13 April 1973, Volume 180, Number 4082 SCIENCE

Carbon-13 Nuclear Magnetic Resonance Spectroscopy

New applications emerge for this powerful method as a result of breakthroughs in instrumentation.

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Carbon-13 nuclear magnetic resonance spectroscopy (abbreviated ^{13}C NMR or CMR, carbon magnetic resonance) is an extremely powerful research tool for the study of organic molecules (*1, 2*). The information derivable from ^{13}C NMR is often complementary to that obtained from proton NMR spectroscopy, which has been available in many chemical laboratories for more than a decade.

The most common isotope of carbon, carbon-12, does not have the proper nuclear characteristics (its magnetic moment is 0) to be used for magnetic resonance experiments. The isotope carbon-13 has favorable characteristics —a nonzero magnetic moment and a nuclear spin of ½—but its natural isotopic abundance is low (1.1 percent). This low isotopic abundance coupled with a low inherent sensitivity for the ^{13}C nucleus relative to ^{1}H makes ^{13}C NMR experiments approximately 6000 times less sensitive than ^{1}H NMR experiments. Thus, other factors being equal, 6000 times larger samples would be required for ^{13}C NMR experiments (however, the geometry of the experiment does not permit this). Despite the low sensitivity of ^{13}C NMR at natural isotopic abundance, it became clear after the initial work (*3*) on neat samples of organic molecules of low molecular weight that ^{13}C NMR could provide new kinds of important structural information. During the middle and late 1960's several breakthroughs in experimental methodology and NMR instrumentation merged to finally allow facile, sensitive, and versatile ^{13}C NMR studies at natural isotopic abundance.

With the new, stable spectrometers of the early 1960's repetitive scans of spectral regions could be coherently added in a hard-wired (special purpose) or general purpose computer. This computer time-averaging extends NMR sensitivity because the coherently added weak NMR signals increase in intensity relative to noise signals, which tend to cancel out. Actually, the spectral signal-to-noise ratio increases with the square root of the number of scans.

The second important new experimental technique, wide-band (or noise-modulated) proton decoupling, resulted in individual carbon resonances appearing as sharp singlet signals, rather than the multiplets due to ^{13}C-^{1}H spin-spin coupling. The ^{1}H decoupling results in approximately an order of magnitude increase in sensitivity because of (i) collapse of the ^{13}C-^{1}H multiplet structure and (ii) a positive nuclear Overhauser effect (NOE) (*4*) that increases the observed signal by as much as 200 percent.

The most significant experimental breakthrough for ^{13}C NMR was the development of pulsed Fourier transform (FT) NMR (*5*), which resulted in effective sensitivity increases of more than an order of magnitude. With a time-averaged accumulation of data from repetitive pulses spaced typically 1 second apart (each pulse is analogous to a slow sweep through the entire spectrum in conventional swept NMR, which is the advantage of the FT method), it is possible to achieve time savings greater than a hundredfold. The FT time advantage, coupled with a similar gain resulting from wide-band ^{1}H decoupling, allows ^{13}C experiments to be performed on solutions that are 0.5 to 1 molar in a few minutes. Experiments on large molecules at very low concentrations (10^{-2} to $10^{-3}M$) can also be performed, at the expense of extended spectrometer acquisition times (typically overnight). The FT method also allows ^{13}C NMR spectra of moderately concentrated solutions to be obtained without proton decoupling. In this case, valuable information on spin-spin coupling between ^{13}C and ^{1}H nuclei is obtained, but the time required to obtain such spectra is, of course, much greater than when wide-band ^{1}H decoupling is used. An increase in sensitivity in "undecoupled" ^{13}C FT experiments may be obtained by irradiating the protons between (but not during) periods of data acquisition (*6*). This results in a significant nuclear Overhauser effect, without any collapse of the ^{13}C multiplet structure. Selective decoupling experiments of various kinds (*1, 2*) can be carried out to determine the number of protons attached to a particular carbon, and to establish a relationship between the resonance frequencies of a carbon and a directly attached proton. In these experiments the ^{13}C multiplets are partially or selectively collapsed and there is a NOE enhancement, so that the sensitivity is quite good.

Dr. Allerhand of Indiana University has recently extended the sensitivity limits with ^{13}C with his own FT spec-

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trometer, which is designed to handle very large sample tubes (20 millimeters in diameter; the usual ^{13}C NMR sample tubes are 10 to 13 mm in diameter) (*7*). With these extra large sample tubes, useful spectra of $10^{-2}M$ solutions can be obtained with less than 1 hour of data acquisition. Figure 1 shows the ^{13}C spectrum of 0.04*M* sucrose (*7*), obtained with a 20-mm tube in 37 minutes. The use of extra large sample tubes is expected to have particular impact on studies of low-concentration solutions of biopolymers such as proteins.

We can demonstrate the power of ^{13}C NMR by comparing proton and carbon spectra of the steroid cholesterol. The proton spectrum obtained at 251 megahertz (Fig. 2) gives significant information about the structure of cholesterol but falls far short of allowing assignment of all the resonance lines to specific types of protons. By contrast, the ^{13}C spectrum at 25 Mhz (Fig. 3) has individually resolved lines for 26 of the 27 carbons in the molecule, despite the fact that the ^{13}C spectrum was obtained at a much lower field than the proton spectrum. Using various decoupling techniques and empirical chemical shift correlations between related compounds, Roberts and coworkers (*8*) were able to assign all the peaks in some 30 steroid spectra, including that of 0.2*M* cholesterol (Roberts' assignments are used in Fig. 3). It is interesting to compare this ^{13}C spectrum obtained by Fourier transform NMR (Fig. 3) with two early (1968!) frequency-swept spectra of 1.1*M* cholesterol reported by Roberts and co-workers (*9*) (Fig. 4). The spectrum obtained without ^{1}H decoupling (Fig. 4a) is unusable. The spectrum obtained with wide-band ^{1}H decoupling (Fig. 4b) in the same time (about 8 hours) has a greatly increased signal-to-noise ratio and shows much spectral detail. At the rapid sweep rate used for this wide spectral scan not all individual carbons are resolved, however. Roberts resolved 26 carbons by using several narrower spectral scans, but this required considerable time. In contrast, the FT spectrum of 0.2*M* cholesterol shown in Fig. 3 was recorded after only 3.4 hours of data accumulation.

Despite the higher spectral resolving power of ^{13}C NMR (evident from an examination of Figs. 2 and 3), there are some situations where ^{1}H NMR is a more useful technique. With Fourier transform ^{1}H NMR (*5*), particularly with spectrometers operating at very high magnetic fields, extremely small amounts (micrograms) of compounds as well as very dilute solutions can be usefully examined. The natural-abundance ^{13}C NMR spectra of such samples cannot be obtained by present methods.

In compounds where the ^{13}C nuclei are spin-coupled to protons, it is possible to observe the ^{13}C spectrum indirectly by double resonance methods on a proton NMR spectrometer equipped for heteronuclear decoupling. This technique has been very useful in investigations of carbonium ions (*10*) and has some sensitivity advantages over direct detection by continuous wave ^{13}C NMR. However, the indirect methods are not applicable in all instances and there is no great sensitivity advantage over pulsed Fourier transform ^{13}C

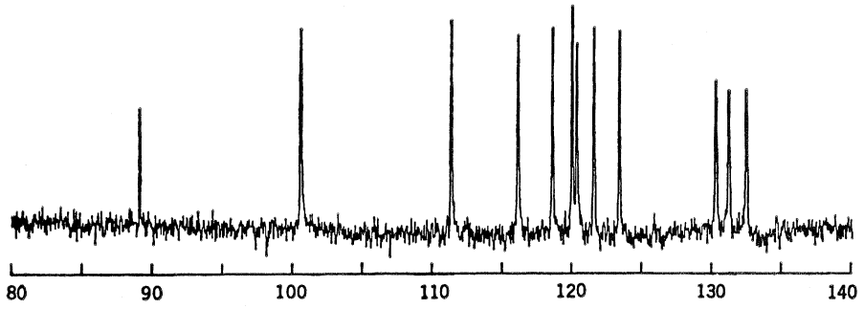


Fig. 1. Natural-abundance ^{13}C Fourier transform NMR spectrum of aqueous 0.04*M* sucrose with proton decoupling. The spectrum was obtained at 15.2 Mhz in a sample tube 20 mm in diameter, with 90° radio-frequency pulses, 1024 scans, and a pulse interval of 2.2 seconds (total accumulation time of 37 minutes). The horizontal scale is in parts per million upfield from $^{13}CS_2$. The 12 different carbons of sucrose are completely resolved (*7*). [Courtesy of A. Allerhand]

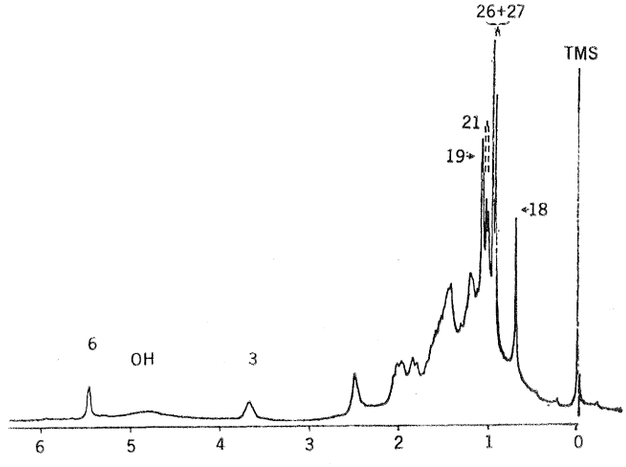


Fig. 2. Proton NMR spectrum of cholesterol (same solution as in Fig. 3) at 251 Mhz with frequency sweeping. The scale is in parts per million downfield from tetramethylsilane (*TMS*). Peaks corresponding to protons on methyl groups (C18, C19, C21, C26, and C27) and on C3, C6, and the OH groups are marked (see Fig. 3 for structure).

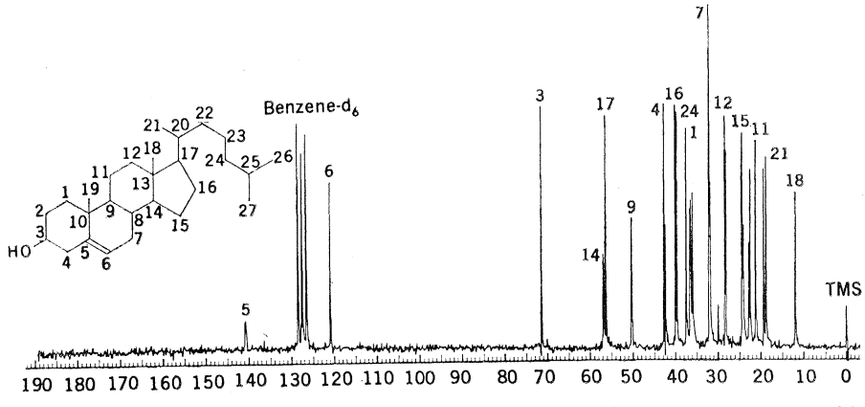


Fig. 3. Fourier transform ^{13}C NMR spectrum of cholesterol (0.2*M* in benzene-d_6) with wide-band proton decoupling and ^{13}C in natural abundance. The spectrum was obtained at 25.1 Mhz with 30,000 scans and a pulse interval of 0.4 second (total time of 3.3 hours). The scale is in parts per million downfield from the tetramethylsilane (*TMS*) carbon resonance (*1*). [Courtesy of John Wiley & Sons, Inc.]

NMR. The normal proton spectrum has been used as a measure of the enrichment of ^{13}C at certain molecular positions, as in biosynthetic studies of the incorporation of labeled [^{13}C]acetate into the fungal tropolone, sepedonin (*11*). In this case also, pulsed Fourier transform ^{13}C NMR offers an attractive alternative when the instrumentation is available.

Commercial NMR instruments providing ^{13}C Fourier transform capabilities are currently available from three sources (*12*). These spectrometers typically operate with magnetic fields of 14,000 or 23,000 gauss, that is, at frequencies of 15 and 25 Mhz, respectively. The corresponding proton frequencies at these fields are 60 and 100 Mhz. Superconducting solenoid spectrometers operating at frequencies up to 75 Mhz for ^{13}C are also available. Although the cost of complete ^{13}C Fourier transform NMR instrumentation including a small computer is high (ranging upward from $130,000), smaller "complete" systems are available (about $80,000), and even lower priced systems (under $50,000) may be on the way.

In this article we present an overview of ^{13}C NMR methods and their application to chemical problems. Because of length restrictions many topics must be omitted. Some emphasis is placed on applications to molecules of biological significance, since recent work in this area has shown much promise.

Special Characteristics

It is clear from the ^{13}C and ^{1}H cholesterol spectra that carbon resonances occur over a wider range of chemical shifts than do corresponding proton signals. The total range of known ^{13}C resonances is approximately 600 parts per million (ppm), while the resonances for most organic molecules appear between carbonyl groups, which are at low fields, and methyl carbons, which are at high fields, in a range of just over 200 ppm. By contrast, most proton resonances occur over a range of about 10 ppm. The apparent increase in "chemical shift dispersion" with ^{13}C NMR is actually understated, since only with carbon resonances is it generally possible to obtain sharp, single lines for individual types of nuclei (with complete ^{1}H decoupling). Figure 5 gives general ranges of ^{13}C chemical shifts for various types of carbon groups, with chemical shifts

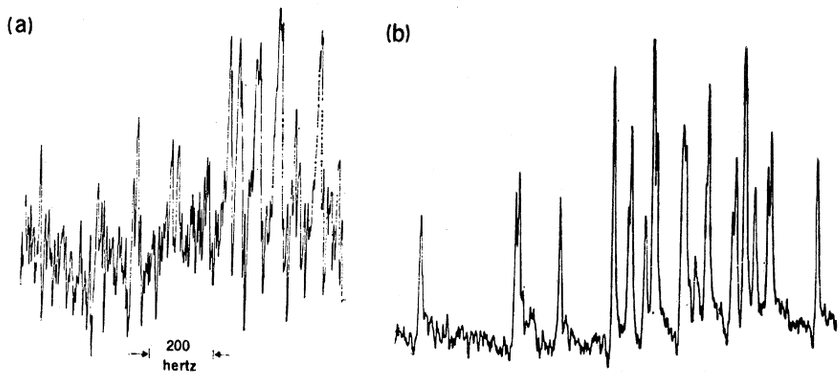


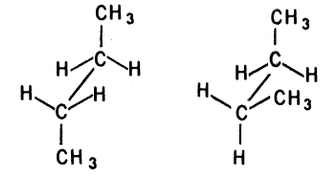
Fig. 4. Natural-abundance ^{13}C NMR spectra of 1.1*M* cholesterol in the spectral region of saturated carbon. The spectra were obtained at 15 Mhz with frequency sweeping and: (a) without proton decoupling, (b) with wide-band proton decoupling. In each spectrum 1000 sweeps were accumulated (*10*). [Courtesy of the National Academy of Sciences]

stated relative to the methyl carbons in the NMR standard, tetramethylsilane.

The main contribution to the chemical shift of a ^{13}C nucleus arises from the local paramagnetic term in the usual quantum mechanical expression for the screening constant (*13*). The local diamagnetic term, which is dominant in proton chemical shifts, is small, although not negligible. Because of the large range of the ^{13}C chemical shifts, contributions from ring currents and magnetically anisotropic groups are relatively unimportant.

Although much progress has been made in theoretical calculations of ^{13}C chemical shifts, practical spectrum analysis depends almost entirely on empirical relationships determined in simple model compounds. Experimentally, it has been found that the effects of substituents on ^{13}C chemical shifts are often more or less additive (*1, 2*). Hybridization and electronic charge have large effects on chemical shifts, as can be seen from Fig. 5. Correlations of ^{13}C NMR chemical shifts for carbons *para* to substituents in monosubstituted benzenes have been made with Hammett-type sigma parameters and with electron densities obtained from molecular orbital calculations.

A very interesting steric effect (the gamma effect) on ^{13}C chemical shifts has been investigated in detail by Grant and co-workers (*14*) (*1, 2, 15*). An axial methyl carbon on a cyclohexane ring, for example, is more shielded than an equatorial methyl carbon by about 4 ppm, other things being equal. The carbons in the 3 and 5 positions, which are involved in the steric interaction with the axial 1-methyl group, are also shifted upfield. Similar effects are found in aliphatic hydrocarbons for four-carbon fragments which exist in the *gauche*-butane conformation. The *trans*-butane type of conformation, on the other hand, does not have any steric repulsion and does not show an unusual upfield shift.



Trans-butane Gauche-butane

Paramagnetic lanthanide shift reagents (*16*), which have proved so useful in proton NMR, promise also to be quite important in ^{13}C NMR spectroscopy (*1*), particularly for spectral assignments, and for structural studies in solution. Complexes of ytterbium are particularly appropriate for ^{13}C NMR studies since the shifts observed are nearly entirely pseudocontact shifts and thus are amenable to geometric analysis (*17*). The more usual europium complexes, by contrast, induce shifts in carbons close to the complexing atom (for example, oxygen for an alcohol) by the contact as well as the pseudocontact mechanism, and thus the analysis becomes quite complex.

Spin-spin coupling between ^{13}C and ^{1}H nuclei has been extensively studied (*18*), particularly ^{13}C-^{1}H coupling through one bond, which can often be observed in ^{1}H NRM spectra. These coupling constants to directly bonded protons range from 125 to about 300 hertz and depend particularly on carbon hybridization and on the electronegativity of substituents. Longer range ^{13}C-^{1}H couplings (over two or more bonds) are generally less than 15 hertz.

Spin-spin couplings between ^{13}C and

^{13}C (*19*) and between ^{13}C and other nuclei (such as ^{19}F, ^{31}P, ^{15}N, and ^{29}Si) (*20*) have been reported. These couplings often find application in structure determinations, supplementing ^{13}C chemical shift information. Couplings between ^{13}C and ^{31}P are being used in the determination of molecular conformations in molecules of biological interest, such as cyclic nucleotides (*21*), and in synthetic heterocyclics (*22*).

Another type of information available from ^{13}C spectra is perhaps somewhat unfamiliar to chemists using proton NMR. In ^{13}C NMR, peak areas (peak integrations) do not necessarily correspond with the number of nuclei making up the individual signals. This results from variations in NOE's and spin-lattice relaxation times for the different carbons in a molecule. The spin-lattice relaxation time (T_1) describes the (normally) first order process of energy exchange between the nuclear spins and the sample "lattice." In most small organic molecules T_1 varies from 10 to 100 seconds; in large molecules it is much shorter. Significant spin-lattice relaxation must occur during the intervals between excitation pulses in a repetitive FT experiment. If the pulses are spaced too closely relative to the T_1's for a particular carbon, that carbon will yield reduced signals after successive pulses (*23*). Thus, in these experiments carbons with relatively short T_1's will give larger signals than carbons whose T_1's are longer and more nearly comparable with the interval between pulses (*24*). Such intensity differences can actually be useful in spectral assignments when gross structural features are known. For example, carbons not having directly attached protons (nonprotonated carbons) have relatively long T_1's (and often low NOE's) and can be differentiated from protonated carbons.

Variations in nuclear Overhauser enhancements for individual carbons occur when the spin-lattice relaxation process occurs by any mechanism other than through ^{13}C-^1H dipole-dipole interactions. In many large organic molecules these dipole-dipole interactions dominate the ^{13}C T_1 behavior, but in smaller molecules several other mechanisms may contribute substantially to the carbon relaxation (*25*). Experimental methods are available to minimize the effects of variations in T_1 and NOE behavior, so that a close correspondence can be obtained between peak areas and molecular structure (*26*).

It is possible with many ^{13}C Fourier transform spectrometer systems to measure directly the T_1's for all the carbons in a molecule (*27*). The most commonly used method, but by no means the only one available, is to apply a two-pulse sequence, in which the first pulse (a π or 180° pulse) inverts the nuclear magnetization. The nuclei recover their normal magnetization with a time constant equal to T_1. After a specific waiting time (t), the magnetization of the various nuclei can be sampled by the application of a second pulse, which is usually a $\pi/2$ or 90° pulse. Data acquisition then proceeds as in a normal FT experiment. If time averaging is required, as it usually is, the system must be allowed to return to equilibrium before the pulse sequence is carried out again, and this takes several T_1's for the nuclei under consideration. The whole experiment is then repeated for several appropriate values of t, and the T_1's for all the carbons can be calculated from the changes in the line intensities with time. Although T_1 measurements require much more time than a normal FT experiment, especially for weak spectra with very long T_1's, they are practical for large biologically important molecules because the T_1's are then quite short.

The T_1 data can be used to describe overall and internal molecular motions in solution and thus probe the symmetry and stereochemistry of organic molecules (*27*). The T_1 data may also be used to facilitate ^{13}C spectral assignments in complex organic molecules (*28*). Carbon-13 spin-spin relaxation times (T_2's) may also be measured (*29, 30*). However, these experiments are very difficult, partly because wide-band ^1H decoupling cannot be used (*29*).

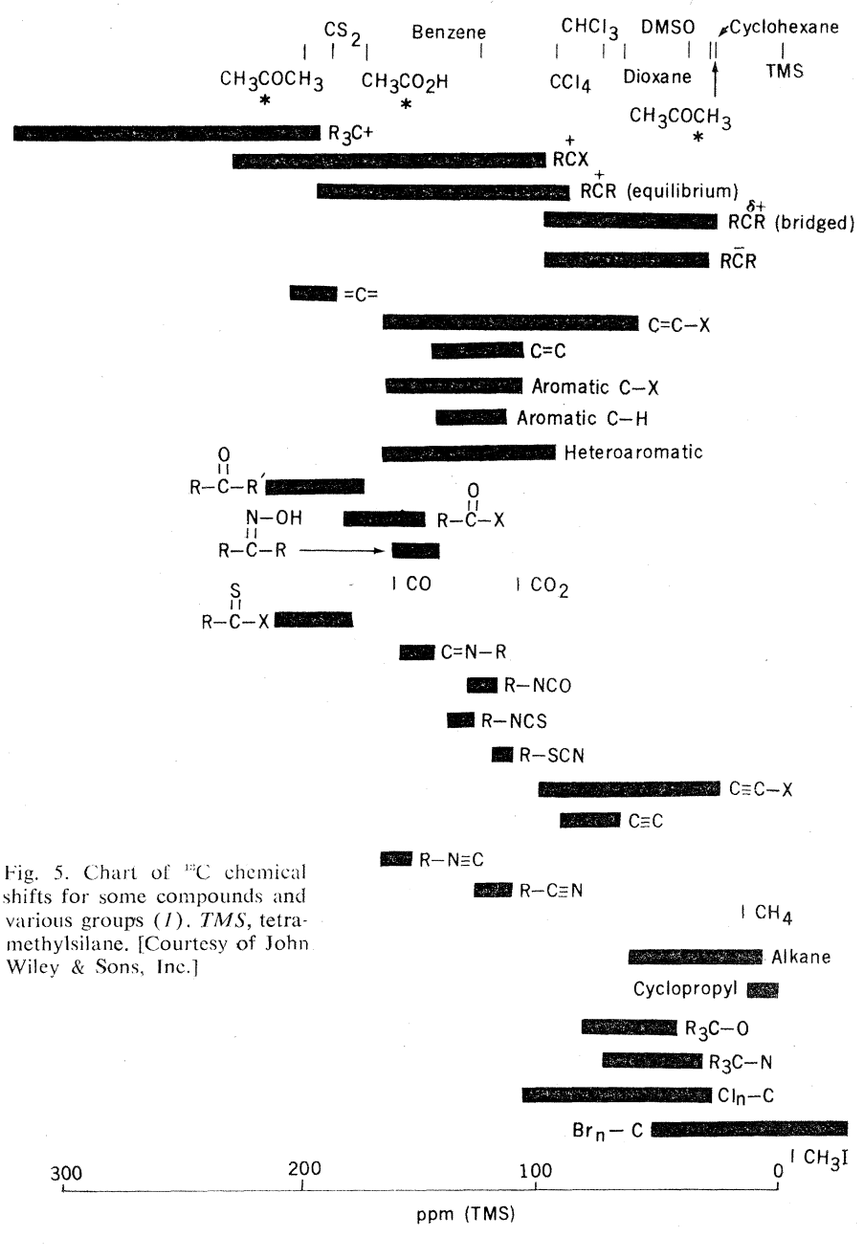
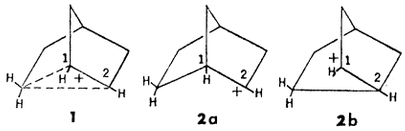


Fig. 5. Chart of ^{13}C chemical shifts for some compounds and various groups (*1*). *TMS*, tetramethylsilane. [Courtesy of John Wiley & Sons, Inc.]

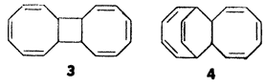
Organic Structure and Conformational Analysis

Much of the recent ^{13}C NMR work has been concerned with determinations of chemical shifts, coupling constants, and relaxation parameters for a very wide variety of compounds of known structures. Because the number of ^{13}C NMR spectrometers in operation is still relatively small, applications to structural determinations are as yet comparatively few, but they are expected to grow rapidly in the near future. Carbon-13 NMR spectroscopy is especially valuable for compounds with many nonprotonated carbons, since the lack of protons prevents the use of ^{1}H NMR. Examples are chlorocarbons, metal carbonyls, and substituted acetylenes and aromatics.

Strong evidence for a nonclassical structure (**1**) for the norbornyl cation has been obtained from ^{13}C chemical shift measurements. Comparisons with model classical carbonium ions show that an explanation in terms of rapidly equilibrating classical ions (**2a** and **2b**) is not satisfactory since the average chemical shift expected at C1 and C2 in **2a** and **2b** is at least 50 ppm different from the observed shift (*10*). Structure **1** has the correct symmetry, and the presence of a special kind of three-membered ring is consistent with an unusual chemical shift for C1 and C2. The dashed lines in structure **1** represent half-bonds.



1 **2a** **2b**



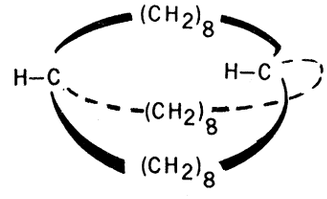
3 **4**

As in the case of proton NMR, a simple but powerful use of ^{13}C NMR is in the determination of the number of different carbons in a molecule. Thus, a choice can be made between isomeric compounds which have different symmetries. For example, a choice can be made between structures **3** and **4** for the cyclooctatetraene dimer of melting point 53°C. The observed ^{1}H-decoupled spectrum has only four sharp lines and is consistent with structure **3**, but would fit structure **4** only with very unlikely coincidences (*31*).

Symmetry considerations are also very useful in applications of ^{13}C NMR to the conformational analysis of cyclic compounds (*32*). For example, cyclononane (*33*) and cyclododecane (*34*) each show two lines in the intensity ratio of 2/1 in their proton-decoupled ^{13}C NMR spectra at temperatures of $-160°$ and $-130°C$, respectively. These results are consistent with conformations of threefold and fourfold symmetries for the C_9 and C_{12} cycloalkanes, respectively. The low observation temperatures are essential because ring pseudorotation (intramolecular conformational rearrangement) must be slow on the NMR time scale for separate resonances to be observed. As the temperature is raised, the lines broaden and coalesce, and finally only a single sharp line is observed. From the temperature dependence of the ^{13}C spectra, the free energy barriers for ring pseudorotation in the C_9 and C_{12} cycloalkanes are calculated to be 6.0 and 7.3 kilocalories per mole, respectively. By contrast, the ^{1}H NMR spectra of the C_9 and C_{12} cycloalkanes are extremely complex at low temperatures and are only partially resolved even at 251 Mhz; such spectra give little information on symmetry.

In cyclohexane, the proton-decoupled ^{13}C NMR spectrum is unaffected by ring inversion, whereas this process is visible and thus can be studied in the ^{1}H NRM spectrum. In substituted cyclohexanes, however, ^{13}C NMR can be used and is advantageous because of the large chemical shifts between, for example, axial and equatorial methyl groups (*35*).

Bicyclo[8.8.8]hexacosane stereoisomers have recently been prepared [in-out isomerism (*36*)]. The in-out isomer has ten different carbons, unlike the in-in or out-out isomers, which have only five different carbons. One of the isomers prepared gives ten peaks in its ^{13}C NMR spectrum and is thus the in-out isomer (**5**). Because of peak overlap, ^{1}H NMR, even at 220 Mhz, could not be used for structural assignments in this series.



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Natural Products and Biosyntheses

The ^{13}C NMR of numerous natural products including alkaloids, amino acids and polypeptides, antibiotics, carbohydrates, lipids, nucleotides, porphyrins, steroids, terpenes, and vitamins have been investigated (*1*, *2*). In all except the most complex compounds it has been possible to make assignments to virtually all the spectral peaks. Once the resonances in a compound have been assigned, it becomes very easy to determine by ^{13}C NMR which carbons have been enriched in ^{13}C as a result of feeding experiments. No chemical degradation, which is often lengthy and tedious, is required, in contrast to the standard ^{14}C tracer technique.

A biosynthetic ^{13}C Fourier transform NMR study of the secondary bacterial metabolite prodigiosin is illustrative. The ^{13}C Fourier transform NMR spectra of prodigiosin, isolated from biogenetically enriched *Serratia marcescens* bacteria (in separate experiments with sodium [1-^{13}C]acetate and sodium [2-^{13}C]acetate) are shown in Fig. 6, along with the spectrum of prodigiosin with ^{13}C in natural isotopic abundance. Once ^{13}C spectral assignments have been made (Fig. 6A), determination of the pattern of [^{13}C]acetate incorporation is trivial (the incorporation pattern is given at the top of Fig. 6).

There are several other examples of the use of ^{13}C NMR in biosynthesis studies (*1*). An interesting recent example concerns the biosynthesis of vitamin B_{12} (*37*). Rather complex spectra are obtained when δ-[5-^{13}C]aminolevulinic acid is used as a precursor for vitamin B_{12}, because the labeled carbons (seven in all) occur in groups of two, two, and three directly bonded together. Thus ^{13}C-^{13}C couplings of 60 to 70 hertz are observed. Nevertheless, the results can be interpreted unambiguously and provide valuable new information on the biosynthesis of this vitamin. The results also support and extend the ^{13}C assignments made by Doddrell and Allerhand (*38*) on vitamin B_{12} and other corrinoids.

Two other biochemical applications of ^{13}C NMR are discussed below under separate headings.

Amino Acids, Peptides, and Proteins

Extensive ^{13}C chemical shift data for the natural amino acids were first reported in 1968 (*39*); they were obtained by a double resonance method from the ^{13}C satellites in the proton spectra. Direct ^{13}C measurements on amino acids with the isotope both in natural abundance and enriched were made soon afterward (*40*). With the introduction of the FT technique, the ^{13}C NMR spectra of large peptides and small proteins, including enzymes, have

been obtained with only a few hours of data acquisition (*41*).

Denatured proteins give rise to well-resolved spectra, in part because of segmental motion and in part because the resonances of the amino acid residues are virtually independent of their positions in a long peptide chain. Such spectra, therefore, give little information on the sequence of amino acids. Only the smallest proteins have as yet been investigated in a native form by ^{13}C NMR. Ribonuclease A and lysozyme both give informative spectra even though most of the carbons cannot be resolved (there are over 500 carbons in each of these enzymes). Measurements of spin-lattice relaxation times (T_1's) on ribonuclease A show that the molecule as a whole tumbles relatively slowly [the correlation time (τ_c) $\simeq 3 \times 10^{-8}$ second] (*42*). Thus, rather broad lines are expected for all protonated carbons except for carbons on side chains, which have additional motional freedom. For example, the carbons in the epsilon positions of the lysine residues in ribonuclease give rise to an exceptionally sharp line because they have a much longer T_1 (0.33 second) than other protonated carbon atoms ($T_1 \simeq 0.03$ second). In native proteins the same kind of amino acid can give rise to different chemical shifts because it can be in different local environments in a fixed polypeptide chain conformation. For example, the nonprotonated indolyl carbons in the beta positions of the six tryptophane residues in lysozyme give well-resolved lines, except for two residues which give overlapped resonances (*7*).

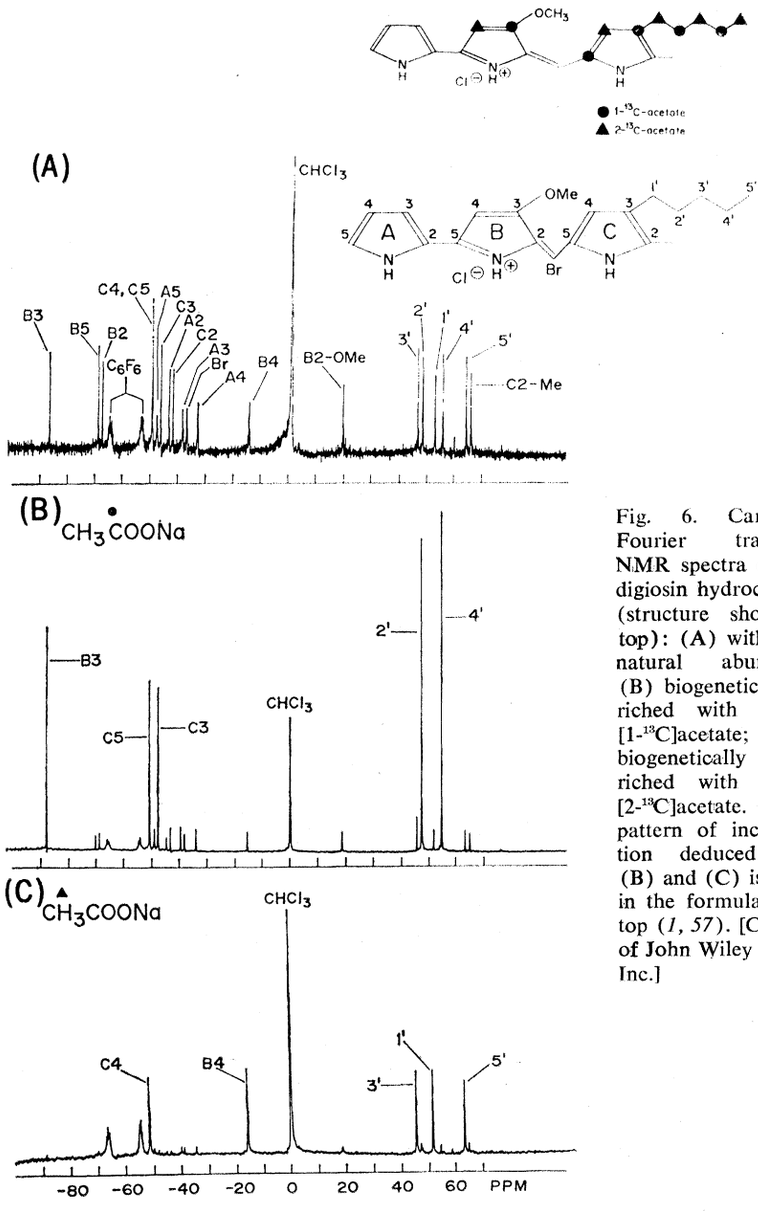


Fig. 6. Carbon-13 Fourier transform NMR spectra of prodigiosin hydrochloride (structure shown at top): (A) with ^{13}C in natural abundance; (B) biogenetically enriched with sodium [1-^{13}C]acetate; (C) biogenetically enriched with sodium [2-^{13}C]acetate. The pattern of incorporation deduced from (B) and (C) is shown in the formula at the top (*1*, *57*). [Courtesy of John Wiley & Sons, Inc.]

Studies of proteins and enzymes with natural abundance ^{13}C have involved very concentrated solutions, and hence relatively large quantities (about 1 gram) of material are needed. Proteins selectively enriched in ^{13}C should be very suitable for ^{13}C NMR studies, since even 10 percent incorporation of a 90 percent ^{13}C-labeled amino acid would reduce the amount of protein required to 100 milligrams or so, while 100 percent incorporation at one carbon in a single residue would allow measurements on about 10 mg of protein.

Synthetic and Natural Membranes

Biological membranes contain relatively large proportions of lipid bilayers as well as other components. Synthetic membranes which show many of the properties of natural membranes can be prepared from aqueous dispersions of lecithins. Membranes have been investigated by various magnetic resonance methods, including ^{13}C resonance. Unsonicated human erythrocyte membranes in D_2O buffer show several broad envelopes of resonances even though the solution is extremely glutinous (*43*). Figure 7 shows ^{13}C NMR spectra of sonicated dipalmitoyllecithin (DPL) (transition temperature of 43°C) in a D_2O buffer, as a function of temperature (*44*). In order to obtain well-resolved NMR spectra it is essential to operate above the transition temperature of the particular lecithin so that the interior of the membrane is liquid-like rather than "crystalline," and also to use sonicated preparations which contain small and homogeneous spherical vesicles (*45*). Below the transition temperature, only the choline methyl groups, which are relatively free to move in the aqueous phase, give rise to a narrow resonance signal (*44*).

The ^{13}C NMR spectrum of sonicated DPL at 64°C (Fig. 7) consists of relatively sharp peaks, most of which have been assigned. The two palmitic ester side chains are sufficiently similar to give indistinguishable chemical shifts. The well-resolved spectra of DPL allow T_1 measurements to be made on many of the carbons (*44*), and the results are given below, together with the structure of the lecithin. Protonated carbons (such as CH_2 groups) near the end of either lipid chains have longer relaxation times than those in the middle of the chain, which in turn have longer T_1's than the carbons near the carbonyl

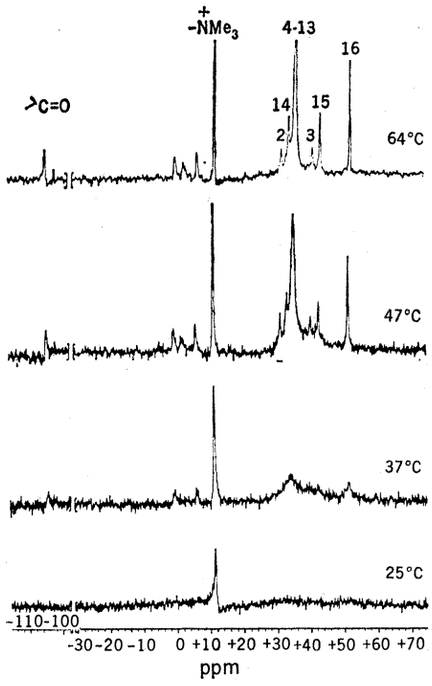
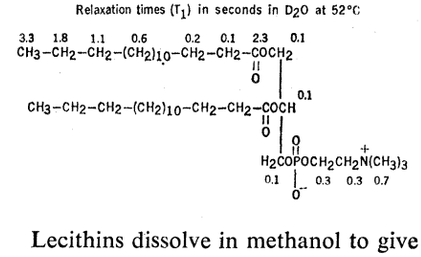


Fig. 7. Carbon-13 NMR spectra of sonicated dipalmitoyllecithin (0.23*M*) in D_2O buffer as a function of temperature. The chemical shift scale is in parts per million with respect to dioxane as a reference (positive shifts upfield) [Reprinted from (*44*) by permission of the American Chemical Society]

group or in the glycerol residue. The variations in T_1 reflect the greater mobility of the methylene groups near the free ends of the chains, due to segmental motion.



Lecithins dissolve in methanol to give ordinary solutions rather than bilayers. The ^{13}C NMR spectra under these conditions consist of very sharp lines, and the variations in T_1 along the lipid chains are substantially less than in the bilayers (*44*). Moderately sharp spectra are obtained for chloroform solutions (*44–46*) where lecithins exist as spherical micelles containing 60 to 70 molecules, with the polar groups on the inside and the hydrocarbon chains on the outside. In such micelles the choline methyl groups are restricted in their motions and have much shorter T_1's than in the bilayers, where these groups are on the outside.

In contrast to the spin-lattice relaxation times, the carbon chemical shifts are only slightly dependent on the state and nature of aggregation of the lecithin. However, the carbon chemical shifts of methylene groups near the ends of the side chains of egg lecithin as vesicles in water are slightly dependent on temperature (about 0.01 ppm/°C) and this effect has been ascribed to an increasing population of *gauche* conformations at higher temperatures (*47*). As a result of the gamma effect discussed earlier, a shift to high fields should occur when the temperature is increased, as observed.

The use of natural membranes selectively enriched in ^{13}C promises to be very fruitful in ^{13}C NMR studies, as shown by the preliminary work of Metcalfe *et al.* (*48*) on *Acholeplasma* membranes. The organisms were grown on a medium which included palmitic acid (1-^{13}C)-labeled at the carbonyl group.

The use of specific ^{13}C enrichment increases the signal-to-noise ratio and allows T_1 measurements to be made on broad or overlapping resonances.

Polymers

Relatively sharp lines are found in the ^{13}C NMR spectra of bulk polymers provided that the experiment is carried out above the glass-transition temperature (*49*). The lines are an order of magnitude narrower than ^1H NMR lines of the same system and resonances of individual carbons can be resolved. Polymers can also be studied by ^{13}C NMR as solutions, and in these cases extremely sharp lines are often observed (*1, 2*).

Carbon-13 magnetic resonance has been used successfully to determine the tacticity of vinyl polymers (*1*). For many polymers, such as poly-(methyl methacrylate), ^{13}C NMR is distinctly superior to ^1H NMR (*50*). In a "pentad" of five monomer residues, a carbon in the central unit has the possibility of having one of ten different chemical shifts, depending on the configurations of the adjacent pair of units on either side of the central unit. Eight of these chemical shifts can be resolved for the carbonyl carbons in poly-(methyl methacrylate). The other carbons in this polymer, however, are significantly affected only by the nearest neighbor unit and thus only give information on the distribution of triads.

Factors which affect the line widths

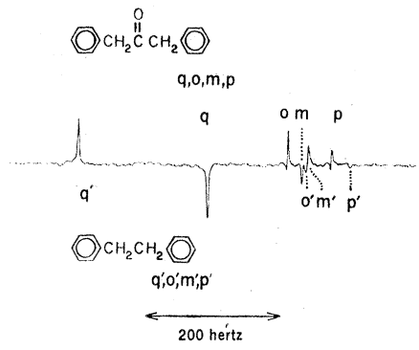


Fig. 8. Aromatic region of the Fourier transform ^{13}C NMR spectrum at 25.1 Mhz. The spectrum was obtained during the photolysis of dibenzyl ketone in $CDCl_3$. The alternating polarization in the ring carbons of both the parent ketone and dibenzyl ketone reflects the alternating spin densities in the dibenzyl radical (*56*). [Courtesy of R. Kaptein]

and spin-lattice relaxation times in polymer samples have been investigated and interpreted in terms of segmental motions and entanglements in the polymer chains (*51*). Relaxation effects in polymers can be more complex than in ordinary molecules because the tumbling rate is not necessarily much higher than the resonance frequency, and the "extreme narrowing" approximation may no longer be valid (*51, 52*).

Solids

In certain solids, particularly where there is some internal motion (as in crystalline benzene at low temperatures) ^{13}C NMR spectra have been obtained by an ingenious method (*53*) which increases the sensitivity over the normal FT procedure by several orders of magnitude. The method makes use of the magnetization of abundant nuclei, such as protons, to observe the resonance of rare nuclei, such as ^{13}C (unfortunately, the technique is not applicable to liquids). In polycrystalline solids, the resonance of a single kind of carbon (as in benzene) is very broad (typically 100 ppm) because of chemical shift anisotropy, that is, the carbon chemical shift is dependent on the orientation of the benzene molecule with respect to the magnetic field (in solution rapid molecular tumbling causes an averaging effect and only a single sharp line is observed). The anisotropy parameters obtained from ^{13}C NMR in solids are extremely useful, as they give a deeper understanding of the normal (isotropic) chemical shifts. Because of the broadness of the

lines, it appears that solid state ^{13}C NMR will be limited to molecules containing only a few different kinds of carbons, unless ^{13}C enrichment is used.

Reaction Mechanisms

Reactions which take place with rearrangements can easily be studied by ^{13}C NMR by appropriate ^{13}C-labeling of the starting compound (*1*, *2*). Labeling with deuterium is also very useful, and in this case natural abundance ^{13}C NMR can be used (*54*). In studies of chemically induced dynamic nuclear polarization ^{13}C NMR gives information which is sometimes not available from ^{1}H NMR (*55*). For example, the ^{13}C magnetic resonance spectrum of dibenzyl ketone obtained during photolysis (Fig. 8) is very clear and can be interpreted as follows (*56*). The triplet state of the molecule, which is formed by intersystem crossing from the excited singlet state, decomposes to give a radical pair ($PhCH_2\cdot$ plus $PhCH_2CO\cdot$). The escape rate of the components from this radical pair is dependent on the nuclear spin states, and thus recombination of the radical pair reforms the parent ketone in a state in which the nuclear spins are polarized. The presence of both emission and absorption lines in the ^{13}C NMR spectrum reflects the alternating spin densities in the benzyl radical.

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