Applications of natural-abundance nitrogen-15 nuclear magnetic resonance to large biochemically important molecules

(chemical shift/spin-lattice relaxation/nuclear Overhauser effect/biopolymers)

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ABSTRACT   Natural-abundance nitrogen-15 nuclear magnetic resonance spectroscopy of enzymes and other biopolymers is found to be feasible using newly available instrumentation. The long correlation times of such molecules result in short spin-lattice relaxation times, and these in turn allow rapid signal accumulation. The advantages of short T_1 values are sometimes offset, however, by unfavorable nuclear Overhauser effects. The dependence of T_1 and nuclear Overhauser effects upon correlation time is discussed, and preliminary nitrogen-15 nuclear magnetic resonance results for several biopolymers, including lysozyme, protamines, pepsin, hemoglobin, vitamin B_{12}, and tRNA, are presented.

Nitrogen is widely distributed in molecules of biochemical interest, and nitrogen atoms are often intimately associated with sites of biological activity and major structural features. Nitrogen-15 nuclear magnetic resonance (NMR) spectroscopy is therefore, in principle, a promising tool for the study of biochemical systems, especially in view of the greater range of chemical shifts for nitrogen as compared to hydrogen and carbon. Until the present, however, ^{15}N NMR studies of large molecules at the natural-abundance level have been severely limited by problems of sensitivity. Although ^{15}N has a spin of $\frac{1}{2}$, and therefore gives rise to sharp resonances, its relative NMR sensitivity for equal numbers of nuclei is only 1.04×10^{-3} times that of the proton. In addition to a small magnetogyric ratio, the natural abundance of ^{15}N is 0.37%. Thus, at the natural-abundance level, the sensitivity of ^{15}N is 3.8×10^{-6} that of the proton. This results in a *difference of about 10^{11}* in the time required to obtain a given signal-to-noise ratio at constant field. The sensitivity problem is often exacerbated by problems with nuclear Overhauser effects and relaxation times as will be detailed below.

The prototype of a new NMR spectrometer, the Bruker WH-180, has recently become operational in our laboratories. This instrument is capable of performing ^{15}N measurements with much greater sensitivity than any other instrument presently available. The gain in sensitivity is achieved primarily by use of large sample volumes (25-mm diameter tubes, 15- to 30-ml samples), quadrature detection, high field (42 kG), and Fourier transform operation. The instrument is capable of measuring ^{15}N spectra at 18.25 MHz and ^{13}C at 45.28 MHz. A superconducting magnet is employed, as is a deuterium field-lock system. Both continuous wave and broadband proton decoupling are provided, and the computer is linked to a disk-storage system which allows convenient automated measurement of spin-lattice relaxation times (T_1) and nuclear Overhauser effects (NOE).

With the availability of the WH-180, we decided to undertake a series of ^{15}N NMR studies of molecules of biological interest. Our goal was to investigate both the potential and the difficulties of ^{15}N NMR spectroscopy for biochemical studies, and to discover areas where specific ^{15}N enrichment would be of value.

LYSOZYME AND RELATED MOLECULES

There are several potential problems facing the nitrogen-15 spectroscopist in addition to low sensitivity. The T_1's of nitrogen nuclei in small molecules are often long (on the order of seconds to even hundreds of seconds), and therefore long pulse delays and/or small pulse angles are necessary. It was expected that, in larger molecules with longer correlation times (τ_c), dipolar relaxation would become more efficient and that this would allow rapid pulsing. However, long correlation times often result in unfavorable NOE's which may cause some resonances to have intensities near zero. Nonetheless, natural-abundance ^{15}N studies of biopolymers are indeed feasible, as is illustrated by Fig. 1a. This figure shows the ^{15}N spectrum of hen egg-white lysozyme in water. The chemical shifts for this and other spectra reported herein are in ppm upfield from external 0.1 M $D^{15}NO_3$ in D_2O.

The resonances in Fig. 1a may be readily assigned by reference to standard texts (see, for example, ref. 1). The amide nitrogen resonances lie in the region 245–266 ppm, the arginine guanidino NH at 291.2 ppm, the guanidino $NH_2^\oplus$ at 304.4 ppm, and the lysine $NH_3^\oplus$ in the region 337–345 ppm. The instrumentally induced spike at about 207 ppm in this and other spectra arises from a slight breakthrough of the carrier frequency which is centered in the spectrum as a result of quadrature detection. Although the amide region contains so many peaks that assignment of resonances is far from straightforward, the arginine guanidino and lysine amino group resonances are well separated from the amide region and therefore amenable to study. Because there are 11 arginine and six lysine residues in lysozyme, which has a total of 129 amino-acid residues, and a molecular weight of about 14,300, most, if not all, of the peaks in Fig. 1a represent multiple resonances.

Nuclear Overhauser Effects. It is evident from Fig. 1a that the amide resonances are in phase with the reference whereas the guanidino and ammonium resonances are inverted. The reason for this behavior is readily apparent from Fig. 2. The two spectra in this figure were taken under identical conditions, with the exception that the upper spectrum was proton decoupled whereas the lower one was not. The guanidino and ammonium nitrogens show a strong negative NOE. The amide region, on the other hand, actually in-
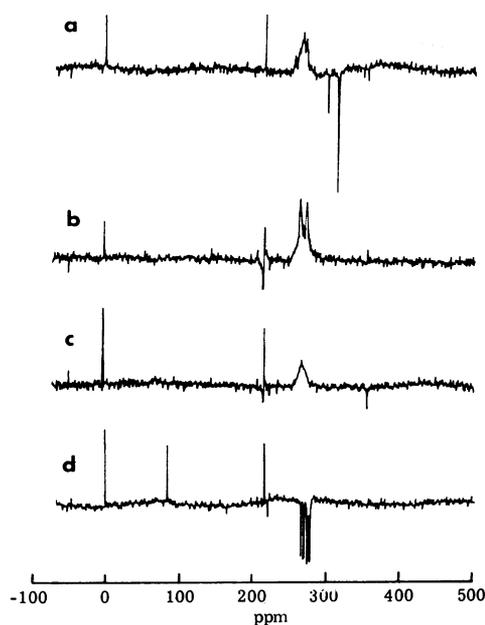
FIG. 1.   (a) Natural-abundance nitrogen-15 NMR spectrum of 9 mM hen egg-white lysozyme in water at pH 3.9. A total of 93,287 transients was accumulated with a 0.819 sec repetition rate, a 90° pulse, and 3 W broadband proton decoupling. (b) Denatured porcine pepsin (10 g/30 ml of solution) at pH 7.5. The spectrum was obtained in 192,278 transients. Other conditions were the same as for lysozyme. (c) Spectrum of 2 mM rabbit cyanomethemoglobin in 0.15 M sodium chloride solution. The cyano group was 99% ^{15}N-enriched. Conditions were the same as for lysozyme, with the exception that 86,692 transients were accumulated and 3.5 W decoupling power was used. (d) Spectrum of 0.08 M cyanocobalamin in 1:1 EtOH:H$_2$O. The cyano group was about 2.4% ^{15}N-enriched. A total of 78,811 transients was employed. Other conditions were the same as those employed for lysozyme.

creases in intensity when the decoupler is turned off. These nitrogen nuclei thus experience NOE values between 0 and +1.

Recognizing that most of these nitrogens have at least one attached proton, it is clear that the NOE is very much influenced by structure, and the structural effect which is important here is the correlation time. On the assumption that only dipolar relaxation to protons is important and that isotropic reorientation depends on the correlation time τ_c, the dependence of NOE on τ_c at 42 kG may be calculated (2). A plot of calculated values of NOE against τ_c appears in Fig. 3. The NOE varies from −3.93 for short correlation times to 0.88 at long τ_c. The enhancement is negative because ^{15}N has a negative magnetogyric ratio. There are several features of Fig. 3 which are of interest in the study of large molecules. Many biopolymers have correlation times on the order of 10^{-9} sec or more. As a result, nitrogen nuclei in such molecules may not experience full Overhauser effects. In fact, in unfavorable cases, signal intensities may be decreased to such an extent that resonances are no longer observable. On the other hand, because the NOE is rapidly changing with τ_c in the region of interest, NOE values are a sensitive measure of correlation time if relaxation is wholly dipolar.

If we assume that relaxation of the amide nitrogens of lysozyme is dipolar, then the average NOE for these nitrogens (estimated as about 0.51 from Fig. 2) may be used in conjunction with Fig. 3 to estimate τ_c as 5 × 10^{-9} sec. Because the majority of the amide nitrogens lie in the "backbone" of
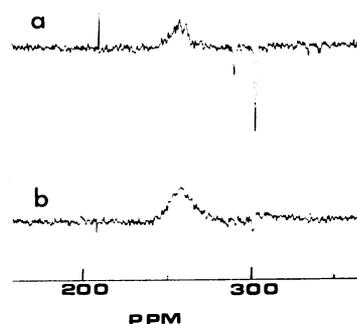

FIG. 2.   Proton decoupled (a) and undecoupled (b) spectra of 9 mM hen egg-white lysozyme. Conditions were the same as in Fig. 1a, with the exception that 50,700 transients were employed for each spectrum.

the protein, this correlation time represents an estimate of τ_c for the protein itself, assuming approximately isotropic reorientation. This τ_c value is a lower limit to τ_c for the protein itself, because a few of the amide nitrogens will have internal segmental motions and hence shorter correlation times than the bulk of the protein. Values of about 8.5 × 10^{-9} sec for τ_c of lysozyme under conditions similar to those employed here have been reported (3, 4). These data are consistent with the assumption of dipolar relaxation for the amide nitrogen nuclei.

In contrast to the amide nitrogens, the guanidino and ammonium nitrogens of lysozyme experience strong negative NOE values. These nitrogen nuclei must therefore have considerably shorter correlation times than the amide nitrogens, and therefore shorter correlation times than the protein as a whole. A more precise estimate of τ_c for these nitrogen nuclei was obtained from T_1 measurements.

Spin-Lattice Relaxation Times. In general, assuming isotropic reorientation and dipolar relaxation to protons, the dependence of T_1 on τ_c at 42 kG may also be calculated (5). Fig. 4 shows T_1 as a function of τ_c for nitrogen nuclei bound to one, two, or three protons. It will be seen that T_1 decreases with increasing τ_c until a minimum is reached at about 8.5 × 10^{-9} sec. Further increases in τ_c result in an increase in T_1. If we assume that on the average the amide nitrogens of lysozyme relax from the influence of one directly attached proton, then the τ_c data discussed above may be used with Fig. 4 to estimate a T_1 for these nitrogens of about 0.16 sec. Thus, rapid pulsing using large pulse angles is the
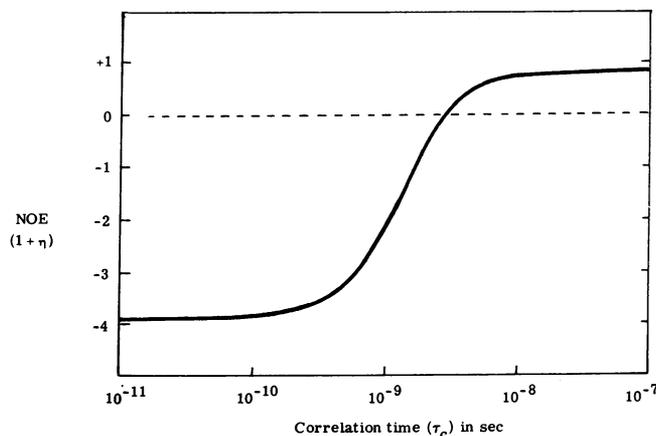

FIG. 3.   Plot of NOE against τ_c for nitrogen-15, assuming dipolar relaxation from protons and isotropic rotation.
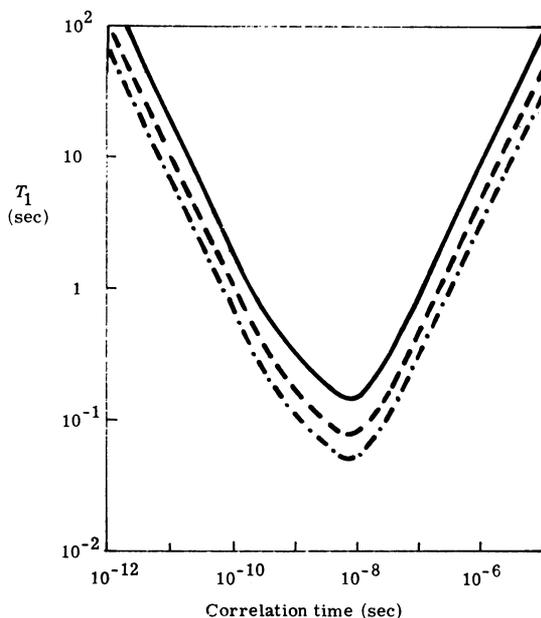
FIG. 4. Plot of T_1 against τ_c for nitrogen-15 at 18.25 MHz, assuming dipolar relaxation and isotropic rotation:—, relaxation to one proton with internuclear distance of 1.02 Å; - - - -, relaxation to two protons with internuclear distance 1.03 Å; - - · - - ·, relaxation to three protons with internuclear distance 1.03 Å.

most efficient way to obtain spectra of the amide nitrogens of lysozyme.

Because the $NH_2^{\oplus}$ guanidino nitrogen resonances of the 11 arginine residues of lysozyme appear as a single intense peak (Fig. 1a), it seems reasonable to conclude that the arginine guanidino groups are in roughly similar environments, and that measurement of an average T_1 value for these groups makes sense chemically. A sample of 9.4 mM lysozyme [concentration measured spectrophotometrically (6)] in 0.1 M citrate buffer at pH 5.0 was prepared, and the T_1
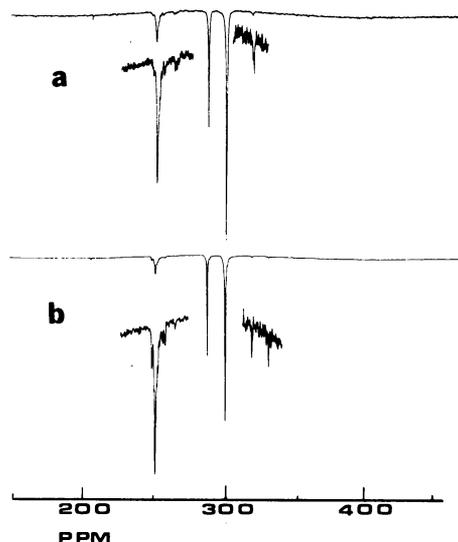

FIG. 5. (a) Natural-abundance nitrogen-15 NMR spectrum of salmine sulfate (7 g/18 ml of solution) at pH 1.0. A total of 50,898 transients was employed with a repetition rate of 0.819 sec and about 3 W proton decoupling power. (b) Nitrogen-15 spectrum of clupeine sulfate (7 g/18 ml of solution) at pH 0.67. A repetition rate of 0.819 sec was employed, and 49,000 transients were accumulated. Decoupling power was 3 W.

of the guanidino $NH_2^{\oplus}$ nitrogen nuclei was measured at 33° using the progressive saturation technique (7). Two measurements yielded values of 0.30 and 0.35 sec, for an average of 0.33 sec for T_1. Assuming dipolar relaxation from the two directly attached protons, Fig. 4 yields an estimate of about 3.5×10^{-10} sec for τ_c. Because the correlation time of the protein as a whole is about 5×10^{-9} sec or greater, the guanidino nitrogen nuclei clearly experience a considerable degree of internal motion. These T_1 results are in qualitative agreement with the NOE observations reported above. Fig. 3 yields a maximum NOE of about −3.5 based on the estimated correlation time of 3.5×10^{-10} sec.

The lysine ammonium nitrogen nuclei are likewise at the ends of long flexible hydrocarbon side chains, and the relatively short τ_c expected for these nuclei explains the large negative NOE values observed.

Protamines. Fig. 4 indicates that nitrogen nuclei in molecules with τ_c shorter than that of lysozyme will have longer T_1 values. Protamines are peptides containing from 30 to 33 amino-acid residues, about ⅔ of which are arginine. These peptides were chosen as examples of molecules having τ_c values between those of enzymes and those of small molecules. Fig. 5 shows the natural-abundance ^{15}N spectra of protamine sulfates from salmon (Fig. 5a) and herring (Fig. 5b) (samples obtained from Sigma Chemical Co.). The resonances centered at about 254 ppm arise from amide nitrogens, whereas the intense peaks at 291 and 304 ppm correspond to the guanidino NH and $HN_2^{\oplus}$ nitrogens, respectively. The resonance at 323 ppm in Fig. 5a corresponds to an NH-terminal proline amino group (8). The protamine derived from salmon (salmine) is known to consist of at least three closely related peptides with NH-terminal proline residues (9).

The protamine isolated from herring (clupeine) shows two resonances in the N-terminal amino region. One resonance corresponds to proline, but the other occurs at the resonance position for NH_2-terminal alanine (10). Clupeine consists of three closely related peptides, one of which has an NH-terminal proline, and two of which have alanine as the NH_2-terminal residue (9).

The excellent signal-to-noise achieved for the protamines illustrates that in favorable cases individual ^{15}N resonances may be readily observed in large molecules. Such observations open the door to the study of specific structural features and specific binding interactions in biopolymers using ^{15}N NMR at the natural-abundance level.

T_1 measurements for the guanidino nitrogens of the clupeine solution at 35° yielded values of 0.47 and 0.86 sec for the $NH_2^{\oplus}$ and NH nitrogen nuclei, respectively. Fig. 4 yields a τ_c of about 2.5×10^{-10} sec for the guanidino group. T_1 values for the amide nitrogen nuclei of clupeine were not determined. However, the intensities of the resonances yield some information about the T_1 values for these nitrogens. Because the amide resonances are inverted, they are exhibiting NOE values algebraically less than 0. Thus, the correlation times for these amides must be shorter than those for the amides of lysozyme. On the other hand, the amide resonances of clupeine are not nearly as intense as the guanidino NH resonance, and this fact indicates that the amide nitrogens experience less NOE than the guanidino nitrogens and hence have longer correlation times.

Arginine Hydrochloride. A progressive saturation study of 3 M arginine hydrochloride in water at pH 5.8 (33°) yielded T_1 values of 2.8, 4.5, and 4.6 sec for the $NH_2^{\oplus}$, NH, and $NH_3^{\oplus}$ nitrogen nuclei, respectively. Assuming dipolar

Chemistry: Gust *et al.*

Proc. Nat. Acad. Sci. USA 72 (1975) 4699

relaxation and isotropic reorientation, these results yield τ_c values of 3.7×10^{-11}, 4.3×10^{-11}, and 1.5×10^{-11} sec. The values for the NH and $NH_2^\oplus$ groups are equivalent, within experimental error, but τ_c for the $NH_3^\oplus$ group is somewhat shorter than for the others. This finding suggests that there is a significant amount of internal rotation about the $C-NH_3^\oplus$ bond.

The T_1 measurements discussed above are relatively crude, and effects due to contamination by trace paramagnetic impurities cannot be ruled out. However, the trends observed do allow some important conclusions to be drawn. The change in τ_c and T_1 for the guanidino group nitrogens upon going from arginine hydrochloride through protamine to lysozyme is about an order of magnitude. This change has a very large effect on the amount of time needed to obtain a spectrum with a given signal-to-noise ratio at a given concentration, because signal-to-noise increases as the square root of the number of transients. It is this dramatic decrease of T_1 with increasing τ_c which makes natural-abundance nitrogen-15 NMR spectroscopy of biopolymers practical.

OTHER BIOPOLYMERS

Pepsin. Fig. 1b shows the ^{15}N NMR spectrum of alkaline pH-denatured porcine pepsin obtained in 43.7 hr. Apart from the reference and the carrier spike, the only resonances which can safely be differentiated from instrumental spikes or random noise occur in the amide region. The molecular weight of pepsin (304 residues) is more than twice that of lysozyme, and the sensitivity is not great enough to permit unambiguous observation of non-amide nitrogens.

Hemoglobin. Hemoglobin is another relatively large globular protein (574 residues) containing 48 lysines. The relatively large negative NOE observed for the lysine ammonium nitrogens of lysozyme suggested that the corresponding nitrogens of hemoglobin should be observable. Fig. 1c shows the spectrum of freshly prepared cyanomethemoglobin containing 99% ^{15}N-enriched cyanide. Apart from the reference and the carrier spike in the center of the spectrum, the only resonances observed are the amide resonances (250–269 ppm) and the lysine ammonium resonances (343 ppm). As was the case with lysozyme, the amide nitrogen nuclei have an average NOE between 0 and $+1$ whereas the lysine ammonium nitrogens experience a strong negative NOE as a result of internal motions. The resonance for the enriched cyano group was not observed. Although it is conceivable that this resonance has been shifted outside our spectral width, it is also possible that the resonance is broadened beyond detectability by the paramagnetic iron(III) of methemoglobin.

The ^{15}N NMR spectrum of ^{15}N-enriched mouse hemoglobin has been reported (11). The signal-to-noise ratio obtained was comparable to that in Fig. 1c. It should be noted that all peaks in the spectrum of the enriched material were depicted as having the same phase, and the problem of negative NOE's was not discussed.

Vitamin B₁₂. Fig. 1d shows the spectrum of cyanocobalamin in which the cyano group has been enriched in nitrogen-15 to about 2.4%. The seven amide nitrogens appear as seven individual resonances in the region 256.8–268.2 ppm. It is interesting to note that the chemical shifts of these seven amide nitrogens span essentially the entire amide region observed in lysozyme, pepsin, and cyanomethemoglobin.

The intense resonance at 80.9 ppm arises from the partially enriched cyano group. Because the cobalt is diamagnetic in this complex, no broadening of this resonance occurs.
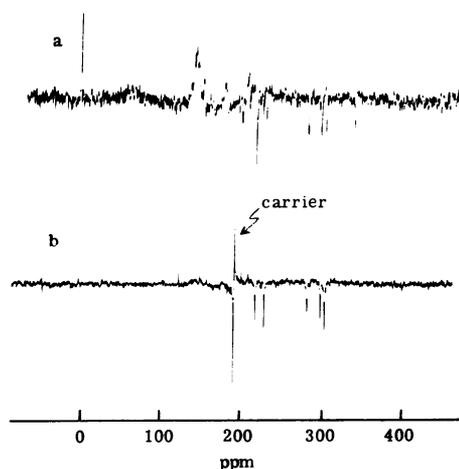

FIG. 6. Proton decoupled spectra of yeast tRNA (5 g/15 ml of solution, about 11 mM) in 0.15 M sodium chloride solution. Spectrum (a) was obtained on a sample at pH 5.6 at about 30° with a 90° pulse, a 0.409 sec repetition rate, and 301,802 transients. Spectrum (b) was obtained at about 80° on a sample at pH 5.4 using a 0.819 sec repetition rate, and 82,810 transients.

The ring nitrogens of cyanocobalamin were not observed. Long relaxation times, small NOE values, and coupling to cobalt may contribute to the lack of signal intensity. The importance of the first two factors is illustrated by the fact that whereas the ^{15}N resonance for 0.04 M tetraphenylporphine dication in chloroform–trifluoroacetic acid is readily observable with decoupling in a few hours (40° pulse, 2 sec delay), the corresponding resonance for a more concentrated solution of zinc tetraphenylporphyrin is detected with a similar signal-to-noise ratio only after more than 43 hr of signal averaging using a 40° pulse, an 8 sec pulse delay, and no decoupling.

Transfer Ribonucleic Acid. Transfer ribonucleic acids are relatively small nucleic acids (molecular weight about 30,000) which are readily available and quite soluble. We chose this class of compounds for our initial investigation into the feasibility of ^{15}N NMR studies of nucleic acids. The spectra obtained for yeast tRNA (a mixture of various tRNA species) at about 30° and 80° are shown in Fig. 6. At 30° all resonances were quite weak and a total of 301,802 transients were required to obtain the spectrum shown. In general, the upfield peaks in this spectrum (corresponding to NH_2 and sugar-bound nitrogens) have NOE values less than 0, whereas the downfield resonances (corresponding to ring nitrogens) have NOE values between 0 and $+1$. When the sample was warmed to 80°, the spectrum in Fig. 6b was obtained. This spectrum required only 82,810 transients. Note that the five major upfield resonances have increased in intensity, whereas the downfield resonances have now decreased greatly in relative intensity. These changes reflect an overall algebraic decrease in NOE for all resonances, which in turn reflects a decrease in τ_c (Fig. 3). This decrease in τ_c is due in part to the increase in temperature. However, another factor may also be important. At 80° tRNA is known to exist in a "melted" form in which secondary structure is lost. The loss of double-helical structure accompanying the loss of base pairing is expected to yield a more flexible molecular structure in which the individual nitrogen nuclei have shorter correlation times.

The resonances observed for tRNA at 80° may be assigned by reference to the corresponding resonances of the

5′ monophosphates of guanosine (G), cytidine (C), adenosine (A), and uridine (U) (as measured here by Dr. V. F. Markowski). These assignments in ppm for tRNA are G, 303.2; A, 297.9; C, 282.0; G, U, 229.0; U, 218.3; C, 176–180; G, A, 137–153. The corresponding assignments for the spectrum at 30° are obvious by inspection. The spectrum at 30° contains a resonance at 338 ppm which is not assignable to any of the major bases, and which falls in the region expected for aliphatic amines or protonated amines. This resonance may reflect the presence of an impurity (such as Tris buffer) or a minor base in tRNA.

CONCLUSIONS

The studies outlined above demonstrate the feasibility of natural-abundance ^{15}N NMR studies of biopolymers and other molecules of biochemical interest. At present, the method requires large sample volumes, high solute concentrations, and a considerable investment of instrument time to achieve useable results. However, even with these limitations, ^{15}N NMR at natural abundance is now applicable to a large variety of biochemical problems.

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