# High-Field, High-Resolution Proton "Magic-Angle" Sample-Spinning Nuclear Magnetic Resonance Spectroscopic Studies of Gel and Liquid Crystalline Lipid Bilayers and the Effects of Cholesterol†

**Jeffrey Forbes, Cynthia Husted, and Eric Oldfield***

*Contribution from the School of Chemical Sciences, University of Illinois at Urbana-Champaign, 505 South Mathews Avenue, Urbana, Illinois 61801.* *Received November 19, 1986*

**Abstract:** We have obtained proton ($^1$H) "magic-angle" sample-spinning (MASS) nuclear magnetic resonance (NMR) spectra of a variety of smectic liquid crystalline phases, including sodium decanoate (30.1 wt %)–decanol (38.9 wt %)–water, potassium oleate (72 wt %)–water, and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (lecithin)(50 wt %)–water, in addition to investigating the effects of temperature and cholesterol (CHOL) addition on the lecithin spectrum. Our results indicate that even relatively slow (~3 kHz) MASS causes averaging of the dipolar interactions causing line broadening in the static NMR spectra, at least for the non-CHOL species. All of the major proton-containing groups are well resolved, the resolution being at least as good as obtained in previous studies of oriented samples or sonicated systems. The methylene chain protons in each liquid crystalline lipid bilayer system give rise to intense, sharp, spinning sidebands (SSBs) due to the special form of the dipolar Hamiltonian. The methyl groups of the lipids, and the trimethylammonium group in lecithin, do not yield intense SSB patterns. Addition of CHOL causes attenuation of the center-band methylene peak of the lecithin, and a corresponding increase in SSB intensity. All or nearly all of the non-CHOL protons present in the samples appear to contribute to the high-resolution spectra, within our experimental error of ~10–20%. Use of a chain-deuterated lecithin allows peaks arising from the side chain of CHOL to be observed. In the gel phase of lecithin, only the trimethylammonium peak is apparent. The high-resolution afforded by MASS of the liquid crystalline phases permits rapid determination of the spin–lattice relaxation times ($T_1$) of all resolved resonances. In addition, the observation of numerous chemically shifted peaks permits the use of two-dimensional (2-D) NMR techniques, which can give information on the spatial proximity of the various groups in the bilayer. Taken together, our results indicate a very promising future for high-field $^1$H MASS NMR studies of other lipid and membrane systems because of the extremely high sensitivity of the $^1$H nucleus and the unique ability to obtain chemical shift, $T_1$, and 2-D information from a single sample, without recourse to isotopic labeling, macroscopic sample orientation, or ultrasonic irradiation.

Nuclear magnetic resonance (NMR) spectroscopy has been used to investigate the structure of model and biological membranes for over 20 years, and studies of molecular motion in simple lipids and hydrocarbons can be traced back even further.[1,2] The earliest studies concentrated on the $^1$H nucleus, which because of its high sensitivity and abundance was expected to be a useful probe of membrane structure. Early studies by Cerbón[3,4] identified mobile lipid components in *Nocardia asteroides*; then Chapman et al. began an extensive series of studies of model[5-8] and biological membrane systems.[9-12] The early $^1$H NMR experiments utilized wide-line methods, and assumed that $^1$H–$^1$H dipolar interactions dominated the observed line widths, since very broad lines were, in general, obtained. These line widths could be reduced by sonicating the (liquid crystalline) lipid bilayers, or membranes, reducing their particle size and permitting faster vesicle tumbling. Shortly after the first papers by Chapman et al., numerous other groups published similar studies on related systems.[13-25]

Three main questions arose from the early studies. These centered around the nature of the line-broadening mechanisms in the liquid crystalline phases, the effects of sonication (does it cause line narrowing due to a change in lipid bilayer structure, or because of increased rates of particle rotation?), and whether spin diffusion occurs in the liquid crystalline phases. These questions have (at least in part) been answered over the past 15 years. Thus, Chan et al.,[26] Tiddy,[27] and Oldfield et al.[28] showed that line broadening in various liquid crystalline phases (at least up to ~90 MHz) was purely dipolar in origin, since the line widths (or effective $T_2$ values, $T_2$*) were field independent. This view was supported by the results of $^1$H "magic-angle" sample-spinning (MASS) NMR,[29] by multiple-pulse line narrowing,[30] and by "magic-angle" alignment of oriented samples.[31-33] The effects of sonication have been widely studied, and the early view, that sonication causes line narrowing only due to increased particle tumbling, has received considerable support,[34-36] although it is not universally accepted.[37] Finally, the occurrence of spin-diffusion is deemed to be unimportant in sonicated liquid crystalline

†This work was supported in part by the U.S. National Science Foundation (Grant DMB 84-16771) and by the University of Illinois.

(1) Andrew, E. R. *J. Chem. Phys.* **1950**, *18*, 607–618.
(2) Chapman, D.; Richards, R. E.; York, R. W. *J. Chem. Soc.* **1960**, 436–444.
(3) Cerbón, J. *Biochim. Biophys. Acta* **1965**, *102*, 449–458.
(4) Cerbón, J. *Biochim. Biophys. Acta* **1970**, *211*, 389–395.
(5) Chapman, D.; Penkett, S. A. *Nature (London)* **1966**, *211*, 1304–1305.
(6) Chapman, D.; Williams, R. M.; Ladbrooke, B. D. *Chem. Phys. Lipids* **1967**, *1*, 445–475.
(7) Penkett, S. A.; Flook, A. G.; Chapman, D. *Chem. Phys. Lipids* **1968**, *2*, 273–290.
(8) Salsbury, N. J.; Chapman, D. *Biochim. Biophys. Acta* **1968**, *163*, 314–324.
(9) Jenkinson, T. J.; Kamat, V. B.; Chapman, D. *Biochim. Biophys. Acta* **1969**, *183*, 427–433.
(10) Phillips, M. C.; Kamat, V. B.; Chapman, D. *Chem. Phys. Lipids* **1970**, *4*, 409–417.
(11) Kamat, V. B.; Chapman, D.; Zwaal, R. F. A.; Van Deenen, L. L. M. *Chem. Phys. Lipids* **1970**, *4*, 323–331.
(12) Chapman, D.; Kamat, V. B.; De Gier, J.; Penkett, S. A. *J. Mol. Biol.* **1968**, *31*, 101–114.
(13) Lecar, H.; Ehrenstein, G.; Stillman, I. *Biophys. Soc. Annu. Meet. Abstr.* **1967**, *1*, A65.
(14) Lecar, H.; Ehrenstein, G.; Stillman, I. *Biophys. J.* **1971**, *11*, 140–145.
(15) Glaser, M.; Simpkins, H.; Singer, S. J.; Sheetz, M.; Chan, S. I. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *65*, 721–728.
(16) Kaufman, S.; Steim, J. M.; Gibbs, J. H. *Nature (London)* **1970**, *225*, 743–744.
(17) Hansen, J. R.; Lawson, K. D. *Nature (London)* **1970**, *225*, 542–544.
(18) Davis, D. G.; Inesi, G. *Biochim. Biophys. Acta* **1971**, *241*, 1–8.
(19) Birdsall, N. J. M.; Lee, A. G.; Levine, Y. K.; Metcalfe, J. C. *J. Chem. Soc., Chem. Commun.* **1971**, 1171–1172.
(20) Finer, E. G.; Flook, A. G.; Hauser, H. *Biochim. Biophys. Acta* **1972**, *260*, 59–69.
(21) Davis, D. G.; Inesi, G. *Biochim. Biophys. Acta* **1972**, *282*, 180–186.
(22) Robinson, J. D.; Birdsall, N. J. M.; Lee, A. G.; Metcalfe, J. C. *Biochemistry* **1972**, *11*, 2903–2909.
(23) Horwitz, A. F.; Horsley, W. J.; Klein, M. P. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 590–593.

0002-7863/88/1510-1059$01.50/0 

systems, although it is more difficult to disprove some occurrence in unsonicated systems, where extensive spectral overlaps occur. Indeed, the early studies were carried out on unsonicated systems, and it is not possible to disprove the occurrence of some spin-diffusion by analysis of systems in which these overlaps are reduced (e.g., by MASS or sonication).

In this paper we report our progress on the $^1H$ NMR of lipids some "20 years on". We find that excellent spectral resolution can now be achieved by combined use of high (8.45 and 11.7 T) magnetic field strengths (corresponding to 360- and 500-MHz $^1H$ resonance frequencies, respectively) and "magic-angle" sample-spinning. *The resulting resolution is approximately the same as that obtained using sonicated lipid vesicles* or macroscopically oriented samples, but the sensitivity of the MASS approach is considerably higher, since neither dilute suspensions (sonication) nor stacks of glass plates (oriented multibilayers) are required. Thus, spectra may be obtained in about 5–10% of the time required for these other techniques. A surprising result is the observation of numerous, intense, sharp, spinning sidebands (SSBs) in the liquid crystalline phases, up to seven being observed in the presence of cholesterol (CHOL).

## Experimental Aspects

**NMR Spectroscopy.** All $^1H$ spectra were obtained on "home-built" NMR spectrometers, which operate at 50, 360, or 500 MHz, using either a Nalorac (Concord, CA) 4.0-in. bore, 3.52-T solenoid, run at 1.17 T, or on Oxford Instruments (Osney Mead, U.K.) 3.5-in. bore, 8.45-T or 2.0-in. bore, 11.7-T solenoids, together with Nicolet (Madison, WI) Model 1180 or 1280 computer systems, Henry Radio (Los Angeles, CA) Model 1002 radio-frequency amplifiers, and either Doty Scientific (Columbia, S.C.) or "home-built" MASS NMR probes. For the experiments done at 360 MHz, the 90° pulse widths varied between 20 and 30 μs, but 5-μs pulses were used to mitigate the possibility of pulse-power falloff. The experiments done at 500 MHz had 90° pulse widths of 9 μs. All spectra were referenced to an external standard of hexamethyldisiloxane (δ 0.055 ppm from tetramethylsilane), and high-frequency, low-field, deshielded, paramagnetic shifts are denoted as positive (IUPAC δ scale).

**Lipid Samples.** Potassium oleate was prepared by titrating oleic acid (octadec-9-en-1-oic acid; Sigma Chemical Co., St. Louis, MO) with reagent grade KOH (J. T. Baker) in EtOH, to a phenolphthalein endpoint, followed by recrystallization from EtOH at −20 °C. The product was dried at 100 °C for 24 h. Sodium decanoate was similarly prepared, and liquid crystalline phases were made by the addition of appropriate quantities of $D_2O$ and decanol, followed by extensive homogenization at ~70 °C. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (Sigma) was used without further purification. Chain perdeuterated DMPC ([$^2H_{54}$]DMPC) (Avanti Polar Lipids, Inc., Birmingham, AL) was also used without further purification. Cholesterol (Aldrich) was recrystallized three times from EtOH before use. DMPC samples were prepared by addition of $D_2O$ (50 wt %). DMPC–CHOL samples were prepared by codissolving the appropriate amounts of DMPC and CHOL in $CHCl_3$ and removing the solvent under an $N_2$ stream at ~40 °C, followed by evacuation overnight. Lecithin purity was monitored periodically, using standard methods.

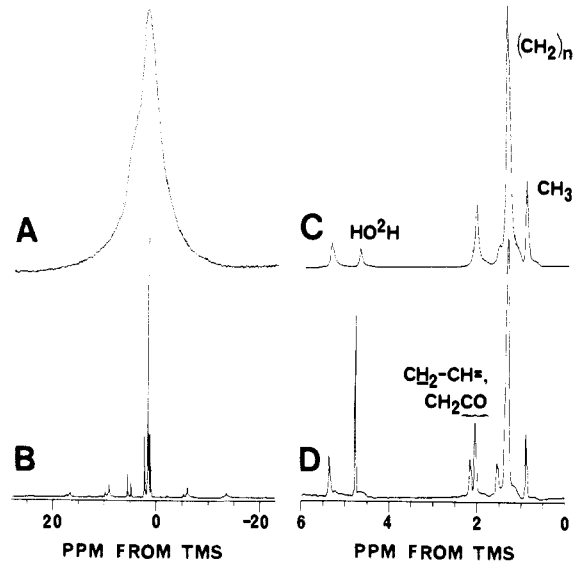


**Figure 1.** 360-MHz static and MASS $^1H$ Fourier transform NMR spectra of potassium oleate(72 wt %)–$D_2O$ liquid crystalline mesophase: (A) static spectrum at 26 °C, (B) 2.7-kHz MASS NMR spectrum, (C) expansion of the center-band region of B, (D) solution spectrum of potassium oleate in $D_2O$.

## Results and Discussion

We show in Figure 1 representative 360-MHz $^1H$ Fourier transform NMR spectra of the potassium oleate (72% wt %)–$D_2O$ smectic liquid crystalline system, at ~25 °C. As expected, the spectrum of a static sample (Figure 1A) consists of a broad, non-Lorentzian line having a half-height width of about 2.2 kHz. The line broadening is due primarily to incompletely motionally averaged proton–proton dipole–dipole interactions. Previously, DeVries and Berendsen[31] investigated the potassium oleate–$D_2O$ system in the smectic liquid crystalline phase, using samples macroscopically oriented on glass plates. In their work (carried out at a proton resonance frequency of only 60 MHz), they were able to show a substantial line narrowing at the "magic-angle", although spectral resolution was poor. Much better results were, however, obtained by Van der Leeuw and Stulen,[33] at a proton resonance frequency of 360 MHz, the same as in most of the present study. At the higher magnetic field strength employed by these workers, a much better resolved static spectrum was obtained at the "magic-angle". We show in Figure 1B the results of a 2.7-kHz MASS experiment at 360 MHz, which clearly indicate a substantial line narrowing upon MASS, even at the relatively low spinning frequency of 2.7 kHz. There are a series of well-resolved center-band resonances, together with several sets of sharp, well-resolved SSBs, which originate from the broad wings of the static spectrum. Figure 1C shows an expansion of the center-band resonances, and it can be seen that well-resolved $CH_3$, $(CH_2)_n$, and CH=CH protons are all apparent. The spectrum is qualitatively similar to that obtained by Van der Leeuw and Stulen; however, our resolution is significantly improved over that obtained in the oriented sample work.[33] For purposes of comparison, we show in Figure 1D the 360-MHz $^1H$ NMR spectrum of the potassium oleate soap dissolved in $D_2O$ to form an isotropic solution. In this case, the methylene groups adjacent to the double bond and the methylene group adjacent to the carbonyl group are now resolved.

We believe that the results shown in Figure 1 are interesting for a number of reasons. First, the MASS spectrum of the smectic liquid crystalline phase (Figure 1C) is quite highly resolved, although we were unable to observe $J$ couplings with our current instrumentation (and technique). Secondly, Figure 1B shows the presence of numerous SSBs. Note, however, that well-resolved SSBs are only obtained from the methylene and olefinic chain protons; the resonances of the water and that of the terminal

(24) Chan, S. I.; Seiter, C. H. A.; Feigenson, G. W. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 1488–1492.
(25) Birdsall, N. J. M.; Feeney, J.; Lee, A. G.; Levine, Y. K.; Metcalfe, J. C. *J. Chem. Soc., Perkin Trans. 2* **1972**, 1441–1445.
(26) Chan, S. I.; Feigenson, G. W.; Seiter, C. H. A. *Nature (London)* **1971**, *231*, 110–112.
(27) Tiddy, G. J. T. *Nature Phys. Sci.* **1971**, *230*, 136–138.
(28) Oldfield, E.; Marsden, J.; Chapman, D. *Chem. Phys. Lipids* **1971**, *7*, 1–8.
(29) Chapman, D.; Oldfield, E.; Doskočilová, D.; Schneider, B. *FEBS Lett.* **1972**, *25*, 261–264.
(30) Seiter, C. H. A.; Chan, S. I. *J. Am. Chem. Soc.* **1973**, *95*, 7541–7553.
(31) DeVries, J. J.; Berendsen, H. J. C. *Nature (London)* **1969**, *221*, 1139–1140.
(32) Hemminga, M. A.; Berendsen, H. J. C. *J. Magn. Reson.* **1972**, *8*, 133–143. Dijkema, C.; Berendsen, H. J. C. *Ibid.* **1974**, *14*, 251–259.
(33) Van der Leeuw, Y. C. W.; Stulen, G. *J. Magn. Reson.* **1981**, *42*, 434–445.
(34) Bloom, M.; Burnell, E. E.; Valic, M. I.; Weeks, G. *Chem. Phys. Lipids* **1975**, *14*, 107–112.
(35) Bloom, M.; Burnell, E. E.; Roeder, S. B. W.; Valic, M. I. *J. Chem. Phys.* **1977**, *66*, 3012–3019.
(36) Bloom, M.; Burnell, E. E.; MacKay, A. L.; Nichol, C. P.; Valic, M. I.; Weeks, G. *Biochemistry* **1978**, *17*, 5750–5762.
(37) Parmer, Y. I.; Wassall, S. R.; Cushley, R. J. *J. Am. Chem. Soc.* **1984**, *106*, 2434–2435.

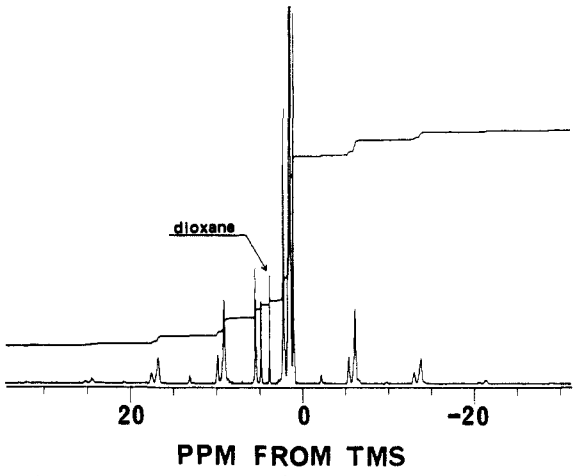


**Figure 2.** Absolute quantitation of potassium oleate spectrum via addition of 5 μL of 1,4-dioxane: 360 MHz, 2.7-kHz MASS spectrum, recycle time 15.0 s, 20-μs pulse excitation (90° solution pulse width = 20 μs).

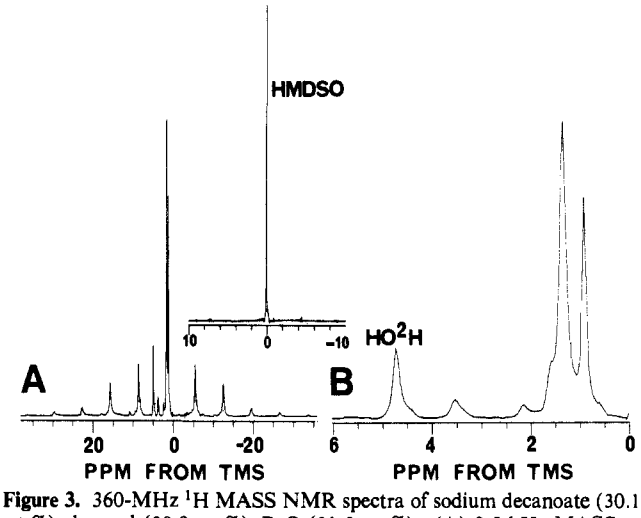


**Figure 3.** 360-MHz $^1H$ MASS NMR spectra of sodium decanoate (30.1 wt %)–decanol (38.9 wt %)–$D_2O$ (31.0 wt %): (A) 2.5-kHz MASS at 26 °C (inset: MASS NMR spectrum of hexamethyldisiloxane, demonstrating field homogeneity), (B) expansion of the center-band region of A.

methyl group do not give rise to such SSBs.

Integration of both the center band and SSB peaks yields good quantitative agreement with the results expected on the basis of chemical composition; i.e., there appears to be little missing signal intensity, within our estimated experimental error of ~10–15%. We have also quantitated these results on an absolute basis using an internal standard comprised of 5 μL of 1,4-dioxane, as shown in Figure 2 and Table I. The results of this experiment again indicate that 90 ± 10% of the protons present in the sample contribute to the high-resolution signal.

We show in Figure 3 similar results for a decanol–sodium decanoate–$D_2O$ liquid crystalline phase, obtained using MASS NMR at ambient probe temperature (~25 °C). The spectrum of Figure 3A exhibits a set of intense center-band resonances averaged by MASS, together with four or five sets of sharp SSBs, due to the methylene chain protons. Spinning sidebands are not observed from the terminal methyl or from the $HO^2H$ water resonance, or from a separate sample of hexamethyldisiloxane (inset spectrum), which indicates adequate $H_0$ field homogeneity. An expansion of the center-band resonances, Figure 3B, shows well-resolved signals from the terminal methyl, chain methylene, $CH_2CO$, and $CH_2OH$ protons, together with a signal from residual $HO^2H$. The methyl resonance in the center-band spectrum, Figure 3B, appears more intense than expected when compared with the methylene proton resonance, because much of the methylene intensity is contained within the envelope of spinning sidebands.

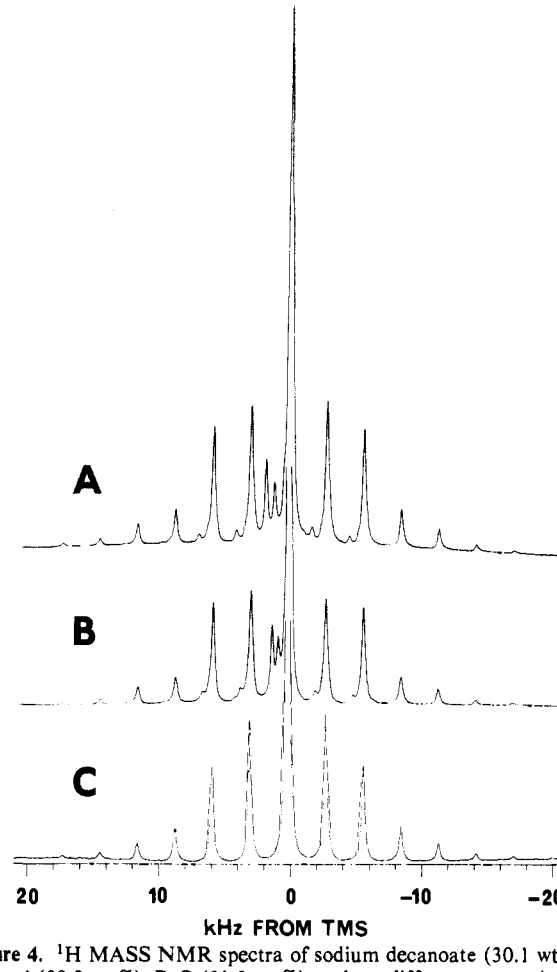


**Figure 4.** $^1H$ MASS NMR spectra of sodium decanoate (30.1 wt %)–decanol (38.9 wt %)–$D_2O$ (31.0 wt %) at three different magnetic field strengths. Each spectrum was taken with a constant spinning speed of 2.86 kHz ± 5 Hz, and at ambient temperature. An exponential line broadening of 200 Hz was used on each: (A) 500 MHz, (B) 360 MHz, (C) 50 MHz.

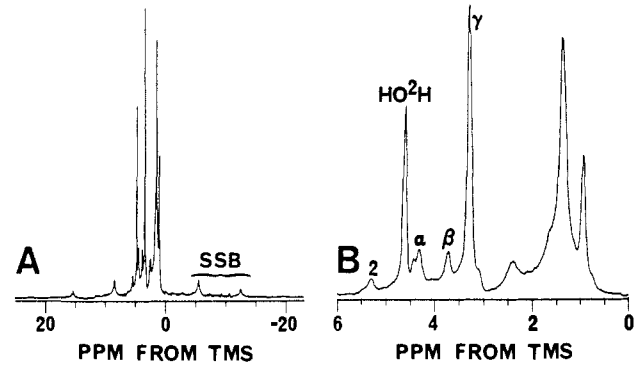


**Figure 5.** 360-MHz $^1H$ MASS NMR spectra of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (50 wt %) in $D_2O$, at 36 °C: (A) 2.5-kHz MASS, (B) expansion of the center-band region of A. Nomenclature as in: Brown, M. F.; Seelig, J. *Biochemistry* **1978**, *17*, 381–384.

There can only be a very small falloff in intensity in the wings of the MASS NMR spectrum, because of our use of a relatively short pulse width. These results indicate that well-resolved proton MASS NMR spectra of smectic liquid crystalline phase lipids may be obtained (at 360 MHz) using relatively modest MASS spinning rates. In addition, results obtained at 50 and 500 MHz, Figure 4, indicate essentially the same pattern of SSBs, ruling out any significant field-dependent (nondipolar) contributions to the overall spectral width, although a very small chemical shift anisotropy might be anticipated.

We show in Figure 5 similar 360-MHz $^1H$ MASS NMR spectra of the smectic liquid crystalline phase of DMPC, dispersed

**Table I.** Absolute Quantitation of Potassium Oleate (72 wt %)-$D_2O$ Spectra Using an Internal 1,4-Dioxane Standard[a]

| chemical shift (ppm)[b] | assignment | theor no. of protons[c] | measd no. of protons | | | error (%)[e] |
|---|---|---|---|---|---|---|
| | | | center | SSB | total[d] | |
| 5.30 | -CH=CH- | 2 | 1.28 | 0.38 | 1.66 | -17.0 |
| 2.02 | $-CH_2CO$ $-CH_2CH=$ | 6 | 3.25 | 1.90 | 5.15 | -14.2 |
| 1.50, 1.29 | $(CH_2)_{11}$ | 22 | 14.78 | 5.99 | 20.77 | -5.6 |
| 0.89 | $CH_3$ | 3 | 2.78 | | 2.78 | -7.3 |
| | overall | 33 | 22.09 | 8.27 | 30.36 | -8.0[f] |

[a] 1,4-Dioxane (5 μL) added to a known weight of a potassium oleate (72 wt %) -$D_2O$ mesophase. Three samples were investigated using 360-MHz $^1H$ MASS. For a typical spectrum, see Figure 2. [b] Error is ~±0.05 ppm. [c] Total number of protons from chemical structure. [d] Number of protons contributing to peak(s) indicated, based on integrated intensity of 5 μL (5.17 mg) of added 1,4-dioxane. Values shown are averages of three experiments. [e] Average of three determinations. [f] Overall experimental error, average of three determinations. The individual errors were -27.2, -17.2, +20.4%.

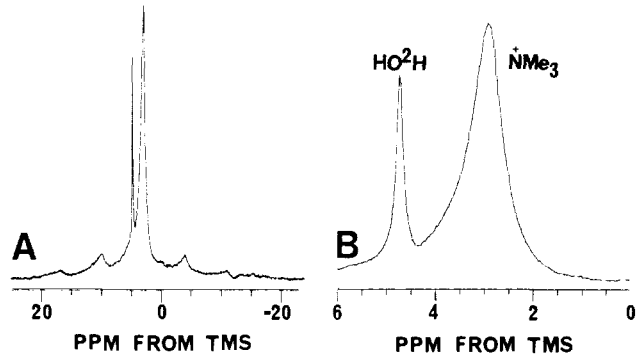


**Figure 6.** 360-MHz $^1H$ MASS NMR spectra of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (50 wt %) in $D_2O$, at -10 °C: (A) 2.5-kHz MASS. (B) expansion of the center-band region of A.

in $D_2O$. There are a series of well-resolved center-band resonances, Figure 5A, together with two or three sets of SSBs. Figure 5B shows an expansion of the center-band resonances, in which the terminal methyl, chain methylene, and $CH_2CO$ chain protons may be resolved, together with the choline headgroup α-methylene, β-methylene, and γ-trimethylammonium protons. There is also a resonance at 5.29 ppm, which on the basis of previous work on sonicated lipid dispersions[38] may be attributed to the isolated *sn*-2 methine proton of the glycerol backbone. Investigation of the SSB intensities reveals, once again, that the most intense feature may be attributed to the chain methylene protons, as in the case of the two previous soap liquid crystal phases. There are only very weak SSBs for the terminal methyl resonance, and from the trimethylammonium and methylene headgroup resonances. Integration of the DMPC data, at 360 and 500 MHz, using as an internal standard the $^+NMe_3$ resonance (9.00 protons from the center-band and 2 SSBs) yields overall intensities some 20–30% below that expected (data not shown). This suggests that some of the chain $CH_2$ protons may not yield high-resolution signals, or alternatively that some of the bilayers may yield very broad lines, but the errors are close to our experimental uncertainties. Nevertheless, the observed intensities are still in quite close accord with those expected on compositional grounds.

On cooling the sample below the (24 °C) gel-to-liquid crystal phase transition temperature, we obtain (at -10 °C) the 360-MHz $^1H$ MASS NMR spectrum of the gel phase of DMPC, as shown in Figure 6. Figure 6A shows a broad spectral width plot in which center-band resonances due to $HO^2H$ and the lipids' $^+NMe_3$ group may be assigned. Spinning sidebands are observed only for the $^+NMe_3$ group, and, as shown in Figure 6A, they are quite broad, in sharp distinction to the previous results on the liquid crystalline phase of DMPC. There are no signals which may be attributed to the hydrocarbon chain protons, or to the methylene groups in the phospholipid headgroup region. This result is in accord with that obtained previously.[29]

A number of explanations of the results of Figures 1–6 have been developed by ourselves, our colleagues, and the referees of this publication. Overall, the results obtained by ourselves, and by previous workers, indicate that the line broadening in this system can be described as a (field-independent) inhomogeneous, dipolar broadening, even though a large number of spins are involved. The inhomogeneous nature of the linear chain of $^{19}F$ spins in fluoroapatite has been discussed previously,[39] and more recently, Vega has observed related inhomogeneous (SSB) behavior in rotating $NH_4^+$ groups in $NH_4^+$-ρ-zeolite, in $CH_3$ groups in a deuterated sodium acetate matrix,[40] and in $Si(CH_3)_3$ groups in a surface silyl residue,[41] and Zumbulyadis has observed similar behavior in the $^{19}F$ NMR of $CF_3CO_2Ag$.[42]

For the case of $NH_4^+$ exchange or rotation, all H or X nuclei became equivalent, so that the static dipolar Hamiltonian

$$\sum_{i<j} D_{ij}(\phi)(3I_{zi}I_{zj} - \mathbf{I}_i\cdot\mathbf{I}_j) \quad (1)$$

which generally does not commute with itself at different rotor orientations, becomes

$$D(\phi)\sum_{i<j}(3I_{zi}I_{zj} - \mathbf{I}_i\cdot\mathbf{I}_j) \quad (2)$$

which does commute at different rotor orientations.[40]

While this is not identical with the situation with liquid crystalline lipid bilayers, Wennerström,[43] Ulmius,[44] and Bloom et al.[36] have shown that the Hamiltonian for the protons in a liquid crystalline membrane sample is qualitatively different from that of protons in a normal solid sample. In the liquid crystalline phase, intermolecular dipole–dipole interactions are reduced to a negligible amount by rapid lateral diffusion, while fast axial rotation reduces the intramolecular dipole–dipole interactions and causes the angular dependence of the Hamiltonian to be the same for *all* of the proton pairs.[43] A result of this is that the shape of the absorption curve is independent of θ, the angle between the director axis and $H_0$. The effect of a variation in θ is only a change in the frequency scale since the couplings between all protons are multiplied by the same factor, $P_2(\cos\theta)$, which causes the familiar super-Lorentzian line shapes observed.[43]

Using this scaling then, we may write

$$D_{ij} = \tfrac{1}{2}(3\cos^2\theta - 1)D_{ij}^0 \quad (3)$$

for the individual dipolar interactions where $D_{ij}^0 = \gamma^2\hbar^2/r_{ij}^3$, in which case the Hamiltonian becomes

$$\tfrac{1}{2}(3\cos^2\theta - 1)\sum_{i<j} D_{ij}^0[3I_{zi}I_{zj} - \mathbf{I}_i\cdot\mathbf{I}_j] \quad (4)$$

basically as in eq 2 above.[40,42] In the gel state, axial rotation and lateral diffusion are presumably too slow to allow this type of motional averaging, and indeed super-Lorentzian lineshapes of

(38) Neumann, J. M.; Zachowski, A.; Tran-Dinh, S.; Devaux, P. F. *Eur. J. Biophys.* **1985**, *11*, 219–223.

(39) Yesinowski, J. P.; Mobley, M. J. *J. Am. Chem. Soc.* **1983**, *105*, 6191–6193 and ref 11–16 cited therein.

(40) Vega, A. J., private communication.

(41) Köhler, J.; Chase, D. B.; Farlee, R. D.; Vega, A. J.; Kirkland, J. J. *J. Chromatogr.* **1985**, *352*, 275–305.

(42) Zumbulyadis, N., private communication.

(43) Wennerström, H. *Chem. Phys. Lett.* **1973**, *18*, 41–44.

(44) Ulmius, J.; Wennerström, H.; Lindblom, G.; Arvidson, G. *Biochim. Biophys. Acta* **1975**, *389*, 197–202.

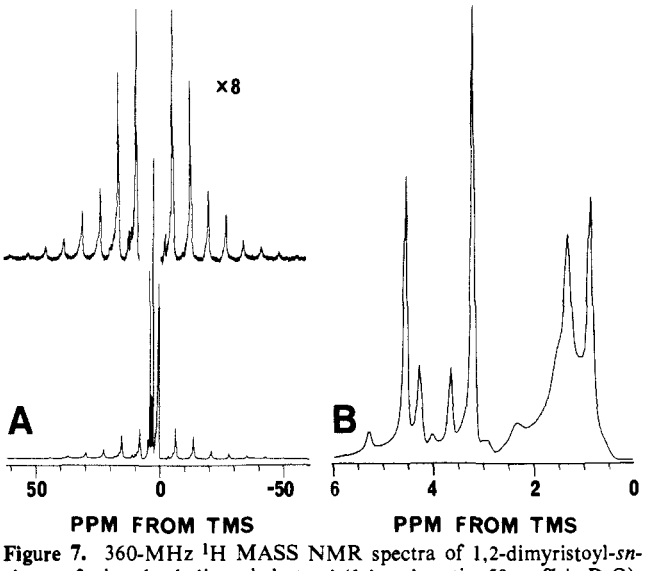


**Figure 7.** 360-MHz $^1H$ MASS NMR spectra of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine–cholesterol (1:1 mole ratio, 50 wt % in $D_2O$), at 36 °C: (A) 2.6 kHz MASS (the top spectrum shows an 8 × vertical expansion of the bottom trace), (B) expansion of the center-band region of A.

the gel state have not been reported, using wide-line $^1H$ NMR methods.

Notably, a prediction of the above ideas is that with very slow spinning, SSBs will still be observed, and the peak intensities of the SSBs should "map out" a well-resolved super-Lorentzian lineshape. This is exactly what is observed experimentally, the 820-Hz MASS rate NMR spectrum of the decanol–sodium decanoate–$D_2O$ system (data not shown) being in very good agreement with the super-Lorentzian lineshape computed by Wennerström[43] and Bloom et al.[35]

In the gel phase, there is presumably a breakdown of the adiabatic approximation, due to slow axial rotation and the presence of strong intermolecular dipolar interactions, due to a lack of fast lateral diffusion, in which case it will likely be necessary to combine MASS with multiple-pulse techniques in order to achieve high-resolution $^1H$ NMR spectra.

The results shown in Figures 1–4 indicate that high-resolution spectra may be obtained from liquid crystalline phase lipids, without orientation or sonication. We show in Figure 7 the effects of the molecule cholesterol (CHOL) on the observed proton MASS NMR spectrum of DMPC. We investigated a 1:1 mole ratio sample of DMPC–CHOL (in order to observe the largest possible effect of CHOL), dispersed in excess water, at 36 °C. Based on extensive previous investigations of the lecithin–CHOL system,[5,45-48] we expected the cholesterol molecule to "condense" the lipid hydrocarbon chains. We thus might expect one of two types of behavior in the proton NMR spectrum, due to the addition of CHOL. The first possibility is that we would have a much more "gel-like" phase, due to the increased strength of the dipole–dipole interactions, as a result of decreased axial rotation, and increased importance of *intermolecular* interactions. In this case the main observable feature might only be that of the trimethylammonium headgroup, as found in the gel state spectrum of lecithin, Figure 6. The second possibility is that cholesterol would only increase $\langle P_2(\cos\theta)\rangle$, which would be reflected in an increase in the number of SSBs present. The latter behavior is the one observed, as shown in Figure 7A. In addition to a series of well-resolved center-band resonances, Figure 7B, Figure 7A shows that there are at least

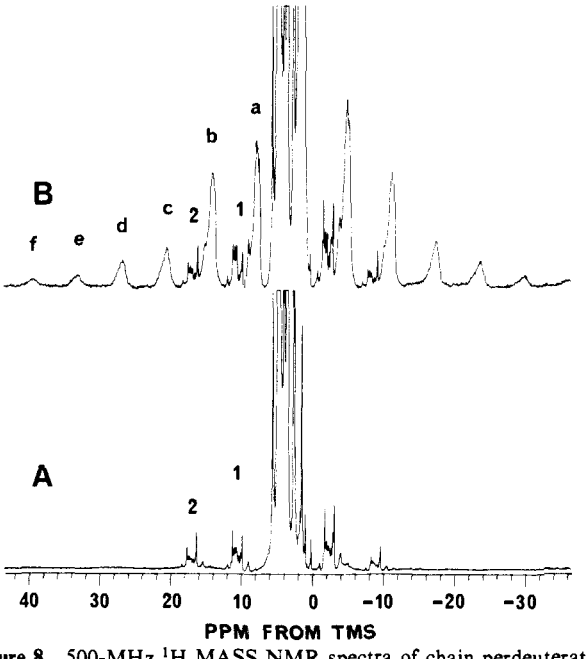


**Figure 8.** 500-MHz $^1H$ MASS NMR spectra of chain perdeuterated DMPC with and without cholesterol: (A) [$^2H_{54}$]DMPC (50 wt %) in $D_2O$, 3.2-kHz MASS, at 26 °C; (B) [$^2H_{54}$]DMPC–CHOL (1:1) (50 wt %) in $D_2O$, 3.1-kHz MASS, at 26 °C.

seven SSBs present. The center-band spectrum, Figure 7B, once again contains signals from the terminal methyl and methylene chain protons, in addition to the $\alpha$, $\beta$, and $\gamma$ methylene and methyl group protons of the choline headgroup, and the signal at about 5.3 ppm, tentatively assigned to the *sn*-2 methine proton.[38] The center-band intensity of the methylene chain resonance is diminished relative to that of the terminal methyl proton resonance, owing to the presence of significant intensity in the spinning sideband manifold (and to a small contribution from pulse-power falloff).

We believe that these results once again demonstrate the utility of high-field proton MASS NMR spectroscopy in studying lipid bilayer structure. The effect of cholesterol is not unexpected, in that it increases the order of the lipid hydrocarbon chains, thereby increasing the number of spinning sidebands observable. Rapid lateral diffusion in this *liquid crystalline* lecithin–cholesterol phase presumably causes substantial averaging of the intermolecular dipole–dipole interactions, which in the gel phase of lecithin likely contributes significantly to the loss of methylene proton signal intensity.

Analysis of integrated intensity results for the DMPC–CHOL sample reveals, assuming that only the DMPC protons are being observed, a +4.6% error in total intensity, versus −32.2% (360 MHz) or −23.3% (500 MHz) values for DMPC alone. It seems surprising that better quantitative results are found in the presence of CHOL, and, in addition, it is surprising that the error for the terminal methyl groups (+122%) is so high. This suggested to us that some of the CHOL protons were contributing to the high-resolution spectrum, since observation of all DMPC and CHOL resonances would yield a −36.2% error, rather greater than that observed for DMPC–CHOL, or DMPC alone.

We thus recorded the $^1H$ MASS NMR spectra of a chain-perdeuterated DMPC, in the presence and absence of an equimolar amount of CHOL, as a 50 wt % dispersion in $D_2O$; the results are shown in Figure 8.

The full spectral width plots of [$^2H_{54}$]DMPC and [$^2H_{54}$]-DMPC–CHOL (1:1) are shown in Figure 8, A and B, respectively. As can be seen, there is an intense center-band plus two sets of SSBs (denoted 1,2) arising from the [$^2H_{54}$]DMPC (headgroup and glycerol backbone region), while in the presence of CHOL, six extra sets of SSBs (denoted a–f) appear. These additional broad SSBs arise from the CHOL molecule. Expansions of the center-bands of the [$^2H_{54}$]DMPC and [$^2H_{54}$]DMPC–CHOL

(45) Hubbell, W. L.; McConnell, H. M. *J. Am. Chem. Soc.* **1971**, *93*, 314–326.
(46) Oldfield, E.; Chapman, D. *Biochem. Biophys. Res. Commun.* **1971**, *43*, 610–616.
(47) Oldfield, E.; Chapman, D.; Derbyshire, W. *FEBS Lett.* **1972**, *16*, 102–104.
(48) Oldfield, E.; Meadows, M.; Rice, D.; Jacobs, R. *Biochemistry* **1978**, *17*, 2727–2740.

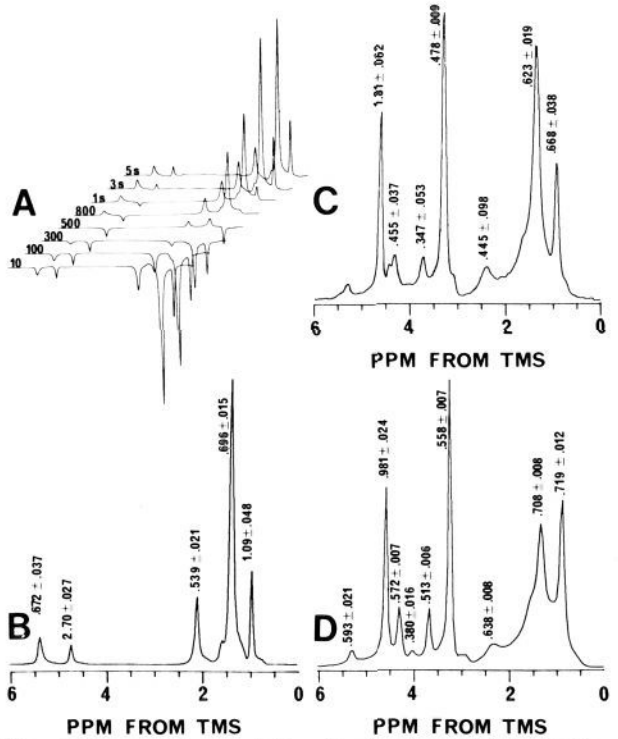


**Figure 9.** Representative spin–lattice relaxation time ($T_1$) determination, and $T_1$ results on liquid crystalline bilayers: (A) partially relaxed Fourier transform NMR spectra of potassium oleate–$D_2O$, at 26 °C, $\tau$ values in milliseconds unless noted; (B) expansion of center-band resonance of potassium oleate–$D_2O$ spectrum, showing $T_1$ values obtained from computer analysis of PRFT data; (C) center-band region of liquid crystalline DMPC spectrum (at 36 °C) showing $T_1$ values; (D) as in C, but for DMPC–CHOL, 1:1. The errors on the $T_1$ values are from a computer program. Realistic errors are ±5–10%.

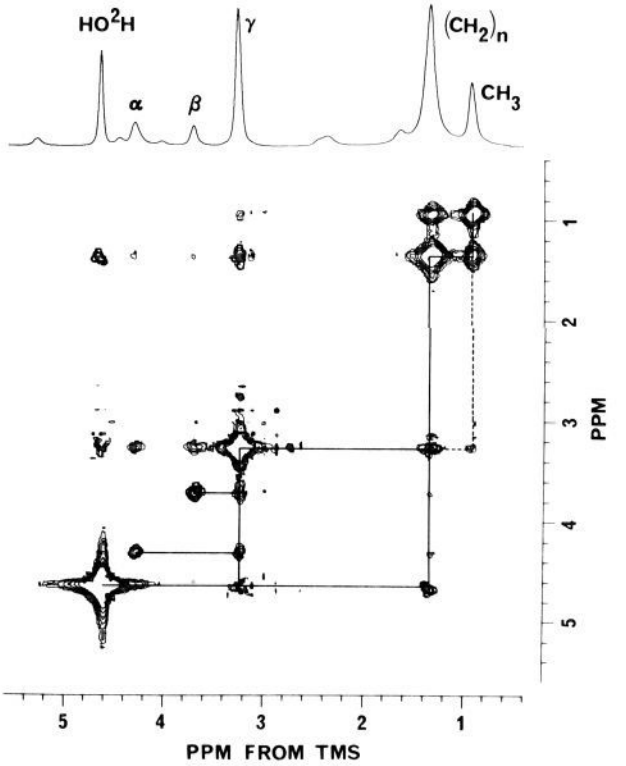


**Figure 10.** 500-MHz two-dimensional cross-relaxation (NOESY) contour plot of DMPC (50 wt %) in $D_2O$, at 26 °C, using a 500-ms mix time (see Xu and Cafiso, 1986, for details of the pulse sequence and phase cycling used), and a 3.1-kHz MASS rate. Assignments of the one-dimensional spectrum are given at the top of the figure.

spectra (data not shown) indicate that the chemical shifts of the new peaks observed are close to those observed previously by Kroon et al.,[49] and can be assigned to protons on the (mobile) CHOL side chain, together with a small feature attributable to residual DMPC chain protons.

These results clearly indicate that the CHOL side chain is mobile and contributes to the observed high-resolution $^1H$ MASS NMR spectrum, and quantitation of the results of Figure 8B, assuming all 17 protons in the $C_8$ CHOL side chain plus the two angular methyl groups are mobile, gives a result more in accord with the pure DMPC results discussed previously; i.e., we obtain a 9% error between experiment and prediction. We believe this result is of particular interest, since it implies that studies of a variety of other species, e.g., drugs, anesthetics, peptides, and other lipid classes, may be carried out using the MASS NMR method, using massively deuterated lipids, much as suggested previously by Kroon et al.,[49] but using unsonicated, as opposed to sonicated, lipid systems.

Unfortunately, we do not currently have a satisfactory explanation for the lack of well-resolved signals from the CHOL nucleus. Excessively long $T_1$ values, or excessively short $T_2$'s (due perhaps to chemical exchange) may be responsible, as may the need for a multiple order parameter description of the CHOL motion. Further work is underway in order to better characterize the NMR response of the CHOL nucleus.

Because of the high sensitivity and resolution of the $^1H$ MASS NMR method, we have been able to rapidly obtain the partially relaxed Fourier transform (PRFT) $^1H$ MASS NMR spectra, of the potassium oleate–water, sodium decanoate–decanol–water, DMPC, and DMPC–CHOL (1:1) systems at 360 MHz, and at several temperatures; representative results are presented in Figure 9. Figure 9A shows typical PRFT spectra, obtained at room temperature, of the potassium oleate (72 wt %)–$D_2O$ liquid crystalline mesophase system, and in Figure 9B we present the expanded center-band region of the normal $^1H$ Fourier transform NMR spectrum of this mesophase, indicating the spin–lattice relaxation times we have determined, using a 180°–$\tau$–90° pulse sequence. Similarly, we show in Figure 9C the $T_1$ values determined for each of the resolved resonances in DMPC (at 36 °C), and in Figure 9D the corresponding values for DMPC–CHOL (1:1), again at 36 °C. While a complete analysis of proton spin–lattice relaxation times is difficult and certainly beyond the scope of this publication, and has been considered for similar systems elsewhere,[50-53] the results of Figure 9 nevertheless clearly indicate that high-quality $T_1$ data sets may be obtained for high-resolution proton spectra of various unsonicated liquid crystalline lipid mesophases using the MASS NMR method. Similarly, $T_2$ and $T_{1\rho}$ data sets should be readily obtained via MASS NMR.

Finally, we show in Figure 10 the results of a two-dimensional (2-D) cross-relaxation (NOESY) experiment on unsonicated DMPC, carried out basically as described by Xu and Cafiso,[54] who investigated a *sonicated* 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) system. Our results are summarized in the 2-D contour plot in Figure 10, in which the spectral assignments are given on the 1-D spectrum at the top of the figure. We find the following. Only one set of cross peaks is observed for the terminal methyl–methylene peaks, unlike the two seen in the sonicated work, due to the equivalence of both sides of the bilayer in the coarse multilamellar system we have investigated. Similarly, there is only a single, weak, cross peak for the $^+NMe_3$–$(CH_2)_n$

(49) Kroon, P. A.; Kainosho, M.; Chan, S. I. *Nature (London)* **1975**, *256*, 582–584.

(50) Feigenson, G. W.; Chan, S. I. *J. Am. Chem. Soc.* **1974**, *96*, 1312–1319.

(51) Kroon, P. A.; Kainosho, M.; Chan, S. I. *Biochim. Biophys. Acta* **1976**, *433*, 282–293.

(52) Brown, M. F. *J. Chem. Phys.* **1984**, *80*, 2808–2831.

(53) Brown, M. F. *J. Chem. Phys.* **1984**, *80*, 2832–2836.

(54) Xu, Z-C.; Cafiso, D. S. *Biophys. J.* **1986**, *49*, 779–783.

interaction, unlike the two observed by Xu and Cafiso. Our terminal methyl–$^{+}NMe_3$ cross peak is exceedingly weak, unlike that seen in the sonicated system, due in that case presumably to lipid interdigitation or chain bends. The diagonal peaks for the $\alpha$-, $\beta$-, and $\gamma$-headgroup peaks are unsplit, owing to equivalence of the two halves of the bilayer. Finally, because our system has much less excess water, loss of magnetization to this large "bath" is attenuated, and we observe cross peaks with $HO^2H$ for $^{+}NMe_3$ and hydrocarbon chain protons, reflecting, we believe, the presence of substantial amounts of $HO^2H$ in the lipid bilayer.

### Conclusions and Future Prospects

We can summarize our conclusions as follows: (1) high-field $^1H$ MASS NMR yields narrow, multiline spectra for many liquid crystalline lipid bilayer systems, from which isotropic chemical shifts can be determined; (2) resolution in $^1H$ MASS is about as good as with sonicated systems, and is much better than with oriented samples; (3) line widths and spin–lattice relaxation times, for individual groups, can be readily measured ($T_2$ and $T_{1\rho}$ determinations should also be feasible); (4) information on order parameters can (probably) be extracted from the spinning-sideband manifold, without isotopic labeling; (5) two-dimensional (2-D) NMR techniques can probably be used to obtain spatial or connectivity information (via cross-relaxation or scalar coupling effects); (6) strongly dipolar coupled systems, e.g., the gel phase of DMPC, may not yield high-resolution spectra at convenient spinning rates; (7) data acquisition is at least an order of magnitude faster than with conventional techniques; (8) there is no requirement to subject samples to ultrasonic irradiation; and (9) there is no requirement for isotopic labeling (as with $^2H$).

In the future, it may be possible to obtain improved resolution, and to use $^1H$ $J$-coupling information. However, such experiments are expected to be somewhat difficult, since a typical $^1H$ high-resolution specification of 0.5 Hz corresponds to only 0.001 ppm at 500 MHz, a value not met by conventional MASS NMR probes. Thus, some efforts will have to be expended on probe construction, shimming, field-locking, and so forth. In addition, relaxation effects may prohibit some such experiments. Alternatively, various resolution enhancement aids may be applied to improve spectral resolution, since signal-to-noise ratios are high.

The ability to record highly resolved $^1H$ (1- and 2-D) spectra implies that a variety of *heteronuclear* 2-D experiments (involving, for example, $^2H$, $^{13}C$, $^{14}N$, $^{15}N$, and $^{31}P$) may be applied to unsonicated lipid (and biological) membranes. We hope the results presented in this publication spur such activities.

**Acknowledgment.** We are very grateful to Professor Myer Bloom and Drs. A. Vega and N. Zumbulyadis for their helpful comments on the origin of spinning sidebands in liquid crystalline systems.

**Registry No.** CHOL, 57-88-5; Na decanoate, 1002-62-6; decanol, 112-30-1; water, 7732-18-5; K oleate, 143-18-0; lecithin, 18194-24-6.

# Observation of a Mobile Molybdenum Carbonyl Fragment on $\gamma$-Alumina by Solid-State Carbon-13 Nuclear Magnetic Resonance Spectroscopy

**Thomas H. Walter, Arthur Thompson, Max Keniry, Sumio Shinoda,† Theodore L. Brown, H. S. Gutowsky, and Eric Oldfield***

*Contribution from the School of Chemical Sciences, University of Illinois at Urbana-Champaign, 505 South Mathews Avenue, Urbana, Illinois 61801.* *Received February 4, 1987*

**Abstract:** High-resolution solid-state carbon-13 nuclear magnetic resonance spectroscopic studies of a chemisorbed molybdenum hexacarbonyl fragment on $\gamma$-alumina are described. It is shown that chemical shift anisotropy information, obtained by analysis of spinning sideband intensities in magic-angle spinning spectra, can be used to study the mobilities of chemisorbed metal carbonyl fragments. Evidence is presented for very facile rotation of a $Mo(CO)_5$(ads) species about the surface–Mo bond.

In the past decade there has been growing interest in the preparation of heterogeneous catalysts by reaction of transition metal carbonyl complexes with high surface area metal oxides, such as transitional aluminas and porous silica.[1-4] In addition to providing novel catalysts, these supported complexes may also afford relatively well-defined surface structures, allowing the identification of important features of heterogeneous catalysts through systematic synthesis and characterization. Unfortunately, in most cases many structural questions remain after characterization by such readily available techniques as infrared spectroscopy, ultraviolet–visible spectroscopy, and temperature-programmed desorption.[3] The availability of solid-state carbon-13 nuclear magnetic resonance (NMR) spectroscopic data on these systems has thus been eagerly anticipated.[3,4] Although only highly mobile physisorbed species could be detected in the earliest $^{13}C$ NMR studies of supported metal carbonyls,[5] recent studies using solid-state NMR techniques such as magic-angle spinning (MAS) and cross polarization (CP) have shown that less mobile chemisorbed species can also be observed.[6-8]

One of the most extensively studied supported complexes is molybdenum hexacarbonyl on $\gamma$-alumina, which has been exam-

† On leave from the Institute of Industrial Science, University of Tokyo, 22-1, Roppongi 7 Chome, Minato-ku Tokyo 106, Japan.

(1) Yermakov, Yu. I.; Kuznetsov, B. N.; Zakharov, V. A. *Catalysis by Supported Complexes*; Elsevier: Amsterdam, 1981.
(2) (a) Bailey, D. C.; Langer, S. H. *Chem. Rev.* **1981**, *81*, 109. (b) Basset, J. M.; Choplin, A. *J. Mol. Catal.* **1983**, *21*, 95.
(3) Hartley, F. R. *Supported Metal Complexes*; D. Reidel: Dordrecht, Holland, 1985.
(4) Gates, B. C. In *Metal Clusters*; Moskovits, M., Ed.; Wiley: New York, 1986; p 283.
(5) (a) Ben Taarit, Y.; Wicker, G.; Naccache, C. In *Magnetic Resonance in Colloid and Interface Science*; Fraissard, J. P., Resing, H. A., Eds.; Reidel: Dordrecht, Holland, 1980; p 497. (b) Nagy, J. B.; Van Eenoo, M.; Derouane, E. G.; Vedrine, J. C. *Ibid.*, p 591. (c) Gelin, P.; LeFebvre, F.; Elleuch, B.; Naccache, C.; Ben Taarit, Y. *ACS Symp. Ser.* **1983**, *No. 218*, 455.
(6) Liu, D. K.; Wrighton, M. S.; McKay, D. R.; Maciel, G. E. *Inorg. Chem.* **1984**, *23*, 212.
(7) Toscano, P. J.; Marks, T. J. *Organometallics* **1986**, *5*, 400.
(8) Hanson, B. E.; Wagner, G. W.; Davis, R. J.; Motell, E. *Inorg. Chem.* 1984, *23*, 1635.

0002-7863/88/1510-1065$01.50/0 