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Carbamyl phosphatidylcholine – cholesterol interactions in unilamellar vesicles *

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Small unilamellar vesicles formed from 1-palmitoyl-2-*O*-(*N*-(heptadec-8-*cis*-enyl)carbamyl)-*sn*-glycero-3-phosphocholine (CMPC) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in the presence of varying amounts of cholesterol have been studied using fluorescence polarization and NMR (^{1}H and ^{13}C) techniques. The fluorescence polarization and ^{1}H-NMR data clearly indicate that the phospholipid packing order in CMPC bilayers is significantly greater than that in the POPC bilayers. The ^{13}C-NMR chemical shift measurements show that this difference between the two phospholipids possibly arises due to the intramolecular hydrogen-bond formation between the -NH and the phosphate residues in the CMPC molecule. It is further shown that unlike POPC, the CMPC packing order is not much affected by including cholesterol in the phospholipid bilayers. These results demonstrate that introduction of one -NH residue adjacent to the C-2 carbonyl carbon in the POPC molecule could make its structure more ordered in the vesicles bilayer, and also would alter its interactions with cholesterol.

Introduction

Carbamyl phosphatidylcholines are synthetic analogs of phosphatidylcholines in which the C-2, or both C-1 and C-2, ester group(s) have been replaced by the carbamyloxy (-NH-C(=O)O-) function [1,2]. These phospholipids are biologically interesting as they resist their degradation by phospholipase A_2 [1,2], and form liposomes which are more stable and longer living, as compared to egg phosphatidylcholine/cholesterol liposomes, in the blood circulation of the injected animals [3,4]. The blood stability of the liposomes formed from monocarbamyl phosphatidylcholine and cholesterol has been shown to result from their ability to resist lysis by the blood components [5,6]. However, the physicochemical characteristics which confer this property on these phospholipids are not yet fully understood.

Differential-scanning calorimetric studies [7,8] have revealed that carbamyl phosphatidylcholines resemble sphingo-myelins, rather than phosphatidylcholines, in their thermal phase transition behavior. Like sphingolipids [9], these compounds exhibit two low-temperature states, one metastable and the other stable [7,8]. This behavior of carbamyl phosphatidylcholines has been attributed to their capacity to form hydrogen bonds. As sphingomyelins also form blood-stable liposomes [10], it would seem that the enhanced stability of the carbamyl phosphatidylcholine/cholesterol liposomes in the blood circulation is probably due to the hydrogen-bond-forming potential of the phospholipid component.

To further analyze the possibility of hydrogen bonding, we have studied the interactions of CMPC with cholesterol in unilamellar vesicles, using NMR and fluorescence polarization techniques. The vesicles were prepared by sonication, and fractionated by centrifugation. The vesicle size was determined by molecular-sieve chromatography. The results of these studies indicate that the CMPC packing order in bilayers is increased, as compared to POPC, due to restricted motion of its headgroup, resulting from the intramolecular hydrogen-bond formation between the NH and the phosphate

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Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; CMPC, 1-palmitoyl-2-*O*-(*N*-(heptadec-8-*cis*-enyl)carbamyl)-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene.

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residues. This CMPC packing order was not significantly influenced by cholesterol as compared to that of POPC in the bilayers.

Materials and Methods

All the reagents and chemical used in the study were of the highest purity available. Cholesterol was purchased from Centron Research Laboratory, Bombay, India, and used after crystallizing it three times from methanol. DPH was bought from Sigma Chemical Company. CMPC was prepared according to the published procedure [1]. POPC was synthesized as described earlier [11]. The purity of phospholipids was checked by thin-layer chromatography (TLC) on silica gel G-60 plates. The plates were developed in chloroform/methanol/water (65 : 25 : 4, v/v), and spots were visualized by staining the plate with iodine vapor followed by Molybdenum blue spray [12]. All the phospholipid samples used in this study exhibited single spots on TLC plates. Total phosphorus was estimated by the method of Ames and Dubin [13].

Unilamellar vesicle preparation

A solution of phospholipid and cholesterol in chloroform/methanol (1 : 1, v/v) mixture was evaporated to dryness in a glass tube under a slow jet of N_2, resulting in the formation of a thin lipid film on the wall of the tube. Final traces of the solvents were removed by leaving the tube in vacuo for 3–4 h. The dried lipid mixture was dispersed in Tris-buffered saline (10 mM Tris/145 mM NaCl (pH 7.4)). For fluorescence polarization experiments, 0.025% EDTA was also included in the dispersion buffer. The mixture was vortexed for 15 min at room temperature (25–35 °C). The lipid dispersion thus obtained was carefully transferred to a water-jacketted cuvette and sonicated (6–8 °C) under N_2 in a probe-type sonicator (Heat systems, W-220 F) to give an optically clear suspension (30–40 min for POPC and 60–80 min for CMPC). The sonicated preparation was centrifuged at 105 000 × *g* (Ti-50 fixed-angle rotor) for 60 min (5 °C) to effect the removal of titanium particles as well as poorly dispersed lipids. Only the vesicles found in the top two-thirds of the supernatant were used in the study.

Vesicle size determination

The vesicle size was determined by analytical molecular-sieve chromatography on Bio-Gel A-50m at 6 ± 2 °C, as described earlier [14].

Fluorescence polarization measurements

Fluorescence polarization of DPH as a function of temperature was measured according to Shinitzky and Barenholz [15], using a Perkin Elmer MPF 44A fluorometer. The DPH was embedded in membranes and excited at 360 nm (10 nm band pass). The fluorescence was viewed through the emission monochromator set at 428 nm (10 nm band pass). In all the experiments, the mole ratio of phospholipid to DPH was about 500 : 1.

Polarizations were calculated according to the equation,

$$P = (I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$$

where I_{vv} is the vertically polarized component of fluorescence and I_{vh} is the horizontally polarized component of fluorescence, the emission is excited at vertically polarized light. G is the grating transmission factor.

In all experiments the fluorescence polarization and total fluorescence intensity of DPH were calculated after correction for light scattering. The correction was made as described by Shinitzky et al. [16]. There was no polarization due to light scattering, since dilution of liposomes labeled with DPH had no effect on the fluorescence polarization.

Labeling of vesicles with DPH was carried out by adding the solution of fluorophore (2 mM, 1 μl) in tetrahydrofuran to the vesicle dispersion (0.8 mM phospholipid, 4.5 ml) under rapid stirring. Similar amounts of tetrahydrofuran, free of DPH, were added to the vesicles which were used as controls. The vesicle samples were incubated at room temperature (above the phospholipid thermal phase transition temperature) for at least 90 min to allow equilibration of DPH with the vesicle wall. The total incorporation of DPH was ensured by measuring the fluorescence intensity after the incubation, and polarization experiments were started after a plateau in fluorescence intensity had been achieved. Under these conditions, undesirable fluorescence polarization due to nonradiative or radiative energy transfer between the probe molecules was practically eliminated.

NMR measurements

^{1}H-NMR spectra were recorded at 400.13 MHz on a Bruker WM 400 Fourier-transform spectrometer equipped with 5 mm ^{1}H/^{13}C dual-probe head and ASPECT 2000 computer. Samples (0.004 M phospholipid) were prepared either in $CDCl_3$ or Tris-buffered saline containing 20% D_2O (v/v). Tetramethylsilane was taken as an internal standard in the case of samples prepared in $CDCl_3$ and dioxane as the external reference for the samples prepared in buffer. The typical spectral conditions were as follows: pulse width 7 μs (flip angle 40 °), sweep width 6410.26, data points 16 K, acquisition time 1.28 s and resolution of 0.78 Hz/point. The number of scans was 1200.

^{13}C-NMR were recorded at 100.57 Hz, using the above NMR spectrometer. The concentration of phospholipid used for these experiments was 0.017 M. The pulse conditions were as follows: pulse width 11 μs (flip

angle 90°), repetition time 1.59 s, data points 32 K and digital resolution 1.7 Hz/point. The broad-band proton-decoupled NMR spectra were recorded with an accuracy of parameters ±0.1 ppm for chemical shifts. Free induction decays were acquired over 27777.78 Hz, and were line-broadened to 6.0 Hz prior to Fourier transformation. The number of scans was 20000–30000.

Results

CMPC and POPC were separately sonicated in the absence as well as in the presence of varying cholesterol concentrations to form small unilamellar vesicles. The vesicles were fractionated by centrifugation, and their size after fractionation determined by molecular-sieve chromatography over Bio-Gel A-50 m [14]. As shown in Fig. 1, the size of the CMPC vesicles was not much different than that of the POPC vesicles. Also, the cholesterol-induced increase in the size of POPC vesicles was similar to that observed for the CMPC vesicles. This increase in the vesicle size was sharp up to the phospholipid-to-cholesterol mol ratio of 2, as compared to that at the higher cholesterol concentrations, which is consistent with the earlier findings [17].

The acyl chain disorder in the phospholipid bilayers was measured by measuring the fluorescence polarization of DPH as a function of temperature. As shown in Fig. 2, the polarization values for the CMPC vesicles were considerably higher than that for the POPC vesicles at least up to 50°C. However, these values for POPC

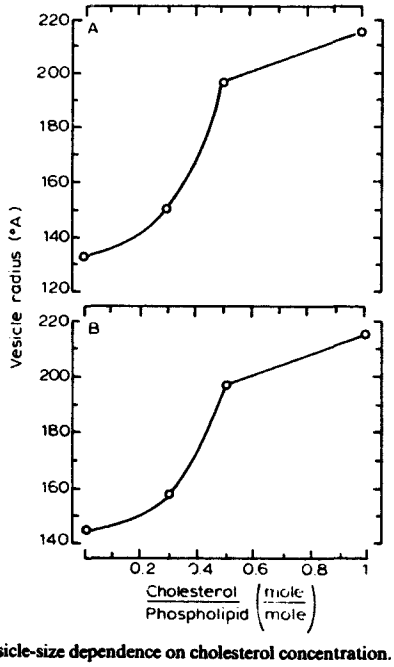

Fig. 1. Vesicle-size dependence on cholesterol concentration. A, POPC; B, CMPC.

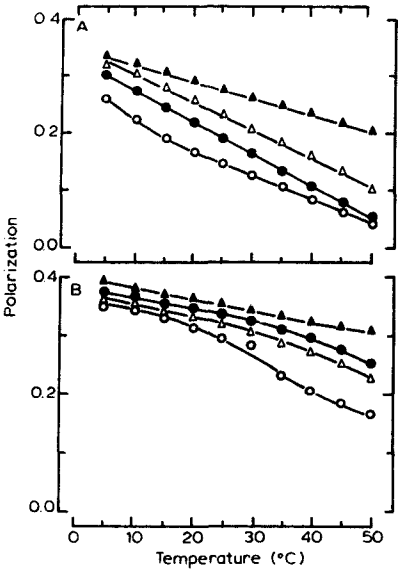

Fig. 2. Temperature-dependence of DPH fluorescence polarization in POPC (A) and CMPC (B) vesicles containing cholesterol. ○, phospholipid/cholesterol (1:0, mol/mol); ●, phospholipid/cholesterol (1:0.3, mol/mol); △, phospholipid/cholesterol (1:0.5, mol/mol); ▲, phospholipid/cholesterol (1:1, mol/mol).

bilayers significantly increased with cholesterol concentration, and decreased linearly with temperature. Unlike POPC bilayers, inclusion of cholesterol in the CMPC bilayers did not significantly enhance the polarization values upto 30°C, and the decrease in these values with temperature was nonlinear up to the phospholipid-to-cholesterol mol ratio of 2. Also, the polarization values for the CMPC/cholesterol (1:0.5, mol/mol) bilayers were smaller than those for the CMPC/cholesterol (1:0.3, mol/mol) bilayers. These observations indicate that acyl chain packing in the CMPC bilayers is more ordered than in the POPC bilayers, and that the effect of cholesterol on the CMPC packing is different from that observed in the case of the POPC bilayers.

To further confirm these results, the various vesicle preparations were analyzed by ^{1}H- and ^{13}C-NMR spectroscopy at 25°C. Fig. 3 shows that the widths at half-height of the terminal methyl and methylene proton signals for the POPC bilayers increased steeply with cholesterol concentration. However, these widths for the CMPC vesicles were not significantly influenced by cholesterol. Also, both the terminal methyl and methylene proton line widths were smaller for the CMPC/cholesterol (1:0.5, mol/mol) vesicles, as compared to that for the CMPC/cholesterol (1:0.3, mol/mol) vesicles. Besides, the methylene protons for the CMPC vesicles appeared as a distorted doublet which upon increasing the cholesterol content to 33 mol%

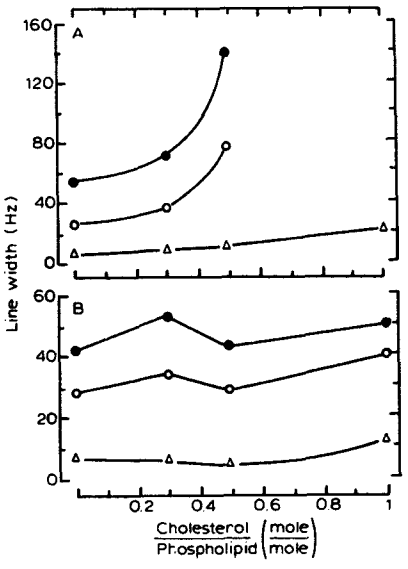

Fig. 3. Line width at half-height for ^{1}H-NMR spectra of POPC (A) and CMPC (B) vesicles containing cholesterol. ●, methylene; ○, acyl chain terminal methyl; △, choline methyl.

(CMPC : cholesterol, 1 : 0.5, mol/mol) in the vesicles bilayer, was resolved into a clear doublet (Fig. 4). But further increase in the amount of cholesterol (CMPC : cholesterol,, 1 : 1, mol/mol) led to the selective broadening of the downfield methylene protons signal. These findings suggest that the interactions of CMPC with cholesterol are weaker than that of POPC, and that the observed differences in the interactions of cholesterol with POPC and CMPC could be due to differences between the structure of these phospholipids in the vesicles bilayer.

In order to examine the validity of the above suggestions, the structures of POPC and CMPC, both in $CDCl_3$ and vesicles, were analyzed by ^{13}C-NMR. The spectra are shown in Fig. 5 and the relevant ^{13}C-chemical shifts are listed in Table I. As may be seen in Table I, the ^{13}C-NMR signals from the CMPC headgroup carbons in $CDCl_3$ appeared downfield, as compared to those from the POPC headgroup carbons. These ^{13}C-chemical shifts for CMPC remained virtually unaltered even when CMPC was present in the vesicles. However, the ^{13}C-NMR signals for the POPC headgroup carbons in vesicles appeared downfield, as compared to that

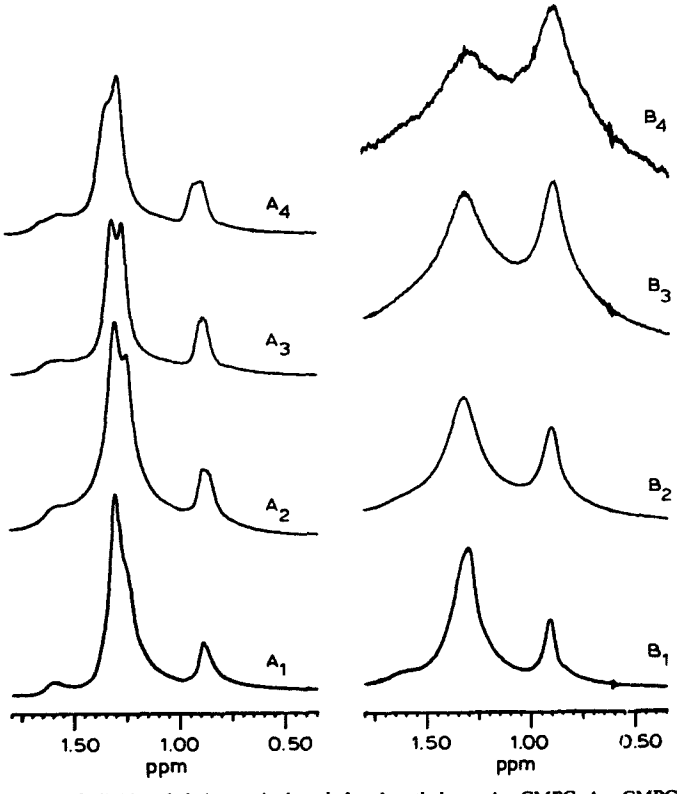

Fig. 4. ^{1}H-NMR spectra of vesicle phospholipid acyl chain terminal methyl and methylenes. A_1, CMPC; A_2, CMPC/cholesterol (1 : 0.3, mol/mol); A_3, CMPC/cholesterol (1 : 0.5, mol/mol); A_4, CMPC/cholesterol (1 : 1, mol/mol). B_1, POPC; B_2, POPC/cholesterol (1 : 0.3, mol/mol); B_3, POPC/cholesterol (1 : 0.5, mol/mol); B_4, POPC/cholesterol (1 : 1, mol/mol).

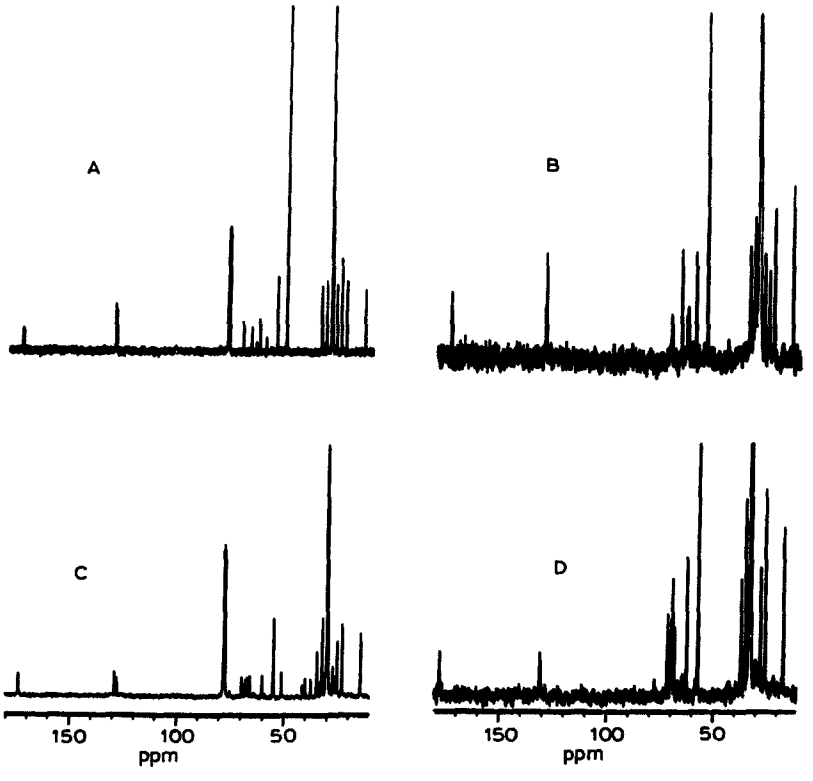

Fig. 5. ^{13}C-NMR Spectra of POPC and CMPC. A, POPC in $CDCl_3$; B, CMPC in $CDCl_3$; C, POPC in vesicles; D, CMPC in vesicles.

observed in $CDCl_3$. Furthermore, two closely spaced signals at around 174 ppm for the carbonyl carbons could be seen for POPC in vesicles (or in $CDCl_3$), whereas only one signal was observed in this region in the case of the CMPC vesicles (Fig. 5). Also, differences were observed between POPC and CMPC in the 127–130 ppm region both in $CDCl_3$ and vesicles. These results clearly demonstrate that the structure of CMPC in bilayers (or in $CDCl_3$) is different from that of POPC, under identical conditions.

TABLE I

^{13}C-Chemical shifts for POPC and CMPC polar headgroup carbons in both $CDCl_3$ and vesicles

Group	POPC (ppm)		CMPC (ppm)	
	$CDCl_3$	vesicles	$CDCl_3$	vesicles
$\overset{+}{N}(\underline{C}H_3)_3$	50.35	54.38	54.36	54.36
$OP\underline{C}H_2$ (choline)	54.55	59.69	59.49	59.40
$\underline{C}H_2OC(=O)$	59.64	63.29	65.24	65.44
$\underline{C}H_2OP$ (glycerol)	62.76	63.88	66.21	66.35
$\underline{C}H_2\overset{+}{N}$ (choline)	66.52	66.38	67.21	66.93
$\underline{C}HOC(=O)$	70.40	71.00	68.89	68.65

Discussion

Carbamyl phosphatidylcholines, despite their hydrogen-bonding potential, possess thermal phase transition temperatures similar to phosphatidylcholines of comparable acyl chain length [7,8]. This is suggestive either of the absence of the intermolecular hydrogen bonding in the gel phase of these lipids or of the possibility of an occurrence of the intermolecular hydrogen bonding equally well in both gel and liquid-crystalline phases [18]. But, based on the fact that the ^{13}C-chemical shifts

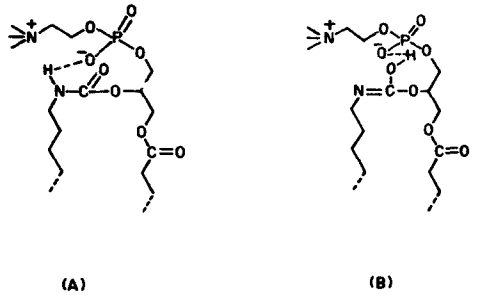

Fig. 6. Schematic representation of intramolecular hydrogen-bonding possibilities in the CMPC molecule.

for the CMPC headgroup carbons as well as the carbonyl carbon in vesicles were similar to those obtained in $CDCl_3$ (Fig. 5 and Table I), the possibility of intermolecular hydrogen bonding in CMPC bilayers may be completely ruled out. The lack of intermolecular hydrogen bonding in the CMPC bilayers could be attributed to the choline headgroup, which may behave as a repulsively charged headgroup as in PC.

The observed downfield ^{13}C-chemical shifts for the CMPC headgroup carbons, as compared to the POPC headgroup carbons in $CDCl_3$ suggest that the headgroup structure of CMPC is different than that of POPC in this organic solvent. As these chemical shifts did not significantly change by transferring CMPC from $CDCl_3$ to vesicles, it may be further inferred that the CMPC headgroup movement is restricted even in $CDCl_3$. Also, it suggests that the environment of the CMPC headgroup carbons is not much influenced by the presence of water. These findings could be fully explained by invoking an intramolecular hydrogen bonding between the -NH and the phosphate residues in the CMPC molecule. Since such a hydrogen bonding would result in the formation of a cyclic structure, as shown in Fig. 6, the headgroup carbons should experience a downfield shift due to their positioning in the deshielding zones of the C=O and the P=O groups. Moreover, this intramolecular hydrogen bonding should be strong in nature, and thus may not be influenced by water.

Intramolecular hydrogen bonding in CMPC would not only restrict the headgroup motion but could also make the molecule perfectly cylindrical, ideal for packing in the bilayer configuration [19]. Therefore, the higher DPH polarization values observed for the CMPC bilayers, as compared to the POPC bilayers in identical conditions, may be attributed to the more ordered structure of CMPC in the vesicles bilayer. That the structure of CMPC is more ordered than that of POPC in bilayers is further evidenced by our observation that inclusion of cholesterol in the CMPC bilayers does not significantly enhance the acyl chain packing order, as it does in the case of the POPC bilayers (Figs. 2 and 3). Also, the anamolous behavior of cholesterol at 33 mol% strongly indicates that cholesterol interacts differently with CMPC, as compared to POPC, in bilayers.

It is interesting to note that the distorted doublet-like shape of the CMPC acyl chain methylene proton's NMR signal was greatly modified with the cholesterol concentration. The pattern of this shape change (Fig. 4) may be taken to suggest that cholesterol interacts differentially with the two acyl chains of CMPC. Though the present data are inadequate to ascertain which of the two acyl chains interacts strongly with cholesterol, but based on the hydrogen-bonding potential of this sterol, it may be inferred that its β-face should preferentially point towards the C-2 acyl chain of CMPC. This preference of cholesterol would perhaps result in weakening of the intramolecular hydrogen bonding in CMPC molecule due to the tendency of the 3β-OH group to form a hydrogen bond with nitrogen of the carbamyl residue, which in turn could adversely affect the CMPC headgroup packing order. The anamolous behavior of CMPC, as compared to POPC, at 33 mol% cholesterol may thus be attributed to the existence of equal amounts of free and cholesterol-bound CMPC molecules, which probably leads to disorder in the overall phospholipid packing.

Cholesterol is known to exert a greater ordering effect on phosphatidylcholines which contain an oleoyl acyl chain in position-2 and a saturated fatty acyl chain in position-1 of their glycerol backbone [20]. This preference of cholesterol for 1-acyl-2-oleoylphosphatidylcholines has been attributed to the C-2 acyl chain 9,10-*cis* double bond, which has been postulated to accommodate the sterol C-18 and C-19 angular methyl groups [21]. Since CMPC is formed by introducing one -NH residue adjacent to the C-2 carbonyl carbon of POPC, it may be envisaged that this change could lead to the reduced phospholipid acyl chain–cholesterol interactions due to a shift in the position of the 9,10-*cis* double bond. This is consistent with our present finding that the cholesterol-induced increase in the acyl chain methylene proton's line width is considerably greater in the case of the POPC bilayers, as compared to the CMPC bilayers.

POPC is known to undergo thermal phase transition at about $-5°C$ [22]. An introduction of one -NH residue adjacent to the carbon atom of the C-2 ester group of this phospholipid did not significantly alter its thermal phase transition behavior, as we repeatedly failed to observe any major phase change between 5 and 50°C in the CMPC bilayers, using 8-anilinonaphthalene-1-sulfonate as the fluorescent probe [5]. The nonlinear temperature-dependence of DPH fluorescence polarization observed in the case of the CMPC bilayers may, therefore, be attributed to some novel but minor change in the CMPC acyl chain packing order at about 30°C. Alternatively, this phase change in these bilayers at 30°C could arise from the weakening of the intramolecular hydrogen bonding in the CMPC molecule at this temperature. The present data are, however, not sufficient to further distinguish between these two possibilities.

In summary, the present study shows that the CMPC headgroup is highly ordered even at temperatures above the phospholipid thermal phase transition temperature [5], possibly due to the strong intramolecular hydrogen-bond formation between the -NH and the phosphate residues. It further demonstrates that the phospholipid packing order in the CMPC bilayers is significantly greater than that observed for the POPC bilayers. This order in the CMPC bilayers is not much affected by cholesterol, whereas under identical conditions, this

sterol markedly enhances the acyl chain order in the POPC bilayers.

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