CHEMICAL AND ENZYMIC STUDIES OF CARBOHYDRATE OXIMES

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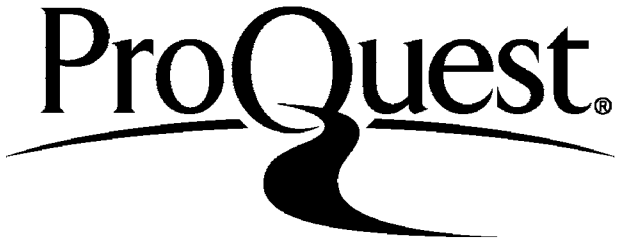

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ABSTRACT

The oximes of D-arabinose, D-galactose, D-mannose and D-ribose have been synthesised and their structures determined in the solid state and in solution by a variety of chemical and physical methods. These carbohydrate oximes can exist in the acyclic anti(Z) or syn(E) form in the solid state, and isomerise to a mixture of anti(Z, 20%) and syn(E, 80%) forms in aqueous solution. D-glucose oxime exists in the cyclic β-pyranose form in the solid state and equilibrates to a mixture of β-pyranose (23%), α-pyranose (7%), syn(E, 56.5%), and anti(Z, 13.5%) forms in solution.

The interconversion of carbohydrate oximes in aqueous solution was studied and found to be simple (first order) except for D-glucose oxime which showed complex isomerisation kinetics. The simple (first order) isomerisation of D-arabinose oxime was shown to be subject to specific and general acid, and specific base catalysis.

D-Arabinose oxime has been shown to act as a substrate of yeast hexokinase, the product D-arabinose oxime-5-phosphate (lithium salt) has been isolated and characterised. This result together with observations of the interaction of other carbohydrate derivatives has allowed the current picture of the substrate specificity of this enzyme to be extended.

By application of transition state theory carbohydrate oximes are predicted to act as inhibitors of enzymes which catalyse the interconversion of aldoses and ketoses. This prediction has been confirmed in the case of D-xylose (D-glucose) isomerase (Streptomyces species), of which D-arabinose and D-glucose oximes are more powerful inhibitors than other compounds previously examined.

DEDICATED

TO

H.H. Dr. Syedna Mohammed Burhanuddin Saheb

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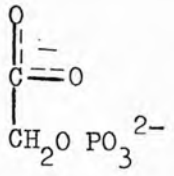
CHAPTER 1 ENZYME INHIBITION BY TRANSITION STATE ANALOGUES AND ITS APPLICATION TO ALDOSE-KETOSE ISOMERISATION

1.1 TRANSITION STATE ANALOGUES AS ANTIMETABOLITES

The recognition of the ability of antimetabolites to act as drugs dates from D.D. Woods[1] discovery in 1940 that the effectiveness of sulphanilamide is due to its antagonism to the bacterial growth factor (para-aminobenzoic acid). Today the term antimetabolite encompasses a multitude of compounds which inhibit the normal operations of enzymes, transport and binding proteins and receptors, by substitution for their usual substrates or cofactors and regulatory agents.

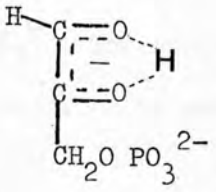
In the case of enzymes, catalysis appears to depend on the development of strong forces of attraction between the enzyme and activated forms of the substrate, which subside as the product is formed and the enzyme returns to its free state. So great is this affinity that a stable substance that is recognised by the enzyme as a substrate in the midst of its conversion to product, rather than as an alternative substrate or product, is expected to be an unusually effective inhibitor.[2-4] Such an inhibitor is called a transition state analogue inhibitor. It is a molecule which structurally resembles the substrate portion of the transition state (s^*) for the enzymic reaction.[5] Such inhibitors are usually of the reversible ('Km') type and do not become covalently altered on interaction with the enzyme. An example is 2-phosphoglycolic acid anion (1) which is a potent inhibitor of triose-phosphate isomerase

(EC 5.3.1.1) due to its resemblance to the proposed high-energy intermediate (2).[6]



1

Ki = 4×10^{-7}M



2

Substrate Km = 3×10^{-4}M

In addition to their possible use as antimetabolites, inhibitors of this kind can provide a useful indication of the particular mechanism by which a substrate is transformed by the enzyme that it inhibits. In certain cases it may be difficult to isolate reaction intermediates and inhibitors can sometimes provide mechanistic information that is not easily accessible by other methods. Structural features of strong inhibitors may also provide an indication of binding determinants at the active site which are important for catalysis. The binding of analogues of activated intermediates in substrate transformation in conjunction with exact structural studies, may help to provide a dynamic picture of the succession of events that occur during the catalytic process, and may be useful for examining the possibility that limited changes in the structure of the active site occur during enzyme action. Such changes have been postulated as a basis for enzyme specificity and regulation,[7] and may contribute to catalysis itself.[8]

1.2 THE THEORY OF INHIBITION BY TRANSITION STATE ANALOGUES

That enzymic catalysis could be described in terms of the activated complex or transition state theory of reaction rates was first suggested by Pauling in 1948.[9] The application of the transition state theory of reaction rates to enzymic catalysis has been discussed by Wolfenden,[10] Lienhard,[11,12] Lindquist,[5] and Jencks[13] and involves a consideration of binding forces which exist during enzymic reactions. According to this theory[14] the rate of any reaction is proportional to the concentration of the reactant in the transition state form. More specifically, the first-order rate constant (kn) for the non-enzymic reaction is given by equation (1)

$$kn = \frac{kT}{h}[K_n^{\ddagger}] \qquad \ldots \qquad (1)$$

where k is Boltzmann's constant, h is Planck's constant, T is the absolute temperature, and $K_n^{\ddagger}$ is the equilibrium constant for the formation of the transition state.

Equation (1) is derived from Scheme 1.1 given below:

$$S \xrightarrow{k_n} P$$

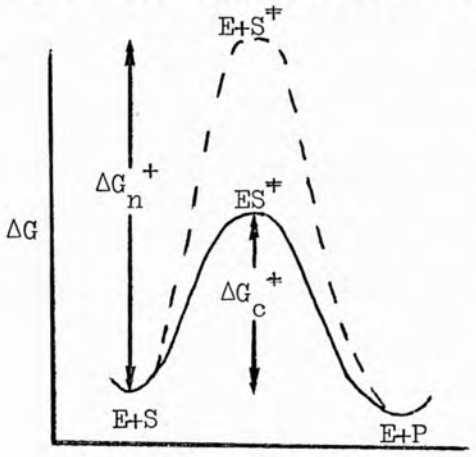
$$S \underset{}{\overset{K_n^{\ddagger}}{\rightleftharpoons}} S^{\ddagger} \xrightarrow{kT/h} P$$

$$\text{rate} = \frac{kT}{h}[S^{\ddagger}] = \frac{kT}{h}[K_n^{\ddagger}][S] = k_n[S]$$

Scheme 1.1

$[S]$ and $[S^{\ddagger}]$ are the concentrations of the substrate and the transition state, respectively, and where the transition state is in

equilibrium with the reactant ($K_n^{\neq} = [S^{\neq}]/[S]$) as predicted by the transition state theory.[14] The relationship in equation (1) between the rate and equilibrium constant is valid for the conversion of a reactant S to a product P, which will take place by the pathway with the lowest energy barrier. The transition state will be the structure of highest energy on this pathway (see Fig. 1.1)



$$\Delta G^{\neq} = -RT\ln K^{\neq}$$

Fig. 1.1 Free energy-reaction pathway profile for a single substrate enzymic reaction and the corresponding non-enzymic reaction.

In the presence of an enzyme or other catalyst a reaction proceeds at a rate that, at low substrate concentrations, is proportional to the concentration of both the substrate and the enzyme. Kinetically it is a second-order reaction with a rate constant, k_{cat}/K_m and a corresponding equilibrium constant ($Ke^{\neq}$) for activation given by Scheme 1.2.

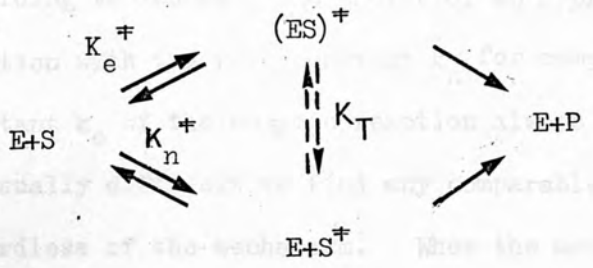
$$E + S \xrightarrow{k_e} E + P \qquad k_e = k_{cat}/K_m$$

$$E + S \underset{}{\overset{K_e^{\neq}}{\rightleftharpoons}} (ES)^{\neq} \xrightarrow{kT/h} E + P$$

$$\text{rate} = \frac{k.T}{h} (K_e^{\neq}) [E][S] = k_e[E][S]$$

Scheme 1.2

Combining Schemes (1) and (2), it is found that K_T, the formal dissociation constant of the enzyme substrate complex in the transition state, $ES^{\neq}$, which yields free enzyme (E) and the altered substrate ($S^{\neq}$) can be estimated from the cycle shown in Scheme 1.3.[15] It is equivalent to the rate constant for the non-enzymic reaction, k_n divided by the rate constant for the enzymic reaction, k_e and appears in units of moles per litre (assuming that the second-order rate constant k_{cat}/K_m employed conventional units of concentration)



$$K_T = \frac{K_n^{\neq}}{K_e^{\neq}} = \frac{k_n}{k_{cat}/K_m}$$

$$\therefore \quad \frac{K_T}{K_m} = \frac{k_n}{k_{cat}}$$

Scheme 1.3

Lienhard[12] has considered five factors which influence the binding constant K_T and has discussed the relative importance of each factor in enzyme catalysis. The factors are (1) transition state structure, (2) entropy change, (3) solvent interaction, (4) enzyme interactions, and (5) conformational changes of the enzyme.

Transition state theory gives no information about the extent of similarity in structure between the transition state of the non-enzymic reaction and the substrate portion of the transition state of the enzymic reaction. In scheme 1.3 they are assumed to be similar. Our knowledge of the mechanism of enzymic reactions and of the corresponding non-enzymic reactions suggests that in most cases there is a basic similarity in the bond-making and bond-breaking processes that the substrate undergoes, so that the transition state for the enzymic and non-enzymic reactions are similar in structure and energy.[16] However, there may be exceptions to this conclusion, in which the mechanism of the enzymic reaction is fundamentally different from the corresponding non-enzymic reaction. According to Jencks[13] the choice of an appropriate non-enzymic reaction with the rate constant k_n for comparison with the rate constant k_e of the enzymic reaction always required discretion as it is usually difficult to find any comparable non-enzymic reaction regardless of the mechanism. When the mechanism of the two reactions are different, this means that the non-enzymic reaction with a mechanism that is the same as that of the enzymic reaction must have a still higher free energy of activation i.e. k_n/k_e will be larger than the true ratio. It is possible, at least in principle

to find analogues for the (correct) transition state that give a ratio that is even smaller than k_n/k_e in this situation.

According to Lienhard[12] the category of entropy change refers largely to loss of entropy of S and $S^{\neq}$ upon their binding to the enzyme. Both species lose translational and overall rotational entropy, and this loss makes an unfavourable contribution to binding for both. In addition the defined geometries of ES and $ES^{\neq}$ complexes requires some loss of entropy of internal rotation. For some reactions the loss of internal rotational entropy upon binding will be less for $S^{\neq}$ than for S. Where this difference in loss of internal rotational entropy exists, it is a factor which contributes to tighter binding of $S^{\neq}$ than of S and so contributes to catalysis.

Additional entropy changes may occur as a result of interaction with water. The association of S and of $S^{\neq}$ with the enzyme are accompanied by the disruption of some of the interactions that occur between water molecules and functional groups of S, $S^{\neq}$ and the enzyme. The water that is released interacts with itself (Scheme 1.4). If S interacts with water more strongly than $S^{\neq}$, then water interactions

$$\begin{array}{ccccc} E & + & S \text{ or } S^{\neq} & \leftrightharpoons & ES \text{ or } ES^{\neq} \quad + H_2O \\ | & & | & & | \\ H_2O & & H_2O & & H_2O \end{array}$$

Scheme 1.4

favour tighter binding of $S^{\neq}$ than S and so contribute to catalysis. If the reverse is true, water interactions hinder catalysis. Moreover, if

the enzymic and uncatalysed reactions in water have similar mechanisms but differ in the point on the reaction coordinate at which the transition state is reached (see Fig. 1.2), this would presumably lead to an underestimation of the ability of the enzyme to stabilise the least stable intermediate on the non-enzymic reaction pathway.

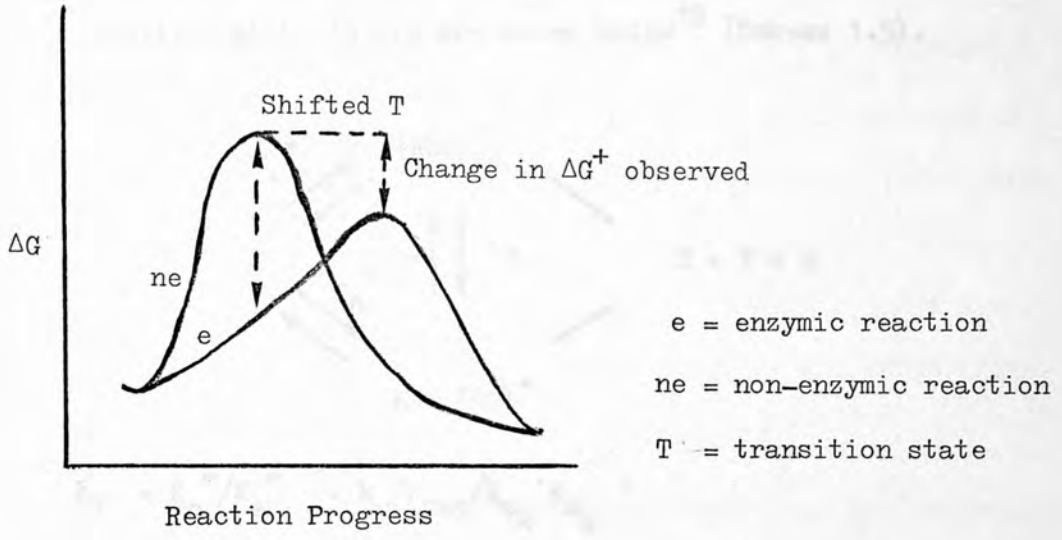


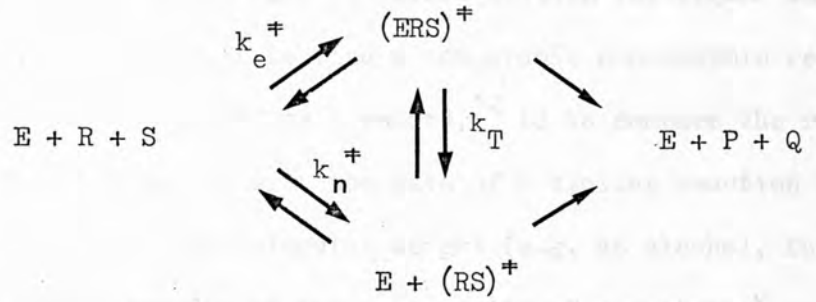
Fig. 1.2 Comparision of free energy profiles for catalysis, for a case in which the transition states are reached at different points on the reaction coordinate.

The contributions from non-covalent interaction between the enzyme and S and $S^{\neq}$ in the ES and $ES^{\neq}$ complexes fall into the same classes as those with water : hydrogen bonding, electrostatic interactions, and van der Waals forces. It may be possible to estimate the relative strength of the specific interactions of an enzyme with S and $S^{\neq}$, by making use of the structures determined by X-ray crystallography.

Finally, it is probable that changes in the conformation of the protein accompany the binding of the substrate to the enzyme.

Such a change must be energetically unfavourable for the enzyme alone because if this were not so, the free enzyme would exist in the altered conformation that it adopts in the ES complex.

In the case of a two substrate reaction which proceeds via a ternary complex of both substrates and enzyme (ERS), then the equilibria describing the non-enzymic and enzymic reactions in terms of the transition state theory are shown below[15] (Scheme 1.5).



$$K_T = K_n^{\neq}/K_e^{\neq} = k_n/k_{cat}/K_{m_R} \cdot K_{m_S}$$

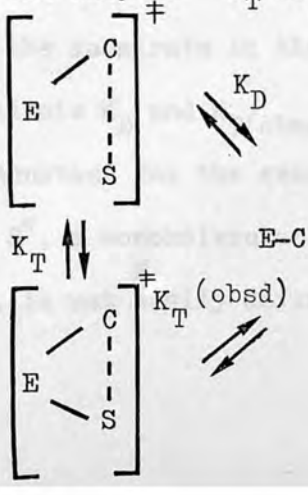
Scheme 1.5

The equilibrium constant for the release of the transition state is then equivalent to the second-order rate constant for the non-enzymic reaction k_n, divided by the third-order rate constant for the enzymic reaction $k_{cat}/K_{m_R} \cdot K_{m_S}$, in dilute solution. If inhibitors bear a useful analogy to activated intermediates in catalysis, rather than to the substrates, their K_i values are usually expected to be much lower than the K_m values of substrates. K_T must be less than K_m in Scheme 1.3 if catalysis is to occur at all. K_m may of course be a complex combination of rate constants with little physical meaning and the same may be true of the term $K_{m_R} \cdot K_{m_S}$ in Scheme 1.5. In the special

case where K_{m_R} and K_{m_S} are true dissociation constants and are expressed as mole fractions, their product ($K_{m_R}.K_{m_S}$) might be considered to be equivalent to the dissociation constant of an enzyme complex with a hypothetical molecule, R.S , in which the reactants are virtually in collision with each other at the active site, but have not yet undergone reaction.

A special case for transition state theory is the reaction in which a covalent bond is formed between the enzyme and substrate. It is very difficult to find a comparable non-enzymic reaction. One recourse suggested by Lienhard,[12] is to compare the rate of an enzymic reaction with the rate of a similar reaction involving a catalyst of low molecular weight (e.g. an alcohol, for comparison with a serine residue at the active site of a protease).

In a re-evaluation of the theoretical aspects of the transition state analogue approach Schray and Klinman[17] have considered the case of an enzyme containing a catalytic group C that interacts with the substrate S. The reactions which are being compared are those in which the group C acts simply as a chemical catalyst, without binding of either the substrate or the transition state to the rest of the enzyme and the same reaction with binding of the substrate and the transition state to the enzyme. K_T in Scheme 1.6 does not represent



Scheme 1.6

the complete dissociation of the transition state from the enzyme and cannot be estimated from the observed binding The actual measured dissociation constant for the transition state (or an analogue thereof) is $K_{T(obsd.)}$ and it is related to K_T by equation (2).

$$K_{T(obsd.)} = K_T \times K_D \quad \ldots \quad (2)$$

Substitution of $K_{T(obsd.)}/K_D$ for K_T in equation (2) gives

$$K_{T(obsd.)} = \frac{k_n}{k_{cat}/K_m} \cdot K_D \quad \ldots \quad (3)$$

Apparently the tightness of binding of a transition state or transition state analogue in this system does not provide a direct measure of the catalytic advantage ($k_{cat.}/k_n$) brought about by the enzyme. The value of $K_{T(obsd.)}$ is the resultant favourable contributions from the intrinsic binding energy and the chemical interactions between the catalyst and the transition state, and an unfavourable contribution from the loss of entropy required for combination with the catalytic group.[13]

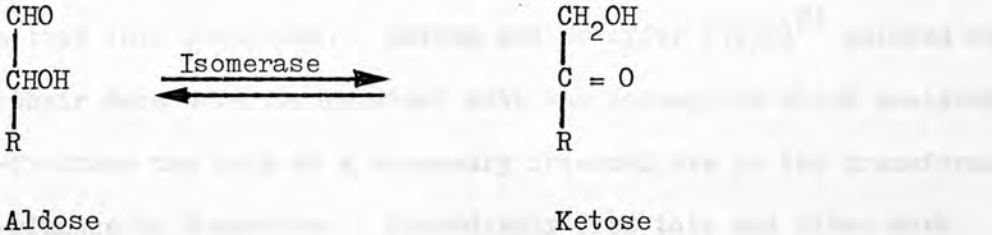
Although this system is a useful one to illustrate the contribution of different factors to catalysis, it is not readily applied to real systems. If there is a covalent bond between the catalytic group and the substrate in the transition state it is difficult or impossible to evaluate K_D and $K_{T(obsd.)}$ and the relationship of k_n, a bimolecular rate constant for the reaction with a catalytic group, to the stability of $S^{\ddagger}$, a monomolecular transition state in the absence of catalyst, is not easily defined.[13]

Thus, for catalysis to be observed, the altered substrate must be tightly bound in activated forms which approach the transition state. Tight binding alone should not be considered a sufficient criterion in studies of this kind. Compounds tested should also bear some mechanistically understandable relationship to substrates and products, as tight binding of transition state analogues does not give an absolute indication of transition state structure, but rather provides an index of the difference between the structures of the substrate and transition state.

A substantial number of proposed transition state anlogue inhibitors have been reported and their properties reviewed.[5,10,11,12,15] For comparison the inhibitor constants, K_i, and the K_m values of related substrates with which the inhibitor is in most cases competitive are used.

1.3 APPLICATION OF THE TRANSITION STATE ANALOGUE APPROACH TO ALDOSE-KETOSE ISOMERASES

In this thesis the application of the transition state analogue approach to the enzyme catalysed interconversion of aldoses and ketoses (Scheme 1.7) is described. Enzymes which catalyse this interconversion



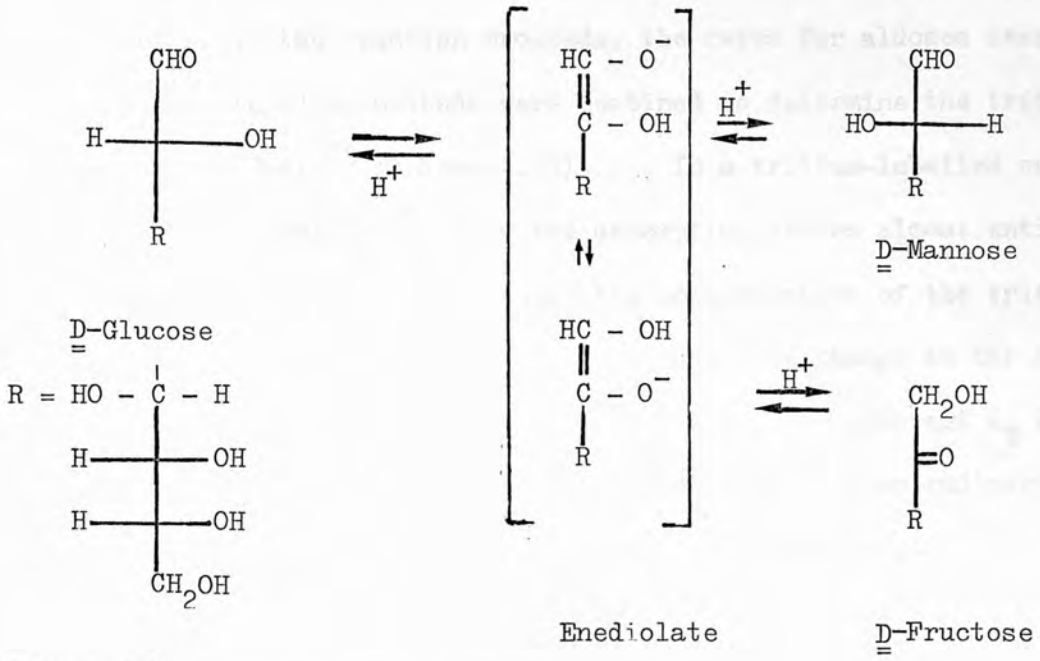
Scheme 1.7

are of widespread biological occurrence (e.g. D-glucose-6-phosphate isomerase EC 5.3.1.9 a component of the glycolytic cycle) and of commercial importance (D-xylose (D-glucose) isomerase, EC 5.3.1.5).

In order to design specific inhibitors aimed at producing a good model for the transition state structure, information about the mechanism of the enzymic reaction, including knowledge of a hypothetical transition state structure would be very useful. In the case of aldose-ketose isomerases some insight into the enzymic reaction mechanism may be obtained first by considering the chemical isomerisation of reducing sugars under alkaline conditions.

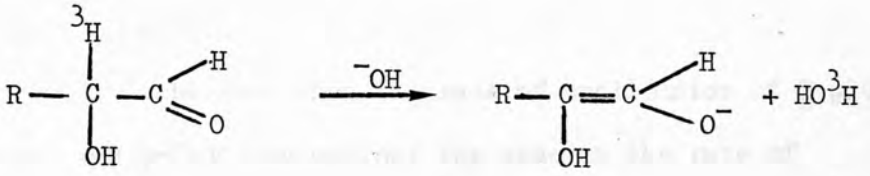
1.3.1 The Base-catalysed Isomerisation of Sugars

These complex and manifold reactions have engaged the attention of carbohydrate chemists ever since their discovery by Lobry de Bruyn and Alberda van Ekenstein in 1895.[18] In 1900 Wohl and Neuberg[19] advanced the concept that interconversion of D-glucose, D-mannose, and D-fructose occurs through reversible enolisation. Topper and Stetten (1951)[20] from experiments using deuterium exchange postulated a trans-enediol intermediate in the transformation of D-fructose into D-glucose, and a cis-enediol for the conversion of D-fructose into D-mannose. Sowden and Schaffer (1952)[21] pointed out that their data were inconsistent with the assumption which assigned to D-fructose the role of a necessary intermediate in the transformation of D-glucose to D-mannose. Accordingly from this and other work reviewed by Speck,[22] D-glucose, D-mannose and D-fructose should give the same enediol (Scheme 1.8).



Scheme 1.8

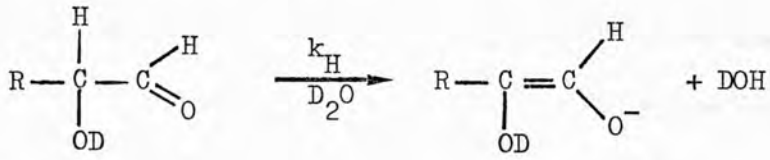
Isbell and co-workers[23,24] tried to test this hypothesis using labelling techniques. One of these methods[23] depends on the rate at which tritium ions are released from aldoses-2-t to the solvent (Scheme 1.9). The method requires tritium-labelled sugars which are difficult to obtain and involves a primary isotope effect.



Scheme 1.9

Isbell and co-workers[24] have developed a convenient method for measuring the rate of enolisation that does not involve a primary isotope effect. It uses infra-red absorption at 2.95 microns for measuring the amount of DOH formed by enolisation of the deuterated sugar in alkaline D_2O.

However, as the reaction proceeds, the rates for aldoses increase. The two preceding methods were combined to determine the tritium isotope effect.[24] (Scheme 1.10). In a tritium-labelled substrate in D_2O, the change in infra-red absorption arises almost entirely from release of protons because the concentration of the tritium species in the reactant is small. Thus, the change in the DOH absorption represents release of protons. The constant k_T for release of tritium to the solvent is determined from radioactivity measurements of water from the same reaction mixture.



Scheme 1.10

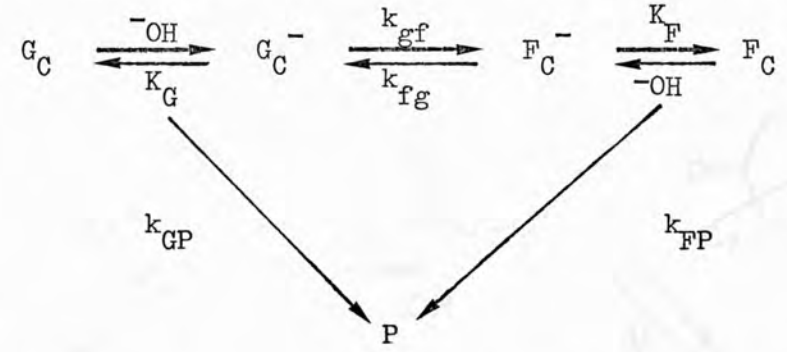
For comparative studies of a large group of sugars another method[23] was used involving tritium incorporation in the sugar by reversible enolisation in water-t. The method measures the overall rate of enolisation and deenolisation. However, the process is complicated by an isotope effect.

They[23,24] observed that the rate of enolisation of D-glucose, D-mannose and D-fructose was not the same as the rate of isomerisation of the corresponding sugar. This suggested that these sugars went through more than one intermediate in the isomerisation reaction.

An alternative mechanism to that of the enediol intermediate, is the hydride transfer mechanism. Isbell[24] excluded this mechanism

by tracer studies. A sample of D-glucose-2-t was rearranged by treatment with calcium hydroxide at 35°. Aldoses were removed by oxidation with bromine; the resulting D-fructose was separated and observed to give only 0.3 count per minute per mg compared to the original D-glucose-2-t which gave 3,400 counts per minute per mg.

Kooyman *et al.*[25] studied the chemical isomerisation of D-glucose into D-fructose in aqueous alkaline solutions. They[25] developed a simplified kinetic model based on the rate constants observed (Scheme 1.11), assuming that the concentration of the enolate-ion was very low and also that the concentration of D-mannose was low relative to that of D-glucose and D-fructose. Also, the kinetic



F_C = D-Fructose F_C^- = D-Fructose anion

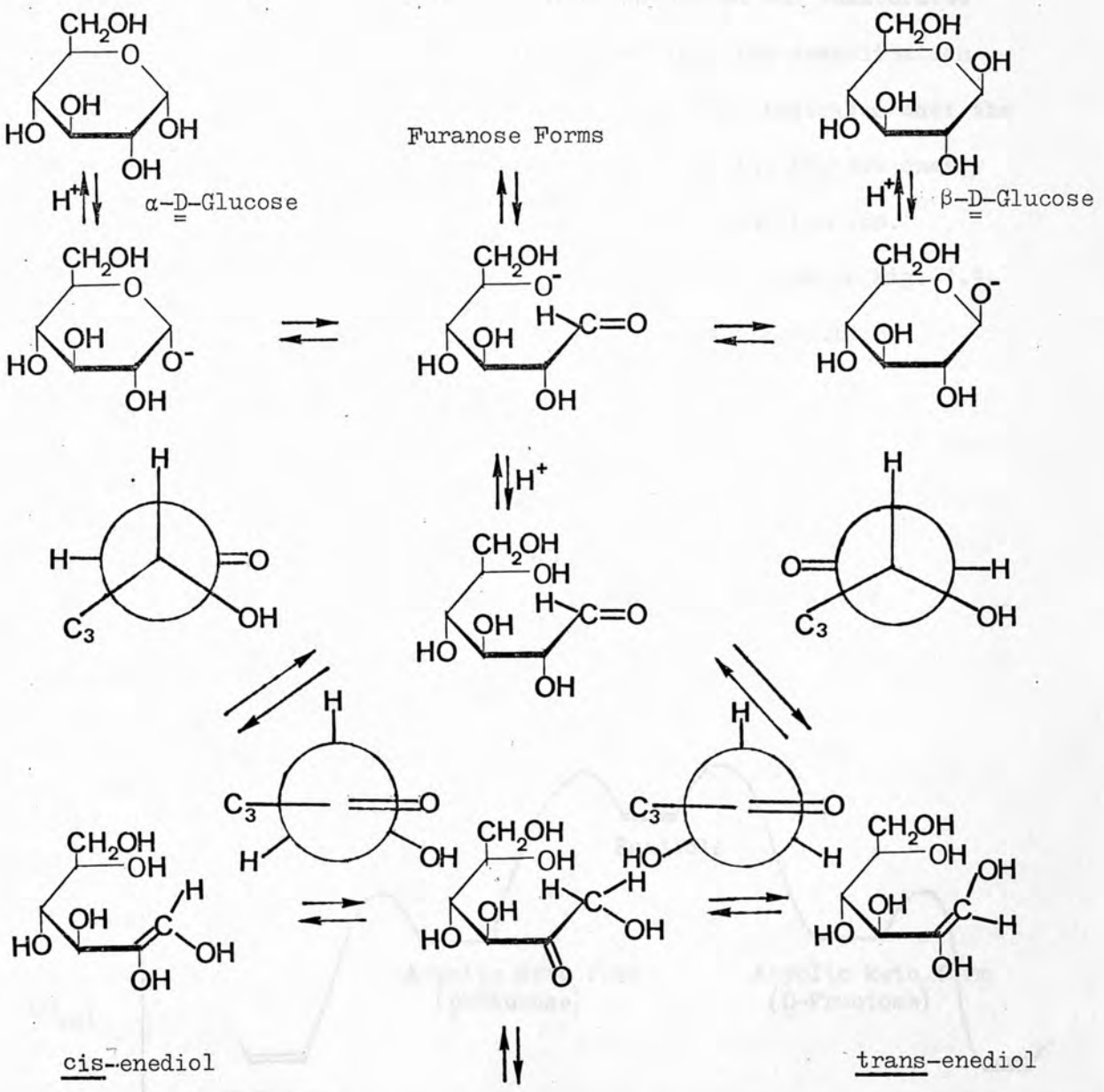
G_C = D-Glucose G_C^- = D-Glucose anion

P = by-products.

Scheme 1.11

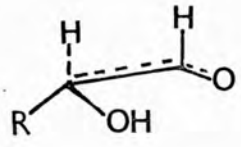
model took the α/β pyranose and α/β furanose forms of the sugars to be in rapid equilibrium with each other under the reaction conditions employed.

From the above evidence a generally acceptable mechanism for the base-catalysed chemical isomerisation of D-glucose is that shown in Scheme 1.12.



Scheme 1.12

From the reaction scheme 1.12 it is apparent that the enolisation proceeds from the acyclic form to the enediol intermediate. The enolisation can occur either by a concerted elimination or by a step-wise mechanism involving an anion.[26] From the proportions of keto:enol forms in a mixture for other saturated and unsaturated compounds ([enol] « [keto]) it is assumed that the deenolisation step is faster than the enolisation step. This indicates that the highest energy intermediate is the enediol and the highest energy transition state is that of the step involving enolisation.
The energetics of the isomerisation pathway are shown in Fig. 1.3. The highest energy transition state in the profile could be represented by (3).



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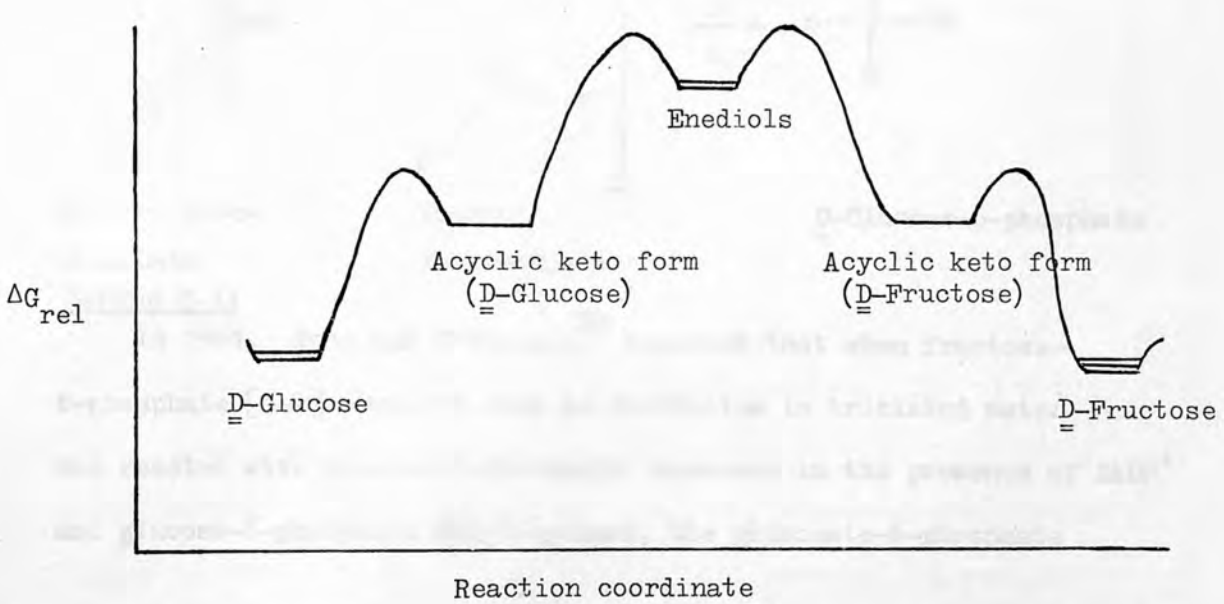
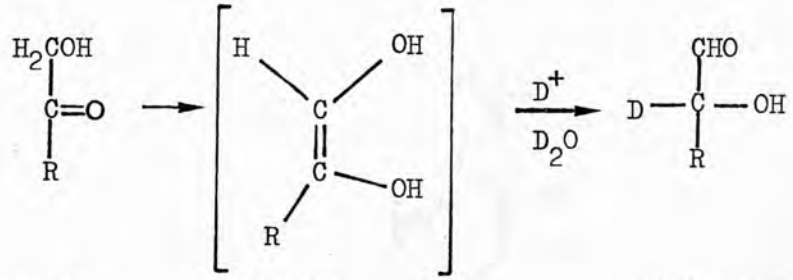


Fig. 1.3 Free energy-reaction pathway profile for the base-catalysed isomerisation of D-glucose to D-fructose.

In addition to the formation of D-fructose the base-catalysed chemical isomerisation of glucose also gives D-mannose and other by-products in small quantities.[18]

1.3.2 The Enzyme-catalysed Isomerisation of Sugars

Unlike chemical isomerisation under alkaline conditions, in enzyme catalysed isomerisation no epimerisation is known to occur. To find out whether the reaction proceeds *via* similar enediol intermediates, evidence has been gathered by various workers. In 1957 Topper[27] examined the glucose-6-phosphate isomerase (D-glucose 6-phosphate ketol-isomerase (EC 5.3.1.9)) reaction in D_2O (95%) in the direction of glucose-6-phosphate formation. The product isolated as the barium salt was observed to contain 0.91 atom excess deuterium at C2. This was interpreted in support of catalysis by way of an enolisation in the first half-reaction and stereospecific deuteration at C2 from the medium (Scheme 1.13).

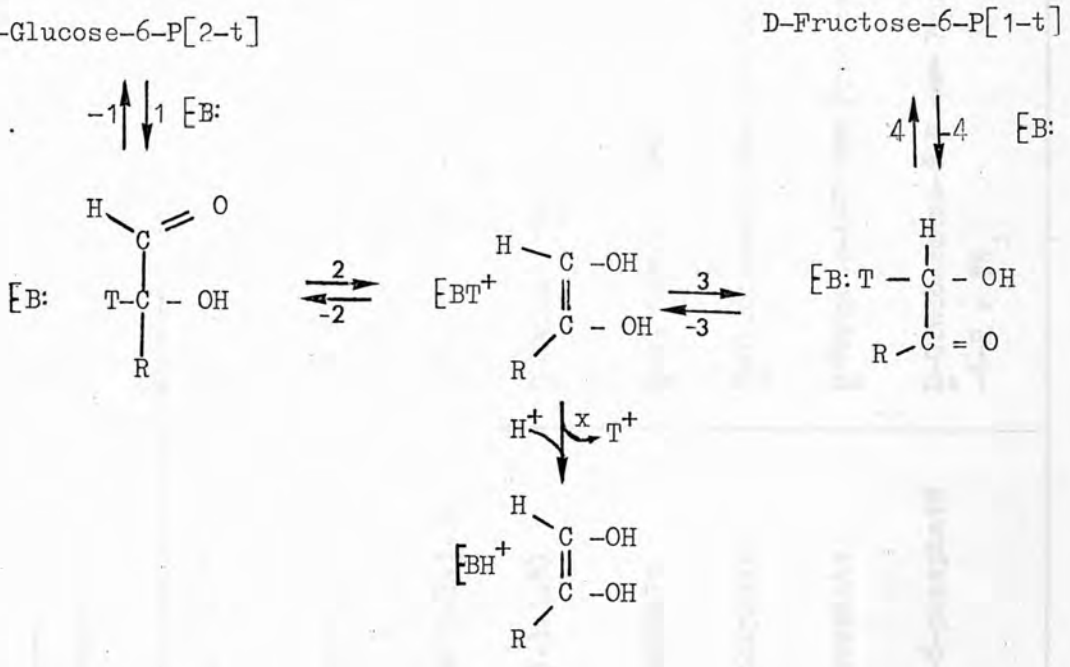


D-Fructose-6-phosphate — Enediol Intermediate — D-Glucose-6-phosphate

Scheme 1.13

In 1961, Rose and O'Connell[30] reported that when fructose-6-phosphate [1-t] prepared from an incubation in tritiated water was reacted with glucose-6-phosphate isomerase in the presence of $NADP^+$ and glucose-6-phosphate dehydrogenase, the gluconate-6-phosphate

contained tritium at C2 in addition to the water. The failure of Topper to observe protium transfer was undoubtedly the result of inadequate trapping of the glucose-6-phosphate. When the Topper experiment is done using the dehydrogenase trap, only about 0.1 deuterium atom is incorporated into C2 of the gluconate-6-phosphate.[29] The occurrence of transfer and exchange was best explained by assuming that the enzyme supplied the base for the initial proton abstraction and that the resulting acid might undergo reversible ionisation, in part, prior to the second proton transfer[28,30] (Scheme 1.14).



Scheme 1.14

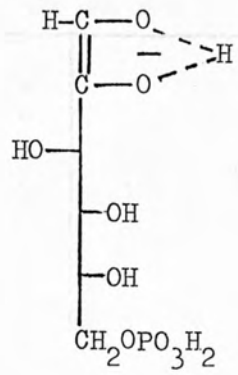
This exchange of proton with the medium has also been observed for other isomerases such as triosephosphate isomerase (EC 5.3.1.1) D-ribose-5-phosphate isomerase (EC 5.3.1.6), D-mannose-6-phosphate isomerase (EC 5.3.1.8).[29] (see Table 1.1). However, in the case

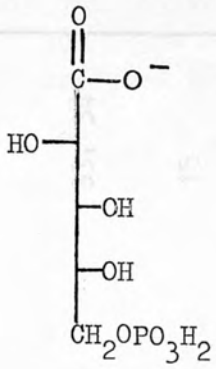
Table 1.1 Catalytic Properties of Some Isomerases

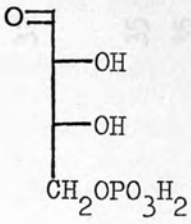
Isomerase	Forward reaction	Cofactors	V_f sec^{-1}	Exchange/ transfer of tritium
Triosephosphate (EC 5.3.1.1)	D-Glyceraldehyde-3-P → Dihydroxyacetone-P	None	2800	~50/1
L-Arabinose (EC 5.3.1.4)	L-Arabinose → Ribulose	Mn^{2+}/Co^{2+}	~200 (37°)	low
D-Arabinose (EC 5.3.1.3)	D-Arabinose → Ribulose	Mn^{2+}	~90 (37°)	low
D-Xylose (EC 5.3.1.5)	D-Xylose → Xylulose	Mn^{2+}/Co^{2+}	~3.5 (25°)	~1/5000
D-Ribose-5-phosphate (EC 5.3.1.6)	D-Ribose-5-P → Ribulose-5-P	None	1040 (37°)	high
D-Glucose-6-phosphate (EC 5.3.1.9)	D-Glucose-6-P → Fructose-6-P	None	1280 (38°)	1/1
D-Mannose-6-phosphate (EC 5.3.1.8)	D-Mannose-6-P → Fructose-6-P	Zn^{2+}	620 (30°)	1/1
D-Glucosamine-6-phosphate (EC 5.3.1.10)	D-Glucosamine-6-P → Fructose-6-P + NH_3	None	> 300 (37°)	100/0.5

of D-xylose isomerase little proton exchange occurs. Rose et al.[31] found that the failure of xylose to incorporate deuterium from a D_2O medium was indicated by the fact that the C1 proton of D-xylose remained a doublet signal in ^1H nmr. They[31] also found that in the case of D-xylose isomerase the incorporation of tritium into an equilibrium mixture occurred at less than 0.4% of the velocity of forward reaction and was probably due to non-enzymic exchange since it was uninhibited by a concentration of xylitol that inhibits the isomerisation rate to the extent of 70%. To introduce tritium into C1 of D-xylulose it was therefore necessary to use tritiated D-xylose. However, according to Rose[29] other aspects of the xylose isomerase relate it to the other Mn^{2+} dependent isomerases (which also show low degrees of exchange). Also with prolonged incubation and large amounts of enzyme some enzyme dependent exchange could be observed with xylose isomerase, hence it seems likely that the base-catalysed enediol mechanism applies generally for the aldose-ketose isomerases.

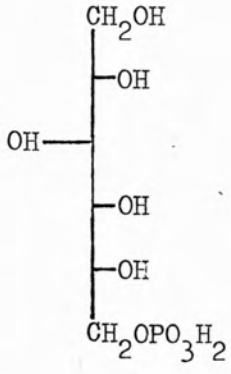
Other evidence in support of the enediol mechanism is the effectiveness of inhibitors that resemble the enediol structure (2,3). This may explain the greater affinity of erythrose-4-phosphate (5) and 5-phosphoarabinonate (6)[15,32,33,34] compared with the polyols, erythritol-4-phosphate (8) and D-glucitol-6-phosphate (7),[32] respectively for D-glucose-6-phosphate isomerase (Table 1.2). A similar proposition can be applied to explain the values for binding to triose-phosphate isomerase by 2-phosphoglycolate (1),[15] and 2-phosphoglycolohydroxamate (9)[15] compared with 2-phosphoethyleneglycol (10)[4] (see Table 1.2). To the extent that the transition state for

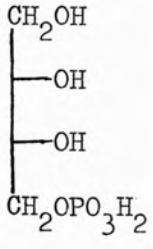






cis-enediolate 4 6 5





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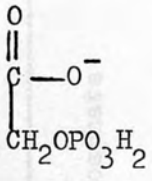
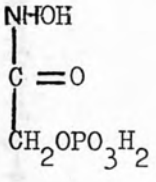
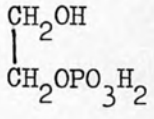
the rate determining step resembles a metastable enediol intermediate (2,3) these analogues may be considered as transition state analogues.[4]







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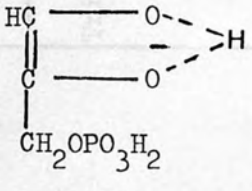


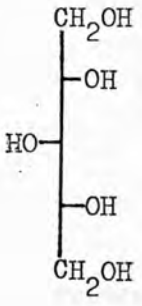
cis-enediol 2

Table 1.2 Potential Transition State Analogues

Isomerase	Inhibitor, K_i	Substrate(s), K_m	Reference(s)
Triosephosphate	2-Phosphoglycolate $K_i = 4 \times 10^{-7}$M	D-Glyceraldehyde-3-phosphate $K_m = 3 \times 10^{-4}$M	15
	2-Phosphoglycolohydroxamate $K_i = 3 \times 10^{-6}$M	"	15
	2-Phosphoethyleneglycol $K_i = 2 \times 10^{-3}$M	"	4
D-Glucose-6-phosphate	Erythrose-4-phosphate $K_i = 2 \times 10^{-6}$M	D-Glucose-6-phosphate $K_m = 6 \times 10^{-5}$M	33, 34
	5-Phosphoarabinonate $K_i = 2.7 \times 10^{-7}$M	"	15
	Erythritol-4-phosphate $K_i = 4 \times 10^{-5}$M	"	32
	Glucitol-6-phosphate $K_i = 2.5 \times 10^{-5}$M	"	32
D-Xylose	D-Xylitol $K_i = 2.7 \times 10^{-3}$M	D-xylose	35
	D-Arabinitol $K_i = 130 \times 10^{-3}$M	$K_m = 5 \times 10^{-3}$M	35
	L-Arabinitol $K_i = 146 \times 10^{-3}$M	"	35
	D-Lyxose $K_i = 70 \times 10^{-3}$M	"	35

As seen in the above two cases two types of compounds have been reported as potential inhibitors, those that resemble the enediol intermediate e.g. carboxylate anions (mimics the enolate anion portion) and those that resemble the acyclic forms of the sugar substrate e.g. polyols. However, the polyols do not bear the correct resemblance to the acyclic sugars which have an aldehyde group at C1 or a ketone group at C2 in the cases of the aldoses and ketoses repsectively. Therefore, these polyols show weaker binding compared to the corresponding acyclic form of the sugar substrates or the analogues resembling the enediol intermediate.

In the case of D-xyloseisomerase (L. brevis)[35] xylitol (11), D- and L- arabinitol (12,13) and D-lyxose (14a, 14b) inhibited competitively (Table 1.2). As discussed earlier the polyols



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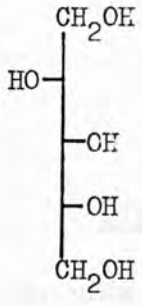

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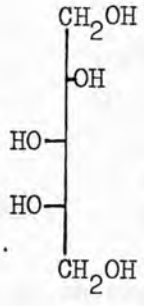

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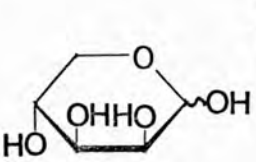
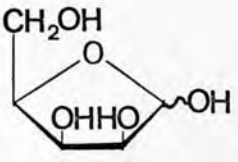


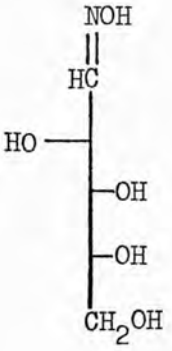
14a



14b

xylitol, D- and L-arabinitol would not be very good models for the acyclic forms of the substrate, also D-lyxose resembles the cyclic form of D-xylose but has the wrong orientation of the OH group at C2, therefore, these inhibitors would not be expected to bind strongly (see Table 1.2). Unlike these compounds carbohydrate oximes offer

a clear opportunity of applying the transition state analogue approach to aldose-ketose isomerases. In the acyclic form and with proper stereochemistry such compounds are convincing models of the proposed metastable enediol intermediates and therefore, potential transition state analogue inhibitors. Thus, D-arabinose oxime (15) is a model for the enediol (16) derived from D-glucose and D-fructose. It possesses a planar enol-type structure at N1, C1 and C2 (although devoid of C2-OH) and the correct stereochemistry at C3 to C5. Because of the possibility of their existing in syn(E)- and anti(Z)-forms, the oximes offer a unique opportunity to examine the predictions made for the stereochemistry of the enediol intermediates.



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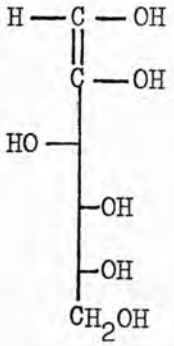
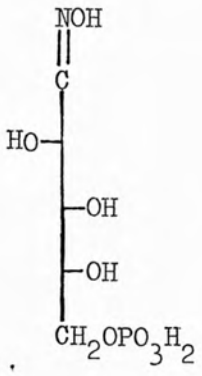


cis-enediol 16

Before these compounds can be interacted with D-xylose isomerase they need to be obtained in a pure crystalline form, their structures characterised, and their behaviour in solution at different pH values determined. The preparation and characterisation of some carbohydrate oximes are described in chapters 2 and 3 and the enzyme inhibitory properties are described in Chapter 5.

Further, in searching for analogue inhibitors for specific enzymic reactions, modification of the basic structure of an analogue for one enzyme to meet specificity requirements for another enzyme

in the same mechanistic class should yield a transition state analogue for the second enzyme. Thus, D-arabinose oxime-5-phosphate (17) could act as a potential transition state analogue inhibitor of the glycolytic enzyme glucose-6-phosphate isomerase.



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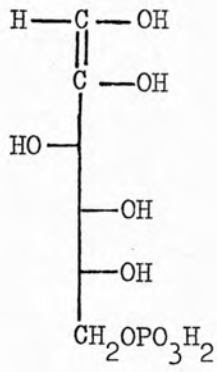

cis-enediol 4

These principles can be used to design oxime inhibitors of other aldose-ketose isomerases.

The phosphorylation of D-arabinose oxime and other compounds catalysed by the first enzyme of the glycolytic cycle, namely hexokinase is described in Chapter 4 of this thesis.

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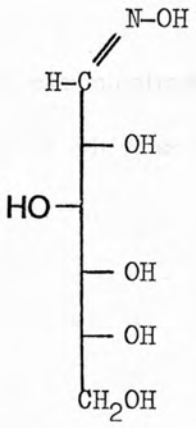
CHAPTER 2 SYNTHESIS AND STRUCTURAL ANALYSIS OF CARBOHYDRATE OXIMES

2.1 INTRODUCTION

Oxime derivatives of carbohydrates prepared by reaction of hydroxylamine at the anomeric centre were first described by P. Richsbieth in 1887[1] who synthesised D-galactose oxime. Other carbohydrate oximes investigated in this work viz D-glucose oxime,[2-7] D-mannose oxime,[2,5,8] D-arabinose oxime[9-11] and D-ribose oxime[12-14] were subsequently isolated by other workers. Oxime derivatives have been used for the characterisation of sugars. However, the structures of these compounds in the solid form and in solution have not been established unequivocally.[15,16] Since aqueous solutions exhibit mutarotation and a number of reactions such as Wohl degradation[3] are best explained in terms of an acyclic structure, it has been widely presumed that equilibrium between cyclic and acyclic forms (18A,B,C,D,E,F) is established in solution.[5,15,17,18] D-Glucose oxime(solid)has been assigned the β-cyclic structure on the basis of methylation,[17] acetylation,[5] optical rotation[5] and i.r. data.[19,20] D-Galactose oxime was thought to be α-cyclic on the basis of acetylation[21] and optical rotation[21] data. D-Arabinose oxime was suggested to have an acyclic structure from infra-red measurements.[19] Haas et al.[22] from the results of polarography experiments concluded that in solution the oximes of D-glucose, D-galactose, D-mannose, D-ribose and D-arabinose exist in the acyclic form to the extent of 47%, 71%, 100%, 100% and 100% respectively. Plenkiewicz and coworkers[23] have described the synthesis and characterisation of O-benzoyloximes of carbohydrates and have assigned acyclic structures on the basis of Raman and ^{1}H nmr data. Kampf and Dimant[24] have characterised 3-O-benzyl-2,4-O-ethylidene-D-erythrose oxime by infra-red and ^{1}H nmr as syn(E) and



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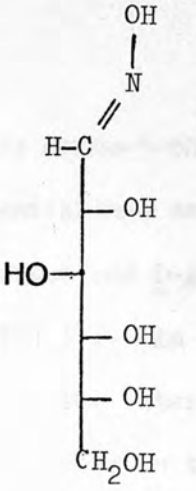
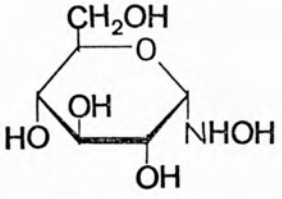


18B



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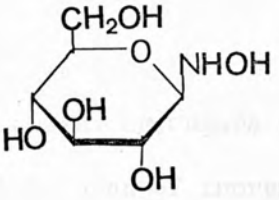

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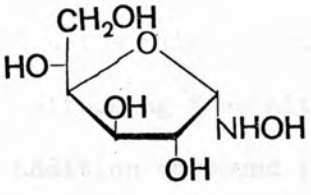

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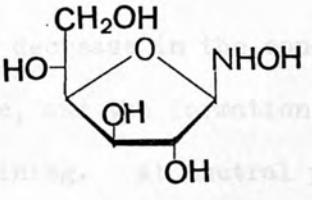


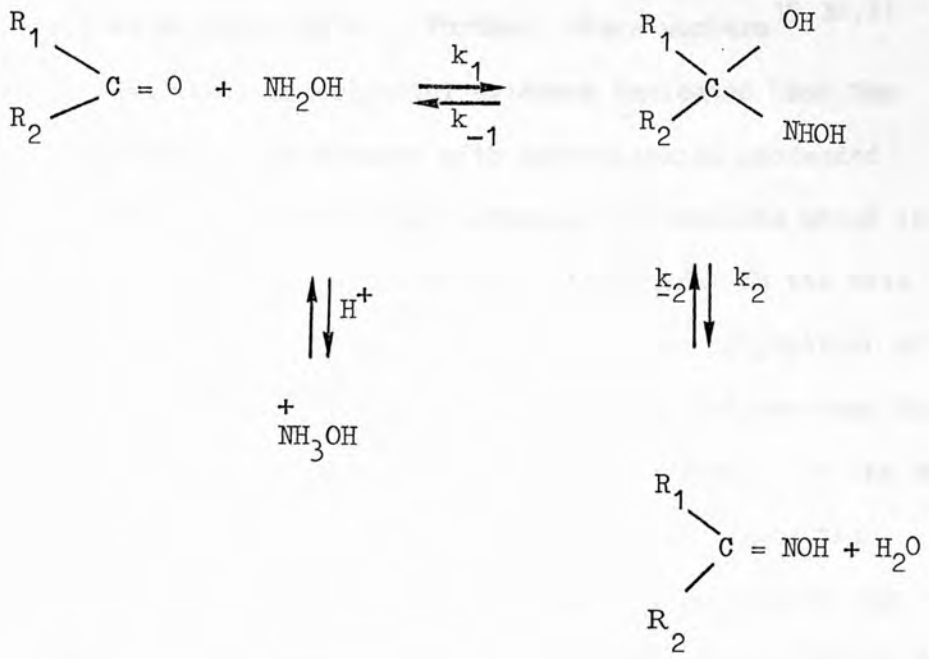
18F

anti(Z) forms. Thus, it is seen that the structures of these carbohydrate oximes in the solid state and the number of isomeric forms existing in solution is not unequivocally established. Further, in the case of D-glucose oxime crystallisation was observed to be difficult to achieve.[25]

The carbohydrate oximes and D-arabinose oxime-5-phosphate were prepared in this work because of their potential use as transition-state-analogue inhibitors of D-xylose isomerase and D-glucose-6-phosphate isomerase respectively (see CHAPTER 1). In order to interpret the results of experiments involving the interaction of enzymes with carbohydrate oximes, their structures in the solid state and in solution need to be established. This was done in this work by the use of infra-red spectroscopy, periodate oxidation, gas liquid chromatography, mass spectroscopy, polarimetry, paper chromatography, electrophoresis, n m r spectroscopy and X-ray crystallography.

2.1.1 Mechanism of Oxime Formation

The derivatives obtained by condensing sugars with compounds of the general formula $R-NH_2$, have been of increasing biochemical interest. Fitzpatrick and Gettler[26] investigated the reaction of oxime formation with other carbonyl compounds and found it to be a second-order reaction. Jencks[27,28] established the pH dependence of the rate-limiting step for oxime formation. At low pH, below the rate maximum, there is a decrease in the concentration of the attacking free nitrogeneous base, and the formation of a carbinol-amine addition compound is rate determining. At neutral pH values general acid-catalysed dehydration leading to oxime formation is rate limiting. The pH rate maximum is the result of a transition between these two opposing effects (Scheme 2.1)

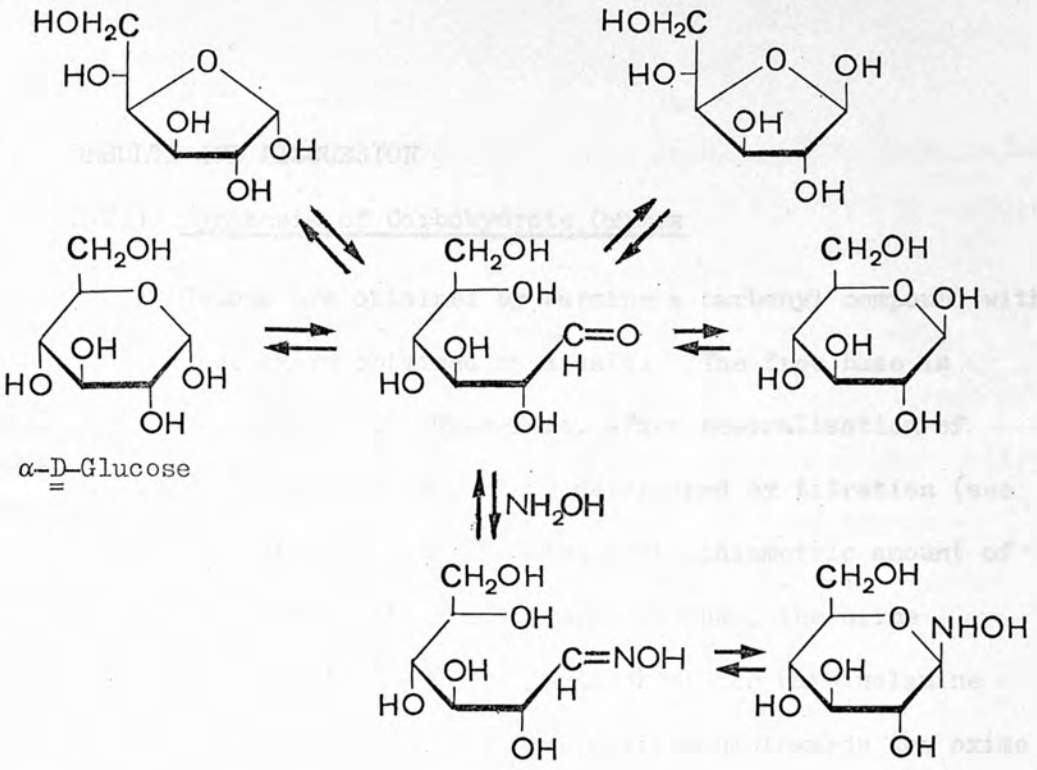


Scheme 2.1

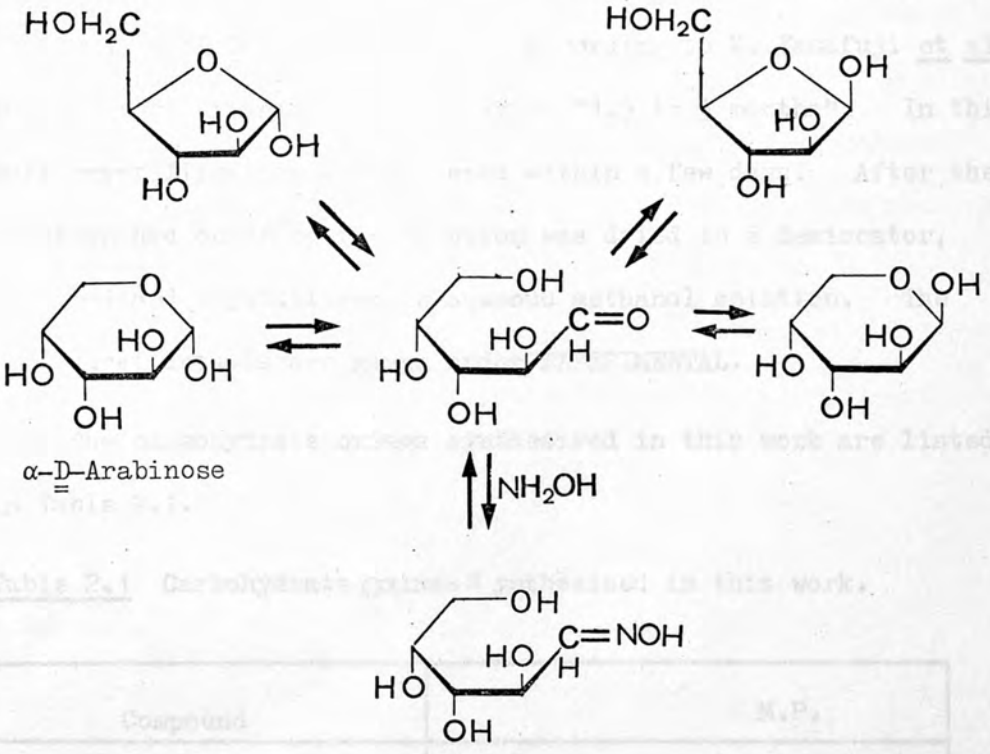
Further, according to Jencks[27] the alkali-catalysed reaction of hydroxylamine with acetone described by Barrett and Lapworth[29] involves a base-catalysed dehydration of the addition compound, or, in the reverse reaction, an attack of hydroxide ion on acetoxime.

The analogous reactions with sugars are complicated by the fact that aldoses in aqueous solution can exist in a number of ring modifications inter-related through an acyclic form. Haas et al.[30] and other workers[15,31] have studied the reaction of several aldoses and some ketoses with nitrogeneous bases and determined equilibrium constants and rate constants. They observed under certain conditions anomeric rate differences which were attributed to different rates of ring opening at pH's where this is partly rate determining and to different standing concentrations of the

aldehydo form at other pH's. Further, these workers[15,30,31] suggested that their experimental evidence indicated that the reaction of aldoses and ketoses with hydroxylamine proceeded through a carbinolamine addition compound intermediate which is subsequently dehydrated to the oxime. At neutral pH the rate determining step in the case of aldoses involves dehydration of the carbinolamine addition product (Scheme 2.1) and the reaction is subject to both general and specific acid catalysis. In the case of aldopentoses and aldohexoses from the above evidence the interaction with hydroxylamine near neutral pH leading to the formation of oxime which may crystallise in acyclic or cyclic forms is depicted in Schemes 2.3 and 2.2.



Scheme 2.2



Scheme 2.3

2.2 RESULTS AND DISCUSSION

2.2.1 Synthesis of Carbohydrate Oximes

Oximes are obtained by warming a carbonyl compound with hydroxylamine which is obtained as a salt. The free base is subject to air oxidation. Therefore, after neutralisation of hydroxyl amine its concentration was determined by titration (see EXPERIMENTAL). This helped in adding a stoichiometric amount of hydroxylamine to the reaction mixture. Further, the oxime formation step (Scheme 2.1) is dehydration of the carbinolamine intermediate, therefore to shift the equilibrium towards the oxime formation water was removed from the reaction mixture, under reduced pressure. In previous reports of carbohydrate oxime synthesis, crystallisation was observed to be difficult to achieve especially in the case of D-glucose oxime. According to K. Yamafuji *et al.*[25] Wohl's procedure gave crystals after "1.5 to 3 months". In this work crystallisation was achieved within a few days. After the reaction had occurred the solution was dried in a desiccator, powdered and crystallised in aqueous methanol solution. The procedural details are given under EXPERIMENTAL.

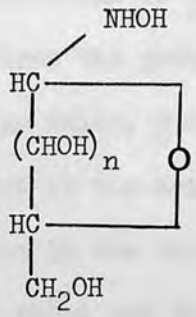
The carbohydrate oximes synthesised in this work are listed in Table 2.1.

Table 2.1 Carbohydrate Oximes Synthesised in this work.

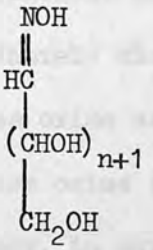
Compound	M.P.
D-Glucose Oxime	141° (Lit.,[6] 141°)
D-Arabinose Oxime	140° (Lit.,[9] 138-139°)
D-Galactose Oxime	176° (Lit.,[1] 175-176°)
D-Mannose Oxime	176.5° (Lit.,[5] 175-177°)
D-Ribose Oxime	138° (Lit.,[13] 140°)

2.2.2 Infra-red and Raman Spectroscopy of Carbohydrate Oximes

The C=N functional group normally gives rise to a strong absorption band near 1660 cm^{-1} in infra-red spectra,[19,32-34] although this band is of only moderate or weak intensity with certain oximes.[35] According to Horton et al.[35] several oximes of sugar derivatives with the oxime group (not at the anomeric position) exhibited negligible absorption in this region of the infra-red spectrum. Their explanation was that as the intensity of an absorption band in the infra-red is related to the extent of polarisation of the bond and is zero when the bond is not polarised, the inductive effects of the substituents at each end of the C=N bond coincidently give rise to little net polarisation of the bond so that absorption in the infra-red region is weak or absent with their oximes. The vibrational transitions of electronically symmetrical bonds which are inactive in the infra-red give rise to strong absorptions in Raman spectra. Therefore, the C=N group of the oximes should be readily detectable by Raman spectroscopy, thereby confirming the presence of a C=N group in the molecule. In the case of the carbohydrate oximes the presence of a C=N group will clearly demonstrate the presence of acyclic as distinct from cyclic isomeric forms.



Cyclic Carbohydrate Oxime



Acyclic Carbohydrate Oxime

It is seen (Table 2.2) that except for D-glucose oxime all the other carbohydrate oximes synthesised *viz* D-arabinose oxime, D-ribose oxime, D-galactose oxime and D-mannose oxime showed a moderate to very weak band in the 1660 cm^{-1} - 1695 cm^{-1} region of the infra-red spectra in the solid state. D-Glucose oxime (solid) showed a sharp band at 3215 cm^{-1} attributed to N-H stretching vibration, also a band at 891 cm^{-1} was observed which according to Barker *et al.*[36] is typical of a β-pyranose configuration of sugars. Previously D-galactose oxime was reported[37] to exist in the α-cyclic form due to the absence of a band at 1650 cm^{-1} to 1695 cm^{-1} in the infra-red and from optical rotation work. In this work there was no band at 844 cm^{-1} characteristic[36] of an α-pyranose form while a very weak band at 1678 cm^{-1} was observed. Since a weak band is commonly observed for the C=N group, and as water also absorbs in this region,[33,34] it is not possible to make a definite assignment from infra-red measurements. In order to confirm the presence of a C=N group Raman spectroscopy was carried out on these oximes.

Moderate intensity bands were observed in the 1660 cm^{-1} - 1695 cm^{-1} region in the Raman spectra (Table 2.2) of the oximes of D-arabinose, D-ribose, D-mannose and D-galactose (solid state). Two bands in the 1660 cm^{-1} - 1695 cm^{-1} region were observed for aqueous solutions of D-glucose oxime and D-arabinose oxime (Table 2.2). This confirms the presence of C=N group and thereby shows that D-arabinose oxime, D-ribose oxime, D-galactose oxime and D-mannose oxime exist in the acyclic form while D-glucose oxime exists in the cyclic form in the crystalline state. Further, in aqueous solution D-glucose oxime and D-arabinose oxime show the presence of two bands

probably due to the presence of syn(E) and anti(Z) forms.

Table 2.2 Infra-red and Raman data for Carbohydrate Oximes

Compound	Infra-red Frequencies	Raman Frequencies	
	Solid	Solid	Solution
D-Glucose Oxime	891 cm^{-1}		1648 cm^{-1}, 1665 cm^{-1}
D-Mannose Oxime	1672 cm^{-1}	1671 cm^{-1}	
D-Galactose Oxime	1678 cm^{-1}	1680 cm^{-1}	
D-Ribose Oxime	1694 cm^{-1}	1691 cm^{-1}	
D-Arabinose Oxime	1680 cm^{-1}	1678 cm^{-1}	1656 cm^{-1}, 1664 cm^{-1}

2.2.3 Periodate Oxidation of Carbohydrate Oximes

D-Glucose oxime and D-arabinose oxime were taken as representative of the cyclic and acyclic forms of the carbohydrate oximes. Periodate oxidation was carried out on freshly dissolved

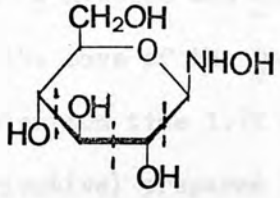
Table 2.3 Periodate Oxidation data for Carbohydrate Oximes.

Compound (1Mole)	No. of Moles of Periodate Consumed	No. of Moles of Formaldehyde formed	No. of Moles of Formic Acid formed
D-Glucose Oxime	4.2	0	1.95
D-Arabinose Oxime	4.7	0.8	2.9

oximes of D-glucose and D-arabinose and the number of moles of periodate consumed along with the number of moles of

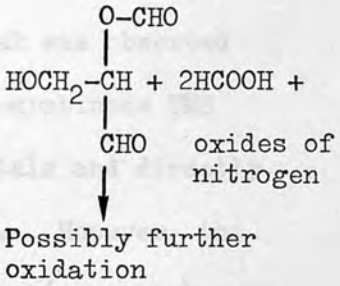
formaldehyde and formic acid liberated were determined.[38-40] (Table 2.3). It is known that periodate cleaves vicinal diols in sugars[39] producing a dialdehyde in addition to formic acid and formaldehyde depending on whether there are more than two vicinal diol groups and whether there is a primary hydroxyl group present in the diol. In the case of carbohydate oximes an additional factor is involved and that is the presence of the oxime group. It has been reported by G.A. Snow[41] and others[42,43] that the oxime group is oxidised to oxides of nitrogen. Therefore, D-glucose oxime (18) and D-arabinose oxime (15) are suggested to show the following degradation pattern from the number of moles of periodate consumed and the number of moles of formaldehyde and formic acid liberated (Scheme 2.4).

D-Glucose Oxime:-

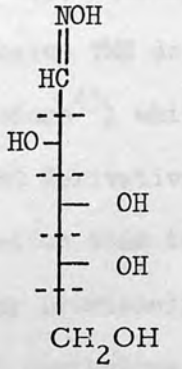


18

IO_4^{1-}, >4 moles



D-arabinose oxime:-



15

IO_4^{1-}, >4 moles

3HCOOH + HCHO + Oxides of nitrogen + CI fragment

Scheme 2.4

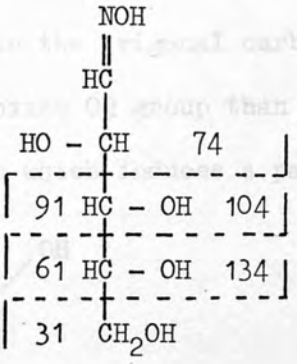
While not conclusive, the results of the periodate oxidation studies support the structural assignments for the oximes as shown in Scheme 2.4.

2.2.4 Gas Liquid Chromatography of Trimethylsilyl Derivatives of Carbohydrate Oximes

A number of workers have proposed[44-46] the use of O-trimethylsilyl (TMS) derivatives of oximes for analysis of carbohydrate mixtures, and in general it has been presumed that each sugar gives rise to a mixture of syn(E) and anti(Z) acyclic forms. The TMS derivatives of D-glucose, D-arabinose, D-glucose oxime (freshly dissolved) and D-arabinose oxime (freshly dissolved) were prepared and compared with the oxime TMS derivatives prepared from D-glucose and D-arabinose by the procedure of G. Petersson.[45] In the case of the D-arabinose oxime a single peak was observed (retention time 1.72 relative to that of the α-D-arabinose TMS derivative) prepared from D-arabinose oxime crystals and directly from D-arabinose (by G. Petersson's procedure[45]). However, the oxime TMS derivative prepared from D-glucose oxime (crystals) gave two peaks (a major peak retention time 1.49 and a minor peak retention time 1.37 relative to the TMS derivative of α-D-glucose) compared to the oxime TMS derivative prepared from D-glucose (G. Petersson's procedure[45]) which gave only one peak (retention time 1.49 relative to TMS derivative of α-D-glucose). The peak at 1.37 relative retention time to TMS derivative of α-D-glucose slowly disappeared as was previously observed by Sweeley,[47] and was presumed to be an O-TMS derivative of cyclic D-glucose oxime.

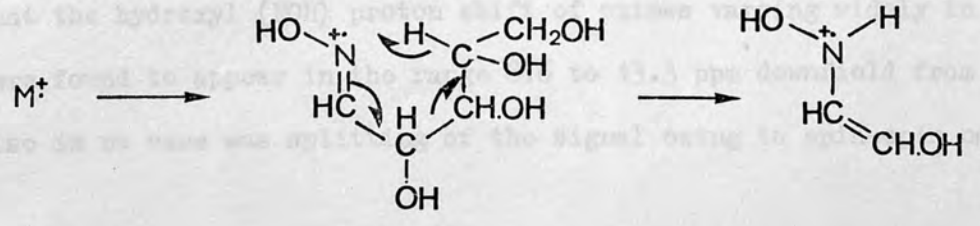
2.2.5 Mass Spectroscopy of Carbohydrate Oximes

The structures of the oximes are supported but not unequivocally confirmed by mass spectroscopy. The carbohydate oximes tested *viz* D-glucose oxime, D-mannose oxime, D-galactose oxime, D-ribose oxime and D-arabinose oxime gave small m + 1 peaks which were shown to have the expected molecular formula by accurate mass measurements. Other peaks could be ascribed based on previous work on carbohydrates[48] and aliphatic oximes.[49] The majority of the peaks for the oximes could be attributed to a simple cleavage as shown for D-arabinose oxime (15)



15

The base peak at m/e 75 for D-arabinose oxime probably arises from a McLafferty rearrangement (Scheme 2.5) as has been observed for other oximes.[49]



Scheme 2.5

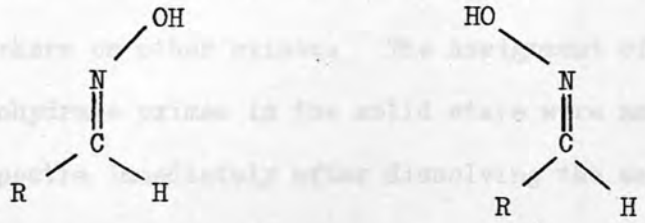
m/e 75

This peak is of different intensity in the mass spectra of other oximes, but the breakdown pattern is similar to that arising from D-arabinose oxime.

2.2.6 Nuclear Magnetic Resonance Spectroscopy of Carbohydrate Oximes

2.2.6.1 ^{1}H nmr:

Acyclic aldoximes are known to exist as two interconverting isomeric forms. The assignment of syn(E) and anti(Z) forms has been made on the basis of chemical shift correlations.[50-57] According to W.D. Phillips[50] in aldoximes the hydrogen atom on the trigonal carbon is more deshielded when cis (syn), to the oxime OH group than when trans (anti) due to the proximity of oxygen which induces a paramagnetic shift. Study



Syn(E) Anti(Z)

of the nmr spectra of syn(E) and anti(Z) p-chlorobenzaldoxime isomers[51] (whose structure was established by crystallographic studies[58]) confirmed the assignments made by Phillips.[50] Kleinspehn[56] showed that the hydroxyl (NOH) proton shift of oximes varying widely in type were found to appear in the range 8.6 to 13.3 ppm downfield from TMS. Also in no case was splitting of the signal owing to spin-spin coupling

detected. The NOH proton signals for the syn(E) isomer were more shielded than for the anti(Z) aldoximes due to magnetic anisotropy effects. Further, Durbetaki and Miles[54] proposed the magnitude of the difference $\delta_{NOH} - \delta_{CH=N}$ of the oxime isomers as a criterion for assigning aldoxime configuration, the smaller difference being characteristic of the syn(E) isomer and the bigger difference to that of the anti(Z) isomer. Such correlations have been used previously[24,59] to assign structures of other carbohydrate oxime derivatives.

From the above evidence and taking into account intensity considerations, the ClH signals and NOH signals (which disappear on adding deuterium oxide to the [$^2H_6$] dimethyl sulphoxide solution of the carbohydrate oximes) of the syn(E) and anti(Z) acyclic forms of the carbohydrate oximes (Table 2.4) were assigned. The assignments for the five carbohydrate oximes fit the correlations made by previous workers on other oximes. The assignment of the structures of the carbohydrate oximes in the solid state were made by observing their nmr spectra immediately after dissolving the sample. (see Figs. 2.1, 2.2).

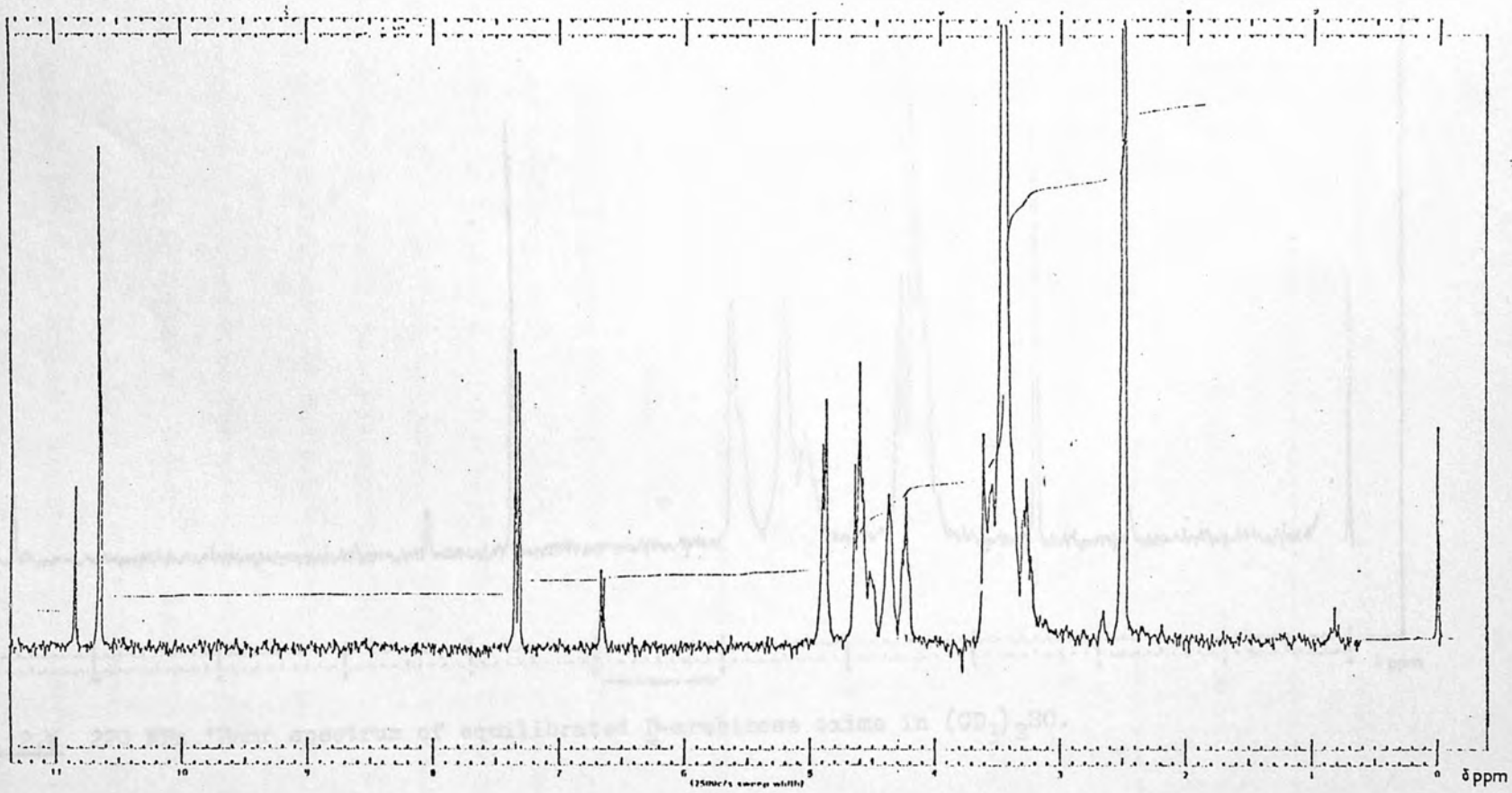


Fig. 2.2 220 MHz ^{1}Hnmr spectrum of D-arabinose oxime taken after equilibration in $(CD_3)_2SO$.

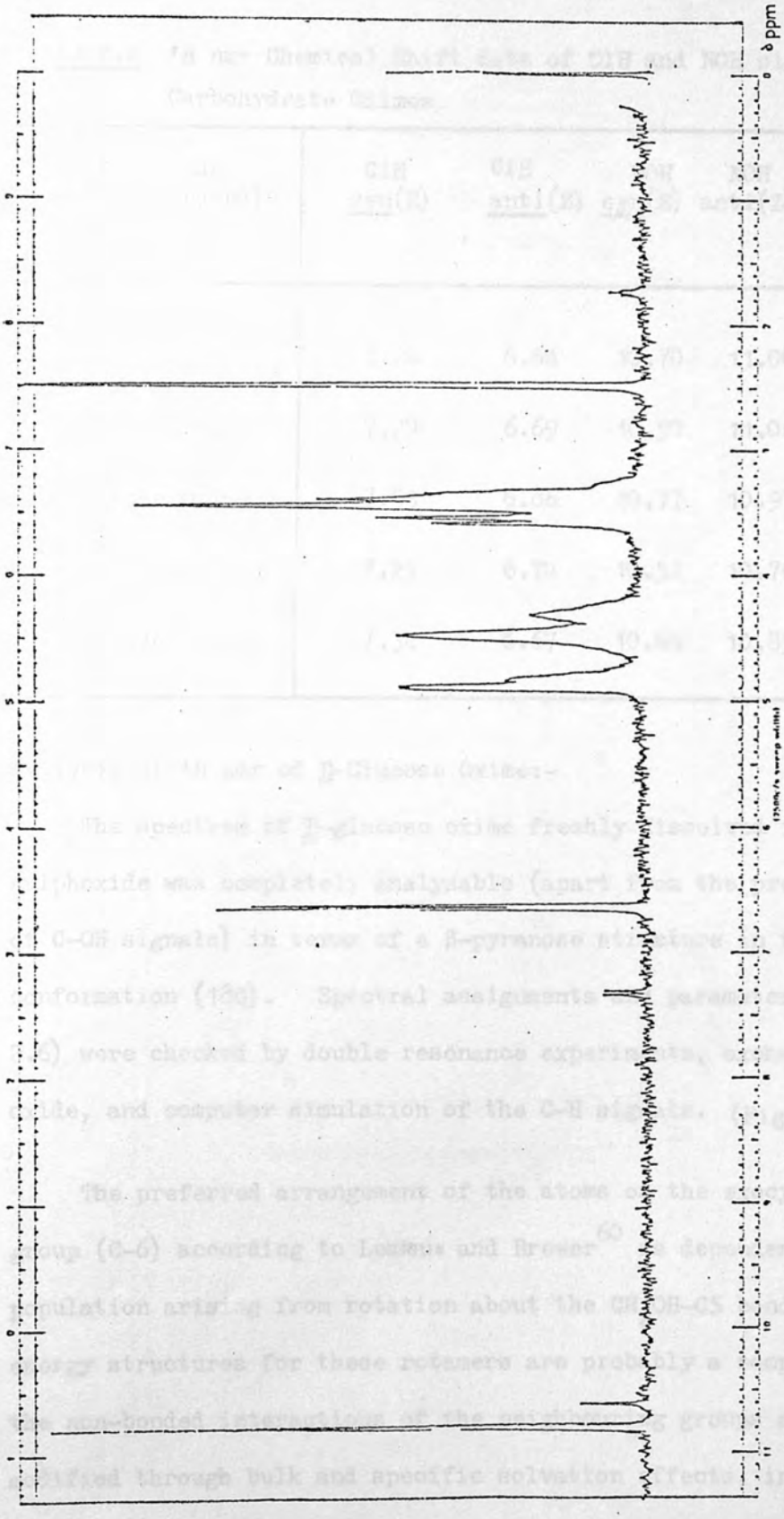


Fig. 2.1 220 MHz ^{1}Hnmr spectrum of equilibrated D-arabinose oxime in $(CD_3)_2SO$.

Table 2.4 'H nmr Chemical Shift data of C1H and NOH signals of Carbohydrate Oximes

Compounds (Equilibrated)	C1H syn(E)	C1H anti(Z)	NOH syn(E)	NOH anti(Z)	$(\delta_{NOH}-\delta_{CH=N})_{anti} - (\delta_{NOH}-\delta_{CH=N})_{syn}$
D-Glucose Oxime	7.24	6.64	10.70	11.00	+ 0.90
D-Mannose Oxime	7.29	6.69	10.50	11.06	+ 1.17
D-Galactose Oxime	7.53	6.86	10.77	10.97	+ 0.87
D-Ribose Oxime	7.29	6.70	10.52	10.76	+ 0.83
D-Arabinose Oxime	7.34	6.67	10.66	10.85	+ 0.86

Analysis of 'H nmr of D-Glucose Oxime:-

The spectrum of D-glucose oxime freshly dissolved in [$^2H_6$] dimethyl sulphoxide was completely analysable (apart from the precise assignment of C-OH signals) in terms of a β-pyranose structure in the normal $^4C_1$ conformation (18G). Spectral assignments and parameters (Tables 2.5 and 2.6) were checked by double resonance experiments, exchange in deuterium oxide, and computer simulation of the C-H signals. (Figs. 2.3, 2.4).

The preferred arrangement of the atoms on the exocyclic hydroxymethyl group (C-6) according to Lemieux and Brewer[60] is dependent on the rotamer population arising from rotation about the CH_2OH-C5 bond. The minimum energy structures for these rotamers are probably a compromise between the non-bonded interactions of the neighbouring groups and how these are modified through bulk and specific solvation effects, including demands for

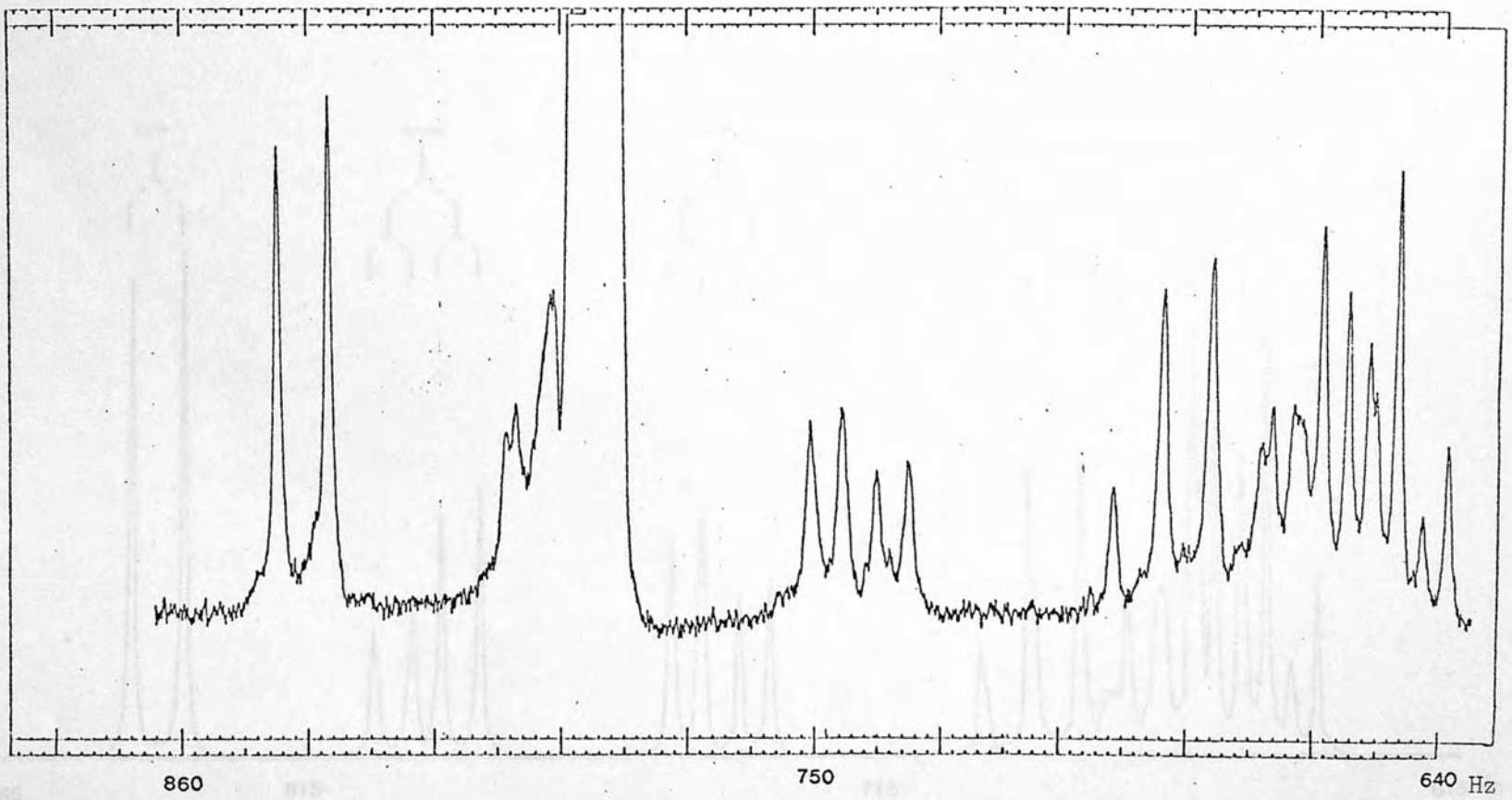


Fig. 2.3 220 MHz ^{1}H nmr spectrum of β-D-glucose oxime in $(CD_3)_2SO$-D_2O

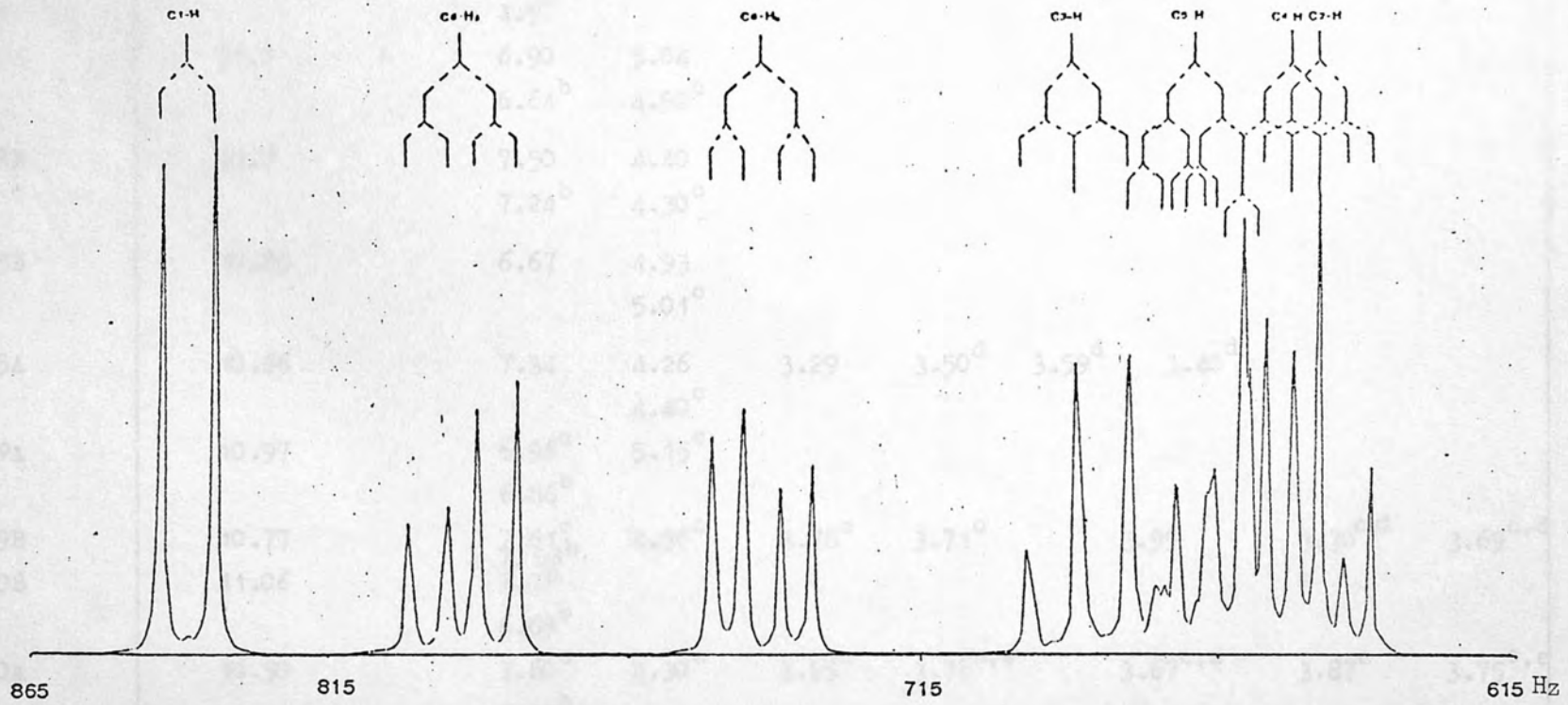


Fig. 2.4 220 MHz Computer simulated ^1H nmr spectrum of β-D-glucose oxime in $(CD_3)_2SO$-D_2O.

Table 2.5 'H Chemical Shifts in ppm (δ values; Me_4Si standard)[a]

Compound	NOH[b]	NH[b]	1-H	2-H	3-H	4-H	5-Ha	5-Hb	6Ha	6Hb
18D	7.36	5.81	3.81	2.95	3.13[d]	2.98[d]		3.04[e]	3.60	3.38
18C			4.5[c]							
18A	11.0		6.90	5.04						
			6.64[b]	4.92[c]						
18B	10.7		7.50	4.40						
			7.24[b]	4.30[c]						
15B	10.85		6.67	4.93						
				5.01[c]						
15A	10.66		7.34	4.26	3.29	3.50[d]	3.59[d]	3.40[d]		
				4.40[c]						
19A	10.97		6.96[c]	5.15[c]						
			6.86[b]							
19B	10.77		7.61[c] 7.53[b]	4.56[c]	3.78[c]	3.71[c]		3.99	3.70[c,d]	3.69[c,d]
20B	11.06		7.0[c]							
			6.69[b]							
20A	10.50		7.60[c]	4.30[c]	3.95[c]	3.78[c,e]		3.67[c,e]	3.87[c]	3.75[c,e]
			7.29[b]							
21A	10.76		6.92[c]	5.10[c]						
			6.70	4.86						
21B	10.52		7.55[c] 7.29	4.46[c]	3.69[c]	3.79[c,e]	3.67[c,e]	3.83[c,e]		

a In $(CD_3)_2SO$-D_2O except where specified. [b] In $(CD_3)_2SO$. [c] In D_2O. [d] Obtained by computer simulation

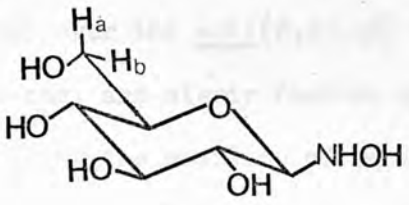
Table 2.6 ^{1}H Coupling Constants (H_z)[a]

Compound	NOH, NH[b]	NH, 1-H[b]	1-H, 2-H	2-H, 3-H	3-H, 4-H	4H, 5-Ha	4-H, 5H$_b$	5-Ha, 5-Hb	5-H, 6-Ha	5-H, 6-Hb	6-Ha, 6-Hb
18D	2.70	3.24	8.95	8.95	8.95[d]		8.95[d]		7.0	5.5	-11.5
			9.1[c]								
18C			ca.4.0[c]								
18A			6.20								
			6.7[c]	6.7[c]							
18B			7.8								
			7.0[c]	7.1[c]							
15B			5.7[c]	2.2[c]							
			5.6[b]								
15A			7.3	2.1	7.8	2.6	5.9	-10.5			
			7.28[c]	2.8[c]	6.0[c]						
19A			5.50[c]	1.7[c]							
19B			5.85[c]	2.03[c]	9.23[c]	1.28[c]			5.76[c,d]	7.2[c,d]	-11.52[c]
20B			6.0[c]								
20A			6.92[c]	8.15[c]	0.79[c]	4.0[c,e]			2.0[c]	7.0[c,e]	-10.54[c]
21A			6.2[c]	3.4[c]							
21B			6.82[c]	4.12[c]							

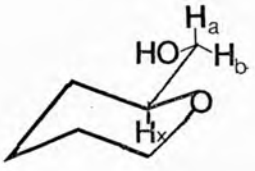
[a] In $(CD_3)_2$ SO-D_2O except where specified. [b] In $(CD_3)_2SO$. [c] In D_2O. [d] Obtained by computer simulation.

[e] Assignments uncertain.

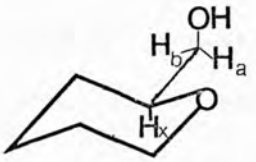
intramolecular H-bonding. Three rotamers are possible for D-glucose oxime.



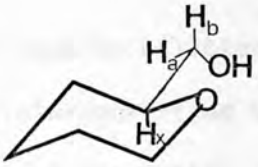
18G



i



ii



iii

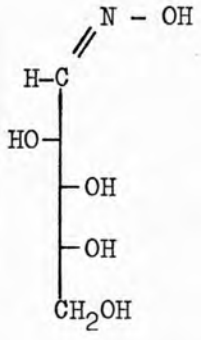
while in (iii) $J_{AX} < J_{BX}$.

In (i) J_{AX} should be greater than J_{BX}, in (ii) $J_{AX} \simeq J_{BX}$ / The fact that one of these vicinal coupling constants $J_{5,6}$ is larger (7.0 Hz) than the other (5.5 Hz) implicates the presence of a rotamer with a diaxial relationship between C5 and C6 protons, and since the smaller coupling is not associated with the downfield signal which would arise from deshielding by the opposing C-4 hydroxyl group,[60] the major rotamer is thought to be (i). The spectrum of the solution in [$^2H_6$] dimethylsulphoxide showed a slow decrease in intensity of the signals attributed to NOH, C1-H and NH, while new signals appeared at δ 11.0, 10.7, 7.24, 6.64, 4.40 and 5.04 ppm and the remaining part of the spectrum became more complex. The signals (doublets) at δ 7.24 and 6.64 ppm were assigned, according to correlations[50-57] referred to above, to C1 protons of the syn(E) and anti(Z) forms of acyclic D-glucose oximes respectively. On addition of deuterium oxide the singlets at δ 11.0 and 10.7 ppm disappeared; they were assigned to NOH of the anti(Z) and syn(E) forms, respectively. At equilibrium in deuterium oxide four doublets attributable to the C1

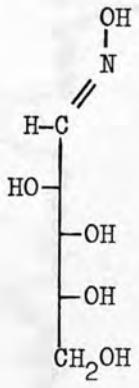
protons of α-pyranose (18C), β-pyranose (18D), syn(E , 18B) and anti(Z , 18A) forms were visible at 220 MHz. The preponderance of the syn(E,56.5%) over the anti(Z,13.5%) form is consistent with steric considerations, and steric factors appear to outweigh electronic factors in controlling the position of equilibrium between β-pyranose (23 %) and α-pyranose (7 %) forms.

Analysis of ^{1}H nmr of D-Arabinose Oxime:-

D-Arabinose oxime in [$^2H_6$] dimethyl sulphoxide solution gave a ^{1}H nmr consistent with the presence of two interconverting isomeric forms (Tables 2.5 and 2.6). The assignment of the anti(Z, 15B) structure to the initial form and of the syn (E, 15A) structure to the preponderant form at equilibrium was made on the basis of chemical shift correlations discussed earlier. Two low field signals at



15B



15A

δ 10.85 and 10.66 ppm which disappeared on addition of deuterium oxide were assigned to the NOH protons of the anti(Z) and syn(E) forms respectively while the two doublets at δ 6.67 and 7.34 ppm were assigned to the C1H protons of the anti(Z) and syn(E) forms. Other assignments were made with the aid of exchange in deuterium oxide, double resonance, and computer simulation procedures. The observed and simulated spectra of D-arabinose oxime syn(E)-form are shown in Figs. 2.5, 2.6.

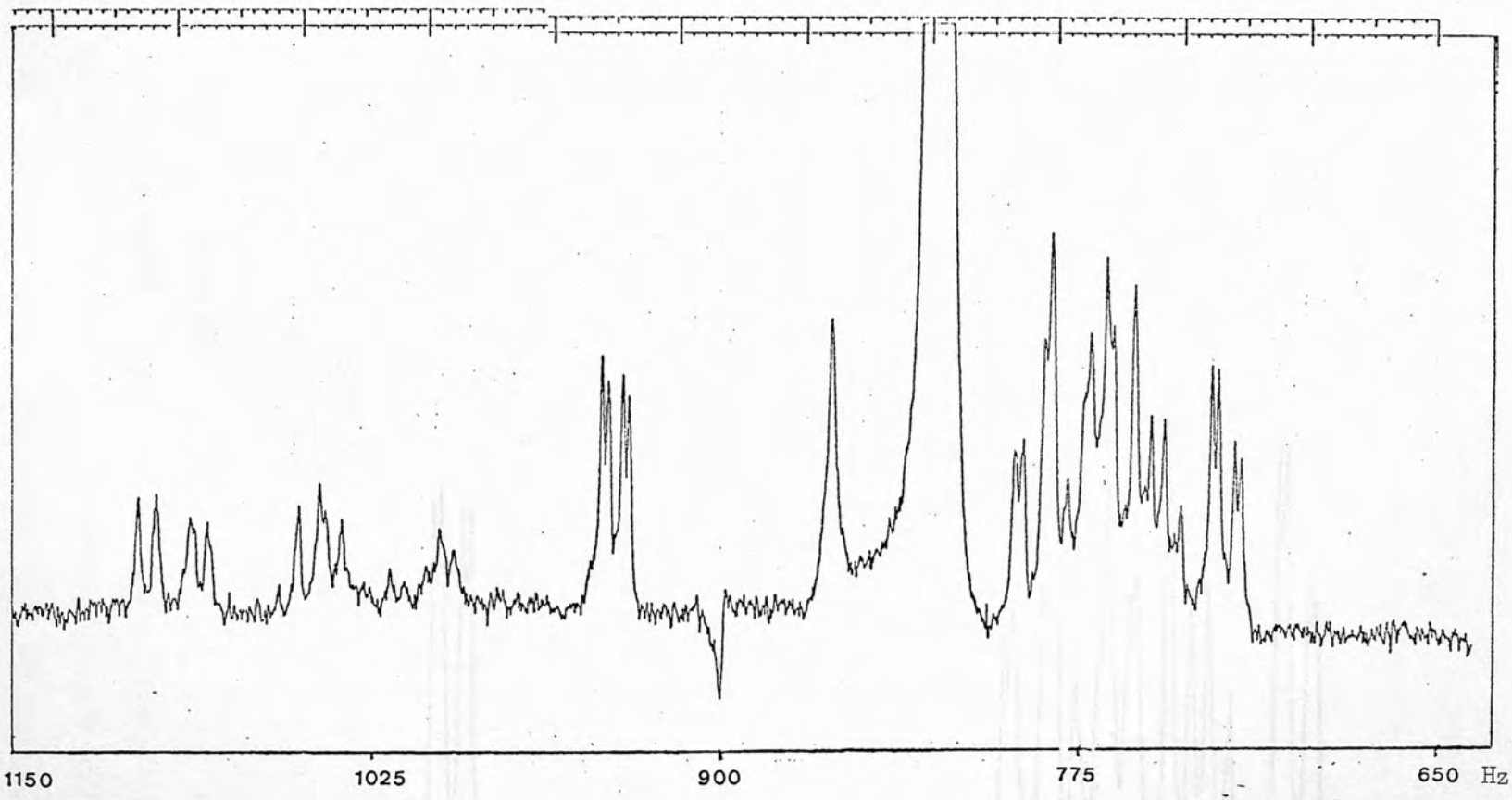


Fig. 2.5 220 MHz ^{1}H nmr spectrum of D-arabinose oxime syn(E)-form in $(CD_3)_2SO$-D_2O

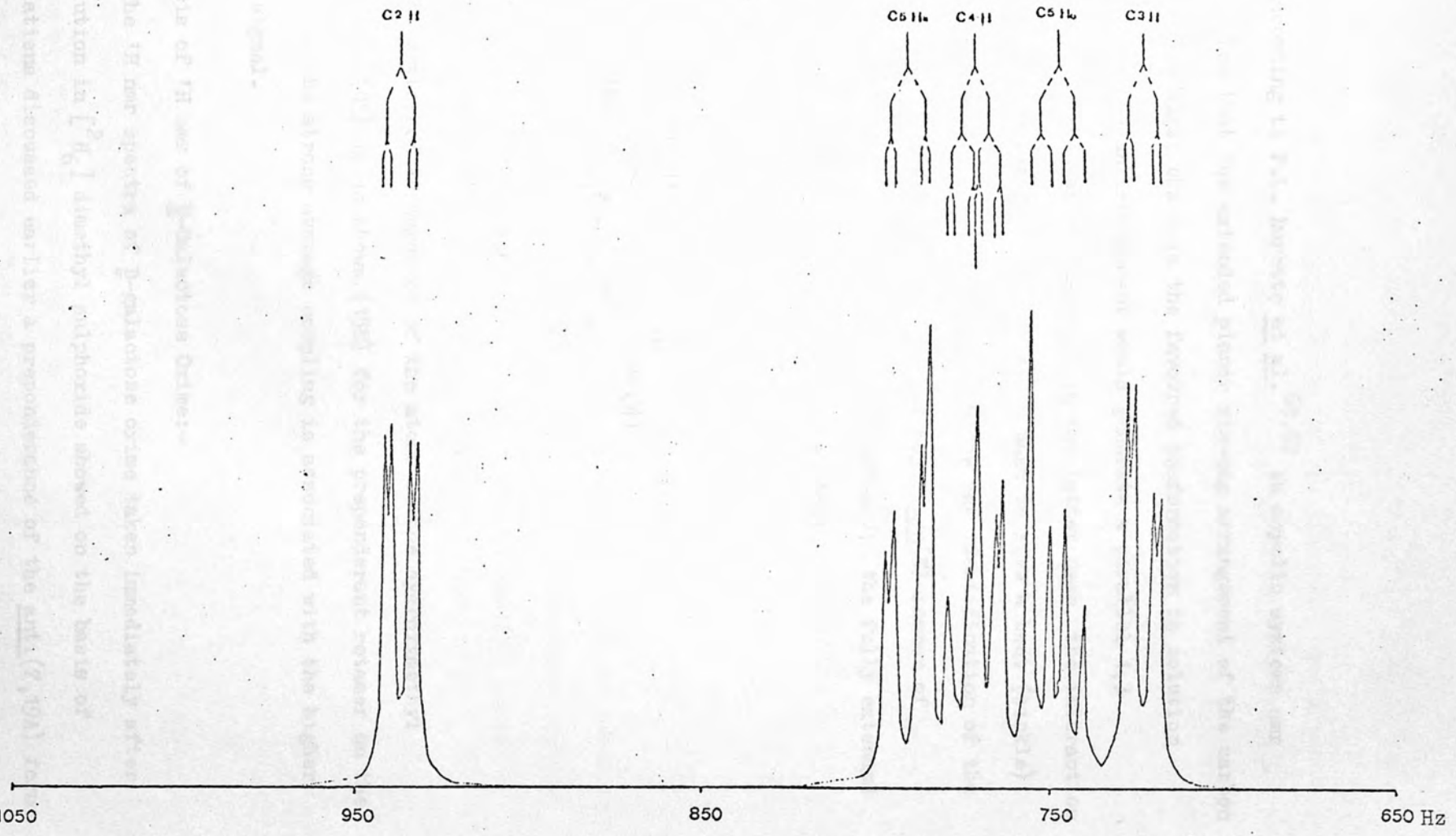
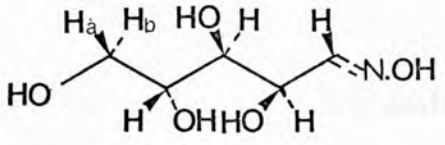


Fig. 2.6 220 MHz Computer simulated ^{1}H nmr spectrum of D-arabinose oxime syn(E)-form in $(CD_3)_2$ SO-D_2O

According to P.L. Durette et al.[61,62] in acyclic systems nmr studies show that the extended planar zig-zag arrangement of the carbon atoms in the sugar chain is the favoured conformation in solution except when such an arrangement would generate a parallel 1,3 interaction between substituents. In the latter case, the interaction is alleviated by rotation about the C-C bond to give a bent (sickle) form. The ^{1}H nmr coupling constants allow the identification of the preponderant conformation (15G) of the major syn(E) isomer of D-arabinose oxime at equilibrium in $(CD_3)_2SO$-D_2O; the fully extended planar zig-zag arrangement is favoured (see 15G).



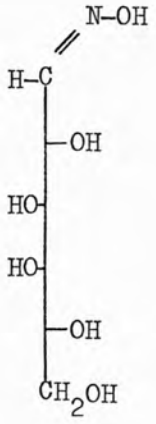
15G

The preferred arrangement of the atoms on the hydroxymethyl carbon atom (C5) is as shown (15G) for the preponderant rotamer on the basis that the strong average coupling is associated with the higher field signal.

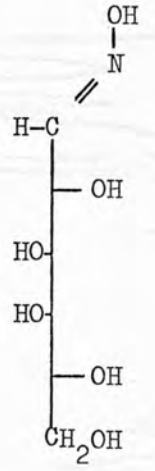
Analysis of ^{1}H nmr of D-Galactose Oxime:-

The ^{1}H nmr spectra of D-galactose oxime taken immediately after dissolution in [$^2H_6$] dimethyl sulphoxide showed on the basis of correlations discussed earlier a preponderance of the anti(Z,19A) form. The equilibrated deuterium oxide solution of D-galactose oxime shows the

presence of two forms viz anti(Z) and syn (E, 19B) with the ^{1}H nmr spectra of the major isomer (syn(E)) at equilibrium being completely analysable. The spectral assignments and parameters (Tables 2.5 and 2.6) were checked by double resonance, exchange in deuterium oxide,

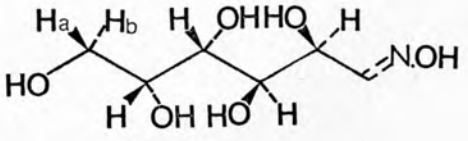


19A



19B

and computer simulation of the C-H signals. On the basis of the observed coupling constants and chemical shifts a fully extended planar zig-zag form (19G) is favoured for the preponderant form at equilibrium of D-galactose oxime. (Figs. 2.7, 2.8).



19G

Analysis of ^{1}H nmr of D-Mannose Oxime

The ^{1}H nmr obtained after immediate dissolution shows almost exclusively the syn(E)-form (20A) on the basis of arguments presented earlier. At equilibrium a mixture of syn(E) (major isomer) and

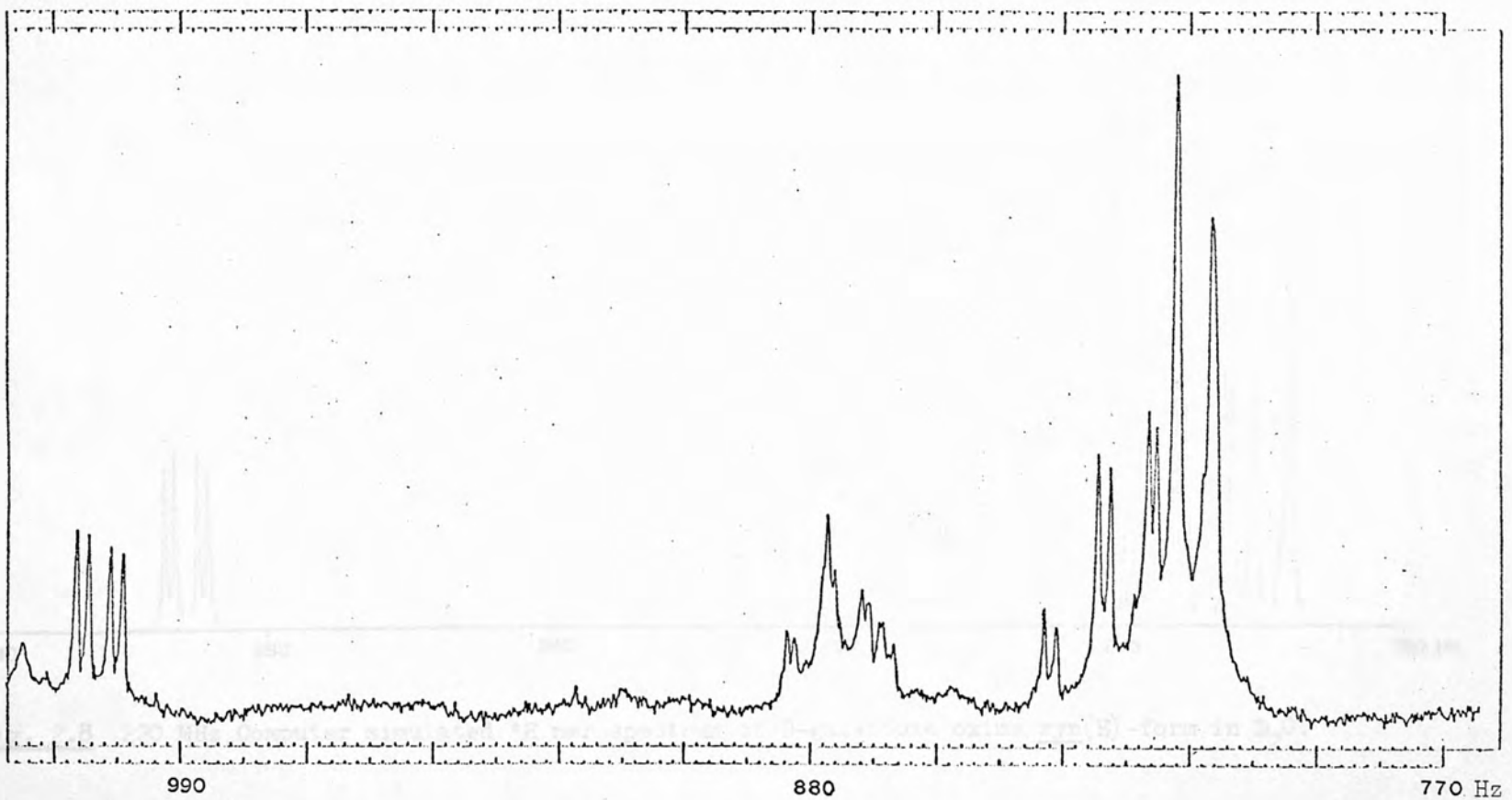


Fig. 2.7 220 MHz ^{1}H nmr spectrum of D-galactose oxime syn(E)-form in D_2O

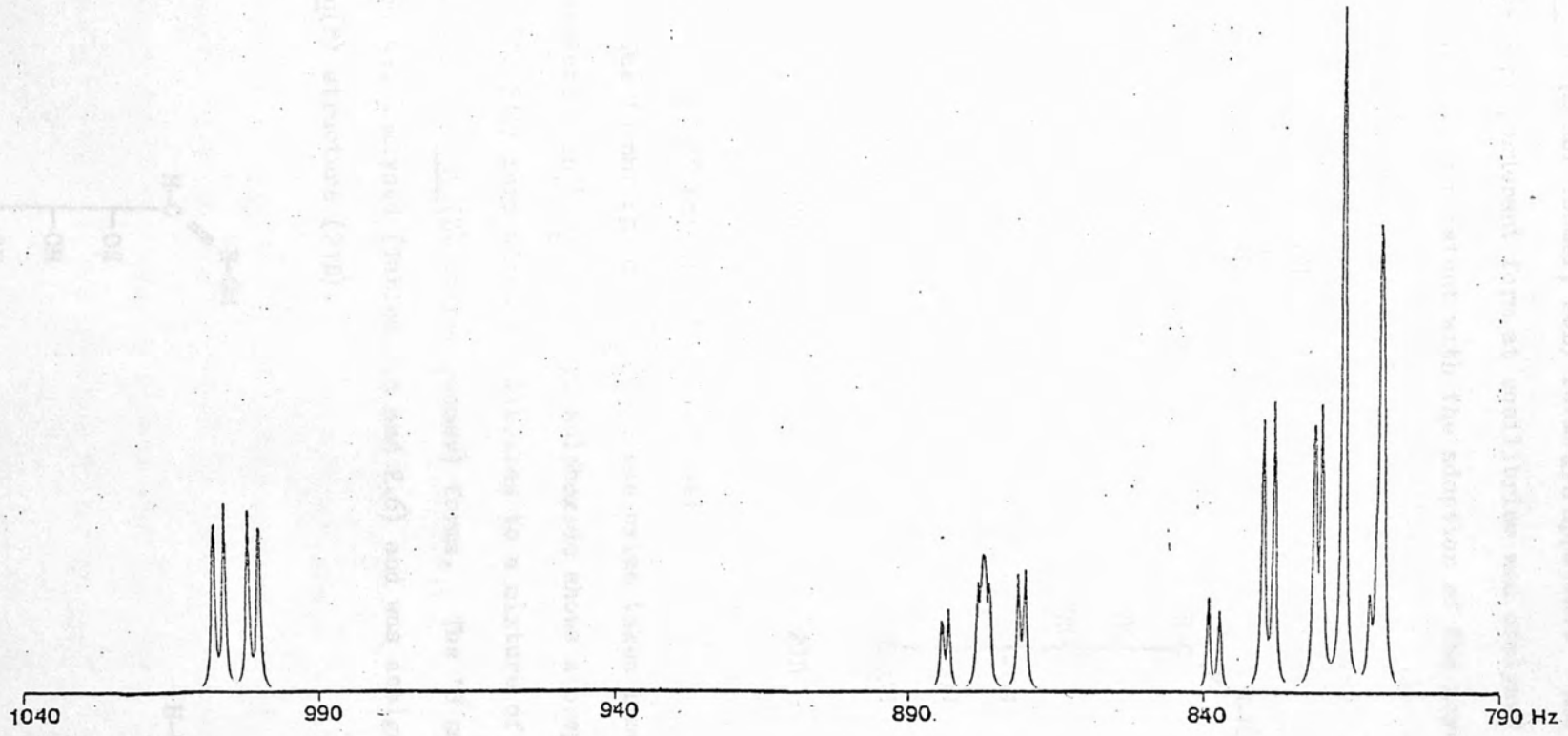
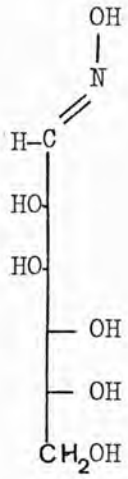
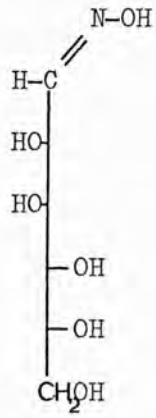


Fig. 2.8 220 MHz Computer simulated ^{1}H nmr spectrum of D-galactose oxime syn(E)-form in D_2O.

anti(Z) (minor isomer, 20B) forms are apparent. The ^{1}H nmr spectrum of the preponderant form at equilibrium was analysed (Tables 2.5 and 2.6) and was consistent with the adoption of the acyclic syn(E) structure (20A)



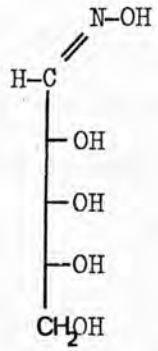
20A



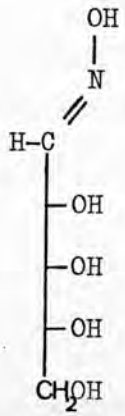
20B

Analysis of ^{1}H nmr of D-Ribose Oxime:

The ^{1}H nmr spectrum of D-ribose oxime taken immediately after dissolution in [$^2H_6$] dimethyl sulphoxide shows a preponderance of the anti(Z, 21A) form which equilibrates to a mixture of syn(E) (major isomer) and anti(Z) (minor isomer) forms. The ^{1}H nmr of the major form was analysed (Tables 2.5 and 2.6) and was assigned the acyclic syn(E) structure (21B).



21A



21B

The equilibrated deuterium oxide solutions of the five carbohydrate oximes showed them to exist in the acyclic syn(E) and anti(Z) forms except for D-glucose oxime which exists as a mixture of syn(E), anti(Z), α-pyranose and β-pyranose forms (Table 2.7). The proportions of the syn(E) and anti(Z) forms of the acyclic oximes are constant at 80:20 and in fact similar to those observed for other sugar oximes and aliphatic oximes (Table 2.7).

After this work was completed the preparation of some pentose oximes (arabinose oxime, ribose oxime and xylose oxime) and structural assignments by ^{1}H nmr spectroscopy were reported by M. Iio et al. (1975).[63] They based their assignments on the magnitude of the C1-H, C2-H coupling constants and assigned the anti(Z)-form a greater value than the syn(E)-form. On this basis the C1 proton of the anti(Z)-form is more deshielded than that of the syn(E)-form, and the NOH group proton of the anti(Z)-form is less deshielded than that of the syn(E)-form. This is contrary to the assignments made by Phillips[50] and other workers[51-57] which in the case of p-benzaldoxime were confirmed by crystallographic studies. Also the assignments of Iio et al.[63] are contrary to those made in this work for D-arabinose oxime and D-ribose oxime. The correlations used in this work have been confirmed by ^{13}C nmr and X-ray crystallographic data (see Sections 2.2.7 and 2.2.8). Further, the coupling constant for the syn(E)-form is observed to be greater than that of the anti(Z)-form for the carbohydrate oximes synthesised in this work (see Table 2.8), i.e. an opposite result to that proposed by Iio et al.[63]

Table 2.7 Proportions of Carbohydrate Oximes and other Oximes in the Solid State and in Equilibrium Solution by ^1H nmr

Compound	Cyclic		Acyclic	
	α-Pyranose	β-Pyranose	Syn-E	Anti-Z
D-Arabinose Oxime				
a) Solid +				100%
b) Equilibrium solution			80%	20%
D-Ribose Oxime				
a) Solid				100%
b) Equilibrium solution			82%	18%
D-Mannose Oxime				
a) Solid			100%	
b) Equilibrium solution			81.5%	18.5%
D-Glucose Oxime				
a) Solid		100%		
b) Equilibrium solution	7%	23%	56.5%	13.5%
D-Galactose Oxime				
a) Solid				100%
b) Equilibrium solution			80%	20%
Methyl ethyl ketoxime[b]				
Equilibrium solution			72%	28%
Methyl propyl ketoxime[b]				
Equilibrium solution			73%	27%
2,3:4,5-di-O-isopropylidene[a] D-Arabinose Oxime.				
Equilibrium solution			80%	20%

[a] Taken from (116). [b] Taken from (115).

+ Also crystallised as a mixture of syn(E) (80%) and anti(Z) (20%) forms.

Table 2.8 Coupling Constants (J_{1H-2H}) of the Carbohydrate Oximes

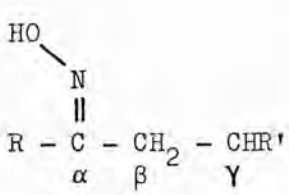
Compound	J_{1H-2H}(syn-E)	J_{1H-2H}(anti Z)
D-Glucose Oxime	7.80	6.20
D-Mannose Oxime	7.60	6.20
D-Galactose Oxime	5.85	5.50
D-Arabinose Oxime	7.30	5.70
D-Ribose Oxime	7.80	6.30

2.2.7 ^{13}C nmr (cmr) Spectroscopy of Carbohydrate Oximes

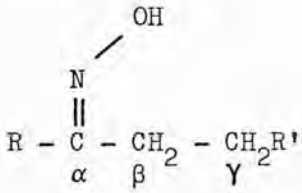
^{13}C nmr (cmr) spectroscopy has proved to be a useful tool in conjunction with ^{1}H nmr for structural elucidation[64,65]. The resonances of the carbon nuclei for monosaccharides were assigned in accord with the general recognition that steric hindrance and proximity effects of substituents are very important factors in the determination of carbon-13 chemical shift differences in related compounds.[66] The anomeric carbon signals were observed[67] to appear at the lowest field in the cyclic monosaccharides due to the two bonded oxygens. Also the hydroxymethyl carbons absorb at the highest field and they are readily identified by "off-resonance decoupling" in which mode they appear as triplets. In the aldopyranoses the hydroxymethyl carbons are equivalent, or nearly so, in each anomeric pair.[67] In monosaccharides due to the occurrence of mutarotation the assignment of the signals for the anomers was made on the basis of their relative intensities, which in turn gave an approximate measure of their proportions. With the introduction of pulse Fourier transform (PFT)

operation, spectra could be taken rapidly enough to permit the observation of individual anomers. The unequivocal assignments of the carbon-13 shifts for the monosaccharides was made by supplementing the substituent effect evidence with that obtained from selected proton decoupling, ^{13}C isotope enrichment, deuteration, and methylation of selective hydroxyl groups.[68-70]

The application of carbon-13 nmr to the determination of the configuration and composition of the syn and anti isomers of aldoximes and ketoximes was made by Levy and Nelson[71] and by Hawkes et al.[72] According to these workers the carbon ($C\alpha$) to which the oxime group is attached appears at δc 150-155 ppm downfield from TMS($\delta_c = 0$) with that of the syn(E)-form being at a slightly higher field than that of the anti(Z)-form.



Syn



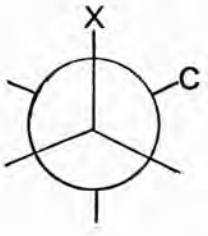
Anti

R,R' = H or alkyl group smaller than propyl

Levy and Nelson[71] observed that the β-carbon experiences a steric compression shift depending on whether the N-OH group is syn or anti to the β-carbon involved ($\delta_{syn} - \delta_{anti} = +6.0$ ppm for ketoximes and $\delta_{syn} - \delta_{anti} = +4$ to $+5$ ppm for aldoximes[72] with the β carbon signal being more shielded for the anti(Z) than the syn(E) isomer. This effect is small for the γ carbons of the syn(E) and anti(Z)-forms. These workers[71,72] observed the presence of both syn(E) and anti(Z) isomers with the major isomer being the syn(E)-form as expected on the

basis of steric hindrance which would favour the OH being *syn* to the least substituted β carbon or hydrogen in the case of aldoximes. Hawkes et al.[72] made use of $Cr(acac)_3$ to diminish differential Overhauser effects and to measure the ratios of *syn*(E) and *anti*(Z) isomers at equilibrium. This gave reasonably satisfactory agreement with proton spectra.[73]

Since the γ -carbon does not show very much difference in the chemical shift for the *syn*(E) and *anti*(Z) isomers one can assign it by making use of the upfield shift caused by a hetero substituent, in this case the oxime group. The upfield shift observed for the resonance of a carbon which is *gauche* to another carbon or hetero atom at the γ -position (22A) relative to the resonance of an analogous carbon in the *anti* conformation (22B) is of particular utility for stereochemical assignment. This γ -effect was ascribed to steric perturbation which was thought to polarise the ^{13}C–H bonds of the encumbered groups such that the carbon nucleus was shielded and the attached protons were deshielded. According to E.L. Eliel *et al.*[74] the results of their study



22A

22B

clearly demonstrate that a carbon atom *anti* to a second-row heteroatom in the γ-position (22B, X = N, O, F) generally resonates at significantly higher field than an analogous nucleus *anti* to a methyl or methylene group

or to a third-row heteroatom (22B, X = CH_3, $-CH_2-$, S, Cl). In addition, their data confirms earlier conclusions that a gauche heteroatom (22A, X = N,O,F,S,Cl) results in an upfield shift of a carbon-13 signal greater than the upfield shift caused by a methyl or methylene group. However, the incremental upfield shift (22A, X = N,O,F,S,Cl as compared with X = CH_3 or CH_2) on the gauche carbon is generally less than the corresponding incremental upfield shift on the anti carbon (22B). Since the γ_c effect of a $-CH_2-$ or $-CH_3$ group is negligible the γ effects of anti-periplanar heteroatoms may be effectively referenced with respect to hydrogen.[74]

In the case of oximes one can assign the carbon resonances for the α, β and γ carbons by making use of the work of Nelson and Levy,[71] Hawkes et al.[72] and Eliel et al's.[74] This is illustrated in Table 2.9 for nbutane and the related aldehyde, 2 - ketone, syn(E) and anti(Z) aldoximes and 2-ketoximes.

Table 2.9 ^{13}C Chemical Shifts in ppm relative to TMS $\delta_c = 0$ of n-Butane and Derivatives

Compound		C_1	C_2	C_3	C_4
$\overset{1}{C}H_3-\overset{2}{C}H_2-\overset{3}{C}H_2-\overset{4}{C}H_3$	a	13.2	25.0	25.0	13.2
$H-\overset{\alpha}{\overset{O}{\overset{\|}{C}}}-\overset{\beta}{C}H_2-\overset{\gamma}{C}H_2-\overset{\delta}{C}H_3$	b	202.5	45.9	15.8	13.8
$H-\overset{\alpha}{\overset{HO-N}{\overset{\|}{C}}}-\overset{\beta}{C}H_2-\overset{\gamma}{C}H_2-\overset{\delta}{C}H_3$	c	152.1	31.5	20.1	13.6
$H-\overset{\alpha}{\overset{N-OH}{\overset{\|}{C}}}-\overset{\beta}{C}H_2-\overset{\gamma}{C}H_2-\overset{\delta}{C}H_3$	d	152.6	27.0	19.5	13.9
$\overset{1}{H_3C}-\overset{\alpha}{\overset{O}{\overset{\|}{C}}}-\overset{\beta}{\overset{3}{C}}H_2-\overset{4}{\overset{\gamma}{C}}H_3$	e	28.9	208.8	36.4	7.4
$H_3C-\overset{\alpha}{\overset{HO-N}{\overset{\|}{C}}}-\overset{\beta}{C}H_2-\overset{\gamma}{C}H_3$	f	13.0	159.1	28.9	10.7
$H_3C-\overset{\alpha}{\overset{N-OH}{\overset{\|}{C}}}-\overset{\beta}{C}H_2-\overset{\gamma}{C}H_3$	g	18.9	159.5	21.7	9.6

(a) δ_c taken from (64)

(b-g) δ_c taken from (73)

Taking the chemical shifts of the n-butane and comparing them with those of the analogous carbons of the aldehyde and the syn(E) and anti(Z) aldoximes, it is observed that for C1 resonances the aldehyde carbon(α) is deshielded the most followed by the anti(Z) and syn(E) aldoximes. For the C2 resonances once again the aldehyde carbon (β) is deshielded more than the syn and anti(Z) aldoxime β-carbons. Further the difference in the chemical shifts of the β-carbons for the syn(E) and anti(Z) aldoximes is 4.5 ppm. Going on to the C3 resonances one observes a shielding effect, with the aldehyde carbon (γ) being the most shielded followed by the anti and the syn aldoxime γ-carbon atoms. In this case there is a negligible difference between the γ-carbons of the syn(E) and anti(Z) aldoximes. The C4 resonances for all four compounds are nearly the same showing that the heteroatom substituent effect for the δ-carbon is negligible. Similar, effects are observed when one compares the corresponding carbons of n-butane, but-2-one, and the syn(E) and anti(Z) ketoximes.

The carbon-13 resonance signals for the carbohydrate oximes, D-arabinose oxime, D-ribose oxime, D-mannose oxime, D-galactose oxime and D-glucose oxime were assigned by comparing the acyclic oximes with the corresponding deoxy polyol* and the polyol,* and the cyclic pyranose oxime with the corresponding 1,5-anhydro polyol and the aldopyranose. Further, the cmr spectra were taken on dissolution of the carbohydrate oxime in D_2O and then after a week. The carbon-13

* The chemical shifts of deoxy polyols and polyols were obtained from Dr D. Lewis and Dr D. Gibson of Chemistry Department, Royal Holloway College. The values were relative to internal TSP (δ_C=0). These shifts were assigned by these workers by means of substituent effects and deuteration studies.

resonances were assigned to the different isomers from the relative intensities of the signals. Finally, to distinguish between the carbons bearing primary hydroxyl from those with secondary hydroxyl groups use was made of the "off resonance" decoupled spectra in which the decoupling frequency is a few hundred hertz outside the range of the Larmor frequencies of the protons. This causes the geminal and long range couplings to collapse, thus enabling one to observe a first order doublet for the hydroxymethine carbon and a first order triplet for the hydroxymethylene carbon.

D-Arabinose oxime (see Table 2.10) showed the presence of two isomeric acyclic forms at equilibrium and gave signals at δ_c 155.13 and δ_c 156.04 ppm, which being at the lowest field were assigned to the C 1 resonances of the acyclic syn(E) (15A) and anti(Z) (15B) forms. The chemical shifts are in accordance with typical values for aldoximes reported previously.[72] The signals for the other carbons were assigned from their relative intensities to the syn(E)-form and anti(Z)-form. The signal at the highest field δ_c 65.40 ppm was common to both the isomers and was assigned to the C5 carbons from the "off resonance" decoupled spectra.[66,67] The remaining three carbon resonances were assigned (Table 2.10) by comparison with 1-deoxy-D-arabinitol (23) and D-arabinitol (12). (See Figs. 2.9, 21.0).

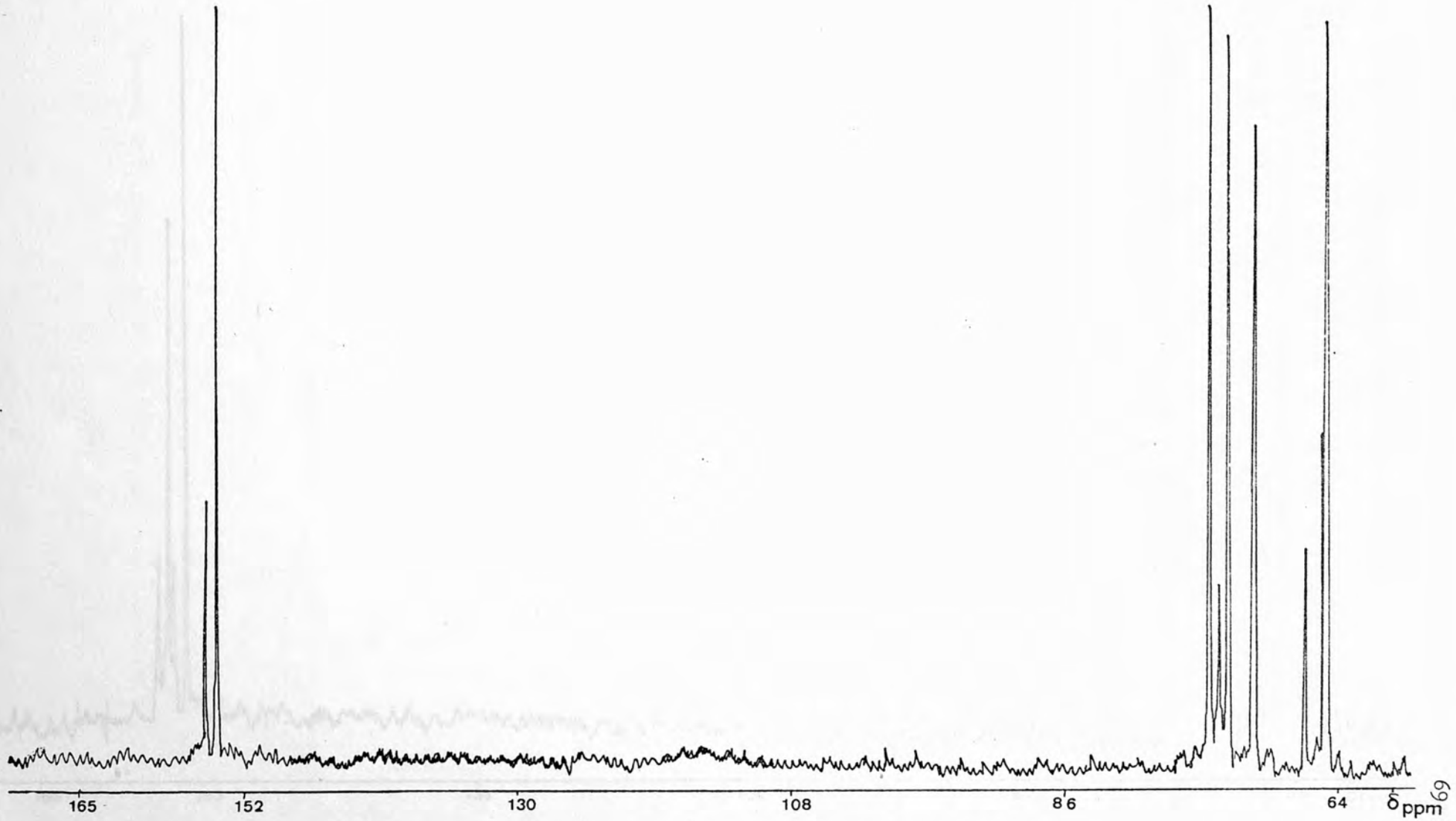


Fig. 2.9 22.63 MHz ^{13}C nmr broad band spectrum of equilibrated D-arabinose oxime in D_2O.

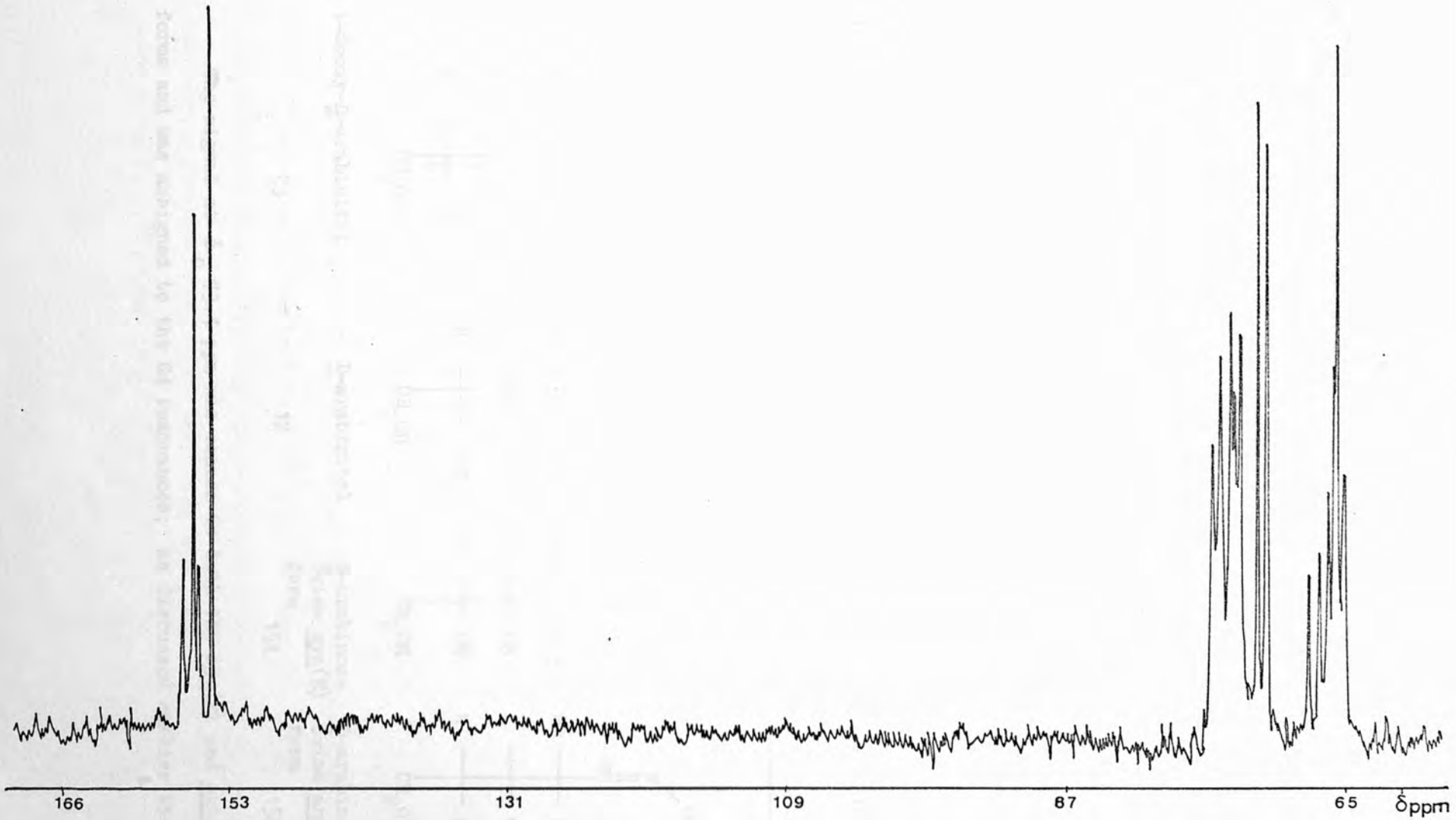
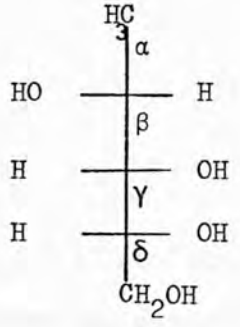


Fig. 2.10 22.63 MHz ^{13}C nmr "off resonance" spectrum of equilibrated D-arabinose oxime in D_2O

Table 2.10 ^{13}C Chemical Shifts in ppm relative to TSP $\delta_c = 0$ (Internal Std.) of D-Arabinose Oxime and Related Compounds

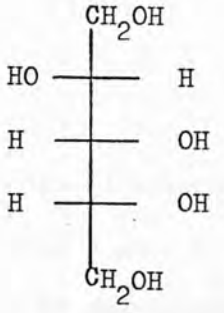
Compound	C-1	C-2	C-3	C-4	C-5
1-deoxy-D-arabinitol	21.1	68.9	77.0	74.0	65.4
D-arabinitol	66.0 or 65.8	73.2[a]	73.5[a]	73.9[a]	65.8 or 66.0
D-arabin ose oxime (syn(E)-form)	155.1	71.1	74.9	73.4	65.4
D-arabinose oxime (anti(Z)-form)	156.0	67.1	74.1	73.4	65.4

[a] = assignments uncertain



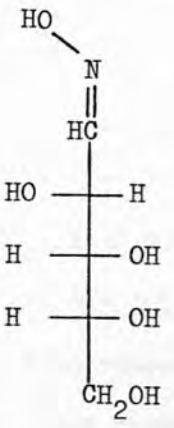
1-deoxy-D-arabinitol

23



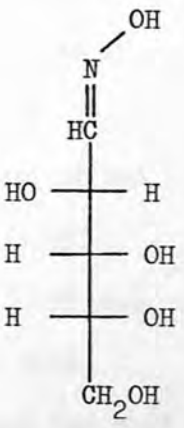
D-arabinitol

12



D-arabinose oxime syn(E) form

15A



D-arabinose oxime anti(Z) form

15B

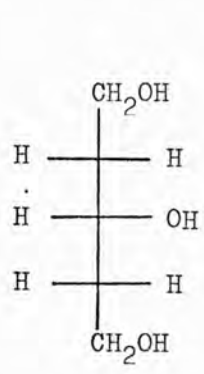
The signal at δ_c 73.4 ppm was common to both the syn(E) and anti(Z) forms and was assigned to the C4 resonances; as discussed earlier the

oxime group has a negligible effect on the chemical shift of the δ carbon atom. The signal at δ_c 71.1 was assigned to the C2 resonance of the syn(E)-form; the proximity of the oxime substituent deshields C2 in 15A . The deshielding effect of the oxime group appears to be less than that of the OH group in D-arabinitol. The signal at δ_c 67.1 ppm was assigned to the C2 resonance of the anti(Z)-form and appears at a higher field because of the shielding effect due to the proximity of the oxime hydroxyl group. The difference in the chemical shifts of the C2 resonances for 15A and 15B is $\delta_{c_{syn}} - \delta_{c_{anti}} = 4.0$ ppm which is comparable to the values obtained by Hawkes et al.[72] for aldoximes. Finally, the signals at δ_c 74.9 and δ_c 74.1 ppm were assigned to the C3 resonances of 15A and 15B; being in the γ -antiperiplanar position to the heterosubstituent[74] these signals are shielded relative to those of 23 but appear to be less so than for 12.

D-Ribose oxime (see Table 2.11) also showed the presence of two isomeric acyclic forms and gave signals at δ_c 154.0 and δ_c 154.3 ppm which were assigned to the C1 resonances of the syn(E) (21B) and anti(Z) (21A) forms, while the signal at δ_c 65.4 common to both isomers was assigned to the C5 resonances of these forms from the "off resonance"decoupled spectra. The remaining three carbon assignments for each isomer were made by comparison with the chemical shifts (Table 2.11) of ribitol (25) and 2-deoxy-D-ribitol (24), (the δ_c values for 1-deoxy-D-ribitol were not available) as well as those of D-arabinose oxime which is a C2 eipmer of D-ribose oxime.

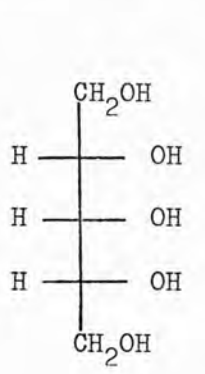
Table 2.11 ^{13}C Chemical Shifts in ppm of D-Ribose Oxime and Related Derivatives relative to TSP $\delta_c = 0$ (Internal Std.)

Compound	C-1	C-2	C-3	C-4	C-5
2-deoxy-D-ribitol	61.4	37.0	71.8	77.5	65.2
ribitol	65.4	75.0	75.0	75.0	65.4
D-ribose oxime syn(E)-form	154.0	72.2	75.7	74.1	65.4
D-ribose oxime anti(Z)-form	154.3	68.1	75.4	73.8	65.4



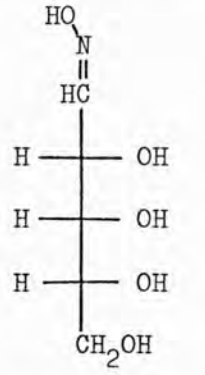
2-deoxy-D-ribitol

24



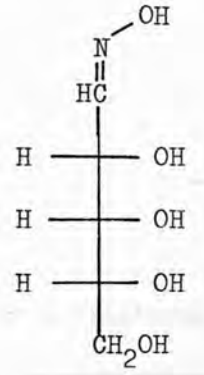
ribitol

25



D-ribose oxime syn(E)-form

21B



D-ribose oxime anti(Z)-form

21A

The signals at δ_c 72.2 and δ_c 68.1 ppm were assigned to the C2 resonances of the syn(E) and anti(Z) forms of D-ribose oxime ($\Delta\delta_{c_{syn-anti}} = 4.1$ ppm). The carbon atom in the γ-position to the deoxy function in 2-deoxy-D-ribitol appears at δ_c 77.5 ppm while the carbon (C3) γ to the hydroxymethyl group in ribitol appears at δ_c 75.0 ppm as it is expected to be shielded being in an antiperiplanar

arrangement. On this basis the Y-carbons (i.e. C3) to the oxime functions are assigned the resonances at δ_c 75.7 and δ_c 75.4 ppm for 21B and 21A, being less shielded than in the case of ribitol. Finally, the signals at δ_c 74.1 and δ_c 73.8 ppm were assigned to C4 resonances of 21B and 21A.

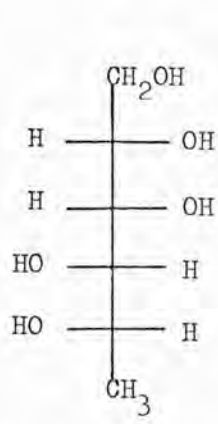
D-mannose oxime showed the presence of predominantly one form from the carbon-13 spectrum taken on dissolution and after a week. The signals at δ_c 155.3 and δ_c 65.7 ppm were assigned to C1 and C6 resonances being at the lowest and highest field respectively, and the identity of C6 was confirmed by "off resonance" decoupling. The remaining carbon resonances were tentatively assigned by analogy with 6-deoxy-L-mannitol (26) and D-mannitol (27) (Table 2.12).

Table 2.12 ^{13}C Chemical Shifts in ppm relative to TSP $\delta_c = 0$ (Internal Std.) of D-Mannose Oxime and Related Derivatives

Compound	C-1	C-2	C-3	C-4	C-5	C-6
6-deoxy-L-mannitol[a]	21.6	69.9	76.1	72.3 or 73.9	73.9 or 72.3	65.9
D-mannitol	66.0	73.8	72.1	72.1	73.8	66.0
D-mannose oxime (syn(E)-form)	155.26	71.8 or 71.4	73.5 or 73.2	71.4* or 71.8	73.2* or 73.5	65.7

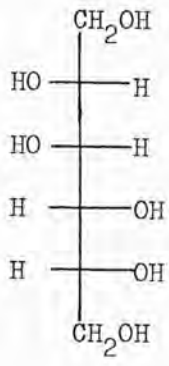
a = The numbering of the molecule is done by turning the molecule through 180° and taking C1 as C6 (ie CH_3)

* = uncertain assignments



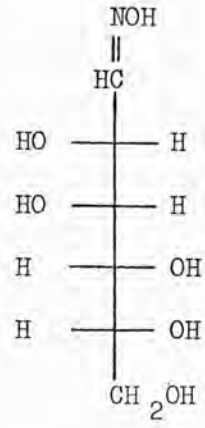
6-deoxy-L-mannitol

26



D-mannitol

27



D-mannose oxime

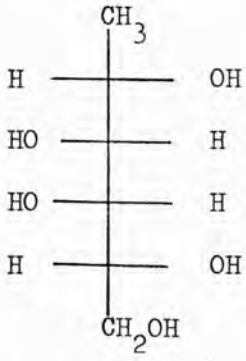
20

The signal at δ_c 71.8 or that at δ_c 71.2 ppm was assigned to the C2 resonance as it is in a β-position to the oxime function and would be expected to be deshielded relative to the β-carbon at δ_c 69.9 ppm to the deoxy function of(26). The signal at δ_c 73.5 or that at δ_c 73.2 ppm was assigned to the C3 resonance being in a γ -antiperiplanar position with respect to the oxime group and therefore shielded relative to the γ-antiperiplanar carbon atom to the deoxy function in(26). The C4 and C5 resonances are uncertain as the corresponding resonances for the deoxy polyol are not definitely assigned. The absence of a resonance at δ_c 67 - 68 ppm and comparison with the ^{1}H nmr data suggest that this is the syn(E)-form (Section 2.2.6.1).

The cmr spectra of D-galactose oxime showed the presence of two isomeric forms at equilibrium. The signals at δ_c 155.5 and δ_c 156.5 ppm were assigned to the C1 resonances of the syn(E) and anti(Z)-forms, and the highest field signal at δ_c 65.8 ppm which is common to both isomers was assigned to C6 resonances of these isomers. The remaining signals were assigned by comparison with the ^{13}C chemical shifts (Table 2.13) of 1-deoxy-D-galactitol (28) and galactitol (29).

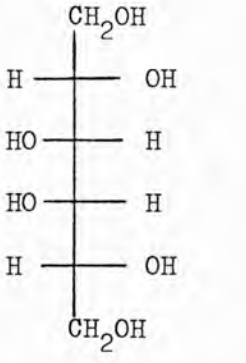
Table 2.13 ^{13}C Chemical Shifts in ppm relative to TSP $\delta_C = 0$ (Internal Std.) of D-Galactose Oxime and Related Compounds

Compound	C-1	C-2	C-3	C-4	C-5	C-6
1-deoxy-D-galactitol	21.4	68.9	75.8	73.0 or 72.7	72.7 or 73.0	66.0
galactitol	66.1	73.0	72.3	72.3	73.0	66.1
D-galactose oxime syn(E)-form	155.5	71.3	74.0	72.7 or 72.0	72.0 or 72.7	65.8
D-galactose oxime anti(Z)-form	156.5	67.3	73.3	72.7 or 72.0	72.0 or 72.7	65.8



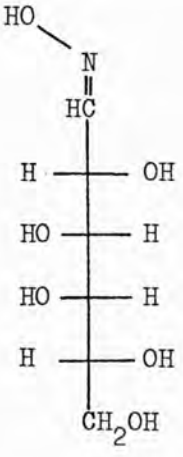
1-deoxy-D-galactitol

28



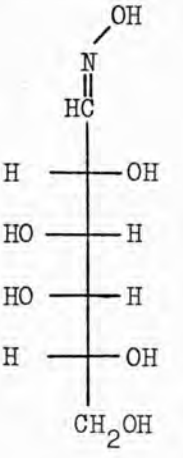
galactitol

29



D-galactose oxime syn(E) form

19B



D-galactose oxime anti(Z) form

19A

The signals at δ_c 71.3 and δ_c 67.3 ppm were assigned to the C2 resonances of the syn(E) (19B) and anti(Z) (19A) forms ($\Delta\delta_{c\ syn-anti}$ = 4 ppm). The signals at δ_c 74.02 and δ_c 73.24 ppm were assigned to the C-3 resonances of (19B)and(19A)on the basis of the γ -effect discussed earlier. The signals at δ_c 72.0 and δ_c 72.7 ppm are common to the C4 and/or C5 resonances of both isomers, as being remote from the oxime group they would experience a negligible differential influence from the syn(E) and anti(Z) oxime configurations. These two signals are not possible to assign definitely to either C4 or C5 as the corresponding signals of 1-deoxy-D-galactitol (28) and galactitol (29) have not been assigned with certainty.

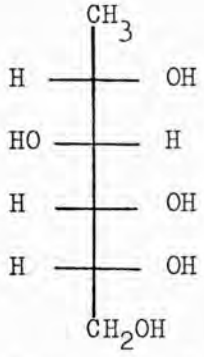
D-glucose oxime from ^{1}H nmr and other evidence discussed in previous sections is known to exist in the crystalline state in the $^4C_1$ chair conformation having the β-anomeric configuration. In solution at equilibrium 4 isomeric forms are present namely the β-pyranose and α-pyranose cyclic forms, and the syn(E) and anti(Z) acyclic forms. By taking the cmr spectrum immediately after dissolution the β-pyranose carbon-13 signals could be identified and then by observing the cmr spectrum after one week the resonances due to the syn(E) and anti(Z) forms could be identified. However, the resonances due to the α-pyranose form could not be seen. Taking the acyclic syn(E) and anti(Z) forms first (Table 2.14), the lowest field signals at δ_c 154.3 and δ_c 154.7 ppm were assigned to the C1 resonances of the syn(E) (18b) and anti(Z) (18a) forms respectively, while the highest field signal at δ_c 65.83 ppm common to both syn(E) and anti(Z) forms was assigned to the C6 resonances of these isomers. The remaining signals were assigned by comparison with the chemical shifts (Table 2.14) of 1-deoxy-D-glucitol (30) and D-glucitol (31).

Table 2.14 ^{13}C Chemical Shifts in ppm relative to TSP $\delta_c = 0$ (Internal Std.) of D-Glucose Oxime and Related Derivatives

Compound	C-1	C-2	C-3	C-4	C-5	C-6
1-deoxy-D-glucitol	21.1	71.6	76.6	73.8 or 73.6	73.6 or 73.8	65.8
D-glucitol	65.3	75.6	72.4	73.9	73.7	65.6
D-glucose oxime syn(E)-form	154.3	72.6	73.1	73.7	73.7	65.6
D-glucose oxime anti(Z)-form	154.7	68.6	73.3	73.7	73.7	65.6
1,5-anhydro-D-glucitol[a]	70.2	70.8	78.8	71.1	81.7	61.9
β-D-glucopyranose[b]	97.1	75.6	77.3	71.2	77.3	62.4
β-D-glucopyranose oxime	93.7	72.1 or 72.3	79.5 or 79.8	72.3 or 72.1	79.8 or 79.5	63.7

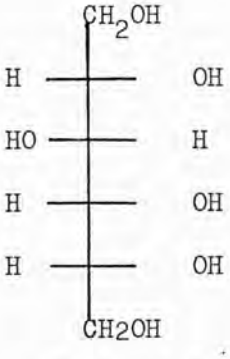
(a) = chem. shifts taken from (75)

(b) = chem. shifts taken from (70)



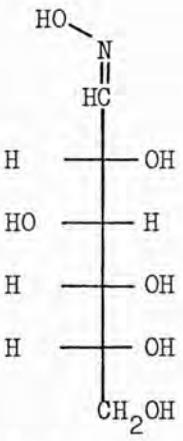
1-deoxy-D-glucitol

30



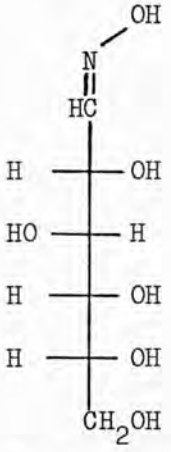
D-glucitol

31



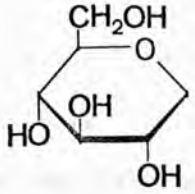
D-glucose oxime syn(E)-form

18B



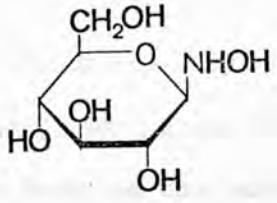
D-glucose oxime anti(Z)-form

18A



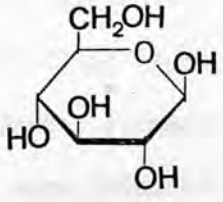
1,5-anhydro-D-glucitol

32



β-D-glucopyranose oxime

18D



β-D-glucopyranose

33

The signals at δ_c 72.6 and δ_c 68.6 ppm were assigned to the C2 resonances of the syn(E) (18b) and anti(Z) (18a) forms ($\Delta\delta_{c\ syn-anti}$ = 4 ppm). The signals at δ_c 73.1 and δ_c 73.3 ppm were assigned to the C3 resonances of (18B) and (18A). The signals for C4 and C5 resonances had the same chemical shift δ_c 73.7 ppm and were common to both (18b) and (18a) being further away from the oxime group they experience a negligible substituent effect and have values very similar to the corresponding carbon resonances (Table 2.14) of 1-deoxy-D-glucitol (30) and D-glucitol (31).

In the case of the cyclic β-D-glucopyranose oxime the lowest and highest field signals at δ_c 93.7 and δ_c 63.7 ppm were assigned to the C1 and C6 resonances. The signal at δ_c 72.3 or that at δ_c 72.1 ppm was assigned to C2 i.e. in the β-position to the -NHOH group. This carbon experiences a deshielding effect relative to the corresponding carbon in 1,5-anhydro-D-glucitol (32), but less so than the C2 carbon in β-D-glucopyranose (33,OH substituent on anomeric carbon) The signals at δ_c 79.5 and δ_c 79.8 ppm were assigned to C3 and/or C5 resonances, both being in the γ -antiperiplanar position to the -NHOH group experience a shielding effect which is less strong than that on the corresponding carbons (Table 2.14) in β-D-glucopyranose (33). Finally, by comparison with data for compounds (32) and (33) the C4 resonances of (18D) is assigned to the signal at δ_c 72.1 or that at δ_c 72.3 ppm.

By taking the cmr (PFT) spectra immediately after dissolution of the carbohydrate oximes and then after one week, it was possible to postulate the isomeric forms these oximes adopt in the crystalline state (Table 2.15). By measuring the C1 peak areas for the different isomeric forms at equilibrium, the propcrtions of the various forms were obtained.

Table 2.15 Equilibrium Proportions of Carbohydrate Oximes by 'H nmr and ^{13}C nmr

Compound	'H nmr				^{13}C nmr			
	Cyclic form		Acyclic form		Cyclic form		Acyclic form	
	β-pyranose	α-pyranose	syn(E)	anti(Z)	β-pyranose	α-pyranose	syn(E)	anti(Z)
1. D-arabinose oxime								
a) solid				100%				100%
b) equilm. soln.			80%	20%			83%	17%
2. D-ribose oxime								
a) solid				100%				100%
b) equilm. soln.			82%	18%			79%	21%
3. D-mannose oxime								
a) solid			100%				100%	
b) equilm. soln.			81.5%	18.5%				
4. D-galactose oxime								
a) solid				100%				100%
b) equilm. soln.			80%	20%			82.6%	17.4%
5. D-glucose oxime								
a) solid	100%				100%			
b) equilm. soln.	23%	7%	56.5%	13.5%	11%		74.5%	14.5%

Comparing these values (Table 2.15) with those obtained by ^{1}H nmr one can see that they are in reasonable agreement.

The assignments made for the carbon resonances are tentative; in order to make unequivocal assignments one would have to carry out ^{13}C isotope enrichment, selective frequency "off-resonance" decoupling, deuteration of specific protons attached to the relevant carbon atoms and comparison with some O-methyl derivatives of these compounds.

Thus, the cmr evidence helps in confirming the structural assignments made to the syn(E) and anti(Z) aldoximes by ^{1}H nmr.

2.2.8 X-Ray Crystallographic Studies of Carbohydrate Oximes

X-ray crystallographic studies on D-glucose oxime, and D-arabinose oxime (syn(E) and anti(Z)) crystals synthesised in this work have been carried out by Dr A. Mostad of the University of Oslo, Norway. The structure of D-glucose oxime he arrived at, was a pyranose form with β-anomeric configuration in the $^4C_1$ chair conformation (Fig. 2.11). The structure derived from one sample of D-arabinose oxime crystals was observed to be acyclic in a planar zig-zag conformation having the anti(Z) orientation of the NOH group. This particular sample gave directly after dissolution ^{1}H and ^{13}C nmr spectra consistent with anti stereochemistry (Fig. 2.12). Further, a D-arabinose oxime crystal grown under different conditions by Dr Mostad was shown to comprise molecules in the syn(E)-form in a planar zig-zag conformation (Fig. 2.13). Thus the X-ray crystallographic studies of the oximes of D-arabinose and and D-glucose confirm the structural assignments which were made by consideration of ^{1}H and ^{13}C nmr data.

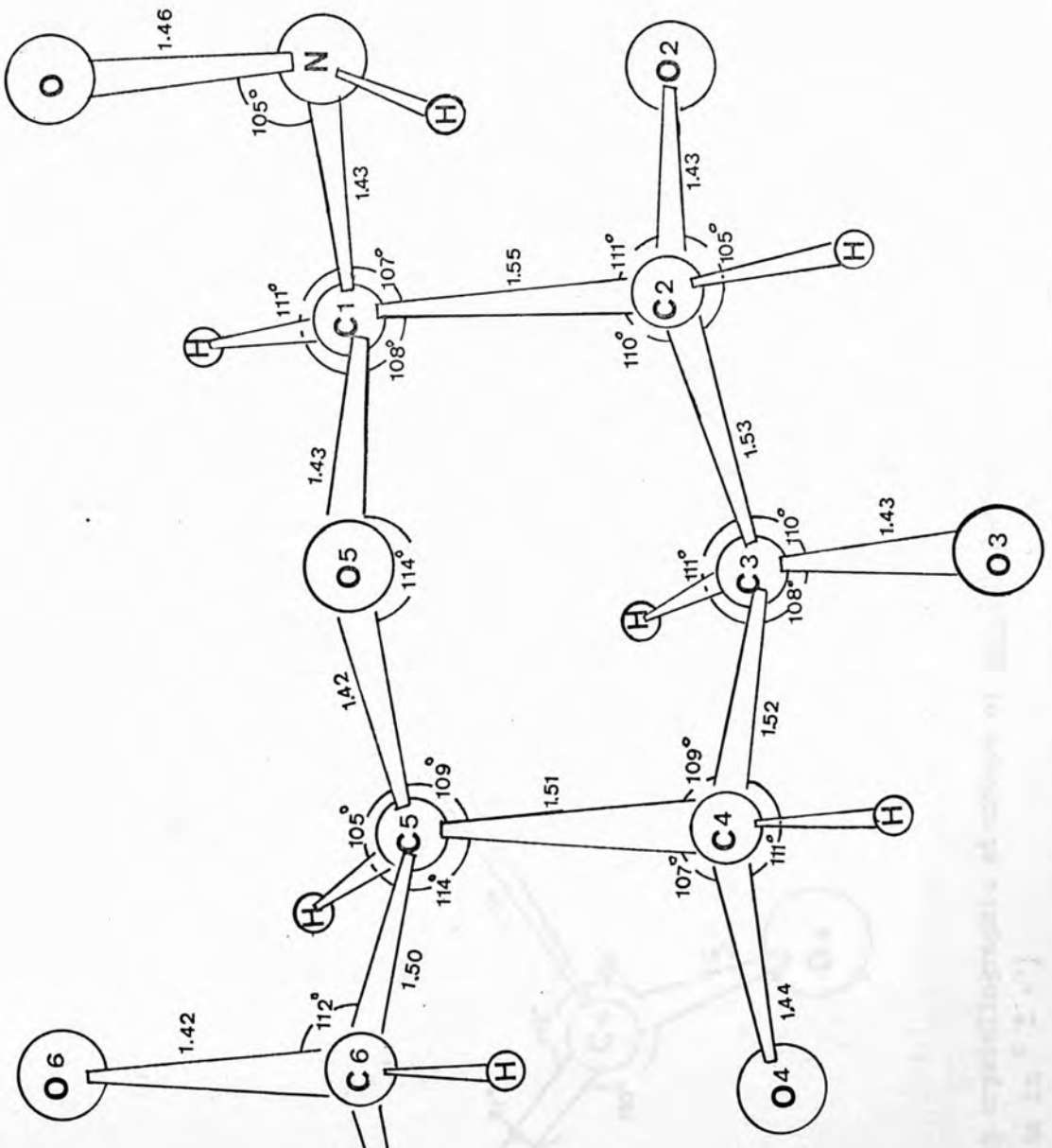


Fig. 2.11 X-Ray crystallographic structure of D-glucose oxime (R = 0.05, bond length in $^{o}_{A} \pm 0.01$; bond angles in $^{o} \pm 1^{o}$)

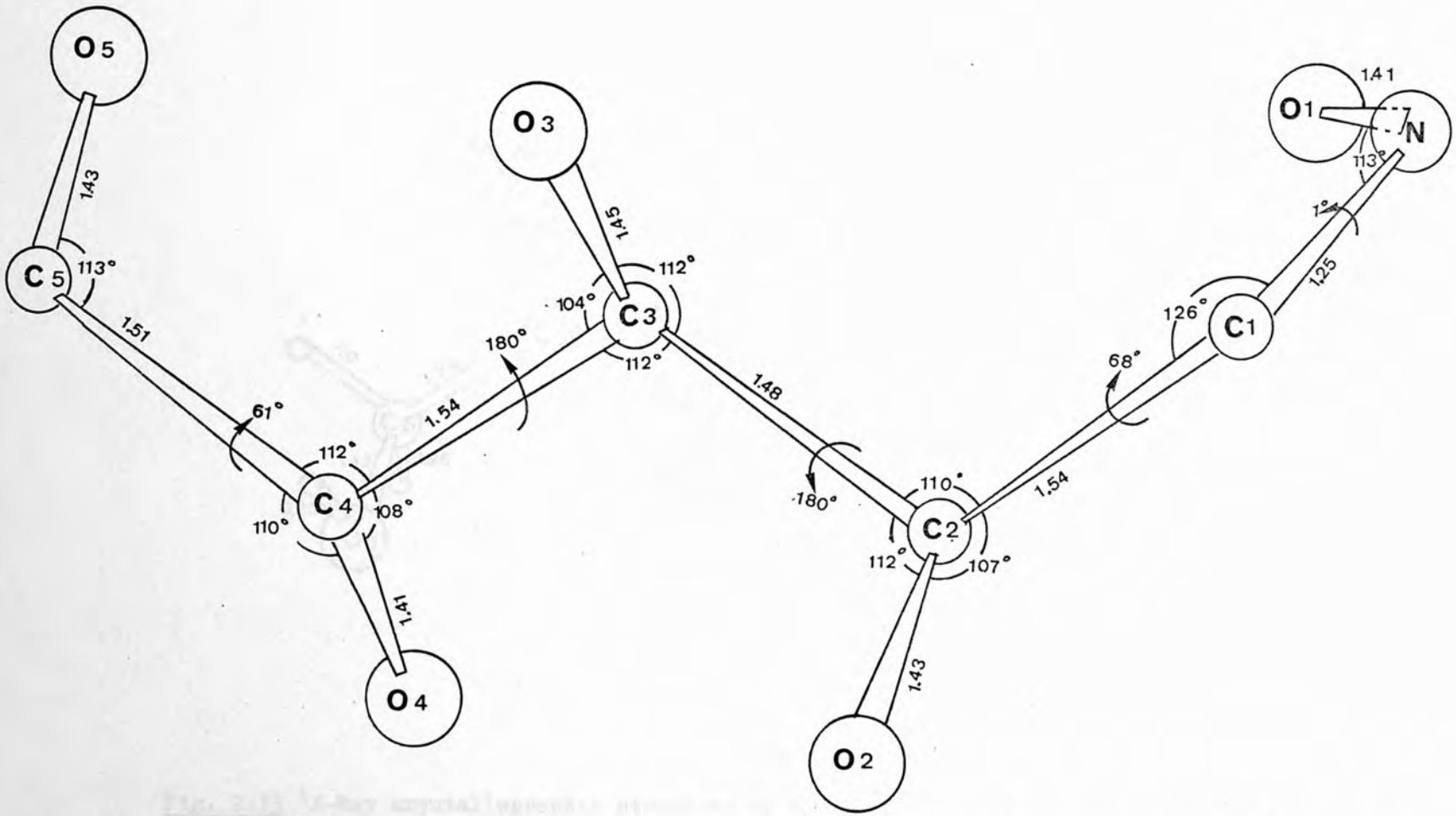


Fig. 2.12 X-Ray crystallographic structure of anti(Z) D-arabinose oxime (bond length in $^{o}A \pm 0.01$; bond angles in $^{o} \pm 1^{o}$)

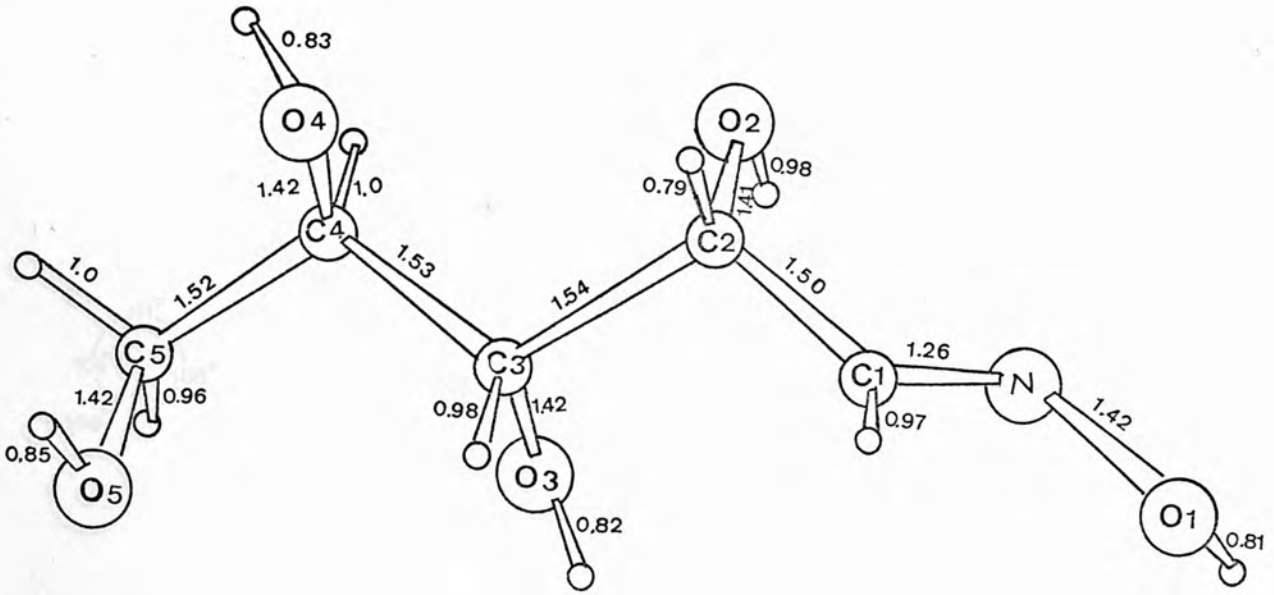


Fig. 2.13 X-Ray crystallographic structure of syn(E) D-arabinose oxime (bond length in $^{o}A \pm 0.01$; bond angles in $^{o} \pm 1^{o}$)

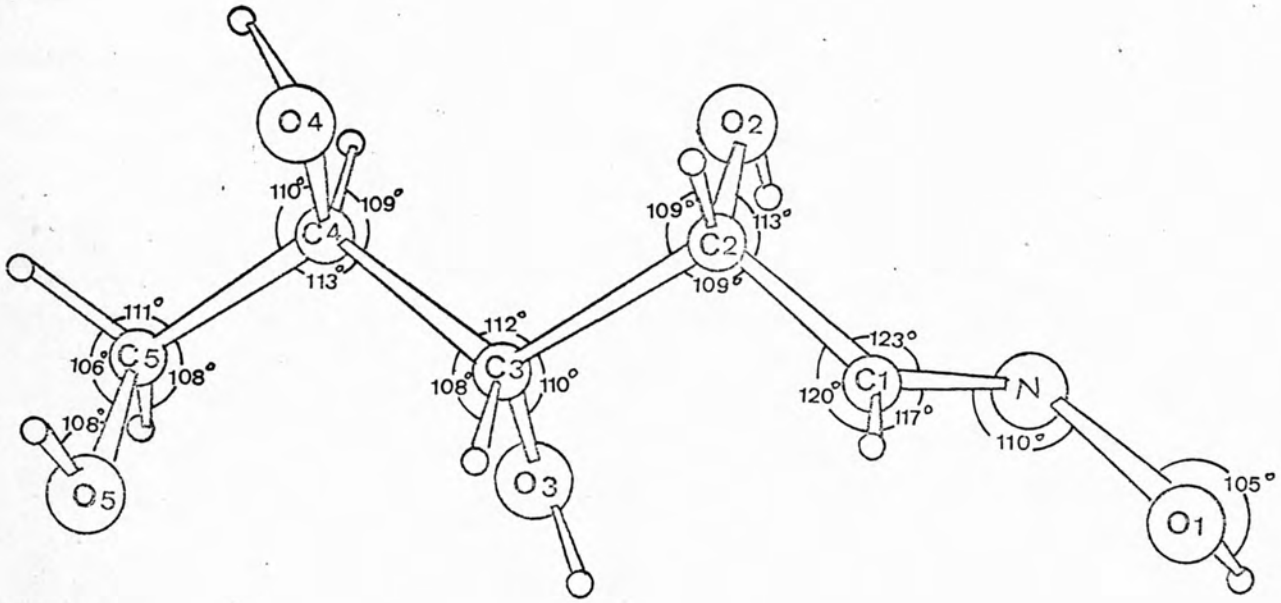


Fig. 2.13 (continued)

In addition, the X-ray data permit a more precise assessment of the appropriateness of an oxime as an analogue of an enediol. The bond lengths and bond angles of the isomers of D-arabinose oxime and of a number of enolic compounds are shown in Table 2.16 In every case the C = C bond length is greater than C=N, while the N-OH bond length is greater than the C-OH bond length. Moreover, the C=C^{-OH} anglesare 120 - 125^{o} while the C=N^{-OH} angles are 126^{o} for anti(Z)-form and 110^{o} for syn(E)-form.

Table 2.16 X-Ray Data of Oximes and Enols

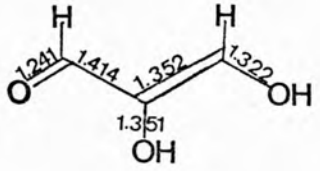
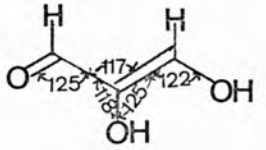
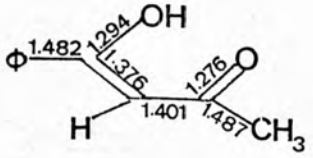
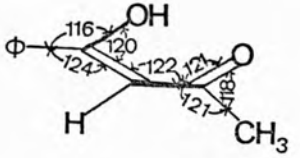
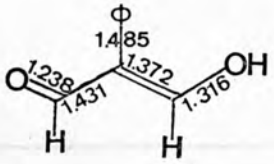
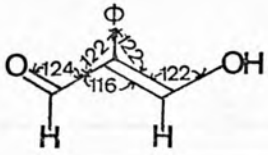
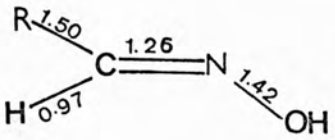
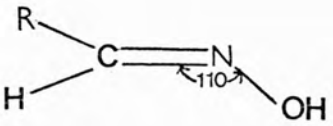
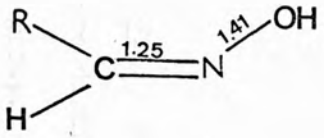
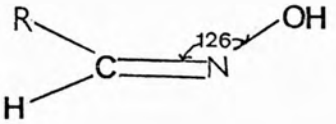
Compound	Bond lengths in °A	Bond angles°	Refs.
Triose reductone			109b
Benzoyl acetone			109a
Phenyl malondialdehyde			117

Table 2.16 (continued)

Compound	Bond lengths in ^{o}A	Bond angles^{o}	Refs.
D-Arabinose Oxime (syn (E)-form)			This work
D-Arabinose Oxime (anti(Z)-form)			This work

2.2.9 Phosphorylation of D-Arabinose Oxime by Yeast Hexokinase

The enzymic phosphorylation of D-arabinose oxime was achieved by using adenosine-5'-triphosphate (ATP) and yeast hexokinase (EC 2.7.1.1). This enzyme is known to selectively phosphorylate the primary hydroxyl group of pentoses and hexoses.

A reaction mixture containing D-arabinose oxime, $Mg.ATP^{2-}$ and hexokinase was prepared. Paper chromatography and electrophoresis experiments carried out on this mixture indicated that phosphorylation had occurred (see EXPERIMENTAL).

2.2.9.1 Analysis by 'H nmr Spectroscopy: The reaction was also followed by 'H nmr spectroscopy at 360 MHz with help of Dr R. Kaptein (University of Groningen, Netherlands, courtesy of Professor H.C. Berendson). The 'H nmr spectrum of D-arabinose oxime has been analysed as discussed earlier (see Section 2.2.6.1). Some predictions from previous work can be made about the changes which might be expected to occur on phosphorylation. According to M.P. Schweizer *et al.*[76] the C-H protons which are adjacent to the phosphoryl ester group are deshielded owing to the electron withdrawing effect of the phosphoryl group. The electron density of the phosphoryl group is expected to be higher for the dianion form than the monoanion form. On comparing the C5-Ha' and C5-Hb' signals of the ribose moiety of adenosine-5'-monophosphate (AMP) with those of adenosine at pD 5.9, they[76] observed chemical shift differences of 0.249 ppm with the C5' protons of the phosphorylated compound at lower field. Other workers[77-79] have found that the 3 bond angular dependence of ^{31}P-O-C-'H coupling for phosphate parallels that established for vicinal 'H-'H and ^{19}F-'H couplings. In the case of a series of phosphate esters the vicinal ^{31}P-O-C-'H couplings were rationalised in terms of varying populations of individual *trans* or *gauche* rotamers. (see Table 2.17 for 'H nmr of phosphorylated and nonphosphorylated sugars).

In this work 360 MHz ^{1}H nmr spectra were taken before the addition of hexokinase to the reactants (D-arabinose oxime and $Mg.ATP^{2-}$ in D_2O at pH 7.6), after the addition and then after 14 h. D-Arabinose oxime proton signals were observed to be separated from $Mg.ATP^{2-}$ signals (Fig. 2.14) and the data are given in Table 2.18. This made it possible to observe any changes in the proton signals of D-arabinose oxime as the reaction proceeded. It was observed that after 1 h and 14 h (Figs 2.15 and 2.16) additional signals had appeared (see Table 2.18) due to the formation of ADP, at the same time the intensity of the ATP signal at δ 8.3 (C8-H), 7.99 (C2-H) and 5.99 ppm (C 1'-H) had decreased. Further, the C5-Ha and C5-Hb signals of D-arabinose oxime had shifted by 0.22 ppm downfield (Table 2.18) and showed increased splitting. The C4-H signal of D-arabinose oxime had also shifted downfield by 0.05 ppm and become more complex. The chemical shifts of the C1-H signals of the syn(E) and anti(Z) forms of D-arabinose oxime were not shifted and there was no change in their proportions. It was concluded that both syn(E) and anti(Z) forms of D-arabinose oxime became phosphorylated and that phosphorylation did not affect the equilibrium proportions of the two forms (nor the chemical shifts of the C1 protons).

2.2.9.2. Analysis by ^{31}P nmr Spectroscopy:

The ATP-dependent phosphorylation of D-arabinose oxime by yeast hexokinase was followed by observing the Fourier transform ^{31}P nmr spectrum. Spectra were taken before the addition of yeast hexokinase (Fig. 2.17) and then after 3.5 h (Fig. 2.17). ^{31}P signals were observed at δ_p -10.39, -19.53 and -6.20 ppm (from H_3PO_4) corresponding to the α, β- and γ- phosphorus resonances of ATP.

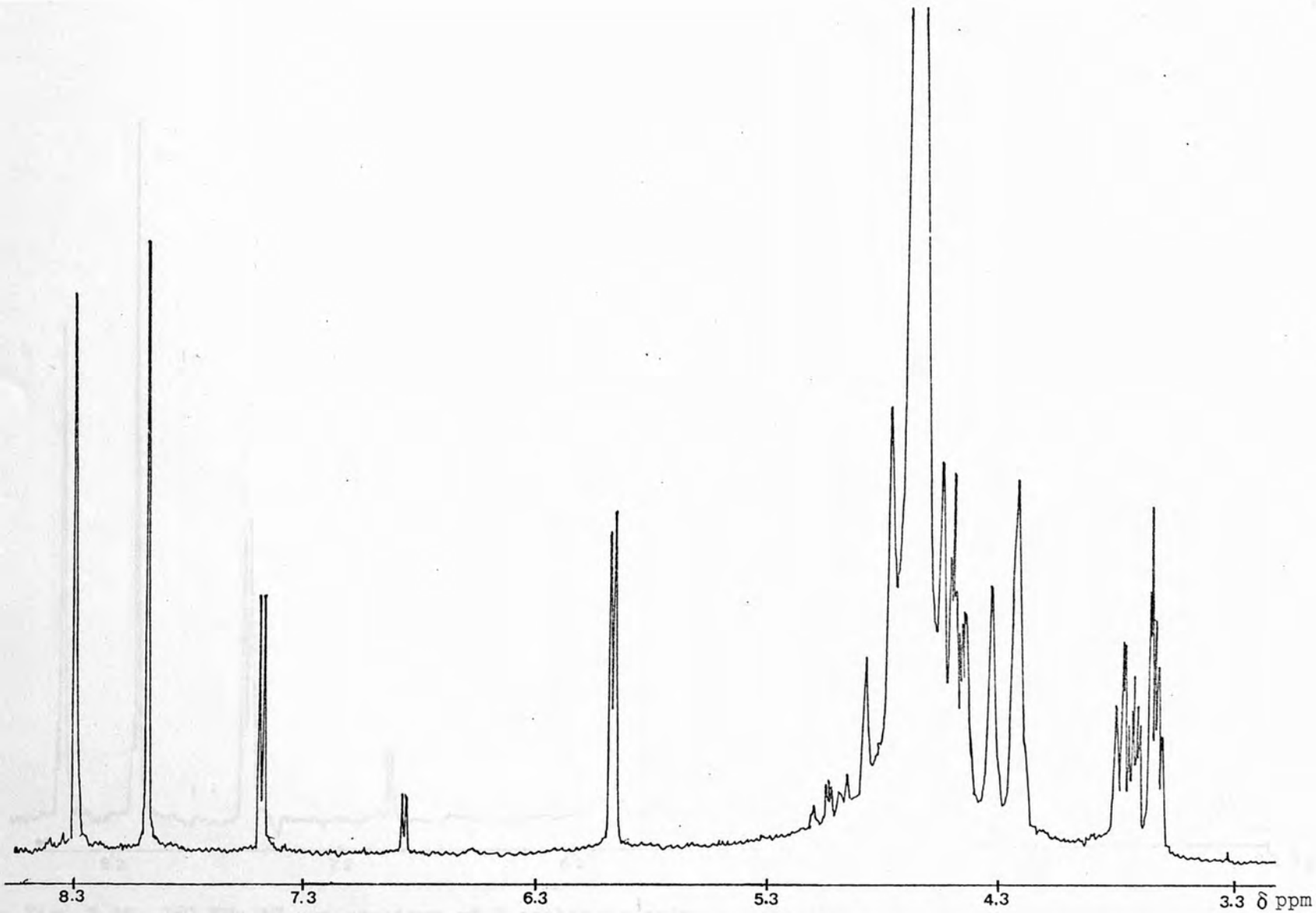


Fig. 2.14 360 MHz ^{1}H nmr spectrum of D-arabinose oxime and $Mg.ATP^{2-}$ before addition of hexokinase in D_2O

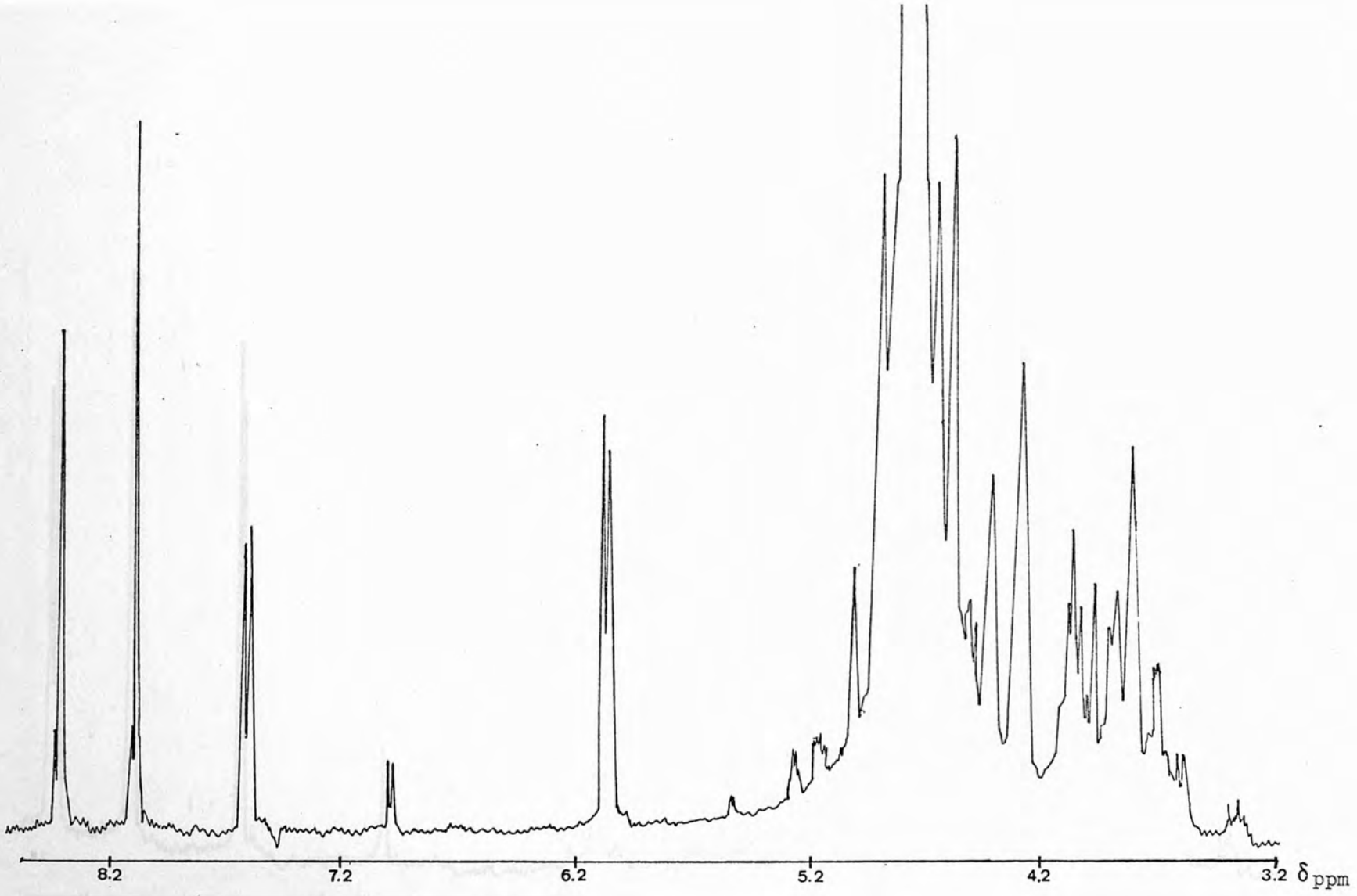


Fig. 2.15 360 MHz ^{1}H nmr spectrum of D-arabinose oxime and $Mg.ATP^{2-}$, 1 h after addition of hexokinase in D_2O

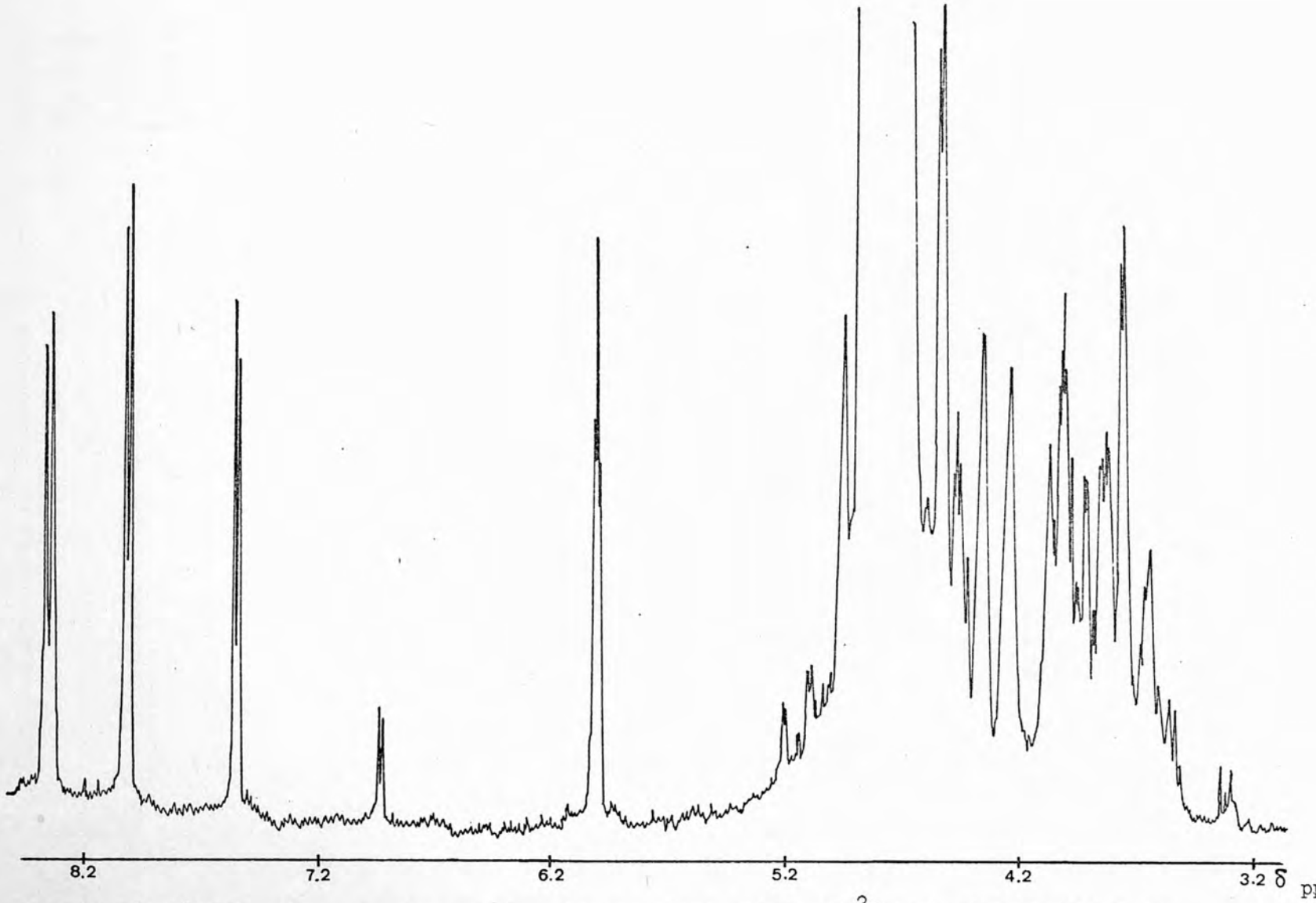


Fig. 2.16 360 MHz ^{1}H nmr spectrum of D-arabinose oxime and Mg.ATP^{2-}, 14 h after addition of hexokinase in D_2O

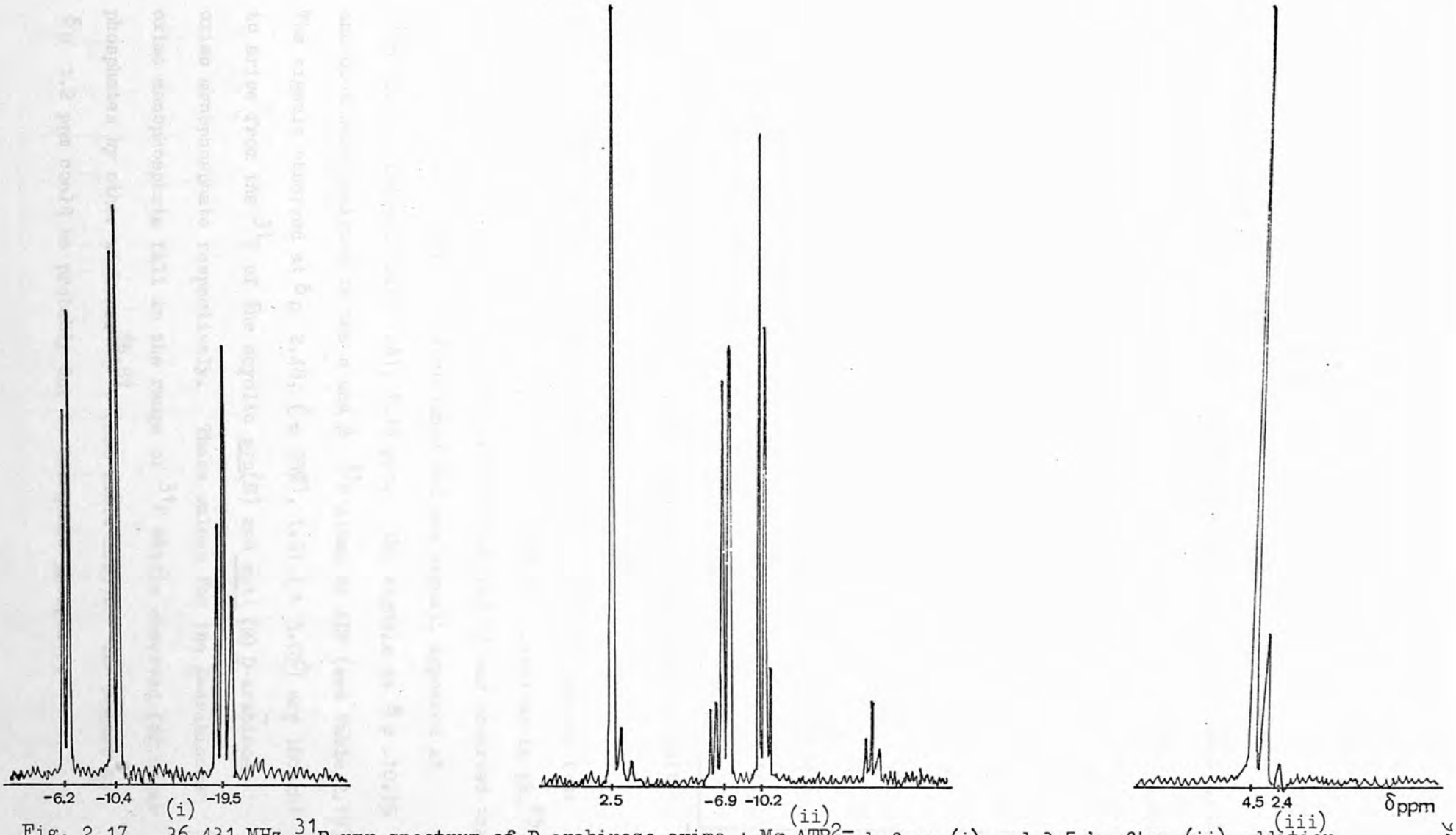


Fig. 2.17 36.431 MHz ^{31}P nmr spectrum of D-arabinose oxime + $Mg.ATP^{2-}$ before (i) and 3.5 h after (ii) addition of hexokinase; isolated D-arabinose oxime-5-phosphate (lithium salt) (iii).

Table 2.17 ^{1}H nmr Parameters in D_2O (δ: Standard TSP)

Compound	Chemical shift, δ ppm H-α	ΔHα	$J_{H-\alpha,p}$ Hz	Reference
α-D-glucopyranose-1-phosphate[a]	5.40 (q)		8.5	80
α-D-glucopyranose	5.32 (d)	0.08		81
α-D-mannopyranose-1-phosphate[b]	5.34 (q)		7.0	80
α-D-mannopyranose	5.25 (d)	0.09		81
α-D-galactopyranose-1-phosphate[c]	5.68 (q)		7.0	80
α-D-galactopyranose	5.34 (d)	0.34		81
5'-AMP[c]	4.60 (m)			76
Adenosine	4.35 (q)	0.25		76

a = potassium salt, b = cyclohexyl ammonium salt, c = sodium salt
d = doublet, q = quartet, m = multiplet.

These values are similar to those observed[83,84] by other workers (see Table 2.19), the small differences were ascribed to variations in pH.[85] In the ^{31}P nmr spectrum taken after 3.5 h (Fig.2.17ii) it was observed that the signals due to ATP were diminished and new signals appeared at δ_p -10.15,-6.94 and 2.48, 1.61, 1.16 ppm. The signals at δ_p -10.15 and-6.94 were assigned to the α and β ^{31}P atoms of ADP (see Table 2.19). The signals observed at δ_p 2.48, (≃ 95%), 1.61 (≃ 5.0%) are thought to arise from the ^{31}P of the acyclic syn(E) and anti (Z) D-arabinose oxime monophosphate respectively. These values for the D-arabinose oxime monophosphate fall in the range of ^{31}P shifts observed for sugar phosphates by other workers.[86,87] (see Table 2.20). The signal at δ_p 1.2 ppm could be probably due to inorganic phosphate.

Table 2.18 Chemical Shifts in D_2O (δ, ppm from Standard DSS)

	Signals from Mg-ATP +						Signals from D-arabinose oxime syn-form *anti-form					
	H-8		H-2		H-1'	H-2', H-3', H-4', H-5a', H-5b'	H-1	H-2	H-3	H-4	H-5a	H-5b
(a)	8.30		7.99		5.99	4.51 - 4.24	7.50 6.89*	4.47 5.04*	3.66	3.75	3.81	3.64
(b)	8.32		8.00		6.00	4.51 - 4.24	7.54 6.92*	4.48 5.07*	3.66	3.79	3.99	3.82
(c)	8.33	8.36	8.00	7.98	6.00	4.51 - 4.24	7.52 6.91*	4.48 5.07*	3.66	3.80	4.03	3.86

+ Assignment of the chemical shifts were taken from (82)

(a) Before addition of hexokinase

(b) 1 hr after addition of hexokinase

(c) 14 h after addition of hexokinase

Table 2.19 ^{31}P nmr Parameters of ATP and ADP (δ , ppm, H_3PO_4 External Std.)

pH	^{31}P chemical shift upfield from 85% H_3PO_4 in ppm			*Coupling constant in Hz		Reference
	α-P	β-P	γ-P	$^2J_{P\alpha OP\beta}$	$^2J_{P\beta OP}$	
10[a]	11.70	22.71	6.62	20.6	21.3	83
7.6[b]	10.39	19.53	6.20	14.65	17.09	This work
7.6[c]	10.15	6.94	-	17.09	-	This work

a = ATP b = Mg.ATP c = MgADP

* The coupling constant $^2J_{pOp}$ is equal to the distance between adjacent peaks measured on a frequency scale.

From these ^{31}P nmr results one can conclude that D-arabinose oxime has been phosphorylated, but it is not possible to determine which hydroxyl group is involved. However from ^1H nmr data it was possible to show with reasonable certainty that it was the C5-OH group which had been phosphorylated to give D-arabinose oxime-5-phosphate. The techniques used could be applied to other substrates if they are available in sufficient amount for ^1H nmr (>20 mg) and ^{31}P nmr (>100mg).

2.2.9.3. Larger Scale Enzymic Synthesis of D-Arabinose Oxime-5-phosphate

The ATP-dependent phosphorylation by yeast hexokinase was next used on a preparative scale. The method of Koerner et al.[88] in a modified form was first used. To separate the products of phosphorylation a Dowex 1X8 ($HCOO^-$) column[89] (pH 2.7) was used and the eluted fractions were tested by the orcinol reaction (see EXPERIMENTAL). However, it was observed from analysis of the sample that hydrolysis of the oxime group

Table 2.20 ^{31}P nmr Chemical Shifts of Sugar Phosphates

Compound	^{31}P Chemical Shift downfield from 85% H_3PO_4 in ppm	pH	Reference
1,3-Dihydroxy-2-propanone phosphate dimethyl acetal	2.8	6.5	86
1,3-Dihydroxy-2-propanone phosphate	1.23*, 3.33 & 4.03	6.5	"
2,5-Anhydro-D-mannitol 1,6-diphosphate	3.46	7.0	"
2,5-Anhydro-D-glucitol 1,6-diphosphate	3.81 & 4.30	7.6	"
Methyl β-D-fructofuranoside 1,6-diphosphate	1.52*, 2.91 & 3.17	7.0	"
Methyl α-D-fructofuranoside 1,6-diphosphate	1.52*, 3.29 & 4.23	7.0	"
β-D-fructofuranose 1,6-diphosphate	2.06 & 2.32	6.15	"
α-D-fructofuranose 1,6-diphosphate	3.17 & 4.23	6.15	"
D-Glyceraldehyde 3,3-d_2 3-phosphate dimethylacetal dicyclohexylammonium salt	4.0		"
D-Arabinose oxime-5-phosphate	1.16, 1.61 & 2.48	7.6	This work

* Inorganic phosphate

had occurred. To overcome this problem the reaction product was passed through a Dowex 1 X 8B (HCO_3^-) column and eluted with a gradient (0 to 2M ammonium bicarbonate pH 7.5 buffer). Once again from analysis and ^1H nmr (absence of C1-H signals at δ_H 7.0 and 6.34 ppm and presence of signal at δ_H 5.60 ppm) of the sample it was suspected that hydrolysis of the oxime group had occurred. Since it was difficult to isolate the product D-arabinose

oxime-5-phosphate from the ADP and ATP present, use was made of an ATP regenerating system which required the addition of only a very small amount of ATP. The ATP is reformed from ADP and phosphoenol pyruvate by pyruvate kinase. The method of Avigad and Englard[90] was used but modified in two respects. The pH of the reaction mixture was not allowed to fall below 4 and the product was isolated as the lithium salt. This is soluble in water and therefore convenient to use for spectroscopic analysis and other experiments. The compound isolated analysed for $C_5H_{10}NO_8P\ Li_2.2H_2O$ (see EXPERIMENTAL). The C, H and N values were acceptable but inconsistent results were given for P content. The purity of the sample was confirmed by paper chromatography (R_f = 0.12) (molybdate spray[91] and silver nitrate[92]) electrophoresis, ($M_{DAO\text{-}5\text{-}P} = 5.0 \times 10^{-9} m^2 s^{-1} V^{-1}$, development with silver nitrate[92]) both gave only one spot. The sample was further examined by ^1H and ^{31}P nmr spectroscopy.

^1H nmr: The ^1H nmr spectrum of D-arabinose oxime monophosphate dilithium salt prepared as mentioned above showed (see Table 2.21 and Fig.2.18) the presence of doublets at δ_H = 6.99 ppm and δ_H = 6.36 ppm (downfield from TMS) corresponding to the C1-H signals (see 2.2.6.1) of the acyclic syn(E) and anti(Z) forms respectively. The integrated values of the two signals revealed that the compound exists in solution in the syn(E)(83%) and anti(Z) (17%) forms. Further, it was observed that the C5-Ha and C5-Hb signals of D-arabinose oxime-5-phosphate had shifted by 0.11 ppm downfield relative to the C5-Ha and C5-Hb signals of D-arabinose oxime. The deshielding effect on C-H protons on phosphorylation of a C-OH group has been observed previously.[77-79] The C4-H signal was also shifted downfield (by 0.04 ppm), and the C5-H and C4-H signals appeared to be further split due to the $^3J_{^1H^{31}P}$ and $^4J_{^1H^{31}P}$ coupling. The magnitudes of these couplings were difficult to measure due to signal broadening. The downfield shifts observed in this case (0.11 ppm for H-5

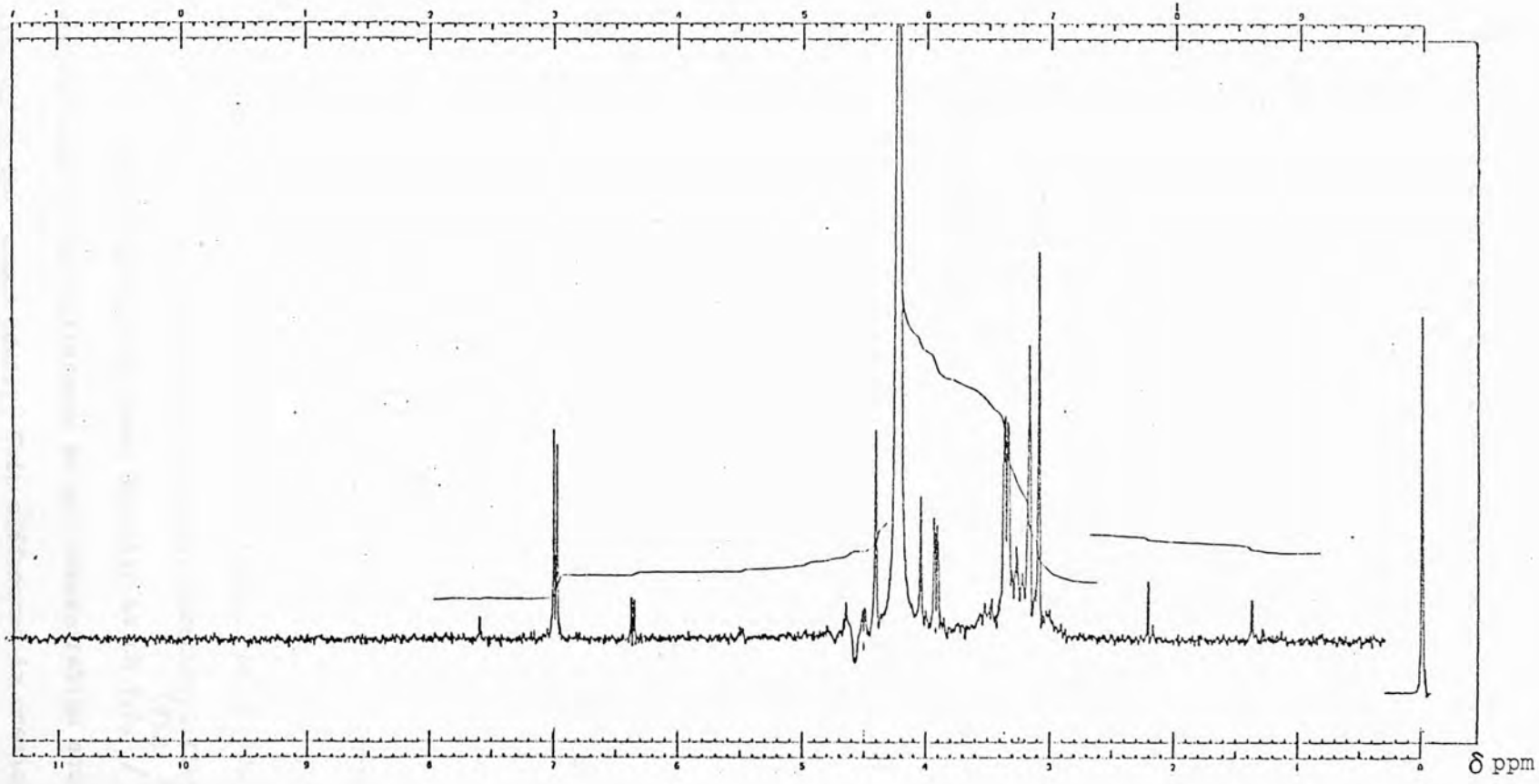


Fig. 2.18 220 MHz ^{1}H nmr spectrum of D-arabinose oxime-5-phosphate (lithium salt) in D_2O

Table 2.21 ^{1}H Chemical Shifts of D-Arabinose Oxime-5-Phosphate in D_2O (δ, ppm; External Std. TMS).

Compound	H-1	H-2	H-3	H-4	H-5a	H-5b
D-arabinose oxime (syn E-form)	6.98	3.93	3.09	3.21	3.26	3.05
D-arabinose oxime (anti Z-form)	6.36	4.50				
D-arabinose oxime 5-phosphate (syn E-form)	6.98	3.93	3.09	3.25	3.37	3.16
D-arabinose oxime 5-phosphate (anti Z-form)	6.36	4.50				

and 0.04 ppm for H-4) on phosphorylation were less than those (0.2 ppm for C5-H and 0.05 ppm for C4-H) observed when the ATP-dependent phosphorylation of D-arabinose oxime by yeast hexokinase was followed directly (section 2.2.9.1). This may be ascribed to differences in pH the solution in the former case being at pH 6.5 while in the latter it was 7.6. At pH 7.6 a greater proportion of the dianionic form will be present than at pH 6.5 however /the dianion may show a greater shielding than the monoanion.[76]

^{31}P nmr: The ^{31}P nmr protondecoupled spectrum of D-arabinose oxime-5-phosphate dilithium salt was taken at pH 6.5 and the ^{31}P signals appeared at δ_p 4.54, 3.67 and 2.40 (Table 2.22), instead of δ_p 2.48, 1.61 and 1.16 ppm in the case when the ATP dependent phosphorylation of D-arabinose oxime by hexokinase was followed directly at pH 7.6. (Fig. 2.17) /This difference is attributed to the difference in pH, concentration and the presence of lithium in the former case. This difference in chemical shift due to change in pH and presence of metal ion has been observed by other workers.[83,85]

e.g. $[O_3POOH]^{2-} + 2H^+$ δ_p -3.1

$[O_3POOPO_3]^{4-} + 4H^+$ δ_p -1.7

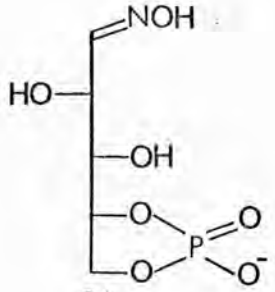
$[O_3POOPO_3]^{4-} + 4Li^+$ δ_p +7.4

$[O_3POOPO_3]^{4-} + 4K^+$ δ_p + 8.6

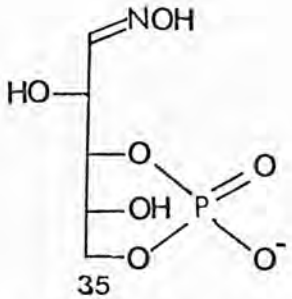
Table 2.22 ^{31}P Chemical Shifts of D-arabinose oxime-5-phosphate

^{31}P Chemical shifts δ ppm down field from 85% H_3PO_4	Integrated ratios of ^{31}P signals
4.54	81%
3.67	17.4%
2.40	1.6%

The undecoupled spectra showed the presence of broad triplets at δ_p 4.54 and δ_p 3.67 ppm. This is thought to be due to $^3J_{,H}{}^{31}P$ coupling with further small splitting due to $^4J_{,H}{}^{31}P$ coupling. The signal at δ_p 4.54 ppm was assigned to the acyclic syn(E)-form of D-arabinose oxime monophosphate dilithium salt, and the phosphate group was presumed to be attached to the C5-OH group since the undecoupled ^{31}P spectrum gave a broad triplet due to $^3J_{,H}{}^{31}P$ and $^4J_{,H}{}^{31}P$ coupling. This $^3J_{,H}{}^{31}P$ coupling has been observed by G.R. Gray[86] for D-fructose 1,6 diphosphate and other related mono- and diphosphates. He observed a 1:2:1 triplet due to $^3J_{,H}{}^{31}P$ coupling. The signal at δ_p 3.67 ppm was assigned to the acyclic anti(Z)-form of D-arabinose oxime monophosphate. Once again it was a triplet and therefore is thought to arise from a primary phosphomonoester attached at the C5 position. This signal cannot arise from a secondary monoester as then it would give a doublet or quartet in the undecoupled spectrum. Also it cannot be due to a cyclic 5-membered (34) or 6-membered (35) phosphate ester. This is because

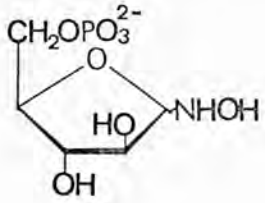


34



35

in the former case the ^{31}P resonance appears at δ_p 15.0 ppm[93,94] and in the latter case at δ_p -8.0 ppm.[93,94]. Also the signal cannot arise from a cyclic isomer (17c) of D-arabinose oxime-5-phosphate as the ^1H nmr showed the presence of syn(E) and anti(Z) forms, so also did the ^{13}C nmr (to be discussed later).



17C

Therefore, it seems that the ^{31}P signal at δ_p 3.67 ppm corresponds to the acyclic anti(Z)-form of D-arabinose oxime-5-phosphate.

The ^{31}P signal at δ_p 2.40 could possibly be due to a cyclic, furanose isomer of D-arabinose oxime-5-phosphate or an inorganic phosphate. It was difficult to assign this signal with certainty as it was present to the extent of only 1.6% and therefore would be very difficult to observe in tbe ^1H and ^{13}C nmr.

The assignment of the signals at δ_p 4.54 and δ_p 3.67 ppm to the acyclic syn(E)(81%) and anti(17.4%) forms of D-arabinose oxime-5-phosphate agrees with the ^1H nmr data, which shows the presence of a mixture of acyclic syn(E) (83%) and anti(Z)(17%) forms.

^{13}C nmr: The (PFT)^{13}C nmr of the synthetic D-arabinose oxime monophosphate dilithium salt prepared was obtained. The broad band proton decoupled spectrum, the "off resonance" spectrum and the proton

undecoupled spectrum were taken.

Appreciable ^{13}C chemical shifts have been observed[95-103] on phosphorylation of hydroxyl groups of sugars and nucleotides (see Table 2.23). Also two bond and three bond couplings between ^{13}C and ^{31}P have been observed[95-103] (Table 2.24). Dorman and Roberts[104] noted that the three bond coupling between ^{13}C and ^{31}P was greater than the two bond coupling. This has been also observed by other workers (see Table 2.24). Further, the three bond couplings between ^{13}C and ^{31}P have been shown previously,[95-98,100] by analogy with the behaviour of three bond couplings for ^{31}P OC'H and 'HCC'H to depend on the dihedral angle between the ^{31}POC and $OC^{13}C$ planes with a larger coupling being expected for trans rotamers and smaller one for gauche rotamers. This provides an estimation of the preferred rotamer.

Table 2.23 ^{13}C Shielding Differences (ppm)[a] between the Phosphorylated Derivatives and the Original Compounds

Compound	C-1	C-2	C-3	C-4	C-5	C-6	References
α-D-Mannose-6-phosphate	+0.3	+0.1	-0.2	-0.8 or -0.4	-0.3	+2.1	99
β-D-Mannose-6-phosphate	+0.3	+0.1	-0.3	0 or -0.4	-0.4	+2.1	99
β-Fructose-6-phosphate	-0.1	0	-0.3	-0.1	-0.7	+2.1	101
5'-Uridine monophosphate[b]	-0.9	-0.8	+0.5	-0.3	+2.5	-	95
3'-Uridine monophosphate[b]	-0.2	-0.2	+2.6	-0.5	0	-	95
2'-Uridine monophosphate[b]	-0.3	+1.7	+0.4	0	0	-	95
5'-Adenosine mono-phosphate[b]	-0.5	+1.2	+1.2	-0.3	+3.5	-	102

[a] = A positive difference indicates that the resonance in the phosphorylated derivative occurs at a lower field.

[b] = ^{13}C Chemical shifts of ribose part of the nucleotide given.

Table 2.24 $^{31}P - ^{13}C$ Coupling Constants of Monophosphates in Hertz

Compound	$^{2}J_{31_{P-}13_{C}}$	$^{3}J_{31_{P-}13_{C}}$	References
α-D-Mannose-6-phosphate	5.0(d)	7.0(d)	99
β-D-Mannose-6-phosphate	5.0(d)	7.0(d)	99
β-Fructose-6-phosphate	4.2(d)	7.3(d)	101
5'-Uridine monophosphate	4.5(d)	8.5(d)	95
5'-Adenosine monophosphate	3.9(d)	7.9(d)	102

Thus, from Table 2.23 it can be seen that the ^{13}C chemical shift of the ^{13}C-OH which is phosphorylated is deshielded by about + 2.0 ppm and also shows splitting due to $^{2}J_{31_P13_C}$ and $^{3}J_{31_P13_C}$ coupling.

In the case of D-arabinose oxime monophosphate, the ^{13}C nmr showed the presence of syn(E)(81.5%) and anti(Z)(18.5%) forms. The ^{13}C chemical shifts (Table 2.25) were assigned by comparison with D-arabinose oxime and as mentioned in (2.27).

Table 2.25 ^{13}C Chemical Shifts of D-Arabinose Oxime Monophosphate in D_2O (δ_c, ppm: TSP, Internal Standard)

Compound	C-1	C-2	C-3	C-4	C-5
D-Arabinose oxime (syn E-form)	155.1	71.1	74.9	73.4	65.4
D-Arabinose oxime monophosphate (syn E-form)	155.6 + 0.5[a]	71.2 +0.1[a]	74.2 −0.7[a]	72.8[b] −0.6[a]	67.7[c] +2.3[a]
D-Arabinose oxime (anti Z-form)	156.0	67.1	74.1	73.4	65.4
D-Arabinose oxime monophosphate (anti Z-form)	156.5 + 0.5[a]	67.1 0 [a]	73.6 −0.5[a]	72.8[b] −0.6[a]	67.7[c] +2.3[a]

[a] Chemical shift difference with positive value indicating downfield shifts.
[b] $^{3}J_{31_{P-}13_C}$ = 5.9 Hz (doublet) [c] $^{2}J_{31_{P-}13_C}$ = 4.4 Hz (doublet).

From the data in Table 2.25 it can be concluded that the C5-OH group has been phosphorylated in D-arabinose oxime monophosphate. The shift difference of +2.3 ppm agrees with the values obtained by other workers shown in Table 2.23. Also the values of the two bond ($^{2}J_{^{31}P-^{13}C}$) coupling of 4.4 Hz and three bond ($^{3}J_{^{31}P-^{13}C}$) coupling of 5.9 Hz are similar to those obtained by other workers (Table 2.24); this further confirms that D-arabinose oxime-5-phosphate has been formed. Moreover, the three bond coupling also confirms the C-4 resonance assignment in D-arabinose oxime.

Thus, from paper chromatography, electrophoresis, ^{1}H nmr, ^{31}P nmr and ^{13}C nmr evidence it can be concluded that D-arabinose oxime-5-phosphate has been formed and it exists predominantly in the acyclic form as a mixture of synE(83%) and anti Z(17%) forms The yield of the product was 66.3%.

2.2.10 Chemical Synthesis of D-Arabinose Oxime-5-Phosphate

A number of chemical techniques to achieve the selective phosphorylation of sugars have been proposed.[105-108] An attempt was made to chemically prepare the 5-phosphate of D-arabinose oxime by K. Schutzner's procedure[108] using a reagent composed of trimethyl phosphate and phosphoryl chloride. The product obtained analysed correctly for D-arabinose oxime monophosphate dilithium salt (see EXPERIMENTAL). Paper chromatography and electrophoresis results showed that phosphate of D-arabinose oxime has been formed. The infra-red spectrum showed thepresence of a C=N group (1680 cm^{-1}) as well as a phosphate group (1100 cm^{-1}). ^{1}H nmr gave a quartet at δ_H 7.58 ppm shifted by 0.5 ppm from the unphosphorylated syn(E) D-arabinose oxime and was assigned to a C1 proton. The splitting was due to $^{3}J_{^{1}H-^{1}H}$ and $^{4}J_{^{31}P-^{1}H}$ coupling.

Table 2.26 Physical Data of D-Arabinose Oxime Monophosphate (Chemically Synthesised).

Paper Chromatography	Electrophoresis Mx	I.R.** cm^{-1}	*^1H nmr δ_H ppm	*^{13}C nmr δ_C ppm	^{31}P nmr δ_P ppm
		3340 s(OH)			
		2940 w(CH)			
0.12	5.0×10^{-9} $m^2 s^{-1} V^{-1}$	1680 w(C=N)	7.58 (q)[a]	155.3[b]	4.96
		1660 m(C=N)		154.5[c]	4.56
		**1100 s(P=O)		154.1[d]	4.29
		**1000 s(POC)		99.2[e]	4.02

* D_2O Internal Standard TSP

** Lit.,[88] 1100 cm^{-1}, s (P=O), 990 cm^{-1}, s (POC), KBr disc

a $^4J_{^{31}P-^1H}$ = 2.95 hertz. $^3J_{^1H_1-^1H_2}$ = 5.0 hertz

b $^3J_{^{31}P-^{13}C}$ = 3.0 Hertz Acyclic anti(Z) D-arabinose oxime N-O-phosphate

c $^3J_{^{31}P-^{13}C}$ = 3.0 Hertz Acyclic syn(E) D-arabinose oxime N-O-phosphate

d Acyclic syn(E) D-arabinose oxime monophosphate

e Cyclic D-arabinose oxime monophosphate.

These results indicated that the oxime group has been phosphorylated in the reaction.

The proton decoupled (PFT) ^{13}C nmr showed the presence of four species. Signals at δ_C 155.3 and δ_C 154.5 ppm (doublets) are thought to correspond to the $^{13}C1$ signals of the acyclic syn (E) and anti (Z) forms of the N-phosphorylated D-arabinose oxime, while the signals at δ_C 154.1 and δ_C 99.2 ppm are thought to correspond to the $^{13}C1$ of acyclic syn(E) form and $^{13}C1$ of cyclic D-arabinose oxime monophosphate with the phosphate group situated on the C5 atom. Schutzner's[108] procedure is supposed to preferentially phosphorylate the primary OH group.

Finally, the proton decoupled ^{31}P nmr (Table 2.26) shows the presence of four main forms which could correspond to the acyclic syn(E) and anti(Z) forms of the D-arabinose oxime N-phosphate the acyclic D-arabinose oxime-5-phosphate and cyclic D-arabinose oxime-5-phosphate.

Thus, it appears from the above evidence that the chemical phosphorylation of D-arabinose oxime has given a mixture of acyclic N- and C-phosphates and/or a cyclic C-phosphate of D-arabinose oxime Therefore, to prepare the 5 or 6-phosphate of the pentose and hexose oximes chemically one would need to use blocking groups to protect the oxime functional group. An alternative approach might be to introduce the oxime function after phosphorylation under conditions such that dephosphorylation will not occur.

Thus, from the above evidence it seems that the ATP-dependent phosphorylation by yeast hexokinase is a convenient method for the preparation of the required primary phosphate esters.

Thus, in the case of the carbohydrate oximes infra-red and Raman spectroscopy helped in distinguishing between the cyclic and acyclic structure while ^{1}H nmr and ^{13}C nmr assisted in arriving at their structures in the solid state and the proportions of isomers present in solution at equilibrium. These results were further supported but not unequivocally confirmed by periodate oxidation, gas liquid chromatography and mass spectroscopy. Further, in the case of D-glucose oxime and D-arabinose oxime (syn(E) and anti(Z) forms) X-ray crystallographic data substantiated their structures arrived at by the above mentioned techniques used in this work, and helped in confirming the ^{1}H nmr and ^{13}C nmr signal assignments of the acyclic syn(E) and anti(Z) forms based on chemical shift correlations. The knowledge of the structures of the carbohydrate oximes in the solid state and of the different isomeric forms present in solution will be of use in biochemical work, especially in studying their interactions with yeast hexokinase (CHAPTER 4) and D-xylose isomerase (CHAPTER 5). Moreover, the isolation and characterisation of D-arabinose oxime-5-phosphate obtained by making use of the ATP-dependent phosphorylation by yeast hexokinase reveals a select ive pathway for phosphorylation of carbohydrate oximes which are substrates of yeast hexokinase, compared to the chemical synthesis which yielded a mixture of D-arabinose oxime monophosphates. The availability of D-arabinose oxime-5-phosphate will allow investigations of its possible action as a transition-state analogue inhibitor of the glycolytic enzyme glucose-6-phosphate isomerase.

2.3 EXPERIMENTAL

2.3.1 General Techniques

'H NMR: 'H nmr spectra were recorded on Varian EM 360, HA 100, HR 220 (PCMU) or Brucker HX 360 (University of Groningen, Netherlands, courtesy of Dr Kaptein) spectrometers.

^{13}C NMR: ^{13}C nmr spectra were recorded on a Brucker HX 90E (PCMU or Kings College, University of London) spectrometer operating at 22.63 MHz.

^{31}P NMR: ^{31}P nmr spectra were recorded on a Brucker HX 90E (Kings College, University of London) spectrometer operating at 36.431 MHz.

All spectra were run at ambient temperature. The reference for deuterium oxide (D_2O) solution was 3-(trimethylsilyl)-propane sulphonic acid sodium salt (TSP) for 'H and ^{13}C nmr, for other solutions tetramethylsilane (TMS) was taken as an internal or external standard while for ^{31}P nmr phosphoric acid (external standard) was used. Spectrum simulation was carried out in some cases using program UEA NMR BAS filed at the University of London Computer Centre in conjunction with a plotting routine LIBRARY RHC PLOT developed at Royal Holloway College Central Computer Services.

Infrared Spectroscopy: Infra-red spectra were recorded with Perkin-Elmer 337 and Perkin-Elmer 225 (PCMU) spectrometers. Discs were prepared using potassium bromide (1% w/w), and spectra were observed in the range 4000 cm^{-1} to 400 cm^{-1} or 4000 cm^{-1} to 200 cm^{-1}.

Raman Spectroscopy: Raman spectra were taken of powdered solids or saturated aqueous solutions using a Coderg PHO spectrometer and CR Argon Krypton mixed gas laser on the red line (6471Å) or a Cary 81 instrument on the yellow line.

Gas Liquid Chromatography: Gas liquid chromatography (g.l.c.) was carried out with a Pye 104 dual column chromatograph equipped with flame ionisation detectors. The retention times and peak areas were measured by a Hewlett Packard 3370B/71B integrator which was connected to the gas chromatograph. Nitrogen was employed as a carrier gas at a flow rate of 40 cm^3 min^{-1}. The glass columns (9' x 0.25") and (6.5' x 0.25") were packed with the following stationary phases:

(i) 3% OV-225 on chromosorb Q, 80-100 mesh, 168°.

(ii) 7.5% OV-17 on chromosorb W, 80-100 mesh, 158°.

The g.l.c. was performed on the trimethylsilyl derivatives of the carbohydrate oximes by (a) direct or (b) indirect procedures of G. Petersson.[45]

(a) To a solution or suspension of the sample (10 mg) in dry pyridine (1 cm^3), hexamethyldisilazane (HMDS, 0.35 cm^3) followed by trimethylsilyl chloride (TMSC, 0.175 cm^3) was added. The reaction was completed by shaking the mixture for at least 2 h at room temperature. The precipitate was removed by centrifugation and the supernatent liquid was used directly for subsequent g.l.c. work.

(b) To a solution or suspension of free sugar (10-12 mg) in dry pyridine (1 cm^3) was added hydroxylamine hydrochloride (6 - 8 mg). The solution was shaken for 2 h at room temperature. This resulted

in the formation of the oxime whose trimethyl silyl derivative was made as described in (a).

Mass Spectrometry: Low and high resolution mass spectra were recorded on a VG Micromass 12F or A.E.I. MS-902 (PCMU) spectrometers. A direct insertion method was used with an ionisation potential of 70 eV and a trap current of 100 uA.

Polarimetry: Optical rotations of aqueous solutions were measured on a Perkin-Elmer 141 polarimeter, operating on the sodium D line (589 nm) using a 1 cm^3 jacketed glass cell.

Paper Chromatography: Descending and/or ascending chromatography was carried out with Whatman No.1 and No.3 MM papers. The solvent systems used were:-

(A) n-butanol, ethanol, water (40:11:19 v/v)
and/or
(B) acetonitrile, 0.1M ammonium acetate pH 7.0 (60:40 v/v)[110]
and/or
(C) diisopropyl ether, formic acid (3:2, v/v).[91]

The components were detected by visualising the spots by (i) silver nitrate dip[92] or (ii) oxime spray[111] or (iii) glucose oxidase spray reagent[112] or (iv) phosphate spray.[91]

(i) Silver nitrate dip reagent[92]: The paper is dipped through three solutions in succession

(a) A saturated aqueous solution of silver nitrate (5 cm^3) in acetone (1000 cm^3) and water (20 cm^3).

(b) Ethanolic sodium hydroxide solution containing sodium hydroxide (2 g), ethanol (98 cm^3) and water (2 cm^3).

(c) An aqueous solution of sodium thiosulphate pentahydrate (10% w/w).

A dark spot indicates reducing sugars.

(ii) Oxime spray[111]: A mixture of ferric chloride ($FeCl_3.6H_2O$, 0.5 g); water (30 cm^3), formalin (10 cm^3) and methanol (10 cm^3) was prepared. A purple colour indicates the presence of an oxime.

(iii) Glucose oxidase spray reagent[112]: 'Glucostat' kit (Worthington Biochemical Company) made up as directed. A pink colour (immediately) indicates the presence of β-D-glucose.

(iv) Phosphate spray[91]: 70% Perchloric acid (9 cm^3) was taken and to this concentrated hydrochloric acid (1.7 cm^3), ammonium molybdate (2 g), ethylene diaminotetraacetate disodium salt (Na_2 EDTA, 0.2 g) were added and the solution was made up to 200 cm^3 with deionised water.

Electrophoresis: Paper electrophoresis was carried out on Whatman No.3 MM paper using a Shandon High Voltage Electrophoresis apparatus L24 for 1.5 h at 3.0 kV with 0.5 M phosphate buffer (pH 7.0) as electrolyte. The paper was developed by silver nitrate dip reagent.[92] D-Glucose and D-arabinose oxime were taken as the non-migrating markers.

Periodate Oxidation:

(A) Determination of periodate consumed[38]:

0.015 M solutions of sodium metaperiodate and sodium iodate were prepared and aliquots (1 cm^3) from each were withdrawn and diluted to 250 cm^3 with deionised water. The absorbance of the resulting solutions were measured at 222.5 nm on an SP 500 spectrophotometer using deionised water as a blank. A linear calibration curve was plotted of absorbance versus percent ion composition of the solution from the readings of these two solutions assuming that the

periodate solution contained 100% IO_4^{1-} and 0% IO_3^{1-} while the iodate solution contained 100% IO_3^{1-} and 0% IO_4^{1-} ions.

The sample under investigation (8–10 mg) was accurately weighed and dissolved in 0.015M sodium metaperiodate solution (10 cm^3). The resulting solution was incubated in the dark at room temperature. Aliquots (1 cm^3) were removed periodically, diluted to 250 cm^3 with deionised water, and the absorbance measured. This was continued till a constant reading was obtained. The calibration curve was used to deduce the number of moles of periodate consumed per mole of sample from the absorbance reading. To check the accuracy of the method 2,5-O-methylene-D-mannitol was used as a standard at the same time.

(B) Estimation of formaldehyde[39]: Formaldehyde estimation was carried out on the periodate-oxidised solution spectrophotometrically by its colour reaction with chromotropic acid reagent (chromotropic acid sodium salt, 1 g; water, 100 cm^3 and the solution was made up to 500 cm^3 with 12.5 M sulphuric acid; the absorbance of chromotropic acid should not exceed 0.1 at 570 nm). After constant reading was obtained (5 h) for the periodate-oxidised solution of the samples and standard, aliquots (1 cm^3) were withdrawn and diluted to 10 cm^3 with deionised water. The standard solution had aliquots (1 cm^3) diluted to 20 cm^3 and 50 cm^3 also. To a portion (1 cm^3) from each of the diluted solutions was added aqueous sodium sulphite solution (20% w/v, 0.1 cm^3) and chromotropic acid reagent (8.4 cm^3). A blank of deionised water (1 cm^3) was similarly treated. The samples were heated on a boiling water bath for 1 h (those samples that contained formaldehyde developed a violet colouration). After cooling, aqueous thiourea solution (0.4% w/v, 0.5 cm^3) was added to each sample. The absorbance was read at 570 nm against the blank

solution as reference. A linear calibration graph was plotted from the three absorbances obtained for the standard 2,5-O-methyl-D-mannitol against the calculated formaldehyde concentrations. The formaldehyde liberated by the test samples were deduced from this graph.

(C) Estimation of formic acid[40]: Two drops ethylene glycol was added (to destroy any excess periodate) to aliquots (5 cm^3) of each of the periodate-oxidised solutions after constant reading was achieved, and this was titrated against 0.01M sodium hydroxide solution, using methyl red as indicator. A sample from the bulk sodium periodate solution (0.015M) was similarly treated. The difference in the titre readings was taken to obtain the formic acid liberated by the periodate oxidation of the test samples.

Melting Points

The melting points were recorded on a Gallenkamp or Thomas-Hoover apparatus (uncorrected).

Elemental Analysis

Micro elemental analysis were performed by the School of Pharmacy (University of London), Butterworths Microanalytical Consultancy (Teddington) and Bernhardts(Germany).

2.3.2 D-Glucose oxime:

Powdered hydroxylamine hydrochloride (70 g) in dry methanol (300 cm^3) was neutralised with a solution of sodium methoxide (20 g sodium in 100 cm^3 methanol). The neutralised solution was cooled, filtered and washed with dry methanol (200 cm^3). The combined filtrate and washings were shown to contain 0.64 mol of hydroxylamine

(64% of the original) by reaction with an excess of 0.016M potassium bromate followed by reduction of the excess bromate with hydrogen iodide and titration of the liberated iodine with 0.1M sodium thiosulphate (the concentration of hydroxylamine can also be determined by F.W. Fitzpatrick and J.D. Gettler[26] and H. Omura et al.[113] procedures). The methanolic hydroxyl amine solution was refluxed in a water-bath and powdered anhydrous D-glucose (110 g, 0.61 mol) was added slowly. The solution was concentrated, left at room temperature overnight and dried in a dessicator. The dry solid was powdered, dissolved in 80% aqueous methanol (v/v) and allowed to crystallise in a freezer. Recrystallisation was carried out from 70% methanol , followed by 60% methanol and gave truncated prisms (53 g, 45%), m.p. 141° (Lit.,[3] 137.5°, Lit.,[2] 136-137°; Lit.,[6] 141°) (Found: C, 36.7; H, 6.8; N, 7.2. Calc. for $C_6H_{13}NO_6$: C, 36.9; H, 6.7; N, 7.2%), $[\alpha]_D^{32.5°}$ initial -8.04°, final -1.92° (c 2.6 in H_2O) [Lit.,[2] $[\alpha]_D$ final -2.2° (c 9.4 in H_2O)] (the mutarotation was observed to be complex, i.e. initially the rotation decreased but after 0.5 h, it increased until it reached a constant value after about 10 h); paper chromatography, D-glucose oxime was spotted on Whatman 1 paper and developed using solvent A. After development the papers were sprayed separately with the oxime spray[111] (violet colouration), and glucose oxidase spray[112] (no immediate pink colouration) and treated with silver nitrate dip reagent[92] using D-glucose as standard (dark spot), R_G of D-glucose oxime = 1.13; i.r. ν_{max} (KBr) 980 cm^{-1} (s, N-O[114]) and 891 cm^{-1} (sharp and m, ring breathing[36]); Raman ν_{max} (saturated aqueous solution) 1648 cm^{-1} and 1653 cm^{-1} (C = N) not given by powdered crystals or water;

periodate oxidation-D-glucose oxime consumed[38] 2.4, 3.2, 4.2 and 4.2 mol. equiv. of periodate after 0.5, 1.25, 4 and 5 h respectively, and liberated no formaldehyde,[39] and 1.95 mol equiv. of formic acid[40] after 4 h; g.l.c. of trimethylsilyl derivative, two peaks having retention times of 1.49 (major) and 1.37 (minor) relative to the trimethylsilyl derivative of α-D-glucose; mass spectra, m/e 196 ($C_6H_{14}NO_6$, M + 1), for ^1H nmr and ^{13}C nmr data see Tables 2.5, 2.6, 2.14 .

2.3.3 D-Arabinose oxime:

D-Arabinose oxime was prepared essentially according to the method of Hockett,[10,11] except 28 g of hydroxylamine hydrochloride was used in 90% ethanol (aqueous, v/v). The pH of the hydroxylamine solution was adjusted to 6.5 with sodium ethoxide solution (10 g sodium in 100 cm^3 dry ethanol). The solution was cooled and filtered. The filtrate and washings (100 cm^3 ethanol) were collected (the concentration of hydroxylamine was found to be 66% of the original as described earlier2.3.2) and refluxed on a water-bath at 60° - 70° and D-arabinose (36 g, 0.24 mol.) was added slowly with stirring (about 3 - 4 h). The crude product was concentrated under vacuum, dried by adding ether (sodium-dried) and distilled under vacuum. The dried solid was powdered and crystallised as plates from 60% ethanol (aqueous, v/v); yield 24 g, 60%; m.p. 140° (Lit.,[9] 138-139°; Lit.,[10] 136 - 137°) (Found: C, 36.7; H, 6.5; N, 8.2. Calc. for $C_5H_{11}NO_5$: C, 36.4; H, 6.7; N, 8.5%); $[\alpha]_D^{25}$ initial -83.6°, final -14.2° (c 1.3 in H_2O) [Lit.,[10] $[\alpha]_D^{20}$ initial -84.0°, final -13.5° (c 2.05 in H_2O)] (simple mutarotation); paper chromatography, n-butanol:ethanol:water, R_G = 1.55 (gave violet colouration with oxime spray[111] and dark spot with silver nitrate dip

reagent[92]); i.r., ν_{max} (KBr) 1680 cm^{-1} (w, C=N); Raman, ν_{max} (solid) 1678 cm^{-1} (m, C=N), (saturated aqueous solution) 1656 cm^{-1} and 1664 cm^{-1} (w, C=N); periodate oxidation - it consumed[38] 4.6, 4.7 and 4.7 mol. equiv. of periodate after 0.5, 2.0,4.2 h respectively, and liberated 0.8 mol. equiv. of formaldehyde[39] and 2.9 mol. equiv. of formic acid[40] after 4 h; g.l.c. of trimethylsilyl derivative, one peak retention time 1.72 relative to the trimethylsilyl derivative of α-D-arabinose; mass spectra, m/e 166 ($C_5H_{12}NO_5$, m+1), 134 ($C_4H_8NO_4$), 104, 103 ($C_4H_7O_3$), 91, 75 ($C_2H_5NO_2$), 74, 61 and 31; ^1H nmr and ^{13}C nmr data are given in Tables 2.5, 2.6, 2.10

2.3.4 D-Ribose oxime

Sodium ethoxide solution (3.5 g sodium in 50 cm^3 ethanol) was added to powdered hydroxylamine hydrochloride (11 g) in ethanol (50 cm^3) till the pH was 6.5. The solution was cooled, filtered and washed with ethanol (50 cm^3). The washings and filtrate were collected (the concentration of hydroxylamine was found to be 66% of the original as discussed earlier 2.3.2) and heated on a water bath (under reflux) at 60°. D-Ribose (15 g, 0.1 mol.) was added slowly with stirring (about 1.5 to 2.0 h). The reaction mixture was left standing at room temperature overnight. The solid obtained was recrystallised with 60% ethanol (aqueous, v/v) yield 10 g,60%; m.p. 138° (Lit.,[12] 141°; Lit.,[13] 140°). (Found C, 36.3; H, 6.8; N, 8.5. Calc. for $C_5H_{11}NO_5$: C, 36.4, H, 6.7; N, 8.5%); $[\alpha]_D^{25°}$ initial +70.1, final +8.0 (c 2.5 in H_2O) [Lit.,[20] $[\alpha]_D^{20°}$ 54° (after 10 min.) final 6.3° (the mutarotation was observed to be simple); paper chromatography, butanol:ethanol:water, R_G = 1.49 (gave a violet

colouration with the oxime spray[111] and a dark spot with silver nitrate dip reagent[92]); i.r. ν_{max} (KBr) 1694 cm^{-1} (C=N); Raman, ν_{max} (solid) 1691 cm^{-1} (C=N); mass spectra m/e 166 ($C_5H_{12}NO_5$, m+1); for ^1H nmr and ^{13}C nmr data see Tables 2.5, 2.6, 2.11

2.3.5 D-Mannose oxime:

Wolfrom and Thompson's method[5] was used in a modified form to prepare D-mannose oxime. Powdered hydroxylamine hydrochloride (6.5 g) in water (25 cm^3) was neutralised with saturated barium hydroxide solution, cooled and filtered. To the filtrate (concentration of hydroxylamine was 64% of the original) D-mannose (10 g, 0.056 mol) was added slowly with stirring at R.T. (about 3 h). The resulting solution was heated for 1 h at 70° and allowed to stand at room temperature overnight. Crystals of D-mannose oxime were obtained (yield 7.3 g, 67.4%); m.p. 176.5° [Lit.,[5] 175 - 177°). (Found, C, 36.8; H, 6.8; N,7.2. Calc. for $C_6H_{13}NO_6$: C, 36.9; H, 6.7; N, 7.2%); $[\alpha]_D^{25}$ initial +8.04, final +0.80 (c 2.5 in H_2O [Lit.,[2] $[\alpha]_D^{20}$ +3.2° (c 4.8 in H_2O) the mutarotation was observed to be simple); paper chromatography, butanol:ethanol:water R_G = 1.063 (violet colouration with oxime spray[111] and a dark spot with silver nitrate dip reagent[92]). i.r. ν_{max} (KBr) 1672 cm^{-1} (w, C=N); Raman ν_{max} (solid) 1671 cm^{-1} (m, C=N); mass spectra m/e 196 ($C_6H_{14}NO_6$, m+1); ^1H nmr and ^{13}C nmr data are given in Tables 2,5, 2.6, 2.12.

2.3.6 D-Galactose oxime

Powdered hydroxylamine (42 g) in ethanol (150 cm^3) was interacted with sodium ethoxide (15 g sodium/150 cm^3 ethanol) and the pH adjusted to 6.5. The solution (concentration of hydroxylamine was 66% of the original) was cooled, filtered and washed with cold ethanol (150 cm^3). The filtrate and washings were collected and

heated at 70° on a water bath (under reflux). D-Galactose (64 g, 0.36 mol) was added slowly with stirring (water (150 cm^3) was added to completely dissolve D-galactose) for about 3 - 4 h. The solution was allowed to stand overnight at room temperature. D-Galactose oxime was recrystallised with 50% and 40% ethanol (aqueous, v/v) (yield 35 g, 51%), m.p. 176° (Lit.,[1] 175 - 176°). (Found; C, 36.9; H, 6.5; N, 7.3. Calc. for $C_6H_{13}NO_6$: C, 36.9; H, 6.7; N, 7.2%); $[\alpha]_D^{25°}$ initial +84.0 , final + 13.8 (c 1.2 in H_2O)[Lit.,[2] $[\alpha]_D$ 14.8° (c 5.1 in H_2O); paper chromatography, butanol:ethanol:water, R_G 1.11 (gave violet colouration with oxime spray,[111] and a dark spot with silver nitrate dip reagent[92]); ir ν_{max} (KBr) 1678 cm^{-1}vw (C=N); Raman ν_{max} 1680 cm^{-1}m (C=N); mass spectra, m/e 196 ($C_6H_{14}NO_6$, m+1); ^{1}H nmr and ^{13}C nmr data are given in Tables 2.5, 2.6, 2.13.

2.3.7 D-Arabinose oxime-5-phosphate (Enzymic method)

The conditions used in preparing D-arabinose oxime-5-phosphate were similar as those used by T.A.W. Koerner, Jr. *et al.*[88] (for making other sugar phosphates) except that the reaction was scaled up. Adenosine 5'-triphosphate disodium salt (ATP, 4.175 g) and magnesium chloride ($MgCl_2.6H_2O$, 1.42 g) were dissolved in tris (hydroxymethyl)aminomethane buffer (0.3M, pH 7.5 , 30 cm^3) and the pH was adjusted to 7.5 with 0.2M sodium hydroxide. D-Arabinose oxime (775 mg) was added under slow magnetic stirring followed by yeast hexokinase (Type F300, 7200 I.U.). The pH of the solution was adjusted to 7.5 by adding 0.2M sodium hydroxide. The reaction was followed by paper chromatography (using diisopropyl ether: formic acid and phosphate spray[91]) and electrophoresis (using silver nitrate dip reagent[92]). The reaction was observed to be complete after 24 h

as seen from the absence of any D-arabinose oxime spot on the electrophoretogram. The resulting solution was concentrated at 40° under reduced pressure to a volume of 5 cm^3. The concentrated solution was filtered and passed down a Dowex 1 X 8 ($HCOO^-$) column (100 x 4 cm) and eluted with a formate buffer (2.5224 g ammonium formate (0.02M) + 15.1 cm^3 formic acid (0.2M) made up to 2000 cm^3 with deionised water, pH 2.7). The eluted fractions (14 cm^3) were analysed by means of the orcinol reaction (1 cm^3 sample plus 3 cm^3 70% sulphuric acid plus 1 cm^3 0.5% orcinol, the mixture heated at 100° for 15 min and the absorbance observed at 420 nm). Fractions 86 - 156 were pooled and concentrated to 5 - 10 cm^3 under reduced pressure at 40° and paper chromatography and electrophoresis were carried out (as described in 5.2.2.1). The residue after concentration gave a single spot (R_F = 0.12, $M(PO_4^{3-}) = 5.0 \times 10^{-9}\ m^2 s^{-1} V^{-1}$) and was converted to its lithium salt by neutralising with lithium carbonate (saturated solution) The solution was filtered and to the filtrate (5 - 10 cm^3) five times the volume of ethanol (40 - 50 cm^3) was added. The solution was allowed to stand overnight in the freezer and the precipitate obtained was collected (yield, 800 mg, 66.3%). (Found; C, 18.79; H, 3.85; N, 0.47; P, 14.68 . $C_5H_{10}NPLi_2$ requires C, 23.37; H, 3.92; N, 5.45; P, 12.05%) ^1H nmr showed no C1H signals at δ 7.34 and 6.67 ppm indicating absence of oxime group. This showed that under the condition of elution, acid hydrolysis of the oxime group had occurred. To overcome this problem of hydrolysis an ATP-regenerating system was used which helped in avoiding the usage of a column for separation.

Preparation of D-arabinose oxime-5-phosphate using an ATP-regenerating system:

The method of Avigad and Englard[90] was used but modified in two respects. Firstly the pH of the reaction mixture was not allowed to fall below pH 4.0 and secondly the product was isolated as the Lithium salt. Tris(hydroxymethyl)aminomethane (5 mmoles) in deionised water (80 cm^3) was taken and the pH was adjusted to 7.0 with 1M hydrochloric acid. To this solution magnesium chloride (0.2 mmoles) and adenosine-5'-triphosphate disodium salt (0.15 mmoles) were added and the pH adjusted to 7.0 with 1M sodium hydroxide. Then phosphoenol pyruvic acid monocyclohexylamine salt (3.98 mmoles), potassium chloride (4 mmoles), and pyruvate kinase (from rabbit muscle, EC 2.7. 1.40; activity approximately 300 uM min^{-1} mg^{-1} protein, 250 I.U.) was added. The pH was readjusted to 7.0 with 1M sodium hydroxide. The solution was slowly stirred, D-arabinose oxime (4.82 mmoles) followed by yeast hexokinase (Type F300, 2400 I.U.) was added. The reaction was followed by withdrawing a small quantity of the reaction mixture and carrying out paper chromatography and electrophoretic studies on the sample. The pH of the reaction mixture was maintained at 7.0. The reaction was complete after 24 h (absence of any spot for D-arabinose oxime on the electrophoretogram). The solution was filtered and the pH adjusted to 4.0 with 0.1M and 1.0M hydrochloric acid. The pH 4.0 solution was freeze dried and dissolved in deionised water (20 cm^3). The pH was found to remain at 4.0. The solution was shaken with ether (5 x 100 cm^3). The aqueous layer obtained after discarding the ether extract was separated and lithium carbonate (4.15 mmoles) was added. To this cold ethanol (60 cm^3) was added and left in a freezer overnight. The precipitate obtained was

collected by centrifuging at -5° on a Beckman J-21 centrifuge at 5000 rpm using JA 20 rotor . The D-arabinose oxime-5-phosphate lithium salt was placed in a desiccator at 0 to 5° to dry. Paper chromatography (silver nitrate[92] and molybdate spray[91]) and electrophoresis (silver nitrate dip reagent[92]) showed a single spot (yield 800 mg, 66.3%). (Found: C, 20.4; H, 4.9; N, 4.4. $C_5H_{10}NO_8PLi_2.2H_2O$.Requires:C, 20.5; H, 4.8; N, 4.7; P, 10.54%). The phosphorus analysis results showed that it was present but there was inconsistency in the same sample sent to BMAC (P, 8.5%) and School of Pharmacy (P, 15.7%); ^1H nmr, ^{13}C nmr and ^{31}P nmr data are given in Tables 2.21, 2.25, 2.22.

2.3.8 D-Arabinose oxime-monophosphate (Chemical synthesis)

Schutzner's[108] procedure was adopted to synthesise D-arabinose oxime-5-phosphate. D-Arabinose oxime (4.125 g) was added to magnetically stirred trimethyl phosphate (40 cm^3) maintained at -15° (acetone-dry ice). Then phosphorus oxychloride (7 cm^3) was added slowly and the reaction mixture stirred at -3° to -6° for 2 h for complete dissolution of the D-arabinose oxime. The excess phosphorus oxychloride was decomposed by adding deionised water (20 cm^3) at -10° to -25°. The resultant syrup was poured into ice (50 g). After the ice had melted, trimethyl phosphate was extracted with chloroform (10 x 15 cm^3). The aqueous layer was neutralised with lithium carbonate (18.5 g) and filtered. To the filtrate cold ethanol (250 cm^3) was added to precipitate D-arabinose oxime-5-phosphate lithium salt overnight. The precipitate was filtered and washed or dissolved in methanol (20 cm^3) and re-precipitated with 80% ethanol (aqueous v/v, 100 cm^3). The precipitate was isolated by

centrifuging at -5^{o}, 5000 rpm (yield 4.5 g, 70%); Found: C, 23.1; H, 3.7; N, 5.7; P, 11.9. $C_5H_{10}O_8NPLi_2$ requires: C, 23.4; H, 3.9; N, 5.4; P, 12.1%); i.r. ^{1}H nmr, ^{13}C nmr and ^{31}P nmr data are given in Table 2.26 .

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CHAPTER 3 ISOMERISATION OF CARBOHYDRATE OXIMES

3.1 INTRODUCTION

The carbohydrate oximes synthesised in this work have been shown (see CHAPTER 2) to isomerise in solution to equilibrium mixtures of acyclic syn(E) and anti(Z) forms (oximes of D-arabinose, D-galactose, D-mannose and D-ribose) together with cyclic pyranose forms (oxime of D-glucose).

In the past, the mutarotation of carbohydrate oximes in water solution has been observed, and the optical rotation values on dissolution, after certain time intervals, and at equilibrium were noted.[1-3] Hockett and Maynard[2] observed that the change in optical rotation for solutions of D-arabinose oxime obeyed first order kinetics and obtained a value of $k_1 + k_{-1}$ (sum of rate constants for forward and back reactions) of $0.167 \times 10^{-4} s^{-1}$ at 20°. The isomerisation of acyclic carbohydrate oximes can formally be classed as an example of restricted rotation about a C=N bond.

The phenomenon of restricted rotation due to the presence of double bonds (C=N, C=C, C=O, etc.) was discovered a long time ago, it is only in the last few years that the development of modern physical methods has enabled the investigation of the mechanism of this type of isomerisation.[4] The isomerisation rates of double bond isomers can vary widely depending on the nature of the substituents, the type of double bond and in some cases the type of medium used.[4]

Le Fevre and Northcott[5] studied the rates of isomerisation of piperonaldoxime (Scheme 3.1) in several media at two different temperatures. They proposed a rotation mechanism and suggested that the action of polar solvents (on the oxime and various azo-derivatives) in slowing the

reaction and raising its energy of activation could be ascribed to a "diminution of resonance in the solute" leading to a destabilisation of the transition state.

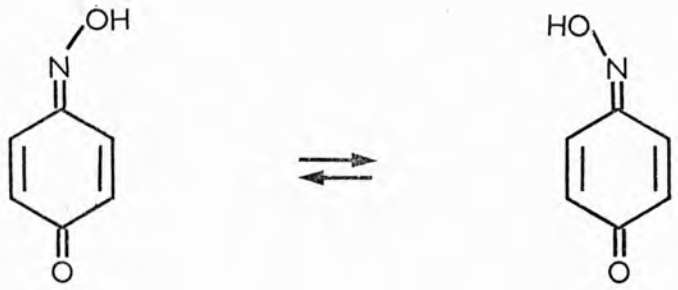
anti(Z) syn(E)

Scheme 3.1

According to I.L. Finar[6] the mechanism of interconversion of oximes involves a 'lateral shift' i.e. an inversion mechanism. Havinga and Schors[7] and Norris and Sternhell[8,9] observed the isomerisation of the p-nitrosophenol-p-benzoquinone monoxime system and suggested that the isomerisation proceeds via a rotation mechanism. They[7-9] proposed mechanisms of isomerisation in dry solvent and in aqueous medium (Scheme 3.2). Also, they observed that aqueous acids catalysed the isomerisation while non-aqueous trifluoroacetic acid inhibited it. This latter effect was ascribed to protonation of the nitroso or phenoxy anions.

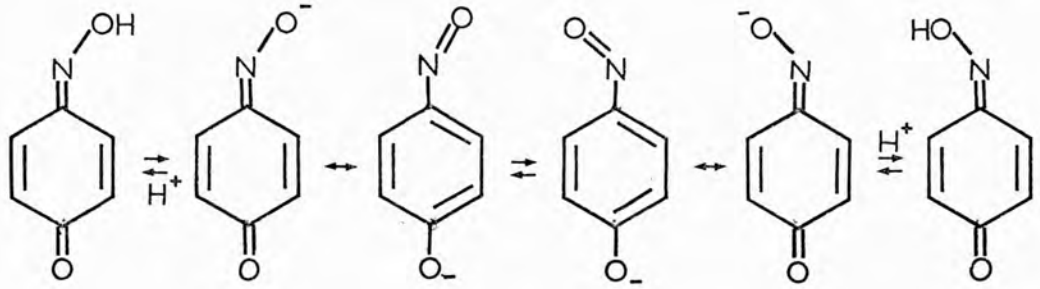
Pejkovic-Tadic et al.[10] observed that all liquid isomeric aldoximes are very unstable, being converted into the equilibrium mixture in 20-60 min. They found great difficulty in separation of liquid acetaldoxime isomer on account of its tendency to interconversion.

The distinction between inversion and rotation depends upon the different pathways taken by the substituent on nitrogen during the isomerisation. For the case where the substituents attached to the imino carbon are identical, the symmetry of the transition state (TS) is

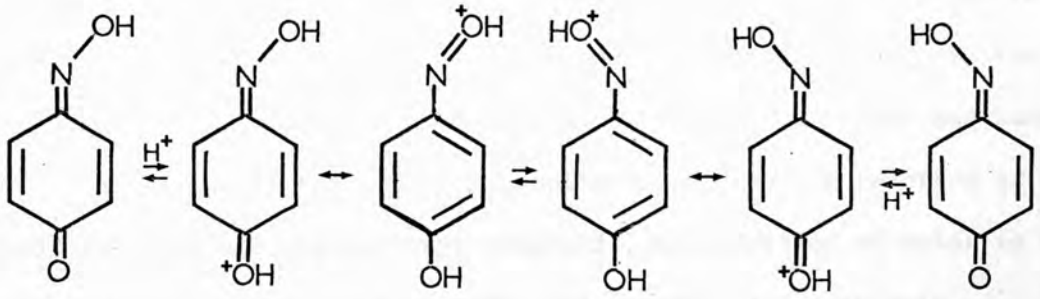


p-Benzoquinone Monoxime (p-Nitrosophenol)

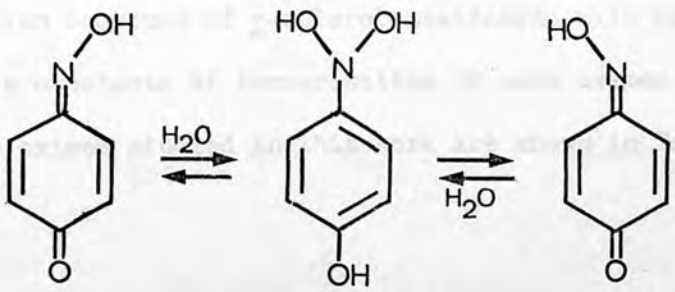
1. In Dry Solvent:



2. In Aqueous Medium:

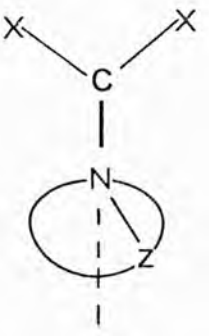


OR



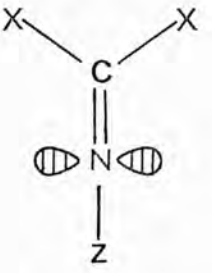
Scheme 3.2

C_{2v} (linear arrangement of CNZ (36)) for inversion, but C_S (the CNZ plane is perpendicular to the XCN plane (37)) for rotation. Huckel



TS
rotation

37



TS
inversion

36

molecular orbital calculations for the simple methyleneimine show a lower energy for C_{2v} than for C_S symmetry.[4] This is in agreement with semiempirical CNDO/2 calculations which give barriers of 31.1 kcal/mol (C_{2v}) and 61.1 kcal/mol (C_S).[11]

An alternative to inversion or rotation in aqueous solution is reversible hydration. W.P. Jencks and co-workers[12-14] studied the reversible hydration of oximes under the influence of acids and bases. The dehydration of the addition complex (formed by the reaction of hydroxylamine and the carbonyl compound), and addition of water to the oxime are subject to both specific and general acid catalysis. Further, Anderson and Jencks[15] observed a base catalysed dehydration of the addition compound of p-chlorobenzaldehyde with hydroxylamine.

The rate constants of isomerisation of some oximes and of the carbohydrate oximes studied in this work are shown in Table 3.1.

Table 3.1 First Order Isomerisation Rate Constants of Oximes

Compound	First Order Rate Constants $k_{obsd.}$ s^{-1} x 10^4 at 25°.[a,b,e]	Reference
Piperonaldoxime	0.16[a], 0.63[b]	5
Arabinose oxime	0.17[c]	2
p-Tolylphenyl ketoxime	0.31[d]	16
Phenyl-2-pyridylketoxime	46.67[f], 3.40[g], 1.72[h]	17
D-Arabinose oxime	0.57[e]	This work
D-Mannose oxime	0.48[e]	"
D-Galactose oxime	1.20[e]	"
D-Ribose oxime	0.83[e]	"

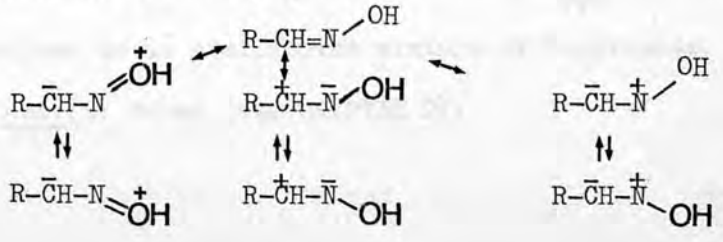
[a] = In cyclohexanone, [b] = In cyclohexane, [c] = In water at 20°,

[d] = In methanol at 144°, [e] = In water, [f] = In the molten state at 175°,

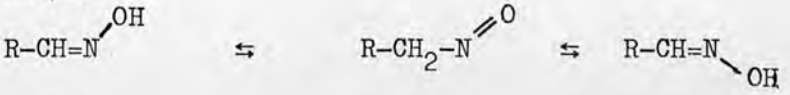
[g] = Syn(E)-form in cyclohexanol at 144°, [h] = Anti(Z)-form in cyclohexanol at 144°

From the above evidence the following possible mechanisms of oxime isomerisation can be proposed:

Rotation:



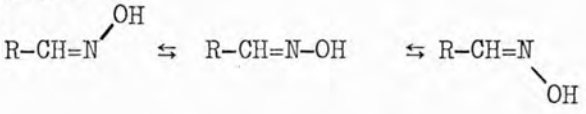
and/or



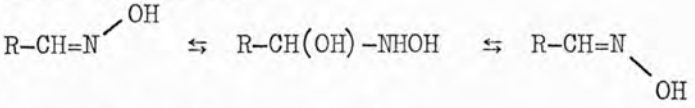
(Primary and secondary nitroso compounds are unstable and rearrange to oximes).

Possibly <u>via</u> $R\bar{C}H-\overset{\overset{O}{\|}}{N}$ (Base catalysis)

Inversion:



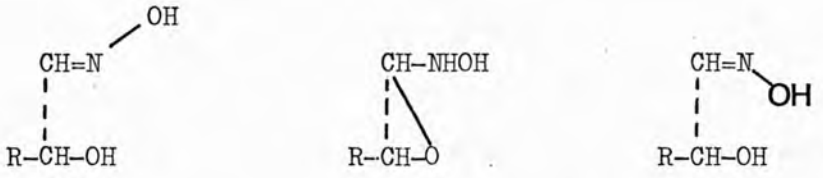
Reversible Hydration:



(Acid and base catalysed)

Reversible Cyclisation:

In the case of the carbohydrate oximes an additional mechanism analogous to reversible hydration can be proposed <u>viz</u>.



<u>syn</u>(E) cyclic (C) <u>anti</u>(Z)

In support of this proposal it has been shown that <u>D</u>-glucose oxime, which exists in the β-pyranose form ($\beta-C_{pyr}$) in the solid state isomerises to an equilibrium mixture of β-pyranose, α-pyranose, <u>syn</u>(E) and <u>anti</u>(Z) forms (see CHAPTER 2).

In order to gain further information about the isomerisation of carbohydrate oximes, the mutarotation of a number of examples have been measured in aqueous solutions, and the effects of added acids and bases investigated.

3.2 RESULTS AND DISCUSSION

3.2.1 Mutarotation of Carbohydrate Oximes in Water

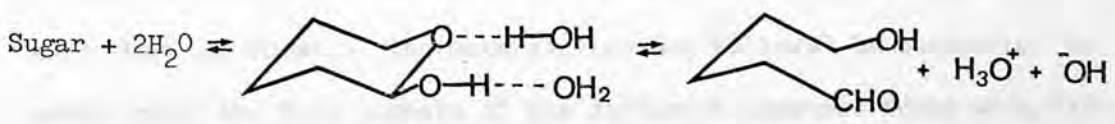
The mutarotation of carbohydrate oximes in water was followed polarimetrically. It was observed that the mutarotation was complex in the case of D-glucose oxime, and simple (first order) with all other oximes (i.e. D-arabinose, D-mannose, D-galactose and D-ribose oximes). The rate constants for these latter oximes were measured in water and found to be of similar magnitude to those observed for some other oximes (see Table 3.1).

3.2.2 Mutarotation of Carbohydrate Oximes in Deuterium Oxide

The mutarotation of D-arabinose oxime and D-glucose oxime was followed in deuterium oxide by polarimetry and ^{1}H nmr. It was observed by polarimetry that the mutarotation rate constant for D-arabinose oxime was the same as that in water (i.e. $0.57 \times 10^{-4} s^{-1}$). In the case of D-glucose oxime the mutarotation was observed to be complex and slower in deuterium oxide (equilibrium $\simeq$ 36 h) compared to that in water (equilibrium $\simeq$ 10 h). Differences in the rate of of mutarotation of sugars in water and in deuterium oxide have been studied by various workers.[18-21] The difference in rates arises from a combination of kinetic and solvent isotope-effects, expressed as k_H/k_D, and called the isotope effect. Kinetic isotope-effects are

caused by differences in the energy required for alteration of the normal and isotopic bonds in the corresponding transition states; solvent isotope-effects can exist when the isotopic compound is used both as a reactant and as a solvent. The observed isotope-effect, k_H/k_D may have values smaller than, equal to, or greater than unity. For elucidation of the course of mutarotation reactions, the timing of the addition and elimination of the proton is important. If the proton transfer occurs after the rate-controlling step, it will have no primary kinetic consequence. If the proton transfer occurs during the rate-controlling step, a large kinetic-isotope effect will be found. If proton addition occurs before the rate-determining step, and no proton transfer occurs in the transition state, the reaction is subject to hydronium ion catalysis, and an inverse isotope-effect (arising from the difference in the basicity of water and deuterium oxide) will be found.

E. Pacsu[18,19] found that the rate of mutarotation of α-D-glucopyranose was higher in water than in deuterium oxide. Nicolle and Weisbuch[20] determined the rate of mutarotation of a large number of sugars and found that the value of k_H/k_D lies in the range 3.0 to 3.8; however, the values were not related to pH or to the type of reaction. The high value of k_H/k_D[21] may arise from a substantial contribution to the overall mutarotation of the concerted reaction mechanism proposed by Lowry (Scheme 3.3). In the transition state for

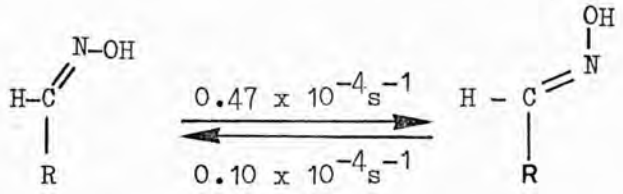


Scheme 3.3

the concerted reaction, an acid catalyst releases a proton to the ring oxygen atom and a base catalyst removes a proton from the anomeric hydroxyl group. The isotope effect is high because two proton transfers occur in the transition state. A possible variation of the concerted mechanism includes a dimeric water molecule as a bifunctional catalyst.

D-Arabinose oxime showed no isotope effect with water and deuterium oxide, indicates either that the opposing solvent and kinetic isotope-effects cancel each other or there is no proton transfer involved in the rate-determining step.

The mutarotation of D-arabinose oxime and of D-glucose oxime were followed by ^{1}H nmr in deuterium oxide. In the case of D-arabinose oxime the ^{1}H nmr data was consistent with the presence of two interconverting isomeric forms (Scheme 3.4). The rate constants of isomerisation for the forward and reverse reactions were



anti(Z) 20% syn(E) 80%

Scheme 3.4

determined from the isomerisation rate constant and the equilibrium constant In the case of D-glucose oxime the ^{1}H nmr results showed the presence of more than two forms. The mutarotation was followed by observing the areas under the Cl-H signals of the different isomeric forms with time

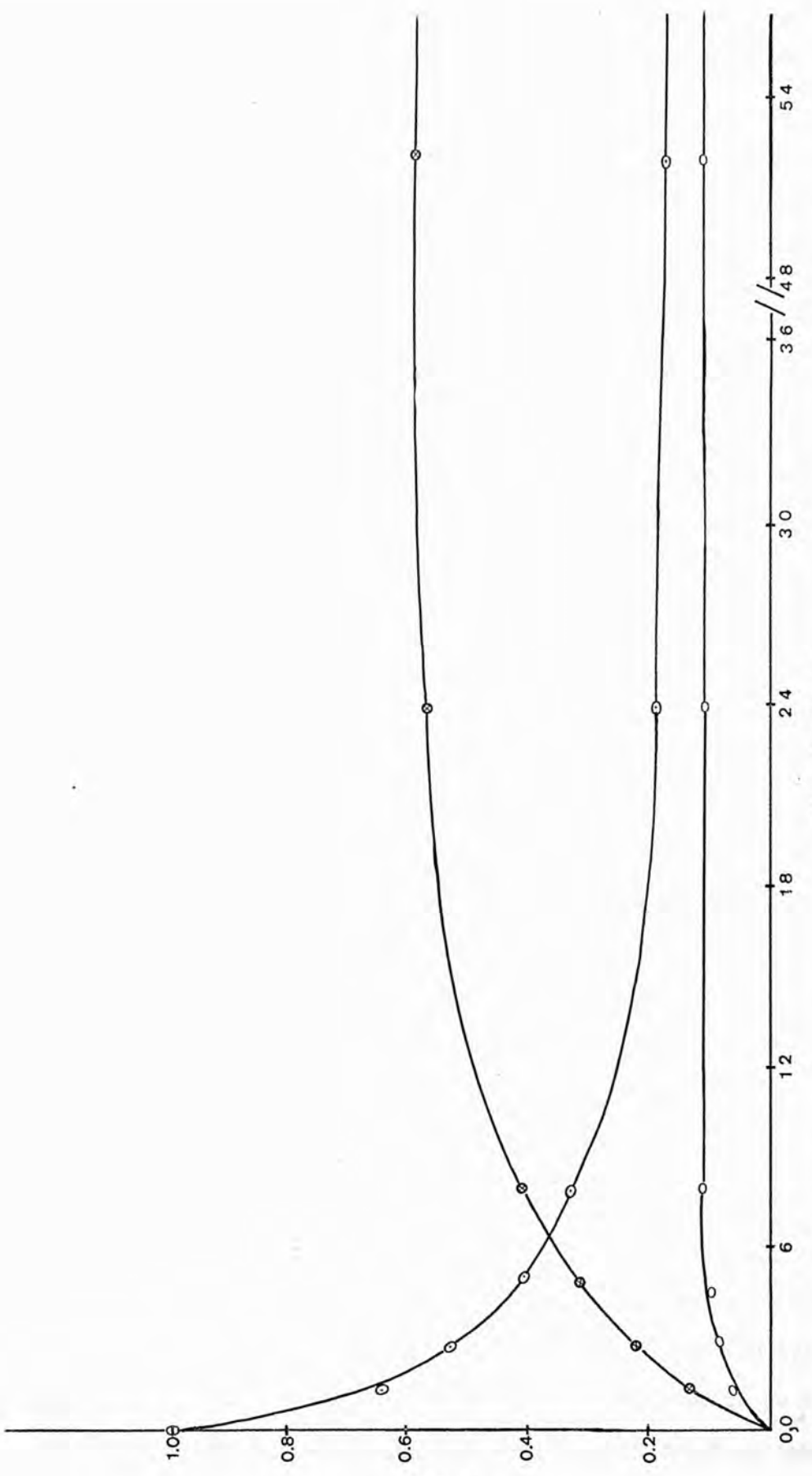
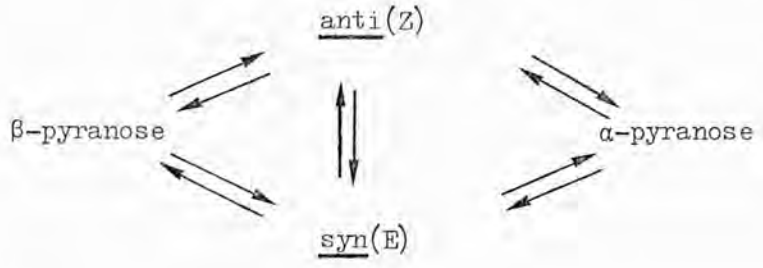


Fig. 3.1 Mutarotation of D-glucose oxime by 220 MHz ^{1}H nmr in D_2O at 23°; O anti(Z)-form, Ø syn(E)-form, ⊙ β-pyranose-form.

(Fig. 3.1). The equilibrium mixture consisted of α-pyranose (7%), β-pyranose (23%), syn(E, 56.5%) and anti(Z, 13.5%) forms. These observations are suggested to be consistent with Scheme 3.5.



Scheme 3.5

The polarimetric readings in water and deuterium oxide of D-glucose oxime isomerisation showed a fast rate followed by a slower rate of mutarotation. ^{1}H nmr data shows (Fig. 3.1) that the anti(Z)-form reaches an equilibrium concentration more rapidly than does the syn(E)-form. This suggests that the β-pyranose → anti(Z) → α-pyranose pathway is the faster process while the β-pyranose → syn(E) → α-pyranose is the slower process. The proportion of the α-pyranose-form was calculated by difference in intensity of the C1-H signals of the β, syn(E) and anti(Z) isomeric forms at equilibrium from that of the β-form initially.

3.2.3 Acid-Base Catalysis of D-Arabinose Oxime Isomerisation

D-Arabinose oxime was taken to study the behaviour of carbohydrate oximes under acidic and basic conditions (pH 4.0 to 10.0). This was done firstly because it is believed to be a potential transition state analogue inhibitor of D-xylose isomerase and secondly as it showed simple (first order) mutarotation.

Table 3.2 First Order Isomerisation Rate Constants of D-Arabinose Oxime under Acidic and Basic Conditions at 25°, I = 0.2

Buffer	Total Buffer concn.[M]	pH	$k_{obsd.}s^{-1} \times 10^2$ [b]
Acetate	0.423	4.6	0.570
	0.212	"	0.398
	0.106	"	0.283
Acetate	0.289	5.0	0.221
	0.145	"	0.150
	0.073	"	0.129
Acetate	0.235	5.4	0.0778
	0.118	"	0.0615
	0.059	"	0.0317
Acetate[a]	0.235	5.4	0.0876
	0.118	"	0.0598
	0.059	"	0.0287
Trimethyl acetate[c]	0.236	4.85	0.323
	0.154	"	0.265
	0.118	"	0.227
	0.059	"	0.177
	0.028	"	0.155
Trimethyl acetate	0.368	5.0	0.313
	0.276	"	0.240
	0.184	"	0.202
	0.092	"	0.130
Trimethyl acetate[c]	0.343	5.17	0.232
	0.206	"	0.162
	0.137	"	0.123
	0.069	"	0.088
Trimethyl acetate	0.300	5.3	0.179
	0.225	"	0.148
	0.150	"	0.115
	0.075	"	0.081

Table 3.2 (continued)

Buffer	Total Buffer concn.[M]	pH	$k_{obsd}s^{-1} \times 10^2$ [b]
Cacodylate [c]	0.499	6.09	0.159
	0.399	"	0.115
	0.299	"	0.098
	0.199	"	0.080
	0.099	"	0.045
Cacodylate [c]	0.4	6.27	0.0665
	0.32	"	0.0652
	0.24	"	0.0627
	0.16	"	0.0340
	0.08	"	0.0202
Cacodylate [c]	0.261	6.47	0.0503
	0.196	"	0.0474
	0.130	"	0.0323
	0.065	"	0.0196
Cacodylate [c]	0.286	6.64	0.0488
	0.229	"	0.0398
	0.172	"	0.0290
	0.114	"	0.0230
	0.057	"	0.0080
Phosphate	0.088	7.0	0.0555
	0.044	"	0.0326
	0.022	"	0.0195
1,2-Dimethylimidazole	0.2	7.1	0.0100
	0.1	"	0.0091
	0.05	"	0.0101
1,2-Dimethylimidazole	0.2	8.0	0.0490
Ammonium bicarbonate	0.05	8.0	0.0559
Tris-HCl	1.656	9.0	0.620
	0.761	"	0. 590
	0.414	"	0.606

a = Deuterated buffer species (pD = pH + 0.41)

b = Regression coefficient ≃ 0.99

c = with technical assistance of Mr B.M. Cockerill

The first order mutarotation rate constants ($k_{obsd.}$) were measured in different buffer systems (Table 3.2) at an ionic strength of 0.2 (addition of potassium chloride). The isomerisation in acetate, trimethylacetate, cacodylate and phosphate buffers is subject to general acid catalysis (i.e. $k_{obsd.}$ increases with increasing buffer concentration at constant pH e.g. trimethylacetate buffer catalysis, see Fig. 3.2). Further, it is observed that in the acid region as the pH falls the catalytic effect of the buffer increases (Fig. 3.2), indicating that it is the acid form which is acting as the catalyst.

At alkaline pH no buffer species catalysis was detected in 1,2-dimethylimidazole (pH 7.1) and tris (pH 9.0) buffers (Fig. 3.3).

The magnitudes of the rate constants extrapolated to zero buffer concentration indicated that specific acid and specific base catalysis of the isomerisation reaction occurs. Moreover, a plot of $k_{obsd.}$ vs. pH (Fig. 3.4) showed that the isomerisation rate falls in the range pH 4.0 to 6.0, remains constant in the range pH 6.0 to 7.5 and then increases in the range pH 7.5 to 10.0.
A-$\log_{10} k_{obsd.}$ vs. pH plot (Fig. 3.5) gave a straight line in the acid region (pH 4.0 - 6.0) with a slope of 1.15 and a straight line in the basic region (pH 7.0 - 10.0) with a slope of 1.00, confirming that both specific acid and specific base catalysis are operating.

In the acid region (pH 4.0 - 6.5) where general acid catalysis was observed, buffer catalytic rate coefficients k_{HA} and k_B for particular buffers were measured by observing the isomerisation rates at varying buffer concentration while maintaining the same buffer ratio (r = the ratio of the concentration of the conjugate base [B] to that of the acid [HA] i.e. r = [B]/[HA]). The data were evaluated using the following equations:

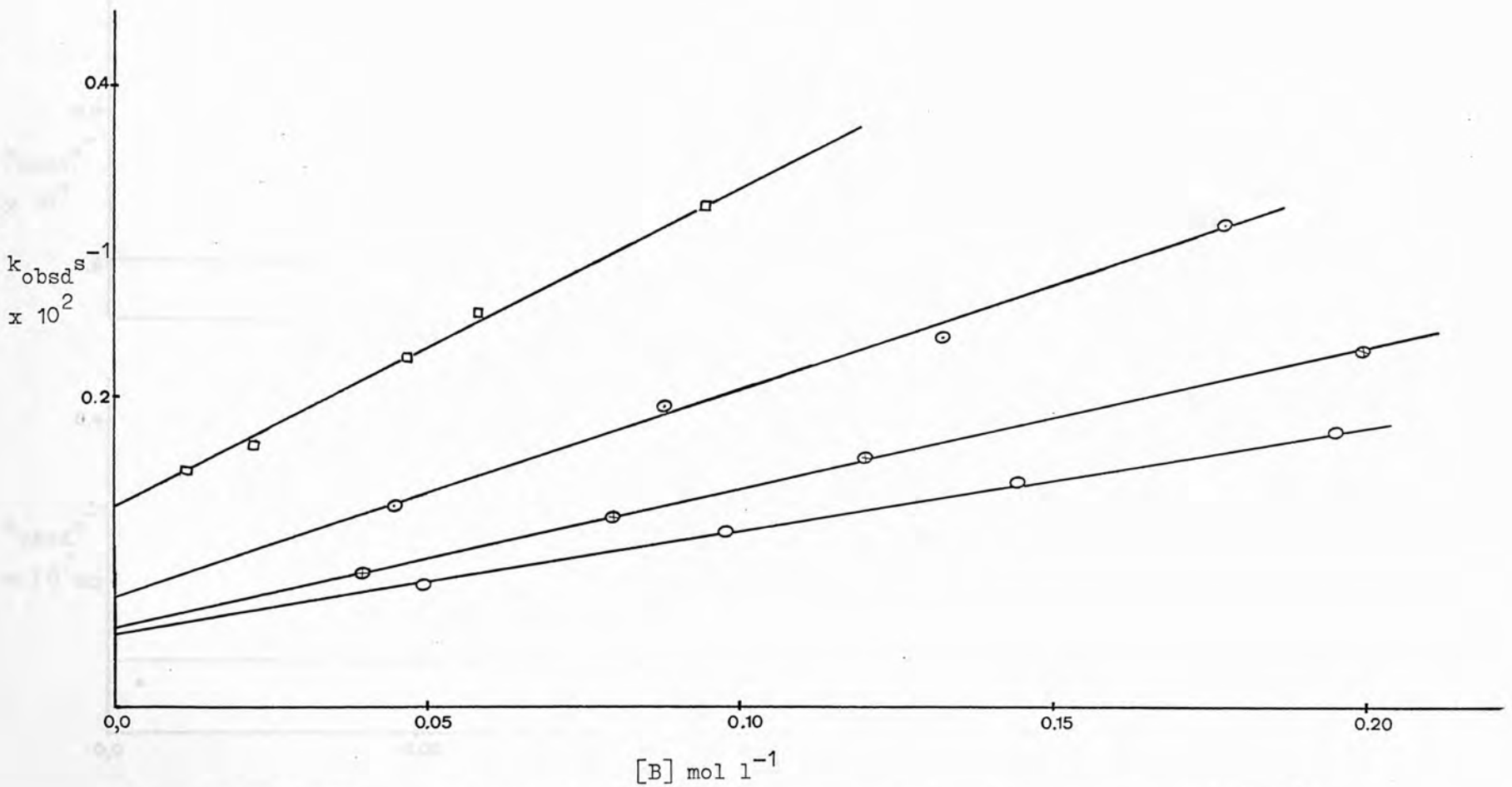


Fig. 3.2 Plot of $k_{obsd.}$ vs buffer (conjugate base) concn. for D-arabinose oxime; □ pH 4.85, ⊙ pH 5.0, ⊗ pH 5.17, ○ pH 5.3 (trimethylacetate).

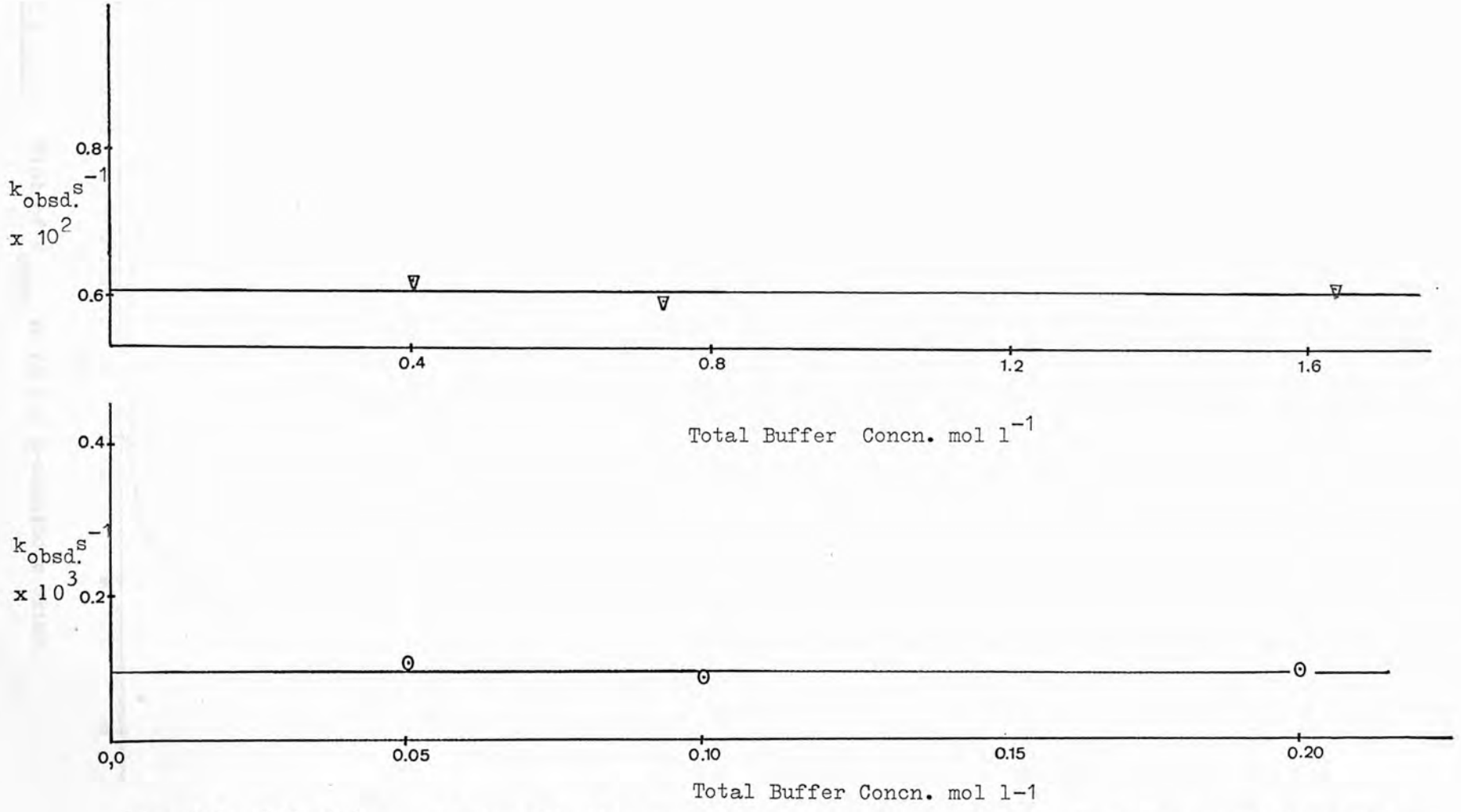


Fig. 3.3 Plot of $k_{obsd.}$ vs total buffer concn. for D-arabinose oxime in ⊙ 1,2-dimethylimidazole, and ∇ tris.HCl.

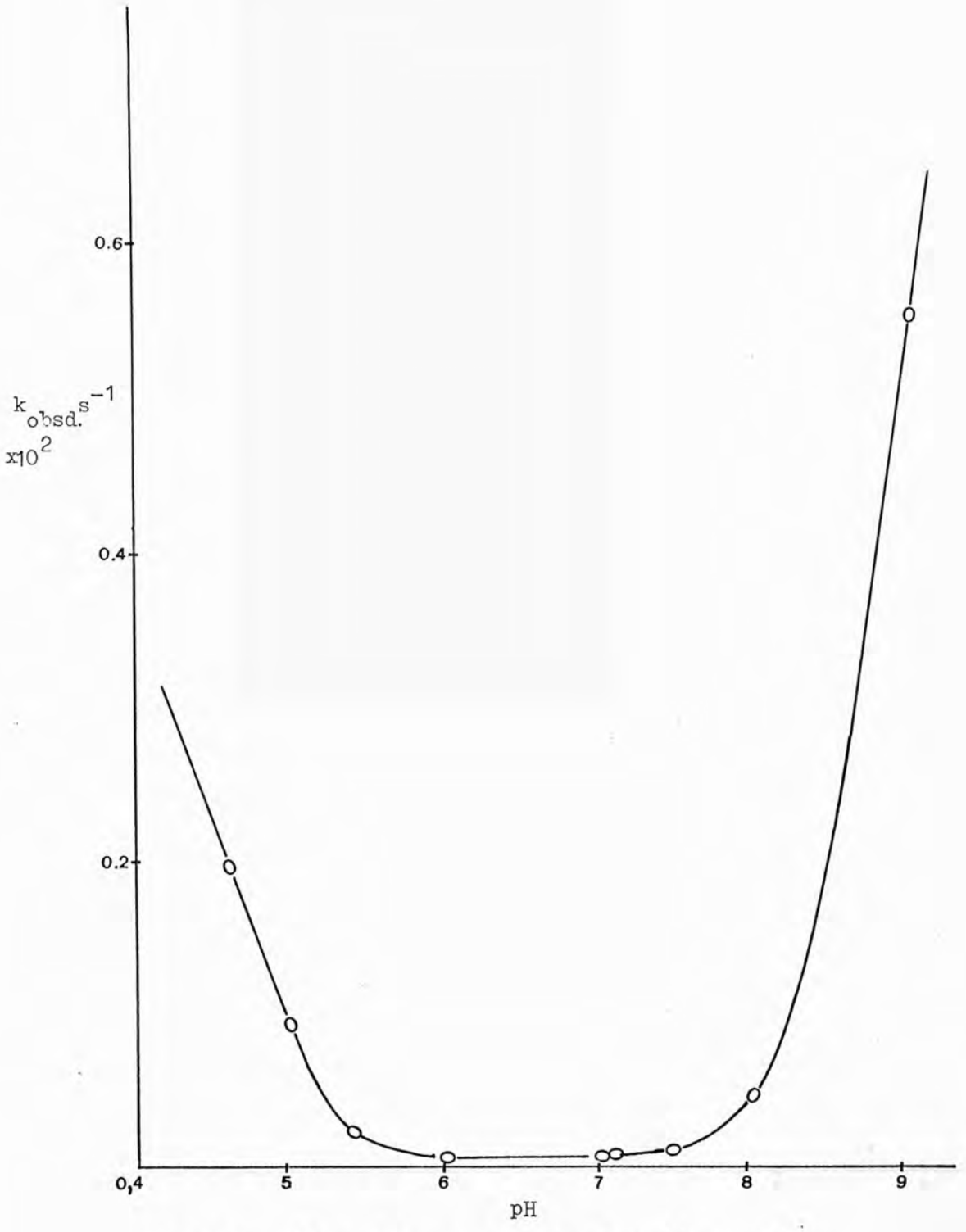


Fig. 3.4 Plot of $k_{obsd.}$ vs pH for D-arabinose oxime.

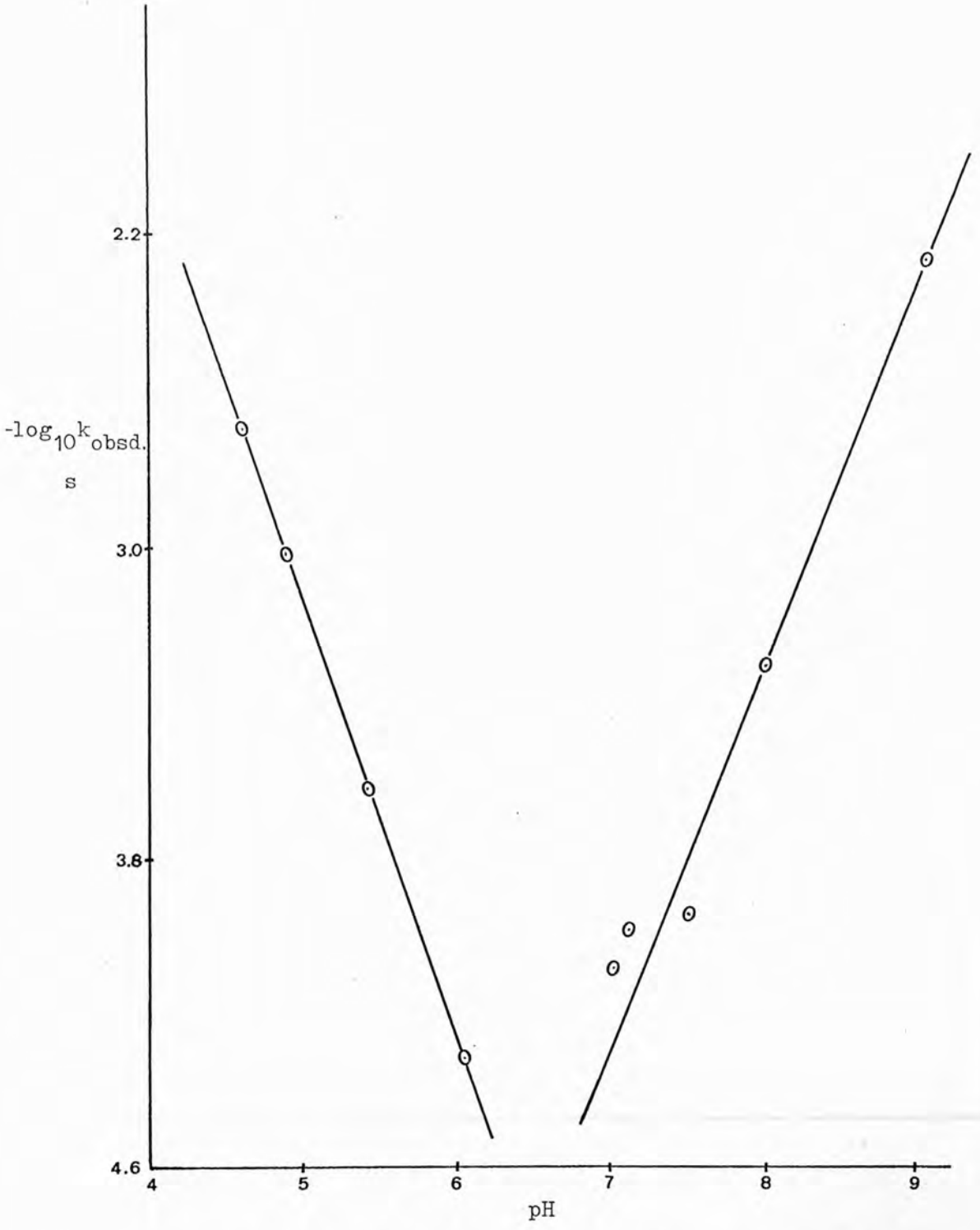


Fig. 3.5 Plot of $-\log k_{obsd.}$ vs pH for D-arabinose oxime.

$$k_{obsd.} = k_{solvent} + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-] + k_{HA}[HA] + k_B[B]$$

On rearranging

$$k_{obsd.} = k_{solvent} + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-] + [B](k_{HA}/r + k_B)$$

Plots of $k_{obsd.}$ against [B] (e.g. trimethylacetate (Fig. 3.2)) were found to be linear with a slope Sr, where $Sr = k_{HA}/r + k_B$ and an intercept Ir,

where $Ir = k_{solvent} + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-]$

A plot of Sr against the reciprocal (1/r) of several different buffer ratios (e.g. trimethyl acetate (Fig. 3.6)) allows k_{HA} and k_B to be evaluated for any buffer (Table 3.3).

Table 3.3 Catalytic Rate Coefficients for Isomerisation of D-Arabinose Oxime and Pteridine

Catalytic Rate Coefficients ($mol^{-1}\ l\ s^{-1}$)	D-Arabinose Oxime	Pteridine[22]
k_{OH^-}	0.68×10^3	0.04×10^3
$k_{H_3O^+}$	0.08×10^3	0.37×10^3
k_{H_2O}	$0.16 \times 10^{-4}/55.5$	$0.2 \times 10^{-4}/55.5$
k_{CH_3COOH}	1.59×10^{-2}	
$k_{(CH_3)_3CCO_2H}$	1.43×10^{-2}	
$k_{(CH_3)_2AsO.OH}$	0.41×10^{-2}	

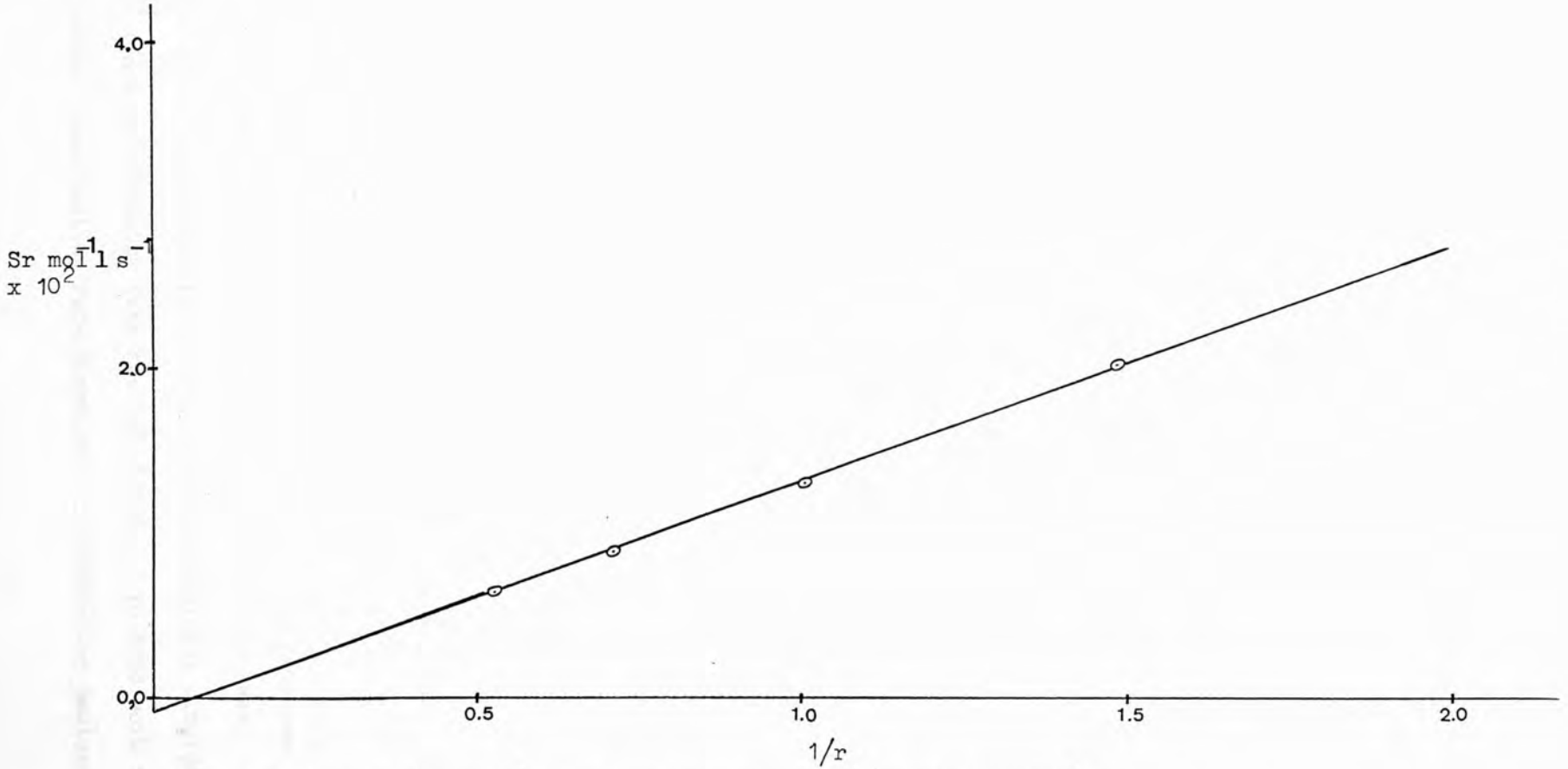


Fig. 3.6 Plot of Sr vs 1/r for D-arabinose oxime (trimethylacetate)

The catalytic rate coefficients for the acids (k_{HA}) of acetate (pK_a 4.76), trimethyl acetate (pK_a 5.05) and cacodylate (pK_a 6.27) are nearly the same for the former two cases and lower for the third case, showing that as the acid gets weaker the k_{HA} value falls. On the other hand for all three cases virtually negligible k_B values were obtained, indicating that the basic component has no effect on the isomerisation rate.

The catalytic coefficients $k_{H_3O^+}$ and k_{H_2O} (Table 3.3) were deduced from the data obtained in acetate buffer, by plotting Ir against the corresponding hydronium ion concentration (Fig. 3.7). The slope of this plot gives $k_{H_3O^+}$ and the intercept $k_{H_2O(solvent)}$. The value of k_{OH^-} (Table 3.3) was determined from the slope of a plot of $k_{obsd.}$ vs. hydroxide ion concentration (Fig. 3.8). The $k_{H_3O^+}$ and k_{OH^-} values are very much greater than the catalytic coefficients of the acids (k_{HA}) and k_{H_2O}. They are of comparable value to those obtained for hydration of pteridine.[22]

In order to distinugish between nucleophilic and basic catalysis in the basic region 1,2-dimethylimidazole and ammoniumbicarbonate buffers of pH 8.0 were used. Nearly equal $k_{obsd.}$ values (Table 3.2) were obtained, suggesting the probable absence of nucelophilic catalysis.

3.2.4 Isotope-Effect on D-Arabinose Oxime Isomerisation

Another aspect of oxime isomerisation which was investigated was the isotope effect. It is useful for elucidation of the reaction mechanism (see section 3.2.2). In this work the first order isomerisation rate constants in acetate and deuterated

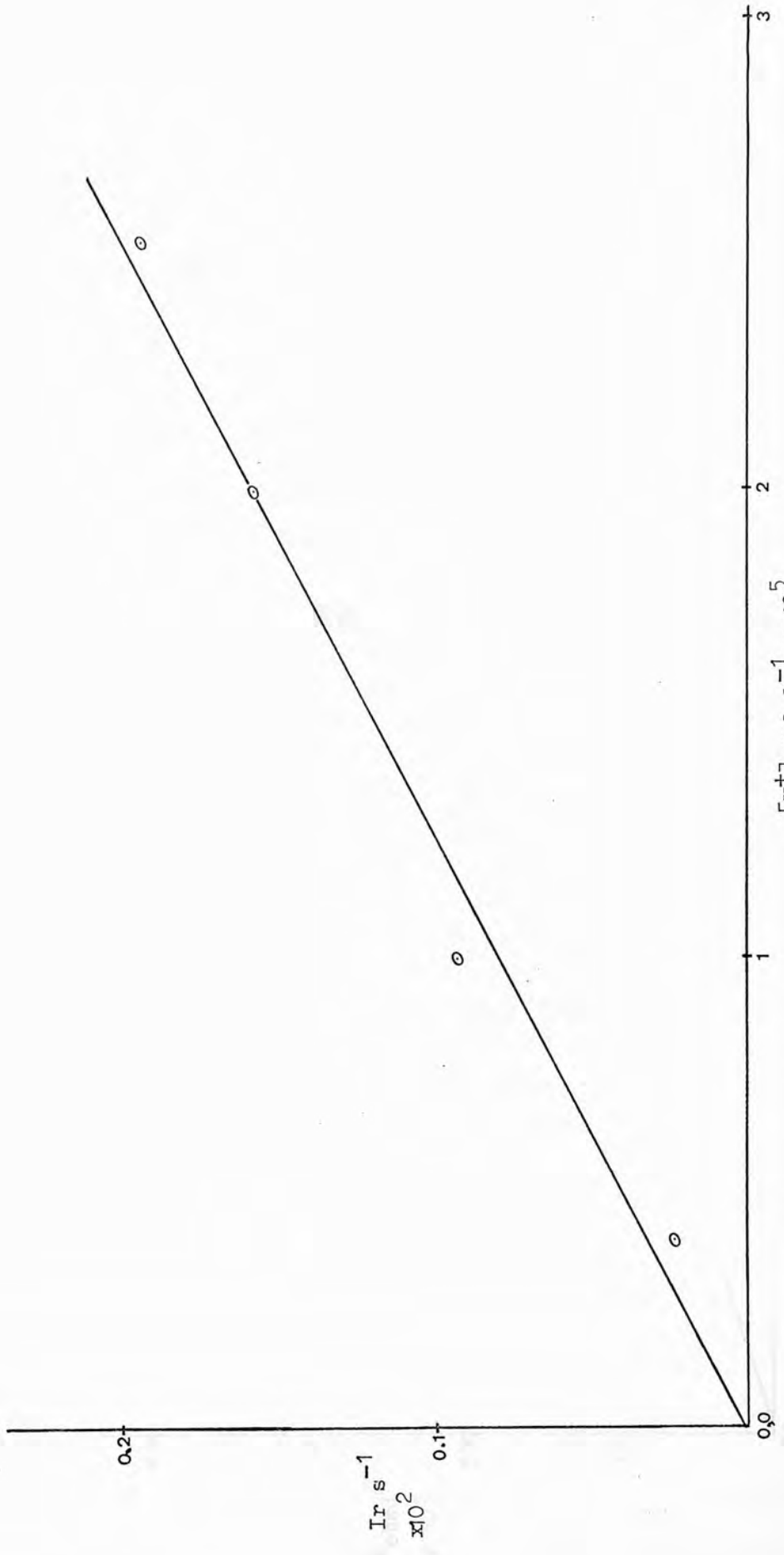


Fig. 3.7 Plot of Ir vs [H^+] for D-arabinose oxime (acetate)

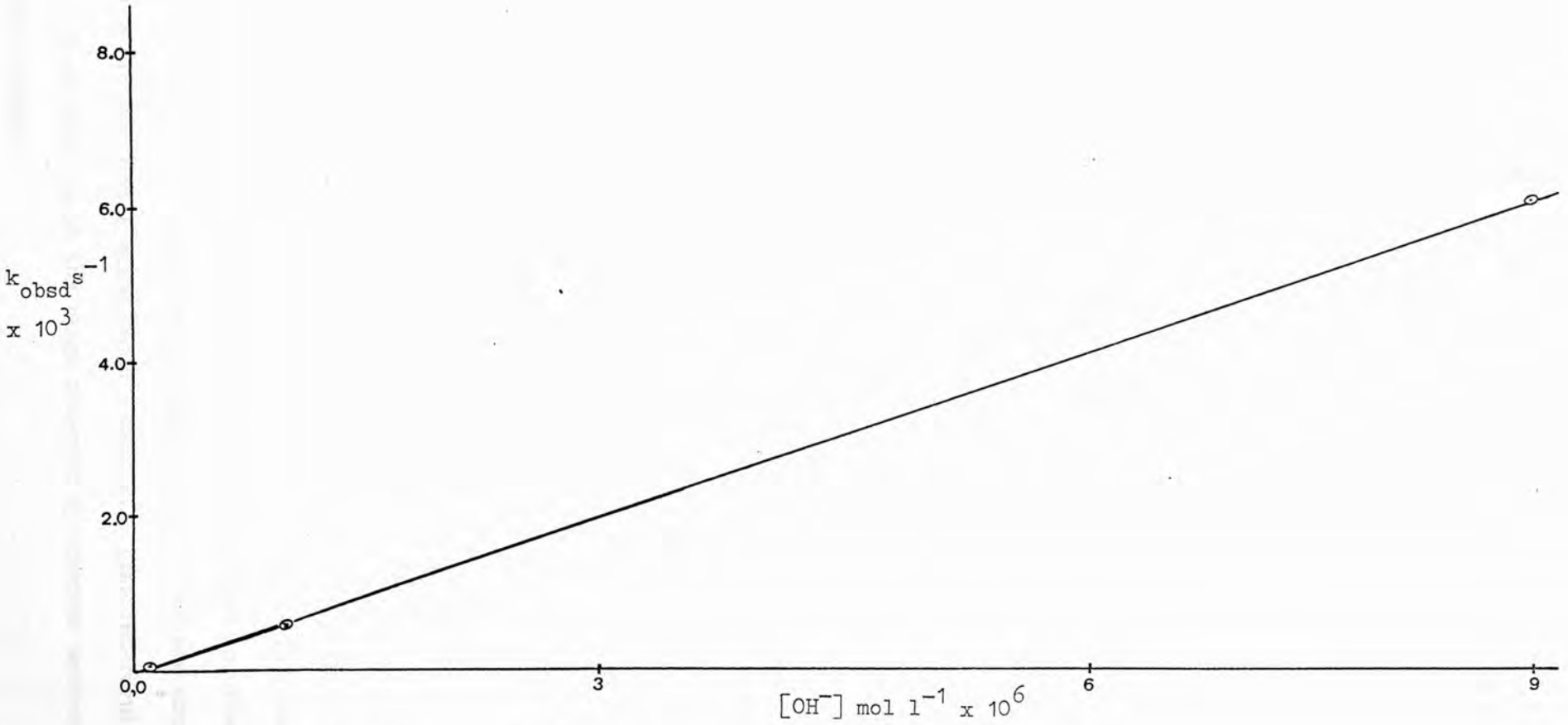


Fig. 3.8 Plot of $k_{obsd.}$ vs [OH^-] for D-arabinose oxime (in 1,2 dimethylimidazole, ammonium bicarbonate and tris. HCl).

acetate buffers at pH 5.4 were compared (Table 3.2). The values of $k_{obsd.}$ were very nearly similar indicating either that there was no proton transfer involved in the rate-determining step or that the opposing solvent and kinetic isotope-effects cancelled each other. More experiments need to be done with various deuterated buffers at different pH's to calculate the catalytic coefficients $k_{D_3O^+}$, k_{D_2O} and k_{OD^-}, which may give information about the proton transfer process occurring before, during or after the rate-determining step.

3.2.5 Measurement of Dissociation Constant of D-Arabinose Oxime

The dissociation constant (pKa) values for simple oximes reported in the literature are in the range 10 to 12[23]; the presence of an α-keto group considerably increases the acid strength (pKa range 7 - 10).[24] The pKa of the conjugate acid lies in the range <-1 to $+3$.[25,26] The individual constants are presumed to apply to the following equilibria:

$$RCH = NOH + H_3O^+ \underset{}{\overset{pKa\ <3}{\rightleftharpoons}} RCH = \overset{+}{N}HOH$$

$$RCH = NOH + H_2O \underset{}{\overset{pKa\ >7}{\rightleftharpoons}} RCH = NO^- + H_3O^+$$

There was no information about the dissociation constants of carbohydrate oximes in the literature, therefore it was decided to determine the pKa value of D-arabinose oxime. The pKa value was measured of D-arabinose oxime syn(E)-form by observing the change in the chemical shift of the C1-H signal in the ^1H nmr spectrum with pH (Fig. 3.9). A dissociation constant value of 11.0 was obtained from the plot of -log ([base]/[acid]) vs. pH. (The intercept is the pKa). This value is in the range observed by previous workers[23] for other oximes.

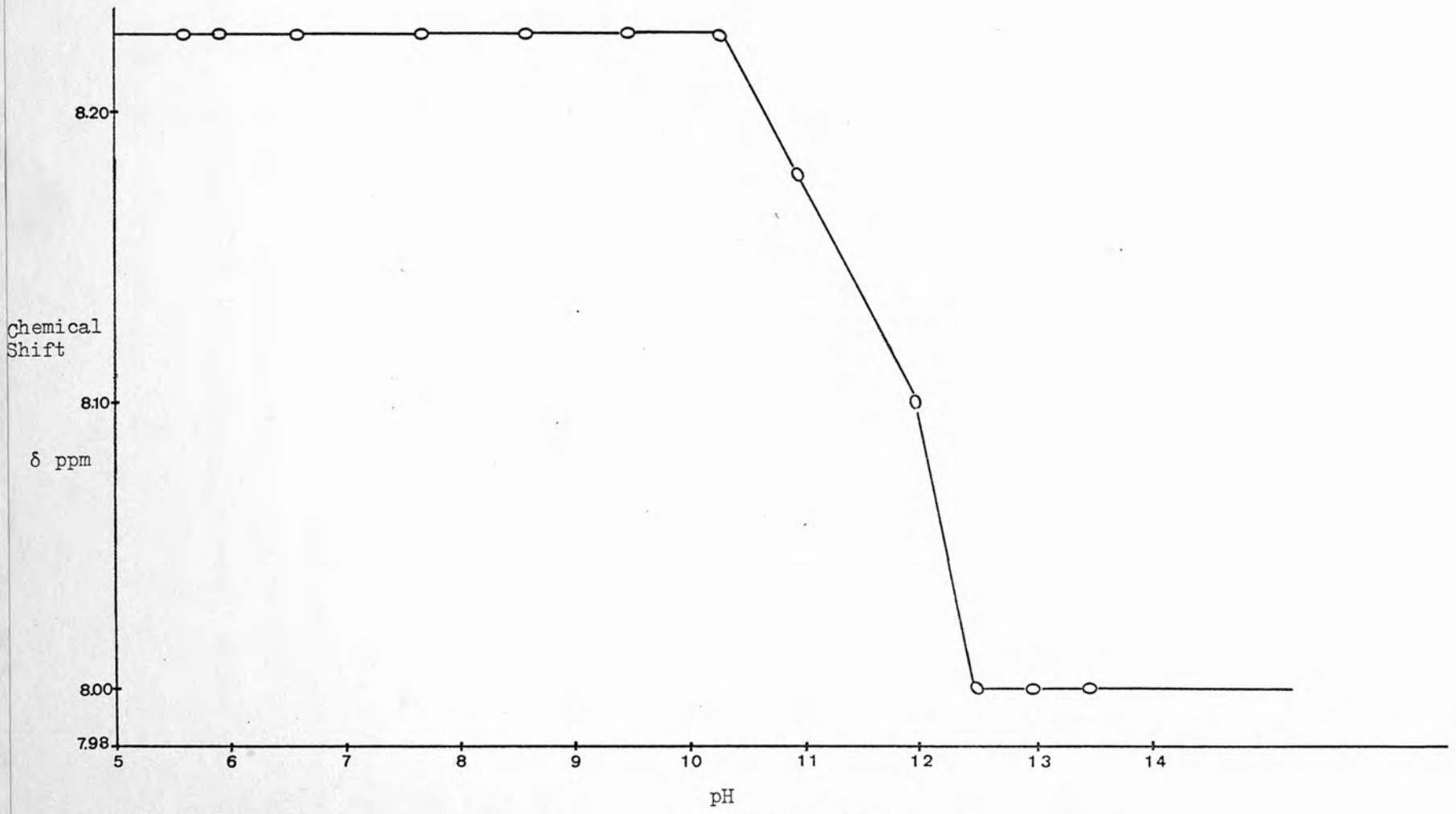
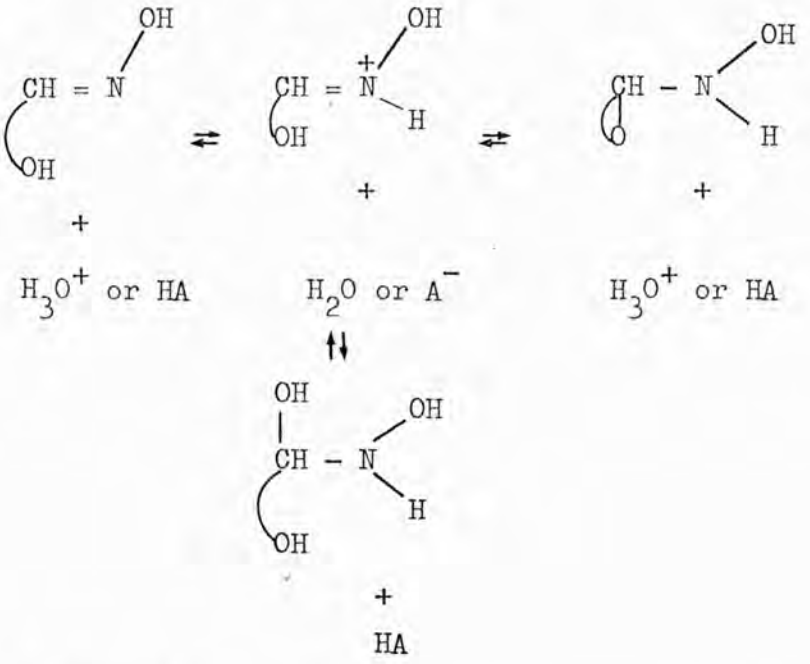


Fig. 3.9 Measurement of dissociation constant (pKa) of syn(E) D-arabinose oxime by ^{1}H nmr.

3.2.6 Mechanisms of Carbohydrate Oxime Isomerisation

3.2.6.1 At pH 4.0 to 6.0

In the acid region where the isomerisation is subject to specific and general acid catalysis, the mechanism shown in Scheme 3.6 is proposed.

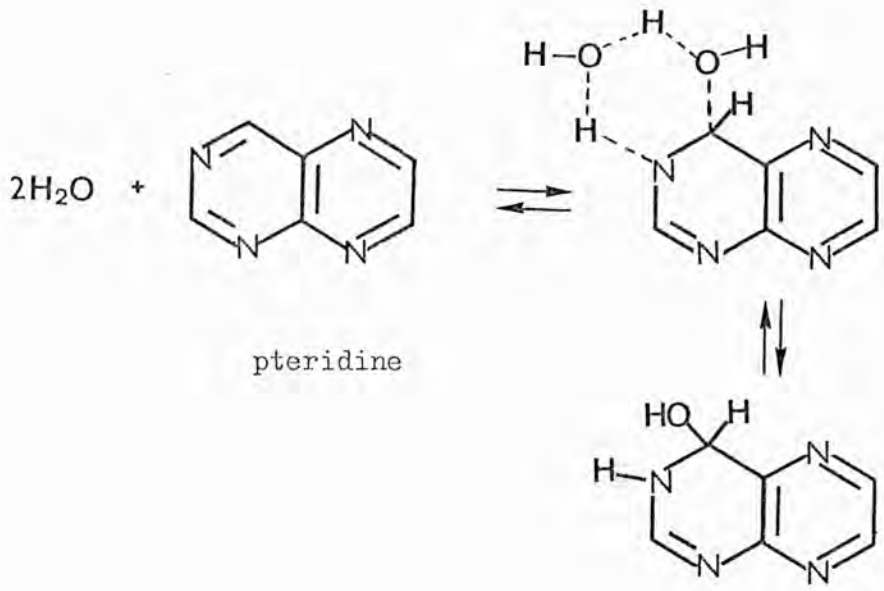


Scheme 3.6

The protonated oxime can undergo either a cyclisation or addition of water. Rotation about the carbon-nitrogen bond and/or inversion at the nitrogen atom can then occur.

3.2.6.2 At pH 6.0 to 7.5

The pH-rate profile (Fig. 3.4) shows a broad minimum in this region suggesting the operation of a concerted mechanism (Scheme 3.8) as has been postulated for the hydration of pteridine[22] (Scheme 3.7) and the mutarotation of D-glucose[21] (Scheme 3.3).



Scheme 3.7

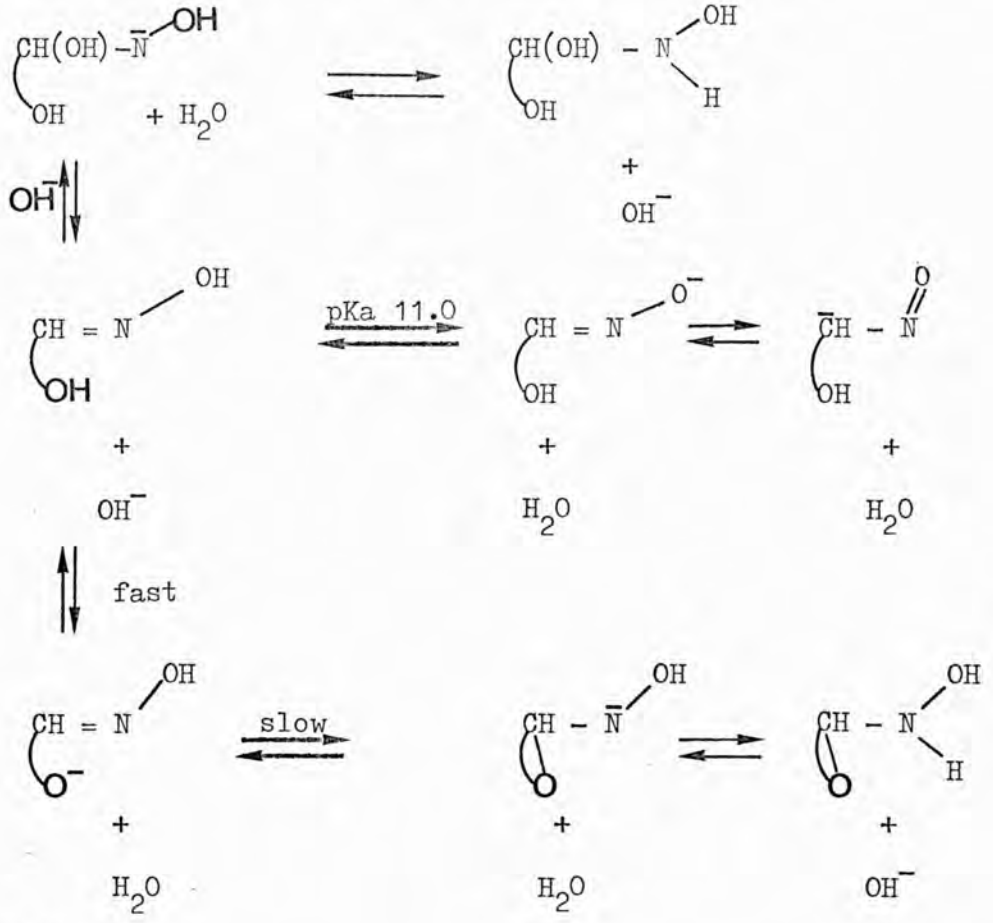
(HA and B can be water molecules)

Scheme 3.8

3.2.6.3 At pH 7.5 to 10.0

In this pH region isomerisation is known to be specific base catalysed. Also, the pKa of D-arabinose oxime syn(E)-form was found to be 11.0 indicating that it will be essentially undissociated at pH <11.0. Also, the rate would be expected to increase to a maximum at pH 11.0. It is observed that the rate is increasing in the pH range 7.5 to 10.0 (Fig. 3.4). It is known[7-9] that nitroso compounds rearrange to the oxime which can interconvert possibly via $R\bar{C}H - N^{=O}$ (base catalysis) or $R\bar{C}H - N^{=\overset{+}{O}H}$ $\longleftrightarrow$ $\overset{+}{R}CH - N^{-OH}$. Anderson and Jencks[15]

have observed a base catalysed reversible hydration of p-chlorobenzaldoxime. From the above evidence the following pathways (involving isomerisation via a nitroso form or reversible hydration followed by rotation) (Scheme 3.9) are suggested for D-arabinose oxime isomerisation in this pH region (7.5 to 10.0)



Scheme 3.9

Circumstantial evidence for the occurrence and possible involvement of cyclic forms of D-arabinose oxime comes from the observation that this compound acts as a substrate for yeast hexokinase (see CHAPTER 4).

3.3 EXPERIMENTAL

The isomerisation of carbohydrate oximes was followed by observing the change in optical rotation at 589 nm in a Perkin-Elmer 141 polarimeter equipped with a constant temperature cell supplied with water at 25 $\pm$ 0.1° by a Tempunit Tecam Model TU 12. Measurements of pH and pD (pD = pH reading + 0.41) were made on a Pye Unicam Model 290 pH Meter, using Pye Unicam E07 combined electrode or EIL Micro Combination pH electrode (0 - 14 pH, 0 - 100°). The pH Meter was standardised using standard buffer solutions at two different pH values. All buffers were made up to an ionic strength of 0.2 using potassium chloride. When required the optical rotation readings at zero time were obtained by extrapolation. In some cases the isomerisation was followed by ^{1}H nmr in deuterium oxide solution. All aqueous solutions were made using deionised water except where otherwise stated.

3.3.1 Isomerisation of Carbohydrate Oximes in Water

The carbohydrate oximes (crystals) were dissolved in water and the optical rotation measured immediately after dissolution (2-3 mins) until a constant equilibrium value was attained. The concentration of the oxime solutions were:

D-Glucose Oxime	(0.128M)
D-Arabinose Oxime	(0.079M)
D-Galactose Oxime	(0.062M)
D-Mannose Oxime	(0.128M)
D-Ribose Oxime	(0.152M)

3.3.2 Isomerisation of D-Glucose Oxime and D-Arabinose Oxime in Deuterium Oxide

Polarimeter: The oximes were dissolved in deuterium oxide and the optical rotation observed until a constant reading was reached. The concentration of the oximes were:

D-Glucose Oxime (0.128M)

D-Arabinose Oxime (0.079M)

^{1}H nmr: ^{1}H nmr spectra were run on an EM360 Varian 60 MHz and HR220 MHz (P C M U) spectrometers at ambient temperatures (22° - 23°) in deuterium oxide and using TSP as standard. The isomerisation was followed by measuring the C1-H peak areas of the different isomeric forms of the oximes. Any deviations in the experimental conditions were noted by observing the peak area of TSP (internal standard) and appropriate corrections were made for the other signals. The concentration of the oximes were:

D-Glucose Oxime (0.342M)

D-Arabinose Oxime (0.303M)

3.3.3 Isomerisation of D-Arabinose Oxime under Acidic and Basic Conditions

D-Arabinose oxime (0.079M) was dissolved in buffer (see Table 3.2) and kinetic measurements made by reading the optical rotation values at different times using a W+W Recorder 1100.

The rate constants were calculated using a least squares computer programme[+] in conjunction with a Texas Instruments Model SR 52 calculator.

[+] Written by Mr R Renaud, RHC Chemistry Department.

3.3.4 Measurement of Dissociation Constant (pKa) of D-Arabinose Oxime

The dissociation constant (pKa) of D-arabinose oxime was measured by ^{1}H nmr spectroscopy at 60 MHz, ambient temperature (22 - 23°), in deuterium oxide using external TMS as standard. The pH was varied from 4.0 to 13.5 with deuterated hydrochloric acid (DCl) and sodium hydroxide (NaOD). The change in the chemical shift of the C1-H signal of the syn(E) (major isomer) form of D-arabinose oxime (0.3M) were measured and plotted against the pH (Fig. 3.9). There was observed to be no shift in the other signals on addition of DCl or NaOD.

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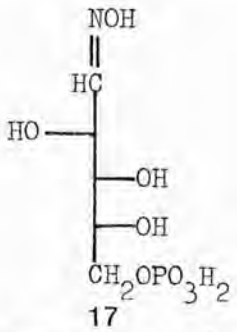
CHAPTER 4 THE INTERACTION OF CARBOHYDRATE OXIMES WITH YEAST HEXOKINASE

4.1 INTRODUCTION

As discussed in Chapter 1, the major objective of this work was the preparation and study of potential transition stage analogue inhibitors of enzymes. Based on arguments also presented in Chapter 1, the compound D-arabinose oxime-5-phosphate(17) was thought



17

to be a potential inhibitor of D-glucose-6-phosphate isomerase (EC 5.3.1.9). One way of obtaining this compound is enzymic phosphorylation catalysed by yeast hexokinase (ATP: D-hexose 6-phosphotransferase) (EC 2.7.1.1) as discussed in Chapter 2. The fact that this reaction proceeds as seen in Chapter 2 means that D-arabinose oxime itself could enter the glycolytic cycle and hence show biological activity. Moreover, it was considered that if such activity was discovered for other oximes and sugars used then its study might lead to new insights into the action of yeast hexokinase itself. As shown in Chapter 2 of this thesis these carbohydrate oximes are acyclic except for D-glucose oxime, and no acyclic substrates of yeast hexokinase have been reported previously. For these reasons the activity of yeast hexokinase towards D-arabinose oxime and other compounds was investigated.

4.2 THE ACTION AND STRUCTURE OF HEXOKINASE

4.2.1 The Reaction Catalysed by Hexokinase

Yeast hexokinase belongs to a class of enzymes which catalyse the phosphorylation of hexoses. This reaction is central to the metabolism of glucose since formation of glucose-6-phosphate makes glucose available for glycolysis and for the synthesis of polysaccharides.

In 1927 O. Meyerhof[1] discovered that aged muscle extracts could regain their ability to ferment sugars by treating them with an extract from yeast. The Meyerhof muscle enzyme, called hexokinase[2] was found to be very similar to the yeast enzyme, heterophosphatese[3,4] and was capable of catalysing the transfer of the γ-phosphoryl group of adenosine triphosphate (ATP)[5,6,7] in the presence of magnesium ions (Mg^{2+}) to a number of hexoses (Fig. 4.1).

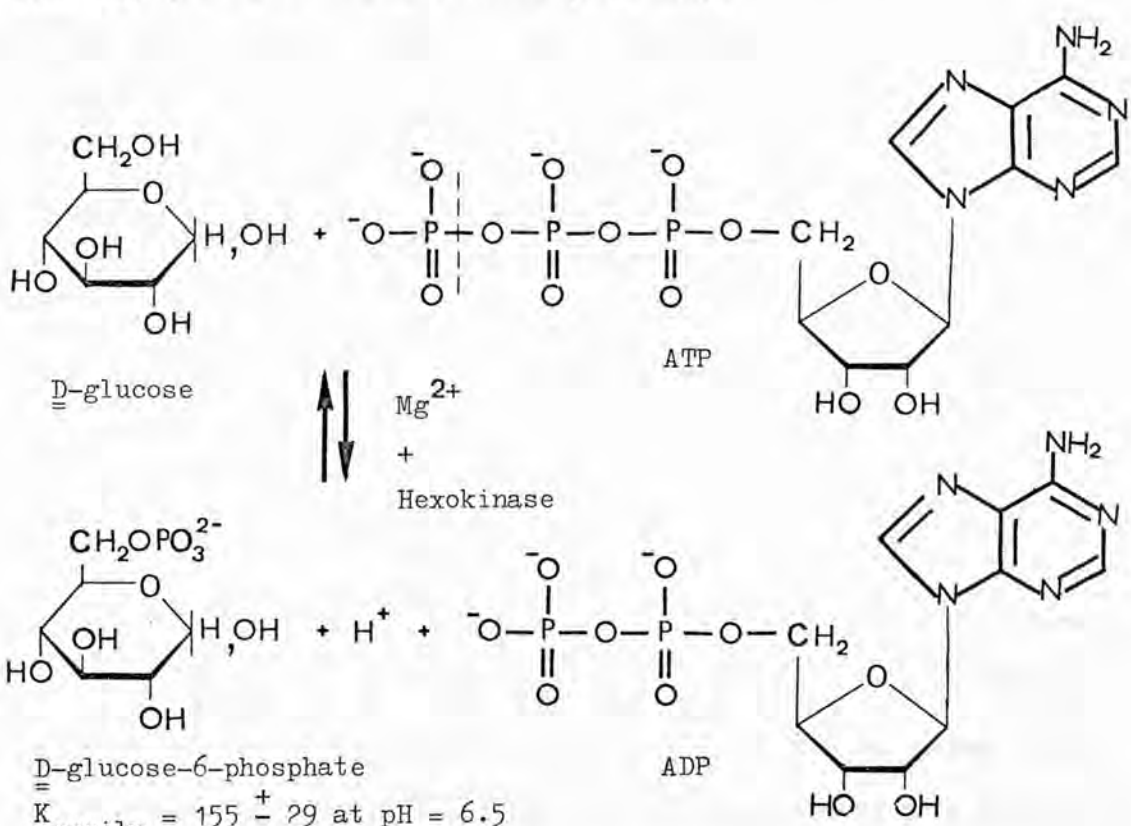


Fig. 4.1 The reaction catalysed by hexokinase

4.2.2 The Purification of Hexokinase

Yeast hexokinase was purified by a number of groups of workers.[2-13] However, these early preparations resulted in poor yields. Therefore, new procedures which gave better yields were devised,[14] but they were shown to give multiple forms of yeast hexokinase.[15-19] A yeast protease is present even in repeatedly crystallised yeast hexokinase, and in order to prevent proteolysis diisopropyl phosphofluoridate, phenyl methyl sulphonyl fluoride and gel filtration of cell-free extracts was carried out.[20-27]

Purified yeast hexokinase has an absorption maximum at 278 nm does not contain any protein bound coenzymes, and is essentially free of bound phosphate or glucose.[16,28]

4.2.3 Isoenzyme Forms

A number of studies have indicated the existence of several isoenzymic forms of yeast hexokinase.[19,22,23,24,27,29] The native isoenzymes are designated as A (or P-I in Colowick's nomenclature), B (P-II) and C according to the order of their elution from DEAE (diethylaminoethyl) cellulose column.[29] The molecular weight of isoenzyme A lies in the region of 102,000[28] to 108,000[29] and of B and C at 104,000.[30] Isoenzyme A is composed of identical alpha (α) subunits and B and C of identical beta (β) subunits.[31] The specific activity towards glucose of isoenzyme A is 200 μ moles/min/mg of protein, of B is 800 μ moles/min/mg of protein and of C is 750 μ moles/min/mg of protein.[21,31] Further the measurements of the ratios of activity towards fructose and glucose is 2.6-3.0 for isoenzyme A and 1.0-1.3 for isoenzyme B,[21,32] while the mannose:glucose activity ratios were 0.6 and 0.3 for isoenzymes A and B respectively.[21] Thus, the

relative amounts of A and B present in crude extracts or in purified preparations can be determined. Further, B and C were observed not to be structural isoenzymes, rather they appeared conformationally different, as C can be converted to B by high ionic strength.[31] An alpha-beta hybrid A' found in fresh yeast cell lysates was obtained[31] and this form changes to the A and B isoenzymes during the purification procedure. These isoenzymes can be dissociated without loss of activity by high ionic strength, elevated temperatures, exposure to slightly alkaline conditions (pH = 8.0), presence of glucose and orthophosphate.[24]

From dissociation studies under different conditions yeast hexokinase was observed to be a dimer having identical polypeptide chains of about 50,000 daltons molecular weight.[19,23,25,26,30,33] Formerly it was thought that the enzyme was a tetramer, with each subunit of molecular weight near 25,000.[12,17,22,34,35,36] The discrepancy in the molecular weight was due to incomplete digestion, inefficient separation of the resulting peptides and contamination by traces of yeast proteases in the preparations.[37]

The amino acid compositions of the native isoenzymes (A and B) were observed to be very similar but do show clear differences in the content of several amino acids.[25,34,38] Incubation of the native isoenzymes (A and B) with trypsin in the presence of high salt concentration, or glucose produces two modified enzyme forms (A' and B') and this involves removal of eleven amino acids from the N-terminus.[24,25,26,34,38] The physiological roles of these different isoenzymic forms remain unclear, but from the dependence of the relative proportion of these forms upon the stage of growth would

suggest that each isoenzyme may perform a specialised function.

Improved isolation techniques, and characterisation of the physical and chemical properties of the enzyme facilitated the kinetic study of the mechanism of the phosphotransferase reaction. In 1955 Gamble and Najjar[39] showed the reversibility of the hexokinase reaction with the rate of the reverse reaction being one fiftieth as fast as the forward reaction. In 1956 Agren and Engstrom[40] reported the isolation of labelled phosphorylserine from an acid hydrolysate of hexokinase incubated with $^{32}P/ATP$ and suggested that the reaction mechanism (Fig. 4.2) involved the participation of a phosphoryl-enzyme intermediate.

$$E + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP + ADP$$

$$EP + \text{glucose} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP.\ \text{glucose} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + \text{glucose-6-P}$$

Fig. 4.2 Mechanism of hexokinase reaction according to Agren and Engstrom.

However Hass et al.[41] could not substantiate this even when pyruvate kinase (EC 2.7.1.40) and 2-phosphoenol pyruvate were added to displace the possibly unfavourable equilibrium for the enzyme phosphorylation, and Najjar and McCoy[42] failed to observe an exchange reaction between [^{14}C]-glucose and glucose-6-phosphate in the absence of ATP and ADP. The latter workers further proposed an alternative mechanism (Fig. 4.3) involving a glucose-enzyme intermediate.

E-glucose + ATP ⇆ EX ⇆ E-glucose-6-P + ADP

E-glucose-6-P + glucose ⇆ EY ⇆ E-glucose + glucose-6-P

Fig. 4.3 Alternative mechanism of hexokinase reaction

From the analysis of purified hexokinase Trayser and Colowick[15, 27] concluded that neither a phosphoryl-enzyme nor a glucose-enzyme intermediate was involved and suggested instead a direct interaction of substrates on the enzyme surface.

In recent years the possible presence of phosphoenzyme intermediates has been studied by investigating the half reaction, i.e. involving either the reaction of the phosphoryl donor with enzyme to form the presumed phosphoryl-enzyme intermediate, or the reaction of the presumed phosphoryl-enzyme with the phosphoryl acceptor. The finding of Kaufman[43] of enzymic-ADP exchange into ATP has been confirmed with pure preparations of yeast hexokinase which were free of any trace of glucose or glucose-6-phosphate.[44] The rate of the ADP⟷ATP exchange reaction was only 0.01% of the rate of glucose phosphorylation, but four times the maximum rate of ATP hydrolysis.[45] Walsh and Spector[46] reported that significant exchange between labelled glucose-6-phosphate and glucose can be demonstrated in the absence of nucleotides provided that one uses a high ratio of glucose-6-phosphate to glucose (400:1), in the presence of magnesium ions and a low pH (6.5). Further, the glucose⟷glucose-6-phosphate exchange rate is the same as the ADP⟷ATP exchange in the presence of polyanion activators like citrate.[47] However, these exchange reactions were suggested not to be involved at the catalytic site of the hexokinase reaction because N-acetyl glucosamine (2-acetamido-2-deoxy-D-

glucopyranose) which inhibits the ATPase reaction and D-lyxose which activates the ATPase reaction have little or no effect on the ADP⟷ATP exchange rate.[44,45] However, if the ADP⟷ATP exchange is relatively fast compared to the hydrolysis of the phosphoryl-enzyme then these activating and inhibiting effects need not be observed.

4.2.4 Kinetic Studies

The first initial rate experiments were carried out independently by Hammes and Kochavi[48] and by Fromm and Zewe.[49] In both these reports, Lineweaver-Burk plots[50] of initial rates with D-glucose and ATP indicated that the extrapolated lines intersected to the left of the $1/v$ axis. They both concluded that the reaction was sequential, however, they disagreed on how the ternary complex was formed. Hammes and Kochavi[48] favoured the ordered mechanism (dark arrows, fig. 4.4) with Mg ATP^{2-} being added to the enzyme-glucose complex. Fromm and Zewe[49] however, suggested a rapid equilibrium random mechanism (Fig. 4.4) in which either substrate could bind first to the enzyme, and further all the steps were at equilibrium except conversion of the ternary complex.

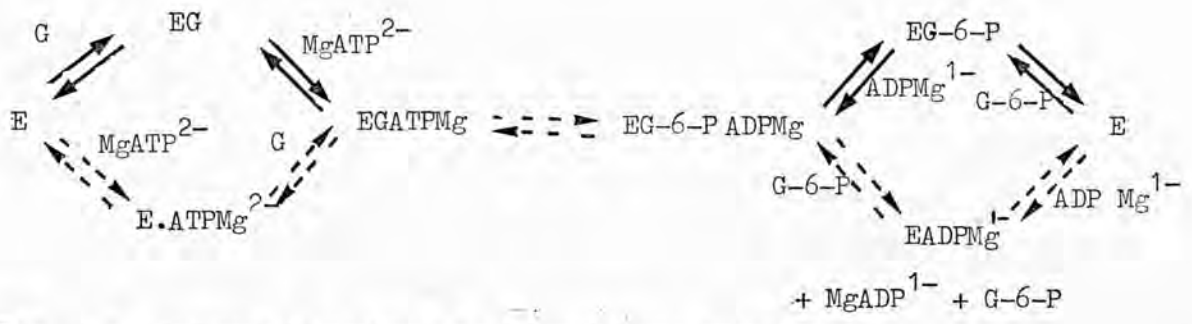
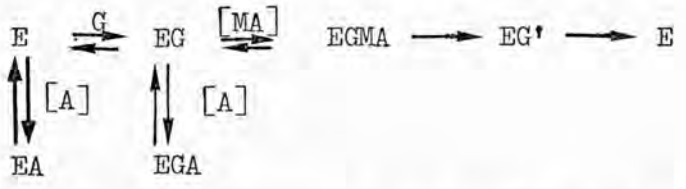


Fig. 4.4 Sequential mechanism of hexokinase reaction

Ottolenghi[51] has since shown that the data of Hammes and Kochavi was consistent with a random kinetic mechanism. Moreover, the initial rate studies of the reverse reaction were also in harmony with a sequential mechanism.[52] It[53] was suggested from an induced fit model that the ternary complex formed by sequential addition of nucleotide to the enzyme-sugar binary complex is active while an abortive ternary complex is formed when sugar binds to the enzyme-nucleotide binary complex which in turn also decomposes rapidly. To distinguish between ordered and random mechanisms various experiments were carried out by different workers.

Noat et al.[54] arrived at the same conclusion as Hammes and Kochavi[48] primarily on the basis that the K_m for ATP depends on which sugar is being phosphorylated, however, these workers[54] obtained different values for the rate constants. Further, they[55] showed by equilibrium dialysis that yeast hexokinase binds one molecule of [^{14}C]-glucose per monomer in the absence of ATP, while the binding of the chelate ($Mg[^{14}C]ATP^{2-}$) could not be detected in the absence of glucose. Later these workers[56] carried out a study of the inhibition of glucose phosphorylation by the ionic species ATP^{4-} and Mg^{2+} and concluded that only one model could account for this inhibition (Fig. 4.5).



(A = ATP^{4-}; G = Glucose; MA = $MgATP^{2-}$; G' = Glucose-6-P; E = Enzyme).

Fig. 4.5 Ordered sequential mechanism of hexokinase reaction according to Hammes and Kochavi.

The inhibition of the hexokinase reaction by the high nucleotide concentrations was suggested to be due to the formation of the "dead-end" complexes, (EA and EGA) which are devoid of any activity, with the affinity for the second form (EG) being greater than the first (E) for the nucleotide. In another study[57] using alternate substrates (D-fructose and D-mannose) they concluded that a random mechanism could be ruled out and the ordered mechanism previously deduced was correct. Hohnadel and Cooper[58] observed that the analogue of ATP in which glucose replaces the ribose moiety was an effective competitive inhibitor of ATP but an uncompetitive inhibitor with respect to glucose. For an ordered pathway a competitive inhibitor for the substrate which binds first will cause mixed or non-competitive inhibition relative to the second substrate. On the other hand a competitive inhibitor for the second substrate will produce uncompetitive inhibition relative to the first substrate. However, Himoe[59] has shown that for a sequential pathway in which one substrate binds slowly non-competitive or uncompetitive inhibition may be observed. Kosow and Rose[60] observed non linear intercept effects in double reciprocal plots of ADP inhibition relative to $MgATP^{2-}$ using undegraded yeast hexokinase isoenzymes. They concluded from these studies that the random equilibrium mechanism was ruled out.

A number of arguments favouring the random mechanism came from initial rate studies carried out in Fromm's laboratory. Fromm and Zewe[49] applied product inhibition studies to the yeast hexokinase reaction. This approach relies on the fact that the presence of a reaction product alters the form of the rate expression in a manner which is unique for each sequential kinetic mechanism. However, the formation of non-productive abortive ternary complexes can influence

the way in which products appear to inhibit the initial reaction velocity with respect to each substrate. They found[61] that glucose-6-phosphate inhibition relative to either substrate was mixed, whereas the ADP inhibition appeared competitive with ATP and non-competitive with glucose. They further used substrate analogues that behaved as linear competitive inhibitors of substrates, and suggested that for a random pathway a competitive inhibitor for either substrate will produce mixed or non-competitive inhibition relative to the other substrate. 2-Acetamido-2-deoxy-D-glucopyranose behaved as a competitive inhibitor for glucose and a non-competitive inhibitor for Mg ATP^{2-}. Also, the alternative substrate D-fructose was a competitive inhibitor for glucose and a non-competitive inhibitor for Mg ATP^{2-}. Additional evidence which in their view supported the random mechanism came from experiments using the inhibitors Cr ATP^{1-} and Cr ADP.[62] It was found that Cr ATP^{1-} and Cr ADP are competitive inhibitors of Mg ATP^{2-} and Mg ADP^{1-} respectively, and non-competitive inhibitors of glucose and glucose-6-phosphate respectively. Rudolph and Fromm[63] studied the hexokinase mechanism by computer simulation. Their data were consistent with the enzyme mechanism being steady state random but approximated by the rapid equilibrium assumption. Further, the systematic comparison of the initial rates and inhibition data of the commercial S-forms and the A(P-I) form of the enzyme showed no qualitative difference in the mechanism occurring.

4.2.5 Isotope Exchange Studies.

The early isotope exchange work described in Section 4.2.3 was carried out using ^{14}C and ^{32}P-labelled compounds to determine

the presence of a phosphoryl-enzyme intermediate and/or a glucose-enzyme intermediate. Later Britton and Clarke[64] using a modified equilibrium flux technique[65] measured the ratio of two fluxes [(flux of material from glucose-6-phosphate to ATP)/(flux from glucose-6-phosphate to glucose)] as a function of increasing concentrations of D-glucose or ATP at equilibrium. In general for random addition the flux ratio plotted against ATP concentration should yield a hyperbola with the ratio increasing from a value less than unity to a plateau greater than unity and a similar curve should be obtained when the reciprocal of the ratio is plotted against D-glucose. For ordered addition with D-glucose adding first the flux ratio should be independent of D-glucose concentration and should increase linearly from unity with ATP concentration. If part of the reaction should proceed via addition of ATP before D-glucose and if the step:

$$\text{ATP} + \text{Enzyme} \leftrightarrows \text{Enzyme.ATP}$$

should be very fast the flux ratio will vary as described for ordered addition except that it will reach a maximum at high ATP concentrations. Flux measurements were made with ^{32}P- and ^{14}C-labelled substrates at pH 6.5, ionic strength (0.2) and concentration of free Mg^{2+} (5.0 mM) kept constant. They found no change in flux ratio as the D-glucose concentration was increased, but a linear rise from 1 to 5 as the ATP concentration was raised from 0 to 1.2 mM. This implies an ordered pathway. The pathway with ATP binding first was not detected and was estimated to be responsible for less than 10% of the total flux. However, Fromm et al.[32] reported from isotope exchange studies (with both substrates and products being present) that equilibrium exchange kinetic measurements were consistent with a

random addition of substrates to the enzyme based on the evidence that ATP⟷ADP exchange rate is approximately 50% greater than the corresponding D-glucose⟷D-glucose-6-phosphate exchange rate and also that high ATP - ADP concentrations failed to depress the glucose⟷ glucose-6-phosphate exchange. For a truly rapid equilibrium random mechanism, these exchanges must be equivalent.[66] This suggested that the interconversion of the enzyme-$MgATP^{2-}$-D-glucose and enzyme-$MgADP^{1-}$-glucose-6-phosphate ternary complexes were not rate limiting, and the data were in agreement with the possibility that glucose-6-phosphate release was partly rate limiting.[60] According to Kosow and Rose[60] an alternative sequence of product release occurred with glucose-6-phosphate release occurring first however, this sequence suggested to be of minor significance except in the presence of high concentration of $MgADP^{1-}$. They suggested a branched pathway (Fig. 4.6).

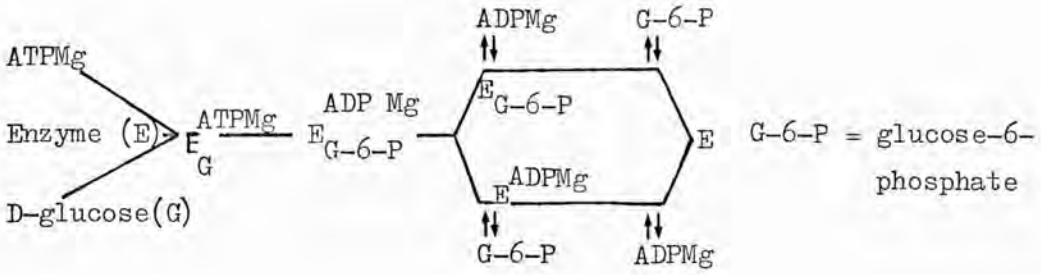
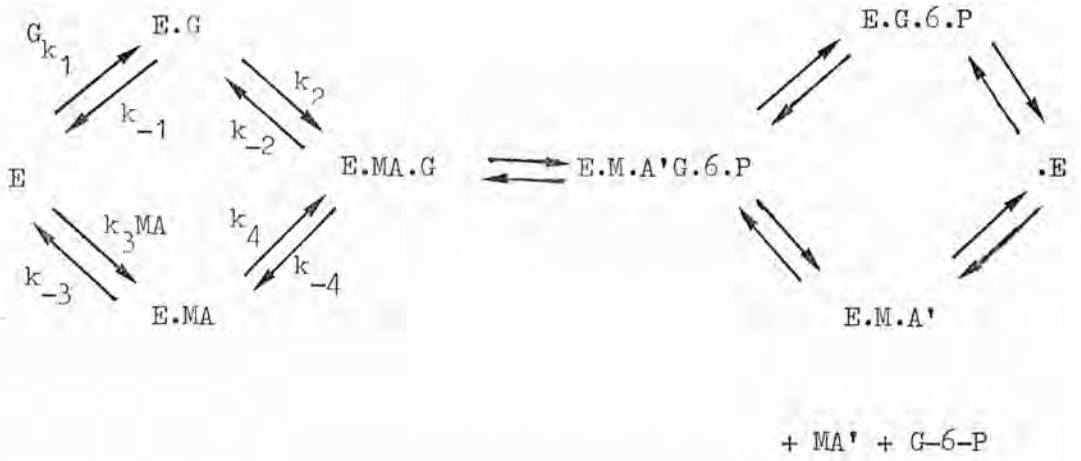


Fig 4.6 Mechanism of hexokinase reaction according to Kosow and Rose.[60]

From the measurement of exchange rates at equilibrium Boyer and Silverstein[66] also concluded that an 'alternative random' and not ordered mode of substrate addition was followed (Fig. 4.7).



k_1 < or > k_4 similarly for product release

k_3 < or > k_2

G = glucose G-6-P = glucose-6-phosphate

MA = $MgATP^{2-}$ E = enzyme

Fig. 4.7 Alternative random mechanism of hexokinase reaction

The results of steady state kinetics including substrate analogue and product inhibition studies, and isotope exchange studies, are consistent with a sequential reaction pathway for the hexokinase catalysed reaction in which initial binding of glucose is preferred when both substrates are present at K_m levels.

4.2.6 Influence of Metal Ions

The influence of metal ions on the yeast hexokinase reaction has been studied by various workers.[48,56,67-72] M. Cohn[69] observed that Mn^{2+} and enzyme or Mn^{2+} and nucleotide or enzyme and Mn^{2+}. ATP or Mn^{2+}. ADP showed no enhancement in proton relaxation rate of water coordinated to the metal thereby showing that the metal ion does not act as a bridge between the enzyme and the substrate. The overall view is that the metal ion (Mg^{2+}) possesses no affinity for the enzyme. It only helps in polarising

the γ-phosphoryl bond at ATP that is split[56] when glucose-6-phosphate is formed. It forms with ATP the reactive species $MgATP^{2-}$ and at excess Mg^{2+} concentration the inhibition of the hexokinase reaction is due to the lowering of the concentration of the reactive $MgATP^{2-}$ species by the formation of the inactive ternary chelate Mg_2ATP.[56] Thus, the differences in kinetic results and interpretations could arise also due to differences in the actual concentration of $MgATP^{2-}$ used by different workers. Excess Mg^{2+} and ATP^{4-} both inhibit the reaction. Therefore it is essential to know the concentration of ATP^{3-}, ATP^{4-}, $MgATP^{2-}$, Mg_2ATP and Mg^{2+} in the assay mixture for proper interpretation of kinetic studies.

Recently, Sigel and Amsler[73] studied the hydrolysis of nucleoside triphosphates promoted by metal ions and suggested that the ideal divalent cation should be one which coordinates initially only to the β- and γ-phosphate group, thus allowing a shift along the phosphate backbone into the α,β coordination without removal of a binding site, a condition which is fulfilled by Mg^{2+} (see Fig. 4.8). Secondly, this metal ion should have a low tendency to interact with the base moiety of a nucleoside triphosphate (NTP) to prevent the formation of self-reactive dimers during the transport of the M^{2+}-substrate complex. An alternative mechanism possible to facilitate the phosphoryl transfer is the activation of the γ-phosphate group by the coordination of metal ion to this group only; this means that the metal ion must again be shifted from its initial NTP coordination.

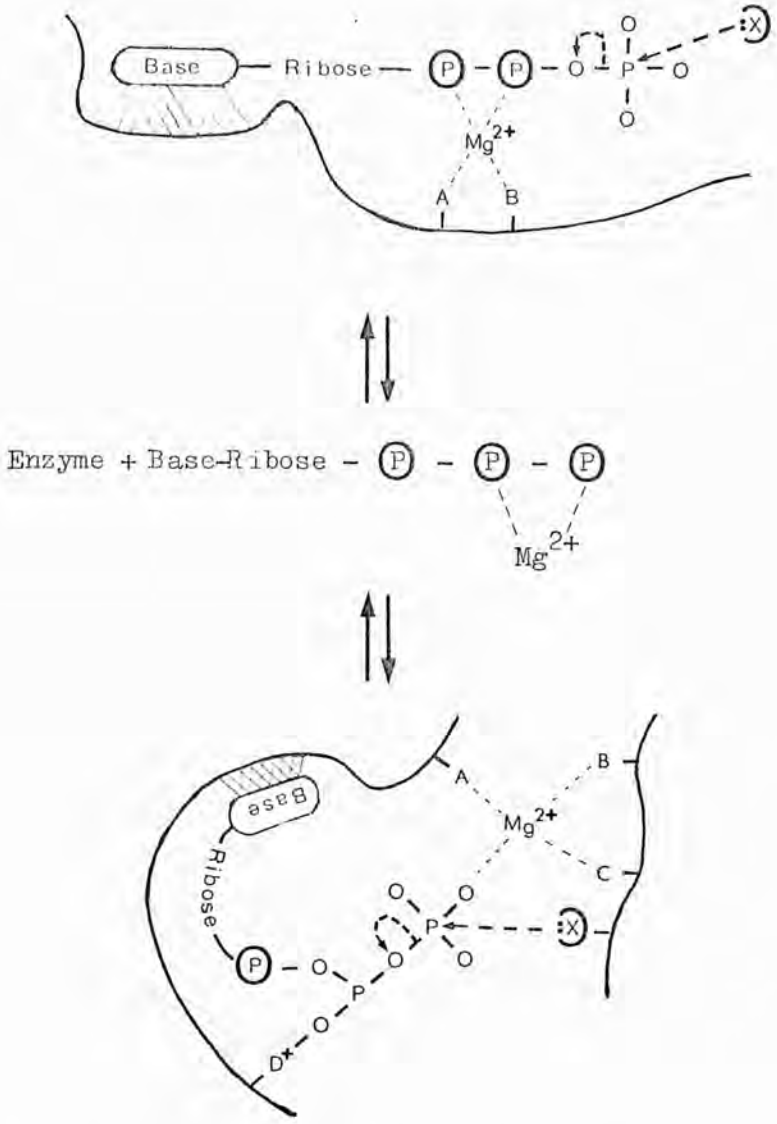


Fig. 4.8 Interaction of Mg^{2+} with the nucleoside triphosphate and enzyme[73]

Cleland and co-workers[74,75,76] have studied the interaction of hexokinase with nucleotide salts in which the magnesium is replaced by chromium III. They found that Cr ATP^{1-} is a competitive inhibitor with respect to $MgATP^{2-}$ and a non-competitive inhibitor with respect to glucose. Initially the reaction rate was time dependent in the presence of Cr ATP^{1-}, and this was attributed to an isomerisation of the initially-formed glucose-Cr ATP^{1-} enzyme complex.

Since bidentate $Cr(NH_3)_4ATP^{1-}$ and monodentate Cr ADP displayed inhibition which was greater with time, whereas bidentate Cr ADP was a poor inhibitor it was concluded that the actual substrates are β-γ-bidentate MgATP and β-monodentate ADP. Barnard and co-workers[77] have studied the binding of Cr ATP^{1-} (and glucose in the presence of Cr ATP^{1-})by pure hexokinase isoenzymes A and B. They found that at an ionic strength I of 0.5 isoenzymes A and B are monomers while at I = 0.05 A is a dimer and B a monomer. All of them bind one molecule of Cr ATP^{1-} and one molecule of glucose per monomer except dimeric A, which binds only one glucose molecule. Further, in absence of glucose, Cr ATP^{1-} does not bind.

4.2.7 ATPase Activity

Besides catalysing the phosphorylation of sugars yeast hexokinase also catalyses the slow hydrolysis of ATP [27,78] (ATPase activity). It has been shown that some inhibitors of the hexokinase reaction, such as 2-acetamido-2-deoxy-D-glucopyranose inhibited the ATPase reaction while others like the pentoses xylose and lyxose (glucose and mannose with the terminal hydroxymethyl groups removed respectively) stimulate the ATPase activity.[79,80] According to Rudolph and Fromm[80] this activation could apparently be due to the pentoses allowing water to replace the 6-hydroxyl of a hexose and place the enzyme in a conformation which facilitates the hydrolysis of ATP. Further, the pentoses xylose and lyxose were observed to lower the K_m for ATP to the same value as that of the K_m value for the hexokinase reaction (phosphorylation).[53,79,81] This has led to the proposal of an induced fit mechanism

in which the binding of some sugars like lyxose and xylose promotes both the binding of the nucleoside triphosphates to yeast hexokinase and also increases the rate of hydrolysis of the nucleotides. Lyxose also promotes the binding of the inhibitor Cr ATP^{1-} to hexokinase but to a lesser extent than does glucose.[77] After the initial activating effect of xylose, it was observed[44] that the enzyme (ATPase) is inactivated, Colowick has since shown that this inactivation is related to the phosphorylation of hexokinase.[37] Cheng et al.[82] reported that the inactive phosphoenzyme was stabilised by thiols and D-xylose, contains one mole of phosphate per monomeric unit, and also that the inactivation of the phosphoenzyme could be reversed by $MgADP^{1-}$ plus D-xylose.

$$\underset{\text{active}}{E^{*}} + Mg\,ATP^{2-} \leftrightarrows \underset{\text{inactive}}{E.P} + Mg\,ADP^{1-}$$

The slower inactivation of the enzyme in the presence of lyxose as compared to xylose was attributed to the higher concentration of Mg ADP^{1-} formed in the ATPase reaction[83] which induces the reactivation of the enzyme. A linear relationship is observed[83] between the inactivation and phosphorylation of the protein. The phosphoryl group (one phosphoryl group incorporated per enzyme subunit of 51,000 molecular weight) is found to be covalently bound by an ester linkage with a primary hydroxyl group of a serine residue of the protein.

In order to obtain a better insight into the effect of various substrates, inhibitors and other species on the hexokinase activity an understanding of the binding mechanism is essential.

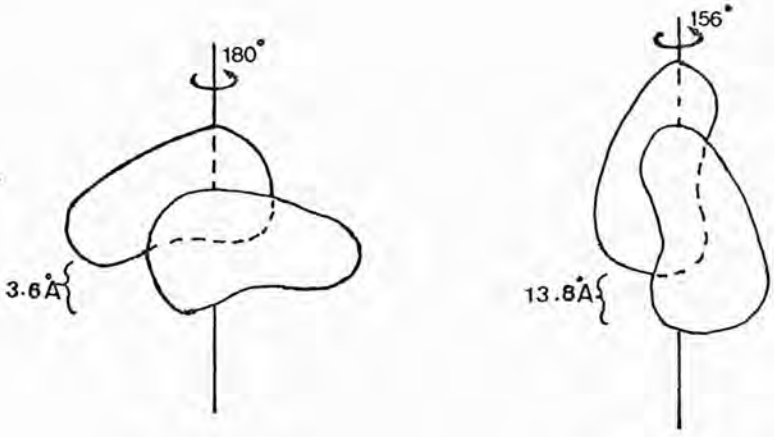
Shill and Neet[84] during a study of kinetic properties of yeast hexokinase B observed transient changes in reaction curves i.e. transients going from an initial high activity to a lower steady state activity. They carried out experiments documenting a slow change in hexokinase activity during assay which suggested that yeast hexokinase B undergoes a slow change in activity under the assay condition. This appeared to be dependent on some reversible change induced by the presence of both substrates ($Mg\ ATP^{2-}$ + D-glucose) and they concluded that a slow conformational change of the enzyme from an initial form to a second less active form would be consistent with the results. Roustan *et al.*[85] from difference spectrophotometry experiments inferred that sugar and phosphorylated sugar binds at the same part of the active site as they induce the same spectral effect. Kosow and Rose[47] proposed that yeast hexokinase B may exist as two conformational isomers, an inactive form which is favoured in the acid range and an active form favoured by various polyanions or by alkaline pH. Shill *et al.*[86] carried out reacting enzyme sedimentation and sedimentation velocity studies of yeast hexokinase B (P-II) at pH 6.75 and pH 8.55 to determine the size of the reacting form of the enzyme and to study the monomer-dimer equilibrium. From the $S_{20,w}$ values obtained they concluded that at low concentration the monomer was favoured while at high concentration of enzyme the $S_{20,w}$ values at the two pH's were interpreted as an association to dimers. In the absence of

substrates the enzyme was present as a dimer at both the pH's. In the presence of glucose alone dissociation of the dimer was observed to a certain extent while the combined effect of both the substrate (glucose + Mg ATP^{2-}) and not the products (glucose-6-phosphate and Mg ADP^{1-}) brought about the observed dimerisation at pH 8.55. Further, the presence of both substrate also enhanced the dimerisation at pH 6.75. Thus, the two conformations equilibrium and reacting steady state, not only have different kinetic properties, but apparently differ markedly in their self association properties. Colowick et al.[87] from their experiments concluded that there is preferential binding of the sugar to the dissociated form of the enzyme, but Hoggett and Kellett[88] observed that a saturation concentration of D-glucose did not bring about complete dissociation of yeast hexokinase. They concluded that both the sugar binding sites in the dimer were occupied and further that these sites were equivalent and non-cooperative at low pH values. Under intermediate conditions of pH (pH 7.0, I = 0.15M) where monomer and dimer co-existed, the binding of D-glucose showed a weak positive cooperativity. In addition, the binding was dependent upon the concentration of enzyme in the direction of stronger binding at lower concentrations. Further Kellett and co-workers[89] found that the binding of D-glucose to hexokinase A is stronger than to hexokinase B. Also D-glucose binding to hexokinase A dimer shows positive cooperativity. Williams et al.[90] found that upon dilution the specific activity of yeast hexokinase A was lowered until an equilibrium value is reached. The lost catalytic activity is recovered by lowering the temperature (from 30° to 25°).

The overall conclusion is that the hexokinase isoenzymes can exist in solution in monomeric and dimeric form.

4.2.8 X-Ray Crystallography

X-ray crystallographic studies have been carried out on yeast hexokinase BI, BII, and BIII crystal forms.[91-97] The BI crystal form (Fig. 4.9)is a dimer has space group $P2_12_12_1$ and molecular weight of 102,000 daltons. The two subunits are related by a 180° rotation plus a translation of 3.6°A along the symmetry axis of one subunit relative to the other. The BII crystal form is (Fig. 4.9) also a dimer, has space group $P\ 2_12_12_1$ and the subunits are related by a 156° rotation and a screw translation of 13.8°A.



BI ($P\ 2_12_12_1$) BII ($P\ 2_12_12_1$)

Does not bind substrates Binds substrates

Fig. 4.9 Diagrammatic representations of hexokinase dimers.

Unlike the other two forms the BIII crystal form is a monomer in the asymmetric unit and has space groups P $2_12_12_1$.[97]

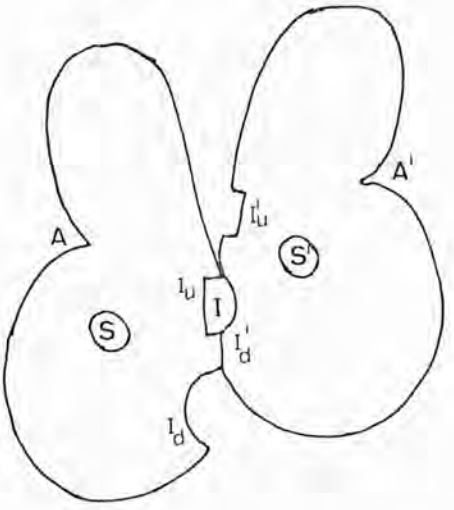


Fig. 4.10 Schematic drawing of the yeast hexokinase dimer showing the locations of observed sugar binding sites (S & S') and nucleotide binding sites (I formed by I_u & I'_d) and symmetry related sites (I'_u ; Id). A and A' are the AMP binding sites observed in B III crystal form.

The sugar (substrates, substrate analogues and inhibitors) bind to each subunit deep in the cleft (S & S') which separates the two lobes (Fig. 4.10), while the nucleotides one per dimer bind in the inter subunit region(I) which is formed by both subunits and lies on the molecular symmetry axis.

It was observed[93] that the BI crystal (crystallised from ammonium sulphate pH 7.0, plus 0.04M phosphate) form does not bind substrates and the region around the sugar binding site is very open to the solvent. On the other hand BII crystal form is crystallised from

concentrated phosphate solution (pH 7.0) and binds substrates. According to Steitz et al.[94] the conversion of BI into BII could be accompanied by a rotation around a single axis of contact between the two subunits and may be the conformational change implied by the hysteretic kinetics.

The two subunits of yeast hexokinase BII were found[95,96] to show unequal affinities for sugar substrates and for nucleotide substrates and analogues. Although the inhibitors O- and p-iodobenzoyl glucosamine and O-toluylglucosamine bind equally to both subunits, the degree of substitution of glucose (preferentially to the lower subunit) or xylose (preferentially to the upper subunit) is very different for the two subunits (Fig. 4.11). This negative cooperativity in substrate binding was suggested probably to result from the heterologous or non-equivalent environments for the two chemically identical subunits. In addition, there is a positive allosteric interaction between the sugar and nucleotide binding sites. Finally they[95,96] suggested that the ATP bound to the intersubunit(I) site could not function in direct phosphoryl transfer, but it might be involved in regulation of hexokinase activity. Recently Steitz et al.[98] observed that there are three binding sites for nucleotide in the dimeric hexokinase B crystals. The one lying between the two subunits is a unique site on the dimer and is presumably responsible for the allosteric activation of this enzyme. The other two nucleotides are bound near the active site and are probably involved in phosphoryltransfer.

Hoggett and Kellett[88] observed from their results that the phenomenon of half-sites reactivity observed in crystalline yeast

hexokinase BII[95,96] does not occur in solution. The simplest explanation of their finding two equivalent sites is that the dimer results from a homologous association of two identical subunits. They[88] suggested that the observation of negative cooperativity in the crystals was probably due to the asymmetric lattice forces

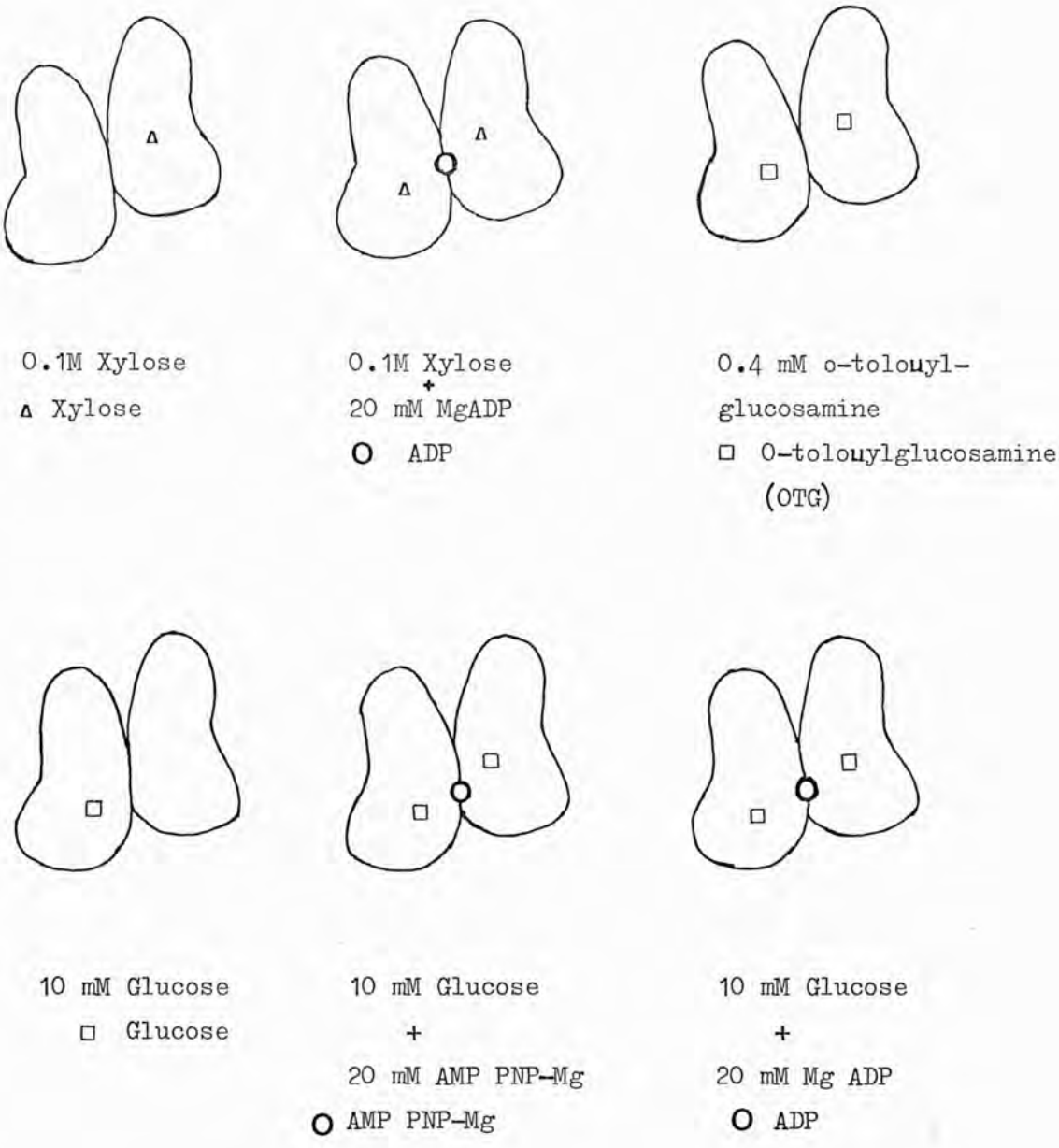


Fig. 4.11 Representation of the interaction with hexokinase B of glucose and xylose in presence and absence of nucleotide.

Thus, from the above evidence it appears that yeast hexokinase is a dimeric enzyme, having a molecular weight of about 100,000 daltons. It exists in various isoenzymic forms (natural and modified). The enzyme reacts with sugar (D-glucose) and nucleotide ($Mg\ ATP^{2-}$) in a random (steady state) sequential manner with the pathway with sugar binding first predominating to give D-glucose-6-phosphate and $Mg\ ADP^{1-}$ with the function of the divalent metal ion being to labilise the terminal phosphoryl group of the nucleoside triphosphate. It also exhibits the ATPase activity. The dimer is the catalytically active form. In solution there is a monomer-dimer equilibrium with the presence of D-glucose, $MgADP^{1-}$, high ionic strength, dilution and alkaline pH shifting the equilibrium towards the monomer, and the presence of D-glucose plus $Mg\ ATP^{2-}$, acid pH (>3.0) shifting it towards the dimer. Under intermediate conditions of pH (ie. pH 7) in solution the enzyme shows positive cooperativity and a burst type of slow transient. This is explained by a substrate induced conformational change depending upon the condition in which the experiment is carried out. In the crystalline state the B isoenzyme shows negative cooperativity due to a heterologous association of the two identical subunits caused probably by the presence of assymmetric lattice forces. The true picture would possibly emerge if the enzyme crystals could be grown with D-glucose rather than soaking the crystalline enzyme in D-glucose solution.

4.2.9 Substrate Specificity

The manifestation of specificity in the maximum velocity of the covalent step of enzymic reactions appears to require the utilisation of free energy that is made available from

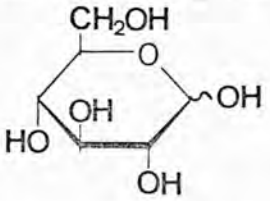
binding interaction with specific substrates. According to W.P. Jencks[99] induced fit and non-productive binding mechanisms are involved with control and specificity while destabilisation and entropy provide specificity as well as decrease the free energy of activation and thus supply the necessary driving force for catalysis.

It was found by earlier workers[100-102] that D-glucose, D-mannose and D-fructose were substrates of yeast hexokinase with D-fructose being proposed to react in the furanose form.[102] In 1958 A. Sols et al.[103] characterised the substrate specificity of yeast hexokinase by using the Michaelis constants and the rates of phosphorylation of a number of related compounds, taking D-glucopyranose as the model substrate. Their aim was to find which groups of the polyfunctional glucose molecule were involved in binding and which groups influence the phosphorylation. They concluded from their results that the hydroxyl groups at positions 1,3,4 and 6 of the D-glucopyranose molecule play an important role in the formation of the enzyme.substrate complex. In 1972 Bessel Foster and Westwood[104] tried to find the precise nature of the role of each of these hydroxyls since they could act as a proton donor and/or proton acceptor in the formation of a hydrogen bond. Also, chemical reactions are a possibility. They[104] used deoxy-fluoro-D-glucopyranose and related compounds, as the replacement of a hydroxyl by a fluorine substituent reduced the number of possibilities for interaction at the active site of yeast hexokinase. They[104] observed the variations in the value of K_m for the sugars and a corresponding change in the value of K_m for $Mg.ATP^{2-}$, showing that the binding of $Mg.ATP^{2-}$ is modified by the binding of the sugar. They[104] concluded from their results

that the hydroxyl groups at positions 3 and 4 could have similar roles in enzyme-substrate binding and that the hydroxyl groups at positions 3,4 and 6 do not function solely as hydrogen bond acceptors when located at the receptor site. Moreover, there was scope for modification at position 2. The replacement of hydroxyl groups at positions 2,3,4 and 6 by a fluorine substituent does not result in complete loss of binding to the enzyme. However, replacement of OH-1 in α-or β-D-glucopyranose by a fluorine substituent resulted in complete loss of binding to the enzyme which they[104] suggested could be explained by postulating either that OH-1 plays a vital role in the binding, or that D-glucose does not bind in its pyranose form. H. Katagiri et al.[105] and Wurster and Hess[106] found that the specificity towards α-and β-D-glucose of yeast hexokinase was not very much different. Since sugars can mutarotate in solution this leaves one with the possibility that the substrate can react in the cyclic pyranose forms and/or the cyclic furanose forms and/or the acyclic form.

The results of previous work which gives information about the specificity of hexokinase towards substrates and inhibitors is summarised in Tables 4.1, 4.2, 4.3 and 4.4. The majority of the work presented was carried out on crude hexokinase rather than on purified isoenzyme forms which may differ in their activities. Thus isoenzyme B has a three-fold greater maximum reaction velocity towards glucose than isoenzyme A (Ramel et al. 1971[29]). However, it is considered that the general conclusions to be drawn from all the data will not be affected by these differences.

Table 4.1 · Substrates of Yeast Hexokinase considered as modified D-Glucopyranose Derivatives



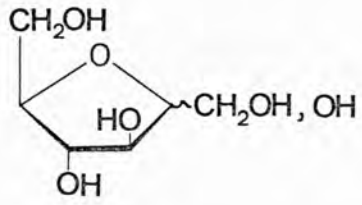
Compound	Modified at Carbon atom	Percentage of pyr-form at equilibrium α-pyranose	β-pyranose	Apparent K_m Moles/litre x 10^5	Relative V_{max} (w.r.t. D-glucose)	Phosphorylation coefficient $\frac{(V_m/K_m)\text{substrate}}{(V_m/K_m)\text{D-glucose}}$	Ref.
D-Glucose		36	64	10.0	1.0	1.000	103
1,5-Anhydro-D-glucitol	1		100	300.0	1×10^{-2}	3.3×10^{-4}	103
D-Manno-2-heptulose	1,2	+	+	15.0	+	$< 10^{-4}$	103
D-Mannose	2	67	33	5.0	0.8	1.500	103
2-Deoxy-D-arabino-hexose	2	55	45	30.0	1.0	0.300	103
2-Amino-2-deoxy-D-glucose	2	61	39	150.0	0.7	0.050	103
D-Arabino-2-hexosulose	2	+	+	2.0	0.2	1.000	103
2-Deoxy-2-fluoro-D-glucose	2	+	+	19.0	0.5	0.450	104
2-Deoxy-2-fluoro-D-mannose	2	+	+	41.0	0.85	0.350	104
2-Deoxy-2,2-difluoro-D-arabino-hexose	2	+	+	13.0	0.53	0.700	104

Table 4.1 (continued)

Compound	Modified at Carbon atom	Percentage of pyr-form at equilibrium α-pyranose	β-pyranose	Apparent K_m Moles/litre x 10^5	Relative V_{max} (w.r.t. D-glucose)	Phosphorylation coefficient $\frac{(V_m/K_m)\text{substrate}}{(V_m/K_m\ \text{D-glucose}}$	Ref.
2-Chloro-2-deoxy-D-glucose	2	+	+	210.0	0.54	0.044	104
D-Allose	3	18	70	$>10^4$	>0.1	1.0×10^{-4}	103
3-Deoxy-3-fluoro-D-glucose	3	+	+	7×10^3	0.1	2.4×10^{-4}	104
D-Galactose	4	27	73	5×10^3	$>2 \times 10^3$	4.0×10^{-6}	103
4-Deoxy-4-fluoro-D-glucose	4	+	+	8.4×10^3	0.1	2.0×10^{-4}	104
D-Threo-2,5-hexodiulose	5 or 2	+	+	60.0	1.4	0.140	107
5-Thio-D-glucose	5	+	+	400.0	0.013	0.0320	108
α-D-Glucose		100		13.0	1.27	1.150	106
β-D-Glucose		-	100	10.5	0.88	0.950	106
D-Glucose (equilibrium)		36	64	11.3	1.00	1.000	106

+ Possibility of it existing in that particular form.

Table 4.2 Substrates of Yeast Hexokinase considered as modified D-Fructofuranose Derivatives



Compound	Modified at Carbon atom	Percentage of furanose form at equilibrium α-F	β-F	Apparent K_m Moles/litre ($M \times 10^5$)	Relative V_{max} (w.r.t. D-fructose)	Phosphorylation coefficient $\frac{(V_m/K_m)\text{substrate}}{(V_m/K_m)\text{D-fructose}}$	Ref.
D-Fructose		9	31	98	1.0	1.0[a]	110
D-Arabinose	2	3		$>10^4$	$>1.1 \times 10^{-2}$	7.8×10^{-4}	103
1-Deoxy-D-fructose	1			1090	+		109
2,5-Anhydro-D-Mannose	1,2	100		31	0.37	1.17	110
2,5-Anhydro-D-Mannitol	2	100		630	0.52	0.08	110
2,5-Anhydro-D-glucitol	2	100		4700	0.37	0.008	110

[a] The phosphorylation coefficient of D-fructose is 0.3 relative to D-glucose (taken as 1.0)[105]

Table 4.3 Inhibitors of Yeast Hexokinase

Compound	Carbon atom modified relative to D-glucopyranose	Percentage pyranose form at equilibrium α-P	β-P	$K_i \times 10^5$ moles/litre	Ref.
1-Amino-1-deoxy-D-fructose	1,5	+	+		11
2-Deoxy-2-C-hydroxymethyl-D-glucose	2	+	+	150	103
2-Acetamido-2-deoxy-D-glucose	2	+	+	100	103
2-(3,5-dinitrobenzamido)-2-deoxy-D-glucose	2	+	+	2.5	104
2-O-methyl-D-glucose	2	+	+	6000	104
2-O-methyl-D-mannose	2	+	+	700	104
D-xylose[a]	5	3 3	67	1000	103
D-lyxose[a]	2,5	71	29	500	111
D-Glucose-6-phosphate	6	40	60	2500	112
6-Deoxy-6-fluoro-D-glucose	6			500	103

[a] = <1% exists in the furanose form

\+ = Possibly can exist in that particular form

Table 4.4 Non-substrates and Non-inhibitors of Yeast Hexokinase

Compound	Carbon atom(s) modified w.r.t. D-glucopyranose	Carbon atom(s) modified w.r.t. D-fructofuranose	Percentage of pyr, fur and acyclic forms at equilibrium α-P	β-P	α-F	β-F	Acyclic	Ref.
D-Glucitol			-	-	-	-	100	103
Methyl-α-D-glucoside	1	2,3,4,5	+					103
Methyl-D-fructoside	1,2,3,5	1			+	+		103
1,4-Anhydro-D-glucitol		2,3,4,5			+	+		103
L-sorbose	1,2,3,4,5	5	+	+	+	+	+	103
3-O-Methyl-D-glucose	3	2,3,4,6	+	+	+	+	+	103
3-O-Methyl-D-fructose	3	2,3,4,6	+	+	+	+	+	103

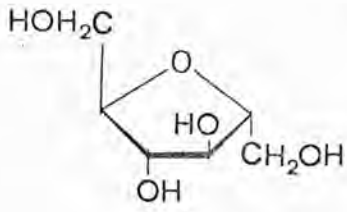
+ Possibly can exist in that particular form

The specificity of a substrate was taken as its phosphorylation coefficient (phosphorylation coefficient $= \frac{(V_{max}/K_m)_{\text{substrate}}}{(V_{max}/K_m)_{\text{glucose or fructose}}}$

with D-glucopyranose taken as the model substrate for the pyranose sugars and D-fructofuranose as a model for the furanose sugars.

It has been reported (see Table 4.3) that D-glucitol is neither a substrate nor an inhibitor of yeast hexokinase. This probably eliminates the possibility that the acyclic form is the reactive species. The evidence that the sugar substrates listed in Table 4.1 react in the pyranose form with yeast hexokinase rests on the observation of Sols *et al.*[103] that 1,5-anhydro-D-glucitol is a substrate. All the other substrates in Table 4.1 can mutarotate to the acyclic form and to cyclic furanose forms. However, the phosphorylation coefficient of 1,5-anhydro-D-glucitol is only 3.3×10^{-4} times that of D-glucose and therefore there must be some doubt regarding the validity of conclusions drawn from this observation. The sugar substrates listed in Table 4.2 are proposed to react in the furanose form with yeast hexokinase since (1) formation of the pyranose forms would block the reacting hydroxyl groups of fructose and arabinose and (2) 2,5-anhydro-D-mannitol and 2,5-anhydro-D-mannose (where the furanose conformation is fixed) are substrates with phosphorylation coefficients of 0.08 and 1.17 respectively relative to D-fructose. It is not known whether yeast hexokinase prefers the α or β furanose form. However, it is possible to suggest which would be the preferred form by consideration of some of the compounds listed in Table 4.2. Taking 2,5-anhydro-D-mannitol(38),2,5-anhydro-D-

glucitol(39)and 2,5-anhydro-D-mannose(40) and comparing them with D-fructose(41A,41B)



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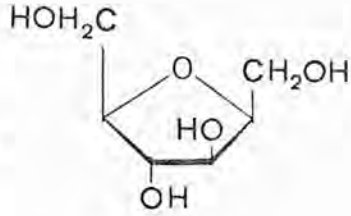

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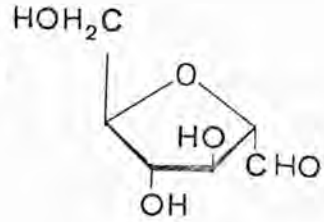
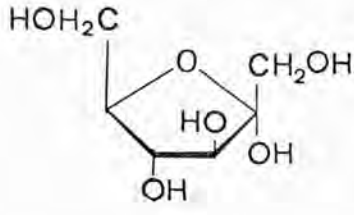
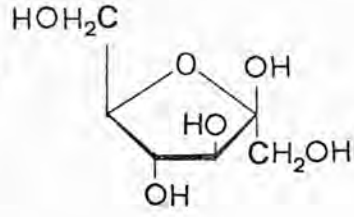


40

1.17



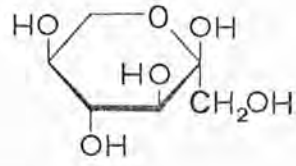
41A



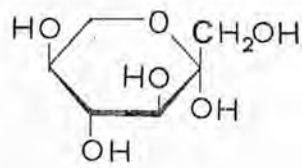
41B

1.0

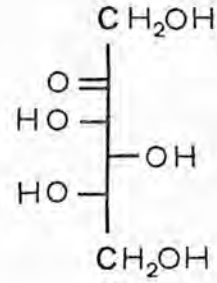
It is seen that the difference between 38 and 39 is that in 38 CH_2OH (C-1) group is <u>trans</u> to OH-3 while in 39 it is <u>cis</u> to OH-3 and this causes a difference in specificity by a factor of 10. This suggests that β-D-fructofuranose which has <u>trans</u> orientation of CH_2OH and OH-3 should be preferred to α-D-fructofuranose. Further, looking at 38 and 40 it is seen that the difference between them is that in 38 there is a CH_2OH (C-1) group while in 40 there is a CHO (C-1) group. However, they differ by a factor of 14.6 in specificity, suggesting that the interaction of a CHO group is more favourable than a CH_2OH group with the enzyme. It has been observed (see Table 4.3)



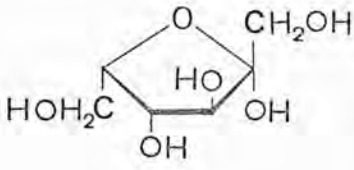
42A



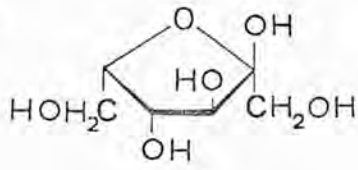
42B



42E

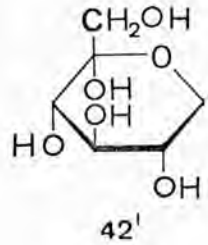


42C



42D

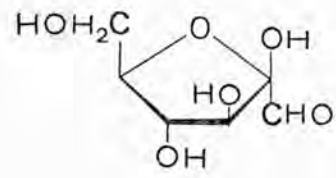
that L-sorbose is neither a substrate nor an inhibitor. L-Sorbose(42A-E) exists in the pyranose, furanose and acyclic forms as shown. It cannot react in the pyranose form as there is no CH_2OH on C-5, however, on reversing the molecule a potentially reactive primary hydroxyl group is present. However there is no hydroxyl group on C-1' but there is a hydroxyl group on C-5'.



42'

This could prevent interaction with yeast hexokinase. In the furanose forms the groups on C-4 (OH) and C-6(CH_2OH) are in a <u>cis</u>

relationship. This suggests that OH-4 and CH_2OH (C-6) in the furanose forms should be trans related otherwise there will be no interaction with yeast hexokinase. All this evidence leads one to conclude that the orientation of the groups in the preferred furanose form should be as shown below.(43)



43

This compound is β-D-arabinofurano-2-hexosulose which can also exist in the pyranose and acyclic forms. This compound shows a phosphorylation coefficient which is the same as D-glucose (i.e. 1.0 Table 4.1) but greater than D-fructose (ie. 1.0:0.3 Table 4.1(a)).

Thus, from previous evidence it is fairly certain that the sugar substrates, listed in Table 4.2, react in the furanose form with yeast hexokinase. However, there is some doubt about the structures of the reactive species of the compounds listed in Table 4.1. Also, the result of A. Sols et al.[103] that D-glucitol does not interact with yeast hexokinase needs confirmation.

4.3 RESULTS AND DISCUSSION

In order to obtain a better insight into the substrate specificity of yeast hexokinase paper chromatography and electrophoresis experiments were designed to observe the interaction of certain specific and poor substrates with yeast hexokinase.

The progress of phosphorylation was followed by treating chromatograms and electrophoretograms with molybdate reagent[113] and silver nitrate dip[114] respectively. D-glucose-6-phosphate was taken as a standard for both paper chromatography and electrophoresis. It was observed on paper chromatography that there were no blue spots given with the molybdate spray by the compounds tested before reaction except for the standard. After different time intervals blue spots were apparent for the various compounds shown in Table 4.5. These compounds possessed similar R_f values to that of the standard D-glucose-6-phosphate in the solvent system used. In order to obtain better resolution of starting materials and products electrophoresis was carried out followed by visualisation using silver nitrate dip reagent.[114] This reagent will readily detect all the reducing carbohydrate compounds whether phosphorylated or not; phosphorylated compounds however, will show a mobility towards the cathode (see Chapter 2) of ca. $5.0 \times 10^{-9}\ m^2 s^{-1}\ V^{-1}$ (taken as 1.0) in the phosphate buffer (pH 7.0, 0.5M) used. Electrophoresis not only confirmed that phosphorylation had occurred as observed by the migration of the sugar phosphates but also indicated the completeness of the reaction.

Having shown by paper chromatography and electrophoresis experiments that certain compounds shown listed in Table 4.5 could

Table 4.5 Paper Chromatography and Electrophoresis Data of the ATP-dependent Phosphorylation with Yeast Hexokinase.

Compound	Paper chromatography		Electrophoresis	
	t = 0	t = 24 h	t = 0	t = 24 h
D-Glucose-6-phosphate	+	+	1.0	1.0
D-Glucose	-	+	-	≃1.0
D-Arabinose oxime	-	+	-	≃1.0
D-Ribose oxime	-	+	-	≃1.0 (nc)
D-Mannose oxime	-	+	-	≃1.0
D-Galactose oxime	-	(a)	-	≃1.0 (nc)
D-Glucose oxime	-	+	-	≃1.0 (nc)
D-Glucitol	-	(a)	-	≃1.0 (a)
Methyl-α-D-glucopyranoside	-	(a)	-	≃1.0 (a)
4-Deoxy-D-xylo-hexose	-	+	-	≃1.0 (nc)
5-Deoxy-D-xylohexose	-	-	-	-

$R_{f_{G-6-P}} = 0.11$ a = a faint spot indicating very little reaction has occurred.

$M_{G-6-P}(PO_4^{3-}) = 1.0$ nc = the phosphorylation reaction is not complete

\+ = blue spot observed indicating that reaction has occurred.

Non-migrating markers for electrophoresis were the compounds at t = 0 and for D-glucose-6-phosphate it was D-glucose.

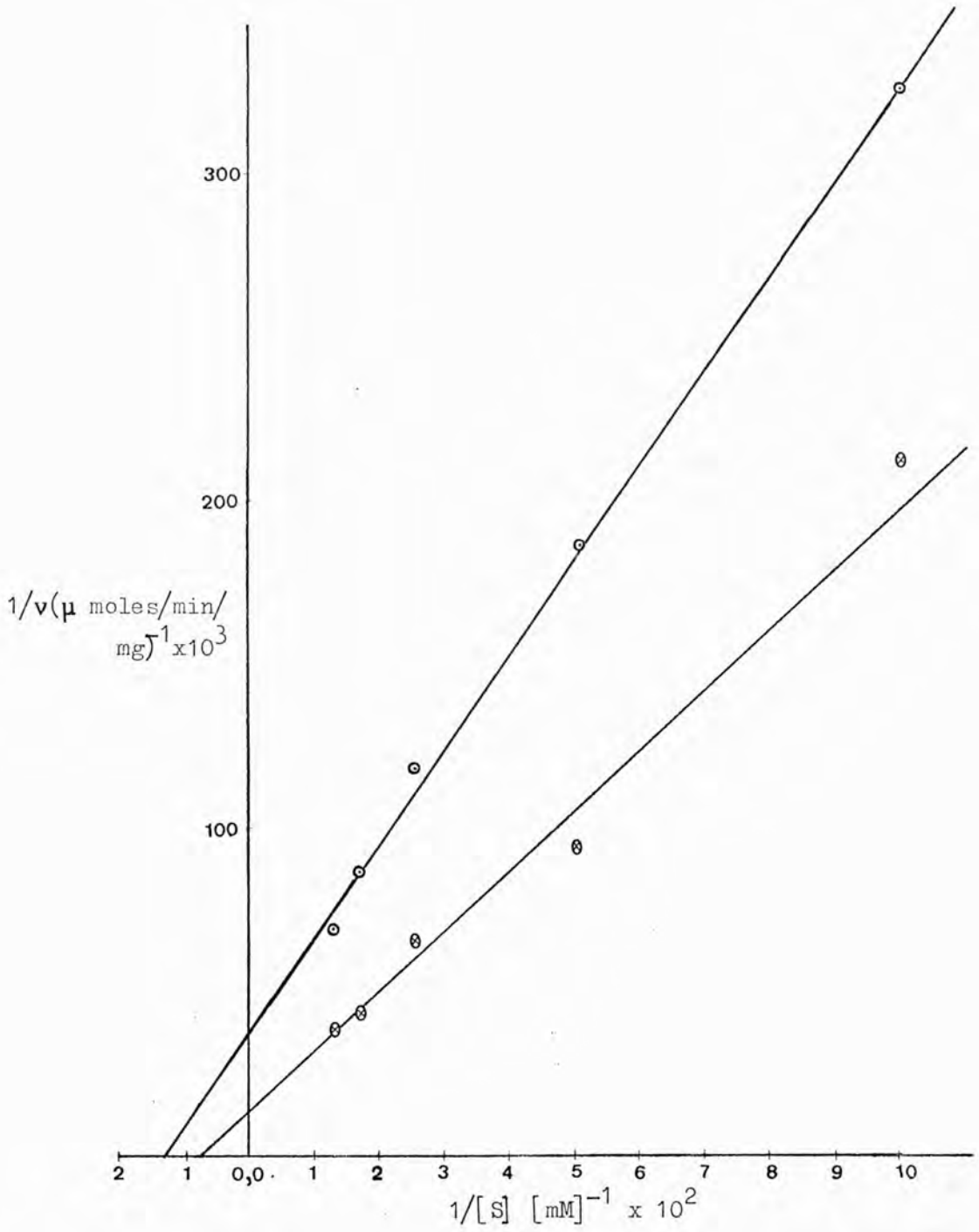


Fig. 4.12 Lineweaver Burk Plot. Interaction of anti(Z) ⊗ and equilibrated (syn(E, 80%) + anti(Z, 20%)) ⊙ D-arabinose oxime with hexokinase.

act as substrates, kinetic experiments were carried out using a coupled assay system to monitor the initial rates of the forward reaction shown below:

$$\underline{D}\text{-glucose} + Mg\ ATP^{2-} \xrightleftharpoons{\text{hexokinase}} \underline{D}\text{-glucose-6-phosphate} + Mg\ ADP^{1-} + H^{+}$$

$$Mg\ ADP^{1-} + \text{phosphoenolpyruvate} \xrightleftharpoons{pK} Mg\ ATP^{2-} + \text{pyruvate}$$

$$\text{Pyruvate} + NADH \xrightleftharpoons{LDH} \text{lactate} + NAD^{+}$$

(PEP = phosphoenol pyruvate, pK = pyruvate kinase, LDH = lactate dehydrogenase).

The initial rates were used to derive the Michaelis parameters for $\underline{D}$-arabinose oxime. The Lineweaver Burk[50] method has been found objectionable as it puts great weight on those points furthest from the ordinate, which are those obtained at lowest values of [s] (substrate concentration) and v (initial velocity). However, it is useful for testing linearity and for observing the type of inhibition involved. The Lineweaver Burk plots of fresh and equilibrated solution of $\underline{D}$-arabinose oxime are shown in Fig. 4.12.

The K_m and V_{max} values were calculated by the graphical method of Eisenthal and Cornish-Bowden[115] as it gives more accurate results. In the case of other compounds listed in Table 4.6 in order to make a semi-quantitative comparison the phosphorylation rates at 10 mM substrate and equal enzyme concentration were measured. The results of paper chromatography (Table 4.5), electrophoresis (Table 4.5) and kinetic (Table 4.6) experiments for yeast hexokinase show that $\underline{D}$-glucitol is not a substrate. A slow rate of

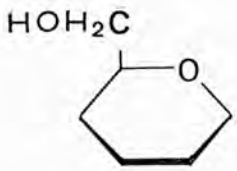
Table 4.6 Initial Rates observed of the ATP-dependent Phosphorylation with Yeast Hexokinase using a Coupled Enzyme Assay System.

Compound (a) (10 mM conc.)	Rate of phosphorylation relative to D-glucose (b)
D-Glucose	1.0
4-Deoxy-D-xylo-hexose	3.6×10^{-4}
5-Deoxy-D-xylo-hexose	-
D-Arabinose	1.53×10^{-3}
D-Arabinose oxime	1.44×10^{-2}
D-Ribose oxime	1.2×10^{-3}
D-Mannose oxime	3.36×10^{-3}
D-Glucose oxime	8.4×10^{-4}
D-Galactose oxime	1.2×10^{-4}
Methyl-α-D-Glucopyranoside	8.0×10^{-5}
D-Glucitol	1.1×10^{-4}

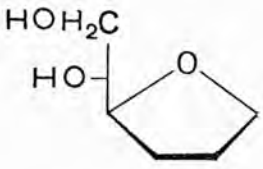
(a) All compounds used are taken as equilibrated solution.

(b) $V_{D\text{-glucose}} = 93.44$ μ Moles/min/mg of enzyme.

phosphorylation was observed in the kinetic experiment (Table 4.6). The change in absorbance corresponded to a conversion of 0.043% in 50 minutes and there was observed to be 0.05% contamination of D-glucose by GLC of the TMS derivative. The slow rate observed was ascribed to the reaction of this D-glucose impurity (ca. 0.005 mM). This confirms the previous result of Sols *et al.*[103] The fact that 4-deoxy-D-xylo-hexose was observed to be phosphorylated shows that a pyranose form can act as a substrate since this compound cannot adopt a furanose form and it is already known from the result of D-glucitol that the acyclic form cannot react. The kinetic result (Table 4.6 shows that 4-deoxy-D- xylo-hexose reacts slower than D-glucose, this could possibly be due to the absence of OH-4 (cf. the unreactivity of D-galactose). It is realised from the start that the sugars which are phosphorylated by yeast hexokinase must have an exocyclic hydroxymethyl group. Now most of the sugars which possess the exocyclic hydroxymethyl group attached at C-5 in the pyranose form (44A)



44A



44B

could also exist in the furanose form with an exocyclic 1,2-dihydroxyethyl ($CHOH-CH_2OH$) group (44B) In order to find out whether or not this grouping is reactive 5-deoxy-D-xylo-hexose was taken. This compound cannot adopt the pyranose form and in the furanose form

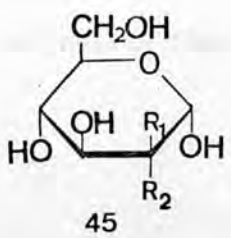
it will have 1(2-hydroxy ethyl) (rather than a hydroxy methyl) group. No reaction in the presence of yeast hexokinase was observed (Table 4.6). Knowing from earlier work[103,104] that a hydroxyl group at position five is not necessary for binding, this suggests that the hydroxymethyl group must be attached directly to the ring in order to be phosphorylated. Thus, from these results it seems that those sugars listed in Table 4.1 which can exist in the pyranose and furanose forms will react in the pyranose form if the exocyclic hydroxymethyl group is attached directly to the ring (i.e. at C-5). Having confirmed that the sugar substrates in Table 4.1 can react in the pyranose form with yeast hexokinase and knowing from earlier results[103,104] that positions 1,3,4, and 6 are important for binding, it was decided to consider the preference in the orientation of the hydroxyl groups. It appears that the presence of a hydroxyl group on C-1 is essential, however, the orientation of this group is not important (see α-and β-D-glucose specificity Table 4.1). The presence or absence of a hydroxyl group at C-2 does not alter the specificity greatly.[103] Also when it is present the orientation of the hydroxyl group as in D-mannose confers a higher specificity (phosphorylation coefficient 1.5) to that observed with D-glucose (1.0) possibly because of the higher concentration of the slightly preferred α-pyranose form (Table 4.1 phosphorylation coefficient of α-D-glucose : β-D-glucose is 1.15 : 0.95). This contribution may be estimated by substituting the specificity values of D-glucose for D-mannose (equilibrium proportions of α-pyranose : β-pyranose are 67 : 33[116]).

Specificity value of D-mannose (equilibrated) = % of α-D-mannose x specificity of α-D-glucose + % of β-D-mannose x specificity of β-D-glucose.

$$\frac{67}{100} \times 1.15 + \frac{33}{100} \times 0.95 = 1.084$$

This suggests that the presence of hydroxyl group at C-2 in D-mannose makes a small contribution towards specificity. This contribution is much smaller than those of the orientations of the hydroxyl groups at C-3 and C-4 (see Table 4.1, relative phosphorylation coefficients of : D-allose and D-galactose are 1.0×10^{-4} and 4×10^{-6}).[103] Further, replacement of the hydroxyl on C-3 in D-glucose by fluorine reduces the specificity considerably (Table 4.1 , D-glucose: 3-deoxy-3-fluoro-D-glucose specificity ratio 1.0: 2.4×10^{-4}). Similarly on replacing the hydroxyl on C-4 in D-glucose by fluorine the specificity is once again greatly reduced (Table 4.1, D-glucose : 4-deoxy-4-fluoro-D-glucose. Specificity ratio 1.0 : 2.0×10^{-4}). This shows that not only the presence of a hydroxyl group on C-3 and C-4 is vital but also its correct orientation is essential.

In the case of the hydroxyl group on C-5 it is seen that on replacing this hydroxyl group by a thio group (Table 4.1, D-glucose: 5-thio-D-glucose specificity ratio 1.0 : 3.2×10^{-2}) reduces the specificity. Finally, the hydroxymethyl group (C-6) should be attached to the ring directly for the reaction to occur (Table 4.5 and 4.6, cf. 5-deoxy-D-xylo-hexose). Therefore, the preferred substrate reacting in the pyranose form with yeast hexokinase should be (45)

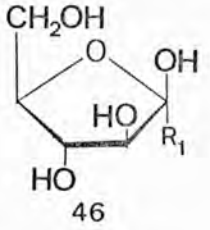


45

R_1 = OH and R_2 = H; D-Mannose (1.5)

R_1 = H and R_2 = OH; D-Glucose (1.0)

while as discussed earlier the preferred substrate reacting in the furanose form with yeast hexokinase should be (46).

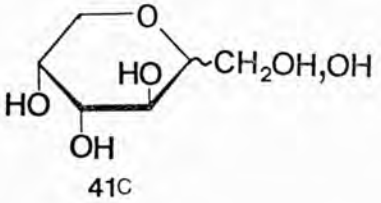


46

β-D-fructofuranose R_1 = CH_2OH (0.3)

D-Arabinofurano-2-hexosulose R_1 = CHO (1.0)

The observed specificities of the preferred pyranose and furanose substrates are very similar. There is a difference in the case of β-D-fructofuranose (specificity 0.3). However D-fructose can exist in solution at equilibrium in the α-furanose (10%), β-furanose (30%), α-pyranose (< 1%) and β-pyranose (60%) forms respectively.[117] In the furanose form it should react preferentially in the β-configuration as discussed earlier, while in the pyranose form (41c) D-fructose would not be expected to react owing to the absence of an exocyclic



41c

hydroxymethyl group on C-5. It has an exocyclic hydroxymethyl group on C-2 but no D-fructose-1-phosphate is formed therefore this possibility is eliminated. Hence, owing to the presence of other unreactive forms, the specificity of yeast hexokinase towards the reactive species of D-fructose (either 30% if β only needed or 40%) is greater than the overall value measured.

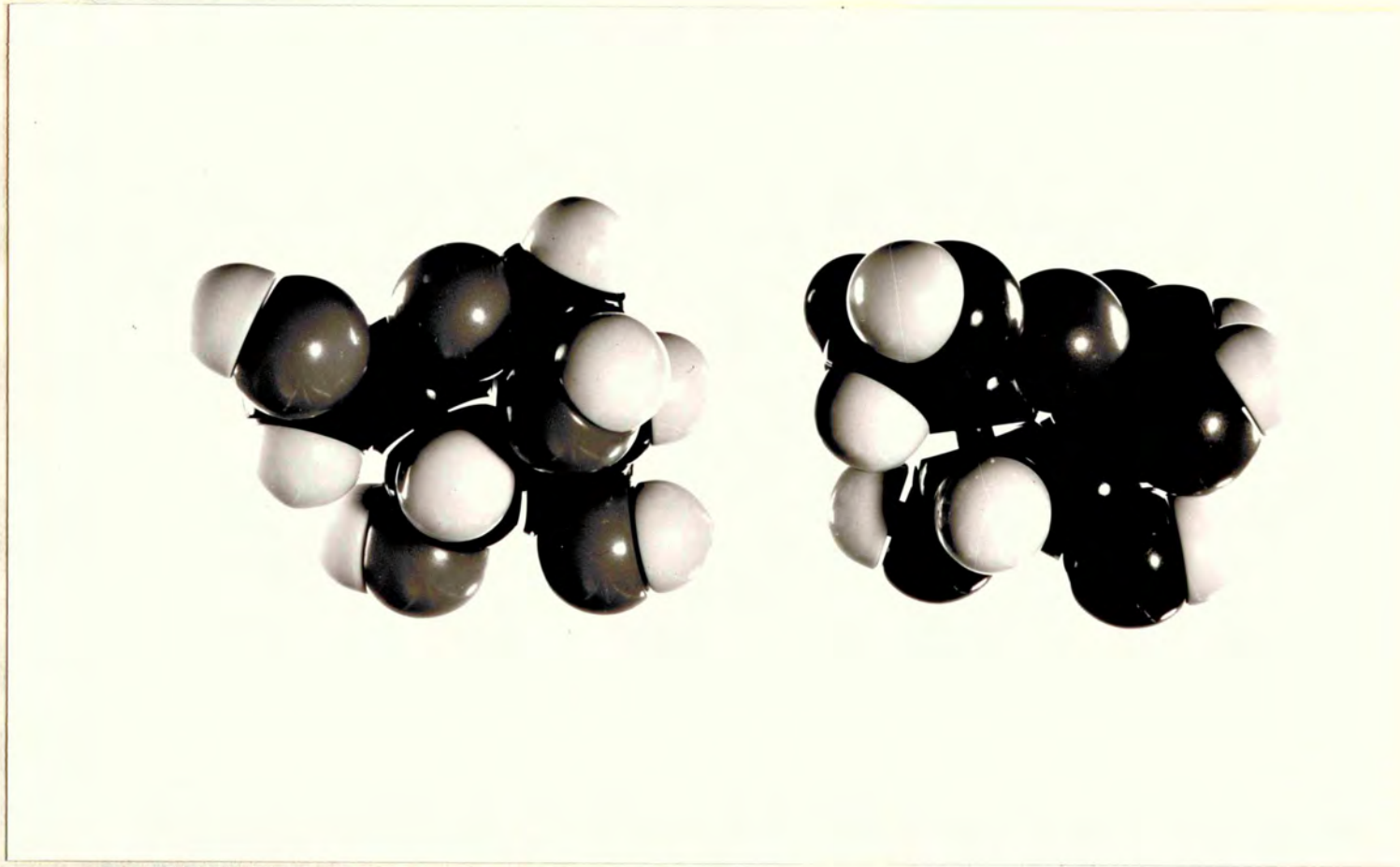
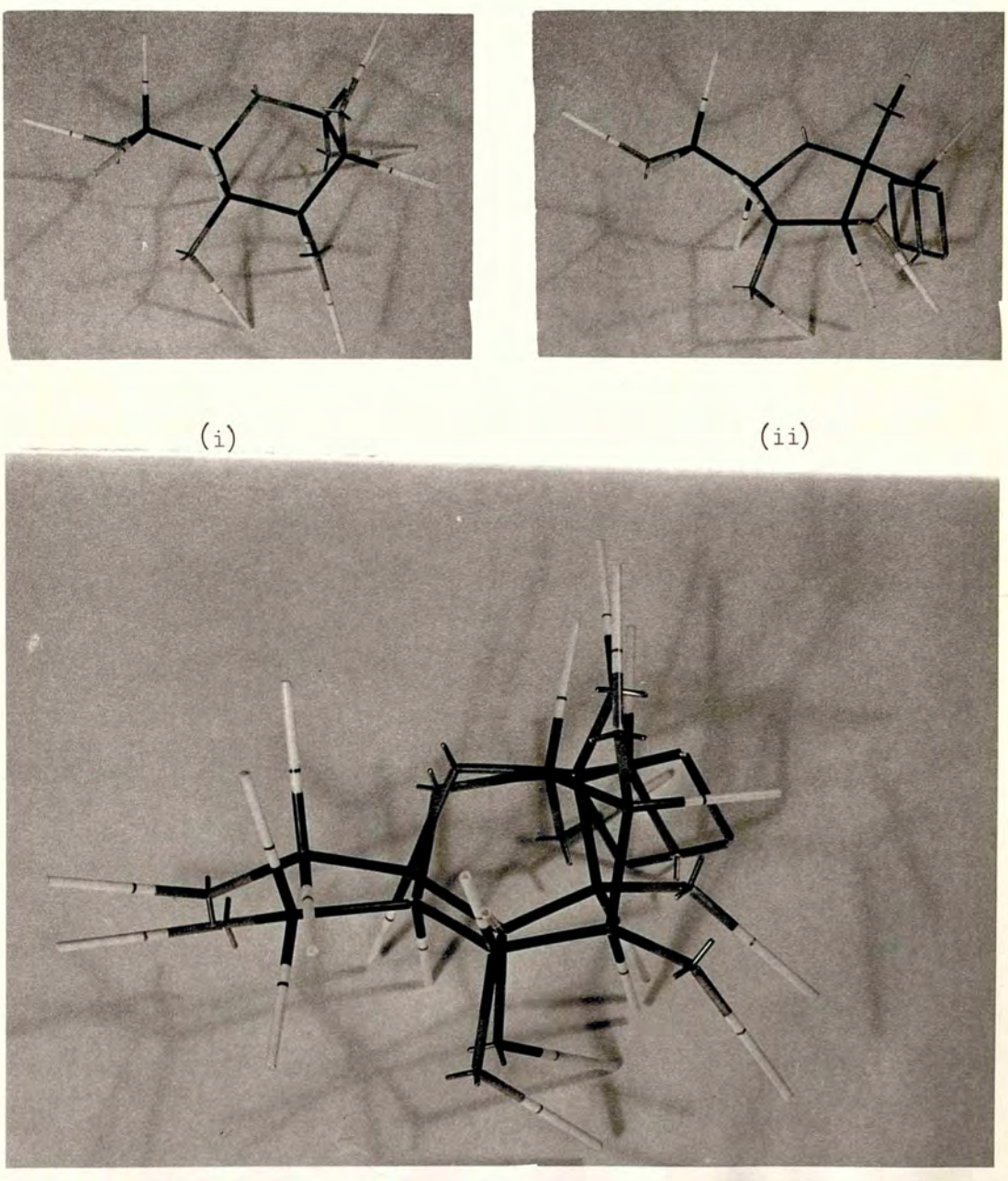


Fig. 4.13 Space filling models of the preferred furanose (D-Arabinofurano-2-hexosulose, left hand side) and pyranose (α-D-mannopyranose, right hand side) substrates of hexokinase. (black = carbon, grey = oxygen, light colour = hydrogen).



(i) (ii)

(iii)

Fig. 4.14 Preferred substrates of hexokinase (i) α-D-mannopyranose, (ii) β-D-arabinofurano-2-hexosulose and (iii) α-D-mannopyranose superimposed on β-D-arabinofurano-2-hexosulose.

Thus, the fact that yeast hexokinase will apparently readily accept both pyranose and furanose substrates prompts a comparison of the two forms on a steric basis. This can be done qualitatively by superimposing the models of the preferred pyranose and furanose forms (Figs. 4.13, 4.14). It is seen that the major difference is in the region of C-2 of the pyranose form, where it is known from earlier evidence (Table 4.1) that the structure is not very critical for binding. In the remainder of the structures a close steric correspondence is apparent between the pyranose and furanose forms. To date no x-ray studies of the binding of furanose substrates or inhibitors, to hexokinase have been reported, but it may be possible for both types of substrate to bind at the same site(s) on the enzyme.

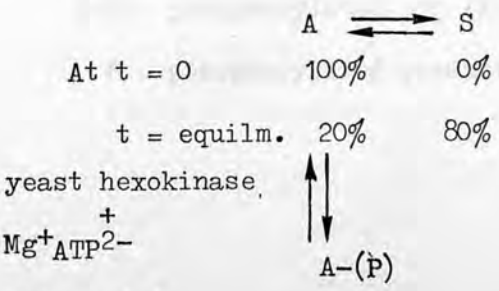
In the case of the carbohydrate oximes listed in Table 4.5, it is observed by paper chromatography (Table 4.5), electrophoresis (Table 4.5) and kinetic (Table 4.6) experiments that they are phosphorylated by yeast hexokinase. These carbohydrate oximes are known (see Chapter 2) to exist in the acyclic syn (E) and anti (Z) forms except for D-glucose oxime which exists in the β-pyranose form in the crystalline state and at equilibrium in the α-pyranose (7%), β-pyranose (23%), acyclic syn (E, 56.5 %) and anti (Z, 13.5 %) forms respectively. However, it has been confirmed that D-glucitol which is acyclic, cannot react with yeast hexokinase. Unlike D-glucitol the carbohydrate oximes used mutarotate in solution (see Chapter 3) and this could proceed via pyranose or furanose forms which act as hexokinase substrates.

Of the carbohydrate oximes tested, D-arabinose oxime was seen to react at the highest rate (Table 4.6). Initial rate data were obtained for equilibrated (*syn* (E) 80% + *anti* (Z) 20%, forms) and fresh (*anti* (Z) 100%) solutions of D-arabinose oxime. The data gave linear Lineweaver Burk plots (see Fig. 4.12) with fresh solution giving higher rates at equivalent concentrations. The Michaelis parameters are given in Table 4.7. Under the assay conditions used less than 1% of the substrates were consumed and about 10% mutarotation occurred (see experimental).

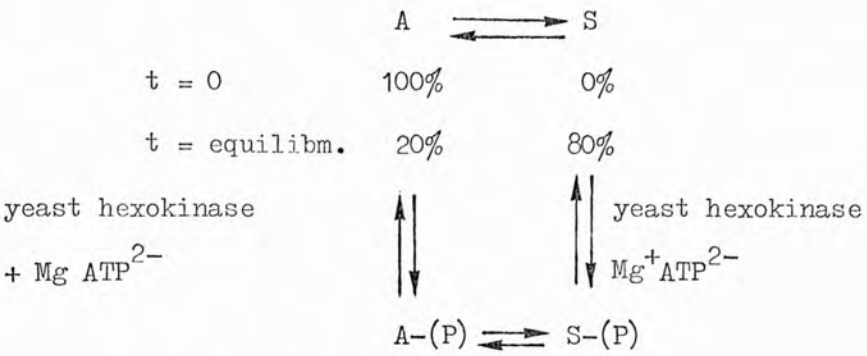
Table 4.7 Michaelis Parameters of D-Arabinose Oxime in the Reaction Catalysed by Yeast Hexokinase

Compound	K_m mM	V_{max} µMoles/min/mg protein
D-Arabinose oxime (fresh, *anti* (Z) 100%)	180	8.5
D-Arabinose oxime (equilibrated, *anti* (Z) 20% + *syn* (E) 80%)	97	3.1
D-Arabinose oxime (*syn* (E) 100%, calc.)	45	1.3

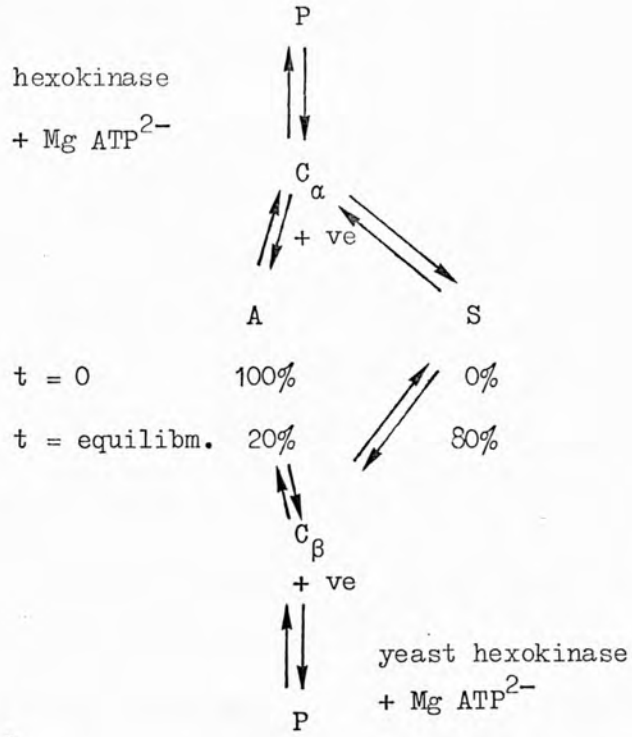
These results can be accounted for in three ways as shown below:



Scheme 4.1



Scheme 4.2



Scheme 4.3

where A = anti (Z)-form of D-arabinose oxime

S = syn (E)-form of D-arabinose oxime

A-(P)= phosphorylated anti (Z)-form of D-arabinose oxime

S-(P)= phosphorylated syn (E) - form of D-arabinose oxime

P = phosphorylated product

In Scheme 4.1 the enzyme reacts with only the anti (Z)-form. If that were the case then,

$$\text{rate equilibrium} = \frac{V_{max}(\text{anti}) \times [S]}{[S] + K_m (\text{anti})}$$

where [S] = concentration of anti (Z)-form present at equilibrium, substituting the values for the various parameters given in Table 4.7 gives:

$$\text{rate equilibrium} = \frac{8.5 \times 2}{2 + 180} = 0.0934 \ \mu \text{ moles/min/mg}$$

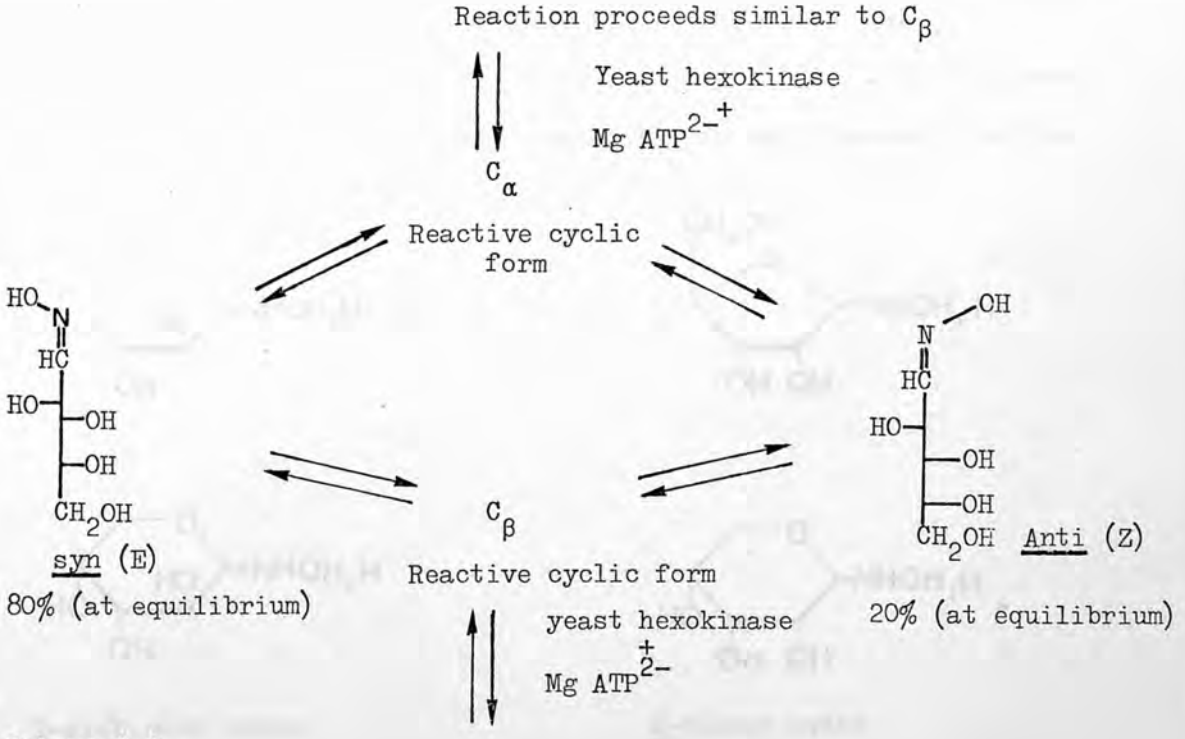
(at 10 mM equilibm. concn.)

The measured rate at equilibrium concentration of 10 mM is 0.3070 μMoles/min/mg. Also the $V_{max.}$ values should be the same for Scheme 4.1, but they are different. Therefore, this rules out Scheme 4.1.

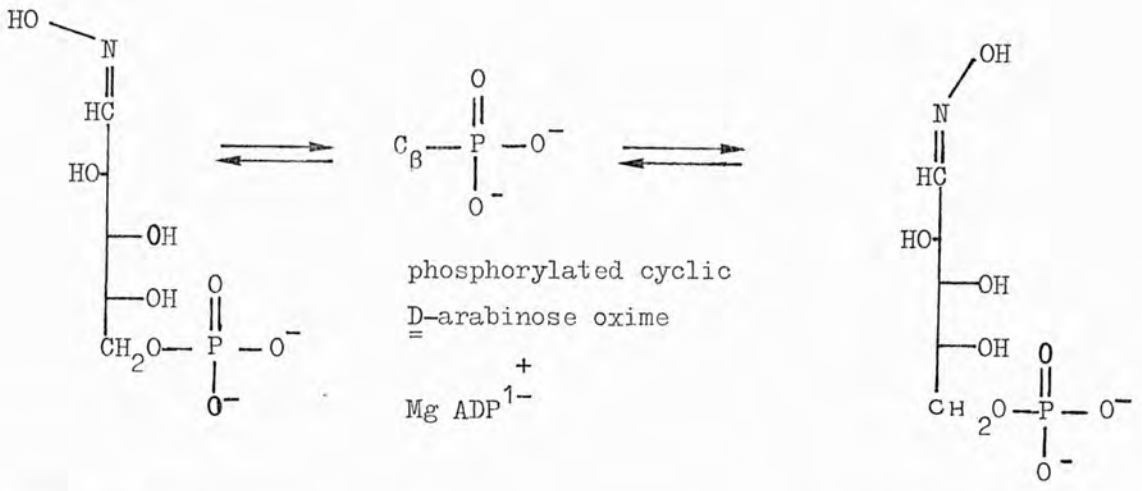
In the case of Scheme 4.2 the enzyme simply shows a preference for the acyclic anti(Z)-form over the syn (E)-form. However, this is considered to be unlikely because of the specificity of hexokinase discussed previously in particular the non-reactivity of acyclic compounds. The alternative explanation (Scheme 4.3) proposes the presence of two reactive cyclic species C_α and C_β which exhibit different reactivities towards yeast hexokinase. If only a single common reactive species were formed then the fresh and equilibrated solutions of D-arabinose oxime should show identical V_{max} values. The two reactive cyclic anomers of D-glucose show values of K_m and V_{max} (Table 4.1) which are different from each other and from an equilibrium mixture of the two.

The ATP-dependent phosphorylation of the equilibrated D-arabinose oxime by yeast hexokinase was followed by ^{1}H nmr at 60 MHz at 10 minute

intervals for the first hour, one hour intervals for 8 h and finally after 24 h. No measurable change in the proportions of the syn (E) and anti (Z) forms was observed from the intensities of these H-1 signals. Also the D-arabinose oxime phosphorylated by yeast hexokinase was isolated and characterised as discussed in Chapter 2. It was found to be a mixture of syn (E) and anti (Z)-D-arabinose oxime-5-phosphate. This shows that the equilibrium constant for interconversion of the phosphorylated products of the enzyme-catalysed reaction is the same as that for the starting oximes, and that the reaction products finally appear as an equilibrium mixture of syn (E) and anti (Z)-forms (Scheme 4.4). The actual K_m values of the reactive species must be at least an order of magnitude smaller than the values measured.



Scheme 4.4

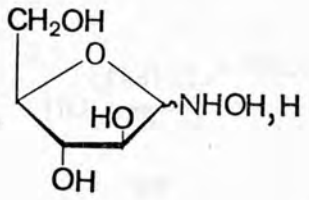


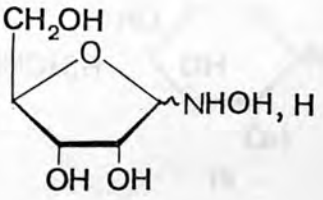
syn (E)-D-arabinose oxime-5-phosphate

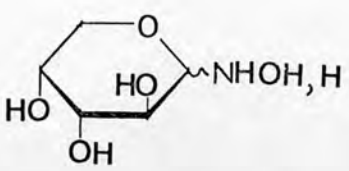
anti (Z) D-arabinose oxime-5-phosphate

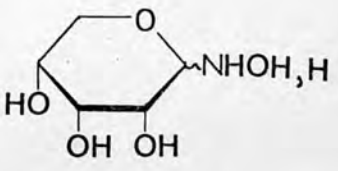
Scheme 4.4 (continued)

On the basis of the previous discussion one can consider possible structures for the reactive cyclic forms in the cases of the pentose and hexose oximes. Taking the pentose oximes first, viz D-arabinose oxime and D-ribose oxime, it is seen from the structures (15 , 21) that reaction is impossible in the pyranose form due





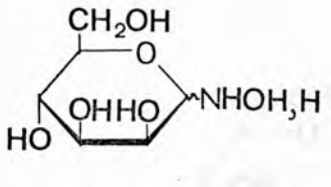




D-arabinose oxime
15

D-ribose oxime
21

to the absence of the exocyclic $-CH_2OH$ group at position 5. In the furanose form D-arabinose oxime is homomorphous with D-fructofuranose except for the presence of H and -NHOH instead of an OH and $-CH_2OH$ groups at C-1. D-ribose oxime is epimeric at C-2 to D-arabinose oxime and as a result it would be expected to react slower than D-arabinose oxime as observed (Table 4.6). In the case of the hexose oximes cyclisation to the pyranose and furanose forms is again possible and has been confirmed for β-D-glucopyranose oxime. However, in the furanose forms they will have an exocyclic glycol group (-CHOH-CHOH) and hence cannot react. Therefore they are likely to react in the pyranose form as they are homomorphous with their parent sugars.



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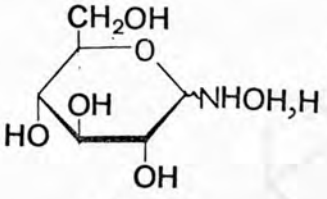

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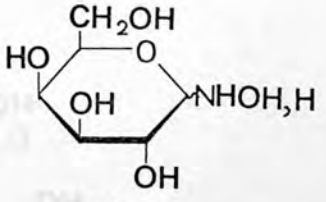

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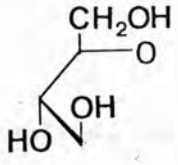
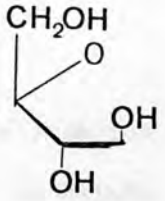
It is seen that the phosphorylation rates of D-glucose oxime (18) and D-mannose oxime (20) are very much smaller than those of the parent sugars. This can only be due to the presence of the -NHOH group on C-1 instead of the OH group in the corresponding unmodified sugar. Therefore, it appears that a bulkier substituent at C-1 than an OH group if all other factors remain the same tends to diminish the rate. This has also been observed in the case of methyl-α-D-glucopyranoside (Table 4.6). Finally, it is seen that the phosphorylation rates of the three hexose oximes tested follow the same order as do those of the parent monosaccharides.

Thus, yeast hexokinase interacts preferentially with sugars homomorphous to D-mannopyranose(47) and β-D-arabinofurano-2-hexosulose(43) and also with those acyclic sugars which can form reactive cyclic form(s) resembling either the preferred pyranose or furanose forms. Consideration of the preferred cyclic pyranose and furanose forms, shows that they both have the D-arabino configuration with the



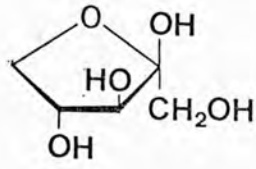
cyclic-pyranose form



cyclic-furanose form

former showing freedom of orientation of the OH on C-1 while the latter shows a preference for the β-furanose form. In addition some compounds (e.g. D-fructose and carbohydrate oximes) give rise to unreactive and/or possibly inhibitory species (besides the reactive species) present in solution. Moreover, the carbohydrate oximes other than D-glucose oxime probably have cyclic forms present in solution (at low concentrations) which have escaped detection by n m r

Finally, it is seen that lyxose and xylose have been used as inhibitors for pyranoses in x-ray crystallographic and kinetic work. It would be quite useful if in the future inhibitors for the furanoses could be used in x-ray and kinetic work. From the considerations presented in this chapter, a suitable potential inhibitor would be D-xylulose in the β-furanose form (48). It would confirm some of



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the above proposals as well as help in the delineation of a stereochemical model of the active site, which in turn will be helpful in the rational design of active site directed reagents.

4.4 EXPERIMENTAL

4.4.1 Materials

The yeast hexokinase used for this work was Sigma Type F300 (specific activity 465 IU/mg protein) and contained both the native and modified isoenzymic forms. All the reagents used except whereotherwise stated were BDH "Analar" grade.

4.4.2 Analysis of the ATP-dependent phosphorylation of sugars with yeast hexokinase by paper chromatography and electrophoresis

The enzymic phosphorylation reaction was carried out under conditions similar to those used by T.A.W. Koerner, Jr. *et al.*[118] Adenosine 5'-triphosphate disodium salt (ATP, 167 mg, 0.268 mMole) and Magnesium chloride ($MgCl_2 \cdot 6H_2O$, 57 mg, 0.28 mMole) were dissolved in 0.3M tris(hydroxymethyl)aminomethane buffer pH 7.5 (3 cm^3). The pH of the solution was adjusted to 7.5 with 0.2M sodium hydroxide. Then the sugar substrate (Table 4.5) (0.19 mMole) was added under slow magnetic stirring, followed by yeast hexokinase (334 I.U.), and the pH was adjustedto 7.5with 0.2M sodium hydroxide. The reaction was followed by spotting the solution on Whatman No.1 and No. 3 MM paper before addition of yeast hexokinase, immediately after its addition, then after 24 h. D-glucose-6-phosphate was used as a standard for both paper chromatography and electrophoresis experiments. Paper chromatography was carried out by the descending technique using diisopropyl ether: formic acid (3:2 v/v) solvent system followed by application of a spray[113] (9 cm^3 70% perchloric acid $HClO_4$, 1.7 cm^3 conc. hydrochloric

acid HCl, 2 g ammonium molybdate, 0.2 g disodium ethylene diamino-tetraacetate Na_2 E.D.T.A. diluted to 200 cm^3 with deionised water) which responded (blue colour) to the presence of phosphates. Electrophoresis was carried out on a Shandon L24 high voltage apparatus with 0.5M phosphate electrolyte (pH 7.0) for 1.5 h at 3kV on Whatman No. 3 MM paper. The standard migrating marker used was D-glucose-6-phosphate and the non-migrating markers employed were D-glucose and the unreacted compounds tested (Table 4.5). The paper (after completion of electrophoresis) was developed by silver nitrate dip reagent.[114]

4.4.3 Analysis of ATP-dependent phosphorylation of D-arabinose oxime with yeast hexokinase by ^{1}H nmr spectroscopy

The ^{1}H nmr spectroscopy at 60 MHz was carried out on a reaction mixture consisting of ATP (90 mg), anhydrous $MgCl_2$ (14 mg), and D_2O (0.5 cm^3). The pH was adjusted to 7.5 with NaOD in D_2O and the final volume made up to 1 cm^3. To this solution D-arabinose oxime (22 mg) was added and the ^{1}H nmr spectra taken. When the D-arabinose oxime had reached equilibrium proportion of *syn* (E) and *anti* (Z) forms (about 1.0 h), yeast hexokinase (280 I.U.) was added and the reaction followed by taking the ^{1}H nmr spectrum initially, after every 10 min for the first hour, then every hour up to 8 hours and finally every 8 hours up to 24 hours.

4.4.4 Measurements of initial rates of phosphorylation by yeast hexokinase

The method used was a coupled enzyme assay system described by D. Jaworck *et al.*[119] in which the following reagents were

added in a sequence to make a final volume of 3 cm^3 for the assay: tris (hydroxymethyl) aminomethane (0.5M, pH 7.5, 1.9 cm^3); phosphoenol pyruvate/potassium chloride (19.5 mM PEP/1.3 M KCl in tris buffer (stock solution), 0.2 cm^3); reduced nicotinamide-adenine-dinucleotide disodium salt (12 mM, in 5% sodium carbonate (stock solution), 0.05 cm^3); rabbit muscle pyrvuate kinase (EC 2.7.1.40) (90 I.U./cm^3 tris buffer, 0.05 cm^3); pig heart lactate dehydrogenase isoenzyme H_4 (EC 1.1.1.27) (1080 IU/cm^3 tris buffer, 0.05 cm^3); ATP/$MgCl_2.6H_2O$ (15 mM ATP/176 mM $MgCl_2$ $6H_2O$ in tris buffer (stock solution), 0.15 cm^3); sugar substrate (0.5 cm^3); yeast hexokinase (20 I.U. + 1 mg bovine serum albumin in 1 cm^3 tris buffer kept at 0-5°, 0.1 cm^3). This mixture (3 cm^3) was placed in a jacketed 1 cm cuvette and the absorbance was monitored against a blank cuvette (containing all reagents except yeast hexokinase, instead of which tris buffer (0.1 cm^3) was added) in a Pye Unicam SP 1800 spectrophotometer at 340 nm and 25°.

The concentrations of the sugar substrates were as follows:

4.4.4.1 To calculate Michaelis Parameters:

D-arabinose oxime (freshly prepared (anti-(Z)-form) 10 mM to 80 mM).

D-arabinose oxime (equilibrated (syn (E): anti (Z) 80:20), 10 mM to 80 mM).

4.4.4.2 To compare the rates of phosphorylation relative to D-glucose

The sugar substrates used were either (a) synthesised (b) from commercial sources or (c) gifts.

D-glucose (10 mM)[b]

D-arabinose (10 mM)[b]

Methyl-α-D-glucopyranoside (recrystallised, 10 mM)[c] Dr P. Finch

4-Deoxy-D-xylo-hexose (10 mM)[c] Dr D.Gibson, as Methyl-α-D-hexoside derivative

5-Deoxy-D-xylo-hexose (10 mM)[c] Professor W.G.Overend, as 1,2-O-isopropylidene derivative

D-arabinose oxime (10 mM)[a]

D-ribose oxime (10 mM)[a]

D-mannose oxime (10 mM)[a]

D-glucose oxime (10 mM)[a]

D-galactose oxime (10 mM)[a]

D-glucitol (10 mM)[b]

A blank correction was made by monitoring the absorbance of a solution containing all reagents mentioned in 4.4.4 except sugar substrate and yeast hexokinase against one containing yeast hexokinase but no 'sugar' substrate. The volume in each case was made up to 3 cm^3 with tris buffer.

4.4.5 Mutarotation of freshly prepared D-arabinose oxime occuring during assay

A correction was made for any isomerisation which could have occurred during the time interval in which the initial rate measurement was carried out with the freshly prepared solution of D-arabinose oxime (anti (Z)-form). The coupled enzyme reaction

mixture (4.4.4) containing no yeast hexokinase and freshly prepared D-arabinose oxime (240 mM) was prepared and the mutarotation followed by polarimetry in a 1 dm jacketed cell at 25° and 589 nm.

4.4.6 Hydrolysis of methyl-4-deoxy-α-D-xylo-hexoside[120]

The sample (50 mg) was dissolved in 90% formic acid (2.5 cm^3) and solid carbon dioxide added to give an inert atmosphere. The flask was sealed and heated for 6 h at 100°. The hydrolysate was diluted with deionised water (25 cm^3) and heated at 100° for a further 2 h. The solution was concentrated to dryness and residual formic acid removed by codistillation with methanol. The resultant oil was analysed by paper chromatography and found to be completely hydrolysed.

4.4.7 Hydrolysis of 1,2-O-isopropylidene-5-deoxy-D-xylo-hexose[121]

The sample (46 mg) was dissolved in deionised water (5 cm^3), then regenerated Amberlite IR 120 (H^+) resin (2 cm^3) was added and the mixture heated at 100° for 2 h. The solution was filtered and the resin washed with hot water (10 cm^3). The resulting filtrate along with the washings was concentrated to dryness, examined by paper chromatography and TLC on silica gel (development by methyl ethyl ketone saturated with water, detection by spraying with 10% ethanolic H_2SO_4); hydrolysis was found to be complete.

4.4.8 Gas-Liquid Chromatography of trimethylsilyl derivative of D-glucitol

D-Glucitol was dissolved in dry pyridine (1.0 cm^3) Hexamethyldisilazane (0.2 cm^3) and trimethylchlorosilane (0.1 cm^3)

were added and the mixture shaken for 1 minute and allowed to stand for 5 minutes. The white precipitate of ammonium chloride was removed by centrifugation, and the GLC carried out on a Pye 104 dual column chromatograph (9' x 0.25" column of 3% OV-225 on Chromosorb A, 80-100 mesh at 168°). The retention time of the trimethylsilyl derivative of D-glucitol was T = 0.76 compared to the trimethylsilyl derivative of α-D-glucose T = 1.00.

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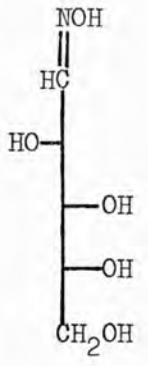
CHAPTER 5 THE INTERACTION OF CARBOHYDRATE OXIMES WITH D-XYLOSE ISOMERASE

5.1 INTRODUCTION

The aim of this work was to test carbohydrate oximes as potential transition state analogue inhibitors of D-xylose isomerase (D-xylose ketol isomerase, EC 5.3.1.5). As shown in arguments presented in chapter 1 of this thesis, D-arabinose oxime (15) is a potential inhibitor of D-xylose isomerase because of its structural relationship to the proposed enediol enzyme intermediate (16).



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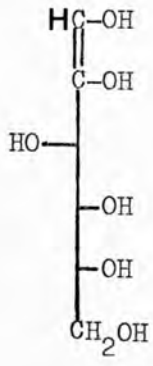

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The substrates for D-xylose isomerase (Streptomyces species) namely D-xylose, D-glucose, D-xylulose and D-fructose exist at equilibrium in aqueous solution in cyclic pyranose and/or furanose forms with a very small amount of acyclic form being present.[1] It has been suggested by Schray and Rose[2] that the enzyme binds the cyclic substrates and the resulting enediol intermediate can be formed either by a concerted β-elimination, or by a ring opening followed by an enolisation step. As shown in chapter 2 of this thesis,

carbohydrate oximes generally exist in the acyclic (syn (E) and anti (Z)) forms except for D-glucose oxime which exists in the cyclic β-pyranose form. The interaction of oximes which have different structures and stereochemistry would give further information about the enzymic isomerisation pathway as well as in the case of D-arabinose oxime support the presence of an enediol intermediate which it resembles.

The ever increasing demand for sugar and its rising price has led to rapid progress in research on alternative sweeteners during the past decade or so.[3-5] The greater inherent sweetness of fructose over that of glucose and sucrose makes it an attractive alternative provided it can be obtained economically. The production of fructose from glucose by using a glucose isomerising enzyme D-xylose isomerase (D-glucose isomerase, EC 5.3.1.18) is thus of practical importance. Large-scale production facilities for fructose are already in operation in the United States, Europe and Japan.[3-5] The estimated production of high fructose corn syrup (HFCS) in the United States alone was 1,400 to 1,800 million pounds in 1976 and production is expected to be 3,500 million pounds in 1977 and 7 to 8 billion pounds annually by 1980.[3]

The conversion of glucose to fructose is one of a group of reactions collectively known as Lobry de Bruyn-Alberda van Ekenstein transformation[6] which may be brought about chemically by alkali. A major limitation to alkaline isomerisation is the production of D-mannose which imparts a bitter taste to the solution. Additionally, a multitude of other rearrangements, fragmentations and dehydrations

occur giving rise to non-metabolisable materials such as psicose and objectionable coloured materials which are costly to remove.[7] The use of D-xylose isomerase (D-glucose isomerase) eliminates some of these problems under the correct conditions. The limitation to the use of enzyme has been the cost of its production and preparation. This is compounded by the solubility of the natural or free form of the enzyme which makes it difficult to remove and also to re-use. Insolubilisation or immobilisation of free enzymes by covalent attachments or by entrapment with inert support materials are the subject of much research[8,9] and insoluble D-xylose isomerase is in terms of scale at least the most successful outcome of this research.

Enzymes capable of isomerising D-glucose to D-fructose have been known since the early 1950's.[10,11] The majority of the organisms investigated require the isomerisation of the phosphorylated substrates. These enzymes have a narrow specificity for the aldose-ketose pair they interconvert. On the other hand those acting on the free sugars are usually characterised by their ability to isomerise several substrates and this at times leads to the same enzyme being called by more than one name. Marshall and Kooi (1957),[11] were the first to report an enzyme from P. hydrophila which was known to catalyse the isomerisation of D-xylose to D-xylulose, and also of D-glucose to D-fructose. A large number of micro-organisms that produce enzymes capable of catalysing the isomerisation of D-glucose to D-fructose have been found.[12-19] Initially, these investigations seemed to indicate the

presence of at least three different types of glucose-isomerising enzymes showing marked difference in certain physical characteristics. These enzymes are (1) an NAD-linked glucose isomerase from P. aerogenosides,[9] (2) a non-NAD-linked glucose isomerase from A. aerogenes which requires arsenate, and (3) the D-glucose isomerase obtained from various bacterial and yeast microorganisms. The Streptomyces species have probably been studied more intensively as sources of D-glucose isomerising activity than those of any other genus. These D-glucose isomerising enzymes have been shown to be, more accurately D-xylose isomerases based on observations that the Michaelis constants for D-xylose are lower than for D-glucose under identical conditions.[14-16]

5.2 PHYSICAL AND CHEMICAL CHARACTERISTICS OF D-XYLOSE ISOMERASE

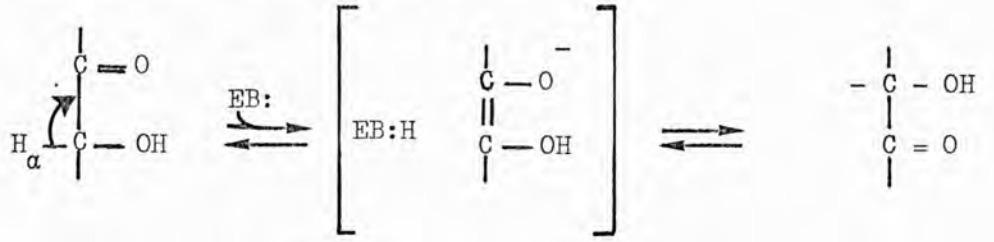
5.2.1 Isolation and General Properties of D-Xylose Isomerase

D-Xylose isomerases from several microorganisms have been studied in detail. The enzymes from Streptomyces species,[17] Bacillus coagulans,[18] and Lactobacillus brevis[19] have been purified to the degree of crystallisation and apparent homogeneity. Takasaki[17] isolated and crystallised D-xylose isomerase from Streptomyces albus. It had a molecular weight of 157,000, the pH optimum at 70° was 8.0-8.5, the temperature optimum 80° and the pH stability was wide (pH 4.0-11.0). The rate of the enzymic reaction was at a maximum in the presence of 1×10^{-3}M cobalt ion (Co^{2+}) plus 1.5×10^{-1}M magnesium ion (Mg^{2+}). The crystalline enzyme contained 1.4 atoms of cobalt and 0.3 atom of magnesium per

molecule of enzyme. It catalyses the isomerisation of D-xylose to D-xylulose and of D-glucose to D-fructose. Km (Michaelis constant) values of 3.2×10^{-2}M, 1.4×10^{-1}M and 2.3×10^{-1}M were observed for D-xylose,[17] D-glucose[14] and D-fructose[14] respectively.

5.2.2 Kinetic Studies of D-Xylose Isomerase

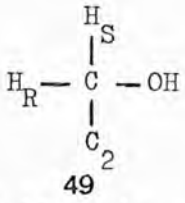
The aldose-ketose isomerases have been shown[20] to function as a base attacking specifically the hydrogen atom alpha to the carbonyl function of the substrate. The resulting conjugate acid-enzyme-enolate substrate complex was thought to serve as an intermediate (see Scheme 5.1).



Scheme 5.1

The occurrence of hydrogen transfer implies that one and the same proton is involved in abstracting the C2 proton of the aldose or C1 proton of the ketose. The proton transfer was demonstrated to be intramolecular.[20] In addition to transfer, the proton is known also to exchange with the medium as was shown in the case of D-glucose-6-phosphate isomerase.[20] This was taken as a criterion to show that the reaction proceeds through an enediol intermediate as discussed in chapter 1 of this thesis. In the case of D-xylose isomerase the rate of exchange with solvent was observed to be very low.

I.A. Rose et al.[21] examined three manganese-dependent bacterial aldose-ketose isomerases to determine the stereochemistry of the C1 proton of the ketose that is abstracted in the formation of the aldose. With D-xylose isomerase (L. brevis) the incorporation of tritium into an equilibrium mixture, occurred at < 0.4% of the rate of the forward reaction. In the case of D-xylose and L-arabinose isomerases it was the pro-R(49) position of the D-xylulose and L-ribulose that was activated, while it is the pro-S(49) position of L-fuculose that is labelled in tritiated water. These stereochemical results conform to those of other sugar



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isomerases listed in table 5.1. A minimal motion interpretation of the intramolecular proton transfer requires that the protonation occurs alternately to C1 or C2 from the same side of the proposed enediol intermediate. Thus, the absolute configurations at C1 of the ketose with hydrogen isotope introduced into the reaction and at C2 of the aldose allows one to determine the geometry of the enediol (see Fig. 5.1) according to Rose (1975).[22] Rose[22] further found that all the isomerases that use 2R-aldoses as substrates produce [1R-1*H] ketose products labelled at C1 in labelled water ($^{*}H_2O$), whereas those that act on 2S-aldoses give [1S-1*H]ketoses (Table 5.1). It follows that both 2R-and 2S-aldoses must proceed through cis-enediols which are oriented oppositely with respect to

Table 5.1 2R and 2S Isomerases

Isomerases	Interconversion		
2R-Aldose			
Triose phosphate	D-glyceraldehyde-3- P	→	Dihydroxy acetone -P
D-Xylose	D-xylose	→	D-xylulose
L-Arabinose	L-arabinose	→	L-ribulose
D-Ribose-5- P	D-ribose-5- P	→	D-ribulose-5- P
D-Glucose-6- P	D-glucose-6- P	→	D-fructose-6- P
2S-Aldose			
D-Arabinose	D-arabinose	→	D-ribulose
D-Mannose-6- P	D-mannose-6- P	→	D-fructose-6- P
P = phosphate group			

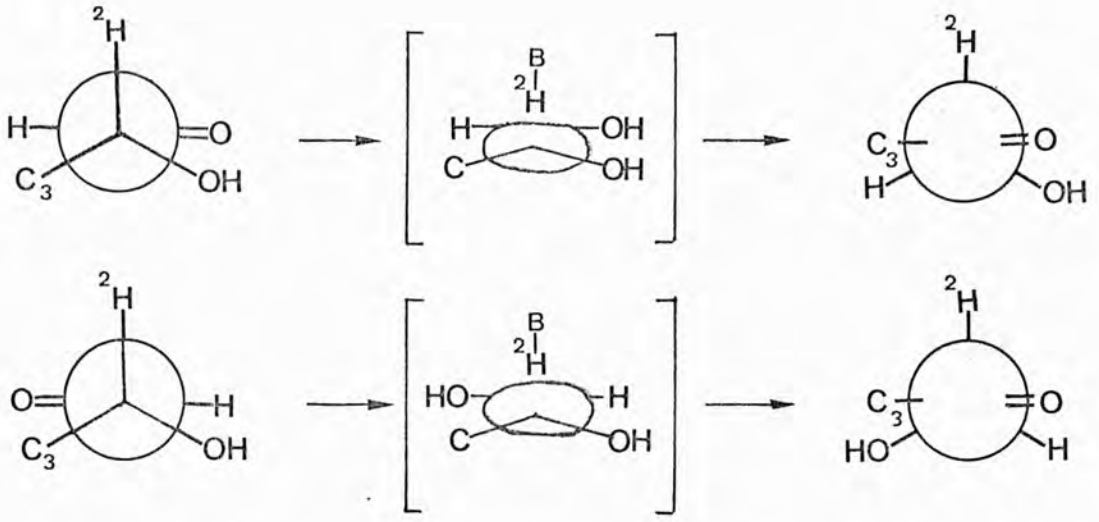


Fig. 5.1 Orientation of acyclic aldoses and ketoses for a (1) cis- or (2) trans-enediol process.

$-BH^+$ of the enzyme (see Fig. 5.2). The absolute stereospecificity of the labelling implies either a restriction on torsional freedom around the C2–C3 bond of the enediol intermediate relative to the

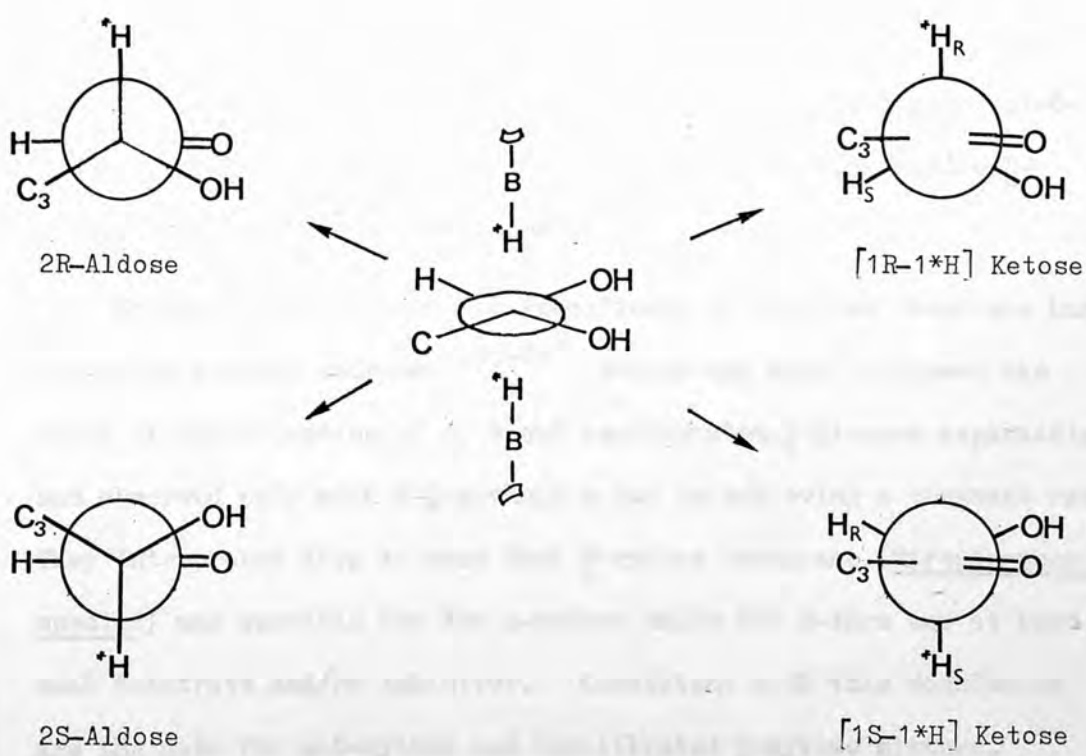


Fig. 5.2 Stereochemical course of 2R and 2S isomerases

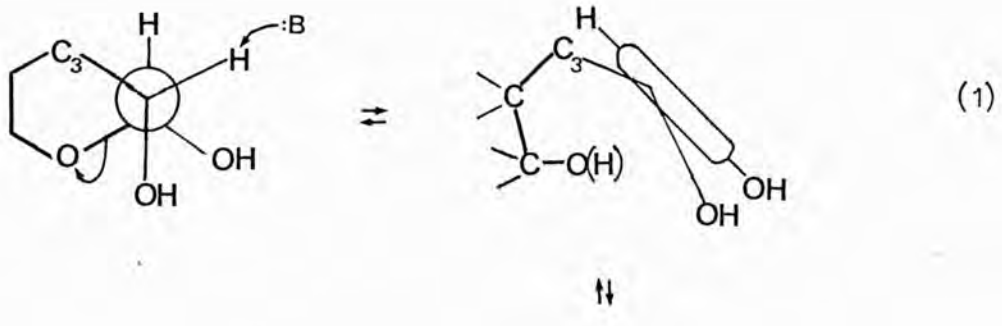
rate of the proton transfer or the presence of additional factors such as catalytic determinants in addition to -BH, the positioning of which are important, or both.

5.2.3 Anomeric specificity of D-Xylose Isomerase

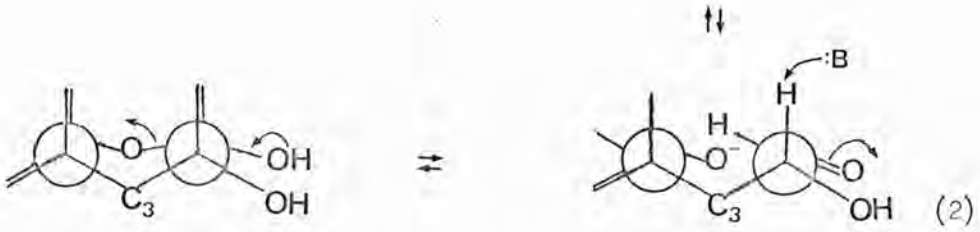
The enediol mechanism making use of the carbonyl function to increase the acidity of the α proton requires either that the carbonyl form of the substrate is used directly or that one of the metastable forms is converted to the carbonyl form as part of the catalysis. In the case of pentoses and hexoses of non-phosphorylated free sugars, the rates of mutarotation at pH = 7.0 are generally slow and the amount of free carbonyl form present in solution is thought to be very small.[23] To raise the level of the acyclic form of an aldose for subsequent enolisation, the isomerase enzyme must not only open the ring but must bind the acyclic form more tightly than the cyclic substrate. This was shown to be the case with D-glucose-6-phosphate isomerase. The inhibitor D-glucitol-6-phosphate (K_i = 25 μM) binds more strongly than 1,5-anhydro-D-glucitol-6-phosphate (K_i = 2.5 mM).[24]

Evidence for the anomeric specificity of D-xylose isomerase has come from several sources.[2,25-29] Schray and Rose[2] followed the rates of isomerisation of α, β and equilibrated D-glucose separately and observed only with β-D-glucose a lag in achieving a constant rate. They interpreted this to mean that D-xylose isomerase (*Streptomyces species*) was specific for the α-anomer while the β-form was at best a weak substrate and/or inhibitor. Consistent with this conclusion are the data for α-D-xylose and equilibrated D-xylose mixture.

The Km's of α-D-xylose and the mutarotated mixture are related as expected, if only the α-anomer was bound and the $V_{max.}$ of the mixture is not less than that for α-D-xylose. Also the enzymically formed D-xylulose anomer is greatly dominant, comprising 81% of the products of the D-xylose isomerase reaction at equilibrium. Moreover, the result of rapid overshoot equilibrium studies indicated that D-xylose isomerase does not catalyse the anomerisation of D-xylose and D-xylulose. They[2] further pointed out that only with the α-anomer could the generally accepted cis-enediol be formed without extensive orientation of the C1 and C2 hydroxyls from their cis relation (i.e. an axial-equatorial relationship) in the most stable chair or eclipsed boat conformation. They[2] suggested a mechanism which takes into account the relationship between the observation of a requirement for the cis structure both in the cyclic reactant and acyclic intermediate. According to this mechanism (scheme 5.3) the proposed cis-enediol intermediate can be reached in two ways: (1) direct elimination of the C2 proton and C1 ring oxygen and (2) ring opening followed by enolisation.



(1)



(2)

Scheme 5.3

The anomeric specificity was rationalised in terms of an elimination mechanism (equation 1, scheme 5.3) based on the argument that only anomers with cis-1,2-hydroxyl configuration are capable of undergoing an anti-elimination to form the cis-enediol. This can occur only in the skew boat conformation. An anti-elimination from the β-anomer would yield a trans-enediol. However, they observed that methyl-α-xylopryanoside which binds to the enzyme was not converted into a ketose. The alternative mechanism shown in equation 2 (Scheme 5.3) accommodates the ring opening with the classical enolisation mechanism. Here the ring closure inevitably leads to a cis relation between the C1 and C2 hydroxyls of the aldose if the cis relation of the enediol is maintained in the acyclic form of the enzyme-bound aldose i.e. if rotation about C1-C2 bond is prevented at the time of ring closure. Further, when the proton adds to C2 the carbon chain beyond C2 must become oriented so that for ring closure to be least sterically hindered C5 hydroxyl can approach the C1 carbonyl only from the opposite direction of the original proton approach to C2.

Another piece of evidence for anomeric specificity came from Kersters-Hilderson et al.[25] They developed an accurate and rapid method for assaying D-xylose isomerase (L. brevis) based on a coupled

reaction with D-glucitol dehydrogenase. The rate of change in absorbance for the mutarotated D-xylose was observed to be slower than the freshly dissolved α-D-xylose.

More evidence for anomeric specificity came from nmr experiments. Feather et al.[26] found by nmr that the first product formed from reaction of D-xylulose with D-xylose isomerase, was the α-anomer of D-xylose. Mildvan and co-workers[27-29] by epr, longitudinal proton relaxation rates of water solution of D-xylose isomerase, and observation of a greater paramagnetic effect of enzyme-bound Mn^{2+} on the C1 proton of α-D-xylose than on the β-anomer, provided independent evidence for the specificity of D-xylose isomerase.

These specificity results indicate that, as concluded for the enediol, either torsional freedom of the aldose (or ketose) intermediate is restricted or that catalysis of ring closure requires additional functional groups with limited mobility. In the case of D-glucose-6-phosphate isomerase[30] these restrictions are appar ently relaxed, where absolute specificity implies restriction at the enediol state but where the ability to catalyse anomerisation implies that the carbonyl group is able to orient either face to the C5 hydroxyl. Thus, the restriction of rotation about the C2 - C3 bond with D-xylose isomerase indicates tighter binding in this region of the substrate.

An alternative possibility for the observed anomeric specificity was suggested to be due to the stereochemistry of the binding sites which were selected in the evolution of the isomerases to have changed only to accommodate different substrates.

5.2.4 Influence of Metal Ions

D-Xylose isomerases from L. brevis and S. species are known to require a divalent cation for activity.[17,19] Yamanaka[31] made a detailed kinetic analysis of the inhibition of D-xylose isomerase (L. brevis) by pentitols and D-lyxose as a function of manganese ion (Mn^{2+}) concentration. From the inhibition pattern it was concluded that the substrate as well as the inhibitors combine with the enzyme through a Mn^{2+} bridge with a compulsory order of Mn^{2+} binding preceding the binding of the substrate. Schray and Mildvan[28] working with D-xylose isomerases from both Streptomyces species and L. brevis by e p r and longitudinal proton relaxation rates of water observed both tight and weak binding sites for Mn^{2+}. They[28] also noted decreased proton relaxation rate enhancements in the presence of substrates and analogues and thought this to be consistent with the existence of an E-Mn^{2+}-S bridge complex. Mildvan and co-workers[27-29] examined the interaction of D-xylose isomerase (S. species and L. brevis) with Mn^{2+} and D-xylose by following the relaxation of water protons and with Mn^{2+} and D-xylose or the competitive inhibitor xylitol by observing their proton relaxation rates. According to Mildvan[27] the calculated Mn^{2+}-to-proton distances and coupling constants are consistent with the enzyme-Mn^{2+} complex binding the cyclic substrate α-D-xylose with the metal ion providing α activation. The next stage proposed is ring opening followed by enolisation with the proton which has been removed from C2 to form the cis-enediol conserved and transfered to C1 from the same side of the double bond to form D-xylulose. Closure of the furanose from above the plane of the

carbonyl group to form α-D-xylulose completes the reaction. Further, the inactive β-anomer of D-xylose binds to enzyme-Mn^{2+} complex as detected by changes in the water relaxation rate,[27] but shows a negligible paramagnetic effect on the C1 proton of the β form in the presence of enzyme-Mn complex.[29]

Further, Young et al.[29] from proton relaxation rates observed comparable effects for the enzyme-bound Mn^{2+} on C1, C2 and C5 protons of α-D-xylose suggesting that these protons are equidistant from the bound Mn^{2+}. Further, they[29] found that the calculated distances between the enzyme-bound Mn^{2+} and C1 proton of α-D-xylose and methylene proton of xylitol from relaxation studies were nearly the same. Also they[29] inferred from the substrate and water relaxation studies that two small ligands such as water molecules or a larger portion of the protein intervene between the bound metal ion and the bound substrate in the active ternary complex. Moreover, it has been proposed[21,22] that in the case of the isomerases the divalent metal ion which may be closely related to the active site functions to polarise the carbonyl group of acyclic substrate for the α-hydrogen abstraction and that evolution converged to the cis-enediol intermediate because of the possibility that a single electrophile such as a metal ion or an amino acid residue could function with either substrate.

5.2.5 Analogue Binding Studies

To support the presence of intermediates in enzyme catalysed aldose-ketose isomerisation reactions an indirect approach avoiding the employment of fast kinetic methods has been the synthesis

and testing of substrate and transition state analogues. The substrate analogues would be those that resemble the acyclic configuration or possess the stereochemistry of either the α- or β-anomer of the substrate but are incapable of anomerisation. The substrate analogues may be examined either as substrates or as competitive inhibitors. If they function as substrates a comparison of their V_{max} and K_m values with those of the natural substrate may suggest the anomeric preference of the enzyme. In the case of analogues which act as inhibitors their K_i values should be lower than the K_m (or K_s) values of the substrate indicating tighter binding.

It is seen from Table 5.2 that the D-xylose isomerases from L. brevis[16,19] and B. coagulans[15] catalyse the isomerisation of D-xylose D-glucose and D-ribose while the enzyme from S. species (S. albus)[17,32] isomerises only D-xylose and D-glucose.

K. Yamanaka[16,19] presented evidence that D-xylose isomerase (L. brevis) catalysed the isomerisation of D-xylose, D-glucose and D-ribose. These three sugars have in common a cis configuration of OH groups at C2 and C4. Pentoses with a trans configuration of OH groups at C2 and C4 do not act as substrates, thus D-arabinose, L-arabinose and D-lyxose are not isomerised by the enzyme. K. Yamanaka[31] found from inhibition studies of D-xylose isomerase that xylitol, D-arabinitol, L-arabinitol and D-lyxose (Table 5.2) are competitive inhibitors whereas ribitol was ineffective. From the difference in K_i values for D-lyxose and D- and L-arabinitol Yamanaka[31] concluded that the difference in structure at C1 must have a significant effect on the binding.

Table 5.2 Substrates and Inhibitors of Isomerases

Isomerase	Substrate, K_m	Inhibitor, K_i	K_i/K_m	Reference
D-Xylose (*L. brevis*)	D-Glucose, 9.2×10^{-1}M			19
Mn^{2+}	D-Ribose, 6.7×10^{-1}M			19,31
	D-Xylose, 50×10^{-3}M	Xylitol, 2.7×10^{-3}M	5.4×10^{-1}	19,31
	"	L-Arabinitol, 1.46×10^{-1}M	29.2	31
	"	D-Arabinitol, 1.3×10^{-1}M	1.9×10^{-1}	31
	"	D-Lyxose, 7.0×10^{-2}M	14.0	31
D-Xylose (*B. coagulans*)	D-Ribose, 7.7×10^{-2}M			15
Co^{2+}, Mn^{2+}, Mg^{2+}.	D-Xylose, 1.1×10^{-3}M	Xylitol, 2.5×10^{-3}M	2.27	15
	D-Glucose, 9.0×10^{-2}M	D-Glucitol, 3.0×10^{-2}M	3.3×10^{-1}	15
	"	D-Mannitol, 7.0×10^{-2}M	7.7×10^{-1}	15
D-Xylose (*S. species*)	D-Xylose, 3.2×10^{-2}M			17
= Co^{2+}, Mg^{2+})	D-Fructose, 2.3×10^{-1}M			17
	D-Glucose, 1.6×10^{-1}M	D-Glucitol, 37% Inhibition (0.01M concn.)		17
	"	D-Mannitol, 32% Inhibition (0.01M concn.)		17
	"	D-Gluconic acid, 15% " (0.01M)		17

Table 5.2 (continued)

Isomerase	Substrate, K_m	Inhibitor, K_i	K_i/K_m	Reference
D-Xylose (S. species) Co^{2+}, Mg^{2+}	D-Glucose, 1.4×10^{-1}M	D-Glucose oxime, 3.0×10^{-4} [a]	2.1×10^{-3}	This work
	"	D-Arabinose oxime, 7.5×10^{-4}M	5.3×10^{-3}	
	D-Fructose, 2.9×10^{-1}M	D-Glucose oxime, 3.0×10^{-4}M[a]	1.0×10^{-3}	"
	"	D-Arabinose oxime, 7.5×10^{-4}M	2.5×10^{-3}	
Triose phosphate	D-Glyceraldehyde-3-phosphate, 3.0×10^{-4}M	2-Phosphoglycolate, 4×10^{-7}M	1.3×10^{-3}	37
		2-Phosphoglycohydroxamate 3.0×10^{-6}	1.0×10^{-2}	37
D-Glucose-6-phosphate	D-Glucose-6-phosphate 6.0×10^{-5}M	5-Phosphoarabinonate, 2.7×10^{-7}	4.5×10^{-3}	24
	"	1,5-Anhydro-D-glucitol-6-phosphate, 2.5×10^{-3}M	42.0	24
	"	D-Glucitol-6-phosphate, 2.5×10^{-5}M.	4.2×10^{-1}	24

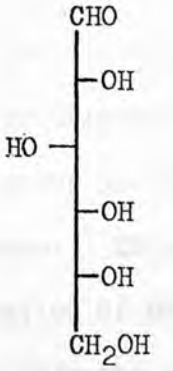
a = value calculated from Dixon Plots (Figs. 5.7, 5.8)

G. Danno[15] from the inhibition studies on D-xylose isomerase (B. coagulans) found that xylitol, D-glucitol, D-mannitol (Table 5.2) inhibited competitively. It was concluded from inhibition studies that although D-glucose, D-xylose- and D-ribose-isomerising activities were activated with different metal ions, the three isomerisation reactions occurred in the same active site of the same enzyme.

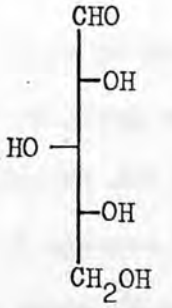
Takasaki and co-workers[17,32] found that at 0.01M concentrations D-glucitol, D-mannitol and D-gluconic acid inhibited the D-xylose isomerase (S. species) catalysed aldose-ketose isomerisation reaction (see Table 5.2). Young et al.[29] studied the interaction of D-xylose isomerase from two sources (L. brevis and S. species) with Mn^{2+} and D-xylose or xylitol by nmr. They[29] found that the amount of xylitol necessary to displace α-D-xylose from the substrate.enzyme. Mn^{2+} complex was consistent with the K_m value for α-D-xylose and the K_i value for xylitol.

The substrates of the three strains of D-xylose isomerase (Table 5.2) can exist in the acyclic and cyclic forms shown below:

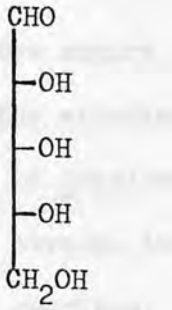
Acyclic forms:



D-glucose

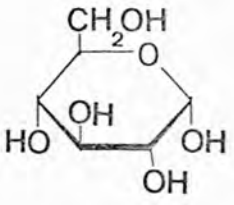


D-xylose

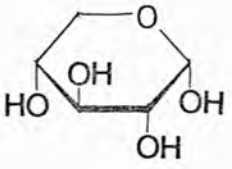


D-ribose

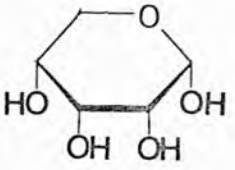
Cyclic pyranose forms:



α-D-glucose

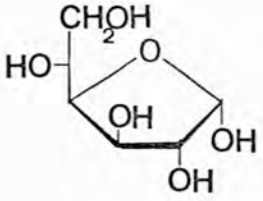


α-D-xylose

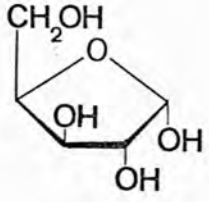


α-D-ribose

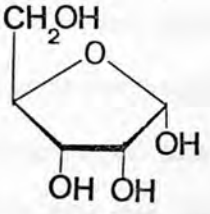
Cyclic furanose forms:



α-D-glucose



α-D-xylose



α-D-ribose

From the above structures it is apparent that all three sugars have similar orientations of OH group on C2 and C4 while the orientation of the OH group on C3 is different for D-ribose from that of D-xylose and D-glucose. Therefore, it appears that for all three strains the cis orientation of OH groups about C2 and C4 is vital for reaction. The enzyme from the S. species does not react with D-ribose indicating that the orientation of OH group about C3 is important also in this case, and should be as in D-xylose and D-glucose.

It may be concluded from the relative magnitudes of K_i value of D-xylitol (2.5 mM) with that of the K_m for D-xylose (5 mM) for L. brevis enzyme and that of D-glucitol (K_i = 29 mM) and D-glucose (K_m = 90 mM) for the B. congulansenzyme, that the enzyme from both sources prefers the acyclic form over the cyclic form. This is observed to be the case with D-glucose-6-phosphate isomerase where D-glucitol-6-phosphate (K_i = 25 μM) exhibits a stronger binding than 1,5-anhydro-D-glucitol-6-phosphate (K_i = 2.5 mM).[24] D-Lyxose (71% α-pyranose +29% β-pyranose)[33] is a C2 epimer of D-xylose and acts as a competitive inhibitor. This could be due to the incorrect orientation of the OH group on C2 precluding reaction but not binding. In the case of D-xylose isomerase from S. species it is seen from Table 5.2 that D-glucitol, D-mannitol, D-lyxose and D-gluconic acid inhibit the enzymic reaction, however, their K_i values are not known. It would be difficult to draw definite conclusions from these results However, owing to the similarity of the D-xylose isomerases of the three strains it would be expected to show a preference for the acyclic inhibitor as observed for the D-xylose isomerases from L. brevis and B. coagulans strains.

Thus, it is apparent from previous work that D-xylose isomerases from L. brevis and B. coagulans catalyse the isomerisation of D-xylose, D-glucose and D-ribose while the D-xylose isomerase from S. species catalyses the isomerisation of D-xylose and D-glucose only. The divalent metal ion is essential for activity and may form a bridge between the enzyme and substrate as well as provide α-activation. The isomerisation reaction is known to proceed via a cis enediol

intermediate from the stereochemical evidence obtained from labelling studies. The α-forms of the substrates are found to be preferred. Schray & Rose[2] proposed two mechanisms for the aldose-ketose isomerisation pathway *via* a *cis* enediol intermediate : (1) A concerted elimination and (2) ring opening followed by enolisation. The inhibition studies indicate a slight preference for acyclic forms by D-xylose isomerase for the *L- brevis* and *B. coagulans* strains, as is the case for D-glucose-6-phosphate isomerase. Moreover, in the case of D-xylose isomerase an inhibitor resembling the *cis*-enediol would be expected to bind more strongly if the reaction is proceeding through a *cis* enediol intermediate. Also, if an inhibitor resembling the acyclic form of the sugar could be interacted then it would be expected to provide information about whether the reaction is proceeding by a concerted elimination or step-wise ring opening followed by enolisation mechanism, because if the acyclic inhibitor binds strongly then it indicates a preference for the step-wise mechanism. With this background in mind the carbohydrate oximes were interacted with D-xylose isomerase (*S. species*). The carbohydrate oximes exist predominantly in the acyclic *syn* (E) and *anti*(Z) forms except for D-glucose oxime which exists in the β-pyranose form in the solid state and in solution at equilibrium as a mixture of α-pyranose (7 %), β-pyranose (23 %), *syn*(E 56.5 %) and *anti*(Z 13.5 %) forms (see Chapters 2 and 3 of this Thesis). D-Arabinose oxime resembles the enediol intermediate in the D-glucose to D-fructose isomerisation reaction while acyclic D-glucose oxime resembles the acyclic form of D-glucose; thus both compounds may act as inhibitors of D-xylose isomerase.

5.3 RESULTS AND DISCUSSION

In this work D-xylose isomerase from Streptomyces species was used. The enzyme requires Mg^{2+} (0.2% w/v) and Co^{2+} (0.02% w/v) as cofactors. For following the reaction kinetics of the isomerisation reaction different techniques have been used in the past.[22,31,32] Schray and Rose[2] used the cysteine-carbazole method[31] for assaying the ketose content in the isomerase reaction.

Kersters-Hilderson et al.[25] used Yamanaka's[34] procedure to investigate the anomeric specificity of D-xylose isomerase from L. brevis. In this method a coupled enzyme assay system is employed, using glutathione, NADH, D-glucitol dehydrogenase (EC 1.1.14) and the reaction kinetics was followed by observing the change in absorbance due to the oxidation of NADH to NAD^+. They[25] observed that the rate of isomerisation was faster with freshly dissolved α-D-xylose than with equilibrated solution of D-xylose.

Takasaki[35] used the cysteine-carbazole method[36] and a method involving direct observation of the change in optical rotation at 300 nm in a spectropolarimeter to follow the isomerisation reaction using either D-glucose or D-fructose as substrates. From the experimental data Michaelis parameters and equilibrium constants at different temperatures were calculated.[35]

The cysteine-carbazole and coupled enzyme assay systems measure the amount of ketose present. The former method suffers from a limitation in the concentration of aldose present, as beyond a certain limit it interferes with the ketose absorbance. The coupled enzyme method it is felt would not be appropriate in this work because of

possible interference with the action of D-glucitol dehydrogenase by the inhibitors to be studied. The direct observation by the kinetic method of Takasaki[35] where the reaction was monitored in a spectropolarimeter at 300 nm overcomes the drawbacks of the previous two methods. It has one possible deficiency namely that D-xylose isomerase is known to be anomer specific in its action (see section 5.2.3) and therefore according to this method one is measuring the enzyme catalysed isomerisation reaction as well as the chemical anomerisation reaction.

In this work the D-xylose isomerase (S. species) catalysed isomerisation of D-glucose and D-fructose was followed by withdrawing aliquots from the reaction mixture, quenching in perchloric acid, allowing the sample to equilibrate, and then the optical rotation values were read in the polarimeter at 365 nm or 436 nm. In this method at any particular polarimeter reading the substrate and product are at anomeric equilibrium. Also, the proportion of the equilibrated aldose and/or ketose present at a particular instant can be calculated from their measured individual equilibrium optical rotation values. The Michaelis parameters were obtained by this method for D-glucose and D-fructose. The K_m and V_{max} values (Table 5.3) were calculated from Lineweaver Burk plots (Figs. 5.3,5.4) using a least squares computer programme. The equilibrium constant value was calculated from the K_m and V_{max} values of D-glucose and D-fructose using the Haldane relationship:

$$K_{eq} = \frac{V_{max}}{K_m}_{\text{glucose}} \Big/ \frac{V_{max}}{K_m}_{\text{fructose}}$$

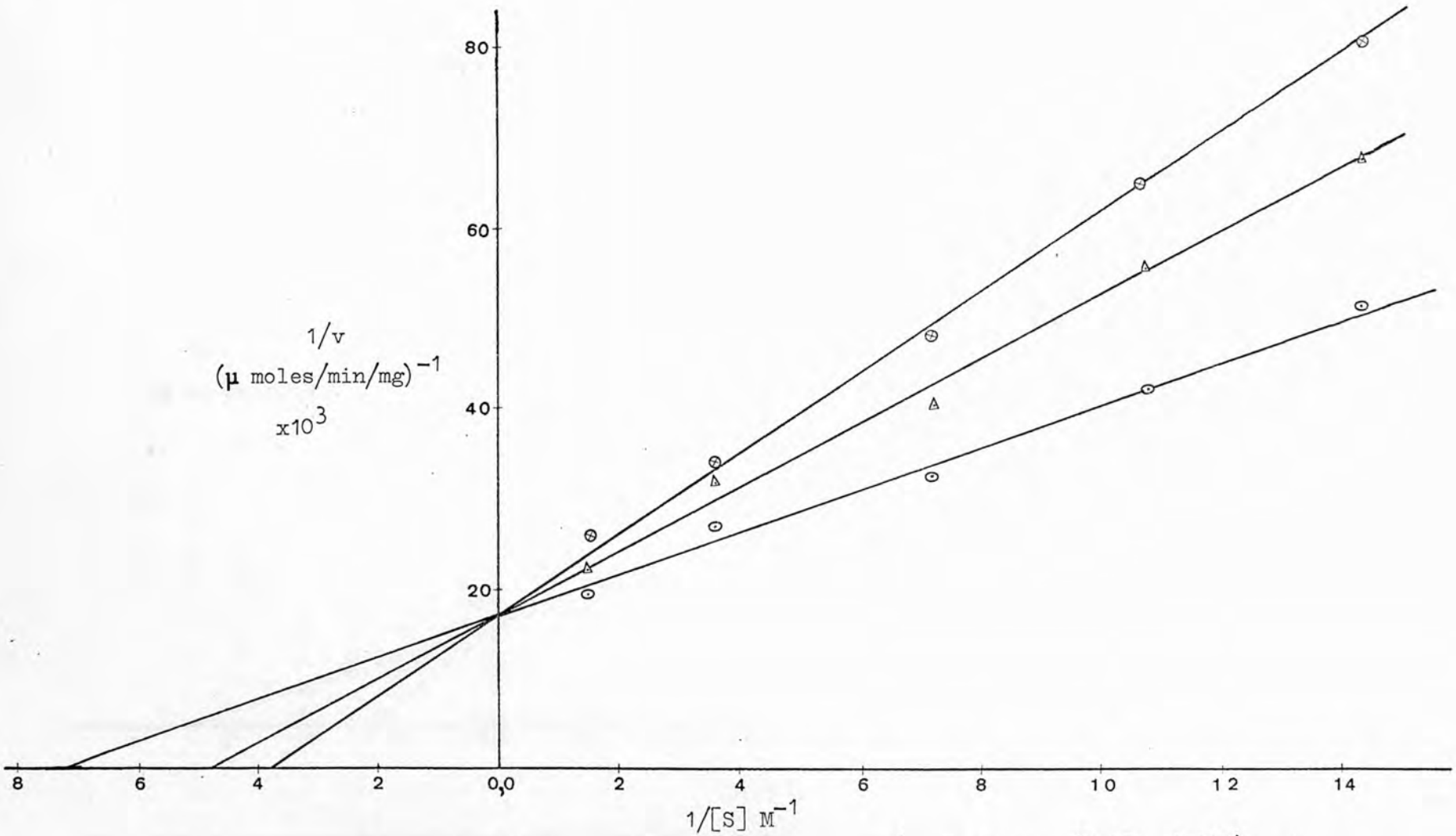


Fig. 5.3 Lineweaver Burk Plot of inhibition of D-xylose isomerase (⊙ D-glucose (0.07 - 0.70M); Δ D-glucose (0.07-0.70M) + 0.833mM equilibrated D-glucose oxime; ⊗ D-glucose (0.07-0.70M) + 1.25mM equilibrated D-arabinose oxime).

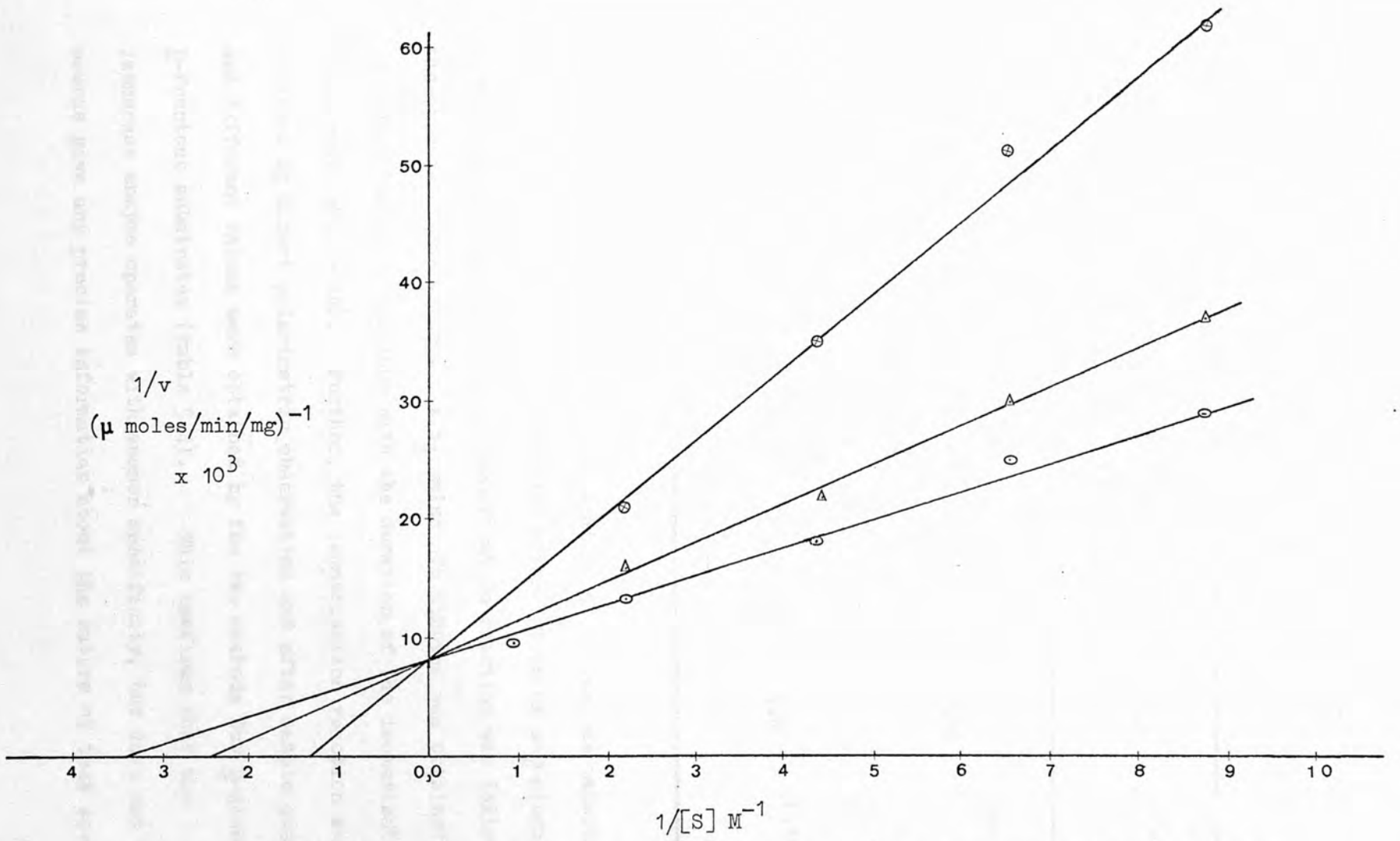


Fig. 5.4 Lineweaver Burk Plot of inhibition of D-xylose isomerase (⊙ D-fructose (0.115 – 1.150 M); ▲ D-fructose (0.115 – 1.150M) + 0.833mM equilibrated D-glucose oxime; ⊘ D-fructose (0.115M – 1.150M) + 1.25mM equilibrated D-arabinose oxime).

Table 5.3 The Michaelis Parameters of D-Glucose and D-Fructose with D-Xylose Isomerase (Streptomyces Species) at 60°.

D-Glucose		D-Fructose			
K_m M	V_{max} µmoles/min/mg protein	K_m M	V_{max} µmoles/min/mg protein	K_{eq}	Reference
0.14	61	0.29	125	1.01	This work
0.14	31	0.23	51	1.0	Litr.[17,32,35]

In order to test whether anomerisation at 60° was an important factor in the polarimetric method, the mutarotation of α-D-glucose under conditions similar to the isomerisation reaction was followed in the absence of the enzyme. A $t_{\frac{1}{2}}$ value of 6 minutes was obtained; this is significant in comparison with the duration of the isomerisation experiments (0.5 - 1h). Further, the isomerisation reaction was followed by direct polarimetric observation and after sample quenching, and different values were obtained by the two methods for D-glucose and D-fructose substrates (Table 5.4). This confirms that the isomerase enzyme operates with anomer specificity, but does not of course give any precise information about the nature of that specificity. (Figs. 5.5, 5.6)

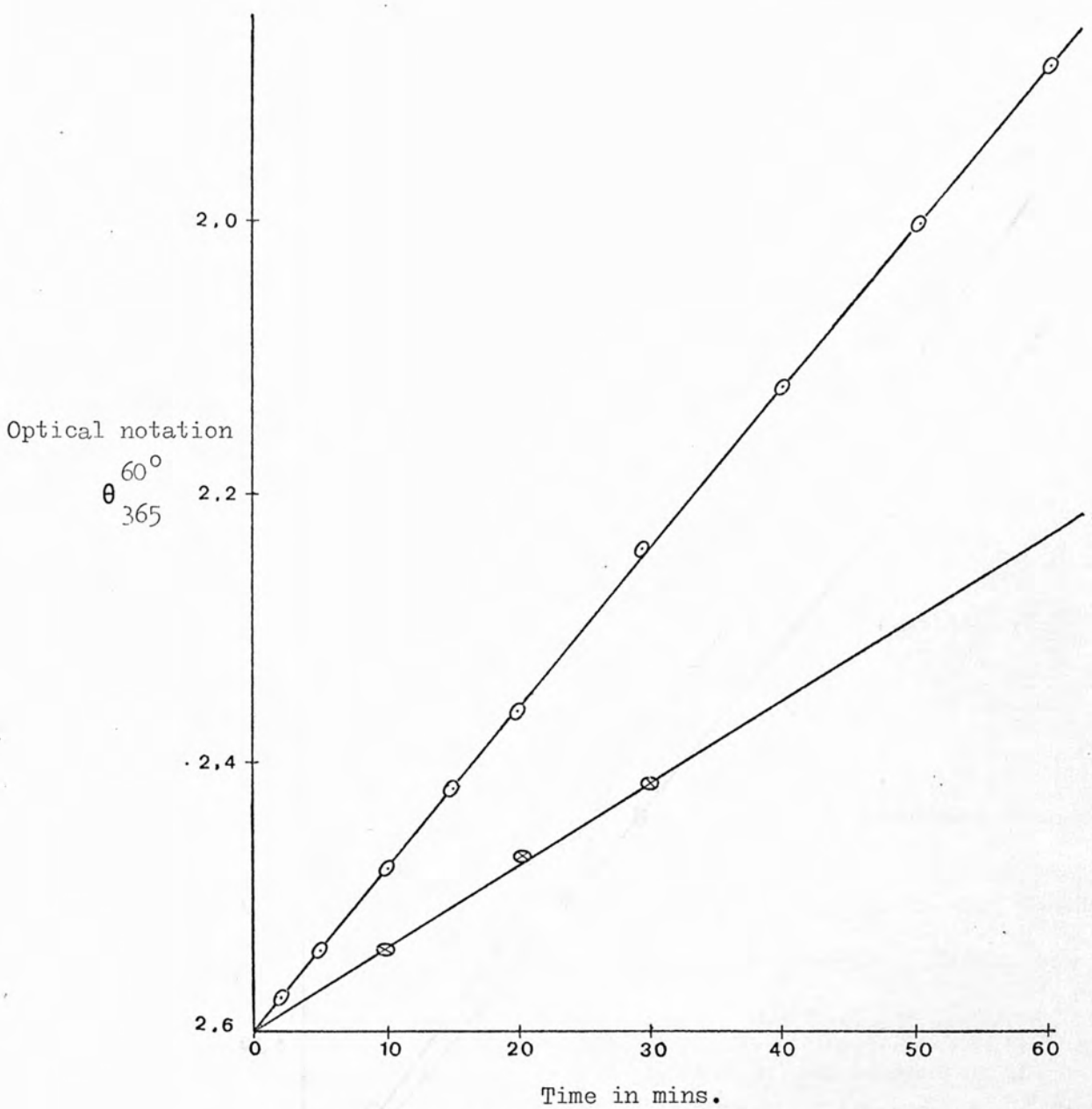


Fig. 5.5 Enzymic rate of isomerisation of D-glucose by ⊗sample quenching and ⊙ direct methods.

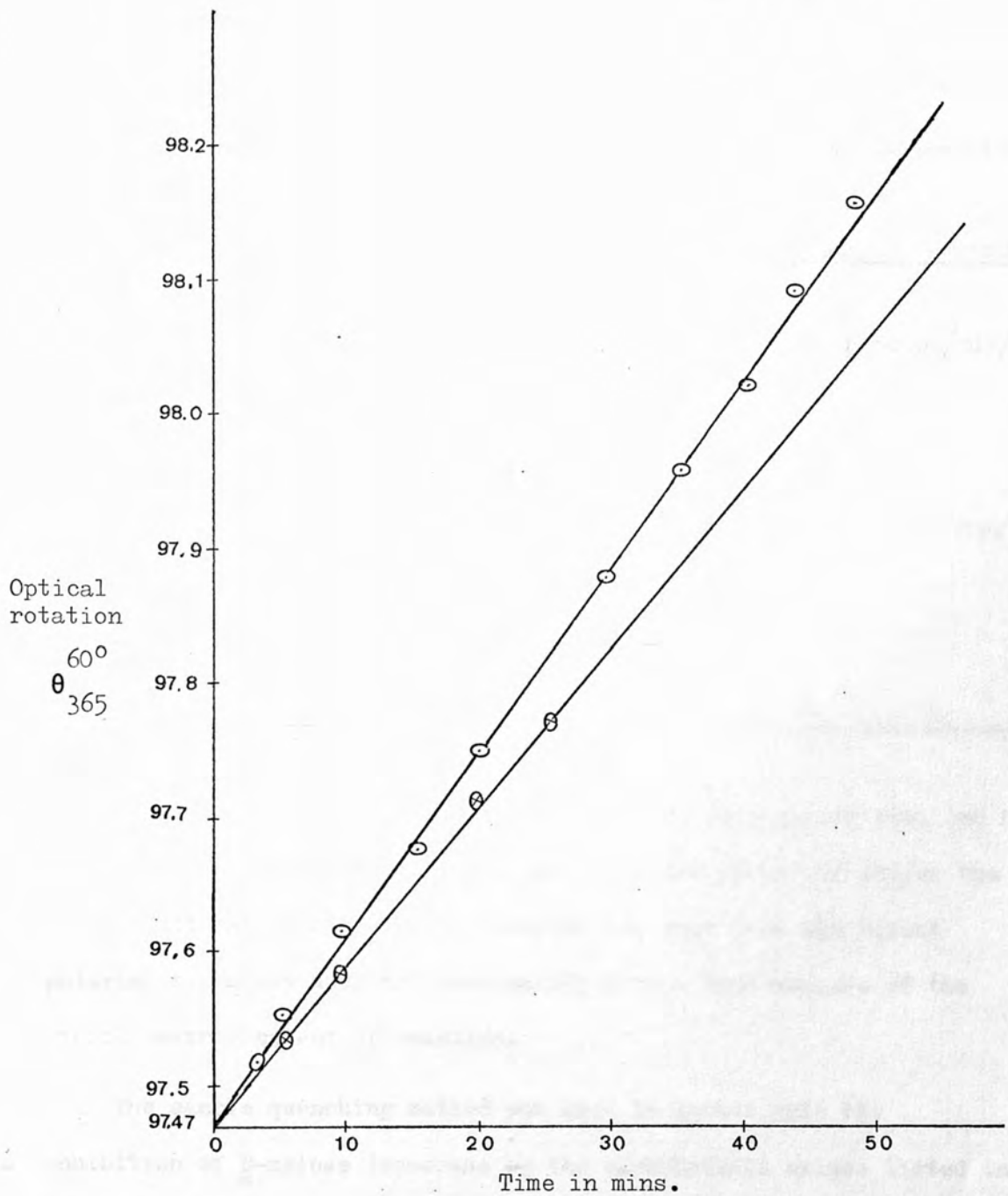


Fig. 5.6 Enzymic rate of isomerisation of D-fructose by ⊗ sample quenching and ⊙ direct methods.

Table 5.4 Rates of Isomerisation of D-Glucose and D-Fructose by D-Xylose Isomerase (S. species) by direct and after sample quenching methods.

Compound	Rate of Isomerisation	
	Direct method	After sample quenching
D-Glucose	22.5 μ moles/min/mg	12.6 μ moles/min/mg
D-Fructose	26.1 μ moles/min/mg	25.0 μ moles/min/mg

The specificity could operate in the substrate utilisation step (as has been shown for D-xylose and D-glucose, see section 5.3.2) and/or the product liberation step. The results also show that the direct polarimetric assay does not necessarily give a true measure of the stoichiometric extent of reaction.

The sample quenching method was used to investigate the inhibition of D-xylose isomerase by the carbohydrate oximes listed in Table 5.5 taking D-fructose as the substrate.

Table 5.5 Inhibition of D-Xylose Isomerase by Carbohydrate Oximes

Inhibitors (1.25 mM)	Percentage Inhibition
D-Arabinose Oxime	45%
D-Glucose Oxime	59%
D-Galactose Oxime	20%
D-Mannose Oxime	17%
D-Ribose Oxime	< 1%
Concentration of Substrate (D-Fructose) = 0.115M	
Initial rate of Isomerisation in absence of Inhibitor = 32.77 μmoles/min/mg protein	

The percentage inhibition values (Table 5.5) indicate that of the carbohydrate oximes (1.25 mM) taken D-glucose oxime and D-arabinose oxime are good inhibitors. D-Arabinose oxime (anti(Z)-form) was taken and its mutarotation followed in the presence and absence of the enzyme. The rate constants obtained in either case were the same (Table 5.6).

Table 5.6 Mutarotation of D-Arabinose Oxime in presence and absence of D-Xylose Isomerase

Rate of Mutarotation in presence of D-Xylose Isomerase	Rate of Mutarotation in absence of D-Xylose Isomerase
$0.473 \pm 0.02\ s^{-1}$	$0.467 \pm 0.02\ s^{-1}$

Therefore, the enzyme has no effect on the mutarotation under the conditions used. Both D-Arabinose oxime and D-glucose oxime were observed not to act as substrate of D-xylose isomerase.

Equilibrated solutions of D-arabinose oxime and D-glucose oximes were taken and their inhibition constants determined taking D-glucose and D-fructose as substrates. Two approaches were used, (1) the concentration of the inhibitor was varied keeping the concentration of the substrate constant and (2) the concentration of the inhibitor was kept constant while the concentration of the substrate was varied. The results are shown in Figs. 5.3, 5.4, 5.7 and 5.8. D-Glucose oxime and D-arabinose oxime were both observed to act as competitive inhibitors for D-xylose isomerase. The inhibition constants determined in the presence of D-glucose and D-fructose as substrates are given in Table 5.2. It can be seen that the values determined for each reaction direction are similar as would be expected. Comparison of the values of the inhibition constants, K_i with the Michaelis constants, K_m for the enzyme substrates, by means of the ratio K_i/K_m, gives an indication of the potency of the inhibitors. The K_i/K_m values for inhibitors of the D-xylose isomerase from various strains studied by other workers are listed in Table 5.2. It is seen that the best inhibitors for this enzyme up to date have been some of the polyols having K_i/K_m values in the range 0.2 to 0.8. In the case of D-arabinose oxime and D-glucose oxime, the inhibitors used in this work, the K_i/K_m values are 2.5×10^{-3} 2.1×10^{-3} respectively. This suggests that these inhibitors are the most potent so far tested against D-xylose isomerases. The closeness of the ratio of K_i/K_m to the ratio of the rate constant of the non-enzymic reaction, k_n to that of the enzymic reaction, k_{cat} (k_n/k_{cat}) is an apparent measure of the resemblance of the inhibitor to the proposed activated complex or transition state of the enzyme catalysed reaction (see Chapter 1). The k_n/k_{cat} value for D-xylose isomerase (S. species)

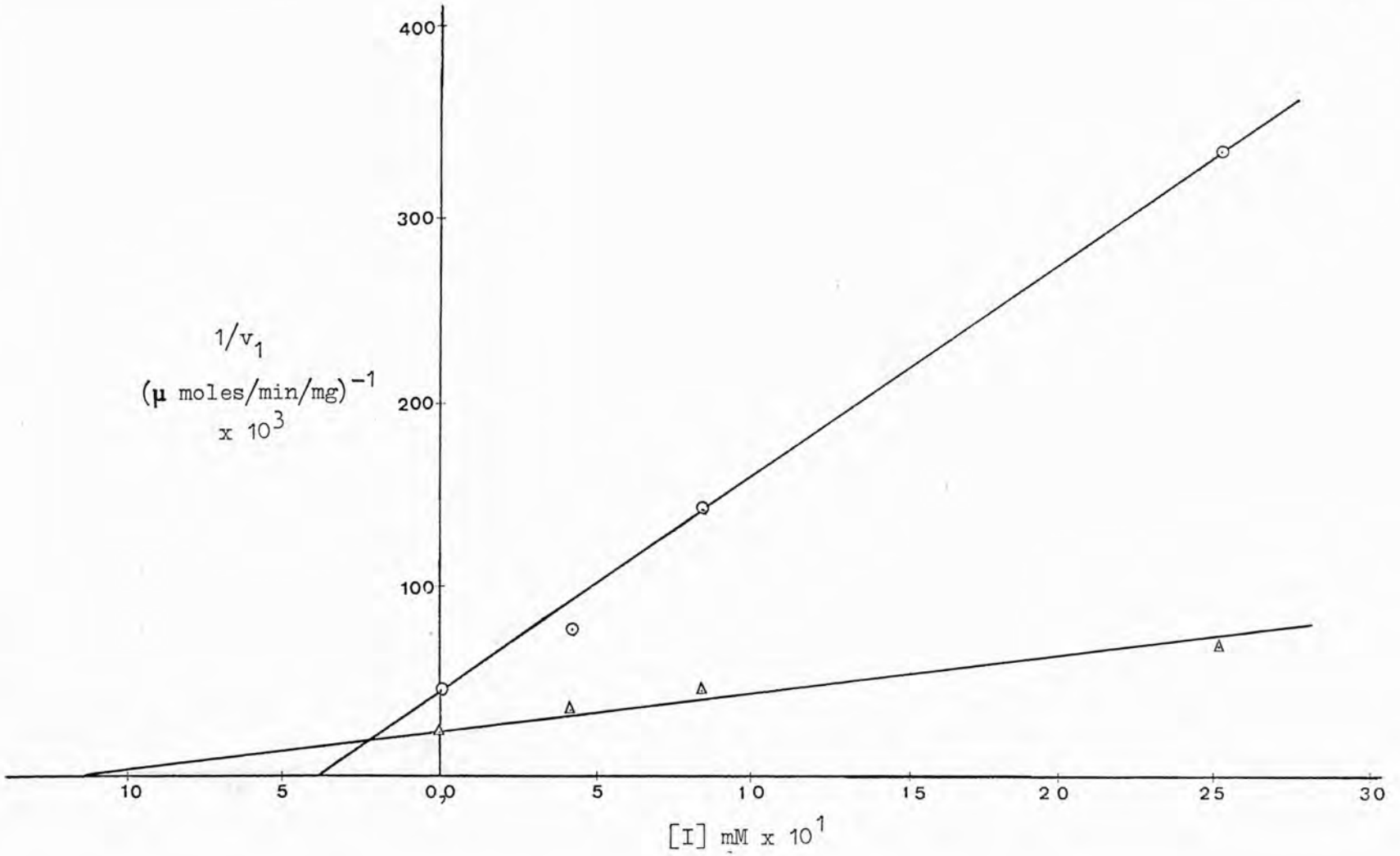


Fig. 5.7 Dixon Plot of inhibition of D-xylose isomerase (D-glucose oxime (0.42 - 2.5 mM) plus 0.07M ▲ and 0.28M⊙D-glucose).

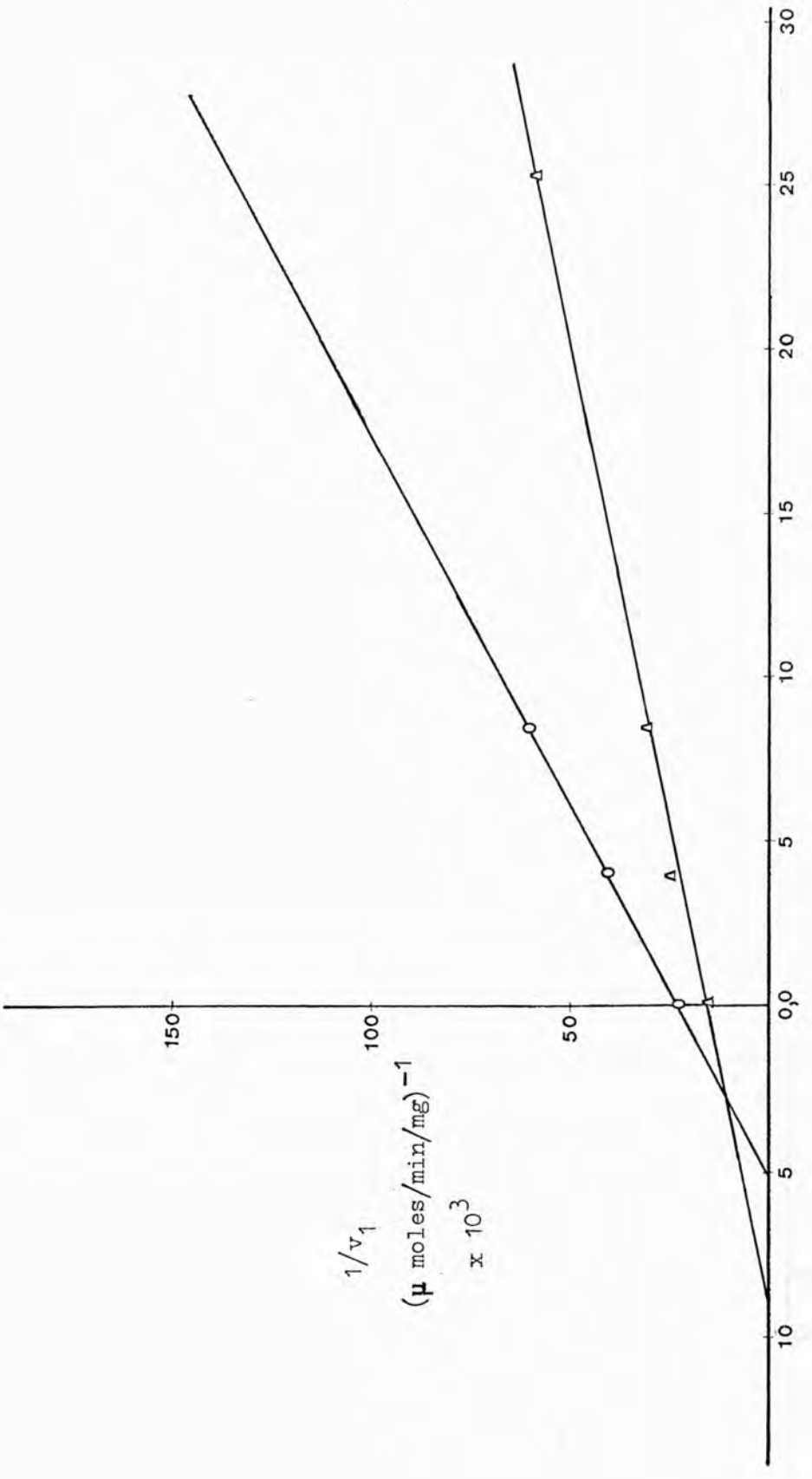


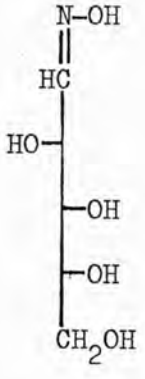
Fig. 5.8 Dixon Plot of inhibition of D-xylose isomerase (D-glucose oxime (0.42 - 2.5 mM) plus 0.115M ⊙ and 0.46M ▲ D-fructose.

was calculated at 60°, by making use of data obtained from other workers[35,37,38] for experiments carried out under similar conditions. (See Appendix in EXPERIMENTAL). A value of k_n/k_{cat} of 3.1×10^{-12} was obtained.

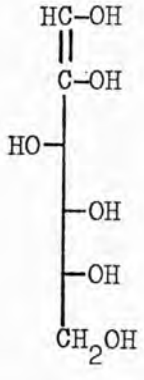
Comparison of the K_i/K_m values of D-arabinose oxime and D-glucose oxime to the k_n/k_{cat} value shows difference in magnitude of 8.1×10^8 x and 6.8×10^8 x respectively. However, these K_i/K_m values of D-arabinose oxime and D-glucose compare favourably with some of the best inhibitors examined against the other isomerases as seen in Table 5.2.

Moreover, the value of k_n/k_{cat} may not necessarily be the true measure of the dissociation constant of the (correct) proposed transition state of D-xylose isomerase reaction (see Chapter 1).

Taking D-arabinose oxime first, it is known (Chapter 3) that in aqueous solution it exists predominantly as a mixture of syn(E , 80%) (15A) and anti(Z , 20%) (15B) forms, with a very small amount being present in the cyclic pyranose (15C) and cyclic furanose (15D) forms.



15B



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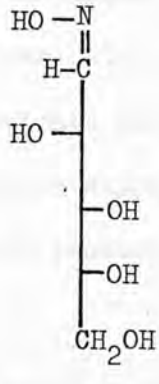
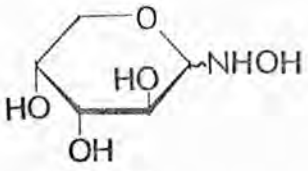
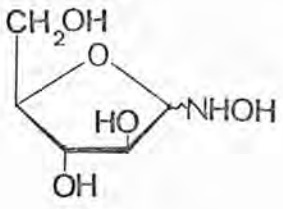


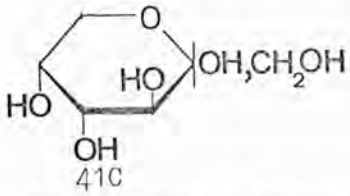
15A



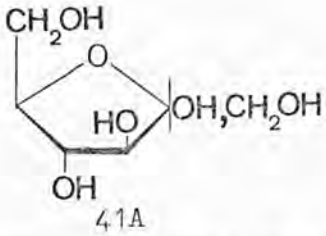
15C



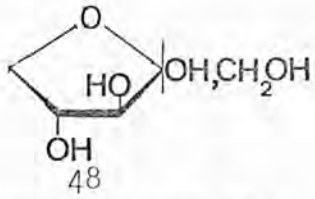
15E



41C



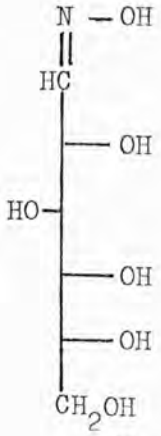
41A



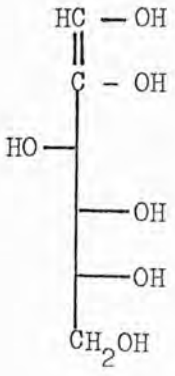
48

In the cyclic forms D-arabinose oxime is isostructural with the pyranose (41C) and furanose (41A) forms of D-fructose apart from the replacement of CH_2OH and OH groups by NHOH and H groups at the anomeric carbon atom. Further, it does not resemble D-xylulose (48). Therefore on this basis it would be expected to have an inhibition constant higher than the K_m of D-fructose. However, D-arabinose oxime has an inhibition constant which is nearly 400 times smaller than the K_m of D-fructose. This suggests strongly that D-arabinose oxime must be binding preferentially in the acyclic form to D-xylose isomerase. In the acyclic forms (15A, B) it resembles the cis-enediol intermediate (16) formed in the D-glucose to D-fructose interconversion. According to the transition state analogue theory compounds which mimic the transition state of an enzyme catalysed reaction will bind more strongly than the normal substrate and this is what happens with D-arabinose oxime. This evidence supports the presence of a cis-enediol intermediate in the D-xylose isomerase reaction pathway.

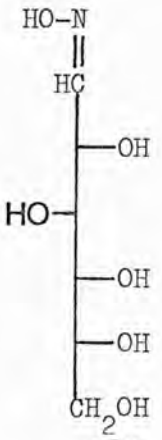
In the case of D-glucose oxime, it is known to exist in the syn(E, 56.5%) (18B), anti(Z, 13.5%) (18A), α-pyranose (7%) (18C) and β-pyranose (23%) (18D) forms at equilibrium (Chapter 3). A very small amount may also exist in the furanose form (18E).



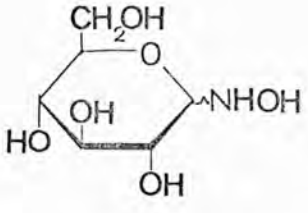
18A



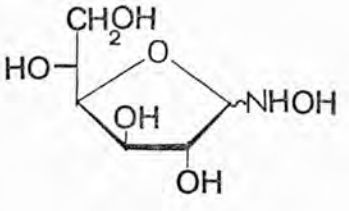
16



18B

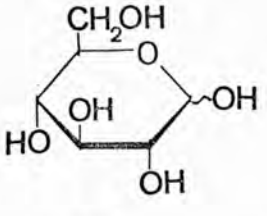


18C

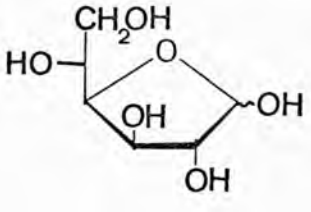


18E

Taking the cyclic forms first, it is seen that the cyclic forms of D-glucose oxime are isostructural with the cyclic forms of D-glucose (33A, 33C) except for the presence of a -NHOH group attached on the



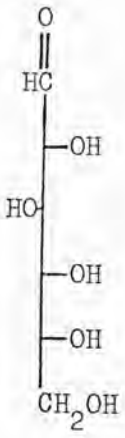
33A



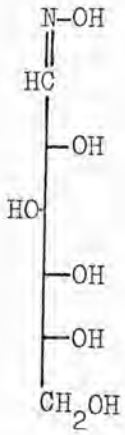
33C

anomeric carbon atom instead of the -OH group. D-glucose oxime is observed to be a competitive inhibitor (K_i = 0.30 mM, D-glucose K_m = 0.14M)of D-xylose isomerase, (Table 5.2). This suggests that either it is the presence of a -NHOH group in the cyclic form or the presence of an acyclic form which is responsible for the K_i value, about

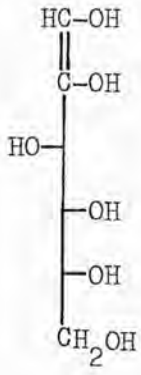
500 times lower than the K_m value of D-glucose. The acyclic forms of D-glucose oxime (8A,B) do not resemble the cis-enediol intermediate (16) postulated for the D-glucose to D-fructose interconversion. The most likely explanation for the tighter binding of D-glucose oxime is its structural analogy to the structure of the acyclic form of D-glucose (33E)



33E



18A,B



16

The equilibrium concentration of the acyclic D-glucose is approximately 0.0026%[33] and as discussed earlier (section 5.2.3), the D-xylose isomerase is shown to prefer acyclic structures over cyclic structures of inhibitors or substrate analogues. This means that the K_m of acyclic D-glucose would be:

$$K_m \text{ (measured)} \times 0.0026\% = 3.9 \times 10^{-6} M$$

In the case of D-glucose oxime by analogy with D-glucose, the K_i value of the acyclic species would be:

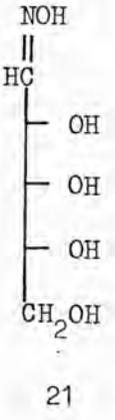
$$K_i \text{ (measured)} \times 70\%. = 2.1 \times 10^{-4} M$$

The tight binding observed with D-glucose oxime supports the step-wise ring opening followed by enolisation pathway rather than the concerted

elimination mechanism. If the reaction proceeded by the concerted elimination mechanism then the enzyme would not be expected to show tight binding for the acyclic form of the inhibitor.

It is seen that D-glucose oxime resembles the acyclic form of D-glucose while D-arabinose oxime mimics the cis-enediol intermediate in the D-glucose to D-fructose isomerisation pathway. The inhibition constants for both the inhibitors are nearly similar (Table 5.2). This indicates that the ring opened form (33E) and the cis-enediol intermediate (16) lie at nearly the same energy levels in the enzyme catalysed isomerisation pathway. It is further seen from Table 5.2 that the K_i/K_m values for D-arabinose oxime and D-glucose oxime compare favourably with some of the best inhibitors of the isomerases.

The other carbohydrate oximes listed in Table 5.5 are known to exist in the syn(E , 80%) and anti(Z , 20%) forms at equilibrium. D-Ribose oxime(21) is a C2 epimer of D-arabinose oxime and shows negliglble inhibition of D-xylose isomerase (Table 5.5). This indicates that the orientation about C2 in D-ribose oxime which corresponds to the orientation about C3 in the cis-enediol intermediate (16) formed from D-glucose is important for binding. It is also known



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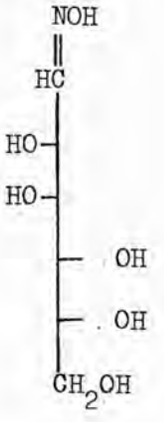

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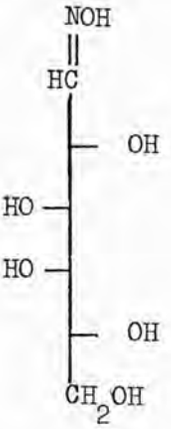

19

that D-xylose isomerase (S. species) does not catalyse the isomerisation of D-ribose (C3 epimer of D-xylose). D-Mannose oxime (20) and D-galactose oxime (19) are C2 and C4 epimers of D-glucose oxime respectively. They show a lower inhibition rate owing to the wrong orientation about C2 and C4 respectively.

From the above discussion it is concluded that D-xylose isomerase (S. species) shows a preference for the α-anomer of the aldose and/or ketose substrate. It favours the pathway involving ring-opening followed by enolisation and the isomerisation proceeds through a cis-enediol intermediate. Further, the orientation about C3 is important for binding with the enzyme.

5.4 EXPERIMENTAL

5.4.1 Materials

D-Xylose isomerase (D-glucose isomerase) of Streptomyces albus strain (Optisweet P) was obtained by courtesy of Miles Laboratories, Stoke Poges, England. It is a coarse yellowish-grey powder, which disperses in water to a turbid solution with an activity of 3000 TGIU[a] (Takasaki Glucose Isomerase Unit) $\pm$ 5% and requires 0.2% $MgSO_4.6H_2O$ and 0.02% $CoCl_2.6H_2O$ as cofactors. All other chemicals were of BDH "Analar" grade except for D-arabinose oxime, D-ribose oxime, D-galactose oxime, D-glucose oxime and D-mannose oxime which were synthesised (Chapter II).

5.4.2 Measurement of Rates of Isomerisation catalysed by D-Xylose Isomerase

Preparation of D-xylose isomerase solution:- D-xylose isomerase (9 mg per cm^3, dry wt.) was added to 0.05M phosphate buffer pH 6.5 containing 0.2% magnesium sulphate ($MgSO_4.6H_2O$) and 0.02% cobalt chloride ($CoCl_2.6H_2O$). The solution was heated at 55° with slow stirring for 0.5h. Then it was centrifuged in a Beckman J-21 model centrifuge using JA 20 rotor for 0.5 h at 5500 r.p.m. and 25°. The supernatant solution was collected and used for the isomerisation reaction. A fresh solution of D-xylose isomerase was prepared each time.

(a) 1 TGIU is defined as the amount of enzyme which, from a 0.1M D-glucose solution, under the given analysis conditions of pH 7.0 and 70° will form 1 mg D-fructose in 1 h.

The isomerisation reaction was followed by taking the substrate solution (in 0.05M phosphate buffer pH 6.5, containing 0.2% $MgSO_4.6H_2O$ and 0.02% $CoCl_2.6H_2O$) and D-xylose isomerase solution (9 mg/cm^3, dry wt.) and keeping them at 60° for 15 minutes before mixing them. The reaction mixture was then maintained at 60° and aliquots (2 cm^3) were transferred immediately and after various time intervals into 1.25M perchloric acid ($HClO_4$, 0.5 cm^3). The quenched sample was left to equilibrate at room temperature and the optical rotation values read at 365 nm in a Perkin Elmer 141 polarimeter in a 1 dm jacketed cell maintained at 27° unless stated otherwise.

The concentrations of the substrates(equilibrated) and inhibitors (equilibrated) were as follows:

5.4.2.1 For obtaining Michaelis Parameters for substrates:

D-Glucose (0.07M to 0.70M)

D-Fructose (0.115M to 1.150M)

(samples taken after zero time and then every 5 minutes for 1 h).

5.4.2.2 For observing inhibition of D-xylose isomerase by carbohydrate oximes:

(a) D-Fructose (0.115M) + D-Arabinose oxime (1.25 mM)

D-Fructose (0.115M) + D-Ribose oxime (1.25 mM)

D-Fructose (0.115M + D-Galactose oxime (1.25 mM)

D-Fructose (0.115M) + D-Glucose oxime (1.25 mM)

D-Fructose (0.115M) + D-Mannose oxime (1.25 mM)

(b) D-Fructose (0.115M) + D-Arabinose oxime (0 to 25 mM)

(c) D-Fructose (0.115M to 1.150M) + D-Arabinose oxime (1.25 mM)

(d) D-Glucose (0.14M) + D-Arabinose oxime (0 to 25 mM)

(e) D-Glucose (0.07M to 0.70M) + D-Arabinose oxime (1.25 mM).

(f) D-Fructose (0.115M) + D-Glucose oxime (0 to 7.5 mM)

(g) D-Fructose (0.460M) + D-Glucose oxime (0 to 7.5 mM)

(h) D-Fructose (0.115M to 1.150M) + D-Glucose oxime (0.833 mM)

(i) D-Glucose (0.07M) + D-Glucose oxime (0 to 7.5 mM)

(j) D-Glucose (0.28M) + D-Glucose oxime (0 to 7.5 mM)

(k) D-Glucose (0.07M to 0.70M) + D-Glucose oxime (0.833 mM)

(Sampling times 0, 10, 20, 30 minutes).

5.4.3 Direct Monitoring of Isomerisation by Polarimetry

5.4.3.1 The substrate or inhibitor solution and D-xylose isomerase solution (9 mg/cm^3, dry wt.) were prepared as mentioned earlier and kept at 60° for 15 minutes before mixing them. Then the reaction mixture was placed in a 1 dm jacketed cell in a polarimeter and the reaction followed directly by observing the optical rotation reading at 436 nm and 60°.

5.4.3.2 To compare the rates of isomerisation by the direct procedure and by the sampling procedure. D-Fructose (0.115M) amd D-glucose (0.140M) in 0.05M phosphate buffer (containing 0.2% $MgSO_4.6H_2O$ and 0.2% $CoCl_2.6H_2O$) pH 6.5 were taken and interacted with D-xylose isomerase (9 mg/cm^3, dry wt.) and the optical rotation values measured by the direct procedure and after sample quenching in a polarimeter at 436 nm and 60°.

5.4.3.3 Direct observation of D-xylose isomerase interaction with inhibitor at 436 nm and 60°. No change in the optical rotation value of D-glucose oxime (0.05M) was observed.

5.4.3.4 Mutarotation of D-arabinose oxime (anti Z-form, 0.067M final concentration) using 0.05M phosphate buffer (containing 0.2% $MgSO_4.6H_2O$ + 0.02% $CoCl_2.6H_2O$), pH 6.5 in the presence and absence of D-xylose isomerase (9 mg/cm^3, dry wt.). The optical rotation readings were taken in a polarimeter at 436 nm and 60°. The initial and final equilibrium readings were the same in either case.

5.4.3.5 Mutarotation of α-D-glucose (0.05M) in 0.05M phosphate buffer (containing 0.2% $MgSO_4.6H_2O$ + 0.02% $CoCl_2.6H_2O$) pH 6.5, was taken in a polarimeter at 365 nm and 60°.

5.5 APPENDIX

5.5.1 Calculation of the Rate of Isomerisation Catalysed by D-Xylose Isomerase (S. species).

$$[\alpha]_t = x[\alpha]_G + (1-x)[\alpha]_F$$

$[\alpha]_t$ = specific rotation of reaction mixture of anomeric equilibrium at time t.

$[\alpha]_G$ = specific rotation of D-glucose at equilibrium.

$[\alpha]_F$ = specific rotation of D-fructose at equilibrium.

For a forward reaction of isomerisation of D-glucose to D-fructose

$$x = \frac{\text{Amount of D-glucose present at time t}}{\text{Total amount of D-glucose taken initially}}$$

For a reverse reaction of isomerisation of D-glucose to D-fructose.

$$x = \frac{\text{Amount of D-fructose present at time t}}{\text{Total amount of D-fructose taken initially}}$$

5.5.2 Calculation of Inhibition constant using Lineweaver Burk plot of $1/v$ vs. $1/[S]$ for competitive inhibitors:

$$\text{Slope} = \frac{K_m}{V_m}\left(1 + \frac{I}{K_i}\right)$$

K_m = Michaelis constant for substrate.

V_m = Maximum velocity for substrate.

I = Inhibitor concentration (kept constant).

K_i = Inhibition constant.

5.5.3 Calculation of Non-enzymic k_n and Enzymic k_e Rate constants for the Isomerisation of D-Glucose to D-Fructose at pH 6.5 and 60°.

$$k_n = k_{gf}\ \frac{K_G[OH^-]}{K_G[OH^-]+1}$$

k_{gf} = rate constant for conversion of D-glucose anion to D-fructose anion

$= 31 \times 10^{-4}\ s^{-1}$ [39]

$$K_G = \frac{[G^-]}{[G][OH^-]} = 15 \times 10^{-3}\ \text{l m mole}^{-1}$$ [38]

G and G^- are the non-ionic and anionic forms of D-glucose.

$k_n = 1.5 \times 10^{-9} s^{-1}$

$k_e = k_{cat}/Km$

$k_{cat} = V_m/[E]_0$

From the work of Y. Takasaki[35]

$$k_{cat} = \frac{31 \times 10^{-6}}{60} \Big/ \frac{0.9}{157 \times 10^{6}}$$

$$= \underline{4.77 \times 10^{2} s^{-1}}$$

Thus $K_T/K_m = k_n/k_{cat} = 1.5 \times 10^{-9}/4.77 \times 10^{2} = \underline{3.1 \times 10^{-12}}$

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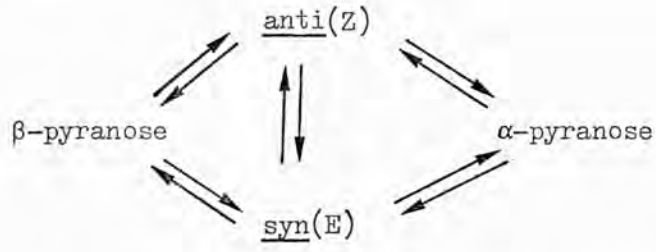
CHAPTER6 GENERAL CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

The carbohydrate oximes synthesised in this work crystallised mainly in the acyclic anti(Z) and/or syn(E) forms except for D-glucose oxime which exists in the β-pyranose $^4C_1$ conformation in the solid state. The preference of one of a number of isomeric forms in the solid state is presumably related to crystal lattice forces; this could be investigated by physical measurements e.g. of heat capacity.

The carbohydrate oximes showed a simple mutarotation except for D-glucose oxime in which case it was complex. The mutarotation of D-glucose oxime was followed by ^1H nmr and showed the β-pyranose form decreasing to an equilibrium value while the syn(E), anti(Z) and α-pyranose forms increased in amount. The mutarotation is suggested to proceed via Scheme 6.1.



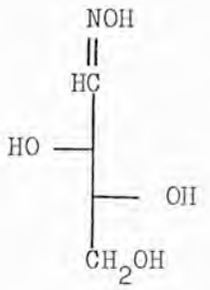
Scheme 6.1

In future one could determine the individual rate constants by more accurate measurements with a view to defining more precisely mutarotation pathway of D-glucose oxime. Also, in the case of the other carbohydrate oximes which showed simple mutarotation, further investigation of the mechanism would be desirable. In the isomerisation of carbohydrate oximes, more information about the mechanism could be obtained by doing further experiments involving isotope effects e.g. evaluation of k_{D_2O}, $k_{D_3O^+}$, k_{OD^-}. Also, there is

a need for more work on simple aliphatic oximes in aqueous solution.

The use of paper chromatography and electrophoresis in conjunction with, ^{1}H nmr, ^{13}C nmr and ^{31}P nmr provides conclusive evidence of the ATP-dependent phosphorylation by yeast hexokinase of a sugar substrate and reveals the site of phosphorylation. The selective phosphorylation of the C5-OH or C6-OH shown by carbohydrate oximes could be utilised in other cases where chemical synthesis is difficult. Consideration of the substrate specificity of yeast hexokinase suggests that the X-ray crystallographic studies carried out on crystals obtained by crystallisation of hexokinase in the presence of α-D-mannopyranose or D-arabinofurano-2-hexosulose or D-xylulose (furanose form inhibitor) would give new information about substrate binding to this enzyme. Also, D-arabinose oxime-5-phosphate prepared in this work is clearly of potential value for inhibition studies of D-glucose-6-phosphate isomerase.

The interaction of carbohydrate oximes with D-xylose isomerase (*Streptomyces species*) supports the theory that the enzyme catalysed isomerisation proceeds by a step-wise pathway *via* an enediol. The proposed *cis*-enediol could be substantiated by studies with *syn*(E) and anti(Z)-D-arabinose oxime instead of with the equilibrated mixture which was used in this work due to low enzyme activity. The theoretical prediction is that the *syn*(E) form will be the better transition state analogue. Further, D-xylose is a better substrate than D-glucose for D-xylose isomerase, synthesis of D-threose oxime ((50) which would mimic the *cis*-enediol of the D-xylose to D-xylulose (51) interconversion) would facilitate further confirmation of the presence of a *cis*-enediol intermediate.



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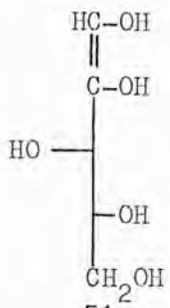
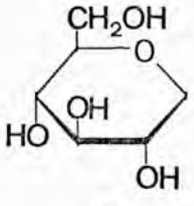


51

It is known from previous work that D-xylose isomerase prefers the α-anomer of the aldose substrate, but there is no information about the preference of a ketose substrate. This could be determined by taking a very active enzyme and following the isomerisation of D-glucose to D-fructose by ^{13}C nmr, where the anomeric signals of the different isomeric forms of D-fructose and D-glucose can be distinguished. Also, the binding of the cyclic form to the enzyme could be confirmed by using 1,5-anhydro-D-glucitol or 1,5-anhydro-D-xylitol.(32 or 52)



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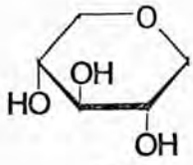

52

Finally, if syn(E)-D-arabinose oxime could be crystallised with D-xylose isomerase, X-ray crystallographic studies could be carried out on the enzyme inhibitor complex in order to define the location and geometry of the enzyme active site. Since the structure of syn(E) D-arabinose oxime is already known, the difference method could be

applied to gain information about the active-site structure of D-xylose isomerase. This would in turn help in design of other inhibitors and of active site directed reagents.

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The Structures of D-Arabinose and D-Glucose Oximes

By **Paul Finch** * and **Z. Merchant**, Chemistry Department, Royal Holloway College, Egham, Surrey TW20 0EX

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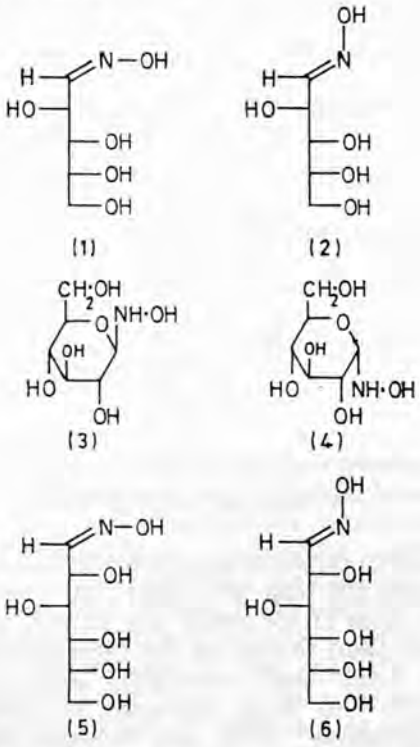
The Structures of D-Arabinose and D-Glucose Oximes

By **Paul Finch** * and **Z. Merchant**, Chemistry Department, Royal Holloway College, Egham, Surrey TW20 0EX

By means of various physical techniques D-arabinose oxime has been shown to exist in the *anti-* (*Z*-) acyclic form in the solid state and to isomerise to a mixture of *anti-* (*Z*)- (20%) and *syn-* (*E*-) (80%) forms in aqueous solution. D-Glucose oxime exists in the cyclic β-pyranose form in the solid state and isomerises to a mixture of β-pyranose (23%), α-pyranose (7%), *anti-* (*Z*-) (13.5%), and *syn-* (*E*-) (56.5%) forms in aqueous solution. Conformational analysis of one cyclic (β-D-*gluco*-) and of one acyclic [(*E*)-D-*arabino*-] form has been carried out by ^{1}H n.m.r. spectroscopy.

OXIME derivatives of carbohydrates prepared by reaction of hydroxylamine at the anomeric centre were first described in 1887,[1] and have been used for the characterisation of sugars; however the structures of these derivatives in the solid form and in solution have not been established unequivocally.[2,3] Since aqueous solutions exhibit mutarotation and a number of reactions such as the Wohl degradation [4] are best explained in terms of an open-chain structure, it has been widely presumed that equilibria between cyclic and acyclic forms exist in solution.[2,5-7] D-Glucose oxime has been assigned a β-cyclic structure on the basis of methylation,[5] optical rotation,[6] and i.r.[8,9] data, and arabinose oxime was thought to possess an acyclic structure on the basis of i.r. measurements.[8] From the results of polarographic measurements Haas *et al.*[10] concluded that in solution the oximes of glucose and arabinose exist in the acyclic form to the extent of 47 and 100%, respectively. Recently Szarek and his co-workers [11] have described the synthesis and characterisation of *O*-benzyloximes of carbohydrates, and have assigned acyclic structures on the basis of Raman and ^{1}H n.m.r. data. We now present evidence to show that the normal † crystalline form of D-arabinose oxime possesses an *anti-* (*Z*-) acyclic structure (1) which isomerises to a mixture of *syn-* (*E*-) (2) and *anti-* (*Z*-) forms in aqueous solution, and that the normal crystalline form of D-glucose oxime has a β-cyclic pyranosyl structure (3) which isomerises to a mixture of interconvertible β-cyclic, α-cyclic (4), *syn-* (*E*-) (6), and *anti-* (*Z*-) (5) forms in aqueous solution.



Solid D-arabinose oxime gave a sharp, weak, i.r. band at 1 680 cm^{-1}, and a band of moderate intensity in the Raman at 1 678 cm^{-1} suggestive of the presence

† *Note added in proof.* It is also possible to prepare crystals of D-arabinose oxime, m.p. 136—137°, which consist of a mixture of *syn-* (80%) and *anti-* (20%) forms.

[1] P. Richsbieth, *Ber.*, 1887, **20**, 2673.
[2] B. Capon, *Chem. Rev.*, 1969, **69**, 407.
[3] R. J. Ferrier and P. M. Collins, ' Monosaccharide Chemistry,' Penguin, Harmondsworth, 1972, p. 72.
[4] A. Wohl, *Ber.*, 1891, **24**, 994; 1893, **26**, 730.
[5] J. C. Irvine and R. Gilmour, *J. Chem. Soc.*, 1908, **93**, 1429.
[6] M. L. Wolfrom and A. Thompson, *J. Amer. Chem. Soc.*, 1931, **53**, 622.
[7] V. Deulofeu, *Adv. Carbohydrate Chem.*, 1949, **4**, 119.
[8] F. Legay, *Compt. rend.*, 1952, **234**, 1612.
[9] H. Bredereck, A. Wagner, D. Hummel, and H. Krieselmeier, *Chem. Ber.*, 1956, **89**, 1532.
[10] J. W. Haas, jun., J. D. Storey, and C. C. Lynch, *Analyt. Chem.*, 1962, **34**, 145.
[11] J. Plenkiewicz, W. A. Szarek, P. A. Sipos, and M. K. Phibbs, *Synthesis*, 1974, 56.

of a C=N grouping.[8,9,12-14] In aqueous solution two weak bands were exhibited in the Raman at 1 656 and 1 664 cm^{-1}, indicating the presence of two C=N species. Mutarotation was observed to be kinetically simple and to proceed at the same rate in water and deuterium oxide, indicating that, in contrast to the situation with free sugars, proton transfer is not involved in the rate-determining step.[15] D-Arabinose oxime in [$^2H_6$]dimethyl sulphoxide solution gave a ^1H n.m.r. spectrum consistent with the presence of two interconverting isomeric forms (see Tables 1 and 2). The assignment of the *Z*-structure to the initial form and of the *E*-structure to the preponderant form at equilibrium was made on the basis of chemical shift correlations. These correlations [16-23] state that in aldoximes the hydrogen atom on the trigonal carbon is more deshielded when *cis* (*syn*) to the oxime OH than when *trans* (*anti*), and that the hydrogen atom of the oxime OH is more deshielded when *trans* (*anti*) to that on the trigonal carbon than when *cis* (*syn*). Such correlations have been used previously [24,25] to assign structures of carbohydrate oxime derivatives. Thus two low-field singlets at δ 10.85 and 10.66 which disappeared on addition of deuterium oxide were assigned to the NOH protons of the *Z*- (*anti*-) and *E*- (*syn*-) forms, respectively, and two doublets at δ 6.67 and 7.34 were assigned to the C-1 protons of the *Z*- (*anti*-) and *E*- (*syn*-) forms. Other assignments were made with the aid of exchange in deuterium oxide, double resonance, and computer simulation procedures. The ^1H n.m.r. coupling constants allow the identification of the preponderant conformation (7) of the major *E*- (*syn*-) isomer at equilibrium in [$^2H_6$]dimethyl sulphoxide–[$^2H_2$]water; the fully extended planar zig-zag form is favoured except where it would lead to parallel 1,3-diaxial interactions between substituent groups.[26,27] The rate of isomerisation of the initial *Z*-form of D-arabinose oxime in deuterium oxide was measured by monitoring the intensities of the C-1 proton n.m.r. signals with time. The same overall rate constant of 0.204 h^{-1} was obtained as that given by observation of mutarotation, and the equilibrium (after *ca.* 20 h) proportions of *E*- (2) (80%) and *Z*- (1) (20%) forms yield interconversion rate

TABLE 1

^1H Chemical shifts (δ values; Me_4Si standard) [a]

Compd.	NOH [b]	NH [b]	1-H	2-H	3-H	4-H	5-H_a	5-H_b	6-H_a	6-H_b
(1)	10.85		6.67	4.93						
				5.01 [c]						
(2)	10.66		7.34	4.26	3.29	3.50 [d]	3.59 [d]	3.40 [d]		
				4.40 [c]						
(3)	7.36	5.81	3.81	2.95	3.13 [d]	2.98 [d]	3.04 [d]		3.6	3.38
(4)			4.5 [c]							
(5)	11.0		6.9	5.04						
			6.64 [b]	4.92 [c]						
(6)	10.7		7.5	4.40						
			7.24 [b]	4.30 [c]						

[a] In $(CD_3)_2SO$–D_2O except where specified. [b] In $(CD_3)_2SO$. [c] In D_2O. [d] Obtained by computer simulation.

TABLE 2

^1H Coupling constants (Hz) [a]

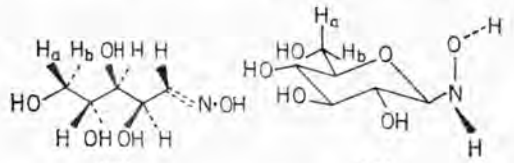
Compd.	NOH,NH [b]	NH,1-H [b]	1-H,2-H	2-H,3-H	3-H,4-H	4-H,5-H_a	4-H,5-H_b	5-H_a,5-H_b	5-H,6-H_a	5-H,6-H_b	6-H_a,6-H_b
(1)			5.7 [c]	2.2 [c]							
			5.60 [b]								
(2)			7.3	2.1	7.8	2.6	5.9	−10.5			
			7.28 [d]	2.8 [c]	6.0 [c]						
(3)	2.70	3.24	8.95	8.95	8.95 [d]		8.95 [d]		7.0	5.5	−11.5
			9.1 [c]								
(4)			*ca.* 4.0 [c]								
(5)			6.2								
			6.7 [c]	6.7 [c]							
(6)			7.8								
			7.0 [c]	7.1 [c]							

[a] $(CD_3)_2SO$–D_2O unless specified. [b] In $(CD_3)_2SO$. [c] In D_2O. [d] Obtained by computer simulation.

[12] H. Spedding, *Adv. Carbohydrate Chem.*, 1964, **19**, 34.
[13] D. Horton, K. Just, and B. Gross, *Carbohydrate Res.*, 1971, **16**, 239.
[14] F. R. Dollish, W. Fateley, and F. F. Bentley, 'Characteristic Raman Frequencies of Organic Compounds,' Wiley, New York, 1974, p. 134.
[15] H. S. Isbell and H. Pigman, *Adv. Carbohydrate Chem.*, 1969, **24**, 13, and references therein.
[16] W. D. Phillips, *Ann. New York Acad. Sci.*, 1958, **70**, 817.
[17] E. Lustig, *J. Phys. Chem.*, 1961, **65**, 491.
[18] G. Slomp and W. J. Wechter, *Chem. and Ind.*, 1962, 41.
[19] G. J. Karabatsos, R. A. Taller, and F. M. Vane, *J. Amer. Chem. Soc.*, 1963, **85**, 2326.
[20] A. J. Durbetaki and C. Miles, 148th Meeting of the American Chemical Society, Chicago, 1964, Abstracts A–47.
[21] A. Daniel and A. A. Pavia, *Tetrahedron Letters*, 1967, 1145.
[22] G. G. Kleinspehn, J. A. Jung, and S. A. Studniarz, *J. Org. Chem.*, 1967, **32**, 460.
[23] A. C. Huitric, D. B. Roll, and J. De Boer, *J. Org. Chem.*, 1967, **32**, 1661.
[24] P. M. Collins, *Chem. Comm.*, 1966, 164.
[25] A. Kampf and E. Dimant, *Carbohydrate Res.*, 1971, **16**, 212.
[26] P. L. Durette, D. Horton, and J. D. Wander, 'Carbohydrates in Solution,' A.C.S. Advances in Chemistry Series No. 117, Washington, 1973, p. 147.
[27] P. L. Durette and D. Horton, *Adv. Carbohydrate Chem.*, 1971, **26**, 49.

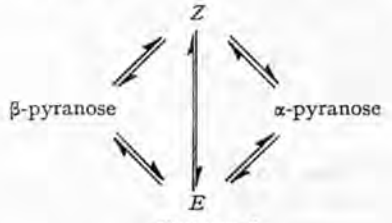
constants of 0.168 and 0.036 h^{-1}. The specific rotations of the diastereoisomers (1) and (2) were calculated to be −83.6 and +3.15°, respectively. The equilibration of non-carbohydrate oximes has been noted previously,[28] and the preponderance of the *E*-form at equilibrium is in accord with steric considerations.

Solid D-glucose oxime gave no band in the 1 650—1 690 cm^{-1} region in i.r. or Raman spectra, suggesting the absence of a C=N group [8,9,12-14] and the presence of a cyclic structure. Some evidence in support of a β-D-configuration was provided by an i.r. band at 891 cm^{-1}.[29] The occurrence of mutarotation in aqueous solution was confirmed and observed to be kinetically complex. As has been observed with other carbohydrates having a cyclic structure [15] mutarotation was slower in deuterium oxide than in water. The formation of acyclic forms was suggested by two bands of moderate intensity in the C=N region of the Raman spectrum of an aqueous 20% solution. The occurrence of isomerisation was confirmed and further studied by ^1H n.m.r. spectroscopy.



(7) (8)

The spectrum of D-glucose oxime freshly dissolved in [$^2H_6$]dimethyl sulphoxide was completely analysable (apart from the precise assignment of C–OH signals) in terms of a β-cyclic structure in the normal $^4C_1$ conformation (8). Spectral assignments and parameters (Tables 1 and 2) were checked by double resonance experiments, exchange in deuterium oxide, and computer simulation of the C–H signals. The preponderant conformation of the exocyclic hydroxymethyl substituent is proposed to be that shown (8) on the basis of the observed coupling constants and chemical shifts of the C-6 protons. The fact that one vicinal coupling constant, $J_{5,6}$, is larger (7.0 Hz) than the other (5.5 Hz) suggests the presence of a rotamer with a diaxial relationship between C-5 and C-6 protons, and since the smaller coupling is not associated with the downfield signal which would arise from deshielding by the opposing C-4 hydroxy-group [30] the major rotamer is thought to be that shown. In a survey [31] of the conformation of the primary alcohol group in 23 carbohydrate structures the arrangement having OH *anti* to C was most favoured. The small value of $J_{NCH,NH}$ (2.7 Hz) suggests that the torsion angle between the NO–H and N–H bonds must be within about 30° of 90°. The rotamer having a diparallel arrangement of the NO–H bond and the nitrogen lone pair as shown in (8) is predicted to have minimum energy by molecular orbital calculations on *N*-methylhydroxylamine.[32] The low value of $J_{NH,1-H}$ (3.24 Hz) suggests a preponderance of a rotamer having a torsion angle between the N–H and C(1)–H bonds of about 60° (as distinct from 180°), and of the two possible rotamers that having a 1,3-diparallel relationship of C(2)–OH and N–OH bonds is proposed to be disfavoured as compared with that shown in (8). The spectrum of the solution in [$^2H_6$]-dimethyl sulphoxide showed a slow decrease in intensity of the signals attributed to NOH, 1-H, and NH while new signals appeared at δ 11.0, 10.7, 7.24, 6.64, 4.40, and 5.04 and the remaining part of the spectrum became more complex. The signals (doublets) at δ 7.24 and 6.64 were assigned, according to correlations [15-22] referred to above, to C-1 protons of the *E*- (6) and *Z*- (5) forms of acyclic D-glucose, respectively. On addition of deuterium oxide the singlets at δ 11.0 and 10.7 disappeared; they were assigned to NOH of the *Z*- and *E*-forms, respectively. At equilibrium in deuterium oxide four doublets attributable to the C-1 protons of α-, β-, *E*-, and *Z*-forms were visible at 200 MHz. The observations are consistent with Scheme 1. The preponderance of the *E*- (*syn*-) (56.5%) over the *Z*- (*anti*-) form (13.5%) is consistent with steric considerations, and steric factors appear to outweigh electronic factors in controlling the position of equilibrium between β- (23%) and α- (7%) cyclic forms.

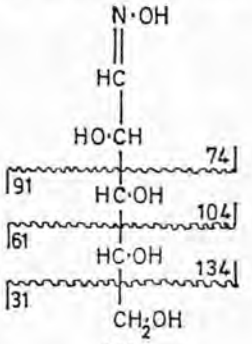


SCHEME 1

The structures of the oximes are supported but not unequivocally confirmed by mass spectrometry and g.l.c. of the per-*O*-trimethylsilyl derivatives. Both oximes gave small $M + 1$ peaks which were shown to have the expected molecular formulae by accurate mass measurements. Other peaks could be accounted for in terms of breakdown patterns based on previous work on carbohydrates [33] and aliphatic oximes.[34] The majority of the peaks in the mass spectrum of arabinose oxime could be attributed to simple cleavage as shown in (9). The base peak at *m/e* 75 probably arises from a McLafferty rearrangement (Scheme 2) as has been observed for other oximes.[34] This

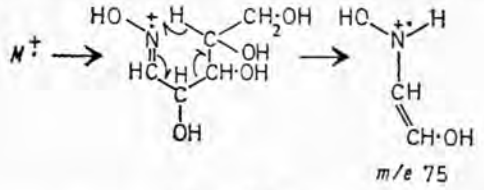
[28] G. E. Hawkes, K. Herwig, and J. D. Roberts, *J. Org. Chem.*, 1974, **39**, 1017.
[29] S. A. Barker, E. J. Bourne, and D. H. Whiffen, *Methods Biochem. Analysis*, 1956, **3**, 213.
[30] R. U. Lemieux and J. T. Brewer, 'Carbohydrates in Solution,' A.C.S. Advances in Chemistry Series No. 117, Washington, 1973, p. 121.
[31] D. C. Fries, S. T. Rao, and M. Sundaralingam, *Acta Cryst.*, 1971, **B27**, 994.
[32] L. Radom, W. J. Hehre, and J. A. Pople, *J. Amer. Chem. Soc.*, 1972, **94**, 2371.
[33] N. K. Kochetkov and O. S. Chizov, *Adv. Carbohydrate Chem.*, 1966, **21**, 39.
[34] H. Budzikiewicz, C. J. Djerassi, and D. H. Williams, 'Mass Spectrometry of Organic Compounds,' Holden-Day, San Francisco, 1967, pp. 367—379.

peak is of lower intensity in the mass spectrum of D-glucose oxime, but the breakdown pattern is similar to that arising from D-arabinose oxime. A number of



(9)

workers have proposed [35-37] the use of *O*-trimethylsilyl derivatives of oximes for analysis of carbohydrate mixtures, and in general it has been presumed that each



SCHEME 2

sugar gives rise to a mixture of *syn*- and *anti*-acyclic forms. However we observed an additional component to be present in the *O*-trimethylsilyl derivative prepared from crystalline D-glucose oxime. This slowly disappeared as was observed by Sweeley,[38] and was presumed to be an *O*-trimethylsilyl derivative of the β-cyclic oxime.

The results (see Experimental section) of periodate oxidation studies on the freshly dissolved oximes supported the structural assignments for the solid compounds; in particular the lower rate of oxidation of D-glucose oxime and the lack of production of formaldehyde were considered to indicate a cyclic structure.

The preference of one of a number of isomeric forms of similar energy in the solid state is presumably related to crystal lattice forces, and it is hoped to investigate the crystal structures in the near future. However there is no obvious reason why a cyclic structure should be more favoured in the case of D-glucose oxime than in the case of D-arabinose oxime.

EXPERIMENTAL

^{1}H N.m.r. spectra were recorded on Varian EM 360 or HR 220 (P.C.M.U.) spectrometers; spectrum simulation was carried out using program UEA NMR BASIC filed at the University of London Computer Centre in conjunction with a plotting routine LIBRARY RHC PLOT developed at Royal Holloway College Central Computer Services. Optical rotations were recorded on a Perkin-Elmer 141 polarimeter. I.r. spectra were recorded with a Perkin-Elmer 337 spectrometer. Raman spectra were taken with a Coderg PHO spectrometer and CR Argon Krypton mixed gas laser at 6 471 Å (D-glucose oxime), and a Cary 81 instrument on the yellow line (D-arabinose oxime). G.l.c. was carried out with a Perkin-Elmer F11 chromatograph (9 ft column of 3% OV-225 on Chromosorb Q, 80—100 mesh, at 168 °C). Mass spectra were taken on an A.E.I. MS 902 spectrometer (P.C.M.U.).

D-Arabinose Oxime.—D-Arabinose oxime was prepared essentially according to the method of Hockett,[39,40] except that 28 g of hydroxylamine hydrochloride was used. The concentration of hydroxylamine after neutralisation was found to be 66% of the original by reaction with an excess of 0.016M-potassium bromate followed by reduction of the excess of bromate with hydrogen iodide and titration of the liberated iodine with 0.1N-sodium thiosulphate. The crude product was concentrated under vacuum, dried by adding sodium-dried ether, and distilled under vacuum. The solid crystallised as plates from 60% aqueous ethanol; yield 60%; m.p. 140° (lit.,[41] 138—139°; lit.,[39] 136—137°) (Found: C, 36.7; H, 6.5; N, 8.2. Calc. for $C_5H_{11}NO_5$: C, 36.4; H, 6.7; N, 8.5%); for ^{1}H n.m.r. data see Tables 1 and 2; $[\alpha]_D^{25}$ initial −83.6°, final −14.2° (*c* 1.3 in H_2O) {lit.,[39] $[\alpha]_D^{20}$ initial −84.0°, final −13.50 (*c* 2.05 in H_2O)}, simple mutarotation constant ($k_1 + k_2$) 0.204 h^{-1} at 25 °C in D_2O and H_2O [lit.,[39] 0.07—0.19 h^{-1} (calculated from data given)]; D-arabinose oxime had consumed [42] 4.6, 4.7, and 4.7 mol. equiv. of periodate after 0.5, 2.0, and 4.2 h respectively and liberated 0.8 mol. equiv. of formaldehyde [43] and 2.9 mol. equiv. of formic acid; *m/e* 166 ($C_5H_{12}NO_5$, $M + 1$), 134 ($C_4H_8NO_4$), 104, 103 ($C_4H_7O_3$), 91, 75 ($C_2H_5NO_2$), 74, 61, and 31; i.r. $\nu_{max.}$ (KBr) 1 680w cm^{-1} (C=N); Raman $\nu_{max.}$ (solid) 1 678m cm^{-1} (C=N), (sat. aq. soln.) 1 656 and 1 664 cm^{-1} (C=N).

D-Glucose Oxime.—Powdered hydroxylamine hydrochloride (70 g) in dry methanol (300 cm^3) was neutralised with a solution of sodium methoxide [from sodium (20 g)] in methanol (100 cm^3). The neutralised solution was cooled in ice and filtered, and the residue was washed with dry methanol (200 cm^3). The combined filtrate and washings were shown to contain 0.64 mol of hydroxylamine (64% of the original) by titration as described above. The methanolic hydroxylamine solution was refluxed in a water-bath and powdered anhydrous D-glucose (110 g, 0.61 mol) was added slowly. The solution was concentrated, left at room temperature overnight, and dried in a desiccator. The dry solid was powdered, dissolved in 80% aqueous methanol, and allowed to crystallise in a freezer. Recrystallisation was carried out from 70% methanol and 60% methanol, and gave truncated prisms (53 g, 45%), m.p. 141° (lit.,[4] 137.5°; lit.,[44] 141°; lit.,[45] 136—137°) (Found: C, 36.7; H, 6.8; N, 7.2. Calc. for

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$C_6H_{13}NO_6$: C, 36.9; H, 6.7; N, 7.2%); for ^1H n.m.r. data see Tables 1 and 2; $[\alpha]_D^{32.5}$ initial −8.04°, final −1.92° (*c* 2.6 in H_2O) {lit.,[45] $[\alpha]_D$ final −2.2° (*c* 9.4 in H_2O)} (the mutarotation was observed to be complex, *i.e.* initially the rotation decreased but after 0.5 h it increased until it reached a constant value after about 10 h; the mutarotation in D_2O was similar except that equilibrium was reached after about 36 h; equilibration was also observed by ^1H n.m.r. to be a complex process; the areas of the 1-H signals of the acyclic forms did not increase at the same rate nor by a simple first-order process); D-glucose oxime consumed [42] 2.4, 3.2, 4.2, and 4.2 mol. equiv. of periodate after 0.5, 1.25, 4, and 5 h, respectively, and liberated no formaldehyde [43] and 1.95 mol. equiv. of formic acid after 4 h; *m/e* 196 ($C_6H_{14}NO_6$, $M + 1$); i.r. $\nu_{max.}$ (KBr) 980s (N–O [46]) and 891sharp and m cm^{-1} (ring breathing [29]); Raman $\nu_{max.}$ (20% aq. solution) 1 648m and 1 653m (C=N) not given by powdered crystals or water alone.

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