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G. Albertini [a] , G. Bossi [c] , B. Dubini [b] , M. G. Ponzi Bossi [b] & F. Rustichelli [b]

[a] Dip. di Scienze dei Materiali e della Terra

[b] Istituto di Fisica Medica

[c] Clinica Dermatologica UNIVERSITA´ DI, ANCONA, 60131, ITALY

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STRUCTURAL MODIFICATIONS IN THE LIQUID CRYSTALLINE L_α PHASE OF DISTEAROYLPHOSPHATIDYLCHOLINE INDUCED BY VARIABLE CHOLESTEROL CONCENTRATION.

G.ALBERTINI[1], G.BOSSI[3], B.DUBINI[2], M.G. PONZI BOSSI[2], F.RUSTICHELLI[2]

[1]Dip. di Scienze dei Materiali e della Terra
[2]Istituto di Fisica Medica
[3]Clinica Dermatologica
UNIVERSITA' DI ANCONA - 60131 - ITALY

ABSTRACT

The melted chain liquid crystal phase of distearoyllecithin liposomes in water was investigated at various cholesterol content. X-ray diffraction and differential scanning calorimetry techniques were used. A fluidifying effect with respect to the chain melting temperature and a stiffening effect, deduced from the progressive increase of the lamellar repeat spacing in the melted chain phase, were deduced for low cholesterol concentrations, in agreement with the previous results in other phospholipids. An increase of the in plane lattice parameter and a decrease of the lamellar repeat spacing, corresponding to an overall non monotonic behaviour, were observed at higher concentrations, below the saturation value. The positional order inside the layers was considered for the first time in a phospholipid in a quantitative way by evaluating the correlation length ξ. That in plane correlation length remains constant for cholesterol concentrations lower than 35 % and then decreases, indicating opposite effects concerning the in plane translational order as compared to the orientational order: the cholesterol increses the last kind of order but decreases the first one.

INTRODUCTION

The importance of cholesterol in biology is well known. In fact mammalian cells need cholesterol for their normal function. In particular the cholesterol concentration influences the membrane stability and permeability trough modification of its structure and dynamics. In additions to these positive aspects cholesterol can have a very negative side because it is contained among other constituents in pathological deposits like those in atherosclerosis and in xanthomas and xantelasmas skin diseases.

In spite of the large amount of experimental work already published on cholesterol-membrane interactions, many basic mechanisms remain unknown, as it is indicated by the continuous scientific production on this subject. Also considering the restricted field of the interaction between cholesterol and model membranes made of lecithin in water, some controversies exist , about the critical concentration able to induce dramatic changes of the physical properties and about the existence of phase segregation [1]. X ray diffraction was used in the past in order to detect changes induced by the cholesterol in the lipidic layer inside the membrane [1,2], but no quantitative information was given about the translational correlation length as a function of the cholesterol content. The present work belongs to a series of investigations performed by our group on cholesterol. At first some structural

properties of individual cholesteryl esters were investigated [4,5] than of a mixture of cholesteryl esters [6,7] and finally of the Γ phase of cholesterol-distearoylphosphatidylcholine multilamellar vesicles [7].

In particular in this last system the Γ phase appears at room temperature at sufficiently high concentration of cholesterol. Two different experiments [7,8] indicate a lateral phase separation in the temperature range of existence of the Γ phase. The other phase Γ' appears to be richer in cholesterol. Moreover the (Γ + Γ') to L_α phase transition was investigated and the temperature dependence of the in plane correlation length was determined. The Γ phase appeared more disordered than the $L_{\beta'}$ and $P_{\beta'}$ phases of pure phosphatidylcholine but more ordered than the L_α phase.

This paper presents the results of an investigation performed after that reported in ref. 7. In fact instead of considering one fixed cholesterol concentration and exploring the temperature dependence of physical quantities related to the structure and order [7], here a fixed phase is considered, namely the liquid crystalline phase L_α and the cholesterol concentration dependence of the same physical quantities is explored. Actually the L_α phase is the most interesting one from a biological point of view, as the cell membranes, made of a mixture of lipids, are in that phase at physiological conditions.

The used lecithin has two saturated 18 carbon chains: at that chain length an interesting deviation was observed [3] in the behaviour of the layer thickness as a function of cholesterol content.

2. MATERIALS AND METHODS

2.1. Sample preparation

1,2-distearoyl-3-sn-phosphatidylcholine (DSPC) was purchased from SIGMA (St. Louis, Mo, USA) and was used without further purification. Cholesterol was purchased from Serva Feinbiochemica (Heidelberg, Germany).

The proper quantities of cholesterol were added to known quantities of DSPC in order to obtain the wanted molar ratios R between cholesterol and lecithin. Lipids were then dissolved in chloroform, dried in nitrogen stream and lyophilized. Distilled water was then added in a weight ratio water to DSPC x=3. Values of x=7, and x=5 were also used in order to test the effect of water.

Liposomes were obtained by incubating these mixtures for some hours at about 60°C and by vortexing several times for some minutes during the incubation.

2.2 Calorimetry

The samples were studied by using differential scanning calorimetry techniques. A DSC2c Perkin

Elmer calorimeter with related processor was used. Containers having 20µl capacity, for volatile samples, were heated and cooled at both 2.5°C/min and 10°C/min scan rates.

2.3 X-ray diffraction

X ray diffraction pictures from both aligned and not aligned samples were obtained by using a rotating anode generator Rigaku Denki RU300 and a flat chamber.

X ray diffraction patterns from not aligned samples were obtained by using a conventional powder diffractometer.

Ni filtered Cu-Kα radiation (λ = 0.154 nm) was used in any case.

2.4 Optical Microscopy.

Optical microscopy observations with crossed polarizers were performed by using a LEITZ ORTOLUX 2POL microscope equipped with a METTLER FP52 hot stage.

3. RESULTS

Although different x-ray diffraction patterns are obtained at room temperature for different cholesterol concentrations, similar profiles are obtained at the highest temperatures (below 100°C):

* in the small angle scattering region a series of peaks appears, which is compatible with a

lamellar phase having a layer thickness similar to - although not the same as - that in the liposomes without cholesterol.

* in the high angle region a diffusive peak is detected, indicating that the aliphatic chains are melted.

The temperature at which the chain melting transition occurs depends on the cholesterol concentration.

In figure 1 the chain melting transition temperatures are reported as a function of the cholesterol concentration, as they were obtained from the maximum position of calorimetric peaks.

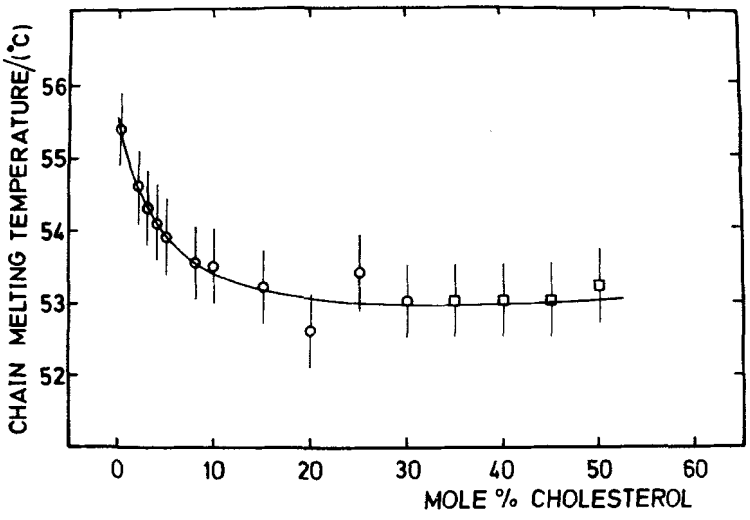

Figure 1. Chain melting temperature vs. cholesterol concentration. (Line is a guide for the eye).

For cholesterol molar fraction greater than ≈30% but not greater than 50% no calorimetric peak was

observed for heating rate of 2.5°C/min: the reported values for these concentrations refer to a 10°C/min heating rate.

A decrease of transition temperature with the cholesterol content can be seen in the range of concentrations between 0% and 50% cholesterol molar fraction.

No calorimetric peak was observed between room temperature and 80°C for higher cholesterol concentration.

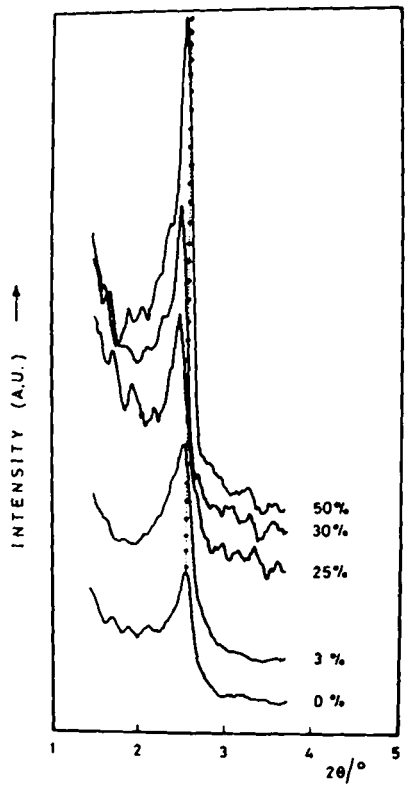

Figure 2a. Low angle x-ray scattering: Diffracted intensity vs. diffraction angle.

The low angle x-ray diffraction patterns of liposomes at 70°C having different cholesterol content are reported in figure 2a: the peak positions are at lower 2θ diffraction angles for higher cholesterol concentration, indicating a larger lamellar thickness; for still higher cholesterol concentrations lower thicknesses are obtained. The behaviour of lamellar thickness obtained by considering the low angle scattering from liposomes at 70°C is reported in figure 2b as a function of cholesterol content.

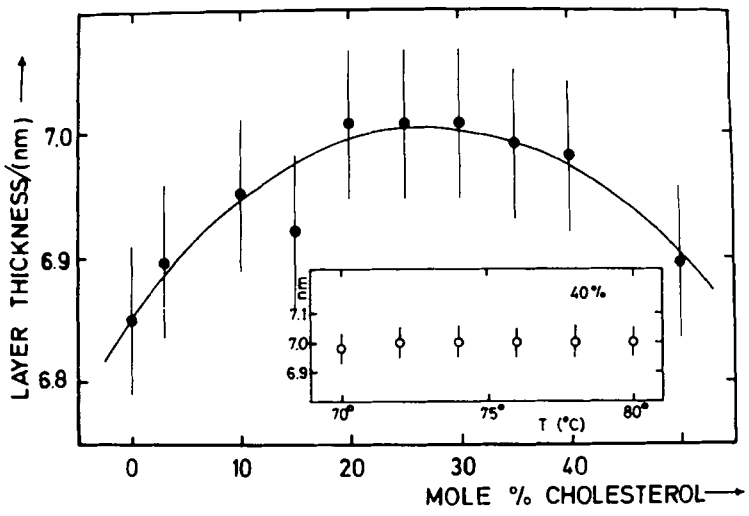

Figure 2b. Lamellar repeat spacing vs. cholesterol content (line is a guide for the eye). Insert: temperature dependence of lamellar repeat spacing at 40% cholesterol.

In order to test if the differences in thickness reported in figure 2b could be attributed to different thermal expansion coefficients and/or

to the differences in the temperature range of existence of the phase, the behaviour of the lamellar thickness as a function of temperature was considered in the temperature range between 70°C and 80°C: constant values equal to those reported in figure 2b were obtained, (as an example, the 40 % cholesterol molar fraction is reported in the insert of figure 2b, with a consequent confirmation of the non monotonic dependence of the repeat unit thickness on the cholesterol concentration).

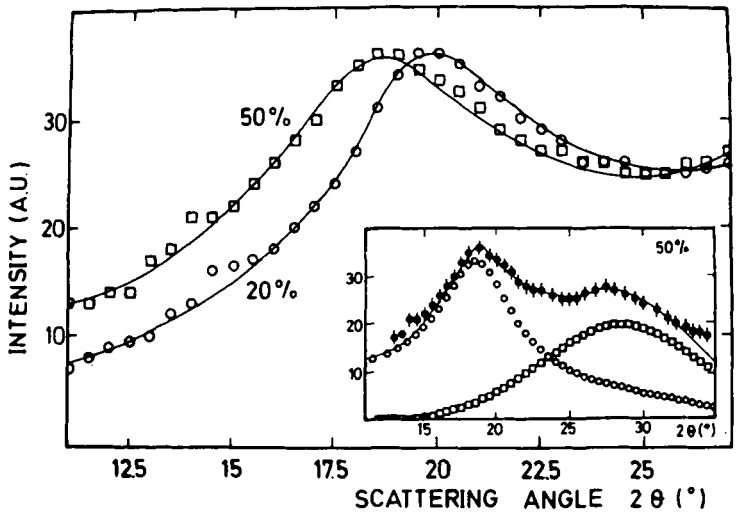

Figure 3. High angle x-ray scattering from 50% (□) and 20% (o) cholesterol molar fraction. Insert: 50% cholesterol curve: (◆) experimental data, (□) water curve, (o) calculated curve for lipids, (—) sum of water and lipid curve.

Differences are also observed in the high angle region, as can be seen in figure 3, where samples

containing 20% and 50% cholesterol molar fraction are compared. In particular the peak corresponding to the sample richer in cholesterol appears to be larger and shifted toward lower values of scattering angle 2θ.

In order to eliminate the water contribution to the high angle scattering and to get structural information from the peak shape about the in plane translational order of aliphatic chains, a data analysis was performed similar to that reported by Albertini et al. [7]: a Gaussian curve was used for the water peak:

$$I_w(2\theta) = I_o \exp[(2\theta - 2\theta_o)/\sigma]^2$$

where $2\theta_o$ =28.5°, σ=7.6° and the I_o value depends on the quantity of water present in the sample; those $2\theta_o$ and σ values were obtained by considering the contribution of water in samples having different lipid-water ratios.

Theoretical curves were then obtained for the x-ray scattering due to the chains, by considering a hexagonal chain packing with short range positional order in planes parallel to the layer surface and attributing a lorentzian shape to the two-dimensional structure factor $f(Q_p)$ inside these planes, as it was previously done in hexatic phases [7,9-10]:

$$f(Q_p) = 1/[(Q_p - Q_o)^2 + \xi^{-2}]$$

being the in plane correlation length, Q_o the (100) vector in reciprocal space and Q_p the in plane component of the scattering vector. That structural organization was assumed in the melted chain phase in analogy to that proposed for the smectic A two-dimensional liquid phase [13].

Concerning the electronic density σ along an axis z normal to the layers and starting from the centre of the bilayer, two different functions were considered: the first one corresponding to a cholesterol rich phase.

$\sigma \approx -12$ for $|z| \leq 3$

$\sigma \approx 5$ for $3 < z \leq 20$

$\sigma \approx 15$ for $20 < z \leq 26.5$

$\sigma \approx 0$ for $26.5 < z \leq D/2$

(where D = lamellar repeat spacing)

the second one corresponding to pure DSPC:

$\sigma \approx -14$ for $|z| \leq 3$

$\sigma \approx 0$ for $3 < z \leq 22$

$\sigma \approx 15$ for $22 < z \leq 30$

$\sigma \approx 0$ for $30 < z \leq D/2$

Both these profiles were deduced from data reported by McIntosh [3].

The x-ray intensity diffracted at an angle 2θ was computed by integrating the intensity in the reciprocal space over a sphere of radius $Q=4\pi\sin(\theta)/\lambda$ and making corrections due to the geometry of the apparatus. No correction was made for instrumental resolution, which is much smaller than the width of the peak [10]

The Q_o and ξ values corresponding to each experimental peak were obtained by a best fit of the experimental results by the theoretical curve so obtained. The difference obtained by using the two electronic density functions are lower than the given experimental uncertainties. An example of high angle scattering decomposition into a water peak and a chain peak is reported in the insert of figure 3. From the Q_o value the interchain distance was obtained by using the relation:

$$\ell = 4 \pi / (\sqrt{3} * Q_o);$$

values at 70 °C as a function of cholesterol molar concentration are reported in figure 4a.

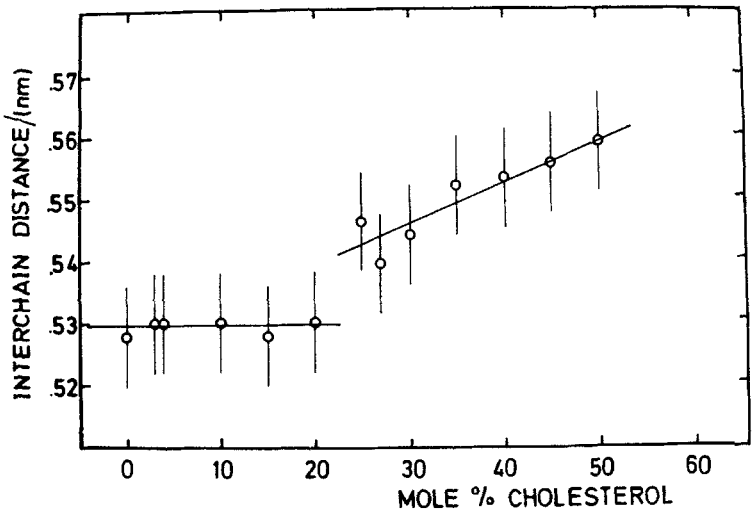

Figure 4a. Hexagonal lattice parameter, vs. the cholesterol content.

The corresponding peak widths at half maximun (HMW) are reported in figure 5a.

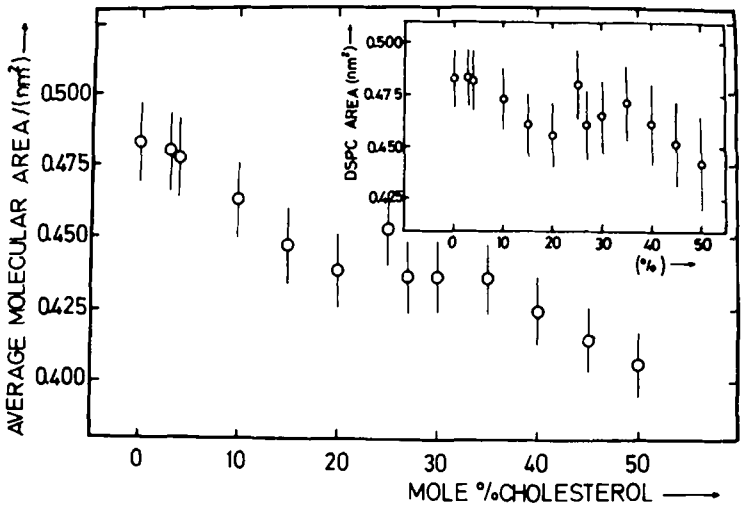

Figure 4b. Mean molecular area and molecular area of lecithin (insert) vs. the cholesterol content.

The behaviour of the correlation length versus cholesterol content at the same temperature of 70°C is reported in figure 5c. An increase of the chain lateral distance together with a broadening of the peak, corresponding to a reduction of correlation length ξ , can be observed for increasing cholesterol concentration.

For cholesterol concentrations greater than 50% the formation of crystals was observed in polarized light microscopy. At this concentrations the structural quantities above reported have the same value, inside the

experimental uncertainties, as those for 50% cholesterol molar fraction.

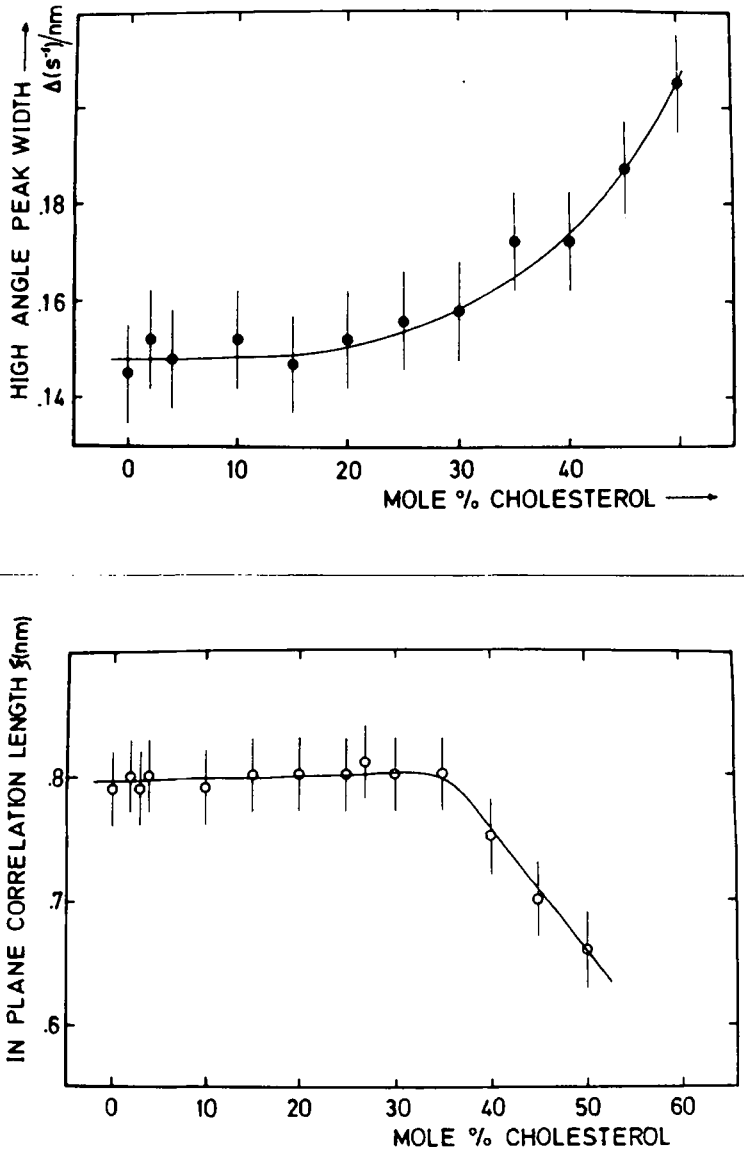

Figure 5. High angle peak width (a) and in plane correlation length (b) vs the cholesterol content.

4. DISCUSSION

The progressive decrease of the transition temperature to L_α phase, corresponding to the temperature at which a chain melting occurs, as a function of the cholesterol concentration is a first indication that the presence of cholesterol in progressive amounts tends to introduce disorder in the intermolecular lateral organization of lipid chains. In fact the matter is not so simple because it is well known that on one hand the cholesterol tends to fluidify the gel phase, introducing disorder inside the compact in-plane intermolecular organization of the lipid chains, and on the other hand tends to increase the orientational order parameter of chains in the L_α phase [14].

This last fact is probably at the origin of the increase of the lamellar repeat distance as a function of cholesterol, observed in figure 2b for low cholesterol concentrations. Probably a saturation effect of this phenomenon occurs at a concentration of about 30%, after which a decrease of the lamellar repeat spacing is observed, which can be attributed to the shorter length of the cholesterol molecule as compared to the lipid molecules: when enough cholesterol molecules are present the average bilayer thickness is reduced. An increase of lamellar repeat spacing for increasing cholesterol concentrations was observed in the melted chain phase of liposomes made of dimiristoyl lecithin

(DMPC) [1] and at 20°C in liposomes of lecithins having two identical saturated chains, shorter than those of DSPC [3]. On the other hand, in DSPC liposomes a decrease of the thickness was observed at 20°C for 33 % cholesterol molar fraction [3], but the behaviour of DSPC liposome thickness in the melted chain phase was still unknown. Our data (figure 2) show that DSPC liposomes in the melted chain phase behave similarly to DMPC for low cholesterol concentrations: an inversion in that behaviour is detected at higher cholesterol content. A similar overall behaviour was observed in the case of insaturated lecithin at 20°C [15]

The dependence of interchain lateral distances seems to confirm this explanation (figure 4a). In fact a relatively sudden increase of this quantity is observed for a cholesterol concentration of 22.5±2.5 %, indicating that at this concentration the cross sectional area of the cholesterol molecule larger than that of a liquid chain, begins to play a role. Instead of such increase of the lateral interchain distance, a decrease of the mean molecular area was reported [16]; in fact only one lecithin molecule corresponds to two aliphatic chains, while one molecule of cholesterol corresponds to one aliphatic chain: we obtained an evaluation of the mean molecular area A from the interchain distance ℓ of figure 4a by considering that

$$A = \frac{A_c \cdot (2 \cdot N_L + N_C)}{N_L + N_C}$$

where $A_c = \ell^2 \sqrt{3}/2$ is the average area per chain
N_L = Number of lecithin molecules
N_C = Number of cholesterol molecules;
the obtained values are reported in fig 4b as a function of cholesterol content. The molecular area of a lecithin molecule is reported in the insert, considering a constant cholesterol area of $0.37nm^2$ [16]. A decrease not only of the mean molecular area but also of the lecithin area is obtained, as it was the case for the egg lecithin[16], that should be related to the reduction of the orientational disorder and therefore to the mean lateral spatial fluctuation of CH_2 segment.

In agreement with the above reported considerations, which refer to a cholesterol molecule in the two-dimensional lattice of the chains, the in-plane correlation length, providing a measure of the extent of the in-plane translational order, drops at cholesterol concentrations larger than 30%, indicating a progressively strong introduction of translational disorder due to the presence of large amounts of cholesterol molecules. For cholesterol concentrations smaller than 30% the presence of the external molecules appears not to affect the already low extent of translational order ($\approx .8nm$).

It cannot be completely excluded that the enlargement of the high angle peak reported in figure 5a and related to a lowering of the correlation length could also be attributed to the presence of lattice distance fluctuations, attributable for instance to segregation of cholesterol rich aggregates. Neutron scattering from samples containing deuterated molecules of cholesterol or lipids should eliminate any doubt. On the other hand low values of positional correlation length at high cholesterol concentrations are in accordance with the other above reported effects of cholesterol.

The critical concentration of cholesterol which can induce detectable effects on the lecithin liposomes is 20% (one cholesterol every four lecithin molecules) for some authors and 33% (one cholesterol every two lecithin molecules) for others [1]. Figures 2 and 4 show that at 20% molar fraction the cholesterol is no more effective on the molecule stiffness, as it can be deduced by the increase of layer thickness (figure 2b) and it has a strong effect on the lateral distance between molecules (figure 4a); this fact can explain the changes observed at this concentration in the lateral diffusion coefficient [17]; also the transition temperature is no more affected from that concentration on (figure 1). At 33% cholesterol molar fraction the effect begin to be on the correlation length inside the layer, which begins to decrease (figure 5b), on the layer thickness (figure 2b)

which, according to our interpretation, decreases for a geometrical reason, and on the dynamics of the transition, as the transition calorimetric peak was not detected at low scan rate (figure 1).

5. CONCLUSIONS

Different effects are induced by increasing cholesterol concentration in DSPC liposomes in the liquid crystal melted chain phase.

At low cholesterol concentrations (cholesterol moles/cholesterol+DSPC moles < 20%) the chain melting temperature decreases, indicating a fluidifying effect of the dopant, and the lamellar repeat spacing increases, due to a chain stiffening whereas the interchain distance remains constant.

At higher cholesterol content, but below the equimolar concentration, the interchain distance increases, due to the cholesterol molecule cross sectional area larger with respect to a chain; the layer thickness does not increase anymore and then decreases slightly, due to the shorter length of cholesterol molecule.

Concerning the in plane translational order, which we considered in a quantitative way by introducing the correlation length ξ , no variation is observed for concentrations lower than about 30 % (corresponding to 1 cholesterol molecule every 2 lecithin molecules). For larger

concentrations a strong decrease in the ξ value is observed, indicating that cholesterol induces two opposite effects in the liquid crystalline phase: on one side it is well known to lower the orientational disorder of lecithins, but on the other side it increases the translational disorder. Saturation is reached at a molar ratio cholesterol:DSPC $\approx$ 1:1, and cholesterol crystals are present in the mixture for higher concentrations.

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