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The ripple phase of phosphatidylcholines: effect of chain length and cholesterol

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Saturated phosphatidylcholine (PC) bilayers display a rippled surface in the temperature region between the pre- and main transitions. Ripple repeat distance was measured from freeze-fracture electron micrographs. All of the lipids examined ($C_{13}PC$ to $C_{16}PC$; $C_{14}C_{16}PC$ and equimolar $C_{14}PC/C_{16}PC$) showed a bimodal distribution of ripple repeat distances with the two dominant values being in the ratio of 1:2. Within this series, chain length was a weak determinant of the actual repeat distance. The introduction of increasing concentrations of cholesterol eliminated the bimodal distribution and led to the appearance of a single distribution of increasing repeat distance and decreasing amplitude. Ripples disappeared above a cholesterol concentration of 15 mol%. These observations are discussed within the framework of a model which links the genesis of the ripples (vertical displacement of lipid molecules) to the *trans-gauche* isomerization known to occur at the pre-transition.

Introduction

Disaturated phosphatidylcholines display a number of distinct thermotropic phase transitions that have been characterized by techniques such as differential scanning calorimetry (DSC) and X-ray diffraction. Three major transitions have been demonstrated in this class of lipid (sub, pre, and main) and therefore, four distinct physical states intervene between these transitions [1]. For dipalmitoylphosphatidylcholine ($C_{16}PC$) these three transitions occur at 22°C, 36°C and 42°C, respectively. Below 22°C, the lipid is packed into a poorly hydrated crystal-like arrangement. Between 22°C and 36°C the chains become tilted, and more disordered and at the pre-transition there is an increase in the population of hydrocarbon chain *gauche* rotamers toward the centre of the bilayer [2]. Between 36°C and 42°C the bilayer surface ceases to be predominantly planar and a periodic ripple pattern appears. Finally, at the main transition the acyl chains become liquid-like and the ripple pattern disappears.

Several models [3–5] have been proposed for the ripple phase of saturated phosphatidylcholines. Generally these models start from the known mismatch in the projected areas of the head group and acyl chains. In addition given the constraints that the hydrocarbon chains are tilted and that the monolayer (or bilayer) has a spontaneous curvature, rippling becomes the accommodating mecha-

Abbreviations: C_nPC, diacylphosphatidylcholine, of n carbons per saturated acyl chain; $C_{14}C_{16}PC$, this lipid has a C_{14} chain on the 1-position of the glycerol backbone and a C_{16} chain on the 2-position.

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nism. The importance of *trans-gauche* isomerization is not explicitly included in any of these models nor do any of them specifically account for the effects of cholesterol. Recently Georgallas and Zuckermann [6] have proposed a model in which the vertical displacement of lipid molecules is coupled to their conformational state i.e. acyl chains are allowed to twist into the free volume created by the vertical displacement of neighbouring chains. Although this model has been solved for a one-dimensional system only, we believe that it furnishes a possible description of the ripple phase and the effects of perturbations such as the inclusion of cholesterol.

We would predict, on the basis on this model, that ripple repeat distance should be only weakly dependent upon acyl chain length and that changes in the distribution rather than amount of free volume within the bilayer should have the more significant effect on ripple repeat distance. The introduction of cholesterol will change the distribution of free volume within the bilayer while lengthening the acyl chains for a fixed concentration of cholesterol will increase the size of individual pockets of free volume (beneath the cholesterol molecules) rather than their distribution. To examine for these predictions, we have performed freeze-fracture experiments and have obtained quantitative data concerning the relationship of ripple repeat distance to both acyl chain length and the presence of different amounts of cholesterol. The experimental results bear out these predictions.

Materials and Methods

Liposome preparation

The phospholipids used in this study, $C_{13}PC$, $C_{14}PC$, $C_{14}C_{16}PC$, $C_{15}PC$ and $C_{16}PC$ were obtained from Avanti Polar Lipids (Birmingham, AL). All gave a single spot on TLC and a narrow endotherm on a DSC scan and were thus considered pure.

Preparation of multilamellar liposomes was done by well documented techniques [7] which included dissolving dry lipid in chloroform, evaporating under reduced pressure and suspending in warm buffer (50 mM NaCl, 5 mM Tris, (pH 7.5)) at least 10 Cdeg above the main transition temperature of the particular lipid. Vortexing was used to ensure complete suspension. The mixture was incubated at room temperature for at least 10 min before preparation for freeze-fracture.

Cholesterol was supplied from the Sigma Chemical Company (St. Louis, MO) and likewise showed a single spot on TLC. For incorporation into a liposome suspension, the appropriate mol% of cholesterol was weighed, dissolved in chloroform, and added to the lipid/chloroform solution.

Freeze-fracture

Freeze-fracture of the lipid dispersions was done on a Balzer's Freeze Etching System BAF (Model 400D). A gold EM grid was dipped into the liposome preparation and then sandwiched between two gold supports. The sample was then incubated for 5 minutes in a thermostatically controlled air medium at a temperature between the pre- and main transition of the lipid. Subsequently the samples were quickly immersed into a Freon slush and frozen. Frozen samples were transferred to a double replica specimen stage and introduced into the pre-cooled vacuum chamber. Fractured surfaces were shadowed with platinum/carbon. Platinum evaporation was done from a 45° angle; carbon from a 90° angle. Replicas were examined in a Hitachi 500 electron microscope.

Measurement of ripple repeat distance and analysis of data

In order to minimize photographic enlargement error, measurements were made directly from the negatives under a Wild Leiz M32 stereomicroscope, using a calibrated graticule (J.B. EM Services Inc. Pointe Claire, Quebec).

Surfaces suitable for measurements should be flat and parallel to the plane of the photographic film. Therefore surfaces that appeared obviously curved were rejected. The degree of curvature was further assessed in the following manner. If a surface curved away from (or for that matter towards) the plane of the film, the measured repeat distance for ripples running across the direction of curvature should become progressively smaller. Surfaces displaying such a feature were not used. To ensure that a given surface was parallel to the photographic film the following procedure was adopted. If a surface is tilted then

for ripples that run across the plane of the tilt, the measured repeat distance would be less than the actual distance. However, for ripples that run along the plane of the tilt, measured and actual repeat distances would be the same. Hence we selected surfaces which contained patches of ripples running at different angles with respect to each other. For example in Figs. 1b and 1d the surfaces contain areas of ripples which run at right angles to each other. If the repeat distance in these different areas were very similar (within 15 Å–see below) then we concluded that the surface was not significantly tilted with respect to the film. The principles underlying this procedure can be appreciated by dividing a sheet of paper into areas and drawing (within each area) equidistant parallel lines with the direction of the lines being different in each area. When the paper is tilted, lines in some of the areas appear closer than lines in other areas depending upon the plane of tilt (to the horizontal) and the direction of a given set of lines. Only when the paper is horizontal will all the lines appear equidistant. Some tilts might not be detected by this method as for example a tilt plane that bisected the angle between two sets of ripples. We believe, however, that most surfaces chosen in this manner will be parallel to the plane of the film.

As more than one measurement of ripple repeat distance could be made from a single vesicle, a method was devised in order that repetitive measurements on the same region would not be made. Each negative was divided into ten sectors. Some sectors (usually 3 to 5 of the 10) contained ripples within them. When a measurement of 3 to 8 ripples could be made within a sector, the average wavelength of these ripples represented a single measurement. The measurement was taken perpendicular to the direction of the ripples. A sector could be rejected for measurement on the basis of insufficient ripples, variance in ripple structure or poor resolution (more prominent at higher cholesterol concentrations). The two main sources of error were the resolution of the markings on the graticule and the accuracy of the magnification of the electron microscope. Measurements made by this method were estimated to be accurate to within 15 Å except for bilayers containing 15 mol% cholesterol. These surfaces contained ripples which were quite broad and indistinct (due to low amplitude) and hence measurements of repeat distance were subject to a larger error than 15 Å. The data have beeen presented in two forms. In Figs. 2, 3 and 5 ripple repeat distances have been organized into 15 Å groups since 15 Å is the estimated error of measurement. The number of measurements are given in the figure legends. In Figs. 4, 6 and Table I the data are presented as the means of the actual measurements. This standard deviation is a measure of the distribution of repeat distances rather than the error involved in making a single measurement.

Results

Representative electron micrographs of $C_{13}PC$ vesicles are illustrated in Fig. 1. Although small vesicles usually showed broad rippling, this was not invariably the case. Many individual large vesicles contained co-existing populations of ripples of different repeat distance (Fig. 1 and Ref. 8, Fig. 4). Incorporation of cholesterol caused a (concentration-dependent) lengthening of the ripple repeat distance as well as a reduction in ripple amplitude (Fig. 1).

Fig. 2 contains histograms of ripple repeat distances for different lipids. As described in the methods section, each measurement was the average for 3 to 8 ripples and was estimated to be accurate to within 15 Å. Hence the measurements have been organized into 15 Å groups for the plot in Fig. 2. All of the lipids display a predominantly bimodal distribution. For example, $C_{13}PC$ has two populations which centre around (most probable) repeat distances of 120–134 Å and 240–254 Å, respectively.

We initially wondered whether the bimodal pattern was related to vesicle size. However, several observations were inconsistent with such a direct relationship. Firstly, as illustrated in Fig. 1, individual vesicles could be found which contained coexisting ripples of different repeat distances. Secondly, all of these lipids ($C_{13}PC$ to $C_{16}PC$) displayed a unimodal rather than bimodal distribution of sizes as measured by quasi-elastic light scattering (QELS) *.

* QELS was done in collaboration with Drs. T. Racey and P. Rochon, Physics, Royal Military College, Kingston. These results are currently being prepared for publication.

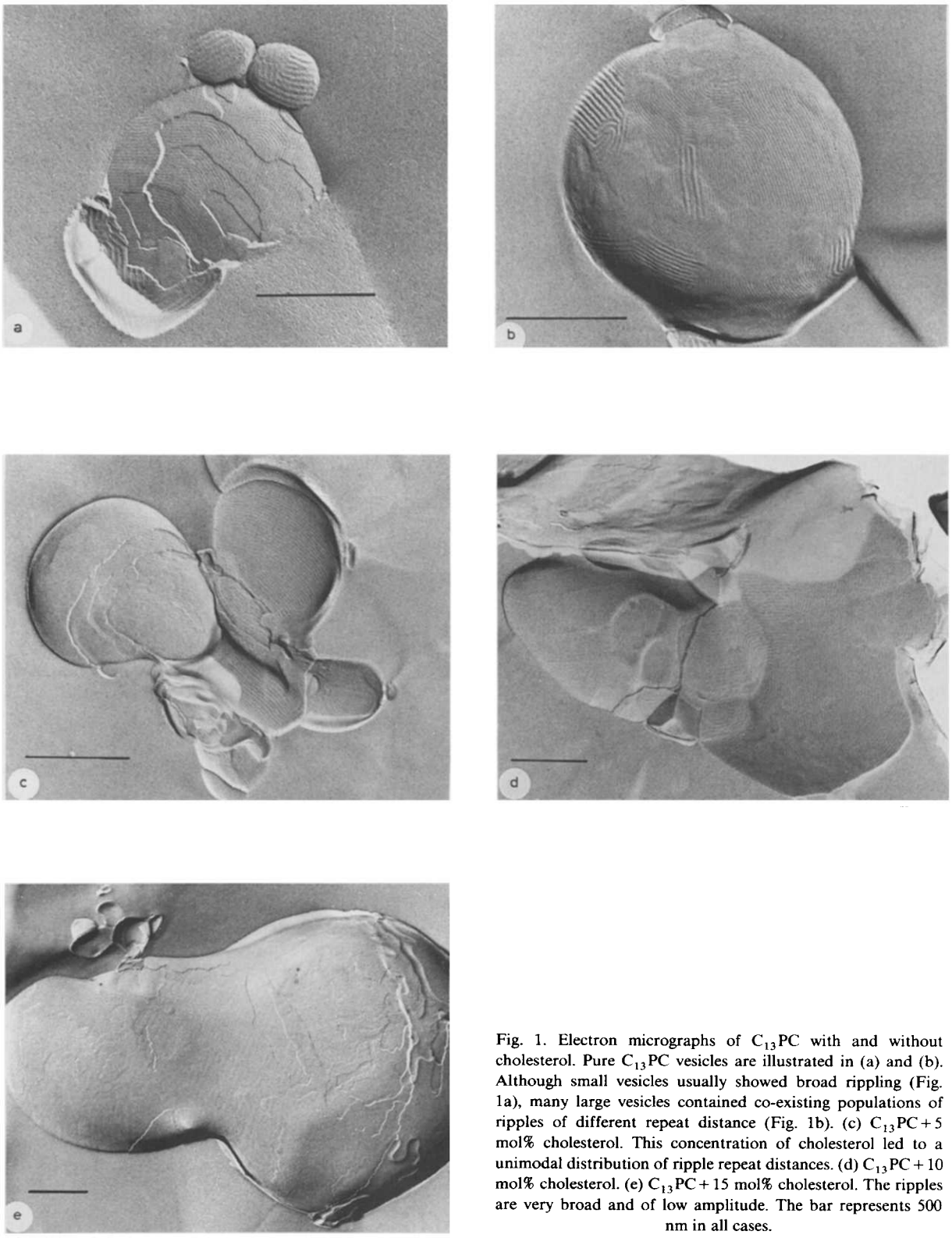

Fig. 1. Electron micrographs of $C_{13}PC$ with and without cholesterol. Pure $C_{13}PC$ vesicles are illustrated in (a) and (b). Although small vesicles usually showed broad rippling (Fig. 1a), many large vesicles contained co-existing populations of ripples of different repeat distance (Fig. 1b). (c) $C_{13}PC + 5$ mol% cholesterol. This concentration of cholesterol led to a unimodal distribution of ripple repeat distances. (d) $C_{13}PC + 10$ mol% cholesterol. (e) $C_{13}PC + 15$ mol% cholesterol. The ripples are very broad and of low amplitude. The bar represents 500 nm in all cases.

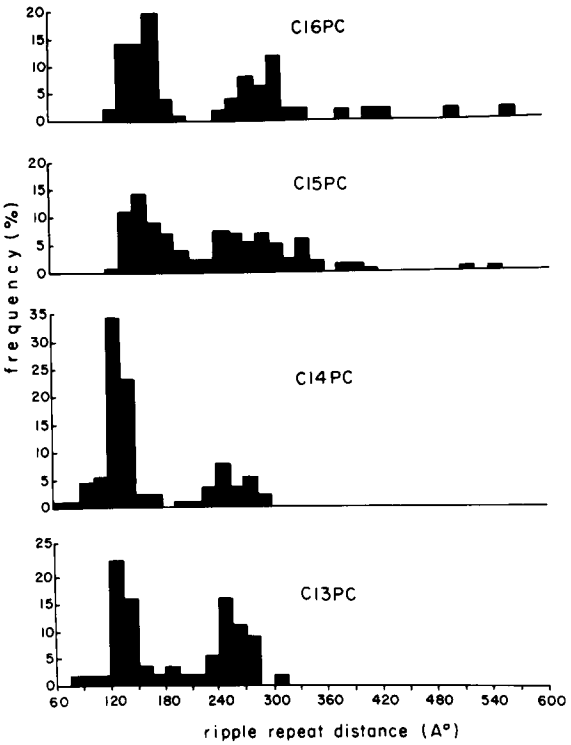

Fig. 2. Histograms of ripple repeat distance versus percent frequency for different lipids. As described in Methods, each measurement consisted of the average width of between 3 to 8 adjacent ripples. These measurements were estimated to be accurate to within 15 Å. Hence the measurements have been organized into 15 Å groups for the purpose of plotting the histograms. The number of measurements were: $C_{13}PC$, 56; $C_{14}PC$, 90; $C_{15}PC$, 132; $C_{16}PC$, 51.

Several other observations are also apparent from Fig. 2. Firstly, the most probable repeat distances of the two populations are in the ratio of 1 : 2 and secondly, the longer chain lipids, ($C_{15}PC$ and $C_{16}PC$), display a small number of ripples of quite long repeat distance, suggesting that a third population might exist in these cases.

Fig. 3 illustrates histograms for the lipid $C_{14}C_{16}PC$ and a 1 : 1 molar mixture of $C_{14}PC$ and $C_{16}PC$. The same bimodal pattern is observed although fewer measurements were made for these lipids than for $C_{13}PC$ to $C_{16}PC$. Furthermore the most probable ripple repeat distances fall within those of the lipids $C_{13}PC$ to $C_{16}PC$.

Fig. 4 illustrates the relationship between ripple repeat distance and acyl chain length. The lines have been drawn by the method of least-squares

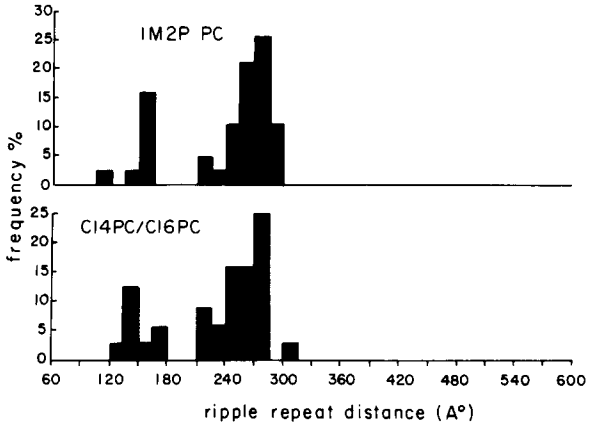

Fig. 3. Histograms of ripple repeat distance versus percent frequency for $C_{14}C_{16}PC$ (1M2P PC) and a 1 : 1 molar mixture of $C_{14}PC/C_{16}PC$. These two liposome systems showed a comparable bimodal distribution to those of $C_{13}PC$ to $C_{16}PC$. The number of measurements were: $C_{14}C_{16}PC$, 37; 1 : 1 $C_{14}PC/C_{16}PC$, 32.

analysis and have slopes of 11.3 Å per carbon for the primary repeat distance and 27.4 Å per carbon for the secondary repeat distance.

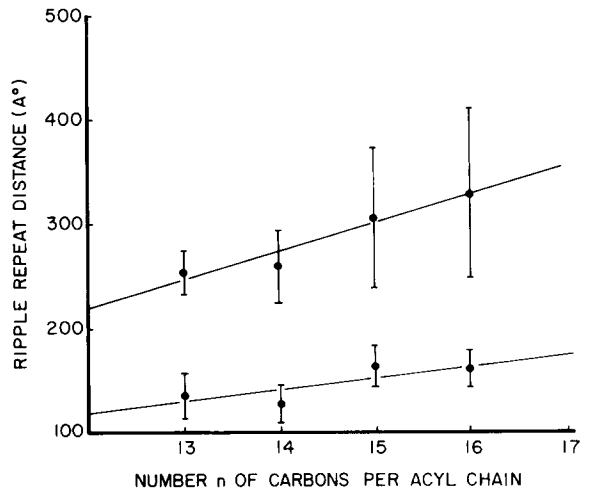

Fig. 4. Relationship of ripple repeat distance to acyl chain length. Experimental points represents means ± S.D. for comparison with a gaussian distribution. The lines have been drawn by the method of least-squares analysis. The ordinate is ripple repeat distance in Å while the abscissa is the number of carbon atoms per acyl chain. The bottom line is for the primary repeat distance ($y = 11.3\ x\ -16.1$) while the upper line is for the secondary repeat distance ($y = 27.4\ x\ -109.8$).

Although the ripple phase has been studied by freeze-fracture microscopy and X-ray diffraction, there are few quantitative data in the literature. Table I summarizes measurements that have been made of the ripple repeat distance. Sackmann et al. [8] reported two populations of ripples for $C_{14}PC$ with repeat distances of 130 and 220 Å, respectively. Using a technique to reconstruct three-dimensional surface profiles they proposed that the longer wavelength ripples were formed from a combination of two of the narrower ripples. Copeland and McConnell [9] reported a re-

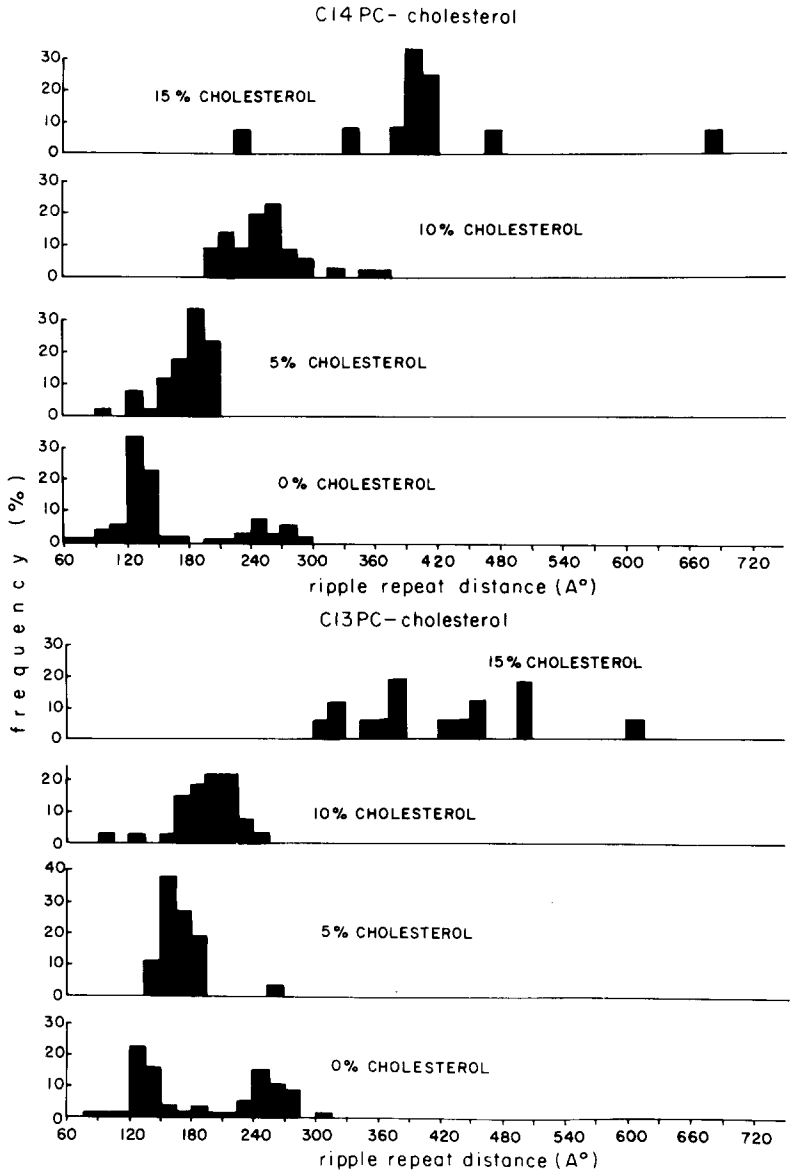

Fig. 5. Histograms of ripple repeat distance versus percent frequency for $C_{13}PC$ with cholesterol and $C_{14}PC$ with cholesterol. The pure $C_{13}PC$ and $C_{14}PC$ data are taken from Fig. 2. The concentrations of cholesterol illustrated are 5, 10, and 15 mol%. Above 15 mol% no ripples could be detected and vesicle surfaces were either smooth or slightly mottled. With 15 mol% cholesterol, few measurements could be made since the ripples were quite broad and indistinct due to the low amplitude. The number of measurements were: $C_{13}PC$, 56; $C_{13}PC$/5 mol% cholesterol, 26; $C_{13}PC$/10 mol% cholesterol, 27; $C_{13}PC$/15 mol% cholesterol, 16; $C_{14}PC$, 90; $C_{14}PC$/5 mol% cholesterol, 50; $C_{14}PC$/10 mol% cholesterol, 34; $C_{14}PC$/15 mol% cholesterol, 12.

TABLE I

RIPPLE REPEAT DISTANCE MEASUREMENTS

F.F. stands for freeze-fracture electron microscopy.

Lipid (C_nPC)	Ripple repeat distance (Å)		Technique	Reference
C_{13}PC	136±22 *	254±21 *	F.F.	this study
C_{14}PC	129±17 *	260±35 *	F.F.	this study
C_{14}PC	130±20	220±20	F.F.	Sackmann [8]
C_{14}PC	120–140;	228–266	F.F.	Copeland [9]
C_{14}PC	130±10		F.F.	Luna [10]
C_{14}PC	120		X-ray	Janiak [11]
C_{15}PC	164±20 *	306±67 *	F.F.	this study
C_{16}PC	162±18 *	330±81 *	F.F.	this study
C_{16}PC	150±20		F.F.	Luna [10]
C_{16}PC	140		X-ray	Janiak [11]
C_{16}PC	147		X-ray	Stamatoff [12]
C_{14}PC/C_{16}PC 1:1 molar ratio	149±20 *	258±23 *	F.F.	this study
$C_{14}C_{16}$PC	147±14 *	265±19 *	F.F.	this study

* Means ± S.D. for comparison with a gaussian distribution.

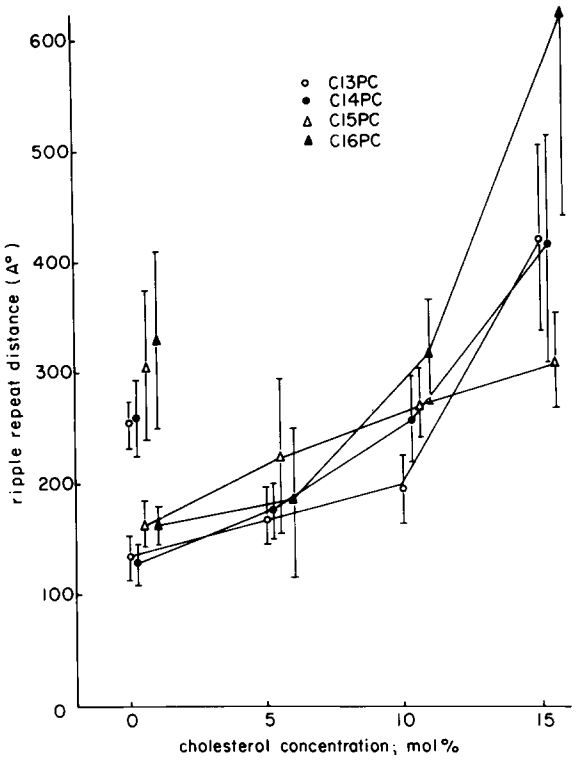

Fig. 6. Relationship between ripple repeat distance and cholesterol concentration for C_{13}PC, C_{14}PC, C_{15}PC, and C_{16}PC. Experimental points represent means ± S.D. for comparison with a gaussian distribution. Arbitrarily, the lines have been connected to the lower populations of the pure lipids.

peat distance of 120–140 Å for C_{14}PC and mention a secondary ripple population with a distance of 1.9–1.95-times the primary pattern. Luna and McConnell [10] observed a repeat distance of 130 ± 10 Å for C_{14}PC and 150 ± 20 Å for C_{16}PC. Using X-ray diffraction Janiak et al. [11] reported a repeat distance of 120 Å for C_{14}PC and 140 Å for C_{16}PC while Stamatoff et al. [12] recorded a repeat distance of 147 Å for C_{16}PC. Neither of these studies refers to secondary populations. The measurements summarized in Figs. 2 and 3 are also listed in Table I for comparison.

Figs. 5 and 6 illustrate the effects of cholesterol incorporation on ripple repeat distance for C_{13}PC to C_{16}PC. The addition of 5 mol% cholesterol eliminated the bimodal distribution and led to the appearance of a single distribution with a most probable repeat distance intermediate between that of the original two. With larger concentrations of cholesterol, the repeat distance of this single distribution becomes longer. As illustrated in Fig. 1, the ripple amplitude also decreased as more cholesterol was incorporated (we were unable to quantitate amplitude). With incorporation of 15 mol% cholesterol, we could make only few measurements, since the ripples were quite broad and indistinct due to the low amplitude. Above 15 mol% cholesterol the vesicle surfaces were smooth or slightly mottled and no measurements could be made. These results are comparable to those reported by Copeland and McConnell [9] and Verkleij et al. [13], although these authors did not give quantitative data. Sackmann et al. [8] examined the effects of small concentrations of cholesterol (< 6 mol%) on the ripple pattern of C_{14}PC. Small amounts in the order of 0.5 mol%, stabilized the ripples of shorter wave length, with the repeat distance not increasing until a concentration of 6 mol% was reached. No mention is made of an effect on the ripples of longer repeat distance. We examined the effects of 1 and 3 mol% cholesterol on C_{16}PC and observed a bimodal pattern comparable to that of pure C_{16}PC (data not illustrated). A unimodal pattern first became apparent at 5 mol% cholesterol and rippling disappeared above 15 mol%.

Discussion

The ripple phase represents an interesting phenomenon in which the packing constraints of the lipid molecules are accommodated by the bilayer surface assuming a regular three-dimensional ripple pattern. The data summarized in Fig. 2 indicate that there is not one unique solution to the ripple distance. Each lipid displays a bimodal distribution as noted by other investigators [8,9,14] and within each distribution a range of repeat distances are observed. For example, the lower population of $C_{14}PC$ has a most probable repeat distance of 120–134 Å and yet distances as short and as long as 60–74 Å and 165–179 Å, respectively, can be found. That is, the range of repeat distances observed (Fig. 2) is greater than the error limits of measurement. Any quantitative model of the ripple phase must be able to account for this observation. In addition the distributions show at least two dominant repeat distances in the ratio 1 : 2. These represent some minima in the system free energy (though possibly not absolute minima). Thus the free energy must have characteristics which permit standing wave solutions (including harmonics) to the packing problems.

Within the series of lipids examined ($C_{13}PC$, $C_{14}PC$, $C_{15}PC$, $C_{16}PC$), chain length correlates with ripple repeat distance (Fig. 4) although the correlation is not a strong one since the distributions for these lipids do overlap (Fig. 2). This lack of a strong correlation is consistent with one of the predictions given in the introduction. Similarly, the inequivalence between the 1- and 2-chains is not a dominant factor since $C_{14}C_{16}PC$ and $C_{14}PC$ show overlapping distributions (Figs. 2 and 3).

Addition of increasing concentrations of cholesterol causes the appearance of a unimodal distribution of ripples followed by a progressive increase in repeat distance and a decrease in ripple amplitude such that ripples disappear above 15 mol% cholesterol. The loss of ripples at high cholesterol concentrations is consistent with the observations of Copeland and McConnell [9] and Verkleij et al. [13]. For a fixed concentration of cholesterol there still appears to be a correlation between chain length and repeat distance (Fig. 6) although not a strong one since the distributions for the different lipids do overlap. This correlation is apparent at 5 and 10 mol% cholesterol. With the addition of 15 mol% cholesterol, measurement errors become large because of the low ripple amplitude and thus obscure any relationship between chain length and repeat distance. In terms of the predictions set out in the introduction, it is clear that for a given lipid, adding increasing amounts of cholesterol has a much more significant effect on ripple repeat distance (with eventual loss of ripples) than varying acyl chain length for a fixed concentration of cholesterol.

In the gel phase below the pre-transition temperature, phosphatidylcholine acyl chains are tilted [1,15]. This tilting is rationalized as an accommodation for the head group/acyl chain mismatch since the head group area is larger than the cross-sectional area of the chains. The consequences of this chain tilt are a reduction in the relative length of the two acyl chains, since the 1-chain is approximately 2–3 CH_2 units longer than the 2-chain in an untilted lipid, and also a reduction in the 'void volume' near the bilayer centre. As the pre-transition temperature is approached, the number of *gauche* rotamers per lipid molecule near the terminal methyl end of the chains increases [2].

Most current models of the pre-transition view the acyl chains as all *trans* and use the out of plane displacement of the lipid molecules (rippling) as the structural solution to the head group/chain area mismatch problem [3,4,5]. The actual molecular mechanism responsible for the vertical displacements is not described. In addition none of these models specifically accounts for the effects of cholesterol.

A brief comment is in order with regard to Copeland and McConnell's interpretation of the effects of cholesterol [9]. These authors suggest that microscopic phase separation occurs within the bilayer such that cholesterol-rich strips are separated by thin strips of pure phosphatidylcholine. Whether or not such phase separation actually occurs, there are several other problems with this explanation. Firstly Fig. 4 of their paper suggests that cholesterol does not decrease the ripple amplitude but only increases the ripple repeat distance. Secondly, and most importantly, these authors offer no molecular explanation as to

why the cholesterol-rich strips are smooth i.e. lacking ripples.

As outlined in the introduction, Georgallas and Zuckermann [6] have proposed a new model in which vertical displacement of lipid molecules is coupled to their conformational state. At present the model has been solved only for a one-dimensional (monolayer) system but we believe this model can be extended qualitatively to the bilayer. Briefly, acyl chains are allowed to twist to simulate the increase in *gauche* rotamers known to occur as the pre-transition temperature is approached. A twist would be facilitated by the presence of a free volume into which the chain can bend. The essence of the model is to create internal free volume by the vertical displacement (out of plane) of a neighbouring lipid molecule. The energy gained by allowing a chain to twist is balanced by the energy cost of moving a neighbouring chain out of the local plane to accommodate this twist. Since the forces resisting vertical displacement are chiefly between the polar ends of the lipid molecules, one would predict that chain length would not be a strong determinant of ripple repeat distance. This is indeed the case as illustrated in Fig. 4. This model also accommodates the effects of cholesterol. Cholesterol creates internal free volume both by acting as a 'spacer' molecule and by causing a loss of chain tilt in the gel phase [15]. The presence of this free volume would allow chain twisting to take place without requiring vertical displacement of a neighbour. The model thus predicts a loss of ripples with the addition of enough cholesterol. The model also predicts that the distribution of free volume is more important than the amount. That is, it is more important to have a distribution of free volume such that many chains can undergo 'twists' rather than have a few large 'pockets' of free volume such that a few chains can undergo multiple twists. This prediction is borne out as illustrated in Fig. 6. For a given lipid, adding increasing amounts of cholesterol has a much more significant effect on ripple repeat distance (with eventual loss of ripples) than varying acyl chain length for a fixed concentration of cholesterol. The introduction of cholesterol will change the distribution of free volume within the bilayer while lengthening the acyl chains for a fixed concentration of cholesterol will increase the size of individual pockets of free volume (beneath the cholesterol molecules) rather than their distribution. In summary, the strengths of this model lie in its more realistic depiction of acyl chains as being capable of *trans-gauche* isomerization (rather than being all trans as in previous models) plus in its supplying a molecular explanation for the out of plane vertical displacement of lipid molecules. However, we do realize that quantitative confirmation of this model must await its solution for a two-dimensional lipid array (bilayer).

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