CHAPTER 3 "APPLICATION OF ISOTOPE FILTERED NMR EXPERIMENTS FOR NUCLEIC ACIDS"

3.1 Summary

In this chapter the NMR spectral editing technique of isotope filtering is used to examine nucleic acids that have been partially isotope labeled. The application of an isotope labeled NOESY experiment on a duplex DNA that has been labeled on one strand is demonstrated and is shown to be an effective method of making assignments.

A new pulse sequence is presented that incorporates an isotope-filter with a pulse field-gradient stimulated echo sequence. This new experiment makes it possible to follow the translational self-diffusion of an isotope-labeled species in solution, independent of other solutes. An example is presented in which the diffusion constant of an isotope-labeled DNA is followed before and after binding a protein.

3.2 Introduction

The concept of the isotope filter in NMR is simple. The one-bond J-coupling between a proton and another magnetically active "X" nucleus (^{13}C or ^{15}N for example) is exploited to control the phase of the observable magnetization of the proton. Using some simple phase cycling methods, data can be collected in which only signal arising from a proton covalently attached to this "X" nucleus is observed. If a NMR sample has been synthesized in which only part of the sample is isotopically labeled, it is possible to use isotope filtering to selectively view the signal arising from either the labeled or the unlabeled portion. Some advantages of this technique include spectral simplification and reduction in assignment ambiguity.

3.2.1 Isotope selection by NMR

Otting and Wüthrich (1990) have reviewed the theoretical and practical applications of isotope-filtered techniques for NMR. The utility of these experiments has been shown for a variety of biologically interesting problems, such as in obtaining strand resolved spectra for duplex RNA (SantaLucia, *et al*., 1995; Cai & Tinoco, 1996), characterization of symmetric protein dimers (Weiss, 1990; Arrowsmith, *et al*.,1990; Folkers, *et al*., 1993; Burgering, *et al*., 1993), a protein-DNA complex (Otting, *et al*., 1990), protein-ligand binding (Fesik, 1988; Fesik *et al*., 1988), spectral simplification by specific amino acid labeling (Fesik *et al*., 1987; Torchia *et al*., 1989) and determination of RNA dimerization (Aboul-ela & Pardi, 1996; Flemming, *et al*., 1996) among others.

A quick overview of the isotope filtering technique is presented. The pulse sequence elements fundamental to isotope selection are shown below in figure 3.1.

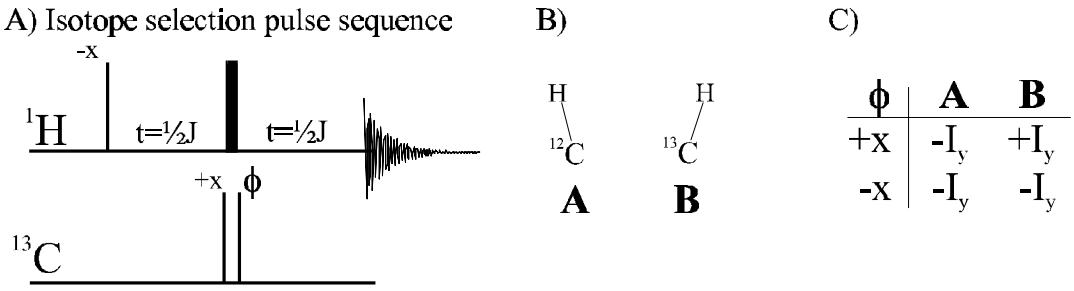


Figure 3. 1 Isotope selection schematic

To illustrate what happens in the isotope selection experiment, follow the magnetization of two protons, as shown in figure 3.1B. The first proton, **A** is attached to a ^{12}C atom, while the second, **B** is attached to a ^{13}C atom. Using the product operator formalism (Sorensen, *et al*., 1983; Harris, 1985; Howarth, *et al*., 1986; Shiver, 1992) to follow the evolution of the pulse sequence. (Chemical shift is included only for

completeness, it clearly will not affect the final proton magnetization since this is a spin-echo pulse sequence). One observes that,

$$I_z \xrightarrow{-90_x(I)} I_y$$

$$\xrightarrow{t=\frac{1}{2J}} I_y \cos\left(w/2J\right) - I_x \sin\left(w/2J\right)$$

$$\xrightarrow{180_x(I)} -I_y \cos\left(w/2J\right) - I_x \sin\left(w/2J\right)$$

$$\xrightarrow{t=\frac{1}{2J}} -\left[I_y \cos\left(w/2J\right) - I_x \sin\left(w/2J\right)\right]\cos\left(w/2J\right) - \left[I_x \cos\left(w/2J\right) + I_y \sin\left(w/2J\right)\right]\sin\left(w/2J\right)$$

$$= -I_y \cos^2\left(w/2J\right) - I_y \sin^2\left(w/2J\right) = I_y\left[\cos^2(wt) + \sin^2(wt)\right] = -I_y. \qquad 5.1$$

For proton **A** there is no 1-bond J coupling and the pulse sequence acts like a spin-echo. Notice that the chemical shift precession terms will always refocus in this type of pulse sequence.

Atom **B** is covalently attached to a ^{13}C isotope and one-bond J-coupling between the carbon and proton is present. If the phase (ϕ) of the second ^{13}C pulse is set to –x, it "cancels out" the effect of the first ^{13}C pulse with phase +x. Thus, with the phase of ϕ set to –x, both the chemical shift and ^{13}C-^{1}H J-coupling will be refocused by the spin-echo leaving the magnetization state of $-I_y$ for proton **B**, giving **B** the same phase as proton **A**.

However, if the phase of ϕ is set to +x, it works in conjunction with the first ^{13}C $\pi/2$ degree pulse to create an "effective" π pulse. With ϕ set to +x, the final magnetization of **B** will be $+I_y$, as shown below (the effects of chemical shift precession have been removed for the sake of brevity),

$$I_z \xrightarrow{-90x(I)} I_y$$

$$\xrightarrow{t=1/2J} I_y \cos(\pi Jt) - 2I_x S_z \sin(\pi Jt) = -2I_x S_z$$

$$\xrightarrow{180x(I)} -2I_x S_z \xrightarrow{180x(S)} 2I_x S_y$$

$$\xrightarrow{t=1/2J} 2I_x S_z \cos(\pi Jt) + I_y \sin(\pi Jt) = +I_y \qquad 5.2$$

The final values obtained are summarized in the table found in figure 5.1C.

Thus, the magnetization of the proton attached to the "X" labeled nucleus can be set to either $+I_y$ or $-I_y$ through the use of the phase ϕ. This can be exploited in an NMR experiment by collecting two sets of data, one in which the phase ϕ is set to +x and one in which the phase ϕ is set to –x. The simulated spectra for the **A** and **B** is shown below in figure 3.2. The ^{13}C and ^{12}C subspectra (figure 3.2 C and D) can then be constructed by respectively subtracting or adding the two original spectra.

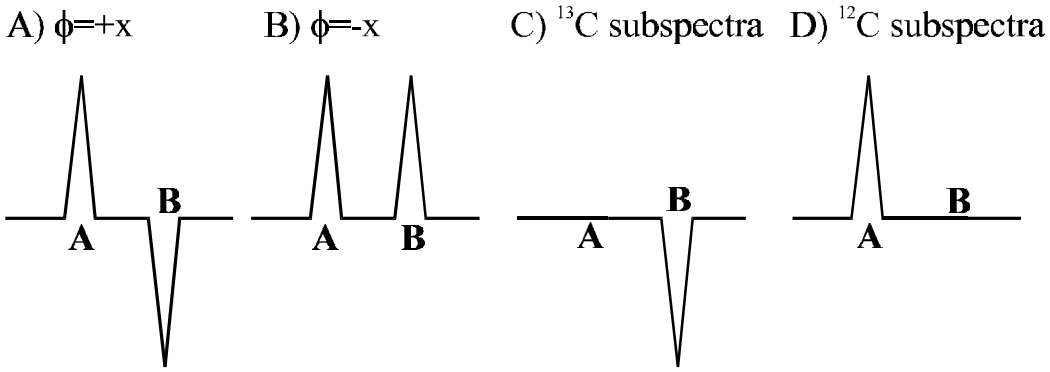


Figure 3. 2 Isotope filtered subspectra

This isotope filter pulse sequence element can be incorporated into some traditional proton NMR experiments.

3.2.2 Isotope filtered NOESY

The "nuclear Overhauser effect spectroscopy" (NOESY) experiment is of fundamental importance in elucidating molecular structure and dynamics information by NMR. The NOESY spectrum contains information on the dipolar relaxation processes occurring in the molecule, and this data can be utilized to calculate proton-proton distances (see Chapter 7). One of the major limitations of the NOESY experiment is finding well resolved cross peaks suitable for volume quantitation. The larger and more homogeneous the molecular structure, the greater this problem can be. For large DNA molecules this can be a formidable obstacle, but the use of isotope selection or filtering experiments can simplify the task. Figure 3.3 demonstrates how the concept of the "isotope subspectrum" presented before can be extended to a two dimensional experiment.

The application of ^{15}N and ^{13}C isotope-filtered NOESY NMR experiments was used for assignment of proton resonances for a DNA molecule in which one strand is uniformly isotope labeled. This procedure utilizes standard isotope-filtered NOESY techniques to assign the exchangeable imino proton spectra and to obtain strand-resolved spectra of the non-exchangeable protons for both the labeled and unlabeled halves of the DNA. Since these experiments can be performed on a single sample, they expedite the process of assigning resonances in large DNA molecules. A comparison between NOESY spectra of an unlabeled sample of the same sequence to those obtained using the filter NOESY experiments on the labeled counterpart will be presented and demonstrates the spectral simplification obtained by this technique.

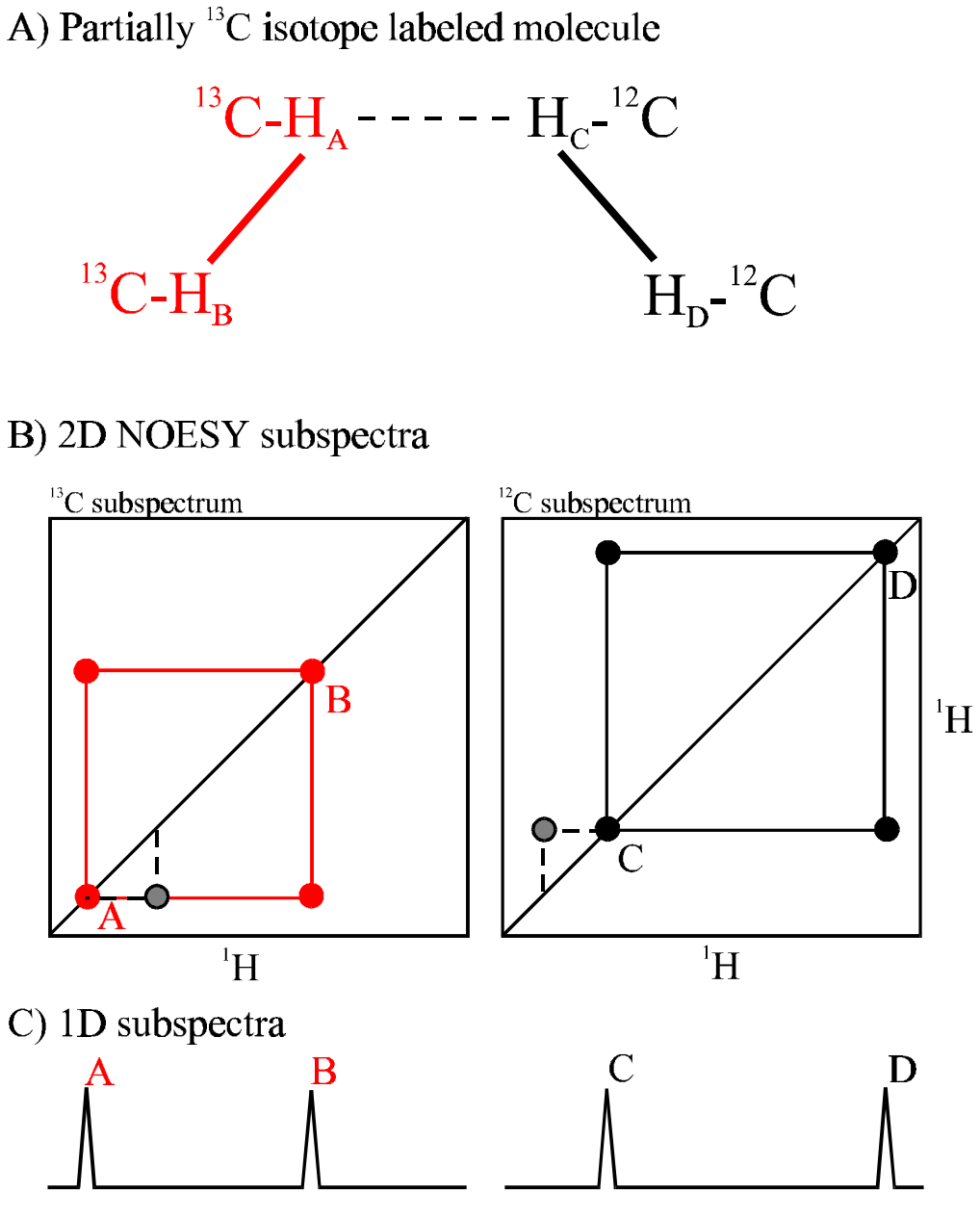


Figure 3. 3 Simulated NOESY subspectra for a partially labeled molecule

A) A partially ^{13}C/^{12}C molecule with proton A and B attached to a ^{13}C and proton C and D attached to a ^{12}C. The spacial arrangement of the protons is such that proton A is within an NOE distance from B and C; proton C is within an NOE distance form A and D. The solid and dashed lines represents the NOE connectivities. **B**) The simulated ^{13}C and ^{12}C subspectra from the 2D isotope filtered NOESY experiment. The dashed line represents the connectivity between proton A and C, notice that the crosspeak between A and C is found in BOTH spectra, because A is ^{13}C labeled and C is ^{12}C labeled. **C**) The 1D subspectra for the sample.

3.2.3 Isotope filtered pulsed field-gradient stimulated echo

Determining the translational diffusion rate of a molecule can give important information on the hydrodynamical shape of that molecule and can be used to estimate its approximate molecular size. One of the NMR experiments used for determining the translational diffusion constant of a molecule is known as the "pulsed field-gradient stimulated echo" (PFG-STE) and has been shown to accurately measure the diffusion constants of nucleic acids (Lapham, *et al*., 1997; Chapter 4). This experiment can be modified to include an isotope filter, allowing for the discrimination between the diffusion rate of a labeled and an unlabeled molecule.

The importance of having the ability to observe the translational diffusion constant of a single species in a complex solution is that it avoids the problems that may arise in interpreting diffusion data for complexes which may not be in a 1:1 molar ratio. For instance, if a DNA-protein complex were constructed in a 1:1.2 ratio (an excess of protein), the measured diffusion rate of the complex would be some average of the diffusion rate of the full complex and the 20% free protein. This would, naturally, give rise to an erroneous diffusion constant.

To address this problem, we created a ^{13}C isotope filtered pulsed field-gradient stimulated echo pulse sequence (^{13}C filtered-PFG-STE). It can be used for monitoring protein-DNA binding by NMR, by measuring the diffusion constant of the isotope-labeled strand of the DNA. The experiment is capable of monitoring the diffusion constant of a single component in a complex mixture, and is the only known method for accomplishing this.

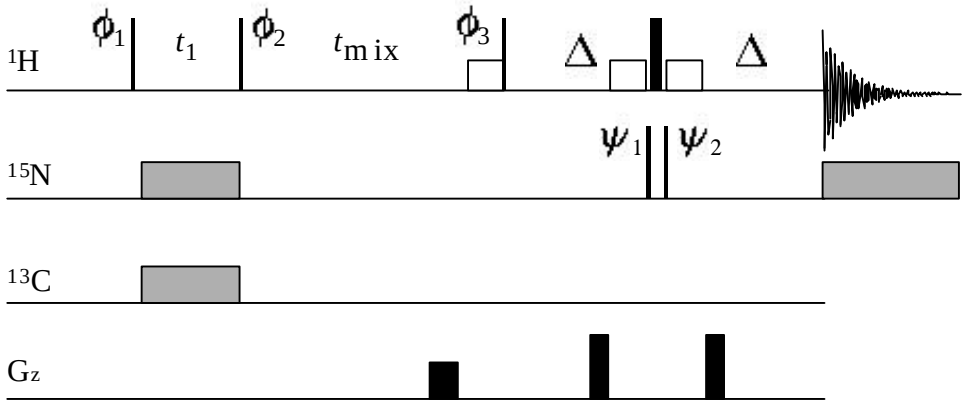
3.3 Results

3.3.1 Exchangeable protons

The NOESY NMR spectroscopy of the imino protons of nucleic acids is of critical importance in assigning the secondary structure of a DNA or RNA molecule (Wüthrich, K., 1986). While it is possible to label one strand of a DNA with ^{15}N and perform an ^{15}N-^{1}H HMQC to identify the iminos from the labeled strand, chemical shift degeneracy, common in standard B-form DNA, may make it impossible to resolve every imino proton. This particular problem can be alleviated by observing the crosspeak patterns between adjacent iminos in the isotope filtered NOESY experiment. The crosspeaks of the imino protons from a NOESY spectrum offer a second dimension to resolve such degeneracy. Using these isotope-filtered NOESY techniques on a single strand labeled heteroduplex DNA allows one to assign an orientation to the imino protons based on the pattern of the crosspeaks found in the two subspectra.

The data for the exchangeable proton spectra were collected using a watergate NOESY pulse sequence for the fully unlabeled DNA and an isotope-filtered watergate NOESY for the single strand isotope labeled DNA (Fig. 3.4a). Comparison of the exchangeable imino proton spectrum of the unlabeled D19 and the isotope-filtered NOESY of the single strand labeled D19 is shown in figure 3.5. All crosspeaks found in the unlabeled spectrum (Fig. 3.5b) are clearly visible in either the ^{14}N or ^{15}N subspectrum from the isotope-filtered NOESY (Figs. 3.5c/d). Interpretation of the data from the two subspectra is quite straightforward. If a crosspeak appears on both sides of the diagonal in the ^{14}N subspectra, then the two imino protons which gave rise to the crosspeak belong

A) Isotope-filtered ^{15}N watergate NOESY



B) Isotope-filtered ^{13}C NOESY

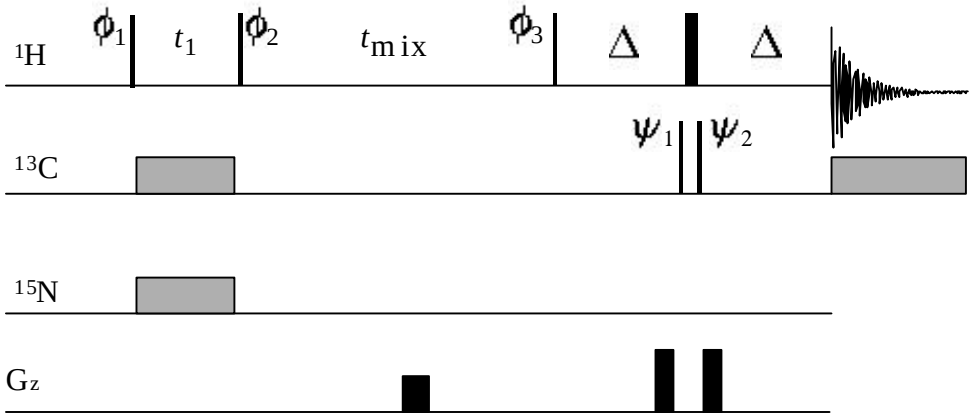
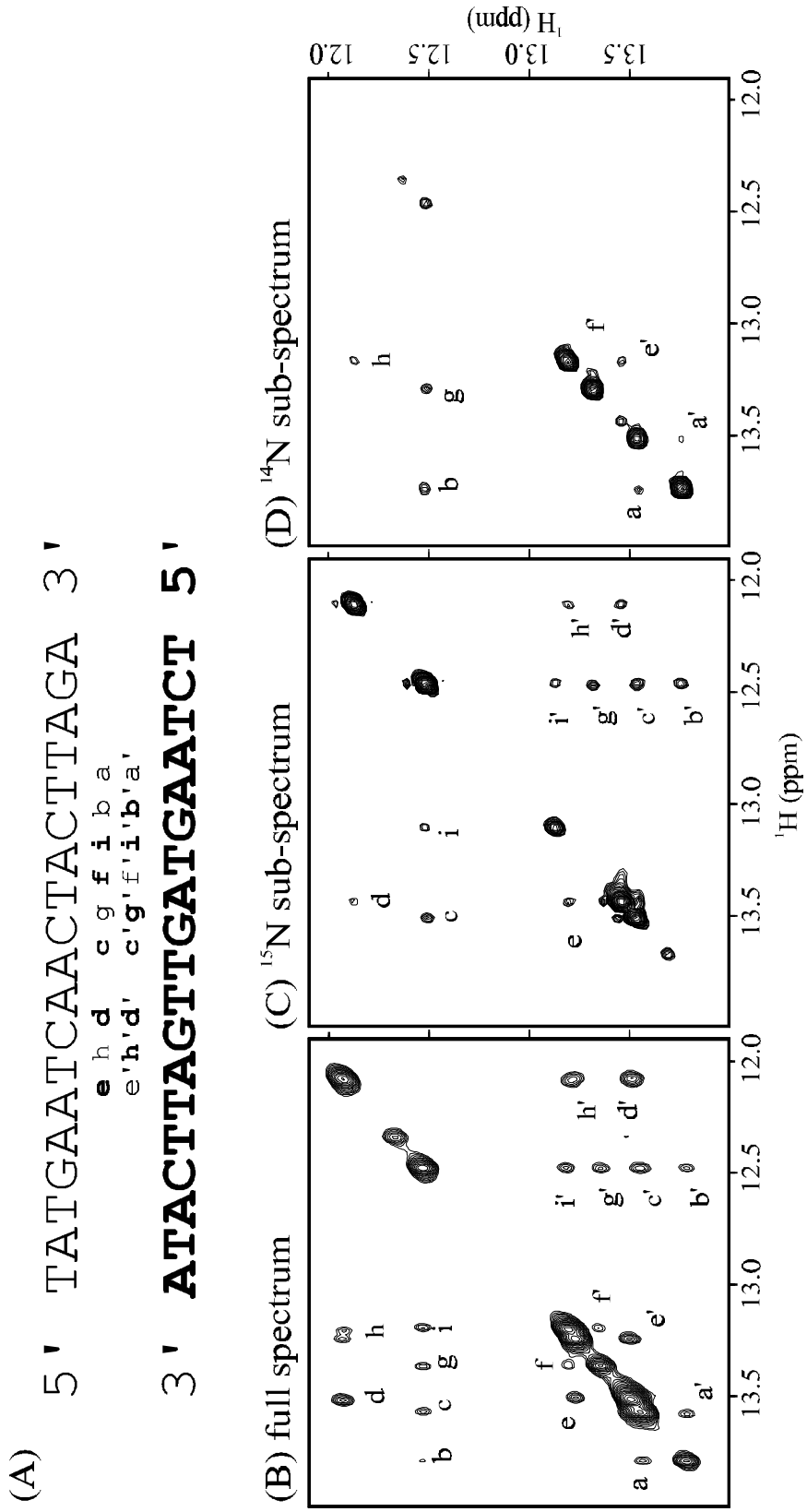


Figure 3. 4 Isotope filtered NOESY pulse sequences

For both the ^{15}N and ^{13}C isotope-filtered experiments, all pulses indicated by the thin lines are $\pi/2$ pulses and the wide lines are π pulses. All hard pulses are phase cycled +x unless otherwise indicated, all the soft pulses are phase cycled -x. Φ_1 is cycled (x, -x) and also includes the States phase cycling for quadature detection (States, et al., 1982). Two experiments are collected for each States cycle, in which the phase of Ψ_1 and Ψ_2 is (+x) for the first experiment, the second experiment is collected with Ψ_1 set to (-x) and Ψ_2 set to (+x). Garp decoupling (Shaka, et al., 1985) was used for both the nitrogen and carbon channels during the t_1 time and acquisition, if indicated. **A)** The isotope filtered pulse sequence used for the exchangeable proton NOESY experiment. **B)** The isotope filtered pulse sequence used for the non-exchangeable proton NOESY experiment.



5 $^{14}N/^{15}N$ isotope filtered watergate NOESY spectra for DNA

The 19 base pair DNA, D19, used in these experiments. The bottom strand, in bold, is the $^{15}N/^{13}C$ labeled strand. **B)** 2D $_2O$ NOESY spectra of the unlabeled D19. The labeling of the crosspeaks begins at the bottom left with "*a*" and *i*", while each symmetry related crosspeak has the same label with a prime. **C)** The ^{15}N **D)** ^{14}N filtered sub-spectrum of the single strand labeled D19, which were processed using the

to the unlabeled strand of DNA. Conversely, if a crosspeak appears on both sides of the diagonal in the ^{15}N subspectra, the two imino protons are located on the labeled strand. Finally, if a crosspeak appears on one side of the diagonal in the ^{14}N subspectra and on the other side of the diagonal in the ^{15}N subspectra, then the two iminos contributing to the crosspeak are on separate strands of the DNA. In this manner, every observable imino proton crosspeak for D19 was assigned, as shown in figure 3.5a.

3.3.2 Non-exchangeable protons

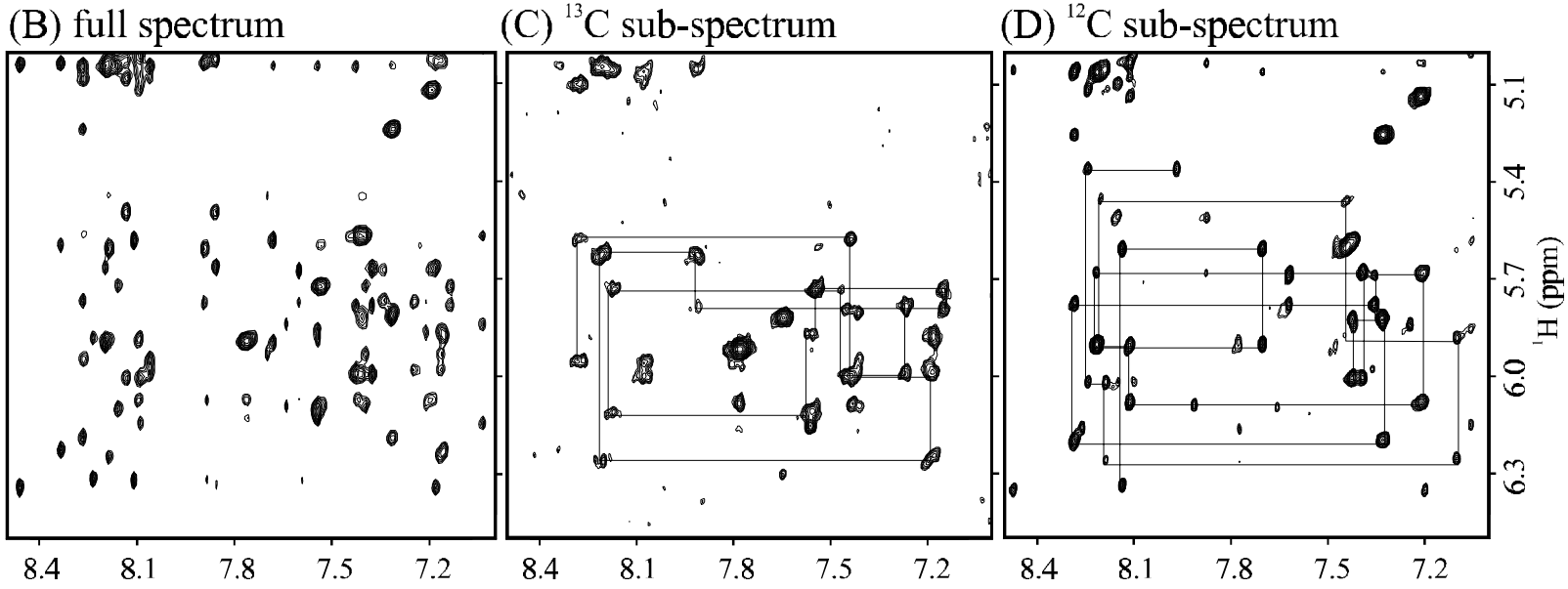
The non-exchangeable protons in DNA are of critical importance in structure determination. For B-form DNA, the sequence specific assignment of these protons can be accomplished by means of the anomeric-aromatic walk found in the 2D NOESY. This connectivity pattern correlates the H6/H8 base proton of a nucleotide to its own H1' sugar proton, and to the H1' sugar proton of the nucleotide in the 5' direction. In a well resolved spectrum, every H6/H8 and H1' can be sequence-specifically assigned in this manner.

The isotope filtered NOESY pulse sequence (figure 3.4b) was used to collect data on the sample with one strand labeled, and the data was compared with that from an unlabeled DNA. The ^{13}C and ^{12}C isotope-filtered subspectra for the single strand labeled sample are shown in figure 3.6c/d respectively. The drawn line in the spectra represents the sequential nucleotide connectivities; the isotope filter clearly separates the two distinct aromatic-anomeric walks. Figure 3.6b demonstrates what the standard 2D NOESY for the fully unlabeled DNA sample looks like.

(A)

5' TATGAATCAACTACTTAGA 3'

3' **ATACTTAGTTGATGAATCT*** 5'



6 ^{12}C/^{13}C isotope filtered NOESY spectra for DNA

The 19 base pair DNA, D19, used in these experiments. The bottom strand, in bold, is the ^{15}N/^{13}C labeled strand. **B)** 2D $_2$O NOESY spectrum of the unlabeled D19. **C)** ^{13}C filtered sub-spectrum and the **D)** ^{12}C selected sub-spectrum of the

3.3.3 PFG diffusion measurements

NMR isotope-filtering techniques offers a unique ability to observe a single molecular species in a complex solution. This is an especially powerful tool for the spectroscopist interested in monitoring the physical behavior of a molecule under the influence of another. We demonstrate this by measuring the translational self-diffusion rate of the isotope-labeled strand of D19 both bound and unbound to a protein. The data is simple to interpret in that the resonances of the unlabeled protein do not complicate the spectrum.

The NMR pulsed field-gradient (PFG) spin-echo technique (Hahn, 1950; Stekjskal & Tanner, 1965) has long been used to measure diffusion constants. Applications to biological systems include determination of the aggregation state of proteins (Alteiri, *et al*., 1995; Dingley, *et al*., 1995), measurement of the bulk movement of hemoglobin in human erythrocytes (Kuchel & Chapman, 1991) and quantitation of processes such as amide proton exchange with water (Andrec & Prestegard, 1996). For the NMR spectroscopist, it provides a simple, accurate method for measuring the diffusion constants of the materials they are investigating under the same conditions as all their other NMR experiments. Chapter 4 of this thesis gives a more exhaustive theoretical and experimental discussion of translational self-diffusion.

We present here a new pulse sequence for measuring the diffusion rate of a single isotope-labeled molecule in a complex solution, an isotope-filtered PFG stimulated echo (filtered-PFG-STE, Fig. 3.7). This pulse sequence was adapted from Tanner's (1970) PFG-STE sequence that maximizes the signal of samples with short T_2 relaxation times.

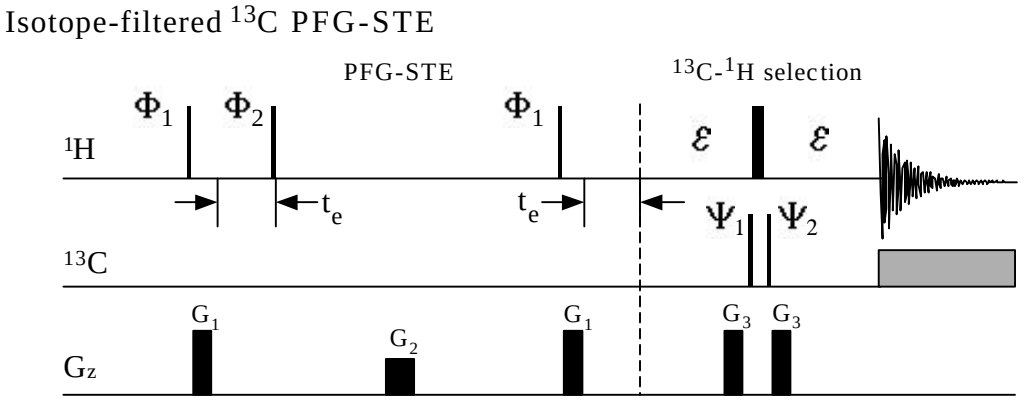


Figure 3. 7 The isotope filtered PFG-STE pulse sequence

Φ_1 was cycled (+x, -x) and Φ_2 was cycled (-x, +x). Two fids were collected for each increment of G_z: the first with the phase Ψ_1 of (+x) and Ψ_2 of (-x), the second with the phase of Ψ_1 of (-x) and Ψ_2 of (-x). ε was set to $1/2J_{H\text{-}C}$ for the methyls in the DNA ($1/2 \bullet 140$ hz) and the carbon carrier was centered at 12 ppm for the methyls. The isotope filtered spectra was generated by linear addition of these two fids.

A comparison of the measured self-diffusion rate of the fully unlabeled DNA using the PFG-STE sequence and the single strand labeled DNA using the filtered-PFG-STE sequence (Fig. 3.8) demonstrates that they both give approximately the same values, $1.10(.01)x10^{-6}$ and $1.12(.01)x10^{-6}$ cm^2/s respectively. IHF was added to the D19 sample in a 1:1 molar ratio, and the diffusion constant was measured from the ^{13}C PFG-STE subspectra and was found to be $0.76(.034)x10^{-6}$ cm^2/s for the isotope labeled strand (Fig. 3.8).

This experiment shows protein binding to the DNA by the change in the translational self-diffusion constant of the isotope labeled strand of the DNA. It does not require assignment of any resonances, and the data is not complicated by the additional protein resonances.

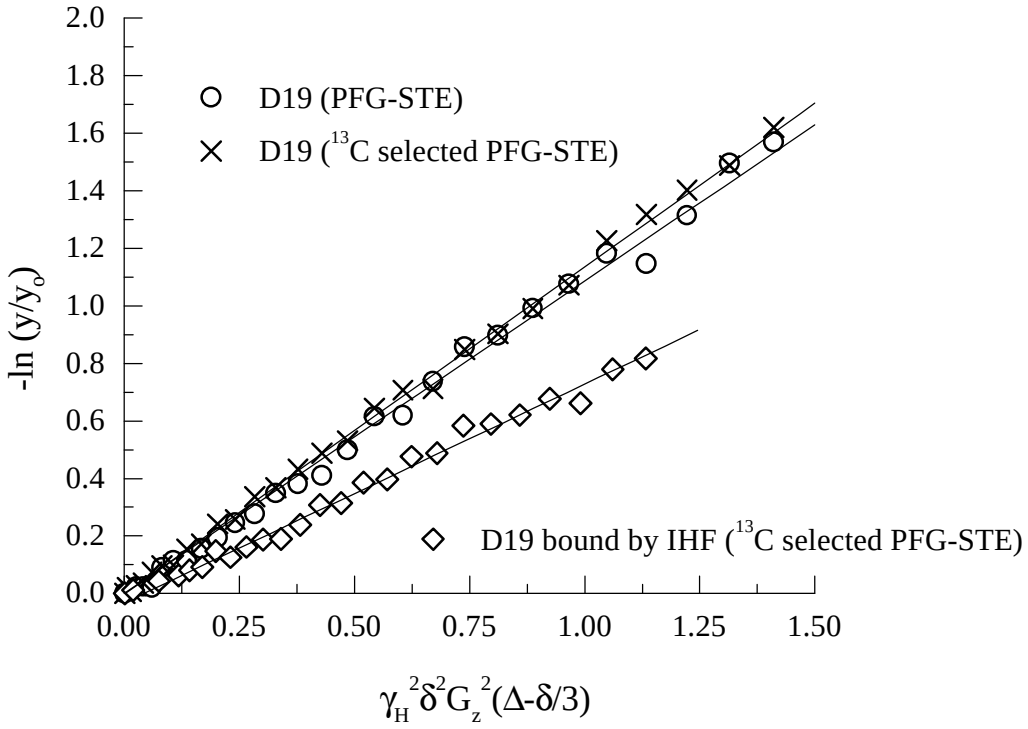


Figure 3. 8 Protein binding DNA as measured by isotope filtered diffusion

Translational diffusion rate for D19 upon binding by IHF. D19 unbound data (circles) was collected using the standard PFG-STE (see Chap. 4) and the isotope-filtered PFG-STE (crosses) as presented in this chapter. The D19 bound by IHF data (diamonds) was collected using the isotope-filtered PFG-STE pulse sequence.

Within the error of the experiment the results from the unbound DNA demonstrate that the isotope-filtered PFG-STE pulse sequence measures the same diffusion rate, as does the standard PFG-STE. The data from the bound D19 demonstrate that the binding of the IHF protein decreases the translational diffusion rate of the isotope-labeled strand of the DNA. This is what would be expected, the protein-DNA complex should have a larger frictional coefficient than the DNA alone.

3.4 Discussion

Traditionally, structural studies of DNA molecules by NMR have been accomplished by means of homonuclear 1D and 2D proton correlation experiments. Our lab has recently published techniques for synthesis of uniformly ^{13}C and ^{15}N isotope labeled DNA molecules (Zimmer & Crothers, 1995) which allows for single strand labeling of any DNA sequence which is not dyad symmetric. Given that most DNAs of biological interest are non-dyad symmetric dimers, we feel that synthesis of these single strand labeled samples, in conjunction with standard isotope filtered NMR experiments, will greatly facilitate the study of larger DNAs. We present this as a general method for obtaining proton assignments for large DNA molecules, while requiring that only one sample be synthesized.

The method of data collection and processing of the isotope filtered NOESY experiments presented allows for obtaining both the labeled and the unlabeled subspectrum at the same time. The same data set is either added together or subtracted to form one subspectrum or the other. In this manner, the experiments are more efficient than the ½-X-filtered type experiments (Otting and Wüthrich, 1990) that require complete data sets be collected for each subspectrum.

In addition to proton assignment, and ultimately structural determination, NMR can be used to measure other physical properties of systems. Recently, pulsed field-gradient (PFG) methods have been employed to measure the translational diffusion constants for nucleic acids (Lapham *et al*., 1997). Use of an isotope-filter in conjunction with these PFG diffusion measurements makes it possible to follow the translational self-diffusion of a single molecular species in a complex solution. This can be used to

monitor any process that will change the hydrodynamic properties of the isotope labeled species, such as protein binding. The advantage of being able to filter away the signals due to the unlabeled DNA strand and other ligands (such as a protein in this case) is that it simplifies the interpretation of the data.

3.5 Materials and methods

3.5.1 DNA sample preparation

The unlabeled DNA strands were synthesized on an Applied Biosystems 380B DNA synthesizer. The ^{15}N and ^{13}C uniformly labeled DNA strands were synthesized enzymatically as previously described (Zimmer & Crothers, 1995). Two samples of the 19 base pair D19 sample were produced, one which was composed of two unlabeled strands and one which was composed of an unlabeled top strand and an isotope labeled bottom strand. The top strand sequence for D19 is 5'-TATGAATCAACTACTTAGA-3' and the complementary bottom strand sequence is 5'-TCTAAGTAGTTGATTCATA-3'.

200 nmoles of each DNA strand was combined in a 1:1 molar ratio, concentrated to 160 uL volume, and dialyzed several times against 10 mM sodium phosphate buffer at pH 6.8, 100 mM NaCl and 0.5 mM EDTA. The sample was placed in a Shigemi NMR tube (Shigemi corp., Tokyo, Japan) with a total volume of 160 µl, with a final duplex concentration of 1.25 mM.

The exchangeable data were collected on a 85% H_2O and 15% D_2O sample, while the non-exchangeable data was collected on a 100% D_2O sample. Prior to the NMR experiments the samples were heated to 90° C then allowed to cool slowly to room temperature to insure complete duplex formation.

3.5.2 Protein sample preparation

Aliquots of an IHF protein stock at 2.3 mM were added to the D19 strand labeled duplex at 1.25 mM and dialyzed against a buffer containing D_2O, 100 mM NaCl, 10 mM

sodium phosphate (pD 6.8) and 0.5 mM EDTA. Complete protein binding was monitored by native condition gel electrophoresis band shift assay.

3.5.3 NMR spectroscopy: filtered NOESY

Standard isotope-filtered pulse sequences were employed. For the exchangeable proton data, an ^{15}N isotope-filtered NOESY with the watergate (Piotto, *et al.*, 1992; Lippens, *et al.*, 1995; Sich, *et al.*, 1996) water suppression technique was utilized (figure 3.4a). The proton carrier was set to the water resonance and the ^{15}N carrier was set to 150 ppm, centered between the N1 of guanidines and N3 of the thymidines. For the non-exchangeable proton data, a ^{13}C isotope-filtered NOESY was used (figure 3.4b). The ^{13}C carrier frequency was set to 190 ppm, centered between the C1' and C6/C8 resonances.

All data were acquired on a Varian Unity 500 MHz NMR spectrometer at 30° C. Both the exchangeable H_2O NOESY and non-exchangeable D_2O NOESY experiments were obtained by collecting 2048 complex t_2 points in 32 scans with 300 t_1 time increments with a total experiment time of 24 hrs for each of the data sets. Two FIDS were collected for each states cycle, and were either added together to produce the ^{14}N (or ^{12}C) sub-spectra, or subtracted from each other to produce the ^{15}N (or ^{13}C) sub-spectra as described elsewhere (Otting & Wüthrich, 1990; SantaLucia, *et al.*, 1995). All data shown were apodized using a 90 degree shifted sine bell function. The data were processed on a Silicon Graphics computer using the Felix95 NMR processing program (Biosym Technologies, San Diego, CA).

3.5.4 NMR spectroscopy: filtered PFG-STE

The translation diffusion constant was measured using an isotope filtered PFG-STE pulse sequence (figure 3.7). 32 experiments were collected in which the strength of the gradients G1 were incremented from 1 to 32 gauss/cm. The data were processed and interpreted as previously described (Lapham, *et al*., 1997). The carbon filter was added to the end of the pulse sequence to allow of the observation of only those resonances on the ^{13}C labeled strand of D19. The carbon carrier was set to 12 ppm to center on the DNA methyls, which gave strong signal in the DNA-protein complex.

3.6 Appendix

The following pages contain the processing pulse sequences and felix95 macros used to process the data shown in this chapter.

3.6.1 Isotope filtered jump-return spin-echo 1D pulse sequence

```
#ifndef LINT
static char SCCSid[] = "@(#)GE_hmqc_jrse.c";
#endif

/*      GE_14n_15n variables:

    mix = mixing time.         (50-300ms)
    deltav = imino_v - h2o_v       (3625 hz)
    tau = (1/(4*deltav))           (~69 us)
    tau_corr = tau-(pw+pw2)-rof1      (~45 us)
    tau_corr2=tau*2-(pw+pw2)-rof1     (~114 us)
    post = gradient settling time         (50-200us)
    d1 = relaxation delay          (0.1 - 1.0 s)
    pw = 1H 90             (6 - 8 us)
    grt = gradient time            (1 ms)
    grl = gradient level           (8000)

    phase = 1,2 for States-TPPI

    -J. Lapham 7/25/95 */

#include <standard.h>

/* Define static integers arrays used to create the AP tables */

static int ph1[1] = {0},
    ph2[1] = {2},
    ph3[1] = {0},
    ph4[1] = {2};

pulsesequence()
{

/* Declare Variables */

    /* char charvar; */

    int phase;

    double  post, tau_corr,    tau_corr2,
        djxh2, pw2, jxh,
        grt, grl;

/* Load Variables */
    ni = getval("ni");
    phase = (int) (getval("phase") + 0.5);
    post = getval("post");
    grt = getval("grt");
    grl = getval("grl");
    tau = getval("tau");
    jxh = getval("jxh");
    pw2 = getval ("pw2");
    pwx2 = getval("pwx2");
```

```
    pwxlvl2 = getval("pwxlvl2");
    dpwr2 = getval("dpwr2");

/* Initialize variables */
    djxh2 = (1.0 / (2.0 * jxh)) - grt - post;
    tau_corr = tau - (pw + pw2) - rof1;
    tau_corr2 = tau*2 - (pw + pw2) - rof1;

/* check validity of parameter range */

    if((dm[A] == 'y' || dm[B] == 'y' || dm[C] == 'y' || dm[D] == 'y'))
        {
        printf("Decoupler must be set as dm=nnnny or n\n");
        abort(1);
    }

    if((dm2[A] == 'y' || dm2[B] == 'y' || dm2[C] == 'y' || dm2[D] == 'y'))
        {
        printf("Second decoupler must be set as dm2=nnnny or n\n");
        abort(1);
    }

    if( dpwr > 50 )
    {
        printf("dpwr too large (must be less than 51)!\n");
        abort(1);
    }

    if( dpwr2 > 50 )
    {
        printf("dpwr2 too large (must be less than 51)!\n");
        abort(1);
    }

/* Define phase cycling tables */
    settable(t1, 1, ph1);     /* t1 = 0,... */
    settable(t2, 1, ph2);     /* t2 = 2,... */
    settable(t3, 1, ph3);     /* t3 = 0,... */
    settable(t4, 1, ph4);     /* t4 = 2,... */

if (phase == 1)
    {
    assign(zero, v1);
    assign(zero, oph);
    }

if (phase == 2)
    {
    assign(two, oph);
    assign(two, v1);
    }

if (phase == 3)       /* 15N spectrum */
    {
    mod2(ct, v1);  /* v1 = 0,1,... */
    dbl(v1, v1);   /* v1 = 0,2,... */

    mod2(ct, oph);  /* oph = 0,1,... */
    dbl(oph, oph);  /* oph = 0,2, ... */
    }

if (phase == 4)       /* 14N spectrum */
    {
    mod2(ct, v1);  /* v1 = 0,1,... */
    dbl(v1, v1);   /* v1 = 0,2,... */

    assign(two, oph);
    }


/* BEGIN THE ACTUAL PULSE SEQUENCE */
```

```
 status(A);

    rcvroff();
    rlpower(pwxlvl2,DO2DEV);  /* Set decoupler power to pwxlvl */
    rlpower(tpwr,TODEV);      /* Set power for hard pulses  */
    delay(d1);

status(B);
    rgpulse(pw, t1, rof1, 0.0);   /* 90x */
    delay(tau_corr);           /* tau_corr delay */
    rgpulse(pw2, t2, rof1, 0.0);  /* 90-x */
    delay(djxh2);

status(C);
    rgradient('z', grl);      /* apply gradient */
    delay(grt);
    rgradient('z', 0.0);
    delay(post);
    dec2rgpulse(pwx2, v1, rof1, 0.0); /* first dec channel*/
    rgpulse(pw, t3, rof1, 0.0);   /* 90x */
    delay(tau_corr2);     /* tau_corr2 delay */
    rgpulse(pw2, t4, rof1, 0.0);  /* 90-x */

status(D);
    dec2rgpulse(pwx2, zero, rof1, 0.0);   /* first dec channel*/
    rgradient('z', grl);      /* Refocus resonances, remove */
    delay(grt);        /* residual water */
    rgradient('z', 0.0);
    delay(post);
    delay(djxh2);

status(E); /* acquire data */
    rlpower(dpwr2,DO2DEV); /* Set decoupler power to dpwr2 */
}
```

3.6.2 Isotope filtered watergate NOESY 2D pulse sequence

The variable "phase" must be set to 1,2,3,4 (a four step array of 1,2,3,4). Four separate FIDs will be collected for each t1 time increment. The linear combination of FID #1 and #2 will give the ^{14}N subspectrum, while the linear subtraction of the same FIDs will give the ^{15}N subspectrum.

```
/* n_sel_w_noesy.c

    Pulse sequences adapted from the
watergate NOESY pulse sequence
    coded by John Diener.

    Last edited 2/12/97  -JPL
*/

#include <standard.h>

/* Define Phase Tables */

   static int phi1[8] = {0,1,2,3,2,3,0,1},
phi2[8] = {0,1,0,1,2,3,2,3},
    phi3[8] = {2,3,2,3,0,1,0,1},
    rec4[8] = {0,1,2,3,2,3,0,1},
```

```
    phi5[2] = {0,2},
    phi6[2] = {0,2},
    phi7[2] = {0,2};

pulsesequence()
{
/* DECLARE VARIABLES */

 double   mix,modmix,tau,modtau,grt,gzlvl1,
sl901,sl902, sl90dif,tpwrsl,stweak,
pshift,djxh1,djxh2,jxh;

 int  phase;

/* LOAD VARIABLES */

  mix = getval("mix");
  gzlvl1 = getval("gzlvl1");
  grt = getval("grt");
  sl901 = getval("sl901");
  sl902 = getval("sl902");
  tau = getval("tau");
  tpwrsl = getval("tpwrsl");
  stweak = getval("stweak");
  phase = (int) (getval("phase") + 0.5);
  pwxlvl = getval("pwxlvl");
  pwxlvl2 = getval("pwxlvl2");
  pwx = getval ("pwx");
  pwx2 = getval ("pwx2");
  jxh=getval("jxh");
/*

/* Set AP Tables */

  settable(t1,8,phi1);
  settable(t2,8,phi2);
  settable(t3,8,phi3);
  settable(t4,8,rec4);
  settable(t5,2,phi5);
  settable(t6,2,phi6);
  settable(t7,2,phi7);


/* Calculate the n_sel phases for the second nitrogen pulse */
    if (phase == 1)
        {
        }
    if (phase == 2)
        {
        tsadd(t7,2,4);
        }
    if (phase == 3)
        {
        tsadd(t1,1,4);
        }
    if (phase == 4)
        {
        tsadd(t1,1,4);
        tsadd(t7,2,4);
        }

/* CHECK VALIDITY OF PARAMETER RANGE */

  if( tpwrsl > 35 )
    {
    printf("TPWRSL too large !!!  ");
    abort(1);
    }

/* Initialize Variables */
```

```
  initval(1.0,v1);
/* required real-time multiplier for
       phase shifts. It is set to 1 so that
       the desired 'pshift' is used as
          determined by 'stweak' */
  pshift = stweak + 360.0;
  modmix = mix - tau - grt - sl901;
  sl90dif = sl901 - sl902;
  modtau = tau + sl90dif;
  djxh1=(1.0 / (2.0 * jxh)) -
2*POWER_DELAY-grt - tau- pwx2-sl901-pw;
  djxh2=(1.0 / (2.0 * jxh)) -
2*POWER_DELAY-grt - modtau- pwx2-sl902-pw;
/* BEGIN ACTUAL PULSE SEQUENCE */

/* Receiver off time */

status(A);
   rcvroff();
   delay(5e-6);
   obsstepsize(pshift);
/* Allows sl90 to be slightly more or
       less than 90 deg. to maximize
       selectivity. On varians this is often
       not necessary so stweak can be set
       to 0.0 */

   rlpower(tpwr, TODEV);
   rlpower(dpwr,DODEV);
   rlpower(dpwr2,DO2DEV);

   delay(d1);
   rgpulse(pw, t1, rof1, 0.0);


status(B);
   delay(d2);


status(C);
   rgpulse(pw, t2, rof1, 0.0);


status(D);
   delay(modmix);
   rlpower(tpwrsl, TODEV);
   rgradient('z', gzlvl1/2);
   delay(grt);
   rgradient('z', 0.0);
   delay(tau);
   xmtrphase(v1);
   rgpulse(sl901, t3, 0.0, 0.0);
   rlpower(tpwr, TODEV);
   xmtrphase(zero);
   rgpulse(pw, t2, 0.0, 0.0);

status(E);
   delay(djxh1);

   delay(2*POWER_DELAY);
   rgradient('z', gzlvl1);
   delay(grt);
   rgradient('z', 0.0);
   delay(tau);

   rlpower(tpwrsl, TODEV);
   rlpower(pwxlvl, DODEV);
   rlpower(pwxlvl2, DO2DEV);
   xmtrphase(v1);
   rgpulse(sl901, t2, 0.0, 0.0);
   rlpower(tpwr, TODEV);
```

```
   xmtrphase(zero);

  /* nitrogen pulse for n_sel */
  dec2rgpulse(pwx2, t6, rof1, 0.0);

   rgpulse(2*pw, t3, 0.0, 0.0);
   rlpower(tpwrsl, TODEV);
   xmtrphase(v1);

  /* nitrogen pulse for n_sel */
  dec2rgpulse(pwx2, t7, rof1, 0.0);

   rgpulse(sl902, t2, 0.0, 0.0);
   rlpower(tpwr, TODEV);
   xmtrphase(zero);

   delay(2*POWER_DELAY);
   rgradient('z', gzlvl1);
   delay(grt);
   rgradient('z', 0.0);
   delay(modtau);

    delay(djxh2);

status(F);
   rcvron();
   rlpower(dpwr,DODEV);
   rlpower(dpwr2,DO2DEV);
   setreceiver(t4);
}
```

3.6.3 Isotope filtered ^{13}C 1D pulse sequence

```
#ifndef LINT
static char SCCSid[] = "@(#)GE_hmqc_jrse.c";
#endif

/*      c_sel_1d variables:

post = gradient settling time (50-200us)
d1 = relaxation delay     (0.1 - 1.0 s)
pw = 1H 90         (6 - 8 us)
grt = gradient time       (1 ms)
grl = gradient level      (8000)

phase = 3 for 13C spectrum
phase = 4 for 12C spectrum

-J. Lapham 9/18/95 */

#include <standard.h>

/* Define static integers arrays
   used to create the AP tables */
static int ph10[8] = {1,1,2,2,3,3,0,0};

pulsesequence()
{

/* Declare Variables */

    /* char charvar; */

    int phase;

    double  djxh2, jxh;
```

```
/* Load Variables */
    ni = getval("ni");
    phase = (int) (getval("phase") + 0.5);
    jxh = getval("jxh");
    pwx = getval("pwx");
    pwxlvl = getval("pwxlvl");
    pwx2 = getval("pwx2");
    pwxlvl2 = getval("pwxlvl2");
    dpwr = getval("dpwr");
    dpwr2 = getval("dpwr2");

/* Initialize variables */
    djxh2 = (1.0 / (2.0 * jxh)) - pwx;

    settable(t10, 8, ph10);

/* check validity of parameter range */
    if((dm[A] == 'y' || dm[B] == 'y' ||
 dm[C] == 'y' || dm[D] == 'y'))
    {
    printf("Decoupler must be set as dm=nnnny or n\n");
    abort(1);
    }

    if((dm2[A] == 'y' || dm2[B] == 'y' ||
 dm2[C] == 'y' || dm2[D] == 'y'))
    {
    printf("Second decoupler must be set as dm2=nnnny or n\n");
    abort(1);
    }

    if( dpwr > 50 )
    {
        printf("dpwr too large (must be less than 51)!\n");
        abort(1);
    }

    if( dpwr2 > 50 )
    {
        printf("dpwr2 too large (must be less than 51)!\n");
        abort(1);
    }

/* real time variable calcs */
    mod2(ct,v3);   /* v3=0,1,0,1,.... */
    dbl(v3,v3);/* v3=0,2,... */

if (phase == 1)
    {
    assign(zero, v1);
    assign(zero, oph);
    }

if (phase == 2)
    {
    assign(two, oph);
    assign(two, v1);
    }

if (phase == 3)
    {
    mod2(ct, v1);  /* v1=0,1,... */
    dbl(v1, v1);   /* v1=0,2,... */

    mod2(ct, oph);  /* oph=0,1,...*/
    dbl(oph, oph);  /* oph=0,2, ...*/
    }

if (phase == 4)
    {
```

```
    mod2(ct, v1);  /* v1=0,1,...*/
    dbl(v1, v1);   /* v1=0,2,...*/

    assign(two, oph);
    }


/* BEGIN THE ACTUAL PULSE SEQUENCE */
 status(A);
    rcvroff();
    delay(d1);

    if (satmode[A] == 'y')
    {
    if (fabs(tof-satfrq)>0.0)
offset(satfrq, TODEV);
    rlpower(satpwr,TODEV); txphase(t10);
    rgpulse(satdly, t10, rof1, rof1);
    rlpower(tpwr,TODEV);
    if (fabs(tof-satfrq)>0.0)
    {  offset(tof,TODEV); delay(40.0e-6); }
    }

    rlpower(tpwr,TODEV);
    rlpower(pwxlvl,DODEV);
    rlpower(pwxlvl2,DO2DEV);

status(B);
    rgpulse(pw, zero, rof1, 0.0);

    delay(djxh2);

status(C);
sim3pulse(pw, pwx, pwx2, v3, v1, v1, rof1, 0.0);
sim3pulse(pw, pwx, pwx2, v3, zero, zero, rof1, 0.0);

status(D);
    delay(djxh2);

status(E); /* acquire data */
    rlpower(dpwr,DODEV);
    rlpower(dpwr2,DO2DEV);
}
```

3.6.4 Isotope filtered ^{13}C 2D NOESY pulse sequence

```
#ifndef LINT
static char SCCSid[] = "@(#)c_sel_noesy.c";
#endif

/*      13/12C selected 2D D2O Noesy:

Carbon or Nitrogen (optional) on second
or third channel


Set phase= 1,2,3,4
phase 1: states off, refocus off
phase 2: states off, refocus on
phase 3: states on, refocus off
phase 4: states on, refocus on

Variables:
mix = mixing time.
d1 = relaxation delay
```

```
pw = 90 degree proton pulse width
jxh = proton - carbon 1 bond coupling
pwx = 90 13C
pwxlvl = 13C hard pulse power
pwx2 = 90 15N
pwxlvl2 = 15N hard pulse power

Water Presaturation:
    satmode='ynnnn'
    satfrq = frequency for presat
    satpwr = saturation power (5-8)
    satdly = saturation delay (0.1 - 1.0 s)

t2 processing:
    addition of fid#1 with fid#2 gives c12
    subtraction of fid#2 from fid#1 gives c13

t1 processing:
    normal states processing
    for phasing use phase0 = 90, phase1 = -180

    -- Jon Lapham 7/25/95
    -- G.M. Dhavan 3/1/96
-- Modified by Anna Lee 4/11/97
 */

#include <standard.h>

static int ph3[2] = {2,0},
    ph4[2] = {0,2},
    ph10[8] = {1,1,2,2,3,3,0,0};

pulsesequence()
{
/* Declare Variables */
        int     phase;
    double  mix, djxh2, jxh, t1_delay,
mix_corr, grt, grl, post;

/* Load Variables */
    phase = (int) (getval("phase") + 0.5);
    mix = getval("mix");
        ni = getval("ni");
    jxh = getval("jxh");
    dpwr = getval("dpwr");
    dpwr2 = getval("dpwr2");
    pwxlvl = getval("pwxlvl");
    pwxlvl2 = getval("pwxlvl2");
    pwx = getval("pwx");
    pwx2 = getval("pwx2");
    grt = getval("grt");
    grl = getval("grl");
    post = getval("post");
    sw1 = getval("sw1");

/* initialize variables */
djxh2 = (1.0 / (2.0 * jxh)) - pwx - rof1;

if  ( pwx2 > pwx )
{ t1_delay = (2*pw/3.1415) + pwx2 + rof1; }

if  ( pwx > pwx2 )
{ t1_delay = (2*pw/3.1415) + pwx + rof1; }

    mix_corr = mix - rof1 - grt - post;

/* Set AP tables */
    settable(t3, 2, ph3);
    settable(t4, 2, ph4);
    settable(t10, 8, ph10);
```

```
/* Real time phase cycling calculations */
/* phase = 1,2,3,4 to collect separate
fids for 12C and 13C data */
    mod2(ct,v1);   /* v1 = 0,1 */
    dbl(v1,v1);/* v1 = 0,2 */

    mod2(ct,oph);  /* oph = 0,1 */
    dbl(oph,oph);  /* oph = 0,2 */

    if ((phase == 3) || (phase == 4))
        incr(v1);


/* BEGIN THE ACTUAL PULSE SEQUENCE */

status(A);
    rcvroff();
    delay(d1);

    if (satmode[A] == 'y')
    {
    if (fabs(tof-satfrq)>0.0) offset(satfrq, TODEV);
    rlpower(satpwr,TODEV); txphase(t10);
    rgpulse(satdly, t10, rof1, rof1);
    rlpower(tpwr,TODEV);
    if (fabs(tof-satfrq)>0.0)
    { offset(tof,TODEV); delay(40.0e-6); }
    }

    rlpower(tpwr,TODEV);
    rlpower(pwxlvl,DODEV);
    rlpower(pwxlvl2,DO2DEV);

status(B);

        if (d2 == 0)
        {
         rgpulse(pw, v1, rof1, 0.0);
              delay(d2);
              rgpulse(pw, t3, rof1, 0.0);
        }

        else
        {
         rgpulse(pw, v1, rof1, 0.0);
     delay(d2/2 - t1_delay);
     sim3pulse(0.0,pwx*2,pwx2*2,t3,t4,t4,rof1,0.0);
     delay(d2/2 - t1_delay);
          rgpulse(pw, t3, 0.0, 0.0);

        }

status(C); /* NOE mixing time */
    rgradient('z', grl);
    delay(grt);
    rgradient('z',0.0);
    delay(post);
    delay(mix_corr);


status(D); /* carbon selected HMQC */
    rgpulse(pw, t4, rof1, 0.0);   /* 90x */

    delay(djxh2);

    if ((phase == 1) || (phase == 3))
        rgpulse(2*pw,t4,rof1,rof1);
    if ((phase == 2) || (phase == 4))
        sim3pulse(2*pw,2*pwx,0.0,t4,t4,t4,rof1,rof1);

    delay(djxh2);
```

```
status(E); /* acquire data */
    rlpower(dpwr,DODEV);
    rlpower(dpwr2,DO2DEV);
}
```

3.6.5 Felix macros for processing NOESY subspectra

Notes: Processing the t1 dimension is identical to that of any other States data set. The difference between processing the labeled and unlabeled subspectra is the "mul -1" statement. The "mul –1" is used to subtract the FIDS, because Felix only has a "add to buffer" statement (adb) one of the FIDs must be multiplied by –1, then added to the other.

```
c**14N_NOESYt2 processing

cmx
cl

def phase0 0
def phase1 0
def file
def nrows 500
def wcor 'cnv 0 32'
def wind1 'sb 512 90'
def wind2 'kw 1024 2'

ty Building the matrix
c**bld &filen14.mat 2 1024 1024 0
mat &filen14.mat w
ty Transform t2

for row 1 &nrows
  re &file.dat
  stb 1

  re &file.dat
  mul -1
  adb 1
  ldb 1

c**  &wcor
  &wind1
c**  &wind2
ft

  ph
  red
  sto 0 &row
  esc escape
  if &escape eq 1 escape
  ty Row #&row$
  next
end


c**15N_NOESYt2 processing

cmx
cl
```

```
def phase0 0
def phase1 0
def file imino
def nrows 500
def wcor 'cnv 0 32'
def wind1 'sb 512 90'
def wind2 'kw 400 10'

ty Building the matrix
c**bld &filen15.mat 2 1024 1024 0
mat &filen15.mat w
ty Transform t2

for row 1 &nrows
  re &file.dat
  stb 1

  re &file.dat
  adb 1
  ldb 1

  &wcor
  &wind1
c**  &wind2
  zf 1024
  ft

  ph
  red
  sto 0 &row
  esc escape
  if &escape eq 1 escape
  ty Row #&row$
  next
end
```

3.7 References

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