Biological Nuclear Magnetic Resonance Spectroscopy

New areas of research have been opened by developments in instrumentation and techniques.

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Nuclear magnetic resonance (NMR) spectroscopy provides information about atoms and molecular fragments that is of interest to physicists, chemists, and biologists. The data from NMR experiments can be interpreted in terms of structural features and dynamical phenomena; in many cases the results can be obtained in no other way. The benefits of NMR are, of course, highly dependent on having good experimental design; not only must the chemical system under study be well chosen to provide a limited problem in spin physics, but also the instrumentation employed must be capable of performing the necessary nuclear spin manipulations, provide adequate chemical shift resolution, and have sufficient sensitivity for detection of the signals.

To interpret NMR results, the data obtained must be for a nuclear site that can be assigned to a particular part of the molecule either chemically or spatially, and that site must have well-defined nuclear spin interactions. Only when there is a single dominant interaction determining the behavior of a nuclear site can the problem be sufficiently limited in scale to be interpreted. Since interactions capable of affecting a nuclear spin are either specific for an individual atomic site or short-range through space effects, a number of different strategies can be employed, depending on the information desired. The localized character of nuclear spin interactions means that spectroscopic data from complex macromolecules are usually as readily interpreted as those from small model compounds. Implicit in any detailed analysis of NMR parameters is the resolution of resonances due to nuclei that are in different environments by virtue of their chemical bonding, neighboring atoms, or geometry or, most significantly for biochemistry, being in chemically identical but different local structural environments on a macromolecule.

In this article I emphasize the aspects of NMR that are most pertinent to the study of biological systems. However, this is a difficult partition to make. In pursuit of biochemical information, many ideas and techniques of physicists and chemists have been exploited. Biological NMR encompasses studies of living organisms and intact organs, isolated macromolecules and cell constituents, and many small molecules, especially water and metal ions, that interact with biomolecules.

Background

Much of the work on biological problems has employed the chemical shift of a particular nucleus, usually hydrogen, to monitor its electronic environment and thus indicate both its covalent structure and any sequence-induced structure. This approach has been particularly fruitful for proteins because they are constructed from about 20 different subunits and a large amount of chemical shift dispersion is induced by their secondary and tertiary structure. Since the spectrum of ribonuclease was reported in 1957 (*1*), an enormous number of papers on protein NMR have been published; the results have been reviewed by Wüthrich (*2*), Roberts and Jardetzky (*3*), and Komoroski *et al.* (*4*), among others. Nuclear magnetic resonance spectra of proteins have been used to determine titration curves of individual residues, distinguish conformational states, measure rates and binding constants for small molecules and metals, determine structure, and describe intramolecular motions. The last is a relatively new area, primarily because it awaited the availability of pulsed spectrometers that are capable of observing nuclei whose relaxation properties are amenable to interpretation.

In the past, NMR studies of membranes and nucleic acids have generally not been as successful as protein studies. This is because it is much more difficult to resolve chemical shifts among phospholipids or the four nucleic acid bases, and because these biological structures are not well approximated by isolated spheres, which is necessary for ready application of liquid state theoretical formulations and instrumentation. Many of the early membrane studies have been reviewed by Lee *et al.* (*5*). Recent NMR experiments on membranes have been extremely successful because the systems have been considered as solids or liquid crystals rather than liquids, and partially averaged characteristics of solids such as deuterium quadrupole splittings and carbon or phosphorus chemical shift anisotropy have been measured. From this work detailed pictures of phospholipid motions and phase transitions have emerged. These newer membrane studies required sophisticated instrumentation that is not generally available and the application of procedures that have been developed recently.

With one major exception, the use of NMR to study polynucleotides has not been successful for chemical and instrumental reasons, although much valuable information has been obtained for oligonucleotides and model systems (*6, 7*). Nuclear magnetic resonance studies of purified transfer RNA have proved extremely important in demonstrating the presence of secondary and tertiary hydrogen bonds in solution and verifying the crystal structure (*8, 9*).

The list of known nuclear spin interactions is short, and for any experimental problem in biochemistry only a few need be considered. The physical basis and chemical applications of these interactions are well understood and are treated in textbooks (*10, 11*) and review series (*12*). These interactions occur between nuclei with like spins, between nuclei with unlike spins, between nuclei and applied magnetic fields, and between nuclei and electrons. The most important site-specific interactions are chemical shielding, which is due to slight alteration of the applied magnetic field by electron fields and is represented by a second rank tensor and the nuclear quadrupole interaction which occurs for nuclei with a spin quantum number greater than 1/2 where there is an electric field gradient at the nucleus. The interactions through

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space are hetero- and homonuclear dipolar couplings, electron-nucleus couplings, and hetero- and homonuclear scalar spin-spin couplings. With the exception of spin-spin couplings, all of the spin interactions are anisotropic; this means that different values are associated with different angles with respect to the applied magnetic field, although in many situations, especially in liquids, only a time-averaged isotropic quantity is observed. Only recently has the anisotropic character of these effects been used to provide biochemical information, and in large part this is due to new instrumental capabilities.

Nuclear magnetic resonance spectroscopy is an extremely active field even though it is 30 years old (*13*), and biological applications at the level of macromolecules began around 20 years ago (*1*). New experiments and applications of old ideas to new systems appear constantly in the literature. In many cases it is the development of more instrumental capability that underlies a new piece of work, and NMR is certainly one of the fields where the leaders are often those with the best instrumentation. In a historical sense and in the case of current investigations, much of the potential of NMR has been obvious, yet the realization in terms of obtaining data has often been restricted by technical limitations. The experimental capabilities are a combination of instrumentation and methodology that it is particularly appropriate to consider in this issue.

A number of laboratories are actively involved in the investigation of fundamental spectroscopic aspects of NMR and the number is increasing. This high level of activity on the part of spectroscopists is important because of the close relationship between the most physical and biological aspects of NMR. Part of the mixture of disciplines is on a personal level, since a number of the leading biological NMR research groups are headed by scientists who have been physicists for a large part of their careers. In addition, much of the information of interest available from NMR is cast in the language of physics, especially with regard to molecular dynamics and nuclear spin interactions.

Spectrometer

An NMR spectrometer is used to detect and manipulate nuclear spins. The presence of nuclear spins that resonate at a particular frequency, along with any fine structure, can be determined quantitatively by a number of detection schemes in either the time domain or the frequency domain. To be observed, spins must be manipulated by applying a strong radio-frequency (RF) pulse; it is also possible to significantly alter the interactions among spins with various pulsed or continuous applications of RF energy or mechanical sample spinning. This capability allows the extraction of much data from chemical systems and is very much a function of the instrumentation in use.

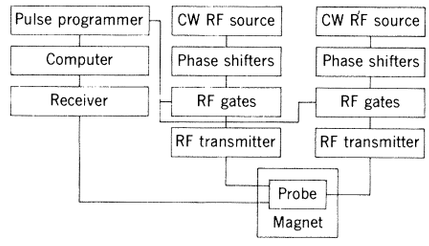


Fig. 1. Block diagram of an NMR spectrometer.

The design and construction of NMR spectrometers have been discussed extensively in the literature. The best introduction to the subject is the textbook by Farrar and Becker (*14*); and some of the most sophisticated equipment has been described in review articles (*15, 16*) and is described in the research literature on a continuing basis. Many types of commercial spectrometers are available which are suitable for routine structure determination or most research applications. One thing that characterizes this instrumentation is its high cost, particularly for research equipment. A "home-built" research spectrometer costs well over $100,000, and a very-high-field multinuclear commercial spectrometer may cost up to $500,000.

Figure 1 is a block diagram of a complete NMR spectrometer. At this level of detail, virtually all NMR spectrometers look the same; however, in many cases one of the channels is used only in a mode of constant irradiation frequency and phase (for decoupling). An important point here is that a wide variety of experiments are performed, and they differ enormously in specific instrumental requirements within the framework of the boxes in Fig. 1. Because of the severe loads placed on individual components, there is no such thing as a typical research spectrometer, much less an optimal spectrometer. For different biochemical experiments the most appropriate instrumentation may range from that of an analytical chemist to that of a solid state physicist.

Because the Zeeman interaction is dominant for most nuclei of interest, a magnet is the central feature of a spectrometer. A large static magnetic field (H_0) that is very homogeneous across the sample volume and very stable is required. Spectrometers currently in use have fields between 1.4 and 8.5 teslas (*17*), and most new research instruments have fields above 3.5 teslas. The most rigorous homogeneity requirements occur in NMR, with high-resolution proton (^{1}H) studies needing better than 1 part in 10^9.

In most research spectrometers constructed today superconducting solenoids are used to generate the magnetic field (*18*), although electromagnets are still in use in many laboratories. Cryomagnets have the advantages that they can generate much higher fields than electromagnets (which are limited to about 2.3 teslas), have an intrinsically very high stability that eliminates the need for field frequency locks, have much lower operating costs, and do not depend on electricity or cooling water. At the present state of superconducting magnet technology, there is a trade-off between the size of the open bore and the field strength, with large 9- or 10-centimeter openings possible only at intermediate field strengths (3.5 to 4.7 teslas). Because of the effects of field strength on resolution and the requirements for sensitivity for rare spins, it is not unusual for a commercial spectrometer to be sold with one console and two magnets so that an optimal magnet configuration can be employed. Very homogeneous magnetic fields are obtained by virture of coil design, superconducting shim coils, and room-temperature shim coils that correct up to 12 direction gradients. Samples are routinely spun along one axis to average residual magnetic field inhomogeneities. A few years ago, when nearly all biological NMR studies involved the detection of protons in very similar chemical environments, it was frequently the case that the larger the magnetic field the better, and new spectrometer models were regarded as advances according to their field strength. This is why more emphasis has been placed on the development of the magnet than of any other part of the spectrometer and reflects the economic pressure on instrument companies from analytical chemistry and biochemistry rather than physics. Today, the choice of field strength depends on the experiments to be performed, particularly on the details of nuclear relaxation mechanisms that are effective and on sample size limitations.

All of the blocks beside the magnet in Fig. 1 involve electronics suitable for the generation or detection of several dif-

ferent radio frequencies, audio frequencies, and digital signals. Most NMR experiments involve pulsed Fourier transform operation, where an intense RF pulse near the nuclear resonance frequency rotates the nuclear spins from the direction parallel to H_0 (z) to the (x, y) plane for detection of the dephasing of the spins as a function of time (free induction decay). The signals from a large number of free induction decays are averaged to improve the signal-to-noise ratio and then Fourier-transformed to provide a frequency domain spectrum. Although continuous wave (CW) NMR has been superseded as a technique for high-resolution studies by pulsed NMR, there are some applications where it is useful. In particular CW/NMR combined with rapid scanning and cross correlation (correlation spectroscopy) has been used to detect weak signals in the presence of nearby strong signals by not exciting the unwanted nuclei. Only the specific instrumental requirements for pulsed spectrometers will be discussed in this article; however, a CW spectrometer can be thought of as a large array of frequency-specific spectrometers hooked together which deliver a weak pulse at a particular frequency. The frequency range of interest is then swept by pulsing the frequency channels sequentially. A pulsed spectrometer excites all frequencies at once rather than one at a time, thus the greater efficiency of the Fourier transform (FT) mode is obvious from this well-known "multichannel spectrometer" analogy (*19*).

Several variable and very stable RF sources are required, and either voltage-controlled crystal oscillators or (preferably) frequency synthesizers can be used. The phase shifting (relative delays of RF waves), gating (turning on and off), and receiving are often done at a fixed intermediate frequency to allow optimal design of tuned components, while a wide range of final frequencies can be obtained by mixing with the variable sources to provide multinuclear capability. The basic signal generation and manipulations are performed at low RF power levels, but the final pulses going to the sample are amplified enormously with a series of driver and final power amplifiers that can be tuned or broadband. A final pulse is usually in the range 100 watts to 1 kilowatt. The RF field at the frequency of the observed spin is designated H_1, and the field at another frequency for decoupling or manipulation of other spins is H_2. In many cases a third irradiation is required as a basis for a field-stabilizing lock, particularly in electromagnets.

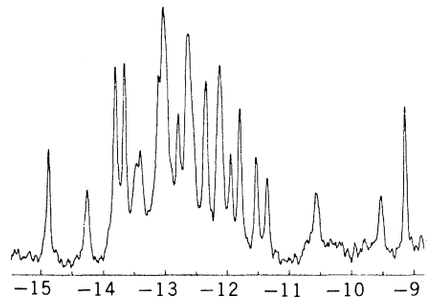


Fig. 2. Proton NMR spectrum of the exchangeable protons in the hydrogen bonds of *Escherichia coli* $tRNA^{Val_1}$ in H_2O, obtained in a very high magnetic field (8.5 teslas) using correlation spectroscopy. [From (*24*)]

Many experiments require considerable sophistication in the low-level RF sections in order to provide the differently timed and phased pulses needed to manipulate the spins. These functions are controlled by the pulse programmer, a digital timing device interfaced to the computer. Pulse programmers are capable of controlling multiple independent outputs with great speed and reliability and in some cases have considerable independent programmability. In the future, microprocessors will be used to control the spectrometer in a very intelligent manner.

The RF generating and receiving sections of the spectrometer are joined at the probe, which is a circuit tuned at the resonance frequency of the nuclei of interest. The coil of the circuit is used to generate the fields for manipulating the spins of the sample. Severe loads are placed on the probe, since it is used for irradiating with large fields and then must switch quickly to receiving weak fields from the sample. In addition, many experiments with pulsed spectrometers require extremely homogeneous RF fields. A number of configurations of probe components are used, the most efficient being a single-coil solenoid arrangement, where the same inductor is used for receiving and transmission. A crossed-coil arrangement uses two orthogonal coils for the individual functions. Both solenoids and Helmholtz saddle coils are used, depending on the sample and magnet geometry. Because the field of a superconducting magnet is vertical in the laboratory, it is almost essential to use Helmholtz coils so the sample tubes can be spun vertically.

Much of the sensitivity of a spectrometer depends on the receiving section, where the signals from the nuclear spins are amplified, filtered, and detected. The RF signals are converted to audio frequencies by mixing with reference signals. The audio signals are then digitized and stored in a computer. Signal averaging is performed by coadding free induction decays. In addition to its important role as a signal-averaging device, the computer is used to Fourier-transform the averaged free induction decays to provide a frequency spectrum. The computer is also used to control the RF signals through the pulse programmer and to manipulate the acquired data.

High-Resolution Nuclear Magnetic Resonance of Inidividual Sites

Much of the interest in high magnetic field strengths has been due to the volume of research on ^1H NMR of highly structured soluble macromolecules. In this case, the higher the field the greater the sensitivity and resolution. Progress in ^1H NMR has not been limited by RF technology because of the low power and low RF homogeneity requirements. In fact, pulsed FT NMR has had much less impact on proton than on other nuclear magnetic resonance spectroscopy because the use of large fields takes care of much of the sensitivity problem, the resonances do not have a wide frequency spread, and by the time very dilute solutions of biological macromolecules are investigated the interference problem from the solvent protons is so severe that a variation of CW signal averaging is often the best procedure.

The study of spin 1/2 nuclei in molecules in solution implies the study of isotropic phenomena. Investigations of nuclei that are relaxed by relatively ineffective mechanisms and dipolar fluctuations benefit greatly from very high fields when the molecules of interest tumble rapidly. The situation is much more complex for relatively slowly reorienting molecules, where the relaxation mechanism may involve chemical shift anisotropy. For nuclear interactions that are fully averaged by motion both resolution and sensitivity are enhanced in very high fields, since more nuclei are polarized and isotropic chemical shifts are further separated. In certain situations, and particularly with macromolecules, the longitudinal relaxation times that dictate how often pulse cycles can be repeated increase at higher field strengths, which results in an effective decrease in sensitivity. Also, if line widths increase no more than peak separations, going to a higher field strength is disadvantageous.

High-resolution NMR is closely associated with the observation of protons in molecules in solution and refers to the capability of distinguishing resonances from each other and measuring scalar

spin-spin couplings. The isotropic averaged chemical shift is used semiempirically in most situations as a parameter monitoring a particular chemical environment, primarily because the chemical shift anisotropy of protons is so small that it is rarely, if ever, evident in solution, and because of the lack of success of most chemical shift calculations. The chemical shift of resolved sites, despite the limited physical information it conveys, has proved useful in many biochemical studies—for example, to distinguish protein or transfer RNA (tRNA) conformations and to monitor the ionization of various residues. Detection of resonances at unusual positions is indicative of protons in unusual environments, such as near unpaired electrons or in hydrogen bonds.

Dynamic range problems occur when the resonances of interest from a dilute sample are near those of the solvent. This difficulty is associated with the finite word length of the analog-to-digital converters and computer, which can be filled with an enormous solvent signal to the exclusion of the signal of interest. It is easier to overcome this problem chemically than instrumentally, by using deuterated solvents and buffers, although even when ^2H_2O is used there are usually enough residual protons to make dynamic range a limiting factor in experimental design. A great deal of effort has been put into the development of solvent elimination techniques. This is a particularly acute problem because some of the most topical and interesting studies involve hydrogen-bonded systems, where H_2O must be present to avoid loss of resonances by chemical exchange.

The most straightforward approaches to handling the dynamic range problem use CW techniques, where only a spectral region far downfield from the H_2O resonance is observed. In pulsed NMR long computer word lengths (20 or 24 bits), double-precision arithmetic, and analog-to-digital converters with word lengths up to 20 bits have been used. Because the solvent protons often have longitudinal relaxation times much longer than those of protons in a macromolecule, it is also possible to selectively enhance the intensity of the resonances of interest relative to the solvent peak by pulsing rapidly so that the pulse recycle time is much less than the relaxation time of the solvent resulting in the solvent resonances being partially saturated.

This significant difference in relaxation times can be exploited more effectively with water elimination FT NMR (*20, 21*). In this variation of the inversion-recovery experiment all of the spins in the sample are inverted with a 180° pulse; then a time delay allows the spins of interest, which have a short relaxation time, to essentially fully recover their magnetization, and while the solvent protons go through a "null" condition the magnetization of interest is sampled with a 90° pulse. There are other possibilities for selectivity in pulsed experiments based on resonance frequency. By effectively tailoring the excitation so that unwanted resonances are not disturbed, either by variations of stochastic resonance (*22*) or by pulse combinations that are capable of shaping the applied RF field (*23*), most of the potential difficulties of dynamic range problems can be bypassed.

One of the most successful applications of high-field proton NMR has been to the study of the structure of tRNA molecules in solution. The secondary and tertiary structure of a tRNA molecule is specified in large part by hydrogen bonding between bases. Studies of tRNA in H_2O have shown that the exchange rates for the hydrogen-bonded protons are sufficiently long that a unique set of downfield resonances can be observed which disappear when the molecules are in ^2H_2O (*8*). The resonances observed in the region 11 to 15 parts per million (ppm) are ascribed to the hydrogen-bonded ring NH protons on the basis of model system studies, comparisons of the spectra of different tRNA's, and the fact that no other resonances are expected in this region.

In addition to adenosine-uridine and guanosine-cytidine base pairs resulting in hydrogen bonds with different chemical shifts, nearby stacking interactions are also reflected in the chemical shifts, showing a great deal of resolution. Figure 2 shows a proton NMR spectrum of tRNA at 360 megahertz (*24*). The chemical shift dispersion reflects the wide variety of local environments monitored by the method, while the areas indicate that all secondary and tertiary hydrogen bonds predicted by a simple cloverleaf model are present in solution.

Nuclear Magnetic Resonance Studies of Isolated Spin Systems

A spin can be isolated in the sense of having very few spin interactions, by being chemically or isotopically dilute, or by having an interaction that is much stronger than competing ones. Some of the nuclei used in biochemical studies are isolated by low natural abundance (^{13}C and ^2H); location in only a few sites per molecular system (^{31}P); isotopic enrichment in selected sites (^{13}C and ^2H); use of a synthetic nuclear spin label (^{19}F); and negative labeling, which blanks out the abundant spins (for example, gross substitution of ^2H for ^1H serves to isolate a few ^1H sites). Many other nuclei under investigation may be similarly characterized—for instance, ^{15}N, ^{14}N, and ^{17}O, as well as many metals.

The dominance of ^1H NMR studies of biological and chemical systems is at an end, and various complementary strategies are now used to characterize a system. Part of the motivation for studying other nuclei is that highly sensitive spectrometers capable of routinely detecting nuclei with resonance frequencies over a wide range are now available. In addition, in many cases information is now available that cannot be obtained from ^1H data.

There are several advantages to studying the NMR properties of nuclei other than protons, with regard to both performing the experimental measurements and interpreting the results in terms of biochemistry. The experimental advantages are related to the removal of the solvent-imposed dynamic range problem, the fact that it is easier to decouple heteronuclear interactions than homonuclear interactions, and the larger chemical shift ranges. When dilute sites are observed homonuclear interactions are minimized, and as a result the nuclear resonance parameters are determined by well-defined spin interactions either at the site (chemical shift anisotropy) or through space (dipolar) to covalently bound neighbors a fixed distance away. Once it has been determined that a dominant spin interaction is present, the well-developed knowledge of spin physics can be exploited in the calculations; the interpretations that result are considerably more valuable than those obtained by monitoring chemical shift changes at individual sites.

There are experimental disadvantages to studying less abundant nuclei, nearly all of which are related to lack of sensitivity. Much effort has been put into optimizing NMR instrumentation and methods for this reason. Pulsed techniques, broad-band proton decoupling, and large sample tubes (20 millimeters or more in diameter compared to 5 millimeters for ^1H) are routine. It may seem obvious that there are significant advantages to be gained from using very high magnetic fields to enhance sensitivity (the observed signal-to-noise ratio increases approximately as H_0^2), but the generally desirable large chemical shift dispersions

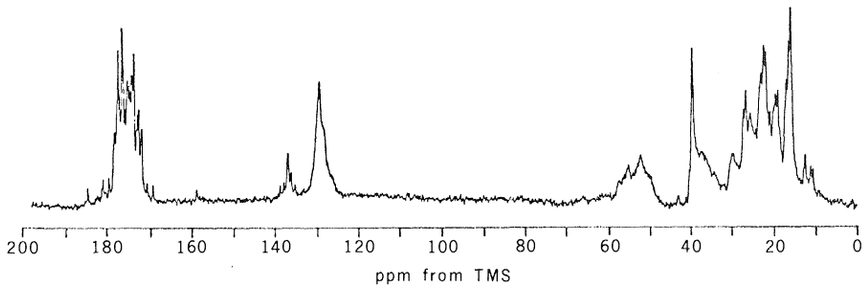


Fig. 3. Proton-decoupled natural-abundance ^{13}C NMR spectrum of muscle calcium-binding protein; obtained with a 2.3-tesla pulsed spectrometer. [From (*28*)]

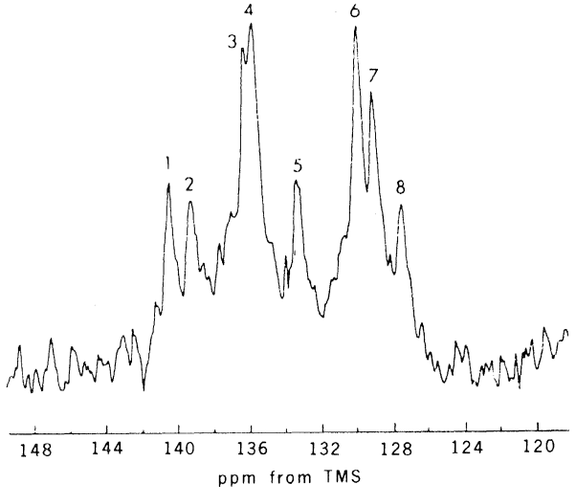
are obtained with nuclei that have large chemical shift anisotropies, and for nuclei without attached protons the shift anisotropy is the dominant line-broadening mechanism. The line width increases as H_0^2 while the separation of peaks increases linearly with H_0; therefore, in many important cases the sensitivity advantages gained by employing a very high magnetic field are lost because the intensity is spread over a wider area. In complex spectra the loss of resolution due to the same effect can also be devastating. For these studies the optimal field strength is intermediate, typically in the range 3.5 to 4.7 teslas. Such fields seem to be a good compromise for line broadening from chemical shift anisotropy in biological macromolecules, and at the present state of magnet technology they can be developed in solenoids with large open bores, allowing the use of large sample tubes.

Carbon-13 NMR of proteins. Carbon-13 NMR is rivaled only by diffraction techniques as a method for obtaining data for all the carbon atoms of a complex molecule. High-resolution ^{13}C NMR of proteins has many attractive features; for example, there is a large (200 ppm) chemical shift range; application of broad-band proton decoupling results in single-line spectra; the 1 percent natural abundance of ^{13}C removes the possibility of homonuclear couplings; and ^{13}C relaxation parameters are often well defined and can be calculated. Double-resonance procedures are important, since the use of proton decoupling makes it possible to define single exponential relaxation times for the ^{13}C spins, enhances the ^{13}C resonances (the nuclear Overhauser effect), and enables the use of a variety of partial decoupling experiments that differentiate among carbon types.

The first natural-abundance ^{13}C spectrum of a protein was that obtained for lysozyme in 1970 with CW signal averaging (*25*). It illustrated the severe instrumental limitations associated with the low gyromagnetic ratio and abundance of ^{13}C, as well as the line broadening arising from chemical shift nonequivalence and reduced motional freedom of the protein structure. Subsequently, Allerhand *et al.* (*26*) reported a ^{13}C spectrum of ribonuclease obtained by pulsed FT methods, demonstrating the feasibility of the method.

Detailed and interpretable ^{13}C NMR studies of proteins have been made possible only by vast improvements in instrumentation (*27–29*). Many spectrometer developments that are generally useful can be traced directly to requirements for ^{13}C NMR studies. A suitable spectrometer relies on quadrature phase detection and large sample volumes for its ^{13}C sensitivity. In general, ^{13}C spectroscopy of macromolecules does not depend on the use of very high magnetic fields because large samples are needed and because of relaxation considerations. Since the number of nuclei to be detected is limited, it is essential to average the signals due to many free induction decays, and the total time of the experiment is reduced by pulsing as rapidly as the ^{13}C longitudinal relaxation times (T_1) will allow (for accurate intensity information the repetition rate must be slower than $3 \times T_1$); therefore, for carbons relaxed by dipolar interactions with protons, the ^{13}C T_1 can become prohibitively long at high fields. For aromatic and carbonyl carbons without attached protons, chemical shift anisotropy makes significant contributions to the relaxation at high fields, and while this has the advantage of reducing T_1 there are detrimental resolution effects because of line broadening. The unsaturated carbons have proved to be the most studied protein sites because there are relatively few of them and at low or intermediate field strengths their line widths are narrow. A typical high-resolution natural-abundance ^{13}C spectrum of a protein is shown in Fig. 3.

Fluorine-19 NMR of highly structured macromolecules. There are many advantages to labeling a protein or other macromolecule with an "NMR-active" nuclear label. When that label is ^{13}C (*29*),



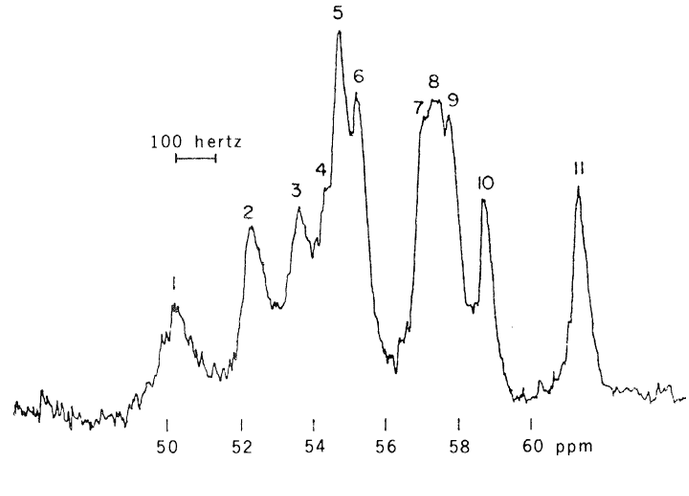


Fig. 4 (left). Proton-decoupled ^{13}C NMR spectrum of alkaline phosphatase with enriched [γ-^{13}C]histidine, obtained with a 2.3-tesla pulsed spectrometer. [From (*29*)] Fig. 5 (right). Pulsed ^{19}F NMR spectrum of fluorotyrosine alkaline phosphatase in a 2.3-tesla field. [From (*30*)]

well-established relaxation mechanisms can be exploited to yield dynamical information while many sensitivity and resolution limitations are obviated (Fig. 4). One of the most popular labeling schemes is to incorporate an ^{19}F-labeled precursor into the molecule of interest—for example, 3-fluorotyrosine into a protein (Fig. 5) (*30*) or fluorouracil into tRNA (*31*). It is also possible to synthetically label a protein with a fluorine-containing reagent.

The current activity in ^{19}F NMR can be explained by the specificity of the label in relatively few sites of the systems chosen. Fluorine-19 is 100 percent abundant and has a high gyromagnetic ratio, so that the sensitivity is 83 percent as good as that for protons, the advantages of a dilute label are present, and there are no dynamic range problems to contend with. Also, spectrometer requirements for these studies are minimal.

Because ^{19}F in aromatic and aliphatic sites have large chemical shift anisotropy the line-broadening effects of this mechanism are made worse by the high gyromagnetic ratio. It has been demonstrated that for ^{19}F-labeled macromolecules there is no advantage to using high fields, and lower fields permit the use of relatively dilute solutions in large sample tubes.

Deuterium NMR of membranes. Nuclear magnetic resonance studies have contributed many ideas to the understanding of membrane dynamics. Conventional high-resolution studies of membrane preparations and model systems are difficult to interpret in detail because of the restricted anisotropic motions that are present. In the last several years it has proved possible to quantitatively interpret membrane experiments in which ^{2}H-labeled lipids, ^{31}P-labeled headgroups, and, in a few cases, ^{13}C-labeled lipids have been used. These successful experiments involve the use of specialized NMR techniques.

The ^{2}H nucleus has a spin of 1 and hence is nonspherical, so that it has a quadrupole moment. The quadrupole interaction is generally dominant and is well understood, leading to sound physical interpretations of ^{2}H NMR spectra. In ordered systems such as liquid crystals or multilamellar liposomes, a partially averaged quadrupole powder pattern results, as shown in Fig. 6 (*32, 33*). Splittings between maximums can be directly related to molecular order parameters. The instrumental requirements for this work are related to the frequency spread of the powder pattern; intense RF pulses are needed to rotate all the magnetization and the data acquisition system must have a fast response. In general, spectrometers set up for liquid samples are not well suited for these experiments.

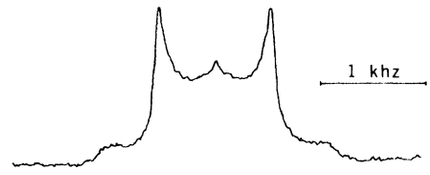


Fig. 6. Deuterium NMR spectrum of bilayers of $CD_3\overset{+}{N}$–dipalmitoyl phosphatidylcholine above the gel-liquid crystal phase transition in a 2.1-tesla field. [From (*33*)]

Phosphorus-31 NMR of proteins. Phosphorus-31 lends itself to biochemical NMR investigations for spectroscopic reasons such as its 100 percent abundance, relatively high gyromagnetic ratio, and large chemical shift range, as well as for chemical reasons related to its key role at the active site in enzymes and the few phosphorus nuclei present in any system. Because ^{31}P has a very large chemical shift anisotropy in almost all chemical environments, its NMR spectroscopy is a prominent case of benefiting from the use of intermediate field strengths. When ^{31}P is bound to a protein there are severe line-broadening and sensitivity problems associated with slow rotational correlation times. Several recent investigations have been successful in exploiting ^{31}P NMR in enzymology by observing phosphorus-containing substrates bound at the active site (*34*).

Phosphorus-31 studies of membranes. Some of the most informative NMR studies of membranes have utilized the ^{31}P resonances of the headgroups (*35*). Partial averaging of the static chemical shift tensor reflects the angles and rates of rotation of the headgroups. Several procedures developed for NMR of solids must be employed to obtain information on ^{31}P chemical shielding. The dominant spin interaction is the incompletely averaged chemical shift anisotropy when high-powered resonant proton decoupling is applied to remove the effects of ^{1}H-^{31}P dipolar interactions. In some cases the sensitivity can be significantly improved by obtaining the ^{31}P magnetization through cross polarization from the protons rather than by directly pulsing the phosphorus resonances (*36*).

These experiments require very large RF fields in order to effectively separate the chemical shift anisotropy from the dipolar interactions. When cross polar-

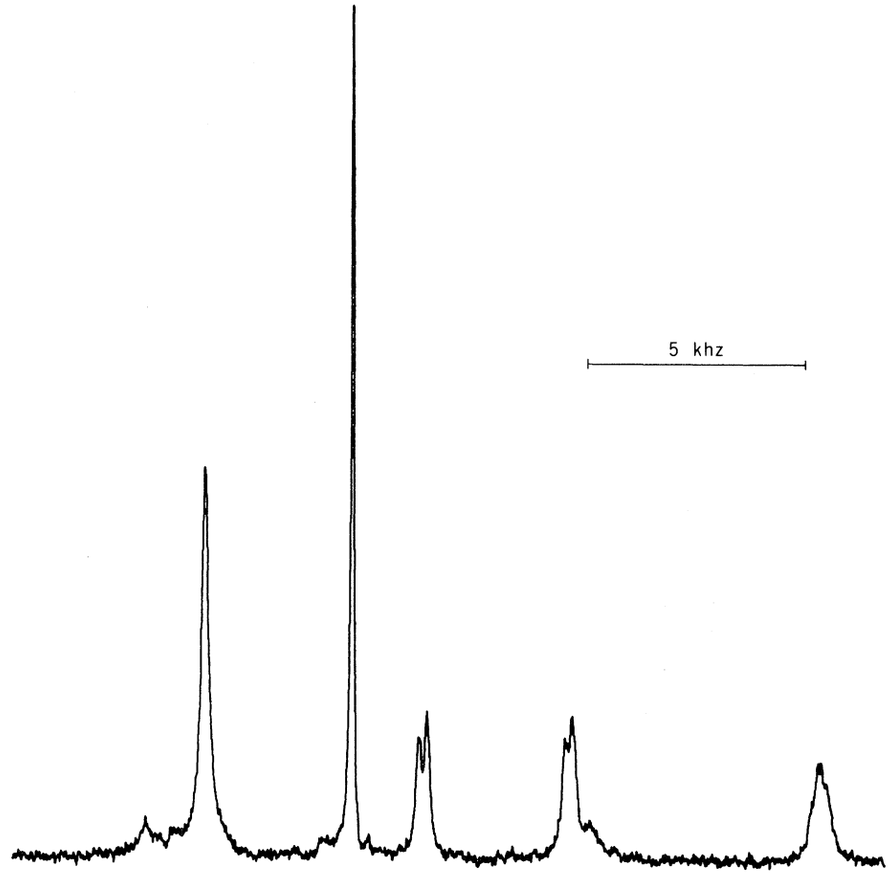


Fig. 7. Proton-decoupled ^{31}P NMR spectrum of chicken pectoralis muscle at 3.5 tesla [Courtesy of L. F. Johnson and T. Glonek]

ization is employed the pulse recycle time is determined by proton parameters, and the long T_1 of ^{31}P is not a problem. Because of this, and the need to distinguish shoulders and discontinuities in powder patterns, high fields are beneficial. In contrast, for molecules in solution any increase in the expressed chemical shift anisotropy is detrimental to resolution. Similar spectroscopic strategies have been used with natural-abundance ^{13}C and enriched sites in membranes (*37*).

Phosphorus-31 NMR of intact organs. In one of the most intriguing new areas in biochemical NMR, ^{13}P has been applied to the study of intact biological specimens such as heart, kidney, and skeletal muscle (Fig. 7) (*38, 39*). This is due to the development of instrumentation capable of resolving ^{31}P resonances of small molecules such as adenosine triphosphate, adenosine diphosphate, creatine phosphate, and inorganic phosphate—the molecules most important in cellular physiology. Fortunately, the *pK*'s of several small molecules such as inorganic phosphate and diphosphoglycerate are around 7; therefore, their ^{31}P chemical shifts are very sensitive to *p*H and can be used to measure intracellular *p*H (*39*). This use of an NMR spectrometer as a *p*H meter is advantageous because of its nondestructive character and potential for kinetic experiments. A large open-bore magnet is often required in these studies in order to physically contain the sample.

Enhancement of Nuclear Magnetic Resonance Information

Convolution difference spectroscopy. A number of mathematical manipulations can be performed on free induction decays and frequency spectra either to enhance the signal-to-noise ratio at the expense of some resolution or to enhance the resolution. Simple weighting schemes are applied routinely. In favorable cases, such as in high-resolution ^{1}H or ^{13}C NMR of proteins, manipulations of the data can effectively differentiate broad and sharp peaks, greatly enhancing the effective resolution.

The procedure of convolution difference spectroscopy is illustrated in Fig. 8 (*40*). The final sharpened spectrum is obtained as the difference between a normal spectrum and a spectrum broadened by multiplying the free induction decay by a negative exponential. It is also possible to do the broadening chemically rather than mathematically by using paramagnetic reagents and to obtain site specificity. These methods involve only the data-handling system and software.

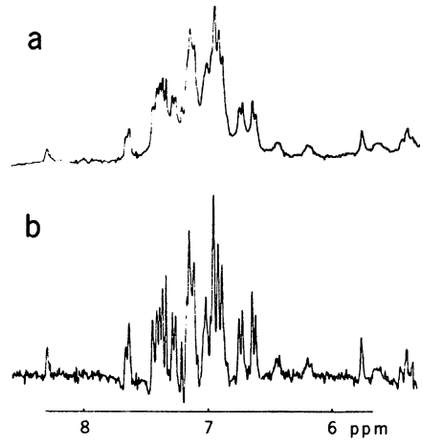


Fig. 8. Comparison of the aromatic region of the ^{1}H NMR spectra of lysozyme obtained at 6.2 teslas: (a) conventional Fourier transform spectrum and (b) the corresponding deconvoluted spectrum. [From (*40*)]

Zeugmatography. Image reconstruction techniques can be applied to NMR data obtained in very inhomogeneous magnetic fields in order to describe macroscopic objects (*41*). Spatial information is available from chemical shifts because the nuclei in different parts of the sample have different resonance frequencies when a linear field gradient is superimposed on the static applied magnetic field. The spectrometer used for these measurements can be a conventional one, and the necessary gradient can be achieved by using a shim coil, which is normally used for field correction.

Two-dimensional pictures are obtained by combining data from a number of gradient directions. Biological objects such as fruits, nuts, and mice have been imaged by proton NMR zeugmatography. Extensions of the technique to other nuclei, particularly to the ^{31}P resonances of cell metabolites, may lead to detailed views of compartmentalizations. Other NMR parameters, including relaxation times, can be spatially correlated by use of this approach.

Two-dimensional Fourier transform NMR. A group of experiments that can be performed without hardware modification have proved to be extremely useful for simplifying complex spectra and measuring otherwise unobtainable spectral parameters. Nuclear magnetic resonance signals that are observed at two different time intervals can be subjected to two-dimensional Fourier analysis. The final display is signal intensity plotted as a function of two different frequencies. Aue *et al.* (*42*) have published a general discussion of two-dimensional NMR.

These techniques, which are basically only methods of collating and manipulating data, have been applied to homo- and heteronuclear coupled systems in liquids and solids. As a result, some scalar couplings and dipolar couplings have been measured for the first time. By using versions that employ spin echoes, it has been possible in certain situations to enhance the resolution over that obtained with a static magnetic field.

Essentially no biochemical two-dimensional NMR experiments have been performed yet; however, several applications are obvious and should be demonstrated soon. For example, it is possible, especially in high fields, to obtain a two-dimensional representation of homonuclear coupled ^{1}H spectra that are completely decoupled (*43*). This could significantly enhance the resolution obtained with proteins and lead to single-line ^{1}H NMR spectra similar to heteronuclear decoupled ^{13}C spectra. Accurate measurements of ^{13}C-^{1}H couplings by two-dimensional methods would be beneficial in studies where the biochemical interpretation relies on them [for example, see (*44*)]. In addition, it is clear that many new experiments can be performed with two-dimensional NMR, with the benefits of spectral simplification particularly helpful for complex macromolecules.

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Geometry of Adsorbates on Solid Surfaces

Angle-resolved photoemission spectroscopy is a promising tool for obtaining structural information.

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The last decade has witnessed a tremendous surge in the growth of surface science, with every indication that this growth will continue for many years (*1*). The interest and activity in the gas-solid interface can be attributed to the coincidence in time of at least three complementary factors. First, the last few years have witnessed the development of several experimental techniques potentially capable of characterizing a surface on a microscopic level. Second, societal pressures have forced many scientists to attempt a closer coupling between basic research and practical problems. Surface science seems at face value easier to relate to the needs of society than many other areas of basic research. For example, it is often argued that the concepts and information obtained from work on well-characterized, idealized surfaces can and will have an impact upon technologically important areas such as corrosion, catalysis, fabrication of microelectronic devices, and lubrication. Third, from a purely conceptual point of view a surface can be seen as a distinct phase of matter, with unique properties differing both from a three-dimensional periodic solid and from free gas molecules (*2*). Many scientists in solid state physics and molecular chemistry have been willing to try to apply the concepts and expertise they have developed to understand surface phenomena.

Among the many questions that one can ask about a surface, one seems important with respect to the motivation outlined above: "How does a foreign atom or molecule interact with and bond to a surface?" This question is far ranging since it encompasses both static bonding and the dynamics of adsorption and desorption. It is obviously the question most relevant to practical surface problems. At the heart of this question is the experimental determination of the geometrical arrangement of the atoms. The present dearth of structural information is, in our view, what is currently impeding progress in this field. Theory is of little help in this regard; it is the exception and not the rule that a calculation can predict the structure of a molecule or bulk solid. Even the calculational schemes that successfully pass this test may not work at a surface. Yet if one knows where the atoms are, many less sophisticated theories can be used, in conjunction with experimental data, to elucidate chemisorption mechanisms.

When a gas phase molecule strikes a surface it may bounce off, maintaining its molecular identity or it may interact more strongly, either bonding or undergoing a chemical reaction induced by the surface and reemerging in the gas phase as a different species. If it bonds to the surface we want to know where it bonded and then how it is bonded. Figure 1 illustrates schematically some possibilities for a carbon monoxide molecule. The molecule may stand straight up on either end, bend over or dissociate into one carbon and one oxygen atom. Either of these two atoms (or possibly both), may penetrate the metal surface, forming the initial stage of an oxide or a carbide. If we are going to understand the interaction of an atom or molecule with the surface we must develop experimental techniques to measure the geometrical configuration of the atoms.

For determining the geometrical structure of surfaces conventional techniques such as x-ray diffraction or high-energy electron scattering are of little use. The power of these techniques arises from the weak interaction of the probe with the sample, which makes analysis relatively simple. For surface problems we face a very different situation. If a single layer of atoms is adsorbed on a single crystal of dimension 1 cm by 1 cm by 1 mm, then the ratio of the number of adatoms to substrate atoms is less than one to a million. This means that we need a probe that is only sensitive to the surface. Such a probe must not penetrate more than a few tens of angstroms, a situation which occurs only when it interacts strongly. Neutrons or x-rays are weakly interacting, whereas low-energy electrons are strongly interacting. The price for these surface-sensitive probes is a much higher degree of complexity in the data analysis. Much of the theoretical effort in surface science has been devoted to calculating the response of the system to experimental probes (*3*).

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