NATURAL ABUNDANCE NITROGEN-15 NMR BY ENHANCED HETERONUCLEAR SPECTROSCOPY

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The detection of NMR spectra of less sensitive nuclei coupled to protons may be significantly improved by a two-dimensional Fourier transform technique involving a double transfer of polarization. The method is adequate to obtain natural abundance ^{15}N spectra in small sample volumes with a commercial spectrometer

1. Introduction

In spite of the obvious biological relevance, nitrogen-15 NMR has been hitherto rather limited in its applications, requiring large, concentrated samples and lengthy accumulations [1]. Nitrogen-15 chemical shifts can provide insight into the sequencing of peptides because the shifts are not merely characteristic of the individual amino acids but also depend on the neighboring residues [2]. Furthermore, the three-bond scalar coupling $^3J_{NH}$ to the α proton of the next residue may provide information about the torsional angle of the peptide bond [3,4]. The sensitivity is limited by the 0 36% isotopic abundance and the gyromagnetic ratio ($\gamma_N/\gamma_H = -0.101$) which entails a relative signal intensity of 0 001 compared to an equal number of protons, disregarding the unfavorable longitudinal relaxation times [5] In addition, the negative Overhauser effect ($+1 > 1 + \eta > -3$ 9) is often incomplete and may lead to accidental signal cancellations for some molecular correlation times [6,7]

A variety of techniques have been proposed to enhance the sensitivity of nuclei like nitrogen-15. An attractive approach consists in observing the proton spectrum of a ^{15}N enriched sample as a function of the offset of a cw nitrogen decoupler, to monitor the collapse of the heteronuclear scalar coupling J_{NH} when the decoupler has the precise frequency of the nitrogen chemical shift [8]. The method is less attractive for samples with natural isotopic abundance, and

tends to be rather time consuming, since the changes in the proton spectrum must be observed while the second rf field is stepped in a point-by-point manner through the spectral range of the nucleus of interest.

Maudsley and Ernst [9] have recently introduced heteronuclear two-dimensional spectroscopy to extend the Fourier advantage not only to the observation of the proton resonances, but to the spectrum of the insensitive nucleus as well. In this experiment, the nitrogen-15 magnetization is generated initially by a non-selective 90° pulse, and hence the sensitivity suffers from the inherently modest Boltzmann polarization, from the unfavorable Overhauser effect, and from saturation if the experiment is repeated before the longitudinal magnetization is fully recovered. On the other hand, there is a considerable gain in sensitivity by observing at the proton frequency after the transfer of the magnetization from the nitrogen to the proton transitions.

A method proposed recently by Morris and Freeman [10] for the detection of insensitive nuclei enhanced by polarization transfer (INEPT) follows an opposite strategy, by transferring magnetization from protons to the less sensitive nucleus. The resulting signal amplitude in the ^{15}N spectra is proportional to the population difference that normally occurs across proton transitions. In N-acetyl valine, the experimental enhancement achieved with the INEPT sequence was found to be seventeen-fold (in comparison with a normal undecoupled ^{15}N spectrum, obtained with a flip angle of

30° found to be optimum for a pulse repetition rate of 0 7 s common to both experiments). A ten-fold enhancement factor arises from the ratio γ_H/γ_N which governs the relative Boltzmann population differences, the additional factor 1.7 is due to the more favorable proton relaxation [11] As in conventional ^{15}N NMR, the inherent disadvantage of the detection at a lower frequency has to be taken into account

This letter describes a two-dimensional experiment which initially generates proton magnetization, transfers the coherence to the nitrogen transitions and, after an interval designed to probe the nitrogen chemical shift, transfers the coherence back to the proton transitions to provide the advantage of the detection at high frequencies The pulse sequence shown in fig. 1 indicates the double transfer of coherence symbolically by the sinusoidal pattern representing the precession of the transverse magnetization. The τ delays are approximately adjusted to $(4J_{NH})^{-1}$ and the nitrogen precession is monitored by incrementing the evolution period t_1 in regular increments The proton decoupling of the nitrogen spectrum, which is achieved by the 180° proton pulse in the middle of the evolution period, may be omitted, the nitrogen decoupling of the proton spectrum during the acquisition period is also optional The details of the pulse sequence and phase alternations will be discussed below, suffice it to say here that the proton spectrum observed experimentally with this technique vanishes if the nitrogen pulses are omitted or set too far off resonance.

The application of this pulse sequence to a 1 M solution of 99% enriched N-acetyl valine in perdeuterated dimethylsulphoxide (DMSO-d_6) produces the proton spectra shown in fig. 2 The amide proton gives rise
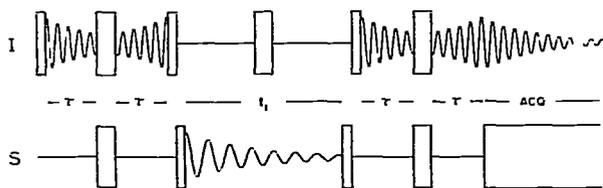

Fig. 1. A total of ten pulses are applied to the I spins (protons or other sensitive nuclei) and to the S spins (^{15}N or other nuclei with low gyromagnetic ratio) The transfer of magnetization is indicated schematically. The different pulse widths represent flip angles of 90° and 180° The proton pulse at $\frac{1}{2}t_1$ and the nitrogen decoupling in the acquisition period are optional.
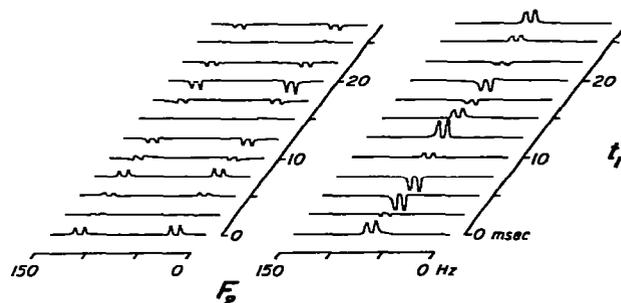

Fig. 2 Proton spectra of the NH region of enriched N-acetyl valine, observed with the ten-pulse sequence as a function of the nitrogen evolution time t_1 To the left, the decoupling has been omitted in both dimensions, leaving a heteronuclear splitting $^1J_{NH} \approx 95$ Hz apart from the smaller splitting $^3J_{HH} \approx 7$ Hz The right side shows the collapse resulting from nitrogen decoupling as well as a simpler t_1-modulation pattern due to proton decoupling of the nitrogen precession. The experimental conditions may be adjusted by maximizing the signal for $t_1 = 0$, provided four transients are averaged as described in table 1.

to a doublet of doublets ($^1J_{NH} \approx 95$ Hz and $^3J_{HH} \approx$ 7 Hz) seen in the left half of fig 2. The heteronuclear splitting collapses when the nitrogen spins are decoupled during signal acquisition (right half of fig 2), the smaller splitting is due to the α proton of the amino acid and is not essential to the experiment To optimize the various delays and flip angles in the pulse sequence, it is sufficient to maximize the signal amplitude of the first spectrum obtained for $t_1 = 0$ Ideally, the signal should be comparable to that obtained after an ordinary 90° proton observation pulse, although the transverse decay, expressed by a factor $\exp(-4\tau/T_2)$, should be taken into account. As the evolution period t_1 is incremented, the proton signals experience an amplitude modulation which reflects the offset of the nitrogen transitions from the low-frequency transmitter. For example, the proton signals appear upside down if both nitrogen magnetization vectors precess by 180° in the t_1 interval The t_1 modulation of the proton peaks in fig 2 may be subjected to a second, real Fourier transformation which results in the F_1 frequency domain shown in fig. 3. This domain is equivalent to a nitrogen-15 spectrum observed at 27.36 MHz. (The proton Larmor frequency occurs at 270 MHz in these experiments) To the left in fig. 3, each of the four traces shows a doublet, because the 180° proton pulse at $\frac{1}{2}t_1$ has been omitted. These proton coupled
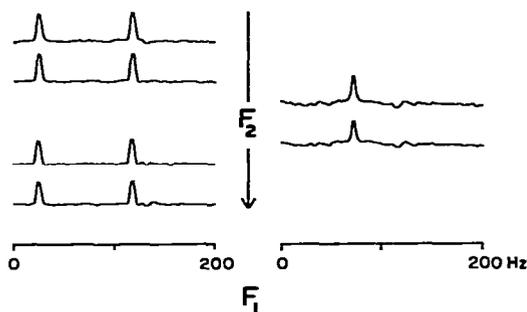
Fig 3. Sections taken from a phase-sensitive two-dimensional frequency plot, obtained from the data shown in fig. 2 after a second Fourier transformation. The F_2 domain corresponds to the peaks in the proton spectra, the F_1 dimension shows ^{15}N spectra. As in fig 2, the decoupling has been omitted in the left half of the figure. All signals are in absorption mode without requiring phase corrections

Fig 4. Spectra of N-acetyl valine with natural nitrogen-15 abundance. Apart from the t_1-modulated ^{15}N satellites, the time—frequency domain (left) shows central doublets which result from the imperfect cancellations of the proton signals of ^{14}N containing molecules. The proton-decoupled nitrogen spectrum (right), obtained by Fourier transformation of the t_1-modulation of the highest-field satellite, provides an accurate measure of the ^{15}N chemical shift, in spite of some artifacts due to imperfect cancellation.

nitrogen spectra may be useful to observe $^3J_{NH}$ couplings to the α protons of neighboring peptide residues [3,4]. All four spectra are in absorption mode, and do not require any phase corrections in the F_1 domain, provided the initial duration of t_1 is vanishingly short. The right half of fig. 3 shows the effect of the second Fourier transformation when the spectra are decoupled in both dimensions. The F_1 or ^{15}N domain is simplified to a single resonance at the nitrogen chemical shift. A peptide would give rise to a two-dimensional map, where the proton and nitrogen shifts of each NH group determine the frequency coordinates, thus providing additional information for the assignment of the signals.

The experiment is designed, in principle, to suppress any proton signal that does not originate from the ^{15}N transitions. The implications for N-acetyl valine in natural isotopic abundance (0.36%) are shown in fig 4. The central proton resonances in the amide region belong to the ^{14}N isotope; this signal, which is normally 280 times stronger than the ^{15}N satellites, has been reduced to about 1%, reflecting short term instabilities of the spectrometer. The interference of the residual central component with the ^{15}N satellites reduces the signal-to-artifact ratio achieved after the second Fourier transformation. Nevertheless, the nitrogen chemical shift is clearly identified in the F_1 domain in fig. 4, which represents a spectrum of 3.6 mM ^{15}N in a 5 mm sample tube. The use of smaller sample volumes opens the way to the study of materials which are not abundantly

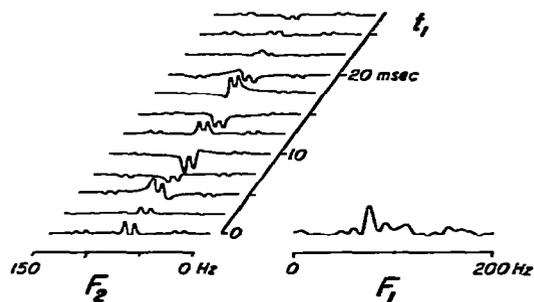available. The technique is suitable both for studies in deuterated organic solvents as well as for non-exchanging NH groups of proteins dissolved in D_2O.

2. Details

The mechanism of the ten-pulse sequence shown in fig 1 is best discussed for a heteronuclear AX system consisting of one proton coupled to one ^{15}N nucleus. Such a unit occurs in all peptide bonds with the exception of proline, with scalar coupling constants J_{NH} typically around 92 Hz [12]. The proton magnetization, consisting of two vectors precessing with frequencies $\delta_H \pm \frac{1}{2}J_{NH}$, is initially rotated into the $+y$ axis of the rotating frame, and refocused by simultaneous $180°$ pulses applied to both nuclei, to form an echo at time $2\tau = (2J)^{-1}$. The heteronuclear coupling causes the two vectors to refocus along opposite $+x$ and $-x$ axes of the rotating frame. As in INEPT, a $90°_y$ proton pulse, applied at the top of the echo, flips one of these vectors back into the equilibrium position, while the other is inverted and ends up along the $-z$ axis. It can be readily shown that the two ^{15}N transitions have become associated with dramatically enhanced population differences, of $+2\Delta + 2\delta$ and $-2\Delta + 2\delta$ respectively (in this conventional notation, the equilibrium population differences are defined to be 2Δ for protons and

2δ for nitrogen transitions, with $\Delta/\delta = 10$). An alternative experiment uses a 90°_{-y} proton pulse at the top of the echo to invert the other proton magnetization vector instead, and yields population differences across the nitrogen transitions of $-2\Delta + 2\delta$ and $+2\Delta + 2\delta$ respectively. The subtraction of the signals obtained from two such experiments cancels the 2δ terms and therefore eliminates the nuclear Overhausser effect (the latter stems from a net redistribution of populations across the nitrogen transitions, and is reflected by a change in δ only).

Now the ^{15}N magnetization is brought into the transverse plane of a frame rotating in synchronism with the nitrogen carrier frequency. Because of the populations prevailing just before the 90°_x nitrogen pulse, the two doublet components point along opposite $+y$ and $-y$ axes. A proton 180° pulse at $\frac{1}{2}t_1$ interchanges the identity of the two nitrogen vectors, a vector initially rotating with the frequency $\delta_N + \frac{1}{2}J_{NH}$ resuming its precession with the frequency $\delta_N - \frac{1}{2}J_{NH}$ in the second half of the evolution period. At the end of the t_1 interval, each vector has accumulated a phase $2\pi\delta_N t_1$ The second 90°_x nitrogen pulse rotates the y components back into the z axis, thus generating population differences which are "read" by a 90°_x proton pulse At this point, the two proton magnetization vectors are in opposite phase, but after an interval $2\tau = (2J_{NH})^{-1}$ both vectors acquire the same phase [13]. The simultaneous application of 180° pulses to both nuclei in the middle of this interval removes the frequency dependent phase shift and eliminates signal losses due to inhomogeneous decay in this interval. Once the proton doublet is in phase, a continuous nitrogen decoupler may be applied.

To cancel the proton signals arising from molecules containing ^{14}N, the phase of the first 90° nitrogen pulse is alternated, which reverses the algebraic sign of the information transferred to the protons [9]. The proton signals are added or subtracted according to the scheme in table 1, which also has the virtue of eliminating the nuclear Overhauser effect from the ^{15}N spectra Any spurious transverse magnetization generated by the first nitrogen "180°" pulse is also cancelled.

The experiments were performed with a Bruker 270 MHz spectrometer. The 5 mm probe, originally designed for proton decoupled fluorine-19 NMR, was tuned for proton observation, the decoupler coil being retuned to resonate for ^{15}N. A frequency synthesizer

Table 1

In a frame rotating in synchronism with the proton carrier frequency, the phases of all proton pulses in fig. 1 are along the x axis except for the phase φ_H of the *third* proton pulse, which alternates between $+y$ and $-y$ as shown. In the nitrogen frame, all phases are along the x axis except for the phase ϕ_N of the *second* ^{15}N pulse Adding and subtracting the free induction decays as shown suppresses all signals except those arising from a double transfer of magnetization from protons to nitrogen and back, and eliminates the nuclear Overhauser effect

Transient	ϕ_H	φ_N	Acquisition
1	y	x	add
2	y	$-x$	sub
3	$-y$	$-x$	add
4	$-y$	x	sub

generated a signal at 27 36 MHz which was gated, phase-shifted with a double balanced mixer, and amplified to 9 W with a Boonton 230A amplifier. The pulse sequence was generated by modifying the computer program controlling a Nicolet 293 pulse programmer. A Nicolet 1080 computer was used for the two-dimensional Fourier transformation. The spectra of the enriched sample were obtained in 1 h, those of the natural abundance sample required about 8 h. In theory, the advantage in sensitivity over conventional ^{15}N spectroscopy is determined by the factor $(\gamma_H/\gamma_N)^{5/2}$ In practice however, the two-dimensional method tends to be rather time consuming, particularly if high resolution is to be achieved in the ^{15}N dimension [14]. Furthermore, the "noise" does not only arise from the receiver coil and the preamplifier, but consists primarily in artifacts, whose intensity is proportional to the amplitude of the signals themselves. The limitations of the sensitivity for dilute solutions should be explored experimentally.

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