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Biochimica et Biophysica Acta 1514 (2001) 318–326

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Attenuated total reflection (ATR) Fourier transform infrared spectroscopy of dimyristoyl phosphatidylserine–cholesterol mixtures

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Received 14 March 2001; received in revised form 27 June 2001; accepted 9 July 2001

Abstract

Mixtures of cholesterol with dimyristoyl phosphatidylserine or deuterated dimyristoyl phosphatidylserine were investigated by polarized and non polarized attenuated total reflection (ATR) Fourier transform infrared (FTIR) Spectroscopy. From polarized spectra the dichroic ratios of various vibrations as a function of cholesterol were calculated. Dichroic ratios of methylene vibration (CH_2) 2934 cm^{-1} of cholesterol decreases with increase of cholesterol concentration leveling off in the region where cholesterol phase separation takes place. The orientation of deuterated methylene (CD_2) symmetric and asymmetric bands of the deuterated dimyristoyl phosphatidylserine is influenced little by cholesterol. In the polar region of dimyristoyl phosphatidylserine no effect of cholesterol on the dichroic ratios of carbonyl (C=O) and asymmetric phosphate (PO_2^-) vibrations were detected. For nonpolarized spectra the broad bands in the polar region of the phospholipid were deconvoluted. The carbonyl band (C=O) in pure dimyristoyl phosphatidylserine is composed of five bands; in the presence of increasing concentrations of cholesterol conformational change of these vibrations takes place evolving into one predominant band. Similar conformational change takes place in the presence of 75 molecules water/molecule DMPS. For the asymmetric phosphate band very small shifts due to interaction with cholesterol were detected.

Keywords: ATR; DMPS; Cholesterol

1. Introduction

Phosphatidylserine and cholesterol are essential constituents of eukaryotic membranes. Phosphatidylserine is negatively charged at neutral pH and cholesterol comprises up to 42 mole% of the total lipid content of the biomembranes. In bilayers of negatively charged phospholipids, the solubility of cholesterol is limited, with concomitant separation into a mixed phospholipid–cholesterol phase and a crystalline cholesterol phase as detected by differential scanning calorimetry (DSC) and X-ray diffraction experiments [1].

It was shown that the onset of phase separation of cholesterol from phospholipid–cholesterol mixtures depends on the charge of the phospholipid on hydrogen bonding in the headgroup region as well as on the length and degree of saturation of the acyl chains. In dimyristoyl phosphatidylserine (DMPS)–cholesterol bilayers the onset of cholesterol phase separation as detected by X-ray diffraction is at mo-

Abbreviations: ATR, attenuated total reflection; FTIR, Fourier transform infrared spectroscopy; DMPS, dimyristoyl phosphatidylserine; DMPS-d54, dimyristoyl phosphatidylserine with fully deuterated acyl chains; *R*, dichroic ratio

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0005-2736/01/$ – see front matter
PII: S0005-2736(01)00388-1

lar ratio DMPS/cholesterol of 2:1 and 1.7:1 in the gel and in the liquid crystalline states of the phospholipid, respectively [1].

Previously we have investigated aqueous dispersions of phosphatidylserine from bovine spinal cord or DMPS with cholesterol by transmission Fourier transform infrared spectroscopy [2]. Small shifts of the peak frequency of the symmetric and asymmetric methylene stretching bands of the phosphatidylserines due to interaction with cholesterol were detected. Effect of cholesterol on the CH_2 bending bands (1465–1457 cm^{-1}) of the phospholipids was also detected.

In the present study we have extended this investigation by using attenuated total reflection Fourier transform infrared spectroscopy (ATR FTIR) and deuterated DMPS. Working with deuterated dimyristoyl phosphatidylserine (DMPS-d54) enables to determine the independent contributions of the interaction between the lipids on the CH_2 vibrations of cholesterol and of DMPS. Dicko et al. [3] used deuterated dimyristoyl phosphatidylcholine in the investigation of this phospholipid with various sterols.

From polarized ATR measurements of oriented multilayers of DMPS-d54 we have calculated the dichroic ratios (R) of the various vibrations in the presence and absence of cholesterol. In the case of some polarized and nonpolarized spectra an attempt was made to deconvolute the bands for detecting vibrations not resolved by the instrument and to investigate the effect of cholesterol on the various bands.

2. Materials and methods

Dimyristoyl phosphatidylserine (DMPS) (sodium salt) and DMPS-d54 (sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was from Nu-Check-Prep. (Elysian, MN). The phospholipids and cholesterol were dissolved in chloroform/methanol (2:1 v/v) and mixed at appropriate ratios.The solvents were driven off by a stream of nitrogen and the samples were kept under high vacuum for 3 h. To the phospholipid or phospholipid–cholesterol mixtures H_2O or in some cases D_2O were added to give concentration of about 10 mg/ml or 40 mg/ml phospholipid for ATR or transmission IR, respectively. The dispersions were incubated for 1 h at 55°C with frequent vortexing.

2.1. ATR

For ATR and polarized ATR experiments aligned layers on the surface of germanium prism (5 cm, 0.2 cm, 45°) were obtained by spreading a measured volume of aqueous suspensions of the phospholipid or phospholipid–cholesterol mixture (about 0.7 mg phospholipid on an area of about 5 cm^2 on each side of the prism) obtaining a surface layer of about 1 μm thick and aligning the lipid layers with plastic tip in the course of water evaporation. Evaporation was carried out in an oven. After short drying about 10–20 molecules of water were left per molecule of phospholipid as obtained from the water–phospholipid calibration curves [4]. The wet lipid layers were sealed with a rubber frame and spectra were recorded. At the second stage water was added into the rubber frame, wetting the lipid film by diffusion of water vapor. When an equilibrium was reached 30–50 molecules of water/phospholipid molecule were detected. It was observed that when water was present in large excess, the orientation of lipid layers was probably lost as erratic polarization ratios were obtained. After spectra were recorded the cell was kept in an oven for 1.5 h at 75°C; the water was completely removed as no water band at 3400 cm^{-1} was detected, and spectra for the dry samples were recorded.

For polarized ATR spectra a Graseby/IR grid polarizer (CBS, Suffolk, UK) was used.

2.2. Transmission IR

To eliminate water interference on IR spectra these measurements were performed in D_2O. The protocol was as follows. About 1 mg of dispersion of DMPS or DMPS–cholesterol mixtures in D_2O was applied to CaF_2 window (Buck Scientific, East Norwalk, CT). The CaF_2 window was kept under a stream of N_2 for 2 h, transferred to vacuum desiccator and kept for additional 2 h at about 40°C. Subsequently 5 μl of D_2O were applied to the disc, and covered with another CaF_2 window with Mylard spacer (DuPont, Geneva, Switzerland) between the windows. The cell was wrapped with parafilm to

eliminate entrance of water vapor, and IR spectra were run immediately.

All the IR experiments were performed on a 1600 Perkin Elmer FTIR spectrophotometer with 4 cm^{-1} resolution; 256 scans between 4000–900 cm^{-1} were collected. The measurements were performed at room temperature, below the melting temperature of the phospholipid.

2.3. Data analysis

The dichroic ratio R was calculated as in [5]

$$R = A(\parallel)/A(\perp) \quad (1)$$

where A denotes absorbance measured at 0° and 90°, respectively.

Separation of overlapping bands was obtained by deconvolution using Jandel Scientific Peakfit programs and Lorentzian function.

As DMPS-d54 contains two CH_2 groups (on the glycerol as only methylene groups of the hydrocarbon chains are deuterated) to obtain CH_2 vibration (about 2934 cm^{-1}) of cholesterol only from the mixtures with DMPS-d54, a correction has to be made. This was achieved in two ways: (i) From the spectra of the mixtures at 0° and 90° the respective spectra of DMPS-d54 were subtracted and the dichroic ratios calculated. (ii) All the spectra were deconvoluted in the range 4000–2250 cm^{-1}, the absorptions of the CH_2 band (2934 cm^{-1}) were measured and dichroic ratios calculated as a function of the input cholesterol concentration. The raw data contain contribution of CH_2 vibration from DMPS-d54 (2/molecule) and from CH_2 of cholesterol (11/molecule). To obtain the dichroic ratio (R_2) of CH_2 vibration of cholesterol the contribution of CH_2 vibration of glycerol has to be taken into account in the measured dichroic ratios. Corrected dichroic ratios R_2 for cholesterol are given by Eq. 2 developed according to [6].

$$R_2 = \frac{R_1 \cdot G(x_2-1) + R(G+x_2)\cdot R_1}{R_1 + (x_2-1)\cdot R + x_2 \cdot G} \quad (2)$$

where R_1 denotes the measured dichroic ratio for DMPS = 2.29; G for germanium prism with an angle of incidence 45° and with refractive index 4 and a lipid layer with a refractive index 1.4 = 1.45 [8]. x_2 denotes molar fraction of cholesterol in the mixture corrected for the number of CH_2 groups in DMPS molecule −2, in cholesterol molecule −11, and R denotes measured dichroic ratio for each mixture.

In this evaluation it is assumed that the dichroic ratio for CH_2 vibrations of glycerol is not affected by the presence of cholesterol. This assumption is not severe as at high cholesterol concentrations where the effect of cholesterol on the measured dichroic ratio of the CH_2 vibrations may become significant, the concentration of the CH_2 vibrations and their contribution to the overall dichroic ratio becomes negligible.

3. Results and discussion

3.1. Summary and comparison of various methods used for preparation of phospholipid–cholesterol mixtures

Recently two papers appeared discussing artifactual phase separation of cholesterol from mixtures with phospholipids prepared by conventional method [7,8]. Huang et al. [7] investigated mixtures of phosphatidylcholines or phosphatidylethanolamines with cholesterol, dissolving the lipids in chloroform only and evaporating the solvent at room temperature produced samples that gave very irreproducible results with respect to phase separation. Huang et al. [7] developed new method for preparation of cholesterol–phospholipid samples, spraying the lipid mixture into water resulting in rapid exchange of organic solvent by water. By this method very high solubility of cholesterol in zwitterionic phospholipids was obtained; however, problems with detection of unstacked cholesterol patches by X-ray diffraction may introduce an overestimation of the solubility limits of cholesterol [9].

McMullen et al. [8] investigated mixtures of saturated phosphatidylserines with cholesterol. The lipids were dissolved in chloroform/methanol 2:1 and the solvents were evaporated by N_2 at temperature of 40–50°C. McMullen et al. [8] claim that cholesterol crystallites were not detected in mixtures of DMPS with cholesterol.

In our experiments we use a mixture of chloroform/methanol 2:1 for dissolving the lipids, evaporating the solvents at room temperature (conventional method) and hydrating the dry lipids by

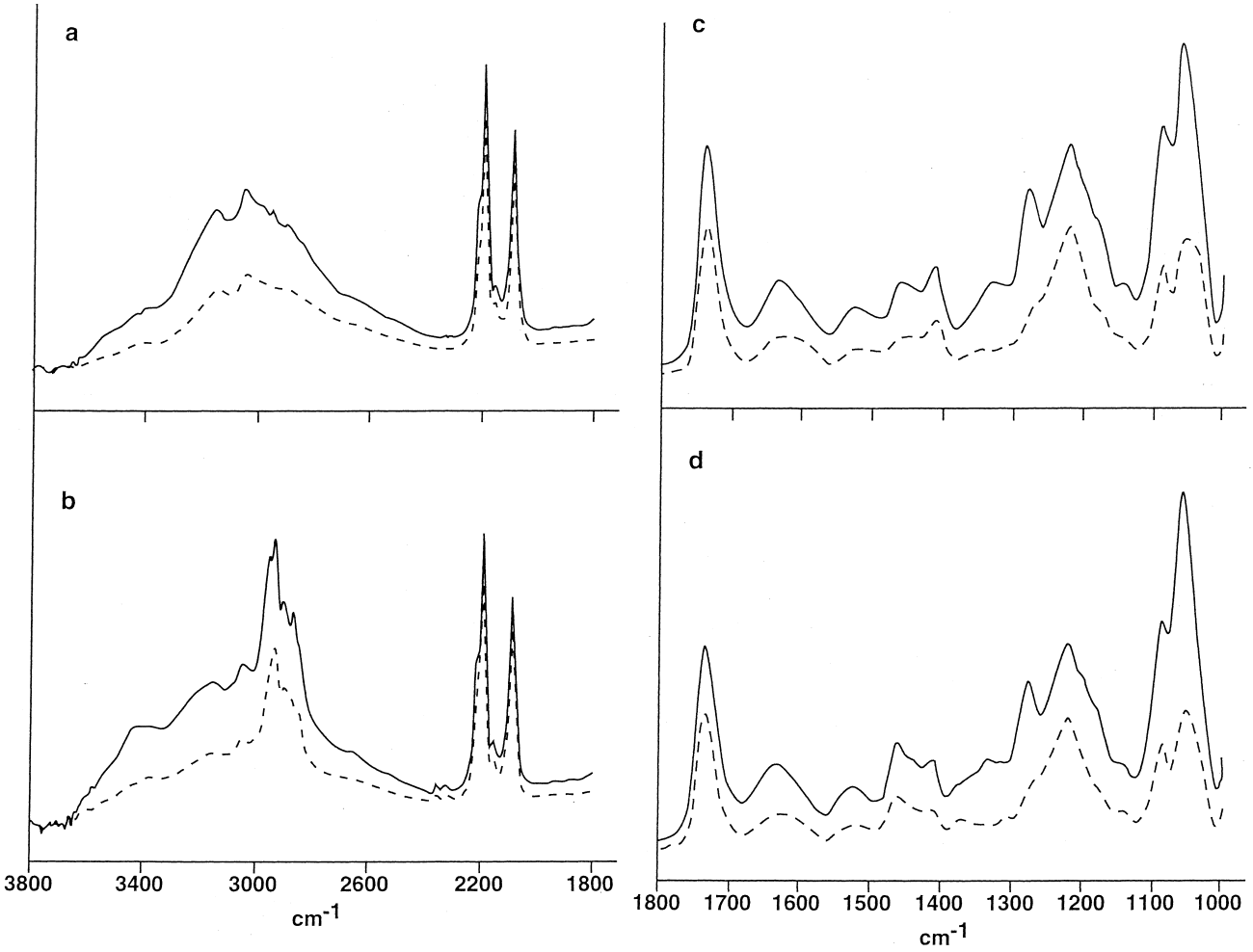

Fig. 1. Polarized ATR spectra of DMPS-d54 alone (a,c) and DMPS-cholesterol mixtures (b,d) DMPS-d54 cholesterol mixture molar fraction of cholesterol X(chol) = 0.4 full line 0°, broken line 90°. Spectra were smoothed using Perkin Elmer software.

incubating for 1 h at temperatures at least 10°C above the melting temperature of the phospholipid. Reproducible results were obtained using this protocol showing that phase separation of cholesterol is a function of the host phospholipid. Miscibility of cholesterol in negatively charged phospholipids can be decreased by adding various components to the hydrating medium, e.g., Ca^{2+} ions [10]. Already in 1992 we have shown that mixtures of 1-stearoyl-2-oleoyl phosphatidylserine with cholesterol dissolved in benzene, quickly frozen and lyophilized for several days, show similar properties with respect to phase separation of cholesterol as mixtures dried from chloroform/methanol 2:1 [11]. As benzene is removed from a solid state the probability that the lipid components will separate during lyophilization is very low.

We have prepared mixtures of several phosphatidylserines with cholesterol–DMPS, 1-palmitoyl-2-oleoyl phosphatidylserine, 1-stearoyl-2-oleoyl phosphatidylserine and natural phosphatidylserine using McMullen's procedure [8] and the conventional method [12]. In all cases cholesterol crystals were detected.

To conclude, phase separation of cholesterol in DMPS–cholesterol mixtures as shown in [1] is a real property of the system.

3.2. *Polarized ATR of DMPS-d54–cholesterol mixtures*

In Fig. 1 are presented polarized ATR spectra of DMPS-d54 alone and in the presence of cholesterol, molar fraction of cholesterol (X(chol)) = 0.4. As the

acyl chains are fully deuterated the asymmetric and symmetric stretching CH_2 vibrations are shifted from 2920 and 2850 cm^{-1} to 2198 and 2091 cm^{-1}, respectively. A very small band at 2940 cm^{-1} due to stretching of CH_2 band of glycerol is also seen. This band is much stronger on Fig. 1b as it also contains the contribution from the stretching vibration of cholesterol. The number of water molecules/molecule of phospholipid in these spectra is about 10–20 as obtained from the ratio of water band at 3400 cm^{-1} to the asymmetric phosphate stretching band and the calibration curves from [4]. The dichroic ratios (R) for various vibrations were calculated from spectra recorded at several molar fractions of cholesterol.

In Table 1 are presented dichroic ratios (R) for several vibrations in DMPS-d54. alone. Brandenburg and Seydel [13] and Hubner and Mantsch [14] reported dichroic ratios for various bands of synthetic and natural phospholipids and sphingolipids but not for phosphatidylserines. The reported dichroic ratios for CD_2 vibrations (Table 1) are similar to those shown in [13,14]; for the corresponding CH_2 vibrations in other phospholipids R for C=O vibrations in Table 1 are also similar to those of other phospholipids [13]. However, dichroic ratios for the asymmetric vibration of PO_2^- as shown in Table 1 are higher than those presented by Hubner and Mantsch [14] for solid dry films of phosphatidylcholines.

The dichroic ratios of the CH_2 stretching band (2934 cm^{-1}) of cholesterol as a function of cholesterol molar fraction are shown in Fig. 2a,b. As cholesterol is very insoluble in water, cholesterol films cannot be formed by spreading aqueous solution on the surface of germanium prism. Cholesterol was dissolved in a mixture of chloroform/methanol and this solution was spread on the germanium prism, and after removing the solvents by drying in an oven spectra at 0° and 90° were recorded. The dichroic ratio (R) for CH_2 band (2934 cm^{-1}) is 1.6. The reported R is for dry cholesterol layer as this film could not be made wet by water. The dichroic ratios presented in Fig. 2a,b are for phospholipid–cholesterol mixtures, in the presence of water about 10–20 molecules H_2O/molecule of phosphatidylserine.

In Fig. 2a the data were obtained by deconvolution and correction for the contribution of CH_2 vibrations of glycerol calculated by Eq. 2 and in Fig. 2b by subtraction of DMPS-d54 spectra (see Section 2). As seen from Fig. 2a, at low cholesterol fractions R decreases strongly with increase in the molar fraction (X) of cholesterol, leveling off at X(chol) between 0.3 and 0.38 where phase separation of cholesterol starts [1]. These results indicate that the organization of cholesterol as inferred from the dichroic ratios is different when dispersed in the phosphatidylserine bilayer than when cholesterol is also in separated cholesterol crystallites.

In our previous IR study of DMPS–cholesterol mixtures we have shown that the frequency of the CH_2 symmetric and asymmetric stretching bands change little due to interaction with cholesterol [2]. The small effects were interpreted as weak interaction between DMPS and cholesterol and superposition of cholesterol bands on those of DMPS. In the present study we used deuterated DMPS, enabling us to investigate separately symmetric and asymmetric CD_2 vibrations without interference from cholesterol bands.

In Fig. 3a,b are presented dichroic ratios (R) of CD_2 asymmetric and CD_2 symmetric bands, respectively, as a function of the molar fraction of cholesterol. No significant effect of cholesterol on R is detected, indicating that the effect of cholesterol on the organization of the CD_2 groups is weak. It is known that phase separation of cholesterol crystallites above 0.3 molar fraction of cholesterol takes place [1]. Even at lower cholesterol concentrations some clustering of cholesterol may occur to minimize its interaction with DMPS.

Orientation of cholesterol in phosphatidylserine bilayers is not known.

In dimyristoyl phosphatidylcholine bilayers cholesterol is situated with its long axis parallel to the acyl

Table 1
Dichroic ratios (R) of various vibrations in DMPS-d54

Vibration	Wavenumber (cm^{-1})	R^a	R^b	R^c
CD_2 (as)	2198	1.1	1.06	1.05
CD_2 (s)	2091	1.09	1.05	1.06
C=O	1734	1.47	1.42	1.44
PO_2^-(as)	1227	1.31	1.36	1.25

[a] 10–20 water molecules/DMPS-d54 molecule.
[b] Completely dry.
[c] 35–50 water molecules/DMPS-d54 molecule.

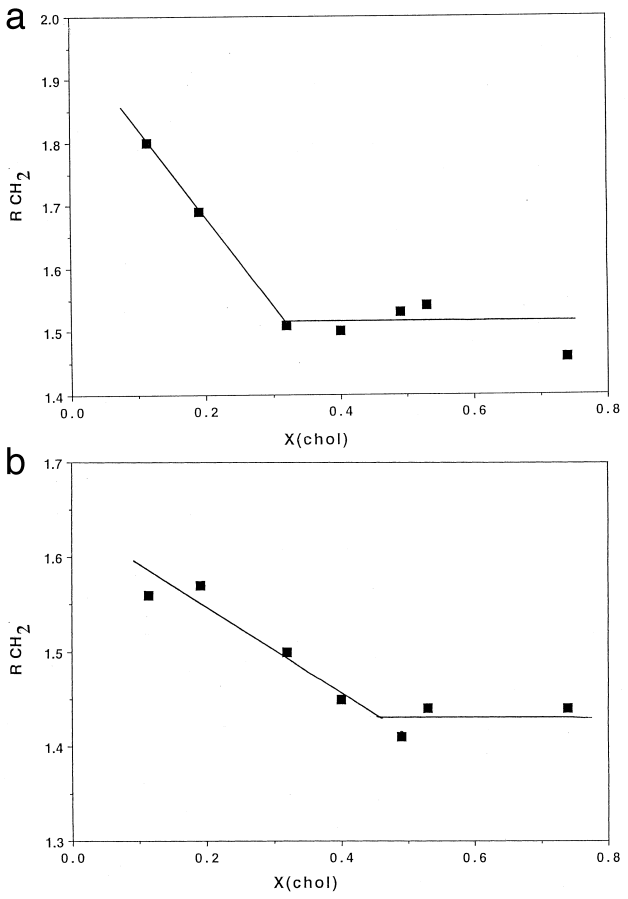

Fig. 2. Dichroic ratios (R) of CH_2 vibration of cholesterol as a function of molar fraction of cholesterol (X(chol)). (a) obtained from deconvolution (see Section 2), (b) obtained from subtraction of DMPS d54 spectra (see Section 2).

chains of the phospholipid. Barnes and Freed [15] investigated by ESR orientation of cholestane which simulates cholesterol in dimyristoyl phosphatidylcholine and DMPS bilayers in the gel state of the phospholipids. In dimyristoyl phosphatidylcholine the spectral parameter implies that the long axis of cholestane is parallel to the bilayer normal, whereas in DMPS the probe sees a local strongly biaxial environment. Inefficient packing between cholesterol and DMPS takes place due to high rigidity of the serine head group [16]. These findings might explain the weak interaction of phosphatidylserine and cholesterol resulting in the small effect of cholesterol on the orientation of the phosphatidylserine groups. Within experimental error no effect of cholesterol on the dichroic ratios of (C=O) and asymmetric phosphate (PO_2^-) vibration was detected. In the completely dry state of the phospholipid (no water band at 3400 cm^{-1} was seen) decrease of R of the carboxylate vibration from 2.2 followed by leveling off to 1.9 in the region of phase separation was seen.

3.3. ATR and transmission IR of DMPS–cholesterol mixtures

The broad bands in the polar region of the phospholipid were deconvoluted to reveal the components of the vibrations not resolved by the Instrument.

3.4. The carbonyl C=O stretching vibration

The vibration of the ester carbonyl group encompasses the region of 1750–1720 cm^{-1}. Bush et al. [17] and Wong et al. [18] deconvoluted this band in syn-

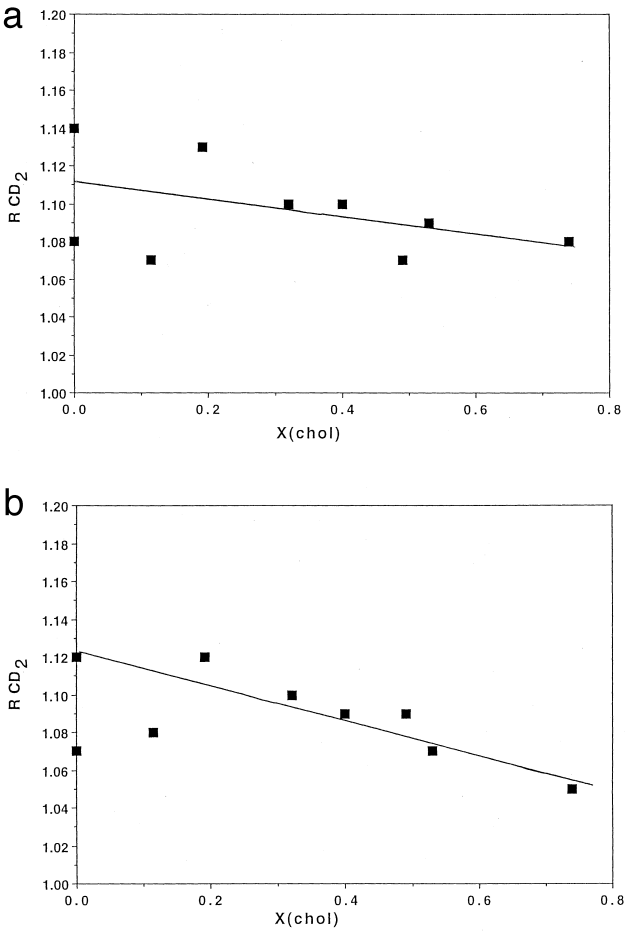

Fig. 3. Dichroic ratios (R) of CD_2 vibrations as a function of molar fraction of cholesterol (X(chol)). (a) Asymmetric vibration, (b) symmetric vibration.

thetic phosphatidylcholines into at least three separate bands and assigned them to the various C=O vibrations based on the known conformation of the headgroup region and of the two acyl chains. Recently Lewis and McElhaney [19] investigated the carbonyl bands in synthetic phosphatidylserines.

We have performed deconvolution of the carbonyl band in the region 1675–1800 cm^{-1}. In Fig. 4 are presented the deconvoluted spectra for DMPS only (Fig. 4a), and for mixtures with cholesterol, X(chol) 0.193 (Fig. 4b) and X(chol) 0.32 (Fig. 4c). The water content in these experiments is about 10 water molecules/molecule DMPS, in addition in Fig. 4d is presented the deconvoluted spectrum for DMPS in the presence of a large excess of water. All spectra were obtained in ATR mode. In Fig. 4a the deconvolution yielded four significant peaks, the two major ones of comparable areas are at about 1728 and 1737 cm^{-1}, two smaller ones are at about 1718 and 1745 cm^{-1}. As the structure of DMPS and the orientation of the carbonyl groups in the bilayer are not known, it was not possible to make an assignment of the various bands to sn_1 and sn_2 carbonyls as was done previously for the bands in synthetic phosphatidylcholines [17,18].

Lewis and McElhaney [19] investigated by transmission FTIR a series of synthetic phosphatidylserines in an excess of D_2O. For the DMPS in the gel phase (L_β phase) the authors reported that the carbonyl band is composed of two bands, a sharp component at about 1743 cm^{-1} and a broad component at about 1728 cm^{-1}. Based on previous research, Lewis and McElhaney [13] concluded that the higher frequency band is due to the free ester and the lower one to the carbonyl group hydrogen bonded to water. In the present work deconvolution was performed with five input peaks giving further separation of the carbonyl bands into sharp components as seen in Fig. 4a. Addition of cholesterol above

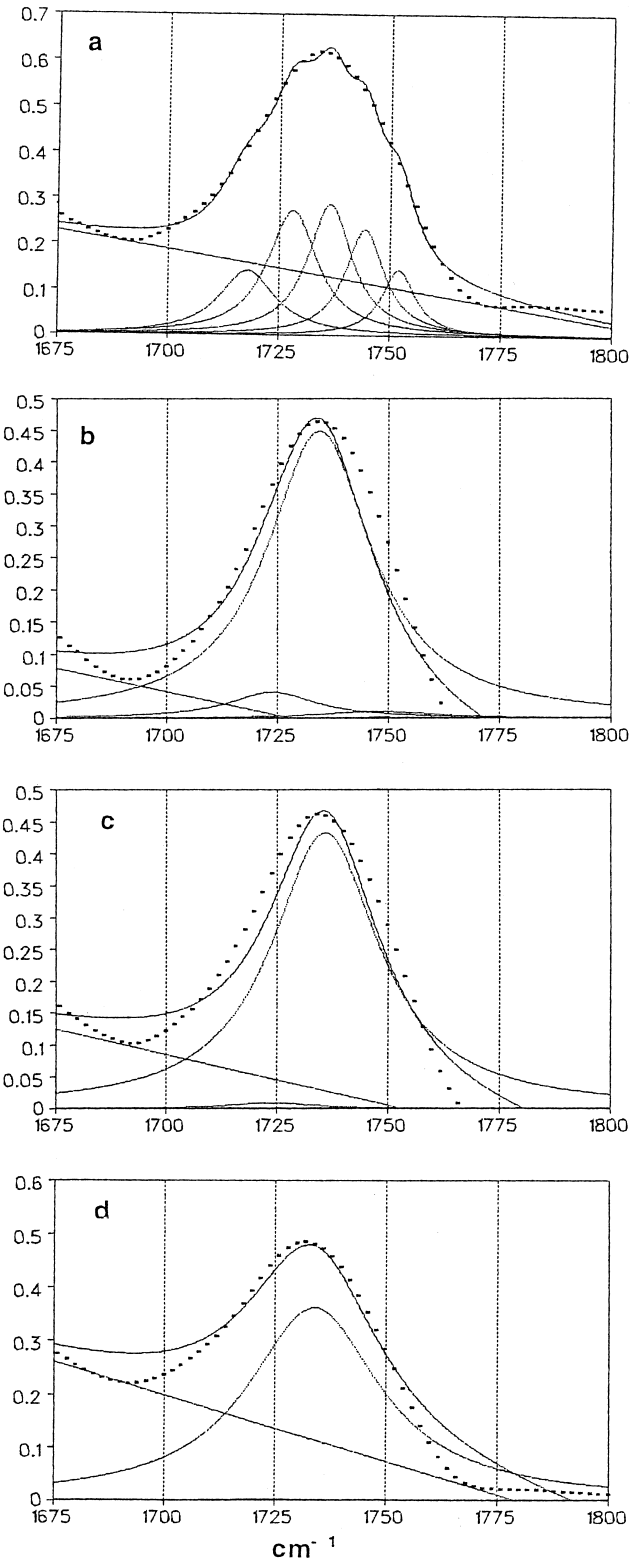

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Fig. 4. Deconvoluted spectra for the carbonyl (C=O) stretching vibration region. (a) DMPS only, (b) DMPS–cholesterol mixture molar fraction of cholesterol (X(chol)) 0.19, (c) DMPS–cholesterol mixture molar fraction of X(chol) 0.32, (d) DMPS only. In a–c water content was about 10 water molecules/DMPS molecule, in d about 75 water molecules/DMPS molecule.

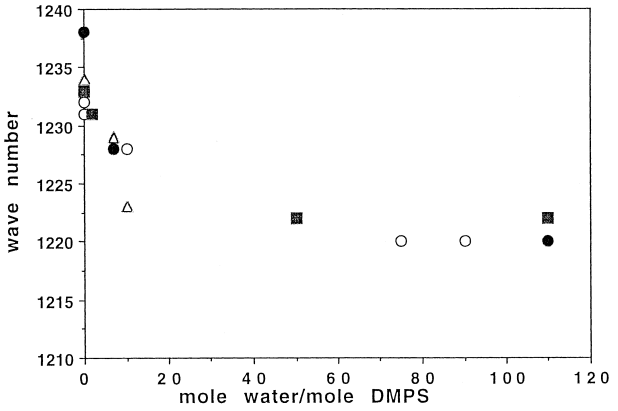

Fig. 5. Wave number of phosphate (PO_2^-) asymmetric stretching vibration as function of water content. (○) DMPS only, (●) X(chol) 0.11, (△) X(ch0l)-0.32, (■) X(chol) 0.44.

X(chol) 0.1 has a dramatic effect on these bands as shown in Fig. 4b,c. Only one band at about 1735 cm^{-1} is seen corresponding to about 95% of the total area of the band. When these samples were dried completely (no water band at 3450 cm^{-1} was detected) the effect of cholesterol was the same. It is possible that a conformational change takes place in DMPS molecule due to interaction with cholesterol; the intensity of the carbonyl band at around 1735 cm^{-1} strongly increases with diminishing intensity of other bands. To investigate this behavior further, the measurements were repeated in a large excess of water. In Fig. 4d the deconvoluted spectrum of DMPS in a large excess of water (about 75 water molecules/molecule of DMPS) is presented. Also here only one predominant peak at about 1734 cm^{-1} is seen. It seems that cholesterol at low water content or in the dry state induces similar conformational changes in DMPS molecules as water does when present in large excess. Bush et al. [17] investigated the effect of water on the carbonyl region of dipalmitoyl phosphatidylcholine. In anhydrous state the carbonyl band is split into several components. Addition of water shifts the band to higher frequencies and abolishes the splitting (at least as seen on the nondeconvoluted spectra: Fig. 2 of [17]). Wong et al. [18] showed that in the absence of water hydroxyl of cholesterol forms hydrogen bond with sn_2 carbonyl of synthetic phosphatidylcholines as detected by the shift of one of the carbonyl bands. By comparison of the strength of the hydrogen bond between the carbonyl band and hydroxyl of cholesterol or the carbonyl band and water, Wong et al. [18] concluded that the hydrogen bond of hydroxyl of cholesterol to the sn_2 of carbonyl should remain even in the presence of water. It is possible that part of the effect of cholesterol on the various carbonyl bands shown in the present work is due to hydrogen bonding between cholesterol and carbonyl of DMPS. In our previous publication [2] we did not find any evidence for hydrogen bonding between cholesterol and DMPS when examining the nondeconvoluted transmission spectra of DMPS–cholesterol mixtures.

3.5. The phosphate–PO_2^- stretching vibrations

The phosphate stretching region in the range 1125–1275 cm^{-1} was deconvoluted for DMPS only and for DMPS–cholesterol mixtures in the dry state and in the presence of water. Five peaks of different frequencies were obtained. Peaks at about 1235 cm^{-1} for the dry lipid and between 1228 and 1220 cm^{-1} in the presence of water were assigned to the asymmetric stretching of PO_2^- band. Addition of cholesterol at increasing concentrations caused small changes of the position of these bands either in the dry state or in the presence of water. As seen in Fig. 5, cholesterol has a very small effect on the phosphate band. These results are in keeping with our results published previously [2].

3.6. The carboxylate–CO_2^- stretching vibration

The carboxylate antisymmetric stretching band occurs at 1640–1620 cm^{-1} depending whether the adjacent ammonium is protonated or deuterated [5,20]. In this region water bending (1645 cm^{-1}) and NH_3^+ antisymmetric bending (1630 cm^{-1}) are also located. To prevent interference from water absorption, either completely dry samples or samples suspended in D_2O were used. When using D_2O the ammonium band shifts to much lower frequencies and the CO_2^- band shifts to 1620 cm^{-1}. In the present work we used the two approaches to evaluate the effect of cholesterol on the carboxylate band.

For ATR in the absence of water the DMPS or DMPS–cholesterol mixtures were dried in an oven (Section 2) and spectra were recorded in a completely dry state as judged by the absence of the water band at 3400 cm^{-1}. The spectra were deconvoluted in the

range 1550–1800 cm^{-1}. To simplify the assignment of ammonium band, spectrum of pure completely dry dimyristoyl phosphatidylethanolamine (DMPE) was also deconvoluted in the range 1600–1680 cm^{-1}. Major band (95% of the area) was detected at 1638 cm^{-1} and was assigned to the NH_3 antisymmetric bending of DMPE. For DMPS and DMPS–cholesterol mixtures in the completely dry state deconvolution produced three peaks: 1631–1635 cm^{-1} and a third band around 1620 cm^{-1}. With addition of cholesterol very small shifts of these bands were detected; however, they did not change systematically with an increase of cholesterol concentration.

3.7. Transmission IR

In the ATR mode it was not possible to get rid of H_2O when working with D_2O. This is the reason why we worked with an excess of D_2O in the transmission mode. Using the protocol described in Section 2, we succeeded to decrease the H_2O content to about 15%. In the deconvoluted spectrum the band at around 1620 cm^{-1} was assigned to carboxylate stretching. Addition of cholesterol shifted this band up by maximum 6 cm^{-1}, but also here the shifts were not consistent. We have reported previously [2] that in the presence of a molar fraction of cholesterol of 0.4, an upward shift of 2 cm^{-1} was detected.

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