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EFFECTS OF CHOLESTEROL ON THE INFRARED DICHROISM OF PHOSPHATIDE MULTIBILAYERS

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SUMMARY

1. We have obtained polarized infrared spectra from multibilayers of lecithin ±cholesterol, lysolecithin and phosphatidylethanolamine deposited on AgCl plates.

2. The ester C=O stretching band (1730 cm^{-1}), the P=O stretching absorption (1250–1220 cm^{-1}) and the [C]–O–P deformation band (960 cm^{-1}) are found to be dichroic.

3. In the presence of cholesterol the P=O frequency shifts to lower frequency (1230 cm^{-1}) suggesting hydrogen bonding between the phosphatide P=O and the cholesterol hydroxyl.

4. Analysis of the dichroism indicates that addition of cholesterol to lecithin multibilayers induces a configurational rearrangement of the polar head groups. In this the transition vectors for the C=O, P=O and [C]–O–P bands change from 45° to 41°, 49° to 53° and 60° to 49°, respectively.

5. Owing to the hydrogen bonding the cholesterol ring portion would extend to carbon 6 from the ester linkages of lecithin, rigidifying the proximal apolar acyl chain segments.

INTRODUCTION

The lipids in the cellular membranes of animal cells consist principally of phosphatides and cholesterol. In the plasma membranes of many cells the molar ratio [cholesterol: phosphatide] approaches 0.5, but the association of cholesterol with membranes tends to be rather loose. Thus, the steroid exchanges rapidly with plasma lipoproteins *in vivo*[1] and with lipoproteins or phosphatide dispersions *in vitro*[2–4].

One generally assumes that membrane cholesterol does not interact significantly with membrane proteins. Such reasoning does not explain the established paradox that in many cells types, the steroid associates primarily with one membrane type, *e.g.* plasma membranes, but not others, *e.g.* mitochondria, while phosphatides not infrequently exhibit a different distribution[5]. In a single cell, all of its membranes should be equally accessible to the cell's lipid biosynthetic machinery over anything but very short time intervals. Anomalous cholesterol distributions thus implicate membrane-associated proteins, acting in either a structural and or catalytic manner.

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Such protein involvement might also explain the apparent clustering of cholesterol tangentially to and normal to the erythrocyte membrane[4].

Nevertheless, the steady-state association of cholesterol with biomembranes probably involves primarily membrane phosphatides, and we have accordingly examined the interaction of this steroid with diverse phosphatides by measuring the infrared dichroism of oriented phosphatide–cholesterol multibilayers. In this we extend earlier studies using nuclear magnetic resonance, differential thermal calorimetry and spin labelling (*cf.* review in ref. 6), documenting that cholesterol acts to reduce the fluidity of the hydrocarbon moieties of artificial phosphatide bilayers. By examining the infrared dichroism of the C=O, P=O and P–O–C vibrations of the phosphatides under diverse conditions, both for phosphatides and for phosphatide cholesterol mixtures, we show that this very likely involves polar interactions between the steroid–OH and phospholipids, as well as a probable rotation of the phospholipid acyl groups about their glycerol ester linkages, bringing the fatty acid chains into closer apposition. The last mechanism has also been recently suggested to explain the increasing spin–spin interaction of nitroxide–cholestane in phospholipid bilayers, with rising cholesterol proportions[7].

THEORY

The group frequencies for diverse intermolecular vibrations in phosphatides have been reported previously[8]. Important infrared-active linkages, which may exhibit infrared dichroism when the phospholipids are oriented with respect to the electrical vector of the polarized beam, are listed in Table I.

TABLE I

Vibration	*Frequency* (cm^{-1})
C= O stretching (glycerol–fatty acyl ester)	1730
P= O stretching (glycerol–phosphoryl–bases)	1250
[P]–O–C (glycerol–phosphoryldeformation)	1080–1050*
P–O–[C] (glycerylphosphoryl deformation)	960**

* The broad absorbance at 1080–1050 cm^{-1} may result from two overlapping bands.
** The 960 cm^{-1}, if of uncertain assignment, exhibits low intensity but strong dichroism.

To interpret the results of infrared polarization dichroism it is convenient to assume a hypothetical two dimensional crystal with frequency-dependent X and Y polarization axes. Then the transition moment of a given vibrational transition, P, and the polarization axes are confined to the X–Y plane (Fig. 1). If k_Y and k_X are the absorption coefficients along the Y and X axes respectively; θ, the angle between transition vector, P, and the Y axis, and ε_Y and ε_X the corresponding dielectric constants, the dichroic ratio, R, can be defined as[9]

$$R = k_Y/k_X = (\varepsilon_X/\varepsilon_Y)^{\frac{1}{2}} \cot^2\theta \qquad (1)$$

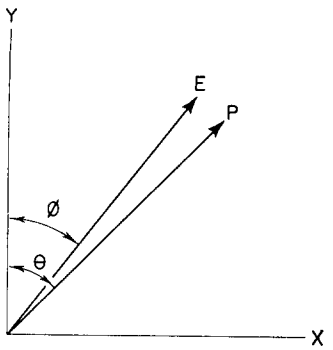


Fig. 1. Relation between the transition moment, P, the electrical vector E of the infrared beam, and the polarization axes X and Y. θ and ϕ are the angles of P and E with axis Y, respectively.

According R tends toward $\cot^2\theta$ as the value of $(\varepsilon_X/\varepsilon_Y)$ approaches unity at the center of an absorption band.

If the electrical vector, E, makes an angle ϕ with the polarization axis, Y, T_X and T_Y are the transmittances along the X and Y axes, and the absorbance, A, is given by

$$A = -\log(T_Y \cos^2\phi + T_X \sin^2\phi) \tag{2}$$

When the P is at 45° ($\theta = 45$ °), R equals unity; also $A = -\log T_Y$ when $T_X = T_Y$. Under such circumstances the absorbing band would be unpolarized. On the other hand, when P and E lie along the Y axis, $\theta = 0$, $R = \infty$, and a polarization maximum is observed. Simplifying, no polarized absorbance would be noticed when E is perpendicular to P and maximum polarized absorbance occurs when E lies parallel to P. At intermediate angles, polarized absorbance will depend on the projection of the P vector upon the Y axis.

To establish these conditions experimentally, one must first determine what polarizer angles yield maximum and minimum absorbance for a given sample. The orientation of the polarization axes, Y and X, can then be defined by the angles of minimum and maximum absorbance. In the case of phospholipid multibilayers, minimal absorbance occurs at 0°, *i.e.* when the electrical vector, E, is vertical, while maximum polarized absorbance occurs at 90°. Hence, the values of θ reported here are the angles between E and P.

We deposit the multibilayers as in many spin-labelling experiments, but using AgCl plates as supports. Our technique does not allow perfect orientation of the headgroups with regard to the polarization axis of the illuminating beam. This fact does not significantly influence our results where we compare the infrared dichroism of various bands with that of the other frequencies, as well as that of cholesterol-free films with multibilayers containing various proportions of the steroid.

When R equals unity, the vibrational dipoles are either non-dichroic or randomly oriented so that their individual components cancel out. An R value approaching 0 indicates that the dipole orientation tends to become perpendicular to the electric vector, and R values greater than 1 indicate an orientation approaching parallel to the electric vector.

To eliminate instrumental artefacts, we make measurements with the long axis of the AgCl plate both parallel and normal to the electric vector. We then calculate the values of θ for different bands, A significant devation of θ from the calculated value is noticed when the plate axis lies parallel to the electric vector. This can be explained as follows (Fig. 2):

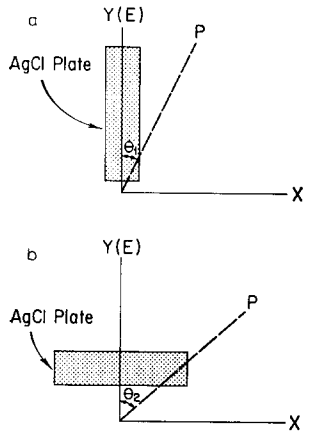


Fig. 2. (a) The long axis of AgCl plate is in parallel orientation and the vector P makes an angle of θ_1 with Y. (b) The long axis lies perpendicular to the Y axis and P is at an angle of θ_2.

Let us suppose that when the plate axis is parallel to the electric vector, θ equals θ_1. Then when the plate is rotated 90° the angle, θ_2, should be

$$\theta_2 = \theta_1 - (90 - \theta_1) + \theta_1 = 3\theta_1 - 90 \tag{3}$$

To test the validity of the above equation, assume $\theta_1 = 45°$, then $\theta_2 = (3 \times 45) - 90 = 45°$, hence no change should occur in the value of θ_1 by rotating the plate. Similarly, no change could be seen by rotating the plate if θ_1 has the value of 90 °. Intermediate angles should show a change in θ_2 as we observe experimentally with multibilayers, within an error of 1–2.

EXPERIMENTAL

Phosphatidylcholine, phosphatidylethanolamine, and lysolecithin are purchased from Lipid Products (Southnut Field, England) while the cholesterol was supplied by Sigma (St. Louis, Mo.). Multibilayers are deposited onto AgCl plates (Harshaw, Solon, Ohio) by evaporating the chloroform solutions of the phosphatides (10 mg/ml ± cholesterol). To provide the films with a smooth surface, we gently stroke the choroform solutions as they dry on the AgCl plates. We then dry the lipid films *in vacuo* for 1 h and follow this by soaking for 1–2 h in 0.1 M KCl. The spectra do not not change markedly upon soaking for more than 1 h. We drain the films after soaking, eliminating all but bound water, which can be detected by absorption (OH deformation) at 1680–1630 cm^{-1} and near 3360 cm^{-1}. The latter, intense band, due to OH stretching, lies at a lower frequency than found in liquid water because of H bonding. Concordantly, the intensities of both OH vibrations depends upon the thickness of the multibilayers. Also, they do not vary during the course of an experiment. We do

not know the extinction coefficient of the bound water but, assuming that it is identical to that of liquid water, our films typically contain water equivalent to a 100-Å thick layer. (Our calculations indicate that we typically deposit about 30 bilayers.)

Infrared spectra are recorded on a Perkin–Elmer 621 infrared spectrophotometer equipped with a common beam wire grid polarizer (Model 186-0187).

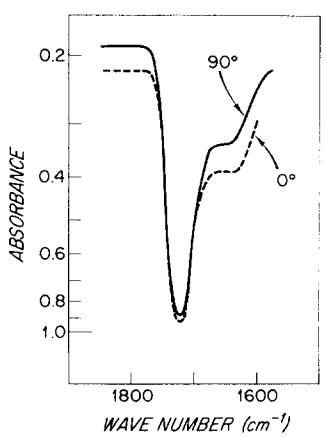
We control sample temperature in the spectrophotometer at 28 ± 1 °C and relative humidity approx. 55% by a stream of air across the film.

Since phosphatides possess considerable axial asymmetry, *i.e.* they are much longer than wide, one might expect these molecules to orient parallel to the shearing direction when stroking is unidirectional during drying. Then their various molecular vibrations should have a shear dependent dichroism. We evaluate this possibility by applying our samples with fluid shear parallel to the long axis of the AgCl plate, but examining this in two, mutually perpendicular directions. We could observe no shear dependent changes in dichroism. This fact cannot be explained by assuming that the lipids deposit as micelles with spherical symmetry, since this would exclude dichroism for any vibration. Two possibilities remain: (1) Our shearing methods, while sufficient to orient large molecules such as synthetic polypeptides, fail to provide a preferential orientation of the lipids. (2) The lipids orient with their molecular axes normal to the plate, as in multilayers deposited by the Blodgett[10] method. In this arrangement one would not detect shear-related dichroism, but bond dichroism would be significant for the vibrations in question, since these inevitably exhibit substantial vectors out of parallel with the molecular axes.

RESULTS

Infrared dichroism of phosphatidylcholine multibilayers

Figs 3 and 4 and Table II illustrate the infrared dichroism of oriented egg



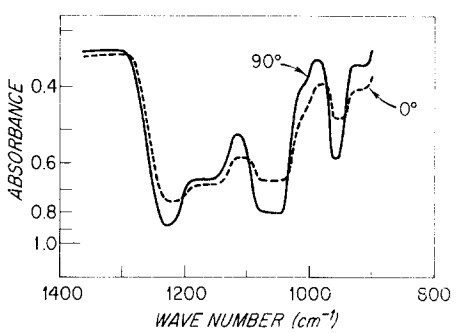


Fig. 3. Polarized infrared spectrum of hydrated egg lecithin multibilayer (from 1800 cm^{-1} to 1600 cm^{-1}). The peak at 1730 cm^{-1} represents the C= O stretching vibration of the ester linkages. The solid line gives the spectrum measured at 90° to the electrical vector E and the dotted line obtains when E is parallel, or $\phi = 0$.

Fig. 4. Polarized absorption infrared spectra of hydrated egg lecithin films. The peaks represent different phosphate bands. – – – –, $\phi = 0$, and ———, $\phi = 90°$.

TABLE II

Presumptive vibration	*Frequencies* (cm^{-1})	*Transition moment* (θ, °)*			
		Lecithin	*Lecithin–cholesterol***	*Lysolecithin*	*Phosphatidyl-ethanolamine*
C=O	1730	45	41	40	43
P=O	1250–1230	49	53	52	52
P–O–[C]****	960	60	49	62	—

* The values of θ reported vary within ± 1–2°.

** Films contain 50 moles percent cholesterol.

*** This band for phosphatidylethanolamine is broad and is near 1000 cm^{-1} in the polarization measurements. See text.

lecithin multibilayers. Poorly hydrated films, *i.e.* those showing minimal water absorption near 3600 and 1600 cm^{-1} demonstrate no significant dichroism at 1730 cm^{-1} and 1250–1230 cm^{-1} (P=O), but yield a dichroic ratio, R, of 0.38 at 960 cm^{-1}. However, after soaking the film for up to 2 h in 0.1 M KCl, the spectra change significantly. First, a broad band appears at 1680–1630 cm^{-1}, presumably due to the –OH deformation vibration of absorbed water. Second, the 1730 cm^{-1} (ester C=O stretch) has a θ value of 45°. Third, the P=O stretching band becomes dichroic between 1250–1220 cm^{-1}, with a θ value of 49°. Fourth, the P=O stretching band shifts to a lower frequency. This effect varies directly with the water absorption at 1680–1630 cm^{-1}. Finally, the dichroism at 960 cm^{-1} shows a θ value of 60°. This band, most reasonably attributed to P–O–[C], thus appears to align in some preferential orientation in a state approaching dehydration, but loses this feature upon exposure to excess water and/or physiologic ionic strength.

Lysolecithin

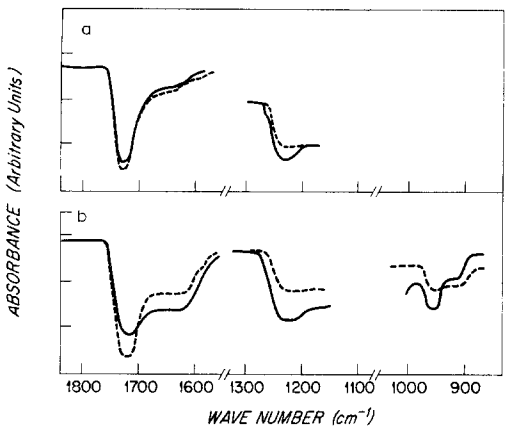
Lysolecithin films exhibit dichroism of the C=O (1730 cm^{-1}), P=O (1240–1230 cm^{-1}) and P–O–[C](960 cm^{-1}) bands, yielding θ values of 40°, 52°, and 62° respectively. Apparently, the head groups arrangement, particularly that of C=O and P=O, is different from that in lecithin multibilayers. The band appearing at 960 cm^{-1} has almost the same θ value as found in lecithin bilayers.

Phosphatidylethanolamine

Phosphatidylethanolamine reveals similar infrared spectra as lecithin. The ester C=O stretching band at 1730 cm^{-1} and P=O at 1230–1220 cm^{-1} show dichroism in hydrated phosphatidylethanolamine films. The band for P–O–[C] is not very well resolved due to the overlapping with other frequencies and weak intensity, both of which prevented dichroism measurements. The θ values (Table II) for C=O and P=O are 43° and 52°, respectively.

Lecithin–cholesterol

Addition of cholesterol to lecithin influences the infrared dichroism (Fig. 5). At 50 mole percent cholesterol θ changes from 45° to 41° for C=O; from 49° to 56°



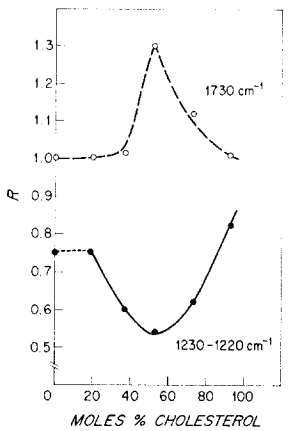


Fig. 5. Polarized infrared absorption curves of multibilayers of egg lecithin–cholesterol (a) before and (b) after treatment with 0.1 M KCl. The cholesterol proportion is 50 moles percent. The bands at 1730 cm^{-1}, 1230–1220 cm^{-1} and 960 cm^{-1} represent C=O stretching, P=O stretching, and P–O–[C] deformation. Solid curves apply when E is at 90° ($\phi = 90°$) and dotted lines when E is at 0° ($\phi = 0°$).

Fig. 6. Relation between R, the dichroic ratio, and cholesterol proportion. The R values have been calculated for the C=O, P=O stretching bands (1730 cm^{-1} and 1230–1220 cm^{-1}). The amount of egg lecithin applied was kept at 11 mg (14 mM) and the cholesterol proportion increased from 0 to 5 mg (0–13 mM). Dichroic spectra were recorded from multibilayers soaked in 0.1 M KCl. We assume a mol. wt of 780 for lecithin.

for the P=O band; and from 60° to 49° for the P–O–[C] band (Table II). The relation between cholesterol proportion and R is shown in Fig. 6. The curve indicates that the value of R and hence θ changes with cholesterol concentration until the proportion of cholesterol in the lecithin multibilayer is 50 moles percent. Lapper *et al.*[11] reported that at this cholesterol proportion maximally orients lecithin cholesterol films. The infrared spectrum of lecithin–cholesterol shows a frequency shift in the P=O band from 1250 cm^{-1} to 1230 cm^{-1}, but the positions of the other bands remain unaltered. The observed change in the P=O frequency is best attributed to a hydrogen bonding as predicted by Zull *et al.*[12]. Then the electrostatic attraction between the phosphatide group of lecithin and hydroxyl group of cholesterol would alter their relative orientations and hence in their values of θ.

DISCUSSION

The calculated angles, θ, of the transition moments for the normal modes of the C=O, P=O, and P–O–[C] vibrations do not necessarily coincide with the bond orientations within the multibilayers. In the case of lecithin the C=O moment lies at 45° with respect to the electrical vector of the polarized beam. For the sake of discussion, let us consider that this also represents the bond orientations. Then our data can be explained in two ways: either both the ester C=O groups are arranged randomly or they are oriented at a net angle of 45°. In the latter case, we must evaluate two further possibilities: (a) both the groups are oriented at 45°, (b) the groups are perpendicular to each other. In both cases the value of θ will be 45°.

P=O and P-O-[C]groups of lecithin multilayers on AgCl plates exhibit transition angles of 49° and 60°, respectively. These appear reasonable values also for the bond angles, assuming an extended orientation of the head groups as recently reported by Phillips *et al.*[13]. The significant changes of θ for the P-O-[C] group upon hydration and dehydration suggest that water binds in the vicinity of the P-O-[C] and P=O linkages.

Addition of cholesterol changes the angle of the C=O transition moment from 45° to 41°, that of P=O from 49° to 53° and that of P-O-[Cz from 60° to 49°, reflecting the intramolecular rearrangements which proceed during the association of cholesterol with phospholipid. Fig. 7 schematizes possible configurational changes occurring in lecithin head groups upon cholesterol addition. A reasonable explanation for these findings is hydrogen bonding between the P=O of lecithin and the -OH of cholesterol. This can alter the P=O angle to 53°, *i.e.* closer to the *X*-axis and would require a concomitant reorientation of the C=O groups. Thus, Rothman and

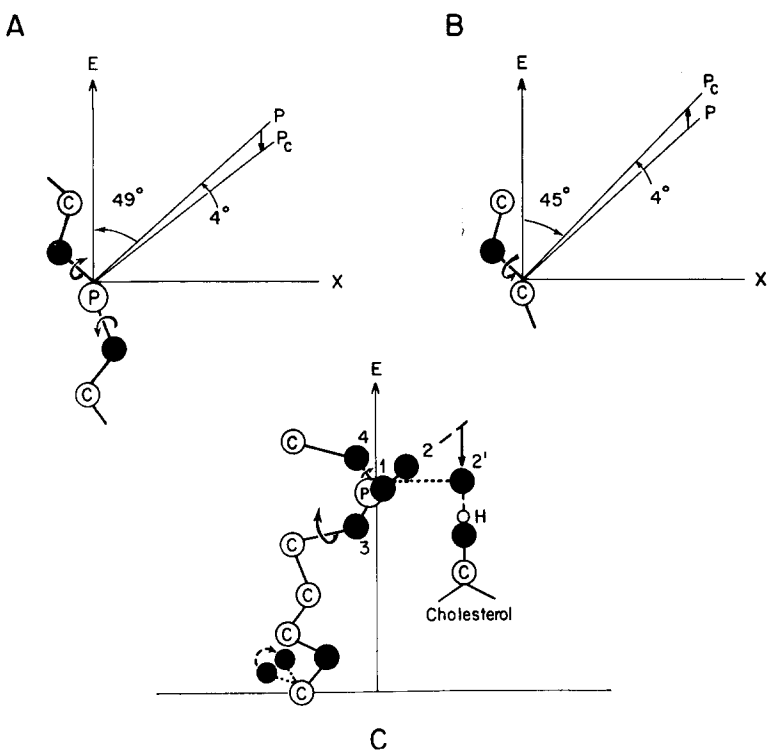


Fig. 7. Schematic representation of the configurational changes occurring in the polar head groups of lecithin upon the addition of cholesterol. In (A) and (B) *P* and P_c denote the transition vectors of P= O and C= O in lecithin and lecithin-cholesterol multibilayers. In (C) we illustrate formation of a hydrogen bond between P= O (oxygen atom No. 2) of lecithin, oriented at an angle of 49° (A), and the -OH of cholesterol. Rotation of two O-P bonds (oxygen atoms marked 3 and 4) takes place concomitantly and causes a change in their transition vector from 60° to 49° (not indicated). ● denotes an oxygen atom. Other atoms are identified by the letters within the circle.

Engleman[14] recently proposed a molecular model for the interaction of cholesterol with lecithin supporting the view [15] for the existence of an intermediate fluid condition. They integrate existing data obtained by nuclear magnetic resonance, differential calorimetry, X-ray diffraction as well as spin-label studies, and conclude that all indicate an association of the ring portion of cholesterol with the upper apolar region of lecithin, rigidifying this, but allowing the fatty acid terminal to remain mobile. Mendelsohn, studying Laser–Raman spectra of egg lecithin and egg lecithin–cholesterol mixtures supports their view[16], but neither he nor Rothman and Engelman specify what molecular features of cholesterol underlie its effect.

Our data provide some of the needed clues for the multibilayer model. They indicate that the –OH of cholesterol interacts with the phosphatide P=O electrostatically. This localizes the steroid ring so that it does not extend beyond C-6 from the C=O groups; concomitantly, the acyl chains rotate about the latter and pack more tightly, as suggested by spin-label experiments[17]. Also, the interaction of the phosphatide with cholesterol very likely causes the polar head groups to assume a *gauche* configuration[18,19]. The net change in θ approximates 4° for the P=O moment and 11° for P–O–[C]. This suggests that the two OdP bonds rotate upon cholesterol addition, correlating with the prediction of Gupta and Govil[20], based on their extended Hückel calculations on phospholipid configurations.

Recently Kruyff *et al.*[21] have explored the interactions of phosphatides with cholesterol and cholesterol analogues in monolayers and liposomes by measurements of force–area curves and differential scanning calorimetry, respectively. They interpret their data to indicate that neither the acyl ester linkages of phosphatides nor the various phosphorous–oxygen bonds are essential for sterol–phosphatide interactions. They further argue against hydrogen bonding between the sterol –OH and any polar part of the phosphatide.

These data, based on the general interactions of cholesterol and its analogues with phosphatides and some of their analogues, do not necessarily contradict our own, which deal very specifically with the reaction of cholesterol with certain phosphatides. Moreover, the NMR data of Darke *et al.*[22] on lecithin–cholesterol–^2H_2O systems clearly suggest the likelihood of hydrogen bonding between the 3β-OH of cholesterol and phosphate–oxygen linkages of lecithin.

We agree with Kruyff *et al.*[21] and others (see review in ref. 5) measuring signals emanating directly from phosphatide acyl chains that these interact strongly with the apolar moiety of cholesterol, but we believe that information as to possible interactions between phospholipid and cholesterol has hitherto been ambiguous.

Assuredly lipids lacking the polar moieties of lecithin can bind sterols strongly by apolar forces. However, we question whether such models properly represent the mutual interactions of cholesterol with a phosphatide. Indeed, our infrared studies and NMR data[22], indicate that in that case polar bonds between the phosphatide phosphorous and sterol –OH modulate the apolar interactions between the two molecular species in a way not dectable by the methods of Kruyff *et al.*[21].

ESR studies indicate that the 3β-OH group is essential for optimal orientation of phosphatide–cholesterol multibilayers[23] and one cannot explain such data without invoking polar phosphatide–cholesterol interactions. It thus appears that a full understanding of the relationship between cholesterol and diverse phospholipids can only be attained through discerning measurements of specific signals emanating

from both the polar and apolar moieties of the two lipid species. We shall shortly present further infrared studies which deal with this matter.

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