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Carbon-13 Fourier Transform NMR at 14.2 kG in a 20 mm Probe*

We have recently developed a probe for 20 mm spinning sample tubes (*1*) as a replacement for the 13 mm probe originally installed in our "home-built" ^{13}C Fourier transform NMR apparatus (*2*, *3*). With the use of the 20 mm probe, a given signal-to-noise ratio in a ^{13}C NMR spectrum can be achieved considerably faster than on presently available commercial instruments (*1*). At the time of our previous report (*1*), we could get a given signal-to-noise ratio (for a given concentration) in about one-quarter to one-ninth the time required using the Varian XL-100-15 Fourier transform NMR spectrometer. Further instrumental developments in our laboratory have given us a sensitivity for concentration limited ^{13}C NMR (in 20 mm sample tubes) that is about 4.5 times that of the Varian XL-100-15 FT NMR spectrometer (with 12 mm sample tubes) and similar instruments from other manufacturers. Thus, we can achieve a given signal-to-noise ratio in about *one-twentieth* the time required on commercial equipment, in spite of the fact that we use a magnetic field strength of 14.2 kG while the commercial instruments operate above 20 kG.

We present here a brief description of key instrumental features of our system, some natural-abundance ^{13}C spectra at very low concentrations, and examples of the range of applications of our Fourier transform NMR spectrometer.

We incorporated the 20 mm probe into out existing home-built ^{13}C Fourier transform NMR spectrometer (*2*, *3*). This instrument is built around a Varian V-3601 12-in. electromagnet (of the type used on Varian HR-60, HA-60, DA-60 and DP-60 spectrometers). To avoid interference with another system operating at the traditional 14.1 kG, our magnetic field strength is 14.2 kG, corresponding to ^{13}C and ^{1}H resonance frequencies of 15.182 MHz and 60.372 MHz, respectively. An external ^{19}F NMR lock is used for field stabilization. The radiofrequency excitation and detection systems are of standard design for pulsed NMR. It is pertinent to the discussion below that our phase-sensitive detector is a double-balanced mixer operating directly at the resonance frequency of 15.182 MHz. No intermediate frequency amplifiers are used in the detection system. The digital processor is a Nicolet 1083 computer, equipped with 8K 20-bit addresses for signal accumulation. Occasionally, we borrow from another Nicolet system an additional 8K of memory which can be plugged in directly for 16K accumulation. The probe contains two coils: an inner one of about 21 mm diameter shared by the ^{13}C excitation and detection systems, and an outer one, orthogonal to the ^{13}C coil, tuned to 60.372 MHz for proton decoupling. With about 300 W of ^{13}C rf power, we get a 90° pulse of about 15 μsec. This corresponds to a value of $\gamma H_1/2\pi$ of about 17 kHz, which is more than adequate for ^{13}C NMR at 15.18 MHz. The spectra presented below were obtained with the use of 90° pulse excitation. The proton rf field strength is about 4 kHz (1 G) when the rf power is 15 W. For studies of biopolymers and other large molecules, about 5 W of decoupling power is usually sufficient.

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The probe body was built in our machine shop. It is 38 mm thick and, thus, fits well into the gap of the Varian V-3601 magnet (about 40 mm). There is no Dewar jacket inside the probe, and, thus, the available temperature range is small; we routinely operate in the range 30°–75°C. Temperatures down to 5°C can be obtained. The source of heat in the probe is the proton-decoupling rf power. The temperature is varied by changing the flow of cooling air into the probe. Temperature stability of ±1°C is routinely achieved. We are now constructing a variable-temperature version of our 20 mm probe, which will incorporate a Dewar-jacketed insert of standard design.

We use precision glass sample tubes of 20 mm outside diameter and 18 mm inside diameter. Sample volume is usually 12 ml. The sample tubes are supplied by Wilmad

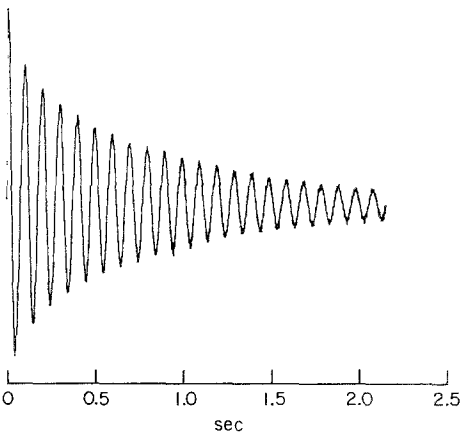

FIG. 1. Proton-decoupled natural-abundance ^{13}C free induction decay of neat ethylene glycol at 50°C, in a spinning 20 mm sample tube, recorded after a single 90° rf pulse at 15.18 MHz. The signal was phase-detected with the reference frequency about 10 Hz off-resonance.

Glass Company, Buena, New Jersey. The probe is equipped with a spinner also supplied by Wilmad. The spinning rate is usually 15–20 Hz. We have encountered no difficulties when spinning 20 mm tubes. Vortex-preventing plugs made out of Teflon are inserted above the sample.

Our initial concern about possible large magnetic field inhomogeneities in the large sample volume was unfounded. We use no homogeneity correction coils other than those supplied by the manufacturer (X, Y, Z, YZ, and curvature corrections). Nevertheless, an instrumental contribution to the line width of only 0.3 Hz can be obtained routinely. With some effort, less than 0.2 Hz inhomogeneity broadening can be achieved. Figure 1 shows the induction decay following a single 90° rf pulse applied to a sample of neat ethylene glycol at 50°C. The decay corresponds to a line width of 0.3 Hz. A natural line width of 0.15 Hz was inferred from T_1 measurements. Thus, the inhomogeneity broadening is only 0.15 Hz. With proper adjustment of the homogeneity corrections, spinning side bands are also very small. In Fig. 2 we show the proton-decoupled natural-abundance ^{13}C spectrum of neat ethylene glycol (17.9 *M*) recorded with a spinning rate of 20 Hz and 128 accumulations. The signal-to-noise ratio[1] is

[1] Ratio of peak height to rms noise.

about 7000, which is sufficient for detailed observations of spinning side bands. The height of each spinning side band (peaks A and B in Fig. 2) is only about 0.5% that of the main peak. Additional spinning sidebands of even smaller amplitude are also present (Fig. 2). It should be noted that both of the main spinning sidebands are displaced about 20 Hz with respect to the main resonance. The apparent asymmetry in the position of peaks A and B is caused by a slight asymmetry in the wings of the main resonance.

The signal-to-noise ratios accessible to us now result not only from the use of the 20 mm probe, but also from the incorporation of a crystal filter designed to discriminate

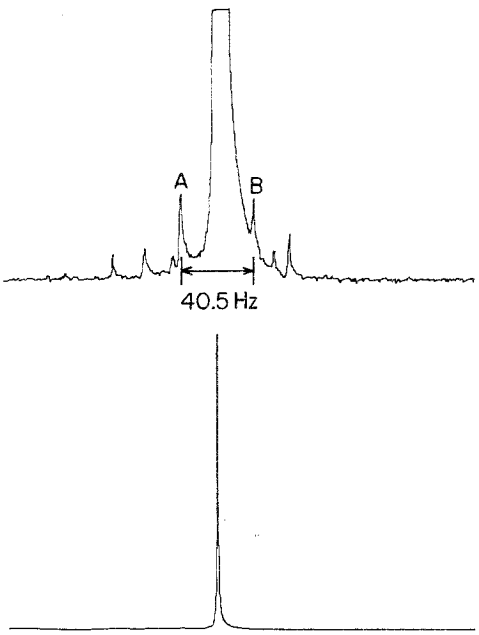

FIG. 2. Proton-decoupled natural-abundance ^{13}C spectrum of neat ethylene glycol (at 35°C) in a 20 mm sample tube spinning at a rate of 20 Hz, recorded at 15.18 MHz using 90° rf pulses, a digital resolution of 0.488 Hz, 128 accumulations, and a recycle time of 3 sec. The vertical gain in the upper spectrum is 64 times that of the lower one.

"positive" from "negative" frequencies relative to the carrier. After suitable amplification of the induction decay most Fourier transform NMR spectrometers transfer the observed rf signal into the audio frequency range by mixing with a suitable reference frequency in a phase-detector. This audio conversion is done either directly at the NMR frequency by mixing with a reference signal at the rf excitation frequency, or at some intermediate frequency. In either case, after ordinary phase detection, the spectrum is imaged about a frequency ν_0 (usually equal to but sometimes slightly displaced from the rf excitation frequency). Thus, for example, a frequency of $\nu_0 + 500$ Hz cannot be distinguished from a frequency of $\nu_0 - 500$ Hz, because after phase detection both signals appear at 500 Hz. One normally avoids this ambiguity by offsetting ν_0 sufficiently far off-resonance to ensure that all NMR signals are below ("upfield") or above

("downfield") from ν_0. However, this procedure does not solve the problem of *noise* imaging about ν_0. Just like any NMR signal, noise at $\nu_0 - 500$ Hz cannot be distinguished from noise at $\nu_0 + 500$ Hz. Thus, the resulting noise level is $\sqrt{2}$ greater than if one could distinguish frequencies below ν_0 from those above ν_0. Dual detection has been used successfully for separating signals above ν_0 from those below ν_0 (*4*, *5*, *6*). In this procedure, the outputs from two phase-detectors in quadrature are treated as the real and imaginary parts of a complex time-domain signal. After complex Fourier transformation, there is no imaging problem. However, if the rf field stregnth ($\gamma H_1/2\pi$, in Hz) is much greater than the spectral range to be covered (a condition easily satisfied in ^{13}C NMR), then one is only concerned with the imaging of *noise*. In this case, instead of using a procedure that distinguishes frequencies above and below ν_0, it is equally satisfactory to employ any procedure that suppresses signals on one side of ν_0. A properly designed crystal filter tuned so that one edge of the pass band is approximately at ν_0 (or some other appropriate frequency if intermediate-frequency detection is used) performs this function. The attractiveness of this method lies in its simplicity, ease of installation, and very low cost. As mentioned above, we phase-detect directly at the resonance frequency. We have placed a crystal filter directly between the output of the final rf amplification stage of the detection system and the input to the phase detector. We use a four-pole Butterworth crystal filter supplied by Bliley Electric Co. of Erie, Pennsylvania. The upper 3 dB point of the filter was chosen at 28 ppm downfield from CS_2 (221 ppm downfield from tetramethylsilane). The lower 3 dB point is at 255 ppm upfield from CS_2. We normally set the rf frequency near the upper 3 dB point of the filter. The actual gain and phase characteristics of this crystal filter are very close to the theoretical response of a four-pole Butterworth design. Thus, the frequency-dependent phase shift is very linear throughout most of the pass band. The linear phase correction available in the software package of the Nicolet-1083 computer is sufficient. By recording one spectrum with and one without the crystal filter, but with otherwise identical instrumental conditions, we have verified that the crystal filter does indeed improve the signal-to-noise ratio by a factor of 1.4. All the spectra reported below were obtained with the use of the crystal filter.

In Fig. 3 we show natural-abundance ^{13}C NMR spectra of 2 and 10 mM aqueous sucrose. When the concentration is 10 mM, spectra with a signal-to-noise ratio of about 10 can be obtained in about 1 hr of signal accumulation. The lowest concentration that yields useful spectra in 10 hr of accumulation time is about 2 mM (Fig. 3a). With commercial NMR instruments, the lower limit is 10 mM (*7*).

The above concentration limits apply only when proton-decoupling produces a nuclear Overhauser enhancement (NOE) of the ^{13}C intensities that is close to the theoretical maximum of 2.988 (*8*). In studies of biopolymers, the NOE may be nearly nonexistent for many carbons because of the slow molecular rotation (*9*). Thus, the lowest practical concentration for observing single-carbon resonances of native biopolymers may actually be 30 mM when using commercial instruments, but only 6 mM with our apparatus. The large molecular weights of biopolymers preclude the use of 30 mM solutions.

We have already reported the first observation of numerous single-carbon resonances in the aromatic-carbon region of the spectrum of hen egg-white lysozyme (*10*). That study was made with a previous version of the 20 mm probe and before the crystal filter

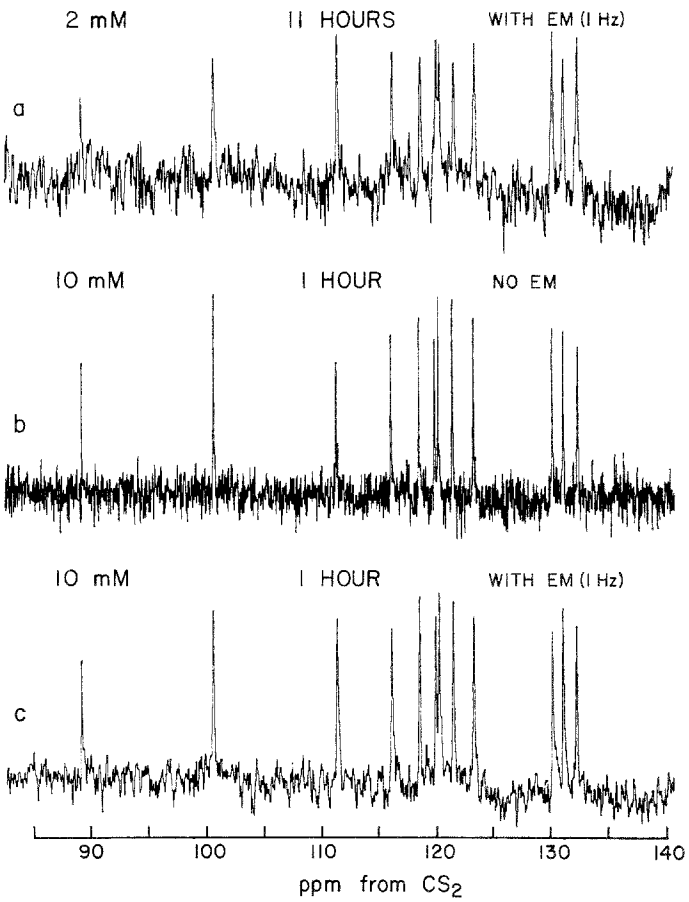

FIG. 3. Proton-decoupled natural-abundance ^{13}C spectra of aqueous sucrose at 33°C. All spectra were recorded at 15.18 MHz using 90° rf pulses, 4096 points in the time-domain, a spectral width of 2202.64 Hz, and a recycle time of 1.005 sec. An additional 4096 points (each equal to zero) were added to each accumulated time-domain signal. In two of the spectra, exponential multiplication (EM), with a negative time constant, was used to increase the signal-to-noise ratio. The resulting line-broadening is indicated above each spectrum. (a) 0.002 *M* sucrose, after 38,659 accumulations. (b) 0.01 *M* sucrose, after 4096 accumulations, without exponential multiplication. (c) 0.01 *M* sucrose, after 4096 accumulations, with 1.027 Hz broadening as a result of exponential multiplication.

was incorporated. Since then, we have considerably reduced the time required to detect single-carbon resonances of lysozyme and other highly soluble proteins of low molecular weight. In Fig. 4 we show a proton-decoupled natural-abundance ^{13}C spectrum of 13 mM lysozyme (19% w/v in water), recorded with 7.4 hr of signal accumulation. Assignments of the resonances have been discussed elsewhere (*10*). We have previously determined (*10*) that the region of aromatic carbons (about 36 ppm to 85 ppm upfield from CS_2) shows the most promise for observing resolved single-carbon resonances in ^{13}C spectra of proteins. In Fig. 5b we show in more detail the unsaturated-carbon region of Fig. 4. For comparison, a spectrum recorded on the Varian XL-100 Fourier transform NMR spectrometer, using 9.5 hr of accumulation time, is shown in

Fig. 5a. Peaks 1–22 of Fig. 5b contain the resonances of the 28 *nonprotonated* aromatic carbons of lysozyme. Details have been given elsewhere (*10*). Here we just wish to point out that peaks 1 and 2 are the resonances of C^{ζ} of the three tyrosine residues, and

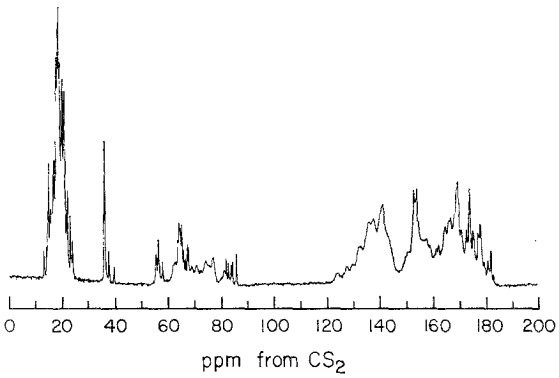

FIG. 4. Proton-decoupled natural-abundance ^{13}C spectrum of 13 mM hen egg-white lysozyme (19% w/v in 0.1 *M* NaCl in H_2O, pH 3.96, 42°C), recorded at 15.18 MHz using a 20 mm sample tube, 90° rf pulses, 8192 points in the time-domain, 1.085 sec recycle time, 24,576 accumulations (7.4 hr total time), a 250 ppm sweep width, and 0.44 Hz broadening from exponential multiplication.

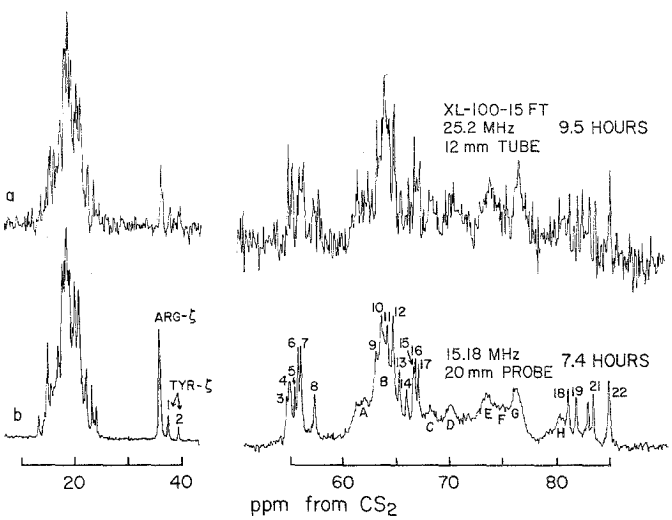

FIG. 5. (a) Proton-decoupled natural-abundance ^{13}C spectrum of 13 mM hen egg-white lysozyme (19% w/v in 0.1 *M* NaCl in D_2O, pD 4, 42°C), recorded at 25.2 MHz on the Varian XL-100-15 Fourier transform NMR spectrometer, using a 12 mm sample tube, 90° rf pulses, 8192 points in the time-domain, 1.035 sec recycle time (acquisition time plus delay), 32,768 accumulations (9.5 hr total time), a 250 ppm sweep width, and 0.6 Hz broadening from exponential multiplication. (b) Expanded downfield region of the spectrum in Fig. 4.

that peaks 18–22 are the resonances of C^{γ} of the six tryptophan residues. Clearly, peaks 2, 18, 19, 20, and 21 are single-carbon resonances, while peaks 1 and 22 are two-carbon resonances. We used 7.4 hr of signal accumulation to obtain the spectrum shown in Figs. 4 and 5b. The signal-to-noise ratio of the single-carbon resonances is at least

twice that required for observation of these resonances. Thus, about 2 hr of signal accumulation is sufficient for observing single-carbon resonances of lysozyme and other proteins of low molecular weight and high solubility.

The broad resonances labeled A–H in Fig. 5b arise from protonated aromatic carbons. These resonances are much broader than those of the non-protonated aromatic carbons because of the dependence of ^{13}C–^{1}H dipolar relaxation on the inverse sixth power of carbon–hydrogen distances. Details have been given elsewhere (*10*).

In addition to studies that require spectra of very dilute solutions, our instrument facilitates quantitative analysis by means of ^{13}C integrated intensities. Examples will be given elsewhere (*11*). Our apparatus is also advantageous for ^{13}C T_1 measurements of individual carbons by means of partially-relaxed Fourier transform (PRFT) spectra (*3*, *12*). Often, the range of ^{13}C T_1 values within a molecule covers more than an order of magnitude (*3*). In such cases, very many PRFT spectra have to be recorded. Obviously, the relatively short accumulation time required with the use of our spectrometer is a distinct advantage.

We believe that the results presented here demonstrate that we have considerably extended the range of applications of natural-abundance ^{13}C Fourier transform NMR, in concentration-limited research. This development required a relatively small investment of funds and effort compared to the cost of a complete spectrometer. We recommend this approach to anyone facing a poor signal-to-noise ratio in concentration limited ^{13}C NMR research.

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