Snell, E. E. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) 19–35, Wiley, New York.
Suelter, C. H., Wang, J., & Snell, E. E. (1976) *Anal. Biochem. 76*, 221–232.
Tanizawa, K., & Miles, E. W. (1983) *Biochemistry 22*, 3594–3603.
Tsai, M.-D., Schleicher, E., Potts, R., Skye, G. E., & Floss, H. G. (1978) *J. Biol. Chem. 253*, 5355–5349.

Role of Head Group Structure in the Phase Behavior of Amino Phospholipids. 1. Hydrated and Dehydrated Lamellar Phases of Saturated Phosphatidylethanolamine Analogues†

John R. Silvius* and Pamela M. Brown
Department of Biochemistry, McGill University, Montréal, Québec, Canada H3G 1Y6
Timothy J. O'Leary
Laboratory of Chemical Physics, National Institutes of Health, Bethesda, Maryland 20205
Received December 27, 1985

ABSTRACT: Analogues of dimyristoylphosphatidylethanolamine (DMPE) have been prepared with head groups modified by N-alkylation, alkylation of carbon 2 of the ethanolamine group, or interposition of extra methylene segments between the phosphoryl and amino groups. The phases formed by these lipids in aqueous dispersions have been examined by high-sensitivity differential scanning calorimetry and Raman spectroscopy. All of the DMPE analogues examined, excepting *N*-methyl-DMPE but including *N*-ethyl-DMPE, form hydrated gel phases that are metastable with respect to a dehydrated "high-melting" solid phase that has been observed previously for DMPE itself. The properties and the conditions of formation of this high-melting phase are qualitatively distinct from those of the "subgel" phase, which is observed for dipalmitoylphosphatidylcholine and for some of the DMPE analogues examined in this study. The high-melting phases of different DMPE analogues all exhibit similarly tight packing of the acyl chains, which however do not pack according to a single type of subcell that can be universally and specifically associated with this phase. Increasing the size of the PE head group invariably decreases the melting temperature of the hydrated gel phase, even when the normal hydrogen-bonding capability of the head group is preserved. By contrast, addition of larger alkyl substituents to either the amino group or carbon 2 of the ethanolamine moiety substantially increases the transition temperature of the high-melting solid phase, indicating that the contributions of the head group to the energies of the hydrated gel and the high-melting phases are fundamentally different. Our results suggest that the head group structural requirements for a neutral phospholipid to form stable hydrated bilayers are rather stringent, a fact that may explain the overwhelming predominance of only a few such head group structures in most natural membranes.

Phosphatidylcholine (PC)[1] and phosphatidylethanolamine (PE) together constitute the majority of the total phospholipids in most animal cell membranes. These two lipids differ markedly in several key physical properties that may be important in determining the stability and the flexibility of the bilayer organization of lipids in natural membranes. Most notably, lamellar phases of PE are less strongly hydrated than are bilayers of PC (Lis et al., 1981), and aqueous dispersions of PE's can adopt nonlamellar configurations much more readily than can PC's with the same acyl composition (Cullis & de Kruijff, 1979; Dekker et al., 1983; Verkleij, 1984). These differing properties of PE and PC can have a profound influence on the stability and the interactions of bilayer membranes containing these lipids (Düzgünes et al., 1981; Sundler et al., 1981; Silvius & Gagné, 1984a,b).

The differences observed in the physical properties of PE and PC can be attributed to two basic differences in the structural characteristics of the head groups of these species. First, of course, the sheer volume of the PC head group is greater than that of PE, and moreover, the non-hydrogen

†This work was supported by grants from the Medical Research Council of Canada (Grants ME-7580 and MA-7776), les Fonds de la recherche en santé du Québec (Grant 820040), and les Fonds pour la formation de chercheurs et l'aide à la recherche du Québec (Grant 86-EQ-2954).

[1] Abbreviations: *t*-Boc, *tert*-butoxycarbonyl; 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; *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; DLPE, dilauroylphosphatidylethanolamine; DM, dimyristoyl; DPPC, dipalmitoylphosphatidylcholine; 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; 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; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; TLC, thin-layer chromatography; T_m(high melting), temperature of transition of a high-melting solid phase to a liquid-crystalline phase.

0006-2960/86/0425-4249$01.50/0

atoms of the choline moiety are joined in a branched structure, while the heavy atoms of the ethanolamine head group are arranged in a linear structure. Second, tri-N-methylation of PE to form PC abolishes the ability of the amino group to participate in hydrogen bonds with the phosphoryl groups of adjacent lipids, a potentially important feature in the organization of PE bilayers (Hitchcock et al., 1974; Eibl & Wooley, 1979; Boggs, 1980). Previous studies of the properties of N-mono- and N-dimethylated PE's (Vaughan & Keough, 1974; Casal & Mantsch, 1983; Mulukutla et al., 1984; Gagné et al., 1985; Chowdhry & Dalziel, 1985) have shown that most properties of these compounds change gradually rather than abruptly from a "PE-like" to a "PC-like" pattern as the extent of methylation of the PE amino group increases. However, these studies have not allowed us to determine whether N-methylation of PE changes the physical properties of this species by increasing the head group bulk per se, by decreasing the hydrogen-bonding capacity of the head group, or by a combination of these factors.

In this study, we have prepared a series of head group modified analogues of PE in which the steric bulk and the hydrogen-bonding capacity of the head group have been varied independently. In this paper, we describe calorimetric and Raman spectroscopic results obtained with a series of analogues of dimyristoyl-PE, which provide information about the structures and thermodynamic properties of the lamellar phases of these compounds and how these properties are affected by the lipid head group structure. In the following paper, we describe results obtained by calorimetry, ^{31}P NMR, and freeze–fracture electron microscopy for a series of unsaturated PE analogues, which allow us to examine in detail the effects of head group structure on the formation of nonlamellar phases as well.

Materials and Methods

Materials

DMPC (grade 1) was obtained from Sigma, and all amino alcohols used in preparing DMPE and its analogues were obtained from Aldrich. Ethanolamine, 2-amino-1-propanol, and 2-amino-2-methyl-1-propanol were recrystallized as the hydrochlorides from methanol/acetone or ethanol, and 3-amino-1-propanol and 4-amino-1-butanol were redistilled in vacuo before use. All common chemicals were of at least reagent grade; all solvents were redistilled before use.

Lipid Preparations. Dimyristoylphosphatidylethanolamine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-3′-amino-1′-propanol, 1,2-dimyristoyl-*sn*-glycero-3-phospho-4′-amino-1′-butanol, 1,2-dimyristoyl-*sn*-glycero-3-phospho-2′-(ethylamino)-1′-ethanol (*N*-ethyl-DMPE), 1,2-dimyristoyl-*sn*-glycero-3-phospho-2′-amino-1′-propanol (C_2-methyl-DMPE), and 1,2-dimyristoyl-*sn*-glycero-3-phospho-2′-amino-1′-butanol (C_2-ethyl-DMPE) were prepared from DMPC by phospholipase D catalyzed transphosphatidylation with the appropriate amino alcohols, essentially as described previously for preparation of PE (Comfurius & Zwaal, 1977; Silvius & Gagné, 1984a), except that the reactions were carried out at pH 6.8 instead of pH 5.6. The lipids were purified by silicic acid column chromatography followed by acetone precipitation as described previously (Silvius & Gagné, 1984a). In some cases, samples were further purified by preparative TLC to ensure against contamination by traces of fluorescent materials.

1,2-Dimyristoyl-*sn*-glycero-3-phospho-2′-amino-2′-methyl-1′-propanol (C_2-dimethyl-DMPE) could not be prepared efficiently from DMPC by enzymic transphosphatidylation, and this lipid was therefore synthesized by coupling the N-protected amino alcohol to dimyristoylphosphatidic acid (Aneja et al., 1970) and then deprotecting the amino group under mild conditions, by procedures we have previously employed to prepare synthetic phosphatidylserines (Silvius & Gagné, 1984a). Briefly, 2-amino-2-methyl-1-propanol was first converted to its *N-t*-Boc derivative with di-*tert*-butyl dicarbonate, as described by Morodor et al. (1976; method B). The protected amino alcohol (2 molar equiv) was then coupled to dimyristoylphosphatidic acid (1 equiv) with triisopropylbenzenesulfonyl chloride (3 equiv) in dry pyridine as a condensing agent, as described previously for the synthesis of protected phosphatidylserine derivatives (Silvius & Gagné, 1984a). The protected lipid was isolated by silicic acid column chromatography (eluting with a gradient of methanol in chloroform, yielding the pure product at ~7% methanol) and then thoroughly dried in vacuo over P_2O_5. The dried lipid was dissolved in dry, alcohol-free chloroform and deprotected by bubbling the solution successively with dry nitrogen for 15 min and with dry HCl for 25 min at 0 °C and then stirring the HCl-saturated solution at 0 °C for 2 h, followed by workup as described previously for isolation of deprotected phosphatidylserines (Silvius & Gagné, 1984a). The deprotected C_2-dimethyl-DMPE was finally purified in the same way as the other amino phospholipids described above.

Methods

Sample Preparation. Lipid samples were lyophilized from cyclohexane or cyclohexene/ethanol (20:1 v/v) and redispersed in buffer (200 mM NaCl, 5 mM histidine, 5 mM Tes, and 1 mM EDTA, pH 7.4) at concentrations of 6–10 mM (for calorimetric samples) or ~100 mM (for Raman samples). Lipids to be dispersed as hydrated samples were then warmed briefly above their transition temperatures (to 65 °C for DMPE, DMPP, DMPB and C_2-methyl-DMPE, and very briefly to 85 °C for C_2-dimethyl-DMPE), cooled quickly to 50 °C, and cooled from this temperature to 2 °C at a rate of ≤0.5 °C/min. These samples were further incubated at 2 °C for varying times, as indicated in the text. Samples to be dispersed below their transition temperatures were briefly bath sonicated at 15 °C to ensure complete wetting of the lipid, then freeze/thawed 3 times with a dry ice–acetone bath, and finally allowed to rewarm to 15 °C, cooled slowly to 2 °C, and incubated at the latter temperature for a minimum of 2 days (the exact incubation periods for each type of experiment are specified in the text).

Calorimetry. Samples were analyzed on a Microcal MC-1 high-sensitivity differential scanning calorimeter, with a temperature scan rate of 25 °C/h. Transition enthalpies were determined from the calorimetric traces as described previously (Silvius & Gagné, 1984a). Lipid phosphorus was assayed by the method of Lowry and Tinsley (1974), with the modification that samples were digested for 6 h to ensure complete release of inorganic phosphate.

Raman Spectroscopy. Raman spectra were acquired on Spex Ramalog 6 and Spex Triplemate Raman spectrometer systems, which have been described previously (O'Leary & Levin, 1984; Silvius et al., 1985). Excitation with 514.5-nm light provided 30–200 mW of power at the sample. Lipid dispersions for Raman experiments were prepared by adding distilled water to the phospholipid to form a 1:10 (w/w) lipid/water dispersion. The sample was then placed in a 1.25-mm Kimax glass capillary tube, sealed, and centrifuged in a hematocrit centrifuge. The resulting dispersions were allowed to remain at room temperature for 1–2 days prior to acquisition of spectra. Spectra for "unhydrated" samples were acquired prior to warming of the samples above the melting tempera-

ture; spectra for "freshly hydrated" and hydrated/preincubated samples were obtained after preparation of samples as described for the calorimetric experiments. Temperature control during data acquisition was provided by placing the sample capillary tube in a thermostatically regulated brass mount. Changes in trans/gauche isomerization and chain packing as a function of temperature and lipid structure were monitored by means of the I_{2935}/I_{2880} and I_{2850}/I_{2880} CH stretching mode ratios (Levin, 1984). Details of chain packing were examined where indicated with information from the 1375–1525-cm^{-1} CH_2 deformation mode region, in which the presence of site group splitting can be used to detect orthorhombic or monoclinic acyl chain packing (Boerio & Koenig, 1970).

Results

Calorimetry. Previous studies of aqueous dispersions of DMPE (Seddon et al., 1983a; Chang & Epand, 1983; Mantsch et al., 1983; Wilkinson & Nagle, 1984) have shown that this lipid can form two quite different types of solid phases in excess water. The first type, a relatively well-hydrated gel phase, forms rapidly when an aqueous dispersion of DMPE is warmed above 57 °C and then cooled to below 50 °C. This hydrated gel phase is, however, metastable with respect to a second, "dehydrated" solid phase that can be generated either by dispersing the lipid in an aqueous medium below 57 °C or by incubating an initially hydrated sample for long periods (ca. days) at 2 °C. To determine whether various head group modified analogues of DMPE can also adopt multiple solid phases at low temperatures, we examined the calorimetric behavior of aqueous dispersions of these analogues that were prepared in three ways: by dispersal at 15 °C and incubation for 12–48 h at 2 °C; by initial dispersal above 57 °C followed by incubation at 2 °C for long periods (ca. days to weeks); or by dispersal above 57 °C followed by rapid cooling to 2 °C (at ~30 °C/min) immediately before the sample was loaded in the calorimeter. For the sake of brevity, we will refer to these three types of preparations as "unhydrated", "hydrated/preincubated", and "freshly hydrated" dispersions, respectively, in the discussion that follows.

In Figure 1 are shown thermograms that were obtained with unhydrated dispersions of DMPE and several analogues. The transition temperatures and enthalpies observed for these transitions are summarized in Table I. Unhydrated dispersions of DMPE and its "head group elongated" analogues DMPP and DMPB show single highly endothermic transitions at 57.3, 52.5, and 51.6 °C, respectively. C_2-methyl-, C_2-dimethyl-, and C_2-ethyl-DMPE dispersions prepared in the same way show transition endotherms with comparably high enthalpies at even higher temperatures. Interestingly, C_2-methyl-DMPE prepared from *l*-2-amino-1-propanol (*l*-C_2-methyl-DMPE) shows a single sharp, high-enthalpy transition at 73.2 °C under these conditions, while the corresponding compound prepared from *dl*-2-amino-1-propanol (*dl*-C_2-methyl-DMPE) shows a broad, multicomponent transition peaking at 60.1 °C. In contrast to the behavior of these compounds, all of which have an unsubstituted amino group, *N*-methyl-DMPE dispersions prepared at low temperatures exhibit a single transition at 42.7 °C, whose heat content (7.6 kcal mol^{-1}) is much less than that measured for the other DMPE analogues under comparable conditions. However, *N*-ethyl-DMPE dispersed at low temperatures shows an endothermic transition with a much higher peak temperature and heat content than is observed for the *N*-methyl species.

The calorimetric behavior of freshly hydrated dispersions of DMPE and its head group elongated analogues is quite different from that just described for unhydrated samples, as the thermograms shown in Figure 2 illustrate. Freshly hydrated samples of DMPE, DMPP, DMPB, and *N*-ethyl-DMPE show single endothermic transitions at 50.1, 41.9, 34.4

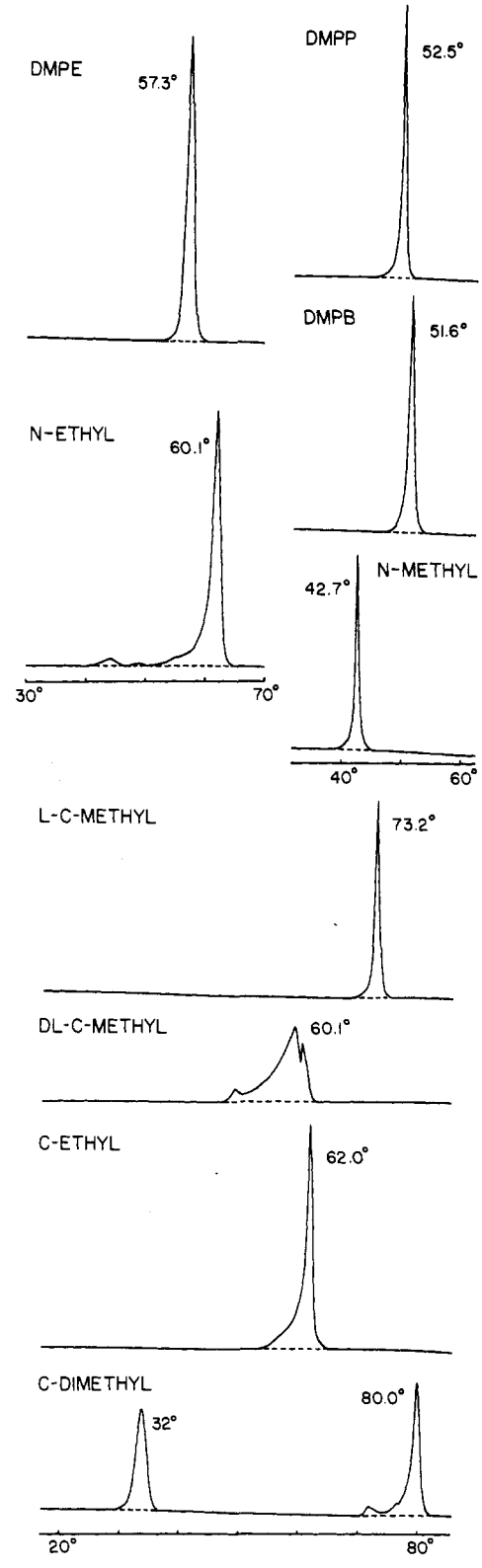

FIGURE 1: Calorimetric thermograms recorded for "unhydrated" samples of DMPE and its analogues, which were dispersed at 2 °C and incubated at this temperature for 8–14 days. The transition shown at 32 °C for C_2-dimethyl-DMPE was seen only in samples that were dispersed at 2 °C and incubated at this temperature for a minimum of 2 weeks. Other lipids gave endotherms comparable to those shown when dispersed at 2 °C and incubated for as little as 24–48 h at this temperature. C_2- or N-alkylated DMPE analogues are designated in this and following figures by the abbreviations C-alkyl or N-alkyl.

Table I: Transition Temperatures and Enthalpies of DMPE Analogues

analogue	T_c(hydrated)	ΔH(hydrated)	T_m(high melting)	ΔH(high melting)
DMPE	50.1	6.1 ± 0.6	57.3	19.5 ± 1.9
DMPP	41.9	7.4 ± 0.8	52.5	20.0 ± 0.1
N-methyl-DMPE	42.7	7.6 ± 0.6	*a*	*a*
dl-C_2-methyl-DMPE	*b*	*b*	60.1	24.1 ± 0.5
l-C_2-methyl-DMPE	43.4	*c*	73.2, 64.4[d]	25.6 ± 1.3, 24.0 ± 0.7[d]
DMPB	34.4	7.2 ± 0.3	51.6	18.4 ± 0.2
N,*N*-dimethyl-DMPE	31.4	7.2 ± 0.3[e]	*a*	*a*
N-ethyl-DMPE	37.7	7.1 ± 0.5	59.3	20.3 ± 1.0
C_2-dimethyl-DMPE	37.5	*c*	80.0/81.0	14.3 ± 0.9
C_2-ethyl-DMPE	34.2	*c*	62.0	19.7 ± 0.7

[a] Samples gave low-melting gel phases, regardless of the method of sample preparation. [b] No melting transition of a hydrated gel could ever be observed. [c] Samples could never be obtained wholly in a hydrated gel phase, precluding determination of ΔH(hydrated). [d] Values given were obtained with samples dispersed at 2 or 75 °C, respectively. [e] From Gagné et al. (1985).

and 37.7 °C, respectively, with molar enthalpies that are less than half those measured for the main transitions of unhydrated samples of these lipids. The order and separation of transition temperatures in the series DMPE, DMPP, and DMPB is similar to that reported by Seddon et al. (1983b) for the corresponding analogues of ditetradecyl-PE. Samples of *dl*-C_2-methyl-DMPE and C_2-ethyl-DMPE that are hydrated above T_c and then quickly cooled to 2 °C show large endothermic transitions that are very similar to those shown for unhydrated dispersions of these compounds in Figure 1. However, the thermograms for freshly hydrated samples of these two species also show heat evolution at lower temperatures during the calorimetric scan (see Figure 2), indicating that at the start of the calorimetric run the samples exist in a state that is metastable with respect to the high-melting solid phase. Freshly hydrated samples of *N*-methyl-DMPE give thermograms indistinguishable from those obtained with unhydrated dispersions (Figure 1). No exothermic activity was ever observed in thermograms for freshly hydrated or hydrated/preincubated samples of this lipid. C_2-Dimethyl-DMPE and *l*-C_2-methyl-DMPE show a still different type of behavior. Freshly hydrated samples of these species show major transitions with high enthalpies similar to those observed for unhydrated dispersions, but the transitions are clearly different for unhydrated vs. freshly hydrated samples. For C_2-dimethyl-DMPE, this difference is simply a variation in the relative amplitudes of the transition components at 80.0 and 81.0 °C, while for *l*-C_2-methyl-DMPE the temperature of the main transition is quite different for the two types of samples (64.4 vs. 73.2 °C).

The results presented to this point suggest that DMPE analogues with an unsubstituted amino group or an *N*-ethylamino group, like DMPE itself, can exist in at least two distinct low-temperature phases, which we will designate for the sake of convenience as "low-melting" and "high-melting" solid phases. Using the solid to liquid-crystalline transition temperatures and enthalpies measured by calorimetry for DMPE, DMPP, and DMPB, we can calculate that the high-melting gel phase is the thermodynamically more stable solid phase for all three of these species. This fact is also quite evident in the case of the C_2-alkylated analogues of DMPE, where liquid-crystalline samples that are cooled rapidly to 2 °C revert spontaneously to the high-melting solid phase on a time scale that is faster than or comparable to that of a calorimetric scan (a few hours). In contrast to the behavior of all these compounds, *N*-methyl-DMPE dispersions prepared in various ways form only one type of solid phase, which appears from its melting temperature and enthalpy to be comparable to the low-melting gel phase of DMPE, DMPP, and DMPB. The behavior of *N*-methyl-DMPE is not a universal characteristic of N-alkylated PE's, however, since *N*-ethyl-DMPE, like DMPE, can form both a high-melting and a low-melting solid phase, depending on the conditions of sample preparation.

As we have already noted above, samples of C_2-alkylated DMPE's appear to form rather unstable low-melting gel phases that relax rapidly to higher-melting gel phases. However, under certain conditions we could obtain samples of these species that exhibited small sharp endothermic transitions at temperatures well below the transition temperatures of the high-melting solid phases (see, for example, Figure 2). These small endotherms could be observed most consistently when samples were cooled from high temperatures to ~20 °C at moderate rates (~2 °C/min) and then cooled only briefly to 5 °C before beginning the calorimetric run. On the basis of the conditions under which these transitions are observed, as well as their small and variable amplitudes, we conclude that they represent the melting of small portions of the samples that have remained in the low-melting gel phase long enough to be detected calorimetrically. The temperatures of these small transitions are entered in Table I as the putative transition temperatures for the low-melting gel phases of the C_2-alkylated DMPE's. We note that the pattern of low-melting gel phase transition temperatures for these compounds and for freshly hydrated DMPE is very similar to the pattern observed for the corresponding dielaidoyl species (Brown et al., 1986), where the C_2-alkylated species form somewhat more stable low-melting gel phases whose transitions can be more easily characterized.

To examine in more detail the stabilities of the low-melting gel phases of DMPE, DMPP, DMPB, and *N*-ethyl-DMPE, we carried out a series of experiments in which initially hydrated samples of these lipids were incubated for varying periods at 2 °C prior to examination by calorimetry. Some representative calorimetric scans obtained for samples prepared in this way are shown in Figure 3. After 21 days of incubation at 2 °C, DMPE showed a complete reversion to the high-melting gel phase (not shown), in agreement with the previous report of Wilkinson and Nagle (1984). When incubated for 12–24 h at 2 °C, initially hydrated dispersions of DMPE showed endotherms at 50.1 and 57.3 °C with an intervening exotherm, as shown in Figure 3. This observation indicates that DMPE relaxes only slowly from the low-melting to the high-melting solid phase when samples are incubated at 2 °C but that the high-melting solid phase forms rapidly when the sample is subsequently heated through the transition temperature of the low-melting gel phase.

DMPP samples that were incubated for 21 days at 2 °C after hydration showed a broad endotherm with a peak at ~36 °C, followed immediately by a shallow exotherm and a large peak at 52.5 °C, the transition temperature of the high-melting solid phase (Figure 3). When incubated for only 4 days at

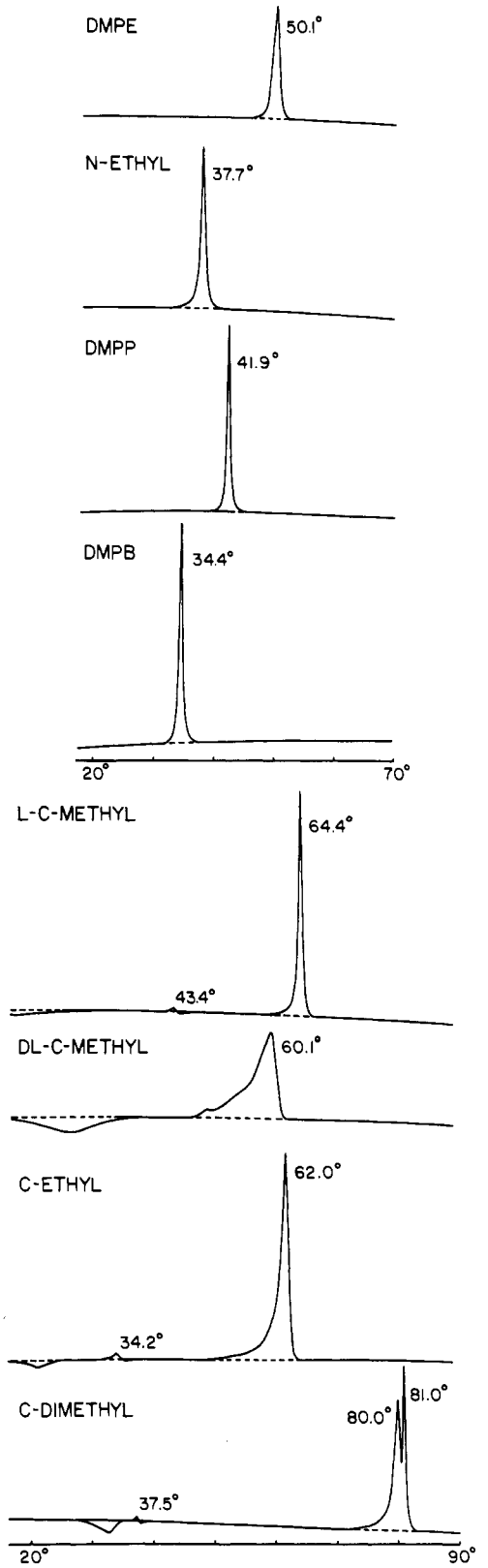

FIGURE 2: Calorimetric thermograms recorded for "freshly hydrated" samples of DMPE and its analogues, which were hydrated above T_m (high melting), then cooled to 2 °C, and analyzed immediately by calorimetry, beginning the heating run ~50 min after hydration of the sample. DMPE analogues are designated by the abbreviations used in Figure 1.

2 °C after initial hydration, samples of this lipid showed a small endotherm centered at 38 °C, followed in succession by the 41.9 °C transition of the low-melting gel phase, a large exotherm, and a second large endotherm at 52.5 °C. As noted

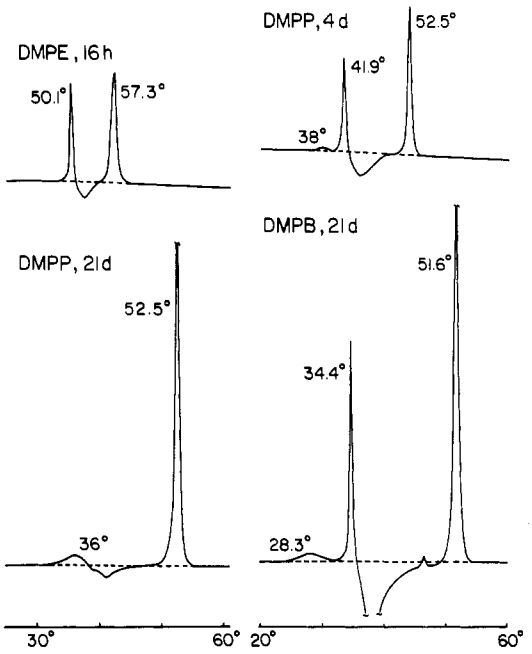

FIGURE 3: Calorimetric thermograms recorded for samples of DMPE, DMPP, and DMPB that were initially hydrated above T_m (high melting) and then incubated at 2 °C for the indicated times prior to calorimetry.

above, freshly hydrated dispersions of DMPP show only a single endothermic transition at 41.9 °C, while unhydrated dispersions, even after lengthy incubation at 2 °C, show only a large endothermic transition at 52.5 °C. From these results, we conclude that prolonged incubation of hydrated DMPP samples at 2 °C produces two types of changes in the state of the sample. First, extensive incubation of hydrated (but not unhydrated) samples at 2 °C leads to the creation of a third solid phase, which converts back to the low-melting gel phase at 38 °C. The calorimetric properties and the conditions of formation of this third solid phase resemble those of subgel phases formed by lipids such as saturated phosphatidylcholines (Chen et al., 1980; Mulukutla & Shipley, 1984; Gagné et al., 1985). Second, however, preincubation of hydrated DMPP at low temperatures appears to promote the formation of nucleation centers for the relaxation of the hydrated lipid to the high-melting solid phase. The actual rate of this relaxation, once nucleation centers are established, is much faster near the 38 °C transition of the third solid phase than it is at 2 °C.

The solid-phase behavior of hydrated dispersions of DMPB is qualitatively very similar to that of hydrated DMPP. After 21 days of incubation at 2 °C, initially hydrated (but not unhydrated) samples of DMPB form a third solid phase that reconverts to the low-melting gel phase in a broad endothermic transition centered at 28.3 °C (ΔH = 1.5 kcal mol^{-1}). Calculations based on the calorimetric data indicate that the high-melting solid phase should be the most stable phase for DMPB below 51.6 °C, but this phase does not form readily until the temperature reaches the vicinity of the transition of the low-melting gel phase. The low-melting gel phase of *N*-ethyl-DMPE shows metastable behavior similar to that of DMPP and DMPB (not shown), but the relaxation to the high-melting solid phase in this case is sufficiently rapid, even at 2 °C, that no third transition was ever observed for this species.

Raman Spectroscopy. The calorimetric results described above indicate that DMPE and its analogues can form a variety of solid phases, depending on the temperature and conditions of sample preparation. To characterize in more detail

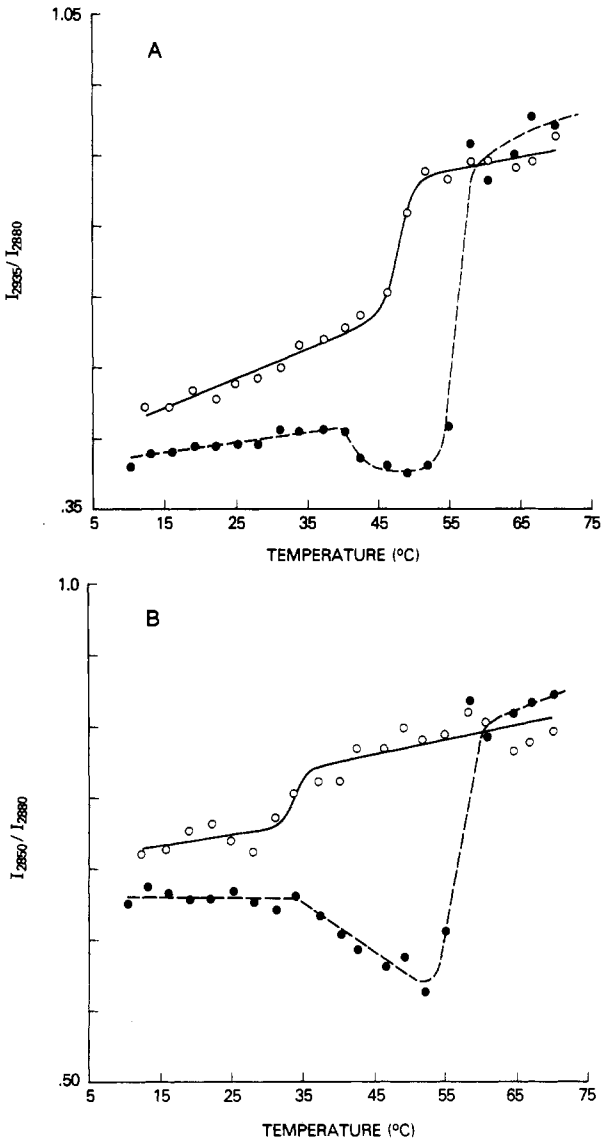

FIGURE 4: Temperature profiles obtained in heating scans for freshly hydrated (solid curves) and hydrated/preincubated (dashed curves) DMPE dispersions, monitoring (A) the I_{2935}/I_{2880} and (B) the I_{2850}/I_{2880} CH stretching mode intensity ratios. Conditions of sample preparation are given in the text.

the organization of the lipid molecules in these phases, we examined the Raman spectra for aqueous dispersions of DMPE and some of its analogues as a function of the temperature and the sample history.

In Figure 4 are shown temperature profiles of the Raman peak intensity ratios I_{2935}/I_{2880} and I_{2850}/I_{2880} for aqueous dispersions of DMPE that were prepared in various ways. On first heating a sample that was dispersed at low temperature, or that was held at 2 °C for several days after initial hydration, a gradual decrease in both peak height ratios, particularly I_{2850}/I_{2880}, is seen over the 30–50 °C temperature range. The magnitude of this decrease is greater for the initially hydrated than for the unhydrated samples. The peak height ratios at these temperatures are typical of polymethylene chains packed in an orthorhombic subcell (Snyder et al., 1978). A spectral feature present at 1421 cm^{-1} (Figure 5) is also typical of this packing (Boerio & Koenig, 1970; Yellin & Levin, 1977). A sharp increase in both spectral intensity ratios at 57 °C corresponds to formation of the liquid-crystal phase. The profile obtained during subsequent cooling of the sample demonstrates a transition from the liquid-crystalline phase to a gel phase

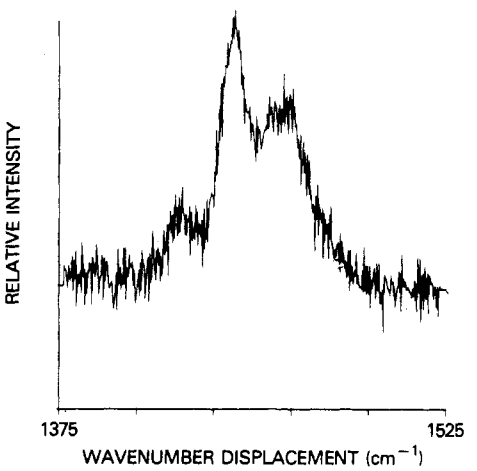

FIGURE 5: Raman spectrum of a hydrated/preincubated sample of DMPE in the 1375–1525-cm^{-1} CH_2 deformation mode region. The spectral feature at ~1421 cm^{-1} is typical of acyl chains packed in an orthorhombic subcell.

with quasihexagonal chain packing at 47 °C. The heating profiles for freshly hydrated samples of DMPE show substantially higher I_{2935}/I_{2880} and I_{2850}/I_{2880} ratios below the melting transition than do unhydrated or hydrated/preincubated dispersions. Curiously, the I_{2935}/I_{2880} ratio profile (Figure 4A) indicates a transition at ~48 °C for freshly hydrated DMPE, while the I_{2850}/I_{2880} ratio shows no abrupt change at this temperature but does suggest a small transition at ~33 °C (Figure 4B). This finding suggests that hydrated DMPE may undergo two transitions, one of which (at 33 °C) is primarily a lattice rearrangement without a substantial absorption of heat or increase in gauche chain conformers while the second (seen at 48 °C by Raman and 50 °C by calorimetry) involves a major uptake of heat and production of gauche conformers.

A generally similar pattern of Raman results is seen when we compare the behavior of freshly hydrated and initially hydrated, extensively preincubated dispersions of DMPB (Figure 6). Temperature profiles obtained from hydrated/preincubated samples indicate increasing acyl chain organization as the melting temperature, 50 °C, is approached, demonstrating that the exothermic features observed in the calorimetric experiments (Figure 3) correspond to decreases in the number of gauche conformers and tighter acyl chain packing. Spectral measurements in the CH_2 deformation region (Figure 7) demonstrate that the observed decrease in the number of gauche conformers is accompanied by the development of orthorhombic chain packing, as for DMPE. The transition that is detected calorimetrically at 28.3 °C was not observed, possibly because relaxation to the "high-melting" solid phase begins below this temperature under the conditions of the Raman experiments. Freshly hydrated samples of DMPB underwent a transition from a quasihexagonally packed gel phase to the liquid-crystalline phase at 34 °C.

In contrast to our results with DMPE and DMPB above, we were unable to observe "annealing" of hydrated/preincubated DMPP samples to an orthorhombic form during prolonged incubations at 2 °C. CH_2 deformation region spectra of DMPP did not demonstrate the presence of site group splitting in anhydrous, "unhydrated", freshly hydrated, or hydrated/preincubated samples. I_{2850}/I_{2880} and I_{2935}/I_{2880} temperature profiles obtained from preincubated samples did, however, demonstrate both the 35 and the 50 °C transitions observed by calorimetry (Figure 8). As with DMPE and DMPB, freshly hydrated samples undergo a transition from

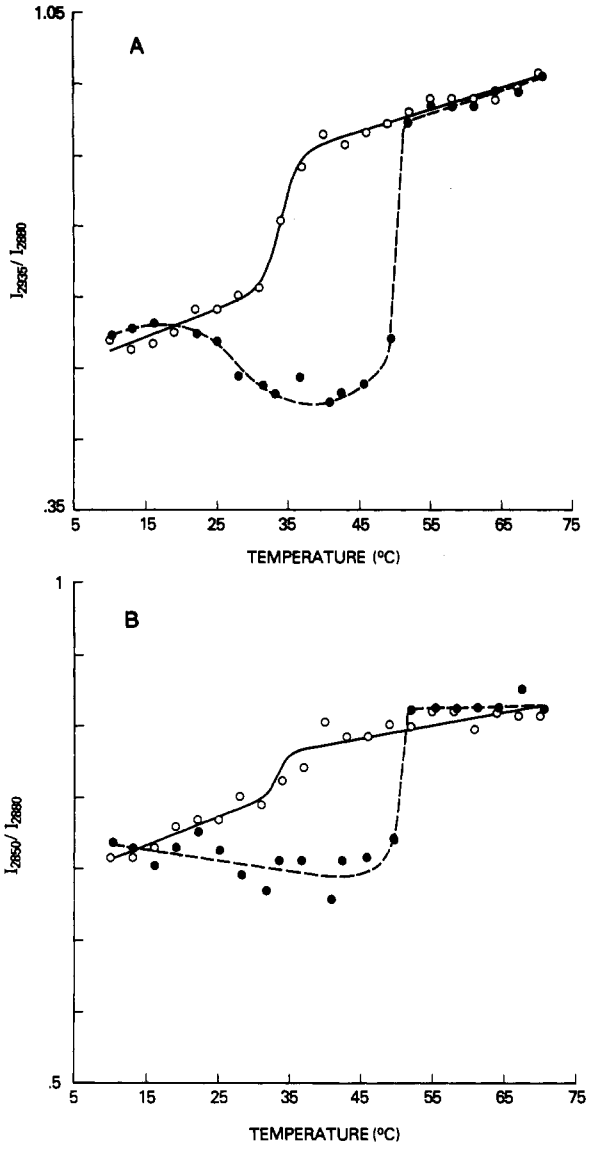

FIGURE 6: Temperature profiles obtained in heating scans for freshly hydrated (solid curve) and hydrated/preincubated (dashed curve) dispersions of DMPB, monitoring (A) the I_{2935}/I_{2880} and (B) the I_{2850}/I_{2880} CH stretching mode intensity ratios.

a quasihexagonally packed gel to the liquid-crystalline phase at a temperature lower than that seen with unhydrated or hydrated/preincubated samples, in this case 41 °C.

Repeated heating and cooling profiles of C_2-dimethyl-DMPE demonstrated only a single melting pattern regardless of sample history (Figure 9). Two distinct transitions were seen in I_{2935}/I_{2880} profiles, one of which was centered at approximately 75 °C and the second of which was centered at approximately 80 °C. Interestingly, only the former transition is seen in the I_{2850}/I_{2880} profiles, suggesting that near-maximal lateral disordering of acyl chains occurs prior to the onset of trans–gauche isomerization.

Discussion

A number of studies appearing in recent years have demonstrated that at low temperatures the normal hydrated gel phases of many saturated PC's and PE's are metastable with respect to more ordered, less well hydrated solid phases. A number of saturated PC's, and the N-methylated derivatives of dimyristoyl-PE, have been shown to form "subgel" phases that convert to other gel phases below the temperature of the main melting transition (Chen et al., 1980; Mulukutla &

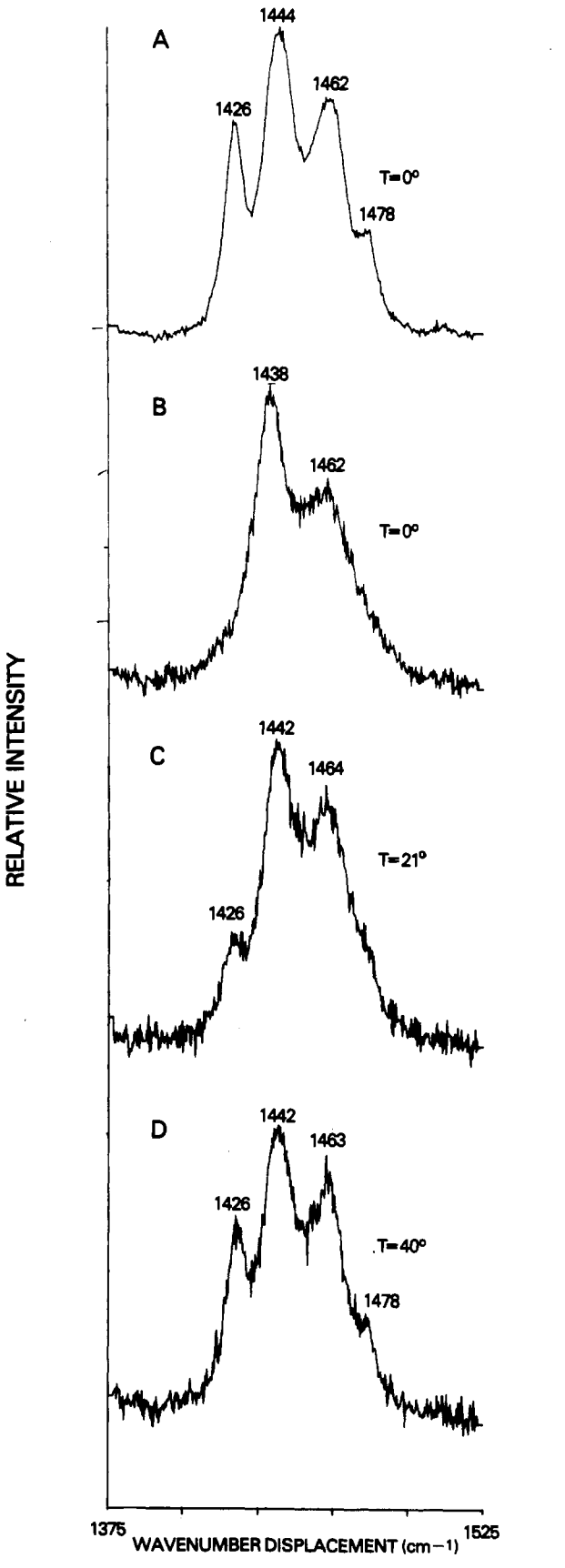

FIGURE 7: Raman spectra of (A) anhydrous and (B–D) hydrated/preincubated samples of DMPB in the CH_2 deformation mode region. The spectra shown in (B–D) were recorded during a single temperature scan. The growth of the 1426-cm^{-1} spectral feature with increasing temperature indicates annealing of the acyl chains from a quasihexagonal to an orthorhombic subcell.

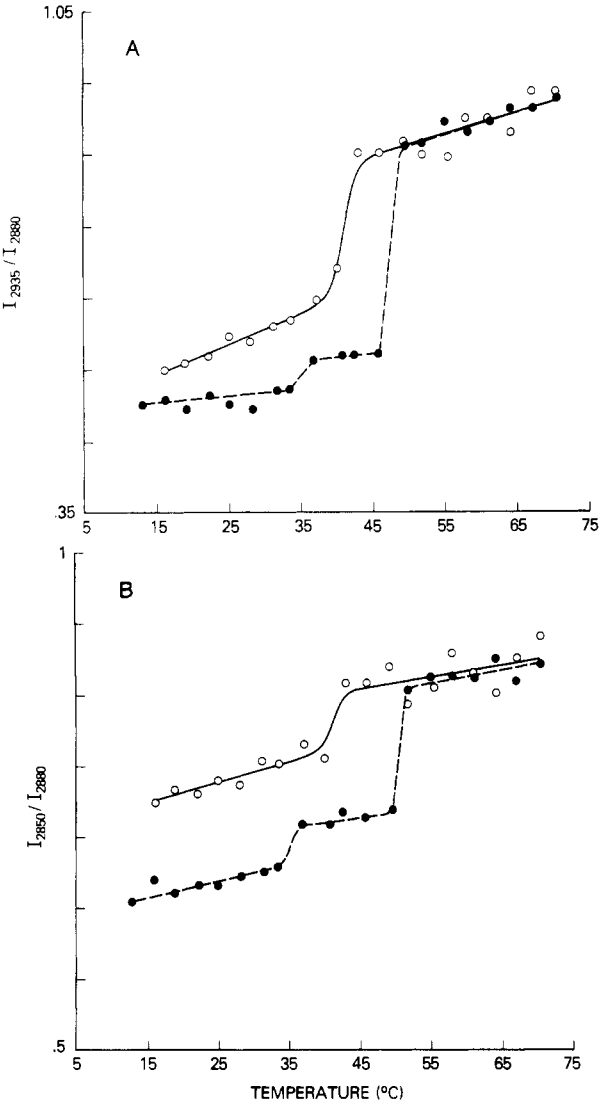

FIGURE 8: Temperature profiles obtained in heating scans for freshly hydrated (solid curve) and hydrated/preincubated (dashed curve) dispersions of DMPP, monitoring (A) the I_{2935}/I_{2880} and (B) the I_{2850}/I_{2880} CH stretching mode intensity ratios.

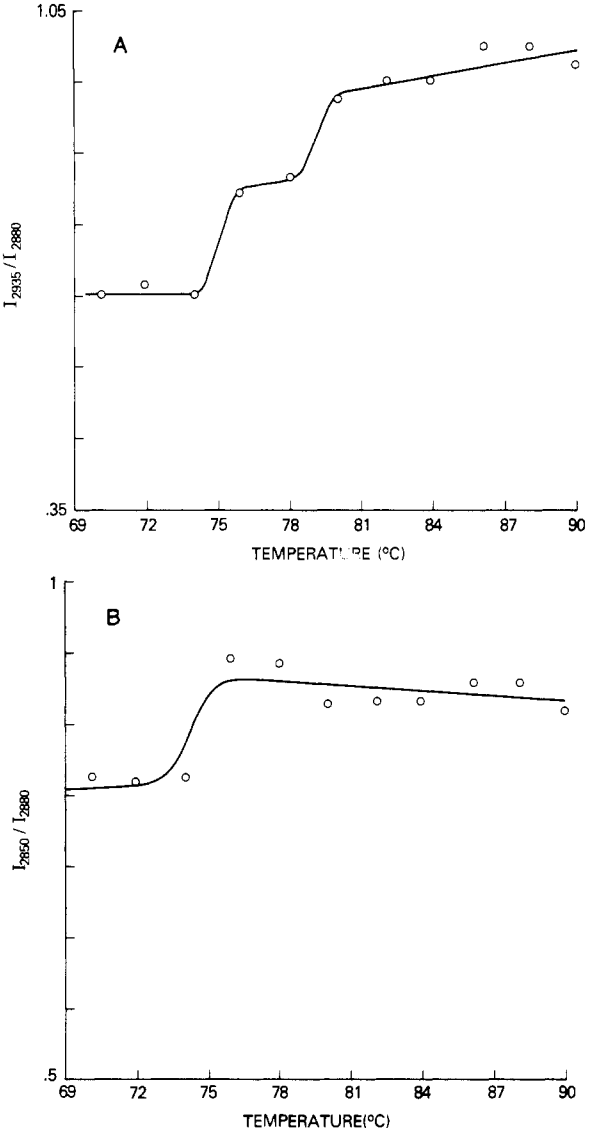

FIGURE 9: Temperature profiles obtained in heating scans for an unhydrated sample of C_2-dimethyl-DMPE, monitoring (A) the I_{2935}/I_{2880} and (B) the I_{2850}/I_{2880} stretching mode intensity ratios. Temperature scans obtained for hydrated/preincubated samples of this lipid were identical with those shown for unhydrated samples.

Shipley, 1984; Finegold & Singer, 1984). By contrast, short-chain-saturated PE's form what we here term "high-melting" solid phases, which remain stable up to a temperature well above the melting transition of the normal hydrated gel phase (Seddon et al., 1983; Chang & Epand, 1983; Mantsch et al., 1983). As discussed by Wilkinson and Nagle (1984), it is conceivable in principle that the high-melting solid phases of some saturated PE's are fundamentally equivalent to the subgel phases of N-methylated PE derivatives, differing only in the extent of the temperature range over which they are stable with respect to the normal hydrated gel phase(s). However, as we discuss below, several lines of evidence obtained in this study and previously suggest that phases of the subgel and high-melting types are in fact distinct in their properties and behavior.

Previous studies of the organization of the subgel phase of DPPC (Ruocco & Shipley, 1982) and the high-melting (β_1) phase of dilauroyl-PE (Seddon et al., 1983) have shown that the subgel phase is much more extensively hydrated than the high-melting phase of PE, which appears to be nearly anhydrous. A second difference between high-melting and subgel phases can be seen in the conditions of preparation required to generate each type of phase. As demonstrated in this paper and previously (Mantsch et al., 1983; Wilkinson & Nagle, 1984), the high-melting solid phase of DMPE and its analogues can be obtained quite readily by dispersing a well-dried lipid sample in cold buffer. By contrast, the subgel phase of *N*-methyl-DMPE cannot be obtained directly by this method, which gives instead the hydrated L_{β}' phase that relaxes to the subgel phase only over a period of weeks at low temperatures (Mulukutla & Shipley, 1984; Gagné et al., 1985). We have observed a similar result for DPPC (J. Silvius, unpublished observation). It thus appears that a lyophilized lipid powder, when dispersed in buffer at low temperatures, can readily adopt the organization characteristic of a high-melting gel phase, but it must undergo a very slow and presumably substantial rearrangement via the L_{β}' phase to form a subgel structure. A final piece of evidence that suggests that high-melting and subgel phases are qualitatively distinct is the observation that DMPP and DMPB can form low-temperature phases of either type, depending on the conditions of sample preparation. This behavior may be also be characteristic of DMPE itself. Mulukutla and Shipley (1984) have reported that DMPE, when hydrated and then incubated at −4 °C, slowly forms a solid phase that on heating ultimately converts to the L_{β}' gel

phase at 41 °C. By contrast, both we and Wilkinson and Nagle (1984) have observed that DMPE dispersions that are incubated for long times at 2 °C tend to relax to the high-melting gel phase. It thus appears that DMPE, like DMPP and DMPB, can adopt either a "subgel-like" or a high-melting solid phase, depending on the conditions of sample preparation. In general, the use of lower incubation temperatures appears to favor the tendency of these species to relax from the $L_{\beta'}$ phase to a subgel as opposed to a high-melting gel phase.

By correlating Raman spectroscopic results with our calorimetric observations, we can extract several useful conclusions regarding the organization of the solid and fluid phases of these DMPE analogues. First, the changes in both the I_{2935}/I_{2880} and the I_{2850}/I_{2880} Raman intensity ratios at the main transition of the hydrated gel phase are almost identical for DMPE, DMPP, and DMPB. This fact suggests that the structural changes at this transition are very similar for all three species. The absolute values of the Raman intensity ratios all are also quite similar for all three compounds above and below this transition. Given that the values of these ratios are affected by small contributions from the lipid head groups (which differ for the different species), it is evident that the nature of the acyl chain packing in the hydrated gel and liquid-crystalline phases of DMPE, DMPP, and DMPB is fundamentally similar (and may be virtually identical) for all three compounds.

Raman spectroscopic results indicate that the high-melting gel phases of various DMPE analogues show lower contents of gauche conformers, and tighter interchain packing, than do conventional hydrated gel phases. This point is made dramatically evident in the temperature scans for hydrated/preincubated dispersions of DMPB and DMPE, where the exothermic relaxation of the hydrated gel phase to the high-melting state that is observed calorimetrically during heating is accompanied by a substantial increase in acyl chain ordering as seen by Raman spectroscopy (Figures 4 and 6). The Raman results indicate clearly that the much higher enthalpies of transition measured for high-melting vs. conventional gel phases can be attributed, at least in part, to the considerably tighter acyl chain packing in the high-melting solid phase.

The changes in the Raman I_{2935}/I_{2880} ratio at the transition of the high-melting gel phase are of similar magnitude for DMPE, DMPP, and DMPB and are significantly less for C_2-dimethyl-DMPE. The enthalpies of the main transitions of the high-melting gel phases for these compounds follow a very similar pattern (Table I). These results might be taken to suggest that the high-melting solid phases of DMPE, DMPP, and DMPB are fundamentally alike in the arrangements of their acyl chains and are different from the high-melting phase of C_2-dimethyl-DMPE. However, the Raman I_{2880}/I_{2850} temperature profiles suggest that there are significant differences in acyl chain packing in the high-melting phases of all four compounds. Examination of the Raman spectral region around 1420 cm^{-1} gives further evidence for this suggestion, as the high-melting phases of DMPE and DMPB reveal a spectral feature indicating an orthorhombic acyl chain packing, while C_2-dimethyl-DMPE and, significantly, DMPP do not. It thus appears that the high-melting phases of various DMPE analogues share a common feature of a tight acyl chain packing, with a relatively low population of gauche chain conformations, but the details of the packing arrangement for the acyl chains can vary from compound to compound. This is true even when we compare compounds that show very similar enthalpies of transition for their high-melting solid phases.

The results just discussed indicate that the high-melting solid phase of DMPE and its analogues cannot be identified with a single type of acyl chain packing, even for structurally similar compounds. Mantsch et al. (1983) have reported a similar conclusion on the basis of results with different polymorphic forms of dilauroyl- and dimyristoyl-PE, which give high-melting solid phases with very similar transition temperatures (enthalpies were not specified), even though infrared spectroscopy reveals substantial differences in chain packing (e.g., orthorhombic vs. quasihexagonal) for the different polymorphs. It is likewise not clear that phases of the subgel type exhibit a single specific type of acyl chain packing. While an orthorhombic packing of the acyl chains is observed for the subgel phases of compounds such as dipalmitoyl-PC (Cameron & Mantsch, 1982), this type of chain packing is also observed for the high-melting phase of DMPE or DMPB, and it is not observed for samples of DMPP that are equilibrated under any conditions, including the conditions that clearly produce the subgel phase of this compound. Therefore, the qualitative differences observed in the behavior of high-melting vs. subgel-type solid phases cannot be simply related to a different packing motif for the acyl chains in the two types of phases.

A comparison of the melting temperatures of the $L_{\beta'}$ gel phase for various DMPE analogues, T_c(hydrated), reveals an interesting pattern: to a first approximation, the single most important determinant of T_c(hydrated) for these species is simply the number of carbon atoms in the alkanolamine head group, regardless of how the atoms are arranged. This observation also holds true for the corresponding analogues of dielaidoyl-PE (DEPE), a group that includes a greater number of species than can form a long-lived hydrated gel phase, as described in the following paper (Brown et al., 1986). Finer details of head group structure do have some influence on the value of T_c(hydrated), as can be seen for example by comparing the transition temperatures for *N*-ethyl- and *N*,*N*-dimethyl-DMPE (Table I). However, we can observe as a general rule that addition of one carbon to the PE head group reduces T_c(hydrated) by ~7–8 °C for DMPE analogues and by ~6–8 °C for DEPE analogues, while addition of two extra carbons decreases T_c(hydrated) by roughly double these amounts. This approximate rule applies to N- and C_2-substituted PE's as well as to analogues with extra methylene units added to the head group. Extrapolating from these observations, we can estimate that T_c(hydrated) should be depressed by ~21–24 °C when three extra carbons are added to the DMPE head group and by a comparable or slightly smaller amount when the head group of DEPE is similarly modified. In fact, the T_c values for DMPC and DEPC both lie ~26 °C below the values of T_c(hydrated) for the corresponding PE's. It thus appears that a large part of the reduction in T_c that is typically observed for PC's when compared to PE's of like acyl composition can be accounted for simply as a consequence of the greater steric bulk of the PC head group, without specific reference to the loss of hydrogen-bonding potential that accompanies the tri-N-methylation of PE to form PC. Eibl (1977) has, in fact, reported that dipalmitoylphosphatidyl-6-amino-1-hexanol has a transition temperature slightly lower than that of dipalmitoyl-PC, a result consistent with this conclusion. This need not imply, of course, that head group hydrogen bonding may not be important in the organization of hydrated PE bilayers but simply that other factors, notably head group size, seem to be of greater importance in determining the high transition temperatures of the $L_{\beta'}$ gel phases of PE's as compared to PC's. The simplest explanation for this result would be that the strength of hydrogen-bonding

interactions between PE head groups, regardless of its absolute magnitude, does not change greatly at the transition of the hydrated L_{β}' gel phase to the liquid-crystalline phase.

When we examine the transition temperatures of the high-melting gel phases of various DMPE analogues, we observe a very different pattern than that just described for the values of T_c(hydrated) for these compounds. First, the transition temperature is no longer clearly a function of the number of extra carbon atoms in the head group but is instead strongly dependent on the details of the head group structure. Both N- and C_2-alkylated species that can form a high-melting gel phase show higher values of T_m (high melting) than does DMPE itself, while the 1-propanolamine and 1-butanolamine analogues give somewhat lower values for this transition temperature. Within the group of C_2-alkylated analogues of DMPE, increases in the extent of C_2-substitution appear to stabilize the high-melting gel phase up to progressively higher temperatures.

In general, the stability of the high-melting gel phase appears to be favored by modifications of the PE head group that add alkyl substituents without decreasing the hydrogen-bonding capacity of the amino group or changing the distance between the phosphoryl and amino groups. However, either of these latter characteristics of the PE head group can be modified without seriously destabilizing the high-melting gel phase so long as the modification also entails a compensatory increase in the overall "hydrocarbon bulk" (alkyl plus methylene groups) of the head group. It is interesting to note that N-methylation, the biologically most important modification of the PE head group, is the only structural modification of DMPE out of all those considered here that can prevent the formation of a high-melting gel phase. This is probably a consequence of the fact that N-methylation of the PE head group substantially reduces its overall hydrogen-bonding capacity while introducing a minimum of additional hydrocarbon bulk into the head group. The range of phospholipid head group structures that can promote formation of stable, well-hydrated bilayers may be more limited than we sometimes assume, a fact that might explain the conservation of relatively few such structures in a wide variety of organisms and cell types.

Registry No. DMPE, 998-07-2; DMPP, 102572-85-0; *N*-methyl-DMPE, 68755-13-5; *dl*-C_2-methyl-DMPE, 102520-93-4; *l*-C_2-methyl-DMPE, 102520-94-5; DMPB, 102572-86-1; *N*,*N*-dimethyl-DMPE, 68755-14-6; *N*-ethyl-DMPE, 102535-15-9; C_2-dimethyl-DMPE, 102520-95-6; C_2-dimethyl-DMPE, 102520-96-7; C_2-ethyl-DMPE, 102520-96-7; *t*-BocNHC$(CH_2OH)(CH_3)_2$, 102520-97-8; dimyristoylphosphatidic acid, 30170-00-4.

References

Aneja, R., Chadha, J. S., & Davies, A. P. (1970) *Biochim. Biophys. Acta 218*, 102.

Boerio, P., & Koenig, J. L. (1970) *J. Chem. Phys. 52*, 3425.

Boggs, J. M. (1980) *Can. J. Biochem. 58*, 755.

Brown, P. M., Steers, J., Hui, S. W., Yeagle, P. L., & Silvius, J. R. (1986) *Biochemistry* (following paper in this issue).

Cameron, D. G., & Mantsch, H. H. (1982) *Biophys. J. 37*, 175.

Casal, H. L., & Mantsch, H. H. (1983) *Biochim. Biophys. Acta 735*, 387.

Chang, H., & Epand, R. M. (1983) *Biochim. Biophys. Acta 728*, 319.

Chowdhry, B. Z., & Dalziel, A. W. (1985) *Biochemistry 24*, 4109.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36.

Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 599*, 399.

Dekker, C. J., Guerts Van Kessel, W. S. M., Klomp, J. P. G., Pieters, J., & de Kruijff, B. (1983) *Chem. Phys. Lipids 33*, 93.

Düzgünes, N., Wilschut, J., Fraley, R., & Papahadjopoulos, D. (1981) *Biochim. Biophys. Acta 642*, 182.

Eibl, H. (1977) in *Polyunsaturated Fatty Acids* (Kunau, W. H., & Holman, R., Eds.) p 229, American Oil Chemists' Society, Champaign, IL.

Eibl, H., & Wooley, P. (1979) *Biophys. Chem. 10*, 261.

Finegold, L., & Singer, M. (1984) *Chem. Phys. Lipids 35*, 291.

Gagné, J., Stamatatos, L., Diacovo, T., Hui, S. W., Yeagle, P. L., & Silvius, J. (1985) *Biochemistry 24*, 4400.

Hitchcock, P. B., Mason, R., Thomas, K. M., & Shipley, G. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3036.

Levin, I. W. (1984) *Prog. Infrared Raman Spectrosc. 11*, 1.

Lis, L. J., McAlister, M., Fuller, N., Rand, R. P., & Parsegian, V. A. (1982) *Biophys. J. 37*, 657.

Lowry, R. J., & Tinsley, I. J. (1974) *Lipids 9*, 491.

Mantsch, H. H., Hsi, S. C., Butler, K. W., & Cameron, D. G. (1983) *Biochim. Biophys. Acta 728*, 325.

Morodor, L., Hallett, A., Wünsch, E., Keller, O., & Wersin, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1651.

Mulukutla, S., & Shipley, G. G. (1984) *Biochemistry 23*, 2514.

O'Leary, T. J., & Levin, I. W. (1984) *J. Phys. Chem. 88*, 1790.

Ruocco, M. J., & Shipley, G. G. (1982) *Biochim. Biophys. Acta 684*, 59.

Seddon, J. M., Harlos, K., & Marsh, D. (1983a) *J. Biol. Chem. 258*, 3850.

Seddon, J. M., Cevc, G., & Marsh, D. (1983b) *Biochemistry 22*, 1280.

Silvius, J. R., & Gagné, J. (1984a) *Biochemistry 23*, 3232.

Silvius, J. R., & Gagné, J. (1984b) *Biochemistry 23*, 3241.

Silvius, J. R., Lyons, M. J., Yeagle, P. L., & O'Leary, T. J. (1985) *Biochemistry 24*, 5388.

Sundler, R., Düzgünes, N., & Papahadjopoulos, D. (1981) *Biochim. Biophys. Acta 649*, 751.

Vaughan, D. J., & Keough, K. M. W. (1974) *FEBS Lett. 47*, 158.

Verkleij, A. J. (1984) *Biochim. Biophys. Acta 779*, 43.

Wilkinson, D. A., & Nagle, J. F. (1984) *Biochemistry 23*, 1538.