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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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ENTITLED Magic Angle Sample Spinning $^{13}$C Nuclear Magnetic
Resonance Spectroscopy of Myelin

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE
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Magic Angle Sample Spinning $^{13}$C Nuclear Magnetic Resonance Spectroscopy of Myelin

By

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Introduction

Membranes have been studied for many years because understanding their structure and dynamics is essential to understanding almost all living systems. On the cellular level, membranes control cell volume, nutrient uptake, and electrical gradients. On higher levels of organization, membranes are responsible for controlling action potentials in the central nervous system, providing exchange surfaces for oxygen uptake in the lungs, regulating specific nutrients passage into the digestive system, and many other vital functions.

Information on the way membranes are constructed and behave aid in understanding how they carry out their various vital functions. In the nervous system, the myelin sheath membrane is a crucial component of higher organisms. The structure of myelin and the extent of myelination are extremely important in understanding neural development and disease. Furthermore, the complexity of myelin is a useful gauge for the complexity of an organism and its central nervous system. The dynamics of membranes are also important for creating more useful anti-cancer agents and radioactive imaging agents. The effectiveness of anti-cancer drugs such as cis-platinin and technetium containing imaging agents are contingent on how well they cross specific membranes. Information about the dielectric nature of the membranes these drugs are targeted at can provide an
intelligent approach to creating better pharmaceuticals. Data on the nature of membranes can provide insight to these and many other medical and biological problems.

Many methods have been used for elucidating the structure and dynamics of membranes. Membranes are mainly constructed from lipids and proteins. In many membranes of interest, these components have been well characterized using simple quantitative techniques such as GC-mass spectrometry for lipids and gel electrophoresis for the characterization of their proteins. Other studies have been done to determine the supramolecular architecture of membranes using X-ray diffraction, electron microscopy, and freeze fracture techniques. In addition to structural studies, many investigations of the dynamics of membranes have been carried out. Until the advent NMR, the dynamics of membranes were probed using ESR, laser bleaching, and differential scanning calorimetry. NMR of 1H, 2H, 13C, 19F, and 31P nuclei have provided much additional insight on membrane systems. Not only do NMR techniques give information on the structure and dynamics of membranes, but they allow investigators to solve problems that were once considered intractable using the previously mentioned methods of examination.

NMR of Membranes
The first experiments that employed NMR to examine membranes were carried out about twenty years ago. In these early studies, it was shown that $^1$H and $^2$H NMR methods could be used to probe the phase characteristics of intact membranes, but this only confirmed what was already known from X-ray diffraction, and differential scanning calorimetry. In subsequent studies using $^1$H, and $^2$H NMR, the order parameters of various positions on the acyl chains in membrane lipids were calculated by selectively deuterating various positions on the lipids. This was possible because isotopic substitution on particular parts of the membrane allowed that part of the membrane to be studied with the remaining proton nuclei being "transparent" because of their very different resonance frequencies.

In addition to showing how specific sites in membranes are ordered, NMR was used to examine how lipid ordering in membranes changed as the chemical content of the membrane changed. Rance et al. showed that cholesterol increased the order of the acyl side chains in the lipids in the membranes of Acholeplasma Laidlawii, using $^2$H-NMR techniques. A similar study done by Oldfield et al. showed that increasing the amount of protein (cytochrome oxidase) in the membrane increased the order of the side chains of the lipids, the effects being largest near the chain termines.

Many investigators have since begun using NMR to study the phospholipid bilayers of membranes. The variety of
nuclei examined, systems looked at, and types of problems have become more diverse. Macdonald and coworkers used $^{19}$F substituted fatty acids to study phase behavior and cis and trans orientations in Acholeplasma Laidlawii B membranes via NMR. Another type of NMR problem was examined by Littlemore et al. In this study, the effect of lysophosphatidylcholine on the spectra of myelin basic protein was followed by $^{1}H$ and $^{31}P$ NMR spectroscopy. They showed that $^{1}H$ line broadening of the methylarginine residues was increased upon the protein's interaction with the lyssolecithin, indicating that the internal domain of the protein had undergone conformational changes. The $^{31}P$ spectra in this study suggests that the average phosphate group in lysophosphatidylcholine becomes more mobile as a result of binding the basic protein to the lipid micelle.

Solid State NMR

Although the previously mentioned NMR techniques have revolutionized the examination of membranes, recently one type of NMR study has proven to be the premiere method. This is Magic Angle Sample Spinning (MASS) NMR. For years $^{13}C$ NMR has been used to study the interaction of lipids with cholesterol. These studies have been carried out on sonicated unimolecular vesicles or on static multimolecular dispersions. This is a very inconvenient method because the creation of vesicles is a time consuming
process. Furthermore, the signal to noise ratio in these experiments was so small the investigators had to resort isotopic labeling to probe the sites they were interested in.28

Oldfield et al. have recently shown that high resolution spectra of lipids in the gel or liquid crystal phase can be taken without creating vesicles or using isotopic labeling.29 This technique involves the macroscopic rotation of the sample at a "magic angle" of 54.7° with respect to $H_0$. Others such as Andrew have used this technique to remove dipolar broadening.30 In 1983, Sefick et al. obtained the first spectra of a liquid-crystalline phase lipid employing MASS techniques.31 These early MASS studies were not very successful because they relied on single air bearing spinners that made sample spinning difficult. The high resolution spectra obtained by Oldfield and coworkers were made possible by the advent of double air bearing sample spinners. These rotors allowed fluid or liquid materials to be easily spun at high speeds.

Some of the advantages of the MASS NMR technique are the following:

1. It will yield narrow, multiline spectra for most fluid liquid-crystalline-phase lipid bilayer systems.

2. Resolution in $1H$ NMR will in general be about the same as with sonicated, liquid crystalline systems.
(3) Resolution in 13C NMR will in general be about the same, or even better, than with sonicated, liquid-crystalline system.

(4) Line width and order parameters for single carbon or proton sites can be measured.

(5) There is possibility to measure intramolecular connectivities and spatial proximities using 2D MASS.

(6) 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.

(7) In some cases, sonication can actually cause lines to "disappear".

(8) The force that the membrane sample is subjected to during macroscopic sample rotation is less than that in sonic disruption. [taken from 29]

It is clear that MASS is a superior way for experimentalists to examine liquid-crystalline lipid systems.

Myelin

Myelin is a multi-layered membrane that surrounds nerves in the nervous systems of higher order organisms. Its function is to increase the speed of action potentials in nerves, while conserving metabolic energy required to create the voltage gradients which drive the action potentials.2 It has high cross membrane resistance since it is comprised mainly of lipids.3
Myelin has been the subject of many studies on natural membranes. This is true due simply to the fact that, unlike most natural membranes, myelin is easily isolated in high purity. This is the case because all myelin, regardless of the source, will be suspended on 0.8 molar sucrose density gradient. One interesting aspect of myelin is it develops in some animals after birth and its chemical composition changes during the early stages of development. A study by Cuzner and Davison showed that at birth rats had little to no myelin. In addition, they showed that the sulfatides and phosphatidyl ethanolamine steadily increases from birth to adulthood, however the lecithin increases from birth but then decreases during adulthood. Another interesting aspect of myelin is that it is lacking in the victims of some genetically transmitted diseases. Several studies have been done to quantify the extent of demyelination in the central nervous systems and peripheral nervous systems of such mice strains as jimpy, shiverer, and quaker. Furthermore, some studies have even isolated the genes in chromosomes that control the production of the proteins in myelin. These genes have analogues in the human genome that manifest themselves as diseases such as multiple sclerosis, and krabbes disease. One other interesting aspect of myelin is that its supramolecular architecture changes with species. A X-ray and Neutron diffraction study by Blaurock showed that the intralayer
spacings and packing vary in animals from invertebrates (shrimp) to terrestrial vertebrates (humans). There is much biological information to be gained from the investigation of myelin including topics such as evolution, disease, development, and the nature membranes in general.

NMR has been used in the past to study myelin, but in a limited manner. These studies were limited to looking at the $^1H$ spectra of the myelin basic protein, and how it changed when incorporated into a lysophosphatidylcholine membrane. The spectra obtained using $^1H$ techniques have wide lines, small signal to noise ratios, and have so many lines it is difficult to make any sense of the spectrum. In the past, NMR of the complicated membrane, myelin, has been a sparingly useful method for probing structure and dynamics, but new technology will allow NMR to be a useful tool for studying myelin in the future.

Experimental (MASS 13C NMR of Myelin)

The method for isolating myelin is well known. In this study the myelin was treated in a consistent manner. Nerve tissue was homogenized and suspended in a 0.25 molar sucrose solution containing 1 mM phenylmethyisulfonyl-fluoride, and the final volume was adjusted to contain about 1 gram wet weight tissue per 10 ml of solution. The suspension was centrifuged at 10,000 X g for ten minutes in a Sorvall Superspeed Refrigerated Centrifuge. Next, the
pellet was resuspended in 0.88 molar sucrose solution containing 1mM phenylmethylsulfonylfluoride, and carefully layered over 15 ml of 1.2 molar in a centrifuge tube. Then, the fraction was covered with a 0.25 molar sucrose solution and the tube was spun by a SW-27 swinging bucket rotor in a Beckman L5-50 Ultracentrifuge at 53,500 X g for 70 min. The myelin collected at the interface between the 0.25 and 0.88 molar sucrose layers. This was carefully collected and washed twice in glass distilled water and resuspended in water. At that point the sample was either frozen or hard pelleted at 113,000 X g for twelve hours to prepare for data collection. All operations were performed at 4oC.

The human myelin sample was the kind gift of Mario A. Moscarello of the Hospital for Sick Children, University of Toronto. The goldfish were obtained from the Sailfin Pet Shop of Champaign, IL. The entire brain of 60 goldfish, weighing a total of 0.27 g, was used in the preparation of the goldfish myelin. The deer was obtained from the wiled, and the white mater from the posterior portion of the brain was used for the myelin preparation.

The central nervous system of a Horseshoe crab was homogenized but the homogenate yielded no myelin. The portion of the homogenate that would not float in water was pelleted and a spectra was taken.

All 13C 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 Henery Radio (Los Angeles, CA) Model 1002 radio frequency amplifier, an Amplifier Research (Souderton, PA) Model 200L radio frequency amplifier and a Doty Scientific (Columbia, SC) MASS NMR probe. The spinning rate were 2.5-3.0 KHz, and the pulse widths were between 8 and 9 usec. (spectra in figures 1 and 2).

Results and Discussion

In the myelin spectra, there are at least 53 unique resonances. These peaks represent either individual carbon sites in the various lipid components (see Table I.) of myelin or groups of carbons with chemical shifts that are so close that they can not be resolved. The signal to noise ratio is very good, and the lines are quite narrow, making these the most highly resolved spectra ever taken of natural membranes. Although the exact ordering of the individual sites is still being calculated, it is apparent that some of the lineshapes are different. One example of this is the peak at 20.4 ppm corresponding to C19 on cholesterol. In the deer myelin sample this line is broader than in the human sample, indicating that that cholesterol carbon at position 19 has less freedom in the deer myelin. Another interesting observation about the various spectra is that the goldfish myelin lacks three peaks in the 70-90 ppm region. These
peaks have been determined to be those of cerebrocides or sugar lipids. The horseshoe crab spectra had no peaks that were distinguishable from background noise indicating that it contained little to no lipids.

With highly resolved $^{13}$C spectra of natural membranes like those obtained in this study, membrane investigators can quickly and easily obtain data on the chemical make up, ordering, and dynamics of membranes. In addition, such spectra may aid in determining how individual lipids pack together in membranes and with what force they attract one another.
References


Figure Captions

Figure 1. A. Human myelin 13C NMR spectra. B. Goldfish myelin 13C NMR spectra.

Figure 2. Deer myelin 13C NMR spectra.
<table>
<thead>
<tr>
<th>Lipid</th>
<th>Composition of Myelin Lipids (% of total lipids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>25</td>
</tr>
<tr>
<td>Phosphatidyl Ethanolamine</td>
<td>14</td>
</tr>
<tr>
<td>Phosphatidyl Serine</td>
<td>7</td>
</tr>
<tr>
<td>Phosphatidyl Choline</td>
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</tr>
<tr>
<td>Sphingomyelin</td>
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<tr>
<td>Ceramide</td>
<td>1</td>
</tr>
<tr>
<td>Cerebrocides</td>
<td>25</td>
</tr>
<tr>
<td>Others</td>
<td>11</td>
</tr>
</tbody>
</table>
Figure 1.
Figure 2.