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THERMAL AND ^{13}C-NMR STUDY OF THE DYNAMIC STRUCTURE OF 1-PALMITOYL-2-OLEYL-*sn*-GLYCERO-3-PHOSPHOCHOLINE AND 1-OLEYL-2-PALMITOYL-*sn*-GLYCERO-3-PHOSPHOCHOLINE IN AQUEOUS DISPERSIONS

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Mixed-acid monounsaturated phosphatidylcholines containing palmitate and oleate chains have been synthesized by phospholipase A_2 digestion of the appropriate single-acid phosphatidylcholine, followed by reacylation of the lysophosphatidylcholine with the desired fatty acid anhydride. The positional isomers 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine and 1-oleyl-2-palmitoyl-*sn*-glycero-3-phosphocholine have been thus obtained. The thermotropic behavior of these lipids dispersed in excess water has been studied by differential scanning calorimetry. Positional isomers of mixed-acid monounsaturated phosphatidylcholines are found to have different gel to liquid-crystalline transition temperatures and enthalpies. It was found that mixtures of 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine or 1,2-distearoyl-*sn*-glycero-3-phosphocholine exhibited inmiscibility in the phosphatidylcholine gel state. The dynamic structure of 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine and 1-oleyl-2-pamitoyl-*sn*-glycero-3-phosphocholine bilayers has been investigated by measuring the ^{13}C nuclear spin-lattice relaxation times of sonicated aqueous dispersions. No difference was found between the two systems, suggesting that above the thermal transition the presence of the unsaturated acyl group in the 1 or 2 position does not affect significantly the dynamic structure of the bilayer.

Introduction

There is considerable evidence for the occurrence of lipid bilayer regions within the mosaic structure of biological membranes [1]. To investigate the details of the dynamic organization of these bilayers, many workers have studied, by different physical techniques, sonicated lipid dispersions, as it is reasonable to consider that the intra- and intermolecular motions of lipid molecules in these bilayer model membranes can be assimilated to those present in bilayer regions of natural membranes. The techniques most frequently used with these aims have been spin-label ESR, nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC) and X-ray diffraction [2,3].

Aqueous dispersions of phosphatidylcholines containing a single type of fatty acyl residue have been studied extensively as models for biological membranes. For example, measurements of ^{1}H and ^{13}C spin-lattice relaxation times (T_1) of dipalmitoylphosphatidylcholine [4] and dioleylphosphatidylcholine [5] in sonicated aqueous

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suspensions have shown that detailed information about the molecular motion of the lipids can be obtained. Single species of mixed-acid phosphatidylcholines have been much less studied. For systems with saturated mixed-acid phosphatidylcholines, it has been shown that an intramolecular structural change in the position of acyl chains has a significant effect on the thermal phase transition of the phospholipid [6,7].

Saturated and unsaturated mixed-acid phosphatidylcholines are the major components of naturally occurring phosphatidylcholines. Although thermal and NMR data are available on dispersions of mixtures of single-acid phosphatidylcholines containing saturated or unsaturated chains [8], no systematic study of dispersions of molecular species with mixed chains has been carried out previously. Here we report a thermal and NMR spectroscopy study of the two possible phosphatidylcholines in which palmitoyl and oleyl chains are bound in definite positions.

From T_1 ^{13}C-NMR data, we observe no significant differences between the dynamic properties of the studied isomers above the phase transition temperature but find that the gel to liquid-crystalline phase transition is determined by the distribution of the two acyl chains between the *sn*-1 and *sn*-2 position of the glycerol backbone.

Materials and Methods

Synthesis of mixed-chain phosphatidylcholines. Briefly, the method employed for the synthesis of mixed-chain phosphatidylcholines was a follows. The starting substrate is a like-chain phosphatidylcholine in which the fatty acyl chain is the one desired to be in the 1-acyl position of the product mixed-chain phosphatidylcholine.

These phosphatidylcholines were synthesized by rapidly stirring a mixture of *sn*-glycero-3-phosphocholine (GPC), with the desired fatty acid anhydride in the presence of the corresponding tetraethylammonium salt. The resulting like-chain phosphatidylcholines were purified by elution from silicic acid employing chloroform/methanol. The 2-acyl fatty acid of this substrate was removed by enzymatic hydrolysis with phospholipase A_2, and the resulting 1-acyllysophosphatidylcholine was purified by crystallization from diethyl ether. The lysophosphatidylcholine was then reacylated with the anhydride of the fatty acid desired to be in the 2-acyl position of the mixed-chain product.

Isolation of glycerophosphocholine (GPC). A mixture of 1 g of the adduct GPC-Cl_2Cd (Sigma) dissolved in 10 ml distilled water/2 g Amberlite IR 45/2.5 g Amberlite IRC 50 was kept at room temperature for 2 h under mechanical stirring, and filtered. The filtrate was tested for the presence of Cd^{2+} and Cl^- with Na_2SO_4 and $AgNO_3$, respectively. When either ion was present, the ion-exchange step was repeated. Once the solution was free of ions, it was lyophilized and immediately used for acylation as described below.

Synthesis of fatty acid anhydrides. Palmitic, stearic and oleic anhydride were prepared by using dicyclohexylcarbodiimide [9].

Synthesis of tetraethylammonium salts. Tetraethylammonium salts of the palmitic, stearic and oleic acids were obtained by adding the required amount of an alcoholic solution of the base to an alcoholic solution of the fatty acid.

Acylation of free glycerophosphocholine. A mixture of 2.25 mmol free GPC and 5.1 mmol of the corresponding tetraethylammonium salt, in 2 ml dry, ethanol-free chloroform was evaporated to dryness under reduced pressure. Next, 6.4 mmol of the correspondent fatty acid anhydride in 5 ml of dry, ethanol-free chloroform and 20 ml glass beads (1 mm diameter) were added. The flask was put on a rotary evaporator under reduced pressure at 60°C for 70 h, while being mechanically rotated slowly to stir the mixture gently.

Thin-layer chromatography of the mixture gave a clear spot of phosphatidylcholine and a faint spot of lysoderivate when chloroform/methanol/water (65:25:4, v/v) was used as the development system. Phosphatidylcholine thus obtained was purified as previously described [10].

Isolation of lysophosphatidylcholine. 100 mg 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) or 1,2-dioleyl-*sn*-glycero-3-phosphocholine (DOPC) prepared as described above were subjected to phospholipase A_2 degradation. Lysoderivate was prepared as previously described [10].

Acylation of lysophosphatidylcholine. The acylation of lysophosphatidylcholine was carried out in the same way as the acylation of free GPC described above, except that in this case the fol-

lowing molar ratio of reactants was used: lysophosphatidylcholine/salt/anhydride (1:2:4). The 'mixed-chain' phosphatidylcholine thus obtained was purified as previously described [10].

Preparation of vesicles. Vesicles were obtained by sonication of coarse dispersions of lipid in ^2H_2O using an MSE sonicator, 150 W, fitted with a microtip [10]. After sonication, no degradation of the sample was detected by thin-layer chromatography.

Differential scanning calorimetry. Calorimetric measurements were performed using a Perkin-Elmer DSC-2 apparatus provided with an Intracooler II unit. Heating and cooling scans were run at scanning rate of 10 K/min at a sensitivity range of 0.2–1.0 mcal/s. All samples were heated and cooled at least twice from −20°C to 40°C to insure that excess water was present and that the transitions were reproducible.

The temperature scale was calibrated using the transition temperatures of C_{11}, C_{13}, C_{15} and C_{19} paraffins, lauric and stearic acids, indium and galium. The indium transition was also used to calibrate the enthalpy per unit area. Weighing was done on a Cahn electrobalance, model G.

Magnetic resonance measurements. The ^{13}C-NMR spectra were obtained in the FT (Fourier transform) mode (50000 scans) on a Varian XL-100-15 spectrometer at 25.16 MHz with proton noise decoupling and a deuterium internal lock. The spinning sample tube had an external diameter of 12 mm. The T_1 relaxation times were measured by the FIRFT (Fast Inversion Recovery Fourier Transform) method [11].

Results

Synthesis and characterization of 1-palmitoyl-2-oleyl -sn-glycero-3-phosphocholine (POPC) and 1-oleyl-2-palmitoyl-sn-glycero-3-phosphocholine (OPPC)

Yields for the different steps in the synthesis of POPC and OPPC described above were as follows: 85% for the preparation of free GPC, 50% for the preparation of single-acid phosphatidylcholines and 60–65% for the preparation of mixed-acid phosphatidylcholines from single-acid phosphatidylcholine. These values are satisfactory and considerably greater than those obtained by Keough and Davis [7] in similar experiments. Moreover, no excessive isomerization occurred during the preparation of these molecules (Table I). OPPC has a perfect distribution of its acyl chains, whereas POPC shows a degree of isomerization of about 10%.

TABLE I

FATTY ACID ANALYSIS OF THE SYNTHETIC PHOSPHATIDYLCHOLINES POPC AND OPPC AFTER PHOSPHOLIPASE A_2 TREATMENT

Synthetic phosphatidylcholines POPC and OPPC were treated with phospholipase A_2 and the mixture was extracted with chloroform/methanol (2:1, v/v) and separated by thin-layer chromatography on Kieselgel H plates using chloroform/methanol/water (65:25:4, v/v) as developing system. Fatty acid methyl esters from free fatty acids and from lysophosphatidylcholines were prepared by the methanolysis procedure. Analysis of methyl esters was performed as previously described [14].

Phosphatidylcholine	Phospholipase A_2 treatment	Fatty acid analysis	
		16:0	18:1
POPC	1 position	94	6
	2 position	12	88
OPPC	1 position	–	100
	2 position	100	–

Differential scanning calorimetry

Measurement of the phase transitions of POPC and OPPC have been carried out by DSC. The DSC data on the melting behavior of these phosphatidylcholines are summarized in Table II together with those of the parent single-acid phosphatidylcholines (DPPC and DOPC). Δt is the interval between the beginning and the end of the transition in the isothermal baseline and $t_{1/2}$ represents the width of the transition at half-height.

DSC measurements were made on samples consisting of lipids dispersed in water containing 50% (v/v) ethylene glycol to prevent the freezing of water. Van Echteld has reported [12] that, depending on the lipid species, the presence of 50% (v/v) ethylene glycol may influence the thermotropic behavior of phospholipids. We have not observed such effects in the transition temperature of DPPC in assays in presence and absence of ethylene glycol, although we found a slight variation (13%)

TABLE II

THERMODYNAMIC DATA FOR THE CRYSTALLINE→LIQUID-CRYSTALLINE TRANSITION OF SYNTHETIC PHOSPHATIDYLCHOLINES

Samples were sealed in aluminium pans and examined in a Perkin-Elmer DSC-2 equipped with an Intracooler II unit. The scan speed was 10 K/min with a sensitivity range of 0.2–1.0 mcal/s. EtG, ethylene glycol.

	Transition			ΔH (kJ·mol^{-1})	ΔS (J·K^{-1}·mol^{-1})
	Range (Δt)(K)	t_t (K)	$t_{1/2}$ (K)		
DPPC/H_2O:EtG (1:1) [b]	40.0–47.0	41.5	1.5	44.1	140
DPPC/H_2O [b]	39.0–50.0	41.5	2.0	38.3	122
DPPC/H_2O [a]	32.0–44.0	41.0	1.75	41.6	132
POPC/H_2O:EtG (1:1) [b]	−16.0– 5.0	−4.5	4.5	26.9	100
POPC/H_2O:EtG (1:1) [a]	−15.0– 2.0	−4.6	4.5	26.2	98
OPPC/H_2O:EtG (1:1) [b]	−20.0–12.0	−8.7	9.0	15.3	58
DOPC/H_2O:EtG (1:1) [b]	−35.0–20.0	−23.4	4.5	15.7	63

[a] Sonicated dispersion.
[b] Multillamelar dispersion.

in the ΔH and ΔS values for the same molecule.

Phase diagrams of mixtures of POPC with either DPPC or 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), constructed from calorimetric transition curves, are shown in Fig. 1. The systems are far from ideal; consequently, monotectic behavior is observed. The initiation temperature remains constant over most of the concentration range for the unsaturated phosphatidylcholine.

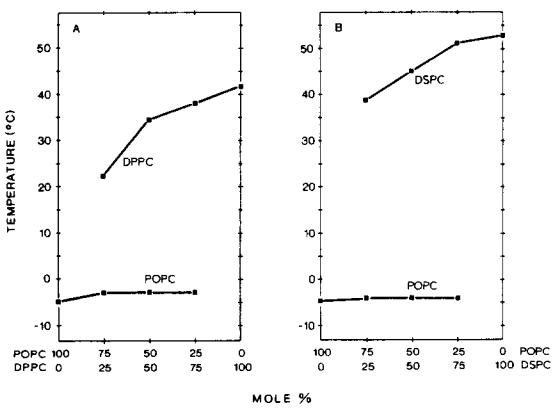

Fig. 1. Temperature composition diagrams for binary mixtures of synthetic phosphatidylcholines dispersed in excess water/ethylene glycol (1:1, v/v). (A) POPC-DPPC systems. (B) POPC-DSPC systems. The mixtures were prepared dissolving the two components in chloroform and then removing the solvent and dispersing the resultant mixture in water/ethylene glycol (1:1, v/v). The scan speed was 10 M K/min with a sensitivity range of 0.2–1.0 mcal/s. ■, Transition temperatures from DSC heating curves.

^{13}C-NMR measurements

The ^{13}C-NMR spectrum of POPC in deuterochloroform and that of the same phosphatidylcholine in a fully sonicated dispersion in deuterated water are shown in Fig. 2. The spectra of OPPC were similar.

The alkyl chain carbon signals were assigned by analogy with the spectra of fatty acids. Other resonances were assigned on the basis of the spectra of DPPC [4]. Table III shows the complete assignment of both spectra.

In the upfield region of the ^{13}C-NMR spectra of POPC in deuterochloroform (expansion in Fig. 2) distinct resonances are observed for the terminal methyl (C16(P) and C18(O)) (Signal a, Fig. 2) and for C2 (P and O), C3 (P and O), C14(P) and C16(O), C15(P) and C17(O), (Signals l, c, k and b), in addition to the main methylene envelope containing the remaining carbons of both chains. Although six sharp maxima can be discerned clearly as components of the envelope, no assignment of the different signals has been established yet. The chemical shift value in Table III corresponds to the highest-intensity signal. In comparison with DPPC spectra, a new resonance appears at 13.17 ppm out of the methylene en-

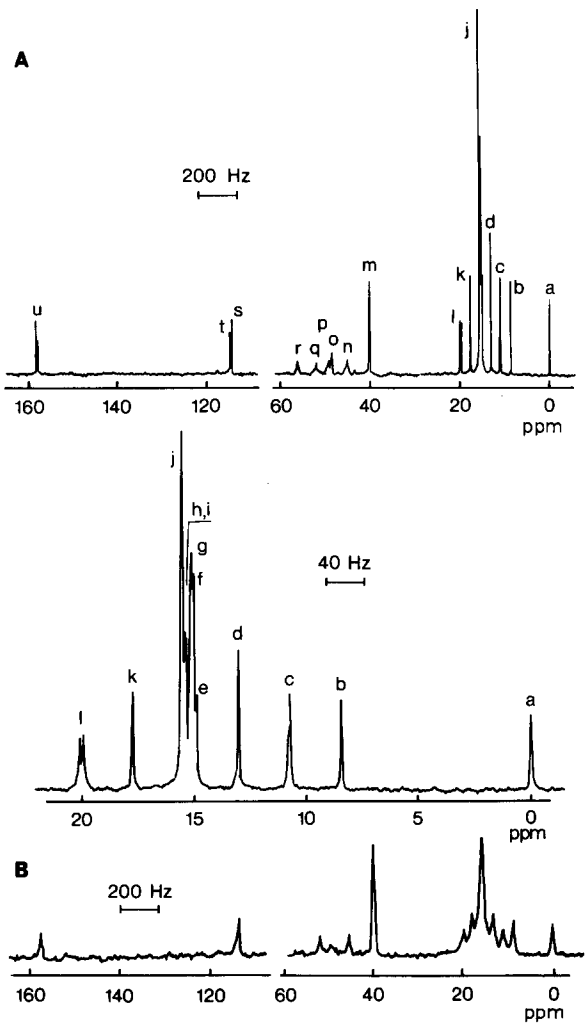

Fig. 2. ^{13}C-NMR spectra of 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC). (A) In deuterochloroform at 40°C. The insert shows an expanded upfield region. (B) Sonicated aqueous dispersions at 28°C. The spectra were obtained operating at 25.16 MHz. The solvent deuterium signal was used as internal lock. Standard conditions were: 90 μs pulse width, 5000 Hz sweep width, 8 K points and 50000 scans. Resonances have been labelled with small letters (a–u) for discussion purposes (see Table II).

velope (signal d), which is assigned to the carbon nuclei close to the olefinic carbons, e.g., carbons 8 and 11 in the oleyl chain.

All six glycerol and choline carbon nuclei are well resolved and assigned. Two resonances are observed for carbons 1 and 2 for POPC in deuterochloroform, corresponding to the α- and β-fatty acid chain, but these resonances are not resolved in the broadened spectra from the POPC bilayer in deuterated water. Olefinic carbons in positions 9 and 10 in the oleic acyl chain are also resolved in the ^{13}C-NMR deuterochloroform spectra but they appear as a single signal in the deuterated water systems.

T_1 measurements were made on most of the observable nuclei of the molecule in the upfield region of the spectra corresponding to POPC and OPPC dispersions in deuterated water, and the results of such measurements at 28°C are shown in Table III.

It should be noted that the T_1 values for all the chain carbons (except C8 and C11 of the oleic chain) are the average values for the two chains, and that the methylene envelope is an unspecified average of the component resonances calculated from the exponential decay of the composite resonances as a function of τ.

Discussion

Values of transition temperatures for water dispersions of POPC and OPPC have been observed at −4.5 and −8.7°C, respectively. The difference between these two values is smaller than that observed by Keough and Davies [7] in saturated mixed-acid phosphatidylcholines but it is in reasonable agreement with their observations. These authors obtained a difference of 8.1°C between the gel to liquid-crystalline transitions of pure palmitoylmyristoylphosphatidylcholine (27.2°C) and myristoylpalmitoylphosphatidylcholine (35.3°C).

Our results with mixed-acid phosphatidylcholines containing palmitic and oleic acids are in contrast with those of Phillips et al. [13], who observed similar monolayer and thermal behavior with a sample of 1-oleyl-2-stearoylphosphatidylcholine and its positional isomer, indicating that the position of the chains within the phospholipid molecule does not affect their packing behavior.

In addition to the differences in the t_t and $t_{1/2}$ values, POPC and OPPC show a strong discrepancy in their enthalpy and entropy values. OPPC exhibits a much smaller value of ΔH, which suggests that the phosphatidylcholine with an unsaturated acyl group at position 1 presents a more

TABLE III

CHEMICAL SHIFT AND ^{13}C-NMR T_1 RELAXATION TIMES OF 1-PALMITOYL-2-OLEYL-*sn*-GLYCERO-3-PHOSPHOCHOLINE (POPC) AND 1-OLEYL-2-PALMITOYL-*sn*-GLYCERO-3-PHOSPHOCHOLINE (OPPC)

^{13}C-NMR spectra were obtained in the FT mode on a Varian XL-100-15 spectrometer at 25.16 MHz with proton noise decoupling and a deuterium internal lock. Standard conditions were: 100 μs pulse width, 5000 Hz sweep width, 8K points and 50000 scans. Sonicated vesicles were 200 mM and organic solutions 300 mM. Palmitic (P) and oleic (O) acid carbons are numbered from the carboxyl (1) to the terminal methyl group (16 or 18). Chemical shifts are downfield from CH_3. The T_1 relaxation times were measured by the FIRFT method [11]. Small letter labelling of resonances corresponds to that shown in Fig. 2.

Resonance (Fig. 2)	Carbon	POPC			OPPC	
		Chemical shift		Relaxation time T_1(s) (^2H_2O (28°C))	Chemical shift (^2H_2O)	Relaxation time T_1(s) (^2H_2O (28°C))
		C^2HCl_3	^2H_2O			
m	$N(CH_3)_3$	40.32	40.16	0.38±0.02	40.10	0.35±0.08
q	CH_2N	52.35	52.25		52.14	
n	CH_2OP (chol.)	45.37	45.61		45.28	
p	CH_2OP (glyc.)	49.49	50.17		49.68	
o	CH_2O (glyc.)	48.99	49.50		48.87	
r	CHO (glyc.)	56.51	56.72		56.75	
u	C1 (P+O)	159.26	159.49		159.19	
l	C2 (P+O)	20.19	20.15	0.10±0.01	20.14	0.11±0.02
c	C3 (P+O)	10.89	11.16	0.17±0.03	11.04	0.19±0.04
e,f,g, h,i,j	$(CH_2)_n$	15.38	15.97	0.35±0.03	15.73	0.32±0.04
s	C9 (O)	115.61	115.30		115.32	
t	C10 (O)	115.95				
d	C8,11 (O)	13.17	13.41	0.36±0.03	13.26	0.27±0.06
k	C14(P)+C16(O)	17.89	18.11	0.65±0.07	18.25	0.59±0.09
b	C15(P)+C17(O)	8.60	8.80	1.1 ±0.1	8.81	1.0 ±0.1
a	C16(P)+C18(O)	0.00	0.00	2.4 ±0.3	0.00	2.1 ±0.3

disordered gel structure than that with the unsaturation in position 2, at least in this particular case. This observation, which can have an important physiological significance, is in good agreement with the results obtained in our laboratory with chick embryo phosphatidylcholines of different organs [14]. Brain phosphatidylcholines show a gel to liquid-crystalline transition with very low ΔH and ΔS values, in agreement with the unusual high content of OPPC of brain (15%).

POPC vesicles prepared by sonication exhibit a phase transition behavior identical to that of multilamellar preparations (Table II). Our results are in contrast with those of Suurkuusk et al. [15] and Kantor et al. [16], but are in agreement with De Kruijff et al. [17], who have reported that the calorimetric characteristics of sonicated and unsonicated liposomes are indistinguishable.

In general, when two phosphatidylcholines with saturated hydrocarbon acyl chains, differing only by two carbons in the chain lengths, are dissolved in an organic solvent, dried, and then dispersed in water they seem to form a nearly ideal mixture with a homogeneous gel phase. In such mixtures a single broad and endothermic gel to liquid-crystal transition is observed. In the case of saturated phosphatidylcholine molecules with greater differences in chain length or having one unsaturated acyl chain, there are two transitions and a heterogeneous gel phase. In this situation, as crystallization starts, phosphatidylcholine molecules migrate within a given bilayer to form separate clusters of the two components, which could lead to regional permeability differences along the plane of the bilayer.

The systems POPC-DPPC and POPC-DSPC

show monotectic behavior. The presence of the double bound must then prevent cocrystallization so that, as the systems are cooled, phosphatidylcholine molecules migrate within the bilayers, forming crystalline regions corresponding to the two components.

High resolution NMR data of POPC and OPPC at 28°C give information on dynamic bilayer properties well above the thermal transition. T_1 ^{13}C relaxation times for the fatty acid chains increase steeply along the alkyl chains away from the glycerol backbone toward the terminal methyl group, showing again that the center of the bilayer is more mobile than the polar surface, since uniform T_1 values would be expected for the methylene carbons if the chain motion were determined by the tumbling of the molecule as a whole. Nevertheless, no significant differences were found between ^{13}C T_1 for the POPC and OPPC systems. It seems that above the thermal transition, the presence of a double bond in a chain attached to the 1 or 2 position does not affect significantly the motion of the fatty acyl chains present in an isomer (Table III).

In conclusion, we observe that the dynamic properties of the studied isomers POPC and OPPC above the phase transition temperature are similar; however, we find that the gel to liquid-crystalline phase transition is determined by the distribution of the two acyl chains between the *sn*-1 and *sn*-2 position of the glycerol backbone.

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