# Nonmonotonic alterations in the fluorescence anisotropy of polar head group labeled fluorophores during the lamellar to hexagonal phase transition of phospholipids

Xianlin Han*‡ and Richard W. Gross*‡§

Division of Bioorganic Chemistry and Molecular Pharmacology, Departments of *Internal Medicine, ‡Chemistry, and §Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110 USA

ABSTRACT The temperature dependence of the fluorescence anisotropy of polar head group labeled fluorophores (i.e., *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-$\alpha$-phosphatidylethanolamine or *N*-(lissamine rhodamine B sulfonyl)dipalmitoyl-L-$\alpha$-phosphatidylethanolamine) incorporated into multiple phosphatidylethanolamine molecular species was parabolic, possessing minima ($dr/dT = 0$) that precisely correlated with the respective lamellar ($L_\alpha$) to hexagonal ($H_{II}$) phase transition temperature of each species. The parabolic alterations in the thermotropic behavior of these fluorophores were due to increased motional constraints in the polar head group region during heating ($dr/dT > 0$), because significant alterations in the fluorescence lifetimes of these probes during the phase transition did not occur. The sensitivity inherent in identification of peak minima was exploited to determine the lamellar to hexagonal phase transition temperatures of several homogeneous molecular species of plasmenylethanolamine (e.g., the transition temperature of 1-*O*-(*Z*)-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphoethanolamine was 28°C). Experiments using ethanolamine glycerophospholipids containing either an ester or a vinyl ether linkage at the *sn*-1 position demonstrated that introduction of the vinyl ether constituent increased the propensity of these species to assume the hexagonal phase. Collectively, these results identify and substantiate a new technique for the characterization of the lamellar to hexagonal phase transition in phospholipids that requires only small amounts of phospholipids present in dilute membrane suspensions.

## INTRODUCTION

Recently, the importance of nonlamellar structures in such diverse processes as membrane fusion and activation of ion transport proteins has generated intense interest (for recent reviews see Gruner et al., 1985; Cullis et al., 1986; Hui, 1987; Yeagle, 1989; Seddon, 1990). Many physical techniques, including nuclear magnetic resonance (NMR),[1] x-ray diffraction, differential scanning calorimetry, freeze-fracture electron microscopy, and infrared spectroscopy (e.g., Deamer et al., 1970; Rand and Sengupta, 1972; Cullis and De Kruijff, 1979; Harlos and Eibl, 1981; Mantsch et al., 1981; Seddon et al., 1983), have been used to document the polymorphic phase behavior of a variety of lipids and lipid mixtures. However, progress in defining the importance of nonlamellar phases in subcellular membrane processes has been hindered by the requirement for substantial amounts of lipid mass to identify lamellar to nonlamellar phase transitions by use of these techniques. Thus, the adaptation of the highly sensitive technique of fluorescence spectroscopy to identify the lamellar ($L_\alpha$) to hexagonal ($H_{II}$) phase transition in lipid aggregates has been enthusiastically received (Ellens et al., 1986*a*, *b*; Hong et al., 1988; Cheng, 1989; Stubbs et al., 1989; Nieva et al., 1990). However, the modest alterations in the thermotropic dependence of the previously used fluorescence parameters have resulted in significant ambiguities in the characterization of the lamellar to hexagonal phase transition in some systems.

The propensity of lipids for adapting the hexagonal phase is directly related to the relative steric bulk of their hydrophobic constituents in comparison with the steric bulk of their polar head groups (Israelachvili et al., 1980). It is largely believed that this results from the relatively greater steric constraints on the polar head group in the hexagonal phase compared with the lamellar phase (e.g., Gruner, 1985; Larsson, 1988). Accordingly, we anticipated that the transition from the lamellar to the hexagonal phase would be accompanied by substantial increases in the fluorescence anisotropy of fluorophores, reflecting the decreased motional freedom of the polar head group in the hexagonal phase, and by substantial alterations in the membrane fluidity gradient. We now report that large alterations in the fluorescence anisotropies of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-dipalmitoyl-L-$\alpha$-phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)dipalmitoyl-L-$\alpha$-phosphatidylethanolamine) (Rh-PE) are present dur-

Address correspondence to Dr. Gross.

[1] *Abbreviations used in this paper:* EE PhosEth, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; EY PhosEth, egg yolk phosphatidylethanolamine; n-AS, n-(9-anthroyloxy)stearic acid ($n$ = 2, 6, 9, 12, 16); NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-$\alpha$-phosphatidylethanolamine; NMR, Nuclear Magnetic Resonance; OO PhosCho, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; OO PhosEth, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; O'O PlasEth, a mixture of 1-*O*-(*Z*)-octadec-1′,9′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphoethanolamine and 1-*O*-(*Z*)-octadec-1′,11′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphoethanolamine; PO PhosEth, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; PO PlasEth, 1-*O*-(*Z*)-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphoethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)dipalmitoyl-L-$\alpha$-phosphatidylethanolamine; SA PhosEth, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine; SA PlasEth, 1-*O*-(*Z*)-octadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycero-3-phosphoethanolamine; SO PhosEth, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; SO PlasEth, 1-*O*-(*Z*)-octadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphoethanolamine.



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ing the lamellar to hexagonal phase transition in a variety of homogeneous lipids and lipid mixtures and that the relatively simple technique described herein can be used to characterize this transition by use of microgram quantities of phospholipids in dilute membrane suspensions. We have exploited this new method to study the lamellar to hexagonal phase transition in homogeneous plasmenylethanolamine molecular species to further characterize the phase behavior of a major phospholipid constituent of electrically active membranes (Gross, 1984, 1985).

## MATERIALS AND METHODS

### Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (OO PhosEth), 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (EE PhosEth), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PO PhosEth), egg yolk phosphatidylethanolamine (EY PhosEth), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (OO PhosCho) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and were used without further purification. NBD-PE, Rh-PE, and n-(9-anthroyloxy) stearic acids (n-AS; $n = 2, 6, 9, 12, 16$) were obtained from Molecular Probes, Inc. (Eugene, OR). Bovine heart ethanolamine glycerophospholipids were extracted by the Bligh and Dyer procedure (Bligh and Dyer, 1959) and purified by straight-phase high-performance liquid chromatography (HPLC) using a Dynamax Macro silica column (21.4 × 250 mm; Ranin Instrument Co, Woburn, MA) by isocratic elution employing a mobile phase comprised of hexane/isopropanol/$H_2O$ (55:45:1, vol/vol/vol). 1-Stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (SA PhosEth) and 1-*O*-(*Z*)-octadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycero-3-phosphoethanolamine (SA PlasEth) were freshly purified from bovine heart ethanolamine glycerophospholipids by reverse-phase HPLC using an Ultrasphere octadecyl silica column (250 × 10 mm, 5-μm particles; Beckman Instruments, Berkeley, CA). Isocratic elution was performed with a mobile phase comprised of methanol/water/acetonitrile (90.5:7:2.5, vol/vol/vol) containing 20 mM choline chloride as previously described (Gross, 1985). Similarly, 1-*O*-(*Z*)-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphoethanolamine (PO PlasEth), 1-*O*-(*Z*)-octadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphoethanolamine (SO PlasEth), and a mixture of 1-*O*-(*Z*)-octadec-1′,9′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3- phosphoethanolamine and 1-*O*-(*Z*)-octadec-1′,11′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphoethanolamine (O′O PlasEth) were separated by reverse-phase HPLC from a mixture of 1-*O*-(*Z*)-alk-1′-enyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphoethanolamines (kindly provided by Paul Glaser, Washington University, St. Louis, MO) as described above. The purities of all lipids were substantiated by both thin-layer chromatography and capillary gas chromatography after acid methanolysis (Fink and Gross, 1984) and were found to be ≥97% pure.

### Preparation of multilamellar vesicles

The preparation of multilamellar vesicles containing 0.4 mol% of fluorescence probe (NBD-PE or Rh-PE) was initiated by codissolving the lipid(s) (typically 100–500 μg total lipid) and the probe in chloroform. The solvent was subsequently evaporated under a nitrogen stream before high vacuum evacuation for a minimum of 2 h. After addition of 2 ml of buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) to the dried lipids, vesicles were prepared by warming (or cooling) the suspensions above the main phase transition temperature (gel to liquid-crystalline state) but below the lamellar to hexagonal phase transition temperature for 2 min before vigorous vortexing for 1 min in the presence of glass beads (all operations were performed under a nitrogen atmosphere). Warming (or cooling) and vortexing cycles were repeated five times, and all dispersions were maintained in the lamellar state before assessment of the thermotropic dependence of their fluorescence anisotropies.

### Measurements of steady-state fluorescence anisotropy and fluorescence lifetime

Measurements of fluorescence steady-state anisotropies were performed with a spectrofluorometer (SLM-Aminco 4800C; SLM Instruments, Urbana, IL) using a T-format essentially as described by Lakowicz (1983). An excitation wavelength of 450 nm (4-nm slit width) and an emission wavelength of 538 nm with a 515-nm cutoff filter (Schott Optical Glass Inc., Duryea, PA) were used for NBD-PE. Measurements using the Rh-PE fluorophore were performed similarly with a 530-nm excitation wavelength (4-nm slit width) and a 588-nm emission wavelength through a 550-nm cutoff filter. An excitation wavelength of 364 nm (4-nm slit width) and an emission wavelength of 450 nm through a 418-nm cut-off filter were used for n-AS. Fluorescence lifetimes were measured by the phase-modulation method (Lakowicz, 1983) using *P*-bis[2-(5-phenyloxazolyl)]benzene as the external lifetime reference (Lakowicz et al., 1981). Modulation frequencies of 18 and 30 MHz were used for the measurements of NBD-PE and Rh-PE lifetimes, respectively. Sample temperature was maintained with an isothermal bath and was monitored with a thermistor (model YSI421; VWR Scientific, Chicago, IL) inserted into the sample chamber (found to be accurate within ±0.1°C).

## RESULTS

### Temperature dependence of the fluorescence anisotropy of NBD-PE or Rh-PE in liposomes comprised of commercially available phosphatidylethanolamines

Initial experiments examined the temperature dependence of the steady-state fluorescence anisotropy of NBD-PE (incorporated as a substitutional impurity) in liposomes comprised of EE PhosEth, PO PhosEth, OO PhosEth, or EY PhosEth. The thermotropic dependence of the polymorphic phase behavior of these commercially available lipids has been documented extensively by a multiplicity of different techniques, including NMR, differential scanning calorimetry and infrared spectroscopy (see Table 1 for references). The temperature dependence of the fluorescence anisotropies of NBD-PE incorporated into liposomes comprised of each of these ethanolamine glycerophospholipids demonstrated the anticipated decrease during the gel to liquid-crystalline phase transition, reflecting an abrupt increase in the motional freedom of the probe in the liquid-crystalline bilayer (the liquid-crystalline phase transition in OO PhosEth was not studied because it is far below 0°C). On further heating, well-defined minima in the thermotropic dependence of fluorescence anisotropy were present in each of the four commercially available samples of ethanolamine glycerophospholipids studied that occurred at their previously reported lamellar to hexagonal phase transition temperatures (Fig. 1 and Table 1). The

TABLE 1 **Gel to liquid-crystalline ($T_c$) and lamellar to hexagonal ($T_h$) phase transition temperatures of some common ethanolamine glycerophospholipids in excess aqueous buffer***

| Phosphatidyl-ethanolamine | $T_c$ This work | $T_c$ Published data‡ | $T_h$ This work | $T_h$ Published data‡ |
|---|---|---|---|---|
| | °C | | °C | |
| OO PhosEth | | | 10 ± 1 | 8–10 |
| EE PhosEth | 35 ± 1 | 38, 35–38.5§ | 58 ± 1 | 55–65.6 |
| PO PhosEth | 21 ± 1 | 20 | 67 ± 1 | 71.4, 65‖ |
| EY PhosEth | 10–15 | 11.3¶ | 39 ± 1 | 32–45¶ |

* The lamellar to hexagonal phase transition temperature was obtained using the fluorometric method from the minimum point of the temperature dependence of fluorescence anisotropy (Fig. 1); ‡All published data were taken from the CRC Handbook of Lipid Bilayers (Marsh, 1990) except those indicated; §Small, 1986; ‖Perly et al., 1985; and ¶Boggs et al., 1981.

abrupt increase in fluorescence anisotropy of NBD-PE in these systems concomitant with the reorganization of the lipid matrix into a hexagonal phase reflects the diminished molecular motion of the polar head group

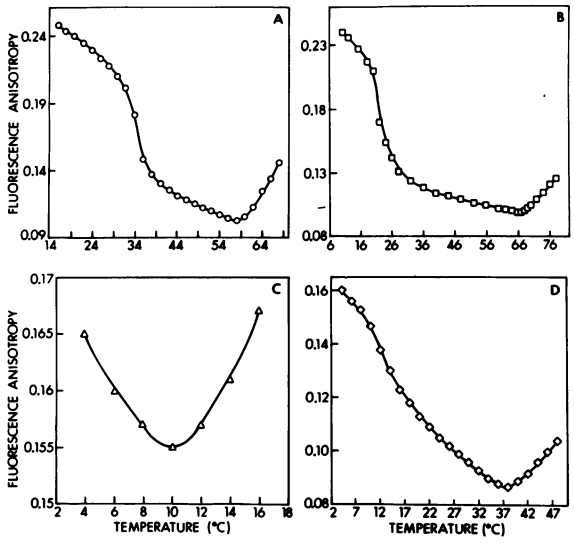


FIGURE 1 Thermotropic dependence of steady-state fluorescence anisotropy of NBD-PE incorporated into EE PhosEth, PO PhosEth, OO PhosEth, or EY PhosEth. Approximately 500 μg of EE PhosEth (*A*), PO PhosEth (*B*), OO PhosEth (*C*), or EY PhosEth (*D*) was evaporated under nitrogen before high vacuum evacuation for ≥2 h. Subsequently, vesicles were prepared by addition of 2 ml of buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) before formation of multilamellar vesicles by vortexing as described in Materials and Methods. Fluorescence anisotropies were calculated from the relative intensities of the parallel and perpendicular components of emitted light at 538 nm by use of the T-format as described in Materials and Methods. The results represent the mean values of at least three independent sample preparations. The standard error of each determination is within the height of each symbol.

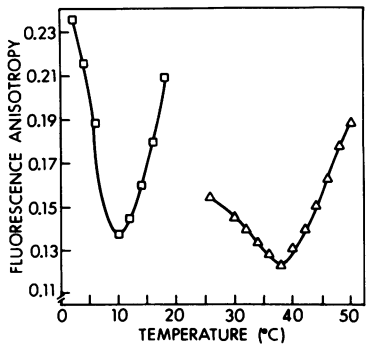


FIGURE 2 Temperature dependence of the steady-state fluorescence anisotropy of Rh-PE incorporated into vesicles comprised of OO PhosEth or EY PhosEth. Vesicles comprised of OO PhosEth (□) or EY PhosEth (△) were prepared by methods identical to those described in Fig. 1 as detailed in Materials and Methods. Each data point represents the means ± SE of at least three independent sample preparations.

probe in the hexagonal phase during the lifetime of the excited state. The observed alterations in the fluorescence anisotropy were due to an alteration in the motional regime of the probe, because the fluorescence lifetime of NBD-PE was not different significantly in the liquid-crystalline vs hexagonal phases (e.g., 6.5 ns at 6°C vs 6.6 ns at 15°C in OO PhosEth vesicles corresponding to liquid-crystalline and hexagonal phases, respectively).

To determine the ubiquity of the diminished polar head group molecular freedom in the hexagonal phase and to exclude the possibility that these results represented idiosyncratic behavior of NBD-PE in the hexagonal phase, additional experiments were performed using Rh-PE incorporated into liposomes comprised of either EY PhosEth or OO PhosEth as the reporter probe. Examination of the thermotropic dependence of the fluorescence anisotropy of the Rh-PE probe demonstrated lamellar to hexagonal phase transition temperatures identical to those determined by use of the NBD-PE probe in each of the systems studied (Fig. 2). The higher fluorescence anisotropies measured with the probe Rh-PE (in both EY PhosEth and OO PhosEth) in comparison with those ascertained by use of the NBD-PE probe reflect, in large part, the differential fluorescence lifetime of each probe (e.g., 6.5 ns for NBD-PE versus 3.0 ns for Rh-PE in OO PhosEth at 6°C). Because both probes gave qualitatively identical profiles and identical minima representing the lamellar to hexagonal phase transition temperature, a single probe (NBD-PE) was used in subsequent experiments.

To establish the foundation for a wider usage of this fluorometric method for determination of the lamellar to hexagonal phase transition in mixed systems, we performed additional experiments to measure the phase transition in binary mixture comprised of 5:1 OO PhosEth/OO PhosCho. Measurements of the fluorescence anisotropy of the NBD-PE probe over a temperature range from 18 to 44°C demonstrated a parabolic profile (Fig.

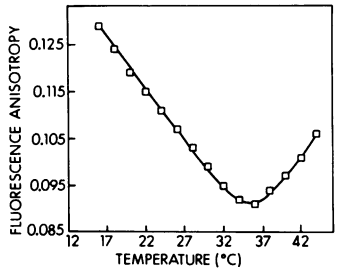


FIGURE 3 Steady-state fluorescence anisotropies of NBD-PE incorporated into binary mixtures of OO PhosEth/OO PhosCho (5:1). Vesicles comprised of 5:1 OO PhosEth/OO PhosCho were prepared by methods identical to those described in Fig. 1 as detailed in Materials and Methods. The minimum of the parabolic profile of 5:1 OO PhosEth/OO PhosCho occurred at 36°C. Each data point represents the means ± SE of at least three independent preparations.

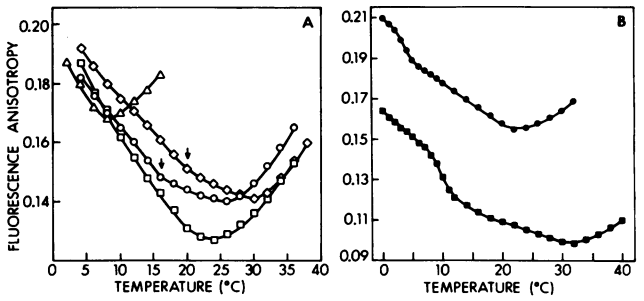


FIGURE 4 Characterization of the thermotropic dependence of homogeneous plasmenylethanolamine molecular species. (*A*) Approximately 500 μg of reverse-phase purified PO PlasEth (○), SO PlasEth (◇), or O'O PlasEth (△) were evaporated under a nitrogen stream and subsequently evacuated exhaustively at low pressure. Vesicles were prepared by addition of buffer and subsequent vortexing in the presence of glass beads as detailed in Materials and Methods. Mixed PlasEth (□) refers to a mixture of 1-*O*-(*Z*)-alk-1'-enyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphoethanolamines (the parent mixture) that was used to resolve each of these individual molecular species of plasmenylethanolamine. (*B*) Vesicles comprised of reverse-phase purified SA PlasEth (●) or SA PhosEth (■) were prepared as described in *A*. Each of the data points represents the means ± SE from at least three measurements.

3) with a well-defined minimum in the thermotropic dependence of fluorescence anisotropy at 36 ± 1°C. These results are in good agreement with the previously reported lamellar to hexagonal phase transition temperatures for OO PhosEth/OO PhosCho mixtures (Tilcock et al., 1982; Tate and Gruner, 1989).

## Lamellar to hexagonal phase transition temperatures of homogeneous plasmenylethanolamines

Although several reports have documented the lamellar to hexagonal phase transition for mixed plasmenylethanolamines (Boggs et al., 1981; Lohner et al., 1984), the lamellar to hexagonal phase transition temperature of any homogeneous plasmenylethanolamine molecular species has never been determined. To examine the lamellar to hexagonal phase transition temperatures of various homogeneous plasmenylethanolamines, a mixture of plasmenylethanolamine molecular species was resolved into individual constituents by reverse-phase column chromatography (as described in Materials and Methods). The lamellar to hexagonal phase transition temperature of liposomes comprised of homogeneous PO PlasEth, O'O PlasEth, or SO PlasEth was determined by characterizing the thermal profile of the fluorescence anisotropy of NBD-PE incorporated into each type of vesicle. The lamellar to hexagonal phase transition temperatures of PO PlasEth, O'O PlasEth, and SO PlasEth were 28, 8, and 30°C, respectively (Fig. 4 *A* and Table 2). Thus, the lamellar to hexagonal phase transition temperature of multiple homogeneous plasmenylethanolamine molecular species is substantially lower than their diacyl ethanolamine glycerophospholipid counterparts.

The gel to liquid-crystalline phase transition temperatures ($T_c$) of PO PlasEth and SO PlasEth can also be estimated from these NBD-PE anisotropy profiles as 15 and 20°C, respectively (Fig. 4 *A*, *arrows*). These values differ substantially from the previously determined values of 28 and 30°C for PO PhosEth and SO PhosEth (Small, 1986). Thus, although only relatively modest differences in the gel to liquid-crystalline phase transition temperature are present in choline glycerophospholipid subclasses (e.g., $\Delta T_c$ = 3°C between plasmenylcholine and phosphatidylcholine) (Han and Gross, 1991), the presence of a vinyl ether linkage in the proximal portion of the *sn*-1 aliphatic chain lowers the gel to liquid-crystalline phase transition temperature of ethanolamine glycerophospholipids considerably.

Because SA PlasEth and SA PhosEth are the two major ethanolamine glycerophospholipid constituents in several mammalian subcellular membranes (Gross, 1984, 1985; Nakagawa et al., 1985), characterization of

**TABLE 2 Lamellar to hexagonal phase transition temperatures ($T_h$) of plasmenylethanolamines and phosphatidylethanolamines***

| Phospholipid(s) | $T_h$ |
|---|---|
| | °C |
| PO PlasEth | 28 ± 1 |
| SO PlasEth | 30 ± 1 |
| O'O PlasEth‡ | 8 ± 1 |
| Mixed PlasEth§ | 24 ± 1 |
| SA PlasEth | 22 ± 1 |
| SA PhosEth | 32 ± 1 |

* The lamellar to hexagonal phase transition temperature was obtained from the minimum point of the thermal profile of fluorescence anisotropy (Fig. 4); ‡O'O PlasEth represents a mixture of 1-*O*-(*Z*)-octadec-1',9'-enyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphoethanolamine and 1-*O*-(*Z*)-octadec-1',11'-enyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphoethanolamine; and §mixed PlasEth represents 1-*O*-(*Z*)-alk-1'-enyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphoethanolamine.

the thermotropic properties of membranes comprised of these constituents was performed. An abrupt decrease of fluorescence anisotropy at ~4°C for SA PlasEth and 10°C for SA PhosEth (corresponding to the gel to liquid-crystalline phase transition, respectively) was determined, and a minimum at 22°C for SA PlasEth and 32°C for SA PhosEth (corresponding to the lamellar to hexagonal phase transition temperature for these molecular species, respectively) was identified (Fig. 4 *B* and Table 2). Thus, for these molecular species, the lamellar to hexagonal phase transition temperature of the homogeneous plasmenylethanolamine is substantially lower than its diacyl counterpart, and the presence of a vinyl ether linkage in the proximal portion of the *sn*-1 aliphatic chain lowers the gel to liquid-crystalline phase transition temperature of ethanolamine glycerophospholipids significantly. Furthermore, direct comparisons of the absolute values of fluorescence anisotropy of the NBD-PE probe in SA PlasEth and SA PhosEth membranes demonstrated that the presence of a vinyl ether linkage resulted in differences in the fluorescence anisotropy of the NBD-PE probe in these two classes in both lamellar state and hexagonal phases. For example, the fluorescence anisotropy of NBD-PE in vesicles comprised of SA PlasEth was 0.16, whereas that of NBD-PE in SA PhosEth vesicles was 0.11 (each at 20°C).

## Differential membrane fluidity gradients in the lamellar and hexagonal phases

One hallmark of the membrane dynamics of liquid-crystalline lipid bilayers is the similarity of the order parameter of deuterium probes on carbon atoms $n = 1$ to $n = 10$ (Seelig, 1977; Seelig and Seelig, 1980). In sharp contrast to dynamics in lipid bilayers, rapid alterations in the order parameter of $^2H$ in the aliphatic chain of lipids in the hexagonal phase has been demonstrated (e.g., Sternin et al., 1988). These differences have been attributed to the rapid diffusion of lipid molecules along the cylinder axis on the NMR time scale (Cullis and De Kruijff, 1979), as well as alterations in acyl chain packing in regions between the lipidic cylinders in the hexagonal phase. The experiments performed in this study demonstrate increased order as a function of temperature in the polar head group region for lipids present in the hexagonal phase. Accordingly, the bilayer to hexagonal phase transition should be accompanied by a substantial increase in the membrane fluidity gradient. With the use of a well-known series of homologous fluorescent probes (i.e., n-AS with $n = 2, 6, 9, 12, 16$) (Thulborn and Sawyer, 1978; Thulborn et al., 1979; Tilley et al., 1979), the fluidity at different positions of the membrane bilayer was quantified as a function of temperature in each phase and compared with alterations in NBD-PE fluorescence anisotropy (Fig. 5). In the bilayer phase (e.g., at 44 and 50°C), vesicles comprised of EE PhosEth possessed

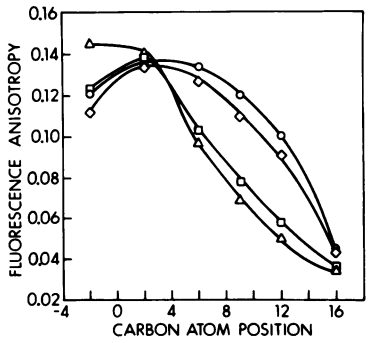


FIGURE 5 Membrane fluidity gradient profiles of EE PhosEth vesicle in the lamellar and hexagonal phases. Vesicles comprised of EE PhosEth were prepared as described in Fig. 1. Fluorescence anisotropies of NBD-PE ($n = -2$) and n-AS ($n = 2, 6, 9, 12, 16$) in EE PhosEth vesicles were determined from the parallel and perpendicular components of emitted light at 44 (○), 50 (◇), 64 (□), and 68°C (△). As shown in Fig. 1, the lamellar to hexagonal phase transition for this phospholipid is 58°C. Each data point represents the means ± SE of at least three independent preparations.

similar fluorescence anisotropies in the polar head group region and along the proximal portion of the aliphatic chain (up to the ninth carbon atom). At regions distal to the ninth carbon atom, a rapid decrease in fluorescence anisotropy was manifest. Thus, measurement of membrane fluidity using this fluorescence anisotropy technique closely corresponds to prior $^2H$ NMR results of bilayers in the liquid-crystalline phase. However, a substantial increase in membrane fluidity as a function of distance from the glycerol backbone was manifest for EE PhosEth in the hexagonal phase, even at the proximal regions of the aliphatic chains.

## DISCUSSION

The present study demonstrates the utility of a new fluorometric method for characterization of the lamellar to hexagonal phase transition and its suitability for determination of phase transition temperatures from as little as 100 μg of total lipid. The sensitivity of this method results largely from the parabolic profile of the thermotropic dependence of fluorescence anisotropy in conjunction with the intrinsic sensitivity of the technique of fluorescence spectroscopy. Other fluorescence methods previously used for detection of the hexagonal phase result in modest changes in the slope of the parameter ($P$) studied with respect to temperature (i.e., $dP/dT$), whereas the present method exploits the sensitivity and precision inherent in phenomena accompanied by changes in the sign of their first derivatives (i.e., increases in anisotropy with increasing temperature are present in the hexagonal phase, whereas decreases in anisotropy with increasing temperature are present in the liquid-crystalline phase). As noted previously (Hong et al., 1988), fluorescence spectroscopy does not offer precise

structural information and therefore cannot be used to identify existing hexagonal phases in membranes with unknown phase behavior. Thus, in the absence of a phase change, this methodology cannot be used to determine either the presence of a hexagonal phase or the existence of mixed phases. However, fluorescence anisotropy profiles offer the added advantage of allowing the simultaneous characterization of membrane dynamics in a wide variety of lipid systems studied as homogeneous dispersions or as mixtures containing physiologically relevant amphiphilic lipid constituents.

The molecular mechanisms mediating the observed alterations in the temperature dependence of the fluorescence anisotropy in systems undergoing sequential phase changes (i.e., gel to liquid-crystalline to hexagonal phase) appear straight-forward, at least in terms of first-order effects. During the gel to liquid-crystalline phase transition, the increased time-averaged spacing between neighboring polar head groups allows faster motion of the fluorescence reporter group, resulting in an accelerated rate of decrease in fluorescence anisotropy as a function of temperature. In contrast, during the lamellar to hexagonal phase transition, increased steric constraints in the polar head group region effectively preclude increased motional freedom of the polar head group as a function of temperature, although it previously has been shown that the order parameter of aliphatic chains of lipids in the hexagonal phase decreases as a function of temperature, demonstrating increased angular fluctuations of aliphatic chains with respect to the membrane director by using $^2$H NMR and fluorescence spectroscopy with probes imbedded in the membrane interior (Gally et al., 1980; Perly et al., 1985; Sternin et al., 1988; Chen et al., 1990; van der Meer et al., 1990). The experimentally observed increase in the fluorescence anisotropy in the head group region as a function of temperature in the hexagonal phase demonstrates that the radius of the internal water cylinder does not increase proportionally to the increased steric volume occupied by the aliphatic chain during heating. Thus, heating lipids in a hexagonal phase amplifies the fluidity gradient between the aliphatic chains and their polar head groups as shown herein. These alterations in relative molecular volume of the aliphatic and polar head group regions likely underlie the parabolic nature of the thermal profile of head group labeled fluorophores in lipid aggregates during the lamellar to hexagonal phase transition.

The measured fluorescence anisotropy is a function of the relative molecular motion of the reporter group and its fluorescence lifetime in each statistically relevant physical state. The alterations in fluorescence anisotropy observed in this study during the lamellar to hexagonal phase transition resulted from changes in the molecular freedom of the reporter group because substantial changes in the fluorescence lifetime of the probe did not occur. Previously, the possibility of alterations in phase separation or self-quenching of NBD-PE during the lamellar to hexagonal phase transition was excluded (Hong et al., 1988; Stubbs et al., 1989), which was further confirmed during this study (data not shown). Collectively, these results demonstrate that the observed alterations reflect bona fide changes in the motional regime of the reporter head group probe.

Using alterations in the fluorescence anisotropy of NBD-PE probe, we have determined the phase transition temperature of a variety of plasmenylethanolamine molecular species in homogeneous systems. In general, the $T_h$ of plasmenylethanolamine is much lower than that of its diacyl phospholipid counterpart (e.g., 28°C for PO PlasEth versus 67°C for PO PhosEth and 22°C for SA PlasEth versus 32°C for SA PhosEth). Increases in the unsaturation index of the aliphatic chains (particularly at the *sn*-1 position) diminish these subclass specific alterations (e.g., Fig. 4). According to the self-assembly theory of lipid aggregates (Israelachvili et al., 1976, 1980), the structure assumed by aqueous dispersions of lipids can qualitatively be related through a geometric parameter ($P$) defined by:

$$P = V/al_c \quad (1)$$

where $V$ is the aliphatic chain volume, $a$ is the hydrophobic-hydrophilic interfacial area of the lipid, and $l_c$ is the aliphatic chain length. The relationship between $P$ and the structure assumed by lipid dispersions is as follows: $\frac{1}{2} < P \leq 1$, bilayer structure and $P > 1$, inverted hexagonal structure. Our previous studies demonstrated that plasmalogens possess a smaller cross-sectional area ($a$) near the hydrophobic-hydrophilic interface, and the position of their head group is more upright in comparison with that of their diacyl phospholipid counterparts (Han and Gross, 1990; Han et al., 1991). Assuming that $l_c$ is the same for both plasmalogen and diacyl phospholipids, then the $P$ value is higher and the $T_h$ value is lower for plasmalogens in comparisons with their diacyl phospholipid counterparts. The presence of a double bond in the *sn*-1 aliphatic chain dramatically reduces the value of $l_c$ and results in a corresponding increase in $P$. This increase diminishes the difference in $T_h$ between OO PhosEth and O'O PlasEth.

Collectively, the present results identify a new fluorometric method for the characterization of the lamellar to hexagonal phase transition in phospholipid systems where limiting amounts of phospholipid mass are present. The utility of this technique lies in the precision inherent in defining peak minima (i.e., $dr/dT = 0$) of the fluorescence anisotropy of head group labeled fluorophores during the lamellar to hexagonal phase transition. It is hoped the application of this method will promote the characterization of the phase behavior of biologically important phospholipids and phospholipid mixtures where limiting amounts of sample are available.

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