# High-resolution proton and carbon-13 NMR of membranes: Why sonicate?

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# High-Resolution Proton and Carbon-13 NMR of Membranes: Why Sonicate?†

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ABSTRACT: We have obtained high-field (11.7-T) proton and carbon-13 Fourier transform (FT) nuclear magnetic resonance (NMR) spectra of egg lecithin and egg lecithin–cholesterol (1:1) multibilayers, using "magic-angle" sample spinning (MASS) techniques, and sonicated egg lecithin and egg lecithin–cholesterol (1:1) vesicles, using conventional FT NMR methods. Resolution of the proton and carbon-13 MASS NMR spectra of the pure egg lecithin samples is essentially identical with that of sonicated samples, but spectra of the unsonicated lipid, using MASS, can be obtained very much faster than with the more dilute, sonicated systems. With the 1:1 lecithin–cholesterol systems, proton MASS NMR spectra are virtually identical with conventional FT spectra of sonicated samples, while with $^{13}C$ NMR, we demonstrate that most $^{13}C$ nuclei in the cholesterol moiety can be monitored, even though these same nuclei are essentially invisible, i.e., are severely broadened, in the corresponding sonicated systems. In addition, $^{13}C$ MASS NMR spectra can again be recorded much faster than with sonicated samples, due to concentration effects. Taken together, these results strongly suggest there will seldom be need in the future to resort to ultrasonic disruption of lipid bilayer membranes in order to obtain high-resolution proton or carbon-13 NMR spectra.

One of the early goals of NMR studies of membrane structure has been to obtain a well-resolved NMR spectrum, which permits assignment of all sites present, allowing relaxation ($T_2$, $T_1$, $T_{1\rho}$) experiments to be carried out, in order to study the dynamics of membrane structure. In addition, more recent goals have included obtaining information on the spatial proximity of various groups, via two-dimensional NMR spectroscopy, and on order parameters, primarily by using $^2H$ spin-echo methods on labeled samples. Unfortunately, conventional NMR spectra of intact, unlabeled cell membranes, lipid–water, or lipid–cholesterol dispersions are basically uninformative lumps, which do not permit these types of study. It was shown early on, however, by Chapman et al. (Chapman & Penkett, 1966; Penkett et al., 1968; Chapman et al., 1968b) that ultrasonic dispersal of liquid-crystalline bilayers and intact biological membranes (Chapman et al., 1968a; Jenkinson et al., 1969; Kamat et al., 1970; Glaser et al., 1970) yielded small membrane fragments, vesicles, which did yield fairly high resolution spectra. Whether the structures of sonicated fragments are the same as unsonicated membranes and whether the line broadening or narrowing observed reflects intermolecular interactions as opposed to changes in vesicle size (Finer et al., 1972; Gent & Prestegard, 1974; Bloom et al., 1978) have, however, been topics of lively debate.

We thus tried some time ago to develop more nonperturbing methods of investigating lipid and membrane structure and reported $^{13}C$ NMR spectra of unsonicated lipids (Oldfield & Chapman, 1971; Keough et al., 1973), $^2H$ NMR spectra of unsonicated gel and liquid-crystalline lipids, and their interaction with cholesterol (Oldfield et al., 1971), and $^1H$ "magic-angle" sample-spinning (MASS) NMR spectra of gel and liquid-crystalline lipid dispersions (Chapman et al., 1972). To date, the $^2H$ NMR method has been quite successful and has seen widespread application to structural studies of gel and liquid-crystalline lipids, to the effects of sterols, peptides, and proteins on lipid structure (Seelig & Seelig, 1978; Kang et al., 1979; Seelig et al., 1981) and to proteins themselves (Smith & Oldfield, 1984, and references cited therein). However, selective deuteriation is often a difficult, expensive, and time-consuming process, so we have recently sought to reinvestigate the other nonperturbing techniques, $^{13}C$ and $^1H$ NMR, using state-of-the-art solid-state NMR instrumentation. In particular, we have followed up on the 1972 prediction that $^{13}C$ MASS NMR at high fields should be particularly informative (Chapman et al., 1972).

In this paper we report the results of our recent $^1H$ and $^{13}C$ MASS NMR studies of two archetypal model membrane systems: egg lecithin and egg lecithin–cholesterol (1:1), hand dispersed in excess water. The results indicate that resolution is at least as good as with sonicated samples, but because dilute samples are not required, spectra may be obtained in much shorter time periods, typically at least an order of magnitude faster, with MASS. In addition, our results demonstrate that numerous resonances in the $^{13}C$ NMR spectrum of cholesterol (in egg lecithin), which are "invisible" in sonicated dispersions, give very narrow lines in $^{13}C$ MASS experiments, so that ultrasonic dispersal of lipids (or other membranes) now seems to be an unnecessary step in obtaining highly resolved $^1H$ and $^{13}C$ NMR spectra of membranes.

## Experimental Procedures

*NMR Spectroscopy.* All $^1H$ and $^{13}C$ NMR spectra were obtained on a "home-built" NMR spectrometer, which operates at 500 MHz for $^1H$, using an Oxford Instruments (Osney Mead, U.K.) 2.0-in. bore, 11.7-T superconducting solenoid, together with a Nicolet (Madison, WI) Model 1280 computer system, a Henry Radio (Los Angeles, CA) Model 1002 radio frequency amplifier, an Amplifier Research (Souderton, PA) Model 200L radiofrequency amplifier, and either a Doty Scientific (Columbia, SC) MASS NMR probe or a 5-mm multinuclear solution NMR probe (Cryomagnet Systems, Indianapolis, IN). For $^1H$ MASS experiments, the 90° pulse widths were 8 μs, and spinning rates were ~2.4–2.8 kHz. For sonicated samples the $^1H$ 90° pulse width was 12 μs. For $^{13}C$ MASS, dipolar decoupled (40-W $^1H$ power) Bloch decays were recorded using 9-μs $^{13}C$ 90° pulse widths. For $^{13}C$ NMR of sonicated samples, 10-μs pulse widths were used,

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together with Waltz-16 proton decoupling (gated at 0.6 and 5 W), to ensure full decoupling and nuclear Overhauser effect, but without appreciable sample heating. Temperature was adjusted via a gas-flow cryostat, and values reported were gas-flow temperatures monitored with a Doric (San Diego, CA) Trendicator. All spectra were referenced to an external standard of tetramethylsilane, and high-frequency, low-field, deshielded, paramagnetic shifts are denoted as positive (IUPAC $\delta$ scale).

*Lipid Samples.* Egg yolk lecithin (EYL) was obtained from Lipid Products (South Nuttfield, Surrey, U.K.) and was used without further purification. Cholesterol (CHOL) was from Aldrich (Milwaukee, WI) and had been recrystallized 3 times from EtOH before use. EYL samples were prepared by addition of the appropriate amount of "100%" $D_2O$ (Sigma, St. Louis, MO) to dried EYL and either dispersed by hand or for three 6-min periods using a Heat Systems-Ultrasonics ultrasonicator (Plainview, NY). Sample temperature was kept low by having the sonicating vial surrounded by ice, and by agitating the sample for 2 min in the ice, between sonic bursts. EYL–CHOL samples were prepared by codissolving the appropriate amounts of EYL and CHOL in $CHCl_3$ and removing the solvent under an $N_2$ stream at ~40 °C, followed by evacuation over $P_4O_{10}$ for 24 h. The samples were then hydrated to the appropriate level (50 wt % lipid for an unsonicated dispersion, 10 wt % for a sonicated dispersion) and dispersed, basically as described above for EYL.

## Results and Discussion

We show in Figure 1 the 500-MHz $^1H$ NMR spectra of EYL and EYL–CHOL (1:1) samples, in excess water, using $^1H$ MASS (Figure 1A,C) or conventional high-resolution NMR of sonicated samples (Figure 1B,D). As can be seen by comparison of parts A and B of Figure 1, the $^1H$ MASS NMR spectrum of a 50 wt % hand dispersion (multilayers, Figure 1A) is virtually identical with that obtained with a dilute, sonicated dispersion (Figure 1B). We discuss the origin of the high-resolution MASS NMR spectrum, which is accompanied by several sharp spinning side bands (not shown), in detail elsewhere (Forbes et al., 1987). For completeness we comment on it briefly again, since the observation of a high-resolution $^1H$ MASS NMR spectrum is rather surprising, because the static dipolar Hamiltonian

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

does not in general commute with itself at different rotor orientations, $\phi$. However, in the liquid-crystalline phase, intermolecular dipole–dipole interactions are averaged by fast lateral diffusion, while fast axial rotation reduces the intramolecular dipole–dipole interaction and causes the angular dependence of the Hamiltonian to be the same for all proton pairs (Wennerstrom, 1973; Valic et al., 1974; Bloom et al., 1977). The dipolar interaction is thus scaled by the term $P_2(\cos\theta)$, where $\theta$ is the angle between the director axis and $H_0$, such that

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

and the Hamiltonian becomes

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

which under MASS commutes with itself at different rotor orientations, $\phi$. The result is that the dipole–dipole interaction is inhomogeneous in the sense that line narrowing, and the generation of sharp spinning side bands, is achieved at sample rotation rates much slower than the static spectral breadth. Thus, the static interactions are averaged by MASS at slow rates (Figure 1A) or by particle tumbling at much faster rates (Figure 1B), and the resultant spectra are very similar in appearance and are probably dominated by similar $T_2$ relaxation processes and scalar coupling effects. In any case, they appear the same, but (for the same probe and sample volume) the $^1H$ MASS NMR spectrum of EYL (or presumably any other fluid, smectic liquid-crystalline phase lipid) can in principle be obtained in a time period on the order of the square of the concentration ratio of the two samples. Thus, the MASS NMR spectrum of a 50 wt % lipid dispersion would be obtained $[(50/10)^2 \times 2/3]$ ~10–20 times faster than the spectrum of a 10 wt % sonicated dispersion, a welcome improvement. The 2/3 term represents the projection of the Zeeman magnetization onto the $x$–$y$ plane, due to the MASS orientation of the radio frequency coil, in terms of data acquisition time, a factor that will generally be mitigated by use of solenoid rather than Helmholtz coil geometry.

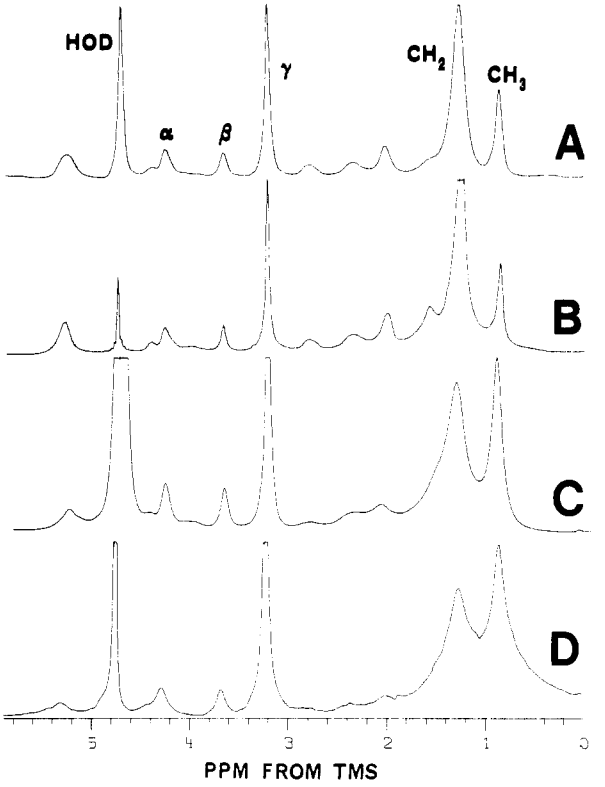


FIGURE 1: 500-MHz $^1H$ Fourier transform NMR spectra at 21 °C of EYL and EYL–CHOL (1:1) samples. (A) $^1H$ MASS (2.4-kHz) NMR spectrum of egg yolk lecithin (50 wt %)–$D_2O$. (B) $^1H$ NMR spectrum of sonicated egg yolk lecithin (12.7 wt %)–$D_2O$. (C) $^1H$ MASS (2.8-kHz) NMR spectrum of egg yolk lecithin (33 wt %)–cholesterol (17 wt %)–$D_2O$ (50 wt %). (D) $^1H$ NMR spectrum of sonicated egg yolk lecithin (8.5 wt %)–cholesterol (4.2 wt %)–$D_2O$ (87.3 wt %). An exponential line broadening of 8 Hz was applied to each spectrum. Recycle times of 5 s were used in all spectra shown.

We show in parts C and D of Figure 1 a similar MASS/sonicated dispersion comparison, which again demonstrates the close similarity between the two types of sample. In the complete MASS NMR spectrum [data not shown, but see Forbes et al. (1987)] we find that there are numerous (~7–8) spinning side bands, which, on the basis of experiments with a chain-deuteriated lecithin, originate in large part from the lipid hydrocarbon chain methylene group, which explains their relatively low intensity in the MASS NMR spectrum (compared with, e.g., the chain's terminal methyl groups). We believe this effect originates in the larger value of $\langle P_2(\cos\theta)\rangle$,

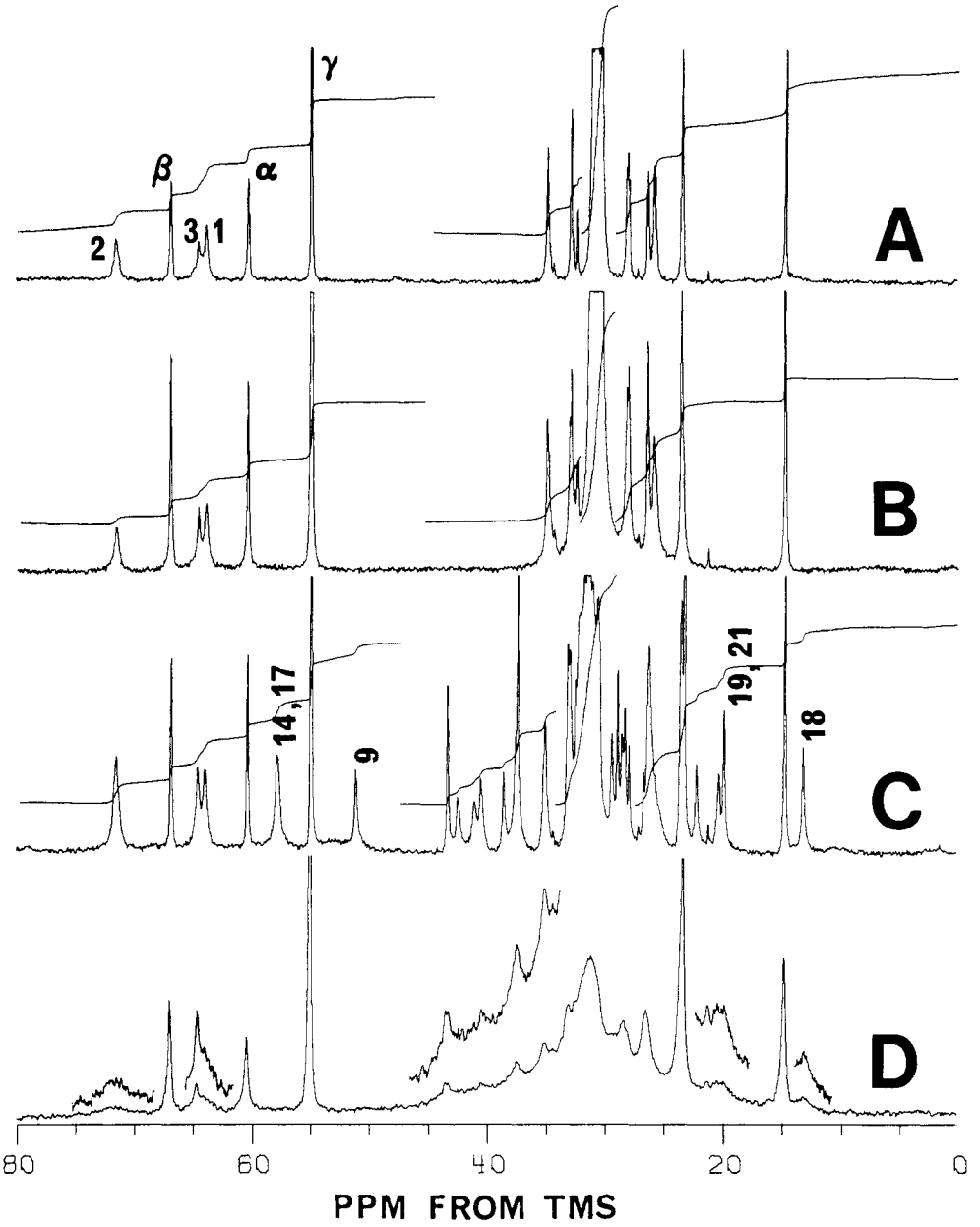


FIGURE 2: 125-MHz proton-decoupled $^{13}C$ Fourier transform NMR spectra at 21 °C of EYL and EYL–CHOL (1:1) samples. (A) Proton-decoupled $^{13}C$ MASS (2.1-kHz) NMR spectrum of egg yolk lecithin (50 wt %)–$D_2O$ at ~40-W CW $^1H$ decoupling. (B) Nuclear Overhauser effect enhanced, proton-decoupled $^{13}C$ NMR spectrum of sonicated egg yolk lecithin (12.7 wt %)–$D_2O$ at ~5-W WALTZ-16 $^1H$ decoupling. (C) Proton-decoupled $^{13}C$ MASS (2.8-kHz) NMR spectrum of egg yolk lecithin (33 wt %)–cholesterol (17 wt %)–$D_2O$ (50 wt %) at ~40-W CW $^1H$ decoupling. (D) Nuclear Overhauser effect enhanced, proton-decoupled $^{13}C$ NMR spectrum of sonicated egg yolk lecithin (8.5 wt %)–cholesterol (4.2 wt %)–$D_2O$ (87.3 wt %) at ~5-W WALTZ-16 $^1H$ decoupling. An exponential line broadening of 6 Hz was applied to each spectrum. Recycle times of 5 s were used in all spectra shown.

due to the condensing effect of cholesterol. What is even more surprising is that, on the basis of these deuteriation experiments, only the cholesterol side chain (and perhaps the angular methyl groups) contributes to the high-resolution spectra, in *both* parts C and D of Figure 1 (Forbes et al., 1987; Darke et al., 1971; Kroon et al., 1975). Presumably, short $T_2$ values for the rigid cholesterol nucleus cause excessive line broadening in both cases, but further work is needed to clarify this point. Nevertheless, the $^1H$ MASS NMR spectrum is essentially identical with that obtained with the more dilute, sonicated, suspension, as seen with the pure EYL system (Figures 1A, B). Given the same sample size, the $^1H$ MASS NMR spectrum can, nevertheless, be obtained much more rapidly than with the sonicated sample, as outlined above.

We now turn to a consideration of the $^{13}C$ NMR spectra of unsonicated EYL and EYL–CHOL (1:1). Parts A and B of Figure 2 show a comparison between the MASS (unsonicated, Figure 2A) and conventional FT (sonicated, Figure 2B) spectra of EYL. As expected on the basis of previous $^{13}C$ MASS NMR spectra of dilauryllecithin (Sefcik et al., 1983) and dimyristoyl- and dipalmitoyllecithin (Haberkorn et al., 1978) and similar $^{13}C$ NMR spectra of sonicated lipid dispersions (Metcalfe et al., 1971; Birdsall et al., 1972), the MASS and sonicated spectra are virtually identical. Thus, the only advantages to the MASS NMR method are that ultrasonic disintegration is unnecessary and spectra can be obtained (with use of cross polarization) about an order of magnitude faster than with conventional FT methods on sonicated dispersions. While these features are very welcome, they do not represent the "quantum" improvements shown in Figure 2C,D.

We show in parts C and D of Figure 2 a comparison between the $^{13}C$ MASS NMR spectrum of EYL–CHOL (1:1) (Figure 2C) and the conventional pulsed FT $^{13}C$ NMR

spectrum of a sonicated dispersion of EYL–CHOL (1:1) (Figure 2D). We believe the improvement in resolution using the MASS NMR technique (Figure 2C) is quite remarkable. In particular, many of the cholesterol carbons give rise to extremely well resolved 1- and 2-carbon peaks (Figure 2C) in the MASS experiment, while only a few broad features (Brainard & Cordes, 1981) can be attributed to cholesterol peaks in the sonicated spectrum (at 1:1 EYL–CHOL mole ratios). Especially obvious are the C9, C14, and C17 carbons, reported by Brainard and Cordes (1981) to be "unobservable" in sonicated dispersions, as exemplified in Figure 2D, where these peaks are, in fact, just not visible. Also obvious are the angular methyl groups (C18, C19, and C21) and a cluster of ring carbons in the 35–45 ppm window, which only give rise to very broad, weak features in the spectrum of the sonicated sample (Figure 2D). Presumably, the broadening in the $^{13}C$ NMR spectrum of Figure 2D is due to breakdown of the adiabatic approximation, due to fast ($\sim$1 MHz) isotropic vesicle rotation, for the "rigid" CH and $CH_2$ groups in the lipid bilayer. This effect may not be restricted to the cholesterol nucleus, since clearly C1($CH_2$) and C2(CH) of the glycerol backbone are similarly affected (as may be a number of lipid methylene chain groups), although further work will be required in order to rule out chemical exchange effects. The result of Figure 2D does show, however, that sonication may well *prevent*, rather than facilitate, acquisition of high-resolution $^{13}C$ NMR spectra of some molecules within lipid bilayers.

## Conclusions

We can draw the following conclusions, and make some predictions, from the results presented in this paper:

(1) High-field $^1H$ MASS NMR will generally yield narrow, multiline spectra for most fluid liquid-crystalline-phase lipid bilayer systems, from which isotropic chemical shifts can be determined.

(2) Other smectic, nematic, cholesteric, isotropic, and hexagonal lipid phases should also be amenable to study, by both $^1H$ and $^{13}C$ MASS, as long as they are reasonably fluid.

(3) Resolution in $^1H$ MASS will in general be about the same as with sonicated, liquid-crystalline systems.

(4) Resolution in $^{13}C$ MASS will in general be the same, *or even better*, than with sonicated, liquid-crystalline systems.

(5) Line width, $T_1$, $T_{1\rho}$, and $T_2$ values for numerous, resolved, single carbon atom, or individual proton, sites can be measured by MASS NMR techniques.

(6) The ability to record both high-resolution $^1H$ and $^{13}C$ MASS NMR spectra, from, e.g., the EYL–CHOL (1:1) system, implies that some homonuclear and heteronuclear two-dimensional NMR techniques may be applicable to nonsonicated liquid-crystalline lipid bilayer membranes. The opportunity may exist for determining both intramolecular connectivities and spatial proximities, in membranes, with 2D MASS NMR methods.

(7) For rigid species (e.g., the gel state of lipids, the cholesterol nucleus), $^{13}C$ MASS NMR methods are definitely preferable over sonication, or proton MASS.

(8) Data acquisition can be an order of magnitude faster with MASS than with conventional FT NMR (on sonicated samples), due simply to increased sample concentrations.

(9) In some cases, sonication can actually cause peaks to "disappear".

(10) MASS typically subjects membranes to an $\approx$40000$g$ force, typical for many biomembrane isolation protocols. While nonzero, this is probably an insignificant perturbing effect compared with the rigors of sonic disruption.

(11) Because of improved spectral signal-to-noise ratios, various resolution-enhancement aids should be applicable.

(12) There is no need to sonicate any lipid or membrane sample in order to achieve a high-resolution NMR spectrum, as far as we can determine on the basis of results we have achieved to date, on intact myelin and on pulmonary surfactant.

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We thank Professor Myer Bloom (University of British Columbia) and Drs. A. Vega (Du Pont) and N. Zumbulyadis (Eastman-Kodak) for helpful discussions on the origins of spinning side bands in $^1H$ MASS NMR spectra of smectic liquid crystals.

**Registry No.** Cholesterol, 57-88-5.

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## *Articles*

# Proton Nuclear Magnetic Resonance Studies of Mast Cell Histamine†

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ABSTRACT: The state of histamine in mast cells was studied by $^1$H NMR spectroscopy. Spectra were measured for histamine in situ in intact mast cells, for histamine in suspensions of mast cell granule matrices that had been stripped of their membranes, and for histamine in solutions of heparin. The $^1$H NMR spectrum of intact mast cells is relatively simple, consisting predominantly of resonances for intracellular histamine superimposed on a weaker background of resonances from heparin and proteins of the cells. All of the intracellular histamine contributes to the NMR signals, indicating it must be relatively mobile and not rigidly associated with the negatively charged granule matrix. Spectra for intracellular histamine and for histamine in granule matrices are similar, indicating the latter to be a reasonable model for the in situ situation. The dynamics of binding of histamine by granule matrices and by heparin are considerably different; exchange of histamine between the bulk water and the granule matrices is slow on the $^1$H NMR time scale, whereas exchange between the free and bound forms in heparin solution is fast. The chemical shifts of resonances for histamine in mast cells are pH dependent, decreasing as the intragranule pH increases without splitting or broadening. The results are interpreted to indicate that histamine in mast cells is relatively labile, with rapid exchange between bound histamine and pools of free histamine in water compartments confined in the granule matrix.

Mast cells are a major tissue storage site of histamine. In the adult rat (5–7 months old) 25 ± 5 μg of histamine is present per $10^6$ mast cells (Kruger & Lagunoff, 1981). To the limits of available methods of measurement, all of the histamine in a normal mast cell is contained in specialized storage granules. The mean volume of a peritoneal mast cell from a 5–7 month old rat is 1080 ± 80 $\mu m^3$, of which the aggregate granule volume represents 50% (Hamel et al., 1983). If the 25 pg of histamine in a mast cell were in solution in the total granule volume, the concentration of histamine would accordingly be 0.42 M.

It is reasonable to believe on the basis of equilibrium sedimentation in Percoll that the aqueous compartment comprises of the order of 10% of the granule volume (Kruger et al., 1980) and that much of the histamine is bound to the granule matrix rather than in solution. However, the binding site of histamine remains controversial, and the actual distribution of histamine between bound and free states in situ is unknown. In consideration of the large amount of intracellular histamine and the location of the chemical shifts of the imidazole protons in a biologically uncluttered portion of the $^1$H NMR[1] spectrum, it seemed likely that $^1$H NMR measurements performed on intact mast cells in suspension could add to an understanding of the status of granule histamine.

## Materials and Methods

Mast cells were washed from the peritoneal cavities of rats decapitated under $CO_2$ anesthesia and separated from the other cells on Percoll (Bauza & Lagunoff, 1983). Immediately before the $^1$H NMR measurements were made, the mast cells were washed twice in balanced salt solution (Bauza & Lagunoff, 1983) prepared in $D_2O$ (BSS-$D_2O$). Several million mast cells were suspended in 0.4 mL of BSS-$D_2O$; 0.25% 2-methyl-2-propanol was usually added as an internal chemical shift reference. Histamine released from the mast cells was assayed (Kruger et al., 1980) on supernatants after collecting the cells by centrifugation at the completion of the NMR experiments. Granule matrices stripped of their histamine

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[1] Abbreviations: NMR, nuclear magnetic resonance; BSS-$D_2O$, balanced salt solution in $D_2O$; Gly-His-Gly, glycylhistidylglycine; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; FID, free induction decay.

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