APPLICATIONS OF PROTON AND FLUORINE
NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY
TO THE STUDY OF LARGE ORGANIC MOLECULES

BY

CHRISTOPHER WILLIAM MAITLAND GRANT
B.Sc. (Hon.), McMaster University, 1968

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in the Department
of
CHEMISTRY

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA
March, 1972
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Chemistry

The University of British Columbia
Vancouver 8, Canada

Date Mar. 29, 1972
ABSTRACT

This thesis is divided into three chapters; each involving a different approach to the use of both proton (¹H) and fluorine (¹⁹F) nuclear magnetic resonance (n.m.r.) to study large organic molecules in solution. To some extent the three chapters represent an evolution in technique of the scientific world in general and of our laboratory in particular.

Previous students in this laboratory have used high resolution n.m.r. spectroscopy (of ¹H, ³¹P and ¹⁹F nuclei) combined with double irradiation techniques to study progressively larger organic molecules. In the first chapter this work has been extended to "natural products" - molecules not previously very susceptible, because of their spectral complexity, to detailed n.m.r. studies. In particular ¹H and ¹⁹F n.m.r. have been employed in conjunction with heteronuclear noise modulated decoupling and ¹H-¹H INDO. A series of steroids substituted with fluorine in the A, B or D rings have been examined as model systems: 2α-fluoro-cholestan-3-one, 6α- and 6β-fluoro-cholest-4-en-3-one and 16,16-difluoro-androst-5-en-3β-ol-17-one. In each case it has been possible to obtain coupling constants and chemical shifts for the nuclei in the area of the fluorine atom and hence to derive structural data from the n.m.r. spectra in spite of their complexity. The ¹H-¹H INDO technique alone has been further applied to several problems posed by organic chemists involved with natural products. In each case this approach has been successful.
Recently very large molecules such as enzymes have been studied by n.m.r. via their effects on the chemical shift and line width of smaller molecules with which they interact. As part of a programme to investigate the application of heteronuclear n.m.r. to problems of biological interest, we have used the above technique to study the interaction of various N-trifluoroacetylated monosaccharides with the enzyme, lysozyme. The $^{19}$F chemical shifts of N-trifluoroacetyl-$D$-glucosamine and its methyl glycosides have been studied as a function of enzyme concentration. The results suggest that the fluorine substituents affect the binding process to some extent but that such effects can be informative. The possibility of using n.m.r. to study the effects of substituents on enzyme-inhibitor interactions led to a study of the monosaccharides, N-acetyl-$D$-glucosamine-$\alpha$-methyl glycoside and its C$_6$-iodo and C$_5$-methyl derivatives, all three of which gave similar results. This work is treated in Chapter II along with a brief discussion of the conformations of the inhibitors involved.

Very recently organic chemists and biochemists have begun employing pulsed n.m.r. equipment in a variety of problems. We have become interested in the applications of relaxation time measurements to structural problems. In Chapter III pulsed $^1$H n.m.r. experiments involving several model systems are reported: mixtures of the cis and trans isomers of 1,2-dichloro and 1,2-dibromoethylene and of the ethyl esters of maleic and fumaric acids have been studied. The results of these experiments are encouraging, indicating that, in this case at least, relaxation times are sensitive to structure and substituent in a consistent fashion. We have experimented almost exclusively with
selective pulse techniques and have built and used a variety of equipment. In the Appendix are described two audiofrequency-pulse units which can be attached to a Varian HA-100 n.m.r. spectrometer and which were used for the experiments discussed in Chapter III. The same audiofrequency-pulse techniques have been applied to the measurement of nuclear relaxation times of individual protons in the alkaloid, vindoline, and in the sugar, 3,4,6-tri-O-acetyl-1-O-benzoyl-2-bromo-2-deoxy-ß-D-glucopyranose and its 2-chloro analogue with less encouraging results. In addition we have reported the use of $^{19}$F pulsed n.m.r. to calculate the rate constants, $k_{-1}$ and $k_{1}$, for the association of the α-anomer of N-trifluoroacetyl-D-glucosamine with lysozyme.
# TABLE OF CONTENTS

## CHAPTER I. FLUORINE N.M.R. AND THE INDOR TECHNIQUE AS

**PROBES OF NATURAL PRODUCT STRUCTURE** .................................. 1

**Introduction** ................................................................. 1

**Results and Discussion** ................................................... 8

A. **Studies of Fluorosteroids** .......................................... 8

B. **Application of $^1$H–$^1$H INDOR to Natural Products** ......... 31

**Experimental** ................................................................. 41

## CHAPTER II. FLUORINE AND PROTON N.M.R. AS APPLIED TO CERTAIN ASPECTS OF THE INTERACTION OF LYSOZYME WITH MONOSACCHARIDE INHIBITORS** ........................................... 42

**Introduction** ................................................................. 42

**Results and Discussion** ................................................... 52

A. **Choice of a Suitable Chemical Shift Reference** .................. 52

B. **Experiments with N-Trifluoroacetyl-$^4$-glucosamine** ......... 53

C. **Experiments with $C_6$-Substituted N-Acetyl-$^4$-glucosamine-$\alpha$-methyl glycosides** ................................. 71

D. **Conformation of Free Monosaccharide Inhibitors** ............... 83

E. **N.M.R. as a Probe for Conformation of Bound Monosaccharide Inhibitors** ....................................................... 92

**Experimental** ................................................................. 98
CHAPTER III. APPLICATION OF THE AUDIOFREQUENCY PULSE TECHNIQUE TO THE STUDY OF LARGE ORGANIC MOLECULES IN SOLUTION

Introduction ................................................................. 105
Results and Discussion ..................................................... 113
  A. A Case for the Applicability of $T_1$ and $T_2$ to Structural Organic Chemistry ...................... 113
  B. Measurement of Nuclear Relaxation Times of an Alkaloid by the Audiofrequency Pulse Technique ......................... 123
  C. Measurement of Nuclear Relaxation Times of Carbohydrate Derivatives by the Audiofrequency Pulse Technique ......................... 131
  D. The Audiofrequency Pulse Technique and Enzyme-Inhibitor Rate Constants ......................... 138

Experimental ............................................................... 145

APPENDIX A. PRINCIPLES OF PULSED N.M.R. SPECTROMETRY WITH PARTICULAR REFERENCE TO HIGH RESOLUTION EXPERIMENTS ... 148

APPENDIX B. AUDIOFREQUENCY PULSE SPECTROMETERS EMPLOYED ... 153

REFERENCES ............................................................... 162
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHAPTER I</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>N.M.R. Parameters for the Ring A Resonances of 2α-Fluorocholesterol-3-one (1)</td>
</tr>
<tr>
<td>2</td>
<td>N.M.R. Parameters for Parts of Rings A and B of 6α- and 6β-Fluorocholest-4-en-3-one (3 and 4)</td>
</tr>
<tr>
<td>3</td>
<td>N.M.R. Parameters for the Ring D of 16,16-Difluoroandrost-5-en-3β-ol-17-one (5)</td>
</tr>
<tr>
<td>4</td>
<td>N.M.R. Parameters for Compounds 6 (Cyano-aldehyde), 9, 10, and 11 (Decalones)</td>
</tr>
<tr>
<td><strong>CHAPTER II</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>¹⁹F Chemical Shift Data for N-Trifluoroacetyl-D-glucosamine (1 and 2) and its Methyl Glycosides (3 and 4) in the Presence of Lysozyme</td>
</tr>
<tr>
<td>2</td>
<td>Linewidth Data for N-Trifluoroacetyl-D-glucosamine (1 and 2) and its Methyl Glycosides (3 and 4) in the Presence of Lysozyme</td>
</tr>
<tr>
<td>3</td>
<td>K_P, K_B and Δ Values for Compounds 1 to 4; also ¹H Chemical Shift Data for 3 and 4 Competing with N-Acetyl-D-glucosamine-α-methyl-glycoside for Lysozyme</td>
</tr>
<tr>
<td>4</td>
<td>Chemical Shift Data for the N-Acetyl Protons of 7, 9, and 10 and for the C_5-Methyl of 10 Interacting with Lysozyme</td>
</tr>
</tbody>
</table>
### Table

<table>
<thead>
<tr>
<th>Page</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>$K_D$, $K_B$, and $\Delta$ Values for Compounds 7, 9, and 10; also $^1$H Chemical Shift of Glycoside Methyl on Binding</td>
</tr>
<tr>
<td>6</td>
<td>N.M.R. Parameters for the O-Acetate Derivatives of 3', 4', 7', 9', and 10'</td>
</tr>
</tbody>
</table>

### CHAPTER III

<table>
<thead>
<tr>
<th>Page</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Relaxation Time Data for 3,4,6-Tri-O-acetyl-1-O-benzoyl-2-bromo-2-deoxy-β-D-glucopyranose (1) and its 2-Chloro analogue (2)</td>
</tr>
<tr>
<td>2</td>
<td>Nuclear Relaxation Time Data for the $\alpha$- and $\beta$-Anomers of the Free Sugar, N-Trifluoroacetyl-β-D-glucosamine</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The $^1$H n.m.r. spectrum (100 MHz) of 2α-fluorocholestan-3-one (1) in CDCl$_3$ solution. A. The normal spectrum; B. The spectrum measured with simultaneous irradiation at the $^{19}$F resonance frequency.</td>
</tr>
<tr>
<td>2</td>
<td>The $^1$H-$^1$H INDOR experiments performed on the $^{19}$F decoupled spectrum of 1 in CDCl$_3$ solution.</td>
</tr>
<tr>
<td>3</td>
<td>The $^1$H-$^1$H INDOR experiments performed on the normal spectrum of 1 in CDCl$_3$ solution.</td>
</tr>
<tr>
<td>4</td>
<td>Partial $^1$H n.m.r. spectrum of the highfield region of 1 in CDCl$_3$ solution. A. The normal spectrum; B. the $^{19}$F decoupled spectrum.</td>
</tr>
<tr>
<td>5</td>
<td>The $^{19}$F n.m.r. spectra (94.071 MHz) of 1 in CDCl$_3$ solution.</td>
</tr>
<tr>
<td>6</td>
<td>The normal $^1$H n.m.r. spectra of 3 (A) and 4 (B) in CDCl$_3$ solution.</td>
</tr>
<tr>
<td>7</td>
<td>The $^{19}$F n.m.r. spectra (94.071 MHz) of 3 (A), 4 (B) and 5 (C) in CDCl$_3$ solution.</td>
</tr>
<tr>
<td>8</td>
<td>The $^1$H-$^1$H INDOR experiments performed on the $^{19}$F decoupled spectrum of 5 in CDCl$_3$ solution.</td>
</tr>
<tr>
<td>9</td>
<td>The $^1$H n.m.r. spectrum (100 MHz) of a steroid (6) reaction mixture in CDCl$_3$ solution.</td>
</tr>
<tr>
<td>10</td>
<td>The $^1$H n.m.r. spectra of decalones 9 (A), 10 (B), and 11 (C) at 100 MHz in CDCl$_3$ solution.</td>
</tr>
</tbody>
</table>
**Figure** | **Page**
---|---
1 | The section of a natural polysaccharide substrate which would occupy lysozyme's active site during lysis ................................. 47
2 | $^{19}$F n.m.r. spectra (94.071 MHz) of a mutarotating solution of N-trifluoroacetyl-$\alpha$-D-glucosamine ($\alpha$-anomer, 1) in pH 5.5 citrate buffer ............. 55
3 | $^{19}$F n.m.r. spectra recorded during a study of 1 and 2 ($\alpha$- and $\beta$-anomers of N-trifluoroacetyl glucosamine) with lysozyme ................................. 57
4 | A. Graph of $^{19}$F chemical shift data from the study of 1 with lysozyme  
B. Increase in $\delta$ as a function of time during the mutarotation of pure $\alpha$-anomer (1) to an equilibrium mixture in the presence of lysozyme .................. 61
5 | $^1$H n.m.r. spectra recorded during a study of C$_5$-methyl-N-acetyl glucosamine-$\alpha$-methyl glycoside (10) with lysozyme ................................. 74
6 | Graphs of chemical shift data for 10, 7 and 9 interacting with lysozyme ................................. 77
7 | A. $^1$H n.m.r. spectrum (100 MHz) of N-acetyl glucosamine $\alpha$-methyl glycoside (7) in D$_2$O  
B. Partial $^1$H n.m.r. spectrum (100 MHz) of C$_6$-iodo-N-acetyl glucosamine-$\alpha$-methyl glycoside diacetate (9') in CDCl$_3$ solution ......................... 84
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Partial $^1$H n.m.r. spectra (100 MHz) of A. N-trifluoroacetyl glucosamine-α-methyl glycoside triacetate (3') and B. N-trifluoroacetyl glucosamine β-methyl glycoside triacetate (4') in CDCl₃ solution</td>
<td>86</td>
</tr>
<tr>
<td>9</td>
<td>Computer-simulated spectra of the H₁ 'doublet' region of a hypothetical N-acetyl glucosamine-α-methyl glycoside in aqueous solution</td>
<td>94</td>
</tr>
</tbody>
</table>

CHAPTER III

1. The alkene proton regions of the n.m.r. spectra (100 MHz, solvent CDCl₃) of A. 1,2-dibromo- and 1,2-dichloroethylene (cis and trans isomers) and B. maleic and fumaric acid diethyl esters (plus cis-1,2-dichloroethylene) | 115   |
2. Effect of a modified Carr-Purcell sequence on the magnetization vector, $M_0$ | 119   |
3. Effect of a $T_1$ sequence on the magnetization vector, $M_0$ | 121   |
4. The $^1$H n.m.r. spectrum (100 MHz) of the alkaloid, vindoline, in CDCl₃ solution | 125   |
5. A. Scope photograph of a typical Carr-Purcell sequence on the C₄-acetate resonance of vindoline. B. Scope photograph of a typical $T_1$ pulse sequence on the same resonance | 126   |
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Relaxation time data obtained on the alkaloid, vindoline, in CDCl₃ solution.</td>
<td>129</td>
</tr>
<tr>
<td>7</td>
<td>Partial ¹H n.m.r. spectra (100 MHz) of 3,4,6-tri-O-acetyl-1-O-benzoyl-2-bromo-2-deoxy-β-D-glucopyranose (A) and its 2-chloro analogue (B) in C₆D₆.</td>
<td>133</td>
</tr>
<tr>
<td>8</td>
<td>A. Plots of 1/T₁ and 1/T₂ vs. [E₀] for the N-trifluoroacetyl group of the α-anomer. B. Plot of (1/T₂ - 1/T₁) vs. [E₀] for the α-anomer.</td>
<td>143</td>
</tr>
</tbody>
</table>

**APPENDIX**

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Scope traces of various pulses on the alkene resonance of the diethyl ester of maleic acid.</td>
<td>151</td>
</tr>
<tr>
<td>2</td>
<td>Block diagram of the components used in the &quot;Mark I&quot; audiofrequency-pulse spectrometer.</td>
<td>154</td>
</tr>
<tr>
<td>3</td>
<td>Block diagram of the components used in the &quot;Mark I&quot; pulse unit.</td>
<td>156</td>
</tr>
<tr>
<td>4</td>
<td>Block diagram of the components used in the &quot;Mark II&quot; audiofrequency-pulse spectrometer.</td>
<td>159</td>
</tr>
<tr>
<td>5</td>
<td>Block diagram of the components used in the &quot;Mark II&quot; pulse unit.</td>
<td>160</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

Firstly, I would like to thank Dr. L.D. Hall for his direction in this work - not only as a source of information and ideas, but also for his guidance in developing a more mature scientific philosophy.

Secondly I would like to acknowledge my numerous helpful discussions with Ben Malcolm and Dr. P. Steiner of this laboratory regarding my early work, with Dr. A.G. Marshall about theoretical aspects and with Roland Burton concerning n.m.r. electronics.
CHAPTER I

FLUORINE N.M.R. AND THE INDORE TECHNIQUE AS
PROBES OF NATURAL PRODUCT STRUCTURE

Introduction

The use of $^1$H nuclear magnetic resonance (n.m.r.) spectroscopy as a method for studying small organic molecules is well known. But with increasing molecular size and complexity, the method becomes considerably less useful simply because the spectrum becomes so complex as to be not readily analysable; indeed it often happens that a resonance of particular interest is obscured by the overlapping transitions of other resonances, which is the so-called "hidden-resonance" problem. A number of methods have evolved to overcome this problem and these include the development of ever larger and more powerful magnets. Also various double resonance techniques have become available in the last decade and these can, in many cases, help considerably in the interpretation of complex n.m.r. spectra.

Another method of obtaining n.m.r. data on molecules with complex $^1$H n.m.r. spectra is to look at the n.m.r. spectrum of some heteronuclear label (i.e. some spin 1/2 nucleus other than protons) which has been

* An acronym coined by Baker$^1$ for InterNuclear Double Resonance.
specifically incorporated for that purpose or of heteronuclei already present in the molecule (e.g. $^{31}\text{P}$ or $^{13}\text{C}$). This laboratory has investigated both of the above techniques and their application to ever-larger molecules, e.g. sugars\textsuperscript{2,3} and cyclic phenyl phosphates.\textsuperscript{4}

Success in such studies encouraged us to investigate the potential of these methods with still larger and more complex molecules and "natural products" presented a next logical step in this direction. Various $^1\text{H}$ n.m.r. methods have already been tried in this area but have met with limited success.\textsuperscript{5} In some areas researchers have looked for empirical chemical shift relationships; for example, in the steroid area the relationship between the $C_{13}$ methyl group and ring geometry has been studied.\textsuperscript{6,7} More recently, however, shift reagents have been applied to natural products\textsuperscript{8} and this approach promises to be quite useful, although little has appeared in the literature on steroids as yet.

"Natural products" are of considerable importance, yet organic chemists who isolate and synthesize them often have considerable difficulty in learning their chemical identity or configuration. The n.m.r. problem in this case is often that transitions of interest are located in the "methylene envelope" which generally extends from $\tau \approx 7.5$ to $\tau \approx 9.0$. Our first experiments involved the use of n.m.r. spectroscopy in an attempt to examine the configuration of the basic steroid skeleton. Sites of interest were labelled with fluorine.\textsuperscript{9} This had two major benefits:

1. the electronegative $^{19}\text{F}$ nucleus tended to deshield one or more neighbouring protons to low field of the methylene envelope where they
could be used for $^1H-\{^1H\}$ INDOR experiments,

2. since fluorine is a 100% spin 1/2 atom, its own n.m.r. spectrum could be readily examined and the $^1H-^{19}F$ coupling constant data used to augment the proton data. Fluorine was also found (see text) to be useful as a subject of spin decoupling experiments which made spectral assignments very easy.

The information obtained by the above methods is basically a set of coupling constants and chemical shifts for the groups of nuclei investigated. Relative signs of coupling constants can in many cases also be obtained by the double resonance techniques used, but were not investigated here. Small differences in chemical shift between different molecules provide a very limited structural probe because of the many un-predictable factors involved. Coupling constants, on the other hand, are a very useful structural probe. Vicinal $^1H-^1H$ and $^1H-^{19}F$ coupling constants are known to follow fairly well-defined relationships as a function of bond angle.$^3,13$

The double resonance techniques employed in this work have been quite thoroughly described in the literature. They all involve the perturbing effect of a second radio-frequency field (i.e., in addition to the extremely weak field being used to observe the spectrum). This effect depends on the strength of the perturbing field, $\gamma H_2/2\pi$ Hz, (where $\gamma$ is the nuclear gyromagnetic ratio and $H_2$ is the perturbing r.f. field) compared with its distance from a given line. Early use of double resonance was confined to perturbing fields such that $(\gamma H_2/2\pi) > J$ where $J$ is the spin coupling constant which is to be removed. Such power levels lead to "spin decoupling" experiments.$^{14,15}$
In many situations however, the high perturbing field power levels necessary for spin decoupling are unsatisfactory because of the low selectivity of the resultant experiment. Freeman and Anderson\textsuperscript{16} have discussed the theory and merits of using weak perturbing fields \((\gamma H_z/2\pi)\) of the order of the transition linewidth at half height. These lower power techniques include "spin tickling" and "INDOR".\textsuperscript{1,17-19}

We have found that for \(^1\text{H}-\text{ }^1\text{H}\) double resonance experiments, the lowest power (and hence most selective) form of INDOR is convenient. This technique involves observing one sharp, well-resolved peak in a spectrum while slowly scanning a weak perturbing field through other regions of the spectrum. The peak being observed corresponds to a transition between two nuclear energy levels:

\[
\begin{array}{c}
\text{E} \\
\uparrow
\end{array}
\quad \alpha
\quad \beta
\begin{array}{c}
\downarrow
\end{array}
\]

The intensity of this peak is related to the ratio, \(\frac{\text{population } \beta}{\text{population } \alpha}\). If the weak perturbing field reduces this ratio for the peak one is observing, the peak will become smaller, and vice versa. A weak perturbing field can cause such population changes to occur when the nucleus being monitored is spin-coupled to other nuclei.

For instance, consider monitoring the peak corresponding to a transition \(A_1\) of the following two spin AX system:\textsuperscript{10}
The peak $A_1$ corresponds to a transition between levels $\beta \alpha$ and $\alpha \alpha$. These levels are also involved with the $X_2$ and $X_1$ lines, but not with the $A_2$ line, of the spectrum. Sweeping a weak perturbing radio-frequency field through $X_1$ and $X_2$ should cause intensity changes in $A_1$: a decrease as the $\alpha \beta - \alpha \alpha$ transition pumps spins into the $\alpha \alpha$ level, and an increase as the $\beta \beta - \beta \alpha$ transition pumps spins into the $\beta \alpha$ level.

A convenient way to run such experiments is to arrange the n.m.r. equipment so that the recorder pen Y-axis monitors the intensity of a particular peak while the X-axis corresponds to the position of the weak perturbing field as it is swept through the spectrum. The result of the above experiment would be:  

\[ \text{A convenient way to run such experiments is to arrange the n.m.r. equipment so that the recorder pen Y-axis monitors the intensity of a particular peak while the X-axis corresponds to the position of the weak perturbing field as it is swept through the spectrum. The result of the above experiment would be.} \]
This model is readily extended to a system of three or more spins. From this then it can be seen that one proton can be monitored and used to find the transitions of other protons with which it is spin coupled (e.g., in the previous diagram, the X lines would have been detected even had they been entirely hidden beneath the methylene envelope of a steroid). The INDOR technique is so selective as to be applicable as long as individual lines can be identified, preferably on a first order basis. The limitation is that some proton connected with the site of interest must be visible for monitoring.

We report here the use of the above approach to study the A-ring of 2α-fluoro-cholestan-3-one, the B-ring of 6α- and 6β-fluoro-cholesterol-4-en-3-one and the D-ring of 16,16-difluoro-androst-5-en-3β-ol-17-one. These steroids all have the commonly-occurring trans 5-10 and 13-14 ring junctions. An additional interest in these particular molecules is associated with the fluorine substituent itself since fluorine as a steroid "label" is interesting from a biological viewpoint (a number of fluorinated steroids have shown biological activity20 - sometimes enhanced21). Partly for this reason, the literature on introduction of such a label is considerable.22

Nevertheless, in terms of the general applicability of the n.m.r. method, one would hope to be able to routinely analyze natural product n.m.r. spectra without having to resort to spin labelling. We have found that in many cases the natural product site of interest, being the reaction site, is marked by a double bond, an oxygen-containing group, or some other electronegative species. Such a group often serves to deshield at least one neighbouring proton so that it appears to low
field of the methylene envelope. It can then usually be employed in INDO
experiments to pick out the transitions of neighbouring protons which are still buried in the methylene envelope. This technique was found to work consistently well in identifying the products of several new reactions on natural products. The results of some typical problems are included at the end of this chapter.
Results and Discussion

A. Studies of Fluorosteroids

2α-Fluoro-cholestan-3-one (1)

This compound will be discussed in some detail as it represents a "typical" case; the other molecules will be more briefly dealt with except where they posed special problems.

The normal \(^1\)H n.m.r. spectrum of 1 (Fig. 1A) clearly shows the
Fig. 1. The $^1$H n.m.r. spectra (100 MHz) of 2α-fluoro-cholestan-3-one (1) in deuterochloroform solution.

A. The normal spectrum
B. The spectrum measured with simultaneous irradiation at the $^{19}$F resonance frequency (94, 076, 140.0 Hz). The assignment of the C-methyl resonances follows previously established criteria.$^5$ CHCl$_3$ was used for the field-frequency lock.
C₂ proton which has been deshielded from the methylene envelope by the electronegative fluorine atom. Irradiation at the \(^{19}\text{F}\) resonance frequency removes the 48 Hz geminal \(^{19}\text{F}-\text{H}_2\) coupling, collapsing this low-field octet to a quartet (Fig. 1B). Changes also occur in the methylene envelope region during fluorine decoupling and these will be discussed later.

Two series of \(^1\text{H}-\{^1\text{H}\}\) INDOR measurements were made while monitoring transitions of the clearly-discernible C₂ proton. The first of these was performed on the \(^{19}\text{F}\) decoupled spectrum. Fig. 2 shows the results of this experiment; thus, Fig. 2A is the INDOR spectrum obtained by scanning a weak radio-frequency field through the methylene envelope while monitoring its effect on transition 12 (using the numbering system of Fig. 1B). Sequential monitoring of transitions 11 and 9 gave the responses shown in Fig. 2B and C. The frequency at which an INDOR response occurs marks the position of a transition connected to the one being monitored. The sense of the response (either up or down) contains information as to the relative signs of the coupling constants involved - but in this work the signs were not of interest. The effective summation of all the INDOR responses (shown diagrammatically above the normal spectrum in Fig. 2) picks out all transitions corresponding to the C₁ protons. The four low-field INDOR responses confirm that the four low-field transitions of the normal spectrum belong to a single proton and indicate that it is one of the C₁ protons. Perhaps more importantly, the higher-field set of INDOR responses now identify the transitions of the other C₁ proton which are normally totally obscured by the resonances of other methylene protons.
The $^1\text{H}-\{^1\text{H}\}$ INDOOR experiments performed on the $^{19}\text{F}$ decoupled spectrum of 1 in deuterochloroform solution. Using the transition numbering system of Fig. 1, the spectrum in A corresponds to monitoring transition 12, B corresponds to monitoring transition 11, and C to transition 9. The composite assignment resulting from these spectra is shown diagrammatically above the normal proton spectrum. CHCl$_3$ was used as the internal-reference signal for the field-frequency lock.
The second series of INDORe measurements (Fig. 3) was done without $^{19}F$ decoupling. Five lines of the $H_{2\beta}$ resonance were monitored in turn to pick out all transitions belonging to the $C_1$ protons. Because of the decrease in intensity of the transitions being monitored, the effective signal-to-noise ratio for these responses is poorer than that of the responses shown in Fig. 2. Nevertheless it is possible to make a summation of the individual responses and obtain the complete set of transition frequencies for both of the $C_1$ protons. Due to the fortuitous, near equality of the spectral splittings, there is considerable degeneracy for the upfield $C_1$ proton.

Inspection of the lower-field portion of the normal and $^{19}F$ decoupled methylene region (Fig. 4) indicated that in addition to the changes associated with the collapse of the known $H_1$ transitions, two further proton multiplets were subject to some decoupling; likely these were from the $H_4$ protons. Several of these transitions were clearly resolved and a series of INDORe experiments in which these transitions were monitored made possible a reasonably accurate assignment of all the resonances of both $C_4$ protons. Further responses were also observed around $\tau 8.4$ and these were assigned to $H_5$. In this latter part of the work the ability to remove or retain $^1H-^{19}F$ couplings at will was very useful for identifying transitions.

Close inspection of the INDORe spectra shown in Fig. 2 and Fig. 3 reveals that some responses occur at frequencies corresponding to the C-methyl resonances. These seem to be instrumental artifacts arising from overloading of some amplifier stage in the spectrometer.

The normal $^{19}F$ spectrum of 1 is shown in Fig. 5. The comparatively
Fig. 3. The $^1\text{H}-(^1\text{H})$ INDOE experiments performed on the normal spectrum of 1 in deuterochloroform containing enough CHCl$_3$ for a lock signal. The $^1\text{H}_2$ transitions were monitored as follows: A transition 8; B transition 6; C transition 7; D transition 2; E transition 3.

A diagrammatic summary of these responses is given above the normal spectrum.
Fig. 4. Partial $^1$H n.m.r. spectrum of the high field region of 1 in deuterochloroform solution (CHCl$_3$ lock). The normal spectrum is shown in A and the $^{19}$F decoupled spectrum in B. The first-order assignments of these spectra were based on the INDOE experiments discussed in the text.
poor resolution of the undecoupled spectrum is due to the fact that the $^{19}$F substituent is part of a fairly highly-coupled proton spectrum and to the large number of small, long-range couplings. In addition to the large geminal coupling with $H_{2\beta}$ (ca. 48 Hz) a number of smaller proton couplings are resolved (Fig. 5).

The foregoing set of experiments served to identify the resonances of all the protons associated with ring A of derivative 1. Comparison of the transitions assigned to the 'normal' and $^{19}$F decoupled resonances of $H_{1\alpha}$ and $H_{1\beta}$ provided an estimate of the magnitudes of the $^1H-^1H$ and $^{19}F-^1H$ couplings, which proved to be in reasonable accord with the estimates obtained by direct measurement of the $^{19}$F spectrum (Fig. 5). These data are included in Table 1. Simulation of the spectra of the $H_1$ and $H_{2\beta}$ resonances using the parameters involving these three nuclei and the $F_{2\alpha}$ substituent, gave calculated transition energies which were in close accord with the experimental values; on this basis it was concluded that a full, iterative analysis was unnecessary.

Interestingly, the $^1H-^1H$ couplings for the $H_1$ and $H_2$ protons listed in Table 1 are identical, within experimental error, with those previously reported by Allinger et al. for 2α-fluoro-5α-androstane-3,17-dione.

Evaluation of the $H_4$ and $H_5$ resonances proved to be more difficult. However, in spite of the small chemical shift separation of the two $C_4$ protons, a satisfactory estimate of the inter-proton couplings was readily made by examination of the $^{19}$F decoupled spectra and by iterative, computer-based analysis involving the $H_{4\alpha}$, $H_{4\beta}$ and $H_5$ resonances. Because of several fortuitous degeneracies, several
Fig. 5. The $^{19}$F n.m.r. spectra (94.071 MHz) of 1 in deuterochloroform solution. The field-frequency lock in this case was CFCl$_3$. The normal spectrum is shown in the lower trace. The insert was recorded at the same gain but with simultaneous irradiation of the entire $^1$H spectrum using a noise-modulated radio-frequency centered at 99, 997, 945.0 Hz. A partial first order assignment of the smaller couplings is shown.
attempts were required before a full analysis which included the $^{19}\text{F}$ couplings could be made. It should be noted that the presence of a spin 1/2 heteronucleus was crucial for these assignments since in its absence it would have been impossible to assign the $H_{4\alpha}$ and $H_{4\beta}$ resonances. The $^{19}\text{F}-H_{4\alpha}$ coupling was assigned an absolutely positive sign on the basis of previous studies.24

We turn now to consider the conformational significance of the coupling constants determined for 1. In a previous study, Abraham and Holker25 had rationalized the vicinal $H_1-H_2$ couplings of the 2α-bromo analog (2) of the derivative we were studying in terms of a slightly distorted chair conformation for ring A. Since the $H_1-H_2$ couplings of 1 are closely similar to those of 2 it seems probable that both systems have similar conformations. The remaining couplings determined for 1 appear to support this contention and hence, indirectly, the validity of previous conclusions. Thus the $H_{4\beta,5}$ coupling of 14.4 Hz clearly accords with a trans-diaxial relationship between these two protons. Furthermore, the magnitudes of the vicinal $^{19}\text{F}-H$ couplings are only consistent with a gauche relationship between the $F_{2\alpha}$ substituent and the two protons at $C_1$. Although there is a paucity of data concerning $J_{F,H}$ couplings, the values listed in Table 1 for the couplings between $F_{2\alpha}$, $H_{4\alpha}$ and $H_{4\beta}$ appear to be in reasonable accord with expectation.24

One of the more interesting points which we wished to evaluate in the course of this study was whether it might be possible to use a fluorine substituent to label a specific site of a steroid molecule for subsequent n.m.r. studies. It is obvious from the normal $^{19}\text{F}$ spectrum of 1 (Fig. 5) that, for this molecule at least, conventional
Table 1. N.M.R. Parameters for the Ring A Resonances of 2α-Fluorocholestan-3-one (1)

**Chemical shifts (τ-values, \( \phi_c \)-values)**

<table>
<thead>
<tr>
<th></th>
<th>( H_{1\alpha} )</th>
<th>( H_{1\beta} )</th>
<th>( H_{2\beta} )</th>
<th>( H_{4\alpha} )</th>
<th>( H_{4\beta} )</th>
<th>( H_5 )</th>
<th>( F )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>8.52</td>
<td>7.52</td>
<td>5.01</td>
<td>7.79</td>
<td>7.65</td>
<td>8.47</td>
<td>( \phi_c + 194.5 )</td>
</tr>
</tbody>
</table>

**Coupling constants (Hz)**

<table>
<thead>
<tr>
<th></th>
<th>( H_{1\alpha}H_{1\beta} )</th>
<th>( H_{1\alpha}H_{2\beta} )</th>
<th>( H_{1\beta}H_{2\beta} )</th>
<th>( H_{2\beta}F )</th>
<th>( H_{1\alpha}F )</th>
<th>( H_{1\beta}F )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>-12.2( ^\dagger )</td>
<td>12.3( ^\dagger )</td>
<td>6.9( ^\dagger )</td>
<td>48.0( ^\dagger )</td>
<td>11.5( ^\dagger )</td>
<td>4.5( ^\dagger )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>( H_{4\alpha}H_{4\beta} )</th>
<th>( H_{4\alpha}H_{5\alpha} )</th>
<th>( H_{4\beta}H_{5\alpha} )</th>
<th>( H_{4\alpha}F )</th>
<th>( H_{4\beta}F )</th>
<th>( H_{5\alpha}F )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>-13.9( ^\ddagger )</td>
<td>2.1( ^\ddagger )</td>
<td>14.4( ^\ddagger )</td>
<td>7.3( ^\ddagger )</td>
<td>-2.0( ^\S )</td>
<td>0</td>
</tr>
</tbody>
</table>

* Measured in deuterochloroform solution containing CDCl\textsubscript{3} or CFCl\textsubscript{3}

\( ^\dagger \) Estimated error \( \pm 0.2 \) Hz

\( ^\ddagger \) Estimated error \( \pm 0.4 \) Hz

\( ^\S \) Estimated error \( \pm 1.0 \) Hz

Note: The error limits indicated by \( ^\dagger \) and \( ^\S \) represent the limits between which the coupling constants were found to vary when the individual transitions were varied over the maximum possible limit.
$^{19}$F measurements have little to recommend them. However, the detection of a $^{19}$F resonance while simultaneously irradiating the proton spectrum appears to be a more attractive proposition. In the case of 1, the $^1$H resonances were spread over ca. 4.5 p.p.m. and continuous-wave $^{19}$F-$^1$H decoupling experiments only resulted in partial collapse of the proton couplings. However, noise-modulated $^{19}$F-$^1$H decoupling effectively removed all of the proton couplings and, as is indicated in the insert of Fig. 5, the $^{19}$F resonance was then detected as a reasonably sharp singlet.

6α- and 6β-Fluoro-cholest-4-en-3-one

The normal $^1$H n.m.r. spectra of 6β-fluoro-cholest-4-en-3-one (compound 3) and 6α-fluoro-cholest-4-en-3-one (compound 4) in deuterochloroform solution are shown for comparison in Fig. 6. A few drops of chloroform was used for the internal field-frequency lock. Both $^1$H₄ and $^1$H₆ appear to low field in each case. $^1$H₆ shows the characteristic geminal $^{19}$F-$^1$H coupling of ca. 50 Hz.

An interesting difference between the two spectra is the splitting of the C₁₀ methyl group into a doublet in the 6β-fluoro steroid (Fig. 6A) whereas it is a sharp singlet in the 6α-fluoro analog (Fig. 6B).
Fig. 6. The normal $^1$H n.m.r. spectra of 3 (A) and 4 (B) in deuterochloroform solution containing CHCl$_3$ for a field-frequency lock.

The inserts are expansions of the C$_{10}$ methyl group region showing the effect of irradiation at the $^{19}$F resonance frequency (94, 078, 509.0 Hz for 3 and 94, 079, 100.0 Hz for 4).
This phenomenon has been investigated and discussed for a number of fluorinated steroids by Cross and Landis. Both \( \text{C}_{10} \) and \( \text{C}_{13} \) methyl groups have been observed to be split into doublets under certain conditions in steroids containing a \( ^{19}\text{F} \) substituent. Cross and Landis rationalized the splittings in terms of a long-range \( ^{19}\text{F}-^{1}\text{H} \) coupling. This explanation has now been confirmed for the first time by \( ^{19}\text{F} \) decoupling (see expanded inserts with and without \( ^{19}\text{F} \) decoupling) in Fig. 6. Such splittings could be quite readily understood if the fluorine were close to the methyl group in question - but when the \( ^{19}\text{F} \) is five or six \( \sigma \) bonds away, the phenomenon seems worth remarking on.

The actual transmission mechanism of the coupling (both 'through bond' and 'through space' have been argued) is still questionable but all known cases have been found to follow the "converging-vector rule": "long range coupling between angular methyl protons and fluorine five or more \( \sigma \)-bonds apart may occur only when a vector directed along the C-F bond, and originating at the carbon atom, converges upon and intersects a vector drawn along an angular methyl C-H bond in the direction of the proton, and originating at the methyl carbon."

In both 3 and 4, the \( \text{H}_4 \) resonance could be readily examined by \( ^{19}\text{F} \) decoupling to determine the \( \text{FH}_4 \) splitting. The \( \text{H}_4\text{H}_6 \) splitting was also clearly assignable during these decoupling experiments. In both anomers part of the highly coupled \( \text{H}_1\text{H}_2 \) proton system is visible at the extreme low-field end of the methylene envelope, but unambiguous INDO work was not possible with this system - it was both second order and difficult to assign.

The systems comprising \( \text{H}_4, \text{H}_6, \text{H}_{7a}, \text{H}_{7b}, \text{H}_8 \) and \( ^{19}\text{F} \) were close
enough to first order to enable INDIR analysis. H₆ was the logical set of transitions to monitor during INDIR experiments designed to find the C₇ protons and H₈. Unfortunately the H₆ lines were broadened and poorly-resolved for both isomers. With $^{19}$F decoupling it was possible to monitor H₆ while running INDIR scans to find both C₇ protons (Fig. 6). However, the quality of the INDIR responses did not allow an unambiguous determination of the position of H₈. For both 3 and 4 the 7α-proton was entirely hidden in the methylene envelope, but several transitions of the 7β-proton could be distinguished.

Without the $^{19}$F decoupling, the INDIR response quality was even lower. However, the H₇/β - F splittings could be picked out for 3 and 4 by direct comparison between the decoupled and undecoupled spectra. A good estimate of the H₇/α - F couplings was also obtained from the $^{19}$F spectra.

At this stage, the next step should have been to analyze the chemical shift and splitting data (with the help of a computer program) to obtain 'true' chemical shifts and coupling constants. This was not done here because a reliable value for the H₈ chemical shift was not found through the INDIR experiments. Nevertheless, the nuclei at C₄, C₆, and C₇ can be seen (Fig. 6) to form a virtually first order system - and the H₇ transitions found by INDIR seem to indicate that H₈ is not very close to either. This means that one can fairly safely take the observed chemical shifts and splittings to represent true, first order values within experimental error (Table 2).

The normal $^{19}$F n.m.r. spectrum of 3 is shown in Fig. 7A, and that of 4 in Fig. 7B. That of compound 3 in particular is broadened by
Table 2. N.M.R. Parameters for Parts of Rings A and B of 6α- and 6β-Fluoro-cholest-4-en-3-one (3 and 4)

### First order chemical shifts (τ-values, φc-values)*

<table>
<thead>
<tr>
<th>Compound</th>
<th>H₄</th>
<th>H₆</th>
<th>F₆</th>
<th>H₇α</th>
<th>H₇β</th>
<th>C₁₀-CH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 (6β-F)</td>
<td>4.16</td>
<td>5.05</td>
<td>φc + 165.61</td>
<td>8.81</td>
<td>7.84</td>
<td>8.70</td>
</tr>
<tr>
<td>4 (6α-F)</td>
<td>3.97</td>
<td>4.94</td>
<td>φc + 183.63</td>
<td>8.79</td>
<td>7.74</td>
<td>8.84</td>
</tr>
</tbody>
</table>

### First order coupling constants (Hz)

<table>
<thead>
<tr>
<th>Compound 3</th>
<th>H₄H₆α</th>
<th>H₆αH₆α</th>
<th>H₆αH₇β</th>
<th>H₆αH₇α</th>
<th>H₇αH₇β</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>49.5</td>
<td>2.5±0.8</td>
<td>2.6±0.8</td>
<td>13.0±1.0</td>
</tr>
<tr>
<td></td>
<td>H₇αH₇β</td>
<td>H₇βH₈</td>
<td>C₁₀-CH₃</td>
<td>H₇αF</td>
<td>H₇βF</td>
</tr>
<tr>
<td></td>
<td>-13.5±1.0</td>
<td>3.0±0.8</td>
<td>1.9</td>
<td>47±3</td>
<td>13.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound 4</th>
<th>H₄H₆β</th>
<th>H₆βH₆β</th>
<th>H₆βH₇β</th>
<th>H₆βH₇α</th>
<th>H₇αH₇β</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.8</td>
<td>47.8</td>
<td>5.8</td>
<td>11.8</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>H₇αH₇β</td>
<td>H₇βH₈</td>
<td>C₁₀-CH₃</td>
<td>H₇αF</td>
<td>H₇βF</td>
</tr>
<tr>
<td></td>
<td>-10.8</td>
<td>2.5</td>
<td>0</td>
<td>8.8</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* Measured in deuterochloroform solution containing CHCl₃ or CFCl₃

Unless otherwise indicated, the errors in the above first order coupling constants are roughly ± 0.3 Hz.
Fig. 7. The $^{19}$F n.m.r. spectra (94.071 MHz) of 3 (A), 4 (B) and 5 (C) in deuterochloroform solution. CFCl$_3$ was used for the internal field-frequency lock.
unresolved splittings - but in both cases, the $^1H-^{19}F$ couplings are recognizable in the fluorine spectra.

In this series of experiments we have been able to find the resonances of nuclei associated with an appreciable portion of rings A and B. Wittstruck et al. have studied several $\Delta^4$-3-keto fluoro steroids by n.m.r. They showed that the $6\beta$-fluorine is more strongly spin-coupled to than is a $6\alpha$-fluorine. We have observed the same phenomenon here.

The INDOM technique in this case supplied several useful points. The location of the $C_7$ proton chemical shifts permitted a check on the first order nature of the observed $H_6-H_7$ splittings and the summation of the INDOM responses gave the $H_7-H_8$ splittings. The combination of a $^{19}F$ probe and noise modulated decoupling gave a set of vicinal $H-F$ couplings as well as making the study possible. Both $^1H-^1H$ and $^1H-^{19}F$ vicinal couplings in compounds 3 and 4 are indicative of an approximate chair conformation for ring B in spite of the double bond in ring A.

With regard to the possibility of using normal $^{19}F$ spectra to study a labelled steroid, the spectrum of compound 4 seems to be more encouraging than that of 1 or 3. Although the $^{19}F$ spectrum of 3 is broad, that of 4 could be useful.

16,16-Difluoro-androst-5-en-3β-ol-17-one

This compound provided, amongst other things, insight into the D-ring conformation - a subject of some speculation. Fig. 8 displays the $^{19}F$ decoupled proton spectrum of 16,16-difluoro-androst-4-en-3,17-dione (5) in deuterochloroform solution: chloroform was used for
the internal field-frequency lock. The insert shows the C_{18} and C_{19} protons: their normal spectrum on the left and their $^{19}$F decoupled spectrum on the right. Here we have another example of the "converging-vector rule" but this time it is the C_{13} methyl group which is split by a five bond coupling to the $\beta$-fluorine. The ability to decouple the $^{19}$F nucleus allowed an accurate measurement of $J_{18, F\beta}$.

\[ \text{\includegraphics[width=0.5\textwidth]{structure.png}} \]

Once again, two sets of data were available due to the simplicity of noise decoupling the $^{19}$F nuclei. $^1$H transitions associated with the region of interest were readily found by switching the decoupling power on and off, and comparing the spectra. Then, a first, simple set of INDO R experiments (Fig. 8A, B, C, D) was run while irradiating both of the $^{19}$F nuclei. In this case, transitions of both $H_{15a}$ and $H_{15b}$ could be monitored while scanning a weak radio-frequency field through the methylene region. $H_{14}$ showed up as a fairly complex set of transitions because even without the $^{19}$F nuclei it is coupled into four other protons. However, its chemical shift was readily found. Note that monitoring line 4 (a degenerate line of $H_{15b}$) leads to
Fig. 8. The $^1$H-$^1$H INDO experiments performed on the $^{19}$F decoupled spectrum of 5 in deuterochloroform solution. $H_{15}$ transitions were monitored as follows: A transition 1; B transition 2; C transition 4; D transition 3.

The assignment resulting from these spectra is shown diagrammatically above the normal proton spectrum.

The insert shows the effect of $^{19}$F decoupling on the C10 and C13 methyl groups.

Decoupling was by noise modulated irradiation at 94, 084, 210.0 Hz.
responses for all four lines of \( H_{15\alpha} \) (Fig. 8C). The effective summation of the INDOOR responses is shown above the spectrum and clearly gives all the information needed for analysis of the D-ring protons.

After switching off the \(^{19}\text{F}\) decoupling it was possible to obtain a second series of INDOOR responses for \( H_{15\alpha} \) and \( H_{15\beta} \) which included \(^1\text{H} - ^{19}\text{F}\) splittings.

Note that the C-methyl resonances give rise to their usual artifact responses in the INDOOR spectra.

The normal \(^{19}\text{F}\) spectrum of 5 is shown in Fig. 7C. The spectrum is sharp and well resolved. It displays the typical large (ca. 284.6 Hz) geminal \(^{19}\text{F} - ^{19}\text{F}\) coupling.

The experiments described above provided enough data to completely analyze the D-ring nuclei of 5. The data was analyzed with the help of a computer program to obtain the true coupling constants and chemical shifts (Table 3).

One would expect to be able to say something about the D-ring conformation at this stage. It is well known that the Karplus-type curves for vicinal \(^1\text{H} - ^1\text{H}\) and \(^1\text{H} - ^{19}\text{F}\) coupling constants must be empirically derived to a large extent and depend upon substituents and hybridization amongst other things; furthermore, applications to five-membered rings appear to be particularly difficult. It seems that the most sensible approach when using such curves to determine ring geometry is to consider the general trend of as large as possible a number of couplings around the ring. Thus \( J_{14,15\beta} = 14.0 \text{ Hz} \) would definitely indicate a trans-diaxial relationship of the two protons and \( J_{14,15\alpha} = 5.9 \text{ Hz} \).
Table 3. N.M.R. Parameters for the Ring D of 16,16-Difluoro-androst-5-en-3β-ol-17-one (5)

Chemical shifts (\(\tau\)-values, \(\phi_c\)-values)*

|   |   |   |   |   |   |   |
|---|---|---|---|---|---|
| \(H_{14}\) | \(H_{15\alpha}\) | \(H_{15\beta}\) | \(F_{16\alpha}\) | \(F_{16\beta}\) | \(C_{13-CH_3}\) |
| 8.55 | 7.60 | 8.04 | \(\phi_c + 109.57\) | \(\phi_c + 100.49\) | 8.95 |

Coupling constants (Hz)

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(H_{14}H_{15\alpha})</td>
<td>(H_{14}H_{15\beta})</td>
<td>(H_{15\alpha}H_{15\beta})</td>
<td>(H_{15\alpha}F_{16\beta})</td>
<td>(H_{15\alpha}F_{16\alpha})</td>
<td></td>
</tr>
<tr>
<td>5.9*</td>
<td>14.0*</td>
<td>-14.2*</td>
<td>-0.7*</td>
<td>19.6*</td>
<td></td>
</tr>
<tr>
<td>(H_{15\beta}F_{16\beta})</td>
<td>(H_{15\beta}F_{16\alpha})</td>
<td>(C_{13-CH_3}F_{16\beta})</td>
<td>(C_{13-CH_3}F_{16\alpha})</td>
<td>(F_{16\alpha}F_{16\beta})</td>
<td></td>
</tr>
<tr>
<td>16.3*</td>
<td>22.6*</td>
<td>0.9*</td>
<td>0</td>
<td>284.6*</td>
<td></td>
</tr>
</tbody>
</table>

* Measured in deuterochloroform containing \(CHCl_3\) or \(CFCl_3\).

† Estimated error ± 0.3 Hz.

‡ Estimated error ± 0.4 Hz.
indicates roughly a 60° dihedral angle for this pair. The $^{19}\text{F}$ resonances are now readily identified as $16\alpha$ upfield and $16\beta$ downfield. The $-0.7$ Hz coupling between $H_{15\alpha}$ and $F_{16\beta}$ suggests a $\sim90°$ dihedral angle between them. The remaining three $H_{15}F_{16}$ couplings agree with the same geometry. It seems then pretty safe to draw the D-ring of 5 as:

where carbons 13, 17, 16 and 15 are in a plane. However, the geometry may well be different when carbon 17 is $sp^3$ hybridized. In fact it has been suggested that carbons 14, 15, 16 and 17 are in a plane for the 17-OH and 17α-acetyl analogues.7,28

It seems that in this compound at least, the $^{19}\text{F}$ labels have very useful "normal" n.m.r. spectra. Nevertheless, they can be reduced to sharp singlets by noise modulated decoupling of the entire proton region. Similarly, the $^{19}\text{F}$ splittings could be readily removed from the proton spectrum by noise modulated decoupling at the $^{19}\text{F}$ frequency.

It can be concluded from the above studies that $^{19}\text{F}$-$\{^1\text{H}\}$ and $^1\text{H}$-$\{^{19}\text{F}\}$ heteronuclear decoupling experiments can considerably facilitate n.m.r. studies of fluorinated steroidal systems. In particular, the observation that noise-modulated irradiation of the entire $^1\text{H}$ spectrum can effectively reduce a $^{19}\text{F}$ resonance to a narrow singlet augurs well
for the future of fluorine as a probe for evaluating the interaction of steroidal derivatives with other systems of biochemical significance such as membranes. We also conclude that $^1$H-$^1$H INDOR measurements should find widespread application in a number of structural problems commonly encountered in natural product chemistry. For, although the combination of $^{19}$F labelling and double resonance can be a powerful technique, the presence of a fluorine substituent is not a necessary prerequisite for such measurements. The next section will discuss this.

B. Application of $^1$H-$^1$H INDOR to Natural Products

The examples discussed in the previous section of this thesis were chosen with a variety of different points in mind; however they all indicated that $^1$H-$^1$H INDOR measurements greatly facilitated the assignment of $^1$H n.m.r. spectra of "natural products". It now remained to show whether or not such measurements could be usefully applied routinely to solve the type of structural problem which often occurs during laboratory syntheses of natural products or of related precursors. The following examples were chosen at random from several of the U.B.C. laboratories concerned with natural product chemistry in order to investigate this point.

(i) A Steroidal Problem

As part of another programme, Weiler and Paisley (private communication) were attempting to perform the conversions indicated in the flow sheet below. The problem with which we were presented was as follows:— the workers had a crude reaction mixture whose n.m.r. spectrum is shown in Fig. 9. The mixture contains starting material and at least
Fig. 9. The $^1$H n.m.r. spectrum (100 MHz) of a steroid (6) reaction mixture in deuterochloroform solution. The position of the CHCl$_3$ internal field-frequency lock is shown. Chemical shift positions of the protons of interest are shown.
one side product. The only clear spectral features associated with the molecular site of interest are two aldehyde groups around $\tau$ 0.2.

Of the several possible alternative products, only the desired product should involve the aldehyde proton as part of an AMX system (i.e. only 3 protons: one at $C_2$ and two at $C_1$). Paisley believed that the more intense aldehyde group represented the desired product while the other was a biproduct. The peaks of the downfield aldehyde group were monitored in turn while sweeping a perturbing radio-frequency field through the methylene envelope. A pair of quartets was obtained by summing the INDO responses: one at $\tau$ 7.93 and the other at $\tau$ 7.48 (Fig. 9 and Table 4). Both of these quartets were otherwise entirely hidden in the methylene envelope.

In this case, the INDO technique provided n.m.r. data which agreed with the proposed structure of the major product. I.R. and mass spectroscopy were of no use in differentiating amongst the various possibilities. Chemical confirmation was doubtful because of the known
possibility of anomerization during reaction. Several other possible products could also have produced an aldehyde quartet e.g.

(8 could have a quartet for the aldehyde via second order coupling to one of the C₆ protons). But neither of these compounds would have produced the simple AMX system found by the INDOR experiments. Thus one very useful feature of the INDOR method is that it can be applied to crude mixtures of compounds.

(ii) Some Decalone Problems

A reaction sequence involving the A-B ring junction was thought to have produced the following two decalones which were subsequently separated by G.L.C. (Piers and Phillips - private communication). It

Proposed minor product  Proposed major product
was hoped that their n.m.r. spectra would provide a check of the above assignment. The $^1$H n.m.r. spectrum of the minor product is given in Fig. 10A and that of the major product in Fig. 10B. Both samples were dissolved in deuterochloroform. In each case the C$_4$ methyl group is readily identified by comparison with other compounds and by double resonance experiments (Fig. 10) as the downfield doublet in the methyl region. In the case of the minor product (9), several protons are partly resolved at the low field end of the methylene envelope. In the case of the major product (10), only one proton is resolved.

For both compounds it was possible to perform INDOR experiments in which the C$_4$ methyl group lines were monitored in turn while sweeping a weak radio-frequency field through the downfield methylene envelope region. In each case a complex series of responses was obtained for H$_4$ due to the degeneracy of the methyl group protons and the overlap of H$_4$ transitions. The information so obtained was a chemical shift value for H$_4$ (Table 4) and the fact that in both compounds the H$_4$ transitions were spread over at least 32 Hz. If the H$_4$-$H_5$ coupling were not axial-axial (e.g. axial-equatorial) the H$_4$ transitions would be spread over only 22-25 Hz at most. This result agrees with the proposed structures given above.

The remaining visible downfield transitions for 9 were assigned, on the basis of their chemical shift and small number of couplings, to H$_{2\alpha}$ and H$_{2\beta}$. By monitoring these transitions in turn while scanning a weak perturbing field through the rest of the spectrum, it was possible to pick out all of the C$_2$ proton transitions and to see that they formed a relatively first order system (Fig. 10A and Table 4). H$_1$ was not unambiguously located for 9 as only 8 mg of sample was available.
Table 4. N.M.R. Parameters for Compounds 6 (Cyano-aldehyde), 9, 10, and 11 (Decalones)

First order chemical shifts (τ-values)*

<table>
<thead>
<tr>
<th>Compound</th>
<th>H&lt;sub&gt;1&lt;/sub&gt;</th>
<th>H&lt;sub&gt;1&lt;/sub&gt;</th>
<th>H&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>7.48</td>
<td>7.93</td>
<td>0.14</td>
</tr>
<tr>
<td>C&lt;sub&gt;4&lt;/sub&gt;-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>H&lt;sub&gt;2α&lt;/sub&gt;</td>
</tr>
<tr>
<td>9</td>
<td>8.97</td>
<td>7.33</td>
<td>7.52</td>
</tr>
<tr>
<td>10</td>
<td>8.99</td>
<td>7.88</td>
<td>7.93</td>
</tr>
<tr>
<td>C&lt;sub&gt;1&lt;/sub&gt;-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>H&lt;sub&gt;1&lt;/sub&gt;</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>11</td>
<td>9.08</td>
<td>8.08</td>
<td>7.20</td>
</tr>
</tbody>
</table>

First order coupling constants (Hz)

<table>
<thead>
<tr>
<th>Compound</th>
<th>H&lt;sub&gt;1&lt;/sub&gt; H&lt;sub&gt;2&lt;/sub&gt;</th>
<th>H&lt;sub&gt;1&lt;/sub&gt; H&lt;sub&gt;2&lt;/sub&gt;</th>
<th>H&lt;sub&gt;1&lt;/sub&gt; H&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1.4&lt;sup&gt;†&lt;/sup&gt;</td>
<td>4.0&lt;sup&gt;†&lt;/sup&gt;</td>
<td>14.5±1.0</td>
</tr>
<tr>
<td>C&lt;sub&gt;4&lt;/sub&gt;-CH&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>H&lt;sub&gt;2α&lt;/sub&gt;H&lt;sub&gt;28&lt;/sub&gt;</td>
<td>H&lt;sub&gt;28&lt;/sub&gt;H&lt;sub&gt;18&lt;/sub&gt;</td>
<td>H&lt;sub&gt;2α&lt;/sub&gt;H&lt;sub&gt;18&lt;/sub&gt;</td>
</tr>
<tr>
<td>9</td>
<td>6.4&lt;sup&gt;±&lt;/sup&gt;</td>
<td>13.0&lt;sup&gt;†&lt;/sup&gt;</td>
<td>3.9&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>C&lt;sub&gt;4&lt;/sub&gt;-CH&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>H&lt;sub&gt;2α&lt;/sub&gt;H&lt;sub&gt;28&lt;/sub&gt;</td>
<td>H&lt;sub&gt;28&lt;/sub&gt;H&lt;sub&gt;1α&lt;/sub&gt;</td>
<td>H&lt;sub&gt;2α&lt;/sub&gt;H&lt;sub&gt;1α&lt;/sub&gt;</td>
</tr>
<tr>
<td>10</td>
<td>6.4&lt;sup&gt;±&lt;/sup&gt;</td>
<td>12.6&lt;sup&gt;†&lt;/sup&gt;</td>
<td>12.6&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>C&lt;sub&gt;1&lt;/sub&gt;-CH&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;1&lt;/sub&gt;</td>
<td>H&lt;sub&gt;1&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>H&lt;sub&gt;1&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>H&lt;sub&gt;1&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>11</td>
<td>7.2&lt;sup&gt;±&lt;/sup&gt;</td>
<td>14.5&lt;sup&gt;†&lt;/sup&gt;</td>
<td>1.4&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Measured in deuterochloroform solution containing CHCl<sub>3</sub>.

<sup>‡</sup> Estimated error ± 0.2 Hz.

<sup>†</sup> Estimated error ± 0.4 Hz.
and the signal-to-noise ratio was low.

About three times as much of 10 was available so that it was possible to locate all of the C₂ proton transitions and a set of weaker INDO\textsc{r} responses gave a chemical shift value for H₁. In the case of 10, only one proton was visible at the low-field end of the methylene envelope and its transitions were monitored during INDO\textsc{r} experiments to find the others (Fig. 10B and Table 4).

The splittings quoted above are certainly close enough to first order values to be used for gross conformational analysis. The results are interesting. From the structure proposed for 9 one would expect J₂α₁β and J₂β₁β < 6 Hz. But the values in Table 4 suggest that H₁ is axial rather than equatorial. Thus the n.m.r. data agree with the structure proposed for 10 but not for 9.

Partly as a result of this conflicting n.m.r. data, Piers and Phillips were led to propose that the AB ring junction had formed cis instead of trans during the Birch reduction of a 4,5 double bond. This would avoid the steric strain caused by an axial isopropyl group.

It should be noted that the n.m.r. INDO\textsc{r} data does not distinguish between 9 and 10. It only says that 9 is not the compound it was
The $^1$H n.m.r. spectra of decalones 9 (A), 10 (B) and 11 (C) at 100 MHz in deuterochloroform containing enough CHCl$_3$ for a field-frequency lock. The first order proton assignments of interest are indicated diagrammatically.

Fig. 10.
originally thought to be. But once again, INDOR provided important structural information.

Fig. 10C shows the $^1$H n.m.r. spectrum of a decalone produced via Birch reduction in a manner very similar to 9 and 10. It was thought to have one of the following structures:

![Diagram of structures](image)

The downfield quartet (Fig. 10C) was made the subject of a set of INDOR experiments. Strong responses were obtained for a geminal partner, and weaker responses for a vicinal proton. Both of these other protons were hidden in the methylene envelope and they fell close together (Table 4). By monitoring the C$_1$ methyl doublet while scanning a weak radio-frequency field through the methylene envelope, the above-mentioned vicinal partner was shown to be H$_1$.

The data shown in Table 4 support structure (a). Structure (b) would be expected to have one of the H$_1$-H$_2$ splittings greater than 10 Hz. The C$_2$ protons have not been assigned as $\alpha$ or $\beta$ in the table. However, it seems quite likely that the downfield proton is H$_{2\beta}$ and the upfield one is H$_{2\alpha}$. The axial C$_1$ methyl group might be expected to distort the A-ring in such a fashion that the dihedral angle between H$_1$ and H$_{2\beta}$ became less than 60° ($\therefore J \approx 6.2$ Hz) and that between H$_1$ and H$_{2\alpha}$ somewhat more than 60° ($\therefore J \approx 1.4$ Hz).
From the above examples, it seem reasonable to conclude that the $^1H-^1H$ INDOR technique should find considerable application as an analytical tool in natural product chemistry.
Experimental

General Methods

(a) All n.m.r. measurements were made with a modified Varian HA-100 spectrometer operating in the frequency-swept mode.

(b) The equipment required for the heteronuclear decoupling experiments has been described elsewhere. An account of the modifications necessary for the measurement of $^1H-{^1H}$ INDOR spectra has also been given previously.

(c) All n.m.r. measurements were made in deuterochloroform solution. Since the spectrometer gain is generally quite high for INDOR experiments it is often desirable to lock onto something as far away from the region of interest as possible. For natural products CHCl$_3$ was found to be useful. Proton chemical shifts are reported in the $\tau$ scale.

(d) Where possible, analyses of the n.m.r. spectra were made with a modified version of the LAOC00N III program and an I.B.M. 360-67 computer in the U.B.C. Computer Centre.

$^{24}$-Fluoro-cholestan-3-one (1) was prepared as described in the literature.

$^6$- and $^6$-Fluoro-cholest-4-en-3-one (3 and 4) were prepared according to general procedures described in the literature.

16,16-Difluoro-androst-5-en-3-ol-17-one (5) was prepared as described in the literature.

The non-fluorinated natural products studied were obtained from L. Weiler and K. Paisley (steroid) and E. Piers and N. Phillips (decalones) — (private communication).
CHAPTER II

FLUORINE AND PROTON N.M.R. AS APPLIED TO CERTAIN ASPECTS OF THE INTERACTION OF LYSOZYME* WITH MONOSACCHARIDE INHIBITORS

Introduction

The applications of n.m.r. spectroscopy to the study of structure and mechanism in biological systems are becoming increasingly widespread. Perhaps this should not be surprising as it is one of the only two methods capable of detecting individual atoms in macromolecular systems - the other method being X-ray diffraction studies of crystals. In cases where a very large molecule (for instance a protein) can be isolated in a pure, crystalline form, X-ray diffraction can yield uniquely valuable information concerning its geometry (lysozyme, carboxypeptidase A, and chymotrypsin being cases in point). N.m.r. is attractive because it offers, at least in principle, straightforward techniques for the detailed investigation of the structure of large molecules in solution as well as techniques for studying the dynamic aspects of molecular structure and interaction.

* An enzyme found in many animal tissues. All work described here was done on lysozyme derived from the whites of hens' eggs.
There are a number of very good review articles in the recent literature dealing with the uses of n.m.r. spectroscopy in biological systems. There is no one, universal n.m.r. approach to such problems. A whole area has grown up around the use of paramagnetic probes in n.m.r. studies of enzyme-substrate complexes. A more obvious approach is the use of normal high resolution n.m.r. spectra to study structure, and pH effects thereon, of 'small' biological building blocks such as amino acids, peptides etc. A recent, but very important, area of endeavour is the application of n.m.r. to the study of rate phenomena in biochemical systems. Chemists have been using n.m.r. to study rate processes for a good ten years, but biological applications have been longer in appearing. Recent technological advances have opened up whole new areas to the n.m.r. spectroscopist interested in biochemical studies. Typical examples include Fourier transform methods for measuring natural abundance $^{13}$C n.m.r. spectra, superconducting magnets for measuring $^1$H spectra at 220 or 300 MHz and also studies of $^{15}$N n.m.r. spectra.

In addition to weak signals, a major difficulty in applying n.m.r. to the study of biopolymers is that the combination of large linewidths due to slow reorientation, and the presence of many closely spaced lines in the spectrum, often produces featureless broad bands. The signal-to-noise problem can be overcome to some extent by time-averaging methods. The other difficulties have inspired a variety of more or less indirect approaches. Obviously spectrometers with higher magnetic fields give better separation of spectral features. Also one may hope to learn about macromolecules by studying the simpler spectra of their
breakdown products or by looking at synthetic model systems (e.g., the use of n.m.r. in cell membrane studies\textsuperscript{46}). In favourable cases, the n.m.r. spectrum of a biopolymer may contain peaks or regions identifiable with some site of interest and such spectral features provide probes of their surroundings (e.g., lysozyme,\textsuperscript{47a,b} ribonuclease\textsuperscript{48}). In less favourable cases, selective deuteration or heteronuclear labelling may be tried.

It is also possible to observe the spectrum of a small molecule or ion which interacts with the biopolymer. Such interactions may produce observable changes in line positions and/or relaxation times\textsuperscript{*} which can be related to phenomena of interest. The use of ions in such experiments generally involves relaxation time measurements - the results being typically treated as outlined in Stengle and Baldeschwieler's ion-probe method.\textsuperscript{49} The technique of comparing the n.m.r. spectrum of a small molecule before and after addition of some biopolymer is an indirect but very promising one and is the method considered in this chapter.

The object of any such experiment is to get information about some facet of a large molecule's geometry or behaviour. A small molecule is chosen which has an observable spectrum and which interacts with the larger molecule in such a way as to reflect upon some site of interest. A good kinetic model for many problems is the "two site case."\textsuperscript{44,50-53} The small molecule is thought of as existing either free in solution (site A) or associated in some way with the biopolymer

\textsuperscript{*} Relaxation phenomena will be discussed in Chapter III.
molecule (site B). Thus

\[
E + I \leftrightarrow EI
\]

\[
K_B = \frac{[EI]}{[E][I]}
\]

Thus

\[
E = \text{enzyme}
\]

\[
I = \text{inhibitor}
\]

\[
K_B = \text{the binding constant}
\]

There may be considerable information on the rates of the exchange processes involved contained in the relaxation phenomena associated with such a system (see Chapter III). In general, the two sites available to a small molecule may be characterized by different resonance frequencies, \( \omega_A \) (bound) and \( \omega_B \) (free). This is so because in general the magnetic properties of site A will differ from those of site B. This may be particularly noticeable if the bound molecule is proximal to an aromatic system or the electric field of a polar group or metal ion. Here it suffices to say that if the rate of exchange (\( k_{-1} \text{sec}^{-1} \)) is very small compared to the difference in frequency, \( (\omega_A - \omega_B) \), then separate resonances will occur at \( \omega_A \) and \( \omega_B \). If the reverse is true, then a single average resonance will appear between the two frequencies at a distance \( \delta = P_B \Delta \) from \( \omega_A / 2\pi \) where \( P_B \) is the fraction of the small molecule which is bound at a given time and \( \Delta \) is the chemical shift \( (\omega_A - \omega_B) / 2\pi \) between bound and unbound species. This latter situation has been found to occur regularly for reversible inhibitors interacting with lysozyme. The chemical shift of an observed inhibitor as a function of concentration, 

\^ See Experimental Section for a more complete description.
pH or temperature yields information which can be used to calculate $K_B$, $A$ and thermodynamic constants.

**Lysozyme**

Like all enzymes, lysozyme is a globular protein\(^{54}\) - a series of amino acids bound together in a chain by peptide bonds and folded into a complex three-dimensional structure. Lysozyme consists of 129 amino acid subunits of 20 different kinds and has a molecular weight of about 14,400.\(^{55}\) Thus it is a very small enzyme. It is also exceptionally stable in aqueous solution and is readily purified and crystallized. These properties contributed to its being the first enzyme to have its complete three dimensional structure determined (X-ray diffraction\(^{36a,b,c,56,57}\)). Lysozyme provides then a very useful model enzyme system for solution study.

Lysozyme's biological function in animals (where it is found in many tissues) is to destroy the cell walls of certain bacteria by catalysing the hydrolysis of the carbohydrate component, specifically a $\beta-1,4$-linked polysaccharide with alternate N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) residues. This reaction proceeds with cleavage of a $C_1$-oxygen bond in the substrate. Fig. 1 shows the section of such a polysaccharide which would be bound to the enzyme during lysis. A, C and E are NAG residues, B, D and F are NAM residues.
The enzyme's ability to perform this catalytic function does not depend so much on the chemical nature of its constituents as on its folded, three-dimensional structure. Several generalizations concerning the gross conformation of lysozyme have been noted in the literature. Peptides with hydrophilic side chains (-acid or base) are found largely on the surface of the enzyme. On the other hand, most of the markedly hydrophobic side chains are shielded from the surrounding aqueous medium by more polar parts of the molecule. The overall folding of the lysozyme polypeptide chain has led to a structure with a deep cleft running up one side. It is this cleft which contains the 'active site'.

It is not possible to perform X-ray studies of lysozyme interacting with a bacterial cell wall. But it has been possible to grow crystals
of the enzyme interacting with small "inhibitor" sugar molecules. These are species which can inhibit the action of lysozyme by themselves binding to the active site in a manner similar to that of the true substrate but which themselves react only slowly if at all.

Lysozyme's action is known to be inhibited by such N-acetate-containing polysaccharide breakdown products as the previously mentioned NAG and NAM and also by the C₆-iodo derivative of NAG, the disaccharide, chitobiose (di-NAG) and the trisaccharide, chitotriose (tri-NAG). It was primarily X-ray studies of lysozyme interacting with tri-NAG that led to the binding array shown in Fig. 1. This array places a monosaccharide subunit in each of 6 subsites (A to F) in the cleft of lysozyme. The bond cleaved is shown by a dotted line. Polysaccharides consisting of NAG up to the tetrasaccharide are known to bind with their reducing end in subsite C and consecutive sugar rings in subsites B and then A (hence the monosaccharide NAG occupies subsite C). From the proximity of the sugar residues to polypeptide side chains in the cleft (as indicated by X-ray studies), modes of bonding have been postulated for all six subsites. Workers have been quite specific about likely hydrogen bonding interactions but less specific about nonpolar ones. Nevertheless nonpolar binding seems likely to play in important part in view of the "oil droplet with a polar coat" nature of lysozyme. More recent solution studies have tended to bear out the X-ray postulates. The proposed mechanism for lysis involves the catalytic effect of the proximate peptide side chains of residue 35 (glutamic acid) and 52 (aspartic acid) on the C-0 bond in question (Fig. 1). It has been suggested that C₁ of
ring D passes through a carbonium ion intermediate and that binding of the substrate distorts ring D. X-ray data on lysozyme has formed a basis for all the more recent solution work.

Lysozyme and the Chemical Shift Technique

Previous studies from this laboratory have shown that $^{19}\text{F}$ n.m.r. parameters are more sensitive to changes in chemical environment than are $^1\text{H}$ n.m.r. parameters. Because of this we have been interested in the possibility of using fluorine substituents as structural probes for investigating a variety of chemical problems. From the n.m.r. viewpoint any such heteronuclear probe has a number of obvious advantages and this is particularly so in the study of biomolecular associations where it is often difficult to identify unequivocally a particular proton resonance.

In view of the strong stereoelectronic preference of the carbon-fluorine bond, it seems likely that in some cases the introduction of a $^{19}\text{F}$ substituent may seriously perturb the system of interest. However, such a perturbation might itself yield valuable information as we shall show. Lysozyme seemed to be an ideal model for studying this phenomenon because of the detailed X-ray and n.m.r. data which are already available. For these studies four N-trifluoroacetyl derivatives (1-4) of $\alpha$-glucosamine were synthesized

* Strictly these are derivatives of 2-deoxy-2-trifluoroacetamido-$\alpha$-glucopyranose.
which correspond to four known inhibitors (5-8) of lysozyme.

These latter compounds (5-8) have been extensively studied via n.m.r. by Raftery et al.\textsuperscript{64-66} and by Sykes et al.\textsuperscript{67,68} (both of whom monitored the N-acetate peaks). Values of $K_B$ and $\Delta$ obtained for such compounds necessarily have a high experimental error (typically $\pm$ 10-20%). Hence one must be careful not to place undue emphasis on small differences in measured values between different compounds.

Our studies of the trifluoro derivatives (in which we have monitored the N-trifluoroacetate peaks) subsequently led us to investigate two other problems:
(a) The effect of substituents at C₆
(b) The conformation of inhibitors.

The former problem was approached by synthesizing and studying the following series of compounds:

Inhibitor conformations were studied by analysis of the ¹H n.m.r. spectra of their O-acetates. We have also briefly considered the possibility of studying the conformation of bound inhibitors.
Results and Discussion

A. Choice of a Suitable Chemical Shift Reference

The most accurate way to measure small chemical shifts in n.m.r. spectroscopy is by comparison with some reference compound. When using organic solvents it is common practice to employ tetramethylsilane (TMS) as an internal reference for measuring proton chemical shifts, and freon 11 (CFCl₃) for fluorine work. However, enzyme studies are carried out in aqueous solution. Theoretically one could use a capillary of TMS or freon 11 held concentric with the n.m.r. tube (see Experimental Section). But it has been shown that chemical shifts measured relative to such external standards are very prone to bulk magnetic susceptibility effects—especially when the temperature is varied between samples.

For proton work it is convenient to use the water peak for a field-frequency lock; but this peak is notoriously temperature and pH dependent. Hence a small quantity of some other species must be added for use as an internal standard. For lysozyme work, both acetone and t-butanol have been employed.

For a ¹⁹F field-frequency lock a capillary of trifluoroacetic acid was used. This was found to be very suitable for work with a trifluoroacetyl label as extensive field offsets were not required. We have considered the possibility of using sodium trifluoroacetate (NaOCOCF₃) as an internal reference. It is very soluble and quite inert. Unfortunately, it was found to interact with lysozyme in such a way as to shift and broaden. Other internal references tried were trifluoroethanol (CF₃CH₂OH) and hexafluoroacetone sesquihydrate. The former
appears as a very sharp triplet quite close to the region of the trifluoroacetate label (Fig. 2). It shows no sign of broadening or shifting on addition of lysozyme (Table 2) and was used in all fluorine chemical shift studies.

Trifluoroethanol is somewhat acidic. For this reason a check was made to see that it was not overloading the 0.1 M pH 5.5 citrate buffer used for this work. Twice the concentration of trifluoroethanol actually employed during enzyme runs was seen to have no noticeable effect on the buffer pH, hence no problem is to be expected.

B. Experiments with N-Trifluoroacetyl-\textsuperscript{D}-glucosamine

The monosaccharide, N-acetyl-\textsuperscript{D}-glucosamine (NAG-anomers 5 and 6) are known\textsuperscript{35,58,60} from X-ray studies to bind specifically and reversibly at subsite C on the enzyme surface (although the \(\alpha\)-anomer is a special case). The methyl glycosides (7 and 8) seem likely\textsuperscript{58,60} to behave in a similar fashion to 6 with respect to binding to lysozyme.\textsuperscript{60,58}

In all four cases, the \(^1\text{H}\) resonance of the N-acetyl group shows a definite upfield shift\textsuperscript{65,66} and broadening on binding of the sugar to lysozyme. This shift has been attributed to the proximity of the N-acetyl methyl protons in the bound sugar to the aromatic portion of a tryptophan residue (residue 108) in lysozyme.\textsuperscript{57,66} In the case of the free sugar, NAG, the \(\alpha\)- and \(\beta\)-anomers have been observed to show a degenerate N-acetyl resonance when no lysozyme is present\textsuperscript{58,59} but this singlet gradually becomes a doublet as enzyme is added. This degeneracy has considerably hampered\textsuperscript{58,59,68} accurate chemical shift
measurements on the system and no doubt partly explains why Raftery et al. and Sykes et al. get somewhat different values for $K_B$ and $\Delta$.

We have synthesized (see Experimental section) the following series of compounds in which the normal N-acetate group of N-acetyl-D-glucosamine has been replaced by an N-trifluoroacetyl group. It was expected that the fluorine resonances of 1 and 2 might not be degenerate as in the $^1H$ case. Moreover, it was hoped that fluorine chemical shifts on association with the enzyme would be greater than proton shifts. At the same time we expected that the $^{19}F$ nuclei might well have some perturbing effect on the interaction.

When a mixture of the $\alpha$- and $\beta$-anomers of the free sugar was allowed to crystallize slowly from water, the $\alpha$-anomer was obtained as a pure, crystalline hydrate. The mutarotation of this species to its equilibrium mixture could be followed by $^{19}F$ n.m.r. (Fig. 2) and was observed to take about 45 minutes in pH 5.5 citrate buffer at 31.5°C. The final ratio of $\alpha$ to $\beta$ was 46% $\alpha$ and 54% $\beta$. The fact that it was indeed the $\alpha$-anomer which was obtained pure could be readily proven from the proton n.m.r. spectrum in which the $H_1$-$H_2$
Fig. 2 $^{19}$F n.m.r. spectra (94.071 MHz) of a mutarotating solution of N-trifluoroacetyl-D-glucosamine (α-anomer, 1) in pH 5.5 citrate buffer (0.1 M). The internal standard, trifluoroethanol, is seen as a sharp triplet some 100 Hz to higher field. A capillary of TFA was used for the field-frequency lock. The time after dissolving the pure α-anomer is shown to the right of the spectra.
splitting is clearly visible in the downfield C^-proton. It should be noted from Fig. 2 that, as was hoped, the N-trifluoroacetyl groups of 1 and 2 are non-degenerate (separation ca. 27.0 Hz), and that they fall in a convenient position relative to the internal reference compound, CF_3CH_2OH. Unfortunately the mutarotation of the pure α-anomer is sufficiently fast to result in an appreciable amount of the β-anomer (2) only several minutes after dissolution. The excellent separation of the ^19F peaks of the two anomers permitted accurate integration of the intensities.

Fig. 3 shows the effect of lysozyme on the ^19F n.m.r. spectra of 1 and 2. The experiment was performed by varying the sugar concentration from 0.10 M to 0.02 M while holding the concentration of lysozyme constant at 3 \times 10^{-3} M (see experimental section). In Fig. 3A no enzyme has been added and the shifts of α- and β-anomers are shown relative to the center line of the trifluoroethanol triplet (204.5 Hz downfield from the trifluoroacetic acid capillary lock signal). Internal standard concentration was 0.05 M. Fig. 3B displays the spectrum obtained when a sample of sugar at the same concentration (0.10 M) is made 3 \times 10^{-3} M in lysozyme. The α-anomer (1) is seen to broaden considerably and to shift upfield toward the internal reference signal. The β-anomer (2) shows no appreciable change. When the sugar concentration is lowered to 0.04 M (Fig. 3C) the compound 1 shows even greater changes in chemical shift and line-width, whereas the spectrum of 2 remains relatively unchanged. The chemical shift results of this experiment are listed in Table 1. The data were treated, as described in the experimental section, according to the method of Dahlquist and Raftery^{66} in order to obtain the dissociation
Fig. 3 $^{19}$F n.m.r. spectra recorded during a study of 1 and 2 (α- and β-anomers of N-trifluoroacetyl glucosamine) with lysozyme in pH 5.5 citrate buffer (0.1 M).

A. Sugar concentration 0.10 M, no enzyme.
B. Sugar concentration 0.10 M, 3 x $10^{-3}$ M enzyme
C. Sugar concentration 0.04 M, 3 x $10^{-3}$ M enzyme

In each case the center line of the trifluoroethanol standard triplet is shown to highfield.
constant, \( K_D = \frac{1}{K_B} \), and the bound chemical shift, \( \Delta \). For this purpose it was initially assumed that because the \( \beta \)-anomer does not shift or broaden appreciably, that therefore it does not bind to lysozyme. Making this assumption (Fig. 4A, filled circles) leads to calculated values of \( K_B = 96.7 \ M^{-1} \) and \( \Delta = 0.947 \ \text{ppm} \) for 1. At this stage it seems appropriate to remark on the changes in linewidth observed for the \( ^{19}F \) resonances of 1 and 2 and for the centre line of the \( \text{CF}_3\text{CH}_2\text{OH} \) triplet. Such changes will be discussed at length in Chapter III, but it should be noted here that although the resonance of 1 broadens considerably (Table 2), that corresponding to 2 broadens negligibly and the reference remains as sharp as it was before lysozyme was added.

However, lack of a detectable chemical shift or broadening in the spectrum of an inhibitor in contact with an enzyme does not preclude the possibility of its binding. For one thing, the fraction of bound inhibitor is low at any one time. Also there are several mechanisms for broadening and, as will be shown in Chapter III, that operating in the case of the \( \alpha \)-anomer (1) is primarily "exchange broadening."\textsuperscript{67} Sykes\textsuperscript{67} and Sykes and Parravano\textsuperscript{68} have shown that this is also the primary broadening mechanism for the \( ^1H \) n.m.r. N-acetate resonances of the proton analogues of compounds 1 to 4. Exchange is a mechanism for broadening if it occurs between sites of different chemical shift. In other words, the observed lack of change in the \( ^{19}F \) spectrum of 2 could be explained by its binding to lysozyme in such a way that the N-trifluoroacyetyl group does not experience a very great change in magnetic field. It might in fact be
a stronger inhibitor of lysozyme than 1 and yet appear not to bind at all from chemical shift and linewidth data.

Dahlquist and Raftery\textsuperscript{66} have derived equations permitting one to calculate $K_D = 1/K_B$ and $\Delta$ for two interconverting anomers, both of which can be seen to shift on binding. They had limited success in applying these equations to the compounds 5 and 6 because of the small shift of the $\beta$-anomer and the near degeneracy of the N-acetyl peaks. In our case, although there was no problem of degeneracy, the $\beta$-anomer gave no information which would permit solution of the equations.

A partial answer to this impasse was to make use of the pure $\alpha$-anomer (1) by dissolving weighed amounts in thermostated solutions just prior to recording their $^{19}F$ n.m.r. spectra (see experimental section). It was then possible to monitor spectral changes during anomeration. This was done repeatedly for several initial concentrations of sugar ($[\alpha_o] = 0.10$ M and $[\alpha_o] = 0.06$ M) and the results were compared with the results at mutarotation equilibrium to decide whether or not the $\beta$-anomer (2) was binding effectively. Perhaps this is best illustrated by example. Suppose an initial concentration, 0.10 M, of pure $\alpha$-anomer with $3 \times 10^{-3}$ M lysozyme shows a chemical shift $\delta = x$ Hz. As mutarotation occurs, the concentration of $\alpha$-anomer
(1) will drop to 0.046 M and hence the bound fraction, \( P_B \), will increase and \( \delta \) will increase (i.e., as shown in Fig. 4A, smaller sugar concentrations give larger shifts). But the \( \beta \)-anomer concentration increases from 0 to 0.054 M as that of the \( \alpha \)-anomer decreases. If this \( \beta \)-anomer (2) binds to lysozyme as strongly as the \( \alpha \)-anomer, it will begin to compete for sites on the enzyme and at anomeric equilibrium the number of available enzyme sites will have been decreased by a quantity \([E_\beta]\) (the concentration of bound \( \beta \)-anomer).

The result will be that although \([\alpha]\) will have decreased by roughly 50\%, so will \([E_\alpha]\) and hence the observed chemical shift \( \delta = \chi \text{ Hz} \) will remain virtually the same (note that at these sugar concentrations nearly all of the sugar is in the unbound form).

Fig. 4B shows the results of these mutarotation experiments. The data so obtained suffered from the fact that a finite time (3-5 minutes) was required to produce an initial spectrum and from the fact that even once the sample was in the probe mutarotation took place rapidly relative to the time required to make careful measurements. Nevertheless, the data show clearly that the \( \alpha \)-anomer (1) does undergo an upfield shift during mutarotation (note that, conversely, a downfield shift would indicate that the \( \beta \)-anomer was bound more strongly than the \( \alpha \)). Moreover the magnitude of this mutarotation shift (\( \sim 2.7 \text{ Hz} \) when \([\alpha_o]\) = 0.06 M and \( \sim 2.1 \text{ Hz} \) when \([\alpha_o]\) = 0.10 M) is roughly within experimental error of the shift that would be expected if the \( \beta \)-anomer did not bind at all (see Table 1). It seems safe then to say that \( K_B \) for 1 is considerably larger than \( K_B \) for 2. But from these experiments it is impossible to realistically
Fig. 4. A. Graph of $^{19}$F chemical shift data from the study of 1 with lysozyme: filled circles - mutarotated mixture; X's - maximum error from the study of pure α-anomer. The Y-axis is in units of molarity of 1.

B. Increase in δ ($\delta'$) as a function of time during the mutarotation of pure α-anomer (1) to an equilibrium mixture in the presence of lysozyme: filled circles - $[\alpha_0] = 0.10$ M, X's - $[\alpha_0] = 0.06$ M.
estimate a $K_B$ for 2. It is possible to use the results of the mutarotation studies to estimate a maximum correction to the original graph for the $\alpha$-anomer. This is done in Fig. 4A: the line through the filled circles representing equilibrium data, and the line through the "X's" indicating an approximate maximum correction to the pure $\alpha$-anomer data. As shown in Fig. 4A, taking the $\beta$-anomer into consideration may change the intercept on the sugar concentration axis by as much as 0.002 M and this will change $K_D$ for 1 from 0.0103 M to roughly 0.012 M. However, in view of the error limits involved in the mutarotation studies (and indeed in the technique itself) such a correction is not warranted.

It should be emphasized here that there remains the possibility that the $\beta$-anomer is binding to the enzyme in such a way that it neither shifts on binding nor interferes with the binding of the $\alpha$-anomer or that it binds non-competitively in the slow exchange limit.

Lysozyme and the Methyl Glycosides of N-Trifluoroacetyl-D-glucosamine

As mentioned previously, the methyl glycosides (3 and 4) were made as part of the series of fluorinated compounds. These were purified and separated by column chromatography on silica gel (see
Table 1. $^{19}$F Chemical Shift Data for N-Trifluoroacetyl-D-glucosamine (1 and 2) and its Methyl Glycosides (3 and 4) in the Presence of Lysozyme ($3 \times 10^{-3}$ M)

<table>
<thead>
<tr>
<th>Total Sugar Concentration $[I_0]$ (M)</th>
<th>Chemical Shift from Trifluoroethanol (Hz)</th>
<th>Changes in Shift for 1</th>
<th>1/δ (Hz$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>δ (Hz)</td>
</tr>
<tr>
<td>0.10 (no lys.)</td>
<td>129.85</td>
<td>102.96</td>
<td>0</td>
</tr>
<tr>
<td>0.10</td>
<td>125.41</td>
<td>103.21</td>
<td>4.57</td>
</tr>
<tr>
<td>0.08</td>
<td>124.72</td>
<td>103.41</td>
<td>5.27</td>
</tr>
<tr>
<td>0.06</td>
<td>123.45</td>
<td>103.47</td>
<td>6.53</td>
</tr>
<tr>
<td>0.04</td>
<td>121.55</td>
<td>103.57</td>
<td>8.43</td>
</tr>
<tr>
<td>0.02</td>
<td>117.88</td>
<td>103.60</td>
<td>12.11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sugar Concentration $[I_0]$ (M)</th>
<th>Chemical Shift from Trifluoroethanol (Hz)</th>
<th>Sugar Concentration $[I_0]$ (M)</th>
<th>Chemical Shift from Trifluoroethanol (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 (no lys.)</td>
<td>130.95</td>
<td>0.10 (no lys.)</td>
<td>99.82</td>
</tr>
<tr>
<td>0.10</td>
<td>131.43</td>
<td>0.10</td>
<td>100.20</td>
</tr>
<tr>
<td>0.08</td>
<td>131.40</td>
<td>0.08</td>
<td>100.68</td>
</tr>
<tr>
<td>0.06</td>
<td>131.46</td>
<td>0.06</td>
<td>100.28</td>
</tr>
<tr>
<td>0.04</td>
<td>131.65</td>
<td>0.04</td>
<td>100.45</td>
</tr>
<tr>
<td>0.02</td>
<td>131.75</td>
<td>0.02</td>
<td>100.35</td>
</tr>
</tbody>
</table>
Table 2. Linewidth Data for the N-Trifluoroacetate resonances of N-Trifluoroacetyl-$D$-glucosamine (1 and 2) and its Methyl Glycosides (3 and 4) in the Presence of Lysozyme ($3 \times 10^{-3}$ M)

<table>
<thead>
<tr>
<th>Total Sugar Concentration $[I_0]$ (M)</th>
<th>Transition Linewidth at Half Height</th>
<th>1</th>
<th>2</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 (no lys.)</td>
<td>0.70</td>
<td>0.70</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>1.10</td>
<td>0.75</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>0.08</td>
<td>1.15</td>
<td>0.76</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>1.28</td>
<td>0.80</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>0.04</td>
<td>1.50</td>
<td>0.80</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>2.00</td>
<td>0.79</td>
<td>0.60</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sugar Concentration $[I_0]$ (M)</th>
<th>Transition Linewidth at Half Height</th>
<th>3</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 (no lys.)</td>
<td>0.74</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>0.77</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>0.08</td>
<td>0.78</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>0.79</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>0.04</td>
<td>0.77</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>0.84</td>
<td>0.47</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 (no lys.)</td>
<td>0.69</td>
</tr>
<tr>
<td>0.10</td>
<td>0.74</td>
</tr>
<tr>
<td>0.08</td>
<td>0.77</td>
</tr>
<tr>
<td>0.06</td>
<td>0.75</td>
</tr>
<tr>
<td>0.04</td>
<td>0.75</td>
</tr>
<tr>
<td>0.02</td>
<td>0.75</td>
</tr>
</tbody>
</table>
experimental section). Hence they could be studied separately in
interaction with lysozyme. This was done (once again using the
method of Dahlquist and Raftery) by monitoring the $^{19}\text{F}$ resonances and
measuring their chemical shift relative to the internal standard,
trifluoroethanol, while varying the concentration of sugar from 0.10
to 0.02 M) in the presence of a constant concentration of enzyme ($3 \times 10^{-3}$ M). The chemical shift results are tabulated (Table 1) with
those for the free sugar. The chemical shift data for 3 and 4 is
very similar to that for 2. That is, there is only a very slight
($\sim 0.5$ Hz) downfield shift as compared to the 12.0 Hz upfield shift of
the $\alpha$-anomer, 1. This similarity amongst 2, 3 and 4 also shows up
in the line broadening data: in each case a very slight broadening
(ca. 0.1 Hz) is apparent over the total range of concentration
data (Table 2) compared to a broadening of well over 1.0 Hz for 1.
The standard (trifluoroethanol) can be seen not to broaden detectably.

In the case of the glycosides it is possible to measure their
binding strength (or lack thereof) against that of some other sugar
of known strength. This was not possible with the free sugar,2,because
all effects would be swamped by those of the strong inhibitor,1.
The known inhibitor, 2-deoxy-2-acetamido-$\alpha$-methyl-glycoside (7), was
chosen for comparison. We have measured $K_D$ for 7 (as will be described
later in this chapter) and found it to be 22.9 M$^{-1}$ ($K_D = 0.0437$ M).
This inhibitor is assumed to bind at subsite $C$.57-60 The experiment
performed in each case involved the same approach. The shift, $\delta$,
of the N-acetate group of 7 at a given concentration of enzyme and
sugar was compared to that of another sample containing the same
Table 3. Dissociation Constants \( (K_D) \), Binding Constants \( (K_B) \) and Bound Chemical Shifts \( (\Delta) \) for Compounds 1 to 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_D ) (M)</th>
<th>( K_B ) ( (M^{-1}) )</th>
<th>( \Delta ) (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0103</td>
<td>96.7</td>
<td>0.947</td>
</tr>
<tr>
<td>2</td>
<td>&gt;&gt;0.0103</td>
<td>&lt;&lt;96.7</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>&gt;0.0437</td>
<td>&lt;22.9</td>
<td>~0</td>
</tr>
<tr>
<td>4</td>
<td>&gt;0.0437</td>
<td>&lt;22.9</td>
<td>~0</td>
</tr>
</tbody>
</table>

Competition of the N-Trifluoroacetyl-\( \alpha \)- and \( \beta \)-methyl Glycosides (3 and 4) for Subsite C as Indicated by the N-Acetyl Proton Chemical Shift of 7

<table>
<thead>
<tr>
<th>Concentration of 7 (M)</th>
<th>([E_o]) (M)</th>
<th>Other Sugar Added</th>
<th>Observed Shift (( \delta )) for 7 (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.49</td>
<td>0.003</td>
<td>none</td>
<td>2.2</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Cpd. 3 ( ~0.49 ) M</td>
<td>1.7</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>none</td>
<td>2.2</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Cpd. 4 ( ~0.49 ) M</td>
<td>1.5</td>
</tr>
</tbody>
</table>
concentration of the sugar, 7, and enzyme plus an equal concentration of either 3 or 4. In each case the observed shift, $\delta$, was seen to decrease by roughly 1/4 (Table 3). This indicates that 3 and 4 bind to subsite C (or at least inhibit binding at subsite C) less strongly than N-acetyl-glucosamine-$\alpha$-methyl glycoside for which we have calculated $K_B = 22.9 \text{ M}^{-1}$ (literature values $\sim 20 \text{ M}^{-1}$).

We have at several points mentioned the problem that it is difficult to separate the phenomenon of binding at one specific site from that of a multiple equilibrium with several sites. Our data cannot resolve this difficulty. Both Dahlquist and Raftery and Sykes and Parravano have been quick to realize the problem. The best information on the subject is that provided by the X-ray work of Blake et al. which indicates that monosaccharide NAG binds at subsite C (although $\alpha$-NAG binds in a different orientation from $\beta$-NAG). It is certainly possible to state from our results that the fluorinated analogues 1, 3 and 4 compete with normal NAG for sites on the enzyme surface - but this is not concrete proof of subsite C occupation.

It seems appropriate at this point to consider our results in the light of present theories regarding binding of inhibitors and substrates to lysozyme. Since the original X-ray studies of Blake et al. a number of solution studies and further X-ray data have appeared which have to a large extent borne out the initial claims. However, there is still uncertainty as to the actual importance of the various possible binding modes. Workers in this area have generally assumed that contributions from each of the six subsites can...
be added to obtain values for the unitary free energy of association
\( \Delta F_u = -RT \ln K \) of an oligosaccharide. In our case, we are
dealing with monosaccharides which seem most likely to bind at
subsite C. A considerable number of inhibitors have been studied
to date and intercomparisons permit several conclusions. It is
generally accepted that the most important contribution to binding at
subsite C arises from interactions of the acetamido group with the
enzyme. In particular it has been postulated to form hydrogen bonds
between its NH and CO groups and the main chain CO and NH groups of
amino acid residues 107 and 59 respectively. Also it has been
suggested that there is a strong nonpolar association of the
acetamido methyl group with the aromatic indole ring of tryptophan
108. It is of course the proximity to this latter side chain which
is proposed to account for the large value of \( \Delta \) for acetamido methyl
protons of saccharides bound at subsite C. There are in fact a total
of 30 proposed Van der Waals contacts (< 4 Å) with the enzyme for a
sugar residue at subsite C. Blake et al. have reported a slightly
different bound orientation for the \( \alpha \)- and \( \beta \)-anomers of NAG. They
suggest that the \( \beta \)-anomer occupies subsite C in a manner typical of
the actual substrates, being subject to the above-mentioned interactions
plus hydrogen bonds between its O(6) and O(3) atoms and the NH groups
of the tryptophan side chains 62 and 63 respectively. The \( \alpha \)-anomer
on the other hand is thought to be subject to the above mentioned
interactions except those involving O(6) and O(3) and is bound in such
a way as to achieve a hydrogen bond between O(1) (which points down
into the enzyme) and the main chain NH of residue 109. On the other hand
the α-methyl glycoside 9 has been shown not to achieve this hydrogen bond via O(1)\(^5\)\(^8\) but rather to bind as the β-anomer of the free sugar - Blake et al.\(^5\)\(^8\) suggest that this is due to the glycoside methyl group's interfering sterically with the formation of such a bond.

Certainly the qualitative trend of our results (that 1 binds differently than do 2, 3 and 4 and that 2, 3 and 4 behave similarly to one another) is in agreement with what would be predicted from known facts. However, there are several discrepancies. Perhaps the most glaring is that substitution of NHCO\(_3\)\(_F\) for NHCO\(_3\)\(_H\) has greatly enhanced the differences between the α-anomer of the free sugar and β-anomer and glycosides. Literature values of \(K_B\) for 5 and 6 show considerable variation as remarked by Chipman and Sharon\(^6\)\(^0\) - nevertheless they fall between 20 and 50 M\(^{-1}\) whereas we arrive at 96.7 M\(^{-1}\) for 1 and something considerably less than 96.7 M\(^{-1}\) for 2, 3 and 4. Average literature values quoted by Chipman and Sharon for 7 and 8 are \(K_B = 20\) and 27 M\(^{-1}\) respectively. Although we have not measured a value for the β-glycoside, 8, our value of \(K_B = 22.9\) M\(^{-1}\) for the α-glycoside (see next section) is within experimental error of the data from other workers.

Our observations for the N-trifluoroacetates 2, 3 and 4 can be explained by postulating that replacement of the normal N-acetate by its \(^19\)F analogue has disrupted (or even destroyed) certain modes of binding via this group (i.e. the two hydrogen bonding sites and the Van der Waals interactions). Our previously mentioned observation that the NH proton is more readily exchanged for deuterium in the trifluoroacetate derivatives would support such a postulate. It is
already widely accepted that binding via the acetamido group of NAG derivatives accounts for most of the free energy of association at subsite C. This same lack of association of the N-trifluoroacetyl group with tryptophan 108 would explain the lack of chemical shift on binding (particularly in the case of 3 and 4 which seem to bind weakly at least).

However, such a simple argument does not explain the relatively strong binding (for monosaccharide inhibitors) of the α-anomer, 1. If replacement of the N-acetyl group of 5 by an N-trifluoroacetyl group served only to weaken bonding via this group we would certainly not expect to observe $K_B = 96.7 \text{ M}^{-1}$. An observation which may provide a clue is that the total bound chemical shift for 1 was found to be $\Delta = 95 \text{ Hz}$ which is not much more than the proton shifts ($\Delta = 70 \text{ Hz}$) observed for monosaccharides binding to subsite C. This apparent lack of $^{19}F$ chemical shift sensitivity to environment may simply reflect (as with 2, 3 and 4) a lack of binding via the trifluoroacetamido group. The fact that a shift is seen here at all may result from a strong O(1) hydrogen bond to the NH of residue 109 which holds the inhibitor in position. This must be regarded as a rather unsatisfactory explanation though as it does not explain the large value of $K_B$ for 1. That this is not a unique phenomenon is suggested in a recent note by Kent and Dwek who report high binding constants for several halogenated monosaccharide inhibitors.

Since we completed our work a communication has appeared which reports the inhibition of lysozyme interacting with an actual substrate by the free sugar, N-trifluoroacetyl-$l\text{-}$glucosamine. These authors
have also looked at the $^{19}\text{F}$ n.m.r. spectra of the species, 1, 2, 3, and 4 interacting with lysozyme and report that $K_D$ for 1 is $0.0091\text{ M}^{-1}$ and $\Delta = 78\text{ Hz}$ (at 100 MHz). Both of these values agree within experimental error with our own. These workers also report no n.m.r. chemical shift for 2, 3, and 4 interacting with the enzyme.

Although there are few examples in the literature\textsuperscript{72,73} of the use of $^{19}\text{F}$ as a probe for investigating enzyme-inhibitor interactions, the technique does seem to have potential benefits. For instance, the $\alpha$- and $\beta$-anomers (1,2) were well separated from one another in their spectra in contrast to their proton analogues (5,6). It also seems likely that the failure of the $^{19}\text{F}$ probe to display a large bound shift, $\Delta$, is a peculiarity of this system as mentioned previously. Certainly the effects observed point out dramatically that in biological systems a CF bond can have specific effects which are different from those of a CH bond, and these effects may throw light on the mechanisms involved.

C. Experiments with $C_6$-Substituted N-Acetyl-$D$-glucosamine-$\alpha$-methyl glycosides

In the previous section we reported studies via $^{19}\text{F}$ n.m.r. of the interaction of lysozyme with various monosaccharide inhibitors. These studies were carried out partly to study the applicability of fluorine labels to such systems and partly in an effort to learn more about the nature of the interaction. One of the conclusions we came to was that the replacement of CH$_3$ by CF$_3$ in the N-acetyl group seemed to have modified (probably lessened) its binding interactions
with the enzyme. Nevertheless, the N-trifluoroacetylated methyl glycosides still showed evidence of weak binding. As mentioned before, saccharide units occupying subsite C in the normal fashion have been postulated to have 30 Van der Waals contacts and 4 hydrogen bonding sites (2 of the latter involving the N-acetyl group). But of all the interactions, those involving the N-acetyl group are known to be the most powerful. We report in this section an attempt to gauge the importance of the hydrogen bonding contact postulated between O(6) and the NH group of tryptophan side chain 62 using ¹H n.m.r.

Our approach has been to synthesize a series of C₆-substituted monosaccharide inhibitors, one of which has been previously studied by X-ray diffraction and is known to bind in the 'typical' way at subsite C (compound 9), another which is assumed to bind at subsite C in the same way (compound 7), and a third which is a previously unknown inhibitor of lysozyme (compound 10). These three inhibitors are shown below:

\[
\begin{align*}
9 & \quad R = \text{I} \\
10 & \quad R = \text{H}
\end{align*}
\]
The proton spectra of each of these compounds contained sharp peaks corresponding to the N-acetyl and methoxyl groups - these were suitable for studies with lysozyme. The new inhibitor, 10, also displayed a sharp doublet corresponding to the C₆ protons and this proved to be useful as a further probe of the bound environment. It was hoped that the changes at C₆ in going from 7 to 9 to 10 would be specific in their effects as measured by $K_B$ and $\Delta$.

**Compound 10**

The study of this compound's interaction with lysozyme may be taken as typical of the proton work done here. The compound itself was produced by catalytic hydrogenation at atmospheric pressure of the C₆-iodo derivative 9, as described in the experimental section. As with the fluoro-derivatives of the previous section, $K_B$ and $\Delta$ were calculated from the results of a study of the chemical shift of sharp inhibitor peaks as a function of sugar concentration (0.10 to 0.02 M) in the presence of a constant enzyme concentration (3 x $10^{-3}$ M) in pH 5.5 citrate buffer (0.1 M).

Sample spectra from the experiment with 10 are shown in Fig. 5. Fig. 5A displays the condition, $\delta = 0$, in which no enzyme is present. Spectral features shown are the N-acetate peak, the C₅-methyl doublet and the internal reference compound, 0.025 M tertiary butanol. The large water peak was used for a field-frequency lock. Note that when 3 x $10^{-3}$ M lysozyme is added (Fig. 5B) both the N-acetyl peak and the doublet due to the C₆ protons experience an upfield shift (and broadening). When the sugar concentration is decreased to 0.04 M, the value of $\delta$ is increased as illustrated in Fig. 5C. Note that
Fig. 5 $^1$H n.m.r. spectra recorded during a study of C$_5$-methyl-N-acetyl glucosamine-$\alpha$-methyl glycoside (10) with lysozyme in pH 5.5 citrate buffer (0.1 M).

A. Sugar concentration 0.10 M, no enzyme
B. Sugar concentration 0.10 M, $3 \times 10^{-3}$ M enzyme
C. Sugar concentration 0.04 M, $3 \times 10^{-3}$ M enzyme

The acetate resonance, C$_5$-methyl resonance and the tertiary butanol internal standard are shown.
the C₆ protons shift less than the acetyl protons.

The shifts (relative to the internal standard) of both the N-acetate peak and the C₅-methyl doublet were large enough to be readily measured during the course of the experiment. In Fig. 6A, 1/δ is plotted vs. the initial sugar concentration for both of these spectral features. It is an encouraging check on the technique that both the graph for the N-acetate peak (filled circles) and that for the C₅-methyl peak (X's) intersect at very nearly the same point. Naturally this is as should be because the intersection point, -(Kᵦ + [Eₙ]), should be the same for all peaks in the same compound as described in the experimental section. The value of Kᵦ as determined from following the N-acetate peaks if 27.0 M⁻¹ and that determined from the C₅-methyl peaks is 26.4 M⁻¹. The former is probably more accurate in view of the larger δ values involved. The bound chemical shift, Δ, for the N-acetate peak was found to be 0.728 ppm and that for the C₅-methyl peak 0.254 ppm (see Tables 4 and 5).

**Compound 7**

This compound, the α-methyl glycoside of NAG, has been studied in solution with lysozyme previously with the result that its values for Kᵦ and Δ are already in the literature. We have repeated the study to make sure that our values do have some generality and that comparison of data amongst 7, 9 and 10 is as meaningful as possible. Data obtained from the study with 3 x 10⁻³ M lysozyme over a sugar concentration range of 0.10-0.02 M are shown plotted in Fig. 6B and are listed in Table 4. Shift values were again measured relative to the internal standard, tertiary butanol. The shifts given in the table and plotted
in Fig. 6B are those observed for the N-acetyl peak. The values calculated for $K_B$ and $\Delta$ (Table 5) from the intercept and slope respectively are $K_B = 22.9 \text{ M}^{-1}$ and $\Delta = 0.734 \text{ ppm}$. The C$_6$-protons of 7 were not suitable for chemical shift studies because of the complexity of their spectrum.

**Compound 9**

Although X-ray studies have been made of the compound, 9, interacting with lysozyme, no solution data seem to have been reported. The compound was synthesized from the $\alpha$-methyl glycoside of NAG (7) via the C$_6$-tosylate by displacement with NaI as described in the experimental section. This particular compound was physically the most difficult to work with because of its low solubility in the citrate buffer used. Partly for this reason it was considered a good system on which to test the repeatability of measurements. The results of two separate studies are shown graphically in Fig. 6C and the data is listed in Tables 4 and 5. As with 7, the N-acetate peak alone was used to calculate $K_B$. The runs were performed in a fashion exactly analogous to that for 7 and 10 (i.e., by varying the sugar concentration, $[I_o]$, while holding $[E_o]$ constant at $3 \times 10^{-3} \text{ M}$). The same internal reference, tertiary butanol, was employed and the water peak was used for a field-frequency lock. All runs were made on the same spectrometer whose probe temperature was found to be 31.5°C. The results calculated from the two separate runs are $K_B = 35.0 \text{ M}^{-1}$ and $\Delta = 0.583 \text{ ppm}$ (filled circles) and $K_B = 31.8 \text{ M}^{-1}$ and $\Delta = 0.631 \text{ ppm}$ (X's).
Fig. 6 Graphs of chemical shift data for the N-acetate protons of 10, 7, and 9 (filled circles - A, B, and C respectively) interacting with lysozyme. In A the X's represent data for the C<sub>5</sub>-methyl resonance. In C the X's represent a run done one week later.
Table 4. Chemical Shift Data for the N-Acetyl Protons of Compounds 7, 9 and 10 and for the C₅-Methyl of 10 Interacting with Lysozyme (3 x 10⁻³ M)

### Compound 7

<table>
<thead>
<tr>
<th>[I₀] (M)</th>
<th>δ (Hz)</th>
<th>1/δ (Hz⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>.10</td>
<td>1.44</td>
<td>0.694</td>
</tr>
<tr>
<td>.08</td>
<td>1.70</td>
<td>0.588</td>
</tr>
<tr>
<td>.06</td>
<td>2.01</td>
<td>0.498</td>
</tr>
<tr>
<td>.04</td>
<td>2.45</td>
<td>0.408</td>
</tr>
<tr>
<td>.02</td>
<td>3.17</td>
<td>0.316</td>
</tr>
</tbody>
</table>

### Compound 9 (data from two runs)

<table>
<thead>
<tr>
<th>[I₀] (M)</th>
<th>δ (Hz)</th>
<th>1/δ (Hz⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>.10</td>
<td>1.34</td>
<td>0.749</td>
</tr>
<tr>
<td>.08</td>
<td>1.57</td>
<td>0.637</td>
</tr>
<tr>
<td>.06</td>
<td>1.95</td>
<td>0.513</td>
</tr>
<tr>
<td>.04</td>
<td>2.43</td>
<td>0.412</td>
</tr>
<tr>
<td>.02</td>
<td>3.25</td>
<td>0.308</td>
</tr>
</tbody>
</table>

### Compound 10

<table>
<thead>
<tr>
<th>[I₀] (M)</th>
<th>δ⁻NHC0CH₃ (Hz)</th>
<th>1/δ⁻NHC0CH₃ (Hz⁻¹)</th>
<th>δ⁻CH₃ (Hz)</th>
<th>1/δ⁻CH₃ (Hz⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>.10</td>
<td>1.51</td>
<td>0.662</td>
<td>0.53</td>
<td>1.888</td>
</tr>
<tr>
<td>.08</td>
<td>1.74</td>
<td>0.575</td>
<td>0.60</td>
<td>1.670</td>
</tr>
<tr>
<td>.06</td>
<td>2.17</td>
<td>0.461</td>
<td>0.74</td>
<td>1.351</td>
</tr>
<tr>
<td>.04</td>
<td>2.62</td>
<td>0.382</td>
<td>0.87</td>
<td>1.149</td>
</tr>
<tr>
<td>.02</td>
<td>3.50</td>
<td>0.286</td>
<td>1.26</td>
<td>0.794</td>
</tr>
</tbody>
</table>
Table 5. Dissociation Constants ($K_D$), Binding Constants ($K_B$) and Bound Chemical Shifts ($\Delta$) for Compounds 7, 9 and 10

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_D$ (M)</th>
<th>$K_B$ (M$^{-1}$)</th>
<th>$\Delta$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.0437</td>
<td>22.9</td>
<td>0.734</td>
</tr>
<tr>
<td>9</td>
<td>0.0314</td>
<td>31.8</td>
<td>0.631</td>
</tr>
<tr>
<td>(data from two runs)</td>
<td>0.0286</td>
<td>35.0</td>
<td>0.583</td>
</tr>
<tr>
<td>10 NHCOCH$_3$</td>
<td>0.0370</td>
<td>27.0</td>
<td>0.728</td>
</tr>
<tr>
<td>C$_5$-CH$_3$</td>
<td>0.0378</td>
<td>26.4</td>
<td>0.254</td>
</tr>
</tbody>
</table>

Shift of Glycoside Methyl on Binding (+ = upfield, - = downfield).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$[I_0]$ (M)</th>
<th>$[E_0]$ (M)</th>
<th>$\delta$-NHCOCH$_3$</th>
<th>$\delta$-OCH$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.003</td>
<td>+ 2.2</td>
<td>- 0.2</td>
</tr>
<tr>
<td>9</td>
<td>0.10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.003</td>
<td>+ 2.2</td>
<td>+ 0.2</td>
</tr>
<tr>
<td>10</td>
<td>0.10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.003</td>
<td>+ 2.4</td>
<td>- 0.1</td>
</tr>
</tbody>
</table>
A further technique-checking experiment performed with this sugar was one designed to test for a sugar concentration dependence in the shift between the standard and the acetate peak. In this experiment no enzyme was added but the sugar concentration was varied over its full range. Without lysozyme being present the chemical shift between the N-acetate peak and the internal standard (tertiary butanol) was found to be invariant within less than 0.1 Hz.

It was mentioned at the beginning of this section that the C$_1$-methoxyl groups of 7, 9, and 10 were potentially suitable for chemical shift measurements (i.e. that they were sharp and readily visible). However, Raftery et al.$^{65}$ have reported that the methoxyl peak of 7 does not shift appreciably on binding to lysozyme. Indeed we found this to be true for all three compounds, 7, 9 and 10 (see Table 5). It is encouraging that the NHCOCH$_3$ $\delta$ values found here fall exactly on graphs of data taken several months earlier.

Since our measurements indicate that 7, 9 and 10 bind with approximately equal affinity to lysozyme, then if they are occupying the same subsite (presumably subsite C) they should be approximately equally affected by competitive inhibitors. Raftery et al.$^{65}$ have used the N-deuteroacetyl analogue of NAG (see experimental section) for
similar problems because, although not a strong inhibitor, it has the advantage of not displaying an interfering \(^1\)H N-acetate resonance. It was indeed found that this compound affected 7, 9 and 10 to roughly the same extent in the presence of lysozyme.

The results of measuring \(K_B\) and \(\Delta\) for the three compounds, 7, 9 and 10 are that the values are quite similar (Table 5). The values of \(\Delta\) for 7 and 10 are identical and the value for 9 is not very different (perhaps not significantly different) - however, it may indicate that the bound orientation of 9 is very slightly different from that of 7 and 10.

The \(K_B\) values for the three compounds are also too similar to allow any clear distinctions. Nevertheless it may be significant that the order of relative magnitudes, \(K_B\) (compound 9) > \(K_B\) (compound 10) > \(K_B\) (compound 7), is the same as the order of lipiphility of the compounds (e.g. during T.L.C. on silica gel using MeOH/CHCl\(_3\) as eluent, 9 ran fastest, then 10 and then 7). Certainly though, it seems safe to say that the reverse order of binding strength (as measured by \(K_B\)) does not exist - that is, \(K_B\) for 7 is not larger than \(K_B\) for 10 and 9. This suggests that either:

(a) hydrogen-bonding via the C\(_6\)-oxygen is unimportant or

(b) there is also some potential for Van der Waals interactions involving the C\(_6\) region and that these make up for the loss of a hydrogen-bonding site.

This latter possibility is attractive in view of the appreciable bound shift seen for the C\(_5\)-methyl group of 10. We have already mentioned that an appreciable Van der Waals interaction is postulated...
between the N-acetate methyl of NAG and the indole ring of tryptophan 108 and that it is the aromatic field of this ring which causes the large bound shift of the N-acetate peak. There are in fact two tryptophan residue indole rings which are close enough to interact to some extent with the C₅-methyl group of 10: tryptophan 62, which is postulated⁶⁰ to be involved in hydrogen-bonding to O(6) of sugars in subsite C, and tryptophan 63 which is postulated⁶⁰ to be involved in hydrogen-bonding to O(3) of sugars in subsite C. The imidazole ring contains an NH group which is the proposed hydrogen-bonding moiety; Van der Waals interactions via the same ring are less specific.⁶⁰

It seems from our studies that the n.m.r. technique does indeed have certain advantages over other solution methods of studying biological systems. These have been pointed out in several review papers mentioned at the beginning of this chapter. One really noteworthy advantage is the ability to investigate individual parts of complex systems (e.g. individual anomers in mixtures and individual protons in the same molecule - examples of both of which have been included in this chapter). A noteworthy drawback to n.m.r. in biological systems is the low signal-to-noise ratio of the technique. The recent introduction of Fourier transform n.m.r. spectroscopy represents a quantum jump in the solution of this latter problem, but it requires expensive equipment. The search for n.m.r. probes such as ¹⁹F which potentially display a large bound chemical shift, Δ, is sensible because a large Δ permits the use of very dilute enzyme solutions in studies such as those performed here.
D. Conformation of Free Monosaccharide Inhibitors

The binding of an inhibitor or substrate to an enzyme active site is often highly specific and depends, not only on the substituent groups, but also on the configuration and conformation of the molecule involved.\textsuperscript{74,75} Also mechanisms for enzyme action have postulated both enzyme and substrate distortion. In these respects, the conformations of monosaccharide subunits of lysozyme substrates are of interest. The conformations in solution of the 2-deoxy-2-amino sugars studied here have not been previously reported. Also, quite apart from determining the absolute conformations of NAG derivatives, we wanted to either confirm or rule out the possibility of conformational differences being the cause of variation in $K_B$ and $\Delta$ between inhibitors.

The n.m.r. spectra of sugars in aqueous solution are generally unsuitable for conformational studies. This is a result of the fact that the bulk of the ring protons (often all but $H_1$) fall close together in the region about $\tau$ 6.0 to $\tau$ 6.5 (e.g. Fig. 7A). A standard method of getting around this problem is to make the completely O-acetylated derivative of the sugar in question. This serves the dual function of increasing its solubility in organic solvents and tending to spread the ring proton spectrum over a wider energy range. It is then often possible to find a solvent in which the n.m.r. spectrum is partly or wholly analysable. The assumption is made that the data so derived can be reasonably extrapolated to the non-acetylated species in water. For the molecules studied here this assumption could be checked via the coupling $J_{1,2}$. We report here the results of such a study on the completely O-acetylated derivatives of
B. Partial $^1$H n.m.r. spectrum (100 MHz) of C$_6$-iodo-N-acetyl glucosamine-$\alpha$-methyl glycoside diacetate (9') in CDCl$_3$-TMS solution. The first order assignment is shown. Note the remnant of the NH coupling into H$_2$. 

Fig. 7 A. $^1$H n.m.r. spectrum (100 MHz) of N-acetyl glucosamine-$\alpha$-methyl glycoside (7) in $D_2$O using a TMS capillary for field-frequency lock.
compounds 3, 4, 7, 9, and 10. These derivatives will be labelled after their parent compounds as follows:

3': \( R_1 = H \quad R_2 = OCH_3 \)

4': \( R_1 = OCH_3 \quad R_2 = H \)

7': \( R = OAc \)

9': \( R = I \)

10': \( R = H \)

The \(^1H\) n.m.r. spectra (100 MHz) of the ring proton regions of 3' and 4' are shown in Fig. 8A and B respectively. In each case the solvent is deuterochloroform-TMS. N-trifluoroacetyl-\(\alpha\)-methoxy-3,4,6-pyranose triacetate (3') and its \(\beta\)-methoxy counterpart (4') both displayed couplings into \(H_2\) due to the proton on the \(C_2\)-nitrogen. These couplings have been removed in the spectra shown in Fig. 8 by exchanging the nitrogen proton with deuterium. This was accomplished by adding several drops of \(D_2O\) to the n.m.r. tube containing the sample dissolved in deuterochloroform-TMS and to this adding a tiny drop
Fig. 8 Partial $^1$H n.m.r. spectra (100 MHz) of A. N-trifluoroacetyl glucosamine-α-methyl glycoside triacetate (3') and B. N-trifluoroacetyl glucosamine-β-methyl glycoside triacetate (4'), both in CDCl$_3$ solution using TMS for a field-frequency lock.
of triethylamine. For the N-trifluoroacetyl compounds this exchange occurred very readily, presumably due to the acidity of the -NH group brought about by the electronegative fluorine atoms.

Both 3' and 4' show a well resolved doublet corresponding to $H_1$ at $\tau 5.17$ and $\tau 5.33$ respectively. It is thus immediately possible to distinguish between $\alpha$- and $\beta$-anomers by the smaller $H_1$ splitting of the $\alpha$-compound. $H_2$ was readily assigned in each case by INDO experiments performed while monitoring $H_1$. Subsequent INDO experiments permitted all transitions to be assigned as shown diagrammatically in Fig. 8A and B. In each case the $C_1$-methoxyl group occurs at highest field. $H_3$ and $H_4$ form a slightly coupled system to lowfield. The $C_6$ protons form a fairly highly coupled system at highfield and $H_5$ can be seen at $\tau 6.02$ (3') and $\tau 6.18$ (4') as an octet.

The line positions for compounds 3' and 4' were used to calculate coupling constants and chemical shifts by means of iterative, computer-based analyses. Both compounds could be treated as seven spin systems. The data so obtained are listed in Table 6.

The completely O-acetylated derivatives of 7, 9 and 10 (7', 9' and 10') were treated in the same way as 3' and 4'. For example, the ring proton spectrum of 9' is shown in Fig. 7B. Once again the characteristic $H_1$ resonance can be identified (at $\tau 5.24$). $H_2$ transitions were then made the subject of INDO experiments to locate $H_3$ and $H_4$. The replacement
of the C₆-O-acetate by iodine has caused the C₆-protons to shift to highfield. 7' and 10' were treated in exactly the same fashion.

As with the N-trifluoroacetyl compounds it was found convenient to simplify the spectrum somewhat by exchanging the C₂-nitrogen proton for deuterium as described earlier. This was considerably more difficult when the adjacent acetate group was a normal acetate as opposed to a trifluoro-substituted group as pointed out previously. In fact a remnant of the H₂-NH coupling can be seen in Fig. 7B as weak satellites of H₂ since the exchange was not 100%. In general the C₂-nitrogen proton appeared to lowfield when not replaced by deuterium (e.g. for 10' -NH occurred at τ 3.99 and the H₂-NH splitting was 9.5 Hz).

There is no point in discussing each of these three compounds in detail. In each case, transitions were assigned and used as the basis of an iterative calculation (by computer) of the true coupling constants and chemical shifts (see Table 6). The proton spectra of all compounds 3', 4', 7', 9' and 10' were very similar with two exceptions:

(a) 4' has a larger H₁-H₂ coupling (axial-axial as opposed to equatorial-axial).

(b) the C₆-protons of 10' appear as a doublet rather than 8 lines as they are equivalent.

The spectra obtained for the acetates should be compared to that shown in Fig. 7A. It is the H₁ n.m.r. spectrum of 7 in D₂O - obviously very little information is available from such a spectrum. The only clearly identifiable features are H₁ (which suffers a degeneracy with
the HOD peak) at \( \tau 4.5 \) and the methoxyl and N-acetate peaks to highfield. The bulk of the ring protons form an extremely highly coupled and degenerate region from \( \tau 6.0 \) to \( \tau 7.0 \). This spectrum was run using a capillary of TMS held concentric with the n.m.r. tube as a field-frequency lock.

The conformations of the 2-deoxy-2-amino sugars studied here are reflected in the coupling constants \( J_{1,2}, J_{2,3}, J_{3,4}, \) and \( J_{4,5} \). The conformations suggested by the data in Table 6 are shown below - one very important feature being that, regardless of their absolute conformation, each of the five sugars studied seems to have the same conformation as indicated by the close similarity of the values.

In each case (except that of 4') \( J_{1,2} \) would suggest an equatorial-axial coupling with a dihedral angle in the neighbourhood of 60°. In the case of 4', the \( H_1-H_2 \) relationship seems to approach axial-axial.

![Diagram of sugars](image-url)
Table 6. N.M.R. Parameters for the O-Acetate Derivatives 3', 4', 7', 9' and 10'

Chemical Shifts (τ-values)*

<table>
<thead>
<tr>
<th></th>
<th>H₁</th>
<th>H₂</th>
<th>H₃</th>
<th>H₄</th>
<th>H₅</th>
<th>H₆₁</th>
<th>H₆₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'</td>
<td>5.17</td>
<td>5.67</td>
<td>4.70</td>
<td>4.86</td>
<td>6.02</td>
<td>5.72</td>
<td>5.87</td>
</tr>
<tr>
<td>4'</td>
<td>5.33</td>
<td>5.98</td>
<td>4.59</td>
<td>4.90</td>
<td>6.18</td>
<td>5.69</td>
<td>5.82</td>
</tr>
<tr>
<td>7'</td>
<td>5.25</td>
<td>5.66</td>
<td>4.78</td>
<td>4.90</td>
<td>6.06</td>
<td>5.74</td>
<td>5.89</td>
</tr>
<tr>
<td>9'</td>
<td>5.24</td>
<td>5.66</td>
<td>4.80</td>
<td>5.09</td>
<td>6.22</td>
<td>6.69</td>
<td>6.85</td>
</tr>
<tr>
<td>10'</td>
<td>5.33</td>
<td>5.68</td>
<td>4.83</td>
<td>5.15</td>
<td>6.17</td>
<td>8.81</td>
<td>-</td>
</tr>
</tbody>
</table>

Coupling Constants (Hz)†

<table>
<thead>
<tr>
<th></th>
<th>H₁H₂</th>
<th>H₂H₃</th>
<th>H₃H₄</th>
<th>H₄H₅</th>
<th>H₅H₆₁</th>
<th>H₆₁H₆₂</th>
<th>H₆₂H₆₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'</td>
<td>3.7</td>
<td>11.3</td>
<td>9.4</td>
<td>10.5</td>
<td>4.7</td>
<td>2.2</td>
<td>-12.4</td>
</tr>
<tr>
<td>4'</td>
<td>8.6</td>
<td>10.8</td>
<td>9.6</td>
<td>9.7</td>
<td>5.2</td>
<td>1.9</td>
<td>-13.0</td>
</tr>
<tr>
<td>7'</td>
<td>3.6</td>
<td>10.9</td>
<td>9.3</td>
<td>9.7</td>
<td>2.4</td>
<td>4.7</td>
<td>-12.2</td>
</tr>
<tr>
<td>9'</td>
<td>3.7</td>
<td>10.5</td>
<td>9.4</td>
<td>9.6</td>
<td>2.2</td>
<td>8.9</td>
<td>-10.7</td>
</tr>
<tr>
<td>10'</td>
<td>3.6</td>
<td>10.4</td>
<td>9.6</td>
<td>9.8</td>
<td>6.2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Measured in deuterochloroform solution containing TMS.

† The errors in these coupling constants are ± 0.2 Hz.
The values of the $\text{H}_2$-$\text{H}_3$, $\text{H}_3$-$\text{H}_4$, and $\text{H}_4$-$\text{H}_5$ couplings (all $\approx 10$ Hz) are in close accord with a trans-diaxial relationship in each case (i.e. the $180^\circ$ vicinal angle indicated in the sugar diagrams).

As mentioned previously, it is not immediately obvious that the conformations arrived at for the above compounds may be meaningfully extrapolated to those of their un-acetylated parent compounds in water. However it is possible to check the observed splitting of $\text{H}_1$ for a sugar dissolved in water (e.g. Fig. 7A) against the $\text{H}_1$-$\text{H}_2$ coupling constant of the O-acetylated system; for instance the compounds 3, 7, 9, and 10 were seen to have $\text{H}_1$ splittings in the neighbourhood of 3.1 Hz. At first sight this would suggest that their conformations are slightly different from those of their O-acetylated derivatives in deuterochloroform. However, as will be discussed in more detail in the next section, $\text{H}_2$ and $\text{H}_3$ fall quite close together in the parent sugars (Fig. 7A) and are strongly coupled ($J_{2,3} = 10-11$ Hz $\approx$ their chemical shift separation). In fact a theoretical calculation of the $\text{H}_1$ spectrum assuming $J_{1,2} = 3.6$ Hz but taking into account the close coupling of $\text{H}_2$ and $\text{H}_3$ gives a splitting for $\text{H}_1$ of 3.1 Hz (see Fig. 8) as observed. This observation would argue favourably for the extrapolation from O-acetylated sugar conformations to those of their unacetylated precursors.

The conformations arrived at for the amino sugars studied are those to be expected on steric grounds and on the basis of previous n.m.r. studies of non-amino sugars.$^2,^3$
E. N.M.R. as a Probe for Conformation of Bound Monosaccharide Inhibitors

There has been considerable speculation concerning the role of conformational changes on binding of substrates (or inhibitors) to enzymes. Perhaps the best known proponent of enzyme conformational changes on binding is Koshland\(^7^5\) whose "induced fit theory" has been put forward as a mechanism of enzyme action. In the case of lysozyme - a relatively small, rigid enzyme - X-ray work has shown that very little distortion of the enzyme occurs on binding.\(^3^5,3^6\) On the other hand, the same workers have postulated that during cleavage of oligosaccharides the sugar unit bound in subsite D of the active site is distorted toward a half-chair conformation. This conclusion was drawn from molecular models. No concrete proposals have been made regarding distortion of sugars at other subsites although it has been suggested\(^6^0\) that such distortions are possible.

In principle n.m.r. chemical shifts and coupling constants could be used to measure distortion on binding. The latter possibility will be considered here.

Since, in the fast exchange limit, the inhibitor resonances represent a population-weighted average of the bound and unbound species, it should be possible to extrapolate from the observed coupling constants to those of the totally bound species. However, as has been emphasized previously (Chapter I), observed splittings can only be taken as true coupling constants in very special circumstances (the basic condition for this assumption to be valid being that all nuclei be separated by shifts large with respect to the coupling constants).
In none of the inhibitors examined here is this true. For instance, the $^1$H n.m.r. spectrum of 7 dissolved in D$_2$O is shown in Fig. 7A. H$_1$ can be seen to lowfield ($\tau$ 5.25) and the splitting due to coupling with H$_2$ is clearly visible. H$_2$ and H$_3$ are known to fall generally in the lowfield region of the ring proton envelope for free sugars dissolved in aqueous media. Indeed INDOE experiments run while monitoring H$_1$ can be used to pick out part of the H$_2$ spectrum at the lowfield end of the envelope (Fig. 7A). The coupling between H$_2$ and H$_3$ is known from the last section to be 10.9 Hz. By inspection it is possible to guess a rough chemical shift position for H$_3$.

Armed with this data it is a simple matter to calculate theoretical spectra for the system formed by H$_1$, H$_2$ and H$_3$. This should give a fairly reliable representation of H$_1$ to be compared with that observable experimentally. A computer plot programme was used which actually drew out the calculated H$_1$ spectrum. Fig. 9A shows the result of such a computer print out for H$_1$ when $J_{1,2} = 3.6$ Hz and $J_{2,3} = 10.9$ Hz (i.e., the coupling constants calculated in the previous section for 7'), and when the separation between H$_2$ and H$_3$ is taken as 12.0 Hz (see Table in Fig. 9). The splitting seen in the simulated spectrum is found to be 3.1 Hz. Fig. 9B shows the result of allowing H$_2$ to shift upfield toward H$_3$ by 1.7 Hz: the observed splitting in H$_1$ has now become 3.0 Hz. In Fig. 9C, H$_2$ has been allowed to shift another 1.0 Hz toward H$_3$ with the result that the H$_1$ splitting becomes 2.9 Hz. The point we have tried to make here is that rather small changes in the chemical shifts of H$_2$ or H$_3$ (changes which are on the scale of those expected from binding to the enzyme) can cause appreciable
Fig. 9. Computer-simulated spectra of the $H_1$ 'doublet' region of a hypothetical N-acetyl glucosamine-\(\alpha\)-methyl glycoside in aqueous solution. The $H_1$ resonance has been generated from a consideration of $H_1$, $H_2$ and $H_3$ by assuming $J_{1,2} = 3.6$ Hz, $J_{1,3} = 0$ Hz, $J_{2,3} = 10.9$ Hz and the chemical shift values shown above. Note that only the chemical shift of $H_2$ is varied.
changes in the splitting observed in $H_1$. Fig. 9D emphasizes the point by showing what happens to the $H_1$ 'doublet' when $H_2$ has shifted 8.0 Hz toward $H_3$ - obviously there is now no simple interpretation of the $H_1$ splitting in terms of $J_{1,2}$. In fact the splitting observed experimentally (Fig. 7A) in $H_1$ was found to be 3.1 Hz which agrees well with the proposed arrangement of $H_1$, $H_2$ and $H_3$ in Fig. 9A.

In spite of what has been said above, it would be good evidence for inhibitor distortion on binding to see a large change in the observed splitting of $H_1$. We have calculated an order of magnitude for errors likely to be introduced by chemical shift changes in $H_2$ and $H_3$ (this error being roughly ± 0.2-0.3 Hz). It is now a simple matter to calculate the changes to be expected in the $H_1$ splitting of an inhibitor with $K_B = 50 \text{ M}^{-1}$ and $\Delta \approx 0.70$ ppm.

$$K_B = \frac{[EI]}{[E][I]} = 50 \text{ M}^{-1}$$

where $[EI]$ = concentration of bound inhibitor

$[E]$ = concentration of free enzyme

$[I]$ = concentration of free inhibitor

if $[I] = [I_0] = 0.04 \text{ M}$ (where $[I_0]$ is the initial inhibitor concentration)

and $[E] = 0.003 - [EI]$

then

$$\frac{[EI]}{(0.003-[EI])(0.04)} = 50$$
\[ [EI] = 0.002 \text{ M} \]

Fraction of inhibitor bound \( = I_B = 1/20 \)

and Fraction of inhibitor free \( = I_F = 19/20 \)

Now in the fast exchange limit the observed splitting, \( J'_{\text{obs.}} \), is a sum of two terms:

\[
J'_{\text{obs.}} = I_B J'_{\text{obs.B}} + I_F J'_{\text{obs.F}}
\]

where \( J'_{\text{obs.B}} \) = splitting in totally bound case

\( J'_{\text{obs.F}} \) = splitting in totally free case.

If we assume quite a large distortion on binding so that

\[
J'_{\text{obs.B}} = 6 \text{ Hz} \quad \text{and} \quad J'_{\text{obs.F}} = 3.1 \text{ Hz}
\]

Then

\[
J'_{\text{obs.}} = (1/20) \cdot 6 + (19/20) \cdot 3.1
\]

\[
= 0.3 + 2.95
\]

\[
= 3.25 \text{ Hz}
\]

It should be obvious then that to use spectral splitting changes to measure inhibitor distortion on binding will require careful spectral analysis. Nevertheless, the technique should be viable provided the following steps are followed:
(a) Obtain a high fraction of bound inhibitor - this will involve dilute solutions and broad lines.

(b) Use a time averaging technique (esp. Fourier transform) to achieve good signal-to-noise.

(c) Choose an inhibitor which shows little exchange broadening (and/or work at higher temperatures) - this will greatly increase line sharpness and accuracy.

(d) Choose a system whose spectrum is either first order or can be analysed to avoid ambiguities due to high order effects.

Another potential technique for the investigation of conformational changes on binding is one which this laboratory has been working toward for some years. It involves the extreme sensitivity of $^{19}\text{F}$ chemical shifts to intramolecular effects (such as conformation or configuration changes). For instance, in the system studied here one would observe a $^{19}\text{F}$ substituent on the ring. The ability to "noise decouple" the ring protons would be very useful in any such project.
Experimental

General Methods

(a) All $^1$H n.m.r. spectra were measured with a Varian HA-100 spectrometer operating in the frequency sweep mode. For enzyme studies the water resonance was used for the field-frequency lock. Sugar solutions in CDCl$_3$ were run locked on tetramethylsilane (TMS). All $^1$H chemical shifts are reported on the $\tau$ scale.

(b) All $^{19}$F spectra were measured at 94.071 MHz using a Varian HA-100 spectrometer operating in the frequency sweep mode. A capillary of trifluoroacetic acid (TFA) held concentric with the n.m.r. tube was used for the field-frequency lock.

(c) Melting points were performed on a Thomas-Hoover capillary m.p. apparatus and are corrected for thermometer error.

(d) The HA-100 probe temperature was 31.5°C for all enzyme experiments.

(e) Two different internal reference compounds were used for the measurement of chemical shifts ($\delta$) in enzyme studies: for proton work, tertiary butanol and for fluorine work, trifluoroethanol (Aldrich).

(f) All pH measurements were performed on an Instrumentation Lab. Inc. pH meter with expandable scale.

(g) All enzyme measurements were made in 0.1 M citrate buffer at pH 5.5 (Documenta Geigy$^{77}$).

(h) The technique of measuring $\delta$ relative to the internal standards in enzyme runs was as follows. The spectrum of the standard was recorded and calibrated followed by that of the sugar resonance of interest. Then the standard spectrum was run again and the results
were averaged. This was repeated several times for each sample.

implicit

(i) All enzyme measurements were made with the same batch of hen egg white lysozyme (Worthington Biochemical - salt-free, twice recrystallized). Solutions were made up freshly immediately prior to use. Enzyme concentrations were calculated by removing a 25 µl aliquot and diluting to 25.00 ml with buffer. The absorbance was then read at 280 µm on a double beam Bausch & Lomb Spectronic 600 and used to calculate \([E_0]\) from the known extinction coefficient.\(^{78}\)

Organic Synthesis

2-Amino-2-deoxy-N-trifluoroacetyl-\(\alpha\)-glucose (1 and 2) was the precursor of all fluoro derivatives studied here and was prepared by the action of S-ethyl trifluorothiolacetate (Pierce Chemical Co.) on \(\alpha\)-glucosamine in methanol as described in the literature.\(^{79}\) \(\alpha\)-glucosamine itself was produced in the reaction vessel from glucosamine hydrochloride (Aldrich) and sodium methoxide prior to adding the trifluorothiolacetate. The \(\alpha\)-anomer (1) crystallized selectively from an aqueous solution of the mutarotated pair as solvent was allowed to evaporate slowly. 1 was obtained as large, clear, colourless crystals which when carefully dried became white and powdery: m.p. 188-188.5°C (with decomposition).

2-Amino-2-deoxy-N-trifluoroacetyl-\(\alpha\)-(and \(\beta\))-methyl glycoside (3 and 4) were prepared from pure, dry N-trifluoroacetyl glucosamine via the general method of acid catalysis in methanol using a resin (Dowex-Ag 50 W X8, Bio Rad) as catalyst. The resin in its acid
form was washed thoroughly with methanol over a period of several
days before use. Sample conditions were: 4 g of starting material
was dissolved in about 50 ml of dry methanol. To this was added 30 ml
of resin and the mixture was refluxed with stirring for 3 hours.
The mixture was then filtered and the resin washed thoroughly.
Stripping of the solvent from the filtrate produced a syrupy mixture
of α- and β-glycosides (in about a 2:3 ratio). Column chromatography
on silica gel (Mallinckrodt "SilicAR" CC-7), using first 10%
MeOH/CHCl₃ and then 20% MeOH/CHCl₃ gave the pure anomers in roughly
60% yield. M.p. of 3 was 196.5-197.5°C (with decomp.), m.p. of 4
was 216.0-217.0°C (with decomp. Both were white solids.

2-Amino-2-deoxy-N-acetyl-D-glucose-α-methyl glycoside (7) was
prepared from N-acetyl glucosamine (Pfanstiel Lab.) as described in
the literature using Dowex resin in the acid form and MeOH.
Purification was achieved by means of column chromatography on
silica gel using 10% MeOH/CHCl₃ as eluent. The m.p. of the white
solid was 192-193°C.

2-Amino-2-deoxy-N-acetyl(d₃)-D-glucose was prepared by selective
N-acylation of glucosamine with acetic anhydride-d₆ (Merck, Sharp
and Dohme) as described in the literature.

2-Amino-2-deoxy-N-acetyl-C₆-O-p-tolylsulfonyl-D-glucose-α-methyl-
glycoside was produced in a fashion similar to that described in the
literature for its glucose analogue. 3.9 g of 7 was dissolved in dry pyridine (55 ml) in a flask equipped with drying tube. 1.1 mole equivalents of p-tolylsulfonyl chloride (Eastman) was dissolved in 8 ml of dry pyridine. The solution of 7 was cooled to 0°C in a salt-ice bath. The p-tolylsulfonyl chloride solution was then added dropwise with stirring over a period of 20 min while maintaining the temperature at or below 0°C. The mixture was then left to warm up to room temperature. After 48 hours the solvent was stripped off. The resulting oil was dissolved in CHCl₃ and washed with an aqueous solution of KHCO₃. The yield was 50-60% of a light tan oil.

2-Amino-2-deoxy-N-acetyl-C₆-ido-D-glucose-α-methyl glycoside (9) was prepared from the C₆-tosylate by a method analogous to that described in the literature for its glucose analogue. The oil obtained as described above (4 g) was dissolved in dry acetone (40 ml). 3 mole equivalents of dry NaI (Allied Chemical) was then added and the solution was refluxed with stirring for 3 hours after which the platey crystals of sodium p-tolylsulfonate were filtered off. Workup afforded a slightly tacky powder. Purification was achieved by column chromatography on silica gel eluted with 5% MeOH/CHCl₃. The result was a good yield of a white solid, m.p. 162-164°C.

2-Amino-2-deoxy-N-acetyl-C₅-methyl-D-glucose-α-methyl glycoside (10) was prepared by catalytic hydrogenation of 9 at atmospheric pressure. The general technique for hydrogenation of alkyl halides is discussed
elsewhere. 2.0 g of 9 was dissolved in 25 ml of MeOH in a 50 ml Erlenmeyer flask. 3-4 equivalents (1 g) of solid KOH was added and 300 mg of palladium on powdered charcoal (Matheson, Coleman and Bell-10% catalyst). The mixture was hydrogenated at room temperature and atmospheric pressure for 3 hours. Workup afforded a clear, colourless glass (yield > 90%).

O-Acetylated derivatives of sugars were found to be extremely useful for product identification via their $^1$H n.m.r. They were produced by reaction with excess acetic anhydride (Baker Chemical Co.) in dry pyridine.

**Treatment of Data**

The correlation of chemical shift ($\delta$) and inhibitor concentration ([I]) with bound chemical shift ($\Delta$) and dissociation constant ($K_D = \frac{1}{K_B}$) has been described by Dahlquist and Raftery in some detail. The basic treatment is shown below for an inhibitor interacting reversibly with an enzyme.

$$ E + I \leftrightarrow EI $$

$$ K_D = \frac{[E][I]}{[EI]} \quad (1) $$

[E] = enzyme concentration

[I] = inhibitor concentration

[ EI] = concentration of enzyme-inhibitor complex

In the fast exchange limit we have:
\[ \delta = P_B \Delta \quad \text{(2)} \]

where \( \delta \) is the observed shift of a particular sugar peak from its unbound position and \( P_B \) is the fraction of total inhibitor bound at a given time.

From (2) \[ \delta = \frac{[EI]}{[I_0]} \Delta \]

\[ \therefore [EI] = [I_0] \frac{\delta}{\Delta} \]

but \[ [E] = [E_0] - [EI] = [E_0] - [I_0] \frac{\delta}{\Delta} \]

and \[ [I] = [I_0] - [EI] = [I_0] - [I_0] \frac{\delta}{\Delta} \]

where the subscript 'o' indicates initial concentration.

Substitution into equation (1) gives

\[ K_D = \frac{[E_0] \Delta}{\delta} - [I_0] - [E_0] + [I_0] \frac{\delta}{\Delta} \]

Making the following approximations that:

(a) \( \delta \ll \Delta \)

and (b) \( K_D \) is of the order of \( [I_0] \)

we may drop the term \( \frac{\delta}{\Delta} [I_0] \) to get:

\[ K_D = \frac{[E_0] \Delta}{\delta} - [E_0] - [I_0] \]

or \[ [I_0] = \frac{[E_0] \Delta}{\delta} - K_D - [E_0] \]
Hence a plot of $[I_o]$ vs. $1/\delta$ should yield a straight line of slope $[E_o]A$ and intercept $-(K_D + [E_o])$. 
CHAPTER III
APPLICATION OF THE AUDIOFREQUENCY PULSE TECHNIQUE TO
THE STUDY OF LARGE ORGANIC MOLECULES IN SOLUTION

Introduction

Several techniques involving the use of n.m.r. spectroscopy to study large organic molecules in solution have already been discussed. Magnetic resonance methods (both e.s.r. and n.m.r.) involve relatively low energy spectroscopic processes and, hence, reflect in their spectra phenomena of great interest to the chemist. Previously in this thesis we have made use of the well known spectral features: chemical shift, scalar coupling and integrated intensity. A fourth feature, the relaxation times associated with a given resonance, will now be considered. Until quite recently this last feature has been used primarily by physicists and physical chemists in problems involving small molecules.

The term relaxation here refers to the transfer of spin populations between nuclear energy levels as a result of their energy differential in a magnetic field. Obviously if there is some mechanism for this transfer there will be a tendency to reach a Boltzmann distribution such that a resultant macroscopic magnetic moment vector will exist along the magnetic field axis. At temperatures greater than a few degrees
Kelvin, the ratio of lower to upper state population can be written

\[ \frac{N_+}{N_-} = 1 + \frac{2\mu H_o}{kT} \]

\( T \) is the Boltzmann spin temperature: the spins are immersed in a thermal bath (the bulk solution) at roughly room temperature. However, there is only a very weak thermal coupling between the nuclear spin system and the bath so that the Boltzmann spin temperature can become very high during the course of some perturbation such as irradiation at the resonance frequency. This weak coupling arises from time-dependent local magnetic fields (the time dependence being derived from molecular motion). These fluctuating local magnetic fields to which a molecule in solution is exposed are responsible for all nuclear relaxation processes but there are some half dozen major mechanisms for production and interaction of such fields.\(^{51,83,84}\) Moreover there are two macroscopically observable features of relaxation processes: the ease of saturation of a transition (related to the above spin-temperature discussion) and the linewidth of the resonance corresponding to the transition (related to the nuclear spin state lifetime).

In dealing with nuclear relaxation, it is generally convenient to consider the above-mentioned macroscopic magnetic moment which is experimentally observable and which at equilibrium lies along the Z-axis (\( H_o \)-axis). The behaviour of this vector can be described by classical mechanics\(^{53}\) and basic relaxation phenomena are readily dealt with using such a model (more complete discussions have been given elsewhere\(^ {50,83-85}\)).
With any transition in a n.m.r. spectrum there is associated such a vector. When this vector is disturbed from its equilibrium position (say by irradiation at its resonance frequency, ω) it recovers exponentially at a characteristic rate upon removal of the perturbation. The time constant for its recovery parallel to the Z-axis is called $T_1$ and that for its collapse in the XY-plane is called $T_2$.

Experimentally both $T_1$ and $T_2$ can be measured by a variety of techniques. $T_2$ is reflected in the linewidth of a single transition according to the relationship $T_2 = 1/\pi \Delta \nu$ (where $\Delta \nu$ is the linewidth at half height). Unfortunately, for most common organic molecules in solution the 'true' value of $\Delta \nu$ is obscured by field inhomogeneity and/or small, unresolved splittings. Spin-echo methods and the $T_{1\rho}$ technique are more generally applicable for measurement of $T_2$. The spin-echo technique was chosen for use in this work, although both other methods have been tried. $T_1$ can be measured by $T_{1\rho}$, adiabatic rapid passage, saturation recovery and progressive saturation techniques. In this work $T_1$ was measured by a very simple pulse technique in which a single pulse is used to invert the magnetization along the Z-axis (see Appendix A) and its return to equilibrium is monitored at intervals.

**Biological Applications**

Because $T_1$ and $T_2$ are determined by time-dependent inter- and intramolecular relationships, they contain information about relative rates of motion, orientations and distances in the molecules involved. Although the biological applications of relaxation studies are not as
numerous to date as those of chemical shift and coupling constant data, they are very important and becoming more so. A very general review of the area has been included in the article by Allerhand and Trull. More specific review articles exist on various aspects of the use of nuclear relaxation rates to study macromolecules. In particular, Mildred Cohn has described in detail the use of paramagnetic probes. For instance considerable work has been done by introducing paramagnetic species at the active site of an enzyme for enzyme-inhibitor interaction studies. The effect of the unpaired electron spins on the relaxation rates of nuclear spins is a function of the distance between the two interacting species and of the motional freedom in the region of the paramagnetic species (the reason for using a paramagnetic probe in the first place is that unpaired electrons are extremely effective in causing rapid relaxation of neighbouring nuclei). Thus the exploitation of relaxation rate changes effected by paramagnetic probes at the active site provides the possibility of estimating interatomic distances and of mapping the substrates at the active site in solution as well as characterizing the molecular motion of highly localized regions of the active site. The same approach may permit calculation of the rate of chemical exchange between the 'enzyme-bound' and 'unbound' substrate conditions. This rate is related to the rate of the first elementary step in the reaction sequence of enzyme catalysis. A well known example of the paramagnetic probe technique is the study of creatine kinase using paramagnetic manganous ions and a stable nitroxide free radical.
Sykes and Scott have produced an excellent review on the use of n.m.r. to study dynamic aspects of molecular structure and interaction in biological systems exclusive of paramagnetic probe methods. They point out that there are two approaches whereby biological systems have been studied for kinetic data. Both approaches involve solution of the phenomenological Bloch equations for the nuclear magnetization, as modified by McConnell to include the effects of chemical exchange, for a spin system where a nucleus is transferred back and forth between two distinct environments, A and B, with first order lifetimes, $\tau_A$ and $\tau_B$. Perhaps the most obvious technique is that applicable when both sites are approximately equally populated. It is then possible to derive equations describing the lineshape for a variety of exchange lifetimes and to compare these to the observed spectrum. With this approach it is desirable to be able to vary the exchange lifetime, $\tau = \frac{\tau_A \tau_B}{\tau_A + \tau_B}$, over a fairly wide range relative to the difference in resonance frequencies ($\omega_A - \omega_B$) if this is non-zero or relative to the relaxation rates of the sites if $\omega_A = \omega_B$.

In the system (lysozyme and inhibitor) studied in this chapter, the condition of equal site populations was not fulfilled. However, an interesting area where the lineshape approach has been applied (with somewhat ambiguous results) is the study of helix-coil transitions in certain synthetic polypeptides. In these studies the resonances corresponding to peptide bond NH groups and $\alpha$-CH groups were monitored.

The other approach described by Sykes and Scott for calculation of forward and reverse exchange rate constants is applicable when
the population of one site is much greater than that of the other site. This is a common situation in enzyme-substrate and enzyme-inhibitor studies where the concentration of free substrate or inhibitor is much larger than the concentration of bound species. In fact in these cases only one resonance is observed even in the slow exchange limit and rate constants must be calculated from a study of $T_1$, $T_2$ and $\omega$ for this resonance as a function of some variable such as temperature, spectrometer frequency or relative population of sites A and B. In our case we have used the latter approach by varying the enzyme concentration. Sykes and Scott\textsuperscript{45} point out that for the case of Population A $>>$ Population B, Swift and Connick\textsuperscript{95} have derived a general solution to the modified Bloch equation for $T_2$ and $\omega$ which is valid for all exchange rates. A corresponding equation for $T_1$ has been derived by Luz and Meiboom\textsuperscript{96a} and by O'Reilly and Poole.\textsuperscript{96b}

Sykes\textsuperscript{97a} and Sykes and Parravano\textsuperscript{97b} have manipulated these equations into forms suitable for dealing (via n.m.r.) with the case of an enzyme-inhibitor system in which the bound magnetic environment of some portion of the inhibitor differs from that of its unbound counterpart. Naturally these equations are most sensitive to exchange rates which are "on the n.m.r. time scale" - to date, all rate constant data reported for monosaccharide inhibitors of lysozyme seems to be in this intermediate range. Sykes\textsuperscript{97a} and Sykes and Parravano\textsuperscript{97b} have applied their equations to the interaction of lysozyme with four N-acetyl-$D$-glucosamine compounds (5-8) of the previous chapter by making use of the N-acetyl resonances, and Sykes\textsuperscript{98} has reported a similar measurement on trifluoroacetyl-$D$-phenylalanine interacting
with α-chymotrypsin.

In this chapter, amongst other uses of the audiofrequency pulse technique, we report the results of rate constant calculations on the system of lysozyme and N-trifluoroacetyl-α-D-glucosamine as measured by pulsed $^{19}$F n.m.r. This data was of particular interest to us because we were curious as to the effects of $^{19}$F labels with regard to the work of Chapter II.

Applications to Large Organic Molecules

Although the phenomenon of nuclear relaxation is of fundamental importance to the measurement of n.m.r. spectra, and has as such received a great deal of attention from physicists, it has been largely neglected by organic chemists. There are several good reasons for this lack of interest, of which the most cogent is that until recently, instrumentation suitable for the routine and selective measurement of the nuclear relaxation times of anything other than very simple systems has been unavailable. However, the development of the selective, audiofrequency-pulse (n.m.r.) technique by Freeman and Wittekoek has made possible, at least in principle, relaxation studies of complex organic systems. This is an extremely new area about which very little is known. As will be seen, 'large' organic molecules such as sugars and steroids have quite different relaxation times from simple molecules such as benzene or chloroform and there are considerable problems to be overcome in dealing with them. The work reported here (and indeed elsewhere in the literature) is only a scratch on the surface of the field of selective relaxation studies of large
organic molecules; eventually it may be possible to relate the results of such studies to intramolecular phenomena of interest. In this regard we describe here some preliminary considerations of the problem of whether $T_1$ and $T_2$ are sensitive to intramolecular phenomena by measuring the relaxation times of a series of closely related simple compounds (the cis and trans isomers of 1,2-dichloroethylene, 1,2-dibromoethylene and the ethyl esters of maleic and fumaric acids). As will be shown, the results of these experiments are encouraging. We have also gone on to apply similar methods to more practical examples: the measurement of proton relaxation times of various nuclei in an alkaloid and several sugars.
Results and Discussion

A. A Case for the Applicability of $T_1$ and $T_2$ to Structural Organic Chemistry

Physicists and physical chemists have measured nuclear relaxation times of small molecules in attempts to elucidate the mechanisms of relaxation. $83,84,50-53,100$ A typical approach is to calculate $T_1$ and $T_2$ for a simple molecule by considering the various possible mechanisms and comparing the calculated values to those observed experimentally. In such cases measurements are usually made in a solvent possessing no hydrogen nuclei or in a deuterated solvent and the data extrapolated to infinite dilution to rule out intermolecular effects. As chemists dealing with the structure of large organic molecules, we were curious as to whether relaxation time measurements are sensitive to molecular parameters of interest to us - and we were willing to settle for empirical relationships. For instance, are $T_1$ and $T_2$ sensitive to changes in substituent groups or orientation and proximity of substituents? If not, a number of potential uses of pulsed n.m.r. would be closed to us.

As a first step in this direction we have measured the relaxation times of a series of substituted cis and trans isomers of ethylene:

* The apparatus used for pulse experiments described in this chapter (with the exception of those in Section B) was the "Mark II" pulse spectrometer (see Appendix B) designed and constructed in collaboration with Mr. Roland Burton of this Department.
Since we were interested primarily in changes in $T_1$ and $T_2$ brought about by intramolecular chemical differences rather than in absolute values, we wished to maintain all other factors as constant as possible. A very simple method of ensuring this is to dissolve all compounds in the same sample—a method which is beautifully compatible with selective pulse techniques. As a typical example Fig. 1A shows the $^1$H n.m.r. spectrum (100 MHz) of a mixture of the cis and trans isomers of 1,2-dichloro- and 1,2-dibromoethylenes. The solvent used was deuterochloroform with a few drops of TMS for a field-frequency lock. Although the bromo-compounds were purchased as a mixture of isomers, the concentrations of the four compounds were approximately equal ($\approx 0.5$ M). The sample was carefully degassed (6 freeze-pump-thaw cycles) before being sealed off to exclude (paramagnetic) oxygen. Each compound gives a single, symmetric, very sharp resonance whose linewidth
Fig. 1. The alkene proton regions of the n.m.r. spectra (100 MHz) of degassed samples (solvent deuterochloroform) of A. 1,2-dibromo- and 1,2-dichloroethylene (cis and trans isomers) and B. maleic and fumaric acid diethyl esters (plus cis 1,2-dichloroethylene as a reference). TMS was used for the internal field-frequency lock. In each case the resonances are numbered and the compounds to which they correspond are indicated. The nuclear relaxation data for each resonance is tabulated below the spectra.
is determined mainly by field inhomogeneity. The proton resonances corresponding to bromo analogues fall to lower field than those of chloro analogues and in each case the cis anomer is to low field of the trans isomer (identification of the bromo compounds was via scalar couplings in the $^{13}$C satellites). It was a relatively simple matter to selectively measure $T_1$ and $T_2$ for each resonance (as will be discussed in more detail at the end of this section) although the separation between the chloro isomer resonances was only 6.3 Hz. The values of $T_1$ and $T_2$ found are listed below Fig. 1. The error limits involved in such measurements are roughly $\pm 10\%$ for the $T_1$ data but are probably considerably larger for $T_2$ in this particular case as will be discussed later in this section. The notable features of the data are that:

(a) in each case $T_1$ is considerably longer than $T_2$ (this difference is more pronounced in the case of the chloro compounds)

(b) in each case the cis isomer has shorter relaxation times than the trans isomer.

Fig. 1B shows the $^1$H n.m.r. spectrum of the alkene region of a second sample containing the esters of maleic and fumaric acids as well as the already measured cis 1,2-dichloroethylene. This sample was made up in an exactly analogous fashion to that of the sample just considered: solvent CDCl$_3$ with a few drops of TMS for a field-frequency lock; and was degassed (6 freeze-pump-thaw cycles) prior to being sealed off. The alkene proton resonance of fumaric acid diethyl ester falls to low field of its maleic acid counterpart.
Note the reduced ringing in the cis-isomer. Both of these acid esters may have very small unresolved couplings between the ethylene protons and the ethyl protons. This second sample (Fig. 1B) contained a somewhat lower concentration of protonated species than the previous one (Fig. 1A) however in each case the sample was roughly 80% CDCl₃ by volume.

That there were no large differences in intermolecular effects between the samples of Fig. 1A and 1B was checked in this case by the inclusion of some common species in each sample. The common species, or internal standard, was cis 1,2-dichloroethylene.

The relaxation data for this new sample is listed below that for the first sample in Fig. 1. Each of the seven spectral transitions is numbered and its corresponding compound indicated above the spectrum. Note that in the second sample, $T_2$ for cis 1,2-dichloroethylene has remained the same as in the first sample within experimental error (however the slight increase may reflect the more dilute solution). This use of a standard permits a certain amount of data comparison between samples. Once again it is obvious that the cis isomer has a shorter relaxation time than the trans isomer. However, in this case, $T_1$ and $T_2$ are much more nearly equal (in fact part of the difference may be experimental, as will be discussed later in this section).

The $T_2$ Experiment

The method of $T_2$ measurement employed here was that commonly referred to as the "Gill-Meiboom modification of the Carr-Purcell pulse
sequence. The result of such a measurement on resonance #7 (the diethyl ester of maleic acid) in Fig. 1B is shown in Fig. 2 together with a diagrammatic explanation of the pulse sequence. This sequence consists of a π/2-pulse (see Appendix A) along the X'-axis followed by a series of π-pulses along the Y'-axis of the rotating reference frame. Experimentally, it was convenient to make the spacing between successive π-pulses equal to twice that between the π/2-pulse and the first π-pulse. The duration of a π/2 pulse in the particular trace shown is 0.2 seconds. The components of such a pulse sequence are discussed in detail in Appendix A. Here it suffices to say that the Carr-Purcell sequence removes the contribution of field inhomogeneity to the measured value of T₂ and the Gill-Meiboom modification makes less crucial the accurate setting of pulse durations. The value of T₂ can be readily calculated from the peak heights of the initial 90°-pulse and the ensuing echoes according to the exponential relationship \( M_t = M_o e^{-t/T_2} \) (Appendix A) where \( M_o \) is the magnitude of the magnetization vector along the Y'-axis immediately after the 90°-pulse and \( M_t \) is the value at some later time, t.

The T₁ Experiment

The pulse sequence used to measure T₁ was considerably simpler and involved less pulsing per unit time as will be discussed later in this section. The sequence consisted of two pulses: the first pulse (a π-pulse along the X'-axis) to prepare the magnetization vector \( M_o \) along the -Z'-axis, and a second pulse along the X'-axis at some later time, t, to force the magnetization vector \( M_t \) into or through
Fig. 2. Effect of a modified Carr-Purcell sequence on the magnetization vector, $M_0$. A. Equilibrium; B. $\pi/2$-pulse along the $X'$-axis; C. Bloch decay; D. $\pi$-pulse along the $Y'$-axis causing refocussing of the field-inhomogeneity component; E. Echo; F. Bloch decay; G. $\pi$-pulse; H. Echo. The scope trace for such a sequence on the maleic acid diethyl ester resonance of Fig. 1B of Chapter III is shown.
the observation plane (X'Y'-plane) so that the receiver coil could detect it. In the set of experiments just described, this second pulse was a 2\(\pi\)-pulse (360°). One then simply waited for the system to return to equilibrium and then repeated the two pulse sequence using a different time delay, \(t\), between the first and second pulses.

The result of such a measurement on resonance #2 (trans 1,2-dibromoethylene) of Fig. 1A is shown in Fig. 3 together with a diagrammatic explanation. The \(\pi\)-pulse length in this case was 0.8 seconds. Note that, for convenience, the initial \(\pi\)-pulse of each pulse pair has been triggered exactly on top of that from the previous pulse pair. Hence the trace shown represents seven pulse pairs with varying values of the delay, \(t\). The value of \(T_1\) was readily calculated from the positive-going recorder peak heights according to the relationship \(M_t = M_o (1-2e^{-t/T_1})\) where \(M_o\) is the initial magnitude of the magnetization vector along the \(-Z'-axis\) and \(M_t\) is its magnitude at some later time, \(t\).

The results of the experiments just described and the techniques used for measurement of \(T_1\) and \(T_2\) deserve several comments. Firstly, the pulse sequence used to measure \(T_1\) is more laborious than others available. For instance, since the 2\(\pi\)-pulse used for monitoring the magnitude of \(M_t\) leaves the vector in its original position, there should be no need to perform a \(T_1\) experiment by a series of pulse pairs with waiting periods in between. Freeman and Wittekoek have pointed this out and suggested that \(T_1\) can be measured by a pulse sequence consisting of one \(\pi\)-pulse followed by a series of equally spaced 2\(\pi\)-pulses (in R.F. pulse work this corresponds to the "triplet sequence"). This technique has the drawback of requiring long,
Fig. 3. Effect of a $T_1$ sequence on the magnetization vector, $M_0$.
A. Equilibrium; B. Preparatory $\pi$-pulse along the X'-axis; C. A $2\pi$-monitoring pulse along the X'-axis; D. E. and F. show $2\pi$-pulses which would occur at progressively longer time delays. A $T_1$ sequence on the trans 1,2-dibromoethylene resonance of Fig. 1A of Chapter III.
frequent pulses and in our experience the slower method gives somewhat better data.

In the case of $T_2$ there is no alternative to measurement with one pulse sequence because diffusion effects in solution cause "phase memory loss" of the spin isochromats which can only be minimized by frequent (relative to the diffusion rate) echo generation. Nevertheless, the Carr-Purcell technique involves a minimum of pulsing. But when $T_1$ is considerably longer than $T_2$, it can contribute appreciably to the observed length of the spin-echo decay during pulses. Hence in the case of samples such as the halo-ethylenes, one has to choose between too many and too few pulses in the $T_2$ sequence. For this reason, as mentioned earlier, the $T_2$ data in this case are likely not as accurate as the $T_1$ data although they were quite reproducible within a reasonable range of pulse repetition rate. Loss of magnetization between pulses can become more serious if a resonance contains small, unresolved splittings and this could account for some of the observed difference between $T_1$ and $T_2$ for the maleic and fumaric acid derivatives where the pulse intervals have been kept long for comparison to the other compounds. It would seem desirable when $T_1 \approx T_2$ to generate frequent echoes.

The very large difference between $T_1$ and $T_2$ in the case of the halogenated species is likely due to scalar coupling of the ethylene protons to the halogen quadrupole moment. The shorter relaxation times of the cis isomers relative to the trans in each case is quite possibly due to the intramolecular dipole-dipole coupling mechanism: i.e., the ethylene protons are closer together in the cis isomer and
the expression for dipole-dipole coupling\(^8^4\) contains a factor of \(1/r^6\). However, without knowing more about the system, it is not possible to rule out chemical shift anisotropy and spin-rotation interactions as sources of the difference.

The point we wish to make is that there are differences amongst the relaxation times and they appear to be systematic. Furthermore, the techniques of dissolving samples in the same solution and of using an internal standard between different samples seem to be useful for purposes of comparison.

B. Measurement of Nuclear Relaxation Times of an Alkaloid by the Audiofrequency Pulse Technique

The above experiments demonstrated that it is possible to selectively pulse small organic molecules with long relaxation times in order to calculate \(T_1\) and \(T_2\) using Freeman and Wittekoek's audio-frequency-pulse technique.\(^9^9\) We found that, in the cases studied at least, chemical changes were systematically reflected in changes in \(T_1\) and \(T_2\). In this section we report the use of selective pulse techniques* to measure \(T_1\) and \(T_2\) for individual protons of an alkaloid, vindoline. Vindoline was chosen to test the suitability of audio-frequency-pulse techniques for relaxation time measurements with

* The apparatus used for experiments on vindoline was the "Mark I" pulse spectrometer (see Appendix B) designed and constructed in collaboration with Mr. Roland Burton of this Department.
substances having the molecular complexity commonly associated with "natural products". It was of additional interest to us because of its relation with the anti-tumour principles obtained from Vinca rosea lin.101 The important point which we wished to establish was whether individual protons of a substance of this complexity would still have differing nuclear relaxation times.

Because we were interested in biological systems where it may often be inconvenient or undesirable to degass samples, this particular set of experiments was performed in non-degassed solution (it was subsequently shown that other relaxation mechanisms were so effective as to make the presence of atmospheric oxygen relatively unimportant). The normal $^1$H n.m.r. spectrum of vindoline in deuterochloroform solution (100 MHz) is shown in Fig. 4 along with a structural formula. TMS dissolved in the same sample was used to generate a signal for the field-frequency lock.

Initially, the relaxation times of the acetate-methyl and N-methyl singlets were determined. Measurement of the spin-spin relaxation times, $T_2$, for these two resonances followed the usual Meiboom-Gill modification of the Carr-Purcell experiment. A typical scope trace from such an experiment on the C$_4$-acetate methyl peak is shown in Fig. 5A ($\pi/2$-pulse duration = 0.10 sec). The echoes are seen as broad humps between the inflections caused by the 180°-pulses. Because of the rather short relaxation times, $T_2$, of these protons, the spin-echo trace falls to zero rather rapidly and only the first few echoes can be used for measurement purposes. However, it was always possible to vary the delay between pulses during the course of several different
Fig. 4. The $^1$H n.m.r. spectrum (100 MHz) of the alkaloid, vindoline, in deuterochloroform solution using TMS for the internal field-frequency lock. A structural formula for vindoline is given and the resonances pulsed are indicated.
Fig. 5 A. Scope photograph of a typical Carr-Purcell sequence on the C₄-acetate resonance of vindoline. Duration of \( \pi/2 \)-pulse = 0.1 sec.

B. Scope photograph of a typical \( T_1 \) pulse sequence on the same resonance. \( \pi/2 \)-pulse duration = 0.1 sec.
Carr-Purcell sequences and so obtain sufficient points for an acceptable determination of $T_2$. Data were found to be consistent over a range of pulse intervals.

The method used to measure $T_1$ was essentially that described in the previous section which involved a repeated series of two-pulse experiments. However, in this case, the monitoring second pulse was a $\pi/2$-pulse. This was chosen as the monitoring pulse since it required the least quantity of energy to be put into the system and hence had the greatest "selectivity". Because of the short relaxation times involved here it was not inconvenient to wait for the system to relax completely between pulse pairs. A sequence of such experiments gives the entire $T_1$-decay envelope including the zero-point as is illustrated with an example in Fig. 5B. This figure shows the result of such a measurement on the $C_4$-acetate methyl resonance ($\pi/2$-pulse duration = 0.10 second).

Instrumentally the measurement of $(T_1)_p$ values is particularly straightforward by the audiofrequency-pulse method. Thus the initial $\pi/2$-pulse is readily phase-shifted by precisely $90^\circ$ and the $(T_1)_p$ decay detected. Unfortunately, these particular measurements appear to be sufficiently prone to interference from neighbouring lines that their general application to complex organic substances may be somewhat limited. For example, the $(T_1)_p$ measurement shown below clearly fails to return to the baseline as it should due to the interference of near neighbour lines. Nevertheless there are potential advantages to the $(T_1)_p$ method as mentioned previously, since the pulse power is maintained constantly. In all the preliminary work
A $\pi/2$ pulse (0.1 sec) and a $(T_1)^p$ experiment on the $C_4$-proton resonance of vindoline. Note the extra height of the $(T_1)^p$ trace (upper) and its failure to return to the baseline.

discussed here we have adhered to those pulse methods requiring the least amount of pulse power in order to achieve high selectivity. Further work on practical samples will be required in order to test the applicability of $(T_1)^p$.

Following the comparatively simple experiments on the intense singlet resonances, attention was next directed to the other resonances. The two methoxyl groups gave two resonances which were only separated by ca. 1 Hz and it proved impossible to effect any recognizable experiments with them. On the other hand, the singlet due to the single $C_4$ proton was readily amenable to measurement of $T_1$ and $T_2$. Of the many spin-multipelets, only the sharp doublet due to the $C_{14}$ proton proved suitable for relaxation measurements. This doublet has a
Fig. 6. Relaxation time data obtained on the alkaloid, vindoline, in deuterochloroform solution. Values are shown associated with the protons to which they correspond.
splitting of 8.0 Hz and it was possible to perform measurements on both of the transitions. We were disappointed that it was not possible to make measurements on the other well resolved multiplets: unfortunately the small peak-separations and the rather short relaxation times (accompanied by large half-height widths and necessitating short pulses) effectively precluded meaningful experiments. Degassing of the solution did very little to lengthen the relaxation times.

It does not seem appropriate to make any lengthy discussion of the relaxation times obtained, beyond a brief comment that even though they are all quite short some differential still remains. As can be seen from the data summarized in Fig. 6, the N-methyl group has the shortest relaxation time. This might have been expected from the scalar coupling effect of the nitrogen quadrupole moment. Note that both transitions of the C₁₄ proton have the same relaxation times, and note also that in every case $T_1 = T_2$. The problem of pulsing individual lines in a multiplet is not trivial from a theoretical viewpoint, especially if the system involves high order coupling. This has been discussed in some detail by Freeman et al.¹⁰² In all cases studied in this work only relatively first order multiplets have been pulsed and the early parts of the $T_1$ decay curves have been used to calculate $T_1$ as suggested by the above workers. The experiments on vindoline gave values which probably have an accuracy in the neighbourhood of ±10% in terms of reproducibility.

In conclusion then, it seems that selective measurements of
relaxation times of individual protons of reasonably complex organic substances are feasible. The audiofrequency pulse technique is compatible with measurements of transitions separated by 5 Hz or more from a neighbouring resonance, providing that the transitions in question are sharp (i.e., $T_2 > 3$ sec.): for broader transitions a separation of 8-10 Hz is a more realistic one.

C. Measurement of Nuclear Relaxation Times of Carbohydrate Derivatives by the Audiofrequency Pulse Technique

We report here a study via pulsed $^1$H n.m.r. of two closely related saccharides. The compounds studied were 3,4,6-tri-O-acetyl-1-$\beta$-benzoyl-2-bromo-2-deoxy-$\alpha$-$D$-glucopyranose (1) and its 2-chloro analogue (2). Both had been previously prepared by Dr. John Manville in this laboratory$^2$ and were crystallized from aqueous ethanol prior to use.

The compounds were studied separately in C$_6$D$_6$ solution using TMS for an internal field-frequency lock. Each sample was carefully filtered and then degassed (6 freeze-pump-thaw cycles) prior to being sealed off. For purposes of intercomparison, both samples were made up to virtually the same concentration (concentration of bromo compound, 0.20 M; concentration of chloro compound, 0.23 M) and were treated identically. In order to confirm that the solution characteristics were the same in each case, a small amount of acetone was added to each sample prior to degassing. By measuring the relaxation time of this internal standard a cross check was possible between samples.
The two compounds, 1 and 2, were chosen for this study because it was already known that they had well resolved $^1$H n.m.r. spectra and because they had the same configuration. Thus these substances provided a further opportunity to test the viability of the audio pulse technique with "complex" organic systems. The ring proton region of the normal $^1$H n.m.r. spectrum of 1 in $C_6D_6$ solution is shown in Fig. 7A.

\[
\begin{align*}
\text{AcO} & \quad \text{CH}_2\text{OAc} & \quad \text{O} & \quad \text{H}_2 & \quad \text{OBz} \\
\text{AcO} & \quad \text{H}_5 & \quad \text{R} & \quad \text{H}_1 \\
& \quad \text{H}_3 & &
\end{align*}
\]

1 \hspace{1em} R = \text{Br} \\
2 \hspace{1em} R = \text{Cl}

The identification of the various multiplets is shown above the spectrum in Fig. 7A. Initially pulse experiments were performed on the resonances of the clearly resolved $H_1$ doublet.

The method of measuring $T_1$ was that previously discussed with reference to the alkaloid, vindoline: a series of $\pi$-pulse, $\pi/2$-pulse pairs interspersed with waiting periods for equilibration.

The scope trace for a Carr-Purcell sequence used to measure $T_2$ (upper field transition #2 in Fig. 7A; $\pi/2$ pulse duration = 0.1 sec) is shown below.
Fig. 7. Partial $^1$H n.m.r. spectra (100 MHz) of 3,4,6-tri-O-acetyl-1-O-benzoyl-2-
 bromo-2-deoxy-$\beta$-$D$-glucopyranose (A) and its 2-chloro analogue (B) in
deuterobenzene (degassed). Proton assignments follow those of
reference 2 and are the same in each case. Transitions are numbered
for discussion in the text. TMS was used for a lock signal.
Note the spikes between echoes. These are caused by interference from near-neighbour lines. However, during the echoes themselves the pulse causing the interference is shut off. As in the case of the alkaloid experiments of the previous section, the need for high selectivity necessitated the use of as long pulses as possible (i.e., low pulse power), and because of the short relaxation times, only a few echoes were obtained in each experiment. Several experiments were performed on each line with different delays between pulses. The values of $T_1$ and $T_2$ so obtained for the resonances of $H_1$ are listed in Table 1.

Similar experiments were performed on the outer transitions, #3 and #6, of the $H_3$ quartet (Fig. 7A). The inner transitions, #4 and #5,
were resolved into separate lines too close for selective pulse work. Similarly, of the four transitions making up $H_4$, only the outer ones, #7 and #10, were made the subject of pulse experiments — the inner transitions, #8 and #9, were nearly degenerate. The values found for $T_1$ and $T_2$ during these experiments are also listed in Table 1.

As observed for the alkaloid of the previous section, these relaxation times are short. Another disappointing feature of these experiments is that it was not possible to make satisfactory measurements of $T_1$ and $T_2$ on any of the other resonances. Even the transitions of the $H_6$ quartet (#11-#14) were too close together for realistic measurements. $T_2$ for the acetone internal standard in this sample was found to be 15.2 seconds.

The partial $^1H$ n.m.r. spectrum of the chloro compound (2) in $C_6D_6$ is shown in Fig. 7B. The assignment is the same as that indicated for 1. Although the spectra in Fig. 7A and B are very similar, the $H_3$ and $H_4$ "quartets" are somewhat closer together in the case of the chloro compound. Pulse experiments on this compound were very similar to those on its bromo analogue. The transitions 6 and 7 of $H_3$ and $H_4$ respectively were deemed too close together (ca. 6 Hz) for good measurements on such broad lines (half height width ca. 0.8-1.0 Hz due to small unresolved splittings). Nevertheless, measurements of $T_1$ and $T_2$ were made on transitions of $H_1$, $H_3$ and $H_4$ and are listed in Table 1 along with those for compound 1. Note that in this case the reasonably sharp peak corresponding to transitions #8 and #9 was pulsed satisfactorily. The value of $T_2$ for the internal standard, acetone, was found to be 15.8 seconds in this second sample. This of
Table 1. Relaxation Time Data for Sugar Derivatives 1 and 2

1 Bromo analogue

<table>
<thead>
<tr>
<th>line #</th>
<th>1/T₂ (sec⁻¹)</th>
<th>T₂ (sec)</th>
<th>1/T₁ (sec⁻¹)</th>
<th>T₁ (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.52</td>
<td>1.9</td>
<td>0.52</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>0.54</td>
<td>1.8</td>
<td>0.55</td>
<td>1.8</td>
</tr>
<tr>
<td>3</td>
<td>0.58</td>
<td>1.7</td>
<td>0.53</td>
<td>1.9</td>
</tr>
<tr>
<td>6</td>
<td>0.66</td>
<td>1.5</td>
<td>0.54</td>
<td>1.8</td>
</tr>
<tr>
<td>7</td>
<td>0.84</td>
<td>1.2</td>
<td>0.70</td>
<td>1.4</td>
</tr>
<tr>
<td>10</td>
<td>0.83</td>
<td>1.2</td>
<td>0.69</td>
<td>1.4</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.0660</td>
<td>15.2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2 Chloro analogue

<table>
<thead>
<tr>
<th>line #</th>
<th>1/T₂ (sec⁻¹)</th>
<th>T₂ (sec)</th>
<th>1/T₁ (sec⁻¹)</th>
<th>T₁ (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.56</td>
<td>1.8</td>
<td>0.51</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>0.54</td>
<td>1.8</td>
<td>0.53</td>
<td>1.9</td>
</tr>
<tr>
<td>3</td>
<td>0.45</td>
<td>2.2</td>
<td>0.50</td>
<td>2.0</td>
</tr>
<tr>
<td>8 &amp; 9</td>
<td>0.92</td>
<td>1.1</td>
<td>0.72</td>
<td>1.3</td>
</tr>
<tr>
<td>10</td>
<td>0.85</td>
<td>1.2</td>
<td>0.80</td>
<td>1.2</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.0634</td>
<td>15.8</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
course is well within the ±10% error limit of that found for the first sample. It should be pointed out that in spin coupled systems of complex molecules each spectral line is usually broadened by unresolved splittings so that the observed value of $T_2$ is generally greater than that indicated by the linewidth from the relationship $\Delta \nu = \frac{1}{\pi T_2}$. For instance if $T_2$ is greater than 1.5 second, a single resonance would be expected to have a half-height width of less than 0.22 Hz. Allowing for a field inhomogeneity of up to 0.3 Hz, one would expect linewidths of 0.5 Hz — in fact most resonances are broader than this.

At first sight the close similarity between the relaxation times of the various protons studied here may seem to be rather disappointing; however it should be noted that all the protons have an axial orientation and this may be a controlling feature of the relaxation processes. It is also possible that the acetate methyl protons contribute significantly to the overall relaxation of the ring protons. Note that the values for compound 1 are closely similar to those for the corresponding protons of compound 2 (Table 1) — this is not surprising in view of the close structural similarity between the molecules. As mentioned previously, one of the penalties for using the low energy Carr-Purcell sequence to measure $T_2$ can be a loss of magnetization between pulses. It may be this phenomenon which accounts for the fact that in the $H_4$ resonances of 1 and 2 (which are broader than those of $H_1$ and $H_3$) $T_1$ is slightly longer than $T_2$. However the differences between $T_1$ and $T_2$ even for the $H_4$ resonances are small relative to the ±10% experimental error involved and may arise from the spin multiplet effects mentioned by Freeman et al.
Clearly it will be necessary to study other examples to see if any differential exists between the relaxation times of protons in more different chemical environments. In this regard a measurement technique having an even higher degree of selectivity would be desirable; if this cannot be developed, then the potential of this area will be severely restricted. It would also seem desirable if any improvements in selectivity could be accompanied by attempts to increase the overall magnitude of the relaxation times. In this regard decoupling experiments should be helpful.\textsuperscript{99}

It is worth remarking that from the data given here it would seem that no great differential exists between the relaxation times of the various resonances associated with any one proton. However, the spectral features studied were fairly first order and in general differences may be expected.\textsuperscript{102}

D. The Audiofrequency Pulse Technique and Enzyme-Inhibitor Rate Constants

We have just discussed to some extent the possibility of deriving structural information from n.m.r. pulse studies of 'large' organic molecules in solution. Although this area is as yet almost totally uninvestigated, there has been considerable introductory work done on the use of pulse techniques in studying rate processes involving biological systems. In particular, enzyme-inhibitor interactions have been studied via the technique of observing nuclear relaxation phenomena associated with the inhibitor.
Sykes has done excellent work in this area and has applied the technique to systems involving chymotrypsin\textsuperscript{98} and lysozyme.\textsuperscript{97a,b} Sykes\textsuperscript{97a} and Sykes and Parravano\textsuperscript{97b} have measured the rate constants, $k_{-1}$ and $k_1$, for the N-acetyl-D-glucosamine derivatives, 5-8, of the previous chapter.

$$k_{-1} = \text{rate constant for dissociation of the enzyme-inhibitor complex}$$

$$k_1 = \text{rate constant for complex formation}$$

$$K_D = \frac{[E][I]}{[EI]} = \frac{k_{-1}}{k_1}$$

We have already shown that $^{19}$F labels used in Chapter II had some effect on the binding of N-trifluoroacetylated monosaccharides to lysozyme. We were curious therefore as to the rate constants for our fluorinated inhibitors.

The exchange of a nucleus, or group of equivalent nuclei, between sites of different local magnetic environment (characterized by different resonance frequencies) is a relaxation mechanism for the nuclear spin system. If the rate of exchange is less than the resonance frequency of the nuclei involved ($\approx 10^2$ MHz here), the exchange will shorten the transverse relaxation time, $T_2$, but will not affect the longitudinal relaxation time, $T_1$. In fact this exchange mechanism has been shown\textsuperscript{97a,b} to be the dominant relaxation mechanism of the N-acetyl protons brought about by addition of lysozyme to aqueous solutions of monosaccharide NAG derivatives. Of the fluorinated NAG derivatives studied in Chapter II, only the $\alpha$-anomer of the free sugar was found to show an appreciable bound chemical shift which would permit an accurate calculation of the rate constants involved.
As mentioned in the introduction, Sykes$^{97a}$ and Sykes and Parravano$^{97b}$ have put the Bloch equation solutions into a form very convenient for relating the difference between $T_1$ and $T_2$ to the rate constant, $k_{-1}$. In the case where $\frac{[E]_I}{[I]} << 1$ and $[I] = [I_0]$ the expression is:

$$\frac{1}{T_2} - \frac{1}{T_1} = \frac{[E_0]}{K_D + [I_0]} \left(\frac{1}{k_{-1}}\right) \Delta^2$$

where $T_1$ and $T_2$ are in seconds

$\Delta$ is in radians/sec

concentrations are in molarity.

Hence if $K_D$ and $\Delta$ have been calculated from chemical shift studies, it is a relatively simple matter to arrive at $k_{-1}$ from a plot of $(\frac{1}{T_2} - \frac{1}{T_1})$ vs. $[E_0]$ while holding $[I_0]$ constant.

In this study, $T_1$ and $T_2$ for the N-trifluoroacetyl group were measured for each of four different values of $[E_0]$ (Fig. 8A and Table 2). All measurements were made in 0.1 M citrate buffer at pH 5.5. The
"Mark II" pulse unit attached to a Varian HA-100 spectrometer operating at 94.071 MHz (probe temperature 31.5°C) was used to measure $T_1$ and $T_2$. A capillary of trifluoroacetic acid held concentric with the n.m.r. sample tube was used for a field-frequency lock. The total sugar concentration (0.0775 M) was the same in each sample. Hence the concentration of $\alpha$-anomer (compound 1 of Chapter II) in each case was 0.0356 M. Since, as was determined Chapter II, the effect of the $\beta$-anomer on the $\alpha$-anomer binding was no larger than experimental error, the effect of the $\beta$-anomer was neglected.

In each case the measurement of $T_1$ was straightforward and followed the conventions described for the 'large' organic molecules of the previous two sections (a series of $\pi$, $\pi/2$-pulse pairs with varying delays between the first and second pulse). As can be seen from the data (Table 2 and Fig. 8A - solid line through X's), $T_1$ decreases only slightly with increasing enzyme concentration. A similar slight decrease has been noted by Sykes.\(^{97a}\)

The measurement of $T_2$ was quite simple but was checked by several methods. The spin-echo method described previously was suitable for measurement of $T_2$ on the first two samples. But $T_2$ became so short on the remaining samples (Table 2) that field inhomogeneity became only a small correction to the Bloch decay generated by a $\pi/2$-pulse. The value of $T_2$ obtained from the time constant for the Bloch decay of a $\pi/2$-pulse is the same as that obtained from the expression $\Delta \nu = \frac{1}{\pi T_2}$ provided there are no unresolved couplings. The actual magnitude of the field inhomogeneity correction term can be estimated from the linewidth of an internal standard for which $T_2$ is long or from the
Table 2. Nuclear Relaxation Time Data for the α- and β-Anomers of the Free Sugar, N-Trifluoroacetyl-β-<br>Glucosamine

<table>
<thead>
<tr>
<th>Sample #</th>
<th>$[E_o]$ (M)</th>
<th>$T_1$ (sec)</th>
<th>$1/T_1$ (sec$^{-1}$)</th>
<th>$T_2$ (sec)</th>
<th>$1/T_2$ (sec$^{-1}$)</th>
<th>$\frac{1}{T_2} - \frac{1}{T_1}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>0</td>
<td>1.87</td>
<td>0.534</td>
<td>1.58</td>
<td>0.634</td>
<td>0.100</td>
</tr>
<tr>
<td>1</td>
<td>β</td>
<td>1.82</td>
<td>0.549</td>
<td>1.52</td>
<td>0.658</td>
<td>-</td>
</tr>
<tr>
<td>2 α</td>
<td>$0.95 \times 10^{-3}$</td>
<td>1.62</td>
<td>0.617</td>
<td>0.830</td>
<td>1.20</td>
<td>0.587</td>
</tr>
<tr>
<td>3 α</td>
<td>$2.85 \times 10^{-3}$</td>
<td>1.32</td>
<td>0.755</td>
<td>0.364</td>
<td>2.75</td>
<td>1.994</td>
</tr>
<tr>
<td>β</td>
<td></td>
<td>1.63</td>
<td>0.612</td>
<td>1.06</td>
<td>0.940</td>
<td>-</td>
</tr>
<tr>
<td>4 α</td>
<td>$4.75 \times 10^{-3}$</td>
<td>0.960</td>
<td>1.04</td>
<td>0.234</td>
<td>4.27</td>
<td>3.226</td>
</tr>
</tbody>
</table>

$[\alpha_o] = 0.0356$ M
$[\beta_o] = 0.0419$ M
Fig. 8. A. Plots of $1/T_1$ (solid line through X's) and $1/T_2$ (solid line through filled circles) vs. $[E_0]$ for the N-trifluoroacetyl group of the $\alpha$-anomer. Several data points for $1/T_1$ (dotted line through triangles) and $1/T_2$ (dotted line through open circles) for the $\beta$-anomer are shown for comparison.

B. Plot of $(1/T_2 - 1/T_1)$ vs. $[E_0]$ for the $\alpha$-anomer N-trifluoroacetate group.
effect of spin-echo generation on the same sample or on a similar sample
with longer $T_2$. Neither technique is extremely accurate in the
intermediate range of linewidths less than 2 Hz but more than 0.8 Hz.

Fig. 8A (solid line through filled circles) clearly shows the
sharp decrease in $T_2$ for the N-trifluoroacetyl resonance of the free
sugar $\alpha$-anomer upon addition of lysozyme. For purposes of comparison,
several points are plotted for the $\beta$-anomer ($1/T_1$ as dotted line
through triangles; $1/T_2$ as dotted line through open circles).

Fig. 8B is a plot of $(1/T_2 - 1/T_1)$ vs. $[E_0]$. The slope of this
line is equal to $\frac{\Delta^2}{K_D + [\alpha]} \frac{1}{k_{-1}}$ and was found to be $6.72 \times 10^3$
sec$^{-1}$M$^{-1}$. The calculated values of $k_{-1}$ and $k_1$ for the $\alpha$-anomer of the
free sugar are

\[
\begin{align*}
    k_{-1} &= 1.1 \times 10^4 \text{ sec}^{-1} \\
    k_1 &= \frac{k_{-1}}{K_D} = 1.1 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}
\end{align*}
\]

The error limits involved are at least $\pm 30\%$ in view of the errors in
the numbers used for the calculation. It is interesting that for the
proton analogue (compound 5 of Chapter II) of the above N-trifluoro-
acetate, Sykes and Parravano$^{97b}$ quote $k_{-1} = 8.5 \pm 2.5 \times 10^3$ secd$^{-1}$ and
for $k_1$ they report $3.5 \pm 1.2 \times 10^5$ molal$^{-1}$ secd$^{-1}$. Hence, the replacement
of the N-acetyl protons by fluorines seems not to have affected $k_{-1}$
but may have slightly increased $k_1$. 

Experimental

General Methods
(a) All $^1H$ and $^{19}F$ spectra were run on the same spectrometer (Varian HA-100) and under the same conditions as described in Chapter I and Chapter II.

(b) Enzyme handling techniques were the same as those described in Chapter II.

(c) The "Mark I" pulse unit was used for the experiments on vindoline. All other pulse work was done with the "Mark II" unit.

Compounds

The alkaloid, vindoline, was obtained from Professor J.P. Kutney of this department.

Ethylene derivatives were obtained from the Aldrich Chemical Co. and were used without further purification.

3,4,6-Tri-O-acetyl-1-O-benzoyl-2-bromo-2-deoxy-β-D-glucopyranose (1) m.p. = 161-162°C and its 2-chloro analogue (2) m.p. = 160-161°C were made previously by Dr. J. Manville of this laboratory and were recrystallized from aqueous ethanol prior to use.

Pulse Unit Operation

Each individual relaxation time measurement requires the sequential adjustment of the correct observing frequency, of the amount of power contained in a single pulse, and of the phase of the irradiating field.
In general it is convenient to set up these parameters in the order:

1. Pulse phasing
2. Exact frequency determination
3. Pulse power

With the apparatus available to us at this juncture we have found it convenient to correctly adjust the phase of the observing field in the fashion generally used in high resolution n.m.r. experiments: by using the sweep-oscillator of the frequency synthesizer it is convenient to sweep repeatedly through the resonance of interest and to make adjustments with the phasing control of the P.A.R. lock-in amplifier. With the "Mark II" pulse spectrometer it is necessary to make this adjustment only once since the pulse frequency is held constant.

Freeman and Wittekoek have described previously how (T\(_1\)\(_\rho\)) experiments can be used for optimising the phasing controls. We have used their method and find it to be quite convenient providing that a (T\(_1\)\(_\rho\)) experiment can be meaningfully performed on the particular sample: however, the difficulties already mentioned when a near neighbour resonance is present tend to complicate the method.

The precise frequency difference between the field-frequency lock and the pulse signal needed for a particular experiment can be obtained in either of two ways. Either by direct measurement and calibration of the high resolution spectrum, or by observation of the decay occurring after a pulse. In the latter case the line position is quickly measured to within 0.5 Hz and the frequency is adjusted in small increments to achieve a smooth exponential decay to baseline after each pulse. The off-resonance condition is marked by the trace
of the magnetization vector either continuing through the baseline before returning to its equilibrium position or by a hump in the otherwise smooth decay curve. This method is very fast and quite general and can give the frequency to within \( \pm 0.02 \) Hz (depending on the linewidth).

The amount of power required to produce a particular pulse is conveniently ascertained by setting up the condition for a \( \pi \)-pulse (or \( 2\pi \)-pulse); it is then a simple matter to arrive at the power level or pulse duration for a \( \pi/2 \)-pulse.
APPENDIX A

PRINCIPLES OF PULSED N.M.R. SPECTROMETRY WITH PARTICULAR
REFERENCE TO HIGH RESOLUTION EXPERIMENTS

Nuclei having a spin quantum number greater than zero possess
both spin angular momentum and a magnetic moment, \( \mu = \gamma h I \) (where \( \gamma \) is
the nuclear gyromagnetic ratio and \( I \) is the nuclear spin quantum number). Magnetic moments give nuclei certain properties characteristic of
gyroscopes. For instance the nuclear spin isochromats precess about
an external magnetic field, \( H_o \), at a frequency characteristic of the

\[ \omega_o = \gamma H_o \text{ radians/sec} \]

\( \omega_o \) = Larmor frequency

In the powerful magnetic field of a n.m.r. spectrometer there is
a resultant macroscopic magnetization (\( M_o \)) along the field axis (Z-axis) at equilibrium. By briefly applying a new magnetic field, \( H_l \),
perpendicular to \( H_o \), it is possible to turn the vector \( M_o \) through a
specific angle away from the Z-axis. The resultant magnetic vector,
\( M_o \), will then precess like the individual spin isochromats about the
Z-axis at the Larmor frequency. For this reason, it is standard
practice to transform from the XYZ laboratory frame to a rotating
reference frame, \( X'Y'Z' \), which still has \( H_o \) along the Z'-axis, but
which rotates about that axis at the Larmor frequency, \( \omega_o \). In this
new reference frame, neither the spin isochromats, nor their resultant
\( M_o \), precess about the Z'-axis. Short-lived \( H_l \) fields can be conveniently
produced by bursts of radiation at the Larmor frequency, $\omega_0$. The radiofrequency magnetic field, $H_1$, can be thought of as made up of two counter-rotating fields; one of which synchronizes with the precessing nuclear magnetic moments and the other of which has a negligible effect.

Thus, if $H_1$ is directed along the $X'$-axis it will cause a rotation of the resultant magnetization vector in the $Y'Z'$ plane at a frequency:

$$\omega_1 = \gamma H_1$$

If $H_1$ is left on for a time, $t$ seconds, then the vector, $M_0$, will turn through an angle:
It is possible to vary the strength of $H_1$ (the pulse power) and/or the duration of irradiation (pulse duration) to achieve varying degrees of vector rotation. In radiofrequency pulse experiments, powerful $H_1$ fields are generally employed: this permits one to pulse extremely broad lines and to measure very short relaxation times. In high resolution n.m.r., $T_1$ and $T_2$ are generally of the order of 1 second or longer and very homogeneous magnetic fields are employed: this permits the use of long but very selective (narrow band) pulses allowing one to measure relaxation times of individual resonances in complex spectra.

The receiver coil of the spectrometer used here is oriented so as to detect any non-zero resultant magnetization in the $X'Y'$ plane — and the larger that component, the larger the recorder response. The pulses employed are so weak that the magnetization vector can actually be monitored during the pulse. Fig. 1 shows a series of scope presentations for the various constituents of all pulse sequences together with a diagrammatic representation of each. The 90°-pulse ($\pi/2$-pulse) of Fig. 1A shows the characteristic sharp rise in recorder response as $M_o$ is forced maximally into the $X'Y'$ plane, followed by an exponential Bloch decay of $M_o$ along the $Y'$-axis. Fig. 1B clearly demonstrates the effect of a 180°-pulse ($\pi$-pulse) on the same resonance after it has been allowed to come to equilibrium: $M_o$ has been forced from the positive $Z'$-axis into the $X'Y'$-plane (giving a sharp recorder response) and then on through the $X'Y'$ plane.
Fig. 1. Scope traces of various pulses on the alkene resonance of the diethyl ester of maleic acid in the sample shown in Fig. 1B of Chapter III. 
A. $\pi/2$-pulse = 0.2 sec; B. $\pi$-pulse = 0.4 sec; C. $2\pi$-pulse = 0.8 sec. The effect of each pulse on the equilibrium magnetization vector, $M_0$, is shown to the right of the trace.
to the negative Z'-axis where it gives a zero recorder response. Note that the recorder response actually returns through the baseline to a slight extent. This is because the resonance pulsed has a finite linewidth and the pulse itself has a bell-shaped power distribution - the result being that the central part of a resonance receives slightly too strong a pulse and the wings of the resonance receive slightly too weak a pulse. Fig. 1C illustrates the effect of a 360°-pulse (2π-pulse) on the $M_0$ vector at equilibrium: note that a maximum positive recorder response is observed as $M_0$ passes through the positive Y'-axis and a maximum negative response as it passes through the negative Y'-axis. Obviously the recorder response is zero again at the end of a 2π-pulse (except for the finite linewidth effect mentioned above) and the overall effect of a perfect 2π-pulse is negligible. All pulses shown in Fig. 1 were performed on the alkene resonance of diethoxy maleic acid (resonance #7 of Fig. 1B of Chapter III) and were done with the same pulse power but differing pulse durations - the pulse durations being 0.2, 0.4 and 0.8 seconds in Fig. 1A, B and C respectively. It should be noted that if the pulse duration is not $\ll T_1$ and $T_2$, then an appreciable amount of decay of the vector $M_0$ can occur during the pulse.
APPENDIX B

AUDIOFREQUENCY PULSE UNITS EMPLOYED

"Mark I"*

The basic format of both "Mark I" and "Mark II" pulse spectrometers used in these experiments follows that of Freeman and Wittekoek. Because we were uncertain as to which pulse durations would be most suitable for studies of complex organic substances we chose to use a small digital computer to control all pulse durations and intervals. It should be emphasized that the components which we used as the basis for the gate and other circuits were selected solely because they were readily available in our laboratory at the time we started these experiments; more versatile, and cheaper, components are now available from a variety of manufacturers.

A block diagram of the instrument, together with the intersystem connections required for interface with a Varian HA-100 spectrometer, is given in Fig. 2. The two major components of the system are a JEOLCO computer (model JRA-5) and a Hewlett Packard Frequency Synthesizer (model 5110B), which is driven by a "master oscillator" (Hewlett-Packard, model 5105B).

The principle components of the pulse-unit are summarized in Fig. 3. Briefly, the circuitry used here serves the following functions:

(a) In the lock-channel, it takes a 1 MHz signal from the master

* This device was designed and built in collaboration with Mr. Roland Burton of this Department.
Fig. 2. Block diagram of the components used in the "Mark I" audio-frequency-pulse spectrometer.
oscillator and reduces it by digital division to a 2.5 KHz square wave. This signal (at 1 V p.p.) is then used in the Varian lock-box (V-4354) instead of the signal normally provided by the manual oscillator (card 910868). We have made this interchange switchable so that it does not require physical removal of the manual oscillator card. All subsequent operations of the lock-circuitry are unaltered by this interchange except that lock-offsets are not possible.

(b) In the observation channel the circuitry provides the audio-modulation frequency required for the detection of resonances. At the same time this channel requires provision for switching, phase shifting and filtering. In order to obtain a sufficiently fine (+ 0.01 Hz) control of the frequency of the observation side-band, the digital frequency synthesizer is generally run at ca. 1 MHz and the required audiofrequency (ca. 2.5 KHz) is obtained by digital division as for the lock-channel. Since the phase-shifts required in pulse experiments are integral units of 90°, these are conveniently obtained during the above division. Sequential division ÷ 10, ÷ 10, ÷ 2, invert, ÷ 2 gives the required audiofrequency square-wave which is used in a line A (Fig. 3) as the reference signal to the Princeton Applied Research (PAR) lock-in amplifier and to the gate. Using the first three stages of the above sequence (÷10, ÷ 10, ÷ 2) followed by a separate ÷ 2 stage gives a second audiofrequency square-wave in line B. This square-wave has precisely the same frequency as that of line A but differs from it and the signal in the lock-in amplifier by a precisely 90° phase-shift. Either of these two audiofrequencies can then be connected to the modulation coils via a filter and an attenuator. The latter is a 10-turn
Fig. 3. Block diagram of the components used in the "Mark I" pulse unit.
200 ohm helipot.

The switching of both line A and line B is controlled by the JEOLCO JRA-5 computer which is programmed to perform the following:-

(a) To accept positive integers (<32,000) from the teletype; nine of these integers may be used. Each of these controls a specific switching operation.

(b) To turn on, or off, either line A or line B of the observation channel. When line A of Fig. 3 is "on" the phase-shift of the irradiating field is zero, and when line B is "on" the phase-shift is 90°.

(c) To pause for a time-interval which is numerically equal to the integer selected in (a) above, multiplied by 10 milliseconds.

It is, of course, a trivial matter to vary the pulse-lengths, pulse-intervals, and phase sequences in any of a wide variety of ways.

The spectra obtained from the pulse spectrometer are conveniently recorded on a storage oscilloscope (Tektronix, model R564 B) and a permanent record obtained with a homemade scope camera using Polaroid Film.

"Mark II"

The "Mark II" pulse spectrometer is an improved version of "Mark I".

* More accurately, the phase-shift between the reference signal going to the PAR-121 lock-in amplifier and the signal going to the modulation coil.

** Derived from a Polaroid camera (model 210). It is necessary to divide the original camera into two parts, the back-film holder and the lens-shutter mechanism. The two parts are then remounted approximately twice as far apart as in the original camera. The total cost of our camera was less than $50.

† This device also was designed and built in collaboration with Mr. Roland Burton of this Department.
It is not only more convenient from the operator's point of view, but it contains its own solid state pulse sequence generator which obviates the need for an expensive computer. The choice of pulse durations (both pulse #1 and pulse #2) is digitally variable over the following range: .025, .050, .10, .20, .40, .80, 1.60, ∞ seconds. The delay between pulse #1 and pulse #2 is continuously variable.

A block diagram of the "Mark II" pulse spectrometer together with the intersystem connections required for interface with a Varian HA-100 spectrometer, is given in Fig. 4. As mentioned above, although the Hewlett-Packard Frequency Synthesizer (model 5110 B) driven by a "master oscillator" (Hewlett Packard, model 5105 B) is still used as a source of stable frequencies, the JEOLCO computer has been replaced with solid state circuitry built into the pulse unit.

The principle components of this new pulse-unit are summarized in Fig. 5. There are basic differences in the circuitry used for "Mark II" as follows:–

(a) The lock channel frequency is variable and hence is taken from the digital frequency synthesizer. For high accuracy in setting the frequencies, the lock signal of ca. 2.5 KHz was obtained by digital division (see Fig. 5) of a radiofrequency in the 1 MHz range and filtering to obtain a sine wave at (1 V p.p.) whose frequency can be set with extreme accuracy and which can be simply switched to replace the ordinary manual oscillator (card 910868). With this arrangement any lock offset is readily achievable.

(b) As in the "Mark I" unit, the observation channel circuitry provides the audio-modulation frequency required for detection of
Fig. 4. Block diagram of the components used in the "Mark II" audio-frequency-pulse spectrometer.
Fig. 5. Block diagram of the components used in the "Mark II" pulse unit.
resonances. The observation frequency of 2.5 KHz is derived by
digital division of the master oscillator (1 MHz) and is fixed. Hence
it is sufficient to tune the PAR lock-in-amplifier to one frequency.
The 2.5 KHz pulse #1 is obtained by a simple sequential division, ÷ 10,
÷10, ÷2, ÷2 of the master oscillator frequency. Pulse #2 has exactly
the same frequency as pulse #1 (see Fig. 5) but may be switched to a
variety of different phase relations to pulse #1 (0°, 90° or 180°).
This switching is manual. The pulses #1 and #2 are gated according
to switch settings on the front panel of the pulse unit to give any
sequence desired. As with the "Mark I" unit, these pulse sequences
are fed to the D.C. modulation coils and the demodulated output of
the receiver coil (demodulated in the V-4311 receiver unit) is fed to
the PAR lock-in-amplifier. The Tektronix, model R 564 B, storage
oscilloscope is used to record the D.C. output. The same Polaroid
camera is used to record storage scope traces.
REFERENCES

26. (a) A.D. Cross and P.W. Landis, J. Amer. Chem. Soc. 84, 1736 (1962); (b) A.D. Cross and P.W. Landis, ibid. 84, 3784 (1962); (c) A.D. Cross and P.W. Landis, ibid. 86, 4005 (1964).
91. (a) A.L. Van Geet and D.N. Hume. Analyt. Chem. 37, 983 (1965); 
     (b) A.L. Van Geet and D.N. Hume. Analyt. Chem. 37, 979 (1965).
96. (a) Z. Luz and S. Meiboom. J. Chem. Phys. 40, 2686 (1964); 


Publications:

E. J. Casey, C. W. M. Grant and C. L. Gardner,

C. L. Gardner, E. J. Casey and C. W. M. Grant,

C. W. M. Grant and L. D. Hall,

R. Burton, C. W. M. Grant and L. D. Hall,

Awards:

1964 - 65 Ontario Scholarship
1964 - 68 The Governor's Scholarship
1964 - 68 Dean's Honour List
1964 - 65 The Beauty Counselors of Canada Scholarship
1966 - 67 The J. L. W. Gill Scholarship
1966 - 67 The C. I. C. Prize
1968 - 72 N.R.C. 1967 Science Scholarship
1972 - N.R.C. Nato Fellowship