PROGRESS IN BIOPHYSICS

AND MOLECULAR BIOLOGY

28

Editors

J. A. V. BUTLER

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NUCLEAR MAGNETIC RESONANCE IN THE STUDY OF BIOPOLYMERS†

H. W. E. RATTLE

Biomolecular Science Laboratories, Portsmouth Polytechnic, Portsmouth, Hants, England

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† Revised September 1973.

I

NUCLEAR MAGNETIC RESONANCE IN THE STUDY OF BIOPOLYMERS†

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NUCLEAR MAGNETIC RESONANCE IN THE STUDY OF BIOPOLYMERS†

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I. INTRODUCTION

In the three years or so since the publication of the previous review under this title (Sheard and Bradbury, 1970), the flow of papers on the applications of nuclear magnetic resonance (NMR) to biological molecules and systems has increased to a flood. Techniques which at that time were only in the experimental stage, such as Fourier transform spectroscopy, are now available to any research worker with access to a sufficiently deep purse, and the large increases in resolution and signal-to-noise ratio obtained mean that NMR is becoming more and more a useful tool in the hands of the molecular biologist. The purpose of this article is to bring up to date the account of the applications of NMR to biological macromolecules and their interactions with small molecules and ions given by Sheard and Bradbury, and in addition to provide a brief introduction to the techniques which have been developed since their review. In an effort to keep the number of references within reasonable bounds, only the later papers in a series are cited where these refer to earlier work.

The discussions which follow assume a fairly minimal level of knowledge about NMR; the reader who is completely unfamiliar with the technique should refer to Sheard and Bradbury for an excellent and concise introduction, or to one of the textbooks on basic NMR which have appeared in the last few years (Becker, 1969; Bovey, 1969; Jackman and Sternhell, 1969; Emsley, Feeney and Sutcliffe, 1965; McLauchlan, 1972). In addition to the basic ideas behind NMR, the reader will find there a description of such phenomena as ring-current shifts, scalar shifts and proton relaxation enhancement, which were described and explained by Sheard and Bradbury and thus need not be covered in the techniques section of this review. For those who would like to be refreshed on the basic observable phenomena of NMR we may state:

1. The spectrum consists of a number of peaks, each having an area which is proportional to the number of nuclei which are giving rise to that peak.

2. The position of the peak in a spectrum, known as its chemical shift and measured relative to the peak of a reference compound, is usually stated as a fraction (parts per million, or ppm) of the nominal resonance frequency. Chemical shifts measured in this way are independent of the resonance frequency. The chemical shift of a proton or other nucleus is a function both of the nature of the chemical group of which it is part and of the proximity of other groups or atoms which modify the local magnetic field. Important examples of the latter are aromatic and other ring structures, which give rise to ring-current shifts, and ions

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† Abbreviations used in this chapter: NMR, nuclear magnetic resonance; PMR, proton magnetic resonance; PRE, proton relaxation enhancement; D_2O, deuterium oxide; Hb, hemoglobin; ORD, optical rotatory dispersion.

belonging to the lanthanide series, which give rise to the lanthanide shifts covered in a later section.

3. The width at half-height of a peak is a measure of the transverse relaxation time T_2 (see section on Fourier transform spectroscopy). In general we may state that the broad peaks are usually an indication of slow molecular motion. Broadening may also be brought about by a shortening of T_2 through the presence of paramagnetic ions (paramagnetic relaxation enhancement). Both mechanisms provide analytical probes.

4. In the spectra of small molecules most peaks are seen to have a multiplet structure. The precise nature of this structure, which depends on the number and spatial arrangement of other magnetic nuclei within a few chemical bonds of the resonating nuclei, can be the source of much valuable information; more often than not, however, the spectra of large biological molecules are so broadened by the slow motion and many differing chemical environments of the molecule that all information from these spin–spin splittings is lost.

In the sections that follow, emphasis will be laid on physical concepts at the cost of some loss in rigour. The references cited will, of course, give a much more complete treatment for those who require it.

II. NEW TECHNIQUES

A. Fourier Transform NMR

1. Basic Concepts

One of the major obstacles to the application of NMR in biology has been lack of sensitivity. Typically, a single-scan proton spectrum on a continuous-wave spectrometer requires a sample concentration of at least 0.01 M, which for a protein of, say, 10,000 daltons means a 10% solution; even the use of a CAT to store and average repeated scans of the spectrum only offers a useful improvement of, perhaps, a factor of 10. For biological macromolecules, which often show a marked tendency to aggregate, even 0.001 M is a very high concentration. Further, the use of a CAT is very time-consuming; times of several hours to obtain a single spectrum are quite common. The root cause for the lack of sensitivity of NMR lies in the small separation of the energy levels of the nuclei, but the problem is compounded by the serial way in which the information is collected. Given a line, say 2 Hz wide in a total spectral width of 2 kHz, we see that data on that peak is only being collected for 0.01% of the time! Fourier transform spectroscopy offers a means of collecting data on all the peaks in a spectrum simultaneously, with a consequent enormous saving in time. This saving means that many more spectra may be collected and averaged in a given time, so that a very much higher signal-to-noise ratio may be achieved. The rapid data collection also means that kinetic studies using NMR are now feasible (Sykes and Scott, 1972). An excellent and very readable introduction to Fourier transform and other pulse methods will be found in Farrar and Becker (1971).

To understand the Fourier transform method it is necessary to understand, or at least accept, the following:

1. Any complex waveform, however complicated, can be analysed into a number of simple harmonic (sine or cosine) waves of different frequencies; this process is known as Fourier analysis.

2. Since the original waveform usually describes the variation of some quantity with time, it is called the time domain function. The *spectrum* of the frequencies into which the waveform is analysed is known as the frequency domain function.

3. Given one of the functions, it is possible to produce the other mathematically; the process is known as Fourier transformation, and the two functions are the Fourier transforms of each other.

A "normal" NMR spectrum, being a plot of the frequencies of absorbance of the various nuclei in the sample, is a frequency domain function. If all these frequencies are excited together, the resultant complex waveform, which is the sum of all the resonant frequencies of the spectrum beating together, is a time domain function which is the Fourier transform of the "normal" spectrum. Thus by causing all the nuclei to resonate together and performing a Fourier transformation on the resultant waveform, a NMR spectrum may be produced in a fraction of the time needed for the excitation and detection of each frequency individually.

2. *Nuclear Magnetization and Free Induction Decay*

Consider first a particular species of nuclei in a sample, all of them precessing at the same frequency (the Larmor frequency) due to an applied magnetic field H_0 (conventionally in the z direction). Under equilibrium conditions, we may say: (1) that the number of nuclear spins aligned with H_0 is somewhat greater than in the opposite direction; there is thus a net magnetization (conventionally M_z) in the direction of H_0, and (2) the nuclear spins will precess independently of each other, so that the x and y components of their magnetization are distributed in the xy plane and average to zero. This condition may be expressed as $M_{xy} = 0$ (Fig. 1(a)).

The application of a radiofrequency field to the spins has various effects, depending on the strength of the field and the length of time for which it is applied. These effects may be summarized as follows:

1. A disturbance of the populations of the two states whose sum gives rise to M_z; this appears as a reduction of M_z or even a reversal of its direction from the $+z$ to the $-z$ direction. In the absence of further disturbances, M_z returns to its equilibrium value exponentially with a characteristic time T_1, known as the longitudinal relaxation time.

2. The nuclear magnets are made to precess in phase with one another; their x and y components no longer cancel, but rather add, so that a net magnetization appears in the xy plane. It is the oscillating field produced by this magnetization (which rotates around the z axis with the Larmor frequency) which is usually detected in NMR spectrometers by means of a small coil perpendicular to the x or y axis. In time, the spins lose the phase coherence which was imposed upon them, and the M_{xy} signal decays exponentially with a characteristic time T_2, the transverse relaxation time.

According to the precise way in which the radiofrequency (rf) radiation is applied, almost any combination of M_z and M_{xy} may be produced. A strong, continuous rf field produces the condition shown in Fig. 1 (b) where M_z and M_{xy} are zero; this is the condition known as saturation, and its occurrence prevents the detection of any NMR signal. More generally useful effects are produced by a strong rf field which is applied only for a very short time—a pulse. The effects produced by a pulse of rf depend on its strength and duration, but in general, applied to a system initially at equilibrium, it produces a reduction in M_z and an increase in M_{xy}. Another way of looking at this is that it tilts the original M_z magnetization out of the z direction (Fig. 1 (c)). Special cases of this, which we shall mention again, are the 90° pulse, in which the magnetization is tipped into the xy plane (Fig. 1 (d)) and the 180° pulse (Fig. 1 (e)).

Suppose now that we have applied a 90° pulse to a group of nuclei in a sample. The radio-
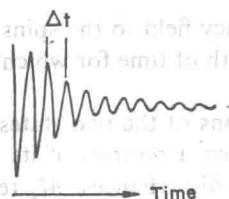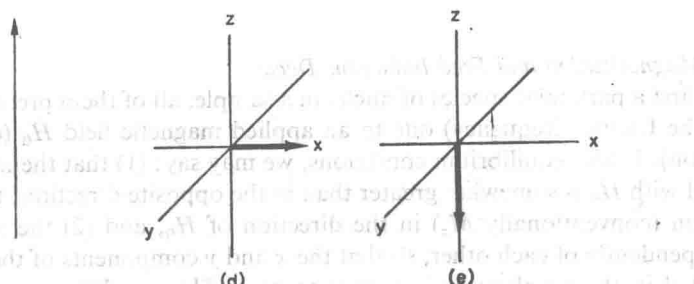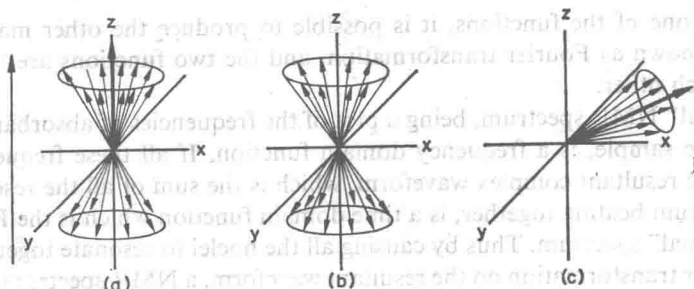
FIG. 1. The effects of radiofrequency (rf) irradiation on a group of spins as seen in a frame of reference rotating at the Larmor frequency. (a) Spins in equilibrium ($M_{xy} = 0$) under conditions of no irradiation. (b) Saturation condition ($M_z = 0$, $M_{xy} = 0$) produced by high continuous rf. (c) M tilted away from the z axis by a pulse of rf. (d) 90° pulse. (e) 180° pulse. (f) Free induction decay for single nucleus. $(\Delta t)^{-1}$ gives the frequency of resonance relative to the main frequency of the pulse. (g) Free induction decay of a complex molecule.

frequency which is being pulsed will not normally be exactly the same as the Larmor frequency of the nuclei, but because the pulse is short, it will contain Fourier component frequencies over quite a wide range, and these frequencies will include the Larmor frequency of the nuclei. A detector coil placed on the x or y axis will, after the end of the pulse, pick up the radiation due to the movement of M_{xy} about the z axis. If a single group of nuclei is involved, the signal produced, which is known as the free induction decay, or FID, will be a sine wave which has a frequency equal to the difference $\Delta\nu$ between the main rf frequency (which is used in the detector circuit) and the Larmor frequency of the nuclei, and which decays at a rate determined by the T_2 of that group of nuclei (Fig. 1 (f)). If more than one group of nuclei is involved, then the resultant pattern (Fig. 1 (g)) will be that produced by adding the FIDs of all the groups. This overall FID is the time-domain function which is then transformed by the computer into the normal NMR spectrum. Typically, it takes anything from 0.1 sec to about 10 sec to record the FID for a proton spectrum; thus many FIDs may be recorded and averaged in quite a short time to improve the signal-to-noise ratio of the spectrum. Herein lies the great advantage of the FT spectrometer over the conventional spectrometer; a single spectrum obtained by recording a FID, transforming it and reading out the result will take almost as long as the recording of a single conventional spectrum, but when several hundred or even several thousand spectra have to be averaged, and each one takes only about 1% of the time on a FT machine as on a conventional machine, the saving in time becomes enormous.

3. The Spectrometer

The requirements for a FT spectrometer are, in general, very similar to those of a conventional one, with the addition of the necessary radiofrequency switches and pulse programmer to produce the pulse. It is of great importance that the radiofrequency amplifiers are powerful enough to give a 90° pulse of short duration; long pulses tend to produce some distortions in the spectrum. It is also a great advantage for the magnetic field locking to be heteronuclear, so that the radiofrequencies used for locking purposes are different from those used for spectra. Most current FT spectrometers have magnets locked on the resonance signal of deuterium. An additional radiofrequency channel will also be needed for decoupling experiments, either homonuclear or heteronuclear.

The main irradiation frequency is normally placed at one end of the spectral range to be measured; this avoids difficulties due to "folding back", a phenomenon which appears if the irradiation is within the spectral range and which is due to the fact that the transformation programme cannot distinguish between positive and negative frequency differences (see Farrar and Becker, 1971).

4. Data Acquisition and Processing

The precision of the Fourier transformation depends on the rate at which the data from the FID is collected and the time over which it is acquired. The general requirements are that the rate of data acquisition for a spectrum covering a total of Δ Hz should be at least 2Δ points per second, while for a resolution of R Hz in the final spectrum, data must be collected for at least $1/R$ sec. Clearly the size of computer required is $2\Delta/R$ words, although the use of filters may reduce this requirement somewhat. Particularly in spectra for which R is small (narrow lines), which implies a long T_2 and hence a slowly decaying FID, the available size of computer may require the cutting off (truncating) of the FID before the decay is complete. Transformation of a truncated FID gives a distorted spectrum; this may

be overcome to some extent by multiplying the cut-off end of the FID by some function which slopes more gently to zero. This process is known as *apodization*. The FID may be manipulated in other ways before transformation; chief among these is the use of the exponential multiplier. If the FID is multiplied by a function which decays exponentially, thus increasing the apparent rate of decay of the FID, the result is an improvement in signal-to-noise ratio at the cost of some increase in effective linewidth. Conversely, multiplication by a function which reduces the apparent decay rate gives an artificial narrowing of the lines and increase in resolution, at a cost in signal-to-noise ratio (Ernst *et al.*, 1967).

After transformation, both the absorption and dispersion mode spectra are available. Usually these are mixed somewhat, a situation similar to that when a conventional spectrometer has its phase control badly adjusted, except that the phase error may vary linearly through the spectrum. Phase correction may be applied to produce the pure absorption spectrum. The computers supplied with FT spectrometers usually require only single commands to perform such processes as exponential multiplication, apodization, Fourier transformation, phase correction, baseline correction, selection of data for recording, integration, and so forth.

5. *Measurement of T_1 and T_2*

The methods for measurement of the longitudinal and transverse relaxation times by pulse methods do not lend themselves to brief and simple explanation, but an indication of how they may be performed using a pulse spectrometer may be found useful.

T_1 may be measured by applying a series of pulse sequences of the type 180°, τ, 90°, where τ is a small time which is varied from one pulse sequence to the next. The first, 180°, pulse inverts M_z, which then proceeds to decay back towards its original value. After a time τ the magnetization will have reached a value M which may be measured by applying a 90° pulse, thus tipping it into the xy plane where it may be detected. M will vary, as τ is increased, from a negative value, through zero, to its original positive value, and it may be shown that the value of τ for which M passes through zero is given by $\tau = 0.69\, T_1$.

Given a Fourier transform facility, the 180-τ-90 method may be extended to give measurements of the T_1 for the individual resonances in the spectrum. A series of FIDs, corresponding to different values of τ, are accumulated and transformed. In the resulting "partially relaxed" spectra, the size of each individual peak is a function of its own T_1 value. Figure 2 shows a series of such partially relaxed spectra obtained by Glushko, Lawson and Gurd (1972) for the ^{13}C resonances of ribonuclease A. Clearly the different peaks pass through zero intensity at different delay times τ; as with the ordinary pulse method, the time at which any peak passes through zero is given by $\tau = 0.69\, T_1$. Given access to spectrometers of different frequencies, it is possible, using these techniques, to detect frequency dependence of T_1. Analysis of this frequency dependence may then give quite important information on the motion and separation of relaxing groups in the molecules (Coates *et al.*, 1973).

T_2 may be measured by "spin-echo" experiment, which may take one of several forms. Basically a 90° pulse, which tips the magnetization into the xy plane, is followed by a decay of M_{xy} (a) by loss of phase coherence of the nuclei through transverse relaxation and (b) by loss of phase coherence through magnetic field inhomogeneities. At a time τ after the 90° pulse, a 180° pulse (or a series of 180° pulses) is applied. The change of 180° means that the spins which were originally dephasing are now regaining their phase coherence, and when they come together again a signal (a spin-echo) is detected. But only the systematic dephasing (b) is reversible by this process; true transverse relaxation (a), which is a random
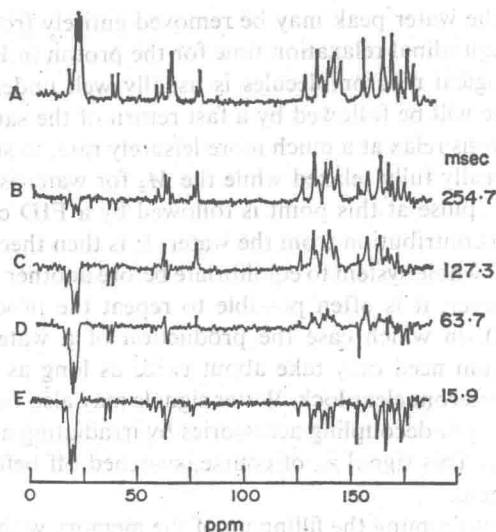
FIG. 2. Set of partially relaxed natural abundance ^{13}C NMR spectra of RNase A, pH 2.12, 15 mM. The delay times τ in the 180°, τ, 90° pulse sequence are shown by the spectra. The normal spectrum is shown as A (Glushko, Lawson and Gurd, 1972).

process, is not reversed and so the 'echo' is smaller than the original signal. From the variation of echo height with time τ, T_2 may be calculated.

More sophisticated pulse methods, which enable measurements to be made selectively on peaks within a spectrum, are described by Farrar and Becker (1971).

A way of 'cleaning up' some biological NMR spectra by using the differences in T_2 between different resonances in the spectrum has been described by Seiter et al. (1972). A delay time $\Delta\tau$ is introduced between the end of the 90° pulse and the start of data collection in an otherwise normal Fourier transform experiment. Each individual component of the FID will have decayed to exp $(-\Delta\tau/T_2)$ of its original value by the time data collection starts. Thus each resonance in the transformed spectrum is weighted by this factor, and very broad (short T_2) resonances are effectively filtered out. The method is applicable in very rigid systems such as membranes, although it can lead to problems with phase corrections and baseline distortion.

6. Water Elimination (WEFT) and Block Averaging

One of the major problems which has always faced the investigator of the proton magnetic resonance of biological molecules is the fact that water, which is the 'natural' solvent for such molecules, gives rise to an enormous resonance which usually swamps the spectrum. The use of D_2O as a solvent alleviates the problem and also simplifies the spectrum by the removal of signals from exchangeable protons, which is desirable provided they are not the peaks of interest! However, the advent of FT adds a new dimension to the problem; even in a well-deuterated sample, the HDO peak usually dominates in terms of height if not in total area, and since the whole height of the FID must be recorded, this water component, arising from the residual HDO molecules in the sample, uses up the available computer space and reduces the number of scans possible as well as cutting down the efficiency of digitization of the FID of the sample resonances.

In some cases at least the water peak may be removed entirely from the spectrum (Patt and Sykes, 1972). The longitudinal relaxation time for the proton in HDO varies from 5 to 15 sec, while T_1 for biological macromolecules is usually well under a second. Thus the application of a 180° pulse will be followed by a fast return of the sample spins to equilibrium, while the water protons relax at a much more leisurely rate, to such an extent that the sample protons are practically fully relaxed while the M_z for water is still passing through zero. Application of a 90° pulse at this point is followed by a FID coming only from the sample protons, with zero contribution from the water. It is then theoretically necessary to wait for a long time for the whole system to equilibrate before another pulse sequence can be applied; in practice, however, it is often possible to repeat the process after a relatively short time ($\sim 5T_1$ (sample)), in which case the production of a water-eliminated Fourier transform (WEFT) spectrum need only take about twice as long as normal. The process requires a magnet with a heteronuclear lock. Water signals may also be reduced in size if the spectrometer is fitted with spin-decoupling accessories by irradiating at the HDO frequency with a saturating intensity. This signal is, of course, switched off before the 90° pulse and data accumulation can begin.

There are two ways of overcoming the filling up of the memory with a FID which mainly comes from solvents which do not involve manipulation of the solvent peak itself. The more common is known as 'block averaging' and is performed by accumulating FIDs until the memory is nearly full, transforming the result and transferring it to another section of the memory. Although the solvent peak still dominates the transformed spectrum, it no longer matters that the top of it overflows the memory. The whole process is then repeated as often as necessary, accumulating transformed spectra in the same way as a CAT accumulates normal continuous-wave spectra. The procedure may be performed manually (a very tedious business), or may be programmed into the computer for automatic block averaging. A disadvantage of this method is that it requires the conditions of Fourier transformation and particularly the exponential multiplier, to be set up before accumulation starts, and cannot be used for any techniques involving manipulation of the FID. This may be overcome by using a method of accumulation currently being developed by Dr. G. E. Chapman, in which the FID is accumulated as before until the memory is nearly full, then converted into a double-precision form (i.e. using twice as many bits of store per spectral point) and storing in the double-precision form. In this way a very large number of scans may be accumulated without fear of over-filling the memory. After accumulation the FID is divided down until it again fits single-precision locations and is then transformed in the usual way.

B. ^{13}C Magnetic Resonance Spectroscopy

1. Introduction

The carbon isotope ^{13}C has a magnetogyric ratio about $\frac{1}{4}$ of that of a proton. This means that its resonance frequency in a given magnetic field is also $\frac{1}{4}$ of that for protons, and further leads to the conclusion that its sensitivity to NMR detection is only about 1.6% [$(\frac{1}{4})^3$] of that of protons. Since ^{13}C occurs naturally only to the extent of about 1.1% of all carbon, it is clear that an NMR experiment entailing observation of natural abundance ^{13}C resonance starts off with about 0.01% of the available signal strength of a proton spectrum of the same sample. Until the advent of Fourier transform spectroscopy, experiments involving such low sensitivities were clearly not a matter of routine; even now, quite high concentrations of sample and large bore sample tubes are required.

Against these difficulties may be placed the vast potential of ^{13}C spectroscopy. The chemical shift range for ^{13}C is much greater than that for protons (about 350 ppm) and the shift values are very sensitive to the electronic environment. Coupling constants with protons directly bound to the carbons are in a range $\sim$ 120–250 Hz. Spin coupling between ^{13}C atoms does not appear in natural abundance spectra because the chance of two ^{13}C atoms being direct neighbours is only about one in 10^4; information is thereby lost, but at the same time the whole spectrum is made very much simpler, a particular advantage in large molecules where many of the problems associated with peak overlap in proton spectra would be much less in the absence of such peak splittings. A useful introduction to ^{13}C spectroscopy is provided by Levy and Nelson (1972) and by Stothers (1972) and Pregosin and Randall (1971). Other references are given in these works.

2. Proton Decoupling

Each carbon in an organic molecule is spin coupled not only to directly bound hydrogen atoms but also to hydrogen atoms up to four bonds distant. The resultant complexity of the spectrum, with its associated apparent loss in signal-to-noise ratio, is generally undesirable, and so it is normal to decouple all the hydrogen nuclei in the sample by irradiating with a range of frequencies which cover the whole proton resonance region. Such 'broad band decoupling' entails the use of quite high rf power levels, and the sample temperature may be raised unless it is controlled by a cooling flow of air past the sample.

A further advantage of proton decoupling is provided by a phenomenon known as the nuclear Overhauser effect. The continual excitation of transitions in the protons which are coupled to the carbon atoms being observed causes a larger difference between the populations of the upper and lower energy states of the carbon atoms than in an undisturbed system; the result is that more carbon transitions are observable. The full value of the nuclear Overhauser enhancement is just under 3; in other words the integrated area of proton-decoupled ^{13}C peaks is about three times that of undecoupled peaks.

Proton decoupling at a single frequency is of considerable value in assigning ^{13}C spectra. It may be used in two different ways:

(a) Selective proton decoupling. This is equivalent to the conventional spin decoupling experiment; a low rf power is used to irradiate the sample at the proton resonance frequency of a given group of protons, and the signals in the ^{13}C spectrum corresponding to carbons bonded to those protons lose their multiplicity. Thus if the assignment of the proton resonance is known, the ^{13}C resonances may be assigned.

(b) Off-resonance proton decoupling. In this type of decoupling a high rf power is applied at a single frequency some 500–1000 Hz away from the protons to be irradiated. The effect on the ^{13}C spectrum is to remove splittings due to long-range coupling but to leave behind a residual coupling between the ^{13}C nuclei and protons bound directly to them. Thus the multiplicity of a ^{13}C resonance immediately identifies whether the carbon is bound to one, two or three protons (doublet, triplet and quartet resonances respectively) and paves the way for the first stage of peak assignment.

3. Peak Area Measurement

Two factors make the measurement of peak areas in ^{13}C spectra difficult; the first is the fact that for small molecules in particular, the nuclear Overhauser enhancement may not reach its full value for all carbons. The second effect, which is perhaps of more importance for the larger molecules of concern here, is due to the long longitudinal relaxation time (T_1)

often found for ^{13}C nuclei. It takes $\sim 5T_1$ for 99% of the original M_z to be restored; FT experiments with pulse repetition rates faster than this will find some at least of the nuclei still not fully relaxed. The only way to overcome this is to allow a long waiting time between pulses, which of course destroys much of the advantage of the FT method. For the above reasons, integrated peak areas are often not used in routine ^{13}C spectroscopy.

4. Chemical Shifts for Biopolymers

Table 1 gives the ^{13}C chemical shift values for amino acids (Horsley, Sternlicht and Cohen, 1970) together with the ranges of chemical shift changes to be expected from incorporation of these amino acids as N and C terminal residues (Christl and Roberts, 1972). Non-terminal amino acids in proteins at neutral pH would be expected to have much the same shifts as the free amino acids. Also shown on Table 1 are the relative chemical shifts of various compounds commonly used as internal references. Data on shifts for other molecules are available (e.g. Johnson and Jankowski, 1972, and references therein).

5. Sample Requirements

For a spectrometer employing 10–12 mm tubes, sample concentrations of the order of 0.5–1 M are required for a normal spectrum at natural abundance; about 1.5 ml of solution would be required, although the use of a microcell can reduce this to about 0.3 ml. A useful spectrum of such a sample could be obtained, using Fourier transform techniques, in about a minute. The practical lower limit of concentration is about 0.01 M; the spectrum of such a sample would take about 12 hr to obtain. Even 0.01 M is a considerable concentration—it corresponds, for example, to about 100 mg/ml for a histone sample or 130 mg/ml for cytochrome C! Clearly the field for ^{13}C-enriched and ^{13}C-labelled biological molecules is very inviting. Saunders and Offord (1972), for example, have attached a ^{13}C-glycine to insulin molecules as a probe. The phenylalanine B-1 residue was removed and replaced by the labelled glycine, whose chemical shift changes with pH could then be readily followed.

6. Some Applications of ^{13}C Spectroscopy

^{13}C resonance, mostly at natural abundance, is already being applied to a wide range of biopolymers. At this early stage, of course, much of the effort has still to be expended on assignment of resonances and assessment of the magnitude of spectral changes brought about by conformational variation in the molecular structure. Various peptides have been studied by several groups (Christl and Roberts, 1972; Gurd et al., 1971, and references therein) and the shifts resulting from neighbour effects and pH changes determined. Paolillo et al. (1972a) have shown a 3 ppm upfield shift for the αC resonance, with a 2 ppm shift for the carbonyl carbon, on formation of α-helical structure in poly-γ-benzyl-L-glutamate, while similar studies have been reported for poly-(Nδ-carbobenzoxy-L-ornithine) by Boccalon, Verdini and Giacometti (1972). The helix-coil transition of poly-L-glutamic acid as a function of pH has been studied by Lyerla et al. (1973) and also showed an α-C shift of about 3 ppm. Assignments of some peaks in the natural abundance ^{13}C spectrum of RNAse peptides and lysozyme have been made by Freedman et al. (1973) who also followed the changes in the spectrum on titration of these proteins. Line sharpening on denaturation of RNAse A, coupled with T_1 measurements for the various resonances from partially relaxed spectra, has been shown by Glushko, Lawson and Gurd (1972).

The natural abundance ^{13}C spectrum of lysozyme gives 28 single-carbon peaks in the aromatic region. Many of these have been assigned (Allerhand, Childres and Oldfield, 1973)