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Novel thermal phase transition behavior of phosphatidylcholine analogs containing 1,2,4-butanetriol as their backbone

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Abstract

The *sn*-glycerol moiety in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was replaced by the *rac*-1,2,4-butanetriol residue, and the aqueous dispersions of the resulting DPPC analogs, viz. 1,2-dihexadecanoyloxy-*rac*-but-4-yl-[2-(trimethylammonium)ethyl]phosphate (1,2-bPC) and 1,3-dihexadecanoyloxy-*rac*-but-4-yl-[2-(trimethylammonium)ethyl]phosphate (1,3-bPC), were characterized by differential scanning calorimetry (DSC) and fluorescence polarization technique. Also, the corresponding (3*R*)-isomer of 1,3-bPC (1,3-(3*R*)bPC) was prepared and characterized by DSC. While the thermal phase transition properties of 1,2-bPC were similar to that of racemic DPPC, 1,3-bPC in identical conditions showed an abnormal property by exhibiting a metastable phase behavior at about 15°C. This abnormal property was associated only with the racemic mixture and was completely absent in its 3*R*-isomer. 1,3-(3*R*)bPC, unlike DPPC, showed only two high enthalpy transitions at about 29.7°C (ΔH, 7.45 kcal/mol) and 35.3°C (ΔH, 6.93 kcal/mol). These results clearly demonstrate that an insertion of one additional methylene residue between the glycerol C1 and C2 carbons in DPPC markedly alters its thermal phase transitional properties, whereas these properties remain virtually unchanged if a similar chemical change is introduced between the glycerol C2 and C3 carbon atoms.

Keywords: Metastable state; Phosphatidylcholine analog; Butanetriol backbone; Differential scanning calorimetry; Fluorescence polarization; Phase behavior

1. Introduction

Glycerophospholipids constitute the major portion of the membrane phospholipids present in mammalian cells. This class of phospholipids generally contain glycerol as their basic backbone to which three substituent chains are linked. The first two carbons contain the two fatty acyl chains while the third carbon is linked to a free or monoesterified phosphate residue. These compounds upon dispersing in water usually form bilayers. Several studies have shown that the preferred conformation of these molecules in micelles or bilayers is determined primarily by their glycerol backbone [1–3].

In order to analyse the effect of the glycerol modifications on the backbone conformation, we have synthesized recently the phosphatidylcholine analogs that have *rac*-1,2,4-butanetriol, instead of *sn*-glycerol residue, as their backbone, and characterized them in

Abbreviations: DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; DPH, 1,6-diphenyl-1,3,5-hexatriene; EDTA, ethylenediamine tetraacetic acid; DMAP, *N*,*N*-dimethyl-4-aminopyridine; *rac*-DPPC, 1,2-dipalmitoyl-*rac*-glycero-3-phosphocholine; 1,3-DPPC,1,3-dipalmitoyl-*sn*-glycero-2-phosphocholine

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chloroform as well as in aqueous dispersions by high-resolution ^{1}H- and ^{13}C-NMR spectroscopy [4]. In continuation of this work, we have now studied the aqueous dispersions of these phospholipid analogs by differential scanning calorimetry and fluorescence polarization technique. Results of these studies indicate that the thermotropic properties of phosphatidylcholines are not much affected by introducing one additional methylene group between the glycerol C2 and C3 carbon atoms, but these properties are significantly altered by introducing a similar chemical change between the glycerol C1 and C2 carbons.

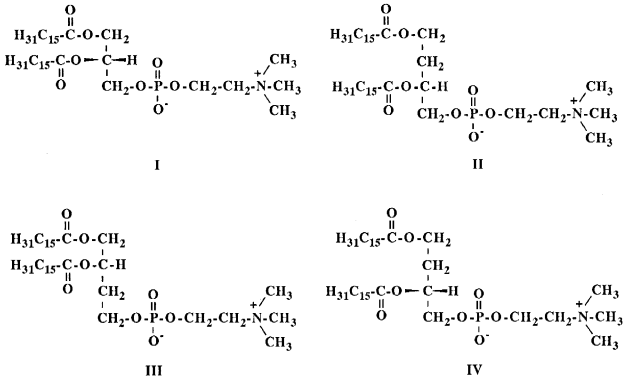

Fig. 1. Molecular structures of phosphatidylcholine analogs wherein the glycerol moiety has been replaced by the butanetriol residue.

2. Materials and methods

2.1. Materials

1,2-Dihexadecanoyl-*sn*-glycero-3-phosphocholine (Fig. 1, **I**), 1,3-dihexadecanoyloxy-*rac*-but-4-yl-[2-(trimethyl-ammonium)ethyl]phosphate (Fig. 1, **II**) and 1,2-dihexadecanoyloxy-*rac*-but-4-yl-[2-(trimethyl-ammonium)ethyl]phosphate (Fig. 1, **III**) were prepared and purified as described elsewhere [4]. Cholesterol (Sisco Research Laboratories, Bombay) was used after crystallizing it three times from methanol. 1,6-Diphenyl-1,3,5-hexatriene (DPH), palmitic acid and *N,N*-dimethyl-4-aminopyridine (DMAP) were procured from Sigma Chemical Co. Crude snake venom from *Naja naja* (Haeffkin Institute, Bombay) was used as the source of phospholipase A_2. 1,3-Dihexadecanoyloxy-(3*R*)-but-4-yl-[2-(trimethylammonium)ethyl]phosphate (Fig. 1, **IV**) was prepared from **II** in two steps as shown in Fig. 2. A brief description of the preparation of **IV** is given below.

Thin films of **II** (100 mg) were treated under shaking with *Naja naja* snake venom (2.5 mg) and $CaCl_2 \cdot 2H_2O$ (64 mg) in 10 ml of Tris buffer (20 mM, pH 8.8) containing 20 ml of methanol/ether mixture (2:98, v/v) at 37°C for 12 h. The organic solvents were evaporated under reduced pressure, and phospholipids from the remaining aqueous suspension were extracted using chloroform/methanol (2:1, v/v). The solvents were removed, and 1-hexadecanoyloxy-3-hydroxy-(3*R*)-but-4-yl-[2-(trimethylammonium)ethyl]-phosphate (**V**) was isolated in about 35% yield by chromatographing the residue over a Sephadex LH-20 column (2.5 × 100 cm) using chloroform/methanol (1:1, v/v) as the eluent. The compound **V** on acylation with palmitic anhydride in presence of DMAP [5] afforded **IV** in about 85% yield.

2.2. General methods

Phospholipids were purified by silica gel column chromatography, Sephadex LH-20 column chromatography, and preparative high-pressure liquid chromatography (HPLC). The Sephadex column was eluted with chloroform/methanol (1:1, v/v) mixture at a flow rate of about 60 ml/h. The preparative HPLC was carried out on an LKB TSK-ODS-120T semiprep column (7.8 × 300 mm, particle size 10 μm) using methanol/chloroform/water (90:10:4, v/v) at a flow rate of 2 ml/min as the eluent. The purity of phospholipids was checked by thin-layer chromatography (TLC) over silica gel G-60 plates using chloroform/methanol/water (65:25:4, v/v) as the developing solvent system, and also by HPLC on an Altex ultrasphere μC_{18} reverse phase column (4.5 × 250 mm, particle size 5 μm) using ethanol/water/hexane (75:13:8, v/v) containing 25 mM choline chloride as the elution system. All the phospholipids samples exhibited single spots on TLC and single peaks on HPLC.

2.3. Differential scanning calorimetry (DSC)

2.3.1. Sample preparation

Known volumes of phospholipid solutions in chloroform/methanol (1:1, v/v) mixture were dried un-

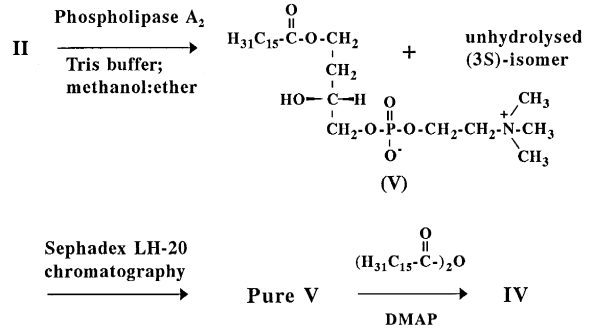

Fig. 2. Conversion of 1,3-dihexadecanoyloxy-*rac*-but-4-yl-[2-(trimethylammonium)-ethyl]phosphate (**II**) into 1,3-dihexadecanoyloxy-(3*R*)-but-4-yl-[2-(trimethyl-ammonium)ethyl]phosphate (**IV**).

der a slow jet of N_2 to give a thin lipid film on the wall of a glass tube. After removing the traces of the solvents under vacuo, the dried lipid films were hydrated at 50°C with phosphate-buffered saline (5 mM phosphate containing 50 mM NaCl, pH 7.4) containing 1 mM EDTA, so as to give a final lipid concentration of 3–4 mM. The dispersion was immediately vortexed at 50°C for 5 min and then slowly cooled to 5°C. This heating/cooling cycle was repeated for two more times and the resulting multilamellar vesicles were stored at 0°C for more than 3 days. Then, equal volumes of the resulting dispersions were degassed at 0°C for 30 min under stirring. Finally, the dispersion was loaded into the sample cell of the calorimeter, and equilibrated at 2°C for 60 min prior to DSC scan [6].

2.3.2. Calorimetry

DSC was carried out on a high sensitivity MC-2 Differential Scanning Microcalorimeter (MicroCal Company, Amherst, MA, USA) equipped with DA-2 digital data acquisition system. All the samples were scanned between 2°C and 50°C at a constant scan rate of 22.85°C/h in an ascending temperature mode. Cooling was initiated at 50°C, and the cell temperature was brought to 2°C in about 50 min. The samples were incubated for 1 h at this temperature after which reheating scans were initiated exactly under similar conditions, as used for the initial heating scans. Phosphate buffer baseline was also collected and subtracted from each thermogram. Transition temperatures were taken from the transition peaks at the maximum height positions. The calorimetric enthalpies (ΔH) were calculated from the peak areas using MicroCal ORIGIN software. The thermograms were normalized from mcal/min to mcal/°C by dividing with the scan rate. The heat capacity in units of kcal/°C/mol was determined by further dividing the normalized thermograms with the number of moles of lipid present in the vesicle dispersion.

2.4. Sonicated vesicles

Measured volumes of phospholipid and cholesterol solutions were mixed in a clean glass tube so as to achieve a desirable phospholipid-to-cholesterol ratio. It was evaporated to dryness under a slow jet of N_2 giving rise to the formation of a thin lipid film on the walls 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 containing 145 mM NaCl (pH 7.4)) at 50°C. For fluorescence polarization experiments, 0.025% EDTA was also included in the dispersion buffer. The mixture was vortexed for 15 min at 50°C. The lipid dispersion thus obtained was sonicated (50°C) for 40 min under N_2 using a probe-type sonicator (Heat Systems Ultrasonic Processor XL) at 15% power setting. The sonicated mixture was centrifuged at $107\,000 \times g$ at 25°C for 60 min to remove titanium particles as well as the poorly dispersed lipids. Only the top two-thirds of the supernatant was used in further experiments.

2.5. Vesicle size

Vesicle size was determined by negative, staining electron microscopy [7] using a Jeol 1200 EXII electron microscope operating at 80 kV. Samples were prepared by placing the grids into a droplet of freshly prepared unilamellar vesicles for 5 min at 25°C, followed by removal of the excess dispersion by blotting the grids with filter paper. Staining was done by placing the grid first into a phosphotungstic acid solution (2% in water, pH 7.0) and then into a uranyl acetate solution (2% in water) [8]. The negatively stained grids were viewed under the microscope at $100\,000\times$ magnification. Vesicle diameter was determined by taking mean of the diameter along the two perpendicular axes.

2.6. Fluorescence polarization

Fluorescence polarization of DPH as a function of temperature was measured according to Shinitzky and Barenholz [9] using a Kontron spectrofluorometer. The DPH was embedded in membranes of sonicated phospholipids vesicles (0.4 mM, 2 ml) and excited at 357 nm (10 nm band pass). The fluorescence was viewed through the emission monochromator set at 428 nm (20 nm band pass). In all the experiments, the mole ratio of phospholipid-to-DPH was about 500:1. Polarization was 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 the experiments fluorescence polarization and total fluorescence intensity were calculated after correction for light scattering, as described by Shinitzky et al. [10]. There was no polarization due to light scattering, since dilution of vesicles labeled with DPH had no effect on the fluorescence polarization.

Labelling of vesicles with DPH was carried out by adding the solution of the fluorophore (1 mM, 1 μl) in tetrahydrofuran to a rapidly vortexed vesicle dispersion (0.4 mM phospholipid, 2 ml). Similar amounts of tetrahydrofuran, free of DPH, were added to the vesicles which were used as controls. The vesicle samples were incubated with DPH at 50°C for 2 h to allow equilibration with the vesicles. The total incorporation of DPH was ensured by measuring the fluorescence intensity after the incubation, and polarization experiments were started after a plateau in the fluorescence intensity had been achieved. Under these conditions, undesirable fluorescence polarization due to nonradiative energy transfer between the probe molecules was practically eliminated.

3. Results

The racemic mixtures of **II** and **III** were synthesized as reported elsewhere [4], and with the hope to isolate the desired (*R*)-isomers, the purified phospholipid samples were treated with phospholipase A_2, as described in Section 2. This enzyme readily hydrolysed 35–40% of **II** (presumably the (*R*)-isomer) to give the corresponding lysolipid **V** (Fig. 2), but in accordance with the earlier report [11], no hydrolysis of **III** was observed in identical conditions. The lysolipid **V** on acylation with palmitic anhydride [5] gave **IV** in high yields. The compound **IV** thus represents a modified version of **I** where an additional methylene residue has been introduced between the glycerol C1 and C2 carbon atoms. All these lipids (**I**–**IV**), in absence as well as in presence of 33 mol% cholesterol, were hydrated and the phase transition properties of thus obtained aqueous dispersions studied by DSC.

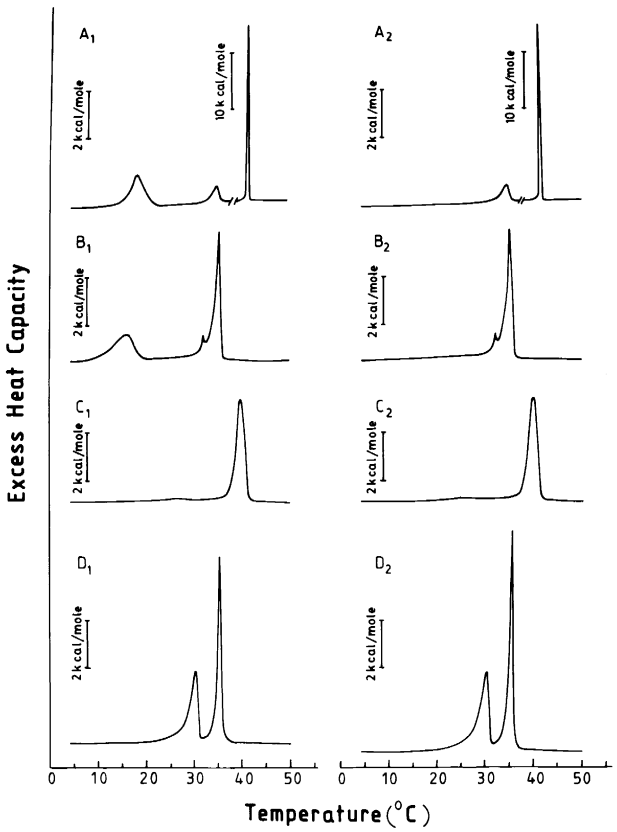

Fig. 3. Calorimetric transition curves of multilamellar phospholipid suspensions. A1 and A2, heating and reheating scans of **I**, respectively; B1 and B2, heating and reheating scans of **II**, respectively; C1 and C2, heating and reheating scans of **III**, respectively; D1 and D2, heating and reheating scans of **IV**, respectively. Heating and reheating were carried out as described in Section 2.

Table 1
Thermodynamic characteristics [a] of the phase transitions of multilamellar suspensions of phospholipids

Phospholipid	Subtransition				Pretransition				Main transition			
dispersion	T_m	$\Delta T_{1/2}$	ΔH	ΔS	T_m	$\Delta T_{1/2}$	ΔH	ΔS	T_m	$\Delta T_{1/2}$	ΔH	ΔS
I	18.35	2.93	3.86	13.23	34.43	1.64	1.20	3.89	41.43	0.25	8.99	28.60
II	15.60	4.22	4.78	16.56	32.17	0.88	1.01	3.31	35.10	1.13	6.83	22.17
III	–	–	–	–	25.45	7.72	0.28	0.95	39.75	1.86	8.66	27.67
IV	–	–	–	–	29.73	1.96	7.47	24.67	35.28	0.68	6.91	22.40

[a] T_m, melting transition temperature (°C); $\Delta T_{1/2}$, transition width at half-peak height (°C); ΔH, transition enthalpy (kcal/mol); ΔS, transition entropy (cal/kmol).

Fig. 3 shows both the initial heating (A1–C1) and reheating (A2–C2) DSC scans of **I**–**III** incubated at 0–1°C for a minimum period of 3 days. The thermodynamic characteristics of the initial scans of each of these three phospholipid species are listed in Table 1. As may be seen in Fig. 3, the aqueous dispersions of **I** exhibited three transitions with thermodynamic characteristics similar to those reported for this phospholipid earlier [6]. Like **I**, phospholipid **II** also exhibited three transitions. However, the main transition in **II** was centered at a lower temperature (35°C) and was broader ($\Delta T_{1/2} = 1.13$°C) than the main transition of **I** ($\Delta T_{1/2} = 0.25$°C). Also, it was not well separated from the pretransition. Unlike **I** and **II**, the PC analog **III** exhibited only two endothermic transitions; a very broad, low enthalpy transition at about 25°C and the main transition at about 40°C. Also, the main transition was much broader ($\Delta T_{1/2} = 1.86$°C) than that observed for **I** and **II**. However, the other thermodynamic characteristics, viz. enthalpy and entropy, of this transition were similar to those observed for **I** (Table 1). These findings are quite in accordance with the earlier studies which showed that 1,2-dipalmitoyl-*rac*-glycero-3-phosphocholine (*rac*-DPPC), unlike **I**, does not exhibit the subtransition at 18°C [12]. In order to better characterize these transitions, the samples were cooled to 2°C at a rate of approximately 60°C/h (unprogrammed adiabatic cooling) and, after incubation for 1 h at 2°C, these were rescanned. Under these conditions, the subtransition could not be observed in case of both **I** and **II** dispersions, indicating that the phases responsible for these peaks were metastable and were observed only on prolonged incubation (several days in this case) at low temperatures. Moreover, on including 33 mol% cholesterol in the phospholipid dispersions, all of the three phospholipids (**I**–**III**) did not exhibit any phase transition up to 50°C (data not shown).

The initial heating and reheating scans of **IV** are shown in Fig. 3 D1 and D2, respectively. This phospholipid, unlike its racemic form (viz. **II**), exhibited only two transitions; the one at lower temperature was centered at around 29°C and had slightly higher entropy and enthalpy as compared to the one observed at the higher temperature (about 35°C), which corresponded to the chain melting transition temperature of **II**. Moreover, none of these transitions was metastable since no change in any of these two peaks was observed upon cooling and subsequently reheating the phospholipid sample (Fig. 3, D2).

To further characterize the phase transition behaviour of **I**–**III**, we studied the acyl chain order–disorder transitions in the sonicated aqueous dispersions of **I**–**III**, employing the fluorescence polarization technique. All these phospholipids, in presence as well as in absence of cholesterol, upon sonication formed vesicle-like structures which had average outer diameters of 24–27 nm and 33–38 nm in absence and presence of cholesterol, respectively (Table 2). The acyl chain disorder in these phospholipid disper-

Table 2
Mean outer diameter of vesicles as determined by negative staining transmission electron microscopy

Vesicle composition	Total number of vesicles sized	Outer diameter [a] (nm)
I	100	27.20 ± 4.38
II	222	25.96 ± 4.38
III	249	24.35 ± 5.09
I/cholesterol [b]	151	33.21 ± 7.76
Il/cholesterol	171	37.10 ± 9.26
III/cholesterol	156	38.20 ± 7.70

[a] values shown are mean ± S.D.
[b] 33 mol%.

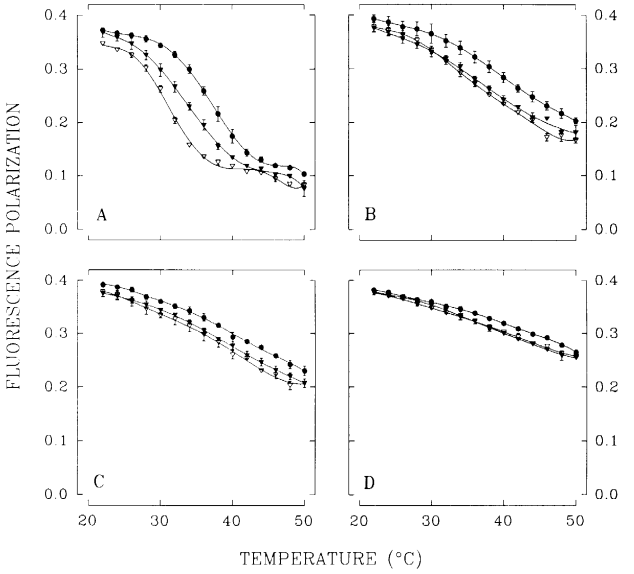

Fig. 4. Temperature-dependence of DPH fluorescence polarization in phospholipid (PL)/cholesterol (CH) vesicles. (A) PL/CH – 1:0, mol/mol; (B) PL/CH – 4:1, mol/mol; (C) PL/CH – 3:1, mol/mol; (D) PL/CH – 2:1, mol/mol. Closed circle, **I**; open triangle, **II**; closed triangle, **III**. Values shown are means ± S.D. of three determinations.

sions was ascertained by measuring the fluorescence depolarization of DPH as a function of temperature. Fig. 4A shows that the polarization values for **II** or **III** aqueous dispersions were smaller as compared to the aqueous dispersions of **I**. However, this difference in the polarization values continuously diminished and ultimately vanished by including increasing amounts of cholesterol in the phospholipid dispersions (Fig. 4B–D). At 33 mol% cholesterol, all the three phospholipids dispersions showed similar fluorescence polarization values and exhibited no detectable phase transition up to 50°C. These results clearly indicate that though the micellar aggregates comprised of **II** and **III** are more fluid (T_m: **II**, 30.7°C; **III**, 34.2°C) than that of **I** (T_m: 37.1°C), this difference between the fluidity gradually disappears by incorporating increasing amounts of cholesterol in the phospholipid dispersions.

4. Discussion

This study shows that the phase transition properties of racemic phosphatidylcholines [12] are not much affected by introducing one additional methylene residue between the C2 and C3 carbons. However, these properties are markedly altered if the extra methylene group is inserted between the glycerol C1 and C2 carbons. The modified phospholipid thus obtained (viz. **II**), unlike *rac*-DPPC [12], not only exhibited a metastable phase behavior at about 15°C but also its main transition temperature as well as the associated enthalpy and entropy values were smaller than that observed for **I** or reported earlier for *rac*-DPPC [12]. Since the absence of subtransition in *rac*-DPPC has been assumed to be the result of the impossibility of the racemic phospholipid to form the low temperature crystal state L_c [12], it may be inferred that the observed low temperature incubation-induced crystallization of chains in **II** could be due to its unique chemical structure [4], which affords a greater structural flexibility to its backbone, as compared to **I** or **III**.

Since the pretransition and main transition in **II** were overlapping, we synthesized the chiral (3*R*)-analog of **II**, which has the same configuration of the secondary acyl chain as that of the natural isomer. It was expected that **IV** would exhibit the three transitions similar to that of **II**, with the main transition being sharper and therefore, well separated from the pretransition. However, only two high enthalpy transitions were observed in case of **IV**. It is pertinent to note that the initial heating scans of **IV** resemble somewhat that of 1,3-dipalmitoyl-*sn*-glycero-2-phosphocholine (1,3-DPPC) [13,14], which also exhibits two high-enthalpy transitions at 27°C and 37°C. But, the reheating scans of **IV** differ from that of 1,3-DPPC; whereas the low temperature endothermic transition in **IV** was not metastable, this transition in case of 1,3-DPPC has been reported to be metastable [14]. Also, except for the sharpening of the peak ($\Delta T_{1/2} = 0.67$°C) there was not much change in the thermodynamic characteristics of the main transition in **IV**, as compared to **II** (Table 1). These results taken together suggest that the phase transition properties of **II** and **IV** are novel and are not comparable with any of the stereochemically equivalent analogs having the glycerol backbone, but seem to be a consequence of the unique acyl chain packing in these phospholipids brought about by virtue of their chemical structure and backbone conformation [4].

High-resolution ^{1}H- and ^{13}C-NMR studies on **I**–**III**

have revealed that the conformational preference around the butanetriol C2–C3 bond in **II** is almost similar to that observed for the glycerol C1–C2 bond in **I** or butanetriol C1–C2 bond in **III** [4]. Based on this observation, it has been suggested that structurally, the C2 methylene in the butanetriol backbone of **II** could perhaps represent the proximal beginning of the primary acyl chain [4]. Since the preferred conformation of phosphatidylcholines in bilayers is such that the C2 ester group aligns at the bilayer interface while the C1 ester group remains perpendicular to the plane of the bilayer [2,3,15], the further pushing of the C1 acyl chain into the bilayer matrix, as envisaged in the case of **II** [4], may in fact adversely affect the acyl chain packing in the phospholipid bilayers. This suitably accounts for the differences seen between **I** (or **III**) and **II** during DSC and DPH fluorescence polarization experiments. Further, the presence of an additional methylene residue between the glycerol C2 and C3 carbons in phosphatidylcholine should, in principle, not affect appreciably the alignments of the fatty acyl chains in the bilayer and therefore, **III** is expected to behave not very differently from **I**. However, the polar headgroup structure in **III** seems to be more ordered than that in **I** [4], which may perhaps explain the minor differences observed between the phase transition characteristics of **I** and **III**.

These results indicate that **II** and **III** in an aqueous environment form large micellar aggregates which have strong tendency to undergo thermal phase transition. This tendency of these phospholipid analogs is quite similar to that of the natural phosphatidylcholines which in presence of water form closed bilayer structures. That the phospholipid **II** and **III**, like **I**, also form the closed bilayer-like structures is evidenced by our observations that (a) sonication of the aqueous dispersions of **II** or **III** yielded vesicles which could entrap 6-carboxyfluorescein (data not shown) and had outer diameters similar to that observed with **I**, (b) cholesterol exhibited similar effects on the 6-carboxyfluorescein permeability (data not shown) and size of the sonicated vesicles in case of all the three phospholipid molecular species, (c) an addition of 33 mol% cholesterol to the multilamellar or sonicated dispersions of **II** or **III** abolished their phase transition [16], and (d) the ^{31}P-NMR spectra recorded of the multilamellar dispersions of **I**–**III** at 50°C exhibited ^{31}P chemical shift anisotropy [17] of about 50–55 ppm (A. Arora and C.M. Gupta, unpublished preliminary observations).

In summary, the present study clearly demonstrates that an introduction of one additional methylene group between the glycerol C2 and C3 carbons in phosphatidylcholines does not affect their phase transition behavior, but this behavior is significantly altered if a similar change is introduced between the glycerol C1 and C2 carbon atoms. It would therefore seem that the proximal vicinal alignment of the acyl chains in these glycerophospholipids is perhaps essential for their normal behavior. Increasing the separation between the two acyl chains by introducing an extra methylene group between the glycerol C1 and C2 carbons resulted not only in a hitherto unknown gel phase behavior but also in the decreased acyl chain packing order in the micellar aggregates of the resulting phospholipid analogs. Thus, study of the phase behavior of the glycerol backbone modified phospholipids may in itself be very important in understanding the interfacial interactions of various membrane components in natural membranes, and its possible functional repercussions.

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