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Structure of Vitaminylated Lipids in Aqueous Dispersion: X-ray Diffraction and ^{31}P NMR Studies of *N*-Biotinylphosphatidylethanolamines

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ABSTRACT: The structures of the phases formed in excess buffer (at pH 7.4) by a homologous series of saturated diacylphosphatidylethanolamines in which the headgroup is N-derivatized with biotin have been investigated for chain lengths of C(12:0) to C(20:0), using both ^{31}P nuclear magnetic resonance (NMR) spectroscopy and small-angle X-ray diffraction. In 1 M NaCl, all lipids display ^{31}P NMR spectra characteristic of a lamellar gel phase at low temperature. In the fluid phase, the lipids of C(12:0) and C(14:0) chain lengths display isotropic ^{31}P NMR spectra, corresponding to aggregated phases with high surface curvature, whereas those with C(18:0) and C(20:0) chain lengths display sharp axial powder patterns characteristic of a lamellar (L_α) phase. The lipid of intermediate C(16:0) chain length displays a more complex temperature dependence of the ^{31}P NMR spectra in the fluid phase. The spectra convert from an axial powder pattern of unusually low chemical shift anisotropy to one characteristic of a fluid lamellar (L_α) phase with increasing temperature. The small-angle X-ray diffraction patterns of the lipids in 1 M NaCl have lamellar repeat spacings in the gel phase which increase linearly with chain length and are consistently lower than those in the fluid phase [for chain lengths of C(16:0) to C(20:0)]. In addition, the gradient in long spacing with chain length in the gel phase is approximately half that expected for a gel phase with untilted, *all-trans* chains, indicating that the lipid chains are interdigitated in the gel phase ($L_\beta{}^i$). The continuous X-ray scatter from those lipid aggregates with an isotropic ^{31}P NMR spectrum [C(12:0) and C(14:0)], or with low chemical shift anisotropy [C(16:0)], in 1 M NaCl suggest that the fluid state for these lipids corresponds to a micellar-like aggregated phase, hitherto not identified for phospholipids. In the absence of salt, the ^{31}P NMR spectra indicate that the lipids with chain lengths of C(16:0), C(18:0), and C(20:0) are in a lamellar gel phase at low temperature which on chain-melting convert to a fluid micellar phase. The small-angle X-ray diffraction patterns as a function of water content indicate that the lamellar gel phase for the lipid of C(18:0) chain length in the absence of salt is also one with interdigitated chains ($L_\beta{}^i$). The lipids with chain lengths of C(12:0) and C(14:0) are found to be in a fluid micellar phase at temperatures above 0 °C, but on prolonged incubation at low temperature the C(14:0) lipid forms a gel phase.

Phosphatidylethanolamines which have been derivatized by attachment of the vitamin biotin at the amine function (biotin-PEs)[1] are of particular interest because the proteins avidin and streptavidin are then capable of binding with high affinity to the phospholipid polar headgroup (cf. Green, 1975; Wilchek & Bayer, 1989). Such phospholipids with derivatized headgroups allow the attachment of further molecules (in this case via the unoccupied biotin sites on the bound avidin) which may then be used either for targetting of liposomes or for constructing molecular biodevices from monolayer lipid arrays [see, e.g., Urdal and Hakomori (1980), Loughrey et al. (1987), and Ahlers et al. (1989)]. In addition, not only are lipid systems with incorporated vitamins of intrinsic biological interest, but also biotinylated phosphatidylethanolamine is of use for ultrastructural localization in biological systems (Bayer et al., 1979) or for the formation of two-dimensional arrays for protein structure determination by electron crystallography (Blankenburg et al., 1989; Darst et al., 1991). For the biological applications of such unusual phospholipids, and in their use for studying molecular recognition at membrane surfaces, it is important to characterize the lyotropic structures formed when they are dispersed in an aqueous environment.

In the present paper we report on determinations of the structures of the various phases formed by the aqueous dispersions of biotin-PEs of different chain lengths. Structural characterization was effected by X-ray diffraction, broad-line ^{31}P NMR spectroscopy and polarizing microscopy. It is found that, in many cases, the stable phases are quite different from those most frequently encountered with diacylphospholipids, namely, the fluid and gel bilayers, and are strongly affected in a chain length-dependent manner by the presence or absence of salt. This indicates that the nature of the phases formed in aqueous dispersion depends sensitively not only on the molecular structure but also on the electrostatic properties of these negatively charged lipids. In particular, a new isotropic fluid phase is found, in certain instances, that has not been identified previously for phospholipid systems.

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[1] Abbreviations: PE, phosphatidylethanolamine; biotin-PE, *N*-biotinylphosphatidylethanolamine; DLBPE, DMBPE, DPBPE, DSBPE, and DABPE, 1,2-dilauroyl-, 1,2-dimyristoyl-, 1,2-dipalmitoyl-, 1,2-distearoyl-, and 1,2-diarachinoyl-*sn*-glycero-3-(*N*-biotinyl)phosphoethanolamine; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulphonic acid; NMR, nuclear magnetic resonance; ESR, electron spin resonance; $L_\beta{}^i$, lamellar gel phase with interdigitated chains; L_β, lamellar gel phase with untilted and noninterdigitated chains; L_α, fluid lamellar phase.

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MATERIALS AND METHODS

Materials. Saturated diacyl *N*-biotinylated phosphatidylethanolamines with symmetrical chains of lengths from 12 to 20 C atoms (DLBPE, DMBPE, DPBPE, DSBPE, and DABPE)[1] were synthesized from the corresponding underivatized phosphatidylethanolamines (from Avanti, Birmingham, AL, and Fluka, Buchs, Switzerland) and biotinyl-*N*-hydroxysuccinimide (from Sigma, St. Louis, MO) according to Bayer and Wilchek (1974) and Bayer et al. (1979).

Sample Preparation. Samples for ^{31}P NMR spectroscopy were prepared by dissolving 20–40 mg of the biotin-PE in dichloromethane, removing the solvent by rotary evaporation, and drying overnight under vacuum. The dry lipid was hydrated with 0.5 mL of 10 mM Hepes, 1 mM EDTA, pH 7.4, buffer at approximately 10 °C above the chain-melting phase transition temperature and transferred to a 10-mm NMR tube. Samples were freeze-thawed five times before measurement. For measurements in the presence of 1 M NaCl, the appropriate volume of 5 M NaCl was added to the sample with vortex mixing. The samples were then pelleted in a bench-top centrifuge, and excess buffer was removed before measurement.

For X-ray diffraction measurements in excess buffer, an aliquot of the samples prepared for NMR measurements was transferred to a 1-mm diameter fine-wall glass capillary and centrifuged at 3000 rpm in a bench top centrifuge for ca. 5 min, and the excess supernatant was removed. The capillary was then flame sealed. For measurements with defined water content, samples were prepared essentially according to Seddon et al. (1984). Dry lipid (2–5 mg) was transferred into a preweighed capillary and was pelleted by centrifugation in a bench top centrifuge. The capillary was weighed again, and then a small aliquot of water was added above the lipid pellet and spun down into the lipid. The capillary was weighed, flame sealed approximately 1 cm above the top of the lipid, and reweighed to ensure that there was no loss of water during sealing. The capillary was then heated in boiling water for several minutes to ensure homogeneous hydration of the sample.

X-ray Diffraction. X-ray diffraction measurements were performed using a Kratky small-angle X-ray scattering apparatus with slit geometry [see Würz (1988)]. Compared with the range of scattering angles covered, the slit can be regarded as of infinite length, and the half-width of the beam (1.5×10^{-3} Å^{-1}) is negligible. Sample capillaries were mounted in a massive copper housing which was thermostated with a Peltier device. Temperature stability and gradients were better than 0.1 K. Nickel-filtered Cu K$_\alpha$ radiation ($\lambda = 0.154$ nm) was obtained from an AEG fine-focus tube, and diffracted intensity was recorded with a linear position-sensitive detector mounted on an extension tube at a distance of 98.35 cm from the sample. To record data at higher scattering angles, the detector was repositioned in a direction orthogonal to the beam. Diffraction patterns are presented as scattering intensity that has not been corrected for the slit geometry.

^{31}P Nuclear Magnetic Resonance Spectroscopy. Proton–dipolar decoupled ^{31}P NMR spectra were recorded at a frequency of 121.5 MHz on a Bruker MSL–300 spectrometer operating in the Fourier transform mode. The $\pi/2$ pulse width was 11 μs, and recycle delays were always in excess of $5T_1$. The decoupling power was approximately 10–15 W, and the duty cycle of the gated decoupling was ca. 0.2%. Temperature was regulated by a thermostated nitrogen gas flow system.

Partial Specific Volume. The apparent specific volume of the biotin lipid DABPE was determined by isopycnic flotation (cf. Nagle & Wilkinson, 1978). The DABPE lipid was dispersed above the phase transition in CsCl solutions of different concentrations, transferred to 1-mm diameter glass capillaries, and centrifuged at 20 °C in a Heraeus Biofuge A with hematocrit rotor. The density of the CsCl solution at which the lipid neither sank nor floated was taken to be the reciprocal of the apparent specific volume. A correction for possible cation binding was not made, but experiments with the negatively charged phospholipid phosphatidylserine (Cevc et al., 1981) suggest that this is unlikely to be large.

Polarized Microscopy. Polarizing microscopy was performed with an Optiphot Type 104 microscope from Nikon, equipped with a THM 600 thermostated stage from Linkam Scientific Instruments.

RESULTS

Saturated symmetrical diacyl biotinylphosphatidylethanolamines of even chain lengths from C12:0 to C20:0, dispersed in neutral buffer both without additional salt and with 1 M NaCl, have been investigated by ^{31}P NMR spectroscopy, polarizing microscopy, and X-ray diffraction. At 1 M NaCl, the electrostatic interactions of these charged lipids will be screened almost totally (Cevc et al., 1980), but binding of salt is unlikely to be appreciable (Cevc et al., 1981).

^{31}P NMR Spectroscopy. The proton–dipolar decoupled ^{31}P NMR spectra of biotin-PEs with chain lengths of C12:0, C14:0, C18:0, and C20:0 dispersed at pH 7.4 in buffer containing 1 M NaCl are given in Figure 1. Typical spectra representative of the gel and fluid phases are shown for each lipid. For all four biotin-PEs the spectra recorded at the lower temperature consist of broad, axially symmetric powder patterns characteristic of phospholipids in a lamellar gel phase. For DLBPE, the spectrum recorded at –5 °C is extremely broad, possibly because the buffer is frozen at this temperature. The spectra recorded at the higher temperature for DLBPE and DMBPE consist of a single, sharp isotropic resonance. In addition, the DMBPE dispersions were also found to be optically isotropic at these temperatures. However, the lack of complete optical clarity for DLBPE at these temperatures and the possibility to sediment both samples by centrifugation indicates that the isotropic spectra do not arise from conventional micellar solutions but correspond to aggregated fluid phases of the lipids which possess high curvature. In contrast, the ^{31}P NMR spectra of DSBPE and DABPE recorded at the higher temperatures consist of sharp, axially anisotropic powder patterns characteristic of phospholipids in fluid lamellar phases.

The temperature dependence of the ^{31}P NMR spectra of DPBPE dispersed in buffer at pH 7.4 with 1 M NaCl is shown in Figure 2 and is considerably more complex than that for the other four lipids. At low temperature (24 °C), the spectrum is a broad, axially symmetric powder pattern indicative of a lamellar gel phase. On warming, the spectra of DPBPE transform at 40 °C to a sharp, axially anisotropic powder pattern, but with a much reduced chemical shift anisotropy relative to that of DSBPE and DABPE in the fluid lamellar phase (cf. Figure 1C,D). At these temperatures, the DPBPE dispersion was also found to be optically isotropic. On a further increase in temperature, the chemical shift anisotropy of the axial powder pattern of DPBPE increases, reaching a value comparable to that for DSBPE and DABPE in the fluid phase, at a temperature of 70 °C.

The temperature dependence of the effective chemical shift anisotropy for the five *N*-biotinylphosphatidylethanolamines in 1 M NaCl at pH 7.4 is given in Figure 3. It is seen from Figure 3A that DLBPE and DMBPE undergo a sharp thermotropic (chain-melting) transition from a lamellar gel

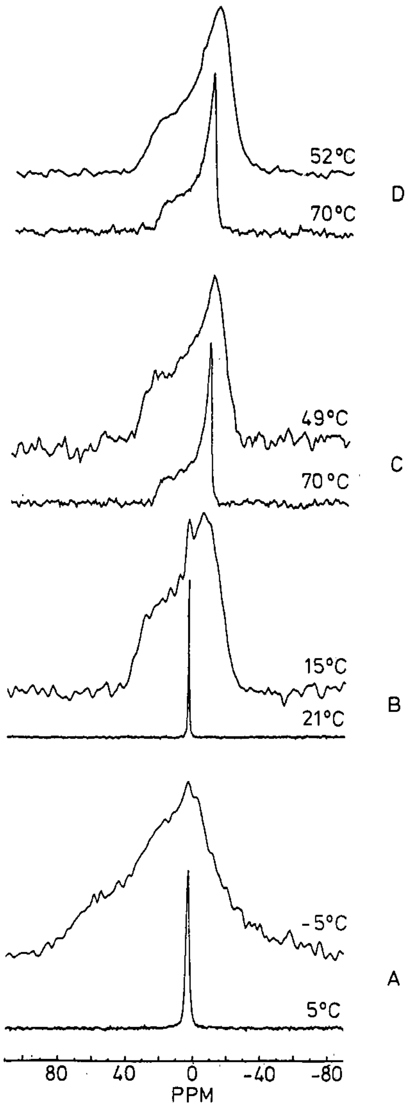

FIGURE 1: Proton–dipolar decoupled 121.5-MHz ^{31}P NMR spectra of *N*-biotinylphosphatidylethanolamines dispersed in 1 M NaCl, 10 mM Hepes, and 1 mM EDTA, pH 7.4, recorded at the temperatures indicated, in the gel and fluid phases (upper and lower spectra in each pair, respectively). (A) Dilauroylbiotin-PE (C12:0), (B) dimyristoylbiotin-PE (C14:0), (C) distearoylbiotin-PE (C18:0), and (D) diarachinoylbiotin-PE (C20:0). Chemical shifts (ppm) are referenced to external 85% phosphoric acid.

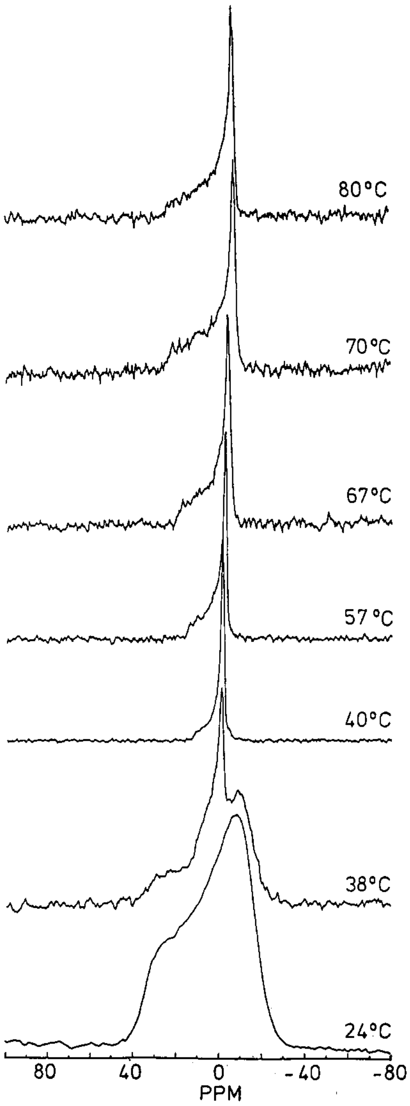

FIGURE 2: Proton–dipolar decoupled 121.5-MHz ^{31}P NMR spectra of dipalmitoylbiotin-PE (C16:0) dispersed in 1 M NaCl, 10 mM Hepes, and 1 mM EDTA, pH 7.4, recorded at the temperatures indicated in both the gel and fluid phases. Chemical shifts (ppm) are referenced to external 85% phosphoric acid.

phase (with effective chemical shift anisotropy of $\Delta\sigma \approx -56$ ppm for DMBPE) to an isotropic fluid phase with $\Delta\sigma \approx 0$ ppm at temperatures of 3 and 19 °C, respectively. DSBPE and DABPE (see Figure 3, panels B and C, respectively), on the other hand, undergo sharp chain-melting transitions from a lamellar gel phase with effective chemical shift anisotropy of $\Delta\sigma \approx -55$ to -51 ppm, to a lamellar fluid phase with $\Delta\sigma \approx -34$ to -33 ppm, at 52 and 58 °C, respectively. DPBPE (see Figure 3A) undergoes a sharp chain-melting transition at 39 °C from a lamellar gel phase with effective chemical shift anisotropy of $\Delta\sigma \approx -53$ ppm to a fluid phase where the lipid has a reduced (absolute) chemical shift anisotropy of $\Delta\sigma \approx -14$ ppm. When the temperature is increased above 60 °C, the chemical shift anisotropy then increases to a limiting value of $\Delta\sigma \approx -32$ ppm at 70 °C, corresponding to a fluid lamellar phase. The transition temperatures found by ^{31}P NMR are comparable to those found by differential scanning calorimetry for samples in 1 M NaCl (Swamy et al., unpublished results), except for the higher temperatures (DSBPE and DABPE) where the NMR determinations are subject to gradients in temperature from the point of measurement, which result in the transitions occurring at apparently somewhat lower temperature.

The ^{31}P NMR spectra of DPBPE, DSBPE, and DABPE dispersed in pH 7.4 buffer in the absence of salt are given in Figure 4. At low temperatures, all three lipids yield broad axially anisotropic powder patterns, characteristic of lamellar gel phases. At higher temperatures, in the fluid phase, all three lipids give sharp isotropic resonances. The optical clarity of all the samples at these temperatures indicates that the isotropic spectra in the absence of salt arise from conventional micellar solutions of the lipids in the fluid phase. For DLBPE (and DMBPE) a sharp isotropic ^{31}P NMR resonance arising from a clear micellar solution was observed at all temperatures above 0 °C. Incubation of DMBPE at low temperature with repeated freeze-thawing cycles gave rise to an aggregated phase which then underwent a chain-melting phase transition to a fluid micellar phase at ca. 7 °C. Clearly, the micellar phase observed at lower temperatures without incubation is metastable for these two lipids.

As expected, the ^{31}P NMR spectra of DMBPE at 25 °C in the fluid phase did not change appreciably with varying salt concentration, since the spectra at both extremes—in the absence of salt and in 1 M NaCl—consist essentially of a

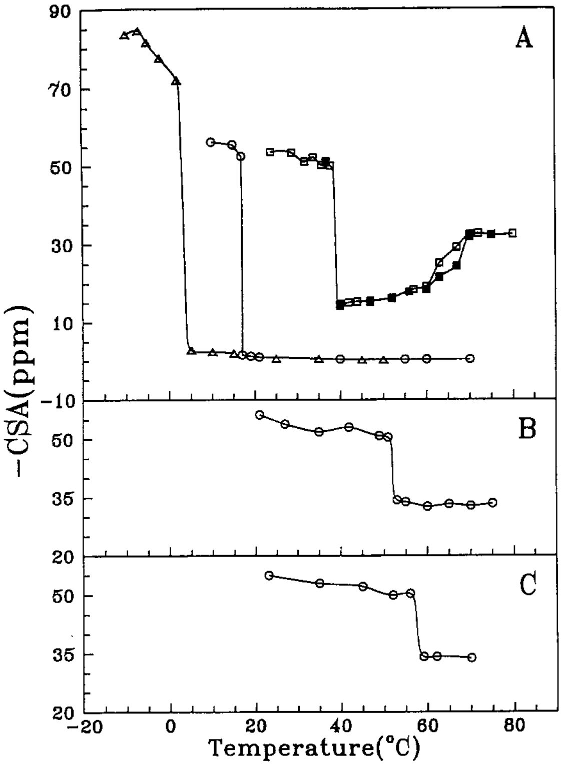

FIGURE 3: Temperature dependence of the apparent chemical shift anisotropy (CSA) in the ^{31}P NMR spectra of *N*-biotinylphosphatidylethanolamines dispersed in 1 M NaCl, 10 mM Hepes, and 1 mM EDTA, pH 7.4. (A) DLBPE (△), DMBPE (○), and DPBPE (□, heating; ■, cooling); (B) DSBPE; and (C) DABPE.

single sharp isotropic peak. However, the optical density (at 330 nm) of the DMBPE dispersions did change with increasing salt concentration at 25 °C. For salt concentrations up to 0.6 M NaCl, the (micellar) suspension remained clear. Between 0.6 and 0.8 M NaCl there was a sharp increase in the turbidity of the suspension, indicating the formation of larger aggregates of the lipid. Thus the fluid micellar phase of the DMBPE dispersion in the absence of salt converts to the aggregated isotropic fluid phase that is characteristic of dispersions of higher ionic strength at salt concentrations in the region of 0.6–0.8 M NaCl.

The temperature dependence of the effective chemical shift anisotropy for the three *N*-biotinylphosphatidylethanolamines of longer chain length in the absence of salt at pH 7.4 is given in Figure 5. It is seen from this figure that the lipids undergo a chain-melting transition from a lamellar gel phase with effective chemical shift anisotropy of $\Delta\sigma \approx -53$ ppm to a fluid micellar phase with $\Delta\sigma \approx 0$ ppm at temperatures of 30, 40.5, and 65 °C for DPBPE, DSBPE, and DABPE, respectively. The transition temperatures found by ^{31}P NMR are comparable to those found independently by differential scanning calorimetry for samples in the absence of salt (Swamy et al., unpublished results).

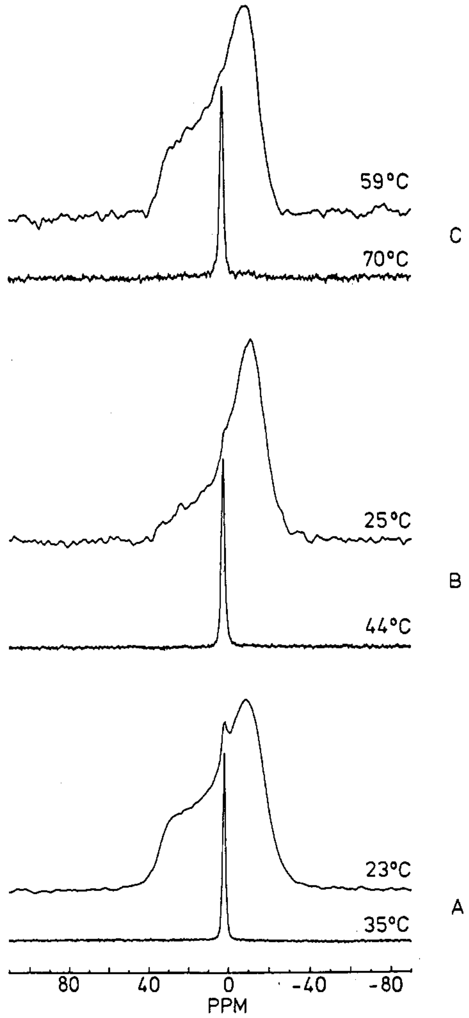

FIGURE 4: Proton–dipolar decoupled 121.5-MHz ^{31}P NMR spectra of *N*-biotinylphosphatidylethanolamines dispersed in 10 mM Hepes and 1 mM EDTA, pH 7.4 (without salt), recorded at the temperatures indicated, in the gel and fluid phases (upper and lower spectra in each pair, respectively). (A) Dipalmitoylbiotin-PE (C16:0), (B) distearoylbiotin-PE (C18:0), (C) diarachinoylbiotin-PE (C20:0). Chemical shifts (ppm) are referenced to external 85% phosphoric acid.

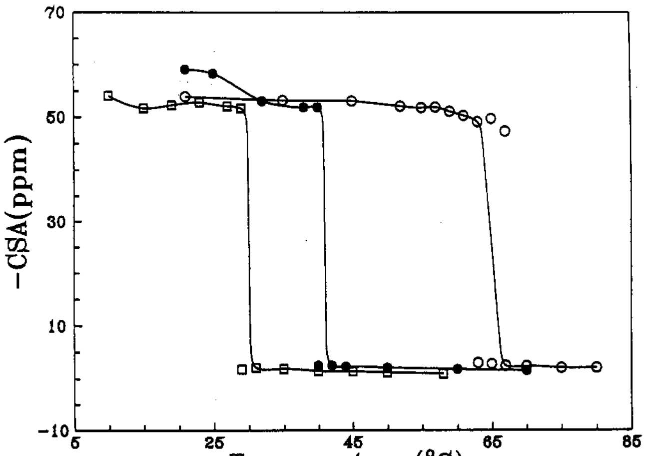

FIGURE 5: Temperature dependence of the apparent chemical shift anisotropy (CSA) in the ^{31}P NMR spectra of *N*-biotinylphosphatidylethanolamines dispersed in 10 mM Hepes and 1 mM EDTA, pH 7.4 (without salt). DPBPE (□), DSBPE (●), and DABPE (○).

X-ray Diffraction. The small-angle X-ray diffraction patterns of *N*-biotinylphosphatidylethanolamines with chain lengths of C14:0, C18:0, and C20:0 dispersed at pH 7.4 in buffer containing 1 M NaCl are given in Figure 6. For these chain lengths, the ^{31}P NMR spectra evidence a simple transition from a gel to a fluid phase (cf. Figure 3), and typical small-angle diffraction patterns in the two phases are given for each lipid in Figure 6. For all three biotin-PEs the diffraction patterns in the gel phase are characteristic of a multilamellar arrangement of the lipid dispersions. For DMBPE, only a broad continuous scatter is observed in the fluid phase, whereas for DSBPE and DABPE in the fluid phase a multilamellar diffraction pattern is observed for which notably the repeat spacings are larger than those in the corresponding gel phases. The temperature dependence of

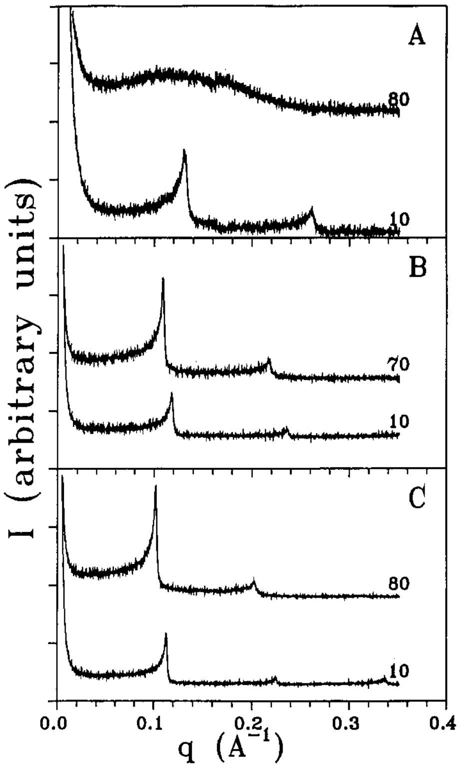

FIGURE 6: Small-angle X-ray diffraction patterns of *N*-biotinylphosphatidylethanolamines dispersed in excess 1 M NaCl, 10 mM Hepes, and 1 mM EDTA, pH 7.4, recorded at temperatures in the fluid and gel phases (upper and lower patterns in each pair, respectively). (A) Dimyristoylbiotin-PE (C14:0), (B) distearoylbiotin-PE (C18:0), and (C) diarachinoylbiotin-PE (C20:0). The temperature (°C) at which the diffraction pattern was recorded is indicated on the right in each case. The magnitude of the scattering vector, q (Å^{-1}), is $2\pi \sin 2\theta/\lambda$, where 2θ is the scattering angle. The discontinuity in diffraction intensity at $q \approx 0.16$ Å^{-1} in panel A corresponds to a mismatch on repositioning the detector to record the second half of the data at higher q values.

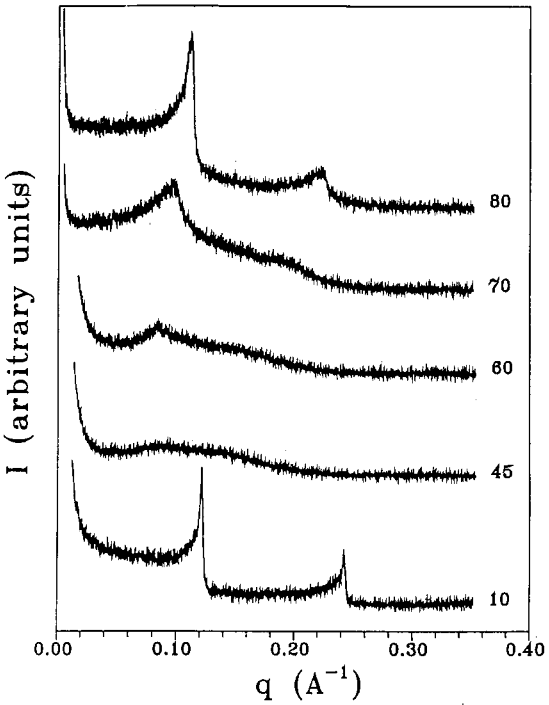

FIGURE 7: Small-angle X-ray diffraction patterns of dipalmitoylbiotin-PE (C16:0) dispersed in excess 1 M NaCl, 10 mM Hepes, and 1 mM EDTA, pH 7.4, recorded at different temperatures in both the gel and fluid phases. The temperature (°C) at which the diffraction pattern was recorded is indicated on the right in each case. The magnitude of the scattering vector, q (Å^{-1}), is $2\pi \sin 2\theta/\lambda$, where 2θ is the scattering angle.

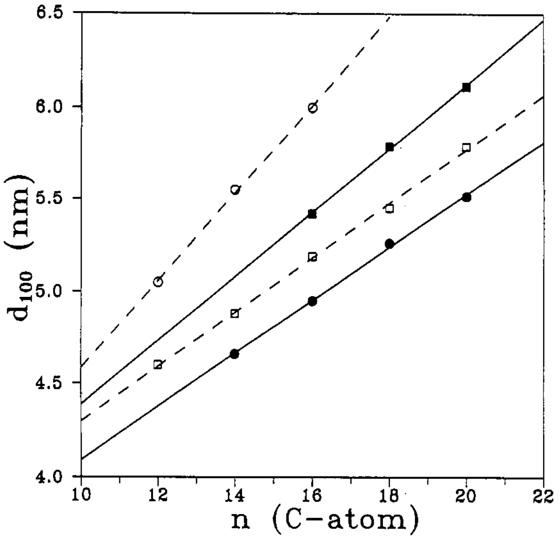

FIGURE 8: Chain length dependence of the X-ray long spacings, d_{100}, of *N*-biotinylphosphatidylethanolamines (dispersed in excess 1 M NaCl, 10 mM Hepes, 1 mM EDTA, pH 7.4) in the gel (L_{β}^{i}, ●) and fluid (L_α, ■) lamellar phases. *n* is the number of C atoms in either of the (symmetrical) acyl chains. Data for symmetrical diacylphosphatidylethanolamines (dispersed in excess water) in the gel (L_β, ○) and fluid (L_α, □) lamellar phases are taken from Seddon et al. (1984). The lines shown are linear regressions with the parameters given in Table I.

the diffraction patterns for DPBPE dispersed in buffer with 1 M NaCl at pH 7.4, which was found to give a more complicated behavior of the ^{31}P NMR spectra in the fluid phase (cf. Figures 2 and 3A), is shown in Figure 7. In the gel phase, the DPBPE gives a multilamellar diffraction pattern which is first replaced by a continuous scatter on heating to the fluid phase. This then transforms to a multilamellar diffraction pattern on further heating, where again the repeat distance is larger than that in the gel phase. This behavior of the temperature dependence of the X-ray diffraction patterns strongly resembles that displayed by the ^{31}P NMR spectra of the same lipid dispersed in excess 1 M NaCl, 10 mM Hepes, and 1 mM EDTA, pH 7.4, which was described above.

The dependence on chain length of the X-ray long spacings for those of the biotinylated phosphatidylethanolamines that give rise to lamellar phases in excess buffer with 1 M NaCl are given in Figure 8. The measurements in the lamellar gel phase were made at various temperatures, and the long spacings were not found to be appreciably temperature dependent. The long spacings in the lamellar fluid phase were found to be temperature dependent in some cases, and the values given in Figure 8 were all obtained at 80 °C. (For *n* = 20, the value at 70 °C is rather similar). The chain length dependence of the long spacings obtained from previous measurements on the lamellar phases of diacyl phosphatidylethanolamines (Seddon et al., 1984) are also included in Figure 8 for comparison. These data were obtained at temperatures 5 °C below and 3 °C above the gel-to-fluid phase transition for the various lipids. In each case, the data show an approximately linear dependence of the long spacing on chain length, and the linear regression parameters are given in Table I.

The data shown in Figure 8 display a clear difference between the biotinylated phosphatidylethanolamines and normal phosphatidylethanolamines. Whereas the long spacings in the gel phases for the underivatized PEs are consistently higher than those in the fluid phase, as is normally the case for hydrated phospholipid bilayers [see, e.g., Marsh (1990)], for the biotin-PEs the opposite is the case. This abnormal

Table I: Parameters from Linear Regressions to the Chain Length Dependences of the Long Spacings, d_{100}, in the X-ray Diffraction Patterns of the Lamellar Phases of Saturated Diacyl-*N*-biotinylphosphatidylethanolamines in Excess 1 M NaCl/10 mM Hepes and 1 mM EDTA, pH 7.4, and of Saturated Diacylphosphatidylethanolamines in Excess Water

lipid	phase	gradient (nm/CH$_2$)	intercept (nm)
biotin-PE	L_β^i	0.144 ± 0.004	2.65 ± 0.07
	L_α	0.175 ± 0.007	2.65 ± 0.12
PE[a]	L_β	0.237 ± 0.007	2.21 ± 0.10
	L_α	0.148 ± 0.003	2.82 ± 0.05

[a] Data from Seddon et al. (1984).

Table II: Long Spacings, d_{100}, Lipid Layer Thicknesses, d_l, and Areas per Lipid Molecule, *S*, Deduced from the X-ray Diffraction Patterns of the Lamellar Phases of Distearoyl-*N*-biotinylphosphatidylethanolamine with Limited Amounts of Water, as a Function of Lipid Concentration, *c* (w/w)

$(1-c)/c$	T (°C)	d_{100} (nm)	d_l (nm)	S (nm^2)
0.261	10	5.0	3.8	0.72
	60	5.1	3.5	0.85
0.414	10	5.4	3.6	0.76
	60	5.2	3.6	0.82
0.455	10	5.3	3.4	0.80
	60	5.2	3.4	0.86

behavior strongly suggests that the chains of the biotin-PEs are interdigitated in the gel phase, giving rise to atypically short repeat spacings in comparison with those in the fluid phase. Further evidence for chain interdigitation of the biotin-PEs comes from the chain length dependence of the long spacings in the two gel phases which are given in Table I. The gradient with chain length in the untilted L_β phase of normal PEs is $d(d_{100})/dn = 0.24$ nm/CH$_2$, which is quite close to the value of 0.25 nm/CH$_2$ expected for the thickness of a normal bilayer with *all-trans* chains, i.e., neglecting changes in the limiting hydration with chain length. In contrast, the gradient with chain length in the gel phase of the biotin-PEs is $d(d_{100})/dn = 0.14$ nm/CH$_2$, which is closer to half the value for the gradient in thickness of a noninterdigitated bilayer with *all-trans* chains.

The X-ray diffraction patterns of the various biotinyl-PEs (DPBPE, DSBPE, and DABPE) dispersed in excess buffer in the absence of salt display broad lamellar diffraction peaks, in the gel phase, whose positions correspond to rather large repeat spacings (data not shown), as would be expected for charged phospholipids at low ionic strength. For DPBPE the repeat spacings are in the range of $d_{100} \approx 15$–22 nm, for DSBPE $d_{100} \approx 20$–26 nm, and for DABPE $d_{100} \approx 19$–26 nm, where the values decrease systematically with increasing temperature in the gel phase. In the fluid phase, a broad continuous diffraction was observed for DPBPE, for DSBPE, and for DABPE, in the absence of salt. These diffraction patterns were somewhat similar to those found for the fluid phase of DMBPE in the presence of salt (cf. Figure 6A), with broad maxima at approximately 5.6, 6.2, and 7.0 nm for DPBPE, DSBPE, and DABPE, respectively, and correspond to the micellar phase. These identifications of the phases of the biotinyl-PEs in the absence of salt are fully consistent with the ^{31}P NMR studies described above.

Samples of DSBPE hydrated with limited amounts of water (in the absence of salt) gave rise to sharp lamellar diffraction peaks in both the gel and fluid phases. The repeat spacings obtained as a function of water content are given in Table II. Surprisingly, the repeat spacing with limited water content was found not to be very different at 10 and 60 °C, i.e., in the gel and fluid phases (cf. Table II). However, a structural change corresponding to the gel-to-fluid phase transition was observed in the intensities of these diffraction peaks which were found to increase abruptly at a temperature of 52 °C, on heating. This transition temperature is higher than that found in excess buffer in the absence of salt (40–42 °C), because of the limited water content for these samples. The effective thickness of the lipid layer, d_l, for these samples was calculated from the following expression which is based on lumped densities (Luzzati, 1968):

$$d_l = d_{100}/[1 + (1/\bar{v}_l)(1-c)/c] \qquad (1)$$

where c is the lipid/water weight ratio and $\bar{v}_l$ is the lipid partial specific volume. The latter was estimated from isopycnic flotation experiments in CsCl solutions of different densities, which gave a value of $\bar{v}_l = 0.842(5)$ mL/g for DABPE at 20 °C (strictly speaking, this is the apparent specific volume). Correcting this for the volume of the extra CH$_2$ groups in DABPE [see Marsh (1990)] yielded a value of $\bar{v}_l = 0.825$ mL/g for DSBPE at 20 °C. Further correction for the thermal expansion, based on measurements for distearoylphosphatidylcholine [see Marsh (1990)], yielded an estimate of $\bar{v}_l = 0.889$ mL/g for DSBPE at 60 °C. The values of the lipid layer thickness obtained in this way were used further to estimate the area per molecule, *S*, at the surface of the lipid layer (Luzzati, 1968):

$$S = 2M_l\bar{v}_l/(N_A d_l) \qquad (2)$$

where N_A is Avogadro's number and M_l is the molecular weight of the lipid. These values are also presented in Table II. The possible systematic errors arising from the neglect of any cation binding in the determination of the partial specific volume are estimated to be maximally ca. 5% in d_l and ca. 15% in *S*. Experiments with other negatively charged phospholipids (Cevc et al., 1981; Cevc & Marsh, 1983) suggest that, at 1 M monovalent salt, the true corrections will be much smaller than these maximal values.

The values of the lipid layer thickness and area per lipid molecule that are given in Table II are consistent with an interdigitated gel phase for DSBPE in the absence of salt, as found already from the repeat spacings for the biotinyl-PEs in the presence of 1 M NaCl. The values found for d_l (ca. 3.4 nm for the higher degree of hydration) are close to half the thickness of the lipid layer in the crystalline lamellar phase of distearoylphosphatidylethanolamine [6.1 nm, obtained by interpolation; see Seddon et al. (1984)], in spite of the smaller headgroup of the latter lipid. The values of the surface area per lipid molecule (ca. 0.8 nm^2 at the higher hydration) are also close to twice the value expected for a diacylphosphatidylethanolamine with fully extended chains [ca. 0.39 nm^2, see Marsh (1990)], which also suggests full interdigitation of the lipid chains of DSBPE in the gel phase in the absence of salt. The values of the lipid layer thickness for the fluid phase of DSBPE with limited amounts of water that are given in Table II are rather small, and the areas per lipid molecule are also rather high, the latter being somewhat larger than the upper limits (ca. 0.7 nm^2) observed for normal fluid L_α phases at high temperature (cf. Marsh, 1990). This most probably indicates that the lipid chains are rather more disordered than in normal fluid lamellar phases, which is consistent with the formation of micellar fluid phases for the biotin-PEs in excess buffer in the absence of salt.

DISCUSSION

The results presented above indicate that the structures of biotin-PEs in aqueous dispersion differ from those of the more common phospholipids, in a manner that depends not only on the lipid chain length but also on the ionic strength of the dispersion. Values were chosen for the latter such that the influence of the surface electrostatics of these charged lipids either would be almost maximal (in the absence of salt) or would be screened almost fully (in 1 M NaCl) but without appreciable ion binding (cf. Cevc & Marsh, 1987). The turbidity measurements with DMBPE indicate that the transition from the low to the high ionic strength regime takes place in the region of approximately 0.6–0.8 M NaCl. The structures of the gel and fluid phases (at both low and high ionic strength) are discussed in detail separately below.

Gel Phase. The ^{31}P NMR spectra indicate that dispersions of the saturated diacyl-*N*-biotinylphosphatidylethanolamines form lamellar gel phases at low temperatures, both in the presence and in the absence of salt, for all chain lengths studied (see Figures 1, 2, and 4). This conclusion is confirmed by the small-angle X-ray diffraction patterns recorded in the gel phase, at least for samples in the presence of 1 M NaCl or at lower water content (see Figures 6 and 7). Only in the case of the C(12:0) and C(14:0) lipids in the absence of salt is metastability (of a micellar phase) observed in the gel phase.

The repeat spacings in the small-angle X-ray diffraction patterns from biotin-PEs of different chain lengths in the presence of salt (see Figure 8 and Table I) strongly suggest that the lamellar gel phase detected by ^{31}P NMR spectroscopy is not of the normal type but corresponds to structures ($L_{\beta}{}^{i}$) in which the lipid chains are fully interdigitated across the bilayer. The evidence for this comes from the shorter repeat spacings in the gel phase than in the fluid phase and from the unusually low increment in long spacing per CH_2 group (cf. Results).

A greater hydration of the lipids in the gel phase than in the fluid phase would be required to explain the difference in long spacings between the two phases, if the chains were not interdigitated in the gel phase, but this is contrary to the situation with other phospholipids (cf. Marsh, 1990). In principle, the low value for the increment per CH_2 group might otherwise be explained either from a (very large) dependence of the limiting hydration on chain length or from an unusually high degree of chain rotational isomerism in the gel phase of the biotin-PEs, or from a tilting of the lipid chains in the gel phase. The first possibility is unlikely when compared with data for other phospholipids in the gel phase (Marsh, 1990). It is also extremely unlikely that the low value of the gradient of the long spacing in the gel phase arises from chain rotational isomerism, since this is even lower than that in the fluid phase, unlike the situation for normal PEs. In addition, electron spin resonance studies of spin-labeled lipid chains provide no evidence for a high degree of rotational isomerism in the biotin-PE gel phases. On the contrary, the conventional electron spin resonance spectra for different positions of chain labeling display essentially no motional averaging of the hyperfine anisotropy in the gel phase (M. J. Swamy, and D. Marsh, unpublished results). Also, it is less likely that the low value for the gradient with chainlength (and overall repeat spacing) could arise from tilting of the chains, since this would require an unusually large angle of tilt (ca. 56°). Instead, electron spin resonance results provide further evidence for chain interdigitation in that spin labels at the terminal methyl end of the chain are equally immobilized to those located closer to the glycerol backbone, unlike the situation in noninterdigitated gel phases for which an increased mobility is found close to the terminal methyl group (M. J. Swamy, and D., Marsh, unpublished results; cf. Boggs & Rangaraj, 1985; Bartucci et al., 1993). Therefore the gradient with chain length of the long spacings and difference in absolute values of the spacing between the gel and fluid phases must be interpreted in terms of an interdigitation of the chains of the biotin-PEs in the gel phase in 1 M NaCl. Such a structure is also consistent with the packing requirements for the rather large (and hydrophobic) biotin-PE headgroup.

In the absence of salt, the biotin-PEs take up large amounts of water, as a result of electrostatic repulsion between the lipid headgroups of adjacent layers. This is evidenced by the broad diffraction peaks and large X-ray repeat spacings found for samples dispersed in excess buffer. X-ray diffraction measurements on DSBPE in the presence of limited amounts of water suggest, however, that the gel phases of biotin-PEs with longer chain lengths in the absence of salt also possess the $L_{\beta}{}^{i}$ structure with interdigitated lipid chains, as was found for these lipids in 1 M NaCl. The evidence for this comes from the small thickness of the lipid layer and the large surface area per lipid molecule (see Table II). The difference between the thickness of the DSBPE lipid layer at $(1 - c)/c = 0.454$ and half the thickness predicted for an anhydrous distearoylphosphatidylethanolamine bilayer in the crystalline phase is 0.35 nm (see Results). This is in good agreement with the effective thickness of the biotin part of the headgroup (0.32 nm) for a surface area per molecule of 0.8 nm^2 (see Table II), which is predicted from the measured density of biotin (Traub, 1956). The extended length of the biotin molecule, based on the X-ray crystal structure, is 0.9–1.0 nm (Traub, 1956), but measurements of the lipid layer thickness based on lumped densities do not allow one to decide whether the biotin moiety is oriented parallel or perpendicular to the surface of the layer. However, the biotin groups from adjacent layers must be interdigitated at limited water contents, if the latter is the case.

Fluid Phase. The biotinyl-PEs of different chain lengths display a variety of different lyotropic structures in the fluid phase. At low ionic strength (in the absence of salt), this phase has been identified as being micellar (M_I) of the normal (oil-in-water) type for lipids of all chain lengths. This is evidenced by the optical clarity, isotropic ^{31}P NMR spectra, and lack of sharp X-ray diffractions from these samples. In 1 M NaCl, the lipids of longer chain length, C(18:0) and C(20:0), display normal fluid lamellar (L_{α}) phases. This is evidenced by the lamellar small-angle X-ray diffractions and the axial ^{31}P NMR powder patterns. The chemical shift anisotropy of the ^{31}P NMR spectra (ca. 33–34 ppm) is somewhat smaller than that normally observed (ca. 40–45 ppm) for underivatized phosphatidylethanolamines or phosphatidylcholines in a fluid lamellar phase. A comparable reduction in chemical shift anisotropy is also observed for cardiolipins [see, e.g., Powell and Marsh (1985)] and on titrating the amine group of PE (Seelig & Gally, 1976). This may point to a rather different conformation about the phosphate of the lipid headgroups in the biotinyl-PEs than in the normal, underivatized PEs.

Most interesting is the behavior of the biotin PEs with chain lengths of C(12:0), C(14:0), and C(16:0) in 1 M NaCl in the fluid phase. The lipids with the two shorter chain lengths display isotropic ^{31}P NMR spectra and give only continuous X-ray scatter at small angles. The fluid phase is not a simple micellar solution, but from the small-angle X-ray scattering results is probably not of the cubic type [or even a sponge phase, cf. Roux et al. (1992)] either, since no long-range order is detected. A possible structure that is consistent with the X-ray scattering patterns is an aggregated phase consisting of micelles with disordered packing. Such an isotropic phase has not been identified previously in aqueous dispersions of phospholipids and tentatively may be given the designation, I_M. The suggested structure is in agreement with preliminary freeze–fracture electron microscopy data for DMBPE in 1 M NaCl, which revealed no long-range order, simply aggregates of small (amorphous) particles. The size of these putative micelles is probably larger than that of normal micelles, since, for example, the broad scattering peak from DMBPE in 1 M NaCl occurs at an effective spacing of ca. 5.7 nm, which is comparable to that from the normal micelles of the longer chain DPBPE (5.6 nm) in the absence of salt. A possible reason for the aggregation of the micelles could be interactions between the biotin headgroups which is overcome by electrostatic repulsion in the absence of salt, resulting in a normal micellar solution in this latter case.

The more complex temperature dependence of C(16:0) DPBPE in 1 M NaCl in the fluid phase (Figures 2, 3, and 7) provides support for the suggestion of an I_M phase consisting of flattened, disc-like micelles and gives further insight into its relation to the lamellar L_α phase. At lower temperatures above the chain-melting phase transition, the small-angle X-ray scattering patterns are similar to those for the biotin-PEs of shorter chain lengths in the fluid phase, but with broad maxima corresponding to considerably larger dimensions, ca. 8 nm. This latter value is greater both than that obtained for the normal micelles formed by the same lipid in the absence of salt (5.6 nm) and than the repeat distance in the lamellar phase (5.5 nm) of DPBPE in salt at higher temperature, suggesting an extended (flattened) structure. In addition, the continous diffraction observed at 45 °C in Figure 7 exhibits a second shoulder, corresponding to a spacing of ca. 4.6 nm, that might be more related to an effective bilayer thickness. At higher temperatures the X-ray pattern and the ^{31}P NMR spectrum convert to those characteristic of the normal lamellar L_α phase, hence establishing the structural relationship between the two phases. At this transition, changes in the electron spin resonance spectra of lipids spin-labeled at different positions throughout the length of the molecule indicate that the lower temperature phase is one of normal curvature, i.e., I_{M_I}. The grounds for this assignment are that an increase in mobility is observed for phospholipids labeled in the headgroup region, whereas a decrease in mobility is observed for phospholipids labeled close to the terminal methyl end of the chains, on transition from the bilayer to the I_M phase (M. J. Swamy, and D. Marsh, unpublished results).

The ^{31}P NMR spectra of DPBPE in 1 M NaCl at lower temperatures in the fluid phase (Figure 2) are particularly interesting. The axial symmetry of the powder pattern and the sign of the chemical shift anisotropy are suggestive of a lamellar motif, but the absolute magnitude of the chemical shift anisotropy is reduced by half relative to that of the normal lamellar L_α phase. The small chemical shift anisotropy in this temperature regime could be explained if the phase at lower temperature (I_M) consisted basically of flattened micelles containing bilayer discs. The volume of the bilayer regions would have to be approximately 50% of that of the micellar edges with normal curvature to account for the reduction in size of the chemical shift anisotropy. The diameter of the disc-shaped micelle would then be ca. 1.8 times its thickness, which is comparable to the ratio (1.7) of the positions of the broad shoulders in the continuous diffraction recorded at 45 °C. For DLBPE and DMBPE in 1 M NaCl, the micelles would have to be considerably closer to a spherical shape than this, in order to account for their isotropic ^{31}P NMR spectra and more symmetrical shape of the X-ray scattering intensity profile.

Conclusions. Aqueous dispersions of biotin-PEs, both in the presence and in the absence of salt, display fluid and gel phases that differ in many cases from the normal bilayer structure. For chain lengths of C(14:0) to C(20:0) the gel phases are interdigitated in 1 M NaCl, as also are the gel phases of the biotin-PEs of longer chainlengths in the absence of salt. The fluid phases are normal micellar in the absence of salt for all chainlengths. In 1 M NaCl, the fluid phases are lamellar for chainlengths of C(18:0) and C(20:0), but possess an isotropic structure, most probably corresponding to normal micellar aggregates, for chain lengths of C(12:0) and C(14:0). For C(16:0) biotin-PE in 1 M NaCl, the structure of the fluid phase converts from that characteristic of the shorter chain length lipids at lower temperatures to that characteristic of the longer chain length lipids at the higher temperatures. The results demonstrate a very strong influence of the unique headgroup of the biotin–PEs on the structural polymorphism of the phases formed in aqueous dispersion, one of which is a phase (I_M) so far not reported for phospholipids.

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