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Fourier transform infrared spectroscopy of aqueous dispersions of phosphatidylserine-cholesterol mixtures

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Abstract. The effect of cholesterol on vibrational spectra in the non polar and in the polar region of dimyristoyl phosphatidylserine (DMPS) and of phosphatidylserine from bovine spinal cord (PS) has been investigated. The small shifts in the methylene CH stretching frequencies after taking into account the contribution of the cholesterol spectrum were interpreted as a combined effect of cholesterol on the conformation of the chains and of the lesser contributions of the cholesterol methyl groups. Cholesterol also influences the ratio of the trans (1465 cm^{-1}) to the lower wavelength (1457 cm^{-1}) CH_2 bending bands. No significant direct effect of cholesterol on the vibration of the polar residues was discerned. The small shift of the carboxylate band observed below the phase transition is probably due to the change in the intermolecular zwitterions when the average distance between the neighboring polar groups increases due to incorporation of cholesterol molecules.

Key words: FTIR – Phosphatidylserine cholesterol

Introduction

The interactions between cholesterol and zwitterionic phospholipids have been investigated extensively (Ladbrooke et al. 1968; Mabrey et al. 1978; Estep et al. 1978, 1979; Blume 1980; Umemura et al. 1980; Cortijo et al. 1982; Davis and Keough 1983; Wong et al. 1989; Yang et al. 1990). These studies showed that cholesterol affects consierably the conformation, the fluidity and the thermotropic properties of the phospholipids. However, only a few studies have been published on the interaction of the negatively charged phospholipid phosphatidylserine, either natural (PS) or synthetic, with cholesterol (Van Dijck 1979; Bach 1984; Wachtel and Bach 1987; Bach and Wachtel 1989). Recently, PS-cholesterol mixtures have been studied using differential scanning calorimetry (DSC) and X-ray diffraction. At a phosphatidylserine/cholesterol molar ratio of about 2:1, phase separation into an almost pure cholesterol phase and a phosphatidylserine/cholesterol phase was detected with both natural (Bach 1984; Wachtel and Bach 1987), and dipalmitoyl phosphatidylserine (Bach and Wachtel 1989). In zwitterionic phospholipid:cholesterol mixtures phase separation of cholesterol takes place at about 1:1 ratio in mixtures with phosphatidylcholine and at about 1.5:1 ratio in mixtures with phosphatidylethanolamine, as inferred from neutron scattering (Knoll et al. 1985) and X-ray diffraction (Finean and Hutchinson 1988; Cheetham et al. 1989). Browning and Seelig (1980) have shown that phosphatidylserine has a more rigid head group than phosphatidylcholine or phosphatidylethanolamine. This is in keeping with the lower miscibility of cholesterol in phosphatidylserine (1:2) than in phosphatidylcholine (1:1).

Vibrational spectroscopy can be used for localization of the particular groups in the lipid molecules affected by phase transition and by interactions with other molecules. Fourier transform infrared (FTIR) spectroscopy has been employed for investigating the effect of hydration (Leberle et al. 1989) and ion binding (Casal et al. 1987a, b, 1989). In this work we investigated the effect of cholesterol on the FTIR spectra of the different residues of phosphatidylserine below and above the phase transition. Above the phase transition we worked with saturated phosphatidylserine DMPS and with bovine spinal cord PS containing nearly 70% of mono- and poly-unsaturated acyl chains. Below the phase transition we studied the interaction of cholesterol with DMPS only.

Abbreviations: PS, phosphatidylserine natural; DMPS, dimyristoyl phosphatidylserine; DPPC, dipalmitoyl phosphatidylcholine; FTIR, Fourier transform infrared spectroscopy; DSC, differential scanning calorimetry; PE, phosphatidylethanolamine

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Materials and methods

Phosphatidylserine from spinal cord (PS), grade 1 monosodium salt, (in chloroform:methanol 2:1) was pur-

chased from Lipid Products (South Nutfield, UK). Cholesterol (extra pure) was obtained from Merck (Darmstadt, FRG) and was twice recrystallized from ethanol. Dimyristoyl phosphatidylserine (DMPS) was purchased from Avanti Polar Lipids-Pelham, AL. The midpoint transition temperature of this phospholipid dispersed in an excess of water was 36 °C as determined by DSC and is similar to reported values (Hauser et al. 1982). PS in chloroform : methanol 2:1 or DMPS dissolved in the same solvents were mixed with appropriate volumes of cholesterol in the same solvents to yield the desired molar ratios. The solvents were removed by a stream of nitrogen and the samples placed in vacuum for 3 h. The lipid films were resuspended either in 150 m*M* NaCl, 10 m*M* HEPES, pH 7.2–7.4 in H_2O or in D_2O or in pure water or D_2O only. In some experiments on DMPS-cholesterol mixtures the lipids were also dispersed in 1 m*M* EDTA pH 7.5. The phospholipid concentration was 50–80 mg/ml. The samples were incubated at 45–47 °C for 30 min with frequent vortexing and immediately used for FTIR spectroscopy. The experiments were performed on a 1600 Perkin Elmer FTIR spectrophotometer with 2 cm^{-1} nominal resolution. In order to reach the desired accuracy (0.4–0.5 cm^{-1}), 400 scans between 4000–1000 cm^{-1} were collected, the solvent was subtracted and the noise smoothed. The measuring cell had CaF_2 windows (BUCK Scientific, E. Norwalk, CT) and a 5 μm Mylar spacer (DuPont, Geneva, Switzerland). Some samples were concentrated by partial evaporation of water from the lipid dispersions placed on one of the windows. In those cases, the second window was put directly on the concentrated sample, without any spacer. The partially dried samples were still fully hydrated, with two to three fold excess of water, as revealed by the strong OH stretching band of water (at about 3400 cm^{-1}). The sample temperature was 30 ± 1 °C for PS-cholesterol mixtures and 24 °C or 47 °C for DMPS-cholesterol experiments.

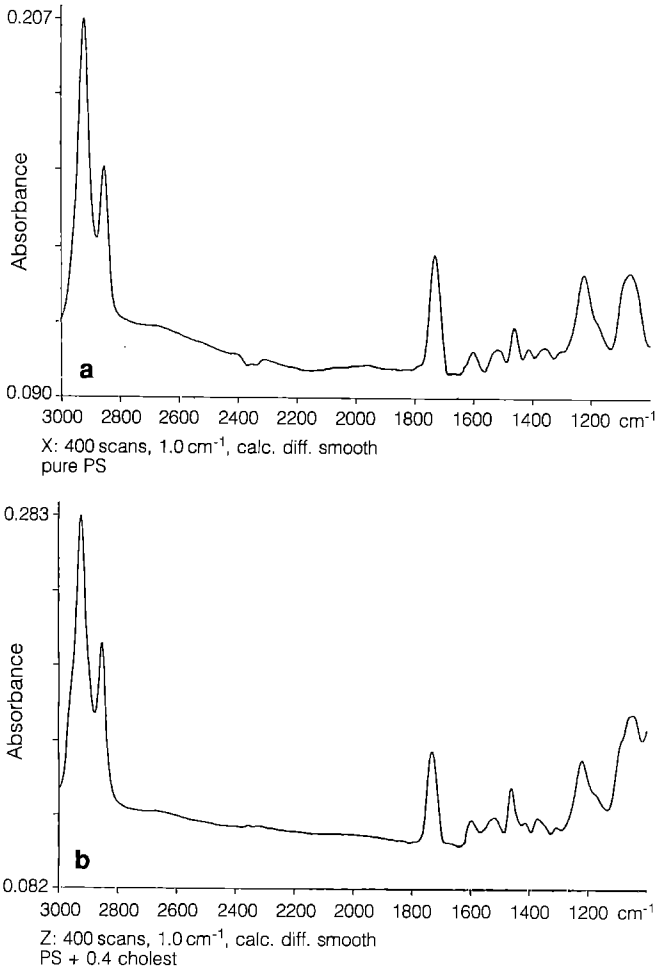

Fig. 1. Overview spectra between 3000 cm^{-1} and 1000 cm^{-1} of PS (**a**) and of PS + cholesterol $X_{chol} = 0.4$ (**b**) in an aqueous dispersion of 60 mg/ml after subtraction of the water spectrum. Each spectrum was averaged over 400 scans. The nominal resolution was 2 cm^{-1}

Results

The PS/cholesterol mixtures at different concentrations in the aqueous dispersions and the samples concentrated by evaporation gave similar spectra, so all the samples were analyzed together. We are treating separately the CH stretching and the CH_2 bending spectra of the acyl chains and the head group region spectra. In every case good quality spectra in the whole spectral region were obtained. An example of two such overview spectra is given in Fig. 1 a and b.

Vibrational spectrum of hydrocarbon chains

CH stretching and CH_2 bending vibrations are sensitive to the order-disorder transition (Umemura et al. 1980; Cortijo et al. 1982; Casal and Mantsch 1984). When the lipid has an all trans conformation, the symmetric CH stretching mode of the acyl chains is at about 2850 cm^{-1}. This absorption band is shifted towards higher wavenumbers with increasing number of gauche conformers in the lipid chains (Casal et al. 1987a). As shown in Fig. 2, in the Na salt of DMPS measured at 24 °C which is below the phase transition, the symmetric stretching band (ν_s) is at 2849 ± 0.2 cm^{-1} and the asymmetric (ν_{as}) one at

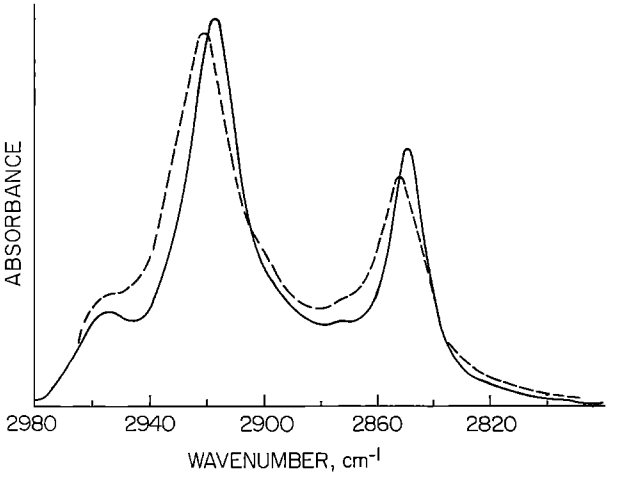

Fig. 2. Methylene CH stretching bands in DMPS below (24 °C) --- and —— above (47 °C) the phase transition

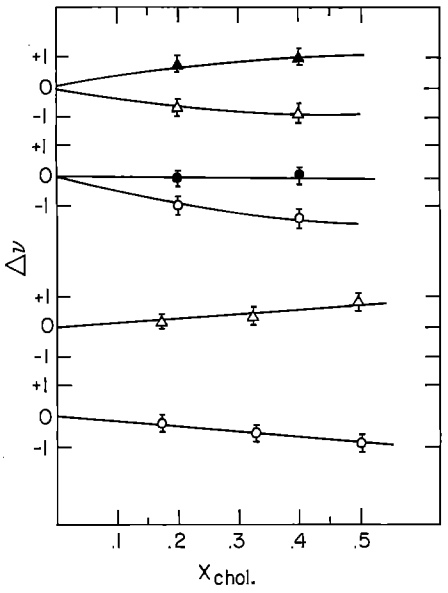

Fig. 3. Shift of the peak frequency $\Delta\nu$ of the symmetric (o—o) and of the asymmetric (Δ—Δ) methylene stretching bands as a function of molar fraction of cholesterol. Open symbols above phase transition, closed symbols below it. Upper four line DMPS, lower two lines PS

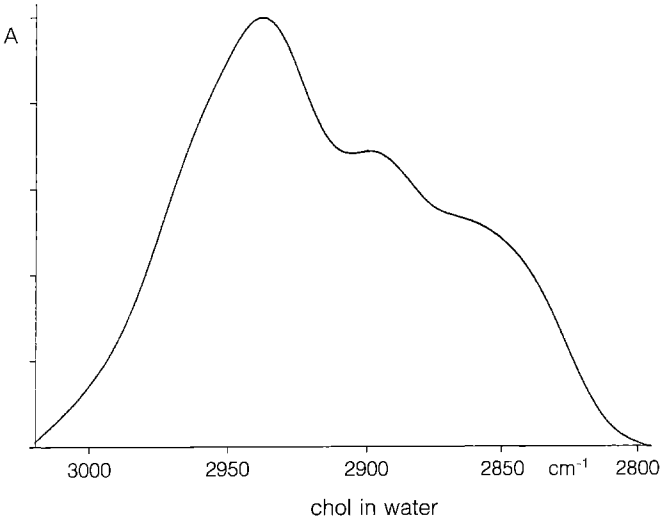

Fig. 4. Spectrum of cholesterol between 3000 cm^{-1} and 2800 cm^{-1}. The only band of sufficient intensity obtained reproducibly from a cholesterol dispersion of 30 mg/ml H_2O in a 5 μm thick layer

2916.8 ± 0.2 cm^{-1}. The two bands are shifted to higher wavenumbers above the phase transition at 47 °C, namely to 2851.7 ± 0.2 cm^{-1} and 2921.4 ± 0.2 cm^{-1}, respectively, due to the contribution of the gauche conformers. No effect of cholesterol was observed below the phase transition on the symmetric stretching band (2849 cm^{-1}). The asymmetric band (ν_{as}) at 2916.8 ± 0.2 cm^{-1} was shifted to 2917.8 ± 0.2 cm^{-1} by a molar fraction of cholesterol of 0.4. Above the phase transition at 47 °C the effect of cholesterol on the symmetric band is quite significant. At a molar cholesterol fraction of 0.4 it shifts from 2851.7 cm^{-1} to 2850.3 ± 0.2 cm^{-1} which is half of the shift due to the transition into the rigid state of the pure phospholipid. On the other hand, the shift of the asymmetric band is only 0.6–0.7 wavenumber from 2921.4 ± 0.2 cm^{-1} to 2920.8 ± 0.2 cm^{-1} which is only about 15% of the total shift from the liquid crystalline to the gel state of pure DMPS. Figure 3 summarizes the shifts of the peaks of the stretching bands of DMPS (below and above the phase transition) and of PS above the phase transition as a function of the molar fraction of added cholesterol. Each point is an average of 3 to 10 independent measurements of 400 scans each. The CH symmetric stretching band of PS above the phase transition is also shifted to smaller wavenumbers, however the shift is smaller than in DMPS. On the other hand, the asymmetric band, in contradistinction to DMPS, is shifted, although slightly, towards higher wavenumbers.

We interpret the apparent discrepancy in the behavior of the two peaks by the different effect of the superposition of the cholesterol spectrum and by the different susceptibility to modulation by the Fermi resonance upon phase transition, when cholesterol is added (Spiker and Levin 1975; Snyder et al. 1978). It seems that the first effect, namely the superposition of the pure cholesterol spectrum (Fig. 4) is predominant below the phospholipid phase transition when its mixing with cholesterol may be restricted even before complete phase separation is observed (Bach and Wachtel 1989). In this region an increase by one wavenumber of the asymmetric band of DMPS is observed. The calculated effect of the superimposed spectrum of cholesterol is nearly the same. At the same time its calculated effect on the symmetric band (ν_s) is about 0.2 wavenumbers and the measured shift is zero. Thus, the effect of DMPS chain disordering below their phase transition by cholesterol seems to be minimal. Cholesterol, situated between the phospholipid chains, affects them in two ways: *i*) it separates the chains, diminishing interchain resonance, *ii*) orders the chains above the phase transition while the disordering below the phase transition is minimal. Moreover, the CH_3 vibration in the cholesterol molecule has a resonative contribution around 2930 cm^{-1} (Spiker and Levin 1975) and may induce upward shifts in the CH_2 asymmetric stretching band. The upward shift of around one wavenumber of the asymmetric band by superposition of the pure cholesterol spectra is balanced below, the phase transition by the expected downward shift due to rigidification and is possibly supplemented by a small Fermi resonance effect. At 30 °C at which the spectra of PS were taken, cholesterol is below its phase transition (Bach 1984) and may not mix completely with PS to rigidify it and the downward shift in wavenumber is smaller. CH_2 stretching is expected to be affected by hydrogen bonding with the hydroxyl of the cholesterol (Wong et al. 1989) and some associated secondary effect in the head group region (Leberle et al. 1983).

No appreciable shift in the frequency of the CH_2 bands assigned to the random and the trans bending at around 1473 cm^{-1} and 1465 cm^{-1} can be observed in DMPS upon phase transition. Neither is there any effect on the band of undetermined nature at 1457 cm^{-1} observed reproducibly in PS and in DMPS. The peak at 1457 cm^{-1} may stem from the gauche defects, responsible for flu-

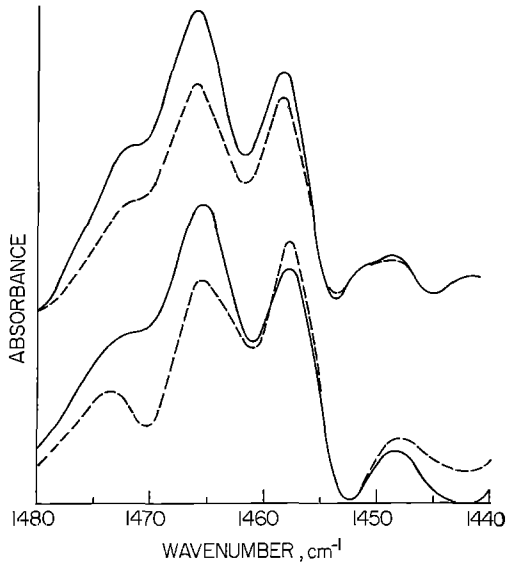

Fig. 5. CH_2 bending band of DMPS in the presence of 1 m*M* EDTA. —— 24 °C, - - - 47 °C. The two lower curves pure DMPS, the two upper curves DMPS with $X_{chol} - 0.4$

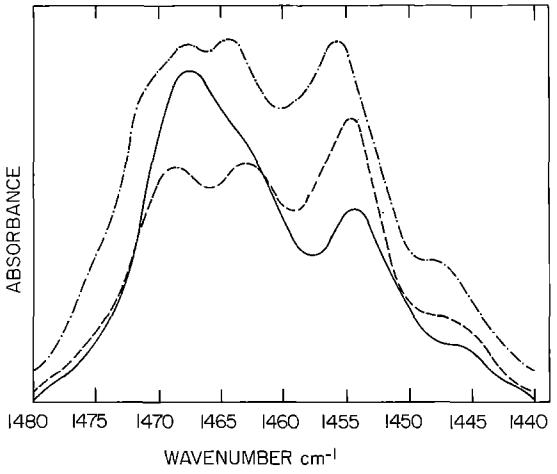

Fig. 6. CH_2 bending of DMPS (no EDTA added). --- Pure DMPS 24 °C, - - - pure DMPS 47 °C, --.-- $X_{chol} - 0.47$ °C. The spectrum in the presence of cholesterol is on a different absorbance scale

idization and downward broadening of the CH_2 bending band (Kopp et al. 1975, Fringeli and Gunthard 1981). There is a very pronounced change in the ratio of the absorbance of the two bands at 1 465 cm^{-1} and 1 457 cm^{-1} (Fig. 5). Below the phase transition the ratio of the absorbance of the 1 465 cm^{-1} to 1 457 cm^{-1} band varies between 1.3 and 1.4 and it is not affected appreciably by the added cholesterol. Above the phase transition this ratio decreases to 0.9 ± 0.05. Cholesterol at a molar fraction of 0.4 causes increase of the ratio to 1.15 ± 0.05. The spectra in Fig. 5 were obtained in the presence of 1 m*M* EDTA. In the absence of EDTA the peak at 1 465 cm^{-1} corresponding to the random bending mode is split into two peaks above the phase transition. Nevertheless, the absorbance ratio of this split peak of PS or DMPS to the peak at 1 457 cm^{-1} increases with added cholesterol just like the ratio in the presence of EDTA. Below the phase transition the split peak converges into

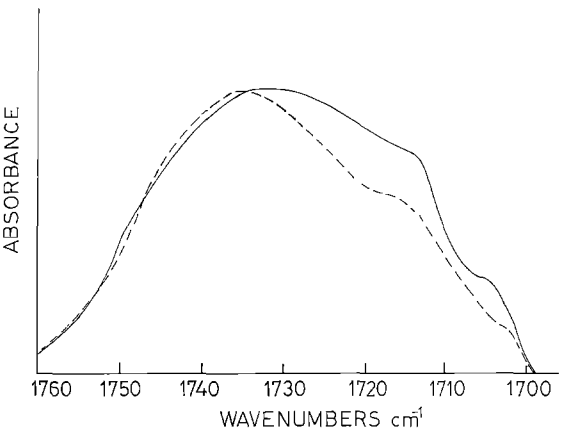

Fig. 7. Carbonyl stretching band of DMPS below --- (24 °C) and —— above (47 °C) the phase transition. Each spectrum was obtained by averaging six independent spectra

one (with a residual shoulder) about 70% higher than the peak at 1 457 cm^{-1} (Fig. 6). Below the phase transition with or without EDTA the peak ratio is practically independent on the added cholesterol.

Vibrational spectra of the polar groups of phosphatidylserine

Wong et al. (1989) have shown that in the dry state the hydrogen bond between the carbonyl sn_2 of phosphatidylcholine and the hydroxyl of cholesterol is formed. As this bond is strong, one would expect that cholesterol should have an effect in aqueous dispersions on the conformation of the carbonyl residue. However, the results presented below show that the effect of cholesterol on the carbonyl was not detected.

The C=O bands in the vibrational spectra of lipids (around 1 730 cm^{-1}) (Levin et al. 1982), are sensitive both to the phospholipid conformation (Dluhy et al. 1983) and to hydrogen bonding (Wong et al. 1989). It is assumed that a shift towards smaller wavenumbers and/or increase of the 1 710–1 715 cm^{-1} band is an indication for hydrogen bonded C–O groups. The spectrum of this region obtained by us differs in some details from that presented by Dluhy et al. (1983), but it shows a similar change upon the phase transition (Fig. 7). The spectra in this figure are obtained by averaging six spectra below and above the phase transition obtained in different experiments in H_2O and in D_2O. No significant effect of cholesterol on this spectrum could be observed either below or above the phase transition.

The phosphate stretching bands at 1 225 cm^{-1}, 1 088 cm^{-1} and 1 065 cm^{-1} (Fig. 8) of PS or DMPS are insensitive to the added cholesterol, in agreement with the results obtained by Umemura et al. (1980) and Wong et al. (1989) who investigated the effect of cholesterol on phosphatidylcholine. A small shift in these bands was observed upon the phase transition of DMPS from the liquid crystalline to the gel phase; however, no significant change within the limits of the reproducibility of our ex-

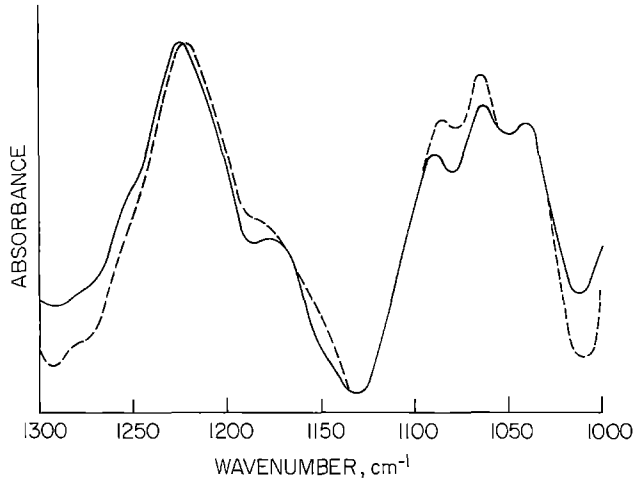

Fig. 8. Symmetric and asymmetric phosphate stretching band at — 24 °C; and - - - 47 °C

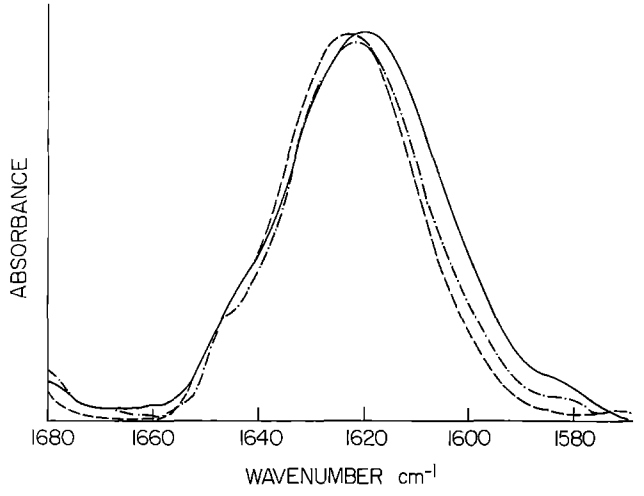

Fig. 9. CO_2^- stretching band of DMPS below — (24 °C) and above --- (47 °C) phase transition. - · - · $X_{chol} - 0.4$ below phase transition (24 °C)

periments could be recorded upon addition of cholesterol either below or above the phase transition.

Casal et al. (1987) reported that the phosphate bands are almost unaffected by temperature or by phase transition. However, we observed a reproducible downward shift of the asymmetric stretching band at 1 226 cm^{-1} in DMPS. This shift is in the opposite direction than that of the symmetric and asymmetric CH bands. The difference may result from the increase of the average distance between the phosphate group and its counterion at elevated temperatures. The two peaks of the PO-symmetric stretching band below 1 100 cm^{-1} shift in opposite directions. The peak at around 1 090 cm^{-1} in DMPS shifts downward by at least 2 wavenumbers after heating the samples from 24 °C to 47 °C, while the peak at 1 063.2 cm^{-1} is shifted upward so that the peak separation of the 1 063 cm^{-1} and the 1 090 cm^{-1} bands decreases by about five wavenumbers when DMPS passes the phase transition temperature at 36 °C. The splitting of this band diminishes with increasing temperature indicating that the two modes of symmetric stretching which generate the split peak intermix at elevated temperatures.

The assignment of the asymmetric COO^- stretching band in PS was done by Dluhy et al. (1983). As this band is specific for phosphatidylserine it was of interest to follow changes in this band due to interaction with cholesterol. In order to be able to investigate this region without interference from H_2O, only dispersions in D_2O were analyzed. The frequency of the asymmetric stretching band at 1 620 cm^{-1} increases with cholesterol addition (Fig. 9) below the phase transition. The increase of the wavenumbers by about 2 cm^{-1} probably stems from the rearrangement in the carboxylate-ammonium zwitterions bonded by a hydrogen bond. The zwitterions may be intra- or intermolecular. The intramolecular hydrogen bond is under bending strain and so it is energetically less favorable than between COO^- and N^+H_3 of neighbouring molecules.

Cholesterol probably cannot affect the carboxylate group directly, it can only increase the average distance between the neighbouring phosphatidylserine molecules, diminishing the number of the ordered intermolecular N^+H_3-COO^- hydrogen bonded entities, and enhancing intramolecular zwitterion formation. Above the phase transition both in PS and DMPS the shift in the COO^- band is within experimental error. There may be a compensating effect of the increase of the PS intermolecular distances by the inserted cholesterol and by their decrease in the adjacent sites due to induced rigidification.

Discussion

Change in temperature, particularly in the phase transition region, affects the vibrational energies of the different groups in phospholipids. An increase in frequency is observed in the symmetric and in the asymmetric CH stretching band, when DMPS is heated above its phase transition temperature. The increase is larger in the asymmetric stretching band from 2916 ± 0.2 to 2921 ± 0.2 cm^{-1}, than in the symmetric one (from 2849 ± 0.2 to 2851.7 ± 0.2 cm^{-1}). A major contribution to the modulation of the CH stretching vibration frequency is from the Fermi resonance interaction with the trans- and random bending frequency at around 1 460 cm^{-1} and from the CH_2 rocking frequency at around 720 cm^{-1} (Snyder et al. 1978). The methylene bending frequency interactions along the chain as well as interchain interactions in the perpendicular direction are to be considered. The interactions in both directions vary with the changing order at the phase transition. For symmetry reasons the interaction of the bending frequency is allowed only with the symmetric stretching band ($d^+ - \pi$) at around 2 850 cm^{-1}; however, this interaction is very weak because of the large distance between this frequency and between the second harmonic of the bending frequency. On the other hand, the symmetric stretching fundamentals in the CH_3 group are split by Fermi resonance into two components: and the one at around 2 930 cm^{-1} can modulate the asymmetric stretching frequency in CH (Spiker and Levin 1975; Snyder et al. 1978 footnote). Addition of cholesterol to

phospholipids above their phase transition is known to rigidify their structure and the order by increasing the trans conformation; below the phase transition the effect is in the opposite direction (albeit much smaller). In both cases cholesterol separates neighbouring hydrocarbon chains, decreasing interchain resonance. Probably the most prominent effect is the simple contribution of the spectrum of the pure cholesterol added to the PS. The calculated upward shift of ν_{as} at 2920 cm^{-1} of CH_2 by 40% cholesterol is up to 1.3 wavenumbers and that of ν_s at 2850 cm^{-1} is less than 0.3 wavenumber. No appreciable shift in the frequency of the symmetric stretching band of DMPS could be detected upon addition of cholesterol up to a molar fraction of 0.4 below the phase transition. However, at the same fraction of added cholesterol the asymmetric band increased by 1 to 1.3 wavenumbers. This increase may be attributed mainly to the direct superposition of the cholesterol spectrum. The effect of cholesterol on the rigidity of the hydrocarbon chains and the associated effect on the spectrum is marginal. Above the phase transition the frequency of both bands is lowered by the added cholesterol. The symmetric band by up to 1.4 wavenumbers and the asymmetric band by only up to 0.8 wavenumbers. Presumably the resonative energy transfers and mainly the contribution of the cholesterol spectrum counteract the change in vibrational energy of ν_{as} due to rigidification. The rigidification of the partially unsaturated spinal cord PS by cholesterol is smaller than that of the DMPS and the maximal decrease in the frequency of the symmetric stretching band is only about 0.8 wavenumbers. The spectral superposition of cholesterol on the asymmetric band overbalances the frequency decrease due to rigidification and the resulting effect is a frequency increase of up to one wavenumber. The effect of cholesterol on CH bands in PS is smaller than the effect of cholesterol on the symmetric and the asymmetric CH bands of DPPC above the phase transition (Umemura et al. 1980; Cortijo et al. 1982) and of PE above phase transition (Yang et al. 1990) probably due to lower miscibility of cholesterol in PS (Bach 1984; Wachtel and Bach 1987).

The temperature dependent change in fluidity does not affect the vibrational energies of the trans- and random scissoring bands at around 1465 and 1457 cm^{-1}, but only their relative intensity (Figs. 5 and 6). Cholesterol also significantly affects the relative intensity of these two bands above the phase transition but hardly at all below it, in agreement with the small effect on the lipid fluidity.

The relative intensity of the C=O vibrational bands at about 1739 1734 and 1718 cm^{-1} varies with the phase transition (Fig. 7) but is not affected by the added cholesterol either below or above phase transition. This indicates that cholesterol does not change the vibrational energy of the carbonyl by hydrogen bonding and its indirect effect is negligible. These results do not agree with the prediction of Wong et al. (1989) who claimed that even in the presence of water hydrogen bonding between the C=O of phosphatidylcholine and cholesterol should take place.

The carboxyl group is probably the most remote from the cholesterol within the lipid bilayer. Nevertheless, it is affected by the added cholesterol albeit only below the phase transition. The frequency of the carboxylate stretching band is higher in DMPS above the phase transition by about three wavenumbers than below it. The shift is in the same direction as in CH and in opposite direction to the shift of phosphate. The frequency of the carboxylate band should depend on the possibility of zwitterion formation with adjacent N^+H_3 by hydrogen bonding. This may occur intramolecularly with a formation of a highly strained hydrogen bonded ring. The other possibility is zwitterion formation with an ammonium from one of the neighbouring molecules. The ratio of intramolecular to intermolecular zwitterion forms is expected to depend on the average distance between the phospholipid molecules and on their mobility and it is therefore expected to be higher above than below the phase transition. The effect of cholesterol, penetrating the PS array below the phase transition and increasing the average inter-phospholipid distances is in the same direction as that caused by an increase in temperature. Above the phase transition the effect of cholesterol on the probability of intermolecular zwitterion formation seems to be unimportant. This is in contrast to the effect of cholesterol on CH_2 vibration. The separation between the head groups by the intercalating cholesterol may be compensated by the closer distances in the rigidified domains.

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