# NUCLEAR MAGNETIC RESONANCE STUDIES OF BIOLOGICALLY RELEVANT ISOTOPES OTHER THAN HYDROGEN

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## INTRODUCTION

The contemporary outpouring of the number and range of nuclear magnetic resonance (NMR) applications to biological and biochemical systems is largely attributed to the observation of isotopes other than hydrogen ($^1H$).[1] In magnetic resonance terminology, these are generally termed heteronuclei. Developments in modern technology, notably the introduction of the pulse Fourier transform technique[2] and the use of dedicated computers,[3] have enabled heteronuclear NMR to be applied to systems of biological interest. Such applications include structural studies of macromolecules[4,5] and model[6] and biological membrane systems,[7] metabolic studies on whole live microorganisms,[8] and analysis of human metabolites.[9] In such applications, the great advantage of the heteronuclear NMR technique is that it utilizes the presence of nondisturbing, natural isotopic probes.

It is the intention of this review to delineate the types of selective biological applications resulting from the inherent NMR properties of each type of heteronucleus. This overview appears to be worthwhile since each type of application using each type of heteronucleus is effectively becoming a separate specialization. This is not intended to be an exhaustive review of the literature; rather, selected and instructive applications are cited.

The information obtained from NMR studies falls into four categories:

1. The local electronic (chemical) microenvironment, reflected in the position of the resonance frequency $\nu_o$ and often expressed as a dimensionless scale of chemical shift ($\delta$)
2. The local mobility as expressed in the correlation time ($\tau_c$), obtained from the measured spin-lattice ($T_1$) and spin-spin ($T_2$) relaxation times
3. The concentration, reflected in the relative areas (intensities) of the signals
4. The conformational relationships of nuclei, from the value of the spin-spin coupling constant (J) between them: the value of J depends on the angular juxtapositions of the nuclei.

It should be emphasized that the parameters measured in NMR experiments are nuclear properties which reflect the environment in the molecule into which the nuclei are incorporated. Of course, all the above parameters are not necessarily measured in a particular NMR application. Further

TABLE 1

**NMR Properties of Heteronuclei of Biological Interest Compared to Those of Hydrogen**

| Nucleus | I[a] (ħ) | Sensitivity[b] | Natural abundance (%) | Chemical shift range (ppm) |
|---|---|---|---|---|
| $^{1}H$ | 1/2 | 1.00 | 99.98 | 10 |
| $^{2}H$ | 1 | $9.7 \times 10^{-3}$ | $1.6 \times 10^{-2}$ | 10 |
| $^{3}H$ | 1/2 | 1.2 | – | 11 |
| $^{13}C$ | 1/2 | $1.6 \times 10^{-2}$ | 1.1 | 200 |
| $^{14}N$ | 1 | $1 \times 10^{-3}$ | 99.6 | 900 |
| $^{15}N$ | 1/2 | $1 \times 10^{-3}$ | 0.4 | 900 |
| $^{17}O$ | 5/2 | $3 \times 10^{-2}$ | $3.7 \times 10^{-2}$ | 600 |
| $^{19}F$ | 1/2 | 0.8 | 100 | 400 |
| $^{23}Na$ | 3/2 | $9.3 \times 10^{-2}$ | 100 | – |
| $^{31}P$ | 1/2 | $6.6 \times 10^{-2}$ | 100 | 400 |
| $^{33}S$ | 3/2 | $2.3 \times 10^{-3}$ | 0.74 | – |

[a]Nuclei with I > 1/2 possess a nuclear quadrupole moment.
[b]For an equal number of nuclei at constant magnetic field strength.

details on the NMR method may be obtained in a number of texts.[2,10,11]

An important question in this field is the relation of the NMR properties of these heteronuclei to those of $^{1}H$, on which most of our experience is based.[10] In this respect, technical questions of resolution and sensitivity must be considered which may require different conclusions than were found for $^{1}H$ NMR. Furthermore, a consideration of the properties of distinct heteronuclei may indicate the future directions of potential biological applications for each.

One of the most significant aspects of this field has been the introduction of stable isotopic enrichment.[12] It should be noted that radioactive isotopes may also have NMR properties, and the most relevant such isotope, tritium[13] ($^{3}H$), is specifically included in this discussion to clarify the nature of the relationship between NMR studies of stable and radioactive nuclei.

## PROPERTIES OF BIOHETERONUCLEI

The relevant properties of the heteronuclei of biological interest are given in Table 1. As will be seen, the range of chemical shifts for most heteronuclei is far greater than those of the three hydrogen isotopes. This results from a greater range of different chemical or electronic environments experienced by those nuclei. A large range of chemical-shift values implies a greater resolution of resonances of the observed nucleus, provided line widths remain comparably narrow. This increased resolution will clearly be a great advantage in studies of macromolecules or complex mixtures containing many such nuclei.

A distinction may be made between those nuclei with a spin nuclear quantum number I = ½ and those with I > ½. The latter possess a nuclear quadrupole moment[11] which usually dominates the nuclear magnetic relaxation processes, giving more efficient relaxation than for the usual dipole-dipole relaxation mechanisms. The origin of quadrupolar relaxation is the fluctuation of electric field gradients at the nucleus as a result of molecular motions; thus, its value is very sensitive to the local symmetry and correlation time. There is a reciprocal relationship between the spin-spin relaxation time, $T_2$, and the width at half the height of a resonance. As a result of the short $T_2$ values, the resonances of quadrupolar nuclei with I > ½ are generally broad. Consequently, only low-resolution NMR studies are usually possible with such nuclei. Therefore, we may distinguish between low- and high-resolution heteronuclear magnetic resonance applications (Table 2). Since most studies of biological systems require high resolution observations, the nuclei which will receive most of our attention will be those with I = ½, i.e., $^{3}H$, $^{13}C$, $^{15}N$, $^{19}F$, and $^{31}P$.

There is a relationship of resonance line width to the correlation time of the nucleus which also holds for nuclei with I > ½. In certain favorable cases such as $^{2}H$, studies of mobility are possible

TABLE 2

**NMR Applications of Bioheteronuclei**

| Category | Property | Isotope | Main types of applications | References |
|---|---|---|---|---|
| High resolution | I = 1/2 | | | |
| Biologically occurring | High abundance | $^{31}P$ | Structural probe for phosphates | 44–50 |
| | | | Metabolic probe | 8, 81–86 |
| | Low abundance | | | |
| | Positive NOE | $^{13}C$ | Structural and dynamic probe | 27, 39, 42, 51–58, 61, 62 |
| | | | Metabolic probe on enrichment | 9, 12, 87, 88 |
| | NOE passes through zero | $^{15}N$ | Structural and dynamic probe of N-containing groups | 33, 36–38, 59, 60, 70, 79 |
| | | | Metabolic probe on enrichment | – |
| Nonbiologically occurring | Stable isotope | $^{19}F$ | Substituent for $^{1}H$ in structural studies | 30, 71–73 |
| | Radioactive | $^{3}H$ | Substituent for $^{1}H$ in metabolic studies | 20, 21 |
| Low resolution | I > 1/2 | $^{2}H$ | Substituent for $^{1}H$ in dynamic studies | 14, 74, 75 |
| | | $^{14}N$ | Limited structural probe | 80 |
| | | $^{17}O$ | Limited structural probe | 78, 79 |
| | | $^{23}Na$, $^{39}K$ | Metal ion binding | 15, 18 |
| | | $^{35}Cl$ | Anion binding | 16, 17 |

where the resonance lines are narrow enough to be readily observed.[14] These applications will be considered later. Other groups of heteronuclei with $I > \frac{1}{2}$ constitute the common metal cations such as $^{23}Na$[15] and halogen anions such as $^{35}Cl$.[16] Although these ions have biological relevance,[15–18] the information they provide on biochemical systems is generally indirect, and they are considered beyond the scope of this review.

The group of high-resolution heteronuclei may be further subdivided (Table 2) on the basis of their natural occurrence and stability. $^{19}F$ is not found in biological systems but nevertheless may be an excellent probe in those systems where it can be incorporated.[19] Tritium also does not occur naturally, may be used as a nuclear probe just as $^{19}F$, and may be a preferable substituent for $^{1}H$.[20,21] But its radioactivity may hamper such applications. At manageable levels of specific activity (approximately 10 mCi) the nature of the applications may be identical to those for $^{19}F$, and the very high NMR sensitivity of $^{3}H$ indicates that concentrations down to 100 $\mu$M may be observed using pulse FT NMR. However, for many applications with small amounts of labeled compounds, high specific activities may be necessary. The safety problems associated with such work result in specialized NMR laboratories being required for tritium NMR studies. In addition, at high levels of specific activity, radiolytic fragmentation may occur.

As a result of the above considerations and by a process of elimination, one is left with three nuclei, $^{13}C$, $^{15}N$, and $^{31}P$ (Table 2),[22,23] as the most attractive heteronuclei for NMR studies of biological systems. Their greatest advantage is the fact that they are stable isotopes of elements that occur naturally in biological systems and as such, constitute nondisturbing intrinsic nuclear probes of these systems. One notable difference between $^{13}C$ and $^{15}N$ on the one hand and $^{31}P$ on the other is their degree of natural abundance (Table 1); those of the former are very low while that of $^{31}P$ is 100%. Therefore, while it is correspondingly easier to observe $^{31}P$ by NMR techniques at natural abundance in biological molecules, it is possible to selectively enrich sites with $^{13}C$ and $^{15}N$, which is not possible with $^{31}P$. This fact naturally results in different types of biological applications for each of these nuclei, as described below.

## SENSITIVITY CONSIDERATIONS

The difficulty in observing NMR spectra for $^{15}N$ nuclei at natural abundance is greater than that for $^{13}C$ by a factor of 44, taking into account the NMR sensitivity and natural abundance (Table

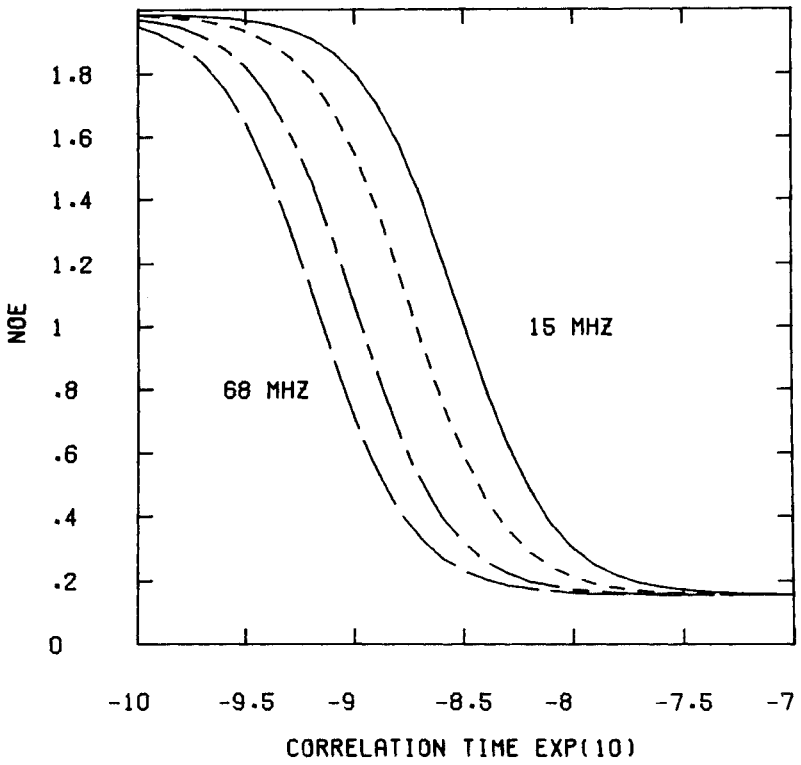


FIGURE 1. Theoretical plot of nuclear Overhauser effect (NOE) as a function of correlation time ($\tau_c$) for four $^{13}C$ NMR observing frequencies (15, 25, 45, and 68 MHz). This assumes an isotropically reorienting C-H vector relaxing exclusively by the dipolar mechanism.

1). This is a sufficiently significant factor to make $^{13}C$ NMR studies at natural abundance much easier than for $^{15}N$. However, although the low sensitivity of observation of these nuclei constitutes a major obstacle, to some extent it has been overcome in order to take advantage of their biological relevance.

One approach removes the couplings of covalently bound hydrogen nuclei, which cause a splitting in the observed heteronuclear signals, by applying a second high-power proton resonance frequency. In order to cover the whole range of proton resonances, this decoupling frequency is usually noise-modulated.[24] There are two results of this decoupling: the simplification of the observed heteronuclear spectrum to a singlet resonance for each heteronucleus and a change in the relative occupations of the energy levels of the heteronuclei bound to $^{1}H$, giving rise to a change in the spectral intensity of the signals. This is the nuclear Overhauser effect (NOE)[25] and is naturally very useful when it results in a net enhancement of the observed signal. Such is the case for $^{13}C$ where the limiting value of NOE for rapid motion ($\nu_0^2\tau_c^2 \ll 1$) is 1.98, and the NOE is always positive (Figure 1).[26]

The NOE, $T_1$, and $T_2$ are all functions of both frequency of observation and $\tau_c$. Due to the loss of the maximum NOE enhancement at higher magnetic field strength (Figure 1), there is a net loss of sensitivity for a $^{13}C$ nucleus in a protein with $\tau_c$ in the range $10^{-8}$ to $10^{-9}$ [26] On the other hand, there is in theory a net gain in sensitivity at least proportional to the applied magnetic field strength, $H_0$. Combining all these factors gives a semiempirical equation for the overall signal intensity at a given noise level:[5]

$$I = K\,(NOE + 1)\,H_0 T_2 \sqrt{1/T_1}$$

where K is a constant including the sample volume, concentration, and quality of the probe. This function is plotted in Figure 2. Apart from the slowest motions ($\tau_c > 10^{-8}$ sec), it can be seen

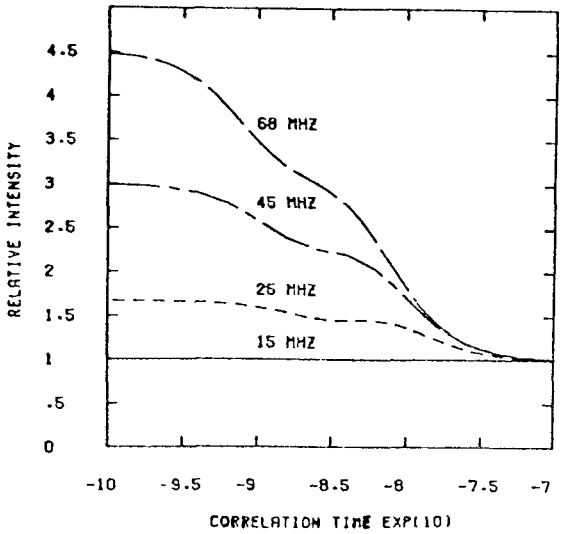


FIGURE 2. Theoretical plot of relative sensitivity as a function of correlation time at four $^{13}C$ observing frequencies with 15 MHz set equal to unity. (From Shindo, H. and Cohen, J. S., *Proc. Natl. Acad. Sci. U.S.A.*, 73, 1979, 1976. With permission.)

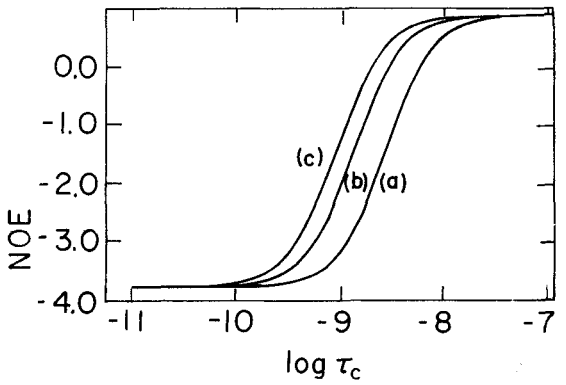


FIGURE 3. Theoretical plot of nuclear Overhauser effect (NOE) as a function of correlation time ($\tau_c$) for three $^{15}N$ NMR observing frequencies (a, 9.12; b, 18.24; and c, 27.36 MHz). This assumes an isotropically reorienting N-H vector relaxing exclusively by the dipolar mechanism.

that it is preferable to work at higher field strength from the point of view of sensitivity of $^{13}C$ observation. Of course, a larger size probe or higher concentration may be used to obtain a greater S/N, but these require larger amounts of material which, in the case of biological systems, may be limited.

While the relationship used in this calculation of S/N $\propto$ Ho for $^{13}C$ is empirical, recent experimental determinations indicate that it is a reasonable relationship[27] and is supported by theoretical considerations.[28] A further assumption is that the NOE, $T_1$, and $T_2$ are calculated for dipole-dipole relaxation only. There are other possible sources of relaxation for heteronuclei, e.g., that resulting from the anisotropy of the spin environment. The relaxation rates arising from this mechanism are given by:

$$1/T_2{}^A \propto \delta_A{}^2 \tau_c$$

and similarly for $T_1{}^A$, where $\delta_A$, the chemical shift separation due to local magnetic anisotropy, is proportional to $H_o$. Since the effect of increase of $1/T_2{}^A$ with $H_o$ is dominant, giving broader resonances, sensitivity and resolution do not increase with magnetic field strength if chemical-shift anisotropy becomes the dominant relaxation mechanism.[29,30]

In the case of $^{15}N$, the NOE value changes its sign from negative at fast correlation times to positive at slow values (Figure 3).[31] This results in $^{15}N$ nuclei in proteins with $\tau_c$ between $10^{-8}$ and $10^{-9}$ showing a nulled resonance,[32] and there are several ways in which $^{15}N$ resonances can be nulled.[33] This means that for many systems it may be worthless to use higher magnetic-field strength to gain sensitivity in $^{15}N$ studies. Alternatively, one can dispense with proton decoupling, thus losing the NOE, but also possibly losing a net enhancement (negative or positive) for some $^{15}N$ resonances. In this respect, $^{15}N$ NMR studies with proton decoupling, because of the change of sign of the NOE value, provide a means of selective discrimination between different correlation time regions of a single molecular species.[32]

Such considerations of NOE are not as important for $^{31}P$ NMR studies due to the greater effective sensitivity in dealing with this nucleus. In addition, $^{31}P$-$^{1}H$ couplings are often measured to aid in resonance assignment.

Several attempts have been made to determine optimum magnetic field strength for studies with a given nucleus taking into account not only the NOE but also relaxation by the chemical shift anisotropy relaxation mechanism.[30,34,35] Since both these factors are functions of the correlation time, it is clearly impossible to have a single optimal field strength for all applications, even of a single nucleus. In addition, such practical considerations as the amount of material available and whether or not it can be isotopically enriched are crucial. The most general statement that can be

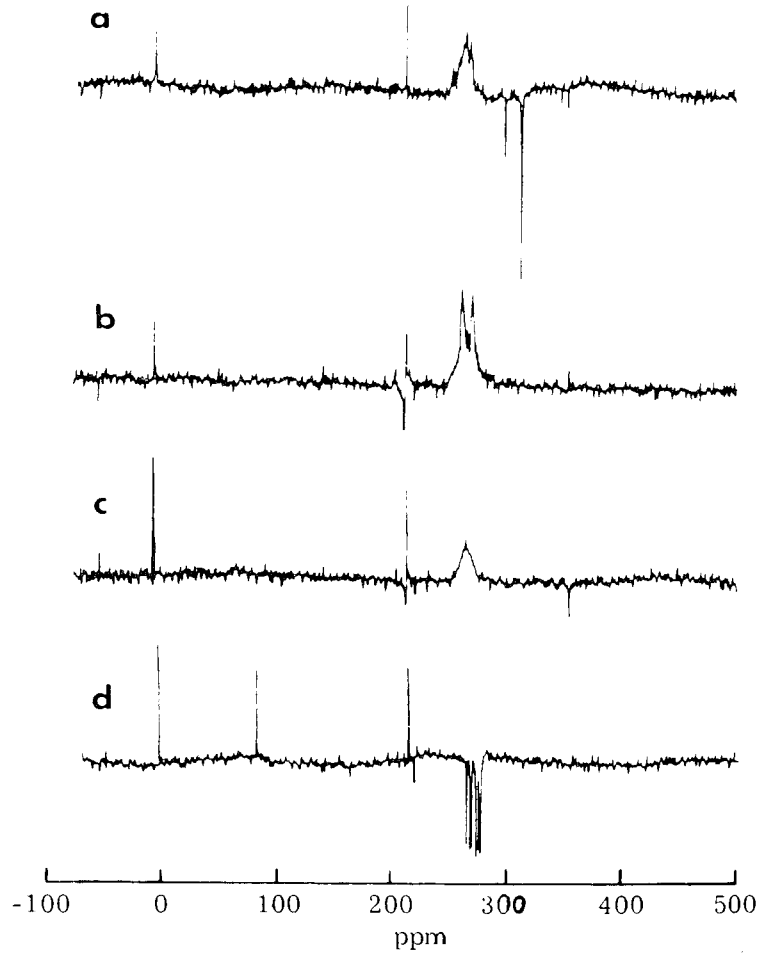


FIGURE 4. Natural abundance $^{15}N$ spectra at 18.25 MHz of (a) hen egg-white lysozyme (9 m*M*) at pH 3.9, (b) denatured porcine pepsin, (c) rabbit cyanomethemoglobin (2 m*M*), and (d) cyanocobalamin (0.08 M) in 50% aq. alcohol. (From Gust, D., Moon, R. B., and Roberts, J. D., *Proc. Natl. Acad. Sci. U.S.A.*, 72, 4696, 1975. With permission.)

made is that for observation of spectra of small molecules, higher fields will usually give a better S/N ratio, while for macromolecules (or slowly moving components), this may not always be the case.

## NATURAL ABUNDANCE STRUCTURAL STUDIES WITH $^{13}C$, $^{15}N$, AND $^{31}P$

$^{15}N$ NMR studies of amino acids have been carried out on $^{15}N$-enriched materials due to sensitivity considerations,[36] although several studies of small peptides at natural abundance $^{15}N$ have been reported[37,38] which have concentrated on the properties of the amino and peptide groups. Studies of peptides using $^{13}C$ NMR have recently been reviewed.[39]

Gust et al.[35] have reported the natural abundance $^{15}N$ spectra of several macromolecules (Figure 4) at 18.25 MHz using a large size probe (25 mm requiring a 15-ml sample). While the sensitivity obtained was greater than previously described for $^{15}N$, the spectra were not as informative as comparable $^{13}C$ NMR spectra, particularly in relation to the resolution of single-atom resonances.

$^{13}C$ NMR studies of proteins at natural abundance have provided evidence of the value of this method.[27] Thus, Allerhand and co-workers have carried out a series of studies at 15 MHz using a large size probe (20 mm requiring 10-ml sample)

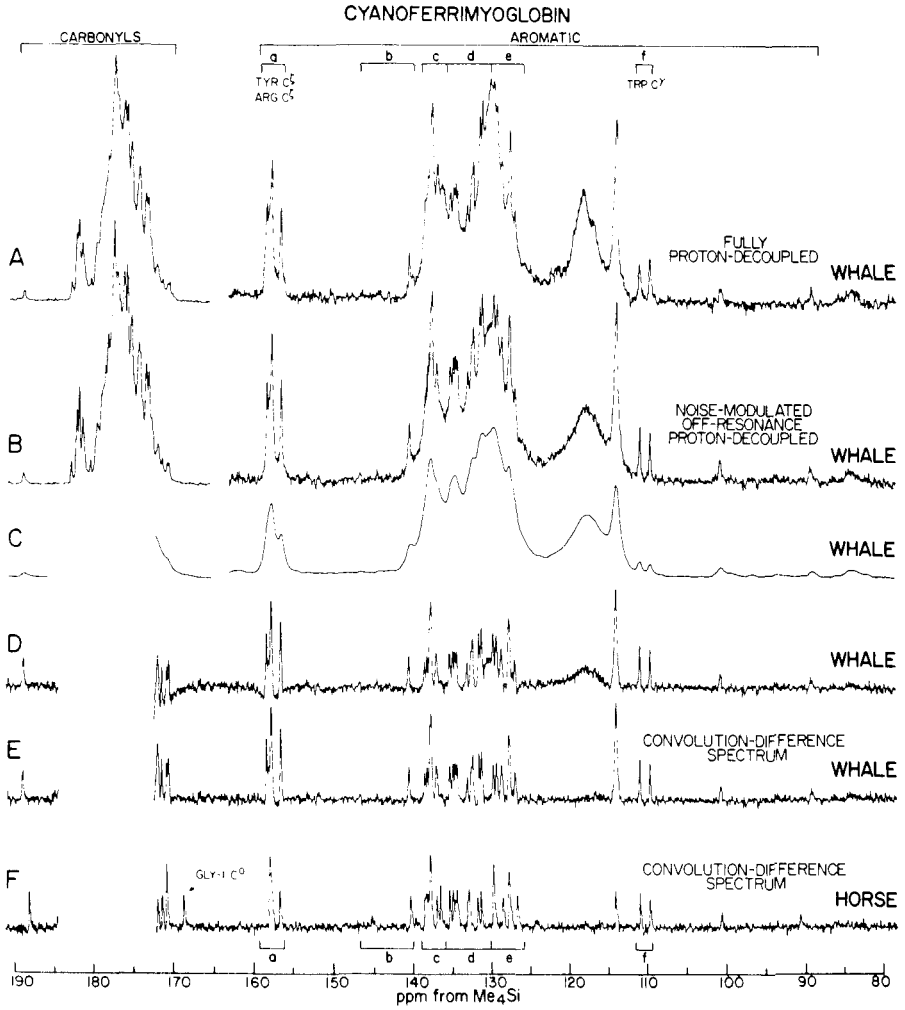


FIGURE 5. Natural abundance $^{13}C$ NMR spectra of sperm-whale (A to E) and horse (F) cyanoferrimyoglobin (8.8 m*M*) in $H_2O$ (0.05 *M* phosphate buffer, pH 6.8) showing the aromatic and carbonyl regions. The spectra were recorded at 15 MHz with proton-decoupling, and the spectral data were manipulated to provide convolution-difference spectra (E and F) as described in the publication. (From Oldfield, E., Norton, R. S., and Allerhand, A., *J. Biol. Chem.*, 250, 6381, 1975. With permission.)

and have resolved individual carbon resonances of tyrosine, tryptophan, phenylalanine, and histidine residues in several small proteins (Figure 5).[4] At 68 MHz with a normal size probe (10 mm requiring a 1.5-ml sample), Cohen and co-workers have resolved individual carboxyl carbon-atom resonances in lysozyme[5] (Figure 6). Individual titration curves of tyrosines and carboxyl groups in several proteins have now been described, as well as assignments to specific residues utilizing a variety of methods.[4,27,40] It is quite likely that similar detailed studies of lysine and arginine will be possible for small proteins at natural abundance of $^{13}C$.

The structure and microdynamics of cell-wall polysaccharides have been investigated using $^{13}C$ NMR at natural abundance.[41] For phospholipid vesicles, the ability to resolve individual lines for most carbon atoms in the fatty acid chain (Figure 7) allows the measurement of the mobility of each atom from its $T_1$ relaxation time.[6,42] These can then be studied in the presence of various perturbants. Nevertheless, such a measurement, while it is averaged over the whole sample for a given $^{13}C$ atom, is much more specific than the "microviscosity" determined from a free fluorescence probe or a spin label and additionally is a nondisturbing probe.

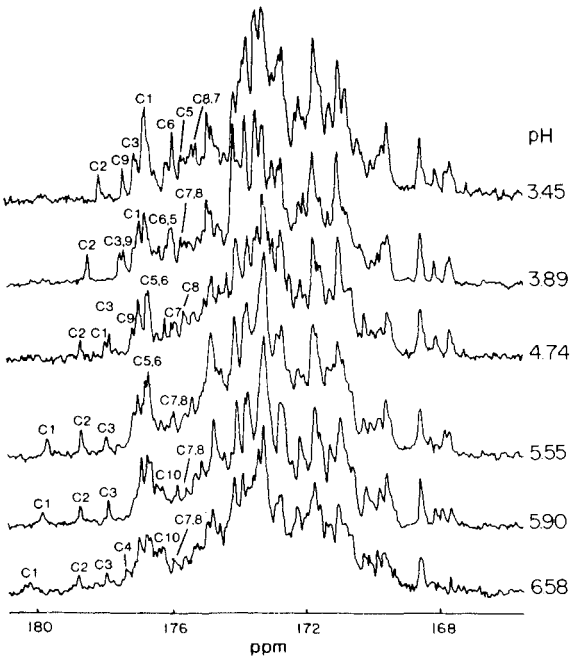


FIGURE 6. Natural abundance proton decoupled carbon-13 NMR spectra at 68 MHz of the carboxyl and carbonyl resonances of hen egg-white lysozyme (10 m*M*) as a function of pH. (From Shindo, H. and Cohen, J. S., *Proc. Natl. Acad. Sci. U.S.A.*, 73, 1979, 1976. With permission.)

In addition to studies of proteins, $^{13}C$ natural-abundance spectra have been reported for unfractionated yeast tRNA in which the resonances of some minor bases were resolved (Figure 8) and some were found to be undergoing fast reorientation.[43] In view of the relatively greater sensitivity of $^{31}P$ NMR compared to $^{13}C$ and $^{15}N$, it is not surprising that $^{31}P$ NMR studies of phosphate-containing macromolecules have been made. These include phosphoproteins,[44] polynucleotides,[45] tRNA (Figure 9),[46] DNA,[47] and phospholipid vesicles.[48-50] On the other hand, it is clear that the information obtained in this way is restricted to only a part of the molecule, e.g., the backbone in nucleic acids or the phosphocholine head group in studies of phospholipid vesicles. Therefore, general conclusions concerning the whole molecule or system may not be possible. It should also be noted that structural studies using $^{31}P$ may be carried out preferably at lower field strength where the broadness of lines due to relaxation by the chemical-shift anisotropy mechanism is less.[46,50]

## ISOTOPIC ENRICHMENT OF $^{13}C$ AND $^{15}N$

There are several ways in which isotopic enrichment of $^{13}C$ and $^{15}N$ may be accomplished in order to facilitate heteronuclear NMR studies.

### Chemical Modification

Chemical modification implies the addition of an isotopically enriched group to a substance, such as a $^{13}C$ glycine onto the carboxyl terminal end of insulin.[51] In general, this method has the disadvantage that it disturbs the system one wishes to study by adding an extrinsic probe much like a spin label and effectively loses one of the main advantages of heteronuclear NMR probes. On the other hand, this is an excellent method by which to study the process and products of the chemical-modification reactions. A good example of this is the work of Matwiyoff and co-workers on carboxymethylation of cytochrome *c*.[52] By comparison of the natural abundance spectrum of cytochrome *c* and that of the enriched chemically modified mixture of products, they were able to resolve and identify several different types of modification (Figure 10) and determine their relative proportions as a function of reaction time. Other examples of $^{13}C$ NMR studies of $^{13}C$ enriched chemically modified proteins have been reported.[53,54]

### Chemical Synthesis

Chemical synthesis is the usual procedure for incorporating radioactive nuclei into chemical substances, generally via a substitution reaction. Using tritium NMR, it becomes readily possible to observe which positions have been substituted in a compound,[20,21] and this should have important implications for the use of radiochemicals in radioimmunoassay and metabolic studies.

For proteins, complete chemical syntheses are generally not possible. However, it is readily possible to synthesize peptides, and $^{13}C$-enriched amino acids have been incorporated into several peptides.[55,56] Individual sites in the intact, fully active, globular protein, ribonuclease S, have been studied utilizing the noncovalent binding of the synthetic amino terminal 1 to 15 peptide to ribonuclease S-protein (residues 21 to 124).[57,58]

### Biosynthesis

With biosynthetic incorporation, all sites may

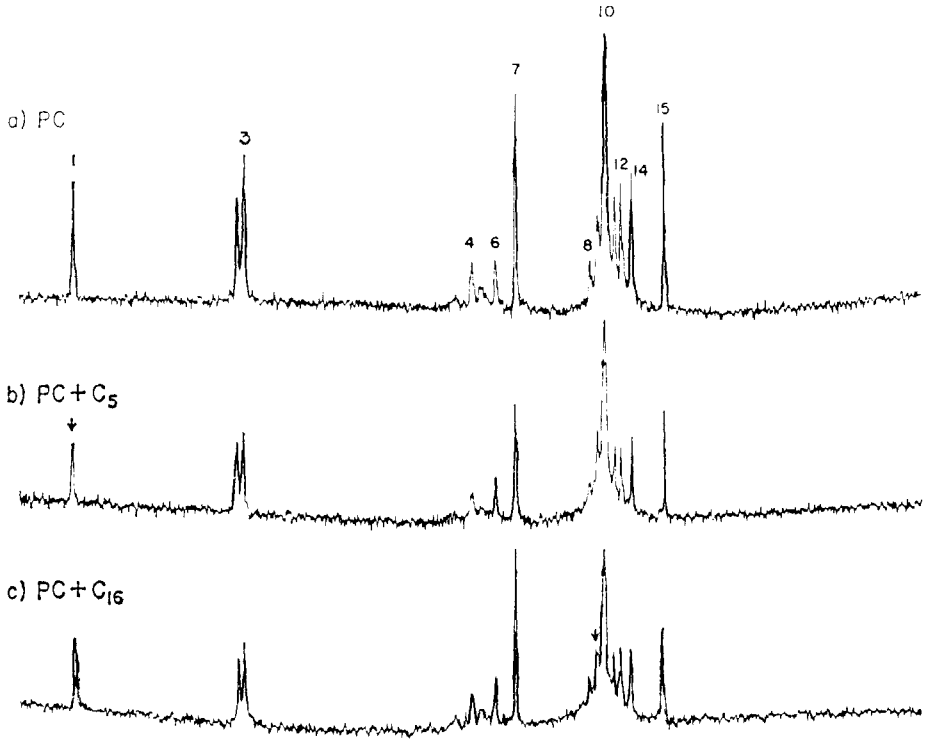


FIGURE 7. Natural abundance proton-decoupled carbon-13 NMR spectra at 15 MHz of sonicated aqueous egg-yolk phosphatidyl choline dispersions: (a) vesicles, (b) vesicles labeled with C5 stearic acid derivative spin label, and (c) vesicles labeled with C16 spin label. The arrows indicate the resonances most affected by the spin label. (Reprinted with permission of Godici, P. E. and Landsberger, F. R., *Biochemistry*, 13, 362, 1974. Copyright by the American Chemical Society.)

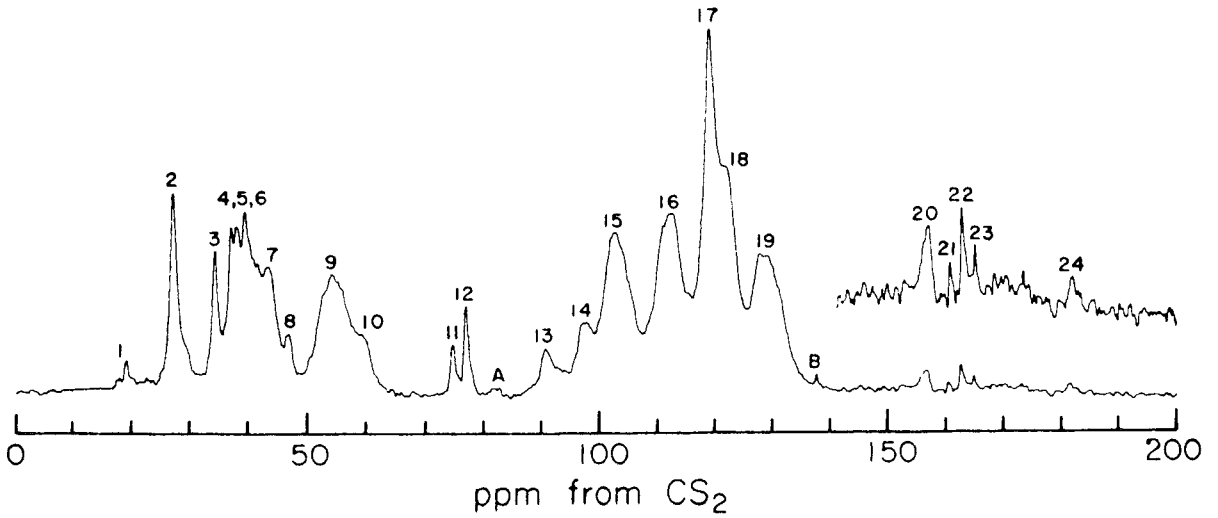


FIGURE 8. Natural abundance proton-decoupled carbon-13 NMR spectrum at 15 MHz of unfractionated baker's yeast tRNA in water (150 mg/ml). Inset: resonance is 20 to 24, derived from rare bases. (Reprinted with permission of Komorski, R. A. and Allerhand, A., *Biochemistry*, 13, 369, 1974. Copyright by the American Chemical Society.)

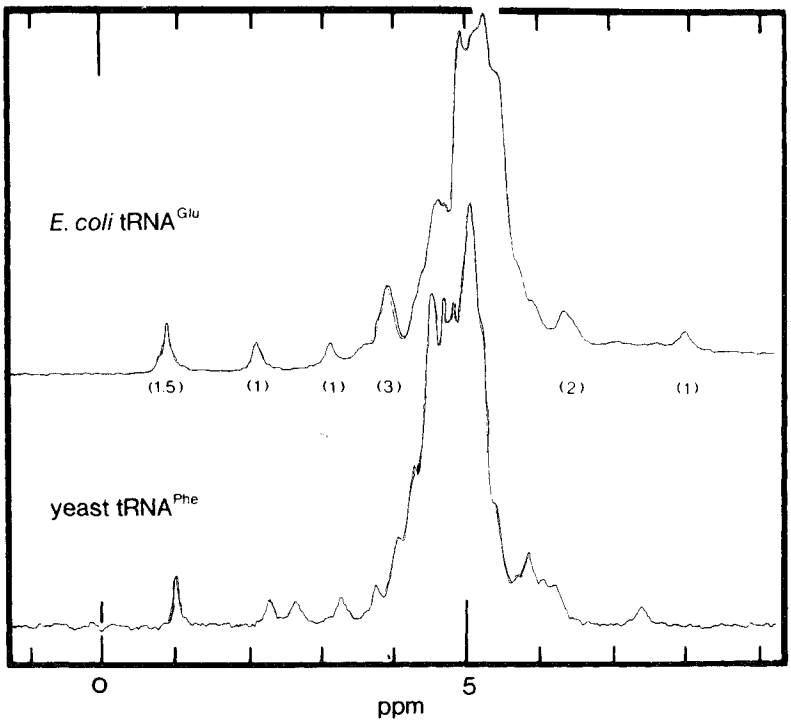


FIGURE 9. Phosphorus-31 NMR spectra at 109 MHz of *E. coli* tRNA species in cacodylate buffer containing magnesium. (From Gueron, M. and Shulman, R. G., *Proc. Natl. Sci. U.S.A.*, 72, 3482, 1975. With permission.)

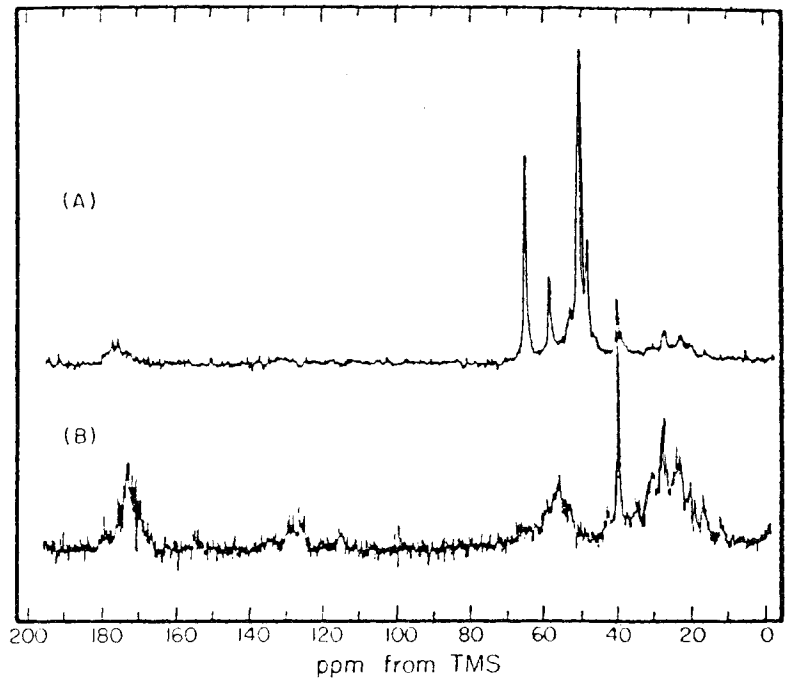


FIGURE 10. Proton decoupled carbon-13 NMR spectra at 25 MHz of A. [2-$^{13}$C] carboxymethylferricytochrome c and B. ferricytochrome c. (Reprinted with permission of Eakin, R. T., Morgan, I. O., and Matwiyoff, N. A., *Biochemistry*, 14, 4538, 1975. Copyright by the American Chemical Society.)

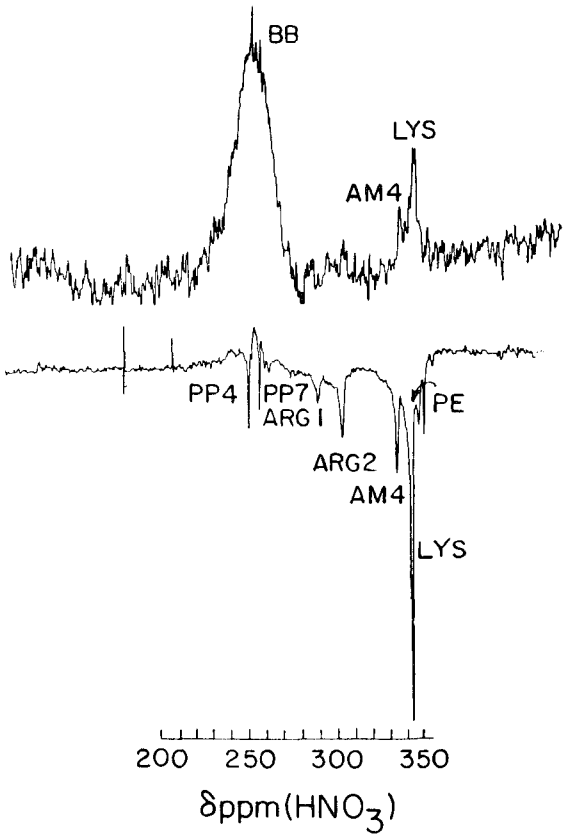


FIGURE 11. Nitrogen-15 NMR spectra at 9 MHz of $^{15}N$ enriched (approximately 90%) whole *E. coli* cells (A) undecoupled and (B) proton decoupled. (From Lapidot, A. and Irving, C. S., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1988, 1977. With permission.)

become enriched from the added enriched precursor, unless specific metabolic blocks occur. This may be an advantage in certain cases, e.g., in the formation of uniformly $^{15}N$-enriched whole cells of bacteria grown in 90% $^{15}N$-ammonium chloride. Lapidot and Irving have found that the nulling of the majority of the protein $^{15}N$ resonances on proton decoupling enabled them to observe sharp inverted resonances (Figure 11) which they have attributed to relatively mobile cell-wall components.[32] Thus, they are able to determine the dynamics of cell-wall materials[59] both isolated and in whole cells.[30] They have also determined the effect of hydrogen-bonding on the $^{15}N$ resonances of [$^{15}N$-gly] hemoglobin derived from Friend leukemic cells grown in $^{15}N$-enriched media.[60] It is also necessary to use biosynthetic $^{13}C$ enrichment in order to study the mobility of phospholipid chains *in situ*.[7,42] Enriched $^{13}C$-methyl methionine cytochrome *c*[61] (MW 12,000) and β-galactosidase[62] (MW 450,000) have been biosynthetically prepared from methionine-requiring auxotrophs grown on enriched $^{13}C$-methyl methionine. The selective oxidation of methionines to methionine sulphoxides was monitored in the case of β-galactosidase, and the degree of conversion was correlated to the degree of enzymatic activity indicating the involvement of at least one methionine in the mechanism of action.[62] More selective biosynthetic incorporation has been obtained where only one residue of the desired type is present in the product.[63]

**Isotope Substitution**

The chemical substitution of a group by one that is chemically identical but isotopically different, in an intact molecule, may be distinguished from the chemical synthesis or biosynthesis of a partially enriched product. The chemical substitution of $^{13}C$-methyl methionine groups in myoglobin has been achieved by treatment with $^{13}CH_3I$. Thus, Jones et al.[64] were able to resolve and study the resonances of the two methionines in myoglobin (Figure 12).

In addition to the above types of studies, $^{13}C$-enriched substrates have been used to detect enzyme complexes[65,66] and an enzyme-substrate intermediate[67] by $^{13}C$ NMR spectroscopy.

The use of isotopic enrichment must be approached with caution, particularly where multiple- or high-level enrichments are used. These result in couplings between the heteronuclei themselves which give splittings and multiple resonances.[68,69] While this reduces the overall sensitivity and increases the complexity of the spectra, these couplings are the source of valuable conformational information (Figure 13). The measurement of $^{13}C$-$^{15}N$ couplings also promises to provide a valuable conformational tool in studies of peptides.[70]

## SUBSTITUENTS FOR $^1H$

Three types of applications utilizing NMR may be delineated in studies of nuclei substituted for $^1H$, depending on the properties of the heteronuclei. These may be roughly divided as follows:

**Substituent for $^1H$ in structural studies** – $^{19}F$ constitutes a reasonable substituent for $^1H$ which shares many of its properties such as chemical bonding characteristics and spin quantum number.

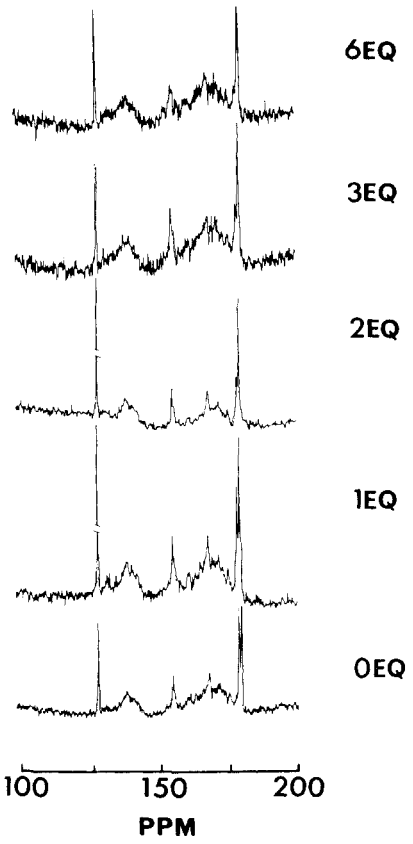


FIGURE 12. Proton decoupled carbon-13 NMR spectra at 25 MHz of $^{13}$C-methyl methionine ferrimyoglobin. The number of equivalents of free radicals is shown on the right. (From Jones, W. C., Rothgeb, T. M., and Gurd, F. R. N., *J. Biol. Chem.*, 251, 7452, 1976. With permission.)

$^{19}$F by contrast to $^{1}$H does not occur in biological systems and gives a wide range of chemical-shift values which are sensitive to the local electronic microenvironment.[71] Therefore, it has been used as a selective stable nuclear substituent for $^{1}$H in amino acids,[72] nucleotides,[73] and proteins.[57] An excellent example of biosynthetic incorporation of $^{19}$F is via fluorotyrosine into alkaline phosphatase[19,30] in which eleven fluorotyrosine resonances were observed by $^{19}$F NMR (Figure 14). However, in general, $^{19}$F-substrates may prove toxic for biological growth, and specific mutants may be required in these studies.

**Substituent for $^{1}$H in metabolic studies** – $^{19}$F is not a sensible probe as a metabolic substituent for $^{1}$H since its nuclear radius is larger and $^{19}$F metabolites are often toxic. $^{3}$H is an excellent metabolic substituent, and its radioactivity ensures that the progress of its incorporation can be followed with great sensitivity. However, the position of incorporation is not directly known from radioactivity measurements but can now be determined by mass spectrometric and by direct nondestructive $^{3}$H NMR observation (Figure 15).[20]

**Substituent for $^{1}$H in dynamic studies** – While $^{2}$H can be observed at natural abundance by NMR,[74] its main value lies in its use as a probe of hydrogen dynamics in biological systems. This results from the fact that since the $^{2}$H relaxation times are dominated by the quadrupolar mechanism, there is no significant intermolecular component as there is, for example, in dipole-dipole relaxation mechanisms. Thus, NMR observation of $^{2}$H provides a probe for the purely intramolecular $\tau_c$ value. This fact has been most exploited in studies of membrane systems where $^{2}$H is incorporated into selective positions on a phospholipid chain and NMR measurements are taken of vesicles or in vivo under different experimental conditions (Figure 16).[14,75]

In addition to direct observation of $^{2}$H, this nucleus may be used as a negative label for removal of resonances in the observation of $^{1}$H NMR. This approach has been used to simplify the $^{1}$H NMR spectra of proteins derived from microorganisms grown on deuterated media.[76,77]

Similar dynamic types of applications are possible for $^{17}$O but, partly because of the extreme broadness of the resonances due to the value of the quadrupole moment (I = 5/2) and the low sensitivity and natural abundance of this nucleus, very few applications have been recorded.[78] One application includes the $^{17}$O (and $^{15}$N) titration properties of enriched glycylglycine.[79] Similarly, the pH-dependence of the $^{14}$N NMR resonances of a series of biologically interesting molecules has been described.[80]

## METABOLIC PROBES

Heteronuclei other than isotopes of hydrogen which may be used as metabolic probes are $^{31}$P, $^{13}$C, and $^{15}$N. Many phosphate esters are vital components of biological systems and it is possible to obtain their relative concentrations at equilibrium from the relative areas (intensities) of their resolved $^{31}$P NMR signals. It is also possible to follow their interconversions as a function of time

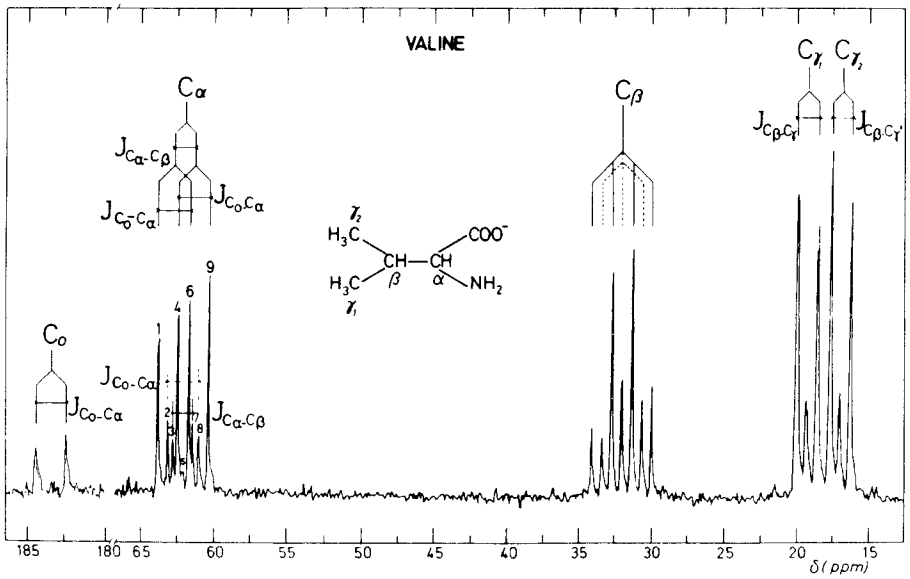


FIGURE 13. Proton decoupled carbon-13 NMR spectrum at 25 MHz of enriched (85%) $^{13}$C-valine. (From Tran-dinh, S., Fermandjian, S., Sala, F., Marmet-Bouvier, R., Cohen, M., and Fromageot, P., *J. Am. Chem. Soc.*, 96, 1484, 1974. With permission.)

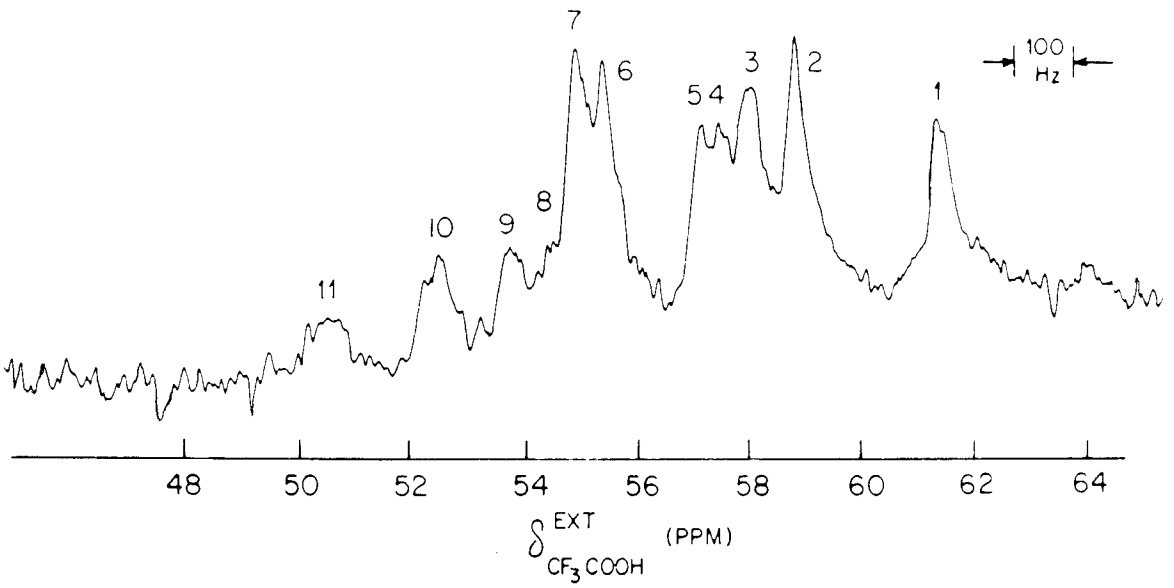


FIGURE 14. Fluorine-19 NMR spectrum at 94 MHz of fluorotyrosine alkaline phosphatase in Tris. HCl buffer (25 mg/ml). Each numbered resonance represents a single fluorotyrosine residue. (From Sykes, B. D., Weingarten, H. I., and Schlesinger, M. J., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 469, 1974. With permission.)

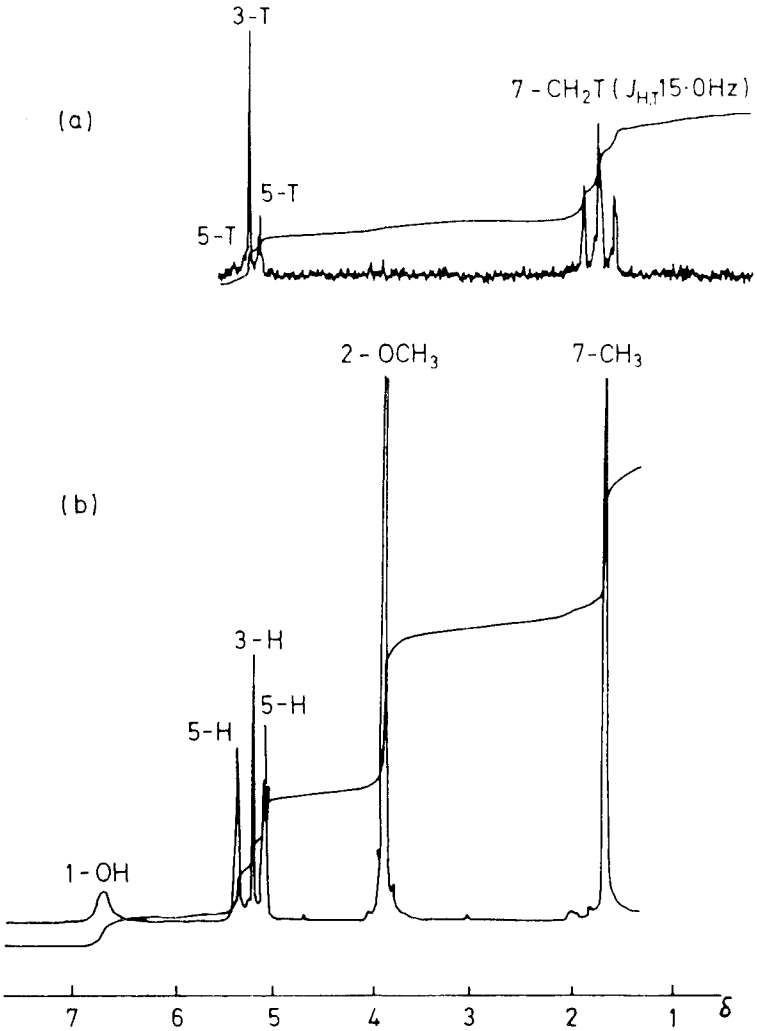


FIGURE 15. (a) Tritium NMR spectrum at 96 MHz penicillic acid (35 mg, approximately 98.3 mCi/mmol) in 100 μl deuteroacetone. (b) $^{1}$H NMR spectrum at 90 MHz. (From Al-Rawi, J. M. A., Elvidge, J. A., Jarswal, D. K., Jones, J. R., and Thomas, R., *Chem. Commun.*, 220, 1974. With permission.)

using $^{31}$P NMR, provided a steady state does not exist. This condition arises from the fact that $^{31}$P exists as 100% natural abundance; thus, no selective isotopic enrichment is possible in order to observe the dynamics of a metabolic equilibrium. Hoult et al.[81] have described the time course of conversions of ATP in glycogen particles (Figure 17) and whole frog muscle by $^{31}$P NMR at high-magnetic field strength to gain sensitivity. Bock and Sheard have observed an intermediate in the reaction of alkaline phosphatase,[82] and Burt et al. have observed unusual resonances in muscles which they were able to attribute to unknown naturally occurring components.[83] There is no doubt that $^{31}$P NMR is an extremely important metabolic probe technique for biological systems[84] enabling, for example, the nondisturbing measurement of internal cell pH[85] and comparative studies of the phosphate metabolism of tumor cells.[86]

Similar applications are possible with $^{13}$C and $^{15}$N, but because of the need for isotopic enrichment, this becomes an expensive and sometimes difficult proposition. Nevertheless, the possibility of direct observation of the metabolic fate of selected carbon and nitrogen atoms under condi-

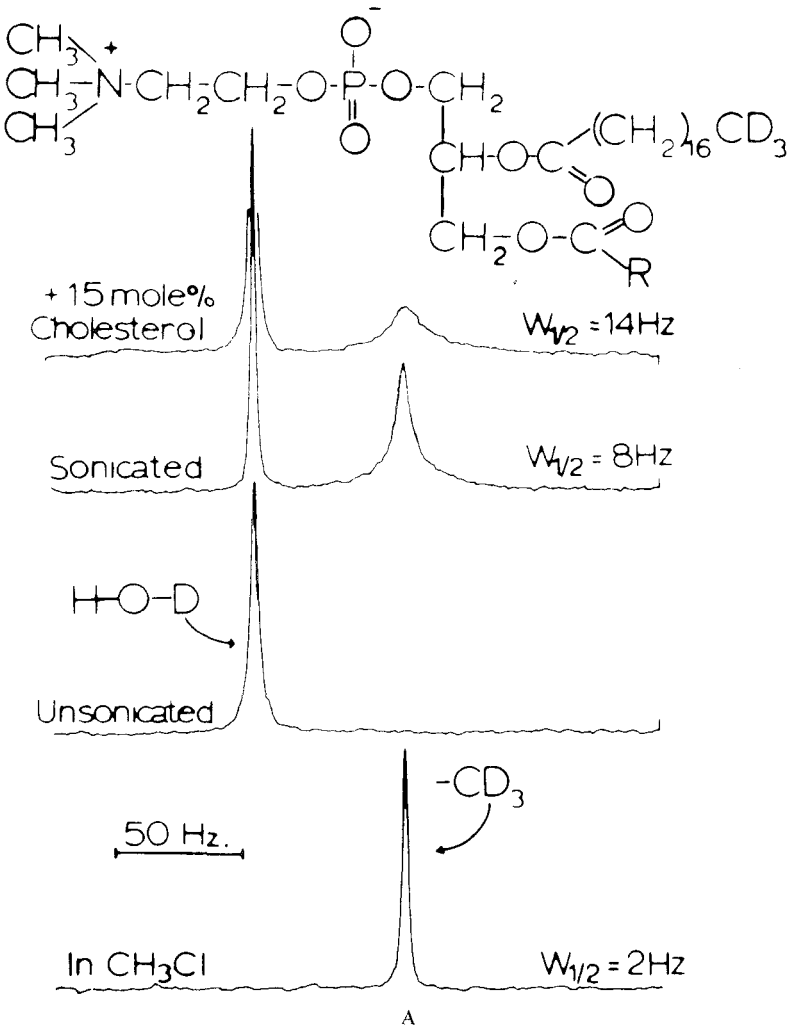


FIGURE 16. A. Deuterium NMR spectrum at 15 MHz of ω-trideuteriostearoylphosphorylcholine in chloroform (15 mg/ml) and as 10 mol % in sonicated aqueous egg phosphorylcholine dispersions. B. Dependence of the $^{2}H$ NMR linewidth ($W_{1/2}$) on cholesterol concentration for lauric and stearic acid probes in sonicated dispersions. (From Stockton, G. W., Polnaszek, C. F., Leitch, L. C., Tulloch, A. P., Hasan, F., and Smith, I. C. P., *Biochem. Biophys. Res. Commun.*, 60, 849, 1974. With permission.)

tions of metabolic equilibrium means that applications of this approach will undoubtedly grow, particularly coupled with mass spectrometric observations.[11,87] Recent applications include, for example, the elucidation of the pattern of incorporation of $^{13}C$-labeled alanine, proline, glycine, and serine into the microbial product prodigiosin by $^{13}C$ NMR,[88] and the metabolic pathway of $^{13}C$-valine in methylmalonicacidemia in man.[9]

It has also been suggested that $^{12}C$ depletion is an alternative or complementary technique to $^{13}C$ enrichment studies.[89]

## CONCLUSION

A wide range of biological applications of heteronuclear NMR techniques are feasible. Selective applications depend upon the nuclear properties and natural abundance of the various biologically relevant heteronuclei.

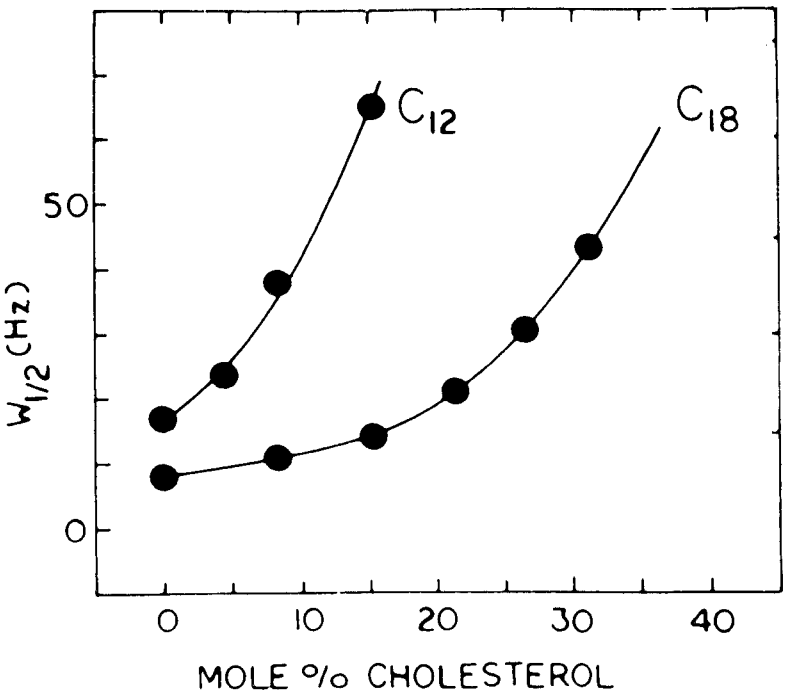


FIGURE 16B

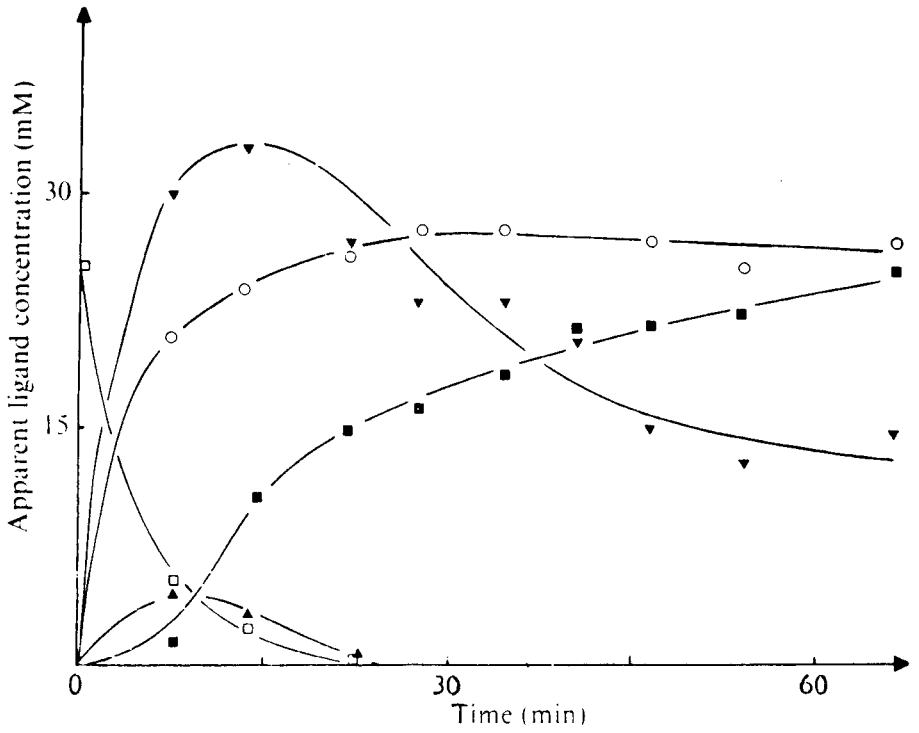


FIGURE 17. Variations of concentrations of metabolites in glycogen particles as indicated by the phosphorus-31 NMR resonance intensities at 129 MHz following the addition of ATP (□) at time zero; ADP, (▲); AMP, (○); glucose-6-phosphate, (■); and Pi, (▼). (From Hoult, D. I., Busby, S. J. W., Gadian, D. G., Radda, G. K., Richards, R. E., and Seeley, P. J., *Nature*, 252, 285, 1974. With permission.)

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