NMR STUDIES OF ENZYMES IN SITU AND IN VITRO

A thesis submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy.

by

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This thesis discusses applications of nuclear magnetic resonance (NMR) to the study of enzyme catalysed reactions and their behaviour in the intact cell.

Chapter 2 describes a method which allows the observation, by NMR, of several magnetic nuclei simultaneously. The increase in information available over that from mononuclear studies was shown to be very valuable in investigations of cellular metabolism. The advantages are particularly apparent in studies involving isotopic labelling. The technique was applied to an investigation of the flux through the enzyme 2,3 bisphosphoglycerate synthase-phosphatase in the human erythrocyte under steady-state conditions. Information about the flux through the pentose-phosphate shunt was also available from the same experiments.

In the experiments outlined in Chapter 3, a temperature-jump relaxation method was developed to observe the flux through aldolase in the intact human erythrocyte. The characterisation of this relaxation in an in vitro model system by $^{31}$P NMR allowed the estimation of the aldolase activity in situ in the erythrocyte. The results indicated that binding of aldolase to the erythrocyte membrane is not a physiologically significant phenomenon.
Chapter 4 explores the application of NMR techniques to the study of the compartmentation of glycolytic intermediates between active and inactive forms. The effects of glycolytic enzymes on the interconversion of these forms was examined and possible implications to metabolic regulation are discussed.
Acknowledgements

A chemist, aspiring to apply nuclear magnetic resonance techniques to biochemical problems, must gain a working knowledge of subjects ranging from physics, through electronics and computing, to cellular biochemistry. I should like to take this opportunity to thank the people who have hastened this learning process.

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# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>DHAP</td>
<td>dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>1,3 DPG</td>
<td>1,3-bisphosphoglycerate</td>
</tr>
<tr>
<td>2,3 DPG</td>
<td>2,3-bisphosphoglycerate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetracetic acid</td>
</tr>
<tr>
<td>F6P</td>
<td>fructose 6-phosphate</td>
</tr>
<tr>
<td>FDP</td>
<td>fructose 1,6-bisphosphate</td>
</tr>
<tr>
<td>G6P</td>
<td>glucose 6-phosphate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MDP</td>
<td>methylene diphosphonic acid</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced NAD⁺</td>
</tr>
<tr>
<td>PFK</td>
<td>phosphofructokinase</td>
</tr>
<tr>
<td>PPM</td>
<td>parts per million</td>
</tr>
<tr>
<td>T₁</td>
<td>longitudinal relaxation time</td>
</tr>
<tr>
<td>T₂</td>
<td>transverse relaxation time</td>
</tr>
<tr>
<td>TPI</td>
<td>triose phosphate isomerase</td>
</tr>
<tr>
<td>GAP</td>
<td>glyceraldehyde 3-phosphate</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>3-PGA</td>
<td>3-phosphoglycerate</td>
</tr>
<tr>
<td>PGK</td>
<td>phosphoglycerate kinase</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
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Aldolase

Triose phosphate isomerase

Glyceraldehyde 3-phosphate dehydrogenase

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NMR temperature jump experiments

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5 SUMMARY
CHAPTER 1

GENERAL INTRODUCTION

1.1 Scope of this thesis

Some of the earliest nuclear magnetic resonance (NMR) experiments attempted after the discovery of the phenomenon in the 1940s were on samples of living tissue. The abundance of protons in biological material and the relative ease of detection of this nucleus meant that most of these observations were of water protons. Early studies included an attempt by Purcell to obtain a spectrum of his finger and possibly the first published observation was of the exchange of D$_2$O and H$_2$O in human red blood cells (Odeblad et al., 1956). These experiments were followed by measurements of T$_2$ relaxation rates (e.g. Bratton et al., 1965) and diffusion rates (Tanner & Stejskal, 1968) of cellular water. Early observations of other nuclei included low resolution $^{23}$Na NMR studies of blood cells (Jardetzky & Wertz, 1956). The first high resolution observations by Eakin et al. (1972) of $^{13}$C NMR spectra of yeast metabolising $^{13}$C enriched glucose and by Moon and Richards (1973) of $^{31}$P spectra of human erythrocytes were the start of extensive applications of NMR methods to biological problems.

Many areas of biochemical interest are now accessible to study by the impressive array of NMR methods available. The applications fall broadly into two classes, those of structural and mechanistic studies of macromolecules and those of the investigation of metabolic processes in intact cells and tissue. NMR has made
The physical nature of the NMR experiment with emphasis on its biochemical applications has been dealt with comprehensively elsewhere (Campbell & Dobson, 1979; Dwek, 1973). The main factor facilitating the extensive use of NMR in the study of biological systems is the transparency of living tissue to radiofrequency radiation and the relatively low energy of this radiation. This enables NMR to be used to detect species in intact tissue non-invasively and also to gain information about the intracellular environment.

The simplest, and possibly most powerful, application of NMR to metabolic studies is as a method of non-invasive assay of the species which are visible in the spectrum of a magnetic nucleus. Observations of qualitative changes are possible without the need for laborious and destructive chemical extraction and assay. There are many examples of these applications using a variety of magnetic nuclei. The most widely used have been $^{31}\text{P}$, $^{13}\text{C}$ and $^1\text{H}$ and to a lesser extent $^{19}\text{F}$, $^{23}\text{Na}$ and $^{39}\text{K}$ (Roberts & Jardetzky, 1981; Gadian, 1982; Gupta et al.,
An area of considerable interest to biochemists which is not easily accessible to study by classical methods is that of intracellular compartmentation. There are several different meanings of this term in biochemistry and here it is taken to mean the existence of different regions in the cell separated by membranes which pose a barrier to the free translation of species between the compartments. NMR has proved to be of use in the investigation of compartmentation because the different microenvironments in each region can affect the magnetic properties of a species such that its location may be determined by NMR experiments.

The clearest case is when the chemical shift of a species is different in two compartments. This has been observed using $^{31}\text{P}$ NMR in several systems where differing pH between compartments leads to a difference in the chemical shift of inorganic phosphate ($P_i$). Examples include the detection of extracellular and intracellular $P_i$ in E. Coli (Navon et al., 1977), vacuolar and cytosolic $P_i$ in higher plant tissue (Kime et al. 1982a) and cytoplasmic and stromal $P_i$ in photosynthesizing Chlorella (Mitsumori & Ito, 1984). In addition to detecting the compartmentation, this method has provided a probe for estimating the pH in the various compartments.

If such a chemical shift difference is not inherent to the system, it may sometimes be induced artificially. This approach has been used to distinguish intracellular and extracellular Na$^+$ ions through the addition of the
paramagnetic shift reagent dysprosium tripolyphosphate, 
\([\text{Dy(PPP)}_2]^{7-}\) which binds to external Na\(^+\) ions producing an 
upfield shift in their resonance in the \(^{23}\text{Na}\) spectrum
(Gupta & Gupta, 1982). Studies of the sequestration of Na\(^+\) ions in the erythrocyte membrane and investigations of 
several other tissues have been carried out (Gupta et al., 1984). The dysprosium shift reagent has also been shown to 
be effective for differentiating intracellular and 
extacellular K\(^+\) ions in \(^{39}\text{K}\) NMR spectra (Ogino et al.,
1983). Recently Boulanger et al. (1985) have discussed the 
problems of quantification of the effects visible in the 
\(^{23}\text{Na}\) spectrum and suggested that owing to the slow 
accumulation of Dy\(^{3+}\) inside certain cells from \([\text{Dy(PPP)}]\)^{7-} 
alternative shift reagents may be preferable in some 
cases.

Less obvious differences in properties between 
compartments have also been employed to detect 
compartmentation. Brown (1983) assigned resonances in the 
spin echo \(^1\text{H}\) spectrum of chicken erythrocytes to 
metabolites in the cytoplasm, nucleus or extracellular 
space on the basis of their \(T_2^*\) values which were dominated 
by differences in magnetic susceptibility between the 
various regions. An extension of this approach is the use 
of spin echo pulse sequences in whole body \(^1\text{H}\) imaging to 
differentiate tissues on the basis of the \(T_2\) values of 
their constituent water (Mansfield & Morris, 1982).

Associated with the concept of compartmentation is 
that of the transport of a species from one region of the 
cell to another. Such effects may sometimes be observed 
directly e.g. in the investigation of the influx and
efflux of Na\(^+\) and K\(^+\) ions in the erythrocyte by \(^{23}\)Na and \(^{39}\)K NMR (Ogino et al., 1985) where signals due to all four species could be seen and changes in their intensity followed. More often the effects of transport are seen indirectly. The transport of H\(^+\) ions across phospholipid vesicle membranes was followed kinetically through the shift in the internal P\(_i\) resonance in \(^{31}\)P NMR spectra (Oxley, 1982) and the transport of Mn\(^{2+}\) into the vacuole of plant tissue was seen through the broadening of the vacuolar P\(_i\) (Kime et al., 1982b). The effects of diffusion of a species between areas of different magnetic susceptibilities may be detected in spin echo experiments (Brindle et al., 1979; Brown et al., 1983). Several studies of the transport of water across cell membranes have also been made by an NMR method based on the different relaxation times on intracellular and extracellular water (e.g. Conlon & Outhred, 1972). It should be noted that all these methods have a requirement for slow exchange between the compartments on the NMR timescale.

The lower concentration limit for direct detection of a species in tissue and cell samples by NMR is of the order of 0.5 mM. It is sometimes possible, however, to use one NMR nucleus as a probe for another which may not itself be detectable directly and in some cases there is a considerable increase in the sensitivity normally expected. An example already quoted is the estimation of pH by \(^{31}\)P NMR which enables the measurement of proton concentrations of the order of 10^{-8} M. Gupta et al (1978) developed a method to measure the free Mg\(^{2+}\) concentration
in the erythrocyte from a study of the effects of magnesium binding on the $^{31}$P spectrum of ATP. This method has since been applied to several tissues (Gupta & Moore, 1980; Wu et al., 1981) and recently Garfinkel & Garfinkel (1984) have discussed the differences in the literature over the interpretation of the observed effects.

Smith et al. (1983) used $^{19}$F NMR studies of the chemical shift of fluorine-labelled chelating agents to measure the concentration of free Ca$^{2+}$ in the sub-micromolar range in mouse thymocytes. Attempts have recently been made to extend this method to the measurement of the calcium levels in perfused rat hearts.

The sensitivity of detection of a magnetic nucleus may also be increased by observing it indirectly through coupling to a more sensitive nucleus. For example $^{13}$C and $^{15}$N labelled metabolites were observed in $^1$H spin echo spectra (Brindle et al., 1982a; 1984). Polarisation transfer methods of enhancing the detection of an insensitive nucleus (Morris & Freeman, 1979) are not generally applicable to biological systems owing to the short $T_2$ values of the resonances of interest.

A further area of application of NMR methods to biochemistry is in the study of enzyme catalysed reactions in intact living systems and this is the main field covered by this thesis.

1.3 The study of enzymes in vitro and in situ

Much of our knowledge about the function and regulation of enzymes has come from extensive kinetic studies of the highly purified enzymes in vitro. There has
been continued interest in recent years in the properties of these enzymes when they are in situ in the intact cell (Sies, 1980). There are many features of the intracellular environment which could modify these properties such as a) compartmentation of substrates either regionally as described above or through binding to proteins (Sols & Marco, 1970; Masters, 1977), and, b) compartmentation of the enzymes themselves by binding to membranes, proteins or other enzymes (Ottaway & Mowbray, 1977; Solti & Friedrich, 1979; Brindle et al., 1985).

Without a complete knowledge of these possible interactions in the intact cell it is impossible to extrapolate, with any confidence, in vitro measurements on enzymes to establish their likely properties in situ. It is therefore desirable to make measurements on enzymes in situ.

Some attempts at non-invasive investigation of enzyme reactions in intact tissue have been reviewed by Sies (1980). Optical techniques such as spectrophotometry and fluorimetry have been used to study specific reactions in whole organs (Sies & Brauser, 1980) but are restricted by the opacity of biological tissue to these wavelengths.

Another method used has been to make cells permeable to their metabolites by cross-linking the membrane proteins with bifunctional reagents prior to the solubilisation of the lipid. Kinetic studies of the constituent enzymes may then be carried out by conventional spectrophotometric methods. This technique has been applied to several systems (e.g. Aragon et al., 1980) but is far from being non-invasive and the cross-
linking reagents may modify the properties of some enzymes.

Radio-isotope tracer studies have been very widely used for investigating metabolism and provide a very powerful tool. Applications have included studies of substrate cycling between fructose 6-phosphate and fructose 1,6-bisphosphate in several tissues (Newsholme & Crabtree, 1976) and the control of glycolysis in the erythrocyte (Rose & Warms, 1970). A disadvantage of these methods is that they require destruction of the tissue for their analysis and often involve lengthy fractionation, extraction, separation and counting procedures.

In recent years NMR has proved to be a very valuable tool in the field of in vivo enzymology because of its ability to detect, and observe changes in, metabolites in intact tissue. Some of the strategies used will be discussed below.

An enzyme whose in situ properties are to be the subject of study will generally be part of a metabolic pathway which is in a chemical steady-state on a cellular level. As such it is difficult to obtain any kinetic information about an individual enzyme and in general it is necessary to introduce some kind of perturbation to the system so that information can be gained from observing how it responds. Ideally this perturbation should be kept to a minimum so that the method employed does not influence the process it is designed to measure. The type of perturbation used will depend on the nature of the particular enzyme catalysed step being isolated for study.
e.g. whether it represents a rate limiting step or is close to equilibrium in the cell. The range of methods employed in NMR studies of in situ enzyme kinetics nearly all fall into one of the following categories:

a) chemical (concentration) perturbation
b) isotopic perturbation
c) magnetic perturbation

Chemical perturbations

The most severe perturbation is to displace the system from its chemical steady-state and follow the concentration changes of metabolites as it responds. This approach has been used quite widely for example in $^{31}\text{P}$ NMR studies of the effects of inhibition of the $F_1-F_0$ ATPase on the energy status and intracellular pH of E. Coli (Ugurbil et al., 1978b), the effects of fructose on metabolism in intact rat liver (Iles et al, 1980) and the effects of ischaemia in perfused hearts (Garlick et al, 1979). It has also been possible to synchronise acquisition of $^{31}\text{P}$ spectra with a regular, repeated stimulation to gain information about muscle contraction (Dawson et al, 1977). One problem with this type of experiment is that it may be difficult to attribute observed changes to the effects of a single enzyme and if possible the perturbation should be made as specific as possible.

These methods of observing concentration changes are only really suitable for studying the enzymes which are rate limiting in a particular pathway and do not give much information about those which are close to equilibrium.
An example of this approach will be seen in Chapter 2 where the irreversible natures of hexokinase and 2,3 bisphosphoglycerate synthase-phosphatase in the glycolytic pathway allow the concentration changes of $^{13}$C labelled metabolites observed by $^{13}$C NMR to be equated with absolute chemical fluxes.

Reactions which are close to equilibrium may be studied by a concentration perturbation method if the equilibrium is disturbed in some way. The system may then be observed as it returns to its new equilibrium position. Such methods are known as relaxation techniques and have been used quite widely in in vitro studies of enzyme kinetics (Malcolm, 1975). The displacement from equilibrium is usually small and is often brought about by a rapid change in temperature or pressure. An example of the use of NMR in a temperature-jump relaxation study of an enzyme is described in Chapter 3.

Isotopic perturbations

Rather than subjecting a system to a chemical perturbation, it may be possible in some cases to let it remain in chemical steady-state and introduce an isotopic perturbation. NMR has the ability to distinguish between isotopic nuclei and this can be a very powerful method, particularly for studying reactions close to equilibrium. In some ways these NMR methods are analogous to the classical radio-isotope methods but do not require extensive and destructive analysis of the tissue.

The most widely used combinations of isotopes in NMR studies have been $^{13}$C/$^{12}$C and $^2$H/$^1$H. In both of these
pairs one nucleus is NMR visible while the other is invisible and this has led to the development of powerful methods of kinetic analysis. The redistribution of $^{13}\text{C}$ label through metabolic pools has been used quite extensively. For example the "scrambling" of $^{13}\text{C}$ label from C-1 to C-6 in fructose 1,6-bisphosphate was observed by $^{13}\text{C}$ NMR in Yeast (den Hollander et al., 1979) and in E. Coli. (Ugurbil et al., 1978) and was found to be more extensive in the former implying that the reactions catalysed by triosephosphate isomerase and aldolase were close to equilibrium in that tissue. This approach has been extended more recently by Cohen (1983) who analysed the $^{13}\text{C}$ labelling patterns of metabolites in perfused rat livers to obtain information on the Krebs cycle and glycolytic pathway.

$^2\text{H}/^1\text{H}$ exchange experiments have been used to follow enzyme catalysed exchange with solvent. For example Brindle et al (1982b) followed the exchange of the C-2 proton of lactate with solvent in human erythrocytes. The process is catalysed by a series of four glycolytic enzymes and the specific inhibition of one of these, glyceraldehyde 3-phosphate dehydrogenase allowed the kinetics of that enzyme to be studied separately. Paul et al (1983) employed a similar method to study the exchange of the C-3 proton of β-hydroxybutyrate with solvent in rat liver mitochondria.

The advantages of observing less sensitive nuclei such as $^{13}\text{C}$ and $^{15}\text{N}$ through their heteronuclear coupling to a more sensitive nucleus has been mentioned above. This may be exploited in isotope exchange experiments and
examples have included the observation of exchange of $^{13}$C label between alanine and pyruvate catalysed *in situ* by alanine aminotransferase (Brindle et al., 1982a). A heteronuclear $^{15}$N/$^{31}$P spin echo experiment to follow the exchange of $^{15}$N label between creatine and phosphocreatine has also been described (Brindle et al., 1984b).

The use of $^1$H NMR to estimate the fractional incorporation of $^{13}$C in the lactate pool in human erythrocytes is described in Chapter 2. A heteronuclear $^{31}$P/$^1$H spin echo experiment to follow solvent exchange of a C-1 proton of fructose 1,6-bisphosphate is described in Chapter 3.

**Magnetic perturbations**

NMR may sometimes be used to make kinetic studies of enzyme reactions at equilibrium which are in both chemical and isotopic steady-state by introducing a magnetic perturbation. Such methods are collectively called magnetisation transfer techniques and may be used to measure rates which fall approximately in the range 0.1-10 s$^{-1}$. The most widely used magnetisation transfer method in *in vivo* studies is saturation transfer and its first application was the determination of the exchange rates between inorganic phosphate and ATP in E. Coli (Brown et al., 1977). There have since been many other applications including studies of adenylate kinase in the human erythrocyte (Gupta, 1979) and creatine kinase in frog muscle (Gadian et al., 1981). These experiments have been the subject of comprehensive reviews (e.g. Meyer et al., 1982; Alger & Shulman, 1984). Theoretical treatments of
the analysis of this type of experiment are also available (Campbell et al., 1978; Brindle et al., 1985). The other magnetisation transfer methods have also been applied to biological problems but to a lesser degree, for example studies of the arginine kinase reaction in crayfish by inversion transfer (Butler et al., 1985) and the enzyme catalysed isomerisation and anomerisation of glucose 6-phosphate by a two-dimensional magnetisation transfer technique (Balaban & Ferretti, 1983).

It has recently become apparent that some caution should be exercised when interpreting the data from magnetisation transfer experiments in vivo. First, in the complex intracellular environment, there may be a number of possible routes by which magnetisation may be transferred from one site to another and these should be taken into account before assigning an observed flux to a particular enzyme. For example, the flux measured between \( P_i \) and ATP in E. Coli. (Brown et al. 1977) was attributed to the rate of the ATP synthase but recent investigations have shown (Mitsumori et al. 1985) that much of this flux may be due to exchange reactions of the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase. This situation highlights the problem outlined above of confining observations made in situ to a specific enzyme.

Further, Brindle & Radda (1985) have recently measured the exchange velocity between ATP and ADP catalysed by creatine kinase in vitro and shown that under certain conditions the rate measured by saturation
transfer and by radioisotope exchange are different. It was proposed that a loss of saturation in an enzyme-bound intermediate may account for the discrepancy and that analysis of the exchange by the simple two-site model may not always be valid.

Magnetisation transfer techniques are used in the experiments described in Chapter 4 to investigate anomeric equilibria in the substrates of some glycolytic enzymes.
1.4 References


2.1 Introduction

The NMR nuclei most commonly used in the study of biological systems are $^{13}$C, $^{31}$P and $^1$H and each of these has certain advantages and disadvantages in its application. Natural abundance $^{13}$C NMR studies of intact tissue are difficult to carry out owing to the low abundance and inherent insensitivity of this nucleus. The metabolism of $^{13}$C labelled substrates in cells, however, may be observed fairly readily and the good spectral dispersion means that considerable information is potentially available. There are numerous examples of qualitative investigations into the redistribution of a $^{13}$C label through various metabolic pools (e.g. Scott & Baxter, 1981; den Hollander et al., 1981) but kinetic information is not so readily available if there is the possibility of unobserved chemical flux superimposed on the observable isotopic flux as this would make interpretation difficult.

$^{31}$P has been very widely used (reviewed in Roberts & Jardetzky, 1981; Gadian & Radda, 1981; Gadian et al., 1979) but its value is restricted by the relatively low information content of the spectra from most tissues. This is largely a consequence of the paucity of phosphorylated metabolites of sufficient concentration and the poor spectral dispersion which results in resonances of
potential interest overlapping with each other. Spectral editing techniques have been of some use in alleviating the latter problem (Brindle et al., 1985) but in general the very short $T_2$'s of intracellular resonances restrict their value.

$^1H$ NMR of biological samples has the opposite problem in that there is an over abundance of resonances which overlap extensively and observations usually have to be restricted to the less crowded regions of the spectrum. Off-setting this are the high sensitivity with which this nucleus can be detected and the versatility of applications (Brindle et al., 1984). $^1H$ NMR is now being used increasingly for biochemical studies (e.g. Nicholson et al., 1984; Ugurbil et al., 1984).

The data available from these three nuclei are highly complementary and there is an advantage in being able to observe more than one nucleus at once. There have been a number of studies combining the use of two nuclei observed separately (Gillies & Benoit, 1983; Yoshizaki et al., 1981) but these suffer the problem of sample variability in the different measurements. The advantages of multinuclear observation are particularly great in the case of isotope exchange studies. For example in the metabolism of a $^{13}C$ labelled substrate, the $^{13}C$ label is often incorporated into a phosphorylated intermediate. The $^{31}P$ spectra may indicate the total concentration of the intermediate while the $^{13}C$ spectra give the concentration of labelled intermediate. For non-phosphorylated intermediates the $^1H$ spectra may yield the same information since protons adjacent to $^{13}C$ may be
distinguished from those adjacent to $^{12}$C (Brindle et al., 1982; 1983). In this case direct observation of the $^{13}$C nucleus is not required and the sensitivity of its detection is enhanced by using $^1$H NMR. A great deal of information about isotopic redistribution may thus be obtained from the observation of several nuclei simultaneously.

The possibility of double-tuning a coil to detect signals from $^{31}$P and $^{13}$C nuclei sequentially has been demonstrated previously (Styles et al., 1979) and Ogino et al. (1983) and Cohen (1983) have used probes tuned to two frequencies to study the stoichiometry of cation exchange in *Saccharomyces cerevisiae* and metabolism in perfused rat liver respectively. This chapter describes the use of a probe tuned to three observable frequencies: $^{31}$P, $^{13}$C and $^1$H and also with a $^2$H channel available for a field-frequency lock. By interleaving the acquisition of signals from the three observed nuclei, a set of essentially simultaneously recorded spectra may be obtained. A time course of metabolic change may thus be followed through up to three different nuclei at once.

The technique is demonstrated by the study of some enzyme catalysed reactions in the human erythrocyte. The main examples are studies of the 2,3 bisphosphoglycerate bypass and the pentose phosphate shunt in the glycolytic pathway of human erythrocytes. The potential of the method for studying other systems and the possible use of other nuclei is discussed.
**Figure 2.1**

Schematic diagram of glycolysis in erythrocytes

Solid lines represent reactions catalysed by a single enzyme and dotted lines represent the sum of two or more individual reactions. The steps at which a molecule of ATP is synthesised are marked with an asterisk (*).
2,3 bisphosphoglycerate bypass

The compound 2,3 bisphosphoglycerate (2,3-DPG) is present in high concentrations (ca. 5 mM) in the human erythrocyte and as well as serving as a cofactor of the enzyme phosphoglyceromutase it has a crucial role in the red cell's major function as the mechanism of oxygen transportation in the body. 2,3 bisphosphoglycerate binds to haemoglobin and has a greater affinity for the deoxy form thus shifting the HbO₂ ↔ Hb + O₂ equilibrium to the right and facilitating the release of oxygen to tissues (Benesch & Benesch, 1967). The levels of 2,3 bisphosphoglycerate are known to be elevated in conditions associated with hypoxia such as anaemia and high-altitude acclimatisation and are depressed in other conditions such as acidosis (Duhm & Gerlach, 1974). The level of 2,3 bisphosphoglycerate in erythrocytes is regulated by a side reaction of the main glycolytic pathway known as the 2,3 bisphosphoglycerate bypass or Rapoport-Luebering cycle (see Figure 2.1). This is unusual in that both synthesis and breakdown of the metabolite are essentially irreversible reactions controlled by different catalytic activities of a single enzyme, bisphosphoglycerate synthase-phosphatase (Rose, 1980; Chiba & Sasaki, 1978, Sasaki et al., 1982). The exact role of the bypass and its relative contribution to the overall glycolytic flux are still in debate. It is possible that, as well as serving to synthesise the allosteric modifier of haemoglobin, the cycle, which bypasses one of the ATP synthesis steps, allows glycolysis to continue at a reduced rate of ATP production while the energy requirements of the cell are
low. It may be significant that 2,3 bisphosphoglycerate has been detected in cereals which have no known requirement for the compound as they lack haemoglobin and their phosphoglyceromutase activity is not dependent on the presence of 2,3 bisphosphoglycerate (Ito & Grisolia, 1959).

Previous estimates of the relative flux through the 2,3 bisphosphoglycerate bypass have only been made indirectly by extrapolation of measurements under non-steady state conditions (Momsen & Vestergaard-Bogind, 1978; Rapoport et al., 1977). Attempts to measure the bypass flux at steady-state by studies of the redistribution of the radioactive $^{32}$P isotope after its addition as inorganic phosphate were not successful owing to the difficulties of interpretation (Snyder et al., 1975).

The simultaneous observation of $^{31}$P, $^{13}$C and $^1$H NMR spectra of erythrocytes metabolising [1-$^{13}$C] glucose described here allows the measurement of the bypass contribution under steady-state conditions. The data from the same experiment also allowed the calculation of the contribution of the pentose-phosphate shunt to the overall glycolytic flux. Other information such as nucleotide status and intracellular pH is also readily available from a single experiment.
2.2 Materials and Methods

2.2.1 Materials

The following chemicals were obtained from Sigma Chemical Company: HEPES, pyruvic acid, lactic acid, 2,3-DPG, ATP, ADP, methylene diphosphonic acid (tetrasodium salt), all enzymes.

[1-\textsuperscript{13}C]glucose of 92% atom purity was obtained from Prochem Ltd. (now Amersham International).

All other chemicals were of analytical grade.

2.2.2 Sample preparation

**Human erythrocytes**

Freshly drawn blood from a healthy donor was washed once with ice-cold phosphate-buffered saline (5 mM NaH\textsubscript{2}PO\textsubscript{4}, 150 mM NaCl, pH 7.4) and four times with Krebs-Ringer buffer (Krebs & Hanseleit, 1932) supplemented with 50 mM HEPES, pH 7.4. All buffers except that for the final wash contained 10 mM glucose.

2.5 ml samples of packed erythrocytes (haematocrit 75-80%) were contained in 10 mm NMR tubes with a coaxial capillary containing 300 mM methylene diphosphonic acid (MDP) buffered in HEPES at pH 7.4 as an external \textsuperscript{31}P chemical shift and intensity reference.

**Rat liver mitochondria**

Mitochondria were prepared by M. Carr from the livers of starved rats killed by cervical dislocation.

The mitochondria were suspended in a buffer containing 75 mM mannitol, 25 mM sucrose, 90 mM KCl, 10 mM KH\textsubscript{2}PO\textsubscript{4}, 10 mM HEPES and 1 mM EDTA, at pH 7.2 to give a
protein concentration of around 0.5-1.0 mg per ml. 2.5 ml of the suspension was contained in a 10 mm NMR tube with a concentric capillary of MDP as described above. Samples were aerated during the NMR experiments by continuous bubbling with the aid of a peristaltic pump. This served both to satisfy the oxygen demands of the mitochondria and to prevent their sedimentation.

2.2.3 NMR spectrometer

In order to be able to switch rapidly between the observation of different nuclei, it is necessary to have a large proportion of the NMR spectrometer functions under computer control. To this end a spectrometer was designed and built in the Department of Biochemistry, Oxford by R. Porteous and N. Soffe based on a 53 mm bore Oxford Instruments 6.4 T superconducting magnet. The spectrometer was interfaced to a Nicolet 293B programmable pulser and 1180 computer and was controlled by the Nicolet NTCFTB operating program with modifications to allow greater software control of spectrometer function.

The ability to carry out a variety of experiments on different nuclei including spin-echos, heteronuclear decoupling and solvent saturation necessitated having computer control of the observe and decoupling frequencies, amplifier selection, power and phase of the transmitter, intermediate-frequency gain of the receiver and the filter settings. The software modifications required to implement these functions were made by R. Porteous. It was also necessary to make changes to the software which controlled the storage and processing of
**Figure 2.2**

Circuit for double-tuning a probe to two different frequencies

$L_1$ is a second inductance whose purpose is to present a high impedance to the higher frequency. $C_1$ and $C_2$ are used to fine-tune the probe to $^{31}P$ and $^{13}C$ respectively.
the data from several different nuclei in rapid succession and these were carried out by the author.

**Probe and tuning circuit**

The probe used in these experiments was of conventional design with two concentric saddle-shaped coils which could accommodate a 10 mm diameter tube. The outer coil was double-tuned to the $^1H$ and $^2H$ frequencies at 269.9 MHz and 41.4 MHz respectively and the inner coil was double-tuned to the $^{31}P$ and $^{13}C$ frequencies at 109.3 MHz and 67.9 MHz respectively. The tuning circuit used is illustrated in Figure 2.2 and is based on the use of a second inductance to reject the higher frequency when observing the lower one.

A double-tuned probe inevitably has lower sensitivity than when singly tuned (Hoult, 1978). Probes are regularly double-tuned to the $^1H$ and $^2H$ frequencies to allow both proton decoupling and a field-frequency lock to be employed and for these nuclei the losses in sensitivity may be kept fairly low owing to the relatively large difference in their frequency (a factor of 6.5). Typically the sensitivity of the higher frequency will be reduced by about 10% and that of the lower frequency by about 20%. When the two frequencies are closer together, however, it becomes more difficult to double-tune the probe without larger losses in sensitivity. In the case of $^{13}C$ and $^{31}P$, whose frequencies differ by a factor of only 1.6, the sensitivity of both will be reduced by about 30%. The values of the components $L_1$, $L_2$, $C_3$ and $C_4$ in the circuit (Figure 2.2) may be chosen to minimise the losses in
sensitivity of the inherently less sensitive nucleus (in this case $^{13}\text{C}$). The variable capacitors $C_1$ and $C_2$ are used to fine tune the probe to $^{31}\text{P}$ and $^{13}\text{C}$ when it is **in situ** in the magnet.

Signal to noise measurements were made on ASTM samples and were found to be 89:1 for $^{13}\text{C}$ (on C$_6$D$_6$) and 33:1 for $^{31}\text{P}$ (on TMP). The proton signal to noise was not measured. The system has recently been implemented on a wide-bore 360 MHz spectrometer and a great increase in sensitivity has been achieved (around 200:1 signal to noise for both $^{13}\text{C}$ and $^{31}\text{P}$ when singly-tuned).

An alternative method of double-tuning a probe is to use quarter-wavelength lines (Hoult, 1973) where the lower frequency tuning is performed at the node in the half-wavelength line of the higher frequency. This method might give an improvement in sensitivity and is at present being assessed on the wide-bore 360 MHz spectrometer.

**Data acquisition**

There are a number of alternative ways of arranging the acquisition of data from several nuclei at the same time. Two nuclei might, in principle, be excited simultaneously and their responses separated in the spectrometer. With our spectrometer design it was easier to observe the different nuclei separately but in rapid sequential order. The detection is thus not truly simultaneous on a short timescale but is effectively so over the period of minutes required to observe a spectrum.

For the observation of two nuclei simultaneously the required pulse sequences and acquisition parameters were
Each nucleus is observed in turn for a short period before the accumulated FID is stored on disc and the acquisition parameters for the next nucleus are loaded. This cycle is continued until sufficient signal to noise has been attained in each spectrum. The time spent observing each nucleus depends on the sensitivity of its detection.
stored on disk in two separate files and could be called by the computer alternately. At the end of each period of observation, the accumulated transients were stored on the disk and the parameters for the second nucleus would be loaded. If a single pulse is used on each nucleus, that nucleus relaxes during the time taken to observe the other nucleus and so the dead time of the spectrometer usually necessary between pulses is usefully employed. In practice, however, the time spent accessing the disk in this approach becomes comparable with the time spent making observations and in order to achieve maximal signal to noise for a given observation period, it was found to be preferable to collect several transients using rapid pulses for each nucleus before switching to the other one. The spectra obtained by this method are not fully relaxed and this had to be taken into account for quantification of the data. Typically the amount of time spent observing each nucleus was varied depending on its sensitivity. The number of scans acquired was chosen to be compatible with the cycling of the quadrature phase employed in these experiments.

For the observation of three nuclei these principles were extended and the organisation of data acquisition in an experiment for the simultaneous detection of a \(^{1}H\) spin echo spectrum with water suppression, and \(^{13}C\) and \(^{31}P\) spectra with broad-band proton decoupling is illustrated in Figure 2.3.
2.2.4 Calibration

While NMR spectra readily give qualitative information about the metabolites present in tissues and allow rough estimates of their relative concentration, more accurate quantification poses some problems. For a given resonance in an NMR spectrum the peak intensity is proportional to the concentration of the metabolite responsible but the constant of proportionality may differ between resonances in the same spectrum. This is due to differences in the spin-lattice relaxation time, nuclear Overhauser enhancement and linewidth of the resonances (Campbell & Dobson, 1979). This problem is particularly pertinent when spectra of different nuclei are being compared and where non-relaxed spectra are being observed to maximise the signal to noise ratio obtained. For these reasons considerable effort was spent in calibration of the peak intensities of the resonances used in the analysis of the results.

Erythrocyte experiments

The $^1H$ spectra of erythrocytes were used to observe lactate and this resonance was calibrated by making extracts of the cells at the beginning and end of the NMR experiment. The lactate in these extracts was assayed spectrophotometrically (see section 2.2.5) and intermediate concentrations could be interpolated from the peak height of the lactate resonance in the $^1H$ spectrum. The same approach was used for the 2,3 DPG resonance in the $^{31}P$ NMR spectrum and the assay for this metabolite is also described in section 2.2.5.
The $^{13}$C spectra were used to compare the concentrations of $[1^{-13}C]$ glucose and $[3^{-13}C]$ 2,3 DPG and it was important to take into account the effect of any differences in the relaxation times or nOe effects on their respective resonances. Perchloric acid extracts of erythrocytes were made during the NMR experiments and after lyophilisation the residue was dissolved in 150 mM KCl, 20 mM HEPES, pH 7.4 to mimic the intracellular conditions. $^{13}$C NMR spectra of these extracts were recorded using identical acquisition conditions to those used in the kinetic studies and these were compared with fully relaxed spectra recorded without nOe enhancement ($^1$H decoupling gated off during the relaxation period). In these latter spectra the intensities of the resonances from glucose and 2,3 DPG were proportional to their relative concentrations. It was found that under the conditions used in the kinetic studies the C-3 resonance of 2,3 DPG was enhanced by 35% relative to the sum of the two C-1 resonances of glucose. This factor was used to correct the 2,3 DPG peak height in the analysis.

**Mitochondria experiments**

These experiments were easier to calibrate as the resonances of interest were all due to extracellular components whose concentrations were adjusted to known values at the start of the experiment.

**2.2.5 Spectrophotometric assays**

**Lactic acid**

Lactic acid was assayed by the method of Henry
(1968) which is based on its conversion to pyruvate by the enzyme lactate dehydrogenase (LDH) in the presence of excess NAD. The reaction is followed by the increase in absorption at 340 nm due to the formation of NADH.

Extracts were made by adding one volume of packed cells to two volumes of ice-cold 10% perchloric acid. The mixture was shaken vigorously and left to stand for 10 minutes before centrifugation in an Eppendorf Microfuge to obtain a clear supernatant.

The assay was carried out in a 1 ml plastic cuvette which contained 1 ml 0.16 mM NAD in 180 mM glycine buffer (pH 9.2) containing 150 mM hydrazine and 15 U/ml LDH. 0.071 ml of the supernatant from the PCA extract were added and the cuvette was incubated at 37°C for 30 minutes before reading the A₃₄₀ against a blank which contained 0.071 ml 10% perchloric acid in place of the extract. The concentration of lactic acid present was calculated using a value of 6.22 x 10⁶ cm² mol⁻¹ for the molar extinction coefficient of NADH at 340 nm.

2,3 bisphosphoglycerate

2,3 DPG was assayed by the method of Lowry et al. (1964) which is based on the following coupled enzyme reactions:

\[
2,3 \text{ DPG} \xrightarrow{2,3 \text{ DPG phosphatase}} 3\text{-PGA} + P_i
\]

\[
3\text{-PGA} + \text{ATP} \xrightarrow{\text{PGK}} 1,3 \text{ DPG} + \text{ADP}
\]

\[
1,3 \text{ DPG} + \text{NADH} \xrightarrow{\text{GAPDH}} \text{GAP} + \text{NAD}
\]
The decrease in absorption at 340 nm caused by the oxidation of NADH to NAD reflects the amount of 2,3 DPG originally present.

The extract used for this assay was the same as that used for the lactic assay described above but had been neutralised by taking 2.5 ml, adjusting the pH to 7.0 with 1 M KOH and making the volume up to 5.0 ml.

The assay was carried out in a 1 ml plastic cuvette which contained 1 ml 0.16 mM NADH in 170 mM triethanolamine buffer pH 8, 0.04 ml 180 mM ATP and 0.1 ml supernatant from the extract. 8 µl of 2,3 DPG phosphatase (2400 U/ml) and 4 µl each of GAPDH (800 U/ml) and PGK (450 U/ml) were added and the $A_{340}$ was read against a water blank. 40 µl of 20 mM phosphoglycolic acid solution was added and after incubation at 37°C for 30 minutes the $A_{340}$ was read again. The difference in the two readings is due to the oxidation of NADH and was used to calculate the concentration of 2,3 DPG initially present.

2.3 Results and Discussion

2.3.1 Human erythrocyte experiments

The spectra shown in Figure 2.4 illustrate the metabolism of [1-$^{13}$C] glucose by human erythrocytes over a period of 15 hours. The time course was followed by the simultaneous observation of $^{13}$C, $^{31}$P and $^1$H NMR signals from the sample as described in section 2.2.

In the experiment illustrated, the cells had been incubated for 48 hours at 4°C with [1-$^{13}$C] glucose prior to the experiment in order to raise the levels of the
A simultaneous multinuclear NMR time course from human erythrocytes metabolising [1-$^{13}$C] glucose.

A 15-h time-course is represented by stacked plots of 40 simultaneous spectra. Each set of spectra from the three nuclei took 22 minutes to record by interleaving the acquisition of blocks of transients in the order $^{31}$P-$^{13}$C-$^1$H- etc. The approximate relative times spent observing each nucleus were 1.5:4:1 respectively.

(A). 67.89 MHz proton decoupled $^{13}$C spectra. Each spectrum is the sum of 640 transients with a recycle time of 1.13 seconds. The spectra show resonances from the α and β anomers of [1-$^{13}$C] glucose and [3-$^{13}$C] 2,3 bisphosphoglycerate.

(B). 109.28 MHz proton decoupled $^{31}$P spectra. Each spectrum is the sum of 320 transients with a recycle time of 0.81 seconds. The spectra show resonances from inorganic phosphate, the 2- and 3-phosphates of 2,3 bisphosphoglycerate and a resonance downfield of the 3-phosphate resonance of 2,3 bisphosphoglycerate, which is assigned to AMP.

(C). 269.96 MHz $^1$H spin echo spectra (τ = 136 ms). Low power on-resonance decoupling was used to saturate the water peak during the delay between successive acquisitions. Each block was the sum of 160 transients with a recycle time of 1.01 seconds. The spectra show resonances from the methyl group of $^{12}$C and $^{13}$C labelled lactate. The coupling constant between the $^{13}$C label and the methyl group protons is approximately 130 Hz.

For all three nuclei, each block was preceded by two "dummy scans" to ensure equilibration of magnetisation during the entire acquisition.
triose phosphates (Eckel et al., 1966). At $t = 0$ sodium pyruvate was added to give a concentration of 6 mM pyruvate which should increase the flux into the 2,3 bisphosphoglycerate bypass (Eckel et al., 1966; Rose & Warms, 1970). The sample was then placed in the probe maintained at 37°C and allowed to equilibrate for five minutes before any spectra were recorded. Simultaneous $^{13}$C, $^{31}$P and $^1$H NMR spectra were then collected automatically over the course of the experiment.

As a result of the simultaneous observation of these three nuclei, the following information can be obtained from a single experiment.

The $^{13}$C spectra (Figure 2.4(A)) show the concentration of [1-$^{13}$C] glucose, [3-$^{13}$C] 2,3 bisphosphoglycerate and [3-$^{13}$C] lactate (not shown in Figure 2.4). These are the only metabolite pools which accumulate sufficient label to be observed under the conditions of the experiment. The observation of two resonances for glucose is due to the presence of $\alpha$ and $\beta$ anomers. The resonance due to [3-$^{13}$C] lactate is difficult to observe in the $^{13}$C NMR spectrum of erythrocytes as it is superimposed on a large, broad, natural abundance signal due to the methyl groups of protein, particularly haemoglobin. This hump could be removed by the use of a spin echo experiment with a short $\tau$ value (ca. 2 ms) owing to the relatively short $T_2$s of the protein resonances. However, the loss of signal to noise in the spectrum as a whole meant that this technique was not used routinely in the experiments described in this chapter.

The $^{31}$P spectra (Figure 2.4(B)) show the
concentration of 2,3 bisphosphoglycerate, $P_i$, ATP and ADP (the last two are not shown in the figure). In addition to this, the intracellular pH may be determined from the chemical shift of the $P_i$ resonance. In the early stages of the time course illustrated in Figure 2.4 (B), the $P_i$ resonance has two components and these are due to signals from both the intracellular and extracellular media at slightly different pH. Under some conditions these resonances were better resolved allowing the measurement of the pH in the two compartments.

The proton spectra (Figure 2.4 (C)) were accumulated with a $90^\circ-\tau-180^\circ-\tau-$ spin echo pulse sequence using a $\tau$ value of 136 ms. This method of reducing the intensity of unwanted resonances due to water and haemoglobin has been described previously (Brown et al., 1977). The water resonance was further suppressed by applying saturating irradiation at the water resonance frequency during the delay between successive acquisitions. Only part of the proton spectrum is illustrated and this shows the resonances due to the C-3 protons of lactate. The $^{13}C - ^1H$ coupling constant is around 130 Hz and so the $^{13}C$ satellites are well resolved. The proton spectra contain a large number of other resonances which can provide further information.

The combination of the information provided by the observation of these metabolites simultaneously allows the investigation of several aspects of glycolysis in erythrocytes. These are discussed below.
Figure 2.5

Anomeric specificity of glucose utilisation

A plot of the peak heights of the C-1 resonance of the α (x) and β (o) anomers of glucose from the $^{13}$C spectra in Figure 2.4. The utilisation of glucose appears to be anomerically symmetrical as a result of the relatively low glycolytic flux in erythrocytes (see text).
Anomeric specificity of glucose utilisation

The peak heights of the α and β anomers of glucose from Figure 2.4 (A) are plotted as a function of time in Figure 2.5. The ratio of the initial intensities of these resonances was 37:63 and this reflects the expected anomeric equilibrium for glucose (Benkovic & Schray, 1976). This ratio does not change significantly during the time course indicating that either there is no anomeric specificity of glucose uptake or that the spontaneous rate of anomerisation is greater than that of glucose utilisation. It is known (Faust, 1960) that the transport mechanism for glucose into the erythrocyte is specific to the β anomer and that the enzyme aldose-1-epimerase (mutarotase), which catalyses the anomerisation of glucose, is absent from the erythrocyte membrane (Kahlenberg & Miller, 1972). This implies that the lack of an observable anomeric asymmetry in glucose utilisation is due to the slow rate of glycolysis (around 2 mM h⁻¹) relative to the rate of spontaneous anomerisation (K_{αβ} = 1.9 \times 10^{-2} \text{ min}^{-1} (den Hollander et al., 1979)). This is in contrast to the situation observed in E. Coli (Ugurbil et al., 1978) and yeast cells (den Hollander et al., 1979) where a preferential utilisation of β glucose was observed. This difference is probably due to the much higher rate of glycolysis in these cells.

The 2,3 bisphosphoglycerate bypass

It is known that on storage at 4°C the ATP concentration in red cells declines rapidly (Rapoport, 1974). This effect was observed in the experiment
Figure 2.6

2,3 DPG as an energy reservoir

Plots of the 3-P resonance of 2,3 DPG and the β resonance of ATP during the time-course illustrated in Figure 2.4. The ATP concentration remains constant at the expense of the 2,3 DPG pool until the latter is exhausted when the ATP level declines rapidly.
illustrated here through the $^{31}$P spectra where the ATP resonances (not shown in Figure 2.4) are less than half the intensity of those observed in fresh cells. As a consequence, at $37^\circ$C the cells begin to metabolise 2,3 bisphosphoglycerate as an energy source and the level of this pool decreases while the level of ATP remains constant (Figure 2.6). Despite this, the $^{13}$C spectra show that while the cells are still metabolising glucose the concentration of $^{13}$C labelled 2,3 bisphosphoglycerate increases initially and then levels off. The fractional labelling of this pool is thus increasing showing that there is still considerable flux into the bypass. This is quite interesting as the 2,3 bisphosphoglycerate cycle bypasses one of the steps of ATP production in glycolysis and under conditions of ATP depletion it might be expected that the flux through the bypass would be greatly reduced.

In another experiment where the pH of the erythrocytes was 7.2 (only 0.2 less than the in vivo value) the 2,3 bisphosphoglycerate declined more rapidly than in the experiment illustrated above even in the presence of glucose. In this experiment it was not possible to detect any flux into the 2,3 bisphosphoglycerate pool from the $^{13}$C spectra and this provides confirmation of the proposal of Rapoport et al. (1977) that the 2,3 bisphosphoglycerate synthase reaction is virtually completely inhibited at low pH. These findings also lend support to the theory that the main controlling factor of the flux into the bypass is the level of 1,3 bisphosphoglycerate (1,3-DPG), an activator of the synthase reaction. Under the conditions of the
experiment illustrated here (high triose phosphate levels and high pyruvate concentration) the concentration of 1,3-DPG would be elevated (Rose & Warms, 1970) and this could account for the high flux through the bypass even when there is also a high 2,3 bisphosphoglycerate-phosphatase activity. Under the conditions of low pH it is known that the concentration of 1,3-DPG is greatly reduced (Minakami & Yoshikawa, 1966; Momsen & Vestergaard-Bogind, 1978) and so the flux into the bypass is repressed.

In contrast, at high pH the concentration of 1,3-DPG would be expected to increase as a result of the observed accumulation of fructose 1,6-diphosphate and the triose phosphates under these conditions (Mitsumori, 1985). Hamasaki & Minakami (1972) did observe an increase in 2,3 bisphosphoglycerate concentration in erythrocytes at pH 7.8 in the presence of 5 mM inosine. In the absence of this additional source of carbon, Mitsumori (1985) observed a constant level of 2,3 bisphosphoglycerate in erythrocytes at pH 7.8 while the ATP levels declined for the first three hours of the incubation. These changes were assigned to the effects of activation of phosphofructokinase at high pH. It is possible, however, that a factor contributing to this phenomenon is an increase in flux through the 2,3 bisphosphoglycerate bypass resulting in a reduced ATP production.

At longer incubation times at pH 7.8 Mitsumori observed a rapid decline in 2,3 bisphosphoglycerate but this was not sufficient to maintain the level of ATP. This is in contrast with the results from the experiment at pH
7.4 described above where the ATP concentration was maintained at the expense of the 2,3 bisphosphoglycerate pool (Figure 2.6). Again, this may be due to a greater relative flux through the bypass at pH 7.8 than at pH 7.4 resulting in a loss of potential ATP production. It would be interesting to measure the flux through the 2,3 bisphosphoglycerate bypass under alkaline conditions using the method described here to see if it was indeed increased.

Figure 2.4 (A) shows that after about six hours into the incubation at pH 7.4 the glucose supply is exhausted and soon after this the ATP rapidly declines. This change is concomittant with the appearance of a resonance just downfield of the 3-phosphate peak of 2,3 bisphosphoglycerate. This is assigned to AMP on the basis of its chemical shift (Burt et al., 1979). These observed changes in the nucleotide balance are in agreement with the findings of Rappoport et al. (1977), who followed by chemical assay the AMP, ADP and ATP levels of erythrocytes in the presence and absence of glucose.

The quantified results of two separate experiments are summarised in Table 2.1. In experiment A, fresh blood was washed as described in section 2.2.2, the final wash containing no glucose. At t = 0, 10mM [1-13C] glucose was added and the sample placed in the NMR probe at 37°C. The final pH was 7.4. Experiment B was identical to experiment A except that 6 mM sodium pyruvate was also added at t = 0. In both these experiments multinuclear NMR spectra similar to those shown in Figure 2.4 were collected over a period of 3 hours. Peak heights of the metabolite
Plot of concentrations (mM) of [1-^{13}C] glucose and [3-^{13}C] 2,3 bisphosphoglycerate as a function of time during steady state glycolysis. Peak intensities were measured from spectra collected as described in the legend to Figure 2.4 and calibrated as described in section 2.2.4. The sum of the α and β[1-^{13}C]glucose peaks (♦) and the 3-^{13}C bisphosphoglycerate peak (◊) were used and are shown here as concentrations. The curves are least-square fits of straight lines to the data.

A comparison of these rates shows that 37% of the glycolytic flux passes through the bypass under these conditions.
resonances were measured and converted to concentrations by the various calibration methods described in section 2.2.4. The rates of chemical or isotopic flux were then calculated and are displayed in Table 2.1. The peak height data from the $^{13}$C spectra in experiment B after conversion to concentrations are illustrated in Figure 2.7.

Table 2.1

<table>
<thead>
<tr>
<th>Metabolic fluxes ($\mu$mol hr$^{-1}$ per ml cells)</th>
<th>2,3 DPG</th>
<th>Pi</th>
<th>ATP</th>
<th>glucose</th>
<th>2,3 DPG</th>
<th>$^{13}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTAL</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-2.3</td>
<td>+0.63</td>
<td></td>
</tr>
<tr>
<td>Experiment A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-0.75</td>
<td>+0.3</td>
<td></td>
</tr>
</tbody>
</table>

Over the period of observation the $^{31}$P spectra showed that the total concentration of 2,3 bisphosphoglycerate was constant and so the increase in intensity of the 3-C resonance of 2,3 bisphosphoglycerate in the $^{13}$C spectra over this period represents the total flux of $^{13}$C label into the 2,3 bisphosphoglycerate pool. This may be compared with the rate of [1-\(^{13}\)C] glucose utilisation (from the sum of the $\alpha$ and $\beta$ anomer peak heights in the $^{13}$C spectra) to give the percentage flux through the bypass under steady-state conditions. The value obtained is 27%. This is in agreement with some of the previous estimates of the bypass contribution e.g.
Rapoport et al., (1977) (24%); Hamasaki & Minakami, (1972) (25-50%) but differs from other estimates e.g. Momsen & Vestergaard-Bogind (1978) (10-15%), Rapoport & Neiradt (1955) (90%). These previous estimates were made from computer simulations or from in vitro measurements under non-steady-state conditions and involved making assumptions about the rate limiting step of the bypass (thought to be the phosphatase step). Possibilities of activation of the phosphatase reaction by inorganic phosphate (Rose & Liebowitz, 1970) or 2-phosphoglycolate (Rose & Salon 1979) were not taken into account in some of these calculations and may affect the interpretation. The method used here is much easier to interpret as it is carried out under true steady-state conditions and no assumptions about the mechanism of the bypass need to be made other than that its two individual steps are essentially irreversible.

In the presence of 6 mM pyruvate (Experiment B) the contribution of the 2,3 bisphosphoglycerate bypass under these conditions was found to be 37% of the overall glycolytic flux. This reflects the expected increase in flux through the bypass in the presence of high pyruvate concentrations (Rose & Warms, 1970) and is due to production, via lactate dehydrogenase, of high concentrations of NAD required for the synthesis of 1,3-DPG in the glyceraldehyde 3-phosphate dehydrogenase reaction.
The appearance of the $^{13}\text{C}$ satellites of the lactate peak in the $^1\text{H}$ spectra (Figure 2.4 (C)) can be seen to lag behind the time course of the $^{12}\text{C}$ peak. This is due to the time taken to flush the residual $^{12}\text{C}$ from the intermediate metabolic pools before $^{13}\text{C}$ label begins to accumulate in the lactate pool. The pool size may be estimated from this time lag and was found to be around 5mM in this experiment. This is in agreement with published values (e.g. Eckel et al., 1966).

Information about the pentose-phosphate pathway may be obtained by comparison of the rates of production of $^{13}\text{C}$ and $^{12}\text{C}$ lactate. Values for these quantities in two separate experiments are shown in Table 2.2. Difficulties in calibration are relatively slight here because both species are observed in the same spectrum and are due to the same compound. There will be no significant difference between the $T_1$ values of the C-3 protons of the $^{12}\text{C}$ and $^{13}\text{C}$ species as dipolar relaxation will be dominated by the adjacent protons rather than by the carbon nucleus.

**Table 2.2**

<table>
<thead>
<tr>
<th>Metabolic fluxes ($\mu\text{mol hr}^{-1} \text{ per ml cells}$)</th>
<th>$^{12}\text{C}$ lactate</th>
<th>$^{13}\text{C}$ lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A</td>
<td>2.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Experiment C</td>
<td>4.1</td>
<td>2.7</td>
</tr>
</tbody>
</table>
Figure 2.8

Estimation of the pentose-phosphate pathway contribution

A) Theoretical variation of the $^{12}\text{C}$ and $^{13}\text{C}$ lactate concentrations with time in the absence (solid lines) and presence (broken line) of a contribution from the pentose-phosphate shunt.

B) Experimental variation of the $^{12}\text{C}$ (o) and $^{13}\text{C}$ (x) lactate peak heights with time from the $^1\text{H}$ spin echo spectra of erythrocytes metabolising [1-$^{13}\text{C}$] glucose.
Figure 2.8 (A) shows the theoretical variation of the concentrations of $^{12}\text{C}$ and $^{13}\text{C}$ labelled lactate with time if all the glucose in the cells is instantaneously replaced with glucose labelled with $^{13}\text{C}$ at the C-1 position. When steady-state has been reached the rate of production of $[3-^{12}\text{C}]$ lactate and $[3-^{13}\text{C}]$ lactate should be equal as half the carbon at the C-3 position comes from the C-6 carbon of glucose which is not labelled. This situation is represented in Figure 2.8 (A) by the solid curve. If, however, there is a leakage of carbon originating from the C-1 position of glucose out of the Embden-Meyerhof pathway, the steady-state rate of $[3-^{13}\text{C}]$ lactate production will be less than that of $[3-^{12}\text{C}]$ lactate and the rate of leakage may be calculated from this difference. This is represented by the broken curve in Figure 2.8 (A). In glycolysis such a mechanism for leakage exists in the form of the pentose-phosphate shunt in which glucose 6-phosphate is oxidised to ribulose 6-phosphate and CO$_2$. All the CO$_2$ originates from the C-1 position of glucose in the first cycle of the shunt and the remaining five carbons are eventually returned to the main glycolytic pathway as fructose 6-phosphate and glyceraldehyde 3-phosphate.

Figure 2.8 (B) shows experimental data from the $^1\text{H}$ spectra of erythrocytes metabolising $[1-^{13}\text{C}]$ glucose and the situation is clearly akin to that where the pentose-phosphate shunt is active.

The quantitative contribution of the shunt may be calculated as follows. The rate of production of $^{13}\text{C}$ lactate is simply the rate of glycolysis minus the rate at
which glucose flows into the pentose phosphate shunt. The rate of production of $^{12}$C lactate is equal to the rate of glycolysis plus the rate at which the remaining carbon returns to the Embden-Meyerhof pathway after C-1 has been lost as $^{13}$CO$_2$.

Thus, if the rate of glucose utilisation = G, the rate of flow of glucose into the pentose phosphate pathway = P and the rates of production of $^{12}$C and $^{13}$C lactate are $[^{12}\text{C}]$ and $[^{13}\text{C}]$ respectively, then:

$$[^{13}\text{C}] = G(1-P)$$

$$[^{12}\text{C}] = G(1-P) + \frac{5GP}{3}$$

Substituting for G:

$$[^{12}\text{C}] =[^{13}\text{C}] + \frac{5[^{13}\text{C}]P}{3(1-P)}$$

Solving for P:

$$P = \frac{3([^12\text{C}] -[^{13}\text{C}])}{5[^{13}\text{C}] + 3([^12\text{C}] -[^{13}\text{C}])}$$

However, the glucose used in these experiments is not isotopically pure but only contains 91% $^{13}$C at C-1 and the above equations are modified as follows:

$$[^{13}\text{C}] = SG(1-P)$$

$$[^{12}\text{C}] = SG(1-P) + \frac{5SGP + 2G(1-S)}{3}$$

where S = the fractional isotopic purity of the glucose.

Then, solving for P:
Using the data in Table 2.2 the contribution of the pentose phosphate pathway to the overall glycolytic flux in experiment A is found to be 25%. This is rather higher than some previous estimates of the contribution of the shunt (Yunis & Yashmineh, 1969) (ca. 10%) but it has been shown that under oxidative stress the flux through the shunt can increase dramatically and in the presence of the oxidising agent methylene blue the pentose phosphate pathway was shown to account for over 80% of the glycolytic flux (Gaetani et al., 1974). It is possible that the existence of oxidative stress in the erythrocytes used in this experiment resulted in a high pentose phosphate shunt contribution. In a similar experiment (Experiment C in Table 2.2) the pentose phosphate shunt contribution was found to be 13% which is closer to the value expected for normal red cells.

It should be possible to check these estimates and also to make an estimate of the extent, if any, of secondary recycling of the pentose-phosphate shunt by repeating this experiment with glucose labelled at the C-6 or C-2 positions. These experiments have not been done owing to the difficulty and expense of obtaining the labelled compounds.

The method presented here provides a very convenient way of estimating the contribution of the pentose phosphate pathway to glycolysis in the erythrocyte. Previously published methods have been based on
Figure 2.9

**Observation of oxidative phosphorylation in mitochondria by simultaneous multinuclear NMR**

A) Series of 109.3 MHz $^{31}$P NMR spectra. Each spectrum represents the sum of 96 transients recorded with a 70 degree pulse and a recycle time of 1.41 s. The spectra show resonances due to the β phosphate of ADP and the γ phosphate of ATP. The chemical shift scale is referenced to MDP at 0.0 PPM.

B) Series of 269.96 MHz $^1$H NMR spectra. Each spectrum represents the sum of 48 transients recorded with a 90-τ-180-τ spin echo sequence using a τ value of 136 ms. The recycle time was 2 s and irradiation was applied at the water frequency during the relaxation delay to saturate this unwanted resonance. The spectra show the resonance due to succinate and are referenced relative to TMS at 0.0 PPM.
radioisotopic techniques using $^{14}\text{C}$ labelled glucose and necessitate the trapping, isolation and counting of metabolites containing the label (e.g. Yunis & Yashmineh, 1969).

Recently a method of measuring the pentose phosphate pathway flux in erythrocytes by the observation of the rate of oxidation of reduced glutathione in the $^{1}\text{H}$ spin echo spectrum has been proposed (Thorburn & Kuchel, 1985). The method presented here, however, should be more sensitive as the concentration of lactate produced in the erythrocyte is an order of magnitude greater than that of glutathione.

2.3.2 Applications to other systems

The wealth of data available from a multinuclear study of metabolism is potentially available from a wide range of biological systems. A further application that has been studied here is in an investigation into oxidative phosphorylation in rat liver mitochondria. These sub-cellular organelles synthesise ATP from ADP via an enzyme called the $F_1/F_0$ ATP synthase situated on the inner mitochondrial membrane. The driving force for this reaction comes from the utilisation of protons that have been pumped out of the mitochondria by the coupled enzymes of the electron transport chain. In this process substrates such as NADH or succinate are oxidised and the energy produced is used to pump protons across the mitochondrial membrane (Nichols, 1982; Ferguson, 1985).

Figure 2.9 shows a time-course of the oxidative phosphorylation process in mitochondria followed by
simultaneous $^1$H and $^{31}$P NMR. The $^{31}$P spectra (Figure 2.9(A)) show the resonances of $\beta$ ADP and $\gamma$ ATP which may be used to estimate the rate of phosphorylation. The $^1$H spectra (Figure 2.9(B)) show the resonance due to succinate which may be used to calculate the rate of substrate utilisation. The combination of these data provide useful information about the stoichiometry of the coupling of oxidative phosphorylation in the mitochondria (Carr et al., 1985).

2.4 Conclusions

The adaptation of an NMR spectrometer to put all the major functions under computer control allowed the simultaneous detection of up to three magnetic nuclei with a fourth frequency available for a field-frequency lock. With modern spectrometers this degree of software control is commonplace and the technique described here could be implemented quite readily.

The advantages of simultaneous detection of more than one nucleus are great in that much more information is available than from the observation of a single nucleus. In this study a combination of $^{31}$P, $^{13}$C and $^1$H NMR was used to investigate some aspects of glycolysis in the human erythrocyte. The advantages of multinuclear observations were particularly apparent in the study of the redistribution of $^{13}$C label originating from [1-$^{13}$C] glucose. Some metabolites which incorporated this label were observable in the spectra of two of the nuclei observed and this allowed the calculation of the
fractional labelling of that metabolic pool. The separation of chemical and isotopic flux would be difficult without this information.

The data were used to calculate the fluxes through the 2,3 bisphosphoglycerate bypass and the pentose-phosphate shunt which were found to vary between 27-37% and 13-25% respectively under various conditions. The relative ease with which these measurements were made compared with the classical techniques available should make a systematic study of these pathways more feasible.

In a second application, the coupling of substrate oxidation and ADP phosphorylation in rat liver mitochondria was observed in a simultaneous $^{31}$P and $^1$H NMR study.

In principle, the method described could be applied to any magnetic nuclei. The main limitation is that of many applications of NMR to biological systems: that of a lack of sensitivity. The sensitivity in this experiment is less than that in a mononuclear study for two reasons. First, there is an unavoidable loss in sensitivity when the probe is double-tuned to two frequencies. This loss is reduced in the extra-inductance method of tuning used here if the two frequencies are very dissimilar. The second source of the loss in sensitivity is the fact that the observation time must be divided between different nuclei. This limitation must be weighed against the gain in information in a particular case.

A further application of the technique presently being considered is a double-tuned $^{23}$Na-$^{31}$P probe which would be useful for studying the compartmentation of Na$^+$
and H\(^+\) ions in biological systems.
2.5 References


CHAPTER 3
TEMPERATURE-JUMP RELAXATION STUDIES OF ALDOLASE

3.1 Introduction

There has been much discussion in the literature about the possible association of some glycolytic enzymes with various sub-cellular structures and the implications of this to metabolic regulation (Ottaway & Mowbray, 1977; Clarke & Masters, 1975). Postulated interactions have ranged from a "glycosome" in Trypanosoma brucei containing many glycolytic enzymes in a single particle (Opperdoes & Borst, 1977) to smaller scale interactions between cell membranes and hexokinase (Rose & Warms, 1967), phosphofructokinase (PFK) (Higashi et al., 1979), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Kliman & Steck, 1980) and aldolase (Solti & Friedrich, 1976; Strapazon & Steck, 1977).

In vitro studies on human erythrocyte ghosts have shown that the most strongly bound enzymes are aldolase and GAPDH. These two enzymes appear to bind to a common site in the N-terminal region of the band III protein of erythrocyte membranes (Tsai et al., 1982) and this site is also shared by PFK (Higashi, 1979). Sufficient sites are available in the band III protein to bind all three enzymes completely (Kliman & Steck, 1980). While PFK seems to be activated by membrane binding (possibly as a result of relief of allosteric inhibition by ATP and 2,3 bisphosphoglycerate (Karadsheh & Uyeda, 1977)), both GAPDH and aldolase are inactivated on membrane binding, this
inactivation being reversed by elution of the enzymes at high ionic strength or millimolar concentrations of their substrates.

Most of the studies of these interactions have been carried out on hypotonically lysed cells and it has been suggested (Maretzki et al., 1974) that at the ionic strengths found in vivo the extent of enzyme binding would be negligible. However, the possibility of large deviations from thermodynamic ideality in the presence of high concentrations of inert macromolecules (Nichol et al., 1981), particularly haemoglobin in the erythrocyte, has led some workers (Kelley & Winzor, 1984) to suggest that binding, and hence inactivation, of the enzyme may still be significant under physiological conditions. Extrapolation of in vitro data to intracellular conditions has lead Kliman & Steck (1980) and Jenkins et al. (1984) to propose that as much as two thirds of GAPDH and 40% of aldolase are membrane-bound in the intact human erythrocyte.

It has also been proposed recently (Offermann et al., 1984) that the intracellular presence of biological disulphides such as glutathione disulphide may cause covalent modification of aldolase leading to an increase in membrane binding and inactivation in situ.

Such in vitro studies, while demonstrating effects that may be of significance in the intact cell, are insufficient to throw much light on the true situation in vivo. Hence there have been efforts to measure the properties of the glycolytic enzymes under conditions more
closely resembling those in the intact cell. Keokitichai & Wrigglesworth (1980) carried out studies on erythrocytes cross-linked by glutaraldehyde prior to lysis and found a high degree of association of GAPDH with membranes. Brindle et al. (1982), however, measured the inhibition kinetics of GAPDH in the intact cell by a non-invasive NMR method and concluded that membrane binding was not likely to be physiologically significant for this enzyme. There are fewer data available for aldolase but Aragon et al. (1980) have made measurements on erythrocytes which have been permeabilized by delipidation and these suggest that the \( V_{\text{max}} \) of aldolase is about 40% of its value in vitro while its \( K_m \) is not significantly different in situ and in vitro.

**Investigation of aldolase kinetics in situ**

There are two general approaches to the study of the kinetics of an enzyme catalysed reaction in situ. The reaction may be investigated near equilibrium by observing isotopic flux under chemical steady-state or by observing chemical flux through the enzyme when the reaction is displaced from equilibrium. In either approach the main problem is isolating the enzyme of interest from others catalysing precursor or subsequent reactions.

Some examples of observing isotopic flux through the glycolytic pathway under biochemical steady-state have been discussed in Chapter 2. Attempts to observe flux through aldolase have been made by both radioisotope studies on human erythrocytes (Rose & Warms, 1970) and NMR studies of \(^{13}\text{C} \) label redistribution in yeast (den
Exchange of the pro-S-C-1 proton of fructose 1,6 bisphosphate with solvent.

The exchange is followed by a $^{31}\text{P}-^1\text{H}$ heteronuclear spin echo experiment described in the text. The sample contained 20mM FDP at pH 7.4 and 20°C and the exchange was initiated by the addition of 15 Units of rabbit muscle aldolase. Each spectrum represents the sum of 128 transients containing 2K data points and took 6.7 minutes to accumulate. A $\tau$ value of 70ms was employed to give inversion of the doublet.
Hollander et al., 1979). It was concluded that aldolase and triosephosphate isomerase (TPI) were close to equilibrium in both tissues.

The isotope exchange method has been applied successfully to the study of GAPDH in the human erythrocyte (Brindle et al., 1982; Foxall et al., 1984). These experiments employed a $^1$H NMR spin-echo method to follow the enzyme-catalysed solvent exchange of $^1$H and $^2$H at the C-2 position of lactate. Iodoacetate, a specific inhibitor of GAPDH, was used to investigate inhibition patterns of the enzyme in the intact cell.

A similar approach might in principle be applied to the aldolase problem. It has been shown (Lowe & Pratt, 1976) that aldolase catalyses the exchange of the pro-S-C-1 proton of fructose 1,6-diphosphate (FDP) with solvent. As this proton is coupled to the 1-phosphate group of FDP with a heteronuclear $^{31}$P-$^1$H coupling constant of 6.8Hz, its exchange with solvent could be followed in D$_2$O solution using a heteronuclear $^{31}$P-$^1$H spin echo experiment of the type proposed by Freeman et al. (1981). In this experiment a $^{31}$P spin echo is observed using a 90°-τ-180°-τ sequence with τ = 1/2$J_H$, where $J_H$ is the $^{31}$P-$^1$H coupling constant. A 180° $^1$H pulse is applied at the same time as the 180° $^{31}$P pulse and modulation of the $^{31}$P resonance is observed with the heteronuclear coupling constant as shown in equation (1).

$$I(2\tau) = I(0) \exp(-\frac{2\pi}{T_2} - \frac{2D\tau^2}{3}) F(J) \quad (1)$$

where $F(J)$ is of the form $\cos(2\pi J\tau)$ for a doublet.
A sample of freshly drawn human erythrocytes pH 7.4 after 3 hours incubation at 37°C with 10mM inosine and 4mM inorganic phosphate. The spectrum represents the sum of 128 transients with a sweep width of 2000Hz, a 90° pulse and an interpulse delay of 1s. The assignments of the resonances are: peak 1, DHAP; peaks 2 and 3, FDP; peaks 4 and 5, 2,3 DPG; peak 6, inorganic phosphate; peak 7,8 and 9, nucleotide phosphates.
I is the resonance intensity in the spin echo spectrum, D is the diffusion coefficient, \( G^2 \) is a field gradient term and \( \gamma \) is the gyromagnetic ratio.

Broad-band \(^1\text{H}\) decoupling may be applied during the acquisition to collapse the \(^{31}\text{P}-^1\text{H}\) coupling in the final spectrum. A time course of the exchange at 20°C in a 20mM sample of FDP was followed using this method and is illustrated in Figure 3.1. At \( t=0 \) the exchange was started by the addition of 15 Units of aldolase and as the pro-S-C-1 proton is replaced by a deuteron from the solvent, the 1-phosphate peak becomes inverted. The rate of exchange should be a function of aldolase activity and the process could, in principle, be followed in the intact erythrocyte and compared with measurements made \textit{in vitro}. There are two obstacles to the application of this method, however. First, the exchange of the pro-S-C-1 proton is not linked directly to the FDP cleavage reaction but is due to a side reaction (Lowe & Pratt, 1976) and it may be difficult to quantify the effects observed. In the case of the GAPDH catalysed reaction the exchange rate is directly related to the flux through the enzyme. The second obstacle is the technical difficulty of observing the exchange in the intact erythrocyte because of a) the proximity of the large 2,3 bisphosphoglycerate resonances to the FDP 1-P signal (Figure 3.2) and b) the short \( T_2 \) relaxation times of the resonances which result in poor sensitivity for the \(^{31}\text{P}\) spin echo experiments. \textit{In situ} inhibitor studies of the sort done by Foxall et al. (1984) would be difficult owing to the lack of a selective inhibitor of aldolase which could cross the cell membrane.
Figure 3.3

Reactions catalysed by aldolase and TPI
These difficulties in making observations of aldolase at equilibrium force us to consider investigating the enzyme when the reaction it catalyses is displaced from equilibrium. The major control enzymes in glycolysis such as hexokinase and PFK, which are normally displaced far from equilibrium, are quite readily studied in situ as their activities have a great influence on the overall glycolytic flux (Rose et al., 1964; Minakami & Yoshikawa, 1965). An enzyme such as aldolase, however, which is near equilibrium in the cell is much less accessible to study. The methods usually used to investigate the kinetics of such enzymes in vitro employ conditions under which the reaction is displaced far from equilibrium. This situation is neither attainable nor desirable in situ. Other methods that have been used in vitro are pressure and temperature jump techniques which induce small perturbations in a system at equilibrium followed by observations of the system "relaxing" to its new equilibrium position (e.g. Malcolm, 1975). Temperature jump experiments have been particularly useful in the study of NADH-linked dehydrogenases where the reaction may be followed conveniently by the absorbance of NADH at 340nm. Such methods have not been employed in situ owing to the difficulty of restricting the effects of the perturbations to a specific enzyme and the problems of monitoring the concentration of substrates in the cell.

The temperature jump method does, however, have potential for studying the aldolase catalysed reaction (illustrated in Figure 3.3) since this is close to
equilibrium in the cell and involves a finite enthalpy change of 54.6 KJ mol\(^{-1}\) (Herbert et al., 1940). The problem of isolating aldolase from the rest of the glycolytic pathway is partly overcome by inhibiting GAPDH with iodoacetate. The phosphofructokinase catalysed reaction (immediately preceding the aldolase step in glycolysis) is essentially irreversible in the red cell (the gluconeogenic enzyme fructosebiphosphatase is absent from the erythrocyte) and the isomerisation of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) catalysed by TPI has a negligible enthalpy change (Herbert et al., 1940) so neither of these reactions will be significantly perturbed by the temperature jump. It has been observed (Dr. K.M. Brindle, unpublished results) that when human erythrocytes at 37°C are cooled to 4°C, the concentrations of FDP, DHAP and GAP adjust to their new equilibrium values over a period of about an hour. The relaxation is therefore slow enough to be studied by methods other than the usual rapid reaction techniques of stopped-flow or rapid quench.

This chapter describes the use of \(^{31}\)P NMR to follow the chemical relaxation of an in vitro model of the reaction catalysed by aldolase subsequent to a rapid temperature jump from 37°C to 4°C. The dependence of the relaxation rate on the aldolase activity is examined in vitro and the results of this study are compared with the relaxation observed in the intact erythrocyte by chemical assay. These measurements are used to estimate the activity of aldolase visible to its substrates in situ in the red cell.
3.2 Materials and Methods

3.2.1 Materials

Triose phosphate isomerase and aldolase from rabbit muscle were obtained from Sigma and dialysed twice against 2.7mM EDTA containing 0.64mM 2-mercaptoethanol to remove ammonium sulphate.

All biochemicals and buffers were obtained from Sigma. All other chemicals were of analytical grade.

3.2.2 Preparation of human erythrocyte aldolase

This purification was based on the method of Strapazon and Steck (1977). 250ml of 6 day old packed cells was washed four times with 8 volumes of phosphate buffered saline (150mM NaCl, 5mM sodium phosphate, pH 7.0) at 4°C. After each wash the cells were spun at 2000 rpm for 15 min. and the buffy coat and cell debris were removed by aspiration. The cells were then lysed by dilution in 36 volumes of 5mM sodium phosphate containing 1mM dithiothreitol, 1mM EDTA, pH 7.0 and the hemolysate was incubated for 2 hours at 37°C. The membranes containing bound aldolase were collected by centrifuging the hemolysate at 10000 rpm for 30 min. at 37°C in a Beckman J2.21 centrifuge with a JA 10 rotor. The supernatant and debris were aspirated off and the membrane fractions were washed four times with 5mM sodium phosphate, pH 7.0 at 4°C. These membranes may be stored for several days at 0-5°C with no loss of aldolase activity (Strapazon and Steck, 1977).

The membrane fraction at this stage was around 110
ml and this was resuspended in 500 ml 5mM sodium phosphate and mixed with 500 ml 2mM fructose 1,6 bisphosphate. The final pH was adjusted to 7.8 and the suspension was stirred for one hour at 0°C to elute the aldolase from the membranes. The membranes were centrifuged off by spinning at 13000 rpm for 100 min at 4°C. The supernatant was collected and centrifuged again to remove the last traces of membrane material.

The total volume of the aldolase suspension at this stage was around 1000 ml and this was reduced by ultrafiltration using a 300 ml capacity Amicon ultrafiltration cell fitted with a PM30 membrane. The final volume of the aldolase concentrate was 11.5 ml and this had an aldolase activity of 4.8 units/ml. This represented a recovery of 40% of the aldolase initially present in the packed erythrocytes. The concentrated enzyme solution was stored frozen at -70°C where it was found to be stable for at least 6 weeks.

### 3.2.3 Enzyme assays

Assays of aldolase, GAPDH and TPI were performed using modifications of methods described by Beutler (1975). The methods all involve coupling the enzyme whose activity is being estimated to an NADH-linked dehydrogenase so that the reaction may be monitored by the change in absorbance at 340nm as NADH is oxidised to NAD⁺ or vice versa. Assays were carried out using a Varian Series 634 UV-Visible spectrophotometer fitted with a thermostatted cell holder maintained at 37°C. The assay
mixture was contained in disposable 1 cm path length plastic cuvettes and absorbance at 340 nm was measured against a water blank.

Enzyme activities were calculated on the basis of an extinction coefficient for NADH of $6.22 \times 10^6$ cm$^2$ mol$^{-1}$ at 340 nm and are expressed in terms of units (µmol of substrate consumed per minute). Blank assays were performed to ensure that the auxiliary enzymes used in the coupled assays did not contain significant activity of the enzyme being measured.

**Aldolase**

The coupled reactions used in this assay are:

\[
\text{aldolase} \quad \text{FDP} \quad \xrightarrow{\text{aldolase}} \quad \text{GAP} + \text{DHAP}
\]

\[
\alpha\text{-CDH} \quad \text{DHAP} + \text{NADH} + \text{H}^+ \quad \xrightarrow{\alpha\text{-CDH}} \quad \alpha\text{-glycerophosphate} + \text{NAD}^+
\]

The reaction mixture contained 100 mM Tris- HCl, pH 8.0, 0.5 mM EDTA, 0.2 mM NADH, 10 mM fructose 1,6 bisphosphate and approximately 1 unit/ml of TPI and 0.3 unit/ml of glycerol-3-phosphate dehydrogenase. The reaction was initiated by the addition of a suitable dilution of the sample containing aldolase and the linear decline in absorbance at 340 nm was followed on a chart recorder. The aldolase activity could then be calculated from this slope.

The FDP concentration used in this assay is greater than that employed by Beutler (1975) but was found necessary under these conditions to ensure maximal rate of the enzyme.
Triose phosphate isomerase

The coupled reactions used in this assay are:

\[
\text{TPI} \\
\text{GAP} \rightleftharpoons \text{DHAP} \\
\text{α-GDH} \\
\text{DHAP} + \text{NADH} + H^+ \rightleftharpoons \text{α-glycerophosphate} + \text{NAD}^+ 
\]

The reaction mixture contained 100mM Tris-HCl, pH 8.0, 0.5mM EDTA, 0.2mM NADH, 3mM D-GAP and approx. 0.2 unit/ml glycerol-3-phosphate dehydrogenase. The D-GAP was prepared from the diethylacetal of DL-GAP as outlined by Sigma and was assayed for D-GAP content by a modification of this assay. The reaction was started by the addition of a suitable dilution of the sample containing TPI. The decline in absorbance at 340nm is biphasic owing to the fact that D-GAP exists in two forms one of which (the diol form, comprising 95% of the equilibrium mixture) is inactive (Trentham et al., 1969). The rapid initial phase is due to the utilisation of the active aldehyde form of GAP and is used to estimate the TPI activity. The slow second phase is due to the rate limiting conversion of GAP(diol) to GAP(aldehyde) and is independent of TPI activity.

Glyceraldehyde 3-phosphate dehydrogenase

The coupled reactions used in this assay are:

\[
\text{PGK} \\
3\text{-PGA} + \text{ATP} \rightleftharpoons 1,3\text{-DPG} + \text{ADP} \\
\text{GAPDH} \\
1,3\text{-DPG} + \text{NADH} + H^+ \rightleftharpoons \text{GAP} + P_i + \text{NAD}^+ 
\]
The reaction mixture contained 100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.2 mM NADH, 10 mM MgCl₂, 2 mM ATP and an appropriate amount of a 1:20 hemolysate of erythrocytes prepared according to Beutler (1975). The reaction was started by the addition of 100 mM 3-phosphoglycerate and the linear decline in absorption at 340 nm was followed.

### 3.2.4 Triose phosphate and Fructose 1,6 bisphosphate Assays

These metabolites were assayed by a modification of the method of Beutler (1975). GAP is determined by measuring the increase in absorbance at 340 nm as NAD⁺ is reduced to NADH in the reaction:

\[
\text{GAPDH} \quad \text{GAP} + \text{NAD}^+ \rightarrow 3\text{-PGA} + \text{NADH} + \text{H}^+
\]

In the presence of arsenate rather than the usual substrate phosphate, the reaction is pulled over by the spontaneous arsenolysis of the arsenic acid anhydride formed.

When TPI is added, any DHAP present is converted to GAP and the absorbance increases again.

On the addition of aldolase, any fructose 1,6 bisphosphate present is converted to DHAP and GAP and the subsequent increase in absorbance allows the amount of fructose 1,6 bisphosphate present to be calculated.

**Assay system**

The cuvette contained 300 mM Tris-HCl pH 8.0, 1.5 mM EDTA, 2 mM NAD⁺, 45 mM sodium arsenate, 1 mM 2-
mercaptoethanol and 0.55 ml of the extract in a total volume of 1.0 ml. The increase in absorbance was measured after the successive addition of 10 µl GAPDH (400 unit/ml), 5 µl TIM (40000 unit/ml) and 10 µl aldolase (120 unit/ml). The results were calculated on the basis of an extinction coefficient for NADH of $6.22 \times 10^{-6}$ cm$^2$ mol$^{-1}$ and are quoted in terms of mM per dm$^3$ of cell water, the conversion being carried out using a measured value for the cell density and assuming that 72% of the cell volume is solvent water (Eilam & Stein, 1974).

3.2.5 Human erythrocyte preparation

Human blood was obtained from the Oxford Regional Blood Transfusion Service in the form of packed cells stored in Citrate Phosphate Dextrose Adenine Anticoagulant solution. These cells were generally 1 to 3 days old. Erythrocytes were prepared by washing the blood once with 5 volumes of phosphate buffered saline (5mM sodium phosphate, 150 mM NaCl, pH 7.4) and removing the buffy coat by aspiration, followed by washing 4 to 5 times in one volume of Krebs-Ringer buffer (Krebs & Henseleit, 1932). The buffers contained 10mM glucose and all washes were carried out at 4°C.

After washing, the cells were suspended in an equal volume of Krebs-Ringer buffer and were incubated at 4°C for 3-4 days to elevate the levels of the triose phosphates (Eckel et al. 1966). At this stage, 200mM iodoacetic acid was added to give a total concentration of 2mM in the suspension and the cells were incubated at 37°C.
for 15 minutes. This procedure produces irreversible and complete inhibition of GAPDH (Webb, 1966; Brindle, 1982). The iodoacetate was subsequently washed out of the cells by washing 3-4 times with Krebs-Ringer buffer containing 10 mM glucose. After the final wash the cells were spun down and stored at 4°C before use.

It has been shown (Saito & Minakami, 1967) that following inhibition of GAPDH in the erythrocyte the levels of FDP and the triose phosphates slowly adjust to new steady-state values over a period of 3-4 hours. For the experiments described in this chapter it was necessary to have constant levels of these metabolites so the erythrocytes were left at 4°C overnight to ensure that a steady-state had been reached.

Immediately prior to use, a freeze-thaw haemolysate of the cells was prepared by adding an equal volume of isotonic saline to a sample of the washed packed cells and adding 0.2 ml of this suspension to 1.8 ml 2.7 mM EDTA solution containing 50 μM 2-mercaptoethanol. After shaking, this haemolysate was frozen in liquid nitrogen and then thawed in a water bath. The aldolase and GAPDH activity of this haemolysate was measured as described above. No detectable GAPDH activity was found.

Cell densities were measured by the microhaematocrit method using a Hawksley microhaematocrit centrifuge.

3.2.6 NMR methods

31P NMR experiments were performed on the 270MHz NMR spectrometer described in Chapter 2. Details of spectral
acquisition parameters are given in the legends to the relevant figures.

Peak heights were measured using a subroutine of the Nicolet NTCFTB operating program and are relative to a baseline calculated from the first and last four data points in the spectrum.

3.2.7 Temperature jump experiments

These were of two types: those done by NMR on an in vitro system and those done on intact human erythrocytes.

NMR temperature jump experiments

A solution of 20mM fructose 1,6 bisphosphate in 50mM hepes, 150mM KCl, 5mM EDTA, pH 7.4 was contained in a 10mm NMR tube and the aldolase and TPI were added in the form of the dialysed enzyme suspensions to obtain the required activity. The tube was then placed in a water bath maintained at 37°C for 40 - 60 min to allow the reaction to reach equilibrium. Longer incubations at 37°C were avoided owing to the TIM catalysed breakdown of DHAP to methyl glyoxal and orthophosphate (Campbell et al 1979). The tube was then plunged into a stirred ice bath for 30s and dried before being placed in the NMR probe which was maintained at 4 ± 1°C. A series of 31P NMR spectra were automatically recorded at regular intervals, typically with a time resolution of 2 - 3 min, over the period required for the system to reach its new equilibrium position. Full details of the spectral acquisition parameters are outlined in the legend to Figure 3.6.

In order to check the efficiency of the temperature jump, a similar NMR tube containing methanol was used as
Figure 3.4

Efficiency of the temperature jump

Plots of the temperature as a function of time in the temperature jump relaxation experiments. The two sets of data represent NMR experiments (o) and bench experiments on intact erythrocytes (+).
an "NMR thermometer". The $^1$H spectrum of methanol contains two peaks whose separation is a function of temperature and between 260 and 320K the following dependence is observed:

$$T(^{\circ}K) = 464.0 - \frac{106.5 \times S}{F}$$

where $S$ = separation of peaks in Hz, and $F = ^1$H frequency in MHz.

The tube was subjected to the same temperature jump as that used in the experiments described above and placed in the NMR probe. $^1$H spectra were recorded every 10 s for 5 min and the temperature change calculated (Figure 3.4). These data demonstrate that the temperature jump is essentially instantaneous.

**Erythrocyte temperature jump experiments**

A 1ml aliquot of packed erythrocytes was transferred from the bulk sample to a water bath maintained at 37°C and was incubated for 12 minutes to allow the FDP/DHAP/GAP system to come to equilibrium. The cells were then withdrawn using a 1 ml disposable syringe (pre-warmed to 37°C) and transferred to a thin-walled glass tube in an ice/water bath maintained at 4°C. After a measured time, 0.5 ml was withdrawn using a 1 ml disposable syringe (pre-cooled to 4°C) and injected into 2ml ice cold 4% perchloric acid. The extract was left on ice for 5 minutes before the supernatant was spun off using an Eppendorf 5414 microcentrifuge. 1.5 ml of the supernatant was
Figure 3.5

109.3 MHz $^{31}\text{P}$ NMR spectra of fructose 1,6 bisphosphate after equilibration with rabbit muscle aldolase at pH 7.4. The spectra represent the sum of 256 transients with a sweep width of 450 Hz collected into 8K data points. A 90° pulse was employed with an interpulse delay of 6 s. Broadband proton decoupling was applied during the acquisition and relaxation periods.

A). spectrum after equilibration at 37°C.
B). spectrum after equilibration at 4°C.

Peak assignments are: 1: 1-P of α FDP, 2: DHAP(hydrate), 3: 1-P of β FDP, 4: 6-P of β FDP, 5: DHAP(keto) + 6-P of α FDP
removed and neutralised by the addition of 0.43 ml 1M K$_2$CO$_3$. After storage in the cold for several hours this neutralised extract was centrifuged and assayed for FDP and the triose phosphates as described above.

The efficiency of the temperature jump was assessed by placing a thermocouple in the tube held in the 4°C water bath and recording the temperature every 10 seconds following the injection of 1 ml of blood which had been equilibrated at 37°C for 12 minutes. The data are shown in Figure 3.4 and demonstrate that the temperature jump was complete within about 45 seconds.

3.3 Results and Discussion

3.3.1 NMR temperature jump

Figure 3.5 A) shows a $^{31}$P nmr spectrum of a 20mM sample of FDP which has been incubated at 37°C with aldolase and TPI for 20 minutes. If the temperature is dropped rapidly to 4°C in the manner described in section 3.2.7, a spectrum recorded immediately has the appearance of Figure 3.5 B). The assignments of the peaks are shown in the legend to Figure 3.5 B). These have been made on the basis of the chemical shifts of the pure compounds and published data (see Chapter 4). It can be seen that some of the resonances shift with temperature. This is mainly due to the temperature dependence of the pK$_a$ values of the various phosphate groups. Resonances due to the various forms of FDP and DHAP are resolved but GAP, which is only present in sub-millimolar concentrations, is not visible in the spectrum.
Temperature jump relaxation of an in vitro model of the aldolase / TPI system

Stacked plot of 109.3 MHz $^{31}$P NMR spectra of 20mM fructose 1,6 bisphosphate equilibrated with 1 unit rabbit muscle aldolase and 2000 units rabbit muscle TPI following a rapid temperature jump from 37°C to 4°C. Each spectrum is the sum of 128 transients collected with a sweep width of 500 Hz into 4K data points using a 90° pulse and an interpulse delay of 1 s. The total time taken to accumulate each spectrum was 5.5 minutes.
Graphical representation of Figure 3.7 showing DHAP(hydrate) (o) and α FDP (x) peak heights as a function of time following a rapid temperature jump from 37°C to 4°C. Also shown are the theoretical values for the respective concentrations at zero and infinite times (□,■) derived in Appendix I.
Although the temperature of the system in the NMR probe is 4°C, owing to the slow rate of relaxation of the system, the spectrum in Figure 3.5 B) represents the equilibrium situation at 37°C. The subsequent relaxation of the system to its new equilibrium position at 4°C may be observed by collecting a series of $^{31}$P NMR spectra at regular time intervals. Such a series is illustrated in Figure 3.6. It can be seen from the decline in the DHAP resonance that the equilibrium shifts in the direction of condensation at the lower temperature as expected from the endothermic nature of the dissociation reaction. The values of the equilibrium constant at the two temperatures is discussed in Appendix I.

In order to characterise the relaxation process, the resonance due to the hydrate form of DHAP was employed as it was quite well resolved in the spectrum. The height of this peak in the experiment illustrated in Figure 3.6 is plotted as a function of time in Figure 3.7. Also shown in this figure is the height of the resonance due to the alpha form of FDP which is also resolvable in the $^{31}$P spectrum.

### 3.3.2 Dependence of relaxation rate on TPI activity

At the enzyme levels found in the erythrocyte, the relaxation of this system should be dominated by the aldolase activity as TPI is present in a large excess. The system used for the in vitro studies was chosen to mimic this situation and accordingly the TPI activity used was approximately 2000 units/ml corresponding to that found in the red blood cell (Beutler, 1975). A sample of TPI from
Figure 3.8

Dependence of relaxation on TPI activity
Relaxation of DHAP concentrations following a temperature jump in a system containing ca. 2000 U/ml TPI (o) and ca. 125 U/ml TPI (x). Both experiments had 20mM total fructose 1,6 bisphosphate and 0.21 U/ml rabbit muscle aldolase.
Sigma was assayed after dialysis and an appropriate amount was added to the 20mM FDP sample to obtain the required activity.

In order to test the dependence of the relaxation rate on the TPI activity, the NMR temperature jump experiment was carried out on two samples with the same aldolase activity but with TPI activities differing by a factor of 16. The relaxation of these two samples is shown in Figure 3.8 and is identical indicating that at the levels of TPI used in these studies the relaxation process is independent of the TPI activity. Subsequent to this finding the TPI activity was not generally assayed in each experiment but was kept fairly constant by the addition of the same volume of the dialysed enzyme suspension.

3.3.3 Dependence of relaxation rate on aldolase activity

A series of relaxation experiments was performed with solutions containing 20mM FDP, 2000 U/ml TPI and varying activities of aldolase. The aldolase activity was measured at least twice during the experiment, usually immediately before the temperature jump and immediately after the end of the NMR experiment. The rate at which the system reached its new equilibrium position was found to increase with increasing aldolase activity and this is illustrated in Figure 3.9 which shows the time dependence of the DHAP hydrate peak height as a function of time in a number of experiments with differing rabbit muscle aldolase activities.

The rate of the relaxation process in these
Figure 3.9

Dependence of relaxation on aldolase activity

Time dependence of DHAP concentration in temperature jump experiments on samples containing 20 mM total fructose 1,6 bisphosphate, pH 7.4, 2000 U/ml rabbit muscle TPI and varying aldolase activity. Aldolase activities are: 0.058 U/ml (o), 0.21 U/ml (x), 0.376 U/ml (●), 0.835 U/ml (+).
experiments was characterised by the initial rate of decline of DHAP and was measured in mM DHAP per minute. In order to facilitate the measurement of the initial rate, an exponential function of the form:

$$A = A_0 e^{-t/\tau} + C$$

was fitted to the DHAP(hydrate) peak height using a program written in PASCAL on a Research Machines 480Z microcomputer employing the simplex algorithm (Caceci & Cacheris, 1984) (see Appendix II).

The initial rate of the decay of DHAP is given by:

$$\left(\frac{d A}{d t}\right)_0 = - \frac{A_0}{\tau}$$

and may be calculated from a knowledge of the initial concentration of DHAP and the relaxation time, $\tau$.

The initial concentration of DHAP is the equilibrium concentration at 37°C and may be calculated for a given total concentration of FDP and triose phosphates by solution of the equilibrium conditions for the aldolase and TPI catalysed reactions (see Appendix I). In all the experiments described in this section the total FDP and triose phosphate concentration was 20mM and the corresponding equilibrium concentration of DHAP at 37°C is 6.06mM.

Although the peak used to follow the relaxation process is that of the hydrate form of DHAP and not the total DHAP pool, the rate of interconversion of the two
Figure 3.10

Plot of initial rate of relaxation against rabbit muscle aldolase activity. The curve is a least squares fit of a straight line to the data.
Figure 3.11

Plot of initial rate of relaxation against human erythrocyte aldolase activity. The curve is a least squares fit of a straight line to the data.
forms is much more rapid than the rate of the relaxation (see Chapter 4) and so the hydrate peak may be taken to represent the total pool in this case.

In this temperature jump experiment the perturbation of the concentrations of the species present is comparable to their total concentrations and so the condition of a negligible perturbation required for a truly exponential relaxation to equilibrium (Hague, 1971) is not fulfilled. However, the use of an exponential function in these calculations is justified on the grounds that it gave a very good fit to the data in all cases and is certainly preferable to estimating an initial rate of decay by eye using perhaps only the first 10% of the available data.

The initial rate of the relaxation process is plotted as a function of aldolase activity in Figures 3.10 and 3.11 for the rabbit muscle and human erythrocyte enzymes respectively. These data show that there is a linear dependence of the initial rate on the aldolase activity in the in vitro system for both the human erythrocyte and the rabbit muscle enzymes.

3.3.4 Temperature jump on intact erythrocytes

A $^{31}P$ NMR spectrum of human erythrocytes is shown in Figure 3.2. Some of the peaks in the region just downfield of inorganic phosphate may be assigned to FDP and triose phosphates but it is clear that neither the resolution nor the signal to noise ratio of the spectrum is good enough to allow the relaxation of the FDP/triose phosphate system to be followed by the NMR method used in the in vitro studies described above. However, it should
**Figure 3.12**

Temperature jump relaxation in intact erythocytes

Plot of concentration of DHAP (x), Fructose 1,6 bisphosphate (o) and sum of FDP and triose phosphate (+) as a function of time following a rapid temperature jump from 37°C to 4°C on a sample of human erythrocytes with an aldolase activity of 0.76 U/ml.
be possible to follow the chemical relaxation of the system by classical techniques involving extraction and assay of the FDP and trioses over a period of time subsequent to a temperature jump. In this way the rate of chemical relaxation in the intact erythrocyte may be measured and compared with that observed in the in vitro system.

Samples of erythrocytes from normal donors were subjected to the temperature jump described in section 3.2.5 and the relaxation to equilibrium was followed by spectrophotometric assay of the metabolites FDP, DHAP and GAP. The concentrations of FDP and DHAP as a function of time in a typical experiment are shown in Figure 3.12. Also shown in this figure is the sum of the triose phosphates and FDP (in hexose equivalents) which remains constant throughout the course of the experiment as a result of the inhibition of GAPDH. The data in Figure 3.12 have been normalised to the mean of the total hexose concentration to reduce errors arising from the sampling of 0.5 ml of blood in the extraction procedure, a process which is very difficult to carry out accurately.

The exponential curve superimposed on the DHAP concentration was calculated using the simplex program described in section 3.3.3. The initial rate of DHAP decay given by this fit is 0.134 mol per dm$^3$ cell water per min. This flux should, of course, be matched by the flux into the FDP pool, taking into account the fact that two moles of triose phosphate are required to make one mole of FDP and neglecting the small contribution from GAP. The increase in FDP with time may also be fitted to an
exponential function and the initial flux into the FDP pool is calculated to be 0.073 mol per dm$^3$ cell water per min. which is in close agreement to the value obtained for the DHAP flux.

Reference to the NMR calibration curve in figure 3.11 shows that the observed initial flux represents an aldolase activity of 0.91 Units per ml. The aldolase activity assayed in a haemolysate of the erythrocytes used in this experiment was 1.41 Units per ml cell water so only 35% of the aldolase activity in the intact erythrocyte is detectable by the relaxation method. This experiment was repeated several times and the results are shown in Table 3.1.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Initial rate of relaxation (mM/min.)</th>
<th>Expected aldolase (U/ml)</th>
<th>Measured aldolase (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.146</td>
<td>0.996</td>
<td>1.44</td>
</tr>
<tr>
<td>2</td>
<td>0.153</td>
<td>1.045</td>
<td>1.44</td>
</tr>
<tr>
<td>3</td>
<td>0.111</td>
<td>0.748</td>
<td>1.41</td>
</tr>
<tr>
<td>4</td>
<td>0.134</td>
<td>0.910</td>
<td>1.41</td>
</tr>
</tbody>
</table>

There is some variation in the proportion of aldolase activity which is not detectable in situ but it falls between 27 and 47% of the total aldolase activity measurable in a haemolysate.

The composition and ionic strength of the system used in the NMR "assay" of aldolase activity were chosen...
to mimic the intracellular conditions as closely as possible but there were some unavoidable differences. In particular the total FDP and triose phosphate concentrations are different in the two systems. Limitations of sensitivity mean that the NMR temperature-jump experiments would be very difficult to carry out at the concentrations of total FDP found in the cell (ca 1-2mM). Conversely, it is not possible to elevate the triose phosphate levels in the erythrocyte to those used in the NMR experiments (20mM). It is therefore necessary to establish whether the reduced flux observed through aldolase in situ is due to non-saturating levels of substrate in the cell.

The value of the $K_m$ for FDP with aldolase reported in the literature ranges from $2.8 \times 10^{-4}$ M (Hartman & Barker, 1977) to $2 \times 10^{-4}$ M (Fersht, 1977). This parameter was redetermined in a number of different buffers using the aldolase assay described in section 3.2.3 over a range of FDP concentrations from 0.001 mM to 10 mM. The results were analysed by fitting the Michaelis-Menten equation to the data by an iterative least-squares regression and values for $K_m$ and $V_{max}$ were obtained. It was found that both $K_m$ and $V_{max}$ were quite dependent on the buffer composition and this accounts for the variation in the published values. In the buffer used for the NMR temperature-jump experiments the $K_m$ for FDP was found to be $1.8 \times 10^{-4}$ M. The chloride present in this buffer acts as a competitive inhibitor and in a glycylglycine buffer in the absence of chloride the $K_m$ was $7 \times 10^{-5}$ M. No difference in the $K_m$ for the rabbit muscle and human
erythrocyte enzyme was observed at 37°C. These $K_m$ values suggest that aldolase is likely to be saturated with FDP both in the intact erythrocyte and in the in vitro experiments described in this chapter. However, in the temperature jump described here, the flux observed through aldolase is predominantly in the direction of the condensation reaction and it is perhaps more relevant to consider the $K_m$ values of the triose phosphates. These quantities are difficult to measure as the formation of FDP from the triose phosphates cannot easily be coupled to an NADH linked reaction. However values of 2 mM and 1 mM for DHAP and GAP respectively have been reported (Morse & Horecker, 1968). In this case there may well be a difference in the extent to which aldolase is saturated with its substrates in the in vitro experiments and in the intact cell.

A further possible cause of a reduction in flux through aldolase to be considered is that the free intracellular concentrations of FDP and the triose phosphates are lower than those estimated from the standard methods of assay. It has been suggested (Garrick et al., 1980; Bunn et al., 1975) that haemoglobin in the red cell may be glycosylated by a number of phosphorylated and non-phosphorylated sugars and that this binding of metabolites results in compartmentation which may have a regulatory effect on glycolysis (Brindle, 1982; Wood, 1983). A value of 2 mM for the dissociation constant of FDP with haemoglobin has been obtained from NMR binding studies (Paul, 1982).
The following experiments were carried out to assess the importance of some of these factors.

3.3.5 Effects of substrate concentration on the relaxation process

The experiments described in this section served two purposes. First it was desirable to assess the comparability of the results from the NMR method and the extraction/assay method employed to follow the relaxation process. The second purpose was to assess the dependence of the relaxation rate on the total substrate concentration as this is different in the two methods used.

In order to make these comparisons, the relaxation of the sugar phosphate concentrations in model solutions of FDP, aldolase and TPI were followed subsequent to a rapid temperature jump from 37°C to 4°C using a modification of the extraction and assay technique described in section 3.2.7. Experiments were carried out using around 20mM or 1.5 mM FDP in a buffer containing 150mM KCl, 50mM HEPES, 5mM EDTA at pH 7.4. Dialysed rabbit muscle enzymes were added to give activities of 0.8 U/ml aldolase and 2000 U/ml TPI. After incubation at 37°C for up to an hour the solution was rapidly cooled to 4°C in an ice/water bath and 0.25ml aliquots were sampled at intervals and the enzyme reactions quenched by injection into ice-cold 20% perchloric acid. These extracts were left standing for 24 hours at 4°C before neutralisation and the sugar phosphate concentrations were then assayed by the methods described in section 3.2.4. It was found
Figure 3.13

Temperature jump relaxation on in vitro system
Plot of concentration of DHAP (o) and fructose 1,6 bisphosphate (x) as a function of time following a rapid temperature jump from 37°C to 4°C on a sample of 1.5 mM FDP in 150 mM KCl, 50 mM HEPES, pH 7.4 with an aldolase activity of 0.76 U/ml and a TPI activity of ca. 2000 U/ml.
that the milder conditions used to quench the relaxation reaction in the intact erythrocyte were insufficient for the in vitro model systems as they resulted in residual aldolase activity which interfered with the assay of the triose phosphates. The reason for this difference may be that the presence of large concentrations of protein (particularly haemoglobin) in the intact erythrocyte assists the co-precipitation of the enzymes on acid treatment.

Typical data from an experiment using 1.5 mM fructose 1,6 bisphosphate are illustrated in Figure 3.13. The experiment was carried out twice at the two different concentrations and the initial rate of the relaxation process was measured by fitting an exponential function to the DHAP concentration in the way described in section 3.3.3. The data from these experiments are shown in Table 3.2.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>FDP Conc. (mM)</th>
<th>Measured aldolase activity (U/ml)</th>
<th>Initial Rate (mM DHAP/min)</th>
<th>Calculated Aldolase activity (Fig.3.10) (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.5</td>
<td>0.91</td>
<td>0.33 ± 0.05</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>16.1</td>
<td>0.79</td>
<td>0.30 ± 0.05</td>
<td>0.75 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>0.81</td>
<td>0.12 ± 0.05</td>
<td>0.34 ± 0.05</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>0.83</td>
<td>0.14 ± 0.05</td>
<td>0.39 ± 0.05</td>
</tr>
</tbody>
</table>
Experiments 1 and 2 show that at the high FDP concentration of 15-20 mM (that used in the NMR experiments) the aldolase activity predicted from the observed rate of relaxation (using Figure 3.10) is in close agreement with the value measured in solution by the standard aldolase assay. This confirms the validity of the comparison between the NMR and extraction and assay methods of measuring the relaxation rate.

Experiments 3 and 4 show that at a concentration of 1.5 mM total FDP, the initial rate of relaxation is considerably less than that observed with the higher concentration. This is most likely due to the fact that at 1.5 mM total FDP the equilibrium concentration of DHAP at 37°C (ca. 1.2mM) is not sufficiently greater than its $K_m$ to saturate the enzyme. The $K_m$ values for the triose phosphates quoted above would be in agreement with the finding here that the enzyme is not saturated at a DHAP concentration of 1.2 mM.

The initial rate of relaxation at the lower concentration is around 50% of the value at the higher concentration and this has an important bearing on the conclusions that can be drawn from the measurements on the intact cell. The values of the aldolase activity in situ in the intact erythrocyte shown in Table 3.1 must be multiplied by a factor of 2 to take into account the effect of non-saturating substrate under the prevalent conditions in the cell and this results in a reversal of the situation apparent from Table 3.1. The activity of aldolase in the intact cell visible to its substrates is certainly not less and may even be greater than that...
expected from the standard aldolase assay of intracellular enzyme activity measured in a haemolysate.

These findings seem to rule out the existence of any significant inhibition of aldolase in the intact human erythrocyte. This in turn suggests that there is no significant degree of binding of the enzyme to the erythrocyte membrane in agreement with the suggestions of Maretzki et al (1974). It therefore seems that although extensive binding and inhibition of the enzyme may be observed in in vitro model systems, for example in erythrocyte ghosts (Strapazon & Steck, 1977), under the conditions found in the intact cell this interaction is much less important. It is likely that factors contributing to this situation are the high ionic strength and FDP concentration of the intracellular medium (150 mM KCl, 1-2 mM FDP) compared with those in the hypotonically lysed cells used in many of the in vitro experiments (typically 5mM sodium phosphate, no FDP). Both of these factors could contribute to the elution of the enzyme from the membrane in the intact cell (Strapazon & Steck, 1977).

These findings do not rule out the possibility that under conditions of depleted substrate (total FDP in the micromolar range) membrane binding of the enzyme could become more significant. It is also possible that a different kind of non-inhibitory association takes place as this would not be detectable by this method.

A further conclusion that may be drawn from these experiments is that any binding of FDP or the triose phosphates to haemoglobin does not have significant
physiological effect on the aldolase reaction in the intact erythrocyte. Again this conclusion is only applicable to the conditions of relatively high substrate concentrations used in these experiments. Wood (1983) has calculated that in erythrocytes containing 6 mM haemoglobin a significant proportion of FDP may be bound at low substrate levels (1-50 μM) and so the situation may well be different in cells depleted of substrate.

The situation found here for aldolase is similar to that discussed by Brindle et al. (1982) who found no evidence for the binding of GAPDH to the erythrocyte membrane in the intact cell despite the existence of extensive binding in vitro. It is worth quoting from the paper of Strapazon & Steck (1977) who conclude that: "... neither the specific binding of aldolase (and G3PD) to band 3 in the isolated red cell membrane nor their ready elution provides adequate grounds for inferring their disposition in vivo. In situ studies on intact cells are needed to answer this question."

The possibility that the intracellular aldolase activity could be greater than that measured in a haemolysate is interesting and is comparable to the observations of Brindle et al. (1982) who found the GAPDH activity in situ higher than expected from in vitro studies. This similarity possibly points to a cooperative interaction between the two enzymes in the cell leading to an enhancement of the overall flux through this section of glycolysis. Such an interaction between aldolase and GAPDH has been postulated by Ovadi & Keleti (1978) on the basis of a comparison between the rate of
the GAPDH catalysed reaction and the rate of interconversion of the diol and free aldehyde forms of the substrate GAP under conditions where the latter should become rate limiting. It was concluded that the active aldehyde form of GAP was passed directly on from aldolase to GAPDH without being allowed to equilibrate with the diol form in free solution. In the temperature jump experiments studied in this chapter the predominant direction of flux through aldolase measured is that of the condensation reaction and it is possible that in the intact cell this is also enhanced by the direct utilisation of the aldehyde form of GAP passed on from GAPDH in some form of loose enzyme complex. The implications of the existence of interconverting active and inactive forms of the substrates of glycolytic enzymes is explored further in the next chapter.

3.4 Conclusions

This chapter has described the development of a technique using $^{31}$P NMR to observe the relaxation of an isolated part of the glycolytic pathway following a rapid temperature jump from 37° to 4°C. This is an unusual application of a relaxation method as previous studies have been restricted to enzyme reactions which can be followed spectrophotometrically via a chromophore on either the enzyme or a substrate (Malcolm, 1975).

The experiment was used to characterise the dependence of the rate of the relaxation on the activities of the enzymes which catalyse the reactions involved in an in vitro model system. The same relaxation process was
observed in intact human erythrocytes by a rapid quench and assay method and the results were compared with those of the in vitro experiments. It was found that the activity of aldolase in situ was actually slightly greater than in a comparable in vitro model system ruling out the existence of extensive membrane binding leading to the inhibition of the enzyme as had been proposed by some workers. The results may, however, lend support to postulated interactions between aldolase and GAPDH in the erythrocyte.

The generality of the method described is restricted by the requirement for a reaction which occurs slowly enough for the relaxation to be followed on a timescale suitable for NMR (tens of minutes in this case). The flux through many enzymes would be too great to enable a similar study to be carried out but there may be other systems where the technique could be used.

A disadvantage of the method was the inability to follow the relaxation process by the NMR method in the intact cell owing to limitations of sensitivity and resolution; the bane of many otherwise highly plausible NMR experiments. It is possible that the experiment might be feasible in cells which did not contain such a high concentration of 2,3 bisphosphoglycerate which tends to dominate the $^{31}$P NMR spectrum of human erythrocytes. Sheep erythrocytes contain very little 2,3 DPG and preliminary experiments suggest that the resolution problem may be overcome in this system. However, the conditions used for elevating the levels of the triose phosphate and FDP in
human erythrocytes are not so effective in the sheep cells and so sensitivity remains a problem unless this can be overcome.
3.5 References


**778**, 67-73.

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Maretzki, D., Groth, J., Tsamaloukas, A.G., Grundel, M.,  

**126**, 18-20.


Aldolase and TPI equilibria

The reactions catalysed by aldolase and TPI are illustrated in Figure 3.3. The concentrations of the substrates at equilibrium are determined by the following relationships:

\[ K_{ald} = \frac{[DHAP][GAP]}{[FDP]_{\text{free}}} \]  

(1)

\[ K_{TPI} = \frac{[DHAP]}{[GAP]} \]  

(2)

\[ [FDP]_{\text{total}} = [FDP]_{\text{free}} + \frac{K_{TPI} [GAP]}{2} \]  

(3)

\[ [FDP]_{\text{free}} = \frac{K_{TPI} [GAP]^2}{K_{ald}} \]  

\[ \frac{K_{TPI} [GAP]^2}{K_{ald}} + \frac{K_{TPI} [GAP]}{2} - [FDP]_{\text{total}} = 0, \]  

(4)

which may be solved for GAP for a given total concentration of FDP and triose phosphates using the general solution of a quadratic equation:

\[ x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \]

where \( a = \frac{K_{TPI}}{K_{ald}} \), \( b = \frac{K_{TPI}}{2} \), \( c = -[FDP]_{\text{total}} \).

At 38°C the equilibrium constants for these reactions are:

\[ K_{ald} = 0.99 \times 10^{-4} \text{ mM} \]
Aldolase and TPI equilibrium at 4°C

Equilibrium concentrations of FDP (open symbols) and DHAP+GAP (solid symbols) at 4°C. The data are from this work (circles) and Brindle (1982) (squares).

The dotted curves represent the theoretical values using a published value for the equilibrium constant of aldolase (Herbert et al., 1940) and the solid curve uses a recalculated value of $1.1 \times 10^{-7} \text{ M}$. 
The enthalpy change for the isomerisation must be very small as no change in $K_{TPI}$ was observed over a 20° temperature range (Herbert et al., 1940) so the value of $K_{TPI}$ at 4°C may also be taken to be 22. Using a published value for the enthalpy change of the FDP cleavage reaction of 54.6 KJmol$^{-1}$ (Herbert et al. 1940), it is possible to derive a value for $K_{ald}$ at 4°C of $7.4 \times 10^{-6}$ mM but with the abundance of data on the equilibrium concentrations of the substrates at 4°C both from this work (the experiments described in section 3.3.4) and that of Dr. K.M. Brindle (1982) it is possible to make a more accurate estimate. Figure 3.14 shows experimentally determined equilibrium concentrations of FDP and the triose phosphates as a function of total FDP concentration together with theoretical curves calculated using a value of $K_{ald}$ of $1.1 \times 10^{-7}$ mM which was obtained from a least squares fit of equation (4).
3.7 Appendix II

PASCAL program used to fit data to an exponential function

This program is written in a very general form which allows a wide variety of functions to be substituted for the single exponential function used in this case. The only changes necessary are to the constants m (the number of parameters in the fitting function), n (\(= m + 1\)) and the function itself.

```pascal
program expn (debug);

(* curve fitting with the simplex algorithm *)
(* this version fits a single exponential function *)
(* STO *)
(* 16/4/85 *)

const
date = '16/4/85';
memoo = 'fit of a single exponential: \(y = A\exp(-x/T) + C\)';
m = 3; (* number of parameters to fit *)
nvpp = 2; (* total no. of vars per data point *)
n = 4; (* = m + 1 *)
mnp = 200; (* maximum no. of data points *)

alpha = 1.0; (* reflection coefficient, >0 *)
beta = 0.5; (* contraction coefficient, 0tol *)
gamma = 2.0; (* expansion coefficient, >1 *)

lw = 5; (* width of line in data fields +1 *)

page = 12;
root2 = 1.414214;
type vector = array[1..n] of real;
datarow = array[1..nvpp] of real;
index = 0..255;

var
done : boolean; (* convergence *)
i,j : index;
h,l : array[1..n] of index;

np, maxiter, niter : integer; (* no. of high/low params *)

next, centre, mean, error, maxerr; (* max error accepted *)
```

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Pq, (* to compute the first simplex *)
step : vector; (* input starting steps *)
simp : array[1..n] of vector; (* the SIMPLEX *)
data : array[1..mnp] of datarow; (* the data *)
fname : packed array[1..14] of char;
din, dout : text; (* input, output *)

function f (x:vector; d:datarow) : real;
(* x(1..m) the parameters, d has the data *)
begin
end;

procedure sum_of_residuals (var x : vector);
(* computes the sum of the square of the residuals *)
(* x(1..m) passes parameters. Result returned in x(n) *)
var i : index;
begin
  x[n] := 0.0;
  for i := 1 to np do
    begin
      x[n] := x[n] + sq(f(x,data[i]) - data[i,2])
    end (* loop *)
end;

procedure enter;
(* enters data from disc file fname
may have to change some of this,
data in the order:
-max no of iterations
-starting point coords
-starting increments
-maximum errors
-data *)
var i, j : index;
begin
  dout := printer;
  write(dout, ' SIMPLEX curve fitting version ');
  write(dout, date);
  writeln(dout, ' STO Oxford');
  writeln(dout);
  writeln(dout,' accessing file: ',fname);
  read(din,maxiter);
  writeln(dout, ' max number of iterations is = ',maxiter:5);
  write(dout, ' starting coord.: ');
  for i := 1 to m do
    begin
      read(din, simp[1,i]);
      if (i mod lw) = 0 then writeln(dout);
      write(dout, simp[1,i])
    end;
  writeln(dout);
  write(dout, ' starting steps: ');

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for i := 1 to m do 
begin 
read(din,step[i]);
if (i mod lw)=0 then writeln(dout);
write(dout,step[i]);
ext;
write(dout);
write(dout,' max. errors: ');
for i := 1 to n do 
begin 
read(din,maxerr[i]);
if (i mod lw) = 0 then writeln(dout);
write(dout,maxerr[i]);
ext;
write(dout); 
writeln(dout,' data:');
writeln(dout ' x':16,'y':12);
np := 0;
while not eof(din) do 
begin 
np := succ(np);
write(dout,' Point ',np:3);
for j := 1 to nvpp do
begin 
read(din,data[np,j]);
write(dout,data[np,j])
ext;
write(dout);
eat

procedure report;
var y, dy,
sigma : real;
i,j : index;
d1, d2 : text;    (* disc out files *)
begin
  dout := printer;  (* report to printer *)
  rewrite(d1,'FIT.DAT');  (* fitted data *)
  rewrite(d2,'ERR.DAT');  (* residuals *)
  writeln(d1,' 1 1 1 1 1 1',np);  (* dummy arguments *)
  writeln(d2,' 1 1 1 1 1 1',np);  (* for PLOT program *)
  writeln(dout,' program exited after',niter:5,' iterations');
  writeln(dout,' the final simplex is');
  for j := 1 to n do 
  begin
    for i := 1 to n do
    begin
      if (i mod lw) = 0 then writeln(dout);
      write(dout,simp[j,i]:12)
ext;
    writeln(dout)       (* do j *)
  writeln(dout);
  writeln(dout,' the mean is');
  for i := 1 to n do
  begin

if (i mod lw) =0 then writeln(dout);
  write(dout,mean[i])
end;
writeln(dout);
writeln(dout,' the fractional error is');
for i := 1 to n do
  begin
    if (i mod lw) =0 then writeln(dout);
    write(dout, error[i])
  end;
writeln(dout);
writeln(dout,' POINT', 'x':10, 'y':15, 'FIT':15, 'DEVIATION':17);
sigma := 0.0;
for i := 1 to np do
  begin
    y := f(mean, data[i]);
    dy:= data[i,2] - y;
    sigma := sigma + sqr(dy);
    writeln(dout, i:4, data[i,1]:15, data[i,2]:15, y:15, dy:15);
    writeln(d1, data[i,1], y);
    writeln(d2, data[i,1], dy)
  end;
writeln(dout);
sigma := sqrt(sigma / np);
writeln(dout,' the standard deviation is', sigma);
sigma := sigma / sqrt(np - m);
write(dout,' the estimated error of the');
writeln(dout,' function is', sigma);
end; (* report *)

_revision_
simp[h[n],i] := next[i];
write(next[i])
end;
writeln(dout)
end; (* new vertex *)

procedure order; (* gives high/low in each parameter in simp *)
(* caution: not initialised *)
var i,j : index;
begin
  for j := 1 to n do (* all dimensions *)
    begin
      for i := 1 to n do (* of all vertices *)
        begin
          (* find best and worst *)
          if simp[i,j] < simp[l[j],j] then l[j] := i;
          if simp[i,j] > simp[h[j],j] then h[j] := i
        end (* i loop *)
      end (* j loop *)
  end;
end;

begin (* main SIMPLEX program *)
  writeln(' Enter data filename ');
  read(f name); (* input filename *)
  reset(din,f name); (* fname is on disc *)
  enter; (* get the data *)
  sum_of_residuals(simp[1]); (* first vertex *)
  for i:=1 to m do (* compute offset of vertices *)
    begin (* of the starting simplex *)
      p[i] := step[i] * (sqrt(n) + m -1 ) / (m * root2);
      q[i] := step[i] * (sqrt(n) - 1) / (m * root2)
    end;
  for i := 2 to n do (* all vertices of the *)
    begin (* starting simplex *)
      for j := 1 to m do simp[i,j] := simp[1,j] + q[j];
      simp[i,i-1] := simp[1,i-1] + p[i-1];
      sum_of_residuals(simp[i]) (* and their residuals *)
    end;
  for i := 1 to n do (* preset *)
    begin
      l[i] := 1; h[i] := 1
    end; (* before calling *)
  order;
  first; (* pass to printer *)
  niter := 0; (* no iterations yet *)
  repeat
    done := true; (* keep iterating *)
    niter := succ(niter);
    done := false;
  until done;
end;
for i := 1 to n do centre[i] := 0.0;
for i := 1 to n do /* compute centroid */
  if i <> h[n] then /* excluding the worst */
    for j := 1 to m do
      centre[j] := centre[j] + simp[i,j];
  end;
for i := 1 to n do /* first attempt to reflect */
begin
  centre[i] := centre[i] / m;
  next[i] :=
    (1.0 + alpha) * centre[i] - alpha * simp[h[n],i]; /* next vertex is the specular reflection of the worst */
end;
sum_of_residuals(next);
if next[n] <= simp[l[n],n] then /* better than the best ? */
begin
  new_vertex; /* accepted ! */
  for i := 1 to m do /* and expanded */
    next[i] :=
      gamma * simp[h[n],i] + (1.0 - gamma) * centre[i]; /* still better ? */
  sum_of_residuals(next);
  if next[n] <= simp[l[n],n] then new_vertex /* expansion accepted */
end; /* if not better than the best */
else
begin
  if next[n] <= simp[h[n],n] then /* better than worst */
    new_vertex /* worst than the worst */
  else /* then contract */
    begin
      for i := 1 to m do
        next[i] :=
          beta * simp[h[n],i] + (1.0 - beta) * centre[i]; /* contraction accepted */
      sum_of_residuals(next);
      if next[n] <= simp[h[n],n] then new_vertex /* if still bad */
        begin
          for i := 1 to n do /* shrink all bad vertices */
            begin
              for j := 1 to m do
                simp[i,j] :=
                  (simp[i,j] + simp[l[n],j]) * beta;
              sum_of_residuals(simp[i]); /* i loop */
            end; /* else */
          end; /* else */
        end; /* else */
    end; /* else */
end; /* else */
order;
for j := 1 to n do /* check for convergence */
begin
  error[j] :=
    (simp[h[j],j] - simp[l[j],j]) / simp[h[j],j];
  if done then
    if error[j] > maxerr[j] then
      done := false
end
until (done or (niter = maxiter));

for i := 1 to n do (* average each parameter *)
    begin
        meant[i] := 0.0;
        for j := 1 to n do
            meant[i] := meant[i] + simp[j,i];
        meant[i] := meant[i] / n
    end;

    report; (* to printer *)
    writeln(dout,chr(page)) (* of SIMPLEX *)
end
Figure 4.1

Anomeric and other equilibria of metabolites in a section of glycolysis.
CHAPTER 4
NMR STUDIES OF ANOMERIC EQUILIBRIA IN THE SUBSTRATES OF GLYCOLYSIS

4.1 Introduction

The scheme that was shown in Figure 3.3 representing the reactions catalysed by aldolase and triosephosphate isomerase is a simplification of the true situation. Each of the participating sugars exists in solution in at least two different forms and a more accurate representation of the system is shown in Figure 4.1. Other components of the glycolytic pathway, including glucose 6-phosphate and fructose 6-phosphate also exist in several forms. The existence of these metabolites in different forms results in a kind of compartmentation and there has been speculation over whether this could have any role in the regulation of glycolysis (Benkovic & Schray, 1976; Koerner et al., 1977; Ottaway & Mowbray, 1977).

The existence of anomeric forms of hexoses in solution is a consequence of their cyclic structure. Cyclic acetal formation introduces an asymmetric carbon atom which is achiral in the open chain structure. It is a change in the configuration of this carbon (C-1 for a pyranose sugar) which distinguishes the α and β anomers. The anomers are freely interconverted in solution with rate constants which vary from $9 \times 10^{-5} \text{s}^{-1}$ for D-glucose to around $10 \text{s}^{-1}$ for a diphosphorylated hexose such as fructose 1,6-bisphosphate (FDP) (Benkovic & Schray, 1976).
The implications of this isomerism to biochemical processes was not widely appreciated until the discovery by Keilin and Hartree (1952a) that the enzyme glucose oxidase from *Penicillium notatum* oxidises β D-glucose 100 times faster than α D-glucose. Searches for other anomerically specific enzymes over the past 20 years demonstrated that this was actually a rather common phenomenon. Examples include hexokinase (Salas et al., 1965; Giroix et al., 1985), phosphoglucose isomerase (Salas et al., 1965), glucose 6-phosphate dehydrogenase (Smith & Butler, 1966), phosphofructokinase (Wurster & Hess, 1974a), fructose bisphosphatase (Frey et al, 1977) and aldolase (Schray et al, 1975) which all show a preference for one anomeric form of their substrate. In addition to this, there are four enzymes which have been shown to exhibit anomerase activity; i.e. the ability to catalyse the interconversion of two anomers. These (with their substrates) are: mutarotase (α and β glucose) (Keilin & Hartree, 1952b), phosphoglucose isomerase and glucose 6-P epimerase (α G6P and β G6P) (Wurster & Hess, 1972; 1974b) and yeast aldolase (α and β FDP) (Schray et al., 1975).

The study of the anomerically specific reactions of glucose was facilitated by the ease of isolation of the pure anomers and their slow rate of interconversion in solution. Studies of reactions involving phosphorylated carbohydrates are more difficult owing to the rapid rates of anomic interconversion resulting from intramolecular catalysis by the phosphate group (Bailey et al., 1970). For example, the half-life of the α → β conversion in
the case of FDP is around 90 ms (Midelfort et al., 1976) and in order to follow reactions on this time scale the techniques most widely used have been stopped-flow and rapid-quench methods (Benkovic & Schray, 1976). The approach is illustrated by the determination of the substrate specificity of muscle aldolase which has been the cause of particular debate.

Initial observations by Wurster & Hess (1973) using stopped flow methods showed that the cleavage of FDP by muscle aldolase at high enzyme to substrate ratios exhibited biphasic kinetics. There was an initial rapid phase, dependent on enzyme activity, in which around 80% of the total FDP was utilised and this was followed by a slower phase, independent of enzyme activity, in which the remaining 20% of the substrate was cleaved. Schray et al. (1975) confirmed this result using rapid-quench techniques. It had been established previously that around 80% of the FDP in free solution is present as the β anomer with most of the remaining 20% being in the α ring form (Gray, 1971; Koerner et al., 1973). It was therefore concluded that it was the β anomer of FDP that was the substrate for muscle aldolase and that the slow secondary phase was due to the rate limiting spontaneous anomerisation of the α form.

The possibility that the open-chain keto form of FDP was exclusively the substrate for aldolase was discounted (Schray et al., 1975) on the basis that the turnover rate for the enzyme (ca. 10 s⁻¹) was much greater than the spontaneous α → β rate measured from the secondary slow
phase of the kinetic experiments \((k = 0.5 \text{ s}^{-1})\). It was assumed that the \(\alpha \rightarrow\) open chain rate would be around four times greater than the \(\beta \rightarrow\) open chain rate (the ratio of the thermodynamic stabilities of the anomers is 1:4) and so the latter rate would be slower than the observed turnover rate of aldolase and could not be a necessary step in the reaction.

However, when the individual ring opening rates of FDP were measured by Midelfort et al. (1976) it was found that the \(\beta \rightarrow\) open chain rate constant was actually around \(35 \text{ s}^{-1}\) and was thus faster than both the turnover rate of the enzyme and the \(\alpha \rightarrow\) open chain rate. This suggested that from the kinetic evidence available, the possibility could not be discounted that the \(\beta\) form is not a substrate of the enzyme and that the cleavage reaction goes entirely through the open chain form. This theory was strengthened by the observation (Midelfort et al., 1976) that 5-deoxyfructose 1,6-bisphosphate, which is trapped in the open chain form, proved to be a much better substrate for aldolase than FDP itself \((V_{\text{max}}/K_{m} \text{ is 56 times greater for the open chain analogue})\).

In order to attempt to resolve whether or not the \(\beta\) form of FDP was a substrate for muscle aldolase Rose & O'Connell (1977) carried out kinetic measurements of the cleavage reaction at very high aldolase concentrations and low temperature. Under these conditions they calculated that 98% of the total FDP present was bound to the enzyme and that the spontaneous \(\beta \rightarrow\) open chain conversion would be restricted to the extent that it would become rate limiting in the cleavage reaction. However the observed
rate of cleavage was found to be 20 times greater than the expected rate if spontaneous ring opening was required and this was interpreted as evidence for the direct utilisation of the β form of FDP as a substrate. This interpretation, however, discounts the possibility that the β → open chain conversion could take place on the enzyme or that aldolase could itself catalyse the ring opening. Yeast aldolase has been shown to possess "anomerase" activity (Schray et al., 1975) but this has not been shown to be a property of the muscle enzyme.

A similar approach has been employed to determine the substrate specificity of the phosphofructokinase reaction which precedes aldolase in the glycolytic pathway. In this case the turnover rate of the enzyme (>100 s⁻¹) is considerably greater than the spontaneous rate of α → β interconversion of around 1.5 s⁻¹ (Wurster & Hess, 1974a). By similar arguments to those outlined above, it was concluded that the enzyme was specific for the β anomer of F6P which was converted directly to the β anomer of FDP (Wurster & Hess, 1974a; Fishbein et al., 1974). The individual ring opening rates of F6P have not so far been published and exclusion of the possibility of participation of the open chain form of F6P in the PFK reaction rests on experiments with a substrate analogue of the open chain form (xylulose 5-phosphate) which was found not to bind to the enzyme (Koerner et al., 1974). The extent to which this is present in the keto form in solution is not known however.

Figure 4.1 shows that the two compounds produced by
the aldolase catalysed cleavage of FDP may also exist in more than one form. DHAP and GAP are phosphorylated ketones and aldehydes respectively and may exist in both the free carbonyl form and as a hydrated species. It has been shown that in their reactions with the enzymes aldolase, TPI and GAPDH both these compounds react in the carbonyl form (Reynolds et al., 1971; Trentham, et al., 1969). In the case of GAP the equilibrium is strongly in favour of the inactive hydrate form and only 3-4% exists in the carbonyl form under normal conditions (Trentham et al., 1969). The equilibrium constant for DHAP is nearer unity (Reynolds et al., 1971). There has been some speculation over the possible significance of these effects to the regulation of glycolysis. If some fraction of these metabolites is trapped in the inactive form, could the dehydration reaction to form the free carbonyl species ever become rate limiting?

Some studies have been carried out on GAP where the situation is potentially more severe owing to the low equilibrium concentration of the active form in free solution. The results of investigations into the kinetics of the consecutive reactions catalysed by aldolase and GAPDH (Ovadi & Keleti, 1978) led to the suggestion that the free carbonyl form of GAP produced in the aldolase reaction could be passed directly to GAPDH via some kind of loose enzyme complex without being released into free solution where it could come to equilibrium with the inactive hydrate form. The possible existence of such an interaction between GAPDH and aldolase was also discussed in Chapter 3.
The fact that DHAP can exist in different forms could also be important. The rapid conversion of DHAP to GAP is essential to the efficiency of glycolysis. The enzyme that catalyses this interconversion, TPI is extremely efficient and has been described as a perfectly evolved enzyme (Albery & Knowles, 1976). The ratio $k_{\text{cat}}/K_m$ is close to the diffusion controlled limit and the question arises as to whether the keto form of DHAP is passed on directly from aldolase to TPI in an enzyme complex or whether the two forms of DHAP are allowed to equilibrate in free solution.

Careful measurement of the interconversion rates of the various forms of some of these metabolites in the absence and presence of glycolytic enzymes could shed light on the importance of this form of compartmentation to the regulation of glycolysis.

**NMR as a method for studying anomeric equilibria**

NMR is a particularly valuable tool in this field because of its ability to detect and quantify the various anomeric and tautomeric forms of metabolites. An example of this was seen in Chapter 3 (see Figure 3.5) where some of the forms of FDP and DHAP are visible in a $^{31}$P NMR spectrum. In addition to providing information about anomeric composition, NMR can potentially give a greater insight into these equilibria because the rates of many of the interconversions fall into the range that is accessible to measurement by NMR techniques (see Chapter 1). Most previous estimates of the spontaneous anomerisation rates have been carried out in the presence
of high concentrations of enzymes (see above) and it is certainly possible that this may influence the anomerisation process. The NMR methods to be described allow the measurement of these rates to be made in the absence and presence of interfering substances, thus allowing their effects to be assessed.

Some workers have already applied NMR techniques to the problems of anomic interconversion and specificity of carbohydrates. Some of the initial studies of the relative proportions of the various anomic forms of phosphorylated sugars present at equilibrium were carried out using $^{31}P$ and $^{13}C$ NMR (Gray, 1971; Koerner et al., 1973). Midelfort et al. (1976) estimated the rate of interconversion of α and β anomers of FDP by line shape analysis in $^{13}C$ spectra. Measurements of the anomerisation of some non-phosphorylated furanose sugars have been made by $^1H$ and $^{13}C$ saturation transfer methods (Serrianni et al., 1982). Balaban & Ferretti (1983) used a 2D NOESY NMR method to study the anomerisation and isomerisation of glucose 6-phosphate catalysed by phosphoglucoisomerase under steady state conditions.

This chapter explores the applications of NMR methods to the study of the anomic equilibria in the section of the glycolytic pathway catalysed by the enzymes aldolase and TPI. The possible implications to metabolic regulation are discussed.
4.2 Materials and Methods

4.2.1 Materials

Fructose 1,6-bisphosphate, fructose 6-phosphate, dihydroxyacetone phosphate, HEPES, EDTA, deuterium oxide and all enzymes were obtained from Sigma Chemical Company. Chelex 100 was obtained from Bio-Rad Laboratories, California. All other chemicals were of analytical grade.

The phosphorylated compounds from Sigma were found to give NMR spectra with rather broad lines. This is due to contamination by paramagnetic ions (probably Mn$^{2+}$ or Fe$^{3+}$) and the addition of a chelating agent such as EDTA produced much sharper lines. In some of the studies described in this chapter, it was desirable to have solutions free of both contaminating ions and EDTA (which may catalyse some of the reactions being observed). In these experiments the contaminating metal ions were removed by treatment of the solutions with Chelex 100 resin. The suspension was stirred for half an hour and the resin filtered off. The supernatant was then lyophilized and the residue redissolved in the solvent required for the NMR experiments.

4.2.2 NMR Spectrometer

The NMR spectrometer used in all these experiments was the 270 MHz machine already described (see Chapter 2).

In the cross-saturation experiments it was necessary to employ secondary irradiation of variable power and frequency. This was achieved by modifications to the Nicolet 1180 operating program carried out by Mr. R. Porteous. The transmitter power and frequency selection
A selective pulse was used to observe a $^{31}$P NMR spectrum of 10 mM inorganic phosphate in D$_2$O at pH 7.2. The intensity of the P$_i$ resonance is plotted as a function of the duration of the selective pulse and this allows the measurement of the B$_2$ field strength (see text).
were put under software control so that they could be switched rapidly by instructions implemented by the Nicolet 293B programable pulser. A delay of 2 ms was necessary to allow for the transmitter power to rise or fall to its new value.

The radio-frequency field ($B_2$) produced by the low-power secondary pulse was calibrated by observing the effect of a selective irradiation of variable duration on the signal from a 10mM sample of inorganic phosphate, pH 7.0 produced by a normal 90 degree observe pulse. The intensity of the $P_i$ resonance is shown in Figure 4.2 as a function of the duration of the secondary irradiation.

The effective $B_2$ field is given by:

$$B_{2\text{eff}} = \frac{\pi}{2\gamma t_p}$$

where $\tau$ is the tip angle in radians produced by a pulse of duration $t_p$ seconds. $\gamma$ is the gyromagnetic ratio of the nucleus observed ($10.83 \times 10^3$ rad G$^{-1}$ s$^{-1}$ for $^{31}$P). For the calibration shown in Figure 4.2, the irradiation time required for a $\pi/2$ pulse is 12 ms and so the effective $B_2$ field is 0.012 G. Figure 4.2 also shows the progressive saturation of the resonance with each inversion. In the saturation transfer experiments irradiation was applied for several seconds to ensure complete saturation.

4.2.3 Line shape analysis

A computer program for the calculation of NMR line shapes broadened by chemical exchange was devised by Kleier & Binsch (1970a) using density matrix formulations.
The program was obtained through the Quantum Chemistry Program Exchange (Kleier & Binsch, 1970b) and was run on a Digital VAX 11/780 computer in Oxford. Graphical output was produced using GHOST graphics implemented by Dr. A. Derome of the Dyson Perrins Laboratory, Oxford.

4.3 Results and Discussion

4.3.1 Experiments on fructose 1,6 bisphosphate

The anomerisation of FDP was studied by a combination of magnetisation transfer, accordion spectroscopy and line shape analysis techniques. The effects of the enzymes aldolase and TPI on the anomerisation process were examined.

Inversion transfer experiments

It is well known that the mutarotation of hexose sugars is usually subject to general acid-base catalysis (Lowry & Richards, 1925) although in the case of phosphorylated hexoses such as FDP the process may be dominated by intramolecular catalysis from suitably situated phosphate groups (Bailey et al., 1970). It is quite likely that the mutarotation is medium-dependant and that buffers or other species present with acidic or basic groups could catalyse the process.

In an effort to measure the anomerisation rate in the absence of medium effects, inversion transfer measurements were carried out on a 30 mM sample of FDP which had been treated with Chelex (see section 4.2.1) and dissolved in water containing 20% D$_2$O for the field-
Figure 4.3

109.3 MHz $^3$P NMR spectrum of 30 mM FDP pH 6.7 at 25°C. The spectrum is the sum of 32 transients recorded into 4K data points with a 90° pulse and a sweep width of $\pm$ 400 Hz. A relaxation delay of 25 s was allowed between pulses. Broad-band $^1$H noise-decoupling was applied during the acquisition and relaxation periods.

The spectrum shows three peaks labeled $\alpha 1P$, $\alpha 6P$, $\beta 1P$, and $\beta 6P$. The peaks are labeled with their respective chemical shifts in Hz.

Hz

-50
frequency lock. The pH was adjusted to 6.7 with HCl. The 109.3 MHz $^{31}\text{P}$ NMR spectrum of this sample at 25°C is shown in Figure 4.3.

The inversion transfer measurements were made by applying a selective 180° pulse to the 1-P resonance of β FDP and observing the effect on the 1-P resonance of α FDP. The pulse sequence used was:

selective 180° - τ - non-selective 90° observe pulse

where τ was a delay whose duration was varied from 1 ms to 60 s.

Chemical exchange between the β and α forms of FDP during the delay τ results in a transient change in intensity of the non-irradiated α form. The intensities of the two resonances were measured as a function of τ using a routine in the NMR spectrometer operating program and data from a typical experiment are illustrated in Figure 4.4. The data from such an inversion transfer experiment may be analysed in a number of ways (Campbell et al., 1978), the simplest being the measurement of the initial rate of decline of the I (non-irradiated) resonance. A more complete treatment is possible by fitting the solution of the Bloch equations under these conditions. For a 2-site case:

$$I \xrightarrow{k_I} S \xrightarrow{k_S} I$$

assuming that the $T_1$ values of the I and S resonances are equal and that there are no cross-relaxation effects, the general solution for the intensity of the I resonance following selective inversion of the S resonance is
Figure 4.4

Inversion transfer experiment on FDP

A selective 180° pulse was used to invert the 1-P resonance of β FDP in the $^{31}$P NMR spectrum and the intensity of the 1-P resonance of α FDP is plotted here as a function of the delay time, $\tau$. The curve is a double exponential function: $y = A \exp(j \tau) + B \exp(k \tau) + C$ which was fitted to the data by least-squares regression analysis. The parameters obtained were: $A = 166$, $B = -179$, $C = 148$, $j = 0.425$, $k = 5.01$. 
(Brindle et al., 1985):

\[ I_z = A e^{(\mu_+ t)} - A e^{(\mu_- t)} + I_0 \]  \hspace{1cm} (1)

where \( \mu_+ = -p; \)

\[ \mu_- = -(p + k_I + k_S); \]

\[ p = 1/T_1; \]

\[ A = \frac{-2S_0 k_S}{k_S + k_I}; \]

The parameters \( \mu_+ \) and \( \mu_- \) may be obtained by fitting the above double exponential function to the intensity of the I resonance and this was achieved using either an iterative least-squares procedure incorporated in the Nicolet 1180 software or by a version of the Simplex program described in Chapter 3, section 3.7.

In the experiment illustrated in Figure 4.4, the values obtained for these parameters are:

\[ \mu_+ = -0.20, \mu_- = -2.35 \]

thus \( T_1 = 5.0 \) s and \( k_I + k_S = 2.15 \) s\(^{-1}\)

Now, \( k_I/k_S = [\beta \text{ FDP}]/[\alpha \text{ FDP}] \)

This is the equilibrium constant which may be measured by integration of the fully relaxed spectrum and was found to be 5.0.

Thus \( k_I = k_{\beta \alpha} = 1.8 \) s\(^{-1}\) and \( k_S = k_{\beta \alpha} = 0.36 \) s\(^{-1}\)

The value of \( T_1 \) obtained here agrees well with values of 5.1 s and 5.0 s obtained for the 1-P resonances
of \( \alpha \) and \( \beta \) FDP respectively in an independent \( T_1 \) measurement on the same sample using an inversion-recovery experiment. This experiment also confirmed the validity of the assumption that the \( T_1 \) values of the two exchanging resonances are the same. In general it is one of the advantages of the inversion transfer method over other magnetisation transfer experiments that it is not generally necessary to make an independant measurement of the \( T_1 \). The effects of chemical exchange on the measurement of \( T_1 \) are discussed below in section 4.3.2. The second assumption made in the derivation of equation 1 is that cross-relaxation is absent and this must also be true in the present case.

The anomerisation of FDP is in fact a three site exchange problem as the reaction goes through the open chain intermediate. The validity of analysing the data with a simplified two-site model is discussed in Appendix I.

Measurements of the mutarotation by Midelfort et al. (1976) at the same temperature as the above experiment gave rather higher rates (\( k_{\alpha \beta} = 8.1 \text{ s}^{-1} \)). This is probably due to a combination of the higher pH of their sample (see below) and to their use of EDTA which may catalyse the mutarotation.

**Accordion spectroscopy experiments**

An alternative method of measuring the anomerisation rate in FDP is to use the three-dimensional "accordion" experiment of Bodenhausen & Ernst (1982). This experiment uses the pulse sequence:
109.3 MHz $^{31}$P accordion spectrum of FDP at 25°C.

The sample used is the one described in the legend to Figure 4.3. A total of 32 transients were recorded for each $\tau_1$ increment with a sweep width of ± 400 Hz. The FID was collected into 2K data points and zero-filled to 4K before the first Fourier transformation. After tranposition of the real part of the spectrum, the data was zero filled to 1K before the second Fourier transformation. The final size of the time domain was 512 X 1024 and the sweep width in the $F_m$ dimension was ± 10 Hz. The mixing time extended from 0 s to 4.8 s.
in which $\tau_1$ and $\tau_m$ are delays which are incremented simultaneously. After Fourier transformation with respect to both $\tau_1$ and $\tau_2$, a two-dimensional NMR spectrum is produced in which the existence of chemical exchange is shown by the occurrence of cross peaks whose intensity is given by:

$$I(\tau_m) = \frac{S \omega k_s}{k_s + k_I} (e^{-p\tau_m} - e^{-(p + k_s + k_I)\tau_m})$$ (2)

This experiment was carried out on the same sample of FDP used in the inversion transfer measurements described above and the resultant spectrum is shown in Figure 4.5. Details of the acquisition parameters are given in the legend to this figure.

The exchange rates may be obtained in a number of ways (Bodenhausen & Ernst, 1982) and the method chosen here was direct line-shape analysis. It may be shown that each diagonal peak in the accordion spectrum consists of the sum of Lorentzian lines with a common central frequency but with different widths and intensities. The number of Lorentzians contributing is equal to the number of possible sites in the exchange pathway and the intensity of each Lorentzian is proportional to the fractional occupancy of the corresponding site. In the case of FDP, the contribution of the intermediate to the overall line-shape will be negligible (2%) and so it was assumed that each peak was the sum of two Lorentzians and the widths of these are $p$ and $p + k_I + k_s$. Similarly, the cross-peaks in the spectrum are described by the
difference of the same two Lorentzians. Possible distortion of the line shapes along the diagonal due to overlapping signals in a crowded spectrum mean that it is usually preferable to restrict fitting to the cross-peaks.

A subroutine of the Nicolet 1180 software was used to superimpose Lorentzian lines over cross sections from the experimental spectrum. The line widths and intensities of these were varied, under the constraint that the areas of the contributing Lorentzians were in the ratio 5:1 \( = \frac{k_{\alpha\beta}}{k_{\beta\alpha}} \), until the best fit was obtained. The line widths found in this fit were used to calculate the following rate constants:

\[ k_{\alpha\beta} = 1.13 \text{ s}^{-1} \quad \text{and} \quad k_{\beta\alpha} = 0.23 \text{ s}^{-1}. \]

These are within 40% of the values found by the inversion transfer experiment and so the agreement is quite good.

In general the inversion transfer method is to be preferred for this type of measurement as it is considerably quicker to perform (the accordion method requires a large amount of processing time) and the error on the measurement was smaller. In a situation such as this, where the equilibrium constant is considerably removed from unity, the line width of the low intensity Lorentzian may be varied considerably without having much effect on the appearance of the composite peak. Its line width thus cannot be estimated accurately.

In conclusion, while the accordion method is very elegant and useful for giving a qualitative description of an exchange pathway, its quantitative usefulness is more debatable in systems which are more complicated than a 2-
site exchange case where the equilibrium constant is unity. Boyd et al. (1984) came to a similar conclusion in the comparison of the saturation transfer and accordion methods in a study of the creatine kinase reaction.

**Line shape analysis**

Even without recourse to a magnetisation transfer experiment the existence of chemical exchange between the α and β forms of FDP is apparent from an examination of the one-dimensional $^{31}$P NMR spectrum at 109.3 MHz (Figure 4.3). In the absence of any exchange, the intensities of the 1-P and 6-P resonances of the β form in a fully relaxed spectrum would be the same. This is clearly not the case and the 6-P resonance is considerably broader than the 1-P resonance, indicating that it is in intermediate chemical exchange. Further, contrary to the claim of Van den Berg & Heerschap (1982), it is immediately apparent that the situation does not reduce to a two-site exchange case as the pair of resonances with the smaller chemical shift separation (the 6-P pair) are broadened more than the pair with the larger chemical shift separation (the 1-P pair). That this situation may not arise from 2-site exchange may be seen from an analysis of the theoretical linewidths in the slow and intermediate exchange limits. In the case of slow exchange between two species:

$$\begin{align*}
    A & \leftrightarrow^{k_1} \leftrightarrow^{k_{-1}} B \\
\end{align*}$$

the linewidth of the $A$ resonance, determined by the effective $T_2$ is given by:
The expression for the B resonance is analogous and it may be seen that the linewidth is independent of the separation of the A and B resonances.

In the case of intermediate exchange, the observed $T_2$ is given by:

$$\frac{1}{T_2} = \frac{1}{T_{2A}} + \frac{1}{T_{2B}} + \frac{4\pi^2 f_A f_B \Delta_0^2}{k_1 + k_{-1}}$$

where $f_A$ and $f_B$ are the fractional occupancies of the A and B sites and $\Delta_0$ is the separation of the resonances in the absence of exchange.

It may be seen from equation (4) that as the separation $\Delta_0$ increases, the linewidth also increases and so the appearance of the $^{31}P$ spectrum of FDP in Figure 4.3 must be due to the presence of an intermediate in the exchange pathway.

This intermediate is the open chain form of FDP shown in Figure 4.1. Previous attempts to observe this species in the $^{31}P$ NMR spectrum (Gray & Barker, 1970) and natural abundance $^{13}C$ NMR spectrum (Koerner et al., 1973) have failed and the authors have placed lower detection limits of 1-2\% on the intermediate. However, Midelfort et al. (1976) did detect a resonance in the $^{13}C$ NMR spectrum of $^{13}C$ enriched FDP which they assigned to the free carbonyl form and they estimated that 1.3 ± 0.4\% of the compound existed in this form. This species would be expected to be visible in the $^{31}P$ spectrum in solutions of
Equations 5 and 6 may be derived as follows:

Consider the equilibrium velocities in the reaction:

\[ V_1 \xrightleftharpoons{\alpha}{\beta} \]

Then the overall velocity from \( \alpha \) to \( \beta \) is given by:

\[
\frac{1}{V_{\alpha\beta}} = \frac{1}{V_1} + \frac{1}{V_2}
\]

(Yagil & Hoberman, 1969)

thus,

\[
V_{\alpha\beta} = \frac{V_1V_2}{V_1 + V_2}
\]

and converting to concentrations:

\[
k_{\alpha\beta}[\alpha] = \frac{k_{\alpha}[\alpha]k_{\beta}[\alpha]}{k_{\alpha}[\alpha] + k_{\beta}[\alpha]}
\]

thus,

\[
k_{\alpha\beta} = \frac{k_{\alpha}k_{\beta}[\alpha]}{k_{\alpha}[\alpha] + k_{\beta}[\alpha]}
\]

and

\[
k_{\alpha\beta} = \frac{k_{\alpha}k_{\beta}}{k_{\alpha} + k_{\beta}}
\]

and similarly for \( k_{\beta\alpha} \).
high concentration and its non-appearance is presumably due to the fact that it is broadened by exchange. The situation in the $^{13}$C spectrum is expected to be more favourable as the chemical shift difference between the open chain carbonyl and the "anomeric" C-2 carbon in the ring forms of FDP (ca. 3000 Hz) is much greater than the likely separation of the resonances due to the open chain and ring forms in the $^{31}$P spectrum (probably less than 500 Hz). Thus it seems likely that the intermediate is in slow exchange in the $^{13}$C spectrum but in the $^{31}$P spectrum the exchange is fast enough for the intermediate resonance to be broadened beyond detectability.

The rate constants for anomerisation of FDP found by the inversion transfer measurements above may be used as a starting point for a more detailed study of the line shapes in the $^{31}$P NMR spectrum in Figure 4.3. The unimolecular rate constants found were $k_{\alpha\beta} = 1.8 \text{ s}^{-1}$ and $k_{\beta\alpha} = 0.36 \text{ s}^{-1}$. From the inversion transfer experiment it was not possible to determine the individual ring opening and closing rates but the overall rate constants are expressed in terms of these as follows:

$$ k_{\alpha\beta} = \frac{k_{\alpha\alpha} k_{\beta\beta}}{k_{\alpha\alpha} + k_{\beta\beta}} \quad (5) $$

$$ k_{\beta\alpha} = \frac{k_{\beta\beta} k_{\alpha\alpha}}{k_{\alpha\alpha} + k_{\beta\beta}} \quad (6) $$

From a knowledge of the proportion of FDP present in the open chain form at equilibrium, and hence the ratios
Figure 4.6

Simulated $^{31}$P NMR spectra using the DNMR3 program.

Frequencies: $\alpha$ -169 Hz and -44 Hz, $\beta$ -83 Hz and -63 Hz, open chain form as marked. $T_2 = 0.11$ s, mole fractions in $\alpha$, $\beta$, $\gamma$ are 0.16, 0.82, 0.02.

A). All exchange rates zero.

B). $K_{\alpha} = 1.79$ s$^{-1}$, $K_{\beta} = 282.0$ s$^{-1}$
\( k_{\beta \beta} / k_{\beta \alpha} \) and \( k_{\alpha \alpha} / k_{\alpha \beta} \), and making the assumption that although the absolute rate constants for anomerisation in the sample used in this study and that of Midelfort are different, the ratio of the two ring opening rates \( k_{\beta \beta} / k_{\alpha \alpha} \) are the same, it is possible to calculate the four individual ring opening and closing rate constants:

\[
k_{\beta \alpha} / k_{\alpha \alpha} = 20 \quad \text{(Midelfort et al., 1976)}
\]

then substitution in equation (5) gives:

\[
k_{\alpha \beta} = \frac{20k_{\alpha \alpha}}{21}
\]

hence, if \( k_{\alpha \beta} = 1.7 \text{ s}^{-1} \),

\[
k_{\alpha \alpha} = 1.79 \text{ s}^{-1}, \quad k_{\alpha \alpha} = 14.7 \text{ s}^{-1}, \quad k_{\beta \alpha} = 6.8 \text{ s}^{-1}, \quad k_{\alpha \beta} = 282 \text{ s}^{-1}
\]

These values may be used in the DNMR3 program to simulate the effects of chemical exchange on the \( ^{31}\text{P} \) NMR spectrum of FDP. Figure 4.6 a) shows a spectrum where no chemical exchange occurs and linewidths are determined solely by \( T_2 \). In this case the chemical shift of the intermediate is irrelevant but has been assumed to be coincident with that of the \( \beta \) form. Figure 4.6 b) shows a situation where chemical exchange occurs with the rate constants derived above where the resonances of the intermediate are assumed to be coincident with those of the \( \beta \) form of FDP in accordance with the prediction of Van den Berg & Heerschap (1982). Neither of these is a good model for the anomerisation represented by the real NMR spectrum in Figure 4.3 and it is useful to re-assess the likely positions of the resonances due to the open chain
Figure 4.7

Comparison of experimental and simulated spectra of FDP

A). DNMR3 simulated spectrum with parameters and rate constants as Figure 4.6 B) and position of open chain form as indicated in the figure.

B). Experimental spectrum of FDP (reproduction of Figure 4.3).
A reasonable model for the 1-P resonance would be the keto form of DHAP and this is known to occur just upfield of the 6-P resonance of α FDP (see Figure 3.6). A model for the 6-P resonance of the open chain form is GAP whose predominant resonance due to the hydrated form occurs some way downfield of the 1-P resonance of β FDP (data not shown). Further support for this estimate is a published spectrum of the dimethyl acetal of D-GAP (Gray, 1971) in which the resonance is virtually degenerate with the 1-P resonance of α FDP. If these frequencies are used for the position of the open chain resonances, the simulated spectrum becomes very similar in appearance to the experimental spectrum. The exact form of the spectrum is quite sensitive to the exact position of the 6-P resonance of the open chain form as it is the separation of this and the 6-P resonance of the β form that leads to the characteristic broadening of the latter. Figure 4.7a shows the best fit obtained and the positions of the open chain resonances are shown. Figure 4.7b is a reproduction of the experimental spectrum for comparison. A test of the accuracy of this model would be to apply selective saturation at the suspected position of the 6-P open chain resonance and look for transfer of magnetisation to the β form. Unfortunately it was not possible to saturate this position selectively enough without also saturating the 1-P resonance of the α form.

These simulations have shown that it is possible to use line shape analysis techniques to detect the presence
A 10 mM sample of FDP in D$_2$O at pH 7.2 was used to acquire this spectrum which represents the sum of 64 transients. The residual signal due to water was reduced with a selective saturation pulse prior to acquisition. The resonances are very broad owing to exchange between the $\alpha$ and $\beta$ forms of the molecule.
and probable chemical shift of a species which is not visible per se in the NMR spectrum. These simulation techniques also proved useful in elucidating the anomerisation process in the related sugar fructose 6-phosphate and this is described in section 4.3.2.

The considerations discussed above about the effects of chemical exchange on the appearance of the $^{13}$C and $^{31}$P NMR spectra of FDP may be extended to the $^1$H spectrum. Here the chemical shift separations of the various forms of FDP will be even less and exchange broadening would be expected to be even more dominant. This was confirmed by recording a $^1$H spectrum of FDP in D$_2$O at 470 MHz (Figure 4.8). The lines in the spectrum are very broad making spectral assignment very difficult and the lack of resolution precludes the use of magnetisation transfer experiments on this nucleus of the kind done by Serianni et al. (1982) on non-phosphorylated sugars in which the ring opening and closing rates are much less.

Temperature dependence of the mutarotation

The mutarotation of FDP was measured in a buffer which modelled the intracellular environment and the effect of temperature was studied. The method chosen to measure the anomerisation rates was the inversion transfer experiment as in this system the errors on the measurement were less than with the accordion or line shape analysis techniques.

The sample used was identical to that employed in the temperature-jump experiments described in Chapter 3 i.e. 20 mM FDP in 150 mM KCl, 50 mM HEPES, 5 mM EDTA at pH
Figure 4.9

Arrhenius plot for the anomeration reaction of FDP.

A plot of the data from Table 4.1 in the form $\ln(K_0)$ against $1/\text{temperature}$. The gradient of the line is $7235 \text{ K}$, given a value for the activation energy of $60 \text{ KJ mol}^{-1}$. 
The inversion transfer experiment was carried out at a number of different temperatures and the mutarotation rate constant, $k_{\alpha\beta}$, was determined as described above. The data are shown in Table 4.1 and in Figure 4.9 in the form of an Arrhenius plot. The curve in Figure 4.9 is a least squares fit of a straight line to the data. The gradient of the straight line is equal to $E_a/R$ where $E_a$ is the activation energy and $R$ is the gas constant.

**Table 4.1**

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>$k_{\alpha\beta}$ (s$^{-1}$)</th>
<th>$1/T$ ($K^{-1} \times 10^3$)</th>
<th>$\ln(k_{\alpha\beta})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>277</td>
<td>0.59</td>
<td>3.61</td>
<td>-0.528</td>
</tr>
<tr>
<td>284</td>
<td>1.24</td>
<td>3.52</td>
<td>0.215</td>
</tr>
<tr>
<td>293</td>
<td>2.72</td>
<td>3.41</td>
<td>1.000</td>
</tr>
<tr>
<td>302</td>
<td>5.19</td>
<td>3.31</td>
<td>1.647</td>
</tr>
<tr>
<td>310</td>
<td>10.76</td>
<td>3.23</td>
<td>2.376</td>
</tr>
</tbody>
</table>

It is interesting to note that in the measurement at 37°C, the extrapolation of the fitted double exponential function to $\tau = 0$ gave a value of $I_0$ rather less than $I_\infty$ whereas the two should be the same (equation (1)). It is thought that at this temperature the duration of the selective 180° pulse (ca. 40 ms) is no longer short compared with the exchange timescale. The selective pulse could be made shorter by increasing the power output but it then starts to become less selective. This situation therefore is approaching the upper limit of rates that can...
be measured accurately by this technique. The use of shaped pulses (Bauer et al., 1984) for the selective excitation may allow that limit to be extended.

The activation energy for the anomerisation was found to be 60 KJ mol\(^{-1}\) which is in good agreement with the value of 16.2 Kcal (67.7 KJ mol\(^{-1}\)) found by Midelfort et al. (1976) for the ring opening step \(\beta \rightarrow \text{open chain}\) (the rate limiting step in the anomerisation).

This activation energy is rather less than that measured for the mutarotation of glucose (Los et al., 1956) and glucose 6-phosphate (Bailey et al., 1970) which was found to be around 92 KJ mol\(^{-1}\) in each case. The absolute rate constants for the mutarotation of glucose and glucose 6-phosphate however differed by a factor of 240 and it was proposed (Bailey et al., 1970) that the introduction of a phosphate group at C-6 accelerated the anomerisation process not by causing increased ring strain and hence lowering the activation energy but by effects embodied in the pre-exponential factor in the Arrhenius equation;

\[
k = A e^{(-E_a/RT)}
\]

These effects are mainly entropic in origin and are due to the increased effective concentration of an intramolecular catalytic group.

In the case of FDP it seems that the increased rate of anomerisation over that in G6P (at least a factor of 10 greater) is due, at least in part, to a reduction in the activation energy brought about by the introduction of ring strain by the transition from a 6 membered to a 5 membered ring and the presence of a bulky phosphate group at C-1. Further conclusions about the anomerisation
reaction of FDP were possible following the experiments on fructose 6-phosphate described in section 4.3.2.

The value found in these experiments for the spontaneous anomerisation rate of FDP at 25°C (4.1 s\(^{-1}\) from Figure 4.9) is rather greater than that estimated from the secondary slow phase of the stopped-flow kinetics (0.55 s\(^{-1}\): Wurster & Hess, 1973). The stopped-flow experiments were carried out at high enzyme/substrate ratios and although the \(\alpha\) anomer of FDP is not a substrate for aldolase, there is good evidence that it does bind to the substrate site of the enzyme (Hartman & Barker, 1965) and it is possible that under these conditions a significant proportion of the \(\alpha\) anomer is bound to the enzyme. In these circumstances the anomerisation may be limited either by the dissociation of the \(\alpha\) anomer from the enzyme or by the ring opening step occurring on the enzyme at a lower rate than in free solution.

**Effects of aldolase and TPI on the mutarotation**

In an attempt to establish what effect, if any, aldolase has on the anomerisation of FDP the inversion transfer measurement was carried out on samples of 20 mM FDP to which 200 Units (ca. 24 mg) of rabbit muscle aldolase had been added. Owing to the poor sensitivity of the NMR method, it was not possible to use the very high enzyme to substrate ratios used in some of the previous kinetic studies (Wurster & Hess, 1973; Rose & O'Connell, 1977) as this would require prohibitively large enzyme concentrations. The enzyme concentration in these experiments was around 1 mM (active sites) and under these
conditions no change in the anomerisation rate of FDP was detectable by the inversion transfer method.

It is not possible to conclude from this experiment whether the lower anomerisation rate observed by Wurster & Hess (1973) is due to binding of the α anomer to aldolase but it does seem clear that the rabbit muscle enzyme has no anomerase activity towards FDP.

In a different experiment on a sample of FDP containing 5000 Units of rabbit muscle TPI, the anomerisation rate was also found to be unchanged relative to that in pure FDP.

The lack of anomerase activity of rabbit muscle aldolase is in contrast to the situation with the yeast enzyme which was shown to catalyse the interconversion of the α and β forms of FDP (Schray et al., 1975). This difference may be rationalised, to some extent, by a comparison of the glycolytic rates in the two tissues.

The glycolytic rate in yeast is around 0.5 μmol s\(^{-1}\) per ml cell water (Brindle & Krikler, 1985) and in muscle it is around \(3 \times 10^{-3}\) μmol s\(^{-1}\) per g wet tissue (Challiss et al., 1983). Approximate values of the FDP concentration in these systems are 500 nmol per ml cell water in yeast and 200 nmol per g wet tissue in muscle (Ottaway & Mowbray, 1977). Using a rate constant of 10 s\(^{-1}\) for the α \(\rightarrow\) β conversion at 37°C (Table 4.1), and assuming that 20% of the total FDP is present in the α form, the spontaneous rate of production of the active β form of FDP is 1 μmol s\(^{-1}\) per ml cell water in yeast and 0.4 μmol s\(^{-1}\) per g wet tissue in muscle. Thus, in yeast the spontaneous
rate of anomerisation of FDP is within a factor of 2 of the glycolytic rate, while in muscle it is more than two orders of magnitude faster. It seems likely, therefore, that yeast aldolase has evolved its anomerase activity to ensure that the α to β interconversion could not become rate limiting but in the case of the muscle enzyme this has not been necessary.

It may also be significant that the gluconeogenic pathway is more important in yeast than in muscle and, since the gluconeogenic enzyme FDPase utilises the α anomer of FDP while the aldolase reaction produces the β form, anomerase activity may be required to prevent limitation of flux in this reverse direction.

In addition to these considerations, it has been proposed that in skeletal muscle the reciprocal anomic specificities of PFK and FDPase provide a mechanism for the regulation of futile cycling through these enzymes (Koerner et al., 1977). In this case anomerase activity of muscle aldolase would be undesirable.

It is interesting that the aldolase found in yeast is a Class II aldolase and contains a metal ion (Zn$^{2+}$ or Mn$^{2+}$) at its active site while the muscle enzyme is a class I, Schiff base-forming aldolase (Horecker et al., 1972). It seems quite likely that the anomerase activity of yeast aldolase is associated with this metal ion.

In the erythrocyte, where the glycolytic rate is much lower than in either muscle or yeast (around 3 x 10$^{-4}$ μmol s$^{-1}$ per ml cell water), and where there is no gluconeogenesis, it seems unlikely that aldolase would need to develop anomerase activity.
4.3.2 Experiments on fructose 6-phosphate

The results of the spectral simulation of exchange broadening in the $^{31}$P NMR spectrum of FDP prompted an investigation into the behaviour of the related sugar F6P which is the substrate for the enzyme phosphofructokinase (PFK). It has been proposed that the additional phosphate group in FDP would catalyse the ring opening and anomerisation would be more than an order of magnitude more rapid in this compound than in the monophosphorylated F6P (Midelfort et al., 1976). However, the stopped flow experiments of Wurster & Hess (1973; 1974a) suggest that the spontaneous anomerisation rate of F6P is actually greater than that of FDP.

Previous studies of the $^{13}$C NMR spectra of F6P (Koerner et al., 1973) have shown that the relative proportions of the $\alpha$ and $\beta$ forms are around 20% and 80% respectively. The intermediate open chain form was detected in the IR spectrum (Swenson & Barker, 1971) and in the $^{13}$C NMR spectrum of $^{13}$C enriched F6P (Midelfort et al., 1976) and was found to contribute around 1-2% to the equilibrium composition.

Initial $^{31}$P NMR studies of F6P at neutral pH were disappointing as the spectrum showed only a single narrow line. The non-appearance of resonance due to both $\alpha$ and $\beta$ forms must be due either to the fact that the resonances are not resolved under these conditions or that one of the resonances is broadened beyond detectability. It turned out that the former factor was responsible and at pH 8.4, shifts in the spectrum occurred such that a smaller resonance became resolvable on the downfield side of the
Comparison of simulated and experimental spectra of F6P.

A). 109.3 MHz $^{31}$P NMR spectrum of 40 mM F6P, pH 8.4 at 4°C. The spectrum is the sum of 200 transients collected into 8K data points with a sweep width of $\pm$ 250 Hz and a recycle time of 6.5 s. Broad-band $^1$H noise decoupling was employed.

B). Spectrum of F6P simulated with the DNMR3 program using the following parameters: Frequencies of $\alpha$, $\beta$ and open chain forms are: -99 Hz, -90 Hz and -190 Hz. Mole fractions are 0.205, 0.780, 0.015. Rate constants are $K_{\alpha\beta} = 0.5 \text{ s}^{-1}$, $K_{\beta\alpha} = 18.0 \text{ s}^{-1}$. 
main signal (Figure 4.10a). This spectrum was recorded at 4°C to reduce any line broadening due to exchange. The two signals integrated to give relative contributions of 1 : 3.8 in agreement with the proportions of the α and β anomers previously reported. Examination of the spectrum in Figure 4.10a also revealed the presence of a much less intense broad resonance about 0.37 ppm downfield of the main resonance and this was tentatively assigned to the open chain form. This assignment was confirmed by applying selective saturation at the frequency of the downfield resonance and observing the effects on the two upfield resonances. It was found that the intensity of the resonance due to β F6P was reduced by 53% and that due to the α form was reduced by 62% relative to those in a control spectrum with an identical saturating pulse applied on the upfield side. This experiment confirms that the species responsible for the broad resonance is in chemical exchange with the α and β forms of F6P and is almost certainly due to the intermediate open chain form.

Attempts to measure the exchange rates between the open chain and ring forms of F6P using the inversion transfer or "accordion" methods so far described would be very difficult. This is because in these experiments the changes in magnetisation observed depend on the mole fractions of both exchanging species (see equations (1) and (2)). Thus, as in this case, when the equilibrium constant is unfavourable, these effects are very small. The saturation transfer experiment does not suffer from this drawback as the intensity of the non-irradiated
resonance is independent of the equilibrium constant (equation (7)). This similarity between the inversion transfer experiment and the two-dimensional techniques and the fundamental difference of the saturation transfer experiment has been discussed previously (Brindle et al., 1985).

For saturation of the S spin in a 2 site exchange case the intensity of the I resonance is given by:

\[ I_z = \frac{pI_0}{p + k_I} \]

(7) (Campbell et al, 1978)

and so the rate constant for the exchange, \( k_I \), may be calculated if \( p ( = 1/T_1) \) is known. The \( T_1 \) values for the \( \alpha \) and \( \beta \) forms of F6P were measured using an inversion-recovery pulse sequence and were found to be \( 3.26 \pm 0.13 \) s and \( 3.19 \pm 0.09 \) s respectively. These are effectively identical and the average value of \( p \) is \( 0.313 \) s\(^{-1}\).

The measurement of \( T_1 \) values by the inversion recovery method may be complicated in the presence of chemical exchange because if the \( T_1 \) values of two species are different, transfer of magnetisation between them as a result of chemical exchange will tend to equalise the observed relaxation times. More accurate estimates of the \( T_1 \)s could be made if the inversion recovery experiment was repeated for each resonance in the presence of saturation of the other species with which it was exchanging. This is impossible to achieve in this case because the \( \alpha \) and \( \beta \) resonances are too close together to allow selective saturation of one of them. However, there is no good reason to suggest that the \( T_1 \) values of the 6-phosphate
resonance would be very different in the various forms of F6P. In support of this, it was found that the \( T_1 \) values of the 1- and 6-phosphate resonances are identical within experimental error (data not shown). The estimation of the \( T_1 \) values by the inversion recovery method is therefore considered to be a justifiable approximation.

Using the values of \( I_z/I_0 \) quoted above for the saturation of the intermediate, the following rate constants may now be derived:

\[
\begin{align*}
    k_{\beta\alpha} &= 0.34 \text{ s}^{-1} \\
    k_{\alpha\beta} &= 0.50 \text{ s}^{-1}
\end{align*}
\]

The equilibrium proportion of the open chain form cannot be determined accurately by integration of the \(^{31}\text{P} \) NMR spectrum but assuming a value of 1.5\% (Swenson & Barker, 1971), the full set of rate constants at 4°C may be calculated:

\[
\begin{align*}
    \alpha & \overset{0.5 \text{ s}^{-1}}{\longrightarrow} \overset{6.6 \text{ s}^{-1}}{\longrightarrow} \overset{18.0 \text{ s}^{-1}}{\longrightarrow} \beta \\
    \alpha & \overset{0.34 \text{ s}^{-1}}{\longrightarrow}
\end{align*}
\]

and

\[
\begin{align*}
    k_{\alpha\beta} &= \frac{k_{\alpha\beta} k_{\alpha\beta}}{k_{\alpha\beta} + k_{\beta\alpha}} = 0.36 \text{ s}^{-1} \\
    k_{\beta\alpha} &= \frac{k_{\beta\alpha} k_{\alpha\beta}}{k_{\alpha\beta} + k_{\beta\alpha}} = 0.095 \text{ s}^{-1}
\end{align*}
\]

These rate constants provide the basis for a simulation of the spectrum using the DNMR3 program and Figure 4.10b shows the spectrum obtained. It may be seen that there is excellent agreement between the experimental and
theoretical spectra providing confirmation of the rate constants obtained from the saturation transfer measurements.

As stated above, at neutral pH the α and β resonances of F6P in the $^{31}$P NMR spectrum were unresolved and were found to remain so as the pH was further reduced. It was noted, however, that as the pH was decreased the resonance due to the open chain form sharpened considerably, indicating that the anomerisation rate is reduced at low pH. This is similar to the situation observed for glucose 6-phosphate (Bailey et al., 1970) where the mutarotation rate constant at pH 8 was around ten times its value at pH 4. This may be attributed to the fact that the triply and doubly charged phosphate ions are the most effective catalysts of the ring opening and closing and their concentrations are much reduced at low pH.

Using the rate constants derived above the ratio of the two ring closing rates, $k_{o\beta}/k_{o\alpha}$ may be calculated to be 2.6. In the two other cases where the unidirectional rates have been measured, this ratio has been found to be 21 for FDP (Midelfort et al., 1976) and around 1 for glucose (Los et al., 1956). Two possible causes of the large $k_{o\beta}/k_{o\alpha}$ in FDP were discussed by Midelfort et al.

Model building shows that the bulky phosphate groups on C-1 and C-6 in FDP would have to approach more closely to catalyse the $\alpha \leftrightarrow o$ ring closure than the $\beta \leftrightarrow o$ step and so $k_{o\alpha}$ is reduced by electrostatic repulsion. Another possibility is that the C-2 carbonyl and C-3 hydroxyl oxygens are gauche in the $\beta \leftrightarrow o$ reaction but trans in
the $\alpha \leftrightarrow \beta$ step. It was proposed (Midelfort et al., 1976) that the enhanced rate of the $\alpha \leftrightarrow \beta$ step may be due to the "gauche effect" (Wolf, 1972) in which interaction between the electron lone pairs on the oxygens produces an acceleration of the rate of ring closure. Midelfort et al. were unable to distinguish between these two alternatives but in the light of the results from F6P it is possible to draw further conclusions.

The $k_{o\beta}/k_{o\alpha}$ for F6P is much less than that for FDP and is quite close to that found in glucose. In F6P, as in glucose, intramolecular phosphate-phosphate interactions are absent and so it would seem that these effects are the cause of the reduced $k_{o\alpha}$ in FDP. The "gauche effect" then does not seem to be an important factor in determining the anomerisation rates in F6P or FDP.

The rate of spontaneous anomerisation of F6P at 4°C is within a factor of 2 of that measured for FDP at the same temperature (Table 4.1). The comparison is confused by the different pH values used in the two determinations (8.4 and 7.4 respectively) but it seems that the rates are much more similar than the factor of more than 10 postulated by Midelfort et al. (1976). In fact, if the activation energies for the two anomerisations are taken to be equal, the rate constant $k_{a\beta}$ for F6P at 20°C may be calculated to be 1.7 s$^{-1}$. This is close to the value of 1.5 s$^{-1}$ estimated for the spontaneous anomerisation by Wurster and Hess (1974a) from their stopped-flow experiments on PFK.

It seems then that in contrast to the situation of
Figure 4.11

$^{31}\text{P}$ and $^1\text{H}$ NMR Spectra of DHAP

A). 109.3 MHz $^{31}\text{P}$ NMR spectrum of 15 mM DHAP in $\text{D}_2\text{O}$, pH 6.7.

B). 270 MHz $^1\text{H}$ NMR spectrum of the same sample.
The frequency scales are in Hz and have arbitrary origins.
FDP and aldolase, the anomerisation of F6P is not restricted at high enzyme concentrations. The reason for this may be that, in the case of F6P, binding of the α form to the enzyme does not inhibit the anomerisation reaction. Experiments with substrate analogues have shown, however, that the α form of F6P probably binds to the substrate site of PFK with the same affinity as the β form although it is not itself a substrate (Koerner et al., 1974). In this case it is possible that the anomerisation of F6P can take place on the enzyme as rapidly as in free solution although there seems to be no evidence for anomerase activity of PFK.

The β → open chain rate constant of 6.8 s⁻¹ obtained in these experiments is considerably less than the observed turnover rate of PFK and this supports the conclusion of Koerner et al. (1974) that the main substrate for the enzyme is not the keto form of F6P.

4.3.3 Experiments on dihydroxyacetone phosphate

A 109.3 MHz 31P NMR spectrum of 10 mM DHAP, pH 6.7 is shown in Figure 4.11a). Figure 4.11b) shows a 270 MHz 1H NMR spectrum of the same sample. The proton spectrum is very similar to a previously published one (Lowe & Pratt, 1976) and the assignments are taken from that work. A comparison of the coupling constants in the 1H and 31P spectra allows the downfield resonance in the latter to be assigned to the hydrate form and the upfield multiplet to the keto form. The third resonance in the 31P spectrum is due to inorganic phosphate which is present in the
Figure 4.12

Van't Hoff plot of the DHAP hydrate-keto equilibrium

A plot of ln( [hydrate]/[keto] ) against 1/temperature. The gradient of the straight line gives an enthalpy change for the dehydration reaction of 110.4 KJmol⁻¹.
commercial DHAP. At this temperature (22°C) it may be seen that the keto form predominates and the equilibrium constant may be found by integration of the fully relaxed \(^1\)H decoupled \(^{31}\)P NMR spectrum. It was known from the temperature-jump experiments described in Chapter 3 that at 4°C the downfield (hydrate) resonance was the more intense and so the equilibrium shifts in favour of the hydrate form at lower temperatures. The equilibrium constant was measured from the NMR spectrum over a range of temperatures and the data are shown in the form of a Van't Hoff plot in Figure 4.12. The gradient of the straight line is equal to \(-\Delta H/R\) and so the enthalpy for the dehydration reaction:

\[
\begin{align*}
\text{hydrate} & \iff \text{keto} \\
& \quad \frac{k_H}{k_K}
\end{align*}
\]

may be calculated to be 110.4 KJ mol\(^{-1}\).

**Magnetisation transfer experiments on DHAP**

The dehydration rate constant for DHAP was measured by the inversion transfer method described in section 4.3.1. Either the keto or hydrate resonance was selectively inverted and the transient transfer of magnetisation to the other resonance was followed as a function of the mixing time, \(T\). In this system the exchange pathway is a true two site case and equation (1) is valid. The fit of a double exponential function provides the parameters \(p\) and \(k_I + k_S\) and the individual rate constants may be calculated using the equilibrium constant at the relevent temperature obtained from Figure 4.12. The data at three different temperatures are shown.
in Table 4.2. In two of the experiments the dehydration rate was measured again after the addition of around 4000 Units of rabbit muscle TPI. These data are also shown in Table 4.2.

<table>
<thead>
<tr>
<th>Temp. (K)</th>
<th>$k_H + k_K$ (s$^{-1}$)</th>
<th>$K_{eq}$</th>
<th>Dehydration rate const. (s$^{-1}$) (no TPI)</th>
<th>Dehydration rate const. (s$^{-1}$) (with TPI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>288</td>
<td>0.36</td>
<td>0.89</td>
<td>0.19</td>
<td>0.38</td>
</tr>
<tr>
<td>303</td>
<td>0.91</td>
<td>0.55</td>
<td>0.59</td>
<td>1.47</td>
</tr>
<tr>
<td>310</td>
<td>1.18</td>
<td>0.45</td>
<td>0.81</td>
<td>-</td>
</tr>
</tbody>
</table>

A plot of $\ln(k_H)$ against $1/\text{temperature}$ for the data in the absence of TPI gives a gradient of $E_a/R$ from which a value of 49.9 KJmol$^{-1}$ was calculated for the activation energy of the dehydration reaction. Interpolation to 20°C gave a dehydration rate constant of 0.44 s$^{-1}$. This is very close to the value of 0.45 s$^{-1}$ obtained by Reynolds et al., (1971) from stopped-flow kinetic experiments on the reaction of DHAP with a glycerophosphatedehydrogenase. This similarity suggests that the interconversion of the two forms of DHAP is unaffected by the presence of high concentrations of that enzyme.

The data in Table 4.2 show that the dehydration rate constant for DHAP is increased by a factor of 2 to 2.5 in the presence of TPI. It cannot be ruled out that this catalysis is due to residual contamination of the enzyme by metal ions but the apparent ability of TPI to catalyse the interconversion of the active and inactive forms of
DHAP may reflect the evolution of the enzyme to perform a useful side reaction.

In contrast to the possible localisation of the aldehyde form of GAP in a glycolytic complex, DHAP seems to diffuse out into the cytosol (in some tissues DHAP is transported between compartments e.g. between chloroplasts and leaf cytosol (Ottaway & Mowbray, 1977)). In this case the two forms of DHAP will come to equilibrium and only about half the total pool will be in the active form. It may be of benefit, therefore, for glycolysis to have a mechanism to accelerate the spontaneous rate of interconversion and the observed catalytic activity of TPI could reflect this.

4.4 Conclusions

A variety of NMR methods have been used to study the compartmentation of glycolytic intermediates between active and inactive forms in greater detail than has previously been possible. Measurements of the rates of interconversion of these forms have been made with greater accuracy than has been achieved with the classical rapid-quench and stopped-flow methods. The interconversions have been studied in the absence and presence of glycolytic enzymes which may influence these processes in situ.

It would seem that under certain circumstances, such compartmentation between active and inactive forms could lead to limitation of flux through some glycolytic enzymes. In the cases where this is possible, two mechanisms have evolved to counteract the problem. Some enzymes have evolved anomerase activity, i.e. the ability
to catalyse the conversion of the inactive to the active form. An example of this is yeast aldolase. Muscle aldolase on the other hand does not have detectable anomerase activity and this can be accounted for by a consideration of the properties required of this enzyme in situ.

Rabbit muscle TPI has been shown to catalyse the interconversion of the active and inactive forms of DHAP thus making the inactive fraction of this metabolite more readily accessible to glycolysis.

The second mechanism which may have evolved is to prevent the active form of a metabolite from equilibrating with its inactive form by ensuring its rapid transfer from one enzyme to another via a glycolytic complex. It is possible that this mechanism has been adopted for GAP which may be passed directly from aldolase to GAPDH or vice versa.
4.5 References


4.6 Appendix I

Validity of the 2-site model in inversion transfer

The use of a two-site model in the analysis of a saturation transfer experiment in which there is, in reality, a third site in the form of a minor intermediate, has been discussed previously (Brindle & Radda, 1985). It was shown that, provided there was no significant loss of saturation from the intermediate, the two-site model was a valid approximation. In order for this approximation to break down, the $T_1$ of the intermediate would need to be unusually short (of the order of 50 ms) and it was postulated that this situation might arise in the case of an enzyme-bound intermediate.

In some of the experiments in this chapter, a two-site model was used to analyse the data from an inversion transfer experiment on a system which was truly a three-site problem. The analytical solution to the three-site inversion transfer experiment is available (Dr. J. Boyd, personal communication) but is complex and not very useful in the analysis of experimental data. Hence, the two-site model was used and it is interesting to test its validity in this case.

It might be expected that similar conditions to those described above would apply; i.e. that if there is no significant loss of magnetisation in the intermediate, the approximation is valid. In order to test this, some simulations of inversion transfer experiments were carried out using numerical integration by computer of the Bloch equations, modified to take account of chemical exchange.
Figure 4.13

Comparison of the 2-site and 3-site models in inversion transfer

The plots are simulated inversion transfer experiments in which the $\alpha$ 1-phosphate has been inverted and the $\beta$ 1-phosphate is being observed. The simulations were carried out using a computer program to integrate the Bloch equations, modified to take account of chemical exchange.

The two sets of data represent a 2-site model (x) and a 3-site model (o) in which the overall $k_{\alpha\beta}$ is identical in each case.
via an intermediate present at low concentration.* see over

Two systems were modelled as follows:

A) A two-site model of \( \alpha \) FDP \( \leftrightarrow \beta \) FDP exchange in which \( k_{A\beta} = 1.8 \text{ s}^{-1} \) and \( k_{\beta A} = 0.36 \text{ s}^{-1} \) as measured in section 4.3.1.

B) A three-site model of

\[ \alpha \text{ FDP} \leftrightarrow \text{open chain} \leftrightarrow \beta \text{ FDP} \]

in which \( k_{A\alpha} = 1.79 \text{ s}^{-1} \) and \( k_{\alpha B} = 282 \text{ s}^{-1} \) with relative populations of the sites \( \alpha : o : \beta \) being 0.163 : 0.02 : 0.817. This gives overall \( k_{A\beta} \) and \( k_{\beta A} \) rates identical to those in A).

In both cases, the \( T_1 \) value of all three sites was assumed to be equal to 5.0 s. The result of these two simulations are shown in Figure 4.13.

It can be seen that in this case, the intermediate does have an effect on the transfer of magnetisation to the \( I \) spin but this effect is not large. In order to assess the importance of the deviation, the data in Figure 4.13 were used to re-calculate the interconversion rates by fitting double exponential functions as described in section 4.3.1.

The data from the two-site simulation, as expected, gave the original rate constants used for the simulation \( (k_{A\beta} = 1.8 \text{ s}^{-1}, k_{\beta A} = 0.36 \text{ s}^{-1}) \).

The data from the three-site simulation gave rate constants \( k_{A\beta} = 1.68 \text{ s}^{-1} \) and \( k_{\beta A} = 0.34 \text{ s}^{-1} \). The deviation between these values and those from the two-site model is only 6% and so it is considered justifiable to use the
two-site general solution in this and similar cases.

The generality of this approximation cannot be guaranteed, however, and it is quite possible that with certain combinations of rate constants the deviations will become greater. In particular, the deviations would be expected to be greater when the intermediate becomes more significant or when its $T_1$ is considerably reduced. More detailed analysis of the problem would require a comparison of the general analytical solutions of the two-site and three-site exchange situations.

In the three site case represented by:

\[
\begin{align*}
A & \xrightarrow{k_1} B \xrightarrow{k_3} C \\
& \xrightarrow{k_2} B \xrightarrow{k_4} C
\end{align*}
\]

the three equations to be solved are:

1. \[
\frac{dA_z}{dt} = -p_A(A_z - A_0) - k_1A_z + k_2B_z
\]
2. \[
\frac{dB_z}{dt} = -p_B(B_z - B_0) - B_z(k_2 + k_3) + k_1A_z + k_4C_z
\]
3. \[
\frac{dC_z}{dt} = -p_C(C_z - C_0) - k_4C_z + k_3B_z
\]

where $p_x$ is the reciprocal $T_1$ of the $X$ nucleus and $X_z$ is the component of the $X$ magnetisation in the $z$ direction at time $t$. In the two site case, $k_3$ and $k_4$ are set to zero.
CHAPTER 5

SUMMARY

This thesis has explored some of the ways in which NMR may be used to study enzyme catalysed reactions, with an emphasis on their behaviour in the intact cell. The particular advantage of NMR in this field is its non-invasive nature which allows intracellular events to be observed with minimal, if any, disturbance to the system under study.

A number of magnetic nuclei occur commonly in molecules of biochemical interest, e.g. $^{13}\text{C}$, $^{31}\text{P}$ and $^{1}\text{H}$. Previous studies have generally been restricted to the observation of these nuclei singly. However, the information available from different nuclei is often highly complementary and there are advantages in being able to observe more than one at once. The experiments in Chapter 2 describe how this may be done and illustrate the large amount of data available from a multinuclear study of cellular metabolism.

The technique is particularly powerful in isotopic labelling studies where fractional labelling of metabolic pools may be calculated from observations of different nuclei in the same molecule.

It is to be expected that such "multiparametric" studies will become more widespread in the future with improvements in sensitivity allowing NMR observations to be made in a shorter time. There is considerable potential for extending the technique by combining simultaneous
multinuclear NMR observations with measurements of other parameters such as oxygen or metal ion concentration using ion-selective electrodes.

The importance of being able to make observations of enzyme catalysed reactions in situ is illustrated in the experiments described in Chapter 3. An NMR method was developed to allow the observation of chemical relaxation subsequent to a rapid temperature-jump on an isolated section of the glycolytic pathway, involving the aldolase and TPI catalysed reactions. The relaxation rate was shown to depend on both the aldolase activity and the substrate concentration. Measurement of the same relaxation process in situ in the human erythrocyte by a chemical assay method, allowed the estimation of the effective aldolase activity in the intact cell.

The results implied that binding of aldolase to the erythrocyte membrane is not physiologically significant in situ, in contrast to predictions made from previous in vitro studies.

Chapter 4 describes the study of the compartmentation of some glycolytic intermediates between inactive and active forms. NMR was shown to be a powerful technique for making both thermodynamic and kinetic measurements on these systems. Glycolytic enzymes were shown, in some cases, to have a catalytic effect on the interconversion of the various forms of substrates present in solution.

An overall impression gained from this work is that the major limitation of NMR in biological studies is its
poor sensitivity. Species present at concentrations less than about 1 mM are difficult to detect in a typical kinetic study of metabolism in cell suspensions. Improvements in both magnet and spectrometer design are still being made, but it is to be expected that sensitivity will remain a major problem. This means that the biological NMR spectroscopist has to choose his area of study fairly carefully and the technique will never be a cure-all for biochemistry. Having said that, in the areas where NMR may be employed practicably, the technique can prove very powerful and informative.