13C NUCLEAR MAGNETIC RESONANCE RELAXATION STUDIES OF UNIFORMLY 13C AND 15N LABELLED PROTEINS

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

AMIRAH CHAUDHRY

Department of Chemistry

University of Leicester

September 1997
ABSTRACT

$^{13}$C NUCLEAR MAGNETIC RESONANCE RELAXATION STUDIES OF UNIFORMLY $^{13}$C AND $^{15}$N LABELLED PROTEINS

by

AMIRAH CHAUDHRY

This thesis examines the problems of making $^{13}$C NMR relaxation measurements in proteins uniformly enriched with $^{13}$C and $^{15}$N isotopes and it presents a solution in the form of a new method of analysing $^{13}$C relaxation data.

In uniformly doubly-labelled proteins a particular $^{13}$C spin can undergo relaxation due to its own CSA and its dipolar interactions with bonded $^{13}$C atoms and bonded and non-bonded protons. The non-bonded protons can be on the same amino acid residue or on other residues which are close in space. To quantify the dipolar interactions the internuclear vector needs to be known to a significant degree of accuracy because of the $1/r^6$ proportionality (where $r$ is the internuclear vector). The non-bonded $^{13}$CH distances cannot usually be determined to such a precision and additionally these distances are constantly changing as a consequence of molecular dynamics. This makes the conventional $^{13}$C $T_1$ and NOE measurements difficult to analyse. Furthermore, homonuclear $^{13}$C scalar coupling evolution renders the traditional $T_2$ measurement problematic.

In this study we have developed a method of analysis which, taking account of all the numerous and difficult to quantify interactions, isolates the mutual dipolar interaction of a set of active spins from all other interactions in an $n$-spin system. This method involves taking a linear combination of the longitudinal relaxation matrix elements pertaining to the relaxation decay rates of various longitudinal modes. This method can be used in conjunction with conventional $^{15}$N relaxation measurements and suitable motional models to model the spectral density function. Alternatively, the isolated $^{13}$C dipolar interactions may be used in conjunction with a less rigorous method of analysis to map the spectral density function.

The new method of analysis involves making five relaxation measurements: $^{13}$C $T_1$, proton $T_1$, $^{13}$CH and HH longitudinal two spin order and $^{13}$CHH longitudinal three spin order. Of these only the $^{13}$C $T_1$ was reported in the literature but it needed to be modified for use on uniformly $^{13}$C labelled samples. The design and development of two-dimensional heteronuclear experiments to make the five necessary measurements as precisely as possible is presented in this thesis.

Relaxation data for a small model molecule and a 64 residue protein are presented and the dynamics information which can be obtained is discussed.
CONTENTS

CHAPTER ONE

INTRODUCTION ................................................................. 1
1.1 BACKGROUND ............................................................ 1
1.2 THE CONTRIBUTION OF THIS THESIS TO NMR RELAXATION OF BIOMOLECULES ........................................... 4
1.3 OVERVIEW OF THIS THESIS ........................................... 5
1.4 REFERENCES ............................................................. 8
CHAPTER TWO

THE FUNDAMENTAL PRINCIPLES OF NUCLEAR MAGNETIC RESONANCE

2.1 INTRODUCTION

2.2 GENERAL CONCEPTS

2.2.1 BASIC THEORY OF NMR

2.2.2 THE PRINCIPLES OF FOURIER TRANSFORM NMR

2.2.3 PRODUCT OPERATORS AND THEIR USES

2.2.3.1 Effects of Applied Radio Frequency Pulses

2.2.3.2 Chemical Shift Evolution

2.2.3.3 Scalar Coupling Evolution

2.2.4 RAISING AND LOWERING OPERATORS

2.2.5 TWO-DIMENSIONAL NMR SPECTROSCOPY

2.2.6 COHERENCE TRANSFER PATHWAYS

2.2.6.1 The Structure of Heteronuclear Experiments

2.2.6.2 Coherence Transfer Pathway Selection by Phase Cycling and Gradient Pulses

2.3 THE THEORY OF NMR RELAXATION

2.3.1 THE PHENOMENOLOGICAL ASPECTS OF RELAXATION

2.3.1.1 Definition of Relaxation

2.3.1.2 The Nature of Relaxation and its Dependence on Molecular Dynamics

2.3.2 THEORY OF RELAXATION

2.3.2.1 Background Theory of Relaxation - The Density Operator

2.3.2.2 The Relaxation Hamiltonian

2.3.2.3 The Relaxation Matrix

2.4 REFERENCES
CHAPTER THREE

NMR RELAXATION AND MOLECULAR DYNAMICS.................................57

3.1 INTRODUCTION .....................................................................................................57

3.2 THE IMPORTANCE OF NMR RELAXATION IN BIOMOLECULES.....58

3.2.1 QUANTIFYING MOLECULAR MOTION ..........................................................60

3.3 MODELLING THE SPECTRAL DENSITY FUNCTION...............................63

3.3.1 MODELS WHICH HAVE GENERAL APPLICATIONS .................................63

3.3.1.1 The “Model-Free” Formalism.................................................................63

3.3.1.2 The Extended “Model-Free” Formalism ....................................................66

3.3.2 SPECIFIC MODELS .........................................................................................67

3.3.2.1 The Rotational Diffusion Model .................................................................67

3.3.2.2 The “Wobbling-in-a-Cone” Model ...............................................................70

3.3.2.3 Rotational Jump Models ............................................................................74

3.4 SPECTRAL DENSITY MAPPING .................................................................77

3.4.1 MAPPING THE SPECTRAL DENSITY FUNCTION ....................................77

3.4.2 REDUCED SPECTRAL DENSITY SET .........................................................80

3.5 PRACTICAL IMPLEMENTATION OF RELAXATION TO EXTRACT
DYNAMICS INFORMATION ...............................................................................81

3.5.1 IMPORTANCE OF RELAXATION IN PROTEIN DYNAMICS ......................81

3.5.2 15N RELAXATION ..........................................................................................82

3.5.3 13C RELAXATION ..........................................................................................84

3.6 DEVELOPMENT OF METHODS TO ANALYSE 13C AND 1H
RELAXATION DATA IN UNIFORMLY 13C ENRICHED MOLECULES 89

3.6.1 ISOLATING THE MUTUAL DIPOLAR RELAXATION OF A SET OF COUPLED
SPINS ......................................................................................................................90

3.6.2 PROTON RELAXATION EXPERIMENTS .....................................................100

3.6.2.1 Experimental Methods .............................................................................100

3.6.2.2 Results and Analysis ...............................................................................105
3.6.2.3 Application to Biomolecules ................................................................. 106

3.6.3 APPLICATION TO UNIFORMLY $^{13}$C LABELLED MOLECULES ................. 109

3.6.3.1 $^{13}$CH Groups ......................................................................................... 110

3.6.3.2 $^{13}$CH$_2$ Groups (Inequivalent Protons) .................................................. 110

3.6.3.3 $^{13}$CH$_2$ Groups (Equivalent Protons) ..................................................... 114

3.6.3.4 $^{13}$CH$_3$ Groups ..................................................................................... 118

3.6.3.5 Uses of The Isolated Relaxation Interactions .......................................... 118

3.6.4 APPROXIMATE METHODS FOR THE ANALYSIS OF $^{13}$C RELAXATION DATA ........ 119

3.6.4.1 $^{13}$CH Groups ......................................................................................... 120

3.6.4.2 $^{13}$CHH' Groups (Inequivalent Protons) ................................................... 123

3.6.4.3 $^{13}$CH$_2$ Groups (Equivalent Protons) ..................................................... 127

3.7 SUMMARY ...................................................................................................... 130

3.8 REFERENCES ................................................................................................. 131

CHAPTER 4

EXPERIMENTS DESIGNED TO MAKE $^{13}$C AND $^1$H RELAXATION MEASUREMENTS ................................................................. 136

4.1 INTRODUCTION ............................................................................................. 136

4.2 GENERAL CONSIDERATIONS ..................................................................... 137

4.2.1 CROSS-RELAXATION AND ITS SUPPRESSION ............................................. 137

4.2.2 THE USE OF GRADIENT PULSES TO SELECT COHERENCE TRANSFER PATHWAYS ................................................................. 145

4.2.3 SUPPRESSING WATER COHERENCE ......................................................... 148

4.3 $^{13}$C LONGITUDINAL RELAXATION RATE [$R_c(S_{cz})$] .................................. 149

4.3.1 AN OVERVIEW OF THE EXPERIMENT ...................................................... 150

4.3.2 COHERENCE TRANSFER PATHWAY SELECTION ....................................... 154

4.3.3 SUPPRESSING WATER COHERENCE ....................................................... 155

4.3.4 SUPPRESSING UNDESIRED COHERENCES ............................................. 157
4.3.4.1 Suppressing Undesired Coherences Resulting from Imperfect RF Pulses. 157
4.3.4.2 Suppressing Undesired Coherences Resulting from Homonuclear $^{13}$C Scalar Coupling Evolution .............................................................. 159
4.3.4.3 Suppressing Undesired Coherences Resulting from Cross-Relaxation .... 159

4.4 PROTON LONGITUDINAL RELAXATION RATE $[R_H (Hz)]$ ................. 164

4.4.1 AN OVERVIEW OF THE EXPERIMENT ............................................. 164
4.4.2 COHERENCE TRANSFER PATHWAY SELECTION ............................ 168
4.4.3 SUPPRESSING WATER COHERENCE .............................................. 169
4.4.4 SUPPRESSING UNDESIRED COHERENCES ....................................... 170
  4.4.4.1 Suppressing Undesired Coherences Resulting from Imperfect RF Pulses. 170
  4.4.4.2 Suppressing Undesired Coherences Resulting from Scalar Coupling Evolution .............................................................. 173
  4.4.4.3 Suppressing Undesired Coherences Resulting from Cross-Relaxation ... 174

4.5 $^{13}$CH LONGITUDINAL TWO SPIN ORDER RELAXATION RATE
  $[R_{CH} (2I_{Hz}S_{Cz})]$ .............................................................................. 177

4.5.1 AN OVERVIEW OF THE EXPERIMENT ............................................. 177
4.5.2 COHERENCE TRANSFER PATHWAY SELECTION ............................ 178
4.5.3 SUPPRESSING WATER COHERENCE .............................................. 179
4.5.4 SUPPRESSING UNDESIRED COHERENCES ....................................... 180
  4.5.4.1 Suppressing Undesired Coherences Resulting from Imperfect RF Pulses. 180
  4.5.4.2 Suppressing Undesired Coherences Resulting from Scalar Coupling Evolution .............................................................. 181
  4.5.4.3 Suppressing Undesired Coherences Resulting from Cross-Relaxation ... 182

4.6 $^{13}$CHH LONGITUDINAL THREE SPIN ORDER RELAXATION RATE
  $[R_{CHH} (4S_{Cz}I_{Hz}I_{Hz})]$ .............................................................................. 186

4.6.1 AN OVERVIEW OF THE EXPERIMENT ............................................. 186
4.6.2 COHERENCE TRANSFER PATHWAY SELECTION ............................ 189
4.6.3 SUPPRESSING WATER COHERENCE .............................................. 190
4.6.4 SUPPRESSING UNDESIRED COHERENCES ....................................... 191
4.6.4.1 Suppressing Undesired Coherences Resulting from Non-Optimal RF Pulses ...............................................................................................................191
4.6.4.2 Suppressing Undesired Coherences Resulting from Scalar Coupling Evolution .........................................................................................................191
4.6.4.3 Suppressing Undesired Coherences Resulting from Cross-Relaxation ....192

4.7 HH LONGITUDINAL TWO SPIN ORDER RELAXATION RATE
\[ R_{hh} (2I_{Hz}I_{Hz})\] ..........................................................................................................................197

4.7.1 AN OVERVIEW OF THE EXPERIMENT ..........................................................197
4.7.2 COHERENCE TRANSFER PATHWAY SELECTION ........................................200
4.7.3 SUPPRESSING WATER COHERENCE.................................................................201
4.7.4 SUPPRESSING UNDESIRED COHERENCES .........................................................202
  4.7.4.1 Suppressing Undesired Coherences Resulting from Non-Optimal RF Pulses..............................................................................................................202
  4.7.4.2 Suppressing Undesired Coherences Resulting from Scalar Coupling Evolution .................................................................206
  4.7.4.3 Suppressing Undesired Coherences Resulting from Cross-Relaxation....207

4.8 AN EXPERIMENTAL DEMONSTRATION OF THE RELAXATION EXPERIMENTS DESIGNED AND DEVELOPED FOR THIS STUDY ...210

4.9 SUMMARY ..............................................................................................................213

4.10 REFERENCES ......................................................................................................214

CHAPTER FIVE

RESULTS AND DISCUSSION ..........................................................................................216

5.1 INTRODUCTION .....................................................................................................216

5.2 PROTEIN G .............................................................................................................216

5.3 EXPERIMENTAL .................................................................................................219
5.4 DATA ANALYSIS ................................................................. 226
5.5 PRACTICAL PROBLEMS ENCOUNTERED .......................... 228
5.6 DISCUSSION OF RESULTS ............................................... 236
5.7 REFERENCES ................................................................. 237

CHAPTER SIX
CONCLUSIONS ................................................................. 238

APPENDIX I
CALCULATION OF GENERAL RELAXATION RATES ............... 243

APPENDIX II
PULSE PROGRAMS DESIGNED AND USED ............................ 245

APPENDIX III
DATA PROCESSING PROGRAM ............................................. 282
LIST OF TABLES

Table 2.1  How the change in the phase of a 90° RF pulse affects wanted and unwanted coherences .................................................................36

Table 2.2  Definitions of the operators $A^{(q)}$ and $F^{(q)}$ given in Eqn. 2.30 ..........47

Table 2.3  Definitions of the elements of the longitudinal relaxation matrix for three weakly coupled spins ..................................................51

Table 3.1  Dipole-dipole interaction constant $D_{ij}^{c}$ between $^{13}$C$^a$ and various adjacent nuclei $j$ .................................................................85

Table 4.1  The suppression of unwanted water coherence in the $R_{C}(S_{Cz})$ experiment .............................................................149

Table 4.2  The fate of undesired coherences present at the end of the evolution delay during the course of the rest of the $R_{C}(S_{Cz})$ pulse sequence...162

Table 4.3  The fate of undesired ZQC (arising from imperfect proton RF pulses) present at the end of the mixing time during the course of the rest of the $R_{H}(I_{Hz})$ pulse sequence ..........................................................172

Table 4.4  The fate of undesired coherences present at the end of the evolution delay during the course of the rest of the $R_{H}(I_{Hz})$ pulse sequence ...173
Table 4.5  The fate of undesired coherences present at the end of the mixing period during the course of the rest of the $R_{CH}(2SCzI_{Hz})$ pulse sequence .................................................................182

Table 4.6  The fate of undesired coherences, present at the end of the evolution period, during the course of the rest of the $R_{HH}(2I_{Hz}I_{Hz})$ pulse sequence .................................................................192

Table 4.7  The fate of desired and undesired coherences, present at the end of the mixing time, during the course of the rest of the $R_{HH}(2I_{Hz}I_{Hz})$ pulse sequence ..............................................................................194

Table 4.8  Typical effect of the two 45°(I) RF pulses on desired and undesired coherences, and their fate during the rest of the $R_{HH}(2I_{Hz}I_{Hz})$ pulse sequence .................................................................203

Table 4.9  The fate of undesired coherences present at the end of the evolution time during the rest of the $R_{HH}(2I_{Hz}I_{Hz})$ pulse sequence ......................206
LIST OF FIGURES

Fig. 2.1 A two cone representation of the α- and β-states for a single spin system with \( I = \frac{1}{2} \) .................................................................11

Fig. 2.2 The net magnetisation vector \( M_0 \) represents the net distribution of the precessing nuclear dipoles at equilibrium ................................................12

Fig. 2.3 Schematic representation of the scalar coupling interaction through the bonding electrons in a two-spin system ........................................14

Fig. 2.4 Motion of a spin \( \frac{1}{2} \) nucleus viewed at the rotating reference frame ....16

Fig. 2.5 Vector representation of the effect of a pulse of coherent radio frequency radiation .............................................................................17

Fig. 2.6 Sign convention for x- and y- pulses, chemical shift evolution and scalar coupling evolution .................................................................22

Fig 2.7 The four main parts of two-dimensional experiments .......................27

Fig 2.8 The preparation period of the \( ^{13}C \) T\(_1\) sequence .........................29

Fig. 2.9 Four possible designs for heteronuclear experiments ......................32

Fig. 2.10 This diagram shows how RF pulses applied along the x, y, -x and -y axis are related to their \( \phi \) values .........................................................33

Fig. 2.11 Gradient pulses can be used to dephase and rephase coherences ....38
| Fig. 2.12 | An example of practically implementing gradient echoes in homonuclear experiments | 39 |
| Fig. 2.13 | The generation of gradient echoes in heteronuclear experiments | 40 |
| Fig. 2.14 | A diagramatic representation of $T_1$ relaxation | 43 |
| Fig. 2.15 | A diagramatic representation of $T_2$ relaxation | 43 |
| Fig. 2.16 | Energy level diagram for two coupled spins, $I$ and $S$ | 49 |
| Fig. 3.1 | The autocorrelation time, $\tau_c$, is related to the length of time taken for a molecule with a known orientation to become completely uncorrelated to its starting orientation | 60 |
| Fig. 3.2 | A profile of the spectral density function, $J(\omega)$, for two different sized molecules | 64 |
| Fig. 3.3 | A profile of the "model-free" spectral density function for biomolecules | 65 |
| Fig. 3.4 | The "wobbling-in-a-cone" model as represented by two coupled spins, $I$ and $S$, in a spherical isotropically rotating molecule | 72 |
| Fig. 3.5 | The peptide group is planar because the carbon-nitrogen bond has partial double bond character | 72 |
| Fig. 3.6 | The structure of the amino acid phenylalanine to show the $C^\beta$-$C'$ bond about which the aromatic ring undergoes $180^\circ$ flips | 73 |
Fig. 3.7  An example of the few possible rotamer states available to a methyl
group............................................................................................................74

Fig. 3.8  An example of the numerous unquantified interactions of a spin
surrounded by many others in a part of a protein.........................82

Fig. 3.9  Relaxation model for $^{15}$N in a protein backbone.................83

Fig. 3.10 Models for the relaxation of $^{13}$C in selectively labelled molecules....84

Fig. 3.11 The possible interactions of a $^{13}$C$^\alpha$ spin in uniformly $^{13}$C labelled
molecules....................................................................................................86

Fig. 3.12 A diagrammatic representation of how taking a linear combination of
three relaxation rates results in the isolation of the mutual dipolar
relaxation of a pair of spins from all other interactions.........................96

Fig. 3.13 A diagrammatic representation of how taking a linear combination of
four relaxation rates results in the isolation of three interactions from all
other interactions.......................................................................................97

Fig. 3.14 A diagrammatic representation of how taking a linear combination of
three relaxation rates results in the isolation of two interactions from all
other interactions.......................................................................................98

Fig. 3.15 Pulse sequences used to measure (a) longitudinal magnetisation and (b)
longitudinal two spin order relaxation rates.......................................101

Fig. 3.16 The structural formula of 7-methoxycoumarin............................104

Fig. 3.17 Plots of relaxation data against time for (a) H3 and H4 and (b) H5 and
H6 for a 0.1 M sample of 7-methoxycoumarin in CDCl3 at 298K...107
Fig. 4.1  A schematic representation of the return to equilibrium of species in both the presence and absence of cross-relaxation processes..........139

Fig. 4.2  The effect of the relative ordering of the mixing period and the evolution time on direct cross-relaxation.................................143

Fig. 4.3  The use of gradient pulses to select coherence transfer pathways with the conventional $^{15}$N (a) and the $^{13}$C (b) pulse sequence structure....147

Fig. 4.4  Pulse sequence used to measure $R_C(S_{CZ})$.............................................161

Fig. 4.5  Pulse sequence used to measure $R_H(I_{HZ})$..................................................176

Fig. 4.6  Cross-relaxation in $^{13}$CHH' groups can be overcome by encoding $^{13}$CH double quantum chemical shift evolution in the $t_j$ period..............166

Fig. 4.7  Pulse sequence used to measure $R_{CH}(2S_{CZ}I_{HZ})$........................................185

Fig. 4.8  Pulse sequence used to measure $R_{CHH}(4S_{CZ}I_{HZ}I_{HZ})$..............................196

Fig. 4.9  Pulse sequence used to measure $R_{HH}(2I_{HZ}I_{HZ})$........................................209

Fig. 4.10 Example plots of relaxation data against time for (a) $R_C(S_{CZ})$, $R_H(I_{SZ})$, $R_{CH}(2S_{CZ}I_{HZ})$, $R_{CHH}(4S_{CZ}I_{HZ}I_{HZ})$ and $R_{HH}(2I_{HZ}I_{HZ})$.................211

Fig. 5.1  A one-dimensional, (a), and two-dimensional, (b), representation of the secondary structure of domain II of Protein G..................................218
Fig. 5.2  The $^{13}$C$^\alpha$ T$_1$ data acquired on the Bruker DRX400 Spectrometer, using the pulse sequence given in Fig. 4.4 .......................................................222

Fig. 5.3  The $^{13}$C$^\beta$ T$_1$ data acquired on the Bruker DRX400 Spectrometer, using the pulse sequence given in Fig. 4.4 ......................................................223

Fig. 5.4  The $^{13}$C$^\alpha$-$^{13}$CH longitudinal two spin order data acquired on the Bruker DRX400 Spectrometer, using the pulse sequence given in Fig. 4.4 ...225

Fig. 5.5  One-dimensional proton spectra of domain II of Protein G ............230

Fig. 5.6  Examples of $^1$H$^\alpha$ T$_1$ data acquired at different times on the Bruker DRX400 Spectrometer using sample 3 of domain II of Protein G at 300K .........................................................................................................232

Fig. 5.7  Examples of one-dimensional, (a), and two-dimensional, (b), proton T$_1$ data of sample 3 of domain II of Protein G acquired on the Bruker DRX400 at 300K .................................................................234
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acq.</td>
<td>Acquisition</td>
</tr>
<tr>
<td>DQC</td>
<td>Double quantum coherence</td>
</tr>
<tr>
<td>FID</td>
<td>Free induction decay</td>
</tr>
<tr>
<td>FT</td>
<td>Fourier transform</td>
</tr>
<tr>
<td>gp</td>
<td>Gradient pulse</td>
</tr>
<tr>
<td>$I^+, I^-$</td>
<td>Raising and lowering operators</td>
</tr>
<tr>
<td>$I$</td>
<td>Sensitive nuclei (typically protons)</td>
</tr>
<tr>
<td>MQC</td>
<td>Multiple quantum coherence</td>
</tr>
<tr>
<td>$S$</td>
<td>Insensitive nuclei (typically heteronuclei such as $^{13}\text{C}$ and $^{15}\text{N}$)</td>
</tr>
<tr>
<td>$r_{kl}$</td>
<td>Internuclear vector between spins $k$ and $l$</td>
</tr>
<tr>
<td>$J_{kl}$</td>
<td>Scalar coupling between spins $k$ and $l$</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>$S^2$</td>
<td>Order parameter</td>
</tr>
<tr>
<td>$t_1$</td>
<td>Incremental delay for a two-dimensional experiment</td>
</tr>
<tr>
<td>$t_2$</td>
<td>Data acquisition time</td>
</tr>
<tr>
<td>$t_m$</td>
<td>Mixing delay in a two-dimensional experiment</td>
</tr>
<tr>
<td>$T_1$</td>
<td>Longitudinal relaxation time</td>
</tr>
<tr>
<td>$T_2$</td>
<td>Transverse relaxation time</td>
</tr>
<tr>
<td>TQC</td>
<td>Triple quantum coherence</td>
</tr>
<tr>
<td>ZQC</td>
<td>Zero quantum coherence</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Gyromagnetic ratio</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Correlation time for molecular tumbling</td>
</tr>
<tr>
<td>$\omega_k$</td>
<td>Lamor frequency of spin $k$ (radians)</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

There are many people who have contributed to this work, both directly and indirectly. They fall into three groups: my colleagues, my friends and my family.

I am particularly grateful to my supervisors Timothy J. Norwood and Lu-Yun Lian for their enthusiasm, motivation and eagerness to help at all hours. I would like to thank past and present members of the NMR groups whose stay in Leicester has coincided with mine: Simon A. Clark, David L. Hardy, Ben S. Hickman, Gisella H. E. Scott, Annette M. Hultaker and Marcus L. Tillet for creating an interesting atmosphere to work in, and Igor L. Barsukov, Kong Hong Sze ("Spud"), Andy Preston and Gerry A. Griffith for technical support.

During the struggles of the past few years I have been blessed with many friends whose encouragement and support have been invaluable. I shall not mention any names but their touch will remain indelible.

I thank all the members of my immediate and extended family for the patience and consideration they have shown.

Amirah Chaudhry
Leicester, 1997
To Mother and Milton
Inspirations may crumble,
what has been taught may vanish
and what has been learnt may fade
in the bowels of time.
What is left is illuminator of countless candles,
without losing a flicker of brilliance.

Ben Emeneonu
CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

High resolution liquid state NMR is a tool of increasing use and importance in Chemistry and Biochemistry. Its uses include identification of compounds, determining the kinetics of processes, measuring ligand binding and ionisation states, obtaining structural information, gaining insight into molecular dynamics and studies of spatial distribution of molecules (1). In its simplest form, it involves determining the resonance spectrum of a given NMR-sensitive species ($^1$H, $^{13}$C, $^{15}$N, $^{19}$F, $^{31}$P,) either by irradiating the sample with low intensity radio-frequency (RF) and sweeping the field through resonance, or in a modern way by Fourier transforming (FT) the free-induction decay (FID) obtained after applying a high intensity RF pulse.

A drastic change has occurred over the last two decades resulting from new methods such as polarisation transfer, multiple quantum coherence and a variety of multi-dimensional spectroscopy methods; and from the availability of high-field spectrometers and fast and powerful computers. These methods and technological advances together with advances in molecular biology and recombinant DNA technologies, have produced a qualitative change in NMR by allowing the detailed study of large and complex molecules: much larger and much more complex than was previously possible.

The theory of NMR relaxation in scalar coupled spin systems has been unambiguously confirmed by experimental work since its formulation forty years ago (2-6). Hence in non-viscous isotropic liquids the framework for spin dynamics is well established. However, considerable efforts continue to be expended in this area. Until
about the mid-nineteen-seventies, it was only possible to analyse the relaxation behaviour of the very simplest spin systems with a few appropriate analytical expressions. Furthermore, most of these expressions were derived under conditions which assume molecular dynamics to be unduly simplified. Since then, a more detailed analysis of relaxation in relatively complex spin systems has been a powerful tool for elucidating molecular dynamics - anisotropic molecular reorientation, internal rotation and intermolecular interactions. Additionally, the information obtained about molecular reorientation can be used to determine other physical properties of the molecule, such as chemical shielding, spin-rotation and electric quadrupole coupling tensors for other spins in the molecule (7).

In macromolecules such as proteins and nucleic acids, the application of NMR relaxation to study dynamics in solution has been a recent focus of increasing attention. This is due to the ready availability of isotopically labelled samples together with the improved resolution and sensitivity of multi-dimensional heteronuclear spectroscopy which have accelerated the study of protein dynamics by two-dimensional NMR (8).

In general, obtaining dynamics information from NMR relaxation data involves three steps, once the structural assignment is complete. First, the relaxation interactions relevant to the system under study are identified. Second, the appropriate experiments are chosen, or designed, to make the necessary measurements as precisely and reproducibly as possible. Finally, the observed relaxation rates, once analysed using the most appropriate method, are related to the dynamics of the system.

Conventionally, the heteronuclear $T_1$, $T_2$ and NOE are measured. These three measurements, in themselves, are not sufficient to completely characterise the motions of the bond and therefore the last step proves to be difficult without making some a priori model assumptions about the dynamics. In solution to this limitation, a variety of motional models for the dynamics of XH (where X is $^{15}$N or $^{13}$C) bonds in proteins have been used (8-18). These models provide a set of motional parameters which are optimised to fit the observed data. The optimised motional parameters are then used to describe the dynamics of the protein. Even in cases where these models may be
reasonable, ideally it should not be necessary to introduce the assumptions which are implicit in them.

An alternative approach (to making model assumptions) that directly exploits the dynamics information contained in the relaxation rates was presented a few years ago (19,20). This approach involves making six independent relaxation measurements to provide additional experimental constraints so as to obtain a set of linear equations relating to the individual spectral density components. Hence the spectral density function can be mapped.

The dynamics of the protein backbone can in theory, be monitored by using a complete set of amide $^{15}$N or $^{13}$C$\alpha$ relaxation parameters. However, in some protein-ligand and protein-protein complexes the relaxation of the amide $^{15}$N or $^{13}$C$\alpha$ spins may only give limited information which might not be sufficient to locate the internuclear interactions. In such cases the desired information may be obtained from the relaxation parameter of $^{13}$C spins along the side-chains of the proteins, since these are directly exposed to the environment. It would be ideal to make these measurements on a uniformly $^{13}$C labelled sample which was also used to elucidate the three-dimensional structure. Unfortunately, $^{13}$C-$^{13}$C scalar coupling evolution makes the T$_2$ measurement problematic. In addition, $^{13}$C dipolar interactions are often numerous, difficult to quantify and therefore difficult to analyse. Consequently, a replacement for the $^{13}$C T$_2$ measurement and other ways of analysing the $^{13}$C data obtained need to be sought.
1.2 THE CONTRIBUTION OF THIS THESIS TO NMR RELAXATION OF BIOMOLECULES

To date, regardless of whether or not a model is used for analysis, the numerous dipolar relaxation interactions of a particular spin are reduced to a few which can be quantified. This is done on the assumption that the neglected interactions make an insignificant contribution to relaxation. We looked at the system from a new perspective; we wanted to make full use of the information implicit in NMR relaxation data and consider all possible dipolar interactions, no matter how small and insignificant, or numerous and complicated they may be. We asked how we could measure the mutual relaxation of an arbitrary set of coupled nuclear spins. To answer this question we found it was necessary to develop a new method to separate the relaxation of a particular set of spins from all other interactions. To this end we have developed a new method which exploits the symmetry inherent in elements of the longitudinal relaxation sub-matrix by taking linear combinations of selected elements in a way that isolates only the dipolar interaction of interest between the active spins (21,22). All other interactions between the active spins and others spins simply cancel out.

Our method of analysis involves making five independent relaxation measurements for $^{13}$CH$_2$ and $^{13}$CH$_3$ groups: only three of these can be made for $^{13}$CH groups. For the latter system, the three measurements can be combined to isolate the dipolar interaction between the $^{13}$C and its bonded proton. For CHH' groups, three dipolar interactions can be isolated by using particular combinations each involving three of the five experiments. The dipolar interactions which can be isolated are the $^{13}$C-H, $^{13}$C-H' and H-H'. Our method can also be applied to methyl groups.

Since the new method involves taking a linear combination of up to three relaxation measurements, errors in the individual measurements can rapidly accumulate to make the isolated interactions relatively inaccurate. Therefore it is imperative that
the necessary measurements are made as accurately as possible. Of the five necessary relaxation measurements only one, the $^{13}\text{C} \ T_1$, was previously available in the literature but it needed modification for use on uniformly $^{13}\text{C}$ labelled samples. The remaining four measurements had not been made previously.

The bulk of this project involved designing new two-dimensional heteronuclear experiments to implement the proposed method of analysis. A prime objective of the experiments is to make the measurement as accurately as possible. Consequently, considerable measures have been taken to ensure that unwanted coherence transfer pathways, be they from imperfect RF pulses or from unwanted cross-relaxation processes, are significantly suppressed; water signal, which may distort any desired peaks close to its resonance in the two-dimensional spectrum, has been reduced; and gradient pulses have been used, where possible, in addition to phase cycling to efficiently select the coherence transfer pathway of interest.

1.3 OVERVIEW OF THIS THESIS

The subject matter of this thesis is organised into five chapters, after this introduction. These are as follows:-

Chapter Two. *The fundamental principles of nuclear magnetic resonance*. This chapter reviews the theory relevant for understanding the ideas which will be developed in later chapters. The theory is divided into two main parts; general concepts of NMR and the theory of relaxation. The former section gives an overview of the basic origins of NMR, the significance of FT in converting a time domain signal into frequency domain and two models for understanding NMR experiments; the product operator formalism and raising and lowering operators. It also includes a short discussion on the importance of two-dimensional experiments and how heteronuclear experiments can be designed with a knowledge of coherence transfer pathway selection. The latter part of this chapter provides the phenomenological aspects of relaxation (the definition of relaxation, the density operator and the
relaxation matrix) followed by the relaxation Hamiltonian and a more detailed overview of the nature of relaxation.

Chapter Three. *NMR relaxation and molecular dynamics.* This chapter is divided into three parts. The first part gives a short and general review of the application of NMR relaxation to proteins, how molecular motion is quantified and how the spectral density function can be “modelled” using some *a priori* assumptions or “mapped” using the Peng and Wagner (19,20) approach. The second section specifically describes the practical implementation of $^{15}$N and $^{13}$C relaxation measurements and highlights the drawbacks of existing analytical methods. Then the derivation of the new method of analysis is described, from complicated general relaxation rates of longitudinal magnetisation and various longitudinal spin orders, to descriptions and diagrammatic representations of the individual dipolar interactions isolated in a set of two and three spins. The third section demonstrates the principle of the new method on a small molecule with two AX spin systems. This chapter concludes with a discussion of the application of the new method to molecules of biological importance and the valuable information which can be obtained with this simple approach.

Chapter Four. *Pulse sequences designed to make $^1$H and $^{13}$C relaxation measurements.* The major part of the work undertaken in this study is presented in detail in this chapter. There is a discussion on which measurements need to be made to implement the new analytical method and why it is important to make them as accurately as possible. All five experiments used in this study have been designed to suppress all possible processes which may increase the inaccuracy of the measurements. These are considered generally in the first main section of chapter four. The following five sections discuss the individual experiments using, mainly, the product operator formalism. For each experiment, the path taken by the desired coherence is described, followed by how unwanted coherences (from imperfect RF pulses, cross-relaxation processes and the water signal) have been suppressed by the sophisticated use of gradient pulses, RF pulses and phase cycling.
Chapter Five. *Results and Discussion.* The practical aspect of this work is described in this chapter. Aspects such as the identity of and the reasons for choosing the biomolecule which was used to develop the pulse sequences and which fit, of the two that were discussed in Chapter Four, fits the data better. Additionally, the limited experimental data is presented followed by an account of the unfortunate problems which were the reason for the shortage of results. Finally, the limited data is analysed to gain information on molecular dynamics.

Chapter Six. *Conclusions.* This chapter aims to provide a brief summary of this thesis and the contribution it has made to the area of $^{13}$C NMR relaxation. The work covered in this thesis will be discussed in terms of meeting the objective of this project. Additionally, a brief overview of the results obtained, the practical problems encountered during this project and possible future work are discussed.
1.4 REFERENCES


CHAPTER TWO

THE FUNDAMENTAL PRINCIPLES OF NUCLEAR MAGNETIC RESONANCE

2.1 INTRODUCTION

This chapter briefly explains the theory relevant for understanding the ideas which will be developed in later chapters. The theory is divided into two parts, general concepts of NMR and the theory of relaxation. The former section gives an overview of the basic origins of NMR, the significance of Fourier Transform in converting a time domain signal into frequency domain and two models for understanding NMR experiments; the product operator formalism and raising and lowering operators. It also includes a short discussion on the importance of two-dimensional experiments and how heteronuclear experiments can be designed with a knowledge of coherence transfer pathway selection. The latter part of this chapter provides the phenomenological aspects of relaxation (the definition of relaxation, the density operator and the relaxation matrix) followed by the relaxation Hamiltonian and a more detailed overview of the nature of relaxation.

2.2 GENERAL CONCEPTS

2.2.1 BASIC THEORY OF NMR

Most nuclei possess a nuclear or intrinsic angular momentum $\mathbf{p}$ \((1-3)\). According to the classical picture the atomic nucleus, assumed to be spherical, rotates about an axis. Quantum mechanical considerations \((4,5)\) show that, like many other atomic quantities, this angular momentum is quantised:-
Here \( \hbar = \frac{\hbar}{2\pi} \), where \( \hbar \) is Planck's constant, and \( I \) is the angular momentum quantum number, usually called simply the nuclear spin. The nuclear spin can have the values \( I = 0, \frac{1}{2}, \frac{3}{2}, 2, \text{ etc.} \)

The NMR phenomenon is a consequence of the existence of nuclear spin. Not all nuclei have spin, but those that do also have an associated magnetic field and hence a nuclear magnetic dipole moment, \( \mu \):

\[
\mu = \gamma \mathbf{P}
\]

where \( \gamma \) is a constant for each nuclide and is called the gyromagnetic ratio, or sometimes the magnetogyric ratio. The detection sensitivity of a nuclide in an NMR experiment depends on \( \gamma \); nuclides with a large \( \gamma \) are said to be sensitive (i.e. easy to observe), while those with a small \( \gamma \) are said to be insensitive.

By combining equations 2.1 and 2.2 we obtain for the magnetic moment:

\[
\mu = \gamma \hbar (I(I+1))^{1/2}
\]

Nuclides with spin \( I = 0 \) therefore have no nuclear magnetic moment. Two very important facts for our purpose are that the \( ^{12}\text{C} \) isotope of carbon and the \( ^{16}\text{O} \) isotope of oxygen belong to this type of nuclides - this means that the main building blocks of organic/biological compounds cannot be observed by NMR spectroscopy.

For most nuclides the \( \mathbf{P} \) and \( \mu \) vectors point along the same direction, i.e. they are parallel. However, in a few cases, for example \( ^{15}\text{N} \), they are anti-parallel. Consequently the gyromagnetic ratio of \( ^{15}\text{N} \) has a negative value.
If a nucleus with angular momentum $\mathbf{P}$ and magnetic moment $\mu$ is placed in a static magnetic field $B_0$, the energy of the nucleus becomes orientation dependent. The angular momentum takes up an orientation such that its component $P_z$ along the direction of the field is an integral or half-integral multiple of $\hbar$:

$$P_z = m\hbar$$  \[2.4\]

Here $m$ is the magnetic or directional quantum number and can take any of the values $m = I$, $(I-1)$, $(I-2)$ ... $-I$. Hence there are $(2I+1)$ possible different orientations of the angular momentum and hence the magnetic moment, in the magnetic field. We shall be directly concerned only with spin $\frac{1}{2}$ nuclei, that is nuclei such as $^1\text{H}$, $^{13}\text{C}$ and $^{15}\text{N}$ for which $I = \frac{1}{2}$. For such nuclei there are only two possible energy levels, often called $\alpha$ and $\beta$. These are low and high energy states, respectively and correspond to two particular orientations of $\mu$ relative to the static magnetic field axis. The $\alpha$ state, being at a lower energy, will have a greater population than the $\beta$ state. These two energy levels are shown schematically, using a two cone representation, in Fig. 2.1.

Fig. 2.1 A two cone representation of the $\alpha$- and $\beta$-states for a single spin system with $I = \frac{1}{2}$, showing the slightly higher population of the lower energy $\alpha$-state.
According to the classical picture (6), a nucleus with $I = \frac{1}{2}$ precesses around the z field axis on the surface of a double cone as shown in Fig. 2.1. Since there is a slight Boltzmann excess of nuclei aligned with the magnetic field, these will give rise to a macroscopic magnetisation $M_0$ along the direction of $B_0$ at thermal equilibrium (Fig. 2.2). The vector $M_0$ plays an important role in the description of all types of pulsed NMR experiments.

In the classical representation the nuclear dipoles precess around the z-axis, which is in the direction of the magnetic field; their behaviour resembles that of a spinning top. The precession frequency $\omega$ is proportional to the applied magnetic field strength: 

$$\omega = \gamma B_0 \quad [2.5]$$

Eqn. 2.5 is not strictly true since the nucleus also experiences a shielding effect from the applied magnetic field. The shielding occurs as a result of the external field inducing a circulation in the electrons surrounding the nucleus. This in turn produces a local magnetic field, at the nucleus, which opposes the external magnetic field. If the amplitude of the opposing field is represented by a shielding constant, $\sigma$, then the nucleus experiences a magnetic field $B_0(1-\sigma)$. Consequently Eqn. 2.5 can be re-written as:-
\[ \omega = \gamma B_0(1-\sigma) \]  

From Eqn. 2.6 it can be seen that the Larmor frequency is determined by \( \sigma \) which depends on the electronic and chemical environment of the spin. This property is known as \textit{chemical shift}.

Atoms which have anisotropic bonds produce a different local field at the nucleus depending on the orientation of the bond relative to \( B_0 \). Therefore chemical shift will change as the orientation of the molecule changes. This effect is known as chemical shift anisotropy (CSA). In liquids, all orientations are adopted by a molecule in a short time; hence CSA usually averages to zero and it is normally ignored.

Like chemical shift, \textit{scalar coupling}, represented by the constant \( J \), is another source of fine structure in NMR spectra. It describes the mutual through-bond interaction of a pair of spins which are separated by a few bonds in the molecule. Fig. 2.3 shows this interaction for a two spin system consisting of spins \( k \) and \( l \). The magnetic moment of nucleus \( k \) causes the bonding electrons to be weakly polarised. This is transmitted to spin \( l \) through the overlapping bonding orbitals. Consequently, the effective magnetic field experienced by spin \( l \) is affected and hence its transition energy changes. Each of the two energy states of spin \( k \), for example, will split the transition energy of the other into two: \( (\omega_k + \frac{J_{kl}}{2}) \) and \( (\omega_k - \frac{J_{kl}}{2}) \), where \( J_{kl} \) is the scalar coupling constant between the two spins. This will be seen as a doublet separated by a coupling constant \( J_{kl} \) in the spectrum. Scalar coupling is particularly useful for investigating the constitution of organic compounds and for spectral assignment. The size of \( J_{kl} \) is dependent on the torsional angle of the intervening bond. Hence it provides important conformational information (7-10).
 Scalar coupling is the interaction of the magnetic moment of two spins through bonding electrons. A dipolar coupling is a similar interaction but it acts through space. The local magnetic field, $B_{loc}$, which is generated at the site of spin $k$ by spin $l$ is given by:

$$B_{loc}^k = \mu_l (3\cos^2 \theta_{kl} - 1) r_{kl}^{-3} \quad [2.7]$$

where $\theta_{kl}$ is the angle between the internuclear vector and the $B_0$ field, and $r_{kl}$ is the internuclear distance.

Since molecules in solution are in rapid reorientation, the average value of dipolar couplings is zero. Nevertheless at any given instant in time the dipolar field is not zero since rapid molecular tumbling leads to a fluctuating magnetic field being
produced at the sites of the two nuclei. This results in the exchange of spin polarisation and causes relaxation which will be discussed later.

Dipolar couplings also generate mutual relaxation between spatially neighbouring nuclei giving rise to the nuclear Overhauser enhancement (NOE) effect (11-16). The NOE is a variation in the intensity of a signal resulting from a disturbance in the populations of another nucleus. It depends on the distance between the nuclei and thereby allows the determination of interatomic distances (17-22) making an important contribution to the elucidation of three-dimensional structures.

2.2.2 THE PRINCIPLES OF FOURIER TRANSFORM NMR

The current sub-section begins by introducing the concept of a rotating reference frame followed by a detailed description of the effect of radio frequency pulses. It concludes with the detection and transformation of a signal into a frequency domain spectrum.

So far $M_0$ has been viewed precessing at the Larmor frequency $\omega_0$, in the laboratory frame. If, however, the laboratory is rotated at $\omega_0$ about $B_0$, then the nuclei would no longer appear to precess but would become stationary as shown in Fig. 2.4. The magnetic behaviour is now completely described by a stationary bulk magnetisation vector $M_0$ acting along $B_0$. In reality a spectrum contains a number of resonances with individual Larmor frequencies that differ slightly from $\omega_0$. This system is referred to as a rotating reference frame.
At equilibrium there is a net magnetisation vector along the direction of $B_0$ (Fig. 2.4) and the spins are pointing in different directions in the cone (Fig. 2.1) i.e. the angle between the spins in the $\alpha$– and $\beta$-states varies across the sample, therefore there is no phase coherence.

In a typical NMR experiment, the magnetisation is perturbed from equilibrium and any transverse signal which is generated is observed during a detection period (23,24). The magnetism is displaced or excited from its equilibrium position by a second, weaker magnetic field $B_1$. This originates from a pulse of coherent radio frequency (RF) radiation. If this is applied along the $y$-axis, a component of the net magnetisation vector, $M_0$, will be rotated onto the $x$-axis as shown in Fig. 2.5. The angle of rotation, $\theta$, will depend on the value of $B_1$ and the time it is applied for. The component rotated onto the $x$-axis is equal to $M_0\sin\theta$ and that remaining along the $z$-axis will be $M_0\cos\theta$. If the spectrometer were only to detect signals along the $x$ axis, the maximum signal will be obtained when $\theta = 90^\circ$. The angle of a pulse and the phase of the axis about which $M_0$ is rotated can be chosen. A pulse which tips the bulk magnetisation vector through $90^\circ$ about the $y$-axis is known as a $90^\circ_y$. This will
rotate \( \mathbf{M}_0 \) onto the x-axis. The angles used in typical NMR experiments are 90° and 180°. These pulses can be applied along any axis in the xy-plane.

\[ \left( \begin{array}{c} \mathbf{M}_0 \\ x \\ y \end{array} \right) \]

**Fig. 2.5** Vector representation of the effect of a pulse of coherent radio frequency radiation. At equilibrium, (a), \( \mathbf{M}_0 \) is along the z-axis and there is no phase coherence in the xy-plane. After applying a 90° pulse, (b), \( \mathbf{M}_0 \) is rotated into the xy-plane initially along the x-axis with an amplitude of \( \mathbf{M}_0 \) sin 90°. There is phase coherence in the xy-plane.

Once the RF pulse has been applied, there is a constant angle between the spins in the \( \alpha \)- and \( \beta \)-states and the system is said to have phase coherence. The perturbed net magnetisation will immediately begin to relax back to its equilibrium position (25) by means of two types of relaxation. The component of magnetisation left along the z-axis relaxes back to its equilibrium value, via transitions between the two states, to its original value \( \mathbf{M}_0 \). This is known as longitudinal or \( T_1 \) relaxation.

In the second type of relaxation the spins exchange energy with each other so that some precess momentarily faster than others. This results in the component of magnetisation along the y-axis "fanning out" into the transverse plane, i.e. the phases of the spins become randomised and there is loss of phase coherence. This type of relaxation is called transverse or \( T_2 \) relaxation.

Both types of relaxation give rise to a roughly exponential decay of the magnetisation to equilibrium. \( T_2 \) relaxation is responsible for the decay of the observed signal in the xy-plane. The decaying transverse magnetisation is detected through the signal it induces in a receiver coil. This signal is known as the *free induction decay* (FID). The FID is a time domain signal, i.e. it is a measurement of
intensity as a function of time. It is converted into a more useful frequency domain
signal by means of a mathematical process known as Fourier Transformation (FT) (26):-

\[ F(\omega) = \int_{-\infty}^{\infty} f(t) \exp^{-i\omega t} \, dt \]  

[2.8]

where \( F(\omega) \) is a function of frequency and \( f(t) \) is the corresponding function of time.

2.2.3 PRODUCT OPERATORS AND THEIR USES

There are a number of models which can be used to describe the desired
cohere transfer pathway in a particular pulse sequence. These range from the
simplified classical or semiclassical vector models to the more complicated density
operator theory. The former are inherently limited when describing sophisticated
techniques (e.g. MQC), while the latter is too complicated. An approach which
follows the middle course is the product operator formalism which is presented here.

The product operator formalism (27-29) can be used to visualise the effect of
a sequence of pulses and delays on nuclear magnetisation. This formalism is based
on the product of the angular momentum operators \( I_x, I_y \) and \( I_z \) of individual spins in
the system. The following is a description of how the net magnetisation vector is
influenced by radio frequency pulses applied to the sample. The effect of chemical
shift and scalar coupling evolution during the delays in a sequence is also discussed.

A system with \( N \) spins will consist of \( 4^N \) product operators. As an example, a
two spin system, with spins \( k \) and \( l \), will have 16 product operators. These are:
\[ q=0 \quad \frac{1}{2} E \quad (E = \text{unity operator}) \]

\[ I_{kz}, I_{lz} \]

Longitudinal Magnetisation.

\[ q=1 \quad I_{kx}, I_{ky}, I_{lx}, I_{ly} \]

In-phase Single Quantum Coherence (SQC).

\[ q=2 \quad 2I_{kx}I_{lx}, 2I_{kx}I_{ly}, 2I_{ky}I_{lx}, 2I_{ky}I_{ly} \]

Two Spin Coherences - Linear Combinations of Zero Quantum Coherence (ZQC) and Double Quantum Coherence (DQC).

\[ 2I_{kx}I_{lz}, 2I_{ky}I_{lz}, 2I_{kz}I_{lx}, 2I_{kz}I_{ly} \]

Anti-phase SQC.

\[ 2I_{kz}I_{lz} \]

Longitudinal Two Spin Order.

where \( q \) is the number of operators in the product. These operators have physical meanings in terms of the signals that can be observed. For example, the operator \( I_{kz} \) describes longitudinal magnetisation of spin \( k \) (present at equilibrium). The transverse operators \( I_{kx} \) and \( I_{ky} \) describe in-phase \( x \)- and \( y \)-magnetisation (SQC), respectively, of spin \( k \). These two operators are observable. The operator \( 2I_{kx}I_{lz} \) describes \( x \)-magnetisation of spin \( k \) anti-phase with respect to spin \( l \), (anti-phase SQC). Anti-phase magnetisation consists of multiplets where individual peaks in the spectrum have opposite phases. Operators like \( 2I_{kx}I_{lx} \) and \( 2I_{kx}I_{ly} \) represent linear combinations of two spin coherences in the xy-plane (mixture of ZQC and DQC). The operator \( 2I_{kz}I_{lz} \) describes longitudinal two spin order of spins \( k \) and \( l \). This is a non-equilibrium population distribution which does not have a net magnetisation vector associated with it.
In addition to the above, an operator which will be useful for later discussions is $4I_{kz}I_{lz}I_{mx}$. This is longitudinal three spin order between spins $k$, $l$ and $m$: it can only occur in systems with three or more spins.

Product operators can also describe a heteronuclear $IS$ system where $S = \frac{1}{2}$ heterospin (e.g. $^{13}$C or $^{15}$N). For example, in a $^{13}$CH group, $S_{Cz}$ is longitudinal magnetisation of $^{13}$C. Transverse magnetisation of $^{13}$C can be described by $S_{Cx}$ and $S_{Cy}$. $2I_{Hz}S_{Cz}$ represents x-coherence of proton anti-phase with respect to $^{13}$C. In a $^{13}$CH$_2$ group $4I_{Hz}I_{Hz}S_{Cz}$ is the longitudinal three spin order of all three spins. Another additional term in a methylene group, which is not possible in a $^{13}$CH group, is longitudinal two spin order of the two protons: $2I_{Hz}I_{Hz}$. The importance of this operator will be considered later.

2.2.3.1 Effects of Applied Radio Frequency Pulses

At equilibrium the magnetisation of a spin, $k$, can be described as $I_{kz}$. When an RF pulse, with a flip angle of $\beta$, is applied about the y-axis (commonly represented as $\beta_y$) the equilibrium state will be perturbed. If an RF pulse is specific for a particular spin, e.g. for $k$ spins, it can be represented by $\beta_y(k)$. On applying an RF pulse $I_{kz}$ will undergo the following transformation:

$$ I_{kz} \xrightarrow{\beta_y} I_{kz}\cos\beta + I_{kx}\sin\beta \quad [2.9] $$
$I_{kz}$ is partially rotated along the $x$-axis giving $I_{kx}$. The size of the components of magnetisation left along the $z$-axis is equal to $\cos \beta$ and that rotated on to the $x$-axis is equal to $\sin \beta$. When $\beta = 90^\circ$, $I_{kz}$ will be completely transformed into $I_{kx}$.

The effects of this pulse on $I_{kx}$ and $I_{ky}$ are given below:

\[ I_{kx} \xrightarrow{\beta_y} I_{kx} \cos \beta - I_{kz} \sin \beta \]  \[2.10\]

\[ I_{ky} \xrightarrow{\beta_y} I_{ky} \]  \[2.11\]

$I_{kx}$ is partially transformed into $-I_{kz}$ and partially left along the $x$-axis. The sign convention observed here is shown in Fig. 2.6. Since this RF pulse is applied along the $y$-axis, any magnetisation already along that axis (i.e. $I_{ky}$) will not be affected by the pulse and therefore remain unchanged.

The effects of rotation about the $x$-axis can be described in a similar fashion.
Fig. 2.6 Sign convention for x- and y- pulses, chemical shift evolution and scalar coupling evolution.

The directions of rotation shown are for nuclei with a positive value of $\gamma$ (e.g. $^1$H and $^{13}$C). The rotations will be in the opposite direction when considering nuclei with negative values of $\gamma$ (e.g. $^{15}$N).

When a pulse is applied to an operator consisting of more than one spin, the overall effect is calculated by first determining the individual single spin transformations and then multiplying the results together. For example:

\[
2I_{x}I_{x} \xrightarrow{\beta_{y}} 2(I_{x} \cos \beta + I_{x} \sin \beta) (I_{x} \cos \beta - I_{z} \sin \beta)
\]

\[
\downarrow
\]

\[
2I_{x}I_{x} \cos^{2} \beta + 2I_{x}I_{x} \sin \cdot \cos \beta - 2I_{x}I_{x} \cos \cdot \sin \beta - 2I_{x}I_{x} \sin^{2} \beta \quad [2.12]
\]
2.2.3.2 Chemical Shift Evolution

During a delay $\tau$, transverse magnetisation $I_{kx}$ will evolve due to its characteristic precession frequency, $\omega_k$, about the z-axis:

$$I_{kx} \xrightarrow{\omega_k \tau} I_{kx} \cos \omega_k \tau + I_{ky} \sin \omega_k \tau$$  \hspace{1cm} [2.13]

One component of $I_{kx}$ magnetisation remains along the x-axis while another component evolves onto the y-axis. The amplitude of these components are $\cos \omega_k \tau$ and $\sin \omega_k \tau$, respectively. The sign conventions for chemical shift evolution are given in Fig. 2.6.

Anti-phase SQC will evolve due to chemical shift evolution as follows:

$$2I_{kx}I_{lz} \xrightarrow{\omega_k \tau} 2I_{kx}I_{lz} \cos \omega_k \tau + 2I_{ky}I_{lz} \sin \omega_k \tau$$  \hspace{1cm} [2.14]

Only the spin in the xy-plane (i.e. spin $k$) will undergo chemical shift evolution. The spin along the z-axis will remain unaffected.

The overall effect due to chemical shift evolution of an operator which has more than one spin in the xy-plane can be calculated by multiplying the individual single spin evolutions as shown above in Eqn. 2.12.
2.2.3.3 Scalar Coupling Evolution

Scalar coupling evolution interconverts components of in-phase and anti-phase coherence. A transverse operator can evolve as a result of scalar couplings during a time \( t \) about the z-axis, \( \pi J_{kl} t^2 I_{kz} I_{lz} \), as follows:

\[
I_{kx} \xrightarrow{\pi J_{kl} t^2 I_{kz} I_{lz}} I_{kx} \cos \pi J_{kl} t + 2I_{ky} I_{lz} \sin \pi J_{kl} t
\]  

[2.15]

After the delay there will be a mixture of in-phase \( (I_{kx}) \) and antiphase \( (2I_{ky} I_{lz}) \) components of magnetisation. Their sizes will depend, respectively, on \( \cos \pi J_{kl} t \) and \( \sin \pi J_{kl} t \). Fig. 2.6 shows the sign convention for scalar coupling evolution.

If a second scalar coupling is present, in-phase and anti-phase magnetisation, if left for a further delay \( t' \), can evolve due to a two bond scalar coupling, \( \pi J_{km} t' I_{kz} I_{mz} \), as shown in transformations 2.16 and 2.17:

\[
I_{kx} \cos \pi J_{kl} t \xrightarrow{\pi J_{km} t' I_{kz} I_{mz}} I_{kx} \cos \pi J_{kl} t \cos \pi J_{km} t' + 2I_{ky} I_{lz} \cos \pi J_{kl} t \sin \pi J_{km} t'
\]  

[2.16]

\[
2I_{ky} I_{lz} \sin \pi J_{kl} t \xrightarrow{\pi J_{km} t' I_{kz} I_{mz}} 2I_{ky} I_{lz} \sin \pi J_{kl} t \cos \pi J_{km} t' - 4I_{kx} I_{lz} I_{mz} \sin \pi J_{kl} \sin \pi J_{km} t'
\]  

[2.17]

This transformation results in \(-4I_{kx} I_{lz} I_{mz}\) which is magnetisation of spin \( k \) antiphase with respect to spins \( l \) and \( m \). As with chemical shift evolution, only transverse operators will evolve under the influence of scalar couplings. However, product operators of two transverse operators (e.g. \( 2I_{kx} I_{lx}, 2I_{kx} I_{ly} \) and \( 2I_{ky} I_{ly} \)) or of two
longitudinal operators (e.g. $2I_{kz}l_{iz}$) will not evolve due to the mutual scalar couplings of the active spins.

$$2I_{kx}l_{ix} \xrightarrow{\pi I_{ix}} 2I_{kx}l_{ix}$$ \[2.18\]

$$2I_{kz}l_{iz} \xrightarrow{i I_{iz}} 2I_{kz}l_{iz}$$ \[2.19\]

Individual couplings and chemicals shifts in a multi-spin system operate separately and in any order in a given period of time.

2.2.4 RAISING AND LOWERING OPERATORS

The effects of a pulse sequence on nuclear magnetisation can be described with the product operator formalism. However, with this formalism the selected coherence transfer pathway and the effect of gradient pulses are not immediately apparent. This limitation of the product operator formalism is overcome by using raising and lowering operators.

Raising and lowering operators (29-34) respectively increase and decrease the quantum number of a coherence order ($p$) by absorbing or emitting a quantum of energy. They are defined in terms of product operators by:

$$I_k^+ = I_{kx} + il_{ky}$$ \[2.19\]

$$I_k^- = I_{kx} - il_{ky}$$ \[2.20\]

The operator $I_k^+$ relates to +SQC of spin $k$ precessing at $+\omega_k$ while $I_k^-$ relates to −SQC of spin $k$ precessing at $-\omega_k$. In an experiment both $I_k^+$ and $I_k^-$ are excited but
only \( I_k^- \) (i.e. \( p = -1 \)) is detected. Raising and lowering operators are especially useful in the description of MQC (e.g. ZQC and DQC). For example, the MQC product operator \( 2I_{kx}I_{ly} \) is a superposition of ±DQC and ZQC which becomes clear when it is written in raising and lowering operators:

\[
2I_{kx}I_{ly} = \frac{1}{2i} \left( I_k^+ I_l^+ - I_k^- I_l^- \right) - \frac{1}{2} \left( I_k^+ I_l^- - I_k^- I_l^+ \right)
\]

\([2.21]\)

\( \left( I_k^+ I_l^+ - I_k^- I_l^- \right) \) is pure DQC \((p=\pm2)\) whereas \( \left( I_k^+ I_l^- - I_k^- I_l^+ \right) \) is pure ZQC \((p=0)\).

The sign of a coherence is explicit when it is written in the raising and lowering operator formalism, unlike with product operators. For example, if the coherence transfer process \( I_k^+ \rightarrow I_k^- \) is to be selected, it is immediately apparent in raising and lowering operators. In the product operator formalism the same process may be represented as \( I_{kx} \rightarrow I_{ky} \) which could equally well be \( I^- \rightarrow I^+ \) or \( I^- \rightarrow I^- \). Although the product operators can be manipulated to determine the coherence transfer pathway selected, it is not immediately apparent.

The explicit representation of coherence by raising and lowering operators makes them particularly useful for understanding the effects of gradient pulses (see sub-section 2.2.6.2).
2.2.5 TWO-DIMENSIONAL NMR SPECTROSCOPY

In general, the information content of NMR spectra depends on the experiment used. If detailed structural and dynamic properties of a molecule are to be understood it is desirable for resonances corresponding to individual spins to be resolved. In one-dimensional experiments, resonances of a small peptide can typically be resolved; in proteins the large number of resonances and increased linewidths severely limit the resolution (35). However, overlapping resonances can be resolved by spreading them out into two or more dimensions. What follows in this sub-section is a brief discussion of the structure of two-dimensional experiments, encoding of the second dimension and two-dimensional Fourier transformation.

Two-dimensional experiments consist of four parts (6):

<table>
<thead>
<tr>
<th>preparation</th>
<th>evolution</th>
<th>mixing</th>
<th>detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_1$</td>
<td>$t_m$</td>
<td>$t_2$</td>
<td></td>
</tr>
</tbody>
</table>

Fig 2.7 The four main parts of two-dimensional experiments. The second dimension is encoded in the evolution time $t_1$, while the first dimension is encoded during $t_2$ (see text).

The preparation period consists of a series of pulses and delays which give rise to the coherence of interest. In 2D experiments the evolution delay is introduced and incremented systematically, in a series of experiments, to encode the second dimension. Each FID differs from another in the amplitude and/or phase of individual signals according to the different $t_1$ increments, the chemical shift and scalar coupling evolution of the coherences present during it. The mixing period can consist of pulses and delays during which the magnetisation of interest, might for example, undergo relaxation. The period $t_m$ can be placed before $t_1$ in some experiments and the significance of this will be discussed later. The acquisition time is the same as that for 1D experiments. The data obtained, $S(t_2)$ will provide, after Fourier transformation (Eqn. 2.8), the $f_2$ dimension of the 2D spectrum. For each $t_1$
increment a separate FID is obtained thus the data is a function of two time variables, \( S(t_1, t_2) \). A second Fourier transform with respect to \( t_1 \) yields the second dimension, \( f_1 \), of the 2D spectrum \((6,28,36)\). This principle can be applied to many dimensions.

### 2.2.6 COHERENCE TRANSFER PATHWAYS

The history of a component of magnetisation during an experiment is known as a **coherence transfer pathway**. It provides an understanding of how a pulse sequence works and hence it can be used as an aid in the design of new experiments.

During a pulse sequence many coherences can be present at any one time. These are classified according to their orders, e.g. zero, single, double, triple, etc. Each coherence order is associated with a particular coherence level in a coherence level diagram (Fig. 2.8(b)). For example, DQC had a coherence level of \( \pm 2 \), SQC has \( \pm 1 \) and longitudinal magnetisation has a coherence level of zero. Generally, at a given point in the experiment, only one or two of these coherences is desired and the other undesired coherences need to be eliminated since they may interfere with the observed signal.

The effects of pulse sequences on coherence order can be encompassed in two rules; coherences are transferred from one level to another by applying RF pulses and orders of coherence are preserved during periods of free precession. In an NMR experiment the starting equilibrium state has a coherence level \( p = 0 \). RF pulses with angles of \( 90^\circ \) and \( 180^\circ \) acting on an equilibrium state create coherence orders \( \Delta p = \pm 1 \) and \( \Delta p = 0 \) respectively. The only signal that is detected has a coherence order \( p = 1 \).

During each stage of a pulse sequence the coherence that will give the desired spectrum is selected while all other coherences are suppressed. Coherence is transformed along a specific pathway by RF pulses in the sequence. The path of the coherence that generates the desired spectrum is the desired **coherence transfer**
pathway. The desired coherence transfer pathway is selected by using appropriately designed phase cycles or by applying an appropriate set of gradient pulses.

The above rules will be illustrated by the following discussion of a part of the $^{13}$C T$_1$ experiment which was developed as a part of this work. This experiment will be described in detail in Chapter Three. The relevant section of the pulse sequence is shown in Fig. 2.8(a).

![Diagram of the preparation period of the $^{13}$C sequence](image)

Fig 2.8 The preparation period of the $^{13}$C sequence is shown in part (a). Part (b) shows the desired coherence pathways. Thin and thick vertical lines represent 90° and 180° pulses respectively. The pulses on the top and middle staff are applied to proton $^{13}$C while those on the bottom staff are gradient pulses. $\phi$ represents points in the pulse sequence.

The product operator formalism is used to show what happens to a $^{13}$C spin in a $^{13}$CH$_2$ group. Only terms which will result in observable magnetisation during $t_2$ are considered:
\[ I_{Hz} \xrightarrow{90^\circ(I)} I_{Hy} \xrightarrow{\frac{1}{4J} \cdot 180^\circ(I) / 180^\circ(S)} I_{Hz} \xrightarrow{\frac{1}{4J}} 2I_{Hz}S_{Cz} \xrightarrow{90^\circ(I) / 90^\circ(S)} 2I_{Hz}S_{Cy} \]

\[ \varphi_1 \xrightarrow{\varphi_2} \varphi_3 \]

\[ \frac{1}{8J}, 180^\circ(I) / 180^\circ(S), \frac{1}{8J} \]

\[ S_{Cz} \]

\[ \varphi_4 \]

\[ [2.22] \]

where \( \frac{1}{4J} \) and \( \frac{1}{8J} \) indicate periods of free precession. In transformation 2.22, proton SQC is excited (\( \varphi_1 \)) which then evolves into anti-phase SQC (\( \varphi_2 \)) during a delay of \( \frac{1}{2J} \). The 180° RF pulses applied to both nuclei in the middle of this delay (half way between points \( \varphi_1 \) and \( \varphi_2 \)) ensure that any proton chemical shift evolution and magnetic field inhomogeneities are reversed and only scalar coupling evolution occurs. Anti-phase SQC (\( \varphi_2 \)) is transferred to \( ^{13}\text{C} \) coherence anti-phase with respect to proton (\( \varphi_3 \)) before evolving into in-phase \( ^{13}\text{C} \) SQC (\( \varphi_4 \)). The coherence transfer pathway for this part of the sequence is shown in Fig. 2.8(b).

At the start of the experiment, the coherence level of a system at equilibrium is \( p = 0 \). When a 90° pulse is applied to proton spins, the proton coherence order \( p = \pm 1 \) is created. Since coherences are only transferred from one level to another by applying RF pulses, the 180° pulse to proton changes the existing coherence by \( \Delta p = \pm 2 \), resulting in \( p = \mp 1 \). So far, \( ^{13}\text{C} \) magnetisation is still at \( p = 0 \). The next two 90° pulses applied simultaneously to both spins change the coherence of proton to \( p = 0 \) and the coherence level of \( ^{13}\text{C} \) changes to \( p = \pm 1 \). The following 180° pulses applied to both spins have no effect on proton coherence (although it does have an effect on scalar coupling evolution) but the coherence level of \( ^{13}\text{C} \) changes to \( p = \mp 1 \).

In addition to the desired coherence transfer pathway shown in Fig. 2.8(b), there are other, unwanted, pathways which can interfere with it. The unwanted
coherence transfer pathways can be eliminated by phase cycling selected RF pulses or by using an appropriate set of gradient pulses (sub-section 2.2.6.2).

2.2.6.1 The Structure Of Heteronuclear Experiments

Four options are available for designing 2D experiments for heteronuclear systems (28). They differ in their relative sensitivities which depend on the gyromagnetic ratios of the nuclei involved. The coherence can be generated from either $I$ or $S$ magnetisation and it can be detected as either $I$ or $S$ coherence. In general the overall sensitivity of an experiment is proportional to the product of the gyromagnetic ratios of the excited and detected nuclei, given by:

$$\frac{S}{N} \propto \gamma_{exc} \gamma_{obs}^2$$  \hspace{1cm} [2.23]

where $\frac{S}{N}$ is the signal to noise ratio, $\gamma_{exc}$ and $\gamma_{obs}$ are the gyromagnetic ratios of the excited and detected nuclei respectively. In general, the optimal sensitivity of the experiment will be achieved when the excited and detected nucleus has the highest $\gamma$.

Some implications of Eqn. 2.23 are shown in Fig. 2.9 along with the relative sensitivities of each option.
Fig. 2.9 Four possible designs for heteronuclear experiments along with their relative sensitivities when $I$ is proton and $S$ is as indicated. The arrows indicate the coherence transfer pathway. In (a), $S$-coherence is excited and detected without being transferred to $I$. In (b), $I$-coherence is excited, transferred to $S$ and detected as $S$-coherence. In (c), $S$-coherence is excited, transferred to $I$ and detected as $I$-coherence. In (d) $I$-coherence is excited, transferred to $S$, transferred back to $I$ and detected as $I$-coherence.

Option (a) excites and detects the heteronucleus. The sensitivity of such a design depends on $\gamma_S$ only and is the worst choice. If the heteronucleus concerned is $^{15}\text{N}$ or $^{13}\text{C}$ then the sensitivity will be $1/32$ or $1/317$, respectively, of that of exciting and detecting proton. Such a low sensitivity does not make this design attractive. Options (b) and (c) are progressively more sensitive. Nonetheless the limited sensitivities and resolutions of these three designs would require unusually large concentrations of sample and long experimental times.

The problem of insufficient sensitivity has been minimised by experiments which enable indirect detection of the heteroatom (Fig. 2.9(d)). This concept forms
the basis of almost all the current heteronuclear experiments with proton detection. Such experiments depend on $\gamma$ of spin $I$ only, which is conventionally taken to be proton; the most magnetically sensitive nucleus.

2.2.6.2 Coherence Transfer Pathway Selection By Phase Cycling And Gradient Pulses

In a pulse sequence a single product operator can give rise to any number of operators as a result of imperfect RF pulses, chemical shift or scalar coupling evolution. It is therefore necessary to select the desired coherence transfer pathway and to suppress any components of observable magnetisation arising through unwanted pathways. This can be done by (a) phase cycling where the phases of RF pulse(s) and the receiver are changed in a systematic manner or (b) by incorporating gradient pulses into the pulse sequence, or (c) by using a combination of both (a) and (b).

In order to construct a phase cycle the change in the phase of the observed magnetisation resulting from changing the phase of a given RF pulse needs to be known. This is calculated by using:

\[
\text{Change in phase of magnetisation} = \phi \cdot \Delta \rho
\]  

[2.24]

where $\phi$ is the change in the phase of the pulse (deduced from Fig. 2.10) and $\Delta \rho$ is the change in coherence order brought about by the pulse.

![Diagram](image)

**Fig. 2.10** This diagram shows how RF pulses applied along the x, y, -x and -y axis are related to their $\phi$ values (in brackets). E.g. when an RF pulse is applied along the x axis $\phi = 0$, etc.
A 90° excitation RF pulse will give a $\Delta p = \pm 1$. A 180° refocusing pulse applied to SQC will give $\Delta p = \pm 2$ but a 180° inversion pulse will give $\Delta p = 0$. When no coherence is excited by an RF pulse, $\Delta p = 0$. The coherence order for thermal equilibrium is zero.

Before continuing any further it is important to note that if an $n$-quantum coherence is excited, both $+n$ and $-n$ quantum coherence will be present. Since only -1 quantum coherence is detected $\pm n$-quantum coherence has to be transferred to -1 quantum coherence before it can be detected.

A simple example of how phase cycling is implemented to observe the signal of interest can be shown with the two-dimensional Correlation Spectroscopy (COSY) experiment. The COSY experiment consists of two 90° pulses separated by a delay $t_1$. The first RF pulse excites in-phase coherence of, for example, spin $k$. This evolves into anti-phase coherence with respect to a bonded spin $l$. The subsequent 90° RF pulse transfers coherence from $k$ to $l$, giving rise to an off-diagonal peak modulated at $(\omega_k, \omega_l)$. The desired transformation arising from the second pulse is:–

$$2I_{kx}I_{lz}^{90°} \rightarrow 2I_{kz}I_{lx}$$

[2.25]

for which $\Delta p = -2$. The change in the coherence order is directly apparent when transformation 2.25 is shown in raising and lowering operators:–

$$I_k^+ I_{lz}^{90°} \rightarrow I_{kz} I_l^-$$

[2.26]

The second 90° pulse in the COSY experiment can also give $\Delta p = 0$ arising from the following transformation:–

$$I_k^- I_{lz}^{90°} \rightarrow I_{kz} I_l^-$$

[2.27]
Both of these transformations (2.26 and 2.27) are desired since combinations of $I_k^+l_z$ and $I_k^-l_z$ give the x and y components of coherence calculated from Eqns. 2.19 and 2.20).

If the phase of the 90° pulse is rotated by 180° from y to -y, it can be predicted by using Eqn. 2.24, that the desired coherences will not be effected. Hence:

$$2I_{kx}l_{iz} \xrightarrow{90°} 2I_{kz}l_{ix}$$ [2.28]

In almost all two-dimensional experiments axial peaks are common artefacts which result from imperfect RF pulses. These occur from the following transformation:

$$I_{kz} \xrightarrow{90°} I_{kx}$$ [2.29]

which can be shown to give $\Delta p = -1$ by utilising raising and lowering operators:

$$I_{kz} \xrightarrow{90°} I_k^-$$ [2.30]

By using Eqn. 2.24, it can be seen that a 90° pulse will change the phase of the magnetisation by 180°. Hence the following transformation will occur:

$$I_{kz} \xrightarrow{90°} -I_{kx}$$ [2.31]

Unlike the desired coherence (transformation 2.28) the unwanted coherence (transformation 2.31) is sensitive to 180° phase shifts of the 90° pulse. As the two FIDs are added together, the signal arising from the desired transfer adds up while the unwanted signal cancels out. The converse may happen in other cases, i.e. the desired
coherence may cancel out as the phase of the RF pulse changes while the unwanted coherence may accumulate. In such a situation the phase of the receiver is changed accordingly.

The two-step phase cycle described above (where the phase of a 90° RF pulse is rotated between y and -y) can select both $\Delta p = -2$ and $\Delta p = 0$. If the 90° pulse has a four-step phase cycle, the effect it will have on the wanted and unwanted coherences are shown in Table 2.1.

<table>
<thead>
<tr>
<th>Change in Phase of Pulse</th>
<th>Change in Phase of Magnetisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\phi$</td>
<td>Unwanted Coherence</td>
</tr>
<tr>
<td></td>
<td>$\Delta p = -1$</td>
</tr>
<tr>
<td>x (0°)</td>
<td>x</td>
</tr>
<tr>
<td>y (90°)</td>
<td>-y</td>
</tr>
<tr>
<td>-x (180°)</td>
<td>-x</td>
</tr>
<tr>
<td>-y (270°)</td>
<td>y</td>
</tr>
</tbody>
</table>

The above two-step phase cycle is equivalent to rows 1 and 3 of Table 2.1 where $\phi = 0°$ and 180°. For a four-step phase cycle, if the phase of the receiver remains constant at x then only $\Delta p = 0$ will be detected. However, if the receiver has a phase cycle of x, -x, x, -x, then only $\Delta p = -2$ will be selected. Hence with a four-step phase cycle only one of either $\Delta p = 0$ or $\Delta p = -2$ can be detected and the other has to be sacrificed. Therefore, for the experiment under discussion, it is best to implement a two-step phase cycle for the second 90° pulse in COSY.

The above is a basic explanation of a phase cycle but longer and more complicated pulse sequences may require complex phase cycles. However these tend to be composites of phase cycles for the individual 90° and 180° RF pulses.
Phase cycling is a difference method and it will be effective only if experimental conditions from one transient to another are constant. Slight variations may occur resulting from changes in the lock circulatory, amplitude/phase changes in RF pulses or temperature fluctuations causing small resonance shifts. Such random variations will reduce the effectiveness of phase cycling and also generate $t_1$ noise in two-dimensional spectra.

Gradient pulses offer a reliable alternative to phase cycling when selecting coherence transfer pathways (37-40). During the application of a gradient pulse, $B_0$ is made inhomogeneous. Transverse magnetisation and other coherences dephase across the sample. Another appropriately placed gradient pulse, in a gradient echo, will rephase the coherence. For example, during the first gradient pulse, a homonuclear coherence at a location $z$ in the sample, will acquire a spatially dependent phase, $\phi$, given by:

$$\phi = z \cdot \gamma \cdot G_z \cdot t \cdot p$$

[2.32]

where $\gamma G_z$ is the frequency of the component, $t$ is the length of time the gradient pulse is applied for and $p$ is the coherence order. The sensitivity of a coherence to gradient pulses is proportional to the order of the coherence and to the gyromagnetic ratios of the active spins. For example, proton SQC is 4 times more sensitive than $^{13}$C SQC to the same gradient pulse. Applying a second gradient pulse with amplitude $-G_z$ will result in an additional phase of $-\phi$. Therefore the overall phase will be zero; this corresponds to the formation of a gradient echo, as shown in Fig. 2.11.
Gradient pulses can be used to select a particular coherence transfer pathway by generating gradient echoes which follow the desired coherence transfer pathway while leaving undesired coherence transfer pathways randomised. As a pair, the gradient pulses are usually chosen so that they have no net effect on the magnetisation of interest.

Consider the spin echo shown in Fig. 2.12(a). Coherence transfer pathways A and B are possible if the 180° pulse is imperfect. The second, unwanted pathway can be eliminated by generating a gradient echo around the imperfect RF pulse as shown in Fig. 2.12(b). Coherence transfer pathway B will continue to dephase under the influence of the second gradient pulse while the wanted coherence will be refocused.
Fig. 2.12 An example of practically implementing gradient echoes in homonuclear experiments. (a) A 90° RF pulse will excite coherence $p = \pm 1$. As only -1 coherence is detected there are two possible coherence transfer pathways, A and B. (b) The first gradient pulse will dephase all coherences. In order to rephase coherence with $p = -1$ from the coherence transfer pathway A, the second gradient pulse needs to be identical to the first, according to Eqn. 2.32.

In heteronuclear experiments the spatially dependent phase acquired by a particular multi-spin coherence is given by a general version of Eqn. 2.32:

$$\phi = z \cdot G_z \cdot t \cdot \sum_i p_i \cdot \gamma_i$$  \[2.33\]

where the coherence order and gyromagnetic ratios are summed over all $i$ spins in the coherence.

Consider the effect of two 90° pulses applied simultaneously to $^{13}$C and proton (Fig. 2.13).
Fig. 2.13 The generation of gradient echoes in heteronuclear experiments. The arrows indicate the transfer of coherence from $^{13}$C to proton and the desired coherence transfer pathway is $+I \rightarrow -I$. Proton SQC coherence is four times more sensitive to gradient pulses than $^{13}$C SQC. Hence gp1, applied to $^{13}$C SQC, has four times the area of gp2, which is applied to proton SQC, to select the coherence transfer pathway shown.

Since $\gamma_C = \frac{1}{4} \gamma_H$, $^{13}$C SQC will only be a quarter as sensitive to gradient pulses as proton SQC. The insensitivity of the heterospin is compensated by applying a gradient pulse to $^{13}$C coherence which will have 4 times the area of that applied to proton coherence. This can be done either by increasing the amplitude of gp1 by a factor of four or by applying it for four times longer than the length of gp2.

The coherence transfer pathway shown in Fig. 2.13 can be described explicitly with raising and lowering operators. In this formalism the coherence transfer pathway of interest can be represented as $S_C^+ I_{Hz} \rightarrow I_H^- S_C$. By applying Eqn. 2.33 where $\gamma$ for $^{13}$C is a quarter of that for proton, $G_z$ for $^{13}$C coherence is four times that for proton coherence, $t$ is constant and the coherence order is +1 and -1 for $^{13}$C and proton respectively, it can be seen that the spatially dependent phases, $\phi_C$ and $\phi_H$, for $^{13}$C and proton are identical except for their signs. Therefore $S_C^+ I_{Hz}$ will be dephased.
during the application of gp1 and $I_H \bar{S}_C z$ will be rephased by gp2 and the desired coherence transfer process will be selected.

When using gradient pulses for coherence transfer pathway selection, signals arising from unwanted pathways are removed in individual transients rather than relying on subtraction processes. Hence artefacts from instrumental instabilities will be significantly smaller compared to experiments using phase cycling. Therefore signal averaging will be done to achieve a better signal to noise ratio. Consequently the experiment will take less time to run if gradient pulses are used for coherence selection. In addition, dynamic range, e.g. a large water signal in the spectrum, will not be a problem.

However, there are two drawbacks to using gradient pulses over phase cycling; half of the signal is lost in some experiments because only one of $+n$ or $-n$ quantum coherence is selected whereas phase cycling selects both $+n$ and $-n$. Signal can also be lost due to diffusion if pairs of gradient pulses are placed about a delay; in addition to decay due to relaxation, the signal will also be diffusion encoded. If these two drawbacks are taken into account in the design of experiments then using gradient pulses can have a distinct advantage over phase cycling.

2.3 THE THEORY OF NMR RELAXATION

This section is divided into two parts: the phenomenological and the theoretical aspects of relaxation. The former part provides a relatively general account of the definition of relaxation, its nature and dependence on molecular dynamics. The latter part of this section gives an account of how macroscopic relaxation behaviour, averaged over all the molecules in the sample, can be described by the density operator. This sub-section also describes the relaxation Hamiltonian and the density matrix.
It should be noted here that sub-section 2.3.2 gives only a brief account necessary to understand the work undertaken in this thesis. There are two reasons for this. First it is not necessary to go into any more detail for the purpose of this work and second, excellent accounts in the literature are already available and the reader is directed to these at the relevant points.

2.3.1 THE PHENOMENOLOGICAL ASPECTS OF RELAXATION

2.3.1.1 DEFINITION OF RELAXATION

Relaxation occurs after a system at equilibrium has been perturbed (25), for example, by the application of an RF pulse. The system will relax back to its equilibrium state with a characteristic time profile. There are two types of relaxation, $T_1$ and $T_2$, which were touched on in section 2.2. $T_1$, the longitudinal relaxation time, is a measure of the time taken for the populations of the $\alpha$- and $\beta$-states to return to the equilibrium Boltzmann distribution. $T_1$ relaxation occurs as a result of processes which induce transitions between the two states, which may also result in a loss of coherence. There must be an interaction between the system and the surroundings, or the lattice, leading to a loss of the excess energy in the spin system.

Similarly, $T_2$, the transverse relaxation time, is a measure of the time taken for coherence in the $xy$-plane to dephase to its equilibrium value of zero. Transverse relaxation involves the loss of phase coherence resulting from the dephasing of individual contributions to the macroscopic transverse magnetisation vector $M_{xy}$, so that their resultant sum is zero. This is not necessarily accompanied by transitions but $T_1$ processes can contribute to $T_2$ relaxation. It will also be brought about by any processes which perturb the Larmor frequencies of individual spins. Therefore more processes contribute to $T_2$ than $T_1$ and consequently the rate of transverse relaxation is
always greater than the rate of longitudinal relaxation, i.e. \( \frac{1}{T_2} > \frac{1}{T_1} \) or \( T_2 < T_1 \). These two types of relaxation are represented in Figs. 2.14 and 2.15.

Fig. 2.14 A diagramatic representation of \( T_1 \) relaxation. \( M_0 \) is perturbed by an inversion pulse (a). The inverted net magnetisation vector immediately begins to relax back to its equilibrium value (b). After an approximate time of \( 5 \times T_1 \) the system returns to equilibrium, as in (a), but when \( \tau < 5 \times T_1 \) the system will be in the process of equilibrating (c).

Fig. 2.15 A diagramatic representation of \( T_2 \) relaxation. \( M_0 \) is perturbed from equilibrium by a 90° pulse into the xy-plane, (b). There is a loss of coherence because the net magnetisation vector \( M_y \) fans out as the individual spins precess at their characteristic Larmor frequencies, (c).

2.3.1.2. The Nature Of Relaxation And Its Dependence On Molecular Dynamics

As mentioned above longitudinal relaxation occurs as a result of processes which induce transitions between the \( \alpha \)- and \( \beta \)-states. Abragam (15) has eliminated two possible mechanisms from making a significant contribution: spontaneous emission by emitting a photon to the surroundings and absorption or stimulated emission of photons from the background radiation field (i.e. black body radiation in
thermal equilibrium with the surroundings). These mechanisms are not viable because they are too slow at NMR frequencies.

NMR relaxation is caused by energy exchange processes where energy is directly exchanged between two sets of energy levels. One set of energy levels is those of the nuclear spin system and the other set, in relaxation in solutions, is generally provided by the "lattice". This latter term constitutes rotations, translations and the various internal motions of the molecules. The lattice possesses a large number of degrees of rotation so for a particular NMR transition there is always a possible change in the lattice involving the same amount of energy. For example, a downward flip of a spin will simultaneously involve a corresponding acceleration of some motion of the molecule. Likewise an upward flip will correspond to an accompanying slowing down of some motion of the molecule.

The energy exchange processes are mediated by the lattice causing a fluctuating magnetic field at the site of the spin involved. This can be envisaged as a "local pulse" (41) similar in effect to an RF pulse. It is localised to such an extent that its affect is limited to just one spin undergoing a particular transition at a given moment in one molecule. The corresponding energy is transmitted into or out of the particular motion, which caused the fluctuation in the local field, by the local pulse.

The local fluctuating field needs to fulfil two requirements. Firstly it must have a component oscillating at the corresponding frequency of a transition between two states (as with RF pulses). Secondly, analogous to only x- and y-pulses rotating longitudinal magnetisation, longitudinal relaxation is only caused by the x- and y-components of the local field (transverse relaxation is caused by the z-component).

Despite these similarities of local fluctuation fields and RF pulses there are two main differences. The RF pulse has a coherent effect across the whole sample but the local field is incoherent, i.e. it varies randomly from one spin to another regardless of them being in the same molecule or in different ones. Relaxation differs from excitation in one fundamental aspect: during the latter only SQ transitions occur but during the former DQ and ZQ transitions can also occur in addition to SQ.
Possible sources of the local fluctuating field include other nuclear dipoles, dipolar fields produced by unpaired electrons, electric charges which interact with the nuclear quadrupole moment for nuclei with spin greater than $\frac{1}{2}$, CSA, fluctuating scalar coupling interactions and molecular (spin) rotations. This discussion will be limited to two main sources of fluctuation in the local magnetic field which give rise to the transitions shown in Fig. 2.16: dipolar couplings and CSA. Dipolar interactions result from the coupling of two magnetic moments of two spins (I and S) i.e. spin I produces a dipole field that is experienced by spin S. The dipolar interaction is proportional to $\frac{\gamma_I \gamma_S}{r_{IS}^3}$, where $r_{IS}$ is the distance between the two spins. The size of the interaction also depends on the orientation of the internuclear vector which will change as the molecule undergoes rotational diffusion or internal motions. The frequency of the fluctuations of the dipole field vector will depend on the frequency of rotational motion of the molecule. CSA is a variation in chemical shift with molecular orientation relative to $B_0$. Both dipolar and CSA interactions have an angular $3\cos^2\theta - 1$ dependence on orientation relative to $B_0$. As the orientation of the molecule changes, the dipolar and CSA interactions also change giving rise to local magnetic fields which fluctuate. According to the time-dependent perturbation theory, if the frequency of these fluctuations correspond to the transition frequency between two energy levels, transitions between the two levels will be induced and the system will undergo relaxation.

2.3.2 THEORY OF RELAXATION

The following is a brief overview of the density operator and the relaxation Hamiltonian. In addition to the references below the reader is also directed to references (1, 28, 42) for more details.
2.3.2.1 Background Theory Of Relaxation - The Density Operator

The following is a brief account of the density operator. More thorough accounts can be found in references 1, 28, 43, 44 and 45 and other references in this section. In order to fully understand the concepts presented later in this chapter, it is necessary to overview the theory of relaxation, in particular of longitudinal relaxation.

Both types of relaxation, $T_1$ and $T_2$, are described by the spin density operator, $\sigma$. Its time dependence can be described in matrix form by:

$$\frac{d\sigma(t)}{dt} = -[iH + \Gamma](\sigma(t) - \sigma_0)$$  \hspace{1cm} [2.34]

where $H$ is the time independent nuclear spin superoperator, $\Gamma$ is the relaxation superoperator and $\sigma$ is the density matrix with elements describing the population of spin states and coherence between them. $\sigma_0$ is the density matrix at equilibrium. $H$ contains information about the characteristic properties of the spin such as chemical shift and scalar couplings. Since we are concerned here with longitudinal relaxation modes, $H$ will not be considered further. For convinience $\sigma(t)$ is usually expanded into a basis set, such as product operators, so that various types of longitudinal magnetisation and spin order of interest to be readily identified and manipulated. For a three spin system for example, $\sigma$ can be expanded in terms of product operators including $I_{kz}$ (longitudinal magnetisation of spin $k$), $2I_{kz}I_{lz}$ (longitudinal two spin order of spins $k$ and $l$) and $4I_{kz}I_{lz}I_{mz}$ (longitudinal three spin order of spins $k$, $l$ and $m$). At equilibrium all modes have zero amplitude except longitudinal magnetisation.
2.3.2.2 The Relaxation Hamiltonian

The relaxation Hamiltonian, $H_1$, of a system with spins $k$ and $l$ can be written as (I, 46):

$$H_1(t) = \sum_q F^{(q)}(t) A^{(q)}$$  \[2.35\]

where, for dipolar relaxation, the spin operators $A^{(q)}$ and $F^{(q)}$ are defined in Table 2.2.

<table>
<thead>
<tr>
<th>$A_{kl}^{(0)}$</th>
<th>$b_{kl} { I_{kz} I_{lz} - \frac{1}{4} (I_{k}^+ I_{l}^- + I_{k}^- I_{l}^+) }$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{kl}^{(1)}$</td>
<td>$-\frac{3}{2} b_{kl} (I_{kz} I_{lz}^+ + I_{k}^+ I_{l}^-)$</td>
</tr>
<tr>
<td>$A_{kl}^{(-1)}$</td>
<td>$-\frac{3}{2} b_{kl} (I_{kz} I_{lz}^- + I_{k}^- I_{l}^+)$</td>
</tr>
<tr>
<td>$A_{kl}^{(2)}$</td>
<td>$-\frac{3}{4} b_{kl} I_{k}^+ I_{l}^+$</td>
</tr>
<tr>
<td>$A_{kl}^{(-2)}$</td>
<td>$-\frac{3}{4} b_{kl} I_{k}^- I_{l}^-$</td>
</tr>
<tr>
<td>$F_{kl}^{(0)}$</td>
<td>$1 - 3 \cos^2 \theta_{kl}$</td>
</tr>
<tr>
<td>$F_{kl}^{(1)}$</td>
<td>$\sin \theta_{kl} \cos \theta_{kl} \exp {-i\phi_{kl}}$</td>
</tr>
<tr>
<td>$F_{kl}^{(-1)}$</td>
<td>$\sin \theta_{kl} \cos \theta_{kl} \exp {+i\phi_{kl}}$</td>
</tr>
<tr>
<td>$F_{kl}^{(2)}$</td>
<td>$\sin^2 \theta_{kl} \exp {-zi\phi_{kl}}$</td>
</tr>
<tr>
<td>$F_{kl}^{(-2)}$</td>
<td>$\sin^2 \theta_{kl} \exp {+zi\phi_{kl}}$</td>
</tr>
</tbody>
</table>

In Eqn. 2.35 the operators $A^{(q)}$ act on the time-independent variables of the spin system such as chemical shifts, scalar couplings and dipolar interactions: and $F^{(q)}(t)$ are random functions of time which describe the orientation of the system. To implement Eqn. 2.35 the operators $A^{(q)}$ are expanded into eigenoperators, $A_p^{(q)}$, of the unperturbed Hamiltonian, evolution resulting from relaxation can be written as:-
\[ \frac{d\sigma(t)}{dt} = -\frac{1}{2} \sum_{p,q} J_{qq'}(\omega_p^{(q')}) \left[ A_p^{(q')}, A_p^{(q)}, \sigma(t) - \sigma_0 \right] \quad [2.36] \]

where the power spectral density function is:

\[ J_{qq'}(\omega_p^{(q)}) = \int \langle F^{(q)}(0) F^{(q')}(\tau) \rangle e^{-i\omega \tau} d\tau \quad [2.37] \]

The matrix resulting from Eqn. 2.36 will contain a column of \( n^2 \) coefficients and \( n^2 \times n^2 \) matrix elements where \( n \) is the number of eigenstates (43, 44). For a multi-spin system the number of matrix elements can increase rapidly with a small increase in the number of spins. Hence the task of calculating relaxation of multi-spin systems becomes tedious. Fortunately it is not always necessary to evaluate all the elements of the matrix. Generally a small subset of the density matrix is sufficient.

A generalised relaxation rate equation can be written, from Eqn. 2.36, for a system with two coupled spins \( I \) and \( S \):

\[ R \approx \frac{\gamma_I^2 \gamma_S^2}{6 \tau_{JS}} \left[ aJ(0) + bJ(\omega_I - \omega_S) + cJ(\omega_I) + dJ(\omega_S) + eJ(\omega_I + \omega_S) \right] + \text{CSA} \quad [2.38] \]

where \( a-e \) are coefficients and \( J(\omega) \), the spectral density function at a particular frequency, for an isotropically tumbling molecule, is given by:

\[ J(\omega) = \frac{1}{4\pi} \left[ \frac{2\tau_c}{1 + \omega^2 \tau_c^2} \right] \quad [2.39] \]

where \( \omega \) is the frequency and \( \tau_c \) is an autocorrelation time discussed in section 3.2. In Eqn. 2.38 term 1 represents the size of the dipolar interaction, terms 2-6 give the amount of motion at the respective frequencies (e.g. \( J(0) \) is the amount of motion at
zero frequency i.e. when the molecule is stationary and $J(\omega_S)$, is the amount of motion at $\omega_S$. Term seven is the contribution to relaxation from chemical shift anisotropy. This is also a function of $J(\omega)$. In some relaxation processes cross-terms between the dipolar-CSA interactions also occur.

Four of the transitions given in Eqn. 2.38 are shown in Fig. 2.16. Spins $I$ and $S$ can undergo individual SQ transitions (e.g. $\alpha \rightarrow \beta$) at the frequencies of $J(\omega_I)$ and $J(\omega_S)$ respectively. If the spins flip together in the same direction (e.g. $\alpha\alpha \rightarrow \beta\beta$) the transition frequency will be $J(\omega_I + \omega_S)$. This is a DQ transition. A ZQ transition will occur when both spins flip together in opposite directions (e.g. $\alpha\beta \rightarrow \beta\alpha$) and the transition frequency is equal to the difference of the individual frequencies (i.e. $J(\omega_I - \omega_S)$). Zero frequency motion does not cause transitions and is not shown in the figure.

![Energy level diagram for two coupled spins $I$ and $S$.](image-url)

**Fig. 2.16** Energy level diagram for two coupled spins $I$ and $S.$
2.3.2.3 THE RELAXATION MATRIX

An example of the application of Eqn. 2.34 for a three spin system, consisting of spins 1, 2 and 3, the longitudinal relaxation sub-matrix is given below:

\[
\begin{pmatrix}
[I_{1z}(t)] \\
[I_{2z}(t)] \\
[I_{3z}(t)] \\
[2I_{1z}I_{2z}(t)] \\
[2I_{1z}I_{3z}(t)] \\
[2I_{2z}I_{3z}(t)] \\
[4I_{1z}I_{2z}I_{3z}(t)]
\end{pmatrix}
= \begin{pmatrix}
a_{11} & c_{12} & c_{13} & g_{14} & g_{15} & 0 & e_{17} \\
c_{21} & a_{22} & c_{23} & g_{24} & 0 & g_{26} & e_{27} \\
c_{31} & c_{32} & a_{33} & 0 & g_{35} & g_{36} & e_{37} \\
g_{41} & g_{42} & 0 & b_{44} & d_{45} & d_{46} & h_{47} \\
g_{51} & 0 & g_{53} & d_{54} & b_{55} & d_{56} & h_{57} \\
0 & g_{62} & 0 & g_{63} & d_{64} & d_{65} & b_{66} & h_{67} \\
e_{71} & e_{72} & e_{73} & h_{74} & h_{75} & h_{76} & f_{77}
\end{pmatrix}
\begin{pmatrix}
[I_{1z}(t)]-I_{1z}(0) \\
[I_{2z}(t)]-I_{2z}(0) \\
[I_{3z}(t)]-I_{3z}(0) \\
[2I_{1z}I_{2z}(t)] \\
[2I_{1z}I_{3z}(t)] \\
[2I_{2z}I_{3z}(t)] \\
[4I_{1z}I_{2z}I_{3z}(t)]
\end{pmatrix}
\]

[2.40]

Definitions of the elements of the matrix are given in Table 2.3.

The matrix given in Eqn 2.40 is a set of coupled differential equations. The left-hand column vector gives the rate of change of the amplitude of the seven longitudinal modes at time \(t\). For example, the expression for the decay of \(I_{1z}\) is:

\[
\begin{align*}
- \frac{d[I_{1z}(t)]}{dt} &= a_{11}([I_{1z}(t)]-[I_{1z}(0)]) + c_{12}([I_{2z}(t)]-[I_{2z}(0)]) + c_{13}([I_{3z}(t)]-[I_{3z}(0)]) \\
&+ g_{14}([2I_{1z}I_{2z}(t)]) + g_{15}([2I_{1z}I_{3z}(t)]) + e_{17}([4I_{1z}I_{2z}I_{3z}(t)])
\end{align*}
\]

[2.41]

where \([\text{operator}(t)]\) and \([\text{operator}(0)]\) are the amplitudes of the particular operators at time \(t\) and at equilibrium respectively. Only term \(a_{11}\) describes the decay of \(I_{1z}\). The remaining terms describe cross-relaxation between \(I_{1z}\) and longitudinal magnetisation of other spins or other longitudinal modes. For example, the cross-correlation between \(I_{1z}\) and \(I_{2z}\) is given by the term \(c_{12}([I_{2z}(t)]-[I_{2z}(0)])\).
Table 2.3 Definitions of the elements of the longitudinal relaxation matrix for three weakly coupled spins. $C_i = \left(\frac{8\pi}{15}\right)^{0.5} \gamma_i B_y \Delta \sigma_i$, $D_{ij} = -\left(\frac{\mu_B}{4\pi}\right)^{0.5} \frac{\gamma_i \gamma_j h}{r_{ij}}$, $\theta_{ij,ik}$ defines the angle between the pair of internuclear vectors $r_{ij}$ and $r_{ik}$, $\theta_{i,ij}$ defines the angle between the principle axis of the (assumed) cylindrical symmetry CSA tensor of spin $i$ and the internuclear vector $r_{ij}$ and $i$, $j$ and $k$ correspond to the three nuclear spins.

### Diagonal Elements

#### Self-relaxation of the longitudinal magnetisation $I_{zz}$:

$$a_{pp} = \frac{1}{2} D_{ij}^2 \left[ \frac{1}{3} J(\omega_i - \omega_j) + J(\omega_i) + 2J(\omega_i + \omega_j) \right] + \frac{1}{2} D_{ik}^2 \left[ \frac{1}{3} J(\omega_i - \omega_k) + J(\omega_i) + 2J(\omega_i + \omega_k) \right] + \frac{1}{2} C_i^2 J(\omega_i)$$

#### Self-relaxation of the longitudinal two spin order $2I_{zz}$:

$$b_{pp} = \frac{1}{2} D_{ik}^2 \left[ \frac{1}{3} J(\omega_i - \omega_k) + J(\omega_i) + 2J(\omega_i + \omega_k) \right] + \frac{1}{2} D_{jk}^2 \left[ \frac{1}{3} J(\omega_j - \omega_k) + J(\omega_j) + 2J(\omega_j + \omega_k) \right] + \frac{1}{2} D_{ij}^2 \left[ J(\omega_i) + J(\omega_j) \right] + \frac{1}{2} C_i^2 J(\omega_i) + \frac{1}{2} C_j^2 J(\omega_j)$$

#### Self-relaxation of the longitudinal three spin order $4I_{zz}$:

$$f_{pp} = \frac{1}{2} D_{ij}^2 \left[ J(\omega_i) + J(\omega_j) \right] + \frac{1}{2} D_{ik}^2 \left[ J(\omega_i) + J(\omega_k) \right] + \frac{1}{2} D_{jk}^2 \left[ J(\omega_j) + J(\omega_k) \right] + \frac{1}{2} C_i^2 J(\omega_i) + \frac{1}{2} C_j^2 J(\omega_j) + \frac{1}{2} C_k^2 J(\omega_k)$$
### Off-Diagonal Elements

<table>
<thead>
<tr>
<th>Cross-relaxation between $I_{iz}$ and $I_{jz}$:</th>
</tr>
</thead>
<tbody>
<tr>
<td>$c_{pq} = \frac{1}{2} D_{ij}^2 \left[ -\frac{1}{3} J(\omega_i - \omega_j) + 2J(\omega_i + \omega_j) \right] $</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cross-relaxation between $2I_{iz}I_{jz}$ and $2I_{iz}I_{kz}$:</th>
</tr>
</thead>
</table>
| $d_{pq} = \frac{1}{2} D_{jk}^2 \left[ -\frac{1}{3} J(\omega_j - \omega_k) + 2J(\omega_i + \omega_k) \right] $  
| $+ \frac{1}{2} D_{ij} D_{ik} \left[ 2J(\omega_i) \right] \left\{ \frac{1}{2} \left[ 3 \cos^2 (\theta_{ij,ik}) - 1 \right] \right\} $ |

<table>
<thead>
<tr>
<th>Cross-relaxation between $I_{iz}$ and $4I_{iz}I_{jz}$:</th>
</tr>
</thead>
<tbody>
<tr>
<td>$e_{pq} = \frac{1}{2} D_{ij} D_{ik} \left[ 2J(\omega_i) \right] \left{ \frac{1}{2} \left[ 3 \cos^2 (\theta_{ij,ik}) - 1 \right] \right} $</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cross-relaxation between $2I_{iz}I_{jz}$ and $I_{iz}$:</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_{pq} = \frac{1}{2} C_i D_{ij} \left[ 2J(\omega_i) \right] \left{ \frac{1}{2} \left[ 3 \cos^2 (\theta_{i,j}) - 1 \right] \right} $</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cross-relaxation between $2I_{iz}I_{jz}$ and $4I_{iz}I_{jz}$:</th>
</tr>
</thead>
</table>
| $h_{pq} = \frac{1}{2} C_i D_{ik} \left[ 2J(\omega_i) \right] \left\{ \frac{1}{2} \left[ 3 \cos^2 (\theta_{i,k}) - 1 \right] \right\} $  
| $+ \frac{1}{2} C_j D_{jk} \left[ 2J(\omega_j) \right] \left\{ \frac{1}{2} \left[ 3 \cos^2 (\theta_{j,k}) - 1 \right] \right\} $ |

The diagonal elements of the sub-matrix, (e.g. $a_{pp}$), give the rates of decay of the components of magnetisation or longitudinal spin order while the off-diagonal elements, (e.g. $c_{pq}$), give the rates of relaxation-induced evolution between them, as shown in Eqn. 2.41. It can be seen from Table 2.3 that the off-diagonal elements...
(c_{pq}, d_{pq}, e_{pq}, g_{pq} and h_{pq}) contain a relatively small number of variables. Elements c_{pq}, e_{pq} and g_{pq} only consist of two variables while elements d_{pq} and h_{pq} contain four. Furthermore these variables are restricted to interactions between the set of spins active in the modes being correlated. For example, element c_{i2} describes the cross-correlation of longitudinal magnetisation from spin 1 to spin 2. The definition of this element only consists of variables which include the spectral density function at the frequencies of both spins, i.e. the difference and the sum of \omega_1 and \omega_2. Clearly the smaller the number of variables the easier it is to analyse that element. Hence the off-diagonal elements appear to be a relatively attractive choice for analysis. This is the case with elements c_{pq} (responsible for NOEs) because they only depend on internuclear distances (which can be obtained from crystallography data) and the parameters associated with molecular motion, others are also dependent on the angles \theta_{i,j,k} and \theta_{i,j,k}' which in a molecule with internal motion, may be constantly changing. Furthermore, all off-diagonal elements are usually relatively difficult to determine accurately since to do so one must measure the rate of formation of a low intensity peak which is itself subject to decay.

The diagonal elements, a_{pp}, b_{pp} and f_{pp}, contain a large number of variables. For example a_{pp} is given by:-

\[
a_{pp} = \frac{1}{2} D^2 \left[ \frac{1}{3} J(\omega_i - \omega_j) + J(\omega_i) + 2J(\omega_i + \omega_j) \right] + \frac{1}{2} D^2_{ik} \left[ \frac{1}{3} J(\omega_i - \omega_k) + J(\omega_i) + 2J(\omega_i + \omega_k) \right] + \frac{1}{2} C_i^2 J(\omega_i)
\]

[2.42]

It depends on two internuclear distances and a value of J(\omega) at seven different frequencies. Nonetheless, since the diagonal elements correspond to the straight
forward decay of a given component of magnetisation or spin order, they are, in principle, relatively easier to measure.

2.4 REFERENCES


CHAPTER THREE

NMR RELAXATION AND MOLECULAR DYNAMICS

3.1 INTRODUCTION

There are three stages in extracting molecular dynamics information from NMR relaxation data: firstly, the relaxation interactions need to be identified, secondly, the appropriate experiments need to be chosen to make the necessary measurements and thirdly, the observed relaxation rates need to be related to the dynamics of the system. The first and the last steps are discussed in this chapter: the second step is considered in Chapter Four.

In this chapter a general introduction to protein relaxation is followed by a review of how molecular motion is quantified. If a specific expression for the spectral density function can be written then physically reasonable models for motion can be implemented to extract the corresponding motional parameters. Both general models (the Lipari and Szabo formalism) and specific ones for particular motions (rotational diffusion, "wobbling-in-a-cone" and rotational jump models) are discussed. The discussion on specific models has been limited to those which have been used to characterise the dynamics of proteins. For systems where the spectral density function cannot be defined, Peng and Wagner's approach of "mapping" as opposed to "modelling" the spectral density function can be used.

The following section concentrates on the practical implementations of \(^{15}\text{N}\) and \(^{13}\text{C}\) relaxation, including a critical discussion on the assumptions made and experimental measurements necessary to use existing analytical methods. To eradicate the limited applications of existing methods of analysis, a new perspective, on relaxation in uniformly \(^{15}\text{N}\) and \(^{13}\text{C}\) labelled molecules, is taken where all dipolar
interactions between the spins of interest and all other spins are considered. How this method is derived from the combination of general relaxation rates of longitudinal magnetisation and various longitudinal spin orders, is described. Diagrammatic representations and descriptions of individual dipolar interactions for a set of two and three spins, which can be isolated with the new method, are also given.

The following section demonstrates how the principle of the new method is implemented practically on a small molecule with two AX spin systems. Additionally, any problems which have arisen and their possible solutions are discussed.

This chapter continues with a discussion of how the new analytical method can be applied to biological molecules, the dipolar interactions which can be isolated for $^{13}$CH$_n$ groups, the dynamics information which can be obtained and how mapping the spectral density function can be implemented for $^{13}$C relaxation.

### 3.2 THE IMPORTANCE OF NMR RELAXATION IN BIOMOLECULES

NMR and x-ray crystallography are both ideal for monitoring individual nuclei in complex biological molecules. Compared to crystal structures, those derived from NMR measurements generally contain less structural detail. However, unlike x-ray data, NMR measurements also contain dynamics information. Hydrogen-deuterium exchange experiments are one way in which dynamics information manifests itself in NMR measurements. In such experiments a folded protein (the structure of which may be deduced from crystallographic data), is in equilibrium with an “open” structure in which the amide protons of the backbone can exchange with deuterons of the deuterated solvent without stearic hindrance (1). As another example, phenylalanine and tyrosine side chains have been observed to undergo rapid 180° flips which contradict rotational barriers calculated from the rigid crystal structure of some proteins. Rotational barriers calculated based on a flexible protein structure are generally in agreement with NMR observations (2).
Thermodynamically, there are sufficient fluctuations of protein energy and volume (3) for significant differences between crystallographic and solution structures of proteins. These can be explained by NMR relaxation measurements which indicate that most biomolecules undergo rapid conformational fluctuations.

NMR can be used to monitor molecular dynamics processes covering rates of many orders of magnitude. For the slowest motions, "time lapse spectroscopy" (i.e. applying a chemical, thermal or magnetic perturbation to the equilibrated system and obtaining successive spectra) has provided insight into the exchange rates of amide protons in peptides (4) and proteins (5). Other slow conformational processes which give rise to spectral changes over many hours have also been observed (6, 7). Intermediate rate processes in the range $1 \times 10^6$ s$^{-1}$ are illustrated by the isomerisation of peptide bonds or the internal diffusion of aromatic protein side chains. These can be treated quantitatively by applying the McConnell equations (8) and described as chemical exchange phenomenon. The fastest processes with rates in the range $10^7 - 10^{12}$ s$^{-1}$ include diffusive motions of the molecule as a whole and internal motions of side chains. These can be studied by analysing nuclear magnetic relaxation rates and nuclear Overhauser enhancements.

Dynamics data is valuable in understanding the structural, motional and therefore functional properties of biological molecules. However not all of the dynamics processes which NMR measurements are sensitive to are of equal importance in determining the biochemical behaviour of proteins. For example, protein methyl groups undergo rapid internal motion which has a high profile as far as the technique is concerned because it will significantly affect the relaxation rates but this motion may not be important in a biological context. However there may be a few cases where the methyl group is highly restricted when it is expected to undergo rapid motion. Such data would indicate intramolecular interactions between the methyl group and other groups.
3.2.1 QUANTIFYING MOLECULAR MOTION

Relaxation rates give information about the motional fluctuations of the bond vector of interest with respect to B\(_0\) (9). These fluctuations occur as a result of overall tumbling of the whole molecule and any internal motion localised to the bond being studied. The fluctuations of each bond vector can be described by an angular autocorrelation function G(\(\tau\)) (10). G(\(\tau\)) pertains to the loss of correlations of the bond vector from a known orientation at two times separated by a delay \(\tau\). The autocorrelation function for proteins in solution is given by (11, 12):

\[
G(\tau) = \frac{1}{2} \left( 3 \langle \cos \theta(\tau) \rangle^2 - 1 \right)
\]  

[3.1]

where \(\theta(\tau)\) is the angle between the internuclear vector at times \(t\) and \(t + \tau\). G(\(\tau\)) is an ensemble averaged for all the IS bonds over all protein molecules. When \(\tau = 0\), G(\(\tau\)) = \(\frac{1}{5}\). As \(\tau\) increases, G(\(\tau\)) decreases since the IS vector undergoes motion and \(\cos \theta < 1\), on average. This motion will result from both overall rotational diffusion and internal motion. G(\(\tau\)) = 0 at, and beyond, a characteristic time called the autocorrelation time, \(\tau_c\). This is related to the time taken for a molecule with a known orientation to become completely uncorrelated to its starting orientation (Fig. 3.1). Small, fast tumbling molecules have short correlation times compared to large, slow moving proteins.

Fig. 3.1 The autocorrelation time, \(\tau_c\), is related to the length of time taken for a molecule with a known orientation to become completely uncorrelated to its starting orientation.
The value of $G(x)$ is independent of specific dynamics since its boundary is constant (i.e. $G(0) = \frac{1}{5}$ and $G(\infty) = 0$). However, the temporal decay of $G(\tau)$ is more useful as it is interlinked with the dynamics of the specific IS vector. Such a description is given by taking a Fourier transform of the autocorrelation function ($I0, I1$):

$$J(\omega) = 2 \int_{0}^{\infty} \cos(\omega \tau) \cdot G(\tau) \cdot d\tau$$

[3.2]

The resulting spectral density function, $J(\omega)$, gives the frequencies ranging from $\omega$ to $\omega + d\omega$ which contribute to $G(\tau)$. Hence the shape for $J(\omega)$ gives a temporal decay of $G(\tau)$. Different shapes for $J(\omega)$ reflect different distributions of frequencies contributing to motional fluctuations. Every IS bond vector that has different mobilities will have a $J(\omega)$ of a particular shape that gives the distribution of frequencies for that particular IS bond. For example, bonds which undergo fast motion will have $G(\tau)$ that decay rapidly and consequently $J(\omega)$ will cover a broader range of frequencies that contribute to the fluctuations. A broader shape for $J(\omega)$ means that a wider band of frequencies, hence higher, contribute to the motion. Regardless of the shape of the spectral density function, the area under the curve remains constant implying that the energy of the dipolar and CSA induced fluctuations is constant over all the molecules in the sample ($I0$).

Each NMR relaxation experiment measures a linear combination of the spectral density function at different contributing frequencies. For example, for an IS spin system, the heteronuclear $T_1(R_S(S_z), T_2(R_S(S_{xy}))$ and NOE ($I3$) can be measured. The $T_1$ and $T_2$ rates correspond to the following combinations of the spectral density function ($I0$):

$$R_S(S_z) = \frac{2 \gamma_I^2 \gamma_S^2 h^2}{6 4 r_{IS}} \left[ J(\omega_I - \omega_S) + 3J(\omega_S) + 6J(\omega_I + \omega_S) \right] + \frac{\Delta \omega_S^2}{3} J(\omega_S)$$

[3.3]
\[ R_S(S_{xy}) = \frac{\gamma_I^2 \gamma_S^2 \hbar^2}{8r_{IS}^6} \left[ 4J(0)+J(\omega_I - \omega_S)+3J(\omega_S)+6J(\omega_I)+6J(\omega_I + \omega_S) \right] \]
\[ + \frac{\Delta \omega_S^2}{6} \left[ J(0)+2J(\omega_S) \right] \]  

where \( \gamma_I \) and \( \gamma_S \) are the gyromagnetic ratios for spins \( I \) and \( S \) and \( r_{IS} \) is the IS bond vector length. The steady-state heteronuclear NOE is given by (13):

\[ \text{NOE} = \frac{I_{\text{sat}} - I_{\text{eq}}}{I_{\text{eq}}} = \frac{\gamma_I}{\gamma_S} \frac{R_S(I_z \rightarrow S_z)}{R_S(S_z)} \]

where \( I_{\text{sat}} \) is the intensity of the cross-peak, \( I_{\text{eq}} \) is the intensity at equilibrium and \( R_S(I_z \rightarrow S_z) \) is given by:

\[ R_S(I_z \rightarrow S_z) = \frac{\gamma_I^2 \gamma_S^2 \hbar^2}{4r_{IS}^6} \left[ 6J(\omega_I + \omega_S) - J(\omega_I - \omega_S) \right] \]

These three measurements (Eqns. 3.3 to 3.5) are a function of \( J(\omega) \) at specific frequencies but the information obtained from them is not sufficient in itself to determine what these values are. To overcome this, a motional model can be used to make assumptions about the dynamics and model the shape of the spectral density function. Four such models are discussed below (section 3.3). Alternatively, by making more measurements, the Peng and Wagner approach of “spectral density mapping” can be used (sub-section 3.4.1).
3.3 MODELLING THE SPECTRAL DENSITY FUNCTION

The three relaxation measurements given in Eqns. 3.3 to 3.5 need to be analysed to characterise the dynamics of the IS bond vector. This is typically carried out by using theoretical models which make a priori assumptions. This section gives an overview of two types of models which can be used for this purpose: those which have general applications and others which are specific for particular groups (e.g. methyl side-chains). The former is the "model free" approach (12, 14). The latter includes rotational diffusion models, "wobbling-in-a-cone" (15, 16), which was used extensively until 1982, and jump models.

3.3.1 MODELS WHICH HAVE GENERAL APPLICATIONS

3.3.1.1 The "Model-Free" Formalism

In a spherical molecule which undergoes isotropic motion, the amount of motion at a given frequency can be described by the spectral density function, \( J(\omega) \):

\[
J(\omega) = \frac{1}{4\pi} \frac{2\tau_c}{1 + \omega^2 \tau_c^2}
\]

[3.7]

where \( \tau_c \) is the overall correlation time defining the motional characteristics of the system. A small fast moving molecule will undergo motion at higher frequencies than larger slower tumbling molecules whose dynamics only involve a small range of low frequency motions. The profile of \( J(\omega) \) for these two examples is shown in Fig. 3.2.
A more detailed parameter model is given by (12, 14):

\[
J(\omega) = \frac{1}{4\pi} \left( S^2 \frac{2\tau_c}{1 + \omega^2 \tau_c^2} \right)
\]  \hspace{1cm} [3.8]

where \( S^2 \) is an order parameter which quantitates the extent of order in the molecule. It is equal to 1 for internuclear vectors which are rigid and 0 for those which are completely flexible.

The simple formalism, given in Eqn. 3.8, has proved to be remarkably successful in accounting for relaxation data of small molecules in simple polymers, as well as for fragmentary data obtained from one-dimensional NMR measurements on peptides and proteins (12, 14, 17-22).

The form of the spectral density function given in Eqn. 3.8 assumes that the molecule undergoes isotropic motion. However, dynamics studies of proteins show that different parts of the molecule have different motional characteristics. For example, the backbone tumbles much more slowly than the side-chains. Clearly Eqn. 3.8 is inadequate for modelling the dynamics of such molecules. A three-parameter form of the spectral density function (given in Eqn. 3.9 and shown in Fig. 3.3) describes motion with two correlation times on different timescales as well as an
order parameter. It is conventionally used to describe the dynamics of proteins (14) and is given by:-

\[ J(\omega) = \frac{1}{4\pi} \left( S^2 \frac{2\tau_e}{1 + \omega^2 \tau_e^2} + \left(1 - S^2\right) \frac{2\tau_e}{1 + \omega^2 \tau_e^2} \right) \]  \[ [3.9] \]

where

\[ \frac{1}{\tau_i} = \frac{1}{\tau_e} + \frac{1}{\tau_c} \]  \[ [3.10] \]

where \( \tau_i \) is the correlation time for internal motion and \( \tau_e \) is the effective correlation time. Eqn. 3.9 makes two assumptions: the fast internal motion \( \tau_i \) is independent of the slow overall motion \( \tau_c \) and that the slow motion is isotropic over the whole molecule.

This approach has formed the basis for numerous studies of peptide and protein backbone dynamics (23-25). The flexible regions of the proteins (e.g. C- and N-termini, binding loops, etc.) have smaller order parameters compared to the regions which are rigidly held in \( \alpha \)-helices, \( \beta \)-sheets or turns from one component of secondary structure to another.

![Diagram](image)

**Fig. 3.3** A profile of the "model-free" spectral density function for biomolecules (e.g. proteins).
While the model-free approach has been used successfully in numerous studies it does not always fit the data well (24).

3.3.1.2 The Extended "Model-Free" Formalism

Clore et al (24) found that the "model-free" approach failed to account for their H-\(^1\)N NOE data. Their calculated values of \(T_2\) based on the best fits to \(T_1\) and NOE were 20-100\% too small for some residues compared to the observed times. They attributed this to the internal correlation function being significantly non-exponential and having slow components outside the extreme narrowing boundary.

Assuming the slow internal motions to be independent of the fast ones, \(\tau_i\) given in Eqn. 3.10 can be redefined to:

\[
\tau_i = \tau_i' = \frac{\tau_i \tau_c}{\tau_i + \tau_c}
\]  

[3.11]

where \(i = f\) (for fast) or \(s\) (for slow) and \(S^2\) in the spectral density function given in Eqn. 3.9 can be redefined to:

\[
S^2 = S_s^2 S_f^2
\]  

[3.12]

where \(S_s^2\) and \(S_f^2\) are the generalised order parameters for characterising slow and fast motions respectively. Assuming \(\tau_i\) to be too small (<10 ps) to make a significant contribution to relaxation, \(J(\omega)\) becomes:

\[
J(\omega) = \frac{S^2 \tau_c}{1 + (\omega \tau_c)^2} + \frac{(S_f^2 - S^2) \tau_s'}{1 + (\omega \tau_s')^2}
\]  

[3.13]
With this form of the spectral density function, Clore and co-workers calculated $\tau_e$ to be an order of magnitude smaller than $\tau_s$. Their results imply the existence of relatively fast and slow internal motions which require at least two exponential fits. This formalism has also been used more recently by LeMaster (26) on a system where internal motion is more rapid than molecular tumbling. It was found that there was a general benefit in using the $(S_s^2, S_f^2)$ representation over the $(S^2, \tau_e)$ Lipari and Szabo model. With this comparison LeMaster has made it clear that there are significant indications that molecular motions on two discrete timescales occur.

Treating $S_s^2$ as $S^2$ is equivalent to introducing a scaling factor in Eqn. 3.9. This is the same as changing the distance, $r_{IS}$, in the dipolar interaction (see subsection 2.3.1.2). The extended model free formalism does not necessarily separate the effects of a change in $r_{IS}$ or of very rapid restricted motion (27). Allard and co-workers found that fitting their relaxation data to Eqn 3.13 did not yield further significant information than using Eqn. 3.9.

3.3.2 SPECIFIC MODELS

3.3.2.1 The Rotational Diffusion Model

This is a widely used model developed by Woessner (15) which considers the effect of free internal diffusion on calculated relaxation parameters. It assumes torsional motion about the bond between adjacent ($^{13}$C) atoms to be unhindered but it can be restricted within an angular range which is defined by diffusion parameters (28).

Unrestricted rotation about the bond where the two atoms, in a chain of $i$ atoms, are at the centres of regular tetrahedrons (15, 29, 30), will have the following spectral density function:-
\[
J(\omega) = \sum_{i=0}^{2} B_{i0} \left( \frac{6D_0 + i^2 D_i}{1 + \omega^2 (6D_0 + i^2 D_i)^2} \right)^{-1}
\]

where \(D_0\) is the diffusion coefficient for atom 0, \(D_i\) is that for the \(i\)th atom and the elements of the \(B\) matrix reduce \(i\) to three values: 0, 1 and 2 (31, 32). Eqn. 3.14 can be applied to methyl groups in most proteins where the \(i = 0\) term only contains \(D_0\). The \(i = 1\) and \(i = 2\) terms will depend on \(D_i\) if \(D_i \gg D_0\). If the condition \(D_i > \omega > D_0\) is met, all three terms will be similar in magnitude and equally important in characterising the motion. Any approximations which are made by using either one or two terms only will be insufficient. Hence, for fast internal motion \((\omega / D_i < 1)\), as \(D_i\) decreases, the dependence of \(J(\omega)\) on internal diffusion processes will increase.

The appropriate diffusion coefficients can be estimated by measuring the heteronuclear NOE. For example, if the overall motion is slow \((\omega > D_0)\) the theoretical NOE for \(D_i = 10^9\) s\(^{-1}\) will be much larger than for \(D_i = 10^{11}\) s\(^{-1}\) (33). However, to determine the diffusion coefficients more accurately, it is valuable to make as many relaxation measurements as feasible to fit the model.

When rotational diffusion is restricted to an angular range of \(-\Phi_0 < \phi > \Phi_0\), the spectral density function will have the form (34):

\[
J(\omega) = \sum_{i=-2}^{2} \sum_{n=0}^{\infty} |d_{i0}(\beta)|^2 \cdot |E(i, n)|^2 \cdot \frac{\tau_i}{1 + \omega^2 \tau_i^2} \quad [3.15]
\]

where \(d_{i0}(\beta)\) are the reduced Wignor rotation matrices (35), the matrices \(E(i, n)\) are functions of the angle \(\theta\) defining the allowed range of motion (34, 36) and
\[ \tau_i = \left( \frac{n^2 \pi^2 D_i}{6D_0 + \frac{n^2 \pi^2 D_i}{4\theta^2}} \right)^{-1} \tag{3.16} \]

and the effective rotational correlation time, \( \tau_{me} \), is:

\[ \frac{1}{\tau_{me}} = \frac{1}{\tau_c} + \frac{1}{\tau_m} \tag{3.17} \]

with

\[ \tau_m = 4 \phi_0^2 / m^2 \pi^2 D_i \tag{3.18} \]

In Eqn. 3.18 \( \phi_0 \rightarrow 0 \) in the limiting case of a rigid molecule as expected. However, \( \phi_0 = 180^\circ \) which is against expectation for the case of free rotation. This is accounted for by the fact that in the narrowing case of \( \phi_0 = 180^\circ \), a motion across the boundary is forbidden (37).

The restricted angular range model can give sufficient information to distinguish between unrestricted internal rotation and internal rotation limited within a restricted angle. Variations of this model have been applied to relaxation measurements of a fully \( ^{13} \text{C} \) labelled decapeptide and protein (37). The ranges for the internal correlation times were calculated and shown to be comparable with those obtained from \( ^{13} \text{C} \) \( T_1 \) measurements. The restricted angular range model has been used to describe motions specific to alanines, valines, serines and lysines as well as other motions general to all amino acid residues (37). Both the restricted and unrestricted angular range models have been used recently to characterize the dynamics of lysine side-chains in di- and tri-peptides (38).

Similar models that can be used to characterise rotational diffusion of side-chains in molecules, other than spherical ones, are described in reference (39).
The rotational diffusion model has been used extensively to describe the internal dynamics of peptides and proteins. This model is widely used to characterize the dynamics of methyl groups in particular. However, such unrestricted motion has only been observed in a few groups other than terminal methyl groups.

3.3.2.2 The "Wobbling-in-a-Cone" Model

In systems where internal diffusion is restricted by local or long-range physical interactions, models other than the ones discussed so far, need to be considered. One of these, which has had extensive applications to methyl groups, is the “wobbling-in-a-cone” model.

It is assumed that spins $I$ and $S$ are in or branch from a spherical molecule which rotates isotropically. Additionally, the vector connecting the two spins is assumed to wobble within a cone. Hence the spins relax as a result of both overall molecular tumbling and internal motion which is experienced only by the two spins.

The cone model is shown diagramatically in Fig. 3.4. The spins relax by dipolar interactions in a co-ordinate system $\sum R$. The system has a diffusion constant $D_R$ to describe its rotational motions relative to the laboratory frame $\sum L$. The spins are connected by a vector $r$ which has a fixed angle $\theta_G$ to a rotation axis fixed in the rotating molecule. Any rotation about this axis by the vector $r$ is described by a diffusion constant $D_F$. Additionally the two spins are allowed to wobble with the rotation axis, relative to $\sum R$, with a diffusion constant $D_w$. The wobbling motion is restricted to a cone defined by a half-angle $\theta_{max}$. The wobble is characterised by the system $\sum W$ while the system $\sum F$ characterises rotations of the vector $r$ about the rotating axis. In summary, the resulting complex motion of $r$ can be described by the four systems $\sum L$, $\sum R$, $\sum W$ and $\sum F$ which in turn can be characterised by the three diffusion constants $D_R$, $D_w$ and $D_F$. 70
The expressions for $J(\omega)$ are very complicated and the reader is directed to some very good explanation in the literature \((15, 16)\). In essence, the wobbling-in-a-cone model involves solving for three correlation times given by:-

\[
\tau_R = \frac{1}{6D_R} \tag{3.19}
\]

\[
\tau_W = \frac{1}{D_W} \tag{3.20}
\]

\[
\tau_F = \frac{1}{D_F} \tag{3.21}
\]

where $\tau_R$, $\tau_W$ and $\tau_F$ are the correlation times for molecular diffusion, diffusional wobbling of the vector $\vec{r}$ and fixed rotational diffusion, respectively. Hence there are three unknown parameters for which values can be determined by making three independent measurements. The three relaxation measurements $T_1$, $T_2$ and NOE, given in Eqns 3.3 to 3.7 can be used to calculate the correlation times. Excellent explanations on how this can be done, which are mathematically involved, are given in the literature \((15, 16)\).

The "wobbling-in-a-cone" model can be modified to characterise the dynamics of protein backbones and side-chains \((40)\). When considering the relaxation of $\alpha$ carbons there is no need to allow rotation of the vector between $C^\alpha$ and the bonded proton about the rotating axis because $C^\alpha$ is held in the plane by a partial double bond with the backbone nitrogen as illustrated in Fig. 3.5 \((41)\). Therefore either $\theta_G = 0$ or $D_p = 0$. When the molecule undergoes isotropic rotational motion either $\tau_w = \infty$ or $\theta_{max} = 0$. If the relaxation data gives a good fit with this model then the backbone can be considered to be "wobbling-in-a-cone".
Fig 3.4 The “wobbling-in-a-cone” model as represented by two dipolar coupled spins, I and S, in a spherical isotropically rotating molecule. \( \Sigma_L, \Sigma_R, \Sigma_W \) and \( \Sigma_F \) are the co-ordinate systems; \( D_R, D_W \) and \( D_F \) are the molecular, wobbling and fixed rotational diffusion constants respectively, as described in the text; \( \mathbf{r} \) is a vector which connects the two spins and \( \theta_0 \) is the half-angle of the wobble in a cone.

Fig. 3.5 The peptide group is planar because the carbon-nitrogen bond has partial double bond character.

For the relaxation of methyl carbons in the side-chains of proteins, rotation of the methyl group about the C-C bond must also be included as well as the wobble of the bond. One can make the realistic assumption that \( \tau_p \), the correlation time for rotational diffusion of the methyl group, is very short compared to its diffusional wobbling, i.e. \( \tau_f \ll \tau_w \). In the limit where \( \tau_w \to \infty \) or \( \theta_{\text{max}} \to 0 \), the rapidly rotating methyl group can be considered to be rigidly attached to a sphere (15, 16).
Although the model presently under discussion cannot be used to determine overall rotational motions of proteins (40), it can be used to determine varying degrees of localised dynamics. Fitting the model about the $C^\alpha-C^\beta$ rotational axis of alanines, for example, across the chain shows that the two termini and other flexible segments of the backbone, undergo larger angular displacements compared to the rest of the chain. Internal dynamics of aromatic rings ($180^\circ$ flips about the $C^\beta-C^\gamma$ bond illustrated in Fig. 3.6) showed identical behaviour to that obtained from other studies (42). When analysing methyl-carbon relaxation it was found that the peripheral carbon was apparently more mobile on longer aliphatic chains. This mobility manifested itself in larger half-angles $\theta_{\text{max}}$ and greater frequencies of motion. Illustrative calculations of the dynamics of these different systems in cholesterol are given by Brainard and Szabo (43). Co-ordinate systems $\sum R$ which are rod- and disk-shaped have been discussed in detail by Kinoshita et al (44). The variety of groups the cone model can be applied to and the detailed dynamics information obtained account for its extensive use.

\[
\begin{diagram}
\text{H} & \text{O} & \text{N} - C^\alpha - C \\
\downarrow & \downarrow & \downarrow \\
& & C^\beta H_2 \\
\end{diagram}
\]

\textbf{Fig 3.6} The structure of the amino acid phenylalanine to show the $C^\beta-C^\gamma$ bond about which the aromatic ring undergoes $180^\circ$ flips.
3.3.2.3 Rotational Jump Models

The previous model considered anisotropic or internal motion as a diffusive process over a continuum of available orientations. If there are either local (intra-residue) or long-range (inter-residue) constraints, the internal motion will become a series of jumps between a restricted number of stable conformations. The relaxation rate is then determined by the rate of jumping among these conformations and their lifetimes. Relaxation is described by jump models which involve relatively few conformations because low-amplitude diffusion is ineffective in causing relaxation.

The three-site jump model corresponds to three equally stable rotamer states being available to a methyl group (37, 45-48). This is illustrated in Fig. 3.7 for a threonine side chain.

(a) Eclipsed Conformations

![Eclipsed Conformations](image)

(b) Staggered Conformations

![Staggered Conformations](image)

Fig. 3.7 An example of the few possible rotamer states available to a methyl group. The illustration is that of a methyl group with respect to its neighbouring C\(^\beta\) atom in a threonine residue. Staggered conformers, (b) are stearically less hindered and are therefore likely to be more energetically stable than eclipsed conformers, (a). Of the former three the anti-conformer is usually more stable than the other two. All three are likely to be very similar for the example illustrated here.
In this model, all three conformations are considered to be equally populated:

\[ p_1 = p_2 = p_3 = \frac{1}{3} \quad [3.22] \]

The spectral density function is given by (36):

\[
J(\omega) = \frac{B_{00}}{1 + \omega^2 (6D_0)^{-2}} + \frac{(6D_0 + 3 / \tau_e)^{-1}}{1 + \omega^2 (6D_0 + 3 / \tau_e)^{-2}}
[3.23]
\]

where the lifetime of the methyl orientation in each conformer is \( \tau_e \), \( D_0 \) is the overall isotropic diffusion constant and the elements of the matrix B are given in (31, 32, 36).

The two-site jump model assumes that only two of the three conformations shown in Fig. 3.7 are occupied and the populations may be unequal:

\[ p_2 = 1 - p_1, \quad p_3 = 0 \quad [3.24] \]

The spectral density function is given by:

\[
J(\omega) = (1 - C) \frac{(6D_0)^{-1}}{1 + \omega^2 (6D_0)^{-2}} + C \frac{(6D_0 + 1 / \tau_c)^{-1}}{1 + \omega^2 (6D_0 + 1 / \tau_c)^{-2}}
[3.25]
\]

where, if \( \tau_A \) and \( \tau_B \) are the lifetimes of the two stable states:

\[
\frac{1}{\tau_c} = \frac{1}{\tau_A} + \frac{1}{\tau_B}
[3.26]
\]

and

75
\[
C = \frac{3 \tau_A \tau_B}{(\tau_A + \tau_B)^2} \left[ \sin^2 \beta(1 - \cos 2\theta) \right] \left[ 2 - \sin^2 \beta(1 - \cos 2\theta) \right]
\]

[3.27]

where \( \beta \) is the angle defined by the internuclear bond and the axis about which the vector jumps and \( \theta \) is half of the jump range i.e. the bond vector jumps between \(+\theta\) and \(-\theta\). In the limit \( \tau_A = \tau_B \), the corresponding models have been derived by (49, 50). Other models which assume jumps between numerous states have been covered in the literature (51-55).

Regardless of the dynamics model used, it is of value to make as many relaxation measurements as are feasible to fit it. This may typically involve measuring the \( T_1 \), \( T_2 \) and NOE at multiple field strengths.

The two-step model has been used in several studies indicating that some biomolecules are bistable. The pyrroolidine ring of proline (56-58) and the tetrahydropyrazine ring of tetrahydrofolate (59) both appear to be bistable. The interconversion rates of the former are fast enough to alter the \( T_1 \) values (34). In proteins, the phenylalanine and tyrosine residues undergo flips of 180°. Although the rates are generally not rapid enough to effect the \( T_1 \) values significantly, they can change the relaxation parameters. In NAD\(^+\) the nicotinamide ring has been shown to jump slowly between the syn and anti conformations (60).
3.4 Spectral Density Mapping

The models discussed so far, depending on the system, may not be reasonably appropriate or they may not fit the data well, indicating that at least one of the assumptions inherent in the model is inapplicable. However, ideally it should not be necessary to introduce the assumptions inherent in models. An approach which offers an alternative to the analysis of relaxation data in terms of particular models of motion is briefly described in this section. The first sub-section looks at the original approach proposed by Peng and Wagner and the second part looks at a reduced version of it.

3.4.1 MAPPING THE SPECTRAL DENSITY FUNCTION

Peng and Wagner have proposed a direct method of extracting dynamics information from relaxation data without the need to use a motional model (61, 62). This method involves measuring a set of six relaxation rates including the heteronuclear $T_1$, in-phase and anti-phase $T_2$ and NOE as well as other longitudinal modes. The measured relaxation rates are used to calculate the spectral density function at five frequencies and characterise the dynamics of internuclear vectors directly from experimental measurements. Peng and Wagner have applied this method to backbone $^{15}\text{N}$ spins in a uniformly $^{15}\text{N}$ labelled protein (61). Hence the discussion here will be restricted to a $^{15}\text{N}$-H system but this does not imply that this method cannot be applied to any other spin systems.

The relaxation rates of a $^{15}\text{NH}$ spin system sample the spectral density function, $J(\omega)$, at the five transition frequencies shown in Fig. 2.16: $\omega_N$, $\omega_H$, $(\omega_H+\omega_N)$ and $(\omega_H-\omega_N)$. In order to measure $J(\omega)$ at these five frequencies, it is necessary to make five independent relaxation measurements. These include the heteronuclear longitudinal relaxation rate ($R_n(N_z)$), in-phase tranverse relaxation rate ($R_n(N_{xy})$) and the cross-relaxation rate ($R_n(H_z\rightarrow N_z)$). The relative contribution of
each frequency to these rates has been given in Eqns. 3.3 and 3.4 above. The heteronuclear NOE is obtained as shown in Eqns. 3.5 and 3.6. The remaining two measurements are the relaxation rates of the longitudinal two spin order, \( R_{\text{NH}}(2H^N_z N_z) \) given in Eqn. 3.28, and the anti-phase \(^{15}N\) transverse relaxation rate \( R_{\text{NH}}(2H^N_z N_{xy}) \) given in Eqn. 3.29:

\[
R_{\text{NH}}(2H^N_z N_z) = \frac{\gamma H^N Y N h}{4r^6_{\text{NH}}} \left[ \frac{1}{2} \frac{2}{J(\omega_N) + 3J(\omega_H^N)} \right] + \frac{\Delta \omega_N}{3} J(\omega_N) + \rho_{H^N H^i}
\]

[3.28]

\[
R_{\text{NH}}(2H^N_z N_{xy}) = \frac{\gamma H^N Y N h}{8r^6_{\text{NH}}} \left[ \frac{1}{2} \frac{2}{J(0) + J(\omega_N - \omega_N) + 3J(\omega_N) + 6J(\omega_H^N + \omega_N)} \right] + \frac{\Delta \omega_N}{3} \left[ \frac{1}{2} J(0) + \frac{1}{2} J(\omega_N) \right] + \rho_{H^N H^i}
\]

[3.29]

where

\[
\rho_{H^N H^i} = \sum_i \frac{\gamma H^N Y N h}{8r^6_{\text{NH}}} \left[ J(\omega_N^H - \omega_H^i) + 3J(\omega_H^N) + 6J(\omega_H^N + \omega_H^i) \right]
\]

[3.30]

The latter two relaxation rates are significantly affected by the relaxation decay of the amide proton. Hence the \( H^N \) longitudinal magnetisation decay rate also needs to be measured. It is given by :

\[
R_{H^N}(H^N_z) = \frac{\gamma H^N Y N h}{4r^6_{\text{NH}}} \left[ \frac{1}{2} \frac{2}{J(\omega_N - \omega_N) + 3J(\omega_H^N) + 6J(\omega_H^N + \omega_N)} \right] + \rho_{H^N H^i}
\]

[3.31]
The linear equations of the six measurements can be solved to give the spectral density values directly from the experimental rates (62). These are given by:—

\[ J(0) = \frac{3}{4} \frac{1}{3d+c} \left[ -\frac{1}{2} R_N(N_z) + R_N(N_{xy}) + R_{NH} \left( 2H_z N_{xy} \right) - \frac{1}{2} R_{NH} \left( 2H_z N_z \right) - \frac{1}{2} R_H \left( H_z \right) \right] \]  

[3.32]

\[ J(\Omega_{\text{H}} - \Omega_N) = \frac{1}{4d} \left[ R_N(N_z) - R_{NH} \left( 2H_z N_z \right) + R_H \left( H_z \right) - 2R_N \left( H_z \rightarrow N_z \right) \right] \]  

[3.33]

\[ J(\Omega_N) = \frac{1}{2} \frac{1}{3d+c} \left[ R_N(N_z) + R_N(N_{xy}) + R_{NH} \left( 2H_z N_z \right) - \frac{1}{2} R_H \left( H_z \right) \right] \]  

[3.34]

\[ J(\Omega_{\text{H}} + \Omega_N) = \frac{1}{24d} \left[ R_N(N_z) - 2R_N \left( N_{xy} \right) - 2R_{NH} \left( 2H_z N_{xy} \right) + R_{NH} \left( 2H_z N_z \right) + R_H \left( H_z \right) \right] \]  

[3.35]

\[ J(\Omega_{\text{H}} + \Omega_N) = \frac{1}{24d} \left[ R_N(N_z) - R_{NH} \left( 2H_z N_z \right) + R_H \left( H_z \right) + 2R_N \left( H_z \rightarrow N_z \right) \right] \]  

[3.36]

where 

\[ d = \frac{\gamma_N^2 \gamma_{NH}^2}{4r^6 N^N}, \quad c = \frac{\Delta \omega_N^2}{3} \]  

and

\[ \rho_{H^N H^N} = \left[ -\frac{1}{4} R_N(N_z) - \frac{1}{2} R_N(N_{xy}) + \frac{1}{2} R_{NH} \left( 2H_z N_{xy} \right) + \frac{1}{4} R_{NH} \left( 2H_z N_z \right) + \frac{1}{4} R_H \left( H_z \right) \right] \]  

[3.37]

quantititates the amide proton dipolar interaction to any other proton.

This approach provides an alternative route to obtain dynamics information when motional models do not fit the relaxation data. Peng and Wagner implemented this to map the spectral density function of eglin c and compared it with the model-
They found that two of the higher frequency spectral density values, $J_1$ and $J_2$, were generally not well determined while the other three spectral density values gave good comparisons.

The drawback of taking a linear combination of the spectral density function is the accumulation of errors. The overall error can rapidly propagate when combining four or five relaxation rates as shown in Eqns. 3.32 to 3.37.

### 3.4.2 REDUCED SPECTRAL DENSITY SET

To overcome the problem of $J_1$ and $J_2$ not being well determined, a reduced spectral density component set has been suggested (63-67). It is assumed that the $J_3$, $J_4$, and $J_5$ components are approximately equal and only $J_0$, $J_1$, and $J_2$ are utilized. This assumption yields the following set of three linear equations of the spectral density components for the heteronuclear $T_1$, $T_2$ and NOE:

\[
R_N(\mathbf{N}_z) = \frac{2}{\gamma H \gamma_N \hbar} \left[ \frac{3}{6} J(\omega_N + \omega_N) + 7J(\omega_H + \omega_N) \right] + \frac{\Delta}{3} \frac{2}{3} \left[ J(\omega_H + \omega_N) \right] \]  

\[
R_N(\mathbf{N}_{xy}) = \frac{2}{\gamma H \gamma_N \hbar} \left[ 4J(\omega_N) + 3J(\omega_H + \omega_N) \right] + \frac{\Delta}{3} \frac{2}{3} \left[ \frac{2}{3} J(\omega_H + \omega_N) \right] \]  

\[
R_N(\mathbf{I}_z \rightarrow \mathbf{N}_z) = \frac{2}{\gamma H \gamma_N \hbar} \left[ 5J(\omega_H + \omega_N) \right] \]

With this assumption the three conventionally measured relaxation rates are sufficient to determine values for the remaining three spectral densities and there is no need to
make any further experimental measurements. However these three relaxation measurements are not sufficient to determine the spectral density function without using a dynamics model. Therefore, the QSDF analysis has been used to extract only primary information on protein dynamics followed by a more detailed analysis by using a motional model.

3.5 PRACTICAL IMPLEMENTATION OF RELAXATION TO EXTRACT DYNAMICS INFORMATION

This section briefly describes the problem of using NMR relaxation measurements to probe molecular dynamics. The main consideration is the large number of relaxation interactions which need to be quantified. This is made difficult in a protein because of internal dynamics: the number and the size of the internuclei vectors are constantly changing as a result of the differential motion of the side-chain. Additionally, each relaxation interaction may undergo dynamics which are different from other interactions giving rise to different expressions for the spectral density function.

In this complicated relaxation scenario there are two special cases: $^{15}$N and $^{13}$C. When analysing the relaxation of these two heteronuclei, sometimes a few assumptions can be made about their relaxation behaviour to simplify the picture. These are looked at below.

3.5.1 IMPORTANCE OF RELAXATION IN PROTEIN DYNAMICS

NMR relaxation properties can provide molecular dynamics information and are an important tool in developing our understanding of protein structure and function (68-77). This potential usually goes unrealised where complex relaxation processes occur. The complexity arises from the large number of interactions that occur and hence the large number of variables for which values must be known (Fig. 3.8). If these numerous unknown interactions can be reduced to a few known ones
then it becomes possible to analyse the relaxation data. Special cases where this has been done are the relaxation of heteronuclei. The two which will be considered below and which are commonly used for elucidating protein dynamics are $^{15}\text{N}$ and $^{13}\text{C}$. Since both of these isotopes occur at low natural abundance (<1%), such studies usually require isotopic labelling.

![Fig. 3.8 An example of the numerous unquantified interactions of a spin surrounded by many others in a part of a protein.](image)

### 3.5.2 $^{15}\text{N}$ RELAXATION

$^{15}\text{N}$ relaxation measurements have been used to probe the dynamics of macromolecules since Roberts and colleagues (72) measured natural abundance $^{15}\text{N}$ T$_1$'s and NOEs for biopolymers and since Hawkes' team (73) made similar measurements of natural abundance $^{13}\text{C}$ and $^{15}\text{N}$ of Gramacidin S. This opened up the way for numerous papers reporting the extraction of molecular dynamics information from NMR relaxation data of these heteronuclei (17, 20, 40, 74-81).

$^{15}\text{N}$ relaxation rates are useful for obtaining dynamics information for two reasons: firstly, $^{15}\text{N}$ can be assumed to relax by dipolar interactions only with its bonded proton and by its own CSA (Fig. 3.9). All other interactions are very small by comparison and, therefore, can be neglected. Secondly, these interactions can be used to obtain dynamics information without the need for prior structural knowledge of the system because the amide bond length has a fairly constant value of 1.01 Å.
The studies mentioned thus far directly observed the heteronucleus in one-dimensional experiments. The limited sensitivity and resolution of such methods necessitated large amounts of sample and long experimental times. These drawbacks have been overcome by making indirect measurements of the insensitive nuclei with one and two-dimensional pulse sequences (82, 83).

Obtaining the dynamics of individual amino acid residues in proteins was made possible by Bax and co-workers (23). They reported the use of inverse detection two-dimensional pulse sequences for the measurement of residue-specific $T_1$, $T_2$ and NOEs of backbone $^{15}$N atoms in a uniformly $^{15}$N labelled protein. They used the relaxation data in conjunction with the model-free approach to precisely extract order parameters for the backbone amides. This information has the potential to give a fuller picture of molecular dynamics compared to static structures which were obtainable previously. As molecular dynamics is interlinked with the structure and hence, the function of the protein, it can potentially provide information to elucidate the mechanism of protein function. Since the initial paper in 1989, there has been an abundance of reported relaxation studies (12, 14, 23-25, 61-63, 65, 85-91).
3.5.3 $^{13}$C RELAXATION

Relaxation experiments on $^{15}$N labelled proteins are mostly confined to probing the dynamics of the protein backbone (23, 61, 62). This is a great limitation in a system where side chains are of particular interest for investigating protein interactions with other molecules (e.g. protein-protein or protein-substrate interactions).

Natural abundance $^{13}$C (74), randomly fractional labelling (93) and selective $^{13}$C labelling has enabled some studies of side chain relaxation to be made (27, 90, 94-103). In such a case the heteroatom relaxes with its bonded proton(s) and with unbonded proton(s) which are close by in space. The latter may be on the same amino acid residue as the heteroatom or on other residues which have been brought close in space as a result of the secondary and tertiary structure. These numerous unquantified $^{13}$C dipolar interactions can be reduced to a few quantified ones by assuming that the $^{13}$C spin, like $^{15}$N, relaxes through dipolar interactions solely with its bonded proton(s) and its own CSA. Unlike with $^{15}$N relaxation, the CSA of $^{13}$C is very small (about 30 ppm for $^{13}$C$^\alpha$ (104)) and can be neglected in some cases, e.g. (100). The dipolar interactions to proton(s) other than the bonded one(s) can be neglected because they are assumed to be too small to make a significant contribution to the relaxation of the $^{13}$C spin. For a $^{13}$CH group there will only be one dipolar interaction between the $^{13}$C spin and its bonded proton whereas for a $^{13}$CH$_2$ group there will be two such interactions. These are shown in Fig. 3.10.

![Fig. 3.10 Models for the relaxation of $^{13}$C in selectively labelled molecules. Apart from the $^{13}$C CSA interaction, there is one dipolar interaction for a $^{13}$CH group, (a), and two for a $^{13}$CH$_2$ group, (b).](image-url)
However, in uniformly $^{13}\text{C}$ labelled proteins the relaxation interactions of $^{13}\text{C}$ atoms are even more numerous and varied than those of $^{15}\text{N}$. In addition to the $^{13}\text{C}$ spin relaxing due to its dipolar interaction with bonded and non-bonded proton(s), which may be on the same amino acid or another residue which is close in space, there will also be dipolar interactions between the $^{13}\text{C}$ of interest and its bonded $^{13}\text{C}$ (or $^{15}\text{N}$) neighbours. A range of these interactions for an alpha $^{13}\text{C}$, along the backbone of a protein, have been quantified in Table 3.1 (104) and illustrated in Fig. 3.11.

Table 3.1 Dipole-dipole interaction constant $D_{Cj}^2$ between $^{13}\text{C}^{\alpha}$ and various adjacent nuclei ($j$)

<table>
<thead>
<tr>
<th>Dipolar Interaction</th>
<th>$r$ (Angstroms)</th>
<th>$D_{Cj}^2$ ($10^9$ s$^{-2}$)</th>
<th>$D_{Cj}^2 / \sum D_{Cj}^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{13}\text{C}^{\alpha}-\text{H}^{\alpha}$</td>
<td>1.10</td>
<td>5.084</td>
<td>0.942</td>
</tr>
<tr>
<td>$^{13}\text{C}^{\alpha}-^{13}\text{C}^{\beta}$</td>
<td>1.54</td>
<td>0.43</td>
<td>0.008</td>
</tr>
<tr>
<td>$^{13}\text{C}^{\alpha}-\text{H}^{\beta}$</td>
<td>2.03</td>
<td>0.129</td>
<td>0.024</td>
</tr>
<tr>
<td>$^{13}\text{C}^{\alpha}-^{15}\text{N}$</td>
<td>1.46</td>
<td>0.009</td>
<td>0.002</td>
</tr>
<tr>
<td>$^{13}\text{C}^{\alpha}-\text{H}^N$</td>
<td>2.15</td>
<td>0.091</td>
<td>0.016</td>
</tr>
<tr>
<td>$^{13}\text{C}^{\alpha}-^{13}\text{C}$</td>
<td>1.53</td>
<td>0.044</td>
<td>0.008</td>
</tr>
</tbody>
</table>

$$D_{Cj}^2 = \frac{\gamma_C^2 \gamma_j^2 \hbar^2}{4r_{Cj}^6 \left( \frac{\mu_0}{4\pi} \right)^2} \left( \frac{\mu_0}{4\pi} \right)$$

where $\gamma_C$ (the magnetogyric ratio of $^{13}\text{C}$) = $6.728 \times 10^7$ s$^{-1}$T$^{-1}$, $\gamma_H = 2.6752 \times 10^8$ s$^{-1}$T$^{-1}$, $\gamma_N = 2.709 \times 10^7$ s$^{-1}$T$^{-1}$, $\hbar$ (Plank's constant divided by $2\pi$) = $2.6752 \times 10^8$ Js and $\mu_0$ (permeability of vacuum) = $4 \pi \times 10^7$ Js$^2$C$^{-2}$m$^{-1}$. 

85
Fig. 3.11 The possible interactions of a $^{13}\text{C}^\alpha$ spin in uniformly $^{13}\text{C}$ labelled molecules. As well as the CSA of the heteronucleus there will be at least five different dipolar interactions: $^{13}\text{C}$-$^{13}\text{C}$, $^{13}\text{C}$-H (bonded), $^{13}\text{C}$-H (non-bonded) on the same residue (intramolecular) and on other residues which are close in space (intermolecular) and $^{13}\text{C}^\alpha$-$^{15}\text{N}$.

The dipolar interactions between a $^{13}\text{C}^\alpha$ and protons other than the bonded proton, are several percent of the interaction between the $^{13}\text{C}^\alpha$ and its bonded proton. For example, if the $^{13}\text{C}^\alpha$ relaxes with the $H^\beta$ and $H^N$, as well as the bonded proton, the former two interactions will make a sum contribution of 4% to the relaxation of the heteronucleus. If the alpha $^{13}\text{C}$ relaxes with two beta-protons and one amide-proton in addition its bonded proton, the former three unwanted interactions will make a 6% contribution to the overall relaxation of the $^{13}\text{C}^\alpha$ spin. Additionally, dipolar interactions between the $^{13}\text{C}^\alpha$ and its bonded $^{13}\text{C}$ and $^{15}\text{N}$ neighbours will make a further 1% contribution. Consequently the contribution to $^{13}\text{C}^\alpha$ relaxation from unwanted dipolar interactions proliferates rapidly and it is not justifiable to assume that the $^{13}\text{C}^\alpha$ spin only relaxes with its bonded proton. The unwanted interactions make a significant contribution and they need to be taken into account in the analysis of $^{13}\text{C}$ relaxation data.

The longitudinal relaxation of $^{13}\text{C}^\alpha$ magnetisation is given by:

$$ R_{C^\alpha} \left( S_{C^\alpha} \right) = \sum_{j \neq C^\alpha}^n \frac{1}{2} D_{C^\alpha j}^2 \left[ \frac{1}{3} J(\omega_{C^\alpha} - \omega_j) + J(\omega_{C^\alpha}) + 2J(\omega_{C^\alpha} + \omega_j) \right] $$

[3.41]
The amplitude of $J(\omega_{C\alpha})$ arises as a result of the heteronuclear $^{13}C^\alpha-H^\alpha$ interaction. The $J(\omega_{C\alpha} - \omega_j)$ term becomes $J(0)$ if $j = ^{13}C$. $J(0)$ arises as a result of the $^{13}C^\alpha-^{13}C^\beta$ and $^{13}C^\alpha-^{13}C'$ interactions. For small fast tumbling molecules the $J(\omega_{C\alpha})$ term is much larger than the $J(0)$ term. However in slow tumbling proteins $J(0)$ is much larger than $J(\omega_{C\alpha})$. Consequently the products $D_{C^\alpha C^\beta}^2J(0)$ and $D_{C^\alpha C'}^2J(0)$ become comparable to the product $D_{C^\alpha H^\alpha}^2J(\omega_{C\alpha})$ (104). It has been reported in the literature that the estimated contribution of the sum of the interactions, except for the heteronuclear $^{13}C^\alpha-H^\alpha$ interaction, may contribute as much as 15% to the $T_1$ values of a protein with an autocorrelation time of 6 ns (104). The contribution of the unwanted interactions increases for proteins with longer correlation times. Consequently the $T_1$ data for uniformly $^{13}C$ labelled proteins cannot be interpreted with an AX or an AX2 spin system as with selectively $^{13}C$ labelled proteins.

The size of the six contributions, given in Table 3.1, to the relaxation rate will depend, to varying degrees, on the motional parameters of the relevant internuclear vectors. Such differential motion will result in fluctuating internuclear distances which will affect relaxation of the $^{13}C$ atom. These need to be taken into account in analysis (sub-section 3.6.1) and in the design of the pulse sequences used to make the necessary relaxation measurements (Chapter Four).

The numerous $^{13}C$-proton interactions can be taken into account by using a fictitious effective distance, $r_{\text{eff}}^{-6}$, given by the sum of all the individual $^{13}C$-proton internuclear vectors to the power of -6 (27):

$$r_{\text{eff}}^{-6} = r_{C^\alpha-H^\alpha}^{-6} + r_{C^\alpha-H^\beta}^{-6} + r_{C^\alpha-H^\gamma}^{-6} + r_{C^\alpha-H^N}^{-6} + \sum_i r_{C^\alpha-H^i}^{-6} \tag{3.42}$$
It is assumed that the dominant relaxation interaction is that of the $^{13}\text{C}^{\alpha}$-$\text{H}^{\alpha}$ vector: any cross-relaxation terms are too small to be significant and the spectral density functions of other vectors are similar to that of $^{13}\text{C}^{\alpha}$-$\text{H}^{\alpha}$. This assumption is justified if the overall motion of the molecule is much larger than and dominant over any local motion. Hence all the nuclei in this system have common dynamics.

In Eqn. 3.42 the number of interactions and their sizes are taken to be the same, on average, for all amino acid residues, e.g. the value of $^{13}\text{C}^{\alpha}$-$\text{H}^{\text{N}}$ is taken to be 2.55 Å regardless of the residue. Clearly, this is an over-estimation in proteins since both the number and sizes of the interactions for any $^{13}\text{C}^{\alpha}$ nucleus will vary with secondary structure and therefore with the type of amino acid. For example, a side-chain which is hydrogen bonded and has restricted motion will be more amenable to analysis with this approach than one that has unrestricted motion. Additionally, in the latter case, the sizes of the internuclear vectors will vary with the dynamics of the side-chain. Hence this approach of using a fictitious effective distance is only suitable for making approximations about the dynamics of proteins and not for obtaining any detailed motional information.

The work to date on $^{13}\text{C}$ relaxation, has developed around reducing the numerous dipolar interactions to a few which can be quantified reliably. This is done by making assumptions to determine the dominant interactions. If assumptions are made to do this, then the traditional relaxation measurements (heteronuclear $T_1$ and NOE) can be made for $^{13}\text{C}$. The data is analysed by using a suitable motional model. An obvious drawback to this assumption is that it may not hold true for the system under study and therefore inaccurate conclusions may be drawn from the data. Peng and Wagner have gone part way to offering a truly model free (independent of model assumptions) method of analysis by determining the amount of motion at five frequencies directly from six independent measurements. However, this has not (yet) been applied to $^{13}\text{C}$ relaxation because of the numerous $^{13}\text{C}^{\alpha}$-proton unquantifiable interactions. Hence there is still a need for a method to analyse $^{13}\text{C}$ relaxation.
data of biomolecules, uniformly enriched with this isotope, to characterize the dynamics of the whole molecule simultaneously.

### 3.6 DEVELOPMENT OF METHODS TO ANALYSE $^{13}$C AND $^1$H RELAXATION DATA IN UNIFORMLY $^{13}$C ENRICHED MOLECULES

The numerous relaxation interactions of a $^{13}$C spin for which values cannot be determined accurately, have limited the progress of heteronuclear relaxation studies of uniformly $^{13}$C labelled proteins. The problematic interactions are $^{13}$C dipolar interactions to neighbouring $^{13}$C spins, non-bonded protons on the same amino acid residue and on nearby residues and the $^{13}$C CSA. $^{13}$C dipolar and CSA interactions are variable: the former changes with the number and size of the internuclear vectors which, in turn, are dependent on the dynamics of the structure. The latter varies with the bonded atom(s), e.g. the CSA for $^{13}$Cα is small but it is large for a carbonyl $^{13}$C. In addition, homonuclear $^{13}$C scalar coupling evolution renders the conventional $T_2$ measurement inaccurate. Therefore we need to concentrate on measuring and analysing longitudinal modes (e.g. $^{13}$C $T_1$'s and NOE's).

The purpose of this study is to develop a rigorous and precise method of analysing $^{13}$C relaxation data from uniformly $^{13}$C and $^{15}$N labelled proteins in a simple and unambiguous manner. To this end we generated general relaxation rate equations for longitudinal magnetisation and various longitudinal spin orders from the longitudinal relaxation sub-matrix (sub-section 2.3.2.3). The inherent symmetry of the sub-matrix enabled us to combine the general relaxation rate equations in such a way as to isolate the mutual relaxation of a set of coupled spins without any contributions from any other spins: the variable dipolar and CSA interactions from uncoupled spins simply cancel out.
This method can be used to isolate dipolar interactions in $^{13}\text{CH}_n$ groups. For example, in a $^{13}\text{CH}$ group, the $^{13}\text{CH}$ interaction can be separated from all others. In a $^{13}\text{CHH}'$ group, where the two protons are inequivalent, the $^{13}\text{CH}$, $^{13}\text{CH}'$ and $\text{HH}'$ dipolar interaction can be isolated. For $^{13}\text{CH}_2$ groups with equivalent protons, the two identical $^{13}\text{CH}$ interactions and the homonuclear $\text{HH}$ dipolar interaction can be separated. This method can be used further to determine the spectral density function at a particular frequency by combining the isolated interactions.

The present section is divided into four sub-sections. In the first sub-section the method of analysis is derived from general relaxation rates of longitudinal magnetisation and various longitudinal spin orders. The principle of the new method is demonstrated in the second sub-section by making the appropriate proton relaxation measurements on a small molecule with two AX spin systems. In the third part of this section the applications of the new method to biomolecules is discussed. In the final part we look at approximate methods for analysing $^{13}\text{C}$ relaxation data and ways of determining the spectral density function at particular frequencies.

3.6.1 ISOLATING THE MUTUAL DIPOLAR RELAXATION OF A SET OF COUPLED SPINS

In this sub-section we generate general relaxation rates for the decay of longitudinal magnetisation, longitudinal two and three spin orders in systems with an arbitrary number of spins. The similarities and differences of these general expressions will then be exploited by taking linear combinations of the general relaxation rates to isolate the dipolar interaction(s) of interest and eliminate all other unwanted interactions. These combinations can be used to probe the dynamics of bond vectors, such as $^{13}\text{C}-\text{H}$ and $^{15}\text{N}-\text{H}$, and of pairs of spins which are close in space, for example H and H in $^{13}\text{CH}_2$. 
groups. Furthermore, these combinations can be used in conjunction with other less rigorous relaxation expressions to map the spectral density function (sub-section 3.6.4).

In a two spin system, consisting of spins 1 and 2, the longitudinal magnetisation of spin 1 is described by element $a_{11}$ of the relaxation matrix given in Eqn. 2.40. The $T_1$ relaxation of spin 1 will relax as a result of a dipolar interaction with the other spin and its own CSA. The rate of decay of longitudinal magnetisation of spin 1 is given by (see Appendix I):

$$R_1(I_{1z}) = \frac{1}{2} D_{12}^2 \left[ \frac{1}{3} J(\omega_1 - \omega_2) + J(\omega_1) + 2 J(\omega_1 + \omega_2) \right] + \frac{1}{2} C_1^2 J(\omega_1) \quad [3.43]$$

where $D$ and $J(\omega)$ are given in Table 2.3 and Eqn. 2.39 respectively. The dipolar interaction between the two spins depends on the spectral density function at three frequencies: the sum and difference of the Larmor frequencies of the two spins and the Larmor frequency of spin 1. If a third spin, 3, is incorporated into the existing two-spin system, there will be an additional term to that of Eqn. 3.43. This term describes the dipolar interaction between spins 1 and 3. In a three spin system the longitudinal relaxation decay rate of spin 1 is:

$$R_1(I_{1z}) = \frac{1}{2} D_{12}^2 \left[ \frac{1}{3} J(\omega_1 - \omega_2) + J(\omega_1) + 2 J(\omega_1 + \omega_2) \right] + \frac{1}{2} C_1^2 J(\omega_1)$$

$$+ \frac{1}{2} D_{13}^2 \left[ \frac{1}{3} J(\omega_1 - \omega_3) + J(\omega_1) + 2 J(\omega_1 + \omega_3) \right] \quad [3.44]$$

The two dipolar interactions involve the same combination of the spectral density function. If a fourth spin is now included in the system, the $T_1$ relaxation of spin 1 will depend on the dipolar interaction between spin 1 and all the other spins, i.e. 1 and 2, 1 and 3 and 1 and 4. And the decay rate equation is as follows:
It can be seen from Eqn. 3.45 that the three dipolar interactions between the active spin 1 and the other spins are proportional to the same combination of the spectral density function. Since all the dipolar interactions are similar we can write a general expression for the rate of relaxation of $I_{1z}$ in a system of $n$ spins as:

$$R_1(I_{1z}) = \frac{1}{2} D_{12}^2 \left[ \frac{1}{3} J(\omega_1 - \omega_2) + J(\omega_1) + 2 J(\omega_1 + \omega_2) \right] + \frac{1}{2} C_1^2 J(\omega_1)$$

$$+ \frac{1}{2} D_{13}^2 \left[ \frac{1}{3} J(\omega_1 - \omega_3) + J(\omega_1) + 2 J(\omega_1 + \omega_3) \right]$$

$$+ \frac{1}{2} D_{14}^2 \left[ \frac{1}{3} J(\omega_1 - \omega_4) + J(\omega_1) + 2 J(\omega_1 + \omega_4) \right]$$

[3.45]

where $j$ can be any bonded or non-bonded spin. According to Eqn. 3.46 the relaxation of the active spin depends only on two types of terms: 1) the sum of all the dipolar interactions between the active spin and each other spin in the system and 2) the CSA of the active spin.

The relaxation rate of longitudinal two spin order of spins 1 and 2 is described by the element $b_{44}$ of the relaxation matrix (Eqn. 2.40). In a similar fashion to the general decay of longitudinal magnetisation, the general relaxation rate of longitudinal two spin order of spins 1 and 2 can be determined to be:

$$R_1(I_{1z}) = \sum_{j \neq 1}^{n} \frac{1}{2} D_{1j}^2 \left[ \frac{1}{3} J(\omega_1 - \omega_j) + J(\omega_1) + 2 J(\omega_1 + \omega_j) \right] + \frac{1}{2} C_1^2 J(\omega_1)$$

[3.46]
There are three different types of interactions here: the dipolar interactions between each active spin to all passive spins, the dipolar interaction between the two active spins and the CSA contribution of both active spins. The first two terms in Eqn. 3.47 which describe each active-passive spin interactions are identical to those of Eqn. 3.46. This similarity, and others like it, will be exploited below. The third term of Eqn. 3.47 shows that the dipolar interaction between the two active spins depends on the spectral density function at the two Larmor frequencies. Hence each active spin makes an equal contribution to this term. Likewise the CSA expressions for each spin are also the same.

The expression for the decay of longitudinal three spin order of spins 1, 2 and 3 is described by the element $f_{77}$ of the relaxation matrix (Eqn. 2.40). A general equation for this longitudinal mode is given by:-

$$
R_{12} (2I_{1z} I_{2z}) = \sum_{j \neq 1,2} \frac{4}{2} D_{ij}^2 \left[ \frac{1}{3} J(\omega_1 - \omega_j) + J(\omega_1) + 2J(\omega_1 + \omega_j) \right] \\
+ \sum_{j \neq 1,2} \frac{1}{2} D_{ij}^2 \left[ \frac{1}{3} J(\omega_2 - \omega_j) + J(\omega_2) + 2J(\omega_2 + \omega_j) \right] \\
+ \frac{1}{2} D_{12}^2 \left[ J(\omega_1) + J(\omega_2) \right] + \frac{1}{2} C_1^2 J(\omega_1) + \frac{1}{2} C_2^2 J(\omega_2)
$$

[3.47]
The first three terms of Eqn. 3.48 describe the dipolar interaction between each active spin to all passive spins. The following three terms describe the dipolar interaction between the three possible pairs of the active spins. And the final three terms give the CSA contribution to relaxation of each active spin. The summation of these nine sets of interactions describes the relaxation of longitudinal three spin order.

It is important to note that Eqns. 3.46, 3.47 and 3.48 correspond to different elements of the relaxation matrix (Eqn. 2.40). Similarities between the expressions for different elements are well known (62, 86, 105-107) and this can be seen from Eqns. 3.43 to 3.48. An active spin, spin i for example, in a longitudinal mode will always have the term \( \frac{1}{2} D^2_{ij} \left[ \frac{1}{3} J(\omega_i - \omega_j) + J(\omega_i) + 2J(\omega_i + \omega_j) \right] \) to a passive spin \( j \). The relaxation of two active spins in a longitudinal mode involving two or more spins is described by \( \frac{1}{2} D^2_{ij} \left[ J(\omega_i) + J(\omega_j) \right] \). Additionally, all longitudinal modes have a similar CSA term \( \frac{1}{2} c_i^2 J(\omega_i) \) for each active spin. This is always the case regardless of the number of spins in the system. Consequently no single relaxation decay rate will correspond to the mutual relaxation of two spins. However, due to the similarities

\[
R_{123}(4I_{12}I_{23}I_{32}) = \sum_{j \neq 1,2,3} \frac{1}{2} D^2_{ij} \left[ \frac{1}{3} J(\omega_1 - \omega_j) + J(\omega_1) + 2J(\omega_1 + \omega_j) \right]
+ \sum_{j \neq 1,2,3} \frac{1}{2} D^2_{ij} \left[ \frac{1}{3} J(\omega_2 - \omega_j) + J(\omega_2) + 2J(\omega_2 + \omega_j) \right]
+ \sum_{j \neq 1,2,3} \frac{1}{2} D^2_{ij} \left[ \frac{1}{3} J(\omega_3 - \omega_j) + J(\omega_3) + 2J(\omega_3 + \omega_j) \right]
+ \frac{1}{2} D^2_{12} [J(\omega_1) + J(\omega_2)] + \frac{1}{2} D^2_{13} [J(\omega_1) + J(\omega_3)]
+ \frac{1}{2} D^2_{23} [J(\omega_2) + J(\omega_3)]
+ \frac{1}{2} c_i^2 J(\omega_1) + \frac{1}{2} c_2^2 J(\omega_2) + \frac{1}{2} c_3^2 J(\omega_3)
\]
between Eqns. 3.46, 3.47 and 3.48, it is reasonable to suppose that the contributions of some interactions may be eliminated by taking a linear combination of the spectral density function given in Eqns. 3.46 to 3.48. By taking a linear combination of three relaxation rates corresponding to the decay rates of \( I_{1z}, I_{2z} \) and \( 2I_{1z}I_{2z} \), it is possible to isolate the mutual relaxation of a pair of spins, 1 and 2, when both also relax with an arbitrary number of other spins:

\[
R_{1}(I_{1z}) + R_{2}(I_{2z}) - R_{12}(2I_{1z}I_{2z}) = \frac{1}{2} D_{12}^{2}CH \left[ \frac{2}{3} J(\omega_1 - \omega_2) + 4J(\omega_1 + \omega_2) \right]
\]

[3.49]

The interactions which are isolated in this combination are illustrated in Fig. 3.12. All the terms except for those corresponding to the mutual dipolar relaxation of the two active spins undergo mutual cancellation. The remaining term depends on the spectral density function at just two frequencies: the sum and the difference of the Larmor frequencies of the active spins in the ratio of 6:1. The CSA terms cancel out completely and none are present in the linear combination.

The only variables left in Eqn. 3.49 are the internuclear distance between the two spins, which is needed to calculate the constant \( D_{12} \), and the parameters associated with molecular motion. The internuclear distance can be obtained from the crystal or calculated structure.
Fig. 3.12 A diagrammatic representation of how taking a linear combination of three relaxation rates results in the isolation of the mutual dipolar relaxation of a pair of spins from all other interactions. This corresponds to the combination given in Eqn. 3.49. The blocked spins are those of interest and the three different types of arrows indicate different combinations of the spectral density function. The single headed black arrows represent the \( \frac{1}{2} D_{12}^2 \left[ \frac{1}{3} J(\omega_1 - \omega_2) + J(\omega_1) + 2J(\omega_1 + \omega_2) \right] \) interactions, the double headed grey arrow represents the \( \frac{1}{2} D_{12}^2 [J(\omega_1) + J(\omega_2)] \) interaction and the double headed unfilled arrow represents the \( \frac{1}{2} D_{12}^2 \left[ \frac{2}{3} J(\omega_1 - \omega_2) + 4J(\omega_1 + \omega_2) \right] \) interaction. The CSA terms are not represented here but they cancel out completely when the linear combination is taken.

By measuring the decay rates corresponding to longitudinal three spin order, \( 4I_{1z}I_{2z}I_{3z} \), as well as the longitudinal magnetisation of the three spins, \( I_{1z}, I_{2z} \) and \( I_{3z} \), another combination becomes possible:

\[
R_1(I_{1z}) + R_2(I_{2z}) + R_3(I_{3z}) - R_{123}(4I_{1z}I_{2z}I_{3z}) = \\
\frac{1}{2} D_{12}^2 \left[ \frac{2}{3} J(\omega_1 - \omega_2) + 4J(\omega_1 + \omega_2) \right] \\
+ \frac{1}{2} D_{13}^2 \left[ \frac{2}{3} J(\omega_1 - \omega_3) + 4J(\omega_1 + \omega_3) \right] \\
+ \frac{1}{2} D_{23}^2 \left[ \frac{2}{3} J(\omega_2 - \omega_3) + 4J(\omega_2 + \omega_3) \right]
\]

\[3.50\]
The linear combination of the spectral density function shown in Eqn. 3.50 is obtained when the longitudinal three spin order decay rate is subtracted from the sum of the longitudinal relaxation rates of the three spins. Dipolar interactions between each of the active spins to other spins in the system cancel out. The only interactions isolated in Eqn. 3.50 are the three pairwise dipolar interactions between the three coupled spins, i.e. the dipolar interaction between spins 1 and 2, 1 and 3 and 2 and 3. Each of these interactions depend on the spectral density function at the sum and difference frequencies of the Larmor frequencies of each spin in the pair. Fig. 3.13 shows the interactions which are isolated by this combination.

![Diagram of interactions](image)

Fig. 3.13 A diagrammatic representation of how taking a linear combination of four relaxation rates results in the isolation of three interactions from all other interactions. This corresponds to the combination given in Eqn. 3.50. The definitions of the arrows are the same as in Fig 3.12.

An additional combination involving the longitudinal two spin order, longitudinal magnetisation and longitudinal three spin order decay rates is given by:

\[
R_{12}(2I_{1z}I_{2z}) + R_3(I_{3z}) - R_{123}(4I_{1z}I_{2z}I_{3z}) = \\
\frac{1}{2} D_{12}^2 [\frac{2}{3} J(\omega_1 - \omega_3) + 4J(\omega_1 + \omega_3)] + \\
\frac{1}{2} D_{23}^2 [\frac{2}{3} J(\omega_2 - \omega_3) + 4J(\omega_2 + \omega_3)]
\]

\[3.51\]
The combination shown in Eqn. 3.51 is an extension of Eqn. 3.49 applied to three coupled spins. It is obtained by subtracting the longitudinal three spin order relaxation rate from the sum of the decay rates of longitudinal two spin order of two of the spins and the longitudinal magnetisation of the third spin. The dipolar interactions between the three active spins and all others in the system cancel out. The mutual dipolar interaction between spins 1 and 2 and all the contributing CSA interactions also cancel out. The two terms each depend on the spectral density function at the familiar sum and difference of the Larmor frequencies of each spin in the pair in the expected 6:1 ratio. This combination isolates two pairwise interactions between spins 1 and 2 and 2 and 3. A diagrammatic representation of the interactions which are isolated by this combination is shown in Fig. 3.14.

Consider applying the three combinations given in Eqns. 3.49 to 3.51 to three spins of interest in a system with \( n \) spins. The first combination (Eqn. 3.49) isolates one dipolar interaction. The second (Eqn. 3.50) isolates three interactions and the third combination (Eqn. 3.51) isolates two. In terms of the dynamics information obtained, there is a considerable amount of overlap. The second combination gives the same information as the first plus the third combinations. Hence, depending on the system, there may be a certain amount of redundancy and it should not be necessary to take all
three linear combinations for the same system. Given the choice, Eqns. 3.49 and 3.51 are to be preferred over Eqn. 3.50 for practical reasons. The former two equations involve a fewer number of relaxation rates and therefore there are fewer opportunities for errors to accumulate. Equation 3.51 requires a larger number of relaxation rates which, when combined linearly, are likely to result in a larger accumulative error.

So far in this section, we have only considered isolating interactions in $n$-spin systems with up to three spins of interest. When there are four or more spins of interest, more longitudinal modes can be measured. Hence, other linear combinations, apart from the ones discussed so far, become possible. However, they all depend on the same linear combination of the spectral density function and no additional dynamics information is obtained. Therefore, these possible combinations are not considered in this work.

Collectively, Eqns. 3.49, 3.50 and 3.51 consist of three different terms describing the pairwise dipolar interactions between spins 1 and 2, 1 and 3 and 2 and 3: one term describes the interaction of one of these pairs. Each of these terms has the same dependence on the linear combination of the spectral density function. Consequently, if the Larmor frequencies of the nuclei involved in each pair is the same, the spectral density function will be sampled at only two frequency, i.e. at $\left[\frac{2}{3}J(\omega_i - \omega_j) + 4J(\omega_i + \omega_j)\right]$. Therefore all three combinations will have the same dependence on the dynamics of the molecule. However, if these combinations are used to isolate the dipolar interactions between different pairs of nuclei (e.g. $^{13}$C and H and H and H in $^{13}$CH$_2$ groups) then each term will have a different dependence on molecular dynamics. Each term will then sample the spectral density function at different frequencies. This will be described in detail in sub-section 3.6.3.

The measurements necessary to evaluate the combinations of Eqns. 3.49 to 3.51 can be made at two or more different field strengths to give two or more evaluations for each combination. For example, Eqn. 3.49 can be used to probe the dynamics of a $^{13}$C
and proton pair in a $^{13}$CH group and the dipolar interaction depends on
\[ \left[ \frac{2}{3} J(\omega_C - \omega_H) + 4J(\omega_C + \omega_H) \right] \] at each field strength. The Larmor frequencies of the two spins will be different at each field strength, therefore the $J(\omega_C - \omega_H)$ and $J(\omega_C + \omega_H)$ terms will have different values at each $B_0$ and the evaluation of Eqn. 3.49, at each field strength, will be different. Hence the spectral density function can be sampled at different frequencies and more information about the dynamics of a particular bonded or non-bonded interaction can be obtained (61).

3.6.2 PROTON RELAXATION EXPERIMENTS

The object of this sub-section is two fold: to demonstrate the principle of the new method derived in the previous sub-section and to identify any possible problems which may arise when implementing the new method to proteins.

3.6.2.1 Experimental Methods

From Eqn. 3.49 it can be seen that three experimental measurements are needed to calculate the mutual dipolar relaxation of a pair of coupled spins: the longitudinal relaxation rate of each spin and their longitudinal two spin order. These cannot be obtained in a single one-dimensional experiment since every mode of magnetisation perturbed from its equilibrium value will affect others through the off-diagonal elements of the relaxation matrix (Eqn. 2.40). The two schemes given in Fig. 3.15 show the pulse sequences used to measure longitudinal magnetisation and longitudinal two spin order decay rates.
Acquire

Fig. 3.15 Pulse sequences used to measure (a) longitudinal magnetisation and (b) longitudinal two spin order relaxation rates. The soft RF pulses excite the species of interest which then relaxes during the delay \( \tau \). The two hard RF pulses in (b) filter out any longitudinal magnetisation present during \( \tau \). The phase cycling used for (a) is \( \phi_1 = x, x, x, y, y, y, -x, -x, -x, -y, -y, -y \), \( \phi_2 \) and Acquisition = \( x, -x, -y, y, -x, -y, x, y, -y, x, y, -x \). The phase cycle for (b) is \( \phi_1 = (8) x, x, x, x, y, y, y, y, -x, -x, -x, -x, -y, -y, -y, -y, -y \), \( \phi_2 = x, y, -x, y, -x, x, y, -y \) and Acquisition = \( x, -y, -x, y, -x, y, x, -y \).

The selective inversion recovery sequence (108) depicted in Fig. 3.15(a) was used to measure the decay rate of longitudinal magnetisation. The spin of interest is inverted by the selective 180° RF pulse and then allowed to relax during the delay \( \tau \). Any magnetisation remaining along the z-axis is then rotated into the xy-plane by the subsequent 90° RF pulse before the FID is acquired. The experiment is repeated with different values of \( \tau \). If \( I_{iz} \) is selectively perturbed from equilibrium, the element \( a_{ii} \) in the relaxation matrix given in Eqn. 2.40 will initially be solely responsible for its decay, i.e.:-
However, the initially excited mode of magnetisation will decay partially into other modes due to the off-diagonal elements of the relaxation matrix. The perturbation of these other modes from their equilibrium values becomes greater as $\tau$ increases. These will themselves cross-relax\textsuperscript{1}, resulting in the partial regeneration of the initially excited mode. For example, $I_{1z}$ will partially decay into $I_{2z}$ as a result of the element $c_{12}$. Due to the element $c_{21}$, some $I_{2z}$ will subsequently be converted back into $I_{1z}$ affecting its rate of decay. Consequently the decay constant of $I_{1z}$ will no longer be $a_{11}$. For short values of $\tau$ this effect is small and it can be assumed to have a negligible effect on the intensity of $I_{1z}$. Under these conditions its decay can, to a good approximation, be described by Eqn. 3.52 and the relaxation decay rate constant, $a_{11}$, can readily be extracted. However, as $\tau$ increases the perturbation of modes not initially excited can no longer be assumed to be negligible and $a_{11}$ cannot be considered in isolation from the other elements of the matrix. Consequently only the initial decay of the magnetisation can be used to measure the autorelaxation\textsuperscript{1} rate of $I_{1z}$ by this method.

Solving Eqn. 3.52 yields:

\[ I(\tau) = I(\infty) \left( 1 - 2 \exp \left( \frac{-\tau}{T_1} \right) \right) \]  

[3.53]

\textsuperscript{1} The terms cross-relaxation and autorelaxation, the processes they involve and their effects on making practical measurements are discussed in sub-section 4.2.1.
where \( I(\tau) \) and \( I(\infty) \) are the intensities at time \( \tau \) and at infinity respectively and \( T_1 \) is the longitudinal relaxation time. The rate of longitudinal relaxation of each spin of interest can be calculated by fitting the data directly with this equation or by plotting a graph of \( \log_e (I(\infty) - I(\tau)) \) against \( \tau \), which has a gradient of \( \frac{1}{T_1} \).

The relaxation decay rate of longitudinal two spin order between spins \( k \) and \( l \) can be measured using the pulse sequence shown in Fig. 3.15(b). The first two pulses are semiselective and excite longitudinal two spin order solely between the perturbed spins and their scalar coupling partners. This is described in the product operator formalism for the two coupled spins, \( k \) and \( l \), below:

\[
I_{kz} \xrightarrow{90^\circ_k} - I_{ky} \xrightarrow{1/2J} 2I_{kx}I_{lz} \xrightarrow{90^\circ_l} 2I_{kx}I_{lz}
\]

where only the relevant product operators are given. The first 90° RF pulse selectively excites one spin from its equilibrium amplitude (\( \varphi_1 \)). This then evolves during the delay \( \frac{1}{2J} \), due to scalar coupling evolution, into antiphase magnetisation (\( \varphi_2 \)). The second 90° RF pulse converts this into longitudinal two spin order (\( \varphi_3 \)) which decays during \( \tau \). The last two pulses in Fig. 3.15(b) comprise a double quantum filter which converts the longitudinal two spin order into observable transverse magnetisation. These two RF pulses also eliminate any longitudinal magnetisation present at the end of \( \tau \) which may be observed and may interfere with the signal of interest. Data is collected over a range of \( \tau \) values.
For similar reasons to those given for the selective inversion recovery experiment, the decay rate of longitudinal two spin order is determined from its initial decay, in this case by fitting it with:

\[ I(\tau) = I(0) \exp\left(-\frac{\tau}{T(2 I_{kz} I_{lz})}\right) \]  

[3.55]

where \( T(2 I_{kz} I_{lz}) \) is the relaxation time of longitudinal two spin order between spins \( k \) and \( l \).

To verify the principle of the method given in sub-section 3.6.1, relaxation measurements were made for 7-methoxycoumarin (Fig. 3.16). For this system two comparable but independent sets of measurements can be made. Two pairs of spins were used. The first consists of \( H_3 \) and \( H_4 \) while \( H_5 \) and \( H_6 \) constitute the second. Each multiplet is resolved in the one-dimensional NMR spectrum enabling the longitudinal magnetisation of each spin to be selectively inverted. Furthermore, at least one spin of each chosen pair has resolved scalar coupling solely to the other spin permitting a single component of longitudinal two-spin order to be selectively excited.

Fig. 3.16 The structural formula of 7-methoxycoumarin. The mutual relaxation of two pairs of spins was investigated, spin pair one consists of \( H_3 \) and \( H_4 \) while the second pair contains \( H_5 \) and \( H_6 \). Six experimental measurements were made: longitudinal magnetisation relaxation rates of \( H_3 \), \( H_4 \), \( H_5 \) and \( H_6 \) and longitudinal two spin order relaxation rates of \( H_3 \) and \( H_4 \), and \( H_5 \) and \( H_6 \).
The sequence shown in Fig. 3.15(b) should not in principle excite zero-quantum coherence (ZQC). However, in practise this was not to be the case when applied to the second spin pair. Some ZQC was excited and manifested itself as a $\tau$-dependent oscillation in the data. This may have arisen as a result of relaxation during the two soft pulses, or the extra coupling between H6 and H8. While ZQC cannot be separated from longitudinal two spin order by phase cycling, presaturation of H6 for 5 seconds was found to be effective in eliminating it. Clearly this would take the longitudinal magnetisation of the spins away from their equilibrium values. However, since $^1$H CSA can be considered to be negligible it should not interfere with the subsequent decay of $2I_{H5z}I_{H6z}$. A side effect of presaturation on the instrument used was to decrease the effectiveness of the phase cycling and hence to increase the amount of noise in the decay of $2I_{H5z}I_{H6z}$. To combat this, values of $\tau$ with a shorter increment than that for the selective inversion recovery experiment were used.

3.6.2.2 Results and Analysis

The relaxation decay of $I_{H3z}$, $I_{H4z}$ and $2I_{H3z}I_{H4z}$ are shown graphically in Fig. 3.17(a). The results of spin pair two are shown in Fig. 3.17(b). Only the data acquired over the first 160 ms, when fitted, was consistent with Eqn. 3.52. As a consequence of the cross-relaxation processes mentioned above, only this part of the data was used to calculate relaxation rates. However, attenuation over this time is small and is therefore particularly susceptible to errors. The longitudinal magnetisation relaxation rates of H3, H4, H5 and H6 were found to be $0.253 \pm 0.006$ s$^{-1}$, $0.242 \pm 0.002$ s$^{-1}$, $0.228 \pm 0.014$ s$^{-1}$ and $0.214 \pm 0.034$ s$^{-1}$ respectively. The decay rates for longitudinal two spin order were found to be $0.431 \pm 0.014$ s$^{-1}$ and $0.398 \pm 0.032$ s$^{-1}$ for spin pair one (H3 and H4) and spin pair two (H5 and H6) respectively. The rates of mutual dipolar relaxation between a single pair of spins according to Eqn. 3.49 are $0.064 \pm 0.015$ s$^{-1}$ for H3 and H4 and $0.044$
± 0.049 s⁻¹ for H5 and H6. Assuming isotropic tumbling and hence using Eqn. 3.7, these values correspond to τₐ = 1.71 ± 0.4×10⁻¹¹ s⁻¹ and 1.29 ± 1.00⁻¹¹ s⁻¹ respectively. As noted above the large error in the decay rate of 2Iₜₜₚ/Iₜₚₜ and hence the resulting τₐ value is associated with the particular method used to acquire the data. Despite this, the two values of τₐ are clearly similar. They are within experimental error of each other and consistent with what would be expected for a molecule of this size.

The relatively large errors of the two τₐ values reflect the fact that the values for the combination (Eqn. 3.49) were small. Many current studies of molecular dynamics in solution with NMR are concerned with biomolecules such as proteins. These are considerably larger than 7-methoxycoumarin and consequently will have longer correlation times. As an outcome of this the values of the combination in Eqn. 3.49 will be larger and the error will therefore be less significant.

3.6.2.3 Application to Biomolecules

In this provisional study, one-dimensional experiments were used. These are relatively quick to set up and run. However, this approach is only practical where the one-dimensional spectrum is well resolved: in many systems of biological interest, such as proteins, this will not be the case. As a consequence two-dimensional experiments will have to be used to improve resolution of the peaks. While these take longer to acquire they have the added advantage that information on the whole molecule is obtained simultaneously.

An additional problem which may be encountered when applying the proposed method to proteins arises due to the generally longer autocorrelation times of these biomolecules. This means that longitudinal modes will take longer to decay to infinity therefore the decay curve will need to be measured at longer times. At longer times,
FIG. 3.17: Plots of relaxation decay data against time for (a) H3 and H4 and (b) H5 and H6 for a 0.1 M sample of 7-methoxycoumarin in CDCl₃ at 295 K. (a) ■, longitudinal two spin of H3 and H4, 2J₃H₂/H₄; Δ, longitudinal magnetisation of H3, I₃; A, longitudinal magnetisation of H4, I₄. (b) ×, longitudinal two spin of H5 and H6, 2J₅H₂/H₆; A, longitudinal magnetisation of H5, I₅; Δ, longitudinal magnetisation of H6, I₆. All data were acquired on a Bruker AM500 NMR spectrometer operating at 500 MHz for ¹H. Selective T₁ data was acquired using the sequence shown in Fig. 3.15(a) for H3, H4, H5 and H6. Gaussian shaped soft pulses were used in all cases with \( \tau \) values of 3 μs to 310 ms in 10 ms increments and a relaxation delay of 20 s. 16 transients were acquired for each experiment. To measure the decay of longitudinal two spin order, the pulse sequence shown in Fig. 3.15(b) was used with a 50 ms gaussian shaped pulse applied at the frequency of H3 and H5 to measure \( T(2J₃H₂/H₄) \) and \( T(2J₅H₂/H₆) \) respectively and \( \Delta = 3 \mu s \) in both cases. Values of \( \tau \) in the range of 3 μs to 310 ms with an increment of 2 ms were used to combat the reduced effectiveness of the phase cycle by presaturation (as discussed in the text). The remaining parameters were as for the selective T₁ experiments.
off-diagonal elements of the relaxation matrix (Eqn. 2.40) will have a greater effect on
the longitudinal mode of interest. Consequently cross-relaxation effects will
significantly distort the peak of interest. These cross-relaxation effects will be too
significant to ignore and will need to be suppressed. Components of longitudinal
modes that have experienced different elements of the relaxation matrix and have
undergone cross-relaxation can be made to generate separate peaks in the two-
dimensional spectrum. This allows the intensities of the peaks of interest to be
determined independently of cross-relaxation effects. Any further unwanted
magnetisation modes and coherences, including heteronuclear zero-quantum coherence
can be removed from the data by phase cycling.

To summarise, the two \( \tau_c \) values for each AX spin system of the molecule are
similar and within experimental error of each other, supporting the efficacy of the
proposed method of analysis. Furthermore, three problems are likely to arise when
implementing this approach to proteins. Firstly, the limited resolution of one-
dimensional experiments necessitates the requirement to use two-dimensional pulse
sequences. Secondly, at longer relaxation times, which are necessary to account for
the slow tumbling of proteins, cross-relaxation effects will significantly distort the
intensities of peaks of interest and therefore they can no longer be ignored. Thirdly,
ZQC arising from scalar coupling evolution has been found to give rise to
unacceptable inaccuracies in the calculated value for \( \tau_c \). These three problems can be
accounted for by designing two-dimensional experiments to make the necessary
measurements precisely by suppressing or, if possible, by eliminating cross-
relaxation. The design and development of such sophisticated two-dimensional
heteronuclear experiments is discussed in detail in Chapter Four.
3.6.3 APPLICATION TO UNIFORMLY $^{13}$C LABELLED MOLECULES

As noted above, $^{13}$C-$^{13}$C scalar coupling evolution, the unquantified $^{13}$C dipolar interactions to non-bonded protons and $^{13}$C CSA are a deterrent to analysing $^{13}$C relaxation data. Nonetheless, $^{13}$C relaxation data of molecules selectively labelled with isolated $^{13}$C has been made possible by making an assumption about the dominant relaxation mechanism. The dominant mechanism can be assumed to be the dipolar interaction of the $^{13}$C spin to its bonded proton(s) and its own CSA. However this assumption cannot be justified in all systems and particularly not in molecules which are uniformly labelled with $^{13}$C. With the latter system, as well as the uncertainty of the dominant relaxation mechanism, the traditional T$_2$ measurement also becomes problematic. This is due to inaccuracies resulting from $^{13}$C-$^{13}$C scalar coupling evolution. However, the measurement of the relaxation of longitudinal modes (e.g. heteronuclear T$_1$ and NOE) is still feasible if the numerous dipolar and CSA interactions, discussed in sub-sections 3.5.1 and 3.5.3, can be taken into account during analysis.

A method of analysis has been developed which takes into account all relaxation interactions of the heteronucleus without making any model assumptions (sub-section 3.6.1). This method, outlined in Eqns. 3.49, 3.50 and 3.51, provides new measurements which are complementary to the conventional heteronuclear relaxation measurements (T$_1$'s, NOE's and T$_2$'s where applicable) since they correspond to new linear combinations of the spectral density function. The principle of the method of analysis has been demonstrated to be efficacious for a small molecule and the possible problems which may arise when implementing it to proteins have been discussed (sub-section 3.6.2). Presently, we will discuss the theory of applying our method to $^{13}$CH$_n$ systems in proteins and the dynamics information that can be usefully obtained. The dipolar interaction(s) which can be isolated with $^{13}$CH groups, $^{13}$CH$_2$ groups with either inequivalent or equivalent protons and $^{13}$CH$_3$ groups is (are) described.
3.6.3.1 $^{13}$CH Groups

For $^{13}$CH groups, three experimental measurements can be made: the decay rates for $^{13}$C and proton longitudinal magnetisations and $^{13}$CH longitudinal two spin order. These can be combined as shown in Eqn. 3.49 to isolate the $^{13}$CH mutual dipolar interaction from all other interactions which may be present in the system. Eqn. 3.49 then becomes:

$$R_C(S_{Cz}) + R_H(J_{Hz}) - R_{CH}(2S_{Cz}J_{Hz}) = \frac{1}{2} D_{CH}^2 \left[ \frac{2}{3} J(\omega_H - \omega_C) + 4J(\omega_H + \omega_C) \right]$$

This isolated interaction has a dependence on the spectral density function at the sum and difference of the $^{13}$C and proton Larmor frequencies.

3.6.3.2 $^{13}$CH\textsubscript{2} Groups (Inequivalent Protons)

With $^{13}$CHH\textsubscript{'} groups, it is possible to measure seven relaxation decay rates. These are of $^{13}$C longitudinal magnetisation, longitudinal magnetisation of each bonded proton, $^{13}$CH and $^{13}$CH\textsubscript{'} longitudinal two spin orders, longitudinal three spin order of all three spins in the group and HH\textsubscript{'} longitudinal two spin order. These seven decay rates can by combined in various different ways to isolate different dipolar interactions.

The $^{13}$CH dipolar interaction given in Eqn. 3.56 can be isolated as well as the $^{13}$CH\textsubscript{'} interaction:

$$R_C(S_{Cz}) + R_H'(J_{Hz}) - R_{CH'}(2S_{Cz}J_{Hz})$$

$$= \frac{1}{2} D_{CH'}^2 \left[ \frac{2}{3} J(\omega_H' - \omega_C) + 4J(\omega_H' + \omega_C) \right]$$

[3.57]
This combination gives the same dependence of the spectral density function as Eqn 3.56 because the two nuclei involved in the interactions isolated by these two combinations are the same, i.e. $^{13}$C and proton.

Eqn. 3.49 can also be used to combine the two proton longitudinal magnetisation decay rates and the decay rate of HH' longitudinal two spin order as shown:

$$R_{HH}'(I_{H_2}) + R_{HH}''(2I_{Hz}J_{H'H_2})$$

$$= \frac{1}{2} D_{HH}' \left[ \frac{2}{3} J(\omega_H - \omega_{H'}) + 4J(\omega_H + \omega_{H'}) \right]$$

[3.58]

The combination of the spectral density function isolated in Eqn. 3.58 is dependent on slower and faster frequencies than the ones we have come across so far in this subsection. The isolated spectral density function is dependent on the sum and difference of the two proton frequencies.

The mutual relaxation interaction of the three coupled spins in a $^{13}$CHH' group can be investigated by using Eqn. 3.50:

$$R_C(S_{Cz}) + R_H(I_{Hz}) + R_{H'}(I_{H'z}) - R_{CHH'}(4S_{Cz}I_{Hz}I_{H'z}) =$$

$$\frac{1}{2} D_{CH}^2 \left[ \frac{2}{3} J(\omega_C - \omega_H) + 4J(\omega_C + \omega_H) \right]$$

$$+ \frac{1}{2} D_{CH'}^2 \left[ \frac{2}{3} J(\omega_C - \omega_{H'}) + 4J(\omega_C + \omega_{H'}) \right]$$

$$+ \frac{1}{2} D_{HH'}^2 \left[ \frac{2}{3} J(\omega_H - \omega_{H'}) + 4J(\omega_H + \omega_{H'}) \right]$$

[3.59]
The longitudinal three spin order of all three spins is subtracted from their individual longitudinal magnetisation decay rates. The interactions isolated in Eqn. 3.59 are the $^{13}\text{C}$ with H, $^{13}\text{C}$ with H' and H with H' dipolar interactions. The individual terms depend on the spectral density function at the sum and difference of the two frequencies involved in each pair. Eqn. 3.59 isolates the three pairwise interactions collectively. But each of these interactions can also be isolated independently of the other two by using Eqns. 3.56, 3.57 and 3.58 for the $^{13}\text{CH}$, $^{13}\text{CH}'$ and HH' dipolar interactions, respectively.

Additionally, for $^{13}\text{CHH}'$ groups, Eqn. 3.51 can be used in three different ways. All of these ways involve subtracting the mutual longitudinal three spin order from the sum of the longitudinal two spin order of two of the spins and the longitudinal magnetisation of the third. One of the applications of Eqn. 3.51 requires the $^{13}\text{CH}$ longitudinal two spin order and the H' longitudinal magnetisation decay rates to isolate the $^{13}\text{C}$ with H' and H with H' dipolar interactions:

\[
R_{\text{CH}}(2I_{\text{C}}S_{\text{Hz}}) + R_{\text{H'}}(I_{\text{H'z}}) - R_{\text{CHH'}}(4S_{\text{C}}I_{\text{Hz}}I_{\text{H'z}}) = \\
\quad \frac{1}{2} D_{\text{CH'}}^{2} \left[ \frac{2}{3} J(\omega_{\text{C}} - \omega_{\text{H'}}) + 4J(\omega_{\text{C}} + \omega_{\text{H'}}) \right] \\
\quad + \frac{1}{2} D_{\text{HH'}}^{2} \left[ \frac{2}{3} J(\omega_{\text{H}} - \omega_{\text{H'}}) + 4J(\omega_{\text{H}} + \omega_{\text{H'}}) \right] \\
[3.60]
\]

Another form of Eqn. 3.51 involves combining the decay rates of longitudinal two spin order of $^{13}\text{CH}'$ and the longitudinal magnetisation of the other bonded proton as well as the mutual longitudinal three spin order:
This combination isolates the $^{13}\text{C}$ with H and H with H' dipolar interactions.

The final application of Eqn. 3.51 uses the relaxation decay rates of $^{13}\text{C}$ longitudinal magnetisation and HH' longitudinal two spin order to isolate the $^{13}\text{C}$ with H and $^{13}\text{C}$ with H' dipolar interactions, as shown:

\[
R_{\text{CH}}(2I_{\text{Cz}}S_{\text{H'H'})} + R_{\text{H}(I_{\text{Hz}})} - R_{\text{CHH'}}(4S_{\text{Cz}I_{\text{Hz}}I_{\text{H'H'})}) =
\frac{1}{2} D_{\text{CH}}^2 \left[ \frac{2}{3} J(\omega_{\text{C}} - \omega_{\text{H}}) + 4J(\omega_{\text{C}} + \omega_{\text{H}}) \right]
+ \frac{1}{2} D_{\text{HH'}}^2 \left[ \frac{2}{3} J(\omega_{\text{H}} - \omega_{\text{H'}}) + 4J(\omega_{\text{H}} + \omega_{\text{H'}}) \right]
\]

[3.61]

The three combinations of Eqns. 3.49, 3.50 and 3.51 can be applied to $^{13}\text{CHH'}$ groups to isolate seven different evaluations of the three pairwise interactions of $^{13}\text{C}$ with H, $^{13}\text{C}$ with H' and H with H'. But the seven evaluations given in Eqns. 3.56 to 3.62 depend only on three unique combinations of the spectral density function:

\[
\left[ \frac{2}{3} J(\omega_{\text{C}} - \omega_{\text{H}}) + 4J(\omega_{\text{C}} + \omega_{\text{H}}) \right], \frac{1}{2} D_{\text{CH}}^2 \left[ \frac{2}{3} J(\omega_{\text{C}} - \omega_{\text{H'}}) + 4J(\omega_{\text{C}} + \omega_{\text{H'}}) \right] \quad \text{and} \quad \left[ \frac{2}{3} J(\omega_{\text{H}} - \omega_{\text{H'}}) + 4J(\omega_{\text{H}} + \omega_{\text{H'}}) \right].
\]
3.6.3.3 $^{13}$CH$_2$ Groups (Equivalent Protons)

In a $^{13}$CH$_2$ group with equivalent protons, both of these can be assumed to relax at the same rate. The proton T$_1$ and the heteronuclear longitudinal two spin order measurements will be affected by cross-relaxation. The proton T$_1$ can cross-relax with the longitudinal magnetisation of the other proton. Similarly the $^{13}$CH longitudinal two spin order can cross-relax with the other $^{13}$CH pair. The cross-relaxation processes will interfere with the decay of interest and change the measured rate (see sub-section 4.2.1). Therefore cross-relaxation needs to be either i) suppressed or separated from the resonance of interest or ii) taken into account during analysis. Cross-relaxation processes between equivalent protons in $^{13}$CH$_2$ groups cannot be separated in the two-dimensional spectrum (see section 4.2.1). Hence it is necessary to account for them during analysis. To do this we need to re-consider the decay rate expressions for proton longitudinal magnetisation and heteronuclear two spin order to include cross-relaxation effects.

The T$_1$ relaxation rate of each proton in a $^{13}$CHH group is given by:

$$R_H(I_{Hz}) = \sum_{j\neq H}^{n} \frac{1}{2} D_H^2 J_H \left[ \frac{1}{3} J(\omega_H - \omega_j) + J(\omega_H) + 2 J(\omega_H + \omega_j) \right] + \frac{1}{2} D_{HH}^2 \left[ J(\omega_H) + 4 J(2\omega_H) \right] + \frac{1}{2} C_H^2 J(\omega_H)$$

[3.63]

where the first term gives the dipolar interaction between the proton of interest and other j spins. The second term between the two protons is different from the first term because the protons are equivalent. The last term in Eqn. 3.63 is the contribution to relaxation resulting from proton CSA.
Similarly, the $^{13}$CH longitudinal two spin order decay rate for $^{13}$CHH groups is:

$$R_{CH}(2S_{Cz}J_{Hz}) = \sum_{j\neq C,H} \frac{1}{2} D_{Cj}^2 \left[ \frac{1}{3} J(\omega_C - \omega_j) + J(\omega_C) + 2J(\omega_C + \omega_j) \right]$$

$$+ \sum_{j\neq C,H} \frac{1}{2} D_{Hj}^2 \left[ \frac{1}{3} J(\omega_H - \omega_j) + J(\omega_H) + 2J(\omega_H + \omega_j) \right]$$

$$+ \frac{1}{2} D_{CH}^2 \left[ J(\omega_C) + J(\omega_H) \right] + \frac{1}{2} C_C^2 J(\omega_C) + \frac{1}{2} C_H^2 J(\omega_H)$$

$$+ \frac{1}{2} D_{HH}^2 \left[ J(\omega_C) + 4J(\omega_H) \right]$$

$$+ \frac{1}{2} D_{CH}^2 \left[ 2J(\omega_C) \right] \left( \frac{3\cos^2 \theta_{CH,CH} - 1}{2} \right)$$

[3.64]

The first three lines of Eqn. 3.64 are the same as the general relaxation of $^{13}$CH longitudinal two spin order, as given in Eqn. 3.47. The fourth line describes proton-proton cross-correlation between the two degenerate protons in the $^{13}$CH$_2$ group. This interaction is identical to the one in Eqn. 3.63 and depends on the spectral density function at the frequency of the two protons. The final term describes cross-correlation between the two $^{13}$C-H vectors. The spectral density function for this term depends only on the Larmor frequency of $^{13}$C and it varies with the H-$^{13}$C-H angle.

The homonuclear proton longitudinal two spin order decay rate for $^{13}$CH$_2$ groups remains unchanged. It has been given in a general form in Eqn. 3.47.

The general expressions for the longitudinal magnetisation of proton (Eqn. 3.63), the longitudinal magnetisation of $^{13}$C (calculated from Eqn. 3.46) and the $^{13}$CH longitudinal two spin order (Eqn. 3.64) can be used with Eqn. 3.49 to give:-
\[
R_C(S_{Cz}) + R_H(I_{Hz}) = R_{CH}(2S_{Cz}I_{Hz}) = \frac{1}{2} D_{CH}^2 \left[ \frac{2}{3} J(\omega_C - \omega_H) + 2 J(\omega_C + \omega_H) \right] + \frac{1}{2} D_{CH}^2 \left[ 2 J(\omega_C) \right] \left( \frac{3 \cos^2 \theta_{CH,CH} - 1}{2} \right)
\]

[3.65]

where the first term describes the \(^{13}\text{CH}\) mutual dipolar interaction and the second term arises as a result of the equivalence of the two protons. It is interesting to note that the second term is identical to the cross-correlation term in Eqn. 3.64. All the CSA terms in the relaxation rate expressions cancel out.

The combination given in Eqn. 3.49 can also be used with the decay rates for proton longitudinal magnetisation (Eqn. 3.63) and homonuclear proton longitudinal two spin order (calculated from Eqn. 3.47) to isolate different interactions from those in Eqn. 3.65:

\[
2R_H(I_{Hz}) - R_{HH}(2I_{Hz}I_{Hz}) = \frac{1}{2} D_{HH}^2 \left[ 8J(2\omega_H) \right]
\]

[3.66]

where all the dipolar and CSA interactions cancel out except for those between the two protons in the \(^{13}\text{CH}_2\) group. The remaining interaction depends on the spectral density function at twice the proton frequency.

Eqn. 3.50, when used for \(^{13}\text{CHH}\) groups, involves combining the \(^{13}\text{C\ T}_1\)'s (calculated from Eqn. 3.44), the proton \(\text{T}_1\)'s (Eqn. 3.63) and the decay rate of their mutual longitudinal three spin order (calculated from Eqn. 3.47) as shown:-
Equation 3.67 consists of two terms which describe three interactions. The first term is the sum of the two $^{13}\text{CH}$ dipolar interactions. The second term describes the dipolar relaxation between the two degenerate protons.

The relaxation decay rates for the $^{13}\text{CH}$ longitudinal two spin order (calculated from Eqn. 3.47), the proton longitudinal magnetisation (Eqn. 3.63) and the $^{13}\text{CHH}$ (calculated from Eqn. 3.48) can be combined according to Eqn. 3.51 to give:

\[
R_{CH}(2S_{Cz}I_{Hz}) + R_{H}(I_{Hz}) - R_{CHH}(4S_{Cz}I_{Hz}I_{Hz}) =
\frac{1}{2}D_{CH}^{2}\left[\frac{4}{3}J(\omega_C - \omega_H) + 4J(\omega_C + \omega_H)\right]
+ \frac{1}{2}D_{HH}^{2}[8J(2\omega_H)]
+ \frac{1}{2}D_{CH}^{2}\left[2J(\omega_C)\left(\frac{3\cos^2\theta_{CH,CH} - 1}{2}\right)\right]
\]

[3.68]

There are three terms here which consist of three dipolar interactions. The first term describes one of the $^{13}\text{CH}$ interactions. The second term isolates the HH dipolar interactions as well as the HH cross-correlation interaction. This term depends on the spectral density function at twice the proton frequency. The final term describes cross-correlation between the two $^{13}\text{CH}$ vectors and it arises as a result of the equivalence of the protons.
3.6.3.4 $^{13}$CH$_3$ Groups

The same approach as that described above for $^{13}$CH and $^{13}$CH$_2$ groups can be taken for $^{13}$CH$_3$ groups. However, the relaxation behaviour of this system is complicated and different to that for $^{13}$CH and $^{13}$CH$_2$ groups. Consequently it has not been covered in this work.

3.6.3.5 Uses Of The Isolated Relaxation Interactions

The relaxation interactions isolated in Eqns. 3.56 to 3.68 (excluding Eqns. 3.63 and 3.64) can be used in conjunction with $^{15}$N relaxation studies to obtain a full and detailed characterisation of the dynamics of individual atoms in proteins. The relaxation measurements of $^{15}$N can be used to calculate $\tau_c$, the overall correlation time. $^{13}$C relaxation data analysed according to the "model-free" approach (sub-section 3.3.1) can be used to calculate the order parameter, $S^2$, and possibly, also the internal correlation time, $\tau_i$, from Eqn. 3.9.

The number of frequencies at which the spectral density function is sampled can be increased by using this method of analysis with measurements made at different field strengths. This will enable motional models to be fitted more precisely or more sophisticated models to be used. The advantage of this is that more detailed dynamics information can be obtained.

There may be some accumulation of error since a linear combination is being taken. However, in many cases it will still compare favourably with a more conventional approach given the problems with assessing all the relevant relaxation interactions mentioned above (sub-section 3.5.3).
3.6.4 APPROXIMATE METHODS FOR THE ANALYSIS OF $^{13}$C RELAXATION DATA

We have designed a method of analysis which rigorously considers all the relaxation interactions in the system of interest and does not require any assumptions to be made. In the previous sub-section we looked at ways of applying the combinations of Eqns. 3.49, 3.50 and 3.51 to $^{13}$CH$_n$ groups in biomolecules. The interactions which are isolated can be used with an appropriate motional model (section 3.3) to model the spectral density function. Ideally it should not be necessary to introduce assumptions inherent in models. However, there are not sufficient experimental measurements to adopt a mapping approach similar to that of Peng and Wagner (61, 62) (section 3.4). Nonetheless, this does become possible if we adopt a less rigorous approach than the one used in developing the linear combinations. This option is pursued in the present sub-section. Here, by assuming that the $^{13}$C relaxes solely with its bonded proton we show how it is possible to measure more linear combinations of the spectral density function and hence to determine values for it at specific frequencies. These combinations are in addition to those isolated previously in sub-section 3.6.3. With this approach there will be enough dynamics information to avoid the use of motional models. Instead, we will then have enough measurements to enable the spectral density function to be mapped.

A less rigorous approach than the one discussed in the previous sub-section assumes that the $^{13}$C spin only relaxes with its bonded proton(s), or a fixed fictitious bond length is calculated (Eqn. 3.42) to take into account other dipolar interactions with bonded and non-bonded nuclei. We will apply the former approximation to $^{13}$CH groups and $^{13}$CH$_2$ groups with equivalent and inequivalent protons.
3.6.4.1 $^{13}$CH Groups

Assuming the $^{13}$C spin only relaxes through dipolar interactions with its bonded proton, and the heteronuclear CSA contribution is small enough to be ignored, the $^{13}$C $T_1$ rate can be calculated from Eqn. 3.46 to be:

$$R_C(S_{Cz}) = \sum_n \frac{1}{2} D_{CH}^2 \left[ \frac{1}{3} J(\omega_C - \omega_H) + J(\omega_C) + 2J(\omega_C + \omega_H) \right]$$  \[3.69\]

where $n = 1$ for $^{13}$CH groups.

The change of $^{13}$C intensity with time as a result of saturating the bonded proton, in $^{13}$CH groups, is given by the following differential equation (109):

$$\frac{-d[S_z]}{dt} = \frac{1}{T_1(S)} [S_z - S_0] + \frac{1}{T_1(I-S)} [-I_0]$$  \[3.70\]

where $S$ is the $^{13}$C spin, $I$ is proton, square brackets represent the concentration of the species within them. $\frac{-d[S_z]}{dt} = 0$ at steady state, $T_1(S)$ is the longitudinal relaxation time for $^{13}$C, $T_1(I-S)$ is the decay time of longitudinal cross-relaxation from proton to $^{13}$C, $t$ is the time and $S_0$ is the amplitude of $S_z$ at $t = 0$. Equation 3.70 can be used as an expression for the steady state $^{13}$C NOE. It can be seen from Eqn. 3.70 that the decay of $^{13}$C magnetisation involves two processes: the relaxation of $S_z$ and the cross-relaxation of $I_z$ to $S_z$. When Eqn. 3.70 is solved, the result is an expression for the steady state $^{13}$C NOE:

$$\frac{[S_z]}{[S_0]} = 1 + \frac{T_1(S)}{T_1(I-S)} \cdot \left[ \frac{I_0}{S_0} \right]$$  \[3.71\]
where the ratio \( \frac{S_z}{S_0} \) gives the NOE, \( T_1(S) \) is given in Eqn. 3.69, \( \frac{I_0}{I_0} = \gamma_C / \gamma_H \) and
\[
T_1(I-S) \text{ is given by: -}
\]

\[
\frac{1}{T_1(I-S)} = \frac{1}{2} D_{IS}^2 \left[ -\frac{1}{3} J (\omega_I - \omega_S) + 2J (\omega_I + \omega_S) \right]
\]

[3.72]

For \(^{13}\text{CH}\) groups, the only relaxation interaction which can be isolated, according to our method of analysis, is given in Eqn. 3.56. Using a reduced form of Eqn. 3.56 with the relaxation decay rates for only \(^{13}\text{CH}\) longitudinal two spin order and \(^{13}\text{C}\) longitudinal magnetisation, the following combination of the spectral density function is obtained: -

\[
R_{\text{CH}}(2I_{Hz}S_{Cz}) - R_{\text{H}}(I_{Hz}) = \sum_{j \neq \text{CH}} \frac{1}{2} D_{Cj}^2 \left[ \frac{1}{3} J (\omega_C - \omega_j) + J(\omega_C) + 2J(\omega_C + \omega_j) \right] + \frac{1}{2} D_{CH}^2 \left[ -\frac{1}{3} J (\omega_C - \omega_H) + J(\omega_C) - 2J(\omega_C + \omega_H) \right] + \frac{1}{2} C_C^2 J(\omega_C)
\]

[3.73]

The first term describes general dipolar interactions between the \(^{13}\text{C}\) spin and any other spins. The second term gives the specific dipolar interaction between the \(^{13}\text{C}\) spin and its bonded proton and the last term is the CSA contribution of \(^{13}\text{C}\). If it is assumed that the \(^{13}\text{C}\) spin only relaxes with its bonded proton and its own CSA, and all other dipolar interactions are too small to make a significant contribution, Eqn. 3.73 can be simplified to just two terms:
\[ R_{CH}(2I_{Hz}S_{C2}) - R_{H}(I_{Hz}) = \frac{1}{2} D_{CH}^2 \left[ -\frac{1}{3} J(\omega_C - \omega_H) + J(\omega_C) - 2J(\omega_C + \omega_H) \right] + \frac{1}{2} C^2 J(\omega_C) \] 

[3.74]

With this assumption we can isolate the $^{13}\text{CH}$ dipolar interaction and the $^{13}\text{C}$ CSA. Eqn. 3.74 is the $^{13}\text{C}$ analogue to the approach made by Peng and Wagner (61, 62) for $^{15}\text{N}$ relaxation. The approximate $^{13}\text{CH}$ interaction isolated in Eqn. 3.74 is different from the rigorous one isolated in Eqn. 3.56: the spectral density function is dependent on a different combination of the Larmor frequencies of the two spins. Hence the dynamics information which can be obtained from Eqn. 3.74 is complementary to that of Eqn. 3.56.

The spectral density function can be determined at three different frequencies (the $^{13}\text{C}$ frequency and the sum and the difference of the $^{13}\text{C}$ and proton frequencies) for $^{13}\text{CH}$ groups. This can be implemented by practically measuring the $^{13}\text{C}$ $T_1$, proton $T_1$ and $^{13}\text{CH}$ longitudinal two spin order (to evaluate the combination given in Eqn. 3.57) and by measuring the heteronuclear NOE (Eqn. 3.57). Equations 3.57 and 3.72 can be combined in two different ways to determine the spectral density function at the sum (Eqn. 3.75) and at the difference (Eqn. 3.76) of the $^{13}\text{C}$ and proton Larmor frequencies, as shown:

\[ (\text{Eqn. 3.57}) + 2 \times (\text{Eqn. 3.72}) = \frac{1}{2} D_{CH}^2 \left[ 8J(\omega_C + \omega_H) \right] \] 

[3.75]

\[ (\text{Eqn. 3.57}) - 2 \times (\text{Eqn. 3.72}) = \frac{1}{2} D_{CH}^2 \left[ \frac{4}{3} J(\omega_C - \omega_H) \right] \] 

[3.76]

The spectral density function at the $^{13}\text{C}$ frequency can be determined by combining Eqns. 3.57 with the expression for the $^{13}\text{C}$ $T_1$ (Eqn. 3.69) in the following way:
\[ 2 \times (\text{Eqn. 3.69}) + (\text{Eqn. 3.57}) = \frac{1}{2} D_{CH}^2 \left[ J(\omega_C) \right] \]  

[3.77]

If these four experiments (\(^{13}\)C \(T_1\), proton \(T_1\), \(^{13}\)CH longitudinal two spin order and the heteronuclear NOE) are made at different field strengths then the spectral density function for the \(^{13}\)CH internuclear bond vector can be determined at more frequencies than those given in Eqns. 3.75 to 3.77, as discussed in sub-section 3.5.1.

### 3.6.4.2 \(^{13}\)CHH' Groups (Inequivalent Protons)

The \(^{13}\)C \(T_1\), assuming the \(^{13}\)C spin only relaxes through dipolar interactions with its two bonded protons, is given by Eqn. 3.69 where \(n = 2\).

The differential equation for the steady-state heteronuclear NOE for \(^{13}\)CH\(_n\) systems is given by:

\[
\frac{-d[S_z]}{dt} = \frac{1}{T_1(S)} [S_z - S_0] + \frac{1}{T_1(I-S)} n[I_0] + \frac{1}{T_1(S-IIS)} [4S_zI_zI_z(t)]
\]  

[3.78]

where square brackets represent concentration of the species within them, \(\frac{-d[S_z]}{dt} = 0\), \(T_1(S)\) is the longitudinal relaxation time for \(^{13}\)C, \(T_1(I-S)\) is the decay time for longitudinal cross-relaxation from proton to \(^{13}\)C, \(T_1(S-IIS)\) is the longitudinal cross-relaxation decay time for \(S_z\) to \(8S_zI_zI_z\), \(t\) is the time and \(S_0\) is the amplitude of \(S_z\) at \(t=0\). It can be seen from Eqn. 3.78 that the \(^{13}\)C NOE is generated from three processes: the relaxation of \(S_z\), the cross-relaxation of \(I_z\) to \(S_z\) and \(S_z\) to \(4S_zI_zI_z\). These two cross-relaxation processes can not be eliminated by the methods discussed in sub-section 4.2.1 therefore they are included in the expression for the heteronuclear
NOE to take them into account during analysis. In Eqn. 3.78, the differential
equation for $4S_z I_z(t)$ is given by:

$$\frac{d[4S_z I_z(t)]}{dt} = \frac{1}{T_1(S - IIS)}[S_z - S_0] + 2 \times \frac{1}{T_1(I - IIS)}[I_z - I_o]$$

$$+ \frac{1}{T_1(IIS)}[4S_z I_z(t) - 4S_z I_z(0)]$$

[3.79]

If $[I_o] = 0$ (i.e. the intensity of protons is saturated), $[4S_z I_z(0)] = 0$ at equilibrium and

$$\frac{d[4S_z I_z(t)]}{dt} = 0$$

Eqn. 3.79 can be solved to give:

$$[4S_z I_z(t)] = \frac{T_1(IIS)}{T_1(S - IIS)}[S_z - S_0] + \frac{T_1(IIS)}{T_1(I - IIS)} n[I_o]$$

[3.80]

When Eqn. 3.80 is substituted into Eqn. 3.78 and solved, the result is an expression for
the $^{13}$C NOE in $^{13}$CH$_n$ groups:

$$\frac{[S_z]}{[S_0]} = 1 + \frac{1}{A} \left( \frac{1}{T_1(I - S)} n - \frac{1}{T_1(S - IIS)} \frac{T_1(IIS)}{T_1(I - IIS)} n \right) \frac{[I_o]}{[S_0]}$$

[3.81]

where the ratio $\frac{[S_z]}{[S_0]}$ gives the NOE, $\frac{[I_o]}{[S_0]} = \frac{\gamma_H}{\gamma_C}$, $T_1(IIS)$ is measured experimentally,

$T_1(I-S)$ is given in Eqn. 3.72 and $A$, $T_1(I-IIS)$ and $T_1(S-IIS)$ are as follows:

$$\frac{1}{A} = \frac{1}{T_1(S)} + \frac{T_1(IIS)}{T_1^2(S - IIS)}$$

[3.82]

$$\frac{1}{T_1(I - IIS)} = \frac{1}{2} D_{II} D_{IIS} [2J(\omega_I)] \left( \frac{3 \cos^2 \theta_{II,IIS} - 1}{2} \right)$$

[3.83]
\[ \frac{1}{T_1(S - II(S))} = \frac{1}{2} D_{SI} D_{SI} \left[ 2J(\omega_S) \right] \left( \frac{3\cos^2 \theta_{SI,SI} - 1}{2} \right) \]  

where \( \theta_{SI,SI} \) is the H-\(^{13}\)C-H angle.

It can be seen from the above discussion that the heteronuclear NOE in \(^{13}\)CH\(_n\) groups can be measured experimentally but its analysis is far more involved.

The dipolar interactions which can be isolated, with our rigorous method of analysis, for a \(^{13}\)CHH' group, are given in Eqns. 3.56 to 3.62. We can also analyse the data less rigorously by assuming that \(^{13}\)C relaxes only with its two bonded protons through dipolar interactions and its own CSA. With this assumption, two further combinations of the spectral density function can be taken:

\[
R_{CHH'}(4I_{Hz}I_{Hz}S_{Cz}) - R_H(I_{Hz}) - R_{H'}(I_{Hz}z) = \frac{1}{2} D_{CH}^2 \left[ -\frac{1}{3} J(\omega_C - \omega_H) + J(\omega_C) - 2J(\omega_C + \omega_H) \right] + \frac{1}{2} D_{H}^2 \left[ -\frac{1}{3} J(\omega_C - \omega_H) + J(\omega_C) - 2J(\omega_C + \omega_H) \right] + \frac{1}{2} D_{H'}^2 \left[ -\frac{1}{3} J(\omega_H - \omega_H) + 2J(\omega_H + \omega_H) \right] + \frac{1}{2} C^2 J(\omega_C)
\]  

[3.85]

As in Eqn. 3.59 the three dipolar interaction of \(^{13}\)C with H, \(^{13}\)C with H' and H with H' are isolated in Eqn. 3.85. But in these two equations the three isolated interactions depend on a different combination of the spectral density function. In Eqn. 3.85 the spectral density function depends on the Larmor frequency of \(^{13}\)C as well as the sum and difference of the \(^{13}\)C and proton frequencies for the interactions of \(^{13}\)C with H and \(^{13}\)C with H'. Although the spectral density function for the HH' interaction depends on the sum and the difference of the two Larmor frequencies in
both Eqns. 3.59 and 3.85, the two contributions differ in their relative ratios. Equation 3.85 also includes the $^{13}$C CSA contribution to relaxation. Hence the approximate combination given in Eqn. 3.85 provides additional dynamics information to that obtained from Eqn. 3.59.

An additional approximated term involving the relaxation decay rate of $^{13}$CHH' longitudinal three spin order and the HH' longitudinal two spin order gives:

$$
R_{CHH'} (4I_{Hz}I_{Hz} z_{Cz}) - R_{HH'} (2I_{Hz}I_{Hz} z) = \frac{1}{2} D_{CH}^2 \left[ -\frac{1}{3} J(\omega_C - \omega_H) + J(\omega_C) - 2J(\omega_C + \omega_H) \right] + \frac{1}{2} D_{CH}^2 \left[ -\frac{1}{3} J(\omega_C - \omega_H) + J(\omega_C) - 2J(\omega_C + \omega_H) \right] + \frac{1}{2} C_{CH}^2 J(\omega_C)
$$

[3.86]

In this combination the two $^{13}$C-proton dipolar interactions are isolated as in Eqn. 3.62. However, there are two differences between Eqns. 3.62 and 3.86: the latter also includes a $^{13}$C CSA term and the dependence of the spectral density function in the two equations is different. In Eqn. 3.86 the spectral density function depends on the sum and difference of the $^{13}$C and proton Larmor frequencies and also on the $^{13}$C frequency. As a consequence of the differences between Eqns. 3.62 and 3.86 the dynamics information obtained from these two equations, for $^{13}$CHH' groups, is complementary.

The spectral density function can be determined at the $^{13}$C frequency and the sum and the difference of the $^{13}$C and proton frequencies for $^{13}$CHH' groups as shown for $^{13}$CH groups in Eqns. 3.75, 3.76 and 3.77 respectively. The spectral density function at twice the frequency of proton is isolated in Eqn. 3.66. The value for $J(\omega_C)$ can be used in combination with the expression for the heteronuclear NOE given in Eqn. 3.81 to determine the spectral density function at the frequency of proton.
By making five experimental measurements for $^{13}$CHH' groups ($^{13}$C T$_1$, proton T$_1$, $^{13}$CH longitudinal two spin order, HH longitudinal two spin order and the heteronuclear NOE) five values for the spectral density function can be determined: $J(\omega_C + \omega_H)$, $J(\omega_C - \omega_H)$, $J(\omega_C) J(2\omega_H)$ and $J(\omega_H)$.

3.6.4.3 $^{13}$CH$_2$ Groups (Equivalent Protons)

For $^{13}$CH$_2$ groups with equivalent protons, if we assume that the dominant dipolar interaction is between $^{13}$C and its two bonded protons, the $^{13}$C T$_1$ and the heteronuclear NOE are given by Eqns. 3.69 and 3.81 respectively where $n = 2$.

The relaxation rate of $^{13}$CH longitudinal two spin order (calculated from Eqn. 3.47) and the proton longitudinal magnetisation (Eqn. 3.63) can be combined in the following way:

$$R_{\text{CH}}(2J\omega_{\text{Lz}}S_{\text{Cz}}) - R_{\text{H}}(J_{\text{Hz}}) = \frac{1}{2} D_{\text{CH}}^2 \left[ \frac{1}{3} J(\omega_C - \omega_H) + J(\omega_C) - 2J(\omega_C + \omega_H) \right]$$

$$+ \frac{1}{2} D_{\text{CH}}^2 [2J(\omega_C)] \left( \frac{3\cos^2 \theta_{\text{CH}, \text{CH}} - 1}{2} \right) + \frac{1}{2} C_C^2 J(\omega_C)$$

[3.87]

This equation isolates one of the $^{13}$CH dipolar interactions, the $^{13}$CH cross-correlation term arising from the equivalence of the protons, as well as the $^{13}$C CSA contribution.

The spectral density function can be determined at the $^{13}$C frequency for $^{13}$CH$_2$ groups as shown for $^{13}$CH groups in Eqn. 3.77. Additionally, the spectral density function at the proton Larmor frequency can be determined by combining Eqn. 3.68 and 3.81 in the following way:
(Eqn. 3.68) \(-2 \times (Eqn. 3.81) = \frac{1}{2} D_{\text{HH}}^2 \left[ 8J(2\omega_H) \right] - \frac{1}{2} D_{\text{CH}}^2 \left[ 2J(\omega_C) \right] \left( \frac{3\cos^2 \theta_{\text{CH,CH}} - 1}{2} \right) - \frac{1}{2} D_{\text{CH}}^2 \left[ 4J(\omega_H) \right] \left( \frac{3\cos^2 \theta_{\text{CH,CH}} - 1}{2} \right) \)

\[3.88\]

where \(J(2\omega_H)\) is calculated from Eqn. 3.66, the value for the spectral density function at the frequency of \(^{13}\)C is known from Eqn. 3.77. Using this value for \(J(\omega_H)\) (calculated from Eqn. 3.88) and the value for \(J(\omega_C)\) calculated from Eqn. 3.77 it is possible to calculate \(J(\omega_C + \omega_H)\) and \(J(\omega_C - \omega_H)\) by using the NOE measurement (Eqn. 3.81) as the following:

\[(\text{Eqn. 3.65}) + 2 \times (\text{Eqn. 3.81}) = \frac{1}{2} D_{\text{CH}}^2 \left[ 8J(\omega_C + \omega_H) \right] + \frac{1}{2} D_{\text{CH}}^2 \left[ 2J(\omega_C) \right] \left( \frac{3\cos^2 \theta_{\text{CH,CH}} - 1}{2} \right) + \frac{1}{2} c_c^2 J(\omega_C) \]

\[3.89\]

\[(\text{Eqn. 3.65}) - 2 \times (\text{Eqn. 3.81}) = \frac{1}{2} D_{\text{CH}}^2 \left[ 4J(\omega_C - \omega_H) \right] - \frac{1}{2} D_{\text{CH}}^2 \left[ 2J(\omega_C) \right] \left( \frac{3\cos^2 \theta_{\text{CH,CH}} - 1}{2} \right) + \frac{1}{2} c_c^2 J(\omega_C) \]

\[3.90\]

\(J(2\omega_H)\) can be also calculated from the following, in addition to Eqn. 3.66:
\[
(Eqn.3.67) + 4 \times (Eqn.3.72) = \frac{1}{2} D_{\text{CH}}^2 \left[ 16J(\omega_C + \omega_H) \right] \\
+ \frac{1}{2} D_{\text{HH}}^2 \left[ 8J(2\omega_H) \right]
\]  

[3.91]

where the value for the spectral density function at the sum of the $^{13}\text{C}$ and proton frequencies is known (from Eqn. 3.89).

By making five experimental measurements for $^{13}\text{CHH}$ groups ($^{13}\text{C} T_1$, proton $T_1$, $^{13}\text{CH}$ longitudinal two spin order, HH longitudinal two spin order and the heteronuclear NOE), five values for the spectral density function can be determined: $J(\omega_C + \omega_H)$, $J(\omega_C - \omega_H)$, $J(\omega_C)$, $J(\omega_H)$ and $J(2\omega_H)$. Some of these values of the spectral density function require the combination of up to four different relaxation measurements. This may give rise to a large error, depending on the size of the interaction isolated. For example, if the sum of the square of the individual errors is 0.1, the error on the value of the spectral density function at a particular frequency will be higher if the isolated interaction is 0.5 than if it is 1. Any further theoretical comments on the precision of the mapping approach cannot be made without implementing it practically.
3.7 SUMMARY

In this chapter we have presented a critical overview of the existing methods of analysing NMR relaxation data. The limitations of these methods have been pointed out, in particular, when applying them to $^{13}$C relaxation data. Based on the limitations of these methods and the importance of $^{13}$C relaxation (in terms of the dynamics information which can be gained), we have developed a new method of analysis which takes account of all the numerous unquantified interactions of a $^{13}$C spin with other spins in the system. The theory of this method and its implementation on a model molecule have been presented and shown to be credible but requiring a few considerations for implementation with proteins.

The new method of analysis can be used in conjunction with existing motional models to model the spectral density function. Alternatively, it can be incorporated with a relatively approximate method to map the spectral density function. The modelling approach requires at least three independent relaxation measurements to be made and then analysed according to an appropriate motional model. The mapping approach requires a larger number of relaxation measurements to be made (at least three, four and five for $^{13}$CH, $^{13}$CHH' and $^{13}$CHH groups, respectively) to calculate the spectral density function at specific frequencies, similar to the work published by Peng & Wagner (61, 62) for $^{15}$N relaxation. However, regardless of the approach, if more measurements are made (e.g. at different field strengths (66)), the relaxation parameters can be calculated more accurately when using simple models. Alternatively, more complicated models, which are beyond the scope of this thesis, can be used to characterise the dynamics of the molecule of interest.
3.8 REFERENCES

CHAPTER 4

EXPERIMENTS DESIGNED TO MAKE $^{13}$C AND $^1$H RELAXATION MEASUREMENTS

4.1 INTRODUCTION

The method shown in Eqns. 3.56 to 3.68 can be implemented by making five independent relaxation measurements. These involve measuring the relaxation rates of $^{13}$C and proton longitudinal magnetisation, their mutual longitudinal two spin order, $^{13}$CHH longitudinal three spin order and HH longitudinal two spin order. The first three measurements can be made for a $^{13}$CH group and all five can be made for a $^{13}$CH$_2$ and $^{13}$CH$_3$ groups.

This chapter gives a detailed account of the pulse sequences designed and used to make the above measurements. The experiments need to comply with general considerations to suppress cross-relaxation, use gradient pulses to select coherence transfer pathways and suppress the water magnetisation. These are discussed in section 4.2. The five sections thereafter give a detailed description of each experiment. Each discussion is divided into four parts: an overview of the desired coherence transfer pathway; which pulses have been phase cycled and why; how and where water magnetisation is dephased; and how unwanted coherences arising from chemical shift evolution, scalar couplings and cross-relaxation processes are suppressed.

The pulse sequences which were designed and developed on the Bruker AMX600 spectrometer and used on the Bruker DRX400 spectrometer are given in Appendix II.
4.2 GENERAL CONSIDERATIONS

Any method which relies upon taking a linear combination of a number of measured relaxation rates is susceptible to propagate errors. The greater the number of relaxation rates used, the larger the accumulative error will be. This error will be significant if the value evaluated for the combination is relatively small. For example, if the value is $0.5 \pm 0.2$, the error is large compared to $1.0 \pm 0.2$. If the number of decay rates required for a given combination of the spectral density function cannot be reduced, then the measurements themselves need to be made in the most precise way. Consequently any unwanted coherence transfer pathways resulting from evolutions or processes which may interfere with the signal of interest need to be eliminated or suppressed to a significant degree. This section discusses general methods for suppressing unwanted coherence transfer pathways and the consequences of these for water suppression.

4.2.1 CROSS-RELAXATION AND ITS SUPPRESSION

The decay rate of a given species, for example $I_{1z}$, in the absence of cross-relaxation is given by its autorelaxation rate. However, cross-relaxation from $I_{2z}$ may perturb $I_{1z}$. If the measured decay rate reflects both auto- and cross-relaxation processes then the decay curve will not decay exponentially. Any fitting procedure which assumes exponential decay will yield erroneous results. Cross-relaxation has been shown to be a prominent source of error in existing pulse sequences which make relaxation measurements in homonuclear systems (1-8). Therefore if an accurate value for the autorelaxation rate is desired then it is imperative to assure that the amplitude of the species of interest is not perturbed by cross-relaxation. To comply with this criterion for accuracy it is necessary first to identify the cross-relaxation processes which are likely to occur and determine the effect they have on the species of interest. And second, to implement methods to suppress, or if possible, to eliminate the effects of these processes. The problem of interference from cross-
relaxation pathways has been encountered and circumvented in heteronuclear $^{15}\text{N}$ relaxation studies (9, 10).

Before designing an appropriate procedure to minimise the effects of cross-relaxation on the apparent autorelaxation rate it is necessary to consider how they arise. The following discussion is limited to two-dimensional experiments since they are relevant to this thesis and because one-dimensional experiments have been covered in the literature (4, 8).

Cross-relaxation can be classified as either direct or in-direct. **Direct cross-relaxation** occurs as a direct result of another species being prepared at a non-equilibrium amplitude. For example, in an inversion recovery experiment all components of longitudinal magnetisation will be excited and the cross-relaxation process $I_{zz} \rightarrow I_{iz}$ will perturb the amplitude of $I_{iz}$. Hence the decay curve obtained will give a rate which is a combination of the autorelaxation and various cross-relaxation rates. At no time will the decay of $I_{iz}$ be due solely to its autorelaxation rate, which therefore becomes difficult to determine.

**Indirect cross-relaxation** occurs as a result of at least two cross-relaxation processes, starting and ending with the species of interest and only requires the species of interest which is initially perturbed from equilibrium. For example, if the species of interest is perturbed from equilibrium, it may cross-relax with another species thereby perturbing it from equilibrium; this species may in turn then undergoes cross-relaxation with the species of interest, perturbing its amplitude. In the case of a selective inversion recovery experiment, where only $I_{iz}$ is initially excited to a non-equilibrium population, the indirect cross-relaxation process $I_{iz} \rightarrow I_{2z} \rightarrow I_{iz}$ will interfere with the amplitude of $I_{iz}$. Initially since the amplitude of $I_{2z}$ is at equilibrium the process $I_{iz} \rightarrow I_{2z}$ will dominate and $I_{2z} \rightarrow I_{iz}$ will be negligible. With time as the amplitude of $I_{2z}$ becomes increasingly perturbed from equilibrium the latter process will become more significant. Consequently the initial decay of $I_{iz}$ in a
selective experiment will be due to its autorelaxation rate and it will be independent of cross-relaxation processes. Therefore an accurate rate for the autorelaxation of $I_{1z}$ can, in principle, be calculated by using only the initial part of the decay curve.

In non-selective experiments the decay of a given species is perturbed by both direct and indirect cross-relaxation, as well as autorelaxation processes. However in selective experiments interference will only be due to indirect cross-relaxation. Where cross-relaxation, be it direct or indirect, occurs, the measured decay curve may be significantly affected leading to non-exponential decay. In general, direct cross-relaxation, when present, usually has a larger effect than indirect cross-relaxation. In cases where direct cross-relaxation takes place, the decay rate extracted will always be inaccurate and the autorelaxation rate cannot be reliably obtained. The relative effects of direct and indirect cross-relaxation on the relaxation decay curve are exemplified in Fig. 4.1.

![Schematic representation of the return to equilibrium of species in both the presence and absence of cross-relaxation processes.](image)

**Fig. 4.1** A schematic representation of the return to equilibrium of species in both the presence and absence of cross-relaxation processes. The measured decay rate is slower in the presence of cross-relaxation than in its absence. Direct cross-relaxation has a larger effect than indirect cross-relaxation.

In the absence of cross-relaxation any species will return to equilibrium exponentially and the relaxation decay rate can be calculated from the following fit:

$$I(t) = I(0) \cdot \exp^{-rt}$$  \[4.1\]
where $I(0)$ and $I(t)$ are the intensities at time zero and $t$ respectively (assuming the intensity at equilibrium to be zero, as is the case for our experiments) and $R$ is the autorelaxation decay rate. A value of $R$ calculated from Eqn. 4.1 will be inaccurate if cross-relaxation processes interfere with the autorelaxation decay. As explained above, indirect cross-relaxation processes are negligible at short relaxation times but become increasingly significant at longer times. Therefore the initial part of the decay curve will fit Eqn. 4.1 but the latter part will deviate from it. In such cases, the decay curve may be approximated to an exponential multiplied by the first few terms of a series fit (11):

$$I(t) = I_0 \cdot \exp^{-tR} \cdot (1 + t^2 \cdot a) \quad [4.2]$$

where $a$ is an additional parameter which takes into account indirect cross-relaxation. At longer values of $t$, a significant proportion of $I(t)$ is likely to be the result of cross-relaxation. This difference is taken into account by the variable offset $a$ in Eqn. 4.2.

In Eqn. 4.2 only the first two terms of the infinite series are considered to give an approximate fit. If more terms are used, more variables will be involved and consequently more points on the decay curve need to be obtained to retain the same degree of accuracy. By using only the first two terms, cross-relaxation processes can be approximated to a significant degree of accuracy without the need to make an impractical number of measurements.

Although indirect cross-relaxation can be taken into account during analysis, the fit of Eqn. 4.2 is more susceptible to errors. This makes the exponential model, given in Eqn. 4.1, a preferential choice where practicable.

When designing new pulse sequences to measure $^{13}$C relaxation it is logical to refer to the general structure of existing analogous $^{15}$N experiments as a starting point. Generally, amide proton coherence is excited, transferred to the heteronucleus to encode the second dimension, the relaxation measurement is made and then $^{15}$N coherence is transferred back to proton for detection (sub-section 2.2.6.1). This gives
greatly enhanced experimental sensitivity compared to direct detection of the heteronucleus.

Measurements commonly made for $^{15}$N (i.e. $T_1$ and $T_2$) encode heteronuclear chemical shift evolution after the mixing time. The consequences of using an experiment of this structure to make $^{13}$C relaxation measurements need to be considered before undertaking any practical implementations.

In uniformly $^{13}$C labelled molecules the longitudinal relaxation decay rate of $^{13}$C spins is given by:

$$R_C(S_{Cz}) = \sum_{j \neq C} \frac{1}{D_{ij}^2} \left[ \frac{1}{3} J(\omega_C - \omega_j) + J(\omega_C) + 2J(\omega_C + \omega_j) \right] + \frac{1}{2} C_C^2 J(\omega_C) \quad [4.3]$$

where $j$ can be a bonded proton or a bonded $^{13}$C as well as non-bonded protons and $^{13}$C spins. The bonded dipolar interactions are dominant over the non-bonded ones, therefore the following discussion will be restricted to the former. The $^{13}$C can relax as a result of two different types of dipolar interactions; $^{13}$C-H and $^{13}$C-$^{13}$C. These give rise to $J(\omega_C)$ and $J(0)$ terms respectively in Eqn. 4.3. The relative large $\gamma$ of protons favours the $^{13}$C-H dipolar interaction. For small molecules $J(0)$ is similar in size to $J(\omega_C)$ and therefore the $^{13}$C-$^{13}$C dipolar contribution to $T_1$ (and NOE) measurements is <1%. In proteins, however, the slow motion at $J(0)$ becomes large compared to $J(\omega_C)$ and therefore the $^{13}$C-$^{12}$C dipolar interaction becomes more significant (11) and cannot be ignored. The consequence of this is that dipolar cross-relaxation between adjacent $^{13}$C spins (sub-section 3.4.3) also become significant. Cross-relaxation between $^{13}$C spins will distort the signal intensity in any longitudinal relaxation measurements. For example when measuring $R_C(S_{Cz}^{\alpha})$, the intensity of $S_{Cz}^{\alpha}$ may be significantly affected by the cross-relaxation process $S_{Cz}^{\beta} \rightarrow S_{Cz}^{\alpha}$ arising from the $^{13}$C-$^{13}$C dipolar interaction. Additional complicated processes involving
$^{13}$C spins further along an amino acid side-chain may also distort the intensity of $S_{C^2}$ although this will have a smaller effect. Overall the $^{13}$C-$^{13}$C dipolar interaction has been reported to contribute as much as 15% to the $T_1$ values when $^{13}$C $T_1$'s of selectively labelled RNase were compared to those of the uniformly $^{13}$C labelled protein (11).

In $^{15}$N relaxation measurements the heteroatom is not bonded to another $^{15}$N and it can be assumed to relax solely with its bonded proton and its CSA. The $T_1$ rate of $^{15}$N is given by:

$$R_N(S_{Nz}) = \frac{1}{2} D_{NH}^2 \left[ \frac{1}{3} J(\omega_N - \omega_H) + J(\omega_N) + 2J(\omega_N + \omega_H) \right] + \frac{1}{2} C_N^2 J(\omega_N) \quad [4.4]$$

Since $^{15}$N spins are not bonded to other $^{15}$N atoms, the dipolar relaxation of the heteroatom is dependent only on the $^{15}$N-H interaction. Therefore the $T_1$ relaxation of the $^{15}$N-H bond vector is predominantly governed by motion at $J(\omega_N)$, regardless of the size of the molecule). Consequently in $^{15}$N relaxation experiments, unlike with $^{13}$C, there is no interference from dipolar cross-relaxation. As a consequence of cross-relaxation the conventional experimental structure used for measuring $^{15}$N relaxation (preparation, mixing, evolution and acquisition) is not suitable for making $^{13}$C relaxation measurements. Hence we need to look at other ways of measuring $^{13}$C relaxation, which suppress dipolar cross-relaxation between adjacent $^{13}$C spins.

If conventional $^{15}$N relaxation experiments, where the mixing delay is before the evolution period, are used to measure $^{13}$C relaxation, direct cross-relaxation processes will distort the measured rates. For example, when measuring the $^{13}$C $T_1$, the cross-relaxation process $S_{C^2} \rightarrow S_{C^2}$ during the mixing time will give rise to $\omega_{C^2}$ being encoded during $t_1$ and $\omega_{H^2}$ during $t_2$. This will be modulated at ($\omega_{C^2}$, $\omega_{H^2}$)
which will be in the same place as the peak arising from the autorelaxation of $S_{Cz}$ (Fig. 4.2(a)). Hence the auto- and cross-relaxation processes will be superimposed and the apparent autorelaxation decay rate will be affected. However if the evolution period is before the mixing time then $\omega_{Cz}$ will be encoded during $t_1$ and $\omega_{H\alpha}$ during $t_2$ for the $S_{Cz} \rightarrow S_{Cz}$ cross-relaxation process. This will give a cross-peak at $(\omega_{Cz}, \omega_{H\alpha})$ which will be independent of the autorelaxation peak at $(\omega_{Cz}, \omega_{H\alpha})$ (see Fig. 4.2(b)). Hence direct cross-relaxation effects will give rise to separate peaks and the autorelaxation decay rate will not be affected by this particular cross-relaxation process.

(a) Mixing Period Before the Evolution Time

<table>
<thead>
<tr>
<th>Relaxation Process</th>
<th>Frequency Encoded During</th>
<th>Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autorelaxation of $S_{Cz}$</td>
<td>$\omega_{Cz}$</td>
<td>$\omega_{H\alpha}$</td>
</tr>
<tr>
<td>Autorelaxation of $S_{Cz}$</td>
<td>$\omega_{Cz}$</td>
<td>$\omega_{H\alpha}$</td>
</tr>
<tr>
<td>$S_{Cz} \rightarrow S_{Cz}$</td>
<td>$\omega_{Cz}$</td>
<td>$\omega_{H\alpha}$</td>
</tr>
<tr>
<td>$S_{Cz} \rightarrow S_{Cz}$</td>
<td>$\omega_{Cz}$</td>
<td>$\omega_{H\alpha}$</td>
</tr>
</tbody>
</table>

(b) Evolution Time Before the Mixing Period

<table>
<thead>
<tr>
<th>Relaxation Process</th>
<th>Frequency Encoded During</th>
<th>Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autorelaxation of $S_{Cz}$</td>
<td>$\omega_{Cz}$</td>
<td>$\omega_{H\alpha}$</td>
</tr>
<tr>
<td>Autorelaxation of $S_{Cz}$</td>
<td>$\omega_{Cz}$</td>
<td>$\omega_{H\alpha}$</td>
</tr>
<tr>
<td>$S_{Cz} \rightarrow S_{Cz}$</td>
<td>$\omega_{Cz}$</td>
<td>$\omega_{H\alpha}$</td>
</tr>
<tr>
<td>$S_{Cz} \rightarrow S_{Cz}$</td>
<td>$\omega_{Cz}$</td>
<td>$\omega_{H\alpha}$</td>
</tr>
</tbody>
</table>

Fig. 4.2 The effect of the relative ordering of the mixing period and the evolution time on direct cross-relaxation. When the mixing period is placed before the evolution time, (a), direct cross-relaxation superimposes on top of the autorelaxation peaks. When these two delays are swapped with each other, (b), signal arising from direct cross-relaxation gives peaks which are independent of the autorelaxation peaks.
When the evolution time is before the mixing period, the "pure" T₁ decay of the spin of interest will be observed, to a first approximation. However, at longer mixing times indirect cross-relaxation between the spin of interest and other spins may have an effect which must be taken into account during analysis by using Eqn. 4.2.

When making relaxation measurements in uniformly ¹³C enriched biomolecules, additional cross-relaxation processes between inter-residue protons may alter the relaxation decay rates. Consider a ¹³CHH' system, where the two protons are inequivalent but they are modulated at the same frequency in f₁ because they are bonded to the same ¹³C. When measuring $R_1(I_{Hz})$ the direct cross-relaxation process $I_{Hz} \rightarrow I_{Hz}$ will manifest itself at ($\omega_H, \omega_H$) which is where the autorelaxation peak of H will be modulated. Hence both the auto- and cross-relaxation processes will still give one signal for each proton. Although placing the evolution period before the mixing time gives separate peaks for direct cross-relaxation processes in ¹³CH systems, for ¹³CHH' groups however, not all such processes are separated. The direct cross-relaxation processes in the latter system which remain uneffected can be separated from autorelaxation by encoding ¹³CH double quantum chemical shift evolution in the second dimension. This method will be discussed below for the ¹H T₁ and ¹³CH longitudinal two spin order experiments in sections 4.4 and 4.5 respectively.

Indirect cross-relaxation can be taken into account during analysis by fitting the decay curve with Eqn. 4.2. Since Eqn. 4.2 will only give an approximate fit other means of suppressing cross-relaxation should be sought and implemented where necessary. One such method involves redesigning the pulse sequence during the mixing time. It has been shown that the effects of a specific cross-relaxation process can be suppressed by changing the sign of one of the species involved (by applying a 180° pulse) halfway through the mixing period (3, 7, 12, 13).
Consider the indirect cross-relaxation process $I_{Hz} \rightarrow 4I_{Hz}I_{Hz}S_{Cz} \rightarrow I_{Hz}$ in a $^{13}$CH$_2$ group (with equivalent or inequivalent protons), arising in the mixing time. This process, and others similar to it, may not be affected by placing the evolution period before the mixing time or by encoding $^{13}$CH double quantum coherence in the second dimension of the spectrum because the starting and ending species are the same. Instead, if either the $I$ or $S$ spin is inverted in the middle of the mixing period the sign of the amplitude of either $I_{Hz}$ or $4I_{Hz}I_{Hz}S_{Cz}$ will be inverted while the amplitude of the other species will remain unaffected. For example, the amplitude of $I_{Hz}$ can be inverted by applying a 180° RF pulse to the $I$ spins. Alternatively if a 180° RF pulse is applied to the $S$ spins, the amplitude of $4I_{Hz}I_{Hz}S_{Cz}$ can be inverted. With this method, the effects of indirect cross-relaxation during the first half of the mixing time can be largely reversed in the second half. Suppression will be most effective when the decay over the time period concerned is small. However, not all cross-relaxation processes are amenable to suppression by this method and the elimination of specific cross-relaxation processes relevent to each measurement will be discussed below in the appropriate section for the particular experiment.

4.2.2 THE USE OF GRADIENT PULSES TO SELECT COHERENCE TRANSFER PATHWAYS

Gradient pulses are increasingly being chosen in preference to phase cycling to select coherence transfer pathways. The latter is a difference method (see sub-section 2.2.6) which is sensitive to slight random variations in temperature, RF pulses, resonance shifts, etc. These variations reduce the effectiveness of phase cycling. An alternative to phase cycling for selecting coherence transfer pathways is offered by gradient pulses. This method does not rely on subtraction processes and therefore artifacts arising from instrumental instabilities will be much smaller than with experiments using phase cycling. However while phase cycling selects both $+n$ and $-n$ quantum coherence, gradient pulses only select one of these, hence resulting in
a poor S:N ratio. This problem has been overcome by using gradient enhanced experiments.

In conventional gradient enhanced $^1$H-$^{15}$N heteronuclear relaxation experiments (Fig. 4.3(a)) (14-18) gradient pulses are used to dephase the signal in $t_f$ and rephase it immediately before acquisition. Such experiments give extremely good solvent suppression. During $t_f$ both $S^+$ and $S^-$ are dephased. The coherence transfer pathways $S^+ \rightarrow I^-$ and $S^- \rightarrow I^-$ are selected. Later just before acquisition, the rephasing gradient pulse will select $I^-$ and none of the signal will be lost. If gradient pulses are used in this way when the evolution period is before the mixing time (Fig. 4.2(b)) then the signal will be subject to diffusion attenuation in the mixing time. The extent to which diffusion will be encoded is given by:

$$I(t) = I(0) \exp\left[-B(t) - (A\delta)^2 D \left(\Delta - \frac{\delta}{3}\right)\right]$$  \hspace{1cm} [4.5]$$

where $B(t)$ takes relaxation into account, the constant $A$ is equal to the product of $\gamma G$, $\delta$ is the length of the gradient pulses, $D$ is the diffusion coefficient and $\Delta$ is the time between the start of the gradient pulses. In traditional $^{15}$N gradient enhanced experiments $\Delta$, $A$ and $\delta$ are all kept constant (Fig. 4.3(a)). However, if the mixing period is placed between the rephasing and dephasing gradient pulses, as for $^{13}$C experiments (Fig. 4.3(b)), the delay $\Delta$ will no longer be constant. At long mixing times $\Delta$ will be long and more diffusion attenuation will occur than at short mixing times. Hence the observed signal will be attenuated by diffusion, as well as relaxation, as a function of mixing time.
Fig. 4.3 The use of gradient pulses to select coherence transfer pathways with the conventional $^{15}$N (a) and the $^{13}$C (b) pulse sequence structure. In (a) the delay $\Delta$ remains constant at different mixing times and therefore diffusion attenuation is also constant. However in (b) $\Delta$, and therefore diffusion attenuation, will vary as a function of the mixing time.

Any diffusion attenuation can be kept constant by avoiding the use of gradient pulses around the mixing time. Consequently in $^{13}$C experiments where the evolution period is before the mixing time, gradient pulses cannot be used for solvent suppression in the conventional way. This restricted use of gradient pulses opens the need to look for other ways of suppressing water coherence.
4.2.3 SUPPRESSING WATER COHERENCE

A large water signal in the spectrum may distort the intensity of peaks close to it. Therefore to obtain precise measurements it is necessary to reduce the intensity of the water signal without affecting the peaks nearby.

Generally $^1\text{H}$-$^{15}\text{N}$ heteronuclear gradient enhanced experiments suppress water very well without affecting the intensities of nearby peaks. The dephasing-rephasing pair of gradient pulses only select the signal of interest. Therefore water does not pose a problem. However since the experiments under discussion here, due to their structure, are not conducive to this procedure, any water magnetisation that recovers during the mixing period (especially at longer times) due to relaxation may be seen in the spectrum. Consequently other ways of suppressing water need to be considered.

One such method involves storing the magnetisation of interest along the z-axis while water magnetisation is in the xy-plane and then applying a gradient pulse to dephase the latter. This needs to be done after the mixing time during which water magnetisation may recover, and as close to the end of the pulse sequence as possible in order to reduce any further delays where water magnetisation may recover before acquisition. For example, in the $^{13}\text{C}$ $T_1$ experiment at the end of the mixing time (Fig. 4.4, point $\varphi_0$) the $^{13}\text{C}$ coherence of interest is $S_{Cz}$ and water coherence may have recovered along the z-axis, $I_{Hz}^{H_2O}$. Here, $I_{Hz}^{H_2O}$ is rotated into the xy-plane by applying a 90°($^1\text{H}$) RF pulse and then dephased by a gradient pulse. This “suppression block” is repeated to dephase any water magnetisation remaining along the z-axis. This is summarised in Table 4.1. After the water suppression part, $S_{Cz}$ is rotated into the xy-plane, transferred to proton coherence and then detected.

By using this water suppression block at the most appropriate place(s) in the pulse sequence, water magnetisation can be dephased while the desired coherence is unaffected.
Table 4.1 The suppression of unwanted water coherence. The example below refers to a part of the pulse sequence designed to measure $R_c(S_{Cz})$, given in Fig. 4.4.

<table>
<thead>
<tr>
<th>Pulse Sequence</th>
<th>Wanted Coherence</th>
<th>Unwanted Coherence</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varphi_0$</td>
<td>$S_{Cz}$</td>
<td>$I_{H2O}^H$</td>
</tr>
<tr>
<td>$90^0 (I)\varphi_{10}$</td>
<td>$S_{Cz}$</td>
<td>$I_{H2O}^H$</td>
</tr>
<tr>
<td>gp4 $\varphi_{11}$</td>
<td>$S_{Cz}$</td>
<td>dephased $I_{H2O}^H$</td>
</tr>
<tr>
<td>$90^0 (I)\varphi_{12}$</td>
<td>$S_{Cz}$</td>
<td>$I_{H2O}^H$</td>
</tr>
<tr>
<td>gp5 $\varphi_{13}$</td>
<td>$S_{Cz}$</td>
<td>dephased</td>
</tr>
</tbody>
</table>

4.3 $^{13}$C LONGITUDINAL RELAXATION RATE [$R_c(S_{Cz})$]

To date $^{13}$C work includes $^{13}$C$^\alpha$ $T_1$ measurements of the Xfin zinc finger (19-21) and leucine methyl carbon studies of staphylococcal nuclease (10). $^{13}$C relaxation work of proteins studies have been fewer compared to those of $^{15}$N as a result of the increased cost of $^{13}$C labelling and because of complications arising from homonuclear $^{13}$C-$^{13}$C scalar coupling evolution in uniformly labelled samples. Hence $^{13}$C relaxation measurements have been largely confined to those at natural abundance with highly concentrated samples (19-22), randomly fractional labelled (23, 24) or to molecules which are selectively labelled (9, 10, 25-33).
The experiment we have designed to measure $^{13}$C $T_1$ in uniformly $^{13}$C labelled molecules is shown in Fig. 4.4. It differs from existing pulse sequences which make the same measurement in selectively $^{13}$C labelled samples in terms of its structure. Published experiments for this measurement use a two-dimensional inversion recovery (19, 20) or they are based on $^{15}$N relaxation experiments (32). In both cases the mixing period is before the evolution time. We have placed the evolution time before the mixing period to separate out direct cross-relaxation effects as discussed above. Additionally we have used proton broad-band decoupling to eliminate any cross-relaxation to proton during the mixing period. As the sample used was dissolved in water (to keep the exchangable protons intact) we have also incorporated water suppression sequences into the experiment to minimise the water signal in the two-dimensional spectrum. All these features are discussed in detail below. An example of $^{13}$C $T_1$ decay rates for a $^{13}$CH and a $^{13}$CHH' group are given in Fig. 4.10(a).

4.3.1 AN OVERVIEW OF THE EXPERIMENT

This sub-section describes what happens to magnetisation following the desired coherence transfer pathway during the course of the experiment.

The pulse sequence designed and used in this study to measure $R_C(S_C)$ is shown in Fig. 4.4. Coherence is transferred from the directly bonded proton(s) to the "insensitive" nucleus. The second dimension is encoded before the mixing period after which the coherence is transferred back to protons for detection. In this way the intensity of the detected signal is independent of $\gamma_r$, giving increased sensitivity compared to $^{13}$C direct detection methods (sub-section 2.2.6.1). The sequence in Fig. 4.4 is designed to measure $^{13}$C longitudinal relaxation of the heteroatom in $^{13}$CH$_n$ systems where $n = 1, 2$ or 3. Gradient pulses are used to select coherence transfer pathways and to dephase water magnetisation.
$^{13}$C coherence is prepared in two steps:-

$$I_{Hz} \xrightarrow{90^0(I)} I_{Hy} \xrightarrow{180^0(I)/180^0(S)} 2I_{Hz}S_{Cz} \xrightarrow{90^0(I)} 2I_{Hz}S_{Cz}$$  \[4.6\]

In-phase proton magnetisation ($\varphi_1$) is intially excited and it then evolves, due to the $^{13}$C-H scalar coupling, during a delay having a total length of $\frac{1}{2J}$, into proton magnetisation anti-phase with respect to $^{13}$C, ($\varphi_2$). The two $180^0$ RF pulses applied simultaneously to both nuclei in the middle of the delay have the effect of reversing unwanted proton chemical shift evolution while leaving heteronuclear scalar coupling evolution unaffected. The product operator $2I_{Hz}S_{Cz}$ is stored along the z-axis ($\varphi_3$) while any water magnetisation remaining in the xy-plane is dephased by a gradient pulse (gp3 in Fig. 4.4) and then the desired magnetisation is rotated back into the xy-plane as $^{13}$C coherence anti-phase with respect to proton by a $90^0_x$ RF pulse ($\varphi_4$):

$$2I_{Hz}S_{Cz} \xrightarrow{gp3} 2I_{Hz}S_{Cz} \xrightarrow{90^0_x(S)} 2I_{Hz}S_{Cy} \xrightarrow{1/4J} S_{Cy}$$  \[4.7\]

During the following $\frac{1}{4J}$ period the evolution of $S_{Cy}$ is the desired process. But during the $\frac{1}{4J}$ delay a mixture of in-phase ($S_{Cx}$) and anti-phase ($2I_{Hz}S_{Cy}$) magnetisation will be formed as a result of $^{13}$C-H scalar coupling evolution in $^{13}$CH groups:

$$2I_{Hz}S_{Cy} \xrightarrow{\varphi_5} 2I_{Hz}S_{Cy} \cos \pi J_{CH} \frac{1}{4J} + S_{Cx} \sin \pi J_{CH} \frac{1}{4J}$$  \[4.8\]
In $^{13}$CHH' groups, $2I_{\text{H}_2\text{S}_\text{Cy}}$, $2I_{\text{H}_z\text{S}_\text{Cy}}$, $4I_{\text{H}_zI_{\text{H}_z}\text{S}_\text{Cy}}$ and $S_{\text{Cx}}$ will be formed as a result of scalar coupling evolution between the $^{13}$C spin with both of its protons:

\[
2I_{\text{H}_2\text{S}_\text{Cy}} \frac{1}{4J} \rightarrow 2I_{\text{H}_2\text{S}_\text{Cy}} \cos^2 \pi J_{\text{CH}} \frac{1}{4J} + S_{\text{Cx}} \cos \cdot \sin \pi J_{\text{CH}} \frac{1}{4J} + 4I_{\text{H}_zI_{\text{H}_z}\text{S}_\text{Cy}} \sin \cdot \cos \pi J_{\text{CH}} \frac{1}{4J}
\]

$\varphi_5$ + $2I_{\text{H}_z\text{S}_\text{Cy}} \sin^2 \pi J_{\text{CH}} \frac{1}{4J}$ $\varphi_6$

[4.9]

In $^{13}$CH$_2$ groups with equivalent protons, transformation 4.9 will give the operators $2I_{\text{H}_2\text{S}_\text{Cy}}$, $4I_{\text{H}_zI_{\text{H}_z}\text{S}_\text{Cy}}$ and $S_{\text{Cx}}$ only. In general terms, for a $^{13}$CH$_n$ system, the amplitude of the signal which evolves into in-phase $^{13}$C magnetisation is:

\[
2I_{\text{H}_2\text{S}_\text{Cy}} \frac{1}{4J} \rightarrow S_{\text{Cx}} \sin \pi J_{\text{CH}} \tau \cdot \cos^{n-1} \pi J_{\text{CH}} \tau
\]

$\varphi_5$ $\varphi_6$

[4.10]

After the $\frac{1}{4J}$ period, the heteronuclear chemical shift evolution is encoded during $t_i$ before being rotated onto the z-axis for the mixing period($\varphi_8$):

\[
S_{\text{Cx}} \xrightarrow{t_i} S_{\text{Cx}} \xrightarrow{90^\circ (S)} S_{\text{C}z} \xrightarrow{t_m} S_{\text{C}z}
\]

$\varphi_6$ $\varphi_7$ $\varphi_8$ $\varphi_9$

[4.11]

In the evolution delay (and the preceding $\frac{1}{4J}$ delay) $^{13}$C-$^{13}$C scalar coupling evolution can give rise to $^{13}$C coherence anti-phase with respect to various $^{13}$C spins. The subsequent $90^\circ (S)$ RF pulse may convert some of these into homonuclear $^{13}$C zero,
single and double quantum coherences. What happens to these in the course of the remaining pulse sequence and how these are removed is discussed below.

The evolution period is placed before the mixing delay to separate direct cross-relaxation from autorelaxation as described above in section 4.2. During the mixing time proton is decoupled to prevent any direct cross-relaxation of $^{13}$C longitudinal magnetisation with various proton longitudinal modes and $^{13}$CH longitudinal two spin order. Indirect cross-relaxation between bonded $^{13}$C atoms may still affect the measured rate and should be taken into account during analysis (see section 4.2).

Any water magnetisation which may recover during the mixing delay is destroyed between points $\phi_9$ and $\phi_{13}$ while the $^{13}$C coherence of interest is still along the z-axis. This will be discussed below.

At point $\phi_{13}$ $^{13}$C magnetisation is transferred back to proton for detection by a procedure which is the reverse of its preparation:

\[
\begin{align*}
S_{C_2} & \xrightarrow{4\,\text{J}} I_{Hy} \xrightarrow{90^\circ Y} I_{Hy} \xrightarrow{2\,\text{J}} I_{Hz} \xrightarrow{90^\circ X} I_{Hz} \xrightarrow{\text{Acq}} \\
\phi_9 & \quad \phi_{13} & \quad 0 & \quad \phi_{14} & \quad \phi_{15} & \quad \phi_{16} & \quad 0 & \quad \phi_{17} & \quad \phi_{18} & \quad \phi_{19}
\end{align*}
\]

$^{13}$C coherence is allowed to become anti-phase with respect to proton ($\phi_{12}$) with the same efficiency as the preparation of $S_{C_2}$, (transformation 4.10). This is transferred to proton coherence anti-phase with respect to $^{13}$C ($\phi_{16}$) by applying a $90^\circ$ RF pulse to both spins simultaneously. The operator $2S_{C_2}I_{Hz}$ evolves into in-phase proton magnetisation ($\phi_{17}$). This is stored along the z-axis between points $\phi_{18}$ and $\phi_{19}$ while $\text{gp5}$ dephases any water magnetisation in the xy-plane. The operator $I_{Hz}$ is rotated back into the xy-plane before acquisition.
In transformation 4.11 at longer values of $t_i$, $S_{Cx}$ will evolve into varying amounts of $S_{Cx}$ and $S_{Cy}$ due to chemical shift evolution. Quadrature detection in $f_1$ is achieved by cycling the phase of the $90^\circ(S)$ RF pulse at the end of the evolution period to observe both $S_{Cx}$ and $S_{Cy}$. This is summarised for $S_{Cy}$ as follows:

$$
S_{Cx} \xrightarrow{t_i \neq 0} S_{Cy} \xrightarrow{90^\circ(S)} S_{Cz} \xrightarrow{t_m} S_{Cz} \rightarrow \text{as with } S_{Cx}
$$

4.3.2 COHERENCE TRANSFER PATHWAY SELECTION

A combination of gradient pulses and phase cycling have been used to select the desired coherence transfer pathway shown in Fig. 4.4. Gradient pulse pairs gp1-gp2 and gp6-gp7 have been used to generate two gradient echoes (see sub-section 2.2.6.2) which follow the desired coherence transfer pathway while leaving undesired coherence transfer pathways randomised. Each pair of gradient pulses do not have any net effect on the magnetisation of interest.

Phase cycling has been used at three points in the pulse sequence. Phase cycle $\phi_1$ selects initial proton coherence transfers with $\Delta p = \pm 1$. Phase cycle $\phi_2$ selects $^{13}$C coherence transfer with $\Delta p = \pm 1$ at the end of the evolution period. This phase cycle ensures that only $^{13}$C coherence which has been transferred from proton coherence is selected.

Phase cycling has also been used to eliminate unwanted coherence transfer pathways during the evolution period. During this delay, imperfect proton RF pulses may produce operators which can be transformed into MQC during the evolution period and distort peak intensities in the spectrum. MQC in this part of the pulse sequence is suppressed by phase cycle $\phi_3$. This is discussed in more detail in sub-section 4.3.4.
The receiver is phase cycled with $\phi_1$ and $\phi_2$ because they affect the phase of the desired signal. Phase cycle $\phi_3$ only affects unwanted MQC's; it has no effect on the desired signal therefore the phase of the receiver is independent of this phase cycle.

4.3.3 SUPPRESSING WATER COHERENCE

In conventional $^{15}$N experiments a dephasing-rephasing pair of gradient pulses is used to dephase all coherences at the end of the evolution time and then only rephase the coherence of interest prior to acquisition, leaving solvent magnetisation randomised. This procedure provides very efficient water suppression. Unfortunately such a procedure cannot be used with the structure of $^{13}$C experiments due to problems with diffusion encoding, as discussed above. Consequently gradient pulses have been used in a different way to suppress unwanted coherences and in particular, water coherence. When magnetisation following the the desired coherence transfer pathway is along the z-axis, gradient pulses are used to dephase any unwanted coherences in the xy-plane, e.g. water, as shown below.

Precautions are taken to minimise signal arising from water at three opportunistic places in the pulse sequence. Water magnetisation excited by the first proton RF pulse at point $\varphi_1$ is dephased by gradient pulse $gp3$ as shown in transformation 4.14:-

\[
\begin{align*}
 I_{H_2O} & \xrightarrow{90^\circ_x(I)} I_{H_2O} & \xrightarrow{\frac{1}{2}J} \xrightarrow{90^\circ_y(I)} \xrightarrow{gp3} \text{dephased} \\
 \varphi_1 & \quad \varphi_2 & \quad \varphi_3 & \quad \varphi_4
\end{align*}
\]

Water magnetisation is dephased during points $\varphi_3$ and $\varphi_4$. At this stage of the pulse sequence the proton coherence of interest is stored along the z-axis and is therefore unaffected by $gp3$. 
The second most likely place that water magnetisation may recover is during the mixing period. More water magnetisation will recover at longer mixing times than at shorter ones. It is dephased by a suppression sequence inserted into the pulse sequence immediately after the mixing time. This suppression block consists of a 90°(I) RF pulse which will rotate any water magnetisation along the z-axis into the xy-plane (φ₁₀), followed by a dephasing gradient pulse, gp4:

\[
\begin{align*}
I_{H_2O}^{90°(I)} &\rightarrow I_{Hy}^{90°(I)} & &\text{dephased} \\
\phi_9 &\quad \phi_{10} &\quad \phi_{11}
\end{align*}
\]

Any water coherence along the x-axis is dephased as follows:

\[
\begin{align*}
I_{H_2O}^{90°(I)} &\rightarrow I_{Hx}^{90°(I)} & &\text{dephased} \\
\phi_9 &\quad \phi_{10} &\quad \phi_{11}
\end{align*}
\]

The suppression block of 90°(I) - gp is repeated to dephase any water magnetisation along the y-axis after the mixing period, which was rotated onto the z-axis by the 90°(I) RF pulse from the first suppression block:

\[
\begin{align*}
I_{H_2O}^{90°(I)} &\rightarrow I_{Hy}^{90°(I)} & &\text{dephased} \\
\phi_9 &\quad \phi_{10} &\quad \phi_{11} &\quad \phi_{12} &\quad \phi_{13}
\end{align*}
\]

The values of gp4 and gp5 are different to avoid rephasing any unwanted coherences.

The 90°(I) RF pulses used in the suppression of water magnetisation need not necessarily be applied along the x-axis. A 90°(I) will be equally as effective as 90°(I). Gradient pulse 4 and gp5 only dephase water magnetisation in the xy-plane.
They have no effect on the selected $^{13}$C coherence because it will still be along the z-axis and unaffected by RF pulses applied to proton.

The third place in the pulse sequence where water magnetisation has been suppressed is immediately before acquisition between points $\phi_{18}$ and $\phi_{19}$. Just before this period, at point $\phi_{17}$, the desired signal will be in-phase proton magnetisation along the y-axis and any water magnetisation will be along the x-axis. A $90^0(I)$ RF pulse will rotate the desired signal onto the z-axis while leaving the unwanted water magnetisation in the xy-plane. The subsequent gradient pulse, gp6, will only dephase water magnetisation and leave the desired signal unaffected. The operator $I_{Hz}$ is then rotated into the xy-plane by a $90^0(I)$ RF pulse before acquisition. This is summarised as follows:-

\[
I_{Hz}^{{H_2O}} \xrightarrow{90^0(I)} I_{Hz}^{{H_2O}} \xrightarrow{gp5} \text{dephased}
\]

4.3.4 SUPPRESSING UNDESIR ED COHERENCES

Undesired coherences can arise from imperfect RF pulses, scalar coupling evolution and from cross-relaxation processes. Only the main undesired coherences from these three causes, and ways of suppressing them, are considered here.

4.3.4.1 Suppressing Undesired Coherences Resulting from Imperfect RF Pulses

Undesired coherences, such as MQC, may result from imperfect RF pulses during the evolution time. MQC can arise if the operators $2I_{Hz}S_{Cx}$ and $2I_{Hz}S_{Cy}$, present during the evolution period (transformations 4.8 and 4.9), are acted on by an imperfect $180^0_y(I)$ RF pulse in the middle of the evolution period:-
where the RF pulse in brackets represents uncertainty in the angle of the pulse. If the product operators $2I_{Hz}S_{Cx}$ and $2I_{Hz}S_{Cy}$ are not removed $^{13}$CH chemical shift evolution will be encoded in addition to $^{13}$C chemical shift evolution. This will result in artifactual peaks in the second dimension of the spectrum.

The solution adopted to minimise MQC in this experiment is to phase cycle the $180^\circ_y(I)$ RF pulse in the evolution period from +y to -y (cycle $\phi_3$). When the $180^\circ_y(I)$ pulse has a phase of +y, the MQC produced will have a particular sign (e.g. $+2I_{Hz}S_{Cx}$). If the phase of the $180^\circ_y(I)$ pulse is changed to -y, the MQC generated will have the opposite sign from before (e.g. $-2I_{Hz}S_{Cx}$) and the two MQC operators will cancel out thereby minimising interference from MQCs.

If the $^{13}$C RF pulses are imperfect then the following transformation, from $\phi_5$ in the pulse sequence (transformation 4.7), may occur:

$$2I_{Hz}S_{Cy} \xrightarrow{\frac{1}{4I}} 2SzS_{Cx} \xrightarrow{t_I} 2SzS_{Cx} \xrightarrow{(90^\circ_y(S))} 2SzS_{Cz} \xrightarrow{t_m} 2SzS_{Cz}$$

$$\phi_5 \quad \phi_6 \quad \phi_7 \quad \phi_8 \quad \phi_9$$

$$\xrightarrow{(90^\circ_y(S))} 2SzS_{Cy} \xrightarrow{\frac{1}{4I}} 2I_{Hz}S_{Cy}$$

$$\phi_{14} \quad \phi_{15}$$

[4.20]
The operator \( 2I_{Hz}S_{Cy} \) at point \( \varphi_{15} \) is the most efficient coherence resulting from imperfect \(^{13}\text{C} \) RF pulses. But this has the wrong phase to be transferred to proton coherence (see transformation 4.12) therefore it cannot be observed.

### 4.3.4.2 Suppressing Undesired Coherences Resulting from Homonuclear \(^{13}\text{C} \) Scalar Coupling Evolution

During the evolution period, \(^{13}\text{C} \)-\(^{13}\text{C} \) scalar coupling evolution may give the operators \( 2S_{Cx}S_{Cz} \), \( 2S_{Cy}S_{Cz} \), \( 4S_{Cx}S_{Cz}S_{Cz} \) and \( 4S_{Cy}S_{Cz}S_{Cz} \). What happens to these four product operators during the course of the pulse sequence is shown in Table 4.2. Apart from the desired coherence there are two examples of undesired coherence which evolve into observable coherence. These are \( 4S_{Cz}S_{Cz}S_{Cy} \) and \( \frac{1}{2}(2S_{Cz}S_{Cx} - 2S_{Cz}S_{Cz}) \) which evolve into ±\( I_{Hy} \). The operators -\( I_{Hy} \) and +\( I_{Hy} \) from undesired processes are likely to be present only at very low amplitudes compared to the desired signal. This is because three spin operators (e.g. \( 4S_{Cz}S_{Cz}S_{Cy} \)) have short \( T_2 \) values and relax very quickly compared to single spin operators. Consequently they will decay very quickly during any delay. Additionally, assuming the \( J_{C-C}^{1,2} \) coupling is 35 Hz, the undesired coherences will be at a maximum when \( t_f = \frac{1}{2J} = 14 \text{ ms} \) (39), i.e. at the end of the data set. Hence they can be suppressed by choosing a weighting function which suppresses the end of the \( t_f \) FID.

### 4.3.4.3 Suppressing Undesired Coherences Resulting from Cross-Relaxation

Direct cross-relaxation is suppressed by placing the evolution time before the mixing period as explained in sections 4.2.1 and 4.3.1.
The following indirect cross-relaxation processes may occur during the mixing time and distort the intensity of the desired species:-

\[ S_{Cz} \leftrightarrow I_{Hz} \]  \[4.21\]

\[ S_{Cz} \leftrightarrow 2I_{Hz}S_{Cz} \]  \[4.22\]

\[ S_{Cz} \leftrightarrow 4I_{Hz}I_{Hz}S_{Cz} \]  \[4.23\]

\[ S_{Cz} \leftrightarrow S_{C_{2}} \]  \[4.24\]

The cross-relaxation process 4.21 occurs as a result of \(^{13}\text{C}\)-proton CSA cross-correlation, 4.22 results from the \(^{13}\text{C}\)-proton dipolar interaction, 4.23 occurs as a result of dipolar interactions between \(^{13}\text{C}\) and both if its bonded protons in \(^{13}\text{CH}_{2}\) groups and 4.24 results from \(^{13}\text{C}-^{13}\text{C}\) CSA cross-correlation. The relaxation rates for these processes are given in the relaxation matrix of Eqn. 2.40.

The relaxation processes given in 4.21 and 4.22 can be eliminated by broadband decoupling at the proton frequency. Broadband decoupling has the effect of inverting the sign of the species involving the decoupled spin (i.e. \(+I_{Hz}\) is inverted to \(-I_{Hz}\) in process 4.21 and \(+2I_{Hz}S_{Cz}\) is inverted to \(-2I_{Hz}S_{Cz}\) in process 4.22). Hence the effects of indirect cross-relaxation during the first half of the mixing time cancel out in the second half.

Processes 4.23 and 4.24 are not removed by the pulse sequence and therefore they need to be taken into account during analysis. An exponential multiplied by a series fit (given in Eqn. 4.2) includes an offset which takes cross-relaxation effects into account. The accuracy of this fit to account for cross-relaxation will be discussed in section 5.4.
Fig 4.4 Pulse sequence to measure $R_C(S_{C_2})$. All the RF pulses on the top staff are applied to protons, those on the second staff are applied to $^{13}$C and the third staff represents gradient pulses. The narrow and broad vertical lines represent 90° and 180° pulses respectively, the grey area represents proton presaturation and the shaded areas represent broadband decoupling periods: waltz16 for proton decoupling (34) and garp for decoupling $^{13}$C (35). The following phase cycle was used: $\phi_1 = y, y, -y, -y$, $\phi_2 = y, -y, \phi_3 = y, y, y, -y, -y, -y$ and $\phi_{rec} = x, -x, -x, x, x, x, -x$. The following gradient pulse values, in ms, were used: $gp1 = gp2 = 0.4, gp3 = 3.5, gp4 = 2.7, gp5 = 1.0, gp6 = gp7 = 0.7$ and $gp8 = 2.8$. 161
Table 4.2 The fate of undesired coherences present at the end of the evolution delay during the course of the rest of the $R_C(S_{Cz})$ pulse sequence. At points $\varphi_7$ and $\varphi_{14}$, the operators which are underlined have been followed through the rest of the pulse sequence.

<table>
<thead>
<tr>
<th>Pulse Sequence</th>
<th>Undesired Coherence</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of $t_f$</td>
<td></td>
</tr>
<tr>
<td>$(\varphi_7)$</td>
<td>$2S_{Cx}S_{Cz}$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$2S_{Cy}S_{Cz}$</td>
</tr>
<tr>
<td>$(\varphi_8)$</td>
<td>$4S_{Cx}S_{Cz}S_{Cz}$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$4S_{Cy}S_{Cz}S_{Cz}$</td>
</tr>
<tr>
<td>$90^\circ(S)$</td>
<td></td>
</tr>
<tr>
<td>$(\varphi_9)$</td>
<td>$(x$-pulse)</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$2S_{Ca}S_{Cy}$</td>
</tr>
<tr>
<td>$(\varphi_{14})$</td>
<td>$(ZQC$ and DQC)</td>
</tr>
<tr>
<td>$(\varphi_{15})$</td>
<td>$2S_{Ca}S_{Cy}$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$(SQC)$</td>
</tr>
<tr>
<td>$(\varphi_{16})$</td>
<td>$(ZQC$ and DQC)</td>
</tr>
</tbody>
</table>
| $\downarrow$   | $4S_{Ca}S_{Cy}S_{Cy}$| $(TQC)$
| $\downarrow$   | $4S_{Cy}S_{Cz}S_{Cz}$| $(ZQC$ and DQC) |

SQC, DQC and TQC destroyed by gradient pulse, $gp_4$, after the mixing time, therefore no need to consider further.

$| t_m | (\varphi_m) |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$1/2 (2S_{Cx}S_{Cz} + 2S_{Cy}S_{Cy}) S_{Cz}$</td>
<td>$(ZQC)<em>{Cz}S</em>{Cz} \cos \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\times \cos \alpha_m \sin \varpi_m$</td>
<td>$(ZQC)_{Cy} \cos \alpha_m \cos \varpi_m$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
</tbody>
</table>

$| t_m | (\varphi_m) |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$1/2 (2S_{Cy}S_{Cz} - 2S_{Cy}S_{Cy}) S_{Cz}$</td>
<td>$(ZQC)<em>{Cz}S</em>{Cz} \sin \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\times \sin \alpha_m \cos \varpi_m$</td>
<td>$(ZQC)_{Cy} \sin \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
</tbody>
</table>

$| t_m | (\varphi_m) |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$1/2 (2S_{Ca}S_{Cx}S_{Cy} + 2S_{Ca}S_{Cz}S_{Cz}) S_{Cy}$</td>
<td>$(ZQC)_{Cx} \cos \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\times \cos \alpha_m \sin \varpi_m$</td>
<td>$(ZQC)_{Cx} \sin \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
</tbody>
</table>

$| t_m | (\varphi_m) |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$1/2 (2S_{Cy}S_{Cz} - 2S_{Cy}S_{Cy}) S_{Cz}$</td>
<td>$(ZQC)<em>{Cz}S</em>{Cz} \sin \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\times \sin \alpha_m \cos \varpi_m$</td>
<td>$(ZQC)_{Cy} \sin \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
</tbody>
</table>

$1/2 (2S_{Ca}S_{Cx} + 2S_{Cy}S_{Cy}) S_{Cz}$ not observable).

$| t_m | (\varphi_m) |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$1/2 (2S_{Cy}S_{Cz} - 2S_{Cy}S_{Cy}) S_{Cz}$</td>
<td>$(ZQC)<em>{Cz}S</em>{Cz} \sin \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\times \sin \alpha_m \cos \varpi_m$</td>
<td>$(ZQC)_{Cy} \sin \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
</tbody>
</table>

$| t_m | (\varphi_m) |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$1/2 (2S_{Ca}S_{Cx}S_{Cy} + 2S_{Ca}S_{Cz}S_{Cz}) S_{Cy}$</td>
<td>$(ZQC)_{Cx} \cos \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\times \cos \alpha_m \sin \varpi_m$</td>
<td>$(ZQC)_{Cx} \sin \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
</tbody>
</table>

$1/2 (2S_{Cy}S_{Cz} - 2S_{Cy}S_{Cy}) S_{Cz}$ not observable).

$| t_m | (\varphi_m) |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$1/2 (2S_{Cy}S_{Cz} - 2S_{Cy}S_{Cy}) S_{Cz}$</td>
<td>$(ZQC)<em>{Cz}S</em>{Cz} \sin \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\times \sin \alpha_m \cos \varpi_m$</td>
<td>$(ZQC)_{Cy} \sin \alpha_m \sin \varpi_m$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
</tbody>
</table>

$1/2 (2S_{Ca}S_{Cx} + 2S_{Cy}S_{Cy}) S_{Cz}$ not observable).
<table>
<thead>
<tr>
<th>$\frac{1}{4J}$ delay ($\tau$)</th>
<th>$\frac{1}{2J}$ delay ($\tau'$)</th>
<th>Acquisition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\phi_{13}$</td>
<td>$\phi_{13}$</td>
<td>Observable</td>
</tr>
<tr>
<td>$90^\circ y(S)$</td>
<td>$90^\circ y(I)$</td>
<td>Observable</td>
</tr>
<tr>
<td>$\phi_{14}$</td>
<td>$\phi_{15}$</td>
<td>Unobservable</td>
</tr>
</tbody>
</table>

| $2S_{C_x}J_{C_x} \cos \phi_{m} \cos \phi_{m} \sin \phi_{C_C \tau}$ | $I_{C_x} \cos \phi_{m} \sin \phi_{C_C \tau'}$ | $I_{C_x} \cos \phi_{m} \sin \phi_{C_C \tau}$ |
| $\times \sin \phi_{C_C \tau}$ | $\times \cos \phi_{C_C \tau'} \sin \phi_{C_C \tau}$ | $\times \cos \phi_{C_C \tau} \sin \phi_{C_C \tau}$ |
| | $\downarrow$ | $\downarrow$ |

| $2S_{C_x}J_{C_x} \cos \phi_{m} \sin \phi_{m} \sin \phi_{C_C \tau}$ | $-2S_{C_x}J_{C_x} \cos \phi_{m} \sin \phi_{m} \sin \phi_{C_C \tau}$ | $-2S_{C_x}J_{C_x} \cos \phi_{m} \sin \phi_{m} \sin \phi_{C_C \tau}$ |
| $\times \cos \phi_{C_C \tau} \sin \phi_{C_C \tau}$ | $\times \cos \phi_{C_C \tau} \sin \phi_{C_C \tau}$ | $\times \cos \phi_{C_C \tau} \sin \phi_{C_C \tau}$ |
| | $\downarrow$ | $\downarrow$ |

| $2S_{C_x}S_{C_x} \sin \phi_{m} \cos \phi_{m}$ | $2S_{C_x}S_{C_x} \sin \phi_{m} \cos \phi_{m}$ | $2S_{C_x}S_{C_x} \sin \phi_{m} \cos \phi_{m}$ |
| $+ 2S_{C_x}S_{C_x} \sin \phi_{m} \cos \phi_{m}$ | $+ 2S_{C_x}S_{C_x} \sin \phi_{m} \cos \phi_{m}$ | $+ 2S_{C_x}S_{C_x} \sin \phi_{m} \cos \phi_{m}$ |
| $\downarrow$ | $\downarrow$ | $\downarrow$ |
4.4 PROTON LONGITUDINAL RELAXATION RATE $[R_H(I_{Hz})]$ 

The measurement of longitudinal relaxation rates of protons bonded to heteronuclei have not been reported in the literature. The pulse sequence which we have designed to make this measurement in $^{13}\text{CH}_n$ systems is shown in Fig. 4.5. It is similar to an HSQC-NOESY (36, 37) experiment. The experiment excites proton coherence, encodes $^{13}\text{CH}$ double quantum chemical shift evolution, monitors the decay of $I_{Hz}$ during the mixing time and then detects proton coherence. The evolution period is placed before the mixing time to separate out the effects of direct cross-relaxation, as discussed in sub-section 4.2.1. In addition, any cross-relaxation processes between $I_{Hz}$ and longitudinal modes involving $^{13}\text{C}$ during the mixing time have been eliminated by $^{13}\text{C}$ broadband decoupling. An example of proton $T_1$ decay rates for a $^{13}\text{CH}$ and a $^{13}\text{CHH'}$ group are given in Fig. 4.10(b).

4.4.1 AN OVERVIEW OF THE EXPERIMENT

In this section the fate of magnetisation following the desired coherence transfer pathway is described using the product operator formalism.

Proton coherence is excited ($\varphi_1$) and transferred to $^{13}\text{CH}$ DQC (points $\varphi_2$ to $\varphi_3$) before encoding $^{13}\text{C}$-$^1\text{H}$ double quantum chemical shift evolution during $t_f$.
In the $^{13}\text{C}$ T$_1$ experiment (section 4.3) direct cross-relaxation can be separated from the desired signal by placing the evolution period before the mixing time and indirect cross-relaxation is suppressed by decoupling $^{13}\text{C}$ during the mixing time. However, when measuring decay rates which include proton magnetisation (e.g. $R(I_{\text{HH}})$ and $R(2I_{\text{HC},I_{\text{HH}}})$) there are additional processes of cross-relaxation which cannot be suppressed by the methods discussed so far. For example in $^{13}\text{CHH}'$ systems both proton peaks will occur at the same $^{13}\text{C}$ frequency in the $f_i$ dimension of the spectrum. Any cross-relaxation between the two protons will give rise to the process $I_{\text{HH}} \rightarrow I_{\text{HH}}$ and the equivalent process for the other proton. These processes will manifest themselves at the frequency of the autorelaxation peaks (Fig. 4.6(a)). If these processes are not suppressed they will give imprecise decay rates.

This problem has been overcome by giving each proton in a $^{13}\text{CHH}'$ group a different frequency in $f_i$ thereby separating out cross-relaxation effects. The two protons in a $^{13}\text{CHH}'$ group can be given a different frequency in $f_i$ by encoding $^{13}\text{CH}$ double quantum chemical shift evolution (as shown in Fig. 4.6(b)) instead of $^{13}\text{C}$ single quantum chemical shift evolution during $t_r$. For example, any cross-relaxation resulting from the process $I_{\text{HH}} \rightarrow I_{\text{HH}}$ will be modulated at $(\omega_{\text{HH}},\omega_{\text{HH}})$ where $\omega_{\text{HH}}$ is the $^{13}\text{CH}$ double quantum frequency. This will give rise to a peak which is independent of the autorelaxation peak at $(\omega_{\text{HH}},\omega_{\text{HH}})$ for the present example.
Fig. 4.6 Cross-relaxation in $^{13}$CHH' groups can be overcome by encoding $^{13}$CH double quantum chemical shift evolution in the $t_1$ period. In (a) $^{13}$C single quantum chemical shift has been encoded and cross-relaxation effects (represented by “x”) in $^{13}$CH$_2$ groups superimpose onto the autorelaxation peaks of interest. These effects can be made to give rise to separate peaks which are independent of the autorelaxation peaks by encoding $^{13}$CH double quantum chemical shift evolution during the $t_1$ period.

After point $\varphi_4$ the desired coherence, $2I_{Hx}S_{Cx}$, is rotated into the z-axis ($\varphi_5$).

Gradient pulse 1 dephases any water magnetisation in the xy-plane before a $90^\circ_x (I)$ RF pulse rotates the desired coherence into proton coherence anti-phase with respect to $^{13}$C ($\varphi_7$):

\[
\frac{1}{2}(2I_{Hx}S_{Cx} - I_{Hy}S_{Cy}) \xrightarrow{90^\circ_y (I)/90^\circ_y (S)} 2I_{Hz}S_{Cz} \xrightarrow{\text{gp1}} 2I_{Hz}S_{Cz} \xrightarrow{90^\circ} 2I_{Hy}S_{Cz}
\]

\[\varphi_4 \quad \varphi_5 \quad \varphi_6 \quad \varphi_7\]

[4.26]

The operator $2I_{Hy}S_{Cz}$ is allowed to evolve into in-phase proton coherence ($\varphi_8$) during a delay of $\frac{1}{2J}$ and then rotated onto the z-axis ($\varphi_9$) to observe its $T_1$ relaxation decay during the mixing period:
At the end of the mixing period (after point $\varphi_{11}$) $I_{Hz}$ is rotated into the xy-plane ($\varphi_{12}$). At this point the signal can be detected but any water magnetisation in the xy-plane which may have recovered during the mixing period will give a large signal, especially at longer $t_m$'s. Therefore, in order to dephase water, an extra $\frac{1}{2J}$ delay is introduced at point $\varphi_{12}$ to allow the desired proton coherence to become anti-phase with respect to $^{13}$C ($\varphi_{13}$) so that it can be stored along the z-axis ($\varphi_{14}$) while water magnetisation in the xy-plane can be dephased by gp7:-

$$I_{Hz} \xrightarrow{90^0_y(I)} I_{Hy} \xrightarrow{\frac{1}{2J}} 2I_{Hz}S_{Cz} \xrightarrow{90^0_y(I)} 2I_{Hz}S_{Cz} \xrightarrow{\text{gp7}} 2I_{Hz}S_{Cz} \quad [4.28]$$

$\varphi_{11}$ $\varphi_{12}$ $\varphi_{13}$ $\varphi_{14}$ $\varphi_{15}$

The operator $2I_{Hz}S_{Cz}$ is transformed into proton coherence anti-phase with respect to $^{13}$C ($\varphi_{16}$) and allowed to evolve due to $^{13}$C-H scalar coupling into observable in-phase proton magnetisation ($\varphi_{17}$):-

$$2I_{Hz}S_{Cz} \xrightarrow{90^0_y(I)} 2I_{Hz}S_{Cz} \xrightarrow{\frac{1}{2J}} I_{Hy} \xrightarrow{\text{Acq.}} \quad [4.29]$$

$\varphi_{15}$ $\varphi_{16}$ $\varphi_{17}$
4.4.2 COHERENCE TRANSFER PATHWAY SELECTION

A combination of gradient pulses and phase cycling have been used to select the desired coherence transfer pathway shown in Fig. 4.6. Gradient pulse pairs gp2-gp3, gp5-gp6 and gp8-gp9 have been used to generate three gradient echoes (see sub-section 2.2.6.2) which follow the desired coherence transfer pathway while leaving undesired coherence transfer pathways randomised.

Three phase cycles have been used in the pulse sequence. Phase cycling the first four RF pulses together selects for double quantum coherence. Phase cycle $\phi_2$ ensures that only $^{13}$CH double quantum coherence is selected. Phase cycling has also been used to eliminate unwanted coherence transfer pathways during the evolution period by cycling the two 90° RF pulses applied simultaneously to both nuclei at the end of $t_j$ with the cycle $\phi_3$.

During the proton $T_1$ experiment double quantum coherence is encoded during $t_r$. Since DQC is twice as sensitive to phase changes and RF pulse angles as SQC (38) the phase of the first four pulses is incremented by 45° to implement tppi. The phases of any RF pulses which are cycled while the desired coherence has $\Delta \phi = \pm 2$ need to have half the angle needed for $\Delta \phi = \pm 1$. For example phase cycles $\phi_1$ and $\phi_2$ change by 45°. The effect if these on DCQ will be the same as a 90° change on SQC.
4.4.3 WATER SUPPRESSION

Water suppression is incorporated at three places in the pulse sequence. At all three places the magnetisation of interest is stored along the z-axis while water magnetisation is dephased by a gradient pulse. The first place is between points \( \phi_5 \) and \( \phi_6 \). Any proton magnetisation from water which may be excited at the beginning of the experiment is dephased by \( g_{pl} \) after the evolution period:

Gradient pulse 1 also dephases any water magnetisation which may have evolved due to chemical shift at longer values of \( t_1 \) and any water magnetisation which may have recovered as a result of relaxation during the evolution time. However it does not affect \( \pm I_{Hz}^{H_2O} \). These remaining components of water coherence are dephased by another gradient pulse further along in the sequence:
Water magnetisation recovering during the mixing time (between points $\phi_{10}$ and $\phi_{11}$), particularly at longer values, may be along the z-axis. It is dephased as shown in transformation 4.32 while the coherence of interest is stored along the z-axis:

\[
\begin{align*}
&\pm I_{H_2O}^H \xrightarrow{90^\circ(I)} \pm I_{H_2O}^H \xrightarrow{\frac{1}{2J}} \pm I_{H_2O}^H \xrightarrow{90^\circ(I)} \pm I_{H_2O}^H \xrightarrow{\text{gp4}} \text{dephased} \\
\phi_6 &\quad \phi_7 &\quad \phi_8 &\quad \phi_9 &\quad \phi_{10}
\end{align*}
\]

4.4.4 SUPPRESSING UNDESIRED COHERENCES

Undesired coherences can arise from imperfect RF pulses, scalar coupling evolution and from cross-relaxation processes. Only the main undesired coherences from these three causes, and ways of suppressing them, are considered here.

4.4.4.1 Suppressing Undesired Coherences Resulting from Imperfect RF Pulses

The only unwanted longitudinal modes which may distort the signal of interest and which may be present during the mixing time are $I_{H_2}^H$ and $2I_{H_2}^H I_{H_2}$ groups and $4I_{H_2}^H I_{H_2} I_{H_2}$ in $^{13}$CH groups and $^{13}$CH groups. The former is separated by the signal of interest by encoding $^{13}$CH double quantum chemical shift evolution during the evolution period.
The operator $2I_{Hz}I_{Hz}$ may arise from imperfect RF pulses applied to $2I_{Hy}S_{Cz}$ (see transformation 4.26 for the generation of $2I_{Hy}S_{Cz}$) in the following way:

$$2I_{Hy}S_{Cz} \xrightarrow{\tau = \frac{\pi}{4}} 2I_{Hz}I_{Hz} \sin \pi J_{HH}^{1,3} \tau \xrightarrow{90^6(t)} 2I_{Hz}I_{Hz} \sin \pi J_{HH}^{1,3} \tau \cdot \cos \sin 90^0$$

where the RF pulse in brackets represents uncertainty in the angle of the pulse. The operator $2I_{Hy}S_{Cz} (\varphi_7)$ may evolve into anti-phase magnetisation with respect to a second proton in $13\text{CH}_2$ and $13\text{CH}_3$ groups to form $2I_{Hz}I_{Hz} (\varphi_8)$. The operator $2I_{Hz}I_{Hz}$ is then rotated onto the z-axis by the subsequent proton RF pulse to form $2I_{Hz}I_{Hz} (\varphi_9)$.

The operator $2I_{Hz}I_{Hz}$ may give rise to ZQC and Table 4.3 shows that ZQC is not destroyed during the course of the rest of the pulse sequence. Hence it needs to be taken into account during analysis by using an exponential multiplied by the first two terms of a series fit given in Eqn. 4.2 (see sections 4.2.1 and 5.4).
Table 4.3 The fate of undesired ZQC (arising from imperfect proton RF pulses) present at the end of the mixing period during the course of the rest of the $R_{Hz}(l_{Hz})$ pulse sequence.

<table>
<thead>
<tr>
<th>Pulse Sequence</th>
<th>Unwanted ZQC Coherence</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of $t_m$ ($\phi_{11}$) ↓</td>
<td>$2I_{Hz}I_{Hz}$ ↓</td>
</tr>
<tr>
<td>$90^\circ_\chi$ ($I$) ($\phi_{12}$) ↓</td>
<td>$2I_{Hy}I_{Hy} \Rightarrow (ZQC)_{Hy}$ ↓</td>
</tr>
<tr>
<td>$\frac{1}{2J} = \tau$ ($\phi_{13}$), ($\phi_{16}$) ↓</td>
<td>$2(ZQC)<em>{Hz}S</em>{Cz}\sin\pi J\tau$ ↓</td>
</tr>
<tr>
<td>$\frac{1}{2J} = \tau$ ($\phi_{17}$)</td>
<td>$2(ZQC)_{Hz}\sin^2\pi J\tau$</td>
</tr>
</tbody>
</table>

The operator $4I_{Hz}I_{Hz}I_{Hz}$ may arise from imperfect RF pulses applied to proton in a combination of the following unlikely events:

$$2I_{Hz}S_{Cz} \frac{1}{4J} = \tau \rightarrow 4I_{Hz}I_{Hz}I_{Hz} \sin^2 \pi J_{HH} \tau \rightarrow 90^\circ_\chi(I) \rightarrow 4I_{Hz}I_{Hz}I_{Hz} \sin^2 \pi J_{HH} \tau \cdot \cos^2 \sin 90^\circ \phi_7 \phi_8 \phi_9 \quad [4.35]$$

In the $\frac{1}{4J}$ delay $2I_{Hz}S_{Cz}$ ($\phi_7$) may evolve into anti-phase magnetisation with respect to two protons ($\phi_8$), in $^{13}$CH$_3$ groups, and then be rotated onto the z-axis ($\phi_9$) by the subsequent imperfect RF pulse. This combination of events is unlikely to occur because $2I_{Hz}S_{Cz}$ is unlikely to become anti-phase with respect to two weak HH couplings ($J_{HH} \leq 12$ Hz) (40) in a short delay such as $\frac{1}{4J}$. However, even if the operator
$4I_{Hx}I_{Hz}I_{Hz}$ is present during the mixing time it will have a $\sin^2 \pi J_{HH}^1 \tau \sin(90^\circ)$ dependence and hence it will be very small.

4.4.4.2 Suppressing Undesired Coherences Resulting from Scalar Coupling Evolution

During the evolution period, $^{13}$CH double quantum chemical shift evolution is encoded. The coherence of interest at this point in the experiment ($\varphi_3$) is $2S_{Cx}I_{Hx}$ (see transformation 4.25). This coherence may develop scalar couplings with its bonded $^{13}$C or bonded proton (in $^{13}$CH$_2$ groups) resulting in unwanted three spin coherences at the end of the evolution period ($\varphi_4$). When the 90° RF pulses are applied simultaneously to both nuclei at the end of the evolution period, the unwanted coherences become single or triple quantum coherences ($\varphi_5$) as shown in Table 4.4. These are all dephased by the subsequent gradient pulse, gpl (at point $\varphi_6$).

Table 4.4 The fate of undesired coherences present at the end of the evolution delay during the course of the rest of the $R_{th}(I_{Hz})$ pulse sequence.

<table>
<thead>
<tr>
<th>Pulse Sequence</th>
<th>Unwanted Coherences</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of $t_1$ ($\varphi_3$)</td>
<td>$4S_{Cx}I_{Hx}S_{Cz}$</td>
</tr>
<tr>
<td>90°(/)/90°(S)($\varphi_5$) x-pulses</td>
<td>$4S_{Cx}I_{Hx}S_{Cy}$ (SQC + TQC) and $4S_{Cz}I_{Hz}S_{Cx}$ (doubly anti-phase SQC)</td>
</tr>
<tr>
<td>y-pulses</td>
<td>$4S_{Cx}I_{Hx}S_{Cy}$ (doubly anti-phase SQC)</td>
</tr>
<tr>
<td>gpl ($\varphi_6$)</td>
<td>All dephased</td>
</tr>
</tbody>
</table>
4.4.4.3 Suppressing Undesired Coherences Resulting from Cross-Relaxation

Direct cross-relaxation is suppressed by placing the evolution time before the mixing period as explained in sections 4.2.1 and 4.3.1.

The following indirect cross-relaxation processes may occur during the mixing time and distort the intensity of the desired species:

\[ I_{Hz} \leftrightarrow S_{Cz} \] \[ 4.36 \]

\[ I_{Hz} \leftrightarrow 4S_{Cz}I_{Hz}I_{Hz} \] \[ 4.37 \]

\[ I_{Hz} \leftrightarrow I_{Hz}^'z \] \[ 4.38 \]

\[ I_{Hz} \leftrightarrow I_{Hz}^{'z} \] \[ 4.39 \]

\[ I_{Hz} \leftrightarrow 8I_{Hz}I_{Hz}I_{Hz} \] \[ 4.40 \]

The cross-relaxation processes 4.36 and 4.37 occur as a result of $^{13}$C-proton CSA cross-correlation, process 4.38 results from the dipolar interaction between HH' in $^{13}$CHH' groups, process 4.39 occurs as a result of dipolar interactions between two non-bonded protons (NOE peaks) and process 4.40 results from HH dipolar interactions in $^{13}$CH$_3$ groups. The relaxation rates for these processes are given in the relaxation matrix of Eqn. 2.40.

The relaxation processes given in 4.36 and 4.37 can be eliminated by broadband decoupling at the $^{13}$C frequency. Broadband decoupling has the effect of inverting the sign of the species involving the decoupled spin (i.e. $+S_{Cz}$ is inverted to $-S_{Cz}$ in process 4.36.
and $+4S_{Cz}I_{Hz}I_{Hz}$ is inverted to $-4S_{Cz}I_{Hz}I_{Hz}$ in process 4.37). Hence the effects of indirect cross-relaxation during the first half of the mixing time cancel out in the second half.

Processes 4.38, 4.39 and 4.40 are not removed by the pulse sequence and therefore they need to be taken into account during analysis. An exponential multiplied by a series fit (given in Eqn. 4.2) includes an offset which takes cross-relaxation effects into account. The accuracy of this fit to account for cross-relaxation will be discussed in section 5.4.
Fig 4.5 Pulse sequence to measure $R_H(I_{HZ})$. All the RF pulses on the top staff are applied to protons, those on the second staff are applied to $^{13}$C and the third staff represents gradient pulses. The narrow and broad vertical lines represent $90^\circ$ and $180^\circ$ pulses respectively, the grey area represents proton presaturation and the shaded areas represent broadband garp (35) decoupling periods. The following phase cycle was used: $\phi_1 = (45)^\circ x$, $\phi_2 = (45)^\circ y$, $\phi_3 = x$, $\phi_4 = -x$, $\phi_5 = y$, $\phi_6 = -y$, $\phi_7 = (45)^\circ x$, $\phi_8 = (45)^\circ y$, $\phi_9 = -y$, $\phi_{10} = (45)^\circ x$, $\phi_{11} = (45)^\circ y$, $\phi_{12} = -y$, $\phi_{13} = (45)^\circ x$, $\phi_{14} = (45)^\circ y$, $\phi_{15} = -y$, $\phi_{16} = (45)^\circ x$ and $\phi_{rec} = x$, $-x$, $-x$, $x$, $x$, $-x$, $-x$, $x$. The following gradient pulse values, in ms, were used: $gp1 = 1.317$, $gp2 = 15.1$, $gp3 = 0.39$, $gp4 = 4$, $gp5 = gp6 = 0.35$, $gp7 = 1.6$ and $gp8 = gp9 = 0.25$. 

176
4.5 \textbf{\textsuperscript{13}CH LONGITUDINAL TWO SPIN ORDER RELAXATION RATE}

\[ R_{\text{CH}}(2J_{\text{Hz},S_{CZ}}) \]

The measurement of \textsuperscript{13}CH longitudinal two spin relaxation rate has not been reported in the literature. The pulse sequence which we have designed to make this measurement in \textsuperscript{13}CH\_\textsubscript{n} systems is shown in Fig. 4.7. The experiment excites proton coherence, encodes \textsuperscript{13}CH double quantum chemical shift evolution, monitors the decay of $S_{CZ,I_{Hz}}$ during the mixing time and then detects proton coherence. The evolution period is placed before the mixing time to separate out the effects of direct cross-relaxation, as discussed in section 4.2.1. Signal arising from in-direct cross-relaxation between $2S_{CZ,I_{Hz}}$ and $2S_{CZ,I_{Hz}}$ in \textsuperscript{13}CHH' groups (with inequivalent protons) is separated from the signal of interest by encoding \textsuperscript{13}CH double quantum chemical shift evolution during $t_1$. An example of \textsuperscript{13}CH longitudinal two spin order decay rates for a \textsuperscript{13}CH and a \textsuperscript{13}CHH' group are given in Fig. 4.10(c).

\textbf{4.5.1 AN OVERVIEW OF THE EXPERIMENT}

In this section the fate of magnetisation following the desired coherence transfer pathway is described using the product operator formalism.

In this experiment proton coherence is excited and transferred to \textsuperscript{13}CH DQC and \textsuperscript{13}CH double quantum chemical shift evolution is encoded during the evolution period (see transformation 4.25). \textsuperscript{13}CH DQC ($\varphi_\text{DQC}$) is transformed into \textsuperscript{13}CH longitudinal two spin order to observe its relaxation behavior during the mixing time (between points $\varphi_5$ and $\varphi_6$). The two gradient pulses, gp1 and gp2 during the mixing time, dephase unwanted components of magnetisation (discussed below in sub-section 4.5.4). After the mixing
time $^{13}$CH longitudinal two spin order ($\varphi_6$) is transformed into proton coherence anti-phase with respect to $^{13}$C ($\varphi_7$) and allowed to go back into in-phase proton coherence ($\varphi_8$) during the succeeding $\frac{1}{2J}$ delay. This is summarised as follows:-

$$
2I_{Hx}S_{Cx} \xrightarrow{90^\circ(I)/90^\circ(S)} 2I_{Hz}S_{Cz} \xrightarrow{\varphi_5} 2I_{Hz}S_{Cz} \xrightarrow{90^\circ} 2I_{Hx}S_{Cz} \xrightarrow{\frac{1}{2J}} I_{Hy}
$$

$I_{Hy}$ ($\varphi_8$) is stored along the z-axis (between points $\varphi_9$ and $\varphi_{10}$), while water magnetisation is dephased, and then brought back into the xy-plane ($\varphi_{11}$) for detection:-

$$
I_y \xrightarrow{90^\circ(I)} I_z \xrightarrow{\text{gp4}} I_z \xrightarrow{90^\circ(I)} I_y \xrightarrow{\text{Acq.}}
$$

4.5.2 COHERENCE TRANSFER PATHWAY SELECTION

A combination of gradient pulses and phase cycling have been used to select the desired coherence transfer pathway shown in Fig. 4.7. Gradient pulse pair gp3 and gp4 has been used to generate a gradient echo (see sub-section 2.2.6.2) which follows the desired coherence transfer pathway while leaving undesired coherence transfer pathways randomised.

Three phase cycles have been used in the pulse sequence in exactly the same way as for the proton $T_1$ experiment (see sub-section 4.4.2).
4.5.3 SUPPRESSING WATER COHERENCE

Water magnetisation is dephased by gradient pulses at two places in the pulse sequence. The first place is at the beginning of the mixing period, between points $\varphi_3$ and $\varphi_{5a}$ while the desired coherence is along the z-axis as $2I_{H_2O}S_z$ (see transformation 4.41 at point $\varphi_3$):

\[
\begin{align*}
I_{H_2O} &\rightarrow -I_{y} \rightarrow -I_{y} \rightarrow -I_{y} \rightarrow -I_{y} \rightarrow -I_{y} \rightarrow -I_{y} \rightarrow +I_{y} \rightarrow +I_{y} \rightarrow +I_{y} \rightarrow \text{dephased} \\
&\rightarrow \text{dephased} \\
&\rightarrow \text{dephased} \\
&\rightarrow \text{dephased} \\
&\rightarrow \text{dephased} \\
&\rightarrow \text{dephased} \\
\varphi_1 &\quad \varphi_2 \quad \varphi_3 \quad \varphi_4 \quad \varphi_5 \quad \varphi_{5a} \quad [4.43]
\end{align*}
\]

Any water magnetisation in the xy-plane is dephased by gp1, between points $\varphi_3$ and $\varphi_{5a}$, while the desired coherence is along the z-axis. Any water magnetisation along the z-axis at point $\varphi_3$ is unaffected by gp1. However, this and any other water magnetisation which may recover during the mixing period can be dephased by gp5 at the end of the pulse sequence:-
4.5.4 SUPPRESSING UNDESIRED COHERENCES

Undesired coherences can arise from imperfect RF pulses, scalar coupling evolution and from cross-relaxation processes. Only the main undesired coherences from these three causes, and ways of suppressing them, are considered here.

4.5.4.1 Suppressing Undesired Coherences Resulting from Imperfect RF Pulses

The following process may arise from imperfect proton RF pulses:

\[ 2I_{Hz}I_{Hz} \rightarrow 90^\circ_{x/y}(I) \rightarrow 2I_{Hz}I_{Hz} \]

The operator \( 2I_{Hz}I_{Hz} \) does not become singly anti-phase with respect to \(^{13}C\) and therefore it does not evolve into observable inphase proton coherence during the course of the rest of the experiment.

Similarly imperfect \(^{13}C\) RF pulses do not give rise to unwanted coherences which evolve into observable coherence.
4.5.4.2 Suppressing Undesired Coherences Resulting from Scalar Coupling Evolution

During the evolution period, $^{13}$CH double quantum chemical shift evolution is encoded, as in the proton $T_1$ experiment. Unwanted coherences resulting from scalar coupling evolution during the $t_i$ period of the present experiment will be identical to those already covered in sub-section 4.4.3.2.

During the mixing time, the unwanted coherences $2S_{C^z}I_{H^x}$, $2S_{C^y}I_{H^y}$, $2S_{C^y}I_{H^z}$, $2S_{C^y}I_{H^z}$, $2S_{C^x}I_{H^y}$, and $2S_{C^x}I_{H^z}$ may evolve due to $^{13}$CH or HH scalar couplings. The former four operators will be dephased by gp2 and therefore there is no need to consider them any further. The fate of the latter four operators, during the rest of the experiment is shown in Table 4.5. It can be seen from Table 4.5 that all unwanted coherences are dephased by gp5, at point $\varphi_{10}$, immediately before acquisition, except ZQC. ZQC present immediately before acquisition can be represented by the operators $4S_{C^y}I_{H^z}I_{H^y}$, $4S_{C^x}I_{H^z}I_{H^y}$, $4S_{C^x}I_{H^z}S_{C^z}$ and $4S_{C^y}I_{H^z}S_{C^z}$. All of these operators represent ZQC which is anti-phase with respect to either $^{13}$C or proton and it is not detected since only SQC is observable during acquisition.
Table 4.5 The fate of undesired coherences, present at the end of the mixing period, during the course of the rest of the $R_{CH}(2S_{Cz}I_{Hz})$ pulse sequence.

<table>
<thead>
<tr>
<th>Pulse Sequence</th>
<th>Unwanted Coherences</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of $t_m$ ($\varphi_6$)</td>
<td>$2S_{Cx}I_{Hx}$</td>
</tr>
<tr>
<td>$90^0_y(I)$ ($\varphi_7$)</td>
<td>$2S_{Cx}I_{Hz}$</td>
</tr>
<tr>
<td>$\frac{1}{2}J$ ($\varphi_8$)</td>
<td>$S_{Cy}$</td>
</tr>
<tr>
<td>$90^0_x(I)$ ($\varphi_9$)</td>
<td>$S_{Cy}$</td>
</tr>
<tr>
<td>gp5 ($\varphi_{10}$)</td>
<td>All dephase except ZQC arising from the operators $4S_{Cx}I_{Hx}S_{Cz}$ and $4S_{Cy}I_{Hx}S_{Cz}$</td>
</tr>
</tbody>
</table>

4.5.4.3 Suppressing Undesired Coherences Resulting from Cross-Relaxation

Direct cross-relaxation is suppressed by placing the evolution time before the mixing period as explained in sections 4.2.1 and 4.3.1.

The following indirect cross-relaxation processes may occur during the mixing time and distort the intensity of the desired species:

$$2S_{Cz}I_{Hz} \leftrightarrow 2S_{Cy}I_{Hz}$$  \[4.46\]
\[ 2S_{\text{C}_2}^I_{\text{H}_z} \leftrightarrow S_{\text{C}_2} \tag{4.47} \]

\[ 2S_{\text{C}_2}^I_{\text{H}_z} \leftrightarrow 2I_{\text{H}_z}^I_{\text{H}_z} \tag{4.48} \]

\[ 2S_{\text{C}_2}^I_{\text{H}_z} \leftrightarrow 8S_{\text{C}_2}^I_{\text{H}_z}I_{\text{H}_z}^I_{\text{H}_z} \tag{4.49} \]

\[ 2S_{\text{C}_2}^I_{\text{H}_z} \leftrightarrow I_{\text{H}_z}^I_{\text{H}_z} \tag{4.50} \]

The cross-relaxation process 4.46 arises due to a dipolar interaction between a \(^{13}\text{CH}\) and a \(^{13}\text{CH}'\) pair in a \(^{13}\text{CHH}'\) group. Processes 4.47 and 4.48 occur as a result of \(^{13}\text{C}\) CSA. Process 4.49 may arise as a result of dipolar interactions between a \(^{13}\text{CH}\) with two other protons close in space but since \(8S_{\text{C}_2}^I_{\text{H}_z}I_{\text{H}_z}^I_{\text{H}_z}\) involves four spins, its fast decay makes it insignificant. Process 4.50 may result from proton CSA but since that is negligible, process 4.50 can ignored. The relaxation rates for these processes are given in the relaxation matrix of Eqn. 2.40.

Process 4.46 can be separated from the peak of interest by encoding \(^{13}\text{CH}\) double quantum chemical shift evolution during the evolution period (as explained in section 4.4.1).

The cross-relaxation process given in transformation 4.47 and 4.48 can be eliminated by applying a \(180^\circ\) proton RF pulse in the middle of the mixing delay. This has the effect of inverting the sign of the operator \(2S_{\text{C}_2}^I_{\text{H}_z}\) while leaving \(S_{\text{C}_2}\) unaffected and having no net effect on \(2I_{\text{H}_z}^I_{\text{H}_z}\). Hence the effects of indirect cross-relaxation during the first half of the mixing time cancel out in the second half.

Processes 4.49 and 4.50 are not removed by the pulse sequence but they are unlikely to be significant because \(8S_{\text{C}_2}^I_{\text{H}_z}I_{\text{H}_z}^I_{\text{H}_z}\) has a fast decay rate and \(I_{\text{H}_z}\) results from proton CSA which is small enough to be considered insignificant. However, even if these two
processes do arise they can be taken into account during analysis by using an exponential multiplied by a series fit (given in Eqn. 4.2) which includes an offset to take cross-relaxation effects into account. The accuracy of this fit to account for cross-relaxation will be discussed in section 5.4.
Fig 4.7 Pulse sequence to measure $R_{CH}(2SCaH_2)$. All the RF pulses on the top staff are applied to protons, those on the second staff are applied to $^{13}C$ and the third staff represents gradient pulses. The narrow and broad vertical lines represent $90^\circ$ and $180^\circ$ pulses respectively, the grey area represents proton presaturation and the shaded area represents garp (35) broadband decoupling. The following phase cycle was used: $\phi_1 = (45)x$, $\phi_2 = (45)y$, $\phi_3 = x$, $\phi_4 = -x$, $\phi_5 = x$, $\phi_6 = -x$, $\phi_7 = x$, $\phi_8 = -x$, $\phi_9 = x$, $\phi_{10} = -x$, $\phi_{11} = x$. The following gradient pulse values, in ms, were used: $gp_1 = 0.25$, $gp_2 = 1.317$, $gp_3 = gp_4 = 0.713$, $gp_5 = 3.103$. 
4.6 $^{13}$CHH LONGITUDINAL THREE SPIN ORDER RELAXATION RATE

\[ R_{\text{CHH}}(4S_{CZ}I_{Hz}I_{Hz}) \]

The measurement of heteronuclear $^{13}$CHH longitudinal three spin order relaxation rate has not been reported in the literature. The pulse sequence which we have designed to make this measurement in $^{13}$CH$_n$ systems is shown in Fig. 4.8. The experiment excites proton coherence, encodes $^{13}$C chemical shift evolution, monitors the decay of $4S_{CZ}I_{Hz}I_{Hz}$ during the mixing time and then detects proton coherence. The evolution period is placed before the mixing time to separate out the effects of direct cross-relaxation, as discussed in section 4.2.1. Signal arising from indirect cross-relaxation of $4S_{CZ}I_{Hz}I_{Hz}$ with $2S_{CZ}I_{Hz}$ is made to cancel out by applying a $180^\circ(I)$ RF pulse in the middle of the mixing time. An example of $^{13}$CHH' longitudinal three spin order decay rate for a $^{13}$CHH' group is given in Fig. 4.10(d).

4.6.1 AN OVERVIEW OF THE EXPERIMENT

In this section the fate of magnetisation following the desired coherence transfer pathway is described using the product operator formalism.

In this experiment proton coherence is excited ($\varphi_1$), transferred to a mixture of ZQC and DQC ($\varphi_2$) and the heteronuclear chemical shift evolution is encoded (between points $\varphi_3$ and $\varphi_4$) as follows:

\[
\begin{align*}
I_{Hz} & \xrightarrow{90^\circ_x(I)} -I_{Hy} \xrightarrow{2I_{Hz}S_{CZ}} 2I_{Hz}S_{Cy} \xrightarrow{90^\circ_y(S)} 2I_{Hz}S_{Cy} \xrightarrow{t_f} 2I_{Hz}S_{Cy} \\
\varphi_1 & \quad \varphi_2 \quad \varphi_3 \quad \varphi_4
\end{align*}
\]

\[ [4.51] \]
The operator $2I_{Hx}S_{Cy}$ at $\varphi_3$ is a mixture of ZQC and DQC. The $90^\circ(I)$ RF pulse in the middle of the evolution period reverses proton chemical shift evolution while $^{13}\text{C}$ chemical shift evolution is encoded, similar to an HMQC experiment.

After the evolution period, at $\varphi_4$, the operator of interest is allowed to develop a $^{13}\text{CH}$ scalar coupling to become $^{13}\text{CH}$ coherence anti-phase with respect to a proton ($\varphi_5$). Between points $\varphi_5$ and $\varphi_6$ the two proton RF pulses have an effective angle of either $45^\circ$ or $135^\circ$ depending on the step of the phase cycle. The RF pulses $45^\circ/135^\circ(I)$ and $90^\circ(I)$ applied simultaneously to the operator $4I_{Hx}S_{Cx}I_{Hz}$ (at point $\varphi_5$) will transform it into $^{13}\text{CHH}$ longitudinal three spin order ($\varphi_6$) with an efficiency of $\sin(45^\circ/135^\circ)\cos(45^\circ/135^\circ)$. Therefore at point $\varphi_6$ only half of the signal is selected by the coherence transfer pathway. The decay of the selected operator, $4I_{Hx}S_{Cx}I_{Hz}$, is monitored during the mixing time. These three steps can be represented as:

$$
2I_{Hx}S_{Cy} \xrightarrow{\varphi_4} 4I_{Hx}S_{Cx}I_{Hz} \xrightarrow{45^\circ(I)\text{or}135^\circ/I} 4I_{Hx}S_{Cx}I_{Hz} \xrightarrow{\tau_m} 4I_{Hx}S_{Cx}I_{Hz} \xrightarrow{\varphi_5} 4I_{Hx}S_{Cx}I_{Hz} \xrightarrow{\varphi_6} 4I_{Hx}S_{Cy}I_{Hz} \xrightarrow{\varphi_7} 4I_{Hx}S_{Cy}I_{Hz}
$$

The significance of the $45^\circ$ or $135^\circ$ proton pulses is described in sub-section 4.6.4.1. The subsequent gradient pulse, gp2, at the end of the mixing time, dephases any unwanted coherences in the xy-plane.

The $90^\circ(S)$ RF pulse (between points $\varphi_7$ and $\varphi_8$) transforms the longitudinal three spin order into $^{13}\text{C}$ coherence anti-phase with respect to two protons. The operator $4I_{Hx}S_{Cx}I_{Hz}$ ($\varphi_8$) evolves into inphase $^{13}\text{C}$ coherence ($\varphi_9$) during the following $\frac{1}{2J}$ period.
The desired coherence is then stored along the z-axis ($\varphi_{10}$) to dephase any unwanted coherences by $gp_5$. These three steps are summarised as follows:

\[ 4I_{Hz}S_{Cz}I_{Hz} \xrightarrow{90^\circ_y(S)} 4I_{Hz}S_{Cx}I_{Hz} \xrightarrow{1/2I} S_{Cx} \xrightarrow{90^\circ_y(I)/90^\circ_y(S)} S_{Cz} \]

$\varphi_7$ $\varphi_8$ $\varphi_9$ $\varphi_{10}$

[4.53]

After $gp_5$ (between points $\varphi_{10}$ and $\varphi_{11}$), the desired coherence is rotated in the xy-plane and allowed to evolve into $^{13}C$ coherence anti-phase with respect to one of its protons in $^{13}CH_n$ groups (see transformation 4.10 and the text relevant text to it), as shown:

\[ S_{Cz} \xrightarrow{90^\circ_y(S)} S_{Cx} \xrightarrow{1/4I=\tau} 2S_{Cy}I_{Hz} \sin \pi J_{CH} \tau \cdot \cos^n \pi J_{CH} \tau \]

$\varphi_{10}$ $\varphi_{11}$ $\varphi_{12}$ $\varphi_{13}$

[4.54]

Half of the signal was lost at point $\varphi_6$ and another half is lost at point $\varphi_{13}$. Hence only a quarter of the signal is selected by the coherence transfer pathway.

$^{13}C$ coherence anti-phase with respect to proton ($\varphi_{13}$) is transformed into proton coherence anti-phase with respect to $^{13}C$ ($\varphi_{14}$), allowed to evolve into inphase proton coherence ($\varphi_{15}$) and detected as follows:

\[ 2S_{Cy}I_{Hz} \xrightarrow{90^\circ_y(I)/90^\circ_y(S)} 2S_{Cz}I_{Hz} \xrightarrow{1/2I} I_{Hy} \xrightarrow{Acq.} \]

$\varphi_{13}$ $\varphi_{14}$ $\varphi_{15}$

[4.55]
4.6.2 COHERENCE TRANSFER PATHWAY SELECTION

A combination of gradient pulses and phase cycling have been used to select the desired coherence transfer pathway shown in Fig. 4.8. Gradient pulse pairs gp3 and gp4 and gp6 and gp7 have been used to generate gradient echoes (see sub-section 2.2.6.2) which follow the desired coherence transfer pathway while leaving undesired coherence transfer pathways randomised.

Phase cycling has been used at four points in the pulse sequence. Phase cycle $\phi_1$ selects initial proton coherence transfers with $\Delta \rho = \pm 1$. Phase cycle $\phi_2$ selects $^{13}$C coherence transfer with $\Delta \rho = \pm 1$ immediately before the evolution period. This phase cycle ensures that only $^{13}$C coherence which has been transferred from proton coherence is selected. Phase cycling has also been used to eliminate unwanted coherence transfer pathways during the evolution period by using phase cycles $\phi_3$ and $\phi_4$.

Phase cycle $\phi_3$ changes from $y$ to $-y$ so that the two proton RF pulses between points $\varphi_5$ and $\varphi_6$ have a total effect of $135^\circ$ and $45^\circ$ pulses, respectively. This is important in a procedure to eliminate $^{13}$C coherence which is anti-phase with respect to just one proton, e.g. $2S_{C,H}I_{H}$ (possibly from $^{13}$CH groups). The unwanted coherence, $2S_{C,H}I_{H}$, has a sine dependence on the $45^\circ/135^\circ(I)$ RF pulse and its sign remains constant when the angle of the pulse changes from $45^\circ$ to $135^\circ$. However, the desired coherence, $4S_{C,H}I_{H}$, has a sine multiplied by cosine dependence and its sign changes when the pulse angle changes from $45^\circ$ to $135^\circ$. The different dependence of these two species, $2S_{C,H}$ and $4S_{C,H}$, on phase cycle $\varphi_3$ is used to select only the desired coherence by phase cycling the receiver with phase cycle $\varphi_3$. In this way the signal from $2S_{C,H}$ cancels out while the desired signal from $4S_{C,H}$ adds up.
Phase cycle $\varphi_4$ only selects coherences with $^{13}$C. Hence any unwanted proton coherences excited by the $45^\circ/135^\circ(I)$ RF pulse will be eliminated by phase cycle $\varphi_4$.

4.6.3 SUPPRESSING WATER COHERENCE

Water suppression is incorporated at two places in the pulse sequence. At both of these places the magnetisation of interest is stored along the z-axis while water magnetisation is dephased by a gradient pulse. The first place is after point $\varphi_6$. Any proton magnetisation from water which may be excited at the beginning of the experiment is dephased by $g_{pl}$:-

\[ I_{2H_2O}^{1H} \rightarrow -I_{2H_2O}^{1H} \rightarrow I_{2H_2O}^{1H} \rightarrow I_{2H_2O}^{1H} \rightarrow \text{dephased} \]

\[ \varphi_1 \rightarrow \varphi_2, \varphi_3 \rightarrow \varphi_4, \varphi_5 \rightarrow \varphi_6 \]

Gradient pulse 1 does not affect $I_{2H_2O}^{1H}$ after point $\varphi_6$. However, this remaining component of water coherence is dephased by another gradient pulse, $g_{pl}$, further along the pulse sequence between points $\varphi_{10}$ and $\varphi_{11}$:-

\[ I_{2H_2O}^{1H} \rightarrow I_{2H_2O}^{1H} \rightarrow I_{2H_2O}^{1H} \rightarrow I_{2H_2O}^{1H} \rightarrow \text{dephased} \]

\[ \varphi_6 \rightarrow \varphi_7 \rightarrow \varphi_8 \rightarrow \varphi_9 \rightarrow \varphi_{10} \rightarrow \varphi_{11} \]

190
4.6.4 SUPPRESSING UNDESIRED COHERENCES

Undesired coherences can arise from non-optimal RF pulses, scalar coupling evolution and from cross-relaxation processes. Only the main undesired coherences from these three causes, and ways of suppressing them, are considered here.

4.6.4.1 Suppressing Undesired Coherences Resulting from Non-Optimal RF Pulses

The following process rises from non-optimal proton RF pulses:

\[
\begin{align*}
4S_{cx}I_{hx}I_{hz} & \xrightarrow{45^\circ y (\text{or } 135^\circ)/90^\circ (S)} 4S_{cz}I_{hz}I_{hz}\sin(45^\circ/135^\circ)\cos(45^\circ/135^\circ) \\
& \xrightarrow{45^\circ y (\text{or } 135^\circ)/90^\circ (S)} 4S_{cx}I_{hx}I_{hz}\cos^2(45^\circ/135^\circ)
\end{align*}
\]

\[\varphi_5 \quad \varphi_6 \quad [4.58]\]

The operator \(4S_{cz}I_{hz}I_{hz}\) is selected by the desired coherence transfer pathway but the undesired operator, \(4S_{cx}I_{hx}I_{hz}\), is dephased by \(g_p1\) at the beginning of the mixing period.

4.6.4.2 Suppressing Undesired Coherences Resulting from Scalar Coupling Evolution

The unwanted coherences \(4S_{cy}I_{hy}S_{cz}\), \(4S_{cx}I_{hx}I_{hz}\) and \(8S_{cy}I_{hx}S_{cz}\) may arise as a result of \(^{13}\text{CH}\) scalar coupling evolution during the evolution time. Table 4.6 shows that all unwanted coherences are dephased by \(g_p1\) at the beginning of the mixing time.
Table 4.6 The fate of undesired coherences, present at the end of the evolution period, during the course of the rest of the $R_{\text{CHH}}(4S_{Cz}I_{Hz}I_{Hz})$ pulse sequence.

<table>
<thead>
<tr>
<th>Pulse Sequence</th>
<th>Undesired Coherences</th>
</tr>
</thead>
<tbody>
<tr>
<td>$(\varphi_3)$</td>
<td>$2S_{Cy}I_{Hx}$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
<tr>
<td>$(\tau_4)$</td>
<td>$4S_{Cy}I_{Hy}S_{Cz}$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
<tr>
<td>$\frac{1}{2J}(\varphi_3)$</td>
<td>$8S_{Cx}I_{Hy}S_{Cz}I_{Hz}$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
<tr>
<td>$45^\circ/135^\circ(I)$ and $90^\circ(S)(\varphi_6)$</td>
<td>$8S_{Cy}I_{Hy}S_{Cx}I_{Hz}$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
<tr>
<td>gp1</td>
<td>All dephased</td>
</tr>
</tbody>
</table>

4.6.4.3 Suppressing Undesired Coherences Resulting from Cross-Relaxation

Direct cross-relaxation is suppressed by placing the evolution time before the mixing period as explained in sections 4.2.1 and 4.3.1.

The following indirect cross-relaxation processes may occur during the mixing time and distort the intensity of the desired species:

$$4S_{Cz}I_{Hz}I_{Hz} \leftrightarrow I_{Hz} \quad [4.59]$$

$$4S_{Cz}I_{Hz}I_{Hz} \leftrightarrow S_{Cz} \quad [4.60]$$
The cross-relaxation processes 4.59 and 4.60 may arise due to dipolar interactions between $^{13}$CHH and one of the protons or $^{13}$C, respectively. Processes 4.61 and 4.62 may occur as a result of proton or $^{13}$C CSA, respectively. Process 4.63 may arise as a result of dipolar interactions between an active proton in the operator $4S_{Cz}I_{Hz}I_{Hz}$ with another proton. The relaxation rates for these processes are given in the relaxation matrix of Eqn. 2.40.

The cross-relaxation processes given in transformations 4.59 and 4.61 can be eliminated by applying a 180°(I) RF pulse in the middle of the mixing delay. This has the effect of inverting the sign of $I_{Hz}$ and $2S_{Cz}I_{Hz}$ while leaving $4S_{Cz}I_{Hz}I_{Hz}$ unaffected. Hence the effects of indirect cross-relaxation during the first half of the mixing time cancel out in the second half.

Process 4.62 is not removed by the pulse sequence but it is unlikely to be significant because proton CSA, which gives rise to $2I_{Hz}I_{Hz}$, can be assumed to be small enough to be ignored. However, even if this process does arise it can be taken into account during analysis by using an exponential multiplied by a series fit (given in Eqn. 4.2) which includes an offset to take in-direct cross-relaxation effects into account. This fit can also take account of the cross-relaxation processes shown in transformations 4.60 and 4.63. The accuracy of this fit to account for cross-relaxation will be discussed in section 5.4.
The unwanted coherence $S_{Cz}$ (from transformation 4.60) may be present at the end of the mixing time and it may evolve into observable coherence if it develops a coupling to a proton. Table 4.7 shows that all unwanted coherences are dephased by gp5, between points $\varphi_{10}$ and $\varphi_{11}$, except ZQC arising from $S_{Cz}$. ZQC present between points $\varphi_{11}$ and $\varphi_{15}$ does not evolve into observable SQC, as shown in Table 4.7.

Table 4.7 The fate of desired and undesired coherences, present at the end of the mixing time, during the course of the rest of the $R_{CHH}(4S_{Cz}I_{Hz}I_{Hz})$ pulse sequence.

<table>
<thead>
<tr>
<th>Pulse Sequence</th>
<th>Desired Coherence</th>
<th>Undesired Coherence</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of $t_m$ ($\varphi_{\gamma}$)</td>
<td>$4S_{Cz}I_{Hz}I_{Hz}$</td>
<td>$S_{Cz}$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
<tr>
<td>$90_\gamma^0 (S) (\varphi_8)$</td>
<td>$4S_{Cz}I_{Hz}I_{Hz}$</td>
<td>$S_{Cz}$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
<tr>
<td>$\frac{1}{2}J (\varphi_9)$</td>
<td>$S_{Cz}$</td>
<td>$4S_{Cz}I_{Hz}I_{Hz}$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
<tr>
<td>$90_\gamma^0 (I)/90_\gamma^0 (S)$</td>
<td>$S_{Cz}$</td>
<td>$4S_{Cz}I_{Hz}I_{Hz}$</td>
</tr>
<tr>
<td>($\varphi_{10}$)</td>
<td>$\downarrow$</td>
<td>(ZQC + DQC)</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>(DQC dephased)</td>
</tr>
<tr>
<td>gp5 ($\varphi_{11}$)</td>
<td>$S_{Cz}$</td>
<td>$ZQC=2S_{Cz}\frac{1}{2}(2I_{Hx}I_{Hx}+2I_{Hy}I_{Hy})$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
<tr>
<td>$90_\gamma^0 (S) (\varphi_{12})$</td>
<td>$S_{Cz}$</td>
<td>$2S_{Cz}\frac{1}{2}(2I_{Hx}I_{Hx}+2I_{Hy}I_{Hy})$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
<tr>
<td>( \frac{1}{4J} (\varphi_{13}) )</td>
<td>( 2S_{Cy} I_{Hz} )</td>
<td>( 2S_{Cx} \frac{1}{2} (2I_{Hx} I_{Hz} + 2I_{Hy} I_{Hy}) )</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>( \downarrow )</td>
<td>( \downarrow )</td>
<td>( \downarrow )</td>
</tr>
<tr>
<td>( 90^\circ_v (I) / 90^\circ_x (S) )</td>
<td>( 2S_{Cz} I_{Hz} )</td>
<td>( 2S_{Cx} \frac{1}{2} (2I_{Hx} I_{Hz} + 2I_{Hy} I_{Hy}) )</td>
</tr>
<tr>
<td>( \varphi_{14} )</td>
<td>( \downarrow )</td>
<td>( \downarrow )</td>
</tr>
<tr>
<td>( \frac{1}{2J} (\varphi_{15}) )</td>
<td>( I_{Hy} )</td>
<td>( 2S_{Cx} \frac{1}{2} (2I_{Hx} I_{Hz} + 2I_{Hy} I_{Hy}) )</td>
</tr>
<tr>
<td>Observable</td>
<td>Not observable</td>
<td></td>
</tr>
</tbody>
</table>
Fig 4.8 Pulse sequence to measure $R_{CHHH} (4S_{CJC}/H_H)$: All the RF pulses on the top staff are applied to protons, those on the second staff are applied to $^{13}\text{C}$ and the third staff represents gradient pulses. The narrow and broad vertical lines represent $90^\circ$ and $180^\circ$ pulses respectively, the grey area represents proton presaturation and the shaded areas represent garp (35) broadband decoupling. The following phase cycle was used: $\phi_1 = x, -x, \phi_2 = x, x, -x, -x, \phi_3 = y, y, y, y, \phi_4 = x, -x, -x, x, x, x, x, -x, -x, -x, \phi_{rec} = x, -x, x, x, x, -x, -x, -x, x, x, -x, -x, x$, and $\phi_{rec} = x, -x, x, x, x, -x, -x, -x, x, x, -x, -x, x$. The following gradient pulse values, in ms, were used: $gp1 = 0.7, gp2 = 2.5, gp3 = gp4 = 0.2, gp5 = 4.4 \text{ and } gp6 = gp7 = 0.3$. It was ensured that the $90^\circ y(l)$ RF pulse in the mixing time was in the middle of the delay.
4.7 HH LONGITUDINAL TWO SPIN ORDER RELAXATION RATE

$[R_{HH}(2I_{Hz}I_{Hz})]$ 

The measurement of homonuclear HH longitudinal two spin order relaxation rate has not been reported in the literature. The pulse sequence which we have designed to make this measurement in $^{13}$CH$_n$ systems is shown in Fig. 4.9. The experiment excites proton coherence, encodes $^{13}$C chemical shift evolution, monitors the decay of $2I_{Hz}I_{Hz}$ during the mixing time and then detects proton coherence. The evolution period is placed before the mixing time to separate out the effects of direct cross-relaxation, as discussed in section 4.2.1. Signal arising from in-direct cross-relaxation of $2I_{Hz}I_{Hz}$ with $2S_{Cz}I_{Hz}$ and $2I_{Hz}I_{Hz}$ with $8S_{Cz}I_{Hz}I_{Hz}$ is made to cancel out by applying a 180°$(S)$ RF pulse in the middle of the mixing time. An example of HH longitudinal two spin order decay curve for a $^{13}$CHH' group is given in Fig. 4.10(e).

4.7.1 AN OVERVIEW OF THE EXPERIMENT

In this section the fate of magnetisation following the desired coherence transfer pathway is described using the product operator formalism.

In this experiment proton coherence is excited ($\phi_1$), transferred to a mixture of $^{13}$CH ZQC and DQC ($\phi_3$) and the heteronuclear chemical shift evolution is encoded (between points $\phi_3$ and $\phi_4$) as follows:

$$I_{Hz} \xrightarrow{90^\circ_x(I)} -I_{Hy} \xrightarrow{\frac{1}{2I}} 2I_{Hz}S_{Cz} \xrightarrow{90^\circ_y(S)} 2I_{Hz}S_{Cx} \xrightarrow{t_f} 2I_{Hz}S_{Cx}$$ [4.64]

$$\phi_1 \quad \phi_2 \quad \phi_3 \quad \phi_4$$
After the evolution period, the operator of interest is allowed to develop a $^{13}\text{CH}$ scalar coupling to become $^{13}\text{CH}$ coherence anti-phase with respect to proton ($\varphi_3$). A $90^\circ$ RF pulse applied at the $^{13}\text{C}$ frequency to the operator $4I_{\text{Hx}}S_{\text{Cz}}I_{\text{Hz}}$ (at point $\varphi_5$) will transform it into proton coherence doubly anti-phase with respect to $^{13}\text{C}$ and proton (at point $\varphi_6$). This operator, $4I_{\text{Hx}}S_{\text{Cz}}I_{\text{Hz}}$, evolves into $2I_{\text{Hy}}I_{\text{Hz}}$ as a result of $^{13}\text{CH}$ scalar coupling evolution. These three steps can be represented as:–

$$2I_{\text{Hx}}S_{\text{Cz}}I_{\text{Hz}} \xrightarrow{\frac{1}{2J}} 4I_{\text{Hx}}S_{\text{Cy}}I_{\text{Hz}} \xrightarrow{90^\circ(S)_{\text{Hy}}I_{\text{Hz}}} 4I_{\text{Hx}}S_{\text{Cz}}I_{\text{Hz}} \xrightarrow{\frac{1}{2I}} 2I_{\text{Hy}}I_{\text{Hz}} \quad [4.65]$$

$\varphi_4 \quad \varphi_5 \quad \varphi_6 \quad \varphi_7$

A $45^\circ(I)$ RF pulse will transform the operator $2I_{\text{Hy}}I_{\text{Hz}}$ into $2I_{\text{Hz}}I_{\text{Hz}}$ with an efficiency proportional to $\cos45^\circ\sin45^\circ$. Hence only half of the signal is selected at point $\varphi_8$ while the other half is lost. The subsequent gradient pulse, $gp7$, dephases any unwanted coherences in the xy-plane. The following $90^\circ(S)$ RF pulse and $gp8$ between points $\varphi_9$ and $\varphi_{11}$ dephase any modes with $^{13}\text{C}$ coherences arising from scalar coupling evolution during the evolution period (see sub-section 4.7.4.2). Between points $\varphi_{11}$ and $\varphi_{12}$ the decay of HH longitudinal two spin order is monitored during the mixing period. These three steps are summarised as follows:–

$$2I_{\text{Hy}}I_{\text{Hz}} \xrightarrow{45^\circ(I)} I_{\text{Hz}}I_{\text{Hz}}\cos45^\circ\sin45^\circ \xrightarrow{gp7/90^\circ(S)/gp8} 2I_{\text{Hz}}I_{\text{Hz}}\cos45^\circ\sin45^\circ$$

$\varphi_7 \quad \varphi_8 \quad \varphi_{11}$

$$\downarrow t_m$$

$$2I_{\text{Hz}}I_{\text{Hz}}\cos45^\circ\sin45^\circ \quad \varphi_{12}$$

$[4.66]$
The structure of the mixing period for this experiment is different from that of the other four pulse sequences (discussed in section 4.3 to 4.6) and the relevance of this will be explained in sub-section 4.7.4.

After the mixing time, the operator $2I_H^2I_H$, is transformed into observable proton magnetisation in a sequence of events in reverse order to the preparation of $2I_H^2I_H$, as shown:

\[
2I_{Hz}^2I_{Hz} \sin 45^\circ \cos 45^\circ \xrightarrow{45^\circ(1)} 2I_{Hy}I_{Hz} \sin^2 45^\circ \cos 2 \times 45^\circ \xrightarrow{1/2} 4I_{Hy}S_{Cz}I_{Hz}
\]

\[
\varphi_{12}, \varphi_{15} \quad \varphi_{16} \quad \varphi_{17}
\]

\[
\downarrow 90^\circ(S)
\]

\[
2I_{Hz}S_{Cz} \xleftarrow{90^\circ(1)} 2I_{Hz}S_{Cz} \xleftarrow{90^\circ(1)/90^\circ(1)} 2I_{Hz}S_{Cx} \xleftarrow{1/2J} 4I_{Hz}S_{Cy}I_{Hz}
\]

\[
\varphi_{22} \quad \varphi_{20}, \varphi_{21} \quad \varphi_{19} \quad \varphi_{18}
\]

\[
\downarrow \frac{1}{2J}
\]

\[
I_{Hy} \xrightarrow{\text{Acq.}} \varphi_{23}
\]

The total efficiency of the two $45^\circ(1)$ RF pulses (between points $\varphi_7$ and $\varphi_8$ and $\varphi_{15}$ and $\varphi_{16}$) is proportional to $\sin^2 45^\circ \cos^2 45^\circ$ hence only 25% of the signal is selected by the desired coherence transfer pathway. The $\sin^2 45^\circ \cos^2 45^\circ$ term has been omitted after point $\varphi_{16}$ for simplicity.
In transformation $4.67 \text{ } 2I_{\text{Hz}}^H (\phi_{16})$ is allowed to evolve into proton coherence doubly anti-phase with respect to $^{13}\text{C}$ and proton ($\phi_{17}$) as a result of $^{13}\text{CH}$ scalar couplings. A $90^\circ(S)$ RF pulse transforms this into $^{13}\text{CH}$ coherence anti-phase with respect to proton ($\phi_{18}$). The subsequent $\frac{1}{2J}$ period allows $4I_{\text{Hz}}^H S_{\text{Cy}}^H (\phi_{18})$ to evolve due to $^{13}\text{CH}$ scalar coupling into $2I_{\text{Hz}}^H S_{\text{Cx}}^H (\phi_{19})$. This operator at point $\phi_{19}$ is stored along the z-axis while any water coherence in the xy-plane is dephased by gradient pulse gp11 between points $\phi_{20}$ to $\phi_{21}$. The operator $2I_{\text{Hz}}^H S_{\text{Cz}}^H (\phi_{21})$ is transformed into proton coherence anti-phase with respect to $^{13}\text{C}$ ($\phi_{22}$) by a $90^\circ(I)$ RF pulse. Finally the operator $2I_{\text{Hz}}^H S_{\text{Cz}}^H (\phi_{22})$ evolves into in-phase proton coherence ($\phi_{23}$) as a result of $^{13}\text{CH}$ scalar couplings in the subsequent $\frac{1}{2J}$ delay, before being detected.

### 4.7.2 COHERENCE TRANSFER PATHWAY SELECTION

A combination of gradient pulses and phase cycling have been used to select the desired coherence transfer pathway shown in Fig. 4.9. Gradient pulse pairs gp1 and gp2, gp3 and gp4 and gp5 and gp6 have been used to generate gradient echoes (see sub-section 2.2.6.2) which follow the desired coherence transfer pathway while leaving undesired coherence transfer pathways randomised.

Phase cycling has been used at four points in the pulse sequence. Phase cycle $\phi_1$ selects initial proton coherence transfers with $\Delta \rho = \pm 1$. Phase cycle $\phi_2$ selects $^{13}\text{C}$ coherence transfer with $\Delta \rho = \pm 1$ immediately before the evolution period. This phase cycle ensures that only $^{13}\text{C}$ coherence which has been transferred from proton coherence is selected. Phase cycling has also been used to reverse unwanted coherence transfer pathways during the evolution period by using phase cycle $\phi_3$ (sub-section 4.7.4).
Phase cycle $\phi_4$ changes the phase of the pulse from $y$ to $-y$ so that the two $90^\circ$ RF pulses between points $\varphi_{13}$ and $\varphi_{14}$ have a total effect of $180^\circ$ and $0^\circ$ pulses, respectively. The is important in a procedure to eliminate unwanted coherences (sub-section 4.7.4).

### 4.7.3 Suppressing Water Coherence

For this homonuclear proton longitudinal two spin order experiment, water suppression is relatively less important than the first three experiments. This is because the operator of interest, $2I_{\text{H}_2}I_{\text{H}_2}$, is generated from $^{13}\text{CH}_2$ groups which have resonances sufficiently up-field of the water resonance to be less susceptible to distortion by the water signal in the two-dimensional spectrum. Due to this reason and the lack of opportunity in this experiment, water suppression has only been incorporated at one place in the pulse sequence. This is near the end of the experiment, prior to acquisition. The magnetisation of interest is stored along the $z$-axis while water magnetisation is dephased by gradient pulse $\text{gp11}$, as follows:

\[ I^{{\text{H}_2}^0}_{\text{Hz}} \xrightarrow{45^\circ(I)} I^{{\text{H}_2}^0}_{\text{Hz}} \xrightarrow{45^\circ(I)} 90^\circ(I)/90^\circ(I) \xrightarrow{\text{gp11}} \text{dephased} \]

\[ I^{{\text{H}_2}^0}_{\text{Hz}} \xrightarrow{45^\circ(I)} I^{{\text{H}_2}^0}_{\text{Hz}} \xrightarrow{45^\circ(I)} 90^\circ(I)/90^\circ(I) \xrightarrow{\text{gp11}} \text{dephased} \]

\[ \varphi_{15} \quad \varphi_{16} = \varphi_{19} \quad \varphi_{20} \quad \varphi_{21} \]

[4.65]
4.7.4 SUPPRESSING UNDESIRIED COHERENCES

Undesired coherences may arise from non-optimal RF pulses, scalar coupling evolution and from cross-relaxation processes. Only the main undesired coherences from these three causes, and ways of suppressing them, are considered here.

4.7.4.1 Suppressing Undesired Coherences Resulting from Non-Optimal RF Pulses

The undesired transformations which may arise from the two 45° proton RF pulses are shown in Table 4.8. Both RF pulses may create a mixture of ZQC and DQC. It can be seen from Table 4.8 that the DQC component resulting from the first 45° pulse is dephased immediately after the pulse is applied by gp7 (between points \( \varphi_8 \) and \( \varphi_9 \)) and the ZQC component is dephased later by gp11 (between points \( \varphi_{20} \) and \( \varphi_{21} \)). The DQC component created by the second 45° pulse (at point \( \varphi_{16} \)) is also dephased by gp11 and the ZQC component (at point \( \varphi_{16} \)) evolves into \(^{13}\text{C}\) coherence which is antiphase with respect to two protons (see point \( \varphi_{23} \)). This coherence is decoupled by \(^{13}\text{C}\) decoupling during acquisition and therefore it is not observed.

Zero quantum coherence created from the first 45° pulse (at points \( \varphi_8 \)) may evolve due to chemical shift in the mixing time (between points \( \varphi_{11} \) and \( \varphi_{12} \)) as shown at point \( \varphi_{12} \) in Table 4.8. One component of this is subsequently converted into antiphase coherence and dephased by gp11 (at point \( \varphi_{21} \)) while the other component may evolve into observable magnetisation. Hence a component of the ZQC operator, \( \frac{1}{2}(2I_{Hx}I_{Hx} + 2I_{Hy}I_{Hy}) \), created during the mixing time, may become observable as the operator \( I_{Hy} \). This is unlikely to be a problem because the operator \( \frac{1}{2}(2I_{Hx}I_{Hx} + 2I_{Hy}I_{Hy}) \) is
Table 4.8 Typical effects of the two 45°(l) RF pulses on desired and undesired coherences, and their fate during the course of the rest of the pulse sequence.

<table>
<thead>
<tr>
<th>Pulse Sequence</th>
<th>Desired Coherence</th>
<th>Undesired Coherence</th>
</tr>
</thead>
<tbody>
<tr>
<td>((\varphi_g))</td>
<td>(2I_{Hy}I_{Hz})</td>
<td>(2I_{Hy}I_{Hz}) (\text{sin}^245^\circ) (ZQC + DQC)</td>
</tr>
<tr>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td>(45^0 (l) (\varphi_8))</td>
<td>(2I_{Hz}I_{Hz}) (\text{cos}45^\circ) (\text{sin}45^\circ)</td>
<td>(2I_{Hy}I_{Hz}) (\text{sin}^245^\circ) (ZQC + DQC)</td>
</tr>
<tr>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td>(\text{gp7 (}\varphi_9)- (\varphi_{11}))</td>
<td>(2I_{Hz}I_{Hz}) (\text{cos}45^\circ) (\text{sin}45^\circ)</td>
<td>(DQC dephased)</td>
</tr>
<tr>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td>(\text{End of } t_m) (\text{(}\varphi_{12})- (\varphi_{15}))</td>
<td>(2I_{Hz}I_{Hz}) (\text{cos}45^\circ) (\text{sin}45^\circ)</td>
<td>(\frac{1}{2} (2I_{Hy}I_{Hz} - 2I_{Hz}I_{Hy}) \text{sin}^245^\circ) (no chemical shift evolution)</td>
</tr>
<tr>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td></td>
<td>(\frac{1}{2} (2I_{Hz}I_{Hz} + 2I_{Hz}I_{Hy}) \text{sin}^245^\circ) (chemical shift evolution)</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td>(45^\circ (I))</td>
<td>(2I_{Hy}I_{Hz})</td>
<td>(2I_{Hy}I_{Hy}\cos45^\circ \sin^345^\circ)</td>
</tr>
<tr>
<td>((\varphi_{16}))</td>
<td>(\cos^245^\circ \sin^245^\circ)</td>
<td>(\cos^245^\circ \sin^245^\circ)</td>
</tr>
<tr>
<td>(\frac{1}{2J} (\varphi_{17}))</td>
<td>(4I_{Hz}S_{Cz}I_{Hz})</td>
<td>(2I_{Hz}I_{Hz}\cos45^\circ \sin^345^\circ)</td>
</tr>
<tr>
<td>(\cos^245^\circ \sin^245^\circ)</td>
<td>(\cos^245^\circ \sin^245^\circ)</td>
<td>(\cos^245^\circ \sin^245^\circ)</td>
</tr>
<tr>
<td>(90^\circ (S))</td>
<td>(4I_{Hz}S_{Cy}I_{Hz})</td>
<td>(2I_{Hz}I_{Hz}\cos45^\circ \sin^345^\circ)</td>
</tr>
<tr>
<td>((\varphi_{18}))</td>
<td>(\cos^245^\circ \sin^245^\circ)</td>
<td>(\cos^245^\circ \sin^245^\circ)</td>
</tr>
<tr>
<td>(\frac{1}{2J} (\varphi_{19}))</td>
<td>(4I_{Hz}S_{Cz})</td>
<td>(2I_{Hz}I_{Hz}\cos45^\circ \sin^345^\circ)</td>
</tr>
<tr>
<td>(\cos^245^\circ \sin^245^\circ)</td>
<td>(\cos^245^\circ \sin^245^\circ)</td>
<td>(\cos^245^\circ \sin^245^\circ)</td>
</tr>
<tr>
<td>(90^\circ (I)/ 90^\circ (S))</td>
<td>(2I_{Hz}S_{Cz})</td>
<td>(2I_{Hz}I_{Hz}\cos45^\circ \sin^345^\circ)</td>
</tr>
<tr>
<td>((\varphi_{20}))</td>
<td>(\cos^245^\circ \sin^245^\circ)</td>
<td>(\cos^245^\circ \sin^245^\circ)</td>
</tr>
<tr>
<td>gp11 ($\varphi_{21}$)</td>
<td>$2I_{Hz}S_{Cz}$</td>
<td>(DQC dephased)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>$\cos^2 45^\circ \sin^2 45^\circ$</td>
<td>$ZQC = \frac{1}{2} (2I_{Hz}I_{Hz} - 2I_{Hz}I_{Hy})$</td>
</tr>
</tbody>
</table>

| $90^\circ_y (l) (\varphi_{22})$ | $2I_{Hz}S_{Cz}$ | $\frac{1}{2} (2I_{Hz}I_{Hz} - 2I_{Hz}I_{Hy})$ | $\cos 45^\circ \sin 3 45^\circ$ | \(\downarrow\) | $2I_{Hz}S_{Cz}$ |
|  | $\cos^2 45^\circ \sin^2 45^\circ$ | | $\cos 45^\circ \sin 3 45^\circ$ | \(\downarrow\) | $\cos 45^\circ \sin 3 45^\circ$ |

| $\frac{1}{2} I (\varphi_{23})$ | $I_{Hy} \cos^2 45^\circ \sin^2 45^\circ$ (Observed) | $\frac{1}{2} (2I_{Hz}I_{Hz} - 2I_{Hz}I_{Hy})2S_{Cz}$ | $\cos 45^\circ \sin^3 45^\circ$ (Decoupled) | \(\downarrow\) | $I_{Hy} \cos 45^\circ \sin^3 45^\circ$ (Observed) |
likely to have a short $T_2$ relaxation time and therefore it will dephase rapidly during the mixing time, hence making an insignificant contribution to the observed signal.

4.7.4.2 Suppressing Undesired Coherences Resulting from Scalar Coupling Evolution

The undesired transformations which may arise from $^{13}$CH scalar coupling evolution during the evolution period are shown in Table 4.9. All of these coherences are dephased by gp7 between points $\phi_8$ and $\phi_9$.

Table 4.9 The fate of undesired coherences, present at the end of the evolution time, during the course of the rest of the $R_{HH}^{(2J_{Hz}I_{He})}$ pulse sequence.

<table>
<thead>
<tr>
<th>Pulse Sequence</th>
<th>Undesired Coherences</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of $t_1$ ($\phi_4$)</td>
<td>$4S_{Cz}I_{Hy}S_{Cz}$</td>
</tr>
<tr>
<td>$\frac{1}{2J}$ ($\phi_5$)</td>
<td>$8S_{Cy}I_{Hy}S_{Cz}I_{Hz}$</td>
</tr>
<tr>
<td>$90^0$ ($\frac{\pi}{2}$) ($\phi_6$)</td>
<td>$8S_{Cz}I_{Hy}S_{Cy}I_{Hz}$</td>
</tr>
<tr>
<td>$\frac{1}{2J}$ ($\phi_7$)</td>
<td>$16S_{Cz}I_{Hz}S_{Cy}I_{Hz}S_{Cz}$</td>
</tr>
<tr>
<td>$45^0$ ($\frac{\pi}{4}$) ($\phi_8$)</td>
<td>$16S_{Cz}I_{Hz}S_{Cy}I_{Hz}S_{Cz}$ and $16S_{Cz}I_{Hz}S_{Cy}I_{Hz}S_{Cz}$</td>
</tr>
<tr>
<td>gp7 ($\phi_8$)</td>
<td>All dephased</td>
</tr>
</tbody>
</table>
4.7.4.3 Suppressing Undesired Coherences Resulting from Cross-Relaxation

Direct cross-relaxation is suppressed by placing the evolution time before the mixing period as explained in sections 4.2.1 and 4.3.1.

The following indirect cross-relaxation processes may occur during the mixing:

\[ 2I_{Hz}^I_{Hz} \leftrightarrow 2I_{Hz}^S_{Cz} \] \[4.66]\n
\[ 2I_{Hz}^I_{Hz} \leftrightarrow 2I_{Hz}^I_{Hz}^I_{Hz} \] \[4.67]\n
\[ 2I_{Hz}^I_{Hz} \leftrightarrow 8I_{Hz}^I_{Hz}^S_{Cz}I_{Hz} \] \[4.68]\n
\[ 2I_{Hz}^I_{Hz} \leftrightarrow 4I_{Hz}^I_{Hz}^I_{Hz}^S_{Cz} \] \[4.69]\n
\[ 2I_{Hz}^I_{Hz} \leftrightarrow I_{Hz} \] \[4.70]\n
The cross-relaxation processes 4.66 and 4.67 may arise due to dipolar interactions between an active proton and a non-active $^{13}$C or a non-active proton respectively. The process shown in transformation 4.68 may arise as a result of dipolar interactions between one of the active protons with a non-active proton and a non-active $^{13}$C. Processes 4.69 and 4.70 may occur as a result of proton CSA, which is assumed to be too small to be significant. The relaxation rates for these processes are given in the relaxation matrix of Eqn. 2.40.

The cross-relaxation processes given in transformations 4.66 and 4.68 can be eliminated by applying a $180^\circ (S)$ RF pulse in the middle of the mixing delay. This has the effect of inverting the sign of $2I_{Hz}^S_{Cz}$ and $4I_{Hz}^I_{Hz}^S_{Cz}$ while leaving $2I_{Hz}^I_{Hz}$ unaffected.
Hence the effects of indirect cross-relaxation during the first half of the mixing time cancel out in the second half.

Processes 4.67 and 4.69 are not removed by the pulse sequence. The latter is likely to be insignificant because proton CSA, which given rise to I\textsubscript{H\textsubscript{2}}, can be assumed to be small enough to be ignored. However, even if this process does arise it can be taken into account during analysis by using an exponential multiplied by a series fit (given in Eqn. 4.2) which includes an offset to take in-direct cross-relaxation effects into account. This fit can also take account of the cross-relaxation processes shown in transformation 4.67. The accuracy of this fit to account for cross-relaxation will be discussed in section 5.4.
Fig 4.9 Pulse sequence to measure $R_{HH}(2J_{Hz}/Hz)$. All the RF pulses on the top staff are applied to protons, those on the second staff are applied to $^{13}$C and the third staff represents gradient pulses. The narrow and broad vertical lines represent $90^\circ$ and $180^\circ$ pulses respectively, the grey areas represent proton presaturation and the shaded area represents garp (35) broadband decoupling. The following phase cycle was used: $\phi_1 = x, x, -x, \phi_2 = y, y, y, -y, -y, -y, \phi_3 = y, y, y, y, -y, -y, -y, -y$.

The following gradient pulse values, in ms, were used: $g_{p1} = g_{p2} = 0.13$, $g_{p3} = g_{p4} = 0.3$, $g_{p5} = g_{p6} = 0.2$, $g_{p7} = 0.7$, $g_{p8} = 0.45$, $g_{p9} = 1.9$, $g_{p10} = 4.4$ and $g_{p11} = 2.5$. 
4.8 AN EXPERIMENTAL DEMONSTRATION OF THE RELAXATION EXPERIMENTS DESIGNED AND DEVELOPED FOR THIS STUDY

This section gives a practical demonstration of the relaxation experiments (given in sections 4.3 to 4.7) designed and developed to implement the analysis method developed in section 3.5.

The relaxation decay curves for the $R_C(S_Cz)$, $R_H(I_Hz)$, $R_{CH}(2S_CzI_Hz)$, $R_{CHH}(4S_CzI_HzI_Hz)$ and $R_{HH}(2I_HzI_Hz)$ experiments for a $^{13}_C$H (T30$^\alpha$) and a $^{13}_C$H$_2$ (P63$^\beta$) group of Protein G (see section 5.2), which are well resolved and sufficiently far enough from the water resonance not to be distorted by it, are shown in Fig 4.10.

(a)

(b)
Fig. 4.10 Example plots of relaxation decay data against time for (a) $^{13}$C longitudinal magnetisation, $R_C(S_Cz)$, (b) proton longitudinal magnetisation, $R_H(I_Hz)$, (c) $^{13}$CH longitudinal two spin order, $R_{CH}(2S_Cz/I_Hz)$, (d) $^{13}$CHH longitudinal three spin order, $R_{CHH}(2S_Cz/I_Hz/I_Hz)$ and (e) HH longitudinal two
spin order, \( R_{HH}(2I_Hz/I_{H_H}) \) for domain II of Protein G at a concentration of 4 mM and a pH of 6.3 acquired on a Bruker AMX600 spectrometer operating at 600.13 MHz for proton, at 298 K. In all five graphs 0 and □ represent the appropriate measurements for \( T_{30}^{O} \) (\(^{13}\text{CH} \) group) and \( P_{63}^{B} \) (\(^{13}\text{CH}' \) group), respectively. The ■ in graphs (b) and (c) represent the decay rates of the \( H' \) longitudinal magnetisation and the \(^{13}\text{CH}' \) longitudinal two spin order of \( P_{63}^{B} \). The x and △ in graph (e) represent the decay of homonuclear longitudinal two spin order for \( P_{3}^{B} \) and \( K_{9}^{B} \), respectively. The pulse sequences and the gradient values used are given in Figs. 4.4 to 4.9 (excluding Fig. 4.6) and in Appendix II. A total relaxation delay of 3 s was used for each experiment including 1.5 s of proton presaturation at a power of 65 dB; the \( J_{CH} \) was taken to be 120 Hz; \( t_{l} \) increment of 22 \( \mu \)s for (a), 19 \( \mu \)s for (b) and (c) and 30 \( \mu \)s for (d) and (e); 1024 points were acquired in the directly observed dimension and 256 points in the indirect dimension for (a) to (e) and 208 for (d) and (e); 8 transients were acquired for experiments (a) to (c) and 32 for (d) and (e). The mixing times used are as follows:- (a) 5, 50, 100, 160, 250, 350, 475, 600 ms, (b) 5, 40, 70, 120, 180, 260, 340, 440, 550, 700 ms, (c) 5, 20, 45, 75, 115, 170, 235, 315, 415, 525 ms, (d) 5, 40, 100, 150, 210, 290, 400, 500 ms and (e) 5, 20, 40, 65, 95, 140, 190, 250 ms. The acquired data was analysed using off-line processing on FELIX (BIOSYM/Molecular Simulations). The data was zero filled to 2048 points in \( f_2 \) and 1024 points in \( f_1 \). An optimised Gaussian function was applied prior to Fourier transformation. A baseline correction routine was used in both dimensions to improve the quality of data by averaging out any baseline imperfections. Peak intensities in the two-dimensional spectra were measured using peak picking on FELIX. All the data, except that for the homonuclear longitudinal two spin order, has been fitted to an exponential model (Eqn. 4.1). The relaxation decay rates calculated are as follows:- (a) \(^{13}\text{C} \) longitudinal magnetisation; \( T_{30}^{O} = 2.47 \pm 0.06 \text{ s}^{-1} \) and \( P_{63}^{B} = 3.36 \pm 0.08 \text{ s}^{-1} \), (b) proton longitudinal magnetisation, \( T_{30}^{O} = 3.17 \pm 0.04 \), \( P_{63}^{B}(□) = 3.64 \pm 0.04 \) and \( P_{63}^{B}(■) = 3.94 \pm 0.05 \), (c) \(^{13}\text{CH} \) longitudinal two spin order, \( T_{30}^{O} = 5.47 \pm 0.09 \), \( P_{63}^{B}(□) = 6.62 \pm 0.09 \) and \( P_{63}^{B}(■) = 8.60 \pm 0.08 \), (d) \(^{13}\text{CHHH} \) longitudinal three spin order, \( P_{63}^{B} = 7.16 \pm 0.09 \).

It can be seen from Fig. 4.10 that the data for the \( R_{H}(I_{HZ}) \), \( R_{CH}(2S_{Cz}I_{HZ}) \) and \( R_{CHH}(4S_{Cz}I_{Hz}I_{H_H}) \) experiments decays exponentially. The decay curves for the \( R_{C}(S_{Cz}) \) experiment are comparatively not as good but the errors of 2% for both the \(^{13}\text{CH} \) and \(^{13}\text{CH}_2 \) examples are acceptable. The exponential and another fitting model are discussed in Chapter Five.
The decay curves for the $R_{HH}(2I_H^2I_H^2)$ experiment give the worst fits. Looking at Fig 4.10(e), it can be seen that the points at 5 ms and 250 ms are the most erroneous. These points are consistently out for all the cuves shown in Fig. 4.10(e) implying that an unwanted coherence transfer pathway may have be selected or there may be a possible hardware problem. This needs to be investigated further before making any more comments.

4.9 SUMMARY

This chapter has given a detailed discussion of the general considerations which need to be made in order to make the necessary measurements to implement the new method of analysis of $^{13}$C relaxation data of a protein. A detailed explanation of cross-relaxation has been followed by a discussion on the use of gradient pulses to select the coherence transfer pathway and suppress magnetisation arising from water. In section 4.3 to 4.7, the desired coherence transfer pathway for each experiment has been described in detail using the product operator formalism. How the desired coherence transfer pathway is chosen and how water and unwanted coherence transfer pathways are suppressed in each experiment is also given in the appropriate section. Finally, an example of the relaxation decay curves for a $^{13}$CH and a $^{13}$CHH' group for each measurement are given in Fig. 4.10. All the pulse sequences which were designed and developed on the Bruker AMX600 and used on the Bruker DRX400 spectrometers are given in Appendix II.
4.10 REFERENCES


CHAPTER FIVE

RESULTS AND DISCUSSION

5.1 INTRODUCTION

The theory of method for analysing $^{13}\text{C}$ relaxation data was developed in section 3.6. In order to implement this method on proteins it is necessary to make up to five relaxation measurements and each of these have been discussed in detail in Chapter Four. We now move on to the practical aspect of this work. Aspects such as the identity of and the reasons for choosing the biomolecule which was used to develop the pulse sequences. Also which fit, of the two that were discussed in Chapter Four, fits the data better. Additionally, the limited experimental data is presented followed by an account of the unfortunate problems which were the reason for the shortage of results. Finally, the limited data is analysed to gain information on the molecular dynamics of the chosen molecule.

5.2 PROTEIN G

The method of analysis described in section 3.6 can be implemented on proteins by making up to five independent relaxation measurements. In order to make these measurements it was necessary to modify existing experiments ($^{13}\text{C}$ and proton $T_1$'s) and to design new ones (heteronuclear $^{13}\text{CH}$ and homonuclear HH longitudinal two spin order and $^{13}\text{CHH}$ longitudinal three spin order). All five experiments needed to be developed for use with uniformly $^{13}\text{C}$ labelled molecules. These experiments, described in Chapter Four, were developed using a domain of Protein G as a model.
This section gives a brief overview of Protein G: where it is found in nature; how it has been expressed, labelled and purified; a general description of its secondary structure from x-ray crystallography and NMR studies; and why it was used for developmental purposes in this work.

Protein G is found in the cell walls of some species of pathogenic bacteria (1), e.g. *Streptococci*. It has a high affinity for immunoglobulin G (IgG) (2). The whole molecule consists of approximately 600 amino acid residues. It has three IgG-binding domains near the carboxy-terminal half. These three domains are highly similar to each other (3, 4) and they are referred to as domains I, II and III. Each of these domains have approximately 70 residues each. They have been expressed in *E. coli* and isolated to show IgG-binding capability is retained (3, 5). The individual domains, unlike the whole protein, are a convenient size for NMR studies. The proton NMR structure of domains II and III (6, 7), the crystal structure of domain III (8) and the model, by $^{13}$C and $^{15}$N NMR, for the complex of domain II with the Fc (9) and the FAb (10, 11) fragment have been determined. The backbone dynamics of domain II of Protein G have been determined by $^{15}$N NMR relaxation measurements (12).

Domain II of Protein G consists of 64 residues in the following order:-

LTPAV$^5$TTTKL$^{10}$VINGK$^{15}$TLKGE$^{20}$TTEA$^{25}$VDAAT$^{30}$AEKVF$^{35}$KQYAN$^{40}$

DNVGD$^{45}$GEWTY$^{50}$DDATK$^{55}$TFTVT$^{60}$EKPE$^{64}$

This chain of amino acids is folded into two antiparallel $\beta$-sheets (involving residues 5 to 26 and 45 to 62) separated by an $\alpha$-helix (involving residues 28 to 44) as shown in Fig. 5.1.
Fig. 5.1 A two-dimensional, (a), and a three-dimensional, (b), representation of the secondary structure of domain II of Protein G. Residues L1 to A4 form the N-terminus, residues V5 to V26 form an antiparallel β-sheet with K15 and T16 at the turn, residues A28 to V44 form an α-helix, residues D45 to K62 form the second antiparallel β-sheet with A53 and T54 at the turn and residues P63 and E64 form the C-terminus.
Domain II of Protein G was used as a model for pulse sequence development for a number of reasons. It is of a convenient size for NMR studies. It readily dissolves in water: the concentration used was as high as 4 mM. It is thermally stable and therefore it is unlikely to deteriorate under long experimental conditions. Depending on which region of the molecule is chosen, the overall motion of Protein G can be assumed to be isotropic and therefore ideal for using the model-free approach for analysing the relaxation data.

The DNA for domain II of Protein G was cloned from plasmid pSPG29 (7, 13) and the isotropically uniformly $^{13}$C and $^{15}$N labelled material was expressed in *E. coli*. The protein was purified according to the published protocol (7, 14).

The uniformly $^{13}$C and $^{15}$N labelled NMR sample of domain II was made up of 16.4 mg of protein dissolved in 0.5 ml of water with a small amount of D$_2$O to give a lock signal. The concentration of the protein was 4 mM at a pH of 6.3.

5.3 EXPERIMENTAL

Due to the problems encountered in the late stages of this project, only two sets of measurements (the $^{13}$C $T_1$ and $^{13}$CH longitudinal two spin order) out of five were obtained successfully. In this section the acquisition and processing of the data is described and the results of these two experiments are presented.

NMR relaxation data for $^{13}$C $T_1$ and $^{13}$CH longitudinal two spin order were acquired on a Bruker DRX400 Spectrometer with a proton resonance frequency of 400.13 MHz. The pulse sequences and the gradient values given in Figs. 4.4 and 4.7 were used, respectively. The measurements were made at a temperature of 300K. For both experiments a total relaxation delay of 4.5 s was used including 2.5 s of proton presaturation at a power of 55 dB for the longitudinal two spin order experiment. $J_{CH}$ was taken to be 120 Hz giving a $1/2J$ time of 4 ms. The delay $t_1$ was set to an initial
value of 3 μs with increments of 25 μs for the $^{13}$C $T_1$ and 21 μs for the longitudinal two spin order measurement. 1024 points were acquired in the directly observed dimension, 256 in the indirect dimension and 8 transients were acquired for each experiment. The mixing times used for the $^{13}$C $T_1$ measurement were 5, 10, 15, 25, 35, 50, 70, 95, 125, 160, 200, 245, 295, 350, 410, 545, 620, 700 and 800 ms and those for the heteronuclear longitudinal two spin order were 5, 10, 20, 40, 60, 75, 95, 115, 140, 170, 200, 235, 275, 315, 365, 415, 470 and 525 ms. The first two mixing times were repeated for each experiment. All the gradient pulse lengths were 1 ms except for gp4 (3 ms), gp5 (2 ms) and gp8 (5 ms) for the $^{13}$C $T_1$ experiment. The following amplitudes were used for the gradient pulses for the $^{13}$C $T_1$ experiment:- $g_{p1}$ = $g_{p2}$ = 27, $g_{p3}$ = 78, $g_{p4}$ = 90, $g_{p5}$ = 70, $g_{p6}$ = $g_{p7}$ = 15 and $g_{p8}$ = 95%, and those for the longitudinal two spin order measurement are:- $g_{p1}$ = 7.2, $g_{p2}$ = 38.2, $g_{p3}$ = 20.7 and $g_{p5}$ = 90%, where 100% = 50 Gcm$^{-1}$.

The acquired data was analysed using off-line processing on FELIX (BIOSYM/Molecular Simulations). The data was zero filled to 2048 points in $f_2$ and 1024 points in $f_1$. An optimised Gaussian function was applied prior to Fourier transformation. A baseline correction routine was used in both dimensions to improve the quality of data by averaging out any baseline imperfections. Peak intensities in the two-dimensional spectra were measured using a combination of peak picking on FELIX and a self-written macro in Fortran 77 (Appendix III).

The relaxation decay curves for each peak were fitted to an exponential fit (Eqn. 4.1) and an exponential multiplied by the first two terms of a series fit (Eqn. 4.2) (15) to calculate the decay rates. These two fits have been discussed in sub-section 4.2.1 and their effects on the decay rates calculated will be discussed in section 5.4. The $^{13}$C $T_1$ relaxation data for the alpha- and beta-$^{13}$C is given in Figs. 5.2 and 5.3 respectively. The relaxation data for the heteronuclear $^{13}$CH longitudinal two spin order is given in Fig. 5.4. This data will be discussed in section 5.6. The remaining
Relaxation measurements have not been made successfully due to problems which were experienced during the course of this work. These are discussed in section 5.5.
Fig. 5.2 The $^{13}\text{C}^{\alpha}T_1$ data acquired on the Bruker DRX400 Spectrometer, using the pulse sequence given in Fig. 4.4. Relaxation rates for 41 out of the 64 $^{13}\text{C}^{\alpha}$ spins of domain II of Protein G were calculated. The intensities of the remaining resonances can not be measured from the two-dimensional spectrum due to either poor resolution or distortion by the water resonance. The relaxation decay rates were calculated by fitting the data to the exponential fit given in Eqn. 4.1, (a), and to the exponential fit multiplied by the first two terms of a series given in Eqn. 4.2, (b). The difference in the two relaxation rates for each $^{13}\text{C}$ spin (the decay rate calculated from the exponential fit subtracted from the rate calculated from the exponential multiplied by a series fit) are given in (c). A positive value means the rate calculated from the exponential multiplied by a series fit is larger than that calculated from the exponential fit. A negative values means the opposite. The % errors of the exponential fit ($\Delta$) and that for the exponential multiplied by a series fit ($\sigma$) are shown in (d).
Fig. 5.3 The $^{13}$C$^\beta$ $T_1$ data acquired on the Bruker DRX400 Spectrometer, using the pulse sequence given in Fig. 4.4. Only the threonine $^{13}$C$^\beta$ have been looked at here because they are well resolved in the two-dimensional spectra. The $^{13}$C$^\beta$ $T_1$'s only are shown in (a) while data for both the $^{13}$C$^\alpha$ and $^{13}$C$^\beta$ are shown in (b).
Fig. 5.4 The heteronuclear $^{13}$CH longitudinal two spin order data acquired on the Bruker DRX400 Spectrometer, using the pulse sequence given in Fig. 4.7. Only the data for $^{13}$C$^{\text{a}}$H of domain II of Protein G is shown. Relaxation rates for 33 out of the 64 $^{13}$C$^{\text{a}}$ spins of domain II of Protein G were calculated. The intensities of the remaining resonances can not be measured from the two-dimensional spectrum due to either poor resolution or distortion by the water resonance. The relaxation decay rates were calculated by fitting the data to the exponential fit given in Eqn. 4.1, (a), and to the exponential fit multiplied by the first two terms of a series given in Eqn. 4.2, (b). The difference in the two relaxation rates for each $^{13}$C spin (the decay rate calculated from the exponential fit subtracted from the rate calculated from the exponential multiplied by a series fit) are given in (c). A positive value means the rate calculated from the exponential fit is larger than that calculated from the exponential multiplied by a series fit. A negative value means the opposite.
5.4 DATA ANALYSIS

The data can be fitted to two possible models to calculate the relaxation decay rate. These two fits are compared and contrasted to each other (in terms of the decay rates calculated form them and the error of the calculated rates) to decide on which fit is more appropriate.

The relaxation decay curves for each peak were fitted to an exponential fit (Eqn. 4.1) and an exponential multiplied by the first two terms of a series fit (Eqn. 4.2) (15) to calculate the decay rates. These two fits have been discussed in sub-section 4.2.1. The exponential fit gives a relatively precise relaxation rate if the data is not distorted by cross-relaxation effects. The experiments designed as part of this project include measures to suppress cross-relaxation. However, it is unlikely that all cross-relaxation processes will be suppressed by the design of the experiment and some processes may still affect the decay rates calculated with an exponential fit. The effects of cross-relaxation can be taken into account by fitting the data to an exponential fit multiplied by the first two terms of a series, as discussed in sub-section 4.2.1. Cross-relaxation will make the apparent relaxation rate for the exponential fit smaller than for the exponential multiplied by a series fit and this difference can be used to gauge how much cross-relaxation is occurring. The $^{13}\text{C}^\alpha T_1$ data was fitted with both of these fits and it is shown in Fig. 5.2.

Graphs (a) and (b) of Fig. 5.2 show the $^{13}\text{C}^\alpha$ relaxation decay rates calculated by fitting the data to Eqns. 4.1 and 4.2 respectively. A general trend, which is apparent and similar in both graphs, can be seen to change with the secondary structure. This will be discussed later, for the time being we will discuss the two fits to determine which is likely to give more precise decay rates. Plot (c) of Fig. 5.2 shows the difference between the decay rates obtained by fitting the data to an exponential fit and that to an exponential multiplied by a series fit. The difference in the decay rates varies dramatically from relatively mobile and disordered parts of the molecule to the rigid and ordered parts. The N- and C- termini, involving residues L1 to A4 and P63
to E64 respectively, are likely to be the least rigid and most mobile parts of the protein, yet the difference between the rates obtained from the two fits is negligible. In rigid parts of the protein, like the β-sheets from residues V5 to V26 and from D45 to K62, the difference in the decay rates is relatively greater. The overall positive value for this difference across these residues shows that the exponential fit is giving a slower rate than the other fit, hence the observed decay has been distorted by cross-relaxation processes. The α-helix part of the molecule, involving residues A28 to V44, appears to be generally more flexible than the β-sheets. Therefore, according to the general trend we have observed, we would expect the difference between the two decay rates to be very small. This can be seen to be generally applicable in Fig. 5.2(c).

The heteronuclear longitudinal two spin order data given in Fig. 5.4 can be interpreted according to the above explanation. However, it is very difficult to make any valuable comments by considering the longitudinal two spin order data in isolation of the other necessary relaxation measurements.

Generally, there appears to be more cross-relaxation in the rigid parts of the protein (α-helix and β-sheets) than in the flexible parts (the two termini and connecting loops). This is because in the flexible regions, at or near the fast tumbling regime, cross-relaxation is less efficient than at the slow tumbling regime in the relatively rigid parts.

Looking at the difference between the fits calculated from the exponential and the exponential multiplied by a series models (Fig. 5.2(c)), we can deduce that, generally, the latter model is more likely to take cross-relaxation processes into account than the former model.

Since the exponential multiplied by a series fit involves more parameters than the exponential fit, it requires that more points be measured to achieve a comparable accuracy. Furthermore, the exponential multiplied by a series fit is found to be more susceptible to any erroneous points than the exponential fit. We can evaluate which fit gives the greater errors by looking at a plot of the errors of the two fits (Fig. 5.2(d)).
Generally, the errors are similar, with the series fit giving slightly larger errors than the exponential fit. In the few peaks where the errors are significantly different between the two fits, e.g. T30 and T23, the series model gives a larger error. However, the errors for T30 and T23, as well as Y8, K9 and D51, are more profound with both fits when compared to the rest of the residues. Hence the large errors for these peaks could be due to other reasons, such as poor resolution of the peaks in the two-dimensional spectrum or distortion by a large water resonance at longer mixing times, rather than just the fit used.

Generally, we can say that on the whole the exponential multiplied by a series model gives a better fit of the relaxation data with errors which are comparable to the traditionally used exponential model.

5.5 PRACTICAL PROBLEMS ENCOUNTERED

In the final stages of developing the pulse sequences discussed in Chapter Four, it was found that the exponential model given in Eqn. 4.1 was not fitting the data as well as expected. Consequently, the calculated relaxation rates had larger errors than expected which in turn resulted in even larger errors for the combinations of Eqns. 3.48 - 3.50, thereby casting doubt on the validity of our method of analysis. The imprecision in the relaxation rates resulted from indirect cross-relaxation processes during the mixing time. These processes can not all be removed by taking them into consideration in the design of the experiment as discussed in section 4.2.1. Therefore it was necessary to take them into account during analysis. This can be done by fitting the data to an exponential multiplied by the first two terms of series, given in Eqn. 4.2 (15). Since this model involves fitting the data to three parameters (Eqn. 4.2) and the susceptibility of this fit to erroneous points in the decay curve is greater, it was necessary to acquire more points on the decay curve. This involved acquiring more two-dimensional spectra at more mixing times for the exponential multiplied by a series fit than for the exponential fit. Consequently, it was necessary to use the spectrometer for longer times for each experiment. It was not possible to use the Bruker AMX600 for such long periods of time because it was already in high demand. The Bruker
DRX400 in the Chemistry Department was available for the lengthy and frequent periods of time we now required. Using this spectrometer, meant re-acquiring all the data for all five experiments. Even though this change of equipment came at such a late stage in the project, the decision was made optimistically because all the pulse sequences were in full working order.

The water suppression parameters for each experiment (e.g. the length and power of gradient pulses and presaturation pulses, where applicable) which had previously been optimised on the AMX were no longer applicable on the DRX. Even after re-calibrating these parameters, water suppression on the DRX was not as effective as it had been on the AMX. Although reluctant to sacrifice some of the $^{13}C\alpha$ resonances which were distorted by the water resonance, we continued on the DRX because of its availability of use.

On the DRX, the $^{13}C$ and proton $T_1$'s and the heteronuclear $^{13}CH$ longitudinal two spin order measurements were made successfully on a sample of uniformly doubly-labelled domain II of Protein G (sample 1). Before the remaining measurements could be made, the sample was accidentally destroyed by another user. The direct implication of this action was to make a new sample, re-acquire the three measurements already made on the DRX and continue making the remaining two measurements.

Sample 2 of domain II was made to the same concentration and pH (4 mM and 6.3 respectively) and it was used to measure the $^{13}C\ T_1$ and the heteronuclear two spin order successfully. Despite the concentration and the pH being the same the rates for these two measurements were found to be different from those of sample 1. The proton $T_1$ decay curves for each resonance gave slightly higher errors than expected. On repeating the experiment after a week the errors were even higher.

The errors of the proton $T_1$'s and other irregularities were partly explained when it was found that sample 2 was contaminated with a small amount of a protease
(a protein digesting enzyme). The protease was degrading the protein into small polypeptides. This is shown in the one-dimensional spectra in Fig. 5.5. After only two months of using sample 2, 70% of the protein was broken down. Therefore, this sample could no longer be used for our purpose.

Fig. 5.5 One-dimensional spectra of domain II of Protein G (sample 2). (b) is the fresh sample and (a) was taken two months later. The broad peak evident in (b) are characteristic of large molecules with short T₂ values. The relatively narrower peaks in (a) indicate that the protein has undergone a change. On further investigation it was found that the change resulted from the protein being broken down into a mixture of small polypeptides and amino acids by a protease. These smaller molecules have relatively longer T₂ values and therefore their resonances are narrower in the one-dimensional spectrum (a).
Sample 3 was made from the same batch of protein as sample 2 but it was stored in the freezer in-between uses to slow down the activity of the contaminating enzyme. The continued use of sample 3 gave decay curves for proton $T_1$'s which progressively, with time, deviated more and more from both the exponential and the exponential multiplied by a series fits. An example of proton $T_1$ decay curves for three $^{13}\text{C}\alpha$ resonances which are well resolved in the two-dimensional spectrum, is shown in Fig. 5.6. It can be seen from the decay curves in Fig. 5.6(a) that relaxation to about 160 ms is relatively exponential but it becomes erratic at longer times. The most erroneous points are not consistently out for each resonance, except for the point at 160 ms, which may be circumstantial. Therefore, at this stage at least, it was unlikely to be just a systematic fault (e.g. environmental change or systematic equipment error).

A few weeks later, the same proton $T_1$ experiment was repeated with exactly the same parameters. It gave the decay rates shown in Fig. 5.6(b). Here the relatively exponential decay occurs in an even narrower range than before (to about 50 ms) and the points at longer times appear to be random. These effects are much larger than the noise and therefore are not due to random noise fluctuations. However, the $^{15}\text{N}$ relaxation experiments (the heteronuclear $T_1$'s, $T_2$'s and NOE's) on the Bruker DMX500 Spectrometer gave good data for sample 3. This implied that sample 3 had not deteriorated and the problems could possibly be due to the spectrometer (DRX400).
Fig. 5.6 Examples of $^1$H $T_1$ data acquired at different times on the Bruker DRX400 Spectrometer using sample 3 of domain II of Protein G at 300K. In both plots T22$^\alpha$, T56$^\alpha$ and F57$^\alpha$ protons are represented by o, □ and Δ respectively. The experimental parameters used to acquire the data were identical but (a) was acquired a few weeks before (b). A total relaxation delay of 4.5 s was used of which 2.5 s was for proton presaturation at a power of 55 dB. The delay $t_J$ was set to 3 μs with increments of 23 μs. 1024 points were acquired in the directly observed dimension, 256 in the
indirect dimension with 8 transients. The value of $J_{CH}$ was taken to be 120 Hz. All gradient pulses were 1 ms except for gp8 which was 5 ms. The gradient power (in %) used for each pulse is as follows:- $gp1 = 29.3, gp2 = gp3 = 8.8, gp4 = 50, gp5 = 95, gp6 = gp7 = 90, gp8 = 36$ and $gp9 = gp10 = 67$. The mixing times used are as follows:- 5, 10, 15, 25, 35, 50, 70, 95, 125, 160, 200, 245, 295, 350, 410, 475, 545 and 620 ms. All data was analysed using off-line processing on FELIX (BIOSYM/Molecular Simulations). The data was zero filled to 2048 points in $f_2$ and 1024 points in $f_1$. An optimised Gaussian function was applied prior to Fourier transformation.

The proton $T_1$ experiment was run again on the DRX with some changes in the values of the gradient pulses. This time, a one-dimensional version of the experiment was carried out to do a quick check on the validity of the changes. This data is shown in Fig. 5.7(a) for three arbitrary peaks from different parts of the one-dimensional spectrum. Since these clearly decay exponentially and there are no erroneous points, a two-dimensional experiment was run. The data for this is given in Fig. 5.7(b). Even though the one-dimensional data is good, the two-dimensional data is completely different and far from expectation. The oscillating effect evident in Fig. 5.7(b) is larger than the noise and therefore it can not be due to random noise fluctuations. These results cast doubt on the long term stability of the hardware. Additionally, this data shows the necessity to run a set of two-dimensional experiments, rather than a quick one-dimensional version, to observe the effects of any changes made to the proton $T_1$ experiment.
Fig. 5.7 Examples of one-dimensional, (a), and two-dimensional, (b), proton $T_1$ data of sample 3 of domain II of Protein G acquired on the Bruker DRX400 at 300K. In plot (a) the data is fitted to an exponential model and three peaks from three different regions of the one-dimensional spectrum are shown: peak 1, peak 2, and peak 3, represented by +, x and *, are in the alpha, beta and methyl regions respectively. In plot (b) the data is not fitted to any model and decay curves for $T22^\alpha$, $T56^\alpha$ and $F57^\alpha$ protons, represented by o, □ and Δ respectively, are shown. The experimental and
processing parameters were similar to those given in the legend to Fig. 5.5 except for a few minor changes in some of the gradient values.

The problem experienced with sample 3 could be due to one, or a combination, of three possible faults. There may be something wrong with the pulse sequence, the sample or the hardware. The pulse sequences, especially the one for measuring proton $T_1$, have been demonstrated to give valid results in Chapter Four. The decay curves of Figs. 5.7(b) and 4.10(b), were both acquired using the same pulse sequence and the same acquisition parameters but on different samples and on different spectrometers. Any differences between these cannot be due to the pulse sequence and therefore this option can be eliminated.

The same sample as that used for $^{13}$C relaxation measurements on the DRX400 was used to make $^{15}$N relaxation measurements on the DMX500. The $^{15}$N experiments gave good data. If the sample had deteriorated, the $^{15}$N data would be affected as well as the $^{13}$C data. Since this is not the case and only the $^{13}$C measurements are affected, the sample can be eliminated.

As for a problem with the hardware, the most erroneous points in Fig. 5.7 are not consistently out for each resonance. However the results shown in Fig. 5.7 point to a possible hardware instability.

In general, the problematic decay curves could be due to the spectrometer being unstable at long experimental times. A spectrometer fault could be due to any one or a combination of faults in the following:- RF (proton and/or $^{13}$C), decoupling, presaturation, gradient pulses, tuning (proton and/or $^{13}$C) or receiver gain. Attempts were made to design experiments to systematically eliminate each of these faults one at a time. The $^{13}$C, proton and decoupling pulses were calibrated thoroughly. The proton $T_1$ experiment was run with and without gradient pulses and presaturation. This experiment was also run with and without broadband decoupling, and with broadband decoupling only either in the mixing time or during acquisition. The tuning, of both
nuclei, and shimming was adjusted precisely. The receiver gain was set carefully using longer mixing times and evolution periods. Unfortunately, the results do not convincingly point to any particular fault and no conclusion can be drawn from them.

5.6 DISCUSSION OF RESULTS

The $^{13}$C $T_1$ data can be used, independently of any other measurements, to make generalisations about the dynamics of the protein backbone. In order to do this it is assumed that the order parameter, $S^2$, is directly proportional to the decay rate of longitudinal magnetisation of $^{13}$C. This assumption may not always be justified but it can give a general insight into molecular dynamics when there is not enough experimental data to carry out a full and rigorous analysis. Unfortunately we do not have enough data to implement the method of analysis, which was developed in section 3.6, with proteins.
5.7 REFERENCES


This chapter aims to provide a brief summary of this thesis and the contribution it has made to the area of $^{13}$C NMR relaxation. The work covered in this thesis will be discussed in terms of meeting the objective of this project. Additionally, a brief overview of the results obtained, the practical problems encountered during this project and possible future work are discussed.

The objective of this thesis was to develop a method to measure $^{13}$C relaxation in molecules uniformly labelled with $^{13}$C and $^{15}$N. $^{13}$C relaxation data is important because it provides dynamics information on the whole molecule (e.g. along the backbone and the side-chains of proteins). $^{13}$C relaxation data of uniformly $^{13}$C and $^{15}$N labelled molecules enables dynamics information about the whole molecule to be obtained from one sample and the same sample as that used for structural studies can be used for dynamics studies.

There are two main problems with analysing $^{13}$C relaxation data of molecules uniformly enriched with this isotope: i) $^{13}$C-$^{13}$C scalar coupling evolution makes the conventional $T_2$ measurement problematic and ii) numerous and unquantified $^{13}$C dipolar interactions make the traditional $^{13}$C $T_1$ and NOE measurements difficult to analyse. These two problems raise the need to obtain a replacement for the $^{13}$C $T_2$ measurement and develop ways of analysing $^{13}$C relaxation data which take account of the numerous dipolar interactions.
This thesis develops the theory of a method which enables the mutual dipolar interaction of a set of spins to be isolated from a system with an arbitrary number of spins. It also provides the experiments to make the measurements necessary to implement the new method.

The new method of analysis involves taking a linear combination of selected elements of the longitudinal relaxation matrix in a way which isolates the dipolar interactions of interest between a set of active spins. All other interactions between the active spins and other spins in the system, cancel out. This method can be used in conjunction with existing $^{15}$N measurements (i.e. $^{15}$N $T_1$, $T_2$ and NOE) because it provides a new combination of the spectral density function. These $^{13}$C and $^{15}$N measurements (i.e. the isolated dipolar interactions from $^{13}$C relaxation data and $^{15}$N $T_1$, $T_2$ and NOE) can be used with a motional model to model the spectral density function for each internuclear vector. Alternatively, the isolated $^{13}$C dipolar interactions may be used in conjunction with a less rigorous method of analysis to map the spectral density function. The mapping approach involves taking a linear combination of up to four relaxation measurements which make it relatively more susceptible to significant errors than the modelling approach.

Regardless of whether the modelling or mapping approach is taken, the new method of analysis involves making five independent relaxation measurements for $^{13}$CH$_n$ groups. These are the $^{13}$C $T_1$, proton $T_1$, heteronuclear $^{13}$CH and homonuclear HH longitudinal two spin order and $^{13}$CHH longitudinal three spin order. Only the first three measurements can be made for $^{13}$CH groups while all five can be made for $^{13}$CH$_2$ and $^{13}$CH$_3$ groups. For $^{13}$CH groups the three measurements can be used to isolate the $^{13}$CH dipolar interaction. For $^{13}$CH$_2$ groups, the appropriate three or four of the five measurements can be used to isolate the two $^{13}$CH dipolar interactions and the HH interaction. Our method can also be applied to $^{13}$CH$_3$ groups.
When this study was initiated, only the $^{13}\text{C} \, T_1$ measurement had been previously reported in the literature. However, this needed to be modified to use on molecules uniformly enriched with $^{13}\text{C}$. The remaining four measurements had not been reported in the literature.

The second contribution of this thesis, which is an extension of the first contribution, is the design and development of two-dimensional heteronuclear experiments to measure $^{13}\text{C} \, T_1$, proton $T_1$, $^{13}\text{CH}$ and HH longitudinal two spin order and $^{13}\text{CHH}$ longitudinal three spin order. These measurements are made as precisely as possible by ensuring that unwanted coherence transfer pathways (form imperfect RF pulses, unwanted $^{13}\text{C} - ^{13}\text{C}$ and $^{13}\text{CH}$ scalar coupling evolution, cross-relaxation and a large water resonance which may distort peaks close to it) have been suppressed by the use of phase cycling and gradient pulses. Where a particular coherence transfer pathway has not been suppressed in the design of the experiment the pathway has been taken into account by fitting the data to an exponential multiplied by the first two terms of a series fit.

The practical aspect of this work beyond the development of the experiments, namely the results, is disappointing. This is a consequence of the unforeseen and unresolved problems experienced possibly with both the sample and the hardware. It was hoped that all five $^{13}\text{C}$ relaxation measurements would be made and analysed in at least two different ways: in conjunction with $^{15}\text{N}$ relaxation data (to calculate an overall correlation time) and the "model-free" approach to calculate an order parameter and a correlation time for each internuclear bond vector involving $^{13}\text{C}$; and to make an attempt at mapping the spectral density function. The two approaches would then have been compared and contrasted to each other to draw conclusions on their feasibility of implementation with proteins. Additionally, the modelling and mapping approaches would have been analysed in terms of the molecular dynamics information they provide.
However, the $^{13}$C $T_1$ and the $^{13}$CH longitudinal two spin order measurements were obtained successfully. The $^{13}$C $T_1$ data has been analysed as best as it can be done in isolation of the other necessary $^{13}$C and $^{15}$N relaxation measurements. General trends can be seen between the $^{13}$C $T_1$ rates and the secondary structure of domain II of Protein G. The C- and N-termini, the turns of the $\beta$-sheets and the connecting loops are generally more mobile than the $\alpha$-helix and the two anti-parallel $\beta$-sheets.

It is hoped that this work will be continued to completion. Firstly, the hardware and sample problems experienced in the final stages of this work need to be resolved and overcome. Secondly all five $^{13}$C relaxation measurements ($^{13}$C $T_1$, proton $T_1$, $^{13}$CH and HH longitudinal two spin order and $^{13}$CHH longitudinal three spin order) need to be made. These measurements may be analysed in conjunction with $^{15}$N relaxation data and the "model-free" formalism to model the spectral density function and/or they may be used with the more approximate mapping approach. This information can be used to characterise the dynamics of the protein. It is also hoped that the relaxation measurements will be made on a protein-ligand complex. Hence, it should be possible to determine the bonding interactions between the protein and its ligand from the difference in the dynamics of the bound and unbound protein.
APPENDIX I

CALCULATION OF GENERAL RELAXATION RATES

AN EXAMPLE TO CALCULATE THE TERMS INVOLVED IN THE RELAXATION OF $I_{iz}$ IN A THREE SPIN SYSTEM WHERE ALL THE SPINS HAVE CSA

___number_of_spins_______
3  *input the number of spins in the system*

___number_of_spins_with_csa_relaxation_and_corresponding_labels____
3  *input the number of spins with CSA*
1  *input the identity of each spin with CSA*
2  *input the identity of each spin with CSA*
3  *input the identity of each spin with CSA*

___linear_combination_of_density_matrix_elements____
-
1.  *input the number of spins in the operator*
-
iz  *this means that magnetisation is along the z-axis*
1  *input the identity of the spin(s) in the operator*

J( 0)  *output: no J(0) terms*
ZERO

J( 1- 2)  *output: J(\omega_1 - \omega_2) terms*
-0.16667 D( 1, 2) D( 1, 2) IZ2  *term due to dipolar interaction between spins 1 and 2*
\[ 0.16667 \, D(1, 2) \, D(1, 2) \, Iz1 \]
\[ -0.16667 \, D(1, 2) \, D(1, 2) \, Iz2 \]
\[ 0.16667 \, D(1, 2) \, D(1, 2) \, Iz1 \]

\[ J(1-3) \]
\[ -0.16667 \, D(1, 3) \, D(1, 3) \, Iz3 \]
\[ 0.16667 \, D(1, 3) \, D(1, 3) \, Iz1 \]
\[ -0.16667 \, D(1, 3) \, D(1, 3) \, Iz3 \]
\[ 0.16667 \, D(1, 3) \, D(1, 3) \, Iz1 \]

\[ J(2-3) \]
\[ \text{ZERO} \]
\[ \text{ZERO} \]

\[ J(1) \]
\[ 2.00000 \, C(1) \, D(1, 2) \, Iz1 \, Iz2 \]
\[ \text{term due to dipolar-CSA cross-correlation (the ratio needs to be divided by 2)} \]
\[ 0.50000 \, C(1) \, C(1) \, Iz1 \]
\[ \text{term due to CSA interaction of spin 1} \]
\[ 2.00000 \, C(1) \, D(1, 3) \, Iz1 \, Iz3 \]
\[ \text{term due to dipolar-CSA cross-correlation (the ratio needs to be divided by 2)} \]
\[ 0.50000 \, D(1, 2) \, D(1, 2) \, Iz1 \]
\[ \text{term due to dipolar interaction between spins 1 and 2} \]
\[ 4.00000 \, D(1, 2) \, D(1, 3) \, Iz1 \, Iz2 \, Iz3 \]
\[ \text{term due to dipolar interaction (the ratio needs to be divided by 2)} \]
\[ 0.50000 \, D(1, 3) \, D(1, 3) \, Iz1 \]
\[ \text{term due to dipolar interaction between spins 1 and 3} \]
\[ 2.00000 \, C(1) \, D(1, 2) \, Iz1 \, Iz2 \]
\[ \text{term due to dipolar-CSA cross-correlation (the ratio needs to be divided by 2)} \]
\[ 0.50000 \, C(1) \, C(1) \, Iz1 \]
\[ \text{term due to CSA interaction of spin 1} \]
\[ 2.00000 \, C(1) \, D(1, 3) \, Iz1 \, Iz3 \]
\[ \text{term due to dipolar-CSA cross-correlation (the ratio needs to be divided by 2)} \]
\[ 0.50000 \, D(1, 2) \, D(1, 2) \, Iz1 \]
\[ \text{term due to dipolar interaction between spins 1 and 2} \]
\[ 4.00000 \, D(1, 2) \, D(1, 3) \, Iz1 \, Iz2 \, Iz3 \]
\[ \text{term due to dipolar interaction (the ratio needs to be divided by 2)} \]
\[ 0.50000 \, D(1, 3) \, D(1, 3) \, Iz1 \]
\[ \text{term due to dipolar interaction between spins 1 and 3} \]
J( 2)  
ZERO  
ZERO  

J( 3)  
ZERO  
ZERO  

J( 1+ 2)  
*output: $J(\omega_1 + \omega_2)$ terms*  
1.00000 D( 1, 2) D( 1, 2) Iz2  
1.00000 D( 1, 2) D( 1, 2) Iz1  
1.00000 D( 1, 2) D( 1, 2) Iz2  
1.00000 D( 1, 2) D( 1, 2) Iz1  

J( 1+ 3)  
*output: $J(\omega_1 + \omega_3)$ terms*  
1.00000 D( 1, 3) D( 1, 3) Iz3  
1.00000 D( 1, 3) D( 1, 3) Iz1  
1.00000 D( 1, 3) D( 1, 3) Iz3  
1.00000 D( 1, 3) D( 1, 3) Iz1  

J( 2+ 3)  
*output: no $J(\omega_2 + \omega_3)$ terms*  
ZERO  
ZERO
APPENDIX II

PULSE PROGRAMS DESIGNED AND USED

$R_C(S_C^2)$ EXPERIMENT ON BRUKER AMX600

;acCT1grad
;for shaped gradients using gradient waveform memory
;for gradient blanking using inverted logic on nmrctrl2 bit 3
;Amirah Chaudhry & Tim J. Norwood, September 1995

\[
\begin{align*}
p_2 &= p_1 \times 2 \\
p_4 &= p_3 \times 2 \\
d_0 &= 3u \\
d_3 &= 0.5m \\
d_4 &= 1s/(cnst2*4) \\
d_5 &= d_4/2 \\
d_8 &= p_2 + 6u \\
d_{11} &= 30m \\
d_{12} &= 20m \\
d_{13} &= 3u \\
d_{16} &= 100u \\
d_{17} &= p_3 - p_1 \\
p_{16} &= 3.5m \\
p_{17} &= 2.7m \\
p_{18} &= 1m \\
p_{19} &= 0.4m \\
d_{22} &= 100u
\end{align*}
\]
d6=d4-p19-d16-d3-2u

d26=p16/l21-2u

d28=p18/l21-2u

d27=p17/l21-2u

d29=p19/l21-2u

1 ze

2 d22 dbo; setf2^3
   d1 hl2
   p5 ph1
   d11 hl1 setf2^3
   d11 db10 setf2^3
   (p1 ph1)

3 2u:ngrad
   d29
   lo to 3 times l21
   d16:ngrad
   d3
   d6
   (d17 p2 ph1) (p4 ph1):db

4 2u:ngrad
   d29
   lo to 4 times l21
   d16:ngrad
   d3
   d6
   p1 ph5
   d13

6 2u:ngrad
   d26
   lo to 6 times l21
   d16:ngrad
d3
(p3 ph9):db
d5
(d17 p2 ph1) (p4 ph1):db
d5
d0
(p2 ph6)
d0
(p3 ph4):db
d12 hl3
d9 cpdts
d13 to
d12 hl1
8 2u:ngrad
d27
lo to 8 times l21
d16:ngrad
d3
p1 ph1
d13
9 2u:ngrad
d28
lo to 9 times l21
d16:ngrad
d3
(p3 ph1):db
d5
(d17 p2 ph1) (p4 ph1):db
d5
(p3 ph3):db
d13
(p1 ph3)


```
d4
(d17 p2 ph1) (p4 ph1):db
d4 db11 setf2l3
go=2 ph31 cpdb
d11 dbo wr #0 if #0 zd
d22 setf2^3
d12 dp4
d12 id0
lo to 2 times td1
d22 setf2l3
dbo
exit

ph1=0
ph3=1
ph4=1 3
ph5=1 1 3 3
ph6=1 1 1 1 3 3 3 3
ph9=0
ph10=0
ph11=0
ph12=0
ph15=(360) 0
ph29=0
ph31=0 2 2 0

;h1: ecoupler high power level
;dl0: power level for decoupler hard pulse
;dl1:power level for decoupler garp pulse
;p1 : 90 degree transmitter high power pulse
;p2 : 180 degree transmitter high power pulse
;p3 : 90 degree decoupler high power pulse
```
;p4: 180 degree decoupler high power pulse
;p16: homospoil/gradient pulse
;p31: 90 degree pulse for slave timer (cpd-sequence)
;d0: incremented delay (2D) [3 usec]
;d1: relaxation delay; 1-5 * T1
;d4: 1/(4J)XH
;d11: delay for disk I/O [30 msec]
;d13: short delay (e.g. to compensate delay line) [3 usec]
;d16: delay for homospoil/gradient recovery[100us]
;d20: p16 + d16
;d21: d4 - p16 - d16 - d13
;d27: delay for shaped gradient[8us]
;L21: loop for shaped gradients[100 normally]
;in0: 1/(4 * SW(X)) = (1/2) DW(X)
;nd0: 4
;NS: 1 * n
;DS: 16
;td1: number of experiments
;MC2: TPPI
;cpd: cpd-decoupling according to sequence defined by cpdprg
\( R_{H}(I_{H2}) \) EXPERIMENT ON BRUKER AMX600

;acCH2T1grad

;Amirah Chaudhry & Tim J. Norwood, Leicester Chemistry Dept., July 1995

\[ p2 = p1 \times 2 \]
\[ p4 = p3 \times 2 \]
\[ p15 = 1.317 \text{m} ; 5.33 \text{m} \text{ lm} \]
\[ p17 = 0.35 \text{m} \]
\[ p18 = 1.6 \text{m} \]
\[ p19 = 0.25 \text{m} \]
\[ p20 = 4 \text{m} \]
\[ p21 = 0.39 \text{m} ; 0.333 \text{m} \]
\[ d3 = 0.5 \text{m} \]
\[ d4 = 1s/(cnst2*4) \]
\[ d11 = 30 \text{m} \]
\[ d13 = 3u \]
\[ d12 = 10 \text{m} \]
\[ d16 = 100u \]
\[ d22 = 100u \]
\[ d5 = d4 - 2u - p17 - d16 - d3 - d13 \]
\[ d6 = d4 - 2u - p19 - d16 - d3 - d13 \]
\[ d7 = d4 - 2u - p21 - d16 - d3 - d13 \]
\[ d8 = d4 - 2u - p19 - d22 - d16 - d3 - d13 \]
\[ d14 = p3 - p1 \]
\[ d15 = p15/l21 - 2u \]
\[ d20 = p20/l21 - 2u \]
\[ d17 = p17/l21 - 2u \]
\[ d18 = p18/l21 - 2u \]
\[ d19 = p19/l21 - 2u \]
\[ d21 = p21/l21 - 2u \]
1 ze
2 d22 dbo setf2^3
   d1 hl2
   p5 ph9
   d11 hl1
   d11 dbl0 setf2^3
   p1 ph1
   d4 dbl0 setf2^3
   (d14 p2 ph1) (p4 ph1):db
   d4
   (p3 ph8):db
   d0
   d0
   (p1 ph3)(p3 ph3):db
   d13
3 2u:ngrad
   d15
   lo to 3 times l21
   d16:ngrad
   d3
   (p1 ph9)
   d13
4 2u:ngrad
   d21
   lo to 4 times l21
   d17:ngrad
   d3
   d7
   (d14 p2 ph9) (p4 ph6):db
   d13
5 2u:ngrad
   d21
lo to 5 times l21
d13:ngrad
d3
d7
p1 ph6
d11 db11
6 2u:ngrad
d20
lo to 6 times l21
d13:ngrad
d3
d9 cpdb
d12 db0 db0
p1 ph9
d13
7 2u:ngrad
d17
lo to 7 times l21
d13:ngrad
d3
d5
(d14 p2 ph6) (p4 ph6):db
d13
9 2u:ngrad
d17
lo to 9 times l21
d13:ngrad
d3
d5
p1 ph6
d13
11 2u:ngrad
d18
lo to 11 times l21
d16:ngrad
d3
p1 ph6
d13
13 2u:ngrad
d19
lo to 13 times l21
d16:ngrad
d3
d6
(d14 p2 ph9) (p4 ph9):db
d13
15 2u:ngrad
d19
lo to 15 times l21
d16:ngrad
d22 db11 setf2l3
d3
d8
go=2 ph7 cpdb
d11 dbo wr #0 if #0 zd
d12 ip1
d12 ip4
d12 ip8
d12 id0
d11
lo to 2 times td1
d22 setf2l3
dbo
exit
\[ \phi_1 = (8)0 \]
\[ \phi_2 = 0 \ 0 \ 0 \ 0 \ 2 \ 2 \ 2 \ 2 \]
\[ \phi_3 = 0 \ 0 \ 1 \ 1 \ 2 \ 2 \ 3 \ 3 \]
\[ \phi_4 = (8)0 \ 0 \ 4 \ 4 \]
\[ \phi_7 = 0 \ 2 \ 2 \ 0 \ 0 \ 2 \ 2 \ 0 \]
\[ \phi_8 = (8)1 \ 5 \]
\[ \phi_9 = 0 \]
\[ \phi_{10} = (360)0 \]

;P1,P2: 90, 180 deg H-1 pulse
;D2: 1/(2J)XH
;P3,P4: 90, 180 deg X pulse
;d11=disk i/o 30ms
;d12=20us
;d13=3us
;D5: DE/2
;D6 = 2 usec
;P0 = 5 usec
;P8: 90 deg pulse for X decoupling
;DS: 2 or 4
;NS: 4 * n
;D0 = 3 usec
;IN1: 1 / 4SW(X) = (1/2) DW(X) of x-nucleus dimension
;ND0 = 4
$R_{CH(2SCzHz)}$ EXPERIMENT ON BRUKER AMX600

;acCH2zzgrst
;Amirah Chaudhry & Tim J. Norwood, Leicester Chemistry Dept., July 1995

p2 = p1 * 2
p4 = p3 * 2
p14 = 1.5s
p15 = 1.9m; 1.317m
p17 = 0.25m
p18 = 0.713m
p19 = 3.103m
d3 = 0.5m
d4 = 1s / (cnst2 * 4)
d11 = 30m
d13 = 3u
d12 = 3m
d16 = 100u
d22 = 100u
d6 = d4 - d13 - 2u - p18 - d16 - d3
d14 = p3 - p1
d15 = p15 / l21 - 2u
d17 = p17 / l21 - 2u
d18 = p18 / l21 - 2u
d19 = p19 / l21 - 2u

1 ze
2 d22 dbo setf2^3
d11 h12 db10
d1
p5 ph1
d11 h11
p1 ph3
d4
(d14 p2 ph3) (p4 ph3):db
d4
(p3 ph8):db
d0
d0
(p1 ph2) (p3 ph2):db
d9

7 2u:ngrad
d17
lo to 7 times l21
d16:ngrad
d3
p2 ph5
d13

8 2u:ngrad
d15
lo to 8 times l21
d16:ngrad
d3
d9
p1 ph6
d13

9 2u:ngrad
d18
lo to 9 times l21
d16:ngrad
d3
d6
(d14 p2 ph6) (p4 ph1):db
d13
11 2u:ngrad
d18
lo to 11 times l21
d16:ngrad
d3
d6
p1 ph1
d13
13 2u:ngrad
d19
lo to 13 times l21
d16:ngrad
d3
d11 db11
p1 ph1
go=2 ph7 cpdb
d11 dbo wr #0 if #0 zd
d12 ip3
d12 ip8
d12 id0
d11
lo to 2 times td1
d22 setf2l3
dbo
exit

ph1=0
ph2=0 0 1 1 2 2 3 3
ph3=(8)0
ph7=0 2 2 0 0 2 2 0
ph8=(8)1 5
ph5=0
ph6 = 1
ph10 = (360)0

;P1, P2 : 90, 180 deg H-1 pulse
;D2 : 1/(2J)XH
;P3, P4 : 90, 180 deg X pulse
;d11 = disk i/o 30 ms
;d12 = 20 us
;d13 = 3 us
;D5 : DE/2
;D6 = 2 usec
;P0 = 5 usec
;P8 : 90 deg pulse for X decoupling
;DS : 2 or 4
;NS : 4 * n
;D0 = 3 usec
;IN : 1/4 SW(X) = (1/2) DW(X) of x-nucleus dimension
;ND0 = 4
$R_{\text{CHH}(4S_{C_{2} H_{2}} H_{2})}$ EXPERIMENT ON BRUKER AMX600

;acCH2zzzgrad
;Amirah Chaudhry & Tim J. Norwood, Leicester Chemistry Dept., July 1995

\[ p_2 = p_1 \times 2 \]
\[ p_4 = p_3 \times 2 \]
\[ p_6 = p_1 / 2 \]
\[ p_{15} = 0.7 \text{m} \]
\[ p_{17} = 0.2 \text{m} \]
\[ p_{18} = 2.5 \text{m} \]
\[ p_{19} = 0.2 \text{m} \]
\[ p_{20} = 4.4 \text{m} \]
\[ p_{21} = 0.3 \text{m} \]
\[ d_3 = 0.5 \text{m} \]
\[ d_4 = 1s/(\text{cnst2} \times 4) \]
\[ d_{10} = d_4 / 2 \]
\[ d_{11} = 30 \text{m} \]
\[ d_{13} = 3 \text{u} \]
\[ d_{12} = 10 \text{m} \]
\[ d_{16} = 100 \text{u} \]
\[ d_{23} = d_4 \times 2 - p_{15} + p_{18} \]
\[ d_{22} = 100 \text{u} \]
\[ d_5 = d_4 - 2u - p_{17} - d_{16} - d_3 - d_{13} \]
\[ d_6 = d_4 - 2u - p_{19} - d_{16} - d_3 - d_{13} \]
\[ d_7 = d_{10} - 2u - p_{21} - d_{16} - d_3 - d_{13} \]
\[ d_{14} = p_3 - p_1 \]
\[ d_{15} = p_{15} / 121 - 2u \]
\[ d_{20} = p_{20} / 121 - 2u \]
\[ d_{17} = p_{17} / 121 - 2u \]
\[ d_{18} = p_{18} / 121 - 2u \]
\[ d_{21} = p_{21} / 121 - 2u \]
1 ze
2 d22 dbo setf2^3
d1 hl2
p5 ph9
d11 hl1 db10 setf2^3
p1 ph1
d4
(d14 p2 ph9) (p4 ph9):db
d4
(p3 ph4):db
d0
p2 ph9
d0
d4
(d14 p2 ph9) (p4 ph9):db
d4
(p1 ph6) (p3 ph9):db
d13
p6 ph3
d13
5 2u:ngrad
d15
lo to 5 times l21
d16:ngrad
d3
d9
p2 ph6
d9
6 2u:ngrad
d18
lo to 6 times l21
d16:ngrad
d3
d23
(p3 ph8):db
d13
7 2u:ngrad
d17
lo to 7 times l21
d16:ngrad
d3
d5
(d14 p2 ph6) (p4 ph6):db
d13
9 2u:ngrad
d17
lo to 9 times l21
d16:ngrad
d3
d5
(p1 ph6) (p3 ph6):db
d13
11 2u:ngrad
d20
lo to 11 times l21
d16:ngrad
d3
(p3 ph6):db
d13
13 2u:ngrad
d21
lo to 13 times l21
d16:ngrad
d3
d7
(d14 p2 ph9) (p4 ph9):db
d13
15 2u:ngrad
d21
lo to 15 times l21
d16:ngrad
d3
d7
(p1 ph6) (p3 ph9):db
d4
(d14 p2 ph6) (p4 ph6):db
d4 dbl1 setf2l3
go=2 ph7 cpdb
d11 dbo wr #0 if #0 zd
d12 ip4
d12 id0
d11
lo to 2 times td1
d22 setf2l3
dbo
exit

ph1=0 2
ph3=1 1 1 1 1 1 1 3 3 3 3 3 3 3
ph4=0 0 2 2
ph7=0 2 2 0 2 0 2 2 0 2 0 2 2 0
ph8=1 1 1 1 3 3 3
ph9=0
ph6=1
ph10=(360)0
;P1,P2 : 90, 180 deg H-1 pulse
;D2 : 1/(2J)XH
;P3,P4 : 90, 180 deg X pulse
;d11=disk i/o 30ms
;d12=20us
;d13=3us
;D5 : DE/2
;D6 = 2 usec
;P0 = 5 usec
;P8 : 90 deg pulse for X decoupling
;DS : 2 or 4
;NS : 4 * n
;D0 = 3 usec
;IN : 1 / 4SW(X) =(1/2) DW(X) of x-nucleus dimension
;ND0 = 4
$R_{HH}(2J_{Hz}I_{Hz})$ EXPERIMENT ON BRUKER AMX600

;acHHzzgrad
;Amirah Chaudhry & Tim J. Norwood, Leicester Chemistry Dept., June 1996

\[ p2 = p1 \times 2 \]
\[ p4 = p3 \times 2 \]
\[ p6 = p1 / 2 \]
\[ p15 = 0.7 \text{m} \]
\[ p17 = 0.13 \text{m} \]
\[ p18 = 2.5 \text{m} \]
\[ p19 = 0.2 \text{m} \]
\[ p20 = 4.4 \text{m} \]
\[ p21 = 0.3 \text{m} \]
\[ p22 = 0.45 \text{m} \]
\[ p23 = 1.9 \text{m} \]
\[ d3 = 0.5 \text{m} \]
\[ d4 = l/s/(\text{cnst2} \times 4) \]
\[ d10 = d4 / 2 \]
\[ d11 = 30 \text{m} \]
\[ d13 = 3 \text{u} \]
\[ d12 = 10 \text{m} \]
\[ d16 = 100 \text{u} \]
\[ d5 = d4 - 2u - p17 - d16 - d3 \]
\[ d6 = d4 - 2u - p19 - d16 - d3 \]
\[ d7 = d4 - 2u - p21 - d16 - d3 \]
\[ d14 = p3 - p1 \]
\[ d15 = p15 / l21 - 2u \]
\[ d20 = p20 / l21 - 2u \]
\[ d17 = p17 / l21 - 2u \]
\[ d18 = p18 / l21 - 2u \]
\[ d19 = p19 / l21 - 2u \]
d21 = \frac{p21}{l21} - 2u \\
d22 = \frac{p22}{l21} - 2u \\
d23 = \frac{p23}{l21} - 2u \\

1 ze \\
2 d16 dbo setf2^3 \\
\quad d1 hl2 \\
\quad p5 ph9 \\
\quad d11 hl1 dbi0 setf2^3 \\
\quad p1 ph1 \\
\quad d5 \\
4 2u:ngrad \\
\quad d17 \\
\quad lo to 4 times l21 \\
\quad d16:ngrad \\
\quad d3 \\
\quad (d14 p2 ph6) (p4 ph6):db \\
\quad d5 \\
5 2u:ngrad \\
\quad d17 \\
\quad lo to 5 times l21 \\
\quad d16:ngrad \\
\quad d3 \\
\quad (p3 ph2):db \\
\quad d0 \\
\quad p2 ph4 \\
\quad d0 \\
\quad d7 \\
6 2u:ngrad \\
\quad d21 \\
\quad lo to 6 times l21 \\
\quad d16:ngrad
d3
(d14 p2 ph9) (p4 ph9):db
d7
7 2u:ngrad
d21
lo to 7 times l21
d16:ngrad
d3
(p3 ph9):db
d6
8 2u:ngrad
d19
lo to 8 times l21
d16:ngrad
d3
(d14 p2 ph9)(p4 ph9):db
d6
9 2u:ngrad
d19
lo to 9 times l21
d16:ngrad
d3
p6 ph9
d13
10 2u:ngrad
d15
lo to 10 times l21
d16:ngrad
d3
d13
(p3 ph9):db
d13
11 2u:ngrad
d22
lo to 11 times l21
d16:ngrad
d3
12 d9
(p4 ph9):db
d9
lo to 12 times l3
d13
13 2u:ngrad
d23
lo to 13 times l21
d16:ngrad
d3
p1 ph6
d13
p1 ph3
d13
14 2u:ngrad
d20
lo to 14 times l21
d16:ngrad
d3
p6 ph9
d4
(d14 p2 ph6)(p4 ph6):db
d4
(p3 ph9):db
d4
(d14 p2 ph9)(p4 ph6):db
d4
(p1 ph6)(p3 ph6):db
d13
19 2u:ngrad
d18
lo to 19 times l21
d16:ngrad
d3
p1 ph6
d4
(d14 p2 ph9) (p4 ph9):db
d4 db1 setf2l3
go=2 ph7 cpdb
d11 dbo wr #0 if #0 zd
d12 ip2
d12 id0
d11
lo to 2 times td1
d16 setf2l3
dbo
exit

ph1=0 0 2 2
ph2=1 1 1 1 3 3 3
ph3=1 3
ph4=1 1 1 1 1 1 3 3 3 3 3 3 3 3 3
ph7=0 0 2 2 2 2 0 0
ph8=1 1 1 1 3 3 3
ph9=0
ph6=1

;P1,P2 : 90, 180 deg H-1 pulse
;D2 : 1/(2J)XH
; P3, P4: 90, 180 deg X pulse
; d11 = disk i/o 30 ms
; d12 = 20 us
; d13 = 3 us
; D5 : DE/2
; D6 = 2 usec
; P0 = 5 usec
; P8 : 90 deg pulse for X decoupling
; D5 : 2 or 4
; NS : 4 * n
; D0 = 3 usec
; IN : 1 / 4 SW(X) = (1/2) DW(X) of x-nucleus dimension
; ND0 = 4
$R_C(S_{C_2})$ EXPERIMENT ON BRUKER DRX400

;acCT1grad1
;Amirah Chaudhry & Tim J. Norwood, September 1995
;Converted to DRX by Benjamin Hickman July 1996

#include <Avance.incl>
#include <Grad.incl>

"p2=p1*2"
"p4=p3*2"
"d0=3u"
"d3=0.5m"
"d4=1s/(cnst2*4)"
"d5=d4/2"
"d11=30m"
"d13=3u"
"d12=20u"
"d16=100u"
"d17=p3-p1"
"d22=100u"
"d6=d4-p16-d16-d3"

1 ze
2 d22 do:f2
   d1
   d11 pl1:f1 pl2:f2
   d22 UNBLKGRAD
p1 ph1
   d13
p1 ph3
3 p16:gp5
  d16
  d3
  d6
  (d17 p2 ph1) (p4 ph1) :f2
  d13
4 p16:gp5
  d16
  d3
  d6
  (p1 ph5) ; (p3 ph9) :f2
  d13
6 p16:gp4
  d16
7 p16:gp4
  d16
8 p16:gp4
  d16
9 p16:gp4
  d16
  d3
  (p3 ph9) :f2
  d5
  (d17 p2 ph1) (p4 ph1) :f2
  d5
  d0
  (p2 ph6)
  d0
  (p3 ph4) :f2
  d12 p19: f1
  d9 cpd2: f1
d13 do:f1
d12  pl1:f1
d13
p1 ph1
d13
p1 ph3
d13
10  p16:gp1
d16
11  p16:gp1
d16
12  p16:gp1
d16
d3
p1 ph1
d13
p1 ph3
d13
13  p16:gp6
d16
14  p16:gp6
d16
d3
(p3 ph1) :f2
d5
(d17 p2 ph1) (p4 ph1) :f2
d5
(p1 ph7) (p3 ph3) :f2
d13
15  p16:gp2
d16
d3

272
d6
(dl7 p2 ph1) (p4 ph1) :f2
16 p16:gp2
dl6
d3
d6
p1 ph1
dl3
17 p16:gp3
dl6
18 p16:gp3
dl6
19 p16:gp3
dl6
20 p16:gp3
dl6
21 p16:gp3
dl6
d3
50u BLKGRAD
dl2 pl12 :f2
pl ph1
go=2 ph31 cpd1:2
ndl do :f2
dl wr #0 if #0 zd
dl2 dp4
dl2 id0
lo to 2 times td1
dl2 do :f2
exit

ph1=0
ph3=1
ph4=1 3
ph5=1133
ph6=1113333
ph7=11113333
ph9=0
ph31=02202002
$R_H(I_H_2)$ EXPERIMENT ON BRUKER DRX400

;accHT1grad
;Amirah Chaudhry & Tim J. Norwood, September 1995
;Converted to DRX by Benjamin Hickman July 1996

#include <Avance.incl>
#include <Grad.incl>

"p2=p1*2"
"p4=p3*2"
"d0=3u"
"d3=0.5m"
"d4=ls/(cnst2*4)"
"d11=30m"
"d13=3u"
"d12=20u"
"d16=100u"
"d17=p3-p1"
"d22=100u"
;"d5=d4-p16-d3-d13-d16"
"d6=d4-p16-d16-d3"

1  ze
2  d22 do:f2 pl9:f1
d1
d13 pl1:f1 pl2:f2
d22 UNBLKGRAD
p1 ph1
d4
(d17 p2 ph1) (p4 ph1) :f2
d16
d3 pl2 :f2
d6
(d17 p2 ph6) (p4 ph6) :f2
d13
9 p16:gp4
d16
d3
d6
p1 ph6
d13
11 p16:gp5
d16
12 p16:gp5
d16
13 p16:gp5
d16
14 p16:gp5
d16
15 p16:gp5
d13
d3
p1 ph6
d13
16 p16:gp6
d16
d3
d6
(d17 p2 ph9) (p4 ph9) :f2
d13
17 p16:gp6
d16 pl12 :f2
d3 BLKGRAD
d6
go=2 ph31 cpd1:f2
d12 do :f2
d11 wr #0 if #0 zd
d12 ip1
d12 ip8
d12 id0
lo to 2 times td1
d12 do :f2
exit

ph1=(8)0
ph2=0 0 0 2 2 2 2
ph3=0 0 1 1 2 2 3 3
ph6=1
ph8=(8)1 5
ph9=0
ph10=(360)0
ph31=0 2 2 0 0 2 2 0
$R_C H(2S_C Z_H_2_2)$ EXPERIMENT ON BRUKER DRX400

;acCH2zzgrad
;Amirah Chaudhry & Tim J. Norwood, September 1995
;Converted to DRX by Benjamin Hickman July 1996

#include <Avance.incl>
#include <Grad.incl>

"p2=p1*2"
"p4=p3*2"
"d0=3u"
"d3=0.5m"
"d4=1s/(cnst2*4)"
"d11=30m"
"d13=3u"
"d12=20u"
"d16=100u"
"d17=p3-p1"
"d22=100u"
"p15=1.317m"
"p17=0.25m"
"p18=0.713m"
"p19=3.103m"
;d5=d4-p15-d3-d13-d16"
"d6=d4-p16-d16-d3"

1 ze
2 d22 do:f2 pl9:f1
d1
 d13 pl9:f1 pl2:f2
p20 ph1
d13 pl1:fl
d22 UNBLKGRAD
p1 ph3
d4
(d17 p2 ph3) (p4 ph3):f2
d4
(p3 ph8):f2
d0
d0
(p1 ph2) (p3 ph2):f2
d9
7 p16:gp1
d16
d3
p2 ph5
d13
8 p16:gp2
d16
d3
d9
p1 ph6
d13
9 p16:gp3
d16
d3
d6
(d17 p2 ph6) (p4 ph1):f2
d13
11 p16:gp3
d16
d3
d6
p1 ph1
d13
l3 p16:gp4
d16 pl12 :f2
d3 BLKGRAD
p1 ph1
go=2 ph31 cpd1:f2
d12 do :f2
d11 wr #0 if #0 zd
d12 ip3
d12 ip8
d12 id0
lo to 2 times td1
d12 do :f2
exit

ph1=0
ph2=0 0 1 1 2 2 3 3
ph3=(8)0
ph5=0
ph6=1
ph8=(8)1 5
ph31=0 2 2 0 0 2 2 0
APPENDIX III

DATA PROCESSING PROGRAM

program to format Felix peaklist output files to individual files for each peak.

program format
integer il, itm, ipk
real s1(25,100), s2(25,100), int(25,100)
character*132 spectra, meas, felix(25), assn(100), junk
real tm(25)

write(6,'(A,$)'), 'Please enter the name of the spectra: '
read(5,* ) spectra
write(6,'(A,$)'), 'Please enter the measurement made: '
read(5,* ) meas
write(6,'(A,$)'), 'Please enter the number of mixing times: '
read(5,* ) itm

do 11 il=1,itm
   write(6,'(a24, i2)'), 'Please enter mixing time',il
   read(5,* )tm(il)
11 continue

do 13 il=1,itm
   write(6,'(a51, i2)'), 'Please enter the name of Felix peaklist output file',il
   read(5,* )felix(il)
13 continue

write(6,'(A,$)'), 'Please enter the number of peaks '
read(5,*) ipk

do 15 i1=1,ipk
   write(6,'(a23, i2)'), 'Please enter assignment',i1
   read(5,*) assn(i1)
15 continue

c READ IN ALL FELIX FILES ****************************************

do 19 i2=1,itm
   open(1, file=felix(i2), status= 'old')
   read(1,*)junk
   read(1,*)junk
   do 17 i 1=1 ,ipk
      read(1,*) y, z, s1(i2,i1), s2(i2,i1), int(i2,i1)
   c write(6,*)int(i2,i1)
17 continue
   close(1)
19 continue

c READ IN ALL FELIX FILES *****************************************

c READING FINISHED ***********************************************

c WRITING A FILE FOR EACH PEAK************************************

do 27 i1=1,ipk
   open(2, file=assn(i1), status='new')
   write(2,21) meas, spectra, "13C & 15N ProtG", assn(i1), s1(1,i1), s2(1,i1)
21 format (a8, a13, a17, 5x, a7, F10.3, F10.3)
   do 25 i2=1,itm
write(2,23) tm(i2), int(i2,i1)

23 format (F8.6, 5x F14.4)

25 continue

    close(2)

27 continue

9999 end