STEREOCHEMICAL ASPECTS OF THE INTERACTION BETWEEN ISOMERASES AND THEIR SUBSTRATES

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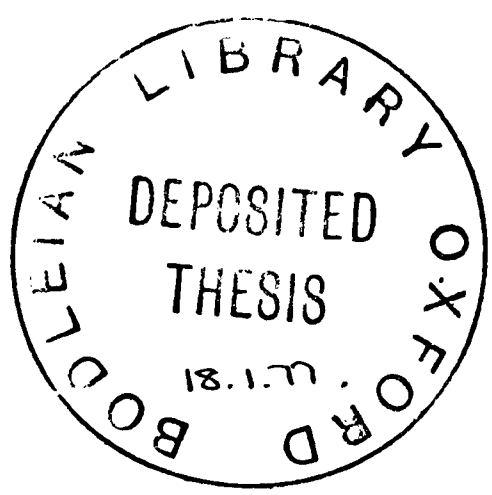
by

Martin R. Webb B.A.

University College
Oxford

The Dyson Perrins Laboratory
Oxford
and
The Department of Chemistry
Harvard

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ABBREVIATIONS

Most of the abbreviations that are used in this thesis conform to the conventions of the Biochemical Journal. In addition, the following have been used:

TIM	triosephosphate isomerase
DHAP	dihydroxyacetone phosphate
GAP	glyceraldehyde 3-phosphate
G6P	glucose 6-phosphate
F6P	fructose 6-phosphate
SDS	sodium dodecyl sulphate

CONTENTS

	Page
FOREWORD	1
PART I TRIOSEPHOSPHATE ISOMERASE	4
CHAPTER 1 INTRODUCTION	4
Irreversible active site inhibitors	5
Mechanism of the catalyzed reaction	8
Enzymically active forms of the substrates	11
Kinetic studies	14
pH studies	19
The energetics of the TIM-catalyzed reaction	21
CHAPTER 2 THE ORIENTATION AND ACCESSIBILITY OF SUBSTRATES ON THE ACTIVE SITE OF TRIOSEPHOSPHATE ISOMERASE	24
The use of sodium borohydride to reduce enzyme bound aldehydes and ketones	24
The aim of this work	27
Analysis of [^{3}H]glycerol 3-phosphate for the D[2-^{3}H] and L-[2-^{3}H] enantiomers	29
Analysis of [^{3}H]glycerol 3-phosphate for D-[1(R)-^{3}H] and D-[1-(S)-^{3}H] isomers	31
Reduction of dihydroxyacetone phosphate	34
Control experiments involving bromohydroxyacetone phosphate	41
Reduction of D-glyceraldehyde 3-phosphate	43
Ratio of reduction at C-1 and C-2	47
Consequences ofthe lack of reduction of enzyme-bound D-GAP	55

CHAPTER 3 TO TRAP AN ENEDIOL 58

Preparation and properties of diimide 58

Diimide with triose phosphates 60

CHAPTER 4 A NUCLEAR MAGNETIC RESONANCE STUDY OF THE BINDING OF TRIOSE PHOSPHATES TO TRIOSEPHOSPHATE ISOMERASE 62

Phosphorus versus proton n.m.r. 62

Ratio of hydrate to keto form of DHAP 64

Stability of triose phosphates in solution 66

Phosphorus n.m.r. of triose phosphate in the presence of triosephosphate isomerase 68

Binding of substrate at pH 5.5 75

CHAPTER 5 EXPERIMENTAL 78

Instruments 78

Enzymes 78

Substrates and coenzymes 79

Column materials 79

Other chemicals 79

Preparations 80

Methods A) Assays 85

B) Effect of sodium borohydride on the activity of triosephosphate isomerase 86

C) Preparation and analysis of tritiated glycerol 3-phosphate 87

D) Analysis of [^{3}H]glycerol 3-phosphate for stereochemistry of labelling at C-2 90

E) Analysis of [^{3}H]glycerol 3-phosphate for steroechemistry of labelling at C-2 92

F) Preparation of deuterated glycerol 3-phosphate and its mass spectral analysis 95

G) Diimide reduction of triose phosphates 96

H) Phosphorus n.m.r. spectra of triose phosphates in the presence of triosephosphate isomerase 97

PART II GLUCOSE-6-PHOSPHATE ISOMERASE 99

CHAPTER 6 INTRODUCTION 99

Thermodynamic and kinetic parameters 102

Mechanism of the hydrogen transfer 104

The forms of hexose 6-phosphates present in solution 109

The forms of the substrates active with glucose-6-phosphate isomerase - anomerase activity 113

CHAPTER 7 THE SUITABILITY OF DEOXY ANALOGUES OF HEXOSE PHOSPHATES TO STUDY THE ANOMERASE ACTIVITY OF GLUCOSE-6-PHOSPHATE ISOMERASE 121

Synthesis and purification of the deoxy analogues 122

Preliminary studies with 2-deoxyglucose 6-phosphate 127

Preliminary studies with 1-deoxyfructose 6-phosphate 130

N.m.r. studies with the deoxyhexose phosphates 132

CHAPTER 8 EXPERIMENTAL 136

Instruments 136

Materials 136

Thin layer chromatography 136

Preparations 137

Assays 142

Tritium exchange experiments with deoxyhexose 6-phosphates 143

REFERENCES 145

SUMMARY i

FOREWORD

Animals obtain much of their energy requirement from the breakdown of carbohydrates. In particular, mammals store carbohydrate as glycogen, which is catabolized for energy production via the phosphorylated intermediate of the glycolytic pathway, ending in pyruvate (see Fig. F.1). This is then used for further energy production in the tricarboxylic acid cyclé. In the breakdown of glycogen to pyruvate, there is net production of ATP: 1 mole (per mole of hexose) is consumed in the conversion of glucose 1-phosphate to fructose 1,6-bisphosphate, and 2 mole (per mole of triose phosphate) is produced between glyceraldehyde 3-phosphate and pyruvate. Thus there would be net formation of only one mole of ATP per mole of hexose, if after breakdown of fructose bisphosphate to two three-carbon units (by the reaction catalyzed by aldolase) only one of these units was used. However, triosephosphate isomerase rapidly interconverts the two three-carbon units and channels almost all the triose phosphate to pyruvate. The net formation of ATP is then close to 3 mole per mole of hexose. A small proportion of triose phosphate may go to form glycerol phosphate and so into fat synthesis.

The glycolytic pathway is the main method of energy production from carbohydrates in all cells from fermenting yeasts to the oxygen-deficient, skeletal muscle of mammals. In yeasts there is a wide variety of final products depending on which fermentation is occurring. In mammals the first-formed product is lactate, which builds up in the muscle until the supply of oxygen is restored. Because of their importance in the rapid formation of ATP, enzymes of the glycolytic pathway are widespread, and present in the cell in remarkably high concentration.

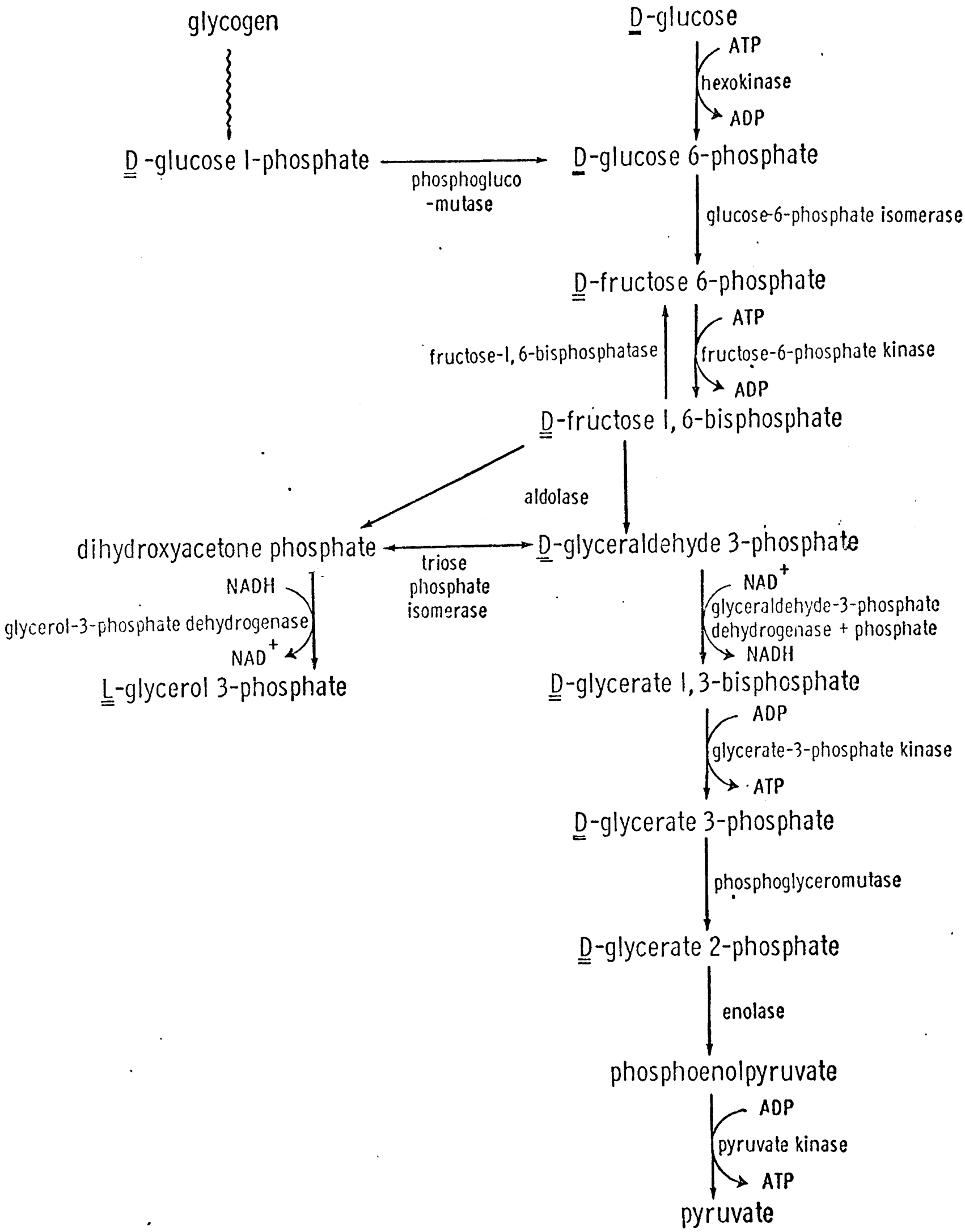


Fig. F.1. The glycolytic pathway.

This thesis is concerned with the two isomerases of the glycolytic pathway, shown in Fig. F.1. Triosephosphate isomerase (EC 5.3.1.1) is responsible for the rapid interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. Phosphoglucose isomerase (EC 5.3.1.9) catalyzes the interconversion of D-glucose 6-phosphate and D-fructose 6-phosphate. The interaction between these enzymes and their substrates has been investigated using both physical and chemical methods, to elucidate the nature of the interaction, and the exact form of the substrate that binds in the active site.

PART I TRIOSEPHOSPHATE ISOMERASE

CHAPTER 1 INTRODUCTION

The enzymes of the glycolytic pathway were first studied in detail by Meyerhof, Embden, Warburg and others in the 1930's and 1940's. The existence of triosephosphate isomerase was first demonstrated in 1934 by Meyerhof and Lehmann.[1] For a long time not much work was done on this enzyme, so that in his review, written in 1970, Noltmann[2] describes it as a "forgotten enzyme." Indeed it was only during the 1960's that extensive work was begun, so that during the last few years, this isomerase has become one of the best understood enzymes in intermediary metabolism.

Crystalline triosephosphate isomerase has been isolated from several sources, including chicken skeletal muscle,[3] calf skeletal muscle,[4] rabbit skeletal muscle,[5] bovine lens,[6] yeast,[5] horse and human liver,[7] pea seedling,[8] human erythrocytes,[9] coelacanth[10] and a thermophilic bacterium.[11] The enzyme forms a large proportion (up to 4% of the soluble protein) of muscle cytoplasm and the purified enzyme has a specific activity of the order of 10^4 unit/mg.* So the enzyme has a very high catalytic activity and the absolute number of units present in the muscle cells is very large.

The isomerase from several sources has been sequenced: chicken,[12] rabbit[13,14] and coelacanth.[15] The enzyme is a dimer of two identical subunits and the sequencing gives a subunit molecular weight of 26,600 (for isomerase from rabbit)[14] and 27,200 (for isomerase from chicken),[12] in

---

*A unit is defined as the enzyme activity that transforms 1 μmol of substrate per minute at 30° under optimum conditions and saturating substrate concentration.

good agreement with other estimations, shown in Table 1.1. Recently, the structure of chicken muscle isomerase has been determined crystallographically to 2.5 Å resolution.[22] The "x-ray structure" shows two identical subunits, each containing an active site (vide infra: irreversible active site inhibitors). The mechanistic importance of amino acid residues in the active site region will be discussed later. At present, the x-ray structure of the enzyme-substrate complex is being determined and this should provide important information on the possible interactions between substrate and enzyme in the active site.

Irreversible active site inhibitors

Irreversible, active-site-directed inhibitors have been used to label a unique residue of the active site and thence to obtain an "active site peptide." Halohydroxyacetone phosphate[23,24,25] and glycidol phosphate (1,3-epoxypropanol 3-phosphate)[26] both react irreversibly with the isomerase in an approximate ratio of 1 mole of inhibitor to 1 mole of subunit, completely destroying enzyme activity. These inhibitors are good substrate analogues and probably react with a residue in the active site. The enzyme inactivated by glycidol phosphate, contains a single modified glutamic acid residue.[26,27,28] The sequence around this residue was determined and is described below.

Bromohydroxyacetone phosphate rapidly inactivates the enzyme and analysis of the product showed that phosphate had been lost and that the modified residue was tyrosine. In order to stabilize the attachment of the phosphate to the inactivated enzyme, the keto group of the inhibitor was reduced by sodium borohydride, immediately after inactivation of the

Table 1.1. Molecular weight estimations for triosephosphate isomerase

Enzyme source	Method of estimation	M.W.	Reference
Bacillus stearothermophilus	S.D.S./polyacrylamide gels	54,000	11
Yeast	Equilibrium centrifugation in S.D.S.	59,000	16
Rabbit muscle	Equilibrium centrifugation in S.D.S.	50,000	16,18
Rabbit muscle	Sedimentation equilibrium	52,900	17
Rabbit muscle	S.D.S./polyacrylamide gels	53,000	17
Rabbit muscle	Gel filtration	48,500	18
Rabbit muscle	X-ray crystallography	52,400	19
Rabbit muscle	Amino-acid sequence	53,200	14
Chicken muscle	X-ray crystallography	49,000	20
Chicken muscle	S.D.S./polyacrylamide gels	53,000 52,000	21 18
Chicken muscle	Equilibrium centrifugation	48,400	18
Chicken muscle	Amino-acid sequence	54,400	12

S.D.S. = sodium dodecyl sulphate

enzyme.[29] In this case, the phosphate remained attached to the modified enzyme and the label now was attached to a glutamate residue.[29] The proposed explanation for these observations is shown in Fig. 1.1. The primary site of attachment of bromohydroxyacetone phosphate is glutamate, but if the keto group remains unreduced a neighbouring tyrosine can cause loss of the phosphate and migration of the label.

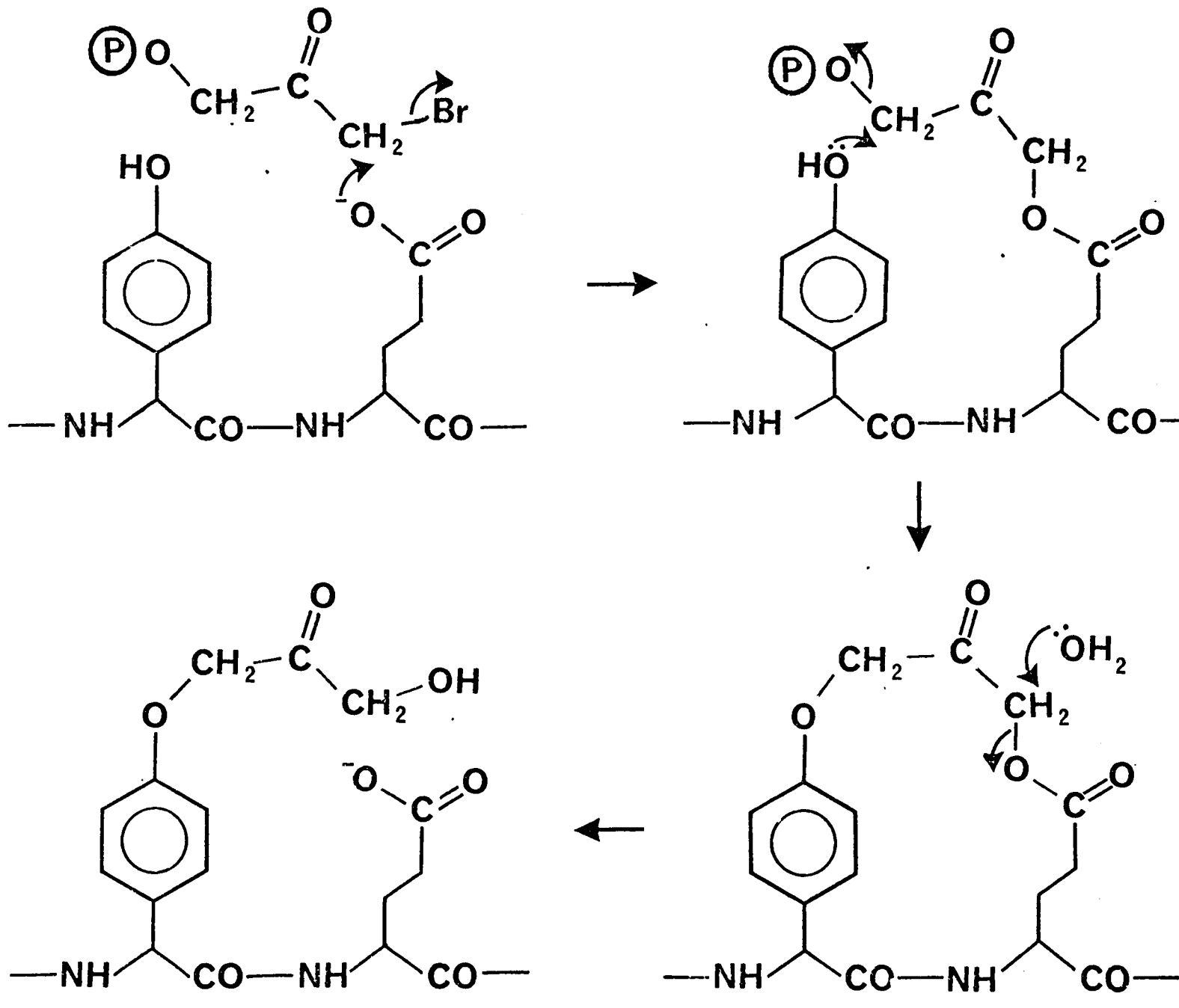


Fig. 1.1. Possible mechanism for the migration of the active-site label

Using chlorohydroxyacetone phosphate, Hartman[30] obtained an active site peptide from rabbit muscle isomerase with the following sequence: Trp-Val-Leu-Ala-Tyr-Glu*-Pro-Val-Trp-Ala-Ile-Gly-Thr-Gly-Lys. This sequence was obtained also from the chicken enzyme, except for a Val rather than a Trp at position 1.[31] Hexapeptides with the sequence from position 4 to 9 have been obtained by Waley et al.[27,28] using glycidol phosphate and by Coulson et al.[29] using bromohydroxyacetone phosphate.

This work has enabled the active site to be partially identified in the x-ray structure[22] and possible amino acids to be implicated in the enzyme mechanism.

Mechanism of the catalyzed reaction

Triosephosphate isomerase (TIM) catalyzes the interconversion of the two triose phosphates, dihydroxyacetone phosphate (DHAP) and D-glyceralde-hyde 3-phosphate (D-GAP), and the reaction involves the cleavage of one C-H bond in the substrate, and the creation of a new C-H bond to form the product. The equilibrium constant is 22 (between 20° and 37°) so that at equilibrium there is ∿96% DHAP and 4% D-GAP.

From early investigations of the reaction, Rose was able to propose a mechanism for the catalysis. If DHAP is incubated with the isomerase in tritiated water, a single tritium becomes incorporated into the substrate.[32] The labelled DHAP was isolated and periodate oxidation showed the tritium to be attached to C-1. Rose determined the absolute stereochemistry of the radioactive species to be [1(R)-^{3}H]DHAP,[33] so that the enzyme labilizes the pro-R-hydrogen of C-1 of DHAP.

The TIM-catalyzed reaction may be studied in either direction by removing the product with a coupling enzyme system. In the direction GAP → DHAP, the DHAP may be removed by an excess of glycerol-3-phosphate dehydrogenase/NADH to oxidize the product to L-glycerol-3-phosphate (see Fig. F.1). This is the basis of the usual assay for TIM activity.[4] In the reverse direction, DHAP → GAP, the product is removed by glyceraldehyde-3-phosphate dehydrogenase/NAD^+/arsenate to give 3-phosphoglyceric acid resulting from oxidation of C-1 of GAP.

Using the latter coupled enzyme system, [1(R)-^{3}H]DHAP (specifically tritiated by TIM) can be converted to phosphoglyceric acid in non-tritiated water. Between 3 and 6% (depending on the extent of the reaction) of the radioactivity is transferred to C-2 of GAP and thence to C-2 of phosphoglyceric acid. The rest of the tritium appears in the solvent.[34,66] This result rules out a hydride-shift mechanism, in which the hydrogen from C-1 of DHAP is transferred directly to C-2 to give GAP. Rose[34] proposed a mechanism shown in Fig. 1.2, with proton transfer and an intermediate

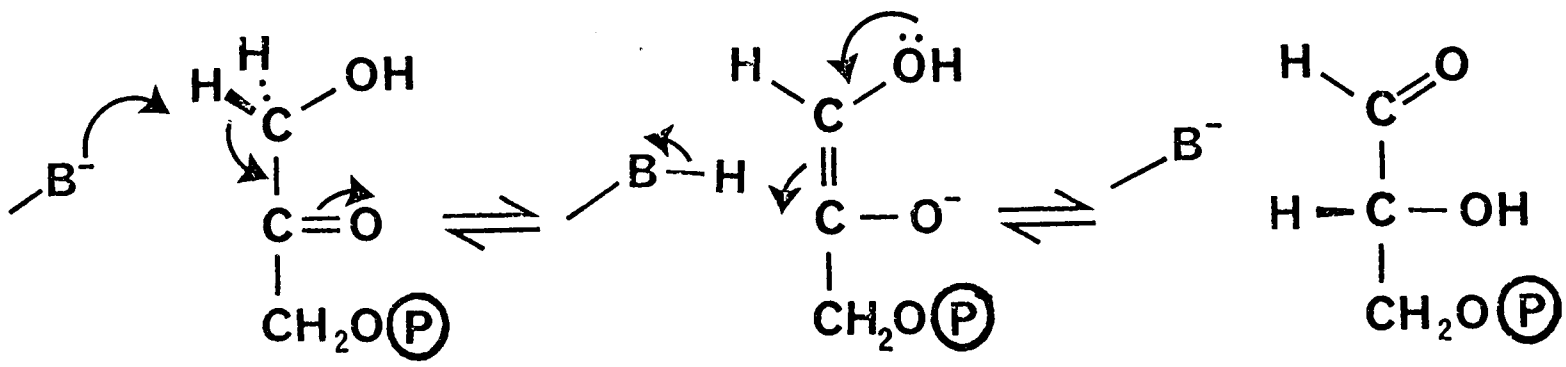
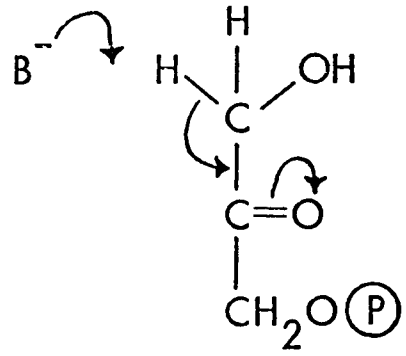


Fig. 1.2. Proposed mechanism of hydrogen transfer catalysed by triosephosphate isomerase[34]

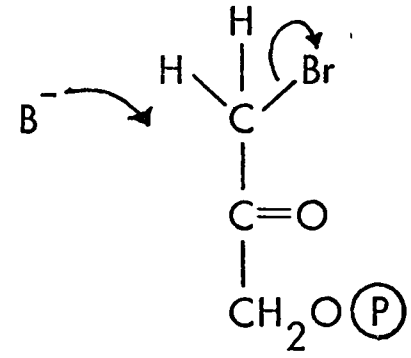
which can rapidly exchange the labilised proton with the solvent. The intermediate is a cis-enediol, which is consistent with a single base (B) on the enzyme, that abstracts the 1-pro-R-hydrogen of DHAP and replaces it on the same (re-re) face of the enediol to give D-GAP.

Because of the work with bromohydroxyacetone phosphate[29] and glycidol phosphate[27] (vide supra), it has been proposed that the glutamate residue that is labelled by these inhibitors, is the base responsible for proton abstraction and transfer. The positioning required of such a base would be similar in the active site and the two reactions proposed for the glutamate can be represented by:



residue (B)

as base



residue (B)

as nucleophile

Fig. 1.3.

It has been proposed by Rose[35,36,37] that the mechanism shown in Fig. 1.2 is a common feature of most aldose-ketose isomerases. Proton exchange and transfer experiments seem to be consistent, in the isomerases studied, with a single base on the enzyme transferring a proton between neighbouring carbon atoms and on the same face of a cis-enediol (either the re-re or the si-si face).

Enzymically active forms of the substrates

Before considering the energetics of the TIM-catalyzed reaction, the different forms of the substrate in solution must be discussed. Compounds of the form $RCH_2.CO.R'$ exist in aqueous solution as an equilibrium mixture of free keto form and the hydrate. In addition, the compound may be in equilibrium with its enol:

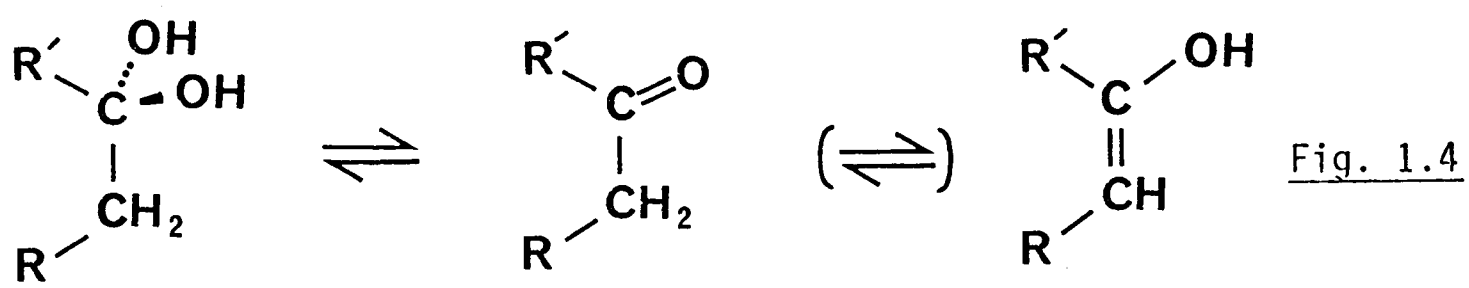
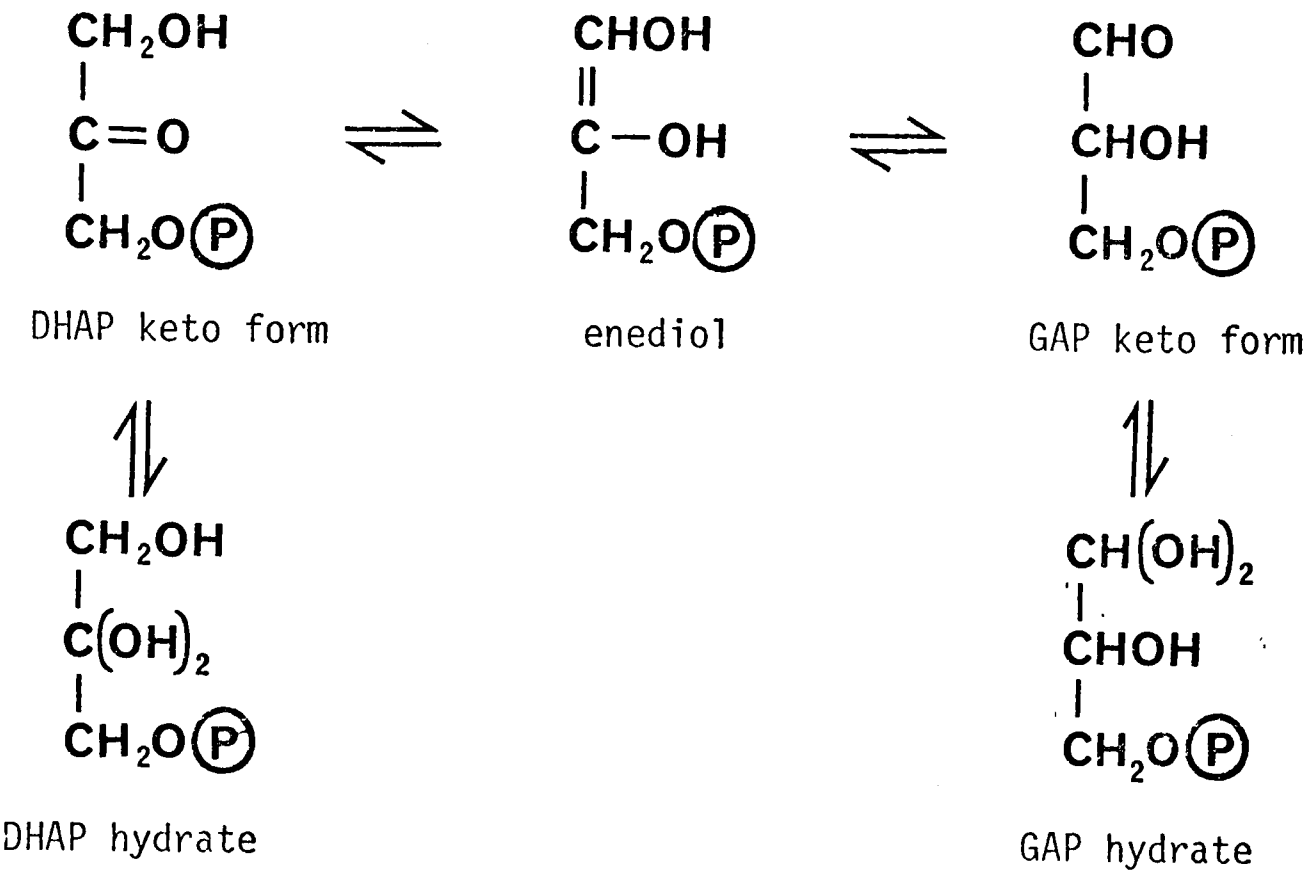


Fig. 1.4

The extent of hydration depends on the nature of R and R', although the hydration-dehydration reaction is generally fast. The extent of enolization also depends on the nature of R and R'. For ordinary aldehydes and ketones this extent is small (for example acetone has $\sim$0.002% enol[38]) and the rate of enol formation is slow.

For both DHAP and GAP the rate of enediol formation is very slow at neutral pH and 30° (the first order rate constant being $\sim 10^{-6} sec^{-1}$) and the measured proportion of enediol is very small (less than 0.1%).[39,40,41] It should be noted that GAP can enolize in one way, and DHAP in two ways. One enediol is common to both triose phosphates (see Fig. 1.5) and represents the most likely intermediate for the non-enzymic interconversion of GAP and DHAP[41] as well as for the TIM-catalyzed reaction. Reynolds et al.[39] have provided evidence against the existence of a DHAP dimer in neutral aqueous solution, and Fig. 1.5 shows the triose phosphate species that are important for the present discussion.

Fig. 1.5. The keto and hydrate forms of triose phosphates present in aqueous solution



The extent of hydration of GAP and DHAP has been determined both enzymically,[3,40] by infrared spectroscopy[42] and n.m.r.[3] The proton n.m.r. spectrum of GAP shows different peaks for the keto form and for the hydrate.[3] This indicated that ∿4% of GAP exists as the free keto form. In the kinetic determination, oxidation of D-GAP was carried out in a stopped-flow apparatus using a high concentration of glyceraldehyde-3-phosphate dehydrogenase and monitoring the change in absorbance at 340 nm due to NADH formation.[3] The reaction was biphasic, 3% of the substrate reacting rapidly, followed

by the slower oxidation of the remainder. The initial burst was taken to be the rapid oxidation of the carbonyl form present at equilibrium and the slow reaction was presumed to be rate-limited by the dehydration of GAP, which was given a first order rate constant of $\sim 10^{-1} sec^{-1}$. Using this system, Trentham et al.[3] also showed that the carbonyl form of GAP was the one released by TIM and by aldolase.

In similar work with DHAP, Reynolds et al.[39] found the ratio of hydrate to keto form at different temperatures. This is summarized in Table 1.2. N.m.r. studies by Gray[43,44] confirm these results. Reynolds et al.[39] showed that the keto form of DHAP is used by glycerol-3-phosphate dehydrogenase and is the form released by TIM and by aldolase. The first order rate constant for dehydration-hydration is $\sim 1\ sec^{-1}$.[39]

Reynolds et al.[39] have discussed the enzymological and metabolic consequences of the above results. It seems that all the glycolytic enzymes that handle the triose phosphates use the carbonyl forms, and there is therefore little problem about the slow rate of dehydration relative to the fast rates of the enzymic reactions. The carbonyl forms of the triose

Table 1.2. Equilibrium data for triose phosphates (taken from ref. 39)

	20°	37°
% unhydrated glyceraldehyde 3-phosphate	3.3	4.3
% unhydrated dihydroxyacetone phosphate	55	82
Overall equilibrium constant (DHAP/D-GAP)	22	22
Equilibrium constant for unhydrated triose phosphates	367	420

phosphates are released by aldolase and can be used directly by TIM and the two dehydrogenases, glycerol-3-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. During glycolysis *in vivo*, there will, of course, be an amount of each of the hydrates in equilibrium with the steady state concentration of carbonyl forms.

These data required that the quantitative features of the reaction catalyzed by TIM must be corrected to accommodate the fact that only a fraction of each substrate is enzymically active. For example, the equilibrium constant for the TIM substrates is 22 in the temperature range 20° to 37°. However, in terms of the active carbonyl forms, the equilibrium constant is 367 at 20° and 420 at 37° (in favour of DHAP). The hydrate forms which are automatically present, would also affect kinetic parameters and energetics, if they can act as competitive inhibitors. These hydrates can be considered as analogues of the true substrates that can bind to the enzyme active site and inhibit enzymic activity. One of the aims of this thesis is to determine the extent of this inhibition by the hydrated substrate species.

Kinetic studies

K_m and k_{cat} for each substrate are shown in Table 1.3. It will be evident that the correction due to presence of hydrate has a large affect on the K_m of GAP, lowering it to the μM level, suggesting that D-GAP binds very tightly to the enzyme. However, in this case, when the rate of binding and the rate of reaction are very similar, K_m is not necessarily a measure of binding.

Table 1.3. Kinetic constants for TIM from rabbit and chicken

Source	Substrate	Reported K_m (mM)	Approximate Recalculated K_m (mM)[a]	k_{cat} (min^{-1})	Conditions	Reference
Rabbit	D-GAP	0.46	0.015	3.35×10^5	25°, pH 7.8	45
Rabbit	D-GAP	0.32	0.011			5
Rabbit	DHAP	0.87	0.485	2.95×10^5	25°, pH 7.8	45
Rabbit	DHAP	0.62	0.34			5
Chicken	D-GAP	0.47	0.019	2.56×10^5	30°, pH 7.42	21
Chicken	DHAP	0.97	0.68	2.59×10^5	30°, pH 7.27	21

[a]The values are corrected for the estimated percentage of keto form of substrate.

Table 1.4 summarizes some of the competitive inhibitors of TIM. First, it may be noted that phosphate, sulphate and arsenate all are competitive inhibitors. Each of the these may be present during the TIM reaction: phosphate from substrate hydrolysis; sulphate from incompletely dialyzed enzyme, that had been stored as a suspension in ammonium sulphate solution; and arsenate, if the isomerase reaction is coupled to glyceraldehyde-3-phosphate dehydrogenase/NAD^+/arsenate.

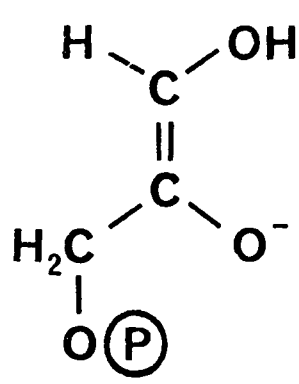
Secondly, there are very few 'good' inhibitors (i.e. with K_i less than mM). D-Glycerol 3-phosphate, a reasonably good structural analogue of triose phosphate does have a low K_i, but monohydroxyacetone phosphate and L-glycerol 3-phosphate, also reasonably close analogues of the substrates, are poor inhibitors. 2-Phosphoglycollate and 2-phosphoglycolohydroxamate are the most effective inhibitors, each with a K_i of the same order as the true K_m of D-GAP. Wolfenden[53] (following Pauling)[54,55,56] has proposed that the transition state should be the most tightly bound configuration of the ligand (substrate-product species), in order to maximize the rate enhancement of the enzyme-catalyzed reaction over the non-enzymic reaction. A transition state analogue, therefore, should be a better inhibitor than an inhibitor that merely mimics the substrate or product. It can be argued that 2-phosphoglycollate and 2-phosphoglycolohydroxamate fall into this category, and their structures are shown in Fig. 1.6 alongside the enediol (which is in fact a reaction intermediate not a transition state). Along with the tight binding, these inhibitors protect the isomerase against inactivation by bromohydroxyacetone phosphate, by glycidol phosphate, and by heat.

Table 1.4. Some competitive inhibitors of TIM

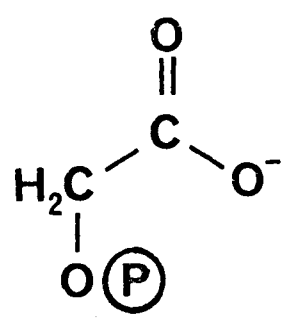
Inhibitor	K_i (mM) (chicken TIM)	K_i (mM) (rabbit TIM)	Reference
Phosphate ion	5.5 (25°, pH 7.7)	4.8 (25°, pH 7.7) 6.0 (25°, pH 7.8)	46 45
Arsenate ion		5.5 (25°, pH 7.8)	45
Sulphate ion	5.0 (25°, pH 7.7)	5.7 (25°, pH 7.7)	47
β-hydroxyethyl phosphate	1.4 (25°, pH 7.7)	2.0 (25°, pH 7.7)	47
Allyl phosphate	1.0 (25°, pH 7.7)	1.3 (25°, pH 7.7)	47
D-ribose 1-phosphate	3.1 (25°, pH 7.7)	4.8 (25°, pH 7.7)	47
DL-glycerol 3-phosphate	 0.84 (30°, pH 7.47) 0.11 (25°, pH 7.7)	0.23 (25°, pH 7.7) 0.23 (25°, pH 7.7)	45 48 47
D-glycerol 3-phosphate	0.46 (30°, pH 7.47)		48
L-glycerol 3-phosphate	4.0 (30°, pH 7.47)		48
monohydroxyacetone phosphate	6.3 (30°, pH 7.51)		48
glyceraldehyde	"∞"[a]		49
dihydroxyacetone	"∞"[a]		49
dihydroxyacetone sulphate	"∞"[a]		50
glycollate	10 (25°, pH 7.7)	5.4 (25°, pH 7.7)	47
2-phosphoglycollate	0.0068 (25°, pH 7.7)	0.006 (25°, pH 7.7)	47
2-phosphoglycolohydroxamate		0.014 (30°, pH 7.5) 0.004 (25°, pH 7.5)	51 52

[a]"∞" indicates that there was no observable inhibition.

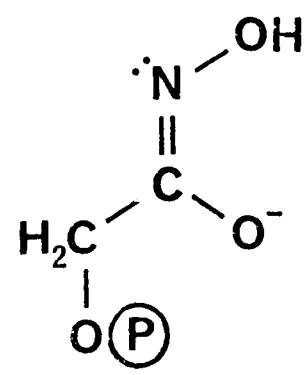
Fig. 1.6. Analogues of the triose phosphate enediol



enediolate



2-phospho-glycollate



2-phospho-glycolohydroxamate

It is noteworthy that glyceraldehyde, dihydroxyacetone and dihydroxyacetone sulphate are not inhibitors of TIM. Indeed, it seems that the only effective inhibitors are those that carry a dianionic group at neutral pH. A number of the phosphate esters listed in Table 1.4 have K_i-values very similar to phosphate ion itself. So, although these phosphate esters were designed to be substrate analogues, it may be that the carbon skeleton is relatively unimportant in the binding of such molecules, and that the binding free energy is dominated by binding of the phosphoryl group. It must therefore be questioned whether these phosphate ester inhibitors bind fully in the active site or whether only the phosphoryl group binds, with the rest of the molecule not taking a particular orientation in the active site. It might be expected that, even if not good inhibitors, the substrate hydrates might bind in this manner and so have K_i-values of the same order as that of phosphate ion.

Although monohydroxyactone phosphate cannot be a substrate for TIM, it has been shown that the enzyme catalyzes the exchange of hydrogen from C-1 with the solvent,[21] albeit at a rate some 10^4 times more slowly than the rate of proton exchange observed with DHAP. Presumably, the exchange with this deoxy-analogue is catalyzed by enolization, just as with DHAP. This is represented by the left hand side of Fig. 1.2.

Three compounds of the form $CHO.CHOH.CH_2X$ have been shown to be substrates for TIM[57] at rates less than that of the true substrate, GAP. These analogues are the phosphonate ($-X = -CH_2PO_3^=$) and the two phosphorothioates ($-X = -SPO_3^=$ and $X = OPO_2S^=$). All three of these species are doubly charged in the phosphoryl position.

pH studies

As more has become known about the structure of TIM and its detailed reaction mechanism, interest has turned to pH studies to clarify certain aspects of the enzymic reaction. Understanding of the protonation state of each residue in the active site will be necessary to understand exactly how the substrates bind in the active site and how the proton transfer is made extremely facile.

The isomerase from chicken muscle has maximal activity (measured by $\frac{k_{cat}}{K_m}$) between pH 7 and 8.[58] The separate titration of K_m shows an apparent pK_a of 5.9. k_{cat} titrates giving an apparent pK_a of 9.2.[58] These pK_a-values are presumably related to protonations in the enzyme-ligand complex. At this stage it is not possible to assign the observed apparent pK_a-values to a particular enzyme residue or to the ligand (at any configuration between substrate and product).

The pH-dependence of binding to TIM by phosphoglycollate has been studied,[59,60] and the change in K_i is consistent with a pK_a of 7.35. Once again, it is not possible to do more than suggest that a protonation occurs with this pK_a in the inhibitor-enzyme complex. Similar problems arise with interpretation of the pH dependence of glycerol 3-phosphate inhibition[49] and the pH dependence of inactivation of TIM by glycidol phosphate. In the latter case, Waley[61] found that the rate of inactivation showed an apparent pK_a at 6.0, with limiting concentrations of the inhibitor; whereas when large concentrations of the inhibitor were present, there was little variation of rate with pH in the range 5.5 to 10.[62]

To overcome the problems due to phosphoryl group ionization in the pH region being studied, Hartman et al.[60] studied the pH-dependence of inhibition of TIM by sulphate ion and inactivation by chlorohydroxyacetone sulphate, which are both monoanions with pK_a-values of less than 2. The K_i for sulphate was constant over the range pH 5.5 to 8.0. Chlorohydroxyacetone sulphate irreversibly inactivates TIM very slowly, in the same manner as bromohydroxyacetone phosphate,[29] forming an ester with the active site glutamate γ-carboxyl group. The rate of inactivation by chlorohydroxyacetone sulphate indicated a pK_a at 3.9 with yeast TIM. It was concluded that this pK_a was due to ionization of the glutamate carboxyl group, since this inhibitor binds very weakly to the isomerase.

A high resolution proton magnetic resonance study of TIM has been carried out.[63] The resonances due to the four histidines of rabbit muscle TIM have been tentatively assigned and the pH dependence of these resonances shows that only one titrates, with an observed pK_a of 5.6. This resonance was shifted slightly by the binding of the competitive inhibitor, glycerol

3-phosphate and was assigned as His_{100}, a residue near to the active site, according to the x-ray structure.[22] As yet no role has been given to this histidine in the reaction mechanism.

The energetics of the TIM-catalyzed reaction

The free energy profile has been determined for the TIM-catalyzed reaction.[64-71] That this has been possible is due to a number of features of the reaction. Firstly, the equilibrium constant is near enough to unity so that the reaction may be studied in either direction. There are straightforward enzymic-coupling reactions with the two dehydrogenases, glyceraldehyde-3-phosphate dehydrogenase and glycerol-3-phosphate dehydrogenase (see Fig. F.1), so that the reaction may be run irreversibly in either direction and a stable product isolated. Each half of the reaction (proton abstraction and transfer) may be separately studied: the proton exchange at the enediol state of the reaction is almost (but not quite) complete (Fig. 1.7). Finally, the form of the profile itself has made accessible most of the rate constants of individual steps.

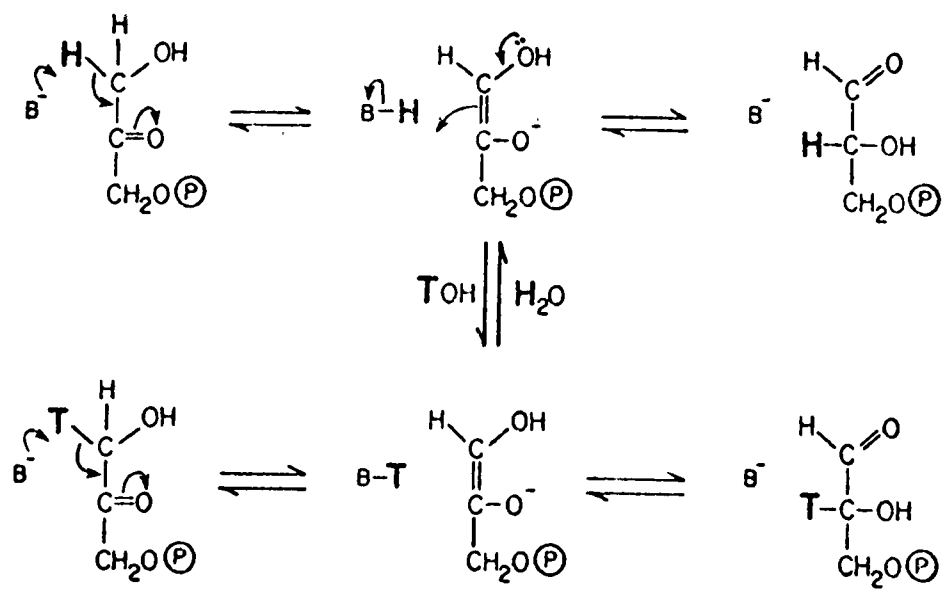


Fig. 1.7. Exchange of tritium label into substrate, catalyzed by triosephosphate isomerase

One class of experiments involved the use of substrates labelled specifically (and stereospecifically) in the appropriate position with deuterium or tritium: the fate of the isotopic label was determined as the reaction was performed in unlabelled water. Alternatively, the reaction of unlabelled substrate was studied in tritiated water and the appearance of tritium in the product and in the remaining substrate was followed. Another class of experiments involved the measurement of the rates of the reaction of specifically deuterium-labelled substrates.

From all these experiments the free energy profile shown in Fig. 1.8 was determined:[64-71] the standard for this profile was chosen as 40 μM, which corresponds to the triose phosphate concentration *in vivo*.[72]

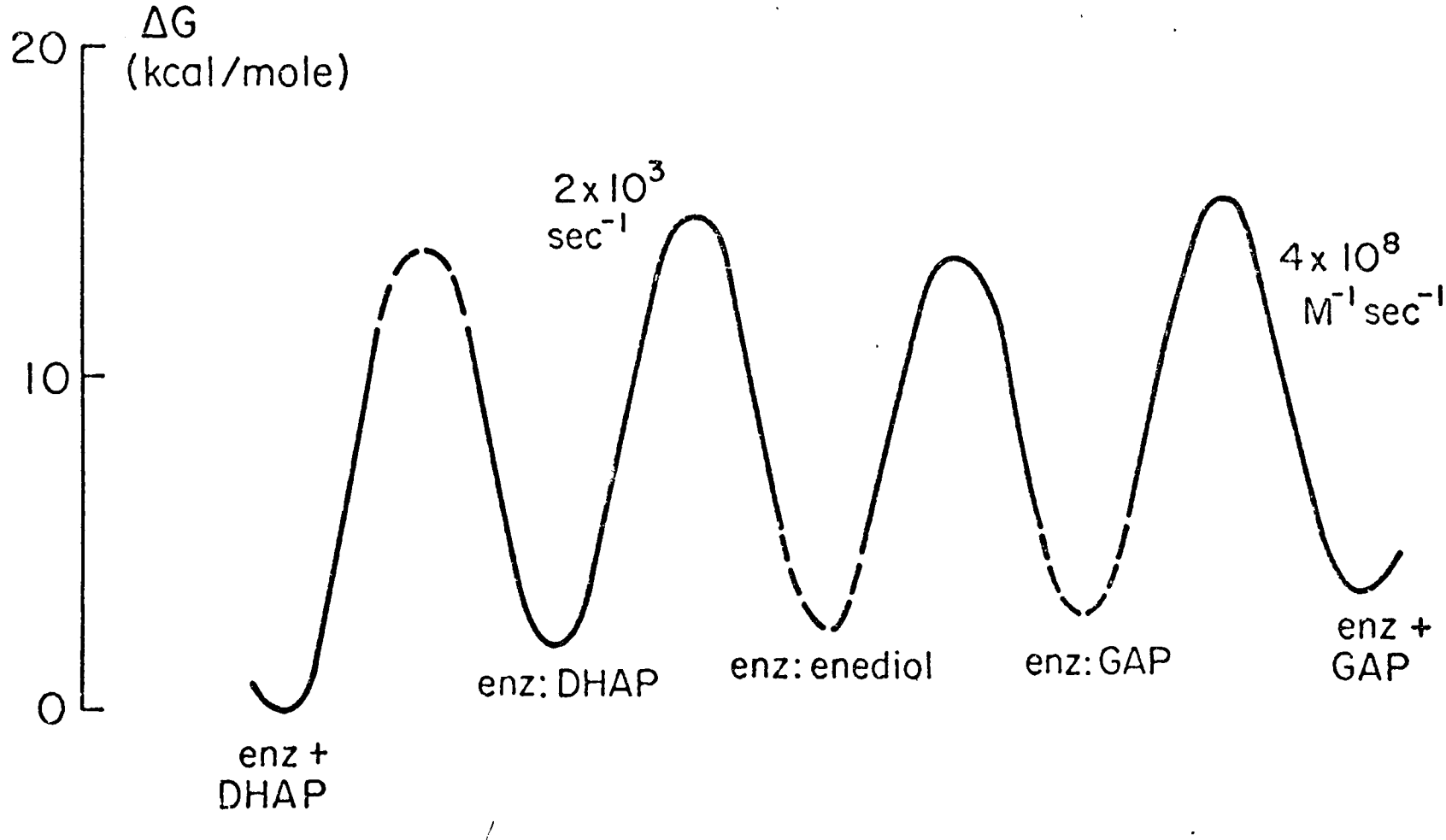


Fig. 1.8. Free energy profile of the reaction catalysed by triosephosphate isomerase. The standard state is 40μM. The dashed line represents limits imposed on the profile.

Albery and Knowles[73] have argued that the free energy profile shows that TIM has reached evolutionary perfection, in terms of the rate of the enzymic reaction. The free energy profile for the non-enzymic reaction has also been determined,[41] and compared with that for the enzyme-catalyzed process.[73] In the latter case, the overall rate of reaction is limited by the diffusion together of enzyme and D-GAP (or, in the reverse direction, by the rate of loss of D-GAP from the enzyme). The fact that the covalency changes that constitute the catalysis are faster than the 'on-off' steps of the one of the substrates, means that the reaction flux in either direction cannot be further improved by refinement of the catalyst.

CHAPTER 2 THE ORIENTATION AND ACCESSIBILITY OF SUBSTRATES ON THE ACTIVE SITE OF TRIOSEPHOSPHATE ISOMERASE

In Chapter 1, the main lines of study that have been carried out on TIM have been discussed. Thus, the three-dimensional structure of the enzyme has been determined[22] and the active site region of the structure has been identified. The reaction mechanism proposed by Rose[34] is generally accepted as the pathway by which hydrogen is transferred between carbon centres of the substrate, and a specific glutamate has been proposed as the base on the enzyme that mediates this transfer. The rate constants for most of the elementary steps of the reaction have been determined[64-71] and the enzyme has been shown to increase the rate of enolization of DHAP 10^9-fold over the non-enzyme-catalyzed rate.[41] However, all this information still leaves unanswered the question of how the enzyme produces the large rate enhancement. Before this question can be answered it is necessary to determine how the substrate interacts with the enzyme, and which enzyme residues are involved in catalysis. One way of approaching these questions is to discover the orientation of the substrate in the active site. Experiments designed to do this are described in the first part of this thesis.

The use of sodium borohydride to reduce enzyme bound aldehydes and ketones

Sodium borohydride is well suited for use with enzyme systems. This reagent is reasonably stable in alkaline aqueous solutions and reduces ketones, aldehydes and imines fairly quickly at neutral pH: these groups are reduced much faster than esters and amides.[74] The stereoselectivity of reduction of carbonyl groups by borohydride has been widely studied and

the topic has been reviewed by Morrison and Mosher.[75] Much of this work was carried out on alkyl substituted cyclohexanones.[76-79] The extent of reduction occurring from each face of the carbonyl group has been explained in terms of "steric approach control" (kinetically favoured) and "product development control" (thermodynamically favoured),[76,80] while Eliel[79] has explained the observed product ratios in terms of consideration of the transition state. However, these explanations have not proved general nor have they allowed a satisfactory level of prediction, although it is clear that if one face of the carbonyl group is blocked then reduction will occur mainly from the opposite (unhindered) face. Reduction of a series of ketones using tritiated sodium borohydride showed that, although the ketone reactivity varied by 10^4-fold, the kinetic isotope effect was relatively constant (between 3.2 and 4.8).[81] This is indicative that the hydride transfer is at least partially rate-limiting for all the substrates used.

Sodium borohydride has been used successfully in several enzyme systems, principally to trap Schiff bases. The first work of this type involved the reduction of the imine formed between pyridoxal phosphate and a lysine residue of phosphorylase a.[82] Other Schiff bases between a substrate carbonyl group and an amine on the enzyme have also been reduced by sodium borohydride.[83-87] Indeed, borohydride reduction has become a "classical" test of Schiff base intermediates in enzyme reactions. A labelled substrate becomes irreversibly attached to the enzyme in the presence of sodium borohydride and all enzyme activity is lost.

Characterization of the acyl-phosphate linkage in phosphoryl-enzymes[89] has also been acheived using borohydride reduction. Enzyme acyl phosphates are reduced by sodium borohydride to give an aminohydroxy-acid residue in the enzyme.

Sodium borohydride has also been used to reduce the free carbonyl group of substrates when bound in enzyme active sites, to determine which face of the carbonyl group is accessible when the substrate is bound. Kosicki and Westheimer[90] first used this reaction to study the interaction between pyruvate and oxaloacetate decarboxylase, which catalyzes the decarboxylation of oxaloacetate to give pyruvate. It was found that in the presence of the enzyme, reduction of pyruvate gave an excess of D-lactate over L-lactate. The ratio of the two enantiomers suggested that the enzyme catalyzes the reduction and that reduction of enzyme-bound pyruvate gives predominantly the D-enantiomer. Reduction of pyruvate in free solution gives, of course, a racemic product. A similar experiment was performed with pyruvate and pyruvate kinase and showed an excess of D-lactate over the L-enantiomer.[91] Similar conclusions were reached. Recently, however, Creighton and Rose[92] have published evidence that oxaloacetate decarboxylase and pyruvate kinase are the same enzyme, and that the two catalytic activities occur in overlapping active sites. When decarboxylation of oxaloacetate was carried out by the enzyme in the presence of sodium borohydride, the product pyruvate was trapped by reduction to lactate.[93] This lactate was found to be predominantly the L-enantiomer. This apparently contradicts the earlier work with this enzyme (in the guise of oxaloacetate decarboxylase and of pyruvate kinase), where, on its own, pyruvate in the presence of the enzyme was reduced to give an excess of D-lactate. The earlier results were explained by the large active site of the enzyme, which can bind oxaloacetate or phosphoenolpyruvate/ATP. The orientation of pyruvate as it is released by the enzyme need not be the same orientation as when it (alone) binds

The aim of this work

The first part of this thesis deals with the orientation and accessibility of the substrates in the active site of triosephosphate isomerase (TIM). The method chosen for this investigation was sodium borohydride to reduce the carbonyl group of each substrate. It is important for the present work, that sodium borohydride does not affect the catalytic activity of the isomerase either in the absence or presence of substrate. This was found to be true, and the results are presented in Table 5.2.

Sodium borohydride will not attack the face of a carbonyl group that is hindered sterically, and as the enzyme is a large, chiral molecule, it seemed likely that the reduction of substrate in the active site of TIM would occur preferentially on one face. Thus, analysis of the product of substrate reduction was expected to show which face of the enzyme-bound substrate is accessible to the solvent.

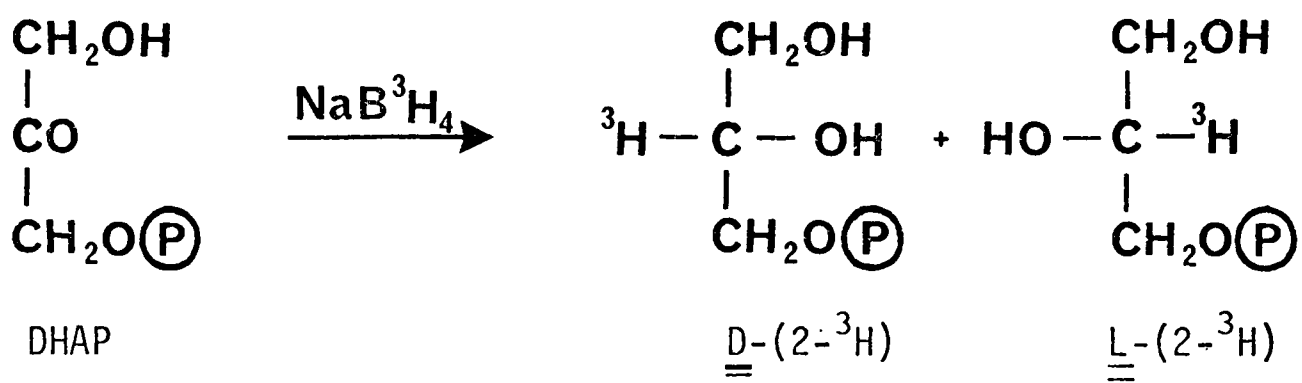
In the presence of triosephosphate isomerase, the following triose phosphate species are in equilibrium in the solution:

$$\underset{\text{hydrate}}{\text{DHAP}} \leftrightarrow \underset{\text{DHAP}}{\text{unhydrated}} \leftrightarrow \text{E.DHAP} \leftrightarrow \text{E.enediol} \leftrightarrow \text{E.}\underline{\underline{\text{D}}}\text{-GAP} \leftrightarrow \underset{\underline{\underline{\text{D}}}\text{-GAP}}{\text{unhydrated}} \leftrightarrow \underset{\text{hydrate}}{\underline{\underline{\text{D}}}\text{-GAP}}$$

(Fig. 2.1)

The species in free solution at 20° are present in the following proportions: keto form of DHAP and its hydrate, 55:45; keto form of $\underline{\underline{\text{D}}}$-GAP and its hydrate, 3.3:96.7; and the equilibrium constant between the keto forms of DHAP and of $\underline{\underline{\text{D}}}$-GAP is 367 in favour of the former.[39] The species that are susceptible to reduction by sodium borohydride are both the free and enzyme-bound forms

of DHAP and of D-GAP. The product of the reduction in each case is glycerol 3-phosphate. In order to distinguish between the reduction on different faces of each species it was necessary to use boro[^{3}H]hydride: the expected reduction products are four isomers of [^{3}H]glycerol 3-phosphate, as shown in Fig. 2.2.



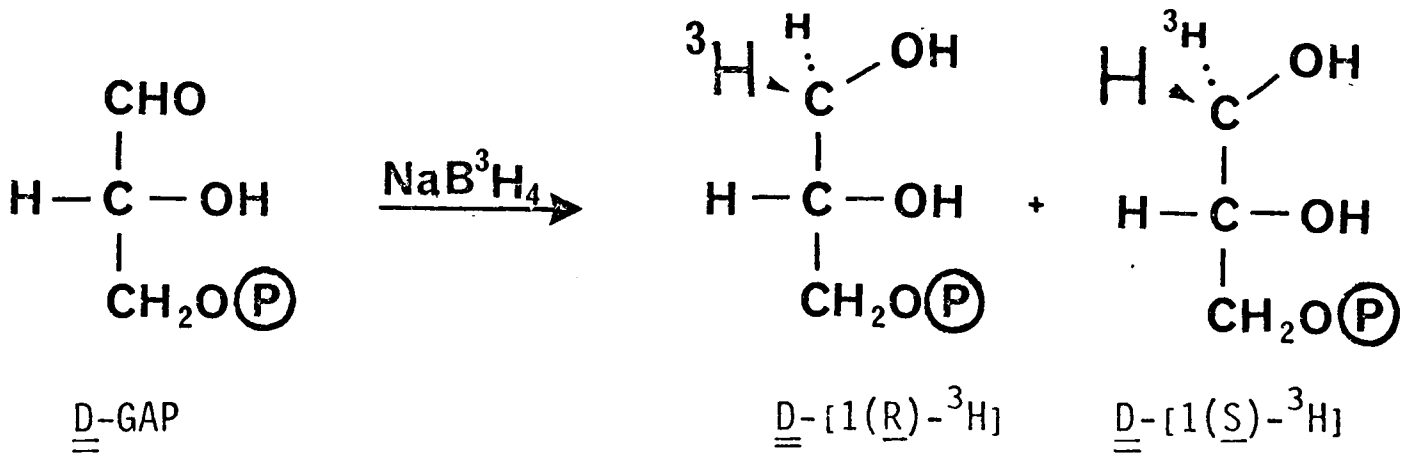


Fig. 2.2. Tritium isomers of glycerol 3-phosphate produced from reduction of triose phosphates by sodium boro[^{3}H]hydride.

In the presence of only a very small concentration of the isomerase, the derived reduction products are due entirely to the reduction of the species

in free solution. As the concentration of the enzyme increases, a significant proportion of the substrates become enzyme-bound and the ratio of products should reflect this proportion. While substrate in free solution is still reduced, the enzyme-bound substrate should also be reduced but most likely stereoselectively. It was therefore necessary to determine the proportion of each tritium isomer in the mixture of [^{3}H]glycerol 3-phosphates obtained from such a reduction. This analysis is described below.

Analysis of [^{3}H]glycerol 3-phosphate for the D-[2-^{3}H] and L-[2-^{3}H] enantiomers

The analysis of the glycerol 3-phosphate with tritium at the 2-position makes use of the oxidation of glycerol 3-phosphate by NAD^+ in the presence of glycerol-3-phosphate dehydrogenase. Only the L-enantiomer is a substrate and the hydrogen at C-2 is transferred to NAD^+, giving NADH. The NADH is separated by ion-exchange chromatography from the remaining glycerol 3-phosphate, which is solely the D-enantiomer (if the reaction has been taken to completion). The specific radioactivity of the NAD^{3}H is the same as the specific radioactivity of the L-glycerol 3-phosphate from which the hydrogen atom derived.

After ensuring that no trace of L-enantiomer remained, the D-glycerol 3-phosphate was treated with acid phosphatase and the resulting glycerol was rephosphorylated using glycerol kinase/Mg^{++}/ATP. These reactions converted the D-enantiomer to the L-enantiomer: in effect, the phosphate group is transferred from C-3 to C-1. This is illustrated in Fig. 2.3. Now the tritium of what was originally D-[2-^{3}H]glycerol 3-phosphate can be transferred to NAD^+ with glycerol-3-phosphate dehydrogenase. The NAD^{3}H

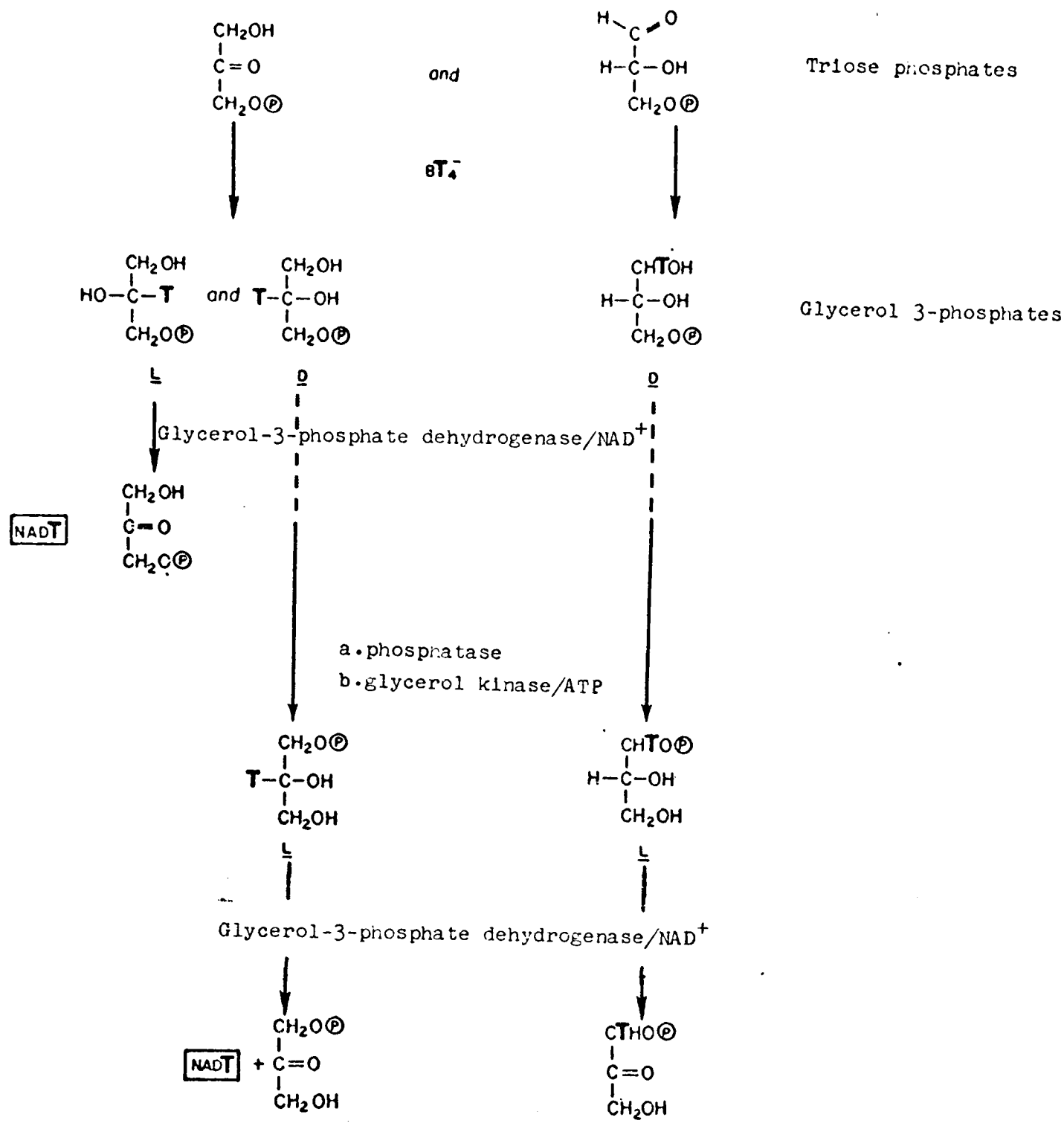


Fig.2.3. Reaction scheme for analysis of tritium at the 2-position of glycerol 3-phosphate

is isolated as before. As shown in Fig. 2.3, the tritium from the C-1 of glycerol 3-phosphate ends up in DHAP and does not affect the analysis for tritium at C-2.

For this analysis, a large molar excess of DL-glycerol 3-phosphate was added to the tritiated material, so that there were equimolar amounts of the D and L enantiomers. Because of this, the ratio of specific radioactivities of the two batches of isolated NAD^{3}H gives the ratio of tritium at C-2 of the L and D-glycerol 3-phosphate.

Analysis of [^{3}H]glycerol 3-phosphate for D-[1(R)-^{3}H] and D-[1(S)-^{3}H]-isomers

The reactions involved in the analysis of tritium at C-1 of glycerol 3-phosphate are outlined in Fig. 2.4. The first step is the transfer of the phosphoryl group from C-3 to the tritiated carbon atom, as described above. This not only produces the L-enantiomer of glycerol 3-phosphate, the form handled by the dehydrogenase, but also protects against the adventitious exchange of these tritium atoms into the solvent.

The two reactions involved in the conversion of L-glycerol 3-phosphate to fructose 1,6-bisphosphate are those catalyzed by glycerol-3-phosphate dehydrogenase and by aldolase. These reactions were coupled and carried out at high pH in order to overcome the unfavourable equilibrium constant of the former reaction. To increase the rate of formation of DHAP, the NADH that was also formed was rapidly reoxidized to NAD^+ by the presence of pyruvate/lactate dehydrogenase. A high concentration of D-glyceraldehyde 3-phosphate was also present to provide carbon atoms 4, 5, and 6 of D-fructose 1,6-bisphosphate (see Fig. 2.4). However, because D-glyceraldehyde 3-phosphate in large concentrations inhibits aldolase

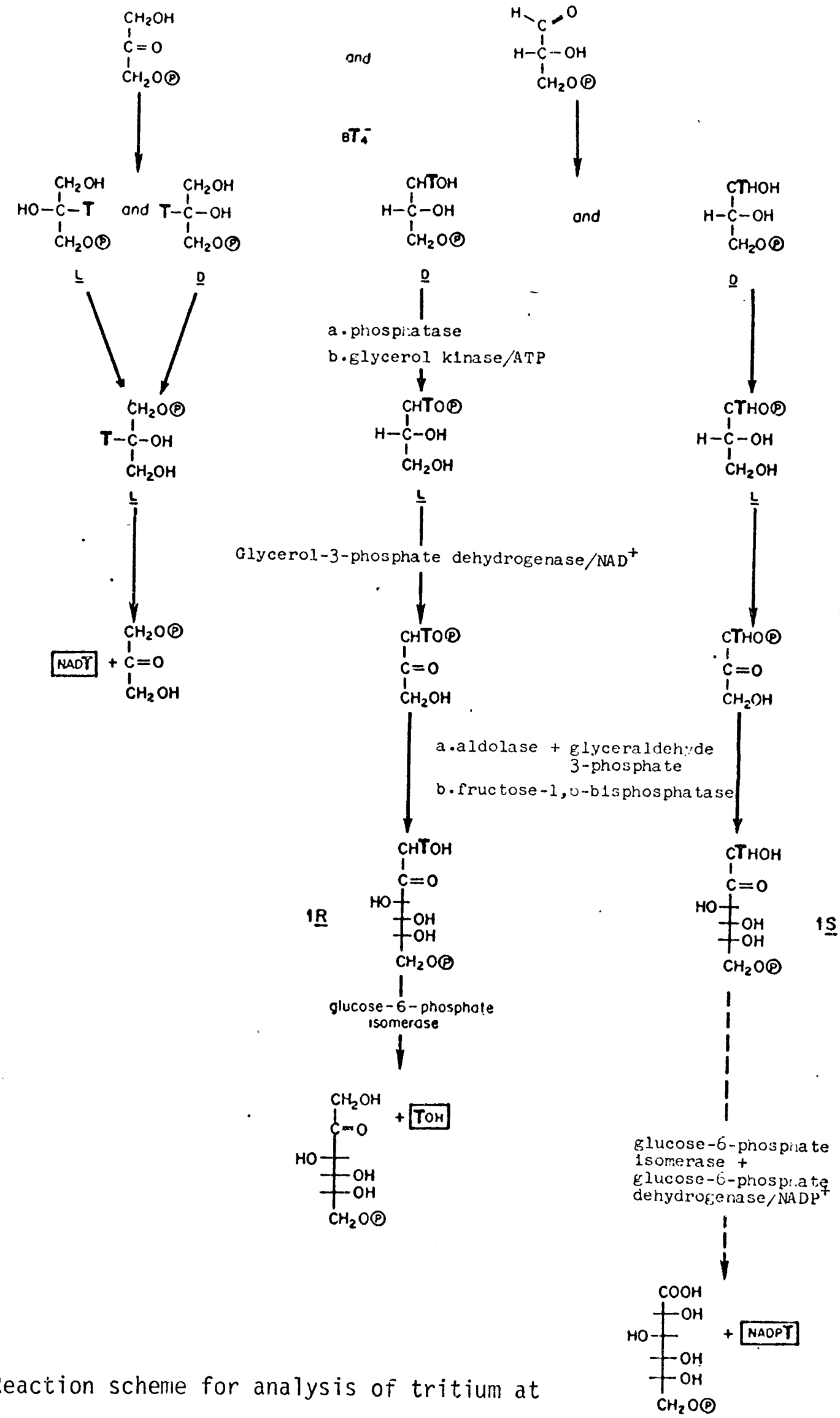


Fig. 2.4. Reaction scheme for analysis of tritium at the 1-position of D-glycerol 3-phosphate

irreversibly,[94] a large concentration of this enzyme was also required. During this two-reaction step, it was crucial that no TIM be present as this would rapidly form DHAP from D-GAP so reversing the glycerol 3-phosphate oxidation and preventing any tritiated material reaching D-fructose 1,6-bisphosphate. All enzymes in this step were therefore treated with bromohydroxyacetone phosphate to inactivate any isomerase present.[29]

It can be seen from Fig. 2.4 that all tritium at C-2 of glycerol 3-phosphate is transferred to NAD^+ during the two-reaction step and does not interfere with this analysis of the tritium at C-1. Treatment of D-fructose 1,6-bisphosphate with fructose 1,6-bisphosphatase specifically removes the phosphate from C-1 to give D-fructose 6-phosphate. The tritium originally derived from C-1 of D-glycerol 3-phosphate is now at C-1 of D-fructose 6-phosphate. Treatment of the D-fructose 6-phosphate with glucose-6-phosphate isomerase labilizes the 1-pro-R-hydrogen[95] and all the 1-R-tritium is exchanged into the solvent. After the treatment, distillation of the solvent from the frozen solution under vacuum ("vacuum sublimation") allows the measurement of both the radioactivity exchanged into the solvent and the radioactivity remaining associated with the residue (a mixture of hexose 6-phosphates). Addition of carrier D-fructose 6-phosphate to the residue and purification by ion-exchange chromatography allows the specific radioactivity of the hexose 6-phosphate to be calculated. The tritium from the [1(S)-^{3}H]glycerol 3-phosphate is now present in the aldehyde hydrogen of D-glucose 6-phosphate and in the 1-pro-S-hydrogen of D-fructose 6-phosphate. These hydrogens are transferred to $NADP^+$ during the reaction catalyzed by glucose-6-phosphate dehydrogenase

in the presence of glucose-6-phosphate isomerase. The resulting NADP^{3}H was isolated by ion-exchange chromatography and the specific radioactivity of the purified material was determined.

The validity of this analytical scheme, shown in Fig. 2.4 depends in part on the purity of the enzymes used. Thus, while glucose-6-phosphate isomerase labilizes the 1-pro-R-hydrogen of D-fructose 6-phosphate, any contaminating mannose-6-phosphate isomerase would labilize the 1-pro-S-hydrogen.[96] Accordingly fructose 1,6-bisphosphatase, glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase were all tested for glucose-6-phosphate isomerase activity and mannose-6-phosphate isomerase activity. No significant contaminating activity (less than 0.05%) was found. Had a mannose-6-phosphate isomerase contaminant been present, the 1-S-tritium of fructose 6-phosphate would have been exchanged into the solvent. Glucose-6-phosphate isomerase activity in the fructose-1,6-bisphosphatase would have labilized the 1-pro-R-hydrogen of the fructose 6-phosphate before the controlled "wash out" procedure.

Reduction of dihydroxyacetone phosphate

As has been shown above, the methods of analysis developed for [^{3}H]glycerol 3-phosphate allow the ratio of reduction products of DHAP to be determined in the presence of the reduction products of D-GAP. So the ratio of product, derived from borohydride attack on each face of DHAP can be determined for reduction of a triose phosphate solution. Triose phosphate was reduced under various conditions and the [^{3}H]glycerol 3-phosphate was purified by gel filtration (to remove protein) and then by ion-exchange chromatography. Table 2.1 shows the specific radioactivities

Table 2.1. Specific radioactivity of glycerol 3-phosphate and of NADH for the analyses of the reduction of dihydroxyacetone phosphate under various conditions

otal riosephosphate somerase (mN)[a]	Dihydroxy-acetone phosphate (mM)	Sodium boro[^{3}H]hydride (mCi/ml)	Unliganded triosephosphate isomerase (mN)[a,b]	10^{-6}x specific radioactivity of [^{3}H]glycerol 3-phosphate from reduction of dihydroxyacetone phosphate (c.p.m./µmol)[c]	10^{-6}x specific radioactivity of NAD^{3}H		R[d]
					From oxidation of L-glycerol 3-phosphate (c.p.m./µmol)	From oxidation of D-glycerol 3-phosphate after inversion of configuration (c.p.m./µmol)	
0	0.40	0.50	-	14.0	13.0	11.6	1.
1.20	0.13	1.25	1.13	2.83	2.70	0.07	38.
0.25	0.13	1.25	0.23	10.90	11.40	2.26	5.
0.060	0.13	1.25	0.055	6.10	5.27	2.54	2.
0.30[e]	0.13	1.25	-	3.31[f]	1.62	1.39	1.
0.28[e]	0	2.0	-	0.135[g]	0.209	0.035	5.

Notes for Table 2.1

[a] The normality of active sites was calculated by using a subunit molecular weight of 25,000.

[b] Calculated by using the thermodynamic data for each step of the triosphosphate isomerase-catalyzed reaction.[71]

[c] After dilution with unlabelled material. This column lists the values of c.p.m./μmol of D- + L-glycerol 3-phosphate.

[d] The ratio of specific radioactivities of the two lots of NAD^{3}H. See the text.

[e] Inactivated by bromohydroxyacetone phosphate.

[f] The glycerol 3-phosphate is derived by reduction of dihydroxyacetone phosphate, with no hydrolysis of the 3-phosphoglyceryl-enzyme. See the text.

[g] The glycerol 3-phosphate is derived by hydrolysis of the 3-phosphoglyceryl-enzyme.

of [^{3}H]glycerol 3-phosphate and of NAD^{3}H for these reductions. From this table various observations can be made:

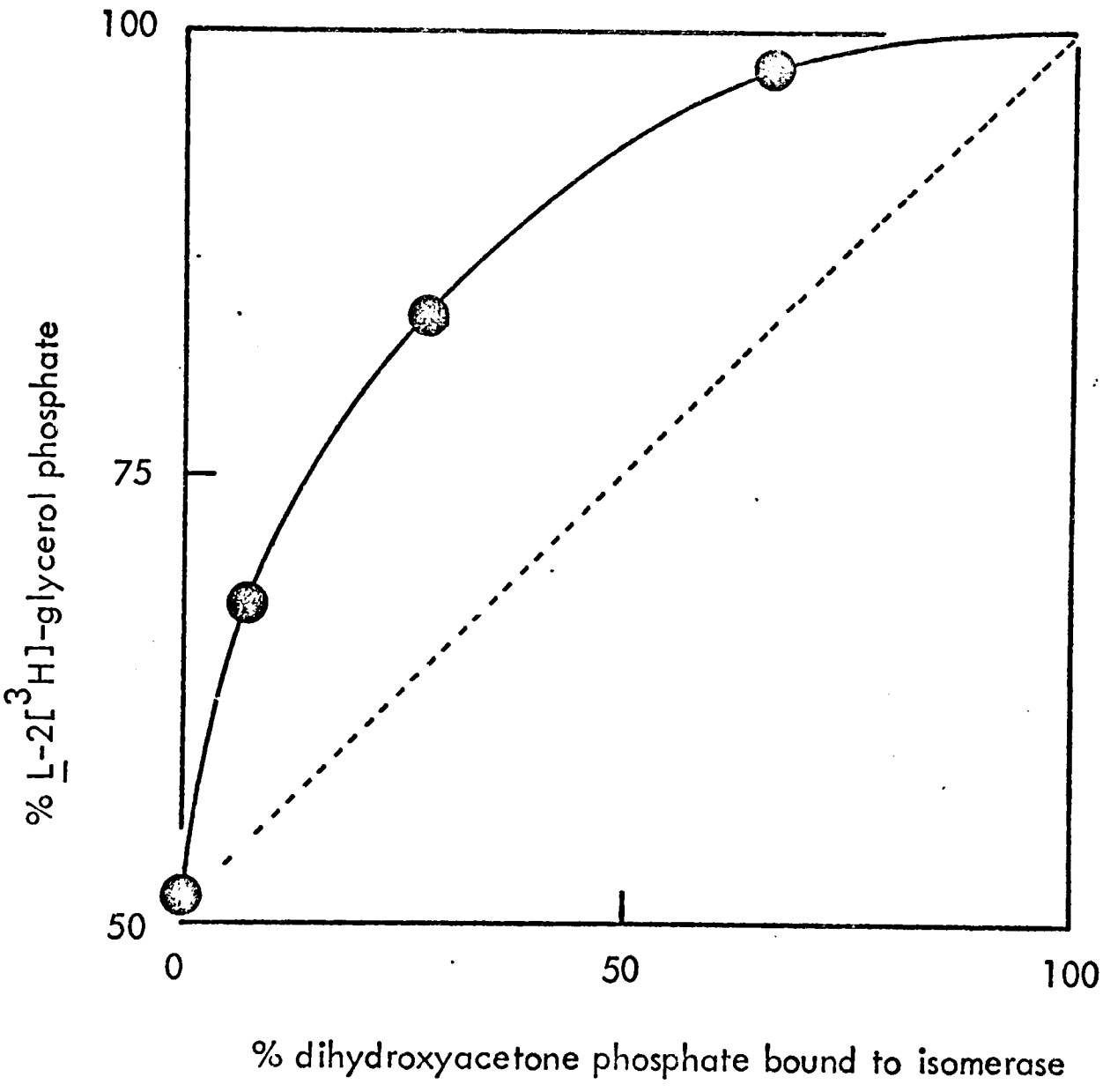
(1) The specific radioactivity of the [^{3}H]glycerol 3-phosphate is high compared with that of NAD^{3}H, due to the presence of tritium on C-1 (deriving from the reduction of D-GAP).

(2) Reduction by sodium borohydride of DHAP alone gives a value of R near unity. R is the ratio of specific radioactivities of the two lots of NADH (see Fig. 2.4) and equals the ratio of L to D-[2-^{3}H]glycerol 3-phosphate present originally. As expected for an achiral substrate, reduction gives a racemic mixture of products.

(3) Reduction of DHAP in the presence of TIM gives values of R greater than unity. More L-[2-^{3}H]glycerol 3-phosphate is formed than the D-enantiomer. These data are represented in Fig. 2.5, in which is plotted the percentage of L-[2-^{3}H]glycerol 3-phosphate *versus* the percentage of DHAP that is bound to the enzyme. This latter number is calculated assuming a binding constant K_s for the keto form of DHAP of 0.7[71] and correcting the triose phosphate concentrations for the presence of the hydrates. This graph shows qualitatively the effect of TIM on the reduction of DHAP. First, it is clear that reduction of enzyme-bound DHAP is completely stereoselective: at very high TIM concentration, the product is entirely L-glycerol 3-phosphate. Secondly, the reduction of enzyme-bound DHAP must be faster than the reduction in free solution. The dashed line shows the result that would be obtained if there were no acceleration.

Assuming that the isomerase keeps all carbonyl forms of substrate at equilibrium throughout the reduction (because of the high concentration of TIM, this is a reasonable assumption that will be validated later in this

Fig. 2.5. Plot of % L-[2-^{3}H]glycerol 3-phosphate against %dihydroxyacetone phosphate bound to the isomerase for the reduction of triose phosphate by sodium boro[^{3}H]hydride in the presence of triosephosphate isomerase



thesis), then the ratio of L to D-[2-^{3}H]glycerol 3-phosphate is equal to the ratio of their rates of formation. If k_1 is the rate constant for the non-sterecselective reduction of unbound DHAP and k_2 and k_3 are the rate

constants for the reduction of enzyme-bound DHAP to L and to D-glycerol 3-phosphate respectively, then the rate ratio (R) for the production of L and D-glycerol 3-phosphates is:

$$\frac{\text{Amount of L}}{\text{Amount of D}} = \frac{k_1[s]/2 + k_2[es]}{k_1[s]/2 + k_3[es]} = R \qquad (2.1)$$

where [s] is the concentration of unbound, unhydrated substrate and [es] is the concentration of the isomerase-DHAP complex. Since:

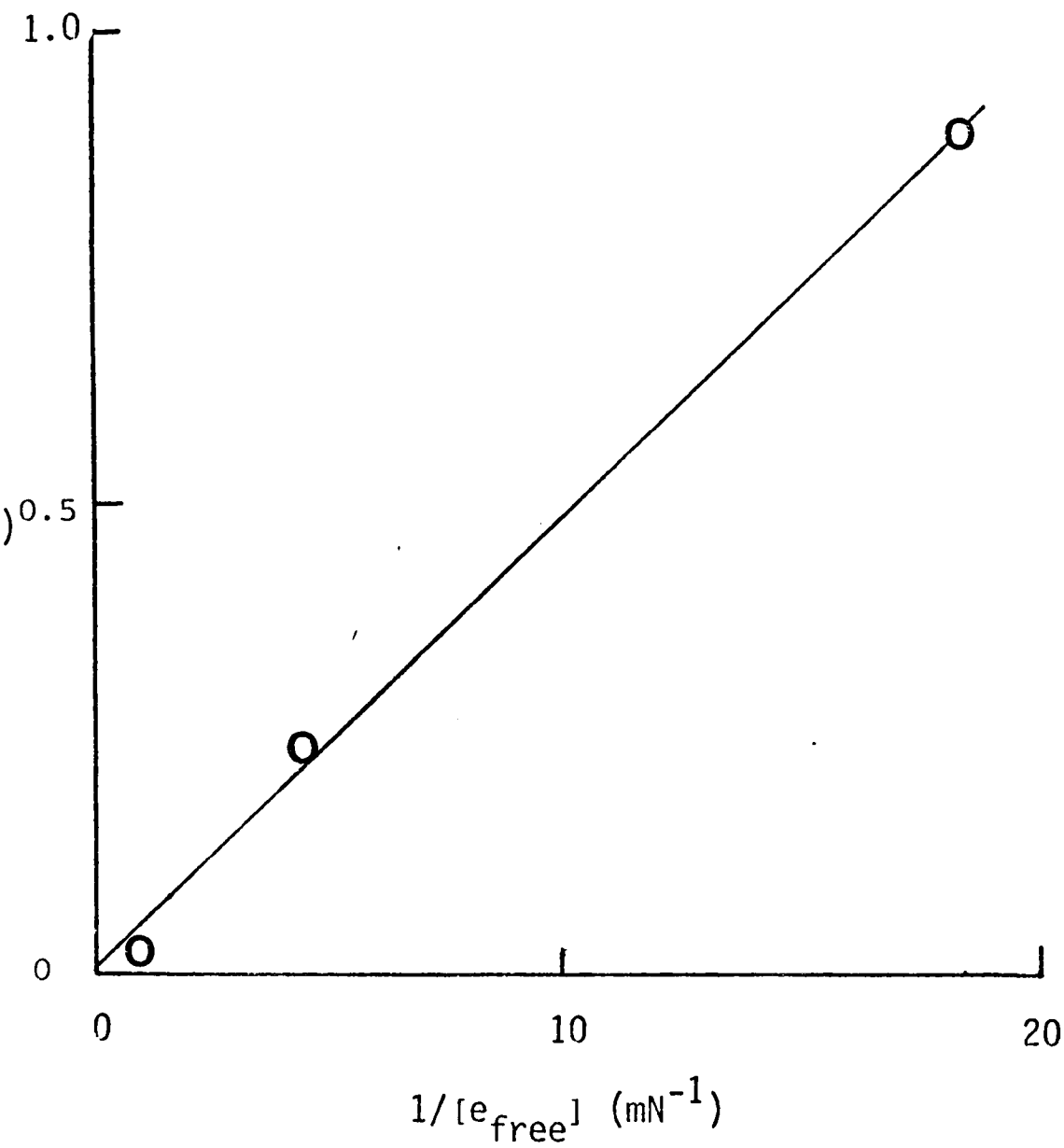
$$K_s = \frac{[s][e_{free}]}{[es]} \qquad (2.2)$$

then:

$$\frac{1}{R-1} = \frac{0.5k_1K_s}{[e_{free}](k_2-k_3)} + \frac{k_3}{k_2-k_3} \qquad (2.3)$$

In Fig. 2.6, $1/(R-1)$ is plotted against $1/[e_{free}]$. From the vertical intercept, k_2/k_3 is approximately 130. This confirms the result obtained qualitatively from Fig. 2.5, that the reduction occurring at the enzyme active site is completely or almost completely stereoselective in favour of the L-enantiomer, and arises by approach of borohydride from the *si* face of the carbonyl group of bound DHAP. On the basis that the borohydride approaches the more accessible face of triose phosphate, the *re* face is the one shielded from the solvent. Since the product of DHAP iosmerization is the D-enantiomer of GAP, the proton is delivered by the enzyme to the *re* face of DHAP: the face that is shielded from borohydride attack. Indeed, the base responsible for proton transfer would be expected to contribute to this shielding.

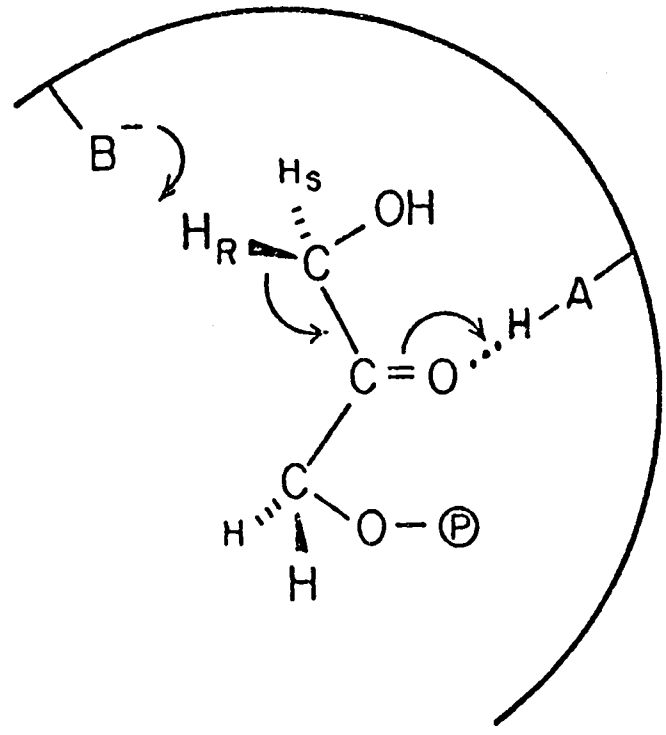
Fig. 2.6. Plot of $1/(R-1)$ against $1/[e_{free}]$, where R is the ratio of the L/D enantiomers of [2-^{3}H]glycerol 3-phosphate and $[e_{free}]$ mN is the concentration of unliganded isomerase.



From the gradient of the line in Fig. 2.6, $(k_2-k_3)/k_1 = 10.1\ K_s$. Since $k_2/k_3 > 100$, the ratio of rate constants $(k_2-k_3)/k_1$ is equal to the ratio $(k_2+k_3)/k_1$, the ratio of rates of reduction on the enzyme active site and in free solution. The reduction occurs approximately seven times faster on the enzyme surface than in free solution. A more helpful way of expressing this result is to consider the rate of reduction of one face

of DHAP: Reduction at the re face is approximately ten times slower on the surface of the enzyme than in free solution, while reduction at the si face is fourteen times faster. So the enzyme is catalyzing the reduction at the si face. This catalysis suggests the existence of an electrophilic group that increases the polarization of the substrate carbonyl group when the substrate is bound to the enzyme. Such an electrophilic group may well be a feature of the triosephosphate isomerase mechanism, increasing the lability of the α-protons, as shown in Fig. 2.7.

Fig.2.7. Proposed involvement of an electrophilic group (AH) in the reaction catalysed by triosephosphate isomerase.



Control experiments involving bromohydroxyacetone phosphate

Bromohydroxyacetone phosphate, a close structural analogue of DHAP, is an irreversible, active-site inhibitor of TIM and reacts rapidly and

stoichiometrically with each enzyme subunit with complete loss of enzyme activity.[29] The resulting phosphohydroxyacetonyl-enzyme can be used in two ways to test features of the triose phosphate reductions described above.

In one experiment, reduction of DHAP by borohydride was carried out in the presence of a high concentration of isomerase whose active site has been blocked by bromohydroxyacetone phosphate. Under these conditions the substrate is reduced with little or no stereoselectivity (Table 2.1). With a fully accessible active site a stereoselectivity of about 6 would be expected at the concentrations of enzyme and substrate employed.

After attachment of bromohydroxyacetone phosphate to the enzyme, the phosphohydroxyacetonyl moiety may be reduced by sodium borohydride and this has been used to stabilize the phosphate ester linkage to hydrolysis.[29] As a check on the validity of the use of borohydride reduction of enzyme-bound carbonyl groups, boro[^{3}H]hydride was used to reduce the covalently-bound phosphohydroxyacetonyl moiety. After purification of the reduced, inactivated enzyme by gel filtration, the ester link of the resulting 3-phosphoglyceryl-enzyme was cleaved by alkaline hydrolysis under conditions where the glycerol-phosphate linkage is stable. This hydrolysis yielded [2-^{3}H]glycerol 3-phosphate, and caused the release of approximately 90% of the radioactivity associated with the protein after reduction.

The [2-^{3}H]glycerol 3-phosphate, with DL-glycerol 3-phosphate added as carrier, was purified and analyzed for tritium at C-2 as outlined in

Fig. 2.3. It was found that at least 80% of the tritium on C-2 was in a position corresponding to L-glycerol 3-phosphate (see Table 2.1). The reduction of the 3-phosphohydroxyacetonyl moiety covalently bound in the active site is therefore highly stereoselective.

These two control experiments show that stereoselective reduction occurs in the active site, and requires the active site to be accessible to the carbonyl-containing species and to the reducing agent.

Reduction of D-glyceraldehyde 3-phosphate

The analytical scheme outlined in Fig. 2.4 enables the reduction products of D-GAP to be analyzed in the presence of the reduction products of DHAP. Table 2.2 shows the results from the C-1 analysis of [^{3}H]glycerol 3-phosphate from reduction of D-GAP alone, of DL-GAP alone, and of DHAP containing the equilibrium proportion (4%) of D-GAP. As a control on the validity of the analytical procedure for tritium at C-1 outlined in Fig. 2.4, DL-[1(S)-^{3}H]glycerol 3-phosphate was analyzed using this sequence. Only the tritium from the D-enantiomer reaches the hexose phosphate stage and only this is therefore analyzed. The tritium in the L-enantiomer stays in the 1-pro-S-hydrogen of DHAP and this hydrogen is removed during the reaction with D-GAP catalyzed by aldolase (see Fig. 2.4). The analysis (Table 2.2) confirms that essentially all of the tritium (approx. 97%) of the D-enantiomer is in the 1-S position, and therefore the stereochemical integrity of the hydrogen atoms at C-1 of glycerol 3-phosphate is unaffected by the reaction sequence involved in the analysis outlined in Fig. 2.4.

Table 2.2. Radioactivity measurements for the analysis of tritium in the 1-R and 1-S positions of D-[^{3}H]glycerol 3-phosphate

Reduction of:	Triose phosphate (mM)	Triose phosphate isomerase (mN)[b]	Sodium boro[^{3}H]-hydride (mM)	Radioactivity measurement x 10^{-5} (c.p.m.) after exchange of 1-R-tritium into the solvent[c]		Ratio of tritium in 1-R-position to tritium in the residue	Specific radioactivities x 10^{-4} (c.p.m. µmol^{-1}) for determination of 1-S-tritium[d]		Ratio of tritium in 1-S-position, to tritium in the D-hexose 6-phosphate	Ratio of tritium in each position of [1-^{3}H]glycerol 3-phosphate R/S
				Residue (D-hexose 6-phosphate)	Solvent (water)		D-hexose 6-phosphate	NADPH		
D-Glyceraldehyde 3-phosphate	1.95	-	1.2	2.473	1.303	0.53	6.76	3.45	0.51	1.04
DL-Glyceraldehyde 3-phosphate	2.10	-	0.6	0.821	0.437	0.53	1.19	0.67	0.56	0.95
Dihydroxyacetone phosphate[d]	0.13	0.29	2.2	1.070	0.375	0.35	4.14	1.44	0.35	1.00
Dihydroxyacetone phosphate[d]	0.13	0.38	2.2	0.375	0.195	0.57	0.85	0.48	0.57	1.00
	[1-(S)-^{3}H]glycerol 3-phosphate[e]			0.414	0.014	0.03	0.92	0.95	1.03	0.03

Notes for Table 2.2

[a] Dihydroxyacetone phosphate, the triose phosphate added, rapidly equilibrates with D-glyceraldehyde 3-phosphate in the presence of the large concentration of triosephosphate isomerase, and both substrates are therefore available for reduction both on and off the enzyme.

[b] The normality of the isomerase active sites was calculated assuming a subunit molecular weight of 25,000.

[c] Incubation of labelled fructose 6-phosphate with glucose 6-phosphate isomerase results in the complete exchange of tritium from the 1-R-position into the solvent water. The amount of radioactivity removed by distillation and the amount of radioactivity remaining in the residue of D-hexose 6-phosphate were each measured. The radioactivity in the water represents the amount of tritium in the 1-R-position of the original fructose 6-phosphate.

[d] The residue of D-hexose 6-phosphate (after removal of the 1-R-tritium) was diluted with a large excess of unlabelled fructose 6-phosphate and its specific radioactivity was determined. Oxidation of this material with glucose-6-phosphate dehydrogenase/$NADP^+$ in the presence of glucose-6-phosphate isomerase results in the transfer of the C-1 tritium of glucose 6-phosphate, to $NADP^+$. The specific radioactivity of the tritiated $NADP^3H$ measures the amount of tritium that derives from the 1-S-position of the original fructose 6-phosphate.

[e] Control experiment in which authentic [1-(S)-^{3}H]glycerol 3-phosphate was subjected to the analytical procedure of Scheme 1. See the text.

On the basis of the stereoselective reduction of both the covalently-bound 3-phosphohydroxyacetonyl moiety and of the noncovalently-bound DHAP, it was expected that the carbonyl group of noncovelently-bound D-GAP would also be reduced stereoselectively by borohydride in the presence of a large concentration of TIM. However, the results in Table 2.2 show that there is no stereoselectivity in the reduction of this aldehydic carbonyl, either in the presence or absence of the isomerase.

Before further consideration of the reduction in the presence of the isomerase, the lack of stereoselectivity observed for reduction in free solution deserves mention. D-GAP, being a chiral molecule, would be expected to yield preferentially one or other of the diastereoisomeric D[1-^{3}H]glycerol 3-phosphates on boro[^{3}H]hydride reduction. In the reduction of free D-GAP in the absence of enzyme, however, no stereochemical preference was observed. Reductions of both D- and of DL-GAP were studied, and in the case of the racemic mixture it should be noted that the reduction of the L-enantiomer causes radioactivity to appear during the analysis in the 3-position of the hexose 6-phosphates, a position where it does not affect the analysis (see Fig. 2.4). Only reduction products from D-GAP are observed in this analytical method. The only observable effect that the presence of the L-enantiomer might have been in the generation of a reducing agent that is a complex between a chiral substrate (or product) molecule and borohydride. Such complexing evidently does not occur and the results for reduction in free solution also preclude the more likely possibility of significant asymmetry in the attack on the carbon atom next to a chiral centre.[97]

Returning to the complete lack of stereoselectivity in the reduction of D-GAP in the presence of TIM, the results in Table 2.2 can be explained in two different ways. The first is that no reduction occurs on the enzyme. The second is that reduction does occur on the enzyme but with complete lack of stereoselectivity. In view of the results from reduction of bound DHAP and of the 3-phosphohydroxyacetonyl moiety the latter is unlikely. However, it can be completely excluded by the experiments described in the next section.

Ratio of reduction at C-1 and C-2

The proportion of reduction of D-GAP occurring on the surface of the enzyme may be determined from the ratio of the amount of reduction of DHAP to the amount of reduction of D-GAP, in the absence of TIM and in the presence of TIM. The ratio of reduction at C-1 (from D-GAP) to reduction at C-2 (from DHAP) is R_{12} such that:

$$R_{12} = \frac{v_g + v_{eg}}{k_d[d] + k_{ed}[ed]} \qquad (2.4)$$

where v_g and v_{eg} are the rates of reduction of free D-GAP and of enzyme-bound D-GAP respectively; k_d and k_{ed} are the rate constants for the reduction of free and enzyme-bound DHAP (pseudo-first order rate constants that include the concentration of reducing agent); and [d] and [ed] are the concentrations of free unhydrated DHAP and DHAP-TIM complex. When the concentration of TIM is very small this equation reduces to the ratio of reduction of free D-GAP and free DHAP:

$$R^{o}_{12} = \frac{v_g}{k_d[d]} = \frac{k_g[g]}{k_d[d]} = \frac{1}{K_{eq}} \frac{k_g}{k_d} \qquad (2.5)$$

where [g] is the concentration of unhydrated D-GAP, k_g is the rate constant for its reduction, and K_{eq} is the equilibrium constant between the unhydrated forms of triose phosphate. In order to use these two equations, there must be sufficient enzyme present to keep all unhydrated species of triose phosphate (free and enzyme-bound) in equilibrium. If this condition is fulfilled, then from Eqs. 2.2, 2.4 and 2.5:

$$\frac{R_{12}}{R^{o}_{12}} = \frac{1 + v_{eg}/v_g}{1 + k_{ed}[e_{free}]/k_d \quad K_s} \qquad (2.6)$$

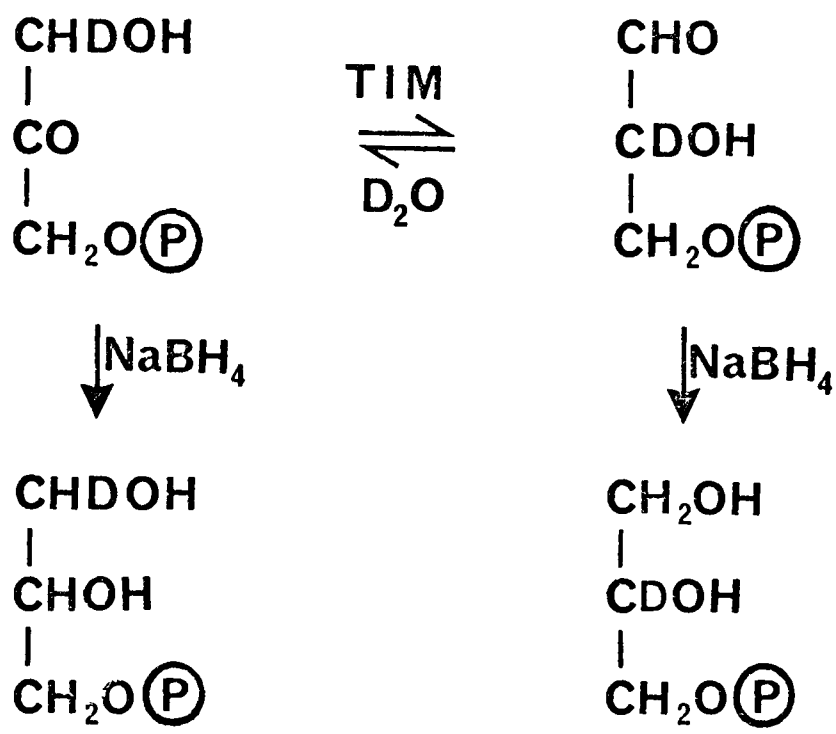
where K_s is the dissociation constant for the DHAP-TIM complex (defined in Eq. 2.2), and $[e_{free}]$ is the concentration of ~~unhydrated~~ unliganded enzyme. From the results of the DHAP reduction studies it was found that $k_{ed}/k_d K_s = 10.1$. So Eq. 2.6 may be rewritten to give the ratio of reduction of enzyme-bound D-GAP and D-GAP in free solution at a particular enzyme concentration:

$$\frac{v_{eg}}{v_g} = \frac{R_{12}}{R^{o}_{12}} \quad (1 + 10.1[e_{free}]) - 1 \qquad (2.7)$$

Assuming that the species being reduced are kept in equilibrium throughout the reduction then R_{12} is given by the ratio of the amounts of product due to reduction at C-1 and at C-2.

In order to find the ratios, R_{12} and R^{o}_{12}, deuterated substrates were used as shown in Fig. 2.8. Incubation of DHAP with TIM in fully deuterated

Fig.2.8. Formation of [^{2}H]glycerol 3-phosphates by reduction of deuterated triose phosphates.



water gives an equilibrium mixture of DHAP, with a deuterium label at C-1, and D-GAP, with a deuterium label at C-2. Reduction by borohydride of DHAP gives [1-^{2}H]glycerol 3-phosphate; reduction of D-GAP gives [2-^{2}H]glycerol 3-phosphate. The ratio of deuterium at C-1 and C-2 of the reduction product may be determined by mass spectroscopy on the volatile tetrakis (trimethylsilyl) derivative of glycerol 3-phosphate (Fig. 2.9).[98,99]

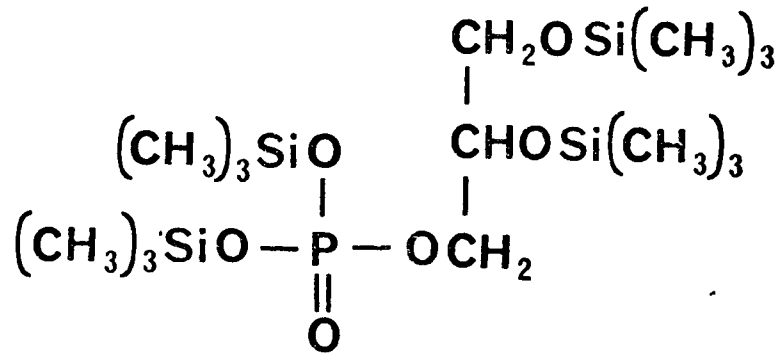


Fig. 2.9. Tetrakis(trimethylsilyl) glycerol 3-phosphate

The spectrum that is obtained is shown in Fig. 2.10(a). Unlabelled tetrakis (trimethylsilyl)-glycerol-3-phosphate fragments give a peak at 445 (M-15), arising from loss of a single methyl group, containing all the hydrogens of glycerol 3-phosphate. The peak at 357 (M-103) arises from cleavage between C-1 and C-2 and contains only the C-2 and C-3 hydrogens of glycerol 3-phosphate. The fragment with m/e = 328 contains the hydrogens of C-3 of glycerol 3-phosphate but none of the C-2 or C-1 hydrogens. Thus, analysis of the ratios of peak intensities 328:329, 357:358 and 445:446 allows the percentage deuteration at each carbon atom of glycerol 3-phosphate to be determined.

Determination of R_{12}^{o}

To determine the ratio of D-GAP reduction to DHAP reduction in free solution (R_{12}^{o}), DHAP was incubated in deuterium oxide with a small amount of the isomerase to obtain an equilibrium mixture of the two triose phosphates fully deuterated in the labile position. The triose phosphate was then reduced by sodium borohydride, with the isomerase serving to keep the equilibrium between DHAP and D-GAP.

The minimum concentration of isomerase was used for this purpose and this concentration was calculated from a knowledge of the rate of reduction by borohydride. By following the rate of formation of glycerol 3-phosphate, during the reduction of D-GAP by borohydride, the rate constant for reduction was observed to be approximately 400 $M^{-1}min^{-1}$.

To find R_{12}^{o}, DHAP was incubated with a small amount of isomerase in tritiated deuterium oxide and then reduced by sodium borohydride. The product, [^{2}H]glycerol 3-phosphate containing some [^{3}H]glycerol 3-phosphate as marker, was purified by ion exchange chromatography and converted to the

volative tetrakis (trimethylsilyl) derivative for analysis by mass spectroscopy (Fig. 2.10(b)).

Any particular ion gives rise to a family of peaks in the mass spectrum at m, m + 1, m + 2, etc., the intensities of which reflect the natural abundances of isotopes of the constituent atoms of the fragment in calculating the percentage deuteration at each carbon position of glycerol 3-phosphate, this natural abundance is taken into account by comparison of the deuterated material with fully protonated material.

The results for glycerol 3-phosphate derived from the reduction of triose phosphate in the presence of a minimal concentration of TIM shows that 96.4% of the product is monodeuterated. However, 13.6% of the material is deuterated at C-3. This is due to the non-specific exchange of hydrogen at this position into DHAP during the period when the DHAP was stored as a solution in fully deuterated water. This position (C-3) has a greater rate of non-enzymic proton exchange than C-1 by a factor of approximately 20.[42] So this non-specific deuteration has no effect on the specific deuteration and reduction, although corrections of the percent deuterations must be made.

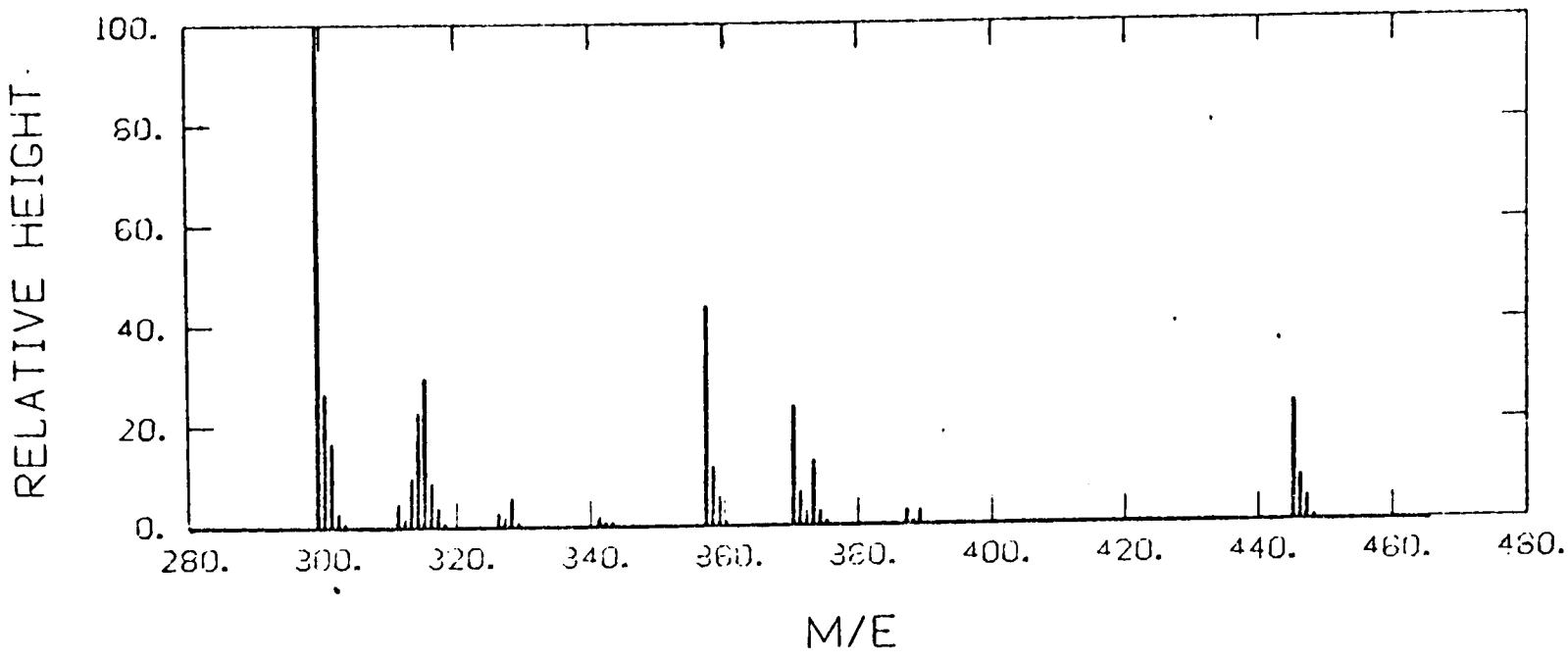
The value of R_{12}^{o} is found to be 0.68 by this method. Then from Eq. 2.5, $k_g/k_d = 250$; i.e. the reduction of D-GAP in free solution occurs some 250 times faster than the reduction of DHAP in free solution. This agrees with the generally greater susceptibility of aldehydes than ketones to reduction by borohydride.

Determination of R_{12}

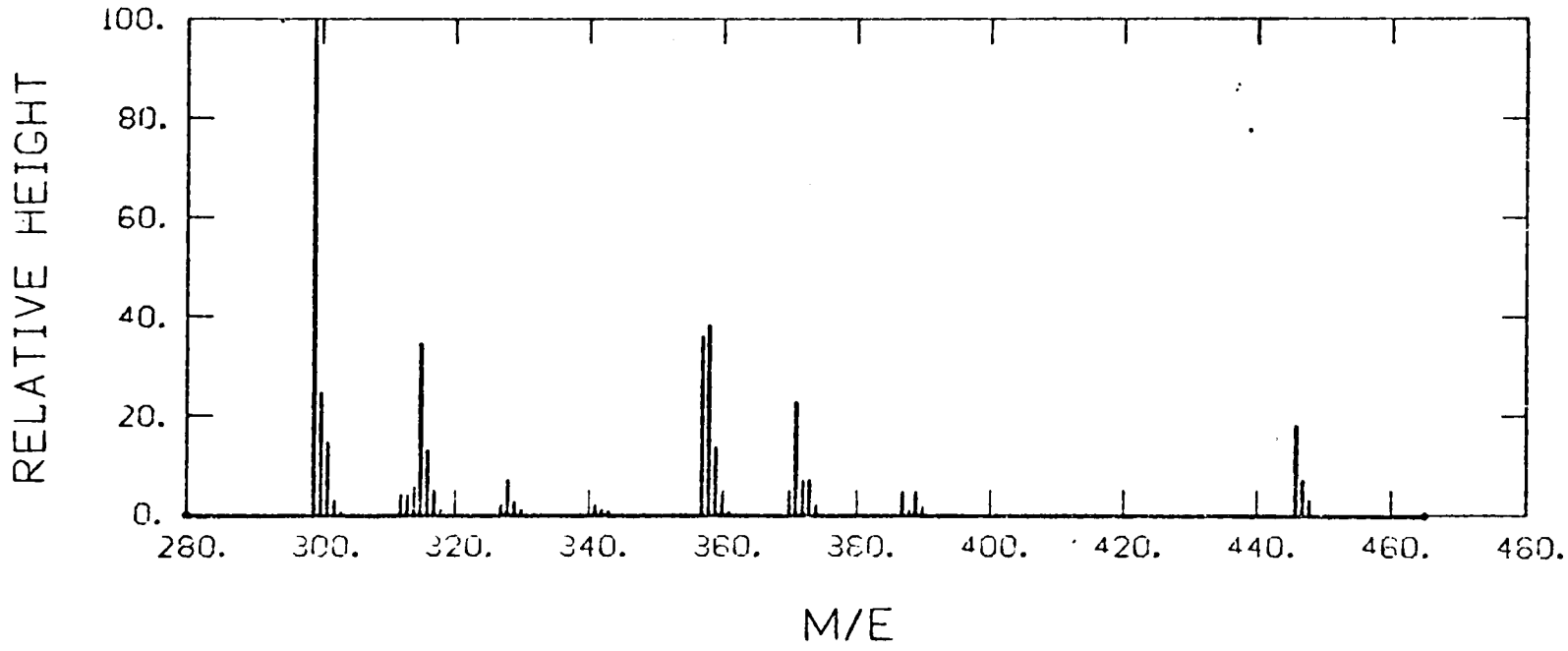
When the experiment was repeated in the presence of 0.24 mN TIM, the results shown in Table 2.4 were obtained. In this case, 96% of

Fig. 2.10. Mass spectra of the tetrakis(trimethylsilyl) derivatives of:

(a) DL-glycerol 3-phosphate.



(b) Deuterated glycerol 3-phosphate from reduction of triose phosphate in the presence of minimum isomerase.



(c) Deuterated glycerol 3-phosphate from reduction of triose phosphate in the presence of 0.24mN isomerase.

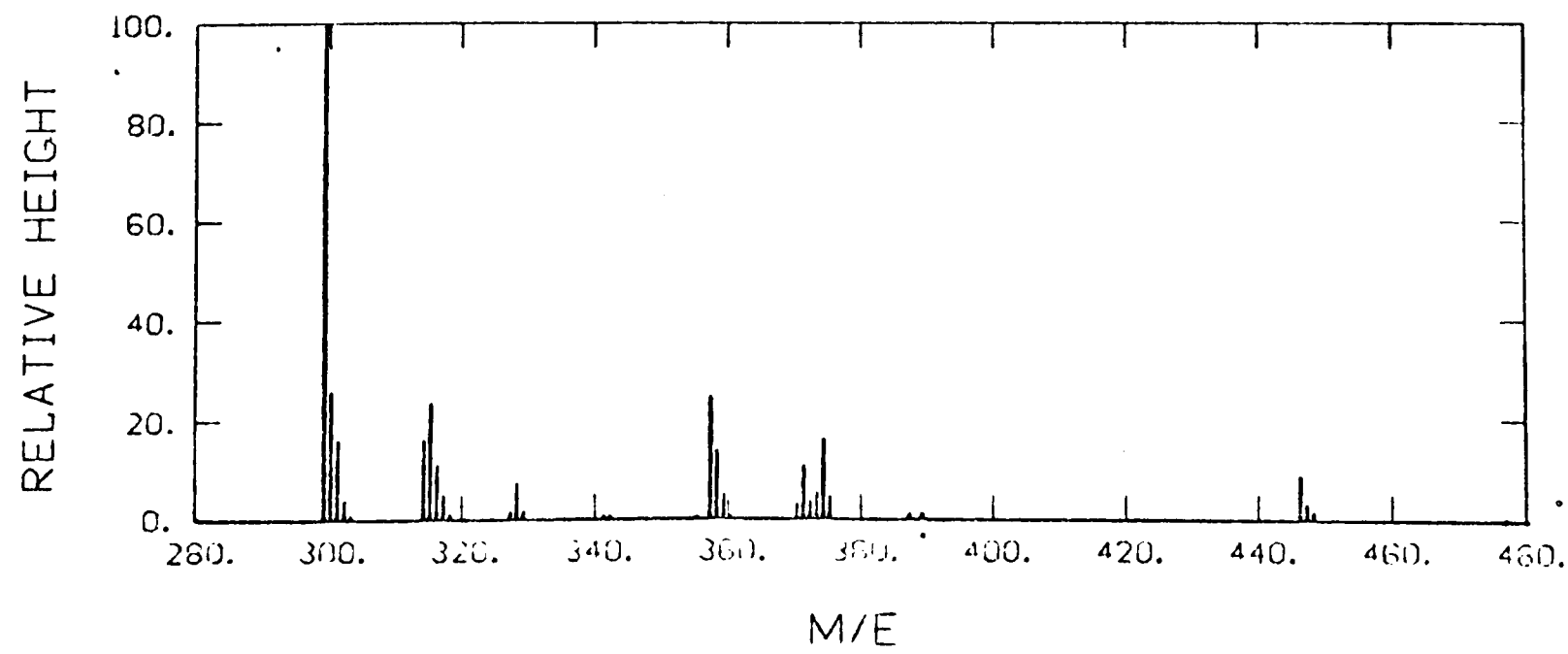


Table 2.4. Mass spectral data for tetrakis (trimethylsilyl)-[^{2}H]glycerol 3-phosphate

Source of glycerol phosphate	Unlabelled DL-glycerol 3-phosphate[1]	Reduction of triose phosphate with minimal TIM	Reduction of triose phosphate with 0.24 mN TIM
Peak intensity ratios:			
I_{446}/I_{445}	0.36	26.9	24.0
I_{358}/I_{357}	0.30	1.10	0.64
I_{329}/I_{328}	0.29	0.45	0.29
No. of spectra averaged	12	7	6
% deuteration[2]	0	96.4	96.0
% deuteration at C-3[3]	0	13.6	0
% deuteration at C-2[4]	0	39.1	25.4
% deuteration at C-1[5]	0	57.3	70.6
R_{12}[6]	-	0.68	0.36

Notes for Table 2.4

1. These ratios express the relative intensity of the m + 1 peak (arising from natural abundance) compared to the m peak.

2. Calculated from the ratio (I_{446}/I_{445}). This is the percentage of molecules containing one deuterium atom. $(I_{446}/I_{445})' = (I_{446}/I_{445}) - 0.36$, i.e. the ratio corrected for the contribution of protonated material in I_{446}.

3. Calculated from $(I_{329}/I_{328})' = (I_{329}/I_{328}) - 0.29$ (see note 2).

4. Calculated from the ratio (I_{358}/I_{357}), corrected for the contribution of protonated material and the contribution of material deuterated at C-3 to the intensity of peak 358. $(I_{358}/I_{357})' = (I_{358}/I_{357}) - 0.30 - (I_{329}/I_{328})'$.

5. The difference between the percentage of molecules containing one deuterium atom and the percentage of molecules deuterated at C-2. Virtually all the deuterium at C-3 will be in doubly labelled material and so does not affect this percentage.

6. R_{12} is the ratio of deuteration at C-2 to deuteration at C-1.

glycerol 3-phosphate was monodeuterated and no deuterium appeared at C-3 (due to the use of a freshly-prepared solution of DHAP). R_{12} was found to be 0.36. The concentration of unliganded enzyme (e_{free}) is calculated to be 0.1 mN, during the reaction, taking into account the binding of substrates[71] and binding of glycerol 3-phosphate.[48] The concentration, $[e_{free}]$ fortuitously remains the same. Using Eq. 2.7, a value for v_{eg}/v_g is calculated as 0.055, i.e. the rate of reduction of enzyme-bound D-GAP is therefore only a fraction (0.055) of the rate of reduction of D-GAP in free solution.

Consequences of the lack of reduction of enzyme-bound D-GAP

The experiment described above precludes the possibility that D-GAP is reduced non-stereoselectively on the surface of the enzyme and so helps to explain why no stereoselective reduction by boro[^{3}H]hydride of D-GAP occurs in the presence of TIM.

There are two ways in which the lack of reduction of enzyme-bound D-GAP may be explained. First, the proportion of unhydrated D-GAP bound to the enzyme may be so small that the reduction product derives exclusively from reduction in free solution. This explanation implies that the dissociation constant (K_s) for the enzyme-unhydrated substrate complex is greater than 1 mM (even assuming that the rate of reduction on the enzyme is only the same as that in free solution). However, it is known that the enediol and unhydrated D-GAP are in rapid equilibrium with each other on the surface of the enzyme[64] and that the combined dissociation constant for these two species is approximately 5 μM.[64]

A dissociation constant of 1 mM for D-GAP alone would then require an equilibrium constant between these two enzyme-bound species of at least 200 in favour of the enediol, which would in turn require a rate constant for the enolization of enzyme-bound D-GAP to be approximately $10^6 sec^{-1}$. [The rate constant for conversion of enzyme-bound DHAP to the enediol is known to be $\sim 2 \times 10^3 sec^{-1}$.[71]] This is an improbably high rate of enolization and therefore it seems unlikely that the failure to observe stereoselectivity in reduction of D-GAP is due to the small concentration of enzyme-bound species.

The second interpretation of the lack of reduction of enzyme-bound D-GAP is simply that the rate of reduction of the enzyme-bound substrate is much less than that in free solution. In the case of DHAP some parallels could be drawn between the enzyme-catalyzed enolization process and the enzyme-catalyzed reduction by borohydride. Both the proton abstraction of the enolization and the hydride attack on the carbonyl of the reduction process are expected to be similarly accelerated by polarization of the carbonyl group, when substrate is bound to the enzyme. However, the steric requirements of the two reactions may be very different. In the reduction of the si face of enzyme-bound DHAP, the polarization of the carbonyl group that leads to a more rapid reduction evidently outweighs any decrease in reactivity due to unaccessibility. For D-GAP however, no acceleration of reduction and no stereoselective reaction by borohydride occur. The probable explanation is that the carbonyl group of D-GAP is, for steric reasons, inaccessible to borohydride. This prevents any reduction in the active site at C-1 of bound substrate.

In summary, of the four possible positions of reduction of the enzyme-substrate complex, only one--the si face of DHAP--is reduced by borohydride. The subtle differences in accessibility of C-1 and C-2 should be clarified by the promising high resolution crystallographic work on the TIM-substrate complex that is in progress.[22] From this work with borohydride, we are able to suggest a further component in the catalysis mediated by TIM: the electrophilic group that aids proton abstraction. Possible candidates for this group are Lys_{13} and His_{95}, both of which are known from the crystallographic work[22] to be in the active site region. Such a residue could interact with both substrate oxygen centres, as shown in Fig. 2.11, and so accommodate the requirement for a cis-enediol (rather than trans).

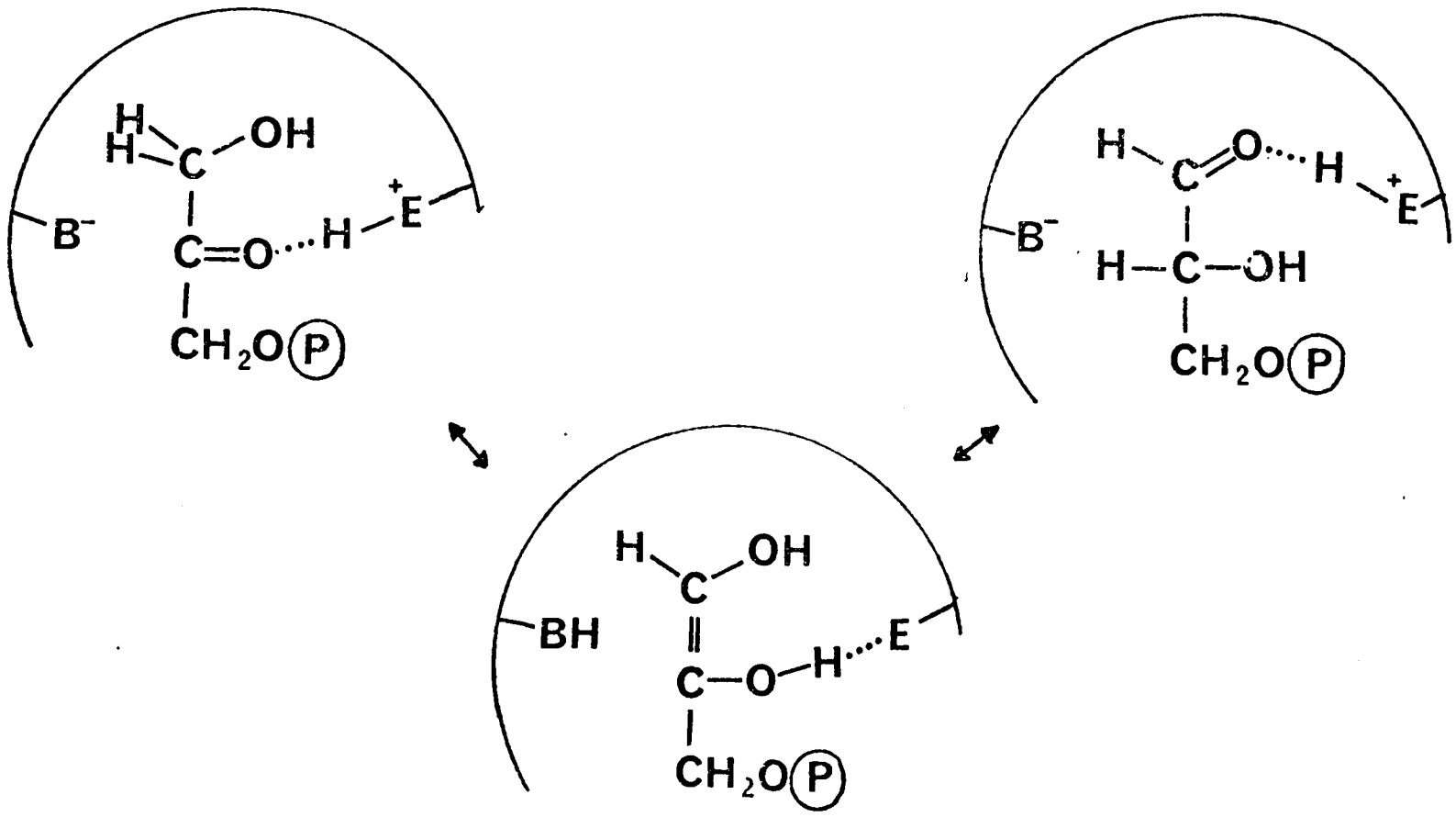


Fig. 2.11. Interaction of proposed electrophilic group in the active site of triosephosphate isomerase with each substrate.

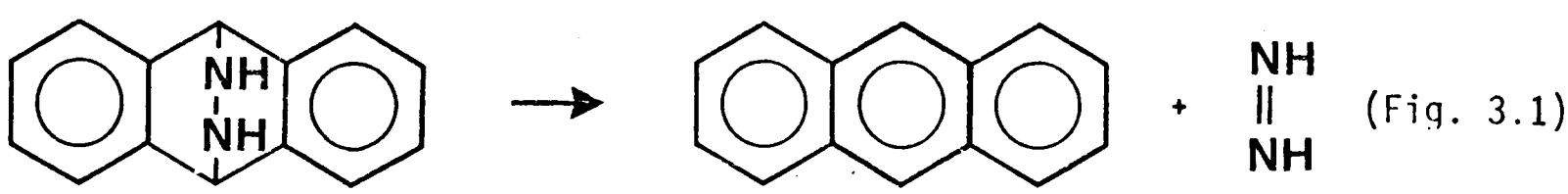
CHAPTER 3 TO TRAP AN ENEDIOL

However likely an enzyme mechanism is, it can never be proved until each elementary step is substantiated and each intermediate has been shown to exist. The mechanism originally proposed by Rose for the reaction mediated by triosephosphate isomerase (TIM) is shown in Fig. 1.2 and involves the transfer of a proton between carbon centres of a cis-enediol to interconvert dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (D-GAP). It would be very good support for this pathway if direct evidence for a cis-enediol could be found.

Following from the work with sodium borohydride described in Chapter 2, it seemed that it might be possible to use a reducing agent to trap the enediol. While borohydride only reduces very polar double bonds, such as carbonyl groups of aldehydes and ketones, reduction of an enediol requires a reducing agent that will not reduce polar double bonds, but will reduce non-polar olefinic bonds. Such a reducing agent is diimide (HN=NH).

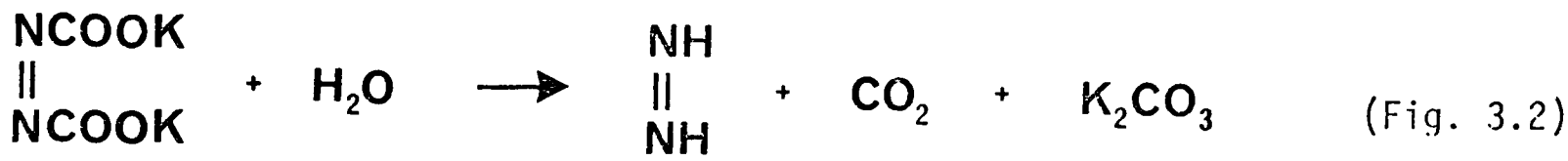
Preparation and properties of diimide

Diimide itself exists as a transient species in solution and must be prepared in situ from a stable precursor.[100] For example, hydrazine in solution can be oxidized under mild conditions to give diimide;[101] anthracene 9,10-diimine decomposes thermally to give diimide:[102]



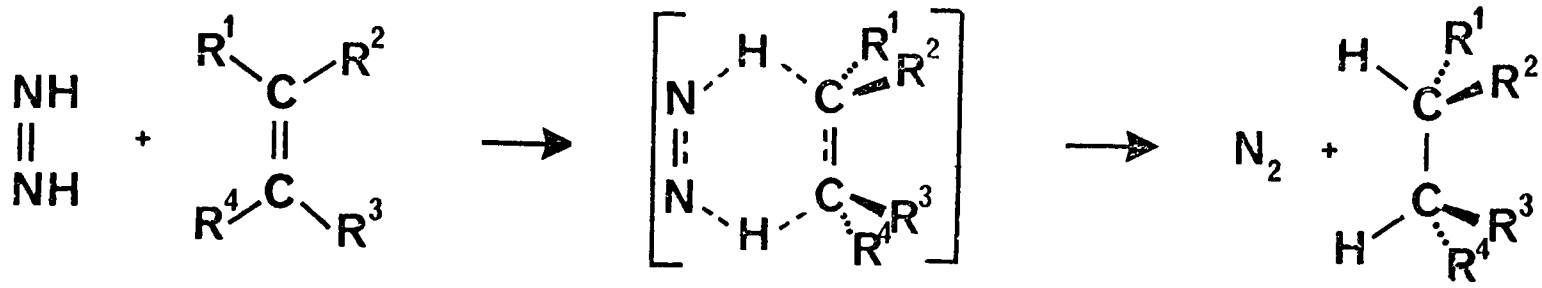
(Fig. 3.1)

However, the method chosen for the present work is the generation of diimide in protonic solvents from potassium azodicarboxylate.[103,104]



(Fig. 3.2)

As can be seen, the hydrogens of diimide when formed from potassium azodicarboxylate originate from water (or other protonic solvent). It has been shown that in reduction of an olefinic double bond, these two hydrogens are transferred in a syn-addition:[105]



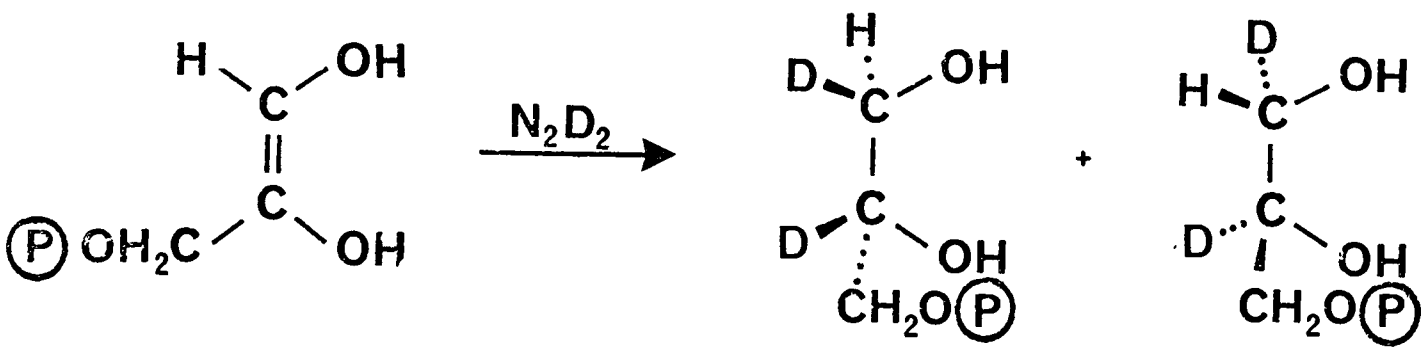
(Fig. 3.3)

Almost all the work with this reducing agent has been with non-aqueous solvents. Studies on the specificity of reduction by diimide have shown that the less polar the double bond, the more easily it is reduced. Thus, aliphatic ketones are reduced very slowly, if at all[106] and hetero atoms attached to the olefinic carbons reduce the rate of reduction of the olefin.[106,107] However, it has been shown that if identical polar groups occur symmetrically on either carbon atom of an olefinic bond, then the bond is easily reduced by diimide.[105,108] Diimide has been shown to be capable of trapping transient species such as cyclodeca-1,3,5,7,9-pentaene,

a Huckel aromatic compound.[109] This augurs well for the possibility of trapping the transient enediol.

Diimide with triose phosphate

Although diimide is very short-lived in aqueous solution, it has a number of features that make it attractive as a trapping agent for the enediol of triose phosphate. The transient diimide is formed rapidly on adding potassium azodicarboxylate to protonic solvents and the decomposition of diimide produces relatively harmless species (hydrazine, nitrogen, carbon dioxide, potassium carbonate). By generating diimide in fully deuterated water, the trapping of the enediol should follow the following scheme:



(Fig.3.4)

The product is deuterated glycerol 3-phosphate, the analysis of which was described in the previous chapter. Similarly, the use of tritiated water should give tritiated glycerol 3-phosphate, so that a combination of these methods would allow complete analysis of the reduction product.

In order to determine if the enzyme-bound enediol could be reduced, the diimide was generated from a large excess of potassium azodicarboxylate in a heavily buffered solution of triose phosphate and TIM in tritiated deuterium oxide. After purification of the reduction product by gel filtration (to remove protein) and ion-exchange chromatography (to remove unaltered substrate), the amount of reduction product was determined from the amount of radioactivity associated with it. Table 3.1 shows that diimide in the absence or presence of TIM gives approximately 0.01% yield of glycerol 3-phosphate from triose phosphate. The presence of [^{2}H]glycerol 3-phosphate was shown by mass spectroscopy. However, the yield is so small that the product might be due to the reduction of the carbonyl group of triose phosphate or the small equilibrium amount of enediol in free solution.

Since the aim of this experiment was both to demonstrate the presence of the enzyme-bound enediol and also to determine its stereochemistry from the labelling pattern of the reduction product, the yield was much too low and this approach to trapping the enediol was therefore not pursued further.

Table 3.1. The yield of glycerol 3-phosphate from reduction of triose phosphate in the presence of TIM in tritiated deuterium oxide

Concentration of TIM (mg/ml)	Amount of DHAP in solution (2.2 ml) (μmol)	Yield of glycerol 3-phosphate ($\times 10^{-3}$ μmol)[1]
7.8	2.4	1.1
0.05	2.4	4.0

1. Measured from the amount of radioactivity associated with the product, assuming it has twice the specific radioactivity of the solvent.

CHAPTER 4 A NUCLEAR MAGNETIC RESONANCE STUDY OF THE BINDING OF TRIOSE PHOSPHATE TO TRIOSEPHOSPHATE ISOMERASE

The major forms of triose phosphates in solution are the hydrate and forms: triosephosphate isomerase has been shown to release the keto form of both dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (D-GAP).[3,39] The carbonyl forms are therefore the true substrates of the isomerase and the kinetic and equilibrium parameters of the catalyzed reaction must be corrected for the proportion of the substrates in the non-active form, the hydrate. However, the hydrates of the substrates are structurally very similar to the carbonyl forms and could bind in the active site of the isomerase as competitive inhibitors. Such inhibition would further complicate the meaning of the observed reaction parameters. It is the purpose of the work described in this chapter to investigate the binding of hydrate and of the keto form of DHAP to the isomerase, by studying the effect of the enzyme on the n.m.r. signals due to each of the substrate species.

Phosphorus versus proton n.m.r.

The two nuclei of the triose phosphates best suited for n.m.r. work are phosphorus and hydrogen: each has certain disadvantages and advantages. The hydrogen signal is sixteen times more sensitive than phosphorus at constant field strength. Generally, peak areas can be used to determine relative concentrations of protons, provided that good signal-to-noise ratio is obtained and that precautions are taken to ensure adequate relaxation in pulse experiments. However, phosphate resonances are not well characterized in this respect and slow relaxation of the phosphorus nucleus

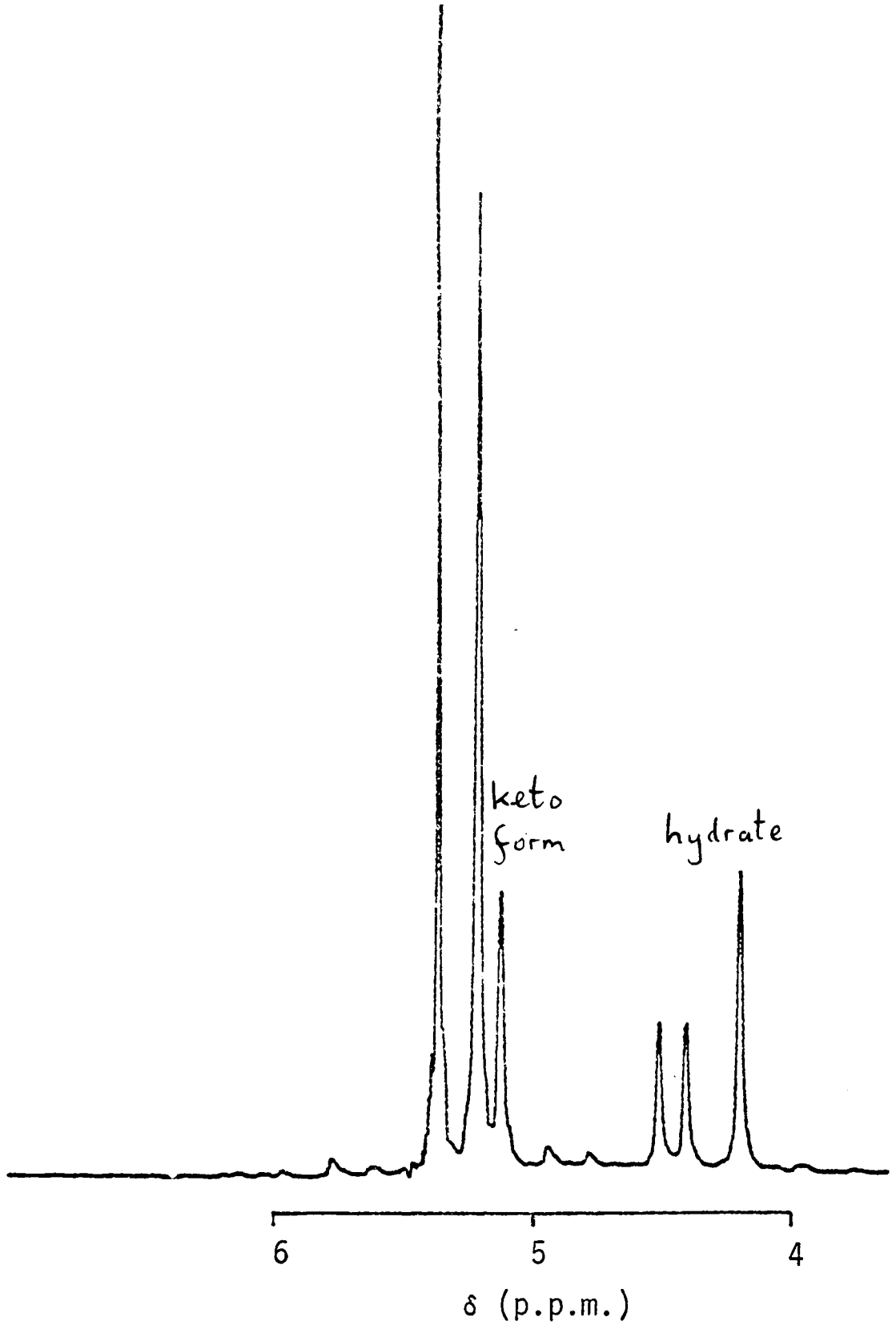


Fig. 4.1. Proton n.m.r. spectrum of dihydroxyacetone phosphate.

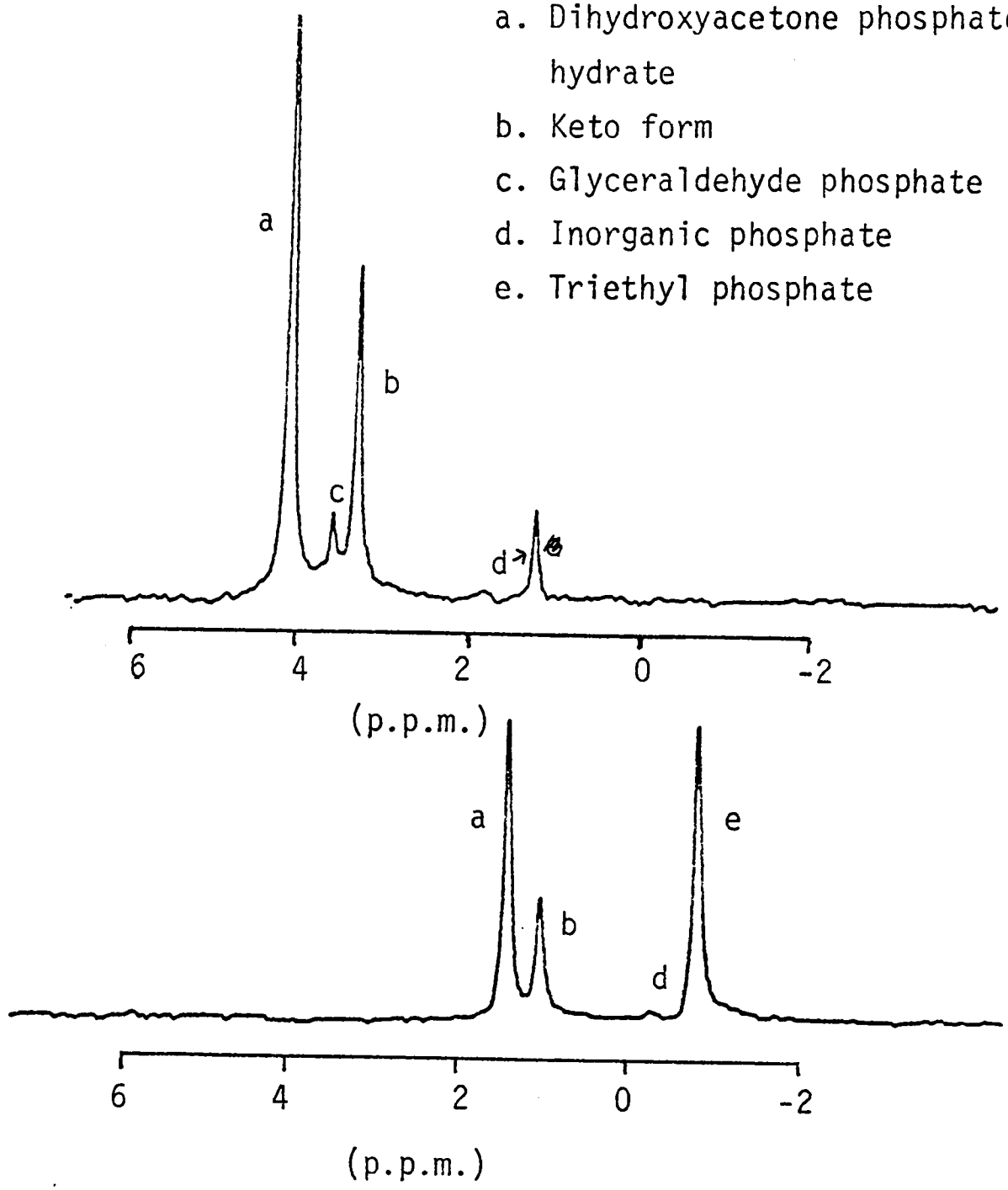


Fig. 4.2. Phoshorus n.m.r. spectra of dihydroxyacetone phosphate at 1°C, (upper) at pH 6.9, containing an equilibrium proportion of glyceraldehyde phosphate; (lower) at pH 5·5.

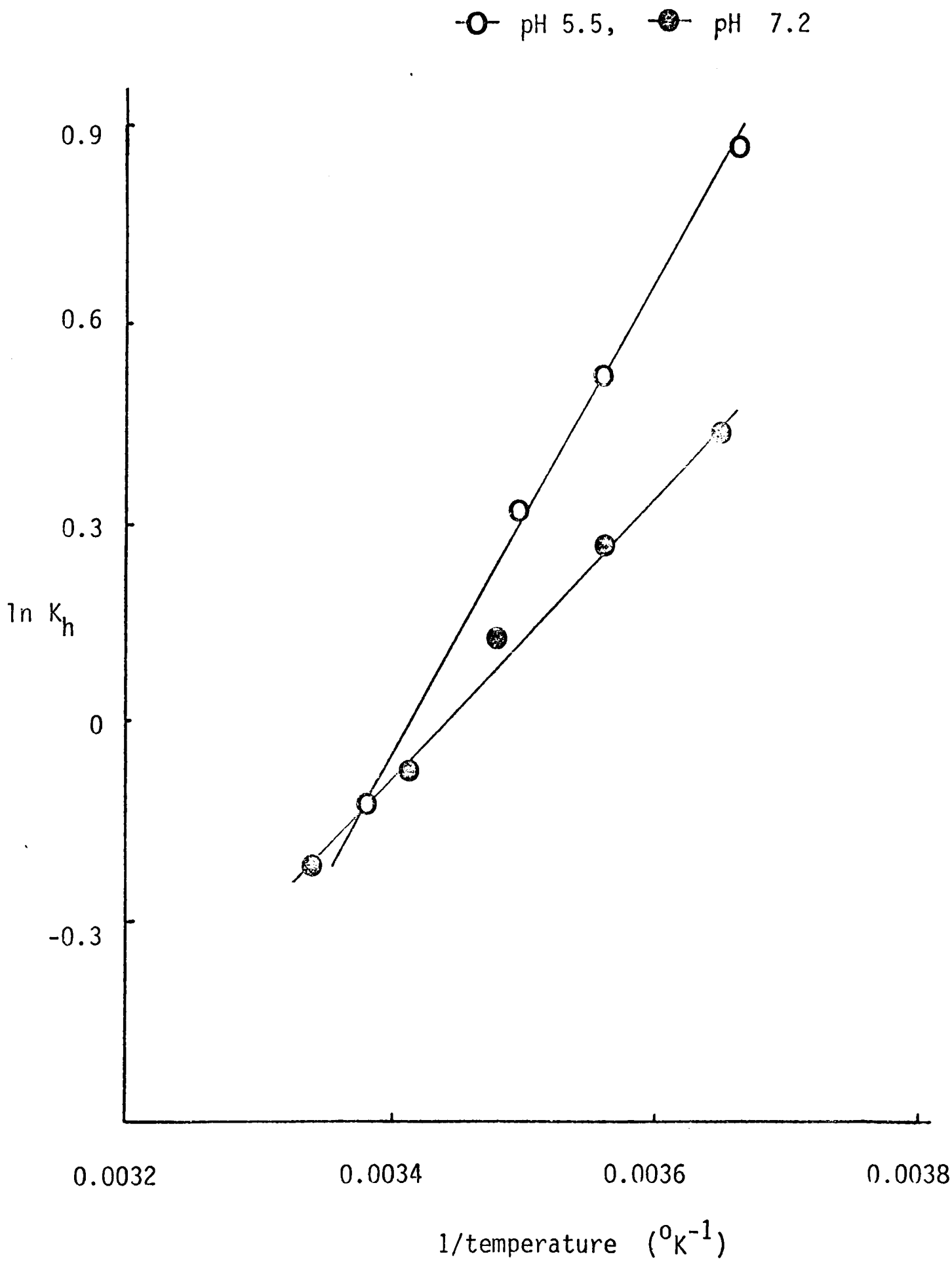
in phosphate esters, together with the consequences of proton decoupling may cause peak areas to deviate considerably from proportionality to concentration. Both proton and phosphorus n.m.r. spectra of DHAP show well-separated peaks for the hydrate and keto forms (see Fig. 4.1 and 4.2). However the proton spectrum has peaks quite close to the HDO peak. The proton spectrum in the presence of a large concentration of isomerase would require the protein to be in pure deuterium oxide. Also, protein proton resonances may require the use of difference spectra to obtain the spectrum due to triose phosphate.

Because of these considerations, proton n.m.r. was used to determine the ratio of hydrate to keto form of DHAP. Phosphorus n.m.r. was used in studies with the isomerase.

Ratio of hydrate to keto form of DHAP

In order to determine the effect of temperature and pH on the equilibrium constant between the hydrate and keto form of DHAP, proton n.m.r. spectra were obtained under different conditions, using a long recycle time for each pulse and aquisition of sufficient pulses to obtain good signal-to-noise ratio. Peak areas were integrated to obtain the ratio of the two forms. The results are presented in Fig. 4.3. The proportion of hydrate decreases as the temperature is increased. The ratio also varies with the protonation state of the phosphate group, and the ratios at the two pH-values studied (5.5 and 7.2) coincide at approximately room temperature. An earlier kinetic determination of the ratio was done at room temperature and the variation in hydrate:keto ratio with pH was thus missed. The relatively large dependence of the ratio on the protonation

Fig.4.3. Variation of the Hydrate/Keto ratio (K_h) of dihydroxyacetone phosphate with temperature.



state of the phosphate, together with the large shift of the phosphate n.m.r. resonance on hydration of the carbonyl group of DHAP (Fig. 4.2) indicates considerable interaction between the phosphate group and the C-2 oxygens.

Stability of triose phosphate in solution

In order to be able to proceed with studies of triose phosphates in the presence of the isomerase, it was necessary not only to know the proportion of hydrate to keto form in free solution, but also to know that the substrate will not decompose during the course of an n.m.r. experiment. The rate of decomposition of triose phosphates in solution was therefore measured under various conditions. The results of this study are presented in Table 4.1, as pseudo-first order rate constants, and the rates relative to that of DHAP at pH 7.4 and 22° in the absence of enzyme. It is evident from these data that the decomposition is faster in the presence of the isomerase at pH 7.4 and 22°. The rate increases with enzyme concentration, which implies some catalysis of the decomposition by the isomerase, other than the effect of maintaining an equilibrium proportion of the more unstable GAP. Changing the buffer concentration, the pH to 6.7 or adding ammonium sulphate or BSA had little effect on the rate of decomposition. Thus the rate cannot be explained by incompletely dialyzed enzyme or by non-specific protein effects. The increased rate of decomposition was not observed if the isomerase was inactivated by bromohydroxyacetone phosphate before incubation with DHAP.

The effect of the isomerase on the rate of decomposition at 22° is too large to allow n.m.r. spectra of triose phosphate in the presence

Table 4.1. The rates of decomposition of triose phosphate

Temperature	pH	Triosephosphate isomerase (mg/ml)	Other additions[a]	"k^1"(hr)	Relative rate
22	7.4	0	-	0.06	0.75
22	7.4	0.05	-	0.08	1.0
22	6.7	0.05	-	0.05	0.62
3	7.0	0.05	-	0.01	0.12
22	7.4	0.90	-	0.51	6.2
22	7.4	1.70	-	0.73	8.7
22	7.4	3.15	-	1.0	12.5
22	6.7	1.70	-	0.40	5.0
3	7.0	1.70	-	0.03	0.37
22	7.4	2.00[b]	-	0.12	1.5
22	7.4	0.05	BSA (5 mg/ml)	0.09	1.1
22	7.4	0.05	c	0.10	1.2
22	7.4	0.05	$(NH_4)_2SO_4$ (0.5 M)	0.11	1.4

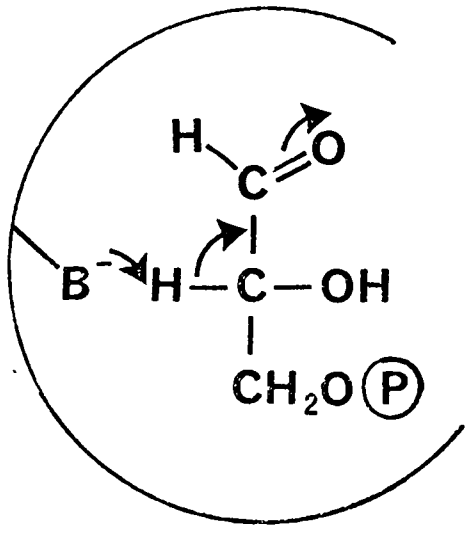
[a]Incubations were in 200 mM triethanolamine-HCl buffer unless otherwise stated. The concentration of DHAP was 25 mM and in the presence of isomerase an equilibrium proportion (4%) was present as GAP.

[b]The enzyme had been totally inactivated by bromohydroxyacetone phosphate

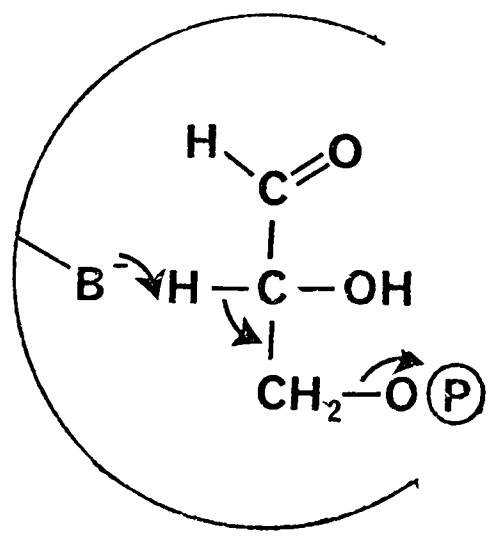
[c]Incubation in 50 mM triethanolamine-HCl buffer

of enzyme to be studied. However, lowering the temperature to 3° stabilized the triose phosphate with respect to decomposition both in the presence and absence of enzyme.

While it seems that the decomposition of triose phosphate is catalyzed in the active site of triosephosphate isomerase, the effect is extremely small compared to the turnover of the normal catalytic activity. No attempt was made to determine the products of decomposition. However, it appears from the phosphorus n.m.r. that inorganic phosphate is formed on incubation of triose phosphate with the isomerase and the enzyme-catalyzed hydrolysis pathway may possibly occur <u>via</u> the β-elimination of GAP:



normal enzymic pathway



enzyme-catalysed hydrolysis

(Fig. 4.4)

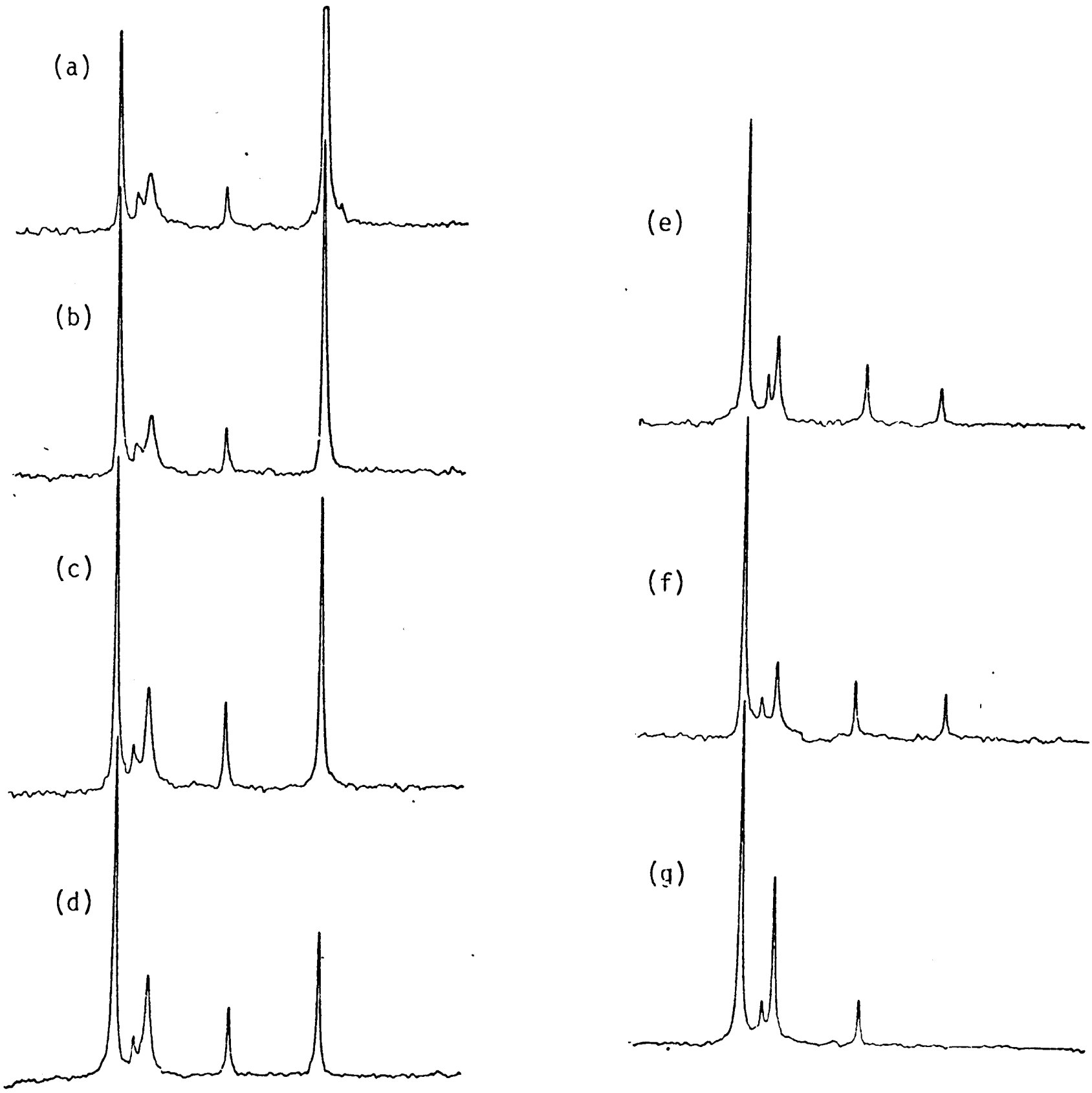
<u>Phosphorus n.m.r. of triose phosphate in the presence of triosphosphate isomerase</u>

N.m.r. spectra of triose phosphate were accumulated at 1°C in 2 hours or less, to ensure that decomposition of substrate did not become significant. The solutions contained concentrations of the isomerase such

Fig. 4.5. Phosphorus n.m.r. spectra of triose phosphates in the presence of triosephosphate isomerase, at pH 6.9 and 1°C.

(a) 7.2mM triose phosphate, 2.0mN isomerase; (b) 11.6mM triose phosphate, 1.8mN isomerase; (c) 15.2mM triose phosphate, 1.5mN isomerase; (d) 21.0mM triose phosphate, 1.2mN isomerase; (e) 30.8mM triose phosphate, 0.59mN isomerase; (f) 13.1mM triose phosphate, 0.14mN isomerase; (g) equilibrium mixture of triose phosphates with no isomerase.

For peak assignments, see Fig. 4.2.



that the species handled by the enzyme were in the fast exchange limit: one resonance was observed for enzyme-bound species, free DHAP and free GAP. Peaks were also observed for the free hydrates of DHAP and of GAP. The peak widths of the triose phosphate species were compared with that of an internal standard, triethyl phosphate, to allow for such effects as instrumental and viscosity broadening. This species has no observable affect on the isomerase activity in the concentration range used (K_i > 40 mM).

A series of proton-decoupled spectra were obtained at pH 6.9 for different ratios of enzyme to triose phosphate and are shown in Fig. 4.5. In Fig. 4.6 and 4.7, the peak widths and peak positions from these spectra are plotted against the ratio of enzyme to total triose phosphate. As a guide to the amount of triose phosphate bound to the enzyme, the K_s-value of 0.7 mM for the keto form of DHAP, determined at pH 7.5 and 30° gives 34% bound keto form at the highest enzyme-substrate ratio. In spite of the change in conditions, (to pH 6.9 and 1°), this is probably a reasonable order of magnitude estimate for the amount of keto form bound.

In the general case, for two species A and B in rapid chemical exchange with each other such that a proportion x is in the form B, then the peak position of the resulting single resonance is given by:

$$w = w_A + (w_B - w_A)\, x \qquad (4.1)$$

where w_A and w_B are the peak positions, when there is no exchange between the two species. Similarly, the peak width at half height, Δw_{obs} of the resulting single peak is a function of the spin-lattice relaxation times, T_2 of the separate species, when the species are in rapid exchange:

$$\Delta w_{obs} = \frac{1 - x}{(T_2)_{A^{\pi}}} + \frac{x}{(T_2)_{B^{\pi}}} + k \qquad (4.2)$$

where k is a constant depending on the characteristics of the instrument and solution. The peak width of an internal standard, s is given by

$$(\Delta w_{obs})_s = \frac{1}{(T_2)_{s^{\pi}}} + k \qquad (4.3)$$

so that from Eqs. 4.2 and 4.3:

$$\Delta w_{obs} - (\Delta w_{obs})_s = \left[\frac{1}{(T_2)_{A^{\pi}}} - \frac{1}{(T_2)_{s^{\pi}}}\right] + x\left[\frac{1}{(T_2)_{B^{\pi}}} - \frac{1}{(T_2)_{A^{\pi}}}\right] \qquad (4.4)$$

if the relaxation rate of species B is slower than that of A, then the broadening of the observed peak relative to the internal standard is proportional to the fraction of material in form B.

It is clear from Fig. 4.6 that as the concentration of enzyme increases, the keto peak broadens markedly. This broadening is best explained by the binding of a fraction of the substrate to the enzyme, such that the enzyme-bound species have a shorter average T_2. Thus Eq. 4.4 may be considered to hold, with species A being the keto form of DHAP and species B being enzyme-bound substrate. The effect of unbound keto form of GAP will be very small as it forms only a very small proportion (approximately 1/400) of the total substrate. The enzyme-bound substrate will give an average signal due to all enzyme bound species which are in rapid equilibirum. The extent of broadening of the keto peak of DHAP is a function of the average T_2 of the enzyme-bound species and the proportion of keto form of substrate that is bound to the enzyme, as in Eq. 4.4.

Fig.4.7. Plot of chemical shifts (w p.p.m.) against isomerase/triose phosphate ratio (E/T) for spectra at pH6.9 and 1°C.

—●—, triethyl phosphate; —△—, inorganic phosphate; —■—, dihydroxyacetone phosphate keto form; —○—, D-glyceraldehyde phosphate hydrate; —▼—, dihydroxyacetone phosphate hydrate.

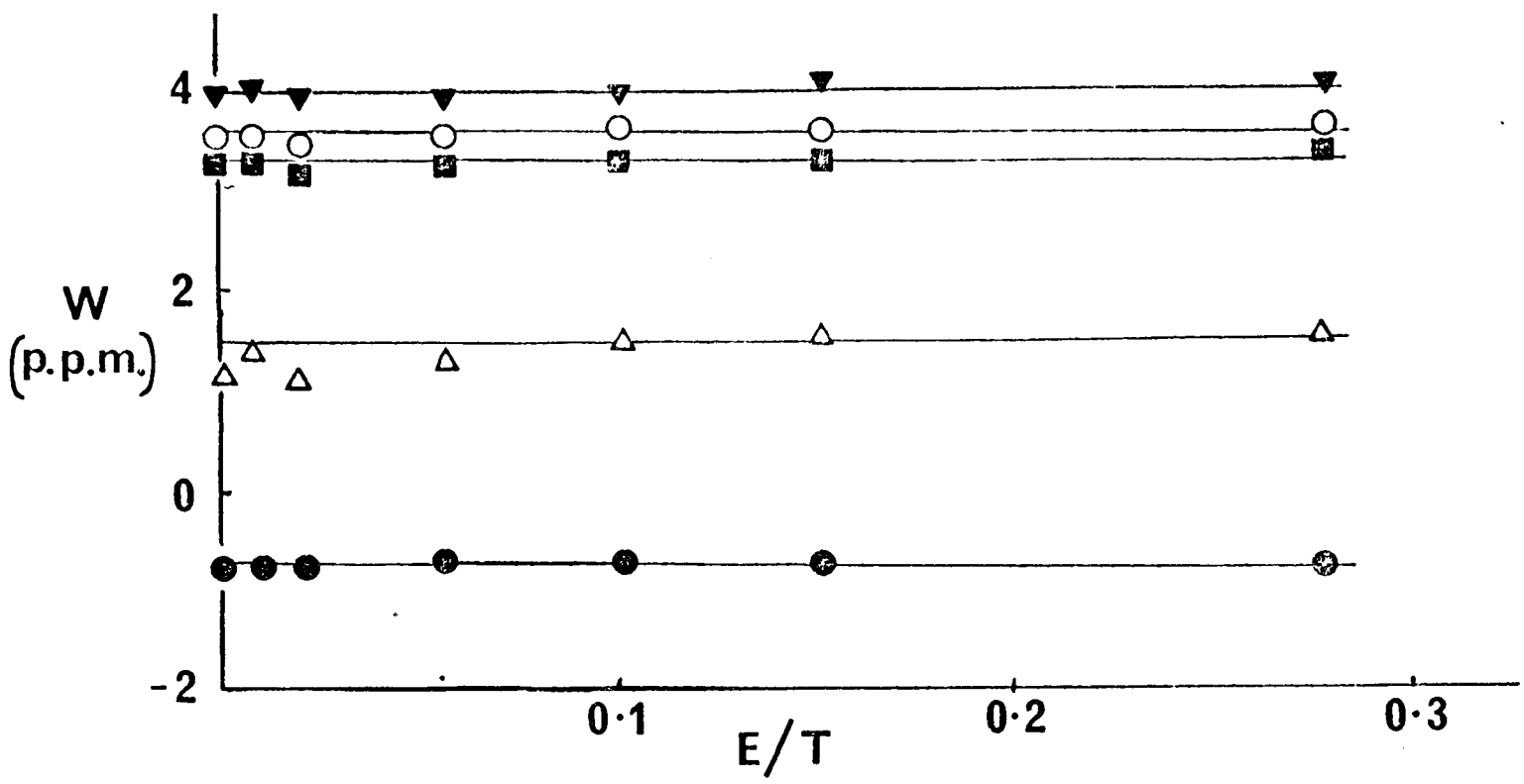
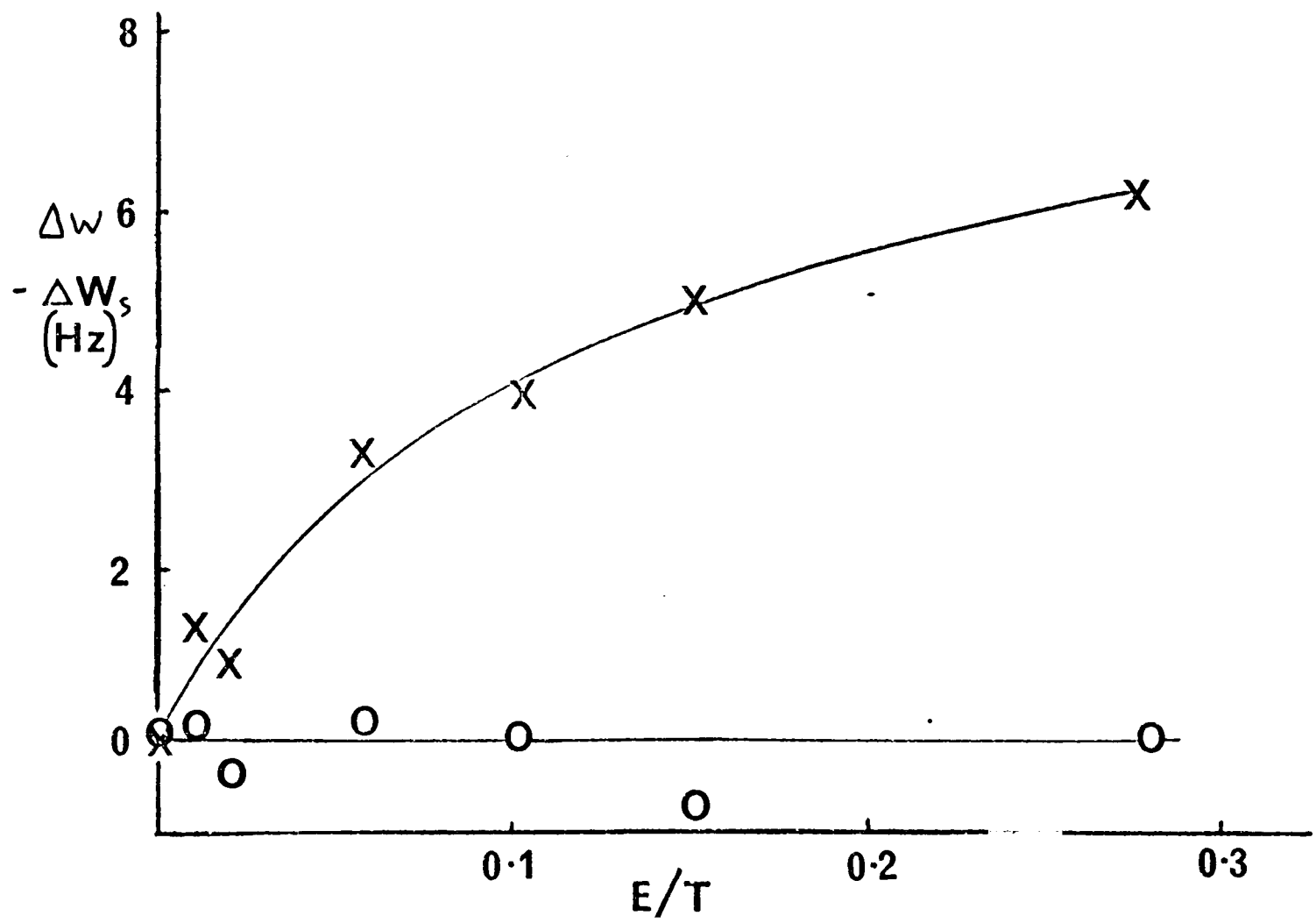


Fig. 4.6. Plot of peak widths (ΔW Hz) against isomerase/ triose phosphate ratio (E/T) for spectra at pH 6.9 and 1°C.

—X—, dihydroxyacetone phosphate keto form; —O—, dihydroxyacetone phosphate hydrate



An approximate value for the average peak width of the enzyme-bound substrate may be obtained by assuming that at large enzyme:substrate ratio (E/T), the concentration of substrate is much larger than K_m so that all the enzyme has substrate bound to it. In this case, the fraction of keto form bound to the enzyme (x) is equal to $E(1 + K_h)/T$ where K_h is the ratio of hydrate to keto form of DHAP in free solution. Thus at low E/T ratio, the graph in Fig. 4.6 represents a plot of Eq. 4.4. Extrapolation of the tangent to the curve at low E/T ratio to a value $1/1 + K_h$ gives the peak width of the enzyme-bound species relative to that of the internal standard. At pH 6.9 and 1°C, an approximate value for this broadening is 20 Hz, whereas the peak width of DHAP in free solution is the same as that of the internal standard.

Such broadening of a phosphate peak on binding to a protein has been observed previously in several systems[110-113] and Hull et al.[113] obtained a similar value for the peak width of inorganic phosphate bound to alkaline phosphatase. Such broadening is generally related to the larger correlation time of the macromolecule-ligand complex than that of the ligand alone. However, such a relationship has not been well studied for phosphorus nuclei.

Fig. 4.6 also shows that there is no broadening of the DHAP hydrate peak, even at high enzyme concentrations. The two explanations of this are either that the hydrate does not bind to the enzyme, or that it does bind but the T_2 of the enzyme-bound species is exactly the same as that of the hydrate in free solution. The latter explanation seems very unlikely in view of the results with the keto form of DHAP and the results of other studies of phosphate species binding to proteins.[110-113]

It would also require a binding mode very different from that of the keto form. The more likely explanation of the lack of broadening of the DHAP hydrate peak is that it does not bind tightly enough to the enzyme to produce detectable broadening. Assuming that the bound hydrate would give a similar peak width to the keto form, then the lower limit of the dissociation constant, K_d, for the hydrate can be estimated to be approximately one order of magnitude larger than the K_s of DHAP under the conditions studied.

It appears, therefore, that the hydrate of DHAP does not bind to the isomerase to any extent that would require modification of the kinetic and equilibrium parameters of the isomerase-catalyzed reaction. The hydrate probably binds to TIM no better than such species as inorganic phosphate, monohydroxyacetone phosphate and L-glycerol 3-phosphate, which all have K_i-values in the range 4 to 6 mM. Very little isomerase is "wasted" in non-productive binding of the hydrate.

In Fig. 4.7 is plotted the chemical shift (relative to 85% H_3PO_4) of the phosphorus resonances as the ratio of enzyme to substrate varies. All the peaks remain at the same chemical shift over the concentration range used. It is slightly surprising that the keto peak does not shift, as a proportion of the material becomes bound to the enzyme. The lack of any shift requires that the average peak position for bound substrate species is the same as that of the keto form of DHAP in free solution. However, the shift of phosphate signals is relatively insensitive to the environment of the group, while very sensitive to the bond angles at the phosphorus atom.[115] It therefore seems likely that the phosphate dianion is bound without distortion in the active site.

Binding of substrate at pH 5.5

The pH-dependence of the triosephosphate isomerase-catalyzed reaction has been studied using the dehydrogenase-coupled reactions in either direction. The value of k_{cat} was found to decrease as the pH was lowered from 7.5 and it titrates with an apparent pK_a of approximately 6. The value of K_m remains constant in the range 5.5 to 7.5. The second pK_a of DHAP is 6.45. The n.m.r. approach used in this work should be useful in studying the binding of DHAP and perhaps give information about the binding of the monoanionic substrate.

An attempt was made to obtain a series of spectra of triose phosphate in the presence of the enzyme at pH 5.5, 1°C (Fig. 4.8). It was found that the enzyme was soluble only to 40 mg/ml and lowering the pH of a more concentrated solution to 5.5 resulted in partial precipitation of the protein. However, after removal of precipitated protein, the solution was reasonably stable (less that 10% loss of activity) when maintained at 1° for 6 hours.

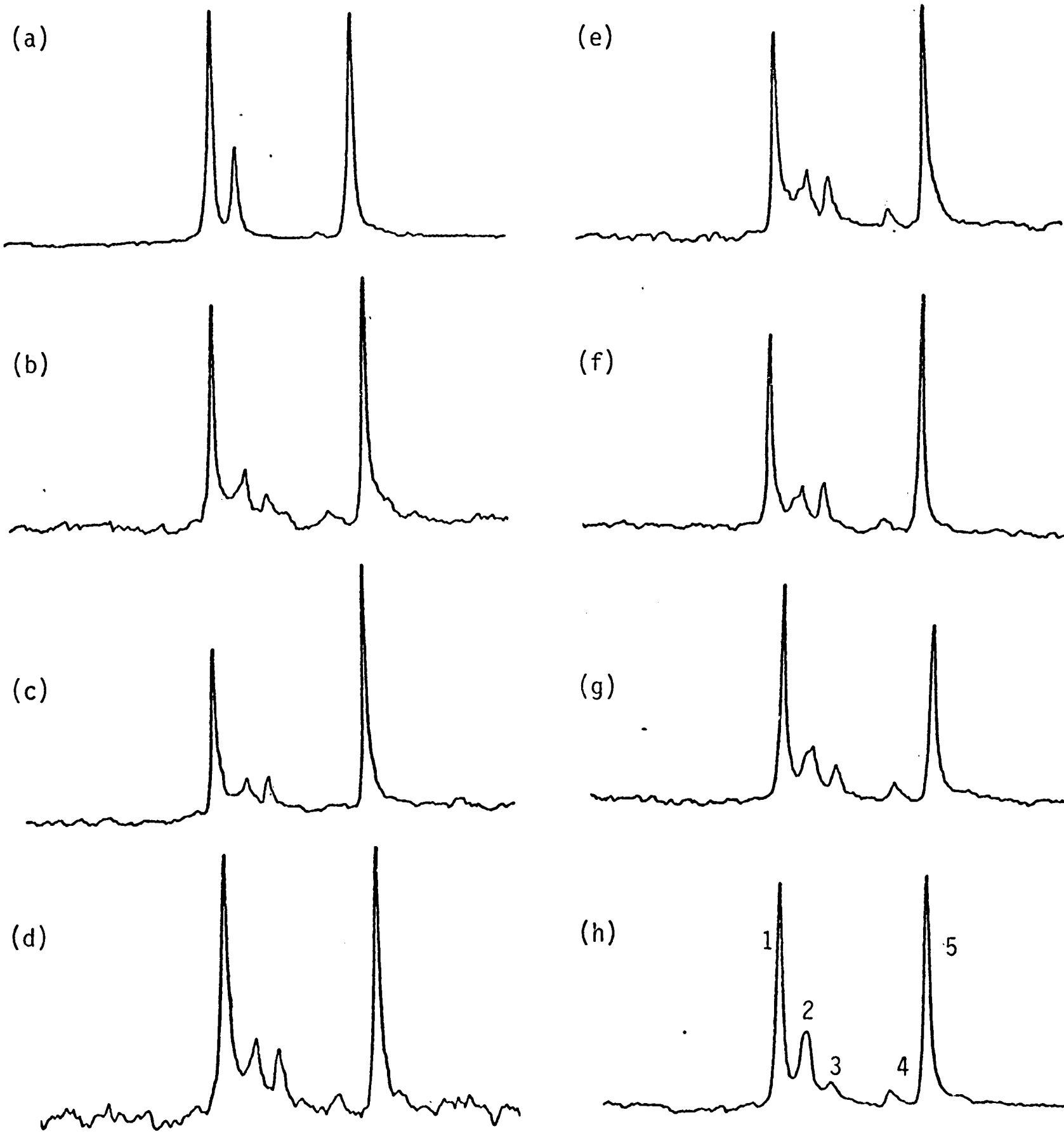
The phosphorus n.m.r. of triose phosphate at 1°, pH 5.5 (Fig. 4.2) shows that all the peaks have shifted upfield due to the protonation of the phosphate group. The GAP hydrate peak has shifted relative to the peak of the keto form of DHAP and so it now lies slightly upfield of it.

When the spectrum was run in the presence of a large concentration of isomerase, a new peak appeared. This probably resulted from a very slight impurity of aldolase in the isomerase, which catalyzed the slow formation of fructose 1,6-bisphosphate. Because this compound was effectively removing triose phosphate from solution, the concentration of the latter was indeterminate. It was not possible, therefore, to obtain a

Fig. 4.8. Phosphorus n.m.r. spectra of triose phosphates in the presence of triosephosphate isomerase at pH 5.5 and 1°C.

(a) Dihydroxyacetone phosphate; (b) 6.9mM triose phosphate, 1.5mN isomerase; (c) 9.1mM triose phosphate, 1.5mN isomerase; (d)11.2mM triose phosphate, 1.4mN isomerase; (e) 13.2mM triose phosphate, 1.4mN isomerase; (f)16.2 triose phosphate, 1.3mN isomerase; (g) 21.8mM triose phosphate, 1.3mN isomerase; (h) 35.1mM triose phosphate, 1.1mN isomerase.

Peak assignments: 1. dihydroxyacetone phosphate hydrate; 2. keto form and glyceraldehyde phosphate hydrate (overlapping); 3. fructose 1,6-bisphosphate (?); 4. inorganic phosphate; 5. triethyl phosphate.



good signal-to-noise ratio, and the peaks were too ill-defined to allow an accurate measurement of the peak width of the keto form of DHAP. Qualitatively it appears that the resonance due to the keto form of DHAP was very broad at high enzyme concentration and became narrower as the enzyme concentration decreased. The DHAP hydrate peak remained at a similar peak width to triethyl phosphate at all enzyme concentrations.

Further work is required at this lower pH to evaluate the effect of pH upon the binding of DHAP to the isomerase.

CHAPTER 5 EXPERIMENTAL

Instruments

Scintillation counting was done on a Beckman LS-233 scintillation counter, using a scintillation cocktail (6.5 ml) containing toluene (4.2 ml), naphthalene (0.37 g), 2,5-diphenyloxazole (18 mg) and 1,4-di [2-(5-phenyloxazolyl)] benzene (0.73 mg).

Measurements of pH were made on a Radiometer PHM 64 pH meter standardized against Fisher Scientific Co. standard buffer solutions.

Ultraviolet absorbance measurements were made with a Unicam SP 1800 instrument fitted with a Unicam AR 25 recorder.

Mass spectrometry was done using an AEI-MS9 instrument.

N.m.r. spectra of triose phosphate were obtained Mr. D. N. Standring at 40.5 MHz (for ^{31}P) and 100.1 MHz (for ^{1}H) on a Varian XL-100-15 spectrometer equipped with a Varian 620-i 16K computer. Other proton n.m.r. spectra were obtained on a Varian T-60 spectrometer.

Polyacrylamide-gel electrophoresis was carried out on a Bio-Phore apparatus from Bio-Rad Laboratories. Stained gels were scanned using the gel scanning attachment of the SP 1800 spectrophotometer.

Chicken muscle was minced by a Waring blender. Centrifugation was on a Sorvall RC-5 centrifuge at 3°.

Enzymes

The following were obtained from Sigma Chemical Company, St. Louis, Missouri 63178, U.S.A.: glycerol kinase, acid phosphatase, glycerolphosphate dehydrogenase, aldolase, fructose-1,6-bisphosphatase, glucose-6-phosphate isomerase, glucose-6-phosphate dehydrogenase, pyruvate kinase, lactate

dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. Mannose-6-phosphate isomerase was obtained from Boehringer Mannheim Corp., New York, New York 10017, U.S.A.

For early experiments, triosephosphate isomerase was prepared from chicken muscle by Mr. J. Law by the method of Putman et al.[21]

Substrates and coenzymes

The following were obtained from Sigma as the sodium salts: phosphoenolpyruvate, ATP, NAD^+, NADH, $NADP^+$, DL-glycerol 3-phosphate, pyruvate, mannose 6-phosphate, fructose 6-phosphate, glucose 6-phosphate and fructose 1,6-bisphosphate. DL-glyceraldehyde 3-phosphate (as the diethyl acetal of the monobarium salt) was from Sigma and D-glyceraldehyde 3-phosphate (as the diethyl acetal of the dicyclohexylammonium salt) was from Boehringer. These were converted to the free substrates according to the manufacturers' data sheets.

Column materials

Diethylaminoethyl cellulose anion exchange resin (DEAE 52), QAE-Sephadex, Sephadex G150, Dowex-1 (chloride form, 200-400 mesh, 8% cross linked) and Dowex-50 (hydrogen form, 100-200 mesh, 4% cross linked) were all from Sigma. Bio-Gel P10 polyacrylamide gel (100-200 mesh) and Bio-Rex-40 (hydrogen form, 100-200 mesh) were obtained from Bio-Rad Laboratories, Rockville Center, New York 11570, U.S.A.

Other chemicals

Triethanolamine hydrochloride, tris (hydroxymethyl) amino methane, ethyldiamine tetraacetic acid (disodium salt) and bis(trimethylsilyl) trifluoroacetamide (BSTFA) were from Sigma.

Tritiated water (5 Ci/ml) was from The Radiochemical Centre, Amersham, Bucks., U.K. Sodium boro[^{3}H]hydride (570 Ci/mol and 8.2 Ci/mmol) were also from the Radiochemical Centre and were stored at -20°C as solutions in 1M NaOH (50 mCi/ml and 200 mCi/ml, respectively).

Ammonium sulphate, especially low in metals for enzyme preparations was purchased from Accurate Chemical and Scientific Corp., Hicksville, New York 11801, U.S.A.

All other chemicals were of the highest grade available.

Bromohydroxyacetone phosphate was prepared by Mr. L. M. Fisher, by the method of de la Mare et al.[29]

Preparations

Triosephosphate isomerase was prepared by a modification of the methods of Putman et al.[21] and McVittie et al.[18] Chicken breast muscle (500 g) was minced at 3° and stirred with 1.3 mM EDTA - 1 mM 2-mercaptoethanol, pH 7.5 (500 ml) at 3° for 30 min. This mixture was centrifuged at 1000 g and 3°C for 30 min and the residue was re-extracted with the EDTA-mercaptoethanol solution (250 ml) for 30 min. After centrifugation, the supernatants were combined and filtered through cheesecloth. Finely ground ammonium sulphate was added slowly with stirring to a concentration of 43 g per 100 ml of supernatant. The solution was left overnight and the precipitate was removed by centrifugation at 8500 g and 3°C for 45 min. The supernatant was dialyzed at 3° for 8 hr against 10 mM triethanolamine-HCl buffer, pH 7.7 containing 1 mM EDTA. The protein solution was concentrated to ∿20 mg/ml by dialysis against this buffer containing 30% (w/v) polyethylene glycol. The concentrated protein solution was dialyzed exhaustively against 10 mM tris-HCl buffer, pH 7.7 at 3° and then

Table 5.1. Purification of triosephosphate isomerase from chicken muscle (500g)

	Total protein[a] (mg)	10^{-6} x total activity[b] (units)	Specific activity (units/mg)
Extract	18,512	3.89	210
$(NH_4)_2SO_4$ fraction	8,535	3.77	441
QAE-Sephadex fraction	475	2.98	6273
G150 gel filtration fraction and crystallization	204	1.69	8255

a. The concentration of protein was measured assuming an $E_{280}^{0.1\%}$ of 1.21

b. The activity of the isomerase was measured at 30°C with glyceraldehyde 3-phosphate as substrate.

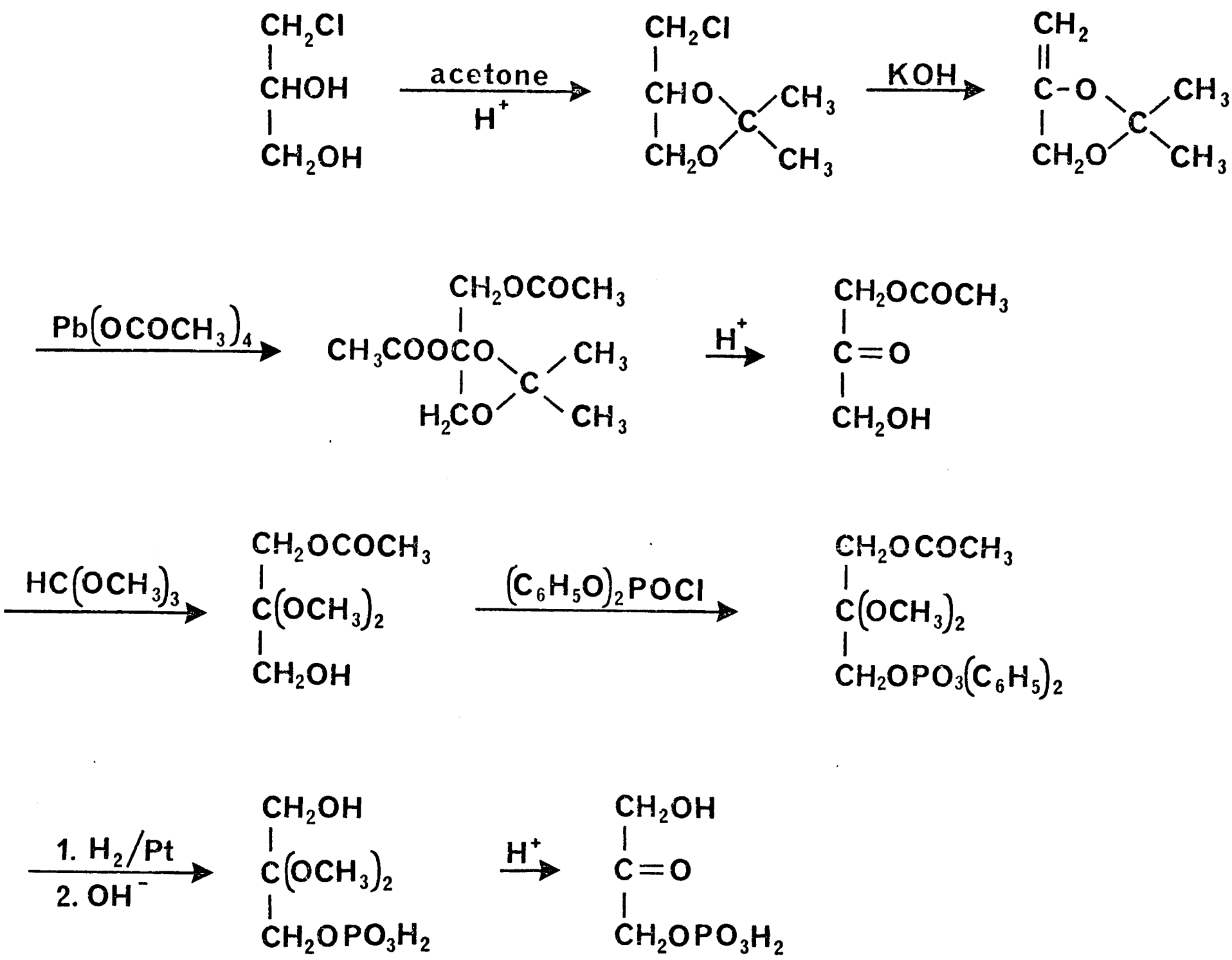


Fig.5.1. Outline of reactions in the preparation of dihydroxyacetone phosphate, by the method of Ballou.[117]

Fig.5.2. Proton n.m.r. of cyclohexylammonium dihydroxyacetone phosphate dimethylketal.

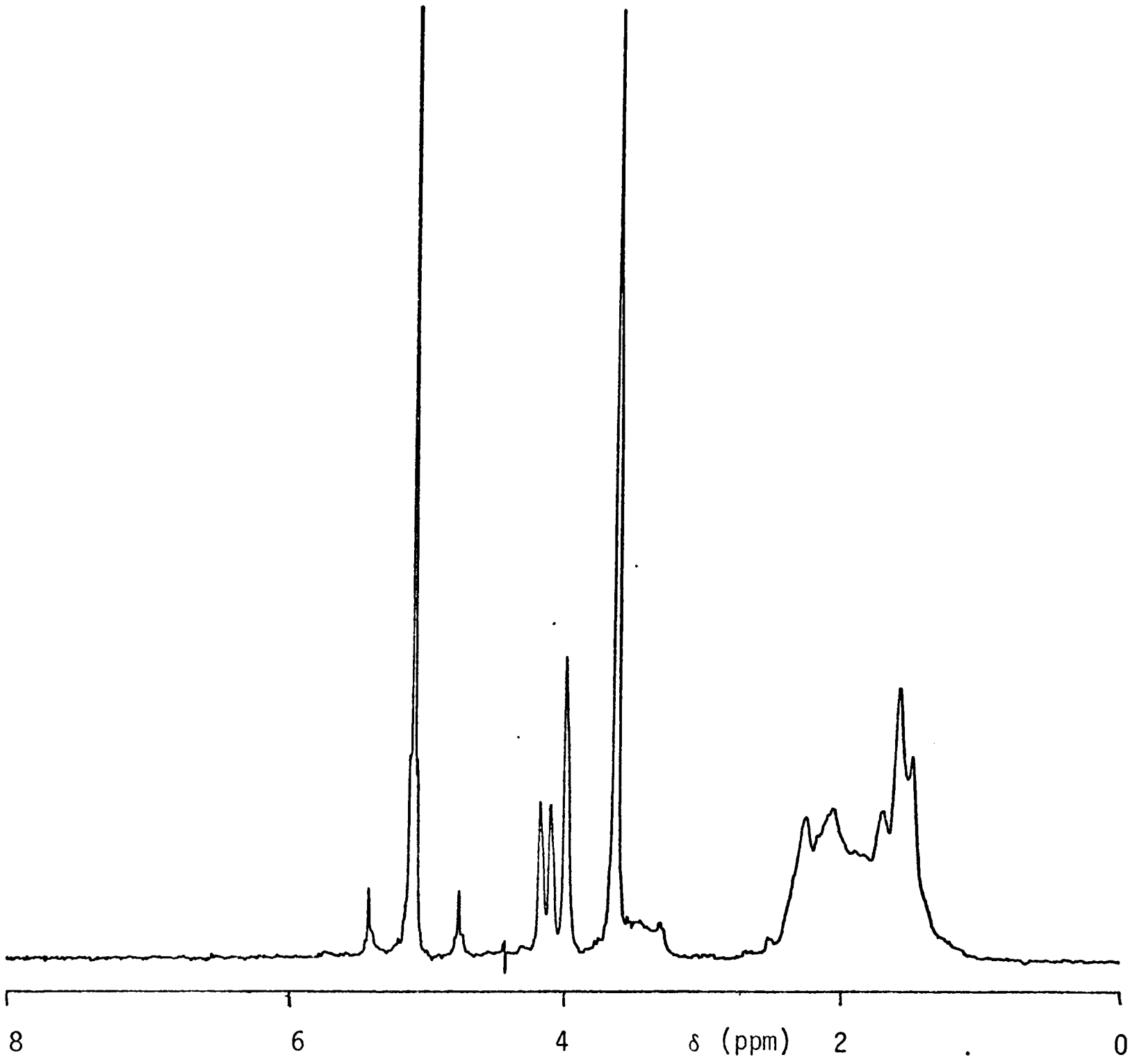
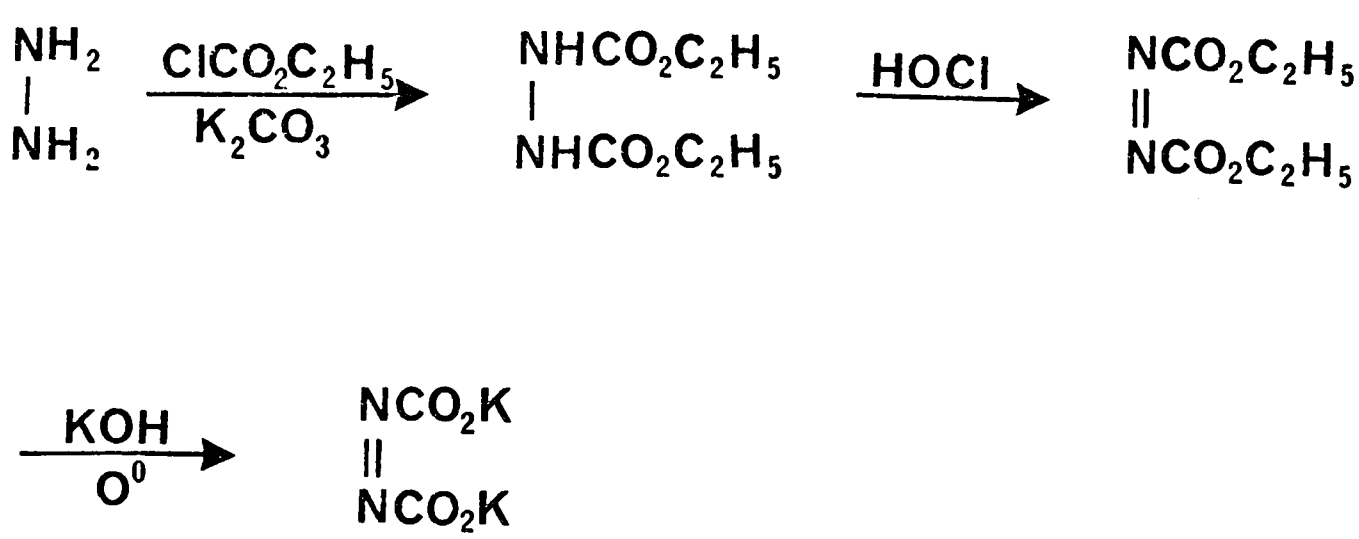


Fig. 5.3. Outline of reactions for the preparation of potassium azodicarboxylate.



subjected to ion-exchange chromatography at room temperature on a column of QAE-Sephadex (5.0 cm x 55 cm) equilibrated with 10 mM tris-HCl buffer, pH 7.2. Protein was eluted by a linear concentration gradient (41) from 10 mM to 100 mM tris-HCl, pH 7.2. Fractions containing triosephosphate isomerase with maximum activity were pooled and concentrated to ∿20 mg/ml by dialysis at 3° against 25 mM-triethanolamine-HCl buffer, pH 7.5 containing 30% (w/v) polyethylene glycol. The solution of the isomerase was then subjected to gel filtration on a column of Sephadex G150 (2.6 cm x 90 cm), equilibrated with 25 mM triethanolamine-HCl buffer, pH 7.5 Fractions containing triosephosphate isomerase with maximum activity were pooled. Finely ground ammonium sulphate was added to this solution until slight turbidity appeared. The solution was left to crystallize. The crystalline enzyme ran as a single band on sodium dodecyl sulphate-polyacrylamide gel electrophoresis at pH 7.1, by the method of Shapiro et al.[116] Table 5.1 shows the yield of isomerase at each step of a preparation.

Dihydroxyacetone phosphate was prepared by the method of Ballou,[117] outlined in Fig. 5.1. The compound was stored as the cyclohexylammonium salt of the dimethyl ketal, the n.m.r. of which is shown in Fig. 5.2. Dihydroxyacetone phosphate was liberated from this precursor by the method of Ballou.[117]

Potassium azodicarboxylate. Diethyl azodicarboxylate was prepared by the method of Rabjohn,[118] outlined in Fig. 5.3. No attempt was made to purify the product by vacuum distillation. Potassium azodicarboxylate was prepared when required by dropwise addition of the ester (0.3 ml) into 5N KOH (2 ml), cooled by an ice bath. A yellow precipitate of the potassium

salt formed as cooled ethanol (2 ml) was added dropwise. The solid was filtered and washed in cooled, dry diethyl ether.

Methods

A) Assays

Triosephosphate isomerase. The concentrations of solutions of triosephosphate isomerase were calculated by assuming an $E_{280}^{0.1\%}$ value of 1.21 for 10 mm light path. The activity of this enzyme was measured by using, slightly modified, the coupled enzyme method of Beisenherz[4] with glyceraldehyde 3-phosphate as substrate.

Glucose-6-phosphate isomerase. The activity of this isomerase was measured by coupling the isomerization of fructose 6-phosphate to glucose 6-phosphate with the latter's oxidation to D-glucono-δ-lactone 6-phosphate catalyzed by glucose-6-phosphate dehydrogenase. The reaction was performed in 200 mM triethanolamine-HCl buffer, pH 7.5 (2.3 ml) containing 10 mM $MgCl_2$, 0.5 mM $NADP^+$, glucose-6-phosphate dehydrogenase (0.1 mg/ml) and 60 mM fructose 6-phosphate. Mannose-6-phosphate isomerase was measured similarly using 60 mM mannose 6-phosphate and glucose-6-phosphate isomerase (0.02 mg/ml).

Dihydroxyacetone phosphate. Concentrations were assayed by measuring the conversion of NADH to NAD^+ during its reduction to L-glycerol 3-phosphate catalyzed by glycerol-3-phosphate dehydrogenase. The assay reaction was performed in 200 mM triethanolamine-HCl buffer, pH 7.5 (2.3 ml) containing 0.4 mM NADH. The decrease in NADH concentration was measured by following the change in absorption at 340 nm. The concentration of D-glyceraldehyde 3-phosphate was measured by a similar method with triosephosphate isomerase (10 μg) in the reaction mixture to catalyze the formation of dihydroxyacetone phosphate.

D-glyceraldehyde 3-phosphate. This was assayed by oxidation by glyceraldehyde-3-phosphate dehydrogenase/NAD^+. The reaction was in 200 mM triethanolamine-HCl buffer, pH 7.5 (2.3 ml) containing 2 mM NAD^+ and 0.3 mM sodium arsenate. The reaction was initiated by addition of 0.25 mg glyceraldehyde-3-phosphate dehydrogenase.

L-glycerol 3-phosphate. The concentration was measured using the oxidation catalyzed by glycerol-3-phosphate dehydrogenase. The reaction was performed in 1 M glycine - 1 M hydrazine buffer, pH 9.3 (2.3 ml) containing NAD^+ (4 mg). The formation of NADH was followed spectrophotometrically.

D-glucose 6-phosphate. This was assayed by its oxidation to D-glucono-δ-lactone 6-phosphate catalyzed by glucose-6-phosphate dehydrogenase/$NADP^+$. The reaction was performed in 200 mM triethanolamine-HCl buffer, pH 7.5 (2.3 ml) containing 1 mM $MgCl_2$ and 0.25 mM $NADP^+$. Addition of glucose-6-phosphate isomerase (0.05 mg) to the assay mixture enabled the concentration of D-fructose 6-phosphate to be measured separately from D-glucose 6-phosphate.

Inorganic phosphate. The concentration of phosphate ions was measured using the method of Briggs.[119]

B) Effect of sodium borohydride on the activity of triosephosphate isomerase

Solutions of the isomerase, glyceraldehyde 3-phosphate and sodium borohydride were made up with concentrations as in Table 5.2 in 200 mM triethanolamine-HCl buffer, pH 7.45. After incubation for 1 hr at room temperature the relative enzyme activity was measured.

Table 5.2. Effect of sodium borohydride on the activity of triosephosphate isomerase

Isomerase (μM)	D-Glyceraldehyde 3-phosphate (mM)	Sodium borohydride(mM)	Relative enzyme activity
55.6	-	-	100
55.6	0.56	-	100
55.6	-	0.25	95
55.6	-	0.50	100
55.6	0.56	0.50	100
55.6	-	1.25	100
55.6	0.56	1.25	100
55.6	-	2.50	96
55.6	0.56	2.50	100

C) Preparation and analysis of tritiated glycerol 3-phosphate

1) DL-[2-^{3}H]Glycerol 3-phosphate was prepared by reduction of dihydroxyacetone phosphate with sodium boro[^{3}H]hydride. Dihydroxyacetone phosphate (0.4 μmol) and sodium boro[^{3}H]hydride were incubated in 200 mM triethanolamine-HCl buffer, pH 7.5 (1.0 ml) at room temperature for 2 hr. DL-Glycerol 3-phosphate (4 mg) was added as carrier and the combined glycerol phosphate was purified on a column (15 cm x 0.9 cm) of DEAE-cellulose, equilibrated with 5 mM NH_4HCO_3 (pH 8.3) and eluted with a linear gradient (50 ml) from 5 mM to 300 mM NH_4HCO_3. The glycerol phosphate (with constant specific radioactivity across the peak) was freeze dried and stored at 4°C.

2) For analysis of the stereochemistry of labelling by tritium at C-2, [^{3}H]glycerol 3-phosphate was prepared in the presence of triosephosphate isomerase by incubating together sodium boro[^{3}H]hydride (3.75 mCi of 570 mCi/mmol material), dihydroxyacetone phosphate (0.4 μmol) and the isomerase (3 ml of a solution 1.5, 6.5 or 30 mg/ml), previously dialyzed against 100 mM NH_4HCO_3 containing 1 mM EDTA, at pH 7.3 to 7.5, 20°C for 3 hr. The catalytic activity of the isomerase was always determined immediately before the incubation. After the incubation, protein was removed by gel filtration through P10 polyacrylamide gel (22 cm x 1.6 cm). DL-Glycerol 3-phosphate was added as carrier, and glycerol phosphate was isolated on a column of DEAE-cellulose as described above. In a control experiment, dihydroxyacetone phosphate was reduced by sodium boro[^{3}H]hydride in the presence of triose-phosphate isomerase that had been completely and specifically inactivated by bromohydroxyacetone phosphate.[29]

3) For the analysis of stereochemistry of labelling at C-1, [^{3}H]glycerol 3-phosphate was prepared as described above except for the following concentrations: sodium boro[^{3}H]hydride (11.71 mCi of 1.6 Ci/mmol material) and triosephosphate isomerase (3 ml of a solution of 7.2 or 9.5 mg/ml). No DL-glycerol 3-phosphate was added prior to isolation.

4) Preparation of [^{3}H]glycerol 3-phosphate from 3-phosphohydroxy-acetonyl-triosphosphate isomerase. Bromohydroxyacetone phosphate concentration was estimated from the rapid stoichiometric inactivation of known concentrations of triosephosphate isomerase. The enzyme activity was measured 15 min after each addition of bromohydroxyacetone phosphate. Triosephosphate isomerase (0.5 ml of a 14 mg/ml solution), previously dialyzed against 100 mM NH_4HCO_3, pH 7.6 was assayed for enzymic activity

and cooled to 0°C. A 1.2 molar excess of bromohydroxyacetone phosphate was added and the pH was adjusted immediately to 7.5 by addition of 2N NaOH. After 10 min at 0°C, the solution was assayed to check that all the enzyme had been inactivated.

Sodium boro[^{3}H]hydride (2 mCi, 15.4 μmol) was added to this solution of 3-phosphohydroxyacetonyl-triosephosphate isomerase, the pH was readjusted to 7.5 by addition of 1N HCl, and the solution was incubated for 3 hr at 20°C. The protein was isolated by gel filtration through a column (22 cm x 1.6 cm) of P10 polyacrylamide gel. 2N NaOH was added to the pooled fractions until the pH was 10.2 and incubation at this pH for 2 hr at 60° achieved hydrolysis of the 3-phospho-[2-^{3}H]glyceryl phosphate isomerase ester linkage. A control experiment showed that the product, 2-[^{3}H]glycerol 3-phosphate is stable under these conditions. The hydrolysate was then subjected to gel filtration as above. The eluate showed two radioactive peaks. The earlier, smaller peak corresponded to protein and contained only 13% of the radioactivity; the later, larger peak corresponded to the expected elution position of glycerol 3-phosphate. To the pooled fractions of this later peak was added DL-glycerol 3-phosphate as carrier and the material was purified on DEAE-cellulose as described above. This product was freeze dried and stored at 4°C.

5) DL-[1(RS)-^{3}H]Glycerol 3-phosphate was prepared by the reduction of DL-glyceraldehyde 3-phosphate (4 μmol) by sodium boro[^{3}H]hydride (1.25 mCi), which were incubated together in 200 mM triethanolamine-HCl buffer, pH 7.5 (0.7 ml) for 2 hr at room temperature. The glycerol 3-phosphate was purified by ion-exchange chromatography as described above. D-[1-(RS)-^{3}H]Glycerol 3-phosphate was prepared by the reduction of

D-glyceraldehyde 3-phosphate (2 μmol) by sodium borohydride (2.5 mCi), from which the product was obtained as above.

6) DL-[1 (S)-^{3}H]Glycerol 3-phosphate was prepared as follows. Aldolase was treated with bromohydroxyacetone phosphate to inactivate any triosephosphate isomerase impurity and the enzyme solution was then dialyzed against 200 mM triethanolamine-HCl buffer, pH 7.5. A portion (0.1 mg) of this solution was added to dihydroxyacetone phosphate (0.5 μmol) in tritiated water (120 μl of a solution, 4 Ci/ml). Aldolase catalyzes the exchange of the 1-pro-S-hydrogen of dihydroxyacetone phosphate with the solvent.[33] After incubation for 2 hr, the solution was freeze-dried to remove tritiated water. The residue was dissolved in 0.1 M HCl (2 ml) and kept at pH 1.5 for 5 min to inactivate the enzyme. The pH of the solution was raised to 8.0 with triethanolamine. Sodium borohydride (10 mg) was added and the solution was left for 3 hr at room temperature to reduce the [1(S)-^{3}H]dihydroxyacetone phosphate to DL-[1(S)-^{3}H]glycerol 3-phosphate. The product was then purified on DEAE-cellulose as described above.

D) Analysis of [^{3}H]glycerol 3-phosphate for stereochemistry of labelling at C-2 (The reaction scheme for this analysis is outlined in Fig. 2.3.)

Analysis of [^{3}H]glycerol 3-phosphate for the L-[2-^{3}H]enantiomer.

The [^{3}H]glycerol phosphate was dissolved in 50 mM NH_4HCO_3 to give a solution, approx. 0.2 mM in L-glycerol phosphate. To a portion (2 ml) of glycerol phosphate solution was added NAD^+ (8 mg) and hydrazine hydrate (200 μl), after which the pH was adjusted to 9.4 with 1 M HCl. The formation of NADH was measured spectrophotometrically after the addition of the glycerol-3-phosphate dehydrogenase (200 μl of 2 mg/ml solution, previously dialyzed

against 20 mM triethanolamine-HCl buffer, pH 7.45). The formation of NADH was quantitative under these conditions. NAD^{3}H and the remaining [^{3}H]-glycerol phosphate (the D-enantiomer) were separated by chromatography on DEAE-cellulose as described above. From the specific radioactivity of NAD^{3}H, the proportion of L-[^{3}H]glycerol phosphate present in the original mixture could be calculated. The D-[^{3}H]glycerol 3-phosphate that remained was freeze-dried.

Analysis of [^{3}H]glycerol 3-phosphate for the D-[2-^{3}H]enantiomer.

The D-glycerol 3-phosphate was dissolved in water (4 ml) and the pH adjusted to 5.6 with 1 M HCl. Incubation with acid phosphatase (5 μg) overnight at 37°C hydrolyzed the ester to glycerol. Enzyme was removed by shaking the solution with DEAE-cellulose (4 ml of slurry). To the filtered solution of glycerol was added $MgCl_2$ (2 mg), ATP (8 mg) and glycerol kinase (0.5 ml of a 0.2 mg/ml solution, previously dialyzed against 20 mM triethanolamine-HCl buffer, pH 7.45). The pH was adjusted to 9.0 with 1 M NaOH and the solution incubated at room temperature for 2 hr. Under these conditions glycerol kinase catalyzes the formation of L-glycerol 3-phosphate from glycerol and ATP.[120] The oxidation of L-glycerol 3-phosphate, formation of NADH and isolation of NAD^{3}H proceeded as above. From the specific radioactivity of NAD^{3}H, the amount of D-[2-^{3}H]glycerol 3-phosphate present in the original mixture was calculated.

E) Analysis of [^{3}H]glycerol 3-phosphate for stereochemistry of labelling at C-1 (The reaction scheme for this analysis is illustrated in Fig. 2.4.)

Analysis of [^{3}H]Glycerol 3-phosphate for the [1(R)-^{3}H] Isomer.

The freeze-dried [^{3}H]glycerol 3-phosphate was dissolved in water (1 ml) to which was added acid phosphatase (10 μg) and then 1 N HCl until the pH of the solution was 5.6. Incubation for 12 hr at 37°C resulted in complete hydrolysis of the phosphate ester. The solution was passed through a small volume (4 ml) of DEAE-cellulose resin, equilibrated in 5 mM NH_4HCO_3 to remove any unreacted glycerol phosphate and enzyme. The glycerol was then phosphorylated by addition of ATP (10 mg), $MgCl_2$ (5 mg), and glycerol kinase (0.04 mg). After raising the pH to 8.8 by addition of 1 N NaOH, the solution was incubated for 2 hr at 25°C. L-[^{3}H]Glycerol 3-phosphate was purified from this solution by chromatography on DEAE-cellulose as described above. The fractions containing glycerol 3-phosphate were freeze-dried and then dissolved in water (3 ml). Portions of this solution were converted to D-fructose 1,6-bisphosphate as described below.

A solution (0.7 ml) containing aldolase (1.5 mg), glycerol-3-phosphate dehydrogenase (0.5 mg), and lactate dehydrogenase (0.25 mg) in 200 mM triethanolamine-HCl buffer, pH 7.5 was treated with bromohydroxyacetone phosphate (0.1 μmol) and then dialyzed against 50 mM triethanolamine-HCl buffer, pH 7.5. An assay for triosephosphate isomerase activity showed that this treatment had inactivated all traces of isomerase.

To each portion (1 ml) of L-[^{3}H]glycerol 3-phosphate were added NAD^+ (15 mg), sodium pyruvate (5 mg), DL-glyceraldehyde 3-phosphate

(0.2 ml of a 45 mM solution), and the isomerase-free enzyme mixture prepared above. After raising the pH to 9.6 by addition of 1 N NaOH, the solution was left for 10 min at room temperature. Under these conditions, dihydroxyacetone phosphate, an intermediate in this enzyme-catalyzed formation of D-fructose 1,6-bisphosphate, is stable. To terminate the reaction, the solution was rapidly mixed with Dowex-50 resin (4 ml, H^+ form). The solution was washed from the resin with approximately 10 ml of water and the pH of the combined washings was raised to 8.0 with 1 N NaOH. Purification on DEAE-cellulose, as above, gave three radioactive peaks, corresponding to glycerol 3-phosphate, NAD^+, and D-fructose 1,6-bisphosphate. The fractions containing D-fructose 1,6-bisphosphate from each portion of [^{3}H]-glycerol phosphate were pooled and freeze-dried.

This preparation of D-fructose 1,6-bisphosphate was dissolved in water (1 ml), to which was added fructose-1,6-bisphosphatase (0.25 mg) and $MgCl_2$ (5 mg). The pH of the solution was rasied to 8.8 with 1 N NaOH and, after incubation for 3 hr at 37°C, D-fructose 6-phosphate was isolated from the solution by ion-exchange chromatography as previously described. This radioactive material was freeze-dried and then redissolved in water (1 ml). Glucose-6-phosphate isomerase (0.1 mg) was added to labilize the 1-*pro*-*R*-hydrogen of D-fructose 6-phosphate and the solution in a sealed flask was incubated for 90 min at 37°C for this exchange to occur. The water was then isolated by vacuum sublimation in a closed system with the sample flask connected to the receiving flask by a short U-tube with a side arm. While the sample was immersed in liquid nitrogen, the system was evacuated and then isolated from the vacuum pump. To effect the sublimation, the liquid nitrogen bath was moved to the receiving flask. After the sublimation was

complete, the residue was dissolved in water (1 ml) and the total radioactivity of the residue and of the distillate was measured. More glucose-6-phosphate isomerase (0.1 mg) was added to the residue, which was again taken through the process of incubation and sublimation to ensure that all exchangeable tritium had been removed from the residue. The proportion of tritium exchanged by the isomerase and removed by distillation gives the proportion of tritium in the 1-pro-R position of the original D-glycerol 3-phosphate (see Fig. 2.4).

Analysis of [^{3}H]glycerol 3-phosphate for the [1(S)-^{3}H] Isomer.

The residue from the above exchange experiment is an equilibrium mixture of labelled D-fructose 6-phosphate and D-glucose 6-phosphate. D-Fructose 6-phosphate (1.5 mg) was added as carrier to this mixture, which was then purified on DEAE-cellulose as previously described. The [^{3}H]hexose 6-phosphate fractions had constant specific radioactivity across the peak and this pooled material was freeze-dried. This residue was redissolved in water (10 ml) to which was added $NADP^+$ (20 mg), $MgCl_2$ (5 mg), glucose-6-phosphate isomerase (0.05 mg), and glucose-6-phosphate dehydrogenase (0.2 mg). The increase in absorbance at 340 nm was followed as $NADP^+$ was reduced to NADPH during the oxidation of hexose 6-phosphate to D-glucono-δ-lactone 6-phosphate, catalyzed by the isomerase (to convert D-fructose 6-phosphate to D-glucose 6-phosphate) and the dehydrogenase. After the reaction was complete, NADPH was isolated on a DEAE-cellulose column (15 cm x 0.9 cm). Elution with a gradient (50 ml) from 150 to 500 mM NH_4HCO_3 gave NADPH with a constant specific radioactivity across the peak. From the specific radioactivity of NADPH and of the hexose 6-phosphate, the proportion of tritium on the 1-pro-S position of D-glycerol 3-phosphate can be calculated.

F) Preparation of deuterated glycerol 3-phosphate and its mass spectral analysis

Dihydroxyacetone phosphate was prepared from its precursor as a solution in deuterium oxide by the method of Ballou,[117] using Dowex-50 (D^+-form) that had been washed in deuterium oxide.

Tris buffer was prepared by freeze-drying 20 mM tris(hydroxymethyl) aminomethane-HCl buffer (pH 7.5) and then freeze-drying deuterium oxide from the residue several times before dissolving this residue in deuterium oxide to give a 20 mM solution of the buffer.

A solution of triosephosphate isomerase in deuterium oxide was prepared. A crystalline suspension of the enzyme (30 mg) was centrifuged and the sediment was dissolved in the minimum volume of the tris buffer in deuterium oxide. This enzyme solution was subjected to gel filtration on a column (16 cm x 1 cm) of P10 polyacrylamide gel previously equilibrated in the tris buffer in deuterium oxide. The eluate (3 ml) containing protein was collected in a sealed flask containing NH_4HCO_3 (80 mg) and assayed. The column was in a system completely sealed from the outside air, except for an air inlet through a drying tube of $CaSO_4$.

To this enzyme solution (0.1 mg/ml or 7 mg/ml) was added tritiated water (20 µl of a sample 5 Ci/ml) and dihydroxyacetone phosphate (2.4 µmole) in deuterium oxide solution. This mixture was left for 30 min to allow complete exchange of the isomerase labile protons and then sodium borohydride (1 mg) was added. After incubation for 3 hr, the mixture was freeze dried. Tritiated [^{2}H]glycerol 3-phosphate was purified by gel filtration on P10 polyacrylamide gel and then by ion exchange chromatography, as previously described. The glycerol 3-phosphate was separated from remaining triose

phosphate by ion-exchange chromatography on a column (15 cm x 1 cm) of Dowex 1-HSO_3^- resin, eluting with a linear HCl gradient (50 ml), pH 3.84 to pH 0. The fractions containing glycerol phosphate were pooled, concentrated and then converted to the free acid form on a column (6 cm x 0.7 cm) of Bio-Rex-H^+ cation exchange resin, eluting with water. The eluate was freeze dried exhaustively.

For mass spectral analysis, the free acid form of [^{2}H]glycerol 3-phosphate was converted to its tetrakis (trimethylsilyl) derivative, by dissolving in dry pyridine (50 µl) and adding bis(trimethylsilyl)-trifluoroacetamide (50 µl). A portion (5 µl) of this solution was sealed in a capillary tube that could be attached to the probe of the mass spectrometer. After 30 min, the seal was broken and the sample was subjected to mass spectroscopy at 60° and 70 eV by direct insertion. The m/e 300 to 450 region was repeatedly scanned.

G) Diimide reduction of triose phosphates

A solution of specifically deuterated triose phosphate (2.4 µmol) and triosephosphate isomerase (0.1 mg/ml or 7 mg/ml) in tritiated deuterium oxide was prepared as described above. Potassium azodicarboxylate (200 mg) was added in portions (20 mg) to this solution, continuously stirred, over a period of 1 hr. Glycerol phosphate was purified from the mixture and subjected to mass spectral analysis as previously described. The amount of glycerol phosphate was determined from the radioactivity associated with it after purification.

H) Phosphorus n.m.r. spectra of triose phosphate in the presence of triosephosphate isomerase

Solutions at pH 6.9 were prepared for use, as follows. All pH measurements were made at 1°C and no correction was made for the presence of deuterium oxide. A solution of dihydroxyacetone phosphate (130 mM) was prepared in 33% (v/v) deuterated water, containing 25 mM triethanolamine-HCl buffer, pH 6.9 and 10 mM EDTA. A concentrated solution of triosephosphate isomerase was obtained by exhaustive dialysis of an ammonium sulphate suspension of the enzyme against 25 mM triethanolamine-HCl buffer, pH 7.5, containing 10 mM EDTA. The enzyme solution was concentrated at 3°C using a Sartorius membrane vacuum filtration apparatus and during this process, it was made 33% (v/v) in deuterium oxide by addition of 25 mM triethanolamine-HCl buffer, pH 6.9 in deuterium oxide, containing 10 mM EDTA. The solution was concentrated to approximately 70 mg/ml in isomerase and then its pH was lowered to 6.9 by dropwise addition of 1N HCl. This solution was finally centrifuged at 2000 g for 30 min to remove any solid particles.

These solutions were combined in various ratios from 2 mM isomerase/7.23 mM triose phosphate to 0.59 mM isomerase/30.8 mM triose phosphate. A small amount of triethyl phosphate was added as an internal standard. Spectra were obtained at 1°C using a sample tube 12 mm in diameter, containing 1.6 to 2.0 ml samples. A vortex plug was used to minimize solution turbulence. Phosphorus spectra were obtained in the Fourier transform mode with 2000 to 8000 accumulations using 15 W broad band proton decoupling and a single side band filter. The pulse width was 20 μsec (corresponding to a 60° tip angle) and the pulse recycle time was 0.5 sec.

For the n.m.r. study at pH 5.5, the solutions were obtained by an analogous method using 25 mM dimethyl glutarate buffer, pH 5.5 containing 10 mM EDTA instead of the triethanolamine-HCl buffer. The isomerase solution, after centrifugation was 40 mg/ml. The solutions were mixed to give ratios of enzyme to substrate from 1.5 mM isomerase/6.9 mM triose phosphate to 1.0 mM isomerase/35.0 mM triose phosphate.

PART II GLUCOSE-6-PHOSPHATE ISOMERASE

CHAPTER 6 INTRODUCTION

Glucose-6-phosphate isomerase catalyzes the interconversion of D-glucose 6-phosphate and D-fructose 6-phosphate and as such the reaction lies directly in the flux of glycolysis (Fig. F.1) After early work to formulate a mechanism for hydrogen transfer, interest has been recently renewed by the realization that this enzyme is not a simple isomerase: its catalytic act is more complicated than the straightforward transfer of hydrogen between carbon centres of the two hexose phosphates. The work described in this part of the thesis is concerned with the way that the catalysis by glucose-6-phosphate isomerase differs from that by triosephosphate isomerase.

Glucose-6-phosphate isomerase has been isolated in crystalline form from such sources as skeletal muscle of rabbit[121] and pig,[122] baker's yeast,[123] brewer's yeast[124] and peas.[125] Most mechanistic and kinetic studies have been on the isomerases from yeast and from rabbit muscle. X-ray crystallographic studies are in progress on the enzyme from pig muscle[126,127,128] and a map has been obtained to 3.5Å resolution,[128] although as yet the amino acid sequence is not available.

Both the crystallographic work[126,127,128] and binding studies[129,130] indicate that glucose-6-phosphate isomerase is a dimer with identical subunits. The results of various molecular weight determinations are shown in Table 6.1 and these indicate that the subunit molecular weight is approximately 63000. The yeast enzyme exists as a mixture of isoenzymes of identical molecular weights,[137,138] while it is uncertain whether there are isoenzymes present in the isomerase from rabbit muscle.[132,139]

Table 4.1. Molecular weight estimations for glucose-6-phosphate isomerase.

Source	Method	Subunit Molecular Weight	Reference
Rabbit	SDS/Ultracentrifugation	64,400	131
	SDS/polyacrylamide gel electrophoresis	64,000	131
	Maleic anhydride/ Equilibrium sedimentation	60,000	131
	Guanidine hydrochloride/ equilibrium sedimentation	70,000	132
	Equilibrium sedimentation	66,000	131
Yeast	Guanidine hydrochloride/ equilibrium sedimentation	70,000	133
	Equilibrium sedimentation	72,000	134
Human Muscle	Ultracentrifugation	61,000	135
	Equilibrium sedimentation	67,000	135
Pig Muscle	X-ray crystallography	60,000	Reported in 136

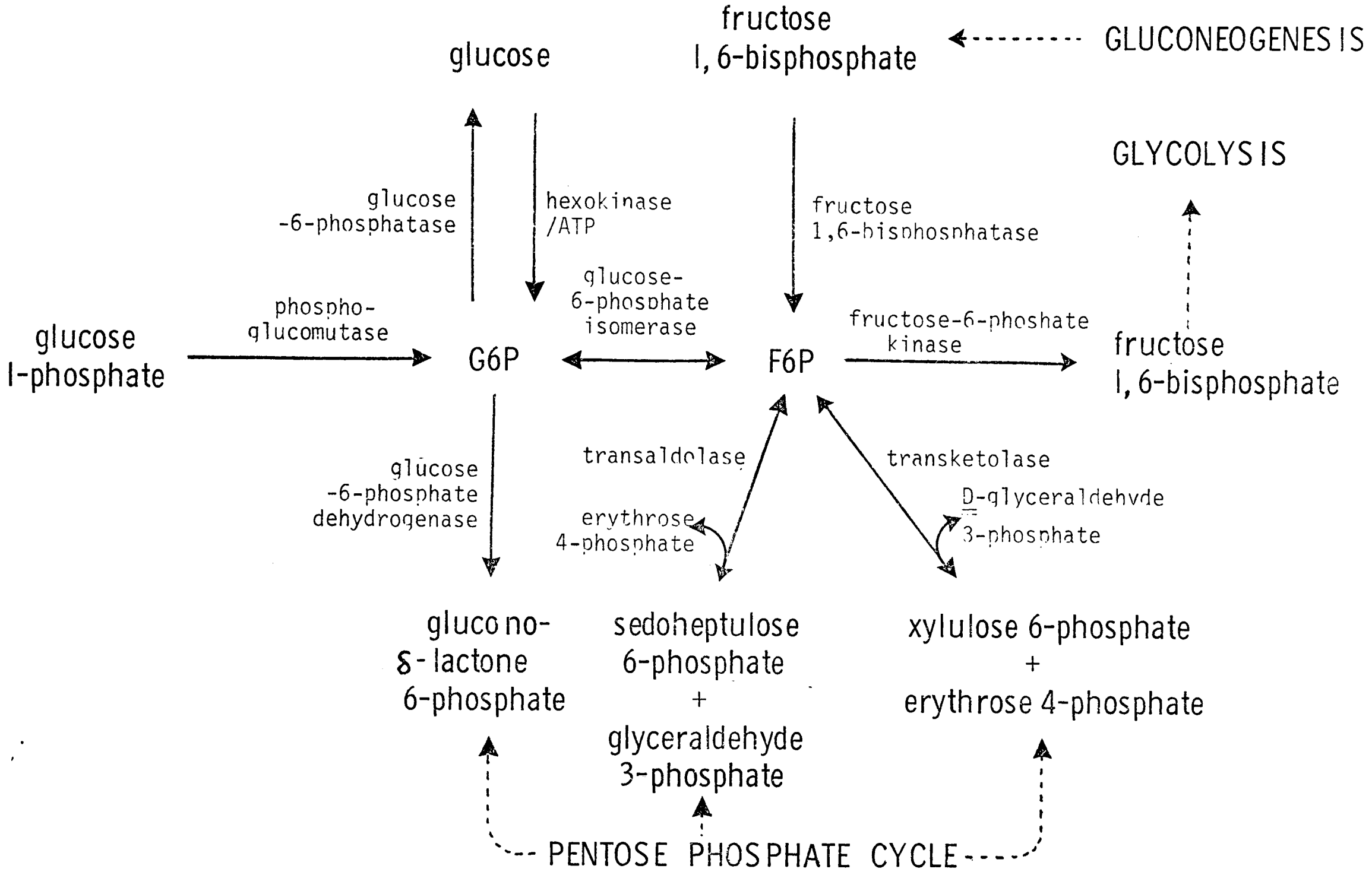


Fig. 6.1. Position of glucose-6-phosphate isomerase at the junction of the pentose phosphate cycle and the glycolytic pathway.

The reaction catalyzed by glucose-6-phosphate isomerase not only lies directly in the glycolytic pathway, but it also stands at the junction with the pentose phosphate cycle. Glucose 6-phosphate is the substrate for glucose-6-phosphate dehydrogenase; and fructose 6-phosphate is the substrate for transaldolase and transketolase (Fig. 6.1). Because of this junction position, it has been postulated that glucose-6-phosphate isomerase may well have some regulatory role dividing the flux of material between the two pathways.[140] Several intermediates of the pentose phosphate cycle are good inhibitors of the isomerase (for example phosphogluconate, erythrose 4-phosphate, and sedoheptulose 7-phosphate: see Table 6.4) and would provide a means for such regulation. However, although the enzyme is not extremely fast (turnover number $\sim 10^5 min^{-1}$, cf, triosephosphate isomerase, $\sim 10^6 min^{-1}$) it has been shown that the rate of glycolysis in vivo is not decreased when glucose-6-phosphate isomerase concentration is only 10% of normal.[141] The question of whether this isomerase is a control enzyme is open, and there has certainly been no specific mechanism proposed for such a function.

Thermodynamic and kinetic parameters

The equilibrium constant between D-glucose 6-phosphate and D-fructose 6-phosphate is 3·3 in favour of the former at 25°. As shown in Table 6.2, it varies somewhat with temperature but is independent of pH in the range 6-10.[142] The values of K_m are approximately 10^{-4} M for each substrate and for glucose-6-phosphate isomerase from a wide

Table 6.2 Equilibrium constant (K_{eq}) (D-glucose 6-phosphate/D-fructose 6-phosphate) under various conditions

K_{eq}	Temperature	pH	Reference
3.70	25	8.5	142
3.33	30	8.5	142
3.85	20	8.0	143
3.36	30	8.0	143
3.06	38	8.0	143
3.57	25	6.4	144

variety of sources (Table 6.3). It is noteworthy that the values of K_{eq} and K_m, using the Haldane relationship, agree with the fact that the maximal velocity is the same in each direction of the isomerase-catalyzed reaction.

The inhibition of glucose-6-phosphate isomerase by structural analogues has received some attention in investigations of whether the

Table 6.3 Michaelis constants for substrates of glucose-6-phosphate isomerase.

	K_m (mM)		
Source	Glucose 6-phosphate	Fructose 6-phosphate	Reference
Rabbit	0.31	0.17	142
	0.8	0.12	145
Bovine mammary gland	0.12	0.07	146
Rabbit liver	0.6	0.12	145
Yeast	0.3	0.15	147
	0.8	0.11	144
Peas	0.27		125

straight-chain form or the cyclic form of the substrates bind better to the enzyme. Table 6.4 shows the dissociation constants for a number of competitive inhibitors. It may be noted that several species inhibit with values of K_i similar to or less than the K_m-values of the substrates, although this tight binding is not necessarily restricted to close structural analogues of the substrate (for example, ATP and phosphoenol pyruvate[143]). The binding of such straight-chain species as erythrose 4-phosphate, sorbitol 6-phosphate, 6-phosphogluconate and ribulose 5-phosphate is tighter than several of the analogues of the cyclic form of hexose 6-phosphate, such as 2-deoxyglucose 6-phosphate, mannose 6-phosphate and 1,5-anhydroglucitol 6-phosphate. This has been taken as evidence that the free keto forms (straight-chain forms) of the substrates themselves bind more tightly to the enzyme than do the cyclic forms.[147] The two best inhibitors are erythrose 4-phosphate and 5-phosphoarabinonate which may be described as more closely analogous to an enediol intermediate than to either substrate,[149] and as such would be expected to bind tightly to the enzyme.[53-56]

It has already been noted that several inhibitors that bind tightly are intermediates of the pentose phosphate cycle, in which the isomerase is also involved. The inhibition by 6-phosphogluconate is very pH-dependent[142,147,149,154] and it has been suggested that regulation by such an intermediate of the pentose phosphate cycle could be aided by temporary changes in pH.[154]

Mechanism of Hydrogen transfer

Glucose-6-phosphate isomerase, like triosephosphate isomerase,

Table 6.4 Competitive inhibitors of glucose-6-phosphate isomerase

Inhibitor	K_i (mM)	Reference
6-phosphogluconate	0.16	142
	0.075	148
	0.015	147
	0.013	125
	0.15	130
5-phosphoarabinonate	0.00027	149
sorbitol 6-phosphate	0.025	147,150
	0.04	149
Mannitol 6-phosphate	0.13	147
Erythritol 4-phosphate	0.10	149
	0.04	147
Erythrose 4-phosphate	0.001	147,151
	0.0007	149
L-xylulose 5-phosphate	0.7	147
Ribulose 5-phosphate	0.05	147
Ribose 5-phosphate	0.5	147
Arabinose 5-phosphate	0.05	149
1,5-anhydroglucitol 6-phosphate	2.5	147,152
Mannose 6-phosphate	0.46	147
2-deoxyglucose 6-phosphate	0.7	125,147
L-sorbose 6-phosphate	4.0	147
Sedoheptulose 7-phosphate	2.0	147,153
Phosphoenolpyruvate	1.1	143
ATP	0.4	143
Inorganic phosphate	1.7	143

requires no metal ion or other cofactor for maximal activity. The mechanism by which the enzyme catalyzes the transfer of hydrogen between carbon centres of fructose 6-phosphate (F6P) and glucose 6-phosphate (G6P) is very similar to that proposed for trisephosphate isomerase[34] and is based on similar evidence.

Glucose-6-phosphate isomerase labilizes the 1-pro R hydrogen of F6P.[95,96] When F6P, labelled in this position with tritium, is isomerized by the enzyme and the G6P produced is rapidly removed by glucose-6-phosphate dehydrogenase /$NADP^+$/Mg^{++}, then approximately 50% of the radioactivity is lost to the solvent. The remaining 50% of the tritium is transferred to C-2 of G6P and so appears in C-2 of phosphogluconate.[138,155] A slightly smaller ratio of exchange/transfer was observed in the conversion of [^{3}H]G6P to F6P.[138,156] The extent of transfer varies with the source of the enzyme and with temperature (Table 6.5), the transfer being greatest at low temperature. This was explained by Rose[155] to be the result of a higher activation energy of the exchange reaction compared to the transfer.

It is an interesting observation that the exchange/transfer ratio, measured for several aldose-ketose isomerases,[37] varies widely from such enzymes as triosephosphate isomerase (where only 3-6% of hydrogen is transferred[34,66]) to xylose isomerase (where no exchange could be found[157]). For isomerases that show a large percentage transfer, it seems feasible that some transfer occurs intermolecularily, i.e. the lifetime of the proton on the enzyme is larger than the lifetime of bound substrate. With glucose-6-phosphate isomerase this is not so: the transfer ([1-^{3}H]F6P to [2-^{3}H]G6P) has been shown to be completely

Table 6.5 Extent of transfer of hydrogen in the reaction catalyzed by glucose-6-phosphate isomerase

Enzyme source	Direction of measurement	Temperature	Exchange Transfer	Reference
Rabbit muscle	F6P → G6P	27	0.78	155
		0	0.21	155
		5	0.30	155
		15	0.45	155
		25	0.85	155
		37	1.31	155
		50	3.44	155
		60	3.17	155
	G6P → F6P	27	0.25	138
Yeast	F6P → G6P	27	0.75	138
	G6P → F6P	27	< 0.47	138
		25	1.0	156
Human Red Cells	F6P → G6P	27	0.97	155

intramolecular.[155]

The mechanism proposed for the reaction catalyzed by this isomerase assumes that hydrogen transfer occurs between the free keto forms of the substrate. The exchange of some of the tritium out of tritiated substrate during the reaction suggested that the reaction occurs by proton transfer (as distinct from the transfer of hydride ions). The stereochemistry of tritium transfer suggested that a proton is transferred between carbon centres on the re-re face of a cis-enediol, as shown in Fig. 6.2.[96,158]

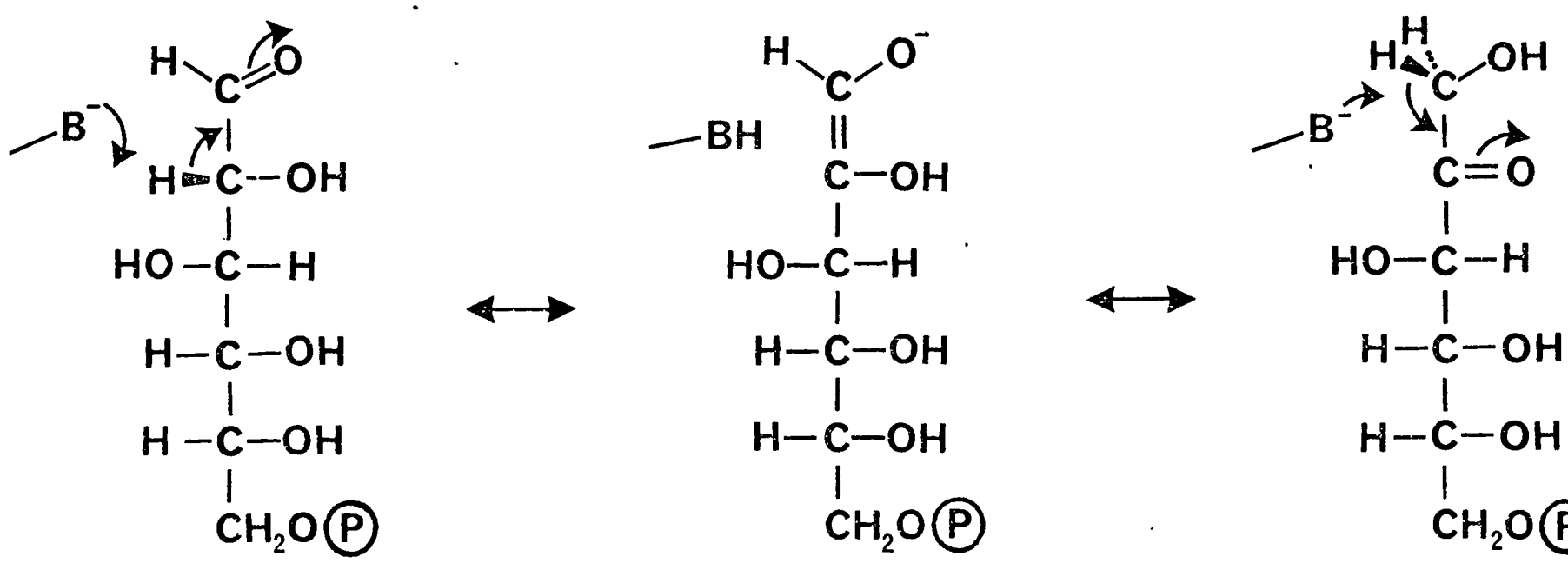


Fig. 6.2. Mechanism proposed for isomerization catalyzed by glucose-6-phosphate isomerase.

Several lines of evidence have been used to implicate amino acid residues on the enzyme in the reaction and in particular, the base responsible for proton transfer. The fact that there is a large percentage of hydrogen transfer during the reaction was considered to be evidence that this base has no proton already attached that could rapidly equilibrate with the proton from the substrate. For

instance, if the base were an amino group, rotation of the conjugate to acid would cause the percent transfer to be at most 33%.[155]

1,2 Anhydro-D-mannitol 6-phosphate reacts irreversibly with a glutamate in the active site of glucose-6-phosphate isomerase[129] and all catalytic activity is lost. As this epoxide is a structural analogue of the substrates, it probably binds in the active rate similarly to the substrate. The glutamate with which it reacts has been proposed as the base responsible for proton transfer.[129] This aspect of glucose-6-phosphate isomerase would then be similar to triosephosphate isomerase, where similar active-site-directed, irreversible inhibitors were used to label a glutamate (Glu_{165}).[23-29]

From studies on the pH dependences of K_m, V_{max} and on K_i-values for 6-phosphogluconate apparent pK_a-values have been obtained between 6.0-6.8 and 9.3-9.9.[142-146] Dyson and Noltmann[142] then studied the temperature dependence of these pK_as, to determine heats of ionization for the deprotonations. The values obtained are consistent with an imidazole (the lower pK_a) and a lysine (the upper pK_a). From these studies, they proposed that a histidine was responsible for proton transfer in the isomerase-catalyzed reaction.

The forms of hexose 6-phosphates present in aqueous solution

Hexose 6-phosphates exist in aqueous solution as an equilibrium mixture of the α and β anomers of the cyclic hemiketals and the straight chain, free keto form. The equilibrium proportions of these three forms are shown in Table 6.6 for fructose 6-phosphate and glucose 6-phosphate. The proportions of α- and β-pyranose forms of glucose 6-

Table 6.6 Equilibrium composition of glucose 6-phosphate and fructose 6-phosphate in aqueous solution.

Compound	Equilibrium Composition	Method of determination	Reference
D-Glucose 6-phosphate	37-39% α pyranose 61-63% β pyranose	kinetic measurement	159,160,161
	<0.4% free aldehyde	i.r.	43
D-Fructose 6-phosphate	20 ± 4% α-furanose 80 ± 10% β-furanose	n.m.r.	162,163
	24% α-furanose 76% β-furanose	kinetic measurements	164
	2.5% ketone	i.r.	43

phosphate are very similar to those for glucose,[165,166] but it has been found that fructose occurs largely in the pyranose form,[167,168] a form which it is impossible for the phosphate ester to adopt. The main method of determining the proportion of cyclic forms is by n.m.r., but this cannot be used for the small percentage of free keto form. The amount of the latter has been determined most accurately by comparison of the intensity of infrared absorption by the carbonyl group with that of a standard ketone such as acetone. Presumably there also exists a small proportion of straight chain hydrate of the hexose 6-phosphates, but this has not been detected as it is likely to be present only at a similar proportion to the free keto form.

The proportion of anomers and the rate of anomerization of G6P has been determined using glucose-6-phosphate dehydrogenase,[159,160]

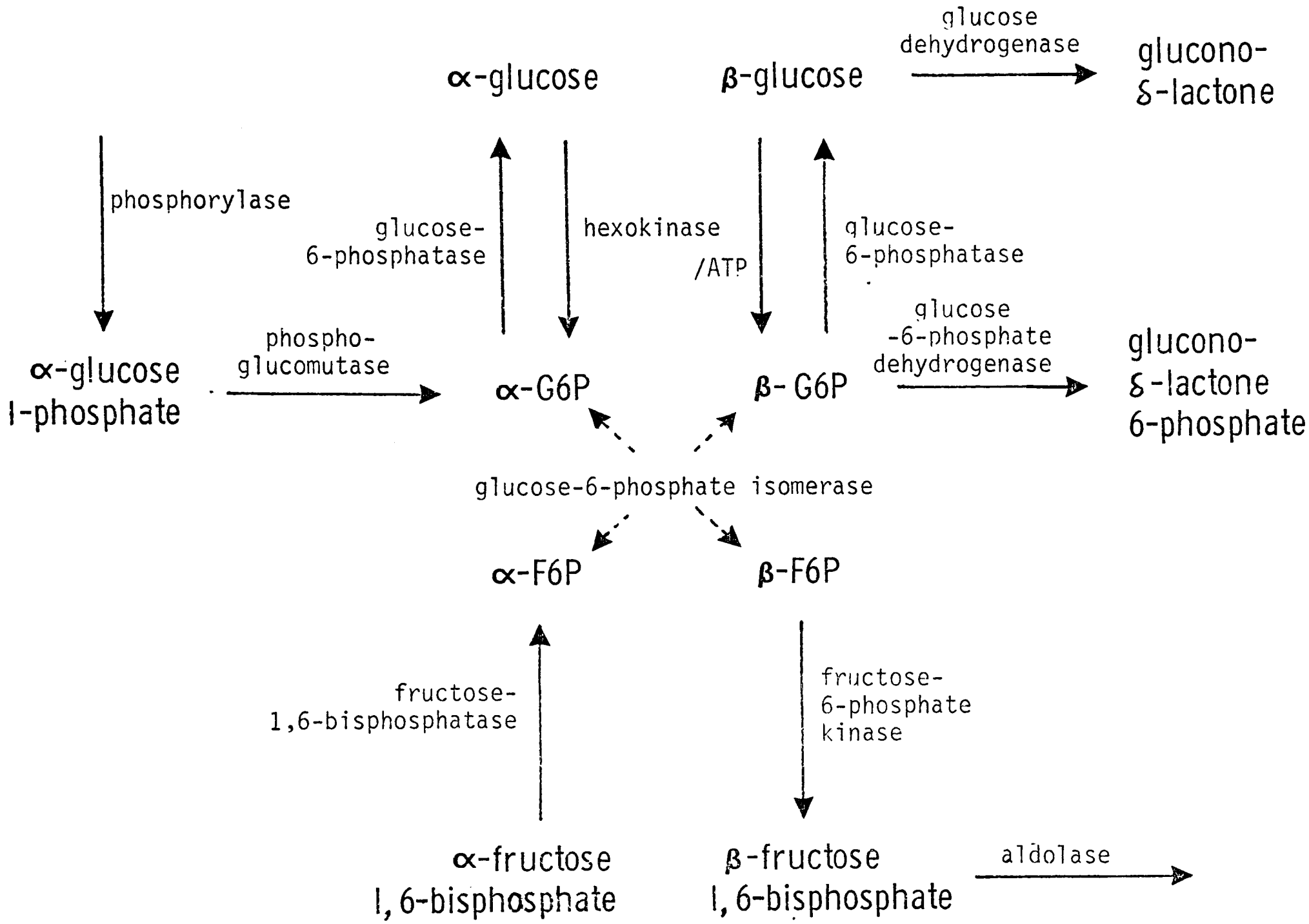


Fig. 6.3. Anomeric specificity of some hexose and hexose phosphate handling enzymes

which handles only the β-anomer of G6P. The rate of anomerization of the α-form to the form handled by the dehydrogenase was found to be 1.1 min^{-1} at 10°.[159,160] Under physiological conditions, the half-life for the anomerization of G6P is 1.5 sec, whereas for glucose it is 7 min.[159,160] The difference was explained by the intramolecular catalysis of this process by the phosphoryl group. Inorganic phosphate itself will catalyse the ring opening of glucose.[160]

Most of the enzymes that handle triosephosphates use only the free keto forms and it has been shown in this thesis that the hydrate of dihydroxyacetone phosphate binds weakly if at all to triosephosphate isomerase. So there appears to be no real problem caused by the presence of multiple forms of the substrates in solution. With hexose 6-phosphates this is not the case. Much effort has been directed towards determining the forms of hexoses and phosphorylated hexoses handled by enzymes.[169,170] Most enzymes seem to handle one anomer of the cyclic form and the anomeric specificity is summarized in Fig. 6.3.

The dehydrogenases that oxidize glucose and G6P use only the β-anomers: that is, they remove the axial hydrogen from C-1 of the substrate.[147,159,160,171,172] Glycogen is converted by phosphorylase to α-glucose 1-phosphate,[173] which in turn is converted to α-G6P by phosphoglucomutase.[147,174,175,176] β-F6P is phosphorylated by fructose-6-phosphate kinase, probably to give β-fructose 1,6-bisphosphate,[164,177,178] which is then the preferred anomer substrate for aldolase.[179,180] However, fructose 1,6-bisphosphatase, which is involved in gluconeogenesis, catalyzes the hydrolysis of the 1-phosphate from preferentially the α-anomer of fructose 1,6-bisphosphate, presumably to give α-F6P.[181]

It seems that enzymes that catalyze a reaction centred on the anomeric carbon have a large preference for one anomer. On the other hand, enzymes that catalyze reactions centred away from the anomeric carbon have little or no anomeric specificity; for example, hexokinase[147,154,182] and glucose-6-phosphatase.[159] Generally, the ability of enzymes to handle the free keto forms of the substrate is not known, as this form is present in such small proportions. Only aldolase has been shown clearly to handle the straight chain form of fructose 1,6-bisphosphate.[180, 183,184,185,186]

In the metabolism of hexose 6-phosphates, the flux of material through certain pathways requires anomerization of the hexose phosphate. An example, shown in Fig. 6.3, is that α-G6P is released by phosphoglucomutase but β-G6P is used by glucose-6-phosphate dehydrogenase. In fact there exist various anomerases that catalyze the interconversion of α and β anomers of glucose or of G6P or of F6P. Glucose-6-phosphate epimerases have been shown to exist in E. Coli,[187] potato tubers[187] and yeast,[161,188] but their presence could not be demonstrated in mammalian muscle or liver.[187]

The forms of substrate active with glucose-6-phosphate isomerase-anomerase activity

The anomeric specificity of glucose-6-phosphate isomerase is not as clearly established as with other enzymes that handle hexoses. Several studies on this specificity have been carried out and sometimes have given contradictory results. It has already been mentioned, that analogues of the straight chain form of hexose 6-phosphate bind more

tightly to the isomerase than do analogues of the cyclic forms. This was taken as evidence that the free keto form of hexose 6-phosphate were likely to be handled by the enzyme perhaps better than the cyclic forms.[147]

The mechanism for aldose-ketose isomerization postulated by Topper[96] and by Rose[158] requires that the substrates bind to the enzyme active site as their keto forms, thereby facilitating the subsequent transfer-reaction. However it was argued that if the free keto form were the sole substrate form accepted by the enzyme, then the true K_m would be improbably small due to the small proportion of hexose 6-phosphate as free keto form. In fact, later estimates of the proportion of free keto form were 1 to 2%[43,44] so that an apparent K_m-value of 10^{-4} M for overall substrate becomes 10^{-6} M in terms of free keto form, and this is within the range thought feasible.

Salas et al[147] used α and β-G6P separately (formed from α and β-glucose respectively with hexokinase/ATP and showed that glucose-6-phosphate isomerase uses α-G6P directly, whereas there was a time lag before β-G6P was used. This was interpreted as being due to α-G6P being the form of substrate handled by the enzyme: the β-anomer has to undergo slow anomerization before being accepted by the enzyme. Both Salas et al[147] and Wurster and Hess[189] used glucose-6-phosphate dehydrogenase to study the anomerization of G6P. This dehydrogenase uses only the β-anomer of G6P: the reaction with an equilibrium mixture of α and β-G6P with a large concentration of enzyme is biphasic. The β anomer is used rapidly, then the α-anomer is used at a rate limited by the anomerization. This provides a method of determining the equilibrium ratio of α and β-G6P

as well as a way of studying the anomerization. When α-G6P alone is used with this dehydrogenase there is a time lag before a steady state rate of oxidation.[189] Addition of glucose-6-phosphate isomerase removed the time lag, indicating that this enzyme was catalyzing the anomerization of G6P,[189] presumably via the ring opening of G6P. The open-chain G6P, thus formed, then undergoes ring closure in free solution.[147]

However, the picture of specificity of isomerization and anomerase activity were modified when Wurster and Hess[190] showed that at high concentrations of the isomerase, β-G6P was handled by the isomerase, and converted to F6P, albeit at less than 10% of the rate of α-G6P. The partitioning of α-G6P between F6P and β-G6P was found to be about 2:1 under the experimental conditions used.[190] In this last experiment, the products, β-G6P and F6P were rapidly removed enzymically so indicating that the two reactions (isomerization and anomerization) are separate - i.e. that anomerization of α to β-G6P does not go via F6P.

Using stopped-flow techniques, Schray et al.[191] studied the anomeric specificity of the isomerase for F6P and showed that in the presence of a large concentration of the isomerase, the approach of F6P to an equilibrium of F6P and G6P was biphasic. The fast, initial reaction was interpreted as the rapid use of α-F6P by the isomerase. The slower part of the reaction was at a rate proportional to the enzyme concentration and was interpreted as being enzyme-catalyzed isomerization of β-F6P. In a similar experiment[191] with G6P, it was shown that βG6P is handled slowly by the isomerase, in agreement with the results of Wurster and Hess.[190]

That the same enzyme catalyzes the anomerization of G6P and the isomerization of α and β-hexose 6-phosphates was indicated by partial

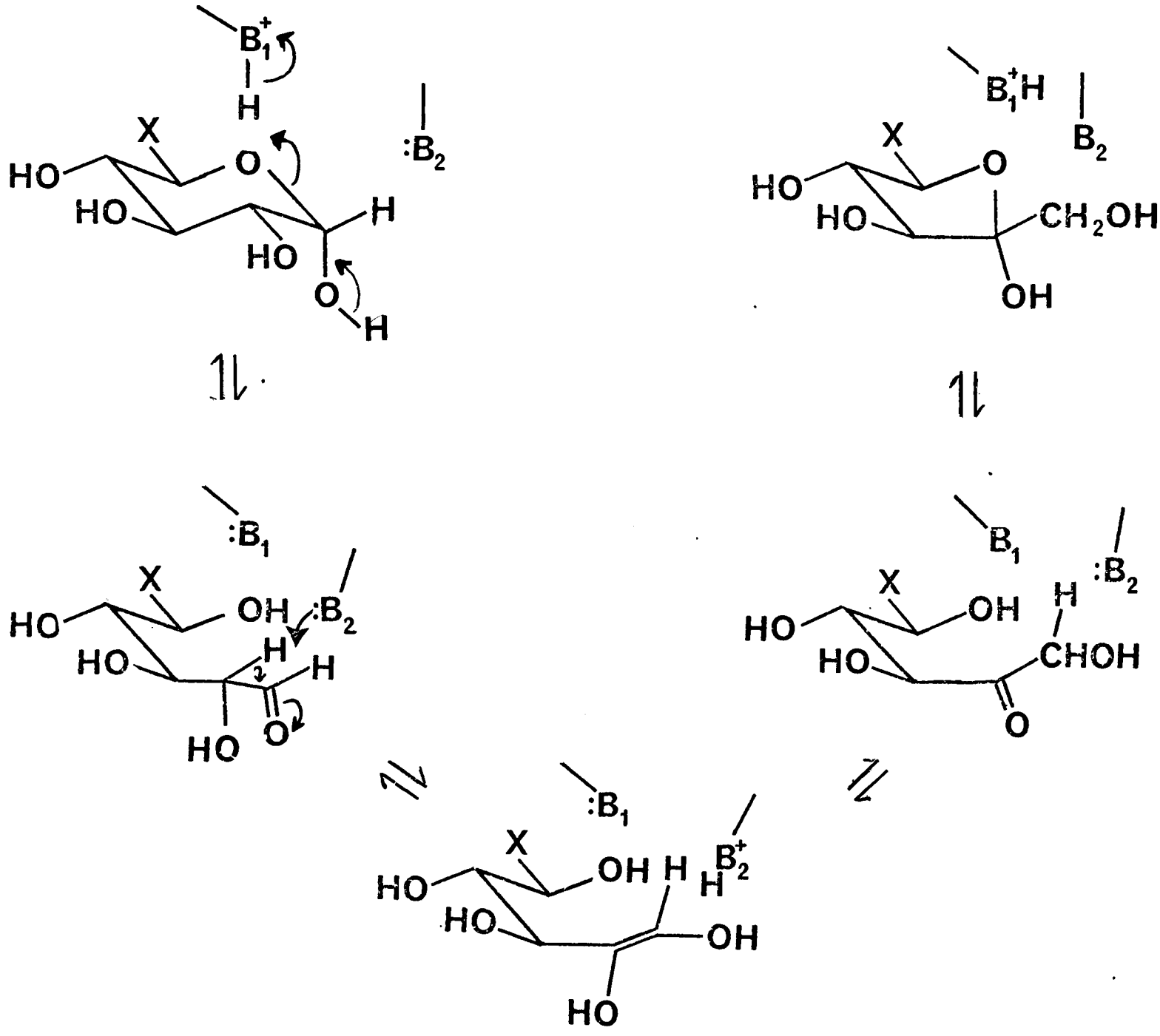


Fig. 6.4. Mechanism proposed by Dyson and Noltmann[142] for the interconversion of α-glucose 6-phosphate and α-fructose 6-phosphate catalyzed by glucose-6-phosphate isomerase. B_1 and B_2 are bases on the enzyme.

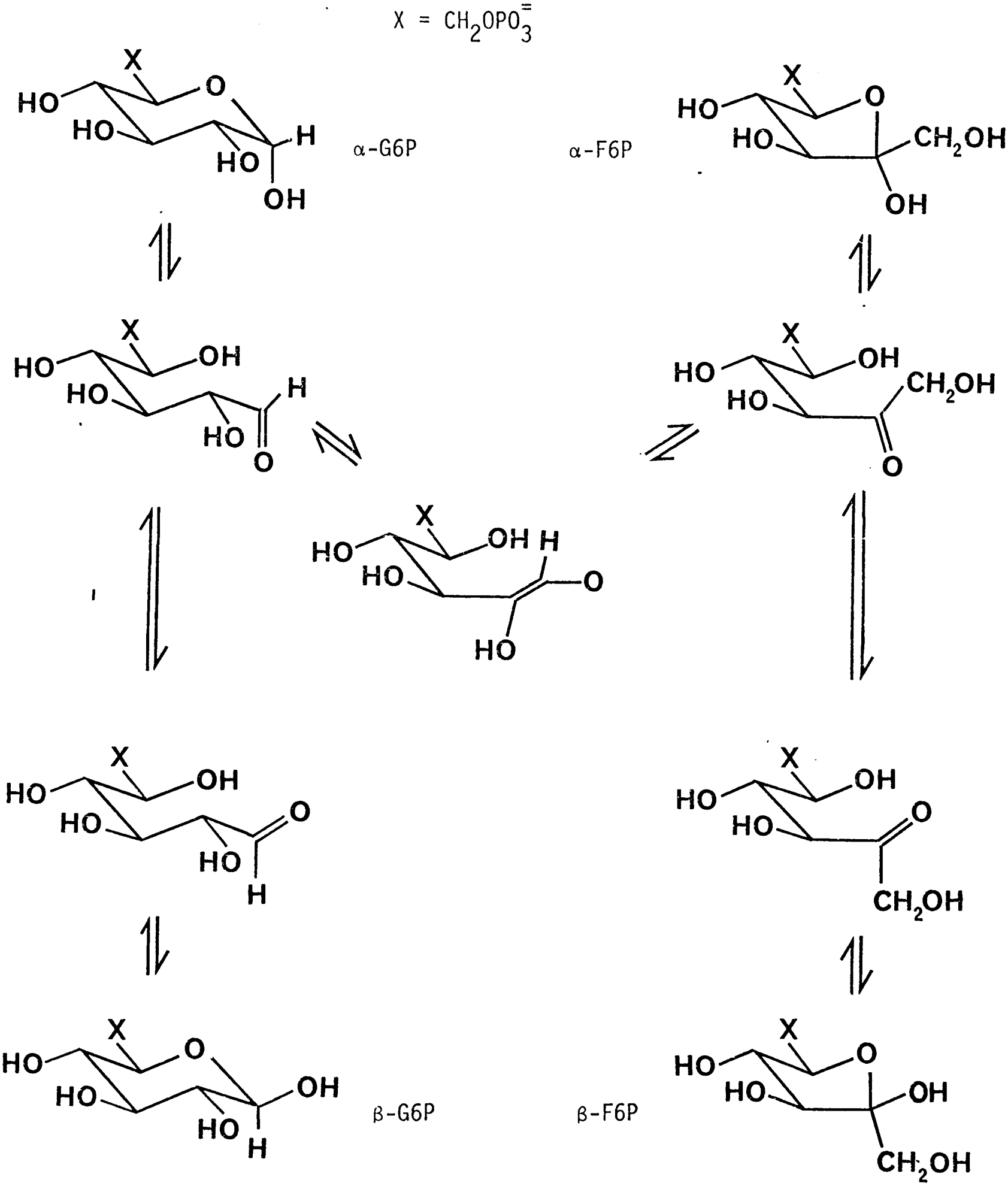
inactivation of the enzyme, using the competitive inhibitor, erythrose 4-phosphate and the inactivator 1,2-anhydro-D-mannitol 6-phosphate.[129] The two activities (anomerase and isomerase) decreased in the same proportion.[147,191]

These studies indicate that the isomerase can handle all three forms (α and β-anomers and free keto form) of each substrate to different extents. An overall mechanism for the reaction catalyzed by glucose-6-phosphate isomerase must include ring-opening and ring-closing steps and must accommodate the anomerase activity. The isomerization of α and β anomers will have very different steric requirements of the enzyme. Some of these problems have been discussed by Dyson and Noltmann[142] and by Schray et al.[191]

Dyson and Noltmann[142] consider only the α-anomer as being opened on the enzyme. They propose this ring opening is catalyzed by a lysine (B_1) on the enzyme and then a histidine (B_2) acts as the base to remove the proton from the acyclic form of substrate to form the enediol (Fig. 6.4).

Schray et al[191] consider three possible mechanisms. In the first, the isomerase accepts only the α-anomer and the free keto form of each substrate. This mechanism is therefore similar to that proposed by Dyson and Noltmann[142] (Fig. 6.4) and is in accord with the suggestion that the anomer used by isomerases can be predicted by reference to a cis-enediol intermediate, the direction of protonation and a requirement of minimal movement of the substrate in the active site.[37,192] Thus, in Fig. 6.4, the movement of the carbon skeleton of the substrate is minimal throughout the reaction. As will be seen, it is not possible

Fig. 6.5. Scheme proposed by Schray et al.[191] for the processes catalyzed by glucose-6-phosphate isomerase, to utilize both anomers of both substrates (α-G6P, β-G6P, α-F6P and β-F6P).



to accommodate the β-anomer without large movement of its carbon skeleton. However, by this mechanism, β-G6P can be used only after non-catalyzed ring opening in free solution. Because of this, the mechanism is dismissed by Schray et al,[191] since the rate of isomerization of β-G6P is directly proportional to the concentration of isomerase and in any case, the observed rate is too large to be limited by spontaneous uncatalyzed ring opening.

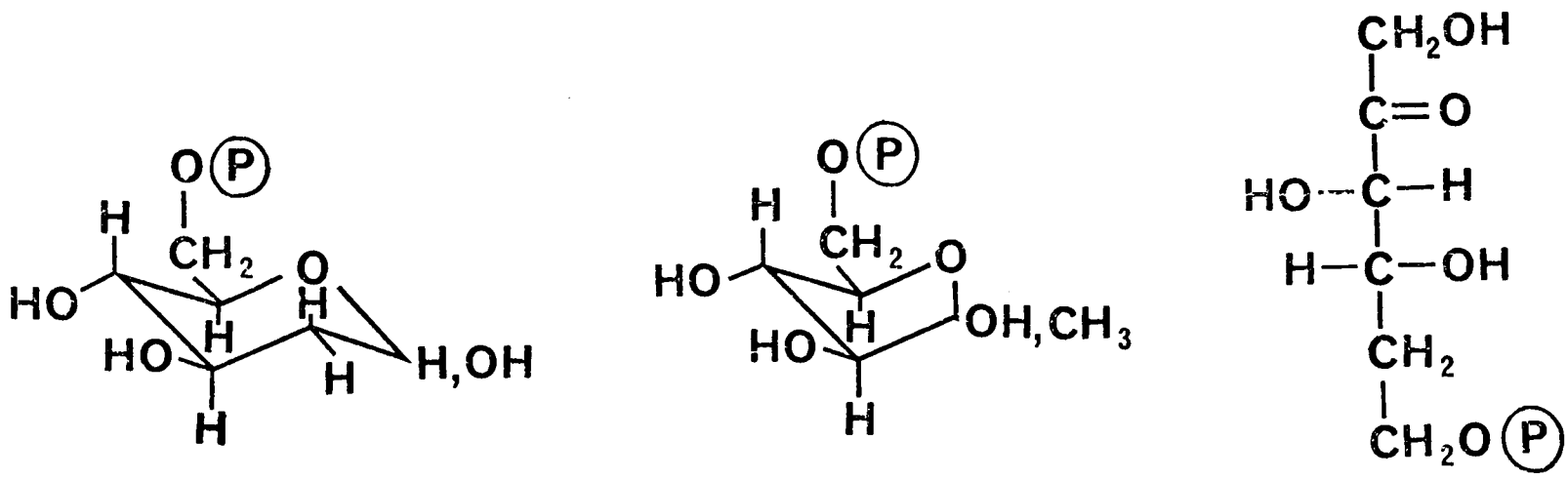
In the other two mechanisms considered by Schray et al.[191] both anomers of the substrates are accepted by the isomerase, and the ring opening of each is catalyzed by the enzyme. This automatically requires a different stereochemistry of catalysis for each anomer. In one proposed mechanism, α-G6P is converted to α-F6P and β-G6P is converted to β-F6P each by a different pathway, involving no common intermediate. The isomerization of α-anomers proceeds via a cis-enediol; the less efficient isomerization of β-anomers is via a trans-enediol. This mechanism cannot accommodate fully the stereochemistry of proton exchange into F6P by the isomerase, nor is anomerase activity possible.

The third mechanism discussed by Schray et al.,[191] and the one favored by them, is outlined in Fig. 6.5. The α-G6P is ring-opened with "minimal motion" to give C1 and C2 in the correct conformation to form a cis-enediol. However, after the β-G6P is ring opened on the enzyme, there must be a conformation change of the substrate and C-C bond rotation to obtain the correct conformation for cis-enediol formation. A similar conformation change must occur to product β-F6P from the keto form on the enzyme. Although this mechanism requires a large degree of motion in the active site, it does accommodate all the stereochemical evidence and

anomeric specificities. So this mechanism, outlined in Fig. 6.5 must be considered as the basis for any further rationalization of the catalysis by glucose-6-phosphate isomerase. It is the aim of this thesis to develop systems to enable parts of the mechanism in Fig. 6.5 to be studied separately from the rest: anomerization without the possibility of isomerization and vice versa.

CHAPTER 3 THE SUITABILITY OF DEOXY ANALOGUES OF HEXOSE 6-PHOSPHATES TO STUDY THE ANOMERASE ACTIVITY OF GLUCOSE-6-PHOSPHATE ISOMERASE

One way to separate the isomerase and anomerase activities of glucose-6-phosphate isomerase is to use anologues of hexose phosphates that can undergo only one of the reactions. Such anologues considered in this chapter, are the deoxyhexose 6-phosphates. 2-Deoxyglucose 6-phosphate (Fig. 7.1(a)) exists in the pyranose form. There is no possibility that



(a) 2-deoxyglucose 6-phosphate

(b) 1-deoxyfructose 6-phosphate

(c) 5-deoxyfructose 6-phosphate

Fig. 7.1. Deoxy analogues of the substrates of glucose-6-phosphate isomerase.

the compound can be isomerized to an analogue of fructose 6-phosphate but it is quite possible that its anomerization would be catalyzed by the isomerase, analogously to the catalyzed anomerization of glucose 6-phosphate. Similarly, 1-deoxyfructose 6-phosphate, which probably exists

in the furanose form as does fructose 6-phosphate (Fig. 7.1(b)), cannot undergo an aldose-ketose isomerization, although an enzyme catalyzed anomerization seems quite feasible. These species should both be good analogues of the cyclic forms of the substrates of glucose-6-phosphate isomerase. A third deoxy-compound, 5-deoxyfructose 6-phosphate (Fig. 7.1(c)) must exist in the straight chain configuration: no pyranose or furanose form is possible. This compound is therefore an analogue of the free keto form of fructose 6-phosphate and should be useful for studying the activity of the free keto form of the substrate with the isomerase.

Synthesis and purification of the deoxy analogues

(a) 2-Deoxyglucose 6-phosphate

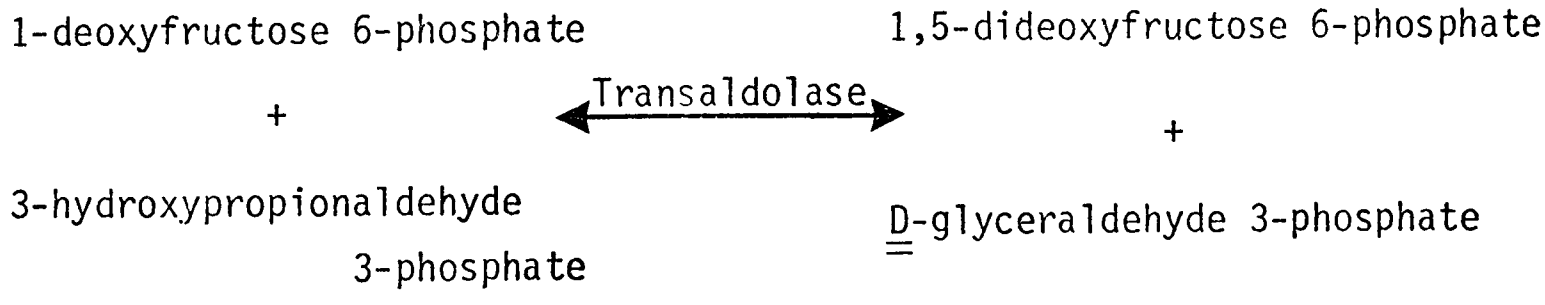
A necessary prerequisite to the use of these analogues of hexose phosphates is that they can be prepared completely free of hexose phosphates: a small proportion of the natural material could invalidate any results obtained. 2-Deoxyglucose was found to contain a small proportion of glucose, but 2-Deoxyglucose 6-phosphate, which is commercially available, was found to contain less than 0.1% of hexose phosphates.

(b) 1-Deoxyfructose 6-phosphate

1-Deoxyfructose was synthesized by a modified version of the method of Haylock et al.[193] This material was phosphorylated using hexokinase/ATP to give 1-deoxyfructose 6-phosphate containing a small amount of fructose 6-phosphate.

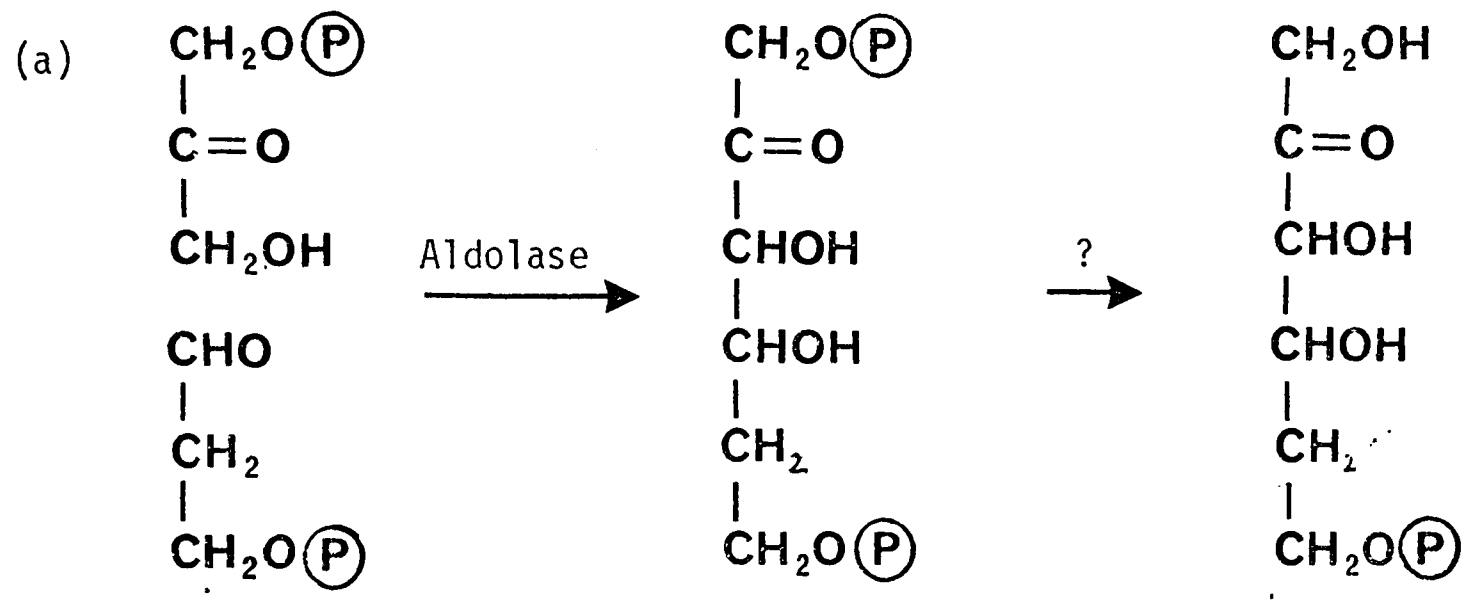
The enzyme-catalyzed phosphorylation of fructose is considerably faster than the phosphorylation of its 1-deoxy analogue, so that fructose 6-phosphate accumulates faster than 1-deoxyfructose 6-phosphate. However, treatment of the phosphorylated mixture with glucose-6-phosphate isomerase/ glucose-6-phosphate dehydrogenase/ $NADP^+$ converts the natural compound to 6-phosphogluconate. Ion exchange chromatography then gave 1-deoxyfructose 6-phosphate containing less than 0.1% of fructose 6-phosphate. Great care was required in freeze drying the material after chromatography because the compound tended to discolour during this process and give very broad peaks in the proton n.m.r. spectrum. Hence it was not possible to completely dry a sample for elemental analysis. The criteria of purity of this compound are discussed later in this thesis.

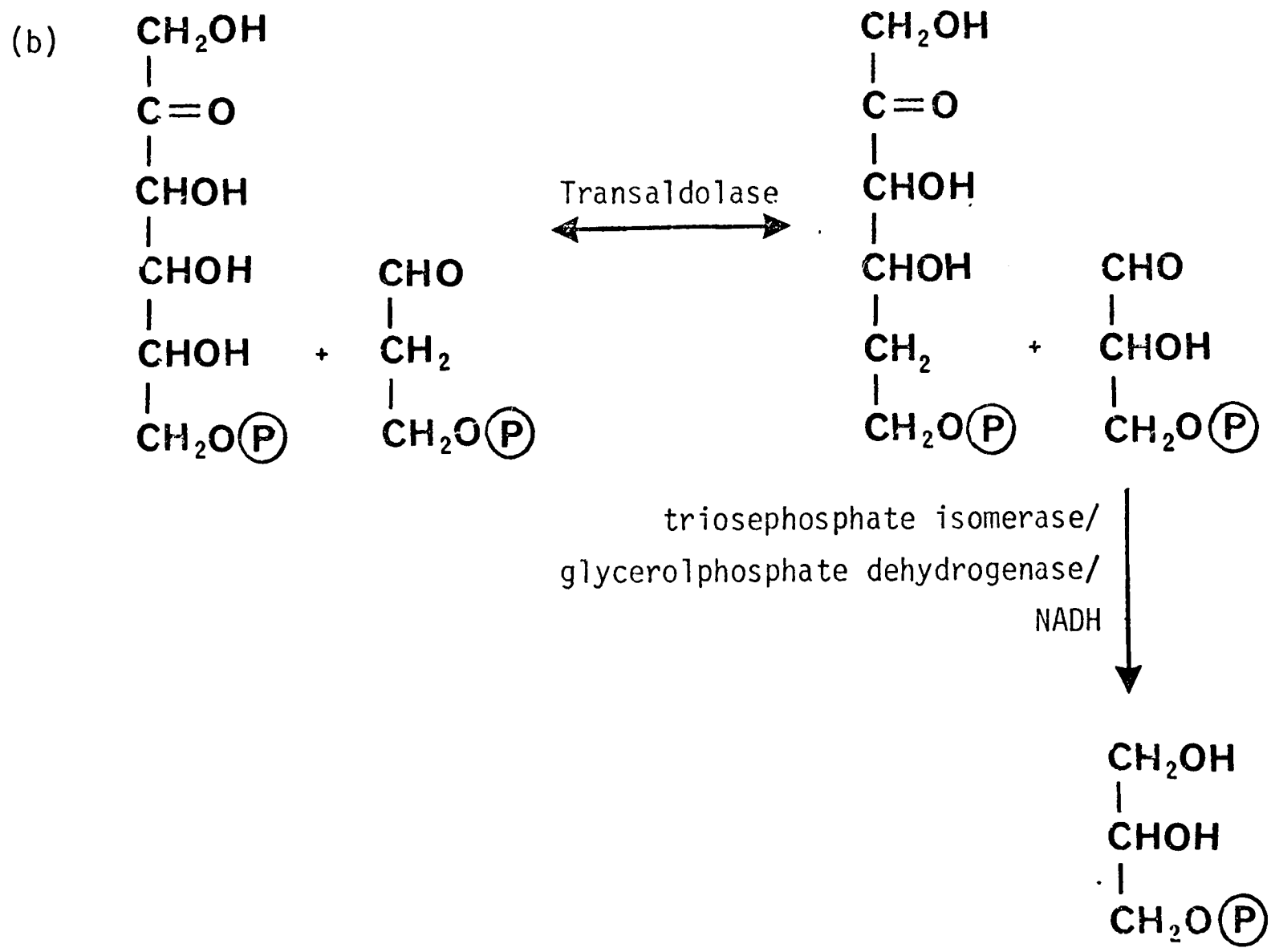
The 1-deoxyfructose 6-phosphate was assayed using the following reaction, catalyzed by the enzyme transaldolase:



This reaction was coupled to the reactions catalyzed by triosephosphate isomerase and glycerolphosphate dehydrogenase/ NADH so that the D-glyceraldehyde 3-phosphate was removed with concomitant consumption of NADH. 3-Hydroxypropionaldehyde 3-phosphate (in effect, 2-deoxy-

Fig. 7.2. Reaction schemes for the attempted syntheses of 5-deoxyfructose 6-phosphate from 3-hydroxypropionaldehyde 3-phosphate.





glyceraldehyde 3-phosphate) cannot undergo an isomerization catalyzed by triosephosphate isomerase.

(c) 5-deoxyfructose 6-phosphate

No synthesis of 5-deoxyfructose 6-phosphate has been published. A suitable starting material seemed to be the 2-deoxy analogue of glyceraldehyde 3-phosphate, 3-hydroxypropionaldehyde 3-phosphate and the preparation of 5-deoxyfructose 6-phosphate from this was attempted by two methods, which are outlined in Fig. 7.2. First, condensation of 3-hydroxypropionaldehyde 3-phosphate with dihydroxyacetone phosphate is catalyzed by aldolase to give 5-deoxyfructose 1,6-bisphosphate. This compound was found not to be a substrate for fructose 1,6-bisphosphatase, so that a chemical method for the removal of the 1-phosphate was required that left the 6-phosphate intact and did not cause any rearrangement (especially aldose-ketose isomerization) of the rest of the molecule. Preliminary studies showed that this hydrolysis was not particularly simple and so this approach was not continued.

The other approach to 5-deoxyfructose 6-phosphate involved the use of transaldolase. This enzyme catalyzes the exchange of C-4,5 and 6 of fructose 6-phosphate with erythrose 4-phosphate to give D-glyceraldehyde 3-phosphate and sedoheptulose 7-phosphate. The enzyme is not particularly specific and will accept a variety of molecules instead of erythrose 4-phosphate.[194] The enzyme was found to catalyze the first reaction in the scheme shown in Fig. 7.2 (b), to give directly 5-deoxyfructose 6-phosphate, together with D-glyceraldehyde 3-phosphate.

The reaction of fructose 6-phosphate, 3-hydroxypropionaldehyde 3-phosphate and transaldolase, in the presence of a coupling system of triosephosphate isomerase/glycerol-3-phosphate dehydrogenase/NADH to remove the glyceraldehyde 3-phosphate, pulled the equilibrium to the right. However, by this method it was difficult to remove the last traces of fructose 6-phosphate due to the instability to long incubation of 3-hydroxypropionaldehyde 3-phosphate. Insufficient 5-deoxyfructose 6-phosphate was prepared to characterize or use with glucose-6-phosphate isomerase. However, the method seems suitable to be scaled up and column ion-exchange chromatography in the presence of borate should separate the product from fructose 6-phosphate impurity.

Recently Rose et al.[195] have demonstrated that 5-deoxyglucose 6-phosphate is a substrate for glucose-6-phosphate isomerase, but have not characterized the reaction or compared the kinetic parameters to those of the true substrate. Even more recently, a synthesis of 5-deoxyfructose 1,6-bisphosphate has been published,[186] similar to the one outlined here. The compound was shown to be a good substrate for muscle aldolase, with a value of V_{max}/K_m that is 56 times that of the true substrate, fructose 1,6-bisphosphate. This was taken as evidence that the keto form of the substrate is the form handled by the enzyme.

Preliminary studies with 2-deoxyglucose 6-phosphate

A Prime consideration for the use of a substrate analogue with an enzyme is how well the analogue binds to the enzyme, compared to the true substrate. The value of K_i for the binding 2-deoxyglucose 6-phosphate to glucose-6-phosphate isomerase has been determined previously to be 0.7mM at 20°C and pH 8. It is therefore clear that the 2-deoxy analogue binds with a K_i-value similar to the K_m of glucose 6-phosphate.

Although this analogue of glucose 6-phosphate cannot undergo isomerization, it seemed possible that it might act as a "virtual" substrate, with the isomerase catalyzing the exchange of the hydrogen at C-2 with protons from the solvent, in the same way as it does with the true substrate. Indeed, incubation of 2-deoxyglucose 6-phosphate in tritiated water in the presence of the isomerase does result in a slow exchange of tritium into the deoxy compound, as shown in Table 7.1. The rate of enzyme-catalyzed tritium exchange is approximately 10^6-times slower than that expected for glucose 6-phosphate under the same conditions, but 18-times the non-enzyme-catalyzed rate. The position of the radioactive label in the [^{3}H]2-deoxyglucose 6-phosphate was not determined and the rate of exchange was too slow to observe a change in the proton n.m.r. spectrum in deuterium oxide in the presence of the isomerase. The catalysis of tritium incorporation presumably requires that the enzyme catalyzes the formation of the enol as shown in Fig. 7.3, but at a very slow rate. This is analogous to the left hand part of the mechanism proposed for the true isomerase-catalyzed reaction shown in Fig. 6.2.

Table 7.1. Tritium exchange into deoxyhexose phosphates from tritiated water[a] in the presence and absence of glucose-6-phosphate isomerase

Deoxyhexose phosphate and concentration (mM)	Glucose 6-phosphate isomerase (units/ml)	10^{-6} x specific radioactivity of water (c.p.m/μequiv)[b]	10^{-6} x specific radioactivity of deoxyhexose phosphate[c] (c.p.m/μmol)	% tritiation of deoxyhexose 6-phosphate[c]
2-deoxyglucose 6-phosphate [60]	0	4.29	0.0069	0.16
2-deoxyglucose 6-phosphate [60]	1200	18.0	0.52	2.9
1-deoxyfructose 6-phosphate [32]	0	9.0	0.76	8.4
1-deoxyfructose 6-phosphate [32]	1600	9.0	0.69	7.6

a. In 100 mM ammonium bicarbonate, pH 8.0 at 22°C.

b. In effect, the specific radioactivity of protons.

c. After 36 hr. incubation.

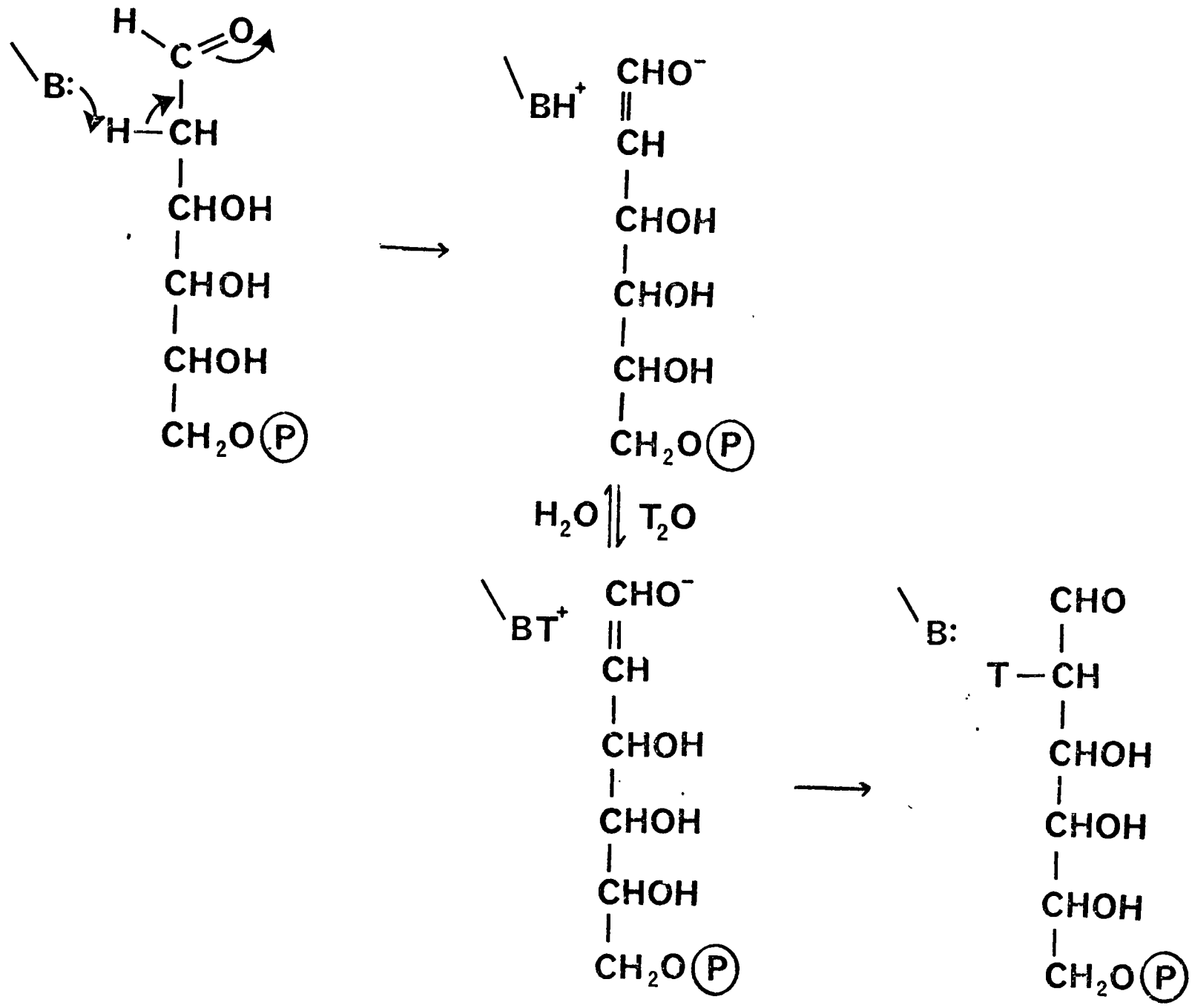


Fig. 7.3. Probable mechanism for exchange of tritium from tritiated water into 2-deoxyglucose 6-phosphate, catalyzed by glucose-6-phosphate isomerase. B is the base in the enzyme active site responsible for proton transfer.

One way of studying the anomeric specificity of 2-deoxyglucose 6-phosphate would be to remove one anomer rapidly from the solution and then follow the possibly slower anomerization reaction in the presence of glucose-6-phosphate isomerase. This is possible only if a reaction

exists that will remove one anomer at a rate considerably faster than the anomerization reaction. In this case it is likely (although not known) that 2-deoxyglucose 6-phosphate anomerizes at a rate similar to glucose 6-phosphate (which has a half-life for anomerization of 1.5 sec at neutral pH and 37°C). Preliminary studies with glucose-6-phosphate dehydrogenase, the most likely enzyme to be able to carry out this reaction, confirmed that the enzyme did handle the deoxy analogue,[196] but at a much slower rate than the true substrate. It was clear that even at very high enzyme concentration, the rate of removal of β-2-deoxyglucose 6-phosphate by the dehydrogenase would not be large enough to make the anomerization rate-limiting.

Preliminary studies with 1-deoxyfructose 6-phosphate

The value of K_i for 1-deoxyfructose 6-phosphate with glucose-6-phosphate isomerase was determined at 30°C and pH 7.5. The results are shown in Fig. 7.4, which yield a value of 0.30 mM for the inhibition constant, a value similar to the K_m for fructose 6-phosphate.

However, the isomerase has no effect on the rate of tritium exchange into 1-deoxyfructose 6-phosphate, as shown in Table 7.1. Thus the rate of enzyme-catalyzed enolization of the deoxy analogue, if it occurs at all, must be at least 10^8-times slower than the rate for fructose 6-phosphate under these conditions. In view of the results with 2-deoxyglucose 6-phosphate (and with monohydroxyacetone phosphate with triosephosphate isomerase, described in Chapter 1) this lack of observable tritium exchange is somewhat surprising. It does not, however, rule out a possible isomerase-catalyzed anomerization of the hexose phosphate analogue.

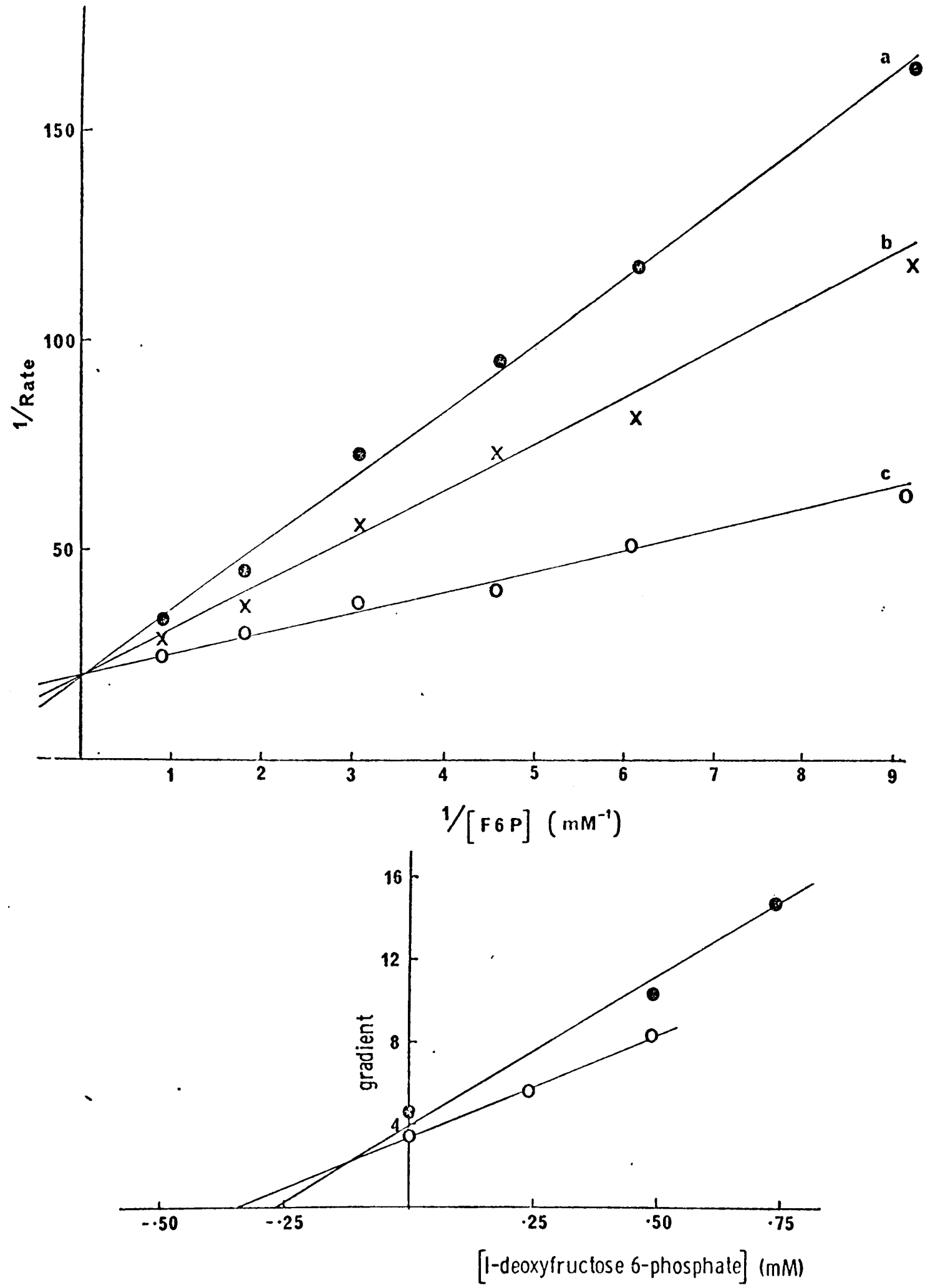


Fig. 7.4. The inhibition of glucose-6-phosphate isomerase by 1-deoxy fructose 6-phosphate at pH 7.5 and 30°C.

The upper graph is a Line-Weaver-Burke plot at (a) 0.74 mM, (b) 0.49 mM and (c) no 1-deoxyfructose 6-phosphate. The lower graph is a plot of inhibitor concentration against gradient, for two series of assays.

N.m.r. studies with the deoxyhexose phosphates.

The proton n.m.r. spectra of 2-deoxyglucose 6-phosphate and 1-deoxyfructose 6-phosphate each show well-defined peaks for the different forms of the compound in solution. The 2-CH_2 group of 2-deoxyglucose 6-phosphate gives multiplets well separated from the rest of the spectrum and some peaks in these multiplets have been assigned by Hall and Manville[197] to α or β anomeric protons. These assignments allow the possibility of studying reaction-broadening of these peaks. The anomeric proton peaks will broaden on addition of glucose-6-phosphate isomerase if the enzyme catalyzes the interconversion of the two anomers. Assuming the rates of anomerization that have been proposed for glucose 6-phosphate with the isomerase[190,191] will obtain for the deoxy analogue, this experimental method should yield measurable peak broadening and hence rates of interconversion of α, β and acyclic forms in the presence of the enzyme.

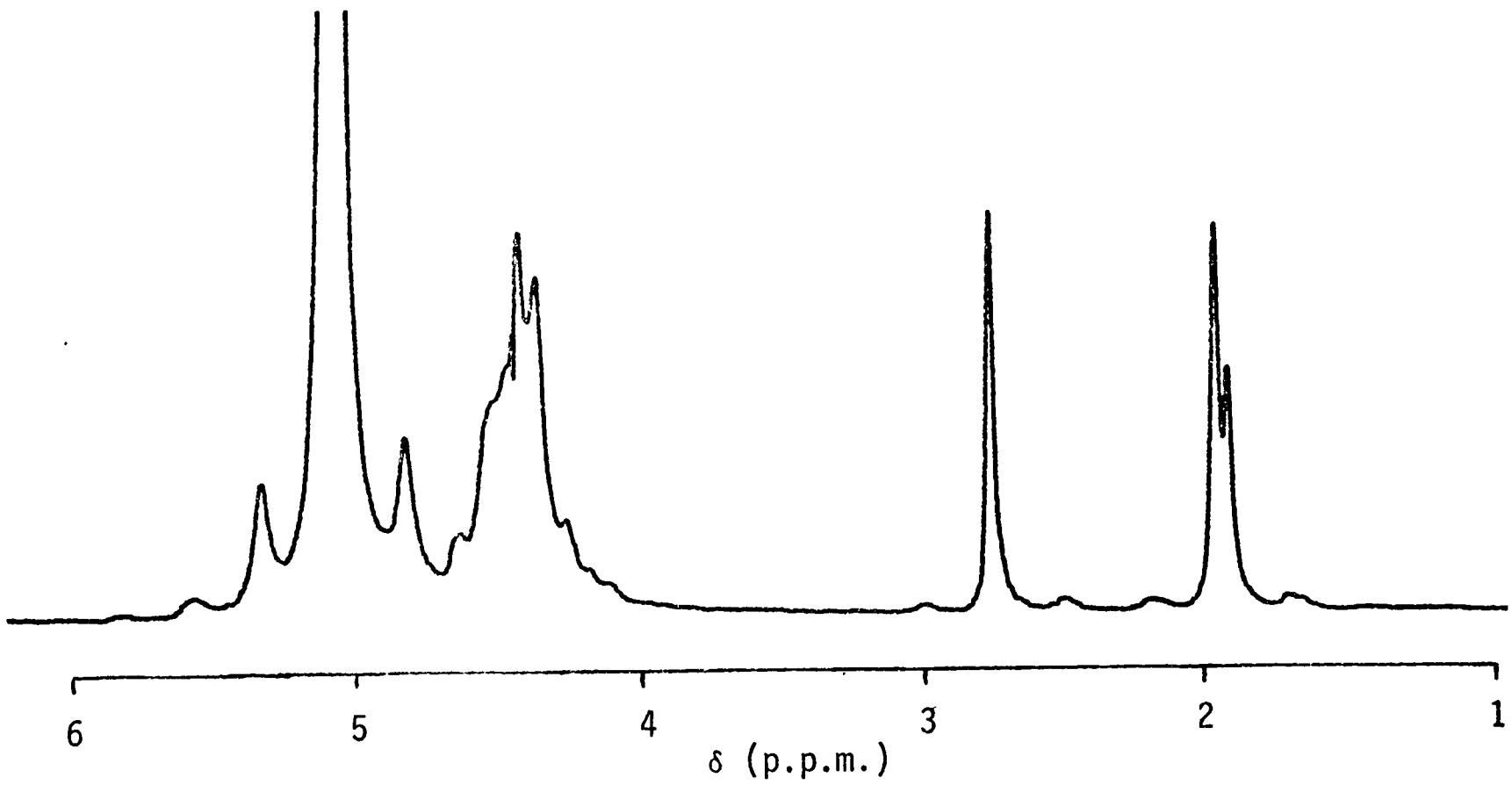


Fig. 7.5. Proton n.m.r. spectrum at 80 MHz of 1-deoxyfructose 6-phosphate at pH 4.5.

The proton n.m.r. spectrum of 1-deoxyfructose 6-phosphate (Fig. 7.5) may be more useful in this respect, since the methyl protons of the different forms of substrate give rise to singlets and their peaks are well separated from other resonances. However, the spectrum is not that expected for an equilibrium mixture of α and β anomers, as there are three peaks in the methyl region. The peaks at 1.95 and 1.91 δ probably represent the anomeric methyl groups of the cyclic forms. The peak at 2.78δ either represents a third form of the compound, or an impurity present in large amount. Several pieces of evidence suggest that the former conclusion is correct:

1) 1-Deoxyfructose 6-phosphate is monodisperse on two t.l.c. systems.

2) 1-deoxyfructose is monodisperse on t.l.c. and a very selective, enzymatic method of phosphorylation was used to produce the 6-phosphate.

3) Integration of the peak areas of the proton n.m.r. of 1-deoxyfructose 6-phosphate gives the expected ratio of 5:2.9 for protons in the alcoholic region to protons in the methyl region. This is reproducible for several preparations of the deoxy compound.

4) A sample of 1-deoxyfructose 6-phosphate was reduced using sodium borohydride. The product of this reaction gave a proton n.m.r. with a sharp doublet in the methyl region, centred at 1.7δ (J = 5.3Hz). The integration of this spectrum was 2.0:0.94 for the alcoholic region to methyl doublet. Thus, the three methyl peaks of 1-deoxyfructose 6-phosphate have merged to a doublet in a spectrum consistent with the expected reduction product, 1 deoxyglucitol 6-phosphate.

Table 7.2 pH-Dependence of methyl group peak widths of the proton n.m.r. spectrum of 1-deoxyfructose 6-phosphate.[a]

	Peak widths at half height	
pH	2.8 δ	1.9 δ[b]
1.2	6.56	6.95
1.8	4.10	6.77
4.5	2.28	2.44,2.60
6.7	4.25	4.67

a. Spectra in deuterium oxide solution at room temperature.

b. Two peaks are unresolved, except at pH 4.5.

The most likely explanation for the methyl peak of relatively low field (2.78δ) is that a proportion of the product is as the free keto form.

The n.m.r. spectrum shown in Fig. 7.5 is of a solution of 1-deoxyfructose 6-phosphate at pH 4.5. The peak widths are pH dependent (Table 7.2) and at both pH 6.7 and pH 1.8 the two methyl peaks at high field appear as a broad singlet. These results are consistent with a pH-dependent interconversion of the three different forms of 1-deoxyfructose 6-phosphate, causing reaction broadening. The relative change in rate with pH parallels that reported for such compounds as glucose, fructose and galactose.[198]

Although confirmation is required for the assignment of different forms of 1-deoxyfructose 6-phosphate, the presence of clearly defined

methyl peaks and the occurrence of a large proportion of the compound in the free keto form holds some promise for the use of this approach in studying the isomerase-catalyzed anomerization.

CHAPTER 8 EXPERIMENTAL

Instruments, materials and assays are as described in Chapter 5 with the additions described below.

Instruments

Proton n.m.r. spectra were obtained on a Varian HFT 80 spectrometer using Fourier transform.

Materials

Hexokinase, 6-phosphogluconate dehydrogenase, fructose, 2-deoxyglucose and 2-deoxyglucose 6-phosphate were obtained from Sigma. Transaldolase was from Boehringer.

3-Hydroxypropionaldehyde 3-phosphate diethyl acetal was prepared as the cyclohexylammonium salt by Mr. I. Farley using the method of Putnam et al.[21] This was converted to the free 3-hydroxypropionaldehyde 3-phosphate by the method of Putnam et al.[21] and stored as a solution at -20°C.

Thin layer chromatography

The progress of reactions and purity of products were monitored by thin layer chromatography. The systems used for phosphate esters were:

Solvent	Stationary phase
IM NaCl in water	Polyethyleneimine cellulose
Methanol:ammonia:water (7:2:1 by volume)	Cellulose

The phosphate-containing spots were located using Hanes-Isherwood spray,[199]

which contains ammonium molybdate (0.5g), water (5 ml) and acetone (40 ml). The plates were developed under uV light. Inorganic phosphate appeared as a yellow spot, organic phosphate as a blue spot.

Preparations

1) 5-deoxyfructose 6-phosphate

(a) Dihydroxyacetone phosphate (40 µmol) and 3-hydroxypropionaldehyde 3-phosphate (90 µmol) were mixed to give a solution (2 ml) whose pH was then raised to 7.5. Aldolase (15 units) was treated with bromohydroxyacetone phosphate to remove all traces of triosephosphate isomerase and then dialyzed against 50 mM triethanolamine-HCl buffer, pH 7.5. This enzyme solution was added to the solution of phosphate esters, which was then left at room temperature for 30 min. The reaction was quenched by passing the solution through a volume (5 ml) of Dowex-50-H^+ form ion-exchange resin. After raising the pH to 8.0, the product was purified by ion exchange on a DEAE-cellulose column (20 cm x 0.9 cm), equilibrated with 10mM NH_4HCO_3 and eluting with a linear gradient (100 ml) of NH_4HCO_3, 10mM to 400mM. 5-deoxyfructose 6-phosphate (16 µmol) was obtained from this column.

Attempts to hydrolyze the 5-deoxyfructose 1,6-bisphosphate (3 µmol) using fructose 1,6-bisphosphatase (incubation with 3.9 units for 24 hr at 37°) or at pH 0.1 or 0.7 (incubation for 24 hr at 37°) appeared to give little or no inorganic phosphate, monitored by phosphorus n.m.r. and by t.l.c. on PEI-cellulose.

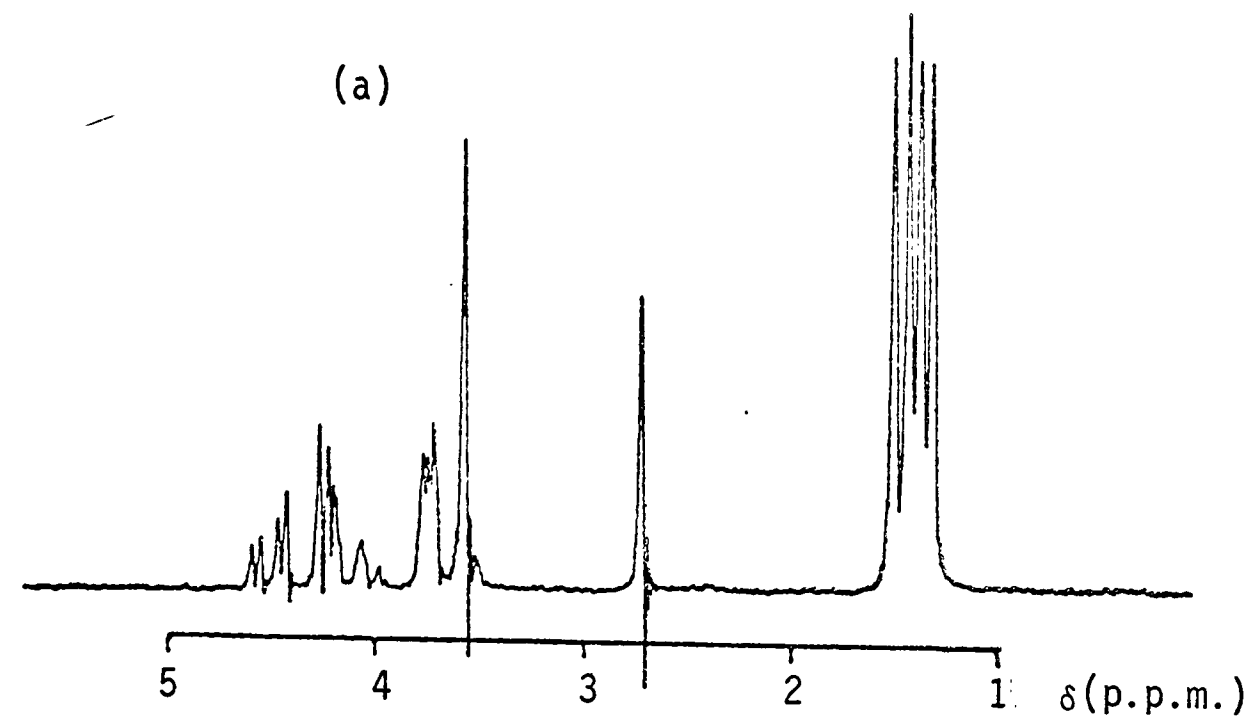
(b) For the second method of preparing 5-deoxyfructose 6-phosphate, the following were added to 200mM triethanolamine-HCl buffer,

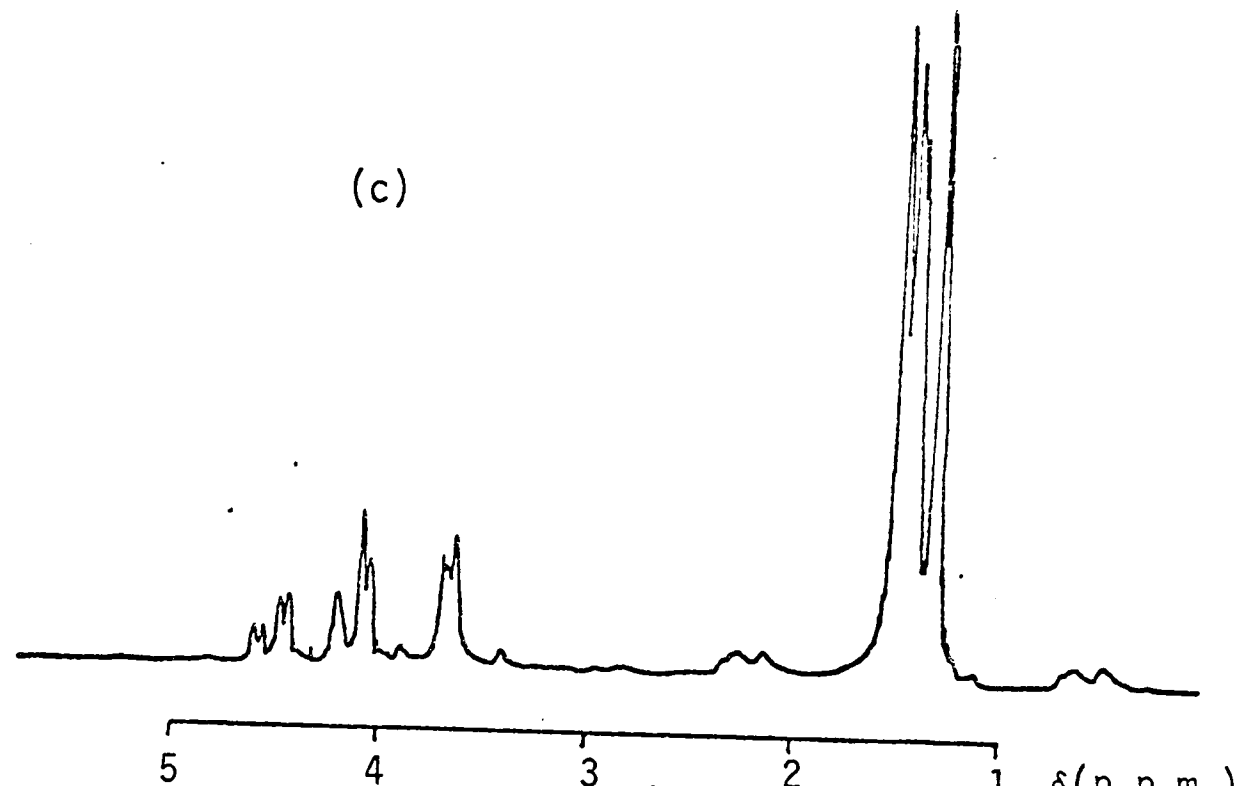
pH 7.5: NADH (50 mg), fructose 6-phosphate (18 mg), glycerol-3-phosphate dehydrogenase (0.1 mg), triosephosphate isomerase (0.05 mg) and transaldolase (5 units). 90 mM 3-hydroxypropionaldehyde 3-phosphate (1.2 ml) was added in 10 portions over 90 min, while the pH was periodically adjusted to remain in the range 7.1 to 7.5. After a further 30 min, there seemed to be no further reaction (monitored by the disappearance of absorption due to NADH at 340 nm). The product was purified on DEAE-cellulose as described above, to give a mixture of 5-deoxyfructose 6-phosphate (33 μmol) and fructose 6-phosphate (14 μmol).

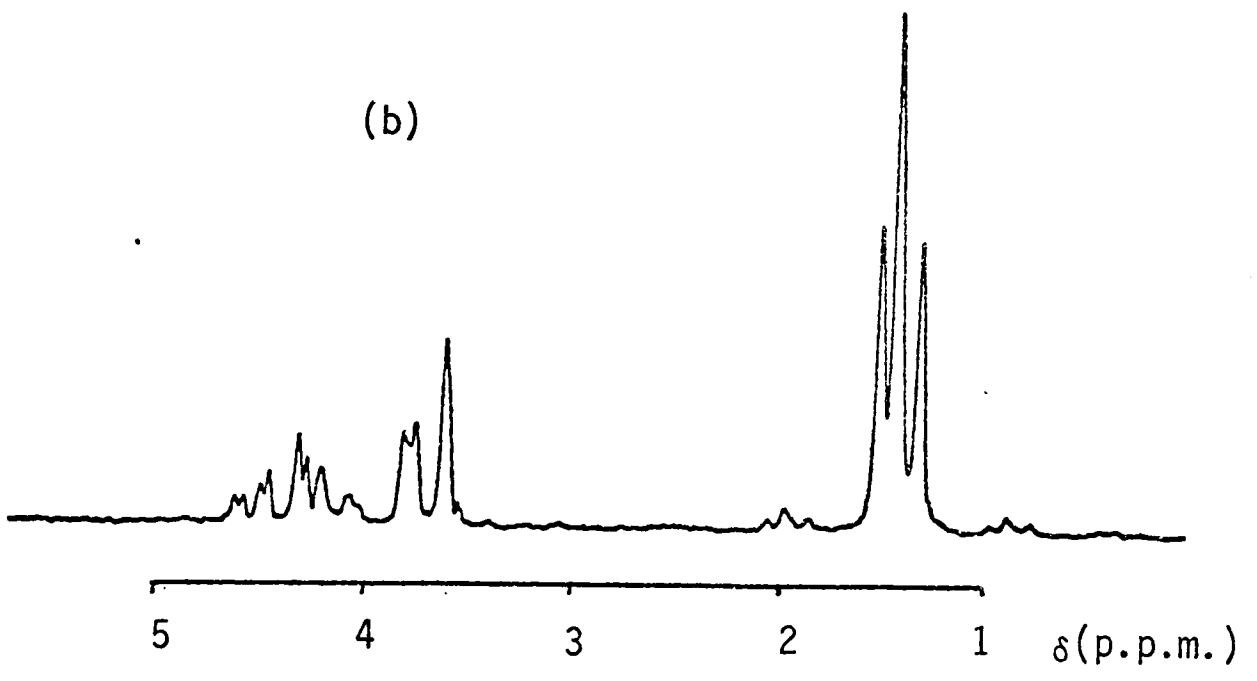
Attempts to remove the fructose 6-phosphate by further reaction with transaldolase, 3-hydroxypropionaldehyde 3-phosphate etc., or by ion exchange chromatography on Dowex-1-HSO_3^- form were not successful.

2) 1-chloro-1-deoxy-2,3:4,5-di-O-isopropylidene D-fructose

2,3:4,5-di-O-isopropylidene β-D-fructose was prepared by the method of Pacsu et al.[200] Concentrated H_2SO_4(40 ml) was added to dry acetone (1 l), stirred magnetically and cooled in an ice bath. Fructose (40 g) was added to the mixture and after stirring for 3 hr, this dissolved as the di-O-isopropylidene derivative. The solution was made slightly alkaline with concentrated ammonium hydroxide and $(NH_4)_2SO_4$ was removed by filtration. The filtrate was evaporated to a white solid which was recrystallized from water:ethanol (80:20 by volume). Further product was obtained by evaporating the supernatant to dryness and recrystallizing material from this. A recrystallization of the combined product from dilute ethanol gave white crystals, m.p. 95-96°C (literature value 96-97°C). The total yield was 39 g and the proton n.m.r. is shown in Fig. 8.1(a).







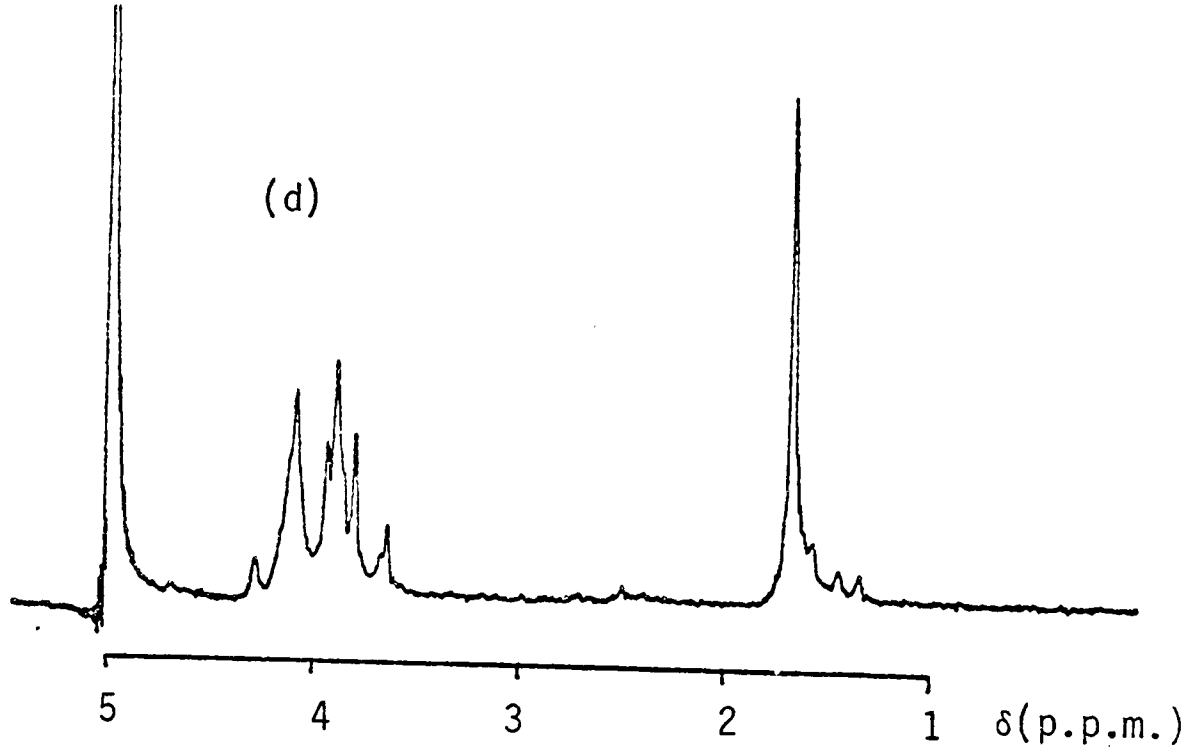


Fig. 8.1. Proton n.m.r. spectra at 60MHz of (a) 2,3:4,5-di-Oisopropylidene β-D-fructose, (b) 1-chloro-1-deoxy-2,3:4,5-di-O-isopropylidene D-fructose, (c) 1-deoxy-2,3:4,5-di-O-isopropylidene D-fructose, (d) 1-deoxyfructose. (a), (b) and (c) were run in deutero-chloroform, (d) was run in deuterium oxide.

1-chloro-1-deoxy-2,3:4,5-di-O-isopropylidene D-fructose was prepared by the method of Haylock et al.[143] 2,3:4,5-di-O-isopropylidene fructose (10 g) was dissolved in a solution of triphenyl phosphine (20 g) in carbon tetrachloride (120 ml). This solution was refluxed for 4 hr and then cooled, diluted with petroleum ether (b.p. range 40°-60°, 2 l) and left overnight at -20°C. The solution was filtered and evaporated to a syrup, which was redissolved in petroleum ether (400 ml) and left at 3°C for 3 hr. The solution was again filtered and evaporated to a yellowish solid. The residue to twice recrystallized from petroleum ether to give white crystals, m.p. 52-53°C (literature value, 53-53.5°C). The proton n.m.r. of this compound is shown in Fig. 8.1(b). The elemental analysis gave C, 51.7%; H, 7.0%; Cl, 12.7% (calculated for $C_{12}H_{14}ClO_5$: C, 51.7%; H, 6.9%; Cl, 12.7%).

3) 1-deoxyfructose 6-phosphate

The protected 1-chlorofructose (8 g) was dissolved in dry glyme (40 ml) and lithium aluminium hydride (4 g) was added. The mixture was refluxed overnight and the excess hydride was destroyed by addition of 10% aqueous ammonium chloride. The resulting mixture was filtered and the residue was washed with acetone. The combined filtrate and washings were evaporated using a water pump and the product was extracted from this residue by 3 portions (20 ml) of chloroform. This chloroform solution was evaporated to give a colourless syrup which was vacuum distilled. The product (6 g) was obtained at a vapour temperature of 75-80°C at 0.4 mM pressure. The n.m.r. of this 1-deoxy-2,3:4,5-di-O-isopropylidene fructose is shown in Fig 8.1(c) and is in good agreement with the published data.[193] It was not possible to crystallize this material.

1-deoxy-D-fructose was prepared by mixing the protected derivative (6 g) with 10% aqueous trifluoracetic acid (50 ml) and stirring overnight at room temperature. The two phases became miscible as the protecting isopropylidene groups were removed. The reaction was monitored by thin layer chromatography on silica gel plates using n-butanol:benzene (10:90 by volume). The spots were visualized by exposure to iodine vapour. The resulting solution was evaporated with water several times to remove all the acid. The residue was partitioned between chloroform (10 ml) and water (10 ml). The aqueous phase was extracted with 2 portions of chloroform (10 ml) and then treated with activated charcoal (200 mg). After filtration, the aqueous solution was evaporated to dryness and the product was freeze-dried to give 1-deoxyfructose as a white amorphous solid. The proton n.m.r. (Fig. 8.1(d)) shows that there are 3 methyl protons to 5 protons in the region 5-6 τ (CHOD, CH_2OD region). The material was monodisperse on thin layer chromatography on silica with 95% ethanol. The spot was visualized by exposure to iodine vapour.

1-deoxyfructose was phosphorylated using hexokinase/ATP. 1-deoxyfructose (100 mg) and ATP (400 mg) were dissolved in 100 mM-tris-HCl buffer (10 ml) containing $MgCl_2$ (10 mg). The pH was adjusted to 8.0 and hexokinase (400 units, previously dialyzed against 100 mM tris-HCl buffer, pH 8.0) was added. The reaction mixture was left for 24 hr at room temperature, with the pH periodically adjusted to 8.0. The solution was repeatedly treated with activated charcoal (500 mg) to remove adenine compounds, until the OD_{260} was less than 2.

During this phosphorylation of deoxyfructose any fructose present is preferentially phosphorylated. So fructose 6-phosphate was removed by addition of $NADP^+$ (20 mg), glucose-6-phosphate dehydrogenase (0.05 mg) and glucose-6-phosphate isomerase (0.01 mg). The conversion of fructose 6-phosphate to 6-phosphogluconate was monitored by the absorbance at 340 nm. After approximately 2 hr the reaction was complete and the solution was treated with activated charcoal (1 g) for 30 min. After filtration the OD_{260} and OD_{340} were both zero. An assay for fructose 6-phosphate showed there was none.

1-deoxyfructose 6-phosphate was purified by ion-exchange chromatography on a DEAE-cellulose column (1.6 cm x 40 cm), equilibrated in 10 mM NH_4HCO_3 and eluting with a linear gradient (100 ml) of NH_4HCO_3 from 10 mM to 300 mM. Fractions containing 1-deoxyfructose 6-phosphate, which were completely separated from the fractions containing 6-phosphogluconate, were pooled and passed through Dowex-50-H^+ form (30 ml), to remove ammonium bicarbonate. The pH of the solution was raised to 69 by addition of IN NaOH. In this state, the phosphate ester could be freeze dried to give a white solid (82 mg). The proton n.m.r., shown in Fig 8.2, gives 3 protons in the methyl region and 5 protons in the CHOH, CH_2OH region. This material was monodisperse on thin layer chromatography on cellulose plates (R_f = 0.60) and PEI-cellulose plates (R_f = 0.45) described above. Elemental analysis gave a P:C ratio of 0.45 (calculated for $C_6H_{11}O_8PNa_2$, 0.43).

Assays

1) D-Glucose was assayed by phosphorylation to glucose 6-phosphate, which

was then oxidized by $NADP^+$/glucose-6-phosphate dehydrogenase. The assay mixture in 100 mM-tris-HCl buffer, pH 8.0 (2.3 ml) contained ATP (1 mg), 1 mM $MgCl_2$, 0.25 mM $NADP^+$, glucose-6-phosphate dehydrogenase (0.05 mg) and hexokinase (0.05 mg).

2) 3-Hydroxypropionaldehyde 3-phosphate was assayed using the method for glyceraldehyde 3-phosphate, described in Chapter 5.

3) 5-deoxyfructose 1,6-bisphosphate and fructose 1,6-bisphosphate were assayed by the method for dihydroxyacetone phosphate, (described in Chapter 5) with the addition of aldolase (0.05 mg) and triosephosphate isomerase (0.01 mg) to the assay mixture.

4) 5-deoxyfructose 6-phosphate was assayed by the method described for fructose 6-phosphate with the addition of glyceraldehyde 3-phosphate (2 μmol) and transaldolase (1 unit) to the reaction mixture.

5) 1-deoxyfructose 6-phosphate was assayed using the following in 200 mM-trethanolamine-HCl buffer, pH 7.5 (2.2 ml): Transaldolase (1 unit), 3-hydroxypropionaldehyde 3-phosphate (2 μmol), triosephosphate isomerase (0.01 mg), glycerol-3-phosphate dehydrogenase (0.05 mg) and NADH (0.4 mg).

6) 6-Phosphogluconate was assayed by using the oxidative decarboxylation reaction, catalyzed by 6-phosphogluconate dehydrogenase/$NADP^+$, giving ribulose 5-phosphate. The assay mixture in 200 mM triethanolamine-HCl buffer, pH 7.5 (2.2 ml) contained $NADP^+$(0.4 mg), $MGCL_2$ (0.1 mg) and 6-phosphogluconate dehydrogenase (1 unit).

Tritium exchange experiments with deoxyhexose 6-phosphates

1) 2-deoxyglucose 6-phosphate

Two lots of 2-deoxyglucose 6-phosphate were dissolved, one in

100 mM NH_4HCO_3, pH 8.0 (200 µl), the other in this buffer (200 µl) containing 1500 units/ml glucose 6-phosphate isomerase to give 60 mM solutions of the phosphate esters. Tritiated water (5 Ci/ml) was added to each solution (10 µl to the former, 50 µl to the latter). The solutions were then incubated at room temperature for 36 hr. The solvent was removed by bulb-to-bulb vacuum sublimation of the frozen solution. Water was removed by this method several times from each sample. The residues were dissolved in 10 mM NH_4HCO_3 and purified on DEAE-cellulose columns (15 cm x 0.9 cm) equilibrated in 5 mM NH_4HCO_3, pH 8.3. Elution was by a linear gradient (50 ml) of 5 mM to 300 mM NH_4HCO_3. The fractions containing 2-deoxyglucose 6-phosphate (assayed by the method for glucose 6-phosphate) were pooled and the specific radioactivity was determined in each case.

2) 1-deoxyfructose 6-phosphate

This experiment was carried out in the same way as above, except the concentration of the 1-deoxyfructose 6-phosphate in the incubation solution was 32 mM. One solution contained 1600 unit/ml of the isomerase. Tritiated water (25 µl) was added to each solution.

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SUMMARY

The work presented in this thesis is concerned with the mechanism of two enzymes of the glycolytic pathway, each of which catalyzes an aldose-ketose isomerization.

The first part of the thesis deals with triosephosphate isomerase, which catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. A mechanism has been proposed for this reaction that involves the transfer of a proton between carbon centres of the substrates, and an enzyme-bound cis-enediol as a reaction intermediate.[1]

The orientation and accessibility of the enzyme-bound substrates was studied by boro[^{3}H]hydride reduction of unlabelled triosephosphates and borohydride reduction of monodeuterated substrates both in the presence and absence of a large concentration of the isomerase. Analysis of the distribution of the hydrogen isotope in the reduction product, glycerol phosphate showed that of the four possible positions of boro-hydride attack on the enzyme-bound species (from the si and re face of each of the substrate carbonyl groups), only one product is formed: that resulting from reduction at the si face of dihydroxyacetone phosphate. Indeed, reduction occurs here some 14-times faster than at the si face of this substrate in free solution. The inaccessibility of the other three faces of the carbonyl groups of the enzyme-bound substrates is presumably due to the steric bulk of the enzyme. Reduction from the si face of dihydroxyacetone phosphate is actually catalyzed by the isomerase in spite of any expected inaccessibility. This catalysis is best

explained by the existence of an electrophilic group at the enzyme active site that polarizes the carbonyl group of dihydroxyacetone phosphate. It is proposed that this electrophilic group is in part responsible for the facile abstraction of the proton from the substrate in the enzyme-mediated isomerization.

In order to obtain direct evidence for the cis-enediol intermediate of the isomerase-catalyzed reaction, deuterated diimide was used in an attempt to trap this species. Since diimide reduces only non-polar double bonds, it was expected to reduce the enediol in a syn manner to give doubly-labelled glycerol phosphate. The stereochemistry of the enediol could then be determined from a knowledge of the position of labelling in the glycerol phosphate product. Unfortunately the yield of glycerol phosphate was too small to perform the necessary stereochemical analysis.

Each of the triose phosphates exists in two forms: the hydrate and keto form.[2,3] The keto forms are known to be the true substrates of the isomerase,[2,3] but the kinetic and equilibrium parameters of the catalyzed reaction must be modified because a proportion of the substrate is present as the inactive hydrate. These parameters would have to be further modified if the hydrates were to bind to the enzyme as competitive inhibitors. This question was investigated by n.m.r. spectroscopy.

Using proton n.m.r., the percentage of dihydroxyacetone phosphate present as the hydrate was found to increase on decreasing the temperature from 23°C to 1°C, or on protonating the substrate. Binding of hydrate and keto forms of dihydroxyacetone phosphate to the isomerase was studied by measurement of peak widths in the phosphorus n.m.r. spectra at

increasing enzyme concentrations. At pH 6.9 and 1°C, the keto peak was considerably broadened due to binding of this species, whereas no broadening of the hydrate peak was observable even at high enzyme concentrations. This indicates that the hydrate binds to the isomerase at least one order magnitude less tightly than does the keto form.

The second part of this thesis is concerned with glucose-6-phosphate isomerase, which catalyzes the interconversion of glucose 6-phosphate and fructose 6-phosphate. The mechanism proposed for this interconversion is analogous to that for triosephosphate isomerase, namely the transfer of a proton between carbon centres of the keto forms of the substrates via a cis-enediol reaction intermediate.[4,5] The α-anomer of each substrate is used preferentially, with the β-anomer being isomerized at a somewhat slower rate.[6,7] The isomerase also catalyzes the anomerization of each substrate,[6,7] but it is not clear how the two activities of the enzyme are related mechanistically. In principle, the use of deoxy analogues of the substrates should allow each enzyme activity to be studied independently from the other, and in this thesis experiments are described that test the feasibility of this approach.

2-Deoxyglucose 6-phosphate and 1-deoxyfructose 6-phosphate are close analogues of the cyclic forms of the substrates. They cannot undergo aldose-ketose isomerization but may undergo enzyme-catalyzed anomerization. A preparation of 1-deoxyfructose 6-phosphate was developed and its K_i-value with the isomerase was determined to be 0.3 mM at 30° and pH 7.5

The isomerase catalyzes the incorporation of tritium into 2-deoxyglucose 6-phosphate from tritiated water at a rate some 10^6-times

slower than into the natural substrate. This enzyme-catalyzed tritium exchange may be explained by enolization of the analogue on the surface of the enzyme in a manner similar to the formation of the enediol in the normal enzymic reaction. No tritium exchange could be observed to occur into 1-deoxyfructose 6-phosphate.

Since these analogues bind to the isomerase with values of K_i similar to the Michaelis constants of the natural substrate, but undergo enolization extremely slowly on the enzyme, they promise well for studies of the anomerization reaction of glucose-6-phosphate isomerase.

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