Deuterium and Phosphorus NMR of Microbial Membranes[1]

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Structure–function relationships in biological membranes present an impressive challenge. Membranes contain a large number of components—proteins, enzymes, lipids, carbohydrates, sterols—of which there are many different types.[2] Some structural information can be obtained from X-ray diffraction, but this is necessarily of low resolution due to the wide variety of diffracting species and the impossibility of obtaining an analogue of a single crystal. The membranes must necessarily be intact during the study, and preferably be in a functioning organism. A wide variety of spectroscopic approaches has been applied to the problem.[3] In early studies much information was obtained by using probe techniques, involving, e.g., ESR spin labels[4] or fluorescent analogues[5] of membrane components. Both techniques suffered from the limitation that the properties of the indigenous components had to be inferred from those observed for the labeled species. In the case of the spin labels this has been shown to be sometimes a misleading analogy.[6] The nonperturbing techniques that have shown the most promise are nuclear magnetic resonance,[7-10] infrared,[11] and Raman[12] spectroscopy.

In this Account we deal only with the phospholipid lipid components of membranes. These are shown schematically in Figure 1 along with some of their possible conformations. The phospholipids in membranes have long been thought to adopt a bilayer structure, with their hydrophilic groups directed toward external and internal aqueous media, and with the acyl chains in a fluid, partially ordered state akin to that of liquid crystals.[13,14] The fatty acyl chains contain a large number of single bonds about which gauche–trans isomerization can occur, as well as cis double bonds which prevent the chains' adopting straight rodlike shapes. One of our goals has been to determine the conformations of individual chain segments as a function of position. A concept that has been very useful in this analysis is that of an order parameter[7] which has found great utility in structural studies of liquid crystals.

If rapid interconversion between allowed conformational states takes place, it is evident from Figure 1 that there exists a direction about which the various states are centered, known as the director of ordering. As a first approximation we take this to be the normal to the bilayer plane. At any instant in time, a given chain segment will make a particular angle θ with this direction. The time average of this angle is related to the effective shape of the molecule at this segment. The bond-order parameter $S_b = (3\cos^2\theta - 1)/2$ gives us a measure of this property. If we account for the different intrinsic bond geometries by projecting this onto the long molecular axis, we have the molecular order parameter $S_{mol} = S_b/S_{geo}$, which has the advantage of a scale varying from 0 for no conformational preference to 1 for a segment perfectly aligned relative to the director (for CH_2 groups this would correspond to a trans conformation with the C–H direction normal to the long molecular axis).

A second useful property of lipids is their rate of molecular motion, including that of gauche–trans interconversion, overall molecular rotation, or translation parallel to the bilayer normal. Both the nature of allowed conformations and the rates of molecular motion determine what has been loosely defined as the "fluidity" of the acyl chains.[15]

For the hydrophilic headgroups of the phospholipids, a wide variety of conformations are possible, and it is much more difficult to estimate about which axis segmental ordering will take place.[9] We expect that dynamical conformational averaging will take place here also.

NMR of Membranes

In the earliest studies of membranes ^1H NMR was the only available technique. While some information

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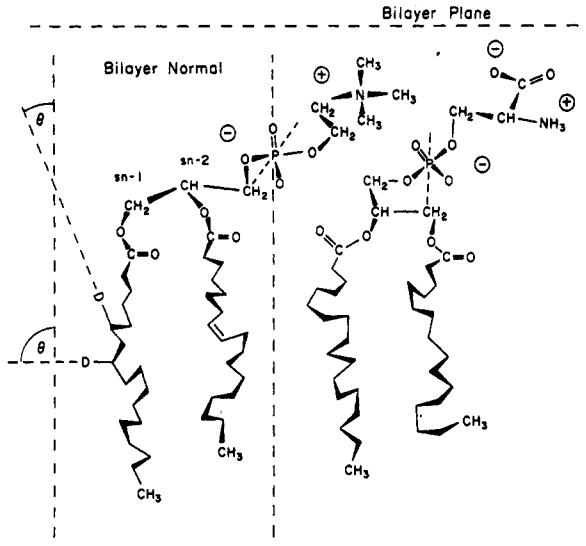

Figure 1. Schematic of the lipid molecules within a membrane bilayer. Both phosphatidylcholine and phosphatidylserine are represented. The angle θ is the instantaneous angle between a carbon–deuterium bond and the normal to the bilayer plane; this angle varies from one conformation to another. The axis of motional averaging may be other than the normal to the membrane, as shown for one of the phosphodiester groups.

can be gained,[16,17] ^{1}H NMR is complicated by the vast number of different types of hydrogen in a membrane and the limited chemical shift range. ^{13}C NMR at natural abundance also has yielded some useful results,[17] but the general utility of both ^{13}C and ^{1}H NMR of biological membranes is restricted by the rather broad lines encountered. Enrichment in ^{13}C,[18] high-power decoupling, cross polarization, and sample spinning at the "magic angle"[19] have helped to overcome some of these difficulties. Nonetheless, ^{13}C NMR of biological membranes remains a complex and expensive approach.

Major strides in the understanding of the properties of the lipids in biological membranes have been made via ^{2}H[7,8] and ^{31}P[9] NMR. Deuterium is employed with specifically labeled components and ^{31}P relies on the 100% naturally occurring isotope. Both make use of partially averaged anisotropy due to rapid motion within an ordered environment.

The magnetic properties of ^{2}H relevant to ordered systems[20] and the instrumental aspects of its observation[19,21] have been reviewed in detail recently. Detection sensitivity is no longer a problem; spectra from the membranes of intact organisms have been obtained in several minutes.[22] The spectra are often broad, and characterized by short spin–spin relaxation times (T_2), necessitating high-power pulses, rapid data acquisition, and the use of echo techniques.[23] Many of the instrumental requirements are not common on conventional high-resolution spectrometers, although instruments designed for the study of solids are usually well-suited for this purpose.

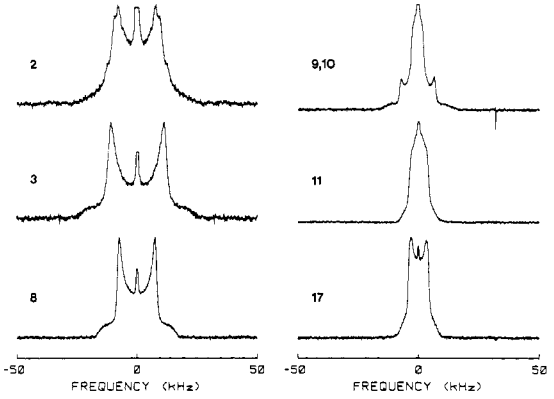

Figure 2. ^{2}H NMR spectra (41.3 MHz) of C18:1^c, specifically deuterated as indicated, in the plasma membranes of *A. laidlawii*, 25 °C. The spectra were obtained by using the quadrupole echo ($90°_x$–τ $90°_y$–τ acquire) technique with a 90° pulse duration of 5.5 μs, and τ = 40–60 μs. The samples comprised 100–150 mg of freeze-dried membrane, hydrated with deuterium-depleted water. The peak at the center of the powder pattern is due to residual deuterium in water.[26]

The property of deuterium that makes it very useful in membrane studies is its small quadrupole moment.[20] This couples with the electrical environment around the deuteron, to yield a quadrupole coupling tensor. Thus, in a rigid system, two NMR signals are observed whose separation depends upon the angles between the applied magnetic field and the principal axes of the quadrupole coupling tensor. The latter are related to the geometry of the molecule, and hence the quadrupole splitting is a measure of the orientation of the molecule relative to the magnetic field. For a small molecule rotating rapidly in solution, this anisotropy is averaged to 0 and only a single resonance is observed. In partially ordered samples the anisotropy is incompletely averaged, and the resulting quadrupole splitting is a direct measure of the segmental order parameter.

In ^{31}P NMR spectra of membranes, the useful anisotropy is that of the chemical shift of phosphorus in the phosphodiester moiety.[9] It is only partially averaged by rapid anisotropic motion, and the width of the spectrum is related to the degree of anisotropy of the motion. Due to the complexity of the geometry in the head group region, it is not possible to calculate an order parameter directly from the spectrum. Order parameters can be estimated by spectral simulations based on motional models.[24]

^{2}H NMR of the Membranes of *Acholeplasma laidlawii*

A large number of ^{2}H NMR studies have been performed on model membrane systems. These have been very useful in defining the properties of relatively simple systems and for developing the NMR techniques. Most of these studies are described in various reviews.[7,8,18,20,21,25] We shall confine our discussion to the intact membranes of the procaryote *Acholeplasma laidlawii*, the system studied in greatest detail to date.

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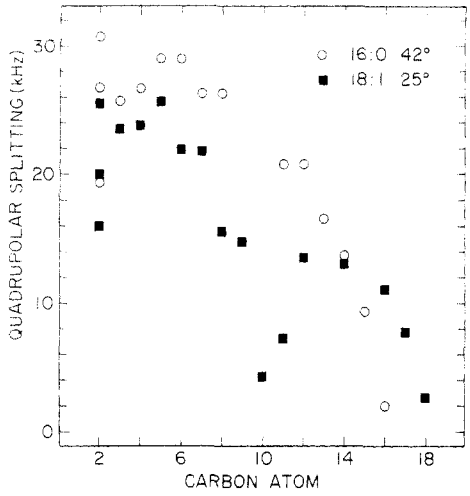

Figure 3. Dependence of the quadrupole splitting (proportional to the order parameter, S_{CD}) on position of labeling of palmitic acid (C16:0, 45 °C)[27] or oleic acid C18:1^c, 25 °C)[26] within the plasma membranes of *A. laidlawii*. Both systems were at temperatures above that of the transition from the gel to the liquid-crystalline state of the lipids.

This organism can be cultured on medium containing particular labeled fatty acids, with incorporation of these acids into membrane lipid to levels as high as 98% of total fatty acid. Thus, the position of labeling and the nature of the labeled fatty acid are well-defined. Many of the results we shall discuss were obtained in collaboration with the research groups of Professors Myer Bloom (Vancouver) and Ken Jeffrey (Guelph), to whom we are deeply indebted.

Figure 2 shows the ^{2}H NMR spectra of *A. laidlawii* membranes enriched in oleic acid, an 18-carbon chain with a cis double bond at position 9 (C18:1^c), labeled at different positions.[26] These are so-called powder spectra, rather than simple two-line spectra, because the membranes undergo rotational diffusion very slowly on the time scale of the ^{2}H quadrupole splittings. They comprise the sum of spectra corresponding to all orientations of the director of ordering with respect to the applied magnetic field. The relatively sharp doublets are due to director orientations of 90°; their separation D_q yields a direct measure of the bond-order parameter, $S_b = 4D_q(3e^2qQ/h)^{-1}$, where e^2qQ/h is the quadrupole coupling constant for deuterium in that particular chemical environment (170 kHz for aliphatic, 175 kHz for olefinic). This analysis assumes rapid, axially symmetric motion of the deuterated position within an ordered environment. If this does not hold, a minimum of two order parameters are required to describe the segment orientation. The quadrupole splittings for all measured positions are shown in Figure 3, where they are compared with the corresponding data for membranes enriched in the 16-carbon, saturated fatty acid, palmitic acid.[27]

The spectrum due to C18:1^c labeled at position 2 shows the presence of multiple quadrupole splittings. This is seen more convincingly after deconvolution to the 90° components only ("de-Pake-ing";[28] vide infra).

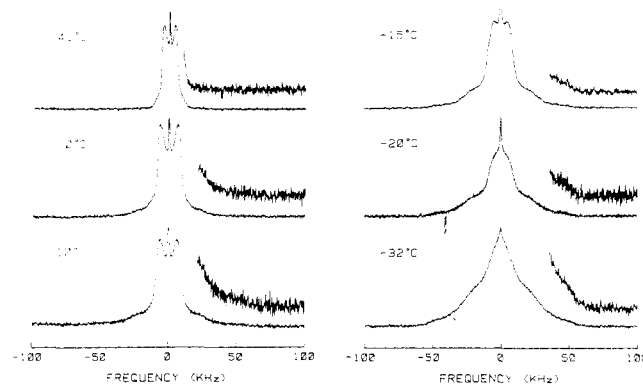

Figure 4. Temperature dependence of the ^{2}H NMR spectra (41.3 MHz) of *A. laidlawii* membranes enriched in [12,12-$^2H_2$]oleic acid.[26] Instrumental conditions were as in Figure 2. The vertical expansions (×4) are presented to demonstrate the presence of signal intensity in the wings of the spectrum.

There are several reasons for the multiple splittings: (i) the chains attached to the *sn*-1 and *sn*-2 positions of glycerol are not equivalent, (ii) the two deuterons on the chain attached at the *sn*-2 position are not equivalent, and (iii) the conformation of the acyl chain at carbon 2 depends on the nature of the lipid head group. These explanations are based in part on earlier studies on model systems.[29,30] Similar behavior for the *A. laidlawii* membranes is seen for C16:0,[27] as well as other fatty acids studied in our laboratory.[22] From position 3 on, no such inequivalences are detectable.

In Figure 3 we notice that the order parameter is high and roughly constant for the first eight to ten carbons of C16:0, whereas a large dip is observed for positions close to the double bond of C18:1^c. Correction for the geometry of the double bond, in the manner suggested by Seelig and Waespe-Šarčevič,[31] demonstrates that the double bond of C18:1^c is almost as well-ordered as the initial methylene segments. In both systems the order parameter decreases rapidly on approaching the end of the fatty acyl chain. These positional dependences of molecular ordering have been observed in a variety of model[7,8,31] and biological[18,22,26,27] systems and have been predicted by theoretical models.[32,33] Thus, lipid bilayers have regions with different degrees of order (populations of gauche conformers), approaching isotropic behavior near the center.

Gel-Liquid Crystal Transition of Membrane Lipids

It is known from both scanning calorimetry and X-ray diffraction studies that lowering the temperature of membranes leads eventually to a phase transition of the lipids from a mobile (liquid crystal) to a relatively immobile (gel) state. ^{2}H NMR has given us further insight into the nature of this state, as has infrared spectroscopy.[34] Figure 4 shows the temperature dependence of the ^{2}H NMR spectra of *A. laidlawii* membranes enriched in 12,12-$^2H_2$-C18:1^c. The 41 °C spectrum is typical for fluid, partially ordered chains. However, from 0 °C downward, a second component

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with a width of ca. 60 kHz appears and increases in relative intensity. This is the spectrum of the gel-state lipid—its width is indicative of highly ordered chains undergoing rapid rotation about their long molecular axes, as was reported earlier for C16:0 chains in *A. laidlawii*.[35] The coexistence of the two phases during the transition demonstrates that the membrane lipid is not in a condition intermediate between the two states, but is a heterogeneous mixture of the two. Further cooling of the sample results in the appearance of components of width ca. 120 kHz, indicative of the slowing down of the motion about the long axes. However, even at −32 °C some motion is still present, since there is a considerable contribution from relatively narrow components in the center of the spectrum. More detailed conclusions from the low-temperature spectra can only be made by resort to spectral simulations based on motional models. Quantitation of the relative amounts of the various phases can be achieved by means of the various moments of the spectra,[36] as has been shown recently for *A. laidlawii* highly enriched in myristic acid (C14:0).[22]

Influence of Cholesterol on Lipid Ordering

Addition of cholesterol to the growth medium results in the incorporation of as much as 35 mol % of total lipid in the membranes of *A. laidlawii*. With both C18:1^c and C16:0 we have found that this leads to large increases in molecular order,[37,38] with the largest absolute increases for the regions of the chains near the carboxyl group. This is in accord with earlier studies on the model system egg lecithin–cholesterol.[39] The larger increases for the upper regions of the chains are thought to be due to the rigidity and large cross section of the A–D ring[40] system of cholesterol, which is located in the bilayers in the region of carbons 2–10 of the fatty acyl chains.[41] Further details of these interactions will come from studies of labeled cholesterol, such as have been reported recently for the egg lecithin–cholesterol system.[42] Surprisingly, very little effect of cholesterol addition was noticed in the ^{2}H spin-lattice relaxation times of labeled oleic acid,[43] indicating that increased ordering does not necessarily lead to decreased rates for the rapid motions (vide infra).

Relaxation and Molecular Mobility

The spin–lattice relaxation time (T_1) of a nucleus is a measure of its rate of return to thermal equilibrium after excitation by radiofrequency.[44] During the 1970s such measurements became popular in the chemical community as indicators of rates of rapid rotational diffusion, both segmental and overall.[20,45] For asymmetric molecules, or molecules partially ordered in an anisotropic environment, calculation of rates of molecular motion or correlation times are much more complex. Various motional models must be invoked for the calculation of rotational rates about individual bonds or directions. Nevertheless, the T_1 values remain as good experimental parameters that can be interpreted by the various theories as they develop.

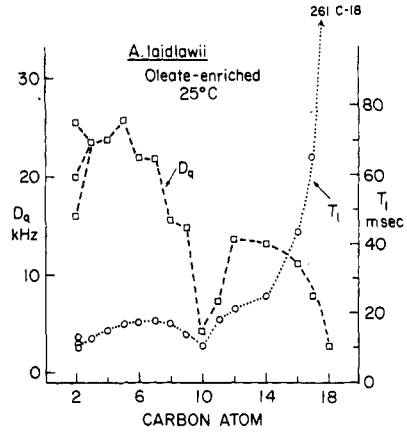

Figure 5. Comparison of the quadrupole splittings[26] (D_q, proportional to the order parameter S_{CD}) and spin-lattice relaxation times[43] (T_1, roughly proportional to the mobility of the segment) for specifically deuterated oleic acid in membranes of *A. laidlawii*, 25 °C.

Figure 5 compares the spin–lattice relaxation times and quadrupole splittings for ^{2}H in the membranes of *A. laidlawii*. The T_1 values are measured at the positions in the spectra corresponding to orientations of 90°; very little angular dependence has been noted, due probably to rapid lateral diffusion. We have considered the T_1 values in terms of a recent formulation,[46] which assumes rapid isotropic rotation within an ordered environment; $T_1^{-1} = (3/8)(e^2qQ/h)^2(1 - S_b^2)\tau_c$, where τ_c is the correlation time for the effectively isotropic motion. A plot of τ_c^{-1} (proportional to the rate of motion) vs. position looks essentially the same as that for T_1 in Figure 5. The qualitative conclusions we can draw from this figure are as follows: (1) from carbons 2–6 of C18:1^c, the rate of motion increases with position from the carboxyl group; (2) with approach toward the double bond at position 9, the rate of rapid motion decreases; (3) from the double bond toward the end of the chain, the rate of motion increases steeply. These data can be compared with the few points available from ^{2}H NMR studies of dipalmitoylphosphatidylcholine at 45 °C in the liquid crystalline phase,[46] where T_1 (54.4 MHz) varied only weakly from positions 3–8 (29 ms), rose significantly for position 12 (40.5 ms), and very strongly for position 14 (94.4 ms). Thus, there was a roughly inverse relationship between the bond-order parameter and the rate of motion. This is clearly not true for the cis double bond in *A. laidlawii*, where the T_1 (mobility) data go through a minimum at the same point as the order parameter, as one might expect due to the rigidity of the double bond. A similar restriction of mobility has been seen by ^{13}C NMR of the unsaturated fatty acyl chains in *Aureobasidium pullulans*.[47]

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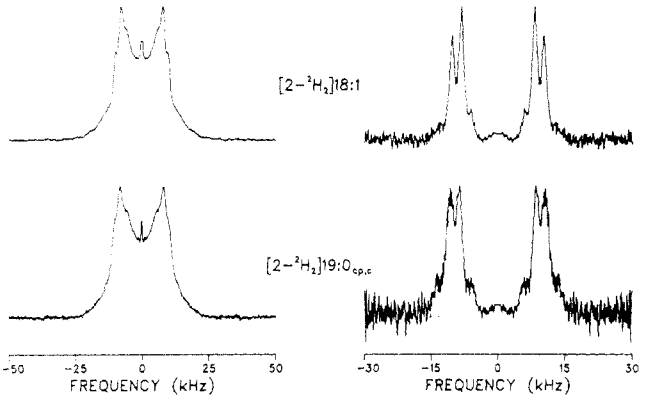

Figure 6. ^2H NMR spectra (left) and their "de-Pake-ed" (90°) analogues (right) for membranes of *A. laidawii* enriched mainly at the *sn*-2 positions of the membrane lipids with oleic acid (upper, 41 °C) or dihydrosterculic acid (lower, 25 °C) labeled with deuterium at carbon 2.[49] To obtain spectra of this quality approximately 400 000 acquisitions were required for 200–300 mg of membranes.

We may ask ourselves at this point, "what is fluidity?". It is widely accepted that unsaturated fatty acids "fluidize" biological membranes;[48] this usually means "lowers the temperature of the gel to liquid crystal transition of the membrane lipids". Our studies have shown that the rates of molecular motion in the liquid crystalline phase are in fact slower in the oleate-enriched membranes than they are in saturated model membranes. They have also shown that within the geometric constraints of a cis double bond, the oleate chains are ordered to a degree comparable with that of saturated chains in *A. laidlawii*. The "fluidizing" effect of C18:1^c must lie in the destabilizing effect of the cis double bond on the properties of the gel state, which for saturated lipids is characterized by all-trans chains, rather than in any significant change in the properties of the liquid crystalline state.

Recently we have turned our attention to transverse relaxation (T_2) of ^2H in lipid systems. The T_2 values are mainly sensitive to the rates and relative populations of slow motions (those with rates less than the ^2H resonance frequency). These can be estimated by the component line widths needed to simulate the ^2H powder patterns, or measured by following the response of the spectrum to different quadrupole echo times. With both methods we find often that T_2 varies strongly across the powder pattern. The source and exact angular dependence of this interaction are now under active study, as they should yield valuable insight into the nature of slow motions of lipids in membranes, which are those most likely to be influenced by other components such as protein and cholesterol.

Spectral Simplification by De-Pake-ing

When more than one ^2H powder pattern is present in a spectrum, it is often difficult to measure the quadrupole splitting or the relative populations of the components without resort to potentially ambiguous simulations. Recently a method, known as "de-Pake-ing", was introduced by Bloom and co-workers[28] to reduce an experimental powder pattern to the spectrum for a particular angle between the director and the applied magnetic field, Figure 1. This method has allowed a much greater resolution in the ^2H NMR method, as demonstrated in Figure 6, where the spectra have been de-Pake-ed to those for the 90° orientation. The spectra are due to *A. laidlawii* membranes whose lipids are enriched mainly at the *sn*-2 position with fatty acid labeled at carbon 2.[49] The powder spectra suggest the presence of three quadrupole splittings, whereas the de-Pake-ed spectra reveal at least four such splittings. It has been demonstrated that the two major splittings are due to glycolipid, whereas the less intense components are due to phospholipid in the membrane.[49] A detailed analysis of the de-Pake-ed spectra indicated the presence of five components, the fifth due to a minor population of lipid labeled at the *sn*-1 position.

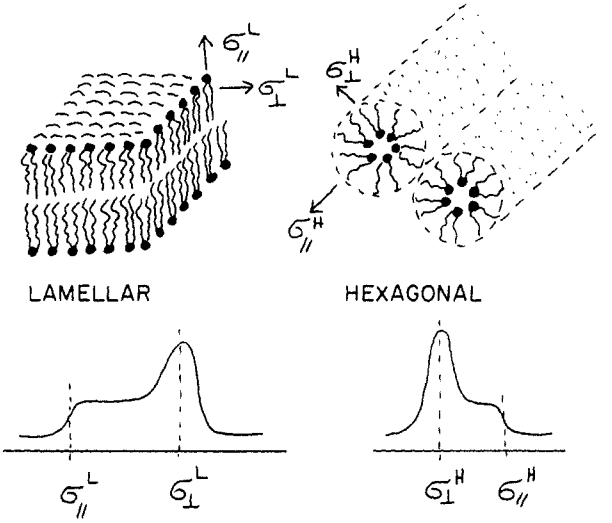

Figure 7. Representation of the lamellar and hexagonal arrangements of lipids, and their expected ^{31}P NMR spectra (proton-decoupled). The symbols $\sigma_\perp$ and $\sigma_\parallel$ refer to the components of the axially symmetric chemical shift tensor which are perpendicular or parallel, respectively, to the unique axis. Magnetic field increases from left to right; frequency decreases from left to right.

^{31}P NMR of Biological Membranes

^2H NMR has been largely used to probe the core of biological membranes at varying depths. ^{31}P NMR, however, provides information about the membrane surface.[9,50] The ^{31}P NMR spectral shape, assuming complete decoupling of the ^1H–^{31}P dipolar interaction, is governed by averaging of the chemical shielding tensor.[9] In phospholipid membranes the axially asymmetric static tensor is averaged to axial symmetry by rapid reorientation of the lipid molecule about a preferred direction, the director (Figure 1). The resonance frequency is determined by the angle between the director and the external magnetic field in a manner that depends upon geometric and motional factors, which may be estimated by spectral simulations.[24,51] However, the residual chemical shift anisotropy $\Delta\sigma$ ($\sigma_\perp - \sigma_\parallel$, Figure 7) has proven to be an exceedingly useful empirical parameter for the comparison of head group motion in different membrane systems,[9,50] for monitoring lipid phase transitions[9] and lipid polymorphism,[52]

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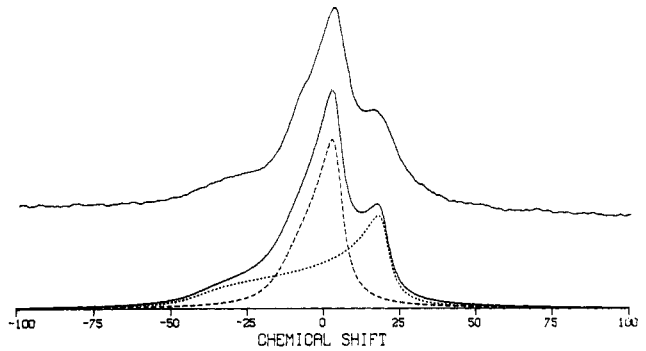

Figure 8. (Upper) ^{31}P NMR spectra (121.5 MHz) of the purple membrane from *Halobacterium cutirubrum*,[60] 15 °C; chemical shifts are expressed relative to phosphoric acid in a capillary tube. (Lower) Computer simulation of the above in terms of two axially symmetric powder patterns characterized by residual chemical shift anisotropies of −61 and −18.5 ppm for the phosphodiester and -monoester moieties of phosphatidylglycerophosphate. The component powder patterns are shown in dashes and dots.

the interaction of ions[53] and drugs[54] at membrane surfaces, and membrane fusion.[55] The assignment of a particular ^{31}P NMR line shape to a specific type of lipid organization, such as bilayer, hexagonal, or isotropic, requires supporting evidence from other techniques such as X-ray diffraction, freeze fracture, or NMR of nuclei in different parts of the molecules.

Figure 7 shows the two types of spectra most commonly seen in membrane systems. The spectrum of a lamellar system is roughly 40 ppm wide, with the $\sigma_\perp$ component on the high-field side. The extra degree of motional averaging in the hexagonal system leads to a $\Delta\sigma$ which is half that seen in the corresponding lamellar system and to a $\sigma_\perp$ on the low-field side of the spectrum. On passing from the fluid state of the fatty acyl chains to the more rigid, so-called gel state, $\Delta\sigma$ increases and the spectrum has broader edges.[9] In circumstances where the phosphate group can undergo rapid isotropic motion, be it in liquid solution or in an effectively isotropic environment in a membrane, a single narrow resonance at the isotropic chemical shift is observed.[52]

The similarity of the ^{31}P NMR spectra for a wide variety of membranes reflects the similarity in preferred head group conformation, motions, and chemical shielding tensors of the lipids involved.[9] Because the phosphate function is ubiquitous in biological systems, the ^{31}P NMR spectra of natural membranes may contain resonances associated with the presence of small molecules such as ATP, inorganic phosphate, or products of membrane degradation.[56] This may lead to difficulties in interpreting spectral characteristics associated with the membrane. Some of these problems may be overcome by selective detection techniques such as saturation transfer,[57,58] in which one of the spectral features is removed, and cross polarization in which one

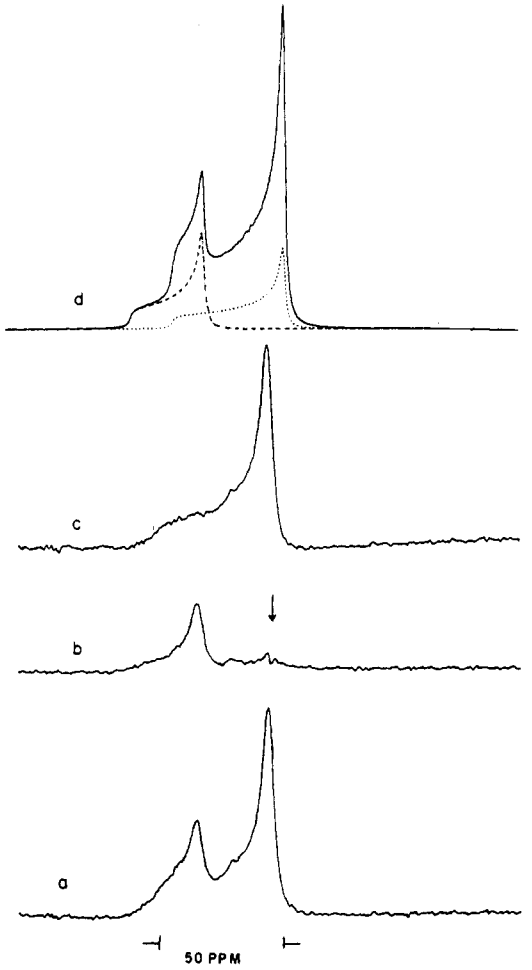

Figure 9. ^{31}P NMR spectra (121.5 MHz) of an aqueous dispersion of a mixture of phosphono- and phospholipids:[58] (a) usual ^1H-decoupled spectrum; (b) as in (a), but with a saturating pulse applied at the frequency indicated by the arrow; (c) spectrum a − spectrum b; (d) simulation of spectrum a in terms of two axially symmetric powder patterns with $\Delta\sigma$ = −46 and −30 ppm for the phospho- and phosphonolipids, respectively.

spectral feature is enhanced relative to the others.[59]

In the case of the purple membrane from *Halobacterium cutirubrum*[60] an additional complication arises in the ^{31}P NMR spectrum from the presence of two types of phosphate esters. The major lipid is phosphatidylglycerophosphate, which contains phosphate di- and monoester functions. Due to the significantly different components of the chemical shielding tensors associated with the two phosphate functions, the residual anisotropies differ greatly. The resulting ^{31}P NMR spectrum consists of two overlapping patterns (Figure 8). The interpretation of the spectrum required simulations of the component powder patterns, as shown in the lower part of the figure. From the anisotropy of the subspectrum attributed to the phosphodiester group of the membrane lipid, a bilayer arrangement of the membrane lipids was inferred.[60]

In another organism under study in our laboratory, *Tetrahymena pyriformis*, an unusual phosphorus-containing lipid is present, in which the phosphate ester is replaced by a phosphonate function.[58] Since the chemical shift tensor elements and their orientation in

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the molecular frame of reference are sensitive to the nature of chemical bonding to the phosphorus atom, the ^{31}P shielding anisotropies of phosphate and phosphonate esters are considerably different.[58] These differences lead to the spectra shown in Figure 9. Unlike the overlapping spectra shown in Figure 8, Figure 9 indicates a significant downfield shift of the phosphonate spectrum relative to that of the phosphate. Deconvolution of the spectra was achieved by saturation of the subspectrum associated with the phospholipid.[58] The separated ^{31}P spectra could then be analyzed and assigned to a bilayer arrangement of the lipids. The ability to change the chemical shielding at the phosphorus nucleus without affecting other physical properties of the lipid molecule in the membrane suggests a method for selectively monitoring a single lipid class in the presence of other phospholipids[58] as has been proposed by other workers.[61]

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^{31}P NMR spectra of membranes are relatively easy to obtain. However, care must be paid to their interpretation since chemical shielding tensor components must be known, and since the angle made by the director with the principal axis of the shielding tensor can alter the spectrum dramatically.[51] Order parameters and correlation times for motions can only be obtained by spectral simulation, and these are often ambiguous. ^{31}P spectra provide a facile first look at a system to see if changes have occurred. Quantitative or speculative conclusions should be confirmed by other methods.

Overview

Magnetic resonance of membranes has reached a high level of sophistication in detection sensitivity and interpretation. Many of the theoretical and technical problems have been solved, and the methods are ready for application to complex biological systems. A high yield of biologically significant conclusions can be expected.

Cobalt(II) as a Probe of the Structure and Function of Carbonic Anhydrase

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Carbonic anhydrase, the premier zinc enzyme, has fascinating biochemical, chemical, and physicochemical properties. Its natural function is that of catalyzing the reversible hydration of carbon dioxide, which it does with one of the highest known turnover numbers.[1] Other catalytic activities are displayed in vitro; in particular, the enzyme is active towards hydration of aldehydes and hydrolysis of esters. The enormous amount of data obtained under different experimental conditions and with the aid of many techniques has often appeared inconsistent within the framework of the proposed models. Striking examples are provided by buffered and unbuffered carbonic anhydrase solutions that may exhibit completely different chemical properties, because small anionic molecules, like sulfate,[2] phosphate,[3] or acetate[4] are capable of interacting at the metal active site. Our purpose here is not to review the field comprehensively, as others[5-7] have but rather to elaborate a structural model of the enzyme that is capable of accounting for its very many physical and biochemical properties.

Ivano Bertini was born in Pisa, Italy, in 1940. He received a Doctor degree at the University of Florence in 1964. After spending a year as research associate, he joined the faculty of the University of Florence and rose to the rank of Professor in 1975. He was research associate at Princeton University, in 1968–1969. His research on the electronic properties of metal ions in systems of biological interest involves spectroscopic investigation of metalloproteins containing paramagnetic metal ions, as well as metal-substituted zinc proteins.

Claudio Luchinat was born in Florence, Italy, in 1952. He received a Doctor degree at the University of Florence in 1976, where he continues to work as research associate.

The physicochemical investigation of the cobalt(II)-substituted enzyme (CoCA) has recently been pursued extensively with gratifying results. High-spin cobalt(II) is being recognized as a powerful spectroscopic probe in biological systems. The cobalt(II) enzyme displays an activity sometimes lower and sometimes larger than the native enzyme, depending on the isoenzyme and on the substrate.[6] The catalytic properties and their pH dependence have been shown to be similar to those of the native enzyme,[8] although the available data on the kinetic studies overwhelmingly are for the native enzyme.

We attempt here to provide a unifying picture of the structure and function of carbonic anhydrase on the basis of recent results obtained on its cobalt derivative. The model rests on the following assumptions: (i) that the kinetic properties of native carbonic anhydrase can be transferred to its cobalt derivative and that (ii) the structural properties obtained from spectroscopical investigations of CoCA can be transferred to the native

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