STABILITY OF YEAST INVERTASE

IN RELATION TO INDUSTRIAL APPLICATION

Being a thesis presented in accordance with the regulations governing the award of the degree of Doctor of Philosophy in the University of Surrey

by

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SUMMARY

The enzyme yeast invertase (E.C. 3.2.1.26.) catalyses the hydrolysis of sucrose to an equimolar mixture of glucose and fructose. Two forms of invertase exist, an extracellular form known as external invertase which is a glycoprotein containing 52% (w/w) mannan, and an intracellular form known as internal invertase which is devoid of mannan.

Thermal inactivation of purified external and internal invertases from Baker's yeast was found to occur at 65°C and 50°C respectively, suggesting that the mannan moiety of the external enzyme is responsible for its thermal stability. Removal of mannan from the external enzyme by digestion with α-mannosidase did not lower its stability to that of the internal enzyme, indicating its ambiguous role in conferring stability on to the invertase tertiary structure.

Candida utilis external invertase has been shown to be much more thermally stable than the corresponding enzyme from Baker's yeast, the latter being used for the production of soft-centred confectionery. The potential usefulness of the C. utilis enzyme to the confectionery industry is discussed.

The improvement of the thermal stability of Baker's yeast invertase to that of the C. utilis enzyme has been attempted by chemical modification using bifunctional cross-linking reagents. Only glutaraldehyde was successful, but at the expense of a loss in specific enzyme activity.
The chemical modification of Baker's yeast invertase using Fremy's salt, iodine, citraconic anhydride, a water-soluble carbodiimide and toluene diisocyanate, resulted in the destabilization of the enzyme activity. The possible amino acid interactions involved in maintaining conformational stability of this enzyme are discussed.

*C. utilis* invertase has been stabilized by its chemical modification using dimethylsuberimidate and by heating in the presence of Ca^{2+}.

The immobilization of Baker's yeast invertase on to several supports and its effect upon thermal stability is described.

Finally, the difference in stability between Baker's yeast and *C. utilis* invertase, and the role of the mannan moiety in stabilizing the external invertase molecule in conjunction with amino acid interactions, is discussed.
TO MY WIFE AND MY MOTHER
ACKNOWLEDGEMENTS

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The determination of standard deviations (S.D.) shown in this thesis were obtained from the results of experiments performed in triplicate.

In all thermal stability studies the concentration of purified invertase activity that was subjected to heat treatment was between 12 - 16 units/ml.
Chapter 1

General Introduction

This chapter reviews the current knowledge on yeast invertase, including its specificity, mechanism of action, purification and properties and the existence of multiple forms of the enzyme. The glycoprotein nature of invertase and the function of its mannan moiety is described. A section is also included on the main uses of yeast invertase. Finally, the principal objectives of this thesis are described.

1.1. - Historical

As early as 1828, Dumas and Boulay had shown that water was required in order for yeast to ferment sucrose. In 1833, Persoz discovered that yeast caused inversion in the optical rotation of sucrose (in Neuberg and Roberts, 1946). Berthelot (1860) isolated the active agent in yeast responsible for this inversion by using alcoholic precipitation and it became known as invertase.

The overall reaction catalysed by invertase is expressed below: glucose and fructose mutarotate producing an inversion of the sign of the optical rotation from positive to negative.

\[
\text{Sucrose} + \text{H}_{2}\text{O} \rightleftharpoons \text{Glucose} + \text{Fructose} \\
(\alpha)_D = +66.5^\circ \quad \rightleftharpoons \quad (\alpha)_D = +52.5^\circ \quad + \quad (\alpha)_D = -93^\circ \\
-21^\circ
\]

This change of rotation is exploited by workers to measure invertase activity polarimetrically (e.g. Lövgren and Sjöblom, 1970)

Studies utilising yeast invertase (E.C. 3.2.1.26) have aided in the elucidation of the details of several fundamental aspects of enzymology. The greater thermostability of invertase in the presence
of its substrate sucrose was explained by O'Sullivan and Thompson in 1890 as being due to the formation of an enzyme-substrate complex (in White et al., 1973). It was also the subject of Sørensen's classic paper on the effect of pH on enzyme activity (Sørensen, 1909). Subsequently Michaelis and Menten (1913) studied the effect of substrate concentration on yeast invertase and proposed their now famous explanation of why, at high substrate concentrations, no further increase in the velocity of an enzyme catalysed reaction occur by increasing the substrate concentration still further.

1.2. The Specificity and reactions of invertase

1.2.1. Specificity

Sucrose is composed of a β-fructofuranoside and an α-glucoside and can therefore be split by two different enzymes - β-fructofuranosidase and α-glucosidase. Both enzymes were differentiated as early as 1923 when Kuhn observed that invertase from yeast would hydrolyse raffinose as well as sucrose but α-glucosidase from Aspergillus oryzae (known as taka-invertase) only hydrolysed sucrose. The difference between the two enzymes was explained on the basis that β-fructofuranosidase attacks the fructose moiety of sucrose whilst α-glucosidase attacks the glucose moiety. This was confirmed by the use of H\textsubscript{2}O\textsuperscript{18} (see Section 1.3.2.) but the enzymes can be best distinguished by the use of two trisaccharides: raffinose and melezitose (see Fig. 1.1.). α-glucosidase attacks the glucose moiety of sucrose and modification of this moiety prevents the enzyme from hydrolysing sucrose. Thus raffinose is not cleaved by α-glucosidase whilst melezitose can be. Conversely β-fructofuranosidase hydrolyses raffinose but not melezitose, indicating that a substrate for this enzyme must possess a terminal unsubstituted fructose residue. Thus β-fructofuranosidase directs its specificity towards the C\textsubscript{(6)} position of the fructose and hydrolyses the bond between C\textsubscript{(2)} - O whilst α-glucosidase directs its specificity towards the C\textsubscript{(6)} position.
Fig. 1.1. - The structure of the trisaccharides
Raffinose and Melezitose
of the glucose molecule and hydrolyses \( C(1)^1 \) - O.

It has been noted that \( \alpha \) - glucosidase is inactive at pH 4.5 towards sucrose hydrolysis (which is the pH at which \( \beta \) - fructofuranosidase is most active) but that it is most active at pH 6.8 (in Neuberg and Roberts, 1946).

\( \beta \) - fructofuranosidase is trivially known as invertase and it hydrolyses methyl \( \beta \)-O fructofuranoside, gentianose (gluc-gluc-fruc) and stachyose (gal-gal-gluc-fruc). As the number of residues on the glucose moiety increases there is a corresponding decrease in the rate of hydrolysis (Adams et al., 1943).

1.2.2. - Transferase action

It is well known that the production of free glucose and free fructose by the hydrolysis of sucrose catalysed by invertase is not the sole reaction. Invertase also possesses transferase activity and is capable of transferring fructose residues to suitable acceptors leading to the formation of mono - , di - and oligosaccharides (Bacon and Edelman, 1950; Blanchard and Albon, 1950). The use of radioactive glucose has shown that the free fructose and the glucose combined in sucrose are exchanged through fructose transfer. Transfer of fructose to free fructose can also occur (Edelman, 1954). Three oligosaccharides are formed by the action of invertase on sucrose (Fischer et al., 1951). The amount of transfer products is usually less than 10% of total sugar but the ratio of transfer to hydrolysis products increases with substrate concentration (Edelman, 1956).

Yeast invertase can transfer fructose to primary alcoholic groups. When invertase, sucrose and methanol are incubated together \( \beta \) - methylfructofuranoside is formed (Bacon, 1952). Internal invertase (see section 1.6.2.) also possesses transferase activity. It can transfer
fructose to methanol, ethanol, n-propanol, isopropanol and benzyl alcohol (Baseer and Shall, 1971). These investigators have also shown that highly purified invertases have transferase activity with methanol, ethanol, n-propanol, isopropanol, benzyl alcohol, aniline, nitroaniline, mercaptoethanol, allyl alcohol and 2-chloroethanol. Thus the possibility that the transfer reactions are caused by contaminating enzymes can be ruled out. Isopropanol was the only secondary alcohol to show transferase activity.

Characterisation of two of the products formed by transfer of fructose to sucrose shows Kestose: (α-glucopyranosyl (1 →2) β-fructofuranosyl (6 →2) β-fructofuranoside and neo-kestose: (β-fructofuranosyl (2 →6) α-glucopyranosyl (1 →2) β-fructofuranoside) (Albone et al., 1953; Gross et al., 1954). Andersen (1967) showed that free fructose was transferred by invertase and two disaccharides formed were identified as 6-β-fructofuranosyl-fructose (levanbiose) and 1-β-fructofuranosylfructose (Inulobiose). He suggested a mechanism for free fructose transfer:

\[
\begin{align*}
EH + FOH & \rightleftharpoons EF + HOH \\
EF + FOH & \rightleftharpoons EH + FOF
\end{align*}
\]

\[F = \text{Fructose} \quad E = \text{Enzyme (protonated)}\]

1.2.3. Effect of transferase activity on the determination of invertase activity

During the reaction between invertase and sucrose, glucose liberation has been shown to be linear with time whereas fructose liberation is not linear due to its involvement in transfer reactions. Consequently monitoring of invertase activity by methods which measure reducing sugar liberation will produce lower values for invertase activity than if glucose was being assayed directly. Polarimetric
measurements of invertase activity assume equal amounts of glucose and
fructose production and neglect any oligosaccharide formation.
Albon et al., (1953) and Gross et al., (1954) have shown that
intermediate oligosaccharides formed by the transferase activity of
invertase introduces a significant error in polarimetric measurements of
initial reaction rates. This error has been shown to be quantitatively
unimportant when short incubation periods of substrate and enzyme are
used (Andersen et al., 1969; Gascon et al., 1968). Bowski et al.,
(1971) compared the rate of hydrolysis of sucrose by invertase at sucrose
concentrations between 0.04M and 2.06M by polarimetry and enzymatic
glucose assay techniques. The rates were not significantly different
between 0.04 - 0.17M sucrose, but above 0.17M sucrose polarimetric assay
produced significant lower reaction rates compared to the method which
assayed glucose enzymatically. Using the latter method they showed that
the peak reaction rate occurred at 0.29M sucrose. This was in contrast
to the well documented peak reaction rate at 0.17M sucrose as previously
determined by polarimetric assay (Nelson and Schubert, 1928).

1.3. – Mechanism of action of yeast invertase
1.3.1. – pH dependence of invertase

The rate of sucrose hydrolysis by invertase is maximal at pH 4.5
although the difference in activity between pH 3.5 and 5.5 is only slight.
The decline in rate on the alkaline side of the pH optimum was suggested
by Michaelis and Davidsohn (1911) to be due to the dissociation of a weak
acid (pKa = 6.6). However, Kuhn (1923) found that the alkaline branch
of the pH activity curve was independent of substrate concentration and
Von Euler et al., (1924) subsequently reported that the group with
pKa = 6.6 was not responsible for substrate binding and has the same Ka
value in the enzyme and enzyme-substrate complex. Myrbäck (1926)
found that the acid branch of the invertase pH activity curve depended
upon substrate concentration and therefore a group in the enzyme with 
pK_b = 3 was responsible for substrate binding.

1.3.2. - Specificity

The specificity of yeast invertase reveals that the substrate 
must possess an unsubstituted β - fructofuranosyl residue (see section 
1.2.1.). A precise spatial orientation between an enzyme and its 
substrate has long been thought and accepted to be the basis of enzyme 
specificity. If the glycosidic oxygen of sucrose is parallel to and 
fac ing the enzyme molecule then this would allow close approximation (1A) 
between substrate and enzyme due to hydrogen bonding between hydroxyl 
residues in the sucrose molecule and hydrophilic groups on the enzyme 
surface. Furthermore, the glycosidic oxygen would have to be at the 
active centre of the enzyme for splitting of the glucose and fructose 
moieties (Gottschalk, 1950).

Koshland and Stein (1954) found that when sucrose was hydrolysed 
by invertase in 0\(^{18}\) - labelled water, splitting of sucrose had taken place 
between the C(1) atom of fructose and the glycosidic oxygen and therefore 
the enzyme had high specificity for fructose and low specificity for 
glucose, the reaction proceeding by a displacement mechanism.

Fischer et al., (1951) proposed that the hydrolysis and transfer 
reactions of invertase should be explained as a two-step reaction in which 
an enzyme - fructose complex was initially formed with the liberation of 
glucose. The subsequent reaction involved the transfer of the fructosyl 
group to either water or sucrose :

\[
\text{Sucrose + enzyme } \leftrightarrow \text{ Fructose - enzyme + glucose} \tag{1}
\]
\[
\text{Fructose - enzyme + sucrose } \leftrightarrow \text{ trisaccharide + enzyme} \tag{2}
\]
\[
\text{Fructose - enzyme + H}_2\text{O } \leftrightarrow \text{ Fructose + enzyme} \tag{3}
\]

Transfer of fructose to primary alcoholic groups would also occur :

\[
\text{Fructose - enzyme + ROH } \leftrightarrow \text{ Fructosyl - O - R + enzyme .OH} \tag{4}
\]
Another hypothesis suggested that an enzyme - sucrose complex was formed and the second step was the simultaneous liberation of glucose and the transfer of fructose to water or sucrose (Edelman and Bacon, 1951).

1.3.3. - Inhibition of yeast invertase

The reaction between low concentrations of iodine (100μM) and invertase has been used to probe the mechanism of action of invertase. Von Euler and Landergren (1922) showed that approximately 50% inhibition of enzyme activity occurred when iodine was reacted with invertase. The product of the reaction was stable and known as iodine - invertase (I⁻ invertase). It was shown that the Km and pH activity curve of I⁻ invertase was identical to that of the native enzyme (Myrbäck, 1926; Von Euler and Josephson, 1923; Myrbäck and Willstaedt, 1958). The nature of I⁻ invertase is unknown. Myrbäck and Willstaedt (1958) have shown that I⁻ invertase is insensitive to silver and mercury concentrations which completely inhibit the native enzyme. This suggested the involvement of SH groups in the iodine reaction but attempts to reactivate I⁻ invertase with the SH compounds cysteine and glutathione were unsuccessful. The reducing agents ascorbic acid, sodium dithionite, sodium amalgam and sodium borohydride also had no effect on I⁻ invertase activity.

Waheed and Shall (1971b) found that mercaptoethanol and mercaptoethylamine reactivated I⁻ invertase whereas cysteine, which is α-carboxyl β-mercaptoethylamine, did not. Since the only difference between cysteine and mercaptoethylamine is a carboxylate anion they concluded that a carboxylate anion was present at, or close to, the active site of the enzyme. Thus cysteine would be kept away from the active site by electrostatic repulsion. They suggested that iodine oxidised a particularly reactive methionine residue. Internal invertase which possesses no cysteine (Gascon et al., 1968) also behaves similarly with iodine (Baseer and Shall, 1971).
Uaheed and Shall (1971b) suggest that either all the active sites are modified decreasing the catalytic activity by half or only half the active sites are modified. This might suggest the presence of iodine-sensitive invertase sub-units.

Invertase is inhibited by Ag\(^+\), Cu\(^{++}\), Cd\(^{++}\), Zn\(^{++}\), Pb\(^{++}\), which is non-competitive and reversed by dialysis. The inhibition by silver is dependent on pH and the group involved has a pKa = 7, probably the imidazole of histidine, although it is likely that other groups also participate in its binding (Myrback, 1960). Thus Myrback (1957) found that histidine, methionine and reduced glutathione in the presence of invertase reduced the inhibitory effect of silver.

Neumann and Lampen (1967) suggested that the sulphydryl groups in invertase were catalytically unimportant since the sulphydryl reagents iodoacetamide, iodoacetic acid and N-ethylmaleimide did not inhibit invertase activity. Waheed and Shall (1971b) found that iodoacetamide but not iodoacetic acid inhibited the enzyme. Again they concluded that a carboxylate anion was close to, or at the active site of invertase and since iodoacetic acid possesses a carboxylate anion electrostatic repulsion would keep this reagent away from the active site. They also postulated that the active site contained a histidine residue as a result of evidence from their kinetic studies on the effect of pH on the rate of the reactivation of invertase after partial inactivation by iodine.

On this basis Waheed and Shall proposed a mechanism of action for yeast invertase (see Fig. 1.2.).

Other amino acids have, however, been implicated in the catalytic activity of invertase. For example, Sizer (1947) found that the enzyme tyrosinase inactivated invertase by 10 - 40% and therefore suggested that tyrosine may be involved in the catalytic activity. However, nitration of tyrosines in the invertase molecule did not affect enzyme activity.
Fig. 1.2. - Proposed mechanism of action of yeast invertase
(from Waheed and Shall, 1971b)

A - Binding of Substrate.

B - Transfer of proton from active site imidazolium cation to glycosidic oxygen.

C - Departure of alcohol. Unstable carbonium ion remains. Active site carboxylate ion stabilizes electrondeficient species.

D - Attack of C-2 cation by nucleophilic oxygen atom of an alcohol or water to yield a fructoside or fructose.

E - Dissociation of Product.
Waheed and Shall, 1971b) which might suggest that tyrosyl residues are not essential for catalysis. Leskovac et al., (1975) showed that oxidation of 5-6 tryptophyl residues/molecule with N-bromosuccinimide completely inactivated the enzyme. They proposed that tryptophan played a role in substrate binding, since 23 out of a total of 34 tryptophyl residues were found to be exposed to oxidation on the enzyme surface. Clearly, the role of tyrosine and tryptophan in catalysis remains to be established.

1.4. Yeast invertase preparations and their purification

Since its isolation in 1860, many workers have extracted and purified yeast invertase. Table 1.1. summarizes the recent preparations of the enzyme. The majority of preparations are associated with yeast mannan although the exact amount can vary. Hence Adams and Hudson (1943) found it to be 7% whilst Fischer and Khotes (1951) reported their preparation to contain 70 - 80% mannan. The varying mannan content appears to be due to the method of extraction of invertase from yeast. Methods which employ prolonged autolysis of yeast cells in order to release the enzyme, frequently yield invertase containing a low mannan content whilst those using mechanical rupture of cells to release the enzyme, generally yield invertase with a mannan content in excess of 50% (w/w).

Previous purification procedures included various precipitations and adsorptions of the enzyme, but the enzyme was not obtained in a pure and homogenous state (Neuberg and Roberts, 1946). Sumner and O’Kane (1948) found that their purified invertase was precipitated by concavalin A, an established glycoprotein precipitant and concluded that invertase itself was a glycoprotein. Earlier Salkowski (1900) and Willstätter and Schneider (1924) had succeeded in removing all the mannan from invertase. Fischer et al., (1951) adsorbed invertase onto bentonite...
and found that all the mannan remained in solution. The adsorbed enzyme was fully active but when eluted it lost all its activity. They concluded that invertase had a strong affinity for mannan which resulted in its stabilization. This finding was contradictory to the finding of Adams and Hudson (1938) who recovered 75% of the adsorbed enzyme activity after its elution from bentonite. Degradation of mannan associated with invertase occurs during prolonged autolysis and is believed to account for the low or negligible mannan content of some invertase preparations (Lampen, 1971).

Procedures currently employed in the purification of invertase include gel filtration and ion exchange chromatography. Some of the highest specific activities have been obtained (see Table 1.1.) using these methods. Purified yeast invertase has subsequently been shown to be a glycoprotein containing 50% (w/w) mannan and 2 - 3% (w/w) glucosamine (Neumann and Lampen, 1967). The purification procedure involved mechanical breakage of cells to release the enzyme; heat treatment at 50°C, pH 5.0; fractional precipitation with ethyl alcohol (50% v/v) and ammonium sulphate (70% - 80% saturation) followed by ion exchange chromatography using SE — and DEAE — Sephadex. Precipitation with ammonium sulphate was the most significant stage of the procedure since most of the inactive protein was precipitated whilst all invertase remained in solution. The specific activity of the preparation was 2,700 units/mg at 30°C and it appeared homogenous by polyacrylamide gel electrophoresis.

Andersen and Jørgensen (1969) purified a commercial yeast invertase concentrate. The purified enzyme contained only 13% (w/w) mannan but still possessed a high specific activity (3780 units/mg at 30°C) suggesting that the mannan was not required for activity. Interestingly, their purified enzyme was precipitated by 75% saturated ammonium sulphate.
**Table 1.1. - Recent preparations of yeast invertase**

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<tr>
<th>Reference</th>
<th>Carbohydrate % (w/v)</th>
<th>Units (30°C) per mg.protein</th>
<th>Method of extraction</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1951-Fischer and Khotès</td>
<td>70 - 80</td>
<td>800 - 1000</td>
<td>A</td>
<td>n.d.</td>
</tr>
<tr>
<td>1965-Myråböck and Schilling</td>
<td>30</td>
<td>3500</td>
<td>A</td>
<td>n.d.</td>
</tr>
<tr>
<td>1967-Neumann and Lampen</td>
<td>50</td>
<td>2700 - 3000^b</td>
<td>M</td>
<td>270,000</td>
</tr>
<tr>
<td>1971 - Lampen</td>
<td>47 - 52</td>
<td>4000 - 5000^b</td>
<td>M</td>
<td>270,000</td>
</tr>
<tr>
<td>1968 - Gascón and Lampen</td>
<td>&lt;3</td>
<td>2900^b</td>
<td>M</td>
<td>135,000</td>
</tr>
<tr>
<td>1971b - Waheed and Shall</td>
<td>50</td>
<td>2770^b</td>
<td>A</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* From Lampen (1971)

a - A, autolysis; M, mechanical breakage
b - homogenous by gel electrophoresis
n.d. - not determined
Uaheed and Shell (1971a) identified several forms (isoenzymes) within a yeast invertase concentrate separated by anion exchange chromatography. The specific activity of the isoenzymes was inversely proportional to their mannan content, the enzyme with the largest mannan content having the highest specific activity. They deduced from this relationship that an invertase devoid of mannan would have approximately 60% of the specific activity of the form possessing a full complement of mannan, suggesting that mannan was not a prerequisite for catalytic activity. More recently Smith and Ballou (1974a) removed all the mannan associated with yeast invertase by digestion of the enzyme with exo-$\alpha$-mannanase isolated from the soil micro-organism Arthobacter G3M-1. Their mannan-free invertase was as active as the native enzyme and thus it could also be concluded that the mannan was not essential for enzyme activity.

Finally, it is worth mentioning that the purification of external invertase has also been performed using invertase-precipitating antibodies (Tkacz and Lampen, 1973).

1.5. - Location and forms of yeast invertase

1.5.1. - Location

Much evidence had accumulated some twenty years ago which disputed the theory that invertase was simply contained within the yeast cell as had previously been thought (Neuberg and Roberts, 1946).

Wilkes and Palmer (1932) showed that the pH-activity curves of invertase in vivo and in vitro were identical and they suggested that the enzyme was peripherally located in the cell. This suggestion gained wide support from many workers. Thus Demis et al., (1954) calculated that sucrose and its hydrolysis products glucose and fructose were located almost exclusively in an extracellular locus. Myrbäck and Willstaedt (1955) found that acid-treatment of Baker's yeast cells
destroyed almost all of the invertase activity, but the ability of the cells to ferment was unimpaired. Myrback (1957) found that invertase was strongly bound to the cell wall and was solubilized only by treatment with proteolytes. Irradiation of yeast with low-voltage electrons showed that invertase was located at a depth of not greater than 1000 Å from the cell periphery (Preiss, 1958). More compelling evidence for the external location of invertase was obtained by workers who demonstrated that almost all invertase activity was released during the conversion of yeast cells to protoplasts with snail gut and microbial enzymes (Friis and Ottolenghi, 1959; Sutton and Lampen, 1962; Burger et al., 1961). Burger et al., (1961) solubilized invertase by mechanically breaking yeast cells with the X-press. More recently Williams and Wiseman (1973) have solubilized invertase from Brewer's yeast cells using the Vibro Mill disintegrator. This suggested that invertase exists in a soluble form outside the protoplast but retained by the cell wall, which is contrary to the idea that the enzyme is itself part of the cell wall structure and can only be released after proteolysis of the cell wall (Myrback, 1957).

Lampen (1960) proposed a model for the yeast cell wall which clearly shows how the mannan moiety may be responsible for the retention of invertase within the matrix of the cell wall (Fig. 1.3.A). Kidby and Davies (1970) found that invertase was released from whole cells of *Saccharomyces fragilis* following treatment with mercaptans and they proposed a model in which invertase was not bonded to the yeast cell wall but retained by structures possessing intact disulphide bridges (Fig. 1.3.B).

1.5.2. **Multiple forms of yeast invertase**

Friis and Ottolenghi (1959) showed that yeast cells grown under repressed conditions (i.e. in glucose) possessed invertase activity that was wholly intracellular whilst derepressed cells (i.e. grown in sucrose) possessed both intracellular and extracellular invertase
Fig. 1.3. — Schematics of the yeast cell wall showing invertase retention

From Lampen (1968)

From Kidby and Davies (1970)

* Photographed from Bull (1972)
activities. Sutton and Lampen (1962) suggested that the internal enzyme represented invertase at its site of formation which would subsequently be secreted and retained in the cell wall.

The intracellular enzyme (known as internal invertase) was shown to be a smaller molecular form than the extracellular enzyme (known as external invertase) as judged by the behaviour of cell free extracts during gel filtration on Sephadex G-200 (Gascon and Ottolenghi, 1967). These authors also showed that the levels of internal invertase activity found in their diploid strain of *Saccharomyces* sp. were independent of the growth conditions, whilst those of the external form varied with changes in the glucose concentration of the medium.

It has been shown that in the yeast *Saccharomyces cerevisiae* there exist six different genes for invertase biosynthesis (known as SUC<sub>1-6</sub>, Mortimer and Hawthorne, 1969). Therefore, multiple forms of invertase are a possibility. However, the existence of different genes is not responsible for the formation of internal and external invertase since these invertases are produced by yeast homozygous for the SUC<sub>2</sub> gene (Gascon and Ottolenghi, 1967). Hackel (1975) showed that a single point mutation in a yeast strain carrying the SUC<sub>3</sub> gene resulted in the loss of formation of both forms of invertase.

Several forms of external invertase have been demonstrated in Baker's yeast. Hoshino et al. (1964) found three distinct forms in which the pH optimum and Km values differed. Similarly, Waheed and Shall (1971a) found several forms in a yeast invertase concentrate. Further evidence for the heterogeneity of external invertase was presented by Colonna et al. (1975) who showed that isoelectric focussing of external invertase from a mutant of *Saccharomyces* sp. distinguished three major protein species with isoelectric points (pI values) of 3.65, 3.32 and < 2.7. Baker's yeast external
invertase yielded four protein species with pI values ranging from 4.40 to 3.96. They also found that the invertases tested were all phosphorylated and differed in their mannan : protein ratios but possessed similar mannose : PO₄ ratios. It was suggested that the differing mannan : protein (and hence PO₄ : protein) ratios were responsible for the multiple forms. Isoelectric focussing of internal invertase (free of phosphate and mannan) revealed a single protein species with a pI value of 4.45.

The significance of the isoenzymic forms of external invertase is unknown. They may be artefacts formed during the extraction of the enzyme from the yeast cell (Lampen, 1971), suggesting that internal and external invertase are the only true isoenzymic forms.

1.6. - *A comparison of the properties of external and internal invertases*

1.6.1. - **Purification of internal invertase**

Gascon and Lampen (1968) purified internal invertase from a mutant strain of *Saccharomyces sp.* which formed high levels of the enzyme. The large scale separation of internal and external invertases in cell-free extracts was achieved by precipitating the internal enzyme with 70% saturated ammonium sulphate whilst most of the external form remained soluble. Further purification of the internal enzyme was done by using DEAE - Sephadex A-50 and SE - Sephadex G-50 ion exchange chromatography. The final product was homogenous and had high specific activity (see table 1.1.)

1.6.2. - **Properties of external and internal invertase**

Internal invertase represents only about 3% of total invertase activity in cell suspensions (Gascon and Lampen, 1968). Gascon *et al.*, (1968) compared the properties of both enzymes. They possess similar kinetic parameters: $K_m$ for each enzyme was 26mM for sucrose and 150mM.
for raffinose and their optimum pH for maximum activity is 4.7. The external enzyme is a glycoprotein containing 50% mannan and 3% glucosamine whilst the internal form contains less than 3% mannan and no glucosamine. External invertase can be highly phosphorylated depending upon the strain of *Saccharomyces* sp. from which it is isolated. The majority of the phosphorus exists in the enzyme molecule as mannose-6-phosphate. Internal invertase lacks phosphorus (Colonna *et al.*, 1975). Phosphorus is not therefore essential for invertase activity.

The molecular weights for the external and internal enzyme are 270,000 and 135,000 respectively as determined by ultracentrifugal analysis. Since half the external molecule is mannan, both enzymes have similar molecular weights for their protein moiety. Tamaki (1974) reported that Baker's yeast external invertase possessed a molecular weight of 340,000 as determined by gel filtration.

Both invertases possess transferase activity (see section 1.2.2.) and were found to be immunologically related. The amino acid compositions of the two enzymes differ markedly (see Table 1.2) especially in the levels of glycine, tyrosine, histidine and cysteine. Cysteine is absent in the internal enzyme (Gascon *et al.*, 1968).

The pH stability of the two enzymes also differs in that the external enzyme is most stable at 30°C between pH 3 and 7.5. Internal invertase is most stable between pH 6.0 and 9.0. The thermal stabilities of both enzymes differ. External invertase is rapidly inactivated by heat above 65°C (Arnold, 1969) whereas internal invertase is rapidly inactivated above 50°C (Goldstein and Lampen, 1975; Woodward and Wiseman, 1976).
Table 1.2. - Amino Acid Compositions of Purified Internal and External Invertases

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>External * Invertase</th>
<th>Internal * Invertase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>71</td>
<td>115</td>
</tr>
<tr>
<td>Alanine</td>
<td>68</td>
<td>84</td>
</tr>
<tr>
<td>Serine</td>
<td>114</td>
<td>151</td>
</tr>
<tr>
<td>Threonine</td>
<td>84</td>
<td>80</td>
</tr>
<tr>
<td>Proline</td>
<td>65</td>
<td>63</td>
</tr>
<tr>
<td>Valine</td>
<td>69</td>
<td>73</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td>Leucine</td>
<td>83</td>
<td>77</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>80</td>
<td>77</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>65</td>
<td>31</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>33</td>
<td>30</td>
</tr>
<tr>
<td>Cysteine</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Methionine</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>178</td>
<td>165</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>115</td>
<td>124</td>
</tr>
<tr>
<td>Arginine</td>
<td>27</td>
<td>32</td>
</tr>
<tr>
<td>Histidine</td>
<td>16</td>
<td>29</td>
</tr>
<tr>
<td>Lysine</td>
<td>60</td>
<td>85</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>38</td>
<td>0</td>
</tr>
</tbody>
</table>

* moles / 135,000 g. of protein

(From Lampen, 1971)
1.7. The relationship between external and internal invertases

The similarities in properties of both enzymes suggested that a biosynthetic relationship existed between them in that internal invertase is the precursor of the external form. Differences in amino acid composition were explained by the presence of sub-units of which one or more was active and identical in both. One sub-unit of external invertase, containing all the cysteine, was absent in the internal enzyme (Gascon et al., 1968). Beteta and Gascon (1971) found that both invertases were present in yeast vacuoles showing therefore, that external invertase also existed within the protoplast. They speculated that internal invertase was probably synthesised in the rough endoplasmic reticulum or by ribosomes attached to the surface of vacuoles. Subsequent addition of carbohydrate onto the internal enzyme would lead to the formation of the external form which would subsequently be secreted through the protoplast. Recently, Moreno et al., (1975) found a continuous spectrum of different molecular forms (as judged by their behaviour on Sephadex G-200) within the protoplast and they suggested that they represented the sequential addition of mannose onto the internal enzyme culminating in the formation of the external form.

Further evidence to support these events came from the observation that internal invertase and intermediate forms accumulated when cells were grown in the presence of 2-deoxy-D-glucose which inhibits glycosylation. There was also an accumulation of external invertase when cells were incubated with cyclohexamide, which prevents de novo invertase synthesis.

When yeast cells were grown with $^3$H amino acids and converted to protoplasts, it was found that more radioactivity was incorporated into the external enzyme compared with the internal form. When the protoplasts were then incubated with unlabelled amino acids, it was found...
that the specific activity of the external enzyme became diluted, whereas the specific activity of the internal enzyme remained the same (Lampen et al., 1972). Lampen et al., (1972) suggested that internal invertase was not the precursor of the external form but appeared to be a metabolic dead-end product. The internal enzyme would be formed from material diverted from the main invertase biosynthetic pathway either before, or during the glycosylation process.

Contrary to the findings by Moreno et al., (1975), Kuo and Lampen (1974) found that tunicamycin, a glucosamine-containing antibiotic, which acts primarily as an inhibitor of glycoprotein synthesis, prevented the synthesis of the external enzyme by yeast protoplasts, but no accumulation of internal invertase occurred.

The exact relationship between the internal and external form of the enzyme still remains to be established.

1.8. Control of invertase biosynthesis

1.8.1. Catabolite repression

It is well known that glucose can play an important role in the catabolite repression of invertase synthesis. Davies, R. (1953) found that when log-phase cells of *Saccharomyces fragilis* were washed and suspended in low glucose concentrations (0.005 - 0.02M) there occurred a tenfold increase in invertase biosynthesis. Higher concentrations of glucose (0.05M) inhibited invertase biosynthesis. Davies, A. (1956) found that glucose concentrations higher than 0.001% inhibited invertase biosynthesis in *S. fragilis* cells grown in continuous culture. Complex inhibition of invertase biosynthesis in *S. fragilis* by 0.02 - 0.04M glucose occurred but cells and protoplasts of *S. cerevisiae* still synthesised invertase in 0.08M glucose. The concentration for optimal invertase synthesis in both strains of yeast appeared to be 0.01M glucose (Islam and Lampen, 1962).
Metzenberg (1962) found that greatest repression of invertase formation in *Neurospora Crassa* occurred by 0.075M mannose whereas highest levels of this enzyme were produced when grown in 0.075M galactose. He also produced a mutant strain of *N. crassa* which produced higher levels of invertase than the parental strain and suggested that a single gene alteration was sufficient to cause this difference.

Dodyk and Rothstein (1964) found that concentrations of glucose higher than 0.01M were inhibitory to the appearance of invertase in *S. cerevisiae*, as were several intermediates of glucose metabolism: acetaldehyde, ethanol, acetate, succinate, fumarate and malate. They found that lactate stimulated invertase formation. Repression of invertase synthesis in *Aspergillus nidulans* by glucose was similar to that found in yeast (Andres and Peabody, 1974).

Invertase is formed by de novo synthesis since its formation is inhibited by cyclohexamide (Lampen, 1968; Liras and Gascon, 1971; Trevithick and Metzenberg, 1966; Eldan and Mayer, 1974).

### 1.8.2. Mechanism of catabolite repression

The antibiotic lomofungin (a phenazine belonging to the class of antimicrobial substances) inhibits m.RNA formation in yeast protoplasts with little effect on total protein synthesis (Lampen *et al.*, 1973a). When lomofungin was added to yeast protoplasts actively synthesizing invertase, there was a delay of some 40 minutes before invertase synthesis was halted. Incubation of protoplasts in high fructose (150mM final concentration) or cyclohexamide halted invertase formation almost immediately. When the protoplasts were transferred to a low fructose (20mM) medium invertase synthesis began immediately and this was not affected by lomofungin. This evidence seemed to suggest that catabolite repression of invertase occurred at the translational
stage. During repression specific mRNA would be produced that could subsequently be translated even in the presence of lomofungin (Lampen et al., 1973b).

It is well known that the low activity of catabolite-sensitive enzymes in the presence of glucose is correlated with a reduced content of cAMP within cells of E. Coli (Wiseman, 1975). External addition of cAMP is able to overcome repression of these enzymes. Intracellular cAMP is increased in derepressed yeast cells (Van Wijk and Konijn, 1971; Sy and Richter, 1972). No correlation, however, was found between cAMP levels and invertase synthesis in yeasts which differed to varying degrees in their susceptibility to hexose repression (Montencort et al., 1973). Lim (1976) found no induction or protection of invertase in yeast by cAMP during deadaptation of the cells in 2% glucose which repressed invertase biosynthesis.

Interestingly, Schlanderer and Dellweg (1974) did find a relationship between intracellular levels of cAMP and activity of invertase in Schizosaccharomyces pombe. A mutant strain partially resistant to catabolite repression synthesized more cAMP and possessed higher levels of invertase activity than did the parent strain. They concluded that cAMP was concerned with the regulation of catabolite-sensitive enzyme synthesis. It would therefore appear that cAMP can affect the rate of invertase production but its role is uncertain.

The synthesis and secretion of external invertase (in an active form at least) is inhibited by 2-deoxy-D-glucose (2dG) whose metabolite 2dG-6-phosphate prevents glycoprotein formation by an unknown mechanism (Lampen et al., 1973c). Kratky et al., (1975) found that there was no close correlation between inhibition of mannan synthesis by 2dG and the appearance of invertase. They found that the inhibition of invertase secretion from yeast protoplasts by 2dG was
1.9. - Invertase as a glycoprotein

1.9.1. - Chemical attachment of carbohydrate to the invertase protein moiety

Studies of the glycoprotein nature of purified external invertase have shown that approximately 30 chains of polysaccharide of varying length are attached to each enzyme molecule and that the carbohydrate-protein linkage involved a glucosylaminyl-asparagine bond (Neumann and Lampen, 1969). The possibility that mannan is linked to invertase protein via an o-glycosyl hydroxyamino acid linkage involving serine or threonine has also been mentioned (Greiling et al., 1969).

Tarentino et al., (1974) showed that invertase contained a neutral oligosaccharide that is attached to the protein by a glycosyl-asparagine bond with the following composition:

\[ \text{Asn} - (\text{GLNAc})_2 \text{Mannan} \]

(GLNAc is N-acetylglucosamine)

Since there are about 38 glucosamine residues per mole of invertase (Neumann and Lampen, 1967) they calculated that there were approximately 20 chains of polysaccharide of varying length attached to the invertase molecule.

1.9.2. - Function of the mannan moiety

Since many enzymes secreted by eukaryotes are glycoproteins the mannan moiety may be important for the secretion process (Elyar, 1966). However, Winterburn and Phelps (1972) have pointed out that many proteins without a carbohydrate moiety are secreted and they proposed that sugars are included in protein structures as a means of coding for the topographical location within an organism.
Since the external invertase was purified and shown to be a glycoprotein (Neumann and Lampen, 1967), some interest has been centred on the function of the mannan moiety. The mannan moiety is not essential for the catalytic activity of invertase since several highly active preparations of invertase have been prepared which have low mannan contents (Andersen and Jørgensen, 1969; Adams and Hudson, 1943; Fischer et al., 1951). Gascon et al., (1968) suggested that the differences in stability between the external and internal enzymes were due to the mannan moiety. The idea was further strengthened when Arnold (1969) fractionated a Baker's yeast invertase preparation on DEAE-cellulose into fractions whose carbohydrate content and stability towards heat treatment at 65°C could be correlated. He concluded that the mannan moiety was responsible for stabilizing the invertase protein by some unknown mechanism.

A somewhat paradoxical situation arose when Smith and Ballou (1974a) removed all the mannan from invertase by exo-α-mannanase treatment and tested the heat stability of the 'mannanless' invertase at 37°C in pHs 1.9, 3.6, 4.7 and 8.2. No difference in stability was observed between the native and 'mannanless' enzymes and they concluded that the mannan moiety was not responsible for the activity nor for the stability of invertase. Smith and Ballou (1974b) suggested that the mannan served to retain the enzyme in the cell wall matrix.

It is generally known that glycoproteins are resistant to denaturation by a variety of agents including heat (Pazur and Arnonson, 1972; Bettelheim - Jevons, 1958). In this respect invertase is typical. Strumeyer and Malin (1970) showed that yeast invertase was resistant to denaturation by tannic acid and Wiseman and Woodward (1975) have noted the resistance of invertase to denaturation by the anionic detergent sodium dodecyl sulphate.
It would therefore seem that the mannan moiety provides protection against a variety of denaturants. Whether the role of mannan is to maintain the conformational stability of external invertase remains to be established.

1.10. - Biotechnology of Yeast Invertase

1.10.1. - Uses of invertase

Neuberg and Roberts (1946) have listed the uses to which invertase has been put. These include the isolation of some di- and trisaccharides, identification of sucrose and higher oligosaccharides of the sucrose type and the determination of the purity of honey. It is also involved in all alcoholic fermentations and in the process of malting. More recently, a method has been described in which invertase is used for the determination of sucrose in cereals and cereal products (Cerning - Beroard, 1975).

One of the most recent events in enzyme biotechnology involves the use of enzyme electrodes for the sensitive assay of biological materials (see New Scientist (1976) 70 No.994 p.16.) Satoh et al., (1976) prepared a sucrose electrode for use in industrial and clinical analysis. The electrode possessed an enzyme-collagen membrane (containing invertase, glucose oxidase and mutarotase) and the principle of the assay was based on monitoring the decrease in dissolved oxygen resulting from the oxidation of β-D-glucose (produced by the action of invertase on sucrose) by glucose oxidase. The decrease in dissolved oxygen was correlated with a decrease in current. Calibration of the electrode was done using known concentration of sucrose and measuring the current generated.

The major industrial use of invertase centres on the production of invert sugar. Highly purified invertase preparations are not used by industry
since these preparations would be more susceptible to physical damage than would cruder preparations (Cochrane, 1961). Commercial invertases are prepared from Baker's yeast by using autolysis followed by alcohol precipitation of invertase from the cell-free extract. The crude enzyme preparation is usually dissolved in 55% glycerol and is sold in this form to industry. The commercial form of invertase is known as 'invertase concentrate'.

The concentrate is used by the confectionery industry in the production of a variety of cream centred sweets. A fondant in its empirical form is essentially a suspension of microscopic sucrose crystals in the syrup from which they have been crystallized. The fondant can be flavoured and coloured but without further modification, gives a firm and rather dense sweet (e.g. pear drops). To make the fondant soften, invertase is used to hydrolyse the sucrose and invert sugar being more soluble than sucrose, dissolves in the water present. Thus the centre softens and because the total amount of solids dissolved is increased (about 80%) the risk of fermentation is reduced. Soft centred chocolates are produced by coating the fondant with chocolate.

During the 'softening' process the fondant and invertase are heated to a temperature dependent upon the final product desired. When high temperatures are used, much of the invertase activity is lost due to denaturation by heat. Consequently, large quantities of invertase are used and lost.

Therefore, it may be more efficient to use invertases from sources other than Baker's yeast which show greater thermal stability. Alternatively, it may be possible to increase the thermal stability of the commercial invertase used in the confectionery industry by means of artificial stabilization, such as by immobilization or by the introduction
of intramolecular cross-linkages. No work on the latter method has appeared in the literature to date.

1.10.2. - Immobilization of invertase

The initial high cost of immobilized enzymes can be overcome by their repeated use and by their ease of removal from the final products of the enzyme reaction. Yeast invertase has been immobilized by many workers with the result that properties of the native enzyme can be changed for practical purposes. Filippusson and Hornby (1970) covalently linked invertase to polystyrene by the azo-coupling reaction. The $K_m$ value (with respect to sucrose) was increased twofold and $V_{\text{max}}$ was increased ninefold compared with these parameters of the native enzyme. Mason and Westall (1972) covalently coupled invertase to porous glass and found that the pH optimum of the immobilized form was 4.0 compared to 3.5 for the native form but $K_m$ and $V_{\text{max}}$ were unchanged. The native enzyme was shown to have greater thermal stability than the immobilized derivative, but the latter was stable at 25°C for 28 days when it was operated continuously. A decrease in thermal stability was also reported when invertase was ionically linked to DEAE-cellulose (Usami et al., 1971). When the enzyme was linked onto bentonite there was an increased resistance to heat in the presence and absence of substrate (Monsan and Durand, 1971). This preparation had a pH optimum of 6.25 compared to 5.2 for the native form.

One of the disadvantages of immobilization is that the bound, insolubilized enzyme may have a lower specific activity than the native enzyme. Kobayashi and Moo-Young (1973) found that 79% of invertase activity was destroyed when the enzyme was covalently bound to anion-exchange resin beads (polyamine type). Maeda et al., (1973) found the recovery of activity was 55 - 70% when the enzyme was immobilized ionically to DEAE-cellulose.
Invertase has also been immobilized by gel entrapment.

O'Driscoll et al., (1972) entrapped invertase in poly *(HEMA)* type gels. Maeda et al., (1973a, 1973b) entrapped the enzyme in poly (vinyl alcohol) gel matrices formed by means of γ-ray and electron beam irradiation of the invertase gel solution. Using sufficient radiation doses no leakage occurred from the gels but 56% and 80-90% loss in enzyme activity occurred by entrapment with electron beam and γ-ray irradiation respectively. Invertase immobilized in polyacrylamide gel retained 90% of its activity (Kreen et al., 1971). Usami and Kuratsu (1973) found that the polyacrylamide gel entrapped enzyme retained only 8-10% the activity of the soluble form but showed greater heat stability. Irradiation treatment of acrylamide and invertase resulted in an entrapped enzyme possessing 69.2% recovery of the activity of the native enzyme. The bound enzyme was more active at 70°C and had a broader pH optimum than the soluble form (Kawashima and Umeda, 1974).

Maeda et al., (1974) irradiated N - vinylpyrrolidone monomer with γ-rays to entrap invertase, but over 90% of activity was lost. Other gels utilised for entrapment of invertase include 2 - hydroxyethyl acrylate and dimethyl acrylamide (Maeda et al., 1975). Marconi et al., (1974) entrapped invertase in cellulose triacetate fibres and found it to be very stable under operating conditions with a half-life value of 5,300 days. The activity of the invertase fibres was 15-65% of the native enzyme, but at very high substrate concentrations (60% w/v) the activities were almost identical.

Invertase has been immobilized on whole cells of *Streptomyces sp.* *Aspergillus niger* and *Rhizopus oryzae* which were treated with the bifunctional reagent toluene diisocyanate and cross-linked to the enzyme, resulting in 65.4% retention of enzyme activity (Takasaki, 1974).

* Abbrev. HEMA - 2 hydroxyethylmethacrylate
Interestingly, Toda and Shoda (1975) entrapped whole cell invertase of \textit{Saccharomyces pastorianus} in spherical agar pellets and found the enzyme activity to be very stable during batch reactions compared with invertase bound on DEAE - cellulose.

One of the major problems in enzyme immobilization is the reduction of enzyme activity after repeated use in a continuous reactor system. Boudrant and Cheftel (1973) hydrolysed sucrose continuously in a stirred tank reactor system limited by an ultrafiltration membrane which retained invertase selectively but remained permeable to solutions of hydrolysed sucrose. There was no loss in activity after 5 days of continuous use. Invertase fibres continuously hydrolysing sucrose did not lose any activity for 5 years (Merconi \textit{et al.}, 1974). Continuous sucrose hydrolysis by invertase immobilized on an Amberlite ion exchange resin remained constant for 8 days and enzyme activity was stable for 1 month at 30°C at pH 3 or 4 (Boudrant and Cheftel, 1975).

In recent years numerous immobilized invertase patents have appeared. These are listed in Table 1.3. Immobilized invertase preparations are required that will have high specific activity, improved thermal stability and a greater resistance to denaturants. Few methods of invertase immobilization so far reported actually increase the thermal stability of the enzyme. It may be possible to increase the stability of invertase after it has already been bound to the support.
<table>
<thead>
<tr>
<th>Inventors</th>
<th>Method of Immobilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Durand et al., (1972)</td>
<td>Solid invertase bentonite complexes</td>
</tr>
<tr>
<td>Emery et al., (1972)</td>
<td>Coupling of invertase to organo-metal derivatives of polysaccharide nylon or glass</td>
</tr>
<tr>
<td>Vieth et al., (1972)</td>
<td>Covalently bound to collagen membranes</td>
</tr>
<tr>
<td>Velzen and Gerardus (1973)</td>
<td>Granular invertase by cross-linking to gelatin or egg white</td>
</tr>
<tr>
<td>Vieth et al., (1973)</td>
<td>Immobilized - invertase membrane complexes</td>
</tr>
<tr>
<td>Kuyama et al., (1974)</td>
<td>Invertase resin formed with Amberlite XAD-12</td>
</tr>
<tr>
<td>Amotz (1974)</td>
<td>Cross-linked invertase admixed with diatomaceous earth, perlite cellulose and plastic materials</td>
</tr>
<tr>
<td>Vieth et al., (1974)</td>
<td>Invertase collagen complex layered on cellulose acetate</td>
</tr>
<tr>
<td>Forgione (1974)</td>
<td>Invertase covalently bound to cross-linked polymer (polyacrolein)</td>
</tr>
<tr>
<td>Takasaki (1974)</td>
<td>Invertase immobilized to microbial cells</td>
</tr>
<tr>
<td>Boudrant and Cheftel (1975)</td>
<td>Invertase immobilized on macro-reticulated resin (Amberlite)</td>
</tr>
</tbody>
</table>
1.11. - Principal objectives of thesis

a) To determine the suitability of Perid reagent (marketed by the Boehringer Corporation for blood sugar determinations) to assay glucose produced by the action of invertase on sucrose and thereby to provide a sensitive assay for yeast invertase activity.

b) To isolate and purify external and internal invertases for studies on their stability and inactivation after chemical modification.

c) To investigate the existence of multiple forms of external invertase and to determine the nature of their separation and their function.

d) To identify thermally stable invertases for possible use in the confectionery industry.

e) To chemically modify commercial invertase in order to improve its thermal stability.

f) To immobilize yeast invertase as a means of improving its thermal stability.

g) To determine whether mannan confers conformational stability onto the yeast invertase protein and if so, to describe the mechanism of how this is achieved.
Basic materials and general methodology

2.1. - Sources of invertase

The following preparations of external invertase were purchased from the Sigma (London) Chemical Company Ltd., London S.W.6.

Grade III invertase (Baker's yeast), a crude preparation (11 units/mg.)*

Grade VI invertase (Baker's yeast), a partially purified preparation (160 units/mg.)*

Grade X invertase (Candida utilis yeast), a highly purified preparation (525 units/mg.)*

The source of the preparation is given in brackets.

Commercial invertase concentrate (Baker's yeast) was a gift from Honeywill & Stein Co. Ltd., Wallington, Surrey.

Slope cultures of yeasts, Saccharomyces cerevisiae (N.C.Y.C. No.525) and Candida utilis (N.C.Y.C. No.708) were purchased from the National Collection of Yeast Cultures, Brewing Industry Research Foundation, Nutfield, Surrey. Baker's yeast was purchased in 1lb. quantities from Cranks Health Shop, 35 Castle Street, Guildford, Surrey.

2.2. - Culturing of yeast cells

Yeast cells were cultured in 100ml. batches of medium in conical flasks which had been pre-sterilised in steam for 15 min. at 120°C at a pressure of 15 p.s.i.

Cells which were subsequently used for the extraction and isolation of external invertase were cultured in the following medium containing:

(1) Yeast extract 1g. (Oxoid Ltd., London S.E.1.)
(2) Peptone 2g. (Oxoid Ltd., London S.E.1.)
(3) Sodium Chloride 0.5g. (B.D.H. laboratories)
(4) Sucrose Analar grade 1g. (B.D.H. laboratories)
(5) Distilled water 100ml.

(Medium 1)

* 1 unit is the amount of enzyme which hydrolyses 1 μmole of sucrose per min. at 55°C (Sigma definition)
For the isolation of internal invertase the cells were grown in the following medium containing:

1. Yeast extract 1g.
2. Ammonium sulphate 0.2g. (B.D.H. laboratories)
3. Glucose Analar grade 4g. (B.D.H. laboratories)
4. Distilled water 100ml.

(Medium 2)

Cells cultured in medium 2 (i.e. in high glucose concentration) were thus grown under conditions which repressed the formation of external invertase without altering the amount of internal invertase formed (Gascon et al., 1973). To obtain conditions of derepression, favouring formation of external invertase (but not internal invertase) cells were cultured in medium 1.

Medium was inoculated aseptically with a wire loop and the yeast was grown at 30°C for 24 h. in shake culture (5 ū cycles/minute Mickle Lab. Engineering Co., Gomshall, Surrey).

The cells were harvested by filtration using a vacuum pump and washed with distilled water. External and internal invertases were isolated and purified (see Chapter 4). Enzyme activity of whole cells was expressed as * units per mg. cells (wet weight)

2.3. Gel filtration studies

2.3.1. Gel filtration on Sephadex.

Glass columns of ** Sephadex G-200 were used to monitor for internal invertase activity during its purification (Gascon and Lampen, 1968), as well as for molecular weight determinations. The gel beads were swollen at room temperature (18°C) for 3 days in 0.1M - sodium acetate buffer pH 4.7, prior to column filling.

* Definition of a unit of enzyme activity quoted in this thesis is given in chapter 3.

2.3.1.1. - Preparation of the column

The glass column was mounted vertically and the dead space under the net was filled with the eluant (0.1M sodium acetate buffer pH 4.7). Prior to packing the column with the gel it was filled with eluant. The outlet was opened and the gel slurry was poured into the column to give gel dimensions of 1.6 x 36 cm. By this method any formation of air bubbles was minimised. The column was transferred to a cold room at 4°C and three column volumes of eluant were passed through the column to stabilize and equilibrate the gel bed. Before sample application the surface of the gel bed was protected with a close-fitting circular piece of filter paper.

2.3.1.2. - Sample application

Most of the eluant above the gel surface was removed by suction using a pasteur pipette. The column outlet was opened in order to drain away the remaining eluant. The outlet was closed and the sample (never more than 5ml in volume) was carefully layered on top of the gel surface so as not to disturb it. The column outlet was then opened and when all the sample had entered the gel, was closed again. The gel surface was then washed with a small volume of eluant and then the column was filled with eluant and connected to the eluant reservoir.

2.3.1.3. - Sample elution

Elution of the sample was achieved either by difference in hydrostatic pressure or by a peristaltic pump to cause the eluant to flow through the column.

2.3.1.4. - Determination of the void volume of the column and the collection of fractions

The void volume of the column was determined with Blue Dextran 2,000 (Pharmacia Fine Chemicals) and fractions were collected using an L.K.B. 7000, Ultrorac fraction collector. Details of the volume of fractions collected are given in the results section of chapter 4.
2.3.2. - Gel filtration on Sepharose **

A glass column of Sepharose 6B was used for molecular weight determinations. Sample application, sample elution, determination of void volume and fraction collection were performed as described for Sephadex G-200.

2.3.2.1. - Preparation of the column

The Sepharose column was packed in the same way as Sephadex G-200. The Sepharose suspension was diluted with 0.1M sodium acetate buffer to facilitate escape of air bubbles prior to being poured into the column. The column (1.4 x 45 cm.) was then equilibrated with the eluant buffer by passing numerous column volumes of the latter through it.

2.3.2.2. - Determination of the void volume

Unlike Sephadex G-200, Sepharose 6B fractionates Blue Dextran 2000. Since Blue Dextran 2000 contains material of very high molecular weight which is excluded from Sepharose the leading fractions containing these large molecules were taken to indicate the void volume.

2.4. - Ion-exchange chromatography on DEAE - Sephadex - A50

DEAE - Sephadex A-50 anion exchange chromatography was used during the purification of external and internal invertases. It was also used to separate invertases whose carbohydrate : protein ratios differed. The dry ion exchanger beads were swollen in 10mM sodium phosphate buffer pH 7.0, at room temperature for 3 days.

2.4.1. - Preparation of the column

The glass column was packed with swollen gel as described for the preparation of the Sephadex G-200 column. To allow the ion exchanger to reach ionic equilibrium with the eluting buffer, eluant (10mM sodium phosphate buffer pH 7.0) was passed through the column until the pH of the effluent was the same as that of the eluant. After equilibration the gel column dimensions were 1.4 x 30 cm.

** Sepharose 6B was purchased from Sigma Chemical Co. Ltd.
2.4.2. - Sample application and elution

Sample application was done as for Sephadex G-200; this resulted in the adsorption of the sample onto the anion exchanger. After adsorption the column was washed with buffer during which time no invertase was detected in the effluent.

Invertase was eluted with stepwise gradients of NaCl in 10mM - sodium phosphate buffer pH 7.0. During this procedure the height of the gel column decreased as the ionic strength of NaCl was increased. Fractions were collected on the L.K.E. fraction collector.

2.5. - Polyacrylamide gel electrophoresis

Polyacrylamide disc gel electrophoresis was performed by using a modification of the method of Davis (1964). Electrophoresis was performed at either pH 8.9 or pH 7.0 in 5% polyacrylamide gels. The materials for preparation of the gels: acrylamide, N,N' - methylenebis-acrylamide (Bis), N,N,N',N' - tetramethylethylenediamine (TEMED) and ammonium persulphate were purchased from B.D.H. 2.5.1

2.5.1. - Polymerization mixture for electrophoresis run at pH 8.9

Stock solutions:

Solution A :  Tris  36.6g
             TEMED  0.23ml.
             in NHCl to pH 8.9
             Distilled water to 100ml.

Solution B :  Acrylamide  20.0g
              BIS  0.53g
              Distilled water to 100ml.

Stock buffer solution :  Tris  6.0g
                        Glycine  28.8g
                        Distilled water to 1 litre, pH 8.3.

(Tris - tris (hydroxymethyl) aminomethane - was purchased from Sigma.
Glycine was a product of B.D.H.)
The glass gel electrophoresis tubes were fitted with polythene end closures and placed in a tube stand. The tubes were filled almost to the top with the polymerization mixture which consisted of 2.0ml. solution A, 4.0ml. solution B, 8.0ml. of freshly prepared ammonium persulphate solution (15mg./ml.) and 2.0ml. of distilled water. Before the gels hardened a few drops of distilled water were carefully layered on top of the gel solution. Polymerization was complete after 15 min. which was indicated by the formation of a refractile water layer. The water layer was discarded and the gel surface was rinsed twice with gel electrophoresis buffer. The polythene end enclosures were removed before the gel tubes were placed in the electrophoresis apparatus.

2.5.2. - Setting up of the electrophoresis apparatus

The gel tubes were inserted into the rubber grommets in the base of the upper buffer reservoir; any unused holes were sealed with rubber stoppers. A drop of buffer was added to the bottom of any running tubes which were not completely filled with gel. The lower buffer reservoir was filled with electrophoresis buffer (diluted 1:10 with distilled water) so that the bottom of the gel tubes were covered with buffer. The lower electrode assembly was inserted. The upper buffer reservoir was placed in position and it was filled with diluted buffer solution. Before electrophoresis was started the +ve lead of the D.C. power supply (Shandon Southern) was connected to the lower electrode and the -ve lead to the upper electrode. Diluting the running buffer had the effect of reducing the time required to run the gel.

2.5.3. - Sample preparation and application

The sample contained 100µg. of purified enzyme in 100µl. of gel buffer, 1 drop of 0.005% bromophenol blue marker (B.D.H.) and 1 drop of glycerol to increase the density of the sample prior to application. The sample was layered on top of the gel using a 100µl. oxford pipette sampler.
2.5.4. - Electrophoresis

The power supply was switched on to give 2 mA per gel tube. The bromophenol blue concentrated as a disc which migrated ahead of all the samples. The current was switched off when the marker was about 1 cm. from the bottom of the gel. This took between 60 - 90 minutes.

2.5.5. - Polymerization mixture for electrophoresis run at pH 7.0

Stock solutions:

Solution A: Acrylamide 11.1g.
BIS 0.6g.
Distilled water to 100ml.

The polymerization mixture consisted of 15ml. gel buffer (diluted 1:10 before use), 13.5ml. solution A, 1.5ml. of freshly prepared ammonium persulphate solution (15mg./ml.) and 50 j j l. of TEMED. For an electrophoresis run the procedure previously described for electrophoresis at pH 8.9 was followed exactly, except that current was switched on to give 3mA per gel tube. Electrophoresis took 4 hrs.

2.5.6. - S.D.S. polyacrylamide gel electrophoresis

S.D.S. gel electrophoresis was used for the determination of the molecular weight of invertase and to determine the number of sub-units existing within the molecule. The method of Weber and Osborne (1969) was used with the modification by Stocklosa and Latz (1974), in which S.D.S. was omitted both from the gel buffer and from the gels themselves.

2.5.6.1. - Sample preparation

The purified invertase sample was incubated at 37°C for 2 hr. in 10mM - sodium phosphate buffer pH 7.0 containing 1% S.D.S. and 1% Dithiothreitol. The sample was then applied to the top of the gel after the usual preparation, and electrophoresis was performed at 3mA per gel tube for 4 hr.

* Abbreviation S.D.S. - Sodium dodecyl sulphate
For an electrophoresis run the procedure previously described for electrophoresis at pH 7.0 was followed exactly.

2.5.6.2. - Determination of molecular weight

Proteins of known molecular weight were used to calibrate the gel. The method is based on the fact that the relative mobility of a protein - S.D.S. complex in a polyacrylamide gel is related to its molecular weight.

\[
\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length of gel after destaining} \times \text{length of gel before staining}} \times \frac{\text{distance of dye migration}}{\text{distance of dye migration}}
\]

Electrophoretic mobility was plotted against the molecular weight of the protein and a smooth curve was obtained. The molecular weight of invertase was extrapolated from this curve.

2.5.7. - Staining and Destaining of the gels

2.5.7.1. - Protein staining

The gels were removed from the gel tubes and placed in screw-capped test tubes containing staining solution. The staining solution consisted of:

- Coomassie Brilliant Blue R-250 0.5g. (purchased from BIO-RAD Laboratories, Richmond, California.)
- Methanol (B.D.H.) 91.0 ml.
- Glacial Acetic Acid (B.D.H.) 18.2 ml.
- Distilled water 91.0 ml.

Gels were stained for 2 hrs. at room temperature after which time they were destained in a solution containing 10% glacial acetic acid and 2.5% ethanol. Destaining was carried out for 2 - 3 days with periodic changes of the destaining solution.

2.5.7.2. - Detection of Invertase activity in the gels.

After electrophoresis the gels were sliced into 2mm lengths and each slice was assayed for invertase activity by the 3,5-dinitrosalicylic
acid reagent method as described in Chapter 3. After assay the gel slices were reassembled and an orange band was taken as the site of location of invertase in the gel.

2.6. - Chemical analyses

2.6.1. - Protein determination

Total protein was estimated according to the method of Lowry et al., (1951).

Stock reagents:

a) 50 ml. 2% Sodium carbonate (in 0.1M Sodium hydroxide) + 1 ml. 0.5% Copper sulphate in 1% potassium sodium tartrate (freshly prepared)

b) Folin - Ciocalteau phenol reagent diluted 1 : 3 before use with distilled water

(All reagents from B.D.H.)

2.6.1.1. - Procedure

2.0 ml. of reagent a) was added to the sample (1.0 ml.) and thoroughly mixed. After 10 min. 0.2 ml. of reagent b) was added and also thoroughly mixed. Colour was allowed to develop for 30 min. at room temperature. Extinction was measured in an Eel colorimeter at 625nm. A standard curve was prepared using bovine serum albumin (B.S.A.) (Sigma) in the range 0 - 500 \( \mu g/ml \) standard sample. Figs. 2.1. and 2.2. distinguish a difference in the standard curves performed using 0.1M sodium acetate pH 4.7 and 0.01M sodium phosphate pH 7.0, buffers.

2.6.2. - Carbohydrate determination

Total carbohydrate was estimated using the anthrone method of Morris (1948) as modified by Chung and Nickerson (1954).

Stock reagent:

0.2 g Anthrone + 100 ml. 75% Sulphuric acid

(Reagents from B.D.H.)

2.6.2.1. - Procedure

5 ml. of the anthrone reagent was added to the samples (1 ml.)
Fig. 2.1. - Protein Standard curve for Samples in 0.1M-Sodium acetate buffer pH 4.7.

Fig. 2.2. - Protein Standard curve for Samples in 0.1M-Sodium phosphate buffer pH 7.0.
which were cooled in an ice bath and thoroughly mixed. The samples were heated at 100°C for 5 min. for colour development. Extinction was measured in an Eel colorimeter (083 filter 600-650nm). A standard curve was prepared using yeast mannan (Sigma) in the range 0-200 µg/ml standard sample. The standard curves (Fig. 2.3.) done for samples in 0.1M - sodium acetate buffer pH 4.7 and 0.01M - sodium phosphate buffer pH 7.0 were identical.

2.7. **Thermal Stability of yeast invertase preparations**

The thermal stability of yeast invertase was investigated using the method of Arnold (1969). Heat treatment of external invertase and internal invertase was carried out at 65°C and 50°C respectively in a thermostated water bath. The standard medium for heat treatment was 0.1M - sodium acetate buffer pH 4.7 for external invertase and 0.01M - sodium phosphate buffer pH 7.0 for the internal enzyme.

2.7.1. **Procedure**

A suitably diluted enzyme solution was added at zero time to a pre-heated test tube (65°C) and at prescribed intervals an aliquot (0.1ml.) was removed and plunged into 2.0ml. of ice-cold 0.1M - sodium acetate buffer pH 4.7. The preparation was subsequently assayed for enzyme activity.

2.7.2. **Treatment of heat inactivation data**

In plotting heat inactivation data the following conventions were used:

a) per cent recovery of maximal enzyme activity at zero time versus t.min. of heat treatment.

b) \[ \log \frac{a}{a - x} \] versus \[ t \] where \( a \) is the enzyme activity at zero time and \( (a - x) \) is the enzyme activity after \( t \) min. of heating from which the pseudo - first order rate inactivation constants could be calculated.
Fig. 2.3. - Mannan Standard Curve
2.8. - The effect of pH on yeast invertase preparations

The determination of pH optimum and pH stability of invertase preparations was investigated using the following buffers:

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M - Glycine - HCL</td>
<td>2.5</td>
</tr>
<tr>
<td>0.1M - Sodium acetate</td>
<td>3.6</td>
</tr>
<tr>
<td>0.1M - Sodium acetate</td>
<td>4.7</td>
</tr>
<tr>
<td>0.1M - Sodium phosphate</td>
<td>6.0</td>
</tr>
<tr>
<td>0.1M - Sodium phosphate</td>
<td>7.0</td>
</tr>
<tr>
<td>0.1M - Sodium phosphate</td>
<td>8.0</td>
</tr>
<tr>
<td>0.1M - Sodium carbonate</td>
<td>10.0</td>
</tr>
</tbody>
</table>

2.8.1. - Determination of pH optimum

The enzyme was assayed by the 3*5 dinitrosalicylic acid reagent method as described in section 3.2.3., using the above buffers to give the required pH o? the reaction. The optimal pH was that at which maximal enzyme activity was achieved.

2.8.2. - Effect of pH on stability

The enzyme was pre-incubated in the same buffers (as used for the pH optimum determination) for 15 min. at 50°C. At the end of the pre-incubation period a 0.1ml. aliquot of enzyme was removed and assayed for activity by the 3*5 dinitrosalicylic acid reagent assay method. The pH conferring the greatest enzyme stability was that at which maximal recovery of original activity was found.

2.9. - Determination of Kinetic parameters Km and Vmax.

The kinetic parameters Km and Vmax were obtained by the commonly-employed procedure proposed by Lineweaver and Burk (Lineweaver and Burk, 1934).
The Michaelis - M\textregistered ment equation is

\[ v = \frac{V_{\text{max}} S}{K_m + S} \quad (1) \]

Equation (1) may be written in reciprocal form:

\[ \frac{1}{v} = \frac{K_m}{V_{\text{max}}} \frac{1}{S} + \frac{1}{V_{\text{max}}} \quad (2) \]

and a plot of \(1/v\) against \(1/S\) gives a straight line of slope \(K_m/V_{\text{max}}\), intercepts \(1/V_{\text{max}}\) on the \(1/v\) axis and \(-1/K_m\) on the \(1/S\) axis.

This is known as the double - reciprocal plot (see Fig. 2.4.).

\(v\) is the initial velocity of the enzyme catalysed reaction, \(V_{\text{max}}\) is the maximal velocity of the reaction, \(S\) is the substrate concentration. \(V_{\text{max}}\) is obtained when the substrate concentration is made so high relative to the enzyme concentration that essentially all enzyme molecules are saturated with substrate. \(K_m\) is equal to that concentration (expressed in moles per litre) of the substrate concentration which gives half the maximal velocity \(V_{\text{max}}\).
Fig. 2.4. - Schematic Lineweaver-Burke plot
(double reciprocal plot)

\[
\frac{1}{V} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}} \left( \frac{1}{[S]} \right)
\]

Slope = \frac{K_m}{V_{\text{max}}}

Intercept = \frac{1}{V_{\text{max}}}

Intercept = -\frac{1}{K_m}
Chapter 3

Measurement of Yeast Invertase Activity

3.1. - Introduction

Invertase activity is estimated by following the hydrolysis of sucrose by polarimetric, reductimetric and colourimetric measurements. The latter measurements are still widely used and colourimetric determination of the reaction products (glucose and fructose) can be performed using the arsenomolybdate method of Nelson (1944), the ferricyanide method of Park and Johnson (1949) or the 3,5 dinitrosalicylic acid method of Bruner (1961). Keilin and Hartree (1948) measured the activity of invertase manometrically by using the enzyme glucose oxidase (E.C. 1.1.1.7) for the determination of glucose. This method was modified by coupling the action of the enzyme peroxidase (E.C. 1.1.3.4) to glucose oxidase. The action of peroxidase results in the oxidation of the chromagen o-dianisidine which becomes coloured (Keston, 1956). This method has become widely used in spectrophotometric determinations of glucose. It has also been used to determine invertase activity by measuring the glucose produced by the action of the enzyme on sucrose (Neumann and Lampen, 1967; Gascon and Lampen, 1968).

The enzyme couple between glucose oxidase and peroxidase for the oxidation of the chromagen Perid was first used to assay for glucose by Werner et al., (1970). The reaction sequence is outlined below:

$$\beta-D\text{-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{gluconic acid}$$

Reduced Perid $\xrightarrow{\text{Peroxidase}}$ Oxidised Perid (coloured)

$$H_2O$$
Formula of Perid:

\[
\begin{array}{c}
\text{O}_3\text{S} \\
\text{C}_2\text{H}_5 \\
\text{N} \\
\text{S} \\
\text{C}_2\text{H}_5 \\
\text{S} \\
\text{O}_3\text{S}
\end{array}
\]

\[
2\text{N} = \text{N} - \text{N} = 2\text{diazo - di - (3- ethyl benzthiazoline - 6 sulphonylic acid)}
\]

Sookias (R.J. Sookias, unpublished work of our laboratory) assayed yeast invertase with Perid. He found that the Perid reagent (containing the enzyme coupled together with the chromagen, Perid, in 0.1M phosphate buffer pH 7.0) contained sucrose activity. This has subsequently been confirmed (J. Woodward and A. Wiseman, unpublished) but even the use of appropriate substrate, enzyme and reagent blanks was unsatisfactory, because batches of Perid reagent were found to have different sucrose activities and some gave substrate blank readings that were extremely high.

Commercial preparations of glucose oxidase can contain a variety of possible contaminant carbohydrases. For example, Blecher and Glassman (1962), found that commercial preparations of glucose oxidase had various amounts of sucrose activity. Dahlqvist (1960) found that glucose oxidase preparations had maltase activity and isomaltase activity. Rerup and Lundquist (1967) claimed that this specific method of glucose determination with glucose oxidase, yielded quantitative reactions with glycogen, starch and maltose. Scharlach et al., (1962) used the glucose oxidase system for the generation of \(\text{H}_2\text{O}_2\) as an oxidative reagent in experiments with thyroid particles, prepared in 0.25M sucrose. They found that the enhancement of iodide organification that occurs from the addition of glucose and crude glucose oxidase also occurred in the absence of glucose.

Unless inhibition of the contaminating carbohydrases of glucose
oxidase is accomplished, then the assay for glucose is inaccurate in systems where the substrates of these carbohydrases are present.

The use of Tris buffer at alkaline pH has been used to overcome this problem. Larner and Gillespie (1956), noticed the inhibition of maltase and 1.6-oligoglucosidase by several amines including Tris at alkaline pH. Friedman (1960) noted that Tris was inhibitory to maltase contaminants in commercial glucose oxidase, but he made no analytical use of this. White and Subers (1961) used Tris buffer, pH 7.6, to inhibit maltase activity in a glucose oxidase-peroxidase coupled reagent and hence provided a suitable reagent for routine maltase assay. Jorgensen and Andersen (1973) used Tris to inhibit sucrase activity in a glucose oxidase-peroxidase coupled assay reagent for determination of yeast invertase activity. None of these authors used Perid.

Inhibition of the contaminating sucrase in the Perid reagent has been achieved and this modified reagent provided a suitable and routine method of determining invertase activity which in fact has several advantages over other methods that are used in measuring reducing sugar. Perid reagent has therefore been compared to the 3,5-dinitrosalicylic acid reagent which has also been used to determine invertase activity by measuring reducing sugar.
3.2. Materials and Methods

3.2.1. Modification of the Perid reagent

Perid reagent was purchased from the Boehringer Corporation (London) Ltd., London WS 2T2, U.K. The reagent is sold as a powder containing 100mM phosphate buffer, pH 7.0, 20μg peroxidase/ml., 180μg glucose oxidase/ml. and 0.5mg chromagen/ml. Before use the reagent needs to be diluted with distilled water. A standard solution of glucose (9.1mg glucose/100ml.) was supplied with the Perid reagent. The Perid powder was diluted with 0.1M - Tris - HCL buffer, pH 7.2 and this solution was used routinely to determine the quantity of glucose formed by the action of invertase on sucrose. To determine whether Tris affected the sensitivity of Perid the standard curve for glucose in Perid - Tris was compared to those standard curves obtained with three other batches of Perid reagent (Perid 1, 2 and 3) made up to the recommended concentration with water.

The glucose standard curve was produced in the range 5 x 10^{-2} to 26.5 x 10^{-2} μmol. of glucose in 5.0ml. of Perid reagent (10 - 53 μM). Colour development was carried out at room temperature (18°C) for 25 min. and extinction was measured in the Eel portable colourimeter at 625 nm.

The effect of different sucrose concentrations on the sucrase activity of Perid - Tris, Perid 1, 2 and 3 was investigated. For this determination a range of sucrose quantities was pipetted into 5.0ml. of Perid reagent and the assay performed as above.

3.2.2. Perid assay of yeast invertase

The recommendations of the latest edition of Enzyme Nomenclature (1973) Elsevier Publishing Co., Amsterdam, London and New York are followed as to the definition of Units of enzyme activity. The unit activity (U) is the amount of invertase which hydrolyses 1 μmole of
sucrose/min. at 25°C.

The reaction mixture found to give a satisfactory assay for invertase linear with invertase dilution in the range 0.8 - 16 units/ml consisted of:

- Analar Sucrose (B.D.H.) 50% (W/V) 0.4 ml.
- 0.1M - Sodium acetate buffer pH 4.7 1.5 ml.
- Diluted invertase solution 0.1 ml.

The enzyme was added to start the reaction and the mixture incubated for 10 min. at 25°C after which a 0.1 ml. aliquot was pipetted into 5.0 ml. of Perid-Tris, pH 7.2. The reaction is stopped by dilution (M.J. Sookias, unpublished work) and also by the change to pH 7.2. Jørgensen and Andersen (1973) found that the activity of invertase in 0.2M-Tris buffer at pH 7.5 was 0.07% of the activity measured under assay conditions. Colour was developed and measured as described above.

3.2.3. - The 3'-5'-dinitrosalicylic acid assay of yeast invertase

The 3'-5'-dinitrosalicylic acid reagent was made up as follows:

- 3'-5'-dinitrosalicylic acid 5g
- Potassium Sodium tartrate 150g
- Sodium hydroxide 24g
- Distilled water 500 ml.

The reagents were purchased from B.D.H.

The mixture was stirred at room temperature for 60 min. and insoluble material was removed by filtration. The resulting orange solution was used routinely to determine the quantity of glucose and fructose formed by the action of invertase on sucrose, by following the method of Bruner (1961).

The reaction mixture found to give a satisfactory assay for invertase linear with invertase dilution in the range 0.8 - 16 units/ml.
consisted of:

Analar Sucrose 10% (w/v), 1.4 ml.
0.1M - Sodium acetate buffer, pH 4.7, 2.0 ml.
Diluted invertase solution 0.1 ml.

The enzyme was added to start the reaction and the mixture incubated for 10 min. at 25°C after which time 2.5 ml. of 3,5-dinitrosalicylic acid reagent was pipetted into the reaction mixture.

This stopped the reaction. The colour was developed for exactly 3 min. at 50°C after which time the test tubes containing the reaction mixture and developed reagent were placed into an ice bath and cooled before extinction was measured. Extinction was measured on the Eel colorimeter at 520nm.
3.3. - Results

3.3.1. - Standard curves for glucose using modified and unmodified Perid reagents

The four standard curves were not significantly different (see Fig. 3.1.) showing that Tris does not affect the sensitivity of Perid to glucose. Pharr and Dickinson (1973) have reported that a convenient dilution procedure was found to decrease β-glucosidase contamination in an assay reagent for glucose determination. Sookias (M.J. Sookias, unpublished work of our laboratory) has noted that if one part of the Perid reagent is diluted by a further eight parts of water, glucose could still be measured quantitatively, although with far less sensitivity. Indeed, this further dilution of Perid also decreased the contaminating sucrase activity. It has subsequently been found, however, (J. Woodward and A. Wiseman, unpublished), that one part of Perid reagent diluted by two parts of water was insensitive to glucose concentrations greater than $30 \mu M$, (see Fig. 3.2.). Presumably at such dilutions the enzyme couple and chromagen becomes limiting.

3.3.2. - The Effect of different sucrose concentrations on the sucrase activity of Perid - Tris, Perid 1, 2 and 3

Fig. 3.3. shows that the Perid reagent had various amounts of sucrase activity as seen with batches 1, 2 and 3. The use of Tris was shown to inhibit the sucrase activity in the Perid reagent. Perid 1 possessed the greatest amount of contaminating sucrase activity. The Boehringer Corporation, who market Perid, found that a particular batch of reagent possessed 0.7% sucrase and stated that this was much too high for the determination of glucose in the presence of sucrose (The Boehringer Corporation (London) Ltd., personal communication). Indeed, it was found that with some Perid reagents, even when made up
Fig. 3.1. - Standard curves for glucose in modified and unmodified Perid reagents

$10^2 \times \text{Concn. of glucose in Perid (µmol.)}$

- - Perid - Tris; O--O Perid 1; △--△ Perid 2; □--□ Perid 3.
Fig. 3.2. - Standard curves for glucose in diluted Perid reagent
Fig. 3.3. - Effect of sucrose concentration on sucrase activity in Perid.
with 0.1M - Tris - HCL buffer, pH 7.2, inhibition of the contaminating sucrase in Perid was not sufficient to give an accurate assay reagent for determining glucose in the presence of sucrose. Frost et al., (1968) assayed French bean and potato tuber invertases by determining the glucose produced by the action of the enzyme on sucrose with a glucose oxidase - peroxidase reagent modified by the addition of Tris to give a final concentration of 0.3M. This modified reagent inhibited interference in the assay by the contaminating maltase.

It was found that inhibition of very high levels of sucrase activity in Perid could be achieved by dissolving the Peric powder in 0.4M - Tris - HCL buffer pH 7.2, giving a reagent (highly contaminated with sucrase activity) suitable for routine assay of yeast invertase. Frost (G.M. Frost personal communication) found that pH 7.6 was better than at pH 7.2. It is therefore suggested that the Perid powder is dissolved in 0.4M - Tris - HCL buffer, pH 7.6 to produce maximal inhibition of the high level of contaminating activity found in certain batches of Perid.

3.3.3. - **Standard curve for reducing sugar done with**

**the 3,5- dinitrosalicylic acid reagent**

The glucose and fructose standard curve (see Fig. 3.4) was performed in the range 10 to 50 μmol. of reducing sugar in 3.5 ml. volume containing 0.1M - Sodium acetate buffer, pH 4.7 (2.0 ml.), 10% W/V sucrose (1.4 ml.) and 0.1 ml. of reducing sugar. 2.5 ml. of 3,5 - dinitrosalicylic acid reagent was added and colour development was performed as described in section 3.2.3. A linear range of extinction was observed.
Fig. 3.4. - Standard curve for reducing sugar in the 3\textsuperscript{15} dinitrosalicylic acid reagent.

Concn. of reducing sugar in 3\textsuperscript{15} dinitrosalicylic acid reagent (\textmu mol.)
3.3.4.1. - Perid assay

a) Let $\propto = \mu$mol. glucose pipetted into 5 ml. Perid
   (obtained from standard curve for glucose)
   
   $\therefore$ in 2.0 of assay mixture there are $20 \times \propto \mu$mol. of glucose

b) $20 \propto \mu$mol. glucose produced in 10 min. by 0.1 ml. of diluted enzyme at 25°C

c) $2 \propto \mu$mol. glucose produced per min. by 0.1 ml. of diluted enzyme at 25°C

d) Since 1 $\mu$mol. sucrose is hydrolysed to 1 $\mu$mol. glucose,
   then $2 \propto \mu$mol. of sucrose are hydrolysed per min. by 0.1 ml.
   of diluted enzyme at 25°C

i.e. $20 \propto \mu$mol. of sucrose hydrolysed/min./ml. diluted enzyme at 25°C

   Activity of diluted enzyme = $20 \propto$ units

3.3.4.2. - 3′5 Dinitrosalicylic acid reagent assay

a) Let $\propto = \mu$mol. of reducing sugar in total assay mixture
   ($\propto$ obtained from standard curve for reducing sugar)
   
   $\therefore \propto/10 \mu$mol. of reducing sugar produced in 10 min. by 0.1 ml.
   of diluted enzyme at 25°C

b) $\propto/10 \mu$mol. reducing sugar produced per min. by 0.1 ml. of diluted enzyme at 25°C
c) Since 1 \mu mol. sucrose is hydrolysed to 2 \mu mol. of reducing sugar (1 \mu mol. glucose + 1 \mu mol. fructose) then \( \frac{x}{20} \) \mu mol. of sucrose are hydrolysed per min. by 0.1 ml. of diluted enzyme at 25°C 

i.e. \( \frac{x}{2} \) \mu mol. of sucrose hydrolysed/min./min./ml. diluted enzyme at 25°C 

Activity of diluted enzyme = \( \frac{x}{2} \) units.

3.3.5. - Determination of invertase activity

The activity of grade \( \text{VI} \) and grade \( \bar{X} \) invertase was determined by the Perid and 3'5 dinitrosalicylic acid assay techniques and the values obtained were similar by both methods.
3.4. - Discussion

Invertase activity has been estimated using a modified Perid reagent which has provided a good method for routine assay. Perid in fact, has several advantages over other methods that are used in measuring reducing sugar. It is a relatively harmless reagent and it is four times more sensitive than the glucose oxidase - peroxidase coupled reagent with 0 - dianisidine as the chromagen (Werner et al., 1970). It is one hundred times more sensitive than the 3‘5’ - dinitrosalicylic acid reagent that has also been used to monitor invertase activity.

The Perid reagent is intended for blood glucose determinations and therefore the contaminating sucrase activity of Perid does not interfere in these measurements. However, even Perid batches containing high sucrase activity may be used satisfactorily when assaying glucose in the presence of sucrose if the Perid powder is dissolved in Tris buffer since Tris is a fully competitive inhibitor of sucrase (Kolinska and Semenza, 1967). These authors studied the kinetics of the inhibition of intestinal sucrase by Tris and found that Ki for Tris was 0.4mM at pH 6.7. Semenza and von Balthazar (1974) suggested that Tris competed with sucrase for the glucosyl subsite of intestinal sucrase. They found that inhibition by Tris was stronger at alkaline pH and showed that the amino group was important in the inhibition since N-Tris (hydroxymethyl) methyl glycine did not inhibit sucrase.

It would therefore seem likely that the inhibition of sucrase in glucose oxidase by Tris is due to competition with sucrose for the substrate binding site, which may involve a carboxylate group (Waheed and Shall, 1971a). It may therefore be that the unprotonated amino group of Tris is important since inhibition is better at alkaline pH values.
The 3,5 dinitrosalicylic acid reagent has also proved to be satisfactory for yeast invertase assay. The one advantage of this method over the Perid assay is that it is cheaper to use. The use of Perid is recommended, however, when very low enzyme concentrations are assayed.
4.1. - Introduction

The purification and a comparison of properties of external and internal invertase by previous investigators have been described (see section 1.6.).

The production of external invertase in some strains of yeast is subject to glucose repression (Gascon and Ottolenghi, 1967). Glucose repression of external invertase formation by S. cerevisiae (N.C.Y.C. No.525) was attempted in order to facilitate the isolation and purification of internal invertase from this strain, so that this strain could be used for the routine production of the internal enzyme.

The presence of multiple forms of invertase is ubiquitous throughout nature. Multiple forms of an enzyme (isoenzymes) in a particular species may be responsible for its ability to withstand milieu-directed changes. The isoenzymes will differ in their properties and their relative activity will be a function of the prevailing environmental conditions. For example, Chhatpar and Modi (1974) found two invertases in crude extracts of Mangifera indica (mango), one with a temperature optimum at 0°C and the other with a temperature optimum at 37°C. The presence of acid and alkaline invertases is often found in higher plants. Lyne and ap Rees (1971) reported two forms of invertase in pea roots, one with a pH optimum of 4 and the other with a pH optimum of 7. Similar invertases were extracted from carrot root (Ricardo and ap Rees, 1970). The location of these invertases is different and each form will therefore be suited to work efficiently under the conditions prevailing in its own locale.
Invertase from the fungus *Neurospora crassa* was shown to occur in two active forms which were separable by gel filtration into a heavy and light form (Metzenberg, 1964). The heavy form could be dissociated into the light form by heating or by high salt concentration. It was suggested that the light form represented four active sub-units of the heavy form (Lampen, 1971). It was subsequently shown that pure invertase from *N. crassa* possessed a molecular weight of 210,000 and upon dissociation by 6M guanidine the molecular weight reduced to 51,500, suggesting that the native enzyme was a tetrameric molecule (Meachum et al., 1971).

The presence of multiple forms of yeast invertase has been described (see section 1.5.2.), although the nature and function of them is uncertain. Similarly, the number of sub-units making up the invertase molecule has not yet been established.

The reaction of sodium dodecylsulphate (S.D.S.) with proteins, frequently causes their dissociation into sub-units and denaturation of the individual polypeptide chains. The individual polypeptide chains assume an overall negative charge because of the binding of S.D.S. to them. In the presence of a reducing agent the molecular weights of the S.D.S. - polypeptide chain complex can be determined by gel electrophoresis since separation is based upon size rather than charge. Weber and Osborn (1969) demonstrated in gels giving significant molecular exclusion, the existence of a near-linear relationship between electrophoretic mobility of a protein - S.D.S. complex and the molecular weight of the protein. Calibration of a gel can be performed using proteins of known molecular weight from which the molecular weight of the protein - S.D.S. complex under investigation can be determined. By this method the number of sub-units in the native enzyme can be determined.
The molecular weight of human erythrocyte 6-phosphogluconate dehydrogenase was shown to be 104,000 by gel filtration, sedimentation and diffusion data. The molecular weight of the S.D.S. protein complex was calculated to be 52,000, indicating that the native enzyme exists as a dimer (Pearse and Rosemeyer, 1974). Similarly, glyceraldehyde 3-phosphate dehydrogenase has been shown to consist of four identical sub-units (Marangos and Constantinides, 1974). Bornmann and Hess (1974) found pyruvate kinase to be composed of four identical sub-units of molecular weight 50,000. However, none of these proteins were glycoproteins.

The work presented in this chapter describes the isolation and purification of external and internal invertases from Baker's yeast and S. cerevisiae (N.C.Y.C. No. 525). The existence of multiple forms of external invertase is established and the nature of their formation, separation and role is discussed. Some of the properties of external, internal and isoenzymic forms of invertases are described and compared to grade X invertase which is a highly purified preparation obtained from Candida utilis yeast. An attempt to determine the size and number of sub-units in the yeast invertase molecule by S.D.S. gel electrophoresis is described. Finally, the nature of the thermal stability of yeast invertases is discussed.
4.2. - Materials and Methods

4.2.1. - Isolation and purification of external and internal invertases

For the large scale preparation of external and internal invertase, 450 g. of Baker's yeast (purchased from Cranks Health Shop) was stirred into 1 litre of 0.1M sodium acetate buffer, pH 4.7 to make a smooth paste of yeast cells. High solubilization of invertase activity from yeast was achieved using the Vibro-Mill disintegrator to disrupt the yeast, following the method of Williams and Wiseman (1973). 45 g. samples of yeast paste were added to the 210 ml. stainless steel container of the Vibro-Mill (E. Buhler, Tubingen, West Germany) and glass beads (diameter 0.15mm) were added to fill the container. The mixture was shaken violently for exactly 10 min. as cold water was pumped around the container. After disruption of the yeast, the beads were decanted off and washed with 0.1M sodium acetate buffer, pH 4.7, and the washings added to the yeast disruptate. Cell debris was removed by centrifugation.

The separation and purification of external and internal invertases from this crude extract was done by following the basic procedure of Gaecon and Lampen (1968).

4.2.1.1. - Ammonium sulphate precipitation

To the yeast disruptate (2,500 ml), 516 g. of solid ammonium sulphate per litre were added (80% saturation) and the solution was stored overnight at 4°C. Separation of external and internal invertase was thus achieved since internal invertase is readily precipitated in 80% saturated ammonium sulphate, whereas external invertase is relatively soluble.

The precipitate, containing internal invertase, was collected by centrifugation at 10,000g for 10 min. The precipitate was suspended in 200 ml. of 10mM sodium phosphate buffer, pH 7.0 into which most of the invertase activity dissolved. The undissolved precipitate was removed.
by centrifugation at 10,000 x g for 10 min. The ammonium sulphate supernatant was stored at 4°C for subsequent extraction of external invertase.

4.2.1.2. - **Dialysis at pH 4.0**

The dissolved precipitate (containing internal invertase) was dialysed against 0.05M sodium acetate buffer, pH 4.0 at 4°C overnight. The inactive precipitate was removed by centrifugation at 10,000 g for 10 min. and the supernatant which contained all the invertase activity was dialysed against 10mM sodium phosphate buffer, pH 7.0 prior to DEAE - Sephadex A-50 chromatography.

4.2.1.3. - **DEAE - Sephadex chromatography**

47.0 ml. of pre-swollen DEAE - Sephadex A-50 was added to the dialysed supernatant and stirred at 4°C for 4 hr. after which time all the invertase activity was adsorbed. The DEAE - Sephadex A-50 was washed twice with 500ml. of equilibrating buffer, then poured in a glass column to give bed dimensions of 1.4 x 30 cm. Enzyme elution was then performed as previously described (see Section 2.4.2.). Internal invertase was eluted with 0.3M NaCl.

4.2.1.4. - **Ammonium Sulphate supernatant**

The ammonium sulphate supernatant containing external invertase activity was dialysed against running tap water to remove all the ammonium sulphate. DEAE - Sephadex A-50 (5g, dry weight, previously equilibrated with 10mM sodium phosphate buffer, pH 7.0) was added to the enzyme solution and stirred at 4°C for 4 hr. All external invertase was adsorbed after this time. The supernatant was discarded and the ion exchange agent was washed twice with 1 litre of fresh buffer. The
external invertase was removed by washing the ion exchanger with 168 ml of 10 mM sodium phosphate buffer, pH 7.0 containing 0.5 M NaCl. The eluted enzyme was dialysed against 0.1 M sodium acetate buffer, pH 4.7 to remove salt and then freeze dried.

4.2.2. - Isolation of internal invertase from Saccharomyces cerevisiae (N.C.Y.C. No. 525)

Cells of S. cerevisiae (N.C.Y.C. No. 525) were cultured in medium 2 as previously described (see Section 2.2). The cells were cultured in high glucose medium since growth under these conditions is known to repress external invertase formation without affecting the levels of internal invertase (Gascon and Ottolenghi, 1959; Gascon et al., 1973). It was therefore supposed that S. cerevisiae (N.C.Y.C. No. 525) would be subject to glucose repression and that most invertase activity would be due to the internal form of the enzyme.

For the isolation and purification of internal invertase, cells were harvested to give 18 g. of cell paste, and the procedure described for large scale preparation of internal invertase was mainly followed except that after precipitation with ammonium sulphate, the precipitate was suspended in 100 ml. of 0.1 M sodium acetate buffer, pH 4.7, and dialysed overnight at 4°C. The dialysate was collected and centrifuged on a bench centrifuge. All the invertase activity was recovered in the supernatant. This supernatant was adjusted to pH 7.0 with 0.5 M disodium phosphate and then applied to a column of DEAE - Sephadex A-50. The enzyme was eluted as described previously (see Section 2.4.2).

4.2.3. - The preparation of yeast invertase isoenzymes

For the preparation of invertase isoenzymes, grade VI invertase was used. 50 mg. of grade VI invertase was dissolved in 5.0 ml. of 10 mM -
sodium phosphate buffer, pH 7.0 and applied to a column of DEAE - Sphadex A-50. Elution of enzyme was carried out as previously described.

4.2.4. - Thermal stability studies

Thermal inactivation of the prepared invertases was carried out as described (see Section 2.7.). Internal invertase was heated at pH 7.0 since the enzyme is stable between pH 6 and pH 9 (Gascon et al., 1968). The other preparations were heated at pH 4.7.
4.3. - Results

4.3.1. - Large scale purification of internal invertase

Table 4.1 summarises the purification of internal invertase from Baker's yeast. Estimation of internal invertase in the yeast disruptate, in the pH 4.0 dialysate and after DEAE - Sephadex A-50 chromatography was done by gel filtration of a small amount of each fraction on Sephadex G-200 (see Fig. 4.1. A.B.C.). The majority of invertase activity was not precipitated by ammonium sulphate and that which was precipitated, consisted of approximately 25% internal invertase activity. The purification obtained in terms of protein was small. After dialysis against pH 4 buffer there was an approximate 200-fold purification in terms of protein. The remainder of contaminating protein was separated from internal invertase by DEAE - Sephadex A-50 chromatography, which resulted in a further 3-fold purification in terms of protein. Fractionation of the pH 4 dialysate on DEAE - Sephadex A-50 resulted in separation of internal invertase from the contaminating external invertase (see Fig. 4.2.). The active fractions of peak 2, containing the internal enzyme, were pooled. The identity of peak 2 as being internal invertase, was confirmed by gel filtration on Sephadex G-200. The eluted enzyme had a Ve/Vo ratio of 1.6, which is indicative of the 'lighter' internal enzyme compared to the external form which is eluted with the void volume, Ve/Vo = 1.05. (Gascon and Lampen, 1968). No mannan was detected in the internal invertase.

The purification obtained overall is in the order of 700-fold in terms of protein which was disappointing since Gascon and Lampen (1968) achieved a 2,500-fold purification. However, they used 15.3 Kg. of yeast from which they isolated and purified internal invertase. In my laboratory, the disruption of such large quantities of yeast with the Vibro-Mill was not practicable. Furthermore, their purification procedure resulted in only a 5% recovery of original crude internal
Table 4.1. - Purification of internal invertase from Baker's yeast

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume ml</th>
<th>Protein mg</th>
<th>Total invertase units</th>
<th>Internal invertase</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast paste</td>
<td>1,000</td>
<td>* n.d.</td>
<td>675,000</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Crude extract</td>
<td>2,500</td>
<td>12,900</td>
<td>531,000</td>
<td>13,385</td>
<td>1.04</td>
</tr>
<tr>
<td>Ammonium Sulphate (precip.)</td>
<td>200</td>
<td>6,375</td>
<td>27,500</td>
<td>7,920</td>
<td>1.24</td>
</tr>
<tr>
<td>Dialysis against pH4 buffer (Supernt.)</td>
<td>350</td>
<td>192</td>
<td>20,300</td>
<td>4,200</td>
<td>224</td>
</tr>
<tr>
<td>DEAE - ** Sephadex A-50 column</td>
<td>150</td>
<td>2.5</td>
<td>1,763</td>
<td>1,763</td>
<td>705</td>
</tr>
</tbody>
</table>

* n.d. - not determined
** corresponding to activity of peak 2 (see Fig. 4.2.)
Fig. 4.1. - Gel filtration in Sephadex G-200 of fractions obtained in the purification of internal invertase from Baker's yeast.

In A, 400 units of crude extract; in B, 160 units of pH4 dialysate and in C, 1,380 units of internal invertase were applied to the column. 11.5ml. fractions collected.
Invertase (l O " x units/fraction)

Fig. 4.2. - Fractionation of the internal invertase
on DEAE - Sephadex A-50

The pH4 dialysate was adjusted to pH7 with 0.5M disodium phosphate and adsorbed on to pre-swollen DEAE - Sephadex A-50.
The adsorbed enzyme was washed with 1 litre of 10mM sodium phosphate buffer pH7 and fractionated in a glass column, by stepwise elution with NaCl in the buffer at the arrows indicated.
Fractions of volume 11.5ml. were collected.
invertase activity. The procedure used in the present study, however, resulted in approximately 13% recovery of original material. Further purification of internal invertase would have drastically reduced the recovery of activity, leaving very little enzyme for studies on its chemical modification and stability.

4.3.2. - **Large scale purification of external invertase**

Table 4.2 summarises the purification of external invertase from Baker's yeast. The starting material was the ammonium sulphate supernatant obtained during the purification of internal invertase. The overall purification was approximately 2,500-fold in terms of protein with a recovery of about 30% of the starting enzyme activity in the yeast paste. The purified external invertase had a specific activity of 2,537 units/mg. of protein at 25°C. This value compares favourably with some of the purest external invertase preparations that have been obtained (see Table 1.1.). The purified enzyme contained 62% (w/v) mannan.

4.3.3. - **Purification of internal invertase from**

*Saccharomyces cerevisiae* (N.C.Y.C. No.525)

Table 4.3. summarises the purification of internal invertase from *S. cerevisiae* (N.C.Y.C. No.525). Gel filtration in Sephadex G-200 of the crude extract and the pH 4.7 dialysate was performed to estimate the internal invertase activity (see Fig. 4.3. A,B.).

Growth of cells in high glucose media did not repress the formation of external invertase, and internal invertase represented only 5% of total activity in the crude cell-free extract. Only about 7% of activity precipitated by ammonium sulphate was due to internal invertase. The overall purification was 1,200-fold in terms of protein. The low yield of internal invertase after ammonium sulphate precipitation may be due to
Table 4.2. - Purification of external invertase from Baker's yeast

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume ml.</th>
<th>Protein mg.</th>
<th>Invertase units</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Sulphate (supernatant)</td>
<td>2,430</td>
<td>n.d.</td>
<td>491,000</td>
<td>n.d.</td>
</tr>
<tr>
<td>Dialysis against running water</td>
<td>3,120</td>
<td>n.d.</td>
<td>448,500</td>
<td>n.d.</td>
</tr>
<tr>
<td>DEAE - Sephadex A-50 Batch</td>
<td>168</td>
<td>105.9</td>
<td>270,900</td>
<td>2,559</td>
</tr>
<tr>
<td>chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialysis against buffer pH 4.7</td>
<td>* 210</td>
<td>86.4</td>
<td>219,200</td>
<td>2,537</td>
</tr>
</tbody>
</table>

* Freeze drying of the desalted material yielded 200mg. of enzyme.
Table 4.3. - Purification of internal invertase from *S. cerevisiae N.C.Y.C. No. 525*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume ml.</th>
<th>Protein mg.</th>
<th>Total invertase units</th>
<th>Internal invertase units</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>193</td>
<td>434</td>
<td>2,160</td>
<td>108</td>
<td>0.25</td>
</tr>
<tr>
<td>Ammonium Sulphate (sediment) after dialysis</td>
<td>100</td>
<td>156</td>
<td>67</td>
<td>4.8</td>
<td>0.03</td>
</tr>
<tr>
<td>DEAE - <em>Sephadex A-50 column</em></td>
<td>18</td>
<td>0.02</td>
<td>6.0</td>
<td>6.0</td>
<td>300</td>
</tr>
</tbody>
</table>

* Corresponding to activity of peak 2 (see Fig. 4.4.)
Fig. 4.3. - Gel filtration in Sephadex G-200 of fractions obtained in the purification of internal invertase from S. cerevisiae (N.C.Y.C. No. 525)

In A 5.6 units of crude extract; in B 0.33 units of pH 4.7 dialysate were applied to the column. 4.5ml. fractions collected by elution with 0.1M sodium acetate buffer pH 4.7.
the instability of invertase in ammonium sulphate. Other investigators have also noted loss of invertase activity when using ammonium sulphate precipitation in their purification procedure (Neumann and Lampen, 1967; Gascon and Lampen, 1968; Smith and Ballou, 1974a). DEAE - Sephadex A-50 column chromatography of internal invertase gave two peaks of activity (see Fig. 4.4). Peak 2 was internal invertase eluted by 0.3M NaCl and contained no mannan. This procedure removed most of the protein that was not part of the internal enzyme.

4.3.4. Preparation of yeast isoenzymes

Grade VI invertase was fractionated into four separate peaks of activity by DEAE - Sephadex A-50 column chromatography (see Fig. 4.5.). The most active fractions of each peak were pooled and the pooled isoenzymes had characteristics given in Table 4.4. The isoenzymes possessed different mannan : protein ratios and were eluted from the column in order of decreasing mannan contents. Thus isoenzyme I (75% (w/v) mannan) was eluted first whilst isoenzyme IV (0% (w/v) mannan), which may have been the internal invertase used, was eluted last, by 0.15M-NaCl and 0.3M-NaCl respectively. Waheed and Shall (1971a) fractionated a yeast invertase concentrate into three peaks of activity on DEAE - Sephadex A-50 whose carbohydrate : protein ratios differed. They found that the material of lowest carbohydrate content was more firmly retained in the column, showing that it had a higher net negative charge which they presumed might be due to the absence of sugar amine groups. In contrast to the work of these authors, the specific activity of isoenzymes I - IV was not inversely correlated with their mannan contents, suggesting that there may be other explanations for the relationship between mannan content and specific activity of invertase preparations. Whilst the work of this thesis was in progress, Colonna et al., (1975) fractionated external invertase by
The pH 4.7 dialysate was adjusted to pH7 with 0.5M disodium phosphate and adsorbed on to a column of DEAE - Sephadex A-50 (1.4 x 30cm). During the adsorption 50mg. inactive protein was eluted. Stepwise elution with NaCl in 10mM - sodium phosphate buffer was begun at the arrows indicated. Fractions of 4.5ml. were collected.
Invertase (50mg.) was applied to a column of DEAE - Sephadex A-50 (1.4 x 30cm.) previously equilibrated with 10mM - sodium phosphate buffer, pH 7.0.

After the enzyme was adsorbed, the column was washed with 144ml. of the same buffer and then stepwise elution with NaCl in the buffer was begun at the arrows indicated. Fractions of volume 4.5ml. were collected.
Table 4.4. - A summary of the characteristics of the isoenzymes obtained after the fractionation of grade VI invertase on DEAE - Sephadex A-50

<table>
<thead>
<tr>
<th>Isoenzymes</th>
<th>Volume ml.</th>
<th>Activity (units)</th>
<th>Protein mg.</th>
<th>Specific activity</th>
<th>Mannan mg.</th>
<th>% Mannan</th>
<th>% Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>33</td>
<td>393.6</td>
<td>0.53</td>
<td>745.4</td>
<td>1.55</td>
<td>74.6</td>
<td>25.4</td>
</tr>
<tr>
<td>II</td>
<td>45</td>
<td>1,896.6</td>
<td>1.44</td>
<td>1,317.0</td>
<td>0.72</td>
<td>33.3</td>
<td>69.7</td>
</tr>
<tr>
<td>III</td>
<td>36</td>
<td>736.4</td>
<td>0.61</td>
<td>1,203.4</td>
<td>0.09</td>
<td>12.8</td>
<td>87.2</td>
</tr>
<tr>
<td>IV</td>
<td>16.5</td>
<td>31.2</td>
<td>0.082</td>
<td>377.2</td>
<td>none</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
hydroxyapatite column chromatography into five isoenzymes whose mannan : protein ratios were different. They also concluded that it was these differing ratios that were responsible for the observed heterogeneity of invertase.

4.3.5. - A comparison of some of the properties of the prepared invertases and grade X invertase

4.3.5.1. - The effect of pH on enzyme activity and stability

The effect of pH on the activity of purified Baker's yeast invertase, internal invertase and Candida utilis grade X invertase (specific activity = 1,013 units/mg. protein at 25°C), is shown in Fig. 4.6. The optimum pH for each enzyme was pH 4.7. It was thought that the differences in the specific activity of isoenzymes I - II might be due to their different pH optima. However, the pH optimum of each isoenzyme was identical to purified external invertase. The effect of pH on the stability of the prepared and grade X invertases is shown in Fig. 4.7. The pH stability of purified external invertase and grade X invertase differs markedly. The external enzyme is stable at 50°C from pH 3.6 to 6.0 whereas grade X invertase is stable at 50°C from pH 3.6 to pH 8.0. Internal invertase is stable at 30°C between pH 6 to pH 9 (Gascon et al., 1968). Since internal invertase is devoid of mannan, low mannan containing invertases may be expected to show similar pH stability to the internal enzyme. The isoenzymes I - IV, however, were stable between pH 3.6 and pH 6.0 suggesting that a low mannan content does not alter the pH stability of the enzyme.

4.3.5.2. - The effect of temperature on the enzyme stability

The thermal stability of external and internal invertase at 65°C in the absence of substrate was measured. Internal invertase was
Fig. 4.6. - Effect of pH on activity of yeast invertase

Key
- O-O Purified Baker's yeast external invertase
- △-△ Purified Baker's yeast internal invertase
- □-□ *Candida utilis* grade X invertase
Fig. 4.7. The effect of pH on the stability of yeast invertase

Key -
- O-O Purified Baker's yeast external invertase
- □-□ Candida utilis grade X invertase
- ●-● Isoenzyme II

% original activity vs. pH
completely inactivated after 1 min. of treatment. Internal invertase was thermally inactivated at temperatures of 50°C and above (Fig. 4.8. A) whereas the external enzyme was thermally inactivated at temperatures of 65°C and above (Fig. 4.8. B). The thermal stabilities of external invertase and grade X invertase were compared (Fig. 4.8. B).

Candida utilis grade X invertase was much more thermally stable than Baker’s yeast external invertase in the absence of the substrate sucrose, the former enzyme losing only 25% of its activity after 10 min. of heating compared to a loss of over 90% of activity for external invertase after the same time of heating.

The reason for the lower thermal stability of internal invertase compared with the other two enzymes, appears to be due to the absence of a mannann moiety. Furthermore, when internal invertase is heated in the presence of mannann at pH 4.7, its thermal stability becomes similar to that of external invertase (Goldstein and Lampen, 1975). This work found that when internal invertase was heated even in the presence of 50mg./ml. of mannann at 50°C and pH 7.0, there was no significant change in its thermal stability. Presumably, the presence of mannann does alter the pH stability of internal invertase. An explanation is therefore required for the finding of this thesis, of why low mannann containing invertases possess similar pH stabilities to high (50% (w/v)) or more) mannann containing invertases.

The rate of thermal inactivation obtained for each of the four isoenzymes (I - IV) was found to be correlated inversely with its mannann content (see Fig. 4.9). The pseudo-first order inactivation rate constants (min⁻¹) of each isoenzyme were I, 0.11 (most stable); II, 0.22; III, 0.55; IV, 0.69 (least stable). These results seem to confirm the stabilizing role of mannann, but contradicting this result is the finding by Smith and Ballou (1974a) that removal of all the mannann
Fig. 4.8. - Thermal inactivation of yeast invertases

In A the temperature of heating was 50°C
In B the temperature of heating was 65°C

Key - ●● internal invertase
○○ external invertase
□□ grade X invertase
Prior to heat treatment, the pH of each isoenzyme was adjusted to pH 4.7 with 0.2M acetic acid and a small portion was desalted on a column of Sephadex G-50 (1.4 x 30cm.) equilibrated with 0.1M - sodium acetate buffer pH 4.7. The protein concentration of each desalted isoenzyme to be heated was 7ug./ml.

Key –  O-O, I;  □-□, II;  ○-○, III;  △-△, IV

Values are mean ± S.D.
from external invertase by digestion with \(\alpha\)-exo-mannanase, did not affect the thermal stability of the enzyme. An explanation is therefore required as to why the removal of mannan from external invertase leaves an active and stable conformation.

4.3.5.3. - **The determination of the kinetic parameters**

**Km and Vmax of yeast invertase preparations**

For the determination of Km and Vmax of each preparation, similar concentrations of enzyme were used. The Km values of each preparation were very similar, as were the values of Vmax (see Table 4.5.). It can be concluded from the similarity in Km values for isoenzymes I - IV that since invertases with different mannan contents possess similar catalytic properties, mannan therefore, has no effect on the catalytic activity of invertase.

4.3.5.4. - **Molecular weight studies**

Since the difference in molecular weight of external and internal invertase is due to the mannan moiety, it was decided to investigate whether isoenzymes I - IV would have different molecular weights because of the difference in their mannan contents. Each isoenzyme was eluted close to the void volume by gel filtration with Sephadex G-200, but it was especially noticeable that isoenzyme IV (which apparently possesses no mannan) appeared a larger molecule than even blue dextran which has a molecular weight of 2,000,000 (see Fig. 4.10). While this work was in progress, Smith and Ballou (1974b) noted that an invertase from which all the mannan had been removed appeared slightly larger than the native glycoenzyme by Sephadex gel filtration. They concluded that the mannan attached to the native enzyme had interacted with the dextran polymer thereby retarding elution. Based upon this reasoning, it is difficult
Table 4.5. - Kinetic parameters for yeast invertase preparations obtained from the double-reciprocal plot analysis

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Sucrose Km (mM)</th>
<th>Vmax μmole min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>External invertase</td>
<td>28.5</td>
<td>20.0</td>
</tr>
<tr>
<td>Internal invertase</td>
<td>25.0</td>
<td>-</td>
</tr>
<tr>
<td>C. utilis Grade X</td>
<td>27.7</td>
<td>20.5</td>
</tr>
<tr>
<td>Isoenzyme I</td>
<td>26.4</td>
<td>20.9</td>
</tr>
<tr>
<td>II</td>
<td>31.2</td>
<td>18.5</td>
</tr>
<tr>
<td>III</td>
<td>28.4</td>
<td>20.0</td>
</tr>
<tr>
<td>IV</td>
<td>30.0</td>
<td>18.9</td>
</tr>
</tbody>
</table>

* Data from Gascon et al., (1968)
Fig. 4.10. - Gel filtration in Sephadex G-200 of isoenzymes I - IV

Blue Dextran

2.75 units isoenzyme I (○○); 6.0 units isoenzyme II (□□)
2.0 units isoenzyme III (●●); 1.25 units isoenzyme IV (ΔΔ)

applied to the column. Fractions of volume 2.8ml. were collected.
to see how internal invertase fits into this situation since it behaved in the usual way on Sephadex, having a $V_e/V_o$ ratio of 1.6 (see Fig. 4.1.C) which corresponds to a molecular weight of about 135,000 (Gascon and Lampen, 1968). Since it is 'mannanless' it might have been expected to behave similarly to isoenzyme IV on gel filtration in Sephadex. Interestingly, Smith and Ballou (1974b) showed that their 'mannanless' external invertase behaved similarly to internal invertase in Sephadex after their preparation had been pre-incubated in the presence of dithiothreitol.

Dithiothreitol may therefore reduce a chemical group(s) on the surface of the external invertase molecule which is exposed by removal of mannan and which interacts with the polymer, this interaction being responsible for repulsion of the enzyme during column elution.

Clearly, Sephadex G-200 is not satisfactory for the molecular weight determinations of native and 'mannanless' external invertase preparations. Neumann and Lampen (1967) determined the molecular weight of external invertase to be 270,000, based upon sedimentation velocity studies. Grade $\overline{X}$ invertase was also eluted close to the void volume and thus its molecular weight could not be estimated. Iizuka et al., (1974) estimated the molecular weight of C. utilis invertase to be 300,000 using Bio-Gel P-150 gel chromatography, and by ultracentrifugal analysis, which is similar to that of external invertase.

Isoenzyme IV was pre-incubated with dithiothreitol (10mM) at 30°C for 1 hr. and then subjected to Sephadex G-200 gel filtration. The enzyme was eluted with the void volume and thus showed no decrease in molecular size by this pre-incubation treatment, which might have been expected from the experimental data of Smith and Ballou (1974b).

Gel filtration of each isoenzyme was also performed in L.K.B.
Ultragel (Aca22) which is a prepared polyacrylamide/agarose gel that fractionates molecular weights in the range 60,000 - 1,000,000. Each enzyme was eluted with the void volume, which showed that similar gel filtration behaviour to that in Sephadex G-200 was occurring.

4.3.5.5. - Polyacrylamide gel electrophoresis of the invertase preparations

Fig. 4.11. shows the gel electrophoresis of the invertase preparations at pH 8.9. Gels were stained for protein and enzyme activity and both were coincident in all the gels. It is apparent that at pH 8.9 the preparations were heterogenous, which may be due to their instability at this pH (Gascon et al., 1968). Grade X invertase appeared to consist of two isoenzymes. This heterogeneity may also be due to invertase molecules possessing mannan chains of varying lengths which results in charge differences. At pH 8.9 internal invertase moves in the gel with the bromophenol blue marker (Gascon et al., 1968). Fig. 4.12 shows the gel electrophoresis of external invertase, internal invertase and grade X invertase at pH 7.0. External invertase was detected at the top of the gel showing that it has very little electrophoretic mobility at this pH. Similar results were obtained for the isoenzymes I - IV. Internal invertase and grade X invertase showed one band of protein of greater mobility than the external enzyme. Protein and enzyme activity in the gels were coincident.

4.3.5.6. - S.D.S. polyacrylamide gel electrophoresis

This study was carried out to determine the molecular weight and hence the number of sub-units in the external invertase molecule. The gels were calibrated with S.D.S. denatured bovine serum albumin B.S.A. (m.w. 68,000), Creatine Kinase (40,000), Trypsin (23,300) and
Fig. 4.11. - Polyacrylamide gel electrophoresis of invertase preparations at pH 8.9

A - purified Baker's yeast external invertase
B - C. utilis grade X invertase
I - IV - Isoenzymes from grade VI invertase

Shaded areas represent protein detected in the gels.
(Enzyme activity in the gels was coincident with the protein)
Fig. 4.12. - Polyacrylamide gel electrophoresis of invertase preparations at pH 7.0

A - purified Baker's yeast external invertase
B - purified Baker's yeast internal invertase
C - C. utilis grade X invertase

Shaded areas represent protein and enzyme activity in the gels which were coincident.
Cytochrome C (11,700). Fig. 4.13 illustrates the electrophoretic mobility of these proteins as a function of their molecular weight. It also shows the extrapolated molecular weights of S.D.S. denatured external invertase and grade X invertase to be 90,000 and 81,000 respectively. The bands of S.D.S. denatured invertase protein, however, were broad and faint suggesting that there was a heterogeneous population of molecules. Therefore, it is unlikely that the values quoted are a true indication of the sub-unit molecular weight. Yeast invertase is resistant to denaturation by S.D.S. and it is likely that S.D.S. does not bind to the protein moiety due to protection by the mannau moiety. Consequently, separation of S.D.S. denatured invertase will be due to charge as well as to size. S.D.S. gel electrophoresis of internal invertase should provide a better indication of the true sub-unit molecular weight. This was not performed due to the lack of sufficient material for detection on the gel.
Fig. 4.13. - Relative mobility of S.D.S. denatured proteins in gels as a function of molecular weight.

The arrows 1 and 2 indicate the mobility of purified external invertase and grade X invertase respectively.
External and internal invertases have been isolated and purified from Baker's yeast in order that they may be used for chemical modification and stability studies. The purity of external invertase in terms of specific activity (units per mg. of protein) is comparable with previously published results (Lampen, 1971). The specific activity of internal invertase was lower than previously reported values (Gascon and Lampen, 1964; Baeer and Shall, 1971), but further purification was foreseen for a high overall yield of activity. The use of *Saccharomyces cerevisiae* (N.C.Y.C. No. 525) proved unsatisfactory for routine use in the isolation and purification of internal invertase because of the low yields of enzyme that were obtained. Furthermore, the formation of external invertase in this strain did not appear to be repressed sufficiently by growth of the cells in high glucose medium. Most of the invertase activity in the crude extract was due to the external enzyme and not the internal enzyme that might have been expected had repression of the external enzyme occurred.

Grade VI invertase (a partially purified external enzyme from Baker's yeast) was fractionated into four isoenzymes whose mannan : protein ratios differed. This is clearly contrary to the belief of Moreno *et al.*, (1975) that external invertase is homogenous. Isoenzymes of external invertase have also been separated by other investigators, (Hoshino *et al.*, 1964; Waheed and Shall, 1971a; Colonna *et al.*, 1975). The basis of separation of external invertase isoenzymes (by anion exchange chromatography and isoelectric focussing) is due to their mannan : protein ratios which result in a different net charge on each form, the forms of lower mannan content being more electronegative, presumably due to the absence of sugar amino groups.
Several distinct physiological functions of isoenzymes have been identified. For example, the function of the lactate dehydrogenase (L.D.H.) isoenzymes LDH₁ and LDH₅ is well established in vitro. LDH₁ and LDH₅ are found predominantly in heart and skeletal muscle tissue respectively. They can be distinguished kinetically. LDH₁ is inhibited by low concentrations of pyruvate which makes it useful to a highly aerobic organ such as the heart. LDH₅ is not inhibited by such low pyruvate concentrations and therefore this isoenzyme can function efficiently in an anaerobic environment such as is found in skeletal muscle during vigorous exercise (Vesel, 1975). There exist two inter-convertible forms of human phosphoribosyl - pyrophosphate amidotransferase which are involved in the regulation of purine biosynthesis. An inactive dimer is split into two active sub-units in the presence of phosphoribosylpyrophosphate, the sub-units recombining in the presence of the nucleotide which provide the rate limiting step in the purine biosynthetic pathway (Holmes et al., 1975).

It is not known whether external invertase isoenzymes have a distinct physiological function, or if they are formed during the course of isolation and purification from the yeast cell. It has already been mentioned that during prolonged autolysis of yeast to extract the enzyme, much of the mannan can be removed (see Section 1.4.). It is likely that a heterogeneous population of enzyme molecules with varying mannan contents may also be achieved by this extraction method.

Isoenzymes with distinct physiological functions can display different kinetic parameters (e.g. The L.D.H. isoenzymes). Since the isoenzymes isolated from grade VI invertase were similar in pH optima, pH stability and Km, it would appear that they are artefacts formed during preparation. Support for this comes from the fact that rapid release of invertase by mechanical breakage of cells usually results in
a homogenous preparation after purification (Neumann and Lampen, 1957). During the purification of internal invertase from Baker's yeast, the external invertase precipitated by ammonium sulphate was clearly homogenous (see Fig. 4.2.). There is therefore no evidence as yet to suggest that the isoenzymes found in Baker's yeast have physiological significance like the multiple forms of invertase found in higher plants (see Section 4.1.).

Studies on the effect of pH on activity and stability of Baker's yeast invertase preparations yielded results which were similar to those found by previous investigators. It was interesting that grade X invertase showed a wider pH stability (pH 3.6 - 8.0) than did the Baker's yeast external invertase. This is in contrast to the work of Izuka et al., (1974) who found C. utilis invertase to be stable only between pH 3 - 6. All invertase preparations tested had similar pH optima and kinetic parameters Km and Vmax.

It is clear that there is a relationship between the mannan content of invertase and thermal stability. Internal invertase, the naturally occurring mannan-free enzyme in yeast is the least stable towards heat. External invertase preparations are much more thermally stable, which appears to be due to the mannan moiety. Since isoenzyme I is more thermally stable than isoenzyme IV this would seem to confirm the stabilizing role of mannan which is in agreement to the work of Arnold (1969). Further support for the idea that mannan stabilizes the protein conformation comes from the work of Neumann et al., (1969), who found that pepsin was more easily denatured than its zymogen, pepsinogen, which is a glycoprotein that loses its carbohydrate moiety to become active.

Recently, the stabilization of enzymes (not glycoenzymes) has been achieved by glycosylation. Marshall and Rabinowitz (1975) coupled trypsin, \( \alpha \) - and \( \beta \) - amylases to cyanogen bromide activated dextran and
found that all three conjugates were more stable to heat than the native enzymes. Lysozyme and β-glucosidase were distinctly more stable towards heat after glycosylation (Christensen et al., 1976). The improved thermal stability of glycosylated enzymes was explained as being caused by cross-linking between protein and polysaccharide (Marshall and Rabinowitz, 1975). In addition to the possible stabilizing role of the mannan moiety, there appears to be little doubt that mannan protects the protein conformation. Glycoproteins, generally, are resistant to hydrolysis and proteolytic enzymes (Pazur and Arnonson, 1972).

Strumeyer and Malin (1970) have shown that external invertase is very resistant to denaturation by tannins. Negoro and Kito (1973) even used tannic acid to precipitate invertase from extracts of Candida Kefyr during its purification. After the removal of tannic acid the invertase was fully active.

A role for the carbohydrate moiety of glycoenzymes in their catalytic activity has also been suggested. This seems unlikely in the case of invertase since the internal enzyme is as active as the external glycoenzyme. Oxidation of the carbohydrate residues with periodate has little effect on the activities of chloroperoxidase (Lee and Hager, 1970), glucose oxidase (Pazur et al., 1965; Nakamura et al., 1976) and glucoamylase I (Pazur et al., 1970).

Some doubt has been shed on the possible stabilizing role of the mannan moiety of glycoenzymes. Smith and Ballou (1974a) showed that the removal of all the mannan from external invertase had no effect on the thermal stability of the enzyme at 37°C, but they heated the mannanless enzyme in the presence of bovine serum albumin (1mg./ml.) which acts as a protein stabilizer (Wiseman, 1973). Nakamura et al., (1976) found that periodate oxidation of glucose oxidase decreased the carbohydrate content to 40% of its original activity but that the heat stability of
the enzyme was unchanged, whilst less resistance to denaturation by S.D.S. was shown. They concluded that carbohydrate protected the enzyme but did not maintain the conformation of the protein. It is interesting to note that isoenzyme IV with an apparently zero mannan content was more heat stable than internal invertase.

If mannan does maintain the stability of the invertase protein conformation, the mechanism by which this is achieved is not clear. Removal of mannan may, therefore, have only small effects on thermal stability of external invertase preparations, which are not enough to claim destabilization by removal of mannan. The mannan-induced thermal stabilization of yeast invertase is discussed in chapter 8.

* C. utilis* grade X invertase was found to be the most thermally stable of all the invertase preparations tested. The greater thermal stability of *C. utilis* invertase over Baker's yeast invertase is industrially advantageous and a possible industrial use of *C. utilis* invertase in the confectionery industry is described in chapter 5. The reason for this increased thermal stability is a matter for speculation. Since increased stabilization of enzymes by glycosylation is thought to be due to cross-linkage formed between the protein and polysaccharide moieties, it may be that inter- and intra-polypeptide chain cross-links of this type are responsible for the stabilization of glycoenzymes. *C. utilis* invertase may therefore show greater thermal stability than Baker's yeast invertase because of an increased number of interchain cross-links between the protein and mannan moiety.

The determination of the molecular weight and hence the number of sub-units in the invertase molecule by S.D.S. gel electrophoresis, was unsuccessful because the binding of S.D.S. to invertase protein is difficult, due to the protection offered by its mannan moiety.
This determination, however, should be possible using a mannanless external invertase which should allow binding of S.D.S. to the protein and subsequent dissociation into sub-units.
Industrial application of thermally stable invertases

5.1. - Introduction

The major source of invertase used in the confectionery industry is Baker's yeast. For over 50 years this enzyme has been used for the production of a wide variety of soft-centred and liquid-centred confectionery (Janssen, 1961). The chocolate coating of soft centres can only be performed by skilful hand-dipping, but incorporation of invertase into the dense fondant (≈ 70% syrup density) enables it to be coated with chocolate by machine. After a fortnight of storage, usually at room temperature, the sucrose is converted into invert sugar which dissolves in the water present, thus producing a soft or liquid centre (see Fig. 5.1.).

Commercial invertase is supplied by its manufacturers as a concentrated solution of the enzyme. It is extracted from a strain of yeast rich in invertase by autolysis and the crude extract obtained is then filtered to remove cell-debris. The enzyme is precipitated from the extract by the addition of an equal volume of chilled industrial alcohol. The precipitate containing the invertase activity is dissolved in 55% glycerol. Finally, the invertase concentrate is filtered for clarification and is stored in sterile containers ready for marketing (Cochrane, 1961).

In the production of cast cream centres, the normal casting temperature is 65°C (150°F) although temperatures of up to 90°C (≈ 200°F) are also used for the production of some special cream centres (Janssen, 1963). At 65°C, Janssen (1964) reported that only 20% of invertase activity is lost after 60 min. incubation with the standard
Fig. 5.1. - Use of invertase in the production of soft-centred confectionery

Fondant (Firm, dense sweet of microscopic sucrose crystals)

- Flavoured and coloured
- Invertase added
- Incubation at desired temperature

Variety of soft centres, e.g. cast, rolled and cordial fruit cream centres

- Coated with chocolate
- Incubation at room temperature

Soft-centred chocolate within 10 - 14 days
fondant of a high sucrose concentration. At 80°C two-thirds of the activity is lost and at 90°C all activity is rapidly lost (see Table 5.1). Compensation for these losses is achieved by using, and losing, large quantities of invertase. At such high casting temperatures some thermally stable invertases may prove to be useful. The greater thermal stability of Candida utilis invertase compared with Baker's yeast invertase was described in chapter 4.

This chapter describes the preparation of technical grade invertase from Saccharomyces cerevisiae (Baker's yeast) and Candida utilis yeast. A comparison of the thermal stability of both invertases in the presence of a high sucrose concentration is made and the usefulness of C. utilis invertase to the confectionery industry is discussed.
Table 5.1. - The effect of high temperature on the efficiency of invertase *

<table>
<thead>
<tr>
<th>Temperature</th>
<th>150°F (65.5°C)</th>
<th>165°F (73.9°C)</th>
<th>175°F (79.3°C)</th>
<th>185°F (85°C)</th>
<th>200°F (93.3°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mins.</td>
<td>15</td>
<td>18-24</td>
<td>33</td>
<td>88</td>
<td>97.5</td>
</tr>
<tr>
<td>60 mins.</td>
<td>20</td>
<td>30-40</td>
<td>61</td>
<td>95</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are % loss of activity

* From Janssen (1964)
5.2. - Materials and Methods

5.2.1. - Isolation of technical grade invertases from
Saccharomyces cerevisiae (N.C.Y.C. No. 525)
and Candida utilis (N.C.Y.C. No. 708)

Yeast, S. cerevisiae (N.C.Y.C. No. 525) and C. utilis
(N.C.Y.C. No. 708) were cultured in medium 1 as described in section 2.2.
Invertase was solubilized from 1g. wet weight of yeast cells by using the
Vibrc Mill disintegrator following the method of Williams and Wiseman
(1973) as described in section 4.2.1 except that the 10ml. stainless
steel container was used.

The disruptate was centrifuged and to the supernatant was added
an equal volume of absolute ethyl alcohol (cooled to -12°C). The
resulting suspension was centrifuged at 10,000 g for 10 min. The
invertase-rich pellet was suspended in 0.1M - sodium acetate buffer
pH 4.7, into which the invertase activity dissolved. Invertase activity
was determined as described in section 3.2.3.

5.2.2. - Thermal stability Studies

Both enzymes were treated at 65°C for 15 min. in the absence of
sucrose and the residual activity determined. The enzymes were heated
at a protein concentration of 28 µg./ml.

Each enzyme was incubated with 60% (w/v) sucrose at 65°C and the
extent of sucrose hydrolysis was determined at intervals. This was
repeated, except that the temperature of incubation was increased to 80°C.
Significant substrate blanks, due to thermal cleavage of sucrose, were
subtracted.
5.3. - Results

5.3.1. - Isolation of Technical grade invertases.

Table 5.2 summarises the isolation of technical grade invertases from \textit{S. cerevisiae} and \textit{C. utilis} yeasts. The low specific activities obtained are characteristic of a technical grade invertase preparation being due to the presence of much contaminating protein. Some of the invertase activity of \textit{C. utilis} yeast was lost by the disruption technique. The reason for this is not known. The invertase activity of \textit{C. utilis} yeast cells may be more accessible to sucrose than \textit{S. cerevisiae} invertase and would consequently show a higher activity \textit{in vivo}. Alternatively and perhaps more importantly, \textit{C. utilis} yeast is a richer source of invertase than the Baker's yeast strain used.

It should be noted that not all the material precipitated from the supernatant by ethyl alcohol could be re-dissolved in the buffer, although all of the invertase activity however, was dissolved.

5.3.2. - Thermal stability of the invertases

Heating in the absence of substrate at 65°C for 15 min. resulted in a total loss of \textit{S. cerevisiae} invertase activity. On the other hand, the \textit{C. utilis} invertase preparation was completely heat stable, losing none of its activity after the same treatment.

The progress of sucrose hydrolysis, performed in 60\% sucrose at 65°C, was linear and identical even after 60 min. of incubation for each enzyme (see fig. 5.2.). The high sucrose concentration protected both enzymes against heat but especially noticeable was the high stability of the \textit{S. cerevisiae} enzyme now equal to that from \textit{C. utilis}.

It was noted that the rates of activity were only about one tenth of the maximum rate. Nelson and Schubert (1928) first noted the decline in the
Table 5.2 - Isolation of technical grade invertases from S. cerevisiae and C. utilis yeasts

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml.)</th>
<th>Invertase units (u)</th>
<th>Sp. Act. (u/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast</td>
<td>1000 (mg.)</td>
<td>583</td>
<td>n.d.</td>
</tr>
<tr>
<td>Vibro Mill disrupta</td>
<td>100</td>
<td>573</td>
<td>n.d.</td>
</tr>
<tr>
<td>Supernatant</td>
<td>76</td>
<td>377</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ethyl 50% Alcohol (v/v)</td>
<td>10</td>
<td>217</td>
<td>54</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml.)</th>
<th>Invertase units (u)</th>
<th>Sp. Act. (u/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast</td>
<td>1000 (mg.)</td>
<td>758</td>
<td>n.d.</td>
</tr>
<tr>
<td>Vibro Mill disrupta</td>
<td>100</td>
<td>537</td>
<td>n.d.</td>
</tr>
<tr>
<td>Supernatant</td>
<td>76</td>
<td>319</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ethyl 50% Alcohol (v/v)</td>
<td>10</td>
<td>191</td>
<td>68</td>
</tr>
</tbody>
</table>

A - S. cerevisiae (Baker's yeast)
B - C. utilis yeast
n.d. - not determined
Fig. 5.2 - Progress of sucrose hydrolysis by invertases at 65°C in 60% (v/v) sucrose.

Key:
- S. cerevisiae invertase
- C. utilis invertase
rate of sucrose hydrolysis by invertase in sucrose concentrations greater than 5% (w/v) and suggested that the decrease in the water content was responsible.

At 80°C the progress curves soon departed from this linearity with a marked loss almost immediately apparent for the *S. cerevisiae* enzyme (see Fig. 5.3.). The *C. utilis* invertase was much more heat stable at 60°C. The recovery of activity of both invertases after treatment at 80°C is shown in table 5.3. The *C. utilis* invertase may be used more efficiently therefore, at high temperatures such as 80°C even in the presence of 60% (w/v) sucrose that protects invertase at the lower temperature due to viscosity or water-removing effects.

Presumably a greater recovery of activity of both invertases can be obtained by using greater quantities of the enzyme, since enzymes are protected at high protein concentration (Wiseman, 1973). Jannsen (1964) found a 39% recovery of invertase activity after 60 min. treatment at 80°C using 1 ounce of invertase / 100 lb. of cream batch.
Fig. 5.3 - Progress of sucrose hydrolysis by invertases at 80°C in 60% (w/w) sucrose
Table 5.3 - % recovery of activity of our prepared invertases after heat treatment at 80°C in 60% sucrose

<table>
<thead>
<tr>
<th>Time of treatment (mins.)</th>
<th>S. Cerevisiae</th>
<th>C. utilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>7.2</td>
<td>32.7</td>
</tr>
<tr>
<td>15</td>
<td>3.8</td>
<td>25.4</td>
</tr>
<tr>
<td>30</td>
<td>1.6</td>
<td>11.6</td>
</tr>
<tr>
<td>60</td>
<td>0.0</td>
<td>3.03</td>
</tr>
</tbody>
</table>

Values are % recovery of activity calculated from the slope of the progress curve; zero when nil recovery.
The optimum temperature for invertase use will depend on the conditions employed. When the enzyme is incubated in 2% (w/v) sucrose it is 55°C (J. Woodward and A. Wiseman, unpublished). When the enzyme is incubated in 60% (w/v) sucrose (industrial concentration) heat inactivation proceeds rapidly at 80°C, indicating that in the presence of high concentrations of sucrose, the optimum temperature is increased. Interestingly, invertase subjected to fluctuating temperatures can show a shift in its optimum temperature. Above 50°C fluctuating treatment shifted the optimum downward (Wu et al., 1974). These authors have also noticed that there is a change in the enzyme conformation when invertase is heated above 50°C. The optimum temperatures found are due to varying degrees of irreversible unfavourable conformational changes occurring in the enzyme under the conditions used.

The reaction rate of invertase is clearly reduced in high sucrose concentration and therefore may be diffusion controlled. McLaren (1963) studied the kinetics of invertase activity in concentrated solutions and concluded that diffusion of reactants in viscous substrate solutions becomes the rate limiting factor. This is contrary to the earlier deduction by Nelson and Schubert (1928) that water content is rate limiting at high sucrose concentrations. Interestingly, Ruchti and McLaren (1964) showed the falling-off of the rate of sucrose hydrolysis by invertase in high sucrose concentrations, was due to substrate inhibition at such high concentrations. They found that constant amounts of sucrose were hydrolysed in aqueous media of varying viscosity at fixed substrate concentration. Therefore, competitive inhibition by sucrose and not diffusion, would be the rate-limiting factor.
The use of invertase in the production of soft-centred confectionery has advantages over acid substances also used in the sugar industry for the production of invert sugar. Using invertase, the product is free from the impurities that may be produced by acid hydrolysis. In particular, flavour can be adversely affected by acid substances (Janssen, 1963).

The use of invertase also increases the shelf-life of the confectionery because the risk of fermentation or mould growth is reduced due to the high syrup densities reached (82 - 83% (w/v) dissolved solids) when using invertase (Cakebread, 1975). By acid hydrolysis the syrup density can reach 75% (w/v) dissolved solids with the addition of the so called 'doctoring' material (e.g. invert sugar, corn syrup) but this is not sufficient to prevent fermentation occurring.

*C. utilis* invertase may prove useful to the confectionery industry due to its greater stability to heat at 80°C in high sucrose concentrations compared to the Baker's yeast invertase normally used. Further experimentation is required, however, to determine whether *C. utilis* is in fact a rich source of invertase. The large scale production of this organism would also have to be a viable and economic proposition for industry.

Invertases from some strains of *S. cerevisiae* may also show greater thermal stability than Baker's yeast invertase, particularly those strains which are thermophilic. Alternatively, it may be possible to chemically modify and hence artificially stabilize, the existing Baker's yeast invertases which are at present used by the confectionery industry.

Recent interest in sucrose and its associated enzymes, stems
from the current high price of the sugar. Wiseman and Woodward (1975) have noted the possibility that invertase, under suitable reaction conditions, may be used to produce sucrose from high glucose and fructose concentrations, since an enzyme can catalyse the reverse reaction as well as the forward reaction. They proposed that invertase variants such as heat-stable and chemically modified forms, may have some use in future industrial processes.
The inactivation and thermal stability of chemically modified yeast invertases

6.1. Introduction

Chemically modified proteins for commercial use have many applications in the pharmaceutical, dyeing and clothing industries. Formaldehyde was used for the modification of bacterial toxins and viruses which would render the toxin or virus pathologically harmless without affecting the immunogenic response to it. Glutaraldehyde is used in the tanning of leather for the cross-linking of collagen, and wool fibres are treated likewise to strengthen the material for clothing (Means and Feeney, 1971).

The identification of 'essential' amino acids for enzyme activity is still performed by chemically-modifying reagents even though most reagents do not have absolute specificity for a particular amino acid. Inferences can usually be made about the amino acids essential for catalytic activity following their modification.

This chapter describes the chemical modification of yeast invertases and relates the modification to their inactivation and thermal stability. The aim of this study was to obtain a chemically modified invertase with increased thermal stability and similar specific activity to the native enzyme. This modified invertase may then be industrially advantageous.
The following reagents were used to modify invertase:

1) Mushroom tyrosinase (E.C.1.10.3.1.)
2) Potassium nitrosyldisulphonate (Fremy's salt)
3) Iodine
4) Citraconic anhydride
5) Maleic anhydride
6) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (E.D.C.)
7) Glutaraldehyde
8) Dimethylsulphurimidate
9) Dimethyladipimidate
10) Methylacetimidate
11) Toluene diisocyanate
12) Sodium periodate

This introduction describes the chemical reactions of the reagents 1-12; some of their uses in protein modification and explains why each one has been chosen for the modification of invertase.

6.1.1. - Mushroom tyrosinase, Fremy's salt

Mushroom tyrosinase catalyses the oxidation of tyrosine to melanin in the presence of oxygen. The most obvious effect of tyrosinase on susceptible proteins is the development of a yellow-brown colour in the protein solution, caused by formation of quinone after oxidation of tyrosine residues. Thus the oxidised proteins absorb strongly in the violet region. The reaction mechanism proposed by Raper (1927) is given in Figure 6.1. Melanin is a polymer formed by the cross-linking of quinone molecules. Sizer (1946; 1947) showed that mushroom tyrosinase oxidised tyrosine groups of trypsin, pepsin, chymotrypsin, accompanied by an increase of absorption in the violet region. He found no loss of enzyme activity for these enzymes, but showed that yeast invertase was susceptible to inactivation by oxidation with tyrosinase with 60 - 90%
Fig. 6.1. - Enzymatic oxidation of tyrosine to melanin (summarised)

Tyrosine \[ \overset{\text{O}_2\text{ tyrosinase}}{\longrightarrow} \text{DOPA quinone (via DOPA)} \]

Indole - 5,6 - quinone \[ \overset{\text{O}_2}{\longrightarrow} \text{DOPA chrome (Red)} \]

MELANIN - a polymer or group of polymers formed by cross-linking of quinone molecules

* DOPA - Dihydroxyphenylalanine

From Raper (1927)
of original enzyme activity recovered after oxidation (Sizer, 1948). This suggested that tyrosine was involved with the catalytic activity of invertase. Contrary to this was the finding that nitration of tyrosine residues in invertase with tetranitromethane had no effect on activity (Waheed and Shall, 1971b). Recently, it was shown that mushroom tyrosinase resulted in the almost complete inactivation of asparaginase indicating the importance of tyrosyl residues in asparaginase to the catalytic activity (Tokushige and Moriya, 1976).

Potassium nitrosydisulphonate (Fremy's salt) was first used to oxidise proteins by Earland et al., (1960). Its known quantitative oxidation of phenols to quinones (see Fig. 6.2.) made it likely that oxidation of tyrosine residues in proteins would perhaps be the almost exclusive reaction. They found that silk fibroin, wool, polytyrosine and insulin were rendered insoluble by Fremy's salt treatment with the formation of intense colouring. The u.v. - absorption spectrum of the oxidised proteins showed a typical melanin-type absorption. They also suggested that Fremy's salt oxidised tyrosine by a similar mechanism to that by which tyrosine is oxidised to melanin by tyrosinase (see Fig. 6.1.). Earland and Stell (1966) concluded that the insolubilization of proteins by treatment with Fremy's salt was caused by the formation of cross-links, perhaps of a melanin-type structure derived from pairs of tyrosine residues.

The action of mushroom tyrosinase and, for the first time, Fremy's salt on yeast invertase has been investigated. The formation of melanin-type cross-links by this action may confer greater conformational stability on to the enzyme, thereby improving the thermal stability. The role of tyrosine in the catalytic activity and conformational stability of invertase is discussed.
Potassium nitrosyldisulphonate, an orange solid, produces a free radical in solution which gives a purple colour. It is the free radical that is responsible for oxidation.
6.1.2. - Iodine

Iodine can react with proteins affecting only tyrosyl, histidyl and cysteinyl residues under mild conditions (see Fig. 6.3.) whereas tryptophanyl and methionyl residues may be affected under harsh conditions (Koshland et al., 1963). Waheed and Shall (1971b) found that low concentrations of iodine (20 - 100 µM) inactivated yeast invertase resulting in the loss of only half the enzyme activity, the reaction being complete within 2 min. The product is called iodine-invertase. They found that iodotyrosines were not formed under the conditions they employed and concluded that iodine oxidised a methionine residue.

Since only half the enzyme activity was lost under the conditions used by Waheed and Shall, this suggested that there may be invertase sub-units that were inactivated by, and sub-units that were insensitive to, low iodine concentrations (J. Woodward and A. Wiseman, unpublished).

The reaction between iodine and invertase has been re-investigated in order to determine whether iodine is a reversible or an irreversible inhibitor of invertase. That iodine can cause partial inactivation of invertase has been confirmed and the thermal stability of iodine-invertase has been investigated.

6.1.3. - Citraconic and maleic anhydrides

Protein amino groups react with citraconic and maleic anhydrides at alkaline pH and are converted to anionic residues (see Fig. 6.4.A). This reaction is known to lead to dissociation of oligomeric proteins through a drastic change of the electrostatic interaction among sub-units and consequently can be used as a method of determining the number of sub-units in a protein (Sia and Horecker, 1968,
Fig. 6.3. - Reaction of iodine with tyrosyl (1) histidyl (2) and cysteine (3) residues in proteins

\[ P \text{-} \text{SH} + I_3^- \rightarrow P \text{-} \text{SI} + H^+ + 2I^- \]  (3)

\[ H_2O \rightarrow P \text{-} \text{SOH} \rightarrow P \text{-} \text{S-S-P} \]  (3)

\[ P = \text{Protein} \]

From Means and Feeney (1971)
Fig. 6.4. - Reaction of maleic anhydride with proteins

\[
\text{P} - \text{NH}_2 + \text{maleic anhydride} \rightarrow \text{P} - \text{NH} - \text{C} - \text{CH} + \text{H}^+ \quad \text{pH 8}
\]

Citraconic anhydride is 2-methylmaleic anhydride which reacts similarly.

Reversal of Reaction

\[
\text{P} - \text{NH} - \text{C} - \text{CH} \rightarrow \text{P} - \text{NH}_2 + \text{maleic anhydride} \quad \text{pH} \leq 4.5
\]

\[\text{P} = \text{Protein}\]

From Means and Feeney (1971)
Citraconylation or maleylation of enzymes can alter their pH optimum and pH stability. Barker (1977) found that the pH optimum of trypsin was displaced from 7.8 to 6.8 after citraconylation. This ability to alter any one enzyme characteristic is of particular importance when a multiple enzyme system is being used in some industrial reactor system. He also found that the modified trypsin showed improved stability to proteolytic attack.

The citraconylated and maleylated proteins are stable at neutral pH but are easily hydrolysed at acidic pH (see Fig. 6.4.B), therefore, the modification can be easily reversed if necessary. Alkylation of sulphhydryl groups by citraconylation and maleylation is an irreversible side reaction, however (Means and Feeney, 1971).

Yeast invertases have been subjected to citraconylation and maleylation and the effect of this modification upon activity and various parameters, has been investigated. The dissociation of invertases into sub-units as seen by polyacrylamide gel electrophoresis is reported.

6.1.4. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (E.D.C.)

The use of the water-soluble carbodiimide, E.D.C., to modify free carboxyl groups of proteins has been reported (Hoare and Koshland, 1967). The initial phase in the reaction appears to be the formation of an o-acylisourea. In aqueous solution this derivative can condense with a variety of nucleophiles to give a large number of different carboxylate derivatives (see Fig. 6.5.). The reaction of a water-soluble carbodiimide with proteins proceeds best between pH 4.5 and 5.0. If the nucleophile is a bifunctional amine such as ethylenediamine, the ionic character of a protein can be changed by such a modification:
Fig. 6.5. The modification of free carboxyl groups of proteins by a carbodiimide in the presence of a nucleophile

\[ \text{Protein} + HX \rightarrow \text{acylisourea} \]

(From Hoare and Koshland, 1967)
Other amines have also been used, for example, glycine methyl ester was
coupled to carboxyl groups of L-glutamate dehydrogenase (Swaisgood
and Natake, 1973). Modification of trypsin carboxyl groups by E.D.C.
in the presence of glycaminide allowed identification of the amino acid
aspartic acid - 177 as the ionic binding site which largely determines
the specificity of trypsin (Eyl and Inagami, 1970). Perfetti et al.,
(1976) irreversibly inactivated papain by its modification with E.D.C.
and glycine ethyl ester and proposed a possible catalytic role for a
carboxyl group in its active centre. Similarly, Semenza et al., (1974)
found that water-soluble carbodiimides irreversibly inhibited intestinal
sucrase activity in the presence of glycine ethyl ester, but that this
inactivation was prevented by the presence of sucrose. They suggested
that one or more carboxyl groups is present in the active site of
intestinal sucrase. Interestingly, Waheed and Shall (1971b) have
postulated the presence of a carboxyl group near, or at the active site,
of yeast invertase (see Section 1.3.3.).

The action of E.D.C. in the presence of ethylenediamine upon
yeast invertase has been investigated with the main aim being to alter
the ionic character of the enzyme in order to displace its pH optimum.
This modified enzyme may be industrially advantageous. The thermal
stability of this modified invertase has also been studied.
6.1.5. - Glutaraldehyde, dimethylsuberimidate (DMS), dimethyl-
adipimidate (DMA), methylacetimidate (MA) and
toluene diisocyanate

Bifunctional reagents possess two reactive groups that are capable of reacting with and forming bridges between and thereby cross-linking, the side chains of the amino acids in a protein. The use of bifunctional reagents has been reviewed by Wold (1972). They can be used for studying the topography of components in sub-cellular organelles, the structure and function relationships in biologically active proteins, the preparation of insoluble enzymes and the determination of the number and arrangement of sub-units in oligomeric proteins.

The particular interest to this thesis is that proteins may be stabilized with bifunctional reagents against drastic conformational changes by cross-linking surface and buried portions of polypeptide chains.

Glutaraldehyde, a 1,5-dialdehyde, has been used as a bifunctional reagent to form cross-links between various side chains in proteins. The reaction of glutaraldehyde with proteins is obscure, but Richards and Knowles (1968) proposed an interesting reaction mechanism based upon the finding that in commercial glutaraldehyde solutions, very little free glutaraldehyde exists, but consists of a polymeric material rich in \( \alpha \) and \( \beta \)-unsaturated aldehydes. This material readily reacts with amino groups of proteins to give stable derivatives (see Fig.6.6.). Although the main reaction appears to involve the \( \varepsilon \)-amino groups of lysine, Habeeb and Hiramoto (1968) have shown that the side chains of cysteine, histidine and tyrosine residues are involved.

Glutaraldehyde has been used to stabilize crystals of carboxypeptidase A by cross-linking. The treated crystals showed
Fig. 6.6. — Proposed reaction mechanism of glutaraldehyde and proteins

\[
\text{OHCCH}_2\text{CH}_2\text{CH}_2\text{CHO}
\]

\text{glutaraldehyde}

\[
\downarrow \text{aldol condensations}
\]

\[
\text{CHO CHO}
\]

\[
\text{OHCCH}_2\text{CH}_2\text{CH}_2\text{CH} = \text{CCH}_2\text{C} = \text{CHCH}_2\text{CH}_2\text{CH}_2\text{CHO}
\]

\[
\downarrow
\]

\[
+ \text{P} - \text{NH}_2
\]

\[
\downarrow \text{pH 8.0}
\]

\[
\text{CHO} \quad \text{CHO}
\]

\[
\text{P} - \text{NH} \quad \text{NH} - \text{P}
\]

\text{cross-linked protein}

\[
P = \text{Protein}
\]

\text{From Richards and Knowles, (1971)}
increased resistance to mechanical breakage (Quiocho and Richards, 1964). It would appear that the major use of glutaraldehyde is to intermolecularly cross-link enzyme molecules that have been immobilized on to colloidal silica particles (Haynes and Walsh, 1969); phenol-formaldehyde resin (Olson and Stanley, 1974); collagen (Bernath and Vieth, 1974); nylon (Reynolds, 1974). This usually prevents the immobilized-enzyme complex from breaking down leading to the re-solubilization of enzyme (Stanley and Olson, 1973).

Monofunctional imidoesters, e.g. methylacetimidate, have been shown to react specifically with free amino groups of proteins (see Fig. 6.7.A) the resulting aminidation preserving the positive charges near these sites (Hunter and Ludwig, 1962). Bifunctional imidoesters have been used as cross-linking agents for proteins and similarly they do not affect the charge distribution of the proteins which they modify (see Fig. 6.7.B). These reagents can have different lengths and can therefore impart cross-linkages of varying sizes into proteins. Hartman and Wold (1967) used dimethyladipimidate (DMA) to determine inter-residue distances in ribonuclease A. They found that two cross-links were incorporated into the enzyme between lysine residues. Zaborsky (1974) studied the thermal stability of DMA - modified RNase A at 65°C and found it more stable than the native enzyme. He also showed that DMA - modified chymotrypsin showed greater thermal stability (at 45°C) than the native enzyme.

The bifunctional imidoesters are also used in the study of the sub-unit structure of oligomeric proteins. Davies and Stark (1970) found that dimethylsuberimidate gave rise to intramolecular cross-links in a number of oligomeric proteins. When each modified protein was subjected to S.D.S. polyacrylamide gel electrophoresis monomers, dimers, trimers and tetramers of the composing sub-units were produced.
Fig. 6.7. - Aminidation of proteins by imidoesters

A. monofunctional imidoester

\[
P - \text{NH}_2 + \text{H}_2\text{N-N}^+ - \text{CH}_3 \xrightarrow{\text{pH 8.0}} P - \text{NH} - \text{C-CH}_3
\]

methyl acetimidate + CH\text{$_3$}OH

B. bifunctional imidoester

\[
2P - \text{NH}_2 + 2\text{H}_2\text{N-N}^+ - \text{C-(CH}_2)\text{n-C-CH}_3 \xrightarrow{\text{pH 8.0}} 2P - \text{NH} - \text{C-(CH}_2)\text{n-C-NH-P} + 2\text{CH}_3\text{OH}
\]

\( P = \text{Protein} \)

When \( n = 4 \) imidoester is Dimethyladipimidate (DMA)

\( n = 6 \) imidoester is Dimethylsuberimidate (DMS)
Kopperschläger et al. (1976) cross-linked yeast phosphorructokinase with diimidoesters of different chain lengths and found the native enzyme to be composed of eight sub-units being arranged in two tetramers. Hajdu et al. (1976) studied the symmetry of aldolase and lactate dehydrogenase by using diimidoesters for cross-linking and also determined the distance between lysyl residues located nearest to each other in different sub-units. Wang et al. (1976) used DMS for the preparation of cross-linked dimers of pancreatic ribonuclease. Interestingly, a disulphide-bridged bifunctional imidoester has been prepared (Ruoho et al., 1975) and therefore cross-linkages introduced into proteins by such a reagent can be broken by mild treatment.

The use of a monofunctional imidoester enables the investigation of the effect of amino group modification without cross-linking.

Several diisocyanates are available that have been used to form covalent bands between two protein molecules (Schick and Singer, 1961). Diisocyanates react with amino groups of proteins to form substituted ureas (Wold, 1972) at neutral to alkaline pH, but reaction with cysteine, tyrosyl, carboxyl and histidyl residues can occur (Means and Feeney, 1971) but only the reaction with amino groups results in the formation of a stable product (see Fig. 6.8.).

Fig. 6.8. - Reaction of proteins with toluene diisocyanate

\[
2P - \text{NH}_2 + \text{H}_3\text{C}-\text{N}=\text{C}=\text{O} \xrightarrow{\text{pH \sim 7}} \text{P - NH}_2 \text{C}_3\text{N}=\text{C}=\text{O} \text{NH-P}
\]

Toluene - 2,4 - diisocyanate

\[
\text{P = Protein}
\]
The conjugation of horseradish peroxidase to IgG. Therefore, it might be possible for this diisocyanate to form intra-molecular cross-links within an enzyme molecule.

Soluble yeast invertases have been chemically modified with the bifunctional reagents described above in order to effect an enhancement of the enzyme's conformational stability.

6.1.6. - Sodium periodate

The oxidation of carbohydrates with sodium periodate leads to the formation of aldehyde groups. The aldehyde groups can then condense with free amino groups of proteins and the adducts so formed can be reduced to form stable alkylamino groups (see Fig. 6.9.). Nakane and Kawaoi (1974) oxidised the carbohydrate portion of horseradish peroxidase (HRPO) with sodium periodate after blocking the α- and ε- amino groups of HRPO with fluorodinitrobenzene to prevent self-coupling. They successfully coupled the HRPO - aldehyde to free amino groups of IgG, providing them with a useful marker for immunohisto-chemistry.

Yeast invertase has been oxidised with sodium periodate without blocking of the amino groups in order to form cross-linkages between the aldehyde groups formed in the mannan moiety by oxidation and amino groups of the protein moiety. This may enhance the conformational stability of the enzyme.
The reaction of aldehyde groups, formed by oxidation of carbohydrates with sodium periodate with protein amino groups

\[ H - C - OH \]  
\[ H - C - OH \]  
\[ HO - C - H \]  
\[ H - C - OH \]  
\[ CH_2OH \]  
\[ 2NaIO_4 \text{ sodium periodate} \]  
\[ \rightarrow \]  
\[ HCO_2H + \]  
\[ H - C - OH \]  
\[ \text{CHO} \]  
\[ CH_2OH \]  
\[ + 2P - NH_2 \]  
\[ \text{pH} \geq 7 \]  
\[ 2H_2O \]  
\[ P - N = CHCH(OH) - O - CH(CH_2OH)CN = N - P \]  
\[ \text{(H)} \]  
\[ P - NH - CH_2CH(OH) - O - CH(CH_2OH)CH_2 - NH - P \]  

\[ P = \text{Protein} \]
6.2. Materials and Methods

Mushroom tyrosinase was purchased from Sigma. Potassium nitrosyldisulphonate (Fremy's salt) was prepared as described by Palmer (1954). 100g. of crushed ice was stirred into a solution of sodium nitrite (17.5g. in 50ml. of water). Stirring was continued whilst sodium bisulphite (25g. in 50ml. of water) and glacial acetic acid (10ml.) were added.

\[
\text{HNO}_2 + 2\text{NaHSO}_3 \rightarrow \text{HO.N} (\text{SO}_3\text{Na})_2 + \text{H}_2\text{O}
\]

The mixture was rendered alkaline by addition of 0.880 concentrated aqueous ammonia (7ml.). whilst this alkaline mixture was stirred continuously, a solution of potassium permanganate was added (6.3g. in 200ml. of water). The precipitate of manganese dioxide formed was removed by filtration through a large pleated paper (without suction). A saturated solution of potassium chloride (250ml. - 33g. KCL/100ml. of water) was added to the violet coloured filtrate. The orange-yellow crystalline precipitate was filtered by suction and washed several times on the filter paper with potassium hydroxide (5% w/v) and then successively with ethanol and acetone. The crystals were dried in warm air and stored in a desiccator.

\[
2 \text{HO.N} (\text{SO}_3\text{Na})_2 + \text{O} \rightarrow \text{O.N} (\text{SO}_3\text{Na})_2
\]

\[
\text{O.N} (\text{SO}_3\text{Na})_2
\]

(converted into the less soluble potassium salt)

Iodine (resublimed) and potassium iodide, glutaraldehyde (25% w/v), analar sodium periodate, sodium borohydride, citraconic anhydride (1.24 - 1.25g./ml.) were purchased from B.D.H. Granular maleic anhydride, ethylenediamine dihydrochloride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were purchased from Sigma. Dimethyladipimidate dihydrochloride and methyl acetimidate hydrochloride were purchased from Pierce Chemicals, Box 117, Rockford, Illinois, 61105,
6.2.1. - Reaction of mushroom tyrosinase and Fremy's salt with invertase

The invertase preparation was diluted with 0.1M - sodium phosphate buffer pH 6.0 (to give 0.8 - 16 units/ml. of invertase activity) and the diluted enzyme was incubated with mushroom tyrosinase (1mg.) in a 1.0 ml. volume at 37°C. At zero time and at various other incubation times, a portion of the incubate (0.1ml.) was assayed for invertase activity. This procedure was also used to investigate the effect of Fremy's salt on invertase activity, except that 0.1M - sodium acetate buffer, pH 4.7, replaced the phosphate buffer. Purified external invertase, internal invertase and commercial invertase were incubated with Fremy's salt at room temperature.

Tyrosine content of the unhydrolysed invertase before and after incubation was determined by using the method of Uehara et al., (1970). To 1.0ml. of the sample containing invertase (1.0mg.), 1.0ml. of 1-nitroso-2-naphthol (0.15% w/v in 0.1N NaOH) and 2.0ml. of the acid-base mixture (prepared by mixing equal volumes of 0.025N HNO₃ and 0.3N NaOH) were added. The solution was heated in a boiling water bath for 10 min. and then placed in a water bath at 50°C until temperature equilibrium was reached. At this stage 4.0ml. of concentrated sulphuric acid was added. After cooling the solution, the red colour developed was measured against water at 520nm. Reagent blank and tyrosine standards were run at the same time.

6.2.2. - Reaction of iodine with invertase

Iodine was dissolved in equimolar solutions of KI; 1.0mM iodine solution contained 1.0mM KI. The reaction between iodine and
invertase was examined at several iodine concentrations (0.02 - 1.0 mM), at 25°C in 0.1 M sodium acetate buffer, pH 4.7. The possibility that iodine gave rise to iodotyrosines was examined by comparing the ultraviolet spectra of equimolar solutions of invertase and iodine-invertase at pH 6.0, 7.8 and 8.6. In alkaline solution, iodotyrosines have absorption maxima near 310 nm and therefore it is possible to distinguish between tyrosine and iodotyrosines (Waheed and Shall, 1971b). The spectra were recorded in a Unicam Sp 800 spectrophotometer.

6.2.3. - Reaction of citraconic and maleic anhydrides with invertase

Commercial invertase (0.1 ml.) and purified Baker's yeast invertase (5 mg. - $18.5 \times 10^{-3}$ μmol.) were diluted to 5 ml. with 0.1 M sodium phosphate buffer, pH 8.0. These enzymes were reacted at room temperature by the addition of a range of citraconic anhydride quantities ($55.75 - 557.5$ μmol.) the pH of the solution being maintained at pH 8.0 by the addition of 1N NaOH. After each addition of citraconic anhydride the effect upon enzyme activity was determined using a suitably diluted aliquot of the reaction mixture. Estimation of free amino groups was performed by using trinitrobenzenesulphonic acid (T.N.B.S.) as described by Goodwin and Choi (1970). Purified Baker's yeast invertase was used as the standard. Polyacrylamide gel electrophoresis at pH 8.9 and 7.0 of the citraconylated purified Baker's yeast invertase was performed (see Section 2.5.). Purified Baker's yeast invertase (5 mg.) was also reacted with a range of maleic anhydride quantities ($10 - 200$ μmol.) as described above.

The citraconylated and maleylated Baker's yeast invertases were subjected to Sepharose 6B chromatography (see Section 2.3.2.).
6.2.4. Reaction of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (E.D.C.), in the presence of ethylenediamine with invertase

Baker's yeast invertase (1mg. - 3.7 x 10^{-3} \mu mol.) was dissolved in 1.0ml. of ethylenediamine (0.5M at pH 4.7) and the reaction initiated by the addition of E.D.C. to a concentration of 0.1M. During the reaction (at 25°C) the pH was maintained at pH 4.7 with 1N HCl. It should be noted that the reaction was done in water (not sodium acetate buffer) and the pH adjusted accordingly as described. At prescribed intervals of time during the reaction, an 0.1ml. aliquot of the incubate was removed and quenched in 0.1M - sodium acetate buffer (1.9ml.) and enzyme activity determined. Quenching in sodium acetate buffer prevented any further reaction with E.D.C. In the control experiment the procedure was the same except that there was no addition of E.D.C.

6.2.5. Reaction of gluteraldehyde, dimethyladipimidate, dimethylsuberimidate and methyl acetimidate with invertase

Commercial invertase (0.1ml.) was diluted with 0.8ml. of 0.1M - sodium phosphate buffer pH 8.0, and reacted with 0.1ml. containing 1.25 - 10 \mu mol. of gluteraldehyde at room temperature for 60 min. An 0.1ml. aliquot of the treated enzyme was then diluted to 2.0ml. with 0.1M - sodium acetate buffer, pH 4.7 and the enzyme activity remaining was determined. Control experiments in which the enzyme was pre-incubated in buffer at pH 4.7 and pH 8.0 without addition of gluteraldehyde were performed at the same time.

Purified Baker's yeast invertase, Candida utilis grade \(X\) invertase (1mg. of each enzyme - 3.7 x 10^{-3} \mu mol.) and commercial invertase (0.1ml.) were dissolved in 0.9ml. of 0.2M - sodium carbonate
of dimethylsuberimidate and the reaction was allowed to proceed for 1 hr. at room temperature. Then an 0.1 ml. aliquot of the DMS - modified invertase was diluted to 1.0 ml. (2.0 ml. for commercial invertase) with 0.1 M sodium acetate buffer, pH 4.7 and 0.1 ml. of this diluted enzyme was assayed for activity. The remainder was dialysed overnight at 4°C against the buffer at pH 4.7 to remove excess salt and to return to the pH of optimal activity and stability. In control experiments, the native enzyme was dissolved in buffer pH 4.7 and in the other the native enzyme was dissolved in buffer at pH 10.0, both without addition of DMS; all other procedures were identical to the test experiment.

This procedure was repeated identically for the reaction of dimethyladipimidate and methyl acetimidate with each invertase except that the latter were reacted with $7.0 \times 10^{-2} \mu$mol. of methyl acetimidate.

6.2.6. - Reaction of toluene diisocyanate with invertase

Commercial invertase (1.0 ml.) was diluted to 9.9 ml. with 0.02 M sodium phosphate buffer pH 8.0. The reaction was initiated by the addition of 0.1 ml. of toluene diisocyanate (712 $\mu$mol.) and it was allowed to proceed at room temperature for 15 min. The solution was then centrifuged at 10,000 for 10 min. to remove unreacted solid toluene diisocyanate. An 0.1 ml. aliquot was diluted to 2.0 ml. with 0.1 M sodium acetate buffer prior to determination of enzyme activity.

6.2.7. - Reaction of sodium periodate with invertase

The reaction of sodium periodate with commercial invertase was performed mainly by following the method of Nakane and Kawaoi (1974), except that blocking of amino groups in invertase was not performed.
Commercial invertase (0.1 ml.) was diluted to 1.0 ml. with distilled water. The enzyme solution was made 0.04 M to sodium periodate and allowed to stand at room temperature for 30 min. The reaction was stopped by the addition of 1.0 ml. of 0.36 M ethylene glycol. An 0.1 ml. aliquot was diluted to 1.0 ml. with distilled water prior to determination of enzyme activity. After 1 hr. the treated enzyme was dialysed overnight at 4°C against 0.01 M — sodium carbonate buffer, pH 9.5.

To stabilize the Schiff base that may have been formed by reaction of invertase amino groups and the carbohydrate aldehyde groups formed by periodate oxidation, the dialysed enzyme was treated with sodium borohydride (2.64 μmol.) overnight at 4°C. After dialysis against distilled water, suitable dilutions were assayed for enzyme activity.

In a control experiment the above procedure was followed exactly except that the oxidation with sodium periodate step was omitted.

6.2.8. — Studies on the modified invertases

The thermal stability and determination of kinetic parameters, Km and Vmax, of some of the modified invertases was investigated.
6.3. - Results

6.3.1. - The effect of mushroom tyrosinase and Fremy's salt on invertase activity

It was found that the mushroom tyrosinase preparation employed in this study had no effect on the activity of any of the prepared invertases tested, including internal invertase. Sizer (1948) showed that not all mushroom tyrosinase preparations are effective in inactivating invertase depending on contaminating activities such as catecholase or creolase. The mushroom tyrosinase used oxidised free tyrosine with the ultimate formation of an intense black colouring, but none of the invertase preparations tested formed any coloured products. One of the reasons for the ineffectiveness of mushroom tyrosinase upon the invertase preparations may be that most of the tyrosine groups in them are inaccessible to a large molecule such as tyrosinase. Alternatively, it may be that the mushroom tyrosinase preparation employed was low in contaminating catecholase activity which Sizer (1948) found was a pre-requisite for the effectiveness of tyrosinase on invertase. Sizer and Fennessey (1951) showed that the inactivation of invertase by tyrosinase was greatly accelerated by the presence of $1 \times 10^{-6} \text{ M }$ copper. However, these studies found no effect of tyrosinase on the invertase preparations even in the presence of similar copper concentrations, to those used by Sizer and Fennessey (1951).

Fremy's salt inactivated all the preparations of invertase tested (see Fig. 6.10.). Grade III, Grade VI and purified Baker's yeast external invertase showed similar behaviour with an initial fast reaction (grade VI invertase lost 25% of its activity in 1 min.), followed by a slower reaction resulting in approximately 60 – 90% inactivation after 20 hr. Control experiments were performed in which the addition of Fremy's salt was the only step omitted from the procedure.
Recovery of activity

Fig. 6.10. - Inactivation of invertase with Fremy's salt

Key

A:  O-O grade III invertase  
     □-□ grade VI invertase

B:  ●-● internal invertase  
     ■-■ Baker's yeast purified external invertase

Invertase (0.8 - 16 units/ml.) incubated with Fremy's salt (1mg.) in 0.1M - sodium acetate buffer, pH 4.7, at room temp.
These experiments showed that none of the invertase activity was lost after 20 hr. at room temperature. Internal invertase behaved differently with Fremy’s salt to the other preparations. There was approximately 10% of activity lost after 4 hr. of incubation, but after 20 hr. over 90% of enzyme activity was lost (see Fig. 6.10.8).

The difference in the reactivity of internal invertase with Fremy’s salt compared to the external preparations, may be due to the fact that internal invertase, unlike external invertases, lacks cysteine (Gascon et al., 1968). Since Fremy’s salt can slowly oxidise cysteine (Earland and Stell, 1966) the initial fast reaction with the external preparations might be due to oxidation of a thiol group rather than tyrosine oxidation. However, partial loss of tyrosine was observed, some within the first minute, with grade VI invertase (see Table 6.1.) showing that there is a correlation between the initial fast loss of enzyme activity and a decrease in tyrosine content.

In separate experiments, using large quantities of grades III and VI invertases, both enzymes formed yellow-brown coloured end-products (which absorbed strongly in the violet region) on incubation with Fremy’s salt, indicating that tyrosine was oxidised.

For the determination of whether the residual activity of invertase after Fremy’s salt oxidation showed greater thermal stability over the native enzyme due to the formation of melanin-type cross-links, commercial invertase was used. Commercial invertase (1.0ml.) was diluted to 10ml. with 0.1M sodium acetate buffer, pH 4.7. The reaction was started by the addition of Fremy’s salt (10mg.). After 20 hr. incubation the colour of the solution had become light brown and the reaction was stopped by the addition of dithiothreitol (1mg.). The enzyme solution was then dialysed overnight at 4°C against 0.1M sodium acetate buffer to remove excess reagents. The recovery of
Table 6.1. - The loss of tyrosine in grade VI invertase during the reaction with Fremy's salt for 1 hr.

<table>
<thead>
<tr>
<th>Time of reaction (min.)</th>
<th>0</th>
<th>1</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine residues per mole</td>
<td>15.4 (65*)</td>
<td>14.5</td>
<td>11.1</td>
<td>10.5</td>
<td>6.8</td>
</tr>
<tr>
<td>% tyrosine loss</td>
<td>0</td>
<td>6</td>
<td>28</td>
<td>32</td>
<td>56</td>
</tr>
</tbody>
</table>

* Theoretical value from Gascon et al., (1968)

**N.B.** The number of tyrosine residues per mole of grade VI invertase is erroneously low compared to the theoretical value. This is probably due to the inhibitory action of carbohydrate in the colour development as shown by Ushara et al., (1970).
original enzyme activity was 35%. Ultraviolet scans of the oxidised commercial invertase showed a reduction in the tyrosine peak at 280 nm with increased absorption towards the violet region (see Fig. 6.11.).

The thermal stability and the determination of $K_m$ and $V_{max}$ of commercial invertase after its modification with Fremy's salt is described in section 6.3.6.

6.3.2. - The effect of iodine on invertase activity

The reaction between grade III, grade VI and grade X (10 $\mu$g./ml.) at several iodine concentrations (0.1mM, 0.5mM and 1.0mM) was examined, at room temperature. Suitable dilutions of invertase were assayed for activity after various incubation times. Each enzyme behaved similarly with each iodine concentration. There was an initial fast reaction resulting in 50% loss of activity after 2 min. and with 0.1mM iodine there was no further loss of activity even after 24 hr. incubation. However, with 0.5mM and 1.0mM iodine, there was an additional slower reaction resulting in further loss of enzyme activity with approximately 15% recovery of activity after 24 hr. incubation (see Fig. 6.12.A). This suggested that iodine could completely inactivate invertase (unlike the findings of Waheed and Shall, 1971b) providing sufficient iodine was present in the reaction mixture.

Thus invertase (10$\mu$g./ml.) was reacted with 0.02mM, 0.05mM and 0.1mM iodine concentration at room temperature, (which have been reported to cause only partial inactivation of invertase) in a 100ml. volume which was 50 times larger than previously used. There was the usual initial fast reaction within 2 min. but a slower reaction resulting in further loss of enzyme activity occurred by continued incubation with these iodine concentrations (see Fig. 6.12.B), resulting in almost complete loss of activity by incubation with 0.1mM iodine for 120 hr. Invertase incubated at room temperature for 120 hr. without iodine showed no
Fig. 6.11. - Ultraviolet spectra of commercial invertase before and after treatment with Fremy's salt

Scanning of commercial invertase was performed between 250 - 500nm in the Unicam Sp 1800 spectrophotometer

Key - unbroken line native commercial invertase
- broken line commercial invertase after treatment with Fremy's salt
Fig. 6.12. - Inactivation of invertase activity by iodine

Key: A - volume of reaction 2ml.; o-o, 0.1mM iodine; △-△, 0.5mM iodine; ■-■, 1.0mM iodine

B - volume of reaction 100ml.; ○-○, 0.02mM iodine; Δ-Δ, 0.05mM iodine; □-□, 0.1mM iodine

Invertase = 10µg./ml.
significant loss in activity. It is concluded that iodine is an irreversible inhibitor of invertase and providing excess iodine is supplied (even at low concentrations) most of the enzyme activity will be lost. Whilst this work was in progress, Bigger and Braymer (1975) reported iodine was an irreversible inhibitor of *Neurospora crassa* invertase.

The nature of the reaction between iodine and invertase is unknown. Waheed and Shall (1971b) have proposed that the fast reaction is due to the oxidation of a particularly reactive methionine residue and found no evidence for the formation of iodothyrosines during this reaction. In agreement with their work it was found that the ultraviolet spectra of grade X invertase and 0.1mM iodine-treated invertase (with 50% loss of original enzyme activity) were identical at pH 6.0, 7.8 and 8.6 with no absorption maxima at 310 nm. However, the ultraviolet spectra of 0.5mM and 1.0mM iodine treated invertase (with 80% loss of original enzyme activity) showed a reduction of the tyrosine peak and evidence of an absorption peak near 310 nm (see Fig. 6.13.) particularly at pH 7.8 and 8.6. It is tempting, therefore, to conclude that the slow inactivation of invertase by iodine after the initial fast inactivation, is due to the iodination of tyrosine residues, but this is not absolutely established by this work. Presumably, low concentrations of iodine can also iodinate tyrosine providing sufficient iodine quantity is present in the reaction mixture.

The determination of the thermal stability of the residual activity of invertase after reaction with iodine was performed using commercial invertase. Commercial invertase (1.0ml.) was diluted to 10ml. with 1.0mM iodine solution in 10mM - sodium acetate buffer, pH 4.7. After 30 min. and 20 hr. of reaction at room temperature, the thermal stability of the residual activity (50% and 19% recovery of original
Fig. 6.13. - Ultraviolet absorption spectra of invertase and iodine-invertase at pH 7.8 and 8.6.

Key:
- - - - , invertase and 0.1mM iodine treated invertase
- - - , 0.5mM iodine treated invertase
- - - - , 1.0mM iodine treated invertase
6.3.3. - The effect of citraconic and maleic anhydrides upon invertase activity

Citraconic and maleic anhydrides inactivated commercial invertase and purified Baker's yeast invertase similarly (see Fig. 6.14.). There was a correlation between loss of enzyme activity and the degree of substitution of amino groups after citraconylation of the purified invertase using the T.N.B.S. reagent (see Table 6.2.). The standard curve obtained using this reagent, was performed using purified invertase (see Fig. 6.15.).

The effect of pH on the activity and stability of citraconylated commercial invertase (50% of original activity) was studied (see Fig. 6.16.). The pH optimum of the modified enzyme was displaced to pH 3.6 compared to pH 4.7 for the native enzyme. Maximal stability of the native and citraconylated enzymes occurred between pH 3.6 - 4.7 but less recovery of original activity of the citraconylated enzyme compared to the native enzyme was achieved, particularly at pH 6.0.

Purified Baker's yeast invertase was subjected to polyacrylamide gel electrophoresis at pH 8.9 and 7.0 after citraconylation and maleylation (21% and 34% recovery of original enzyme activity respectively). At pH 8.9, the modified enzymes were clearly more diffuse than the native enzyme but there was no evidence for sub-units. At pH 7.0, however, the modified enzymes were separated into at least 4 major bands of protein, each of which was clearly homogenous. Other fainter bands of protein were also present (see Fig. 6.17.). The four main bands separated at pH 7.0 may represent sub-units of
Fig. 6.14. - Inactivation of invertases with citraconic and maleic anhydrides

Key: A - citraconic anhydride
     B - maleic anhydride

-• commercial invertase (0.1ml./5ml.)
-• Baker's yeast purified invertase (5mg./5ml.)

volume of reaction 5ml.
Fig. 6.15. - Standard curve for purified invertase using the T.N.B.S. reagent

(for details see Goodwin and Choi, 1970)

Table 6.2. - Correlation between the activity of invertase and the degree of substitution of amino groups after citraconylation

<table>
<thead>
<tr>
<th>Citraconic anhydride (µmol.)</th>
<th>55.75</th>
<th>223</th>
<th>557.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (%)</td>
<td>65</td>
<td>41</td>
<td>8</td>
</tr>
<tr>
<td>Free amino groups (%)</td>
<td>20</td>
<td>12</td>
<td>2</td>
</tr>
</tbody>
</table>
Fig. 6.16. - The effect of pH on the activity and stability of citraconylated commercial invertase.

Key:
- Native enzyme
- Citraconylated enzyme

A - Effect upon activity
B - Effect upon stability
Fig. 6.17. - Polyacrylamide gel electrophoresis of native and citraconylated invertase

A - pH 8.9

Key: 1 - native invertase
2 - citraconylated invertase
3 - maleylated invertase

B - pH 7.0
yeast invertase, in which case the yeast invertase molecule would be
a tetramer. On the other hand, the bands may represent a mixture of
invertase molecules which are citraconylated and maleylated to varying
degrees.

Attempts were made to determine whether the molecular weight
of citraconylated and maleylated invertases (21% and 34% recovery of
original enzyme activity respectively) was smaller, as judged by their
behaviour in Sepharose, compared to the native, unmodified enzyme.
This might be due to the dissociation of invertase sub-units by
acylation. The native and modified enzymes possessed the same elution
volumes which suggested that the recoverable activity after acylation
was not dissociated invertase. There was also no evidence for
inactive dissociated invertase protein eluted at a greater elution
volume either (see Fig. 6.18). Similar results were obtained using
Sephadex G-200. Consequently, the possibility that yeast invertase
exists as a tetrameric molecule still remains to be established.

The thermal stability and the determination of the kinetic
parameters $K_m$ $V_{max}$ of citraconylated commercial invertase, has been
performed (see section 6.3.6.).

6.3.4. - The effect of the carbodilmide, E.D.C., in the presence
of ethylenediamine upon invertase activity

The reaction of purified Baker's yeast invertase with the
water-soluble carbodilmide E.D.C. in the presence of ethylenediamine
resulted in the loss of approximately 90% of the enzyme activity within
3.5 hr. (see Fig. 6.19.). No loss of enzyme activity occurred by
incubation with ethylenediamine only. Since the presence of a
carboxyl group has been implicated in the active site of yeast invertase
(Waheed and Shall, 1971b) it was interesting to determine whether the
Fig. 6.18. - Gel filtration of native and acylated invertases in Sepharose 6B

300 units of citraconylated (○—○) and maleyalted (△—△) invertase were applied to the column.

400 units of native invertase were applied (●—●).

Protein (□—□) shown is of the modified invertases only.
Recovery of activity

Fig. 6.19 - Inactivation of invertase by E.D.C. in the presence of ethylenediamine

Key:
- ○ inactivation with no additions
- □ inactivation in the presence of 0.3M sucrose
- △ inactivation in the presence of 1.0mM dithiothreitol

Purified Baker's yeast invertase (1mg.) dissolved in 1.0ml. water (0.1M to E.D.C. and 0.5M to ethylenediamine)
inactivation by E.D.C. could be prevented if sucrose was present in the reaction mixture. Surprisingly, it was found that the presence of 0.3M sucrose enhanced the rate of inactivation (see Fig. 6.19.). The reason for this is not known at present. The reaction was also performed in the presence of 1mM dithiothreitol since carbodiimides can also react with thiol groups (Means and Feeney, 1971). Some protection was offered by the presence of dithiothreitol, but not enough to claim that the major reaction is with thiol groups (see Fig. 6.19.).

The main aim of this experiment was to incorporate amino groups into the invertase molecule in order to effect a displacement of the pH optimum of the enzyme. An attempt to measure the incorporation of amino groups into the enzyme by using the T.N.B.S. reagent was unsuccessful and no increase of free amino groups could be detected. The pH optimum of the modified invertase with 50% recovery of original enzyme activity was identical to that of the native enzyme. Further modification, resulting in a greater loss of enzyme activity, did not effect a displacement of the pH optimum. Since there are at least 293 carboxyl groups in the native enzyme (178 Asp. and 115 Glu.), it would seem likely that many of these groups are accessible to the modifying reagent. However, this work has presented no evidence for any incorporation of amino groups into these carboxyl groups. This needs to be established before it can be claimed that either incorporation of amino groups does not displace the pH optimum (which seems unlikely in view of the results of the previous section) or that there were too few amino group incorporations, which is not sufficient to alter the gross ionic charge of the enzyme, which would be necessary in order to effect a displacement of the pH optimum.

The thermal stability and the determination of kinetic parameters $K_m$ and $V_{max}$ of the carbodiimide modified Baker's yeast
The effect of glutaraldehyde, dimethyladipimidate (DMA), dimethylsuberimidate (DMS), methylacetimidate (MA), toluene diisocyanate and sodium periodate upon invertase activity

Glutaraldehyde inactivated commercial invertase, the recovery of enzyme activity depending upon the quantity of glutaraldehyde used in the reaction (see Fig. 6.20.). Pre-incubation of invertase for 60 min. in 0.1M - sodium phosphate buffer, pH 8.0 had no effect upon the enzyme activity which was the same as for the enzyme pre-incubated in 0.1M - sodium acetate buffer, pH 4.7. During the reaction the enzyme solutions became yellow, the intensity of the colour increasing with increasing quantities of glutaraldehyde used. Interestingly, Quirocho and Richards (1964) found that crystals of carboxypeptidase A, kept in 6% aqueous glutaraldehyde solution, became yellow and were very resistant to mechanical breakage, suggesting that extensive cross-linking had occurred. Therefore, the yellow colour of the glutaraldehyde treated invertase solutions may have been an indication that cross-linking of the enzyme had occurred.

The reaction between the invertase preparations and the imidoesters, DMS, DMA and MA, resulted in no loss of enzyme activity during any incubation for 60 min. at room temperature. Similarly, the reaction between commercial invertase and toluene diisocyanate for 15 min. at room temperature resulted in no loss of enzyme activity.

The oxidation of commercial invertase with 0.04M sodium periodate resulted in no loss of enzyme activity. The production of aldehyde groups was achieved since it was shown that the oxidised enzyme reduced 3,5 dinitrosalicylic acid reagent which turned orange,
Commercial invertase (0.1ml.) diluted to 0.8ml. with 0.1M sodium phosphate buffer pH 8.0. Glutaraldehyde solution (0.1ml.) was added to initiate the reaction.
Fig. 6.20. - Inactivation of invertase by glutaraldehyde

Commercial invertase (0.1ml.) diluted to 0.8ml. with 0.1M sodium phosphate buffer pH 8.0. Glutaraldehyde solution (0.1ml.) was added to initiate the reaction.
whereas the non-oxidised enzyme had no effect. After the dialysis step, both the control and test experiment lost approximately 50% of activity but no further loss in activity resulted from the reductive alkylation step of the procedure. Presumably, prolonged treatment with 0.01M sodium carbonate buffer, pH 9.5, even at 4°C, results in the loss of activity due to the instability of commercial invertase at this pH (see Fig. 6.16.8). After the dialysis step against distilled water no recovery of activity in the control and test experiment was observed.

The thermal stability and determination of the kinetic parameters $K_m$, $V_{max}$, of the modified invertases has been performed and the results are described in the next section.

6.3.6. - The determination of the thermal stability, $K_m$ and $V_{max}$ of the chemically modified invertases

In summary, the results indicated that significant increased thermal stability was achieved in *Candida utilis* grade $X$ invertase by its chemical modification with DMS but not DMA. Purified Baker's yeast invertase was not stabilized against heat by DMS or DMA. The modification of grade $X$ invertase with MA resulted in no significant effect upon their thermal stability. Commercial invertase was significantly stabilized against heat by its modification with glutaraldehyde and DMS. A dramatic loss in thermal stability of the residual activity of commercial invertase or purified Baker's yeast invertase, was observed after its modification with Fremy's salt, iodine, citraconic and maleic anhydrides, E.D.C. and toluene diisocyanate.
and purified Baker's yeast invertase after their modification with DMS, DMA and MA.

The thermal stability studies on grade X invertase were carried out at 70°C on the test and control experiments as previously described in section 2.7. After 10 min. of heating the native enzyme (prior incubation at pH 4.7) showed 48.4 ± 3.6% recovery of activity. The native enzyme (prior incubation at pH 10.0) showed 68.8 ± 7.9% recovery of activity after 10 min. heating, which indicated significant increased thermal stability (p<.01) over the native enzyme with prior incubation at pH 4.7. DMS modified grade X invertase (prior incubation necessarily at pH 10.0 for the DMS reaction to occur) showed a significantly increased thermal stability (p<.01) compared with the native enzyme (even after prior incubation at pH 10.0) showing 93.4 ± 8.9% recovery of activity after 10 min. heating (see Fig. 6.21.). DMA and MA modified grade X invertase showed similar thermal stability to the native enzyme (prior incubation at pH 10.0).

Similar experiments performed with Baker's yeast purified invertase showed that the DMS-, DMA- and MA- modified enzyme was less stable at 65°C compared to the native enzyme (prior incubation either at pH 4.7 or pH 10.0). The native enzyme (prior incubation either at pH 4.7 or 10.0) showed similar stability after 10 min. of heating (see Fig. 6.22.).

Therefore, under the conditions used in this experiment, it can be concluded that C. utilis grade X invertase is stabilized against heat by its chemical modification (probably through cross-linking within polypeptide chains) with DMS. Surprisingly, Baker's yeast invertase, the least stable of the two enzymes, was destabilized against heat by its chemical modification with the various imidoesters.
Fig. 6.21. - Thermal inactivation at 70°C of grade X invertase after its chemical modification with dimethylsuberimidate

Key:
- invertase, prior incubation with DMS at pH 10.0
- invertase, prior incubation pH 10.0
- invertase, prior incubation pH 4.7

1 mg. invertase (3.7 x 10^{-3} \text{\mu mol.}) reacted with 3.5 x 10^{-2} \text{\mu mol. DMS in 1.0ml. volume}

Values are mean ± S.D.
Fig. 6.22. - Thermal inactivation at 65°C of Baker's yeast invertase after its chemical modification with the various imidoesters

Key:
- O--O invertase, prior incubation pH 4.7
- △--△ invertase, prior incubation pH 10.0
- □--□ invertase, prior incubation with MA, pH 10.0
- ◊--◊ invertase, prior incubation with DMA, pH 10.0
- ▲--▲ invertase, prior incubation with DMS, pH 10.0

1 mg. invertase ($3.7 \times 10^{-3}$ μmol.) reacted with $3.5 \times 10^{-2}$ μmol. DMS or DMA, or $7.0 \times 10^{-2}$ MA, in 1 ml. volume
The pH optimum and the pH at which maximal stability of the native Baker's yeast preparation occurs was not changed by this modification. The reason for this apparent destabilization remains to be established.

6.3.6.2. - The thermal stability of commercial invertase after its chemical modification

The thermal stability of commercial invertase chemically modified with Fremy's salt, iodine, citraconic anhydride, glutaraldehyde, DMS, DMA, MA, toluene diisocyanate and sodium periodate has been investigated. The glutaraldehyde and DMS - modified enzymes were the only ones to show increased thermal stability against heat compared to the native enzyme. Maximal thermal stability of glutaraldehyde modified invertase was achieved at the expense of a loss in specific activity with the maximal stability and the greatest loss in specific activity occurring with the most quantity of glutaraldehyde used in the reaction (see Fig. 6.23.). There was a 60% and 75% recovery of activity (1.25 μmol. and 5.0 μmol glutaraldehyde used in the reaction respectively) after 5 min. of heating at 65°C compared to a 5% recovery of activity for the native enzyme. The DMS - modified enzyme also showed a significant increase in thermal stability compared to the native enzyme with a 20% recovery of enzyme activity after 5 min. of heating at 65°C. There was no loss in specific activity, however (see Fig. 6.23.).

The rate inactivation constants, k (min⁻¹), were 0.22; 0.14; 0.05 and 0.03 for the native, DMS - modified, glutaraldehyde (1.25 μmol.) and glutaraldehyde (5.0 μmol.) modified commercial invertase respectively.

Using the conditions described in this thesis, glutaraldehyde is better than DMS in effecting an enhancement of conformational stability of the enzyme. This is achieved presumably by extensive
Fig. 6.23. - Thermal inactivation at 65°C of gluteraldehyde - modified and DMS - modified commercial invertase.

Key:

- O-O native commercial invertase
- DMS - modified invertase
- Δ-Δ gluteraldehyde (1.25μmol.) modified invertase
- □-□ gluteraldehyde (5.0 μmol.) modified invertase

Values are mean ± S.D.
Purified Baker's yeast invertase was destabilized against heat by its modification with E.D.C. in the presence of ethylenediamine (see Fig. 6.24.). The residual activity remaining after 45 min. reaction (50% recovery of original enzyme activity), however, was more stable than the residual activity remaining after 90 min. reaction (30% recovery of original enzyme activity) which suggests that there is a correlation between destabilization against heat and the extent of
Fig. 6.24. - Thermal inactivation (65°C) of Purified
Baker's yeast external invertase after
its modification with E.D.C. in the
presence of ethylenediamine

Key:  ○○ control no E.D.C. treatment (ethylenediamine present)
△△ 45 min. reaction with E.D.C.
□□ 90 min. reaction with E.D.C.

Values are mean ± S.D.
carboxyl group modification. The rate inactivation constants, 
\( k \) (min\(^{-1}\)), for the native and the modified enzymes after 45 min. and
90 min. were 0.104, 0.38 and 0.65 respectively.

6.3.6.3. - The determination of the kinetic parameters \( K_m \) and \( V_{max} \) of the residual enzyme activity of commercial invertase and purified Baker's yeast external invertase after chemical modification

The effect of chemically modifying invertase upon the kinetic parameters \( K_m \) and \( V_{max} \) is shown in Table 6.3. Modification of commercial invertase with citraconic anhydride and glutaraldehyde resulted in an apparent increase of \( K_m \) by approximately fourfold and threefold respectively. No significant change in \( K_m \) was observed for commercial invertase modified with Fremy's salt, DMS and toluene diisocyanate. There was, however, a significant decrease in \( V_{max} \) of Fremy's salt and toluene diisocyanate modified commercial invertase. The apparent \( K_m \) of purified external invertase was increased by approximately twofold and fourfold depending upon the extent of the modification.
Table 6.3. - The effect of chemically modifying invertase upon Km and Vmax

### A

<table>
<thead>
<tr>
<th>Modifying reagent</th>
<th>Recovery of enzyme activity (%)</th>
<th>k(min⁻¹)</th>
<th>Km (mM)</th>
<th>Vmax (μmol min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>0.22</td>
<td>28</td>
<td>21.0</td>
</tr>
<tr>
<td>Fremy's salt</td>
<td>35</td>
<td>0.55</td>
<td>32</td>
<td>3.3</td>
</tr>
<tr>
<td>Citraconic anhydride</td>
<td>50</td>
<td>0.7</td>
<td>100</td>
<td>19.6</td>
</tr>
<tr>
<td>DMS</td>
<td>100</td>
<td>0.14</td>
<td>27</td>
<td>16.7</td>
</tr>
<tr>
<td>Toluene diisocyanate</td>
<td>100</td>
<td>&gt; 2.0</td>
<td>33</td>
<td>5.8</td>
</tr>
<tr>
<td>Glutaraldehyde (1.25 umol.)</td>
<td>59</td>
<td>0.05</td>
<td>83</td>
<td>20.0</td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>Modifying reagent</th>
<th>Recovery of enzyme activity (%)</th>
<th>k(min⁻¹)</th>
<th>Km (mM)</th>
<th>Vmax (μmol min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>0.104</td>
<td>28</td>
<td>20.0</td>
</tr>
<tr>
<td>E.D.C.</td>
<td>50</td>
<td>0.38</td>
<td>60</td>
<td>19.6</td>
</tr>
<tr>
<td>E.D.C.</td>
<td>30</td>
<td>0.65</td>
<td>100</td>
<td>17.0</td>
</tr>
</tbody>
</table>

Key:  
A - commercial invertase values  
B - Purified Baker's yeast external invertase values
The chemical modification of the various invertase preparations with Fremy's salt resulted in the loss of enzyme activity. Although the oxidation of tyrosine by this reagent is demonstrated even after 1 min. of reaction, whether or not tyrosine is important catalytically remains to be established. Furthermore, it is unlikely that this reagent has absolute specificity for tyrosine (Earland and Stell, 1966) and therefore oxidation of other amino acids (especially during the first minute of reaction) may be responsible for an initial fast inactivation. The obvious choice is cysteine, since internal invertase (which lacks cysteine) did not undergo the fast initial inactivation of enzyme activity characteristic of the reaction between the external invertase preparations and Fremy's salt. However, cysteine is not implicated in the active site of invertase (Waheed and Shall, 1971b). These investigators have also found that nitration of tyrosine groups in invertase by tetranitromethane has no effect upon activity. This reagent also attacks cysteine (Sokolovsky et al., 1966) and therefore provides further evidence that cysteine thiol is not essential for invertase activity. However, since the unmodified phenolic hydroxyl group is unaffected by nitration (Sokolovsky et al., 1966) it is possible that this group is important in the catalytic activity of invertase in view of the inactivation on treatment with Fremy's salt that is associated with ortho-quinone formation and possible subsequent cross-linking. Therefore, it seems reasonable at this stage not to completely discount tyrosine as being catalytically important.

The loss of enzyme activity associated with tyrosine oxidation may also be due to a deleterious change in the conformation of the enzyme, perhaps through a cross-linking reaction. Conformational studies are required to confirm this.
Since the introduction of cross-links into enzymes has resulted in their increased thermal stability (see Section 6.1.5.) the hypothesis that the residual invertase activity remaining, after oxidation with Fremy's salt, might show increased thermal stability due to cross-linking, was tested. Even though the U.V. absorption spectra of the oxidised commercial invertase showed a typical melanin-type absorption, the modified enzyme was found to be destabilized against heat. Interestingly, the residual activity of commercial invertase, modified with 1.0 mM iodine, after the slow inactivation, with the possible formation of iodotyrosines, was drastically destabilised against heat, unlike the residual activity after the fast initial inactivation, apparently not involving tyrosines, which showed similar thermal stability to the native commercial enzyme.

Destabilization against heat was also observed when invertase was modified with citraconic anhydride, toluene diisocyanate (which modify amino groups) and E.D.C. in the presence of ethylenediamine (which modifies carboxyl groups). Similarly, purified Baker's yeast external invertase (but not \textit{C. utilis} grade \textit{X} invertase) modified with DMS, DMA and MA (which modify amino groups) also showed destabilization against heat compared to the native enzyme.

It is proposed, on the evidence presented, that certain amino and carboxyl groups in the invertase molecule may play an important role (probably involving salt-linkages) in maintaining the conformational stability of the enzyme. Modification of these important amino and carboxyl groups would therefore result in a destruction of the salt-linkage which would lead to a destabilization of the enzyme conformation. Since the charge on these groups would obviously be necessary in such a linkage, it might be expected that reagents such as DMS, DMA and MA (which do not change the charge at the site of
modification; would not alter the conformational stability of invertase by preserving the salt-linkage. Indeed, the destabilization caused by DMS, DMA and MA was not nearly so drastic as that caused by toluene diisocyanate, citraconic anhydride and E.D.C. which do not preserve the charge at the site of the modification. Presumably the number of effective salt-linkages, or more likely, the effectiveness of each salt-linkage, is reduced by the modification of invertase with DMS, DMA and MA. No destabilization of C. utilis grade X invertase was shown by its modification with DMS, DMA and MA, suggesting that the effectiveness of each salt-linkage was not reduced as seems to be the case with Baker's yeast external invertase preparations. It is presumed that these salt-linkages would be on or near the external surface of the enzyme molecule. Perutz and Raidt (1975) suggested that the greater thermal stability of thermophilic ferredoxins, compared to their mesophilic counterparts, arises mainly from external salt bridges linking residues near the N-terminus to others near the C-terminus. Colman et al., (1972) found that the structure of thermolysin (the thermally stable proteolytic enzyme from Bacillus thermoproteolyticus) incorporated an unusual number of salt linkages, suggesting that these interactions contributed to the overall thermal stability.

There is much interest, at present, in the determination of the basis for the enhanced thermostability of proteins obtained from thermophilic organisms (Zuber, 1976). Weaver et al., (1976) have proposed that the three-dimensional structures of thermostable proteins will, in general, be very similar to their mesophilic counterparts and therefore the difference between thermolability and thermostability will be due to subtle changes in the protein primary structure, which result in greater interactions contributing to the overall conformational stability (e.g. hydrogen bonding, hydrophobic interactions, ionic interactions). Cass and Stellwagen (1975) found no substantive
differences in the amino acid compositions of phosphofructokinase from the thermophile *Thermus*, *E. Coli* and *CL. pasteurianum* but suggested that the enhanced thermal stability of *Thermus* phosphofructokinase is the result of a small number of amino acid replacements which are likely to produce a variety of additional stabilization interactions. Tanaka *et al.*, (1973) compared the amino acid sequences in mesophilic and thermophilic *Clostridium sp.* ferredoxins and found that glutamic acid occurred in positions 31 and 44 only in the sequences of the thermophilic ferredoxins. They suggested that these residues could be hydrogen-bonded to other amino acid side chains which might account for the increased thermal stability of these ferredoxins.

Interestingly, Yasunobu and Tanaka (1973) found that the thermal stability of these ferredoxins could be correlated with their glutamic acid content in the interior of the molecule. Hase *et al.*, (1976) have pointed out that there may therefore be a correlation between the number of glutamic acid residues present and thermal stability based upon the helical content of the protein tertiary structure, since glutamic acid residues are the best helix markers in proteins (Robson and Pain, 1971). However, the high thermal stability of α-amylase from *Bacillus stearothermophilus* was explained as being due to the low degree of α-helical structure in the enzyme structure and the finding that it appeared to be a relatively unfolded molecule (Mannig *et al.*, 1961). Hase *et al.*, (1976) have postulated that the high thermal stability of *B. stearothermophilus* ferredoxin may be due to its low cysteine content.

The differences in thermal stability of proteins from mesophilic and thermophilic sources can be explained in terms of their differences in $\Delta^* G$ (free energy of activation). Hocking and Harris (1976) have pointed out that a 30-fold difference in the thermal stability between rabbit muscle and *B. stearothermophilus* triose
phosphate isomerase at 60°C results from only a small difference in \( \Delta G \) (2.2 Kcal/mole) between the two enzymes. The extra salt-linkages and hydrogen bonds in the structure of thermophilic ferredoxins, which contribute to their increased thermal stability, amounts to between only 4.5 - 8.5 Kcals/mole of extra stabilization energy (Perutz and Raidt, 1975). Therefore, it seems that the formation of low energy bonds of up to 10 Kcal/mole (like those provided by salt-linkages, hydrogen bonds and hydrophobic bonds) are responsible for the stabilization of proteins, particularly in thermophiles.

The loss of thermal stability of invertase after its modification by citraconic anhydride and E.D.C. may therefore be the result of a partial loss in bond energy provided by salt-linkages. Similarly, the loss in thermal stability of invertase after its modification with Fremy's salt and iodine, may be due to a loss in bond energy provided by tyrosine in possible hydrogen bonding and hydrophobic interactions. The effect upon the thermal stability of proteins by specific chemically modifying reagents is therefore suggested as a probe for understanding the nature of the stability of a three-dimensional protein structure.

Since disulphide bonds can enhance the conformational stability of enzymes, the introduction of additional intramolecular bonds or cross-links may have the same effect (Zaborsky, 1972). Several reports on the enhanced stability of bifunctionally modified proteins relative to the native enzyme have appeared in the literature. Wold (1961) cross-linked bovine serum albumin with \( p,p' \)-di-fluoro-m, m'-dinitro-diphenylsulphone and found that the cross-linked protein showed a marked resistance to denaturation by heat and urea compared to the native protein. Saidel et al., (1964) found that chymotrypsin was stabilized against heat and urea by cross-linking with formaldehyde. Wang and Tu (1969) modified rabbit muscle glycogen
phosphorylase b with glutaraldehyde and found that the modified enzyme exhibited enhanced conformational stability relative to the native enzyme. Unsuccessful attempts at inducing enhanced conformational stability of proteins by their modification with bifunctional reagents have been reported (Zaborsky, 1972). Darlington and Keay (1965) found no evidence of thermal stabilization of mercuripapain when it was cross-linked with either formaldehyde or thioformaldehyde.

One of the aims of this thesis was to increase the thermal stability of invertase for possible industrial use. This has been achieved for commercial invertase using glutaraldehyde and DMS. It can therefore be presumed that new bonds have been formed that increase the overall stability of the enzyme. For DMS-modified invertase at least, these bonds are probably formed between inactive and invertase protein since highly purified external invertase was not stabilized by DMS. Barker and Kay (1975) have stated that cross-linking an enzyme to itself is expensive and inefficient resulting in low enzyme activity. Indeed, glutaraldehyde stabilized invertase at the expense of a loss in enzyme activity, but milder reagents such as imidoesters like DMS can stabilize soluble invertase without loss in enzyme activity. Although toluene diisocyanate is a bifunctional reagent, it is concluded that this reagent did not form cross-links in commercial invertase that may have resulted in its stabilization.

Candida utilis grade X invertase, which is naturally more thermally stable than Baker's yeast invertase preparations, was significantly stabilized by DMS. It is concluded that the longer cross-linking bond that is provided by DMS, compared to DMA, is necessary for intra-molecular cross-linking to occur within this enzyme. One of the reasons why Baker's yeast purified external invertase was not stabilized by DMS may therefore be because this cross-linking bond is
not long enough for intra-molecular cross-linking to occur. The results of this thesis suggest that a pH induced conformational change of *C. utilis* invertase occurs within 1 hr. at pH 10.0 which is not reversed when the pH is lowered to 4.7 by overnight dialysis. The new conformation presumably represents the enzyme conformation in a lower state of free energy. Introduction of cross-links into the invertase molecule may therefore result in increased stabilization of this new conformation even further, or cause the formation of another, more stable conformational variant.

The differences in thermal stability between *C. utilis* and Baker's yeast invertases may be due to amino acid sequence differences in relation to interaction with mannan. Further research is required in order to determine the amino acid composition of *C. utilis* invertase. It is possible that the alkali induced conformational change of *C. utilis* invertase is accompanied by new bond formation (salt-links, hydrogen bonding and hydrophobic interactions) which enhances stability and therefore the amino acid side chains involved would have to be readily exposed for these new interactions to occur, suggesting that this enzyme has high conformational flexibility. In Baker's yeast invertase, the mannan may be positioned such that new bonding is prevented and it displays less conformational flexibility. Alternatively, differences in amino acid sequence may prevent the formation of new cross-links with alkali of DMS.

In considering the nature of the stability of proteins, it is worth remembering that millions of years of evolution contribute to the folding of newly synthesized polypeptides resulting in tertiary and quaternary structures with thermodynamic and kinetic stability. Attempts to enhance the stability by chemical modification (and by immobilization techniques) may not always be successful if the natural mechanism of stability is interfered with.
metabolism has gained much attention. For example, Tyrrell and Ryman (1976) have entrapped invertase in erythrocyte ghosts for possible use in the treatment of intestinal sucrose intolerances. Clearly the entrapment of invertases stabilized by chemical modification against proteolytic attack and thermal denaturation would increase their therapeutic effectiveness. The entrapment of naturally occurring stable invertases such as from *Candida utilis* is also recommended.
Chapter 7

Immobilization of yeast invertase

7.1 - Introduction

Wingard (1972) has written: "Immobilization refers to the modification of an enzyme so as to restrict its gross movement and keep it in a relatively defined region of space".

Since the development of immobilization techniques, there have appeared in the literature several reviews on immobilized - enzymes (Barker et al., 1971; Melrose, 1971; Falb, 1972; Goheer, 1974; Weetall, 1975; Barker and Kay, 1975). There are four main methods that are used for the immobilization of enzymes:

a) physical adsorption on to an inert carrier

b) entrapment within the lattices of natural and synthetic polymers

c) cross-linking of the enzyme with a bifunctional reagent either to functional groups on an insoluble support matrix or to itself after adsorption on to an inert carrier

d) covalent binding to a reactive insoluble support

Barker and Kay (1975) have written a comprehensive review on these methods and therefore they have not been fully described in this thesis.

This chapter describes the immobilization of commercial yeast invertase by a variety of techniques which includes the first description of the use of concanavalin A, insolubilized on agarose, for the immobilization of invertase. The aim of this study was to obtain an immobilized invertase preparation with high retention of specific enzyme activity, enhanced conformational stability and which is stable to continuous inversion of sucrose in a fixed bed reactor. Such a preparation may have potential industrial and therapeutic usage.
Attempts have been made to immobilize invertase on to:

1) DEAE - Sephadex A-50 (adsorption)
2) CM - Sephadex C-50 (adsorption)
3) Microcrystalline cellulose (metal-link method)
4) Porous glass beads (metal-link method)
5) Enzacryl AA (covalent-link)
6) Enzacryl AH (covalent-link)
7) Concanavalin A, insolubilized on agarose (adsorption)

This introduction briefly reviews the immobilization of enzymes by the above mentioned and similar techniques and compares the properties of the immobilized and native enzymes.

7.1.1. - Immobilization by ionic adsorption on to DEAE - Sephadex and CM - Sephadex

The adsorption of enzymes on to an insoluble matrix such as DEAE - Sephadex, DEAE - cellulose and CM - cellulose is mainly due to multiple salt-linkages. The enzyme α-chymotrypsin has been covalently bound on to CM - Sephadex (in Zaborsky, 1973).

Numerous examples of enzymes adsorbed on to these inert carriers appear in the literature (for a review see Zaborsky, 1973). The specific activity of an enzyme immobilized by adsorption is usually lowered. For example, Suzuki et al., (1966, 1967) found only 33% retention of the specific activity of the native enzyme when invertase was bound on to DEAE - cellulose. Similar results were demonstrated by Usami et al., (1971). Maeda et al., (1973) found that approximately 55 - 70% of invertase activity was lost on binding to DEAE - cellulose. Interestingly, no loss in the original enzyme activity was found when

* CM - carboxy methyl
tomato invertase was adsorbed on to CM - cellulose (Nakagawa et al., 1975). Tosa et al., (1969) immobilized aminoacylase upon DEAE - Sephadex and reported an approximately 50% loss in specific enzyme activity.

Immobilization of enzymes by adsorption can result in an increased or decreased thermal stability relative to the native enzyme. Enhanced thermal stability was found for aminoacylase on DEAE - Sephadex and DEAE - cellulose (Tosa et al., 1969), for lactate dehydrogenase attached to DEAE - cellulose (Wilson et al., 1968) and for phospho-monoesterase adsorbed on to CM - cellulose (in Zaborsky, 1973). On the contrary, decreased thermal stability was observed for ATP deaminase and invertase adsorbed on to DEAE - cellulose (Chung et al., 1968; Suzuki et al., 1966, 1967). Interestingly, purified tomato invertase that had been adsorbed on to CM - cellulose was more heat stable than the soluble form (Nakagawa et al., 1975).

When an enzyme is immobilized on a charged carrier, changes can occur in its pH optimum (Barker and Kay, 1975). The pH optimum was decreased (shifted towards more acid pH's) for glucoamylase on DEAE - Sephadex (in Zaborsky, 1973) and for ATP deaminase, aminoacylase and invertase on DEAE - cellulose (Chung et al., 1968; Tosa et al., 1967; Suzuki et al., 1966, 1967). Conversely the pH optimum can be increased (shifted towards more alkaline pH's) for enzymes bound on to a negatively charged support such as CM - cellulose or CM - Sephadex.

The reason for the displacement of the pH in either direction is attributable to the creation of a microenvironment around the water-insoluble adsorbed enzyme. Goldstein et al., (1964) have made a detailed explanation of this behaviour which is summarized by Westall (1975) and Zaborsky (1973). It is based upon a qualitative and quantitative
Changes in the value of the apparent Michaelis constant (Km) can also occur upon immobilization. The apparent Km for aminoacylase adsorbed on to DEAE-Sephadex or DEAE-cellulose was either increased or decreased relative to the Km of the native enzyme depending upon the substrate used (Tosa et al., 1967, 1969). Nakagawa et al., (1975) found that the Km of tomato invertase, adsorbed on to CM-cellulose, was increased two-fold relative to that of the native enzyme.

The apparent Km of an adsorbed enzyme is increased relative to the Km of the soluble enzyme, being due to the creation of a microenvironment around the water-insoluble adsorbed enzyme. Lilly et al., (1968) have explained that a diffusion barrier surrounding the insoluble particle will be the limiting factor in the rate of reaction because the substrate concentration within this barrier will be lower than in the external solution. These diffusional restrictions of substrate into, and products out of the adsorbed enzyme can be overcome by reducing the physical size of the inert particle (Kay and Lilly, 1970), rapid stirring of the immobilized enzyme suspension (Hornby et al., 1966) and by increasing the flow rate through a fixed bed reactor column containing the immobilized enzyme (Weetall, 1975), which return the apparent Km of an immobilized enzyme to that of the soluble enzyme (Barker and Kay, 1975). The difference between the apparent Km of an ionically adsorbed enzyme and the Km of the soluble enzyme can also be explained in terms of the effect of the electrostatic potential in the vicinity of the adsorbed enzyme on the charged substrate molecules in the microenvironment (Goldstein et al., 1964). Hornby et al., (1968) derived an equation which satisfactorily explains the effects of diffusion and electrostatic interaction on the apparent Km of an enzyme immobilized on a charged support.
Generally, like charges on substrate and support increase the apparent
Km and unlike charges decrease the apparent Km relative to the Km of the
soluble enzyme. The effect of diffusion always increases the apparent Km.

Commercial invertase has been immobilized on to DEAE - Sephadex
A-50 and CM - Sephadex C-50 and a comparison of the properties of the
bound enzyme to the soluble enzyme has been made. The suitability of
these supports for the immobilization of invertase is discussed.

7.1.2. - Immobilization to microcrystalline cellulose and
porous glass beads by the metal-link method

The immobilization of enzymes on to cellulose, nylon or glass
surfaces activated with the salts of titanium and other transition metals
was developed by Novais (1971). The enzymes, amyloglucosidase,
α-amylase, trypsin, glucose oxidase and invertase, have been coupled on
to microcrystalline cellulose activated by titanium with 45-75, 54.0, 35.2,
61.5 and 3.6% recovery of original specific enzyme activity respectively
(Barker et al., 1971). The particular interest to this thesis was the
very low retention of specific activity when invertase was immobilized
by this method. Thornton et al., (1974a) successfully immobilized
lactoperoxidase and invertase on to glass beads, porous glass and sand
by the metal-link method (activated by titanium). They found that for
these two enzymes, immobilization was pH dependent, occurring most
efficiently at pH 4.5. It was inferred from this that the metal-link
method for the immobilization of enzymes is restricted to enzymes stable
at pH 4.5. Indeed, attempts to immobilize xanthine oxidase, lactate
dehydrogenase and alcohol dehydrogenase by this method using alkaline
pH values were unsuccessful (Coughlan and Johnson, 1973; Thornton
et al., 1974a). By using the metal-link method, enzymes have been
coupled to numerous supports. Amyloglucosidase has been immobilized
on to various forms of cellulose (microcrystalline, wood flour and sawdust, filter paper), glass, terylene and nylon fibres (Emery et al., 1972), β - galactosidase, invertase and lactoperoxidase on to the minerals hornblende, biotite and muscovite (Thornton et al., 1974b; Johnson et al., 1974). This method is more efficient than the silane method, which is also used for the coupling of enzymes on to inorganic and supports (Woetall, 1975), for the immobilization of lactoperoxidase and invertase on to hornblende and muscovite (Johnson et al., 1974; Thornton et al., 1974b).

The thermostability of amyloglucosidase coupled to microcrystalline cellulose and lactoperoxidase coupled to sand by the metal-link method, has been improved relative to the soluble enzymes (Emery et al., 1972; Thornton et al., 1974a). There seems to be little effect on the pH optimum curves of these enzymes, except that slight spreading of the curve for amyloglucosidase was observed (Emery et al., 1972). For invertase, however, it was reported that the pH optimum of the enzyme bound to sand was 4.6 compared to 4.4 for the soluble enzyme, (Thornton et al., 1974a). These investigators also reported that the apparent Km of lactoperoxidase bound to sand was slightly increased.

Commercial invertase has been successfully immobilized on to microcrystalline cellulose but not on to porous glass beads. Some of the properties of the bound enzyme are compared to those of the native enzyme.

7.1.3. Immobilization by covalent binding to

* Enzacryl AA and * Enzacryl AH

Enzacryls are polyacrylamide copolymers containing functional groups which after activation can covalently bind proteins. Enzacryls

* Registered trade name of Koch-Light Laboratories Ltd.
AA and AH contain the aromatic amino residue and the acid hydrazide residue respectively, which can be activated and covalently bind proteins as depicted in Fig. 7.1.

Barker et al., (1970) covalently bound $\alpha -$ amylase on to Enzacryls AA and AH by using the methods depicted in Fig. 7.1. and found that only 6.1% and 16% recovery of specific activity was obtained upon binding respectively. These investigators also coupled $\beta -$ amylase on to Enzacryl AA and AH and obtained 1.5% and 0.0% recovery of specific activity respectively. Wiseman and Thacker (A. Wiseman and F.E. Thacker, unpublished) recovered only 2.5% of the specific activity of alcohol dehydrogenase bound to Enzacryl AA whilst this enzyme bound to Enzacryl AH was completely inactive.

Barker et al., (1970) reported that $\alpha -$ amylase covalently bound to both Enzacryls showed enhanced thermal stability relative to the soluble enzyme, whereas $\beta -$ amylase similarly bound exhibited lower thermal stability, the soluble enzyme being the most stable in this case.

An attempt has been made to immobilize commercial invertase on to Enzacryls AA and AH, activated as described.

7.1.4. - Immobilization by the group specific adsorbent concanavalin A - agarose

The protein concanavalin A, isolated from jack bean (Canavalia ensiformis), is a phytohaemagglutinin capable of reacting with and binding to polysaccharides and to the carbohydrate moiety of a glycoprotein. Goldstein et al., (1974) reported that concanavalin A binds to saccharide chain ends of $\alpha -$ D - glucans, $\alpha -$ D - mannans and $\beta -$ D - fructans but also binds to 2 - O - substituted $\alpha -$ D - mannopyranosyl units. Studies on the inhibition of concanavalin A - dextran precipitation
Fig. 7.1. - The activation of Enzacryls AA and AH followed by covalent binding of protein

A

\[
\text{Enzacryl AA} \xrightarrow{\text{HNO}_2 \text{ diazotisation}} \text{activated Enzacryl AA}
\]

\[
\text{Enzyme} \xrightarrow{\theta} \text{Covalently bound enzyme}
\]

B

\[
\text{Enzacryl AH} \xrightarrow{\text{HNO}_2 \text{ diazotisation}} \text{activated Enzacryl AH}
\]

\[
\text{Enzyme} \xrightarrow{\theta} \text{Covalently bound enzyme}
\]
revealed that certain hydrogen and oxygen atoms of sugars were involved in binding to the protein, perhaps involving hydrogen bonding (Doyle et al., 1968; So and Goldstein, 1967). Concanavalin A possesses a tetrameric structure, each sub-unit having a sugar binding site (Lis and Sharon, 1973). These investigators reported that X-ray crystallographic studies on a concanavalin A - (o - iodophenyl) β - D - glucopyranoside complex revealed that the iodine atom was located in a deep cleft surrounded by hydrophobic residues. Between this region and the molecular surface is a region of hydrophilic groups proposed to be the carbohydrate binding site. Hardman and Ainsworth (1976) studied the concanavalin A - methyl - α - mannopyranoside complex at 6Å resolution and calculated that the carbohydrate binding site is 35 Å from the iodophenyl binding site. They found that two tyrosyls (Tyr -12 and -100) were present in the carbohydrate binding region. Also present is a charged residue, Glu -102, and a cis peptide bond between Ala -207 and Asp -208 (the only one in the structure). They suggested that these two features destabilized the structure in this region suggesting that conformational flexibility is required in this site during carbohydrate binding.

Concanavalin A bound to Sepharose and agarose is available commercially and recommended for the isolation of polysaccharides and glycoproteins. For example, Gallili (1975) separated external and internal invertases by affinity chromatography using concanavalin A - Sepharose. It is therefore possible to utilise an insolubilized concanavalin A for the preparation of immobilized external invertase. High retention of specific activity should be achieved since immobilization is through the mannan moiety which is not essential for activity.

Sulkowski and Laskowski (1974) reported that the immobilization of venom exonuclease (phosphodiesterase) on to concanavalin A - Sepharose
resulted in the thermal stabilization of the enzyme at room temperature. Interestingly, Davey et al., (1976) have reported the immobilization of human fibroblast interferon on to concanavalin A - agarose which is dependent on carbohydrate recognition exclusively.

Purified Baker's yeast external invertase has been successfully immobilized on to concanavalin A - agarose and the properties of the immobilized-enzyme have been investigated.
7.2. - Materials and Methods

DEAE - Sephadex A-50 and CM - Sephadex C-50 were purchased from Pharmacia. Microcrystalline cellulose (Sigma cell type 20) and a suspension of concanavalin A - insolubilized on agarose were purchased from Sigma. Porous glass beads (pore diameter 13 50A; particle diameter 177-840 microns) were a gift from Corning Biological Products Dept., Medfield, Mass. 02052, U.S.A. Enzacryls AA and AH were purchased from Korh-Light Laboratories Ltd.

7.2.1. - Immobilization of invertase on to DEAE - Sephadex A-50 and CM - Sephadex C-50

0.5g dry weight of DEAE - Sephadex A-50 and CM - Sephadex C-50 were equilibrated in 100ml. of 10mM - sodium phosphate buffer, pH 7.0 and 10mM - sodium acetate buffer, pH 3.6 respectively for 3 days at room temperature. For the preparation of the Sephadex - invertase complexes, 0.1ml. of commercial invertase (≈ 400 units) was added to 10ml. of each equilibrated Sephadex (≈ 50mg. dry weight) and mixed (end to end rotation) for 30 min. at room temperature. The Sephadex - invertase complexes were centrifuged and washed with their respective buffers until no enzyme activity could be detected in the washings. The complexes were then resuspended in their equilibrating buffers (10ml. total volume). A 0.1ml. aliquot of the complex, (previously washed in either pH 7.0 or pH 3.6 buffer) was assayed for activity.

The stability of the complexes to various pH's was investigated by shaking 0.1ml. of each complex in 1.9ml. of buffer at a particular pH for 5 min. at room temperature and measuring the activity of the eluant.
7.2.2. - Immobilization of invertase on to microcrystalline cellulose and glass beads by the metal-link method

The method of Emery et al., (1972) was mainly followed for this procedure. 0.5g of microcrystalline cellulose and 0.5g of glass beads were each shaken (end to end rotation) with 10ml. of titanous chloride solution (15% w/v - purchased from B.D.H.) for 3 hr. at room temperature. The activated supports were then washed exhaustively in 10mM - sodium acetate buffer, pH 4.7. They were then suspended in 9.6ml. of the same buffer and 0.4ml. of commercial invertase (≥ 1600 units of enzyme activity) was added. The enzyme and support were mixed overnight at 4°C by using rotation on rollers.

The enzyme - support complexes were then centrifuged using a bench centrifuge and the supernatants removed and assayed for invertase activity. The complexes were washed and resuspended in 10ml. of buffer, pH 4.7. A 0.1ml. aliquot of the microcrystalline cellulose - invertase complex and 5mg. of the dried glass beads - invertase complex were assayed for enzyme activity.

The microcrystalline cellulose - invertase complex was also washed with 10ml. of buffer, pH 4.7, 1.0M to NaCl, in order to remove any enzyme that may have adsorbed on to the cellulose.

7.2.3. - Immobilization of invertase on to Enzacryls AA and AH

Immobilization of commercial invertase on to Enzacryls AA and AH was performed according to the method of Barker et al., (1970).

Enzacryls AA and AH (100mg. of each) were mixed with 5ml. of 2N HCl at 4°C (mixing performed by rotation on rollers) for 15 min. An ice cold solution of sodium nitrite solution (4ml. 2% w/v) was added
and mixing was continued for a further 20 min. The diazotised Enzacryls AA and AH were then washed thoroughly with 10mM sodium phosphate buffer, pH 8.0 and 10mM sodium carbonate buffer, pH 9.2, respectively, after which the diazo-Enzacryls were resuspended in 9.9ml. of the respective buffers. To each of the activated Enzacryls was added 0.1ml. of commercial invertase (~400 units) and coupling was allowed to proceed for 48 hr. at 4°C with rotary mixing.

Each Enzacryl-enzyme suspension was centrifuged and the supernatant was removed and assayed for invertase activity. The complexes were resuspended in 10ml. of 1M NaCl solution (in 10mM sodium acetate buffer, pH 4.7) and the suspensions shaken vigorously for 5 min. at room temperature. Finally, the Enzacryl-enzyme complexes were resuspended in 10ml. of buffer at pH 4.7 and 0.1ml. of each complex was assayed for invertase activity.

7.2.4. - Immobilization of purified Baker's yeast external invertase on to concanavalin A insolubilized on agarose

For this immobilization procedure, a purified preparation of external invertase was used since the commercial invertase was likely to contain 'non-invertase' mannan which might preferentially bind to the carrier.

For the coupling procedure, 1ml. of the concanavalin A-agarose suspension was added to the external invertase solution (3mg. of enzyme (891 units) in 5.0ml. 10mM sodium phosphate buffer, pH 7.2). The mixture was shaken (end to end rotation) for 30 min. at room temperature. The enzyme-concanavalin A-agarose complex was centrifuged and the supernatant assayed for invertase activity. The complex was washed with buffer at pH 7.2 until no activity was detectable in the washings, and finally resuspended in buffer at pH 7.2 so that the total volume of the
complex suspension was 6.0ml. A 0.1ml aliquot of the complex suspension was assayed for invertase activity.

(* Concanavalin A - insolubilized on beaded agarose in 1.0M NaCl solution, containing 6.18mg. protein/ml. 1mg. of the protein will complex 0.25 - 0.5mg. of yeast mannan at pH 7.2 at 25°C.)

7.2.5. - Studies on the immobilized invertases

The various studies performed on the immobilized - invertases (as described in section 7.3.) e.g. effect of pH on activity and stability, determination of Km and Vmax and thermal stability studies, were carried out as for the studies with the soluble enzyme except that the immobilized - invertase suspension replaced the soluble enzyme. Where standard deviations are shown, the experiment was performed in triplicate.
7.3. - Results

7.3.1. - Immobilization of commercial invertase on to DEAE - Sephadex A-50 and CM - Sephadex C-50

DEAE - Sephadex A-50 and CM - Sephadex C-50 readily bound invertase (see Table 7.1.). Apparently all the invertase added to DEAE - Sephadex was adsorbed but approximately 57 - 80% of the enzyme activity was lost on binding. Invertase was not as efficiently adsorbed on to CM - Sephadex but only 0 - 30% of enzyme activity was lost on binding. It was found that 800 units and 3200 units of invertase was most efficiently bound to 50mg. of DEAE - Sephadex and CM - Sephadex respectively.

Since the adsorption of a protein on to an ion-exchanger is known to be affected by altering the pH at which adsorption readily occurs (i.e. pH 7.0 and 3.6 for DEAE - Sephadex and CM - Sephadex respectively) the binding-stability of the complexes in buffers at different pH's was investigated. A high molarity of the buffer used is also known to elute an ionically bound protein, hence the molarity of each buffer used in this study was 10mM. It was found that the DEAE - Sephadex complex was stably-bound between pH 3.6 and 7.0, whereas the CM - Sephadex complex was stably-bound only between pH 2.5 and 4.0 (see Table 7.2.). Under the conditions used in this experiment, the DEAE - Sephadex complex remained stably-bound over a greater range of pH than either the CM - Sephadex complex or the DEAA - cellulose invertase complex formed by Maeda et al., (1973).

The effect of pH (in buffers of ionic strength, 10mM) on the activity and stability of the complexes was investigated even though elution of some enzyme activity was likely at some pH values. There was shift towards a more acid pH and a more neutral pH of the pH optima.
Table 7.1. - Preparation of DEAE - Sephadex A-50 and CM - Sephadex C-50 invertase complexes

<table>
<thead>
<tr>
<th>Invertase added (units of enzyme activity)</th>
<th>Invertase in washings (u)</th>
<th>Bound invertase theoretical A (u)</th>
<th>Activity of complex B (u)</th>
<th>B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE - 400 u</td>
<td>0</td>
<td>400</td>
<td>164</td>
<td>41</td>
</tr>
<tr>
<td>DEAE - 800 u</td>
<td>0</td>
<td>800</td>
<td>342</td>
<td>43</td>
</tr>
<tr>
<td>DEAE - 1600 u</td>
<td>0</td>
<td>1600</td>
<td>507</td>
<td>32</td>
</tr>
<tr>
<td>DEAE - 3200 u</td>
<td>0</td>
<td>3200</td>
<td>980</td>
<td>31</td>
</tr>
<tr>
<td>DEAE - 6400 u</td>
<td>0</td>
<td>6400</td>
<td>1300</td>
<td>20</td>
</tr>
<tr>
<td>CM - 400 u</td>
<td>100</td>
<td>300</td>
<td>210</td>
<td>70</td>
</tr>
<tr>
<td>CM - 800 u</td>
<td>152</td>
<td>648</td>
<td>498</td>
<td>77</td>
</tr>
<tr>
<td>CM - 1600 u</td>
<td>430</td>
<td>1170</td>
<td>895</td>
<td>77</td>
</tr>
<tr>
<td>CM - 3200 u</td>
<td>824</td>
<td>2376</td>
<td>2476</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CM - 6400 u</td>
<td>2704</td>
<td>3696</td>
<td>3436</td>
<td>93</td>
</tr>
</tbody>
</table>
**Table 7.2.** Elution of invertase activity from DEAE - Sephadex and CM - Sephadex by buffers at various pHs

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Eluted activity (u)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DEAE - Sephadex</td>
<td>CM - Sephadex</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>3.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.7</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>5.6</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>6.0</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>7.0</td>
<td>0</td>
<td>22</td>
</tr>
</tbody>
</table>

13 units of DEAE - Sephadex - invertase and 34 units of CM - Sephadex - invertase complexes were shaken for 5 min. at room temperature in 1.9 ml. of buffer (see below) and the activity of the eluant was measured.

(Buffers: 10mM - glycine - HCl, pH 2.5;
10mM - sodium acetate, pHs 3.6, 4.0, 4.7, 5.6;
10mM - sodium phosphate, pHs 6.0, 7.0).
of the DEAE - Sephadex and CM - Sephadex complexes respectively compared to the native enzyme. The noticeable effect of pH on stability at 50°C was that the CM - Sephadex complex was apparently stable between pH's 5.6 and 7.0, compared to the native enzyme which was stable between pH's 3.6 and 6.0. It would seem, however, that the practical use of these ionically-bound invertases with modified properties, is severely restricted due to their elution from the support at, or near their pH optima for activity and stability.

Thermal stability studies, at 65°C in the absence of substrate, on the DEAE - Sephadex and CM - Sephadex complexes at pH 4.0 and 5.6 respectively, revealed that both complexes were very unstable towards heat, compared to the native commercial invertase, losing all activity within 1 min. of treatment. This is in agreement with the findings of previous investigators who have also found that ionically-bound invertase shows less stability towards heat than does the native enzyme (Chung et al., 1968; Suzuki et al., 1966, 1967). The possible importance of salt-linkages in maintaining the conformational stability of invertase has been mentioned in chapter 6. The adsorption of proteins on to ion - exchangers such as DEAE - Sephadex and CM - Sephadex, involves their carboxyl and amino groups respectively and some of these groups would be involved in salt-linkages in the invertase molecules. It follows, therefore, that the adsorption of invertase on to these supports involving amino and carboxyl groups of the enzyme could lead to a conformationally unstable immobilized - invertase preparation because of the disruption of the conformationally-stabilizing salt-linkages occurring in the enzyme molecule. Thus, some of the amino and carboxyl groups naturally forming these stabilizing salt-linkages would ionically link the enzyme on to the support, the newly formed salt-linkages between the enzyme and support conferring no conformational stability on to the immobilized invertase preparation.
Fig. 7.2. - The effect of pH on the activity and stability of the DEAE - Sephadex and CM - Sephadex - invertase complexes.

Key:

A - Effect of pH on activity
B - Effect of pH on stability

- native commercial invertase
- DEAE - Sephadex - invertase
- CM - Sephadex - invertase
The ionic adsorption of invertase on to DEAE - Sephadex and CM - Sephadex would result in amino and carboxyl groups being exposed on the surface of the enzyme molecule, respectively. If these exposed groups were involved in salt-linkages in the enzyme molecule prior to immobilization, it is reasoned that the addition of reagents such as succinic acid and ethylenediamine to the DEAE - Sephadex and CM - Sephadex complexes respectively, could stabilize them by forming salt-linkages with the exposed amino and carboxyl groups of the bound enzyme (see Fig. 7.3.). Support for this hypothesis came from the finding that the thermal stability of the DEAE - Sephadex and CM - Sephadex invertase complexes, at 65°C in the absence of substrate was significantly improved by their heating in the presence of 20mM succinic acid and 20mM ethylenediamine respectively (see Fig. 7.4.). Trial experiments in which the bound enzymes were shaken in similar concentrations of ethylenediamine or succinic acid at room temperature for 5 min. resulted in no elution of the bound enzymes. Interestingly, Goheer et al., (1976) found that glucose 6 - phosphate dehydrogenase, immobilized on *CNBr - activated Sepharose 4B, showed increased stabilization against heat when the carrier was made positively charged 30 min. after enzyme immobilization, by the addition of ethylenediamine, compared to the enzyme immobilized with no further additions. Again, it is presumed that this increased stabilization is due to the formation of salt-linkages.

It is therefore concluded that a decreased thermal stability of an immobilized enzyme relative to that of the native enzyme, will be expected if the amino acids used in the linkage to the carrier are involved in stabilizing interactions (e.g. salt-linkages, hydrogen bonding etc.). Clearly the understanding of specific amino acid

* CNBr  -  Cyanogen bromide
Fig. 7.3. - A hypothetical approach to the thermal -stabilization of invertase immobilized on DEAE - Sephadex and CM - Sephadex

1. Stabilizing salt linkage

2. Destabilized DEAE - Sephadex - invertase complex

3. Destabilized CM - Sephadex - invertase complex

4. Newly formed stabilizing salt-link in the DEAE - Sephadex - invertase complex

5. Newly formed stabilizing salt-link in the CM - Sephadex - invertase complex
Fig. 7.4. - Thermal inactivation at 65°C of DEAE - Sephadex and CM - Sephadex - invertase complexes in the presence of 20mM succinic acid and 20mM ethylenediamine respectively.

Key:  
- O-O, CM - Sephadex - invertase complex heated in 20mM ethylenediamine  
- ■-■, DEAE - Sephadex - invertase complex heated in 20mM succinic acid

Dashed line represents the thermal inactivation of both complexes in the absence of ethylenediamine or succinic acid, showing total loss of enzyme activity within 1 min. of heating.

Values are mean ± S.D.
interactions involved in maintaining the conformational stability of a
protein will enable a method of immobilization to be chosen that has
little effect on the stability. Alternatively, the addition of reagents
to the external environment of an immobilized enzyme could impart
stability to such a preparation.

The effect of substrate concentration on the activity of the
complexes is shown in Fig. 7.5. The Km of the CM - Sephadex - invertase
complex was increased four-fold relative to that of the native enzyme.
Interestingly, it was found that the DEAE - Sephadex - invertase behaved
similarly to an allosteric enzyme. Apparent activation of the complex
occurred at 0.06M sucrose. The reason for this behaviour is not known
but may reflect a competition between the substrate sucrose and the
positively charged groups of the support for the active site carboxylate
group proposed by Waheed and Shall (1971b). Consequently, under these
conditions, the Km of this complex could not be measured.

Finally, attempts to cross-link the enzyme molecules inter-
molecularly once adsorbed on to the supports using glutaraldehyde, were
abandoned due to almost complete enzyme inactivation of the complex even
when very low glutaraldehyde concentrations were used (1.25 μmol./ml.).

7.3.2. - **Immobilization of commercial invertase on to**
**microcrystalline cellulose and porous glass beads**
**by the metal-link method**

It was found that titanium activitated microcrystalline
cellulose, but not the glass beads used in this study, readily bound
invertase (see Table 7.3.). Invertase bound to microcrystalline
cellulose was less active than the native enzyme and 54% of the enzyme
activity was lost on binding. The washing of the enzyme complex with
1.0M NaCl resulted in no elution of invertase activity. Therefore,
Fig. 7.5. - The effect of substrate concentration on the activity of the DEAE - Sephadex and CM - Sephadex invertase complexes (Lineweaver - Burk plots)

Key: O-O, CM - Sephadex - invertase complex, $K_m = 100\text{mM}$

-■-■, DEAE - Sephadex - invertase complex
Table 7.3. - Preparation of microcrystalline cellulose and glass beads invertase complex using the metal-link method

<table>
<thead>
<tr>
<th>Invertase added (units of enzyme activity)</th>
<th>Invertase in washings (u)</th>
<th>Bound invertase theoretical A (u)</th>
<th>Activity of complex B (u)</th>
<th>B/A %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcrystalline cellulose</td>
<td>1600</td>
<td>1000</td>
<td>600</td>
<td>275</td>
</tr>
<tr>
<td>Glass beads</td>
<td>1600</td>
<td>1600</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
greater retention of specific activity of invertase immobilized by this method was obtained (46%) compared to the finding of Barker et al., (1971) who found that there was only 3.6% retention of specific activity when invertase was similarly immobilized.

Emery et al., (1972) reported the spreading of the pH optimum curve which was obtained when amyloglucosidase is immobilized by the metal-link method. A similar effect of pH activity of the microcrystalline cellulose - invertase complex was observed by this work (see Fig. 7.6.A). The complex exhibited over 90% of maximal activity at pH 2.5 compared to 38% for the native enzyme. Similarly, there was 67% of maximal activity at pH 7.0 compared to 26% for the native enzyme. There was no change in the pH optimum of the invertase complex. The effect of pH on stability at 50°C showed that greatest stability occurred between pH 4.0 and 6.0 and that there was improved stability at pH 2.5 and 7.0 compared to the native enzyme (see Fig. 7.6.B).

The complex exhibited significantly improved thermal stability at 65°C relative to the native enzyme both in the absence and presence of 0.12M sucrose (see Figs. 7.7. and 7.8.). In the absence of substrate there was a 12% and 43% recovery of enzyme activity for the native and complexed enzymes respectively after 5 min. of treatment. In the presence of substrate there was no loss in activity of the complex after 60 min. of incubation, whereas only approximately 30% of the native enzyme activity was recovered after the same treatment (calculated from the slope of the progress curve). It is therefore concluded that this immobilized - invertase preparation could be efficiently used in some industrial reactor process involving the production of invert sugar.

Interestingly, the Km and Vmax of the microcrystalline cellulose - invertase complex were similar to those of the native commercial enzyme, being 28.5mM and 33 μmol. min⁻¹ respectively.
Fig. 7.6. - The effect of pH on the activity and stability of microcrystalline cellulose - invertase complex

Key:  
A - effect of pH on activity  
B - effect of pH on stability  
O--O, native commercial enzyme  
□-□, microcrystalline cellulose - invertase complex
Fig. 7.7. - Thermal inactivation (65°C) of the microcrystalline cellulose - invertase complex in the absence of substrate.

Key:
- ○○, native commercial invertase
- ○-○, microcrystalline cellulose - invertase complex

Values are mean ± S.D.
Fig. 7.8. - Progress of sucrose hydrolysis by native invertase and microcrystalline cellulose - invertase at 65°C in 0.12M sucrose

Commercial invertase (0.1 unit) and microcrystalline cellulose - invertase (0.13 unit) were added to 3.4ml. of 0.12M sucrose in 0.1M sodium acetate buffer, pH 4.7, for initiation of the reaction. At the indicated times 2.5ml. of 3'5 - dinitrosalicylic acid reagent was added and reducing sugar formed was measured as previously described.

Key: as for Fig. 7.7.

Values are mean ± S.D.
7.3.3. - Immobilization of commercial invertase on to Enzacryls AA and AH

Enzacryls AA and AH readily bound invertase, but the bound enzyme on each support was inactive. Using the conditions described in section 7.2.3. Enzacryls AA and AH bound 64% and 46% of the invertase activity available, but all activity was lost on coupling. The reason for this total loss of activity on coupling is not known, but very low recoveries of specific enzyme activity coupled by these methods have been previously reported (see section 7.1.3.).

Since tyrosine residues may be involved in the catalytic activity of invertase (see chapter 6) then coupling of this enzyme to Enzacryl AA could result in the loss of activity. Alternatively, the access of substrate to the active site may be prevented due to steric hindrance. The coupling of invertase to Enzacryl AH may result in an increase of the overall negative charge of the enzyme. This may result in an unfavourable conformational change rendering the enzyme inactive.

7.3.4. - Immobilization of purified Baker's yeast external invertase on to concanavalin A insolubilized on agarose

Concanavalin A - insolubilized on agarose readily bound invertase (see Table 7.4.). Bound invertase was less active than the native enzyme and 27% of the enzyme activity was lost on binding. No enzyme activity was eluted when a 0.1ml. portion of the complex was washed in buffers of 10mM ionic strength between pH 2.5 and 7.0.

The effect of pH on the activity and stability of the complex indicated that there was a slight spreading of the pH optimum curve, the complex showing greater activity at pH 2.5 and 7.0 and a greater stability at 50°C in pH 2.5, compared to the native enzyme (see Fig. 7.9.A.B.). The complex exhibited significantly improved thermal stability at 65°C in
<table>
<thead>
<tr>
<th>Invertase added (units of enzyme activity)</th>
<th>Invertase in washings (u)</th>
<th>Bound invertase A (u)</th>
<th>Activity of complex B (u)</th>
<th>B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>891</td>
<td>85</td>
<td>806</td>
<td>588</td>
<td>73</td>
</tr>
</tbody>
</table>

Table 7.4. - Preparation of insolubilized - concanavalin A - invertase complex
Fig. 7.9. - The effect of pH on the activity and stability of the insolubilized concanavalin - invertase complex.

Key:  
A - effect of pH on activity  
B - effect of pH on stability  
●●, native purified external invertase  
▲▲, insolubilized concanavalin - invertase complex
the absence and presence of 0.12M sucrose (see Figs. 7.10 and 7.11). These results were similar to those obtained with the microcrystalline - cellulose - invertase complex. Heating in the presence of 0.12M sucrose, even at 65°C, considerably protected the soluble and immobilized - invertase preparations against thermal inactivation. This phenomenon has been considered in chapter 5.

The kinetic parameters, Km and Vmax, of the insolubilized concanavalin A - invertase complex, were similar to those of the native enzyme being 28.5mM and 19.6 μmol. min⁻¹ respectively.

7.3.5. - The stability of the microcrystalline cellulose - invertase and insolubilized concanavalin A - invertase complexes to continuous inversion

The stability of these immobilized - invertase complexes during continual sucrose hydrolysis at room temperature, was studied in a fixed-bed reactor as depicted in Fig. 7.12. It was found that both complexes were completely stable to continuous inversion of sucrose in a fixed-bed reactor at room temperature for 14.6 hr. (see Fig. 7.13.). During this time the ratio of sucrose hydrolysis remained constant and was close to 100% inversion, for each complex.
Fig. 7.10. - Thermal inactivation (65°C) of the insolubilized concanavalin A - invertase complex in the absence of substrate.

Key: as Fig. 7.9.

Values are mean ± S.D.
Fig. 7.11. - Progress of sucrose hydrolysis by native invertase and insolubilized concanavalin A - invertase complex at 65°C in 0.12M sucrose.

Key as Fig. 7.9.

For details see Fig. 7.8.

Approximately 0.1 unit of activity was used in the reaction of the native and complexed enzymes with sucrose.

Values are mean ± S.D.
Fig. 7.12. - Schematic diagram of the fixed-bed reactor used to study the stability of immobilized invertases to continuous inversion

160mg. (92.8 units) of the microcrystalline cellulose - invertase complex and 2.5ml. (262.5 units) of the insolubilized concavalin A - invertase complex were used in the formation of the immobilized invertase beds. For the continual hydrolysis of sucrose, sucrose (4% w/v in 10mM - sodium acetate buffer, pH 4.7) was pumped through each bed at a flow rate of 6.63mls hr⁻¹. 4ml. fractions were collected and each was assayed for reducing sugar using the 3'5 dinitrosalicylic acid reagent.

* Bed dimensions = 0.7mm. x 10mm.
Fig. 7.13. Continuous hydrolysis of sucrose by microcrystalline - cellulose - invertase and insolubilized concanavalin A - invertase complexes in a fixed-bed reactor.

Key: ▲▲, microcrystalline cellulose - invertase
     ▲▲, insolubilized concanavalin A - invertase
Studies on immobilized forms of yeast invertase have been performed in order to determine whether such derivatives have high retention of original specific enzyme activity, improved thermal stability relative to the native enzyme and whether they would be stable in a suitable reactor system for the continuous hydrolysis of sucrose under optimal conditions for hydrolysis to occur. It is the view of this thesis that the microcrystalline cellulose - invertase and insolubilized concanavalin A - invertase complexes potentially fulfil these objectives. Both these complexes exhibited enhanced thermal stability in the absence and presence of substrate, were stable to continuous hydrolysis of sucrose and possessed 46% and 73% retention of specific enzyme activity respectively.

Immobilized enzyme engineering uses two main types of reactor design, the continuous stirred tank and batch reactors and the fixed-bed or packed column reactor (Gould, 1975; Westall, 1975; Filbert and Pitcher, 1976). The choice of a particular reactor for use in an immobilized enzyme system depends upon a number of factors including reaction kinetics, the nature of the material being fed into the reactor, stability of the carrier material to the reactor system, substrate and product inhibition and diffusion limitations (Gould, 1975; Filbert and Pitcher, 1976). Further investigation into the efficient use of the microcrystalline cellulose - invertase and insolubilized concanavalin A - invertase complexes in continuous stirred tank and fixed-bed reactors, for the continuous inversion of sucrose, is certainly warranted but some general comments can be made. Since high sucrose concentrations inhibit invertase activity (see section 5.3.2.) a continuous stirred tank reactor (CSTR) may be more efficient in hydrolysing industrial sucrose concentrations (60 - 80% w/v). This is because the amount of sucrose in
contact with the enzyme would be lower in a CSTR. Interestingly, Boudrant and Cheftel (1975) found that continuous hydrolysis of sucrose (80% w/v) in a tubular reactor system resulted in only a 25% inversion using a flow rate of 0.5ml. min.$^{-1}$. It is generally recommended that fixed-bed reactors are used with substrates of low viscosity to prevent clogging or high pressure drops across the length of the reactor (Weetall, 1975). Since high sucrose concentrations are extremely viscous, fixed-bed reactors may be more suitable than CSTR for the continuous hydrolysis of low sucrose concentrations.

Heating in the presence of low sucrose concentrations (0.12M) considerably protects the soluble and immobilized invertase preparations (see section 7.3.4.). Consequently, the continuous inversion of such low sucrose concentrations in a CSTR at high temperatures would not offer as much protection as would a fixed-bed reactor because of the reduction in the amount of sucrose in contact with the enzyme in a CSTR.

It has been established that the low efficiency of immobilized enzymes can be due to film diffusional resistances by increasing their apparent Km values relative to those of the soluble enzymes (Lilly et al., 1968) and that such resistances may be significant in either type of reactor (Zaborsky, 1973). Therefore, the use of these two immobilized invertase preparations may be efficiently used in reactor systems since the apparent Km values of these preparations, in a batch system at least, were unchanged from the Km values of the native soluble enzymes. Filbert and Pitcher (1976) have described some methods of determining whether diffusional effects are significant in a reactor containing an immobilized enzyme. For example, if the observed reaction rate is limited by the rate of diffusion to the whole of the support structure in a fixed-bed reactor on which the enzyme is immobilized (known as
external mass transfer limitation) then the height of the bed ($Z$) necessary for mass transfer to occur is given by the equation:

$$Z = \frac{\sum N \text{Re}^{\frac{2}{3}} N \text{Sc}^{\frac{2}{3}} \ln Y_1}{1.09 a_v Y_2}$$

Where $N \text{Re} =$ Reynolds number $N \text{Sc} =$ Schmidt number $a_v = $ ratio of particle surface area to reactor volume, $Y_1 =$ mole fraction substrate in the feed, $Y_2 =$ mole fraction substrate in the product, $\xi =$ void fraction. If $Z$ is much less than the actual bed height, then no important diffusional effect is present. Filbert and Pitcher (1976) quoted the example of Havewala and Pitcher (1974) who found that the estimated bed height of an immobilized glucose isomerase column was 3% less than the actual bed height, indicating that diffusional effects were not important.

Filbert and Pitcher (1976) have also described a method by which the effect of diffusion resistance limitation inside the pores of an immobilized-enzyme support can be estimated by determining the effect of temperature on the reaction rate of an immobilized-enzyme in a fixed-bed reactor. If the Arrhenius plot produces a straight line (similar to that obtained for a soluble enzyme) this usually implies that no pore diffusion resistance exists. If the plot results in a curve, particularly at high temperatures, then diffusion resistance inside the pores exists.

The microcrystalline - cellulose - invertase and insolubilized concanavalin A - invertase complexes exhibited enhanced stability to thermal inactivation at $65^\circ C$ in the absence and presence of sucrose. This enhanced stability could be explained by diffusional limitations which would result in apparent decrease of the rate constant for inactivation ($k \text{ min}^{-1}$). Interestingly, Ollis (1972) theoretically described the
influence of diffusion on the apparent thermal stability immobilized in a porous solid and showed that the immobilized enzyme would be more thermally stable than the soluble enzyme, even though the kinetic constants $K_m$ and $V_{\text{max}}$ were similar for the soluble and immobilized enzymes. He showed that as diffusion limits the reaction rate of an immobilized enzyme, there will be an increased difference in the temperature dependence between the rates of reaction of a soluble and immobilized enzyme. The rate of diminution of activity at high temperatures would be less for the immobilized enzyme because the effectiveness factor (actual rate of reaction in pore/maximum rate possible without diffusion influences) increases even though there is a decrease in the active enzyme concentration.

Korus and O'Driscoll (1975) have produced a numerical solution for the kinetics of enzymes immobilized by entrapment in spherical gel particles of radius $r_0$ over a wide range of $[S]$ and of the Damköhler number $\beta$:

$$\beta = \frac{k_{\text{cat}} E_i r_0^2}{D K_m^1}$$

Where $D$ is the substrate diffusivity, $E_i$ is the concentration of immobilized enzyme in the gel, $k_{\text{cat}}$ is the rate constant, and $K_m^1$ the Michaelis constant for the immobilized enzyme. They were able to study the denaturation process for an immobilized enzyme under any condition of diffusion control and substrate concentration. Apparent enhanced stability for gel entrapped enzymes could be achieved by increasing the enzyme concentration, the particle size or membrane thickness or by decreasing the substrate diffusivity thereby increasing $\beta$.

It is not known whether diffusional control is responsible for the enhanced thermal stability of the microcrystalline cellulose-invertase
and insolubilized concanavalin A - invertase complexes. This seems unlikely, however, due to the similarity in some kinetic behaviour to the native enzymes. Therefore, cross-linking between the support and enzyme would be responsible for the enhanced thermal stability, presumably by restricting unfavourable conformational changes.

The use of a fixed-bed reactor, containing the two complexes, for the continuous hydrolysis of low sucrose concentration was found to give 100% inversion at a flow rate of 6.63 ml.m⁻¹.min⁻¹. Using a greater quantity of enzyme (by increasing the bed height) and by keeping the same flow rate, should result in an increased residence time which would favour the hydrolysis of higher sucrose concentrations. Filbert and Pitcher (1976) have defined the normalized residence time as:

\[ \frac{kE}{KmF} \]

where \( k \) = turnover number, \( E \) = enzyme quantity, \( Km \) = Michaelis constant and \( F \) = volumetric flow rate. In such a system, however, the \( Km \) of the immobilized enzyme may increase due to diffusional restrictions encountered with a high bed height which would lead to an apparent reduction in the residence time and consequently a reduction in the % conversion of substrate. An increase in the flow rate may be detrimental by not only reducing the residence time but by causing blocking of the reactor bed and by wear and tear. Barker et al., (1971) have reported examples of blockage occurring using fixed columns of immobilized amyloglucosidase. They have reported the use of fluidized bed reactors to overcome these problems. In such a system the particles of immobilized enzyme are suspended in an upward flow of substrate. This prevents blocking of the reactor bed, improves mass and heat transfer properties (reducing the diffusional limitations) and increases the reacting surface area available. Such a reactor is recommended for the continual hydrolysis of sucrose by insolubilized concanavalin A - invertase complex since it is well known that columns of gel material such as
Sephadex G-200 and DEAE-Sephadex A-50 are susceptible to blockage by prolonged passage of fluid (usually buffer) through these columns.

Interestingly, the microcrystalline-cellulose-invertase and insolubilized concanavalin A-invertase complexes both exhibited broader pH optimum curves than the respective native enzymes (see Figs. 7.6. and 7.9.). This finding could have potential application in some reactor system involving the immobilization of several enzymes for consecutive reactions to occur. Zaborsky (1973) has reported on the finding that the simultaneous immobilization of hexokinase and glucose-6-phosphate dehydrogenase resulted in the enhanced formation of NADPH compared to that produced by the freely soluble systems. Filbert and Pitcher (1976) reported that Kent and Emery (1974) immobilized glucoamylase and glucose isomerase simultaneously for the production of invert sugar from starch using optimized conditions. Clearly the immobilization of several enzymes on a single support which optimizes pH, temperature dependence and stability for each enzyme is desirable but ideal.

Finally, therefore, further experimentation is anticipated in order to obtain a suitable reactor system for the hydrolysis of sucrose by immobilized invertase. The potential usefulness of invertase immobilized upon microcrystalline cellulose and insolubilized Concanavalin A has been noted.
Chapter 8

The mannan-induced thermal stabilization of yeast invertase

8.1. - Introduction

There has been no confirmatory evidence in the literature to date on whether mannan is responsible for maintaining the conformational stability of yeast invertase. Although Smith and Ballou (1974b) claimed that removal of all the mannan had no effect on the stability of invertase, thereby refuting Arnold’s hypothesis (Arnold, 1969) that mannan stabilized the invertase protein against heat, they heated their 'mannanless' invertase at 37°C, a temperature at which external invertase is completely stable, as are most enzymes that are not glycoproteins. Therefore, it is the view of this thesis that the evidence presented by Smith and Ballou is not sufficient to claim that mannan has no effect on the stability of invertase especially in view of the fact that they heated their 'mannanless' invertase at 37°C in the presence of 1.0 mg. per ml. of bovine serum albumin - an established protein stabilizer (Wiseman, 1973). It was therefore deemed necessary to re-investigate the claim of Smith and Ballou by comparing the thermal stability of external and 'mannanless' external invertases at 65°C in the absence of any externally added stabilizers such as bovine serum albumin, in order to determine whether covalently attached mannan stabilized the invertase molecule.

Goldstein and Lampen (1975) have reported that internal invertase (which possesses no mannan) is unstable to heat at 56°C. However, if heated at 56°C in the presence of non-covalently attached bovine serum albumin, mannan or external invertase, internal invertase
is stable for 10 min. at pH 4.9, showing identical stability to the external enzyme at this temperature and pH. These results would appear to endorse the view of Arnold (1969) that mannan stabilizes the invertase protein against heat, but what is not clear is whether the enhanced thermal stability of internal invertase is identical to the native thermal stability of external invertase which is rapidly inactivated by heat at temperatures of 65°C and above. This enhanced stability of internal invertase (by heating in the presence of mannan) is presumably due to an interaction between the invertase protein and the externally added mannan. Since the mannan moiety of external invertase from many yeast strains is phosphorylated (Colonna et al., 1975) then the interaction between internal invertase and externally added mannan may be ionic in nature, the positively charged groups on the protein moiety interacting with the negatively charged phosphate groups of the phosphomannan. Since mannan did not enhance the thermal stability of internal invertase at pH 6.0 (Goldstein and Lampen, 1975) then it is clear that these interactions depend upon pH, greatest interaction occurring at acid pH.

Since the mannan moiety of external invertase is covalently bound to the protein moiety via a glycosyl-asparagine bond (Tarentino et al., 1974) whether or not covalently bound mannan is a better stabilizer of invertase than ionically bound mannan is not known.

The finding that the carbohydrate-rich mucoproteins are less susceptible to heat denaturation, compared to other proteins containing no carbohydrate, has been established (Bettelheim-Jevons, 1958). There is also evidence in the literature indicating that the role of the carbohydrate moiety is in stabilizing the tertiary structure of glycoenzymes. For example, Everse and Kaplan (1968)
have found that a number of bacterial diposphopyridine nucleosidases are heat stable. Dey and Pridham (1969) purified the isoenzymes $\alpha$-galactosidase I and II from *Vicia faba* (broad bean) whose carbohydrate contents were 25% and 2.8% respectively. They found that $\alpha$-galactosidase I exhibited greater thermal stability at temperatures above 60°C than did $\alpha$-galactosidase II. Since the mucoproteins and diposphopyridine nucleosidases contain carbohydrate contents in excess of 50% (w/w) (Spiro, 1973) and since the work of Dey and Pridham (1969) suggests that there is a correlation between carbohydrate content and thermal stability, it would appear that a high carbohydrate content is necessary for glycoproteins to show enhanced thermal stability. Indeed, Bettelheim-Jevons (1958) has pointed out that ovalbumin and fibrinogen which contain only 3% carbohydrate are easily denatured. Also, the glycoenzyme human parotid amylase containing 3.7% (w/w) carbohydrate shows similar heat sensitivity to human parotid amylase which possesses no carbohydrate (Keller et al., 1971).

The correlation between the thermal stability of yeast external invertase isoenzymes and their carbohydrate content has been noted by this thesis, but it has been pointed out that the partial loss of thermal stability at 65°C of external invertase with low mannan contents, compared to preparations with high mannan contents, may not necessarily be a true indication of the role of mannan in protecting the tertiary structure of invertase protein, since a 'mannaless' external invertase might be expected to be thermally inactivated at 50°C as is the internal enzyme (see chapter 4).

The experiments described in this chapter have been performed in order to determine whether the mannan moiety stabilizes the protein moiety of invertase. The mannan-induced thermal stabilization is discussed.
8.2. Materials and Methods

Baker's yeast external and internal invertases were purified as previously described (see section 4.2.1.). Yeast mannan was purchased from Sigma and \( \alpha \)-mannosidase isolated from Canavalia ensiformis was purchased from the Boehringer Corporation.

8.2.1. Digestion of external invertase with \( \alpha \)-mannosidase

External invertase (5mg.) was dissolved in 0.9ml. of 0.1M sodium acetate buffer, pH 4.7. The digestion, performed in order to effect removal of the mannan moiety from the enzyme, was begun by the addition of 0.1ml. of the \( \alpha \)-mannosidase suspension (containing 0.5mg. in 3.2M ammonium sulphate). Digestion was allowed to proceed for at least 72 hr. at 25°C (see section 8.3.) and the digested enzyme was then subjected to gel filtration in Sephadex G-200 as previously described (see section 2.3.). The fractions collected were assayed for invertase activity and for their mannan content.

8.2.2. Thermal stability of external, 'mannanless' external and internal invertases in the absence and presence of non-covalently attached yeast mannan

The thermal stability of external and 'mannanless' external invertases was tested at 65°C in the absence of non-covalently attached yeast mannan as previously described (see section 2.7.). This procedure was followed exactly for the thermal inactivation of both invertases in the presence of non-covalently attached mannan, *10 units/mg. 1 unit hydrolyses 1 umole p-nitrophenol - \( \alpha \)-D-mannosidase to p-nitrophenol and \( \alpha \)-D-mannose per minute at pH 4.5 at 25°C.*
but both enzymes were heated in the presence of 5mg./ml. mannan. The thermal stability of external invertase after covalent attachment of yeast mannan on to the protein moiety of invertase (see section 8.2.3.) was also tested at 65°C by the procedure described.

The thermal stability of internal invertase at different p\(H\)'s was tested similarly, but at 50°C in the absence and presence of non-covalently attached yeast mannan.

8.2.3. - Covalent attachment of yeast mannan on to external invertase

Yeast mannan (10mg.) was dissolved in 1.0ml. of sodium periodate (0.04M) and oxidation was allowed to proceed for 30 min. at room temperature. The reaction was stopped by the addition of 1.0ml. of ethylene glycol (0.36M). The coupling of invertase to mannan was performed by adding 1.0ml. of the oxidised yeast mannan solution to 5mg. of Baker's yeast external invertase in 1.0ml. of 10mM - sodium carbonate buffer, pH 9.5, and was allowed to proceed for 1 hr. at room temperature. This reaction mechanism has been previously described (see Fig. 6.9.). After the coupling procedure, a suitably diluted aliquot of the enzyme solution was tested for its thermal stability at 65°C in the usual way.
8.3. - Results

8.3.1. - Digestion of Baker's yeast external invertase with α- mannosidase

Samples of external invertase (5mg.) were digested with α- mannosidase for 24, 72, 96 and 120 hr. After these times the digested enzyme (5mg.) was subjected to Sephadex G-200 gel filtration. The elution of enzyme activity is shown in Fig. 8.1. It is apparent that digestion of external invertase by the α- mannosidase used resulted in the formation of some invertase with a lower molecular weight comparable to that of internal invertase which lacks mannan. The fractions containing invertase activity were also assayed for their mannan contents. No mannan was detectable in fractions 14 - 25 providing evidence that these fractions contained 'mannanless' external invertase formed by the digestion of external invertase with α- mannosidase. It seems, however, that this α- mannosidase preparation is not suitable for the bulk removal of mannan from external invertase. Neumann and Lampen (1967) attempted to remove the mannan from external invertase by digestion with α- mannosidase from jack bean meal, but found that only a few per cent of the mannan was removed. Smith and Ballou (1974a) have noted that phosphate, present in some invertases, can prevent complete digestion and hence removal of carbohydrate from invertase by exo - α- mannanase treatment. They were able to remove all the carbohydrate by such treatment from an invertase isolated from a yeast strain which lacks phosphate. Therefore, since Baker's yeast external invertase contains phosphate (Colonna et al., 1975) removal of most of the carbohydrate by digestion may be prevented. The fractions containing the 'mannanless' external invertase were pooled and the pooled enzyme
Fig. 8.1. - Behaviour of Baker's yeast external invertase in Sephadex G-200 after digestion with α - mannosidase

Invertase sample 5mg. was applied to the top of the column. Fractions of 3.0ml. were collected at a constant flow rate of 12ml. hr.⁻¹

Key:
- ○○, invertase, no digestion
- △△, invertase, 24 hr. digestion
- ■■, invertase, 72 hr. digestion
- ■■, invertase, 96 hr. digestion
- △△, invertase, 120 hr. digestion
used for thermal stability studies. The results indicated that
digestion was mainly completed after the 72 hr. incubation period.

8.3.2. - Thermal stability studies on external, 'mannanless'
external and internal invertases in the absence and
presence of non-covalently attached yeast mannan

The removal of mannan from external invertase by enzymic
digestion significantly lowered its thermal stability (see Fig. 8.2.).
Heating the native enzyme in the presence of non-covalently attached
yeast mannan (5mg./ml.) did not affect its thermal stability but the
'mannanless' external invertase when heated similarly showed a further
loss in thermal stability (see Fig. 8.2.). Although the 'mannanless'
external invertase showed a significantly lower thermal stability than
the native enzyme, it still exhibited much greater thermal stability
than internal invertase (the naturally occurring mannan-free invertase)
which loses all its activity within 1 minute of heating at 65°C
(see section 4.3.5.2.).

There was an increased thermal stability of internal invertase
at 50°C when heated in the presence of non-covalently attached yeast
mannan (50mg./ml.). The ability of mannan to stabilize internal
invertase was clearly pH dependent, stability being achieved when the
enzyme was heated at pH's 3.6 and 4.0, but not at pH's 4.7 or 7.0, in
the presence of non-covalently attached yeast mannan (50mg./ml.).
In the absence of mannan, internal invertase was rapidly inactivated
at 50°C at all pH's (see Fig. 8.3.). It should be noted that the
heating of internal invertase in the presence of non-covalently
attached mannan at pH's 3.6 and 4.0 did not improve its stability to
that of the external enzyme which is completely stable at 50°C for
10 min. This is in contrast to the work of Goldstein and Lampen (1975).
Fig. 8.2. - Thermal stability (65°C) of external and 'mannanless' external invertase in the absence and presence of non-covalently attached yeast mannan (5mg./ml.)

Key:  o—o, native external invertase  
△△, 'mannanless' external invertase  
□□, 'mannanless' external invertase heated in the presence of non-covalently attached yeast mannan

Values are mean ± S.D.
Fig. 8.3. - Thermal stability (50°C) of internal invertase at different pH values in the presence and absence of non-covalently attached yeast mannan (50mg./ml.)

Key:

- ○○ - internal invertase at pHs 3.6, 4.0, 4.7 and 7.0 in the absence of yeast mannan and at pHs 4.7 and 7.0 in the presence of non-covalently attached yeast mannan (50mg./ml.)

- △△ - internal invertase at pHs 3.6 and 4.0 in the presence of non-covalently attached yeast mannan (50mg./ml.)
These results confirm that an acid pH of pH 3.6 or 4.0 is necessary for the stabilization of internal invertase by yeast mannan suggesting that the interaction between them is ionic in nature.

8.3.3. - The effect of covalently attached yeast mannan on the thermal stability of external invertase

Since external invertase is eluted with the void volume when subjected to gel filtration on Sephadex G-200, the determination that mannan had been covalently linked on to external invertase by following an increase in size as judged by its behaviour in Sephadex G-200, was impossible. Consequently, since heating in the presence of non-covalently linked mannan does not affect the thermal stability of external invertase (see section 8.3.2.), then any change in the thermal stability of external invertase, after attempted covalent linking of mannan on to the enzyme, was taken as an indication that mannan had been covalently linked on to the invertase protein.

Incubation of external invertase at pH 9.5 for 1 hr. at room temperature in the absence and presence of periodate oxidized yeast mannan, resulted in a 30% loss of enzyme activity relative to activity of the enzyme in buffer at pH 4.7 (the pH for maximal stability). This indicated that the covalent coupling of mannan on to external invertase had no effect on the enzyme activity. However, the thermal stability of external invertase was significantly lowered after the covalent attachment of mannan to it (see Fig. 8.4.). The amount of mannan covalently linked on to the invertase protein was not determined.

8.3.4. - The determination of the pseudo - first order rate inactivation constants from the heat inactivation data

The pseudo - first order rate inactivation constants ($k \text{ min}^{-1}$)
Fig. 8.4. - Thermal stability (65°C) of external invertase after the covalent attachment of yeast mannan

Key:  ——, external invertase pre-incubated for 1 hr. at room temperature in buffer pH 9.5

—-—, external invertase pre-incubated for 1 hr. at room temperature with 5mg. of periodate oxidized yeast mannan in buffer pH 9.5

Values are mean ± S.D.
for external invertase, external invertase with covalent attachment of yeast mannan, 'mannanless' external invertase, 'mannanless' external invertase heated in the presence of non-covalently attached yeast mannan, internal invertase and internal invertase heated in the presence of non-covalently attached mannan at pH's 3.6 and 4.0 are given in Table 8.1.
Table 8.1. - Pseudo-first order rate inactivation constants for invertase

<table>
<thead>
<tr>
<th>Enzyme preparation and conditions used</th>
<th>k (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>External invertase (65°C)</td>
<td>0.12</td>
</tr>
<tr>
<td>'Mannanless' external invertase (65°C)</td>
<td>0.21</td>
</tr>
<tr>
<td>'Mannanless' external invertase in the presence of non-covalently attached mannan (65°C)</td>
<td>0.33</td>
</tr>
<tr>
<td>External invertase pre-incubated in buffer, pH 9.5 (65°C)</td>
<td>0.10</td>
</tr>
<tr>
<td>External invertase with covalently attached mannan (65°C)</td>
<td>0.17</td>
</tr>
<tr>
<td>Internal invertase (50°C)</td>
<td>0.40</td>
</tr>
<tr>
<td>Internal invertase in the presence of non-covalently attached mannan (50°C)</td>
<td>0.07</td>
</tr>
</tbody>
</table>
8.4. - Discussion

The inactivation rate constant at 65°C of the 'mannanless' external invertase was 0.21 (min\(^{-1}\)) and was significantly different to 0.12 (min\(^{-1}\)) for the native enzyme, thus indicating that the removal of mannan from external invertase did affect the thermal stability. However, internal invertase, which possesses no mannan, has an inactivation rate constant at 65°C of > 2.0 (min\(^{-1}\)). It is therefore argued that if external invertase is formed from the internal enzyme by sequential addition of mannan on to the latter (Moreno et al., 1975) the role of mannan, once added on to the protein moiety, would be to stabilize (or 'hold') the protein conformation. However, removal of this mannan has little or no effect on the conformational stability of external invertase (Smith and Ballou, 1974a; Woodward and Wiseman, 1976), and the results of this thesis agree with the work of these authors.

It is the view of this thesis, on the evidence presented, that there is no biosynthetic relationship between the internal and external invertases, in view of the finding that removal of mannan from external invertase has very little effect on its thermal stability at 65°C.

The basis for the enhanced thermostability of proteins obtained from thermophilic organisms has been discussed (see section 6.4.) and appears to be due to subtle changes in the protein primary structure which result in greater interactions contributing to the overall conformational stability (e.g. hydrogen bonding, hydrophobic interactions, ionic interactions) in the protein tertiary structure. In view of the differences in amino acid composition between internal and external invertases (Gascon et al., 1968) then the greater
conformational stability of the external enzyme may be due to such interactions which are not present in the tertiary structure of the internal enzyme. The evidence presented in chapter 6 suggested that salt-links played an important role in maintaining the conformational stability of the external enzyme. Such ionic interactions would provide the extra stabilization energy necessary to account for the difference in stability between external and internal invertase.

The role of mannan in maintaining the conformational stability of external invertase is therefore not clear. Goldstein and Lampen (1975) found that non-covalently attached mannan stabilized internal invertase (from a *Saccharomyces* mutant Sp.) at 56°C in buffer pH 4.9, its stability being improved to that of the external enzyme at this temperature and pH. The results of this thesis found that internal invertase from Baker's yeast was also stabilized by non-covalently attached yeast mannan at 50°C in buffer at pH's 3.6 or 4.0, but that this stability was not improved to that of the external enzyme. This increased stability of internal invertase may not be due to the stabilizing effect of mannan as such, but rather to the formation of ionic-linkages between the phosphate in the mannan and the protein amino groups which would be prevalent at low pH. Interestingly, the mannan moiety of the invertase from the *Saccharomyces* mutant Sp. isolated by Goldstein and Lampen (1975) is highly phosphorylated containing 27 mol. P0₄ per mol. invertase compared with the mannan moiety of invertase isolated from Baker's yeast which contains a low level of phosphate, 7 mol. P0₄ per mol. invertase (Colonna et al., 1975). Therefore, greater ionic interactions between phosphate and the protein amino groups of internal invertase may be possible when the latter is heated in the presence of non-covalently attached mannan which is highly phosphorylated, giving rise to extra stability than if
the enzyme is heated in the presence of non-covalently attached mannan which has a low phosphate content. This would support the hypothesis that salt-links play an important role in maintaining the conformational stability of external invertase. Presumably, the yeast mannan used in this thesis would have a low phosphate content compared to that used by Goldstein and Lampen (1975).

The thermal stability of 'mannanless' external invertase was lowered when heated in the presence of non-covalently attached yeast mannan. Since the 'mannanless' external invertase would still be stabilized by salt-links, even when the mannan had been removed, heating in the presence of yeast mannan may disturb such salt-links due to ionic interference with them by phosphate in the mannan. Similarly, the presence of covalently-attached yeast mannan decreased the thermal stability of external invertase. Since free amino groups would be involved in the covalent link between external invertase and yeast mannan, then destabilization might be expected if these amino groups were involved in stabilizing salt-linkages.

The possibility that phosphate may play a role in contributing stabilizing salt-links to the invertase molecule must also be noted but it seems unlikely that these interactions would be the major factor contributing to the stability of invertase. The removal of mannan could therefore lead to a slight decrease in stability. It has been previously noted (see chapter 4) that there is an inverse relationship between the rate of thermal inactivation obtained for invertase isoenzymes, I, II, III, and IV and their mannan contents. Isoenzyme IV, with apparently no mannan, is the least stable, whilst isoenzyme I containing 75% (w/v) of mannan, is the most stable. A low or zero phosphate content of isoenzyme IV may be responsible for its lower thermal stability due to the absence of salt-links.
It is generally known that the folding of a polypeptide chain into its three-dimensional conformation is dictated by its amino acid sequence and that the folding will lead to the formation of the most natural stable conformation (Epstein, 1970). Creighton (1975) believes that there is a unique and obligatory pathway for the stable folding of protein, a view which is becoming widely accepted (Robson, 1976). Ultimately, the most natural stable conformation of a protein will depend upon the stabilization energy provided by the amino acid interactions in the protein's tertiary structure.

Ruiz-Herrera and Sentandreu (1975) showed that the poly-ribosomes were the initial site of the glycosylation of mannoproteins from Saccharomyces cerevisiae since short pulses of [U-\textsuperscript{14}C] mannose labelled the ribosomal fraction of the yeast. Their results indicated that initial sugar binding occurs while the nascent polypeptide chains are still growing on the ribosomes. It is therefore the view of this thesis that the sequential addition of mannan on to the unfolded invertase protein while it is still attached to, and even growing on the ribosomes, controls the folding pathway of invertase after it is released from the ribosomes. It may be that it is the initial stages of glycosylation that provide the necessary control for stable folding and once released from the ribosomes and following folding, further glycosylation may occur by stepwise addition of mannose units. Removal of mannan from external invertase, therefore, would have little effect on the stability of the invertase protein since most of the stabilizing bonds (e.g. salt-links) would not be affected upon the mannan removal.

The continuous spectrum of different molecular sizes of
invertase within the yeast protoplast (Moreno et al., 1975) probably represent the enzyme with different mannann contents due to the stepwise addition of mannose units on to the folded invertase protein after it has been released from the ribosomes. Apart from controlling the folding pathway of invertase protein the glycosylation of invertase may be necessary for its secretion in agreement with the hypothesis of Eyal (1966). Once secreted, the mannann may then act as an anchorage point to the cell wall.

The stabilization of enzymes by glycosylation has been achieved (see section 4.4.). Marshall and Rabinowitz (1975) have pointed out that the increased thermal stability of enzymes that have been glycosylated may be due to cross-linking between the protein and polysaccharide. Therefore, it should be noted that although the bulk of the mannann may be removed from external invertase by enzymic digestion, it is possible that intra-polypeptide cross-links between invertase protein and mannose units remain which maintain the conformational stability. Nevertheless, such cross-links would be formed during the initial glycosylation of invertase while it is still unfolded on the ribosomes and may be only partially responsible for maintaining the conformational stability of external invertase from Baker's yeast.

Finally, therefore, it is concluded that mannann does affect the thermal stability of yeast invertase by controlling the folding pathway of invertase protein which leads to the formation of a stable tertiary structure. Formation of intra-molecular cross-links between mannose units and the polypeptide chain during the initial glycosylation may also be responsible for maintaining conformational stability. The major stabilizing energy, however, would be due to amino acid interactions probably involving salt-linkages.
Chapter 9

Final discussion and further work envisaged

This thesis has established that invertase from the yeast *Candida utilis* is much more thermally stable than Baker's yeast invertase (*Saccharomyces cerevisiae*). Thus *C. utilis* invertase may therefore be more efficiently used by the confectionery industry for the production of special cream centres where high temperatures are necessarily employed (see chapter 5). Alternatively, the chemical modification of Baker's yeast invertase, with the incorporation of intra-molecular cross-links into the enzyme molecule, may improve the thermal stability of this enzyme to that of the *C. utilis* enzyme. Such an artificially-stabilized Baker's yeast invertase could also be used more efficiently than the native enzyme. However, it was found that only the chemical modification of Baker's yeast invertase with glutaraldehyde improved its thermal stability close to that of *C. utilis* invertase. This artificially-stabilized enzyme, however, possessed a much lower specific enzyme activity than did the native enzyme, so therefore stabilization of Baker's yeast invertase was achieved at the expense of the enzyme activity.

The native Baker's yeast invertase is also less susceptible to heat denaturation than other proteins that do not have a carbohydrate moiety (see section 8.1.). The reason for the thermal stability of this enzyme has been considered and thought to be due to amino acid interactions, possibly involving salt-linkages, in the protein tertiary structure (see chapter 6). The role of the mannan moiety in contributing to such interactions has also been discussed (see chapter 8). The reason for the greater thermal stability of
C. utilis invertase is a matter for speculation. It is possible that the amino acid composition and sequence is different to that of the Baker's yeast enzyme. Consequently there may be extra amino acid interactions (e.g. salt-linkages, hydrogen bonds) in the C. utilis enzyme that provide the extra stabilization energy necessary for its increased thermal stability over the Baker's yeast enzyme. Therefore, the determination of the amino acid composition of C. utilis invertase would reveal differences between the two enzymes. Clearly amino acid sequencing and the determination of the tertiary structure of the C. utilis and Baker's yeast invertases would enable the precise amino acid interactions and hence possible salt-linkage involvement to be identified. The results of such a study could also provide an understanding for the basis of the thermal stability of both enzymes and reveal the mechanism of the higher thermal stability of C. utilis invertase.

It has been well established that most α - amylases require calcium ions for their stability (Fischer and Stein, 1960; Hasegawa and Imahori, 1976). Similarly, calcium is present in the thermolysin molecule contributing to its thermal stability (Colman et al., 1972). Hasegawa and Imahori (1976) showed that α - amylase from the bacterial thermophile V - 2 was markedly stabilized against heat by the presence of 10mM calcium chloride. This thesis has not determined whether calcium is present in the invertase molecules of Baker's yeast and C. utilis yeast. However, the effect of calcium chloride on the thermal stability of both invertases was studied and the results are shown in Figs. 9.1. and 9.2.

It is evident from Fig. 9.1. that by heating C. utilis grade X invertase in the presence of 10mM and 100mM calcium chloride
Fig. 9.1. - The effect of calcium chloride on the thermal stability (70°C) of C. utilis grade X invertase

The enzyme solution (100μg./ml.) containing various concentrations of CaCl₂ was heated at 70°C as previously described (see section 2.7.)

Key:
- ○─○, control, no addition
- △△, 1μM. CaCl₂
- □□, 10μM. CaCl₂
- ■■, 100μM. CaCl₂
- ▲▲, 1mM CaCl₂
- ■■, 10mM CaCl₂
- ○○, 100mM CaCl₂
Fig. 9.2. - The effect of calcium chloride on the thermal stability (65°C) of Baker’s yeast external invertase

The details and key are identical to those described in Fig. 9.1.

Heating in the presence of 1μM - 10mM CaCl₂ gave results that were not significantly different to those of A, (i.e. the control). In B, the enzyme is heated in the presence of 100mM CaCl₂.

Values are mean ± S.D.
there is a marked enhancement of thermal stability. On the contrary, by heating Baker's yeast external invertase in the presence of 100mM calcium chloride, there is a significant lowering of the thermal stability of this enzyme. The reason for this destabilization of Baker's yeast invertase may be that the calcium ions bind carboxylate residues that are involved in stabilizing salt-linkages in the enzyme molecule. The reason for the stabilization of \textit{C. utilis} invertase is a matter for speculation. The results suggest that salt-linkages may not be important in maintaining the conformational stability of this enzyme. It is possible that stabilizing calcium bridges are formed between two carboxylate residues or between two phosphate molecules present in the mannan moiety. These bridges could confer structural rigidity on to \textit{C. utilis} invertase molecules. If these bridges are between two phosphate molecules, then this may reflect a difference between the phosphate content of the Baker's yeast invertase and \textit{C. utilis} invertase molecules. Since the phosphate content of the former enzyme is low (Colonna et al., 1975) this may reflect an inability to form such stabilizing bridges. The determination of the phosphate content of the latter enzyme is necessary in order to determine whether a possible higher phosphate content would be necessary for such bridges to be formed. It is interesting to note in this respect that calcium ions are essential for flocculation to occur (Williams and Wiseman, 1973a). Flocculating yeast cells bind twice as much calcium as do yeast cells containing phosphomannan - proteins of lower phosphate content, which are non-flocculent. This suggested that calcium - phosphate bridges formed between adjacent yeast cell walls were the cause of the floc formation observed with flocculent yeasts (Lyons and Hough, 1970, 1971).

There may be other reasons why the native \textit{C. utilis} invertase
molecule is much more thermally stable than the Baker's yeast invertase molecule as well as the extra stabilization energy that may be provided by amino acid interactions in the former enzyme molecule. Reference has already been made to the possibility that mannose units may cross-link different parts of the Baker's yeast invertase polypeptide chain, thereby conferring stability on to the enzyme (see section 8.4.). Therefore, the greater thermal stability of the \textit{C. utilis} invertase may be due to a greater number of such cross-links in the enzyme molecule. It is suggested that this is determined by isolating the glycopeptides of this enzyme and comparing the number found with those found in the Baker's yeast invertase molecule. Tarentino \textit{et al.}, (1974) have reported that there are 41 moles of glucosamine per mole of invertase from a mutant strain of \textit{Saccharomyces}. They found that the glycosyl-asparagine had the following composition: \text{Asn - (GlcNAc)\textsubscript{2} (Man)\textsubscript{x}}. Consequently there would be approximately 20 glycopeptides in this invertase molecule.

\textit{C. utilis} grade X invertase (containing \(\sim 60\% \text{(w/w)}\) mannan) was also subjected to digestion with \text{\(\alpha\)-mannosidase} as described in section 8.2. The results obtained with this enzyme were very similar to those obtained with the Baker's yeast enzyme (see Fig. 8.1.), in that only a small amount of 'mannanless' grade X invertase was released. The thermal stability of the 'mannanless' grade X invertase was similar to that of the native enzyme, suggesting that the presence of bulk mannan does not 'hold' or confer conformational stability on to the tertiary protein structure (see Fig. 9.3.).

To determine whether the initial glycosylation of nascent invertase protein whilst still attached to the ribosomes, controls the folding pathway of the invertase protein leading to conformational stability of the protein tertiary structure, requires further
Fig. 9.3. - Thermal stability (70°C) of grade X invertase and 'mannotless' grade X invertase

Values are mean ± S.D.
experimentation. It is suggested that the native invertase and 'mannanless' invertase molecules are subjected to denaturation conditions which would unfold the enzyme molecules, resulting in their inactivation. Subsequent removal of the denaturant would hopefully allow the unfolded enzyme to re-fold and hence to be re-activated. Urea is one such denaturant, which in low concentrations, reversibly inactivates yeast invertase (Chase and Krotkov, 1956). The thermal stability of the re-folded native and 'mannanless' invertase molecules may provide evidence for the hypothesis that mannan controls the folding pathway leading to conformational stability. However, it should be mentioned that although the 'mannanless' invertase lacks the bulk of its mannan moiety, mannose-protein cross-links may still remain which enable the 'mannanless' enzyme to retain some of its three-dimensional structure before re-folding. It may be that it is the initial glycosylation, which leads to such cross-links, that is instrumental in controlling the folding pathway. It may be possible, therefore, to glycosylate unfolded internal invertase during its re-folding process to determine whether this glycosylation would lead to the formation of an active enzyme with conformational stability similar to that of the native enzyme.

The isoenzymes of grade VI invertase behaved anomalously on Sephadex G-200 in that isoenzyme IV with an apparent zero mannan content was eluted with the void volume (see section 4.3.5.4.) unlike internal invertase which possesses no mannan which is eluted with a Ve/Vo ratio of 1.75 (Moreno et al., 1975). Interestingly, the 'mannanless' invertases of Baker's yeast external and grade X invertases behaved normally in Sephadex G-200 with a Ve/Vo similar to that expected for invertase without a mannan moiety. It is possible that conformational differences between isoenzyme IV and the
'mannanless' invertases are responsible for their behaviour in Sephadex G-200.

Finally, an understanding of the basis of the thermal stability of enzymes is necessary for their efficient use industrially and clinically, since their chemical modification and immobilization may result in their destabilization if such processes interfere with the natural mechanism of stabilization. The main conclusion of this thesis is that it is preferable to use naturally occurring thermally stable enzymes such as \textit{C. utilis} invertase for industrial and clinical usage where prolonged stability may be required. Artificial stabilization of enzymes in current use is anticipated to be replaced by the same enzymes that are naturally stable. The eventual use of such enzymes would become economically advantageous.
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