SYNTHESIS AND PROPERTIES OF SELECTED ALPHA AMYLASE INHIBITORS.

ROBERT JOHN PEEK.

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The Bourne Laboratory
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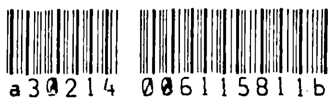

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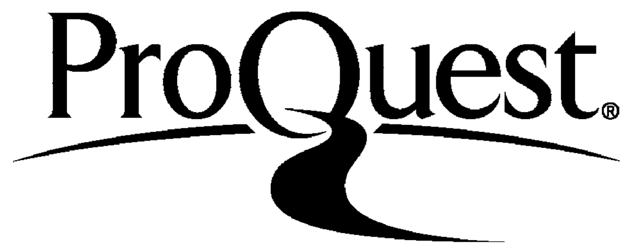

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ABSTRACT.

A logical approach to the design of α-amylase inhibitors is developed from the known features of catalysis by glycosidases in general and α-amylases in particular.

Four potential inhibitors of wheat α-amylase namely maltobiono-δ-lactone, maltal , N-D-glucosylbenzylamine and N-D-glucosylpiperidine have been synthesised and their structure established by a variety of physical methods. ^{1}H NMR has shown maltal to exist either as an equilibrium system involving the $^4H_5$ and $^5H_4$ conformations, with the $^4H_5$ predominating, or possibly as a single conformation slightly distorted from the classical $^4H_5$ half chair. Both N-D-glucosylbenzylamine and N-D-glucosylpiperidine were shown to exist in the β-form with the normal chair conformation of the sugar ring. The substituted piperidine ring undergoes rapid ring inversion at ambient temperature.

For the inhibition studies, wheat α-amylase was extracted from a sample of malted Champlein wheat and obtained with a specific activity of 44 Units per mg of protein.

Inhibition by maltobiono-δ-lactone and maltal was examined at pH 5.0 by analysing for reducing sugar

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production using the alkaline ferricyanide method. Maltobiono-δ-lactone was shown to be a non-competitive inhibitor with an inhibition constant of 2.5 mM. Maltal behaved as a slow-binding inhibitor, and at equilibrium (which was reached after about one hour under test conditions) acted competitively with an inhibition constant of 4.4 mM.

Inhibition by the N-glycosylamines was complicated by their significant hydrolysis at pH 5, and was examined at pH 6 using an iodine stain technique. N-D-glucosylbenzylamine (K_i = 1.5 mM) and N-D-glucosylpiperidine (K_i = 4.9 mM) were moderate inhibitors of wheat α-amylase. Their inhibition behaviour was complex (non-linear), possibly because of concomitant hydrolysis.

DEDICATED TO MY PARENTS

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CHAPTER 1

ALPHA-AMYLASE, THE CHEMISTRY OF ITS ACTION AND INHIBITION

1.1 Enzymes: Their Structure and Specific Catalytic Power.

An enzyme has been defined as, ' a protein molecule having both catalytic activity and specificity for its substrate or substrates; it does not perform a reaction, but it catalyses the performance of the reaction'[1]. This can be represented diagramatically by reaction profiles for uncatalysed and enzyme-catalysed reactions (top and bottom curves respectively of Fig.1.1.)

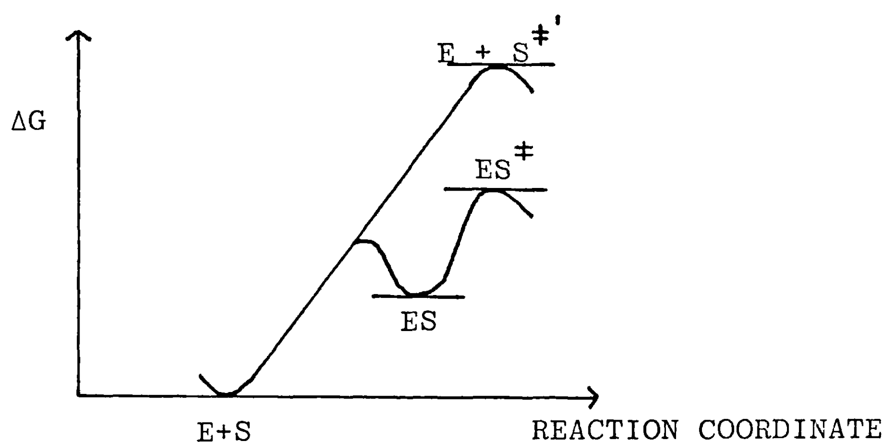
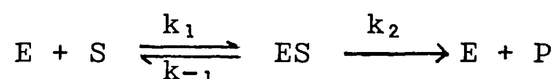


Fig.1.1. Free energy diagram for uncatalysed and enzyme-catalysed reactions, where the concentration of substrate (S) is small compared to Km, so that no substantial concentration of enzyme-substrate (ES) complex is present.

In the late nineteenth century, the ability of yeast extracts to catalyse the conversion of

sugar to ethanol or of body fluids to digest polymers in neutral solution and at low temperature was found astonishing. Fischer (1894) in trying to explain the substrate specificity of enzymes, proposed a simple hypothesis.He saw the enzyme as a rigid, well defined structure, to which a suitably shaped substrate could bind to form an enzyme-substrate (ES) complex, (like a key fitting into a lock). This theory explains the majority of catalytic phenomena, but it is too simple to account for all features of enzyme behaviour. The idea of an ES complex, which breaks down to product and unchanged enzyme, was used in the mathematical approach of Michaelis and Menten[2] on the following reaction scheme (Scheme 1.1).



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Scheme 1.1.

The following assumptions:

1) Rapid equilibrium is achieved and maintained at all times between the enzyme and its substrate.

2) The rate limiting step is the breakdown of ES complex to product.

3) A negligible proportion of the initial concentration of substrate is used up in achieving the equilibrium.

4) Enzyme is not 'used up' and can exist only as free E, or as the complex ES.

led to the Michaelis-Menten equation (shown here with the generally accepted notation).

$$v = \frac{V\ max}{(1 + Ks/\{S\})}$$

where v = reaction rate

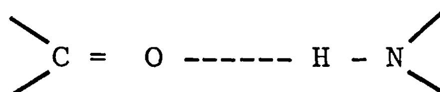
V = maximum reaction rate at saturating substrate levels.

K_S= dissociation constant for the ES complex (under these conditions K_S = Km, the Michaelis constant, often defined as the substrate concentration which produces half maximal velocity). While the mechanisms of real enzyme catalysed reactions may be more complicated than the reaction scheme given above, the Michaelis-Menten equation is often obeyed and the Michaelis-Menten parameters Km and Vmax are important and useful quantities.

That enzymes are, in fact, protein in nature was initially established by Sumner,[3] who succeeded in obtaining the enzyme urease in crystalline form from jack bean meal and subsequently identifying it as a protein. This met with considerable scepticism, until Northrup et al[4] crystallised and characterised several more enzymes; all were proteins, as have proved to be all enzymes purified to date. With the realisation of this fact, the true source of the remarkable rate accelerations (under optimal conditions, most enzymatic reactions proceed 10^8 - 10^{11} times faster than the corresponding nonenzymatic reactions) achieved by enzymes,

can be more readily understood. But first the structural properties of proteins[5] must be considered.

Proteins are linear (i.e. unbranched) polymers made up of about 20 different amino acids, and their conformations and properties are decided by the nature and order of the amino acid monomers in the polymer chain i.e. the primary structure. Non-covalent bonds, much weaker than the covalent bonds, are responsible for both secondary and tertiary structure. Secondary Structure is a result of the formation of hydrogen bonds between carbonyl oxygens and amide NH groups of the polypeptide backbone:



The amino acid residues interacting to form these hydrogen bonds are not adjacent, but are separated in the polypeptide chain. The two common secondary structures are the helix and the sheet. In the most stable helix, the α-helix, each hydrogen bond is formed between a carbonyl group and the amide group of the fourth amino acid residue toward the end of the protein having a free carboxyl group (i.e. there are 3.6 amino acid residues per turn of the helix).
A minority of the residues of enzymes are in helical conformations (0-35% of the molecules generally). In sheets, the interchain hydrogen

bonds are formed between sequentially distinct regions of the protein, which lie alongside each other. Tertiary Structure is the folding of the protein due to interactions of amino acid side chains. Already coiled and partially stablised, the protein can fold further into a compact globular shape, resembling a sphere. For many enzymes, e.g. lysozyme[6] this sphere is divided into two or more domains, which can move relative to one another. This movement allows the protein to entrap a small volume between domains. The connecting strands are called a hinge and contain a high content of the more polar amino acids and/or a high content of glycine, accounting for the greater mobility.[7] The area between domains is the 'enzyme active site'.

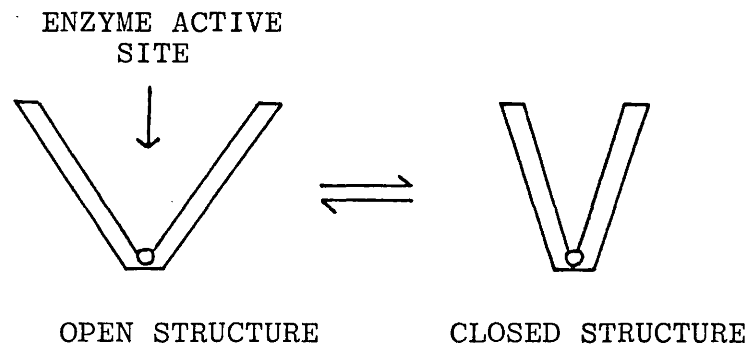


Fig.1.2. A schematic diagram of a hinged protein which has relatively rigid regions and a more mobile loop region. The discussion of real examples is to be found in Ref.8.

In the folded protein all of the ionic side chains, on the amino acids, are exposed to solvent,

except for a few 'buried' groups which often participate in the catalytic reaction. The apolar side chains tend to turn inwards, making the interior of the enzyme hydrophobic. This leads to the formation of further ionic bonds, hydrogen bonds between side chains and also to the formation of -S-S- crosslinks, all of which help to maintain the tertiary structure of the enzyme. We can now envisage the enzyme active site as a low dielectric, hydrophobic environment in which charged groups and permanent dipoles are arranged in such a way that they contribute to the stabilisation of the reaction transition state. Since these groups are unable to rearrange their orientation (to any large extent) they are able to provide a large electrostatic stabilisation which may enable a reaction to proceed faster than in polar solutions. It has been suggested[9,10] that this non-covalent stabilisation is the major source of enzyme catalytic power and this notion will be discussed further below. Other theories of the source of enzyme catalytic power have of course been expounded and these are discussed, in great detail, in the recent reviews of Jencks[11] and Page[12].

The,so called,'central dogma'[13] of molecular biology assumes that the conformation actually adopted by a protein, in its native state, is the

one of lowest free energy, the most stable in the natural environment. This really is an assumption at the present time. Although it would undoubtedly be true at equilibrium, living systems are not at equilibrium and there may not always be time for a polypeptide chain to search out the most stable conformation. The assumption is certainly true for some protein molecules,[14] but it will remain a hypothesis until many more proteins have been studied in detail. (However it cannot be true in the special case of proteins which are biosynthesised by fragmentation of precursors containing disulphide bridges e.g. insulin). Although the protein as a whole may be at a free energy minimum, there are local regions of strain. Vallee and Williams[15] pointed out that the metal ions in many metalloenzymes have unusual spectroscopic properties and coordination geometries. From this they derived the concept of an 'entatic state'. The protein as a whole is at a free energy minimum, but local regions were stressed; metal ions in particular have ligands disposed in a way that in a small complex would not be at a free energy minimum. These local regions of strain (or stress) are concentrated in the active sites of catalytic proteins. In order to achieve such a local state of affairs, whilst the enzyme as a whole is

at a free energy minimum, requires a large mass of subtly disposed protein, which explains the reason for such a large molecule. Insofar as the distortion of a metal ion prosthetic group towards the transition state for, for example, electron transfer constitutes a stabilisation of that state, then the ideas of transition state stabilisation and of 'entatic states' are identical. However, the distinction between the two concepts has been emphasised.[16]

But what of the enzymes' specificity for its substrate? This falls into three main categories. Reaction specificity, that is to say an enzyme catalyses a particular type of reaction, e.g. the oxidation of primary alcohols to aldehydes. Substrate specificity, which denotes the range of different structures which will act as substrates. This may be very broad, in that a number of chemically similar substrates can be accepted e.g. by the proteolytic enzymes or very narrow so as to encompass only a single substrate e.g. urease,which catalyses the hydrolysis of urea to ammonia and carbonate and will act on no known substituted urea. Finally, enzymes may possess stereospecificity.[17] Different types of enzyme stereospecificity may be distinguished; thus reaction specificity and substrate specificity can be extended to include the reaction stereo-specificity (e.g. inversion or retention of

anomeric configuration in the action of glycosidases) and substrate stereospecificity (e.g. the absolute preference for D-glucose rather than L-glucose derivatives). Since enzymes are themselves chiral reagents, reaction specificity can be very subtle i.e. very high (or possibly even absolute) specificity is directed towards prochiral centres and faces.

1.2. The Action of Wheat Alpha-Amylase.

Alpha-amylases (α - 1,4-glucanhydrolase E.C.3.2.1.1.) are hydrolytic enzymes produced by all types of life:animals, plants and micro-organisms, and catalyse the hydrolysis of the glycosidic linkages in α-D-(1 $\rightarrow$ 4)-glucans (amylose, amylopectin, glycogen). However, the linkage in maltose is not broken and the linkage nearest the non-reducing end-group of a larger glucan is cleaved at a very low rate.[18] α-Amylases are so named because the newly formed reducing groups have an α-anomeric configuration i.e. the enzyme acts with retention of configuration. It is found as a corollary that all α-amylases are also endo-ases i.e. they are able to attack the interior of starch chains, in contrast to β-amylase, an exo-ase, which attacks at non-reducing termini of chains. The action pattern of α-amylases, however, varies from source to source, but this survey will concentrate on the catalytic hydrolysis

of amylopectin by cereal α-amylases.

Cereal α-amylases, like all α-amylases, are believed to be calcium metalloenzymes, although the metal-protein stability constants depend on the source of the enzyme and follow the sequence higher plant < mammalian < bacterial < fungal.[19] Removal of the calcium by such chelating agents as (ethylenedinitrilo)-tetraacetic acid, or dialysis, leads to reversible inactivation, which may be restored on the addition of the metal ion.[20] Calcium deficient α-amylases are, however, susceptible to attack by proteases, whereas the native enzymes are remarkably resistant.[21] The amino acid compositions of some α-amylases, e.g. malted sorghum[22,23] have been determined, but the exact sequence or three dimensional structure of any one α-amylase is not yet known.* Different cereal α-amylases have similar molecular weights of about 45 000[24] (this will be a minimum size, as it was found by gel permeation and am ylases tend to be more compact than other enzymes). The effects of pH[24,25] and temperature[24] on the activity of wheat α-amylase has been determined by various groups (Fig.1.3).

*A low resolution (5 Å) x-ray crystallographic study has now been completed by Payan et al, for porcine pancreas alpha-amylase.

Acta. Crystallogr. Sect.B, B36(2), 416 (1980) Payan F, Haser R, Pierrot M, Frey M, Astier J P, Abadie B, Duee E, and Buisson G.

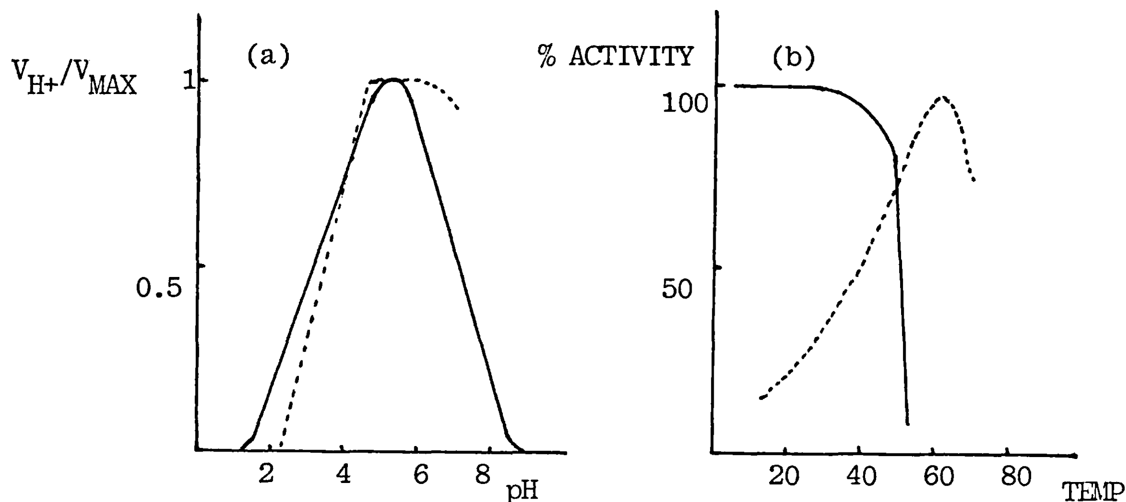


Fig.1.3.(a) Effect of pH on the activity of wheat α-amylase; (——) Milne 1968, (------) Irshad 1978.

(b) Effect of temperature (^{o}C) on the activity (-----) and stability (——) of wheat α-amylase.

The exact role of amino acid side chains at the active site is, as yet, not fully understood, but a considerable amount of research is in progress in this area.[26,27] It is known however that the enzyme must contain binding sites for 8 or 9 glucose units[28,29] and that any substrate smaller than this undergoes hydrolysis at a reduced rate (e.g. it has been shown that plant amylase hydrolyse maltohexaose approximately six times slower than they do starch[30] and for B.Subtilis this figure is nearer ninety[31])

Greenwood and Milne[32] postulated the following favourable and unfavourable interactions of various maltodextrins with the hypothetical active site of cereal α-amylases (Fig.1.4.)

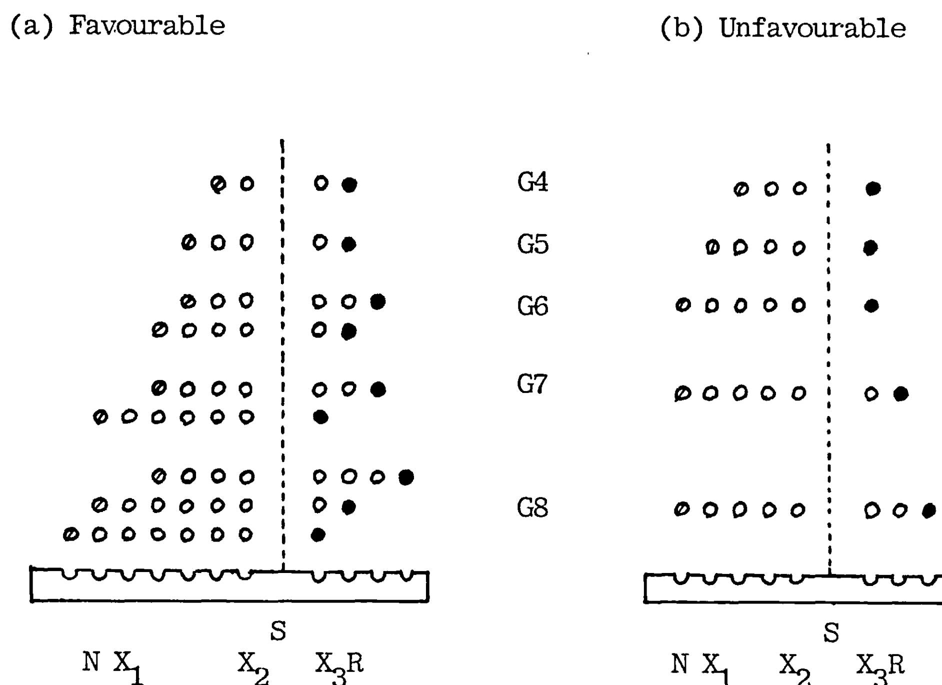


Fig.1.4. Interaction of various maltodextrins with the hypothetical active site of cereal alpha-amylase after Greenwood and Milne, 1968: (a) favourable complex formation; (b) unfavourable complex formation.

N-site that preferentially binds a non-reducing chain-end.

X_1,X_2- sites that are specifically unfavourable for binding non-reducing end groups.

S-point at which hydrolysis occurs.

X_3 - site that is specifically unfavourable for binding a reducing end group.

R - site that preferentially binds a reducing chain end.

Robyt and French[33] pointed out the assymetry of the catalytic binding site relative to the binding sites, the cleavage point being located adjacent to the non-reducing end of the oligo-saccharide. Immediately after a hydrolytic scission, the region of the active site having a greater affinity for a chain fragment will tend to retain it, whilst the other fragment diffuses away(Fig.1.5)[32].

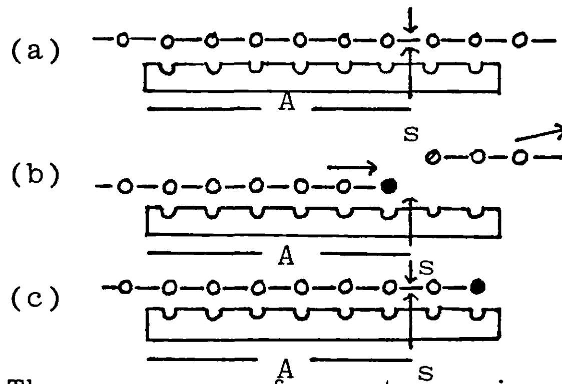


Fig.1.5.The sequence of events envisaged in multiple attack: (a) an internal segment of amylose is bound to the active site of the enzyme, and hydrolytic scission occurs at point S; (b) after scission, the fragment bearing the newly-exposed non-reducing chain-end diffuses away, but the remaining fragment is more strongly bound to portion A of the enzyme, and hence remains longer on the surface; (c) the substrate rearranges itself in the active site, and a second hydrolytic event occurs. It has been assumed, arbitrarily, that maltose is the product of this repetitive attack.

This retention mechanism also implies that the glycosyl fragment will be retained in the active site, while the 'aglycone' diffuses away and is replaced by water, or some other acceptor, in a double displacement reaction. The remaining fragment may then rearrange in the active site, thus giving an opportunity for a second hydrolytic event. Thus the direction (polarity) of any multiple attack will be towards the non-reducing end group in the case of α-amylase, though this has only been established as true for porcine pancreatic α-amylase[34]. (In contrast, the polarity is towards the reducing end for β-amylase[31]).

Discussion so far has dealt only with the later stages of enzyme action on oligosaccharides, but what of the initial action on amylopectin? Amylopectin with a molecular weight of the order 10^7- 10^8 (Banks et al[35] give wheat amylopectin as having a molecular weight of 4×10^8), is one of the largest natural polymers. It is a branched structure containing some 4-5% of α-1 → 6 - branch points. These branch points are stable to hydrolysis by α-amylase and also confer some stability to α-1→ 4-D-glucosidic linkages,[36] thus making hydrolysis of amylopectin incomplete and leading to the formation of limit dextrins.[37] In the vicinity of a branch point (Fig.1.6) only two of

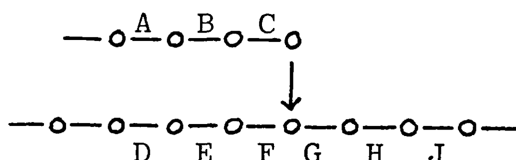


Fig.1.6. Typical branch point of amylopectin.

the α-1→ 4-D-glucosidic linkages are resistant *per se* to α-amylolysis, namely G and H. Apart from these, in the initial stages the attack is totally random (as shown by Greenwood and Milne[24], using the viscometric technique of Vink[38]) leading mainly to the maltodextrins of degree of polymerisation larger than eight (G_8), with smaller amounts of G_2-G_8. The larger sizes then gradually decrease until in the case of amylose only traces of G_8 remain, but in the case of amylopectin more significant amounts. A non-random pattern (as shown previously) then takes over, but at a much slower rate. A typical composition after exhaustive α-amylolysis of amylopectin would be[37]:

G_1, 9%; G_2, 53%; G_3, 6%; G_4, 1%; G_5, 3%; G_6, 3%; G_7, 7%; Higher, 19%.

1.3. The Practical Significance of Alpha-Amylase Inhibition

In the food processing and related industries it is desirable in certain circumstances to control or inhibit completely the activity of a particular

enzyme. Thus the activity of one enzyme of a number used sequentially, or of a number present in a particular process, may be required to be inhibited, while retaining the ability to use other enzymes subsequently. General inhibitors such as acid or heavy metals or general treatments such as elevated temperature may not be attractive or even possible in such circumstances. The requirement is for a selective and powerful inhibitor of the particular enzyme activity concerned.

Wheat α-amylase is of considerable interest in cereal chemistry, as it is directly involved in the absorption properties and gassing power of dough and in the final properties of bread. Excess α-amylase, such as that arising from sprout-damaged wheat, can lead to bread having a sticky, excessively moist, and inadequate crumb. On the other hand, the addition of a small amount of α-amylase to a sound flour may lead to such breadmaking improvements as higher loaf volume, improved crumb colour, increased moistness of the crumb and keeping power, and better flavour.[39] Wheat from the U.K. (because of adverse weather conditions) tends to have high α-amylase activity and the only means of controlling this at present is by selecting and blending with European wheats to give grists with

acceptable activity. A selective and powerful food acceptable, inhibitor of α-amylase could prove very useful in overcoming this problem.

Inhibition of α-amylases has also been investigated as a means of treating diabetes, by slowing the rate of glucose absorption from the gut. Most work so far has centred on the use of dietary fibre, especially guar gum, to modify glucose tolerance in healthy and diabetic volunteers and to reduce glycosuria in diabetics. It has been suggested that the mechanism of action is related to physical properties such as viscosity, which both delays gastric emptying and reduces the rate of uptake of glucose from the lumen of the small intestine. Some recent work has been done with Acarbose (BAY g 5421, Bayer U.K.Ltd., Haywards Heath, Sussex) a highly effective inhibitor of the α-glucosidases in the human digestive tract. With this inhibitor, it is possible to delay the breakdown of ingested carbohydrates (sucrose, starch) and consequently to control the resorption of glucose from the intestines. Clinical studies with Acarbose have been able to prove the usefulness of this new therapeutic principle through the treatment of diabetes mellitus.[40] Its use at present, however, has been limited by common and often unacceptable side effects, including flatulence

and diarrhoea, secondary to colonic fermentation of malabsorbed sugars.

Amylolytic enzymes are also used in key steps in brewing and the manufacture of starch syrups (concentrated aqueous solutions of starch hydrolysates) and crystalline glucose (dextrose). The employment of suitable combinations of α- and β-amylases, amyloglucosidases, isoamylases or maltases, makes it possible to obtain an infinite variety of starch hydrolysis products and hence syrups with any desired properties e.g. with a maltose content exceeding 80%.[41]

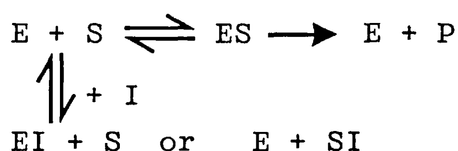
In the textile industry, bacterial amylases are used both for the preparation of modified starch adhesives and for the removal of adhesives from fabrics. Preparations of bacterial amylases, which retain their activity at much higher temperatures than amylases from moulds, have to a large extent replaced malt and pancreatin. In the paper industry, certain bacterial amylases are used to modify starch for coating paper.

1.4. Classification of Specific Enzyme Inhibitors.

The differences between various types of enzyme inhibition pattern will be described in this section in simple kinetic terms (for a more complete discussion see Wong[42]). They can be sub-divided into three main types:

1) Reversible; a.Competitive, b. Uncompetitive, c.Noncompetitive;
2) Irreversible, including so-called suicide or k_{cat} inhibitors;
3) Transition State Analogue, which may be reversible or irreversible.

Two types of ligand binding can bring about reversible competitive inhibition. Firstly, the inhibitor may resemble the substrate sufficiently so as to be bound in its place at the active site. This enzyme-inhibitor (EI) complex is either incapable of being converted to any product, dead-end inhibition, or the rate may be so slow as to be kinetically insignificant. Although many competitive inhibitors have a structural resemblance to the substrate and bind to the same site on the enzyme (e.g. malonate and succinate with succinate dehydrogenase), this is not a necessary criterion for competitive inhibition. Negative feedback inhibitors of allosteric enzymes can be competitive with substrate, even though they bind at different sites on the enzyme. Secondly, an inhibitor may bind to the substrate to form an SI complex, that will not bind to the enzyme (Scheme 1.2).



Scheme 1.2.

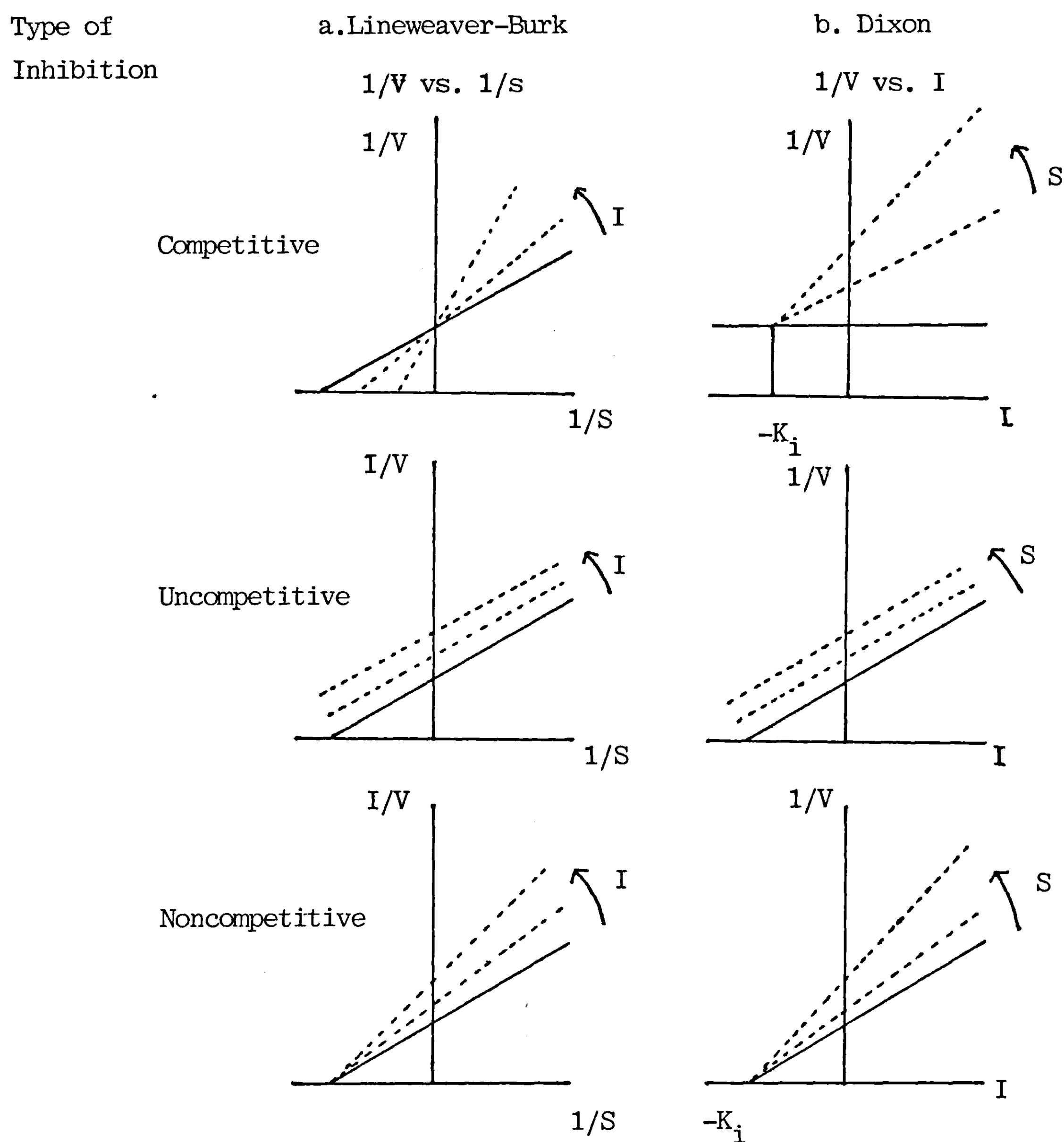


Fig.1.7. Kinetic patterns for various inhibition types

a. The solid lines are the reciprocal plots in the absence of inhibitor, and the dashed lines in the presence of two different concentrations of inhibitor. The arrows show the direction of increase in {I}.

b. The solid lines are the reciprocal plots in the presence of saturating substrate and the dashed lines in the presence of two different concentrations of substrate. The arrows show the direction of decrease in{S}.

The kinetic diagnostic for competitive reversible inhibition is that in the double-reciprocal plot (the Lineweaver-Burk plot[43]) only the slope is affected, i.e. the apparent K_m changes (Fig.1.7). The introduction of saturating substrate will leave the maximal velocity and hence the intercept on the 1/V axis unaffected. Further to this, a plot of 1/V vs. I (the Dixon plot[44]) will give the inhibitor constant K_i, which is a measure of the binding strength of the inhibitor.

$$K_i = \{E\}\{I\} / \{EI\}$$

In reversible uncompetitive inhibition, the inhibitor behaves as if it binds to the ES complex only to form an inactive EIS complex. A double reciprocal plot (Fig.1.7) shows parallel lines i.e. both K_m and V are decreased to give a constant slope, but the intercepts vary with I. It is very rare in single substrate reactions and usually only occurs in multi-substrate cases. In reversible noncompetitive inhibition the inhibitor binds to a site on the enzyme that is not identical to the substrate-binding site, and does not affect the affinity of the enzyme for the substrate. Both EI and EIS complexes can form, but the EIS complex cannot be converted into product. A double reciprocal plot (Fig.1.7) shows that I/V intercepts on the ordinate are increased i.e. maximal velocity is altered but

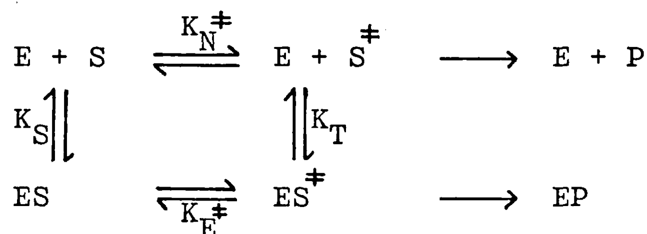
K_m remains unaffected. A noncompetitive inhibitor can be envisaged as removing active enzyme from solution. Its inhibition cannot be reversed by an increase in the concentration of substrate. Noncompetitive inhibitors are rare.

An irreversible inhibitor[45] usually functions by means of a chemical reaction at the active site of the enzyme and either prevents the substrate from binding or removes from an essential amino acid side chain the ability to function normally in the catalytic process. The inhibitor species in this type of inhibition is usually bound to the enzyme by a covalent bond, does not dissociate from the enzyme and cannot be removed by a process such as dialysis. It is important to note that a chemical reagent which brings about inactivation of the protein is not necessarily regarded as an irreversible inhibitor. In addition, the effect of certain irreversible inhibitors may be reversed by chemical means e.g. acetylation of carboxypeptidase causes loss of activity, but treatment of the acetyl-enzyme with hydroxylamine removes the acetyl group and reactivates the enzyme. The original acetylating agent must nevertheless be regarded as an irreversible inhibitor. The process can be represented as:

covalent modification

The transition state analogue theory began with the speculations of Pauling[46] and Haldane[47] that the 'specificity of the catalytic activity of enzymes is due to a surface configuration of the enzyme, such as to make the enzyme complementary to the substrate molecule, or rather, to the strained state that occurs during the catalysed reaction'.[46] The function of an enzyme, after all, like that of any other catalyst is to reduce the relative free energy of the transition state(s) for the reaction and it is difficult to imagine how this could be achieved if the enzyme did not possess an unusual affinity for the altered substrate in the transition state, exceeding that for the substrate itself.

For simplicity, the transition state theory[48,49,50] is applied to a unimolecular process (Scheme 1.3).



Scheme 1.3

Where:

K_S = equilibrium constant for the association of the substrate S, with the enzyme E

$K_N^{\ddagger}$, $K_E^{\ddagger}$ = equilibrium constants for the formation of the transition states of the non-enzymatic and enzymatic reactions, $S^{\ddagger}$ and $ES^{\ddagger}$ respectively.

K_T = equilibrium constant for the binding of $S^{\ddagger}$ to E to form $ES^{\ddagger}$.

Using the thermodynamic cycle Wolfenden[48] obtained $K_T/K_S = K_N^{\ddagger}/K_E^{\ddagger} = k_n/k_e$ which gives the ratio of nonenzymic rate to enzymic (true if transmission coefficients are unity). Generally, the results found for this ratio[51] from kinetic data (k_n,k_e) are in the region 10^{-8} - 10^{-14}, so that K_T should be lower than K_S by this factor. Hence even a quite poor attempt to mimic the activated substrate can give good inhibitory properties. Rate comparisons of this type are likely only to provide a minimum estimate of the binding ratio expected for an ideal transition state analogue, as will be shown below.

The first, and major question, is which non-enzymic reaction is really suitable for comparison with the enzymic reaction. It has been assumed that the substrate has a comparable structure in the transition state for both the enzymic and non-enzymatic reactions. It is very probable, however, that the transition state for the non-enzymatic reaction in water (at the

same pH) will differ, at some level of structural detail, from the substrate portion of the enzyme-substrate complex in the transition state.
The non-enzymic reaction must, of course, follow the pathway with the lowest free energy of activation,which is not true for the enzymic system, and would lead to an underestimate of the rate enhancement produced. Similarly, if the mechanisms are alike, but differ in the point along the reaction coordinate at which the transition state is reached, the ratio will be underestimated (Fig.1.8).

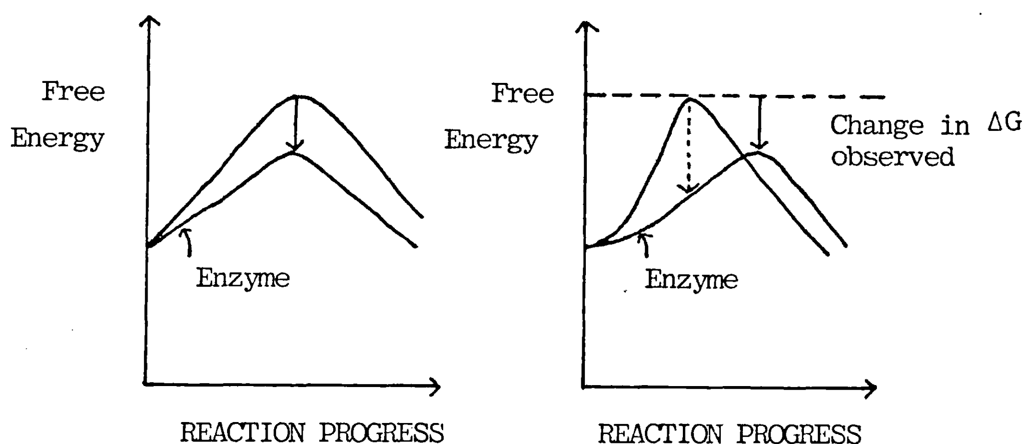


Fig.1.8 Comparison of free energy profiles for catalysis, for cases in which the transition state is reached at the same (left) or a different (right) point on the reaction coordinate for the enzymic and nonenzymic reactions.

This type of situation will lead to an overestimate of K_T and hence an underestimate of the binding ratios. As Wolfenden[49] has

pointed out, the possibility of underestimating K_T is unlikely, which makes this a very useful principle on which to base the design of potent inhibitors.

Clearly the transition state analogue principle can apply to both reversible and (the binding part of) irreversible inhibition. A particular category of irreversible inhibitors have been termed suicide or K_{cat} inhibitors.[52] These are compounds which are selectively activated by the target enzyme specific catalytic action. The inhibitor, thus modified, then binds irreversibly to the enzyme leading to inactivation.

1.5. The Active Site of Alpha-Amylase.

Information about the functional groups essential for substrate binding and catalysis by α-amylases, for which the amino acid sequence and 3D structure are not known, must come from more indirect methods, such as pH dependence of enzyme kinetics, group specific inactivation reactions or the use of active site directed inhibitors.

Studies of the effect of pH on the activity of α-amylases[53,54], have indicated that imidazole and carboxyl groups are necessary for activity. This type of work can give only tentative results, however, as perturbations of buried residues of

up to 3 or 4 pH units have been noted[55]; caused possibly by the hydrophobic environment or neighbouring group stabilisation.

A considerable number of chemical studies[56] have been carried out on α-amylases in order to determine which amino acid residues may be present at the active site of the enzyme. For the most part these have consisted of treating the enzymes with chemical reagents, which react with amino acid side chains and testing the modified enzyme for activity. If the modification has destroyed or decreased the activity of the enzyme, then it has usually been deduced that the amino acid residue reacted was present at the active site. Such a deduction is not always justified. For example p-chloromercuribenzoate, considered to be specific for sulphydryl groups, was reported as inhibiting <u>B.subtilis</u> α-amylase[57], which does not contain such groups. The principles and limitations of this method have been reviewed[58] and a book[59] is available summarising the methods used for the chemical modification of active sites.

The most important reactive side chains in proteins are amino, imidazolium, carboxyl, sulphydryl, phenol, indole, amide, thio ether and aliphatic hydroxyl groups. A study of the action of iodine and diazobenzenesulphonic acid on the enzymes of soya beans[53], broad beans[60]

and cereals[24,32] has helped to confirm the importance of histidine for the catalytic activity. The results of acetylation and modification of the enzyme with, for example, nitrous acid or dinitro-fluorobenzene, indicated that amino groups are probably necessary for the activity of higher plants[24,32,53,60], mammalian[61,62,63], bacterial[64] and fungal[63] alpha amylases. Sulphydryl groups are not believed to form part of the active centre of these enzymes,[65] although it has been claimed that masked sulphydryl groups are necessary for the full activity of porcine pancreatic alpha amylase;[66] their function being to hold the enzyme in the correct conformation for maximum activity.

The role of the tyrosine groups has been studied for various types of alpha amylase and the contradictory results obtained, demonstrate the problems associated with using non-selective reagents. Tyrosine side-chains are reported to have no catalytic effect for alpha amylase of bacterial origin[63,67] and pancreatic alpha amylase.[68,69,70] On the other hand, stabilisation of the optimum steric structure has been attributed to tyrosine groups.[71,72] In order to establish unambiguously whether the subsites present in the active centre play a role in the binding of the substrate and in the formation of products, or if they affect only the stability of the tertiary

structure of the protein, the change in k_{-1}/k_{+1} occurring upon modification must be known. The change in binding affinity ($\Delta k_{-1}/k_{+1}$) due to modification can be determined by a relatively simple spectrophotometric method using cyclohexa-amylose bound to Sepharose 6B as a substrate analogue.[73] Laszlo et al[27] applied this method to the tyrosine groups in porcine pancreatic alpha amylase using N-acetylimidazole[74] as the modifying reagent, which avoided undesirable side-reactions[75] (mainly acetylation of SH and NH_2 groups). Their results indicated that one of the tyrosine groups was located in the active centre, presumably near the catalytic site, and participated in the formation of the enzyme-substrate complex, while the rest were necessary for the maintenance of the tertiary structure of the enzyme.

The reagents which give the most useful and direct data are compounds which, because of their similarity to substrates, can bind reversibly at the active site and then react covalently with a reactive amino acid at the catalytic site. These inhibitors have been called either active-site-directed irreversible inhibitors[45] or affinity labels.[76] Rando[52] has pointed out, that most of the active-site-directed inhibitors studied rely solely on the binding properties of the enzyme.

He has suggested that compounds, which he calls k_{cat} inhibitors (mentioned previously in Section 1.4),selectively activated by the target enzyme's specific catalytic action, may be more useful still. This type of inhibition is made more complicated for α-amylase, because its natural substrate, starch, has such a high molecular weight.

In recent years protein inhibitors of α-amylase have received much attention. They were first described in wheat seed by Kneen et al[77,78,79] and since then many papers have appeared, including a review,[80] and very recent paper.[81]

1.6 The Design of Alpha-Amylase Inhibitors.

As discussed in Section 1.4, a logical approach to the design of enzyme inhibitors is via the specific modification of substrate structure. The interaction of such modified substrates with the enzyme can give insight into the chemistry of enzyme catalysis, however, where the substrate is polymeric, as in the case of starch, this is difficult and a modified approach is more appropriate. The transition state analogue theory could provide a rational basis for the design of inhibitors, but a requirement for its use is a clear idea of the mechanism by which the enzyme acts.

A possible mechanism for catalysis by α-amylase, consistent with the results of studies of non-enzymic hydrolysis,[82] retention of configuration in the enzyme-catalysed reaction and based upon the mechanism proposed for lysozyme, is shown below (Fig.1.9).

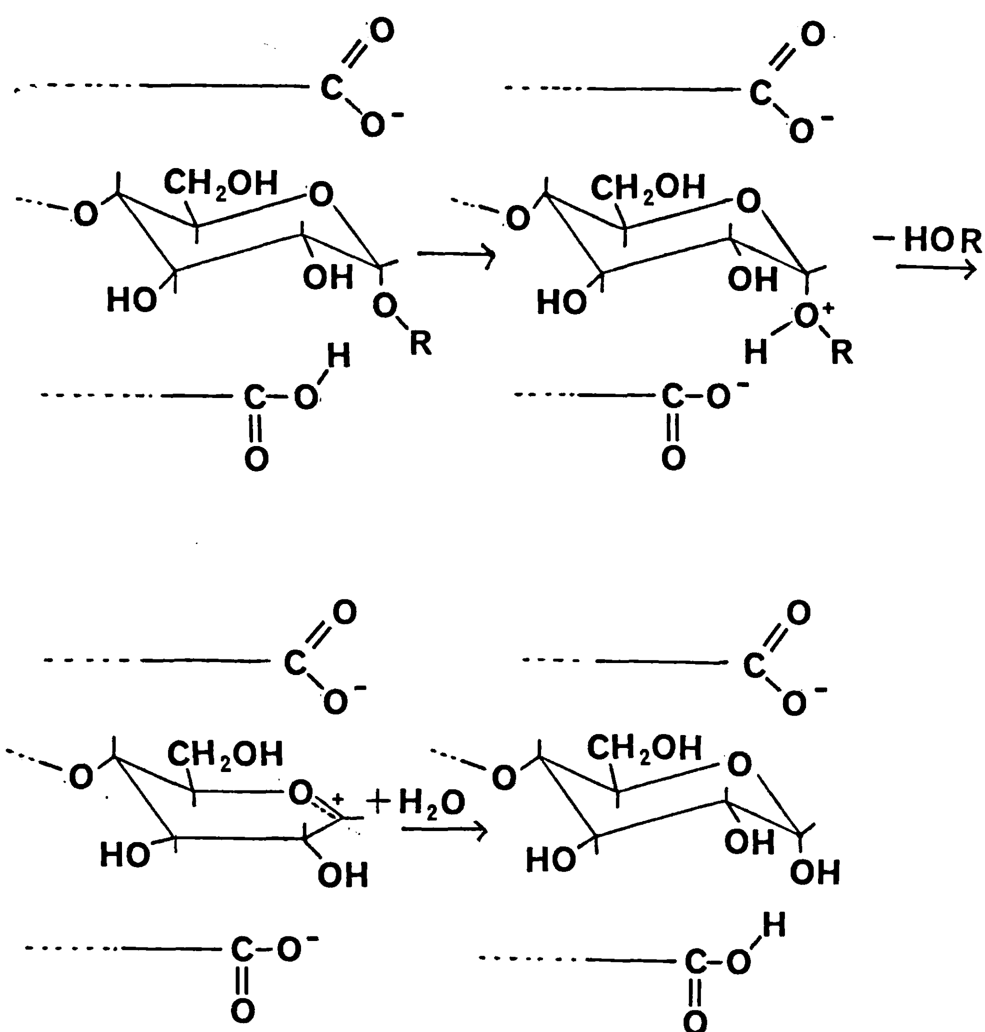


Fig.1.9. Part of the mechanism for an α-D-glucosidase acting on an α-D-glucoside substrate. One carboxyl group is pictured as existing in a protonated state and located adjacent to the glycosidic oxygen atom; the second is proposed to exist as a carboxylate anion located above the D-glucopyranosyl ring. There is some evidence [53,54] for the presence and involvement of carboxyl groups at the active site of cereal α-amylases.

The reaction can be summarised as follows. After binding of the substrate to the active site, the first chemical step is proton transfer to form the conjugate acid. The second step is the loss of aglycone and the formation of a glycosyl oxocarbonium ion, which may be stabilised by charge interaction.[83,84] From the work of Warshel and Levitt[85,86] on the general acid catalysis of the cleavage of a glycosidic bond by lysozyme, the conclusion was drawn that charge stabilisation must be the major factor in enzymic catalysis. Also Sinnott[10] has stated that 'selective non-covalent stabilisation of the transition state accounts for probably most of the catalytic activity of glycosidases.' The final step is stereospecific hydrolysis by water. It is possible, although this is not agreed,[85] that the transition state of the reaction, namely the

glycosyl oxocarbonium ion, exists in the half-chair form. In this mechanism, the loss of aglycone is subject to general acid catalysis and the hydration of the oxocarbonium ion is subject to general base catalysis, both by the same enzyme side chain functional group.

It is unrealistic to suppose that an ideal transition state analogue, perfectly resembling in binding properties the altered substrate in the transition state, can ever be synthesised. Stable analogues cannot reproduce the bond actually being formed or broken in the catalysed reaction and there are likely to be minor differences between the energies of solvation of any inhibitor and of the transition state, which will disturb the binding equilibria. Nevertheless, such tight binding is predicted for transition state analogues that even molecules possessing only a part of the total binding interactions, which normally result in catalysis, should be extraordinarily potent inhibitors with affinities far exceeding that of the substrate. If, as in this case, an enzyme catalyses a reaction by stabilising an oxocarbonium ion, it is reasonable to suppose that a stable analogue possessing similar steric or electrostatic properties could be devised.

Therefore, the transition state analogue approach applied to α-amylase would suggest that,

in addition to substrate-like compounds, inhibitor design should be based on the structure of the glycosyl oxocarbonium ion and the conjugate acid of the glycosidic substrate. The theory suggests that the more effective inhibitors would be those based on the transition state of highest energy i.e. that which leads to the oxocarbonium ion rather than the conjugate acid.[86]

The structure of the conjugate acid is readily visualised, but what is the structure of the oxocarbonium ion? This species was first proposed as an intermediate in glycoside hydrolysis by Vernon et al[87] (1954) and its conformation was alluded to ("coplanar configuration {sic } for O, C1, C2 and C5 .... partially planar carbonium ion") by Chapman and Laird,[88] also in 1954. However the conformation of the oxocarbonium ion was clearly described and represented as a half chair by J.T.Edward[89] in 1955, following the concepts and terminology developed by Barton and coworkers.[90] The conformation of six-membered rings involving a planar group has been further studied by Mathieson.[91] With a planar restraint on four of the six ring atoms, either a boat or half-chair conformation is possible. From experimental data on similar compounds, it seems likely that the glucosyl oxocarbonium ion, in which C1,C2,C5

ring O are in one plane, will exist in the half-chair conformation (Fig.1.10). For example,

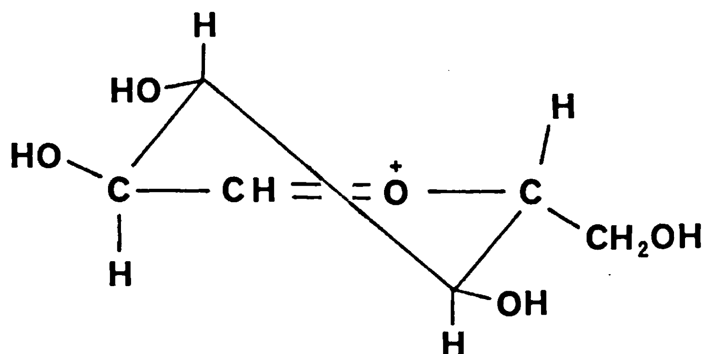


Fig.1.10 Possible conformation ($^{3}H_{4}$) of glucosyl oxocarbonium ion.

2,3-dihydropyran has been shown to exist in the half-chair form by many different methods: IR[92], Raman[93], Microwave[94] and NMR[95]. The activation energy for the interconversion of the different half-chair forms was found to be low (about 30 kJ mol^{-1}), so this process will occur readily at ordinary temperatures. The precise conformation may therefore help binding, but not be vital. It must also be noted, that in this ring system the relationship between the double bond and oxygen are in fact different (Fig.1.11).

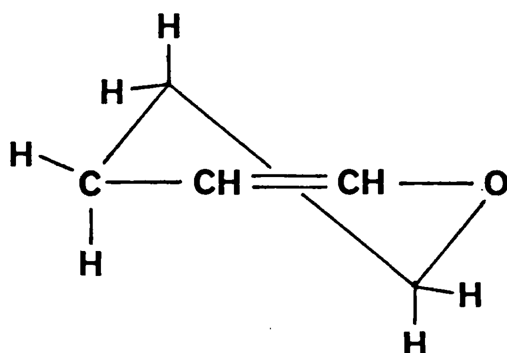


Fig.1.11. Conformation of 2,3-dihydropyran.

Mathematical calculations[85] have indicated that the most stable form of the glucosyl oxocarbonium ion is a slightly distorted half-chair, the 'sofa' conformation (Fig.1.12).

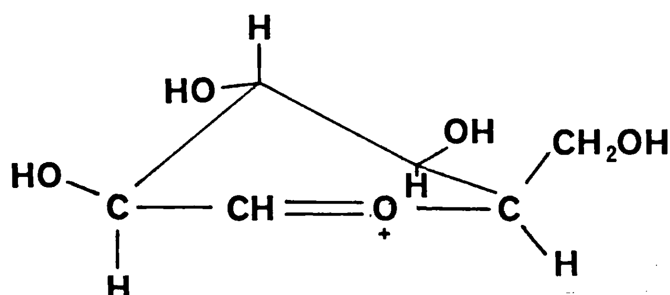


Fig.1.12. Mathematically deduced conformation of the glucosyl oxocarbonium ion, the 'sofa'.

The question now arises as to whether known glycosidase inhibitors emulate the structural and electrostatic features indicated above. A few general examples will be given now to show that this is in fact the case, and the examples will be discussed in greater detail in Chapter 3.

Representative of 'conjugate acid' like inhibitors are the glycosylamines, which inhibit their corresponding glycosidases. They are presumably (pKa ≈ 8) protonated at the active site, and the conjugate acid would then be tightly bound by electrostatic interaction (Fig.1.13)

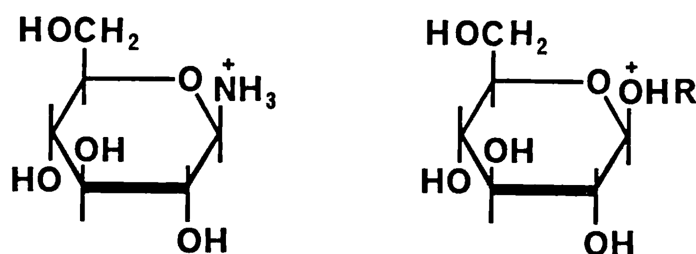


Fig.1.13 Comparison of protonated glucosylamine with the 'conjugate acid' intermediate suggested for glycoside hydrolysis.

An example of the 'oxocarbonium ion' like inhibitors are the glyconic acid lactones, which are powerful inhibitors of their corresponding glycosidases. Their potency has been ascribed to their conformational resemblance to the glucosyl oxocarbonium ion, the resonance structure shown below (Fig.1.14) shows the congruency of structure of the lactone with the glucosyl oxocarbonium ion, however the presence of a small positive charge at CI may afford additional binding energy.

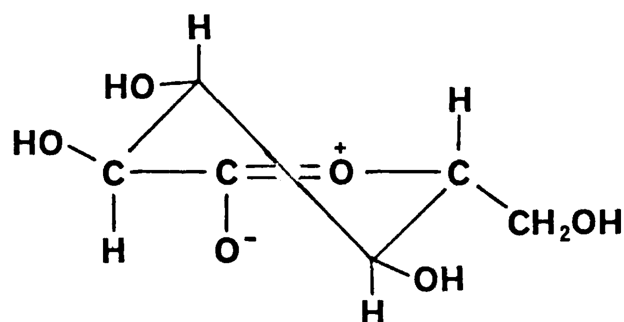


Fig.1.14. Resonance structure of glucono-σ-lactone.

A second example is that of the glycals, which similarly inhibit their corresponding glycosidases. NMR of this type of system (e. g. D-glucal triacetate[96]) has shown them to exist, like 2,3-dihydropyran, in a half-chair conformation with 05, C1, C2 and C3 atoms coplanar (Fig.1.15).

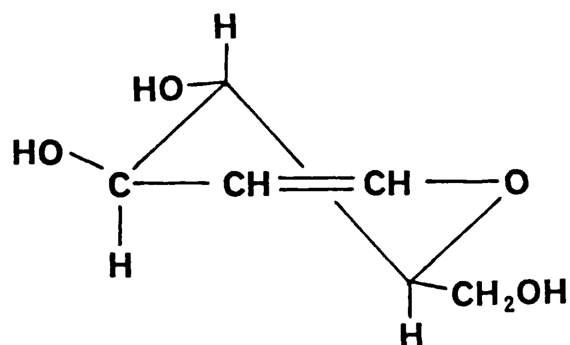


Fig.1.15 Conformation ($^4H_5$) of D-glucal.

However since the form of the half-chair ($^4H_5$) is different from that of the oxocarbonium ion ($^3H_4$) it is not clear that a glucal should show a particularly high binding affinity. In fact the strong inhibition of glycosidases by glycals is a consequence of their chemical reactivity which is characterised by a slow "off rate" (see Chapter 3).

Another very interesting example is that of the antibiotic nojirimycin, which is an inhibitor of glucosidases.[97] It differs from D-glucose only by the substitution of an NH group for oxygen in the ring and is thought to be effective because it forms an imine by elimination of H_2O between N and Cl. The imine (Fig.1.16), presumably resembling the glucosyl oxocarbonium ion.

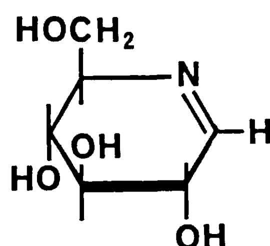


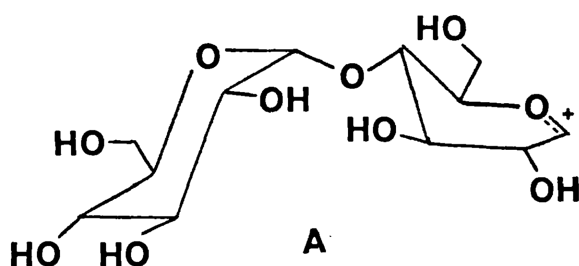
Fig.1.16 The imine formed from the antibiotic nojirimycin and thought possibly to be the inhibitory species.

For reasons based on the substrate and product specificities of the amylases (see discussion in Section 1.2) it is considered that

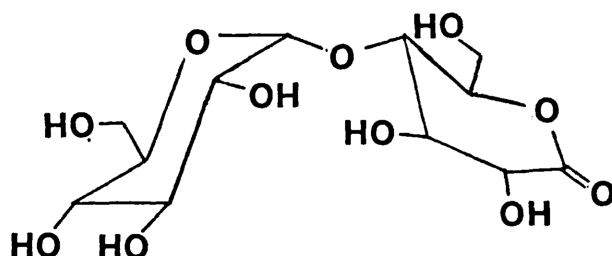
maltose and indeed oligosaccharide derivatives will possess higher affinities for the enzyme active sites than will monosaccharide derivatives. Therefore in this work it was decided to synthesise a number of models of maltose derived intermediates and to examine their interactions with wheat α-amylase. The structures of the derivatives are to embody some of the features which seem to be important according to the previous work with glycoside splitting enzymes, and are shown below.

Synthesis Targets for α-Amylase Inhibition Studies

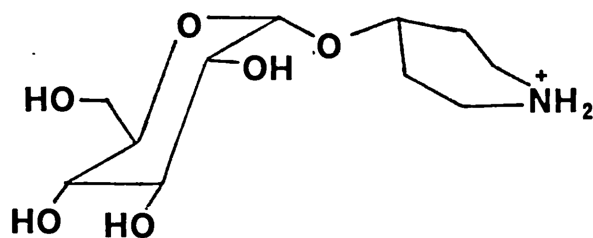
Models of the glycosyl oxocarbonium ion - A



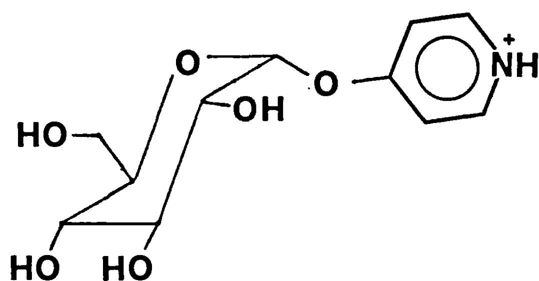
A1 Maltobiono-1,5-lactone



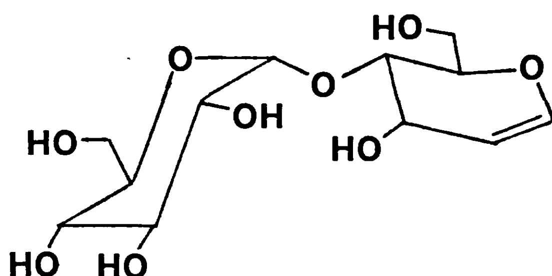
A2 O-(α-D-glucopyranosyl)-4-hydroxypiperidine
(shown as acid form)



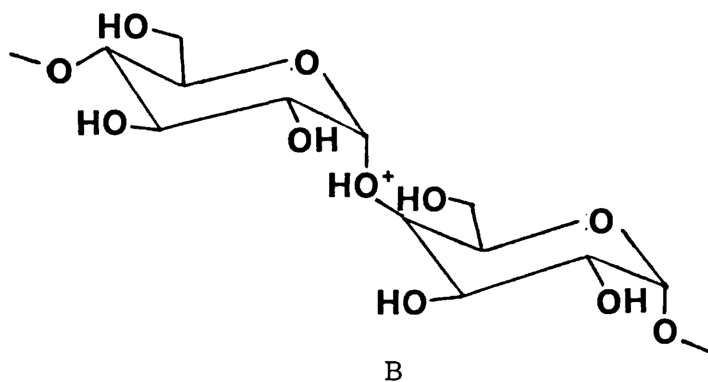
A3 O-(α-D-glucopyranosyl)-4-hydroxypyridine
(shown as acid form)



A4 4-O-(α-D-glucopyranosyl)-D-glucal ('maltal')

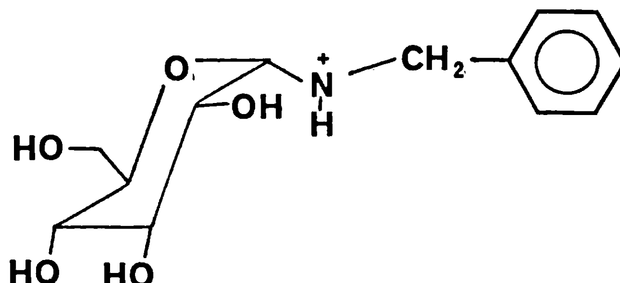


Models of the maltose-4-oxonium ion - B

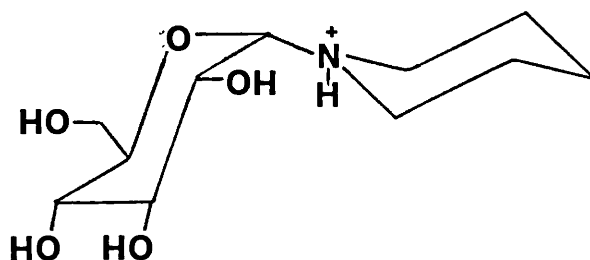


B

B1 N-α-D-glucopyranosylbenzylamine



B2 N-α-D-glucopyranosylpiperidine



Compounds A2,A3 and A4 form a series in which the models for the sugar residue at which glycosyl cleavage occurs are undistorted and charged, distorted and charged and distorted and uncharged. The examination of such a series of compounds with an enzyme may allow a separation of the relative contributions to enzyme activity of substrate distortion and charge interactions. This has been the subject of considerable debate in the case of lysozyme.[98,99,100,101]

The models B1 and B2 may not appear to show a great resemblance to the conjugate acid B, but were chosen for examination because of the potency previously reported[102] of these glycosylamine inhibitors.

CHAPTER 2

SYNTHESIS AND STRUCTURAL ANALYSIS OF POTENTIAL ALPHA-AMYLASE INHIBITORS.

2.1. Introduction

The requirement of the bread industry for a specific and potent inhibitor of α-amylase led (for the reasons discussed in Chapter 1) to the choice of six compounds for synthesis: maltobiono-δ-lactone, 4-O-α-D-glucopyranosyl-1,2-dideoxy-D-arabino-hex-1-enopyranose (maltal), N-β-D-glucosylpiperidine, N-β-D-glucosylbenzylamine, O-(α-D-glucopyranosyl)-4-hydroxypiperidine and O-(α-D-glucopyranosyl)-4-hydroxypyridine The first four of these compounds have been synthesised previously, but only one, maltobiono-δ-lactone had (during the course of this work) been examined as an α-amylase inhibitor and that was of porcine pancreatic α-amylase, rather than wheat α-amylase. This chapter will discuss the synthesis and properties of the potential wheat α-amylase inhibitors.

2.2. Results and Discussion

2.2.1. Maltobiono-δ-lactone

The first synthesis of this lactone was carried out by Fischer[103], who performed a bromine oxidation of maltose to obtain an almost colourless syrup. It was first obtained in

crystalline form by Isbell[104], who prepared lithium maltobionate trihydrate (by an electrolytic method) as a crystalline solid, a solution of which was then passed down an ice cold acid resin column {Amberlite 1R-120(H^+)} , freeze-dried and the lactone crystallised from 2-methoxyethanol, maintained at 50° for 2 weeks.

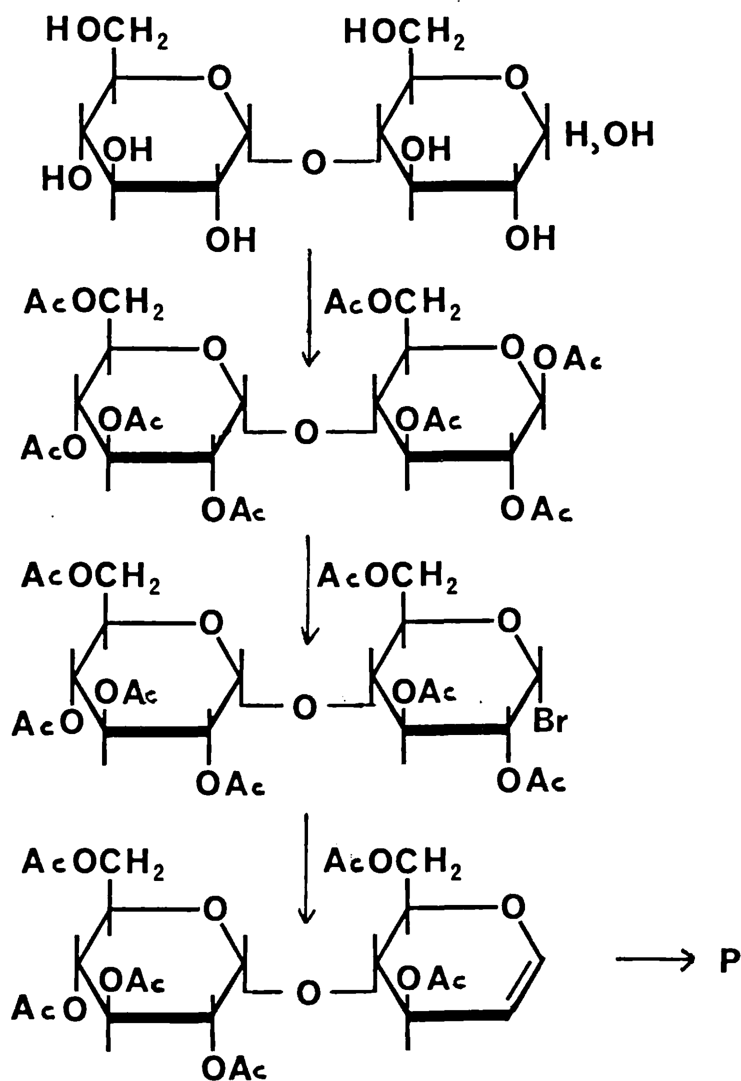
This work followed the method of Diehl et al[105], who prepared cellobiono-δ-lactone by bromine oxidation in a buffered solution. However, instead of using hydrogen sulphide gas to remove metal ions, the solution was passed down an ice cold acid resin column {Amberlite 1R-120(H^+)}, freeze-dried and crystallisation attempted following the method of Isbell. Even with a seed however (kindly supplied by Professor H.S.Isbell), the sample would not crystallise. Thin layer and paper chromatography of the sample, obtained after freeze drying, gave the same results as for the authentic sample and its composition was comparable with a sample obtained by Laszlo et al[106], which similarly failed to crystallise i.e. Lactone, 40%; Acid, 41% ; Reducing substances, 88 mg/g. This work: Lactone, 60% Acid, 17% ; Reducing substances, 47 mg/g.

2.2.2. Maltal (4-O-α-D-Glucopyranosyl-1,2-dideoxy-D-arabino-hex-1-enopyranose).

A new class of compounds, the glycals, were discovered in 1913, by Fischer and Zach[107]. They have become very important in carbohydrate chemistry because of their unusual reactivity and their ease of transformation and isolation. Their chemistry was extensively investigated by Bergmann and Schotte and has been reviewed by Helferlich.[108] They are generally synthesised via their acetyl derivatives, by the treatment of the acetylglycosyl halide with zinc and acetic acid, which brings about the reductive removal of hydrogen bromide and a molecule of acetic acid.

The synthesis of hexaacetyl maltal monohydrate was first described by Bergmann and Kobel,[109] but this was later shown to be octaacetyl maltose by Haworth[110], who reported the isolation of true hexaacetyl maltal. Haworth stated, in that work, that maltal could be obtained as a pale yellow syrup by deacetylation in methanolic ammonia, but gave no further details. Later work by Gakhokidze[111] claimed to have isolated maltal as a crystalline solid, but the optical rotation given for hexaacetyl maltal (M Pt = 132-136°, $\{\alpha\}_{D}^{18}$ = -22.5^{o} Chloroform) does not agree with that reported by Haworth (M Pt = 131-133°, $\{\alpha\}_{D}$ = + 68° C 0.8, Chloroform).

In this work, maltal was synthesised via a route used previously for glycals (Scheme 2.1). Maltose was first acetylated by the sodium acetate method,[112,113] then a bromine group introduced at C1 by hydrogen bromide in glacial acetic acid.[114] This was followed by reaction with zinc dust in glacial acetic acid to give the C1-C2 double band[115] and finally deacetylation to give the final product.[116,117]



Scheme 2.1

The method used for the acetylation of maltose gave the β form (in 50% yield) as shown by the melting point and optical rotation.[118] When this was brominated a white solid was obtained, which could not be crystallised, but due to the low melting point and slightly low optical rotation was thought to be the non-crystalline (amorphous) solid form of maltosyl bromide, which had been previously reported.[119] Hexaacetyl maltal was obtained as colourless short rods, the physical constants for which agreed with those reported by Haworth. Deacetylation of hexaacetyl maltal with methanolic ammonia gave a pale yellow oil, which was characterised by ^{1}H NMR spectroscopy (Fig.2.1.).

The spectral features shown were as follows:

σ 3.44 ppm, 1H, triplet ; 3.58, 1H, quadruplet ; 3.72, 1H,triplet ; 3.76, 1H, octet ; 3.84, 2H, octet ; 3.92, 2H, octet ; 3.93, 1H, quadruplet ; 4.10, 1H, septet ; 4.42, 1H, octet ; 4.88,1H, quadruplet ; 5.47, 1H, doublet ; 6.48, 1H, quadruplet. The assignments for the dihydropyran ring were mainly achieved by decoupling experiments, whilst NOE and INDOR difference spectroscopy were used for the pyranose ring. The starting point, was the assumption that the olefinic protons H1,2 would

give rise to two quadruplets at approximately σ 6.5 ppm (H1) and σ 4.9 ppm (H2), typical for the glycal structure.[120] The quadruplet at σ 6.48 ppm, assigned as H1 was easily seen, but that at σ 4.88 ppm, assigned as H2 was partially hidden beneath the water peak. Decoupling at the latter transition simplified the octet at σ 4.42 ppm to a doublet, which was hence assigned as H3. Decoupling at this transition showed H4 to be at about σ 3.9 ppm, but not easy to pick out until an INDOR experiment was carried out, which gave four negative peaks centred at σ 3.92 ppm. Decoupling here showed H5 to be the septet at σ 4.10 ppm. Decoupling at this transition could not give H4, 6a, 6b unequivocably and two further experiments were therefore required. The first (Fig.2.2) was an inversion-recovery experiment ($180^{o} - \tau - 90^{o}$) in which τ had been adjusted such that the methylene groups were nulled (since methylene protons have shorter T_1's than methine). H4, could now again, be clearly seen as four negative peaks. In the second experiment (Fig.2.3) τ was adjusted to null the methines, thus giving rise to a series of positive peaks at σ 3.77-3.95 ppm due to the two methylene groups. The doublet at σ 5.47 ppm was assigned as H1'. Decoupling at this transition then gave H2' as the quadruplet

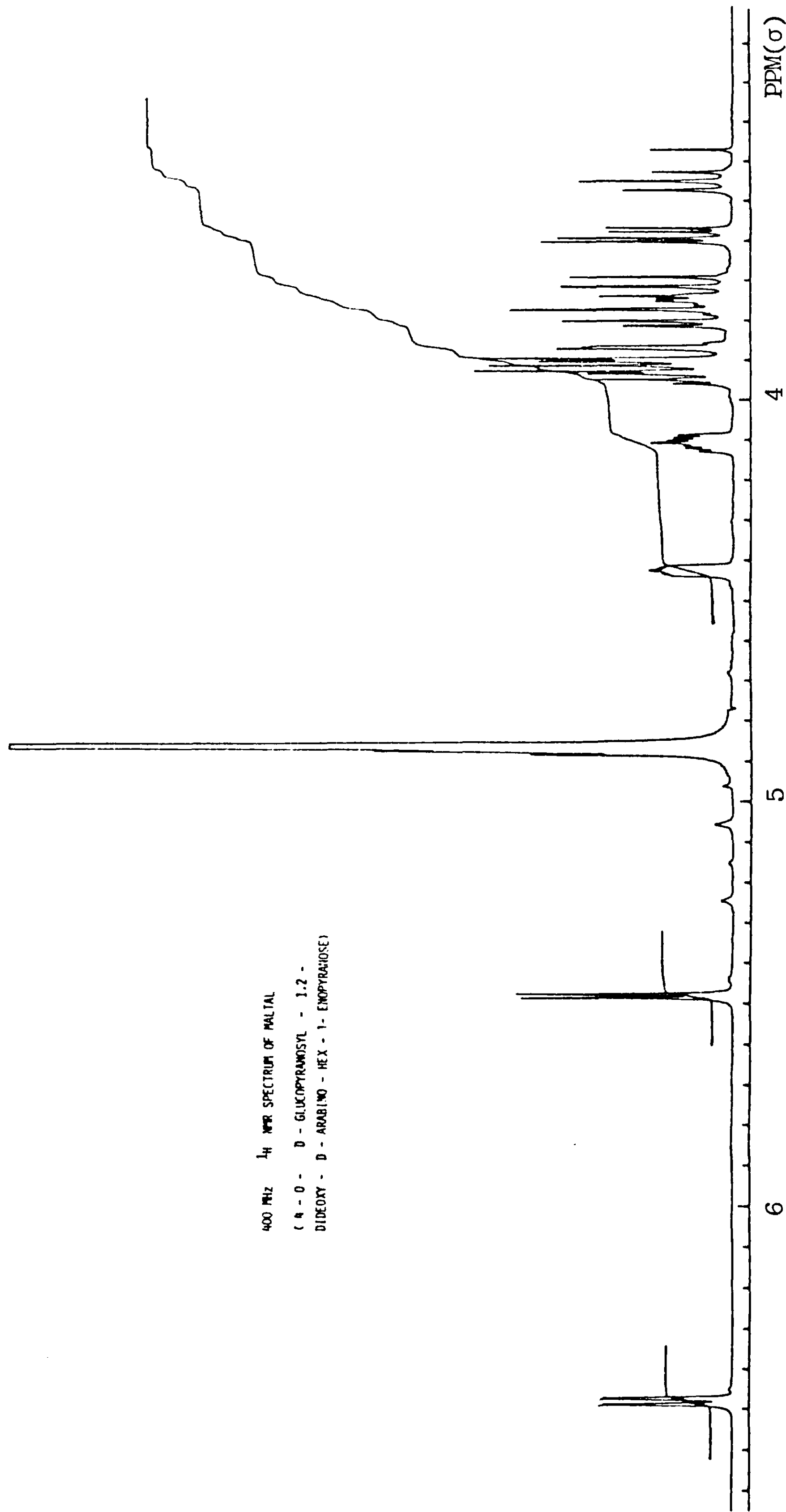


Fig. 2.1. 400 MHz ^{1}H NMR spectrum of maltal

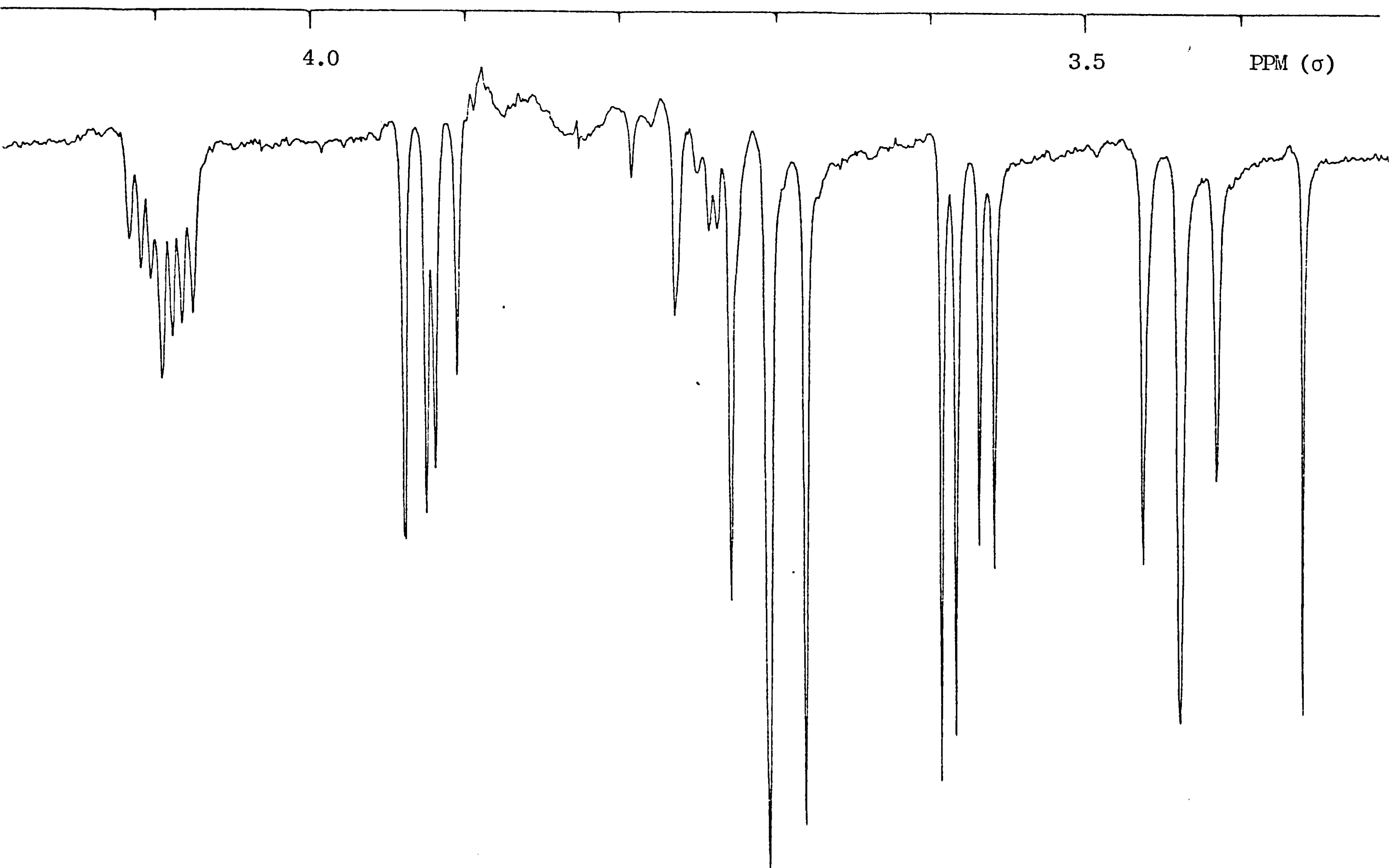


Fig.2.2. Maltal inversion-recovery experiment with methylene groups nulled.

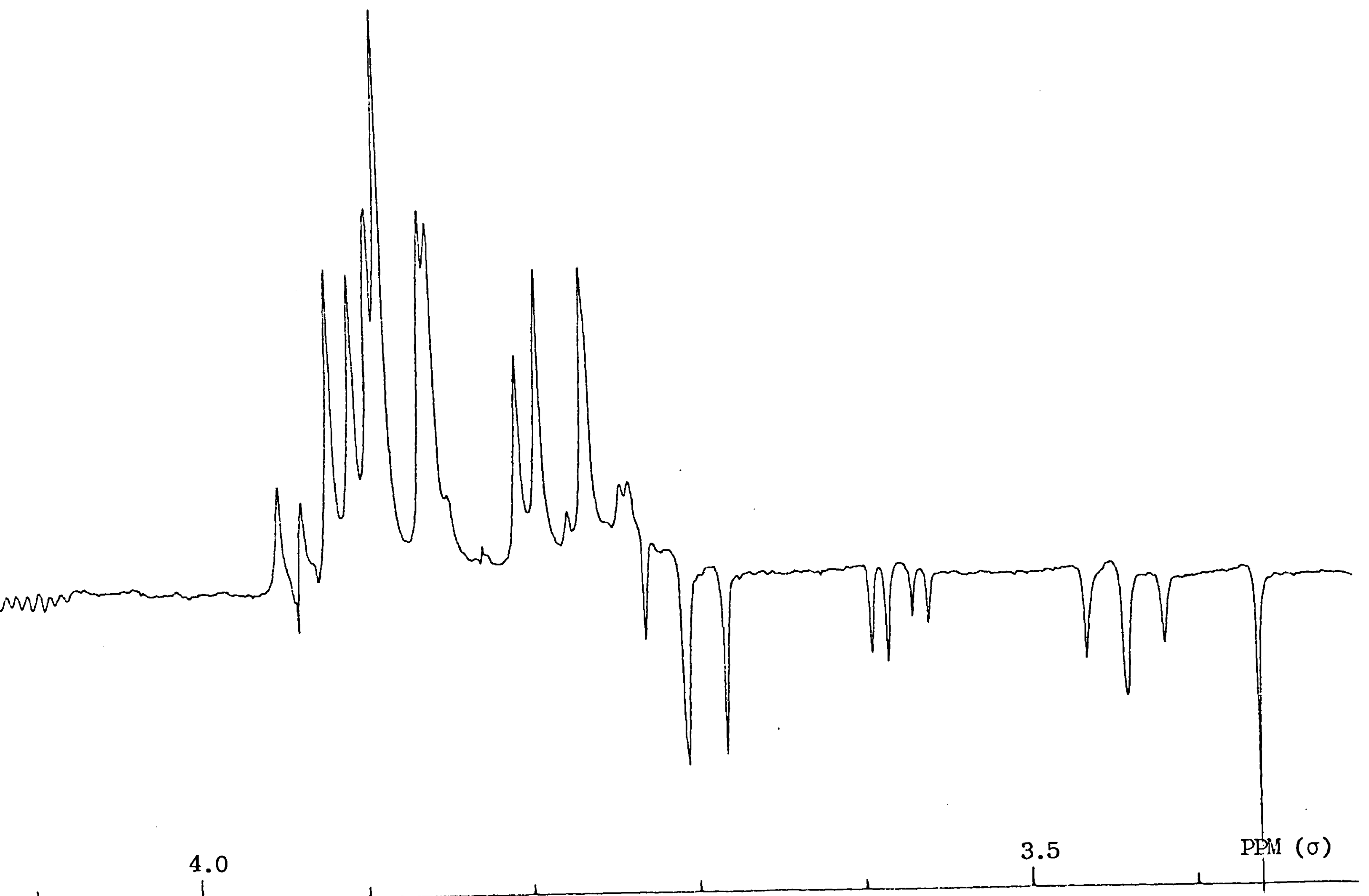


Fig. 2.3. Maltal inversion-recovery experiment with methine groups nulled.

at σ 3.58 ppm. The triplet at σ 3.44 ppm gave an NOE at H2' and was hence H4' (1,3-diaxial proximity). INDOR at this transition gave two multiplets (H3', H5'), the higher of which was a clean doublet of doublets. INDOR at H2' also gave this doublet of doublets, which was therefore due to H3' (seen on the main spectrum as a triplet at σ3.72 ppm). The second multiplet an octet at σ3.76 ppm, was therefore due to H5'. Of the four methylene protons, one, either 6'a or 6'b, is believed to be confused with H5', whilst the other is in the same region as H4, 6a and 6b.

The two quadruplets at σ6.48 and 4.88 ppm attributable to olefinic protons (H1,H2), with a coupling constant ($J_{1,2}$) of 5.92 Hz, are excellent proof of the glycal structure.[120] The magnitude of the coupling constants for the hex-1-enopyranose or dihydropyran system ($J_{1,2}$ 5.92Hz, $J_{2,3}$ 2.96 Hz, $J_{1,3}$ - 1.31 Hz) are in fact similar to those given for D-glucal triacetate[121] ($J_{1,2}$ 6.4Hz, $J_{2,3}$ 3.2 Hz, $J_{1,3}$ - 1.3 Hz). A negative value ($J_{1,3}$) is typical for four bond couplings of unsaturated systems. The analysis and assignment of the spectrum was confirmed by computer simulation and the spectral parameters, given in Table 2.1, are those which give a satisfactory visual fit of computed and observed spectra.

Proton	Chemical Shift		Coupling Constant	Coupling Constant	
	Observed	Simulated		Observed	Simulated
H1	6.48	6.48	$J_{1,2}$	5.92	5.92
H2	4.88	4.88	$J_{1,3}$	-1.31	-1.31
H3	4.42	4.42	$J_{2,3}$	2.96	2.96
H4	3.93	3.93	$J_{3,4}$	5.70	5.70
H5	4.10	4.11	$J_{4,5}$	7.79	7.79
H 6a,6b	3.92	3.91	$J_{5,6a}$	5.39	5.39
			$J_{5,6b}$	2.73	2.73
			$J_{6a,6b}$	-12.26	-12.26
H1'	5.47	5.48	$J_{1'2'}$	3.91	3.82
H2'	3.58	3.59	$J_{2'3'}$	10.02	9.95
H3'	3.72	3.71	$J_{3'4'}$	9.84	9.68
H5'	3.76	3.75	$J_{5'6'a}$	5.00	4.60
H $6'_a$,$6'_b$	3.84	3.84	$J_{5',6'b}$	2.50	2.95
			$J_{6'a,6'b}$	-11.40	-13.04

Table 2.1. The observed and computer simulated parameters for the ^{1}H NMR spectrum of maltal.

As to the conformation of the dihydropyran ring, only the two half-chairs $^4H_5$ and $^5H_4$ (Fig.2.4) will be considered, since alternative conformation will be of considerably higher energy.[95]

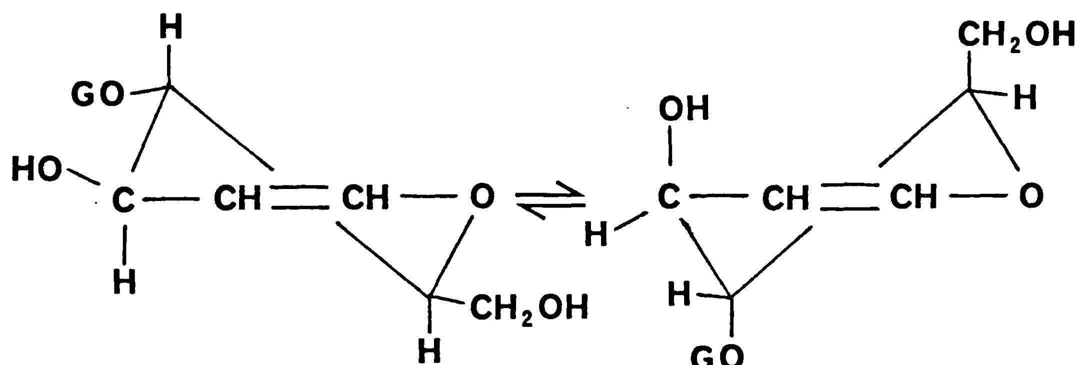


Fig.2.4. Possible half-chair conformations of the dihydropyran ring of maltal (G = α-D-glucosyl).

Two coupling constants $J_{3,4}$ and $J_{4,5}$ are of major importance in deciding which of these conformations is preferred. It is know that vicinal protons in an anti-parallel (axial/axial) relationship give rise to large coupling constants (7.5 - 10 Hz), whereas those in a gauche (equatorial/equatorial) relationship give rise to small coupling constants (~ 2Hz). In the $^4H_5$ conformation, H3 and H4 and H4 and H5 have a pseudo-diaxial orientation and would therefore be expected to exhibit high J-values. The values observed viz. 5.70 and 7.79 Hz support

the assignment of this conformation, but are however not as high as could possibly be expected. This may indicate the presence of an equilibrium mixture of the two forms ($^4H_5$, $^5H_4$) with the $^4H_5$ system predominating or alternatively there may be a single conformation distorted from the classical $^4H_5$ half chair. The occurrence of the $^5H_4$ form is not without precedent; examination of the NMR data in the literature shows that all xylals[122,123] adopt the $^5H_4$ conformation e.g. for the D-threo-isomer, 1,2-dideoxy-2,4-di-O-acetyl-pent-1-enopyranose (di-O-acetyl-D-xylal), the $^5H_4$ form was estimated[124] to be more stable than the $^4H_5$ form by 3.3 kJ mol^{-1}. Generally however, the $^4H_5$ conformation is the most stable and with the exception of 2-C-nitro-D-galactal[123], 2-C-nitro-D-glucal[123] and the glucuronal methyl ester[125] all gulals[126], allals[127], arabinals[128], galactals[129] and glucals[130] adopt this form. All other shifts and coupling constants are consistent with the proposed structure.

2.2.3. Synthesis of Glycosylamines.

Reducing sugars react readily, under mild conditions, with compounds containing the primary amino group, e.g. ammonia, hydroxylamine and a wide variety of amines to give glycosylamines, a well known class of compound.[131,132] The first synthetic compounds of this type were

prepared by Schiff[133,134], who obtained glass-like products by heating aniline or p-toluidine with dry D-glucose. After using a lower reaction temperature, Sorokin[135,136,137,138,139,140] isolated crystalline N-phenyl-D-glucosylamine, -D-galactosylamine and -D-fructosylamine. He also described the most generally used method of preparation, which consists in heating the amine and the reducing sugar in boiling methanol or ethanol, containing up to 10% of water. Small amounts of acids have been used successfully to catalyse the condensations. The products often crystallise on cooling or after evaporation, if necessary, of some of the solvent, followed by the addition of ether. The vast majority of glycosylamines are colourless crystalline solids, but a few have been prepared in the amorphous state only. Their stabilities vary considerably, depending upon the nature of the amine function.[141]

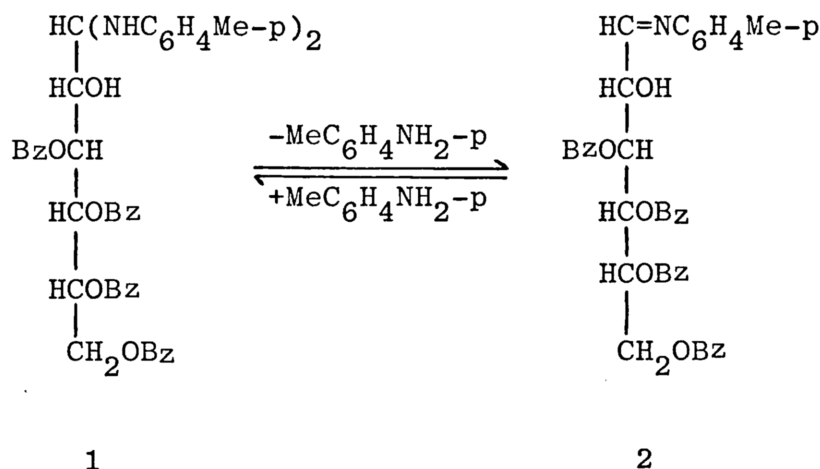
In this work, N-D-glucosylbenzylamine[142] was synthesised by reacting glucose and benzylamine in 95% ethanol, and N-β-D-glucosylpiperidine[143] was synthesised by reacting glucose and piperidine with no solvent. Despite three recrystallisations the melting point of N-D-glucosylbenzylamine was always lower (73-74°) than that (81.5°) given in the literature. This was thought to be due to the fact that the compound was the monohydrate, though this does not fit the analysis perfectly

(see Experimental). In principle glycosylamines of primary and secondary amines can adopt cyclic and acyclic tautomeric forms i.e. α and β glycosylamine structures and E and Z Schiff base structures. The early workers[134,135] prepared anilides whose structures were considered to be of the Schiff's base type[144] or of the cyclic glycosidic type[145]. The methylation studies of Irvine and his co-workers from 1908-1911 (e.g. Irvine and Hynd[146], 1911) furnished strong evidence of the cyclic nature of these compounds. Their isolation of tetramethyl aniline-N-glucopyranoside was held to be evidence for considering the mutarotating aniline-N-glucoside to be α- and β-aniline-N-glucopyranoside. Continuing work in this field, as shown by the review of Paulsen and Pflughaupt[147], has demonstrated that aldosyl- and ketosyl- amines generally favour the pyranoid structure. Certainly the other possible forms do exist and although the assumed furanoid structures of the O-nitroaniline derivatives of D-ribose and L-arabinose[148] and also an N-phenyl-D-ribosylamine[149,150] have not been firmly verified, an undoubtedly authentic D-glucofuranosylamine results from the treatment of 5,6-di-O-methyl-D-glucofuranose with p-toluidine.[151]

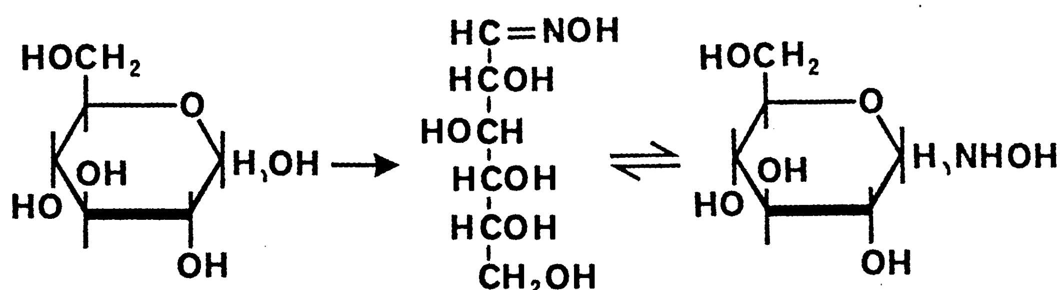
A ribofuranosyl glycosylamine, N-(5'-phospho-

D-ribosyl)-anthranilate, and its Amadori Rearrangement product, are proposed intermiediates in the biosynthesis of tryptophan.[152]

Authentic Schiff bases are normally obtainable only from acylated aldehydo sugars. 3,4,5,6-Tetra-O-benzoyl-aldehydo-D-glucose reacts with p-toluidine to yield the 1,1-bis(p-toluidino) derivative (1), which on heating in ether, loses one mole of amine to give the Schiff base[153](2).



Other related type of compounds which sometimes react to give Schiff bases are hydrazine derivatives,[154] an example of which is hydroxylamine. This reacts with reducing sugars to give oximes in the acyclic form, both in the solid state and in solution. An exception to this is D-glucose oxime which exists as the cyclic β-pyranosyl form in the solid state, but forms an equilibrium of four observable species in solution (α-pyranose,7%; β-pyranose, 23% ; Syn-E, 56.5% ; Anti-Z, 13.5%)[155] (Scheme 2.2).



Scheme 2.2

The structures of N-D-glucosylbenzylamine and N-D-glucosylpiperidine were both confirmed by ^{1}H NMR.

400 MHz ^{1}H NMR of N-D-glucosylbenzylamine taken shortly after dissolution in $DMSOd_6$ + D_2O showed, inter alia, the following spectral features: σ2.96 ppm, 1H, triplet ; 3.05, 1H, triplet ; 3.14, 1H, triplet ; 3.17, 2H, singlet ; 3.31, 1H, quadruplet ; 3.44, 1H, multiplet ; 3.67, 1H, doublet ; 3.78, 3.94, 1H, doublet of doublets ; 4.35, 1H, doublet ; 7.28, 5H, multiplet. The majority of the peaks listed above would appear to be associated with the N-β-D-glucopyranosyl form of the sugar, with H1 being assigned as the doublet at σ3.67 ppm, $J_{1,2}$8.9Hz. The triplets at σ2.96, 3.05 and 3.14 ppm are H2, 3 and 4, all with coupling constants around 9Hz which together with the large coupling constant for H1 (8.9Hz), is indicative of the β-form of the normal chair

conformation. H5 can be seen as a multiplet at σ3.44 ppm, whilst H6a and 6b are partially obscured by the water peak, but a splitting of 14.0 Hz can clearly be seen, typical for a geminal coupling of H6a and 6b. The spectrum is complicated however by the presence of a second sugar form (~ 10%) probably the α-D-glucopyranosyl form with H1 at σ4.35 ppm, $J_{1,2}$ 5.0Hz. The only other feature of this form clearly visible is the quadruplet at σ3.31 ppm which can be assigned as H2 with coupling constants $J_{1,2}$ 5.0Hz and $J_{2,3}$ 9.9 Hz. The only remaining protons are the benzyl methylene assigned as the singlet at σ3.17 ppm and the benzyl ring protons assigned as the multiplet at σ7.28 ppm.

250 MHz ^{1}H NMR of N-D-glucosylpiperidine in D_2O showed the following spectral features: σ1.5 ppm, 6H, multiplet ; 2.62,2H, multiplet ; 2.90,2H, multiplet ; 3.32,2H, multiplet ; 3.46,1H,triplet ; 3.58,1H,triplet ; 3.69,1H, doublet of doublets ; 3.88,1H, doublet of doublets ; 3.91, 1H, doublet. This final doublet at σ3.91 ppm, with a coupling constant of 8.5 Hz, can be readily assigned as H1. The triplets at σ3.58 and 3.46 ppm are H2 and H3 respectively (confirmed by double resonance - irradiation at 3.9 ppm, H1, collapsed the triplet at σ3.58 ppm to a doublet) both with coupling constants around 9Hz ($J_{2,3}$ 8.6 Hz, $J_{3,4}$ 9.4 Hz) which, together

with the large coupling constant for H1 ($J_{1,2}$ 8.5Hz), establishes that the β-glucopyranosyl ring is in the normal $^4C_1$ conformation. H4 and H5 give rise to the multiplet at 3.32 ppm. H6a can be seen as one doublet of doublets with coupling constants 1.8 Hz and 12.5 Hz, the other hydroxylmethylene proton shows $J_{5,6b}$ = 5.7 Hz.

The remaining signals arise from the piperidine ring and were assigned as follows. The two-proton multiplets at lower field (2.62 and 2.90 ppm in D_2O) appeared as five line features in $DMSOd_6$ (2.49 and 2.81 ppm) whose separation did not change markedly on increasing the temperature from 297 to 387°K. Irradiation of the multiplet at 1.54 ppm (D_2O) collapsed these resonances to a pair of doublets, revealing a geminal coupling of 11.8 Hz. The multiplet at 1.5 ppm thus arises from the four C3 and C5 and the two C4 methylene protons, and the features at 2.62 and 2.90 ppm (D_2O) from the different methylene protons adjacent to the nitrogen atom and chemically shifted by the asymmetry of the glucosyl unit. The five lines arise from two overlapping triplets given by a geminal coupling and an average coupling (≃ 5.3 Hz) with the adjacent C3 and C5 methylene protons. This implies a rapid inversion of the piperidine ring at ambient temperature, consistent with the

observations of Lambert and co-workers[156] on piperidine itself and its N-methyl and N-tert-butyl derivatives.

2.2.4. Synthesis of O-Glycosides

The oldest and simplest method of glycoside synthesis is the Fischer[157] reaction, in which the sugar is condensed directly with an alcohol in the presence of hydrogen ion. One or other, or both, anomers may be separated from the reaction mixture. Unfortunately, this method is applicable only to the lowest aliphatic alcohols and to monosaccharides. There is also no way of altering the α/β ratio in the final equilibrium mixture. Anhydrous alcoholic hydrogen chloride (0.25 - 0.30% w/v) was the catalyst of choice, until an elegant improvement in technique was introduced by Cadotte et al[158] with the use of cation exchange resins, which could be simply filtered off at the end of the reaction. (A good yield for methyl-α-D-glucopyranoside was obtained by this method).

The technique which has probably received most attention in recent years,[159,160] is the Koenigs-Knorr[161] reaction of substituted glycosyl halides with hydroxyl functions in the presence of an 'acid acceptor'. Trans glycosides are generally formed by this method i.e. compounds in which the incoming nucleophile is stereo-

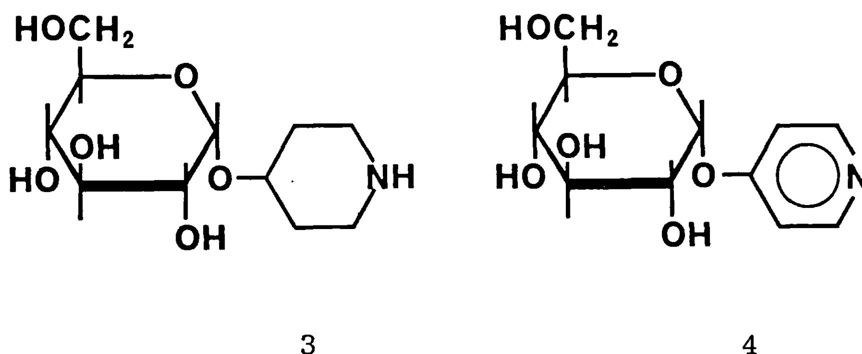
-specifically directed trans to an acyl group at C-2. Occasionally cis anomers have been isolated[162] (e.g. α-D-glucopyranosides), but the configuration of the product may be affected by a number of factors, including the structure of the reacting halide, the ionic strength of the solvent and the nature of the acid acceptor. When the trans form was predominant, one method of improving the cis yield was anomerisation with an agent such as titanium tetrachloride,[163] which was used with some success. A more recent development due to Lemieux[123] is the halide ion catalysis technique, in which a β-glycosyl halide is brought into rapid equilibrium with its more stable α anomer by way of an ion-pair intermediate formed in the presence of an agent such as tetraethylammonium bromide. The β anomer, which can react up to 100 times faster than its α anomer, is thus readily available for reaction, albeit in low concentration, with inversion to the α-glycoside.

For the synthesis of α-D-glucopyranosides directly, it is preferable to use D-glucosyl halides having a non-participating group at C-2 such as a 2-O-nitro[164] or 2-O-benzyl[165] group, but the yield is usually not high. The replacement of the halogen with an imidate group[166,167,168] (to drive the reaction cis) has

also met with some success and could prove particularly useful when the halogen compound is difficult to handle.

2.2.4.1. Fischer's Method

This technique was used to prepare the simple alkyl glycoside cyclohexyl α-D-glucopyranoside which was to serve as a reference compound for the more complex target molecules, 3 and 4:-



3 4

A number of acid catalysts were used and the best (based on the yield of α-D-glucopyranoside in the reaction mixture, 45% cf 20-30%) was found to be the cation exchange resin Amberlite 1R-120 (H^+), which is the variation of the Fischer method introduced by Cadotte. The pure α-glycoside could not however be separated from its β-anomer.

2.2.4.2. Wing's Method[169]

A second attempt at the synthesis of cyclohexyl α-D-glucopyranoside was made using

a trans-esterification process, in which cyclohexanol was reacted with β-D-glucose pentaacetate in the presence of an acid catalyst. This gave a syrup which by GLC analysis appeared to be a mixture of the α and β glycosides in the ratio 77:23 and from which the pure α form could not be obtained. The 250 MHz ^{1}H NMR spectrum was consistent with the presence of a mixture and showed the α(H1 σ5.06 ppm, $J_{1,2}$4.04Hz) and β(H1 σ4.57 ppm, $J_{1,2}$ 7.72 Hz) anomers in the same ratio as obtained by GLC. Elemental analysis was in slight error (carbon being low by 1.7% and hydrogen high by 2.6%), this is thought to be due to the presence of water since alkyl glycosides are extremely hygroscopic. With the apparent success of this method, (giving a higher ratio of α-glycoside) it was repeated using the more complex alcohol 4-hydroxypiperidine, after protection by N-acetylation.[170] In this case however the reaction would not proceed and it was found, by repeating the original reaction with varying amounts of cyclohexanol, that it would give a substantial yield only in the presence of a large excess of alcohol, and the procedure was abandoned as a general method of synthesis.

2.2.4.3. Classical Bromosugar Method

This route has been used frequently to

obtain O-glycosides. Starting from β-D-glucose pentaacetate, the first step was the replacement of the acetyl group on C1 with bromine,[114] followed by reaction with the required alcohol[171] (in this case 4-hydroxypiperidine) and deacetylation.[116,117]

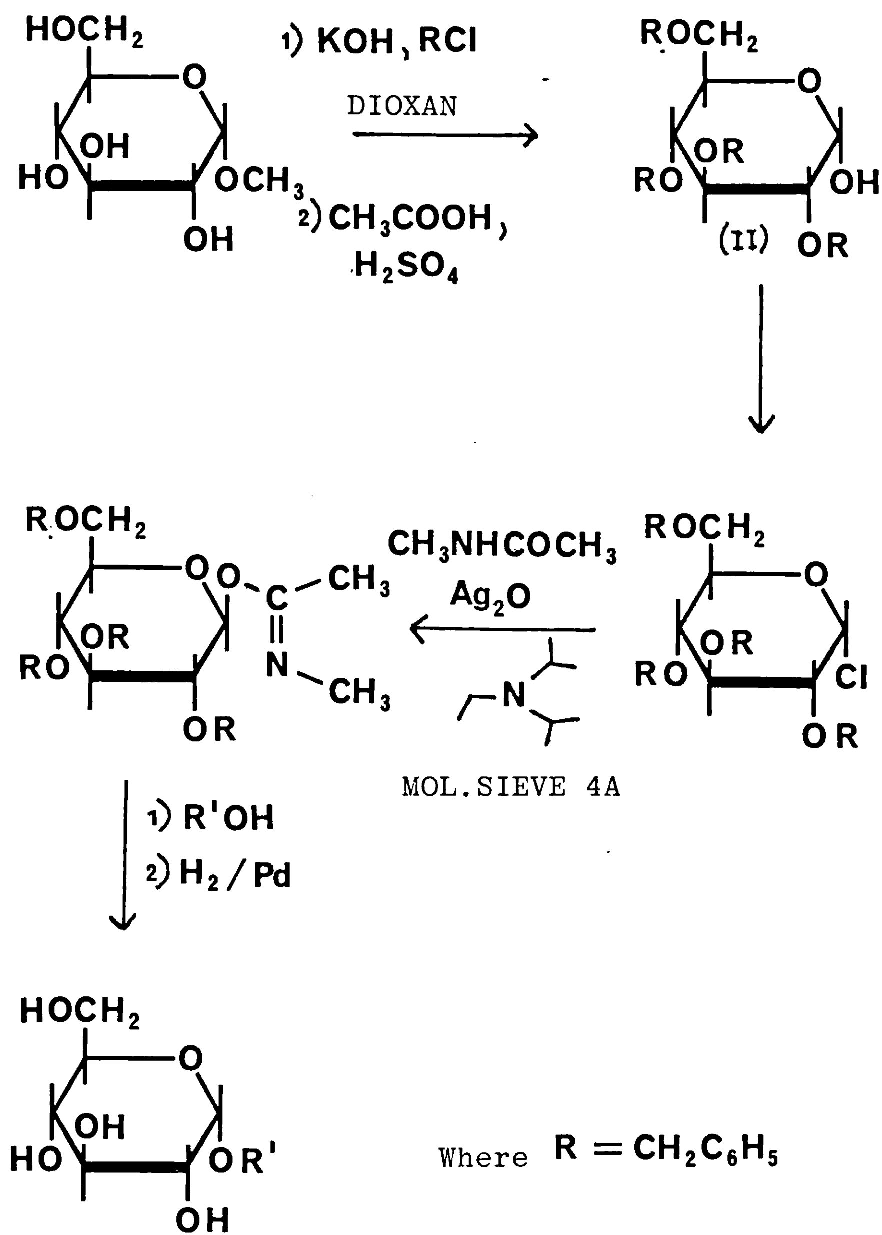
The synthesis proceeded without problem to give a crystalline acetylated glycosyl derivative in 19% yield after recrystallisation. The 400 MHz ^{1}H NMR spectrum in deuteriochloroform solution showed, inter alia, the following spectral features: σ1.39 ppm, 1H,multiplet ; 1.74,2H, multiplet ; 1.85, 2H, multiplet ; 2.44,1H, multiplet ; 2.82,2H, multiplet ; 3.12, 1H, octet ; 3.60, 1H, octet ; 3.68, 1H, multiplet ; 4.02, 1H, doublet ; 4.17, 2H, multiplet ; 5.00, 1H, triplet ; 5.13,1H, triplet ; 5.22,1H, triplet. The doublet at σ4.02 ppm with a coupling constant of 9.1 Hz can readily be assigned to H1. The three triplets at σ5.00, 5.13 and 5.22 ppm are H2,3 and 4. By double resonance these were assigned as σ5.00 ppm, $J_{4,5}$ 9.7 Hz, H4 ; σ5.13 ppm, $J_{3,4}$ 9.4 Hz, H2 ; σ5.22 ppm, $J_{2,3}$ 9.4 Hz, H3. Further double resonance experiments then gave the octet at σ3.60 ppm as H5 and the multiplet at σ4.17 ppm as H6. The multiplet at σ3.68 ppm was assigned as either NH or OH. The remaining features

listed above were attributable to the piperidine ring structure and very similar to that found for N-β-D-glucosylpiperidine.

The large coupling constant for H1 ($J_{1,2}$ 9.1 Hz) together with the coupling constants for H2,3 and 4 (9.4 - 9.7 Hz) would appear to confirm the β-form of the normal chair conformation. That this doublet occurs with the other ring protons and not to lower field, would seem to indicate that the compound is an acetylated glycosylamine structure[172] and not the O-glycoside which was hoped for.

2.2.4.4. Sinay's Method

Another method investigated for the synthesis of α-glycosides was the procedure developed by Sinay and co-workers[167,173] and presented in Scheme 2.3.



Scheme 2.3

The first step of the synthesis gave compound (II) as a white crystalline solid, the physical constants for which were in good agreement with the literature values. Steps 2 and 3 gave yellow syrups which were not fully characterised. The final stage (debenzylation) carried out at 60 psi hydrogen pressure failed, due to rupture of the reaction vessel. To avoid this physical problem, the reaction can be carried out at atmospheric pressure by catalytic transfer hydrogenation.[174] This method is thought to be the most promising for the synthesis of the α-glycosides required.

2.3 General Experimental Techniques

^{1}H NMR: ^{1}H nmr spectra were recorded on HR220 (PCMU); Brucker HX90, HX250 (King's College, University of London); WH-400 (Queen Mary College, University of London). All spectra were run at ambient temperature in either D_2O, $DMSOd_6$ or $CDCl_3$ with TSP, DSS or TMS as internal standards.

GLC: Gas liquid chromatography was carried out with a Pye 104 dual chromatograph (modified for a capillary column) equipped with a flame ionisation detector. Peaks were recorded on a Servoscribe RE 511.20 potentiometric recorder. The glass capillary column was packed with SE30 WCOT stationary phase. Helium was

employed as a carrier gas at a flow rate of $1.2\ cm^3\ min^{-1}$. Temperature: 3 mins at 185° then $6^o\ min^{-1}$ rise to 221°. The glc was performed on the trimethylsilyl derivatives[175] which were prepared as follows:

To a solution or suspension of the sample (10mg) in dry pyridine ($1\ cm^3$) was added hexamethyldisilazane (HMDS, $0.2\ cm^3$) and trimethylchlorosilane (TMCS, $0.1\ cm^3$). The mixture was shaken vigorously for about 30 secs then left 1 hr at room temperature. The precipitate was removed by centrifugation and the supernatant liquid used directly for glc analysis.

Spectrophotometry: All readings in the visible/ultra violet range were carried out on a Perkin Elmer SP550S spectrophotometer.

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 dm jacketed cell.

Paper Chromatography: Descending paper chromatography was carried out with Whatman No.1 paper. The solvent systems used were:

(A) n-Butanol, ethanol, water (40:11:19 v/v)

(B) Ethyl acetate, acetic acid, formic acid, water (18:3:1:4 v/v)

Carbohydrate components were detected by visualising the spots with silver nitrate dip

reagent.[176] 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/v).

TLC: Thin layer chromatography was carried out on 'Polygram Sil G', 5 x 20 cm pre-coated plastic sheets (Machery-Nagel and Company, Duren, W.Germany). The solvent systems used were

(A) 2-Methoxyethanol, dichloromethane (1:1 v/v)

(B) Hexane, ethyl acetate (1:3 v/v)

(C) n-Butanol, ethanol, water (40:11:19 v/v)

(D) Acetone.

The spots were visualised by spraying the plates with a solution of sulphuric acid in ethanol (5% w/v) and heating at 100°.

Ion-Exchange Chromatography: Ion-exchange chromatography of aqueous solutions were carried out using Dowex 1-X8 (OH^-) {Dow Chemical Company, Michigan, USA} or Amberlite IRA-400 (OH^-), IR-45(OH^-), IR-120(H^+) {Rohm and Haas Company, Philadelphia, USA} resins. For the strong

base resins, carbon dioxide free water was used throughout.

Carbohydrates: Carbohydrate content was assayed by the phenol-sulphuric acid method.[177] A 4% phenol solution (1 cm^3) was added to water (1 cm^3) containing 10-100 μg of sugar and concentrated sulphuric acid (5 cm^3) added rapidly. The colour which developed was read at 487 nm.

Lyophilisation: Freeze-drying was carried out on a 'Chem Lab' freeze-drier. Samples were first frozen in a cardice-acetone mixture before being placed on the freeze-drier.

Evaporations: All evaporations were carried out under reduced pressure, between 30° and 40°, with a 'Buchi' rotary evaporator.

Melting Points: The melting points (uncorrected) were recorded on a Thomas-Hoover melting point apparatus.

Elemental Analysis: Elemental micro-analysis was performed by Elemental Micro-Analysis Limited (Beaworthy, Devon) or Butterworth Laboratories Limited (Teddington, Middlesex).

Molecular Sieves: 3A and 4A molecular sieves (BDH) were activated by heating at 400° for 4 hr.

Water: Deionised, distilled water was used in all experiments unless stated otherwise.

Solvents: Anhydrous solvents were prepared by

standard techniques.[178]

Pyridine was distilled from sodium hydroxide pellets and stored over sodium hydroxide or potassium hydroxide pellets (B.D.H.Analar).

Hydrogen bromide in glacial acetic acid (about 45% w/v) was supplied by Hopkins and Williams.

2.4. Experimental

Maltobiono-δ-lactone:-

To a solution of maltose (15.0 g, 42 m moles) in water (560 cm^3) was added cadmium carbonate (23.4 g, 130 m moles), bromine (3.1 cm^3, 12.5 m moles) and the whole stirred in a stoppered 1 l.conical flask, covered in tinfoil to exclude light, for 48 hr at room temperature. To the suspension was added a portion of activated charcoal, the mixture was stirred and filtered through a thin bed of celite analytical filter aid. The filtrate was aerated to remove excess bromine, treated with silver carbonate (15 g- stir for 20 mins), then activated charcoal and filtered as before. The filtrate was cooled to 4° and passed down an ion-exchange column {Amberlite 1R-120 (H^+)} maintained at the same temperature. The eluant was collected and freeze-dried. Part of the sample thus obtained was dissolved in 2-methoxyethanol, heated for 5 mins at 90°, then cooled to 50° in a water

bath. After the volume of solution had been reduced in a gentle stream of air, it was seeded and kept at 50° for 2 weeks. More solvent was added occasionally, followed by reduction as before. No crystals were obtained by this or any other method of crystallisation attempted. Various tests were carried out on the remaining sample.

$\{\alpha\}_D^{25}$ = +105° going to +99° C2, water (12 hrs)

Lit[104] $\{\alpha\}_D^{25}$ = +123° going to +111° C2, water (12 hrs)

Acid/base titrations gave: Lactone, 60% ;
Acid, 17% and reducing substances, 47 mg g^{-1}(5%).

TLC (System A) R_F = 0.67 (plus acid-stationary spot).

{same result as for authentic sample of lactone kindly provided by Professor H.S.Isbell}.

TLC (System C) R_F = 0.31 (plus acid-stationary spot)

Paper (System B) R_G = 0.49

<u>Maltose octaacetate:-</u>

To acetic anhydride (100 cm^3) at about 130° was added sodium acetate (7.5 g, 91.4 m moles) and, carefully over the course of 1 hr, maltose (20.0 g, 58.4 m moles). The mixture was stirred a further 1 hr at 130°, then allowed to cool to room temperature, poured into ice water (300 cm^3) and stirred overnight. The resultant white crystalline solid was filtered at the pump and dried in a vacuum desiccator (37.4 g, 94%).

The crude product was dissolved in the minimum of boiling ethanol, treated with activated charcoal and filtered hot. The filtrate was left overnight to crystallise (19.7 g, 50%).
mp = 157 - 158° (Lit.[110] 159-160°C).
$\{\alpha\}_D^{25}$ = +63.7° C1, Chloroform (Lit.[118] $\{\alpha\}_D^{20}$ = +62.6° C5 Chloroform)
TLC (System B) R_F = 0.40
Anal.Calc. for $C_{28}H_{38}O_{19}$: C,49.56 ; H, 5.59.
Found: C,49.26 ; H, 5.64.

Heptaacetyl maltosyl bromide:-

To a solution of maltose octaacetate (10.0g, 14.7 m moles) in glacial acetic acid (34 cm³) cooled to 15° was added hydrogen bromide in glacial acetic acid (22 cm³, 45% w/v) with stirring. The reaction mixture was kept at 15° for 15-20 mins, then at 5° for 90 mins. After this time, chloroform (80 cm³) was added and the whole poured into ice water (300 cm³) with vigorous stirring. The organic layer was separated and the aqueous further extracted with chloroform (20 cm³). The combined organic layers were washed with ice water (twice), dried over calcium chloride and reduced to a thick syrup, which was taken up in ether (50 cm³) the petroleum ether added until the formation of a second layer. On standing at 4° the bromosugar separated as a thick

white gum, which was collected and placed in a vacuum desiccator (7.1 g, 69%)
mp = 76 - 79° (Lit.[119] = 112 - 113°)
$\{\alpha\}_D^{25}$ = +173.9° C1, Chloroform (Lit.[119] $\{\alpha\}_D^{20}$ = + 180.1° C2 Chloroform).
TLC (System B) R_F = 0.43
Anal.Calc. for $C_{26}H_{35}BrO_{17}$: C,44.65 ; H, 5.04; Br, 11.42. Found: C,45.17 ; H,5.11 ; Br, 11.27.

<u>Hexaacetyl maltal:-</u>

To the bromosugar (7.0g, 10 m moles) was added glacial acetic acid (70 cm^3), zinc dust (14.0 g) and the whole shaken 1 hr at room temperature. The reaction mixture was filtered, cooled in an ice bath and neutralised with sodium bicarbonate (solid sodium bicarbonate was added, followed by a little water and this repeated until neutralisation). Near neutrality, ether was added to extract the product. The ether layer was collected and the aqueous layer further extracted with ether. The combined ether layers were washed with ice water, then reduced to an amorphous white solid, which was dried in a vacuum desiccator (3.7 g, 66%).
This was dissolved in the minimum of hot methanol, cooled to room temperature, seeded and placed in the fridge to crystallise (0.95 g, 17%).
mp = 130 - 131° (Lit.[110] = 131-133°)

$\{\alpha\}_D^{25}$ = +68.2° C 0.9, Chloroform (Lit[110]$\{\alpha\}_D$ = +68° C0.8, Chloroform).

TLC (System B) R_F = 0.38

Anal.Calc. for $C_{24}H_{32}O_{15}$: C,51.43 ; H, 5.75.

Found: C, 51.49 ; H,5.62.

N.M.R. data (chloroform-d): σ6.44 (dd, 1 proton, $J_{1,2}$ 6.16Hz, $J_{1,3}$ 1.17Hz, H1) ; 5.50 (d, 1 proton, $J_{1,2}$ 4.11Hz, H1') ; 4.95 - 5.43 (m, 3 protons, H-2,3,4) ; 4.75 - 4.90 (octet, 2 protons H-5,5'); 4.04-4.36 (m, 7 protons, H-6,2',3'4',6') ; 2.13, 2.11, 2.05, 2.03, 2.01 (18 protons, -OAc).

As the yield was low another method of crystallisation was attempted. The reaction was carried out as before then, after filtration, water added to turbidity, the sample seeded and allowed to crystallise (1.44g, 26%), M.Pt. = 129 - 130°.

The first seed was obtained by following the ether extraction with a chloroform extraction. This solution, was washed, dried over calcium chloride, filtered then left standing in an open conical flask. The sample crystallised overnight.

Maltal:-

Two methods were used for the deacetylation of hexaacetyl maltal viz sodium methoxide and methanolic ammonia. The former caused slight hydrolysis and decomposition, so the latter

method was generally used.

A fairly rapid stream of dry ammonia (passed through a tower of soda lime) was bubbled through a solution of hexaacetyl maltal (0.58g, 1.03 m moles - does not all go into solution immediately) in dry methanol, cooling in an ice/salt bath, for 15 mins. The reaction mixture was placed in a fridge until all of the solid had gone into solution (about 36 hr). The solution was concentrated on the rotary evaporator to a syrup, dry methanol was added and the solution again concentrated. The sample was further purified by pumping under high vacuum (1 Torr, 40°) to sublime any acetamide still present. The final product was a pale yellow oil (0.31g, 97%).

TLC (System C) R_F = 0.32

Paper (System A) R_G = 1.59

N.M.R. data (D_2O): σ6.480 (q, 1 proton, $J_{1,2}$ 5.920 Hz, $J_{1.3}$ -1.310 Hz, H1) ; 5.482 (d, 1 proton, $J_{1'2'}$ 3.820 Hz , H1') ; 4875 (q, 1 proton, $J_{2,3}$ 2.960 Hz, H2) ; 4.423 (octet, 1 proton, $J_{3,4}$ 5.700 Hz, H3) ; 4.105 (septet, 1 proton, $J_{5,6a}$ 5.390 Hz, $J_{5,6b}$ 2.730 Hz, H5) ; 3.933 q, 1 proton, $J_{4,5}$ 7.790 Hz, H4) ; 3.911 (octet, 2 protons, $J_{6a,6b}$-12.260 Hz, H-6_a,6_b) ; 3.839 (octet, 2 protons, $J_{6'a,6'b}$ -13.038 Hz,

H-6'$_a$6'$_b$) ; 3.754 (octet, 1 proton, $J_{5'.6'a}$ 4.603 Hz, $J_{5',6'b}$ 2.951Hz, H5') ; 3.714(t, 1 proton, $J_{3'4'}$ 9.678 Hz, H3') ; 3.588 (q, 1 proton, $J_{2'3'}$ 9.952 Hz,H2'); 3.451 (t, 1 proton, $J_{4'5'}$ 9.992 Hz, H4').

N-β-D-glucosylbenzylamine:-

To a solution of glucose (10g, 56 m moles) in ethanol (200 cm^3, 95%) was added benzylamine (17.8g, 18.1 cm^3, 166 m moles). The mixture was heated for 10 mins at boiling, filtered hot and allowed to stand at room temperature for 48 hr. It was then cooled in ice water. After 1 hr the solution had formed into a gelatinous solid, to which dry ether was added and the whole filtered at the pump. The resulting solid was dried in a vacuum desiccator (11.2g, 75%). This was recrystallised three times from methanol/ether to give a pale yellow solid.

mp = 73-74° (Lit.[142] = 81.5^o)

$\{\alpha\}_D^{25}$ = -39.6^o going to -22.5^o (24 hr) C2,Methanol

Lit.[142] $\{\alpha\}_D^{25}$ = -42.67^o going to -22.66^o (24 hr) C2, Methanol.

TLC (System A) R_F = 0.75

Paper (System A) R_G = 3.53 (with hydrolysis)

Anal.Calc. for $C_{13}H_{19}NO_5.H_2O$ = C, 54.34 ; H, 7.37, N, 4.88. Found: C, 54.78; H,7.52 ; N 5.11.

N.M.R. data (DMSO-d^6 + trace D_2O): σ7.28 (m,

5 protons, $-C_6H_5$) ; 4.35 (d, 1 proton, $J_{1,2}$ 5.0Hz, $H1^{\alpha}$) ; 3.94, 3.78 (dd, 1 proton, $J_{6a,6b}$ -14.0Hz, H6a or 6b); 3.67 (d, 1 proton, $J_{1,2}$ 8.9Hz, H1) ; 3.44 (m, 1 proton, H5) ; 3.31 (q, 1 proton, $J_{2,3}$ 9.9Hz, $H2^{\alpha}$); 3.17 (s, 2 protons, $-CH_2-$) ; 3.14, 3.05, 2.96 (t,t,t, 3 protons, $J_{2,3} \sim J_{3,4} \sim J_{4,5}$ 9Hz, H2, 3 and 4).

On storage in solution or in the solid form TLC and NMR showed that decomposition occurred; one obvious possibility is the Amadori Rearrangement.

N-β-D-glucosylpiperidine

Finely powdered anhydrous α-D-glucose (18.0g, 0.10 moles) and piperidine (17.2g, 20cm^3, 0.20 moles) were stirred together for a few minutes until heat was evolved. The flask was then placed into a water bath (maintained at 70-80°) until a clear, amber coloured syrup was obtained (about 15 mins). Absolute methanol (25 cm^3) and acetone (25 cm^3) were then added and the solution was filtered. To the filtrate was added acetone (225 cm^3) and the flask placed in the fridge (1° - if higher, no crystals could be obtained). After 48 hr, the crystals which had formed were filtered at the pump, washed with methanol/acetone (1:4) and dried in a vacuum desiccator (13.7g, 55%). The solid was

dissolved in methanol/acetone (300 cm^3, 1:3), seeded and again placed into the fridge for 48 hr. White needle-like crystals were obtained and dried as before (7.8g, 32%).

M Pt = 131-132°d (Lit.[143] = 130°d)

$\{\alpha\}_D^{25}$ = +1.8^o C2, Methanol (Lit.[143] $\{\alpha\}_D^{25}$ = +3.0^o C2, Methanol)

TLC (System A) R_F = 0.76

Paper (System A) R_G = 3.48

Anal.Calc. for $C_{11}H_{21}NO_5$: C,53.44 ; H,8.56; N, 5.66. Found: C,53.55 ; H,8.35; N,5.56.

N.M.R. data (D_2O): σ 3.91 (d, 1 proton, $J_{1,2}$ 8.5Hz, H1) ; 3.88 (dd, 1 proton, $J_{5,6a}$ 1.8Hz, $J_{6a,6b}$ - 12.5Hz, H6a) ; 3.69 (dd, 1 proton, $J_{5,6b}$ 5.7Hz, $H6_b$) ; 3.58 (t, 1 proton, $J_{2,3}$ 8.6Hz,H2) ; 3.46 (t, 1 proton, $J_{3,4}$ 9.4Hz,H3) ; 3.32 (m, 2 protons, H4 and 5) ; 2.90, 2.60 (m,m, 4 protons, H2' and 6') ; 1.54 (m, 4 protons,H3' and 5') ; 1.45 (m, 2 protons, H4')

NMR samples showed evidence of decomposition on storage, but the crystalline form is stable if stored at low temperatures under anhydrous conditions.

Cyclohexyl α-D-glucopyranoside:-

(i) A mixture of anhydrous α-D-glucose (20.0g, 0.11 moles), cyclohexanol (100 cm^3) and acid resin {IR-120(H^+)} were stirred under reflux for 24 hr. The resin was filtered off and

washed with more cyclohexanol. The combined solutions were concentrated on the rotary (bath temperature 80°) and allowed to cool to room temperature. No crystals formed in this or any other solvents tried (acetone and ether are generally used for recrystallisation of alkyl glycosides[169]). TLC details revealed the presence of product plus unreacted glucose.

TLC (System C) R_F = 0.53

Analysis by capillary GLC suggested

α-D-glucoside 45%, β-D-glucoside 28%,

α-D-glucose 20%, β-D-glucose 7%

The experiment was repeated with other acid catalysts (e.g. methanesulphonic acid, trifluoroacetic acid), but these gave even smaller yields of required product.

(ii) A solution of β-D-glucose pentaacetate (10g, 25.6 m moles) p-toluenesulphonic acid monohydrate (2.0g, 10.5 m moles) and cyclohexanol (100 cm^3) in chloroform (80 cm^3) was refluxed at $84\text{-}89^{o}$ for 72 hr. After this time, it was allowed to cool to room temperature, neutralised with 1R-45(OH^-) which was then filtered off and washed with aqueous ethanol (400 cm^3,50% v/v). The combined solutions were treated with activated charcoal, filtered through a thin bed of celite analytical filter aid and concentrated on the rotary evaporator. The resulting syrup was

dissolved in water and extracted with hexane to remove impurities. The aqueous solution, thus obtained, was again concentrated to a syrup (4.8g, 71%).

Analysis by capillary GLC suggested

α-D-glucoside 77%, β-D-glucoside 23%

Anal.Calc. for $C_{12}H_{22}O_6$: C, 54.95 ; H, 8.45.

Found: C,54.00 ; H, 8.67.

N.M.R. data (D_2O): σ5.06 (d, 1 proton, $J_{1,2}$ 4.04Hz, $H^1α$) ; 4.57 (d, 1 proton, $J_{1.2}$ 7.72 Hz, $H^1β$) ; 3.18 - 3.92 (m, 6 protons, H-2,3,4,5,6) ; 1.0 - 2.0 (broad peaks due to cyclohexane ring protons).

N-Acetyl-piperidino α-D-glucopyranoside:-

N-acetyl-4-hydroxypiperidine:-

To hydroxypiperidine (5.0g, 50 m moles) in water (15 cm^3) was added acetic anhydride (6 cm^3, 60 m moles). After stirring vigorously the mixture was heated on a water bath for 15 mins (the solid dissolved after a few minutes). On cooling, the sample was concentrated and then purified by passing down a short silica column (10 cm) using acetone as eluant (starting material remains on the top of the column). Concentration of the acetone solution, followed by drying in a vacuum desiccator, gave a white solid (5.8g, 82%)

TLC (System D) R_F = 0.30

Anal.Calc. for $C_7H_{13}NO_2$: C, 58.72 ; H, 9.15 ; N.9.78. Found: C, 58.89 ; H, 9.39 ; N,9.76.

Reaction of N-acetyl4-hydroxypiperidine with β-D-glucose pentaacetate.

A solution of β-D-glucose pentaacetate (2.0g, 5.1 m moles), N-acetyl-4-hydroxypiperidine (2.5g, 24.6 m moles) and p-toluenesulphonic acid monohydrate (0.4g, 2.1 m moles) in chloroform (25 cm^3) was refluxed for 72 hr on a water bath. On cooling to room temperature a small amount of solid formed (81 mg), which was collected. The filtrate was neutralised with 1R-45(OH^-). This was then filtered off washed with aqueous ethanol (80 cm^3, 50% v/v) and the combined solutions concentrated. TLC (System C) showed the product to be mainly unreacted starting material.

1-Bromoglucose tetraacetate:-

To a stirred solution of β-D-glucose pentaacetate (10.0g, 25.6 m moles) in glacial acetic acid (50 cm^3), cooled to 15°, was added hydrogen bromide in glacial acetic acid (45 cm^3, 45% w/v). The reaction was kept at 15° for 15-20 mins, then at 5° for 90 mins. Chloroform was added (80 cm^3) and the whole poured into ice water (300 cm^3) with vigorous stirring. The chloroform layer was separated and the aqueous layer again extracted with chloroform.

The combined organic layers were washed with ice water, dried over calcium chloride, filtered and concentrated to a thick syrup, which was taken up in hot ether. On cooling, a white crystalline solid formed and was collected (6.7g, 64%).

mp = 88-89° (Lit.[179] 88-89°)

$\{\alpha\}_{D}^{25}$= +196.4 C2, Chloroform (Lit.[179] $\{\alpha\}_{D}^{25}$= +198 C2, Chloroform).

Anal.Calc. for $C_{14}H_{19}O_{9}Br$: C, 40.89 ; H, 4.66 ; Br, 19.43. Found: C, 40.34 ; H, 4.44 ; Br, 19.44.

Reaction of 4-hydroxypiperidine with 1-bromo-glucose tetraacetate:-

A solution of the bromosugar (3g, 7.3 m moles) in dried dichloromethane (50 cm^{3}) was stirred overnight at room temperature with molecular sieve (13.6g, type 4A, 1.59 mm pellets). The molecular sieve was filtered off and the filtrate washed with water (until the washings were neutral), dried with sodium sulphate then reduced on the rotary to a thick syrup, which was dissolved in hot ethyl acetate and hexane added. On cooling to room temperature a white crystalline solid appeared and was collected (0.70g, 23%). This was recrystallised to give (0.58g, 19%).

M Pt = 147-8°

$\{\alpha\}_{D}^{25}$ = +7.13^{o} C1, Chloroform.

TLC (System B) $R_F = 0.16$

Anal. Required for $C_{19}H_{29}NO_{10}$: C,52.89 ; H,6.78; N, 3.25. Found: C,52.79 ; H,6.95 ; N, 3.31

N.M.R. data (chloroform-d) : σ5.22 (t, 1 proton $J_{2,3} \simeq J_{3,4}$ 9.4Hz, H3) ; 5.13 (t, 1 proton, $J_{1,2} \simeq J_{2,3}$ 9.4Hz, H2) ; 5.00 (t,1 proton, $J_{3,4} \simeq J_{4,5}$ 9.7 Hz, H4); 4.09-4.25 (m, 2 protons, H6) ; 4.02 (d, 1 proton, $J_{1,2}$ 9.1Hz, H1) ; 3.68 (m, 1 proton, OH) ; 3.57-3.62 (octet, 1 proton, H5) ; 3.09-3.14, 2.76-2.88 2.41-2.47 (octet, 1 proton; m, 2 protons ; m, 1 proton; $-CH_2N-CH_2-$); 2.01, 2.02, 2.03, 2.08 (12 protons, OAc) ; 1.81-1.88, 1.71-1.77, 1.34-1.43 (m, 2 protons; m,2 protons; m, 1 proton; $-CH_2CH-CH_2$).

N,N-Dimethylchloroformimium chloride[180]
$\{(CH_3)_2NCHCl\}^+Cl^-$:-

To dry dimethylformamide (30 cm^3, excess) in a 100 cm^3, 3 necked round bottomed flask fitted with drying tube, was added, cautiously, phosphorus pentachloride (16g) allowing the temperature to rise to 100°. When the addition was complete, the mixture was cooled to 0° and the resultant solid filtered at the pump, quickly washed with dry dimethylformamide, dry ether then placed in a vacuum desiccator over phosphorus pentoxide.

Anhydrous p-toluenesulphonic acid[178]:-

p-Toluenesulphonic acid was dehydrated by heating for 4 hr at 100° under water pump vacuum, followed by recrystallisation from chloroform and finally drying under vacuum at 50°.

2,3,4,6-tetra-O-benzyl α-D-glucopyranoside[181](II):-

To a mixture of methyl α-D-glucopyranoside (25g, 0.13 moles), powdered potassium hydroxide (125g) and dioxan (100 cm^3) being vigorously stirred (mechanical stirrer) in a 1 l, 3 necked, round bottomed flask was added benzyl chloride (40 cm^3), dropwise, while bringing the mixture to the boil. More benzyl chloride (85 cm^3) was added to the boiling mixture and heating continued for 2 hr after the addition was complete. The reaction mixture was allowed to cool to about 70°, then steam distilled until about 1 l had been collected (this is to remove dioxan, benzyl chloride and benzyl alcohol). The organic layer was separated, washed with water and mixed with glacial acetic acid (1050 cm^3) and sulphuric acid (600 cm^3,2M). The hydrolysis mixture was heated on a steam bath with continual stirring for 24 hr, the product crystallised towards the end of this time. Water (270 cm^3) was added, the mixture cooled in an ice bath and the product filtered at the pump. The crystals were washed successively with aqueous acetic acid (200 cm^3, 50% v/v), water, aqueous methanol (200 cm^3,

75% v/v) and finally dried in a vacuum dessicator (22.4g, 32%). The sample was dissolved in ten parts of n-propanol, treated with activated charcoal and filtered hot. The white crystalline solid which formed was filtered at the pump and dried (14.9, 21%).

mp = 149-150° (Lit. = 150-151°)

TLC (System B) R_F = 0.64

Anal.Calc. for $C_{34}H_{36}O_6$: C,75.53 ; H, 6.71.

Found: C,75.20 ; H, 6.75.

<u>1-Chloro-2,3,4,6-tetra-O-benzyl α-D-glucopyranose</u>[180] <u>(III):-</u>

(i) To a suspension of N,N-dimethylchloroformimium chloride (1.3g, 10 m moles) in dioxan (12.0g, 12cm^3, 135 m moles) was added II (2.7g, 10 m moles). The reaction was brought to 100° for 15 mins, allowed to cool to room temperature then poured into ice water (50 cm^3) and extracted with chloroform (3 times). The combined organic layers were washed with water, dried over calcium chloride and concentrated to a dark yellow group (2.5g, 91%).

$\{\alpha\}_D^{25}$ = +101.7^o C2.1, Benzene (Lit.$\{\alpha\}_D^{24}$ = +93° C3.2, Benzene)

TLC (System B) R_F = 0.68

A second method was also attempted to make this compound, following the work of Baddiley et al.[165]

(ii) To II (2.9g, 5.4 m moles) was added thionyl chloride (10 cm^3). The temperature was brought to 70° and maintained for 3 hr. After this time the reaction mixture was reduced on the rotary, then toluene added and again reduced to remove traces of thionyl chloride. A second aliquot of thionyl chloride (10 cm^3) was added and the process repeated. This was repeated a third time, except that when the toluene was added so too was activated charcoal. The mixture was heated on a water bath, filtered through a bed of celite analytical filter aid and concentrated to a dark yellow syrup (3.2g, 107%).

$\{\alpha\}_D^{25} = +78.9^o$ C2, Benzene

As the halogen compound is not very stable, it was decided to use the product formed from the first reaction without further purification.

1-O-(N-methyl)acetimidyl-2,3,4,6-tetra-O-benzyl β-D-glucopyranose[173] (IV):-

A solution of III (2.50g, 4.5 m moles) in anhydrous benzene (160 cm^3) was stirred at room temperature for 20 hr, with the exclusion of moisture, in the presence of N-methylacetamide (0.42g, 5.7 m moles), diisopropylethylamine (1.35 cm^3), silver oxide (3.15 g) and powdered molecular sieve (type 4A). After filtration through a bed of neutral. alumina, the resultant

solution was concentrated to a dark yellow syrup (2.3g, 86%).

$\{\alpha\}_D^{25}$ = +29.7° C1.4, Chloroform (Lit. $\{\alpha\}_D^{20}$ = +28.6° C1.5, Chloroform).

Anal.Calc. for $C_{37}H_{41}NO_6$: C,74.60 ; H,6.94 ; N, 2.35.

Found: C,73.00 ; H,6.58 ; N, 2.26.

Cyclohexyl-2,3,4,6-tetra-O-benzyl α-D-glucopyranoside(V):-

To a solution of cyclohexanol (0.22g, 0.23 cm^3, 2.24 m moles) stirring in dry benzene (90 cm^3) under vigorously anhydrous conditions was added IV (2.0 g, 3.36 m moles) and anhydrous p-toluenesulphonic acid (0.39g, 2.24 m moles). After 24 hr the reaction mixture was neutralised with triethylamine (0.23g, 0.31 cm^3, 2.24 m moles), diluted with benzene, washed with water, dried with anhydrous sodium sulphate then reduced to a thin oil which was passed down a silica column (Merck 7734, eluant - hexane: ethyl acetate, 1:3 v/v). The first fraction eluted was collected and concentrated to a yellow syrup (1.4g, 100%)

$\{\alpha\}_D^{25}$ = +39.6° C0.8, Chloroform

TLC (System B) R_F = 0.71

Cyclohexyl α-D-glucopyranoside[182]:-

To a mixture of V (1.4g, 2.35 m moles) and ethanol (100 cm^3, 95%) was added palladium on carbon (2g). The reaction vessel was then

pressurised with hydrogen (60 p.s.i.) and left vigorously shaking at room temperature. Due to fracture of the vessel, most of the sample was lost. The small amount that was saved, revealed that complete debenzylation had not occurred.

2.5 Separation of α- and β-Glycosides

It was shown by Baddiley et al[183] that anomeric mixtures of alkyl glycosides could be separated on strong base resins. Towards this end, both Amberlite IRA - 400 (OH^-) and Dowex 1- X 8 (OH^-) were used to purify the cyclohexyl-D-glucopyranoside obtained previously (by the method of Wing). In a typical experiment, a Dowex column (20 x 1.5 cm, BV = 35 cm^3) was regenerated by passing a solution of sodium hydroxide (105 cm^3, 3BV, 4%) at 10 cm^3/min followed by water (700 cm^3, 20 BV) at 10 cm^3/min for 1BV then at 20 cm^3/min for the remainder. The sample (2.0g in 4 cm^3) was passed down the column at 20 cm^3/hr. Fractions (5 cm^3) were collected and analysed for carbohydrate content by the phenol/sulphuric method. Neither resin achieved separation of the glycosides.

A second method used to obtain pure α-glucoside followed the method of Wing[169], which involved hydrolysis of the β-D-glucoside with β-glucosidase and removal of the glucose formed

on a strong base resin.

To a solution of cyclohexyl-D-glucoside (1.67g) in acetate buffer (40cm^3,pH5.0, 0.1M) was added β-glucosidase (2.5mg, 11 units). The reaction was kept at 30° for 2 days, then treated with a mixture of Amberlite {IR-45(OH$^-$), IR-120 (H$^+$)} resins (5g + 5g) and stirred for 20 mins. The resins were filtered off and washed with aqueous alcohol (100 cm^3,50%). The filtrate and washings were reduced on the rotary to give a syrup (1.46g), which was dissolved in water (4 cm^3) and passed down a Dowex I column. On concentration a syrup was obtained (0.55g). $\{\alpha\}_D^{25}$ = +93.8^o Cl, Water (Lit.[184] $\{\alpha\}_D^{20}$ = +137.5^o Cl.44, Water).

Insufficient enzyme was probably used in this attempt. A second reaction of this oil with more enzyme should improve the purity of the glucoside.

CHAPTER 3

INHIBITION OF WHEAT ALPHA-AMYLASE

3.1 Introduction

Four possible inhibitors of α-amylase: maltobiono-δ-lactone, maltal, N-β-D-glucosylbenzylamine and N-β-D-glucosylpiperidine, synthesised as described in Chapter 2, have been examined as inhibitors of wheat α-amylase. The reasons for their choice and the different bases for their possible activity, have been described in Chapter 1. As was stated previously, the main motive behind this work was the synthesis of a specific and potent inhibitor for the bread industry, therefore, in order to make the experiments as relevant as possible, the inhibitors were tested against wheat α-amylase, with wheat amylopectin as the substrate.

In this chapter will be described the extraction of α-amylase from a sample of malted Champlein wheat and its inhibition by the four compounds listed above.

3.2. Results and Discussion

3.2.1. Extraction of Wheat α-Amylase

On the advice of the scientists from Rank Hovis McDougall, wheat α-amylase was extracted following the technique of Kruger and Tkachuk.[185] The first step in the procedure was the extraction

of the crude α-amylase from a sample of malted Champlein wheat with 0.2% calcium acetate. This extract was then subjected to heat treatment ($70^{o}C$ for 15 mins at pH 6.6 - under which conditions α-amylase is stable[186]) to inactivate β-amylase and any other heat-labile proteins which were present. The next step was acetone fractionation followed by complexing with glycogen (this method of complexing the enzyme was first reported by Schramm and Loyter[187] as a means of obtaining essentially pure α-amylase). This complex was left overnight at 15° to $20^{o}C$ to permit the glycogen to be digested by the α-amylase. In the final step the digest was passed down a Bio-gel P10 column to remove low molecular weight carbohydrates resulting from the glycogen digestion. (Table 3.1 and Fig.3.1).

PROTEIN			CARBOHYDRATE					
F	A		F	A	F	A	F	A
9	0.00		8	0.00	16	0.60	26	0.79
10	0.66		9	0.36	17	0.50	27	1.26
11	0.51		10	>2.0	18	0.45	28	>2.0
12	0.20		11	1.84	19	0.47	29	>2.0
13	0.11		12	1.18	21	0.47	31	0.37
14	0.05		13	0.95	22	0.53	32	0.14
15	0.03		14	0.81	23	0.53	33	0.05
16	0.02		15	0.71	24	0.68	34	0.03

Table 3.1 Absorbances at 280 nm (protein) and 487 nm (carbohydrate) given by fractions (F) from Bio-gel P10 column chromatography of wheat α-amylase extract.

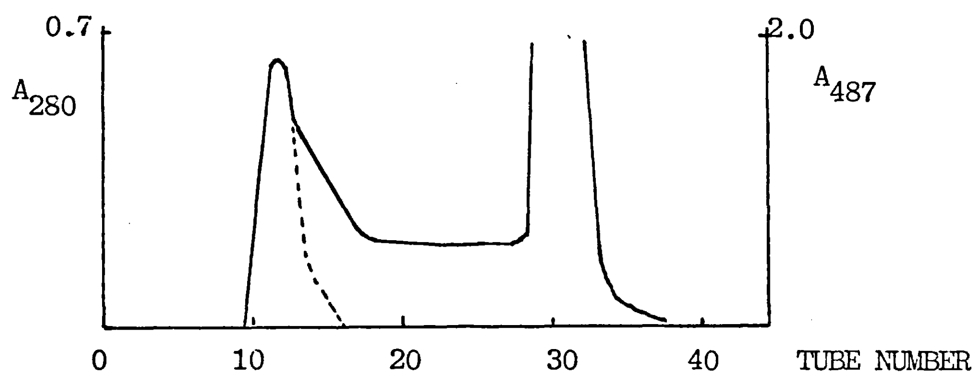


Fig.3.1 Gel-filtration of wheat α-amylase on P10 column, showing carbohydrate (——) and protein (------) content of the fractions.

Tubes 10-14, which contained protein, were assayed for α-amylase activity at pH 5.0, 30°C with a substrate concentration of 1%. The relative activities found were: tube 10, 25.6; 11,21.3; 12,2.9; 13,1.4; 14,1.0. Tubes 10 and 11 were combined to give a solution which had a specific activity of 44 Units per mg of protein where one unit of activity will liberate 1 μmole of maltose monohydrate per cm^{3} per min from a 1% solution of wheat amylopectin at pH 5.0 and 30°. A similar result was obtained by Manners and Marshall[37], who isolated α-amylase from malted rye flour with a specific activity of 61 units per mg of protein where one unit of activity will liberate 1 μmole of maltose monohydrate per cm^{3} per min from a 1% solution of starch at pH 5.0 and 37°.

The Michaelis parameters Km and V_{max} were determined from a Lineweaver-Burk plot (Fig.3.2) of initial rate data (Table 3.2). The values obtained viz 0.25% w/v and 18.2 x 10^{-2} μmol cm $^{-3}$ min^{-1} are comparable with many α-amylases[188] e.g. a bacterial amylase from Bacillus sp. 11-1S was reported[189] with Km and V_{max} of 1.64 mg ml^{-1} (i.e. 0.16% w/v)and 50.2 x 10^{-2} μmol min^{-1} respectively.

Sample number	{S}%	ΔA_{420}	μg Maltose	v x 10^{-2} (μmol/cm^3/min)	1/V	1/S
1	1.0	0.719	106.36	14.76	6.78	1
2	0.5	0.575	85.06	11.80	8.47	2
3	0.2	0.397	58.73	8.15	12.27	5
4	0.1	0.269	39.79	5.52	18.12	10
5	0.067	0.185	27.37	3.80	26.32	15
6	0.05	0.147	21.75	3.02	33.11	20

Ignoring sample number 4, which is low, gives

Km = 0.25%, V_{max} = 18.18 x 10^{-2} μmol/cm^3/min

Table 3.2. Initial rate data for wheat α-amylase at pH 5.0, 30°C.

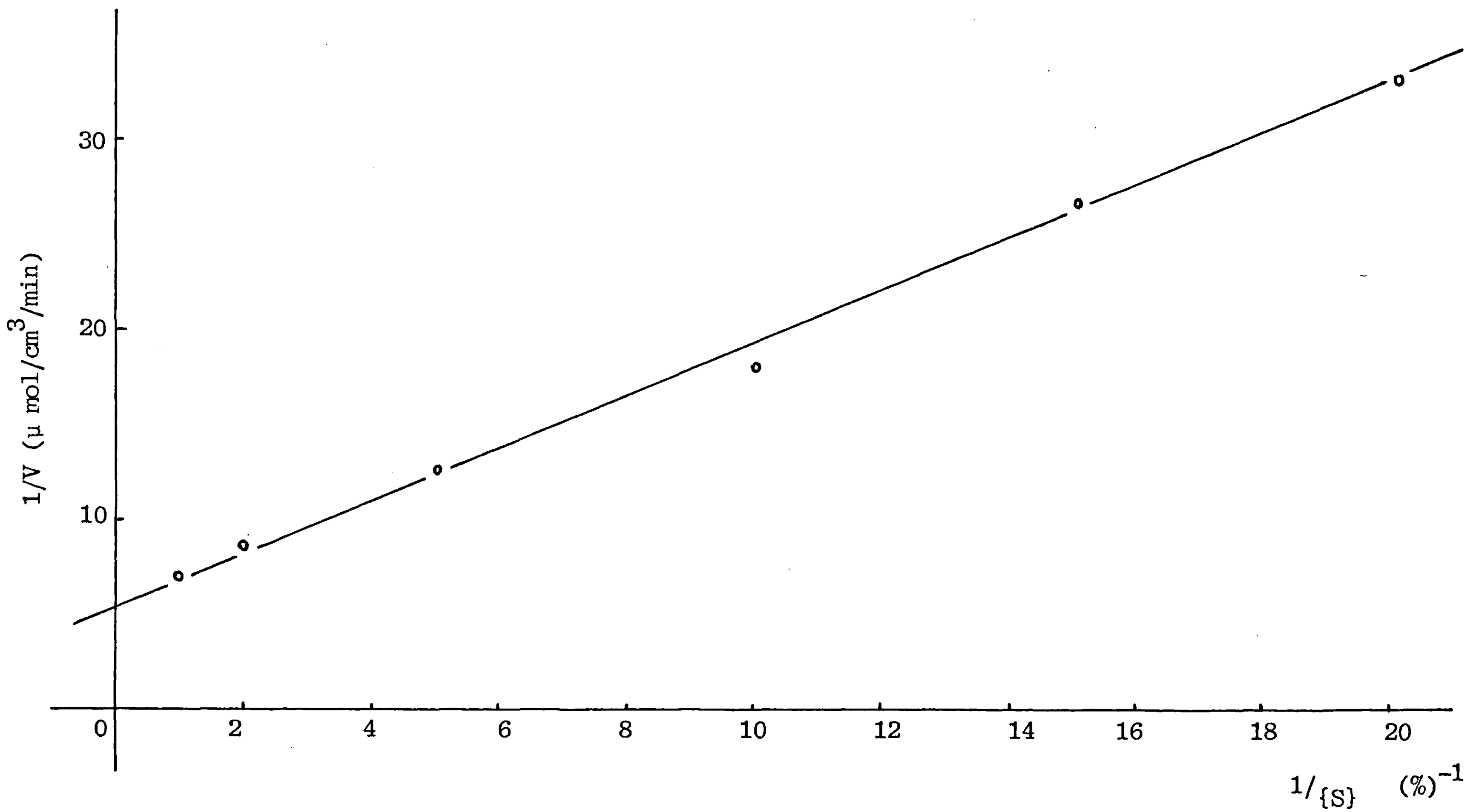
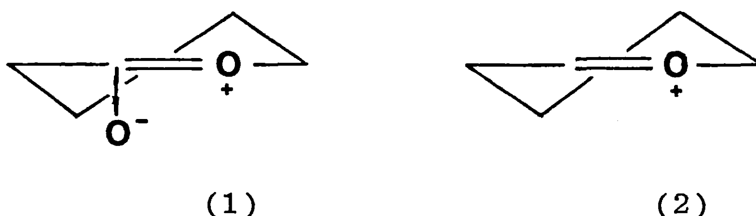


Fig.3.2. Lineweaver-Burk plot for wheat α-amylase.

3.2.2. Inhibition of Wheat α-Amylase by Maltobiono-σ-lactone.

Since the studies of Levvy[190] it has been known that glycosidases are inhibited by D-aldono-1,5-lactones. This was attributed by Leaback[191] to the fact that, for example, D-glucono-1,5-lactone (1) can assume, as it in fact does in the crystal,[192] a half chair conformation similar to that of a D-glucopyranosyl cation (2)



(1) (2)

The activation of glycosides for hydrolysis, as suggested by secondary α-kinetic isotope effects,[193] is thought likely to occur through intermediates in which the glycosidic carbon atom acquires sp^2 hybridisation, showing a similar electronic and structural resemblance to (2). This ring distortion decreases the enthalpy of activation and increases the susceptibility of the glucosyl moiety to nucleophilic attack by a water molecule or a carboxylate group. As shown by the transition state analogue theory earlier, a stable compound resembling this intermediate should be a very

effective inhibitor. Work on D-glucono-1,5-lactone[194] has proved it to be a powerful inhibitor of glucosidases (e.g. in tests against apricot emulsin, Niwa et al. reported it to be a competitive inhibitor with a Ki of 49μM), as are many other lactones.[195,196] During the course of this work maltobiono-1,5-lactone was reported[106] to be a competitive inhibitor of porcine pancreatic α-amylase with a Ki of 0.31 mM. In this work, the lactone was tested against the cereal α-amylase from malted Champlein wheat.

Inhibition by the lactone was to be studied at pH 5.0. The sample used was the pure crystalline compound kindly donated by Professor H S Isbell. According to Pocker[197] the hydrolysis of D-glucono-δ-lactone is subject to intermolecular general acid and general base catalysis and that for a pH $\geqslant$ 5 the reaction proceeds to completion. At 30°, in 0.1M sodium acetate buffer pH 5.0, the hydrolysis of maltobiono-δ-lactone was followed by the ferric hydroxamate method[198] (see Experimental, 3.3), and was similarly found to go to completion with a first order rate constant of 1.61×10^{-4} s^{-1} (Table 3.3, Fig.3.3). This is of the same order of magnitude as that which could be predicted from Pocker's data on gluconolactone.

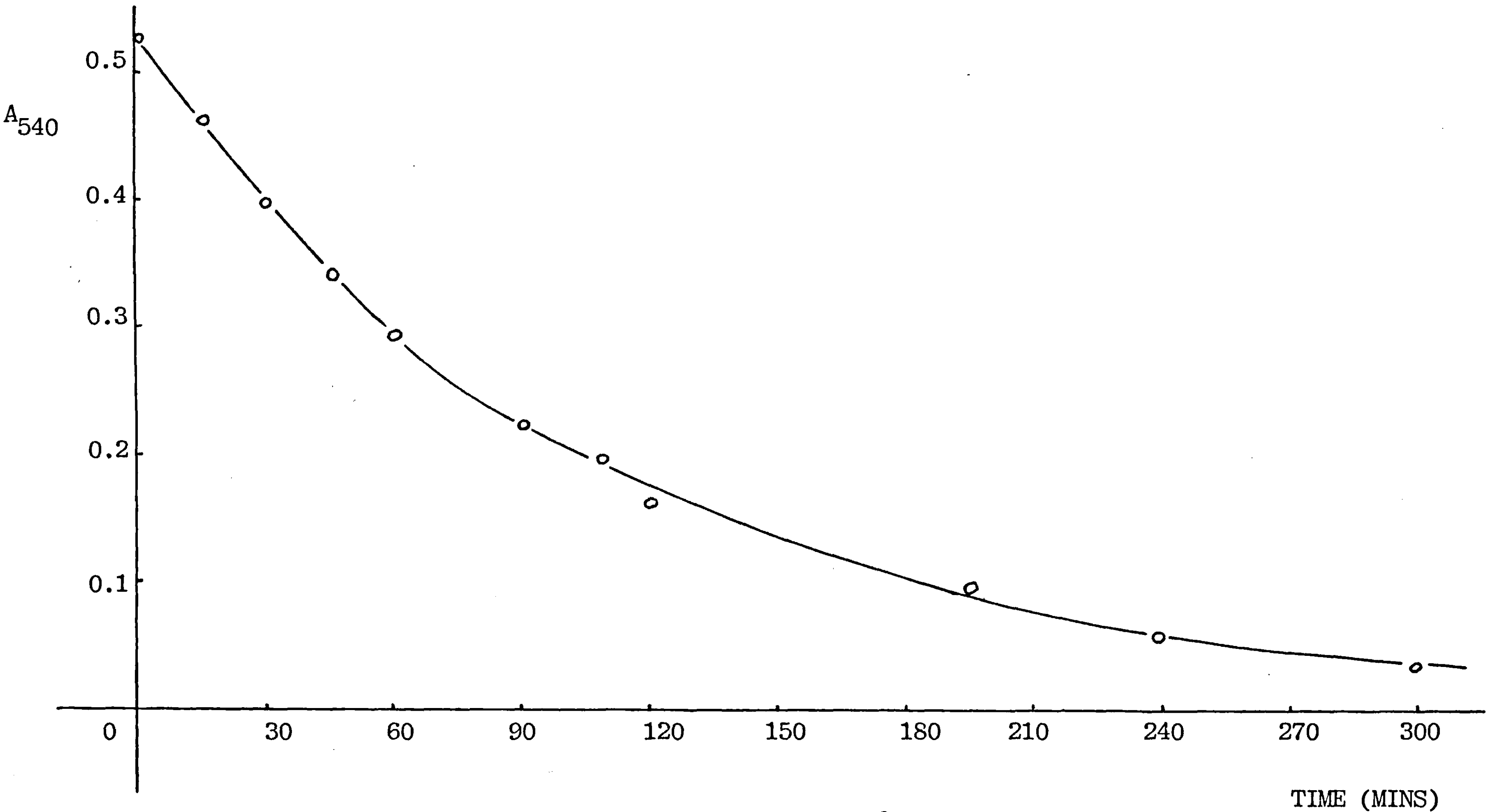


Fig.3.3. First order plot for the hydrolysis of maltobiono-δ-lactone

Time (Mins)	A_{540}	$-\ln(A_t - A_\infty)$
1	0.525	0.644
15	0.462	0.772
30	0.398	0.921
45	0.344	1.067
60	0.296	1.217
90	0.223	1.501
105	0.193	1.645
120	0.166	1.797
195	0.095	2.345
240	0.055	2.900
300	0.037	3.297
T ∞	0.000	

Table 3.3 Hydrolysis of maltobiono-δ-lactone at pH 5.0, 30°.

In order to keep hydrolysis to a minimum, the lactone was dissolved in an inert solvent, freshly distilled trimethylphosphate, and the reaction times kept as small as possible (5 mins - up to this time, the hydrolysis will be less than 5% therefore no correction was made). A Dixon plot (1/V vs.I) of the results showed the inhibition to be perfectly non-competitive[199], i.e. the inhibitor had no effect on Km, with a Ki of 2.15 mM (Table 3.4, Fig.3.4).

{I} (mM)	ΔA_{420}	Maltose (μg)	v x 10^{-2} (μmol/cm^3/min)	1/V
A. {S}	= 0.5%			
0	0.630	93.20	12.93	7.73
0.5	0.545	80.62	11.19	8.94
1.0	0.432	63.91	8.87	11.27
1.5	0.393	58.14	8.07	12.39
B. {S}	= 0.1%			
0	0.279	41.27	5.73	17.45
0.5	0.222	32.84	4.56	21.93
1.0	0.188	27.81	3.86	25.91
1.5	0.164	24.26	3.37	29.67

Table 3.4. Inhibition of wheat α-amylase by maltobiono-δ-lactone; pH 5.0, 0.1M acetate buffer, 30° ± 0.1.

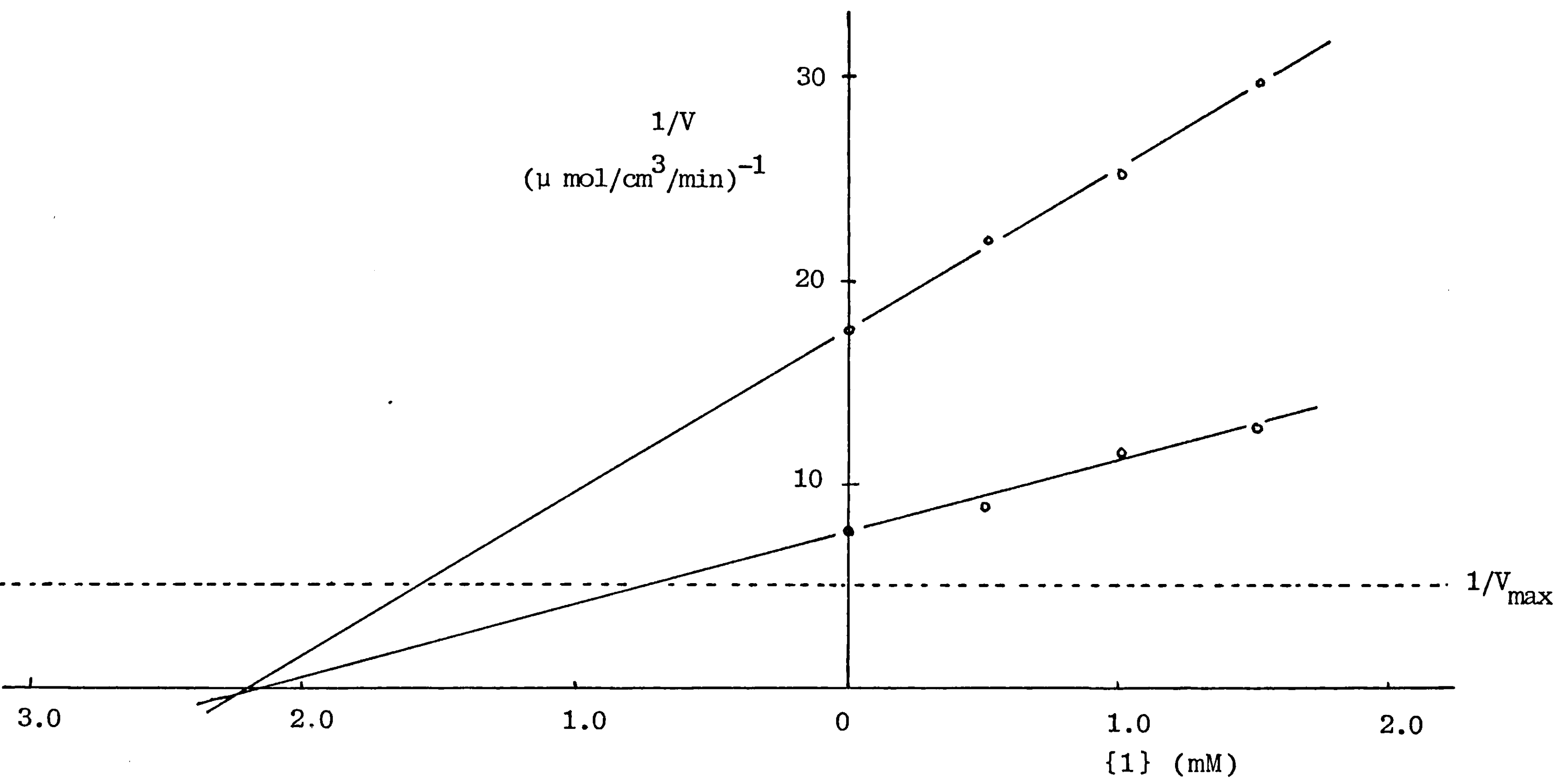


Fig.3.4. Dixon plot for the inhibition of wheat α-amylase by maltobiono-δ-lactone

This is a common type of inhibition with multisubstrate enzymes in which the inhibitor binds to a site other than the actual active site. The simple rationalisation of this type of inhibition is that the substrate can also bind to give a ternary (EIS) complex, which will not then react to give product. The observation of non-competitive inhibition for this supposed transition state analogue is an unexpected result. A possible explanation is that the inhibitor binds to an acceptor site and thus prevents access by water. This could be regarded as a form of substrate inhibition and might lead to transglycosylation products containing terminal lactone residues; whether these are formed is not known. The observed Ki, 2.15 mM, is of the same order as Km viz 0.25% or 15mM per anhydroglucose unit or 2.5 mM per hexasaccharide unit.

In similar inhibition studies of porcine pancreatic alpha-amylase by Laszlo et al with D-glucono-1,5-lactone and maltobiono-1,5-lactone, the inhibition by maltobiono-1,5-lactone was found to be competitive, but that of D-glucono-1,5-lactone to be partially non-competitive. Their explanation of this non-competitive behaviour was basically the same as given above. They envisaged a water-binding site (an old idea[200] that has been neglected because of lack of

evidence) from where the water molecule may attack the activated, transition-state complex.

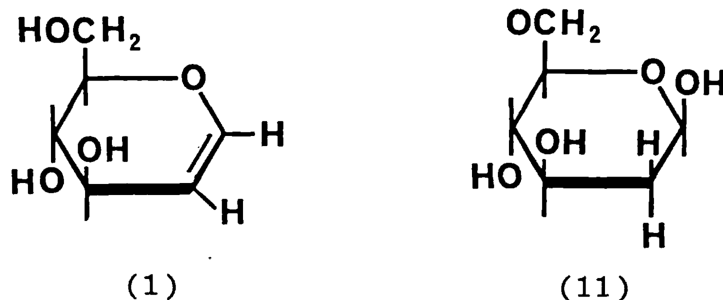
If D-glucono-1,5-lactone is bound near the water site and the formation of an ES complex is not affected by this binding, an EIS complex may form, thus either attack by the water molecule or distortion corresponding to the transition state is hindered.

The different forms of inhibition exhibited by maltobiono-1,5-lactone towards wheat and porcine pancreatic α-amylases was a surprising result, but in fact there is an interesting parallel involving the α-1,4-glucan (starch and glycogen) phosphorylases. The animal (e.g. rabbit muscle) and plant (e.g. potato) enzymes show quite different regulatory behaviour and substrate preferences. Thus rabbit muscle phosphorylase, in both its forms (a and b) is allosterically activated by adenosine monophosphate, whereas the potato phosphorylase is not[201]; and mammalian phosphorylases show a preference for (1,6) branched substrates whereas the potato enzyme shows no such preference.[202]

3.2.3. Inhibition of Wheat α-Amylase by Maltal.

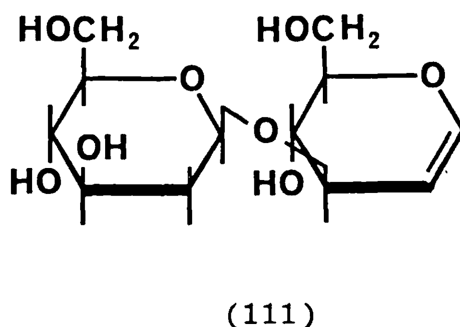
The interaction of glycals with their corresponding glycosidase was first noted by Lee[203], who observed that D-galactal is a powerful and highly specific inhibitor of

β-galactosidases. He showed that D-glucal (1) is specifically hydrated to 2-deoxy-D-glucose (11) by sweet almond β-glucosidase, but



(1) (11)

that it is not attacked by yeast α-glucosidase (maltase). More recent work[204] however has shown that this is not generally the case and that in fact both α-and β-glucosidases will attack the same substrate, D-glucal (which lacks α or β anomeric configuration) and to convert it to 2-deoxy-α(or β)-D-glucose respectively, thus creating anomeric configuration de novo. Lehmann and Schroter[205] have also reported the formation of a disaccharide (111) as a by-product,



(111)

in appreciable quantity, during the enzymic reaction. They envisage it as being produced by the transfer of an allylic D-pseudoglucal

cation to HO-1 of 2-deoxy-D-arabino-hexopyranose (aglycon transfer), which implies the presence of a specific binding-site for the aglycon moiety, having at least two functional groups (A-H and B) situated on either side of the aglyconic substrate (D-glucal). This ensures a conformation of the aglyconic substrate in which HO-3 and HO-4 are anti. An alternative and simpler explanation of the formation of (111), is transfer of a 2-deoxy-D-arabinohexopyranosyl cation to HO-3 of 2-deoxy-D-arabinohexopyranose. This hydration of glycals is interpreted by Hehre[206] by regarding the (π) bond between C1 and C2, which is attacked by glycosidases, as meeting the definition of a glycosylic bond i.e. the union joining the anomeric carbon atom of the cyclical form of a sugar to the anomeric hydroxyl or to any group replacing this hydroxyl. A new view of the glycals as internal C-glycosyl compounds is then obtained, D-glucal, for example, was visualised as an internal 2-deoxy-D-glucosyl compound.[207]

The exact mechanism for the inhibition is not known. Most authors regard the glycals as inhibiting by reacting with an enzyme active site carboxyl, or other group, to form covalent derivatives. It has been shown[204] that the catalytic activity of *Candida* α-glucosidase towards D-glucal is strongly inhibited by excess

substrate. Kinetic analysis shows that this is dead-end inhibition, due to the formation of complexes of enzyme with two D-glucal molecules, whose apparent equilibrium constants (Km = 14.2 mM; Kss = 20.9 mM) have an exceptionally close relationship - Kss/Km = 1.48 (cf. Bray[208]).

As with maltobiono-δ-lactone, maltal was to be tested at pH 5.0. The first experiment was to investigate the effect of pre-incubating the inhibitor (9.54 mM) with the enzyme (0.14U) { total volume of solution - 1 cm^3 } under the test conditions (pH 5.0, 0.1M acetate buffer, $30^oC \pm 0.1$). After the set pre-incubation time the substrate (1 cm^3, 0.5% at 30°) was added (to give final concentrations of {I} = 4.77 mM, {S} = 0.25%) and a sample (0.5cm^3) taken after 4 mins and analysed for reducing sugars by the alkaline ferricyanide method as before (Table 3.5, Fig. 3.5).

Incubation Time (Mins)	ΔA_{420}	Inhibition (%)
0	0.535	0
15	0.495	7.4
30	0.470	12.1
45	0.445	16.8
60	0.360	32.7
120	0.340	36.4

Table 3.5 Effect of pre-incubating maltal with wheat alpha-amylase.

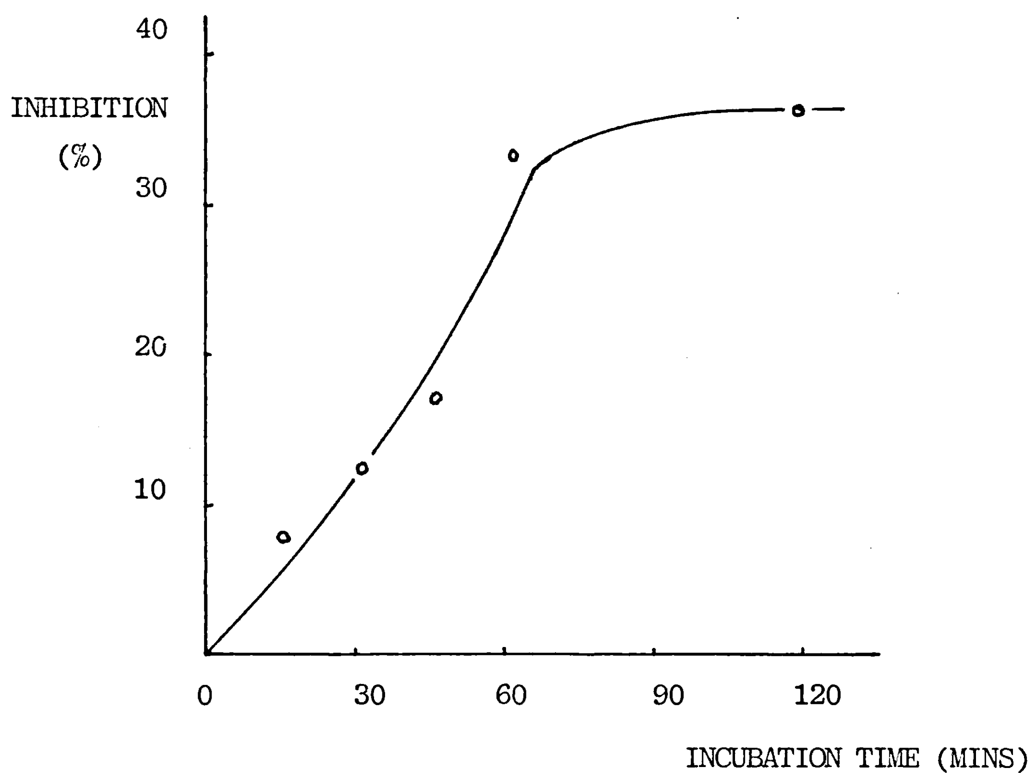


Fig.3.5 Plot of inhibition versus time, for the pre-incubation of maltal with wheat α-amylase.

From these results, a pre-incubation time of 60 mins (for the enzyme plus inhibitor prior to mixing with substrate) was chosen for the inhibition studies, which were otherwise carried out using essentially the same technique as for the lactone. A Dixon plot of 1/V versus I showed the inhibition to be competitive with a Ki of 4.4 mM (Table 3.6, Fig.3.6).

{I} (mM)	ΔA_{420}	Maltose (μg)	V x 10^{-2} (μmol/cm^3/min	1/V
A. {S}	= 0.5%			
0	0.631	93.34	12.95	7.72
1.91	0.560	82.84	11.50	8.70
4.77	0.477	70.56	9.79	10.21
9.50	0.380	56.21	7.80	12.82
B. {S}	= 0.25%			
0	0.535	79.14	10.98	9.11
1.91	0.471	69.67	9.67	10.34
4.77	0.360	53.25	7.39	13.53
9.50	0.285	42.16	5.85	17.09

Table 3.6. Inhibition of wheat α-amylase by maltal; pH 5.0, 0.1M acetate buffer, $30^{o} \pm 0.1$

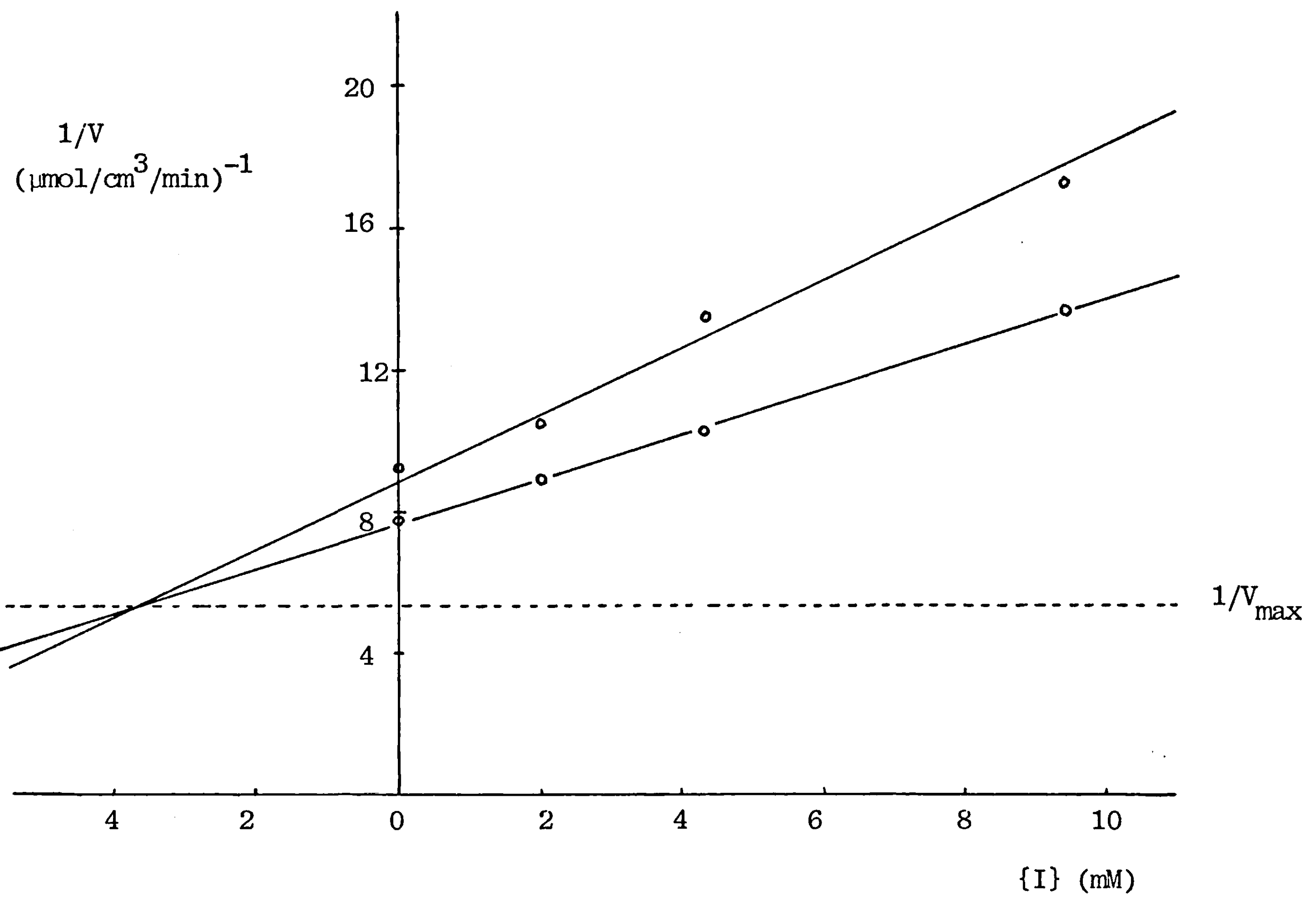


Fig.3.6. Dixon plot for the inhibition of wheat α-amylase by maltal.

A further experiment was carried out to investigate the reversibility of the inhibition by maltal. A sample (EI) of the enzyme (0.14U) was pre-incubated with maltal (9.4 mM) for 120 mins then substrate added (to give a total volume of 2 cm^3 and a substrate concentration of 1.75%). After 5 mins a sample (200 μl) was taken and analysed for reducing sugars. A second sample (E) of the enzyme (0.14U) was similarly treated, but with no inhibitor. The solutions were left at room temperature for 24 hr. Two 3 cm^3 P10 columns were then prepared and centrifuged to remove solvent. On top of one column was placed enzyme solution (E, 1 cm^3) and on to the other enzyme plus inhibitor solution (EI,1 cm^3). The columns were again centrifuged and the solutions (now free of oligosaccharides) collected and used for a further assay. Each sample was pre-incubated at 30° for 10 mins then mixed with substrate (to give a total volume of 2 cm^3 and a substrate concentration of 1%). In order to correct for any zero time blank, the maltose production between 5 and 10 mins was estimated by removing a sample (0.5 cm^3) at each time and analysing for reducing sugars. As can be seen from the results (Table 3.7), only 12% of the enzyme activity was recovered by the passage

through the P10 molecular sieve column.

Sample	ΔA_{420}	Maltose (μg)	v x 10^{-2} (μmol/cm^3/min	Relative Activity
E	0.415	61.39	17.04	1
EI	0.189	27.96	7.76	0.46
After 24 hours				
E	0.057	8.43	1.17	1
EI	0.033	4.88	0.68	0.58

Table 3.7 Reversibility study of the inhibition of wheat α-amylase by maltal.

It is obvious that a lot more work must be carried out on the inhibition of wheat α-amylase by maltal before any definitive answer to its mode of action can be given. Some conclusions can however be drawn from the results obtained so far. As has been shown, under the conditions of these experiments,

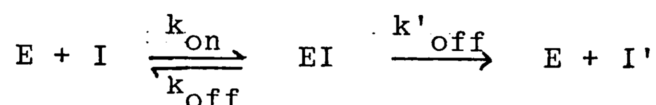
(1) enzyme inhibition is kinetically slow;

(2) inhibition approaches an equilibrium value of about 40%;

(3) reversal of enzyme inhibition is slow;

(4) at equilibrium inhibition is kinetically competitive.

All this suggests that maltal is a slow binding inhibitor[209] of α-amylase (Scheme 3.1)



Scheme 3.1

In this case k_{on} is very small and so is k_{off} (or k'_{off}), but there must be an off rate or the inhibition would reach 100% with sufficient (1 active site equivalent) inhibitor. The picture is very similar therefore to the result for the inhibition of bacterial β-galactosidase by D-galactal[210], shown by Wentworth and Wolfenden. They found the inhibition to be reversible and time dependent, with observed rate constants for binding and release as respecitively 2.7×10^2 s^{-1} M^{-1} and 4.6×10^{-3} s^{-1}. They concluded that the slow rate of inhibitor binding and release was because the process involved a chemical reaction (with appropriate energy barriers) with the enzyme, possibly the formation and breakdown of a covalent 2-deoxygalactosyl-galactosidase intermediate. They envisaged a reaction (hydration) to product, 2-deoxy-β-D-galactose (k'_{off}) and release to original form (k_{off}). In this work, no reaction to a new product could be observed but it is assumed that there is a slow formation of a covalent intermediate, followed by breakdown in

an as yet unknown pathway.

A recent review by Morrison[209] has shown that this type of slow-binding inhibition is in fact a fairly common occurrence. He does not however distinguish between covalent and non-covalent binding interactions. Examples such as galactal,[210] glucal[204] and clavulanic acid[211] have a centre available for covalent binding, but deoxynojirimycin reported by Semenza et al[212] to be a slow-binding inhibitor of sucrase cannot react in this way. Inhibition in this case is possibly caused by a slow enzyme conformational change (analogous to that associated with the formation of the transition state in enzymic catalysis) the mechanism of which is at present unknown. Slow-binding inhibitors may be of particular value in X-ray crystallographic studies of enzymes; if enzyme inhibitor complexes can be crystallised they could reveal active site location and conformation information which is presently inaccessible because complexes with conventional inhibitors do not adopt 'active' conformations e.g. rabbit muscle phosphorylase, hexokinase.

3.2.4. Inhibition of Wheat α-Amylase by N-β-D-Glucosylbenzylamine and N-β-D-Glucosylpiperdine.

That nitrogen containing sugar derivatives inhibit glycosidases has been demonstrated

consistently in the literature. Glycosylamines[213] have been reported to constitute a class of specific (i.e. glucosylamine inhibits glucosidase but not galactosidase, while galactosylamine inhibits galactosidase but not glucosidase), and relatively potent, inhibitors of glycosidases. When acting on β-D-glucosidase from yeast, D-glucosylamine has a Ki of 2.2 μM making it one of the most potent, competitive glycosidase inhibitors reported.[213] It was suggested in this paper that glycosylamines owe their striking specificity and effectiveness to the possibility that they have a double attraction for the enzyme. They have an amino group,which when protonated interacts electrostatically with the essential anionic site of the enzyme, and they also possess a glycosyl moiety which is specifically adapted to the substrate binding region of the enzyme. It was assumed that the normal fit of the glycosyl residue directed the amino group precisely into position for the electrostatic interaction.

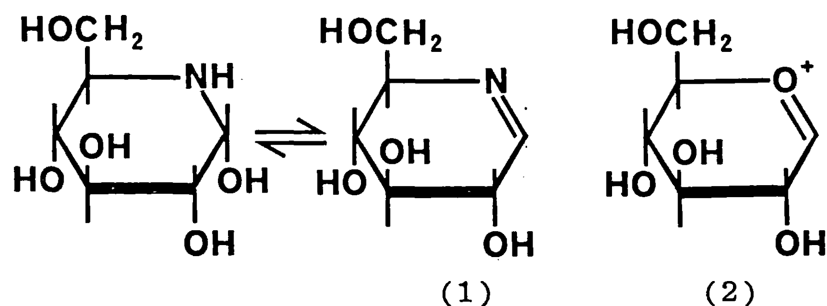
Legler[214] similarly tested substituted glucosylamines against sweet or bitter almond β-glucosidase. Two compounds in particular showed exceptional inhibitory properties; N-β-D-glucosylpiperidine, Ki = 3.5μM and N-β-D-glucosylbenzylamine, Ki = 0.26μM.(cf. From the same paper, β-glucosylamine has

Ki = 2.0mM). This was the reason for their synthesis as potential inhibitors of α-amylase.

Some work has already been done in this area, with the attempted inhibition of amylases by glycosylamines. It has been shown that glucosylamine does not significantly inhibit β-amylase[215] (an exo-enzyme, which catalyses the release of β-maltose[216], from its natural substrate, α-1,4-glucan, hence acting with inversion[217]). Maltosylamine, however, did inhibit this enzyme.[218] It was shown to be uncompetitive in nature, with a concentration of approximately 3×10^{-4}M maltosylamine causing 50% inhibition, when a 0.5% starch solution was used as substrate. The paper suggests that the substrate binds first, possibly causing a conformational change in structure, before the inhibitor can bind. When tested against pancreatic α-amylase and _B.subtilis_ α-amylase (dextrinising), however, little inhibition was noted (2.0mM maltosylamine caused 16% inhibition of _B.subtilis_ enzyme).

Another class of inhibitor, are sugar derivatives with a different heteroatom from oxygen within the ring. It has been shown[219] that D-xylose derivatives with sulphur or nitrogen within the ring act as inhibitors of some glycosidases. Similarly, the antibiotic nojirimycin

which differs from D-glucose only by the substitution of an NH group for oxygen in the ring, has been reported[97,194] as a very potent inhibitor of glycosidases, e.g. it showed competitive inhibition against the apricot emulsin enzyme, with Ki = 5.5μM. It has been suggested that nojirimycin is effective because it forms on imine (1) by elimination of water between N and C1, thus resembling the glucosyl cation (2) in its geometry (Scheme 3.2).



Scheme 3.2

The pKa's of nojirimycin and the imine are 8.5 and 3.5-4 respectively, while the pKa in the bulk phase is approximately 5.3.If the conjugate acid is an inhibitory species therefore, it must be that of the amine form. Amylases are however, little affected by this drug.[220]

Both N-β-D-glucosylpiperidine and N-β-D glucosylbenzylamine (the compounds to be tested in this work) have been reported as very prone to acid catalysed hydrolysis[221,222] and Legler[214] in his paper, specifically stated that'N-benzyl-β-glucosylamine could not be used at pH 5 or below

due to the acid catalysed decomposition of the inhibitor, which caused a considerable rate increase after 1-2 min.' Both compounds were therefore tested at pH 6.0 (0.1M acetate buffer) whilst all other conditions remained as before. By extrapolation from the work of Palm[221] to the test conditions, the following rate constants for hydrolysis are obtained: N-β-D-glucosylpiperidine, $k = 8.0 \times 10^{-4}\ s^{-1}$, i.e. $t_{\frac{1}{2}} = 14.5$ min ; N-β-D-glucosylbenzylamine, $k = 2.7 \times 10^{-3}\ s^{-1}$, i.e. $t_{\frac{1}{2}} = 4.25$ min. The amount of hydrolysis will be small for β-D-glucosylpiperidine, but for β-D-glucosylbenzylamine the concentration of inhibitor after 4 mins will only be approximately 50% of that introduced. The reducing sugar assay could not be used in these tests because of a large blank, so the reaction was monitored by the iodine stain method.[223]

The first experiment carried out was a calibration experiment to correlate iodine staining power with production of maltose at the substrate concentrations required for the inhibition runs (Tables 3.8, 3.9, Fig. 3.7).

Time (Mins)	Iodine Stain A_{550}	Time (Mins)	Reducing Sugar ΔA_{420}	Maltose (μg)
1	0.322	2	0.069	10.21
2	0.267	4	0.133	19.67
4	0.185	8	0.252	37.28
5	0.158	10	0.285	42.16
7	0.116	15	0.304	44.97
10	0.086			
15	0.058			

Table 3.8 Correlation between iodine stain and maltose production in α-amylolysis at a substrate concentration of 0.25%.

Time (Mins)	Iodine Stain A_{550}	Time (Mins)	Reducing Sugar ΔA_{420}	Maltose (μg)
1	0.678	2	0.084	12.43
2	0.599	4	0.164	24.26
3	0.552	6	0.244	36.09
4	0.498	8	0.309	45.71
5	0.432	10	0.364	53.85
7	0.348	12	0.401	59.32
10	0.257	15	0.487	72.04
15	0.168			

Table 3.9 Correlation between iodine stain and maltose production in α-amylolysis at a substrate concentration of 0.50%.

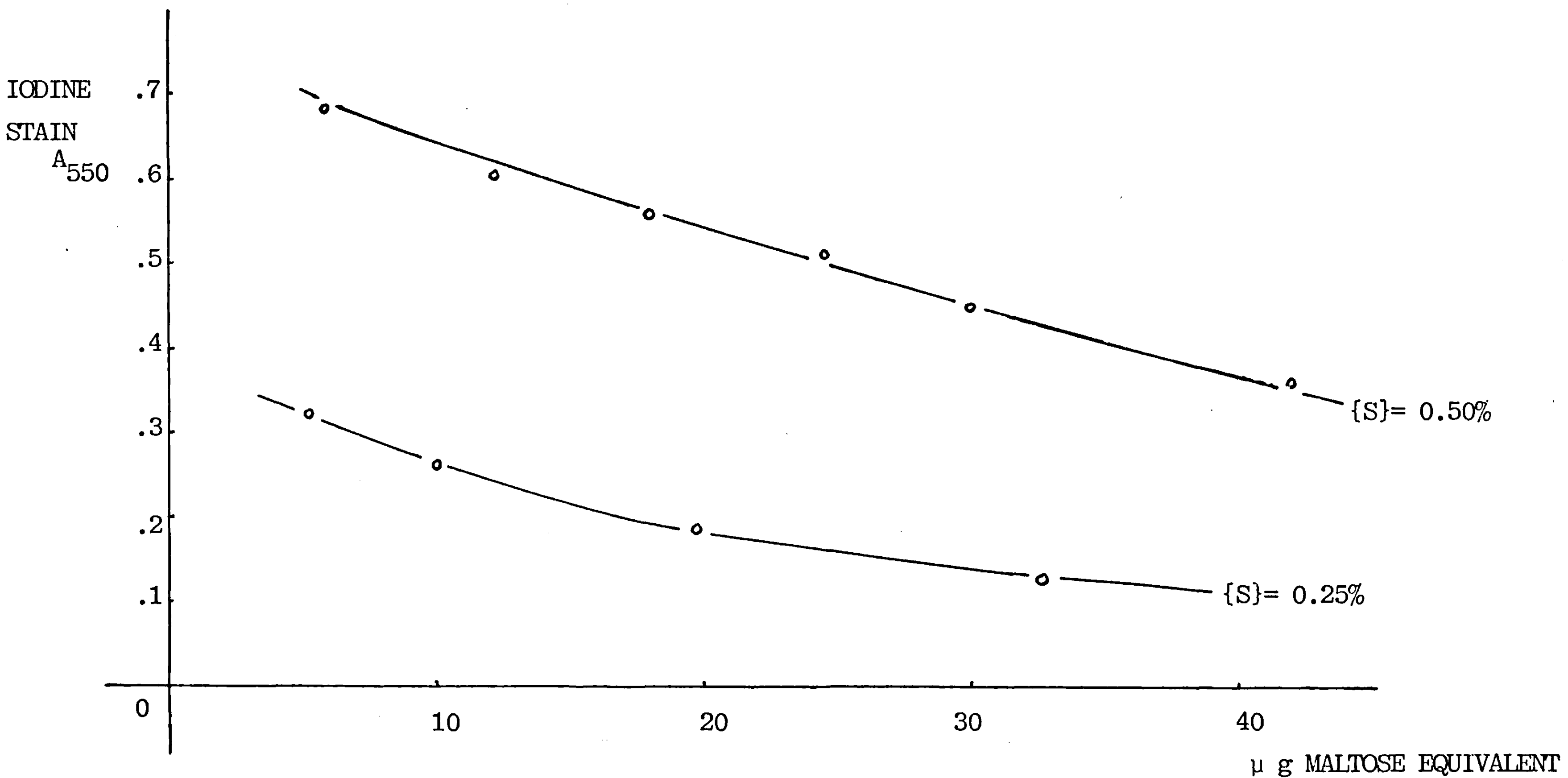


Fig. 3.7. Correlation between iodine stain and maltose production in α-amylolysis at given substrate concentrations.

The next experiments were inhibition studies at pH 6.0. As with the lactone, N-β-D-glucosylbenzylamine and N-β-D-glucosylpiperidine were dissolved in an inert solvent, namely freshly distilled trimethylphosphate, just prior to use and the reaction times were kept as short as possible (4 min.). Dixon Plots of the results showed both compounds to be moderate inhibitors of wheat α-amylase and to display complex behaviour giving curved Dixon Plots. Extrapolation of the linear, initial regions of the Dixon Plots gave for N-β-D-glucosylpiperidine, Ki = 4.9mM (Table 3.10, Fig.3.8) and for N-β-D-glucosylbenzylamine, Ki = 3.0 mM, 1.5 mM with correction (Table 3.11, Fig.3.9). The potency of these inhibitors thus resembles that of typical substrate analogues, rather than transition state analogues.

{I} (mM)	Iodine Stain A_{550}	Maltose (μg)	V x 10^{-2} (μmol/cm^3/min	1/V
A. {S}	= 0.5%			
0	0.536	26.36	6.10	16.39
2.5	0.560	23.58	5.48	18.35
5.0	0.618	17.38	4.02	24.88
7.5	0.662	13.10	3.03	33.00
10.0	0.632	15.98	3.70	27.03
B. {S}	= 0.25%			
0	0.260	20.31	4.70	21.28
2.5	0.266	16.23	3.75	26.67
5.0	0.302	13.59	3.18	31.85
7.5	0.298	10.63	2.46	40.65
10.0	0.350	7.80	1.80	55.56

Table 3.10. Inhibition of wheat α-amylase by N-β-D-glucosylpiperidine ; pH 6.0, 0.1M acetate buffer, $30^{o} \pm 0.1$.

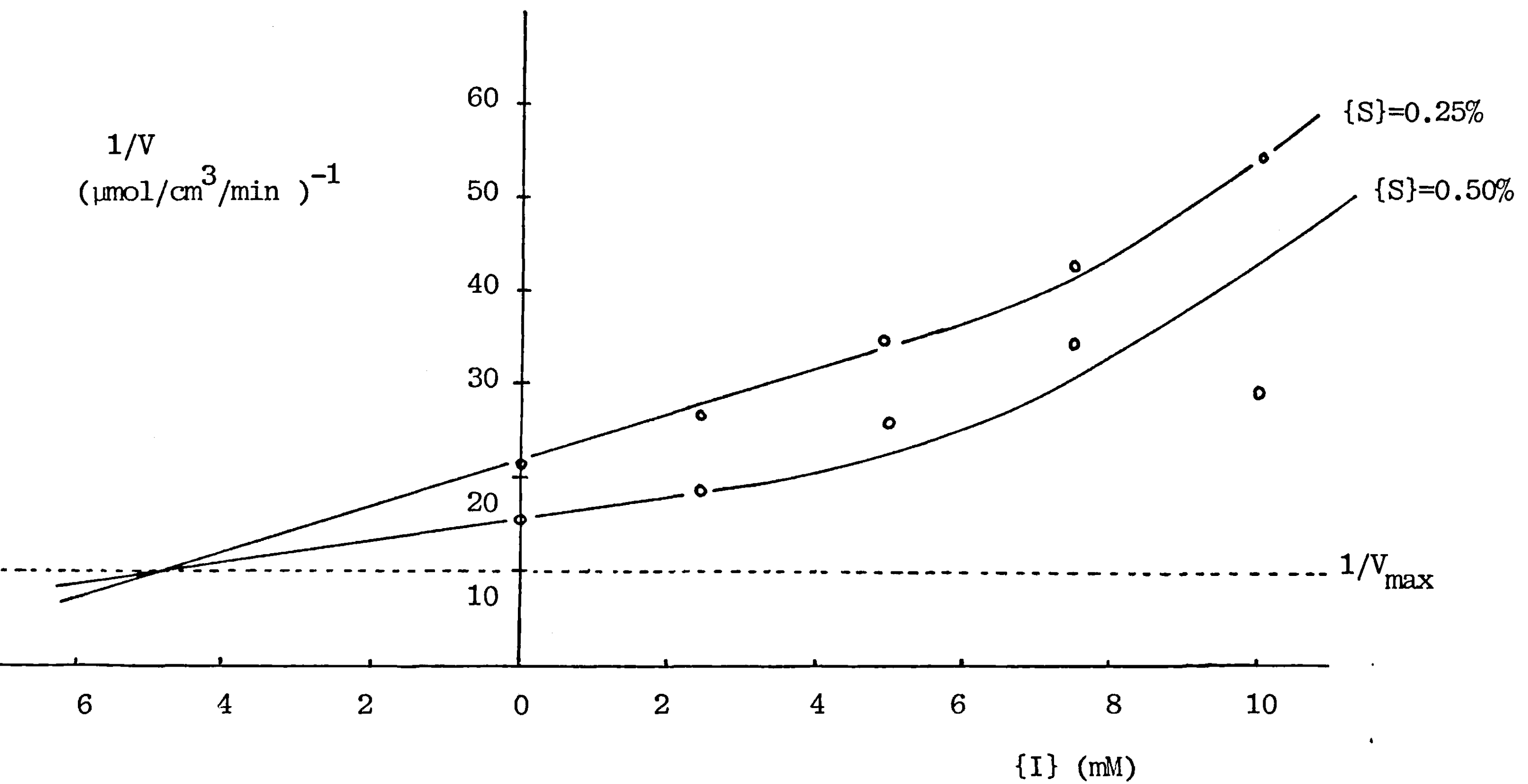


Fig.3.8. Dixon plot for the inhibition of wheat α-amylase by N-β-D-glucosylpiperidine.

{I} (mM)	Iodine Stain A_{550}	Maltose (μg)	V x 10^{-2} (μmol/cm^3/min	1/V
A. {S}	= 0.5%			
0	0.534	26.60	6.15	16.26
2.5	0.550	24.72	5.72	17.48
5.0	0.563	23.24	5.37	18.62
7.5	0.595	19.65	4.54	22.03
10.0	0.626	16.58	3.83	26.11
B. {S}	= 0.25%			
0	0.253	20.31	4.70	21.28
2.5	0.278	16.23	3.75	26.67
5.0	0.296	13.59	3.14	31.85
7.5	0.318	10.63	2.46	40.65
10.0	0.341	7.80	1.80	55.56

Table 3.11. Inhibition of wheat α-amylase by N-β-D-glucosylbenzylamine ; pH 6.0, 0.1M acetate buffer, $30^{o} \pm 0.1$

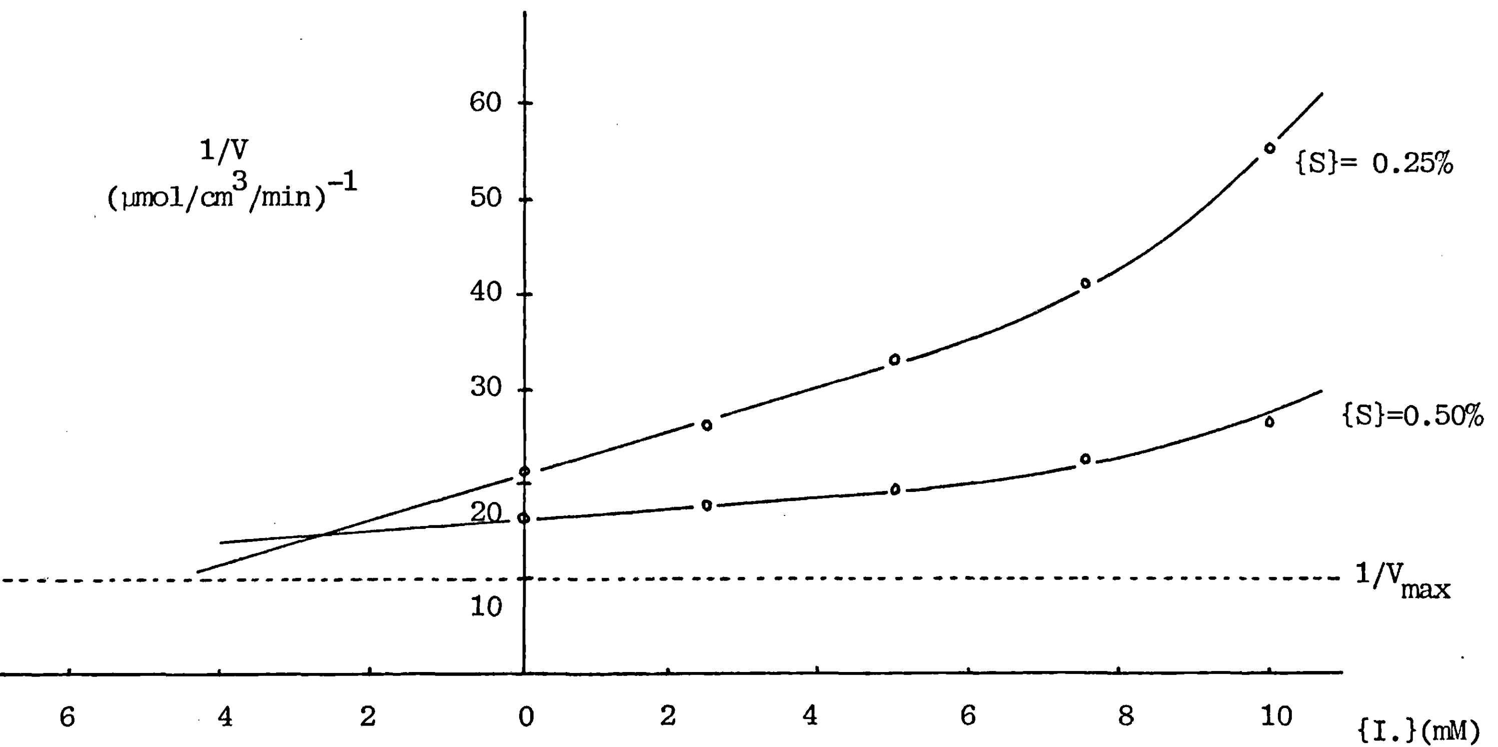


Fig. 3.9. Dixon plot for the inhibition of wheat α-amylase by N-β-D-glucosylbenzylamine

3.3. General Experimental Techniques.

Reducing sugars were assayed by the alkaline ferricyanide method.[224] Two stock solutions were first made:

Solution 1: 0.5 g Potassium cyanide in 100 cm^3 water

Solution 2: 0.1 g Potassium ferricyanide + 5.0 g sodium carbonate in 250 cm^3 water.

Solution 1 (2 cm^3) and solution 2 (3 cm^3) were mixed just prior to use, then the sample (0.5 cm^3) containing 10-100μg reducing sugars was added. The colour developed on heating at 100° for 15 mins was read at 420 nm. The colour was found to be stable at room temperature for at least 6 hr. The test was calibrated for maltose monohydrate (Table 3.12) 100μg giving a colour change (ΔA_{420}) of 0.676

μg Maltose	A_{420}
0	0.675
20	0.547
50	0.331
70	0.186
100	0.000
Int	0.676
Slope	-0.684×10^{-2}
L.R.	0.9995

Table 3.12. Standardisation of reducing sugar test for maltose monohydrate.

Wheat amylopectin was assayed for by an iodine staining method.[223] The stock solution consisted of 0.1% of iodine in 1% potassium iodide. A sample of the enzyme digests (0.2 cm^3) was added to M hydrochloric acid (0.2 cm^3) to stop the reaction, followed by standard iodine reagent (0.2 cm^3) and water (4 cm^3). The colour which developed was read at 550 nm.

Protein was initially detected by measurement of absorbance at 280 nm, then quantitatively determined by the Bio-Rad protein assay at 595 nm. The dye reagent concentrate provided (20 cm^3) was diluted with water (80 cm^3), then filtered through Whatman No.1 filter paper. The standard protein solution was made using lysozyme (1.4 mg/cm^3, supplied by Sigma, Poole, Dorset) in a physiologically buffered saline solution made as follows: Potassium dihydrogen phosphate (6.8 g) was dissolved in water (approximately 60 cm^3), sodium chloride (8.76 g) was added, then the pH was adjusted to 7.2 with concentrated potassium hydroxide solution and the whole diluted to 100 cm^3. To a series of protein standards (0 -70μg) was added the diluted dye reagent (5 cm^3) and the samples vortexed (avoid excessive frothing). After 30 mins the colour which developed was measured at 550 nm.(Table 3.13).

µg Protein	A_{595}
0	0.000
14	0.052
42	0.176
70	0.279
Int	-0.508×10^{-3}
Slope	0.404×10^{-2}
L.R.	0.9992

Table 3.13. Standardisation of protein assay with lysozyme.

Lactone concentration was determined by the method of Jencks.[198] The solution of the lactone (0.2 cm^3, 0.01-0.02M) was added to a 1:1 mix of hydroxylamine hydrochloride and 3.5M sodium hydroxide (0.2 cm^3). After 5 mins a solution of 0.46M ferric chloride in 0.48M hydrochloric acid (0.8 cm^3) was added and water (4 cm^3). The colour which developed was read at 540 nm after a further 5 mins.

All kinetic experiments were carried out at $30^o \pm 0.1^o$ in a thermostatically controlled water bath. The thermostat was a 'Julabo' Paratherm II Electronic, supplied by Scienco Western Ltd., Trowbridge, Wiltshire.

The buffer system used for enzyme assays was 0.1M acetic acid/sodium hydroxide (pH 5 or 6)

with calcium chloride (1 g/dm^3) and human serum albumin (1 mg/dm^3). The stock solution of substrate was a 2% (w/v) solution of wheat amylopectin made up in buffer. The general technique used was as follows: 2 test tubes (3" x ½"); one containing substrate (0-1 cm^3) + buffer (0-1 cm^3) {total 1 cm^3} and the other enzyme (0.14U) + inhibitor + buffer {total 1 cm^3} were incubated for 1 min then gently mixed {final volume 2 cm^3}. A sample (0.5 cm^3) was than taken after 4 mins and analysed for reducing sugars, or a sample (0.2 cm^3) was taken and analysed for wheat amylopectin.

Other buffer systems are as described in the text. Analar reagents were used throughout. Gel-filtration was carried out using Bio-gel P10 (minus 400 mesh), supplied by Bio-Rad Laboratories Ltd., Bromley, Kent.

Preparation of P10 column.

a) Packing the column.

The Bio-gel P10 was first swollen, then deaerated by means of a water pump until evolution of bubbles ceased. The column to be packed was first filled with eluant, until the dead space was taken up, and then the gel slurry was poured down the edge of the column until filled with gel. The column was then allowed to flow at a rate dictated by the working pressure. When very slow

flow rates were required a peristaltic pump was used for packing the column. Equilibration was carried out by letting the column flow for at least two column volumes.

b) Determination of the void volume (Vo) and the total volume (Vt) of the column packed with gel filtration media.

Blue dextran (2 mg/cm^3, Pharmacia) and glucose (5 mg/cm^3) were dissolved in elution buffer and applied to the column. The column was eluted with buffer at the desired flow rate and fractions collected. Glucose was determined by the phenol/sulphuric method. From the elution volumes (Ve) of the samples, Vo and Vt could be found where:

Ve dextran blue = Vo (interstitial volume between the gel granules).

Ve glucose = Vt (total volume of the packed column).

Samples of Champlein wheat, malt and flour were provided by Rank Hovis McDougall Ltd., High Wycombe, Buckinghamshire.

Wheat amylopectin was prepared in this department by P.A. Leonard, following the method of Gilbert et al.[225]

All other experimental techniques were as described in Chapter 2.

3.4 Experimental

3.4.1. Extraction of Wheat Alpha-Amylase.

The α-amylase activity of Champlein wheat flour and malted wheat was first checked, following the extraction method of Macgregor.[226] Malted wheat kernels (20, 0.61 g) were homogenised in a mortar and pestle with acetate buffer (5 cm^3, 0.2M, pH 5.5, containing 0.001M calcium chloride). The extract was centrifuged and the supernatant used for the activity tests. The wheat flour was similarly treated. At pH 5.0 the malted wheat extract showed reasonable activity, the flour very little. The main extraction was therefore carried out on the malted wheat, following the method of Kruger.[185]

The remainder of the malted wheat kernels (about 45 g) were ground in a mortar and pestle. The husks were removed using a 1mm mesh sieve leaving the flour (28.5 g), which was added slowly, with stirring, to a 0.2% solution of calcium acetate (54 cm^3) at room temperature over 15 mins. Stirring was continued a further 2 hr after the addition was completed. The suspension remaining was centrifuged (4°, 9500 rpm) for 20 mins. The dark brown supernatant was adjusted to pH 6.7 with a cold solution of 4% ammonium hydroxide. The stirred extract was heated rapidly to 69° in a water bath maintained at about 85°, then transferred

to a water bath at 72 - 74°. The extract was maintained at 70° for 15 mins, rapidly cooled to 2-3° and acetone (4.0 cm^3) at -10° added dropwise with stirring (i.e. 10-15% v/v of extract volume - 36 cm^3). The temperature was reduced to -5° and more acetone (19 cm^3) at -10° added dropwise (39% v/v). The mixture was allowed to stand for 20 mins then centrifuged (-5°, 9500 rpm) for 10 mins. To the supernatant was added more acetone (21.7 cm^3) at -5° (54% v/v) and after standing 20 mins, centrifuged as before. This time the supernatant was discarded and the precipitate dissolved in Tris-HCl buffer (2.8 cm^3, 5mM, pH 8.0).

The extract was cooled to 2° and a solution of glycogen (40 µl of stock solution - 22.9 mg of oyster glycogen in Tris-HCl buffer, 0.35 cm^3, 5 mM, pH 8.0) also at 2°, added dropwise with stirring. The solution was stirred for a further 10 mins, during which time it became cloudy, then was centrifuged (2°, 4000 rpm) for 10 mins. The supernatant was decanted leaving a brownish residue, which was dissolved in Tris-HCl buffer (0.3 cm^3, 5mM, pH 6.7, containing 5 mM calcium chloride). The supernatant was cooled to 2° and the glycogen precipitation repeated as before. The slight film obtained on centrifugation was dissolved in Tris-HCl buffer (0.25 cm^3) as above, combined with the first solution and left at 20°

overnight for the enzyme to completely digest the glycogen.

Once the digestion of the glycogen was complete, the extract was centrifuged to remove a slight precipitation which had formed, then passed down a Bio-Gel P10 column eluted with a solution of 0.1M sodium chloride, 0.5mM calcium chloride at 4°. The column used was a Pharmacia K15/90 with a bed volume of 154 cm^{3}. The column was run at a flow rate of 10 cm^{3}/hr, collecting fractions of 3 cm^{3}. Protein was detected by measurement of absorbance at 280 nm and carbohydrate by the phenol/sulphuric method.

3.4.2. Inhibition of Wheat α-Amylase by Maltobiono-δ-lactone.

Two tubes (3" x ½"), one containing substrate ($1cm^{3}$) the other enzyme + inhibitor (total 1 cm^{3}) were pre-incubated at 30° for 1 min, then mixed. After a further 4 mins, a sample (0.5 cm^{3}) was taken and added to the ferricyanide solution to stop the reaction. The reagent was placed in a boiling water bath for 15 mins, allowed to cool to room temperature and the colour read at 420 nm. The difference between this reading and one where the enzyme had been denatured prior to reaction was then used to obtain the rate of reaction.

3.4.3. Inhibition of Wheat α-Amylase by Maltal.

Except for using a pre-incubation time of 60 mins, the inhibition studies were carried out using exactly the same technique as for maltobiono-σ-lactone (see Section 3.4.2.).

3.4.4. Inhibition of Wheat α-Amylase by N-β-D-Glucosylbenzylamine and N-β-D-Glucosylpiperidine.

Two tubes (3" x ½"), one containing substrate (1 cm^3) the other enzyme and inhibitor (total 1 cm^3) were pre-incubated at 30° for 1 min, then mixed. After a further 3 mins, a sample (0.2 cm^3) was taken and added to 1M hydrochloric acid (0.2 cm^3) to stop the reaction, followed by standard iodine reagent (0.2 cm^3, see Section 3.3.) and water (4.0 cm^3). The colour which developed was read at 550 nm and this result converted to μg maltose produced. The difference between this reading and one where the enzyme had been denatured prior to reaction was then used to obtain the rate of reaction.

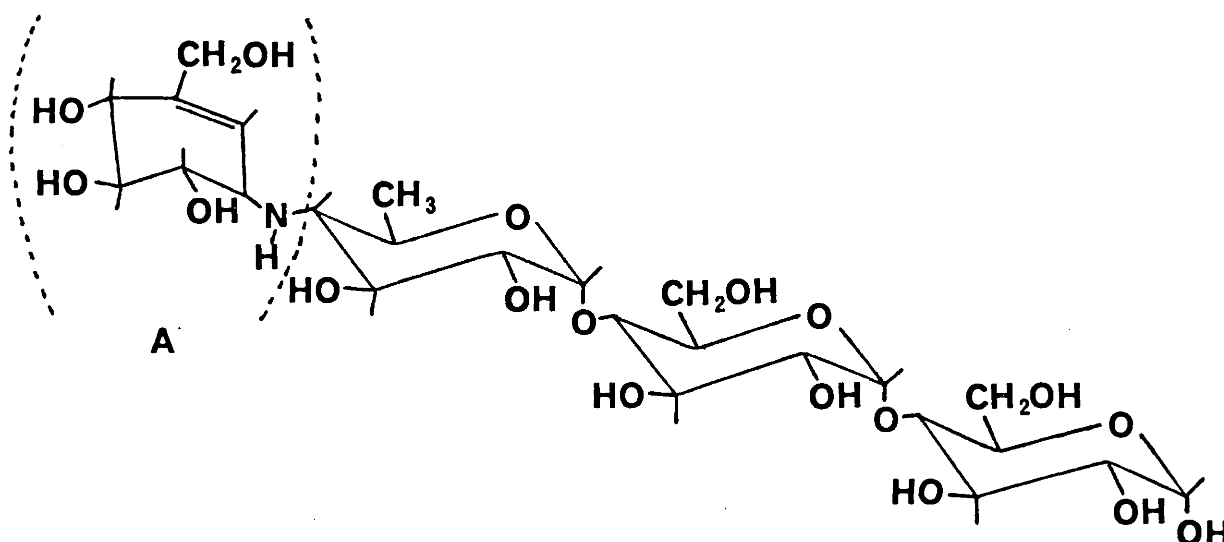
CHAPTER 4

GENERAL CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK.

Four of the six compounds listed as targets for synthesis (see Section 1.6) have been prepared successfully and all shown to be inhibitors of wheat α-amylase. Two of these, maltobiono-δ-lactone and 4-O-(α-D-glucopyranosyl)-D-glucal ('maltal') being representative as models for the glycosyl oxocarbonium ion and the other two, N-D-glucosylbenzylamine and N-D-glucosylpiperidine (both crystallised as the β-form) being representative as models for the maltose-4-oxonium ion. The hoped for series of models for the sugar residue, at which glycosyl cleavage occurs, as undistorted and charged {O-(α-D-glucopyranosyl)-4-hydroxypiperidine, A2 }, distorted and charged {O-α-D-glucopyranosyl)-4-hydroxypiperidine, A3 }, distorted and uncharged {4-O-(α-D-glucopyranosyl)-D-glucal, A4 } could not be achieved in this work because of the difficulty in synthesising the α-glycosides, A2 and A3. More effort could therefore be put into producing these two compounds. The best route would probably be via the imidate route as devised by Sinay. As was stated in Chapter 1, these compounds could theoretically enable a distinction to be made between distortion and charge contributions to binding.

Other target compounds which should be synthesised, could be based upon 'Acarbose' (1)

{ BAY g 5421 } shown by Dr.E.Truscheit and co-workers at the Bayer Co.[227] to be an inhibitor of intestinal α-glucosidases and of pancreatic α-amylase both _in vitro_ and _in vivo_.



(1)

Interestingly, this natural compound incorporates in a single molecule the two features viz ring unsaturation and (pseudo) glycosidic basic nitrogen which were designed features arrived at in this work. If the 'active' unit is assumed to be the portion shown in brackets and designated A, then the position of this unit within a small chain of glucose units could be varied to see if there is any change in inhibition.

Simpler molecules worthy of effort are substituted hydroxypiperidine derivatives, which could again be included in a short glucose chain in an effort to increase binding and hence inhibitory activity.

The compounds that were made and examined in this work tended to produce more questions than supply answers. Maltobiono-δ-lactone was shown to be a non-competitive inhibitor of wheat α-amylase, an unexpected result. Whether this is true for glucono-δ-lactone or amylases from different sources could be tested and also the possibility of increasing inhibition by increasing the chain length of the lactone e.g. the maltotriose analogue. This may improve binding at the enzyme active site, which can accommodate 8-10 glucose units.

Maltal in particular will require considerably more detailed examination in order to fully understand its mode of action. The initial stages of the reaction, as the inhibitor binds to the enzyme and the system equilibrates must be followed continuously. A turbidimetric assay, or the use of a chromophoric substrate are probably the best approaches to this. The possibility of an 'off' reaction with chemical conversion of the inhibitor should be carefully investigated. This could be done by standard chromatographic techniques, isotopic labelling or possibly by NMR of the reaction mixture. Once the full details of this system have been determined other similar compounds could be examined e.g. with the double bond in other positions or oligosacc(h)arides of different chain length.

The two glucosylamines synthesised were both shown to be simple competitive inhibitors of wheat α-amylase. Further work with this type of compound could follow the lines already stated i.e. as for 'Acarbose', with the nitrogen as a linking atom within a small oligosaccharide or with the nitrogen incorporated in a ring to give substituted piperidine derivatives.

REFERENCES

1. Scrimgeour K.G., 'Chemistry and Control of Enzyme Reactions', Academic Press, London (1977).

2. Michaelis L. and Menten M.L., Biochem.Z., 49, 333 (1913).

3. Sumner J.B., J.Biol.Chem., 69, 435 (1926).

4. Northrup J.H., Kunitz M. and Herriott R.M., 'Crystalline Enzymes', 2nd.edn., Columbia University Press, New York (1948).

5. Gutfreund H. and Knowles J.R., in 'Essays in Biochemistry' (Campbell P.N. and Greville G.D. eds.), Vol.3, pp.25-72, Academic Press, London (1967).

6. Blake C.C.F., Grace D.E.P., Johnson L.N., Perkins S.J., Phillips D.C., Cassels R., Dobson C.M., Poulsen F.M. and Williams R.J.P., Ciba Foundation Symposium, 60, 137 (1978).

7. Williams R.J.P., Chem.Soc.Rev., 9, 325 (1980).

8. Williams R.J.P., Biol.Rev., 54, 389 (1979).

9. Warshel A., American Peptide Symposium (5th) San Diego 574 (1977); Acc.Chem.Res., 14, 284 (1981).

10. Sinnott M.L., Chem.in Britain, 293 (1979).

11. Jencks W.P., Adv. Enzymol., 43, 220 (1975).

12. Page M.I., Angew.Chem.Int.Ed., 16, 449 (1977).

13. Ferdinand W., 'The Enzyme Molecule', p.75, John Wiley and Sons, London (1976).

14. Anfinsen C.B., Biochem.J., 128, 737 (1972).

15. Vallee B.L. and Williams R.J.P., Proc.Nat.Acad.Sci. U.S.A., 59, 498 (1968).

16. Campbell I.D., Dobson C.M. and Williams R.J.P., Adv.Chem.Phys., 39, 55 (1978).

17. Hanson K.R. and Rose I.A., Acc.Chem.Res., 8, 1 (1975).

18. Bird R. and Hopkins R.H., Biochem.J., 56, 86 (1954).

19. Stein E.A., Hsiu J. and Fischer E.H., Biochemistry, 3, 56 (1964).

20. Vallee B.L., Stein E.A., Sumerwell W.N. and Fischer E.H., J.Biol.Chem., 234, 2901 (1959).

21. Stein E.A. and Fischer E.H., J.Biol.Chem., 232, 867 (1958).

22. Bates D.P., Joubert F.J. and Novellie L., J.Sci.Food Agr., 18, 409 (1967).

23. Bates D.P., Joubert F.J. and Novellie L., J.Sci. Food Agr., 18, 415 (1967).

24. Greenwood C.T. and Milne E.A., Staerke, 20, 101, (1968).

25. Sharma C.B., Goel M. and Irshad M., Phytochemistry, 17, 201 (1978).

26. Hoschke A., Laszlo E. and Hollo J., Carbohyd. Res., 81, 145 (1980).

27. Hoschke A., Laszlo E. and Hollo J., Carbohyd. Res., 81, 157 (1980).

28. Myrback K. and Johansson N.O., Arkiv. Kemi Mineral Geol., 20A, 1 (1945).

29. Robyt J.F. and French D., Arch.Biochem. Biophys., 100, 451 (1963).

30. Svanborg K. and Myrback K., Arkiv.Kemi, 6, 113 (1953).

31. French D. and Youngquist R.W., Staerke, 15, 425 (1963).

32. Greenwood C.T. and Milne E.A., Staerke, 20, 139 (1968).

33. Robyt J.F. and French D., J.Biol.Chem., 245, 3917 (1970).

34. Robyt J.F. and French D., Arch.Biochem.Biophys., 138, 662 (1971).

35. Banks W., Geddes R., Greenwood C.T. and Jones I.G., Staerke, 24, 245 (1972).

36. Manners D.J., Advan.Carbohyd.Chem., 17, 371 (1962).

37. Manners D.J. and Marshall J.J., Carbohyd.Res., 18, 203 (1971).

38. Vink H., Makromol.Chem., 67, 105 (1963).

39. Paper No.275 of the Board of Grain Commissioners for Canada, Grain Research Laboratory, Winnipeg 2, Manitoba, Canada. Presented in part at the 53rd Annual Meeting, Washington D.C. April 1968.

40. Puls W., Keup U., Krause H.P. Thomas G. and Hoffmeister F., Naturwissenschaften, 64, 536 (1977).

41. Underkofler I.A. and Ferracone W.J., Food Eng., 123, April (1957).

42. Wong J.T-F., 'Kinetics of Enzyme Mechanisms', Academic Press, London (1975).

43. Lineweaver H. and Burk D., J.Amer.Chem.Soc., 56, 658 (1934).

44. Dixon M., Biochem.J., 55, 170 (1953).

45. Baker B.R., 'Design of Active Site Directed Irreversible Enzyme Inhibitors', John Wiley and Sons, New York (1967).

46. Pauling L., Amer.Sci., 36, 58 (1948).

47. Haldane J.B.S., 'Enzymes', Longmans, Green and Company, London (1930).

48. Wolfenden R., Acc. Chem.Res., 5, 10 (1972).

49. Wolfenden R., Ann.Rev. Biophys.Bioeng., 5, 271, (1976).

50. Lienhard G.E., Science, 180, 149 (1973).

51. Koshland D.E.Jr., J.Cell.Comp.Physiol., 47 (Suppl.1), 217 (1956).

52. Rando R.R., Science N.Y., 185, 320 (1974).

53. Greenwood C.T., MacGregor A.W. and Milne E.A., Carbohyd.Res., 1, 229 (1965).

54. Thoma J.A., Wakim J. and Stewart L., Biochem. Biophys. Res. Commun., 12, 350 (1963).

55. Mahler H.R. and Cordes E.H., 'Biological Chemistry', 2nd edn., Chapters 3-4, Harper and Row, New York (1971).

56. Greenwood C.T. and Milne E.A., Adv. Carbohyd. Chem., 23, 281 (1968).

57. Di Carlo F.J. and Redfern S., Arch.Biochem., 15, 343 (1947).

58. Cohen L.A., 'The Enzymes' (P.D.Boyer, ed.), 3rd edn., Vol.1, pp.147-211, Academic Press, New York (1970).

59. Means G.E. and Feeney R.E., 'Chemical Modification of Proteins', Holden-Day, San Francisco (1971).

60. Greenwood C.T., MacGregor A.W. and Milne E.A., Arch.Biochem.Biophys., 112, 459 (1965).

61. Little J.E. and Caldwell M.L., J.Biol.Chem., 147, 229 (1943).

62. Benner K. and Myrback K., Arkiv.Kemi., 4, 7 (1952).

63. Radichevitch I., Becker M.M., Eitingon M., Gettler V.H., Turaballa G.C. and Caldwell M.L., J.Amer.Chem.Soc., 81, 2845 (1959).

64. Di Carlo F.J. and Redfern S., Arch.Biochem., 15, 343 (1947).

65. Caldwell H.L., Weill C.E. and Weill R.S., J.Amer. Chem.Soc., 67, 1079 (1945).

66. Schramm M., Biochemistry, 3, 1231 (1964).

67. Ohnishi M., Suganuma T. and Hiromi K., J.Biochem. (Tokyo), 76, 7 (1974).

68. Little J.E. and Caldwell M.L., J.Biol.Chem., 142, 585 (1942).

69. Thoma J.A., Wakim J. and Stewart L., Biochem. Biophys.Res.Commun., 12, 350 (1963).

70. Wakim J., Robinson M. and Thoma J.A., Carbohyd. Res., 10, 487 (1969).

71. Takagi T. and Isemura T., J.Biochem.(Tokyo), 49, 43 (1961).

72. Omichi K., Kasai S. and Matsuhima Y., J.Biochem.(Tokyo), 78, 493 (1975).

73. Hoschke A., Laszlo E. and Hollo J., Staerke, 28, 426 (1976).

74. Riordan J.F., Wacker W.E.C. and Vallee B.L., Biochemistry, 4, 1758 (1965).

75. Simpson R.T., Riordan J.F. and Vallee B.L., Biochemistry, 2, 616 (1963).

76. Singer S.J., Adv.Protein Chem., 22, 1 (1967).

77. Kneen E. and Sanstedt R.M., Arch.Biochem., 9, 235 (1946).

78. Militzer W., Ikeda C. and Kneen E., Arch.Biochem., 9, 309 (1946).

79. Militzer W., Ikeda C. and Kneen E., Arch.Biochem., 9, 321 (1946).

80. Silano V., Cereal Chem., 55, 722 (1978).

81. Irshad M. and Sharma C.B., Biochim.Biophys.Acta., 659, 326 (1981).

82. Bamford C., Capon B. and Overend W.G., J.Chem.Soc., 5138 (1962).

83. Phillips D.C., Proc.Natl.Acad.Sci. U.S.A., 57, 484 (1967).

84. Imoto T., Johnson L.N., North A.C.T., Phillips D.C. and Rupley J.A. , in 'The Enzymes' (Boyer P.D. ed.), 3rd edn., Vol.7, pp.665-868, Academic Press, New York (1972).

85. Warshel A. and Levitt M., J.Mol.Biol., 103, 227 (1976).

86. Warshel A. and Weiss R.M., J.Amer.Chem.Soc., 102, 6218 (1981).

87. Vernon C.A., Bunton C.A., Lewis T.A., Llewellyn D.R. and Tristram H., Nature, 174, 560 (1954).

88. Chapman N.B. and Laird W.E., Chem. and Ind., 20 (1954).

89. Edward J.T., Chem. and Ind., 1102 (1955).

90. Barton D.H.R., Cookson R.C., Klyne W. and Shoppee R., Chem. and Ind., 21 (1954).

91. Mathieson A.McL., Tetrahedron Lett., 2, 81 (1963).

92. Lord R.C., Rounds T.C. and Toyotoshi U., J.Chem. Phys., 57, 2572 (1972).

93. Durig J.R., Carter R.O. and Carreira L.A., J.Chem. Phys., 60, 3098 (1974).

94. Williams V.E., PhD Thesis, Bangor (1969).

95. Bushweller C.H. and O'Neil J.W., Tetrahedron Lett., 53, 4713 (1969).

96. Hall L.D. and Johnson L.F., Tetrahedron, 20, 883 (1964).

97. Niwa T., Inouye S., Tsuruoka T., Koaze Y. and Niida T., Agr.Biol.Chem., 34, 966 (1970).

98. Phillips D.C., Sci.Amer., 215, 78 (1966).

99. Blake C.C.F., Johnson L.N., Mair G.A., North A.C.T., Phillips D.C. and Sarma V.R., Proc.Roy. Soc. ser.B., 167, 378 (1967).

100. Vernon C.A., Proc.Roy.Soc. ser.B., 167, 389 (1967).

101. Doonan S., Vernon C.A. and Banks B.E.C., Prog. Biophys. Mol.Biol., 20, 249 (1970).

102. Legler G., Biochem.Biophys.Acta., 524, 94 (1978).

103. Fischer E. and Meyer J., Ber., 2, 1941 (1889).

104. Isbell H.S. and Schaffer R., J.Amer.Chem.Soc., 78, 1887 (1956).

105. Diehl H.W., Pokorny M., Zissis E., Ness R.K. and Fletcher H.G.Jr., Carbohyd.Res., 38, 364 (1974).

106. Laszlo E., Hollo J., Hoschke A. and Sarosi G., Carbohyd.Res., 61, 387 (1978).

107. Fischer E. and Zach K., Sitzber.kgl.preuss.Akad. Wiss., 16,311 (1913).

108. Helfer ich B., Adv.Carbohyd.Chem., 7, 209 (1952).

109. Bergmann M. and Kobel M., Ann., 434, 109 (1923).

110. Haworth W.N., Hirst E.L. and Reynolds R.J., J.Chem.Soc. Trans.1, 302, (1934).

111. Gakhokidze A.M., J.Gen.Chem. (U.S.S.R.), 18, 60 (1948).

112. Bates F.J. and others; 'Polarimetry, Saccharimetry and the Sugars', U.S. Department of Commerce, National Bureau of Standards, p.488, circular C440, Washington, U.S.Government Printing Office (1942).

113. Whistler R.L. and Wolfram M.L., eds.,Methods in Carbohydrate Chemistry, Vol.1, p.344 (1962).

114. Helfer ich B. and Steinpreis R., Ber., 91, 1794 (1958).

115. Fischer E., Ber., 47, 196 (1914).

116. Bates F.J. and others; 'Polarimetry, Saccharimetry and the Sugars', U.S. Department of Commerce, National Bureau of Standards, p.493, circular C440, Washington, U.S. Government Printing Office (1942).

117. Whistler R.L. and Wolfrom M.L., eds., Methods in Carbohydrate Chemistry, Vol.2., p.218 (1963).

118. Hudson C.S. and Johnson J.M., J.Amer.Chem.Soc., 37, 1276 (1915).

119. Brauns D.H., J.Amer.Chem.Soc., 51, 1820 (1920).

120. Fraser-Reid B. and Radatus B., Canad.J.Chem., 47, 4095 (1969).

121. Hall L.D. and Manville J.F., Carbohyd.Res., 8, 295 (1968).

122. Ferrier R.J. and Sankey G.H., J.Chem.Soc.(C), 2345 (1966).

123. Lemieux R.U., Nagabhushan T.L. and O'Neil I.K., Canad.J.Chem., 46, 413 (1968).

124. Chalmers A.A. and Hall R.H., J. Chem.Soc. Perk.II, 728 (1974).

125. Thiem J., unpublished.

126. Lemieux R.U., Fraga E. and Watanabe K.A., Canad.J.Chem., 46, 91 (1968).

127. Robins R.K. J.Org.Chem., 37, 3695 (1972).

128. Hughes N.A., Carbohyd.Res., 25, 242 (1972).

129. Lundt I. and Pedersen C., Acta Chem.Scand., 25, 2749 (1971).

130. Lundt I. and Pedersen C., Acta.Chem.Scand., 25, 2320 (1971).

131. Ellis G.P. and Honeyman J., Adv.Carbohyd.Chem., 10, 95 (1955).

132. Hough L. and Richardson A.C. in S.Coffey (ed.), Rodd's Chemistry of Carbon Compounds, Vol.1F, pp.432-437, Elsevier, Amsterdam (1967).

133. Schiff H., Ann.Chem.Pharm., 140, 123 (1866).

134. Schiff H., Ann.Chem.Pharm., 154, 30 (1870).

135. Sorokin B., Ber., 19, 513 (1886).

136. Sorokin B., J.Chem.Soc., 50, 526 (1886).

137. Sorokin B., J.Russ.Phys.- Chem.Soc., Chem.Pt., 1, 377 (1887).

138. Sorokin B., J.Chem.Soc., 54, 807 (1887).

139. Sorokin B., Ber., 20, 783 (1887).

140. Sorokin B., J.prakt.Chem., 37, 291 (1888). (N.B. This author is also referred to as W. or V. Sorokin in some publications).

141. Pigman W.W., 'The Carbohydrates', pp.407-424, Academic Press, New York (1957).

142. Cameron C.N., J.Amer.Chem.Soc., 49, 1759 (1927).

143. Hodge J.E. and Rist C.E., J.Amer.Chem.Soc., 74, 1494 (1952).

144. Schiff H. and Straus P., Ber., 27, 1287 (1894).

145. Marchlewski L., J.prakt.Chem., 50 {ii}, 95 (1894).

146. Irvine J.C. and Hynd A., J., 99 161 (1911).

147. Paulsen H. and Pflughaupt K.-W. in W.Pigman and D.Horton (eds.), The Carbohydrates, Chemistry and Biochemistry, Vol.1B, 2nd Edn., pp.881-927. Academic Press, London (1980).

148. Kuhn R. and Ströbele R., Ber., 70, 773 (1937).

149. Berger L. and Lee J., J.Org.Chem., 11, 75 (1946).

150. Ellis G.P., J.Chem.Soc.(B), 572 (1966).

151. Nánási P., Nemes-Nánási E.and Cerletti P., Gazz. Chim.Ital., 95, 966 (1965).

152. Price N.C. and Stevens L., Fundamentals of Enzymology, p.282, Oxford, New York (1982).

153. Tipson, R.S., Cerezo A.S., Deulofeu V. and Cohen A., J.Res.Nat.Bur.Stand., 71, 73 (1967).

154. Mester L. and Khadem H.S. El., in W. Pigman and D.Horton (eds.), The Carbohydrates, Chemistry and Biochemistry, Vol.1B, 2nd edn., pp.929-988, Academic Press, London (1980).

155. Merchant Z.M., PhD.Thesis, University of London (1978).

156. Lambert J.B., Keske R.G., Cahart R.E. and Johanovich A.P., J.Amer.Chem.Soc., 89, 3761 (1967).

157. Fischer E., Ber., 26, 2400 (1893).

158. Cadotte J.E., Smith F. and Spriestersbach J., J.Amer.Chem.Soc., 74, 1501 (1952).

159. Igarishi K., Adv.Carbohyd.Chem.Biochem., 34, 243 (1977).

160. Paulsen H., Ang.Chem.Int.Ed.Engl., 21, 155 (1982).

161. Koenigs W. and Knorr E., Ber.Dtsch.Chem.Ges., 34, 957 (1901).

162. Helfer ich B. and Zirner J., Ber., 95, 2604 (1962).

163. Lemieux R.U., Adv.Carbohyd.Chem., 9, 1 (1954).

164. Wolfrom M.L., Pittet A.D. and Gillam I.C., Proc.Nat.Acad.Sci, U.S.A., 47, 700 (1961).

165. Austin P.W., Hardy F.E., Buchanan J.G. and Baddiley J., J.Chem.Soc., 2128 (1964).

166. Pougny J.R., Jacquinet J.C., Nasser M., Duchet M.N.D., M.-L.Milat and Sinay P., J.Amer.Chem.Soc., 99, 6762 (1977).

167. Sinay P., Pure Appl.Chem., 50, 1437 (1978).

168. M.-L.Milat and Sinay P., Angew. Chem., Int.Ed.Engl., 18, 464 (1979).

169. Wing R.E. and Miller J.N., Carbohyd.Res., 10, 441 (1969).

170. Vogel A.I., Textbook of Practical Organic Chemistry, 4th edn., Longman, London (1978).

171. Zissis E. and Glaudemans C.P.J., Carbohyd.Res., 50, 292 (1976).

172. Capon B. and Connett B.E., J.Chem.Soc., 4492 (1965).

173. Sinay P. and Pougny J.R., Tetrahedron Lett., 4073 (1976).

174. Rao V.S. and Perlin A.S., Carbohyd.Res., 83, 175 (1980).

175. Sweeley C.C., Bentley R., Makita M. and Wells W.W., J.Amer.Chem.Soc., 85, 2497 (1963).

176. Trevelyan W.E., Proctor D.R. and Harrison J.S., Nature, 166, 444 (1950).

177. Dubois M., Gilles K.A., Hamilton J.K., Rebers D.A. and Smith F., Analyt.Chem., 28, 350 (1956).

178. Perrin W.D., Armarego W.L.F. and Perrin D.W., 'Purification of Laboratory Chemicals', Pergamon Press, London (1966).

179. Whistler R.L. and Wolfrom M.L., eds., Methods in Carbohydrate Chemistry, Vol.2., p.221 (1963).

180. Hepburn D.R. and Hudson H.R., Chem. and Ind., 664 (1974).

181. Perrine T.D., Glaudemans C.P.J., Ness R.K., Kyle J. and Fletcher H.G.Jr., J.Org.Chem., 32, 664 (1966).

182. Lemieux R.U., Hendriks K.B., Stick R.V. and James K., J.Amer.Chem.Soc., 97, 4056 (1975).

183. Austin P., Hardy F.E., Buchanan J.G. and Baddiley J., J.Chem.Soc., 5350 (1963).

184. Reeves R.E. and Mazzeno L.W. Jr., J.Amer.Chem.Soc., 76, 2219 (1954).

185. Kruger J.E. and Tkachuk R.T., Cereal Chem., 46, 219 (1969).

186. Walden C.C. PhD. Thesis, University of Minnesota (1954).

187. Schramm M. and Loyter A., Biochim.Biophys.Acta, 65, 200 (1962).

188. Takagi T., Toda H. and Isemura T., The Enzymes (Boyer P.D. ed.), Vol.V, 3rd edn.,p.235, Academic Press, New York (1971).

189. Uchino F., Agric.Biol.Chem., 46, 7 (1982).

190. Levvy G.A., Biochem.J., 52, 464 (1952).

191. Leaback D.H., Biochem.Biophys.Res.Comm., 32, 1025 (1968).

192. Hackert M.L. and Jacobson R.A., Chem.Commun., 1179 (1969).

193. Dahlquist F.W., Rand-Meir T. and Raftery M.A., Biochemistry, 8, 4212 (1969)

194. Reese E.T., Parrish E.W. and Ettlinger M., Carbohyd.Res., 18, 381 (1971).

195. Levvy G.A. and Snaith S.M., Adv.Enzymol., 36, 151 (1972).

196. Dutta S.K., Bangladesh Pharm. J., 4, 5 (1975).

197. Pocker Y. and Green E., J.Amer.Chem.Soc., 95, 113 (1973).

198. Jencks W.P. and Carriuolo J., J.Amer.Chem.Soc., 83, 1743 (1961).

199. Friedenwald J.S. and Maengwyn-Davies G.D., A Symposium on the Mechanism of Enzyme Action, (McElroy W.D. and Glass B. eds.), p.159. The Johns Hopkins Press, Baltimore (1954).

200. Thoma J.A., J.Theor.Biol., 19, 297 (1968).

201. Graves D.J. and Wang J.H., The Enzymes (Boyer P.D. ed.), Vol.VII, 3rd edn.,p.435, Academic Press (1972).

202. Brown D.H. and Cori C.F., The Enzymes (Boyer P.D. ed.), Vol.V, 2nd edn.,p.207, Academic Press (1961).

203. Lee Y.C., Biochem.Biophys.Res.Comm., 35, 161 (1969).

204. Hehre E.J., Biochemistry, 16, 1780 (1977).

205. Lehmann J. and Schroter E., Carbohyd.Res., 58, 65 (1977).

206. Hehre E.J., Okada G. and Genghof D.S., Adv.Chem. Ser., 117, 309 (1973).

207. Hehre E.J., Abstracts of the International Symposium on Carbohydrate Chemistry, 7th, Bratislava, Czechoslovakia, Aug.5-9, p.158 (1974).

208. Bray H.G. and White K., Kinetics and Thermodynamics in Biochemistry, 2nd edn.,p.295, Churchill, London (1966).

209. Morrison J.F., T.I.B.S., 102, March (1982).

210. Wentworth D.F. and Wolfenden R., Biochemistry, 23, 4715 (1974).

211. Fisher J., Charnas R.L. and Knowles J.R., Biochemistry, 17, 2180 (1978).

212. Hanozet G., Pircher H.-P., Vanni P., Desch B. and Semenza G., J.Biol.Chem., 3703 (1981).

213. Lai H -Y. and Axelrod B., Biochem.Biophys.Res. Commun., 54, 463 (1973).

214. Legler G., Biochim.Biophys.Acta, 524, 94 (1978).

215. Lai H -Y., Ph.D. Thesis, Purdue University (1974).

216. Thoma J.A. and Koshland D.E., J.Biol.Chem., 235, 2511 (1960).

217. Thoma J.A., Spradlin J.E., and Dygert S., The Enzymes, (Boyer P.D. ed.), Vol.V, pp.115-191, Academic Press, New York (1971).

218. Walker D.E. and Axelrod B., Arch.Biochem.Biophys., 195, 392 (1979).

219. Cleayssens M. and De Bruyne C.K., Naturwiss., 52, 515 (1965).

220. Yamamoto T. and Fukumoto J., Bull.Agr.Chem.Soc., 19, 121 (1955).

221. Simon H. and Palm D., Chem.Ber., 98, 433 (1965).

222. Capon B., Chem.Rev., 69, 407 (1969).

223. Dunn G. and Manners D.J., Carbohyd.Res., 39, 283 (1975).

224. Robyt J.F., Ackerman R.J. and Keng J.G., Anal. Biochem., 45, 517 (1972).

225. Whistler R.L. and Wolfrom M.L., eds., Methods in Carbohydrate Chemistry, Vol.4, p.26 (1965).

226. Macgregor A.W., Cereal Chem., 48, 255 (1971).

227. Muller L., Junge B., Fromner W., Schmidt D. and Truscheit E., Enzyme Inhibitors (Brodbeck U., ed.) pp. 109-122, Verlag Chemie, Weinheim, BRD (1980).