Tritium NMR Studies of Protein-Ligand Interactions

A Thesis presented to the University of Surrey for the degree of Doctor of Philosophy in the Faculty of Science

By

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To Mum, Dad, Lisa and Simon
ABSTRACT

Tritium NMR studies provide a convenient way of obtaining detailed information about conformational equilibria, dynamic processes and specific interactions in protein-ligand complexes provided that suitably $^3$H-labelled molecules are available. In this study [7,9-$^3$H]- and [3',5',7-$^3$H]folic acid, and [3',5',7-$^3$H]methotrexate were synthesised and the $^3$H NMR spectra of their complexes with Lactobacillus casei dihydrofolate reductase (DHFR) were assigned and analysed as a function of pH (DHFR-folate complexes) and temperature (DHFR-methotrexate complexes). From these data it was possible to obtain further evidence about the orientation of the pteridine ring in the complexes, and to monitor the dynamic processes in the bound ligands. In the $^3$H NMR spectra of the ternary complexes of the $^3$H-labelled folic acids with DHFR and NADP$^+$, each labelled tritium gave rise to multiple signals, confirming previous findings that there are three interconverting, pH dependent, conformational forms of bound folate (forms I, IIA and IIB) in the ternary complex. The folate benzoyl ring could be shown to be in essentially the same environment in the different forms with the major differences being associated with the pterin ring. The appearance of a single resonance for the 3',5'-tritons showed that the benzoyl ring is flipping rapidly in all three forms. In contrast, the methotrexate binary complex and also the ternary complex with NADPH were shown to exist as a single conformational state with the benzoyl ring flipping rate being too slow to give a single averaged signal for the 3',5'-tritium nuclei over the temperature range 283 - 313 K. $^3$H-$^1$H Nuclear Overhauser enhancement experiments have been conducted on the small molecules, [$^3$H]dimethyl sulphoxide, [3',5',7-$^3$H]folic acid and [3',5',7-$^3$H]methotrexate as a prelude to $^3$H-$^1$H heteronuclear NOE experiments on binary and ternary complexes formed using Lactobacillus casei DHFR and the ligands [3',5',7-$^3$H]methotrexate and [3',5',7-$^3$H]folic acid.
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### Abbreviations

- T: Tritium
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>CIDNP</td>
<td>Chemically induced dynamic nuclear polarisation</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlated spectroscopy (2D)</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>d</td>
<td>Doublet</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>FH₂</td>
<td>7,8-Dihydrofolate</td>
</tr>
<tr>
<td>FH₄</td>
<td>5,6,7,8-Tetrahydrofolate</td>
</tr>
<tr>
<td>FID</td>
<td>Free induction decay</td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>H₂folate</td>
<td>7,8-Dihydrofolate</td>
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<tr>
<td>H₄folate</td>
<td>5,6,7,8-Tetrahydrofolate</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HMQC</td>
<td>Heteronuclear multiple quantum coherence spectroscopy</td>
</tr>
<tr>
<td>HOHAHA</td>
<td>Homonuclear Hartman-Hahn condition</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Ile</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>J</td>
<td>Coupling constant</td>
</tr>
<tr>
<td>L. casei</td>
<td>Lactobacillus casei</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>m</td>
<td>Multiplet</td>
</tr>
<tr>
<td>mM</td>
<td>mmol dm$^{-3}$</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose binding protein</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>NADP$^+$</td>
<td>Nicotinamide adenine dinucleotide phosphate, oxidised</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser enhancement spectroscopy</td>
</tr>
<tr>
<td>NOESY</td>
<td>2D Nuclear Overhauser enhancement spectroscopy</td>
</tr>
<tr>
<td>PGB</td>
<td>Porphobilinogen</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>q</td>
<td>Quartet</td>
</tr>
<tr>
<td>r-RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>s</td>
<td>Singlet</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>T</td>
<td>Tritium ($^3$H)</td>
</tr>
<tr>
<td>t</td>
<td>Triplet</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>tlc</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TMP</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>TMQ</td>
<td>Trimetrexate</td>
</tr>
<tr>
<td>TNOE</td>
<td>Transferred nuclear Overhauser enhancement spectroscopy</td>
</tr>
<tr>
<td>TOCSY</td>
<td>Total Correlated Spectroscopy (2D)</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
</tbody>
</table>
Introduction

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1. INTRODUCTION

The aim of this Thesis is to explore methods of studying ligand binding to the enzyme dihydrofolate reductase (DHFR) using tritium labelled ligands in conjunction with tritium nuclear magnetic resonance (NMR) spectroscopy. The ligands are selectively labelled with tritium (\(^3\)H) and the signals from these sensitive nuclei detected in the NMR spectrum, assigned and used to monitor the interactions and dynamic processes in protein-ligand complexes. The aim is to understand the molecular factors controlling the specificity of ligand binding. More explicitly, the objectives are as follows:

- To explore the potential of tritium nuclear magnetic resonance spectroscopy in protein-ligand complexes
- To selectively label the ligands, folate and methotrexate, to high specific activities with tritium
- To characterise the specific interactions between groups on the ligand and protein
- To characterise the conformational changes accompanying ligand binding
- To measure the conformational equilibria in the various complexes
- To monitor the changes in intramolecular dynamic processes

This chapter provides an introduction to tritium and its uses. The properties of the radioisotope and methods currently employed for the synthesis, purification and analysis of tritiated compounds are reviewed. Other areas of literature are covered in the subsequent chapters in which the topics to which they relate are introduced.

The application of tritium NMR spectroscopy is discussed in relation to determining the extent and distribution of tritium labelling in simple and complex molecules, whilst examples from the literature illustrate how the technique has found use in addressing the questions of mechanism and stereochemistry in small molecules and biochemical and biological systems.
1.1. GENERAL INTRODUCTION TO TRITIUM

Isotopically labelled compounds find widespread use in chemical and biochemical research. Generally speaking, isotopes can be divided into two classes, stable and radioactive. The stable isotopes $\text{H}^2$, $\text{C}^{13}$, $\text{N}^{15}$, $\text{O}^{17}$ and $\text{O}^{18}$ can be detected and measured by a variety of spectroscopic techniques. Mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy are the most important although high performance liquid chromatography (HPLC) and infra-red spectroscopy have also been used. For many applications stable isotopes are incorporated into a compound at close to 100% isotopic enrichment.

However, radioisotopes have found more widespread uses partly because of their detection at tracer levels by liquid scintillation counting (Birks, 1964). Consequently, this allows isotope incorporations to be much lower than for their stable counterparts. From the time that they began to be freely available radioisotopes as tracers have been widely and successfully used as essential tools for research problems in biological studies and organic chemistry (Evans, 1992).

Compounds have been labelled with radioisotopes such as $\text{P}^{32}$, $\text{S}^{35}$ and $\text{Cl}^{36}$ but the most widely used radiolabelled compounds are those labelled with $\text{C}^{14}$ and tritium ($\text{H}^3$) (Evans, 1981). Although $\text{C}^{14}$ compounds are normally preferred for studies of metabolism, and in pharmacology, the relative ease of labelling complex molecules with tritium, together with the properties of tritium (Table 1.1) and the enormous versatility of the isotope, often makes tritium labelled compounds the tracers of choice for many investigations (Evans, 1974a).
1.2. TRITIUM AND ITS USES

There are three established isotopes of hydrogen namely hydrogen or protium (\(^1\text{H}\)), deuterium (\(^2\text{H}\)) and tritium (\(^3\text{H}\)). The discovery of higher isotopes has been reported (New Scientist, 1963a, b), however their existence is a controversial matter in view of their highly unstable nature. Much has been written on tritium and deuterium and a newcomer to the subject would be advised to study the respective standard reference works of Evans (1974a) and Thomas (1971). Both isotopes effectively duplicate the highly diversified chemistry of the parent element hydrogen and not only act as tracers for that element, but also as labels for a carbon skeleton. In the latter case they simply replace hydrogens in an organic or biological compound.

Unlike hydrogen and deuterium, tritium is a radioactive isotope and as such decays by the emission of low energy \(\beta\) radiation as follows,

\[ ^3\text{H} \rightarrow ^3\text{He}^+ + ^0\beta^- \]

Table 1.1 summarises some important physical properties of tritium.

<table>
<thead>
<tr>
<th>Production</th>
<th>[^6\text{Li}(n,\alpha)^3\text{H}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiation</td>
<td>(\beta) (100%)</td>
</tr>
<tr>
<td>Half-life</td>
<td>12.3 years</td>
</tr>
<tr>
<td>Decay constant</td>
<td>(1.8 \times 10^{-9}\text{s}^{-1})</td>
</tr>
<tr>
<td>Max. (\beta)-energy, (E_{\beta\text{max}})</td>
<td>18.6 KeV</td>
</tr>
<tr>
<td>Mean (\beta)-energy, (E_{\beta\text{mean}})</td>
<td>5.7 KeV</td>
</tr>
<tr>
<td>Max. specific activity (per site)</td>
<td>29.12 Ci/mmol</td>
</tr>
<tr>
<td>Dissociation energy, (T_2 \rightarrow 2T)</td>
<td>4.59 eV</td>
</tr>
<tr>
<td>Ionisation energy, (T \rightarrow T^+ + e^-)</td>
<td>13.55 eV</td>
</tr>
</tbody>
</table>

Table 1.1.
Tritium has certain unique advantages over other useful radioisotopes, especially its closest rival $^{14}$C. It is the cheapest radioisotope of its kind and this makes large-scale tracer experiments possible. The half-life of 12.3 years is conveniently long as not to introduce experimental difficulties although short enough to reduce concern over production and the storage of large quantities. The extremely high specific activities (max. 29.3 Ci/mmol per site) compared for example to $^{14}$C (max. 62 mCi/mmol per site) make it an excellent choice for use by the pharmaceutical industry, for example in the fields of drug metabolism and receptor binding studies (Hawkins, 1988). Although $^{14}$C is still dominant in the former field, the use of tritium is necessary for compounds whose normal physiological concentrations are extremely low without significantly affecting the normal metabolic process. However, in receptor-ligand binding studies, where high specific activities are required (O'Connell et al., 1993) the use of tritium is essential.

The excellent autoradiographic properties, due to the weakness of the $\beta$-radiation and the high resolution obtained, makes tritium the first choice in much biological tracer work. The low energy and low penetrating power ($10^{-4}$ cm in photographic film) of the tritium $\beta$-radiation permits precise localisation and measurement on autoradiographs (to within less than 1 $\mu$m) of the atom that disintegrates. In practice a sample containing the radioactive material is brought into contact with a photographic emulsion and the $\beta$-particles from the tritium disintegration cause the same effect on the silver halide grains as light. This leads to a photographic image on the emulsion which allows the radioactivity to be located. This technique may also be used as a means of chemical purification when used in conjunction with thin layer chromatography (tlc). However, a radiochromatogram scanner provides a much quicker and more accurate identification of the distribution of radioactivity on a tlc plate or paper.

Low toxicity and comparative ease of labelling procedures are other obvious reasons for the extensive use of tritium when studying biochemical and chemical reaction pathways and mechanisms (Evans, 1974a).
1.3. TRITIATION PROCEDURES

Compounds labelled to high specific activities with tritium are increasingly required for studies of interactions of biologically active compounds with macromolecules such as receptors and enzymes. The traditional methods of preparing such labelled compounds most commonly fall into two categories,

(1) Hydrogen isotope exchange reactions
(2) Direct chemical synthesis

In practice, tritium attached to atoms other than carbon is often labile because of its acidic or basic nature and consequently only the preparation of those compounds containing carbon-tritium bonds are considered.

1.3.1. Hydrogen Isotope Exchange Reactions

The preparation of tritium-labelled compounds by isotope exchange reactions invariably results in a 'generally' labelled compound, i.e. a compound in which the tritium atoms are distributed in a general or random pattern throughout the molecule. The labelling is seldom uniform.

There are two general exchange labelling procedures which are currently applied to the preparation of tritiated compounds,

(i) Isotope exchange with tritium gas
(ii) Isotope exchange with tritiated solvents

and the procedure selected depends on the nature and behaviour of the compound to be labelled under the experimental conditions (Myasoedov, 1993). A comparative study of platinum catalysed reactions using tritium gas and tritiated water as the isotope sources
was undertaken by Williams and his group (Williams et al., 1991) in conjunction with \( ^3\)H NMR spectroscopy. The group were able to compare detailed incorporation patterns obtained by the two methods. They concluded that alkyl exchange in alkylbenzenes was generally less when HTO rather than tritium gas was the isotope source. A feature of the tritium gas results was the very high incorporation in alkyl groups, especially hydrogens on a carbon atom adjacent to an aromatic ring. In contrast, the total amount of tritium in the alkyl position of straight-chained alkylbenzenes labelled by HTO exchange increased as the number of alkyl vs aromatic protons increased. It was observed that the alkyl and aromatic tritiation rates were more nearly equal in the case of HTO exchange than with \( T_2 \) gas, and this could be a reflection of a lower sensitivity of the HTO exchange process to the structure of the alkylbenzene substrate. It was also shown that in contrast to the products of HTO exchange reactions being free from any hydrogenation or dehalogenation by-products, hydrogenation products from tritium gas reactions were present and usually constituted a maximum of 25% of the radioactivity yield.

**1.3.1.1. Isotope Exchange with Tritium Gas**

Hydrogen isotope exchange of organic compounds with tritium gas was first recognised by Wilzbach in 1957 (Wilzbach, 1957) and has been described in some detail elsewhere (Wenzel, 1962). Although there have been numerous modifications of this radiation-catalysed exchange procedure, the problems associated with the purification of the products and the relatively low molar specific activities normally achieved have made the method of limited use. Furthermore, the rate of labelling for this procedure has been quoted as taking place at the rate of one percent of tritium per day (Ziffero, 1957) and this is inconveniently slow in most instances where longer exposure times increase the risk of radiation damage to the compound. Recent publications (Myasoedov et al., 1977, 1992; Zolotarev et al., 1991) have shown that the application of catalysts to the
Wilzbach method considerably increases the degree of label incorporation as well as its selectivity, and suggest that this technique of solid state isotope exchange may open new avenues in the preparation of tritium-labelled organic compounds.

A more widely used method involving the use of gaseous tritium for heterogeneous catalytic isotope exchange is that developed by Evans in the early 1970's (Evans et al., 1974b). This procedure is based on the ability of hydrogen atoms occupying certain positions in a molecule to exchange with tritium gas in the presence of a metal hydrogen transfer catalyst (usually Pd or Pt) in neutral or basic solution. Palladium oxide supported on barium sulphate has been shown to be the most useful catalyst (Kuhn and Hass, 1955), although palladium supported on calcium carbonate or charcoal are also effective. This method has found applications with a wide variety of organic compounds as reflected in numerous publications (Buncel and Jones, 1987, 1990) and is especially suitable for labelling benzylic compounds, steroids and drugs (Evans, 1974a, b). The results obtained by this catalysed gas-exposure technique show the tritiated products to be of high specific activity and purity and specific labelling may often be obtained.

1.3.1.2. Isotope Exchange with Tritiated Solvents

Hydrogen-tritium isotope exchange reactions in solution are normally catalysed by homogenous or heterogeneous catalysts. The applicability of this general technique is restricted only by the stability of the substrate under the exchange conditions and it is admirably suitable for compounds that are moderately stable in solution at temperatures up to 150°C.

Exchange reactions in solution under the influence of heterogeneous hydrogen transfer catalysts, the most widely used of the methods for obtaining generally labelled organic compounds, involves heating the substrate for a few hours (5 - 20 hrs) in a
tritiated solvent (tritiated acetic acid or water) in the presence of a pre-reduced catalyst. A wide range of heterogeneous catalysts (Pt, Pd, Ni, Co, Fe, Rh etc.) in supported or unsupported forms have been employed (Evans, 1974a), platinum being the most active. Compounds labelled by this procedure include for example amino acids (Gravel and Hundeshagen, 1959), pyrimidines and nucleosides (Taylor et al., 1957) and steroids (Fukushima and Gallagher, 1952; Simmonds, 1993). The adaptation of heterogeneous metal catalysed hydrogen isotope exchange to heterocyclic compounds has been thoroughly reviewed by Calf and Garnett (1973).

A large number of organic compounds can also be specifically labelled under homogeneous conditions by taking advantage of the weakly acidic character exhibited by some carbon-hydrogen bonds (Jones, 1973). Thus in the presence of a suitable base, specific ionisation may occur allowing the intermediate carbanion to abstract a triton from an appropriately tritiated solvent to yield the radiolabelled product. Some compounds such as the β-diketones are sufficiently acidic to permit labelling to take place even in neutral solution at room temperature. For weaker acids such as ketones, strong bases such as hydroxide ions are required to produce the intermediate carbanion (Thomas, 1971).

Treatment of many organic compounds with strong acid results in protonation. Subsequent proton loss (if it involves other than the introduced hydrogen atoms) can provide a means of exchange. A wide range of acids have been employed to label essentially electron rich aromatic compounds and many such studies of acid-catalysed tritiations have been reported (Norman and Taylor, 1965; Evans, 1974a; Orchin and Bollinger, 1975).

Many kinds of Lewis acids have been used to induce exchange and of these ethylaluminium dichloride (EtAlCl₂) is amongst the most reactive (Long et al., 1975; Elvidge et al., 1977).
1.3.2. Direct Chemical Synthesis

Catalytic exchange procedures are expedient methods which rarely give the sufficiently high specific activity necessary for radioimmuno-assay and binding studies. This can be facilitated by direct chemical synthesis, which is the most accurate and reliable way of labelling compounds with tritium. Starting materials for the syntheses of tritium-labelled compounds by this route are tritium gas, tritiated water and tritiated metal hydrides. Reduction reactions are the most significant and even complex organic molecules are labelled by simple one step reductions. Reactions of this type fall into three main categories,

(i) Reduction of unsaturated compounds
(ii) Reduction with tritiated metal hydrides
(iii) Reduction by catalytic halogen-tritium replacement

1.3.2.1. Reduction of Unsaturated Compounds

Hydrogenation of unsaturated compounds by the catalytic addition of tritium gas is one of the most convenient and simplest methods for the introduction of tritium into a molecule. Carbon-carbon aliphatic double and triple bonds are reduced by a metal catalyst in the presence of tritium gas.

\[
\text{CH}_3(\text{CH}_2)_4\text{C}==\text{CCH}_2\text{C}==\text{C(CH}_2)_7\text{COOH} \\
\downarrow \text{T}_2, \text{Lindlar} \\
\text{CH}_3(\text{CH}_2)_4\text{CT}==\text{CTCH}_2\text{CT}==\text{CT(CH}_2)_7\text{COOH}
\]
Using catalyst systems such as Pd/C, Pd/BaSO₄ and Rh/Al₂O₃ a wide variety of tritiated compounds have been prepared, for example, the partial reduction of octadeca-9,12-diynoic acid (1) gives tritiated linoleic acid (2) (Lemmon et al., 1959).

1.3.2.2. Reduction with Tritiated Metal Hydrides

Unsaturated groups other than carbon-carbon bonds can also be reduced to give tritiated products and in these cases tritiated reducing agents are employed. Most widely used are the metal tritides or mixed metal hydrides such as sodium or lithium borotritides and lithium aluminium tritide. Typically, the groups which are reduced include aldehydes, ketones, carboxylic acids, esters and nitriles. This method gives strictly specific labelling and the tritium atoms are located only on the carbon atom forming the unsaturated group. For example the hormone adrenalin (4) is labelled specifically at the 7-position by the reduction of the corresponding ketone (3) with sodium borotritide (Kaplan, 1954).

\[
\begin{align*}
\text{(3)} & \xrightarrow{\text{NaBH₃T}} \text{(4)}
\end{align*}
\]

In recent publications Andres et al. (1990, 1992) have detailed a method for the simple and facile generation "in situ" of lithium tritide, an excellent starting material for the carrier-free preparation of many labelled reagents including LiAl₄T, LiBT₃BT, LiBT₄ and Bu₃SnT. Compounds labelled by this route are tritiated at close to maximum specific activity and if this work could be extended to the tritides of sodium and potassium, as well as the use of other Lewis acids, the range of tritiated reagents would be significantly increased.
1.3.2.3. Reduction by Catalytic Halogen-Tritium Replacement

The final important method of direct chemical synthesis involves the replacement of a halogen atom by tritium under catalytic conditions (similar to those used in hydrogenation reactions).

\[
\text{RX} + \text{T}_2 \rightarrow \text{TX} + \text{RT}
\]

\[
\text{TX} + \text{OH}^- \rightarrow \text{THO} + \text{X}^- \quad \text{X} = \text{Halogen}
\]

A number of experimental aspects have to be considered when undertaking this type of reaction. Firstly, it can be seen that only half of the tritium employed can be incorporated into the molecule, the remainder is lost as the tritiated halide, TX, which is likely to seriously poison the catalyst. This is a particular problem when iodine is involved (Baltzly, 1967). Usually such problems can be prevented by neutralising the tritium halide formed during the reaction with an excess of a weak base such as triethylamine.

A high degree of specific labelling is normally achieved if the rate of the halogen-tritium replacement reaction is fast. Reaction times vary according to the substrate and catalyst, but as a general rule, iodinated compounds react faster than the corresponding bromo- and chloro-derivatives with fluorinated compounds seldom being employed. In the case of slow reactions, the build-up of tritium as tritiated water in solution may result in metal catalysed hydrogen isotope exchange reactions. Although such exchange with compounds in solution is usually slow at room temperature, this phenomenon can result in some non-specific labelling and has been observed with halogenated derivatives of folic acid (Zakrzewski et al., 1970, Evans et al., 1979).
1.4. PURIFICATION AND ANALYSIS OF TRITIUM COMPOUNDS

The ability to isolate the tracer compound in high radiochemical and chemical purity is a measure of the success of any isotopic labelling procedures. This is dependent on the availability of reliable, rapid and accurate methods for radioactive measurement of the isotope and techniques for the identification of the labelled compound.

Tritium is usually detected and measured by liquid scintillation counting (Birks, 1964; Evans, 1974a). The method offers a high detection efficiency, ease of sample preparation, absence of self-absorption and reproducibility of results. Two problems associated with it, namely quenching effects and sample solubility can frequently be overcome (Jaiswal, 1974).

The principle of the method is the conversion of the $\beta$-radiation energy into photoelectrons producing charge pulses which can be amplified and counted by a scaling circuit. In practice, the radioactive sample is dissolved in a solution containing a scintillation solvent and solute. As the tritium undergoes nuclear disintegration the $\beta$-energy transferred to the scintillant solvent can appear as energy of ionisation, dissociation or excitation of the solvent molecules. The solvent excitation energy is then transferred to the molecules of a fluorescent solute which return to the ground state by emitting quanta of light in the visible or near ultra-violet region.

Because of the decomposition of radioactive compounds by self-irradiation, particularly those at the high specific activities required for many investigations, impurities are always likely to be present. It is therefore important to be able to perform quick and reliable checks on the purity of the labelled compounds after purification, and before use.

Techniques involving chromatography (Sheppard, 1972) are well suited to radiolabelled compounds because of the small chemical weights involved. The types of
chromatography normally employed include paper and thin-layer plate chromatography (tlc) and gas-liquid chromatography. The former two methods are restricted to analysing non-volatile compounds while the latter may be used to analyse gases or volatile liquids or solids.

Quantitative determination of tritium in paper and thin-layer plate chromatography may be achieved when the chromatograms are assessed in conjunction with autoradiography, chromatogram scanning or direct counting of the activity by β-liquid scintillation measurement. The most widely used and accurate method for quantitative interpretation of radiochromatograms is by scanning; both paper and thin-layer chromatograms can be scanned (Carleton and Roberts, 1961). The same precautions as those for paper and thin-layer plate chromatography of the corresponding unlabelled compounds must be taken. However, a further problem which must be considered when using these techniques is the possible radiochemical decomposition of the compound on the paper (Moses, 1962) or on the thin-layer plate, particularly when high specific activity compounds are involved.

Gas-liquid or gas-solid chromatographic methods are often used for the analysis and separation of tritiated compounds, particularly the more volatile compounds to which paper or thin-layer methods cannot be applied.

More recently, high performance liquid chromatography (HPLC) (Hawk, 1979; Pryde and Gilbert, 1979) has played an ever more important role for obtaining radiolabelled compounds of very high quality. HPLC plays an equally important role in the analysis of tritium labelled compounds and can often detect trace impurities undetected by paper or thin-layer chromatographic processes.
1.5. TRITIUM NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Since the first report of tritium nuclear magnetic resonance (NMR) spectroscopy by Tiers in the early 1960's (Tiers et al., 1964), the technique has been developed by the pioneering work of the Amersham-Surrey University group (Evans et al., 1985) into a powerful and essential technique in the analysis of tritiated material. Although NMR spectroscopy is an inherently insensitive method, by comparison with, for example, ultraviolet spectroscopy (useful for determination of mass and to confirm structure), it has proved to be the most powerful and far reaching method for investigating molecular processes as well as routine determination of $^3$H labelling patterns.

The triton, like the proton, has a spin quantum number $I = 1/2$, therefore it is a suitable nucleus for high resolution magnetic resonance spectroscopy. Selected magnetic properties of tritium along with those of other radionuclides used in the life sciences are shown in Table 1.2. Some consequences of these magnetic properties of the tritium nucleus make $^3$H NMR spectroscopy particularly useful. Firstly, tritium has a high nuclear magnetic moment ($\mu_T$) which causes the value of the magnetogyric ratio ($\gamma_T = \mu_T/I_T$) to be higher than for any other nucleus in the Periodic table. Consequently at a field of 2.114 T, the $^1$H NMR frequency is 90 MHz and the $^3$H NMR frequency is 96 MHz. In practice this results in a slightly larger spectral dispersion (by the factor $\gamma_T/\gamma_H = 1.06664$) in the $^3$H NMR spectra compared with the $^1$H NMR spectra at the same field and, moreover, the high magnetogyric constant renders the triton the most receptive and sensitive nucleus to NMR detection. The high receptivity, along with effectively zero natural background abundance of the isotope results in detection of the isotope at very low isotopic abundances. In practice, a sample containing 0.5 mCi at a single site (maximum 29.1 Ci) will give a satisfactory signal to noise ratio after one hours accumulation (at 320 MHz) (Evans et al., 1985).
<table>
<thead>
<tr>
<th>Isotope</th>
<th>Natural Abundance (%)</th>
<th>Nuclear Spin</th>
<th>Magnetic Moment $\mu/\mu_N$</th>
<th>Magnetogyratic Ratio $(\gamma)/10^7$ rad T$^{-1}$s$^{-1}$</th>
<th>Resonance Frequency at 2.114 T(MHz)</th>
<th>Relative Sensitivity</th>
<th>Radiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$H</td>
<td>99.984</td>
<td>1/2</td>
<td>4.837</td>
<td>26.752</td>
<td>90.0</td>
<td>1.0</td>
<td>Stable</td>
</tr>
<tr>
<td>$^2$H</td>
<td>0.016</td>
<td>1</td>
<td>1.212</td>
<td>4.106</td>
<td>13.8</td>
<td>10$^{-4}$</td>
<td>Stable</td>
</tr>
<tr>
<td>$^3$H</td>
<td>&lt;10$^{-16}$</td>
<td>1/2</td>
<td>5.159</td>
<td>28.534</td>
<td>96.0</td>
<td>1.21</td>
<td>$\beta^-$</td>
</tr>
<tr>
<td>$^13$C</td>
<td>1.11</td>
<td>1/2</td>
<td>1.216</td>
<td>6.726</td>
<td>22.6</td>
<td>1.6 x 10$^{-2}$</td>
<td>Stable</td>
</tr>
<tr>
<td>$^14$C</td>
<td>&lt;10$^{-10}$</td>
<td>0</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>$\beta^-$</td>
</tr>
<tr>
<td>$^31$P</td>
<td>100</td>
<td>1/2</td>
<td>1.958</td>
<td>10.829</td>
<td>36.43</td>
<td>6.6 x 10$^{-2}$</td>
<td>Stable</td>
</tr>
<tr>
<td>$^32$P</td>
<td>0</td>
<td>1</td>
<td>-0.357</td>
<td>-1.208</td>
<td>4.06</td>
<td>2.5 x 10$^{-4}$</td>
<td>$\beta^-$</td>
</tr>
</tbody>
</table>

Table 1.2. Magnetic Properties of Selected Isotopes used in the Life Sciences
Furthermore, the chemical shifts of tritium nuclei should be closely similar to those of hydrogen nuclei, in corresponding environments (Al-Rawi et al., 1974). This is particularly important because the vast body of data available on proton chemical shifts can then be applied directly to $^3$H NMR, thus greatly facilitating interpretation.

Tritium NMR spectroscopy, unlike for example proton NMR spectroscopy, is not in general used to establish the structure of an unknown compound. More usually, it is used to establish the position and distribution of tritium atoms within a tritiated compound of known structure. The intensity of the tritium signals give directly the relative amounts of the isotope in each position. Signal splitting may give information on the stereochemistry of tritium atoms in nearby positions, because the magnetic interaction between a proton and triton nuclei on adjacent carbon atoms will depend on their relative geometry in space. This information is of special importance, for example, in the study of the biochemical transformation of steroids (Altman & Silberman, 1977a, b; Chambers, 1978). In some cases, further stereochemical information may be obtained by measuring triton-proton couplings in $^3$H NMR spectra without proton spin decoupling, or with specific proton decoupling. The use of spin decoupling techniques effectively destroy couplings between adjacent magnetic nuclei and greatly reduce the complexity of multiple signals but can cause selective signal enhancement in an NMR spectrum. The phenomenon of signal enhancement is known as nuclear Overhauser enhancement (NOE) and is discussed in detail, in general terms, in the literature (Noggle and Schirmer, 1971) and more recently Kaspersen et al. have discussed nuclear Overhauser effects in $^3$H NMR spectroscopy (Kaspersen et al., 1987).

Tritium NMR spectroscopy is undoubtedly the best method for locating the position(s) of the $^3$H label in tritiated compounds and for determining the stereochemistry and relative extent of labelling (Bloxsidge et al., 1981). This is important for many tracer applications of tritium compounds where a knowledge of the
precise position and configuration at the position of the label is essential (Kaspersen et al., 1993).

Although $^3$H has been utilised extensively as a tracer, it has received relatively little use as an NMR label in biological systems. However, with the advent of Fourier transform NMR spectrometers (Al-Rawi, 1974) and quadrature detection, the detection sensitivity has increased and the technique has found use in addressing the questions of mechanism and stereochemistry in small molecules and is increasingly being applied to biochemical (Libor et al., 1980; Dive et al., 1991) and biological studies (Newmark et al., 1990).

The configuration of tritium labelled methyl groups in small molecules has been studied by $^3$H NMR spectroscopy. Ever since the initial disclosure, by Arigoni and co-workers (Lüthey et al., 1969), of methods to synthesise the enantiomers of a methyl group carrying one atom each of $^1$H, $^2$H and $^3$H and to distinguish the $R$ from the $S$ enantiomer of such an entity, there has been considerable fascination with chiral methyl groups and their use to analyse stereochemical problems in chemistry and biochemistry. Examples of this type of study include the stereochemistry of proton elimination in the biosynthesis of tritiated cycloartenol (Altman et al., 1978), the stereochemistry of the incorporation of the methyl groups of 'chiral methyl valine' into methylene groups in Cephalosporin C (Abraham et al., 1983), and more recently, Floss and his group demonstrated for the first time that the configuration of stereogenic (chiral) methyl groups could be assigned by direct $^3$H NMR analysis of a molecule containing an intact CHDT group (Anet et al., 1989).

Applications of $^3$H NMR spectroscopy have extended to studies of conformational analysis and proton-exchange kinetics in simple molecules. As an illustration of the usefulness of tritium in isotopic perturbation studies, Anet described results obtained in a low temperature integration study of $[^3$H]cyclohexane (Anet et al., 1990). Integration of the axial and equatorial tritium signals in the $^3$H NMR spectrum of
the labelled substrate at -88°C showed that in agreement with previous $^2$H NMR work with $[^2$H]cyclohexane, tritium preferred the equatorial site. A year later, Dixon published the first "in situ" measurements of acid- and base-catalysed proton exchange kinetics using $^3$H NMR spectroscopy (Dixon et al., 1991). The method allowed the measurement, in the same NMR tube, of the kinetic acidities of several compounds with a broad range of rates, examples included benzene and $p$-xylene reacting with caesium cyclohexylamide in cyclohexylamine.

As the power of the instrumentation increased, so the application of $^3$H NMR spectroscopy progressed to the study of larger biochemical molecules, however there have been relatively few examples of tritium NMR studies with protein systems (Evans J. et al., 1984).

An essential requirement for understanding protein structure and function is knowledge of the three dimensional structure. Historically, crystallographic methods have been used towards this end, but recently these have been supplemented by NMR studies of proteins at high magnetic fields, in one, two and three dimensions, usually in conjunction with specific isotope enrichment. Only very recently, Gehring and Williams utilised $^3$H NMR spectroscopy to probe protein structure and dynamics (O'Connell et al., 1993a, b). The group used $^3$H NMR studies of specifically tritiated derivatives of tosylchymotrypsin to examine the properties of the tosyl group in this protein. Irradiation at the proton frequency of samples of the specifically tritium labelled enzymes produced a $^3$H{$^1$H} NOE, consistent with dipolar interactions with neighbouring protons being an important component of tritium relaxation. The curly brackets { } are used to indicate the resonance which has been irradiated in the NOE experiment. The nature of the tritium-proton interactions was explored further by means of two-dimensional proton-tritium nuclear Overhauser experiments. The results obtained indicated that multidimensional NMR studies of fairly large molecules by the use of introduced tritium as a heteronuclear probe were feasible.
Chapter 1: Introduction

Tritium NMR spectroscopy has also extended the capability of NMR spectroscopy for studying, for example, anomeric specificity to slowly exchanging processes at low stoichiometries of tritiated ligand to binding studies. The first detection of an enzyme-substrate complex by $^3$H NMR spectroscopy was described by Evans J. et al. in 1986. The group attempted to elucidate the mechanism and structure of the labelled porphobilinogen, [${}^3$H]PBG, with deaminase using $^3$H NMR spectroscopy. From the results they were able to suggest the involvement of a sulfhydryl group as the nucleophilic enzyme group that binds the substrate and also demonstrated the catalytic competence of this complex by displacing bound [${}^3$H]PBG with unlabelled PBG. However, the $^3$H NMR spectra of this study have subsequently been reinterpreted to accommodate the unexpected findings of a C-C bond between substrate and enzyme, in terms of a broad $^3$H signal at $\delta$ 3.28 ppm (Evans J. et al., 1986), which was thought to be due to initial (and transitory) attachment of the substrate at the second cysteine residue conserved in human and *Escherichia coli* (*E. coli*) deaminase (Scott et al., 1988).

The technique has also been used to study the binding of tritium labelled maltose to its transport protein from *E. coli*, maltose binding protein (MBP) (Williams et al., 1989). The sugars were labelled specifically at the reducing end and titrated into a sample of the protein. The high sensitivity of $^3$H NMR spectroscopy along with the vastly simplified $^3$H NMR spectrum, when compared to the $^1$H NMR spectrum of the same complex, gave invaluable information as to the location of the tritium labels in the bound complex. As the mole ratio, maltose to MBP, was increased, signals from the free maltose were also observed in the $^3$H NMR spectrum. Heteronuclear NOE experiments added to the information already available from the $^3$H NMR studies.

This study was extended to the family of linear maltodextrins (of which maltose is the smallest) (Gehring et al., 1991) and the authors were able to show the existence of two bound $\beta$-maltotriose resonances in rapid exchange and assigned them as two distinct sugar-protein complexes.
Clearly, it can be seen that relatively little has been published in this area and the work presented in this thesis will help to confirm the suitability of tritium NMR spectroscopy to studying protein-ligand complexes.
1.6. THESIS OUTLINE

Following this introductory chapter, the thesis is divided into three main experimental chapters followed by a final summary. The layout is as follows:

- Chapter 2 describes the labelling and purification of the ligands, folate and methotrexate, to high specific activity with tritium. The distribution and extent of the tritium labelling is determined by tritium NMR spectroscopy.

- Chapter 3 presents information relating to substrate, inhibitor and coenzyme binding to \textit{Lactobacillus casei} (\textit{L. casei}) dihydrofolate reductase, with particular regard to using selectively tritiated ligands in conjunction with tritium NMR spectroscopy. Tritium NMR studies of \([3',5',7-^3\text{H}]\)folate, \([7,9-^3\text{H}]\)folate and \([3',5',7-^3\text{H}]\)methotrexate binding to \textit{L. casei} DHFR, with NADP\(^+\) and NADPH as coenzyme, are presented. The effects of pH and temperature on the binary and ternary complexes have been investigated and the results discussed in relation to existing \(^1\text{H},\ 13\text{C},\ 15\text{N}\) NMR and X-ray crystallographic data for the DHFR complexes.

- Chapter 4 details the use of the nuclear Overhauser effect (NOE) in small molecules and macromolecular systems. The results from \(^3\text{H}\{^1\text{H}\}\) heteronuclear NOE experiments on small molecules are presented and the feasibility of the technique for observing \(^3\text{H}\{^1\text{H}\}\) NOEs between protein and tritiated ligands is discussed.

- Chapter 5 is devoted to providing a brief summary of the results in previous chapters, with further discussion on the implications of these results. Some suggestions on possible areas of future work are also included.
Labelling of Ligands

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2. LABELLING OF LIGANDS

2.1. INTRODUCTION

2.1.1. Background

The term 'molecular recognition' is one which is increasingly used to describe specific interactions between molecules. It refers to the ability of a receptor molecule to bind some molecules more tightly than others, due to the fact that non-covalent interactions between the receptor and these substrate molecules are more favourable, i.e., the receptor molecules have the ability to discriminate between closely related substrates. This is a key determinant of chemical reactivity, enzyme catalysis and many other fundamental processes, e.g., DNA synthesis and protein biosynthesis.

There are four types of interaction that contribute to the binding of the receptor and substrate molecules: electrostatic (ionic), hydrogen bonding, van der Waals and hydrophobic (Kollman, 1989) and these will be discussed in more detail in subsequent chapters. Broadly speaking, most of the work carried out in this area has been involved with determining and understanding small molecule/large molecule interactions. The interaction of an enzyme with a substrate (or ligand) is a typical example of such molecular recognition. A detailed understanding of this type of recognition is often the vital component for the design of novel drugs and pharmaceutical agents based on the inhibition of a key enzyme in a pathogenic organism. Enzyme inhibition also has implications for the design of new agro-chemicals, for example as herbicides (Cooper et al., 1987).

The design of new molecules which specifically bind very tightly to a selected protein is an important area of increasing interest within the domain of molecular recognition. Thus, by studying the various properties of receptor-ligand complexes, it
should be possible not only to illuminate specific biological problems, but also to gain insight into the general rules governing intermolecular interaction thereby leading to the design of new molecules.

The use of powerful spectroscopic methods has been a key feature in much of the recent work. The employment of NMR spectroscopy has been the most popular and this technique has provided considerable insight into the regions of complexes of small molecules with complicated proteins. The first protein whose binding site was analysed by studying the NMR spectra of the protein, as well as that of the ligand, was bovine pancreatic ribonuclease A (Meadows & Jardetzky, 1968; Meadows et al., 1969). The inhibitor used in this complex was cytidine 3′-monophosphate. From that time on there have been many receptor-ligand complexes that have been studied by NMR spectroscopy (Roberts & Jardetzky, 1970; Fesik, 1991).

![Figure 2.1. Reduction of dihydrofolate (FH₂, 6) to tetrahydrofolate (FH₄, 7) by NADPH as catalysed by dihydrofolate reductase (DHFR)](image-url)
In the present study the receptor of interest is the enzyme dihydrofolate reductase (DHFR) which catalyses the reduction of 7,8-dihydrofolate (FH₂, 6) (Figure 2.1) (and folate (5a)) to 5,6,7,8-tetrahydrofolate (FH₄, 7) using NADPH as a coenzyme (Blakley, 1985). Tetrahydrofolate (7) is a one carbon carrier in various synthetic pathways, including the synthesis of thymidylate from uridine, a step essential for DNA replication and therefore for cell division. Thus, DHFR is required to maintain the cellular pool of reduced tetrahydrofolate in cells that are synthesising DNA and antifolate drugs act by selectively inhibiting this key enzyme in invasive cells. Typical antifolate drugs in clinical use are methotrexate (8) and trimethoprim (9).

**Figure 2.2.** Structures of the substrate folate (5a) and antifolate drugs, methotrexate (8) and trimethoprim (9)
The amino acid sequences for the enzyme have been determined from a number of species (Bolin et al., 1982; Matthews et al., 1985). Structural studies have also been performed on several complexes of dihydrofolate reductase (Antonjuk et al., 1984; Hammond et al., 1987), with the result that DHFR is well characterised and an ideal choice for interpreting the molecular basis of the specificity of protein-ligand interactions.

Since its introduction in 1963 by Forsén and Hoffman (1963), the double resonance transfer of saturation NMR experiment has been widely used to study relatively slow chemical exchange processes in organic and biological systems (Cayley et al., 1979; Clore et al., 1981). In biological applications, e.g., for a ligand binding to an enzyme, saturation transfer experiments have been particularly useful for locating the resonances of bound ligands, which cannot be observed directly in the NMR spectrum, by finding the irradiation frequencies at which transfer of saturation to the corresponding resonances of the free ligand is observed. Saturation transfer experiments have been used to study the binary complex of *L. casei* dihydrofolate reductase with NADP⁺ (Hyde et al., 1980a) and the ternary complex with folate (Hyde et al., 1980a; Clore et al., 1981).

Feeney and colleagues (Birdsall et al., 1982; Cheung et al., 1993) have shown that it is often necessary to resort to isotopic labelling techniques to assign resonances in ligands which are so tightly bound to the protein that transfer of saturation methods cannot be used to connect signals from bound and free species. In the past, ¹³C- and ¹⁵N-labelled ligands have been used (Birdsall et al., 1982, 1987) for example, the use of ¹³C selectively enriched folates has provided a direct way of monitoring the different conformational states in the DHFR-folate-NADP⁺ complex (Cheung, 1993). However, this approach suffers from the fact that the synthesis of the isotopically labelled ligands requires considerable effort (Cheung et al., 1993; Cowart et al., 1994) and also that the nuclei are relatively insensitive to NMR detection. The latter factor makes it difficult to carry out kinetic measurements using line shape analysis.
The present work explores the use of tritium NMR spectroscopy in combination with specifically tritiated ligands in studies of protein-receptor interactions. In principle, the use of tritium NMR spectroscopy to examine tritiated bound ligands offers a useful additional method of directly studying protein-ligand complexes. Although the attractive features of the $^3$H nucleus for NMR studies have been amply demonstrated in studies of small molecules (Evans et al., 1985), the potential value of this nucleus for studies of protein-ligand interactions has hardly been exploited (Gehring et al., 1991; O'Connell et al., 1993a, b). In contrast to the time consuming and expensive $^{13}$C and $^{15}$N syntheses, the incorporation of tritium at very high specific activity can usually be achieved in a one- or two-step process (see Section 1.3 and Evans et al., 1985). Furthermore, it is often possible to modify the synthesis in order to incorporate the label at different sites. The tritiated sites can then be detected directly in the $^3$H NMR spectra, furthermore there are no background signals present.

The work presented in this thesis is concerned with examining the tritium NMR spectra of complexes of *Lactobacillus casei* DHFR and selectively tritiated folates and methotrexate labelled at sites designed to probe multiconformational behaviour and dynamic processes in these complexes. Hence, this chapter is concerned with the labelling of the ligands, folate (5a) and methotrexate (8), to high specific activity with tritium, and the determination of the distribution and extent of tritium labelling, by tritium NMR spectroscopy.
2.1.2. Literature Overview

Tritium labelled folic acid (5b) and methotrexate (8) are both extensively used in biomedical research. For studies in which the tritium is used as a tracer to follow the fate of the intact molecule, the specificity of tritium labelling is normally unimportant. However, in studies where the possibility of metabolism or chemical alteration of the compound arises, it is very important to know the distribution of tritium in the tracer compound (Evans, 1974a). In the present study, a knowledge of the position and extent of tritium labelling is imperative when assigning the signals in the $^3$H NMR spectra for the different conformations of the tritium labelled ligand/enzyme complexes.

The preparation of the 'specifically' labelled compounds by catalytic dehalogenation of 3',5'-dibromofolic acid (10) and 3',5'-dichloromethotrexate (11) with tritium gas (Zakrzewski et al., 1970) is perhaps the classical example of non-specific labelling. Chemical degradation (Zakrzewski et al., 1970) indicated that a substantial proportion of the tritium was located at the C-9 position of both folic acid (5b) and methotrexate (8).

Several other methods have been used for the isotopic labelling of folic acid and are as follows:

(a) exposure to tritium gas (Wilzbach method) (Johns, 1961)
(b) platinum (Pt) or palladium (Pd) catalysed H/T exchange in HTO
   or acetic acid (Zakrzewski et al., 1970)
(c) Pd catalysed dehalogenation using tritium gas (Evans, 1979)
(d) acid catalysed H/D exchange in D$_2$O (Hachey et al., 1978)

All give different patterns of labelling and the earlier studies were investigated by time consuming chemical degradation and physical methods. However, subsequent studies (Evans et al., 1979, 1985) have reinforced the benefits of the direct and non
destructive $^3$H NMR spectroscopic technique for establishing patterns of tritium labelling and this is now the method chosen by most groups.

**Figure 2.3.** Structure of folic acid analogues

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>R</th>
<th>R'</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic Acid (5b)</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Methotrexate (8)</td>
<td>NH$_2$</td>
<td>CH$_3$</td>
<td>H</td>
</tr>
<tr>
<td>3',5'-Dibromofolic Acid (10)</td>
<td>OH</td>
<td>H</td>
<td>Br</td>
</tr>
<tr>
<td>3',5'-Dichloromethotrexate (11)</td>
<td>NH$_2$</td>
<td>CH$_3$</td>
<td>Cl</td>
</tr>
</tbody>
</table>

For methotrexate (8) labelled by catalytic dehalogenation of 3',5'-dichloromethotrexate (11) the $^3$H NMR data (Evans, 1985) shows that in contrast to the results obtained by chemical degradation methods (Zakrezweski et al., 1970), the 3',5'- and 7-positions are exclusively labelled, with no tritium at the 9-methylene position.

As previously stated, contradictory results have been obtained in research concerning the specificity of isotopic labelling by the procedure of catalytic
dehalogenation of 3',5'-dibromofolic acid (10). In studies of tritium labelling Zakrzewski et al. (1970) reported that only 35 - 42% of the label was at the 3',5'-positions with the remainder of the isotope incorporated at C-7 and C-9. In later research, Evans et al. (1979) observed similar isotopic distribution although 3',5'-labelling ranged from 42 to 73% of the total tritium. Gregory and Toth (1988, 1989) reported that the preparation of [3',5'-2H]folic acid by catalytic dehalogenation with deuterium gas yielded little or no incorporation of the isotope at C-7 or C-9, and as a follow up to this study Gregory (1990) reported modifications to the previous catalytic dehalogenation procedure that permitted complete deuterium labelling of folic acid at the 3',5'-positions. The major procedural changes reported were the preliminary removal of exchangeable protons from the substrate 3',5'-dibromofolic acid and the catalytic dehalogenation reaction conducted in D2O. In this study of Gregory (1990), NMR analysis was employed to provide a direct indication of the extent of deuterium labelling of the 3',5'-positions and other sites within the molecule. The spectral data obtained confirmed the high degree of specificity of labelling of the 3',5'-positions which is in contrast to the distribution of tritium obtained under analogous reaction conditions (Zakrzewski et al., 1970, Evans et al., 1979). Although Evans observed, by NMR spectroscopy, that the extent of tritium labelling of C-7 and C-9 varied markedly among batches of palladium on calcium carbonate employed, consistent labelling of these positions was observed. The NMR data for the deuterium labelling study confirmed previous observations (Gregory and Toth, 1988, 1989) that little or no labelling of C-7, C-9 or other positions occurred by deuterium-proton exchange under the conditions of the reaction. It is still unclear whether the difference in labelling specificity between tritium and deuterium reflects a true isotopic effect or a difference in the nature of the catalysts employed.

In this study, where high specific activities are required, folic acid (5b) and methotrexate (8) were labelled with tritium by catalytic halogen-tritium replacement of 3',5'-dibromofolic acid (10) and 3',5'-dichloromethotrexate (11) respectively, using
tritium gas with palladium on calcium carbonate as the catalyst (Evans et al., 1985). 
3',5'-Dichloromethotrexate (11) was first synthesised according to Martinelli & 
Chaykovsky (1980). Labelling with tritium was also achieved by a procedure using 
catalysed exchange between tritium gas and folic acid (5b) in solution to give folic acid 
labelled exclusively in the 7- and 9-positions (Evans et al., 1979). Tritium NMR 
spectroscopy was used to determine the patterns and extent of labelling in the tritiated 
compounds.
2.2. EXPERIMENTAL

2.2.1. Materials and Equipment

Tritium gas was supplied by Amersham International, folic acid, 3',5'-dibromofolic acid and methotrexate were obtained from Sigma Chemical Company. 5% Pd/CaCO₃ was obtained from Aldrich Chemical Company.

The pH measurements were carried out using a Hanna Instruments HI8417 pH meter equipped with a combination glass electrode. Tritiated samples were counted with a Beckman LS 100 liquid scintillation counter by using Unisolve E liquid scintillant.

The ultra-violet (UV) absorption spectra were recorded on a Cecil CE272 spectrophotometer. The UV absorbance of the tritiated folic acid (12, 13) in 0.1 M NaOH was measured at 282 nm, ε₂₈₂ = 25 100 M⁻¹cm⁻¹ (Waller et al., 1948). For methotrexate (14) in 0.1 M NaOH, three UV absorbances were measured: 257 nm, ε₂₅₇ = 23 000 M⁻¹cm⁻¹; 302 nm, ε₃₀₂ = 22 000 M⁻¹cm⁻¹; 370 nm, ε₃₇₀ = 7000 M⁻¹cm⁻¹ (Seeger et al., 1949).

The radiochemical purity was determined by radioc chromatogram scanning (Berthold LB 2842 Radiochromatogram Scanner) of the tlc plates (precoated fluorescent silica gel, 5 x 20 cm, Schleicher & Schuell F1500/LS 254). Known standards were used on the tlc plates for visual comparison with the radiolabelled samples.

The developing tank was purged with nitrogen and kept in the dark. The plate was allowed to run for three hours. The solvent system used was propan-1-ol (3): ammonium hydroxide, 0.3 M (1), folic acid, Rₐ = 0.6; methotrexate, Rₐ = 0.5.

¹H and ³H(¹H-decoupled) NMR spectra were recorded in deuterated solvents using a Bruker AC-300E (300 MHz) spectrometer. Typically, 128 transients and 30 000 transients were collected for ¹H and ³H spectra respectively, with widths of 3846 Hz and using 16K data points, zero filled to 32 K data points.
2.2.2. Experimental Methods

2.2.2.1. The Tritium Gas Line

The synthesis of tritiated ligands by either catalytic exchange in solution or direct chemical synthesis with tritium gas was carried out on the tritium gas line (Figure 2.4). Hydrogen or deuterium may, of course, be substituted for tritium gas as starting material for the preliminary studies. The apparatus is designed to transfer a measured quantity of tritium gas to the reaction vessel. It is constructed from stainless steel high performance liquid chromatography equipment which is connected to Pyrex glassware via glass-metal seals.

The 'business end' of the apparatus is the tritiation tree which is shown in detail in Figure 2.5. It consists of a burette (5.0 cm$^3$, 28 cm length) with a 250 cm$^3$ bulb at the base. The top of the burette is connected, via a four way junction piece (behind the plate on the diagram), to the manifold, tritium gas ampoule and the reaction flask, each of which may be isolated via Whitey swagelock$^\text{TM}$ SS-4181 taps 1, 2, 3 and 4. The tree is connected to the main manifold at tap 1. Connected to the base of the tree is a mercury reservoir (300 cm$^3$) which is in turn connected to the main manifold at tap 5.

A vacuum pump is connected to the apparatus at A and the vacuum attained may be read from the vacuum gauge (C). Volatile tritiated material is collected in the liquid nitrogen trap which is connected to the main manifold by silicon rubber high vacuum tubing. A helium balloon, connected via a teflon 3-way interflow tap, may be attached to the apparatus by the ground glass joint, D.

All glass joints are lubricated with Apeizon grease, all stopcocks are made of teflon and the tubing connecting the manifold to the tritium tree is silicon rubber high vacuum tubing.
Figure 2.4. Tritium gas-line for handling multi-Curie quantities of tritium gas
Figure 2.5. Detail of the tritiation tree
2.2.2.2. Operation of the Tritium Gas Line

The vacuum pump is started when all taps except for A and 5 are closed. Liquid nitrogen should be present in the trap dewar to contain any solvent vapour from the reaction vessel. A balloon of helium is then attached to the apparatus at D and tap E is opened. The reaction vessel containing catalyst, substrate, solvent and a stirrer bar is attached to 3. The stirrer bar is a short piece of paper clip encapsulated in a glass tube manufactured from the tip of a disposable pipette. An ampoule of tritium gas (4.0 Ci, 1.68 cm$^3$) is now attached to 2. A 20 cm$^3$ round bottomed glass flask with a ground glass tap and filled with helium is attached to the other side arm at tap 2. Air can now be evacuated from the burette by closing tap 5 and by opening tap 1 to the manifold and carefully opening tap 4 to the burette; the mercury level should be very close to the tap.

Air is most effectively removed from the reaction flask by freezing the contents with liquid nitrogen before opening tap 3. Tap 2 is now opened to evacuate the entire manifold, reaction vessel and glassware connected to the tritium ampoule.

The vacuum is then closed off at tap A and helium gas allowed into the system until the Edwards vacuum gauge reads atmospheric pressure. Tap 4 is opened carefully to allow about 2 cm$^3$ of helium into the burette. The helium is then closed off via the 3-way tap F and the system re-evacuated by opening A. Tap 4 is carefully opened to evacuate the burette, the mercury level should again be very close to the tap.

This 'sweeping' process is repeated twice more with helium, then all of the taps in the tritium tree, 1, 2, 3 and 4 are closed and the air is now evacuated from the mercury reservoir by opening tap 5. The line behind the tritium tree, leading to the reservoir, is then swept with helium as before and evacuated.

Taps 1, 2, 3 and 5 are now opened and left to evacuate for a further 20 minutes. After this time taps 1, 2, 3 and 5 are closed and the liquid nitrogen removed from around the reaction vessel.
Chapter 2: Labelling of Ligands

The glass breakseal of the tritium ampoule is broken by the magnet contained in the side-arm at 2. Taps 2 and 4 are opened and a drop in the mercury level in the burette provides visual confirmation that the breakseal has broken and tritium gas is being transferred into the burette. Tap 5 is opened and as the mercury flows to fill the vacuum in the reservoir it draws the tritium gas out of the ampoule and into the 250 cm$^3$ bulb below the burette. When the flow has ceased 2 and 4 should be closed. Tap 5 is now very CAREFULLY opened to the atmosphere, to prevent breakage of the graduated tube. Once the pressures have equilibrated the volume of tritium gas that has been transferred may be measured from the graduations on the burette. If insufficient tritium has been transferred, the process may be repeated.

The gas can now be transferred to the reaction mixture by opening taps 3 and 4. Because there is a vacuum in this region, the mercury level has a tendency to rush into the four way junction and reaction vessel. Hence, care must be taken when transferring the gas. Tap 4 can be closed when the desired quantity of gas has been transferred over and the remaining gas can be safely stored in the burette for future use. The stirrer can now be switched on, A closed, the vacuum pump shut off (air bleed) and the dewar removed from the trap.

After the desired reaction time, helium may be introduced into the reaction vessel to ensure maximum incorporation of the tritium into the substrate. The helium is initially transferred from the round bottom flask into the burette as before, and then with taps 3 and 4 open and 5 open to the atmosphere the helium is transferred into the reaction vessel. Stirring is recommenced with taps 3, 4 and 5 open; the level in the burette can then be monitored, to check if any further tritium gas is taken up in the reaction.

After the desired time taps 3, 4 and 5 are closed and the reaction vessel is re-frozen in liquid nitrogen. Tap 5 is then open to the vacuum to evacuate the mercury reservoir. The tritium-helium mixture is transferred back into the burette by opening taps 3 and 4. When all of the tritium-helium gas has been transferred, taps 3, 4 and 5 are
closed, the reaction vessel allowed to warm to room temperature and removed from the tritiation tree and the manifold shut down as described previously. The catalyst may now be filtered out and purification of the tritiated products carried out.

A summary of weights, reaction times and yields is given in Table 2.1. The reaction times for the dehalogenation of 3',5'-dibromofolic acid (10, 40 minutes) and 3',5'-dichloromethotrexate (11, overnight) reflect the relative reactivities of these halogens to hydrogenolysis (Freifelder, 1971). Deoxygenated solvents and nitrogen-flushed tanks (in the dark) were used throughout to prevent decomposition of the light-sensitive compounds.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Starting Material</th>
<th>Catalyst</th>
<th>Tritium</th>
<th>Gas</th>
<th>Specific Activity (Ci/mmol)</th>
<th>Reaction Time</th>
<th>Total Yield (mCi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3',5',7',9'-H]Folic acid (12)</td>
<td>3,5-Dibromofolic acid (10)</td>
<td>5% Pd/CaCO₃ (10.0 mg)</td>
<td>40 mins</td>
<td>2 Ci</td>
<td>32</td>
<td>5 hrs</td>
<td>30</td>
</tr>
<tr>
<td>[7',9',H]Folic acid (13)</td>
<td>Folic acid (5b)</td>
<td>5% Pd/CaCO₃ (40.0 mg)</td>
<td>2 hrs</td>
<td>2 Ci</td>
<td>20</td>
<td>16 hrs</td>
<td>10</td>
</tr>
<tr>
<td>[3',5',7',H]Methotrexate (14)</td>
<td>3,5-Dichloro-Methotrexate (11)</td>
<td>5% Pd/CaCO₃ (24.0 mg)</td>
<td>2 Ci</td>
<td>2 hrs</td>
<td>9</td>
<td>10 hrs</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1. Reaction conditions and yields for the preparation of tritium labelled folic acid and methotrexate.
2.2.2.3. Preparation of \([3',5',7,9\text{-}^3\text{H}]\)Folic Acid (12)

3',5'-Dibromofolic acid (10, 5.0 mg, 0.078 mmol) and 5% palladium on calcium carbonate (10.2 mg) in 4% sodium hydroxide (0.5 cm\(^3\)) were stirred for 40 minutes in the presence of tritium gas (2 Ci) on the tritium gas line. After this time, helium gas was allowed into the reaction vessel and the reaction mixture was stirred for a further ten minutes to ensure the maximum incorporation of tritium gas. The catalyst was immediately filtered out through a cotton wool/celite pad and the filtrate was adjusted to pH 7.0 ± 1.0 pH unit, using dilute HCl. The solution was then frozen in liquid nitrogen and lyophilised. The crude folic acid was taken up in distilled water (0.5 cm\(^3\)) and any insoluble material was solubilised with 4% NaOH; the solution was then chromatographed for five hours on Whatmann 3MM chromatography paper using 0.5% K\(_2\)CO\(_3\) solution as the developing solvent. The folic acid band (R\(_f\) = 0.6) was identified by a marker (yellow in white light and UV absorbing) and by autoradiography. The folic acid band was extracted with warm water and its concentration in solution determined spectrophotometrically (\(\epsilon_{282} = 25\ 100\ \text{M}^{-1}\text{cm}^{-1}\) in 0.1 M NaOH), the solution was then lyophilised. The average radiochemical yield was about 50 mCi and the estimated specific activity at this stage was 40 - 50 Ci/mmol. The folic acid K\(^+\) salt was ion-exchanged on a DEAE cellulose column (1 x 25 cm) to remove radiochemical impurities formed on storing the preparation (Zakrzewski, 1966). The column was eluted with a linear gradient consisting of water (250 cm\(^3\)) in the mixing flask and 2 M ammonium acetate, pH 6.9 (250 cm\(^3\)) in the reservoir. The ultra-violet absorption of the effluent was monitored at 288 nm (\(\epsilon_{288} = 26\ 000\text{M}^{-1}\text{cm}^{-1}\) in H\(_2\)O (1): NH\(_4\)OAc, 2M(1)). Fractions of about 7 cm\(^3\) were collected with an automated sample collector, the folic acid typically eluting between fractions 49 - 59. The fractions were tested for radioactivity by taking 10 L aliquots, pipetting into 5 cm\(^3\) of Unisolve E scintillant and counting. The fractions containing folic acid were pooled and lyophilised until all the
ammonium acetate was removed. The residue was dissolved in water and the content of folic acid was determined spectrophotometrically. The average yield was 30 mCi of folic acid (12) of specific radioactivity 32 Ci/mmol. The folic acid (12) obtained was purged with nitrogen, sealed and stored at -18°C.

$^1$H NMR (D$_2$O, Figure 2.7(a)):- 8 8.6 (s, 1 H, H-7), 7.7 (d, 2 H, J 8.5 Hz, H-2',6'), 6.8 (d, 2 H, J 8.5 Hz, H-3',5'), 4.4 (q, 1 H, J 4.5 Hz, α-CH), 4.2 (s, 2 H, H-9), 2.1 (m, 2 H, γ-CH$_2$), 2.0 (m, 2 H, β-CH$_2$).

$^3$H NMR (D$_2$O, Figure 2.7(b)):- 8 8.7 (s, 1 T, T-7), 6.9 (s, 2 T, T-3',5'), 4.5 (s, 1 T, T-9).

Radio-tlc (Figure 2.9):- 98.5% pure.

2.2.2.4. Preparation of [7,9-$^3$H]Folic Acid (13)

Folic acid (5b, 10.0 mg, 0.023 mmol) and 5% palladium on calcium carbonate (37 mg) in 4% NaOH (0.75 cm$^3$) were stirred for 1.5 hours in the presence of tritium gas (2 Ci) on the tritium gas line. Helium gas was allowed into the reaction vessel and the reaction mixture stirred for a further one hour to ensure maximum incorporation of the tritium gas. The folic acid was then extracted from the solution following the procedure described above. After the Whatmann 3MM chromatography stage the average yield was about 60 mCi and the estimated specific activity was 10 - 20 Ci/mmol. The folic acid (13) was further purified as described earlier and the average yield was 15 - 20 mCi of folic acid (13) of specific radioactivity 5 Ci/mmol. The folic acid (13) obtained was purged with nitrogen, sealed and stored at -18°C.

$^1$H NMR (D$_2$O, Figure 2.7(a)):- 8 8.6 (s, 1 H, H-7), 7.7 (d, 2 H, J 8.5 Hz, H-2',6'), 6.8 (d, 2 H, J 8.5 Hz, H-3',5'), 4.4 (q, 1 H, J 4.5 Hz, α-CH), 4.2 (s, 2 H, H-9), 2.1 (m, 2 H, γ-CH$_2$), 2.0 (m, 2 H, β-CH$_2$).
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3H NMR (D$_2$O, Figure 2.7(c)): - $\delta$ 8.7 (s, 1 T, T-7), 4.6 (s, 1 T, T-9).

Radio-tlc: - 99.9% pure.

2.2.2.5. Preparation of [3',5',7-$^3$H]Methotrexate (14)

3',5'-Dichloromethotrexate (11, 6.5 mg, 0.012 mmol) and 5% palladium on calcium carbonate (24.3 mg) in 4% NaOH (0.75 cm$^3$) were stirred for 16 hours in the presence of tritium gas (2 Ci) on the tritium gas line. Helium gas was allowed into the reaction vessel and the reaction mixture was stirred for a further one hour to ensure maximum incorporation of tritium gas. The methotrexate was then extracted from the solution following the procedure described previously. After the Whatmann 3MM chromatography stage the average yield was about 40 mCi and estimated specific activity was 8 - 10 Ci/mmol. The methotrexate (14) was further purified as described earlier for folic acid (12) and the average yield was 5 - 10 mCi of methotrexate (14) of specific radioactivity 9 Ci/mmol. The methotrexate (14) typically elutes from the DEAE cellulose column in fractions 32 - 42. The methotrexate (14) obtained was purged with nitrogen, sealed and stored at -18°C.

$^1$H NMR (D$_2$O, Figure 2.8(a)): - $\delta$ 8.5 (s, 1 H, H-7), 7.7 (d, 2 H, J 8.9 Hz, H-2',6'), 6.9 (d, 2 H, J 9.0 Hz, H-3',5'), 4.8 (s, 2 H, H-9), 4.2 (q, 1 H, J 4.6 Hz, $\alpha$-CH), 3.1 (s, 3 H, -CH$_3$), 2.2 (m, 2 H, $\gamma$-CH$_2$), 2.0 (m, 2H, $\beta$-CH$_2$).

$^3$H NMR (D$_2$O, Figure 2.8(b)): - $\delta$ 8.7 (s, 1 T, T-7), 7.0 (s, 2 T, T-3',5').

Radio-tlc (Figure 2.10): - 96.8% pure.
2.3. RESULTS

The results (Figures 2.7 to 2.10 and Table 2.2) indicate that [3',5',7,9-^3H]folic acid (12) and [3',5',7-^3H]methotrexate (14) have been prepared by the catalytic tritium-halogen replacement of the corresponding 3',5'-dibromofolic acid (10) and 3',5'-dichloromethotrexate (11), respectively. [7,9-^3H]Folic acid (13) was prepared in solution by catalytic exchange of folic acid (5b) with tritium gas.

Figures 2.7 to 2.8 show the $^1$H and $^3$H NMR spectra of the labelled ligands. The $^3$H signals in each case have been assigned from the corresponding $^1$H spectrum. Tritium incorporation and distribution data are given in Table 2.2 and the radio-tlc data for tritium labelled folic acid (13) and methotrexate (14) are given in Figures 2.9 and 2.10.
Figure 2.6. Structures of the tritiated ligands
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Figure 2.7. Proton and tritium NMR spectra of Folic acid
Chapter 2: Labelling of Ligands

(a) $^1$H NMR spectrum of Methotrexate (8) in D$_2$O at 300 MHz

(b) $^3$H NMR spectrum ($^1$H-decoupled) of [3',5',7-$^3$H]Methotrexate (14) in D$_2$O

Figure 2.8. Proton and tritium NMR spectra of Methotrexate
Table 2.2. Patterns of Labelling, Relative Incorporation (%) and Radiochemical Purity (%) of the Ligands

<table>
<thead>
<tr>
<th>Compound (NMR Solvent)</th>
<th>Chemical Shift $\delta$ (ppm)</th>
<th>Position(s) Labelled</th>
<th>Relative Incorporation (%)</th>
<th>Purity by Radio-tlc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3',5',7,9-3H]Folate (12)</td>
<td>8.50</td>
<td>7</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>pH 7.6 (D$_2$O)</td>
<td>6.74</td>
<td>3', 5'</td>
<td>64</td>
<td>98.5</td>
</tr>
<tr>
<td>[7,9-3H]Folate (13)</td>
<td>8.57</td>
<td>7</td>
<td>42</td>
<td>99.9</td>
</tr>
<tr>
<td>pH 7.8 (D$_2$O)</td>
<td>4.52</td>
<td>9</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>[3',5',7-3H]-Methotrexate (14)</td>
<td>8.66</td>
<td>7</td>
<td>57</td>
<td>96.8</td>
</tr>
<tr>
<td>pH 7.4 (D$_2$O)</td>
<td>6.98</td>
<td>3', 5'</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.9. Radio-tlc of $^3$H Folic Acid (13)

Figure 2.10. Radio-tlc of $^3$H Methotrexate (14)
Figure 2.11. $^1$H NMR spectrum of 3',5'-Dibromofolic acid (10) in D$_2$O at 300 MHz

Figure 2.12. $^1$H NMR spectrum of 3',5'-Dichloromethotrexate (11) in D$_2$O at 300 MHz
2.4. DISCUSSION

2.4.1. Tritium NMR Spectra

The tritium NMR spectra (Figures 2.7(b) and 2.8(b)) show that catalytic halogen/tritium replacement in 3',5'-dibromofolic acid (10) and 3',5'-dichloromethotrexate (11) gave, in addition to the label at these sites a substantial amount of label at the 7-, and in the case of folic acid the 9-position, as expected. Steric hindrance of the N-10 methyl group to the binding of the methotrexate to the catalyst is the probable cause of the lack of label at the 9-position in this compound. That these positions are catalytically labile is shown by the fact that catalytic exchange in solution with tritium gas of folic acid (5b) and methotrexate (8) introduces a label into these 'non-specific' positions. Also, it has been suggested that the proportion of tritium in these general positions increases with respect to the specific positions with increasing time.

2.4.2. Variation of Catalyst

It has been reported that the use of different batches of catalyst leads to totally different patterns of labelling in folic acid and methotrexate (Lenk, 1979). Figures 2.13, 2.14 & 2.15 show the tritium NMR spectra of folic acid (12 and 13) and methotrexate (14) labelled by halogen replacement with a different batch of 5% palladium on calcium carbonate to that used in the main work. As might be predicted from a tritium labelling procedure in which two different reactions occur (i.e., hydrogen-tritium exchange and halogen-tritium replacement), the relative distribution of the tritium label can vary greatly depending on the activity of the catalyst, time of reaction, etc. Table 2.3 shows the results obtained using different batches of 5% Pd/CaCO₃ and the effect on the
distribution of the label in [3',5',7,9-^3H]folic acid (12), [7,9-^3H]folic acid (13) and
[3',5',7-^3H]methotrexate (14) with the reaction time being kept constant.

Table 2.3. Distribution of tritium label as a function of catalyst batch

<table>
<thead>
<tr>
<th>Compound</th>
<th>Batch No.</th>
<th>Age of 5% Pd/CaCO₃ NMR Spectroscopy (%)</th>
<th>Distribution of Label by Tritium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(years)</td>
<td>3', 5'</td>
</tr>
<tr>
<td>[3',5',7,9-^3H]Folic acid</td>
<td>1</td>
<td>New batch</td>
<td>64</td>
</tr>
<tr>
<td>(12)</td>
<td>2</td>
<td>1</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>[7,9-^3H]Folic acid</td>
<td>1</td>
<td>New batch</td>
<td>42</td>
</tr>
<tr>
<td>(13)</td>
<td>2</td>
<td>1</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>[3',5',7-^3H]Methotrexate</td>
<td>1</td>
<td>New batch</td>
<td>43</td>
</tr>
<tr>
<td>(14)</td>
<td>2</td>
<td>1</td>
<td>70</td>
</tr>
</tbody>
</table>

The tritium NMR spectra and the data detailed in Table 2.3. show that the greatest variation in incorporation of the label occurs at the 7- and 9-positions suggesting that these sites are very sensitive to changes in catalyst morphology. Heterogeneous catalysts are very ill-defined materials, the active sites often being associated with surface irregularities. A larger molecule, such as folic acid has fewer degrees of freedom in adsorption onto these active sites which must have just the right geometry. Hence, when labelling folic acid (5b) and methotrexate (8) to high specific activities it is a wise precaution to ensure that the catalyst gives the desired labelling pattern. For the present
study the catalyst was chosen to give good incorporation into the 3',5'-positions (64%) whilst ensuring a reasonable quantity in the 7-position of $[3',5',7,9-^{3}\text{H}]$folic acid (12). This enabled the signals from the 3',5'- and 7-positions to be readily observed in the tritium NMR binding studies, whereas for $[7,9-^{3}\text{H}]$folic acid (13) it was necessary to maintain a balance between incorporation into the 7- and 9-positions. As very little is known about the binding of the H-9 region, the $^{3}\text{H}$ incorporation at this site needed to be higher than at the 7-position. This would ensure a good signal to noise ratio in the $^{3}\text{H}$ NMR spectrum of the enzyme-folate-NADP$^{+}$ complex, in this region of interest. For $[3',5',7-^{3}\text{H}]$methotrexate (14) the area of interest is the benzoyl ring. Previous $^{1}\text{H}$ NMR studies of methotrexate complexes with dihydrofolate reductase have never been able to detect signals for the 3',5'-protons for either the binary or ternary complexes, therefore maximum incorporation of the label into the 3',5'-positions was desirable to increase the chances of observing the tritium signals, if possible, in the $^{3}\text{H}$ NMR spectrum. The maximum level of incorporation into these positions with any batch of catalyst was 57%.
Figure 2.13. $^3$H NMR spectrum ($^1$H-decoupled) of [3',5',7-$^3$H]folic acid (12) prepared with 5% Pd/CaCO$_3$ (batch 3, five years old)

Figure 2.14. $^3$H NMR spectrum ($^1$H-decoupled) of [7,9-$^3$H]folic acid (13) prepared with 5% Pd/CaCO$_3$ (batch 2, one year old)

Figure 2.15. $^3$H NMR spectrum ($^1$H-decoupled) of [3',5',7-$^3$H]methotrexate (14) prepared with 5% Pd/CaCO$_3$ (batch 2, one year old)
2.4.3. Decomposition of $^{3}$H-Folic Acid

All radiochemicals are prone to decomposition - tritium compounds more so than carbon-14 (Evans, 1984). In order to assess the stability of a sample of $[3',5',7,9-^{3}$H]folic acid (specific activity 30 Ci/mmol) under the conditions employed for storage of the DHFR-$[^{3}$H]folate-NADP$^{+}$ complex between overnight $^{3}$H NMR experiments, the tritium NMR spectra of the sample were obtained after 2, 7 and 14 days (Figure 2.16). The sample was stored in a sealed NMR tube at +4°C. The $^{3}$H NMR spectra show the appearance and growth of three new signals as the storage time increases. This suggests that decomposition has occurred via cleavage of the C-9, N-10 bond to give the pteridine (15) and $p$-aminobenzoylglutamic acid (16) moieties. The chemical shift of the postulated pteridine fragment ($\delta$ 8.95 ppm) compares favourably with that reported for the 7-tritium in [7-$^{3}$H]pterin-6-carboxylic acid (Lenk, 1979). The possible structures for the decomposition products are given in Figure 2.17.

![Figure 2.17. Structures of possible decomposition products]

The Y group arises from the solvent (D$_{2}$O) addition to the appropriate radical produced by radiation-induced bond cleavage.
Figure 2.16. $^3$H NMR spectra ($^1$H-decoupled) of $[3',5',7,9,^3$H]Folate (12) in $D_2O$ obtained following storage at $4^\circ$C for (a) two days, (b) seven days & (c) 14 days.
Figure 2.18. Radioactivity profile of $[3',5',7,9-^3\text{H}]$folate (12) from an analytical column of DEAE-cellulose. Decomposition products formed on storing at 4°C for 14 days.

In order to assist in the determination of the tritiated contaminants present in the 14 day old sample of $^3\text{H}$ folic acid (12), analytical column chromatography was carried out. The DEAE-cellulose column was prepared in the same way as previously described. Elution was accomplished with a linear gradient of 250 cm$^3$ of water in the mixing flask and 250 cm$^3$ of 2M ammonium acetate (pH 6.9) in the reservoir. Fractions of 7 cm$^3$ were collected at a flow rate of 0.8 cm$^3$/min. Figure 2.18. shows the radioactivity profile of the fractions as they emerged from the column. 10 μL Aliquots of the eluent were added to 4 cm$^3$ portions of Unisolve E liquid scintillant and counted. Three radioactivity peaks are noted. The folic acid peak (peak I) is known to elute from the column in fractions 45 - 55, while labelled decomposition products were present in fractions 20 - 22 (peak III) and 27 - 29 (peak II). The latter components comprised 4.8 and 9.4% respectively. It is thought that peak II corresponds to tritiated $p$-aminobenzoylglutamic acid (16) as identified by Zakrzewski et al. (1970) and peak III could be due to the other postulated decomposition product, $[7-^3\text{H}]$pterin-6-carboxylic acid (15) (Zakrzewski, 1966). In this particular case, decomposition has been accelerated by the relatively high specific activity of the sample (30 Ci/mmol) and the storage temperature (+4°C).
2.5. CONCLUSIONS

Heterogeneous exchange labelling methods are often regarded as irreproducible and this may be as a result of the variety of destructive techniques formally used for analysis of the reaction products. It is clear that the direct observation of labelled positions through the application of tritium NMR spectroscopy is a much more satisfactory approach (Evans et al., 1985; Jaiswal et al., 1992).

It has been shown by tritium NMR spectroscopy that folic acid (5b) and methotrexate (8) can be selectively labelled with tritium. Through careful choice of experimental procedure and catalyst batch, it has been possible to incorporate tritium labels into positions in the ligands pertinent to studies of the binding in ligand-DHFR complexes. Preparation of [3',5',7,9-^3H]folic acid (12) and [3',5',7-^3H]methotrexate (14) was achieved by catalytic halogen/tritium replacement in 3',5'-dibromofolic acid (10) and 3',5'-dichloromethotrexate (11), respectively, and incorporation of the label into the 7- and 9-positions exclusively of folic acid (13) was achieved by catalytic H2/T2 exchange of folic acid (5b) in solution with tritium gas. The present results compare favourably with previous work (Evans et al., 1979), even though some of the experimental procedures have been modified.

For the present work, it was important to achieve selective labelling of the ligand chosen. For methotrexate (8), ^3H labelling was needed at the pteridine H-7 and benzoyl 3',5'-positions. Although 'ring flipping' of the benzoyl ring has been characterised in the enzyme complex with 3',5'-difluoromethotrexate, it has proved impossible to obtain similar information for the parent compound because the 2',3',5' & 6' proton signals of bound methotrexate have never been detected directly in 1H spectra (this results from a combination of broad methotrexate 1H signals and the presence of overlapping protein signals). The tritium labelled compound offers a means of directly observing nuclei at these positions and of studying their line shape changes as a function of temperature.
without the usual interference from overlapping signals. Such measurements will allow the measurement of the rate of ring-flipping of the benzoyl ring within the protein-ligand complex. The $^3$H chemical shifts of the benzoyl ring protons of bound methotrexate will aid in assessing whether or not the ring is occupying the same binding site as does the benzoyl ring of folate where the $^1$H chemical shift data are already known for the benzoyl ring protons in the bound ligand.

Folate (5) has been labelled at the same positions as those for methotrexate (8) and a parallel set of NMR experiments will be carried out aimed at the problems outlined above. The results will provide the basis for interesting structural comparisons between the substrate and inhibitor binary and ternary complexes.

In order to monitor the multiple conformations (seen in slow exchange) for the DHFR-folate-NADP$^+$ complex by tritium NMR, folic acid has been labelled in the 3', 5', 7-positions and 7', 9-positions. Selective labelling of [7,9-$^3$H]folic acid (13) will allow the binding of the H-9 region to be studied, as very little information is available on this area of the molecule, from previous studies.

The results presented in this chapter clearly show that the pattern of labelling obtained is crucially dependent upon the batch of catalyst employed in the labelling procedure. Hence, care must be taken to ensure that the catalyst chosen gives the desired labelling patterns for the relevant studies, as discussed previously for folate and methotrexate.

Furthermore, when undertaking the $^3$H NMR binding studies, the decomposition of $^3$H-folic acid must be considered. The DHFR-[3$^H$]-folate-NADP$^+$ samples will be stored in D$_2$O, in a sealed NMR tube at 4°C, during the day prior to pH change and the series of NMR experiments over consecutive nights. The $^3$H NMR results shown in Figure 2.16 indicate that [3',5',7,9-$^3$H]folate (12) undergoes decomposition over a period of 14 days, when stored at 4°C in a sealed NMR tube. It will be important to note the positions, in the NMR spectrum, of the signals arising from the decomposition products,
to ensure that these signals are not confused with those resulting from the different conformations observed for bound $^3$H-folate in the DHFR-[3',5',7,9-$^3$H]folate-NADP$^+$ ternary complex.

Clearly tritium NMR spectroscopy provides a relatively cheap and convenient technique not only of determining the proportion of tritium in the labelled positions, but is also an excellent method for monitoring decomposition of the labelled ligands.
Tritium NMR Studies of Folate and Methotrexate Binding to *Lactobacillus casei* Dihydrofolate Reductase

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3. TRITIUM NMR STUDIES OF FOLATE AND METHOTREXATE BINDING TO *LACTOBACILLUS CASEI* DIHYDROFOLATE REDUCTASE

3.1. INTRODUCTION

The enzyme dihydrofolate reductase (DHFR) has become a well-established target for drug action since it was identified about 30 years ago. Clinically useful drugs whose activity originates from DHFR inhibition include the antibacterial agent trimethoprim (9), and methotrexate (MTX, 8) which has extensive use as an antineoplastic agent. The pharmacological importance of DHFR together with its conveniently small size (M$_r$ 18 - 25 000 in most species) has led to intensive studies of the enzyme over the last 10 - 15 years.

The literature covering DHFR-related research is extensive and there have been a number of excellent reviews (Blakley, 1984; Champness et al., 1986; Kraut & Matthews, 1987) recently published. Considerable effort has gone into structural studies involving the enzyme from various sources and high-resolution crystal structures have been determined for DHFR from *Lactobacillus casei* (*L. casei*) and other organisms in a number of ligand complexes (Matthews et al., 1978; Baker et al., 1983; Bolin et al., 1982; Filman et al., 1982). In the NMR spectrum, a substantial number of the $^1$H resonances of *L. casei* DHFR have been assigned (Hammond et al., 1986; Carr et al., 1991; Soteriou et al., 1993), and in addition resonances of bound ligands have been used to characterise their modes of binding (Birdsall et al., 1982, 1983; Feeney et al., 1983, 1991; Cheung et al., 1993). A number of studies of DHFR by site-directed mutagenesis have been reported (Birdsall et al., 1989a; Andrews et al., 1991), while an enormous range of DHFR inhibitors has been synthesised over the last 40 years (Roth & Cheng, 1982). This considerable background of information has helped provide an improved
understanding of the factors which control the specificity of ligand binding and hence, the insight necessary for the rational design of novel ligands.

Much of the work has been directed at defining the conformations of the bound inhibitors and in characterising specific interactions between the ligands and the protein. The techniques available for addressing these questions; mutagenesis, NMR and kinetic studies of the protein are all well developed and documented. The information and understanding available thus make this an ideal system in which to investigate the molecular basis of the specificity of protein-ligand interactions.

The aim of this chapter is to present information relating to substrate, inhibitor and coenzyme binding to *L. casei* DHFR, with particular regard to using selectively tritiated ligands in conjunction with tritium NMR spectroscopy. Firstly, the relevant background information is reviewed. This is followed by $^3$H NMR studies of $[3',5',7-^3H]$folate, $[7,9-^3H]$folate and $[3',5',7-^3H]$methotrexate binding to *L. casei* DHFR, with NADP$^+$ and NADPH as coenzyme. These experiments were conducted as a function of pH and temperature. Finally, the results are explained in relation to understanding the molecular factors controlling the specificity of ligand binding which involves (i) characterising the specific interactions between groups on the ligand and protein, (ii) characterising the conformational changes accompanying ligand binding, (iii) measuring the conformational equilibria in the various complexes and (iv) monitoring the changes in intramolecular dynamic processes.
3.2. BACKGROUND INFORMATION ON DHFR

3.2.1. Role in Cellular Metabolism

Dihydrofolate reductase is a widely distributed enzyme which catalyses the reduction of 7,8-dihydrofolate (FH$_2$, 6) to 5,6,7,8-tetrahydrofolate (FH$_4$, 7) using nicotinamide adenine dinucleotide phosphate (NADPH, 17) as the coenzyme, as follows:

\[
\text{NADPH} + \text{H}^+ + \text{H}_2\text{folate} \rightarrow \text{NADP}^+ + \text{H}_4\text{folate}
\]

The enzyme from some sources, also catalyses a much slower reduction of folate (5):

\[
2\text{NADPH} + 2\text{H}^+ + \text{folate} \rightarrow 2\text{NADP}^+ + \text{H}_4\text{folate}
\]

The product of this reduction, tetrahydrofolate is used in a number of important biosynthetic pathways, in which it takes part in a shuttle mechanism, alternately accepting and donating various one-carbon functionalities (Hitchings, 1983). For example, in the biosynthesis of methionine, homocysteine is methylated using the methyl group of 10-methylFH$_4$.

\[\text{NADPH (17)}\]

Figure 3.1. Schematic representation of NADPH
The fate of FH₄ is different in the biosynthesis of thymidylate as shown in Figure 3.2. The 5,10-methylene derivative of FH₄ acts as a cofactor for thymidylate synthase in its methylation of deoxyuridylate. The methyl group of thymidylate is formed from the 5,10-methylene moiety of the cofactor, along with the hydrogen at C-6, FH₂ being produced in the process. This enzyme is unique in that it represents the sole de novo path for thymidylate synthesis and is the only enzyme that uses a cofactor in which the H₄folate is oxidised during one-carbon transfer. Thymidylate is an essential building block for DNA. Inhibition of DHFR can thus lead to disruption of DNA synthesis and death in rapidly proliferating cells. This property has made DHFR such an important clinical target, especially in the treatment of certain bacterial infections and cancers.

![Figure 3.2. Schematic representation of the role of dihydrofolate reductase in the biosynthesis of thymidylate](image-url)
3.2.2. Structural Studies

The structure of DHFR isolated from various sources has been the subject of such intensive study over the last 20 years that DHFR is rapidly becoming one of the best understood enzyme structures. Many tools have been used in elucidating the structure of DHFR, with the greatest advances in insight resulting from X-ray crystallography, sequencing, and more recently, NMR studies.

3.2.2.1. X-Ray Crystallographic Studies

3.2.2.1.1. Primary Sequence of DHFR

The amino acid sequences of many different species of DHFR are known (Champness et al., 1986); a representative set is shown in Table 3.1. It can be seen that there is greater homology between DHFRs from vertebrate sources (75 - 90%) than between bacterial DHFRs (25 - 40%). The similarity between animal and bacterial DHFR is 20 - 30% and enzyme from animals is generally about 25 residues longer than the bacterial proteins.

Crystallographic studies (Freisheim & Matthews, 1984) of the enzyme architecture have revealed a conservation of three dimensional structure far more pronounced than the extent of residue identity between amino acid sequences. Kraut and Matthews (1987) have shown that in all cases the substrate binding cleft is highly conserved: linear alignment of DHFR from 11 different sources shows that 12 residues are strictly conserved, and each of them is located in or near the active site. It would appear that the insertion/deletion differences between the enzymes have little effect on the overall geometry of their structure.
<table>
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<tr>
<td>Table 3.1. Amino Acid Sequences of DHFR from Selected Organisms$^a,b$</td>
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</table>

| Cl  | 1V 2R 3S 4L 5I 6I 7G 8I 9A 10Q 11M 12J 13N 14M 15N 16I 17G 18K 19F 20N 21G 22G 23P 24G 25P 26G |
| Mo  | 1V 2R 3S 4L 5I 6I 7G 8I 9A 10Q 11M 12I 13N 14M 15N 16I 17G 18K 19F 20N 21G 22G 23P 24G 25P 26G |
| Ec  | 28L 29A 30W 31P 32K 33R 34N 35T 36P 37N 38K 39D 40V 41I 42M 43G 44R 45H 46T 47W 48E |
| Lc  | 27L 28H 29Y 30P 31A 32Q 33Q 34V 35V 36Q 37E 38Q 39S 40T 41S 42R 43V 44E 45G 46K 47Q 48N 49A 50V 51I 52M 53G 54K 55K 56N 57W 58F |
| Cl  | 35Y 32K 33Y 34P 35Q 36R 37M 38T 39S 40T 41S 42K 43V 44E 45G 46K 47Q 48N 49A 50V 51I 52M 53G 54K 55K 56N 57W 58F |
| Hu  | 31F 32R 33Y 34P 35Q 36R 37M 38T 39S 40T 41S 42R 43V 44E 45G 46K 47Q 48N 49A 50V 51I 52M 53G 54K 55K 56N 57W 58F |
| Mo  | 31F 32K 33Y 34P 35Q 36R 37M 38T 39S 40T 41S 42R 43V 44E 45G 46K 47Q 48N 49A 50V 51I 52M 53G 54K 55K 56N 57W 58F |

continued
Chapter 3: Tritium NMR Binding Studies

| Ec  | 111_Y 112_L 113_T 114_H 115_I 116_D 117_A 118_Q 119_V 120_E 121_G 122_D 123_T 124_H 125_P 126_T 127_D 128_Y 129_E 130_P 131_D 132_D |
| Lc  | 114_L 115_V 116_T 117_R 118_L 119_A 120_G 121_S 122_R 123_E 124_G 125_P 126_T 127_K 128_W 129_T 130_P 131_L 132_N 133_I 134_D 135_D |
| Cl  | 134_F 135_V 136_T 137_R 138_I 139_L 140_H 141_E 142_P 143_E 144_S 145_P 146_T 147_F 148_F 149_P 150_E 151_I 152_D 153_Y 154_K 155_D |
| Hu  | 134_F 135_V 136_T 137_R 138_I 139_K 140_Q 141_D 142_P 143_E 144_S 145_P 146_T 147_F 148_F 149_P 150_E 151_I 152_D 153_L 154_E 155_K |
| Mo  | 134_F 135_V 136_T 137_R 138_I 139_K 140_Q 141_D 142_P 143_E 144_S 145_P 146_T 147_F 148_F 149_P 150_E 151_I 152_D 153_L 154_E 155_K |

| Ec  | 133_W 134_E 135_S 136_V 137_F 138_S |
| Lc  | 136_F 137_T 138_K 139_Y 140_S 141_S |
| Cl  | 156_P 157_K 158_L 159_L 160_T 161_E 162_Y 163_P 164_G 165_V 166_P 167_A 168_D 169_I 170_Q 171_E 172_E 173_D 174_G |

Ec = E. coli (Buccinari et al., 1982); Lc = L. casei (Bittar et al., 1977); Cl = chicken liver (Kumar et al., 1980); Hu = human (Masters & Attardi, 1983); Mo = mouse (Stone et al., 1979). A-Alanine, R-Arginine, N-Asparagine, D-Aspartic Acid, C-Cysteine, Q-Glutamine, E-Glutamic Acid, G-Glycine, H-Histidine, I-Isoleucine, L-Leucine, K-Lysine, M-Methionine, F-Phenylalanine, P-Proline, S-Serine, T-Threonine, W-Tryptophan, Y-Tryptosine, V-Valine.

Sequence alignment for Ec, Lc and Cl enzymes follows that of Volz et al. (1982), which was based on the corresponding three-dimensional structures. Alignment of the Hu and Mo sequences were based on maximal homology.

Table 3.1. Amino Acid Sequences of DHFR from Selected Organisms

| Ec  | 149_H 150_S 151_Y 152_C 153_F 154_E 155_I 156_L 157_E 158_R 159_R |
| Lc  | 151_L 152_T 153_H 154_T 155_Y 156_E 157_W 158_Q 160_K 161_K 162_A |
| Cl  | 175_I 176_Q 177_Y 178_K 179_F 180_E 181_Y 182_Y 183_R 184_K 185_S 186_E 187_L 188_A 189_Q |
| Hu  | 175_I 176_K 177_Y 178_K 179_F 180_E 181_Y 182_Y 183_R 184_K 185_S 186_E 187_L 188_A 189_Q |
| Mo  | 175_I 176_K 177_Y 178_K 179_F 180_E 181_Y 182_Y 183_R 184_K 185_S 186_E 187_L 188_A 189_Q |
3.2.2.1.2. Three Dimensional Structure

The first X-ray diffraction investigation of DHFR was carried out on crystals of the Escherichia coli (E. coli) enzyme complexed with MTX (Matthews et al., 1977); this was followed shortly by a study of the ternary complex of L. casei DHFR containing bound NADPH and MTX (Matthews et al., 1978, 1979). The structures originally deduced have subsequently been refined against 1.7 Å diffraction data (Bolin et al., 1982; Filman et al., 1982). A schematic representation of the polypeptide backbone folding for the L. casei DHFR-methotrexate-NADPH ternary complex is shown in Figure 3.3. The dominant feature of the DHFR structure is a central eight-stranded β-sheet flanked on either side by alpha helices. These strands have been designated by Matthews et al. as βA to βH, βA being nearest the amino terminus and βH nearest the carboxyl terminus, the alpha helices are assigned αB, αC, αE, αF, the letter designating the β-strand that follows the α-helix in the primary sequence. The remainder of the residues consist of loops, some quite large, that join the elements of secondary structure.

The enzyme surface is marked by a large cavity, 15 Å deep, cutting across one face, between the B and C helices. This cavity is known to be the active site of binding and is lined by hydrophobic side chains, indicating the importance of hydrophobic and van der Waals interactions in the binding of substrates and inhibitors. The only polar side chain in this cavity is Asp-26, which has been shown by mutagenesis experiments (Jimenez et al., 1989) to participate in inhibitor and substrate binding. The cofactor, NADPH, occupies the lower half of the active site, with its nicotinamide ring presumably positioned for hydride transfer to FH₂ in the catalysed reduction (Filman et al., 1982). In its extended conformation, the cofactor is bound with the diphosphate bridge between the N-termini of helices C and F near the lower edge of the β-sheet and with the adenosine moiety on the opposite side of the sheet.
Figure 3.3. Backbone ribbon drawing of *L. casei* DHFR-methotrexate-NADPH ternary complex. β-Strands (represented by arrows) and α-helices are labelled. The approximate position of every tenth residue is indicated (Bolin *et al.*, 1982)
Methotrexate is bound in the upper part of the cleft in much the same manner reported for folic acid (Oefner et al., 1988). This is not surprising considering that methotrexate is a close structural analogue of folic acid. The $p$-aminobenzoylglutamate portions of the two ligands interact similarly with DHFR, and the pteridine ring system of each compound binds in the same region of the protein. However, the pteridine rings are flipped approximately 180° about the C-2 to 2-amino bond with respect to one another (Bolin et al., 1982; Charlton et al., 1985). The differences in capability of the two ligands ($4$-oxo vs $4$-amino) for hydrogen-bonding is thought to explain these differences in their modes of binding, coupled with the observation that, unlike folate or FH$_2$, the N-1 atom of methotrexate is protonated in its complex with DHFR and can interact ionically with an active site carboxyl group e.g., a side chain from an aspartate or a glutamate.

X-Ray crystallographic data (Filman et al., 1982) indicate that methotrexate binds to bacterial enzymes in an open conformation with the pteridine ring nearly perpendicular to the aromatic ring of its $p$-aminobenzoyl group; the overall conformation is similar in both the binary and ternary complexes (Matthews et al., 1978). The pyrimidine edge of the pteridine ring is deeply buried in the active site cavity, but the $p$-aminobenzoylglutamate side chain extends out of the cavity to the surface, and the glutamate moiety is draped across $\alpha B$. The $\alpha$-carboxylate is positioned to interact ionically with an invariant arginine residue, Arg-57, through a pair of hydrogen bonds. In the *L. casei* enzyme the inhibitor's $\gamma$-carboxylate is within hydrogen bonding distance of His-28 (Bolin et al., 1982), and nuclear magnetic resonance data are consistent with this interaction (Antonjuk et al., 1984).

The pteridine ring of methotrexate makes extensive non polar contacts with protein backbone and side chain atoms of $\beta A$, the loop $\beta A \rightarrow \alpha B$, and $\alpha B$. In the *L. casei* DHFR-MTX-NADPH ternary complex (Figure 3.4) additional van der Waals contact with the pteridine ring are made by the side chain of Leu-19 and the nicotinamide
The pteridine ring of MTX is protonated at N-1 (Cocco et al., 1983) and forms an ionic bond with the carboxyl group of Asp-26. The carboxyl group is nearly coplanar with the pteridine ring and this interaction is mediated through hydrogen bonds donated by the N-1 hydrogen and a hydrogen of the 2-amino group. The second hydrogen of the 2-amino group associates with a fixed water molecule, which in turn is hydrogen bonded to the side chain hydroxyl of Thr-116. This threonine is strictly conserved in all species of DHFR, and the bridging water molecule has also been observed in every refined DHFR structure studied to date. Therefore, it is a reasonable assumption that this interaction is an important structural component for substrate binding. Besides N-1, the
only other endocyclic ring nitrogen involved in hydrogen bonding is N-8. There is a structurally conserved fixed water molecule, water-253, 3.3 Å from N-8, lying directly in line with its lone pair orbital. In turn, this water molecule is hydrogen bonded to Asp-26, Trp-21 and to another fixed water molecule. The 4-amino group of methotrexate is hydrogen bonded to backbone carbonyl oxygens of Leu-4 and Ala-97 (Bolin et al., 1982).

3.2.2.2. Nuclear Magnetic Resonance Studies

The power of NMR spectroscopy as a tool for the study of protein-ligand interactions arises first from the fact that the spectrum contains at least one signal from each amino acid residue in the protein, and secondly from the considerable sensitivity of the spectral parameters, such as chemical shift, to molecular interactions and conformation. However, there are some disadvantages of using this particular technique. The proton NMR spectra from the protein are very complex because of the many atoms involved, and it is usually difficult to resolve the resonances of more than a small fraction of the amino acid residues, though this problem can be partially overcome by various methods of spectral simplification, notably selective deuteration. While X-ray crystallographic studies, as discussed previously, provide very detailed structural information they do require the samples to be studied as crystals. NMR has the advantage that it examines the complexes in solution and does not require crystals. However, the availability of X-ray structural data on some complexes is often crucial for achieving a detailed interpretation of the NMR data obtained either on the same or on related complexes for which no X-ray structural data are yet available. In addition to the studies aimed at understanding the specificity of ligand binding, there have been several efforts (Howell et al., 1986; Andrews et al., 1989) to determine the details of the enzyme mechanism, the understanding of which is still not complete.
Feeney and co-workers (Way et al., 1975; Feeney et al., 1981; Birdsall et al., 1982, 1987; Clore et al., 1984; Charlton et al., 1985; Searle et al., 1986; Cheung et al., 1993) have employed NMR spectroscopy extensively to study complexes of \( L.\) casei DHFR with various substrates and inhibitors in order to obtain detailed information about the protein-ligand binding in these complexes. Such investigations contribute to the understanding of both binding specificity and catalytic mechanism by providing information about individual interactions between groups on the ligand and on the protein and by characterising the conformational changes in the ligand and protein which accompany ligand binding. For complexes where multiconformational forms are present, the conformational equilibria in these complexes may be measured and changes in intra- and inter-molecular dynamic processes observed on complex formation (such as rates of flipping in bound inhibitors e.g., methotrexate (Clore et al., 1984) and trimethoprim (Cheung et al., 1986)) can then be defined.

Because of the rapid developments in methods of assigning resonances in complex protein spectra and in methods of interpreting NOE data in structural terms it seems likely that NMR will become increasingly used for providing detailed conformational information in the solution state.

### 3.2.2.2.1. Assignment of Protein Signals

In order to obtain detailed information from NMR spectra of protein complexes it is first necessary to make specific resonance assignments for the ligand and protein signals. Ligand resonance assignments are relatively easy to achieve either directly by using selective isotopic labelling \((^{13}\text{C}, \, ^{15}\text{N} \text{ or } ^2\text{H})\) (Way et al., 1975; Cheung et al., 1993) or indirectly from transfer of saturation or 2D exchange experiments which connect signals in bound and free ligand species (Hyde et al., 1980 a, b).
Until recently it was much more difficult to obtain protein resonance assignments for moderately sized proteins. Early assignment studies on *L. casei* DHFR relied on correlating X-ray structural data with NOE data from protons in close proximity, thus assuming that the crystal and solution conformations are similar. Birdsall et al. (1977a) showed that it was possible to resolve the C-2 peaks of the histidine imidazole rings in DHFR, by $^{1}$H NMR spectroscopy at 100 MHz, and the effects of pH and the binding of substrates and inhibitors on the chemical shifts of the C-2 protons were reported. It was shown that the C-2 proton resonances of four of the histidine residues were perturbed by the binding of substrates or inhibitors. Matthews (1979) proposed assignments for the six histidine residues observed by Birdsall and colleagues (1977a), based on the three-dimensional structure of the ternary complex (Matthews *et al.*, 1978, 1979) and the chemical shift changes produced by the formation of the binary substrate or inhibitor complexes (Birdsall *et al.*, 1977a). Subsequent determination of the amino acid sequence of the enzyme (Freisheim *et al.*, 1978) showed that it contained seven histidine residues and later NMR studies at 270 MHz (Wyeth *et al.*, 1980) described the identification of the seventh resonance at very low field in the enzyme-methotrexate and enzyme-trimethoprim complexes.

In order to examine other aromatic residues it was necessary to resort to isotopic incorporation experiments to allow the signals for the individual amino acids to be resolved. For example, London and co-workers (1979) incorporated [γ-$^{13}$C]tryptophan into dihydrofolate reductase from *Streptococcus faecium* and detected the $^{13}$C resonances from tryptophans in the labelled enzyme. Several workers (Kimber *et al.*, 1978; Feeney *et al.*, 1980; Searle *et al.*, 1986) have shown how protein $^{1}$H NMR spectra can be simplified by selective deuteration, and this method has been used to study the five tyrosine residues in *L. casei* DHFR (Feeney *et al.*, 1977a).
In this study, a selectively deuterated enzyme containing deuterated phenylalanine, tryptophan and histidine and \([3,5-^2\text{H}_2]\)tyrosine gave a \(^1\text{H}\) NMR spectrum in which the only signals in the aromatic region were those of the 2,6-protons of the five tyrosine residues, and the effects of ligand binding on individual tyrosines were then studied in detail. As shown in Figure 3.5., replacement of all the aromatic protons except the 2,6-protons of the tyrosine residues in DHFR, leads to a dramatic simplification of the aromatic region of the \(^1\text{H}\) NMR spectrum. The overlapping absorption bands of 91 aromatic protons are now reduced to resonances from 10 protons (two from each tyrosine residue).

In subsequent work, Feeney et al. (1980a) reported how tryptophan residues could be monitored in a similar manner by examining a sample of dihydrofolate reductase in which all the aromatic protons except the C-2 protons of tryptophan were replaced by deuterium; this enabled the signals from the four tryptophan residues to be detected directly.
Another approach leading to the resolution of resonances from individual amino acid residues is to introduce a fluorine atom ($^{19}\text{F}$) into the system as an NMR probe, either by using fluorine containing ligands or by the incorporation of fluorine labelled amino acids into the protein (Kimber et al., 1977). Feeney and co-workers characterised the effects of ligand binding on individual tyrosine and tryptophan residues in the protein by measuring the $^{19}\text{F}$ spectra at 94.1 MHz of *L. casei* DHFR containing either 3-fluorotyrosine or 6-fluorotryptophan (Kimber et al., 1978). Although this procedure provided a convenient and sensitive method for monitoring the effects of ligand binding on tryptophan and tyrosine residues, there was always the possibility that the introduction of fluorinated residues could perturb the enzyme structure and its interactions with the ligands. Moreover, the selective deuteration approach has the important advantage that it is unlikely to cause any problems of this type.

More recent studies have taken advantage of the increasingly powerful NMR spectrometers available. Hammond and co-workers (1986) studied *Lactobacillus casei* dihydrofolate reductase in solution by one- and two-dimensional $^1\text{H}$ NMR spectroscopy at 500 MHz. By using a combination of NMR methods in conjunction with the refined crystal structure of the enzyme-methotrexate-NADPH complex (Bolin et al., 1982; Filman et al., 1982), resonances were assigned for 32 of the 162 residues of the enzyme. These were widely distributed through the structure of the protein and included all the histidine and tyrosine residues, as well as several valine, leucine, isoleucine and phenylalanine residues. The general procedure adopted by Hammond and co-workers for making $^1\text{H}$ NMR resonance assignments consisted of three stages. First, resonances connected by spin-spin coupling (and hence arising from the same residue) were identified by spin-decoupling or COSY experiments. In some cases it was possible to identify the type of residue using the pattern of spin-spin connectivities, although selective deuteration (Birdsall et al., 1984) was used to provide unambiguous identification. Secondly, the resonances from protons that were close together in space
(within about 5 Å) were identified by one- and two-dimensional nuclear Overhauser enhancement (NOE) experiments. Selective deuteration again proved valuable in ensuring the necessary selectivity, particularly in the one-dimensional experiments (Birdsall et al., 1984). Finally, the crystal structure was searched for assignments consistent with all data. For resonances having chemical shifts substantially different from the "random coil" values, calculations from the crystal structure of the effects of the magnetic anisotropy of aromatic rings on the chemical shifts were used as an additional constraint on possible assignments. Both the NOE-derived proximity relationships and the "ring current" shift calculations were used only in a semi-quantitative way, in view of the limitations to the precision of the NOE measurements and the accuracy of the shift calculations.

*Lactobacillus casei* DHFR has 24 aromatic residues (seven His, five Tyr, eight Phe and four Trp). At least four of these (His-28, Phe-30, Phe-49 and Trp-21) are directly involved in the active site and others, such as His-22 are nearby. As a follow up to their previous studies, the Mill Hill group reported the complete assignment of the resonances of these residues for the *L. casei* DHFR-methotrexate complex (Birdsall et al., 1990), using a combination of selective deuteration and 2D-NMR techniques at 600 MHz, similar to those previously employed. These methods relied upon the assumption that the crystal and solution structures are similar. At that time, the sequential assignment method, which is based on correlating NOE data with sequence information (Wuthrich, 1986) could not easily be applied to proteins the size of DHFR (162 residues) due to severe $^1$H chemical degeneracy.

The introduction of methods involving $^{15}$N and $^{13}$C isotope editing of $^1$H spectra provided new possibilities for assigning $^1$H spectra of larger proteins. Over the last few years several relayed 2D and 3D $^{15}$N/$^1$H and $^{13}$C/$^1$H NMR experiments have been developed and proved to be useful for assigning $^1$H spectra of isotopically labelled proteins comparable in size to DHFR (Driscoll et al., 1990). These 3D heteronuclear
NMR techniques have been applied to *L. casei* dihydrofolate reductase, in order to make sequential $^1H$, $^{15}N$ and $^{13}C$ resonance assignments for most of the protein residues (Frenkel *et al*., 1990; Carr *et al*., 1991, Soteriou *et al*., 1993). A uniformly $^{15}N$-labelled sample of *L. casei* DHFR was prepared and subjected to NOESY-HMQC and HOHAHA-HMQC 3D experiments (HMQC is a heteronuclear multiple quantum coherence experiment which detects protons directly bonded to $^{15}N$ and characterises them by their appropriate $^{15}N$ chemical shift). These experiments gave the NOESY and HOHAHA crosspeaks involving the relevant NH protons edited in slices according to the $^{15}N$ chemical shifts of each particular amide nitrogen (Carr *et al*., 1991).

By applying these various methods to the DHFR-MTX complex backbone resonance assignments were obtained for 147 of the 162 residues in DHFR and side-chain assignments for more than 80 residues (Carr *et al*., 1991). Later experiments using HCCH-COSY and HCCH-TOCSY methods on a $^{15}N/^{13}C$ labelled sample of *L. casei* DHFR gave essentially all the back-bone and side chain assignments (Soteriou *et al*., 1993). It was revealed, by an analysis of the data (NOE patterns and non-exchanging NH protons) that the β-sheet and most of the helical secondary structure seen in the crystal is essentially retained intact in solution. It was also shown that assignments made previously using the crystal structure/NOE data correlation method were all in agreement with those obtained using the sequential assignment method. Sequence-specific assignments for the γ-, δ- and δ'- protons of the 13 leucine residues in DHFR-MTX were obtained by correlating NOE and crystal structure data (Hammond *et al*., 1986; Carr *et al*., 1991). In addition, this approach was used to make stereospecific assignments for the methyl resonances from 12 of the 13 leucine residues (confirmed later by examining selectively deuterated DHFR incorporating (2S,4R)[5,5,5-$^3$H$_3$]leucine (Ostler *et al*., 1993)). Soteriou and coworkers (1993) confirmed all these previous leucine assignments, using solely protein-sequence data and the highly sensitive NMR techniques of 3D $^{13}C/^{1}H$ HCCH-COSY and HCCH-TOCSY with a uniformly $^{13}C/^{15}N$
labelled DHFR-MTX sample. It was observed that since the original leucine assignments, including the stereospecific assignments for the methyls, were dependent upon networks of long-range interresidue NOEs, the agreement indicated that the overall tertiary structure of *L. casei* DHFR must be similar in solution and in the crystal state.

Comparison of the leucine residue assignments obtained from the crystal-based method with those from the sequential assignment method indicates that as well as the β-sheet and most of the helical secondary structure, the tertiary structure is also retained. In addition, inter-residue NOEs involving many other assigned side-chain protons (particularly aromatic resonances (Birdsall *et al.*, 1990)) also confirm the observation that the crystal and solution structures are universally similar.

The assigned signals have been used as reporter groups to provide information on conformational changes in several complexes formed by substrate analogues binding to wild-type and mutant dihydrofolate reductases (Hammond *et al.*, 1986).

### 3.2.2.2.2. Assignment of Ligand signals

For weakly binding ligands which show fast exchange behaviour on the NMR chemical shift time scale, there is no problem with signal assignment. As increasing amounts of the ligand are added to the protein solution, the ligand signals (which are averaged signals from the free and bound ligand signals) appear in the spectrum with increasing intensities and shift progressively towards the chemical shifts of the free ligand. A detailed analysis of the binding curve obtained by plotting the observed averaged chemical shifts for the ligand signal against the ligand concentration yields the bound and free chemical shifts and the binding constant. NMR signals from nuclei in fragments of methotrexate and trimethoprim such as *p*-aminobenzoyl-L-glutamic acid and 2,4-diaminopyrimidine are usually in fast exchange (Birdsall *et al.*, 1980).
For tightly bound ligands such as methotrexate and trimethoprim, slow exchange behaviour is observed and separate signals are detected for free and bound ligands. In these cases the assignments can be made directly by using analogues containing selective isotopic substituents ($^2$H, $^{13}$C or $^{15}$N): the differences in the NMR spectra of the complexes formed with normal and isotopically labelled ligands can be used to locate the positions of the bound ligand signals (Cheung et al., 1986, 1993).

Transfer of saturation measurements have been used to connect signals from bound and free ligands in spectra from complexes showing slow exchange behaviour if they have sufficiently rapid dissociation rates (> 1 sec$^{-1}$) to allow the transfer of saturation to compete favourably with relaxation mechanisms (Way et al., 1975; Hyde et al., 1980a; Clore et al., 1981).

Studies of bound ligands have been shown to be particularly useful in monitoring ionisation states (Birdsall et al., 1977b; Feeney et al., 1977b; Cheung et al., 1993), multiple conformations (Gronenborn et al., 1981; Birdsall et al., 1982; 1987) and dynamic processes within the binding site of the complex (Clore et al., 1984; Cheung et al., 1986; Searle et al., 1988).

### 3.2.3. The Binding of Ligands to Dihydrofolate Reductase

#### 3.2.3.1. Measuring Ionisation States

X-Ray diffraction has provided detailed information on the conformation of bound inhibitors and these have been discussed in detail in section 3.2.2. The X-ray data of Matthews et al. (1978) for the structure of the ternary complex of *L. casei* DHFR with NADPH and methotrexate clearly indicated the existence of interaction between the N-1 of MTX with the side-chain carboxyl of Asp-26. Exactly the same interaction was also observed in the binary complex of *E. coli* DHFR with MTX (Matthews et al., 1977).
These results strongly suggest interaction of the protonated N-1 of MTX with the carboxylate group of Asp-26 (Asp-27 for E. coli). Since this Asp is invariant in bacterial DHFR and there is a corresponding Glu carboxyl in vertebrate DHFR (Blakley, 1981) the interaction makes an important contribution to the binding of methotrexate to DHFR from all these sources.

There is also extensive evidence on this subject from other techniques, particularly concerning the ionic interaction between the protonated ring of the inhibitor and a negatively charged group on the enzyme. The first indication of this interaction was reported by Baker and Jordan (1965) who studied the effect of pH on the inhibition produced by several diamino pyrimidines and triazines with a range of pK\textsubscript{a} values. Attention was drawn to the increased basicity of N-1 and of the 2-amino group in 2,4-diaminopteridines as compared with 2-amino-4-oxopteridines. Baker (1967) suggested that the former compounds bind to the enzyme in the protonated form and that an additional coulombic interaction is the basis of their substantially greater affinity for the enzyme.

UV Difference spectra have proved to be useful for demonstrating that the bound inhibitor is protonated (Erickson & Mathews, 1972; Poe et al., 1974; Hood & Roberts, 1978). It was shown by Hood and Roberts (1978), that difference spectra associated with complex formation at pH 7.0 between L. casei DHFR and methotrexate, are similar to difference spectra obtained for the free compound at pH 1.0 versus pH 7.0, that is between the protonated and neutral forms. However, no spectral evidence could be obtained for protonation of bound folate. And, in a summary of the folate results it was suggested that folate is bound in the normal keto form, and at pH 7.0 is more than 90% in the neutral ionisation state. It was shown that at pH 7.5, where MTX is bound 2000 times more tightly than folate, one third of the difference in binding energy between the two compounds arises from the differences in charge state (Hood & Roberts, 1978). However, subsequent mutagenesis studies, where the Asp-26→Asn mutant enzyme was
examined, indicated the contribution to be somewhat smaller (Howell et al., 1986; Louden et al., 1986; Jimenez et al., 1989). Similar results were obtained by others with DHFR from *E. coli* (Poe et al., 1974) and chicken liver (Subramanian & Kaufman, 1978). Saperstein et al. (1978), reported that the Raman solution spectrum at pH 7.0 for the complex MTX with *E. coli* DHFR exhibits features similar to those in the spectrum of free methotrexate at pH 2.0, again suggesting protonation of the bound inhibitor.

Further evidence of the protonated state of bound MTX and trimethoprim has been obtained by NMR spectroscopy. $^{13}$C- and $^{15}$N-labelled substrate analogues have proved to be particularly useful for investigating ionisation states in the ligand. For example, measurements of $^{13}$C chemical shifts of bound $[^{13}$C-2]methotrexate (Cocco et al., 1981a) have shown that the N-1 position of bound methotrexate is protonated in its complex with the enzyme. The chemical shift of C-2 is very sensitive to protonation of N-1, moving about 6 ppm as the pH is adjusted through the $pK_a$, the data defining a $pK_a$ for N-1 of 5.73 ± 0.02 (Cocco et al., 1981b). When the $^{13}$C NMR spectrum of the labelled ligand was observed in the presence of DHFR, two distinct resonances were observed, one of which had a chemical shift varying with pH in the same manner as the chemical shift of free MTX. This resonance therefore was assigned to the free MTX in slow exchange with the MTX-DHFR complex. It was observed that since the dissociation constant for the MTX-DHFR binary complex was very low (Gleisner & Blakley, 1975), exchange slow enough to produce a distinct, sharp resonance for the free methotrexate was to be expected.

The second resonance, which did not have a chemical shift at the same position as free MTX, evidently corresponded to MTX bound to the enzyme. It was shown that the chemical shift of this resonance did not change over the accessible pH range pH 6 - 9 and was close to but not identical with that for free protonated inhibitor. Given the sensitivity of the chemical shift of C-2 to proton dissociation from the pteridine ring, this clearly indicated that over the pH range pH 6 - 9, there was no change in the state of
protonation of the pteridine ring of MTX when it was bound to DHFR. However, folate bound to the reductase in either the binary or ternary complex showed the same protonation behaviour as in the free state. Hence, it must be concluded that N-1 of folate does not interact with the active site carboxyl, a conclusion reached from other data (Bolin \textit{et al.}, 1982). The data indicated that the pK\textsubscript{a} of N-1 for MTX is markedly increased (by at least 5 pH units) on binding, showing that the charged form binds as much as $10^5$-fold more tightly to the enzyme than does the neutral form.

By examining complexes of the enzyme with $^{13}$C-2 trimethoprim it has been shown, similarly, that the N-1 of bound trimethoprim is also protonated in its bound state (Roberts \textit{et al.}, 1981). In this case the signal for the NH proton could be detected directly in the $^1$H NMR spectra by examining enzyme complexes formed with $^{15}$N-1-trimethoprim in H$_2$O solution (Bevan \textit{et al.}, 1985).

Schegel and co-workers (1981) have used \textit{ab initio} molecular orbital calculations on neutral and protonated 2,4-diamino-6-methylpteridine, free and in a highly simplified model of the DHFR binding site. They concluded that the ionic bond between N-1 and the aspartate carboxyl is the most important component of the binding of protonated methotrexate. However, their treatment did not include hydrophobic interactions or the binding of the p-aminobenzoylglutamate side chain.

Ionisation states of the protein His residues have also been measured (Birdsall \textit{et al.}, 1977a; Feeney \textit{et al.}, 1980a). It was shown that the pK of His-28 is increased by approximately 1 unit when its charged imidazole ring interacts with the $\gamma$-CO$_2^{-}$ of the glutamate moiety of methotrexate (or indeed of any folate analogue): NMR studies were used to monitor this pK in complexes formed with MTX analogues and thus it was possible to detect whether or not the interaction between the $\gamma$-CO$_2^{-}$ and His-28 was taking place (Antonjuk \textit{et al.}, 1984).
3.2.3.2. Multiple Conformations in Complexes of Substrates with DHFR

Methotrexate is a close structural analogue of the substrate folate, and it was initially assumed that the inhibitor and substrate would bind to the enzyme in a very similar manner. However, stereochemical studies (Charlton et al., 1979, 1985; Hitchings & Roth, 1980) have shown that although the inhibitor and substrate occupy essentially the same binding site in their complexes with dihydrofolate reductase, the orientation of the pteridine ring in the catalytically active form of the substrate-enzyme complex is "turned over" by approximately 180° compared with that in the methotrexate-enzyme crystal structure (Roberts et al., 1980). Subsequent NMR studies on complexes of folate with Lactobacillus casei dihydrofolate reductase have shown that in the DHFR-folate binary complex at least two different conformational states exist, and in the ternary complex DHFR-folate-NADP⁺ three slowly interconverting conformational states are found to be present (Birdsall et al., 1981, 1987). The equilibrium between the three states was shown to be pH dependent, and the proportions of the states was estimated by measuring the intensities of the ¹³C resonances observed in the complex containing [3-carboxamide-¹³C]NADP⁺. At low pH, form I predominates, whereas at high pH, forms IIa and IIb predominate, with the ratio of these forms being relatively insensitive to changes in pH. The different conformational states have been characterised using ¹H, ¹³C and ³¹P NMR studies which indicate that forms I and IIa have conformations similar to each other (Birdsall et al., 1982, 1989b; Cheung et al., 1993). Resonances have been assigned for all the aromatic protons in the bound ligands in the different conformational states, and it has been shown, from the observed chemical shift differences for protons in the ligand and protein, that the structural differences between the different conformations are localised in the binding site of the enzyme. The COSY spectra of the protein in all three forms are very similar to that observed for the enzyme-methotrexate-NADP⁺ complex, indicating that there can only be small differences in
protein conformation in the various complexes. Subsequent $^1$H NOESY experiments on
the DHFR-folate-NADP$^+$ ternary complex detected connections between the pteridine
ring H-7 proton of folate, in the low pH form (form I) and in form IIa (but not form IIb),
and the methyl protons of Leu-19 and Leu-27 (Birdsall et al., 1989b). Similar NOE
connections have been detected in the enzyme-methotrexate-NADPH ternary complex
where the H-7-pteridine is known to be less than 4 Å from the methyl protons of Leu-19
and Leu-27 in the crystal structure (Bolin et al., 1982). In form IIb, no NOE connections
could be detected between the folate H-7 and the Leu methyl protons. More recently
Cheung and co-workers (Cheung et al., 1993) have reported the use of $^{13}$C NMR
spectroscopy in combination with folates selectively enriched with $^{13}$C at positions
sensitive to the tautomeric and ionisation states of the pteridine ring system in the
different bound forms of folates. In the study, $^{13}$C labelling at the proton-bearing C-7
and C-9 carbons of folate facilitated $^1$H-$^{13}$C HMQC-NOESY experiments which
allowed detection of protein protons near the folate H-7 and H-9 and provided additional
characterisation of the different conformational states. These experiments not only
confirmed the earlier work showing the pteridine ring in a methotrexate-like orientation
in forms I and IIa, but also provided structural evidence that the pteridine ring of folate in
form IIb has the orientation expected for the active folate complex, that is, turned over
with respect to the methotrexate orientation. In the $^1$H-$^{13}$C HMQC-NOESY spectrum of
DHFR-[2,4a,7,9-$^{13}$C]folate-NADP$^+$, the H-7 proton in form IIa showed an NOE
connection to the methyl protons of Leu-27 (a similar connection was also observed in
the $^1$H-$^{13}$C HMQC-NOESY spectrum of the methotrexate complex). The H-7 proton in
form IIb showed a connection with the Ala-97 methyl proton signal, indicating the
proximity of the protons. Consideration of the crystal structure data of Bolin and co-
workers (1982) for the L. casei DHFR-MTX-NADPH complex indicated that a "turned
over" pteridine ring occupying essentially the same binding site would have its H-7
proton approximately 3 Å from the Ala-97 methyl protons. One-dimensional NOE
difference spectra selectively irradiating the H-7 proton signals in form I, IIa and IIb for the DHFR-folate-NADP$^+$ complex at pH 6.5 confirmed that only form IIb gave a large NOE to Ala-97 CH$_3$.

![Figure 3.6](image)

**Figure 3.6.** (a) Proposed conformation of the pteridine ring in the "productive" conformation of the folate-DHFR complex (b) Conformation of the methotrexate pteridine ring in its binding site in DHFR (from the data of Matthews and co-workers, 1978).

These observations all suggest that forms I and IIa have the pteridine ring in a similar orientation to that in the methotrexate complex while form IIb has the pteridine ring turned over by 180° about an axis approximately coincident with the C-2-NH$_2$ bond. Such an orientation would allow the catalytic reduction to proceed with the correct stereochemistry and has been considered the "productive" conformation (Figure 3.6(a)). Forms I and IIa with the methotrexate-like orientation of the pteridine ring correspond to the "non-productive" conformation (Figure 3.6(b)).

The ionisable group responsible for the pH dependence of the equilibrium between the three forms has been estimated to have a pK $< 5$ in states IIa and IIb and pK $> 7$ in state I (Birdsall *et al*., 1987). Site-directed mutagenesis studies involving Asp-26
have provided direct evidence that this is the residue involved in controlling the pH
dependence of the conformational equilibrium (Jimenez et al., 1989).

3.2.3.3. Evidence from Complexes Formed with Mutant DHFRs

Site-directed mutagenesis has been used to prepare several mutant *Lactobacillus
casei* dihydrofolate reductases and studies have been undertaken to compare their kinetic
behaviour, ligand binding properties and structures with those of the wild-type enzyme
(London et al., 1986; Jimenez et al., 1989; Birdsall et al., 1989a).

3.2.3.3.1. Asp-26 → Glu and Asp-26 → Asn Mutants

Because of the potentially important role of Asp-26 (Asp-27 in *E. coli*) in both
catalysis and inhibitor binding, much attention has been focused on site-directed
mutagenesis involving substitution of this residue (Howell et al., 1986 & Birdsall et al.,
1989a).

Birdsall and colleagues (1989a) prepared the mutant of *L. casei* DHFR, Asp-26
→ Glu by site-directed mutagenesis, and reported the use of NMR spectroscopy to study
ligand binding, conformation and the dynamic properties of the bound ligand in the
binary complex with the inhibitor, trimethoprim and the ternary complex with folate and
NADP⁺ (Birdsall et al., 1989a). The results indicated that some conformational
adjustments were required to allow the carboxylate of Glu-26 to bind effectively to the
N-1 proton of inhibitors such as methotrexate and trimethoprim. These indications came
from the observation of a change in the dynamics of the bound trimethoprim molecule as
seen from the increased rate of the flipping of the 13C-labelled benzyl ring and the
increased rate of the N-1-H-carboxylate bond breaking.
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The substitution of Asp-26 by Glu did not result in a major perturbation in trimethoprim binding, thus showing that the sequence difference at residue 26, which is a conserved Asp residue in bacterial enzymes but a Glu residue in vertebrate enzymes (Matthews et al., 1985), is not in itself responsible for the specificity of binding of trimethoprim to bacterial over vertebrate enzymes. It was reported that the binding constant for trimethoprim, the conformation of the bound trimethoprim molecule, and the trimethoprim $^{13}$C and its N-1 $^1$H chemical shifts are very similar in the complexes with DHFR and the mutant enzyme. Although the bound chemical shifts of the trimethoprim in its complex with the Asp-26 → Glu enzyme indicated no large changes in the overall bound conformation, the mutation was sufficient to perturb some of the dynamic processes within the complex. For example, the rate of breaking and reforming of the hydrogen bond between the pyrimidine N-1 proton and the carboxylate group at residue 26 was increased 50-fold in the complex with the Asp-26 → Glu mutant. Another dynamic process that was reported to be perturbed in this complex is the flipping of the trimethoprim benzoyl ring, which is four times faster in the Asp-26 → Glu complex. Since it is likely that transient conformational rearrangements of the protein structure are required to permit these dynamic processes to occur, the conformational fluctuations in the protein must be very sensitive indicators of the structural differences between the mutant and wild-type enzyme.

The DHFR-folate-NADP$^+$ complex has been shown by NMR spectroscopy to exist in solution as a mixture of three conformational forms (forms I, IIa and IIb)(Birdsall et al., 1982, 1987). The complex formed with the Asp-26 → Glu enzyme was also shown to exist in the same forms showing essentially the same pH dependence with respect to the ratios of the different forms (Birdsall et al., 1989b). However, for the folate-NADP$^+$ complex formed with the Asp-26 → Asn mutant, very different behaviour has been observed.
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NMR studies (Jimenez et al., 1989) of the mutant enzyme-folate-NADP⁺ complex showed that essentially only one form (> 90%) was detected over the pH range 5.0 to 7.1; this was demonstrated, for example, by the behaviour of the methyl resonances of Leu-118 shown in Figure 3.7. The single conformation seen for this mutant was reported to correspond to the low-pH conformer (form I, the non-productive, "methotrexate-like" conformation) of the wild-type complex, strongly suggesting that Asp-26 is the ionisable group controlling the pH dependence of the equilibrium in the complex formed with the wild-type enzyme.

It has been shown by several workers (Howell et al., 1986; London et al., 1986) that the Asp-26 → Asn mutant (Asp-27 → Asn for E. coli) results in very low catalytic
activity. These results provide further evidence that Asp-26 is either directly or indirectly involved in assisting in the initial proton donation to N-5 of 7,8-dihydrofolate. However, the possibility that the Asp-26 → Asn mutation could result in an unfavourable unproductive conformation of the substrate which would result in low catalytic activity, can not be discounted.

3.2.3.3.2. Trp-21 → Leu Mutant

Trp-21 is a conserved residue in all the reported sequences of DHFR from bacterial and vertebrate sources. In the X-ray crystal structure of the *L. casei* DHFR-methotrexate-NADPH complex (Bolin *et al.*, 1982), this residue was shown to be fairly close to the coenzyme (3.9 Å from Trp-21 C-2 proton to the amide nitrogen of NADPH) and inhibitor binding sites (4.6 and 4.7 Å from the indole NH of Trp-21 to the N-1 proton and N-8 of MTX). Birdsall and co-workers (Birdsall *et al.*, 1989a) used site-directed mutagenesis to change residue Trp-21 to a leucine in order to investigate the role of Trp-21 in its complexes with substrates and substrate analogues.

The results from this study indicated that replacement of the conserved Trp-21 by a Leu residue caused a decrease in the activity of the enzyme and a reduction of the NADPH binding constant by a factor of 400, but the binding of substrates and substrate analogues was only slightly effected. The inhibitor, methotrexate, was shown by NMR spectroscopy to bind to Trp-21 → Leu DHFR by forming the same ionic interactions as in the wild-type enzyme; $^{13}$C chemical shifts of MTX bound to the mutant enzyme indicated that the inhibitor was protonated at N-1 in the complex and the chemical shift of the imidazole 2-proton of His-28 in the same complex was consistent with that measured in the complex with DHFR (Birdsall *et al.*, 1977a), thus indicating that the methotrexate γ-carboxylate group makes the same interaction with His-28 in both complexes.
Evidence that methotrexate adopts essentially the same conformation in the mutant and wild-type enzymes was obtained from comparison of the chemical shifts of the pteridine H-7 of bound methotrexate, which were shown to be virtually identical whether bound to DHFR or Trp-21 → Leu DHFR. Further comparison of the complexes of methotrexate with the mutant and wild-type enzymes was achieved from their COSY spectra. In the aliphatic regions of these spectra, several assigned signals for particular protons were observed to have different chemical shifts. The assigned signals that had shifted could be grouped into two categories. The first group arises from those protons that are in close proximity to the aromatic rings of Trp-21 and these had different chemical shifts in the mutant enzyme, compared with the wild-type, as a result of removal of the Trp ring current effects: these included resonances from residues Leu-118, Leu-19 and Leu-23. The second group comprised signals from protons in residues that are remote from Trp-21 (up to 14 Å from the site of substitution): for example, Leu-27, Leu-54, Leu-4, Val-110 and Val-115, all have protons that were shifted by more than 0.05 ppm between the two complexes. From these results it was concluded that although methotrexate binds to Trp-21 → Leu DHFR in essentially the same conformation as observed in the wild-type enzyme, the amino acid residues in the binding site were not arranged around MTX in an identical manner, and this led to small perturbations in 13C chemical shifts of bound MTX and also to small propagated conformational changes in the protein structure that could be detected from the changes in chemical shift of residues remote from Trp-21. These small shift differences could be explained by differences in atomic position of only about 0.2 Å (Birdsall et al., 1989a).

Measurements of the 1H chemical shifts of bound NADPH in the mutant and wild-type enzyme complexes indicated that the reduced nicotinamide ring binds differently in the two complexes (Birdsall et al., 1989a). In contrast, 13C and 1H chemical shift measurements for nuclei in bound substrate and substrate analogues showed that these were binding similarly to the mutant and wild-type enzymes. The
differences in catalysis and coenzyme binding were attributed by Birdsall and colleagues to structural differences involving the coenzyme binding site. It was shown, by analysis of the $^1$H chemical shifts observed for NADPH bound to DHFR and Trp-21 → Leu DHFR, that most parts of the coenzyme structure bind similarly in the two cases, with the notable exception of the reduced nicotinamide ring itself. This suggested that the reduced nicotinamide ring was probably still binding to the mutant protein within the same binding pocket but in a modified manner. The crystal structure data of Matthews and co-workers (1979) shows that the C-Ç2 proton of Trp-21 has a hydrophobic interaction with the carboxamide nitrogen of bound NADPH. It was shown that the substitution of Trp-21 by Leu leads to loss of this interaction and to a change in orientation of the nicotinamide ring within the binding site. The reduced nicotinamide ring is known to contribute a large amount to the overall binding energy of NADPH, and a perturbation of its interaction with the enzyme could account for the 400-fold reduction in coenzyme binding. It could also account for the substantial decrease observed in the rate of hydride transfer, the rate limiting step in the catalytic reaction for Trp → Leu DHFR (Andrews et al., 1989). Similar behaviour for the coenzyme binding was observed with reduced thio-NADPH, where the interaction between the coenzyme carboxamide group and the enzyme was altered by modification of the ligand rather than the enzyme (Birdsall et al., 1980).

### 3.2.3.4. Substrate Binding and Catalytic Activity

In contrast to the detailed structural information available about the binding of inhibitors to the enzyme DHFR, much less is known about the catalytic activity of the enzyme. Both ultra-violet difference spectroscopy (Hood & Roberts, 1978) and $^{13}$C NMR spectroscopy (Cocco et al., 1981a) show that when bound to the enzyme, folate is not protonated on N-1, as discussed previously. It has also been shown that there is a
major difference in orientation of the pteridine ring of the substrates folate and
dihydrofolate when bound to the enzyme compared with that observed for the inhibitor
methotrexate in the crystal (Birdsall et al., 1977b). This evidence comes from the
stereochemistry of the catalytic reaction (Charlton et al., 1979, 1985). It was first noted
by Matthews et al., from X-ray crystallographic studies that the orientation of the
pteridine ring of folate would give an absolute $R$-configuration at C-6 for the product
5,6,7,8-tetrahydrofolate if substrate 7,8-dihydrofolate was bound like methotrexate, but
an $S$-configuration if its pteridine ring was rotated by 180°. Shortly thereafter,
Fonticella-Camps and colleagues (1979a, b) showed, using X-ray diffraction methods,
that the biologically active diastereoisomer tetrahydrofolate, in fact, did have the absolute
$S$-configuration at C-6. Subsequently, reports by Charlton and co-workers confirmed
these findings for dihydrofolate and demonstrated that the same was also true for folate,
by demonstrating that hydride transfer occurs from the 4-pro $R$ hydrogen of NADPH to
the same corresponding face of these two pteridines (Charlton et al., 1979, 1985). The
nicotinamide ring of NADPH, therefore, must be positioned against the re face of 7,8-
dihydrofolate (the back of the pteridine ring in the orientation of the structure shown on
below):

![Figure 3.8. Schematic representation of 5,6,7,8-tetrahydrofolate (left) and the dihydronicotinamide ring of NADPH (right).](image)

It was shown by the use of (4$R$)-[4-$^2$H$_1$]-NADPH, in conjunction with $^1$H NMR
spectroscopy that reduction of folate with DHFR had transferred the 4-pro $R$ hydrogen of
NADPH to the si face at C-7 of folate. Therefore, hydrogen transfer at both C-6 and C-7
involved the same face of NADPH (4-HG) and the same face of the pteridine system in folate (C-6 re, C-7 si). The relationship between the pteridine and nicotinamide rings implied by these observations shows that folate and dihydrofolate, on the one hand, and methotrexate, on the other, must bind to DHFR to project opposite faces toward the cofactor binding site, and hence, the active site on the enzyme. Figure 3.9. shows a schematic representation of (a) proposed interaction of 7,8-dihydrofolate and (b) methotrexate with active site residues in L. casei DHFR.

Figure 3.9. Schematic representation of hydrogen bonding between L. casei dihydrofolate reductase and the pteridine portions of (a) 7,8-dihydrofolate (hypothetical) and (b) methotrexate. "R" represents p-aminobenzoyl-L-glutamate. Evidence for the interactions with methotrexate (b) are discussed in Section 3.2.2.1.2. The unlabelled carbonyl oxygen hydrogen bonded to N8 in the proposed enzyme-substrate complex might be Leu-4, Ala-97, or both. (Bolin et al., 1982).

It is generally accepted that reduction of dihydrofolate requires N-5 of the pteridine ring to be protonated prior to or in conjunction with hydride transfer from C-4 of NADPH. While it is known that the 4-pro R hydrogen is transferred directly from C-4 (Charlton et al., 1985) the mechanism of N-5 protonation remains obscure. The aspartate side chain of Asp-26 (Asp-27 in E. coli), the sole acidic residue in the pteridine binding
site is thought to be involved in protonation of N-5. Mutation of this residue to asparagine results in greatly diminished activity toward dihydrofolate at pH 7, but activity comparable to native *L. casei* DHFR at pH values sufficiently low to protonate dihydrofolate in solution (Howell *et al.*, 1986; London *et al.*, 1986). However, because the distance between the carboxyl group of Asp-26 and N-5 is approximately 6.3 Å, proton transfer from Asp-26 can not occur directly, and various mechanisms for proton shuttling between Asp-26 and N-5 have been proposed (Morrison & Stone, 1988; Uchimaru *et al.*, 1989; Bystroff *et al.*, 1990). Each of these mechanisms involves enolisation of the pteridine O-4 keto group aided by proton transfer to O-4, via water-253 (water-403, *E. coli*), from the side chain of Asp-26. At the time of these studies, it was unclear how the final proton transfer could take place, since none of the previously reported structures of DHFR contained any group capable of transferring a proton from O-4 to N-5.

Some authors have proposed that the lone pair on N-5 abstracts a proton from the enol form of O-4 directly (Morrison and Stone, 1988), while it has been argued that transfer occurs by way of a transiently-bound water molecule hydrogen-bonded to both the O-4 and N-5 (Bystroff *et al.*, 1990; Brown and Kraut, 1992). It was proposed by Brown and Kraut (1992) that proton transfer via this intervening water molecule was more likely for several reasons. First, the geometry between O-4 and N-5 is not favourable for proton transfer (McTigue *et al.*, 1992), second, a water molecule that makes ideal hydrogen bonds to O-4 and N-5 could be modelled into both the human DHFR-folate and *E. coli* DHFR-folate-NADP*⁺* structures with very slight repositionings of Phe-31 or Met-20, respectively. Finally, a water molecule, water-756, was observed at this position forming just such a hydrogen-bonded link between O-4 and N-5 in the recently solved crystal structure of a ternary complex of chicken DHFR containing biopterin and NADP⁺ (McTigue *et al.*, 1992). The proposed mechanism for proton transfer in this complex involved small conformational changes in residues 31 of
vertebrate DHFR's or in Met-20 of *E. coli* DHFR, to assist in transiently binding this water molecule for protonation of N-5 and then the water is displaced to allow for positive charge build up at C-6 in the transition state (McTigue *et al.*, 1992).

If we now consider the cofactor: refinement of the *L. casei* DHFR-NADPH-MTX ternary complex revealed several rather subtle structural features of the nicotinamide binding geometry that influence the nature of the transition state and how it may be stabilised by the enzyme (Filman *et al.*, 1982). It was noted that there are three oxygen atoms O-13, O-97 and O-45 that are situated in the plane of the nicotinamide ring and close to ring carbons C-2, C-4 and C-6 (Figure 3.10). From the placement of these polar groups it has been suggested that the enzyme stabilises a C-4 carbonium electronic isomer of oxidised nicotinamide in the transition state.

![Figure 3.10](image)

**Figure 3.10.** Schematic representation of the proposed enzyme-dihydrofolate-NADPH complex. Polar interactions between *L. casei* dihydrofolate reductase and the nicotinamide group of bound NADPH are emphasised. Also depicted are hydrogen bonds between the side chain of Asp-26 (shown here as the anion) and the pteridine portion of dihydrofolate. Adapted from Filman *et al.* (1982).
It was also suggested that pyramidalisation of the ring nitrogen (N-1) in the transition state might be promoted by a fixed water molecule (water-439) positioned to donate a hydrogen bond to the N-1 lone pair orbital. Thus Filman et al. (1982) favoured a view in which the principal catalytic effect of the enzyme is to promote hydride donation by NADPH, rather than to facilitate hydride acceptance by dihydrofolate. Clearly, the precise mechanism by which the protein assists hydride transfer is, therefore, still unclear, and additional information is required to provide an unambiguous definition.


3.3. EXPERIMENTAL

3.3.1. Materials and Equipment

Dihydrofolate reductase was isolated from an *Escherichia coli* strain into which the *L. casei* gene had been cloned (Andrews et al., 1985) and the enzyme was purified using methods described previously (Dann et al., 1976). NADP⁺ and NADPH were obtained from Sigma Chemical Company and DCI and NaOD were obtained from Aldrich. The pH measurements were carried out using a Hanna Instruments HI 8417 pH meter equipped with a combination glass electrode. The reported pH values are meter readings uncorrected for deuterium isotope effects.

The tritium NMR experiments were carried out on a Bruker AC-300E spectrometer, operating at 320 MHz for tritium. Typically 30,000 transients were collected for spectra with widths of 3846 Hz and using 16 K data points, zero filled to 32 K data points. As a precaution against sample spillage, 4 mm Teflon liners with stoppers were used inside 5 mm glass screw-capped NMR tubes. The tritium chemical shifts were referenced to the highest field methyl resonance in the proton spectrum (calculated to be at -4.7 ppm) and the tritium chemical shift calculated by multiplying the proton resonance frequency by the ratio of triton to proton magnetogyratic ratios (1.06663974, Bloxsidge et al., 1979). Thus the chemical shifts are reported in ppm, downfield of dioxan, in order to compare the experimental results with ¹H literature values. Tritium Free Induction Decays (FIDs) were filtered with 5 Hz of Lorentzian line broadening before Fourier transformation and phasing. The NMR operating parameters were optimised for the enzyme samples (pulse flip angle, 72°; acquisition time, 1.1 secs.; total recycle time, 1.6 secs.). Unless otherwise stated, all NMR spectra were recorded in D₂O.
3.3.2. Experimental Methods

3.3.2.1. L. casei DHFR-[7,9-^3H]Folate-NADP+ Ternary Complex

In order to study the pH dependence of the three reported conformational states of the L. casei DHFR-folate-NADP+ complex, the 3H NMR spectra of the ternary complex containing [7,9-3H]folate (13) were obtained over the pH range pH 7.4 - 5.3, at 25°C.

The initial experiment was conducted at pH 7.4. A 4 mM aliquot of [7,9-3H]folate with specific activity 4.1 Ci/mmol, and a 2 mM aliquot of DHFR-NADP+ containing 100 mM KCl and 50 mM potassium phosphate (stored at -18°C) were allowed to warm to room temperature. The solution of DHFR-NADP+ binary complex was prepared by redissolving the solid in 0.5 cm³ of D2O and carefully rotating to ensure all of the enzyme was in solution. The enzyme-NADP+ solution was then added to the solid tritiated folic acid and when all the folic acid had dissolved the pH was measured and adjusted with DCl/NaOD to pH 7.4. The DHFR-[7,9-3H]folate-NADP+ solution was then transferred to an NMR tube, sealed and 1H and 3H (1H-decoupled) (Figure 3.11(a)) NMR spectra were recorded at 25°C to see if any of the 3H signals could be observed when the 3H-folate was bound to the enzyme.

When the NMR experiment was complete, the NMR tube containing the DHFR-[7,9-3H]folate-NADP+ complex was stored at 4°C prior to the next NMR experiment. The NMR experiments were conducted overnight, to allow for long scanning times, on consecutive nights and the solution of the ternary complex was stored at 4°C during the intervening daytime periods.

In order to study the DHFR-[7,9-3H]folate-NADP+ complex at pH 6.6, the pH of the solution was altered immediately prior to the NMR experiment. The ternary complex solution was transferred from the NMR tube into an Eppendorf vial, a tiny glass-encased stirrer bar was added and with very gentle stirring the pH of the solution was carefully...
adjusted to pH 6.6 with DCl. The solution was then transferred back into the NMR tube, sealed and the $^1$H and $^3$H ($^1$H-decoupled) (Figure 3.11(b)) NMR spectra were recorded, keeping the spectral parameters consistent with those employed for the initial NMR experiment. Subsequent NMR experiments were carried out at pH 6.0 (Figure 3.11(c)) and pH 5.3 (Figure 3.11(d)).

As a final experiment in this series, the pH of the sample was readjusted from pH 5.3 to pH 7.4 and the $^3$H NMR spectrum (Figure 3.12(b)) recorded to assess if the pH changes were reversible. This final spectrum was recorded at a lower temperature ($10^\circ C$) in order to examine the effects of exchange.

### 3.3.2.2. L. casei DHFR-[3',5',7-$^3$H]Folate-NADP$^+$ Ternary Complex

The sample of DHFR-[3',5',7-$^3$H]folate-NADP$^+$ ternary complex was prepared as described above for the DHFR-[7,9-$^3$H]folate-NADP$^+$ complex by adding a solution of the DHFR-NADP$^+$ binary complex to an aliquot of solid tritiated folic acid. The ternary complex contained 4 mM [3',5',7-$^3$H]folic acid (12, specific activity 32 Ci/nmol) and 2 mM DHFR containing 100 mM KCl and 50 mM potassium phosphate in 0.5 cm$^3$ D$_2$O.

The $^1$H and $^3$H ($^1$H-decoupled) NMR spectra were recorded using the same spectral parameters as those employed for the DHFR-[7,9-$^3$H]folate-NADP$^+$ complex in solution and the pH adjustments made in the same manner as described previously. The pH values used were pH 7.4, 6.86, 6.3 and 5.3 (Figure 3.13).

### 3.3.2.3. L. casei DHFR-[3',5',7-$^3$H]Methotrexate Complex and Ternary Complex with NADPH

A 1.1 mM aliquot of [3',5',7-$^3$H]methotrexate (14) with specific activity 9 Ci/nmol and a 1.1 mM aliquot of DHFR containing 50 mM potassium phosphate and
500 mM KCl, were allowed to warm to room temperature. The DHFR was dissolved in
0.6 cm³ D₂O and this was then added to the solid [3',5',7-³H]methotrexate (14) and
gently swirled to encourage the tritiated methotrexate to dissolve. The pH of the binary
complex solution was adjusted to pH 6.5 with DCl/NaOD and transferred to an NMR
tube, sealed and the ¹H and ³H (¹H-decoupled) NMR spectra were run at 25°C.

Subsequent ³H NMR spectra of the DHFR-[3',5',7-³H]methotrexate binary
complex were recorded, at pH 6.5, at 10°C (Figure 3.14(b)) and 40°C, in an attempt to
detect signals for the 3',5'-triton nuclei in the bound ligand, as well as the signal observed
for T-7.

The DHFR-[3',5',7-³H]methotrexate ternary complex was prepared by the
addition of the DHFR-[3',5',7-³H]methotrexate binary complex solution to a solid sample
of 1.5 equivalents of NADPH, in an Eppendorf vial. The sample was gently stirred to
encourage the NADPH to dissolve. When all of the solid NADPH had dissolved, the
sample was transferred back into the NMR tube and the ¹H and ³H (¹H-decoupled)
spectrum run at pH 6.53, at 25°C, 10°C and 40°C (Figure 3.14(c)).

In a further attempt to detect the signals from the 3',5'-tritium nuclei, the ³H
NMR spectrum of the ternary complex was recorded for a longer period of time (167,
000 scans) at 10°C.
3.4. RESULTS

The tritium chemical shifts of the $^3$H-labelled folic acids and methotrexate in their binary and ternary complexes with *L. casei* DHFR are given in Table 3.2. The tritium chemical shifts are also given for free $[3',5',7-^3$H]folate (12), $[7,9-^3$H]folate (13) and $[3',5',7-^3$H]methotrexate (14), recorded prior to the binding studies.

3.4.1. $^3$H NMR Spectra of the Folate Ternary Complexes Formed with *L. casei* DHFR

The $^3$H NMR spectra of the DHFR-$[7,9-^3$H]folate-NADP$^+$ and DHFR-$[3',5',7-^3$H]folate-NADP$^+$ ternary complexes show multiple signals for each labelled hydrogen in the bound folate, thus confirming the presence of multiconformational states seen in earlier studies (Birdsall et al., 1981, 1982, 1987, 1989b). The pH dependence of the three conformational states in the ternary complex has already been well characterised and this information can be used to assign the multiple signals for the $3',5'$- and 7-tritons to form I, Ila or IIb, simply by monitoring the signal intensities as a function of pH for the ternary complexes. At low pH ($< 5.5$) a single conformation, denoted form I, is observed, while at high pH ($> 7.5$) two other forms, denoted IIa and IIb have been found to coexist in a ratio which is pH independent (Birdsall et al., 1989b). At intermediate pH values, all three forms are present. In the samples studied, excess tritiated folate was present, and separate signals for bound and free species were detected, thus indicating the presence of slow exchange between the species.
3.4.1.1. L. casei DHFR-[7,9-^3H]Folate-NADP^+ Ternary complex

This complex was examined at a series of pH values in the presence of two equivalents of [7,9-^3H]folate and the ^3H spectra are shown in Figure 3.11. At pH 7.4, (Figure 3.11(a)) two signals were observed for bound T-7 (4.18 and 5.67 ppm) corresponding in chemical shift to the H-7 signals of the two conformations Ila and IIb observed previously at high pH in ^1H 2D exchange experiments.

The T-7 signals of both the bound and free folate are broad (ca. 40 Hz) and this reflects exchange between the two species. As the pH is lowered, a new signal appears in the T-7 region and this corresponds to the H-7 proton signals detected previously (4.50 ppm) for form I of the complex. Figure 3.11(d) shows that T-7 signals for all three forms are present, at pH 5.3.

Signals for the bound T-9 were also detected in the spectra. At pH 7.4 (Figure 3.11(a)) these signals appeared at 0.31, 0.08 and -0.59 ppm. The observed signals are even broader than the T-7 signals: part of the line broadening is from ^1H-^3H spin coupling (estimated to be ca. 20 Hz), but there could be a further contribution to the line width from overlap of signals with different chemical shifts. The two tritons at the 9-position will be non-equivalent in bound folate and thus it can be anticipated that there will be four signals from the T-9 nuclei, corresponding to two different conformations. Johnson and Bovey's (1958) ring current shift calculations, based on the crystal structure data of Bolin et al. (1982) (for the L. casei DHFR-MTX-NADPH complex), predict that one of the two protons (or tritons) on C-9 has an upfield shielding contribution of approximately 0.9 ppm. This would correspond closely to the signal observed at -0.59 ppm.

At pH 5.3, where all three conformational forms are present, T-9 signals for bound folate are also observed (at 0.13 and -0.56 ppm). There is a similarity in chemical
shifts at pH 7.4 and pH 5.3, and this indicates that the T-9 nuclei are in similar environments in forms I, IIa and IIb.

As a final experiment in the series, the pH of the sample was readjusted from pH 5.3 to pH 7.4 and the $^3$H NMR spectrum re-recorded (Figure 3.12(b)). This spectrum indicates that the signals from the different conformational forms show the same intensities evident in the earlier spectra recorded at pH 7.4. This indicates that the pH-induced interconversion between the conformational states is reversible. The spectrum shown in Figure 3.12(b) was recorded at a lower temperature ($10^\circ$C), in order to examine the effects of exchange. As expected, the observed lines are narrower due to the decrease in exchange rates between the bound and free species at a lower temperature.

The sample used to obtain Figure 3.12(b) had been prepared for 6 days and its $^3$H spectrum indicates that there is a small amount of folate decomposition. This gives rise to a small signal at 5.48 ppm (marked * on Figure 3.12(b)).

3.4.1.2. *L. casei DHFR-[3',5',7-$^3$H]Folate-NADP$^+$ Ternary Complex*

The $^3$H NMR spectrum of this complex recorded at pH 7.4 is shown in Figure 3.13(a). Again the two distinct T-7 bound signals were detected (at 4.18 and 5.73 ppm) confirming the existence of the two conformations present at high pH in this complex (forms IIa and IIb). In contrast only a single resonance was detected for the 3',5'-$^3$H nuclei from the benzoyl ring of bound folate (1.89 ppm) and there is a substantial chemical shift difference between the bound and free species (the signal from the bound is 1.3 ppm upfield of the free). The chemical shift of the 3',5'-$^3$H nuclei in bound folate at pH 5.3 (1.89 ppm)(Figure 3.13(d)) is the same as observed at pH 7.4. The appearance of a single resonance, well shifted from the position of the free, indicates that the benzoyl ring is binding in essentially the same binding site in all three conformations.
Furthermore, the equivalence of the 3'- and 5'-T chemical shifts shows that this ring is undergoing relatively rapid ring flipping.

As the pH is lowered, the intensity of the two T-7 resonances decreases and that of another resonance (4.53 ppm), in the same region, increases. By pH 5.3, the resonances corresponding to forms Ila and IIb (4.18 and 5.73 ppm) are barely detectable. The signal observed at 4.53 ppm corresponds to the H-7 proton signals detected previously for form I of the complex. As for the DHFR-[7,9-3H]folate-NADP⁺ complex the chemical shifts of the three resonances in the T-7 region of this complex are also independent of pH; only their relative intensities varies. The observation that the relative intensities, but not the chemical shifts, of the three resonances are pH-dependent shows that the three states of the complex which they represent interconvert slowly on the NMR time scale.

In the spectrum recorded at pH 6.86 (Figure 3.13(b)) two new signals are present (2.5 and 1.1 ppm). The sample used to obtain Figure 3.13(b) had been prepared for 4 days and its spectrum indicates a large amount of folate decomposition to be present. The signal at 1.1 ppm corresponds to HTO. The position of the other signals (2.5 ppm) corresponds closely to the 3',5'-H position observed previously at low pH in the ¹H 2D exchange experiments for form I (Birdsall et al., 1989b). However, comparison of the spectra recorded over the pH range pH 6.86 - 5.30, indicates that the peak height of this resonance at 2.5 ppm is too large and anomalous when compared with the T-7 peak heights. Furthermore, the intensity of this resonance is observed to remain constant over the pH range. This is also the case for the signal, at 1.1 ppm assigned to HTO, thus suggesting that these two resonances correspond to signals from decomposition products rather than to the 3',5'-T signals from one of the conformational forms whose signal intensities are pH dependent.
3.4.2. \textsuperscript{3}H NMR Spectra of the Binary and Ternary [3',5',7-\textsuperscript{3}H]Methotrexate Complexes Formed with \textit{L. casei} DHFR

The \textsuperscript{3}H NMR spectra of the binary and ternary DHFR complexes containing [3',5',7-\textsuperscript{3}H]methotrexate and [3',5',7-\textsuperscript{3}H]methotrexate-NADPH were recorded and the chemical shifts of the bound methotrexate measured (Table 3.2). Each tritium gives rise to only a single resonance for bound methotrexate, confirming that the inhibitor is bound in a single conformational state in the protein (Birdsall \textit{et al}., 1989a) in contrast to the situation with the \textit{E. coli} DHFR-methotrexate complex (Falzone \textit{et al}., 1991 & Cheung \textit{et al}., 1992). In the samples studied, there was no excess methotrexate present, hence, only signals for the bound methotrexate were observed.

Figure 3.14(a) shows the \textsuperscript{3}H NMR spectrum of free [3',5',7-\textsuperscript{3}H]methotrexate (14). A comparison with the spectrum of the binary complex of DHFR-[3',5',7-\textsuperscript{3}H]methotrexate is shown in Figure 3.14(b) and indicates that the only bound ligand signal observed is T-7, no signal being seen for the 3',5'-T nuclei. Similar results were obtained for the ternary complex with NADPH (Figure 3.14(c)). Varying the sample temperature over the range 10 to 40 \textdegree C did not noticeably change the \textsuperscript{3}H spectra of the binary or ternary complex. The most obvious explanation for the absence of the 3',5'-T signal(s) is that they have been broadened by some exchange process such as, for example, flipping of the benzoyl ring. This is consistent with previous \textsuperscript{1}H NMR studies of methotrexate complexes with \textit{L. casei} DHFR where signals for the 3',5'-protons have never been detected for either the binary or ternary complexes. Ring current chemical shift calculations (Johnson and Bovey, 1958) based on the crystal structure data of Bolin and co-workers (1982) for the DHFR-MTX-NADPH complex, estimate that the difference in chemical shifts of the 3'- and 5'-protons is 1.36 ppm (435 Hz at 320 MHz). McConnell's modified Bloch equations (McConnell, 1958) have been used to calculate the spectra for signals corresponding to exchange at various rates between two equally
populated non-equivalent sites having a chemical shift difference of 435 Hz. A noise level corresponding to that observed in the experimental spectrum was included in the simulation. Figure 3.16 shows the spectra calculated for various rates over the range 250 s$^{-1}$ to $10^5$ s$^{-1}$: for rates between 500 to 4000 s$^{-1}$ the signals are so broad that they would not have been detected at the prevailing signal to noise ratio.

The calculated spectra are characteristic of nuclei undergoing exchange between two equally populated magnetically non-equivalent sites. Because of the chiral nature of the binding site, any fixed orientation of the benzoyl ring would be expected to result in magnetic non-equivalence of the 3'- and 5'-tritium nuclei. The observed spectra are explained by the exchange process that has the effect of interchanging the positions of the 3'- and 5'-tritons so that at high temperature they experience an average environment. The exchange process has been shown to involve ring flipping of the benzoyl ring about the N-10-C-4' and C-1'-CO bonds (Clore et al., 1984). Similar motions about the symmetry axis have been detected for other ligands bound to the protein (Cayley et al., 1979; Feeney et al., 1981).
Table 3.2. $^3$H Chemical shifts of ligand nuclei in the three conformational states of *L. casei* DHFR-$^3$H-folate-NADP$^+$ complexes and in the *L. casei* DHFR-$^3$H-methotrexate binary, and the ternary complex with NADPH. $^a$ $^1$H data from Birdsall *et al.*, 1989b; $^b$ $^1$H data from Wyeth *et al.*, 1980.

<table>
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<th>Chemical Shift (ppm from dioxan)</th>
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<th>T-3',5'</th>
<th>T-9</th>
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<tbody>
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<td>Free [7,9-$^3$H]Folate, pH 7.8</td>
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<tr>
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Figure 3.11. $^3$H NMR spectra ($^1$H-decoupled) at 320 MHz of [7,9-$^3$H]folic acid in a 1:1:1 complex with L. casei DHFR and NADP⁺ at 25°C, at different pH values (a) pH 7.4, (b) pH 6.6, (c) pH 6.0, (d) pH 5.3. (2 mM DHFR, 4 mM [7,9-$^3$H]folic acid, 100 mM KCl, 50 mM potassium phosphate in D₂O).
Figure 3.12. $^3$H NMR spectra ($^1$H-decoupled) at 320 MHz of [7,9-$^3$H]folic acid in a 1:1:1 complex with \textit{L. casei} DHFR and NAD$^+$ at pH 7.4 at different temperatures (a) 25°C, (b) 10°C.
Figure 3.13. $^3$H NMR spectra ($^1$H-decoupled) at 320 MHz of [3',5'-$^3$H]folic acid in a 1:1:1 complex with *L. casei* DHFR and NADP$^+$ at 25°C at different pH values. (a) pH 7.4, (b) pH 6.86, (c) pH 6.3, (d) pH 5.3.
Figure 3.14. $^3$H NMR spectra ($^1$H-decoupled) at 320 MHz of [3',5',7-$^3$H]methotrexate (a) alone, (b) in a 1:1 complex with *L. casei* DHFR (1.1 mM) at pH 6.5, 283 K, (c) in a 1:1:1 complex with *L. casei* DHFR and NADPH at pH 6.5, 313 K.
Figure 3.15. A comparison of (a) $^1$H NMR spectrum at 300 MHz and (b) $^3$H NMR spectrum ($^1$H-decoupled) at 320 MHz of [7,9-$^3$H]folic acid in a 1:1:1 complex with L. casei DHFR and NADP$^+$ at pH 7.4, 25°C.


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**Figure 3.16.** Calculated spectra for various exchange rates between two equally populated non-equivalent sites in exchange with each other.
3.5. DISCUSSION

If a ligand is strongly bound to an enzyme such that the dissociation rate constant is less than \( k_B - k_F \) (the shift difference between the bound and free species), separate NMR absorption signals are usually obtained for the free and bound states. In \(^1\)H NMR studies, it is often difficult to identify the resonances from the strongly bound ligand in the 1:1 complex because of the presence of the spectrum of the protein. This has been the case, for example, in previous studies of NADPH and folate binding to dihydrofolate reductase (Roberts et al., 1974). Even if resonances from the bound ligand can be detected, there is often the problem of assigning them to the individual protons of the ligand. One general solution to this problem is to examine unlabelled ligands bound to deuterated proteins. In tritium NMR work, on the other hand, the problem can be circumvented by selectively labelling sites in the ligand with tritium and then examining the \(^3\)H signals from the ligand. In the present study \([3',5',7-\text{\(^3\)H}]\text{folate (12)}, [7,9-\text{\(^3\)H}]\text{folate (13)}\) and \([3',5',7-\text{\(^3\)H}]\text{methotrexate (14)}\) have been selectively labelled as described in Chapter 2.

Tritium has a relatively high natural sensitivity to NMR detection (1.2 times greater than that of \(^1\)H nuclei) and because its chemical shifts are directly related to \(^1\)H chemical shifts, it is easy to compare the \(^3\)H and \(^1\)H results, the latter often being obtained from complex spectra where ambiguities in assignment can arise (Evans et al., 1985). There is no difficulty in distinguishing the resonance of the \(^3\)H, since tritium has effectively zero natural background abundance and of course, there is no assignment problem since the site(s) of labelling are known.

Figure 3.15 shows a comparison of the \(^1\)H and \(^3\)H NMR spectra of the DHFR-\([7,9-\text{\(^3\)H}]\text{folate-NADP}\) ternary complex. The tritium spectrum (Figure 3.15(b)) indicates that the only resonances observed are those of the \(^3\)H labelled 7- and 9-positions of the ligand in the different conformational states, whereas the \(^1\)H NMR spectrum (Figure
3.15(a)) of the same complex is complicated by severe spectral overlap. Hence, the $^1$H spectrum has been greatly simplified by the use of $^3$H labelled ligands in conjunction with $^3$H NMR spectroscopy.

3.5.1. Folate Ternary Complexes Formed with *L. casei* DHFR

The observation of multiple $^3$H signals for the labelled tritium nuclei in the pteridine ring of folate bound to *L. casei* DHFR confirms the presence of the multiple conformations detected in earlier studies (Birdsall *et al.*, 1981, 1982, 1987, 1989b). The observed changes in signal intensities as a function of pH confirm the pH dependence of the three forms of the complex (forms I, IIa and IIb) in the ternary complex with NADP$^+$. 

A model for the differences between the three conformational states has been proposed by Birdsall and colleagues from chemical shift data and NOE experiments of the DHFR-folate-NADP$^+$ complex (Birdsall *et al.*, 1982, 1987, 1989b). $^{13}$C and $^1$H chemical shift data for the coenzyme have shown that the only nuclei whose environments differ between the three conformational states are those at the nicotinamide end of the bound NADP$^+$ molecule. The carboxamide $^{13}$C and N-2 and N-4 $^1$H resonances of the nicotinamide ring have similar chemical shifts in conformations I and IIa, but quite different shifts in conformation IIb. Although fewer signals from the bound folate molecule have been identified, a similar trend within its binding site has been observed, since there is a substantial difference in chemical shift between the different chemical states for the pteridine 7-proton and $^{15}$N-5, but no detectable difference for the benzoyl carbonyl carbon (Pastore *et al.*, 1976). In the present study, the T-7 $^3$H resonance of the pteridine ring has similar chemical shifts in conformations I and IIa, but quite a different shift in conformation IIb (shifted downfield ca. 1 ppm), this is consistent with the chemical shifts of the $^1$H resonances previously observed for the H-7 of the...
same complex (Birdsall et al., 1989b). These data clearly suggest that the surroundings of the nicotinamide and pteridine rings - that is, the centre of the active site - is the likely source of the conformational differences between the three conformational states of the complex.

Subsequent ligand-protein NOESY experiments confirmed unambiguously the indications from the chemical shifts that, while the environment of the pteridine 7-proton is similar in states I and IIa, it is quite different in state IIIb. In the former states the 7-proton is within 4 Å of two (conformation IIa) or three (conformation I) methyl groups, from Leu-19 and Leu-27, whereas in state IIIb it is much further from any leucine methyl proton. The same NOEs had previously been detected in NOESY spectra of DHFR complexes with methotrexate (Birdsall et al., 1989a) and are those expected from consideration of the crystal structure studies of Bolin and co-workers (1982). Therefore it was proposed that in states I and IIa folate has its pteridine ring in a methotrexate-like orientation.

In state IIIb no NOE was observed between the folate 7-proton and the methyl groups of Leu-19 or Leu-27, thus indicating that the distance between the pteridine H-7 and leucine residues must be greater than 4 Å.

More recent studies (Cheung et al., 1993) have used 2D $^1$H-$^13$C HMQC-NOESY experiments to further confirm the characterisation of the pteridine ring orientation in the different bound forms of folate. In this study, an NOE was observed between the pteridine H-7 proton in form IIa, of the DHFR-[2,4a,7,9-$^13$C]folate-NADP$^+$ ternary complex and the methyl protons of Leu-27 (a similar connection was observed in the $^1$H-$^13$C HMQC-NOESY spectrum of the methotrexate complex). The H-7 proton in form IIb showed a connection with the Ala-97 methyl proton signal, thus indicating the proximity of these protons. These observations are consistent with previous results and consideration of the crystal structure data of Bolin and co-workers (1982) for the L. casei DHFR-methotrexate-NADPH complex indicated that if the pteridine ring occupied
essentially the same binding site, but was 'turned over' by 180°, the H-7 proton would be approximately 3 Å from the Ala-97 methyl protons. The results from subsequent NOE difference spectra selectively irradiating the H-7 proton signals in forms I, IIa and IIb for the DHFR-folate-NADP⁺ complex at pH 6.5 confirmed that only form IIb gave a large NOE to the Ala-97 methyl group.

Some further support for this proposed reorientation comes from the fact that a qualitative explanation for the pattern of ligand ¹H chemical shift differences between conformations IIa and IIb could be provided. In the binding site the nicotinamide ring of the coenzyme is close to the pteridine ring, and the ring current anisotropy of the latter will contribute to the chemical shifts of the nicotinamide protons. Taking the position of the pteridine and nicotinamide rings in conformation IIa as those observed in the crystal structure of the enzyme-methotrexate-NADPH complex (Bolin et al., 1982) and the position of the pteridine ring in conformation IIb as described above, Birdsall et al. showed that the calculated difference in the ring current contribution to the chemical shift of the nicotinamide protons between the two conformations was in general agreement with their observed experimental values (Birdsall et al., 1989a). The chemical shift of the pteridine H-7 has large shielding contributions from the anisotropy of the benzoyl ring of methotrexate or folate and from the ring currents of neighbouring aromatic residues in the protein (Hammond et al., 1987). Therefore, the change in the mutual orientation of these two rings involved in the suggested mode of binding of folate in conformation IIb would certainly lead to a substantial downfield shift in the resonance of this proton (or triton), as observed experimentally. Similar arguments can be applied to the upfield shift observed for the folate T-7, in the current study, in forms I and IIa from the free T-7 resonance (Hammond et al., 1987).

The proposed 180° reorientation of the pteridine ring in conformation IIb relative to that in the crystal structure of the enzyme-methotrexate-NADPH complex is exactly that required to account for the observed stereochemistry of reduction of folate
(Hitchings & Roth, 1980; Charlton et al., 1979, 1985). The mode of binding of folate that has been proposed for conformation IIb of the enzyme-folate-NADP$^+$ complex thus corresponds to the 'productive' mode of binding in the catalytically functional enzyme-folate-NADPH complex. The alternative orientation seen in conformations I and IIa is said to represent a non-productive mode of binding of the substrate, since it has been shown that the stereochemistry of folate reduction at low pH, where conformation I predominates, is the same as that determined previously at neutral pH. The difference between the binding of methotrexate seen in the crystal and the mode of binding of folate implied by the stereochemistry of reduction shows that instead of methotrexate binding in a unique way which is unavailable to the substrate, the inhibitor binds essentially exclusively in only one of the two modes of binding available to the substrate, that corresponds to 'non-productive' substrate binding. The preference of methotrexate for this orientation must arise, at least in part, from the formation of an ion pair between the protonated pteridine ring and the carboxylate of Asp-26 (Bolin et al., 1982; Cocco et al., 1981b) which is possible only in this orientation.

In a recent study, Cheung et al., (1993) investigated the tautomeric and ionisable states of the pteridine rings in forms I, IIa and IIb. The authors compared the $^{13}$C chemical shifts of the pteridine ring carbons in the different forms with values obtained from 'model' compounds with known tautomeric and ionisation states. Conclusions were formed from these results and by consideration of $^1$H chemical shifts of the enzyme complexes. Folate in form IIb, the catalytically active conformation, was shown to exist in the keto form with N-1 unprotonated, whereas folate in form IIa and form I exist in enolic forms. The postulated keto form (N-1 unprotonated) for form IIb had one less proton than form I, the enol (N-1 protonated), which could account for the observed pH dependent conformational equilibrium. The proposed structure for form IIa, the enolate (N-1 protonated), would also agree with this explanation.
In the present study, it has been possible to detect the signals for the bound T-9 in the DHFR-[7,9-3H]folate-NADP⁺ ternary complex. At high pH, three separate signals (shifted upfield from the free resonance) are present in the T-9 region of the spectrum. The resonance of one of these signals (-0.59 ppm) corresponds to that predicted by Johnson and Bovey ring shift calculations for one of the tritons on C-9, i.e., an upfield shielding contribution of ca. 0.9 ppm. The two tritons at the 9-position will be non-equivalent in bound folate and thus, at pH 7.4 where forms IIa and IIb predominate, four signals for the T-9 nuclei are predicted. If one of these T-9 nuclei in its bound environment gives rise to only one broad signal at the position predicted by Johnson and Bovey calculations, then the other two signals (at 0.31 and 0.08 ppm) could correspond to forms IIb and IIa for the other T-9 nuclei.

At pH 5.3, the T-9 signals for the bound folate still feature the high ring current shifted signal at -0.61 ppm, but the lower field signals are now centred at 0.13 ppm. The close similarity in position of the resonance signals at 0.08 ppm (pH 7.4) and 0.13 ppm (pH 5.3) suggests that the T-9 nuclei in the two forms could be in a similar environment in the binding site, i.e., in forms IIa and I. Thus, the signals at 0.31 and 0.08 ppm could correspond to form IIb and IIa, respectively, and the signal at 0.13 ppm, at pH 5.3 corresponds to form I of this particular T-9 nuclei. However, it can be seen that the similarity in all of the chemical shifts at pH 7.4 and 5.3 indicates that both the T-9 nuclei are in similar environments in forms I, IIa and IIb.

These data are consistent with the observation that for the DHFR-folate-NADP⁺ ternary complex, the pteridine ring of folate has different orientations in the binding site, in the different forms, whilst the rest of the molecule binds in essentially the same orientation in all three forms.

Further evidence for the similarity in binding site of the p-aminobenzoylglutamic acid portion of the molecule is provided by the ³H NMR spectra of the DHFR-[3',5',7-³H]folate-NADP⁺ ternary complex. At pH 7.4, the existence of the two conformations
present at high pH was confirmed by the observation of two distinct T-7 bound signals. Their resonance positions (4.18 and 5.73 ppm) corresponding to those observed for the DHFR-[7,9-3H]folate-NADP\(^+\) complex at high pH (Figure 3.11a). In contrast, only a single resonance was detected for the 3',5'-T nuclei from the benzoyl ring of bound folate (at 1.89 ppm). This is consistent with the results obtained by Birdsall et al. (1989b), who observed only a single \(^1\)H resonance (at 1.84 ppm) for forms IIa and IIb at high pH, for the enzyme-folate-NADP\(^+\) ternary complex. The appearance of a single peak, well shifted from the position of the free (ca. 1.3 ppm upfield of the free), indicates that the benzoyl ring is occupying essentially the same binding site in both conformations.

From the \(^3\)H NMR results obtained at lower pH values, it is impossible to comment on the presence of form I of the complex at low pH. Birdsall and co-workers (1989b) identified a resonance at 2.25 ppm, as that from form I of the ternary complex at low pH. However, the signal at 2.5 ppm in the \(^3\)H spectra (Figures 3.14 (b-d)) has been shown to arise from a tritium decomposition product. It is possible that the signal observed at pH 5.3 at the same resonance position (1.89 ppm) as that observed at pH 7.4 corresponds to form I of the complex, hence, indicating that the benzoyl ring has the same binding site in all three conformations. Furthermore, the equivalence of the 3', and 5', chemical shifts shows that the benzoyl ring is undergoing relatively rapid ring flipping.

These observations are consistent with the results obtained for the binding of the benzoyl ring of folinic acid (5-formyl-5,6,7,8-tetrahydrofolic acid, Feeney et al., 1981). Identical shifts for the H-3' and H-5' benzoyl protons (and for the H-2' and H-6' protons) were observed for folinic acid in its complexes with DHFR. This equivalence was attributed to rapid ring flipping of the benzoyl ring about the N-10-C4' and C-1'-CO bonds in the bound state, which would lead to the observed equivalence of shielding effects and hence chemical shift. Similar effects have also been observed for the trimethoxy benzyl ring of trimethoprim bound to DHFR (Cayley et al., 1979).
However, ring flipping will be impeded by the steric interactions between the benzoyl ring and residues on the protein. If, as is expected, the benzoyl ring is binding in a site similar to that in methotrexate in the enzyme-methotrexate-NADPH complex (Matthews et al., 1978; Bolin et al., 1982) then the side chains of Leu-27, Phe-49 and Leu-54 will be in contact with the ring, and there are also other multiple contacts involving both glutamate and pteridine ends of the molecule which contribute to the tight binding to the enzyme. It is known that conformational fluctuations can take place within protein structures which can momentarily remove the steric interactions impeding ring flipping (Wuthrich and Wagner, 1975). For folinic acid, it has been proposed that the flipping motion leading to the equivalence of the aromatic protons in the p-aminobenzoyl moiety takes place when the folinic acid is fully bound to the enzyme and requires a transient "breathing" of the structure in the vicinity of the bound ligand.

Hence, from the chemical shifts of the tritons on C-9 and C-3' and C-5' of the benzoyl ring in bound folate, it is proposed that the non-pterin part of folate is binding in a very similar way in the different forms, with the benzoyl ring undergoing ring flipping at a rate sufficiently fast to give an average signal for the 3',5'-tritons.

3.5.2. Binary and Ternary [3',5',7-3H]Methotrexate Complexes Formed with L. casei DHFR

The observation of a single resonance in the 3H NMR spectra for bound methotrexate in both the binary and ternary complex with L. casei DHFR confirms the presence of a single conformational state for the inhibitor binding. The observed resonance corresponds to the bound pteridine T-7, with no signal being seen for the 3',5'-nuclei.
Chapter 3: Tritium NMR Binding Studies

The availability of crystal structure data on the ternary complex of *L. casei* DHFR with methotrexate and NADPH (Matthews *et al.*, 1978; Bolin *et al.*, 1982) has proved to be of vital importance as an aid to assigning the protein and ligand chemical signals and in assisting the interpretation of the chemical shift perturbations seen in related complexes. From the crystal structure, the amino acid residues close to methotrexate can be identified. The shieldings of several of the protons in the amino acid residues in or close to the binding site of MTX are directly influenced by ring current contributions from the pteridine and benzoyl rings of MTX. Any small relative movements of the methotrexate aromatic rings and amino acid residues will thus result in chemical shift changes of the protons in these protein residues. Similarly, changes in chemical shifts observed for ligand protons may have shielding contributions from ring currents of neighbouring aromatic residues in the protein and also from aromatic rings within the ligand itself. For example, the H-7 proton of MTX is expected to have ring current shift contributions from Phe-30 (-0.248 ppm), from Phe-49 (0.045 ppm) and from the benzoyl ring of MTX (-0.365 ppm) (Hammond *et al.*, 1987). This gives a total shift of -0.59 ppm. The observed experimental chemical shift of T-7 in the bound enzyme ternary complex is shifted upfield from the T-7 signal of the free ligand by 0.57 ppm. Thus, there is good agreement between the calculated and experimental values, indicating that the pteridine part of methotrexate binds to DHFR in the same way in the crystal and in solution. Further evidence for this observation was obtained from NOE experiments as described previously.

Although ring flipping of the benzoyl ring has been characterised in the complex with 3',5'-difluoromethotrexate, similar information for the parent compound has never been obtained. A combination of broad methotrexate $^1$H signals and the presence of overlapping protein signals have made it impossible to detect directly signals for the 2',3',5',6'-proton signals of bound methotrexate in the $^1$H spectra. In the present study, the absence of the 3',5'-signal(s) in the tritium spectra has been explained by the
suggestion that the exchange process leading to extensive line broadening of these signals, is the intermediate ring flipping rates of the benzoyl ring. Using McConnell's modified Bloch equations (McConnell, 1958) the line shapes have been calculated for the enzyme-[3',5',7-3H]methotrexate complex, assuming exchange between two equally populated non-equivalent sites. It is seen from the spectra (Figure 3.16) that at rates between 500 to 4000 s⁻¹ the signals are so broad that they would not have been detected at the prevailing signal to noise ratio. The rates of flipping of the benzoyl ring are not known for these complexes but they have been measured for binary and ternary complexes of DHFR with 3',5'-difluoromethotrexate and NADPH (Clore et al., 1984) where values of 7000 s⁻¹ at 298 K (binary) and 20 000 s⁻¹ at 298 K (ternary) were obtained. From the activation energies determined in this study, one can estimate that an eight fold increase in rate (from 500 to 4000 s⁻¹) would be achieved by increasing the temperature by 30⁰ (from 283 - 313 K). Thus, the observed rates of ring flipping over the temperature range used to examine the DHFR complexes with the tritiated methotrexate are somewhat lower, but not too dissimilar from those measured earlier for the complexes with 3',5'-difluoromethotrexate.
3.6. CONCLUSIONS

The findings presented in this chapter indicate that tritium NMR spectroscopy provides a very direct method for examining multiconformational states and dynamic processes in protein-ligand complexes. The presence of the three pH dependent forms of bound folate has been confirmed.

The major differences in the conformation of the pterin ring in the different forms have been characterised previously. The present results, by providing information on the similar chemical shifts of the tritons C-9 and C-3' and C-5' of the benzoyl ring in bound folate, indicate that the non-pterin part of folate is binding in a similar way in the different forms. Ring flipping rates for the benzoyl ring in bound folate are sufficiently fast to give an averaged signal for the 3',5'-tritons.

The results for the complexes of L. casei DHFR with methotrexate and NADPH indicate that a single major conformation exists for the binary DHFR-methotrexate complex and also for the ternary complex with NADPH. The line broadening which prevents observation of a signal for the 3',5'-tritons of the benzoyl ring in bound methotrexate is consistent with the ring flipping being insufficiently fast to give chemical shift averaging of the 3',5'-signals.
Nuclear Overhauser Effects in Tritium NMR Spectroscopy

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Chapter 4: NOEs in Tritium NMR

4. NUCLEAR OVERHAUSER EFFECTS IN TRITIUM NMR SPECTROSCOPY

4.1. INTRODUCTION

The assignments of resonances in NMR spectra of complex molecules is often far from trivial. A variety of chemical perturbations can be helpful in this context (Dwek, 1973). One way however, that requires only an instrumental perturbation is to introduce a second radio-frequency pulse in the NMR experiment. A subtle example of this double irradiation effect is the nuclear Overhauser enhancement/effect (NOE), which can lead to structural information as well as providing an increase in signal-to-noise ratio, which is particularly important in $^{13}$C NMR spectroscopy.

The nuclear Overhauser effect is a change of NMR signal intensity brought about by perturbation of the equilibrium populations of nuclear spin states; the perturbation is usually achieved by selective irradiation of the signal(s) for a particular spin in a double irradiation experiment. The spins involved may be either heteronuclear or chemically shifted homonuclear spins. NOEs are due to dipolar interactions (through-space) between different nuclei and are correlated with the inverse sixth power of the internuclear distance. They are customarily quoted in percent of the unperturbed resonance intensity. Often, one proton resonance in the spectrum of the material under study is irradiated continuously while changes in the intensities of other proton signals are observed after the spin system comes to equilibrium.

The potential of the NOE for providing information on the conformation and configuration of molecules in solution was first demonstrated by Anet and Boun (1965) and since that time applications in this area have grown rapidly. When combined with
difference spectroscopy or a two dimensional approach, the nuclear Overhauser effect provides one of the most easily exploited methods of structure determination available.

Observations of $^1\text{H}(^1\text{H})$ nuclear Overhauser enhancements play a central role in studies of the structures of biological macromolecules in solution (Wüthrich, 1986). The approaches used to provide the requisite assignment of the proton spectrum of the system of interest, and the estimation of internuclear distances needed for secondary and tertiary structure determination from multidimensional NOE experiments have developed rapidly. However, these methods begin to falter when proteins are larger than ca. $M_r$ 15000, because, with increasing size, spectral crowding and the linewidths of individual resonances both increase, thereby reducing resolution. With larger structures it is necessary to enrich specific positions with $^{13}\text{C}$ or $^{15}\text{N}$ (Fesik and Zuiderweg, 1990) or introduce an extrinsic nucleus such as $^{19}\text{F}$ (Gerig, 1977, 1980) to obtain structural information.

Tritium NMR spectra from appropriately labelled materials are useful for addressing many questions in a small molecule context (Evans et al., 1985), but there have been few examples of tritium NMR studies with protein systems (Gehring et al., 1991; O’Connell et al., 1993a, b). O’Connell and co-workers (1990) first demonstrated the feasibility of $^3\text{H}(^1\text{H})$ NOE experiments with materials of $M_r$ 25 000 by conducting 2D $^3\text{H}(^1\text{H})$ NOE experiments on an $\alpha$-tritiated tosyl derivative of chymotrypsin, thus revealing through-space dipolar interaction at the active site of the enzyme. Since this report very little has been published on the exploitation of this $^3\text{H}(^1\text{H})$ NOE technique with protein systems.

This chapter presents a description of spin-lattice relaxation ($T_1$), and its relationship to the nuclear Overhauser enhancement in systems of small molecules and the macromolecular system. For more detailed derivations and explanations the reader is referred to Noggle and Schirmer (1971).
First, the theory and experimental considerations of the NOE and spin-lattice relaxation rate are reviewed together, as their origins and interpretation are related. Next, the applications of the NOE difference, heteronuclear NOE and 2D NOESY experiments are presented with specific attention to protein-ligand systems, and finally, the use of $^3\text{H}^1\text{H}$ heteronuclear NOEs is discussed in terms of its current use in small molecules and its future applications.
4.2. THEORY

In the NMR experiment, excess spin population is moved from one energy level to another by electromagnetic radiation of the appropriate frequency. This is excitation. The radiationless return to equilibrium is called spin-lattice relaxation ($T_1$), because the excess energy passes from the spins to the lattice (or surroundings) as heat. These can arise from many sources, but for both protons and carbons in diamagnetic molecules, the dominant fields are due to the magnetic moments of protons in the same molecules as they tumble in solution. This is a dipole-dipole interaction and is the dominant relaxation mechanism associated with the nuclear Overhauser effect. The rate, or efficiency ($R = 1/T_1$) of dipole-dipole relaxation depends on both the frequency and strength of the fluctuating magnetic fields. These, in turn, depend on three factors (i) the distance between the nuclei involved, $r_{IS}$, for nuclei $I$ and $S$, (ii) the effective correlation time, $\tau_c$, of the vector joining the nuclei (this corresponds to the reciprocal of the rate of tumbling in solution of the relevant part of the molecule) and (iii) the nature of the nuclei themselves. Nuclear Overhauser enhancements and the rates at which they increase and decay, are also measures of the strength of the dipole-dipole interaction between two spins, and hence are dependent on internuclear distances and correlation times. This dependence of NOEs on internuclear distances and correlation times leads to their usefulness as structural probes.

4.2.1. Nuclear Overhauser Enhancement in a Two Spin System

4.2.1.1. Steady-State NOEs

The simplest system which can exhibit the NOE consists of two isolated spins which are mutually relaxing, but are not $J$-coupled. Figure 4.1 shows the energy level
diagram for this system. $W_{1l}$ and $W_{1s}$ are the single quantum transition probabilities for $I$ and $S$ spins respectively; they lead to the normal spectrum. $W_2$ is a double quantum transition probability for the two spins to relax simultaneously in the same direction, while $W_0$ is a zero quantum transition probability corresponding to a mutual spin flip. The latter process gives no net relaxation, but leads to the excess energy being moved from one spin to another. This process is particularly important in large molecules. The double and zero quantum processes are not detected directly in the NMR spectra, but they can be detected through their effects on relaxation.

Figure 4.1. Energy level diagram for dipole-dipole relaxation in a homonuclear two-spin system. The $W$s are spin relaxation possibilities.

Spin $S$ is saturated by irradiation of the spin. The populations in the levels linked by $W_{1s}$ are rapidly equalised but initially there is no net change in the differences between the populations linked by $W_{1l}$, levels 1 and 3 having lost equal amounts of populations to levels 2 and 4, respectively. If the state of the spin system is read by a pulse immediately after such a short pre-irradiation is turned off, there will be no $S$ signal and the $I$ signal will have its normal intensity. A new population distribution is generated however by relaxation through $W_0$ and $W_2$ as irradiation of $S$ is continued. $W_2$ increases the intensity
of the $I$ transitions by attempting to establish a Boltzmann distribution between levels 1 and 4, while $W_6$ decreases the $I$ intensity by equilibrating levels 2 and 3. The resulting net change in the intensity of $I$ as a result of the competition between $W_2$ and $W_6$ is the nuclear Overhauser enhancement. An increase in intensity is a positive NOE and a decrease is a negative NOE. Experimentally, the steady-state NOEs are detected without interference from decoupling effects using the inverse gated decoupling sequence shown in Figure 4.2, the irradiation time is long compared with any of the relaxation times.

$$I_{\text{H}}$$

Decoupler

$$AQ$$

Figure 4.2. Pulse sequence for steady-state NOE experiments

If the initial intensity of $I$ before irradiation is $A_0$, then the fractional nuclear Overhauser enhancement $f_I(S)$, of the total intensity of the resonance of spin $I$ when spin $S$ is saturated is defined as:

$$f_I(S) = (A_S - A_0) / A_0 \quad (4.1)$$

where $A_S$ is the intensity of $I$ when $S$ is irradiated. Noggle and Schirmer (1971) have shown by considering the details of population movements between the four levels, that if 100% of the relaxation of $I$ is via dipole-dipole relaxation by $S$, and if the irradiation is
continued for a very long time compared with all relaxation times so that a steady state is reached, then the full equation is

$$f_i \{S\} = \frac{S_0}{A_0}(\sigma_{JS}/\rho_i)$$

(4.2)

where

$$\sigma_{JS} = W_2 - W_0$$

(4.3)

and

$$\rho_i = 2W_{11} + W_0 + W_2$$

(4.4)

where $S_0$ is the initial intensity of the $S$ resonance. $\sigma_{JS}$, and the NOE may be either positive or negative depending on the relative sizes of $W_2$ and $W_0$ and they in turn depend on the correlation time, $\tau_c$.

Relaxation through $W_1$ requires magnetic field fluctuations or magnetic noise which has a component at, or close to the Lamor precession frequency of the nucleus concerned, $\nu_0$, i.e. $10^8$-$10^9$ Hz, while $W_2$ requires field fluctuations around $2\nu_0$. Because the necessary field fluctuations are produced by proton tumbling at a rate $(\tau_c)^{-1}$, $W_1$ and $W_2$ are most efficient when $\nu_0 \tau_c \approx 1$. The correlation time, $\tau_c$, is a measure of how rapidly the molecule undergoes reorientation in solution and for a small molecule such as methanol, $\tau_c$ is about $10^{-12}$ seconds, thus it tumbles in solution rapidly.

The spectral density $S(\omega)$ is the intensity, or concentration of fields at a particular frequency, $\omega$. For molecules, such as methanol, the spectral density near $10^9$ Hz is very low, $W_1$ and $W_2$ are inefficient and the relaxation is very slow, around 0.01 - 0.1 s$^{-1}$ for both protons and carbons. Similarly, in large molecules or viscous solvents where $\nu_0 \tau_c > 1$, slow tumbling cannot give rise to fields at the appropriate frequency for $W_1$ and $W_2$. These are then inefficient relaxation processes. When $\nu_0 \tau_c$ is close to 1, the spectral density or intensity of appropriate field fluctuations, is at a maximum and hence the relaxation is most efficient. Thus, spin-lattice relaxation is most rapid at the $R_1$-maximum where $\nu_0 \tau_c \approx 1$. ($R_1$-maximum traditionally known as $T_1$-minimum).
$v_0$ depends on the field strength, the relaxation rate of a particular spin is field dependent
in molecules where $v_0 \tau_c \approx 1$.

In contrast, $W_0$ connects two levels that are very close in energy. Fields of low
frequency are required and it is most efficient when molecular tumbling is very slow. In
rapidly tumbling molecules $W_0$ is relatively unimportant and the lifetime of the excited
state is determined almost completely by the $R_1$ processes, $W_1$ and $W_2$. When $v_0 \tau_c > 1$,
{$W_1$ and $W_2$ are ineffective but $W_0$ is efficient, although spin-lattice relaxation is slow, the
lifetime of an individual spin state is short because the excitation passes via mutual spin
flips ($W_0$) to other spins. Therefore, when $v_0 \tau_c > 1$, the spin-lattice relaxation, $T_1$, is
long, but the spin-spin relaxation, $T_2$, is short. As a result of the short $T_2$, the NMR
signals are broad. In contrast, when $v_0 \tau_c < 1$ the molecules are said to be in the extreme
narrowing limit (the linewidth is determined by instrumental rather than fundamental
factors).

In the limit of fast tumbling and complete dipole-dipole relaxation of $I$ by $S$, Eqn.
(4.2) simplifies to:

$$ f_I(S) = \frac{\gamma_s}{2\gamma_i} \quad \text{(4.5)} $$

where $\gamma_i$ and $\gamma_s$ are the magnetogyric ratios of nuclei $S$ and $I$. For any homonuclear
decoupling experiment, $\gamma_i = \gamma_s$, i.e., when both are protons, the Eqn. (4.5) gives a
maximum NOE of 0.5, i.e. 50% enhancement, irrespective of the distance between
nuclei.

In 1D experiments with relative line intensities 1 in the absence of NOEs, the line
intensities with NOE then become:

$$ I_I = 1 + f_I(S) \quad \text{(4.6)} $$
In the observation of $^{13}$C spectra, irradiation of directly bonded hydrogens can result in a maximum NOE of 1.987, i.e., a signal increase of $(1 + f_{c}(H))$ or 2.987. For the analogous case of observing $^{15}$N and decoupling $^1$H, the maximum NOE is -4.93, as the $^{15}$N isotope has a negative value of $\gamma$. Thus, on proton noise decoupling a $^{15}$N signal with the maximum NOE, the signal is reversed and is 3.93 times as large. However, in the case where the dipole-dipole contribution is less than 100% it is possible for the NOE to be close to -1, leading to a complete absence of signal.

In the limit of very slow tumbling, Eqn. (4.2) simplifies for the homonuclear case to:

$$f_{s}(s) = -1$$  \hspace{1cm} (4.7)

which corresponds to a complete loss of the $I$ signal when $S$ is irradiated and results from $W_{0}$ transferring the saturation of $S$ to $I$. In such a very slowly tumbling multi-spin system this phenomenon can be a useful means of suppressing macromolecule signals (Stoez et al., 1978). This is possible if the excitation of $I$ passes to the next spin and the next etc., irradiating a single resonance thus can abolish the entire spectrum of the molecule. The propagation of population disturbance from one spin to another in this way is known as spin-diffusion, it is the population information which is actually diffusing, rather than the spins themselves. In the extreme slow tumbling limit mentioned above, spin-diffusion is both rapid and far-reaching, however in the fast tumbling extreme it is more limited.

It has been seen that steady-state NOE in a two spin system contains no distance information. For a fast tumbling homonuclear case a 50% enhancement will always be seen, whilst in a slow tumbling system the signal should disappear. However, for a rigid molecule tumbling isotropically, the rate at which the enhancement develops (and the spins relax) is dependent on the internuclear distance, $r_{IS}$, as shown:

$$\rho_{r} = \gamma_{S}^{2} \gamma_{I}^{2} r_{IS}^{-6}$$ \hspace{1cm} (4.8)
The important terms in Eqn. (4.8) are the IS distance \( r_{IS} \), and the correlation time, \( \tau_c \). As the internuclear separation, \( r_{IS} \) increases, the intensity of the field produced by their interaction decreases and so the efficiency of relaxation also decreases. Thus the NOEs grow more slowly and the protons will have longer values of \( T_1 \). The \( r_{IS}^{-6} \) dependence is a crucial point in the correct application of the NOE.

Any process that induces changes in the population of one spin will lead to a NOE at the other spin. Saturation by irradiation is one way in which this population change may be brought about. Chemically Induced Dynamic Nuclear Polarisation (CIDNP) is another. This procedure involves the formation of radicals in a solution by chemical or photochemical processes and can lead to massive population changes for those protons that are coupled to the unpaired electrons. These abnormal populations then induce NOEs at nearby spins in the usual way (Hore et al., 1982; Feeney et al., 1980b).

### 4.2.1.2. Transient NOEs

An alternative method for inducing NOEs is to apply a selective 180° pulse on one of the resonances. This may be applied using a soft pulse from the decoupler. The result is to invert, within a few milliseconds, the populations of the levels associated with the \( S \) spin. As in the steady-state experiment, \( W_0 \) and \( W_2 \) begin to redistribute this non-equivalent population, and the NOE at \( I \) starts to build up as before. However, in this experiment, the non-equilibrium distribution is not being continuously increased, and within a few \( T_1 \)'s both spins will have relaxed back to their original population distribution. The NOE at \( I \) therefore is transient; it builds up to a maximum after about one \( T_1 \), and then declines. The pulse sequence for observing transient NOEs is shown in Figure 4.3.
The NOE growth and decay can be measured by a series of experiments in which the delay between selective $\pi$ pulse and non-selective $\pi/2$ read pulse is varied. The evolution is not observed directly; it is mapped by a series of experiments. The resulting NOE growth and decay rates can be analysed to give the usual $\tau$ and $r_{ls}$ information (Noggle and Schirmer, 1971; Sanders and Mersh, 1982a).

The main importance for the transient NOE is as a conceptual introduction to the two-dimensional NOE, or NOESY, experiment.

In summary, therefore, the crucial difference between the two experiments, steady-state and transient NOEs, is that no radiofrequency (rf) field is applied during the NOE build up in the transient NOE scheme, whereas the build up is driven by continuous irradiation in the steady-state NOE experiment. Therefore, the decay of the NOEs by spin relaxation is readily observable in the transient NOE experiment, where no further energy is transferred to the nuclear spins after the initial $180^\circ$ pulse. In contrast, a steady-state NOE build up, by the continuous irradiation and NOE decay by spin relaxation, is attained for long pre-irradiation times in the steady-state NOE.
4.2.1.3. Two-Dimensional NOEs - NOESY

The NOESY experiment is transient in nature and the pulse sequence is presented in Figure 4.4 and consists of three $90^\circ$ pulses.

![NOESY pulse sequence](image)

Figure 4.4. NOESY pulse sequence

where $t_m$ is a 'mixing' time, during which the NOEs develop and $\tau$ is the delay period. Of key importance in the NOESY experiment is the time delay (mixing time, $t_m$) which follows the second pulse. The nuclear Overhauser effects require a certain amount of time to build up and the length of time chosen for this mixing period depends quite dramatically on the properties of the molecule being investigated. Both size and shape are important as both of these affect the correlation time, which in turn affects the spin-lattice relaxation time and hence the NOE observed. For small rapidly tumbling molecules, with long $T_1$'s, $t_m$ is of the order of 1 - 5 s, whereas for large slowly tumbling molecules with short $T_1$'s, NOEs build up in a much shorter time and therefore the mixing times of the order of 50 - 200 ms are more realistic (Abraham et al., 1988).

The two-dimensional experiment in its simplest form is carried out by incrementing $\tau$, while keeping $t_m$ constant during the experiment, and employing phase cycling to suppress axial peaks and other artefacts. The cross-peaks consist of responses
corresponding to the size of the transient NOE which has built up during \( t_m \). It is possible, by analysis of how the cross-peak intensity varies with \( t_m \), to extract distance information from the spectra (Keepers and James, 1984).

A heteronuclear 2D NOE spectrum can be obtained by applying the third, read, pulse to the X-spin of interest (Yu and Levy, 1984). This could be \(^{13}\text{C}, ^{15}\text{N}, ^{31}\text{P}\) or any other nucleus which relaxes mainly by dipole-dipole interaction with protons.

### 4.2.2. The NOE in a System with Three Protons

In a three-spin system the combined effects of \( \tau_c \) and distance can become quite complicated.

#### 4.2.2.1. The Linear System

Considering three spins with geometry such that \( r_{AB} = r_{BC} = 1 \) (Figure 4.5):

\[ f_A(B) \approx \rho_{AB} / 2R_A \]  \( (4.9a) \)
\[ f_C(B) \approx \rho_{BC} / 2R_C \]  \( (4.9b) \)
\[ f_B(A) \approx (\rho_{AB} / 2R_y) / (1 - \rho_{BC}^2 / 4R_B R_C) \]  \( (4.10) \)

**Figure 4.5.** Linear three spin system where \( r_{AB} = r_{BC} = 1 \).
Using these values the steady state NOEs are calculated using the Equations (4.9), (4.10) and (4.11) (Noggle and Schirmer, 1971) and are given in Table 4.1.

\[
\begin{align*}
\rho_{AB} & = 1.0 & \rho_{BC} & = -1.0 & \rho_{AC} & = 0.016 & R_1 & = 1.016 \\
\rho_{AB} & = 1.0 & \rho_{BC} & = 1.0 & \rho_{AC} & = 0.016 & R_1 & = 1.016 \\
\rho_{AB} & = 0.016 & \rho_{BC} & = -1.0 & \rho_{AC} & = -11.5 & R_1 & = 2.0 \\
\rho_{AB} & = 0.016 & \rho_{BC} & = 2.0 & \rho_{AC} & = 1.016 & R_1 & = 1.016 \\
\end{align*}
\]

Table 4.1. Relaxation and steady-state NOEs in a linear three-spin system with \( r_{AB} = r_{BC} = 1 \).

From the data in Table 4.1 it can be seen that the central proton B relaxes almost twice as fast as A and C, because it has two equidistant protons providing relaxation pathways. This leads to a maximum enhancement for B of 25% when either A or C is saturated, as A and C each provide only half of the relaxation of B. Proton C is twice as far away from A as B, and so is only \( 2^{-5} \) (i.e. \( 1/64 \)) as effective (and vice versa). Therefore A and C relax more slowly, and saturating B gives 49.2% NOEs to each, according to the equations (Noggle and Schirmer, 1971):

\[
\begin{align*}
  f_A(B) & \approx \rho_{AB} / 2R_A \\
  f_C(B) & \approx \rho_{BC} / 2R_C \\
\end{align*}
\]

The rates \( \rho_{AB} \) and \( \rho_{BC} \) at which the NOEs grow are the same.
If the distance $r_{AB}$ is doubled in the three spin-system, the relaxation rates and steady-state NOEs change and are given in Table 4.2. When B is saturated, the NOEs experienced by A and C are relatively unchanged from the previous case. This is because B is still the nearest spin to both A and C, and hence dominates their relaxation. However, the NOEs at B when A and C are saturated have changed dramatically. $\rho_{AB}$ now contributes very little to the relaxation of B relative to the contribution of $\rho_{BC}$ and hence saturation of A gives a very small NOE at B; by contrast, saturation of C enhances B by almost 50%.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>$f_A$</th>
<th>$f_B$</th>
<th>$f_C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\rho_{AB}$</td>
<td>0.016</td>
<td>0.016</td>
<td>-</td>
<td>{A}</td>
<td>0.8</td>
<td>-0.4</td>
</tr>
<tr>
<td>$\rho_{BC}$</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>{B}</td>
<td>46.0</td>
<td>&gt;49.9</td>
</tr>
<tr>
<td>$\rho_{AC}$</td>
<td>0.001</td>
<td>-</td>
<td>0.001</td>
<td>{C}</td>
<td>-18.6</td>
<td>49.2</td>
</tr>
<tr>
<td>$R_1$</td>
<td>0.017</td>
<td>1.016</td>
<td>1.001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2. Relaxation and steady-state NOEs in a three-spin system with $r_{AB} = 2, r_{BC} = 1$.

Because relaxation is a competitive phenomenon, the NOEs between two protons X and Y depend as much on their distances from other protons as on $r_{XY}$. The NOE observed at B when C is saturated, in the above case, is radically affected by variations in $r_{AB}$ even when $r_{BC}$ is constant. A reduction in $r_{AB}$ to 0.5, would give a NOE of only 0.8% to B, when C was saturated. All that can be measured from the steady-state experiment is relative distance.

The negative enhancements shown in Tables 4.1 and 4.2 are indirect, geometry-induced effects, quite different from the general negative NOEs that occur when $\gamma_i$ is negative or $\omega \tau_i > 1$. In the fast tumbling three-spin system the result of saturating the A spin is to increase the net magnetisation of the B and C spins. Hence, decreasing the
population difference in A, increases the population difference in B and C. The increased population difference in B will lead to a decreased population difference in C i.e., a negative NOE resulting from spin diffusion. This indirect negative contribution to the NOE at C is in competition with the direct positive contribution from A. The size of the indirect contribution is the negative product of the two NOEs involved (Eqn. 4.11). Thus the -11.5% enhancement for $f_c(A)$ in Table 4.1 is the sum of the direct contribution of 0.8% and a negative contribution of 12.3% (25% x 49.2%). Increasing $r_{AB}$ has a dramatic effect on the indirect NOEs. $f_c(A)$ is very small because $f_B(A)$ is so small, but the reverse experiment of irradiating C would give an NOE to A of -18.6% because both $f_B(A)$ and $f_C(B)$ are large.

The linear three-spin system is a favourable geometry because the enhancements are large and can be measured accurately. Relative distances between adjacent spins can be determined easily and accurately. The most important enhancements for determining the ratio $r_{AB}/r_{AC}$, where spin B is between spins A and C are those when spin B is observed. Enhancements between next-nearest neighbours will be small and often negative in this geometry; distances between next-nearest neighbours cannot be determined accurately.

4.2.2.2. Non-Linear Systems

Considering the non-linear system where the A-B-C angle is $\theta$ and $r_{AB} = r_{AC} = 1$ (Figure 4.6). As $\theta$ decreases from 180°, $r_{AC}$ decreases and the direct positive NOE between A and C increases. As a result of this more effective A-C relaxation, the B-C relaxation process becomes relatively less important and the indirect negative A-C contribution correspondingly decreases. When $\theta = 78°$, $r_{AC} = 1.26$ and there is no net NOE between A and C even though the spins are very close together; the direct positive and indirect negative effects are equal and opposite. Compared to the linear case, the
enhancements of the non-linear case are smaller. In the limiting case of three spins on an equilateral triangle, all of the enhancements approach 20% as a maximum.

![Diagram of a non-linear spin system with spins A, B, and C, showing distances r_{AB}, r_{BC}, and r_{AC} and angle θ between A-B-C.](image)

**Figure 4.6.** Non-linear spin system where A-B-C angle is θ and r_{AB} = r_{BC} = 1.

In slowly tumbling systems of three spins, all direct NOEs are negative and the indirect contribution to \( f_{4}\{C\} \), calculated using Eqn. (4.11), is negative also. The direct and indirect contributions are in the same direction and larger as a result of Eqn. (4.5), than in the small molecule case. It is not possible to distinguish between direct negative and indirect negative NOEs by appearance alone. As with the small molecule case, however, they can be distinguished on the basis of their kinetic behaviour. The direct NOE grows without a lag, and initially follows simple kinetics according to \( r^{-6} \), whereas the indirect NOE grows only after a lag phase.

For very slowly tumbling systems, if irradiation is continued indefinitely, Eqn. (4.5) shows that the whole system will experience 100% negative NOE and no signal is observed in the spectrum. This spin diffusion removes all the useful distance specificity from the NOE experiment in large molecules. However, if the irradiation is restricted to a short time, before spin diffusion becomes effective, then the distance specificity is restored. This is known as truncated NOE. Transient and two-dimensional experiments also restore distance specificity provided \( t_m \) is sufficiently short.
4.2.3. Heteronuclear NOEs

The theory for heteronuclear NOEs between spin-1/2 nuclei is similar to that of homonuclear NOEs. However, the ratio of $\gamma$s given by Eqn. (4.4) indicates that (a) unless the $\gamma$s are nearly equal, only experiments where the spin of lowest $\gamma$ is observed are practical; (b) when the spin of lowest $\gamma$ is observed, enhancements may be very large compared to those found in the homonuclear cases. The latter point is particularly important since spins of low $\gamma$ typically have poor signal-to-noise ratios which can be suitably improved by the NOE. This was first demonstrated by Kulilmann and Grant (1968) when they observed the $^{13}\text{C}{^1\text{H}}$ NOE in formic acid and obtained $f_{\text{c}}{^1\text{H}} = 1.98 \pm 0.15$ compared to the theoretical maximum ratio of 1.988. The maximum NOE enhancements for some experiments are listed in Table 4.3.

<table>
<thead>
<tr>
<th>Observe</th>
<th>$^1\text{H}$</th>
<th>$^{19}\text{F}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1\text{H}$</td>
<td>0.50</td>
<td>0.47</td>
</tr>
<tr>
<td>$^3\text{H}$</td>
<td>0.47</td>
<td>0.44</td>
</tr>
<tr>
<td>$^{13}\text{C}$</td>
<td>1.99</td>
<td>1.87</td>
</tr>
<tr>
<td>$^{15}\text{N}$</td>
<td>-4.93</td>
<td>-4.64</td>
</tr>
<tr>
<td>$^{19}\text{F}$</td>
<td>0.53</td>
<td>0.50</td>
</tr>
<tr>
<td>$^{31}\text{P}$</td>
<td>1.24</td>
<td>1.16</td>
</tr>
</tbody>
</table>

Table 4.3. Maximum NOE enhancements for various heteronuclear experiments.

4.2.4. NOE Difference Spectroscopy

The difference method is the most sensitive and rigorous of experiments by which NOEs may be detected and spatial and structural information deduced. The theoretical
lower detection limit is set only by the available dynamic range; in practice the limit is usually set by signal-to-noise considerations and instrument stability. Furthermore, the signals to be monitored do not even need to be resolved in the normal spectrum. It is possible routinely to observe 1% enhancements of completely hidden multiplets. This methodology extends from the fact that NOEs are easier to detect by the subtraction of one spectrum or FID from another, resulting in the so-called difference spectrum in which unaffected signals disappear leaving only the enhancement itself and the saturated resonance. In the first spectrum a signal is saturated, using gated decoupling, whilst in the second spectrum irradiation is performed away from any signals. Subtraction of the second spectrum from the first gives the difference spectrum that uncovers any NOEs.
4.3. EXPERIMENTAL CONSIDERATIONS

4.3.1. Optimising NOE Difference Spectra

The ideal NOE experiment gives the maximum intensity of enhancements with total sensitivity in the irradiation and no artefacts from imperfect subtraction. In practice there are certain factors which are not perfect e.g., frequency selectivity and subtraction, however careful design and setting-up of the experiment can dramatically improve both the appearance of the spectrum and the chance of a successful solution to the problem.

4.3.1.1. Maximising Enhancements

In order to obtain the largest possible useful NOE, the contribution of intramolecular dipole-dipole relaxation should be maximised. This can be achieved by using dilute solutions in solvents without high concentrations of protons (to avoid problems from intermolecular interactions). In the case of very small molecules the presence of paramagnetic molecules can have a profound effect on the spectrum. Where the paramagnetic species are metal ions the result may simply be very broad or unobservable NMR signals. However, the most common paramagnetic species is dissolved oxygen and its effects can be extremely severe for the relaxation or NOE experiment. Because $\gamma_{\text{electron}}$ is about 1000 times bigger than $\gamma_{\text{n}}$, nuclear relaxation, being dominated by the unpaired electron, will be rapid. Intramolecular dipole-dipole relaxation will be relatively less important and there is a resulting loss of useful proton-proton information. In very small molecules, where intrinsic relaxation rates are slow, the contribution of dissolved oxygen may dominate relaxation (in organic solvents its contribution is of the order of 0.1 s$^{-1}$) whereas, in contrast its effects will be insignificant in larger molecules which relax rapidly. Therefore, for very small molecules, it is
necessary to remove as much dissolved oxygen as possible by de-gassing the sample either by the 'freeze-pump-thaw' method or more simply by bubbling dry (oxygen-free) nitrogen or argon through the solution in the NMR tube for a few minutes.

These techniques help to remove external sources of relaxation which slow down the overall relaxation rate, but do nothing to increase the rate of dipole-dipole relaxation and NOE growth. Where $\rho_{NN}$ is very slow, the use of a higher viscosity solvent can help to increase $\tau_e$.

Hence, when relaxation delays and irradiation times are being set for NOE experiments, relaxation times are an important consideration. For the steady-state where NOE signal/noise is to be maximised, it is most efficient for the irradiation time to be 1-2 average $T_1$'s for the acquisition time to be the minimum consistent with digital resolution requirements and for the start of the next irradiation, at the same frequency, to begin immediately after the end of the acquisition, with no relaxation delay in-between. However, the use of this technique for NOEs between distant nuclei and indirect three-spin and spin diffusion effects is unsatisfactory. For these effects to be observable, irradiation times of up to $10T_1$'s would be more appropriate in order to maximise these long range and indirect enhancements.

If the acquisition time is short relative to decay rates, then the enhancements will build up from one acquisition to the next, eventually approaching the steady-state value. In this case, the irradiation time between pulses is virtually irrelevant, provided that a few dummy scans are inserted at the beginning of the sequence to allow enhancements to build up before the first acquisition.

4.3.1.2 Acquisition Strategies

In a NOE difference experiment it is necessary to acquire the control and irradiated spectrum as nearly simultaneously as possible, this helps to eliminate any
artefacts present due to minute phase or frequency shifts between the two spectra which can ruin the resulting difference spectrum. To achieve this, the separate irradiations are interleaved. When phase cycling and quadrature detection are employed, at least eight transients are acquired with irradiation at the first frequency $v_1$, then eight irradiating at $v_2$, the decoupler frequency being shifted automatically under computer control. The control spectrum is generated by irradiating at a frequency which is in a chemical shift region that contains no signals. When several different irradiations are required, two different experimental methods are available. In the original method (Richarz and Wüthrich, 1978) the FIDs from the control irradiation are subtracted during acquisition so that only the difference accumulates. After a pre-determined number of transients the difference FID is stored and the next irradiation experiment begins. In the second method (Hall and Sanders, 1980) FIDs from each irradiation are stored separately for later processing. After eight transients have been acquired for irradiation at each frequency $v_1 - v_2$, the whole experiment is repeated until adequate signal-to-noise has built up. The second method has several advantages:

(i) Only a single control spectrum for all the different $n$ irradiations is required, making $n + 1$ experiments in total. If the difference is directly acquired then the control is required $n$ times, requiring $2n$ experiments. This leads to increased lengths of time for a given signal-to-noise ratio, for example, when $n$ is 10 - 20, which is often the case in natural product work.

(ii) The entire sequence can be stopped at any time in the knowledge that each desired irradiation has been completed, as time elapses the signal-to-noise ratio of each experiment improves.

(iii) The availability of the control spectrum allows calibration of intensity of responses in the difference spectrum.

(iv) With only one control spectrum being acquired it is possible to use a 'hit and miss' approach to analyse complex spectra. The maximum number of different
irradiations is carried out in one experiment (consistent with adequate signal-to-noise for each) and the results are analysed afterwards. It is possible that some were unnecessary or uninformative, but this is less demanding of instrument time than returning for additional experiments and the necessary acquisition of a new control spectrum; it is also possible that the NOE can be observed in both directions, along with the possibility that the extra irradiations will provide unexpected information (Mersh and Sanders, 1981).

4.3.1.3. Minimising Subtraction Errors

For the difference experiment to be successful the utmost performance of the NMR spectrometer is necessary. To eliminate the unaffected signals entirely, the two spectra must coincide exactly, with the signals being at the same absolute frequencies.

\[ \text{Figure 4.7. Control and NOE displays of a small portion of a 400 MHz spectrum of heptamethyl cobyrrinate. (a) Control spectrum (b) uncorrected NOE spectrum (c) the corrected difference spectrum obtained after a 0.005 Hz relative shift of the two spectra (Sanders and Mersh, 1982b).} \]
Imperfect subtraction of signals leads to artefacts which look like dispersion responses. In population experiments where the decoupler is off during acquisition, the artefacts arise from small random phase or frequency shifts between acquisitions, the effects of a few millihertz or tenths of a degree shift can be quite severe (Sanders and Mersh, 1982b). Figure 4.7 shows the effect of corrected and uncorrected NOE difference spectra of heptamethyl cobyricinate.

Hence, the frequency generator of the instrument must be stable to within narrow limits throughout the course of the experiment. A further precaution is to acquire a large number of transients, preferably overnight. This helps because the artefacts, like other random noise, decrease relative to useful signals as the number of transients is increased.

4.3.2. Optimising NOESY Spectra

In general, $^1$H-$^1$H NOESY is not the method of choice for very small molecules, although it has been successfully applied to the relatively small cytochalasin B (Graden and Lynn, 1984). Detailed guides have been given for the application of NOESY and other 2D techniques to proteins (Wider et al., 1984) and to t-RNA (Hare et al., 1985).

The mixing time, $t_m$, between the mixing and read pulses should be set to around $T_1$ for the protons involved, in order to maximise the transient NOE which can be recorded. In most molecules there is quite a substantial range of relaxation times, and one is usually interested in NOEs across a range of distances, so it is frequently necessary to acquire two or three spectra with different mixing times.
4.4. APPLICATIONS OF THE NOE EXPERIMENT

The most important property of proton-proton NOE difference and 2D NOE spectroscopy is the ability to make connections between nuclei through space, independently of any chemical bonding considerations. A second valuable feature is its ability to extract, from a mass of overlapping resonances, a single multiplet whose chemical shifts and coupling constants can then be measured. The chemical and biological problems to which NOEs have been applied fall into four broad categories: the assignment of spectra, determination of covalent structure, the determination of conformation and the study of molecular motion. There is much overlap between these and to a large extent, a problem in one area can be solved only by making assumptions about the other three.

4.4.1. Assignment of Spectra

NOE difference spectroscopy was originally developed in order to help assign protein spectra, but it has proved to be a powerful method for assigning both simple and complex organic molecules (Richardz and Wüthrich, 1978). The ability to reveal hidden multiplets is often as important as making through-space connections.

For the study of protein-ligand complexes, chemical shift degeneracies not only make it difficult to assign the NMR spectra to residue types, but also complicate the analysis of 2D NOESY experiments. This is particularly difficult when one proton has NOE connectivities to several others; any ambiguity in the assignment of the nuclei contributing to the NOESY cross-peaks makes it very difficult to apply the sequential assignment method (Wüthrich, 1986) in a straightforward manner. Although 2D or 3D methods (e.g. combining HOHAHA and NOESY) contribute to removing some of the ambiguities, the short $T_2$ values of larger proteins make these methods difficult to use.
Selective deuteration has often been used to simplify the $^1$H spectra in these cases (Birdsall et al., 1984; Feeney et al., 1989). By careful choice of the deuteration pattern it is possible to simplify 2D NOESY spectra so that unambiguous connections can be made between the remaining protons in the molecule (Feeney et al., 1989; Birdsall et al., 1990). For proteins which are too large to assign by using the direct sequential assignation method, the NOE data can be used in conjunction with available crystallographic structural information to allow specific assignments to be made (Hammond et al., 1986, 1987). The observed NOE cross-peaks can be correlated with anticipated distances $r$ (or $r^{-6}$ values) taken from the crystal structure data (assuming the solution and crystal structures are the same). This approach has been used for *L. casei* dihydrofolate reductase ($Mr$ 18 300) based on the refined crystal structure of the enzyme-methotrexate-NADPH complex (Matthews et al., 1979; Bolin et al., 1982).

Birdsall and co-workers (1984) reported the combined use of selective deuteration and NOE difference experiments to assign the valine and tyrosine resonances of *L. casei* DHFR. Selective deuteration of the enzyme provided information on the resonances of the 16 valine residues in the enzyme, but to assign $^1$H resonances to individual valine residues, the use of NOE experiments was essential to identify residues close in space.

By using a combination of 1D and 2D NOE methods in conjunction with the enzyme-methotrexate-NADPH crystal structure data, Hammond et al. (1986) were able to assign resonances for all the histidine and tyrosine residues, as well as several valine, leucine, isoleucine and phenylalanine residues in *L. casei* DHFR. Selective deuteration of the enzyme provided the necessary selectivity, particularly in 1D experiments. Further examples of the use of selective deuteration in combination with NOESY experiments to assign protein resonances in *L. casei* DHFR include the identification of signals from all eight Phe (Feeney et al., 1989; Birdsall et al., 1990) and four Trp (Birdsall et al., 1990) residues.
One other approach for further simplifying spectra involves 3D $^{15}$N or $^{13}$C isotope editing of $^1$H NMR spectra and this method has provided new possibilities for assigning $^1$H NMR spectra of larger proteins (Fesik and Zuiderweg, 1988). The utility of heteronuclear 3D NMR spectroscopy for resolving spectral overlap is illustrated by the studies of Carr and co-workers (1991) who used 3D NOESY-HMQC experiments on uniformly $^{15}$N-labelled DHFR (as its binary complex with methotrexate) in order to obtain sequential resonance assignments and to extract secondary structure information for the protein in solution (Carr et al., 1991).

### 4.4.2. Determination of Structure

For small molecules, these have generally been of the kind that require the fitting together of several known fragments of a molecule. The problem may include determining the relative stereochemistry of a tertiary or quaternary carbon (Chan and Matlin, 1981) or a double bond which is tri or tetra substituted. It may even be that of finding the correct substitution order of several substituents around an aromatic system. There have been many recent uses of the NOE to solve structural and stereochemical problems, some of which are mentioned in reviews (Noggle and Schirmer, 1971; Sanders and Mersh, 1982a).

In relation to the present work, NOESY spectra have been employed to define the molecular structure of folic acid in DMSO-d$_6$ solution. The solid state structure of folic acid has previously been defined, but there have been few investigations on the structure of the substrate in solution (Rossi et al., 1992). Rossi and co-workers combined magnetic dipolar connectivities and energy minimisation molecular mechanics calculations in order to propose the solution structure of folic acid. Likewise, structural information gained by measuring $^{13}$C-$^1$H NOEs on selective saturation of the
glutamate NH and H-3' of methotrexate (Gaggelli et al., 1993) led to the proposal of a 'preferred' conformation of methotrexate in DMSO-d$_6$ solution.

One- and two-dimensional NOE experiments can also provide structural information about the enzyme binding site by means of the NOEs between protons of the ligand and receptor. For example, in the _L. casei_ DHFR-folate-NADP$^+$ complex, NOEs are observed between the nicotinamide 5- and 6-protons of NADP$^+$ and the methyl resonances of Thr-45 at both low and high pH, indicating the position of the nicotinamide ring in the enzyme binding site (Birdsall et al., 1989b).

In the DHFR-methotrexate-NADP$^+$ ternary complex, NOEs are observed between the pteridine 7-proton of the inhibitor and the methyl protons of Leu-19 and Leu-27 (Hammond et al., 1986). Subsequent 2D $^1$H-$^{13}$C HMQC-NOESY experiments on the DHFR-[4,7,8a,9-$^{13}$C]methotrexate complex were used to obtain further information on the orientation of the inhibitor in the binding site (Cheung et al., 1993). Connections were observed between the C-7 proton and signals at the assigned positions for protons in the methyl groups of Leu-19 and Leu-27 as well as those in the N$_{10}$CH$_3$ group of bound methotrexate. The C-9 protons showed connections to Thr-45(CH$_3$) and Phe-49(ε, ζ) and an intramolecular NOE to N$_{10}$CH$_3$ of methotrexate. No connections were seen between H-9 and the benzoyl ring protons and this was reported to be because the latter have broad signals due to slow ring flipping.

The DHFR-folate-NADP$^+$ ternary complex is known to exist in three interconverting conformational states (Birdsall et al., 1981, 1987) which are pH dependent. At low pH, form I predominates whereas, at high pH forms IIa and IIb predominate. The results from $^1$H NOESY experiments have been used in conjunction with $^1$H, $^{13}$C and $^{31}$P NMR studies to characterise the conformation of the three forms at the binding site. From NOESY experiments it has been shown that forms I and IIa have the pteridine ring in a methotrexate-like orientation (Birdsall et al., 1989a). NOE connections were established between the pteridine H-7 and the assigned methyl protons...
of Leu-19 and Leu-27, the same as observed in the NOESY spectra of DHFR complexes with methotrexate.

More recently, 2D \(^{1}\text{H},^{13}\text{C}\) HMQC-NOESY experiments on ternary complexes formed using \([2,4a,7,9-{^{13}\text{C}}]\)folic acid have been used to establish intermolecular NOEs between the folate H-7 and protons on the protein, and these provide further characterisation of the orientations of the pteridine ring in the different bound forms of folate (Cheung \textit{et al.}, 1993). In the \(^{1}\text{H},^{13}\text{C}\) HMQC-NOESY spectrum of DHFR-[2,4a,7,9-{^{13}\text{C}}]folic acid-NADP\(^{+}\), the H-7 in form IIa shows an NOE connection to the methyl protons of Leu-27 (a similar connection is seen in the \(^{1}\text{H},^{13}\text{C}\) HMQC-NOESY spectrum of the methotrexate complex). The H-7 in form IIb shows a connection with the Ala-97 methyl proton signal, indicating the proximity of these protons. One-dimensional NOE difference spectra selectively irradiating the H-7 signals in forms I, IIa and IIb for the DHFR-folate-NADP\(^{+}\) complex, at pH 6.5, was used to confirm that only form IIb gives a large NOE to the methyl group of Ala-97 (Cheung \textit{et al.}, 1993).

Another technique for studying the structure of proteins and protein-ligand systems is the use of \(^{19}\text{F}\)-labelled proteins, and employing heteronuclear \(^{19}\text{F}\{^{1}\text{H}\}\) NOEs. Heteronuclear \(^{19}\text{F}\{^{1}\text{H}\}\) nuclear Overhauser enhancement experiments have been reported for studying \((4-(\text{trifluoromethyl})\text{benzenesulfonyl})\alpha\)-chymotrypsin (Gerig, 1977, 1980, 1981). Transient \(^{19}\text{F}\{^{1}\text{H}\}\) NOEs have been used to explore the protein environment of the trifluoromethyl group of the modified enzyme. Computational results for a small model spin system which had dynamic properties appropriate for a protein the size of chymotrypsin were presented and these suggested that the NOE experiments should indicate the chemical shifts of those protons of the protein closest to the CF\(_3\) group. Results from this study and those of a \(^{19}\text{F}\{^{1}\text{H}\}\) time development study on the same system (Gerig, 1981) were shown to be consistent with the structural model for the immediate environment of the CF\(_3\) group, proposed in earlier work (Gerig \textit{et al.}, 1979).
4.4.3. Conformational Investigations

If a molecule is flexible, or can take up several distinct conformations in solution then NOEs can be used to identify which spins are close to each other. Many early examples of small molecules were given by Noggle and Schirmer (1971). More recently, the bulk of conformational studies using 1D and 2D NOEs have concentrated on large molecules such as peptides and proteins (Sanders and Mersh, 1982a; Feeney et al., 1983; Wider et al., 1984), polynucleotides and polysaccharides (Sanders and Mersh, 1982a). These tend to be qualitative rather than quantitative, distinguishing, for example between β-pleated sheets and α-helices in proteins (Zuiderweg et al., 1983).

For weakly bound ligands, experimental approaches have been developed to circumvent the problems associated with selectively detecting the resonances of the ligand in the presence of many signals of the macromolecule to which it is bound. These techniques involve the measurement of transferred nuclear Overhauser effects (TNOEs) for ligands that exchange rapidly from the bound to the free state (Albrand et al., 1979; Cayley et al., 1979; Clore and Gronenborn, 1982). Negative NOEs due to the bound state are observed on the free or averaged resonances of the ligand which are narrower than the signals of the macromolecule and easily identified. From an analysis of these NOEs the conformation of a ligand bound to a macromolecule can be determined. In fact, from the measurements of TNOEs the conformation of ligands bound to proteins (Hyde et al., 1980a; Feeney et al., 1983; Birdsall et al., 1987), phospholipids (Wakamatsu et al., 1987) and nucleic acids (Gronenborn et al., 1984) have been reported. The TNOE method can provide quantitative conformational information about a ligand bound to a protein without the necessity for direct observation of the signals of the bound ligand.

Transferred NOE experiments have been used to determine the conformation of the inhibitor trimethoprim bound to L. casei (Cayley et al., 1979), E. coli and L1210 (Birdsall et al., 1983) DHFR.
The chemical shifts of the aromatic proton resonances of trimethoprim in its complex with L1210 DHFR have previously been determined by the saturation-transfer method (Forsen and Hoffman, 1963). When excess ligand is present, the protons of trimethoprim exist in two environments corresponding to the free and bound states; if the rate of exchange between these states is sufficiently slow, separate signals will be seen for each state. In this experiment (Birdsall et al., 1983) the resonances from the excess free trimethoprim were clearly visible, while those from the bound ligand were not.

Figure 4.7. (Lower) Aromatic region of 270 MHz $^1$H spectrum of a sample containing 1 mM trimethoprim and 0.2 mM L1210 DHFR. The aromatic proton resonances of the excess, free trimethoprim are labelled. (Upper) Difference between this spectrum and one in which a saturating radio frequency field has been applied at 2.41 ppm, the resonance frequency of H-2',6', in bound trimethoprim. The marked decrease in intensity of the H-2',6' resonance of free trimethoprim arises from transfer of saturation and the smaller effect on the H-6 resonance from a transferred NOE effect.

Saturating the resonance of a proton in the bound state, transfers this saturation to the free state by the exchange process and a decrease in intensity of the corresponding resonance of free trimethoprim would be observed. Hence, the NOE difference spectrum of L1210 DHFR-trimethoprim (Figure 4.7) showed that irradiation at the resonance frequency of the H-2',6' bound trimethoprim leads to a small decrease in the intensity of the H-6 resonance of free trimethoprim as well as a large decrease in intensity of the H-
2',6' resonance due to a transfer of saturation. Thus, irradiation at the resonance frequency of H-2',6' of bound trimethoprim leads to a decrease in the intensity of the H-6 resonance of the bound ligand by cross-relaxation and this effect is transferred to the H-6 signal of free trimethoprim by the exchange process - the TNOE. Since the sign of the NOE depends on the correlation time of the internuclear vector the observation of a negative TNOE (a decrease in intensity) demonstrates that the effect originated from the bound ligand. Therefore, it was concluded that the conformation of trimethoprim bound to L1210 DHFR is such that H-6 is close to H-2' and/or H-6'; this is also the case for the complex with the \textit{L. casei} enzyme (Cayley \textit{et al.}, 1979).

A further example of the TNOE has been the use of this technique to provide information about the glycosidic bond conformations of NADP⁺ and thioNADP⁺ in their complexes with \textit{L. casei} DHFR (Feeney \textit{et al.}, 1983). Both NADP⁺ and thioNADP⁺ were shown to have very similar anti conformations about their adenine glycosidic bonds when bound to the enzyme. However, their nicotinamide glycosidic bond conformations were shown to be very different; while NADP⁺ binds in an exclusively anti conformation, thioNADP⁺ binds with a distribution of syn/anti conformations very similar to that observed in nicotinamide mononucleotides (Egan \textit{et al.}, 1975).

Another NOE technique that has been used to provide evidence for the existence of multiple conformations in protein-ligand systems is two-dimensional/exchange spectroscopy (Birdsall \textit{et al.}, 1987). Figure 4.8 shows the NOESY/exchange spectrum of the binary complex of \textit{L. casei} DHFR with excess folate. The 7-proton resonance of free folate is seen at 4.95 ppm, whilst the cross-peaks (off-diagonal peaks) from this resonance arise from the exchange of folate between free and bound states. As can be seen in Figure 4.8 there are two such cross-peaks indicating that there are two bound states for the folate molecule, with 7-proton resonances at 4.4 and 5.6 ppm, respectively.
**4.4.4. Mobility Studies**

Where assignments and interatomic distances are reliably known the $\rho$ values can be used to measure $\tau_c$ i.e. effective mobilities. For larger molecules, where $\omega \tau_c > 1$ then the sign and size of the steady-state enhancement will allow elucidation of an effective $\tau_c$. Thus NOEs from directly attached $^1$H to $^{13}$C or to $^{15}$N range from maximum values of $+198\%$ and $-493\%$ in the rapid tumbling case to $0\%$ when $\tau_c$ is very long. The main focus of this type of mobility study has been the use of $^{13}$C{$^1$H} NOEs (and $R_1$s) to probe motion in synthetic polymers (Heatley, 1981) and proteins (McFarlane and Rycroft, 1981). The use of $^1$H-$^1$H NOEs to measure effective values of $\tau_c$ has been much less common, probably because the important internuclear distances are rarely known with accuracy.
Searle and co-workers (1988) have reported the use of $^1$H and $^{13}$C NMR lineshape and relaxation measurements to study the fluctuations in conformation of the antibacterial drug, trimethoprim when bound to *L. casei* DHFR. $^{13}$C-$^1$H NOEs were used to calculate the $^{13}$C-$^1$H cross relaxation rate ($\sigma_{ch}$), which was, in turn, used to calculate the spectral densities which are an expression of the effects of molecular motion on the four measured relaxation parameters $R_1$, $R_2$, $\sigma_{ch}$ and NOE.

### 4.4.5. $^3$H-$^1$H Nuclear Overhauser Enhancements

Nuclear Overhauser enhancement of tritium signals measured with proton noise-decoupling would be expected to exhibit a maximum value of 0.47, assuming 100% dipole-dipole relaxation (Eqn. 4.5). Elvidge and co-workers (Al-Rawi et al., 1976) when first utilising proton noise-decoupling, carried out inverse gating experiments, with a delay time of $5 \times T_1$ (proton value) and observed no significant difference in intensity or total relative integrated intensity as compared with the normal decoupled spectrum. They concluded the NOE defects were absent, possibly due to the presence of radical species in solution (derived from radiolytic decay). Altman and Silberman (1977a) referring to the work of Canet (1976) (who pointed out that, where the relaxation times of the indicated and observed nuclei are similar, cross-relaxation effects require a delay of at least $10 \times T_1$, for decay of the NOE), reported NOE values of up to 0.4. Elvidge and co-workers (Bloxsidge et al., 1977) confirmed these findings of Altman and Silberman, but rejected their conclusion that $T_1$ and NOE measurements had to be made for every sample before a useful analysis could be attempted. It is now generally accepted that for $^3$H-$^1$H NOE experiments, a waiting time of $10T_1$ is essential for observing the maximum possible NOE.

$^3$H-$^1$H NOEs have been measured for small molecules such as steroids (Altman and Silberman, 1977b; Kaspersen et al., 1987) and macromolecular systems where the
enzyme is specifically labelled with tritium (O'Connell et al., 1990, 1993a, b). One- and two-dimensional $^3$H-$^1$H NOE experiments on specifically tritiated tosylchymotrypsin have been used to examine the properties of the tosyl group in this protein. Irradiation at the proton frequency of samples of the tritium labelled enzymes produced a $^3$H-$^1$H NOE which was substantial and negative, consistent with dipolar interactions with neighbouring protons being an important component of tritium relaxation. The same experiment with enzyme containing a tritiated tosyl group having the remaining tosyl protons replaced by deuterium showed a $^3$H-$^1$H NOE of nearly the same magnitude. Thus, it was reported that the proton-tritium dipole-dipole interactions must take place with protons attached to amino acids of the protein that are adjacent to the tosyl group, since protons in the methyl group and on the aromatic ring of the tosyl moiety had been replaced by deuterium. The authors used transient $^3$H-$^1$H NOE measurements in order to help define the parameters appropriate for multidimensional NOE experiments on the protein. Hence, the nature of the tritium-protein proton interactions was explored further by means of 2D proton-tritium Overhauser experiments. The presence of several tritiated isotopomers complicated analysis of the experiments and extensive computer simulations of relaxation behaviour of tritiated species present were used in conjunction with models developed from crystallographic results to interpret the observations made (O'Connell et al., 1993a, b).

The application of 1D and 2D $^3$H-$^1$H NOE experiments to this system has clearly demonstrated the usefulness of $^3$H-$^1$H NOEs in a system where tritium atoms are present. These positive results together with the vast wealth of NMR and crystallographic information available on the enzyme DHFR in its complexes with folate and methotrexate suggest that $^3$H-$^1$H NOEs could be employed to clarify and further confirm some of the interactions between the tritium labelled ligand and the amino acid residues on the enzyme. In particular, measuring heteronuclear $^3$H-$^1$H NOEs from the T-7 of methotrexate to nearby protons on the protein would enable us to unambiguously
identify the protein contact residues. Information from $^1$H NOE studies is already available which indicates that H-7 is near to the methyl groups of Leu-19 and Leu-27; however, the interpretation of such information is always complicated by the possibility of chemical shift degeneracy in the H-7 region of the proton spectrum. Clearly the tritiated H-7 would remove this ambiguity.

As a prelude to the experiments with the macromolecular system, $^3$H{$^1$H} NOE experiments were carried out on small molecules. $[^3$H]DMSO was chosen to optimise the spectrometer conditions employed for observing $^3$H{$^1$H} NOEs in the steady-state experiments. $[^3,5',7-^3$H]folic acid and $[^3,5',7-^3$H]methotrexate were then irradiated at pre-selected proton frequencies and the $^3$H{$^1$H} NOEs produced were measured and recorded.
4.5. EXPERIMENTAL

[3',5',7,3H]Folic acid and [3',5',7,3H]methotrexate were prepared as described in Chapter 2 and the 1H and 3H NMR spectra recorded in D2O. [3H]DMSO was prepared according to Stewart and Jones (1967).

The tritiated samples were placed in 4 mm Teflon liners with stoppers which were then placed inside 5 mm screw-cap glass NMR tubes. To remove any dissolved oxygen, dry (oxygen-free) nitrogen was bubbled through the solution in the NMR tube prior to the NMR experiment.

Tritium NMR spectroscopy was carried out on the Bruker AC-300E system mentioned in previous chapters. All spectra were obtained with the sample spinning and with the sample temperature regulated at 298 K by the instrument controller.

Spin-lattice times ($T_1$) were determined by the inversion recovery method using the $(180^\circ-\tau-90^\circ-\tau)_n$ pulse sequence.

Steady-state 3H{1H} nuclear Overhauser enhancements were determined with gated decoupling techniques upon pre-saturation of pre-determined proton resonances. In order to account for isotope effects in the 3H NMR spectrum, the 1H spectrum was irradiated ca. 9 Hz upfield of the required 1H signal. Typically 32 transients were collected for each of three 1H resonance frequencies (one blank and two pre-selected proton resonances), using four dummy scans before each accumulation with a delay of at least 10x the tritium $T_1$ between each accumulation (Altman and Silberman, 1977a). About 18 hours were required for each NOE experiment.
4.6. RESULTS


A sample of $[^3]H$DMSO (20 mCi) in $d_6$-DMSO surrounded by CDCl$_3$ was used as a test sample to optimise the conditions necessary for observing the steady-state $^3H\{^1H\}$ NOE in a small molecule. Figure 4.9 shows the $^3H\{^1H\}$ NMR spectrum of CH$_3$SOCH$_2$T with (a) irradiation at 5541.67 Hz (b) irradiation at a blank and (c) the $^3H\{^1H\}$ NOE difference spectrum showing an enhancement of 0.20.

4.6.2. $[^3',5',7-^3]H$Folic Acid

Irradiation at the proton frequency of a sample of $[^3',5',7-^3]H$folic acid ($T-3',5'$ 63%, $T-7$ 37%) of specific activity 22.3 Ci/mmol, produces a $^3H\{^1H\}$ NOE which is small and positive (Table 4.4) consistent with dipolar interactions with neighbouring protons being an important component of tritium relaxation. Figure 4.10 shows (a) the $^3H\{^1H\}$ NMR spectrum of $[^3',5',7-^3]H$folic acid irradiated at H-9, (b) the $^3H\{^1H\}$ NMR control spectrum and (c) the $^3H\{^1H\}$ NOE difference spectrum. Two resonances are present in each $^3H$ NMR spectrum at $\delta$ 8.5 and 6.7 ppm, corresponding to T-7 and T-3',5' respectively. The presence of these two resonances in the $^3H\{^1H\}$ NOE difference spectrum indicates that irradiation at H-9 leads to enhancements of both the T-7 and T-3',5' signals. Whereas, irradiation at the H-2',6' frequency leads to a $^3H\{^1H\}$ NOE at T-3',5' but no enhancement at the T-7 position (Figure 4.11).
4.6.3. [3',5',7-3H]Methotrexate

Irradiation at selective proton frequencies corresponding to H-7, H-3',5', H-2',6' and H-9 of a sample of [3',5',7-3H]methotrexate (T-3',5' 45%, T-7 55%), of specific activity 2.1 Ci/mmol, gave no observable $^3$H/$^1$H NOEs, under the conditions of the experiment. The signal-to-noise ratio in the individual tritium spectra was low in all cases, due to the relatively low activity of the sample and this is almost certainly a contributory factor to the absence of any observable enhancements.

Table 4.4. $^3$H/$^1$H NOE enhancement factors measured on selective pre-saturation of H-2',6' and H-9 folic acid

<table>
<thead>
<tr>
<th>Observed $^3$H</th>
<th>H-2',6' Saturated</th>
<th>H-9 Saturated</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-7</td>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td>T-3',5'</td>
<td>0.04</td>
<td>0.07</td>
</tr>
</tbody>
</table>
(a) $^3$H($^1$H) NMR spectrum of $[^3]$H]DMSO in which a saturating radio frequency field had been applied at 5541.7 Hz

(b) Control spectrum

(c) $^3$H($^1$H) NOE Difference spectrum

**Figure 4.9.** Control and $^3$H($^1$H) NOE spectra of $[^3]$H]DMSO at 320 MHz
3.5 ti.a 6  S  En 5 . 5  5 . 3
(a) $^3$H{$^1$H} NMR spectrum of [3',5',7'-$^3$H]folic acid in which a saturating radio frequency had been applied at 4.4 ppm, the resonance frequency of H-9.

(b) Control spectrum

(c) $^3$H{$^1$H} NOE difference spectrum

Figure 4.10. Control and $^3$H{$^1$H} NOE spectra of [3',5',7'-$^3$H]folic acid at 320 MHz
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(a) $^3H\{^1H\}$ NMR spectrum of $[3',5',7-^3H]$folic acid in which a saturating radio frequency field had been applied at 7.7 ppm, the resonance frequency of H-2',6'

(b) Control spectrum

(c) $^3H\{^1H\}$ NOE difference spectrum

Figure 4.11. Control and $^3H\{^1H\}$ NOE spectra of $[3',5',7-^3H]$folic acid at 320 MHz
4.7. DISCUSSION

The NOEs observed for $[^3H]DMSO$ and $[3',5',7-^3H]folic$ acid are consistent with the results previously seen for small molecules of high activities (Altman and Silberman, 1977b). However, the absence of the NOE in the $[3',5',7-^3H]methotrexate$ experiment does not necessarily mean that the interatomic distances between the pre-selected irradiated proton and the tritium atoms are too large for the NOEs to be observable. The most probable cause for the lack of observable enhancement is the relatively low activity of the $[3',5',7-^3H]methotrexate$ sample. The experiment was conducted over a period of 18 hours, the same as for the $[3',5',7-^3H]folic$ acid experiment and this is clearly not long enough to observe a reasonable signal-to-noise level. To suitably improve the signal-to-noise level, the experiment should ideally be conducted over a weekend, but this was not possible at the time.

Gaggelli and co-workers (1993) used $^{13}C\{^1H\}$ heteronuclear NOEs with $^1H$ and $^{13}C$ NMR relaxation measurements to gain structural information on methotrexate. $^{13}C\{^1H\}$ NOEs were measured on selective saturation of the glutamate NH and H-3'. Saturation of H-3' gave NOEs to C-9, C-4', C-3' and C-2' and from $^1H$ NMR relaxation parameters, interatomic distances were calculated, $r_{6,7} = 2.2\AA$ and $r_{7,9} = 2.4\AA$. The nature of the proton-proton interactions were explored further by means of 2D $^1H$-$^1H$ Overhauser experiments (Moore, 1990). Under the conditions of the experiment, NOEs were observed between H-7 and H-9, H-7 and N$^{10}$-CH$_3$, H-2',6' and H-3',5', H-7 and H-3',5', H-3',5' and N$^{10}$-CH$_3$, H-3' and H-5' and H-6' and H-2'. From these 2D results and those of Gaggelli et al. (1993), several $^{3}H\{^1H\}$ NOEs would be expected to be seen at the T-7 and T-3',5' positions in the $^3H$ NMR spectra.

It is therefore concluded that the absence of observable $^{3}H\{^1H\}$ NOEs in the experiment with $[3',5',7-^3H]methotrexate$ is due to poor signal-to-noise levels, due to short overall experimental times for the given activity of the sample.
The \([^{3}\mathrm{H}]\text{DMSO}\) is an ideal system in which to optimise the spectrometer conditions for the steady-state \(^3\mathrm{H}\{^{1}\mathrm{H}\}\) NOE experiment; there is only one tritium atom present in the molecule therefore all of the activity in the sample is present in a single \(^3\mathrm{H}\) signal in the \(^3\mathrm{H}\) NMR spectrum, hence the signal-to-noise ratio is increased relative to the same activity in two non-equivalent sites. The signal-to-noise can be further increased in the \(^3\mathrm{H}\{^{1}\mathrm{H}\}\) NOE difference experiments because the \([^{3}\mathrm{H}]\text{DMSO}\) gives only one resonance in the \(^1\mathrm{H}\) NMR spectrum. Irradiation at this \(^1\mathrm{H}\) resonance frequency and irradiation at a control point in the \(^1\mathrm{H}\) NMR spectrum means that the total number of transients for each of the two frequencies will be greater in a given time, than for irradiation at three \(^1\mathrm{H}\) resonances, i.e., for \([3',5',7-^{3}\mathrm{H}]\text{folic acid}\), hence increasing the signal-to-noise. However, to simulate the \([3',5',7-^{3}\mathrm{H}]\text{folic acid}\) experiment a second blank irradiation point was used, thus leading to similar signal-to-noise for the two experiments.

The \(^3\mathrm{H}\{^{1}\mathrm{H}\}\) NOEs observed upon irradiation of the pre-selected \(^1\mathrm{H}\) resonances of \([3',5',7-^{3}\mathrm{H}]\text{folic acid}\) were small and positive (Table 4.4). The maximum value of enhancement of tritium signals measured with proton noise-decoupling is 0.47 (Eqn 4.5), assuming 100% dipole-dipole relaxation. The irradiation positions were selected by consideration of the data of Rossi et al. (1992), who used NOESY spectra to provide information on the network of proton-proton dipolar interactions of folic acid which are in turn related to internuclear distances. Analysis of the NOESY spectra provided identification of proton pairs in which cross-relaxation contribution was significant. These include NH-10 - H-3',5', NH-10 - H-9, H-3',5' - H-9, H-3',5' - H-2',6' and H-7 - H-9. Therefore with the tritium label at the T-7 and T-3',5' positions in \([3',5',7-^{3}\mathrm{H}]\text{folic acid}\), irradiations were made at the H-9 and H-2',6' positions. As expected \(^3\mathrm{H}\{^{1}\mathrm{H}\}\) NOEs were observed between H-9 and T-7, H-9 and T-3',5' (Figure 4.10) and H-2',6' and T-3',5' (Figure 4.11), whereas there was no enhancement at T-7 when H-2',6' was
saturated, thus indicating that the interatomic distance $r_{7-2,6}$ is greater than ca. 0.4 nm, the maximum value for NOEs to be observed.
4.8. CONCLUSIONS

It is evident from the work with small molecules that $^3 \text{H} \{ ^1 \text{H} \}$ NOEs are observed for tritium obtained under conditions of proton decoupling. One very important limitation of the current study has been the relatively weak signals of the [3',5',7-$^3 \text{H}$]methotrexate leading to the absence of Overhauser enhancements. This can be improved by using higher levels of tritium gas when labelling the methotrexate, to achieve maximum specific activities and by running the $^3 \text{H} \{ ^1 \text{H} \}$ NOE experiment over a long weekend to improve the signal-to-noise ratio. However, the use of very high activity samples would also lead to increased levels of radiochemical decomposition which could, in turn lead to spurious results. It is therefore important to achieve a balance between the high levels of radioactivity needed to observe the NOEs and the resulting increased levels of radiochemical decomposition possible over the long experimental times employed.

The feasibility of the $^3 \text{H} \{ ^1 \text{H} \}$ heteronuclear 1D and 2D NOE technique in protein systems has been demonstrated by O'Connell et al. (1990, 1993a, b). The group reported the detection of $^3 \text{H} \{ ^1 \text{H} \}$ Overhauser effects from $[^3 \text{H}]$tosylchymotrypsin, a $M_r$ 25 000 protein. From these results and those presented in this chapter for small molecules, it is reasonable to assume that it could be possible to use $^3 \text{H} \cdot ^1 \text{H}$ nuclear Overhauser enhancements to study the $L. casei$ DHFR-[3',5',7-$^3 \text{H}$]methotrexate and $L. casei$ DHFR-[3',5,7-$^3 \text{H}$]folic acid binary and ternary systems in order to identify specific interactions between the tritiated ligands and amino acid residues on the protein.
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5. CONCLUSIONS AND FUTURE WORK

5.1. INTRODUCTION

This final chapter will provide a brief summary of the results in previous chapters. These will be discussed in terms of the original aims of the project and the goals achieved. Finally, the implications of the results and how they lead to future work are described.

5.2. ORIGINAL AIMS

The main objective of this study was to explore the potential of tritium NMR spectroscopy as a tool for providing information about protein-ligand complexes. The aims were to synthesise selectively tritium labelled ligands and use them in conjunction with tritium NMR spectroscopy (a) to characterise the specific interactions between groups on the ligand and protein, (b) to characterise conformational changes accompanying ligand binding, (c) to measure conformational equilibria in various complexes and (d) to monitor the changes in intramolecular dynamic processes.

5.3. ACHIEVEMENTS

The original aims have been achieved by performing work in two areas, namely selectively labelling the ligands with tritium and using $^3$H NMR spectroscopy to detect and assign the signals from the $^3$H nuclei. These were then used to monitor the interactions and dynamic processes in the complexes of the labelled ligands with the enzyme *Lactobacillus casei* dihydrofolate reductase.
From the literature (Cheung et al., 1993; Soteriou et al., 1993) it is clear that a variety of approaches have emerged for studying large molecular complexes by NMR. These recently developed methods rely on the use of isotopically labelled ligands or receptors for simplifying complicated NMR spectra which facilitates the data analysis and allows detailed structural information to be obtained. In the past $^{13}$C- or $^{15}$N-labelled ligands have been employed (Birdsall et al., 1982), however, this approach suffers from the fact that the synthesis requires considerable effort. The results in Chapter 2 show that in contrast to the time consuming and expensive $^{13}$C and $^{15}$N syntheses, the incorporation of tritium at very high specific activity has been achieved in a facile one-step process. By careful choice of the experimental procedure and catalyst employed, it has been possible to incorporate tritium labels selectively, into folic acid (5) and methotrexate (8) at sites designed to probe multiconformational behaviour and dynamic processes in the complexes of these ligands with *L. casei* DHFR, when studied by $^3$H NMR spectroscopy.

The results presented in Chapter 3 indicate that tritium NMR spectroscopy provides a very direct method for examining multi-conformational states and dynamic processes in protein-ligand complexes. In the $^3$H NMR spectra (Figure 3.11 and 3.13) of the ternary complexes of tritium labelled folic acids with DHFR and NADP+, each labelled tritium gave rise to multiple signals confirming previous findings that there are three interconverting conformational forms of bound folate (forms I, IIa and IIb) in the ternary complex (Birdsall et al., 1989b). The pH dependence of the three forms (Birdsall et al., 1982; Cheung et al., 1993) was confirmed by the observation of changes in signal intensities as a function of pH.

The major differences in the conformation of the pterin ring in the different forms have been characterised previously (Birdsall et al., 1987). The results presented here, by providing information on similar chemical shifts of the T-9 and T-3' and T-5' indicated that the non-pterin part of folate is binding in a similar manner in the different forms.
The appearance of a single resonance for the 3',5'-tritons showed that the benzoyl ring was flipping rapidly in all three forms.

The results for the complexes of _L. casei_ DHFR with methotrexate and NADPH (Figure 3.14) indicated that a single major conformation exists for the binary DHFR-methotrexate complex and also for the ternary complex with NADPH. The line broadening which prevents observation of a signal for the 3',5'-tritons of the benzoyl ring in bound methotrexate was consistent with the ring flipping being insufficiently fast to give chemical shift averaging of the 3',5'-signals.

In order to characterise the specific interactions between groups on the tritium labelled ligand and protein by $^3$H NMR spectroscopy, it is necessary to measure $^3$H-$^1$H heteronuclear NOE effects from the tritium nuclei on the ligand to nearby protons on the protein. Chapter 4 details preliminary $^3$H-$^1$H NOE experiments on the tritium labelled ligands alone. The results indicated that it is possible to detect $^3$H-$^1$H nuclear Overhauser enhancements with the accessible instrumentation, provided that $^3$H-labelled molecules are available at sufficiently high levels of radioactivity. However, a balance must be maintained between the requirement for high levels of activity of the sample and the resulting increased radiochemical decomposition during lengthy experimental times which are necessary for the enhancements to be observed. This is one area where further work would provide unambiguous information about the proximity of the tritons on the ligand and protons on the protein residues.

Clearly, it has been shown that $^3$H NMR spectroscopy provides a useful additional method of directly studying protein-ligand complexes. A wealth of information is waiting to be unveiled as soon as the full range of 2D $^3$H and $^1$H NOE experiments can be undertaken.
5.4. FUTURE WORK

5.4.1. Introduction

While the general aims of the project have been met a number of areas which require further study have been identified. Brief outlines of three areas of future work are given in this section.

5.4.2. The Use of $^3$H-$^1$H Heteronuclear NOE Experiments

In previous studies of *L. casei* DHFR-folate complexes the different conformations have been characterised in terms of the orientation of the pteridine ring in the binding site. From NOESY experiments it has been shown that forms I and IIa had the pteridine ring in a 'methotrexate-like' orientation by establishing NOE connections between the pteridine H-7 proton and the assigned methyl protons of Leu-27. These NOEs had previously been detected in NOESY spectra of DHFR complexes with methotrexate (Birdsall et al., 1989a). Using $^3$H-labelled folates in conjunction with $^3$H NMR spectroscopy, heteronuclear NOE information from the tritium at the pteridine H-7 position to the assigned protein $^1$H signals would assist in defining the H-7 environment in the different conformations.

Furthermore, $^3$H-$^1$H heteronuclear NOE experiments could be used to characterise the orientation of the pteridine ring of methotrexate in the binding site. Measurement of heteronuclear NOE effects from the T-7 of methotrexate to nearby protons on the protein would help to identify the protein contact residues. The absence of other methotrexate protons close to T-7 should make it easier to detect NOE effects on nearby protons in the protein. Information from $^1$H NOE studies is already available which indicates that H-7 is near to the methyl groups of Leu-19 and Leu-27; however, the interpretation of such information is always complicated by the possibility of
chemical shift degeneracy in the H-7 region of the proton spectrum. Clearly the tritiated H-7 methotrexate will remove this ambiguity.

5.4.3. $^3$H NMR Studies of Asp-26 $\rightarrow$ Asn DHFR-$^3$H-Folate Complexes

Preliminary information is already available indicating that the mutant enzyme Asp-26 $\rightarrow$ Asn strongly perturbs the multi-conformational equilibria involving folate (Jimenez et al., 1989). The use of $^3$H-labelled folate would allow such effects to be explored in detail by $^3$H NMR spectroscopy.

5.4.4. $^3$H NMR Studies of *L. casei* DHFR-Trimethoprim complexes

The antibacterial drug trimethoprim (TMP, 9) acts by selectively inhibiting the enzyme DHFR in bacterial cells. Considerable efforts have been made to understand the factors controlling the specificity of trimethoprim binding to the bacterial enzyme and numerous structure-activity studies using trimethoprim-related inhibitors have been reported (Roth & Cheng, 1982). Previously NMR methods have been used to investigate the protonation state of the pyrimidine ring of bound trimethoprim (Roberts et al., 1981; Bevan et al., 1985), to determine its conformation in complexes with both bacterial and mammalian DHFR (Cayley et al., 1979; Birdsall et al., 1983) and to investigate the presence of multiple conformations in these complexes (Gronenborn et al., 1981). However, as previously stated, one of the major problems encountered in studying complex NMR spectra is that of assigning the signals to particular nuclei in the protein-ligand complexes. This can be largely overcome by using isotopic labelling to simplify the NMR spectra of the protein or their bound ligand. An example of this type of study has been reported by Cheung and colleagues (1986), who used $^{13}$C NMR spectroscopy to study the binding of selectively $^{13}$C-enriched trimethoprim to *L. casei* DHFR. The
work presented in this thesis illustrates the usefulness of using $^3$H-labelled ligands and $^3$H NMR spectroscopy to study such interactions. It is therefore reasonable to assume that $^3$H NMR methods could be applied to the DHFR-trimethoprim system provided that trimethoprim could be labelled with tritium, in positions of interest.

![Trimethoprim (9)](image)

Trimethoprim could be selectively labelled with tritium at the H-6, H-7 and/or H-2', H-6' positions. The use of $^3$H NMR spectroscopy would provide the opportunity to characterise multiple conformations and dynamic processes in its binary complexes with the enzyme and in the ternary complexes with DHFR and coenzyme. Two conformations have previously been detected in the DHFR-TMP-NADP$^+$ complex (Cheung et al., 1986) and if this complex was formed with tritiated trimethoprim then separate signals for the two conformations would be detectable: this would allow the part of the molecule which is most influenced by the conformational differences to be determined. Heteronuclear NOE measurements could be used in an attempt to identify the trimethoprim contact residues on the protein in the two conformations.

The use of specifically tritiated trimethoprim could also offer some advantages in studies of dynamic processes in complexes. With tritium at the H-2' and H-6' positions it should be possible to use $^3$H NMR spectroscopy to monitor their chemical shifts in bound trimethoprim at low temperatures (ca. 273 K) when the benzyl ring is flipping only slowly and two separate signals for each triton should be observed.
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