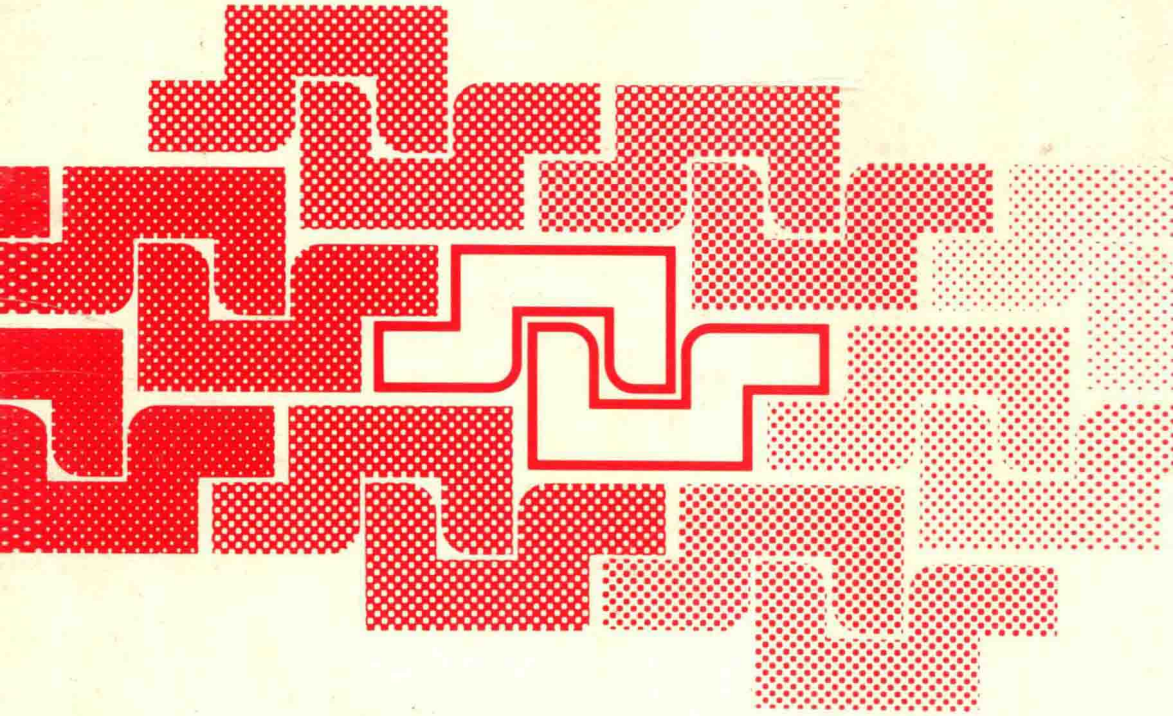

NMR in the Life Sciences

Edited by

E. Morton Bradbury and

Claudio Nicolini

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Series A: Life Sciences

PREFACE

This NATO Double Jump Program, held at Erice, Italy, on NMR in the Life Sciences was supported in part by contributions from Oxford Research Systems, Philips International, Technicare Corporation, Varian Instruments, Sciemens Medical, and ESA Control. This program brought together three major research activities in biomedical applications of NMR: high resolution NMR studies of proteins and nucleic acids, in vivo studies of animals, and NMR imaging. Whereas in the development of in vivo NMR and NMR imaging the major technological advances came initially from high resolution NMR spectroscopy, this is no longer the situation. The importance of in vivo NMR and NMR imaging in biomedical science and medical diagnosis has resulted in an explosion of growth in these areas involving schools of medicine, hospitals and instrument manufacturers. Major advances in NMR technology now come from biomedical applications of NMR as well as from high resolution NMR.

Applications of high resolution NMR to the solutions structures of proteins and nucleic acids have been revolutionized by the development of two dimensional NMR Fourier transform techniques and the techniques of biotechnology. Now it is possible, with small proteins up to 10,000-12,000 daltons, by 2D FT NMR techniques to follow the path of the polypeptide backbone through the molecule. The combination of 2D FT NMR techniques with genetically engineered proteins provides one of the most powerful approaches to understanding the principles of protein folding, protein stucture and enzyme catalysis. By site-directed mutagenesis single amino acids in a known protein sequence can be replaced by other amino acids and the effects of those changes assessed on the structure and function of the protein. Major applications of high resolution NMR techniques have been to study the dynamics of proteins and the interactions of ligands with proteins. These have considerably extended our understanding of protein behaviors based on the largely static structures from X-ray crystallography. Major advances have been made in our understanding of the structures and dynamics of nucleic acids in solution by the development of oligonucleotide synthetic techniques. Now it is possible to synthesize oligonucleotides with known sequences and specific substitutions for 2D FT NMR studies of their conformations and conformational behaviors. Strategies have been developed for the assignments of resonances in both small proteins and oligonucleotides. Major questions concerning the functions of nucleic acids which involve the effects of sequence on structure and how specific sequences are recognized by regulatory proteins can now be addressed by the techniques of 2D FT NMR and biotechnology.

In vivo NMR studies of cells in suspension and of organs and tissues are an extension of high resolution NMR techniques but applied to more complex systems. NMR signals derive from the more abundant small molecule metabolites. Determinations of the relative levels of these metabolities provide information on normal and abnormal states of tissues and organs.

Technological questions are concerned with the precise location and spatial resolution of the region in the animal which gives rise to the in vivo spectrum. A major goal is to define the metabolite "profile" of any organ in an animal. In vivo ^{31}P NMR has been widely used for metabolic studies in a wide range of cells and tissues and organs in small animals. Although very useful, these are restricted by relatively small numbers of phosphate metabolites which can be identified. Major efforts are now being made to extend in vivo NMR by using ^{13}C and ^{19}F labelled molecules to answer specific questions concerning metabolic pathways.

NMR imaging is now established as a major technique in medicine for the imaging of soft tissues in normal and diseased states. Major objectives are the development of more inexpensive imagers at lower magnetic fields, the development of real time sequences of images of the beating heart and of blood flow and the coupling of NMR imaging with in vivo NMR so that the regions giving rise to the in vivo spectra can be identified in the NMR image. Major steps have been taken in reaching these objectives.

Many of the objectives outlined above were discussed in detail at the NATO Double Jump Program on NMR in the Life Sciences.

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CONTENTS

SPECIAL PROBLEMS OF NMR IN H_2O SOLUTION

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INTRODUCTION

Most biochemical NMR is performed in aqueous solutions, most often in D_2O containing typically 1% proton impurity as HDO, but also quite often in 80-100% H_2O. The latter is necessary if you want to observe exchangeable protons in a macromolecule, or if for some reason replacement of H_2O by D_2O is impractical. The large narrow HDO or H_2O signal, equivalent to up to 100 molar proton concentration, presents various problems which will be outlined here, together with partial solutions.

We have already published several articles on this and related topics,[1,2,3] and in the present article we will avoid repeating the contents of these papers as far as possible. Furthermore, the emphasis will be on techniques that we have used in our own laboratory.

The problem is often presented in terms of the ratios of H_2O molarity to typical solute molarity. This is incorrect since solute signals are often masked by thermal noise in a single free induction decay (FID). The more appropriate comparison is the proton signal-to-thermal-noise amplitude ratio for a typical proton bandwidth into the digitizer of 5 kHz (or 10 ppm at 500 MHz). This ratio is around 2×10^5 for a 0.2 ml sample at 500 MHz. The digitizer must have dynamic range greater than this, but even a 16 bit digitizer has a signal-to-digitization error of the sixteenth power of two, or 6×10^4.

Furthermore, another problem arises for macromolecules of size more than about 15 kDalton in H_2O. If H_2O is saturated most of the time, either intentionally as in solvent saturation methods (see below) or because the fast repetition of pulses keeps its magnetization well below normal, this saturation can be transferred to the macromolecule and spin-diffuses through it, causing a costly loss in macromolecule NMR signal.[4] Therefore, any method that perturbs the H_2O signal requires a low pulse rate, at a rate less than T_1^{-1} for H_2O, or about once every two seconds.

ENGINEERING AND RELATED ASPECTS

Sixteen bit A/D Converters have sometimes been promoted by instrument makers as a solution to this problem and a necessity for work with H_2O, as compared to 12 bit converters. However, it is clear from the last two

paragraphs that this is not a complete solution to the problem. There is no question that this and other engineering improvements that will improve the performance of instruments should be encouraged. However, improving linearity and dynamic range of the entire amplifier system to take advantage of the 16 bit A/D may be difficult, and the improvement in actual performance should be evaluated as carefully as possible. Probably there is some advantage to having a 16 bit converter, but not as much as the expected 16-fold improvement over a 12 bit system.

Analog filtering of the signal before A/D conversion to reduce the H_2O signal before conversion is almost always desirable. We have described one carefully thought-out methodology for doing this including computer correction for filter amplitude, as well as phase, varation.[1,2] While first-order phase rotation is generally adequate for this correction, amplitude correction is better because NMR lines close to the filter cut-off can be displayed undistorted. The effect of an analog filter (unlike that of semiselective pulses, described later) can be fully corrected by subsequent data processing. The only disadvantage is loss of dynamic range for peaks close to the cutoff. For selective pulses that discriminate against zero frequency (i.e. the J-R pulse, described later), a dual high pass filter could be used, but this has not been done as far as we know.

Radio and low frequency amplifiers must be arranged with flexibility. Generally the noise level of an amplifier should be about 1/10 the level of the amplified input noise in order to contribute negligible noise power (<1%) while retaining high signal capability. We believe it advantageous to convert a low center frequency (100 kHz in our case) before final (quadrature) conversion to audio frequency because, though more complicated, this allows us to use highly precise analog FET switch conversion in the last stage, to reduce nonlinearity and consequent intermodulation signals (at sums and differences of strong lines). We use triple conversion, to 110 MHz, 6.15, and 100 kHz in the receiver system, and double conversion, from 116.15 MHz, to 110 MHz, and then to 500 MHz in the receiver system. This permits excellent gating by use of multiple gating before mixing, and inexpensive phase shifting of pulses at 6.15 MHz using digital electronics. However, there are workers who believe that multiple down-conversion and low-frequency final conversion is unnecessary and could lead to spurious responses. We also have a fully-linear transmitter system after the 110 MHz level to facilitate precise control of pulse level, including shaped pulses in the future. Instead of the usual non-linear diode arrays we use a Hoult-Richards switch[5] preceeded, in the transmitter line, by a PIN diode switch of standard design (S. Kunz, unpublished), to completely eliminate noise originating in the linear output pulse amplifier.

Software methods[6] to reduce the amplitude of the stored solvent signal serve only a cosmetic purpose in our opinion, except that they do increase the dynamic range of the Fourier Transform (FT) program. However, if the FT program is written in floating point numbers, and/or attention is paid to shift out accumulated roundoff errors in its, dynamic range should not be a problem. These methods should still be useful for compression of 2D data from 32 bit to 16 bit integers, to save storage space and time. We have generalized these methods for arbitrary solvent frequency, and also remove the distortion they produce by post-FT correction (unpublished), for use with a 16-bit FT program; but with a modern (8086-8087 based) computer we no longer need this method of data compression for one-D NMR.

Zero-filling of NMR data is more than a method of interpolation of an N-point spectrum to produce a better looking 2N-point spectra. Rather, it uses the information in the imaginary part of the data set to produce a

better value of odd spectral data points (the even points are identical to the points of the N-point FT without zerofilling). However, the sharp end of a long-lasting signal like that of H_2O (See Figure 1 at time T) will produce annoying displacement of the odd points unless the stored data is apodized. We do this, after multiplying the data set by an upward or downward tiling trapezoid ($F_1(t)$ in Fig. 1) which serves to eliminate unwanted data at the end of the FID while approximating resolution

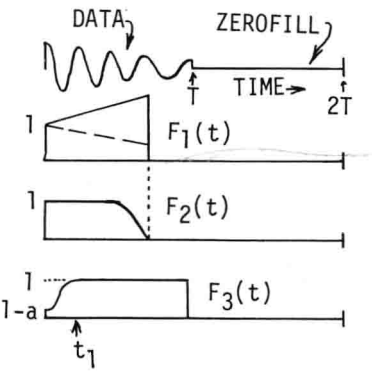

Figure 1. Zerofilled data, and weighting functions described in the text. In F_1, the slope can be varied from positive (solid) to negative, as can the cutoff time, under user control. Likewise the parameters a and t_1 can be varied. In our system F_2 is always goes to zero at the same time as F_1, and its cosine cutoff always has the same width of T/4, where T is one fourth the aquisition time.

enhancement or line broadening. The data are then multiplied by an apodization function $F_2(t)$ which effectively multiplies the remaining data by the first quarter of a cosine function. Finally the data are multiplied by a function F_3 which reduces or removes the first part of the FID. It is actually the function $F_3 = 1-a[1/2 + 1/2\ \cos(t/t_1)]^2$ for $t < \pi t_1$ and $F_2 = 1$ otherwise, where $a<1$ and often $a=1$, and πt_1 is short compared to the acquisition time. This weighting function gives an approximation to a Gausian convolution-difference spectrum. We believe that Gaussian, rather than Lorenzian (exponential, in the time domain) convolution, is desirable because in theory it produces less far-reaching sag of the baseline away from a strong line. However, Gaussian convolution is the only kind with which we have experience. We use simple cosines, and squares thereof, to speed computation of the weighting function. Actually, these three

functions are first computed, then multiplied together, and stored in memory as a single function, and the FID is routinely multiplied by this product function. This weighting is part of the initial subroutine of the FT routine which also includes shuffling of the data according to bit-reversed addresses, a two fold zerofill of the data, and the first set of "butterflies" of the FT. Incidentally, this FT routine is as fast as any we know of for a microcomputer lacking an array processor (1K complex points, 2K total, in about one sec: 8K/16K in ~10 sec). Multiplications are floating point (8087) but additions are time shared 32 bit integer (8086) and butterfly execution is ordered to group together butterflies that use the same sine/cosine values. The 8086/8087 are clocked at 8 MHz.

Details of handling data conversion timing may, or may not, be important. The first pair of complex data points are stored, in analog form, for subsequent conversion within microseconds, exactly τ_d after the end of the observation pulse, and subsequent samples were taken in intervals τ_d later, where τ_d is the dwell time. However, the first conversion pair at time τ_d is stored as the second point of the FID, while zero is stored as the first point. This is done because the analog filter would certainly put out zero signal at the zero since finite time is required for its output to respond to its input. The receiver system is also gated off for about 50 microsec after the end of the RF pulse, to block the probe tuned circuit ringing from the transmitter pulse.

The NMR tube and shimming can influence the results, and ease of obtaining them, in H_2O solvent. Samples in typical 5 mm NMR tubes having traditional volumes of 0.5 ml or more often give poor baselines because an appreciable part of the sample is in a "bad" part of the field, away from the sensitive region of the probe, but still in region where fringing radio frequency field of the probe will excite H_2O, and the probe will pick up the resulting weak signal. The obvious solution is to improve the shimming or get a larger magnet. However, it may be easier to "cheat" on the shimming if one is only interested in, or observing, either only upfield or only downfield of the water resonance: the z^2 and z^4 shims can often be displaced somewhat to give good lineshape, yet move the field above and below the sample to a lower value, for example, lower value if one is observing downfield of water, thus moving the bad baseline to the upfield side of water where one is not interested. The NMR tube can also be chosen to reduce this problem, by using a semimicro type (Wilmad 510 cp) which confines the sample to the sensitive region of the probe only, and thus eliminates the problem of poor magnet line shape. We generally use a 5 mm tube with 10 mm sample length (volume 0.2 ml). This also optimises sensitivity for a given number of mg of sample, and is a safer way to store samples than is an open 5 mm NMR tube. The disadvantage is that the glass-to-H_2O static magnetic susceptibility mismatch produces line broadening which may be as large as 5 Hz at 500 MHz. We have attempted to replace these tubes with 5 mm tubes having plastic end-filters to continue the sample but with less susceptibility mismatch, without success.

Preirradiation of lines in the proton spectrum for NOE, T_1, or exchange rate studies presents problems in H_2O because it stimulates a signal from the water which can be larger than the signal from the observation pulse. This produces baseline curvature in short runs and, more important, it requires running at reduced gain. This problem can be reduced by using a homogeneity spoil pulse, but in our 500 MHz magnet the spoil pulse applied through the shim system must be several msec long, and we have to wait ~10 msec after the spoil to let the eddy currents produced by it to die away. So we asked to the maker of our 500 MHz probes (Cryomagnetic Systems, Indianapolis) to put a spoil coil in our probe, and it works very well. It consists of many turns of fine wire wound on a bobbin on the outside of the glass dewar. This is desirable if you want to

measure short T_1's. A homogeneity spoil pulse interferes with the deuteron lock and to avoid side effects from this interference we turn off the lock system during the time from the start of the spoil pulse to the end of aquisition. During this time the lock field offset is effectively frozen in the equivalent of a track-and-hold circuit.

SOLVENT SUPPRESSION

So far we have outlined various technical strategies to permit a spectrometer to cope better with a strong solvent or other signal without degraded performance. As indicated at the beginning, these methods are doomed to be inadequate in a modern spectrometer, and we now turn to methods that seek also to drastically reduce selected strong signals such as solvent before they escape from the sample. These can be grouped into three main classes: chemical, which means primarily use of deuterated solvents and, sometimes, other buffer components; solvent saturation methods that seek to reduce the solvent proton signal, or other strong signals, by selectively destroying their magnetization just before pulse excitation; and semiselective pulses that seek not to excite water while appreciably exciting other interesting signals.

Chemical methods

These have been discussed elsewhere[3] in slight detail. There is not much to say except that the method cannot be used to observe protons that exchange with solvent in a time that is not long compared to the time needed to change solvent and do an experiment, whatever that may be. This precludes its use for most nucleic acid nitrogen protons, and for proton amide protons close to or on the protein surface.

Solvent saturation

Strategies for solvent saturation can be grouped into two categories. First are more or less continuous wave (CW) methods which use long ($>\sim$0.1 sec) monochromatic pulses to saturate water during some section of the pulse cycle, generally just after acquisition during the time needed for other spins to recover equilibrium. Doing so is usually possible on modern spectrometers, and, if the power can be suitably reduced, can be very selective and reduce water spinning sidebands as well as the main water signal. This method is most useful as a simple way to selectively eliminate the HDO signal in nominal D_2O, to eliminate signals from high concentration protonated buffer components other than H_2O, in conjunction with selective pulses to eliminate H_2O; and for $\sim$ small macromolecules (MW $<$ 15,000) where spin-diffusion from solvated H_2O will not occur rapidly enough to wipe out the spectrum. Saturation methods have the advantage over selective excitation methods (below) that they are more selective than the latter, as lines within only 10-50 Herz of solvent are directly affected; and they are simple. Disadvantages are that they are difficult to use for observation of protons that are rapidly-exchanging with solvent, or for larger macromolecules.

A related second set of pulse preparation techniques use the generally long T_1 of H_2O to discriminate and eliminate the H_2O magnetization. We have no experience with these methods; they are rather complicated to set up, are not very selective, and are not widely used.

Selective excitation

These methods can be grouped into three classes. The first of these includes CW excitation, and correlation or rapid scan NMR which is similar to CW excitation except that rapid frequency sweep excitation is used. In

both cases water is largely not excited, by simply stopping the sweep before water is reached. We have discussed these elsewhere.[3] They have largely fallen into disuse probably because they have proven difficult to implement routinely and with high sensitivity.

Soft Pulse Sequences. The remaining selective excitation methods are pulse methods. Long pulse methods[1,2,3] include the plain long (or soft) pulse first introduced long before FT NMR by S. Alexander, and the 214 pulse which is really the composite of an Alexander soft pulse and a 1-1 pulse (below) combined to give a broader region over which the H_2O resonance and its spinning sidebands are selectively unexcited. We have discussed soft pulse methods repeatedly; here we will simply point out relative virtues. The principle advantage is that when well implimented they are easier to use than hard pulse methods and may be more forgiving of some spectrometer defects. These methods do not require excellent rf field inhomogeneity. The principle disadvantages are that they cannot now be implemented on commercial spectrometers without addition of an attenuator in the transmitter line; fine phase control of parts of the pulse is very desirable for the 214 pulse; and pulse amplitude stability (including a lack of pulse-amplitude droop) is required. The latter problem can be circumvented on some spectrometers by eliminating the final amplifier, since less than one watt of power is usually required. Finally, the simple Alexander soft pulse is often forgotten but is still potentially very useful because of its simplicity. At modern high fields radiation damping might prevent a good H_2O null but this could be achieved by appending a short pulse 90° phase-shifted from the long pulse.

Common Problems. A few general comments about selective pulse methods will be inserted here. Most of these methods are unusable for observing both signals very far from solvent, and those close to solvent, in the same run; and many are not suitable for observing both signals upfield from H_2O, and downfield, in the same run. Repeated runs with different pulse parameters may then be required. However, this lack of selectivity can be an advantage if uselessly crowded sections of macromolecule spectra can be partly discriminated against, because their signals can also overload the spectrometer, and because the entire set of protons in a macromolecule relax more slowly than do a subset of the protons semiselectively excited, since in the latter case spin diffusion is more effective for the recovery of the excited spins.

The problem of amplitude and phase instability is a serious one that generally requires detailed trouble shooting. The symptom is an unacceptably large variation in solvent signal at the best null point, or a poor null point. In using these sequences it is extremely useful to have a loudspeaker to audibly output the signal and an oscilloscope x-y display to display the two outputs of the quadrature detecter. The excellence of the null is a parameter sometimes given as a figure of merit, and we have heard claims made of a few thousand fold for the reduction of the signal. The best we routinely acheive is about a 200-fold reduction, estimated by comparing the size of the null signal into the A/D computer to the size of the H_2O signal after a 90° pulse. The latter is not measured directly, but is inferred from the signal from a shorter pulse, typically a 1° pulse. A 200-fold null is adequate since it reduces the water signal to within the A/D converter range at a gain setting where the digitizer error is at least one-third the thermal noise.

All selective pulse sequences except the J-R and 1-1 sequences (below) produce serious first order phase distortion of the spectrum. This can be corrected by a first-order correction but this correction produces baseline droop, as discussed elsewhere[2], at the edges of strong lines. Convolution--difference treatment of the data helps cosmetically in some

cases, but can distort total intensities of overlapping lines. However, clever successive-approximation, computer correction of the baseline is possible without subjective human intervention.[7] Incidentally, in my opinion this problem is sometimes exaggerated. Delay of the start of data acquisition could produce reported baseline effects called "baseline roll" or "phase roll". We never observe such strong baseline effects (see above, under "timing"), only a very broad curvature due to imperfect H_2O nulling and to the wings of the major aromatic or aliphatic groups of resonances.

Our final general remark concerns the use of phase shifts as well as length adjustment in selective pulses. As described elsewhere we use both fixed and continuously variable phase shifters to get a good H_2O null.[2,3] For a variety of reasons it is desirable to shift the entire phase of selective proton pulses relative to each other, for example in spin-echo or 2D sequences, while maintaining good nulling. It is then by far most flexible and convenient to have completely separate phase shifters for these latter overall shifts. This permits, for example, the use of variable relative phase shifters within a sequence which is very convenient even if not absolutely necessary. And it avoids the necessity of having phase shifts that are precisely 90°, 180°, and 270° as would otherwise be necessary.

HARD PULSE SEQUENCES. We now discuss one class of hard composite pulses. By this we mean composites of short high-power pulses. A large number of these have been demonstrated by now, starting with time-shared versions of Alexander and 214 pulses, and including pulse sequences designed to have broad nulls, narrow nulls, or uniform excitation over a broad band (see, for example, reference 8). We have experience with only one of these which we call a modified J-R pulse, which is essentially the same as sequences called either J-R pulse[7] or 1-1 pulse[9], and limit our discussion to these sequences.

Hard pulse sequences have the advantage that they can be set up and tried (though not necessarily made to work well) on a modern instrument. They are as prone to many of the difficulties outlined above as is the 214 pulse with the possible exception of pulse amplitude droop. Based on our very limited experience, it is somewhat harder to find and optimise the null for hard pulses compared to the soft 214 pulse, and random variations in the null are greater probably because the same nanosecond random variation is bound to be more important for a short high power pulse than for a weak long pulse.

The J-R or 1-1 pulses consist of two theoretically short hard 90° pulses separated by a short time τ_p. The first pulse flips the water magnetization by 90° and the second is suitably phased and timed to return it exactly to the z-axis. This property is independent of whether the two pulses are 90° pulses, or are both some other but equal length, and this is an advantage since the null is rf field independent. More remarkable and useful is that in the case of 90° pulses there is theoretically no first order phase shift in the resulting spectrum although there is an important amplitude distortion. The first pulse puts the entire magnetization in the x-y plane; τ_p is then picked to allow spins of different frequency to spread apart appreciably in a pizza-shaped distribution in the x-y plane; and the second pulse flips the pizza exactly 90°, so that the H_2O magnetization is back along the z-axis while the other spins all have different z components but their projection is in the same direction. It is this projection which gives the relative phase of different signals and this phase is therefore constant for all sets of protons.

The size of this transverse projection gives the relative sizes of the NMR signals and this is easily shown to be proportional to $\sin(2\pi\Delta f\ \tau_p)$

where Δf is the distance in Herz of a line from solvent. Thus τ_p is set at one half the inverse of the distance in Hertz from the H_2O resonance, of the most interesting part of the spectrum. An added advantage is that the pulse sequence is probably the shortest semiselective sequence available for looking at a given set of lines; a soft pulse or 214 pulse is twice as long.

The J-R pulse[7] is a $90_x-\tau_p-90_{-x}$ sequence and water is jumped down in the first pulse, stays fixed in the rotating frame during τ_p, and returned in an exactly reverse path to the z-axis (J-R stands for "Jump and Return"). The carrier frequency is set equal to the water resonance frequency. The 1-1 pulse[9] is a $90_x-\tau_p-90_x$ sequence and the carrier is placed a distance $2/\tau_p$ in Hertz from water resonance, downfield or upfield of desired part of both water and the desired part of the spectrum. The water magnetization in the rotating frame rotates exactly 180° in the x-y plane during τ_p. The advantage of this variant is that no 180° phase shift is required and it is more straightforward to filter out the computer residual water signal using commercial spectrometers. A disadvantage is that half the storage is wasted though this can easily be fixed by software; also more power is required to see lines on both sides of water. The modified JR sequence that we use[10] is $90_x-\tau_d-90\pm y$. The carrier is placed at the center of a sensitive region of the pulse, $2/\tau_p$ Hertz upfield or downfield of water, and the water magnetization rotates one quarter turn in the x-y plane during τ_d. Whether the second pulse is 90_{+y} or 90_{-y} depends on whether the carrier frequency is up-or downfield of water. We chose this variant because we do not have much power, and it seemed better to be able to place the carrier at the center of the spectrum. We are generally only interested in the region well downfield of water.

A brief description of our experience with this JR pulse may be useful. Our 3 watt final transmitter provides 50 μsec 90° pulses but for some reason when we use the modified J-R pulse it is much easier to operate with ~35 μsec pulses and it is generally possible to get a flatter baseline after some extensive trial adjustment, with the J-R pulse than with the 214 pulse. Actually, the best J-R baseline sometimes has a linear slope, whereas the best 214 baseline is curved (Fig. 2). Dr. Richard Griffey (unpublished) has programmed our Varian XL-300 to use this modified JR pulse, and found it to work better than the JR or 1-1 pulses on that spectrometer.

The real reason we were interested in the J-R pulse was its lack of a first order phase shift, for use in 2D NMR. In H_2O solutions of larger macromolecules it is obviously highly desirable that all proton pulses be selective against water, to permit higher repetition rates as we described at the beginning. But the first-order phase shifts produced by most such pulses seemed an undesirable complication since we also wished to do pure-phase 2D. The modified J-R pulse has been used successfully by Richard Griffey in our laboratory to obtain 2D NOE (NOESY) spectra of tRNA.

Somewhat surprising was our experience with Hahn spin echoes in H_2O using 90°-180° sequences. What worked best for the 180° pulse was, inelegantly, two modified 90° JR pulses immediately after each other, as compared to a single "180° J-R" pulse in which the spacing is twice that of the composite 90° JR pulse (ie $2\tau_p$). We have not investigated why this is, or worked with other pulses such as a soft 180° pulse.

OTHER AREAS OF NMR

For completeness we mention two related developments. First, remarkable results have recently been obtained in observation of