By Adam Allerhand, Ray F. Childers, Roy A. Goodman, Eric Oldfield, and Xavier Ysern

Increased sensitivity in ^{13}C FT nmr using 20-mm SAMPLE TUBES

FOURIER TRANSFORM (FT) nmr is rapidly becoming as familiar in the chemical laboratory as continuous-wave (cw) nmr. The advantages of FT nmr, first described in detail by Ernst and Anderson in 1966,[1] have already been demonstrated in numerous published studies.[2-4] To this date, the greatest incentive for the development of FT nmr instrumentation has been natural-abundance ^{13}C nmr spectroscopy of large molecules of chemical and biological interest. The pioneering work of Grant[5] and Roberts[6] demonstrated the resolution and simplicity (relative to proton nmr) of proton-decoupled ^{13}C spectra of *complex molecules*. The development of FT instruments has made ^{13}C nmr a truly practical spectroscopic tool. Nevertheless, the sensitivity of currently available equipment is not sufficient for many important chemical and biological studies. This report describes a simple and inexpensive procedure for increasing the sensitivity in ^{13}C FT nmr by a factor of about three, with respect to currently available equipment — the use of spinning sample tubes with an outside diameter of 20 mm (18-mm i.d.). It is interesting to speculate as to why a high-resolution nmr probe for such tubes was not developed earlier and incorporated into commercial FT nmr spectrometers (which were described in a series of articles in *American Laboratory* in March, 1972). Perhaps there was undue pessimism about possible technical difficulties, such as poor magnetic field homogeneity over a large sample volume and problems with large diameter coils. We have encountered no such problems, however.

To overcome as much as possible the inherently poor sensitivity and low natural abundance (1.1%) of the ^{13}C nucleus, existing high-resolution nmr instruments utilize not only the FT technique, but depend also on three other factors: 1) *The nuclear Overhauser effect* (NOE),[7] which here results in an increase in spectral intensity when strong irradiation at the proton resonance frequency is introduced during the ^{13}C nmr experiment. The NOE attains a maximum value of 2.988 when the rate of molecular rotation is much greater than the resonance frequency,[7-9] and the ^{13}C nuclei are undergoing purely $^{13}C-^{1}H$ dipolar relaxation.[7] The latter is nearly always the case for protonated carbons,[8] but is often not true for nonprotonated ones. 2) *Signal averaging:* In order to achieve satisfactory signal-to-noise ratios with currently available equipment, it is nearly always necessary to digitally accumulate an appreciable number of spectral scans. We will consider a total accumulation time of about one day to be a reasonable upper limit for a single spectrum. 3) *Large sample tubes:* While most proton nmr studies are made in sample tubes with an outside diameter of 5 mm, most existing ^{13}C nmr instruments utilize tubes with a diameter of 12 or 13 mm.

With currently available instrumentation, 0.01 M is now generally accepted as the lowest practical concentration for observing *single-carbon resonances* in natural abundance[2,4] when the maximum NOE factor of 2.988 occurs. For carbons without any NOE, the lower limit is, of course, about 0.03 M. Frequently, effective molar concentrations are much lower. This is particularly true in studies of biopolymer solutions where large molecular weights preclude high molar concentrations and where the NOE may be nearly nonexistent because of the slow molecular rotation.[9] For example, in the ^{13}C FT nmr studies of proteins that have been reported so far[3,10-12] single-carbon resonances are either not observed or barely detectable.

We incorporated the 20-mm probe into an existing "home-built" ^{13}C FT nmr spectrometer which originally had a 13-mm probe.[8] It is pertinent to our discussion that this spectrometer was

Dr. Allerhand is Professor of Chemistry, Dr. Childers and Dr. Oldfield are Research Associates, Mr. Goodman and Mr. Ysern are Graduate Students, Indiana University. *This research was supported by the National Science Foundation (grant GP-17966), the donors of the Petroleum Research Fund administered by the American Chemical Society (grant 4559-AC5), and by Eli Lilly and Company. Dr. Oldfield thanks the European Molecular Biology Organization for partial support. The authors thank Messrs. A. O. Clouse, R. Sporleder, R. Landerebe, M. Williams, and Ms. E. Moe for their help. This paper is publication no. 2121 from the Department of Chemistry, Indiana University.*

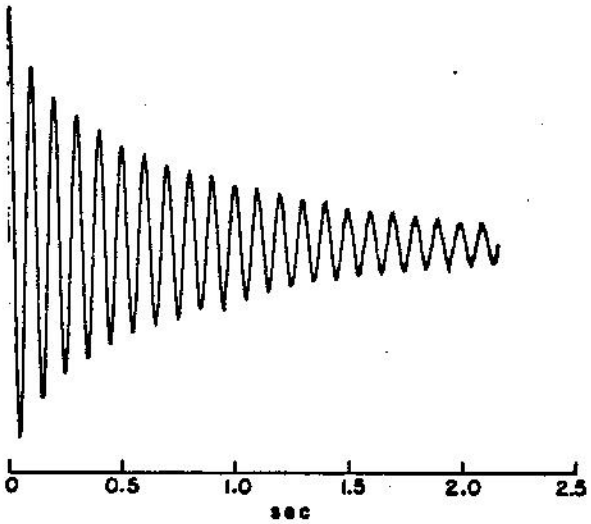


Figure 1 *Homogeneity test of the 20-mm probe. Natural-abundance ^{13}C free induction decay (15.18 MHz) of neat liquid ethylene glycol, single scan, with proton-decoupling, phase detected about 10 Hz off resonance.*

built around a Varian 12-in. high-resolution electromagnet with a field strength of 14.2 kG, corresponding to ^{13}C and ^{1}H resonance frequencies of 15.18 MHz and 60.37 MHz, respectively. The same type of magnet is part of the Varian HR-60, DP-60, and DA-60 series high-resolution nmr spectrometers. The ^{13}C excitation (radiofrequency pulse) and signal detection are shared in a single coil. A separate coil is used 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 γH_1 of 17 kHz, which is more than adequate for ^{13}C nmr at 15.18 MHz. 10 w of ^{1}H rf power is sufficient for proton-decoupling in most cases. The probe body (38-mm thick) was built in our machine shop, except for the spinner assembly, which was made by Wilmad Glass Company. Wilmad is also the supplier of our 20-mm precision glass tubes, which we routinely spin at about 20 Hz. One limitation of our 20-mm probe for some applications is the small available temperature range of 5°–85°C. Furthermore, about 9 ml of sample is required, as opposed to 2 ml with a 12-mm tube.

Our first spectra indicated that the homogeneity of the magnetic field was remarkably good. *Figure 1* shows the time-domain signal (induction decay) following a single 90° pulse applied to a sample of ethylene glycol. This decay corresponds to a line width of 0.3 Hz. A natural line width of 0.15 Hz was inferred from spin-lattice relaxation measurements. Thus, the inhomogeneity broadening is only 0.15 Hz. It is apparent that the Varian 12-in. electromagnet provides an extremely homogeneous magnetic field. We must add that an inhomogeneity broadening of 0.15

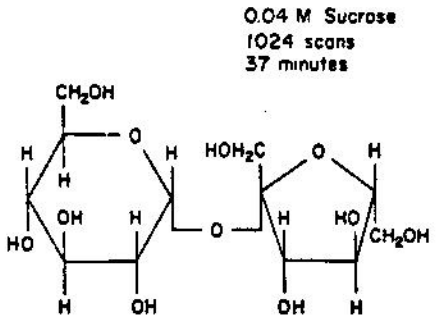
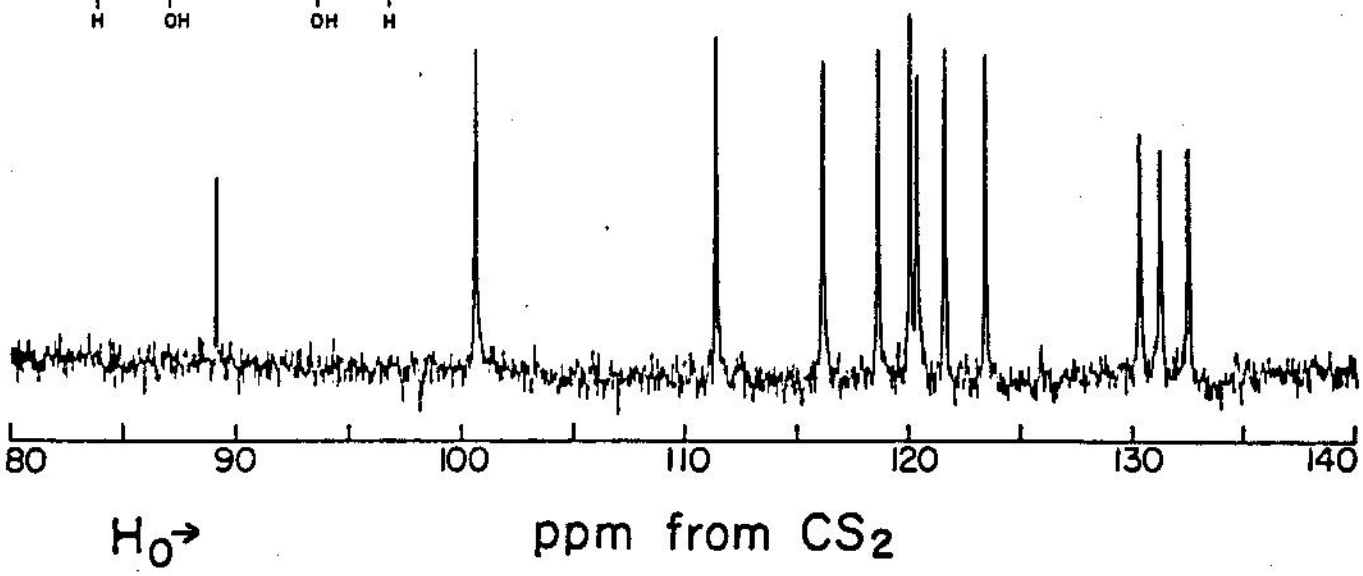


Figure 2 *Proton-decoupled natural-abundance ^{13}C FT nmr spectrum of aqueous 0.04 M sucrose at 35°C obtained at 15.18 MHz with 4096 points in the time domain, a 62.5-ppm spectral width, 2.17-sec recycle time, and 1024 accumulations. A digital filter added 0.3 Hz to the line widths.*



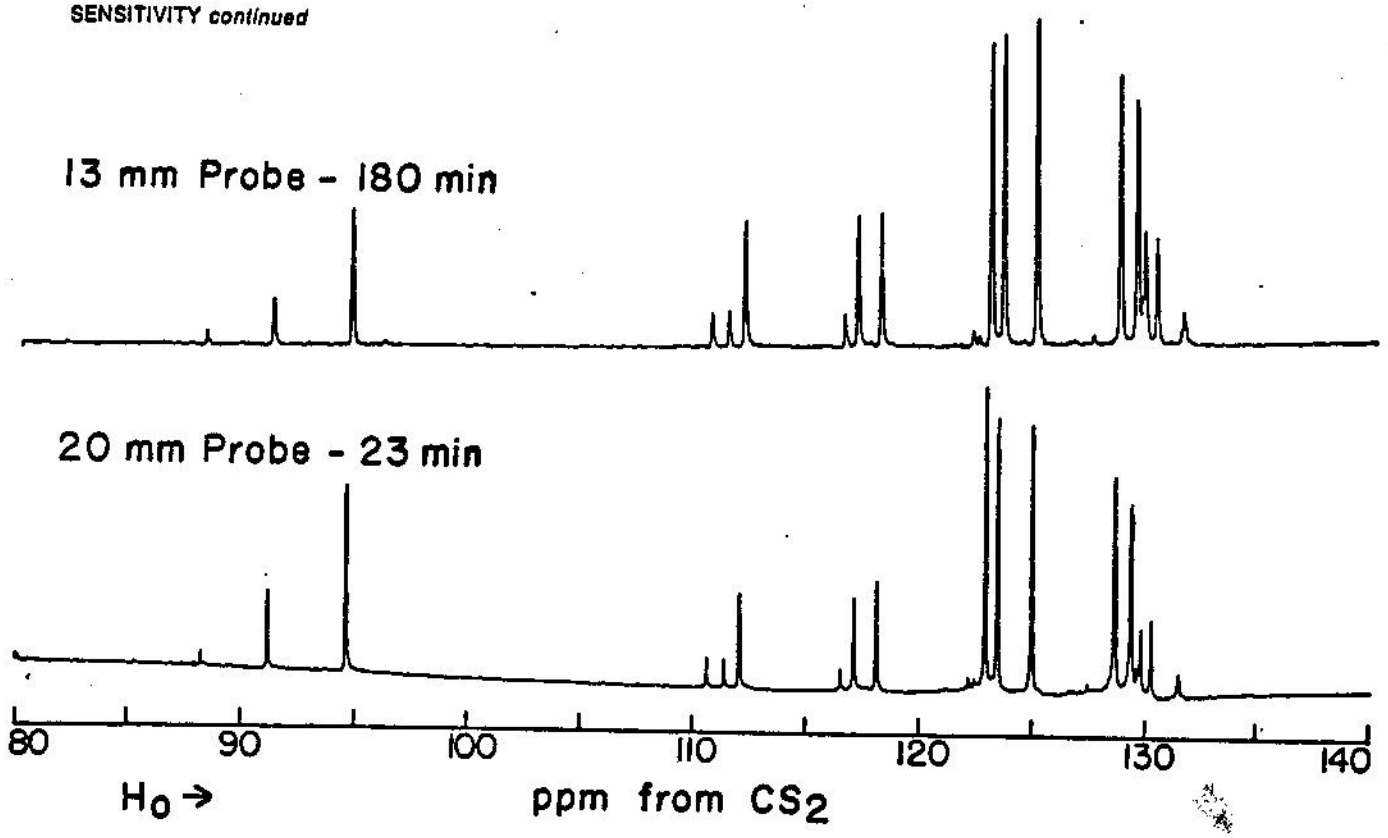


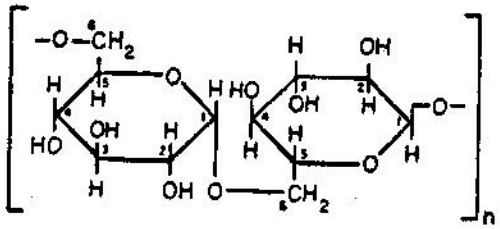
Figure 3 *Proton-decoupled natural-abundance ^{13}C FT nmr spectra of fructose (0.35 g/ml water) obtained at 15.18 MHz with 4096 points in the time domain, a 62.5-ppm spectral width, 5.28-sec recycle time, and a digital filter broadening of 0.15 Hz. Spectra were recorded more than 8 hr after fructose was dissolved. Top: 13-mm probe, 42°C, 2048 accumulations. Bottom: 20-mm probe, 35°C, 256 accumulations.*

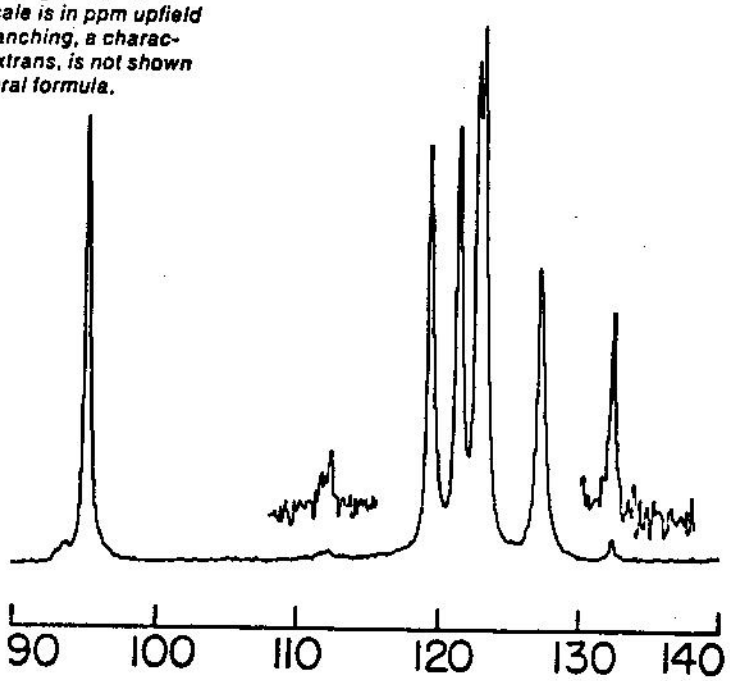
Hz is achieved under optimum conditions (temperature, etc.), but 0.3 Hz is obtained routinely.

Figures 2, 3, 4, 5, and *6* show some representative results obtained with the use of 20-mm sample tubes. The signal-to-noise ratio (peak height-to-rms noise ratio) of the protonated carbons of 0.04 M sucrose in water is about 20 after less than 40 min of signal accumulation. There is thus a three-fold improvement in sensitivity with respect to 12-mm or 13-mm sample tubes.[3] This means that a given signal-to-noise ratio is achieved with the 20-mm probe in about one-ninth of the time required with a 12-mm or 13-mm probe. For example, more than 5 hr of signal accumulation would be needed to get the spectrum of Figure 2 with the use of existing commercial FT nmr equipment. This estimate is based on published data[2] and spectra of 0.04 M sucrose kindly supplied to us by several manufacturers.

Figure 3 compares directly two spectra taken under the same con-

Figure 4 *Proton-decoupled natural-abundance ^{13}C FT nmr spectrum of dextran [NRRL B-512(F) clinical size, 0.19 g/ml water] at 50°C, obtained at 15.18 MHz in a 20-mm probe, with 2048 points in the time domain, 125-ppm spectral width, 0.574-sec recycle time, 4096 accumulations, and a digital broadening of 1.2 Hz. Horizontal scale is in ppm upfield from CS_2. Branching, a characteristic of dextrans, is not shown in the structural formula.*





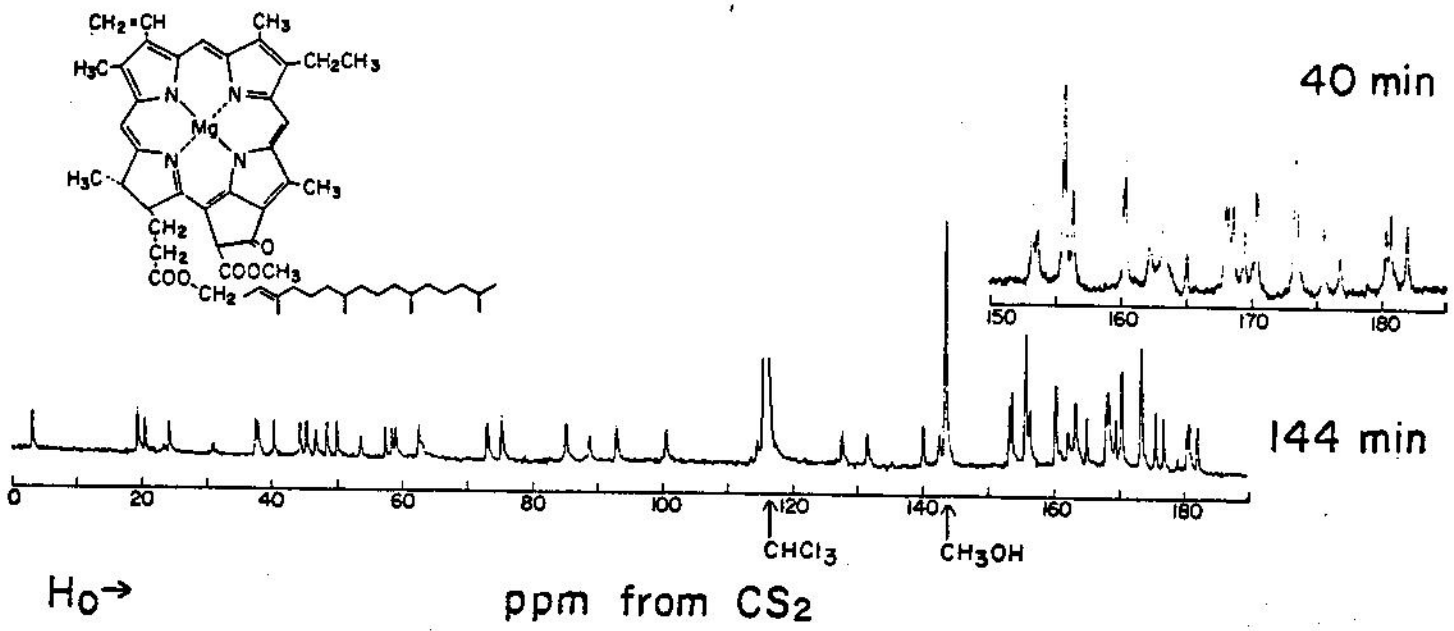


Figure 5 *Proton-decoupled natural-abundance ^{13}C FT nmr spectra of chlorophyll a (1.45 g in 7.9 ml chloroform and 0.6 ml methanol) at 35°C, obtained at 15.18 MHz in a 20-mm probe, with 4096 points in the time domain, 1024 accumulations, and 0.3-Hz digital broadening. Main spectrum: 250-ppm spectral width, 8.44-sec recycle time. Insert (upfield): 62.5-ppm sweep width, 2.32-sec recycle time.*

ditions on our spectrometer, with the use of 13-mm and 20-mm probes. The sample is fructose, more than 8 hr after dissolving it in water, so that anomeric equilibrium is nearly reached. Assignments have been given elsewhere.[13] The 20-mm probe should be very useful for studying minor anomers in equilibrium solutions of saccharides, and for following the kinetics of anomerization of the predominant species.

Another profitable area for the 20-mm probe is in studies of polysaccharides. Figure 4 shows a spectrum of dextran strain NRRL B-512(F), clinical size (molecular weight about 75,000). The six large resonances come from the main backbone of α-glucopyranose units. Branching gives rise to the small resonances. In particular, the small resonance at about 132 ppm upfield from CS_2 represents free CH_2OH groups. Relative integrated intensities can be used for quantitative studies of polysaccharide branching.

Figure 5 shows natural-abundance ^{13}C spectra of chlorophyll *a*. Nearly all of the 55 carbons are resolved into individual resonances. Previous ^{13}C nmr studies of chlorophyll[14–16] were made on ^{13}C-enriched materials.

Our primary motivation for developing the 20-mm probe was the desire to observe single-carbon resonances of proteins in solution. Even small proteins, such as ribonuclease A and hen egg-white (HEW) lysozyme, contain more than 500 carbons. In spite of this, ^{13}C nmr studies[3,10,11] of these proteins indicate that there may be sufficient chemical shift differences to resolve many single-carbon resonances, especially in the aromatic region of the spectrum. Figure 6A shows a ^{13}C spectrum of native HEW lysozyme ($C_{613}H_{950}O_{186}N_{192}S_{16}$). Figure 6B shows the unsaturated carbon region, observed with greater digital resolution than in Figure 6A. The region of the spectrum from peak 1 to peak 22 in Figure 6B encompasses the aromatic resonances (Figure 6C). We have shown[17] that the numbered (narrow) resonances 1–22 are caused by nonprotonated carbons only. There are 28 nonprotonated aromatic carbons in lysozyme (Figure 6C). The majority of peaks 1–22 are single-carbon resonances, observed with a signal-to-noise ratio of about 9. Assignments will be given elsewhere.[17] As an example, peaks 18–22 can be assigned to the γ-carbons of the six tryptophan residues. As expected, denaturation removes their large chemical shift nonequivalence.

It should be noted that 40 hr of signal accumulation was used to get the highly resolved spectrum of Figure 6B. Many biochemical studies will require greater sensitivity than we now have. However, our experience with 20-mm sample tubes suggests that development of nmr probes with much larger tubes is a practical and relatively inexpensive answer to the problem of low sensitivity in ^{13}C nmr spectroscopy of large molecules. We have already started to develop a probe for "extremely large" nmr tubes (we hesitate to commit ourselves yet to an exact size). A large-gap electromagnet (or large-bore superconducting magnet) may be required. In the meantime, the 20-mm probe has significantly increased the range of applications of ^{13}C FT nmr in our laboratory.

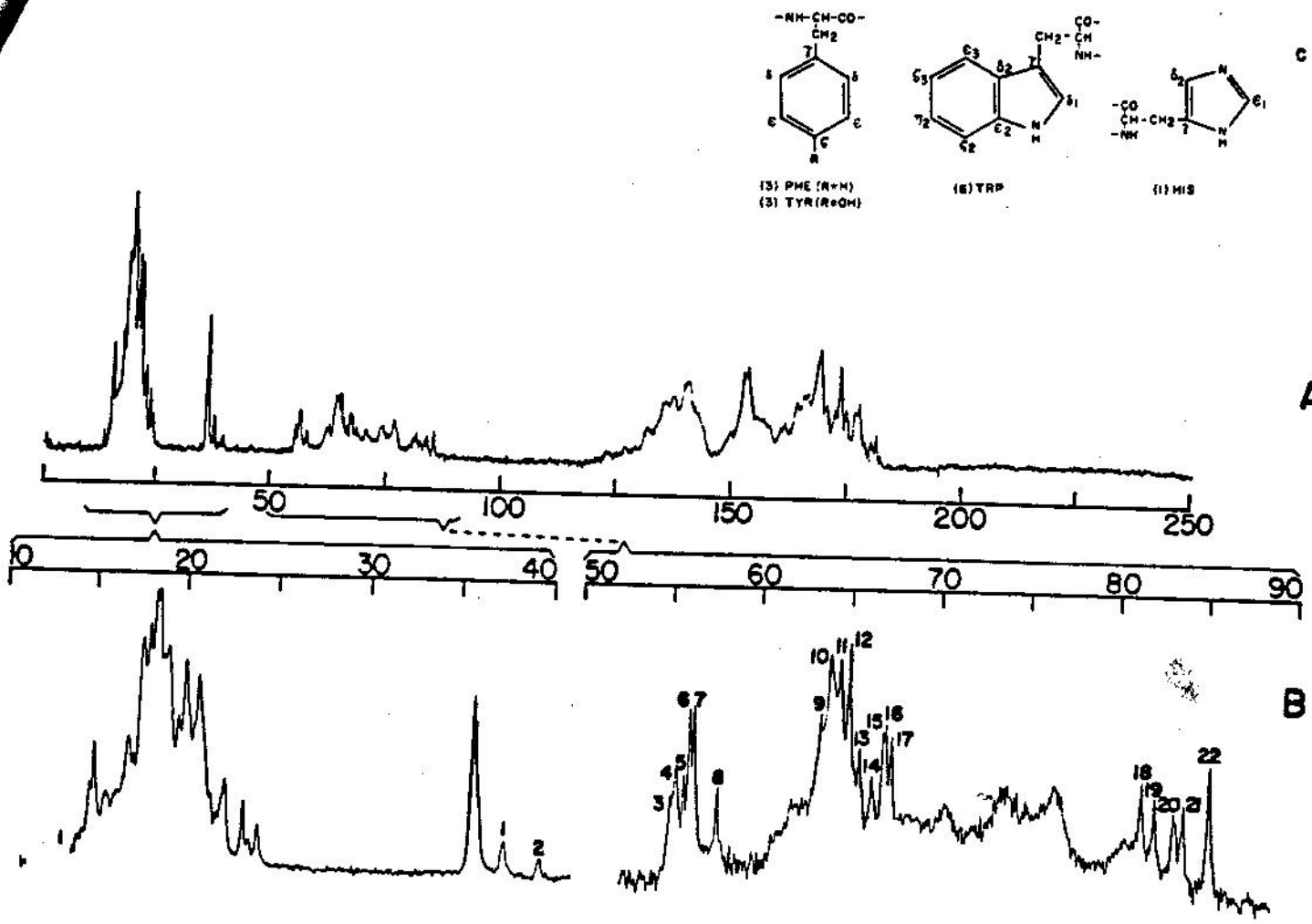


Figure 6 *A Proton-decoupled natural-abundance ^{13}C FT nmr spectrum of native HEW lysozyme (about 20% w/v in 0.1 M NaCl, pH 4.1, 44.5°C) obtained at 15.18 MHz in a 20-mm probe, with 4096 points in the time domain, 250-ppm spectral width, 1.38-sec recycle time, 32,768 accumulations (12 hr total time), and 1.2-Hz digital broadening. Horizontal scale is in ppm upfield from CS_2. B ^{13}C spectrum of the unsaturated carbons of HEW lysozyme (about 25% w/v in 0.1 M NaCl, pH 4.1, 39.5°C) obtained as in (A), but with a 125-ppm spectral width, 1.09-sec recycle time, 131,072 accumulations (40 hr total time), and 0.15-Hz digital broadening. C Structure of aromatic amino acid residues, with standard biochemical nomenclature. Quantity of each residue in lysozyme is shown in parentheses.*

References

1. ERNST, R. R. and ANDERSON, W. A., *Rev. Sci. Instrum. 37*, 93 (1966).
2. LEVY, G. C., *Carbon-13 Nuclear Magnetic Resonance for Organic Chemists* (John Wiley and Sons, Inc., New York, 1972), and references cited therein.
3. ALLERHAND, A., COCHRAN, D. W., and DODDRELL, D., *Proc. Nat. Acad. Sci. U.S. 67*, 1093 (1970); ALLERHAND, A., DODDRELL, D., GLUSHKO, V., COCHRAN, D. W., WENKERT, E., LAWSON, P. J., and GURD, F. R. N., *J. Amer. Chem. Soc. 93*, 544 (1971).
4. DODDRELL, D. and ALLERHAND, A., *Proc. Nat. Acad. Sci. U. S. 68*, 1083 (1971).
5. See, for example, PAUL, E. G. and GRANT, D. M., *J. Amer. Chem. Soc. 86*, 2977 (1964); JONES, A. J., GRANT, D. M., WINKLEY, M. W., and ROBINS, R. K., *J. Amer. Chem. Soc. 92*, 4079 (1970).
6. See, for example, WEIGERT, F. J., JAUTELAT, M., and ROBERTS, J. D., *Proc. Nat. Acad. Sci. U.S. 60*, 302 (1968); REICH, H. J., JAUTELAT, M., MESSE, M. T., WEIGERT, F. J., and ROBERTS, J. D., *J. Amer. Chem. Soc. 91*, 7445 (1969).
7. KUHLMANN, K. F. and GRANT, D. M., *J. Amer. Chem. Soc. 90*, 7355 (1968); KUHLMANN, K. F., GRANT, D. M., and HARRIS, R. K., *J. Chem. Phys. 52*, 3439 (1970).
8. ALLERHAND, A., DODDRELL, D., and KOMOROSKI, R., *J. Chem. Phys. 55*, 189 (1971).
9. DODDRELL, D., GLUSHKO, V., and ALLERHAND, A., *J. Chem. Phys. 56*, 3683 (1972).
10. CHIEN, J. C. W. and BRANDTS, J. F., *Nature New Biol. 230*, 209 (1971).
11. GLUSHKO, V., LAWSON, P. J., and GURD, F. R. N., *J. Biol. Chem. 247*, 3176 (1972).
12. MOON, R. B. and RICHARDS, J. H., *Proc. Nat. Acad. Sci. U.S. 69*, 2193 (1972).
13. DODDRELL, D. and ALLERHAND, A., *J. Amer. Chem. Soc. 93*, 2779 (1971).
14. STROUSE, C. E., KOLLMAN, V. H., and MATWIYOFF, N. A., *Biochem. Biophys. Res. Commun. 46*, 328 (1972).
15. KATZ, J. J., JANSON, T. R., KOSTKA, A. G., UPHAUS, R. A., and CLOSS, G. L., *J. Amer. Chem. Soc. 94*, 2883 (1972).
16. KATZ, J. J. and JANSON, T. R., private communication.
17. ALLERHAND, A., CHILDERS, R. F., and OLDFIELD, E. (to be published).