Effect of Fatty Acyl Chain Length and Structure on the Lamellar Gel to Liquid-Crystalline and Lamellar to Reversed Hexagonal Phase Transitions of Aqueous Phosphatidylethanolamine Dispersions†

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Received June 6, 1988; Revised Manuscript Received August 24, 1988

ABSTRACT: The lamellar gel/liquid-crystalline and the lamellar liquid-crystalline/reversed hexagonal phase transitions of aqueous dispersions of a number of synthetic phosphatidylethanolamines containing linear saturated, branched chain, and alicyclic fatty acyl chains of varying length were studied by differential scanning calorimetry, ^{31}P nuclear magnetic resonance spectroscopy, and X-ray diffraction. For any given homologous series of phosphatidylethanolamines containing a single chemical class of fatty acids, the lamellar gel/liquid-crystalline phase transition temperature increases and the lamellar liquid-crystalline/reversed hexagonal phase transition temperature decreases with increases in hydrocarbon chain length. For a series of phosphatidylethanolamines of the same hydrocarbon chain length but with different chemical structures, both the lamellar gel/liquid-crystalline and the lamellar liquid-crystalline/reversed hexagonal phase transition temperatures vary markedly and in the same direction. In particular, at comparable effective hydrocarbon chain lengths, both the lamellar gel/liquid-crystalline and the lamellar liquid-crystalline/reversed hexagonal phase transition temperatures vary in parallel, such that the temperature difference between these two phase transitions is nearly constant. Moreover, at comparable effective acyl chain lengths, the *d* spacings of the lamellar liquid-crystalline phases and of the inverted hexagonal phases are all similar, implying that the thickness of the phosphatidylethanolamine bilayers at the onset of the lamellar liquid-crystalline/reversed hexagonal phase transition and the diameter of the water-filled cylinders formed at the completion of this phase transition are comparable and independent of the chemical structure of the acyl chain. These results suggest that for any given hydrocarbon chain length, there may be a critical thickness at which the liquid-crystalline phosphatidylethanolamine bilayer becomes unstable with respect to inverted nonbilayer phases such as the H_{II} phase and that the temperature at which this critical thickness is reached is dependent upon that bilayers proximity to the hydrocarbon chain-melting phase transition temperature.

The lipids of all biological membranes studied to date appear to exist exclusively or nearly exclusively in the lamellar or bilayer state under physiologically relevant conditions of temperature and hydration [see Singer and Nicolson (1972) and McElhaney (1984)]. Nevertheless, many biological membranes contain one or more lipid components which, in isolation, prefer to exist in a nonbilayer state [see Cullis et al. (1983), Rilfors et al. (1984), and Gruner et al. (1985)]. Although non-bilayer-forming phospho- and glycolipids comprise a significant fraction of the total membrane lipids in some biological membranes, the biological functions of such lipids remain unclear at present. Some workers have postulated that the transient formation of nonlamellar structures induced by such non-bilayer-forming lipids could play a key role in processes such as membrane fusion (Verkleij et al., 1979; Siegel, 1986a,b) or in the transmembrane movement of ions and macromolecules (Cullis et al., 1983). Other workers, however, argue that the actual formation of nonlamellar lipid structures in biological membranes is unlikely and that the role of non-bilayer-forming lipids is actually to impart some special (though as yet undefined) properties to the lipid bilayer phase [see Gruner (1985) and Hui (1987)]. These considerations and others pertinent to the definition of the function of non-bilayer-forming lipids in biological membranes are currently the focus of intense research interest (Cullis et al., 1983; Rilfors et al., 1984; Gruner et al., 1985).

The phosphatidylethanolamines (PEs)[1] are currently the best studied of the naturally occurring, non-bilayer-forming lipids. Fully hydrated dispersions of many PEs exhibit two thermotropic phase transitions: a lower temperature, higher enthalpy lamellar gel (L_β) to lamellar liquid-crystalline (L_α) transition and a higher temperature, lower enthalpy lamellar liquid-crystalline (L_α) to reversed hexagonal (H_{II}) transition (Seddon et al., 1983, 1984). Although the above pattern is typical of the thermotropic behavior of many of the PEs so far studied, there are some conditions under which PE bilayers can undergo a "direct" conversion from the L_β phase to the H_{II} phase without the formation of a stable, long-lived L_α phase (Marsh & Seddon, 1982; Seddon et al., 1984). Moreover, under certain conditions, some PEs can also form structures with cubic symmetry (Seddon et al., 1984; Shyamsunder et al.,

† This work was supported by operating and major equipment grants from the Medical Research Council of Canada (R.N.M.), by a postdoctoral research fellowship (D.A.M.) and major equipment grants from the Alberta Heritage Foundation for Medical Research (R.N.M.), by NIH Grant GM32614 (S.M.G.), and by DOE Grant DE-FG02-87ER60522 (S.M.G.). D.C.T. acknowledges the support of a NIH traineeship (Grant 5T32GM07312) and a New Jersey Garden State fellowship.

[1] Abbreviations: PE, phosphatidylethanolamine; H_{II}, inverted hexagonal; NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; T_m, gel/liquid-crystalline phase transition temperature; T_h, lamellar/H_{II} phase transition temperature.

0006-2960/89/0428-0541$01.50/0

1988) or structures which may be cubic precursors (Veiro & Rowe, 1988).

The thermotropic phase properties of only a rather limited number of synthetic PEs have been examined to date. The most thorough investigations reported so far are those of Seddon et al. (1983, 1984), who studied the thermotropic and lyotropic phase behavior of a homologous series of PEs containing even-numbered, saturated *n*-acyl or *n*-alkyl chains. Those studies established that the preference of the PEs studied for forming nonlamellar structures is enhanced by increases in hydrocarbon length, by substitution of ether for ester linkages between the hydrocarbon chains and the glycerol backbone, by reductions in the content and chemical activity of water, and by decreases in the pH of the aqueous phase. In contrast, structural modifications of the PE polar headgroup which increase in size or reduce its capacity to form hydrogen bonds reduce the tendency of the resultant PE analogues to form nonlamellar phases (Seddon et al., 1983, 1984; Gagne et al., 1985; Gruner et al., 1988). To date, there have been relatively few systematic studies of the effect of variations in the chemical structure of the fatty acyl chains on the thermotropic properties of synthetic PEs in general and on their preference for nonbilayer phases in particular. However, it has been established that an increase in the degree of unsaturation of the hydrocarbon chains promotes the formation of nonlamellar phases (Cullis et al., 1983; Rilfors et al., 1984).

The molecular basis for the lamellar and nonlamellar phase preferences of membrane lipids is only partly understood at present. The thermotropic and lyotropic phase behavior of lipid/water systems is usually explained by reference either to the "shape" of the lipid molecule, with cylindrically shaped molecules preferring lamellar phases and inverted cone-shaped molecules preferring reversed hexagonal or cubic phases (Israelachvilli et al., 1977, 1980; Cullis et al., 1983; Rilfors et al., 1984; Cevc & Marsh, 1987), or to the "spontaneous curvature" of the monolayers formed by that lipid molecule (Kirk et al., 1984; Gruner, 1985). Clearly, additional information on the relationship between hydrocarbon chain structure and the phase preferences of synthetic lipids would be useful in the testing and refinement of these concepts, since the effective adopted shape preferred by any given class of phospho- or glycolipid molecules, and the spontaneous curvature of the monolayers that they would tend to form, must be strongly influenced by the length and chemical structure of the acyl or alkyl chains. To this end, we have studied the thermotropic properties of a number of diacyl-PEs, varying with respect to the length and structure of their fatty acyl chains, by DSC, ^{31}P NMR spectroscopy, and X-ray diffraction. Since the phase behavior of a limited number of synthetic PEs containing linear saturated and unsaturated hydrocarbon chains has been examined previously, we concentrate here on PEs containing branched chain or alicylic fatty acyl groups. Specifically, both the L_β/L_α and L_α/H_{II} phase transition temperatures of aqueous dispersions of PEs containing methyl and dimethyl isobranched, methyl and ethyl anteisobranched, and ω-cyclohexyl fatty acyl chains are determined here and compared with those of the linear saturated and unsaturated PEs of comparable effective chain length. The dependence of the dimensions of the L_α and H_{II} lipid phase on fatty acyl structure and temperature is also investigated. This comparative approach permits an evaluation of the effect of variations in the number, size, and position of substituents on a linear hydrocarbon chain on the phase preference of PEs.

Materials and Methods

The PEs used in this study were synthesized from their respective phosphatidylcholines by transphosphatidylation using savoy cabbage phospholipase D (Comfurius & Zwaal, 1977) and purified by silicic acid column chromatography. The source phosphatidylcholines were themselves synthesized from their respective fatty acids and purified by methods previously used in this laboratory (Lewis & McElhaney, 1985a,b; Lewis et al., 1987a,b). The differential scanning calorimetric measurements were performed with a Perkin-Elmer DSC-2C calorimeter equipped with a Perkin-Elmer 3700 thermal analysis data station. The sample preparation methodology for the DSC experiments was as follows: samples (3–4 mg) of the dry lipid were placed in a large stainless-steel sample capsule, and, after the addition of 50 μL of distilled water, the capsule was then placed on a heated stage to facilitate the absorption of water by the sample. The capsule was then sealed and repeatedly heated and cooled at 10 °C/min to ensure the complete hydration of the PE. The heating and cooling DSC thermograms were recorded at a scan rate of 1 °C/min. ^{31}P NMR spectra were recorded with a Nicolet NT300-WB spectrometer using the data acquisition and data processing parameters previously reported (Lewis et al., 1988). The small-angle X-ray scattering patterns were recorded with the Princeton SIV X-ray detector beam lines; 3–5 mg of the dry lipid sample was loaded into a 1.5-mm glass X-ray capillary and mechanically mixed with 5–7 mg of deionized water, and the top of the capillary was sealed with 5-min epoxy. During the experiment, a 0.4 mm × 0.4 mm X-ray beam was passed through the sample while the temperature was controlled with a precision of ±0.5 °C by a thermoelectrically controlled sample stage [see Gruner et al. (1988)]. The diffraction patterns were recorded by using an image-intensified, slow-scan TV detector with which good diffraction patterns could be recorded in less than 5 min [see Gruner et al. (1982, 1988)]. The *d* spacings cited are the unit cell basis vector lengths and are reported with an accuracy of ±0.5 Å.

Results

In this study, DSC, ^{31}P NMR spectroscopy, and X-ray diffraction techniques were used to characterize the L_β/L_α and L_α/H_{II} transitions of a number of diacyl-PEs which vary with respect to the length and structure of the acyl chains. The lipids studied here include the di-*n*-acyl-PEs as well as those containing methyl isobranched, methyl anteisobranched, ω-cyclohexyl, ω-*tert*-butyl, and a few other structurally different acyl chains. Of the various diacyl-PEs studied to date, reversed hexagonal phases are only observed in the experimentally accessible temperature range (−40 to +135 °C by DSC) when the acyl chains of the lipids contain 16 or more linear carbon segments. Illustrated in Figure 1 is a representative example of the DSC thermograms recorded for those compounds for which L_α/H_{II} phase transitions are observed. The thermotropic phase behavior exhibited by the majority of the PEs studied is very complex, and in many instances, lower temperature endotherms, attributed to gel/gel phase transitions, in addition to the usual higher temperature L_β/L_α and L_α/H_{II} phase transitions, are observed. A detailed characterization of the gel-state thermotropic phase behavior of the various classes of PEs used in this study is not the focus of this paper and will be presented elsewhere.

The L_β/L_α and L_α/H_{II} phase transitions were assigned by X-ray diffraction and ^{31}P NMR spectroscopy. Typical data sets displaying the temperature-dependent changes in the ^{31}P NMR spectra and low-angle X-ray scattering patterns are illustrated in Figures 2 and 3. As shown in Figure 2, at low temperatures, aqueous dispersions of most of these lipids ex-

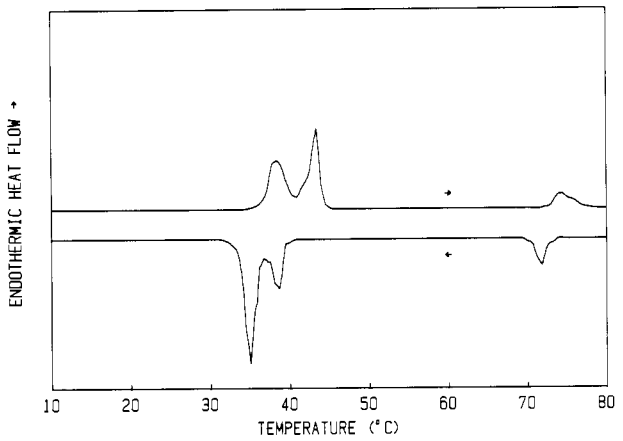

FIGURE 1: DSC heating and cooling thermograms of 20:0_{dmi}-PE. The thermograms were recorded at a scan rate of 1 °C/min. The endotherms centered near 38, 43, and 74 °C observed upon heating correspond to L_c/L_β, L_β/L_α, and L_α/H_{II} phase transitions, respectively (see the text).

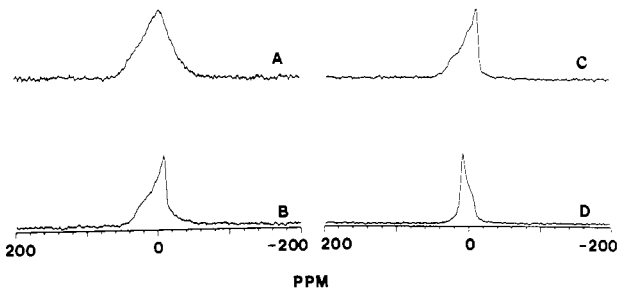

FIGURE 2: Proton-decoupled ^{31}P NMR spectra of an aqueous dispersion of 20:0_{dmi}-PE. (A) L_c phase at 25 °C; (B) L_β phase at 39 °C; (C) L_α phase at 60 °C; (D) H_{II} phase at 85 °C.

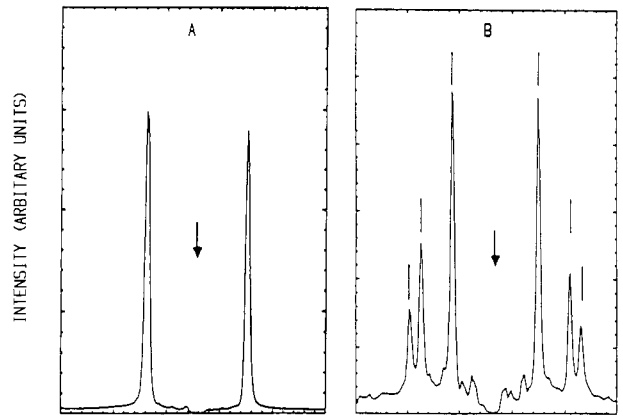

FIGURE 3: Radially integrated X-ray powder diffraction patterns of 20:0_{dmi}-PE. The arrows indicate the position of the unscattered beam, which was blocked by a lead stop. (A) L_α phase at 50 °C; peaks correspond to a d spacing of 55.6 Å. (B) H_{II} phase at 84 °C; tic marks indicate expected peak positions for a lattice with a water core distance of 74.7 Å. The weak peaks immediately adjacent to the beam stop are camera artifacts, while the adjacent weak peaks are due to the presence of a small amount (<1%) of L_α phase.

hibit broad ^{31}P NMR powder patterns that are indicative of slow axially asymmetric motion on the ^{31}P NMR time scale. Such spectra typify the so-called "subgel" phases (L_c phases) of phospholipid dispersions and have been observed in ^{31}P NMR spectroscopic studies of a number of phosphatidylcholines (Fuldner, 1981; Mantsch et al., 1985; Lewis & McElhaney, 1985b; Lewis et al., 1987a,b, 1988). Upon heating, the observed powder patterns undergo abrupt changes at temperatures which coincide with the heating endothermic events reported by DSC. Typically, the broad axially asymmetric powder pattern changes to a narrower pattern (basal line width ≈ 90 ppm) as the lipid undergoes a L_c/L_β (subgel/gel) phase transition. The powder pattern then observed exhibits the features expected of a phospholipid undergoing slow axially symmetric motion in a lipid bilayer assembly [see Seelig (1978)]. Upon further heating, the L_β phase converts to the L_α phase, and there is a concomitant sharp narrowing of the spectrum as it approaches the limiting spectral line shape generally exhibited by phospholipid molecules in a bilayer assembly undergoing fast axially symmetric motion [see Campbell et al. (1979)]. Upon further heating, the typical liquid-crystalline bilayer powder pattern changes abruptly to a powder pattern of reduced width (indicative of reduced anisotropy) and reversed asymmetry. Such powder patterns have been correlated with the formation of H_{II} phases in phospholipid assemblies [see Cullis and de Kruijff (1976) and Gruner et al. (1985) and references cited therein], and its formation coincides with the high-temperature endotherm reported by DSC. The identification of the weakly energetic, high-temperature DSC endotherms as L_α/H_{II} phase transitions was confirmed by the small-angle X-ray scattering data (see Figure 3). These data show that such calorimetric transitions coincide with a change in the small-angle X-ray scattering pattern from that typical of the lamellar phase of lipid bilayers (Figure 3A) to that expected of an H_{II} phase (Figure 3B). In addition, the thermotropic changes detected by both NMR spectroscopy and DSC coincide with discontinuities in the temperature-dependent changes in the d spacings. In particular, the L_β/L_α transitions coincide with a sharp decrease in the d spacing, while the L_α/H_{II} transitions coincide with a large increase in the observed d spacing (see Table II). The magnitude of the d spacings observed in the lamellar and inverted hexagonal phases at the onset and completion temperatures of the L_α/H_{II} phase transition is of special interest with respect to the theme of this paper. From such measurements, information about the thickness of the lipid bilayer in the L_α phase and about the diameters of the cylindrical structures formed in the H_{II} phase can be derived.

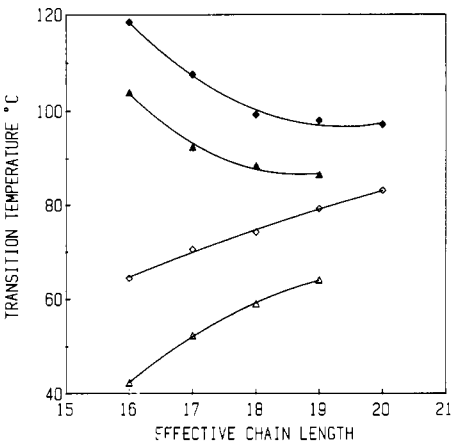

FIGURE 4: Chain length dependence of the gel/liquid-crystalline and lamellar/H_{II} phase transition temperatures of the n-acyl- and isoacyl-PEs. (♦) T_h of n-acyl-PEs; (◇) T_m of n-acyl-PEs; (▲) T_h of isoacyl-PEs; (△) T_m of isoacyl-PEs.

Listed in Table I are the L_β/L_α phase transition temperatures (T_m) and the L_α/H_{II} phase transition temperatures (T_h) for all of the PEs used in this study, along with literature values for other diacyl-PEs for which both values are available. As expected, for any homologous series of compounds, T_m increases with increasing acyl chain length, but as shown in Figure 4, T_h decreases as the length of the acyl chains increases. It is also clear that both T_m and T_h values describe

Table I: Lamellar Gel/Liquid-Crystalline and Lamellar Liquid-Crystalline/Reversed Hexagonal Phase Transition Temperatures of Phosphatidylethanolamines Containing a Variety of Different Hydrocarbon Chains

phosphatidylethanolamine	T_m (°C)	T_h (°C)
linear saturated PEs		
16:0-PE[a]	64.4 (64)[b]	118.5 (123)[b]
17:0-PE	70.5	107.6
18:0-PE	74.2 (74)[b]	99.2 (101)[b]
19:0-PE	79.2	98
20:0-PE[b]	83.1 (82.5)[b]	97.2 (96)[b]
methyl isobranched PEs		
17:0_i-PE	42.3	104
18:0_i-PE	52	94
19:0_i-PE	59	88
20:0_i-PE	64	86.5
dimethyl isobranched PEs		
19:0_{dmi}-PE	38.6	76.8
20:0_{dmi}-PE	43.2	74
methyl anteisobranched PEs		
19:0_{ai}-PE	44.6	80.5
20:0_{ai}-PE	51.5	75
ethyl anteisobranched PE		
20:0_{eai}-PE	24	52
ω-cyclohexyl-PEs		
19:0_{ch}-PE	35	106
20:0_{ch}-PE	45.9	84.2
21:0_{ch}-PE	54	82.8
unsaturated PEs		
18:1$t\Delta$9-PE[c]	38.3	63.5
16:1$c\Delta$9-PE[d]	−30	42.5
18:1$c\Delta$9-PE[e]	−16	10
18:2c,$c\Delta$9,12-PE[f]	−40	−15
mixed-acid PEs		
1-16:0,2-18:1$c\Delta$9-PE[g]	24.8	71.4
egg PE[e]	5	30

[a] Fatty acyl groups are denoted by the total number of carbon atoms present followed, after a colon, by the number of double bonds present. This term is in turn followed by the subscript "i" for methyl isobranched, "ai" for methyl anteisobranched, "dmi" for dimethyl isobranched, "eai" for ethyl anteisobranched, or "ch" for ω-cyclohexyl. In addition, unsaturated fatty acyl groups are denoted "c" or "t" for the cis and trans configuration of the double bond, and the position of this double bond in the hydrocarbon chain is denoted by the symbol Δ plus the number of the first carbon atom bearing the double bond. Where more than one value is listed, the literature values are listed in parentheses. [b] Seddon et al. (1983). [c] Gagne et al. (1985). [d] Silvius et al. (1985). [e] Cullis and de Kruijff (1978). [f] Tilcock and Cullis (1982). [g] Epand (1985).

Table II: d Spacings of a Series of Synthetic Phosphatidylethanolamines Having the Same Effective Chain Length (18 Carbons) As Measured by X-ray Diffraction at the Onset (L_α Phase) and Completion (H_{II} Phase) of the L_α/H_{II} Phase Transition

phosphatidyl-ethanolamine	L_α phase (Å)	H_{II} phase (Å)	T_h (°C)
19:0_i-PE	52.5	77.5	88
19:0_{ai}-PE	52.5	77.7	80.5
20:0_{dmi}-PE	52.5	77.5	74
18:1$c\Delta$9-PE[a]	52.5	78	10
18:1$t\Delta$9-PE[b]	52.5	77	63.5

[a] Kirk & Gruner (1985). [b] Tate (1987).

smooth curves which would probably extrapolate to some limiting high-temperature asymptote (see Figure 4). Moreover, an extrapolation of the trends displayed in Figure 4 suggests that for the longer chain compounds (probably those of 22 or more linear carbons), discrete L_β/L_α and L_α/H_{II} phase transitions would not be observed. Instead, these two transitions would be replaced by a single L_β/H_{II} transition. In fact, Seddon et al. (1984) have reported that 22:0-PE undergoes a direct conversion from the L_β to the H_{II} phase.

While it is clear from Table I that the length of the acyl chain is an important determinant of both T_m and T_h, it is also

Table III: Lamellar Gel/Liquid-Crystalline and Lamellar Liquid-Crystalline/Reversed Hexagonal Phase Transition Temperatures of Aqueous Dispersions of Phosphatidylethanolamines of Comparable Effective Chain Length (18 Carbons)

phosphatidylethanolamine	T_m (°C)	T_h (°C)	ΔT ($T_h - T_m$) (°C)
18:0-PE	74.2	99.2	25.0
19:0_i-PE	59.0	88.0	29.0
21:0_{ch}-PE	54.0	82.8	28.8
19:0_{ai}-PE	44.6	80.5	35.9
20:0_{dmi}-PE	43.2	74.0	30.8
18:1$t\Delta$9-PE[a]	38.3	63.5	25.2
20:0_{eai}-PE	24	52	28
18:1$c\Delta$9-PE[b]	−16.0	10.0	26.0
18:2c,$c\Delta$9,12-PE[c]	−40	−15	25

[a] Gagne et al. (1985). [b] Cullis & de Kruijff (1978). [c] Tilcock & Cullis (1982).

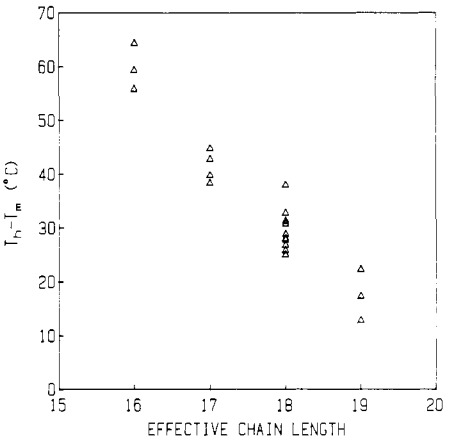

FIGURE 5: Effect of acyl chain length on the reduced temperatures ($T_h - T_m$) at which PEs undergo their L_α/H_{II} phase transitions. Data points are shown for the various structural classes of fatty acyl chains.

clear that both phase transition temperatures are also affected by the structure of the acyl chain. This is vividly demonstrated by Table III, which lists the T_m and T_h values for all of the PEs (taken from this study and the literature) with effective hydrocarbon chain lengths[2] equivalent to 18 linear carbon atoms. It is clear that for a given effective acyl chain length, any modification of the basic linear saturated fatty acyl chain results in a comparable lowering of both T_m and T_h. More specifically, the relative T_m and T_h values for the PEs of the various structural classes of fatty acids studied both decrease in the following order: linear saturated > methyl isobranched > ω-cyclohexyl > methyl anteiosbranched > dimethyl isobranched (ω-*tert*-butyl) > trans monounsaturated > ethyl anteisobranched > cis monounsaturated > cis diunsaturated. An examination of the data pertaining to the branched and alicyclic chain PEs (see Table I) indicates that both T_m and T_h decrease as the size of the substituent near the methyl terminus increases (e.g., 18:0 > 18:0_i > 21:0_{ch} and 19:0_{ai} > 20:0_{eai}), as the number of substituents at any given position increases (e.g., 18:0 > 19:0_i > 20:0_{dm}), as the position of the

[2] The effective chain length of a hydrocarbon chain is defined by the total number of carbon atoms in the "main chain". For linear saturated and unsaturated fatty acyl groups, the effective chain length equals the total number of carbon atoms present, while for branched chain fatty acyl groups the effective chain length equals that of the total number of carbon atoms minus those present in the branch (or branches). For ω-cyclohexyl fatty acyl groups, where three carbon atoms of the terminal six-membered ring actually form part of the main chain, the effective chain length is equal to the total number of carbon atoms present minus three.

substituent is moved toward the center of the acyl chain (e.g., $19{:}0_{i} > 19{:}0_{ai}$), or as the flexibility of the substituent is increased (e.g., the T_m and T_h values of $21{:}0_{ch}$-PE are higher than the comparable parameters of its open-chain analogue, $20{:}0_{eai}$).

It is clear that for any given effective acyl chain length, the variations in the structure of the acyl chains result in comparable changes in both T_m and T_h of the various PE bilayers studied such that irrespective of the wide range of absolute temperatures covered the difference between T_m and T_h remains nearly constant. However, the magnitude of the difference between T_m and T_h for any homologous series of PEs containing a single chemical class of fatty acid is primarily determined by the length of the acyl chain (see Figure 5), while the detailed structure of the acyl chain exerts a considerably smaller effect on the *difference* between T_m and T_h. Thus, for PEs with an effective chain length of 18 carbon atoms, the difference between T_m and T_h is about 25–30 °C (except $19{:}0_{ai}$-PE; see below). However, a closer inspection of the data shown in Table III also reveals a further structural correlation. The PEs which exhibit the smallest differences between T_m and T_h all have unbranched chains (e.g., 18:0, 18:1*t*, 18:1*c*, and 18:2*c*,*c*, which have ΔT = 25–26 °C), while those with branched chain or alicyclic substituents near the methyl terminus exhibit somewhat larger differences ($\Delta T \approx$ 29–31 °C), except the methyl anteisobranched compound which exhibits a higher value (see below). At this stage, it is not clear whether these differences are a reflection of the type of structural modification or of its position on the acyl chain. Nevertheless, the dominant feature remains the relatively constant difference between T_m and T_h despite the wide variations in the structure of the acyl chains and the sizable differences in the absolute temperatures at which these phase transitions occur.

Another consistent feature observed in these studies is the fact that for the methyl anteisobranched PEs, the difference between T_m and T_h tends to be significantly greater than the mean value typical of the given effective chain length (see Table III). However, it has been recognized that there is most likely a dynamic equilibrium between two inequivalent conformations of the methyl anteiso acyl chain [see Lewis et al. (1987b) and Balthasar et al. (1988)]. One of these has the methyl branch offset from the "zig-zag line" of the main polymethylene chain, in which case the effective chain length would be maximal, while in the other conformation the methyl branch is aligned along the zig-zag line of the main polymethylene chain (and in effect forms an ethyl branched substituent at the penultimate carbon), with the result that the effective chain length would be one carbon shorter. The existence of such a dynamic equilibrium is very significant with respect to these studies. Since the existence of such a conformational equilibrium would shorten the effective acyl chain length of the methyl anteisobranched PEs, and since we have shown that the difference between T_m and T_h increases markedly with a decrease in acyl chain length (see Figure 5), the fact that the difference between the T_m and T_h of the methyl anteisobranched PEs tends to be significantly greater than the mean value typical of the other PEs of supposedly comparable chain length is consistent with the rest of our observations. Such a explanation seems all the more plausible when viewed against the fact that when the conformational inequivalence induced by the *methyl* anteisobranch is removed by the replacement of the methyl group by an *ethyl* group, the observed difference between T_m and T_h (28 °C) is reduced to what is typical of PEs with effective chain lengths of 18 carbon atoms (compare $19{:}0_{ai}$-PE with $20{:}0_{eia}$-PE in Table III).

The thermotropic transitions exhibited by the various PEs used in this study were detected in the X-ray diffraction experiments by the abrupt changes in the temperature dependence of the *d* spacings (all transitions) and by the marked changes in the symmetry of the observed small-angle scattering pattern (L_α/H_{II} transitions). As has been reported for phosphatidylcholines, the conversion from the L_β to the L_α phase is accompanied by a change in the *d* spacing, and further heating of the L_α phase results in a monotonic decrease in that parameter. This progressive decrease in the *d* spacing is usually attributed to a temperature-dependent decrease in the thickness of the liquid-crystalline bilayer as a result of increases in the number of gauche bonds and in the frequency of trans–gauche isomerism in the acyl chains that occurs upon heating. As expected, the measured *d* spacings are chain length dependent, but as shown in Table II, the *d* spacings measured for the L_α phases of the PEs of the same equivalent acyl chain length (18 linear carbons in this case) are all similar. Moreover, irrespective of the absolute temperatures at which the L_α/H_{II} transitions of those lipids occur, the observed *d* spacings of their L_α phases at the onset temperatures of their respective L_α/H_{II} transition are all the same (≈52.5 Å). The data listed in Table II also show that at the completion temperatures of their respective L_α/H_{II} transitions, the *d* spacings (X-ray lattice vector lengths, corresponding to the center to center distances between the aqueous channels) of the H_{II} phases of the PEs listed are also all the same (≈77.5 Å). The former result suggests that for PEs of any given effective acyl chain length, there may be a critical bilayer thickness at which their L_α phases become unstable with respect to a nonlamellar structure like the H_{II} phase, irrespective of the detailed structure of the acyl chain. Moreover, the latter result suggests that in spite of the structural variations in the acyl chains, the effective diameters of the cylindrical assemblies formed at the completion temperatures of the L_α to H_{II} phase transition of PEs with similar acyl chain length also appear to be similar.

Discussion

These studies provide data relevant to an evaluation of some of the structural parameters which affect the L_α/H_{II} phase transition of aqueous PE dispersions. First, it is clear that the length of the acyl chain is one of the major factors which affect the conversion from the L_α to the H_{II} phase. Previous studies with the saturated di-*n*-acyl-PEs and their diether analogues (Seddon et al., 1983) also indicated that the L_α/H_{II} phase transition temperatures decrease as the length of hydrocarbon chains increases. An examination of the data presented here and those compiled from the literature (see Table I) shows that the same is also true for all of the other PE classes so far investigated. These observations clearly demonstrate that the formation of inverted nonbilayer phases by PEs tends to be favored by an increase in the length of the hydrocarbon chain [see Gruner et al., 1985) and Israelachvili et al. (1980)].

In these studies, we failed to detect lamellar to H_{II} transitions with those PEs which had acyl chains of 15 or fewer carbon atoms. Given that the L_α/H_{II} transition temperatures of these lipids increase markedly with decreasing acyl chain length, it is logical to assume that the bilayer to nonbilayer transitions of the shorter chain lipids may occur at temperatures that are above the range examined in this study, especially since studies using dialkyl-PEs (Seddon et al., 1983) and some dialkyl monoglycosyl glycerolipids (Jarrell et al., 1986, 1987a,b, unpublished experiments from this laboratory) have shown that short hydrocarbon chains as such are not a barrier to the formation of H_{II} or other nonlamellar phases. However, with some of the shorter chain PEs that were synthesized, we

failed to detect the formation of nonlamellar phases even though we were able to approach temperatures that are as high as 100 °C above that of their L_β/L_α phase transition temperatures. While this is not sufficient evidence to infer that the lamellar phases of the shorter chain diacyl-PE do not undergo transitions to H_{II} or other nonlamellar phases, such a possibility is compatible with our results.

Second, we have also shown that once allowances are made for acyl chain length, the major effect of acyl chain structure on T_h is directly related to the change in the T_m of the PE concerned. Our results have clearly shown that PEs of a given effective acyl chain length exhibit their L_α/H_{II} phase transitions at a relatively constant temperature interval above the gel/liquid-crystalline phase transition temperature, a finding which suggests that the conversion from the L_α to the H_{II} phase is in some way coupled to the chain-melting phase transition. Previous studies have shown that the L_α/H_{II} phase transition temperatures of unsaturated PEs are generally lower than those of their saturated counterparts (see Table I and references cited therein), and this finding has led some workers to suggest that the lower T_h values observed may be the result of a greater disparity between the cross-sectional areas of the polar headgroup and the acyl chains as a result of the presence of carbon–carbon double bonds [see Rilfors et al. (1984) and references cited therein]. However, our data suggest that the lowering of the T_h values observed with the unsaturated non-bilayer-forming lipids is more probably simply the result of substantial reduction in the L_β/L_α phase transition temperatures coincident with the presence of double bonds in the acyl chains. In fact, our results indicate that after the length of the acyl chain and the absolute L_β/L_α phase transition temperature are considered, the specific effects of acyl chain structure on H_{II} phase formation are relatively modest. However, the fact that there is a small variation in the magnitude of the temperature differential between T_h and T_m for PEs of a given acyl chain length does suggest that there must be some other minor effect(s) specific to the structure of the acyl chain. It is possible that the specific effects of acyl chain structure on the L_α/H_{II} transition to PEs (i.e., those effects not attributable to changes in acyl chain length and the L_β/L_α phase transition temperature) may be attributable to small differences in the packing energy of the chains and/or to differences in the temperature-dependent changes in the dynamic properties of the liquid-crystalline lipid bilayer. It has been shown previously that acyl chain substitutions of the types described in these studies can have significant effects on the packing properties of the liquid-crystalline state of lipid bilayers (MacDonald et al., 1983, 1984, 1985a,b; Suzuki & Cadenhead, 1985; Rice et al., 1987; Balthasar et al., 1988). In fact, the interpretation of such data implicitly assumes that one of the major effects of the various structural substituents so far studied is a reduction in the frequency of trans–gauche isomerization in the hydrocarbon chains, with the result that the liquid-crystalline states of such bilayers are, at comparable temperatures relative to their respective gel/liquid-crystalline phase transition temperatures, somewhat more ordered than bilayers composed of unsubstituted acyl chains.

Third, we have shown that for a series of PEs of similar effective chain length but different hydrocarbon structures, the *d* spacings at the onset and completion temperatures of the L_α/H_{II} transition are the same. If for such PEs we assume that at temperatures near T_h the dimensions of the aqueous domains of both the lamellar and H_{II} phases are invariant with respect to hydrocarbon chain structure, then the above observation indicates that despite the variations in their acyl chain structures, the PEs of similar acyl chain length all have a similar bilayer thickness (L_α phase) and a comparable annular monolayer thickness (H_{II} phase) at the L_α/H_{II} transition temperature. If this is true, then following the reasoning given in Gruner et al. (1988), the balance between curvature energy and chain stretch energy must occur at the same critical dimensions for PEs with all the various types of fatty acyl chains tested, providing only that the effective chain lengths are comparable. This interpretation of the data can be verified by direct measurements of the actual thickness of the L_α bilayers and the average annular monolayer thickness of the H_{II} cylinders of these various PEs at temperatures near their respective L_α/H_{II} transition temperatures when sufficiently large quantities of these materials become available. Moreover, such measurements can also provide valuable information on the bilayer thickness near the T_m and on the (negative) coefficient of thermal expansion of the thickness of the L_α phases of these compounds. Such experiments may help to explain why the L_α/H_{II} phase transition occurs at constant reduced temperature relative to the chain-melting phase transition when acyl chain length is held constant.

Our results are broadly compatible with the standard molecular shape arguments often used to explain the lamellar/nonlamellar phase preferences of PEs and other nonbilayer-forming lipids. If the thickness of the L_α bilayer near T_h is indeed comparable for all PEs of the same effective chain length, then the length of the hydrocarbon chain (l_c) in the critical packing parameter equation[3] used by Israelachvilli et al. (1977, 1980) to predict the phase preferences of lipid molecules must also be the same for these molecules at their respective L_α/H_{II} phase transition temperatures. In addition, since the compounds used in this study all have the same polar headgroup (and thus the same a_0 values), it is logical to expect that in a series of PEs with comparable effective chain lengths (but different structures), the hydrophobic volume (V) would be the critical term in defining the packing parameter. Thus, this concept correctly predicts that the addition of a single methyl branch to a saturated hydrocarbon chain will increase its preference for forming a reversed nonbilayer phase (i.e., T_h will be reduced). Indeed, it also correctly predicts that the preference for forming such a phase will be further augmented by increases in the size or the number of branched or alicyclic substituents, since in all such cases the hydrophobic volume term will be increased in magnitude. However, the critical packing parameter *by itself* does not predict that the introduction of a double bond into a saturated hydrocarbon chain will increase the nonbilayer-forming tendency of a PE, nor that the geometrical configuration of that double bond, or the position of substitution of a methyl branch, will be important factors in determining T_h as well. To account for these observations, it would be necessary to replace the van der Waals volume term in the critical packing parameter equation[3] with the "effective" or "dynamic" volume occupied by the PE hydrocarbon chains. In other words, one must consider not only the "static" shape of the molecule itself but also the shape of the average volume which the molecule occupies when its free energy with respect to its static shape is minimized. This concept of effective shape under a particular set of conditions is essentially the basis of the spontaneous curvature concept initially proposed by Helfrich (1973) and later refined by

[3] The critical packing parameter P is defined by the equation $P = V/(a_0 l_c)$ where V is the volume of the hydrocarbon chains, a_0 is the area per molecule at the polar–apolar interface, and l_c is the length of the hydrocarbon chain. P values greater than 1 increasingly favor the formation of reversed phases.

Gruner et al. (Kirk et al., 1984; Gruner, 1985).

It should be emphasized that although fatty acyl chain structural variations at a fixed chain length have only a small effect on the lamellar/nonlamellar phase behavior on the *reduced temperature* scale (i.e., when normalized relative to the chain-melting phase transition temperature), such variations in hydrocarbon chain structure do produce large variations on the more biologically relevant *absolute temperature* scale. Thus, for example, the T_h values of the PEs of effective chain length of 18 carbons studied vary over a range of nearly 115 °C. Hence, in the biologically relevant fatty acyl chain length range (typically 16–18 or 20 carbon atoms for biomembranes), variations in structure can produce much larger changes in the absolute value of T_h than do variations in chain length. Nevertheless, both variables are important and should be considered from the perspective of both experimental design and data analysis in studies of the effect of variations in fatty acid composition on the structure and function of biological membranes. In addition, the fact that at comparable effective chain lengths variations in structure cause T_m and T_h to vary in parallel, while variations in chain length within a given structural type of hydrocarbon chain cause T_m and T_h to vary in opposite directions, can be exploited in experiments designed to determine the relative contributions of lipid homoeoviscous effects (related to T_m) and lamellar/nonlamellar phase preference effects (related to T_h) in biological systems.

Acknowledgments

We thank Dr. Brian D. Sykes for the generous availability of time on the NMR spectrometer.

Registry No. 16:0-PE, 3026-45-7; 17:0-PE, 86388-25-2; 18:0-PE, 4537-76-2; 19:0-PE, 86388-26-3; 20:0-PE, 87136-19-4; 17:0_i-PE, 114833-80-6; 18:0_i-PE, 114833-81-7; 19:0_i-PE, 114833-82-8; 20:0_i-PE, 117581-63-2; 19:0_{dmi}-PE, 117581-64-3; 20:0_{dmi}-PE, 117581-65-4; 19:0_{ai}-PE, 117581-66-5; 20:0_{ai}-PE, 117581-67-6; 20:0_{eai}-PE, 117581-68-7; 19:0_{ch}-PE, 114833-84-0; 20:0_{ch}-PE, 117581-69-8; 21:0_{ch}-PE, 117581-70-1; 18:1*t*Δ9-PE, 16777-83-6; 16:1*c*Δ9-PE, 74524-11-1; 18:1*c*Δ9-PE, 2462-63-7; 18:2*c*,*c*Δ9,12-PE, 55252-82-9; 1-16:0,2-18:1*c*Δ9-PE, 10015-88-0; 16:0-phosphatidylcholine, 2644-64-6; 17:0-phosphatidylcholine, 67896-64-4; 18:0-phosphatidylcholine, 4539-70-2; 19:0-phosphatidylcholine, 85800-07-3; 20:0-phosphatidylcholine, 71259-34-2; 17:0_i-phosphatidylcholine, 87403-01-8; 18:0_i-phosphatidylcholine, 58045-79-7; 19:0_i-phosphatidylcholine, 114849-11-5; 20:0_i-phosphatidylcholine, 117581-71-2; 19:0_{dmi}-phosphatidylcholine, 117581-72-3; 20:0_{dmi}-phosphatidylcholine, 117581-73-4; 19:0_{ai}-phosphatidylcholine, 73641-13-1; 20:0_{ai}-phosphatidylcholine, 108320-55-4; 20:0_{eai}-phosphatidylcholine, 117581-74-5; 19:0_{ch}-phosphatidylcholine, 81326-18-3; 20:0_{ch}-phosphatidylcholine, 97352-46-0; 21:0_{ch}-phosphatidylcholine, 97352-47-1; 18:1*t*Δ9-phosphatidylcholine, 52088-89-8; 16:1*c*Δ9-phosphatidylcholine, 65206-87-3; 18:1*c*Δ9-phosphatidylcholine, 10015-85-7; 18:2*c*,*c*Δ9,12-phosphatidylcholine, 6542-05-8; 1-16:0,2-18:1*c*Δ9-phosphatidylcholine, 6753-55-5.

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Time-Dependent ^{31}P Saturation Transfer in the Phosphoglucomutase Reaction. Characterization of the Spin System for the Cd(II) Enzyme and Evaluation of Rate Constants for the Transfer Process†

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Received April 29, 1988; Revised Manuscript Received August 16, 1988

ABSTRACT: Time-dependent ^{31}P saturation-transfer studies were conducted with the Cd^{2+}-activated form of muscle phosphoglucomutase to probe the origin of the 100-fold difference between its catalytic efficiency (in terms of k_{cat}) and that of the more efficient Mg^{2+}-activated enzyme. The present paper describes the equilibrium mixture of phosphoglucomutase and its substrate/product pair when the concentration of the Cd^{2+} enzyme approaches that of the substrate and how the nine-spin ^{31}P NMR system provided by this mixture was treated. It shows that the presence of abortive complexes is not a significant factor in the reduced activity of the Cd^{2+} enzyme since the complex of the dephosphoenzyme and glucose 1,6-bisphosphate, which accounts for a large majority of the enzyme present at equilibrium, is catalytically competent. It also shows that rate constants for saturation transfer obtained at three different ratios of enzyme to free substrate are mutually compatible. These constants, which were measured at chemical equilibrium, can be used to provide a quantitative kinetic rationale for the reduced *steady-state* activity elicited by Cd^{2+} relative to Mg^{2+} [cf. Ray, W. J., Post, C. B., & Puvathingal, J. M. (1989) *Biochemistry* (following paper in this issue)]. They also provide minimal estimates of 350 and 150 s^{-1} for the rate constants describing (PO_3^-) transfer from the Cd^{2+} phosphoenzyme to the 6-position of bound glucose 1-phosphate and to the 1-position of bound glucose 6-phosphate, respectively. These minimal estimates are compared with analogous estimates for the Mg^{2+} and Li^+ forms of the enzyme in the accompanying paper.

Phosphoglucomutase is one of a group of enzymes that conducts multiple and distinctly different bond-breaking/bond-making operations on a bound substrate before releasing its product. Thus, in the thermodynamically favorable direction (Glc-1-P → Glc-6-P),[1] the enzyme transfers its active-site (PO_3^-) group to the 6-hydroxyl group of Glc-1-P to produce bound Glc-1,6-P_2 (i.e., Glc-P_2); in a second subsequent step the enzyme accepts the 1-(PO_3^-) group from the still bound bisphosphate, thereby producing Glc-6-P and regenerating the phosphoenzyme (Ray & Peck, 1972):

$$E_P\cdot\text{Glc-1-P} \rightleftharpoons E_D\cdot\text{Glc-}P_2 \rightleftharpoons E_P\cdot\text{Glc-6-P}$$

(E_P and E_D are the phospho and dephospho forms of the enzyme, respectively.)

Efficient catalysis by muscle phosphoglucomutase requires a bound metal ion activator. Although Mg^{2+} is the most efficient activator as well as the activator that is important physiologically, a number of bivalent metal ions produce varying degrees of activation (Ray, 1969). One approach to evaluating the role of the metal ion in the bond-breaking/bond-making process is to study the origin of metal-specific differences in enzyme activation. This approach is particularly attractive for the phosphoglucomutase reaction since the equilibrium binding of the glucose monophosphates, viz., the substrate/product pair, is essentially independent of metal ion identity (Ray & Long, 1976). Hence, studies of metal-specific differences can focus on effects that the metal ion produces on the subsequent bond-breaking/bond-making process.[2]

At equilibrium in the enzymic reaction involving Mg^{2+}, the intermediate $E_D\cdot Mg\cdot$Glc-P_2 complex accounts for about half of the total enzyme, while the monophosphate complexes,

† This work was supported in part by a research grant from the U.S. Public Health Services (GM08963, to W.J.R.), by grants from the Biotechnology Resources Program of the Division of Research Resources, National Institutes of Health (RR01077, to the Purdue University Biochemical Magnetic Resources Laboratory), and by grants from the NSF Biological Instrumentation Division (8714258 and BMS8614177, to the Purdue Biological Facilities Center on Biomolecular NMR, Structure and Design).
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[1] Abbreviations: Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, α-D-glucose 6-phosphate, unless the β-anomer is specified; Glc-P_2 or Glc-1,6-P_2, α-D-glucose 1,6-bisphosphate; E_P and E_D, the phospho and dephospho forms of rabbit muscle phosphoglucomutase; M, a bivalent metal ion activator (of phosphoglucomutase).

[2] The insensitivity of glucose monophosphate binding to metal ion identity finds a reasonable rationale in observations which indicate that the bound bivalent metal ion interacts directly with the enzymic phosphate group (Rhyu, 1984). The metal ion thus is unlikely to act as a bridge between the enzyme and glucose monophosphate, where a metal specific effect on binding would be likely.

0006-2960/89/0428-0548$01.50/0