Cubic liquid crystalline phase with phosphatidyl-ethanolamine from *Bacillus megaterium* containing branched acyl chains

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Phosphatidylethanolamine (PE) was isolated from membranes of *Bacillus megaterium*. The organism was grown at 20°C and 55°C. The phase equilibria in PE/water systems were studied by ^{2}H and ^{31}P nuclear magnetic resonance, and by polarized light microscopy. PE isolated from *B. megaterium* grown at 20°C forms a lamellar liquid crystalline phase at the growth temperature, and at low water contents a cubic liquid crystalline phase at 58°C. The ratio iso/*ante*-iso acyl chains was 0.3 in this lipid. PE isolated from this organism grown at 55°C forms only a lamellar liquid crystalline phase up to at least 65°C. In this lipid the ratio iso/*ante*-iso acyl chains was 3.2.

Lipid polymorphism *Cubic phase* *Phosphatidylethanolamine* *Branched acyl chain* Bacillus megaterium *NMR*

1. INTRODUCTION

A large number of procaryotic and eucaryotic organisms, respond to variations in the environmental temperature by changing the acyl chain composition of the membrane lipids [1–6]. The most emphasized regulation mechanism is by far the increase in acyl chain unsaturation when the temperature is decreased. Alternative regulation mechanisms are expected to operate in organisms synthesizing small amounts of unsaturated fatty acids and large amounts of branched-chain fatty acids. This was found in investigations of the acyl chain composition of *Bacillus megaterium* membranes [7]: the ratio iso/*ante*-iso acyl chains is decreased and the hydrocarbon chain length is reduced when the growth temperature is lowered.

The basic structure of all biological membranes is the lipid bilayer matrix. In spite of this, most membranes contain at least one lipid that forms a non-lamellar phase structure with water. Phosphatidylethanolamine (PE) [8,9], phosphatidylserine [10], monogalactosyldiglyceride [11] and monoglucosyldiglyceride [12] can form a reversed hexagonal (H_{II}) liquid crystalline phase in equilibrium with a free water phase. The transition from a lamellar to a non-lamellar phase can be triggered by several environmental factors such as temperature, pH and concentration of ions [8–10, 13,14]. Even if a transition to a non-lamellar phase does not occur, the temperature affects the packing of lipid molecules [3]. An organism is consequently often forced to regulate the membrane lipid composition in response to changes in growth temperature in order to avoid disturbances in membrane functions [3].

Here we show that PE isolated from *B. megaterium* grown at 20°C forms a lamellar liquid crystalline phase at the growth temperature, and at low water contents an isotropic (cubic) liquid crystalline phase at 58°C. This lipid has a low ratio iso/*ante*-iso acyl chains. PE isolated from this organism grown at 55°C forms only a lamellar liquid crystalline phase up to at least 65°C. The ratio iso/*ante*-iso acyl chains is 10-times higher for this lipid.

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2. EXPERIMENTAL

A facultatively thermophilic strain (Ft R32) and an obligately thermophilic strain (Ot 32) of *Bacillus megaterium* were grown at 20°C and 55°C, respectively, in tryptone starch broth [7]. The temperature ranges of the strains were tested before the culture vessel was inoculated. Cells were harvested by centrifugation for 20 min at 8000 × *g* and cell membranes were prepared as in [7]. Lipids were extracted from the dried membranes with chloroform–methanol (2:1, v/v), methanol–chloroform (2:1, v/v) and methanol. During the extractions the membrane suspensions were sonicated in a Bransonic bath for 30 min. The membrane residues were collected on a filter paper and non-lipid contaminants in the extract were removed by passage through a Sephadex® G25 Fine (Pharmacia) column [15]. PE was isolated by thin-layer chromatography (TLC) on Silica gel 60H (Merck) [7]. The purity of the lipid preparations was >95% as judged by TLC. The lipids were stored at concentrated solutions in chloroform–methanol (2:1, v/v) at −70°C. The acyl chain composition of the lipids was determined by gas–liquid chromatography (GLC) on a diethylene glycol succinate column operating at 160°C [7].

Samples for nuclear magnetic resonance (NMR) studies were prepared as in [16,17]. The heavy water concentrations were chosen both above and below the maximum hydration capacity of PE. ^{2}H NMR spectra were recorded at 15.35 MHz on a modified Varian XL-100-15, and analyzed as in [12,17,18]. ^{31}P NMR measurements were taken at 101.27 MHz on a Bruker WM-250 NMR spectrometer equipped with a superconducting magnet. To remove the dipolar interaction between the phosphorus and the protons, the latter were irradiated with an intense decoupling field. The samples were thermally equilibrated within 1°C for ⩾1 h before the spectra were taken. After the recording of the spectra the samples were analyzed for lipid degradation by TLC and GLC. No degradation products were found. The lipid samples were also studied between two crossed polarizers and by polarized light microscopy. Each phase shows a typical microscopic texture [19].

The translational diffusion coefficient of PE in the cubic phase was measured with the NMR diffusion method in [16].

3. RESULTS

The acyl chain composition of the two different PE preparations is shown in table 1. *Ante*-iso-C_{15} was the dominating acyl chain in PE isolated from Ft R32. The ratio iso/*ante*-iso was very low and the C_{15} chains made up nearly 85 mol% of the total amount of acyl chains. In Ot 32 the ratio iso/*ante*-iso was increased by 10-fold as compared to Ft R32 and the chain length parameter was ~8-times higher.

PE isolated from Ft R32 formed a lamellar liquid crystalline phase with water within 3–14 mol $^{2}H_2O$/mol lipid (table 2). The samples were optically birefringent, exhibited a fine mosaic microscopic texture [19], and showed a ^{2}H NMR quadrupole splitting (fig. 1d) [16,17]. ^{31}P NMR spectra exhibited chemical shielding anisotropy of

Table 1

Acyl chain composition in PE isolated from *Bacillus megaterium* strain Ft R32 grown at 20°C and strain Ot 32 grown at 55°C

Acyl chain	Ft R32 20°C	Ot 32 55°C
Iso-C_{14}	0.9	0.3
Normal-C_{14}	0.1	0.5
Iso-C_{15}	18.2	35.8
Ante-iso-C_{15}	65.3	4.7
Normal-C_{15}	–	0.3
Iso-C_{16}	0.9	6.6
Normal-C_{16}	0.2	3.7
Iso-C_{17}	0.6	28.5
Ante-iso-C_{17}	2.7	17.6
Normal-C_{17}	–	2.1
Unidentified acyl chains (C_{16}–C_{18})	11.2	–
Σ normal acyl chains	0.3	6.6
Σ branched acyl chains	88.5	93.4
Acyl chain ratios		
$\frac{\Sigma \text{ iso}}{\Sigma \textit{ ante}\text{-iso}}$	0.30	3.19
$\frac{\Sigma\ C_{16}\text{–}C_{18}}{\Sigma\ C_{14}\text{–}C_{15}}$	0.18	1.41

The values are given as mol%

the order of −40 ppm with a high field peak and a low field shoulder (fig. 1a), indicating a lamellar liquid crystalline phase [20,21]. The lamellar phase was stable up to 50°C with 3 mol ^2H_2O/mol lipid (table 2). Above this temperature the sample consisted of two liquid crystalline phases, one lamellar and one viscous isotropic (cubic) phase. This was evident from the ^{31}P and ^2H NMR spectra, both of which revealed an anisotropic part together with a central isotropic peak (fig. 1b,e) [16,17]. Ocular inspection of the sample between two crossed polarizers showed an anisotropic phase along with an optically isotropic phase. Above 57°C, a pure cubic liquid crystalline phase (one-phase system) was formed with 3 mol ^2H_2O/mol lipid (fig. 1c,f). The transition temperature for the lamellar phase

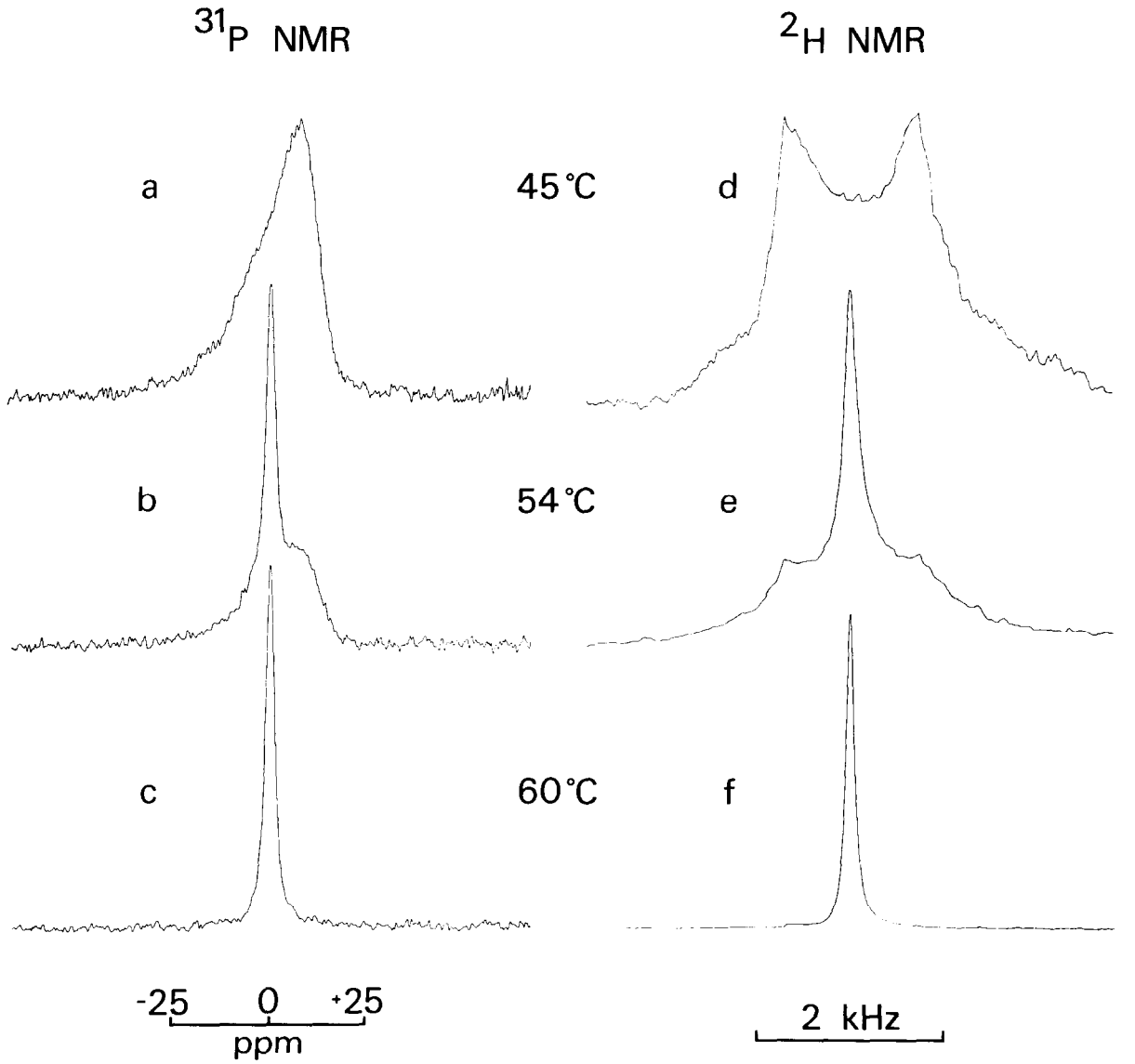

Fig. 1. ^{31}P NMR (a–c) and ^2H NMR (d–f) spectra of PE isolated from *Bacillus megaterium* strain Ft R32 grown at 20°C. The water content was 3 mol ^2H_2O/mol lipid: (a,d) lamellar liquid crystalline phase; (b,e) two-phase sample consisting of lamellar + cubic liquid crystalline phases; (c,f) cubic liquid crystalline phase.

increased by increasing the water content of the samples, and with 12.5 mol ^2H_2O/mol lipid the cubic phase began to appear at 63°C (table 2). A preliminary study with polarizing light microscopy of the sample with 14 mol ^2H_2O/mol lipid revealed that the transition between the lamellar and cubic phases began at ~70°C. A pure cubic phase formed at ~90°C, as shown by the dark microscopic background. Above 105°C a shiny angular texture, typical for hexagonal liquid crystalline phases [19], began to appear. The cubic phase gradually transformed into the hexagonal (probably H_{II}) phase between 105–115°C. When PE isolated from Ft R32 was mixed with >14 mol ^2H_2O/mol lipid, two-phase systems were formed, consisting of a lamellar phase and a free water phase. The lamellar phase was stable up to at least 65°C. The free water phase was evident from a central peak with very narrow line width in the ^2H NMR spectra.

Table 2

Quadrupole splittings (Δ^2H) and phases obtained for different PE/water mixtures

mol ^2H_2O/ mol lipid	Temp. (°C)	Δ^2H (kHz)	Phase(s)
3.0	26	1.6	Lamellar
	45	1.4	Lamellar
	50	1.4	Lamellar + cubic
	54	1.4	Lamellar + cubic
	58	–	Cubic
4.5	26	2.2	Lamellar
	47	1.8	Lamellar
	50	2.0	Lamellar + cubic
	57	–	Cubic
	64	–	Cubic
6.0	26	1.6	Lamellar
	53	2.0	Lamellar
	58	1.8	Lamellar + cubic
	65	–	Cubic
11.0	26	0.9	Lamellar
	55	1.1	Lamellar
	58	1.1	Lamellar + cubic
	60	1.2	Lamellar + cubic
	66	–	Cubic
12.5	26	0.8	Lamellar
	60	1.0	Lamellar
	63	1.1	Lamellar + cubic
14.0	26	0.8	Lamellar
	65	1.0	Lamellar
15.0	26	0.8	Lamellar + free water
	65	0.9	Lamellar + free water
19.0	26	0.6	Lamellar + free water
	65	0.7	Lamellar + free water

PE was isolated from *Bacillus megaterium* strain Ft R32 grown at 20°C

Table 3

Quadrupole splittings (Δ^2H) and phases obtained for different PE/water mixtures

Mol ^2H_2O/ mol lipid	Temp. (°C)	Δ^2H (kHz)	Phase(s)
3.0	26	1.4	Lamellar
	65	1.6	Lamellar
4.5	26	1.6	Lamellar
	47	2.6	Lamellar
	64	2.7	Lamellar
7.0	26	1.2	Lamellar
	62	2.2	Lamellar
8.5	26	1.1	lamellar + free water
	64	2.1	lamellar + free water
10.0	26	1.1	lamellar + free water
	65	2.1	lamellar + free water
11.5	26	1.1	lamellar + free water
	51	1.4	lamellar + free water
	65	1.7	lamellar + free water

PE was isolated from *Bacillus megaterium* strain Ot 32 grown at 55°C

PE isolated from Ot 32 formed a lamellar liquid-crystalline phase with water between 26–65°C, both below and above the maximum hydration capacity of the lipid (table 3). NMR spectra of the type shown in fig. 1a,d was obtained, and the samples were optically birefringent. A two-phase system consisting of a lamellar phase and a free water phase was achieved when the lipid samples contained >8 mol ^2H_2O/mol lipid.

In a preliminary investigation of the diffusion coefficient of PE in the cubic phase at 65°C, it was found that $D_L \approx 5 \times 10^{-12}$ m^2.s^{-1}. This value is of the same order of magnitude as the lateral diffusion coefficient observed for lipids in bilayers, strongly indicating that the cubic phase is bicontinuous and built up of continuous hydrocarbon regions [16,29].

4. DISCUSSION

It is shown that the phase equilibria in the

PE/water system are influenced by changes in the ratio iso/*ante*-iso acyl chains of the lipid molecules. The phase equilibria for PE also depend on the degree of unsaturation of straight acyl chains [20]. As demonstrated in [3,22] the geometry of the lipid molecules is of particular importance for determination of the lipid aggregate shape. Three quantities can be used to describe the shape of lipid molecules: the hydrocarbon chain volume (v); the hydrocarbon–water interfacial area (a); and the hydrocarbon chain length (l). When the packing parameter $v(a \cdot l)^{-1} = ½–1$ the bilayer is the preferred structure. A non-lamellar structure composed of long water rods embedded in a hydrocarbon matrix (water-in-oil aggregates) may form if the packing parameter exceeds unity. An increase in the degree of unsaturation of the acyl chains results in a higher value of $v(a \cdot l)^{-1}$ for a lipid molecule [3,22]. It can therefore be predicted that the most unsaturated PE's should form an H_{II} phase while the more saturated species should form a lamellar phase at a certain temperature. This has also been found experimentally [20].

Iso-acids have their branching point at the penultimate carbon atom while *ante*-iso acids have this point displaced one carbon atom towards the carboxyl group. *Ante*-iso acids can accordingly not be as closely packed as iso acids. This is supported by experimental observations:

(1) *Ante*-iso acids occupy a larger area than iso acids in a monolayer [23];
(2) *Ante*-iso acids have lower melting points than the corresponding iso acids [24].

Synthetic phsophatidylcholines containing *ante*-iso acyl chains have considerably lower gel to liquid crytalline transition temperatures than the corresponding iso derivatives [25,26]. When the ratio iso/*ante*-iso acyl chains is decreased the effective hydrophobic volume (v) of PE is probably increased. The packing parameter $v(a \cdot l)^{-1}$ assumes a higher value and PE isolated from strain Ft R32 can be postulated to have an increased tendency to form cubic and H_{II} phases as compared to PE isolated from strain Ot 32. This was actually found (tables 2,3). These results further support the conclusion [7] that iso and *ante*-iso acyl chains play the roles of straight saturated and unsaturated acyl chains, respectively, in a membrane containing large amounts of these components. However, the difference in physical properties between iso and *ante*-iso acyl chains is less than the difference between straight saturated and *cis*-unsaturated acyl chains [25,26]. Dioleoylphosphatidylethanolamine forms an H_{II} phase in excess water already at 15°C [20].

The capacity to take up water is different for the two PE preparations (tables 2,3). This reflects the different packing properties of iso and *ante*-iso acids. Since the area occupied by the phosphorylethanolamine group is smaller than that occupied by the two acyl chains [27] an increase in the width of the hydrophobic part of PE results in an increased distance between the polar head groups. PE isolated from strain Ft R32, with a large amount of the more loosely packed *ante*-iso acyl chains, is accordingly able to take up larger amounts of water.

Some biological membrane lipids have earlier been found to form cubic phases together with water. These phases are formed either by single lipids such as lecithin [28,29], PE with straight acyl chains at a very low pH [9] and dioleoylmonoglucosyldiglyceride [30], or by lipid mixtures such as monogalactosyldiglyceride/digalactosyldiglyceride [31] and monoglucosyldiglyceride/diglucosyldiglyceride [16,17].

Although both the PE preparations used in this work form a lamellar phase in excess water up to at least 65°C, they have quite different packing properties. It has been shown that the permeability barrier of the bilayer is destroyed before the lamellar phase is tranformed into micelles or an H_{II} phase [32,33]. Moreover, in *Escherichia cold* membranes the increase in permeability as a result of enhanced growth temperature is counteracted by reducing the degree of unsaturation of the phospholipid acyl chains [34]. Since an increase in temperature will increase the tendency of PE to form cubic and H_{II} phases, the temperature-induced regulation of acyl chain composition in *B. megaterium* membranes [7] is most probably necessary in order to maintain an optimal packing of the lipid molecules. Similar regulation mechanisms, related to the phase structures formed by membrane lipids, have been found to operate in *Pseudomonas fluorescens* [35] and *Acholeplasma laidlawii* [3,36]. Here, PE isolated from two different strains of *B. megaterium* was used. However, the same qualitative difference in acyl chain composition as noticed between the

strains is obtained within the growth temperature interval of Ft R32 [7]. Furthermore, a shift in growth temperature when this strain is in the early logarithmic phase of growth elicits a rapid and profound analogous change in acyl chain composition (T. Clementz, L. R., unpublished), indicating that the change is vital for proper membrane function. The regulation of the ratio iso/*ante*-iso acyl chains in the membrane lipids is relevant not only to the genus *Bacillus*. Several other procaryotes (e.g., many extreme thermophiles) contain lipids with mainly branched acyl chains [24]. A temperature-dependent alteration of the ratio iso/*ante*-iso acyl chains like the one found for *B. megaterium* [7] has also been reported [24].

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