^{15}N NMR Studies of Biological Systems

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Nitrogen is widely distributed in biological systems and is often directly involved in biochemical processes of interest. Nitrogen-15 nuclear magnetic resonance (^{15}N NMR) spectroscopy is, therefore, potentially a very valuable technique for the study of biological systems. Within the last several years, a number of exciting, pioneering ^{15}N NMR studies of biomolecules have been reported, and this review will describe several of these in three areas of biochemistry: (1) proteins including proteolytic enzymes and hemoproteins, (2) nucleic acids and nucleotide bases and their interactions in base pairing and drug binding, and (3) in vivo metabolism and cellular dynamics in intact microorganisms and plants. ^{15}N NMR studies of amino acids, oligopeptides, and nucleotides have been reviewed in an excellent book by Levy and Lichter[1] as well as in recent comprehensive reviews by Witanowski, Stefaniak, and Webb,[2] and Martin, Martin, and Gouesnard[3] and will not be included here. First, we will outline some features of ^{15}N NMR that are important in the studies of large molecules.

One of the advantages of ^{15}N NMR is the wider range of chemical shifts for nitrogen, approximately 900 ppm, compared to hydrogen and carbon. Structural and environmental factors that influence nitrogen chemical shifts have been extensively discussed elsewhere.[1,2] Until recently, however, ^{15}N NMR studies of biomolecules have been limited by the low sensitivity of the ^{15}N nucleus, 1.04×10^{-3} times that of proton, and by its low natural abundance, 0.37%. At the natural-abundance level, the theoretical sensitivity of ^{15}N is 3.8×10^{-6} that of the proton at constant magnetic field. Although natural-abundance ^{15}N NMR studies of some biomolecules have been reported,[4] selective ^{15}N enrichment is often essential for obtaining ^{15}N spectra of biomolecules within practical time limits.

Other factors that affect the feasibility of ^{15}N NMR experiments are the relaxation times and nuclear Overhauser effects (NOE) of the ^{15}N nucleus, which, in turn, depend on the correlation time, τ_c, of the molecule.

The dependence of spin–lattice relaxation times, T_1, on τ_c has been calculated,[4,5] assuming dipolar relaxation to protons and isotropic reorientation. Significantly, T_1 rapidly decreases with increasing τ_c until a minimum is reached at about 8.5×10^{-9} s for ^{15}N at 18.25 MHz. (With stronger magnetic fields, T_1 will reach a minimum at smaller τ_c values.[1]) This correlation time corresponds roughly to that of a globular protein with a molecular weight of approximately 14 000 in aqueous solution.[4,6] Further increases in τ_c result in an increase in T_1. Thus, in biomolecules of moderate size, T_1 is much shorter than in small molecules. This dramatic decrease of T_1 with increasing τ_c allows rapid signal accumulation for biomolecules.

The spin–spin relaxation time, T_2, is related to the spectral line width, $\nu_{1/2}$, by $T_2 = 1/\pi\nu_{1/2}$. The width of a ^{15}N resonance rapidly increases as the correlation time exceeds 10^{-9} s.[7] Such line broadening severely limits the observation of ^{15}N resonances from very large molecules.

The dependence of NOE on τ_c has been calculated.[7] The relative signal intensity changes from −3.93 for short correlation times to 1.0 for long τ_c values. The nuclear Overhauser enhancement factor, η, is negative because ^{15}N has a negative gyromagnetic ratio. The large negative enhancement results in a nearly fourfold increase in signal intensity for many protonated nitrogens in small molecules. However, for biomolecules with correlation times on the order of 10^{-9} s or more, nitrogen nuclei may not experience full Overhauser effects and, in unfavorable cases, signal intensities may be nulled. On the other hand, because the NOE changes rapidly with τ_c in the region of interest, NOE values can be a sensitive probe of molecular mobility, and some examples of this will be described in the next section.

Application of ^{15}N NMR to Biological Systems

Proteins. The first natural-abundance ^{15}N NMR spectra of proteins were obtained by Gust, Moon, and Roberts[4] with a Bruker WH 180 spectrometer operating at 18.25 MHz. The proton-decoupled ^{15}N spectrum of lysozyme (molecular weight 14 300) showed intense multiple resonances for the guanidino nitrogens of arginyl and the amine nitrogens of lysyl residues as well as a broad resonance for the backbone peptide nitrogen. The correlation time, τ_c, of the lysozyme molecule as a whole (considered as a spherical molecule) was esti-

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† Contribution No. 6635.

(1) G. C. Levy and R. L. Lichter, "Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy", Wiley, New York, 1979.
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(7) C. S. Irving and A. Lapidot, *Biochim. Biophys. Acta*, **470**, 251 (1977), and references therein.

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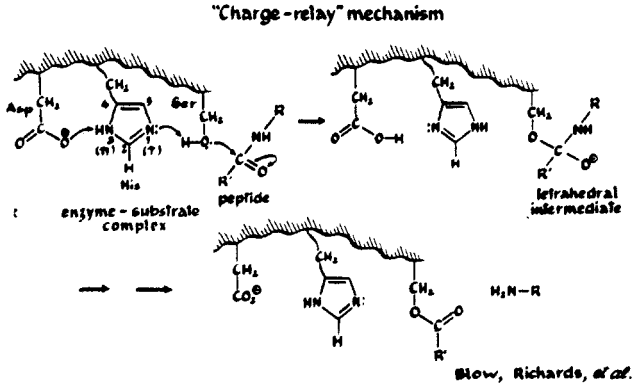
mated to be approximately 5×10^{-9} s from the observed NOE of the amide nitrogens, while a τ_c of 3.5×10^{-10} s was obtained for the guanidino nitrogens of its arginyl residues from their average T_1 values, indicating considerable segmental motion for these side chains.

The first ^{15}N NMR study of a selectively ^{15}N-enriched native enzyme was carried out by Bachovchin and Roberts[8] on α-lytic protease of *myxobacter* 495. α-Lytic protease belongs to a family of serine proteases characterized by the presence at the active site of a "catalytic triad" comprised of serinyl, histidyl, and aspartyl residues. A "charge-relay" mechanism (eq 1) has



(1)

been proposed to explain the role of the catalytic triad in the cleavage of peptide bonds by serine proteases.[9] This mechanism requires the nitrogens of the histidyl imidazole ring to be weaker bases than the aspartate carboxylate. Hunkapiller, Smallcombe, Whitaker, and Richards[9] reported, on the basis of the ^{13}C NMR spectrum of α-lytic protease enriched in ^{13}C at C2 of its single histidine, that this histidyl imidazole has $pK_a < 4$. An active-site group with an apparent pK_a of 6.7, whose ionization controls catalytic activity, was assigned to the aspartyl carboxylate.

However, the problems remained, because different pK_a values have been reported for histidyl residues of serine proteases from proton NMR.[10,11] To gain further insight into this question, the ionization behavior of the histidyl residue in α-lytic protease was studied by ^{15}N NMR using a ^{15}N-labeled enzyme prepared with histidine, enriched in ^{15}N in the imidazole ring. Figure 1 shows representative ^{15}N NMR spectra of ^{15}N-enriched α-lytic protease. The pK_a of this histidyl residue, as determined by the changes of its ^{15}N chemical shift with pH, was found to be 7.0 ± 0.1 at 26 °C. This finding is contrary to the previous report that the histidyl residue at the active site of α-lytic protease is an abnormally weak base while the "buried" aspartyl residue is likely to be an abnormally weak acid. Thus, the ^{15}N NMR results offered no support for the charge-relay mechanism as formulated in eq 1. Furthermore, the ^{15}N chemical shifts showed that, at catalytically active pH values, the tautomer with hydrogen on N3 (the π nitrogen) of the imidazole ring predominates. This is an unusual tautomeric state both for simple 4-substituted imidazole derivatives and for histidyl residues in proteins. Its predominance in α-lytic protease was attributed to a hydrogen-bonded interaction between NH at the 3-position and the adjacent "buried" carboxylate group of aspartic acid.

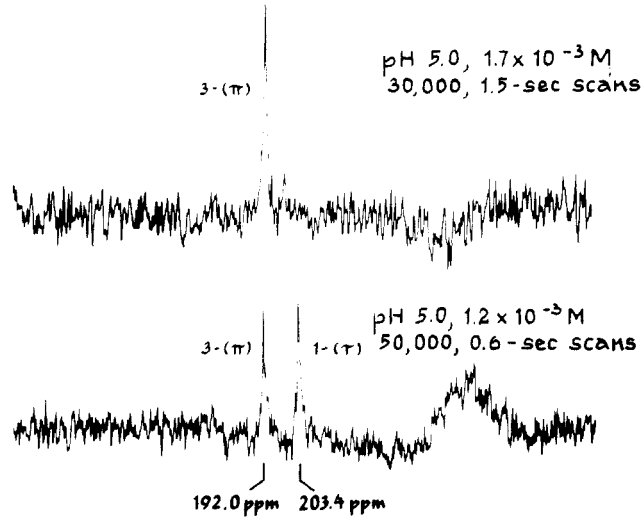
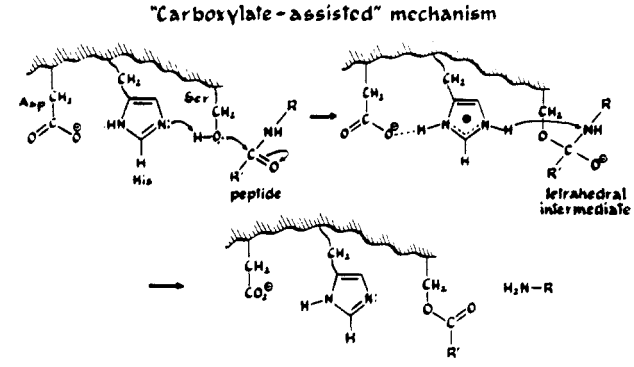


Figure 1. Proton-coupled ^{15}N NMR spectra, at 18.2 MHz of α-lytic protease from an auxotroph of *myxobacter* 495 + ^{15}N-labeled histidine (1 His/molecule, M_r 19 860). Top: ^{15}N-enriched at N3(π). Bottom: ^{15}N-enriched at both N1(τ) and N3(π).

On the basis of these findings, an alternative mechanism was proposed for the catalytic triad of serine protease. This mechanism (eq 2) featured (i) a sufficient degree of hydrogen bonding of the histidyl imidazole in the tautomeric form with the proton on N3 and the aspartate carboxylate to make this tautomer the predominant one, and (ii) assistance of transfer of the serine proton to N1 of the imidazole by both hydrogen and electrostatic bonding between the resulting imidazolium cation and aspartate carboxylate. This mechanism might progress to the serine ester stage by transfer of a proton from the imidazolium cation to the nitrogen of the leaving RNH^- group.



(2)

Recently, Roberts, Yu, Flanagan, and Birdseye[12] studied hydrogen-bond interactions between imidazole and carboxylate groups and their effects on the tautomeric equilibrium of the former, using *cis*-urocanic acid as a model for the histidyl–aspartyl carboxylate pair at the active site of serine proteases. ^{15}N NMR studies of *cis*- and *trans*-urocanic acid in aqueous solution showed that, in the cis isomer, wherein the imidazole ring and the carboxylate group can be correctly aligned for the formation of a hydrogen bond, the tau-

(8) W. W. Bachovchin and J. D. Roberts, *J. Am. Chem. Soc.*, **100**, 8041 (1978).
(9) M. W. Hunkapiller, S. H. Smallcombe, D. R. Whitaker, and J. H. Richards, *Biochemistry*, **12**, 4732 (1973), and references therein.
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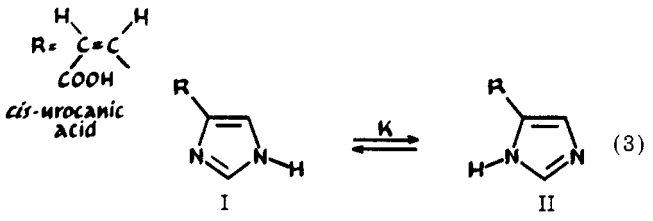
(12) J. D. Roberts, Chun Yu, C. Flanagan, and T. Birdseye, *J. Am. Chem. Soc.*, **104**, 3945 (1982).

tomeric form with the proton on N3 (II) predominates, whereas for the trans isomer and imidazole-4-propionic acid, I is the predominant tautomer. The equilibrium constant K of eq 3 was calculated to be 5 for *cis*-uro-



(3)

canic acid compared to 6.7 for the histidyl imidazole in α-lytic protease. The pH dependence of the ^{15}N shifts of *cis*-urocanic acid, with pK_a of 7.3, was found to be remarkably similar to that of the histidyl imidazole in α-lytic protease. These results provide strong support for the interpretation[8] that the unusual tautomeric state of the histidyl imidazole of α-lytic protease is caused by a hydrogen-bond interaction between NH at the 3-position and the adjacent aspartyl carboxylate.

^{15}N NMR has also been employed to probe the active-site conformation of a metalloenzyme, carboxypeptidase A, in solution. A long-standing controversy regarding this active site is centered on whether its conformation in solution is different from the conformation in crystals. The major conformation of the crystalline enzyme, in the absence of substrate, is the one in which its active-site tyrosine hydroxyl is distant (17 Å) from the zinc atom.[13] On substrate binding, the tyrosine hydroxyl moves closer to the peptide bond of the substrate and to within 4–5 Å of the catalytic zinc.[14] This large inward movement of tyrosine has been regarded as one of the most striking examples of the induced-fit model of enzyme–substrate binding.

On the other hand, Johansen and Vallee[15] have proposed that, in solution, the tyrosine hydroxyl is complexed to Zn in the free enzyme in the absence of substrate, and thus has a conformation significantly different from that of the crystalline enzyme. Their proposal was based on studies of a modified, but catalytically active, carboxypeptidase, arsanilazocarboxypeptidase,[16] which showed visible absorption[15] and resonance Raman spectra[17] similar to those of a model azotyrosine–Zn complex around pH 8.5. However, the equilibrium constant for formation of such a tyrosyl–Zn complex in the enzyme and the precise structure of the complex were not established.

^{15}N NMR gives quite pertinent information as to the structure and the extent of formation of azotyrosyl–Zn complex in the enzyme, because the chemical shifts of azine-type nitrogens are highly sensitive to coordination with metal ions. Bachovchin, Kanamori, Vallee, and Roberts[18] prepared arsanilazocarboxypeptidase enriched in ^{15}N at both azo nitrogens (Zn–AzoCPA, Figure 2) and

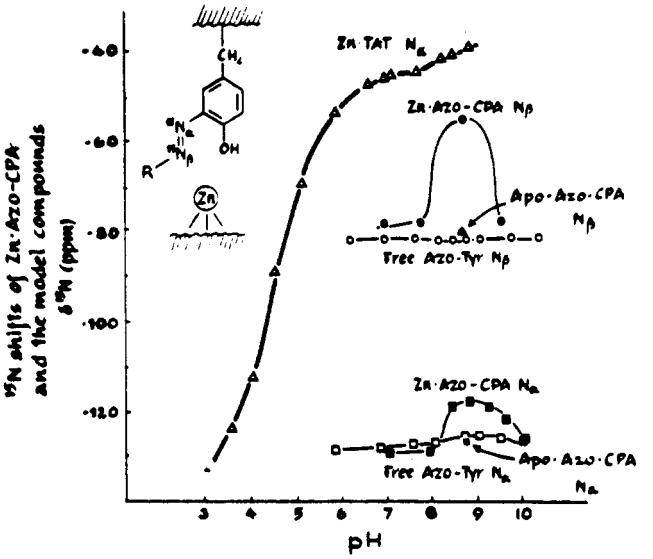


Figure 2. pH dependence of the ^{15}N shifts of the two azo nitrogens of arsanilazocarboxypeptidase (Zn–AzoCPA: ■, N_α; ●, N_β); apoarsanilazocarboxypeptidase (Apo–AzoCPA: ☆, N_α; ▲, N_β); and the model compounds arsanilazo-*N*-acetyltyrosine (free Azo–Tyr: □, N_α, ○, N_β) and tetrazolylazo-*N*-acetyltyrosine–Zn complex (Zn–Azo–Tyr: △, N_α). R = $PhAsO_3H^-$.

studied the ^{15}N chemical shifts as functions of pH (i) for the free enzyme in solution and (ii) on removal of Zn from the enzyme. These shifts were compared to those of a model azotyrosine (arsanilazo-*N*-acetyltyrosine) and an azotyrosine–Zn complex (tetrazolylazo-*N*-acetyltyrosine–Zn complex). The results are summarized in Figure 2. At pH 7.0, the positions of the resonances of the two azo nitrogens, N_α and N_β, of Zn–AzoCPA were found to be close to those of the free model azotyrosine, and, therefore, there is no significant coordination with zinc at this pH. However, at pH 8.8, N_β of the enzyme showed 26.9 ppm shielding relative to N_β of free azotyrosine, while a smaller shielding of 8.1 ppm was observed for N_α. By comparison with the shifts of the model azotyrosine–Zn complex, these shieldings indicate substantial coordination of zinc with the azotyrosyl at pH 8.8. As the pH is further raised to 10.3, both azo nitrogens shift downfield again, because a hydroxide ion binds to zinc and breaks up the azotyrosine–Zn complex. When zinc is removed from the enzyme (Apo–AzoCPA in Figure 2) at pH 8.8, its azo nitrogens are deshielded to values characteristic of free azotyrosine. These results provide independent evidence for the formation of the Zn–azotyrosine complex and establish N_β as the site of coordination to zinc. Furthermore, the high sensitivity of ^{15}N shifts to coordination with zinc permits a semiquantitative estimate of the degree of azotyrosine–Zn complex and shows the extent of coordination to be approximately 55% at pH 8.8.

Because selective ^{15}N enrichment of an enzyme is often difficult and time consuming, consideration should be given to whether useful information about active sites can also be obtained by studying small ^{15}N-enriched molecules (ligands, coenzymes, inhibitors) bound to the protein. Morishima and Inubushi[19–22]

(13) G. N. Reeke, J. A. Hartsuck, M. L. Ludwig, F. A. Quiocho, T. A. Steitz, and W. N. Lipscomb, *Proc. Natl. Acad. Sci. U.S.A.*, **58**, 2220 (1967).
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(18) W. W. Bachovchin, K. Kanamori, B. L. Vallee, and J. D. Roberts, *Biochemistry*, **21**, 2885 (1982).
(19) I. Morishima and T. Inubushi, *FEBS Lett.*, **81**, 57 (1977).
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(21) I. Morishima and T. Inubushi, *J. Am. Chem. Soc.*, **100**, 3658 (1978).

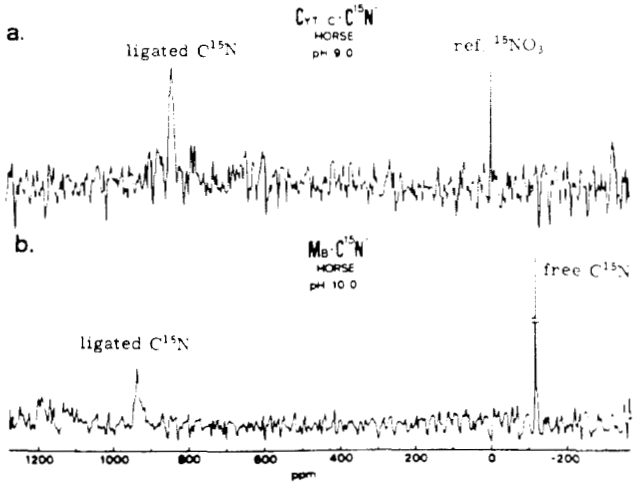

Figure 3. Cyanide nitrogen resonance spectra of $C^{15}N$ complexes of myoglobin and cytochrome *c*. (a) Horse heart cytochrome *c* at pH 9; (b) horse myoglobin at pH 10. (Reproduced with permission from ref 19. Copyright 1977, Elsevier (Amsterdam).)

studied the ^{15}N NMR spectra of ^{15}N-enriched cyanide bound to low-spin ferric iron in various porphyrin derivatives and hemoproteins to probe heme environmental structure and ligand–heme interactions. Figure 3 shows some representative spectra of cyanide–hemoprotein complexes. Relative to free cyanide, iron-bound cyanides show large downfield shifts, presumably of paramagnetic origin. The effects on the cyanide ^{15}N shifts of cis ligands (porphyrin derivatives) and trans ligands (pyridine derivatives) were studied in model complexes. While the cis effect caused by substitution of the porphyrin peripheral groups was small, the trans effect induced by a change in the axial ligand was found to be much more pronounced; the increased basicity of the trans ligand causing a downfield shift of the cyanide ^{15}N resonance. A substantial difference in the iron-bound $C^{15}N$ shifts has been observed in the cyanide complexes of cytochrome *c*, myoglobin, and hemoglobin, ranging from 957 to 1172 ppm relative to free CN in the order cytochrome *c* < myoglobin < hemoglobin. This was primarily interpreted in terms of the trans effect, i.e., the relative binding strengths of the trans ligand, namely the imidazole nitrogen of proximal histidine, to the heme iron. Here, the proximal histidine is considered to be most strongly bound to the heme iron in hemoglobin and least strongly bound in cytochrome *c*.

The ^{15}N NMR of ^{15}N-labeled compounds bound to a large enzyme has been used by Becker and Roberts[23] to assist in determining the structure of a liver alcohol dehydrogenase–inhibitor complex. Alcohol dehydrogenase from horse liver (LADH) is a dimeric, zinc-containing enzyme of M_r 80 000 that catalyzes the oxidation of ethanol to acetaldehyde with NAD^+ as the coenzyme. LADH forms a tight ternary complex with NAD^+ and the inhibitor pyrazole. It has been proposed[24] that, in the ternary complex, N2 of pyrazole is complexed to the active-site zinc, facilitating the formation of a pyrazole–NAD^+ adduct where N1 of pyrazole is covalently bonded to C4 of the coenzyme, producing a reduced coenzyme. Spectrophotometric evi-

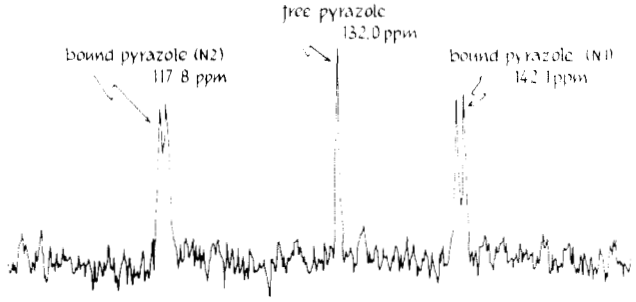

Figure 4. Proton-decoupled 18.25-MHz, ^{15}N NMR spectrum of the LADH–pyrazole–NAD^+ ternary complex, showing ^{15}N-labeled pyrazole resonances in ppm upfield of 1 M $H^{15}NO_3$.

dence suggests formation of a reduced form of coenzyme; however, no very direct proof of this has been previously reported. Becker and Roberts[23] determined the ^{15}N chemical shifts of the ternary complex prepared with N1- and N2-^{15}N-labeled pyrazole and N1-^{15}N-labeled NAD^+. The ^{15}N resonance of the coenzyme showed a 91-ppm upfield shift from NAD^+, analogous to the 109-ppm upfield shift of NADH from NAD^+.[25] The ^{15}N resonances from pyrazole showed two nonequivalent nitrogens with a ^{15}N–^{15}N coupling constant of about 10 Hz (Figure 4). These results demonstrate formation of a *covalent* adduct within the active site of the enzyme, with bond formation between N1 of pyrazole and the nicotinamide ring of the coenzyme. The N2 resonance of the complexed pyrazole is 40–50 ppm upfield from free 1-substituted pyrazoles, a value that seems only explicable through complexation of N2 to the active-site zinc atom. Of unusual interest is the fact that, although broad ^{15}N resonances might be expected from such a high molecular weight enzyme complex, rather narrow line widths (5–20 Hz) were actually observed, which indicates a high degree of segmental mobility, at least of that part of the enzyme that contains the pyrazole–coenzyme adduct. Because the line widths were those expected of a low molecular weight complex, it was important that the pyrazole could not be removed by dialysis.

Nucleotides and Nucleic Acids. The nitrogens of the purine and pyrimidine bases in nucleic acids are directly involved in the formation of hydrogen bonds between complementary base pairs.[26] ^{15}N NMR spectroscopy is useful for studying such interactions between base pairs because ^{15}N shifts of azine-type nitrogens are especially sensitive to hydrogen bonding. The ^{15}N chemical shifts of the major nucleosides and nucleotides have been measured and assigned by Markowski, Sullivan, and Roberts[27] and by Hawkes, Randall, and Hull[28] using natural-abundance samples as well as by Büchner, Mauer, and Rüterjans[29] and Büchner, Blomberg, and Rüterjans[30] using ^{15}N-enriched samples. These results have been reviewed.[1-3] Here, we will focus on more recent works in which the for-

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(24) H. Theorell and T. Yonetani, *Biochem. Z.*, **338**, 537 (1963).
(25) N. J. Oppenheimer and R. M. Davidson, *Org. Magn. Reson.*, **13**, 14 (1980).
(26) J. D. Watson and F. H. C. Crick, *Nature (London)*, **171**, 737 (1953).
(27) V. Markowski, G. R. Sullivan, and J. D. Roberts, *J. Am. Chem. Soc.*, **99**, 714 (1977).
(28) G. E. Hawkes, E. W. Randall, and W. N. Hull, *J. Chem. Soc., Perkin Trans. 2*, 1268 (1977).
(29) P. Büchner, W. Maurer, and H. Rüterjans, *J. Magn. Reson.*, **29**, 45 (1978).
(30) P. Büchner, F. Blomberg, and H. Rüterjans, *Proc. 11th Jerusalem Symp. Quant. Chem. Biochem.*, 1978

mation of hydrogen bonds between base pairs has been investigated by ^{15}N NMR spectroscopy.

Poulter and Livingston[31] studied a mixture of (3-^{15}N)-2′,3′,5′-tri-*O*-benzoyluridine and 5′-*O*-acetyl-2′,3′-*O*-isopropylideneadenosine in deuteriochloroform and observed a 4.73 ppm downfield shift in N3 of the substituted uridine in the presence of a three molar excess of the substituted adenosine. This shift change was reasonably ascribed to a base-pair interaction. Dyllick-Brenzinger, Sullivan, Pang, and Roberts[32] measured shifts for mixtures of cytidine and guanosine in dimethyl sulfoxide and observed a 6.5 ppm upfield shift for N3 and a 1.1 ppm downfield shift for the NH_2 nitrogen of cytidine in the presence of a twofold excess of guanosine. The NH_2 nitrogen of guanosine also showed a 1.0 ppm downfield shift in the presence of equimolar cytidine. These results are consistent with the formation of a guanosine–cytidine base pair.

Recently, James, James, and Lapidot[33] obtained NMR spectra of uniformly ^{15}N-enriched native DNA from *Escherichia coli*. The cytosine N3 in double-stranded DNA was shifted about 10 ppm upfield, relative to the monomer, which is in accord with the N3 being a proton acceptor in a hydrogen bond. Gonnella, Birdseye, Nee, and Roberts[34] have obtained spectra of uniformly ^{15}N-enriched transfer RNA from yeast. Formation of the random-coil conformation by heating resulted in no very large changes in ^{15}N chemical shifts, presumably because (1) interbase hydrogen bonds are not as extensive in native tRNA with its "cloverleaf" structure as in double-stranded DNA, and (2) interbase hydrogen bonds are replaced by hydrogen bonds to water on denaturation. Marked changes in proton–^{15}N Overhauser effects have been observed in both DNA and tRNA on denaturation. In DNA, the NOE of protonated nitrogens became negative on changing to the single-stranded conformation.[33] In tRNA, the nonprotonated ring nitrogens, which have NOE of 0∼1 in the native conformation, showed an algebraic decrease in NOE, resulting in weaker resonances.[34] Both phenomena are surely the result of greater chain mobility and, hence, shorter correlation times on denaturation.

Another promising area of investigation by ^{15}N NMR is the study of binding sites of anticancer drugs to DNA. Nee and Roberts[35] have investigated the binding of the anticancer drug *cis*-diamminedichloroplatinum(II), *cis*-DDP, to nucleosides and found a large upfield coordination shift of the nitrogen resonances. In the bis(guanosine) complex, *cis*-$[Pt(NH_3)_2guanosine_2]^{2+}$, the platinum is bound at N7 as indicated by the 92 ppm upfield shift of this resonance relative to the uncomplexed nucleoside. Cytidine is bound by *cis*-DDP at N3, complexation resulting in an upfield shift of 76 ppm. Adenosine appears to bind *cis*-DDP, to at least some degree, through N1, N3, N7, and $-NH_2(6')$. The sensitivity of ^{15}N shifts to coordination with metal ions in those mononucleosides is encouraging. Further studies using complementary oligonucleotide strands as a model for DNA would be particularly useful.

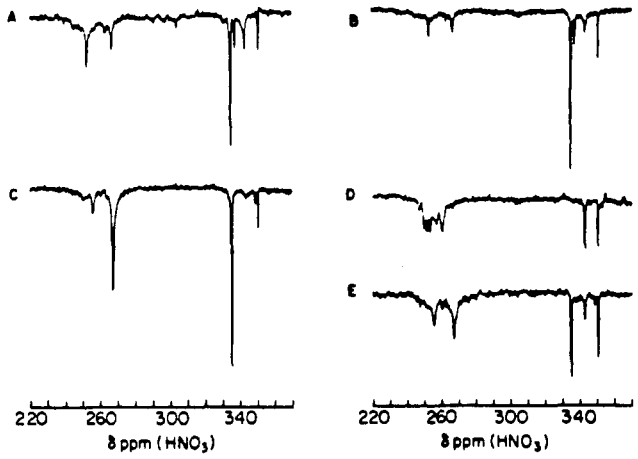

Figure 5. Proton-decoupled, 9.12-MHz, ^{15}N NMR spectra at 27 °C of five ^{15}N-enriched intact Gram-positive bacterial cells. (A) *Bacillus licheniformis*; (B) *Bacillus subtilis*; (C) *Staphylococcus aureus*; (D) *Micrococcus lysodeikticus*; (E) *Streptococcus faecalis*. (Reproduced by permission from ref 39.)

Intact Organisms. NMR studies of intact organisms can provide valuable information on the pathways and rates of metabolism in vivo. Isotopic tracers, radioactive and stable, of carbon, hydrogen, phosphorus, and sulfur have been the most valuable tools for studying metabolic processes. For nitrogen, which lacks a reasonably long-lived radioisotope, ^{15}N is especially important as a potential tracer of nitrogenous metabolites. High-resolution NMR allows observation of intact organisms with little or no perturbation, and ^{15}N NMR can be very powerful for studying nitrogen metabolism in vivo as well as serving as a useful probe of the dynamic properties of nitrogenous cellular components in intact organisms.

a. Solution NMR Techniques. The first ^{15}N NMR spectra of intact living cells were reported by Llinas, Wüthrich, Schwotzer, and von Philipsborn,[36] who grew a fungus, *Ustilago sphaerogena*, with (^{15}N)ammonium acetate as the sole nitrogen source and obtained a proton-decoupled ^{15}N spectrum of the cell slurry at 10.1 MHz. The four ^{15}N resonances observed had chemical shifts characteristic of peptidyl amides and were attributed to substances in the low molecular weight peptide pool of the cell.

Irving and Lapidot, in a series of recent papers,[7,37-41] have reported on the dynamic properties of cell-wall components in uniformly ^{15}N-enriched intact bacteria by ^{15}N NMR. The major resonances observed were those of mobile groups of cell-wall components (Figure 5). In a representative work,[39] the mobilities of various components of bacterial cell walls were estimated from the NOE values of their ^{15}N resonances, which are functions of correlation times. In Gram-positive bac-

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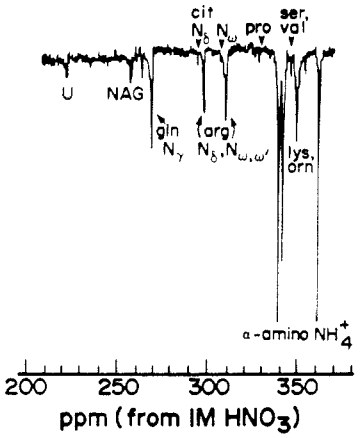

Figure 6. Proton-decoupled, 18.25-MHz, ^{15}N NMR spectrum in ppm upfield of 1 M $H^{15}NO_3$ of intact *N. crassa* mycelia germinated on minimal medium containing 0.2% $^{15}NH_4Cl$. Cit, citrulline; NAG, *N*-acetyl-D-glucosamine; U, uridine.

teria whose cell-wall peptidoglycan consists of glycan strands, peptide stems, and cross-linking groups, all the peptidoglycan ^{15}N resonances observed in proton-decoupled ^{15}N spectra of intact cells were those of cross-linking groups. It was concluded that only the cross-linking groups have a high degree of motional freedom while the glycan strands and peptide stems are rigid, resulting in the nulling of their resonances.

Legerton, Kanamori, Weiss, and Roberts[42] obtained ^{15}N spectra from suspensions of intact fungus *Neurospora crassa*. Well-resolved resonances of amino acids were observed; these include the amide nitrogen of glutamine, the guanidino nitrogen of arginine, the ureido nitrogens of citrulline, side-chain amine nitrogens of ornithine and/or lysine, and α-amino nitrogens of alanine, glutamic acid, and other amino acids (Figure 6).

The ^{15}N spin–lattice relaxation time, T_1, and the nuclear Overhauser effects (NOE) were measured for intracellular glutamine, alanine, and arginine in *N. crassa* to probe their various intracellular environments.[43] The relaxations of $^{15}N_\gamma$ of glutamine, $^{15}N_\alpha$ of alanine, and $^{15}N_{\omega,\omega'}$ of arginine in *N. crassa* were found, on the basis of the NOE values, to be predominantly the result of ^{15}N–H dipolar relaxation. These relaxations are therefore related to the microviscosities of the various environments and associations of the respective molecules with other cellular components that act to increase the effective molecular sizes.

For $^{15}N_\gamma$ of glutamine in the cytoplasm, the intracellular T_1 (4.1 s) was only slightly shorter than that in the culture medium (4.9 s). By contrast, for $^{15}N_{\omega,\omega'}$ of arginine, which is sequestered in vacuoles containing polyphosphates, the intracellular T_1 (1.1 s) was only one-fourth of that in the medium (4.6 s). By comparison with the T_1 values of these nitrogens in model systems of various viscosities and in those containing polyphosphates, it was concluded that either the vacuolar viscosity is substantially above 2.8 cP, a viscosity considerably greater than that of the cytoplasm, or the ω,ω' nitrogens of arginine are highly associated with a polyanion, possibly polyphosphate, in the vacuole.

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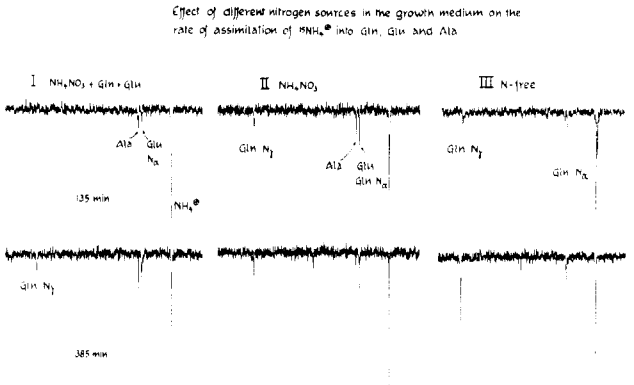

Figure 7. Proton-decoupled ^{15}N NMR spectra of intact *N. crassa* mycelia at 135 min, and at 385 min after $^{15}NH_4Cl$ addition. The mycelia were first grown on different unlabeled nitrogen sources [(I) NH_4NO_3 supplemented with glutamic acid and glutamine; (II) NH_4NO_3; or (III) NH_4NO_3 followed by 3 h of nitrogen starvation] and subsequently transferred to $^{15}NH_4Cl$ (0.2% w/v) medium. Each spectrum represents 250 transients accumulated in 8 min.

Kanamori, Legerton, Weiss, and Roberts[44] also studied the effect of different nitrogen sources in the growth medium on the rate of assimilation of $^{15}NH_4^+$ into glutamine, glutamic acid, and alanine (Figure 7). The rate of glutamine synthesis was fastest after growth in media deficient in free ammonium ion, whereas it was slowest following growth in media containing both glutamic acid and glutamine. The reverse trend was observed for the biosynthesis of alanine. A competition between the two biosynthetic pathways for the same substrate, glutamic acid, was found to limit the rate of alanine synthesis when glutamine synthesis was rapid. These results suggest that ^{15}N NMR could be a useful technique for studying cellular regulation of nitrogen metabolism in vivo.

b. Solid-State NMR. Schaefer, Stejskal, and McKay[45] have used solid-state ^{15}N NMR spectroscopy to study nitrogen metabolism in plants. Magic-angle spinning[46] and cross-polarization[47] techniques were used to enhance, respectively, the resolution and the sensitivity of the ^{15}N resonance. ^{15}N spectra of intact seed and lyophilized pods and leaves of soybeans grown on ^{15}N-enriched ammonium nitrate were obtained. Resonances from amide nitrogens of proteins and α-amino nitrogens of amino acids as well as imidazole nitrogen of histidine and guanidino nitrogens of arginine were observed (Figure 8). In a recent paper, Jacob, Schaefer, Stejskal, and McKay[48] reported studies of the uptake and metabolism of ^{15}N nitrate by *N. crassa* by solid-state NMR. *N. crassa* were first grown on either ammonia or nitrate (unlabeled) media, transferred to ^{15}N nitrate media, and harvested at various times, and ^{15}N spectra were taken of lyophilized *N. crassa* mycelia. The assimilation of ^{15}N nitrogen into the amide nitrogen of proteins and α-amino nitrogens of amino acids was delayed in those cultures that were initially grown on

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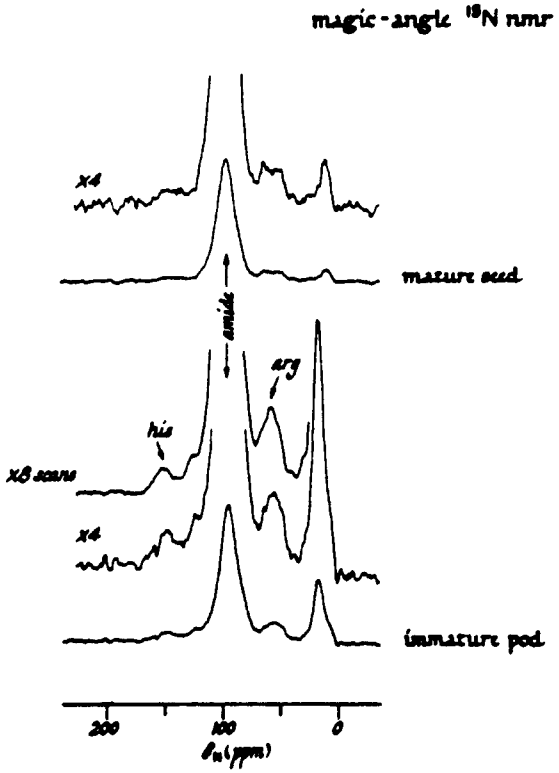

Figure 8. Cross-polarization, 9.12-MHz, ^{15}N NMR spectra of ^{15}N-labeled soybean obtained with magic-angle spinning at 1.7 kHz. The scale is in ppm from solid ammonium sulfate. (Reproduced with permission from ref 45. Copyright 1979, Academic Press.)

the ammonia medium. This is consistent with the results of enzyme assays in crude mycelia extracts that showed that nitrate reductase was initially absent from mycelia grown on ammonia medium and was not maximally induced until after 3-h growth in (^{15}N)nitrate medium. In higher plants, the amount of nitrate reductase that can be extracted from various tissues varies with plant species, age, and cultural techniques. Thus, accurate determinations of reductase activity are generally difficult, and ^{15}N NMR provides a method for directly observing the metabolism of ^{15}N nitrate supplied to a cultured intact tissue.

Concluding Remarks

As in other new areas of science, ^{15}N NMR of biomolecules were initially beset with difficulties and uncertainties; yet, they have come along and have been enlivened by unexpected and rewarding results. Progress, such as has been described here, has been made possible by a combination of outstanding technological advances in NMR instrumentation, increased availability of ^{15}N-enriched compounds, and a clearer delineation of what can be studied in biochemical systems.

In the area of protein chemistry and enzymology, ^{15}N NMR has proved to be particularly useful for studying acid–base and tautomeric equilibria of active-site residues such as histidine, and subtle interactions such as hydrogen bonding, which play important roles in catalysis but are difficult to detect by other techniques. Investigations of ligand–metal ion interactions in metalloproteins have also proved to be fruitful. Another promising area of study is the interaction of ^{15}N-enriched quasisubstrates, coenzymes, and inhibitors with the active sites of enzymes.

With nucleic acids, ^{15}N NMR studies of mononucleotides have shown that ^{15}N chemical shifts, especially of azine-type nitrogens, are sensitive to hydrogen-bonding interactions such as are involved in base pairing and coordination to metal ions, which are important in certain anticancer drugs. Extension of drug-binding studies to oligo- and polynucleotides as models for DNA is particularly important.

In the study of intact organisms, solution ^{15}N NMR studies of suspensions of intact, uniformly ^{15}N-enriched microorganisms have shown that well-resolved resonances can be obtained from mobile, cellular components such as structural peptidoglycans with high segmental motions and small metabolites as amino acids, if present in reasonably high concentration. ^{15}N NMR is especially valuable for studying the cellular regulations of nitrogen metabolism as they occur in vivo through observation of the flux of metabolites through competing pathways. Another promising area of study is the incorporation of selectively ^{15}N-enriched precursors and observation of their metabolic fate in vivo.

^{15}N NMR is also a useful probe for studying the intracellular dynamics and environments of metabolites and cellular components. From measurements of T_1 and NOE values, it is possible to estimate correlation times and microviscosities of molecular environments.

Solid-state ^{15}N NMR studies of plants using magic-angle spinning cross-polarization NMR experiments on in vivo biological systems will surely also prove practical for those organisms that metabolize relatively slowly, such as bacterial spores or plant tissue in cell culture. Cross-polarization signals will arise from cellular components that are completely, or partially, immobilized. The technique, therefore, complements the use of solution ^{15}N NMR to measure the flux of stable isotopes in in vivo cellular systems.

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