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Differential scanning calorimetric studies of aqueous dispersions of phosphatidylcholines containing two polyenoic chains

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The thermotropic behavior of aqueous dispersions of phosphatidylcholines (PC) that contain two identical unsaturated acyl chains, either linoleoyl, α-linolenoyl, or arachidonoyl chains, has been investigated by differential scanning calorimetry. These dipolyenoic PC displayed very broad thermal transitions, which occurred over a range of approximately 40 C deg, that started between −77°C (20:4-20:4 PC) and −71°C (18:2-18:2 PC). The enthalpy changes associated with the transitions were small (1 to 2 kcal/mol, 4 to 8 kJ/mol). The broad, low-enthalpy transitions suggest that these lipids might have a very disordered gel phase, or that the transitions from the gel to the liquid crystal might proceed through some intermediate states. The temperatures over which the transitions occurred were influenced only slightly by the number of double bonds between two and four per chain.

Introduction

Some membranes contain phospholipid molecules in which both chains are unsaturated, and, more particularly, in which each unsaturated chain contains two or more double bonds – lipids which we shall call dipolyenoic phospholipids. Substantial quantities of these lipids have been found in the disc membranes of retinal rods [1–3]; small amounts have been described in other membranes.

Replacement of the normal phosphatidylcholines of human erythrocyte membranes with a substantial amount of dilinoleoyl phosphatidylcholine (18:2-18:2 PC) resulted in an increase in osmotic fragility, whereas replacement with 1-palmitoyl-2-oleoyl-PC or 1-palmitoyl-2-linoleoyl-PC caused no change in osmotic fragility [4]. Enrichment of erythrocyte membranes with 18:2-18:2 PC (to 25% of the total PC) produced a reduction in the rate of cholesterol efflux from the cells in the presence of liposomes made of egg PC [5]. While these observations indicate that dipolyenoic phospholipids can modify the properties of membranes, little is known of the physical chemistry of membranes which contain such lipids. We

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Abbreviations: DSC, differential scanning calorimetry(ic); PC, phosphatidylcholine; $T_{\text{G-LC}}$, temperature of the gel to liquid-crystalline phase transition; T_f, temperature of the finish of the gel to liquid-crystalline phase transition; $T_{\max}$, temperature at which the maximum rate of heat flow into or out of a sample occurred during a phase transition; T_o, temperature of onset of the gel to liquid-crystalline phase transition; 18:0-18:0 PC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; 18:1-18:1 PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; 18:2-18:2 PC, 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine; 18:3-18:3 PC, 1,2-di-α-linolenoyl-*sn*-glycero-3-phosphocholine; 20:4-20:4 PC, 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine.

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have therefore carried out a differential scanning calorimetric investigation of dispersions of a group of PC each of which contained two identical polyunsaturated acyl chains in order to obtain fundamental information about their thermotropic properties. The study has shown that model membranes composed of these PC have unusual gel to liquid-crystalline transitions.

Double bonds in the hydrocarbon chains of phospholipids cause a reduction in the temperature of the gel to liquid-crystalline phase transition ($T_{\text{G-LC}}$) in comparison to that of the saturated lipid of equal chain length [6], and the position of the double bond along the chain influences the magnitude of the reduction in $T_{\text{G-LC}}$ [7]. In heteroacid PC (mixed acid PC) containing a saturated chain and an unsaturated one, the addition of double bonds in excess of two per unsaturated chain caused a much smaller reduction in $T_{\text{G-LC}}$ than did the introduction of the first two double bonds [8–11]. We suggested that as chains become more unsaturated there may be a compensation between the perturbing effects of the double bonds on chain packing in the gel and the restricted motion of the chains in the liquid crystal since fewer bonds can rotate as unsaturation increases. This balance of forces was suggested to lead to the leveling-off of the $T_{\text{G-LC}}$ of the heteroacid PC as unsaturation increased [9]. It is possible that in membranes composed of heteroacid PC having one polyenoic chain the adjacent saturated chain could constrain the polyenoic chain in some way so that this explanation might not be valid. Since there was no constraining saturated chain in the lipids used in this investigation, we had a further test of the validity of this proposal.

Materials and Methods

Materials

Lipids were obtained as follows: 18:0-18:0 PC from Sigma Chemical Co. (St. Louis, MO); 18:1-18:1 PC from Sigma; 18:2-18:2 PC from Avanti Polar Lipids, Inc. (Birmingham, AL), Serdary Research Laboratories Inc. (London, Ontario) and Sigma; 18:3-18:3 PC from Avanti and Serdary; and 20:4-20:4 PC from Avanti, Serdary and Calbiochem-Behring (San Diego, CA).

Ten percent SP-2330 (68% cyanopropylsilicone) on 100/120 Chromasorb$^{\text{R}}$ W AW was purchased from Sulpelco, Inc. (Bellefonte, PA) and silica gel G (Kieselgel 60 G, Merck) from British Drug Houses (Toronto, Ontario). Other chemicals and solvents were reagent grade or better, and were purchased from Sigma, Fisher Chemical Co. (Dartmouth, Nova Scotia) and GSW Consolidated Alcohols, Ltd. (Toronto, Ontario).

Solvents were distilled from glass before use. Water was deionized and distilled twice from glass, the second time from dilute $KMnO_4$ solution.

Methods

Differential scanning calorimetry. DSC was performed with a Perkin-Elmer DSC-2. Solvents were removed from the lipids under a stream of O_2-free N_2 followed by evacuation overnight in the dark over P_2O_5. Just prior to DSC analysis the lipids were dispersed at 25–33% by weight in doubly-distilled water by vortexing at room temperature. All samples were heated and cooled over the range of -125 to $27\,^{\circ}C$ to ensure that excess water was present and that transitions were reproducible. Water was used in the reference pans. High sensitivities (0.1 to 0.5 mcal/s) were necessary. Scanning rates will be given where appropriate in the Results section.

Lipid analyses. After DSC analysis, the DSC pans were opened and the contents extracted a number of times with $CHCl_3/CH_3OH$ (1:1, v/v) and made up to a final volume of 10 ml. The following procedures were carried out on the extracts.

Phosphorus analysis. The following modification of the procedures of Fiske and SubbaRow [12], Dawson [13] and Bartlett [14] was used. After evaporation of any solvent, samples were digested in 1.0 ml of 70% $HClO_4$ until they were colorless. To the digested samples 8.0 ml of H_2O was added, followed by 0.5 ml of 5% ammonium molybdate and 0.5 ml of aminonaphthol sulfonic acid prepared as described by Fiske and SubbaRow [12]. The solutions were immediately heated in a boiling water bath for 12 min. When the solutions had cooled their optical densities were determined at 815 nm.

After thin-layer chromatography, samples of gel that contained phospholipids in addition to lipid-free control gel blanks were digested and subjected to color development as described above. Prior to measuring the absorbance, gel was removed from the samples by centrifugation in a bench-top centrifuge for approximately five minutes.

Ultraviolet spectra. Samples of 100 μl of the lipid extract of the DSC sample were dried under O_2-free N_2, and the residues were redissolved in 1.0 ml of 95% ethanol. Spectra were obtained from 350 to 190 nm with 95% ethanol as a reference using a Cary 118 spectrophotometer.

Thin-layer chromatography. Thin-layer chromatography was carried out on silica gel G using chloroform/methanol/water (65 : 25:4, v/v). The plates were sprayed with 70% H_2SO_4 and the organic material charred by heating the plates at about 180 ° C for approx. 16 h.

Fatty acid analysis. Samples containing 30–60 μg of lipid were transmethylated for 3.5–4.5 h at 65–70 ° C in 6% H_2SO_4 in 99.9 mol% methanol which contained a small amount of recrystallized (from ethanol) hydroquinone. After extraction of the methyl esters and their dissolution in CS_2 [15], the samples were analyzed on 10% SP2330 in a 2 mm × 2 m silanized glass column at 170 ° C in a Packard 7400 series gas chromatograph with a flame ionization detector. Ester masses were quantitated with a Hewlett Packard 3390A integrator.

Results

Shapes of DSC endotherms

The endotherms from the gel to liquid-crystalline transitions of the dispersions of the dipolyenoic PC (Fig. 1) were substantially broader than those usually found for saturated PC, or for dimonoenoic PC. Under our operating conditions, the baseline widths of transitions of dipolyenoic PC were in the range of 38–44 Cdeg (Table I) in contrast to values in the range of 8–10 Cdeg for 18 : 0-18 : 0 PC and 18 : 1-18 : 1 PC (data not shown). Widths of the transitions of dipolyenoic PC at half the maximum excess heat capacity were difficult to measure accurately, especially since some transitions were bimodal (see below), but they extended over about 20 Cdeg in comparison to about 3 Cdeg for those of the disaturated and dimonoenoic PC.

During cooling scans at 5 Cdeg/min, exotherms which were appropriate to the corresponding endotherms were observed for all the 18 : 2-18 : 2 PC samples except one and for most of the 18 : 3-18 : 3 PC samples (data not shown). For 20 : 4-20 : 4 PC samples it was very difficult to discern exotherms during the cooling scans. This may have been due as much to operational problems as to some special characteristic of 20 : 4-20 : 4 PC. Because it was necessary to operate the calorimeter at very high sensitivity, curving baselines resulted from which it was difficult to differentiate the very uncooperative transitions.

Our ability to discern some heating endotherms could be improved by scanning at 20 Cdeg/min and altering the chart speed of the recorder (Fig. 1). This process had its own attendant drawbacks such as difficulties in finding ending temperatures on very rapidly rising baseline curves or discerning baselines for transitions which occurred in the 'valleys' of some traces (Fig. 1). Given the number of different specimens we have analyzed, however, and the large number of scans performed on each sample, we are confident, that the calorimetric data given in Table I are reliable estimates of the properties of these lipids.

A number of the samples displayed endotherms which were bimodal or which had shoulders, often on the high temperature side. Almost all samples of 20 : 4-20 : 4 PC showed this tendency, as did some of the 18 : 3-18 : 3 PC samples. Rapid cooling of the sample (up to 160 Cdeg/min) prior to the heating scan induced bimodality in the 18 : 2-18 : 2 PC samples. In the more unsaturated samples where bimodal endotherms were apparent at the slower programming rates, the endotherms shapes were not further affected by the cooling rate (5 to 160 Cdeg/min). Generally samples of 20 : 4-20 : 4 PC were most oxidized and 18 : 2-18 : 2 PC least oxidized (Table I), so that oxidation might be suspected as contributing to this behavior. However, we have not seen a systematic correlation between oxidation and the appearance of bimodal endotherms.

DSC and analytical data

The main features of the scanning calorimetric data on the dipolyenoic PC are summarized in Table I. The transitions, which extended over

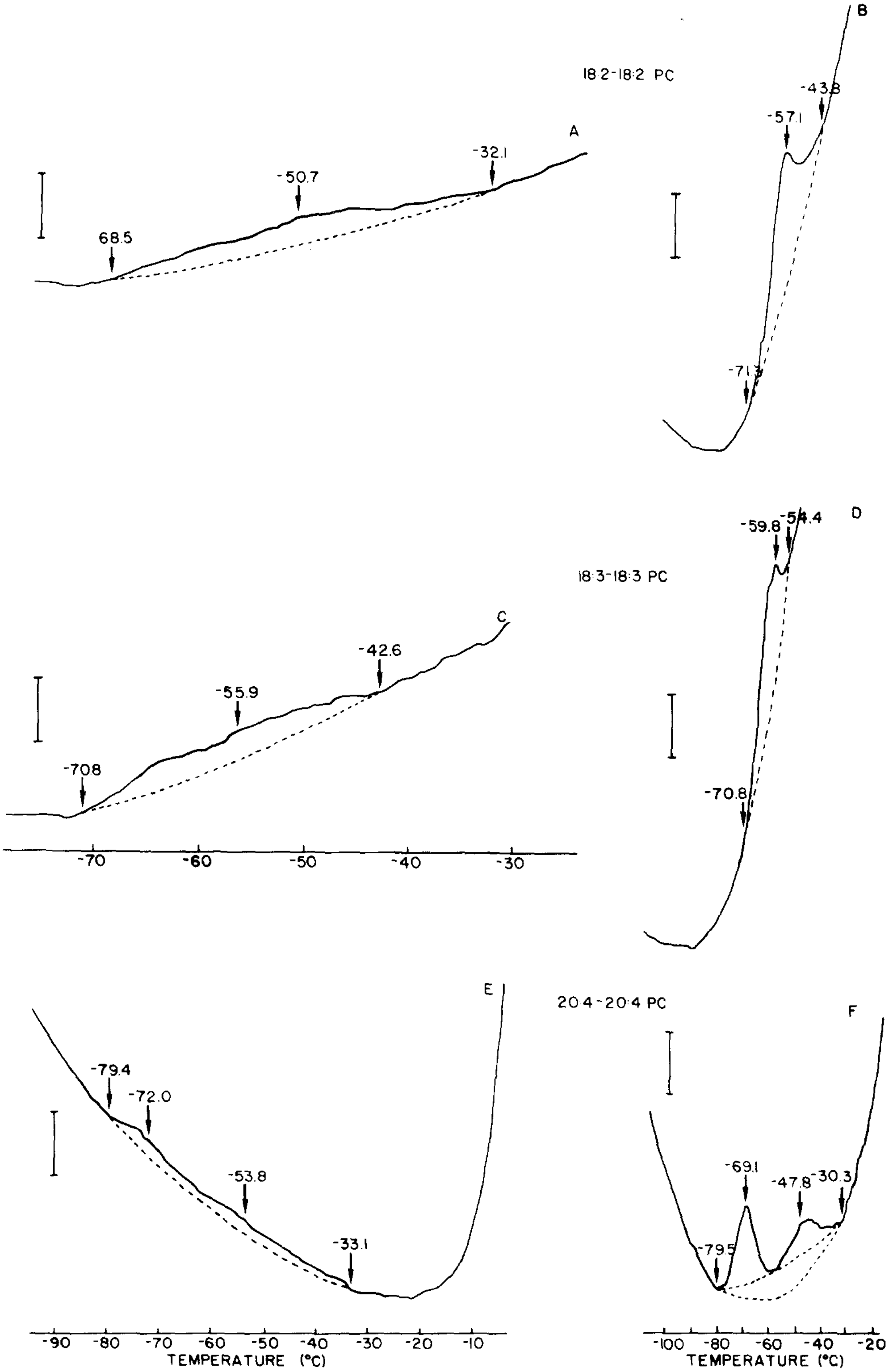

Fig. 1. Differential scanning calorimetric heating thermograms of dispersions of homoacid dipolyenoic phosphatidylcholines obtained under different scan rates in the calorimeter. Scans A, C, and E were at 5 Cdeg/min and B, D, and F were at 20 Cdeg/min. The bars on the left-hand side represent 0.6 mcal/Cdeg and on the right-hand site, 0.15 mcal/Cdeg.

about 40 Cdeg, started between −77 and −71°C, and the onset temperatures decreased slightly with increasing unsaturation. The T_{max} of the three lipids decreased from −52°C for 18:2-18:2 PC to −70°C for 20:4-20:4 PC, but the latter lipid had bimodal transitions with the second T_{max} near −51°C. The enthalpy changes associated with the transitions decreased slightly as the number of double bonds per chain increased. The enthalpy changes associated with the transitions of the three polyenoic lipids varied from 1 to 2 kcal · mol^{-1}.

Fig. 2 shows the thin-layer chromatograms of samples of the dipolyenoic PC after they had been extracted from the dispersions in the DSC pans. Visual inspection of the chromatograms showed that a variable amount of oxidative and hydrolytic

TABLE I

DSC AND ANALYTICAL DATA FOR DISPERSIONS OF DIPOLYENOIC PC

Lipid	Sample	DSC data							Analytical data [a]						
		Scan rate (Cdeg/min)	T_0 (°C)	T_{max} (°C)	T_f (°C)	ΔT_{base} [b] (Cdeg)	$\Delta T_{1/2}$ (Cdeg)	ΔH (kcal·mol^{-1})	Thin-layer chromatography (% of recovered phosphorus)					Ultraviolet spectra	
									PC	oxid. prod. [c]	lyso PC	origin	other	A^{230}_{mol}	A^{270}_{mol}
18:2-18:2 PC	A	5	−70.2	−55.2	−38.2	32	15	2	97.4	–	0.3	1.7	0.5	1980	462
		20	−70.7	−51.7	−31.7	39	21	3							
	B	5	−70.7	−50.4	−33.1	38	26	2	99.7	–	0.3	–	–	2640	477
		20	−71.6	−56.9	−44.8	27	14	2							
	C	5	−69.2	−51.0	−35.3	34	18	1	99.0	0.2	0.4	0.2	–	1830	370
	D	5	−73.2	−55.7	−35.0	38	12	2	99.6	–	0.2	0.2	–	1420	423
		20	−71.9	−53.2	−28.4	44	17	3							
	E	5	−70.6	−53.1	−31.5	39	23	2	95.7	–	0.2	–	4.1	6550	926
	$\bar{x}$ [d]		−70.9	−52.4	−33.3	38	20	2							
18:3-18:3 PC	A	5	−71.9	−52.4	−35.3	37	25	1	94.8	3.6	1.6	0.1	0.1	3910	1380
	B	5	−72.8	−64.2	−33.2	40	26	1	93.9	3.3	1.4	0.6	0.7	3450	884
	C	20	−71.6	−60.6	−30.4	41	27	2	93.2	1.3	0.8	1.4	3.2	3350	1090
	D	20	−74.4	−61.9	−19.2	55	n.d. [e]	1	87.8	10.4	–	1.3	0.5	3030	881
	E	5	−72.2	−63.8	−42.3	30	19	1	85.8	9.2	2.2	3.0	–	2890	995
	$\bar{x}$ [f]		−73.1	−60.0	−29.6	44		1							
20:4-20:4 PC	A	5	−74.4	−68.7	−37.5	37	n.d. [e]	1	87.6	6.6	1.5	1.9	2.4	4800	2540
	B	5	−69.1	−65.8	−21.9	47	n.d.	1	24.8	22.5	13.6	19.4	19.5	11600	6320
	C	5	−79.3	−71.9	−33.0	46	n.d.	1	86.0	6.4	4.6	2.2	0.7	3810	1580
		20	−79.8	−69.4 [−48.0]	−30.5	49	n.d.	2							
	D	20	−79.3	−63.8	−51.1	28	n.d.	– [h]	70.1	14.9	5.6	8.2	1.2	4320	1860
	E	5	−69.9	– [i]	−21.2	49	n.d.	– [h]	62.1	16.4	4.0	11.8	5.6	6960	2670
		20	−76.9	– [i]	−18.2	60	n.d.	– [h]							
	$\bar{x}$ [j]		−76.9	−70.3 [−51.1] [(2.6)]	−35.3	42	n.d.	1							

[a] Analytical data obtained on the samples after calorimetric analyses.

[b] ΔT_{base}, total width of transition ($T_f - T_o$).

[c] Oxid. prod., materials which chromatographed between PC and lyso PC which are likely products of oxidation [16,23].

[d] $\bar{x}$ for A at 20 Cdeg/min and B to E at 5 Cdeg/min.

[e] n.d., not determined because of the presence of bimodal peaks.

[f] $\bar{x}$ for A, B and E at 5 Cdeg/min and C and D at 20 Cdeg/min. If only samples A to C were considered the values were $T_o = -72.1$; $T_{max} = -59.1$; $T_f = -33.0$; $\Delta T_{base} = 39$; $\Delta T_{1/2} = 26$; $\Delta H = 1$.

[g] The values in square brackets are the higher T_{max} seen in bimodal transitions.

[h] Values not determined.

[i] Transition so broad that determination of T_{max} is subject to high error.

[j] Includes samples A and C at 5 Cdeg/min only.

Fig. 2. Thin-layer chromatographic plates of the dipolyenoic phosphatidylcholines obtained after calorimetric analyses. This figure is a composite of lanes from chromatograms run at different times under different conditions of humidity and plate activation. The major spot of all samples had the mobility of PC as judged from the mobility of a PC standard in an adjacent lane. The labelling corresponds to that used in Table I.

breakdown of the samples had occurred. Quantitative data on the state of the various samples after calorimetry are given in Table I. With increasing unsaturation there was also greater oxidation as indicated by the ultraviolet spectra and the thin-layer analyses. Fatty acid analyses of the samples after DSC showed that the only observable fatty acid methyl ester was the one appropriate to the lipid under investigation.

Discussion

Previous work on the effect of oxidation on the transition of 18:0-18:2 PC, suggests that the large widths of the transitions of the dipolyenoic PC are not due only to the presence of oxidation products in these lipids [16]. The amount of oxidation products running just slower than PC itself, the molar extinction coefficients and the amount of lysoPC present in the most oxidized samples which were included in the average data in Table I (Sample E of 18:3-18:3 PC and Samples A and C of 20:4-20:4 PC) suggested that the samples considered in the averages of the DSC data had undergone oxidation equivalent to 2.5 h or less of ultraviolet light exposure. This amount of oxidation would result in a maximum of a 6 Cdeg rise in $T_{\max}$ and a reduction in ΔH of up to 50% at worst [16]. Samples which had undergone very little oxidation also had broad transitions with low enthalpy changes. Analysis of variance showed that there was no statistically significant difference between the widths of the transitions of the three lipids.

The large temperature ranges indicate that these transitions occur with less intermolecular cooperation than do those of disaturated and dimonoenoic PC, assuming that they undergo two-state transitions [17]. It is expected that the transitions observed are associated with melting of chains in an extended bilayer array. Previous studies on the properties of these polyunsaturated PC employing ^{31}P nuclear magnetic resonance spectroscopy indicated that these lipids do form bilayers which are in the liquid-crystalline state at temperatures above 0°C [18]. Membranes composed of dipolyenoic PC may be quite disordered in the gel state in comparison to those made from more saturated phospholipids. The large number of double bonds would reduce chain rotations in the liquid-crystal state in comparison to lipids with fewer double bonds. Both factors would be expected to lead to

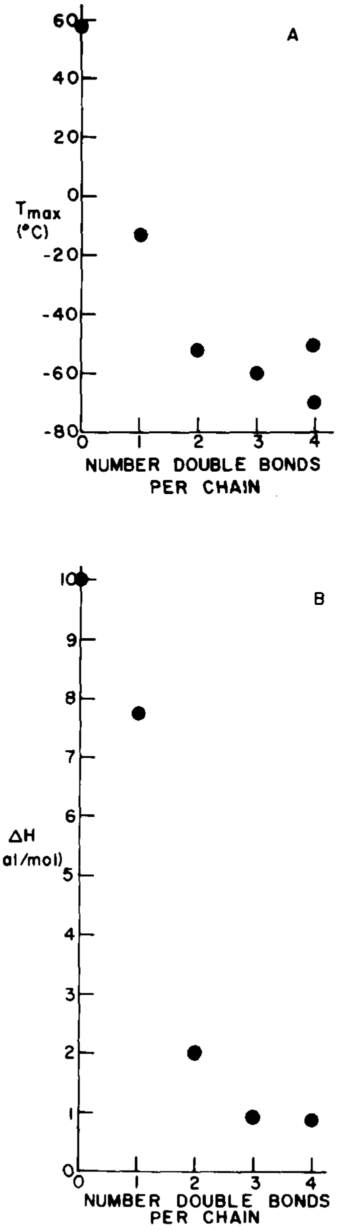

Fig. 3. T_{max} and ΔH for the series of homoacid PC.

low values of ΔH and low cooperativity for the gel to liquid-crystalline transition of the dipolyenoic lipids.

The possibility exists that the transitions of the dipolyenoic PC are not simple two-state transitions [9]. Perhaps these lipids assume one or more intermediate states in the temperature ranges of the calorimetrically-observed transitions. The tendency for the transitions to be bimodal is not inconsistent with this possibility. The observation that some 18:2-18:2 PC samples showed bimodality of the endotherms when they were heated after rapid cooling suggests that there may be some kinetic hindrance to the transformation of putative intermediate states. Unfortunately we could not adjust the operation of the calorimeter sufficiently to be able to study the shapes of the exotherms during rapid cooling scans.

Fig. 3 shows the transition temperatures and enthalpy changes for a series of homoacid (single acid) PC containing from zero to four double bonds per chain. The patterns are reminiscent of those for two series of heteroacid PC that each contained a saturated chain and an unsaturated chain with increasing numbers of double bonds [9–11]. For both series of heteroacid PC and for the series of homoacid PC described here, the introduction of the first double bond per chain caused the greatest reduction in $T_{G\text{-}LC}$, the second double bond per chain caused about half the reduction as did the first, and additional double bonds per chain caused only small changes in comparison to the first two. If each double bond acted independently to reduce $T_{G\text{-}LC}$ by an amount equivalent to that found by Barton and Gunstone [7] for double bonds at various positions along the chains, the predicted $T_{G\text{-}LC}$ would be much lower than the observed values. The T_{max} of both components of the endotherms of 20:4-20:4 PC are shown in Fig. 3. In cases where bimodal transitions were observed for the other two PC, the T_{max} of the upper component corresponded to the upper one always seen with 20:4-20:4 PC.

Fig. 3B shows the enthalpy changes observed for these transitions. The pattern of enthalpy changes is similar to the one for T_{max}. Double bonds in excess of two per chain caused only small changes in ΔH in both the dipolyenoic PC and in the heteroacid PC [9–11]. The similar patterns for transition temperatures and enthalpy changes for all three series of PC (Refs. 9–11; and this work) reinforce the suggestion that the transitions are governed by a balance between the perturbing effects of the double bonds on the area per molecule in the gel and the restricted rotational freedom of polyunsaturated chains in the liquid crystal [9].

The large number of double bonds in these lipids could lead to large volumes for the chains of the lipids in the hydrocarbon core of the membrane, and this could influence interactions between these phospholipids and other components of membranes. In the disc membranes of retinal rods, for example, dipolyenoic phospholipids might provide a hospitable environment for proteins either through direct contact with the proteins, or indirectly by the way in which they influence the packing properties of the lipids in the bilayer. There are dipolyenoic lipids of other classes, for example, phosphatidylethanolamine, in the disc membranes of retinal rods [1–3]. Dipolyenoic phosphatidylethanolamines might have very wide conical bases and a high tendency to form hexagonal phase, a factor which could enhance any dynamic transformations that might be associated with pumping in the membrane.

The increase in osmotic fragility of erythrocytes caused by enrichment with 18:2-18:2 PC [4] might have been caused by a 'loosening' of the normal lipid packing in the membrane. Dipolyenoic PC and phosphatidylethanolamines, while present in very small amounts, are very rapidly metabolized molecular species in rat testes [19]. Perhaps a 'loose' packing in the region of the dipolyenoic lipid enhances its accessability to metabolic enzymes.

The results of this work and of other studies [9–11,20,21] indicate that the continued addition of double bonds to lipid chains does not result in the continued decrease of $T_{\text{G-LC}}$ or chain order, nor in a continual increase in chain mobility. Thus in adaptive strategies for the cell progressive addition of double bonds to one chain of a lipid may not continually enhance 'fluidity'. In contrast, altering the usual chain composition of most phospholipids, a pairing of a saturated and an unsaturated chain, to the combination of two unsaturated chains, especially polyenoic chains, may be a useful response to 'rigidifying' stress. Since dipolyenoic lipids do not appear to readily pack tightly in a bilayer they might loosen lipid packing quite effectively in a membrane.

Perhaps dipolyunsaturated lipids, and even heterochain lipids with one polyunsaturated chain, are useful in the homeostatic control of the monolayer intrinsic radius of curvature that has been suggested by Gruner [22] to be an important property of biological membranes.

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