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Use of spin labels to determine the percentage of interdigitated lipid in complexes with polymyxin B and polymyxin B nonapeptide

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Long chain spin labels with the nitroxide group located near the terminal methyl of the chain were used to determine the percentage interdigitated lipid in complexes of polymyxin B (PMB) and polymyxin B nonapeptide (PMBN) with the acidic lipids dipalmitoylphosphatidylglycerol (DPPG) and dipalmitoylphosphatidic acid (DPPA) at varying mole ratios of drug to lipid and at different pH values. These spin labels are more motionally restricted in the interdigitated than in the non-interdigitated gel phase bilayer. This allows determination of the percentage interdigitated lipid by resolution of the spectrum into motionally restricted and more mobile components. At nonsaturating concentrations of PMB, significantly more DPPG than that which can be maximally PMB-bound, becomes interdigitated. As the temperature approaches the gel to liquid crystalline phase transition temperature, the bilayer becomes progressively non-interdigitated. The ESR spectrum indicates that PMB also causes interdigitation of DPPA. However, in contrast to DPPG, the amount of DPPA which is interdigitated at pH 6, is less than the amount which is expected to be PMB-bound. This is attributed to the ability of DPPA to participate in lateral interlipid hydrogen bonding interactions. Such lateral interactions would be abolished in the interdigitated bilayer and thus they are expected to inhibit its formation. At pH 9, where the interlipid interactions of DPPA are weakened, PMB induces even more lipid than that which is PMB-bound to become interdigitated. Indeed, the percentage interdigitated lipid is even greater than found for DPPG. This may be partly a result of the greater negative charge of DPPA at this pH. A greater repulsive negative charge is expected to favor interdigitation. PMBN is less effective than PMB at inducing interdigitation of DPPG and causes little or no interdigitation of DPPA at pH 6, even at saturating concentrations. PMBN also does not lower the phase transition temperature of DPPA at pH 6 as much as PMB. At pH 9, the effect of PMBN on DPPA is more similar to the effect of PMB. However, even for DPPG, and DPPA at pH 9, PMBN does not maintain interdigitation of the lipids at higher temperatures as effectively as PMB. PMBN's smaller perturbing effect and greatly decreased ability to cause interdigitation of DPPA at pH values below 9 may be related to a decreased ability to cause lateral separation of the lipid molecules, which is necessary in order to weaken the interlipid interactions. PMBN lacks the fatty acyl chain of PMB, which may help cause lateral separation of the lipid molecules. These results may be related to the differences in physiological effects of the antibiotic PMB and the nonbactericidal PMBN on Gram-negative bacteria.

Introduction

A number of amphipathic substances, such as the antibiotic polymyxin B (PMB), have been shown by X-ray diffraction to induce an interdigitated gel phase bilayer when added to dipalmitoylphosphatidylglycerol (DPPG) [1]. In this kind of bilayer the fatty acid chains of lipid on one side of the bilayer interdigitate into and interact with the fatty acid chains of the lipid monolayer on the other side of the bilayer, resulting in a decrease in bilayer thickness. Small domains of this

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Abbreviations: PMB, polymyxin B; PMBN, polymyxin B nonapeptide; DPPG, dipalmitoylphosphatidylglycerol; DPPA, dipalmitoylphosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; ESR, electron spin resonance; DSC, differential scanning calorimetry.

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thinner interdigitated bilayer might not be very compatible with small domains of non-interdigitated lipid bilayer of greater thickness within the same bilayer. In order to avoid exposing hydrophobic regions of the fatty acid chains to water, the lipid which tends to be interdigitated might either (i) phase separate into a large domain of interdigitated bilayer, or (ii) influence the non-interdigitated lipid to become interdigitated, or (iii) become non-interdigitated. The PMB-lipid system is a useful model system to investigate the influence of lipids which tend to be interdigitated and lipids which tend to be non-interdigitated on each other's organization. PMB binds electrostatistically to acidic lipids in a 1 : 5 mole ratio and induces interdigitation of the bound lipid. Excess lipid above this ratio will not be bound to PMB and thus will not be directly induced to interdigitate.

Study of the conditions under which interdigitation can occur will help to understand the molecular forces which influence it and is also necessary in order to evaluate the probability of its occurrence in biological membranes. Complete interdigitation of symmetric lipids has so far been detected only in the gel state. Therefore its relevance to biological membranes is unclear. It could be argued that small populations of saturated lipids might form small domains of interdigitated bilayer in biological membranes, particularly in those such as bacterial membranes which have larger amounts of saturated fatty acid than mammalian membranes. However, these membranes also contain mixtures of lipids, some of which may be induced to interdigitate by a compound such as PMB and some which may not.

An X-ray diffraction study showed that at nonsaturating concentrations of PMB, i.e. mole ratios of DPPG to PMB greater than 5 : 1, non-interdigitated lipid as well as interdigitated lipid is present, but did not reveal what proportion of the lipid is in either type of bilayer [1]. However, in dimyristoylphosphatidylcholine/DPPG/PMB of mole ratio 5 : 5 : 1, where only half the lipid is PMB bound (only the DPPG binds), all or almost all of the lipid is interdigitated indicating that unbound lipid can also be induced to interdigitate along with the PMB-bound DPPG [2].

We have shown that lipid or fatty acid spin labels are a useful method to detect interdigitated bilayers. Formation of this kind of bilayer results in motional restriction and/or ordering of a fatty acid spin label in which the nitroxyl group is near the end of the acyl chain, such as 16-doxyl-stearic acid or a phosphatidylglycerol spin label which has 16-doxyl-stearic acid as one of its acyl chains [3–5]. By resolving spectra of incompletely interdigitated samples, at nonsaturating ratios of PMB, into components resulting from interdigitated and non-interdigitated domains, the percentage of lipid in each can be estimated. We have used this technique to determine whether the unbound DPPG also becomes interdigitated or phase separates into a domain of non-interdigitated bilayer.

Unbound lipids which interact intermolecularly by hydrogen bonding (such as phosphatidylethanolamine (PE) and phosphatidic acid (PA) (reviewed in Ref. 6)) may resist becoming interdigitated, and show a greater tendency to phase separate from an interdigitated bilayer than non-hydrogen bonding lipids (such as phosphatidylglycerol (PG) and phosphatidylcholine (PC)). The greater separation of the head groups in the interdigitated bilayer would inhibit lateral intermolecular hydrogen bonding. Therefore we have also investigated the dependence of the tendency to interdigitate on the intermolecular hydrogen bonding properties of the lipid by using complexes of PMB with dipalmitoylphosphatidic acid (DPPA) at varying mole ratios. PMB bound to PA weakens the interlipid hydrogen bonding, as is evident from the large decrease in transition temperature it causes [7], since it binds to the hydrogen bond accepting site ($P\text{-}O^-$). However, unbound PA can continue to hydrogen bond intermolecularly, if it separates into its own domain. Therefore at nonsaturating ratios of PMB, DPPA consists of a mixture of a PMB-bound, non-hydrogen bonding species and an unbound, hydrogen bonding species.

We also compare the ability of PMB to cause interdigitation in these systems with that of polymyxin B nonapeptide (PMBN), a derivative of PMB which lacks the fatty acyl chain. PMBN is not bactericidal although it sensitizes Gram-negative bacteria to other antimicrobial agents [8]. We have found that, like PMB, it causes motional restriction of 16-doxyl-stearic acid in DPPG, suggesting that it induces interdigitation, but it does not maintain it to as high a temperature as PMB [7]. In the present study we use spin labels to further compare the abilities of PMB and PMBN to maintain interdigitation of bound and unbound lipid at various temperatures. These results may help to understand the different pharmacological properties of these two drugs.

Materials and Methods

Dipalmitoylphosphatidylglycerol (DPPG) was from Calbiochem. La Jolla, CA, and dipalmitoylphosphatidic acid (DPPA) was from Avanti Polar Lipids, Birmingham, AL. Polymyxin B sulfate was from Burroughs Wellcome, Inc., Kirkland, Quebec, Canada. Polymyxin B Nonapeptide (PMBN) was prepared as described previously [7], and was 47% pure peptide and 53% NaCl. The weight used was adjusted accordingly. The fatty acid spin labels, 16-doxyl-stearic acid and methyl 16-doxyl-stearate were from Syva, Palo Alto, CA.

Preparation of lipid-peptide complexes. Spin-labeled complexes of DPPG or DPPA with PMB or PMBN were prepared by dissolving the lipid, spin label, and

peptide together in chloroform/methanol (1:1, v/v), evaporating the solvent under a stream of nitrogen, and evacuating in a lyophilizer for at least 2 h at 0.1 Torr. The mole ratio of lipid to spin label was 150:1 and various mole ratios of lipid to peptide were used. The sample, containing 2 mg lipid, was hydrated with 1 ml of 10 mM buffer containing 100 mM NaCl. The buffer used was sodium acetate for pH 6, Hepes for pH 7.4, and sodium borate for pH 9. The sample was dispersed by vigorous vortex mixing at a temperature above the lipid phase transition temperature. The 5:1 complex tends to stick to the sides of the tube. Therefore several small glass beads were added to each tube to facilitate dispersal of the lipid complexes. The sample was freeze-thawed followed by further vortex mixing and incubation at a temperature above the phase transition temperature for 15 min to ensure complete hydration and mixing of the lipid. This procedure was repeated twice. The samples were divided in half, and each half was centrifuged in an Eppendorf bench centrifuge for 5 min. One half was used for differential scanning calorimetry (DSC) and the other half for electron spin resonance (ESR) measurements.

Some samples were also prepared by dissolving the peptide in the buffer and hydrating the lipid with the peptide solution. Similar results were obtained. In order to determine the concentration dependence of the effect of PMB on DPPG, PMB solutions at concentrations of 0.2 μmol per 0.5 ml down to 0.2 μmol per 100 ml, prepared in plastic containers, were added to a suspension of 1 μmol spin-labeled DPPG in 1 ml buffer. After incubation at 4°C overnight, the resulting precipitate was collected by centrifugation at 2000 rpm.

In order to determine if the spin label could bind to PMB in solution, chloroform/methanol solutions of the spin label and PMB were evaporated together in a test tube and dispersed in buffer at a concentration of 0.2 μmol per 50 μl. The sample was taken up in a capillary tube and the ESR spectrum measured at 9°C. Only a low amplitude sharp 3-line spectrum similar to that in the absence of PMB was observed. A sample of DPPG/PMB 1:1, with excess PMB, was also prepared as described above, centrifuged, and the supernatant removed. The spectra of both the lipid-PMB pellet and the supernatant were measured separately. The spectrum of the pellet was identical to that of the 5:1 sample and the supernatant had no signal.

The supernatant of some samples was analysed for phosphorus content. The results indicated that virtually all of the lipd was in the pellet even in the absence of PMB or PMBN. The percentage of the lipid affected by PMB is generally used as a measure of the percentage bound in most studies of the effect of this compound on lipids [12,13]. Thus if PMB added to lipid at a 1:5 mole ratio affects all of the lipid, it is concluded that virtually all of the PMB added is bound to the lipid. The percentage of DPPA affected in this study was determined from the percentage whose phase transition temperature was lowered by PMB. This could not be done for DPPG since the transition temperature of PMB-bound and unbound DPPG is similar.

For DSC most of the supernatant was removed and the wet pellet was loaded into an aluminum DSC pan. For ESR, all but about 50 μl of the supernatant was removed and the pellet with some supernatant was taken up in a 50 μl capillary tube. The tube was sealed at one end with a flame and centrifuged at 2000 rpm. The end with the pellet was positioned in the ESR spectrometer cavity.

Sucrose density gradient centrifugation. Samples of lipid at varying concentrations of PMB, containing 20000 cpm of ^{14}C-labeled lipid, were layered on a discontinuous sucrose density gradient (20, 25, 30, 35, and 40% sucrose prepared in the same buffer used for the samples) and centrifuged in a SW 50.1 rotor at 40000 rpm at 4°C for 3 h in a Beckmann L3-50 ultracentrifuge. Each layer was removed with a pipet and transferred to a scintillation vial, 15 ml of Biofluor scintillation fluid was added and the samples were counted in a Tracor Analytic Betatrac liquid scintillation system. In some cases where a sharp band was observed in the middle of a sucrose layer, the band and the sucrose layers above and below it were each removed separately and counted.

Uptake of spin label by interdigitated and non-interdigitated bilayers. Samples of DPPG-PMB 5:1 (interdigitated bilayer) or DPPG (non-interdigitated bilayer) containing 1–2 μmol lipid were transferred to glass tubes containing a thin film of 13 nmol 16-S-SL which had been dried on the surface. The tubes were incubated while shaking at 4°C overnight. The samples were pelleted and taken up in capillary tubes for measurement of the ESR spectra as described above. The spectra were measured at 9°C and integrated twice to give the area of the absorbance spectrum, proportional to the amount of spin label in the sample. Since it proved impossible to transfer the DPPG-PMB 5:1 sample quantitatively, the pellets in the capillary tubes were then recovered, redispersed in buffer and aliquots taken for phosphorus analysis. The ratio of the area of the absorption spectrum of the spin label to the amount of lipid phosphorus in the sample was compared for the DPPG and DPPG-PMB 5:1 samples.

Differential scanning calorimetry. Samples were run on a Perkin-Elmer DSC-2 equipped with a Perkin-Elmer data station, at heating and cooling rates of 5–10 C°/min. The temperature of maximum heat absorption was defined as the phase transition temperature, T_m. The areas of the peaks were obtained using the data station. For those samples for which the enthalpy, ΔH, of the phase transition was determined, the amount of lipid in the pan was obtained by phosphorus analysis

[9], after opening the pan, dropping it into 1 ml chloroform/methanol (1 : 1, v/v), and sonicating briefly with a bath sonicator to dissolve the lipid.

Electron spin resonance measurements. ESR spectra were measured on a Varian E-104B spectrometer equipped with a Varian temperature controller and a DEC LSI-11 based microcomputer system. The maximum hyperfine splitting, T_{max}, of the ESR spectra was measured as described earlier [3], and used as a measure of the motional restriction or degree of order of the spin label. The microwave power used was 10 mW. The spectra of samples containing varying amounts of peptide were resolved into components characteristic of interdigitated and non-interdigitated lipid bilayers by pairwise subtraction of spectra of samples of different peptide to lipid ratios from each other by the method of Brotherus et al. [10], except that one spin label at different peptide/lipid ratios was used rather than two spin labels at the same protein/lipid ratio. We have also used this method to resolve spectra of DPPG-Mg^{2+} complexes in different organizational states [11]. The spectra shown in the figures were normalized to the same center peak height for visual comparison. However, they were not normalized for spectral resolution.

The percentage of the probe giving each spectral component was determined by using the equations of Brotherus et al. [10] where A and B are experimental spectra, with integrated intensities a and b, of two samples at different PMB to lipid ratios. If both contain spectral components X and Y, where X is more mobile and characteristic of the non-interdigitated lipid bilayer and Y is motionally restricted and characteristic of the interdigitated lipid bilayer, respectively, the spectral component X can be obtained by subtracting increasing amounts of spectrum B from spectrum A, and the spectral component Y can be obtained by subtracting increasing amounts of spectrum A from B. At the endpoint, $A - u(B) = X$ and $B - v(A) = Y$, where u and v are the amounts subtracted. The integrated intensities x and y of the two end-point spectra X and Y are given by $x = a - ub$ and $y = b - va$. Solving for a and b yield

$$a = (1 - uv)^{-1}x + u(1 - uv)^{-1}v$$

$$b = v(1 - uv)^{-1}x + (1 - uv)^{-1}y$$

in which the first term of each expression gives the contribution of X (i.e. the mobile spectrum) and the second term gives that of Y (i.e. the motionally restricted spectrum). The fractions of the mobile spectrum F_A^X and F_B^X in the two composite spectra A and B are given by

$$F_A^X = (1 - uv)^{-1}x/a = (a - ub)/(1 - uv)a$$

$$F_B^X = v(1 - uv)^{-1}x/b = v(a - ub)/b(1 - uv)$$

The fractions of the motionally restricted spectrum F_A^Y and F_B^Y are then given by $F_A^Y = 1 - F_A^X$ and $F_B^Y = 1 - F_B^X$ and are taken to be equal to the fractions of interdigitated lipid in the samples giving spectra A and B. They were multiplied by 100 to give the percent interdigitated lipid in Table IV. The spectra were baseline corrected as described [24] and integrated twice to give the values a and b. All of the spectra for each lipid at different PMB concentrations were subtracted pairwise in every possible combination to give several values of F^Y for each sample. The range of the values obtained is given in Table IV.

Criteria for judging the endpoint of the subtraction were the appearance of the spectrum, and the maintenance of a zero baseline in the absorption spectrum as described by Jost and Griffith [24]. The endpoint spectra were integrated in order to examine the baseline. With regard to appearance, a single component spectrum which resembled either a motionally restricted powder type spectrum or a more mobile spectrum similar to that of the pure lipid bilayer was sought. Spectra which appeared unrealistic or distorted (e.g. departing from the baseline too sharply) were rejected. The error involved in judging the endpoint was estimated in two different ways. After reaching the best endpoint by varying u or v, these values were increased and decreased again by small amounts until the point at which the result spectrum was clearly not a reasonable endpoint spectrum was reached. The range of values for u and v over which all result spectra appeared to be equally reasonable and valid endpoints was then used to estimate the error of the values for F^Y, the fraction interdigitated lipid, as ± 0.08. The range of values over which the endpoint could be clearly distinguished was also estimated by normalizing the best endpoint spectra giving the motionally restricted and mobile components to the same value of the second integral, and adding them by varying F^X and F^Y in 0.05 increments and comparing to the original spectra. Values of F^X and F^Y which differed by 0.05 from the values obtained from the endpoint subtraction and shown in Table IV, gave spectra which were clearly different from the original spectra, indicating that the effect of differences in the values of F^X and F^Y of ± 0.05 can clearly be distinguished. However, since the choice of the endpoint involves judgement as to what is a reasonable spectrum, we consider the larger error of ± 0.08 obtained by over- and under-subtraction to be more valid.

The mol% PMB-unbound lipid which becomes interdigitated along with the PMB-bound lipid was determined as $F_u^Y/F^Y \times 100$, where F^Y is the mole fraction of the total lipid which is interdigitated, and $F_u^Y = F^Y - F_b^Y$ is the contribution of the PMB-unbound lipid to the mole fraction of the total lipid which is interdigitated. F_b^Y, the contribution of the PMB-bound lipid to the mole fraction of the total lipid which is interdig-

itated, is assumed equal to the mole fraction of total lipid which is PMB-bound.

Results

Effect of varying concentrations of PMB on DPPG and DPPA

The effect of varying concentrations of PMB on the gel to liquid crystalline phase transitions of DPPG and DPPA was determined by DSC, in order to determine what proportion of the total lipid was affected, if phase separation of PMB-bound and unbound lipid occurred, and to determine if PMB has a biphasic effect on the transition temperature as does ethanol, another substance which induces interdigitation [22]. Thermograms for samples of DPPG at pH 7.4 and 9 and DPPA at pH 6 and 9 are compared in Fig. 1 and thermodynamic parameters are given in Table I. When PMB completely saturates DPPG at pH 7.4 or 9, and DPPA at pH 9 (5 : 1 mole ratio of lipid to PMB) it results in two transitions (labeled peaks 1 and 2 in Fig. 1) separated by a few degrees as reported earlier [2,4,12,13] and shown in Fig. 1b, g, m. Transitions 1 and 2 occur at a

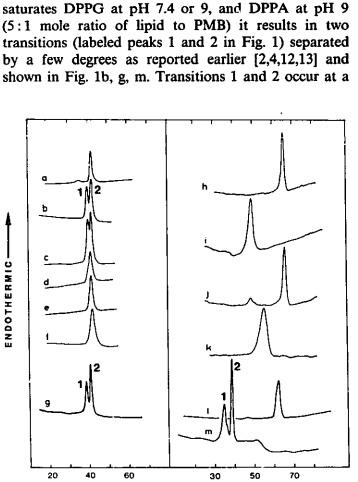

Fig. 1. DSC thermograms of (a) DPPG, pH 7.4; (b) DPPG/PMB 5 : 1, pH 7.4; (c) DPPG/PMB 10 : 1, pH 7.4;(d) DPPG/PMB 25 : 1, pH 7.4; (e) DPPG/PMB 50 : 1, pH 7.4; (f) DPPG/PMB 100 : 1, pH 7.4; (g) DPPG/PMB 5 : 1, pH 9; (h) DPPA, pH 6; (i) DPPA/PMB 5 : 1, pH 6; (j) DPPA/PMB 25 : 1, pH 6; (k) DPPA/PMBN 5 : 1, pH 6; (l) DPPA, pH 9; (m) DPPA/PMB 5 : 1, pH 9. Heating rate 10 C°/min. Sensitivity settings in mcal/s are (a) 1.5; (b) 2.0; (c) 2.5; (d–f) 0.8; (g, h, j) 0.3; (d, i) 0.5. Different amounts of sample were used so the peak areas cannot be directly compared. Enthalpies of some samples are given in Table I.

TABLE I

Effect of PMB and PMBN on the temperatures and enthalpies of the phase transition of DPPG and DPPA

Measured from heating and cooling scans at 10 C°/min.

Sample (mole ratio)		pH	Heating		Cooling [a]
			T_m (°C)	ΔH (kcal/mole)	T_m (°C)
DPPG		7.4	41.1	8.6	41.3
DPPG/PMB	100 : 1	7.4	42.2	8.6	40.1
DPPG/PMB	50 : 1	7.4	41.7	8.5	41.9
DPPG/PMB	25 : 1	7.4	41.6	–	41.3
DPPG/PMB	10 : 1	7.4	39.8, 41.5	10.6	39.3
DPPG/PMB	5 : 1	7.4	40.2, 42.3	10.6	38.9, 40.4
DPPG/PMBN	5 : 1	7.4	42.1	12.3	41.9
DPPA		6	65.7	9.4	64.8, 66.3
DPPA/PMB	25 : 1	6	48.6, <u>66.1</u> [b]	–	66.3
DPPA/PMB	5 : 1	6	49.3	10.5	44.4
DPPA/PMBN	5 : 1	6	55.3	11.3	52.6
DPPG/PMB	5 : 1	9	38.5, 40.7	–	37.9, 40.4
DPPA		9	58.5	–	58.6
DPPA/PMB	5 : 1	9	<u>35, 38, 51</u> [b]	–	<u>33, 35, 51</u> [b]
DPPA/PMBN	5 : 1	9	<u>39.3</u>	–	<u>36.5, 39.2</u> [b]

[a] T_m on cooling corrected for instrumental hysteresis by addition of 4.5°.

[b] Major peak is underlined.

4–5 C° lower temperature for the DPPA-PMB complex than for the DPPG-PMB complex at pH 7.4. However, at pH 9 transitions 1 and 2 of the DPPG-PMB complex occur at a 1.5 – 2 C° lower temperature than at pH 7.4. Thus at pH 9, the transitions of the DPPG-PMB complex are only 2.7–3.5 C° higher than for DPPA-PMB.

Although transition 2 of the PMB-DPPG complex occurs at a similar temperature as the gel to liquid crystaline phase transition of unbound DPPG (Fig. 1a), it is not caused by unbound DPPG. This is evident from the fact that transition 2 of the DPPA-PMB complex at pH 9 occurs at a much different temperature from unbound DPPA (Fig. 1l) and from the fact that an increase in pH lowers the transition temperature of both transitions 1 and 2 of the DPPG-PMB complex (Table I), even though it has no effect on the transition temperature of pure DPPG. Transition 1 of the PMB-DPPA complex has been attributed to PMB molecules interacting only hydrophobically with lipid, while transition 2 has been attributed to PMB molecules interacting both hydrophobically and electrostatically with lipid [12]. A similar explanation seems reasonable for DPPG. Both transitions 1 and 2 are also observed for DPPG/PMB at a 10 : 1 mole ratio (Fig. 1c). However, at lower concentrations of PMB, only one peak is observed at a similar temperature as pure DPPG but of greater width (Fig. 1d–f). The greater width is probably due to the contribution of peak 1 of the PMB-bound lipid, while peak 2 of the bound lipid coincides with the transition of unbound lipid. This suggests that at non-

TABLE II

Distribution of lipid on a sucrose density gradient

Sample (mole ratio)	% lipid at different sucrose levels			
	0–25% [a] sucrose	30% sucrose	35% sucrose	40% sucrose
DPPG [b]	94.0	3.0	1.4	1.0
DPPG/PMB 50:1	94.0	3.7	0.8	1.2
DPPG/PMB 25:1	84.0	11.6	2.8	1.2
DPPG/PMB 10:1	38.2	57.6	2.6	1.6
DPPG/PMB 5:1	0	1.1	96.2	2.1

	% lipid at different sucrose levels					
	0–20% sucrose	25% sucrose	top of 30% sucrose	middle of 30% sucrose	bottom of 30% sucrose	35% sucrose
DPPA [c]	4.5	4.2	88.0	1.6	1.0	0.7
DPPA/PMB 25:1	0.2	0.4	94.0	2.5	1.3	1.5
DPPA/PMB 10:1	0.3	0.2	2.2	94.0	2.0	1.3
DPPA/PMB 5:1	0.6	0.2	2.0	14.4	80.0	2.8

[a] Was collected as 1 layer because some DPPG was distributed throughout it.
[b] pH 7.4.
[c] pH 6. Samples at pH 9 behaved similarly.

saturating concentrations, PMB has the same effect on DPPG as at saturating concentrations, but only a fraction of the lipid is affected.

Because of the similarity of the temperatures of transition 2 of the PMB-bound DPPG and the transition of unbound DPPG it could not be determined if phase separation of two populations of this lipid occurs from the DSC scans. However, centrifugation on a discontinuous sucrose gradient revealed that there were at least two populations of vesicles of different density in the 10:1 and 25:1 samples (Table II). 62% and 16% of the lipid in these samples, respectively, sedimented to or below the 30% sucrose level, in contrast to 98% for the 5:1 sample. This indicates that some phase separation does occur. The density of the heavier population in the 10:1 and 25:1 samples was not as great as that in the 5:1 sample, however. It sedimented to the 30% sucrose level for the 10:1 and 25:1 samples in contrast to the 35% level for the 5:1 sample. Also, the density of at least some of the remaining 38% and 84% of the lipid of the 10:1 and 25:1 samples, respectively, which remained above the 30% sucrose level, appeared greater than that of most of the DPPG, indicating that PMB was bound to some of this population also. However, unbound DPPG could not be separated from this population on the sucrose gradient sufficiently to quantitate the contribution of PMB-unbound and bound lipid to it. DPPG by itself was distributed throughout the 20% and 25% sucrose levels. Furthermore, since these are multilayered complexes, bilayers of pure DPPG may be trapped inside bilayers of DPPG-PMB altering the density, and making it impossible to completely separate these populations. Thus sucrose density gradient centrifugation reveals that some phase separation of two populations containing different amounts of PMB occurs in the 10:1 and 25:1 samples but does not delineate the degree of this phase separation or demonstrate if separate bilayers of unbound DPPG are also present.

At pH 6, the gel to liquid crystalline phase transition temperature of pure DPPA is 7 C° higher than at pH 9 as a result of increased intermolecular hydrogen bonding interactions [19]. Interestingly, the complex of DPPA with PMB at a 5:1 mole ratio (Fig. 1i) has only a single gel to liquid crystalline phase transition, in contrast to the double transition observed for DPPG-PMB and for DPPA-PMB at pH 9. On heating, its transition temperature is 7–9 C° higher than that of the DPPG-PMB complex. There is greater hysteresis between heating and cooling scans for the DPPA-PMB samples than for DPPG, so that on cooling the difference in transition temperature between the PMB complexes of DPPG and DPPA is reduced to 4–6 C° (Table I). Although the transition temperature of the DPPA-PMB complex is higher than that of the DPPG complex, it is considerably less than that of pure DPPA (Fig. 1h), so that the transition of the PMB-bound lipid can be distinguished from that of free lipid. The fact that only the transition of the DPPA-PMB complex is observed and there is no peak at the transition temperature of pure DPPA at a 5:1 ratio indicates that all of the lipid is affected, suggesting that all of the PMB added is bound. At lower concentrations of PMB, two transitions are observed, the lower temperature one corrresponding to the

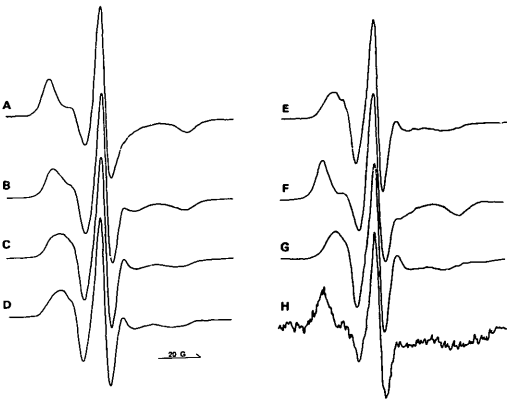

Fig. 2. ESR spectra of 16-doxyl-stearic acid at 9°C and pH 7.4 in (A) DPPG/PMB 5:1; (B) DPPG/PMB 10:1; (C) DPPG/PMB 25:1; (D) DPPG/PMB 50:1; (E) DPPG only; (F) the results of subtracting the spectrum of the 25:1 sample from that of the 10:1 sample; (G) the result of subtracting the spectrum of the 10:1 sample from that of the 25:1 sample; (H) the result of subtracting the spectrum of the 50:1 sample from that of the 25:1 sample. All spectra shown are plotted normalized to the same center peak height.

DPPA-PMB complex and the higher temperature one to pure DPPA, as shown for a 25:1 mole ratio in Fig. 1j, indicating phase separation of free and bound DPPA. Similar results occurred at pH 9.

At this mole ratio a maximum of 20% of the DPPA should be PMB-bound, assuming that PMB binds to the lipid in a 1:5 mole ratio. The area of the lower temperature transition in Fig. 1j is about 23% of the total. After correction for the difference in enthalpy of the PMB-bound DPPA and unbound DPPA (Table I), this indicates that 20% of the lipid contributes to the lower temperature peak. Centrifugation of these samples on a discontinuous sucrose density gradient revealed that almost none of the lipid in the 25:1 sample had a density similar to that of the 5:1 complex (Table II), however. Almost all of the lipid sedimented in a single sharp band to the same level as unbound DPPA. The 10:1 sample, which gave two transitions of approximately equal area at 49°C and 66°C (not shown), sedimented as a single sharp band halfway between those corresponding to unbound DPPA and the 5:1 complex. This suggests that either the populations giving the two peaks in the thermograms are not in separate bilayers, or that bilayers of DPPA/PMB 5:1 are traped within bilayers of pure DPPA in the multilayered complexes, thus decreasing the density.

In order to determine if these samples contain some interdigitated bilayer, they were labeled with 16-doxyl-stearic acid and the spectra were measured at 9°C, well below the temperatures of their gel to liquid crystalline phase transitions. We have already shown that we can detect the interdigitated bilayer formed by DPPG/PMB 5:1 using this probe [4,7,25]. Fig. 2A shows the characteristic powder spectrum typical of 16-doxyl-stearic acid in an interdigitated bilayer of DPPG-PMB at a 5:1 mole ratio. The large T_{max} value of 30.3 G, compared to 24 G for pure DPPG (Fig. 2E), indicates that the spin label is either more motionally restricted or more ordered than in the pure lipid. In this type of bilayer this probe behaves similarly to a spin label with the nitroxide group much closer to the carboxyl group. This occurs because the nitroxide group on 16-doxyl-stearic acid is located in the thinner interdigitated bilayer relatively close to the apolar/polar interface. PMB causes this motional restriction of the spin label only in the gel phase of the lipid, not in the liquid crystalline phase [4]. PMB free in solution, in the presence or absence of a limited amount of lipid, does not affect its spectrum, suggesting it does not even bind to it.

PMB added to DPPG in a 1:5 mole ratio over the concentration range 2 μM to 4 mM, caused similar motional restriction of the probe, indicating that it

TABLE III

T_{max} values of motionally restricted (R) and mobile (M) components of ESR spectra after spectral resolution by pairwise subtraction

16-Doxyl-stearic acid was used at pH 6 and 7.4, and methyl 16-doxyl-stearate was used at pH 9. n.d., not determined.

Sample		T_{max} (G)					
Lipid/drug (mol/mol):		5:1 [a]	10:1		25:1		50:1 [a]
		R	R	M	R	M	M
DPPG/PMB	pH 7.4	30.3	29.7	25.7	29.0	24.0	23.6
DPPG/PMBN	pH 7.4	29.9	28.7	24.3	26.2	23.9	23.8
DPPA/PMB	pH 6	30.8	28.4	22.9	none	22.9	23.0
DPPA/PMBN	pH 6	27.1	26.3	23.2	none	23.0	23.1
DPPA/PMB	pH 9	30.9	30.7	27.2	30.8	27.2	26.5
DPPA/PMBN	pH 9	30.8	30.7	29.0	30.7	26.7	25.6
DPPG/PMB	pH 9	30.7	29.7	26.0	[a]	24.6	n.d.

n.d. not determined.

[a] A mobile component cannot be obtained from the spectrum of the 5:1 sample and a restricted component cannot be obtained from the spectrum of the 50:1 sample by pairwise subtraction using only the concentrations shown.

caused interdigitation over this concentration range. However, the percentage of lipid which was interdigitated decreased somewhat at the lower PMB concentrations. The lowest concentration used is similar to that which completely inhibits bacterial growth [29]. The apparent dissociation constant for PMB interacting with PG monolayers has been reported as less than $2.5 \cdot 10^{-7}$ M [30].

Spectra of the 10:1, 25:1, and 50:1 complexes are shown in Fig. 2B–D. The spectra of the 10:1 and 25:1 complexes appear to have a significant amount of a motionally restricted component also. Since there is no reason why this spin label should become motionally restricted in these samples by a different mechanism than for the 5:1 ratio sample, this motionally restricted component in the spectra at lower PMB concentrations indicates that a large percentage of the lipid in these samples forms an interdigitated bilayer. However, it is difficult to tell by visual inspection of the spectrum of the 50:1 complex if it also has a motionally restricted component.

Quantitation of percentage of interdigitated lipid

The motionally restricted spectrum characteristic of the interdigitated bilayer (Fig. 2A) and the more mobile spectrum characteristic of the non-interdigitated bilayer (Fig. 2E) are sufficiently different from each other that the composite spectra at lower PMB concentrations can be resolved into their motionally restricted and more mobile spectral components by spectral subtraction. In order to avoid finding suitable reference spectra for these spectral components, the spectra of the DPPG/PMB 5:1, 10:1, 25:1, and 50:1 samples were resolved by subtracting them from each other in a pairwise incremental fashion until a spectrum was obtained which appeared to be single component and relatively similar to the spectrum of either the pure lipid or the 5:1 complex, as described in Methods. For example, the results of subtracting the spectra of the 10:1 and 25:1 complexes from each other are shown in Fig. 2F and G and clearly show that both samples have a significant amount of a motionally restricted component (R) as well as a more mobile component (M). The T_{max} values of the mobile component of the 25:1 and 50:1 complexes are similar to that of the pure lipid, while that of the 10:1 complex is a little greater (Table III). The motionally restricted component in the 10:1 sample is also not identical to that in the 5:1 sample; it has a smaller T_{max} value. However, subtraction of larger amounts of the other spectra from the 10:1 spectrum did not give a realistic mobile spectral component with a smaller T_{max} value nor a realistic motionally restricted component with a larger T_{max} value. The motionally restricted component present in the 25:1 sample, obtained by subtraction of the spectrum of the 50:1 complex, is shown in Fig. 2H. It has an even smaller T_{max} value than that in Fig. 2F. However, the motionally restricted spectral components in both the 10:1 and 25:1 samples indicate the presence of an interdigitated domain of lipid in these samples. The pairwise subtraction method gave more realistic endpoint spectra than subtraction of the spectra of the 5:1 complex or pure DPPG from the other spectra because of these small variations in the T_{max} values of the spectral components with PMB concentration.

The spectral resolution allowed quantitation of the percentage of each spectral component in the sample spectrum. The percent of the motionally restricted component in the spectrum can be taken to give the fraction of the total lipid which is interdigitated, provided that the probe distributes equally in the interdigitated and non-interdigitated domains. Attempts were made to verify this assumption by adding non-interdigitated DPPG vesicles to interdigitated DPPG-PMB 5:1

TABLE IV

Percentage of DPPG or DPPA which is interdigitated at varying ratios of PMB or PMBN, at 9°C

Sample	% motionally restricted spin label = % interdigitated lipid			
Lipid/drug (m/m)	5:1	10:1	25:1	50:1
Mol% bound lipid [a]	100	50	20	10
DPPG/PMB pH 7.4 [b]	93–95	66–67	30–35	15–24
DPPA/PMB pH 6 [c]	65–73	41–46	0	0
DPPG/PMBN pH 7.4 [c]	64–69	28–34	[d]	0
DPPA/PMBN pH 6 [c]	[d]	[d]	0	0
DPPG/PMB pH 9 [b]	90–91	62–67	43–44	n.d. [e]
DPPA/PMB pH 9 [b]	90–97	76–79	59–62	38–40
DPPA/PMBN pH 9 [b]	80–84	64–68	44–46	24–28

[a] Mol% lipid which is maximally bound to PMB or PMBN, assuming they bind to all lipids in a 1:5 mole ratio.

[b] The range of values shown was obtained by subtraction of different combinations of spectra.

[c] The range of values shown was obtained by subtraction of the 25:1 and 50:1 spectra from the spectrum indicated.

[d] Inconclusive. See text.

[e] n.d., not determined.

vesicles and measuring the transfer of 16-S-SL from one to the other. However, the DPPG vesicles rapidly aggregated with the DPPG-PMB 5:1 vesicles. The ESR spectrum of the mixture was identical to that of the 10:1 complex rather than the sum of the spectra of the DPPG and DPPG-PMB 5:1 samples, regardless of whether the probe was initially in the DPPG or the DPPG-PMB 5:1 vesicles. The mixture also behaved like the 10:1 complex on sucrose density centrifugation. These results suggest that fusion of the two types of vesicles occurred. DPPC vesicles were then used as the non-interdigitated bilayers and added to DPPG-PMB 5:1 vesicles. Sucrose density gradient centrifugation verified that the two types of vesicles remained separate. However, the transfer of the probe from one type to the other was so slow that very little transfer had occurred even after overnight incubation with shaking at 4°C, regardless of which type of vesicle initially contained the spin label. Transfer of the spin label from the surface of a glass tube to each type of vesicles, DPPG or DPPG/PMB 5:1, was then measured. This transfer takes place rapidly. The DPPG vesicles took up only 1.25-times more spin label per μmol of lipid after overnight incubation at 4°C than the DPPG/PMB 5:1 vesicles.

The percent interdigitated lipid determined by spectral resolution is given in Table IV. The subtraction was often not perfect due to differences in the restricted and mobile components at different PMB concentrations, and it was sometimes difficult to determine the end point, leading to the range of values given in Table IV. However, repetition with a different set of samples gave a similar range of values. As discussed in the Methods the accuracy of the values for % interdigitated lipid in Table IV is estimated as ±8%. The results indicate that at less than saturating concentrations of PMB, considerably more DPPG than that which is PMB-bound is interdigitated. Thus a significant amount of the unbound lipid becomes interdigitated along with the PMB-bound lipid. At a 10:1 mole ratio, the amount of PMB-unbound DPPG which can be incorporated into and become interdigitated in the interdigitated bilayer of PMB-bound lipid is 24 mol%.

Spectra of 16-doxyl-stearic acid in complexes of DPPA with PMB at pH 6 at 9°C are shown in Fig. 3. Visual comparison of Fig. 2A–C with Fig. 3B–D for 5:1, 10:1, and 25:1 samples clearly indicates that less

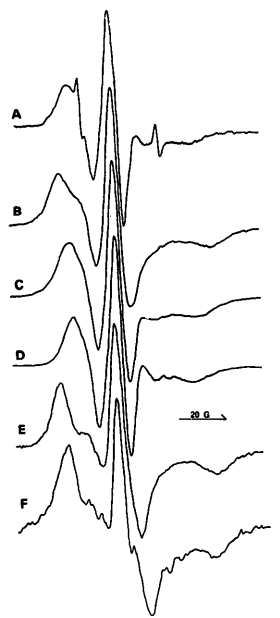

Fig. 3. ESR spectra of 16-doxyl-stearic acid at 9°C and pH 6 in (A) DPPA; (B) DPPA/PMB 5:1; (C) DPPA/PMB 10:1; (D) DPPA/PMB 25:1; (E) the result of subtracting the spectrum of the 25:1 sample from that of the 5:1 sample; (F) the result of subtracting the spectrum of the 25:1 sample from that of the 10:1 sample. All spectra shown are normalized to the same center peak height.

probe is motionally restricted in DPPA-PMB at pH 6 than in DPPG-PMB samples. Only the spectrum of the 5:1 complex (Fig. 3B) clearly reveals a motionally restricted component. This component, resolved by subtraction of the spectrum of the 25:1 complex (Fig. 3D), is shown in Fig. 3E. The T_{max} value is 30.8 G, similar to that for the 5:1 complex of DPPG, and much different from the T_{max} value of 23 G in pure DPPA at pH 6 (Fig. 3A). This motional restriction indicates that PMB also induces interdigitation of DPPA at pH 6. It is unlikely that motional restriction of this probe could occur in this sample by any other mechanism. Subtraction of the 50:1 spectrum from the 25:1 spectrum did not reveal a motionally restricted component indicating that none of the lipid in the 25:1 sample is interdigitated. However, subtraction of the 25:1 or 50:1 spectra from the 10:1 spectrum (Fig. 3C) gave the motionally restricted component shown in Fig. 3F. Its T_{max} value is less than that of the 5:1 complex (Table III) but still characteristic of motional restriction, suggesting that some of the lipid in the 10:1 sample may be interdigitated. The percentage interdigitated lipid obtained from the amount of motionally restricted spin label in the 5:1 and 10:1 complexes is shown in Table IV. It is considerably less than found for DPPG and is even less than the maximum amount of DPPA which is PMB-bound and contributing to the phase transition at 49°C in Fig. 1.

Motional restriction of 16-doxyl-stearic acid in the 5:1 DPPA-PMB complexes was also observed at pH 9. However, the T_{max} value is difficult to measure at this pH because the fatty acid spin label becomes ionized and much more water soluble, resulting in a low concentration of spin label in the bilayer. Therefore, the methyl ester of 16-doxyl-stearic acid, was also used at pH 9. The spectra of this spin label in DPPA and DPPA/PMB 5:1 at pH 9 are shown in Fig. 4. The T_{max} value of methyl 16-doxyl stearate in PMB/DPPA at pH 9 is similar to its value in this complex at pH 6 and similar to that of 16-doxyl-stearic acid in these samples (Table III). This motional restriction indicates that PMB induces interdigitation of DPPA at pH 9. The spectrum of the 5:1 complex in Fig. 4B indicates that at pH 9, almost all of the spin label is motionally restricted at 9°C.

The spectra of methyl 16-doxyl-stearate in 25:1 and 50:1 DPPA-PMB complexes at pH 9 are also shown in Fig. 4C and D and reveal a significant amount of a motionally restricted component in these samples also, in contrast to results at pH 6. Visual comparison of the spectra in Fig. 4B–D with Fig. 2A, C, D for the 5:1, 25:1, and 50:1 samples of DPPG, suggests that even more probe is motionally restricted in DPPA at pH 9 than in DPPG at pH 7.4. The spectra were resolved into components as described above. The motionally restricted component present in the 25:1 sample, obtained by subtraction of the spectrum of the 50:1 sample is shown in Fig. 4E. The T_{max} values of the motionally restricted and more mobile components were similar at all PMB concentrations (Table III). However, the T_{max} value of the more mobile component (e.g. Fig. 4F) is greater than that of pure DPPA at pH 9 (shown in Fig. 4A). The mobile component shown in Fig. 4F was obtained by subtraction of the spectrum of the 5:1 sample from the spectrum of the 50:1 sample, and may still contain more than one component. However, subtraction of greater amounts of the 5:1 spectrum did not yield a realistic appearing spectrum nor one which more closely resembled that of the pure lipid in Fig. 4A.

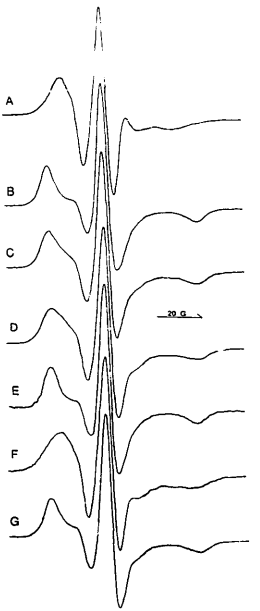

Fig. 4. ESR spectra of methyl 16-doxyl-stearate at 9°C, in (A) DPPA; (B) DPPA/PMB 5:1; (C) DPPA/PMB 25:1; (D) DPPA/PMB 50:1; (E) result of subtracting the spectrum of the 50:1 sample from that of the 25:1 sample; (F) result of subtracting the spectrum of the 5:1 sample from that of the 50:1 sample; (G) DPPA/PMBN 5:1. All spectra shown are normalized to the same center peak height.

Subtraction of the spectra of the 10:1 and 25:1 samples from the 50:1 sample also yielded a similar mobile spectral component to that in Fig. 4F. The mobile component present in the 10:1 and 25:1 spectra was also similar. Thus, the unbound lipid in the samples with PMB is more ordered than the pure lipid. This may be a result of differences in the content of singly and double ionized species of DPPA in the presence and absence of PMB at this pH.

The spectral resolution allowed determination of the percentage motionally restricted probe and interdigitated lipid, shown in Table IV. The results confirm that at pH 9 much more DPPA is interdigitated than at pH 6 or than for DPPG at pH 7.4. At non-saturating PMB concentrations considerably more lipid than that which is PMB-bound is interdigitated. At a 10:1 mole ratio the amount of PMB-unbound DPPA which can be incorporated into and become interdigitated in the interdigitated bilayer of PMB-bound lipid is 35 mol%. In order to determine the contribution of the difference in the spin labels used and the effect of pH on PMB itself to the difference between DPPG at pH 7.4 and DPPA at pH 9, the percentage interdigitated lipid in DPPG-PMB samples at pH 9 was also determined using methyl 16-doxyl-stearate. The results shown in Table IV indicate that the percentage interdigitated DPPG at pH 9 is similar to that at pH 7.4 except at the lowest concentration studied, where it is greater at pH 9.

Effect of PMBN on DPPG and DPPA

We reported previously that the complex of PMBN with DPPG results in motional restriction of 16-doxyl-stearic acid, indicating that it also causes interdigitation at low temperatures. The effect of different concentrations of PMBN on the amount of DPPG induced to interdigitate was studied for further comparison of its ability to induce interdigitation with that of PMB. Spectra of 16-S-SL in DPPG-PMBN at mole ratios of 5:1, 10:1, and 25:1 are shown in Fig. 5A–C. The spectrum of the 50:1 sample resembled that of the 25:1 sample. Comparison with spectra of DPPG-PMB at similar mole ratios in Fig. 2A–C clearly indicate that there is less motionally restricted component present in the spectra of the DPPG-PMBN samples than in the DPPG-PMB samples, even at a 5:1 ratio. This is confirmed by resolution of the spectra into motionally restricted and mobile components and quantitation of the fraction of the spin label contributing to each. The motionally restricted and more mobile components present in the 5:1 and 10:1 spectra, obtained by subtraction of these spectra from each other, are shown in Fig. 5D, E, respectively, and are similar to the motionally restricted and mobile components found in the DPPG-PMB samples.

The percentage interdigitated lipid (equivalent to the percentage motionally restricted probe) in PMBN-DPPG complexes at different concentrations of PMBN is compared to that for PMB in Table IV and indicates that PMBN does not cause as much lipid to become interdigitated as PMB. Indeed in the case of the 25:1 complex, although two components were present in the spectrum, the T_{max} values of both were well below those of the motionally restricted components of the 5:1 or 10:1 complexes (Table III), suggesting that neither component in the 25:1 complex results from interdigitated lipid. However, if it is assumed that the component with T_{max} value of 26.2 G in the 25:1 complex is caused by interdigitated lipid, then the maximum amount of this phase could be only 15% (the percent of the probe giving this component). In any case, the percentage of interdigitated DPPG in the PMBN com-

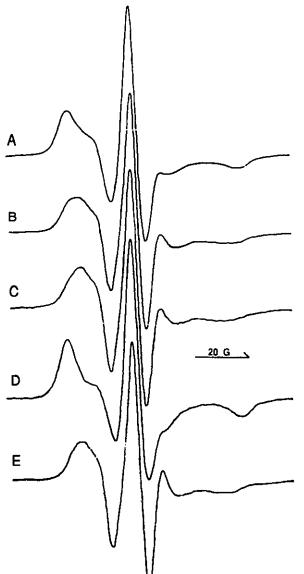

Fig. 5. ESR spectra of 16-doxyl-stearic acid at 9 °C and pH 7.4 in (A) DPPG/PMBN 5:1; (B) DPPG/PMBN 10:1; (C) DPPG/PMBN 25:1; (D) result of subtracting the spectrum of the 10:1 sample from that of the 5:1 sample; and (E) result of subtracting the spectrum of the 5:1 sample from that of the 10:1 sample. All spectra shown are normalized to the same center peak height.

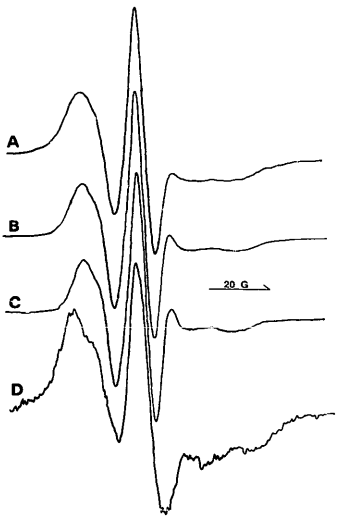

Fig. 6. ESR spectra of 16-doxyl-stearic acid at 9°C, pH 6 in (A) DPPA/PMBN 5:1; (B) DPPA/PMBN 10:1; (C) DPPA/PMBN 25:1; (D) the result of subtracting the spectrum of the 25:1 sample from that of the 5:1 sample. All spectra shown are normalized to the same center peak height.

plexes is considerably less than the percentage of PMBN-bound lipid even at a 5:1 ratio.

The spectrum of methyl 16-doxyl-stearate in PMBN-DPPA at pH 9 is shown in Fig. 4G. The T_{max} value is 30.8 G (Table III) indicating that PMBN also causes interdigitation of DPPA at pH 9. Spectral resolution indicates that the proportion of DPPA induced to interdigitate at pH 9 is a little less than that with PMB but considerably more than for DPPG (Table IV).

The complex of PMBN with DPPG or DPPA has only a single gel to liquid crystalline phase transition at all pH values. In the case of DPPG or DPPA at pH 9, its temperature is similar or a little higher than that of peak 2 of the PMB complex [7]. In contrast, at pH 6 its temperature for the PMBN-DPPA complex is 55°C (Fig. 1k), 6 C° higher than for the PMB complex in this pH range (Fig. 1i). The spectra of 16-doxyl-stearic acid in complexes of PMBN with DPPA at pH 6 at 9°C are shown in Fig. 6A–C. The apparent T_{max} value of the unresolved spectrum of the 5:1 complex (Fig. 6A) is 24.5 G, considerably less than for this complex at pH 9 or the PMB-DPPA complex at pH 6, indicating that much less of the spin label is motionally restricted by PMBN at pH 6. The spectra at lower concentrations of PMBN resemble that of the pure lipid (Fig. 3A). Thus visual inspection of the spectra and comparison with those of DPPA-PMB at pH 6 (Fig. 3B, C) clearly indicate that at 5:1 and 10:1 ratios less DPPA is interdigitated with PMBN than with PMB at pH 6. Subtraction of the spectrum of the 25:1 complex (Fig. 6C) from that of the 5:1 complex (Fig. 6A) gave the spectrum shown in Fig. 6D. This spectrum is indicative of exchange broadening and is still multicomponent, but has a component with a T_{max} value of at least 27.2 G. This may be sufficient motional restriction to indicate the presence of an interdigitated domain. If this motional restriction is indeed caused by an interdigitated domain, quantitation of the amount of spin label giving this spectral component indicates that the maximum size of this domain is 59% of the total lipid. This is probably an over-estimate because of the presence of another more mobile component in Fig. 6D. Resolution of the spectrum of the 10:1 complex also gave two components (not shown), one typical of the pure lipid, the other with a T_{max} value of only 26.2 G (Table III). This is less likely to be sufficient motional restriction to result from interdigitation than that in Fig. 6D. If it does result from interdigitation, however, the maximum amount of interdigitated lipid is 23% of the total lipid. These maximal values for % interdigitated lipid are less than those found for PMB at 5:1 and 10:1 ratios. Therefore, PMBN is less effective at inducing interdigitation of DPPA at pH 6 than PMB. No component other than that of the pure lipid could be detected at lower concentrations of PMBN similar to results found with PMB.

Effect of increase in temperature

We reported earlier that the apparent T_{max} value of the complexes of DPPG with PMB and PMBN approaches that of the pure lipid with increase in temperature as the phase transition temperature is approached, suggesting that these drugs do not maintain complete interdigitation up to the gel to liquid crystalline phase transition [7]. This contrasts with the effect of glycerol, another amphipathic substance which induces interdigitation [26]; it maintains motional restriction up to the phase transition temperature [4]. We showed that the temperature at which the T_{max} value decreases is lower for the PMBN complex than for PMB suggesting that PMBN does not maintain interdigitation at higher temperatures as effectively as PMB [7]. In this study we resolve the spectra to confirm that this drop in T_{max} value is caused mainly by a decrease in the percentage of the lipid which is interdigitated and only partly by a decrease in the T_{max} value of the spin label in the

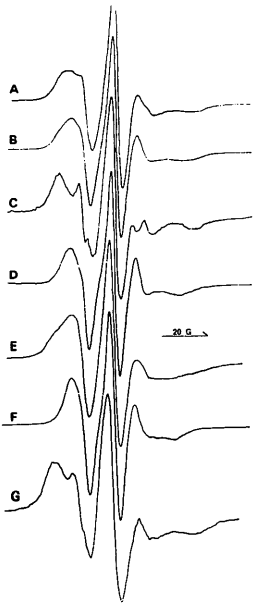

Fig. 7. (A–D) ESR spectra of 16-doxyl-stearic acid at 29°C, pH 7.4 in (A) DPPG/PMB 5:1; (B) DPPG/PMBN 5:1; (C) the result of subtracting the spectrum of the PMBN sample from that of the PMB sample; (D) the result of subtracting the spectrum of the PMB sample from that of the PMBN sample. (E–G) ESR spectra at 29°C of 16-doxyl-stearic acid at pH 6 in (E) DPPA/PMB 5:1; (F) DPPA/PMBN 5:1; (G) the result of subtracting the spectrum of the PMBN sample from that of the PMB sample. All spectra shown are normalized to the same center peak height.

interdigitated bilayer at higher temperatures. The spectra of 16-doxyl-stearic acid in the 1:5 complexes of PMB-DPPG and PMBN-DPPG at 29°C are shown in Fig. 7A and B, respectively. Substraction of the spectra in A and B from each other gives the motionally restricted and mobile components shown in Fig. 7C and D, respectively. The T_{max} value of the motionally restricted component is 27.7 G which is similar to that which was found in the interdigitated bilayer of DPPC in the presence of glycerol at this temperature [4]. The T_{max} value of the mobile component is 22.2 G, similar to that of the pure lipid at this temperature. The results indicate that the PMB sample is still 64% interdigitated at this temperature while the PMBN sample is only 38% interdigitated. This result confirms that much more DPPG remains interdigitated at higher temperatures in the PMB sample than in the PMBN sample.

At 29°C, spectra of the 1:5 PMB-DPPA and PMBN-DPPA complexes at pH 6 indicate that a motionally restricted component is still present for the former but is not apparent for the latter (Fig. 7E and F). Subtraction of the spectrum in Fig. 7F from than in E gives the motionally restricted component shown in Fig. 7G with T_{max} value of 28.1 G. If it is assumed that the PMBN sample is not at all interdigiated at this temperature, then the PMB sample is still 63% interdigitated at 29°C, not much less than at 9°C. At higher temperatures, however, the percent interdigitated lipid in the PMB-DPPA sample decreases as found for DPPG.

Discussion

If a lipid which tends to be interdigitated is mixed with a lipid which normaly tends to be non-interdigitated, the bilayer may not be stable if both interdigitated and non-interdigitated domains remain in the same bilayer, particularly if there are many small domains of each type. This would result in exposure of portions of the acyl chains to water. One of three events is likely to occur as shown in Fig. 8. (i) The presence of the interdigitated lipid influences some of the other lipid to also become interdigitated in order to remain in the same bilayer domain (Fig. 8a). Or (ii) the presence of the non-interdigitated lipid influences some of the other lipid to also become non-interdigitated (Fig. 8b). Or (iii) the interdigitated lipid and the non-interdig-

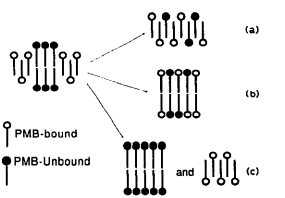

Fig. 8. Diagrammatic representation of changes in bilayer organization of symmetric 2-chain lipids (only 1 chain is shown for simplicity) which may occur following mixing of a lipid which, by itself, tends to form an interdigitated bilayer (PMB-bound lipid, open headgroups) with a lipid which, by itself, tends to form a non-interdigitated bilayer (unbound lipid, closed headgroups). Either (a) the unbound lipid interdigitates along with the PMB-bound lipid; or (b) the bound lipid becomes non-interdigitated along with the unbound lipid; or (c) the two lipids become phase separated into their own bilayer structures.

itated lipid phase separate into distinct large domains or into separate bilayer structures (Fig. 8c). Which of these events occurs will depend on the strength of the forces stabilizing the non-interdigitated and the interdigitated bilayers. In this study we have attempted to investigate these forces using PMB-bound lipid as the interdigitated lipid, and unbound lipid, either DPPG or DPPA, as the non-interdigitated lipid. In the following study we use phosphatidylcholine as the non-interdigitated lipid [27]. Interdigitation of the unbound lipid should be stabilized by increasing repulsive negative charge of the lipid since the surface charge density is lower in the interdigitated bilayer. Formation of a non-interdigitated bilayer by the unbound lipid, on the other hand, should be stabilized by participation of the lipid in lateral interlipid hydrogen bonding or by other means of cross-linking the lipid headgroups, e.g. with divalent cations, since this requires closer packing of the headgroups than can occur in the interdigitated bilayer.

We have used the motional restriction of 16-doxyl-stearic acid in the interdigitated bilayer as a means of quantitating the amount of the interdigitated lipid. We have shown previously that this spin label is motionally restricted in a number of interdigitated bilayers [4,5,7,25] and argue that it is very unlikely that motional restriction of this degree could be caused by any other mechanism in the samples studied here. PMB has been shown to cause interdigitation using X-ray diffraction, even in lipid mixtures [1,2]. Although there is a large error involved in resolution of the spectra and quantitation of the percentage of the probe which is motionally restricted, the quantitative results of the spectral resolution are consistent with conclusions resulting from qualitative comparison of the spectra of different lipid-PMB complexes. These are that PMB induces more DPPG to become interdigitated than DPPA at pH 6, more DPPA to become interdigitated at pH 9 than at pH 6, and more DPPA to become interdigitated than DPPG at pH 9.

Determination of the percentage interdigitated lipid from the percentage of the spin label which is motionally restricted requires the assumption that the spin label is randomly distributed in the PMB-bound and unbound lipid and in interdigitated and non-interdigitated bilayer domains. At neutral pH, 16-doxyl-stearic acid is readily soluble in both interdigitated and non-interdigitated bilayers of DPPG and PMB-DPPG while at high pH, methyl 16-doxyl-stearate is readily soluble in DPPA and PMB-DPPA. There are no sharp peaks indicating spin label in the aqueous phase and no indication of exchange broadening in these samples. The spin label transferred from the surface of a glass tube 25% less into interdigitated DPPG-PMB vesicles than into non-interdigitated DPPG vesicles. This relatively small difference is not necessarily due to a solubility difference of the probe in interdigitated and non-interdigitated bilayers, however. It may be caused by other factors such as a greater exposed surface area of the DPPG vesicles relative to the DPPG-PMB vesicles, which form a highly aggregated multilayered complex. Under the conditions of the measurement of the spectra of the DPPG-PMB complexes at varying concentrations of PMB, where the spin label is present initially in all of the lipid, it seems unlikely that the small solubility difference observed from the transfer experiment, would cause the probe to preferentially distribute to the non-interdigitated domains. If it does, however, then the results of the spectral resolution may underestimate the amount of interdigitated lipid for DPPG-PMB samples.

The spin labels are not as soluble in DPPA at neutral pH because of the intermolecular hydrogen bonding interactions and possible closer packing of this lipid. This leads to a lower order parameter than expected for this lipid and freezing out of some spin label into the aqueous phase or into domains, causing some degree of exchange broadening of the spectrum. The spin labels are more soluble in the PMB-DPPA complex, however, as a result of the weakening of the interlipid hydrogen bonding interactions after binding of PMB. At non-saturating concentrations of PMB, this lower solubility of the probe in unbound DPPA would, if anything, lead to its preferential distribution in the interdigitated PMB-DPPA bilayer at the expense of unbound DPPA and an overestimation of the amount of interdigitated bilayer for this sample. Since the results indicate that DPPA is less interdigitated at pH 6 than DPPG, non-random distribution of the probe in these samples cannot be the cause of the larger value for the % interdigitated lipid in DPPG or DPPA at pH 9 relative to DPPA at pH 6.

The quantitative results of the spectral resolution further indicate that PMB can induce significantly more DPPG and DPPA (at pH 9) to interdigitate than that to which it can bind maximally (model a in Fig. 8). X-ray diffraction results showed that at a 10:1 mole ratio of PMB to DPPG, where 50 mol% of the lipid is not bound to PMB, both non-interdigitated and interdigitated lipid are present [1]. However, the relative amounts of lipid in the two phases was not determined. Incorporation of significant amounts of other normally non-interdigitating lipids into an interdigitated bilayer formed by a different lipid has been reported in other studies. Using X-ray diffraction, Theretz et al [2] showed that 50 mol% dimyristoylphosphatidylcholine can be incorporated into an interdigitated bilayer of PMB-DPPG. Lohner et al. [14] and Kim et al. [15] showed that 30–50 mol% of the normally non-interdigitating dipalmitoylphosphatidylcholine can be incorporated into an interdigitated bilayer of dihexadecylphosphatidylcholine. Phase diagrams determined by DSC of mixtures of distearoylphosphatidylcholine with the asymmetric 1-stearoyl-2-caproyl-phosphatidylcholine sug-

gested that some DSPC could be incorporated into the mixed interdigitated bilayer formed by the latter [28].

The fact that unbound lipid can be incorporated into the interdigitated bilayer of PMB-bound lipid indicates that if the two lipids are present together in the same bilayer structure, it takes less energy for the unbound lipid to interdigitate, even though this may result in some exposure of the hydrophobic terminal methyl groups of its acyl chains to the aqueous phase, than for the PMB-bound lipid to be noninterdigitated, since this would decrease the van der Waals interactions between the acyl chains. The repulsive negative charge on the unbound DPPG may help stabilize its interdigitation. However, most of the unbound lipid becomes phase separated into its own non-interdigitated domain as in Fig. 8c. Sucrose density gradient centrifugation at nonsaturating concentrations of PMB indicated that phase separation of DPPG vesicles containing high concentrations of PMB from vesicles containing low concentrations of PMB occurs suggesting that some macroscopic separation of the interdigitated and non-interdigitated domains into different bilayer structures occurs. However, interdigitated, PMB-bound lipid can not be completely purified from non-interdigitated, PMB-unbound lipid using this technique since bilayers of one type can become entrapped within vesicles consisting of bilayers of the other type. Babin and Pezolet [23] have also detected macroscopic phase separation of PMB-bound and unbound lipid into separate bilayers under some conditions.

The ability of PMB to cause interdigitation of DPPA has not been thoroughly studied previously. Theretz et al. [2] have referred to unpublished X-ray diffraction results indicating that PMB does not cause interdigitation of DPPA. However, no experimental details were given. Mushayakarara and Levin [16] found that at pH 8 and a 1:10 mole ratio of PMB to dimyristoylphosphatidic acid, Raman spectra suggested that the bilayer was not interdigitated. The spin label results indicate that at pH values below 9, only some of the DPPA is interdigitated even at saturating concentrations of PMB, and the amount which is interdigitated decreases greatly at lower concentrations. Thus the presence of unbound DPPA inhibits even the PMB-bound lipid from forming an interdigitated bilayer, as in model b in Fig. 8. DSC studies indicated that the unbound DPPA phase separates into its own non-interdigitated domain. However, sucrose density centrifugation did not reveal whether phase separation into separate structures occurs.

We attribute this greater tendency of DPPA to be non-interdigitated at pH values below 9, to its ability to interact intermolecularly by lateral hydrogen bonding between $P\text{-}O^-$ and P-OH groups of neighboring lipid molecules when the state of dissociation of PA is less than 1.5 [6,19–21]. Lateral inter-lipid hydrogen bonding cannot occur in an interdigitated bilayer because of the greater lateral separation of the lipid headgroups and thus these interactions favor the non-interdigitated bilayer. PMB weakens interlipid hydrogen bonding of the lipid molecules to which it is bound electrostatically, however, since protons from the protonated amino groups of the peptide hydrogen bond with the hydrogen accepting $P\text{-}O^-$ of the lipid instead. Thus the PMB-bound lipid can form an interdigitated bilayer if it can phase separate away from the unbound non-interdigitated lipid. This is more likely to occur at high concentrations of PMB than at low. The fact that the presence of PMB-unbound DPPA can inhibit interdigitation of PMB-bound DPPA, in contrast to results with DPPG (or DPPA at pH 9), indicates that it takes more energy to break the interlipid hydrogen bonds of the unbound DPPA than for the PMB-bound lipid to form a non-interdigitated bilayer.

Rowe [22] found that in mixtures of dipalmitoylphosphatidylcholine with the hydrogen bonding lipid, dilauroylphosphatidylethanolamine, when interdigitation of the PC was induced with ethanol, the PE phase separated into a non-interdigitated domain. This could also be caused by the intermolecular hydrogen bonding of PE. Alternatively, it might be caused by the large chain length difference between the species of PE and PC used. The present study shows that even when the fatty acid chain lengths of the hydrogen bonding lipid (PMB-unbound PA) and non-hydrogen bonding lipid (PMB-bound PA) are identical, the hydrogen bonding species does not become incorporated into an interdigitated bilayer to a significant extent, but phase separates into its own domain.

At pH 9, DPPA behaves more like DPPG than at lower pH values. Indeed, PMB and PMBN cause even more unbound DPPA to become interdigitated at pH 9 than found for DPPG. When PMB binds to DPPA at pH 9, close to the pK_2 of the lipid, it probably causes further dissociation of protons, so that most of the lipid is doubly ionized. This is suggested by the fact that the phase transition temperature decreases from its value at pH 6 and is even a little lower than that of the PMB-DPPG complex at the same pH. Increasing dissociation weakens the inter-lipid hydrogen bonding interactions [6,19,20]. This allows a greater percentage of unbound DPPA to become interdigitated at nonsaturating concentrations of PMB at pH 9 than at lower pH, and even more than for DPPG at pH 7.4.

The greater amount of unbound DPPA which can be incorporated into the interdigitated bilayer of PMB- and PMBN-bound DPPA at pH 9 relative to DPPG at pH 7.4 could be caused by (i) partial deprotonation of the peptides at pH 9 [13] and/or by (ii) the greater negative charge of DPPA at this pH. An increase in pH also increases the amount of DPPG induced to interdigitate but not as much as for DPPA, suggesting that both

factors contribute. Partial deprotonation of the peptides would increase their hydrophobicity and their ability to induce lateral expansion of the lipids, resulting in their interdigitation. The fact that PMB decreases the phase transition temperature of DPPG more at pH 9 than at pH 7.4 suggests this may occur. In addition, partial deprotonation may decrease the ability of the peptides to cause phase separation of bound and unbound lipid. If phase separation does not occur as readily, interdigitation of the unbound lipid is favored for non-hydrogen bonding lipids. With regard to the second factor, an increase of the negative charge of a lipid will also increase its tendency to become incorporated into an interdigitated bilayer, since this will allow the negatively charged headgroups to be separated while maintaining close interactions between the acyl chains.

Qualitative comparison of the spectra of the PMBN samples, as well as the quantitative results of the spectral resolution also indicate that PMBN causes less lipid to be interdigitated than PMB for both DPPG and DPPA. Indeed, PMBN may not induce interdigitation of DPPA at pH 6 at all. The motional restriction of the spin label observed in these samples at pH 6 was not large enough to be able to conclude that an interdigitated bilayer domain was present. PMBN also did not maintain interdigitation of DPPG or DPPA (pH 9) to as high a temperature as PMB. This difference may be due to the smaller perturbing effect of PMBN on the bilayer as reflected in its effect on the lipid phase transition temperature.

The lower of the two transitions found for DPPG and DPPA at pH 9 has been attributed to hydrophobic interactions with PMB, while the upper temperature transition has been attributed to the combination of hydrophobic plus electrostatic interactions [12]. The fact that PMBN results in only the upper temperature transition with these lipids supports this hypothesis. The fact that interaction of PMB with DPPA at pH 6 results in only a single transition in contrast to the double transition found at pH 9 or with DPPG, suggests that PMB interacts with DPPA by the combination of hydrophobic and electrostatic interactions at this pH, and not just hydrophobic interactions. The higher temperature of this transition, compared to DPPG or DPPA at pH 9 suggests that some interlipid hydrogen bonding must still occur for the DPPA samples causing resistance to the bilayer perturbing effects due to the hydrophobic interaction of PMB. Breaking or weakening of these hydrogen bonds in the liquid crystalline phase may allow greater perturbation of the bilayer by PMB and results in the hysteresis observed between heating and cooling scans for the DPPA samples.

The transition temperature of the PMBN-DPPA complex at these pH values is even higher than for PMB, indicating stronger hydrogen-bonding interactions for the former. There is also less hysteresis between heating and cooling scans. Thus even after interacting with the liquid crystalline phase, PMBN does not perturb the lipid as much as PMB. This indicates a decreased ability of PMBN to cause lateral separation of the lipids and may be the reason why PMBN maintains the interdigitated bilayer less well than PMB. The difference between the abilities of PMBN and PMB to lower the lipid phase transition temperature and to maintain interdigitation is greater for the hydrogen bonding DPPA (pH 6) than DPPG. Thus for lipids which can interact intermolecularly by hydrogen bonding, PMB has a significantly greater ability to perturb the lipid and/or to cause interdigitation than PMBN. Along with other differences in behavior reported earlier [7], this may help account for its greater bactericidal effects on Gram-negative bacteria, which contain PG as well as the hydrogen bonding lipid, PE.

Conclusion

Quantitation of the amount of interdigitated lipid in mixtures of a lipid which tends to be interdigitated and a lipid which tends to be non-interdigitated has allowed us to determine several factors which influence the ability of both lipids to form an interdigitated bilayer. (i) The presence of an interdigitated domain of a lipid promotes interdigitation of another lipid if the latter has a repulsive negative charge. (ii) Interlipid hydrogen bonding of one of the lipids inhibits its formation of an interdigitated bilayer and also inhibits formation of an interdigitated bilayer by the other lipid. (iii) Increased negative charge favors formation of an interdigitated bilayer.

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