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The effect of high external pressure on DPPC-cholesterol multilamellar vesicles: a pressure-tuning Fourier transform infrared spectroscopy study

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Abstract

We have investigated the effect of incorporation of cholesterol on the barotropic phase behavior of aqueous dispersions of 1,2-dipalmitoylphosphatidylcholine (DPPC) using Fourier-transform infrared spectroscopy (FTIR) in combination with the diamond anvil technique. Infrared spectral parameters, such as the frequencies, intensities, bandshapes and band splittings have been used to detect structural and dynamical changes upon incorporation of cholesterol into the DPPC bilayer. Analysis of these spectral parameters yields information on conformer population, reorientational fluctuations, interchain interaction, hydrogen bonding, interdigitation packing, and phase transformations of the DPPC/cholesterol mixtures. We present FTIR data of aqueous DPPC dispersions at 0, 10, 20, 30, 40 and 50 mol% cholesterol in the pressure range from 0.001 to 20 kbar at two temperatures, 25°C and 55°C. In addition, comprehensive temperature dependent measurements in the range from 20°C to 80°C were performed at ambient pressure. Analysis of the CH_2 symmetric and antisymmetric stretching modes yields information of the effect of cholesterol concentration on the phase transition phenomena occurring in the lipid bilayer. Observation of the correlation field splittings of the CH_2 bending and rocking modes monitors structural changes and dynamical properties of the lipid mixtures. Cholesterol induces more orientational disorder of the lipid molecules in terms of an increase of the reorientational fluctuations of the molecules and twisting/torsion motions of the acyl chains in the gel phase even at elevated pressures. It therefore appears that one important role of cholesterol is to make the membrane insensitive to changes in external environment, such as high hydrostatic pressure. Increase of pressure leads to a decrease in half width of the C = O band contour of pure DPPC and of DPPC/cholesterol mixtures, especially for cholesterol concentrations equal and higher than 30 mol%, which might be due to a marked increase in free carbonyl groups. At high pressure part of the bound water from the interfacial zone of the membrane is withdrawn. Increase of cholesterol concentration and increase in pressure have opposite effects on the population of free and hydrated carbonyl ester groups of DPPC in the gel phases.

Keywords: Pressure effect; DPPC; Cholesterol; Multilamellar vesicle; FTIR

1. Introduction

Interactions of cholesterol with model membranes have been recently reviewed by Finegold [1]. It is now well established that cholesterol causes disorder in phospholipids below their gel to liquid-crystalline phase transition temperature (T_m) and increases rigidity in phospholipids at temperatures above T_m. This effect has been observed with a variety of experimental techniques, including NMR [2–4], EPR [5], FTIR [6–9], Raman [10–12], fluorescence polarization [13,14], X-ray [15,16], and neutron diffraction [17,18]. In the presence of high cholesterol concentrations, the gel to liquid-crystalline phase transition is strongly suppressed and the fluid bilayer retains many of its phase properties well below the temperature of the normal transition to the gel phase. However, the effect of cholesterol is not limited only to the population of *trans* and *gauche* conformers but also affects the dynamics of phospholipids and the phase equilibria [1,18–20]. The dynamics of phospholipids is quite complex, as many different conformational states are available for both the head group and the acyl chains. In addition, the motions are not independent, a *trans-gauche* transition occurring around a C_i–C_j bond influences the orientation of not only the C-H bonds directly bonded to the C_i and C_j carbon atoms, but also of the neighboring methylene groups. This means that there is a coupling between *trans-gauche* isomerization and acyl chain reorientation. Other motions include, for example, diffusion and fluctuation of the orientation of chain axes.

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The average correlation times for those motions are often found to be larger in the presence of cholesterol than in the pure lipid liquid-crystalline phase, but shorter than in the pure lipid gel phase [19].

While most investigations on lipid-cholesterol mixtures have been conducted at atmospheric pressure, to our knowledge none was done at high pressures. Besides the physico-chemical interest in using pressure as a thermodynamic variable to understand the structure, phase behavior and dynamics of biological molecules and to test theoretical approaches, high-pressure studies on these systems are also of considerable physiological and biotechnological interest [21,22]. Increased pressure reduces intermolecular distances and thus enhances intermolecular interactions. The goal of this high-pressure study is to provide new information on molecular interactions within DPPC-cholesterol mixtures and on the degree of perturbation of the membrane by cholesterol at high pressures. DPPC constitutes most of the lipid lining inside the lung and is the phospholipid molecule that has been studied most intensively using thermodynamic, diffraction and spectroscopic methods [23]. We use Fourier transform infrared spectroscopy (FTIR) to obtain structural and dynamical information on the DPPC-cholesterol system. Infrared spectroscopy is a nonperturbing technique that monitors molecular vibrations and thus operates on a very short time scale. Vibrational spectra of lipid systems consist of bands arising from the transitions between vibrational energy levels of various types of intramolecular and intermolecular vibrations in the ground electronic state. It has been extensively shown in the literature, that many infrared spectral parameters, particularly the frequencies, widths, intensities, shapes and splittings of the IR bands, are very sensitive to the structural and dynamical properties of membrane lipid molecules, as well as functional groups in the molecules [9,24,25]. In particular cases, e.g., by analyzing the CH_2 wagging vibrations, even quantitative conformational information can be obtained [26–28]. In the pioneering work of Wong, the FTIR technique has also been developed for performing pressure dependent studies using the diamond anvil technique [29,30]. In a series of studies, Wong et al. have shown that the high-pressure FTIR method yields valuable information about the perturbation of pressure on the physical properties of membrane systems, as well as the interactions between these systems and other biomolecules, additives and water [29–33]. We present FTIR data of aqueous DPPC dispersions at different cholesterol concentrations in the pressure range from 0.001 to 20 kbar at two temperatures, 25°C and 55°C.

2. Experimental

High-purity 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) was purchased from Sygena (Liestal, Switzerland), and cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Both were used without further purification.

The solid DPPC/cholesterol mixtures were prepared by co-dissolving the solid components in chloroform, vortexing the solution in sealed containers, and drying the solution under vacuum. The samples were then kept under vacuum for at least 16 h. Fully hydrated (70–90 wt.% D_2O) cholesterol/lipid bilayer dispersions were prepared for the infrared experiments by heating the lipid/D_2O mixtures in a closed vessel to a temperature well above the gel to liquid-crystalline phase transition temperature of DPPC ($T_m \approx 42$°C), vortexing the heated samples and immediately freezing the samples in liquid nitrogen. This freeze/thaw cycle was repeated five times to ensure equilibration of cholesterol in the lipid bilayer. For the temperature-dependent studies, lipid dispersions were filled into a 25 μm thick infrared cell with CaF_2 windows. For the pressure-dependent studies, small amounts (typically 0.01 μl) of the homogeneous dispersions resulting from the freeze/thaw cycles were then placed, together with powdered α-quartz, in a 0.5 mm diameter hole of a 0.05 mm thick stainless steel gasket mounted on a diamond anvil cell (Type IIa diamonds from Diamond Optics, Tucson, USA), which was temperature-controlled by an external water thermostat. Pressure was determined from the 695 cm^{-1} phonon band of α-quartz [33,34]. The pressure can be determined to within ± 100 bar using this method.

Infrared spectra were collected on a Nicolet Magna 550 Fourier transform infrared spectrophotometer with a liquid nitrogen-cooled mercury cadmium telluride detector. The infrared beam was condensed by a spectra bench onto the diamond anvil cell. For each spectrum, 512 interferograms were co-added, at a spectral resolution of 2 cm^{-1}, and apodized with a Happ-Genzel function. The total measuring time per spectrum was about 8 min. The sample chamber was purged with dry, carbon dioxide-free air during data collection in order to minimize spectral contributions from atmospheric gases. The linewidths $\Delta\nu^*_{1/2}$ were defined at 50% of the peak maxima (ν frequency, ν^* wavenumber). All of the data analysis, including determination of the vibrational frequencies, was made with the OMNIC™ software developed by the Nicolet Instrument Corporation.

3. Pressure-induced vibrational frequency shifts

In the liquid phase, bond properties are not much affected by moderate pressures up to several kbars, so that vibrational frequencies should be affected mainly by changes in the environment, such as solvation and the intermolecular potential. The application of pressure may shift the position of the band and cause band broadening. The pressure compresses the liquid and decreases the average distance between the atoms but the structure of the molecules remains unchanged. The position of the band is

a result of the balance of the opposite effects of repulsive (blue shift, ν_R) and attractive (red shift, ν_A) interactions [35–37]

$$\nu_{exp} = \nu_R + \nu_A \quad (1)$$

The repulsive contribution can be calculated from

$$\nu_R \approx \hbar^{-1}\Delta q\langle F_R\rangle \quad (2)$$

where Δq is the vibrational amplitude and $\langle F_R\rangle$ is the average repulsive force exerted on the solute molecule by the solvent [36]. Neglecting surface effects it can be shown [36] that the solvent-induced repulsive force is given by the pressure times the volume change with respect to bond length

$$F_R = p\left(\frac{dV}{dl}\right) \quad (3)$$

When repulsive forces dominate in the system, a blue shift is observed. Typically it increases linearly with pressure. Such an effect has been previously observed for the C–H stretching mode of methanol [37], and the C–C stretching mode of isobutylene [36].

For attractive forces dominating in the intermolecular interactions, a pressure-induced red shift is observed. For example, it has been observed previously for the C = O stretching vibration in ethylene carbonate [38], the C–O stretching mode in methanol [37], and the N–H stretching mode in ammonia [39], i.e., for bonds readily participating in hydrogen bond formation.

For lipid bilayers the situation is more complex, as these elastic pressure effects discussed above are accompanied by pressure induced conformational changes. Each of the conformational states is characterized by a different intramolecular potential and thus gives rise to a slightly shifted vibrational absorption band. The overall band is usually best described by a Voight function, a convolution of Gaussian and Lorentzian functions [40]. Pressure-induced conformational effects modify both the band position and its shape.

Elastic and conformational effects cannot be easily distinguished. Fortunately, for some bonds, selective isotope substitution of certain conformational states results in distinctly different molecular vibrational frequencies, so that the bands due to different conformers can be studied separately. Such studies have been performed for the C = O *sn*-1 and *sn*-2 chains of phospholipids, for selected methylene groups, and for the terminal methyl group [26,41,42]. However, no general conclusions have been made and each vibrational mode needs to be discussed separately.

At quite high pressures, intermolecular effects might become significant and lead to the splitting of vibrational bands. It has been reported earlier that the bending and the rocking modes of the methylene chains of phospholipids split into two components when high external pressure is applied to hydrated DPPC bilayers [29,30,43]. The pressure reorients the hydrocarbon chains and reduces the number of *gauche* conformers along the acyl chains, which results in increased order in the structure of the chains and thus in increased intermolecular interaction potential. According to a first-order perturbation theory [43], the energy levels of the excited vibrational state of a lipid bilayer system with, for example, two non-equivalent methylene chains per unit cell will split into two branches. The splitting of each frequency of the isolated chains in the bilayers is the so-called correlation field splitting, which is

$$\Delta\nu = \frac{1}{4\pi^2 \nu_o c} \sum_{b_j=1}^{N} \left(\frac{\partial^2 U_{ab_j}}{\partial q_a \partial q_{b_j}}\right)_o \quad (4)$$

where ν_o is the harmonic frequency of the chain vibrational mode, q is the normal coordinate, and U_{ab_j} is the pair perturbation potential function between nonequivalent methylene chains a and b. This potential is a function of the interchain distance and the relative orientation of the chains in the lipid bilayer.

The correlation field splitting of the modes thus originates from the vibrational coupling interaction between fully extended methylene chains with different site symmetry along each bilayer leaflet. The interchain interactions of methylene chains with the same site symmetry contribute to frequency shifts of the vibrational modes. There will be no correlation field splitting if (i) the methylene chains are highly disordered due to the presence of a large number of *gauche* rotamers and kinks, so that the coupling of the vibrational modes between neighboring chains is random and weak, and (ii) if the methylene chains are conformationally highly ordered and fully extended, but the orientation of these fully extended chains is disordered due to reorientational fluctuations and torsion/twisting motions of the chains [43]. In these cases, only broadening rather than correlation field splitting of the vibrational bands will be observed by increase of pressure.

At high enough pressures, the conformational disorder and orientational fluctuations of the methylene chains can be ordered and dampened, however, due to the lateral compression perpendicular to the chain axis, and correlation field splitting in the vibrational modes of the methylene chains appears, provided that the equilibrium orientations of neighboring chains are non-equivalent. For more orientationally disordered chains, a higher pressure is required to stop these fluctuations and motions. Thus, the correlation field splitting pressure, p_{cf}, at which the splitting appears, is higher. Consequently, the order/disorder dynamics of the methylene chains in lipid bilayers can be determined by the magnitude of the correlation field splitting pressure. Moreover, the magnitude of the correlation field splitting, $\Delta\nu_{cf}$, is a measure of the degree of interchain-interactions in lipid bilayers. The size of the splitting depends on the type of vibration involved and is greater for those modes that involve motions predominantly per-

pendicular to the chain direction, because the interchain interaction is stronger.

Recently, Wong [43] has shown that the correlation field splitting observed is mainly contributed by the intermolecular interchain interaction among neighboring phospholipid molecules rather than the intramolecular interchain interactions within individual lipid molecules. He has shown that the correlation field interactions only take place among the nearest neighbors and that the long-range interactions with the second or higher neighboring molecules are insignificant.

4. Results and discussion

4.1. The acyl chain region

4.1.1. CH_2 stretching mode

The internal vibrational modes of the lipid acyl chains are assigned on the basis of the well-known studies on polymethylenes and polymethylene-chain compounds [44–47]. In the 2800–3100 cm^{-1} region there are infrared absorption bands due to symmetric and antisymmetric modes of the methylene chain, at about 2850 and 2920 cm^{-1}, respectively. The wavenumbers of these bands are conformation-sensitive and thus respond to temperature- and pressure-induced changes of the *trans/gauche* ratio in acyl chains. The vibrational mode (antisymmetric stretch) of the terminal CH_3 group occurs at about 2960 cm^{-1}. Infrared bands due to bending vibrations of the methylene and methyl groups are found in the 1500–1350 cm^{-1} region. The CH_2 bending or scissoring mode (δCH_2) gives rise to a series of bands around 1470 cm^{-1}. The number of these bands, as well as their exact frequencies, are dependent on the acyl chain packing and conformation. The deformation modes of the CH_3 groups occur around 1465 cm^{-1} (antisymmetric) and 1380 cm^{-1} (symmetric). The IR spectra of polymethylene chains also show a series of bands in the region 1380–1190 cm^{-1}, the number and frequencies of which are characteristic of the chain length (CH_2 wagging band progression). There is also a CH_2 rocking band progression in the 1150–700 cm^{-1} region.

The main, or gel to liquid-crystalline phase transition of DPPC multilamellar vesicles occurs at $T_m \approx 42°C$ [23]. The transition involves structural rearrangements which lead to considerable changes in the IR spectrum. Fig. 1 shows the temperature dependence of the wavenumber of the CH_2 symmetric stretching band at 2850 cm^{-1}. It is seen that at T_m a sharp increase in the band position is observed. At 35°C, where the $L_{\beta'}$–$P_{\beta'}$ gel to gel pretransition of DPPC takes place, a slight change in the wavenumber of the symmetric CH_2 stretch can be noticed. It is worth noticing that similar frequency shifts have been observed for the CH_2 antisymmetric stretching mode at 2920 cm^{-1}, but they will not be discussed here.

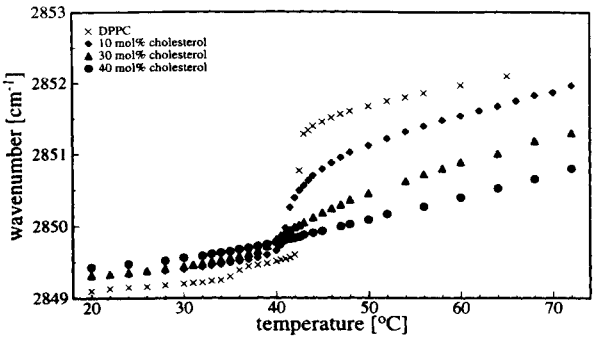

Fig. 1. Temperature dependence of the wavenumber of the CH_2 symmetric stretching band in the IR spectra of aqueous dispersions of DPPC and of selected mixtures of cholesterol with DPPC.

The position of the symmetric C–H stretching vibration is a measure of the number of *gauche* conformers in the acyl chains. When all methylene groups are in *trans* conformation, the band is observed around 2849 cm^{-1} [6]. Addition of *gauche* conformers results in a shift to higher frequency. At the main transition from the gel to the liquid-crystalline phase, which results in melting of the acyl chains, the band shifts by about 2 cm^{-1}. Since the pretransition requires smaller conformational changes than the main transition, the magnitudes of wavenumber shifts at both transitions are drastically different. Though a quantitative analysis of the number of *gauche* bonds is difficult to assess, the data in Fig. 1 clearly show that, with increasing cholesterol content, the number of *gauche* conformers in the liquid-crystalline phase markedly decreases, whereas the average number of *gauche* bonds in the gel phase increases. As a result, the band shift at the main transition is reduced at increased cholesterol concentrations. This effect was previously observed by Lippert and Peticolas [11] and Umemura et al. [6]. At 40 mol% cholesterol, the main transition is almost undetectable.

Fig. 2 shows the CH_2 symmetric and antisymmetric stretching modes of DPPC at 55°C as a function of pressure. The spectrum at ambient pressure corresponds to the

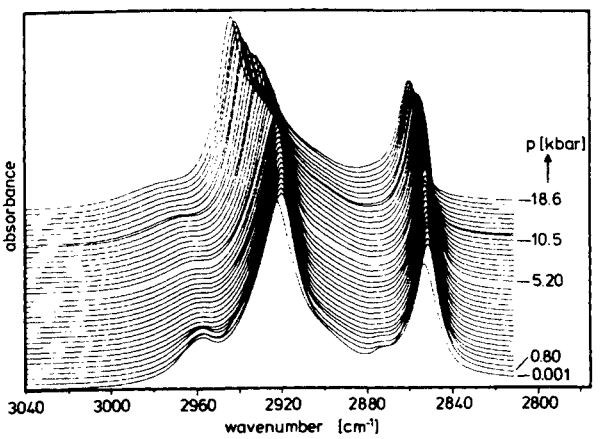

Fig. 2. Pressure dependence of the CH_2 symmetric and antisymmetric stretching mode of aqueous DPPC at $T = 55°C$.

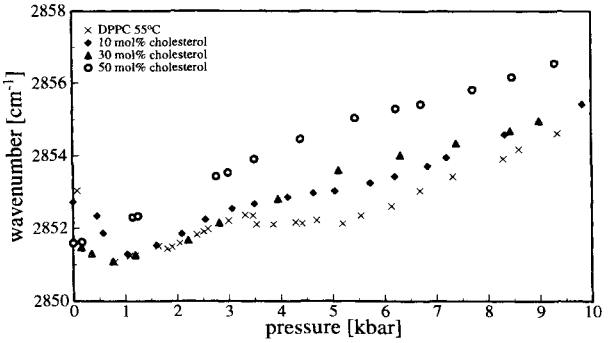

Fig. 3. The pressure-dependent wavenumber of the symmetric CH_2 stretching mode of aqueous DPPC and a few selected DPPC/cholesterol mixtures at $T = 55°C$.

spectrum of DPPC in the liquid-crystalline phase. Increase of pressure to 800 bar leads to the pressure-induced transition to the gel state [48,49] and shifts the CH_2 mode bands to lower wavenumbers. Further increase of pressure sends the bands towards higher wavenumbers. Another important observation, previously not reported in the literature, is the band splitting of the antisymmetric CH_2 stretching mode observed at pressures higher than 15 kbar. The origin of the splitting might be due to correlation field effects.

Fig. 3 exhibits the pressure dependence of the wavenumber of the symmetric stretching CH_2 mode for DPPC and for selected DPPC/cholesterol mixtures at $T = 55°C$. For pure DPPC, ν^*_{sym} (CH_2) initially decreases with pressure, but above 0.7 kbar the trend reverses, and the band shifts towards higher wavenumbers. However, different rates have been observed for different pressure regions. The changes in the slope can be observed at about 1.7 kbar, 3.4 kbar and 5.3 kbar. At these pressures, different phase transitions have been previously identified by neutron diffraction, NMR and turbidity experiments [50–53]. The transition from the L_α phase to the $P_{\beta'}$ (or GI) gel phase takes place at 0.7 kbar. Around 1.7 kbar, a high-pressure interdigitated gel phase $L_{\beta i}$ is formed, where the acyl chains of the opposing leaflets are partially intercalated. Above 3.4 kbar, the transition to a further gel phase (GIII) takes place. In this phase, the chains are expected to be tilted similar to the $L_{\beta'}$ gel phase. Not only the intrachain configuration, but also the interchain orientation is highly ordered in the GIII phase [29,30]. Above 5.3 kbar, a further gel phase (Gx) is formed. In this gel phase, tilting of the methylene chains is probably no longer required.

Addition of cholesterol leads to a decrease in the wavenumber of the symmetric stretch at ambient pressure, which indicates a reduction in the number of *gauche* bonds at 55°C upon incorporation of cholesterol into the lipid bilayer. The pressure dependence of $\nu^*_{sym}(CH_2)$ is only slightly affected by addition of 10 mol% cholesterol (see Fig. 3). For sterol concentrations higher than 20 mol%, the various transitions are smeared out, however, and the wavenumbers increase at different rates depending on the sterol concentration.

The band position of the symmetric stretching mode of the CH_2 groups of pure DPPC at 25°C (data not shown) increases linearly with pressure and only one small change in the slope around 3.2 kbar was observed. At this pressure, recent high-pressure Synchrotron-X-ray diffraction experiments using the diamond anvil technique [53] point to the transition from a lamellar gel phase with tilted chains ($L_{\beta'}$) to a lamellar gel phase with the chains being oriented parallel to the bilayer normal (L_β-phase). The corrresponding data for the samples containing cholesterol did not show significant changes in the whole pressure range covered.

The wavenumber of the terminal CH_3 group of DPPC at 2956 cm^{-1} also increases with pressure for both temperatures. But at higher pressures this band overlaps with the intense antisymmetric CH_2 mode and it cannot be resolved. Slight changes in the band shape are observed in the $P_{\beta'}$–$L_{\beta i}$ transition region at 55°C.

4.1.2. The wagging progression region

The assignment of the bands constituting the so called wagging progression has been based on a study by Snyder [45–47] on hydrocarbon chains. The wagging progression extends from 1300 cm^{-1} to 1400 cm^{-1}, and the bands present there are due to various forms of *gauche* defects in the acyl chains. *Gauche-trans-gauche* sequences (gtg′ (kinks) and gtg) give a band at 1368 cm^{-1}, end-*gauche* conformers at the very end of the acyl chains a band at 1342 cm^{-1}, and double-*gauche* conformers a band at 1356 cm^{-1}. The intensities are determined and normalized to the intensity of the conformation-insensitive band due to the symmetric methyl bending at 1378 cm^{-1}. Single-*gauche* conformers in the interior of the hydrocarbon chain cannot be monitored in this analysis. Their concentration is very low, however [45–47]. The integral band intensities were converted to the number of *gauche* conformers per acyl chain using the calibration factors published by Senak et al. [54]. We find that for pure DPPC in the liquid-crystalline state at 44°C, the total number of *gauche* bonds per chains is about 2.0, which increases up to about 2.5 at 65°C, in agreement with the data recently published by Tuchtenhagen et al. [28]. Casal and McElhaney obtained somewhat higher values [27]. Increase of pressure of 0.6 kbar in the liquid-crystalline state leads to a ca. 14% decrease of the total number of *gauche* conformers. As cholesterol has several bands in the CH_2 wagging region with significant intensity, we did not attempt to analyse the wagging band region in terms of the various conformational states.

4.1.3. Correlation field splitting

The main aim of this work was to study the pressure-induced correlation field splitting of the CH_2 bending mode (δCH_2) and the CH_2 rocking mode (τCH_2), which have

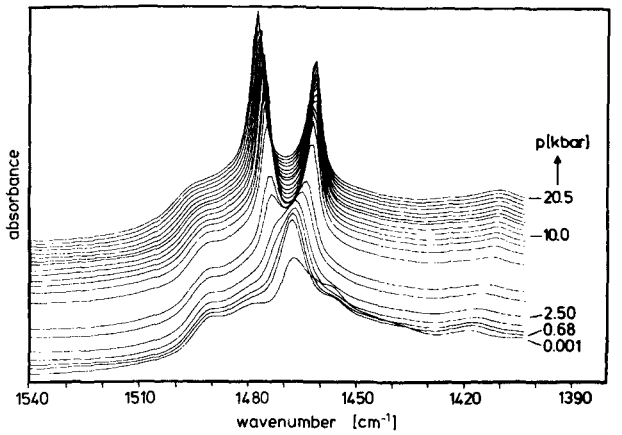

Fig. 4. Pressure contours of the CH_2 bending vibration of the CH_2 groups of the methylene chains of DPPC dispersions at $T = 55°C$.

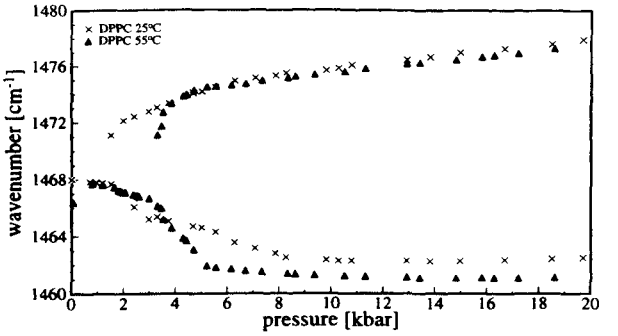

Fig. 5. Pressure dependence of the δCH_2 mode wavenumber of aqueous DPPC at $T = 25°C$ and $T = 55°C$.

been used to monitor the structural changes and dynamic properties of lipid bilayers [30,31]. The CH_2 bending vibrations of the acyl chains of DPPC are located in the region between 1460 and 1480 cm^{-1}. Fig. 4 shows the pressure contours of the IR spectra of the CH_2 bending mode of DPPC dispersion in D_2O at 55°C. At ambient pressure, the spectrum in this region consists of a central band at 1467 cm^{-1}, and three shoulders at 1490, 1479 and 1457 cm^{-1}. The central band at 1467 cm^{-1} is due to the out of phase δCH_2 mode of the methylene chains. The shoulders at 1490 and 1479 cm^{-1} are due to the symmetric and antisymmetric CH_3 bending modes (δCH_3) of the choline groups, respectively, and the shoulder at 1457 cm^{-1} is due mainly to the bending mode of the *gauche* CH_2 groups in the methylene chains [55,56,43,61]. The antisymmetric bending mode of the end methyl group also contributes to the band at 1457 cm^{-1}, but its contribution is very weak [43].

As mentioned before, at a pressure of about 0.7 kbar, the liquid-crystalline to gel transition takes place at $T =$ 55°C. In the wavenumber region shown in Fig. 4, the transition is visible by the decrease in the intensity of the *gauche* CH_2 bending band. Generally, the bands and shoulders of the bands of the spectra of the liquid-crystalline phase are broader, which is due to the conformational disorder of the methylene chains in the liquid-crystalline phase.

It is clearly seen in Fig. 4 that the δCH_2 mode splits into two at pressures above about 3 kbar, and the intensities of the correlation field component bands increase with increasing pressure. The intensity of the small band assigned to *gauche* CH_2 bending vibrations decreases with pressure and it disappears after the splitting starts. The disappearance of the band reflects the fact that at this pressure most of the disordered *gauche* bonds are removed from the chains in both the pure system and the mixtures with cholesterol. As shown by Wong [43], the correlation between the *gauche* bonds removal and bending mode splitting supports the assumption that the band splitting results from pressure enhanced interactions between orientationally oriented acyl chains.

Similar correlation field splitting of the CH_2 band has been observed for DPPC dispersions at $T = 25°C$ (data not shown). At this temperature, the DPPC bilayer is in the gel phase at ambient pressure. In Fig. 5, the pressure dependencies of the δCH_2 mode at the two temperatures are shown. As can be clearly seen, the separation between the two components increases with increasing pressure and at about 20 kbar they are centered at 1462 and 1478 cm^{-1} for DPPC at 25°C. It is interesting to note that the center of gravity of both components shifts to higher frequencies. At 55°C the splitting starts at a 1.7 kbar higher pressure. Above about 5 kbar at $T = 55°C$, and 7 kbar at $T = 25°C$, the frequencies of the two components increase linearly with pressure. As pressure increases, the disordered *gauche* bonds are gradually removed and the increase in the magnitude of the correlation field splitting is explained in terms of an increased intermolecular interaction. When the *gauche* bonds are completely removed, the frequencies shift linearly with pressure as predicted by Eqs. (2), (3).

Fig. 6 depicts a typical correlation field splitting observed for mixtures of DPPC with cholesterol at $T = 55°C$. The spectral shapes of the IR spectra are similar to those of the pure lipid system, except that the onset of the

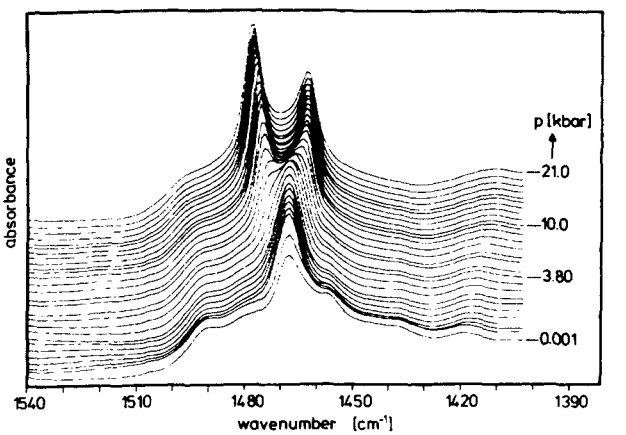

Fig. 6. Correlation field spectra of a DPPC/20 mol% cholesterol mixture at $T = 55°C$.

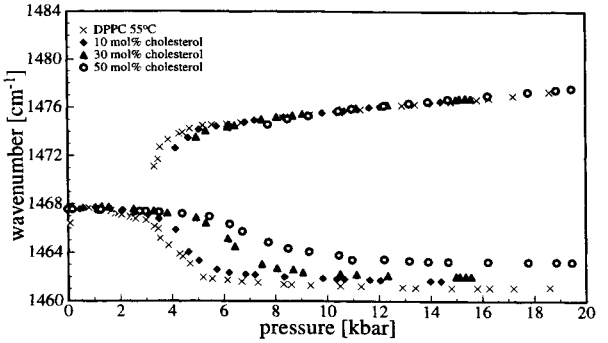

Fig. 7. Pressure dependence of the δCH_2 mode wavenumber of DPPC and DPPC/cholesterol mixtures at $T = 55°C$.

correlation field splitting starts at a somewhat higher pressure, and the 'valley' between the two component bands is particularly less pronounced in the presence of cholesterol. This difference is diminished in the corresponding spectra at $T = 25°C$. From neutron diffraction experiments [48,49] it is known, that for, for example, $T = 55°C$ a so-called interdigitated gel phase forms, with a partial intercalation of the opposing chains in the lipid bilayer at pressures above about 1.7 kbar. It has been shown by Wong [30,43] that an interdigitation leads to a different form of the correlation splitting curve and a much more pronounced valley between the correlation field component bands, which we also observe in Fig. 4. The shallower valley for the systems of cholesterol concentrations greater than 20 mol% indicates that the addition of cholesterol leads to the vanishing of the interdigitated gel phase in DPPC. This conclusion is further supported by the analysis of the CH_2 stretching mode (Fig. 3).

The splitting of the bending CH_2 band has been observed at different pressures in the pure lipid system and in cholesterol mixtures. For example, at 55°C in pure DPPC the splitting starts just below 3 kbar, but when the cholesterol concentration is 10 mol% it starts at 3.5 kbar, and for 30 mol% at 5.1 kbar (see Fig. 7). Fig. 8 depicts the onset of the correlation field splitting, p_{cf}, with increasing cholesterol concentration for both temperatures. Decrease in temperature by 30°C leads to a decrease in p_{cf} of about

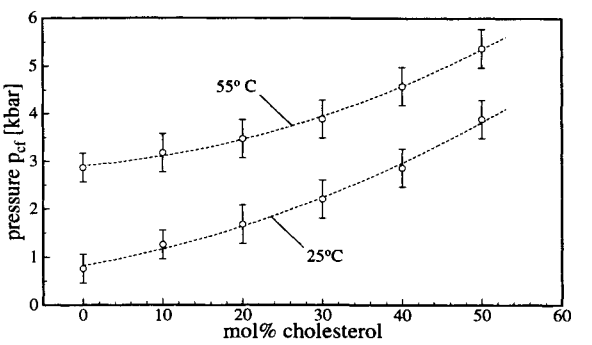

Fig. 8. The onset of the correlation field splitting (p_{cf}) of the δCH_2 band of aqueous DPPC as a function of cholesterol concentration.

2 kbar. The increase of p_{cf} with sterol concentration results from greater conformational disorder and orientational fluctuations in the lipid acyl chains upon incorporation of cholesterol. Higher pressure is required to stop these fluctuations and motions. At ambient pressure, this effect has been observed by spectroscopic methods as already discussed in the Introduction, and the contributions of different classes of *gauche* conformers has been studied by Davies et al. [58]. Interestingly, p_{cf} changes more drastically for concentrations higher than about 20 mol% cholesterol.

With increasing cholesterol contents from 0 to 50 mol% phase separation into cholesterol-rich and cholesterol-poor domains has to be envisaged, and thus phase boundaries might be crossed as a function of cholesterol concentration for both temperatures studied. There have been a number of attempts to study the phase equilibria of the system DPPC/cholesterol and to construct partial phase diagrams (see, for example, [59,60] and references therein). Recent calorimetric studies reveal, for instance, a change in phase state around 24 mol% and 30 mol% cholesterol for 55°C and 25°C, respectively [60]. However, the debate on the phase diagram and molecular organization of cholesterol in DPPC is still a matter of debate, and almost nothing is known about the pressure dependent phase behavior of DPPC/cholesterol mixtures. Therefore, no attempt has been undertaken here to discuss our findings in terms of phase equilibria and phase separation phenomena occurring in the system DPPC/cholesterol. Interestingly, however, the significant increase in correlation field splitting pressure above about 20 mol% cholesterol coincides with the cholesterol concentration, where phase separation into fluid- and gel-like domains occurs at ambient pressure.

The fact that the pressure at which the correlation field splitting starts is cholesterol concentration dependent suggests that the mechanism of the splitting is a function of intermolecular interchain interactions and the contribution of the intramolecular interchain coupling is of lesser importance. The finding that the intramolecular coupling of the vibrational transition dipole moments between the *sn*-1 and *sn*-2 chains is weak supports a similar conclusion reached by Wong for deuterated DMPC [43].

In Figs. 9 and 10 we compare the pressure contours of the terminal CH_2 rocking bands at 720 cm^{-1} [59] of pure DPPC and DPPC/20 mol% cholesterol at $T = 55°C$. Similar spectra have been obtained for other concentrations and also for studies conducted at room temperature. The band at 695 cm^{-1} is the phonon band of α-quartz which was used for the pressure calibration (see Experimental section). The CH_2 rocking band also exhibits the pressure-induced splitting due to correlation field effects. The low-frequency component of the doublet is almost pressure-insensitive, but the position of the high-frequency component shifts linearly with pressure. As observed earlier for the bending mode, the addition of 20 mol% cholesterol and more leads to a less pronounced valley between the two components.

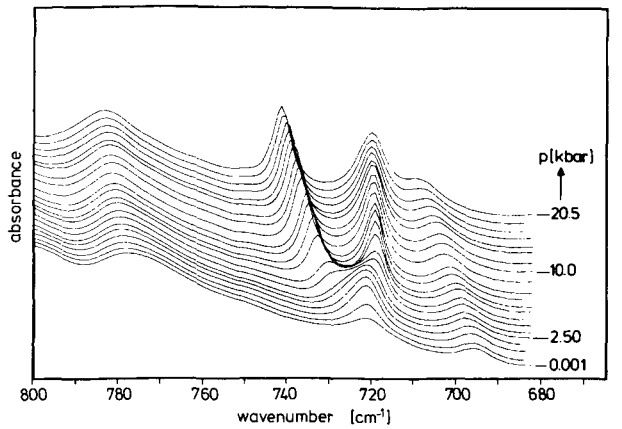

Fig. 9. Pressure contours of the CH_2 rocking band of aqueous DPPC at $T = 55°C$.

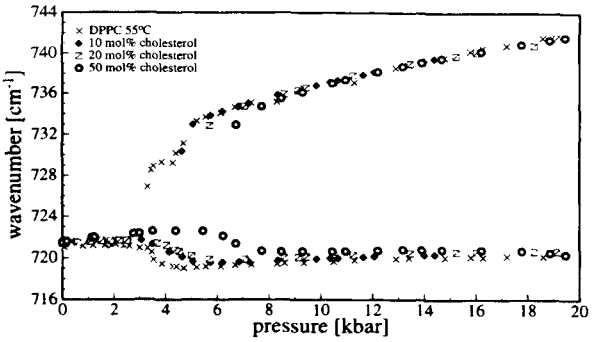

Fig. 11. Pressure dependence of the wavenumber of the CH_2 rocking mode of aqueous DPPC and DPPC/cholesterol mixtures at $T = 55°C$.

Fig. 11 summarizes our findings for the pressure dependencies of the maxima of the CH_2 rocking mode for both temperatures. The correlation field splitting starts at various pressures depending strongly on cholesterol concentrations. Comparing Figs. 7 and 11, one can see that the absolute values of the correlation field pressures $p_{cf}(x_{chol})$ are similar for the bending and rocking modes. But, the magnitude of the correlation field splitting of the rocking mode does not decrease for the samples containing 40 and 50 mol% cholesterol. This indicates that the intermolecular interactions of the terminal CH_2 groups are less affected at these high cholesterol concentrations.

The relative band intensity ratio of the two components provides information on the relative orientation of the coupled chains. The intensities of the two infrared active correlation field components of the CH_2 bending and rocking mode have been shown to be related to the angle θ between the methylene plane and the unit cell axis, as $\gamma = I_1/I_2 = \tan^2 \theta$, where I_1 and I_2 are the intensities of the components of the correlation field bands [44,30]. The height ratio γ' – as a rough estimate of the integrated intensities – of the two bands of aqueous DPPC has been appraised over the pressure range from 2 to 20 kbar: γ' decreases with increasing pressure and shows a change in slope at about 5.3 kbar, where the GIII/Gx transition takes place, and then only slightly decreases up to 20 kbar. It has been shown by Wong that, in the GIII phase of DPPC, the methylene chains in the unit cell are oriented alternately nearly perpendicular and parallel to one another, and that the high intensity ratio is mainly due to the correlation between two acyl chains within each DPPC molecule, which orient almost perpendicular to each other, while the correlation between the parallel chains of neighboring lipid molecules is insignificant due to the reorientational fluctuations of the molecules. The decrease in γ' in the Gx gel phase may be due to an increase in the parallel correlation between chains of neighboring DPPC molecules and/or a decrease in the plane orientational angle between two chains within each DPPC molecule. At low pressures, the contribution of the first effect may be significant, whereas at higher pressures the second effect may dominate. Addition of cholesterol shifts the change in slope of the intensity ratio to higher pressures, to 5.6 kbar at 10 mol% and 6 kbar at 20 mol% cholesterol. For cholesterol concentrations higher than 20 mol%, the change in slope is smeared out, but the $\gamma'(p)$ values are significantly higher. The latter finding suggests that the addition of cholesterol at these higher concentrations enhances the orientational fluctuations of the neighboring lipid molecules, thus leading to the observed larger values of γ' at higher pressures.

Interestingly, the ice formation, which occurs at about 9 kbar at 25°C and 14 kbar at 55°C in pure D_2O [57], has no influence on the spectral shapes of the bands.

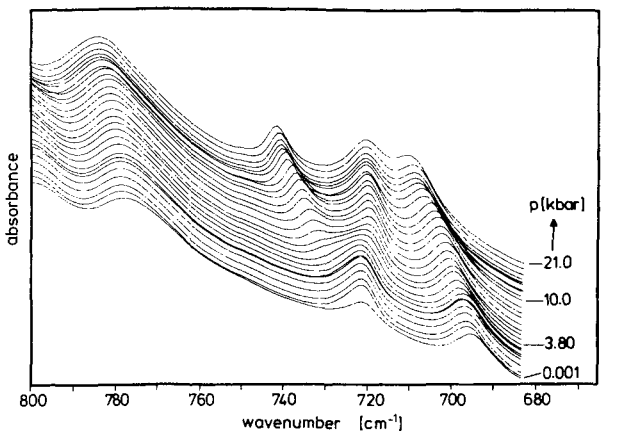

Fig. 10. Pressure contours of the CH_2 rocking band of aqueous DPPC/20 mol% cholesterol at $T = 55°C$.

4.2. The interfacial region

Structural information on the interface region of lipid bilayers is reflected by the C = O stretching mode observed at about 1740 cm^{-1}. The C = O absorption band is conformationally sensitive and responsive to changes in the polarity of the local environment of the C = O groups, and is influenced by hydrogen bonding and other interactions with neighboring molecules. The contour of the

absorption band can be interpreted in terms of changes in the conformational structure and/or hydration of the bilayer/apolar interface [41,42,32]. The interpretation of spectral changes of this band is not straightforward, however, because the C = O contour arises from the *sn*-1 and *sn*-2 chain carbonyl groups of the lipid molecule. The two carbonyl groups are not equivalent and might contribute differently to the observed band shape. The early interpretation of the band changes was based on the assumption that the immediate environments of the *sn*-1 and *sn*-2 C = O moieties result in their different vibrational frequencies. This interpretation was later questioned by a series of experiments in which the *sn*-1 or *sn*-2 chain carbonyl groups had their carbon atoms selectively substituted with ^{13}C isotope [41,42]. The ^{13}C-labeled groups have vibrational frequencies shifted by about 42 cm^{-1} which means that it was possible to analyze separately changes induced in the *sn*-1 and *sn*-2 C = O groups. Based on those experiments it was proposed that for unlabeled DPPC the 1740 cm^{-1} band is composed of several bands. The C = O modes of the *sn*-1 and *sn*-2 chain carbonyl moieties not engaged in hydrogen bond formation have their frequencies separated by only 3 to 4 wavenumbers, therefore they are observed as one broad component which at ambient conditions is centered at about 1742 cm^{-1}. The C = O bonds, both *sn*-1 and *sn*-2 chains, which participate in hydrogen bonding with water have their frequencies red-shifted and are observed as one broad component centered at about 1728 cm^{-1}. It is reasonable to assume that interfacial water is the primary source of hydrogen donors to which the carbonyl groups can be bonded. Thus, the underlying components resolved in the C = O vibrational band of fully hydrated DPPC are assumed to consist mainly of the summation of comparable contributions from both of the ester carbonyl groups rather than arising from the stretching vibrations of the individual *sn*-1 and *sn*-2 ester carbonyl groups. The high- and low-frequency sides that are observed may be attributable to subpopulations of free, mono-, and di-, tri- etc. hydrogen-bonded ester carbonyl groups, respectively [41].

The carbonyl stretching band of DPPC is not symmetric but shows a shoulder at lower wavenumbers, the height of this shoulder depending on the temperature and phase state of the lipid. Fourier self-deconvolution of the band shows that at least three overlapping bands of different frequency, intensity and half-width are present. The band at lower wavenumber increases in intensity, when the lipid passes into the liquid-crystalline state at 42°C.

Fig. 12 shows the C = O band at, for example, 38°C as a function of cholesterol concentration. Clearly, a marked broadening of the low frequency component of the band is observed with increasing cholesterol concentration. The half width at half maximum, $\Delta\nu^*_{1/2}$, of the C = O band significantly increases after incorporating cholesterol into the DPPC bilayer, e.g., from 33 cm^{-1} to 34 cm^{-1} upon addition of 10 mol% cholesterol, and from 33 cm^{-1} to 38 cm^{-1} by addition of 40 mol% cholesterol. The broadening takes place at the low frequency side of the broad C = O band only. This indicates that the contribution of hydrated carbonyl groups significantly increases and the amount of free carbonyl groups decreases by inclusion of cholesterol. In a recent IR spectroscopic study using isotope-labelled 2-[1-^{13}C]DPPC [62], Green et al. found that incorporation of cholesterol causes a significant broadening of both the *sn*-1 and *sn*-2 chain bands, the effect on the *sn*-2 chain being relatively greater than the effect on the *sn*-1 chain feature.

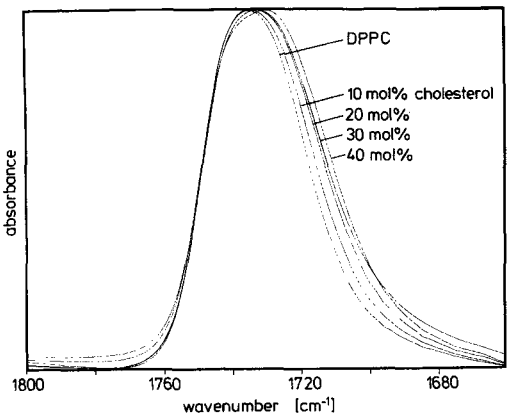

Fig. 12. The C = O band contour of aqueous DPPC at $T = 38°C$ as a function of cholesterol concentration.

We have tried to extract semiquantitative information on the extent of hydrogen bonding to the two ester C = O groups by simulating the observed band shapes with Gaussian-Lorentzian functions. Fitting of the C = O contour band using three peaks, which represent free (1744 cm^{-1}), mono- (1725 cm^{-1}), and higher hydrated carbonyl species (1697 cm^{-1}), has been applied to yield a rough estimate of the relative contributions of free and hydrated C = O groups. For a quantitative determination of the relative contributions one would have to know the transition dipole moments for the different hydrated forms, which are unknown, however. Fig. 13 exhibits the temperature dependence of the high frequency component around

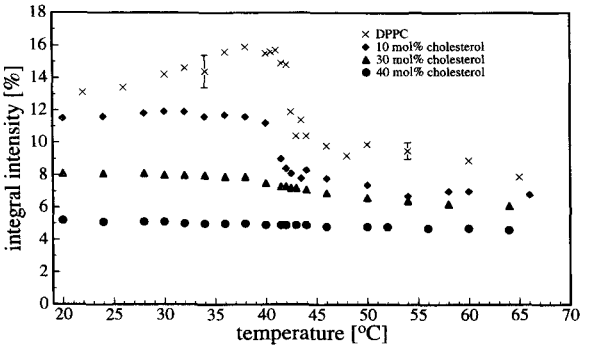

Fig. 13. Intensity of the high frequency component of the carbonyl stretching contour band of DPPC as a function of temperature and cholesterol concentration.

1744 cm^{-1} of the C = O stretching band contour of aqueous DPPC and DPPC/cholesterol mixtures, which is assumed to be due to non-hydrated *sn*-1 and *sn*-2 ester carbonyl groups [41,42]. At the main transition of pure DPPC, the hydration level significantly increases by the lateral expansion of the lipid lattice. It is evident that with increased cholesterol concentration the contribution of the free *sn*-1 and *sn*-2 C = O bands of DPPC below T_m becomes progressively less important, while the distribution of vibrational frequencies of hydrogen bonded carbonyl groups is significantly broadened. The addition of cholesterol leads to an increase in the number of hydrated carbonyl ester groups, predominantly at high cholesterol concentrations and at temperatures below the main transition temperature.

In pure DPPC, the peak maximum of the C = O stretching band is observed at about 1735 cm^{-1}. Increased pressure results in a small band broadening on both low and high wavenumber sides: $\Delta\nu^*_{1/2}$ increases about 1.5 cm^{-1} up to 5 kbar, a larger broadening of about 5 cm^{-1} is observed by application of 10 kbar pressure. The decrease in C = O stretching wavenumber is probably due to the strengthening of the hydrogen-bonding by pressure. The frequency shift of the C = O band is difficult to interpret because it may arise from relative intensity changes of individual hydrated components as well as from pressure induced shifts and conformational changes. For these reasons the following discussion concentrates on the observed band shape changes and not on the peak maximum shifts.

The C = O band of DPPC in, for instance, the 40 mol% cholesterol mixture (see Fig. 14) broadens at the following rates at 25°C: about 4.5 cm^{-1} in the pressure range 1 bar to 1 kbar, 2 cm^{-1} to 3 kbar, and 1.5 cm^{-1} to 5 kbar. Up to 5 kbar, the broadening takes place only on the low-frequency side, the high-frequency side remains unaltered.

Analysis of the band shape in terms of the contributions of free and hydrated C = O groups reveals that increase of pressure leads to a significant increase in free carbonyl groups, e.g. about 10% for DPPC and all DPPC/cholesterol mixtures by application of 1 kbar pressure. Thus, increase of cholesterol concentration and increase of hydrostatic pressure lead to opposite changes in the population of free and hydrated carbonyl ester groups of DPPC in the gel phase. The higher the cholesterol concentration, the more pronounced is the pressure effect on the population of free C = O groups. For instance, 5 kbar pressure leads to an increase of approx. 13% in free carbonyl groups in pure DPPC, of 16% in DPPC/30 mol% cholesterol, and of 25% in DPPC/50 mol% cholesterol.

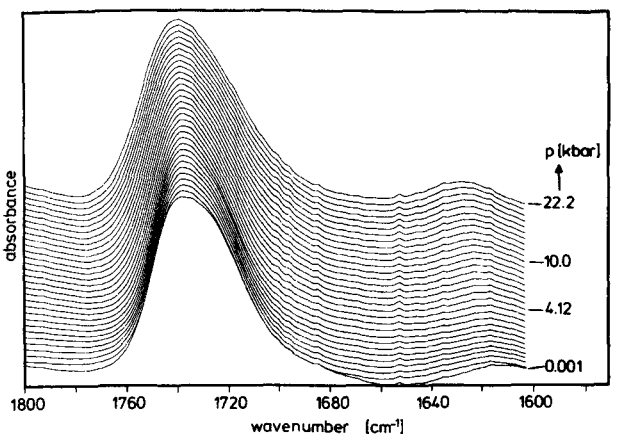

Fig. 14. Effect of pressure on the band shape of the C = O carbonyl band of an aqueous DPPC/40 mol% cholesterol mixture at T = 25°C.

The interpretation given above must be taken with great caution, however. Possible conformational changes in the glycerol backbone conformation, which might occur at higher pressures, have been neglected in these considerations. Although conformational changes between the *sn*-1 and *sn*-2 ester carbonyl groups of DPPC do not seem to be significant determinants of the C = O absorption band position at ambient pressure, they could be a more significant determinant of the substructure of the lipid C = O absorption band of the lipids at high pressures.

5. Conclusions

Detection and analysis of the infrared spectral parameters of the acyl chain and interface region modes have been used to detect structural and dynamical changes upon incorporation of cholesterol into the DPPC bilayer at 25°C and 55°C and pressure values up to 20 kbar. The spectral parameters yield information on the conformer population, reorientational fluctuations, interchain interaction, hydrogen bonding, and phase transformations of the DPPC/cholesterol mixtures.

From the CH_2 symmetric and antisymmetric stretching modes of DPPC, a series of phase transformations has been detected in the gel phase. For example, at T = 55°C the transition from the L_α to the $P_{\beta'}$ (GI) gel phase occurs at 0.7 kbar, the GI-$L_{\beta i}$ transition at 1.7 kbar, at 3.4–5.3 kbar the GIII gel phase exists, and above 5.3 kbar a further gel phase (Gx) is formed. The series of pressure-induced structural phase transitions involve modifications in the acyl chain packing. These modifications are mainly driven by changes in the mutual accommodation between the area of the headgroup and the acyl chains as a result of the pressure-induced intrachain conformational and interchain reorientational ordering process. Concentrations higher than about 20 mol% cholesterol lead to a smearing out or vanishing of the high-pressure gel phase transformations, such as the vanishing of the interdigitated high-pressure gel phase at, for example, T = 55°C.

Analysis of the pressure-induced correlation field splitting of the CH_2 bending and terminal CH_2 rocking mode have been used to monitor structural changes and dynamical properties of the lipid/cholesterol systems. The splitting of these bands arises from pressure enhanced interac-

tions between orientationally oriented acyl chains. With increasing cholesterol concentration, a significant increase of the onset of the correlation field splitting pressure is observed, which results from an increase of disorder from the intercalation of cholesterol between the lipid acyl chains in their gel state. Cholesterol induces more orientational disorder of the lipid molecules in terms of an increase of the reorientational fluctuations of the molecules and twisting/torsion motions of the acyl chains also at elevated pressures. This effect is more pronounced at concentrations above about 20 mol% cholesterol. The CH_2 rocking band exhibits a similar pressure-induced splitting due to correlation field effects as the CH_2 bending band, with the correlation field splitting occurring at the same absolute pressure values, which seems to be reasonable because both splittings monitor a similar intermolecular potential. From the magnitude of the correlation field splitting, which is a measure of the degree of interchain interactions, one can deduce that the disordering effect cholesterol imposes on the acyl chains is less severe with the inner chain region of the bilayer at high external pressures. The relative band intensity ratio of the two correlation field components provides information on the relative orientation of the coupled chains. Incorporation of cholesterol at concentrations higher than about 20 mol% leads to a marked enhancement of the orientational fluctuations, so that pressure-induced parallel orientation of neighboring lipid molecules in the GIII and Gx gel phases are prohibited. These findings support the hypothesis that one biological effect of cholesterol is to minimize changes in the cell membrane that occur as a results of thermal and other perturbations, such as high external pressure.

Structural information on the interface region of the lipid bilayer is reflected by the C = O stretching mode observed at about 1740 cm^{-1}. The C = O absorption band is sensitive to changes in conformation and is strongly influenced by hydrogen bonding. Assuming the conformational changes being of less significance, one can conclude that with increasing cholesterol concentration, the contribution of hydrated carbonyl ester groups increases, predominantly at sterol concentrations above 20 mol% and at temperatures below the main transition temperature of the pure lipid system. Increase of pressure leads to a decrease in half widths of the C = O band contour which is due to a marked increase in free carbonyl groups of pure DPPC and DPPC/cholesterol mixtures, especially for cholesterol concentrations equal and higher than 30 mol%. Thus, increase of cholesterol concentration and increase in pressure have opposite effects on the hydration level of the carbonyl ester groups of DPPC in the gel phase. Increase of pressure withdraws part of the bound water from the interfacial zone of the membrane. The C = O bond of the *sn*-1 chain of aqueous DPPC is located deeper in the bilayer than the *sn*-2 carbonyl and thus is less hydrophilic. One might speculate that the β-OH group of cholesterol is located near the *sn*-1 chain and steric conditions make possible for water to remain in close proximity of the *sn*-2 carbonyl group even at the highest pressures. However, the *sn*-1 chain may loose its hydrated water when the pressure is increased, and the close packing of the head groups requires some of the hydrated water to leave the interface region. After the *sn*-1 chain carbonyl groups lose water, and the intermolecular distances are reduced, they experience an increasingly stronger contribution of the repulsive component of the intermolecular potential which explains the observed strong blue shift of this component at high pressures of 4–20 kbar.

Acknowledgements

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