Role of Head Group Structure in the Phase Behavior of Amino Phospholipids. 2. Lamellar and Nonlamellar Phases of Unsaturated Phosphatidylethanolamine Analogues†

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ABSTRACT: Three types of analogues of unsaturated phosphatidylethanolamines (PE) have been prepared: phosphatidyl-ω-amino-1-alkanols, *N*-alkyl-PE's, and C_2-alkyl-PE's, with alkyl substitution of carbon-2 of the ethanolamine head group. The physical properties of dioleoyl, dielaidoyl, and 1-palmitoyl-2-oleoyl phospholipids with these head groups have been examined by calorimetry, ^{31}P NMR, freeze–fracture electron microscopy, and X-ray diffraction. N-Alkylation of PE, or substitution of the ethanolamine moiety by 3-amino-1-propanol or 4-amino-1-butanol, decreases the transition temperature of the hydrated gel phase (T_c) and considerably increases the temperature of the lamellar to hexagonal II transition (T_H). The pattern of these effects for various PE analogues suggests that head group size and hydrophobicity as well as hydrogen bonding are important determinants of the phase behavior of these lipids. C_2-Alkylated PE analogues exhibit several rather surprising properties, notably the ready formation of a quasi-crystalline "high-melting" solid phase even for di-cis-unsaturated species and substantially *lower* T_H values than are observed for the parent PE species. The behavior of these compounds suggests that "hydration forces" can be more important than considerations of lipid "dynamic shape" in predicting the relative stabilities of lamellar vs. nonlamellar phases for at least some zwitterionic phospholipids.

One of the most intriguing characteristics of the lipids found in biological membranes is that most membranes contain significant proportions of lipids that do not readily form hydrated lamellar structures when dispersed in aqueous media in pure form. The presence of such lipids in membranes may be of considerable importance in supporting transient local fluctuations in membrane curvature and topology in processes such as membrane enzymatic functions (Navarro et al., 1984), transmembrane movement of large molecules (Jensen & Schutzbach, 1984), and membrane fusion (Cullis & de Kruijff, 1979; Duzgunes et al., 1981; Verkleij, 1984).

In animal and many bacterial cell membranes, ethanolamine phospholipids represent the major phospholipid class favoring the formation of nonlamellar phases, while most other major phospholipid classes normally form hydrated bilayer structures spontaneously when dispersed in aqueous media at physiological ionic concentrations. The phosphoethanolamine head group has several distinctive features that differentiate it from most other membrane glycerolipids, including a relatively small size and a high capacity to participate in amino to phosphoryl hydrogen bonds as both a donor and an acceptor. To understand what specific structural features of the ethanolamine phospholipid head group are most important in determining its distinctive physical properties, we have synthesized a series of structural analogues of phosphatidylethanolamine (PE)[1] incorporating small structural modifications of the ethanolamine moiety. In the preceding paper (Silvius et al., 1986), we examined the properties of a series of analogues of dimyristoyl-PE by calorimetry and Raman spectroscopy. These studies suggested that the unusual ability of saturated PE's to form a dehydrated, highly ordered gel phase depends primarily on the high hydrogen-bonding capacity of the PE head group rather than on the small size of the head group or even the conservation of a normal intramolecular separation between the phosphoryl and the amino group. In this study, we have combined calorimetry, ^{31}P NMR, freeze–fracture electron microscopy, and X-ray diffraction to investigate the phase behavior of unsaturated PE analogues. Our results indicate that structural modifications of the PE head group can affect the relative stabilities of lamellar and nonlamellar phases in sometimes surprising ways, and they suggest that the strength of hydration of PE, rather than any specific details of the head group structure (including overall size), is of paramount importance in determining the ability of ethanolamine phos-

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[1] Abbreviations: C_2-dimethyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-2′-amino-2′-methyl-1′-propanol; C_2-ethyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-*dl*-2′-amino-1′-butanol; C_2-isopropyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-*dl*-2′-amino-3′-methyl-1′-butanol; *dl*-C_2-methyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-*dl*-2′-amino-1′-propanol; *l*-C_2-methyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-*l*-2′-amino-1′-propanol; DE, dielaidoyl; DO, dioleoyl; EDTA, ethylenediaminetetraacetic acid disodium salt; *N*-ethyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-2′-(ethylamino)-1′-ethanol; *N*-methyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-2′-(methylamino)-1′-ethanol; NMR, nuclear magnetic resonance; PB, 1,2-diacyl-*sn*-glycero-3-phospho-4′-amino-1′-butanol; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PO, 1-palmitoyl-2-oleoyl; PP, 1,2-diacyl-*sn*-glycero-3-phospho-3′-amino-1′-propanol; T_c and T_c(hydrated), hydrated gel to liquid-crystalline transition temperature; T_H, lamellar liquid-crystalline to hexagonal II transition temperature; T_m(high melting), temperature of transition of a high-melting solid phase to a liquid-crystalline phase.

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Table I: Transition Parameters of Unsaturated Phosphatidylethanolamine Analogues

	T_c(hydrated) (°C)	ΔH(hydrated) (kcal mol^{-1})	T_m(high melting) (°C)	ΔH(high melting) (kcal mol^{-1})	T_H (°C)
DEPE	38.3	9.1	*a*	*a*	63.5
DEPP	30.7	9.1	34.7	17.7	88.5
N-methyl-DEPE	31.7	11.1	*a*	*a*	
dl-C_2-methyl-DEPE	32.3	9.6	40.0	18.4	57.5
l-C_2-methyl-DEPE	32.2	*b*	43.4	24.7	57.3
DEPB	23.7	9.1	35.4/36.6	13.7	
N,*N*-dimethyl-DEPE	21.2	12.0[e]	*a*	*a*	
N-ethyl-DEPE	27.0	9.9	43.2	20.4	
C_2-ethyl-DEPE	23.8	*b*	47.8	17.0	
C_2-dimethyl-POPE	26.2	12.9	61.3	16.1	55.5
C_2-isopropyl-DEPE	*c*	*c*	62.0	22.7	
POPE	25.6	6.4	*a*	*a*	69.0
dl-C_2-methyl-POPE	19.3	6.5	*a*	*a*	62.3
C_2-ethyl-POPE	8.2	*b*	32.1/33.5	12.3	47.2
C_2-ethyl-DOPE	*d*	*d*	27.5	15.4	
C_2-dimethyl-DOPE	*d*	*d*	36.1	11.1	
C_2-isopropyl-DOPE	*d*	*d*	48.7	18.1	

[a] No high-melting solid phase transition could be observed for this compound. [b] Samples could not be obtained entirely in the hydrated phase over the duration of the calorimetric scan. [c] Samples rapidly relaxed to the high-melting phase during preparation. [d] Transition of hydrated gel could not be seen above 5 °C. [e] From Gagné et al. (1985).

pholipids to form nonlamellar phases at temperatures not far from physiological.

Materials and Methods

Materials. Dielaidoyl- and dioleoylphosphatidylcholine were prepared as described previously (Silvius & Gagné, 1984). All amino alcohols used in the preparation of PE analogues were obtained from Aldrich. Ethanolamine, *l*- and *dl*-2-amino-1-propanol, *dl*-2-amino-1-butanol, 2-amino-2-methyl-1-propanol, and *dl*-2-amino-3-methyl-1-butanol were recrystallized as hydrochlorides from methanol/acetone or ethanol, and 3-amino-1-propanol and 4-amino-1-butanol were fractionally redistilled in vacuo before use. All other common chemicals were of reagent grade or better, and all solvents were redistilled.

Dioleoyl- and dielaidoylphosphatidylethanolamine, phosphatidyl-3-amino-1-propanol, phosphatidyl-4-amino-1-butanol, phosphatidyl-*l*- and phosphatidyl-*dl*-2-amino-1-propanol (*l*- and *dl*-C_2-methyl-PE), and phosphatidyl-*dl*-2-amino-1-butanol (C_2-ethyl-PE) were synthesized by enzymatic transphosphatidylation of DOPC and DEPC as outlined in the preceding paper (Silvius et al., 1986). Phosphatidyl-2-amino-2-methyl-1-propanol (C_2-dimethyl-PE) and phosphatidyl-*dl*-2-amino-3-methyl-1-butanol (C_2-isopropyl-PE) were synthesized chemically by condensing the *N*-*t*-Boc-protected amino alcohols with dioleoyl- or dielaidoylphosphatidic acid and then by deprotecting under mild acidic conditions, as described in the preceding paper for the synthesis of C_2-dimethyl-DMPE.

Methods. Lipid samples (typically containing 3.5–7 μmol of phosphorus) were lyophilized from cyclohexane or 19:1 cyclohexane/ethanol and then dispersed in 0.85 mL of 200 mM NaCl, 5 mM histidine, 5 mM Tes, and 0.1 mM EDTA, pH 7.4. "Hydrated" samples, and samples dispersed below their transition temperature, were prepared as described in the preceding paper (Silvius et al., 1986). Conditions for sample analysis by calorimetry, ^{31}P NMR, and freeze–fracture electron microscopy were as described previously (Gagné et al., 1985; Silvius et al., 1986). X-ray diffraction analysis of lipid dispersions was carried out as described by Hui et al. (1980).

Results

Calorimetric Studies. In Figure 1 is shown a series of calorimetric thermograms obtained for dispersions of DEPE and various analogues that were heated to the liquid-crystalline state, then slowly cooled to 4 °C, and incubated overnight at this temperature. The thermodynamic parameters for the phase transitions of these compounds are summarized in Table I. DEPE shows a sharp major endotherm at 38.3 °C, corresponding to the melting of a hydrated gel phase to the liquid-crystalline state, and a small endotherm at 63.5 °C that corresponds to a lamellar to hexagonal II phase transition (Cullis & de Kruijff, 1978). The "head group elongated" analogue DEPP, with one extra methylene segment between the phosphoryl and amino groups, shows a major endothermic transition at 30.7 °C, and DEPB, with two extra methylene segments, shows a similar transition at 23.7 °C. The small endotherm observed at 63.5 °C for DEPE is shifted to 88.5 °C for DEPP, and no similar endothermic transition can be detected up to 100 °C for DEPB. The effects of N-methylation and -ethylation on the main transition of DEPE appear similar to the effects of one or two methylene additions: *N*-methyl-DEPE shows a major transition at 31.7 °C, as reported previously (Gagné et al., 1985), while *N*-ethyl-DEPE exhibits a major transition at 27.0 °C. However, neither N-alkylated DEPE derivative shows any endothermic transition above the main transition up to at least 98 °C.

The thermotropic behavior of aqueous dispersions of C_2-alkylated derivatives of DEPE is quite different from that observed for the other types of analogues just discussed. In Figure 1 are shown thermograms obtained for samples of C_2-alkylated DEPE's that were dispersed above their transition temperatures and then incubated overnight at 2 °C before calorimetry. C_2-methyl-DEPE prepared from racemic 2-amino-1-propanol (*dl*-C_2-methyl-DEPE) shows a major transition at 32.3 °C followed by a smaller transition at 57.5 °C, and C_2-dimethyl-DEPE shows similar transitions at 26.2 and 55.5 °C, respectively. The temperatures of both transitions for these compounds are lower than the temperatures of the corresponding transitions of DEPE. C_2-ethyl- and C_2-isopropyl-DEPE exhibit a different pattern of thermotropic behavior, showing large endotherms at temperatures higher than the main transition of DEPE (47.8 and 62.0 °C, respectively). The major transitions observed for these two C_2-alkylated species are broader and considerably more endothermic than the main transitions of the other DEPE analogues examined (Table I).

C_2-Ethyl-DEPE dispersed under the conditions described above shows, in addition to a large endotherm at 47.8 °C, a

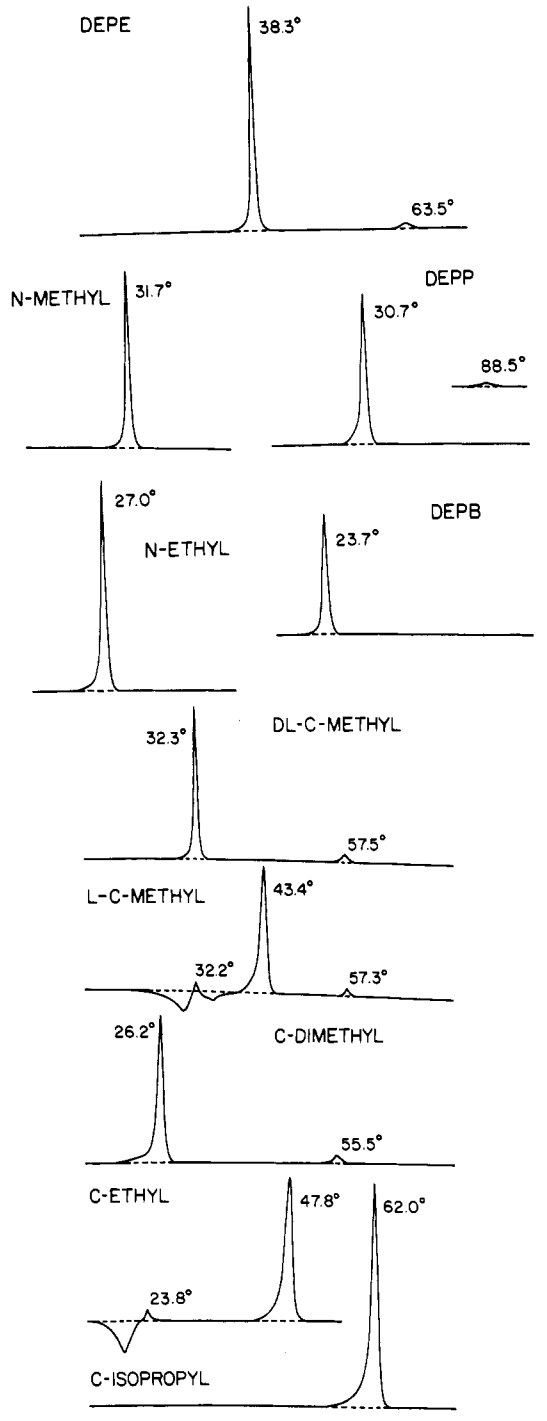

FIGURE 1: Calorimetric thermograms for dispersions of DEPE and its analogues after hydration above the transition temperature and overnight incubation at 2 °C. C_2- and N-alkylated derivatives of DEPE are denoted in the figure simply as C-alkyl or N-alkyl for economy of space. Transition temperatures and enthalpies are summarized in Table I.

small sharp endotherm of varying amplitude at 23.8 °C, which is superimposed on a shallow exotherm. We attribute this feature to an endothermic transition of a small fraction of the lipid that exists in a second, metastable gel phase in these samples at low temperatures. Similar behavior is seen for samples of *l*-C_2-methyl-DEPE (prepared from *l*-2-amino-1-propanol). This species when freshly hydrated shows a sharp endothermic transition at 32.2 °C superimposed on a broad exotherm, which is followed by a large endotherm at 43.4 °C and a small endotherm at 57.3 °C. The amplitude of the first endothermic transition decreases in samples incubated for longer times at 2 °C, as does the magnitude of the observed exotherm, while the amplitudes of the endotherms at 43.4 and 57.3 °C are virtually constant regardless of the conditions of sample preparation.

The relatively high temperatures and heat contents of the major transitions observed for *l*-C_2-methyl-, C_2-ethyl-, and C_2-isopropyl-DEPE suggest that these compounds readily form a low-temperature phase of considerably lower enthalpy than the normal hydrated gel phase of DEPE. To determine whether other analogues of DEPE can form similar solid phases, DEPE derivatives were dispersed at 0 °C by brief bath sonication followed by freeze–thawing and then incubated at 2 °C for 7–10 days prior to calorimetry. Representative calorimetric results obtained with such dispersions are shown in Figure 2. Dispersions of DEPE and *N*-methyl-DEPE that were prepared at low temperatures gave thermograms comparable to those observed for dispersions initially hydrated above the transition temperature. This finding suggests that the hydrated gel phase is the most stable low-temperature phase for these compounds. By contrast, *N*-ethyl-DEPE dispersed at low temperatures shows a new, highly endothermic transition at 43.2 °C, indicating that this compound can form a second, higher melting gel phase under some conditions. DEPP dispersed at 2 °C also shows a new, large endothermic transition at 34.7 °C. Samples of DEPB that are dispersed at 2 °C show a double transition with peaks at 35.4 and 36.6 °C, indicating that this species also forms a high-melting gel phase.

Both *dl*-C_2-methyl- and C_2-dimethyl-DEPE, when dispersed at 2 °C, show major transitions with peak temperatures and heat contents that are substantially higher than those observed for freshly hydrated dispersions of these lipids (Figure 2). *l*-C_2-methyl-DEPE prepared under these conditions shows a similar major transition at 43.4 °C. From the measured enthalpies and temperatures of these transitions, we can calculate that the high-melting solid phase that forms when these species are dispersed at 2 °C is more stable at low temperatures than is the gel phase found in freshly hydrated samples of these lipids. Interestingly, C_2-dimethyl-DEPE dispersions prepared at low temperatures show a single transition at 61.3 °C, while freshly hydrated samples of this compound show a major transition at 26.2 °C and a small transition at 55.5 °C. It appears, therefore, that on heating, the high-melting solid phase of C_2-dimethyl-DEPE passes directly to the high-temperature phase of this compound, without forming as an intermediate a stable liquid-crystalline lamellar phase.

To verify directly that the low-melting gel phases of DEPP, DEPB, *N*-ethyl-DEPE, and C_2-methyl- and C_2-dimethyl-DEPE are in fact metastable with respect to the high-melting phases, we hydrated samples of these lipids above their transition temperature and then incubated the samples for 8–14 days at 2 °C. Dispersions of the C_2-methylated DEPE's prepared in this way gave thermograms very similar to those shown in Figure 2, confirming that the samples relaxed to the high-melting solid phase during the prolonged incubation at 2 °C. *N*-ethyl-DEPE, DEPP, and DEPB prepared in this way still showed some heat absorption at the transition temperatures of the low-melting gel phases, but this was followed immediately by heat evolution, and large endotherms were then observed at the transition temperatures of the high-melting solid phases (not shown). Therefore, the low-melting gel phase is metastable with respect to the high-melting solid phase for all DEPE analogues that can exhibit both types of phases.

The main phase transitions of most dioleoyl phospholipids lie well below 0 °C (Silvius, 1982) and thus are not readily

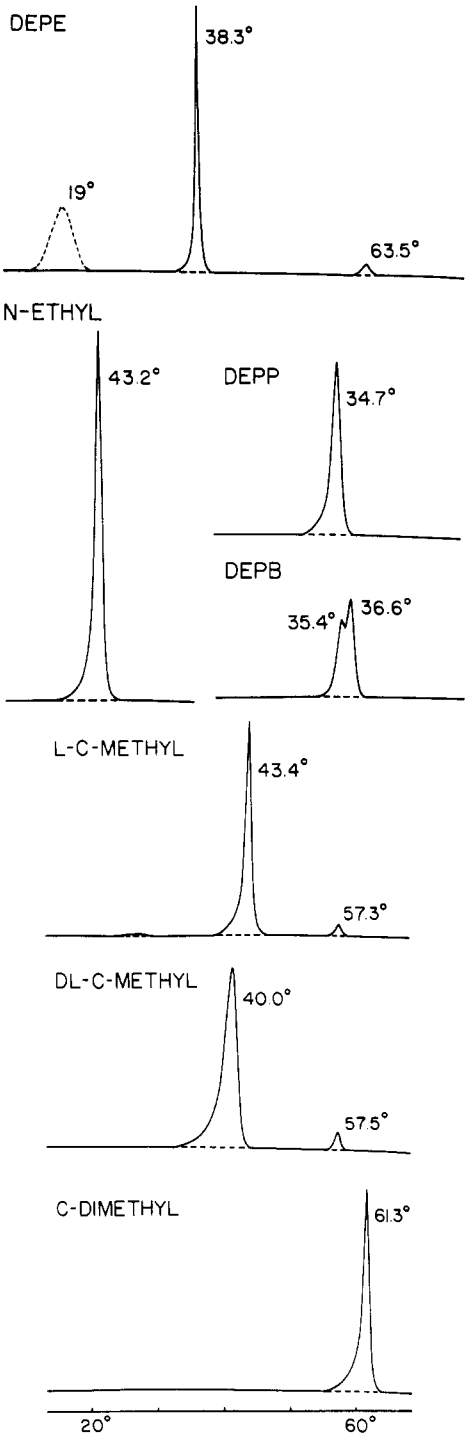

FIGURE 2: Calorimetric thermograms for dispersions of DEPE and its analogues after dispersing at 2 °C, freeze–thawing, and incubating for several days at 2 °C. The dashed peak shown in the thermogram for DEPE was seen only in samples incubated for long periods at 2 °C. Compound names are abbreviated as in Figure 1, and transition temperatures and enthalpies are summarized in Table I.

monitored with our calorimetric apparatus for samples in purely aqueous media. However, since C_2-alkylated PE's quite readily form unusually high-melting solid phases, we examined the calorimetric behavior of dispersions of C_2-alkyl-DOPE's that were dispersed at 2 °C and then incubated at this temperature for periods from one to several days. Representative results from such experiments are shown in Figure 3. C_2-methyl-DOPE shows no transition above 5 °C when dispersed at 2 °C. However, C_2-ethyl-DOPE shows a large endothermic transition peaking at 27.5 °C when dispersed at low temperatures. This transition was also observed when the lipid was dispersed at 37 °C and then incubated for 1 week at 2 °C. C_2-dimethyl-DOPE and C_2-isopropyl-DOPE dispersed under these conditions also show major transitions, at 36.1 and 48.7 °C, respectively. The molar enthalpies of these transitions, summarized in Table I, are comparable to but slightly lower than those of the corresponding transitions for dielaidoyl compounds. In contrast to the behavior of these species, DOPE itself, when dispersed at 2 °C, shows only a small endothermic transition at 9 °C corresponding to the conversion of the liquid-crystalline lamellar phase to the hexagonal II phase (Gagné et al., 1985; Epand, 1985). Likewise, DOPP and *N*-ethyl-DOPE samples prepared in this way showed small, broad transitions centered at 35 and 71.5 °C, respectively, while DOPB showed no clear transition up to at least 98 °C (Figure 3).

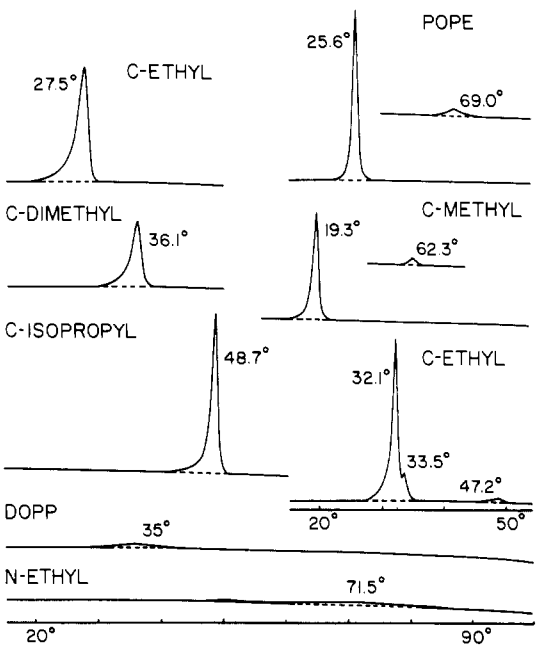

FIGURE 3: Calorimetric thermograms for dispersions of DOPE or POPE analogues that were prepared as were the samples in Figure 2. Thermograms for all DOPE analogues (with traces beginning at the left-hand side of the figure) are plotted on the lower temperature axis, and those for DOPE, *dl*-C_2-methyl-POPE, and C_2-ethyl-POPE (right-hand traces) are plotted on the temperature axis shown beneath the trace for C_2-ethyl-POPE. Other details are as in Figures 1 and 2.

The major glycerolipid species found in most natural membranes carry a long-chain saturated fatty acyl chain at the 1-position of the glycerol backbone and a cis-unsaturated acyl chain at the 2-position. To determine whether PE analogues with this type of acyl chain structure can also form high-melting gel phases, we examined the thermotropic behavior of 1-palmitoyl-2-oleoyl-PE (POPE) and its C_2-methyl and C_2-ethyl derivatives. Calorimetric thermograms obtained for dispersions of these lipids prepared under various conditions are shown in Figure 3. POPE dispersed above or below T_c shows two endothermic transitions at 25.6 and 69.0 °C, corresponding to a hydrated gel to liquid-crystalline transition and a lamellar to hexagonal II transition, respectively (Dekker et al., 1983; Epand, 1985). C_2-methyl-POPE dispersed and preincubated at 2 °C shows two similar transitions, at 19.3 and 62.3 °C, respectively. After being warmed above 20 °C and rapidly cooled to 2 °C, this species exhibited transitions with very similar temperatures and enthalpies, but the major transition was now somewhat broadened on the low-temperature side. C_2-ethyl-POPE, when dispersed at 2 °C, shows

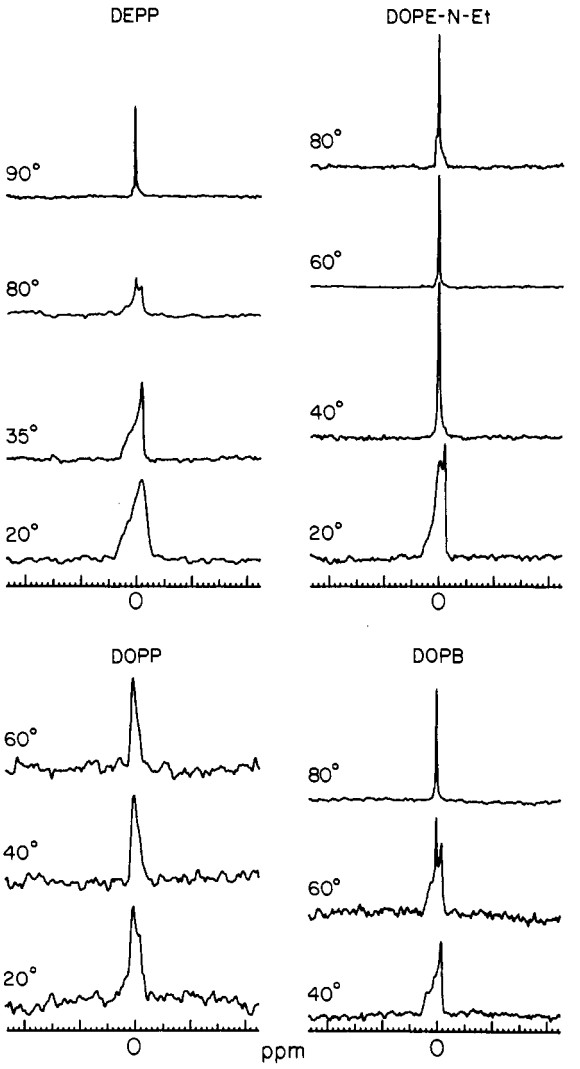

FIGURE 4: ^{31}P NMR spectra recorded for DEPP, *N*-ethyl-DOPE, DOPP, and DOPB at the indicated temperatures. Details of spectral collection were as described by Gagné et al. (1985). The major divisions of the chemical shift scale represent 100 ppm and the minor divisions 10 ppm.

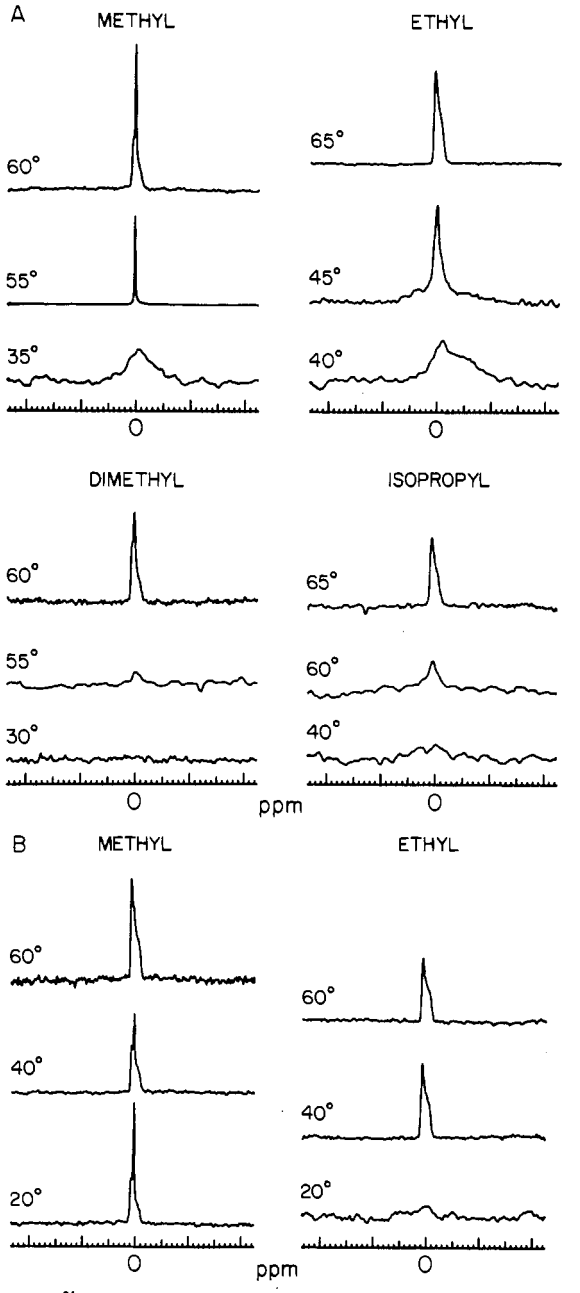

FIGURE 5: ^{31}P NMR spectra recorded for (A) *dl*-C_2-methyl-DEPE, C_2-ethyl-DEPE, C_2-dimethyl-DEPE, and C_2-isopropyl-DEPE and (B) *dl*-C_2-methyl-DOPE and C_2-ethyl-DOPE at the indicated temperatures. Chemical shift scales are as for Figure 5.

a large transition at 32.1 °C with a second, less endothermic component at 33.5 °C, followed by a small endotherm at 47.2 °C. After warming to 55 °C and rapid cooling to 2 °C, heating scans show a small endotherm at 8 °C and no endotherms at 32.1 or 33.5 °C, although the 47.2 °C transition is still observed (not shown). However, when C_2-ethyl-POPE is heated to 55 °C and then cooled to 2 °C and left at this temperature overnight, the major endothermic transitions are again observed at 32.1 and 33.5 °C, with a total enthalpy comparable to that observed for samples dispersed at low temperatures.

^{31}P NMR Studies. In the calorimetric experiments described above, three quite different types of endothermic transitions were observed with various analogues of unsaturated PE's. The first type, exemplified by the main transition of DEPE itself, gives a narrow, symmetrical endotherm with a heat of transition of ~9–12 kcal mol^{-1} and occurs at or below ~38 °C for dielaidoyl lipids. The second type of transition, which is also observed for DEPE, is of much lower enthalpy and occurs above the major transition in those cases where it can be observed as a distinct endothermic component in the calorimetric thermogram. The third type of transition, which is not observed for DEPE, is of considerably higher enthalpy than the other two ($\Delta H \geq 15$ kcal mol^{-1}), is characterized by a somewhat broader and less symmetrical endotherm, and generally occurs at temperatures above 35 °C (for DEPE analogues) or 25 °C (for DOPE analogues). To investigate the molecular organization of the phases involved in these transitions, we collected ^{31}P NMR spectra for a series of DOPE and DEPE analogues at various temperatures. Representative results obtained in these experiments are shown in Figures 4 and 5.

Previous ^{31}P NMR studies of DEPE and *N*-methyl-DEPE (Gagné et al., 1985) have shown that the major calorimetric transitions observed for these compounds represent a transformation from a gel phase that permits substantial rotational motion of the head group phosphate to a lamellar liquid-crystalline phase. As noted above, hydrated dispersions of DEPP and DEPB show major calorimetric transitions similar to those of DEPE and *N*-methyl-DEPE, suggesting that the nature of the main transition is basically similar for all four

compounds. The ^{31}P NMR spectra shown in Figure 4 for DEPP support this conclusion. As DEPP passes through its transition at 30.7 °C, the ^{31}P NMR spectrum shows a dramatic decrease in the intrinsic line width, accompanied by a modest reduction in the chemical shift anisotropy without major changes in the overall line shape. The reductions in spectral line width and chemical shift anisotropy above the transition are indicative of an increased rate of head group rotation and overall motion above 30.7 °C. However, the ^{31}P NMR spectral line shape indicates that the predominant motion is axial rotation, which is observable both above and below the 30.7 °C transition. Similar behavior was observed for hydrated samples of DEPB on warming through the 23.7 °C transition of this species (not shown). These spectral changes are entirely comparable to those observed at the main transitions of DEPE and *N*-methyl-DEPE (Gagné et al., 1985). As the temperature is increased beyond the main transition region, DEPB shows no further substantial changes in its ^{31}P NMR spectrum, consistent with the absence of any calorimetrically detectable phase transition for this compound above 23.7 °C up to at least 98 °C. By contrast, DEPP shows the appearance of a narrow, symmetric resonance in its spectrum at 80 °C, indicative of rapid isotropic reorientation. At 90 °C, the spectrum is a composite of resonances whose line shapes are characteristic of hexagonal structures and structures permitting isotropic motion. The changes observed in the ^{31}P NMR spectrum of DEPP in this temperature range are consistent with the calorimetric observation of a small endothermic transition at 88.5 °C for this compound.

Further insight into the high-temperature phase behavior of phosphatidylpropanolamines and phosphatidylbutanolamines can be obtained from the ^{31}P NMR spectra of DOPP and DOPB, whose cis-unsaturated chain configuration promotes the formation of nonlamellar phases at lower temperatures than in the case of the corresponding dielaidoyl compounds. As shown in Figure 4, DOPP at 20 °C shows a composite spectral line shape in which a lamellar-phase component can still be discerned. At 40 °C and higher temperatures, a spectrum characteristic of the hexagonal II phase is seen for this species. DOPB dispersions give a purely lamellar-phase spectral line shape up to ~60 °C, at which point an isotropic component begins to appear in the spectrum. At 80 °C, the spectrum of DOPB shows essentially only an isotropic resonance. For comparison, we also examined the ^{31}P NMR spectrum at various temperatures for *N*-ethyl-DOPE, whose head group bulk and gross shape resemble those of DOPB. Dispersions of *N*-ethyl-DOPE show a small isotropic spectral resonance even at 20 °C, and the ^{31}P NMR spectra at 40 and 60 °C are largely isotropic, with suggestions of a small underlying lamellar component at 40 °C and a minor hexagonal component at 60 °C. At 80 °C, the spectrum of *N*-ethyl-DOPE is clearly a composite of isotropic and hexagonal-phase components.

As we have discussed above, calorimetry shows that C_2-alkyl-PE's show a quite different thermotropic behavior from the other PE analogues examined, notably in the ready ability of these compounds to form solid phases with unusually high transition temperatures and enthalpies. The distinctive properties of C_2-alkylated PE's are also evident in their ^{31}P NMR spectra. As shown in Figure 5A, none of the four C_2-alkyl-DEPE's examined shows a typical hydrated gel-phase spectrum at low temperatures. At temperatures well below the main phase transition, these species show spectra much broader than those normally observed for gel-phase lipids. For C_2-dimethyl- and C_2-isopropyl-DEPE, no clear ^{31}P NMR spectrum could be collected at temperatures below ~50 °C, even when 20000 spectral decays were collected instead of the usual 5000. For these compounds at 55 °C, and for *dl*-C_2-methyl- and *dl*-C_2-ethyl-DEPE at somewhat lower temperatures, very broad spectra can be observed. The line shapes of these spectra indicate a highly restricted head group motion, which leads to both a fuller expression of the ^{31}P chemical shift anisotropy and an increase in the strength of ^{1}H–^{31}P dipolar interactions, which hampers spectral acquisition with the available instrumentation. Interestingly, these broadened spectra are still observed for *dl*-C_2-methyl-DEPE at 35 °C and for C_2-dimethyl-DEPE at 55 °C. These temperatures lie above the main transition temperatures measured for freshly hydrated dispersions of these lipids, but they lie below the main transition temperatures recorded when these species were dispersed at low temperatures. From this result, we may conclude that C_2-methyl- and C_2-dimethyl-DEPE (as well as C_2-ethyl- and C_2-isopropyl-DEPE) have relaxed to the more stable, higher melting solid phase under the conditions of sample preparation for ^{31}P NMR.

As C_2-isopropyl-DEPE is heated through the transition temperature of the stable solid phase, the ^{31}P NMR spectrum suggests that this compound melts directly from the solid phase to a hexagonal phase (see Figure 5A). Similar results are obtained for C_2-dimethyl- and C_2-ethyl-DEPE, but a small isotropic component is observed in the spectra of these compounds at temperatures very near the melting transition of the stable solid phase. For all three of these compounds, the ^{31}P NMR spectroscopic results agree with the calorimetric results inasmuch as both techniques show a single melting transition for these compounds, with no second transition at higher temperatures. For *dl*-C_2-methyl-DEPE, calorimetry of samples dispersed at low temperatures shows two endothermic transitions, a major one at 40 °C and a smaller one at 57.5 °C. Correspondingly, ^{31}P NMR shows a change from a solid-phase spectrum at 35 °C to a predominantly hexagonal-phase spectrum at 60 °C. Interestingly, however, the intermediate phase that exists between the 40 and 57.5 °C transitions of *dl*-C_2-methyl-DEPE exhibits a purely isotropic spectrum rather than a spectrum characteristic of a liquid-crystalline lamellar phase.

^{31}P NMR spectra recorded for C_2-methyl- and C_2-ethyl-DOPE at various temperatures reveal that the phase behavior of these compounds is quite similar to that of the corresponding dielaidoyl species, although the phase transformations occur at lower temperatures. The spectrum of *dl*-C_2-methyl-DOPE at 20 °C, like that of *dl*-C_2-methyl-DEPE at 60 °C, is a composite of hexagonal-phase and isotropic components, which gradually converts to a purely hexagonal-phase spectrum between 40 and 60 °C. C_2-ethyl-DOPE shows no clear ^{31}P NMR spectrum at 20 °C, in agreement with the calorimetric finding that this compound forms a stable solid phase with a high-enthalpy melting transition at 27.5 °C. Above this transition, a ^{31}P NMR spectrum characteristic of a pure hexagonal II phase is obtained for this lipid.

The ^{31}P NMR spectral results described above provide one further useful finding: the chemical shift anisotropies recorded for the liquid-crystalline lamellar phases of the various PE analogues are all quite similar at similar temperatures, and the same is true for the hexagonal-phase spectra of various analogues at comparable temperatures. The head group conformations around the phosphoryl group therefore appear to be very similar for all the various PE analogues examined (Yeagle, 1978).

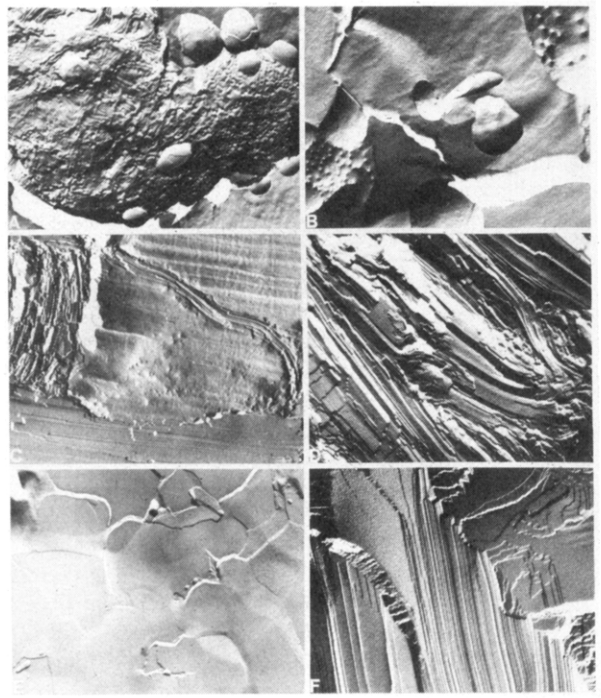

FIGURE 6: Freeze–fracture electron micrographs obtained for samples of (A) DOPP at 20 °C, (B) *N*-ethyl-DOPE at 20 °C, (C) *dl*-C_2-methyl-DOPE at 20 °C, (D) *dl*-C_2-methyl-DOPE at 46 °C, (E) C_2-ethyl-DOPE at 20 °C, and (F) C_2-ethyl-DOPE at 46 °C. Details of sample preparation were as in Gagné et al. (1985).

Freeze–Fracture Electron Microscopy. The isotropic or very broad resonances observed by ^{31}P NMR for some of the PE analogues described above can in principle arise from a variety of lipid structures (Cullis & de Kruijff, 1979). To assign more reliably the structures of dispersions of these species at various temperatures, we examined by freeze–fracture electron microscopy the morphologies of samples of several DOPE analogues in the temperature range 0–50 °C. Some representative results of these studies are shown in Figure 6.

The freeze–fracture morphologies of aqueous dispersions of DOPP and *N*-ethyl-DOPE are generally very similar to those of unmodified PE samples, allowing for differences in the phase transition temperatures of the different species. In Figure 6A,B are shown freeze–fracture images for dispersions of DOPP and *N*-ethyl-DOPE at 20 °C. Both samples are undergoing a bilayer to nonbilayer transition. DOPP at this temperature is farther along in the transition, with numerous point defects (lipidic particles) and line defects outlining the broad tubes that are the precursor of the thinner hexagonal II tubes (Hui et al., 1983). Lipidic particles are also apparent in the sample of *N*-ethyl-DOPE although not as numerous. These features account for the isotropic resonances seen in NMR spectra of these samples. However, the basic structure of these samples is still lamellar, and many areas remain smooth and vesicle-like. The mixed morphology agrees well with the NMR results.

C_2-Alkylated derivatives of DOPE behave quite differently from the above analogues, in agreement with our calorimetric and ^{31}P NMR results. Dispersions of *dl*-C_2-methyl-DOPE are already predominantly in the hexagonal II state at 20 °C with very few lipidic particles (Figure 6C). Upon being heated to 46 °C, the sample becomes entirely hexagonal II (Figure 6D). It appears that the transition from bilayer to hexagonal II involves few point defects (lipidic particles) as an intermediate structure. This may explain the less prominent isotropic peak in the NMR spectra for this compound compared to, e.g., those of DOPP or *N*-ethyl-DOPE. C_2-ethyl-DOPE shows a more

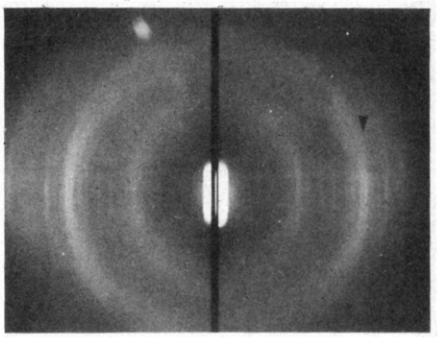

FIGURE 7: X-ray diffraction image recorded for C_2-ethyl-DOPE at 20 °C. The arrow indicates a pair of strong wide-angle reflections at 1/4.5 Å^{-1} and 1/4.4 Å^{-1}. Details of sample preparation and analysis were as in Hui et al. (1980).

dramatic thermotropic behavior. At 20 °C, the sample is in the form of a rigid crystal, with very extensive flat planes (Figure 6E). Upon being heated, the crystalline layers transform directly into hexagonal II structures (Figure 6F). The arrangement of the hexagonal II tubes at 46 °C appears to follow the organization of their precursors, the rigid planes seen at lower temperatures. As a result, the arrays of hexagonal II cylinders are much more ordered in directions perpendicular to the cylinder axes than is the case for, e.g., C_2-methyl-DOPE (Figure 6D). Samples of C_2-ethyl-DOPE show no intermediate structures such as point defects, nor are any vesicles observed that would indicate a flexible bilayer. The direct transformation observed from a (dehydrated) rigid crystal to a hexagonal II form is consistent with the NMR results.

X-ray diffraction measurements were performed on the crystalline form of C_2-ethyl-DOPE to investigate the molecular packing. At 20 °C, the sample gives a lamellar repeat of 54 Å, indicating an extremely dehydrated bilayer packing. Numerous sharp wide angle lines were detected, indicating that the acyl chains are much better ordered than in a typical hydrated gel-state bilayer and instead are packed in an array resembling a crystalline form (Ruocco & Shipley, 1982). The wide-angle spacings start from 7.8 to 3.4 Å (Figure 7) with the strongest lines at 4.4 and 4.5 Å. Upon heating to 50 °C, the wide angle lines disappear, and the small angle pattern shows a hexagonal array with a 66-Å center-to-center distance between the tubes (not shown). This value is only slightly smaller than the hexagonal lattice repeat distance measured by Kirk and Gruner (1985) for DOPE at this temperature.

DISCUSSION

Several recent theoretical and experimental studies have sought to define the factors that determine the relative stabilities of lamellar and nonlamellar phases of polar lipids. Israelachvili et al. (1976, 1980) have described the organization of lipid aggregates as a consequence of the interplay of entropic factors and packing constraints determined by the effective "shapes" of the lipid molecules. The effective shape of a lipid cannot be predicted simply from the structure of the molecule in isolation but depends also on the details of lipid–lipid and lipid–solvent interactions in the lipid aggregate. Rand et al. (1985) and Kirk et al. (1984) have explicitly considered various interactions of these types in recent analyses of the stabilities and dimensions of inverted phases of phospholipids. At present, however, there exists no straightforward method to predict the phase preferences of a particular lipid species simply from its chemical structure. While the very different tendencies of diacylphosphatidylethanolamines and the corresponding phosphatidylcholines to form nonlamellar phases

are well documented (Cullis & de Kruijff, 1979), it has not yet been possible to define precisely the relative importance of head group size, shape, hydrogen-bonding ability, and hydration in determining the very different behavior of these two lipids.

Three types of structural analogues of DOPE and DEPE were prepared and examined in this study: phosphatidyl-*n*-alkanolamines with one or two extra methylene units between the phosphoryl and amino groups; *N*-methyl- and *N*-ethyl-PE's; and C_2-alkylated PE's. All three types of analogues have larger head groups than that of PE itself, but the phosphatidyl-*n*-alkanolamines and *N*-alkyl-PE's retain a linear arrangement of carbon and nitrogen atoms in the head group, while the C_2-alkyl-PE's have a branched arrangement of these atoms. Conversely, the C_2-alkyl-PE's retain both the full hydrogen-bonding capacity of the PE amino group and a normal separation of the phosphoryl and amino groups, while *N*-alkyl-PE's have a diminished hydrogen-bonding capacity and the phosphatidyl-*n*-alkanolamines have an altered spacing between the phosphoryl and amino groups. These compounds thus allow us to examine what specific structural features of the PE head group (e.g., small head group size vs. a high hydrogen-bonding capacity) are the most important determinants of the distinctive physical properties of PE.

As we observed for the dimyristoyl lipids studied in the previous paper, various unsaturated PE analogues can form either conventional hydrated gel phases or "high-melting" solid phases, depending on the conditions of sample preparation, the acyl chain composition, and, of greatest interest here, the structure of the lipid head group. The features of head group structure that influence the relative stabilities of hydrated gel and high-melting solid phases have been discussed in the previous paper (Silvius et al., 1986) for analogues of dimyristoyl-PE, and the results obtained here for unsaturated PE analogues are generally quite comparable. What is most noteworthy for this discussion is the fact that the stabilities of these two types of phases can respond quite differently to a given change in the structure of the PE head group.

The high-melting solid phase formed by some PE's and PE analogues is stabilized by modifications of the PE head group that increase its overall hydrophobicity and that favor the establishment of lipid–lipid over lipid–water hydrogen bonds. This phase is characterized by a strong immobilization of lipid head groups and a very short lamellar repeat distance that suggests a virtual absence of water between the bilayers (this work; Seddon et al., 1983; Chang & Epand, 1983; Mantsch et al., 1983). It is thus understandable that the transition temperature of the high-melting solid phase is sensitive to fine details of the head group structure, particularly insofar as these influence the energy of hydration of the head groups. More surprising is the fact that, for all of the series of modified diacyl-PE's that were examined in this study, the transition temperature for the hydrated gel phase is most sensitive to the head group size and shows a much smaller influence from fine details of the head group structure, including putative hydrogen-bonding capacity. Normal hydrated gel phases of phospholipids differ from the high-melting solid phase in that the lipid head groups in hydrated gel phases exhibit appreciable rates of rotation about the bilayer normal, as well as possible facile interconversions of different head group conformations of similar energies (Seelig & Gally, 1976; Frischleder et al., 1981; Blume et al., 1982; note also the DEPP spectra in Figure 4 of this paper). While the rates of these rotational motions increase considerably at the melting transition of the hydrated gel phase, the nature and extent of such motions change much less dramatically at this transition. Therefore, the basic nature and the energetics of lateral interactions of lipid head groups in hydrated phases may be less sensitive to the exact arrangement of atoms in the head group than to "coarse" features of the head group structure that influence the mean molecular area, such as net charge and excluded-volume effects related to overall steric bulk.

In the light of the above discussion, we would expect that, in a hydrated phase allowing rapid axial motion, increasing alkyl substitution of the PE head group should progressively increase its effective size, altering the "dynamic shape" of the molecule in a way that would decrease its tendency to form hexagonal II or other structures with inverted geometries. Kumar and Gupta (1983) have reported a similar conclusion from measurements of the asymmetric transbilayer distribution of a variety of PE analogues in highly curved lipid vesicles. In fact, however, our experimental results do not appear to fit the pattern we would predict simply from considerations of molecular dynamic shape. C_2-alkylated PE's show lower values of T_H, the temperature of the lamellar liquid-crystalline to hexagonal II phase transition, than do the parent PE's, and the value of T_H decreases with increasing extents of C_2-alkylation. Likewise, *N*-ethyl-DOPE converts to the hexagonal II phase at virtually the same temperature as does *N*-methyl-DOPE, and the *N*-ethyl species can actually form "isotropic" structures in predominantly lamellar phases at lower temperatures than can the *N*-methyl species (this work; Gagné et al., 1985). In general, if we consider a series of analogues of a given diacyl-PE for which the head group "hydrogen-bonding capacity" is maintained constant by fixing the phosphate to amino group distance and the number of *N*-alkyl substitutents, we find that species with larger, more hydrophobic substituents more readily form nonlamellar structures at lower temperatures. These effects of the head group structure on the value of T_H for a PE analogue are quite similar to the effects of the head group structure on the tendency of the analogue to form a high-melting solid phase at lower temperatures. This pattern of results, which suggests that the strength of head group hydration plays a major role in determining the ability of these lipids to form nonlamellar phases, can be explained in the light of the considerations discussed below.

Current models of the organization of aqueous dispersions of polar lipids (Israelachvili et al., 1976; Kirk & Gruner, 1984; Rand et al., 1985) consider two major contributions of the head group structure in determining the relative stabilities of lamellar and nonlamellar phases of zwitterionic phospholipids. First, the head group structure is important in determining the optimal surface area per head group [a_0 in the terminology of Israelachvili et al. (1976)] and the elastic energy associated with variation of the head group area from this optimal value in different lipid packing geometries (Kirk et al., 1984). These "shape" effects have been discussed above. Second, however, the head group structure has a strong influence on the strength of the "hydration force", which falls off exponentially from the lipid surface with a decay length of $\sim$2.5 Å (Lis et al., 1981) and which tends to antagonize the approach of two lipid surfaces, particularly at separations of $\leq$20 Å. Since these distances are of the same order as the dimensions of the internal aqueous cavities of nonlamellar lipid phases, the strength of hydration forces can be an important determinant of the optimal geometry (and the energy) of the hexagonal phase for a phospholipid dispersion (Rand et al., 1985; Kirk et al., 1984).

In the light of these considerations, the results described above suggest that for PE and at least some of its derivatives,

notably, the C_2-alkylated derivatives, the magnitude of the hydration force, rather than the lateral interactions of the lipid head groups, may be the dominant factor through which the head group structure influences the relative stabilities of the lamellar liquid-crystalline and the hexagonal II phases. For these species, we can readily estimate the relative magnitudes of hydration vs. molecular shape effects in determining the stabilities of lamellar vs. nonlamellar phases, since the two factors act in opposite directions. For other types of analogues of PE, including the biologically important N-methylated derivatives, structural modifications that increase the optimal surface area of the head group also increase the strength of the hydration force between surfaces at small separations. It is therefore difficult to assess independently the relative importance of these two factors in determining the overall energy of the hexagonal II phase for these latter species. However, recent theoretical experimental studies (Kirk et al., 1984; Rand et al., 1985) have suggested that both factors may be of importance.

As a final note, we would observe that certain of the analogues of PE examined in this study can be introduced into the membranes of living animal cells by supplementation of the growth medium with the appropriate amino alcohols (Lee et al., 1975; Ferguson et al., 1975, Schroeder et al., 1976). It may therefore be possible to develop new approaches to manipulate the composition of cell membranes in such a way that the strength of surface hydration of the lipid phase, and its proclivity to form nonlamellar phases, may be altered significantly and with some selectivity. Such studies, if they prove feasible, may provide much valuable new information regarding the roles of membrane lipid polymorphism and surface properties in the normal function of living cells.

Registry No. DEPE, 19805-18-6; DEPP, 102587-99-5; *N*-methyl-DEPE, 96647-98-2; *dl*-C_2-methyl-DEPE, 102588-00-1; *l*-C_2-methyl-DEPE, 102588-01-2; DEPB, 102588-02-3; *N*,*N*-dimethyl-DEPE, 96647-99-3; *N*-ethyl-DEPE, 102588-03-4; C_2-ethyl-DEPE, 102588-04-5; C_2-dimethyl-DEPE, 102588-05-6; C_2-isopropyl-DEPE, 102588-06-7; POPE, 26662-94-2; C_2-methyl-POPE, 102588-07-8; C_2-ethyl-POPE, 102588-08-9; C_2-ethyl-DOPE, 102679-63-0; C_2-dimethyl-DOPE, 102679-64-1; C_2-isopropyl-DOPE, 102679-65-2.

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