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Interdigitated gel phase bilayers formed by unsaturated synthetic and bacterial glycerolipids in the presence of polymyxin B and glycerol

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The ability of synthetic phosphoglycerolipids with a *cis* mono-unsaturated acyl chain in the 2-position and a saturated chain in the 1-position of glycerol to form interdigitated gel phase bilayers in the presence of amphipathic substances was monitored using a fatty acid spin label, 16-doxylstearic acid, and a phosphatidylglycerol spin label containing 16-doxylstearic acid. These spin labels become significantly more motionally restricted in an interdigitated gel phase bilayer than in a non-interdigitated gel phase bilayer. The results indicated that polymyxin B and polymyxin B nonapeptide caused interdigitation of 1-palmitoyl,2-oleoyl-phosphatidylglycerol (POPG) and glycerol caused interdigitation of 1-stearoyl,2-oleoyl-phosphatidylcholine (SOPC), similar to their effects on disaturated lipids. The fluidity gradient present in non-interdigitated gel phase bilayers was abolished. However, glycerol did not cause POPG to become interdigitated, in contrast to SOPC. We reported earlier that there is a kinetic barrier to interdigitation of saturated PG in the presence of glycerol, in contrast to saturated PC. This barrier is even greater for the unsaturated species of PG. Furthermore, these compounds lowered the gel to liquid-crystalline phase transition temperatures of the unsaturated lipids more than of saturated lipids suggesting that the interdigitated bilayer of the former may be less ordered or less stable than that of the latter. Since polymyxin B is an antibiotic we also examined its effect on a lipid extract from the Gram-negative bacteria *Pseudomonas aeruginosa* in order to assess whether interdigitation might be involved in its mechanism of bactericidal or bacteriostatic effect. Polymyxin B and polymyxin B nonapeptide also caused motional restriction of a small percentage (about 13% at −2°C and 25% at −14°C for polymyxin B) of the spin label in the lipid extract at low temperatures, where the lipid is in the gel phase, consistent with formation of a small domain of interdigitated bilayer lipid. However, the degree of immobilization was less than that in the interdigitated bilayers of the synthetic unsaturated lipids. This may be a result of the heterogeneous nature of the lipids in the extract. However, it cannot be ruled out that the motional restriction of the spin label in this extract may be caused by something other than interdigitation. Thus the results with the lipid extract are less conclusive of interdigitation than for the synthetic lipids. A motionally restricted population was not detectable at higher temperatures. This study shows that a *cis*-double bond in the acyl chain of phosphoglycerolipids does not prevent formation of an interdigitated gel phase bilayer. However, such lipids are in the liquid-crystalline phase at physiological temperature and thus will not form interdigitated domains in biological membranes.

Introduction

A number of amphipathic substances such as the antibiotic polymyxin B (PMB), glycerol, and ethanol are known to cause synthetic, saturated lipids to form interdigitated gel phase bilayers [1–3]. This has been demonstrated by X-ray or neutron diffraction since it results in a concomitant decrease in bilayer thickness and increase in the bilayer surface area per headgroup [1–5]. Interdigitation compensates for the otherwise

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Abbreviations: PMB, polymyxin B; PMBN, polymyxin B nonapeptide; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPG, 1-palmitoyl,2-oleoylphosphatidylglycerol; SOPC, 1-stearoyl,2-oleoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; 16-S-SL, 16-doxylstearic acid; 5-S-SL, 5-doxylstearic acid; 12-S-SL, 12-doxylstearic acid; 16-MeS-SL, methyl ester of 16-S-SL; PG-SL, 1-palmitoyl,2-(16-doxylstearoyl)-phosphatidylglycerol; DSC, differential scanning calorimeter; EPR, electron paramagnetic resonance.

disordering effect these compounds would have if they penetrated into the apolar/polar interface of a non-interdigitated bilayer, thus causing lateral separation of the lipids. However, most of the lipids in biological membranes contain an unsaturated fatty acid in the 2-position of glycerol. To our knowledge the ability of such unsaturated lipids to form interdigitated bilayers has not yet been reported. In the present study we use a fatty acid spin label, 16-doxylstearate, and a lipid spin label containing 16-doxylstearate to study the ability of synthetic lipids with a saturated fatty acid in the 1-position and an unsaturated fatty acid, oleic acid, in the 2-position, to form an interdigitated bilayer in the presence of polymyxin B and glycerol. We also investigate the ability of polymyxin B to induce the interdigitated bilayer in a lipid extract from the Gram-negative bacteria *Pseudomonas aeruginosa*.

We have shown that lipid or fatty acid spin labels can detect interdigitated bilayers formed by a number of saturated lipids in the presence of a variety of amphipathic compounds and proteins, including polymyxin B and glycerol [6–13]. If the nitroxide moiety is near the terminal methyl of the fatty acid chain, the label becomes motionally restricted in the interdigitated bilayer. This occurs not because an interdigitated bilayer is inherently more ordered than a non-interdigitated bilayer, but because of the dimensions of the interdigitated bilayer. The smaller thickness allows the fatty acid to span the interdigitated bilayer causing the spin label group to be located near the more ordered apolar/polar interface, just as the carboxyl group is located near the same interface on the opposite side of the bilayer. This is a more ordered region of the bilayer than encountered by this spin label in a non-interdigitated bilayer, where the nitroxide group locates at the center of the bilayer.

The results indicate that lipids with one *cis* monounsaturated fatty acid do form an interdigitated bilayer in the presence of polymyxin B and glycerol at low temperatures in the gel phase. However, interdigitation was not detected at physiological temperatures where these lipids are in the liquid-crystalline phase.

Materials and Methods

1-Palmitoyl,2-oleoyl-phosphatidylglycerol (POPG) and 1-stearoyl,2-oleoyl-phosphatidylcholine (SOPC) were from Avanti Polar Lipids, Birmingham, AL. Polymyxin B sulfate was from Burroughs Wellcome, Kirkland, Quebec, Canada. Polymyxin B nonapeptide (PMBN) was prepared as described previously [13], and was 47% pure peptide and 53% NaCl. The weight used was adjusted accordingly. The fatty acid spin labels 16-doxylstearic acid (16-S-SL), 5-doxylstearic acid (5-S-SL), 12-doxylstearic acid (12-S-SL), and the methyl ester of 16-S-SL (16-MeS-SL) were from Molecular Probes, Eugene, OR. The lipid spin label, 1-palmitoyl,2-(16-doxylstearoyl)-phosphatidylglycerol (PG-SL) was a generous gift from Dr. A. Watts, University of Oxford. Glycerol was from Matheson, Coleman, and Bell.

Preparation of lipid extract. Two *Pseudomonas aeruginosa* strains PACF10 and PACF121 isolated from cystic fibrosis patients were grown in 800 ml tryptone broth for 18 h, washed twice with PBS by centrifugation at $10000 \times g$ for 20 min and lyophilized. The readily extractable lipids were extracted with chloroform/methanol according to Folch [14]. The extract was applied to a TLC plate, run at 4°C in chloroform/methanol/water/acetic acid (65:25:4:1, v/v), dipped in 10% sulfuric acid containing 8% $CuSO_4$ and charred for 20 min at 160°C [15]. The lipids were quantitated by one-dimensional laser densitometry of the TLC plate. For fatty acid analysis, the lipid extract was hydrolyzed by alkaline methanolysis. After neutralization with H_3PO_4, the fatty acid methyl esters were extracted with n-hexane, methylated with diazomethane according to Schlenk and Gellerman [16], and then separated by quantitative capillary gas chromatography.

Preparation of spin-labeled samples. The lipids were prepared for EPR spectroscopy and DSC as described earlier [7,9–12]. Briefly, lipid and spin label were combined in chloroform/methanol (1:1, v/v) at a mole ratio of 150:1. If PMB or PMBN were used they were also dissolved in the choloroform/methanol solution at a mole ratio to lipid of 1:5 for POPG and at a ratio of 0.14–0.28 μmol PMB or PMBN to 1 mg of *P. aeruginosa* extract. The solvent was evaporated under a stream of nitrogen followed by evacuation in a lyophilizer at about 0.1 Torr for 2 h. The POPG and lipid extract, with or without PMB were hydrated with 2 mM Hepes containing 0.1 M NaCl and 0.1 mM EDTA (pH 7.4). Some samples of lipid extract were also hydrated with 2 mM Hepes, 1 mM $CaCl_2$ (pH 7.4). When glycerol was used, it was added directly to the dried lipid. Samples with glycerol were compared to samples prepared in distilled water. The lipid concentration of the final suspension was 2 mg/50 μl glycerol or 2 mg/500 μl aqueous solution. The samples were dispersed by vigorous vortex mixing at room temperature. When PMB or PMBN were used it was usually necessary to scrape the dried lipid-drug complex from the sides of the tube. The aqueous samples were centrifuged in an Eppendorf bench centrifuge. For DSC measurements most of the supernatant was removed and the wet pellet was loaded into an aluminum DSC pan. For EPR measurements, all but about 50 μl of the supernatant was removed and the suspension was loaded into a 50 μl capillary tube. The tube was sealed at one end with a torch and centrifuged at 2000 rpm. Glycerol samples were treated as described previously [7].

Calorimetry. Samples were examined on a Perkin-Elmer DSC-2 equipped with a Perkin-Elmer data station. POPG with PMB or PMBN samples were scanned (heated or cooled) between 250 and 271 K at 5 K/min to avoid the ice peak at 273 K. POPG alone and SOPC samples were scanned between 260–320 K. On heating, the heating scan was started immediately after equilibration at 260 K to avoid freezing of the aqueous solution. The temperature of the main gel to liquid-crystalline phase transition (T_m) was measured as the temperature of maximum heat absorption.

EPR spectroscopy. EPR spectra were measured on a Varian E-104B spectrometer equipped with a Varian temperature controller and a DEC LSI-11 based microcomputer system. For samples at temperatures below 0°C the water was allowed to freeze before measurement of the spectrum. The maximum hyperfine splitting, $2T_{max}$, of the EPR spectra, measured as described previously [10], was used as a measure of the motional restriction of the probe in the gel phase of the lipids. The motional parameter, τ_0, measured as described earlier [6], was used as a measure of the motion in the liquid-crystalline phase. The order parameter S, measured as in Ref. 6, was used to determine the phase transition temperature of the lipid extract and as a measure of the effect of PMB and PMBN on the order of the non-interdigitated gel phase population in the extracts. The microwave power used was 10 mW. Spectra of the lipid extract were subtracted as described previously [12]. They were integrated twice in order to determine the percentage of motionally restricted component induced by PMB.

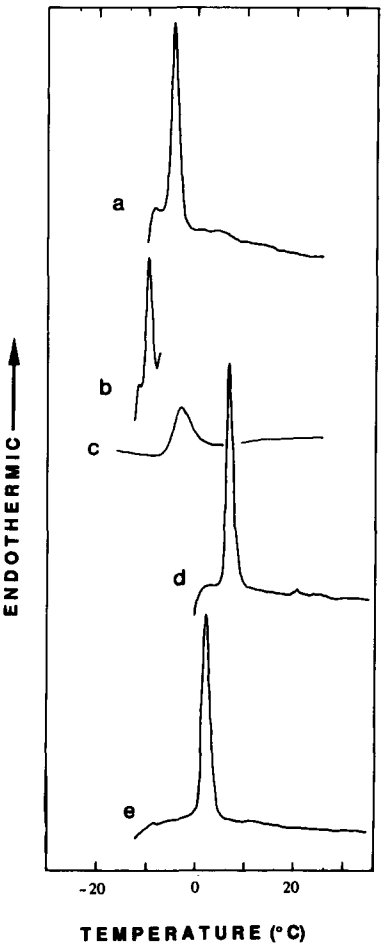

Fig. 1. DSC thermograms at a heating rate of 5 C°/min of (a) POPG; (b) POPG/PMB 5:1 (m/m); (c) POPG in glycerol; (d) SOPC in distilled water; (e) SOPC in glycerol. Different amounts of lipid were present in each case so the peak areas cannot be compared.

Results

Synthetic unsaturated lipids

The phase transition temperatures of POPG and SOPC, determined by DSC, were −4°C and 6.6°C, respectively (Fig. 1a,d, Table I). PMB decreased the T_m of POPG by 5.4 C° (Fig. 1b). Only a single peak was observed. PMBN had a somewhat smaller effect, causing a decrease of 4.5 C° (not shown). Glycerol de-

TABLE I

Effect of PMB, PMBN, and glycerol on the gel to liquid-crystalline phase transition temperature and on T_{max} values of a number of spin labels in unsaturated lipids

Sample	Phase transition temperature (°C) [a]	T_{max} (G) [b]				
		16-S-SL	12-S-SL	5-S-SL	16-MeS-SL	PG-SL
POPG/buffer	−4	24.8	29.4	32.0	~21, 29 [c]	29.9
POPG/PMB	−9.4	31.1	31.4	32.8	31.9	31.0
POPG/PMBN	−8.5	30.8	–	–	–	–
POPG/glycerol	−2.8	24.8	30.9	32.0	–	28.0
SOPC/water	6.6	~21, 28 [c]	28.8	31.1	~21, 28 [c]	29.1
SOPC/glycerol	2.2	31.0	30.6	31.6	31.0	31.2

[a] Determined by DSC at a heating rate of 5 C°/min.

[b] Gel phase spectra of POPG samples were measured at −14°C and of SOPC samples at −6°C.

[c] Two components were present in the spectrum. The amount of the more mobile component (lower T_{max}) increased with increase in temperature. The approximate T_{max} values were estimated from the composite spectrum.

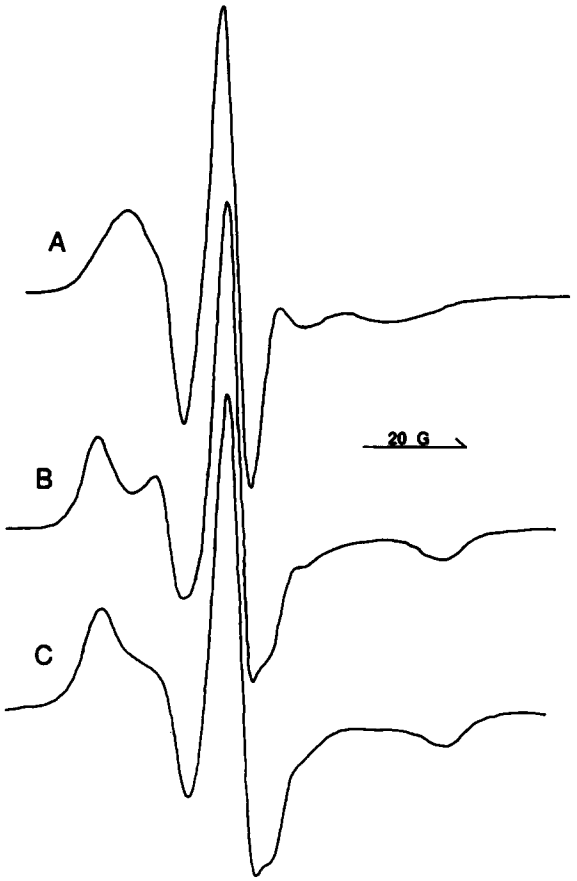

Fig. 2. EPR spectra of 16-S-SL at −14°C in (A) POPG in buffer at pH 7.4; (B) POPG/PMB 5:1 (m/m); (C) POPG/PMBN 5:1 (m/m).

creased the T_m of SOPC by 4.4 C° (Table I, Fig. 1e). Similar effects were observed on both heating and cooling scans for all three compounds. This differs from the effects of these compounds on saturated species of PG and PC. PMBN had little effect on the T_m of dipalmitoylphosphatidylglycerol (DPPG) [9], and PMB resulted in two peaks, one of which was at a similar temperature as the pure lipid and one which was 1.5 C° lower [7,17]. Glycerol increased the T_m of saturated PC by 1–2 C° [2,7]. However, it decreased the T_m of DPPG by about 3 C° when it formed an interdigitated bilayer and by 12 C° when it formed a metastable phase which was not interdigitated [7]. Glycerol increased the T_m of POPG slightly on heating (Table I), but not on cooling, and broadened the transition in both cases (shown for heating in Fig. 1c). There was no evidence of the metastable phase behavior observed with DPPG.

EPR spectra of 16-S-SL in POPG with or without PMB and PMBN were measured at temperatures from 2°C down to −14°C. At −14°C when all three samples were completely in the gel phase, the spin label was significantly more motionally restricted in the PMB and PMBN samples than in pure POPG (Fig. 2). The T_{max} value of 16-S-SL in the PMB and PMBN samples was about 31 G, as found for DPPG with these drugs, in contrast to a value of 24 G for the pure lipid. It was thus increased to almost the value found for 5-S-SL (Table I), which monitors the more ordered region near the apolar/polar interface. In pure POPG as in other lipids, there is a fluidity gradient toward the center of the bilayer [18] with the motion of the probes increasing in the order 16-S-SL > 12-S-SL > 5-S-SL. In the presence of PMB, however, this fluidity gradient is abolished and the motion is similar at all points along the fatty acid chain. PG-SL was also more motionally restricted in the presence of PMB although it has less motion than 16-S-SL in the gel phase of the pure lipid resulting in a smaller difference (Table I). This occurs in saturated lipids also, probably as a result of a different vertical location of the lipid-bound fatty acid compared to the free fatty acid [10].

At a higher temperature, −10°C, a component characteristic of similar motional restriction was still present in the sample with PMBN (not shown) but the PMB sample was partly in the liquid-crystalline phase at this temperature. At 2°C, when all three samples were completely in the liquid-crystalline phase, the spectra of 16-S-SL were all characteristic of nearly isotropic motion and no evidence of a population of lipid of restricted motion was apparent in the spectra (not shown). However, the motional parameter τ_0 was increased by PMB from 1.7 to 2.5 ns indicating a small reduction in motion. PMB and PMBN had similar effects on the liquid-crystalline phase of DPPG [8,9].

At temperatures of −2°C to −14°C, well below the T_m of SOPC, 16-S-SL was significantly more motionally restricted in SOPC-glycerol samples than in SOPC in water. Spectra of 16-S-SL at −14°C, are shown in Figs. 3A,B. The T_{max} value for SOPC-glycerol is 31 G. The spectrum of 16-S-SL in SOPC had two components but the T_{max} value of neither was as large as in the presence of glycerol. At higher temperatures up to −2°C, the T_{max} value of 16-S-SL in SOPC-glycerol remained constant at about 31 G (see Fig. 3D) while both components in the spectrum of the pure lipid had more motion (Fig. 3C), resulting in a decrease in T_{max} for both (T_{max} values at −6°C are shown in Table I). Glycerol increased the T_{max} value of 16-S-SL to almost the value found for 5-S-SL (Table I). The spectrum of PG-SL in SOPC/water had only one component as shown at −6°C in Fig. 3E. It also was significantly more motionally restricted in the presence of glycerol (Fig. 3F, Table I). Glycerol, however, had no effect on 16-S-SL in POPG and decreased T_{max} of PG-SL in contrast to the increase caused in SOPC. Glycerol also does not significantly restrict the motion of 16-S-SL in a metastable phase of DPPG, although it does in a more stable phase [7].

Only one component was observed for 16-S-SL in the gel phase of POPG and saturated forms of PG and

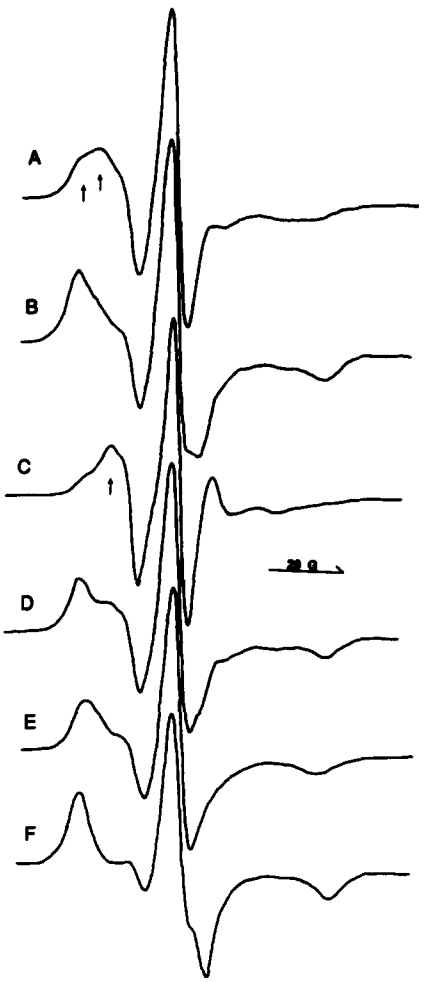

Fig. 3. EPR spectra of (A) 16-S-SL at −14°C in SOPC/water; (B) 16-S-SL at −14°C in SOPC/glycerol; (C) 16-S-SL at −2°C in SOPC/water; (D) 16-S-SL at −2°C in SOPC/glycerol; (E) PG-SL at −6°C in SOPC/water; (F) PG-SL at −6°C in SOPC/glycerol. The two components in the spectrum in (A) and the more mobile component in the spectrum in (C) are indicated by vertical arrows on the low field side.

PC [7], in contrast to its behavior in SOPC. These two components in SOPC are not caused by partial ionization of the fatty acid spin label because an increase in pH from 4 to 9.5 has only a small effect on the T_{max} value of 16-S-SL in this lipid (not shown) or in saturated lipids [7]. Furthermore, the spectrum of 16-MeS-SL has two similar components in both POPG and SOPC (Table I) as well as in saturated lipids [7,10]. The cause of this behavior is not known but could be orientation of the less polar 16-MeS-SL in the bilayer in two directions, one with the ester group near the bilayer/aqueous interface, and the other upside down with the relatively polar spin label group near this interface and the ester group in the center of the bilayer. Possibly the protonated form of 16-S-SL can behave similarly in the gel phase of the unsaturated PC. This spin label is expected to be completely protonated at the pH of the distilled water used to hydrate SOPC [19]. However, identical results are observed at pH 9.5 where some of the probe should be ionized. An alternative possibility is that 16-S-SL may have a greater amplitude of flexing motion about the perpendicular to the bilayer in SOPC compared to saturated lipids, allowing the nitroxide group to be located at the interface at least some of the time. 16-MeS-SL may have this greater motion in both unsaturated and saturated lipids since it is less well anchored at the interface.

Although the motional restriction observed in the presence of PMB and glycerol is even greater than that of the more immobilized component of 16-S-SL and 16-MeS-SL in the pure lipids, it could be argued that it is also caused by a greater amplitude of motion of the spin label so that the nitroxide group spends more time at the interface. It is unlikely that this would result in motional restriction of all of the probe molecules, however. The fact that PG-SL results in a spectrum with a single component and is also more motionally restricted in POPG by PMB and in SOPC by glycerol supports the conclusion that the motional restriction is not caused by location of the probe in the polar/apolar interface of a non-interdigitated bilayer. Its fatty acid spin label is better anchored, has less flexing motion, and has been shown to be located near the depth expected on the basis of its chain length [20]. Neither it nor 16-S-SL are motionally restricted by glycerol in POPG, nor in the metastable state of DPPG induced by glycerol [7], indicating that the effect is specific. Furthermore, the effect of PMB and glycerol on the behavior of these probes in the unsaturated lipids is similar to that in saturated lipids where the cause has been shown by X-ray diffraction to be formation of an interdigitated bilayer [1,2].

At 10°C, in the liquid-crystalline phase, there was no evidence of a motionally restricted component for 16-S-SL but glycerol increased the motional parameter, τ_0, from 1.5 to 1.9 ns as reported earlier for saturated PC [7].

Bacterial lipid extract

The predominant readily extractable lipids from *P. aeruginosa* were phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and cardiolipin (D-PG) (Table II). The major fatty acids in the lipids were 16:0 and 18:1 (Table III). Some free fatty acids (FFA) were also present in the extract but were a minor component (Table II). The extracts from both strains PACF10 and PACF121 were similar with regard to composition and spin label behavior.

A phase transition was not detected in the lipid extract by low sensitivity DSC, probably because of the low enthalpy and cooperativity of the phase transition of the heterogeneous mixture of lipids present. Therefore, the gel to liquid-crystalline phase transition temperature of the lipid extract was monitored from

TABLE II

Densitometric quantitation of lipid content in readily extractable lipid from P. aeruginosa strains

Values are means of three separate preparations. The individual values differed by ±3% or less.

	% of total lipid	
	strain PACF10	strain PACF 121
PG	23	20
PE	64	64
D-PG	12	13
FFA	1	3

changes in the order parameter S of 16-S-SL with temperature. The motion changed from anisotropic at 2°C to predominantly isotropic at 12°C indicating that the transition temperature was inbetween these values in the presence or absence of PMB (Fig. 4). Spectra are compared at −2°C and 16°C in Figs. 5A and D, respectively. The spectrum at −2°C is characteristic of anisotropic motion and is similar to the more mobile component in the gel phase of SOPC at −2°C, indicated by an arrow in Fig. 3C. The spectrum at −14°C, measured in another sample, is shown in Fig. 7A and resembles that in the gel phase of POPG (Fig. 2A).

Below the transition temperature, a component characteristic of motional restriction was apparent in the spectrum in the presence of PMB at −2°C (Fig. 5B). PMBN had a similar effect (not shown). The remaining lipid population was more disordered than the pure lipid as indicated by a lower value of the average order parameter measured from the total spectrum (Table IV). In the presence of Ca^{2+} and PMB the motionally restricted component is more apparent in the spectrum (Fig. 6B) because the remaining lipid is disordered even more than in the absence of Ca^{2+} (Table IV) and thus the two components are better separated. Note that Ca^{2+} alone did not cause motional restriction of a population of the lipid. Thus the effect is not caused simply by electrostatic interactions

TABLE III

Fatty acid composition of readily extractable lipid from P. aeruginosa

Two preparations of lipid from each strain PACF10 and PACF121 were analyzed. The range of values obtained is shown.

Fatty acid	Amount (weight % of total)
14:0	0.8– 1.2
16:0	38 –43
16:1	5 –10
17:1	5.8– 6.2
18:0	0.6– 1.1
18:1	25 –35
19:1	9 –18

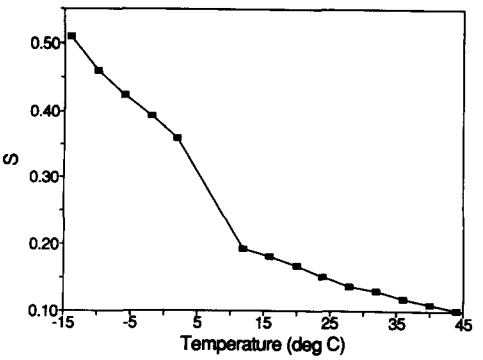

Fig. 4. Temperature dependence of the order parameter *S* of 16-S-SL in *P. aeruginosa* lipid extract.

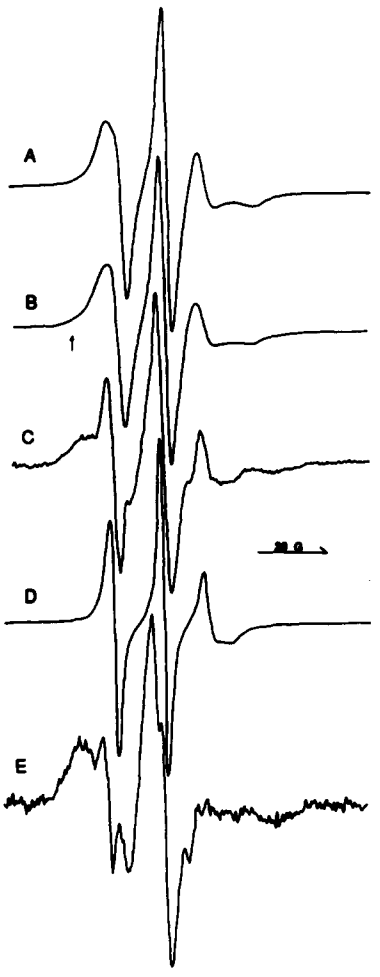

Fig. 5. EPR spectra of 16-S-SL in *P. aeruginosa* PACF 121 lipid extract in buffer containing EDTA (A) alone at −2°C; (B) plus PMB (0.28 μmol/mg lipid) at −2°C; and (D) alone at 16°C. The low-field peak of the motionally restricted component in (B) is indicated by an arrow; the corresponding high-field peak cannot be seen. (C) is the difference spectrum obtained by subtracting a fractional amount of the spectrum in (A) from that in (B). The motionally restricted component is better resolved and both the low- and high-field peaks are apparent. (E) is the difference spectrum obtained by subtracting a fractional amount of the spectrum in (D) from that in (C) further resolving the motionally restricted component. The spectra are shown normalized to the same center peak height but were not normalized for the subtraction and for integration.

TABLE IV

Effect of PMB on the spectral parameters of 16-S-SL in the non-interdigitated bilayer population of P. aeruginosa lipid extract in the presence and absence of Ca^{2+}

Sample	Gel phase		Liquid-crystalline phase
	T_{max} (G)	S	τ_0 (ns) [a]
Lipid (EDTA)	20.1	0.402 [b]	1.07
Lipid/PMB (EDTA)	19.2	0.364 [b]	1.12
Lipid (EDTA)	22.2	0.511 [c]	–
Lipid/PMB (EDTA)	22.3	0.513 [c]	–
Lipid (Ca^{2+})	19.8	0.394 [d]	1.13
Lipid/PMB (Ca^{2+})	17.8	0.278 [d]	1.16

[a] Measured at 37°C.
[b] Extract PACF 121 with or without 0.28 μmol PMB/mg lipid, measured at −2°C.
[c] Extract PACF 10 with or without 0.14 μmol PMB/mg lipid, measured at −14°C.
[d] Extract PACF 121 with or without 0.14 μmol PMB/mg lipid, measured at −2°C.

of the lipid with a polyvalent cation. The motionally restricted component in the spectrum in Fig. 5B was resolved by subtracting the spectrum of the pure lipid measured at the same temperature (Fig. 5A). This gave a spectrum with the motionally restricted component plus a component more fluid than the pure lipid at this temperature (Fig. 5C). Subtraction of spectra of the pure lipid measured at higher temperatures (−1 to 3°C) from that in Fig. 5B did not improve the result. The more fluid component in Fig. 5C was partially removed by subtraction of the spectrum of the pure lipid measured at 16°C (Fig. 5D) giving the spectrum of the motionally restricted component shown in Fig. 5E. The residual peaks in this result spectrum show that the 16°C spectrum clearly must not be identical to the mobile spectral component present in Fig. 5C. Nevertheless, it must be similar. These subtractions reveal the presence of a motionally restricted component in Fig. 5A and suggest that there are also spectral components arising from two other lipid populations of greater fluidity in the original spectrum of the lipid/PMB sample. The motionally restricted component was about 13% of the total spectrum in Fig. 5B. The T_{max} value was 26.8 G. We were not able to similarly resolve the motionally restricted component from the spectrum in the presence of Ca^{2+} (Fig. 6B) because the more mobile component in this spectrum did not resemble the pure lipid sufficiently at any temperature used.

At −14°C the presence of a motionally restricted

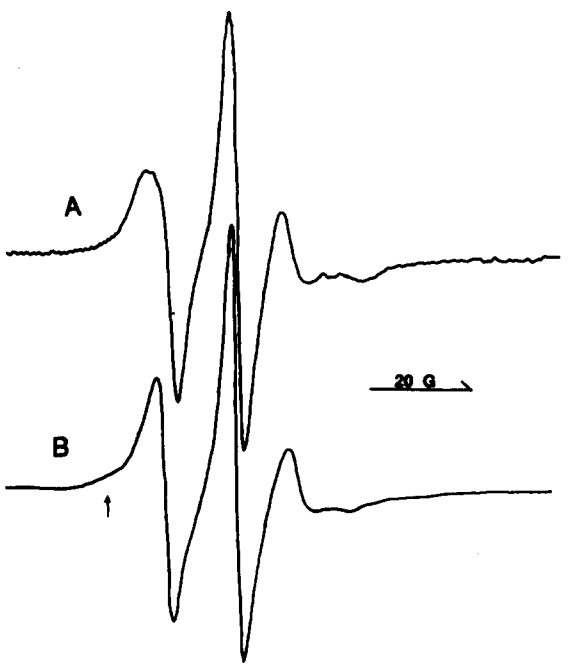

Fig. 6. EPR spectra of 16-S-SL in *P. aeruginosa* PACF 121 lipid extract at −2°C in buffer containing 1 mM Ca^{2+} (A) alone; (B) plus PMB (0.14 μmol/mg lipid). The low-field peak of the motionally restricted component indicated by an arrow is better resolved from the component characteristic of more motion than in Fig. 4B.

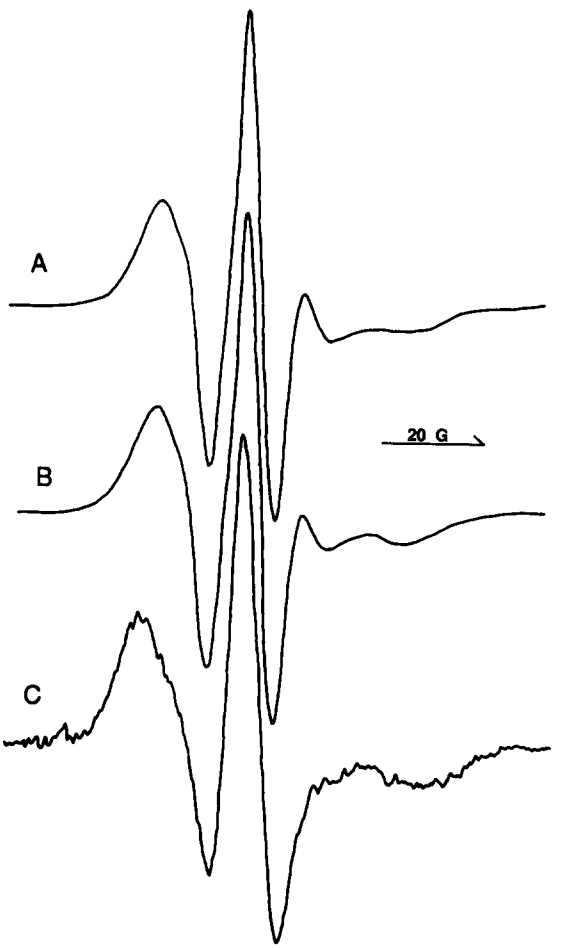

Fig. 7. EPR spectra of 16-S-SL in *P. aeruginosa* PACF 10 lipid extract in buffer containing EDTA (A) alone at −14°C; (B) plus PMB (0.14 μmol/mg lipid) at −14°C; (C) the difference spectrum obtained by subtracting a fractional amount of the spectrum in (A) from that in (B). The spectra are shown normalized to the same center peak height but were not normalized for the subtraction and for integration.

component was less readily apparent from inspection of the spectrum (compare Figs. 7A and B) and the order parameter of the bulk lipid was similar to that in the absence of PMB (Table IV). However, a motionally restricted component could be extracted from the spectrum in Fig. 7B by subtraction of a fractional amount of that in Fig. 7A. The result shown in Fig. 7C has a T_{max} value of 26.2 G and resembles that in Fig. 5E but is cleaner. At this lower temperature it was not necessary to also subtract a more fluid spectrum. The motionally restricted component is 25% of the spectrum in Fig. 7B.

Discussion

PMB and PMBN clearly restrict the motion of 16-S-SL and PG-SL in POPG, while glycerol has a similar effect in SOPC indicating that these compounds cause interdigitation of these lipids with a monounsaturated fatty acid in the gel phase as they do for disaturated fatty acid lipids. The degree of motional restriction of the spin label is similar to that found in the saturated lipids. Glycerol was expected not to cause interdigitation of POPG since there is a kinetic barrier to formation of the interdigitated bilayer even by DPPG in the presence of glycerol. Indeed, glycerol did not restrict the motion of these probes in POPG in contrast to its effect on SOPC, indicating that motional restriction is not caused by any compound which interacts with the bilayer, but rather is a specific effect. Thus a *cis*-double bond in one chain does not prevent formation of the interdigitated bilayer, except in cases such as POPG/glycerol where there is a kinetic barrier to interdigitation even for the saturated lipid.

It is difficult to see how the long range 2-dimensional packing order could be as great in the interdigitated gel phase bilayer as in the non-interdigitated gel phase bilayer formed by these unsaturated lipids. Indeed, the transition temperatures of POPG and SOPC are reduced by PMB and glycerol, respectively, considerably more than that of the saturated lipids. We have reported previously that PMB does not maintain interdigitation of saturated lipids up to the gel to liquid-crystalline phase transition temperature [7,12]. The larger decrease in transition temperature found for unsaturated POPG may indicate that it converts to a non-interdigitated gel phase bilayer more readily than the saturated lipids because of lower order and stability in the interdigitated phase. However, glycerol maintains interdigitation of saturated lipids up to the transition temperature, which is unaltered or even increased a little relative to the pure lipid. Thus indicates that glycerol has similar solubility in the interdigitated gel phase as in the liquid-crystalline phase. In contrast, glycerol lowers the transition temperature of SOPC by about 5 C° but appears to maintain interdigitation up to this transition in this case also. Thus the reduced transition temperature of the unsaturated lipids with PMG or glycerol may be a consequence of less ordered packing of the unsaturated lipid in the interdigitated bilayer relative to a non-interdigitated gel phase bilayer. Alternatively it may be a result of even greater solubility of glycerol in the liquid-crystalline phase than the interdigitated gel phase, i.e., less stabilization of the interdigitated gel phase of unsaturated lipids relative to the liquid-crystalline phase than occurs with saturated lipids.

A motionally restricted component was also observed in the spectrum of this spin label in the lipid extract from *P. aeruginosa* in the presence of PMB and PMBN. This component was about 13% of the total spectrum at −2°C, a few degrees below the transition temperature of the lipid extract, and 25% for a different sample at −14°C. The motional restriction, as indicated by a T_{max} of about 26–27 G was less than that usually observed for this spin label in interdigitated bilayers (T_{max} of at least 30 G), unless the interdigitated bilayer is disordered or loosely packed as a result of the lipid structure or the type of compound used to cause interdigitation [10]. The bilayer formed by these lipid extracts may be disordered as a result of the heterogeneous lipid composition. Thus the motionally restricted component observed is consistent with but does not prove the occurrence of some domains of interdigitated bilayer in the gel phase of this extract in the presence of PMB and PMBN. The extract consists primarily of PG and PE. Although dipalmitoylphosphatidylethanolamine by itself does not bind PMB, it does bind and can become interdigitated in mixtures with DPPG [11]. Thus both the PG and the PE in the bacterial lipid extract may contribute to the putative interdigitated domain. Although the spectral resolution at −2°C was not very good, it was much better at −14°C, where the spectrum of the bulk lipid more closely resembled that of the pure lipid extract at the same temperature. The similarity of the motionally restricted component obtained at both temperatures suggests that the resolution at −2°C by subtraction of two different lipid spectra is realistic even though the spectra subtracted were not identical to the spectral components present in the composite spectrum. This indicates that at the higher temperature there are three different lipid populations present in the presence of PMB, one little affected by PMB, one motionally restricted by PMB, and one induced to go into the liquid-crystalline phase by PMB. The liquid-crystalline lipid may be a small lipid population bound to PMB which has a lower T_m than the rest, possibly because both chains are unsaturated or shorter in chain length. These lipid species may not be able to pack into the interdigitated gel phase domain and thus separate into a non-interdigitated liquid-crystalline phase domain.

PMB is bactericidal to Gram-negative bacteria such as *P. aeruginosa* and causes interdigitation of DPPG at bacteriostatic concentrations [12]. However, interdigitation of saturated and unsaturated lipids only occurs in the gel phase. Gram-negative bacteria have been reported to contain some di-saturated lipids [21] and thus could conceivably have some small domains of gel phase at physiological temperature. No motionally restricted component was detected in the spectrum of this lipid extract at physiological temperatures but domains of less than 5% of the total lipid would be difficult to detect by this technique. Thus the role of PMB-induced interdigitation in the mechanism of its bacteriostatic and bactericidal effects is not known.

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