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Partition of parinaroylphosphatidylethanolamines and parinaroylphosphatidylglycerols in immiscible phospholipid mixtures

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Partitioning of two parinaroyl phosphatidylethanolamines and two parinaroyl phosphatidylglycerols between solid and fluid phase phospholipids was examined. Fluorescence quantum yields and fluorescence polarization measurements were used to calculate $K_p^{s/f}$, the solid to fluid partition coefficient of each probe (Sklar, L.A., Miljanich, G.P. and Dratz, E.A. (1979) Biochemistry 18, 1707–1716). In the immiscible mixture dipalmitoylphosphatidylcholine and dilinoleylphosphatidylcholine, both 1-palmitoyl-2-*trans*-parinaroylphosphatidylethanolamine and 1-palmitoyl-2-*trans*-parinaroylphosphatidylglycerol partitioned preferentially into solid phase lipid with mean $K_p^{s/f}$ values (calculated from quantum yields) of 3.4 ± 1.5 and 2.1 ± 0.7, respectively. In contrast, 1-oleoyl-2-*cis*-parinaroylphosphatidylethanolamine and 1-oleoyl-2-*cis*-parinaroylphosphatidylglycerol partitioned preferentially into fluid phase lipid in the same model system with mean $K_p^{s/f}$ values (calculated from quantum yields) of 0.44 ± 0.26 and 0.16 ± 0.07, respectively. Fluorescence polarization data on the same four parinaroyl phospholipids in mixtures of solid-phase dimyristoylphosphatidyl ethanolamine and fluid-phase dilinoleoylphosphatidylglycerol were similar to those obtained in the immiscible phosphatidylcholine system, demonstrating that the partitioning of these probes is not strongly dependent on head group. Knowledge of the partition properties of these fluorescent probes is relevant to use of these probes in investigation of the phase behavior of *Escherichia coli* inner membrane lipids, since phosphatidylethanolamine and phosphatidylglycerol species account for approximately 95% of these lipids.

Introduction

Previous work has characterized the fluid-solid lipid partition properties of four parinaroyl PCs and four parinaroyl PEs in an immiscible fluid-solid phosphatidylcholine system [1]. Furthermore, the partitioning of two parinaroyl PCs and two parinaroyl PEs between solid phase PC and fluid phase PE and between solid phase PE and fluid phase PC was investigated [2]. These studies demonstrated that the partition properties of the PC and PE probes were almost completely unaffected by the head group of the bulk-phase lipids.

The inner membrane lipids of *Escherichia coli* are comprised of about 80% PE and 15% PG species. Because our long-term goal is to gather information about the organization and phase behavior of specific lipid species in the *E. coli* inner membrane, we embarked on studies similar to those described for PC and PE, using PG and PE probes and mixtures.

Materials and Methods

Phospholipids. DPPC, DLiPC, DMPE and DLiPG were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). The parinaroyl phospholipids were synthesized, using tPnA and cPnA from Molecular Probes, Inc. (Eugene, OR), as described by Welti and Silbert [1] with the following modifications. Lyso PCs were dried at 68°C over phosphorus pentoxide under vacuum. Phospholipase D was prepared from Savoy cabbage as de-

Abbreviations: PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PG, phosphatidylglycerol; cPnA, *cis*-parinaric acid or 9,11,13,15-*cis,trans,trans,cis*-octadecatetraenoic acid; cPn, *cis*-parinaroyl; tPnA, *trans*-parinaric acid or 9,11,13,15-all-*trans*-octadecatetraenoic acid; tPn, *trans*-parinaroyl; 16:0, palmitoyl; 18:1, oleoyl; DPPC, dipalmitoyl PC; DLiPC, dilinoleoyl PC; DMPE, dimyristoyl PE; DLiPG, dilinoleoyl PG; PDPC, 1-palmitoyl-2-docosahexaenoyl PC.

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scribed by Christie [3]. This preparation was partially purified according to a modification of the procedure of Davidson and Long [4], in which the phospholipase D solution was adjusted to pH 5.6, heated to 56°C in a 90°C water bath, cooled quickly to 0°C, and centrifuged at 160 × *g* for 15 min. The supernatant then was lyophilized. This preparation was used to convert PC to PE and PC to PG under the conditions described as optimal by Comfurius and Zwaal [5]. The parinaroyl phospholipids were purified by preparative TLC in chloroform/methanol/ammonium hydroxide (65 : 25 : 4, v/v). All PGs used in these studies were L-α-phosphatidyl-DL-glycerol.

Phospholipid dispersions. Dispersions were prepared by the ethanol injection method. For each sample, 88 μl of 2 mM bulk-phase (non-fluorescent) phospholipid(s) in ethanol was mixed with 16 μl of 0.1 mM parinaroyl phospholipid containing 0.01 mM butylated hydroxytoluene in ethanol. 87 μl of this solution was injected into 3.2 ml 115 mM NaCl, 1 mM EDTA, 10 mM Hepes (pH 7.5) at 55°C as the sample was vortexed. This produced a dispersion which contained 45 μM phospholipid, 0.41 μM parinaroyl phospholipid and 2.6% ethanol. Dispersions were used immediately for absorbance and fluorescence measurements.

Absorption spectroscopy. Spectra were recorded before and after fluorescence measurements on a Beckman Model 25 spectrophotometer. The absorbance used in calculation of quantum yield at each temperature was based on a linear (with temperature) interpolation of the absorbance before and after fluorescence temperature scans. For the PC/PC mixtures for which partition coefficients were calculated, losses of absorbance during fluorescence measurements ranged from 0% to 32%, with an average loss of 12%. Losses in DMPE were higher, as discussed in Results, precluding calculation of partition coefficients in the PE/PG system.

Fluorescence spectroscopy. The method of data collection was as described by Welti and Silbert [1], except that the cooling rate was 1.25 C°/min and the emission bandpass was 20 mm. Shutters were closed at all times except when measurements were being taken. Data shown in all plots in this paper and used in partition coefficient calculations are unsmoothed data. The polarization ratio obtained with fluorescein in alkaline glycerol [6] was 2.65. This value represented the highest value practically obtainable with our instrument, given the alignment of the polarizers when the experiments were performed. Fluorescence quantum yields and partial quantum yields also were calculated as described by Welti and Silbert [1] and Sklar et al. [7], using an ethanolic solution of cPnA at 25°C, excited at 320 nm with a quantum yield of 0.02, as a reference.

Partition coefficients. The solid-fluid partition coefficient, $K_p^{s/f}$, which describes the distribution of fluorescent probe between coexisting solid and fluid phases, can be defined as

$$K_p^{s/f} = (\chi_s^p/\chi_s)/(\chi_f^p/\chi_f) \quad (1)$$

where χ_s and χ_f represent the mole fractions of solid-phase and fluid-phase lipid present, while χ_s^p and χ_f^p represent the mole fractions of the probe in the solid and fluid phases [7]. Solid-fluid partition coefficients were determined by measuring the total quantum yields or polarization ratio, $P = I_\parallel/I_\perp$, of each probe in mixtures of DPPC and DLiPC. $K_p^{s/f}$ can be calculated at temperatures where solid and fluid phase coexist. The solid-fluid partition coefficient of each probe is calculated according to two equations, one employing total quantum yields and the other employing partial quantum yields. The equations, developed by Sklar et al. [7], are

$$K_p^{s/f} = [(Q_{tot}^{mix} - Q_{tot}^{f})\chi_f]/[(Q_{tot}^{s} - Q_{tot}^{mix})\chi_s] \quad (2)$$

and

$$K_p^{s/f} = [(P^{mix}Q_\perp^{f} - Q_\parallel^{f})\chi_f]/[(Q_\parallel^{s} - P^{mix}Q_\perp^{s})\chi_s] \quad (3)$$

where $Q_\perp^f$, $Q_\parallel^f$, and Q_{tot}^f are partial or total quantum yields of the probe in DLiPC (pure fluid phase) at the temperature of the measurement. $Q_\perp^s$, $Q_\parallel^s$, and Q_{tot}^s are the partial or total quantum yields of the probe in DPPC (pure solid phase) at the same temperature. Q_{tot}^{mix} is the experimental total quantum yield of the DPPC/DLiPC mixture. P^{mix} is the experimental polarization ratio, $Q_\parallel^{mix}/Q_\perp^{mix}$, of the DPPC/DLiPC mixture at a particular temperature. For partition coefficient calculations, this ratio is uncorrected for instrumental anisotropy. Values of χ_f and χ_s, the fractions of fluid and solid phase at each temperature, were derived from the phase diagram shown in Fig. 2, following the lever rule [7,8].

Results

Fluorescence polarization of probes in DPPC/DLiPC mixtures

The fluorescence polarization ratio, $I_\parallel/I_\perp$, of 1-16 : 0,2-tPnPE, 1-16 : 0,2-tPnPG, 1-18 : 1,2-cPnPE, and 1-18 : 1,2-cPnPG, as a function of temperature in mixtures of DPPC and DLiPC is shown in Fig. 1. It is clear from these data that the 1-16 : 0,2-tPnPE and 1-16 : 0,2-tPnPG respond strongly to small amounts of solid-phase lipid with increased polarization ratios. For example, in the mixtures of 20% DPPC/80% DLiPC at 4°C (19% solid phase), the polarization ratios of these two probes are nearly as high as in pure DPPC. In contrast, 1-18 : 1,2-cPnPE and 1-18 : 1,2-cPnPG are less sensitive to the presence of solid-phase lipid. For example, in 50% DPPC/50% DLiPC at 4°C (49.4% solid

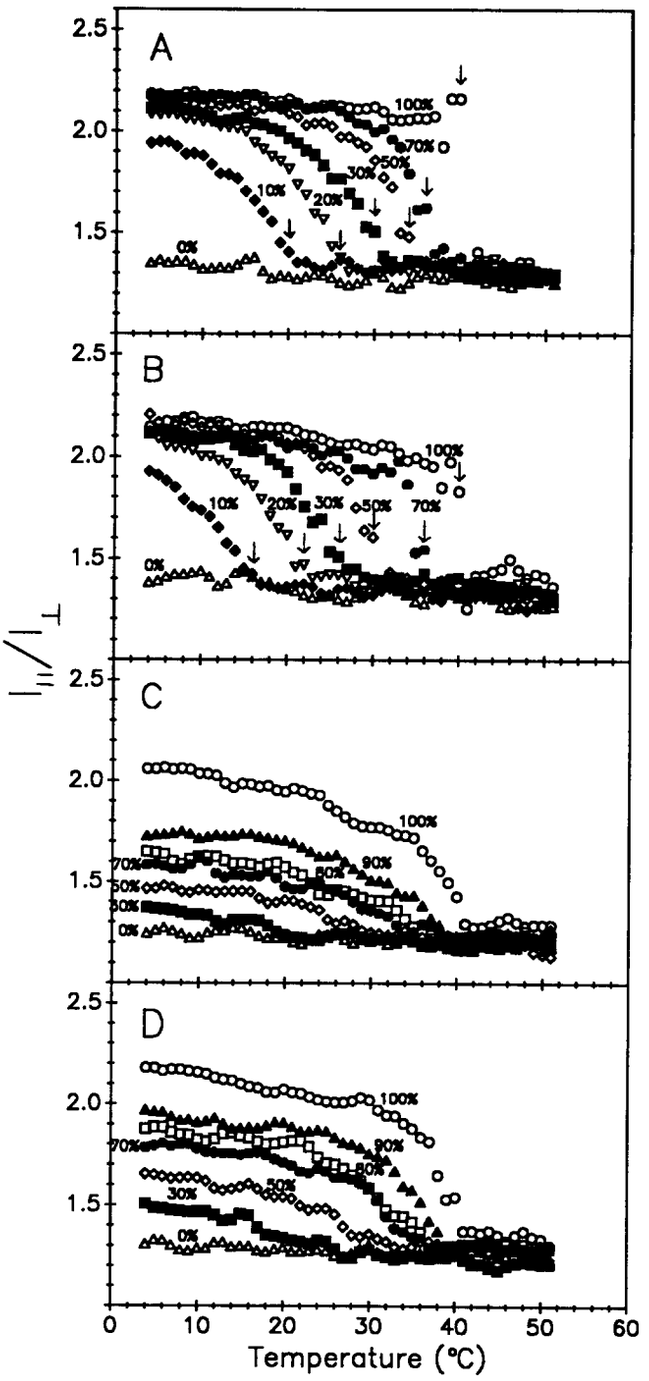

Fig. 1. Fluorescence polarization ratio, $P = I_{\parallel}/I_{\perp}$, of parinaroyl phospholipids as a function of temperature in DPPC/DLiPC mixtures. Percentages are the amount of DPPC in the mixture. Panel A represents 1-16:0,2-tPnPE; panel B, 1-16:0,2-tPnPG; panel C, 1-18:1,2-cPnPE; and panel D, 1-18:1,2-cPnPG. In all graphs, ○ represents 100% DPPC; ▲, 90%; □, 80%, ●, 70%, ◇, 50%; ■, 30%; ▽ 20%; ◆, 10%; △, 0%. The arrows indicate the points taken to represent the onset of solid-phase formation in the phase diagram (Fig. 2).

phase), the polarization ratios of these two probes are closer to those of pure DLiPC than to those of pure DPPC.

The DPPC / DLiPC phase diagram

A phase diagram for DPPC/DLiPC, constructed by the method of Sklar et al. [7] is shown in Fig. 2. The fluidus curve represents the highest temperatures at which solid phase formation could be detected by an increase in the polarization ratio of 1-16:0,2-tPnPE and 1-16:0,2-tPnPG, the probes which are most sensitive to small amounts of solid phase. The transition onset temperatures (cooling) are indicated in Fig. 1 by the arrows. The solidus curve should represent the highest temperature at which no fluid phase can be detected. We were unable to cool our samples to temperatures at which no fluid phase could be detected. Thus the true solidus line lies below the line labeled S_1 and is likely to be approximated by the line labeled S_2, which represents complete solid-phase immiscibility of DPPC and DLiPC [7]. S_2 is used in the partition coefficient calculations described below.

Partition of coefficients of the probes in DPPC / DLiPC mixtures

Partition coefficients calculated according to Eqns. 2 and 3 are shown in Table I. Both mean and median partition coefficients were calculated [1]. The $K_p^{s/f}$ values determined for the PE probes are in good agreement with those previously reported for these probes in a DPPC/PDPC mixture. These data concur with the qualitative observations made from examination of the polarization ratios shown in Fig. 1. 1-16:0,2-tPnPE and 1-16:0,2-tPnPG partition several times more strongly into the solid phase PC as into the fluid phase PC, while 1-18:1,2-cPnPE and 1-18:1,2-cPnPG partition more strongly into fluid-phase PC than into solid-phase PC.

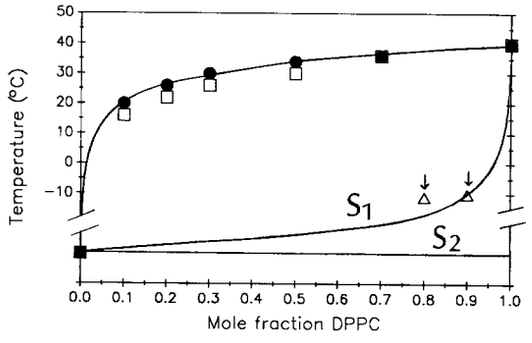

Fig. 2. DPPC/DLiPC phase diagram. The transition temperature of pure DLiPC is not known, but is believed to be less than −20 °C. The fluidus line represents the onset of solid-phase formation as detected by 1-16:0,2-tPnPE, ●, and 1-16:0,2-tPnPG, □. The solidus line should represent the completion of solid-phase formation. S_1 is an upper limit for this line. The points, △, on S_1 were derived by cooling samples with 1-18:1,2-cPnPG to the indicated temperatures, at which they had not obtained polarization ratios characteristic of this probe in solid phase lipid. S_2, the solidus line used for partition coefficient calculations, represents complete solid-phase immiscibility of DPPC and DLiPC.

Fluorescence polarization of probes in DMPE/DLiPG mixtures

The polarization ratio, $I_{\parallel}/I_{\perp}$, as a function of temperature for 1-16:0,2-tPnPE, 1-16:0,2-tPnPG, 1-18:1,2-cPnPE, and 1-18:1,2-cPnPG in mixtures of DMPE and DLiPG is shown in Fig. 3. In general, these data are qualitatively similar to those shown for the probes in the DPPC/DLiPC system. Again, 1-16:0,2-tPnPE and 1-16:0,2-tPnPG can be seen to respond strongly to the presence of solid phase lipid, although the data suggest that the partition coefficients may be slightly lower in the DMPE/DLiPG system. For example, at 4°C in 30% DMPE (22.1% solid phase lipid), the polarization ratios of these two probes are approaching the highest ratio obtained for these mixtures, but appear to be slightly lower, relative to pure solid phase lipid, that that seen in the DPPC/DLiPC system at 19% solid phase (20% DPPC). Similarly, at 4°C, the polarization ratio of these probes in 20% DMPE in DLiPG (11% solid phase) was somewhat lower than that in 10% DPPC in DLiPC (9% solid phase).

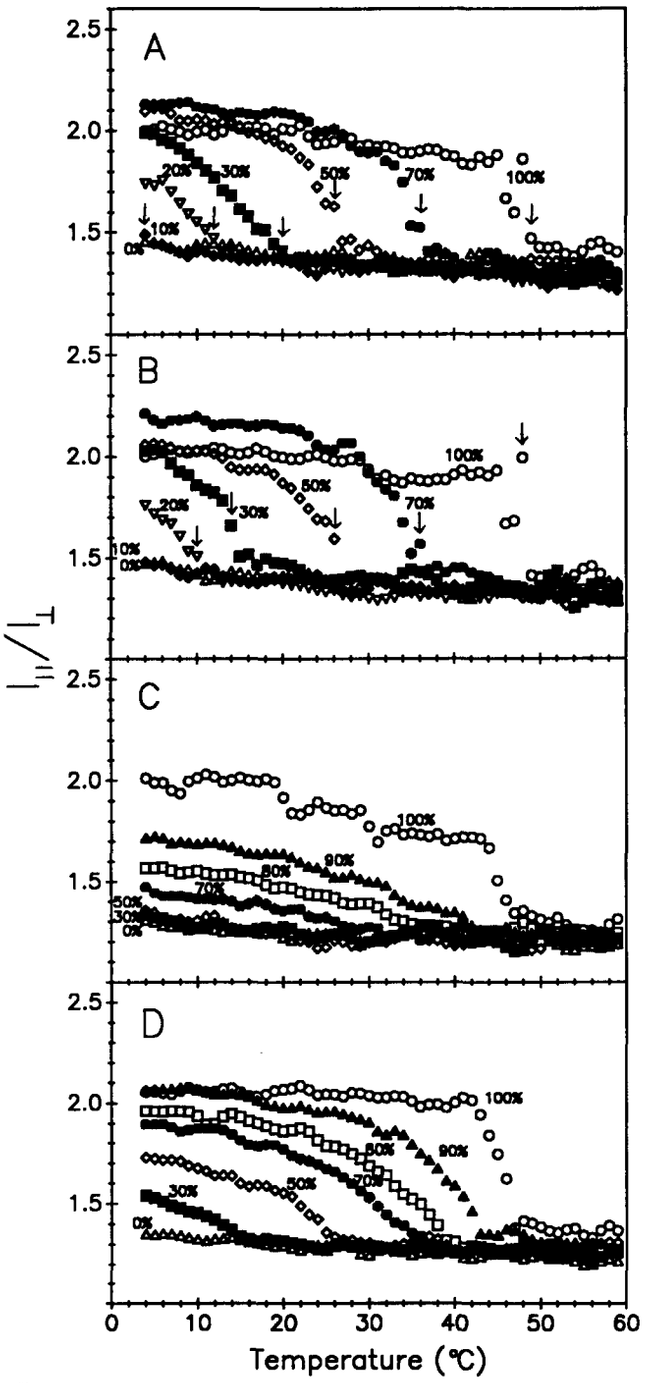

Fig. 3. Fluorescence polarization ratio, $P = I_{\parallel}/I_{\perp}$, of parinaroyl phospholipids as a function of temperature in DMPE/DLiPG mixtures. Percentages are the amount of DMPE in the mixture. Panel A represents 1-16:0,2-tPnPE; panel B, 1-16:0,2-tPnPG; panel C, 1-18:1,2-cPnPE; and panel D, 1-18:1,2-cPnPG. In all graphs, ○ represents 100% DMPE; ▲, 90%, □, 80%; ●, 70%; ◇, 50%; ■, 30%; ▽, 20%; ◆, 10%; △, 0%. The arrows indicate the points taken to represent the onset of solid-phase formation in the phase diagram (Fig. 4).

The data for 1-18:1,cPnPE and 1-18:1,2-cPnPG again suggest that these probes have lower solid-fluid phase partition coefficients than the 1-16:0,2-tPn probes. Like the 1-16:0,2-tPn probes, 1-18:1,2-cPnPE also appears to have a slightly lower partition coefficient in the DMPE/DLiPG system than in the DPPC/DLiPC system. For example, in 70% DMPE in DLiPG at 4°C (66.6% solid phase), the polarization ratio of 1-18:1,2-tPnPE is detectably lower, relative to the pure solid and fluid phase lipids, than in 70% DPPC in DLiPC (69.4% solid phase). The polarization curves for 1-18:1,2-cPnPG suggest that it partitions similarly between the solid and fluid phases in the DMPE/DLiPG and DPPC/DLiPC systems.

Partition coefficients were not calculated for the DMPE/DLiPG mixtures because of probe loss in pure DMPE. In mixtures of 70% or less DMPE in DLiPG, the loss of probe absorbance during fluorescence measurements ranged from 0% to 29%, averaging 11%. In mixtures of 80% and 90% DMPE in DLiPG, the probe loss during fluorescence measurements averaged 31%. When the 100% DMPE samples were subjected to fluorescence measurements from 60°C to 3°C, there was virtually no probe absorbance left (data not shown). Thus the data for each probe in 100% DMPE shown in Fig. 3 were obtained from six different samples. Fluorescence polarization of each sample was measured over an approximately 10 C° interval and the data were compiled to produce one polarization ratio curve. Using this method, probe loss still averaged 40% per sample.

The DMPE/DLiPG phase diagram

The DMPE/DLiPG phase diagram is shown in Fig. 4. The diagram was constructed in the same manner as Fig. 2. The transition onset temperatures (cooling) are indicated by the arrows in Fig. 3.

Discussion

The solid-fluid partition characteristics of both parinaroyl PEs and parinaroyl PGs in both PC/PC

TABLE I

Solid-fluid phase partition coefficients

Partition coefficients are calculated according to Eqns. 2 and 3. Each value used in the calculations was the quantum yield or polarization ratio of a particular DPPC/DLiPC mixture at a particular temperature. Data from the first 5C° below the onset of the phase transition, as indicated by the phase diagram fluidus line, were excluded from all calculations. The median values included data obtained from all mixtures of DPPC/DLiPC. The mean values excluded data from 70% DPPC and 50% DPPC for 1-16:0,2-tPnPE and 1-16:0,2-tPnPG and 30% DPPC for 1-18:1,2-cPnPE and 1-18:1,2-cPnPG, because these data sets, which have polarization ratios very close to those of pure DPPC or DLiPC, had more error associated with them.

Probe	Mean $K_p^{s/f}$		Median $K_p^{s/f}$	
	Eqn. 2	Eqn. 3	Eqn. 2	Eqn. 3
16:0tPnPE	3.4 ±1.5 (47)	4.5 ±1.4 (47)	3.3 (101)	3.1 (101)
Previous [1]	13.8 ±6.9 (23)	5.0 ±2.5 (23)	9.5 (81)	3.2 (51)
16:0tPnPG	2.1 ±0.7 (47)	7.2 ±14.0 (47)	1.9 (101)	3.9 (101)
18:1cPnPE	0.44±0.26 (115)	0.13±0.03 (115)	0.49 (135)	0.13 (135)
Previous [1]	0.50±0.54 (77)	0.36±0.44 (77)	0.28 (102)	0.31 (102)
18:1cPnPG	0.16±0.07 (115)	0.26±0.07 (115)	0.12 (135)	0.27 (135)

mixtures and PE/PG mixtures are dependent on the acyl chains of the phospholipids, rather than on the head groups. Indeed, no evidence was obtained to suggest that the probes are sequestered to any extent in bulk-phase lipid with like head groups. These data suggest that acyl chain identity rather than head group identity is the primary factor affecting the organization of PE/PG mixtures, such as *E. coli* phospholipids, at least in the absence of proteins. Thus, knowing the probe partition coefficients, it should be possible to use these probes, without regard to their head group, to obtain reasonably accurate estimates of the amounts of fluid and solid phases present in *E. coli* lipid mixtures.

It appears that three of the four probes have slightly lower partition coefficients in the DMPE/DLiPG system as compared to the DPPC/DLiPC system. Again this aspect of the data does not suggest head group dependence of partitioning since the probe which did *not* favor the fluid DLiPG phase was a parinaroyl PG. One explanation for the lowered partition coefficients observed is less-than-optimal fit of the probes into the bulk DMPE, with its shorter acyl chains as compared to DPPC. It is unclear why a slightly decreased $K_p^{s/f}$ was not observed with 1-18:1,2-cPnPG, as well as with the other probes.

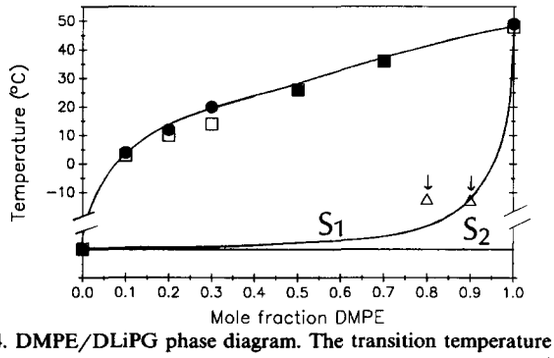

Fig. 4. DMPE/DLiPG phase diagram. The transition temperature of pure DLiPG is not known, but is believed to be less than −20°C. The fluidus line represents the onset of solid-phase formation as detected by 1-16:0,2-tPnPE (●) and 1-16:0,2-tPnPG (□). The solidus line should represent the completion of solid-phase formation. S_1 is an upper limit for this line. The points, △, on S_1 were derived by cooling samples with 1-18:1,2-cPnPG to the indicated temperatures, at which they had not obtained polarization ratios characteristic of this probe in solid phase lipid. S_2 represents complete solid-phase immiscibility of DMPE and DLiPG.

The reason for the high losses of parinaroyl probes during fluorescence measurements in DMPE remains unclear. A similar problem had previously been noted with probe measurements in DPPE [2]. The losses do not seem to be correlated with exposure of the DMPE/probe samples to any particular temperature, as we experienced relatively large losses when samples were subjected to short periods of measurements at any of various temperatures. Previously we determined that losses were decreased somewhat by the addition of butylated hydroxytoluene and EDTA to the DMPE/probe samples. DMPE vesicles have been shown to undergo an EDTA-reversible aggregation in the presence of Ca^{2+} [9], but the relationship of the aggregation to the present phenomenon is unclear.

Acknowledgements

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