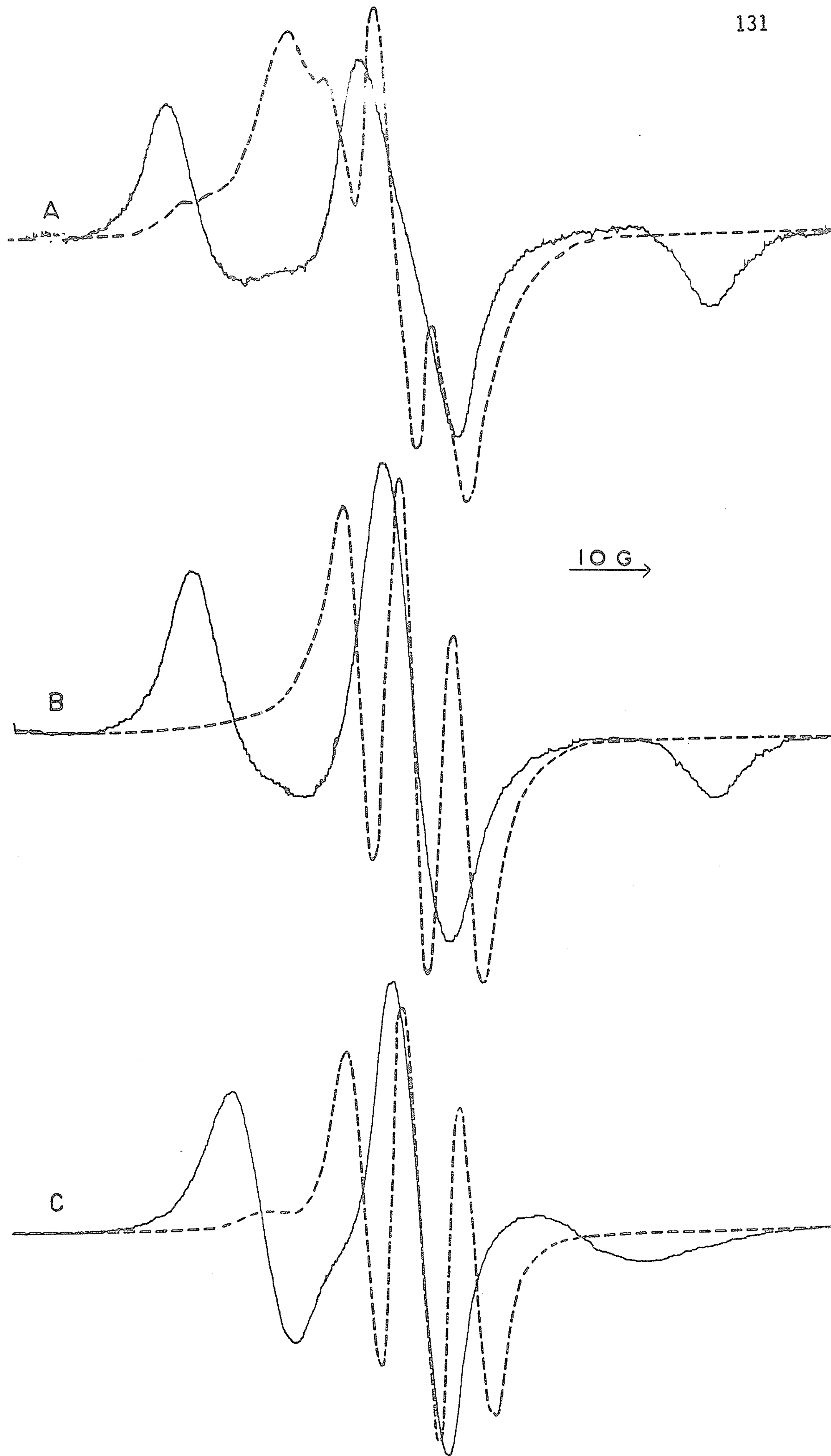
Steroid	Mole %	20 °C			37 °C				
		$T_{//}$	$T_{\perp}$	A/B	$T_{//}$	$T_{\perp}$	A/B	$\langle O_2 \rangle$ †	$\langle \Theta_2 \rangle$ §
cholesterol	0	7.3	31.8	0.32	16.8	16.8	---	---	---
	50	6.3	33.0	0.78	6.4	19.7	0.74	6.5	5.9
cholesteryl methyl ether	50	6.6	32.6	0.71	6.9	19.7	0.72	11.0	11.9
cholesteryl chloride	50	6.8	32.3	0.54	7.4	19.5	0.65	15.0	16.2
thiocholesterol	50	7.6	31.4	0.35	7.8	18.5	0.20	---	---
epicholesterol	50	6.9	32.3	0.22	7.3	19.0	0.25	---	---
5α-cholestane-3β-ol	50	6.5	31.8	0.76	6.8	19.9	0.68	10.3	10.2
Δ^7-cholesten-3β-ol	50	6.6	31.2	0.65	6.6	19.7	0.72	8.8	9.1
7-dehydrocholesterol	50	6.9	32.9	0.72	6.9	19.5	0.71	11.0	12.4
β-sitosterol	50	6.5	32.8	0.84	6.6	19.7	0.79	8.8	9.0
ergosterol	50	6.7	33.0	0.82	6.7	20.7	0.85	9.5	5.5
5α-androstane-3β-ol	50	6.8	32.3	0.36	7.8	18.3	0.35	---	---
5α-androstane-3β-ol	50	7.1	32.1	0.35	7.6	18.5	0.38	---	---

† calculated from equation 19

§ calculated from equation 15

Figure 3.31

ESR spectra of the cholestane spin label in a sphingomyelin film containing 50 mole % cholesteryl methyl ether. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, 20 °C, (B) hydrated film, 20 °C, (C) hydrated film, 37 °C.


A
B
C
10 G

the nitroxides. However, in sphingomyelin, there is no decrease in the signal intensity of the nitroxide spectra which indicates that the spin label and thiocholesterol may have different spatial arrangements in sphingomyelin and phosphatidylcholine, or that the rigidity of the sphingomyelin lattice prevents the sulfhydryl and nitroxides from interacting. Upon hydration of the film, the spectra become angular dependant demonstrating that the thiol derivative has some ordering effect. At 37 °C, the axial rotational frequency of aligned labels is >> $7.5 \times 10^7 sec^{-1}$ and there is no destruction of nitroxides even at this elevated temperature. The absence of powder spectra may indicate that thiocholesterol is soluble in sphingomyelin up to 50 mole % in contrast to a solubility of approximately 9 mole % in phosphatidylcholine. The spectra of the spin label in films containing epicholesterol, (Figure 3.33), indicate that the C_3 substituent must be in the β configuration for the steroid to have the maximum ordering effect in the film.

B - Ring Derivatives

No orientation of the cholestane spin label could be obtained in dry sphingomyelin films containing 50 mole % of 5α-cholestane-3β-ol, Δ^7-cholesten-3β-ol or 7-dehydrocholesterol which points out that the sphingomyelin molecules are sensitive to the structural differences between these sterols and cholesterol in the dry state. In hydrated films, all three derivatives are almost as effective as cholesterol in ordering the lipids at 20 °C and 37 °C. The spectra of the label in these hydrated films are similar to Figure 3.27B and 3.27C. Therefore, changing the number and position of double bonds in the B-ring

Figure 3.32

ESR spectra of the cholestane spin label in a sphingomyelin film containing 50 mole % thiocholesterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, 20 °C, (B) hydrated film, 20 °C, (C) hydrated film, 37 °C.

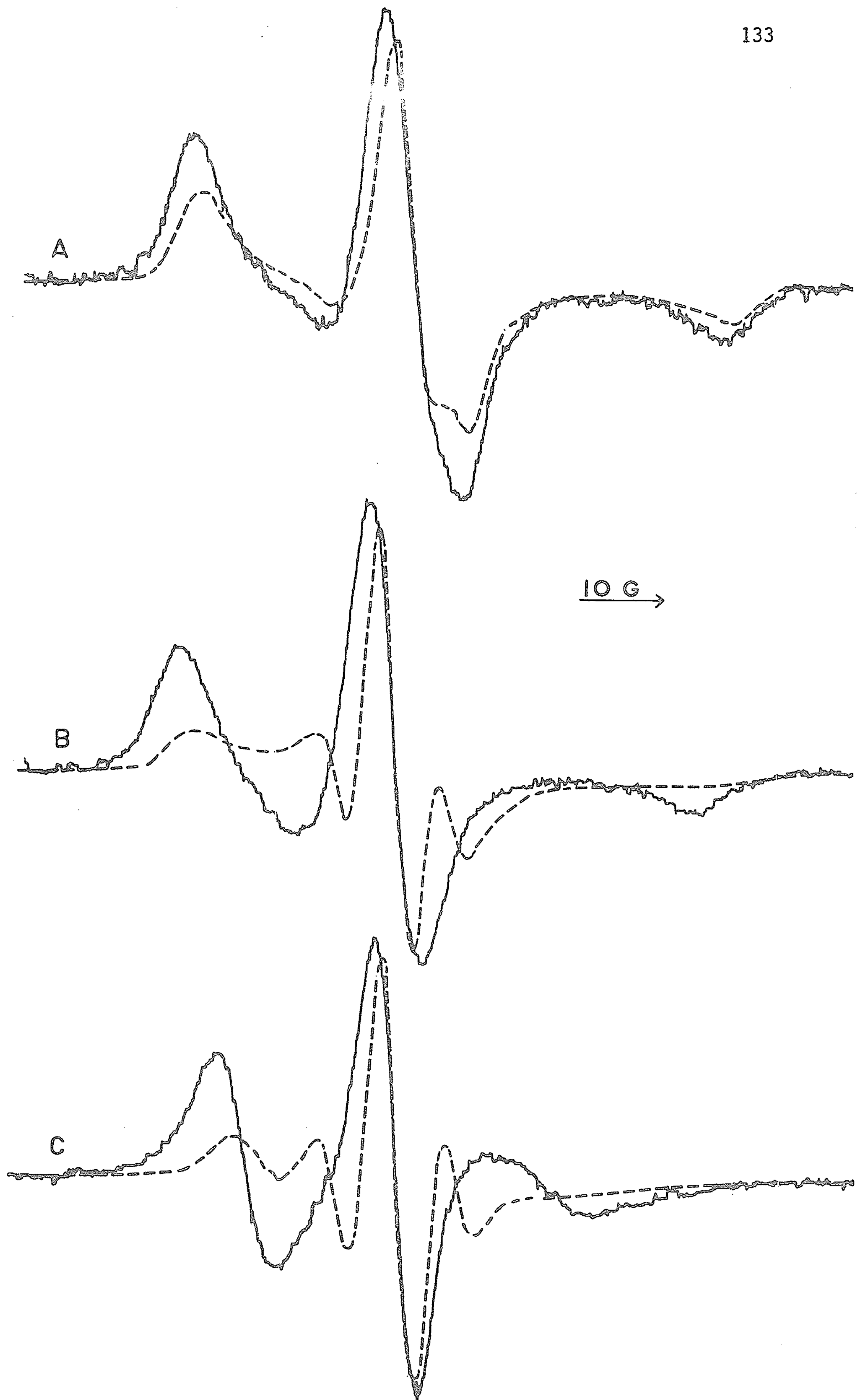
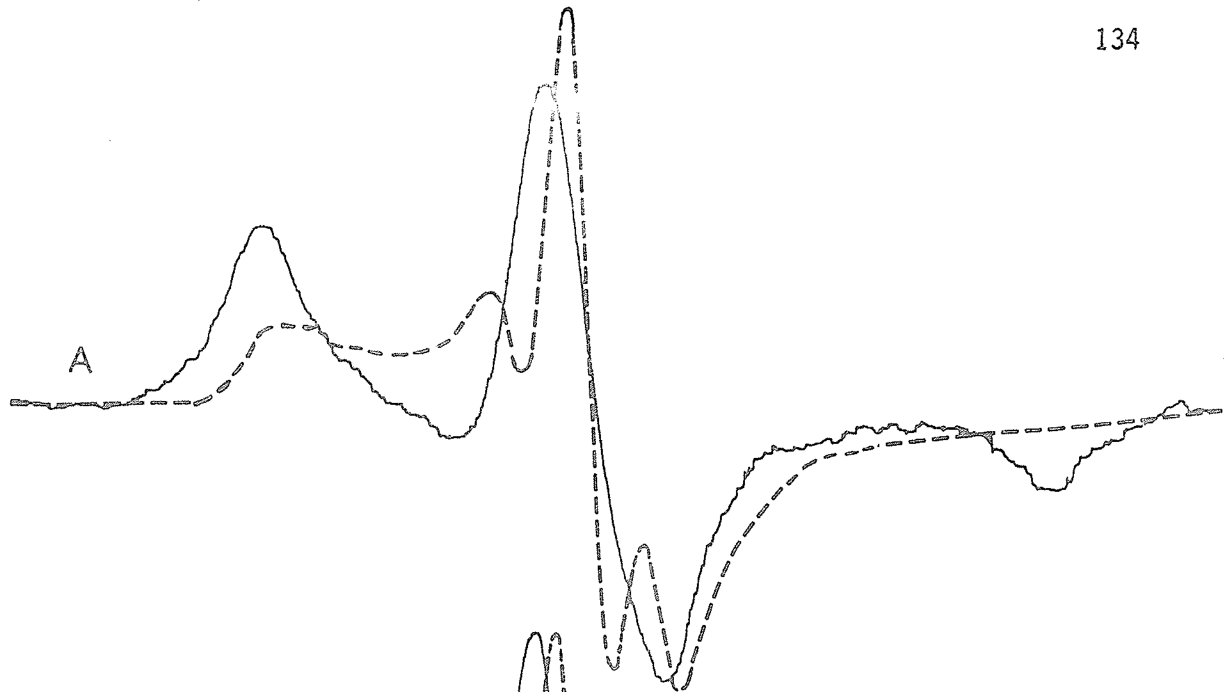
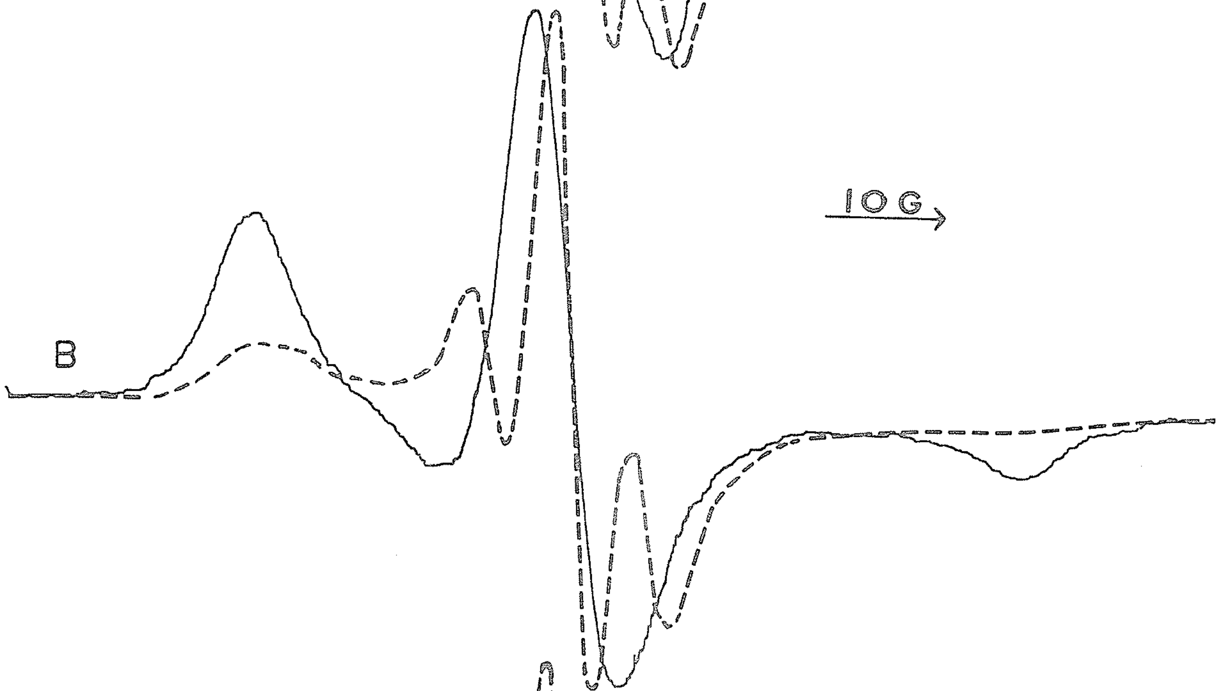
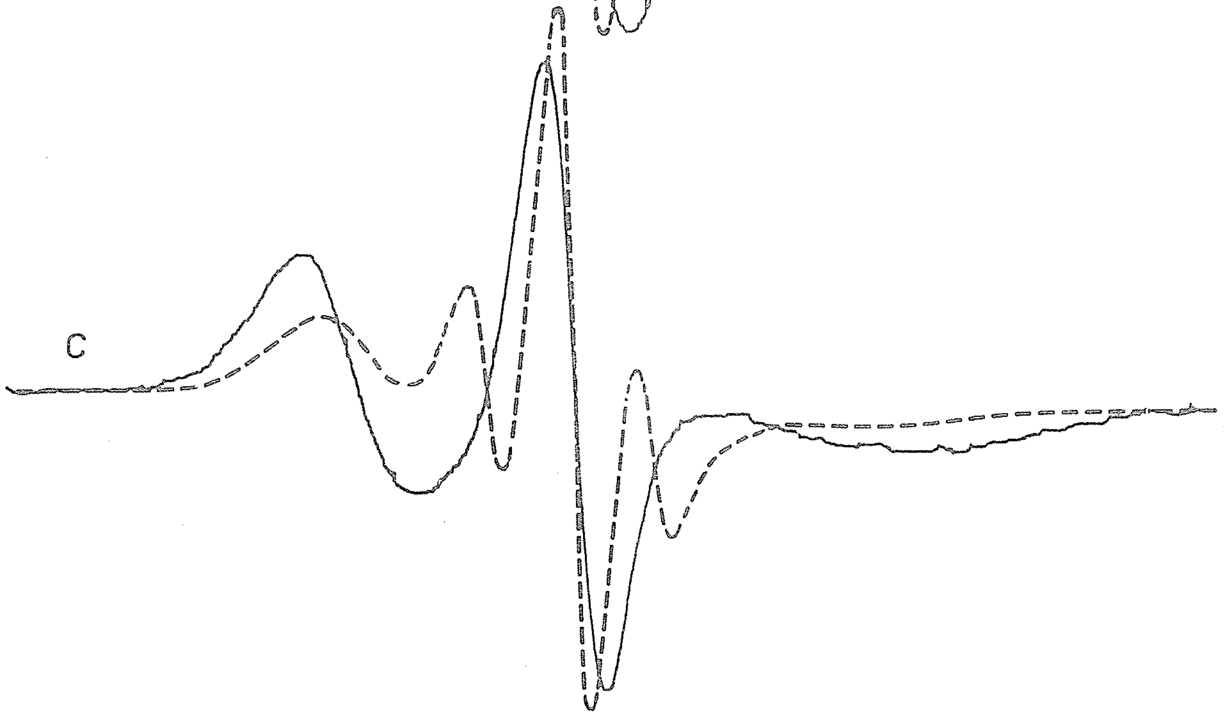

A
B
C
10 G

Figure 3.33

ESR spectra of the cholestane spin label in a sphingomyelin film containing 50 mole % epicholesterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, 20 °C, (B) hydrated film, 20 °C, (C) hydrated film, 37 °C.


A


B
10 G


C

of the steroid nucleus has little effect on the interaction of a membrane ordering sterol and sphingomyelin.

C_{17} Derivatives

Both ergosterol and β-sitosterol are essentially equivalent to cholesterol in ordering hydrated sphingomyelin multibilayers at 20 °C and 37 °C. The spectra for a sphingomyelin film containing 50 mole % ergosterol are shown in Figure 3.34. Unlike the results obtained with phosphatidylcholine-ergosterol films, there is no decrease in the order of the hydrated sphingomyelin bilayers containing ergosterol even at 37 °. It should be noted that both these sterols are more effective in decreasing the average deviation angle of sphingomyelin than phosphatidylcholine which gives support to the suggestion that hydrophobic interactions involving the C_{17} side chains of these molecules are sensitive to the fatty acid composition of the phospholipid.

5α-Androstane-3β-ol and 5α-androstane-3β-ol-17-one can induce some order in the hydrated sphingomyelin films but they are much less effective than an equivalent amount of 5α-cholestane-3β-ol, or cholesterol. Figure 3.35 shows the spectra of the cholestane spin label in a sphingomyelin film containing 50 mole % 5α-androstane-3β-ol-17-one. Similar spectra are obtained with 5α-androstane-3β-ol. Therefore, a C_{17} sidechain is necessary for inducing maximum order in sphingomyelin pointing out the necessity of hydrophobic interactions between the C_{17} group and the sphingolipid.

The results of Table 3.5 indicate that there are subtle differences in the nature of the interaction of cholesterol and sphingomyelin

Figure 3.34

ESR spectra of the cholestane spin label in a sphingomyelin film containing 50 mole % ergosterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, 20 °C, (B) hydrated film, 20 °C, (C) hydrated film, 37 °C.

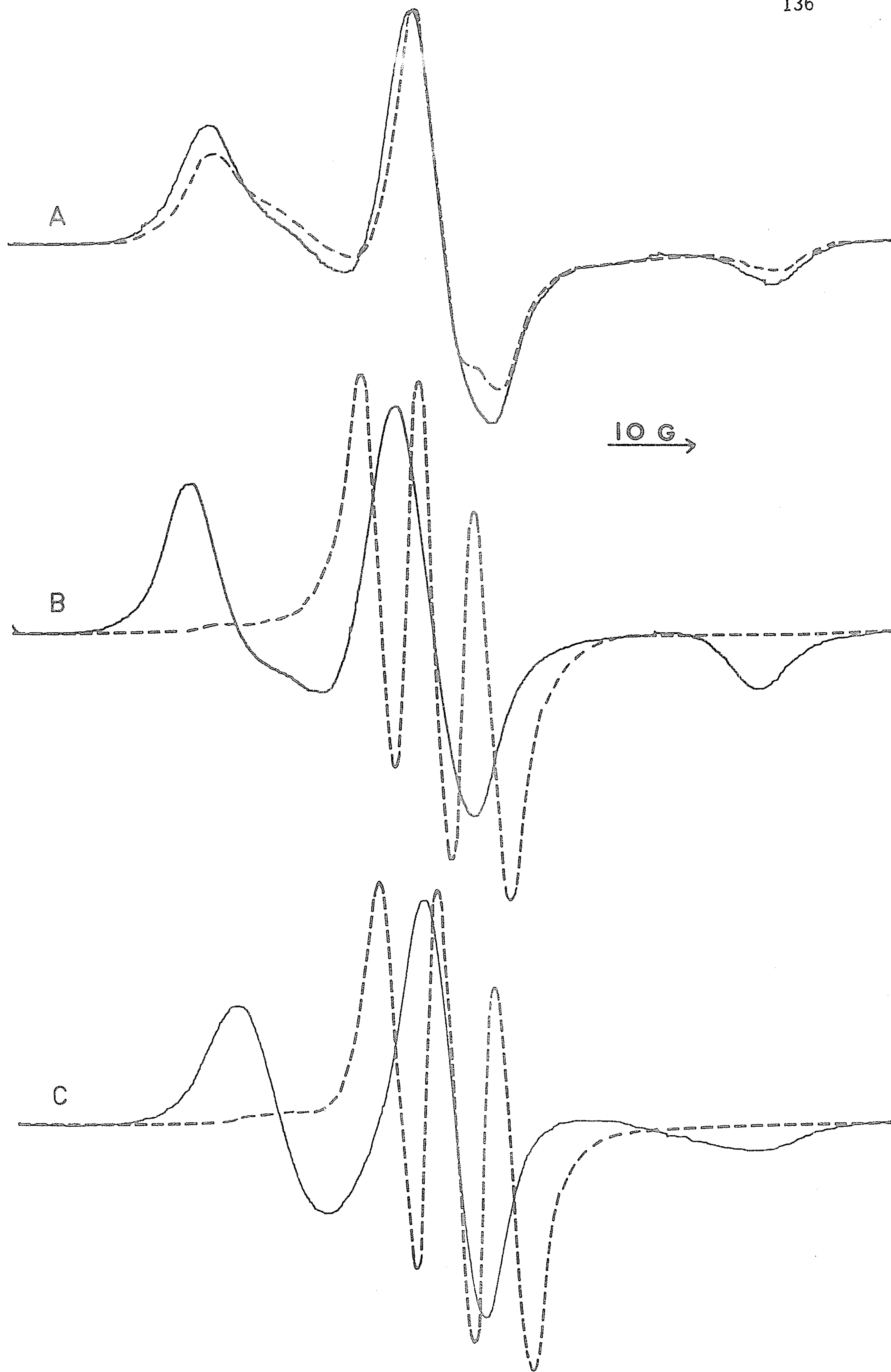
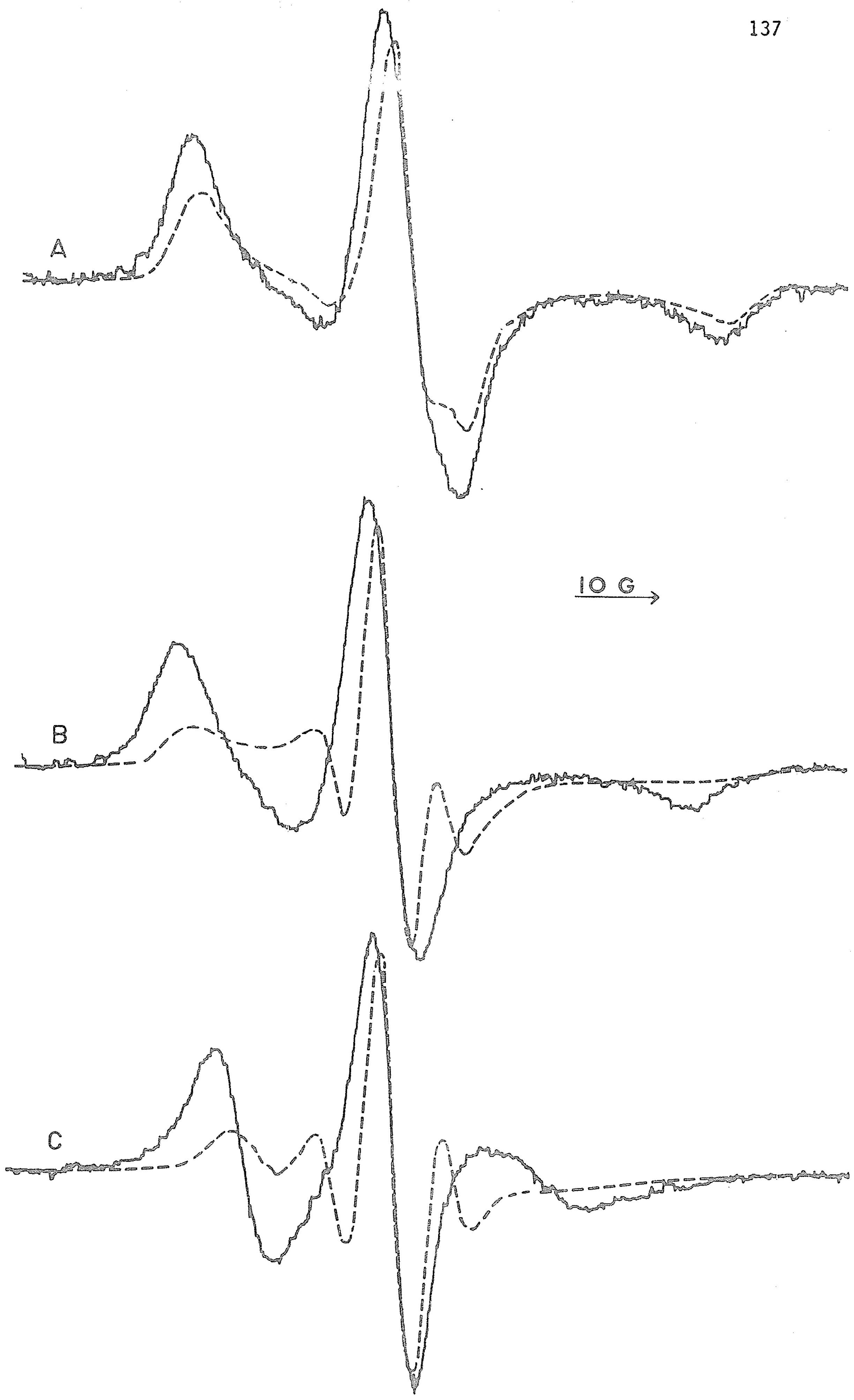

A
B
C
10 G

Figure 3.35

ESR spectra of the cholestane spin label in a sphingomyelin film containing 50 mole % 5α-androstane-3β-ol-17-one. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, 20 °C, (B) hydrated film, 20 °C, (C) hydrated film, 37 °C.


A
B
C
10 G

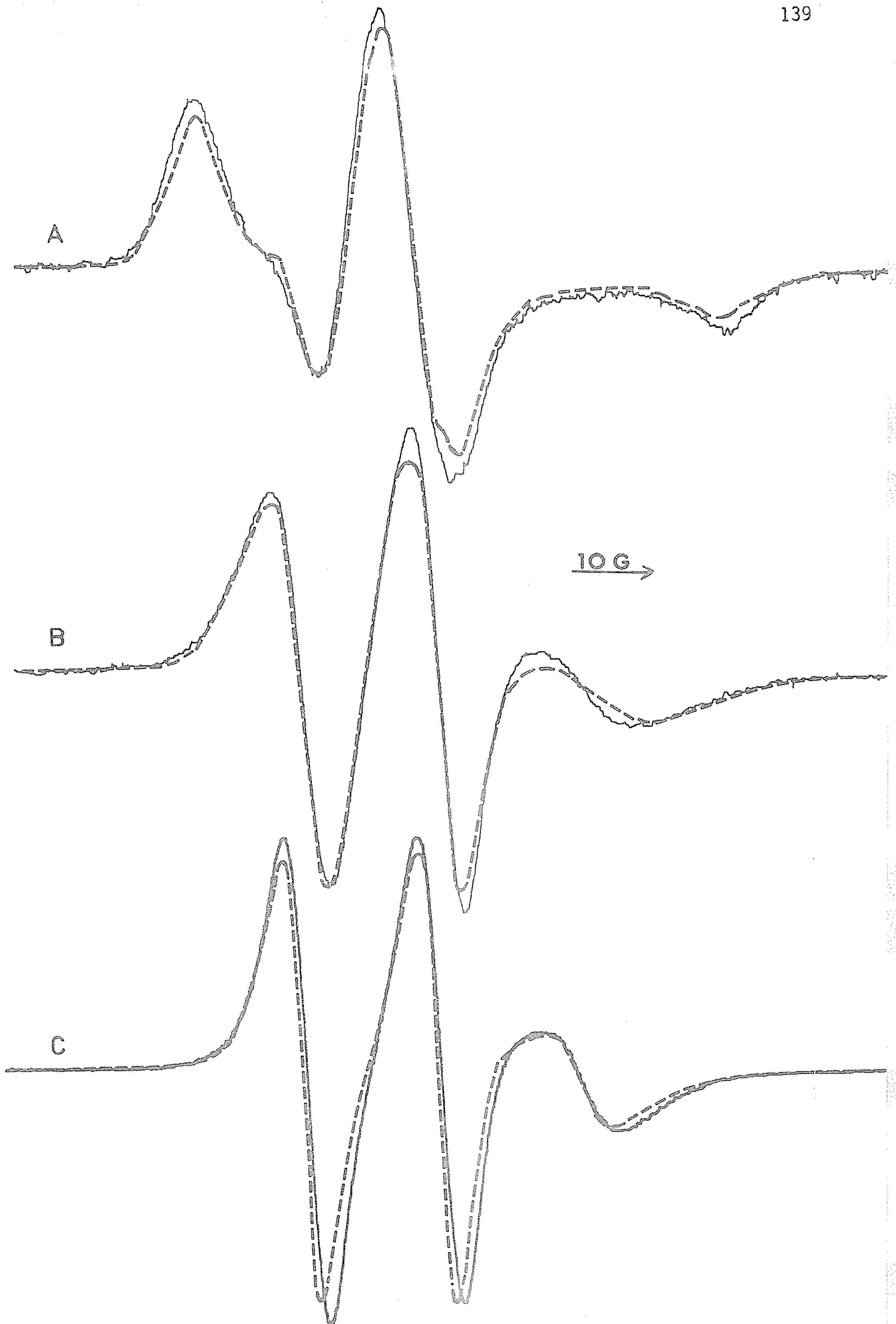
compared to the cholesterol-phosphatidylcholine interaction. To be most effective in aligning the molecules of the sphingomyelin films and maintaining their orientation above 30 °C, it is necessary for the C_3 substituent to be in the β configuration but the substituent need not be a hydroxyl group. Also, the solubility of a steroid in the sphingomyelin films does not appear to require the presence of a 3β-OH group. These observations strongly suggest that forces involving the 3β-OH group and sphingomyelin play a very minor role in the interaction and are of a much different nature and magnitude than the forces between the 3β-OH and phosphatidylcholine. These differences clearly must involve the differences in the chemical structure of the two phospholipids although the exact relationship is not clear. Intermolecular hydrogen bonding between sphingomyelin molecules may preclude any polar interactions between the sterol and sphingolipid. It would appear that hydrophobic forces between cholesterol and sphingomyelin involving the steroid nucleus and the C_{17} sidechain are most important for reducing the average deviation angle of the lipids and maintaining the bilayers in planar arrays at elevated temperatures.

(c) Stearamide Spin Label Results

The spectra of the stearamide spin label in a sphingomyelin thin film are displayed in Figure 3.36. The angular independent strongly immobilized spectra of the label indicate no alignment of the nitroxides and very slow motion of the piperidinyl rings, ($T_{//} = T_{\perp} = 32.8$ gauss). The slow motion of the nitroxides demonstrates the high viscosity of the local environment of the piperidinyl rings. Upon hydration

Figure 3.36

ESR spectra of the stearamide spin label in a sphingomyelin film. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, 20 °C, (B) hydrated film, 20 °C, (C) hydrated film, 37 °C.


A
B
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10 G

at 20 °C, there is a dramatic increase in the motion of the nitroxides. The nitroxides appear to be tumbling almost randomly at a rate sufficient to essentially average out anisotropic tensor components, ($T_{\parallel}$ = 17.4 gauss, $T_{\perp}$ = 16.8 gauss). The broadness of the lines compared with those of the spectra of the label in hydrated phosphatidylcholine indicate the motion of the nitroxides is slower in sphingomyelin.

The relative line width ratio of the spectrum is the same in both orientations with $\Delta H_{M-1} > \Delta H_{M+1} > \Delta H_{M\,0}$. Warming the hydrated film increases the motion of the nitroxides suggested by the narrowing of the spectra lines but there is little change in the orientation of the labels or hyperfine splittings. Figure 3.36C shows the spectra of the label at 37 °C, ($T_{\parallel}$ = 17.1 gauss, $T_{\perp}$ = 16.8 gauss). The hyperfine splitting and the relative line width ratio of the nitroxide spectrum is independent of the angle between the plane of the cell and the magnetic field. This is not surprising since the cholestane spin label results indicated that the film has an isotropic structure at 37 °C. The relative line width ratio found, $\Delta H_{M-1} > \Delta H_{M\,0} > \Delta H_{M+1}$, is typical of a distribution of nitroxides between hydrophilic and hydrophobic environments, having correlation times in the range of 10^{-9} seconds.

The spectra of the stearamide spin label in an equimolar sphingomyelin-cholesterol film are shown in Figure 3.37. The dry state spectra of the label are similar to those in sphingomyelin alone, indicating very slow motion and no preferred alignment of the nitroxides, ($T_{\parallel} = T_{\perp}$ = 33.5 gauss). Upon hydration of the film at

20 °C, the motion of the labels increases and the spectra become

Table 3.6

Spectral Parameters of the Stearamide Spin Label in Hydrated Sphingomyelin Films

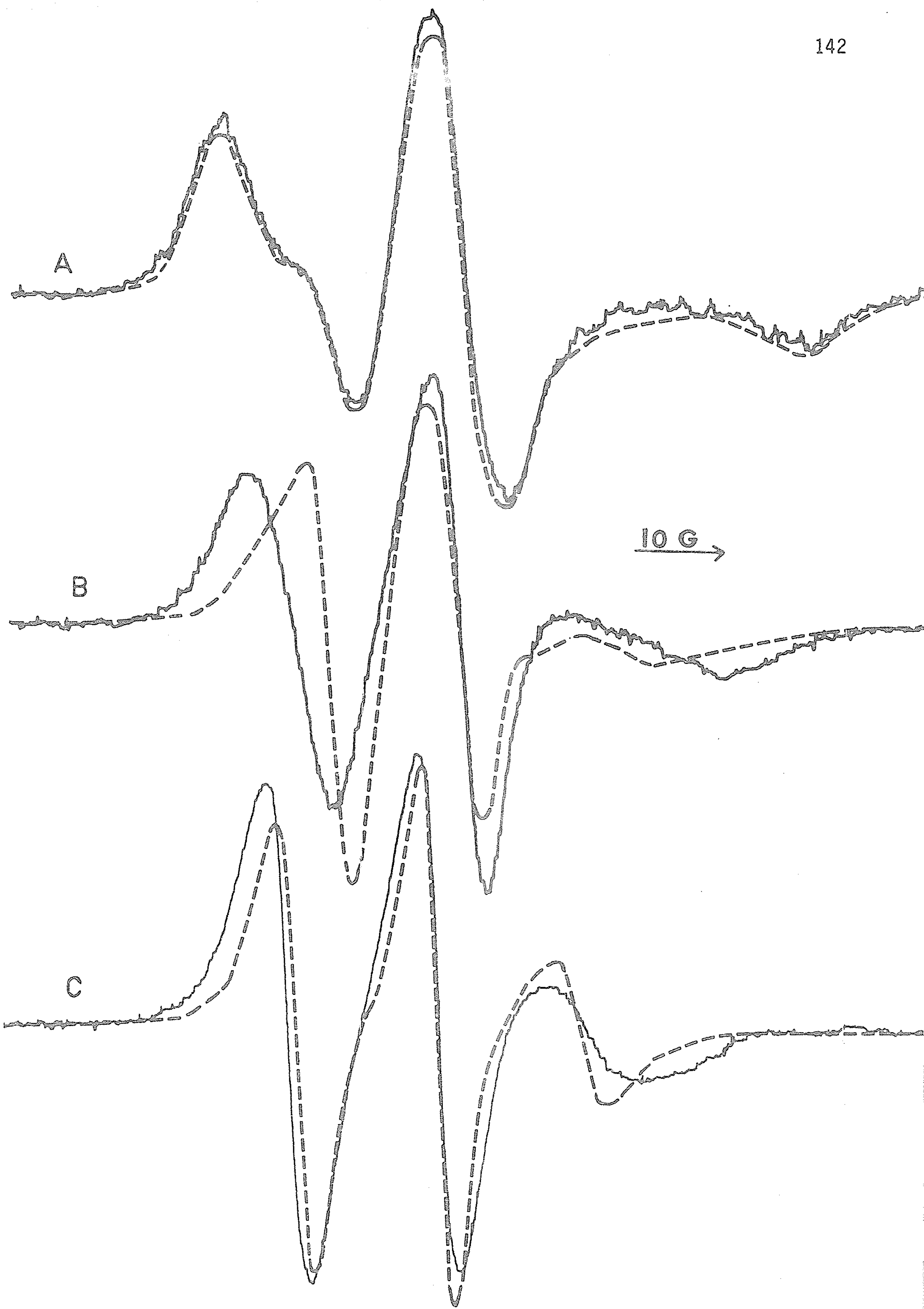
Mole % Cholesterol	Temp.	$T_{//}$	ΔH_{M+1}	$\Delta H_{M\,0}$	ΔH_{M-1}	$T_{\perp}$	ΔH_{M+1}	$\Delta H_{M\,0}$	ΔH_{M-1}
0	20 °C	17.4	6.4	5.8	14.2	16.8	6.6	6.1	12.3
0	37 °C	17.1	4.5	4.7	8.5	16.8	4.9	5.7	9.9
50	20 °C	14.2	5.2	6.6	9.9	18.5	9.5	5.9	19.5
50	37 °C	15.7	5.2	4.7	6.9	16.6	7.1	7.1	10.2

angular dependent, ($T_{//} \simeq 14.2$ gauss, $T_{\perp} \simeq 18.5$ gauss). Since $T_{\perp}$ is greater than $T_{//}$, we can conclude that the nitroxides are preferentially aligned with the direction of the N-O bond parallel to the symmetry axis of the multibilayers. As the temperature is increased, the lines sharpen denoting increased motion of the piperidinyl rings. At 37 °C, the spectra indicate some remaining degree of anisotropic motion, ($T_{//} = 15.7$ gauss, $T_{\perp} = 16.6$ gauss). For the parallel orientation, $\Delta H_{M-1} > \Delta H_{M+1} \simeq \Delta H_{M\,0}$ and for the perpendicular orientation, $\Delta H_{M-1} > \Delta H_{M+1} > \Delta H_{M\,0}$. Therefore, even at this elevated temperature, the relative line width ratio of the spectrum remains asymmetric but in this case, it is not apparent if this asymmetry arises from differences in environment polarity or not.

The stearamide spin label results corroborate the findings using

Figure 3.37

ESR spectra of the stearamide spin label in a sphingomyelin film containing 50 mole % cholesterol. The spectra were recorded with the plane of the film parallel, (———), and perpendicular, (-----), to the direction of the external magnetic field: (A) dry film, 20 °C, (B) hydrated film, 20 °C, (C) hydrated film, 37 °C.


A
B
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10 G

the cholestane spin label. In films of sphingomyelin and those incorporating cholesterol, the sphingomyelin molecules are more tightly packed in the headgroup regions than phosphatidylcholine at room temperature but as the temperature is increased, these regions become more fluid.

CHAPTER IV

SUMMARY AND CONCLUSIONS

In this study it has been found that:

(1) the motional freedom of a spin label in a phospholipid thin film is dependant on the extent of hydration of the phospholipids.

(2) cholesterol decreases the thermal motion of egg phosphatidylcholine molecules.

(3) for maximum ordering effect in egg phosphatidylcholine films, a steroid with a planar nucleus requires a hydroxyl group at C_3 in the β configuration and an eight carbon fully saturated chain at C_{17}.

(4) the solubility of a steroid in egg phosphatidylcholine bilayers depends on the polarity of the C_3 substituent.

(5) sterols having a polar substituent at C_{17} cause local disordering effects in hydrated egg phosphatidylcholine films.

(6) below approximately 37 °C, the intermolecular distance in sphingomyelin films is less than the intermolecular distance in phosphatidylcholine films at room temperature.

(7) planar multilayer arrays of hydrated sphingomyelin undergo an irreversible rearrangement to an isotropic structure over the temperature range ≈ 30 - 36 °C.

(8) cholesterol increases the fluidity of the paraffin chains of hydrated sphingomyelin at 20 °C but causes them to be more rigid at 37 °C.

(9) cholesterol increases the alignment of the sphingomyelin molecules near the polar regions of the bilayers at room tempera-

ture and maintains the bilayers in planar arrays up to at least 42 °C.

(10) the solubility of a steroid in sphingomyelin films does not depend on the polarity of the C_3 substituent.

(11) to be most effective in increasing the order in sphingomyelin films, a steroid with a planar nucleus requires a C_3 substituent, (not necessarily a hydroxyl group), in the β configuration, and a hydrocarbon chain at C_{17}.

(12) the interaction of cholesterol with a phospholipid is dependant on the chemical nature of the phospholipid.

CHAPTER V

SUGGESTIONS FOR FURTHER RESEARCH

The following studies would provide further information concerning the results of this work:

1) a further investigation of the disordering effect of ergosterol in hydrated phosphatidylcholine by chemical analysis.

2) a study of the permeability properties of sphingomyelin-cholesterol liposomes as a function of temperature may indicate a correlation between the thermal phase transition detected by the spin label technique and the permeability properties of the lipids.

3) a comparative study of the membrane properties of sphingomyelin derivatives in which the polar groups were systematically modified may clarify the nature of any intermolecular interactions.

4) nuclear relaxation studies of aqueous dispersions of sphingomyelin would provide further data on the motion of the polar and hydrophobic portions of the sphingomyelin molecule.

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