NMR of a Phospholipid: Modules for Advanced Laboratory Courses

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NMR of a Phospholipid

Modules for Advanced Laboratory Courses

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Rationale

A recent survey of chemistry departments offering ACS-certified degrees reveals that 98.8% of the respondents include ^{1}H NMR spectroscopy instruction in organic chemistry lecture or laboratory, and 86.6% include ^{13}C NMR spectroscopy. However, the percentage of respondents who instruct their organic students in 2D spectroscopy drops to 13.7%, despite the technique's routine research use throughout chemistry (*1*) and the availability of suitable experiments (*2*–*5*).

The following exercise builds upon the NMR experience undergraduates receive in organic chemistry with a battery of NMR experiments designed to investigate egg phosphatidylcholine (egg PC). This material, often labeled in health food stores as lecithin, is a major constituent of mammalian cell membranes (*6*). The NMR experiments may be used to make resonance assignments, to study molecular organization in model membranes, to test the effects of instrumental parameters, and to investigate the physics of nuclear spin systems. A modular suite of NMR exercises is described, allowing the instructor to tailor the laboratory sessions to biochemistry, instrumental analysis, or physical chemistry. If desired, these experiments may be coupled with isolation of PC from egg yolks (*7*).

Introduction

The major component of egg PC is 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (see structures in Fig. 1) (*8*), a nutritional fuel and energy storage molecule (6). High-resolution NMR spectra of egg PC can be obtained by dissolving the PC in deuterated chloroform. Preliminary structural information on this sample is available from simple one-dimensional (1D) ^{1}H, ^{13}C, and ^{31}P spectra. Using multinuclear NMR illustrates the versatility of the technique but also the trade-offs among spectral simplicity, molecular information,

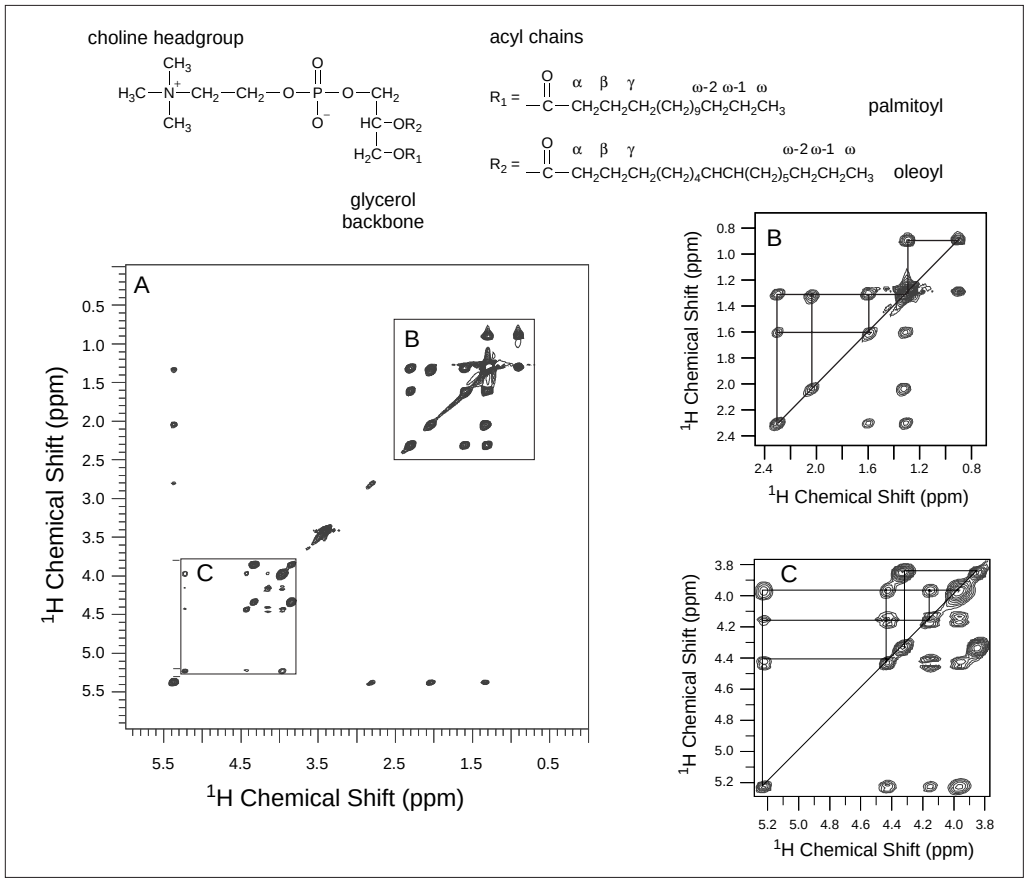

Figure 1. Top: Structure of POPC. Bottom: (A) TOCSY contour plot of egg PC in $CDCl_3$. In the directly detected dimension, 4 transients of 1K data points were acquired with a recycle delay of 2 s and a mixing time of 0.07 s. 128 t_1 increments and a total time of 40 min were required. The data were multiplied by Gaussian apodization functions (*12*) in both dimensions before double Fourier transformation. (B) Enlargement of acyl chain region. (C) Enlargement of headgroup region.

and signal sensitivity. Further information is gathered through *D*istortionless *E*nhancement by *P*olarization *T*ransfer (DEPT), a spectral editing experiment that distinguishes among C, CH, CH_2, and CH_3 groupings (*9*). Finally, two-dimensional spectroscopy (2D *TO*tal *C*orrelation *S*pectroscop*Y*, TOCSY) is used to reveal *J*-coupled spin networks and thus covalent connectivities within the phospholipid structure (*10*). TOCSY is preferable to the more familiar COSY experiment: it is less time consuming, and it provides off-diagonal (cross) peaks either between directly coupled nuclei or throughout a bonded network, depending on the choice of mixing time in the 2D pulse sequence. The theory and practice of 2D spectroscopy are described in a number of references and will not be discussed further here (*11, 12*).

Although the spectral assignment of egg PC is facilitated by dissolving it in chloroform, phospholipids are found naturally in biomembranes, where molecular motion and reorientational excursions are more limited. A membrane environment may be approximated by an aqueous dispersion of the egg PC. The resulting sample is then examined with solid-state NMR techniques. In the solid state, additional structural information is available through dipolar coupling and chemical shift anisotropy (CSA), two interactions that are averaged in solution studies. However, these interactions are dependent on the orientation of the nuclei with respect to the magnetic field, and since typically many nuclear orientations are present, broad lines are observed. The broadening due to dipolar interactions can be removed by high-power decoupling. Additionally, the isotropic (average) value of the chemical shift can be observed by removing the CSA through a technique known as magic-angle spinning (MAS) (*13, 14*). Since the CSA has an orientation dependence of $3\cos^2\theta - 1$ (where θ is the angle between the magnetic field and the principal axis about which the chemical shift is defined), spinning the sample at an angle of 54.7° with respect to the magnetic field reduces the CSA to zero, seeming to "magically" cause it to disappear. The combination of high-power decoupling and MAS allows for the observation of solution-like ^1H, ^{13}C, and ^{31}P spectra of egg PC in a membrane environment. Further information about the theory and application of solid-state NMR is available elsewhere (*12*).

Experimental Procedures

A 2-mL solution of 100 mg of egg PC per milliliter of $CDCl_3$ was prepared in a 10-mm o.d. NMR tube. Aqueous dispersions of egg PC were prepared by dissolving the powder in a minimum amount of chloroform, then removing the solvent under a stream of dry nitrogen and pumping under reduced pressure overnight (*15*). The resulting lipid film was hydrated with an equal mass of D_2O, vortex-mixed, and then subjected to several freeze–thaw cycles to obtain a uniform paste. The samples were loaded into either 5- or 7-mm o.d. rotors equipped with O-rings to avoid leakage.

All spectra were obtained using a Varian Unity*plus*-300 wide-bore spectrometer, equipped for both liquid-state and solid-state NMR. The resonance frequencies were 300.001 MHz for ^1H , 75.442 MHz for ^{13}C, and 121.442 MHz for ^{31}P. The solution experiments were carried out with a Varian 10-mm broadband probe. The solids experiments were conducted with either a Varian 7-mm MAS probe or a Doty 5-mm MAS probe, with the rotor spinning speed maintained to ±4 Hz in both cases by a Varian speed controller. The temperature was adjusted to 30.0 ± 0.1 °C. All NMR data were analyzed with VNMR software, version 6.1b.

Hazards

Spinning rotors can act as projectiles. Safety glasses must be worn during all aspects of this experiment. Chloroform is toxic and irritating. A fume hood should be used when preparing the sample. People with medical implants should stay beyond the fringe field of the superconducting magnet.

Results and Discussion

Spectral Assignments from Solution-State NMR

The ^1H NMR assignments for egg PC in $CDCl_3$ are summarized in Table 1. A first-pass interpretation of the ^1H NMR spectrum can be made with the help of standard chemical shift tables (*16*) and measured integrals. These assignments are then confirmed and refined using the TOCSY data (Fig. 1). Cross peaks are apparent among hydrogens along the acyl chain (1–3 ppm) and among hydrogens in the head group region (3.5–5.5 ppm), but some interpretations are left ambiguous owing to limited spectral dispersion for protons in the central portion of each acyl chain.

The ^{13}C assignments are displayed in Table 2. The DEPT subspectra are used to distinguish among CH_3, CH_2, CH, and quaternary carbons.

Another aid in spectral assignment is the clear splitting of some choline resonances, reflecting 2- or 3-bond C–P couplings and values of J_{CP} from 5.0 to 7.4 Hz. The final assignments given in Table 2 were made by comparison to published spectra (*15*).

Finally, the ^{31}P NMR spectrum shows one peak that is split into a pentet in the absence of ^1H decoupling, attributable to 3-bond coupling with J_{PH} = 6.8 Hz. As expected when considering the natural abundance and gyromagnetic ratio

Table 1. ^1H Resonance Assignments for Egg Phosphatidylcholine

δ/ppm[a]	Integral Value[b]	Proton Type
0.90	5.74	ω-CH_3
1.30	37.79	$(CH_2)_n$
1.60	5.76	β-CH_2
2.04	4.26	$CH_2CH{=}CHCH_2$
2.30	4.47	α-CH_2
2.79	1.59	=CH–CH_2–CH=[c]
3.41	9.00	$N(CH_3)_3$
3.84	1.86	CH_2N
3.97	2.60	PO_3CH_2 (glycerol)
4.14	1.02	CH_2O (glycerol)
4.32	1.64	CH_2O (choline)
4.42	0.91	CH_2O (glycerol)
5.22	1.03	CHO
5.37	2.67	HC=CH

[a]Referenced to the terminal methyl group (ω-CH_3) at 0.9 ppm.
[b]A value of 9.00 was assigned to the peak at 3.4 ppm. The average value of three integrations is recorded here.
[c]Contribution from the 17.7% linoleic acid acyl chains present in the egg PC mixture.

of each nucleus, the ^{31}P nucleus shows a sensitivity that is greater than ^{13}C, but less than ^{1}H.

Solid-State NMR

The extra (unaveraged) interactions present in NMR of solids are immediately apparent by comparing the solution and solid-state ^{1}H and ^{13}C NMR spectra. Even under high-power decoupling and MAS conditions, the liquid crystalline aqueous dispersions exhibit broader lines and fewer resolved spectral features. The solid ^{1}H NMR line-widths range from 15 to 46 Hz, compared to typical solution line-widths of 5 Hz. The solid ^{13}C line-widths range from 22 to 60 Hz, compared to line-widths in solution of 2 Hz.

Table 2. ^{13}C Resonance Assignments for Egg Phosphatidylcholine

δ/ppm[a]	Carbon Type
14.10	ω-CH_3
22.68, 22.57	(ω – 1)-CH_2
24.92, 24.98	β-CH_2
25.65	=CH–**C**H_2–CH=[b]
27.21	**C**H_2CH=CH**C**H_2
29.60, 29.85, 29.98	$(CH_2)_n$
31.53	unassigned
31.93	(ω – 2)-CH_2
34.16, 34.55	α-CH_2
54.40	$N(CH_3)_3$
59.30 (d, J_{CP} = 5.2 Hz)	PO_3CH_2
63.04	CH_2O (glycerol)
63.31 (d, J_{CP} = 5.0 Hz)	CH_2O (choline)
66.22 (d, J_{CP} = 5.5 Hz)	CH_2N
70.65 (d, J_{CP} = 7.4 Hz)	CHO
127.91, 128.10	=CHCH_2**C**H=[b]
129.69, 130.02	HC=CH
129.98, 130.24	H**C**=CHCH_2CH=[b]
173.12, 173.51	C=O

[a]Referenced to the terminal methyl group (ω-CH_3) at 14.10 ppm.
[b]Contribution from the 17.7% of linoleic acid acyl chains present in the egg PC mixture.

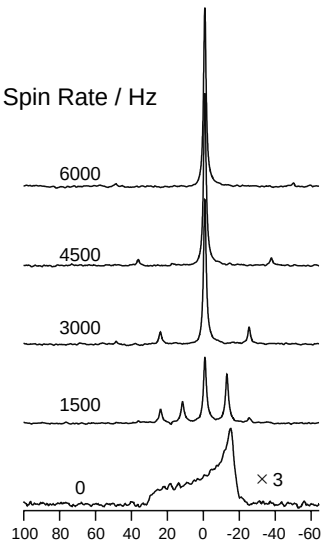

Figure 2. ^{31}P MAS spectra acquired using a delay of 2 s between each of 32 transients in which 1K time domain points covered a spectral range of 160 ppm. The time domain data were zero-filled to 2K points, multiplied by a decaying exponential (line broadening = 120 Hz), and Fourier transformed. The center band was arbitrarily set to 0 ppm.

The effects of CSA are best illustrated by comparing ^{31}P NMR spectra at several MAS spinning speeds (Fig. 2). The static spectrum exhibits a so-called axially symmetric chemical shielding pattern with a breadth of 49 ppm, indicating partial averaging of the CSA by rapid axial rotation and a shape that is usually diagnostic for a bilayered phospholipid arrangement (*17, 18*). Spinning at the magic angle produces a manifold of sharp spectral lines that map out the static spectral pattern; the center band becomes predominant as the spinning speed is increased but complete averaging requires a speed of 6 kHz (49 ppm).

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[w]Supplemental Material

A detailed student handout including questions, notes and answers for the instructor, and additional sample NMR spectra are available in this issue of *JCE Online*.

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