BRIEF COMMUNICATION

CHEMICAL SHIFT ANISOTROPIES OBTAINED FROM ALIGNED EGG YOLK PHOSPHATIDYLCHOLINE BY SOLID-STATE ^{13}C NUCLEAR MAGNETIC RESONANCE

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ABSTRACT Natural abundance solid-state ^{13}C NMR spectra were obtained from orientated egg yolk phosphatidylcholine multilayers in which peaks from the different types of carbon in the lipid were resolved. The residual chemical shift anisotropy of the choline, glycerol, and olefinic carbons, as well as the carbonyl and acyl chain methylene carbons, were estimated. This information provided the basis for a qualitative description of the order and conformation of egg yolk phosphatidylcholine in the L_α phase. The results suggested the gauche conformation for the C_α—C_β bond in the choline moiety, a constrained glycerol region, a magic angle orientation for the *sn-2* carbonyl, and a preferred orientation close to the bilayer normal for the plane of the *sn-1* carbonyl bond and acyl chain C=C bond. The orientations of the carbon nuclei are in accord with the molecular conformation derived from previous ^2H, ^{31}P, and ^{13}C NMR studies.

INTRODUCTION

Solid-state NMR techniques have proven useful in elucidating the molecular conformation and order within phospholipid membranes.^2H NMR of selectively labeled aqueous dispersions of lipid provided many of the early insights in this area, whereas more recent studies have included the use of ^{31}P, ^{13}C, and ^{14}N nuclei (1–5). However, details of the configurations of the carbon atoms in lipid molecules are still limited due to the poor resolution of the spectra obtained with natural abundance ^{13}C NMR. Overlapping residual chemical shift anisotropies (CSA) of adjacent peaks have confined the information obtained from these spectra to the distinctive carbonyl resonances (6) and the large acyl chain methylene resonance (7).

Improvements in natural abundance ^{13}C NMR spectral resolution had been achieved either through the combination of cross-polarization (8) and magic angle spinning (9) techniques, or through the use of micellar or liposomal sample preparations. Unfortunately this resolution has been gained at the expense of the anisotropic information, because only the isotropic value of the shielding tensor is obtained in these analyses.

In the present study we have used cross-polarization solid state ^{13}C NMR to investigate aligned dispersions of egg yolk phosphatidylcholine. Aligned multilayers of L_α phase lipid yielded resolved spectra from which the residual ^{13}C anisotropies could be obtained. The rapid molecular reorientations in these systems reduce the shielding tensor to axial symmetry with a component $\sigma_\parallel$ projected onto the bilayer normal and a component $\sigma_\perp$ perpendicular to the bilayer. It was possible to resolve the resonances from most of the different types of carbon in the lipid and to estimate both the value and sign of the residual CSA. These data were used to qualitatively describe the conformation of the molecule in the aligned dispersions.

MATERIALS AND METHODS

A chloroform solution of 60 mg of egg yolk phosphatidyl-choline (EYPC), prepared by the method of Singleton et al. (10), was deposited onto ~50 glass cover slips cut to fit the cross-section of a 10-mm NMR tube. A vacuum pump applied for 14 h removed the solvent. A total of 35 μl of glass-distilled deionized water was added to the cover slips, which were then stacked and flame-sealed inside the NMR tube. Excess lipid accumulated on the outside of the stack was removed before sealing. Samples were equilibrated for 1–2 h at room temperature before commencing the NMR experiment.

The sample was placed in the magnet, and a home-made goniometer was used to select the orientation of the stacked cover slips with respect to the magnetic field. Alignment of the lipid was confirmed by observing the change in duration of the free induction decay of the ^1H NMR signal as a function of the goniometer angle θ. θ is the angle between the bilayer normal (perpendicular to the stacked cover slips) and the magnetic field. The goniometer angle was calibrated to within 2° by observing the

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characteristic free induction decay at the magic angle [$\theta = \cos^{-1} \cdot (1/\sqrt{3})$].

Solid-state proton-enhanced ^{13}C NMR spectra (8) were obtained at 20°C using a CXP-300 MHz spectrometer (Bruker Analytische Messtechnik, Karlsruhe, FRG) and a B-VT-1000 heating unit. Each spectrum was obtained with 3,000 transients using a 7-μs 90° pulse, 5 ms contact time, and 1 s recycle time. A total of 39 spectra were obtained between the angles of −90 and 120° in increments of 5.4°.

Spectral simulations were performed using computer-generated superpositions of Lorentzian line shapes. The degree of hydration in the samples was checked by measuring the relative integrated intensity of the water peak in the ^1H NMR spectrum obtained at the magic angle. Each preparation contained 31–34% by weight of water. During the experiment beads of water collected at the ends of the NMR tube. Thin layer chromatography using a chloroform:methanol:water (100:25:4) solvent system showed no lipid degradation after the NMR experiments. All chemical shifts were referenced to tetramethylsilane.

RESULTS

Fig. 1 shows an example of a solid-state ^{13}C NMR spectrum from a series of aligned EYPC spectra obtained between the goniometer angles −90 to 120°. Peak assignments were made by comparing the spectrum obtained at the magic angle with a previously reported ^{13}C NMR spectrum of an EYPC solution (11). Most of the different types of carbon were resolved. Carbonyls appeared between 200 and 165 ppm, methene carbons at ~130 ppm, glycerol and choline carbons between 50 and 70 ppm, and acyl chain C4–C15 methylenes and terminal methyl carbons were centered on 30 and 12 ppm, respectively. The resolution achieved in the spectra is shown in Fig. 2, which compares the crowded choline and glycerol regions with the spectral simulations obtained using Lorentzian line shapes.

In Fig. 3 a plot of the peak positions is shown as a function of the goniometer angle for every carbon resonance resolved. The reduced chemical shift anisotropy (CSA) ($\Delta\sigma$) is given by

$$\sigma_{observed} = \sigma_{isotropic} + (\Delta\sigma/3)(3\cos^2\theta - 1),$$

where σ is the chemical shift. The sign of the reduced CSA was chosen according to the conventions $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$ and $\sigma_{11} > \sigma_{33}$. Table I summarizes this information for each resolved carbon in EYPC.

The reduced ^{13}C CSA for the glycerol C1 and C3 carbons were found to be the same. The 0.5-ppm separation between the C1 and C3 peaks observed in the high-resolution solution spectrum was not resolved in the solid-state spectrum obtained at the magic angle, and the complete orientation dependence failed to produce resolved peaks with different anisotropies.

The reduced CSA of 1.5 ppm obtained for the *sn-2* carbonyl was smaller than previous measurements of −5 ppm for dimyristoylphosphatidylcholine (12) and −7 ppm for dipalmitoylphosphatidylcholine (6). However the latter studies estimated the CSA from lineshape analyses of powder spectra. The *sn-1* carbonyl reduced CSA of −27 ppm is comparable with previous estimates (12).

Spectral linewidths were in the range 2 to 4 ppm, with

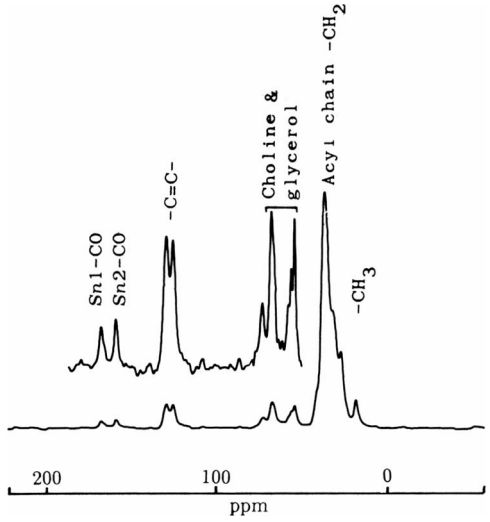

FIGURE 1. Natural abundance solid-state ^{13}C NMR spectrum of aligned EYPC obtained at the goniometer angle of 0°. The spectrum was acquired at 20°C with 3,000 transients using a 7-μs 90° pulse, 5-ms contact time, and 1-s recycle time. The inset shows a ×5 vertical increase in amplitude.

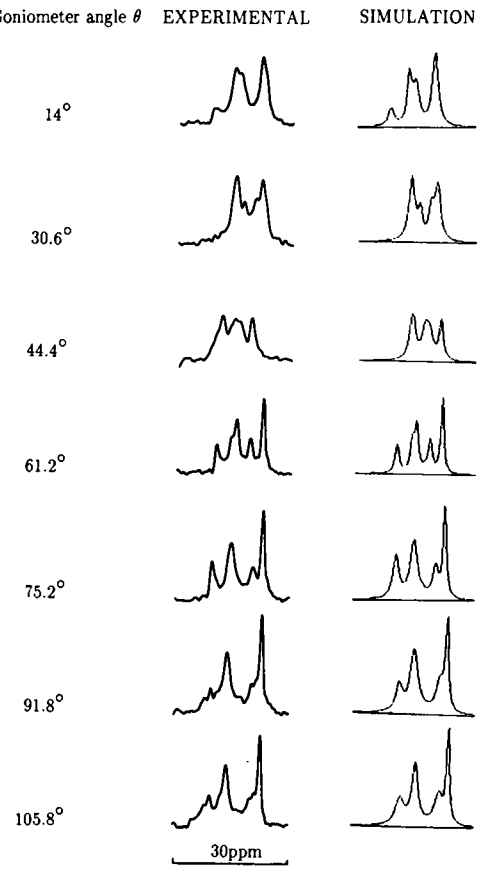

FIGURE 2 A comparison of the ^{13}C NMR spectra with spectral simulations using computer generated superpositions of Lorentzian line shapes. The region containing the peaks corresponding to the choline and glycerol carbons in aligned EYPC are shown.

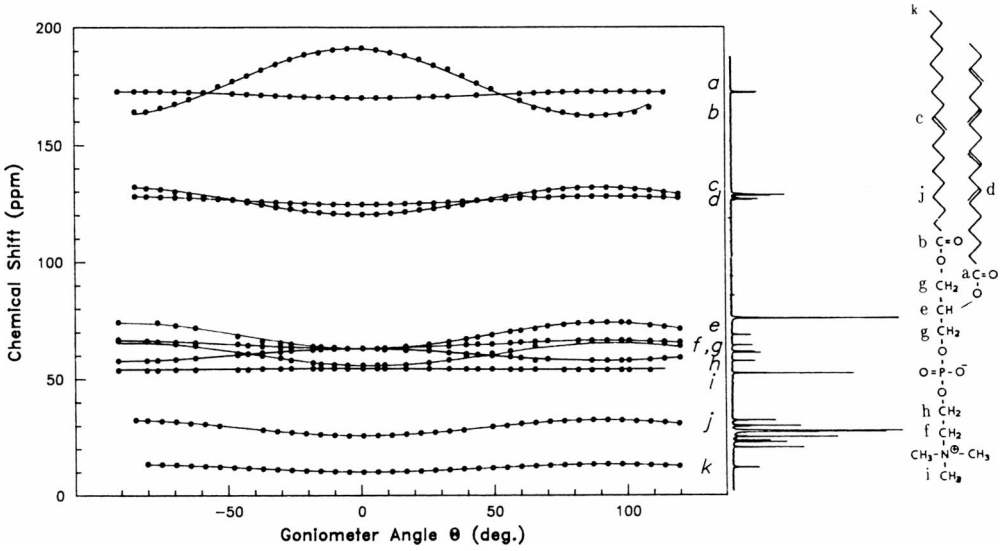

FIGURE 3 The chemical shift dependence of aligned EYPC ^{13}C resonances on the goniometer angle, θ. The isotropic positions are indicated by the ^{13}C NMR spectrum of a chloroform:methanol (1:1) solution of EYPC. The reduced chemical shift anisotropy ($\Delta\sigma$) for each carbon was estimated from $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$ The solid lines represent the theoretical fit of the data to $f(\theta) = A + B \cos 2\theta + C \sin 2\theta$. The error for these data points is ±1 ppm.

the exception of the C4–C15 methylene peak. These methylene resonances were crowded into a region of ~12 ppm, appearing as a single peak with linewidth of 7 ppm. At some orientations individual acyl chain methylenes were resolved, however, this occurred over an insufficient range of angles to determine their reduced CSA. The complexity of the methene region in the spectra in Fig. 1 reflects the heterogeneity of the acyl chains in EYPC. The reduced chemical shift anisotropies were resolved for the monounsaturated and conjugated methenes.

The stability of the aligned lipid dispersions was indicated by the lack of change in the spectra with time. Reproducible peak positions were obtained to a precision of ±1 ppm when the experiment was repeated, and after the sample had been stored for 2–3 yr at −20°C.

TABLE I
REDUCED CHEMICAL SHIFT ANISOTROPIES ($\Delta\sigma$) FOR EYPC CARBONS OBTAINED FROM $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$ IN FIG. 3

	Type of carbon	Chemical shift anisotropy
		ppm
Choline	C_γ(i)*	1.6
	C_β(f)	4.2
	C_α(h)	−8.6
Interfacial region	C3 (g)	11.0
	C2 (e)	11.3
	C1 (g)	11.0
	*Sn*2—CO (a)	1.5
	*Sn*1—CO (b)	−27.0
Hydrocarbon chains	Acyl chain—CH_2 (j)	10.0
	—C═C— (c)	11.0
	—C═C—C═C— (d)	3.0
	—CH_3 (k)	3.0

*Error for these values are ±1 ppm. Letters correspond to the carbon atom labeling on the EYPC molecule shown in Fig. 3.

DISCUSSION

In a single experiment the reduced chemical shift anisotropy for most of the resolved carbons in EYPC bilayers has been obtained. ^{13}C NMR yields both the sign and magnitude of the CSA. By contrast only the magnitude of the quadrupole interaction is available by ^2H NMR. These data permit an estimate of the time-averaged orientation of major segments of the phospholipid molecule. The tensors are all very small which explains why ^{13}C magic angle spinning works so well on these systems (13).

Head Group Region

Natural abundance solid-state ^{13}C NMR spectra yielded choline and glycerol resonances that are accessible to conformational analysis. Reduced chemical shift anisotropies have been estimated for the choline C_α, C_β, and C_γ carbons and for the glycerol C1, C2, and C3 carbons (Table I). With one exception, all methylene carbons had the same sign for their reduced CSA, independent of whether they were main chain, glycerol, or choline methylenes. The exception was the choline C_α (carbon *f* in Fig. 3). For the limited number of nonaromatic and noncyclic model compounds for which the shielding tensor is known, the σ_{33} axis is orientated perpendicular to the H–C_α–H plane, with the directions of the σ_{11} and σ_{22} axes varying according to the local chemical environment (14). Using this orientation for the EYPC methylene shielding tensor axes, these results indicate that on average the σ_{33} axis of C_α lies in a different plane with respect to the other methylene groups in the molecule and has a preferred orientation parallel rather than perpendicular to the bilayer surface. The σ_{11} and σ_{22} axes in the H–C_α–H plane are orthogonal to the direction of the σ_{33} axis. The gauche conformation proposed for the choline C_α–C_β bond from Raman spectroscopy (15), ^2H NMR (16) and x-ray diffraction studies (17), are consistent with the present results.

The decrease in degrees of freedom, from the choline moiety to the glycerol backbone, suggested by ^2H NMR studies for L_α phase lipid (16) was indicated in the ^{13}C NMR spectra by the increase in magnitude of the reduced methylene CSAs from C_β to C3. However despite the rapid motion of the head group the results show a preferred orientation for the C_α and C_β carbons. The C_β carbon favours an orientation in which the H–C_β–H plane lies close to the bilayer surface, whereas the H–C_α–H plane of the C_α carbon favours an orientation close to perpendicular to the bilayer plane. This is consistent with previous ^2H NMR results (18).

Interfacial Region

Assuming the above methylene shielding tensor orientation, a common orientation for C1 and C3 is suggested by their unresolved reduced CSA. To interpret the axially symmetric lineshapes observed in ^{31}P NMR spectra from powder dispersions of phosphatidylcholine lipid, it has been suggested that rotation occurs about the C3–C2 bond (19). A difference in the rotation rate between the two glycerol C–C bonds might be expected to result in a difference in the CSA of C1 and C3. This difference is not observed. Our results support the alternative suggestion that this region of the molecule is essentially rigid, constraining the glycerol conformation to one which produces motionally inequivalent quadrupole splittings of the deuterons on C1 and C3 (20, 21) while keeping the same C1 and C3 methylene carbon orientations.

The *sn-1* and *sn-2* carbonyl shielding tensor orientations were modeled on the dimethyl oxalate carbonyl tensor (22). The small reduced CSA of the *sn-2* carbonyl is consistent with the tensor lying near the magic angle relative to the symmetry axis for motion (6). The larger negative CSA of the *sn-1* carbonyl indicates that the carbonyl plane adopts a preferred orientation near to the bilayer normal, with the C=O bond close to the plane of the bilayer surface. These carbonyl orientations are consistent with those seen for the carbonyls in the x-ray crystal structure (17).

Olefinic Region

The small reduced CSA of the olefinic carbons may arise from two mechanisms. The first is from rotations at the magic angle that, independent of the shielding tensor, will cause a collapse of the CSA. The second is dependent on the shielding tensor and in the present case involves rotation about an axis close to the direction of the σ_{22} axis. Based on a comparison with the ^2H NMR results (23), we conclude that the double bond is orientated approximately perpendicular to the bilayer surface. In the model compound dihydromuconic acid (24), σ_{22} points along the double bond. At $\theta = 90°$ the principal values of the σ_{11} and σ_{33} axes would be averaged by the long axis reorientational motion of the lipid molecule in the L_α phase to 14 ppm, resembling that of 11 ppm obtained from aligned bilayers. This is to be compared with 181 ppm in a static powder.

In summary, ^{13}C NMR spectra of aligned EYPC dispersions resolved most of the carbon sites in the lipid. We have obtained estimates of the reduced CSAs for the choline, glycerol, carbonyl, acyl chain methylene, and olefinic carbons which provided a qualitative description of the conformation of the molecule. The results concur with ^2H NMR data which suggest a preferred gauche conformation for the choline C_α–C_β bond. The backbone region appears rigid with a common orientation for the C1 and C3 glycerol carbons and the magic angle conformation for the *sn-2* carbonyl. The plane of the *sn-1* carbonyl and the acyl chain double bonds have a preferred orientation near to the bilayer normal.

This technique promises to be useful in exploring the conformation of many hitherto uncharacterized regions of model membranes that are difficult to synthesize with NMR visible probes.

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