Effect of Cholesterol on the Bilayer Thickness in Unilamellar Extruded DLPC and DOPC Liposomes: SANS Contrast Variation Study

J. Gallová[1], D. Uhríková[1], A. Islamov[2], A. Kuklin[2] and P. Balgavý[1]

[1] *Department of Physical Chemistry of Drugs, Faculty of Pharmacy, Comenius University, Bratislava, Slovakia*
[2] *Condensed Matter Division, Frank Laboratory of Neutron Physics, Joint Institute for Nuclear Research, 141980 Dubna, Moscow Region, Russia*

Abstract. Small-angle neutron scattering on extruded unilamellar vesicles in water was used to study bilayer thickness when cholesterol (CHOL) was added to dilauroylphosphatidylcholine (DLPC) and dioleoylphosphatidylcholine (DOPC) bilayers in molar fraction 0.44. Using the $H_2O/^2H_2O$ contrast variation and the small-angle form of Kratky–Porod approximation, the bilayer gyration radius at infinite contrast $R_{g,\infty}$ and the bilayer thickness parameter $d_{g,\infty} = 12^{0.5} R_{g,\infty}$ were obtained at 25 ℃. Addition of CHOL to DLPC increased the $d_{g,\infty}$ from 4.058 ± 0.028 nm to 4.62 ± 0.114 nm, while in case of DOPC the $d_{g,\infty}$ values were the same in the absence (4.618 ± 0.148 nm) and in the presence (4.577 ± 0.144 nm) of CHOL within experimental errors. The role of CHOL-induced changes of bilayer thickness in the protein insertion, orientation and function in membranes is discussed.

Key words: Bilayer thickness — Cholesterol — Phosphatidylcholines — Liposomes — Small-angle neutron scattering

Introduction

Cholesterol (CHOL), the main sterol component of eukaryotic membranes, plays an important role in membranes as a modulator of physical and functional properties of lipid bilayers (Yeagle 1985). CHOL is distributed heterogeneously among cellular membranes. Its molar fraction is lowest (<0.05) in the endoplasmic reticulum (the site of CHOL biosynthesis) and increases progressively along the Golgi apparatus to the highest molar fraction (approx. 0.3–0.5) in plasma membranes; concomitantly, the length of transmembrane hydrophobic protein segments significantly increases from about 15 amino acid residues in Golgi proteins to about 20 residues in plasma proteins (Bretscher and Munro 1993; Masibay et al. 1993). It has been suggested

Correspondence to: Jana Gallová, Department of Physical Chemistry of Drugs, Faculty of Pharmacy, Comenius University, Odbojárov 10, 832 32 Bratislava 3, Slovakia
E-mail: jana.gallova@fpharm.uniba.sk

that this correlation between the CHOL content of membranes and the hydrophobic sequence lengths of transmembrane proteins could play a role in membrane protein biogenesis, presumably due to changes in the bilayer thickness caused by CHOL (Bretscher and Munro 1993). This hypothesis has been tested in experiments with model phospholipid membranes. Ren et al. (1997) studied the incorporation of the synthetic α-helical peptide Ac-$K_2GL_7DLWL_9K_2A$-amide in unilamellar liposomes prepared from synthetic phosphatidylcholines (PCs) with *cis*-monounsaturated acyl chains. Using fluorescence of the central Trp residue, they have found that the peptide orientation was trans-bilayer in dioleoylphosphatidylcholine (DOPC) bilayers matching presumably the hydrophobic peptide length, while the orientation was nontrans-bilayer in thickest bilayers prepared from dinervonoylphosphatidylcholine (DNPC). Between these two extremes, the authors suppose an exchange of peptide between trans and nontrans orientations. In bilayers thinner than DOPC, the peptide orientation was trans, but the peptide was most probably tilted to the bilayer normal. The presence of CHOL in bilayers up to molar fraction 0.3 caused a decrease in the tilt angle in thin bilayers and a shift of the equilibrium to the trans orientation in thick bilayers. CHOL displayed no effect on peptide orientation in bilayers with optimal thickness (DOPC). The authors conclude that the observed effects could be caused by CHOL-induced changes in the bilayer thickness. CHOL should thus increase the thickness in bilayers thinner than DOPC and decrease it in those thicker than DOPC; for DOPC bilayers they expect no effect of CHOL on the thickness. Webb et al. (1998) used fluorescence methods to study the interaction of Ac-$K_2GL_7WL_9K_2A$-amide peptide with bilayers of diacylPCs with monounsaturated chain lengths between 14 and 24 carbons. They have observed that the peptide does not incorporate into DNPC bilayers and only partly incorporates into dierucoylphosphatidylcholine (DEPC) bilayers. The strongest binding of peptide to lipid was observed for DOPC bilayers. The presence of CHOL in the DOPC bilayer up to molar fraction 0.5 resulted in a marked reduction in the peptide incorporation into bilayers and the authors suggest an increase in the DOPC bilayer thickness and a reduced peptide partitioning into bilayers as possible causes of this effect. Similar effect of CHOL on the peptide insertion into bilayers has been observed by Ridder et al. (2002). These authors studied the insertion of 3L-4N mutant of Pf3 coat protein with the single hydrophobic $IT_2IG_2AIL_3IVLA_2V_2LGI$ α-helical segment into bilayers of unilamellar liposomes containing diacylPC and diacylphosphatidic acid (diacylPA) with monounsaturated chain lengths between 14 and 22 carbons, in molar fraction 0.75 and 0.25, respectively. The most efficient insertion was observed for DOPC+DOPA bilayers; the replacement of DOPC by CHOL up to molar fraction 0.4 resulted in a marked reduction in the peptide incorporation into bilayers.

The results shortly reviewed above prompted us to investigate the effect of CHOL on the thickness of bilayers in unilamellar liposomes prepared by extrusion from synthetic diacylPCs. We believe that this model membrane system is more relevant than bilayers in partially or fully hydrated multilamellar model membrane systems used in previous diffraction and NMR studies of the CHOL effects on lipid

bilayers (Franks 1976; Worcester and Franks 1976; McIntosh 1978; Oldfield et al. 1978; Sankaram and Thompson 1990; Nezil and Bloom 1992; Léonard et al. 2001; Richter et al. 2001; Gandhavadi et al. 2002) because the peptide or protein interactions with bilayers are studied mostly in unilamellar liposomes. We use the small-angle neutron scattering (SANS) experimental method which is suitable for the estimation of bilayer thickness changes in unilamellar liposomes (see Balgavý et al. 2001 and references therein). In our recent SANS experiments (Gallová et al. 2002), we have found that CHOL increases the thickness in dilauroylphosphatidylcholine (DLPC) liposomes dispersed in heavy water. The present study is an extension of this work – we used the contrast variation SANS method by changing the molar fraction of H_2O in ^2H_2O, and besides the DLPC liposomes we studied also DOPC liposomes, at 25 ℃ and at CHOL : PC = 0.8 molar ratio (approx. molar fraction 0.44). Bilayers from pure synthetic PCs can exist in two main states – in the liquid disordered l_d state above the melting temperature t_m and in the solid-like ordered s_o state below it, a third liquid ordered l_o state forms at CHOL contents above molar fraction 0.3 which is stable both below and above t_m of pure bilayers; in the l_o state the lipid acyl chains have a high degree of conformational order as in the s_o state, but at the same time they have a high lateral and rotational mobility as in the l_d state (Ipsen et al. 1987; Halstenberg et al. 1998; Miao et al. 2002). The values of t_m are approx. 5–7 ℃ for DLPC (see Uhríková et al. 2002 and references therein) and −17.3 ℃ for DOPC (Lewis et al. 1988) in multilamellar liposomes; the bilayers studied in the present work should be thus in the l_d state in the absence of CHOL and in the l_o state in its presence. At high CHOL concentration in the bilayer, CHOL could become immiscible with PC and CHOL-rich and PC-rich domains could separate, especially in PCs with unsaturated acyl chains (Brzustowicz et al. 2002a,b). In the unilamellar dimyristoylphosphatidylcholine (DMPC) liposomes in the l_o state, the complete (static) miscibility has been established in the SANS experiments by Knoll et al. (1985) up to molar fraction of CHOL in bilayers 0.31. In DOPC bilayers, the spin label ESR experiments indicated a formation of small CHOL-rich domains with a lifetime of 1–100 ns (Pasenkiewicz-Gierula et al. 1990, 1991; Wiśniewska et al. 2003). In our static SANS experiments, we expect that these effects will be averaged in smeared scattering curves.

Materials and Methods

DLPC and DOPC were purchased from Avanti Polar Lipids (Alabaster, USA), CHOL was from Serva (Heidelberg, Germany) and heavy water (99.98 % ^2H_2O) was obtained from Izotop (Moscow, Russia). The other chemicals were obtained from Slavus (Bratislava, Slovakia). Organic solvents and H_2O were redistilled before use. Weighted amounts of PC and CHOL were dissolved in chloroform and mixed in solution in the CHOL : PC = 0.8 molar ratio. Solvent was evaporated to dryness under a stream of pure gaseous nitrogen, followed by evacuation in a vacuum chamber. Heavy water was added, the tube was flushed with pure gaseous

nitrogen and sealed with Parafilm M (American National Can, Greenwich, USA). PC+CHOL mixture in ^2H_2O was dispersed by hand shaking and brief sonication in a bath sonicator and vortexing. This dispersion was extruded through one polycarbonate filter (Nuclepore, Plesanton, USA) with pores of diameter 50 nm, using the LiposoFast Basic extruder (Avestin, Ottawa, Canada) fitted with two gas-tight Hamilton syringes (Hamilton, Reno, USA) as described by MacDonald at al. (1991). The samples were subjected to 51 passes through the filters at room temperature. This dispersion of extruded liposomes was diluted with a mixture of ^2H_2O and H_2O to reach the molar fraction x_H of H_2O in the sample aqueous phase and the PC+CHOL concentration 10 g/l. The amounts of sample constituents were gravimerically controlled.

The samples thus prepared were flushed with the pure gaseous nitrogen, filled into 1 mm quartz cells (Hellma, Müllheim, Germany), closed and stored at room temperature. The period between the sample preparation and its measurement was not less than 2 h. During this time the isotopic composition of the aqueous phase inside and outside of liposomes equilibrated due to a very fast permeation of water molecules through the liposome bilayer (Engelbert and Lawaczeck 1985; Deamer and Bramhall 1986; Jansen and Blume 1995; Paula et al. 1996; Huster et al. 1997).

The SANS measurements were performed at the small-angle time-of-flight axially symmetric neutron scattering spectrometer YuMO at the IBR-2 fast pulsed reactor (Vagov et al. 1983; Ostanevich 1988). The sample temperature was set and controlled electronically at 25.0 ± 0.1 ℃. The sample in quartz cell was equilibrated for 1 h at this temperature before measurement. The measuring time varied depending on the x_H value. As a rule, the samples with $x_H \approx 0$ were measured for 30 min, the $x_H \approx 0.3$ samples for 40 min, and the $x_H \approx 0.5$ samples for 60 min. The scattered intensity was normalized by using a vanadium standard scatterer as described by Ostanevich (1988) and corrected for the background effects by using the blank samples containing the same ^2H_2O + H_2O mixtures but not PC+CHOL. The experimental data were evaluated by using the function minimization and error analysis program Minuit (James 2002) and the nonlinear least squares curve fitter (Pezzullo 2002).

Results and Discussion

Typical experimental dependence of the scattered intensity $I_{exp}(q)$ on the scattering vector modulus $q = 4\pi \sin\theta/\lambda$ (2θ is the scattering angle and λ the wavelength of neutrons) is shown in Fig. 1. Experimental errors of $I_{exp}(q)$ are indicated by vertical bars and the scatter of experimental points increase with the increase of q; this is caused by the reduction of small-angle scattering with the increase in scattering angle.

The liposomes extruded through 50 nm filters are spherical with a broad distribution of radii (MacDonald et al. 1991; Balgavý et al. 1998). At the PC concentrations used, the interparticle structure factor for these liposomes is equal to one

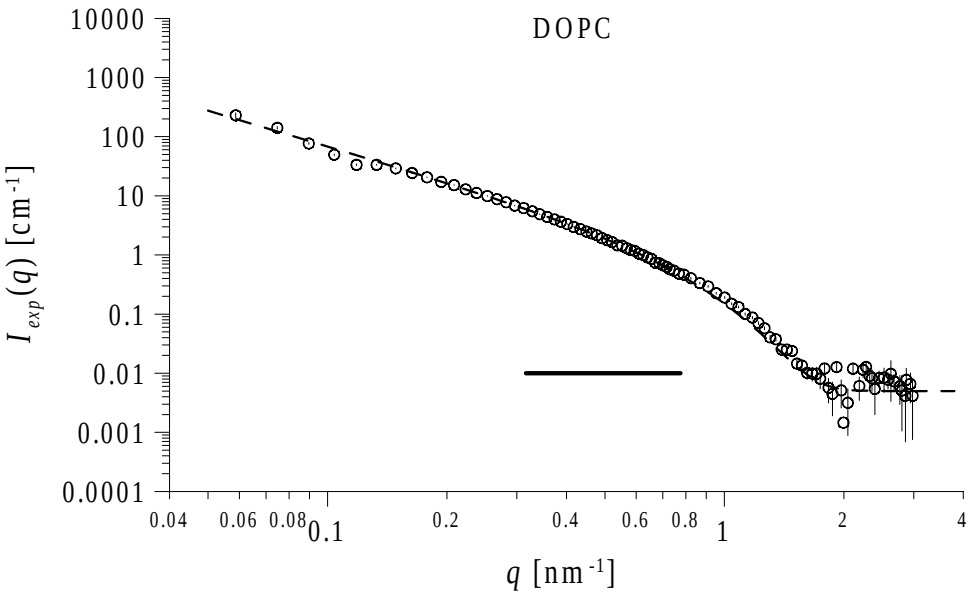

Figure 1. The SANS curve of DOPC liposomes dispersed in the aqueous phase at $x_{\mathrm{H}} = 0$.

(Nawroth et al. 1989), and the scattered intensity can be approximated by scattering on randomly oriented planar bilayers using the small-angle form of Kratky–Porod approximation

$$I(q) = A(\rho_{\mathrm{mean}} t/q)^2 \exp[-R_{\mathrm{g}}^2 q^2 0] \tag{1}$$

where A is the total area of bilayers in dispersion, $\rho_{\mathrm{mean}} = \rho_{\mathrm{L}} - \rho_{\mathrm{w}}$ is the value of contrast (the difference of the mean neutron scattering length density of the bilayer ρ_{L} and that of the aqueous phase ρ_{w}), and t and R_{g} are the one dimensional analogues of volume and radius of gyration (Sadler et al. 1990). We have shown earlier (Balgavý et al. 1998; Kučerka et al. 2003) that the Kratky–Porod approximation can be used safely to evaluate SANS data in the range of q values corresponding to interval 0.1 nm$^{-2} < q^2 < 0.6$ nm^{-2} (indicated in Fig. 1 by a horizontal abscissa) for unilamellar PC liposomes extruded through 50 nm filters. The dashed line in Fig. 1 represents the result of weighted nonlinear fitting of experimental values in this interval by using equation

$$I_{\mathrm{exp}}(q)q^2 = I(q)q^2 + I_{\mathrm{b}}q^2 \tag{2}$$

where I_{b} is the background intensity not removed when using the blank sample. The main contribution to I_{b} is the incoherent neutron scattering on PC+CHOL. If not indicated otherwise, we used $I_{\mathrm{b}} = 0.005$ cm^{-2} in the present work. It is seen that

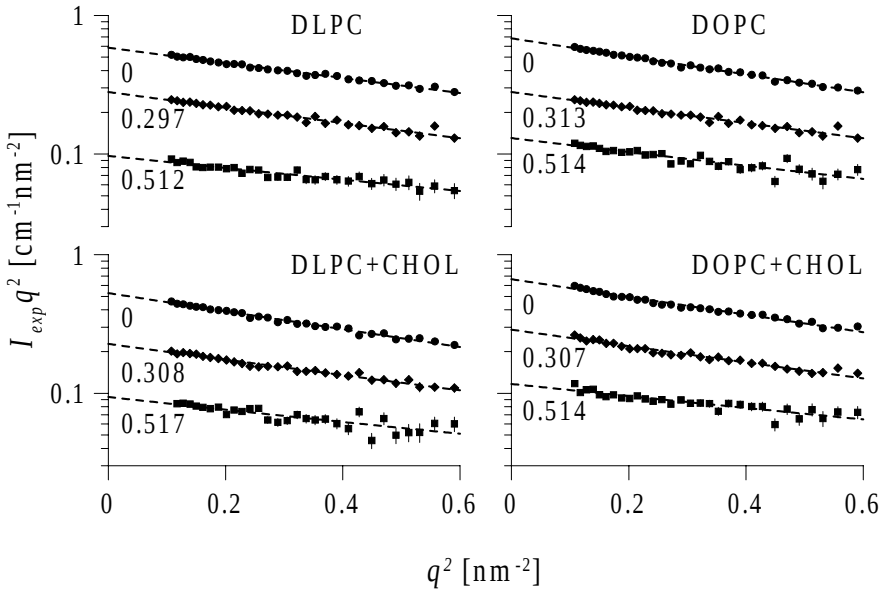

Figure 2. Kratky–Porod plots of experimental SANS data. The values of molar fraction x_{H} are shown inside the figure at each curve. Dashed lines – results of fitting by using Eq. (2).

Eq. (2) approximates the experimental data fairly well not only inside the fitted interval, but also outside of it. The deviations seen outside of the fitted interval are caused by that fact that the bilayers are not planar and that the liposomes are polydisperse.

From the Kratky–Porod plots of experimental data, the gyration radius R_{g} and the limiting value $\lim\,(Iq)q^2$ at $q^2 \to 0$ were obtained using the Eq. (2) and a weighted nonlinear least squares fitting method. The data were fitted in the $0.1\ \mathrm{nm}^{-2} < q^2 < 0.6\ \mathrm{nm}^{-2}$ region. It is seen in Fig. 2 that the Eq. (2) approximates the experimental data excellently in this region. The increase in scatter of experimental points and in experimental errors with the increasing x_{H}, seen in Fig. 2, is caused by the decrease in neutron scattering density contrast between the bilayer and the aqueous phase. The values of R_{g} and $\lim\,(Iq)q^2$ evaluated depend on x_{H} and can be used to obtain several structural parameters of the bilayer. In the following, we use the evaluation method and parameters elaborated and extensively described by Sadler et al. (1990).

From the weighted linear fit of $[\lim(Iq)q^2]^{0.5}$ on x_{H}, the average scattering length density of the lipid bilayer ρ_{L} was calculated from the match point x_{HM} at which $\lim\,(Iq)q^2 = 0$; the obtained values are shown in Table 1. For DOPC liposomes without CHOL we have obtained $\rho_{\mathrm{L}} = (1.94 \pm 0.87) \times 10^{-5}\ \mathrm{nm}^{-2}$. This experimental value can be compared with the calculated scattering length density

Table 1. Bilayer structural parameters obtained from SANS data

Liposomes	$10^5\rho_{\mathrm{L}}$ [nm^{-2}]	$10^4\alpha$	$R^2_{\mathrm{g},\infty}$ [nm^2]	$d_{\mathrm{g},\infty}$ [nm]
DLPC	3.534 ± 0.068	0.467 ± 0.086	1.372 ± 0.019	4.058 ± 0.028
DLPC+CHOL	1.012 ± 0.349	1.497 ± 0.426	1.781 ± 0.088	4.623 ± 0.114
DOPC	1.941 ± 0.872	1.415 ± 0.053	1.746 ± 0.110	4.577 ± 0.144
DOPC+CHOL	1.914 ± 0.523	1.653 ± 0.052	1.777 ± 0.114	4.618 ± 0.148

of DOPC molecule ρ_{DOPC}. The molecular volume of DOPC is $V_{\mathrm{DOPC}} = 1.3033$ nm^3 at 30 ℃ (Tristram-Nagle et al. 1998). Since our experiments were done at 25 ℃, this volume must be corrected for the temperature effect using the temperature volume expansivity $\beta = \partial(\ln V_{\mathrm{L}})/\partial T$, where T is the absolute temperature. When the value $\beta = 0.008$ K^{-1} found experimentally for DOPC (Tristram-Nagle et al. 1998) is used, one obtains $V_{\mathrm{DOPC}} = 1.298$ nm^3 at 25 ℃. Using this molecular volume and the known values of scattering lengths for the DOPC nuclei (Sears 1986; Munter 2002), one calculates $\rho_{\mathrm{DOPC}} = 3.025 \times 10^{-5}$ nm^{-2}. This is close to the estimated ρ_{L}, when taking into account the experimental uncertainty. For DOPC+CHOL liposomes we obtained $\rho_{\mathrm{L}} = (1.91 \pm 0.52) \times 10^{-5}$ nm^{-2}. The molecular volume of CHOL $V_{\mathrm{CHOL}} = 0.6234$ nm^3 can be calculated from its mass density 1.03 g/cm^3 determined by flotation of its crystals in aqueous KCl (Shieh et al. 1981). When supposing that the molecular volumes of DOPC and CHOL are additive in liposomes, one calculates the scattering length density $\rho_{\mathrm{DOPC+CHOL}} = 2.775 \times 10^{-5}$ nm^{-2}. Gershfeld (1978) has reported the mass density of CHOL 1.022 g/cm^3 in $^2H_2O/H_2O$ density gradient between 5 and 30 ℃; one obtains from this $V_{\mathrm{CHOL}} = 0.628$ nm^3 and $\rho_{\mathrm{DOPC+CHOL}} = 2.769 \times 10^{-5}$ nm^{-2}. It can be seen that also the value of ρ_{L} estimated in the present work for DOPC+CHOL liposomes is close to the theoretically calculated values of $\rho_{\mathrm{DOPC+CHOL}}$. Similar calculations can be done for DLPC and DLPC+CHOL liposomes. The differences between the experimental values ρ_{L} and those theoretically calculated could be caused by the location of water molecules inside bilayers (Pencer et al. 2003, submitted).

The gyration radius $R_{\mathrm{g}}(x_H)$ evaluated from the Kratky–Porod plot depends on the molar fraction x_{H} as

$$R^2_{\mathrm{g}}(x_{\mathrm{H}}) = R^2_{\mathrm{g},\infty} + \alpha/\rho_{\mathrm{mean}}(x_{\mathrm{H}}) \tag{3}$$

where $R_{\mathrm{g},\infty}$ is the gyration radius at infinite contrast, $\rho_{\mathrm{mean}}(x_{\mathrm{H}}) = \rho_{\mathrm{L}} - \rho_{\mathrm{w}}(x_{\mathrm{H}})$ is the value of contrast at x_{H}, and α is the dimensionless parameter (Sadler et al. 1990). The experimental R_{g} *vs.* ρ_{mean} data (Fig. 3) were fitted using the weighted linear least squares method and Eq. (3); the values of $R_{\mathrm{g},\infty}$ and α thus obtained are shown in Table 1. The parameter α in Eq. (3) describes the degree of variation of the scattering length density within the bilayer around ρ_{L}; it is governed mainly by the scattering length density differences between the bilayer polar regions and

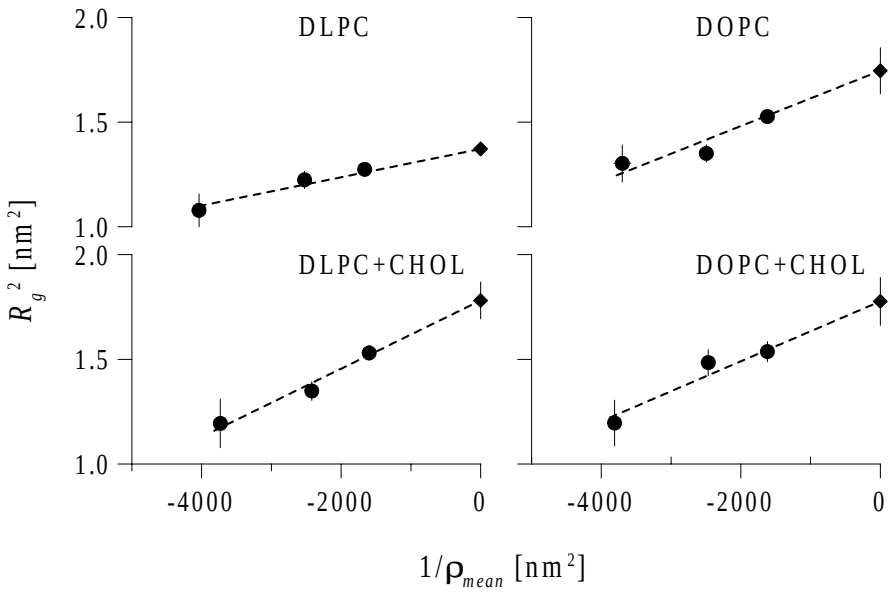

Figure 3. Dependence of the gyration radius on the mean coherent neutron scattering length density contrast. • experimental values; ◆ extrapolated values.

hydrophobic region (Sadler et al. 1990). The value of α obtained for the bilayers in the absence of CHOL is significantly lower than that in its presence (Table 1). This difference can be caused by several factors – e.g., by the differences in lateral packing density caused by the CHOL insertion between phospholipid molecules, by changes in the water molecules penetration into the bilayer, and by the location of CHOL within the bilayer.

The value of $R_{g,\infty}$ in Eq. (2) characterizes the bilayer thickness and is given by

$$R_{g,\infty}^2 = \frac{\int_{-\infty}^{\infty} z^2 \delta(z) \mathrm{d}z}{\int_{-\infty}^{\infty} \delta(z) \mathrm{d}z} \tag{4}$$

where $\delta(z)$ is a dimensionless parameter changing between 0 and 1 in the direction z perpendicular to the bilayer. For the "dry" bilayer model with no water molecules penetrated into the bilayer polar region, $\delta(z)$ is a step function with $\delta(z) = 0$ in the aqueous phase and $\delta(z) = 1$ inside the bilayer. For this special case with a homogeneous distribution of the scattering length density in the bilayer, the bilayer thickness $d_{g,\infty}$ can be obtained from

$$R_{g,\infty} = 12^{-0.5} d_{g,\infty} \tag{5}$$

Since phospholipid bilayers contain some amount of water located in their polar regions, the value of $\delta(z)$ changes smoothly in the interfacial boundary regions.

The values of $d_{g,\infty}$ could underestimate thus the real bilayer thickness. Moreover, the scattering length density within bilayer is not homogeneous. To find the extent of $d_{g,\infty}$ deviations from the real bilayer thickness, we will compare now the $d_{g,\infty}$ values obtained for DOPC directly from experimental $I_{exp}(q)q^2$ data (by using Eqs. (1) and (2) after $R_{g,\infty}$ substitution from Eq. (5) and a nonlinear weighted fitting method) with results of other recent bilayer thickness studies. Using small-angle X-ray diffraction (SAXD), Tristram-Nagle et al. (1998) have found the bilayer hydrocarbon region thickness $d_C = 2.72$ nm in fully hydrated DOPC in multilamellar samples at 30 ℃. The polar region thickness in PC bilayers is $d_P = 0.90 \pm 0.12$ nm as estimated from results of neutron diffraction studies of selectively deuterated DPPC in oriented and partially hydrated multilamellar samples (Büldt et al. 1978; Zaccai et al. 1979; Nagle and Tristram-Nagle 2000; Pabst et al. 2000), the steric bilayer thickness in multilamellar system is thus $d_L = d_C + 2d_P = 4.52 \pm 0.24$ nm. This coincides with the $d_{g,\infty} = 4.58 \pm 0.14$ nm found in the present paper. The resulting mean $d_{g,\infty}$ value slightly depends on the I_b value used. For DOPC, we have evaluated $d_{g,\infty} = 4.61 \pm 0.13$ nm at $I_b = 0$ cm^{-2} and $d_{g,\infty} = 4.54 \pm 0.15$ nm at $I_b = 0.01$ cm^{-2}. Likewise, when the resolution function of the YuMO spectrometer (Ostanevich 1988) was used in the evaluation procedure, $d_{g,\infty}$ values were changed but only within the experimental error. One can conclude thus that the $d_{g,\infty}$ deviation (if any) from the steric bilayer thickness is superimposed by the temperature effects and errors of SANS and SAXD experimental methods and evaluation procedures used. Similar comparisons can be done for DLPC bilayers. Using SAXD, Harroun et al. (1999) have measured the transbilayer distance of phosphate groups $d_{HH} = 3.08$ nm in the fluid lamellar DLPC phase at 20 ℃. The distance between the phosphate group and the boundary between the polar and hydrocarbon region is $d_{H1} = 0.43 \pm 0.02$ nm in DLPC at 20 ℃ (see Balgavý et al. 2001 and references therein). A combination of these data gives the DLPC bilayer hydrocarbon region thickness $d_C = d_{HH} - 2d_{H1} = 2.22 \pm 0.04$ nm and the bilayer thickness $d_L = d_C + 2d_P = 4.02 \pm 0.28$ nm. In the present study we have found at 25 ℃ following values for $d_{g,\infty}$: 4.11 ± 0.04 nm, 4.06 ± 0.03 nm and 4.00 ± 0.01 nm for $I_b = 0$ cm^{-2}; 0.005 cm^{-2} and 0.01 cm^{-2}, respectively. It is evident that the SANS results coincide again within experimental uncertainty with the SAXD result. From the comparisons above, one can conclude that the approximate contrast variation method of Sadler et al. (1990) gives reliable $d_{g,\infty}$ values which can be used safely as a measure of bilayer thickness.

It is seen from the values obtained (Table 1) that CHOL increases the thickness $d_{g,\infty}$ in DLPC vesicles by 0.57 ± 0.14 nm. This is comparable to the bilayer thickness increase by approx. 0.4 ± 0.2 nm in oriented and partially hydrated (at 75 % relative humidity) DLPC multilayers at molar ratio DLPC : CHOL = 2, calculated from the data in Fig. 5 in the paper of McIntosh (1978). From the X-ray diffraction data of CHOL monocrystal (Shieh et al. 1981) one calculates the longest distance 1.76 nm between the O and C(27) atoms in CHOL molecule in case of fully extended all-trans side chain; this distance is shortened to 1.67 nm in case of *gauche-trans-gauche* sequence at C(20)-C(22)-C(23)-C(24) side chain atoms. The

DLPC bilayer hydrophobic region thickness in the presence of CHOL obtained from SANS data is $d_C = d_{g,\infty} - 2d_P = 2.82 \pm 0.35$ nm; the hydrophobic thickness of one monolayer (1.41 ± 0.18 nm) can be compared to the 1.38 nm distance between C(1) and C(12) atoms in fully extended all-*trans* lauroyl chain in dilauroylphosphatidylethanolamine (DLPE) monocrystal (Elder et al. 1977). Visually, these lengths can be compared in Fig. 4 where the spacefill models of DLPE and CHOL molecules are depicted; they were constructed using the atom coordinates of DLPE and CHOL published (Elder et al. 1977; Shieh et al. 1981). Neutron diffraction study of CHOL location in partially hydrated and oriented DMPC bilayers has shown that hydroxyl group of CHOL binds to the phospholipid molecule *via* a hydrogen bond to the acyl chain carbonyl group (Léonard et al. 2001). In the case of such location (binding site A) also in the DLPC bilayers, the CHOL would penetrate 0.2–0.8 nm into the opposite monolayer. The second possibility is the shift of CHOL towards the DLPC headgroup region and formation of the hydrogen bond between the CHOL-OH group and the DLPC phosphate group (binding site B). The exchange of the CHOL molecule between these two locations can be a dynamic process; it has been observed by using the incoherent quasielastic neutron scattering (QENS) on oriented DPPC+CHOL bilayers in the l_o phase that the CHOL molecule exhibits a high-frequency confined diffusion in the direction parallel to the bilayer normal ("out-of-plane motion") with an amplitude 1.0–1.1 nm (Gliss et al. 1999; Endress et al. 2002). The increased probability of CHOL-OH group location in the close proximity of DLPC phosphate group would result in a large redistribution of the scattering length density in the bilayer polar region and this could be responsible for the large change in the parameter α observed (Table 1).

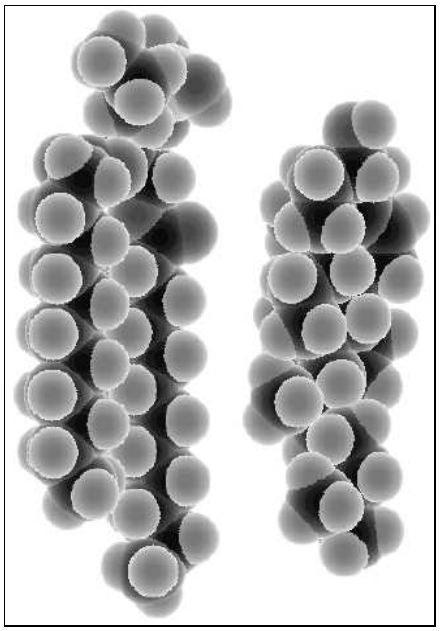

Figure 4. Spacefill models of DLPE with all-*trans* acyl chains (left) and CHOL with the *gauche-trans-gauche* sequence at C(20)-C(22)-C(23)-C(24) side chain atoms (right).

In the DOPC bilayers, where the acyl chains are substantially longer than in the DLPC bilayers, the effect of CHOL is not so clear. Because of relatively large experimental errors, we cannot exclude neither a bilayer thickness increase by about 0.33 nm nor its decrease by 0.25 nm; or in other words, when comparing the mean values – the resulting bilayer thickness change due to presence of CHOL (if any) is less than the relatively large experimental uncertainty (Table 1). It is notable that

the mean DOPC+CHOL bilayer thickness coincides with the DLPC+CHOL bilayer thickness 4.6 nm within experimental uncertainty (Table 1). The bilayer thickness of 4.5–4.7 nm has been measured by neutron reflectivity on oriented fully hydrated DMPC bilayers containing CHOL in molar fraction 0.3 (Watts et al. 2000). Thus, one could speculate that CHOL "buffers" the thickness close to about 4.6 nm in the l_o state in case of bilayers whith thicknesses being less than or equal to this value in the l_d state in the CHOL absence. A small but statistically significant increase in the parameter α due to the presence of CHOL in DOPC bilayers (Table 1) indicates that CHOL could shift between the two positions in the DOPC bilayer as well as in DLPC bilayer. However, small change in α comparing to DLPC bilayers suggests that the population of the binding site B is rather low in DOPC bilayers and that the amplitude of CHOL "out-of-plane motion" is smaller. Nevertheless, the SANS method used in the present paper is suitable to obtain just averaged bilayer structural parameters. For the investigation of CHOL and lipid dynamics in the bilayer, other experimental methods are needed such as QENS (Gliss et al. 1999; Endress et al. 2002) and inelastic X-ray scattering (Weiss et al. 2003). Furthermore, the evaluation of the SANS experimental data as used in the present paper provides rather crude measures of the bilayer structural properties. This could be improved by using more realistic models of the neutron coherent scattering length density in the bilayer in the presence of CHOL inspired by e.g. molecular dynamics simulation (Pasenkiewicz-Gierula et al. 2000; Róg and Pasenkiewicz-Gierula 2001; Chiu et al. 2002; Hofsäss et al. 2003) as done in the contributions of our group for bilayers without CHOL (Kučerka et al. 2004).

Our results are not in contradiction with the assumption of Ren et al. (1997) that the absence of CHOL effect on the incorporation of synthetic α-helical peptide Ac-$K_2GL_7DLWL_9K_2A$-amide in DOPC bilayers could be due to the absence of CHOL effect on the DOPC bilayer thickness. Unfortunately, we cannot exclude also the possibility that the reduction in the incorporation of other peptides into bilayers containing DOPC and CHOL observed by other authors (Webb et al. 1998; Ridder et al. 2002) was caused by the DOPC thickness change. If true, the explanation of the data of Ren et al. (1997) has to involve other CHOL effects than the simple "hydrophobic mismatch" scheme. Even more complicated is the situation in case of large transmembrane proteins. The length of monounsaturated acyl chain of diacylPCs has a marked effect on the activity of several membrane proteins. For example, when reconstituted in unilamellar liposomes from these lipids, the *Escherichia coli* diacylglycerol kinase (DGK) and the sarcoplasmic reticulum Ca,Mg-ATPase (SERCA) display a maximum activity in DOPC liposomes (Johannsson et al. 1981; Lee et al. 1991; Pilot et al. 2001), while the Na,K-ATPase from shark *Scualus achantia* rectal glands in DEPC liposomes (Cornelius 2001); the activities of all three transmembrane enzymes decrease in unilamellar liposomes from PCs with shorter and longer acyl chain lengths. When reconstituted in DOPC liposomes containing CHOL, the activity of DGK decreased (Pilot et al. 2001) and that of Na,K-ATPase increased (Cornelius 2001) and this seems to be in line with the CHOL-induced bilayer thickness increase. Practically no effect of CHOL on

the SERCA activity was observed when reconstituted in DOPC liposomes, but the activity increased more than 3 times when this enzyme was reconstituted in dimyristoleoylPC liposomes (Simmonds et al. 1982; Michelangeli 1986) and this indicates no effect of CHOL on the DOPC bilayer thickness. Most probably, mechanisms of CHOL effects on transmembrane protein properties must be thus more complex. In case of the SERCA, the CHOL effects on the activity could follow from direct CHOL binding to the protein, at the non-annular binding sites which have been postulated to exist at the protein-protein interfaces (Simmonds et al. 1982). Cornelius (2001) has suggested, that the CHOL-induced increase in the dipole potential of the bilayer resulting in large differences in the electric field strength within the bilayer may modify electrogenic steps in the Na,K-ATPase reaction cycle and this could be the reason for the changes in the enzyme activation entropy observed. Cantor (1999) calculated that the addition of CHOL to bilayers containing diacylPCs with unsaturated acyl chains can result in large redistributions of lateral pressure towards the bilayer interior with no changes in the bilayer thickness; he has suggested that this could modulate the transmembrane protein conformational or/and aggregation equilibria.

In conclusion, we have found that CHOL increases the DLPC bilayer thickness and that it has no measurable effect on the thickness of DOPC bilayers due to large experimental errors. The CHOL effects on properties of transbilayer peptides and proteins located in bilayers are suggested to be caused not only by the intuitively simple "hydrophobic mismatch" but also by more complicated physical interactions.

Note added at the proof. The peculiar properties of CHOL+DOPC mixtures could also be caused by the polymorphism observed recently by Epand R. M., Hughes D. W., Sayer B. G., Borochov N., Bach D., Wachtel E. (2003): Novel properties of cholesterol-dioleoylphosphatidylcholine mixtures. Biochim. Biophys. Acta. **1616**, 196—208.

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