THE PROPERTIES AND ACTION OF GLUCOAMYLASE FROM an Aspergillus Species

A Thesis submitted by

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ABSTRACT

Different techniques of enzyme active site labelling have been classified and discussed with particular reference to the glycoside hydrolases and glucoamylase. Glucoamylase from Aspergillus niger has been purified and fractionated into 2 major forms. The physical properties of the forms have been measured and compared. Both purified enzyme forms have similar sedimentation coefficients, behave similarly on disc-gel electrophoresis and have similar molecular weights (80,000). The purified enzyme forms showed distinct amino acid compositions, but both had carbohydrate contents of about 15%.

The specific activities of the enzyme fractions toward wheat amylopectin were similar and about four times greater than those towards maltose. Both purified enzyme forms converted wheat amylopectin to glucose to the extent of virtually 100%. The phenomenon of reversion has been discussed and analysed from a thermodynamic point of view. Extents of reversion theoretically expected have been calculated from available thermodynamic data and compared with experiment.

Both purified enzyme forms have a pH optima of 4.6 and are devoid of "endo"-activity. The chemical modification of protein carboxyl groups has been reviewed, and the technique of differential modification has been applied to the two purified glucoamylases. Carboxyl groups were deactivated by reaction with 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride and labelled with glycine methyl ester, glycine ethyl ester and 2-amino ethane sulphonic acid (taurine). It was found that deactivation was most rapid in the case

of taurine, and the deactivation is restricted to ca. 20% in the presence of maltose. The use of taurine also facilitates determination of the extent of modification by amino acid analysis. The partially deactivated enzyme was stable towards subsequent purification procedures and could be fully deactivated by a second treatment in the absence of maltose. The use of ^{14}C radio-labelled taurine in the second stage showed that maltose protected an enzyme substrate site which contained 6 and 5 modifiable groups in glucoamylase I and II respectively. Peptide maps of chemically modified glucoamylase I and glucoamylase II showed distinct differences.

TO MUM AND DAD

ACKNOWLEDGEMENTS

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CHAPTER 1

GENERAL INTRODUCTION TO STARCH DEGRADING ENZYMES AND CHEMICAL STUDIES OF ENZYME ACTIVE SITES

1.1 General Introduction

In living organisms the existence of enzymes which will catalyse the hydrolysis of starch into smaller units are of vital importance in the utilization of this naturally occurring energy store. In industry the starch hydrolases play a major role in producing sugars which have many uses ranging from brewing to confectionery. In the fields of chemistry and biochemistry the hydrolases have played a part in characterising the structures of starch[1] and glycogen[2] polymers from various sources.

In the initial work using the starch hydrolases, the purity of the preparations was not deemed to be of vital importance because they were used for a particular purpose say for example in industry to reduce the viscosity of starch.[3-5] It was with the application of enzymes for hydrolysing specific linkages in polysaccharide structural determinations[6] that the degree of purity became important. Since the advent of isoelectric focussing[7] it has been shown that enzymes which were previously considered to be pure may themselves split up into many slightly different forms[8, 129] and it is difficult therefore to define the absolute purity of an enzyme. It has been reported in the literature[9-11] that there are at least eight enzymes which will degrade starch and these are shown in TABLE 1.1 together with the usual products obtained after their action on this substrate. Other enzymes, for example Z enzyme,[12] which have also been reported were later shown to be one of the eight.[13, 28] It appears that the physical properties of these enzymes depend upon the micro-organism or organ from which they are derived. Thus the α-amylase

TABLE 1.1

The Starch degrading enzymes

ENZYME COMMISSION NUMBER[11]	TRIVIAL NAME	SYSTEMATIC NAME[11]	PRODUCTS AFTER ACTION ON STARCH AND REFERENCE
E.C. 3.2.1.1	α-amylase	[α-1,4-glucan 4-glucohydrolase]	α-Maltose,[9,18-21] limit dextrin[26,27,33-35,41,43,44] α-D-glucose[24,25]
E.C. 3.2.1.2	β-amylase	[α-1,4-glucan maltohydrolase]	β-Maltose,[21-23,28] limit dextrin[9,29,33,35,36,41-43]
E.C.3.2.1.3	Glucoamylase	[α-1,4-glucan glucohydrolase]	β-D-glucose[20,30,31,33,35,37,41-43]
E.C. 3.2.1.9	Pullulanase	[α-1,4-glucan 6-glucohydrolase]	Almost all α-(1,4)-linked linear polymer chains.[9,10,32,33,38,39,45]
E.C. 3.2.1.9	Amylo-1,6-glucosidase	[Starch 6-gluconohydrolase]	α-(1,4)-linked linear polymer chains.[10,33,35,40,41]
E.C. 3.2.1.9	R-enzyme	[Amylpectin 6-glucoanohydrolase]	α-(1,4)-linked linear polymer chains.[1,9,10,33,35,40,41,45]
E.C. 3.2.1.9	Isoamylase	[Glycogen 6-glucoanohydrolase]	α-(1,4)-linked linear polymer chains.[9,10,35,41,45-50]
E.C. 2.4.1.1	Phosphorylase	[α-1,4-glucan: orthophosphate glucosyltransferase]	α-D-glucose-1-phosphate and polymeric α-(1,4) branched α-(1,6) polymer.[9,33,35,40,41,51]

TABLE 1.2

Alternative Names used for the Enzymes

(listed in TABLE 1.1)

TRIVIAL NAME	ALTERNATIVE NAME	REFERENCE
	Diastase	52
α-amylase	Taka - Diastase	53
	Taka - amylase	24,54
	Taka - amylase A	19,55
	Taka - amylase B	56-58
	Amyloglucosidase	59
	Saccharogenic Amylase	60
Glucoamylase	Gluc - amylase	30, 61, 64, 76
	Glucamylase	62, 63
	Alpha - Amyloglucosidase	31
	γ -amylase	9, 40, 41, 69
	Maltase	70-74
Pullulanase	Bacterial R-enzyme	9, 40
Phosphorylase	P-enzyme	40, 75

from porcine pancreas has a reported molecular weight of 45,000[14] and a pH optimum of 6.8[15] whereas that obtained from Bacillus stearothermophilus has an average molecular weight of 15,404[16] and a pH optimum of 5.0.[17] Before considering aspects of the glucoamylase enzyme which is the subject of this Thesis; to avoid confusion it is useful to list other alternate trivial names which have been used for the enzymes in TABLE 1.1, these are shown in TABLE 1.2. Studies on the properties and on the mechanism of action of these glucan hydrolases and phosphorylase have been reviewed in recent volumes on enzymes.[9, 43-45, 77] α-Amylase and glucoamylase are used in industry to produce high and low glucose equivalent sugars from wheat[78] and other starches and it was with a view to increasing the knowledge about glucoamylase that this work was undertaken with cooperation from Ranks Hovis McDougall Ltd.

In addition to studies of the purification and physical and enzymic properties of glucoamylase, the major part of this Thesis will be concerned with investigations of the chemical nature of the enzyme active site. Information about the active sites of enzymes is gained via three main techniques, X-ray crystallography, chemical modification of the functional groups, and by fast reaction kinetics. Very little X-ray work has been carried out on the glycoside hydrolases apart from the pioneering work on lysozyme, although results of studies on α-amylase are now being reported.[79] The glycosidase, lysozyme, from hens' egg white was the first enzyme to have its complete three dimensional crystal structure determined.[80-86, 88, 89] Virtually no chemical modification[87] had been carried out on this enzyme prior to the X-ray studies. The complete application of the X-ray crystallographic technique is limited by the necessity of obtaining amino acid sequence information and of suitable crystals. However provided the latter

condition is fulfilled, the X-ray technique yields the conformation of the protein chain in the absence of any sequence information, and partial sequence information can then be used to locate amino acid side chains in the three dimensional structure. The second approach which next to X-ray studies has been the most informative involves the chemical labelling of enzyme active sites. In this technique a molecule, perhaps bearing a label such as a radioisotope, is covalently attached to an amino acid residue or residues within the active site. Upon enzymatic or chemical degradation of the protein, peptide fragments bearing the molecule may be detected, isolated and characterised. The catalytic sites of many enzymes which have so far been investigated usually contain some chemically unique or particularly reactive amino acid residue important for their catalytic activity. It is possible to take advantage of their unusual reactivity to label the groups involved in a number of ways. There are five methods of chemically labelling active sites of enzymes, these are labelling by substrate, labelling by nonspecific reagents, labelling by quasi-substrate, differential labelling and affinity labelling.

Examples chosen from the hydrolase enzymes to illustrate these methods are as follows:-

(i) labelling by substrate. Silverstein et al.[91] have isolated a denatured form of enzyme substrate complex from sucrose phosphorylase. They were able to show that one mole of glucose originating from a sucrose molecule complexed with one mole of enzyme. A later hydrolysis study[93] on a glycosyl peptide obtained after pepsin digestion of the glucose-enzyme complex revealed that the glucose linkage was β indicating that the formation of the complex proceeded with inversion. An example of the isolation of an enzyme covalent intermediate demonstrated using

"Sephadex" G-25 column chromatography under both denaturing and non-denaturing conditions has been carried out by Bell et al.[92] The enzyme used for their investigation was phosphoribosyl - adenosine triphosphate: pyrophosphate phosphoribosyltransferase [E.C. 4.2.1c]. In this case enzyme labelled with radioactive phosphorous was obtained. In general however, labelling by substrate is not practical since an enzyme/substrate intermediate complex has only a short lifetime.

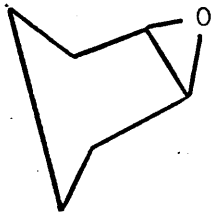
(ii) labelling by nonspecific reagent

Parsons et al.[94-98] have used triethyloxonium fluroborate as the nonspecific reagent in the formation of the β-ethyl ester of the Asp 52 residue in lysozyme. Although it would appear that the reagent is specific for this particular residue, when used in a high concentration 6.3 carboxyl residues were modified out of a possible 11.[94] The reagent discriminates on the pKa value of the acid group being modified and on steric grounds; it is known that the oxonium ion attacks anions more readily than neutral species.[99] A study of the nonspecific carboxyl modification of enzymes using carbodiimide reagents will be given in CHAPTER 6 of this Thesis with particular reference to glucoamylase.

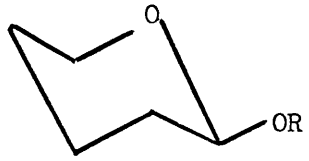
(iii) labelling by quasi-substrate

Labelling by quasi-substrate can be considered as a special case of affinity labelling. A quasi-substrate is an active site directed irreversible inhibitor which both specifically binds and reacts with an enzyme in a very similar way to that of a true substrate. It is so designed that it reacts covalently with a particular group or groups within the enzyme active site which are most likely to be involved during the catalytic reaction. It has to be stable under the conditions of the experiment including the subsequent degradation of the labelled

1D-1,2-anhydro-myo-inositol

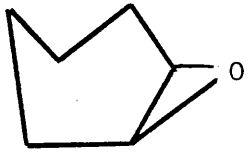


inactivates β

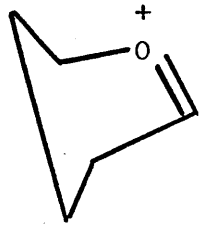


true β-glycoside substrate

1L-1,2-anhydro-myo-inositol

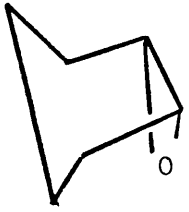


inhibits β?

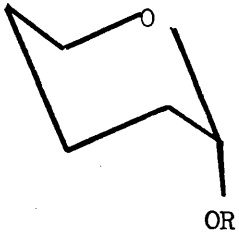


½ chair glucosyl-oxonium ion

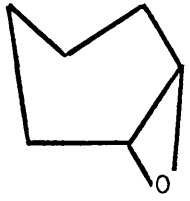
7



slowly inactivates α



true α-glucoside substrate



inhibits α

(OH groups have been omitted for simplicity)

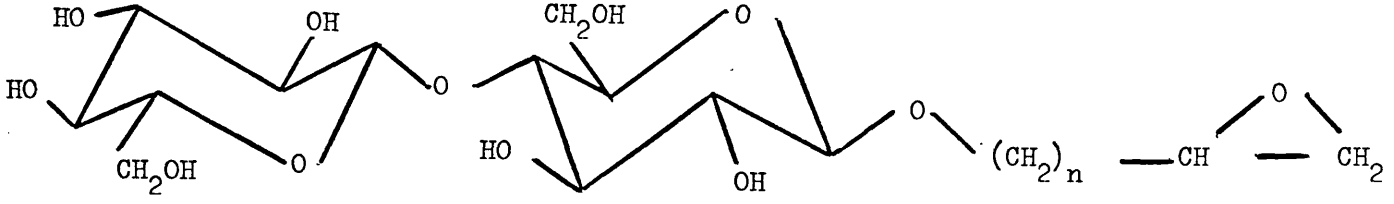
SCHEME 1.1

enzyme in order to reveal information about the functional groups at the active site. This type of study has been carried out on a α-[100] and β-D-glucosidase[101,102,105,106] and on β-D-galactosidase[103] using inositol epoxides as the quasi-substrate. It was shown[101,102] that DL-1,2-anhydro-myo-inositol combined with the active site of β-D-glucosidase and upon hydrolysis of the ester(presumed) formed during the reaction, the product obtained was 1D-chiro-inositol suggesting that it was the D-isomer which was the inactivator. The 1L-isomer was synthesised[104] and reacted with yeast α-D-glucosidase,[100] but it was found only to act as an inhibitor and not an inactivator. The inhibition was lower than that of D-glucose. The 1L-isomer was also tested for inactivation of β-D-glucosidase[100] but none was observed. This supports the suggestion by Legler,[101] that it is the 1D-1,2-anhydro-myo-inositol which inactivates the β-D-glucosidase from the racemic mixture that he used. It has also been shown[106,107] that the D-isomer slowly inactivates yeast α-D-glucosidases. Published interpretations of these results[100] have regarded the C-C inositol bond as a "substitute" for the C1-O5 bond in normal substrates, but a more complete interpretation is possible in which the C-C inositol bond may be regarded as a "substitute" for the C1-C2 bond with no loss of stereochemical correspondence at other positions. Molecular models were made to study the stereochemical analogy of the two racemates of 1,2-anhydro-myo-inositol to the carbonium/oxonium ion half chair transition state intermediate widely supposed to be formed during the authentic enzyme hydrolysis and the situation is shown in SCHEME 1.1. It is observed that when the direction of the C-C epoxide bond follows the direction of the C1-O5 substrate bond, the conformation of the inositol ring closely resembles

that of the half chair derivable from (both α and β) glycosides, whereas when the C-C epoxide bond follows the C1-C2 substrate bond the conformation of the inositol ring differs from the half chair for the same orientation of the rings. The inactivation data is thus rationalised and it may be predicted on this basis that 1L-1,2-anhydro-myo-inositol will only act as an inhibitor of β-D-glucosidase. Although this interaction has been studied[100] and no inactivation occurred, no inhibition data was given. It has already been shown that 1D-1,2-anhydro-myo-inositol inactivates both α-and β-D-glucosidase although the reason for the differences in rate is not apparent. It appears that the quasi-substrate has to have some particular correspondence to the true substrate in some cases before it will react. This is illustrated in the case of the β-D-galactosides. The β-D-galactosidase obtained from Escherichia coli which is very specific for β-D-galactosides[108] was not inactivated by 1D-1,2-anhydro-myo-inositol even at high concentration.[103] The hexosidases from Helix pomatia which hydrolyse β-D-galactosides and β-D-glucosides at at approximately the same rate[109] reacted with 1D-1,2-anhydro-myo-inositol because they are non specific with respect to the configuration at C4 of the hexose.

Cellulase and lysozyme have also been studied using epoxy-substrates. Cellulase will act usually on a β-1,4- linked poly-glucan however it also reacts with a similar oligosaccharide containing four linked glucose residues.[110] Experiments with cellulase have been carried out using 1D-1,2-anhydro-myo-inositol and other epoxides combined to sugar oligosaccharides.[111] It was found that no inactivation occurred with 1D-1,2-anhydro-myo-inositol although a

4,5-epoxypentyl-β-cellobioside



n = 3

STRUCTURE 1.1. An example of an affinity label for cellulase.

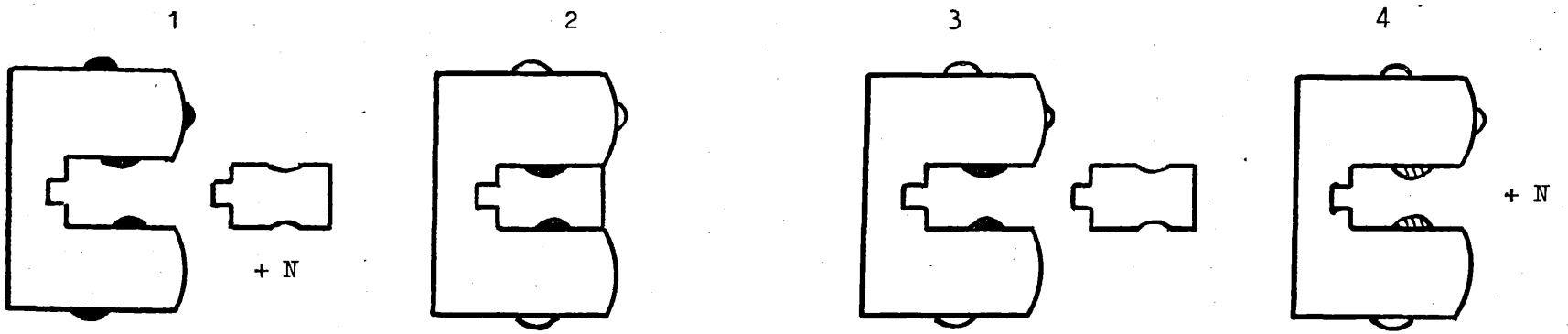
TABLE 1.3

A summary of the results published from the reaction of quasi-substrates on hydrolase enzymes

No.	ENZYME	QUASI-SUBSTRATE	INHIBITION	INACTIVATION
1	β-glucosidase from *Aspergillus wentii* 101, 105	1D≡-1,2-anhydro-*myo*-inositol		observed: 103, 106
2	β-glucosidase from almond emulsion 100	1L≡-1,2-anhydro-*myo*-inositol	? see text	not observed: 100
3	α-glucosidase from yeast 103	1D≡-1,2-anhydro-*myo*-inositol		observed: 103, 106, 107
4	α-glucosidase from yeast 100	1L≡-1,2-anhydro-*myo*-inositol	observed: 100, 103, 104	not observed: 100
5	β-glucosidase from almond emulsion 102	1D≡-1,2-anhydro, 6 deoxy-6-bromo-*myo*-inositol		observed: 103
6	β-galactosidase from *Helix pomatia*	1D≡-1,2-anhydro-*myo*-inositol		observed: 103
7	β-galactosidase from *Escherichia coli*. 103	1D≡-1,2-anhydro-*myo*-inositol		not observed: 103

SCHEME 1.2

Diagramatic representation of the differential labelling of enzyme active sites



Unreacted enzyme, substrate and nucleophile.

Partially reacted enzyme, showing substrate protection of active site.

Partially modified enzyme separated from substrate by dialysis or gel-column chromatography.

Fully modified enzyme bearing label (shaded portion) after reaction with labelled nucleophile without substrate protection of active site.

Key.

- Unreacted groups of enzyme.
- Reacted groups of enzyme.
- Labelled reacted groups of enzyme.

progressive inactivation of cellulase was observed when cellobiosides of the type shown in STRUCTURE 1.1 were used. Unlike 1D-1,2-anhydro myo-inositol, these molecules do not have the reactive functional group as an integral part of the quasi-substrate and therefore will be considered later under affinity labelling.

Peptide sequence studies to identify the labelled residue after reaction with radio-labelled quasi-substrate are underway with Aspergillus wentii β-glucosidase A_3, and a labelled peptide has been isolated and characterised.[103]

It can be concluded that the use of quasi-substrates in the investigation of the hydrolase enzymes is still at an early stage. A brief summary of the results published so far is given in TABLE 1.3.

(iv) differential labelling

This type of labelling was initially described by Cohen et al.[115] in 1953. In this technique the catalytic functional groups of the enzyme are first of all protected by substrate while other non-catalytic groups outside the active site are modified by a non-specific reagent such as a carbodiimide in the presence of a suitable nucleophile. After removal of the substrate and reagents by gel-permeation chromatography or exhaustive dialysis, the partially modified enzyme is further reacted with the non-specific reagent in the presence of radio-labelled nucleophile but in the absence of any protective substrate. After removal of unbound excess labelled nucleophile and other reagents from the enzyme, it is degraded either chemically or enzymatically and the peptide(s) bearing the radio-label isolated and characterised.

There are three points to be noted in this technique if errors are to be avoided. As there is an equilibrium between the enzyme and its substrate, a large excess of the latter must be used during the initial reaction if unlabelled nucleophile is to be prevented from reacting with the active site functional groups. Secondly incomplete removal of the

labelled nucleophile after reaction may lead to incorrect results by indicating too many labelled peptides or give a false number of catalytic groups in the active site. Finally it may be found that when the enzyme is modified it changes character and,for example, precipitates from solution. Very little work has been published on the use of differential labelling applied to glycoside hydrolases, however, it has been reported by Gray et al.[113] that using the procedure essentially described by Hoare and Koshland[114] it was possible to modify glucoamylase with about 80% retention of activity after the initial modification and a considerable loss of activity occurred after subsequent reaction without substrate protection of the active site. A major part of this Thesis is concerned with the differential labelling of glucoamylase, and further discussion of this technique is given in CHAPTER 6.

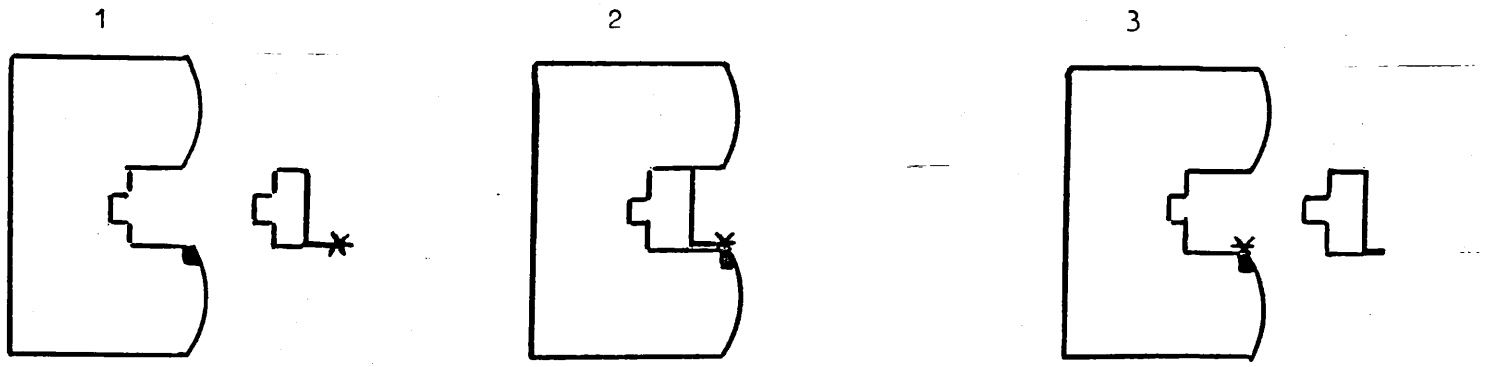
A diagrammatic representation of differential labelling is given in SCHEME 1.2.

(v) affinity labelling

As previously stated this technique of labelling enzyme active sites is similar to that of labelling by quasi-substrate. The essential difference is that the reactive group on the inactivator or inhibitor molecule is external to it and not an integral part as in the case of the quasi-substrate. The molecule used for affinity labelling is essentially made up of two distinct parts. The first is that which resembles the true substrate of the enzyme. This facilitates the access of the substrate into the active site of the enzyme and binds to it in a similar way as the genuine substrate. It also helps to bring about the correct conformational change which may occur within the

SCHEME 1.3

Diagramatic representation of the affinity labelling of enzyme active sites



Unreacted enzyme, and labelled substrate.

Enzyme binds labelled substrate to active site allowing label to react with a group near its catalytic site.

Substrate portion removed from active site after hydrolysis leaving labelled group attached to the enzyme.

Key.

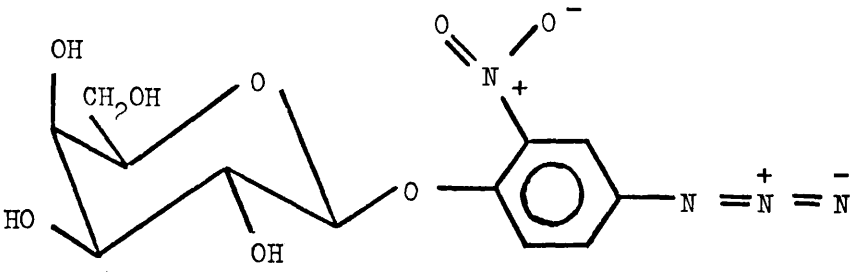
- Unreacted group on enzyme.
- Reactive label.
- Reactive label attached to enzyme.

enzyme when a substrate binds to it. The second part of the molecule is designed to carry a reactive group which will covalently attach itself to a group within the active site of the enzyme once it has been carried in by the other substrate "decoying" part. The disadvantage of this technique is that one cannot be sure that the reactive group on the affinity label actually reacts with a group within the enzyme which is responsible for the catalytic activity. A group on the enzyme which because of the conformational structure is brought into the proximity of, but not part of, the active site may react with the affinity label. This would yield some information about the three dimensional structure around the active site and in conjunction with other data would be useful. Once an affinity label has been attached to an enzyme the substrate portion can generally be removed in some way leaving a group, perhaps radio-labelled, covalently bound to a group on the enzyme. Upon degradation the peptide(s) bearing the label may be isolated and characterised as in the previous cases. A diagrammatic representation of affinity labelling is given in SCHEME 1.3.

Once again although several reviews in the field of affinity labelling are available[116,117] little work has been published in the field of the hydrolases, cellulase,[103] lysozyme,[103] β-D-glucosidase[103] and β-D-galactosidase[123] have however been studied. As stated in the section on quasi-substrates, cellulase is progressively inactivated by cellobiosides of the type shown in STRUCTURE 1.1.

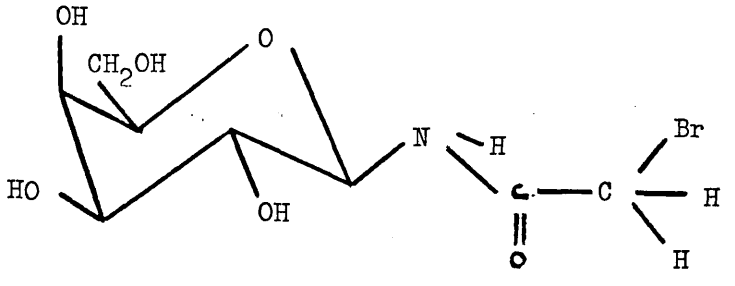
Variation of the length of the alkyl side chain showed a maximum inactivation when five carbon atoms were present in the aglycone. This suggests that with this chain length the epoxide is able to reach a

2-Nitro-4-azidophenyl-β-D-galactopyranoside



STRUCTURE 1.2. An example of an affinity label for β-D-galactosidase

N-bromoacetyl β-D-galactopyranosylamine



STRUCTURE 1.3. An example of an affinity label for β-D-galactosidase from Escherichia coli, and for various permeases.

position on the enzyme which corresponds to the glycosyl oxygen of the last glucose unit of a cellotriose section in the cellulose chain. The rate of inactivation was increased approximately four fold on going from the cellobioside to the cellotrioside. A much higher concentration was used in this work[103] than in the quasi-substrate modification of other hydrolases mentioned earlier. In the study of the inactivation of lysozyme with 2,3-epoxypropyl-β-chitobioside[118] and -trioside,[112] it was observed that with the former epoxide the rate of inactivation was similar to that of the 4,5-epoxypentyl-β-cellobioside and cellulase, and, fifty times higher with the chito-trioside. It was shown by using a radioactive inhibitor that one mole of enzyme was completely inactivated by one mole of inhibitor. In the case of hens' egg white lysozyme a radioactive peptide containing fifteen units was isolated after pepsin digestion, and aspartic acid residue fifty-two identified as the point of attachment of the affinity label.[118]

Diastereomeric epoxy-(β-D-glucopyranosyl) ethanes and 1,2-epoxy-3-(β-D-glucopyranosyl)propanes were synthesised and reacted with β-D-glucosidase from sweet almonds.[119] The enzyme was irreversibly inactivated. 1,2-epoxy-3-(β-D-glucopyranosyl)propane has been synthesised and reacted with yeast hexokinase.[142] It was found that this active site directed inhibitor significantly inactivated the enzyme. Perry *et al.*[120] have synthesised several 2-nitro-4-azidophenyl glycosidase affinity labelling agents (e.g. STRUCTURE 1.2) which may be used for studying β-D-galactosidase, β-D-glucosidase, and cellulase.

As part of an investigation into the lactose transport system of *Escherichia coli*,[122] N-bromoacetyl β-D-galactopyranosylamine (STRUCTURE 1.3) was synthesised together with other related compounds and these were

TABLE 1.4

A summary of the results published of affinity labelling of hydrolase enzymes.

No.	ENZYME	AFFINITY LABEL	INACTIVATION	RATE OF INACTIVATION
1	Cellulase from *Oxyporus species*	4,5-epoxypentyl-β-cellobioside	observed : 103	with cellotrioside increases x 4.0 with 2,3 epoxypropyl decreases x 4.5 with 3,4 epoxybutyl decreases x 4.0 with 5,6 epoxyhexyl decreases x 4.0
2	lysozyme *Oxyporus sp.*	2,3-epoxypropyl-β-chitobioside	observed: 103, 112	comparable to that of cellulase inactivated with 4,5-epoxypentyl-β-cellobioside
3	lysozyme	2,3-epoxypropyl-β-chitotrioside	observed: 103, 112	increased x 50.0 compared with 2.
4	β-D-galactosidase from *Escherichia coli*	N-Bromoacetyl β-D-galactopyranosylamine	observed: 123	At 10^{-3}M concentration of label, enzyme activity reduced to 1% of initial after 60 mins.
5	*lac*-Permease and MG-Permease	N-Bromoacetyl β-D-galactopyranosylamine	observed: 143 (unpublished result cited in 123)	$> 10^{-2}$M concentration of label required before any inactivation observed.

then tested as irreversible inhibitors of lactose uptake using lac-permease employing the method described by Yariv et al.[124] It was found that N-bromoacetyl β-D-galactopyranosylamine was inactive at concentrations up to 10^{-2}M presumably because the inhibitor could not reach the enzyme in vivo. However in an investigation of the active site of β-D-galactosidase from Escherichia coli,[123] N-bromoacetyl β-D-galactopyranosylamine was used at 10^{-3}M concentration as an affinity label and found to totally inactivate this enzyme. The kinetics of the inactivation were first order and a methionine sulphur was found to be the point of attachment of the labelled molecule. A summary of the results of affinity labelling of glycoside hydrolases is given in TABLE 1.4.

Before concluding this section on the chemical modification of enzymes, it is worth noting that this technique may be very useful in the future as a tool to camouflage enzymes for therapeutic purposes.[121] By attaching certain oligosaccharides[130] or glycopeptides around an enzyme by chemical modification and thus camouflaging it, the resistance to proteolytic attack or to immune reaction has been increased in vivo.[125] In this work glycopeptides from a normal circulating protein, fetuin, were attached using glycosyl transferases to lysozyme and albumin. When these modified proteins were injected into rats they remained in circulation. If the sialic acid was first removed to expose the terminal galactose then they were removed by the liver within ten minutes and destroyed by the hepatic cells in two hours. If the terminal galactose residues were also removed the hepatic uptake was depressed. Thus the terminal galactose served as an address label

to direct transport to and destruction in the liver. Blocking the terminal galactose with sialic acid permitted the proteins to stay in circulation and prolonged their lifetime in the organism. Similar work[126,127] using plasma glycoproteins has shown the importance of terminal sialic acid residues keeping the proteins in circulation. Thus it may be possible to differentially modify a hydrolase in such a way that it becomes useful in the treatment of human glycosidase deficiency diseases such as glycogen and lipid storage diseases.

As in the case of the technique of X-ray crystallography little application has been made of rapid reaction kinetic techniques to glycoside hydrolases. A partial explanation may lie in the fact that the natural substrates are polysaccharides and are therefore somewhat ill-defined (e.g. in terms of concentration). Pre-steady state kinetics of lysozyme - substrate interactions have been reported.[128] Two interaction processes were found when chitohexaose was used as substrate while only one was found for chitotriose. The temperature jump and stopped flow methods were used for this investigation, the reactions being followed by proton indicators or fluorescence of tryptophane residues. The technique is useful for obtaining information of the size of the substrate binding cavity.

It has been mentioned earlier in the chapter that hydrolase enzymes such as α- and β-amylase, and glucoamylase play a major part in industry e.g. to produce fermentable sugars in the brewing industry and in providing a non-stop liquefaction and conversion of starch to D-glucose. All industrial processes, to be commercially viable, are operated on a profit making basis and any measures which may reduce operating costs are usually investigated.

Various commercial organisations, and in particular those connected with the food industry have in recent years examined the possibility of replacing soluble enzymes by those attached to a support thus rendering them immobile. In the petrochemical industry immobilised catalysts are commonplace and operate satisfactorily. At first sight it would appear that the use of an immobilised glucoamylase in the industrial starch conversion process would have several advantages over the soluble form, namely, lower overall cost because the enzyme is re-used and reduced contamination of the product by protein. The saving facilitated by the use of immobilised glucoamylase may be quite considerable considering that in 1973 the amount of glucose produced from starch in the United States of America exceeded 1 billion pounds and that during the conversion the glucoamylase employed was partially deactivated and the remainder was not economically recoverable.[137]

In real terms however, the advantages may not be so great because in many cases[131-136] the conversion of starch to glucose did not exceed 85%, however Li et al.[139] reported a 93.9% conversion. Most of the conversions and hence glucose concentrations fall appreciably short of the commercial requirement for the production of crystalline glucose.[137] In the industrial conversion of starch to glucose the major expenditure is on heavy fuel oil and electricity, the expenditure on glucoamylase being only a fraction (2%) of the total "in house" costs. In view of the cost of converting existing industrial starch conversion plants to facilitate the use of immobilised enzymes and that these enzymes slowly lose activity and need periodic replacement the saving of less than 2% in expenditure is not at present worthwhile. There are also technical

problems associated with the large scale use of immobilised enzymes both in batch and column processes. Despite these however, immobilised enzymes may be used extensively in newly constructed starch conversion plants and while the incorporation of minute amounts of enzyme in the glucose produced from industrial processes using soluble glucoamylase is at present unimportant, Government legislation may prohibit it at sometime in the future and thus increase the attractiveness to industry of immobilised enzymes.

For these reasons it has been considered worthwhile to mention briefly some immobilised enzymes. Factors such as physical robustness, chemical inertness, intraparticle diffusion[135] as well as the cost of support material have to be examined before attempting to attach an enzyme and maintain its catalytic activity. Some supports chosen for the immobilisation of glucoamylase are given in TABLE 1.5 together with, where reported, the activities and stabilities of the products relative to the native enzyme.

In addition to glucoamylase, the following enzymes have also been successfully bound to γ -isothiocyanatopropyl-diethoxysilyl glass, the percentage activity of the immobilised enzyme relative to the soluble form being shown in parenthesis. Trypsin (16-24%), horseradish peroxidase (27%), kidney aminoacylase (~1%) and alkaline phosphatase (65%). The adsorptive power of the glass support was less than that of cellulose, cross linked dextran, or polystyrene.[134]

The conditions used to bind an enzyme to a support must be mild enough to prevent a denaturation of the protein while the operating conditions of the immobilised enzyme must not effect its removal from

TABLE 1.5

Supports used activities and stabilities of some immobilised glucoamylases.

Support	Activity % of soluble enzyme	Stability of immobilised glucoamylase	Ref.
D.E.A.E. Sephadex A-50	35		151
D.E.A.E. cellulose	16-55		136
D.E.A.E. cellulose			133
D.E.A.E. cellulose			141
D.E.A.E. cellulose			144
Amberlite CG-50 Type II	45		138
Amberlite IR-45 (OH)		15 days	145
Microcrystalline cellulose, Ground borosilicate glass, Nylon 66 fibre — Impregnated with titanium chloride			131
Activated charcoal			146
Polystyrene Beads	2.4		147
γ-isothiocyanatopropyl-diethoxysilyl glass	68		134
Porous Glass			148
Cellulosic Fibres			149
Copolymer cation exchanges			150
Ethylene - maleic acid, Styrene - maleic acid — copolymers adsorbed on cationic resins		21 days	151
Acid clay or Active charcoal			156
Cross linked acrylamide			157, 137
p-aminobenzenesulphonylethyl cellulose			139
Derivatives of imidazolidine and piperidine			140

the support. It has been fortunate in the work carried out on the attachment of glucoamylase to D.E.A.E. cellulose by Smiley[133] that the enzyme has an isoelectric point which allowed it to bind to the ion exchanger at a pH at which it is still highly active. Barker et al.[156] have attached several of the starch degrading enzymes to supports sufficiently strongly to enable them to be separated from a reaction mixture by centrifugation.

Gruesback[152] described an insolubilised enzyme system for the continuous production of glucose using glucoamylase, while Marshall et al.[9, 153] and Butterworth et al.[154] have developed methods in which glucoamylase and starch are retained above a membrane which is permeable to products. Application of the latter procedure to the degradation of cellulose has also been reported by Ghose et al.[155]

In this Thesis the results of various studies of the starch degrading enzyme glucoamylase from Aspergillus niger are presented. These studies were undertaken in order to (1) obtain information which would assist in the more efficient industrial utilisation of the enzyme (2) investigate the chemistry of the structure of the enzyme and of its interaction with substrate (3) extend the field of knowledge of chemical modification of enzymes in general and of differential labelling of glycoside hydrolases in particular.

References CHAPTER 1

1. HOBSON, P.N., WHELAN, W.J., PEAT, S., J. Chem. Soc. 1451, 1951.

2. PEAT, S., WHELAN, W.J., HOBSON, P.N., THOMAS, G.J., J. Chem. Soc., 4440, 1954.

3. FISHER, E.E., U.S. PATENT 3,720, 583, 1973.

4. WHITE, W.H., DWORSCHACK, R.G., U.S. PATENT 3,725,202, 1973.

5. HAYES, L.P. U.S. PATENT 3,806,415, 1974

6. ABBOTT, D., BOURNE, E.J., WEIGEL, H., J. Chem. Soc., 827, 1966.

7. SVENSSON, H., Acta. Chem. Scand., 15 325, 1961.

8. SCHARPE, S., LAUWERS, A., COAREMAN, W., J. Pharm. Belg., 28, 705, 1973.

9. MARSHALL, J.J., Wallerstein Communications, 116, 49, 1972.

10. MANNERS, D.J., Nature New Biology, 234, 150, 1971.

11. Report of the Commission on Enzymes of the International Union of Biochemistry, 1961 p.110 Pergamon Press Ltd.

12. NEUFELD, E.F., HASSID, W.Z., Arch. Biochem. Biophys., 59, 405, 1955.

13. CUNNINGHAM, W.L., MANNERS, D.J., WRIGHT, A., FLEMING, I.D., J. Chem. Soc., 2602, 1960.

14. DANIELSSON, C.E., Nature, 160, 899, 1947.

15. MEYER, K.H., FISCHER, E.H., BERNFELD, P., Helv. Chim. Acta., 30, 64, 1947.

16. MANNING, G.B., CAMPBELL, L.L., FOSTER, R.J., J. Biol. Chem., 236, 2958, 1961.

17. CAMPBELL, L.L., CLEVELAND, P.D., J. Biol. Chem., 236, 2952, 1961.

18. THOMA, J.A., WAKIM, J., STEWART, L., Biochem. Biophys. Res. Comm., 12, 350, 1963.

19. HAMAUZU, Z., HIROMI, K., ONO, S., J. Biochem. Tokyo, 57, 42, 1965.

20. EVELEIGH, D.E., PERLIN, A.S., Carbohyd. Res., 10, 87, 1969.

21. FREEMAN, G.C., HOPKINS, R.H., Biochem. J., 30, 451, 1936.

22. KUHN, R., Ber., 57, 1965, 1924.

23. THOMA, J.A. KOSHLAND, D.E. Jr., J. Amer. Chem. Soc., 82, 3329, 1960.

24. HANRAHAN, V.M., CALDWELL, M.L., J. Amer. Chem. Soc., 75, 2191, 1953.

25. BIRD, R., HOPKINS, R.H., Biochem. J., 56, 86, 1954.

26. WHELAN, W.J., Die Starke, 12, 358, 1960.

27. HUGHES, R.C., SMITH, E.E., WHELAN, W.J., Biochem. J., 88, 63P, 1963.

28. BANKS, W., GREENWOOD, C.T., Die Starke, 19, 197, 1967.

29. PEAT, S., WHELAN, W.J., THOMAS, G.T., J. Chem. Soc., 4546, 1952.

30. ONO, S., HIROMI, K., HAMAUZU, Z., J. Biochem. Tokyo, 57, 34, 1965.

31. WEILL, C.E., BURCH, R.J., VAN DYK, J.W., Cereal Chem., 31, 150, 1954.

32. ABDULLAH, M., CATLEY, B.J., LEE, E.Y.C., ROBYT, J., WALLENFELS, K., WHELAN, W.J., Cereal Chem., 43, 111, 1966.

33. GREENWOOD, C.T., MILNE, E.A., Adv. Carbohyd. Chem., 23, 281, 1968.

34. ROBYT, J.F., WHELAN, W.J., In "Starch and its Derivatives" (RADLEY, J.A., Ed.) p.470, 1968. Chapman and Hall, London.

35. PAZUR, J.H., In "Starch: Chemistry and Technology" (WHISTLER, R.L., PASCHALL, E.F., Eds.) Vol. 1, p.133, 1965 Academic Press, N.Y.

36. ROBYT, J.F., WHELAN, W.J., In "Starch and its Derivatives" (RADLEY, J.A., Ed.) p.486, 1968. Chapman and Hall, London.

37. ROBYT, J.F., WHELAN, W.J., In "Starch and its Derivatives" (RADLEY, J.A., Ed.) p.499, 1968. Chapman and Hall, London.

38. ROBYT, J.F., WHELAN, W.J., In "Starch and its Derivatives" (RADLEY, J.A., Ed.) p.518, 1968. Chapman and Hall, London.

39. BENDER, H. WALLENFELS, K., Biochem. Z., 334, 79, 1961.

40. ROBYT, J.F., WHELAN, W.J., In "Starch and its Derivatives" (RADLEY, J.A., Ed.) p.69 1968. Chapman and Hall, London.

41. MANNERS, D.J., Adv. Carbohyd. Chem., 17, 371, 1962.

42. PAZUR, J.H., DROPKIN, D.J., HETZLER, C.E., In "Carbohydrates in Solution" (GOULD, R.F., Ed.) p.374, 1973. Advances in Chemistry Series 117, American Chemical Society.

43. THOMA, J.A., SPRADLIN, J.E., DYGERT, S., "The Enzymes", Vol. V, (BOYER, P.D., Ed.) p.115 1972. Academic Press, N.Y.

44. TAKAGI, T., TODA, H., ISEMURA T., "The Enzymes", Vol. V., (BOYER, P.D., Ed.) p.235, 1972. Academic Press, N.Y.

45. LEE, E.Y.C., WHELAN, W.J., "The Enzymes", Vol. V, (BOYER, P.D., Ed) p. 191. 1972. Academic Press, N.Y.

46. ROBYT, J.F., WHELAN, W.J., In "Starch and its Derivatives" (RADLEY, J.A., Ed.) p.107. 1968. Chapman and Hall, London.

64. HIROMI, K., HAMAUZU, Z., TAKAHASHI, K., ONO, S., J. Biochem. Tokyo, 59, 411, 1966.

65. KITAHARA, K., KURUSHIMA, M., J. Ferment. Tech. (Japan), 27, 254, 1949.

66. BRIMACOMBE, J.S., In "Carbohydrate Chemistry" - A Specialist periodical report Vol. 6. p.443, 1973. The Chemical Society, London.

67. RUTTLOFF, H., Ann. Technol. agric., 21, 287, 1972.

68. RUTTLOFF, H., FRIESE, R., TAUFEL, K., Nahrung, 11, 205, 1967.

69. RUTTLOFF, H., FRIESE, R., KUPKE, G., TAUFEL, A., Z. allg. Mikrobiol., 9, 39, 1969.

70. CORMAN, J., LANGLYKKE, A.F., Cereal Chem., 25, 190, 1948.

71. ROY, D.K., UNDERKOFLER, L.A., Cereal Chem., 28, 72, 1951.

72. SCHWIMMER, S., J. Biol. Chem., 161, 219, 1945.

73. COOK, A.H., PHILLIPS, A.W., Arch. Biochem. Biophys., 69, 1, 1957.

74. FRENCH, D., KNAPP, D.W., J. Biol. Chem., 187, 463, 1950.

75. DIXON, M., WEBB, E.C., In "Enzymes" p.238, 1958. Longmans London.

76. PHILLIPS, L.L., CALDWELL, M.L., J. Amer. Chem. Soc., 73, 3563, 1951.

77. GRAVES, D.J., WANG, J.H., "The Enzymes", Vol. VII, (BOYER, P.D., Ed.) p.435, 1972. Academic Press, N.Y.

78. SELBY, K., WOOKEY, N., In "Glucose syrups and related carbohydrates" (BIRCH, G.G., GREEN, L.F., COULSON, C.B., Eds.) p.46, 1970. Elsivier Pub. Co.

79. NORITA, Y., WADANO, A., Mem. Res. Inst. Food. Sci., Kyoto Univ., 36, 6, 1973. Chem. Abs. 79, 123075s.

47. MARUO, B., KOBAYASHI, T., Nature, 167, 606, 1951.

48. GUNJA, Z.H., MANNERS, D.J., MAUNG, K., Biochem. J., 81, 392, 1961.

49. KJOLBERG, O., MANNERS, D.J., Biochem. J., 84, 50p, 1962.

50. KJOLBERG, O., MANNERS, D.J., Biochem. J., 86, 258, 1963.

51. PREISS, J., "The Enzymes", Vol. VIII, (BOYER, P.D., Ed.) p.73 1973. Academic Press, N.Y.

52. PAYEN, A., PERSOZ, J., Ann. Chim. (Phys.) 53, 73, 1833.

53. NISHIKAWA, K., Biochem. Z., 188, 386, 1927.

54. KITANO, T., J. Soc. Chem. Ind., Japan, 39, Suppl. Binding 22, 1936.

55. OHNISHI, M., SUGANUMA, T., FUJIMORI, H., HIROMI, K., J. Biochem., 74, 1271, 1973.

56. OKAZAKI, H., Symp. on Enzyme Chem. (Japan), 9, 43, 1954.

57. OKAZAKI, H., Arch. Biochem. Biophys., 63, 322, 1956.

58. OKAZAKI, H., J. Agr. Chem. Soc. Japan, 29, 273, 1955.

59. KERR, R.W., CLEVELAND, F.C., KATZBECK, W.J., J. Amer. Chem. Soc., 73, 3916, 1951.

60. WATANABE, K., FUKIMBARA, T., J. Ferm. Technol., 43, 690, 1965.

61. HAMAUZU, Z., HIROMI, K., ONO, S., J. Biochem. Tokyo, 57, 39, 1965.

62. BARKER, S.A., FLEETWOOD, J.G., J. Chem. Soc., 4857, 1957.

63. BARKER, S.A., BOURNE, E.J., FLEETWOOD, J.G., J. Chem. Soc., 4865, 1957.

80. BLAKE, C.C.F., FENN, R.H., NORTH, A.C.T., PHILLIPS, D.C., POLJAK, R.J., Nature, 196, 1173, 1962.

81. BLAKE, C.C.F., MAIR, G.A., NORTH, A.C.T., PHILLIPS, D.C., SARMA, V.R., Proc. Roy. Soc., B167, 365, 1967.

82. BLAKE, C.C.F., JOHNSON, L.N., MAIR, G.A., NORTH, A.C.T., PHILLIPS, D.C., SARMA, V.R., Proc. Roy. Soc. B167, 378, 1967.

83. PHILLIPS, D.C., Proc. Natl. Acad. Sci. U.S., 57, 484, 1967.

84. PHILLIPS, D.C., Sci. Ann., 215, 78, 1966.

85. BLAKE, C.C.F., KOENIG, D.F., MAIR, G.A., NORTH, A.C.T., PHILLIPS, D.C., SARMA, V.R., Nature, 206, 757, 1965.

86. REES, A.R., OFFORD, R.E., Biochem. J., 130, 965, 1972.

87. LIN, T.Y., KOSHLAND, D.E., Jr., J. Biol. Chem. 244, 505, 1969.

88. JOHNSON, L.N., PHILLIPS, D.C., RUPLEY, J.A., U.S. At. En. Comm BNL 501116 (Vol. 1), 120-B8. CFSTI 1968, (Pubs. 1969).

89. BEDDELL, C.R., MOULT, J., PHILLIPS, D.C., In "Molecular Properties of Drug Receptors" (PORTER, R., C'CONNOR, M., Eds.) p.85 1970. Churchill, London.

90. TIMASHEFF, S.N., RUPLEY, J.A., Arch. Biochem. Biophys., 150, 318, 1972.

91. SILVERSTEIN, R., VOET, J., REEDS, D., ABELES, R.H., J. Biol. Chem. 242, 1338, 1967.

92. BELL, R.M., KOSHLAND, D.E., Jr., Biochem. Biophys. Res. Commun., 38, 539, 1970.

93. VOET, J.G., ABELES, R.H., J. Biol. Chem., 245, 1020, 1970.

94. PARSONS, S.M., JAO, L., DAHLQUIST, F.W., BORDERS, C.L., Jr., GROFF, T., RACS, J., RAFTERY, M.A., Biochemistry, 8, 700, 1969.

95. PARSONS, S.M., RAFTERY, M.A., Biochemistry, 8, 4199, 1969.

96. PARSONS, S.M., RAFTERY, M.A., Biochem. Biophys. Res. Commun., 41, 45, 1970.

97. PARSONS, S.M., RAFTERY, M.A., Biochemistry, 11, 1623, 1972.

98. PARSONS, S.M., RAFTERY, M.A., Biochemistry, 11, 1633, 1972.

99. BOOTH, H.S., MARTIN, D.R., In "Boron Trifluoride and Its Derivatives", 1949. New York, N.Y., Wiley.

100. BARNETT, J.E.G., MERCIER, D., GERO, S.D., F.E.B.S. letters, 16, 37, 1971.

101. LEGLER, G., Z. Physiol. Chem., 349, 767, 1968.

102. LEGLER, G., HASNAIN, S.N., Z. Physiol. Chem., 351, 25, 1970.

103. LEGLER, G., Molecular and cellular Biochemistry, 2, 31, 1973.

104. MERCIER, D., OLESKER, A., GERO, S.D., BARNETT, J.E.G., Carbohyd. Res., 18, 227, 1971.

105. LEGLER, G., Biochim. Biophys. Acta., 151, 728, 1968.

106. LEGLER, G., Z. Physiol.Chem., 345, 197, 1966.

107. LOTZ, W., Thesis, Univ. Bonn, 1971.

108. WALLENFELS, K., LEHMANN, J., MALHOTRA, O.P., Biochem. Z. 333, 209, 1960.

109. GOT, R., MARNAY, A., Europ. J. Biochem., 4, 240, 1968.

110. WHITAKER, D.R., Arch. Biochem. Biophys., 53, 439, 1954.

111. LECLER, G., BAUSE, E., Carbohyd. Res., 28 45 1973.

112. THOMAS, E.W., McKELVY, J.F., SHARON, N., Nature, 222, 485, 1969.

113. GRAY, C.J., JOLLEY, M.E., F.E.B.S. letters, 29, 197, 1973.

114. HOARE, D.G., KOSHLAND, D.E., Jr., J. Biol. Chem. 242, 2447, 1967.

115. COHEN, J.A., OOSTERBAAN, R., WARRINGA, M.P.G.J. Biochim Biophys Acta 11 52 1953.

116. SHAW, E., In "The Enzymes", Vol. I, (BOYER, P.D. Ed.) p.91, 1970. Academic Press, N.Y.

117. BAKER, B.R., In "Design of active-site directed irreversible enzyme inhibitors", John Wiley & Sons, New York, 1967.

118. ESHDAT, Y., McKELVY, J.F., SHARON, N., J. Biol. Chem., 248, 5892, 1973.

119. SCHULMAN, M.L., SHIYAN, S.D., YA-KHARLIN, A., Carbohyd. Res., 33, 229, 1974.

120. PERRY, M.B., HAENG, L.L.W., Can. J. Biochem., 50, 510, 1972.

121. WOLD, F., Birth Defects, Vol. 9, 46, 1973.

122. THOMAS, E.W., J. Med. Chem., 13, 755, 1970.

123. YARIV, J., WILSON, K.J., HILDESHEIM, J., BLUMBERG, S., F.E.B.S. letters, 15, 24, 1971.

124. YARIV, J., KALB, A.J., KATCHALSKI, E., GOLDMAN, R., THOMAS, E.W., F.E.B.S. letters, 5, 173, 1969.

125. ROGERS, J.C., KORNFELD, S., Biochem. Biophys. Res. Commun., 45, 622, 1971.

126. MORELL, A.G., GREGORIADIS, G., SCHEINBERG, I.H., HICKMAN, J., ASHWELL, G., J. Biol. Chem., 246, 1461, 1971.

127. PRICER, W.E., Jr., ASHWELL, G., J. Biol. Chem., 246, 4825, 1971.

128. HOLLER, E., RUPLEY, J.A., HESS, G.P., Biochem. Biophys. Res. Commun., 40, 166, 1970.

129. BERNDT, W., KOLHOFF, H., STAUDT, V., In "Science tools. The L.K.B. instrument journal, 17, 45, 1970.

130. MOCZAR, E., Experientia, 29, 1576, 1973.

131. BARKER, S.A., EMERY , A.N., NOVAIS, T.M., Process Biochem., 11, Oct., 1971.

132. GRUESBECK, C., RASE, H.F., Ind. Eng. Chem. Prod. Res. Develop., 11, 74, 1972.

133. SMILEY, K.L., Biotechnol. Bioeng., 13, 309, 1971.

134. FLEMMING, C., GABERT, A., WAND, H., Acta. Biologia. Medica. Germanica, 32, 135, 1974.

135. MIYAMOTO, K., FUJII, T., TAMAOKI, N., OKAZAKI, M., MIURA, Y., J. Ferment. Technol., 51, 566, 1973.

136. BACHLER, M.J., STRANDBERG, G.W., SMILEY, K.L., Biotechnol. Bioeng., 12, 85, 1970.

137. WALTON, H.M., EASTMAN, J.E., Biotechnol. Bioeng., 15, 951, 1973.

138. MIYAMOTO, K., FUJII, T., MIURA, Y., J. Ferment Technol. 49, 565, 1971.

139. LI, K.H., CHANG, S.K., SUN, W.R., KU, S.F., YANG, L.W., LI, S.F., YANG, K.Y., Wei. Shing. Wu. Hsueh. Pao., 13, 31, 1973. Chem. Abs., 80, 24313g.

140. MATSUMOTO, A., FUJITA, Y., NOMOTO, M., Japan Patent 7,302,793, 1973, Chem. Abs.,80,1149u.

141. SMILEY, K.L., Division of microbiol chemistry meeting cited in Chem. and Eng. News., Sept., 28, 48, 54, 1970.

142. BESSELL, E.M., THOMAS, P., WESTWOOD, J.H., Chem.-Biol. Interactions, 7, 327, 1973.

143. YARIV, J., KALB, A.J., THOMAS, E.W., umpublished results.

144. WILSON, R.J.H. LILLY, M.D., Biotechnol. Bioeng., 11, 349, 1969.

145. PARK, Y.K., LIMA, D.C., J. Food Sic., 38, 358, 1973.

146. USAMI, S., MATSUBARA, M., NODA, J., Hakko Kyokaishi, 29, 195, 1971.

147. LEDINGHAM, W.M., DO, M., FERREIRA, S.S., Carbohyd. Res., 30, 196, 1973.

148. WEETALL, H.H., HAVEWALA, N.B., GARFINKEL, H.M., BUEHL, W.M., BAUM, G., Biotechnol. Bioeng., 16, 169, 1974.

149. CORNO, C., GALLI, G., MORISI, F., BETTONTE, M., STOPPONI, A., Die Starke, 24, 420, 1972.

150. MATSUMOTO, A., FUJITA, Y., NOMOTO, M., Japan Patent 7,308, 834, 1973. Chem. Abs., 78, 146289h.

151. SOLOMON, B., LEVIN, Y., Biotechnol. Bioeng., 16, 1161, 1974.

152. GRUESBACK, C., Ph. D. Thesis, 1970. Diss. Abstr. Int. B., 32, 5609, 1972.

153. MARSHALL, J.J., WHELAN, W.J., Chem. Ind., 701, 1971.

154. BUTTERWORTH, T.A., WANG, D.I.C., SINSKEY, A.J., Biotechnol. Bioeng., 12, 615, 1970.

155. GHOSE, T.K., KOSTICK, J.A., Biotechnol. Bioeng., 12, 921, 1970.

156. USAMI, S., SHIRASAKI, H., J. Ferm. Technol., 48, 506, 1970.

157. BARKER, S.A., SOMERS P.J., EPTON, R., MacLAREN, J.V. Carbohyd. Res. 14,287,1970.

CHAPTER 2

THE PURIFICATION OF GLUCOAMYLASE

2.1 Introduction

When enzymes were first used the purity of the preparations was not thought to be of great importance, and early reports in the literature quoting results obtained after the action of a certain enzyme on a substrate probably resulted from the action of several enzymes on that substrate. It was not until the late 1950's that the importance of using "pure" enzymes was realised. With the development of gel-permeation chromatography, ion-exchange chromatography, gel-electrophoresis and isoelectric focussing, the purification of enzymes was much easier to achieve.[1] In the case of glucoamylase, many workers[2-15] have reported the existence of multiple forms after ion-exchange chromatography of a crude enzyme preparation. The number of forms obtained varied with the origin of the enzyme and with the technique and conditions used for the separation.

In an early purification of glucoamylase[16] from Aspergillus niger, ammonium sulphate precipitation was used to obtain an enzyme which was essentially free from α-amylase activity after acid treatment[17] at pH 2.9 for 12 days at 10°C. This preparation was also reported to be substantially free from other enzymes such as glucose oxidase and transglucosidase which were capable of acting upon starch or its degradation products.

It appears that the use of D.E.A.E. ion-exchange cellulose as a chromatographic medium for the purification of glucoamylase from Aspergillus niger was first carried out by Pazur et al.[2] These workers,

having adsorbed the crude enzyme solution on an ion-exchange column, used a pH gradient from 8.0 to 4.0 for fractional elution. Three fractions were obtained which showed saccharogenic activity, these being eluted in the pH ranges 6.8 to 7.3, 6.0, and 4.5 to 5.2. After analysis it was found that only the fractions obtained in the lower pH ranges showed glucoamylase activity while the initial fraction appeared to be an α-amylase.

Ion-exchange chromatography was used by Fleming et al.,[3] in their study of glucoamylase from Aspergillus niger. After the crude enzyme solution had been adsorbed on the D.E.A.E. cellulose ion-exchange medium, a linear gradient of sodium chloride in 5 mM TRIS/HCl buffer, pH 7.5 was applied to the column. After the initial elution of a polysaccharide at the gradient front, several α-glucosidase components were eluted between 0.05 - 0.12M chloride followed by two glucoamylase components between 0.15 - 0.18M chloride and 0.21-0.29M chloride. The latter fraction accounted for 90% of the total glucoamylase activity of the preparation.

To illustrate the effect of culture medium upon the two forms of glucoamylase obtained after ion-exchange chromatography, Watanabe et al.,[4,18] produced enzymes from Aspergillus awamori using unusual conditions of pH. It was observed that when a normal culture of glucoamylase was subjected to acid at pH 2.5 at 30°C, about 40% activity was lost within one hour and the residual activity was almost unaltered over twenty-four hours. Using D.E.A.E. cellulose ion-exchange chromatography the enzyme culture produced under normal conditions was separated into two fractions using a pH gradient from 8.0 to 3.0. The fractions containing glucoamylase activity were eluted between pH 8.0 to 7.0 and pH 5.1 to 3.9 the latter containing about 1.25 times the activity of the former. It was observed that when crude enzyme was produced at a culture pH of 2.0 to 2,5, it was

scarcely inactivated by the acid treatment at pH 2.5 and was shown by ion-exchange chromatography to comprise only the fraction obtained between pH 5.1 - 3.9 described above. The crude enzyme produced at a culture pH between 6.0 to 6.5 was almost completely inactivated by the acid treatment at pH 2.5 within one hour. This enzyme was shown by ion-exchange chromatography to be composed mainly of the fraction obtained between pH 8.0 to 7.0 in the initial study. This work indicated that the two forms of glucoamylase from Aspergillus awamori had different acid stabilities and each could be produced individually by suitably adjusting the pH of the culture. The less acid stable glucoamylase has been investigated further in a later publication.[5] From an abstract of a Russian paper[6] it was reported that α-amylase glucoamylase and transferase enzymes from Aspergillus awamori had been separated on a D.E.A.E. cellulose column. The specific activity of the glucoamylase was reported to have increased between 10-15 fold after gel-permeation chromatography using "Sephadex" G-25.

Ruttloff et al.,[7] have reported two forms of glucoamylase from Endomycopsis bispora after adsorbing the crude enzyme on D.E.A.E. cellulose and eluting with a continuous salt gradient between 0 to 1.0M-chloride. The glucoamylase activity was predominantly found in the first eluted peak.

Lineback et al.,[8] have purified glucoamylase from Aspergillus niger using D.E.A.E. cellulose. After adsorption of the crude enzyme on D.E.A.E. cellulose, the components of the preparation were eluted by increasing concentration of buffer at constant pH followed by constant buffer concentration with change of pH. It was reported that the results obtained using this technique were comparable to those obtained using a

linear pH gradient. Three major protein peaks were obtained, the first occurring at pH 8.0, 0.035M buffer concentration, showed α-glucosidase activity and no glucoamylase activity while the others, occurring at pH 6.8, 0.05M buffer concentration and pH 4.9, 0.05M buffer concentration showed only glucoamylase activity. Although the glucoamylase eluted at pH 6.8 appeared as two peaks, they were reported to have been shown to be the same enzyme.

In a similar study by Lineback et al.,[9] using a crude glucoamylase enzyme preparation from Aspergillus phoenicis, two forms were purified by elution from D.E.A.E. cellulose at pH 6.3 and pH 4.7 respectively.

Using "D.E.A.E. - Sephadex" as an ion-exchange medium, the glucoamylase from liver and spleen of Macaca mullata monkeys was purified by using a buffer concentration gradient for elution from 10 mM to 200 mM at pH 7.0. Only one form of glucoamylase was reported.[10]

The crude glucoamylase from Aspergillus niger (Agidex 3,000) was purified by chromatography on D.E. 32 ion-exchange cellulose by Jolley[12] using a pH gradient from 8.0 to 4.0 followed by a NaCl salt gradient from 0 to 1M in pH 4.0 phosphate-citrate buffer 0.1M. Two forms of the enzyme were obtained, the first being eluted at pH 6.0, the second being eluted at pH 4.0 at a salt concentration of 1M NaCl. The ratio of the amount of protein obtained by the salt gradient to that obtained by the pH gradient was 5:1. The former protein was identified to be glucoamylase I.[2,8,11] Although the protein peak eluted by the salt gradient was not symmetrical, the protein was found to be homogeneous on Bio-Gel P-150 chromatography in 8M urea.

For a study of the carbohydrate moieties attached to glucoamylase from Aspergillus niger,[13] radio labelled isoenzymes were produced and

purified on D.E.A.E. cellulose as described earlier.[2]

It was reported by Smiley et al.,[14] that by using a method adapted from that described by Todu et al.,[15] it was possible to obtain two glucoamylase forms from a crude Aspergillus species by buffer concentration gradient elution from D.E.A.E. cellulose at pH 4.2. The first glucoamylase peak emerged shortly after the acetate buffer gradient beginning at 0.10M was applied and was completely eluted when the gradient was between 0.35 to 0.40M. The second glucoamylase peak was eluted over the gradient range 0.45M to 0.55M. After the isolation of the two forms of glucoamylase, each was subjected to rechromatography on D.E.A.E. cellulose. It was found that each form gave rise to only one peak in its expected position.

It has been observed[19] that the glucoamylase from Endomycopsis bispora comprises several isoenzymes whose participation in the total activity is very different. A staggered distribution of the different enzymes present during the evolution of the culture was also observed. By using D.E.A.E. cellulose ion exchange chromatography and "Sephadex" G-200 gel-permeation chromatography, it was possible to separate three forms of glucoamylase from this source.

It has been recently reported[20] that the crystalline glucoamylase obtained from Mucor rouxianus[21] is a mixture of two forms. It has been found that both forms of the glucoamylase from this source could be separated by disc-gel electrophoresis or by chromatography on SP-"Sephadex" C-50. Furthermore, both forms of the purified glucoamylase have been obtained in crystalline form.

Fukui et al.,[22] have used C.M-cellulose cation exchange chromatography for the purification of glucoamylase from Endomyces species IFO 0111 prior to crystallisation. The C.M-cellulose was treated with 0.1M

acetate buffer pH 4.0 to facilitate the adsorption of the crude enzyme and elution was effected with 0.2M NaCl dissolved in this buffer. The enzyme was then reported to be free from α-amylase activity which was present in the crude culture.

Durmishidze et al.,[23] isolated two isoenzymes from Aspergillus awamori one of which was stated to be homogeneous, similarly, Ryzhakova et al.,[24] have reported the isolation of a highly purified glucoamylase from this source. Park et al.,[25] have investigated thirty Aspergillus strains for the production of glucoamylase. Aspergillus awamori was found to produce the largest amount of glucoamylase but α-amylase and transglycosidase activities were also present in the crude enzyme. D.E.A.E. cellulose chromatography separated the crude glucoamylase into two forms. In a study by Qureshi,[26] using D.E.A.E. cellulose chromatography in TRIS buffer, the enzymes from Aspergillus niger were separated into two forms. Glucoamylase from "Agidex" has been fractionated on "D.E.A.E.-Sephadex 50" and was reported to be free from β-glucanase activity.[27]

Glucoamylases from Rhizopus delemar,[28] Dog serum,[29] Coniophora cerebella,[30] Endomycopsis capsularis,[31] Human intestine[32] and Aspergillus oryzae,[33,34] have also been purified using either ion-exchange or gel-permeation chromatography and in the latter two cases, two and four multiple enzyme forms respectively have been observed.

All results have been summarised in TABLE 2.1.

In this work it was necessary to purify the crude glucoamylase from Aspergillus niger ("Agidex 3,000") to enable an investigation into the properties and action of this enzyme to be carried out.

TABLE 2.1

Summary of previous work on the purification of glucoamylase

AUTHOR AND REFERENCE	SOURCE OF ENZYME	CHROMATOGRAPHIC MEDIUM	NUMBER OF ENZYME FORMS AND RANGE OF ELUTION	ELUTION METHOD
BARKER 16	Aspergillus niger	Ammonium sulphate precipitation.	One	
PAZUR 2	Aspergillus niger	D.E.A.E. cellulose (Brown)	Two forms. pH 6.0; pH 4.5 - 5.2	pH buffer gradient 8.0 - 4.0.
FLEMING 3	Aspergillus niger	D.E.A.E. cellulose	Two forms. 0.15-0.18M; 0.21-0.29M Chloride	5 mM TRIS/HCl buffer pH 7.5
WATANABE 4	Aspergillus awamori	D.E.A.E. cellulose (Serva)	Two forms. pH 8.0-7.0; pH 5.1-3.9	pH buffer gradient 8.0 - 3.0.
FENIKSOVA 6	Aspergillus awamori	D.E.A.E. cellulose G-25 "Sephadex"	One form	
RUTTLOFF 7	Endomycopsis bispora	D.E.A.E. cellulose	Two forms	Salt gradient (in 0.01M phosphate) 0-1.0M NaCl.
LINEBACK 8	Aspergillus niger	D.E.A.E. cellulose (Brown)	Two forms. pH 6.8, 0.05M; pH 4.9, 0.05M.	pH gradient 8.0 - 4.0. Buffer gradient concentration 0.025M-0.05M.
LINEBACK 9	Aspergillus phoenicis	D.E.A.E. cellulose (Brown)	Two forms. pH 6.3, 0.05M; pH 4.7, 0.05M.	pH gradient 8.0 - 4.0. Buffer gradient concentration 0.025M-0.05M.
SEETHARAM 10	Intestinal	"D.E.A.E. - Sephadex" A50.	One form.	pH 7.0. Buffer gradient concentration 10mM - 200 mM.

TABLE 2.1 (continued)

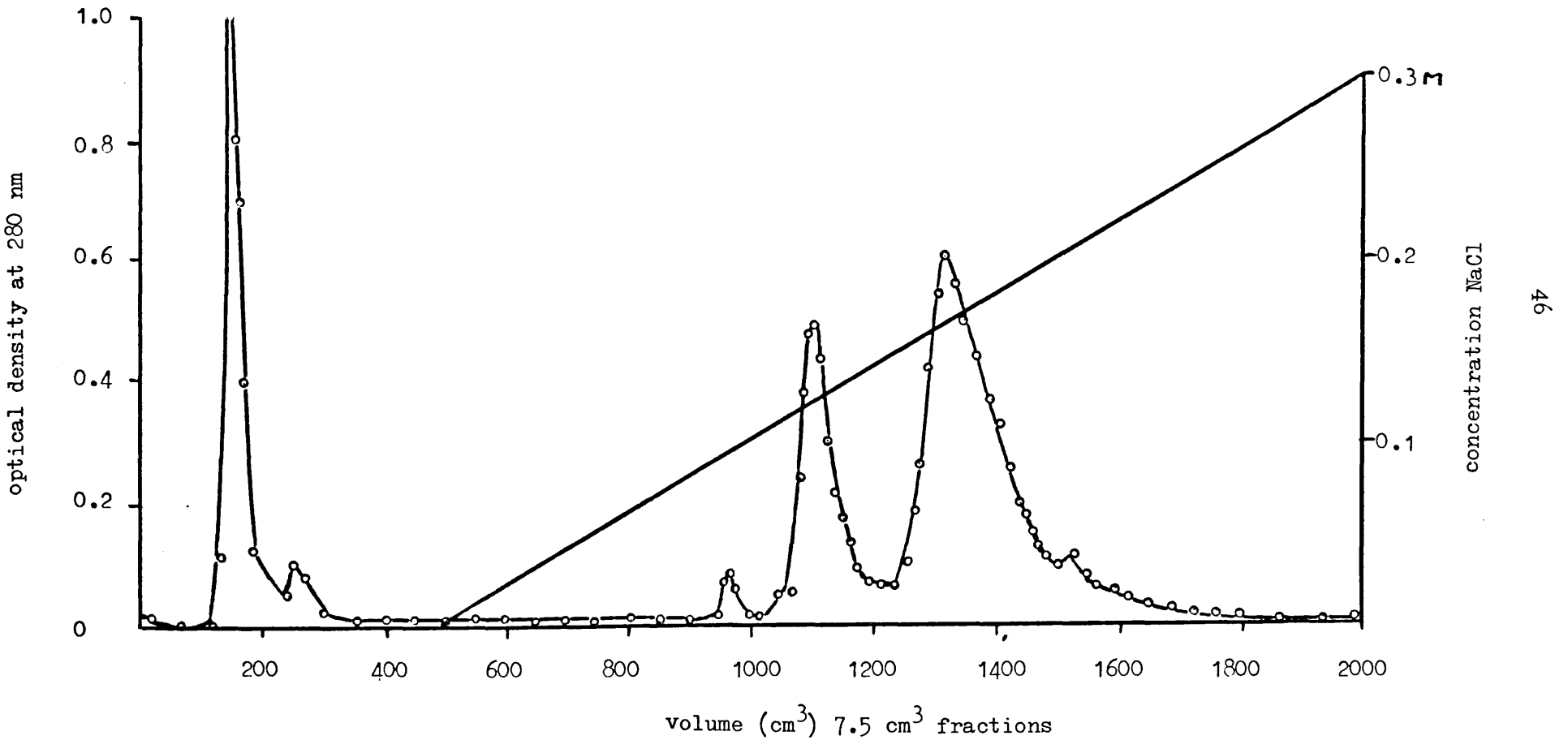
AUTHOR AND REFERENCE		SOURCE OF ENZYME	CHROMATOGRAPHIC MEDIUM	NUMBER OF ENZYME FORMS AND RANGE OF ELUTION	ELUTION METHOD
BARKER	11	Aspergillus niger	D.E.A.E. cellulose (D.E 32 Whatman)	Two forms. pH 5.5; pH 4.0, 1.0M salt.	pH gradient 8.0 - 4.0. NaCl gradient 0 - 1.0M.
JOLLEY	12	"			
SMILEY	14	Aspergillus	D.E.A.E. cellulose (Brown)	Two forms. 0.35 - 0.4M; 0.45 - 0.55M.	Buffer gradient 0.1M - 0.6M.
RUTTLOFF	19	Endomycopsis bispora	D.E.A.E. cellulose "Sephadex" G-200	Three forms.	Salt gradient 0-1M 0.01M phosphate
TSUBOI	20	Mucor rouxianus	SP-"Sephadex" C50	Two forms.	pH gradient 4.0 - 6.0
FUKUI	22	Endomyces species IFO 0111	C.M-cellulose (Brown)	One form. pH 4.0, 0.05-0.10M NaCl.	Salt gradient 0-0.2M NaCl in acetate buffer 0.1M pH 4.0.
PAZUR	28	Rhizopus delemar		One form.	
Mc.GEENEY	29	Dog serum	Bio-Gel P-300 "Sephadex" G-200	One form.	
KELLY	32	Human Intestine		Two forms.	
MORITA	33	Aspergillus oryzae		Four forms.	
OHGA	34				
KING	30	Conisphora cerebella		One form.	
EBERTOVA	31	Endomycopsis capsularis		One form.	
DURMISHIDZE	23	Aspergillus awamori		Two forms - one purified.	

TABLE 2.1 (continued)

AUTHOR AND REFERENCE	SOURCE OF ENZYME	CHROMATOGRAPHIC MEDIUM	NUMBER OF ENZYME FORMS AND RANGE OF ELUTION	ELUTION METHOD
RYZHAKOVA '24	Aspergillus awamori		One form purified.	
PARK 25	30 Aspergillus strains of awamori.	D.E.A.E. cellulose.	Two forms.	
QURESHI 26	Aspergillus niger	D.E.A.E. cellulose.	Two forms.	TRIS
MACREA 27	Agidex	D.E.A.E. "Sephadex-50"	One form purified.	
THIS WORK	Aspergillus niger (Agidex 3,000)	D.E.A.E. cellulose (Whatman DE 52)	Two forms pH 7.7, 0.12 - 0.16M; pH 7.7, 0.18 - 0.24M chloride.	Salt gradient 0-0.3M NaCl in 0.05M Phosphate buffer pH 7.7.

The crude glucoamylase from "Agidex 3,000" was purified by ion-exchange chromatography on D.E. 52 cellulose. Glucoamylase activities were monitored using wheat amylopectin because this was the soluble component of the starch used in the R.H.M. "Tenstar" process for the production of glucose. It was assumed therefore, that the subsequent work on glucoamylase covered in this Thesis would bear a closer relationship to its action within the industrial process than it would if another source of soluble starch had been used.

FIGURE 2.1. Small scale fractionation of Agidex 3,000 on D.E.A.E. cellulose using 0-0.3M NaCl gradient in 0.05M phosphate buffer pH 7.7.



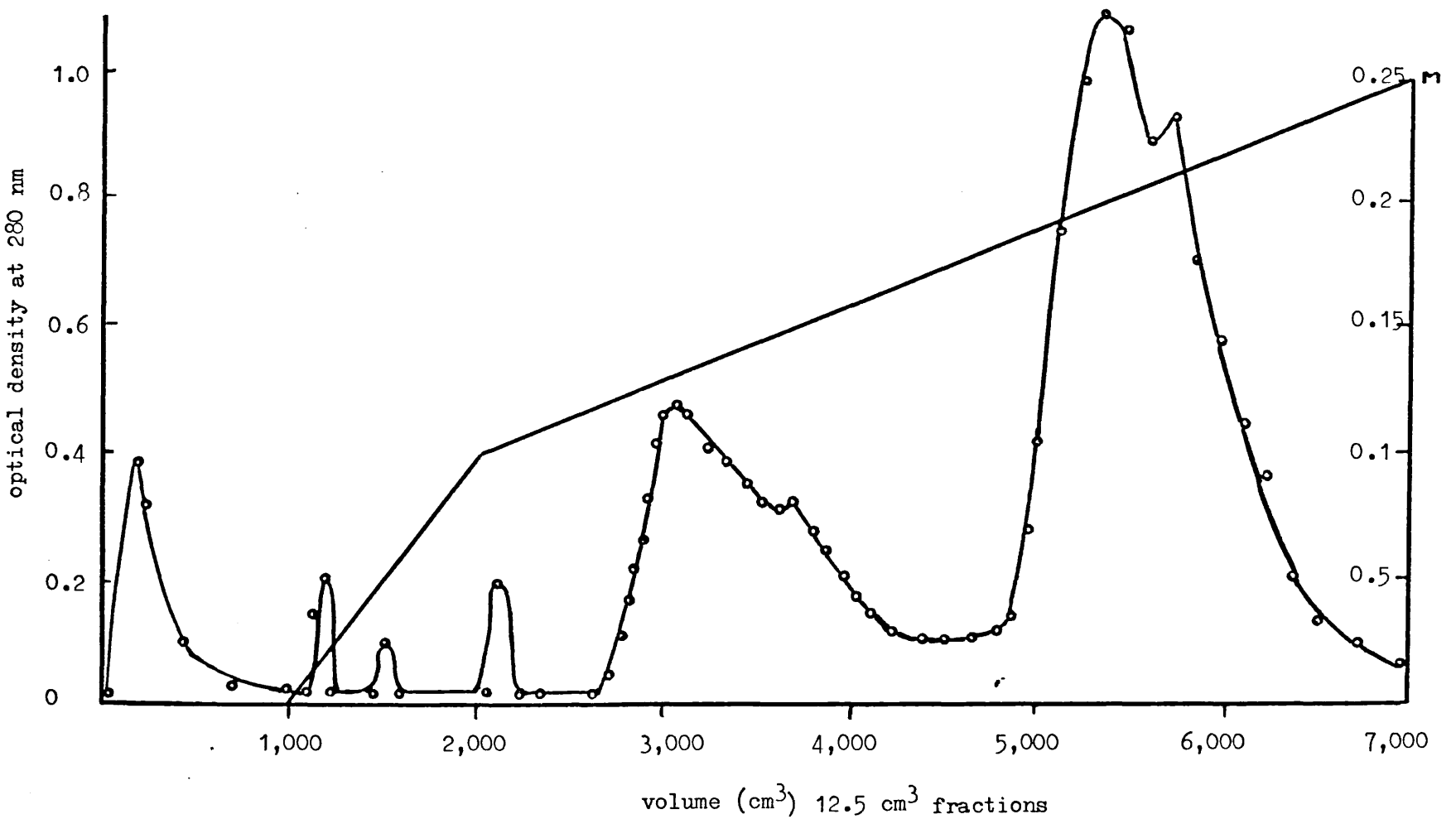
2.2 Results and discussion

The crude glucoamylase from Aspergillus niger (Agidex 3,000) was adsorbed on a small chromatography column and washed with phosphate buffer pH 7.7, 0.05M. A sodium chloride gradient 0–0.25M in the same buffer was then applied to the column. From FIGURE 2.1, it can be seen that essentially three protein peaks were eluted from the column. The first protein peak being eluted before the salt gradient was applied. The second and third protein peaks were eluted between 0.12 - 0.16M chloride and 0.18 - 0.24M chloride, respectively. The protein content of these three peaks collectively represented 87% of the total amount applied and agrees with the recovery found by Lineback et al.[8] After testing each of the fractions for glucoamylase activity it was found that only the latter two gave a positive result while the first, representing 2.7% of the protein recovered, showed none of this activity. The fractions comprising each protein peak were separately pooled.

From results obtained using Cibachron Blue amylose[35–37] as a substrate for glucoamylase (CHAPTER 5 of this Thesis), it was suggested that the protein obtained in the first peak may have been an enzyme having "endo" activity, possibly α-amylase. Glucoamylase has been reported to possess only "exo" activity toward substrates.[38]

Based on electrophoresis studies carried out on glucoamylase by other workers,[8] the enzyme bearing the higher charge was assigned glucoamylase I and that with lower charge glucoamylase II.[52] Lineback et al.,[9] referred to the glucoamylases obtained from Aspergillus phoenicis as A and B respectively, A bearing the lower charge. On ion-exchange chromatography, the glucoamylase carrying the lower charge should be eluted first and is therefore glucoamylase II. It was observed that two small peaks were obtained on the

FIGURE 2.2. Large scale fractionation of Agidex 3,000 on D.E.A.E. cellulose using 0-0.25M NaCl gradient in 0.05M phosphate buffer pH 7.7.



elution profile which corresponded to the point at which the gradient had been changed during the experiment. These had occurred at 0.1M and 0.2M sodium chloride concentrations. In a later experiment, conditions were used which allowed a gradient change to occur at 0.05M and 0.25M chloride. These small peaks did not then occur in the fractionation of the glucoamylases I and II and confirmed them to be experimental artifacts.

The specific activity of glucoamylase I and II from this purification measured at 25°C using 1% w/v wheat amylopectin in citrate buffer (0.05M, pH 5.0) was 12.0 I.U./mg of protein and 16.0 I.U./mg of protein respectively. The specific activity of crude dialysed "Agidex 3,000" glucoamylase was 2.6 I.U./mg of protein, so purification factors of 4.6 and 6.2 were achieved for glucoamylase I and II respectively.

The results of a large scale fractionation of glucoamylase by anion-exchange chromatography on DE-52 are shown in FIGURE 2.2. It can be seen that the elution profile was very similar to that obtained from the small scale purification. As no glucoamylase was eluted between 0-0.10M and 0.25-0.30M chloride in the small scale fractionation, a steep gradient was applied between 0-0.10M chloride and the shallower elution gradient terminated at 0.25M chloride in this purification. The small peaks occurring at 1,200 cm^3 and 2,200 cm^3 and the shoulders on glucoamylase II and I occurring at 3,750 cm^3 and 5,750 cm^3 were considered to be artifacts caused by the salt gradient changes. Because of the large volume of liquid involved in this purification, it was not possible to remove the artifacts as in the case of the small scale purification described previously. From the total protein applied to the column $\approx$62.5% was recovered as freeze-dried glucoamylases. The lower recovery obtained from this purification than that (84.3%) from the small scale fractionation may have resulted from a combination of the following factors:

TABLE 2.2

A comparison of some Specific Activities of glucoamylases

GLUCOAMYLASE SOURCE	REFERENCE	FRACTIONATION METHOD AND ENZYME	SUBSTRATE	TEMPERATURE °C	TIME (MINS)	pH	SPECIFIC ACTIVITY UNITS/mg PROTEIN
		Crude dialysed glucoamylase	3% lintner soluble starch	30	60	4.8	12.0
Aspergillus niger	8	D.E.A.E.-cellulose glucoamylase I	3% lintner soluble starch	30	60	4.8	12.0
		D.E.A.E.-cellulose glucoamylase II	3% lintner soluble starch	30	60	4.8	15.0
		Crude dialysed glucoamylase	1% wheat amylopectin	25	15	5.0	2.6 I.U.
		D.E.A.E.-cellulose small scale glucoamylase I	1% wheat amylopectin	25	15	5.0	12.0 I.U.
Aspergillus niger	THIS WORK	D.E.A.E.-cellulose small scale glucoamylase II	1% wheat amylopectin	25	15	5.0	16.0 I.U.
		*D.E.A.E.-cellulose large scale glucoamylase I	1% wheat amylopectin 1% MERCK Maltose	25	15	5.0 4.6	15.3 I.U. 4.1
		D.E.A.E.-cellulose large scale glucoamylase II	1% wheat amylopectin 1% MERCK Maltose	25	15	5.0 4.6	17.2 I.U. 3.3
		Crude dialysed glucoamylase	3% lintner soluble starch	30	60	4.8	3.0
		D.E.A.E.-cellulose glucoamylase A	3% lintner soluble starch	30	60	4.8	3.3
Aspergillus phoenicis	9	D.E.A.E.-cellulose glucoamylase B	3% lintner soluble starch	30	60	4.8	1.3
		D.E.A.E.-cellulose rechromatographed glucoamylase A	3% lintner soluble starch	30	60	4.8	12.2
		D.E.A.E.-cellulose rechromatographed glucoamylase B	3% lintner soluble starch	30	60	4.8	6.7

I.U. = INTERNATIONAL UNIT

* CHAPTER 5

(a) A larger portion of the glucoamylases in the overlap region between the two peaks was discarded.

(b) Glucoamylase was lost on the surface of the ultrafiltration membrane or on the surface of the dialysis tube after the protein concentration stage.

(c) Traces of solid glucoamylase remained on the glass surface of the flask used for freeze-drying.

(d) An error in the experimental determination of protein being either high for crude glucoamylase or low for purified glucoamylase.

(e) A portion of crude glucoamylase remained on the ion-exchange column.

The latter was considered to be unlikely because of the similarity of this fractionation to that obtained on the small scale. The specific activities of glucoamylase I and II from this purification were found to be 15.3 I.U./mg of protein and 17.2 I.U./mg of protein respectively. This represented purification factors of 5.9 and 6.6 for glucoamylase I and II respectively. Although specific activity has been discussed in CHAPTER 5 of this Thesis, some specific activities obtained in this and previous work have been given in TABLE 2.2 for purposes of comparison.

The results obtained from this purification agree with those obtained by other workers.[3,12] The ratio of glucoamylase I to glucoamylase II was found to be approximately 3 to 1 in this purification. Both forms were shown to be homogeneous by several techniques (see CHAPTER 3 of this Thesis).

It is of interest to note that the purification procedure described earlier for Aspergillus niger glucoamylase, bears a close relationship to that used by Fleming et al.,[3] in that only a salt gradient at constant pH

was used for enzyme elution. These workers however used a 5mM TRIS/HCl buffer of pH 7.5, whereas in this work a phosphate buffer 0.05M, pH 7.7 was used. This was considered to be an advantage because it had been reported that polyols may inhibit enzyme activity.[39]

An experiment was carried out on crude glucoamylase from (Agidex 3,000) in an attempt to use the purification procedure described by Smiley et al.,[14] but it was found that at pH 4.2, the enzyme could not be adsorbed on the DE-52 ion-exchange cellulose.

It has been reported by Lineback et al.,[40] that the levels of glucoamylase, α-amylase and glucosyltransferase produced by Aspergillus niger were influenced by the kind of nitrogen, the concentration of both nitrogen and carbohydrate, the concentration of trace elements and as already mentioned,[4] the pH of the culture medium. Conditions were established for the production of glucoamylase with minimal quantities of α-amylase and glucosyltransferase. The use of a purified glucoamylase for the industrial starch conversion process was the subject of a United States patent.[41] It was claimed that by using the purified enzyme, the yield of glucose in the hydrolysate was increased over that produced using crude enzyme. However the increased cost of producing a glucoamylase for use in industry would have to be balanced by the increase in product production. As will be discussed in CHAPTER 5, some transglucosylase activity may be considered to be a thermodynamic inevitability of catalytic action.

2.3 The fractionation of wheat 'A' starch

Freeze-dried wheat 'A' starch from the R.H.M. "Tenstar" process was fractionated to obtain a sample of wheat amylopectin using the method of Schoch[42] and Lansky et al.[43] A dry sample of amylopectin was obtained

TABLE 2.3

The fractionation of wheat 'A' starch

Source of starch and fractionation method reference		Starch weight g	Amylose Weight g	Amylopectin Weight g	"Blue Value" [44,45] Experimental Amylose	Experimental Amylopectin	Literature Values[45] Amylose	Literature Values[45] Amylopectin
Potato Starch	46	160	6–7	24			1.35–1.41	0.04–0.06
Commercial BDH potato Amylopectin						0.128		
Wheat 'A' starch R.H.M. "Tenstar"	46	160	11	22.5	0.640	0.054		
Wheat 'A' starch R.H.M. "Tenstar"	42	75	not recovered	11	–	0.058		

which had a "blue value" [44,45] of 0.058. In a second fractionation using the method of Gilbert et al.,[46] the amylopectin had a "blue value" of 0.054. The results of the fractionations are shown in TABLE 2.3. The rather high "blue value" of B.D.H. potato amylopectin (0.128) may suggest an incomplete fractionation of the starch, the amylose component contributing to the higher value. It should be noted that the results quoted in TABLE 2.3 are not necessarily those representing the "true" composition of the wheat 'A' starch because the experiment was neither carried out analytically nor repeated for consistency.

The "blue value" of a starch is a measure of the absorption value observed when a polysaccharide is stained with iodine under the standard conditions prescribed by Hassid et al.[47] One would expect a low "blue value" from the highly branched polysaccharides such as glycogen and amylopectin and a high "blue value" for the essentially straight chain amylose.

It can be seen from TABLE 2.3 that the weights of amylopectin and amylose obtained in the fractionation of wheat 'A' starch are comparable to those quoted by Gilbert et al.,[46] for potato starch. Although the amylose obtained in the fractionation was not used in the study of glucoamylase, it was noted that its "blue value" was lower than reported.[46] This may be attributed to one of the following:

(a) The amylose was contaminated by amylopectin.

(b) The amylose was contaminated by sodium chloride used in the fractionation.

(c) Structural alteration occurred within the amylose molecules on freeze-drying after precipitation with butanol, or during the conditions used for dissolution in the "blue value" assay.

2.4 Experimental

All chemicals used were B.D.H. "Analar" grade except where otherwise stated.

2.4.1 Small scale purification of "Agidex 3,000" glucoamylase

5,000 cm^3, 0.05M Phosphate buffer pH 7.7 was prepared[48] using deionised water and was boiled prior to use. 60 x 2.54 cm dialysis tubing was boiled with deionised water (300 cm^3) for five minutes, the water then being discarded. The process was repeated three times. Crude "Agidex 3,000", (10 cm^3) was poured into the dialysis tube and dialysed against phosphate buffer (0.05M, pH 7.7) (500 cm^3) for two days with four buffer changes. The surface of the dialysis buffer was layered with toluene to prevent bacterial contamination. Whatman D.E.-52 anion exchange cellulose (20 g) was washed with deionised water (150 cm^3) and 'fines' removed by suction from a water pump. The washing process was repeated three times. 0.5M HCl was added to the washed suspension until the pH was 7.7. The ion-exchange cellulose was washed with phosphate buffer (pH 7.7) (500 cm^3) and packed into a glass column (9 x 1.0 cm). Dialysed "Agidex 3,000" (7.5 cm^3) was applied to the ion exchange column. Elution was started using phosphate buffer pH 7.7 (500 cm^3). Fractions (7.5 cm^3) were collected using a Gilson Escargot fraction collector. A linear salt gradient (0 - 0.3M NaCl) was then applied in phosphate buffer, pH 7.7, over 1,500 cm^3. Samples from each fraction tube were withdrawn and the optical density (280 nm) recorded automatically using a Pye Unicam SP 1,800 spectrometer. Three peaks were observed, one occurring after washing the column with phosphate buffer, pH 7.7, (500 cm^3), the others occurring between 0.12 - 0.13M chloride and 0.16 - 0.19M chloride. Each fraction

tube was tested for glucoamylase activity and from the results, fractions occurring between 0 - 100 cm^3, 1,000 - 1,200 cm^3, and 1,300 - 1,500 cm^3 pooled separately. All three peaks showed positive results for protein but only the latter two showed glucoamylase activity.

2.4.2 Protein Determination

This was carried out using the method described by Layne.[49] The reagents were kept in separate containers until required, a 2% (w/v) solution of sodium potassium tartrate, and a 1% (w/v) solution of cupric sulphate being mixed in the ratio 1:1 (v/v) prior to use. Folin-Ciocalteu reagent (B.D.H.) was diluted with deionised water in the ratio 1:1 (v/v) before use. The optical densities of the samples were read at 750 nm using a Pye Unicam SP 500 spectrometer and the results related to a standard graph obtained using Bovine serum albumin (Sigma).

2.4.3 Glucoamylase determination

Citrate buffer, (0.05M, pH 5.0)(2,000 cm^3) was prepared[48] using deionised water and boiled prior to use. A stock solution of 2% wheat amylopectin was prepared using this buffer. Glucose oxidase reagent was made up essentially as described by Lloyd *et al.*,[50] except that the 'O'-dianisidine hydrochloride was dissolved in deionised water (1.0 cm^3) before addition to the other reagent components.

Using the results obtained from the protein determination, the enzyme solutions to be tested for glucoamylase activity were diluted to give approximately 35 $\mu g/cm^3$ protein.

2% w/v wheat amylopectin (1.0 cm^3), citrate buffer 0.05M, pH 5.0, (0.95 cm^3) and enzyme (0.05 cm^3) were reacted in a Pyrex 6" x 5/8" test tube for 15 minutes at 25°C and enzyme reaction terminated by heating to 100°C for 5 minutes in a boiling water bath. After cooling, 1.0 cm^3 was

sampled and analysed for D-glucose, using the glucose oxidase reagent[50], and results related to a standard D-glucose graph. A sample containing 0.05 cm^3 buffer to replace the enzyme was used as a blank.

The International Unit (I.U.) of enzyme activity is defined[51] as that amount of enzyme which would catalyse the transformation of one micro equivalent of the group concerned per minute under defined conditions at 25°C.

2.4.4 Large scale preparative purification of "Agidex 3,000" glucoamylase

The method used was essentially that described in 2.4.1. Phosphate buffer, 0.05M, pH 7.7, (10,000 cm^3) was prepared as before. 100 x 2.54 cm dialysis tubing was prepared as previously described and crude "Agidex 3,000", (100 cm^3) dialysed against buffer, pH 7.7, (2,000 cm^3) for 2 days with five buffer changes. The pH of the dialysed "Agidex 3,000" was then checked with a pH meter and if not equal to that of the starting buffer, further dialysis carried out until the pH's were identical. D.E.-52 ion exchange cellulose (400 g) was prepared as described in (2.4.1) and packed into a "Pharmacia" type K26/40, 2.6 x 40 cm, 210 cm^3 capacity glass column. The column was then washed with buffer pH 7.7 (1,000 cm^3). Dialysed "Agidex 3,000" (100 cm^3) was applied to the column and elution begun using phosphate buffer, pH 7.7, (1,000 cm^3) followed by a linear sodium chloride gradient (0 - 0.1M NaCl) in phosphate buffer, pH 7.7, (1,000 cm^3). Fractions (12.5 cm^3) were collected as before. A second linear salt gradient (0.1 - 0.25M NaCl) was then applied over 6,000 cm^3 at pH 7.7. Samples from each fraction tube were withdrawn and the optical density (280 nm) recorded as before. Three peaks were observed, occurring at 0.00M chloride, 0.12 - 0.16M chloride and

0.18 - 0.24M chloride respectively. Each fraction tube was tested for glucoamylase activity and from the results fractions occurring between 0 - 500 cm^3, 2,800 - 4,000 cm^3, and 4,900 - 6,500 cm^3 pooled separately. As discovered previously only the latter two fractions showed glucoamylase activity. These fractions were then concentrated to 50 cm^3 by membrane ultrafiltration using an "Amicon PM-10" membrane at a nitrogen pressure of 40 lb/sq in in a "Chemlab" C 100 ultrafiltration cell. Each sample was dialysed against deionised water (15,000 cm^3) and freeze-dried in a 1,000 cm^3 flask on a "Chemlab" freeze drier. The solid glucoamylase I (0.55 g) and II (0.20 g) were stored in the ice compartment of a refrigerator until required. The initial fraction was stored in an amber glass bottle in a refrigerator.

2.4.5 The fractionation of wheat 'A' starch

The method used was essentially that described by Schoch.[42] The experiment was carried out on a quarter scale. In a round bottom flask, wheat 'A' starch (75 g) from R.H.M. "Tenstar" was suspended in a mixture of deionised water (3,750 cm^3) and amyl alcohol (250 cm^3); anhydrous potassium di hydrogen orthophosphate (2.05 g) and anhydrous di potassium hydrogen orthophosphate (0.45 g) were added to buffer the pH at 6.2 - 6.3. The flask was fitted with a reflux condenser and a high speed propeller agitator and placed in a large heating mantle. The suspension was first stirred in the cold to facilitate a uniform distribution of the starch throughout the flask and then slowly heated to boiling point. The mixture was gently refluxed for 3 hours, allowed to cool overnight and refrigerated for 24 hours, continuous agitation being maintained throughout the operations. The fractionation was completed by centrifugation at 5,000 r.p.m. using a Beckman model C100

centrifuge. The linear fraction was collected as the precipitate and the branched fraction as the clear supernatant. The branched fraction was then precipitated with an equal volume of methanol and allowed to stand at 0°C overnight. The precipitate was collected and then dehydrated with fresh methanol in a Waring blender. The precipitate was then filtered on a Buchner flask and dried at 50°C for 24 hours. Starting from 75 g starch, 11 g amylopectin was obtained. The linear fraction was not purified.

In a second fractionation using the method of Gilbert et al.,[46] starting from 160 g wet starch, 11 g amylose, and 22.5 g amylopectin were obtained.

Determination of "blue values" was carried out as described by Gilbert et al.,[45] optical densities were measured using a Pye Unicam SP 500 spectrometer.

References

1. MARSHALL, J.J., Wallerstein Labs. Commun., 35, 49, 1972.

2. PAZUR, J.H., ANDO, T., J. Biol. Chem., 234, 1966, 1959.

3. FLEMING, I.D., STONE, B.A., Biochem. J., 97, 13p, 1965.

4. WATANABE, K., FUKIMBARA, T., J. Ferment. Technol., 43, 690, 1965.

5. WATANABE, K., FUKIMBARA, T., J. Ferment. Technol., 44, 392, 1966.

6. FENIKSOVA, R.V., RYZHAKOVA, V.G., Prikl. Biokhim. Mikrobiol., 4, 270, 1968.

7. RUTTLOFF, H., FRIESE, R., KUPKE, G., TAUFEL, A., Zeitschrift. fur. Allg. Mikrobiologie., 9, 39, 1969.

8. LINEBACK, D.R., RUSSEL, I.J., RASMUSSEN, C., Arch. Biochem. Biophys. 134, 539, 1969.

9. LINEBACK, D.R., BAUMANN, W.E., Carbohyd. Res., 14, 341,1970.

10. SEETHARAM, B., SWAMINATHAN, N., RADHAKRISHNAN, A.N., Biochem. J., 117, 939, 1970.

11. BARKER, S.A., GRAY, C.J., JOLLEY, M.E., Biochem. Biophys. Res. Commun., 45, 654, 1971.

12. JOLLEY, M.E., Ph. D. Thesis, Univ. Birmingham,1972.

13. PAZUR, J.H. KNULL, H.R., CEPURE, A., Carbohyd. Res., 20, 83, 1971.

14. SMILEY, K.L., HENSLEY, D.E., SMILEY, M.J., GASDORF, H.J., Arch. Biochem. Biophys., 144, 694, 1971.

15. TODU, H., AKABORI, S., J. Biochem., 53, 102, 1963.

16. BARKER, S.A., FLEETWOOD, J.G., J. Chem. Soc., 4857, 1957.

17. PHILLIPS, L.L., CALDWELL, M.L., J. Am. Chem. Soc., 73, 3559, 1951.

18. WATANABE, K., FUKIMBARA, T., J. Ferment. Technol., 43, 864, 1965.

19. RUTTLOFF, H., Ann. Technol. agric., 21, 287, 1972.

20. TSUBOI, A., YAMASAKI, Y., SUZUKI, Y., Agr. Biol. Chem., 38, 543, 1974.

21. TSUBOI, A., YAMASAKI, Y., SUZUKI, Y., Abstract of papers, Annual meeting of Agr. Chem. Soc. Japan, Tokyo, April, 1, 1973.

22. FUKUI, T., NIKUNI, Z., Agr. Biol. Chem., 33, 884, 1969.

23. DURMISHIDZE, S.V., KVESITADZE, G.I., KOKONASHVILI, G.N., NUTSUBIDZE, N.N.,Soobschch. Akad. Nauk. Gruz. S.S.R. 69, 461, 1973.

24. RYZHAKOVA, V.G., FENIKSOVA, R.V., Biokhimiya., 37, 1019, 1972.

25. PARK, K.Y., DE-LIMA, D.C., Rev. Brasil. Technol., 3, 67, 1972. Chem. Abs., 78 54844g.

26. QURESHI, M.Y., Ph. D. Thesis Univ. London, 1967.

27. MACREA, J.C., Planta, 96, 101, 1971.

28. PAZUR, J.H., OKADA, S., Carbohyd. Res., 4, 371, 1967.

29. Mc.GEENEY, K.F., O'DONNEL, D., Biochem. J., 125, 115p, 1971.

30. KING, N.J., Biochem. J., 105, 577, 1967.

31. EBERTOVA, H., Folia Microbiol. (Prague), 11, 422, 1966.

32. KELLY, J.J., ALPERS, D.H., Biochim. Biophys. Acta., 315, 113, 1973.

33. MORITA, Y., SHIMIZU, K., OHGA, M., KORENAGA, T., Agr. Biol. Chem., 30, 114, 1966.

34. OHGA, M., SHIMIZU, K., MORITA, Y., Agr. Biol. Chem., 30, 967, 1966.

35. MARSHALL, J.J., Anal. Biochem., 37, 466, 1970.

36. KLEIN, B., FOREMAN, J.A., SEARCY, R.L., Anal. Biochem., 31, 412, 1969.

37. EWEN, L.M., Clinica. Chimica. Acta., 47, 233, 1973.

38. BARKER, S.A., BOURNE, E.J., FLEETWOOD, J.G., J. Chem. Soc., 4865, 1957.

39. LARNER, J., GILLESPIE, R.E., J. Biol. Chem., 223, 709, 1956.

40. LINEBACK, D.R., GEORGI, C.E., DOTY, R.L., J. Gen. Appl. Microbiol., 12, 27, 1966.

41. KERR, R.W., U.S. Patent 2, 967, 804.

42. SCHOCH, T.J., In "Methods in Enxymology", Vol. 3, p 5-17, (COLOWICK, S.P., KAPLAN, N.O., Eds.,) Academic Press, N.Y., 1957.

43. LANSKY, S., KOOI, M., SCHOCH, T.J., J. Am. Chem. Soc., 71, 4066, 1949.

44. BOURNE, E.J., HAWORTH, W.N., MACEY, A., PEAT, S., J. Chem. Soc., 924, 1948.

45. GILBERT, G.A., SPRAGG, S.P., In "Methods in Carbohyd. Chem", Vol. 4, 168, 1964.

46. GILBERT, L.M., GILBERT, G.A., SPRAGG, S.P., In "Methods in Carbohyd. Chem., Vol. 4, 25, 1964.

47. HASSID, W.Z., Mc.CREADY, R.M., J. Am. Chem. Soc., 65, 1154, 1943.

48. Biochemists Handbook, (LONG, C. Ed.,) p 28-41, Spon E. & F.N., London, 1961.

49. LAYNE, E., In "Methods in Enzymology", Vol. 3, p.447-454, (COLOWICK, S.P., KAPLIN, N.O.,.Eds.,)Academic Press, N.Y. 1957.

50. LLOYD, J.B., WHELAN, W.J., Anal. Biochem., 30, 467, 1969.

51. ROBYT, J.F., WHELAN, W.J., In "Starch and its derivatives", (RADLEY, J.A., Ed.) p.431. Chapman and Hall, 1968.

52. WEBB, E.C., Nature, 203, 821, 1964.

CHAPTER 3

THE PHYSICAL PROPERTIES AND TESTS FOR HOMOGENEITY OF PURIFIED GLUCOAMYLASES

3.1 Introduction

It has been usual practice, having purified a crude glucoamylase enzyme as described in CHAPTER 2 of this Thesis, to test each form for homogeneity, and report their physical properties.[1-15] It is of vital importance in modern enzyme work to establish as far as possible the degree of purity of the enzyme preparation. In the case of glycoproteins, of which glucoamylase is one,[8,11,16-18] this may be difficult to achieve completely because of possible microheterogeneity occurring within each enzyme form.[49] This problem may be partially overcome in the future with the application of isoelectric focussing to the purification of glycoproteins.

In this work, three techniques have been used to examine glucoamylases I and II for homogeneity. These were ultracentrifugation, column chromatography and gel-electrophoresis. Each technique will be considered separately and the results compared with that of previous workers. In addition to yielding information on the purity of glucoamylases I and II these techniques also provide information on the physical properties of the enzymes. The extinction coefficients for glucoamylase I and II were also measured and the results reported.

3.2 Results and Discussion

3.2.1 Ultracentrifugation - Sedimentation Velocity

Both, sedimentation velocity and high speed equilibrium experiments were carried out on glucoamylase I and II and photographs obtained from each method.

From the schlieren sedimentation velocity photographs, PLATE 3.1, it could be seen that the single peaks obtained for both glucoamylase I and II were essentially symmetrical about the vertical axis. This indicated that glucoamylase I and II did not contain any substantial amount of material having either a higher or lower molecular weight than itself. However, the appearance of a single sharp peak did not indicate necessarily a single homogeneous protein.

Other possibilities were as follows:

(a) Two proteins with very similar sedimentation coefficients.

(b) A major component of protein together with a minor component having a not too dissimilar sedimentation value(s).

(c) A polydisperse solute with a range of sedimentation coefficients having pronounced concentration dependence.

(d) Several solutes interacting strongly and rapidly with each other.

However, using experimental results obtained from column chromatography which will be described later (3.2.3), it was considered that the above possibilities probably did not account for the single peaks obtained for glucoamylase I and II.

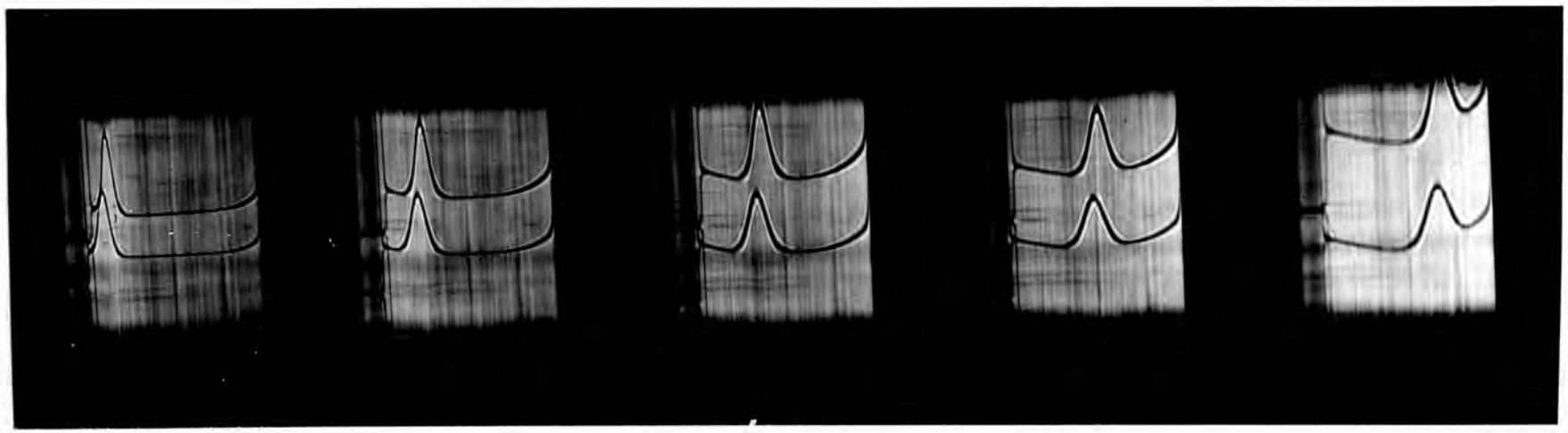
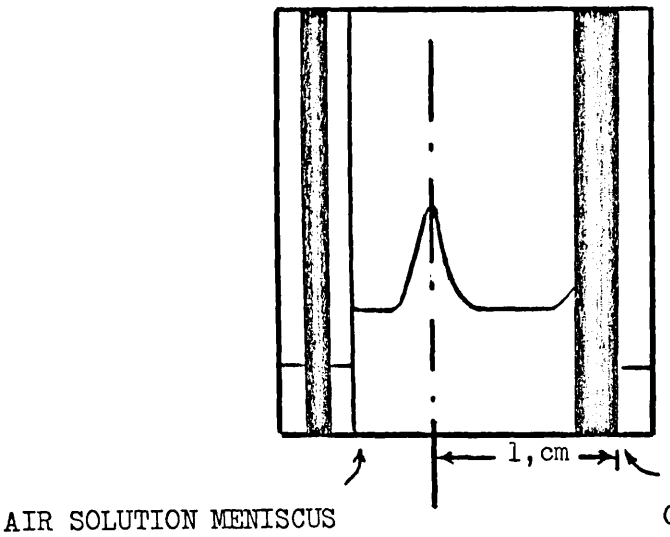


Plate 3.1 Ultracentrifugation of glucoamylase I (upper trace) and glucoamylase II (lower trace) at 59780 rpm. Schlieren photographs taken after 29, 52, 78, 104 and 134 minutes from start.

3.2.2 Measurement of the sedimentation coefficient S.

Photographic plates similar to those shown in PLATE 3.1 were measured using a travelling microscope and the distance (l, cm) of the peak ordinate from the outer reference edge recorded for each frame - see FIGURE 3.1 and TABLE 3.1.

Diagram of Schlieren ultracentrifuge sedimentation velocity plate



SINGLE SECTOR CELL

FIGURE 3.1

From the formula $x = 7.3 - \frac{l}{m}$

where 7.3 is the rotor radius in centimeters,
m is the magnification factor = 2.224,
l is the distance moved by the peak (cm),
x is the distance of the boundary from the centre,

x values were calculated and given in TABLE 3.1.

TABLE 3.1

Table of results used for calculation of sedimentation coefficients of glucoamylase I and II

GLUCOAMYLASE I	SPEED SETTING 52,640 r.p.m.		GLUCOAMYLASE II	SPEED SETTING 59,780 r.p.m.	
x value/cm	$\log_{10}x$	TIME IN SECONDS OF PHOTOGRAPH MEASURED AFTER REACHING SPEED	x value/cm	$\log_{10}x$	TIME IN SECONDS OF PHOTOGRAPH MEASURED AFTER REACHING SPEED
6.4190	0.8075	2070	6.2434	0.7954	2520
6.5725	0.8178	3750	6.4455	0.8093	4230
6.7349	0.8283	5700	6.6816	0.8249	6420
6.8738	0.8373	7260	6.8443	0.8354	7920
7.0163	0.8461	8910	6.9877	0.8444	9660

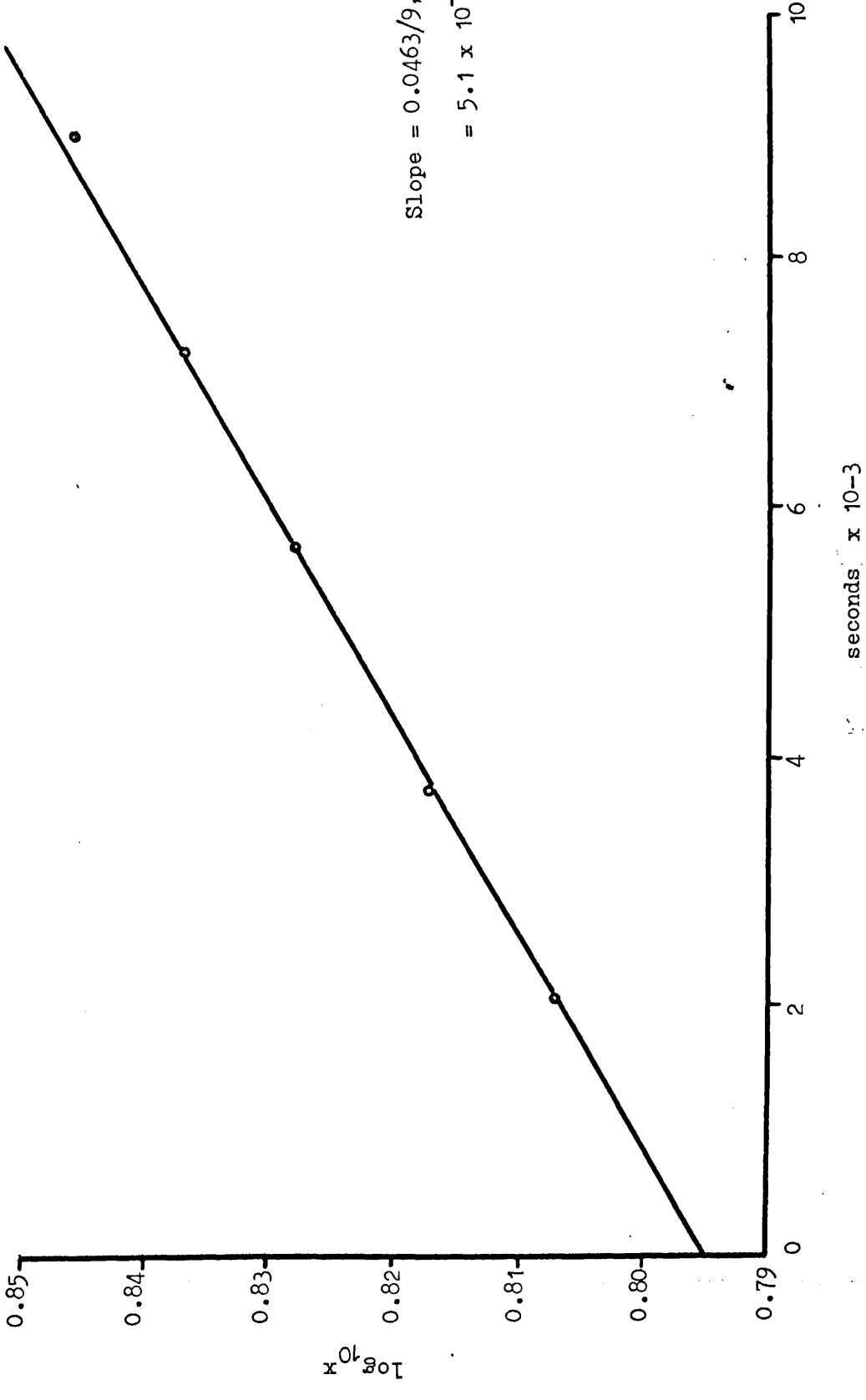


FIGURE 3.2 Plot of $\log_{10}x$ against time for glucoamylase I.

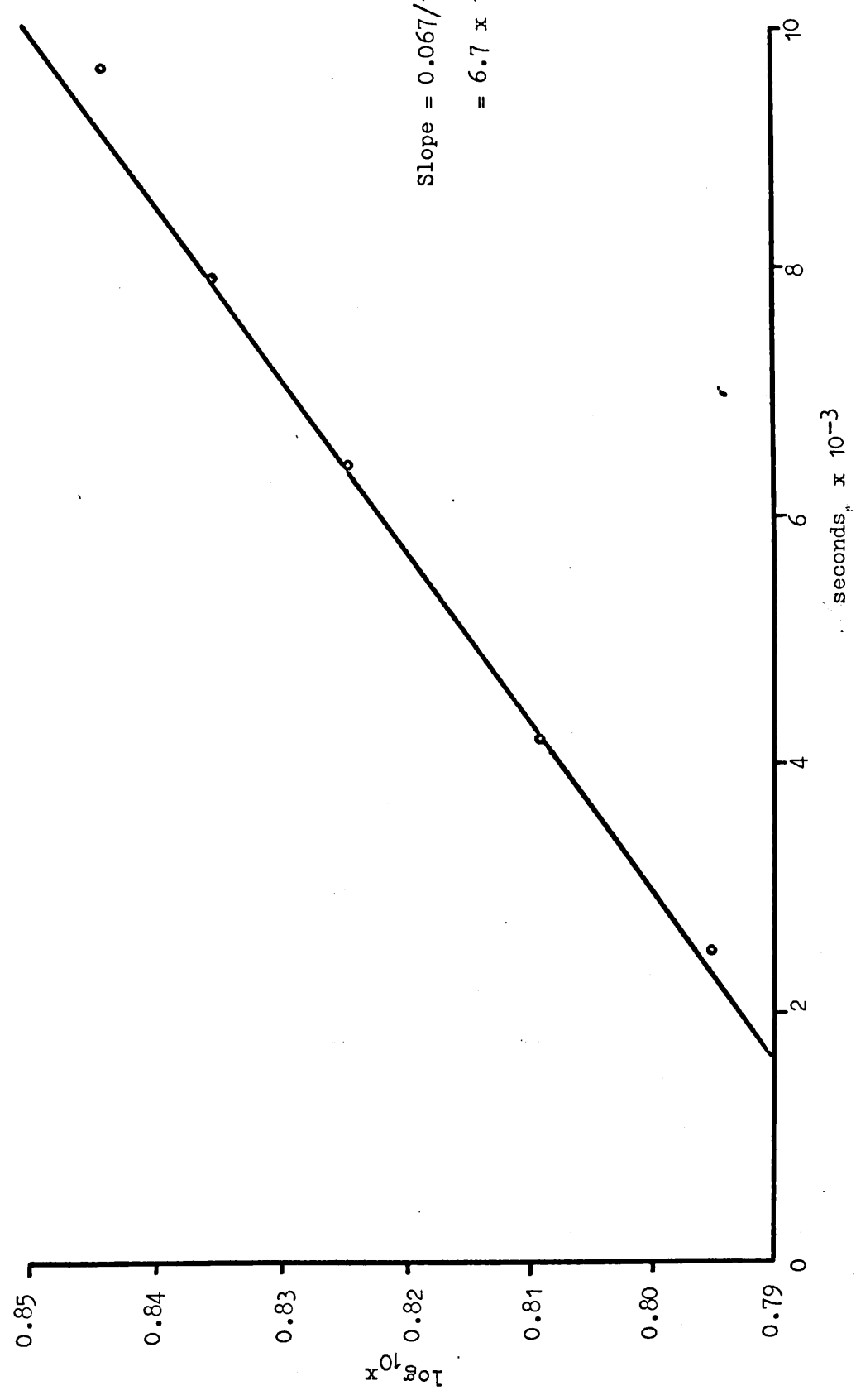


FIGURE 3.3 Plot of $\log_{10}x$ against time for glucoamylase II.

From the figures obtained a graph of $\log_{10}x$ against time in seconds was plotted, FIGURES 3.2 and 3.3, and the slopes used to calculate S using the formula

$$S = \frac{2.303 \, \delta \log_{10} x}{w^2 \, \delta t}$$

where w is the angular velocity in radians per second.

The values obtained were as follows:

Glucoamylase I: S = 3.87 Svedbergs = 3.87×10^{-13} s rad^{-2}

Glucoamylase II: S = 3.94 Svedbergs = 3.94×10^{-13} s rad^{-2}

These values were then corrected to standard conditions using the equation:

$$S_{20^\circ,w} = S \frac{n}{n_{20^\circ,w}} \quad \frac{(1-\bar{v})}{(1-\bar{v}\rho)} \, 20^\circ,w$$

where n = viscosity of the buffer in millipoise at 20°C.

$n_{20^\circ,w}$ = viscosity of water in millipoise at 20°C.

ρ = density of buffer at 20°C.

$\bar{v}$ = partial specific volume of the enzyme.

$\rho_{20^\circ,w}$ = density of water at 20°C.

$\bar{v}$ was calculated using specific volume values of each amino acid[19,20,22] and the values obtained from the amino acid analyses in CHAPTER 4 of this Thesis. The value was assumed to be 0.7157 and no correction was made for the possible existence of amide nitrogen from glutamine or asparagine. Kelly et al.[48] used a value of 0.684 for Human intestinal glucoamylase.

ρ was found by experiment using a specific gravity bottle.

n was found by experiment using a viscometer.

$n_{20^\circ,w}$ and $\rho_{20^\circ,w}$ were found in The International critical Tables.

The corrected S values for glucoamylsse I and II were:

Glucoamylase I: $S_{20^\circ,w} = \frac{(3.87)(10.59)(0.2855)}{(10.08)(0.2699)} = 4.30$ Svedbergs.

Glucoamylase II: $S_{20^\circ,w} = \frac{(3.94)(10.59)(0.2855)}{(10.08)(0.2699)} = 4.38$ Svedbergs.

These results agree well with those obtained by other workers - see TABLE 3.2.

TABLE 3.2

The sedimentation coefficients obtained for glucoamylase

GLUCOAMYLASE TYPE AND SOURCE		SEDIMENTATION COEFFICIENT (S) SVEDBERGS	REFERENCE
GLUCOAMYLASE I	*A. niger*	4.30 $S_{20,w}$	THIS WORK
GLUCOAMYLASE II	*A. niger*	4.38 $S_{20,w}$	THIS WORK
GLUCOAMYLASE I	*Mucor rouxianus*	4.39 $S_{20,w}$	7
GLUCOAMYLASE II	*Mucor rouxianus*	4.29 $S_{20,w}$	7
GLUCOAMYLASE	*Endomyces* species IFO 0111	4.37 $S_{20,w}$	6
GLUCOAMYLASE	*A. awamori*	4.65	4
GLUCOAMYLASE	*A. awamori*	4.19	9

Using the high speed sedimentation equilibrium method described by Yphantis,[21] Rayleight interference fringes produced on photographic plates were measured for glucoamylase I and II using a travelling microscope. A graph of $\log_{10}$ of the fringe displacement against the square of the actual distance from the rotor centre was plotted, and the

value of the slope substituted into the equation:

$$\bar{M}w = \frac{2.3\ (2RT)}{(1-\bar{\upsilon}\rho)w^2} \quad \frac{\partial \log_{10} C}{\partial x^2}$$

where $\bar{M}w$ is the average molecular weight,

R is the gas constant in ergs/mole/°K,

T is the absolute temperature,

C is the concentration and is proportional to the displacement distance of the Rayleigh interference fringes.

Other symbols as previously defined.

The experiment was carried out at 0.01% and 0.10% concentrations of glucoamylase I and II. The results obtained from 0.01% glucoamylase solutions together with those of other workers are given in TABLE 3.3 Similar molecular weight determinations were carried out using 0.1% and 0.01% solutions of Bovine Serum Albumin and Ovalbumin, the results of which are shown in TABLE 3.4. The values were found to be higher than those reported in the literature and this was attributed to protein aggregation. A molecular weight value of 80,000 has been used throughout this work for both glucoamylase I and II. This value lay between the experimental limits of error, but no correction was made which allowed for possible aggregation, as the extent of this for glucoamylase was unknown. These molecular weight determinations were carried out as part of an undergraduate project.[22]

Several detailed reviews and books have been written on the subject of ultracentrifugation and the reader is referred to these for the derivation of equations which have been used, and an explanation of the theory.[24-27]

TABLE 3.3

Molecular weights of glucoamylases

GLUCOAMYLASE AND SOURCE	METHOD OF MOLECULAR WEIGHT DETERMINATION	MOLECULAR WEIGHT	REFERENCE
GLUCOAMYLASE I Aspergillus niger	Ultracentrifugation. High speed equilibrium	80,000 ± 2,500	
	"Sephadex" G-200 gel chromatography	~72,000	THIS
GLUCOAMYLASE II Aspergillus niger	Ultracentrifugation. High speed equilibrium	82,500 ± 2,000	WORK
	"Sephadex" G-200 gel chromatography	~72,000	
GLUCOAMYLASE I Mucor rouxianus	Sodium dodecyl sulphatepolyacrylamide gel electrophoresis (41)	59,000	7
GLUCOAMYLASE II Mucor rouxianus	"	49,000	7
GLUCOAMYLASE Aspergillus awamori	Possibly ultracentrifugation	64,000	4
GLUCOAMYLASE I Aspergillus niger	Not stated	61,500	Lineback D.R. cited in 42
GLUCOAMYLASE I Aspergillus awamori	Bio-gel P-100 chromatography	71,600	10
GLUCOAMYLASE II Aspergillus awamori	"	57,500	
GLUCOAMYLASE I Aspergillus awamori	Ultracentrifugation - Archibald method of sedimentation equilibrium	74,900	Lineback D.R. cited in 10
GLUCOAMYLASE II Aspergillus awamori	"	54,300	
GLUCOAMYLASE Dog serum	Bio-Gel P-300 chromatography and "Sephadex" G-200 gel chromatography	180,000	5
GLUCOAMYLASE I Aspergillus niger	Density gradient ultracentrifugation	99,000	3
GLUCOAMYLASE II Aspergillus niger	"	112,000	

TABLE 3.3 (continued)

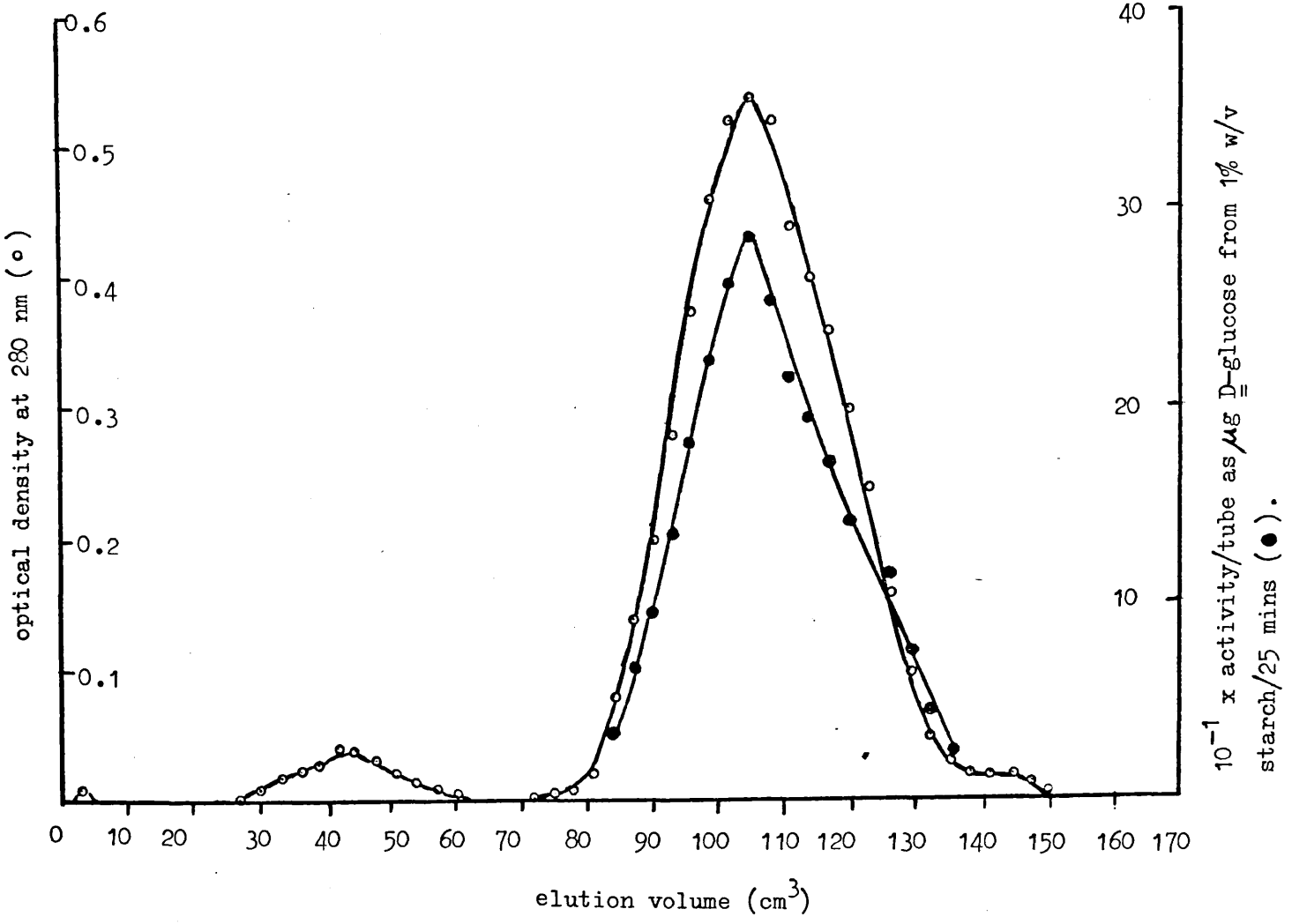
GLUCOAMYLASE AND SOURCE	METHOD OF MOLECULAR WEIGHT DETERMINATION	MOLECULAR WEIGHT	REFERENCE
GLUCOAMYLASE A Aspergillus phoenicis	Sedimentation equilibrium ultracentrifugation	63,600 ± 6,800	8
GLUCOAMYLASE A Aspergillus phoenicis	"Sephadex" G-100 gel chromatography	62,000	
GLUCOAMYLASE Endomyces species IFO 0111	Ultracentrifugation, Archibald method	55,000	6
GLUCOAMYLASE I Aspergillus niger	Ultracentrifugation, Archibald equilibrium method	74,900 ± 1,550	2
GLUCOAMYLASE II Aspergillus niger	"	54,300 ± 6,690	
GLUCOAMYLASE I Aspergillus niger	Bio-Gel P-100 chromatography	70,000	2
GLUCOAMYLASE II Aspergillus niger	"	59,000	
GLUCOAMYLASE I Aspergillus niger	Ultracentrifugation - from sedimentation and diffusion coefficients	97,000	43
GLUCOAMYLASE I Aspergillus niger	Ultracentrifugation - density gradient	110,000	44
GLUCOAMYLASE R. delemar	Ultracentrifugation - sedimentation and diffusion measurements	100,000 100,000	11 cited in 45
GLUCOAMYLASE I Endomycopsis bispora	"Sephadex" G-200 gel filtration	47,800	47
GLUCOAMYLASE II Endomycopsis bispora	"Sephadex" G-200 gel filtration	50,100	
GLUCOAMYLASE Human intestine	Ultracentrifugation - equilibrium sedimentation	210,000	48

TABLE 3.4

Experimental and literature molecular weight values obtained for protein solutions used in this work.

PROTEIN	CONCENTRATION PER CENT	MOLECULAR WEIGHT EXPERIMENTAL	LITERATURE	REFERENCE
BOVINE SERUM ALBUMIN	0.01	79,500 ±2,000	69,600	25
	0.10	79,000 ±2,500		
OVALBUMIN	0.01	54,000 ±1,500	45,000	28
	0.10	55,500 ±3,500		
GLUCOAMYLASE I	0.01	80,000 ±2,500		THIS WORK
	0.10	82,500 ±5,000		
GLUCOAMYLASE II	0.01	82,500 ±2,000		THIS WORK
	0.10	80,000 ±2,000		

FIGURE 3.4. Chromatography of purified glucoamylase I on G-200 "Sephadex" at pH 5.15.



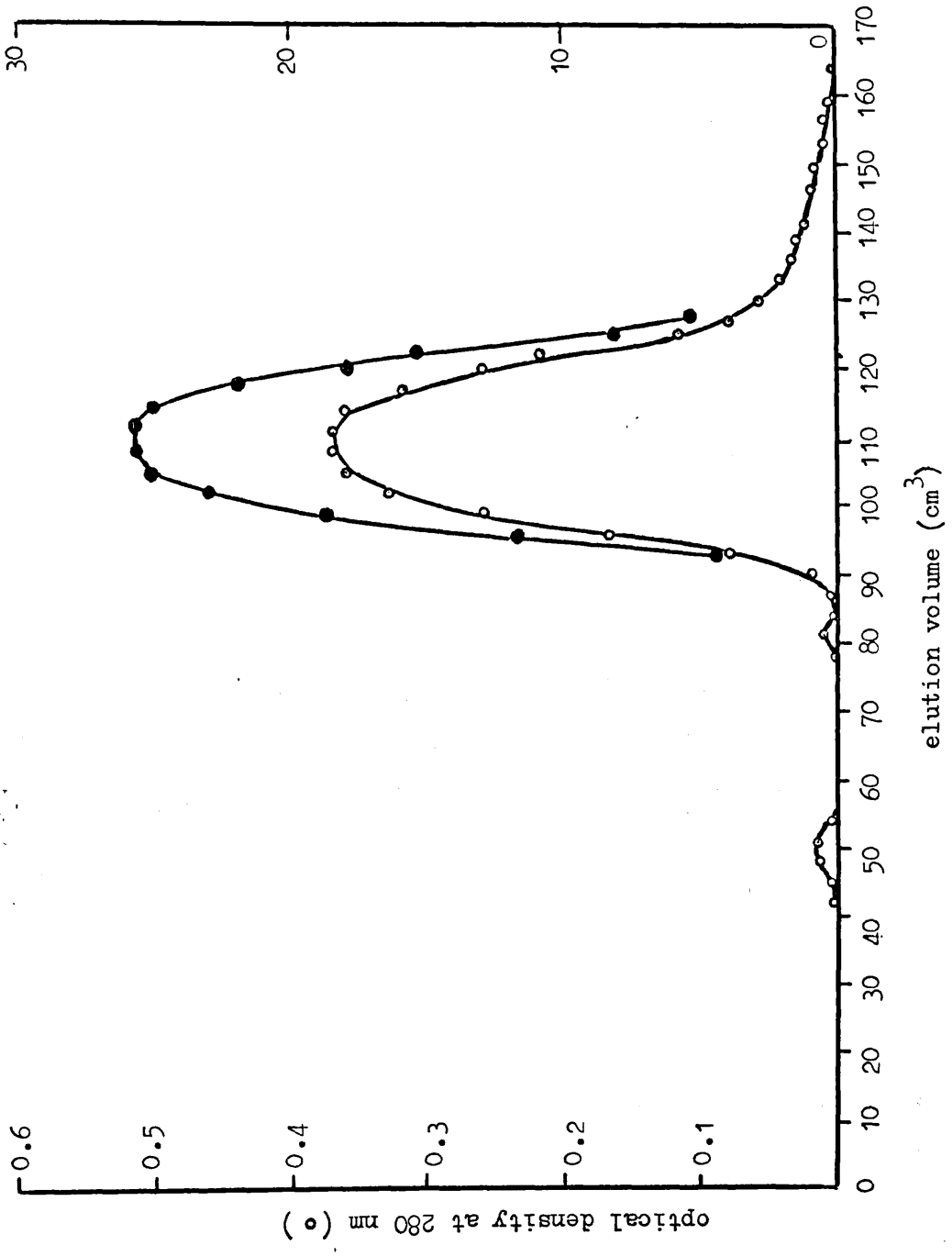


FIGURE 3.5. Chromatography of purified glucoamylase II on G-200 "Sephadex" at pH 5.15.

3.23 Gel-permeation column chromatography

A gel-permeation column using "Sephadex" G-200 in pH 5.15, 0.05M citrate buffer[23] was calibrated using "Blue Dextran 2000", * Bovine Serum albumin (BSA), and DL-tyrosine. Glucoamylase I and II were then passed down the column and from the K_{av} value, (partition coefficient between the liquid and gel phase) a molecular weight obtained for each form.[29,30] From calculation of molecular weight using elution volume it was observed that the value obtained for BSA was much lower than reported in the literature.[29,30]

Glucoamylase I and II was eluted before BSA indicating that the enzymes had a higher molecular weight. Using a literature value for BSA[29,30] of between 68-70 x 10^3, the glucoamylase I and II would have corrected values of approximately 72 x 10^3.

From the peaks obtained (FIGURES 3.4 and 3.5) from glucoamylase I and II on "Sephadex" G-200 gel-permeation chromatography it could be seen that both enzyme forms had almost equal elution volumes (between 106-110 cm^3). This was further evidence to suggest that both enzyme forms had similar molecular size. The elution profiles of each glucoamylase peak were symmetrical. This suggested that each purified enzyme form was essentially homogeneous with respect to molecular size.

Protein content and glucoamylase activity were measured for each eluted peak as described in CHAPTER 2 of this Thesis. Both contours, shown in FIGURES 3.4 and 3.5, followed the optical density measurement at 280 nm for each glucoamylase form. Greater than 92% protein recovery was obtained from the gel-permeation chromatography.

*"Blue Dextran 2000" is a high molecular weight dye - Pharmacia Fine chemicals.

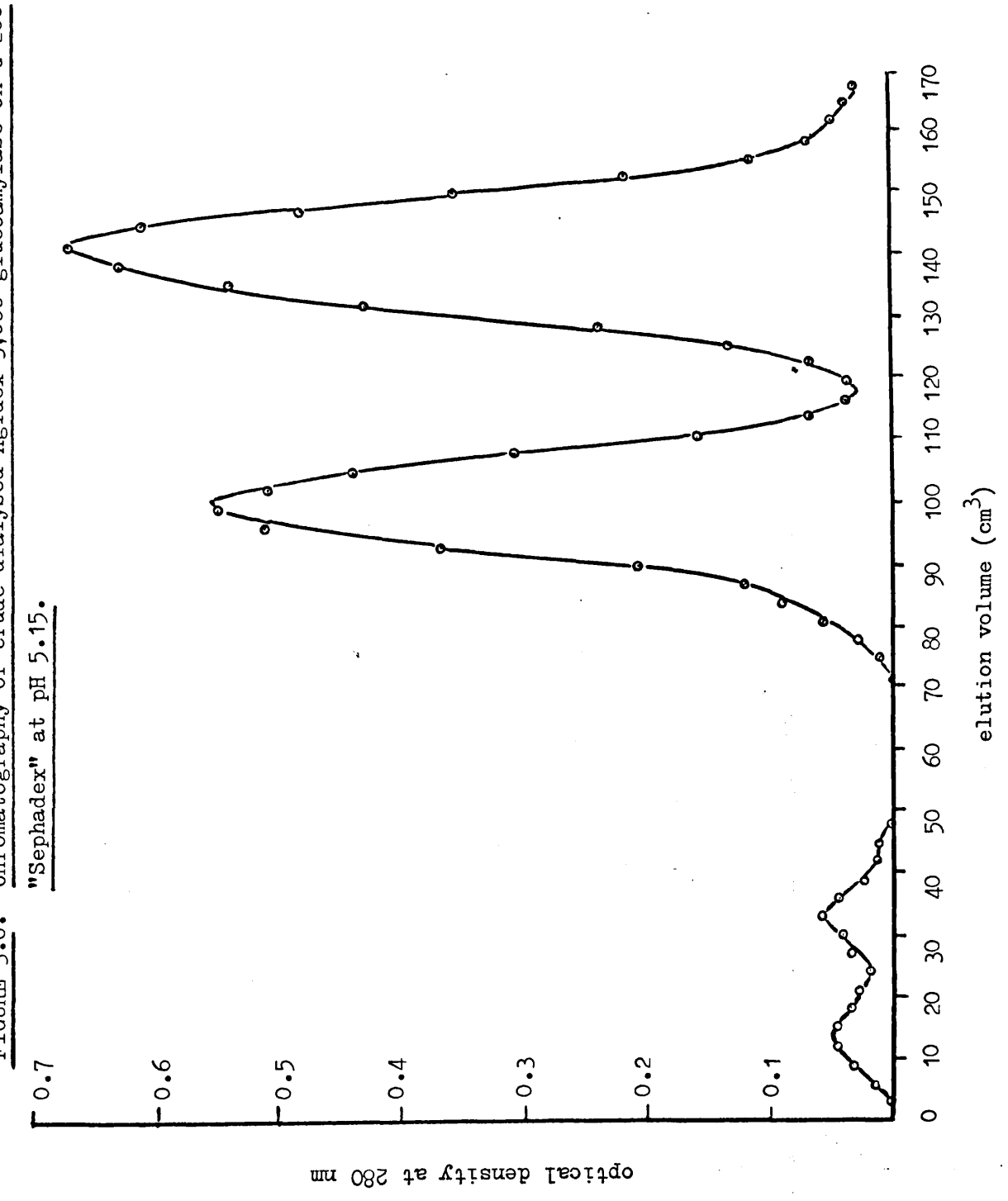


FIGURE 3.6. Chromatography of crude dialysed Agidex 3,000 glucoamylase on G-200 "Sephadex" at pH 5.15.

FIGURE 3.7. Small scale chromatography of glucoamylase I on D.E.A.E. cellulose using 0-0.3M NaCl gradient for elution in 0.05M phosphate buffer pH 7.7.

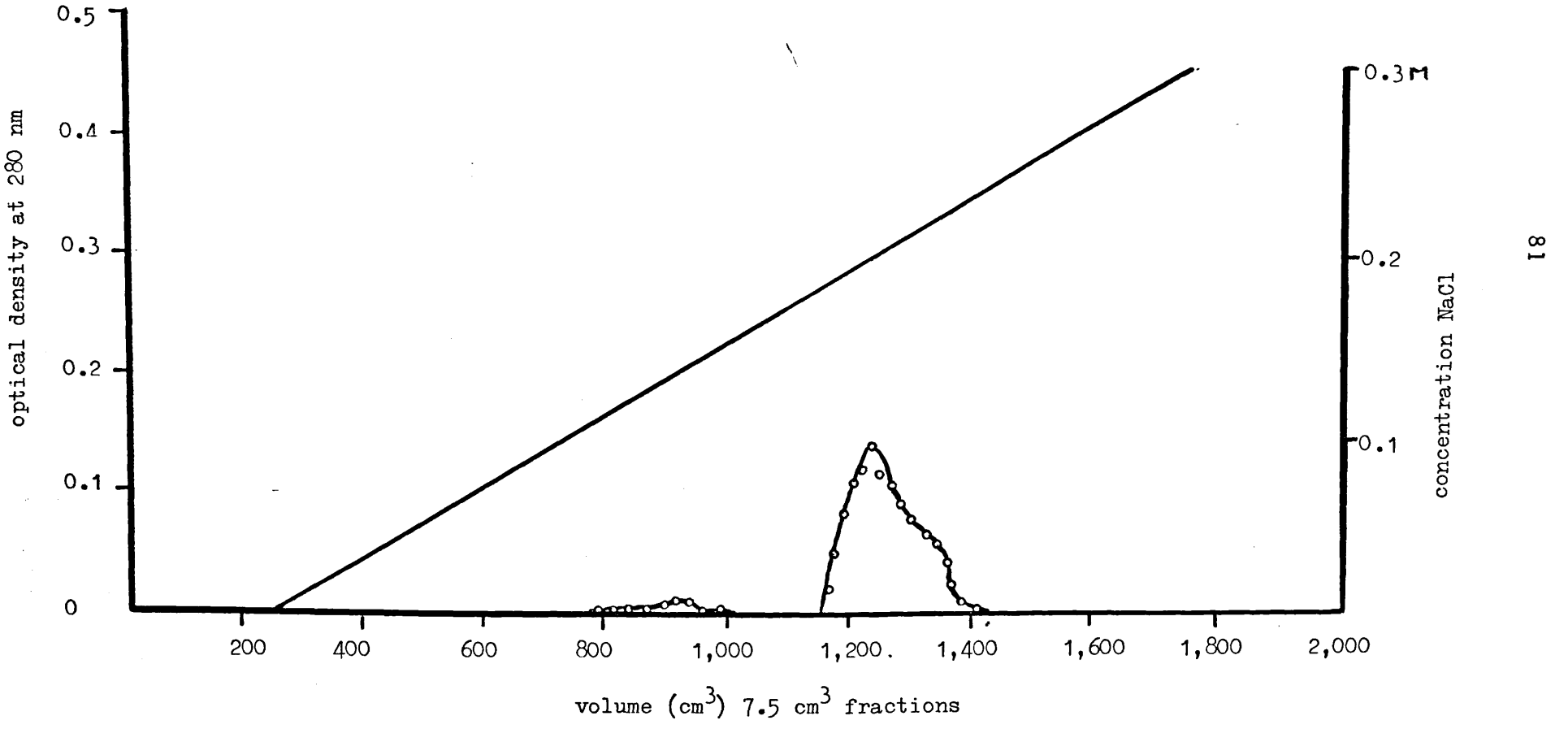
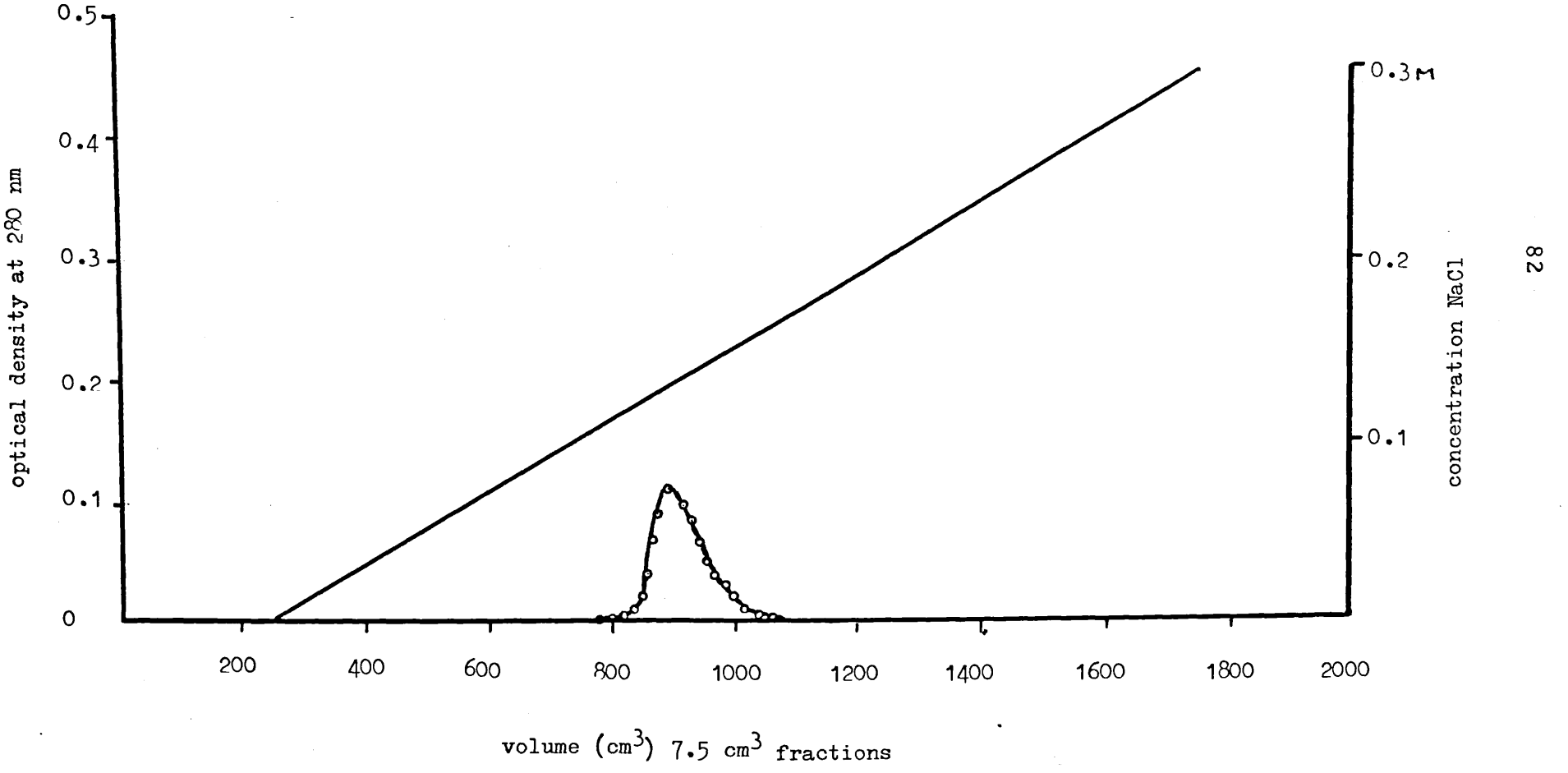


FIGURE 3.8. Small scale chromatography of glucoamylase II on D.E.A.E. cellulose using 0-0.3M NaCl gradient for elution in 0.05M phosphate buffer pH 7.7.



A sample of crude dialysed "Agidex 3,000" glucoamylase was subjected to "Sephadex" G-200 gel-permeation chromatography. This produced two peaks, FIGURE 3.6, one of which occurred in an elution volume of 100 cm^3 and showed glucoamylase activity while the second occurring at an elution volume of 141 cm^3 showed no glucoamylase activity.

3.2.4 D.E.A.E. anion-exchange chromatography

After the freeze-dried glucoamylase I and II had been stored for about three months, a sample from each form was subjected to D.E.A.E. anion exchange chromatography. The purpose of this experiment was to determine whether any interconversion of the two forms had occurred during this period. The experimental conditions were essentially those used for the small scale fractionation of "Agidex 3,000" described in CHAPTER 2 of this Thesis. The results are shown in FIGURES 3.7 and 3.8. It was observed during the salt gradient elution that glucoamylase I contained a trace amount of glucoamylase II; while glucoamylase II did not contain any glucoamylase I. It was concluded that no major interconversion of the two glucoamylase forms had occurred during storage, and the trace amount of glucoamylase II which was present in glucoamylase I had probably resulted from a slight overlap of the peaks from the large scale purification.

3.2.5 Disc-gel electrophoresis

In order to achieve satisfactory results from this technique, the polyacrylamide gel concentration, buffer pH, and buffer composition were varied until optimum conditions were found giving a partial resolution without severe band broadening, in a reasonable time. These conditions were found to be 7.5% acrylamide, in TRIS/HCl buffer, pH 8.9. From the

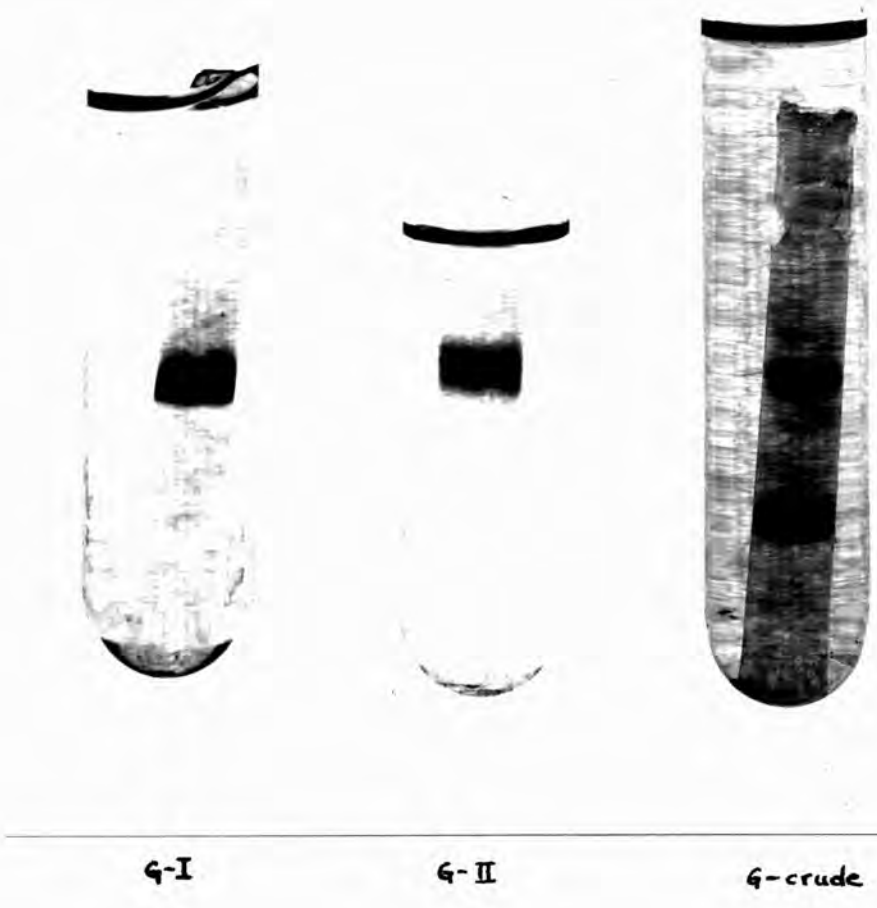


Plate 3.2 Electrophoresis of glucoamylase I (G–I), glucoamylase II (G–II) and crude glucoamylase (G–crude) in 7.5% polyacrylamide gel at pH 8.9 (TRIS/HCl) and 5mA per tube.

gels, PLATE 3.2, it was observed that the two forms of glucoamylase ran at essentially the same rate and that trace impurities were still present in the purified preparations although some may have been from denatured glucoamylase produced during the experiment. The crude dialysed "Agidex 3,000" glucoamylase sample showed two distinct bands one of which corresponded to the purified glucoamylase position. From the "Sephadex" G-200 gel-permeation experiment described earlier it could be seen that the crude enzyme comprised two major fractions one of which corresponded to that obtained from the purified glucoamylase enzyme forms with respect to molecular size. The second fraction eluted from the gel column was of small molecular weight and moved faster on gel-electrophoresis.

These findings agree with those of Lineback et al.,[1] Jolley,[15] and Fukui et al.,[6] the latter workers using glucoamylase from Endomyces species IFO 0111. The buffer systems were formulated according to Ornstein,[33] and Williams,[31] other useful buffer and gel systems have also been described by Maurer et al.[32] An apparatus similar to that described by Davies[34] was used for the experiments. It may be possible to completely resolve glucoamylase I and II using a buffer system of lower pH as described by Orr et al.[35]

Paper electrophoresis has been carried out by Okazaki,[36] using glucoamylase from Aspergillus oryzae, Rodzevich et al.,[37] using glucoamylase from Aspergillus awamori, and by Pazur et al.,[3] using glucoamylase from Aspergillus niger, the latter workers resolving both glucoamylase forms. It is interesting to note that although glucoamylase from Aspergillus oryzae and Aspergillus awamori have been reported by

Morita et al.,[39] and Watanabe et al.,[38] respectively to exist in two forms, only one form was found by Okazaki[36] and Rodzevich et al.,[37] using paper electrophoresis.

3.2.6 Extinction Coefficient

When the protein determinations were carried out on glucoamylase using the method of Layne[40] described in CHAPTER 2 of this Thesis, the concentrations of protein were related to a standard of BSA. If the colourimetric response of the test differed for BSA and glucoamylase, then an erroneous protein concentration would be obtained for the latter. In order to overcome this problem pistol dried and desiccated samples of glucoamylase I and II were dissolved in buffer and their optical densities at 280 nm recorded.

From these, the extinction coefficients could be obtained using the Beer/Lambert equation:-

$$\text{Optical Density} = \log_{10}(Io)/(I) = \varepsilon.l.c.$$

where I_o and I are the intensities of the incident and transmitted light respectively at 280 nm for proteins,
l is the length of the absorbing solution (cm),
c is the concentration in moles/litre,
ε is the extinction coefficient.

Hence, by measuring the optical density of a glucoamylase solution at 280 nm an exact protein concentration could be obtained.

Using a value of 80,000 for the molecular weight of glucoamylase I and II, the extinction coefficients were calculated to be 134,200 l $mol^{-1}cm^{-1}$ and 124,100 l $mol^{-1}cm^{-1}$, respectively.

The extinction coefficients ($E^{1\%}_{1\ cm}$) have also been calculated for a 1% concentration of glucoamylase I and II. These were 16.777 and 15.505 respectively. These values overcome the uncertainty in molecular weight which arises when unsequenced proteins are being studied.

3.3 Experimental

3.3.1 Ultracentrifugation

Sedimentation velocity

1% w/v solutions of glucoamylase I and II were made up using citrate buffer[46] (0.05M, pH 5.15). A single sector ultracentrifuge cell was made up according to the instructions supplied with the ultracentrifuge. The cell was filled with one of the glucoamylase solutions and placed in the AN-D ultracentrifuge rotor together with a counterbalance of the correct weight. The rotor was then placed in the Beckman model E ultracentrifuge and accelerated to 59,780 r.p.m. When this speed had been reached, photographs were taken using Ilford G30 chromatic plates, 15 second exposure, at the times given in PLATE 3.1 and the Schlieren angle recorded. The temperature was maintained at 20°C throughout the experiment. At the end of the ultracentrifugation, the glucoamylase solution was recovered, dialysed against deionised water and freeze-dried. The cell was then cleaned and dried before repeating the experiment with the other glucoamylase solution. The photographic plates after development in Contrast FF, diluted 18:1 with water, were measured as described.

High speed sedimentation equilibrium

0.1% and 0.01% solutions of glucoamylase I and II were made up using citrate buffer[46] (0.05M, pH 5.15) and were then separately dialysed against the buffer solution. 0.1% and 0.01% solutions of ovalbumin and BSA were made up using acetate buffer containing 0.2M sodium chloride and left to dialyse for 24 hours.

A 12 mm double sector ultracentrifuge cell was made up using an Al-Epon centrepiece according to the manufacturers instructions supplied with the ultracentrifuge. The cell was filled such that the solution

channel, the left hand side of the cell viewed from the rotor centre, contained fluoro carbon oil FC-43 (0.01 cm^3) and protein solution (0.11 cm^3). The solvent channel, right hand side, contained FC-43 (0.005 cm^3) and dialyzate buffer (0.11 cm^3). The column height was 3 mm. The cell and suitable counterbalance were inserted into the AN-D rotor and spun for 22 hours at 25,980 r.p.m. except for ovalbumin which was spun at 31,410 r.p.m. The temperature of the ultracentrifugation chamber was kept constant at 20°C throughout the experiment. The Rayleigh interference patterns obtained on Ilford G30 photographic plates after 150 seconds exposure, were measured using a travelling microscope.

3.3.2 Gel-permeation chromatography

"Sephadex" G-200 (7 g) was added to citrate buffer[46] (0.05M, pH 5.15) (200 cm^3) and placed on a boiling water bath for 5 hours and allowed to cool to room temperature. The gel-slurry was then packed in a "Pharmacia" K15/90 (1.5 x 90 cm) glass column, 154 cm^3 bed volume. The head of buffer was kept below 20 cm to prevent compression of the gel, and the column washed with buffer (200 cm^3). Blue dextran 2,000 (5 mg) was made up in citrate buffer, pH 5.15 (1.0 cm^3), and applied to the gel column. Fractions (3.0 cm^3) were collected using a Gilson Escargot fraction collector. A small sample of each fraction was withdrawn and the optical density at 600 nm automatically recorded from a Pye-Unicam SP 1,800 spectrometer. From the peak obtained the void volume of the column was calculated to be 30 cm^3. Similarly the volume of elution of DL-tyrosine (5 mg) applied in buffer (1.0 cm^3) was found to be 145 cm^3 from the optical density peak recorded at 280 nm. This represented the total volume of the gel-column available for sample

permeation. Samples (8 mg) of BSA, glucoamylase I and II and crude dialysed "Agidex 3,000" glucoamylase (1.5 cm^3) were also passed down the gel column and the elution profiles of the latter three are shown in FIGURES 3.4, 3.5 and 3.6.

3.3.3 D.E.A.E. anion exchange chromatography

A small scale ion-exchange column was prepared as described in CHAPTER 2 of this Thesis. A sample of glucoamylase I (5 mg) was dissolved in phosphate buffer (0.05M, pH 7.7) (1.0 cm^3) and was absorbed on the ion-exchange column. The column was then eluted as described in CHAPTER 2 of this Thesis. The elution profile is shown in FIGURE 3.7 The column was then unpacked and repacked with fresh ion-exchange cellulose. The procedure was repeated for a sample of glucoamylase II.

The column was then unpacked and the ion-exchange cellulose regenerated, together with that used for glucoamylase I.

3.3.4 Disc-gel electrophoresis

This was carried out essentially as described by Davis[34] using a "Shandon" small scale gel-electrophoresis apparatus. Eight "precibore" running tubes were cleaned using 5% "Decon 90" liquid detergent, rinsed with tap water followed by distilled water and dried. The following stock solutions were then made up and stored in amber glass bottles in a refrigerator, except (F) which was made just prior to use.

(A) RUNNING BUFFER (small pore gel)

1N HCl	48 cm^3
2-amino 2-hydroxymethyl-1,3-propanediol (TRIS) added until pH was 8.9	
N,N,N',N' -tetramethylethylenediamine (TEMED)	0.46 cm^3
deionised water to	100 cm^3

(B) STACKING BUFFER (large pore gel)

1N HCl	48 cm^3
TRIS added until pH was	7.3
TEMED	0.46 cm^3
deionised water to	100 cm^3

(C) ELECTRODE BUFFER

TRIS	6 g
L-glycine	28.8 g
deionised water to	1000 cm^3
Diluted 1:10 for use	550 cm^3 required per run.

(D) SMALL PORE GEL ACRYLAMIDE SOLUTION

acrylamide	30 g
N,N'-methylenebisacrylamide (BIS)	0.735 g
deionised water to	100 cm^3

(E) LARGE PORE GEL ACRYLAMIDE SOLUTION

acrylamide	10 g
N,N'-methylenebisacrylamide (BIS)	2.5 g
deionised water to	100 cm^3

(F) AMMONIUM PERSULPHATE INITIATOR
(made up just prior to a run)

ammonium persulphate	0.14 g
deionised water to	100 cm^3

(G) RIBOFLAVIN INITIATOR

riboflavin	4 mg
deionised water to	100 cm^3

(H) SUCROSE

sucrose	40 g
deionised water to	100 cm^3

(I) BROMOPHENOL BLUE

bromophenol blue	1 mg
deionised water to	100 cm^3

(J) TRICHLOROACETIC ACID

trichloroacetic acid	125 g
deionised water to	100 cm^3

(K) COOMASSIE BLUE STAIN

coomassie blue	0.5 g
deionised water to	50 cm^3

Diluted 1:20 with J before use

The working gel solutions were made up from the stock solutions in the following proportions.

Small pore gel (7.5% acrylamide)

A	6 cm^3
D	6 cm^3
F	12 cm^3

Large pore gel (2.5% acrylamide)

B	3 cm^3
E	6 cm^3
G	3 cm^3
H	12 cm^3

Each working solution was then degassed on a water pump for 2 minutes. Six running tubes were filled to within 1.5 cm of the top with small pore gel solution and layered with 0.5 cm of deionised water. After 30 minutes the water layer was removed and the large pore gel solution was layered on each running tube to within 0.5 cm of the top. The florescent lamp was then used to initiate the polymerisation of this gel. Samples of glucoamylase I and II (2 mg) were weighed out and made up in deionised water (0.1 cm^3). Glucoamylase I (0.01 cm^3) was withdrawn and added to sucrose (H) (0.2 cm^3) and mixed. This solution was then carefully layered on top of a prepared gel and the tube labelled. The process was repeated for a second gel. Two gels were prepared in a similar way for both glucoamylase II and crude dialysed glucoamylase (0.01 cm^3). The six gel tubes were inserted into the electrophoresis apparatus and electrode buffer (C) added to the upper (250 cm^3) and lower (300 cm^3) reservoirs. Bromophenol blue (I) (1.0 cm^3) was added to the upper reservoir. Constant current (5 mA/tube) was passed through each gel for

40 minutes or until the bromophenol blue marker had migrated to the end of the gel tube. The gels were then removed, fixed in 12.5% trichloroacetic acid (J) for 10 minutes and then stained with coomassie blue (K) for 30 minutes. The gels were then destained using 7% acetic acid for 12 hours.

3.3.5 Extinction coefficient

Samples of glucoamylase I and II were dried at 30°C for 12 hours in a pistol drier and then stored in a vacuum desiccator. Glucoamylase I (1.46 mg) and II (1.04 mg) were weighed out and dissolved in citrate buffer(0.05M, pH 5.15),(5.0 cm^3). The optical densities of glucoamylase I and II were then measured in 1 cm quartz cells at 280 nm using a Pye-Unicam SP 500 spectrometer.

References

1. LINEBACK, D.R., RUSSELL, I.J., RASMUSSEN, C., Arch. Biochem. Biophys., 134, 539, 1969.

2. LINEBACK, D.R., AIRA, L.A., Cereal Chem., 49, 283, 1972.

3. PAZUR, J.H., KNULL, H.R., CEPURE, A., Carbohyd. Res., 20, 83, 1971.

4. DURMISHIDZE, S.V., KVESITADZE, G.I., KOKONASHVILI, G.N., NUTSUBIDZE, N.N., Soobschch. Akad. Nauk. Gruz. SSR., 69, 461, 1973.

5. Mc.GEENEY, K.F., O'DONNELL, D., Biochem. J., 125, 115p, 1971.

6. FUKUI, T., NIKUNI, Z., Agr. Biol. Chem., 33, 884, 1969.

7. TSUBOI, A., YAMASAKI, Y., SUZUKI, Y., Agr. Biol. Chem., 38, 543, 1974.

8. LINEBACK, D.R., BAUMANN, W.E., Carbohyd. Res., 14, 341, 1970.

9. WATANABE, K., FUKIMBARA, T., J. Ferm. Technol., 44, 392, 1966.

10. SMILEY, K.L., HENSLEY, D.E., SMILEY, M.J., GASDORF, H.J., Arch. Biochem. Biophys., 144, 694, 1971.

11. PAZUR, J.H., OKADA, S., Carbohyd. Res., 4, 371, 1967.

12. PAZUR, J.H., ANDO, T., J. Biol. Chem., 234, 1966, 1959.

13. ROSENFELD, E.L., LULOMSKAYA, I.S., POPOVA, I.A., Enzymologia, 30, 1, 1966.

14. QURESHI, M.Y., Ph. D. Thesis, University of London, 1967.

15. JOLLEY, M.E., Ph. D. Thesis, University of Birmingham, 1972.

16. PAZUR, J.H., KLEPPE, K., BALL, E.M., Arch. Biochem. Biophys., 103, 515, 1963.

17. FLEMING, I.D., STONE, B.A., Biochem. J., 97, 13p, 1965.

18. OGHA, M., SHIMIZU, K., MORITA, Y., Agr. Biol. Chem., 30, 967, 1966.

19. COHN, E.J., EDSALL, J.T., In "Proteins, Amino Acids and Peptides" p.375. Elsivier Pub. Co., Amsterdam, 1946.

20. Cited in In "Methods in Enzymology", Vol. 4, p.70.

21. YPHANTIS, D.A., Biochemistry, 3, 297, 1964.

22. KIDWELL, P.F., GILL, E.B., Unpublished work.

23. In "Biochemists Handbook" (LONG C., Ed) p.28-41, Spon E. & F.N. London, 1961.

24. COATES, J.H. In "The physical principles and techniques of Protein chemistry", Part B. Academic Press, N.Y. 1970.

25. BOWEN, C., "Ultracentrifugation"

26. SCHACHMAN, H.K., "Ultracentrifugation in Biochemistry" Academic Press, N.Y. 1959.

27. ADAMS, E.T., Jr., FUJITA, H., In "Ultracentrifugal Analysis in Theory and Experiment", (WILLIAMS, J.W., Ed.,), Academic Press, N.Y. 1963.

28. TANFORD, C., "The Physical Chemistry of Macromolecules", Wiley, New York, 1961.

29. "Sephadex" - Gel filtration in theory and practice, Pharmacia Fine Chemicals Publication, 1973.

30. ANDREWS, P., Methods of Biochemical Analysis, 18, 1-53, 1970.

31. WILLIAMS, D.E., RESIFELD, R.A., Ann. N.Y. Acad. Sci., 121, 373, 1964.

32. MARUER, H.R., ALLEN, R.C., Z. clin. Chem. & clin. Biochem., 10, 220, 1972.

33. ORNSTEIN, L., Ann. N.Y. Acad. Sci., 121, 321, 1964.

34. DAVIS, B.J., Ann. N.Y. Acad. Sci., 121, 404, 1964.

35. ORR, M.D., BLAKLEY, R.L., PANAGOU, D., Anal. Biochem., 45, 68. 1972.

36. OKAZAKI, H., J. Agr. Chem. Soc. Japan, 29, 273, 1955.

37. RODZEVICH, B.I., DOBROLINSKAYA, G.M., Fermentnya. i. spirt. prom., 32, 5, 1966.

38. WATANABE, K., FUKIMBARA, T., J. Ferm. Technol., 43, 690, 1965.

39. MORITA, Y., SHIMIZU, K., OHGA, M., KORENAGA, T., Agr. Biol. Chem. (Tokyo), 30, 114, 1966.

40. LAYNE, E., In "Methods in Enzymology", Vol. 3., 447-454, (COLOWICK, S.P., KAPLAN, N.O., Eds.) Academic Press, N.Y. 1957.

41. WEBER, K., OSBORN, M., J. Biol. Chem., 244, 4406, 1969.

42. BARKER, S.A., GRAY, C.J., JOLLEY, M.E., Biochem. Biophys. Res. Commun., 45, 654, 1971.

43. PAZUR, J.H., KLEPPE, K., J. Biol. Chem., 237, 1002, 1962.

44. PAZUR, J.H., KNULL, H.R., SIMPSON, D.L., Biochem. Biophys. Res. Commun., 40, 110, 1970.

45. GREENWOOD, C.T., MILNE, E.A., Adv. Carbohyd. Chem., 23, 281, 1968.

46. LONG, C., Ed. "Biochemists Handbook", p. 28-41, Spon E. & F.N., London, 1961.

47. RUTTLOFF, H., FRIESE, R., KAUPKE, G., TAUFEL, A., Z. allg. Mikrobiol., 9, 39, 1969.

48. KELLY, J.J., ALPERS, D.H., Biochim. Biophys. Acta., 315, 113, 1973.

49. MONTGOMERY, R., "Glycoproteins", B.B.A. Library Vol. 5 2nd Ed., Part A. (GOTTSCHALK, A., ed.,) p. 518, Elsiver, Amsterdam, 1972.

CHAPTER 4

AMINO-ACID AND CARBOHYDRATE ANALYSES AND PROTEIN CONTENT OF PURIFIED GLUCOAMYLASE I AND II

4.1 Introduction

Amino-acid analyses have been carried out on glucoamylase from *Aspergillus niger*,[1-4] *Aspergillus phoenicis*,[5] *Endomyces* species IFO 0111,[6] *Rhizopus delemar*,[2] and *Rhizopus javanicus*.[7,25] Lineback *et al.*,[1] and Pazur *et al.*,[2] report values for both glucoamylase I and II using different molecular weights. In this work amino-acid analyses have been carried out on both glucoamylase I and II and the results expressed as g/100 g protein, % of total nitrogen, and residues per 80,000 molecular weight. A comparison with the results obtained by other workers has been made.

Glucoamylases from *Aspergillus niger*, *Aspergillus phoenicis*, *Aspergillus oryzae* and *Rhizopus delemar* have been reported to be glycoproteins.[5] The carbohydrate moieties have been identified from *Aspergillus niger* glucoamylase by other workers[1,4,8] and their results compared to this work.

The protein content of glucoamylase has been measured by the method described by Layne,[9] and the results related to Bovine serum albumin.

TABLE 4.1

Amino Acid analysis of glucoamylase I and II

AMINO ACID	MOLECULAR WEIGHT	GLUCOAMYLASE I 16 HOURS	GLUCOAMYLASE II 16 HOURS	GLUCOAMYLASE I 24 HOURS	GLUCOAMYLASE II 24 HOURS	GLUCOAMYLASE I 72 HOURS	GLUCOAMYLASE II 72 HOURS
LYSINE	146.19	2.06	2.10	1.72	2.02	1.88	2.42
HISTIDINE	155.16	0.37	0.45	0.38	0.62	0.45	0.74
AMMONIA	17.00	0.60	0.79	0.78	1.09	0.73	1.03
ARGININE	174.20	2.67	2.86	1.93	2.87	2.52	3.02
ASPARTIC ACID	133.10	8.24	8.70	6.27	8.27	7.82	9.19
THREONINE	119.12	7.40	7.07	5.55	6.89	7.03	7.59
SERINE	105.09	7.64	8.10	5.67	7.62	6.66	7.63
GLUTAMIC ACID	147.13	6.02	5.79	4.64	5.56	5.75	6.39
PROLINE	115.13	2.44	2.25	1.98	2.68	2.31	2.61
GLYCINE	75.07	3.14	3.51	2.46	3.47	3.03	3.82
ALANINE	89.09	5.26	5.68	4.08	5.57	4.82	6.06
CYSTINE/2	120.15	0.53	0.51	0.37	0.48	0.57	0.45
VALINE	117.15	3.42	3.48	3.10	3.64	3.79	4.36
METHIONINE	149.21	0.23	0.27	0.16	0.33	0.27	0.35
ISOLEUCINE	131.17	1.98	2.93	1.81	2.18	2.41	2.90
LEUCINE	131.17	5.07	5.34	4.01	5.30	4.85	6.02
TYROSINE	181.19	3.79	1.43	2.70	3.46	3.61	4.14
PHENYLALANINE	165.19	2.84	3.22	2.16	3.00	2.69	2.57
TRYPTOPHAN	204.23	1.91	1.66	1.91	1.66	1.91	1.66
α-AMINO BUTYRIC ACID	103.00	TRACE	TRACE	TRACE	TRACE	TRACE	TRACE
TOTAL		65.08	65.61	51.66	66.70	63.11	72.92 g/100 g PROTEIN

Figures are averages of three determinations in each case except for glucoamylase I 24 hours for which only 2 determinations were made.

TABLE 4.2

Amino Acid analysis of glucoamylase I and II

AMINO ACID	MOLECULAR WEIGHT	GLUCOAMYLASE I 16 HOURS	GLUCOAMYLASE II 16 HOURS	GLUCOAMYLASE I 24 HOURS	GLUCOAMYLASE II 24 HOURS	GLUCOAMYLASE I 72 HOURS	GLUCOAMYLASE II 72 HOURS
LYSINE	146.19	1.73	1.71	1.44	1.73	1.57	2.07
HISTIDINE	155.16	0.29	0.36	0.30	0.50	0.36	0.60
AMMONIA	17.00	4.30	5.78	5.58	8.02	5.26	7.54
ARGININE	174.20	1.87	2.06	1.35	2.06	1.77	2.17
ASPARTIC ACID	133.10	7.57	8.17	5.76	7.77	7.18	8.63
THREONINE	119.12	7.60	7.41	5.70	7.23	7.21	7.97
SERINE	105.09	8.88	9.64	6.59	9.06	7.75	9.07
GLUTAMIC ACID	147.13	5.00	4.92	3.86	4.72	4.78	5.43
PROLINE	115.13	2.59	2.45	2.11	2.91	2.45	2.84
GLYCINE	75.07	5.11	5.84	4.01	5.78	4.93	6.35
ALANINE	89.09	7.22	7.97	5.61	7.82	6.61	8.50
CYSTINE/2	120.15	0.55	0.53	0.37	0.50	0.59	0.46
VALINE	117.15	3.57	3.71	3.24	3.88	3.95	4.65
METHIONINE	149.21	0.19	0.22	0.13	0.28	0.22	0.29
ISOLEUCINE	131.17	1.85	2.79	1.68	2.08	2.25	2.70
LEUCINE	131.17	4.72	5.08	3.74	5.05	4.52	5.74
TYROSINE	181.19	2.56	0.99	1.82	2.39	2.44	2.86
PHENYLALANINE	165.19	2.10	2.44	1.60	2.27	1.99	1.95
TRYPTOPHAN	204.23	1.14	0.98	1.14	0.98	1.14	0.98
α-AMINO BUTYRIC ACID	103.00	TRACE	TRACE	TRACE	TRACE	TRACE	TRACE
TOTAL		68.84	73.05	56.03	75.01	66.98	80.77 % of total nitrogen

Total nitrogen content 11.45% for glucoamylase I
11.25% " " II

TABLE 4.3

Amino Acid composition of glucoamylase I and II

AMINO ACID	GLUCOAMYLASE I g/100 g PROTEIN HIGHEST AVERAGE	% OF TOTAL NITROGEN	RESIDUES PER 80,000 M.Wt.	GLUCOAMYLASE II g/100 g PROTEIN HIGHEST AVERAGE	% OF TOTAL NITROGEN	RESIDUES PER 80,000 M.Wt.
LYSINE	2.06	1.72	11.30	2.42	2.07	13.26
HISTIDINE	0.45	0.36	2.32	0.74	0.60	3.82
AMMONIA	0.78	5.61	36.70	1.09	8.01	51.30
ARGININE	2.67	1.87	12.27	3.02	2.17	13.87
ASPARTIC ACID	8.24	7.57	49.52	9.19	8.63	55.24
THREONINE	7.40	7.60	49.70	7.59	7.96	50.98
SERINE	7.64	8.89	58.21	8.10	9.63	61.72
GLUTAMIC ACID	6.02	5.00	32.74	6.39	5.43	34.75
PROLINE	2.44	2.59	17.01	2.68	2.91	18.64
GLYCINE	3.14	5.11	32.72	3.82	6.36	40.74
ALANINE	5.26	7.22	47.23	6.05	8.49	54.33
*CYSTINE/2	1.26	0.59	5.96	1.27	0.53	6.01
VALINE	3.79	3.96	25.87	4.36	4.65	29.77
METHIONINE	0.27	0.22	1 45	0.35	0.29	1.87
ISOLEUCINE	2.41	2.25	14.69	2.93	2.79	17.86
LEUCINE	5.07	4.73	30.91	6.02	5.74	36.70
TYROSINE	3.79	2.56	16.73	4.14	2.86	18.28
PHENYLALANINE	2.84	2.10	13.75	3.22	2.44	15.60
TRYPTOPHAN	1.91	1.14	7.48	1.66	0.99	6.50
TOTAL	67.44	71.09	466.56	75.04	82.55	561.24

* Measured as cysteic acid.

TABLE 4.4

The amino acid compositions of various glucoamylases, - number of amino acid residues to nearest integer

GLUCOAMYLASE AND SOURCE	REFERENCE	AMINO ACIDS																				MOLECULAR WEIGHT
		LYSINE	HISTIDINE	AMMONIA	ARGININE	ASPARTIC ACID	THREONINE	SERINE	GLUTAMIC ACID	PROLINE	GLYCINE	ALANINE	CYSTINE/2	VALINE	METHIONINE	ISOLEUCINE	LEUCINE	TYROSINE	PHENYLALANINE	TRYPTOPHAN	γ-AMINO BUTYRIC ACID	TOTAL	
GLUCOAMYLASE Endomyces species IFO 0111.	6	24	10	41	11	62	27	37	30	15	27	29	12	20	0	18	38	27	15	8		410	55,000
SACCHAROGENIC AMYLASE Rhizopus javanicus	7	21	2	18	8	49	44	44	21	15	30	48	6	31	2	19	28	18	17	7		410	48,000
GLUCOAMYLASE I Aspergillus niger	1	12	3	63	18	75	79	84	46	22	47	65	9	45	2	23	44	26	21			684	74,900±1,550
GLUCOAMYLASE II Aspergillus niger		9	3	50	11	57	53	62	31	16	36	50	9	32	2	16	33	17	13			500	54,300±6,690
GLUCOAMYLASE A Aspergillus phoenicis	5	11	4	105	15	62	59	73	38	20	44	58	5	35	1	18	39	19	19	0		625	63,600±6,800
GLUCOAMYLASE I Aspergillus niger	2	16	6	21	23	83	104	119	56	29	59	78	8	46	4	27	53	30	27	30		819	99,000±3%
GLUCOAMYLASE II Aspergillus niger		16	6	53	22	85	103	121	54	29	63	82	8	46	4	25	56	29	28	32		862	112,000±3%
GLUCOAMYLASE R Rhizopus delemar		39	8		18	104	88	108	48	31	77	87	6	58	6	36	57	44	34	36		885	100,000
GLUCOAMYLASE I Aspergillus niger	4	19	6		25	93	119	132	65	33	66	90	8	55	4	32	61	34	32	33		907	110,000
GLUCOAMYLASE I Aspergillus niger	3	13	4		18	69	72	77	45	23	47	64	7	38	1	22	46	26	22	19		613.4	61,500
GLUCOAMYLASE I Aspergillus niger	THIS WORK	11	2	37	12	50	50	58	33	17	33	47	6	26	2	15	31	17	14	8	TRACE	468	80,000±2,500
GLUCOAMYLASE II Aspergillus niger		13	4	51	14	55	51	62	35	19	41	54	6	30	2	18	37	18	16	7	TRACE	533	80,000±2,000

4.2 Results and discussion

4.2.1 Amino-Acid Analysis

Weighed samples of dry glucoamylase I and II were hydrolysed in sealed ampoules under nitrogen at 110°C for various periods of time up to 72 hours using constant boiling 6M hydrochloric acid. After hydrolysis the samples were evaporated to dryness and made up to a standard volume with buffer of pH 2.0 prior to analysis. Cysteic acid was measured from glucoamylase similarly after treatment with performic acid and later made up to a standard volume using 0·1M hydrochloric acid. The asparagine and glutamine residues could have been in either the amide or carboxyl form in the protein and for calculations the carboxyl form was assumed. Tryptophan was determined after hydrolysis with 6M NaOH for 6 hours at 121°C. In each case the hydrolyses were carried out in triplicate, except for glucoamylase I, 24 hours, which were carried out in duplicate. The results were averaged and are shown in TABLES 4.1 and 4.2. TABLE 4.3 shows the number of amino-acid residues based on a molecular weight of 80,000 for both glucoamylase I and II determined as described in CHAPTER 3 (3.3) of this Thesis, and has been compiled using the highest values obtained for each amino-acid in TABLE 4.1.

Because the acid hydrolysis of the bonds between different amino-acids in the polypeptide chain of a protein do not occur at the same rate, a time course of hydrolysis was carried out. An attempt to measure the methionine and cystine in glucoamylase I and II after conversion to methionine sulphone and cysteic acid respectively was carried out, but the methionine sulphone peak was obscured under the aspartic acid peak from both glucoamylases and the standard could not be resolved. The values obtained for cysteic acid were 1.26, and 1.27 g/100 g protein for glucoamylase I and II, respectively after 16 hours hydrolysis. These values give 6 residues for the cystine/2 shown in TABLES 4.3 and 4.4.

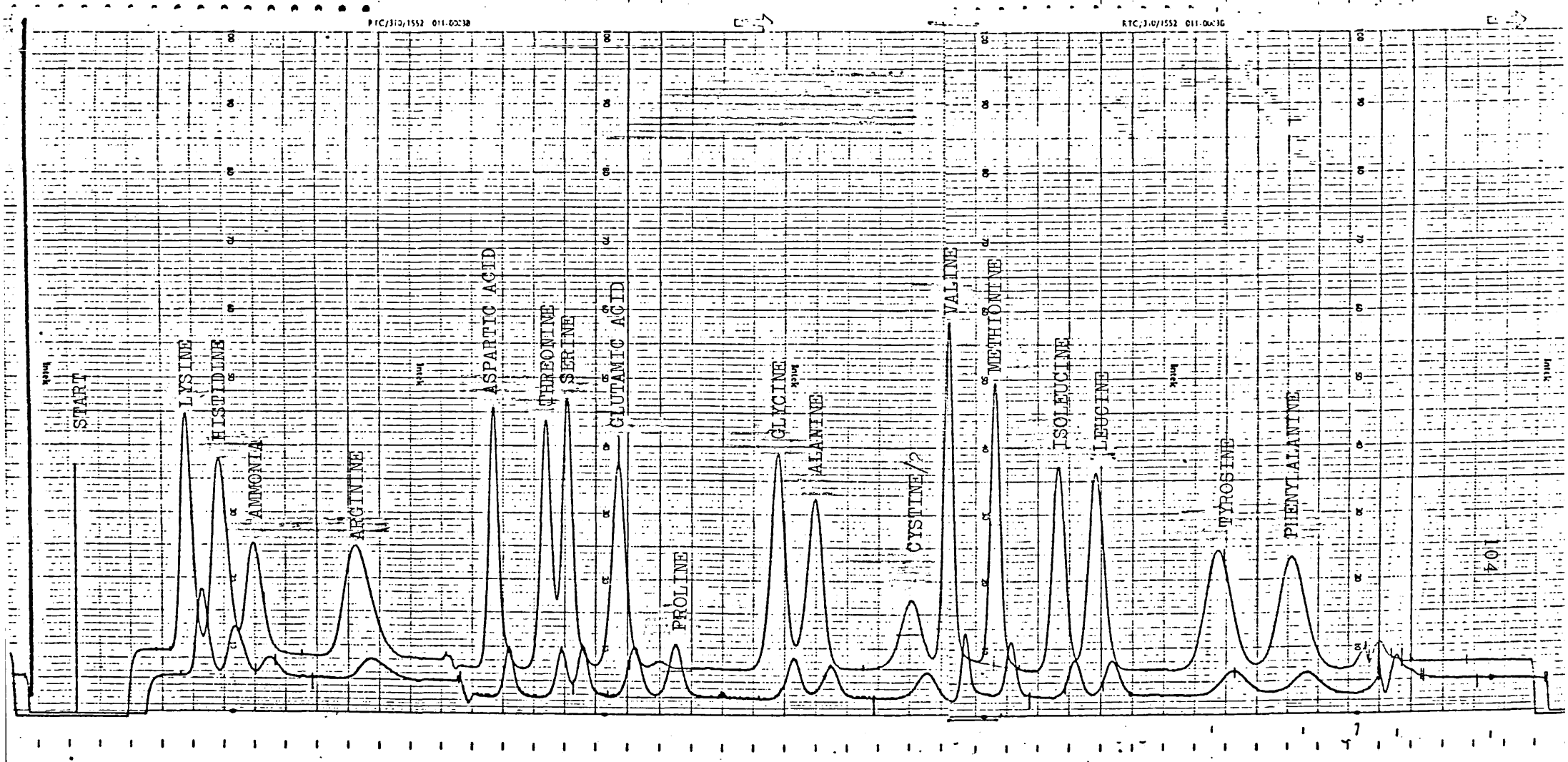


FIGURE 4.1. Amino-acid analysis standards. Upper trace recorded at 580 nm, lower trace 440 nm .25 nMoles of each acid.

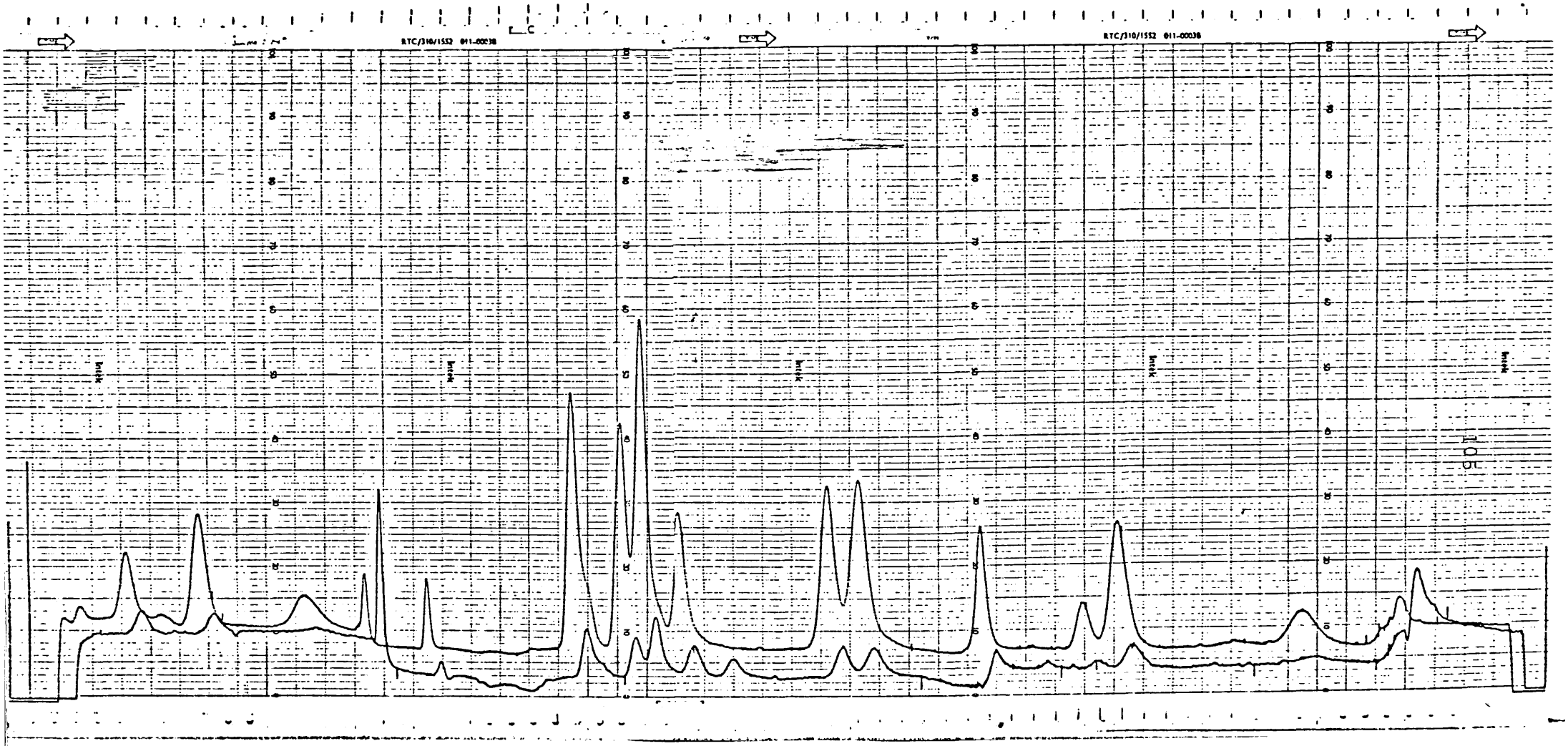


FIGURE 4.6. Amino-acid analysis of oxidised glucoamylase II after 16 hour acid hydrolysis. Upper trace recorded at 580 nm, lower trace 440 nm.

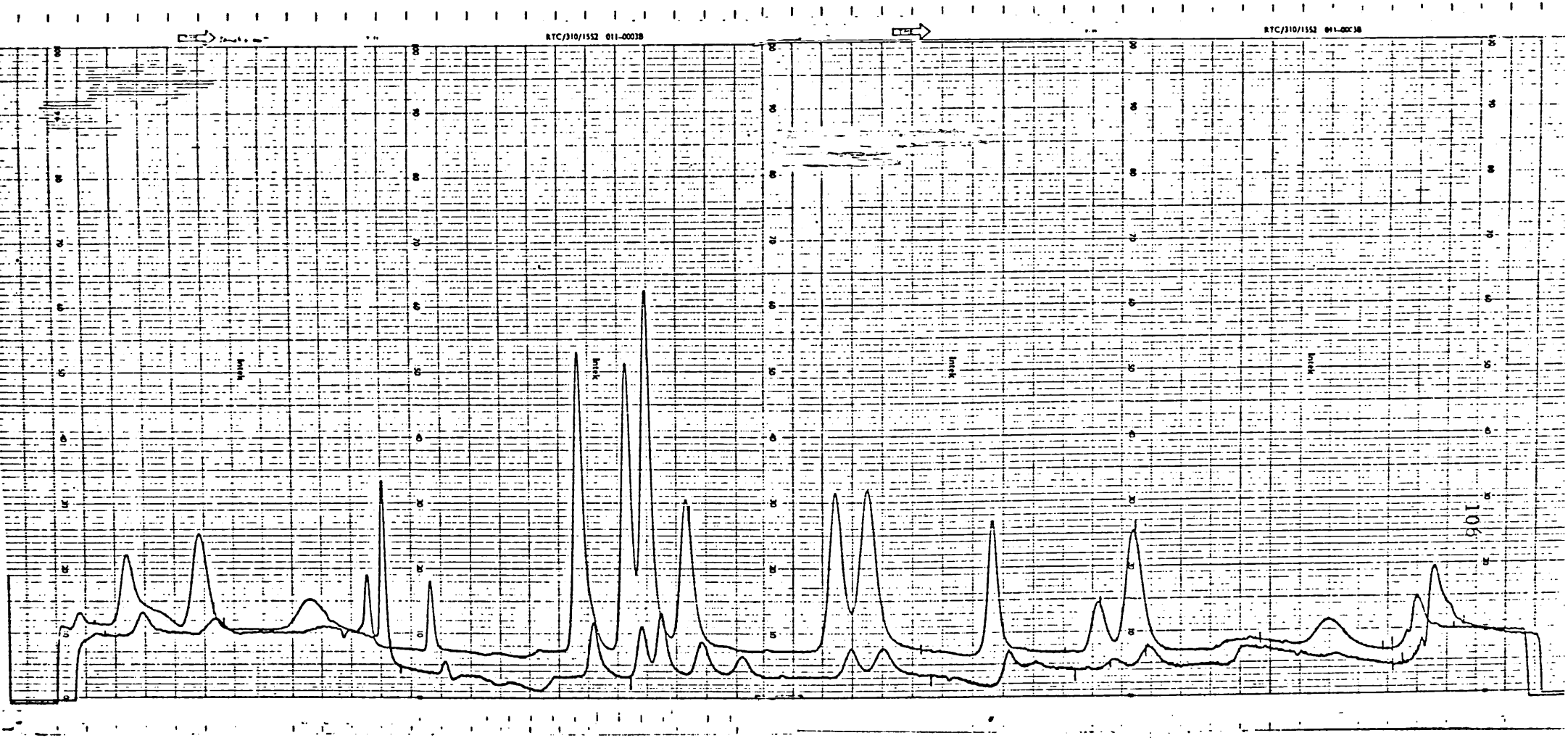


FIGURE 4.5. Amino-acid analysis of oxidised glucoamylase I after 16 hour acid hydrolysis. Upper trace recorded at 580 nm, lower trace 440 nm.

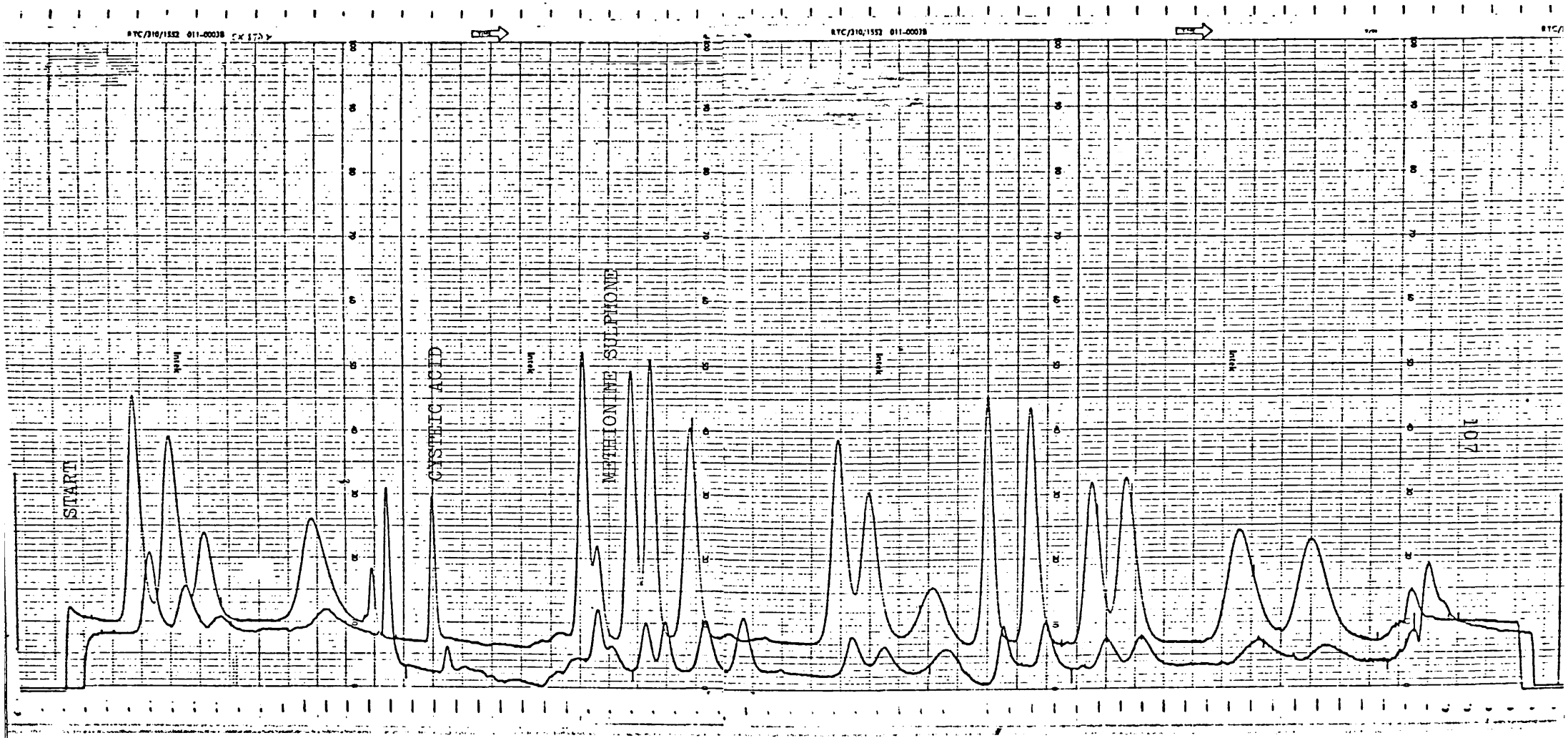


FIGURE 4.4. Amino-acid analysis standards for acid hydrolysis of glucoamylase I and II after performic acid oxidation. Upper trace recorded at 580 nm, lower trace 440 nm. 5.90 nMoles of labelled acids.

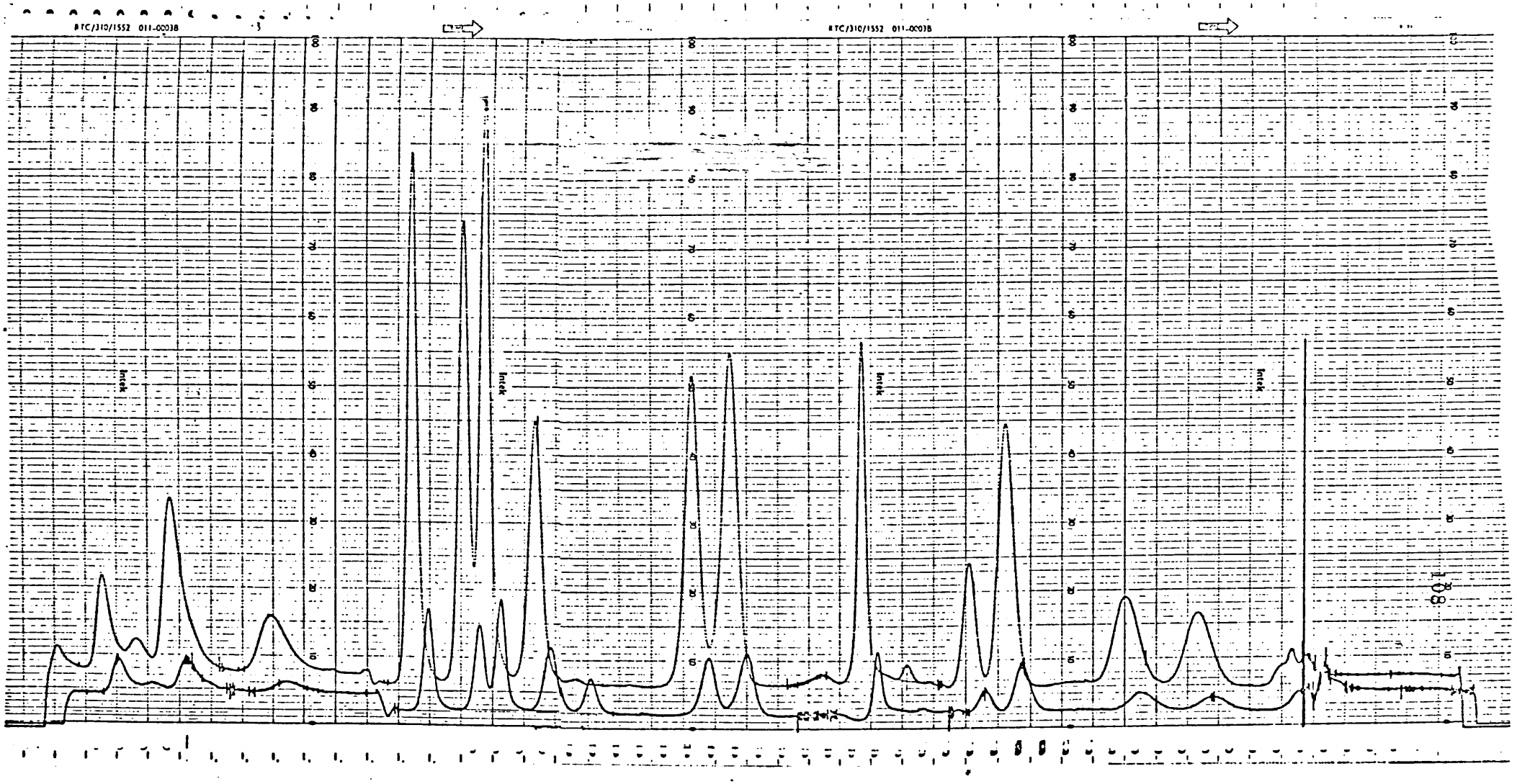


FIGURE 1.3. Amino-acid analysis of glucoamylase II after 72 hour acid hydrolysis. Upper trace recorded at 580 nm, lower trace 440 nm.

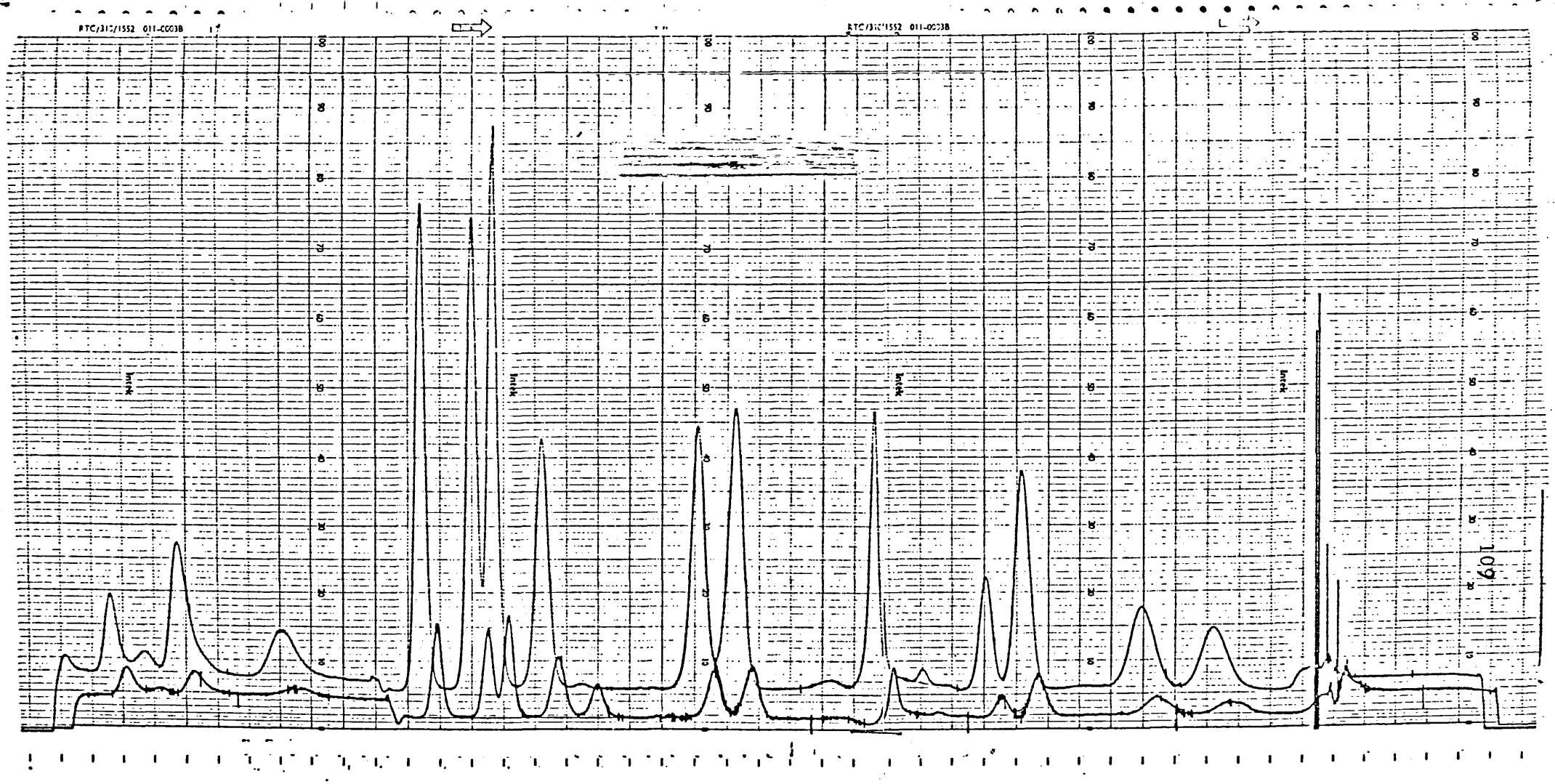


FIGURE 4.2. Amino-acid analysis of glucoamylase I after 72 hour acid hydrolysis. Upper trace recorded at 580 nm, lower trace 440 nm.

Some amino acid analyses obtained for glucoamylase I and II together with standards are shown in FIGURES 4.1 to 4.6.

The total nitrogen content of glucoamylase I and II, 11.45% and 11.20%, respectively, was measured by the micro Kjeldahl method. Both, the amino-acid analyses and nitrogen determinations were carried out at the Lord Rank Research Centre.

It can be seen that the two forms of glucoamylase have similar amino-acid compositions. The values obtained for glucoamylase I are generally lower than those obtained for glucoamylase II. The maximum difference between the two glucoamylase forms occurs in aspartic acid, serine, glycine, alanine and leucine. The results agree broadly with the amino acid compositions found by other workers[2,11] who used molecular weights of 99,000 and 112,000 for glucoamylase I and II, respectively. The compositions found in this work are also similar to those of Lineback et al.,[1] who used 74,900 ± 1,550 and 54,300 ± 6,690 for the molecular weights of glucoamylase I and II respectively, except that their values for glucoamylase I are always larger than those of glucoamylase II. TABLE 4.4 lists the number of amino-acid residues to the nearest integer found in this and other work on glucoamylase.

Pazur et al.,[2] have reported that the hydrolysates of glucoamylase II used for amino-acid analysis contained considerably more ammonia than the hydrolysate of glucoamylase I. These results agree with those found in this work. In order to explain the results Pazur et al.,[2] suggested that glucoamylase II may possess a higher number of amide or perhaps glucosylamine linkages.

A method of hydrolysis of proteins described by Bennett et al,[10] using "Sepharose" bound peptidases may yield the values of glutamine and asparagine present in the native glucoamylase which were unobtainable from the acid hydrolysis because the labile groups were converted into

their parent amino acids. A trial experiment using this method of hydrolysis for glucoamylase I proved unsuccessful because of the low activities of the "Sepharose" bound enzymes which were used.

4.2.2 Protein content of purified glucoamylase I and II

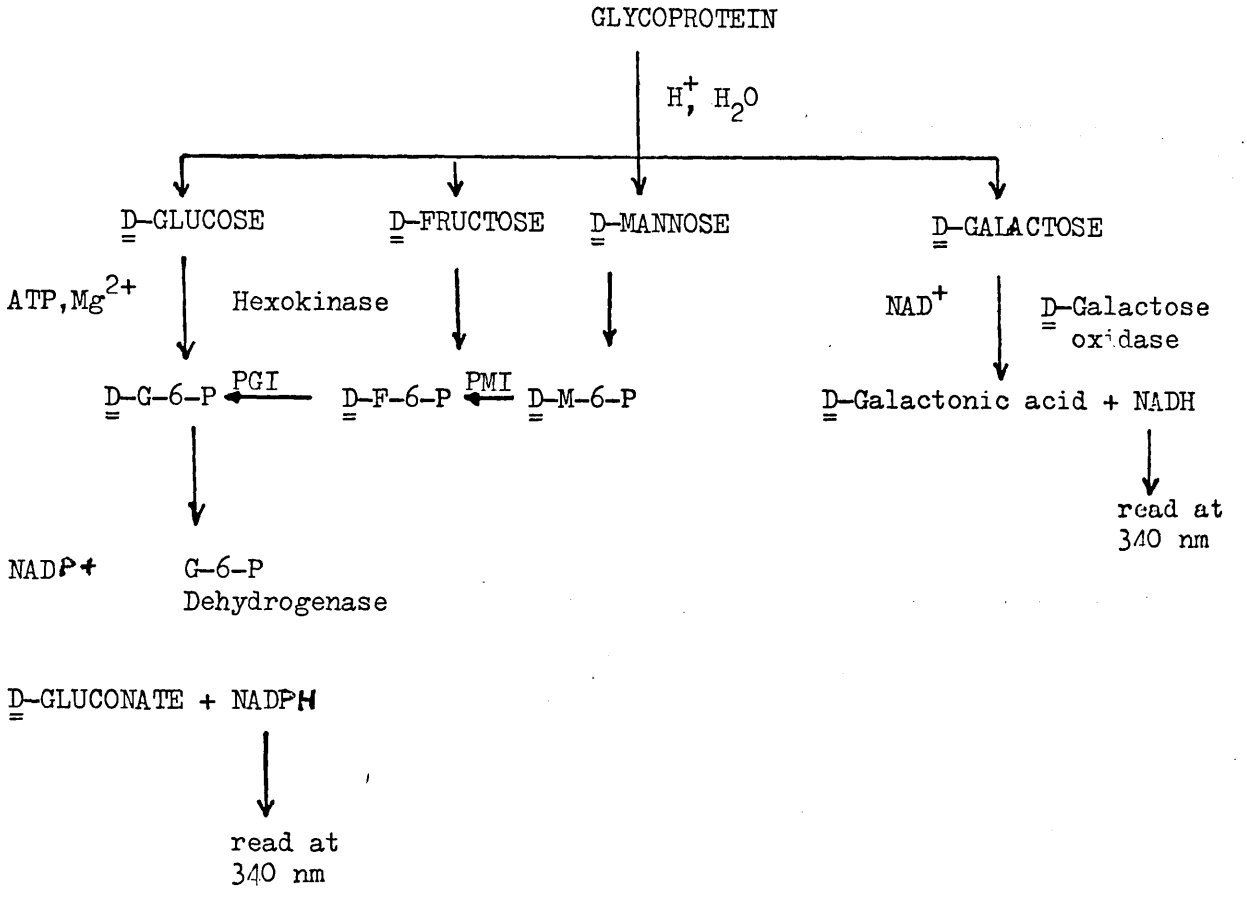
The protein content has been measured with respect to bovine serum albumin (BSA) using the method described by Layne.[9] The method is only exact if BSA gives an identical colour response to that of glucoamylase. The total protein content of the glucoamylases could not be obtained from their extinction coefficients (CHAPTER 3, (3.35) of this Thesis) in this case because these values were themselves obtained from dried glucoamylase.

The results were that glucoamylase I contained 83% protein and glucoamylase II 90% protein. These results agree with the amino-acid analysis in that glucoamylase II contained more amino-acid residues than glucoamylase I for similar molecular weight values. The reason for the protein contents of glucoamylase I and II being lower than 100% was attributed to the glycoprotein nature of these enzymes. The discrepancies should be similar therefore to the carbohydrate content of each glucoamylase. The same glucoamylase stock solutions were used to measure the carbohydrate content.

4.2.3 Carbohydrate content of purified glucoamylase I and II

The carbohydrate content of glucoamylase I and II has been measured by two methods, each of which will be discussed separately.

The first method measured the carbohydrate content of glucoamylase I and II with respect to D-mannose using the phenol sulphuric acid assay described by Dubois et al.[12] This method would only be exact if all the carbohydrate present in the glycoprotein was D-mannose. As the



SCHEME 4.1 DIAGRAMATIC REPRESENTATION OF PROCEDURE FOR CARBOHYDRATE ANALYSIS IN GLYCOPROTEINS DEVISED BY STURGEON.[18]

literature stated that the majority of the carbohydrate in glucoamylase I and II was D-mannose[1,2,4,5,11,13,20,21] it seemed reasonable to take this as the standard for the phenol sulphuric acid carbohydrate measurement. To correct the test for any colour produced from the protein itself an equal concentration of lysozyme, which was known not to be a glycoprotein, was reacted under identical conditions and the small contribution (3%) subtracted from the glycoprotein figures. It has been reported by Beaupoil-Abadie et al.,[14] that tyrosine residues give strong colourations with phenol sulphuric acid and this may give rise to an error in the figures obtained. It may have been more correct therefore to have used a synthetic mixture of amino-acids corresponding to the amino-acid analysis for glucoamylase I and II as a blank. The results obtained, glucoamylase I 16% carbohydrate, glucoamylase II 18% carbohydrate, were in close agreement with those quoted in the literature, see TABLE 4.5.

The second method used to analyse the carbohydrate present in glucoamylase I and II was devised by Sturgeon,[18] and made use of specific enzymes to measure spectroscopically D-glucose, D-mannose and D-galactose after liberation from the glycoprotein by mild acid hydrolysis. D-Fructose can also be measured using this technique.

The technique (shown in SCHEME 4.1) was essentially as follows. D-Glucose was phosphorylated in the six position with hexokinase in the presence of adenosine triphosphate (ATP). The phosphorylated glucose was then dehydrogenated with D-glucose-6-phosphate dehydrogenase in the presence of nicotinamide - adenine dinucleotide phosphate (NADP+). The reduced co-enzyme NADPH formed during the reaction had a strong absorption at 340 nm and the optical density increase used to measure the D-glucose concentration in the original solution. Similarly the addition of

TABLE 4.5

Carbohydrate Contents of glucoamylase I and II

GLUCOAMYLASE, SOURCE, AUTHOR AND REFERENCE	PROCEDURE	MANNOSE %	GLUCOSE %	GALACTOSE %	TOTAL %
GLUCOAMYLASE A Aspergillus phoenicis LINEBACK, 5	METHANOLYSIS	7.3	3.9	0.5	11.7
	HYDROLYSIS	8.0	2.8	1.2	12.0
	PHENOL SULPHURIC ACID ASSAY				16.9
GLUCOAMYLASE Human intestine KELLEY, 22	NOT GIVEN				32-38
GLUCOAMYLASE I and II Aspergillus niger PAZUR, 2	ORCINOL SULPHURIC ACID ASSAY	GLUCOAMYLASE I			14.3
		GLUCOAMYLASE II			23.5
	HYDROLYSIS GLUCOAMYLASE I	69 RESIDUES/ MOLECULE	16 RESIDUES/ MOLECULE	2 RESIDUES/ MOLECULE	15.8
	HYDROLYSIS GLUCOAMYLASE II	128 RESIDUES/ MOLECULE	20 RESIDUES/ MOLECULE	3 RESIDUES/ MOLECULE	24.3
GLUCOAMYLASE I Aspergillus niger PAZUR, 15	PHENOL SULPHURIC ACID ASSAY				15.0
GLUCOAMYLASE I and II Aspergillus niger PAZUR, 13	METHANOLYSIS GLUCOAMYLASE A	8.0	2.0	0.2	10.2
	METHANOLYSIS GLUCOAMYLASE B	15.0	3.0	0.2	18.2

TABLE 4.5 (continued)

GLUCOAMYLASE, SOURCE, AUTHOR AND REFERENCE	PROCEDURE	MANNOSE %	GLUCOSE %	GALACTOSE %	TOTAL %
GLUCOAMYLASE I and II *Aspergillus niger* LINEBACK, 1	PHENOL SULPHURIC ACID ASSAY	GLUCOAMYLASE I			10.0
		GLUCOAMYLASE II			18.0
	METHANOLYSIS GLUCO-AMYLASE I	6.8	2.1	0.3	9.2
	METHANOLYSIS GLUCO-AMYLASE II	10.6	4.4	0.7	15.7
GLUCOAMYLASE I *Aspergillus niger* PAZUR, 4	NOT GIVEN	≈ 80 RESIDUES/ MOLE ENZYME	≈ 20 RESIDUES/ MOLE ENZYME	≈ 2 RESIDUES/ MOLE ENZYME	16.7
GLUCOAMYLASE GREENWOOD, 16	NOT GIVEN	GLUCOAMYLASE from *Aspergillus niger* and *Rhizopus delemar*			13.0
GLUCOAMYLASE I and II *Aspergillus niger* PAZUR, 11	HYDROLYSIS GLUCO-AMYLASE I	69 RESIDUES	16 RESIDUES	2 RESIDUES	15.8
	HYDROLYSIS GLUCO-AMYLASE II	128 RESIDUES	20 RESIDUES	3 RESIDUES	24.3
SACCHAROGENIC AMYLASE *Rhizopus javanicus* WATANABE, 7	PHENOL SULPHURIC ACID ASSAY				9.3
	HYDROLYSIS	27 RESIDUES	GLUCOSAMINE 4 RESIDUES		10.4
GLUCOAMYLASE I and II *Aspergillus niger* THIS WORK	PHENOL SULPHURIC ACID ASSAY	GLUCOAMYLASE I			16.0
		GLUCOAMYLASE II			18.0
	HYDROLYSIS GLUCOAMYLASE I	12.52	1.57	0.04	14.13
	HYDROLYSIS GLUCO-AMYLASE II	12.95	1.78	0.07	14.80

FIGURE 4.7. Spectroscopic measurement of D-glucose and D-mannose in glucoamylase I and II ($0.1\ cm^3$ samples) by enzymes after acid hydrolysis.

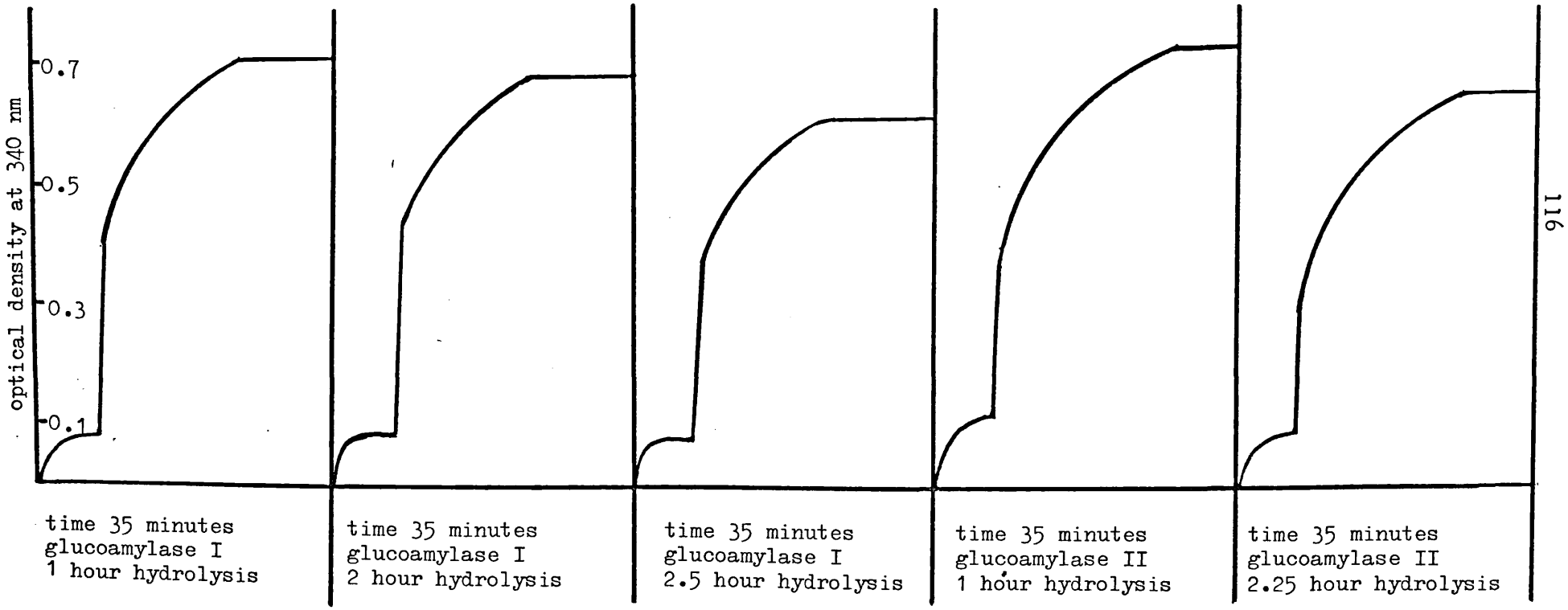
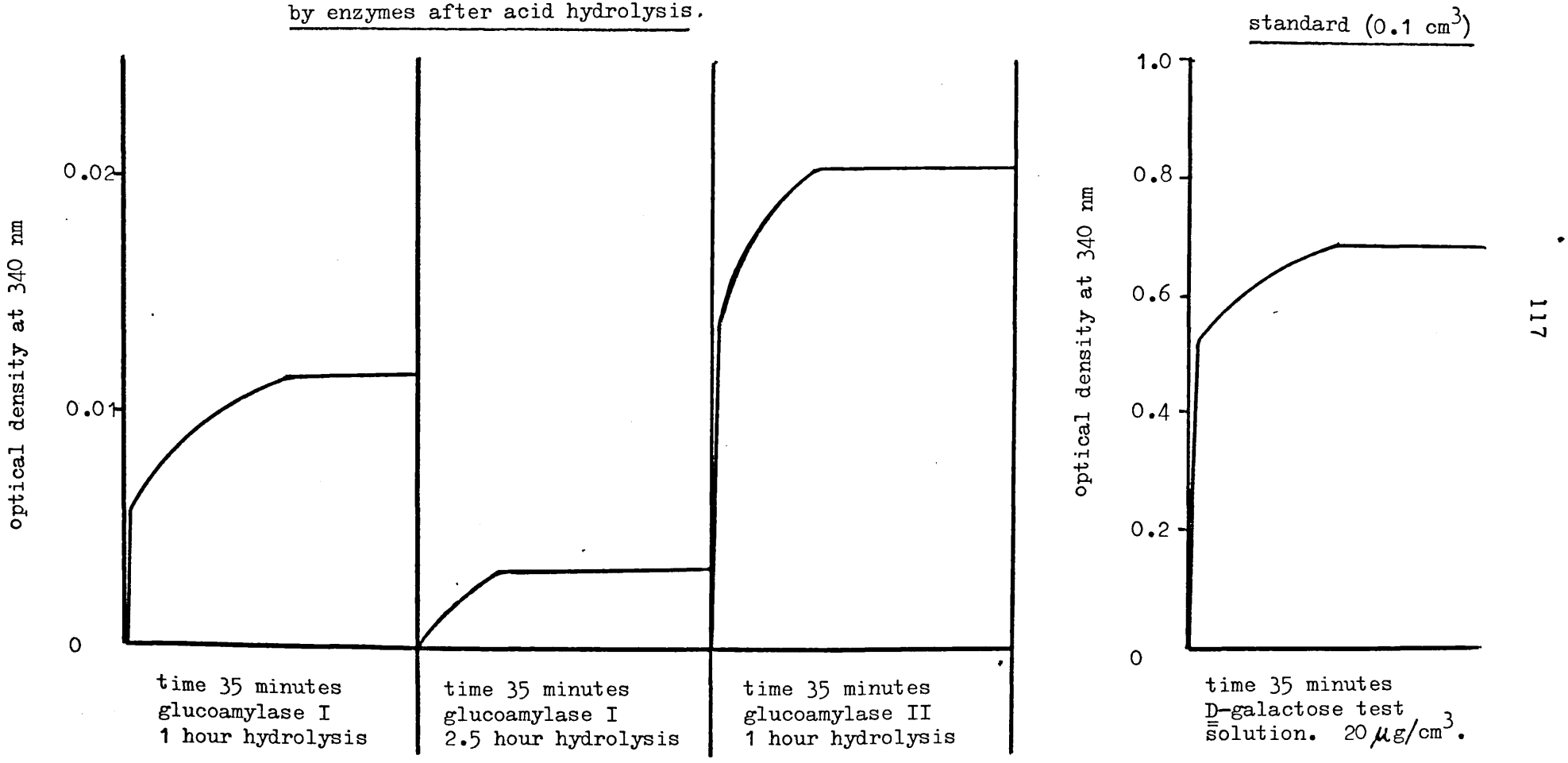


FIGURE 4.8. Spectroscopic measurement of D-galactose in glucoamylase I and II (0.5 cm^3 samples) by enzymes after acid hydrolysis.



phosphoglucose isomerase to the reaction solution converted D-fructose phosphate to D-glucose-6-phosphate and the reaction then proceeded as before. The increase in optical density would therefore be a measure of the D-fructose content of the solution. The addition of phosphomannose isomerase to the reaction solution converted D-mannose-6-phosphate to D-fructose-6-phosphate and the increase in optical density which occurred after the addition of the enzyme was a measure of the D-mannose in the solution. The D-galactose concentration was measured separately using β-D-galactose dehydrogenase and its coenzyme nicotinamide-adenine dinucleotide (NAD+). The optical density measured at 340 nm was a measure of the D-galactose present in the reaction solution. As the optical densities were very low, the reaction was tested with a solution of D-galactose and found to give the correct response. It was concluded therefore that very little D-galactose was present in the acid hydrolysates of glucoamylase I and II which agreed with other workers - see TABLE 4.5.

The spectra obtained from the enzyme assays are shown in FIGURES 4.7 and 4.8. The concentrations of NADP+ and ATP were calculated to be in excess of the test requirement and a change of 0.1 in the optical density at 340 nm corresponded to 0.0161 $\mu M/cm^3$ of sugar. In the test for D-glucose and D-mannose, a crystal of magnesium chloride was added to the test solution to provide magnesium ions necessary for the enzymic phosphorylation of the sugars. The results presented in TABLE 4.5 were calculated from FIGURES 4.7 and 4.8 using the maximum optical density values from the time course after mild acid hydrolysis of glucoamylase I and II and were expressed as percentages of their dry weights.

It has been reported that the carbohydrate moieties in glucoamylase I and II from Aspergillus niger were O-glycosidically linked through

D-mannose to the hydroxyl groups of L-serine and L-threonine in the polypeptide chain.[1,4,8] D-Mannose is the preponderent sugar component of all the glucoamylases reported with lesser proportions of D-glucose and D-galactose. Sialic acid which is present in some plasma glycoproteins,[17] has been reported to be absent from glucoamylase.[1,5] Hexosamines have been reported to be absent in the glucoamylase from Aspergillus phoenicis[5] and Aspergillus niger,[1,13] but have been reported present in the form of 2-Amino 2-deoxy-D-glucose (glucosamine) in glucoamylase from Rhizopus delemar[2] and Rhizopus javanicus.[7,25]

From the protein content of glucoamylase I and II it may have been expected that glucoamylase I would contain more carbohydrate than glucoamylase II. However this was not found. Glucoamylase II was found to possess more carbohydrate than glucoamylase I from both the phenol sulphuric acid, and hydrolysis assays. These findings agree with those of other workers - see TABLE 4.5. The discrepancy between the total carbohydrate as determined by the phenol-sulphuric acid procedure and by hydrolysis could be attributed to the following:

(a) A portion of the sugars being destroyed under the conditions of hydrolysis.

(b) Incomplete release of the hexoses from the glucoamylases.

(c) A larger protein colour contribution to the phenol sulphuric acid assay than corrected for in the lysozyme blanks.

(d) L-sugars present in the glycoproteins.

Aira[23] and Lineback et al.,[1] reported that the carbohydrate moieties of glucoamylase I and II were predominantly trisaccharide units with at least two different sequences: Man-Man-Man-, Man-Glu-Man -.

Other units containing D-galactose must also be present, and it has been suggested by Pazur *et al.*,[15] that the synthesis of the carbohydrate moieties was apparently not under genetic control. The role of the carbohydrate moieties in glycoproteins is not understood but it is thought that they may give rise to immunological properties,[17] and also that they may act as stabilizers of the tertiary structure of the enzyme.[4] It is possible that the presence of carbohydrate confers resistance to proteolytic degradation. Glycoproteins appear to be characteristic of the secreted products of eukaryotes (and not prokaryotes), and it has been proposed[24] that the advent of the endoplasmic reticulum made more probable the transfer of sugars to proteins in the course of secretion. In other words, attachment of sugars may occur "by accident" during secretion and may not have a specialised role.

4.3 Experimental

4.3.1 Amino-acid analysis

Three dry samples (2 mg) of glucoamylase I and II were weighed separately into six labelled "Pyrex" glass ampoules 6" x 5/8". 6M Hydrochloric acid (2.0 cm^3) was added to each ampoule. Each sample was degassed and the ampoules filled with oxygen free nitrogen prior to sealing. The samples were then placed in an oven at 110°C for 16 hours. The process was repeated and the samples hydrolysed for 24 and 72 hours. (Except for glucoamylase I, 24 hour hydrolysis for which only two samples were used). After hydrolysis, the ampoules were cooled and the contents washed into separate pear shaped flasks (25 cm^3) with deionised water (5 cm^3). The samples were evaporated to dryness on a Buchi rotary evaporator. Deionised water (5 cm^3) was added to each sample flask and rotary evaporated to dryness. The process was repeated until the samples had no smell of acid vapour. Amino acid analysis buffer pH 2.0 (2.0 cm^3) was added to each sample and 80 μdm^3 applied to the Technicon auto-analyser.

Performic acid was made as described by Hirs.[19] Three dry samples (2 mg) of glucoamylase I and II were weighed out as before and performic acid (2.0 cm^3) added to each sample. After four hours at 0°C the reaction was terminated by the addition of concentrated hydrobromic acid (1.0 cm^3) and the samples rotary evaporated to dryness over sodium hydroxide pellets. The samples were then acid hydrolysed for 16 hours as described previously. After hydrolysis and evaporation, 0·1M hydrochloric acid (2.0 cm^3) was added to the sample and 40 μdm^3 applied to the auto-analyser.

Standard amino-acid samples were analysed after each sample had been run, 25nMoles being used for each acid except cysteic, for which 5.91nMoles were used. The amino-acid peaks were electronically integrated except for

methionine, cysteine/2 and cysteic acid which were hand integrated using the triangulation method.

4.3.2 Protein estimation

Dry samples of glucoamylase I and II (17.5 mg) were made up to 25 cm^3 with deionised water and diluted 1:1 v/v with deionised water prior to use. Bovine serum albumin (4 mg) was made up to 10 cm^3 with deionised water. Using the method described by Layne,[9] the protein content of glucoamylase I and II was determined as in CHAPTER 2, (2.4.2) of this Thesis.

4.3.3 Carbohydrate estimation using the phenol sulphuric acid procedure

D-Mannose (7 mg) was made up to 100 cm^3 with deionised water. Lysozyme (7 mg) was made up to 10 cm^3 with deionised water. The stock glucoamylase solutions (700 $\mu g/cm^3$) used for the protein estimation were also used in this determination. 5% (w/v) solution of phenol was made up using deionised water. The carbohydrate contents were determined using the phenol-sulphuric acid procedure described by Dubois *et al.*[12]

D-Mannose, (1.0, 0.75, 0.50, 0.25 and 0.10 cm^3) were pipetted into five "Pyrex" glass boiling tubes 6" x 1", followed by deionised water (0.0, 0.25, 0.50, 0.75, 0.90 cm^3). 5% phenol solution (1.0 cm^3) was then added to each tube and mixed thoroughly. Concentrated sulphuric acid* (5.0 cm^3) was then added rapidly to each reaction tube using an "Oxford Pipettor". After 30 minutes the optical density of the solutions were read against a blank, made from deionised water, at 490 nm using a Pye Unicam SP 500 spectrometer. A standard graph 0-70 μg was then constructed.

*Micro-analytical Grade - B.D.H.

The experiment was repeated for glucoamylase I and II using 0.1, 0.2, 0.4 cm^3 enzyme stock solution and 0.9, 0.8, 0.6 cm^3 deionised water. The optical densities of each reaction tube were read against corresponding blanks made from the lysozyme solution.

4.3.4 Carbohydrate estimation using hydrolysis followed by enzymic assay

Glucoamylase I (5 mg) was weighed into three "Pyrex" glass test tubes 3" x 3/8" the tops of which were drawn out to form ampoules. The procedure was repeated for two samples of glucoamylase II. 2M Hydrochloric acid (1.0 cm^3) was added and air purged from each ampoule using oxygen free nitrogen. The ampoules were sealed and placed in an oven at 100°C. Ampoules were removed after 1.0, 2.0, and 2.5 hours for glucoamylase I, and after 1.0 and 2.25 hours for glucoamylase II. The solutions were withdrawn from the ampoules and passed through a Millipore syringe filter into five volumetric flasks (3.0 cm^3). One drop of phenolphthalein solution was added to each flask and 4M NaOH added dropwise until the pink colour was just visible. The colour was then discharged by 1M acetic acid and the solution made up with deionised water.

For D-glucose and D-mannose determinations, a silica cell (1.0 cm^3 capacity) was filled with TRIS buffer, pH 7.5, (0.7 cm^3), NADP, (0.1 cm^3), ATP (10 mg/cm^3) (0.1 cm^3), and hydrolysed glucoamylase (0.1 cm^3). A small crystal of magnesium chloride was then added. The cell was then placed in a Pye Unicam SP 1800 spectrometer and the optical density at 340 nm blanked against TRIS buffer. A baseline was obtained for optical density ranges 0 to 0.5 and 0 to 1.0. Using the 0 to 0.5 range the enzymic determination of D-glucose was begun by adding hexokinase/glucose

6-phosphate dehydrogenase ($10\,\mu dm^3$). For D-mannose determination the 0 to 1.0 optical density range was selected and Phosphoglucose isomerase ($10\,\mu dm^3$), and Phosphomannose isomerase ($10\,\mu dm^3$) added. The maximum optical densities were reached after 35 minutes.

For D-galactose determination, a similar procedure was used. The spectrometer was blanked against TRIS buffer, pH 8.5 using the 0 to 0.2 optical density range. The cell was filled with TRIS buffer, pH 8.5, ($0.4\ cm^3$), NAD, ($0.1\ cm^3$) and hydrolysed glucoamylase ($0.5\ cm^3$). The reaction was begun by adding β-D-galactose dehydrogenase ($10\,\mu dm^3$). The enzymes and coenzymes used were Sigma except phosphomannose isomerase and β-D-galactose dehydrogenase which were Boehringer.

References

1. LINEBACK, D.R., AIRA, L.A., Cereal Chem., 49, 283, 1972.

2. PAZUR, J.H., KNULL, H.R., CEPURE, A., Carbohyd. Res., 20, 83, 1971.

3. BARKER, S.A., GRAY, C.J., JOLLEY, M.E., Biochem. Biophys. Res. Commun., 45, 654, 1971.

4. PAZUR, J.H., KNULL, H.R., SIMPSON, D.L., Biochem. Biophys. Res. Commun., 40, 110, 1970.

5. LINEBACK, D.R., BAUMANN, W.E., Carbohyd. Res., 14, 341, 1970.

6. FUKUI, T., NIKUNI, Z., Agr. Biol. Chem., 33, 884, 1969.

7. WATANABE, K., FUKIMBARA, T., Agr. Biol. Chem., 37, 2755, 1973.

8. LINEBACK, D.R., Carbohyd. Res., 7, 106, 1968.

9. LAYNE, E., In "Methods in Enzymology", Vol. 3, p.447-454 (COLOWICK, S.P., KAPLAN, N.O., Eds.) Academic Press, N.Y. 1955.

10. BENNETT, H.P.J., ELLIOTT, D.F., EVANS, B.E., LOWRY, P.J., McMARTIN, C., Biochem. J., 129, 695, 1972.

11. PAZUR, J.H., ARONSON, N.N., Adv. Carbohyd. Chem. Biochem., 27, 301-341 1972.

12. DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS, P.A., SMITH, F., Anal. Chem., 28, 350, 1956.

13. PAZUR, J.H., KLEPPE, K., BALL, E.M., Arch. Biochem. Biophys., 103, 515, 1963.

14. BEAUPOIL-ABADIE, B., RAFFALLI, M., COZZONE, P., MARCHIS-MOUREN, G., Biochim. Biophys. Acta., 297, 436, 1973.

15. PAZUR, J.H., KNULL, H.R., SIMPSON, D.L., Biochem. Biophys. Res. Commun., 36, 394, 1969.

16. GREENWOOD, C.T., MILNE, E.A., Adv. Carbohyd. Chem., 23, 359, 1968.

17. WOLD, F., Birth Defects, 9, 46, 1973.

18. STURGEON, R., Unpublished work.

19. HIRS, C.H.W., In "Methods in Enzymology", Vol.XI (COLOWICK, S.P., KAPLAN, N.O., Eds)., p.197-199. Academic Press, N.Y. 19 .

20. FLEMING, I.D., STONE, B.A., Biochem. J., 97, 13p, 1965.

21. LINEBACK, D.R., GEORGI, C.E., DOTY, R.L., J. Gen. Appl. Microbiol., 12, 27, 1966.

22. KELLY, J.J., ALPERS, D.H., Biochim. Biophys. Acta., 315, 113, 1973.

23. AIRA, L.A., Diss. Abstr. Int. B. 32, 1982, 1971.

24. LEABACK, D.H., Biochem. J., 129, 17p, 1972.

25. WATANABE, K., FUKIMBARA, T., Agr. Biol. Chem., 38, 1643, 1974.

CHAPTER 5

THE ACTIVITY OF PURIFIED GLUCOAMYLASES I AND II

5.1.1 The specificity and activity of glucoamylase

The range of substrates attacked by an enzyme yields information about its specificity, while activity is a measure of its degree of purity. Glucoamylase has been reported to catalyse the hydrolysis of both α-(1,4)- and α-(1,6)-D-glucopyranosyl linkages in starch, and the activities of glucoamylases from various sources have been determined using substrates containing α-(1,4)-, α(1,6)-, and both α-(1,4)- and α-(1,6)-D-glucopyranosyl linkages in order to investigate the specificity of this enzyme. It has been the normal practice having purified an enzyme to measure its activity toward various substrates. In this Thesis therefore, a selection of the results reported by various authors together with those obtained in this work have been discussed and compared. The majority of previous work on the specificity and activity of glucoamylase has been carried out using mixtures of the now readily isolatable forms I and II.

The activity of glucoamylase is dependent upon the following factors: the source of the enzyme, the substrate, and reaction conditions such as temperature, pH and buffer concentration. A knowledge of the activity of the glucoamylase preparation used in the food industry is important because while sufficient enzyme must be employed to facilitate the conversion of starch into D-glucose in a reasonable time, an excess would not only be wasteful and decrease product purity, but may decrease the glucose content by increasing the rate of reversion.

Isomaltose has been reported by several workers[1-9] to act as a substrate for glucoamylase while others[10-12] have reported that the enzyme does not hydrolyse the α-(1,6)-D-glucopyranosyl linkage in this compound. Barker et al.,[11] however, did report a very slow attack on isomaltotetraose by glucoamylase from Aspergillus niger. Glucoamylase from Aspergillus niger and Endomycopsis bispora had been incubated with dextran by Barker et al.,[11] and Ruttloff et al.,[1,2] respectively, with the result that this polysaccharide was not attacked by this enzyme. Abdullah et al.[5] carried out activity tests on several α-(1,6)-D-glucopyranosyl linked oligosaccharides with glucoamylase from Aspergillus niger and although the rate of hydrolysis appeared to increase with increasing degree of substrate polymerisation, dextran was not tested. It was for this reason that purified glucoamylases I and II from this work were tested for activity toward dextran, and to compare the activity to that using wheat amylopectin. The activity of the purified glucoamylases were also measured using maltose as substrate. This was carried out to determine whether maltose was bound to the enzyme active site but reacted sufficiently slowly to enable it to be used as an active site protector in the labelling study discussed in CHAPTER 6 of this Thesis.

Pazur et al.[13] reported that purified glucoamylase from Aspergillus niger was capable of hydrolysing the α-(1,4)- and α-(1,6)-D-glucopyranosyl linkages in a variety of D-glucopyranosyl oligosaccharides and showed that the smallest structural entity necessary in a substrate for glucoamylase action was a D-glucosyl unit linked through an α-(1,4)- or an α-(1,6)-bond to a D-glucose molecule

which was either free or substituted at position 1. The initial hydrolysis of the substrate by glucoamylase occurred at the non-reducing end and proceeded at a faster rate if the terminal unit was linked by an α-(1,4)- bond rather than *via* an α-(1,6)- bond to the remainder of the oligosaccharide molecule. These results have subsequently been confirmed by Abdullah *et al.*[5]

Kawamura *et al.*,[7-9] have reported the relative rates of hydrolysis of several glucobioses by glucoamylase from different sources. However care must be taken if these results are to be applied to larger molecules. It has been suggested by Abdullah *et al.*[5] that the α-(1,6)-D-glucopyranosyl linkages present in amylopectin are likely to be hydrolysed rapidly, and an erroneous prediction of the rate of hydrolysis of these linkages would be obtained by a simple comparison of the rate of hydrolysis of maltose and isomaltose by glucoamylase. However, merely having a larger molecule is not sufficient in itself to bring about hydrolysis as demonstrated by the use of dextran as a substrate for glucoamylase discussed previously.

Fleming *et al.*,[14] and Suetsugu *et al.*,[15] have reported that phenyl-α-D-glucoside is a substrate for glucoamylases from *Aspergillus niger* (Agidex 3,000) and *Rhizopus delemar* respectively. It was reported in the latter work that the rate of hydrolysis of phenyl α-D-maltoside catalysed by glucoamylase occurred at a much higher rate than that of the corresponding glucoside, the former substrate being used by Ono *et al.*[16] in a study of the anomeric form of sugar produced by the action of glucoamylase from *Rhizopus delmar*. The Michaelis constants (K_m) and the molecular activities (k_o) were determined by Suetsugu *et al.*[15]

for the glucoamylase catalysed hydrolysis of phenyl α-D-glucosides with substituents p-NO_2, H, p-CH_3 and p-$C(CH_3)_3$, and phenyl α-D-maltosides with p-NO_2, H, p-CH_3, p-C_2H_5 and p-$C(CH_3)_3$. It was found that the rate of hydrolysis of phenyl α-D-maltoside was approximately equal to the value reported by Nagao[17] for the hydrolysis of maltose by glucoamylase, but the rate of hydrolysis of phenyl α-D-glucoside by this enzyme was 490 times slower than maltose. Pazur et al.[18] also reported that glucoamylase from Aspergillus niger catalysed the hydrolysis of phenyl α-D-glucoside. These results are of some interest because they open up the possibility of carrying out precise kinetic measurements using fast reaction techniques on the reaction catalysed by glucoamylase. Very few such studies have been carried out on glycoside hydrolases because of the lack of suitable substrates or the occurrence of competing transfer reactions. The difference in k_o observed between the glucosides and maltosides could not be explained by the chemical nature of the bond being hydrolysed. However, it was reasonably accounted for in terms of the statistical weight of productive and nonproductive complexes as determined by the subsite affinity of this enzyme as described by Hiromi[19] which will be discussed later.

It would appear that the ability of glucoamylase to hydrolyse an α-(1,3)-D-glucopyranosyl linkage has not been determined conclusively. Several workers[5,18,20,21-23] have reported that such a linkage can be hydrolysed by glucoamylase while others[11,24] have suggested that the enzyme is unable to facilitate hydrolysis. Thivend et al.,[21] have reported that the rate of hydrolysis of an α-(1,4)-D-glucopyranosyl

TABLE 5.1

The specific activity of purified glucoamylase preparations from various sources

GLUCOAMYLASE	SOURCE OF ENZYME	REPORTED SPECIFIC ACTIVITY	SUBSTRATE	pH	TEMP. °C	REF.
PURIFIED GLUCOAMYLASE I	Aspergillus niger	2,270 units/mg	AMYLOSE	5.0	23	96
PURIFIED GLUCOAMYLASE II		2,015 units/mg				
RECHROMATOGRAPHED GLUCOAMYLASE A ON DEAE-CELLULOSE	Aspergillus phoenicis	12.2 units/mg protein	3% w/v LINTNER SOLUBLE STARCH	4.8	ROOM TEMP.	90
RECHROMATOGRAPHED GLUCOAMYLASE B ON DEAE-CELLULOSE		6.7 units/mg protein				
RECHROMATOGRAPHED GLUCOAMYLASE I ON DEAE-CELLULOSE	Aspergillus niger	12.0 units/mg protein	3% w/v LINTNER SOLUBLE STARCH	4.8	30	4
RECHROMATOGRAPHED GLUCOAMYLASE II ON DEAE-CELLULOSE		16.0 units/mg protein				
LESS ACID STABLE SACCHAROGENIC AMYLASE (FINAL PREPARATION)	Aspergillus awamori	101.0 units/mg protein	-	-	-	88
CRYSTALLINE GLUCO-AMYLASE I	Mucor rouxianus	25.5 units/mg	0.5% SOLUBLE STARCH	4.5	40	97
CRYSTALLINE GLUCO-AMYLASE II		25.3 units/mg				

TABLE 5.1 (continued)

GLUCOAMYLASE	SOURCE OF ENZYME	REPORTED SPECIFIC ACTIVITY	SUBSTRATE	pH	TEMP. °C	REF.
CRYSTALLINE GLUCOAMYLASE	Rhizopus delemar	228 arbitrary units	-	4.5	25	15
CHROMATOGRAPHED GLUCOAMYLASE ON CM-CELLULOSE	Endomyces species IFO 0111	34 units/mg protein	0.5% LINTNER SOLUBLE STARCH	4.8	30	98
TWICE CRYSTALLISED GLUCOAMYLASE	Aspergillus awamori	44.2 units/mg protein	-	-	-	99
HIGHLY PURIFIED GLUCOAMYLASE	Aspergillus awamori	990 units/mg protein	-	-	-	85
PURIFIED ACID GLUCO-AMYLASE	Calf heart muscle	1,100 units	-	-	-	91
PURIFIED GLUCOAMYLASE	Macaca mullata monkeys	ORGAN ACTIVITY INTESTINE 70 m-units/mg protein LIVER 8 " KIDNEY 4 " SPLEEN 4 "	'MERCK' STARCH	4.8	37	92
PURIFIED GLUCOAMYLASE I AFTER DEAE-CELLULOSE CHROMATOGRAPHY	Aspergillus niger (Agidex 3,000)	15.3 International units/mg protein	1% WHEAT AMYLOPECTIN	5.0	25	THIS WORK
		4.1 " "	1% 'MERCK' MALTOSE	4.6		
PURIFIED GLUCOAMYLASE II AFTER DEAE-CELLULOSE CHROMATOGRAPHY		17.2 " "	1% WHEAT AMYLOPECTIN	5.0		
		3.3 " "	1% 'MERCK' MALTOSE	4.6		

linkage by glucoamylase is faster than either the hydrolyses of an α-(1,6)- or an α-(1,3)-linkage; moreover in the latter case the rate of hydrolysis has been reported to be independent of the size of the molecule. Fleetwood et al.,[25] reported that it was the C1-oxygen bond of the glycoside substrate which was cleaved by this enzyme, while it had already been shown by Weill et al.[26] that the D-glucose unit liberated by glucoamylase had undergone a Walden inversion resulting in a change of configuration at C1. This latter discovery was subsequently confirmed by Ono et al.[16] and Eveleigh et al.[27]

The specific activity of an enzyme preparation is a measure of its degree of purity and is defined as the number of micro moles of product produced per minute per milli gram of protein under defined conditions. (Normally the conditions are chosen to be at the optimum pH for maximum enzyme activity at 25°C, and the substrate concentration should be high enough to saturate the enzyme so that the kinetics of the assay will approach zero order with respect to substrate.)

The specific activities of various glucoamylase preparations reported in the literature are presented in TABLE 5.1. Unfortunately not all have been measured under similar conditions and therefore a direct comparison for each preparation cannot be made. The specific activities of the crude enzyme preparations have been excluded from TABLE 5.1 because they may represent the action of more than one hydrolase on the substrate as has been pointed out by Lineback et al.[4]

Because of the large discrepancy in the specific activity values reported, Kujawski et al.,[28] proposed a unified method of glucoamylase activity determination from microbial material. The conditions specified were similar to those suggested for specific activity determination but unlike the latter, pH, temperature, substrate and reaction time were defined precisely.

To test for the presence of an 'endo' acting enzyme in purified glucoamylase, an experiment was carried out by Marshall[29] using Cibachron Blue F3GA-amylose.[30,31] This substrate carries a dye on some of the primary alcoholic groups[32] of the anhydroglucose units comprising the amylose, this is in contrast to the Remazolbrilliant Blue R starch[33] which carries the dye on the C2 position of the polymer units. The action of glucoamylase on Cibachron Blue F3GA-amylose proceeds to release D-glucose from the nonreducing end of the substrate but on reaching a glucose unit bearing the dye, enzyme activity is terminated because the enzyme is unable to by-pass the anomalous unit. The action of an 'endo' acting enzyme such as α-amylase on this substrate cleaves the dyed amylose chain in a random manner, the small oligosaccharides thus produced are soluble in aqueous media and their colour can be measured spectrophotometrically. If glucoamylase is contaminated with an 'endo' acting enzyme the oligosaccharides may be further degraded to D-glucose. Other sensitive methods to detect 'endo' acting carbohydrases in the presence of 'exo' acting enzymes on the same substrate have been reported in the literature.[34,35] These methods have the advantage that they use a soluble substrate, 5% w/w periodate oxidised amylose, and therefore

facilitate a homogeneous enzyme reaction. Cibachron Blue F3GA-amylose is insoluble in aqueous media and therefore the enzyme reaction is heterogeneous with respect to substrate.

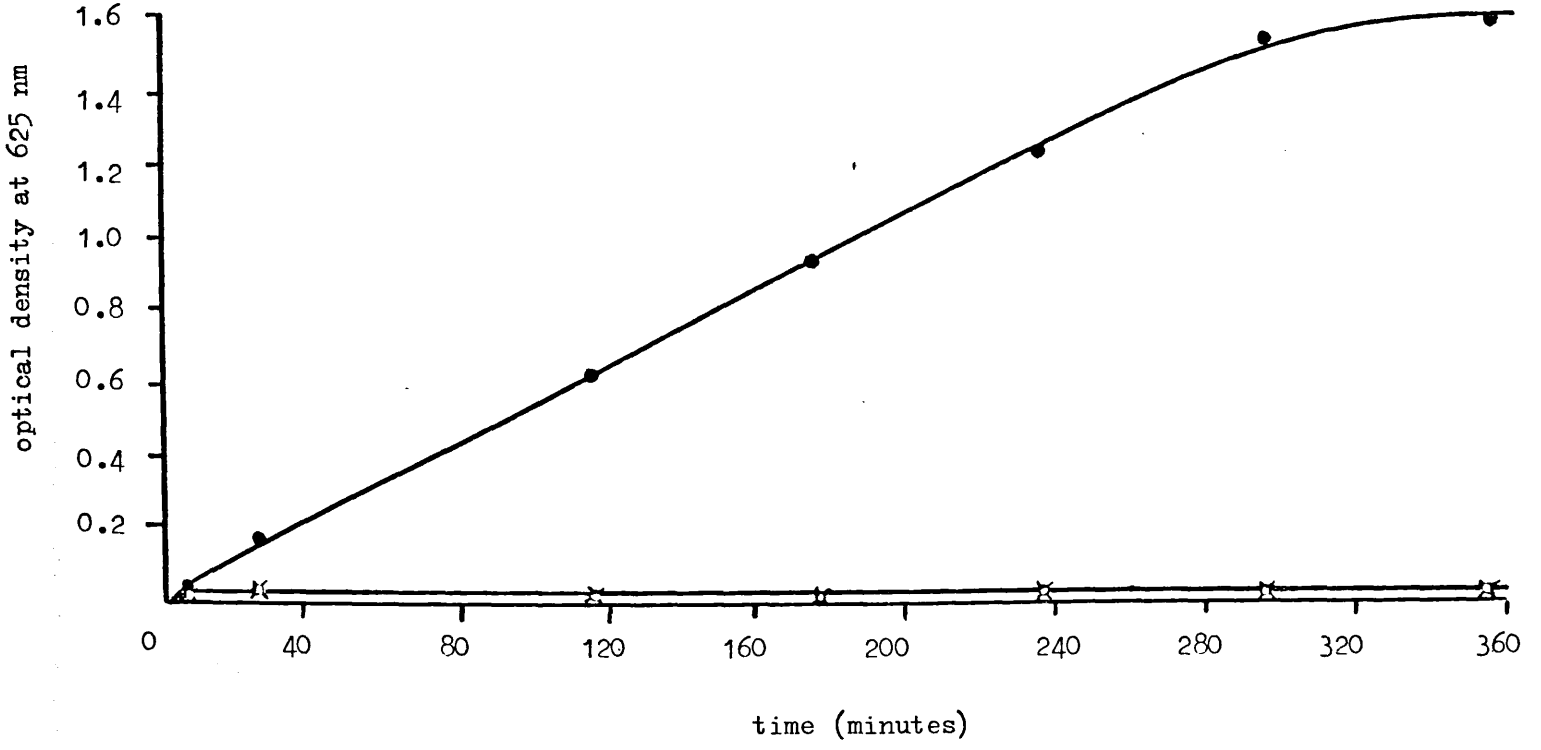
5.1.2 Results and Discussion

In this work it was found that glucoamylase facilitated almost complete hydrolysis of wheat amylopectin to D-glucose, (see Section 5.3.2) suggesting that this enzyme was capable of hydrolysing both α-(1,4)-, and α-(1,6)-D-glucopyranosyl linkages in this substrate.

To test the ability of glucoamylase to hydrolyse an α-(1,6)-D-glucopyranosyl linkage in dextran to produce D-glucose, samples of "Sephadex" G-100 and G-200 were incubated with glucoamylase I and II at pH 5.0. After 24 hours, no D-glucose could be detected in the digests using the glucose oxidase assay procedure described by Lloyd *et al.*[41] The dextran used for this test was highly cross linked and insoluble so it was decided to repeat the experiment using a soluble dextran produced by "Glaxo" for blood filling. Paper chromatograms were run of each digest together with dextran and glucoamylase blanks over a period of 72 hours but no reducing sugars were detected. It was concluded therefore that probably no hydrolysis of the dextran had occurred and if any reducing sugar had been produced it was not detected by the chromatography procedure employed.

Thus the specificity of glucoamylase is apparently such that it catalyses the hydrolysis of nonreducing end groups linked preferentially through an α-(1,4)-bond to an aglycone which in decreasing order of activity is: a chain of α-1,4-linked D-glucosyl residues, a single

FIGURE 5.1 Graph of colour released from Cibachron blue F3GA - amylose by purified glucoamylase I (o), glucoamylase II (x), and crude dialysed Agidex 3,000 (•).



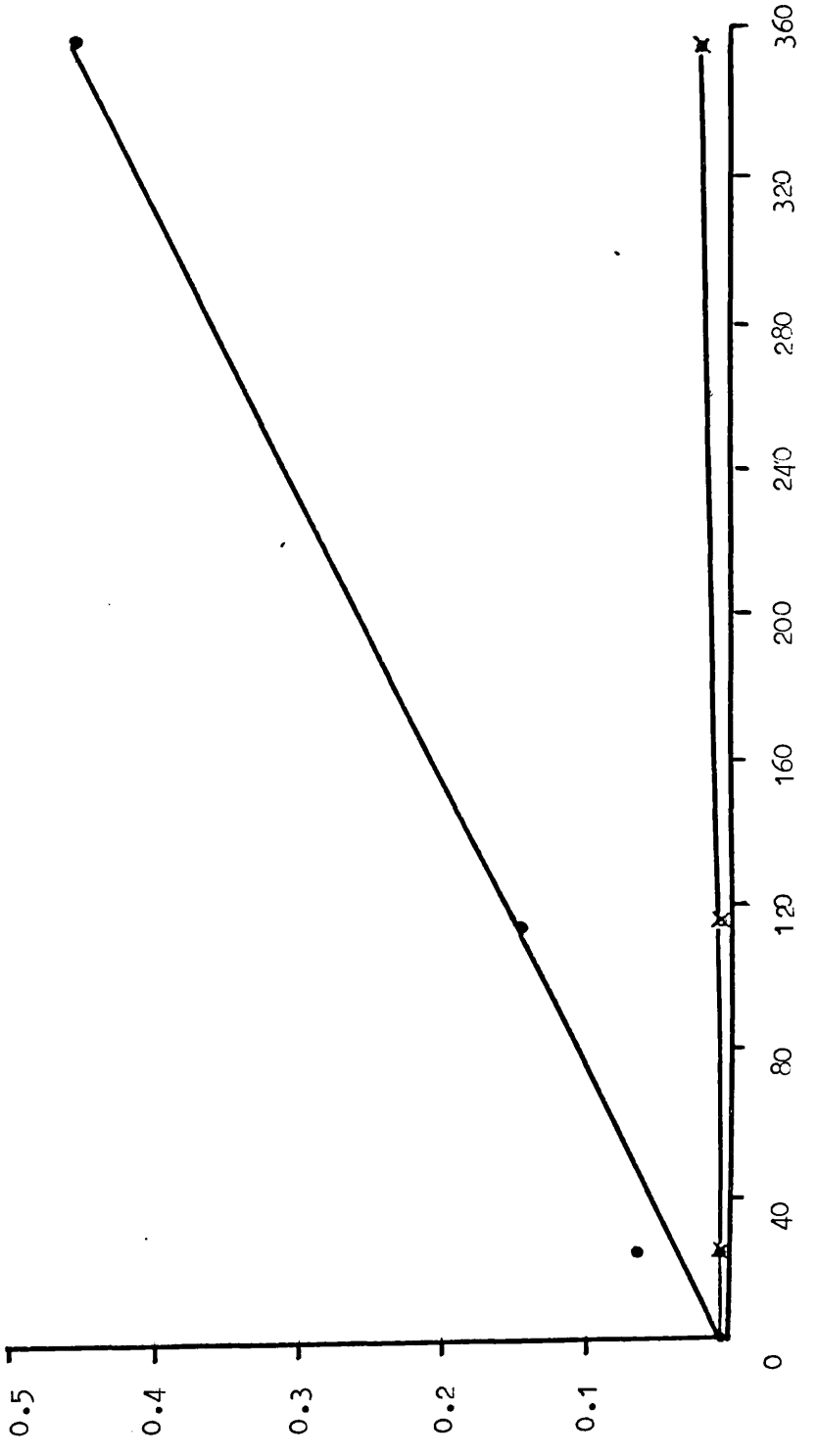


FIGURE 5.2 Graph of D-glucose release from Cibachron blue F3GA - amylose by purified glucoamylse I (•), glucoamylase II (x) and crude dialysed Agidex 3,000 (•).

D-glucose residue, phenol or a substituted phenol. Polysaccharide chains of anhydroglucose residues linked through other than α-(1,4)-bonds (with the possible exception of α-(1,3)-) are not attacked.

The results of this work are in agreement with previous work reported in the literature.[1,2,11,38]

From FIGURE 5.1 it can be seen that no colour was released from Cibachron Blue F3GA-amylose when incubated with purified glucoamylase I and II over 360 minutes while the crude dialysed enzyme produced a colour at essentially a linear rate with respect to time. FIGURE 5.2 shows the D-glucose produced in each digest during the incubation of Cibachron Blue F3GA-amylose with the various glucoamylases used in this work. It can be seen that the release of D-glucose by the purified glucoamylases I and II is small compared to that released by the crude dialysed "Agidex 3,000" over the same period of time. It was concluded therefore that an 'endo' acting enzyme was present in the crude "Agidex 3,000" enzyme but it had been removed from glucoamylases I and II during the purification procedure described in CHAPTER 2 (2.4.4) of this Thesis.

The specific activity of glucoamylase was measured using both wheat amylopectin and maltose as substrates. A 1% w/v wheat amylopectin solution buffered at pH 5.0 was reacted with glucoamylase for 15 minutes at 25°C (25°C was the temperature suggested for enzyme activity by the International enzyme commission).[36,37] When 1% w/v maltose was used as a substrate under identical conditions, the

specific activities of glucoamylases I and II were 4.1 and 3.3, respectively, representing approximately 25% and 17% of the values obtained from wheat amylopectin. Fukumoto[38] has reported that the activity of glucoamylase from Rhizopus delemar and Aspergillus niger toward maltose was in the order of 10% of that exhibited toward amylopectin. The lower activities of glucoamylase with maltose than with higher α-(1,4)-D-glucopyranosyl linked substrates agrees with the findings of other workers.[1,2,4,5,39,40] The maltose used in this work was tested for the presence of other reducing sugar contaminants such as glucose and maltotetraose using paper chromatography. It was found that the sample ran as a single spot and was therefore free from such impurities, hence the specific activity value reported did not represent a composite figure obtained from the action of glucoamylase I and II on a heterogeneous substrate.

5.2.1 The Influence of substrate chain length on the Activity of glucoamylase

In studies of glucoamylase from Aspergillus niger and Rhizopus delemar carried out by Abdullah et al.,[5] and Ono et al.,[42] respectively, it was discovered that the overall rate of reaction was largely dependent on the chain length of the substrate, maltopentaose reacting faster than either maltotriose, amylodextrin, amylose or maltose. In a later work by Ono et al.,[43] it was reported that glucoamylase exhibited a high affinity for α-D-glucose and it was suggested that this molecule was buried in the active centre of the enzyme. In a study on the mechanism of action of glucoamylase from Aspergillus niger by Barker et al.,[11] thirty oligo- and polysaccharides were used as enzyme

substrates. It was found that the attack of glucoamylase on the substrates was a multi-chain process consisting of the stepwise removal of single D-glucose units from their nonreducing chain ends, and is in agreement with that proposed by Pazur et al.[39] Bendetskii[44] carried out a kinetic analysis of the time dependence of the decomposition of a linear homopolymer under the action of an exohydrolase by single and multichain mechanisms. The equations were reported to describe well the hydrolysis of amylose by glucoamylase from Rhizopus delemar. In later work Bendetskii et al.,[45] reported that the mechanism of action of glucoamylase from Aspergillus awamori depended upon the degree of polymerisation of the substrate. When depolymerised amylose, or a linear malto-dextrin with a degree of polymerisation of 15 were used as substrates, a multichain mechanism was observed. However, when amylose was used, a combined mechanism was observed. It was concluded therefore, that the greater the average chain length of the substrate, the closer the hydrolysis resembled a single-chain mechanism. In a review of amylases and their properties by Allen et al.,[46] glucoamylase was reported to have a multiple attack mechanism of action on starch. Multiple attack corresponds to two or more bonds (but not all the bonds in a single chain) hydrolysed per enzyme-substrate encounter, and therefore corresponds to the combined mechanism suggested by Bendetskii et al.[45] for the hydrolysis of amylose.

Kinetics of 'exo' enzyme catalysed degradation of linear polymer substrates have been discussed by Hiromi et al.[47] The variation of k_o and K_m with the degree of substrate polymerisation was used to

evaluate subsite affinities A_i, and an intrinsic rate constant k_{int}, of linkage hydrolysis assuming independency of k_{int} on chain length and the additivity of A_i. The calculated values of k_o and K_m agree with the experimental values for substrates having a degree of polymerisation between 2 and 7. It was concluded that the apparent dependency of velocity of hydrolysis on degree of polymerisation of substrate could be accounted for in terms of the probability of productive complex formation, even though the intrinsic rate of hydrolysis k_{int}, was independent of the substrate chain length. In theoretical calculations of rate parameters, A_i values were taken to be equal for each subsite but when a comparison was made between the experimental values of rate parameters for glucoamylase catalysed hydrolysis of linear malto-oligosaccharide substrates, it was apparent that the A_i values should not have been equal, but should be greatest near the catalytic site and decrease with the distance from it. The subsite affinity values shown in TABLE 5.2 give rate parameters consistent with the experimental results for glucoamylse.

TABLE 5.2

The subsite affinities used by Hiromi[19] for the calculation of rate parameters for the hydrolysis of linear substrates by glucoamylase.

Subsite affinity	Subsite affinity value kJ/mole
$A_1 - D^*$	0
A_2	20.29
A_3	6.65
A_4	1.80
A_5	0.92
A_6	0.46
A_7	0.42

* D = strain free energy.

Watanabe et al.[20] also confirmed that the velocity of D-glucose production catalysed by both forms of purified glucoamylase was influenced by substrate chain length.

Although it had been well established that glucoamylase could hydrolyse both α-(1,4)-and α-(1,6)-D-glucopyranosyl linkages in various substrates, it was of interest to determine whether both linkages were hydrolysed in the same active site of the enzyme or whether two distinct active sites existed each being specific for a particular linkage. An alternate explanation was that two enzymes existed each hydrolysing a specific linkage.

The usual way of investigating such a problem is to measure the pH/activity profile and the heat stability for each type of activity to determine whether they are coincident.

Hiromi et al.,[48] carried out this type of investigation for glucoamylase from Rhizopus delemar using maltose and panose as substrates. The ionisation constants of the essential ionizable groups of the free enzyme for maltose and panose were established and their pKa's found to be 2.9 and 5.9 for both substrates. This information together with that obtained in the study on the competition between the two types of substrates,[58] suggested that both the α-(1,4)- and α-(1,6)-D-glucopyranosyl linkages were hydrolysed in the same single active centre of the enzyme. Hiromi et al.[100] have further shown that isomaltose is hydrolysed in the same active centre as maltose and panose. This is in agreement with the reversion reaction catalysed by glucoamylase discussed in Section 5.4.1.

5.2.2 Results and Discussion

A detailed quantitative kinetic analysis using substrates of different chain lengths was not carried out in this work. However it was shown during specific activity tests that glucoamylases I and II hydrolysed maltose at approximately 25% and 17% of the respective rate of hydrolysis of wheat amylopectin. This agrees with the general result reported by Abdullah et al.[5] and other workers[38,49] that maltose is hydrolysed at a slower rate than amylopectin.

5.3.1 The degree of conversion of starch to D-glucose by glucoamylase

The production of D-glucose from starch is the unique and characteristic activity of glucoamylase and has been of considerable importance in the food industry for some time. It is hardly surprising therefore that there are many reports in the literature which refer to the degree of conversion and action of glucoamylase on this polysaccharide.

Although high yields of D-glucose are obtained from starch and amylopectin, by catalytic hydrolysis using glucoamylase, reports on the ability of this enzyme to hydrolyse the α-(1,6)-D-glucopyranosyl linkages present in these substrates were conflicting.[50,51] Meyer[52] found that the source of glucoamylase influenced the ability to hydrolyse α-(1,6)-D-glucopyranosyl linkages in starch. It was reported that "amyloglucosidases" from autolysed yeast[53] and muscle[54] were able to facilitate hydrolysis of such linkages whereas glucoamylase from Aspergillus niger and Rhizopus delemar were unable to facilitate their hydrolysis. In later reports by Fukumoto et al.,[55,56]

TABLE 5.3

The extent of hydrolysis of starch catalysed by glucoamylases from various sources

ENZYME SOURCE	NAME USED FOR ENZYME	% HYDROLYSIS OF STARCH	REF.
Clostridium acetobutylium	MALTASE	100	72
Rhizopus delemar	GLUCAMYLASE	92	50
" "	"	100	73
Aspergillus niger	AMYLOGLUCOSIDASE	100	26
Aspergillus awamori	DEBRANCHING ENZYME	100	74
Aspergillus niger	AMYLOGLUCOSIDASE	100	13, 39
Aspergillus usamii	ɣ-AMYLASE	70	75
Rhizopus tonkinensis	GLUCOGENIC ENZYME	80	76
Aspergillus niger	AMYLOGLUCOSIDASE	80	77
Aspergillus oryzae	TAKA-AMYLASE B	78	78
Aspergillus niger	AMYLOGLUCOSIDASE	78	79
Aspergillus awamori	SACCHAROGENIC AMYLASE	80	80
Aspergillus niger	GLUCAMYLASE	80	11
Neurospora species	AMYLOGLUCOSIDASE	75	81
Monascus purpureus	AMYLOGLUCOSIDASE	74	81
Endomyces species	AMYLOGLUCOSIDASE	80	82

it was found that glucoamylase from Rhizopus delemar was capable of catalysing the complete hydrolysis of starch into D-glucose from its nonreducing ends. Similarly, Dobrolinskaya et al.,[57] reported that purified glucoamylase from Aspergillus awamori catalysed the complete conversion of the starch in corn meal to D-glucose, and Pazur et al.,[39] reported that purified glucoamylase I from Aspergillus niger converted starch, amylose, amylopectin and amylodextrin to D-glucose in yields approximating complete conversion. Barker et al.,[11] reported that glucoamylase from Aspergillus niger facilitated a rapid and complete conversion of amylose to D-glucose. Hiromi et al.,[58] cited two works by Tsujisaka et al.,[10, 59] in which glucoamylase from Rhizopus delemar was reported to catalyse complete hydrolysis of starch to D-glucose. Fleming et al.,[14] reported that purified glucoamylase from Aspergillus niger (Agidex 3,000) catalysed the hydrolysis of starch. However, closer examination of the literature reveals that the completeness of conversion of starch to D-glucose has not been precisely determined, and when this has been determined the degree of conversion has in many cases approached, but not reached 100%. Both Marshall et al.,[24] and Fleming et al.,[61] have documented examples of incomplete starch conversion to D-glucose by glucoamylases. Some results are shown in TABLE 5.3. In a study of starch conversion by glucoamylase from Aspergillus niger and Rhizopus niveus by Marshall et al,[24] it was suggested that the incomplete conversion of Floridean starch may have resulted from this substrate having a relatively high proportion of α-(1,3)-D-glucopyranosyl linkages present as reported by Peat et al.[60] The action of glucoamylase from Aspergillus awamori on starches from various sources have been reported by Watanabe et al.[20] The extent of hydrolysis of

the starch samples ranged from 92% for soluble potato starch to 99% for glutinous rice starch. In a study of starch hydrolysis by Smiley et al.,[40] using glucoamylase from Aspergillus awamori, it was found that waxy maize starch had a greater initial reaction rate than the starches from rice, potato, or corn. The hydrolysis of both intact and gelatinized starch grains from clover leaves and potato by a purified glucoamylase from Aspergillus niger (Agidex 3,000) was reported by Bailey et al.[62] It was found that the intact leaf starch had the greatest reaction rate. Similar results were also found using starches from tobacco leaves and maize. Shetty et al.[63] using glucoamylase from Aspergillus niger and Rhizopus niveus to produce D-glucose, reported 83.9% and 31.9% conversion from each respective enzyme source after 64 hours incubation with intact wheat starch. The literature contains conflicting reports concerning the action of glucoamylase on intact starch granules. Macrae et al.[64] reported that commercial glucoamylase from Aspergillus niger did not attack raw starch, and Manners,[65] that glucoamylase had only a limited action on starch granules. On the other hand, Evers et al.,[66] presented scanning electron microscopic evidence of attack on starch granules by "Agidex". However, it is possible that the attack observed by Evers et al.[66] resulted from α-amylase in commercial preparations of glucoamylase from Aspergillus niger.[24, 35, 66] It has been well established that α-amylase attacks granular starch.[67]

Japanese workers[68] reported that a commercial glucoamylase from an Endomyces species gave 6.1 and 1.5% digestion of native corn starch and potato starch, respectively, when 25 mg of starch was treated with 6 I.U. of enzyme for 1 hour at 37°C. Again, previous workers[69] had

shown that this commercial enzyme preparation contained an α-amylase which was extremely difficult to separate from the glucoamylase. Leach et al.[70] also used an impure glucoamylase to partially digest granular corn starch.

It is interesting to note that the percentage conversion obtained by Shetty et al.,[63] using glucoamylase from Rhizopus niveus which has been shown to be devoid of α-amylase[29, 35, 66] still effects an extensive conversion of intact wheat starch. Scanning electron microscopy of the partially digested, large wheat granules revealed different morphological modes of attack by glucoamylase depending upon its source.

On the evidence of Bailey et al.,[62] Manners,[65] and Shetty et al.,[63] it was concluded that glucoamylase as well as α-amylase was capable of attacking intact starches.

The percentage conversion of various substrates given relative to a value of 100% for soluble starch have been reported by Ruttloff et al.,[1,2] using purified glucoamylase I and II from Endomycopsis bispora.

In the majority of studies on glucoamylase, starch has been used as a substrate in order to obtain information about the enzyme. In contrast to this, Thivend et al.[21] describe a method for the determination of starch using certain glucoamylases from Aspergillus niger, Rhizopus delemar, and Endomyces species. The method essentially uses the 'exo' action of glucoamylase to discriminate between modified and unmodified starches. By measuring the amount of D-glucose produced on hydrolysis by this enzyme to the total hexose present, the amount of normal and modified starch products in a sample could be

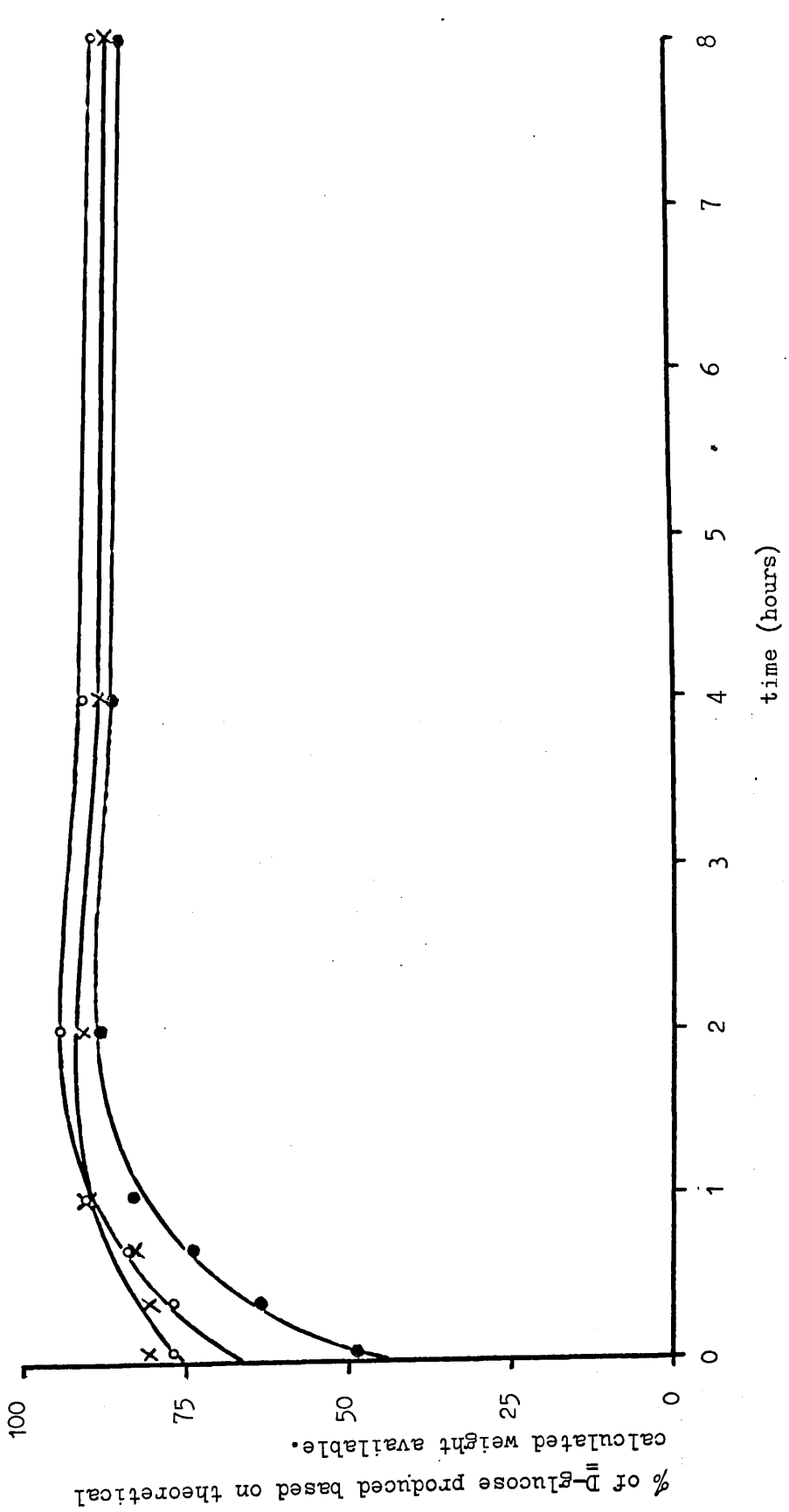
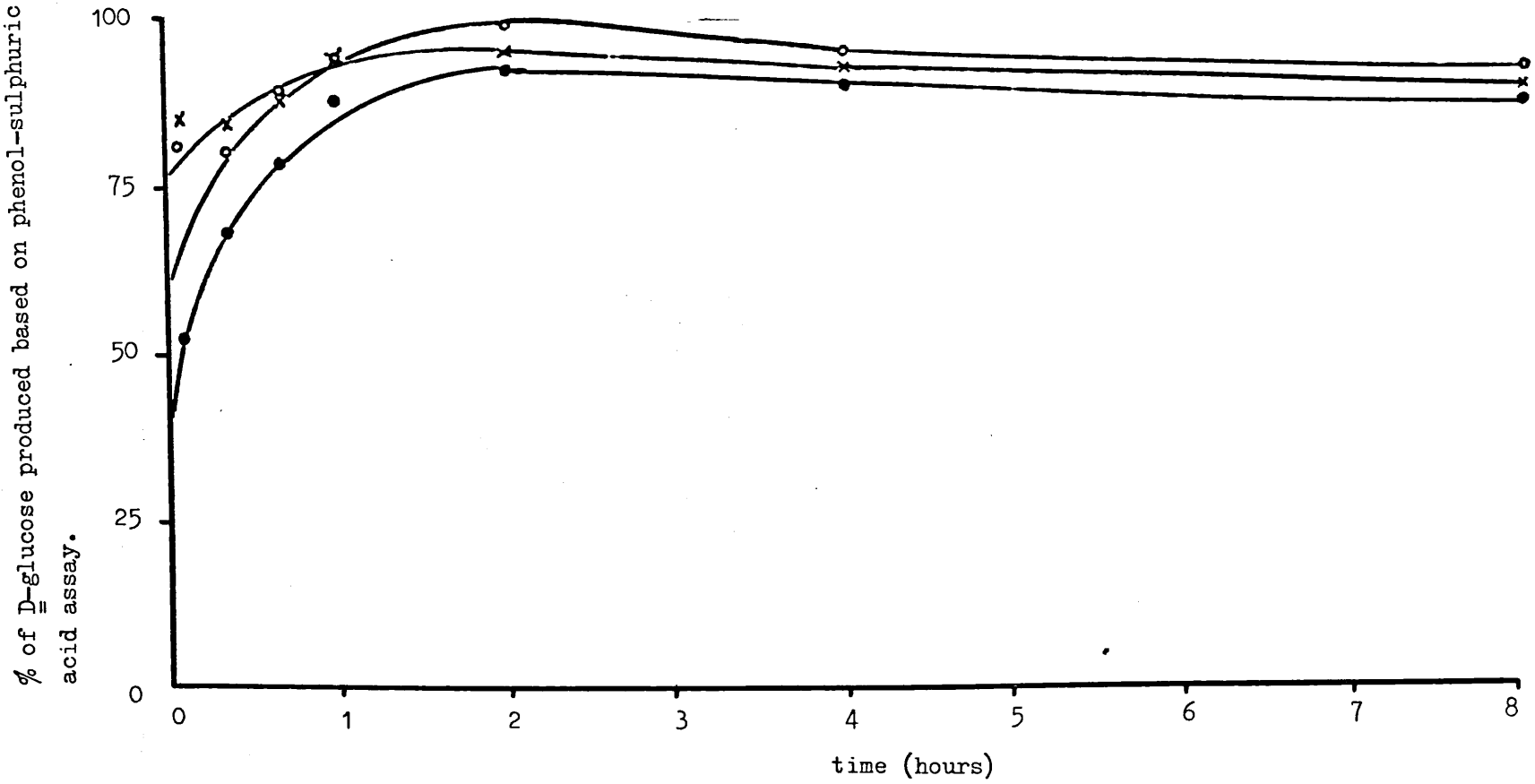


FIGURE 5.3 Conversion of wheat amylopectin to D-glucose by glucoamylose at pH 5.0, 25°C
glucoamylase I (x), glucoamylase II (•), and crude dialysed Agidex 3,000 (o).

FIGURE 5.4 Conversion of wheat amylopectin to D-glucose by glucoamylase at pH 5.0, 25°C glucoamylase I (×), glucoamylase II (•), and crude dialysed Agidex 3,000 (○).



determined. In this work, glucoamylase was reported[21] to catalyse the hydrolysis of α-(1,3)-D-glucopyranosyl linkages, and is therefore in contrast to the report by Marshall et al.,[24] who suggested that it may have been the presence of such linkages which led to an incomplete conversion of Floridean starch.

5.3.2 Results and Discussion

In this work, the production of D-glucose from wheat amylopectin (101×10^{-6} g/cm^3) by purified glucoamylases I and II and crude dialysed glucoamylase from Aspergillus niger (Agidex 3,000) has been studied. Separate digests were prepared at pH 5.0 for each glucoamylase and incubated at 25°C. Aliquots were withdrawn from each digest over a period of 8 hours and enzyme activity terminated by heating in a boiling water bath for 5 minutes. The D-glucose content of each sample was determined by the glucose oxidase method described by Lloyd et al.,[41] and related to a standard graph. The percentage of amylopectin converted to D-glucose was calculated by two independent methods. The first was based on a theoretical weight of D-glucose available from the amylopectin. The value of 162.2 was taken as the molecular weight of every anhydroglucose unit comprising the amylopectin polymer, no correction was made for the lower value (161.2) for the glucose units occurring at the branch points. The second method was based on the total amount of D-glucose available from the amylopectin as measured using the phenol sulphuric acid procedure described by Dubois et al.[71] relative to a D-glucose standard graph. Four amylopectin aliquots were used and the results averaged. The degrees of conversion at various times are shown in TABLE 5.4 and FIGURES 5.3 and 5.4. It was found (TABLE 5.4) that the

degree of amylopectin conversion based on the phenol sulphuric acid assay procedure was higher than that based on the weight of amylopectin taken. The reason for this may be attributed to an error in the weight of the amylopectin; the actual weight of amylopectin present being perhaps lower than the weight taken. The slightly high value (162.2) for the weight of an anhydroglucose unit mentioned previously would give rise to a high value for the degree of conversion, and the better estimate for the amount of convertible amylopectin present is probably that given by the phenol-sulphuric acid assay.

The conversion of wheat amylopectin by glucoamylase in this work was less than 100% as shown in TABLE 5.4. These results agree with the results of several other workers as shown in TABLE 5.3. It would appear therefore that either there is an obstacle within the starch molecule which effectively blocks the complete hydrolysis by glucoamylase or that a "reversion" reaction was taking place in competition to the hydrolysis. In fact, as can be seen from the results, the degrees of conversion reach a maximum after about 2 hours under the conditions of the experiment, and thereafter fell by about 5%. This fall may be attributed to a reversion process which is slower than hydrolysis. The incomplete hydrolysis of starch by glucoamylase is of significance in the industrial conversion process because it represents a loss of glucose theoretically available and causes a contamination of the product with the non-degradable material.

It is of interest to note that the maximum degree of conversion catalysed by the crude dialysed glucoamylase was higher than that obtained with either glucoamylase I or II. This may perhaps be due

TABLE 5.4

Percentage conversions of wheat amylopectin into D-glucose by glucoamylase

INCUBATION TIME IN HOURS AT pH 5.0	% CONVERSION TO D-GLUCOSE OF WHEAT AMYLOPECTIN BASED ON THE WEIGHT OF AMYLOPECTIN TAKEN			% CONVERSION TO D-GLUCOSE OF WHEAT AMYLOPECTIN BASED ON PHENOL-SULPHURIC ACID ASSAY		
	GLUCOAMYLASE I	GLUCOAMYLASE II	CRUDE DIALYSED GLUCOAMYLASE	GLUCOAMYLASE I	GLUCOAMYLASE II	CRUDE DIALYSED GLUCOAMYLASE
0.08	81.2	49.6	77.1	85.6	52.3	81.3
0.35	80.3	64.7	77.1	84.6	68.2	81.3
0.67	83.4	74.8	84.3	87.9	78.9	88.9
1.00	90.2	83.4	90.2	95.1	87.9	95.1
2.00	90.2	87.9	94.7	95.1	92.7	99.8
4.00	87.9	85.7	90.6	92.7	90.3	95.5
8.00	86.1	83.9	88.4	90.8	88.4	93.2

to the presence of an 'endo' acting enzyme shown to be present in the crude dialysed glucoamylase (see Section 5.1.2) which can by-pass obstacles in the starch structure. In cases where glucoamylase has been reported to effect 100% conversion of starch to D-glucose, this has been attributed by Marshall *et al.*[24] to the presence of a contaminating α-amylase. The finding of an incomplete conversion of

an amyloaceous polymer by a highly purified 'exo' enxyme has its parallel in the observation that while a crude preparation of β-amylase had been found to convert amylose to maltose, the crystalline enzyme gave conversions which were far from complete.[83] It was subsequently shown that amorphous β-amylase was contaminated with α-amylase.[84]

To determine whether the extent of starch conversion catalysed by purified glucoamylase could be increased by the addition of α-amylase, an experiment was carried out in which purified glucoamylases I and II, artificially doped with α-amylase, were used to convert wheat amylopectin to D-glucose under identical conditions to those previously used for starch conversion. The results are shown in TABLE 5.5, and by comparing the degree of conversion with the values obtained in the previous experiment (TABLE 5.4), it can be seen that purified glucoamylase in the presence of B. subtilis α-amylase effects a higher degree of starch conversion during the period of the experiment than purified glucoamylase alone. These results are in general agreement with those reported by Marshall et al.[24] However the presence of α-amylase did not prevent a decline in the degree of conversion after the maximum had been reached.

The addition of α-amylase to crude dialysed glucoamylase had relatively little effect on starch conversion presumably because the crude enzyme already contained an 'endo' acting amylase.

TABLE 5.5

Percentage conversions of wheat amylopectin into D-glucose by glucoamylase doped with B. subtilis α-amylase

INCUBATION TIME IN HOURS AT pH 5.0	% CONVERSION TO D-GLUCOSE OF WHEAT AMYLOPECTIN BASED ON WEIGHT OF AMYLOPECTIN TAKEN			% CONVERSION TO D-GLUCOSE OF WHEAT AMYLOPECTIN BASED ON PHENOL-SULPHURIC ACID ASSAY		
	GLUCOAMYLASE I + α-AMYLASE	GLUCOAMYLASE II + α-AMYLASE	CRUDE DIALYSED GLUCOAMYLASE + α-AMYLASE	GLUCOAMYLASE I + α-AMYLASE	GLUCOAMYLASE II + α-AMYLASE	CRUDE DIALYSED GLUCOAMYLASE + α-AMYLASE
0.08	64.5	69.8	67.6	68.0	73.7	71.3
0.35	83.4	78.9	78.0	87.9	83.2	82.2
0.67	85.7	85.7	83.4	90.3	90.3	87.9
1.00	94.0	97.0	92.4	99.1	100.0	97.4
2.00	92.4	92.9	95.1	97.4	97.9	100.0
4.00	92.4	92.0	92.0	97.4	97.0	97.0
8.00	90.2	90.4	85.7	95.1	95.3	90.3

From FIGURES 5.3 and 5.4 it can be seen that the rate of hydrolysis catalysed by glucoamylase I was slightly higher than that catalysed by glucoamylase II using wheat amylopectin as a substrate. This is in agreement with the work of Smiley et al.,[40] who reported a difference in the initial reaction rates between glucoamylase I and II from Aspergillus awamori.

5.4.1 The reversion reactions catalysed by glucoamylase

The normal role of glucoamylase is the catalysis of the hydrolysis of starch to produce D-glucose. In this reaction the enzyme could be said to transfer glucopyranosyl units comprising the starch polymer to molecules of water. If however a solution of D-glucose in the presence of a catalyst gives rise to di- and higher saccharides, the process is termed reversion.

This process may be formally distinguished from transglucosylation which is best defined as the transfer of a D-glucosyl (or higher saccharide) unit from a di- or higher saccharide to another molecule of D-glucose or a higher saccharide. Both reversion and transglucosylation arise from transfer to another saccharide instead of water. The difference is that reversion leads to an increase in the average degree of polymerisation whereas transglucosylation leaves it unaltered. However at a given average degree of polymerisation a (normal) distribution of different degrees of polymerisation is entropically favoured over a single unique value.

In most commercial glucoamylase preparations of fungal origin, certain amounts of α-amylase, maltase, reversion and transglucosylase activities have been reported to accompany the glucoamylase.[101, 102] Because of its adverse effect on glucose production from starch, reversion activity in commercial glucoamylase has been extensively studied and some of the results are presented in TABLE 5.6. Glucose loss due to reversion may amount to 7-8% depending on the conditions of saccharification.[103] As can be seen from TABLE 5.6, contradictory data have been reported for the reversion activity of glucoamylase preparations. Some authors consider the amount of "transglucosidase"

TABLE 5.6

Reported data on reversion catalysed mainly by glucoamylase and α-glucosidase preparations

ENZYME AND SOURCE	SUBSTRATE	PRODUCTS	LOSS OF GLUCOSE YIELD %	CONDITIONS USED				REF.
				TEMP °C	pH	REACTION TIME h	CONCN. OF SUBSTRATE	
GLUCOAMYLASE COMMERCIAL PREPARATION - Aspergilli	PARTIAL STARCH HYDROLYSATE	GLUCOSE, MALTOTRIOSE, MALTOTETRAOSE	7-8	56	4.5	72	30% SOLIDS D.E.* 10.44.	103
TRANSGLUCOSIDASE PURIFIED PREPARATION - Aspergillus niger	MALTOSE	GLUCOSE, ISOMALTOSE, PANOSE	-	30	3.5	24	0.15M	
	STARCH	GLUCOSE, ISOMALTOSE	-	30	3.5	24	1%	113
	STARCH, GLUCOSE, MALTOSE	GLUCOSE, MALTOSE TRI- AND TETRA- SACCHARIDES	-	30	3.5	24	1% STARCH 1% GLUCOSE 0.1M MALTOSE	
	ISOMALTOSE	GLUCOSE, ISOMALTOTRIOSE	-	30	3.5	24	0.15M	
	METHYL MALTOSIDE	GLUCOSE, METHYL-α-D-GLUCOSIDE		30	3.5	24	0.1M	
GLUCOAMYLASE CRUDE PREPARATION - Aspergillus niger NRRL 3112.	MALTOSE	GLUCOSE (98%) MALTOSE (1.6%) ISOMALTOSE(0.4%)	2	60	-	24 .	0.5M	119

* D.E. DEXTROSE EQUIVALENTS (REDUCING SUGARS)

TABLE 5.6 (continued)

ENZYME AND SOURCE	SUBSTRATE	PRODUCTS	LOSS OF GLUCOSE YIELD %	CONDITIONS USED				REF.
				TEMP °C	pH	REACTION TIME h	CONCN. OF SUBSTRATE	
GLUCOAMYLASE COMMERCIAL PREPARATION - Aspergillus species	MALTOSE	GLUCOSE, ISOMALTOSE	-	60	4.0	72	30% w/v	
	GLUCOSE	NONE	-	60	4.0	72	10% w/v	111
	GLUCOSE	ISOMALTOSE	-	60	4.0	72	30% w/v	
	GLUCOSE	ISOMALTOSE ISOMALTOTRIOSE	-	60	4.0	72	50% w/v	
PURIFIED GLUCOAMYLASE - Aspergillus awamori	STARCH	-	6.5	-	-	24	12%	120
GLUCOAMYLASE FREE FROM TRANSGLUCOSIDASE - Aspergillus awamori	MALTOSE	GLUCOSE, ISOMALTOSE, PANOSE, HIGHER OLIGOSACCHARIDES	-	55	-	24	25%	109
PURIFIED GLUCOAMYLASE - Aspergillus awamori	MALTOSE	GLUCOSE, ISOMALTOSE, MALTOTRIOSE, MALTOTETRAOSE, ISOMALTOTRIOSE, PANOSE, PENTASACCHARIDES	-	50	4.5-4.7	24	30%	112
	GLUCOSE	NONE	-	-	-	-	-	

TABLE 5.6 (continued)

ENZYME AND SOURCE	SUBSTRATE	PRODUCTS	LOSS OF GLUCOSE YIELD %	CONDITIONS USED				REF.
				TEMP °C	pH	REACTION TIME h	CONCN. OF SUBSTRATE	
CRUDE GLUCOAMYLASE - Rhizopus niveus	GENTIOBIOSE	TRISACCHARIDE (GENTIOTRIOSE)	-	55	5.0	24	2% w/v	7
PURIFIED GLUCOAMYLASE - Rhizopus niveus	GENTIOBIOSE	NONE	-	55	5.0	24	2% w/v	
PURIFIED GLUCOAMYLASE Rhizopus niveus	GLUCOSE	NIGEROSE-MALTOSE (1.3%) ISOMALTOSE (2.2%) OLIGOSACCHARIDES (0.5%) PANOSE, ISO-PANOSE, ISO-MALTOTRIOSE	4.0	55	-	96	40% w/v	110
PURIFIED GLUCOAMYLASE - Rhizopus niveus	GLUCOSE	NIGEROSE, ISO-MALTOSE, ISO-MALTOTRIOSE	-	55	-	24-120	30% w/v	108
PURIFIED GLUCOAMYLASE Endomycopsis species	GLUCOSE	NIGEROSE-MALTOSE (1.5%) ISO-MALTOSE (3.1%) OLIGOSACCHARIDE (0.6%)	5.2	55	-	96	40% w/v	107

TABLE 5.6 (continued)

ENZYME AND SOURCE	SUBSTRATE	PRODUCTS	LOSS OF GLUCOSE YIELD %	CONDITIONS USED				REF.
				TEMP °C	pH	REACTION TIME h	CONCN. OF SUBSTRATE	
PURIFIED GLUCOAMYLASE - Endomycopsis species 20-9.	MALTOSE	GLUCOSE, ISOMALTOSE, PANOSE	-	50	-	24	40% w/v	106
PURIFIED GLUCOAMYLASE - Aspergillus awamori	GLUCOSE	ISOMALTOSE	-	40	4.6	0-72	10% w/v	20
CRUDE GLUCOAMYLASE - Endomyces species	GENTIOBIOSE	GLUCOSE, SOPHOROSE, LAMINARIBIOSE, GENTIOTRIOSE, 6-O-β-LAMINARIBIOSYL GLUCOSE, 6-O-β-CELLOBIOSYL GLUCOSE, GENTIOTETRAOSE	-	55	5.0	12	10% w/v	8, 121
	LAMINARIBIOSE	GENTIOBIOSE, CELLOBIOSE, GENTIOTRIOSE, 3-O-β-GENTIOBIOSYL GLUCOSE, 6-O-β-GENTIOBIOSYL LAMINARIBIOSE	-	55	5.0	12	1% w/v	121
	CELLOBIOSE	4-O-β-GENTIOBIOSYL GLUCOSE	-	55	5.0	9	1% w/v	

TABLE 5.6 (continued)

ENZYME AND SOURCE	SUBSTRATE	PRODUCTS	LOSS OF GLUCOSE YIELD %	CONDITIONS USED				REF.
				TEMP °C	pH	REACTION TIME h	CONCN. OF SUBSTRATE	
TRANSGLUCOSIDASE FROM *Aspergillus usamii*	MALTOSE	GLUCOSE, ISO-MALTOSE, PANOSE, HIGHER OLIGO-SACCHARIDES		30	4.4	0-24	0.17M	51
TRANSGLUCOSIDASE ISOLATED FROM *Aspergillus niger*	MALTOSE	ISOMALTOSE, PANOSE		ROOM TEMP.	4.8	0-24	10%	4
CRUDE GLUCOAMYLASE - Source not given	GLUCOSE	NIGEROSE, MALTOSE, ISO-MALTOSE, LAM-INARIBIOSE, GENTIOBIOSE, PANOSE, ISO-PANOSE, ISO-MALTOTRIOSE		55	5.0	72-96	40%	117
ACID α-GLUCOSIDASE - Rabbit Muscle	[U-^{14}C] LABELLED MALTOSE GLYCOGEN PULLULAN	^{14}C LABELLED GLYCOGEN AND PULLULAN		-	4.8	0-5	GLYCOGEN AND PULLULAN, 80 mg/cm^3	121

TABLE 5.6 (continued)

ENZYME AND SOURCE	SUBSTRATE	PRODUCTS	LOSS OF GLUCOSE YIELD %	CONDITIONS USED				REF.
				TEMP °C	pH	REACTION TIME h	CONCN. OF SUBSTRATE	
ACID α-GLUCOSIDASE	MALTOSE	GLUCOSE, TRI-, AND HIGHER OLIGO-SACCHARIDES						122-124
ACID α-GLUCOSIDASE	OLIGO- AND POLY-SACCHARIDES	DISPROPORTIONATED SUBSTRATES						123, 125
α-GLUCOSIDASE FROM Mucor javanicus	MALTOSE AND RIBOFLAVIN	B_2-5'-α-GLUCOSE		50	5.3	0.5	5% w/v MALTOSE 0.06% w/v RIBOFLAVIN	126
α-GLUCOSIDASE FROM Mucor javanicus	MALTOSE	MALTOTRIOSE AND UNKNOWN COMPOUND X_2		37	5.3	1	5% w/v MALTOSE	127
GLUCOAMYLASE FROM Rhizopus niveus	β-D-GLUCOSE AND α-D-GLUCOSE	MALTOSE, ISOMALTOSE AND OTHER HIGHER OLIGOSACCHARIDES		30	4.5	5-24	30% w/v GLUCOSE	115
α-AMYLASE FROM six sources	α-D-GLUCO-PYRANOSYL FLUORIDE. α-D-MALTOSE	α-GLUCOSACCHARIDES		30-40	5.6-6.5	0.17	20 μ MOLE SAMPLES	128

present in commercial glucoamylase to be greatly influenced by the fermentation conditions used to produce the glucoamylase,[104,105] or by precipitation and chromatographic enzyme purification steps.[106] "Transglucosidase" activity has also been reported in highly purified glucoamylase preparations.[20, 106-110] The discrepancies in the literature as to substrate specificity,[107, 110-112] the inactivation,[103,109,112,113] and the kinds and number of products of "transglucosidase" action would suggest that "transglucosidase" was a collective term covering different enzymes. "Transglucosidase activity" is generally demonstrated by chromatographic techniques which isolate the products of the enzyme reaction. As these techniques are both time consuming and difficult to quantitate, Rose et al.,[102, 114] developed a simple and rapid enzyme assay method allowing the amount of D-glucose transformed to oligosaccharides by glucoamylase preparations to be expressed in terms of International enzyme units, irrespective of whether the reaction was accounted for by glucoamylase, by "transglucosidase" or by the joint action of both enzymes.

Although Pazur et al.[39] reported that their purified glucoamylase was devoid of "transglucosidase" activity using maltose as a substrate, many other workers have observed reversion reactions from D-glucose. Hehre et al.[115] reported that the initial reversion product from β-D-glucopyranose was maltose. However, upon longer incubation (5 h - 24 h) isomaltose accumulated as the major product. This agrees with the results reported by Watanabe et al.,[107, 110] and Fukumoto et al.,[116] that isomaltose is the major reversion product from the action of glucoamylase on D-glucose. Isomaltose has also been reported by several workers[20,106,108,109,111-113, 117,118] to be a reversion product from the action of glucoamylase on various substrates

although the amount produced relative to other reversion products have not been given.

5.4.2 Experimental results and discussion

In this work the reversion catalysed by glucoamylase was carried out using conditions corresponding as closely as possible to those employed in an industrial starch conversion process with respect to the amount of enzyme present and the final concentration of D-glucose.

When 40% w/v D-glucose solutions at pH 5.0, 25°C, were incubated with glucoamylases at concentrations of 70 $\mu g/cm^3$, loss of D-glucose, (shown in TABLE 5.7) of 11%, 15%, and 5% using glucoamylase I, glucoamylase II and crude dialysed glucoamylase, respectively, were observed after 23 h. In these experiments, a control solution of

TABLE 5.7

Percentage loss of 40% w/v D-glucose by reversion catalysed by glucoamylases

GLUCOAMYLASE	TIME (hours)	OPTICAL DENSITY AT 525 nm FROM GLUCOSE OXIDASE ASSAY		PERCENTAGE LOSS OF D-GLUCOSE
		GLUCOSE	GLUCOSE + ENZYME	
GLUCOAMYLASE I	0.33	0.3920	0.3724	5
	2.83		0.3683	6
	23.00		0.3489	11
GLUCOAMYLASE II	0.33	0.3825	0.3638	5
	2.83		0.3519	8
	23.00		0.3250	15
CRUDE DIALYSED	0.33	0.3650	0.3504	4
	2.83		0.3500	4
	23.00		0.3470	5

40% w/v D-glucose was taken as a blank to correct for any variation of D-glucose which did not result from the action of glucoamylase.

Because of the large dilution required to facilitate D-glucose analysis, the percentages of reversion may be subject to error. However, it is apparent that purified glucoamylase I and II effect a higher degree of reversion than the crude dialysed glucoamylase over 23 hours.

In order to test whether the presence of an 'endo' acting enzyme in the crude dialysed glucoamylase was responsible for the lowering in the amount of reversion product produced, D-glucose (112×10^{-6} g/cm^3) was incubated at pH 5.0, 25°C for 45 hours with both purified and α-amylase doped glucoamylase and the results shown in TABLE 5.8.

TABLE 5.8

Percentage loss of D-glucose (112×10^{-6} g/cm^3) by reversion catalysed by glucoamylases

GLUCOAMYLASE	TIME (hours)	OPTICAL DENSITY AT 525 nm FROM GLUCOSE OXIDASE ASSAY	PERCENTAGE LOSS OF D-GLUCOSE
GLUCOAMYLASE I	1.0	0.7677	TAKEN AS 0
	24.5	0.7575	2
	45.0	0.7500	3
GLUCOAMYLASE I AND α-AMYLASE	1.0	0.7650	TAKEN AS 0
	24.5	0.7575	1
	45.0	0.7560	1
GLUCOAMYLASE II	1.0	0.7950	TAKEN AS 0
	24.5	0.7800	2
	45.0	0.7300	8
GLUCOAMYLASE II AND α-AMYLASE	1.0	0.7750	TAKEN AS 0
	24.5	0.7725	1
	45.0	0.7500	3

The concentration of D-glucose was equal to that representing 100% conversion of wheat amylopectin in the previous experiments described

in Section 5.3.2, and could be readily assayed for D-glucose using glucose oxidase reagent without prior dilution. It was found that losses in D-glucose, (shown in TABLE 5.8) corresponding to 3% and 8% were observed from the purified glucoamylase I and II (100 $\mu g/cm^3$) respectively after 45 hours, while the digests artificially doped with α-amylase (16 $\mu g/cm^3$) showed losses in D-glucose corresponding to 1% and 3% respectively over the same period of time.

The results of glucoamylase catalysed reversion from D-glucose observed in this work are shown in TABLE 5.9.

TABLE 5.9

The reversion catalysed by glucoamylase observed in this work

GLUCOAMYLASE	SUBSTRATE	LOSS OF GLUCOSE YIELD %	CONDITIONS USED			
			TEMPERATURE	pH	TIME OF REACTION h	CONCENTRATION OF SUBSTRATE
GLUCOAMYLASE I GLUCOAMYLASE II CRUDE DIALYSED GLUCOAMYLASE	GLUCOSE FROM WHEAT AMYLOPECTIN	4 4 7	25	5.1	8	112×10^{-6} g/cm^3
GLUCOAMYLASE I GLUCOAMYLASE II CRUDE DIALYSED GLUCOAMYLASE	GLUCOSE	11 15 5	25	5.1	23	40% w/v
GLUCOAMYLASE I AND α-AMYLASE GLUCOAMYLASE II AND α-AMYLASE	GLUCOSE	1 3	25	5.1	45	112×10^{-6} g/cm^3
GLUCOAMYLASE I GLUCOAMYLASE II	GLUCOSE	3 8	25	5.1	45	112×10^{-6} g/cm^3

It was therefore shown that at both high, and low D-glucose concentrations, the presence of α-amylase was beneficial in preventing a loss in D-glucose by reversion catalysed by purified glucoamylases I and II. The explanation of this result is difficult to make because the final composition should be independent of the catalyst. Furthermore the lower reversion observed from D-glucose in the presence of α-amylase-doped glucoamylase is not in agreement with the reversion properties exhibited by B. subtilis α-amylase which is reported to give rise to reversion products from D-glucose[129] and maltose[115] in its own right. As isomaltose has been reported to be the major reversion product of glucoamylase action on D-glucose (see TABLE 5.6), one would expect the presence of α-amylase to have no effect on this reversion reaction because it is unable to hydrolyse the α-(1,6)-D-glucopyranosyl linkage in isomaltose or amylopectin. If the amount of reversion were dependent on the presence of a transglycosylase, then one may suppose that the purified enzymes would show a loss of this activity. One can ascribe the greater rate of starch degradation in the presence of the crude glucoamylase to the presence of other enzyme(s) (see Section 5.1.2), but these should not affect the final position of equilibrium unless they allow the operation of a new reaction (i.e. different reactants and/or products) which cannot proceed in their absence. It can only be postulated that in the cases of the crude dialysed glucoamylase, and the α-amylase-doped glucoamylases I and II, equilibrium had not been reached.

The reversion reactions catalysed by glucoamylase and α-amylase are shown in SCHEME 5.1.

SCHEME 5.1

Reactions catalysed by glucoamylase

$$\beta\text{-G} + (G)_n \leftrightarrows (G)_{n+1} + H_2O \quad (1)$$

$$\beta\text{-G} + (G)_n \leftrightarrows \begin{matrix} G \\ \downarrow \\ (G)_n \end{matrix} + H_2O \quad (2)$$

$$\beta\text{-G} + \begin{matrix} G \\ \downarrow \\ (G)_n \end{matrix} \leftrightarrows G \rightarrow \begin{matrix} G \\ \downarrow \\ G_n \end{matrix} + H_2O \quad (3)$$

$$G \rightarrow (G)_n + (G)_m \leftrightarrows (G)_{m+1} + (G)_n \quad (4)$$

$$\begin{matrix} G \\ \downarrow \\ (G)_n \end{matrix} + (G)_m \leftrightarrows (G)_{m+1} + (G)_n \quad (5)$$

$$G \rightarrow (G)_n + (G)_m \leftrightarrows \begin{matrix} G \\ \downarrow \\ (G)_m \end{matrix} + (G)_n \quad (6)$$

$$\begin{matrix} G \\ \downarrow \\ (G)_n \end{matrix} + (G)_m \leftrightarrows \begin{matrix} G \\ \downarrow \\ (G)_m \end{matrix} + (G)_n \quad (7)$$

$$G \rightarrow (G)_n + \begin{matrix} G \\ \downarrow \\ (G)_m \end{matrix} \leftrightarrows G \rightarrow \begin{matrix} G \\ \downarrow \\ (G)_m \end{matrix} + (G)_n \quad (8)$$

$$\begin{matrix} G \\ \downarrow \\ (G)_n \end{matrix} + \begin{matrix} G \\ \downarrow \\ (G)_m \end{matrix} \leftrightarrows G \rightarrow \begin{matrix} G \\ \downarrow \\ (G)_m \end{matrix} + (G)_n \quad (9)$$

Reactions catalysed by α-amylase

$$\alpha\text{-G} + (G)_n \leftrightharpoons (G)_{n+1} + H_2O \qquad (10)$$

$$G\text{-}\alpha G + (G)_n \leftrightharpoons (G)_{n+2} + H_2O \qquad (11)$$

$$(G)_n + (G)_m \leftrightharpoons (G)_o + (G)_p \qquad (12)$$

where $n+m = o + p$

In all these equations G is D-glucose

G_n, G_m are chains of 1,4 linked α-D-glucopyranosyl units n and $m \geq 1$

$\longrightarrow$ is an α-1,4 link

$\downarrow$ is an α-1,6 link

All reactions except (8) and (9) may lead to a consumption of glucose. Reactions (1), (2) and (10) may consume two glucose molecules. Reactions (4) - (9) and (12) may not consume glucose or heat but a random redistribution of linkages is entropically favoured. Reactions (1) - (7), (10) and (11) in reverse, and (12) may lead to a production of glucose.

Because of the results already reported in the literature on the characterisation of the reversion products produced from the action of glucoamylase on D-glucose, and in view of the experimental difficulty in isolating a small amount of reversion material in a large excess of glucose, it was decided not to characterise the reversion products produced in this work, particularly after an attempt to do so on a small scale using gas liquid chromatography had failed. It was decided, however, using results reported by Hehre et al.,[115] to consider the glucoamylase catalysed reversion process on a theoretical basis assuming isomaltose to be the major reversion product.

5.4.3 Thermodynamic analysis and discussion

By the principle of microscopic reversibility, it would be expected that an equilibrium would be set up between D-glucose produced from the glucoamylase catalysed hydrolysis and its reversion products. As glucoamylase catalyses the hydrolysis of both α-(1,4)- and α-(1,6)-D-glucopyranosyl linkages in amylopectin and other substrates discussed in Section 5.1.1, one may expect the reversion products formed by this enzyme to contain one or both of these linkages. When a situation occurs in which a reactant can yield several different products, the possibility arises that their formation may be either kinetically or thermodynamically controlled.

The reversion equilibrium can be generally expressed by the following equation:-

$$Glu_n + Glu \leftrightarrows Glu_{n+1} + H_2O$$

For the particular case where n = 1, the two D-glucose molecules can combine together to form either maltose or isomaltose containing an α-(1,4)- and α-(1,6)-D-glucopyranosyl linkage respectively. This can be expressed by the following equilibrium equation:-

$$\underset{\text{maltose}}{\text{Glu-Glu}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2Glu \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{isomaltose}}{\text{Glu-Glu}}$$

If (k_1-k_{-1}) is larger than (k_2-k_{-2}), but k_2/k_{-2} is larger than k_1/k_{-1} then isomaltose will be the equilibrium reversion product. However, in such a case, if the reaction is terminated before equilibrium is reached, the principal product will be maltose since it will be formed more rapidly. Kinetically and thermodynamically controlled reactions have already been well established in the field of carbohydrate chemistry,

particularly in the case of acetal formation. It would appear that maltose and isomaltose are the kinetic and thermoydnamic products respectively from the reverse reaction catalysed by glucoamylase on D-glucose.

Another interpretation for the reversion reaction observed during the action of glucoamylase on starch is that a transglucosylase enzyme is present in the glucoamylase, which catalyses the transfer of one D-glucose molecule to another at a higher rate and possibly to give different products than does glucoamylase. However, it should be emphasised that the position of thermodynamic equilibrium will be independent of the type of enzyme (catalyst) present, and will depend solely on the relative free energies of reactants (D-glucose) and products (higher saccharides and water). For the reversion reaction catalysed by glucoamylase the anomeric form of the donor D-glucose molecule is of importance. This arises from the law of microscopic reversibility which states that the mechanism of the reverse reaction is the same, in microscopic detail for the reaction in one direction as it is in the other under a given set of conditions, it must therefore be the β-anomeric form of D-glucose which is the donor for the reverse reaction, as it is this form which is produced by glucoamylase in the hydrolytic reaction.[3,16,26,27]

The current literature appears to be somewhat deficient in reports of thermodynamic quantities such as the heats and free energies of hydrolysis of various glucopyranosyl linkages. However, Burton et al.[130] have reported a value of 17.99 kJ mol^{-1} for the reaction 2 D-glucose ⇆ maltose + H_2O and Kalchkar[131] has reported a value of 8.37 kJ mol^{-1} for the reaction 2 D-glucose ⇆ isomaltose + H_2O.

Both values have been obtained by combining data for coupled reactions and may be subject to error. Hehre et al.[115] have calculated a value of 5.02 kJ mol^{-1} for the reaction D-glucose + β-D-glucopyranose ⇆ maltose + H_2O by estimating initial reversion product maltose produced by the action of glucoamylase on a 30% w/v D-glucose solution. As this method employed the measurement of maltose after elution from chromatographic paper, the result was not considered to be very quantitative.

From the free energy values of hydrolysis reported for maltose[130] and isomaltose,[131] it seemed reasonable to assume that the concentration of other reversion products produced by the action of glucoamylase on D-glucose would be very small compared with that of isomaltose. If maltose and isomaltose were the only possible products at equilibrium then their ratio would be given by the Boltzmann distribution as exp $(17.99 - 8.37)/8.37 \times 10^{-3} \times 298$ i.e. 1:49. For calculations of reversion in this work, the reversion product has been assumed to be composed entirely of isomaltose.

The difference between the free energies of hydrolysis of maltose and isomaltose is consistent with the reports[107,110,115,116] that isomaltose is the major reversion product resulting from the action of glucoamylase on D-glucose.

The physical chemistry of reversion can be considered as follows:

$$Glu_n + Glu \leftrightharpoons Glu_{n+1} + H_2O$$

The free energy change associated with the forward reaction is given by:

$$\Delta G_{final} - \Delta G_{initial} = \sum RT \ln \frac{a_{final}}{a_{initial}}$$

$$-\Delta G^{o} = \sum RT \ln a_{final}$$

At equilibrium $\Delta G_{final} = \Delta G_{initial} = 0$

$$\therefore \quad 0 = -\Delta G^{o} - \sum RT \ln a_{initial}$$

$$\therefore \quad \Delta G^{o} = -RT \ln K_{c}$$

(van't Hoff Isotherm)

For the case of formation of isomaltose from glucose we have:

$$Glu + Glu \leftrightarrows \text{isomaltose} + H_2O$$

Initial activities (a) (a) (0) (ω_o)

Final activities (a-r) (a-r) (r) $(\omega_o + r)$

$$K_c = \frac{(r)(\omega_o + r)}{(a-r)^2} \quad \text{[in favour of isomaltose formation]}$$

$$\Delta G^{o} = -RT \ln K_c = -RT \ln \frac{(r)(\omega_o + r)}{(a-r)^2}$$

$\Delta G^{o} = 8.37$ kJ mol^{-1} for α-(1,6)-D-glucopyranosyl bond formation.[131]

$$\therefore \quad 8.37 = -RT \ln K_c$$

$$= \frac{-(8.37)(298) \ln K_c}{1{,}000}$$

$$K_c = 0.0341 \text{ at } 25^{o}C$$

As the value of ΔG^{o} (8.37 kJ mol^{-1}) was reported to be the standard free energy of formation of an α-(1,6)-D-glucopyranosyl linkage,[131] no allowance was made for the anomeric configuration of the donor D-glucose molecule, and hence in the subsequent calculations

FIGURE 5.5 Plot of % water present in D-glucose solution against activity from data given by Burton et al.[130]

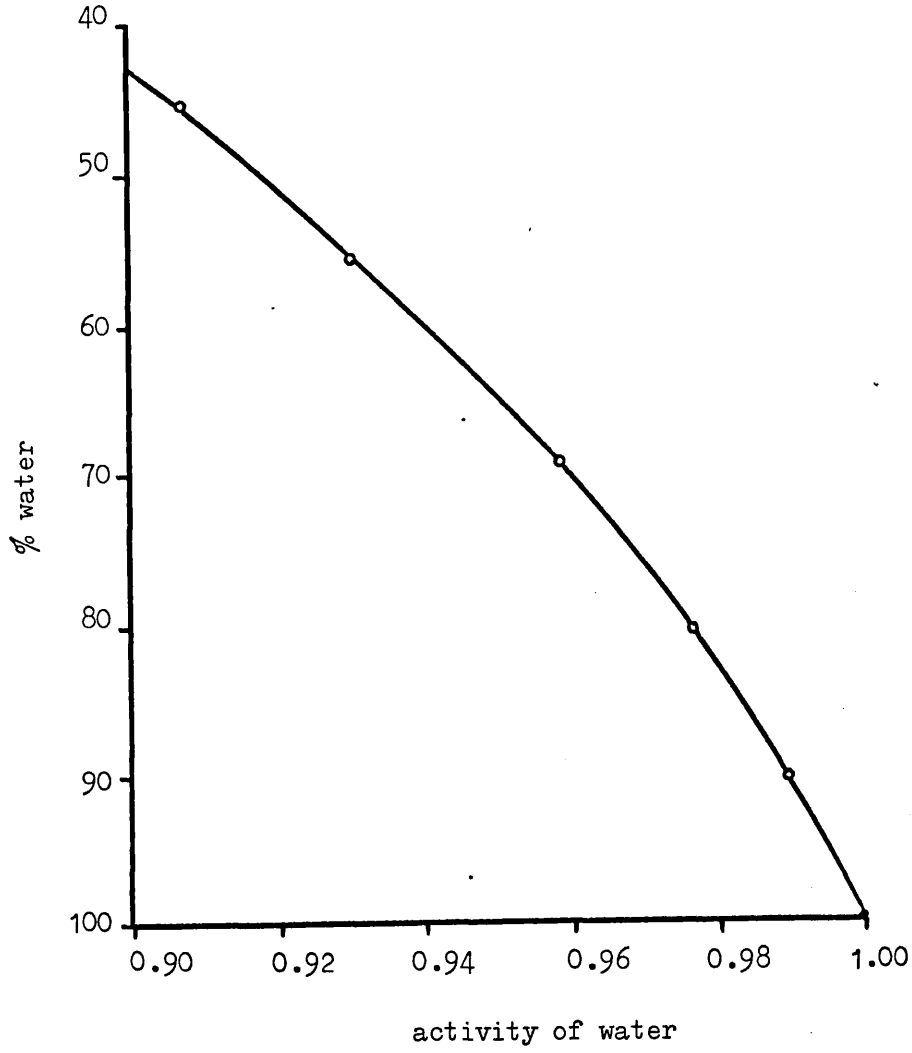
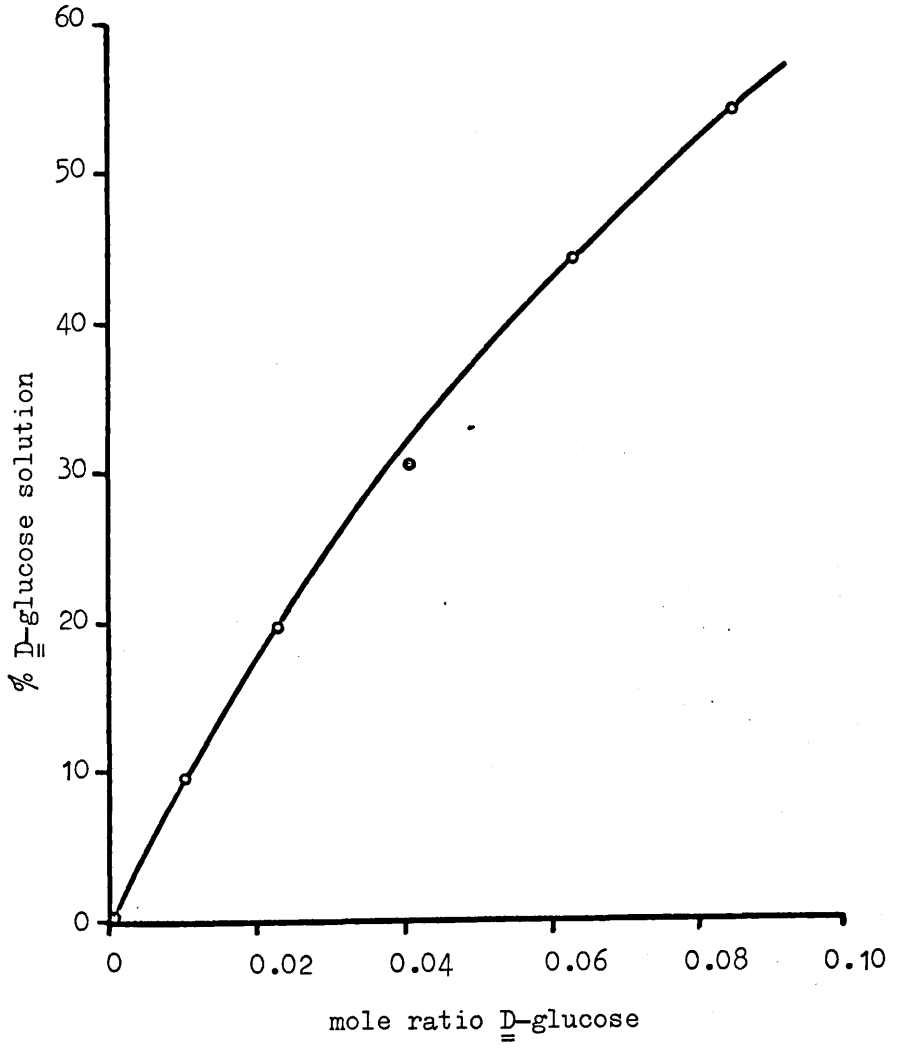


FIGURE 5.6 Plot of percentage D-glucose concentration against mole ratio from data given by Burton et al.[130]



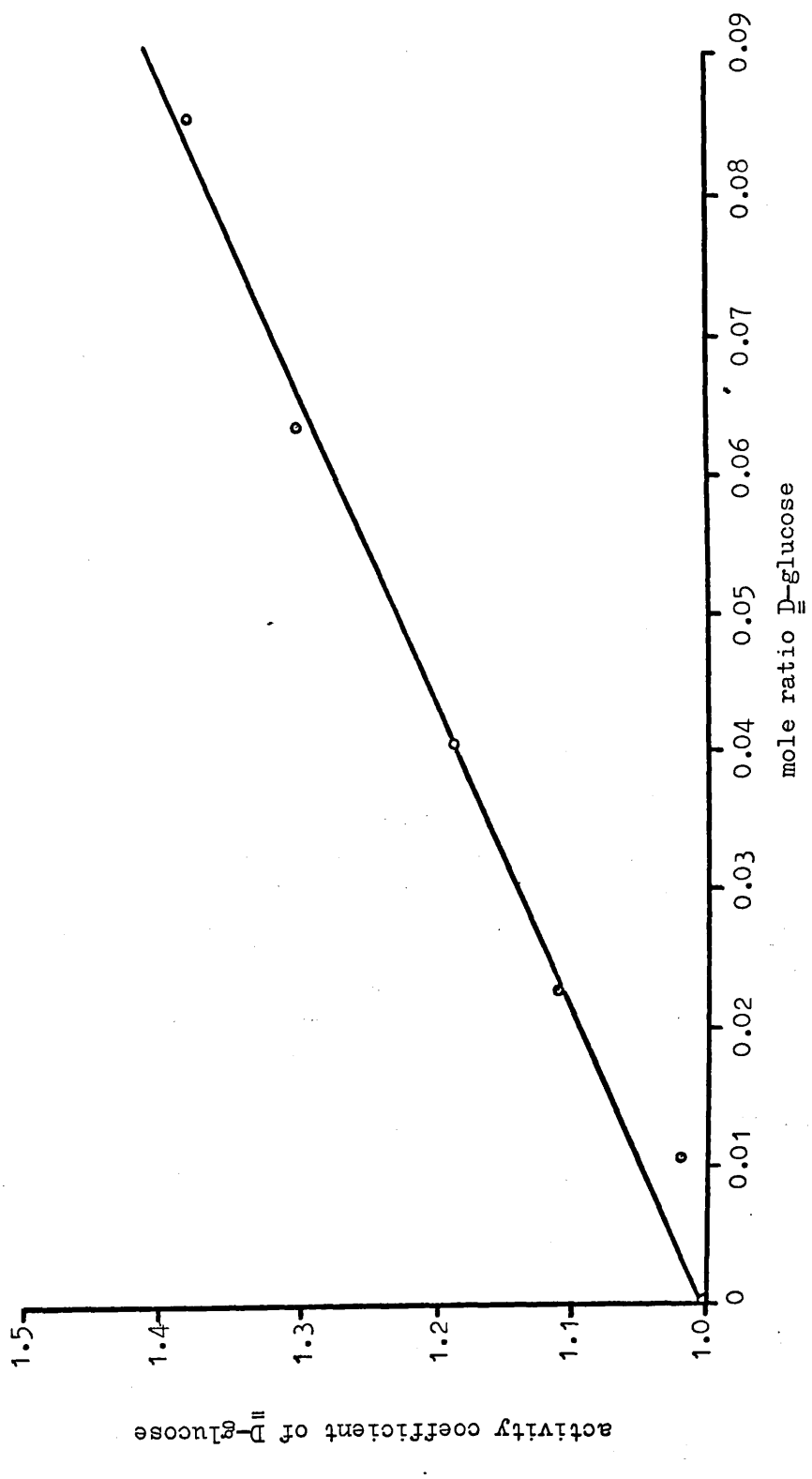


FIGURE 5.7 Plot of activity coefficient against mole ratio of D-glucose from data given by Burton et al.[130]

enzyme specificity has not been taken into account.

TABLE 5.10 shows data taken from Burton et al.[130] which has been used to produce FIGURES 5.5, 5.6 and 5.7 from which numerical values required for calculations of percentage reversion from D-glucose by glucoamylase have been taken.

TABLE 5.10

Thermodynamic data reported by Burton et al.[130]

D-GLUCOSE CONCENTRATION g/cm^3	MOLE RATIO D-GLUCOSE	ACTIVITY OF WATER	D-GLUCOSE ACTIVITY COEFFICIENT
9.98	0.0106	0.989	1.02
19.95	0.0226	0.976	1.11
31.92	0.0401	0.958	1.16
44.82	0.0630	0.930	1.30
54.86	0.0850	0.907	1.38

Calculation of percentage reversion to isomaltose from an initial 40% w/v D-glucose solution using a value of K_c derived from ΔG^{o}_{HYD} = 8.37 kJ mol^{-1}

As the solutions were not dilute, deviations from ideality have been corrected for by the use of activities, giving rise to "effective concentrations" of the species involved in the equilibrium

$$\frac{1}{K_c} = \frac{(a_o - r)^2}{(r)(\omega_o + r)}$$

where a_o = initial activity of D-glucose,

r = activity of reversion product (isomaltose),

ω_o = initial activity of water,

$$\frac{1}{0.0341} = 29.3 = \frac{[0.050678 \times 1.262 - r]^2}{[0.941 + r]r}$$

$$(29.3)(r)(0.941 + r) = [0.050678 \times 1.262 - r]^2$$

$$28.3r^2 + 27.6992r - 0.004093 = 0$$

$$r = \frac{-27.699 \pm \sqrt{(27.699)^2 + (4)(28.3)(0.004093)}}{(2)(28.3)}$$

$$= \frac{-27.699 + 27.6908}{56.6}$$

$$= \frac{0.0082}{56.6}$$

$$= \underline{\underline{0.000148}}$$

As the concentration of reversion product (isomaltose) was so small its activity coefficient was assumed to be unity.

$$\text{Percentage reversion to isomaltose} = \frac{\text{activity reversion product}}{\text{activity initial D-glucose}} \times 100\%$$

$$= \frac{0.000148}{0.06396} \times 100\%$$

$$= \underline{\underline{0.2314\%}}$$

Similarly the percentages of reversion product (isomaltose) calculated at other initial D-glucose concentrations using the appropriate thermodynamic data are given in TABLE 5.11.

FIGURE 5.8 Calculated theoretical % reversion of D-glucose to isomaltose catalysed by glucoamylase using a ΔG^{o}_{HYD} value of -8.37 kJ mol^{-1}

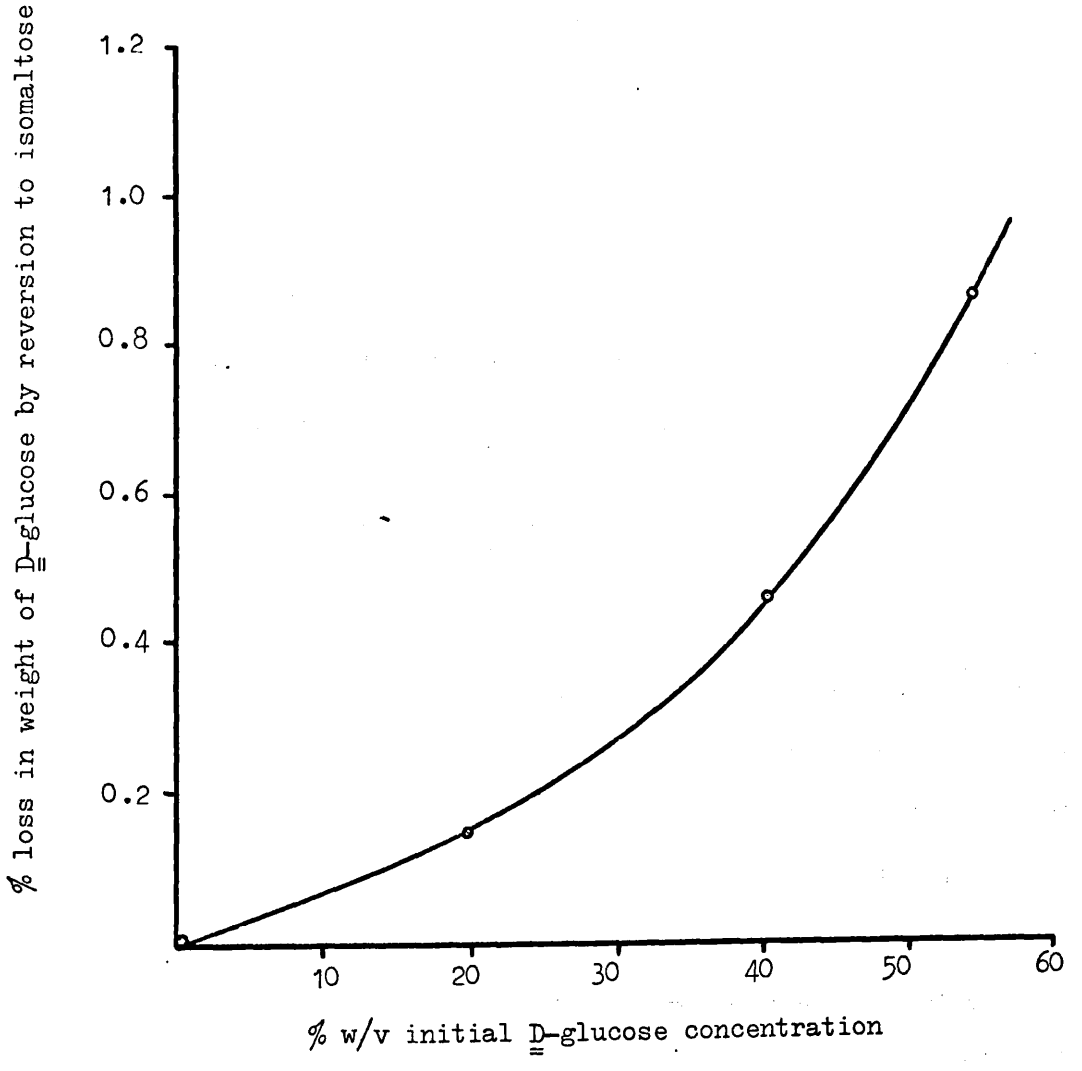


TABLE 5.11

Data used to calculate percentage reversion from D-glucose, based on the literature value of ΔG^{o}_{HYD} for isomaltose.[131]

INITIAL D-GLUCOSE PER CENT	FREE ENERGY OF HYDROLYSIS $kJmol^{-1}$	ACTIVITY OF WATER	ACTIVITY OF D-GLUCOSE	EQUILIBRIUM CONSTANT $1/K_c$	CALCULATED PERCENTAGE REVERSION TO ISOMALTOSE	PERCENTAGE LOSS IN WEIGHT OF D-GLUCOSE BY REVERSION TO ISOMALTOSE
19.95	-8.37	0.976	0.025086	29.3	0.0837	0.1674
40.00	-8.37	0.941	0.063955	29.3	0.231	0.4620
54.86	-8.37	0.907	0 117300	29.3	0.437	0.8740

It can be seen from TABLE 5.11 and FIGURE 5.8 that the percentage of reversion products produced by the action of glucoamylase on several D-glucose solutions of varying concentration using the literature value[131] for the free energy of hydrolysis of isomaltose were not consistent with either that reported by Hehre et al.[115] for 30% w/v D-glucose, nor with that observed in this work at 40% w/v D-glucose. In both cases the calculated values are lower than those observed.

In order to obtain values of percentage reversion which were nearer to those observed by experiment, data given by Hehre et al.[115] was used to calculate an equilibrium constant in favour of reversion from D-glucose to isomaltose. Using this equilibrium constant a value of percentage reversion at 40% w/v D-glucose could then be calculated and compared with the experimental value obtained in this work. In order to use the data reported by Hehre et al.,[115] glucoamylase specificity for β-D-glucose as the substrate for reversion has to be taken into account.

For the formation of isomaltose from D-glucose catalysed by glucoamylase we have:

$$\beta\text{-D-Glu} + \text{either } \alpha\text{- or } \beta\text{-D-Glu} \underset{K_c'}{\overset{\text{glucoamylase}}{\rightleftharpoons}} \text{isomaltose}^{*} + H_2O$$

The equilibrium constant in favour of reversion to isomaltose is given by:

$$K_c' = \frac{\{\text{mole ratio isomaltose}\}\{\text{mole ratio water} + \text{mole ratio isomaltose}\}}{\left[\{\text{mole ratio } \beta\text{-D-glucose}\}\{\text{activity coefficient}\} - \text{mole ratio isomaltose}\right]\left[\overline{\text{mole ratio total glucose}}\ \overline{\text{activity co-efficient}} - \text{mole ratio isomaltose}\right]}$$

Using equilibrium mole ratios from data reported by Hehre et al.,[115] and activity coefficients given by Burton et al.[130] a value of K_c' was calculated as follows:

$$K_c' = \frac{\left\{\frac{0.086}{46.15}\right\}\left\{\frac{44.40}{46.15} + \frac{0.086}{46.15}\right\}}{\left\{\frac{(1.665)(62.2)(1.11)}{(46.15)(100)} - \frac{0.086}{46.15}\right\}\left\{\frac{(1.665)(1.18)}{46.15} - \frac{0.086}{46.15}\right\}}$$

(activity coefficients of isomaltose and water assumed to be unity).

$$\underline{\underline{K_c' = 1.912}}$$

* The anomeric configuration of isomaltose is unimportant.

Calculation of percentage reversion to isomaltose from an initial 40% w/v D-glucose solution using K_c'.

$$\frac{1}{K_c'} = 0.5229 = \frac{[(0.05068)(\frac{62.2}{100})(1.16)-r][(0.05068)(1.262)-r]}{[0.941+r]r}$$

$$0.4771r^2 - 0.5926r + 0.002340 = 0$$

$$r = \frac{0.5926 \pm \sqrt{(0.5926)^2-4(0.4771)(0.002340)}}{(2)(0.4771)}$$

$$r = \frac{0.5926 - 0.5887}{0.9541}$$

$$= \frac{0.0039}{0.9541}$$

$$= \underline{0.004088}$$

$$\text{Percentage reversion to isomaltose} = \frac{\text{activity reversion product}}{\text{initial activity D-glucose}} \times 100\%$$

$$= \frac{0.004088}{0.06396} \times 100\%$$

$$= \underline{6.390\%}$$

Similarly the percentages of reversion product (isomaltose) calculated at other initial D-glucose concentrations using the appropriate thermodynamic data are given in TABLE 5.12 and FIGURE 5.9.

FIGURE 5.9 Calculated theoretical % reversion of D-glucose to isomaltose catalysed by glucoamylase using an equilibrium constant (K_c) of 1.912.

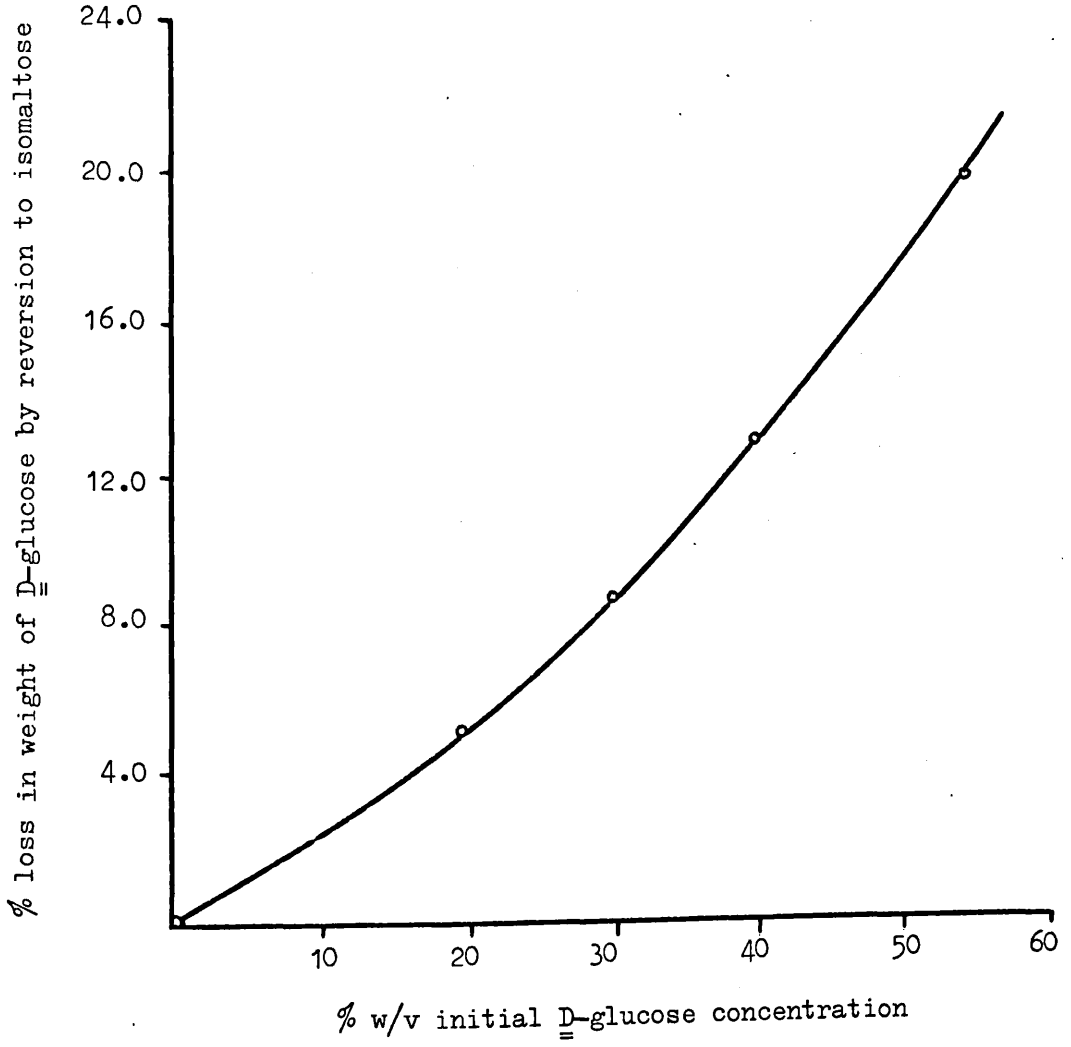


TABLE 5.12

Data used to calculate percentage reversion from D-glucose based on the calculated value of K_c' for isomaltose formation.

INITIAL D-GLUCOSE PER CENT	FREE ENERGY OF HYDROLYSIS kJ mol^{-1}	ACTIVITY OF WATER	ACTIVITY OF TOTAL D-GLUCOSE	EQUILIBRIUM CONSTANT $1/K_c'$	CALCULATED PERCENTAGE REVERSION TO ISOMALTOSE	PERCENTAGE LOSS IN WEIGHT OF D-GLUCOSE BY REVERSION TO ISOMALTOSE
19.95	1.616	0.976	0.02508	0.5229	2.507	5.014
30.00	1.616	0.961	0.03700	0.5229	4.291	8.582
40.00	1.616	0.941	0.06397	0.5229	6.390	12.780
54.86	1.616	0.907	0.11730	0.5229	9.917	19.834

FIGURE 5.9 shows the calculated percentage of reversion products produced by the action of glucoamylase on D-glucose solutions of differing concentration. As expected the percentage reversion at 30% w/v D-glucose agrees with that reported by Hehre *et al.*,[115] while the value (13%) at 40% w/v D-glucose is in closer agreement with that observed (11%) in this work for glucoamylase I.

Although the results taken from the work of Hehre *et al.*[115] were only semi-quantitative, a value for the free energy change of isomaltose formation from D-glucose was calculated from this data to compare with the value reported by Kalckar.[131]

In order to carry out the calculation it was first necessary to obtain an equilibrium constant (K_c'') which did not take into account the anomeric conformation of the substrate. This was dervied from K_c' as follows:

$$K_c'' = \frac{\left\{\begin{matrix}\text{mole ratio}\\ \text{isomaltose}\end{matrix}\right\}\left\{\begin{matrix}\text{mole ratio}\\ \text{water}\end{matrix} + \begin{matrix}\text{mole ratio}\\ \text{isomaltose}\end{matrix}\right\}}{\left[\left\{\begin{matrix}\text{mole ratio}\\ \text{total glucose}\end{matrix}\right\}\left\{\begin{matrix}\text{activity}\\ \text{coefficient}\end{matrix}\right\} - \left\{\begin{matrix}\text{mole ratio}\\ \text{isomaltose}\end{matrix}\right\}\right]^2}$$

$$\text{Hence } K_c'' = K_c'\frac{\left[\left\{\begin{matrix}\text{mole ratio}\\ \beta\text{-D-glucose}\end{matrix}\right\}\left\{\begin{matrix}\text{activity}\\ \text{coefficient}\end{matrix}\right\} - \left\{\begin{matrix}\text{mole ratio}\\ \text{isomaltose}\end{matrix}\right\}\right]}{\left[\left\{\begin{matrix}\text{mole ratio}\\ \text{total glucose}\end{matrix}\right\}\left\{\begin{matrix}\text{activity}\\ \text{coefficient}\end{matrix}\right\} - \left\{\begin{matrix}\text{mole ratio}\\ \text{isomaltose}\end{matrix}\right\}\right]}$$

$$K_c'' = 1.92\frac{\left\{\frac{(1.665)(62.2)(1.11)}{(46.15)(100)} - \frac{0.086}{46.15}\right\}}{\left\{\frac{(1.665)(1.18)}{46.15} - \frac{0.086}{46.15}\right\}}$$

$$= \underline{\underline{1.086}}$$

By applying the van't Hoff isotherm to obtain a value for the free energy of formation of isomaltose at 25°C we have:

$$\Delta G^\circ = -RT \ln K_c''$$

$$= -\frac{(8.314)(298)(0.0826)}{1,000}$$

$$= \underline{\underline{-0.2046 \text{ kJ mol}^{-1}}}$$

The result is somewhat surprising in that it is negative, however, considering the qualitative nature of the data used for its derivation it is possibly subject to error.

It is considered that FIGURE 5.9 may be of use industrially, because, by lowering the concentration of D-glucose present during starch conversion, the amount of reversion product is reduced, and it may be possible to calculate a commercially favourable optimum which allows for an economic glucose concentration with a specified degree of

TABLE 5.13

The pH optima of glucoamylase preparations

GLUCOAMYLASE	SOURCE	pH OPTIMUM	REF.
GLUCOAMYLASE	Aspergillus niger	4.0	86
GLUCOAMYLASE	Aspergillus niger	4.8	39
GLUCOAMYLASE	Aspergillus niger	5.0	13
GLUCOAMYLASE	Aspergillus niger	3.5-5.0	23
ACID STABLE SACCHAROGENIC AMYLASE	Aspergillus awamori	2.4-7.5	87
LESS ACID STABLE SACCHAROGENIC AMYLASE	Aspergillus awamori	3.9-8.2	
LESS ACID STABLE SACCHAROGENIC AMYLASE	Aspergillus awamori	4.8	88
GLUCOAMYLASE	ɣ-amylase	4.0	22,89
GLUCOAMYLASE		6.5	
GLUCOAMYLASE I	Endomycopsis bispora	6.0-7.0	1
GLUCOAMYLASE II		5.0-6.0	
GLUCOAMYLASE I	Aspergillus niger	4.5-5.0	4
GLUCOAMYLASE II			
GLUCOAMYLASE A	Aspergillus phoenicis	4.6	90
ACID GLUCOAMYLASE	Calf heart muscle	5.2	91
GLUCOAMYLASE FROM LIVER AND SPLEEN	Macaca mullata monkeys	4.8	92
GLUCOAMYLASE FROM INTESTINE	"	5.8	
GLUCOAMYLASE	Dog Serum	6.1	93
GLUCOAMYLASE	Aspergillus niger	4.5-4.9	
GLUCOAMYLASE	Rhizopus delemar	4.9	21
GLUCOAMYLASE	Endomyces species	4.9	

TABLE 5.13 (continued)

GLUCOAMYLASE	SOURCE	pH OPTIMUM	REF.
GLUCOAMYLASE	Aspergillus awamori	4.7	94
GLUCOAMYLASE	Aspergillus awamori	4.5-4.7	85
GLUCOAMYLASE	Aspergillus niger	4.0-4.5	46
GLUCOAMYLASE I	Aspergillus niger	4.8	95
GLUCOAMYLASE II	(Agidex 3,000)	4.7	
GLUCOAMYLASE I	Aspergillus niger	4.6	THIS WORK
GLUCOAMYLASE II	(Agidex 3,000)	4.7	

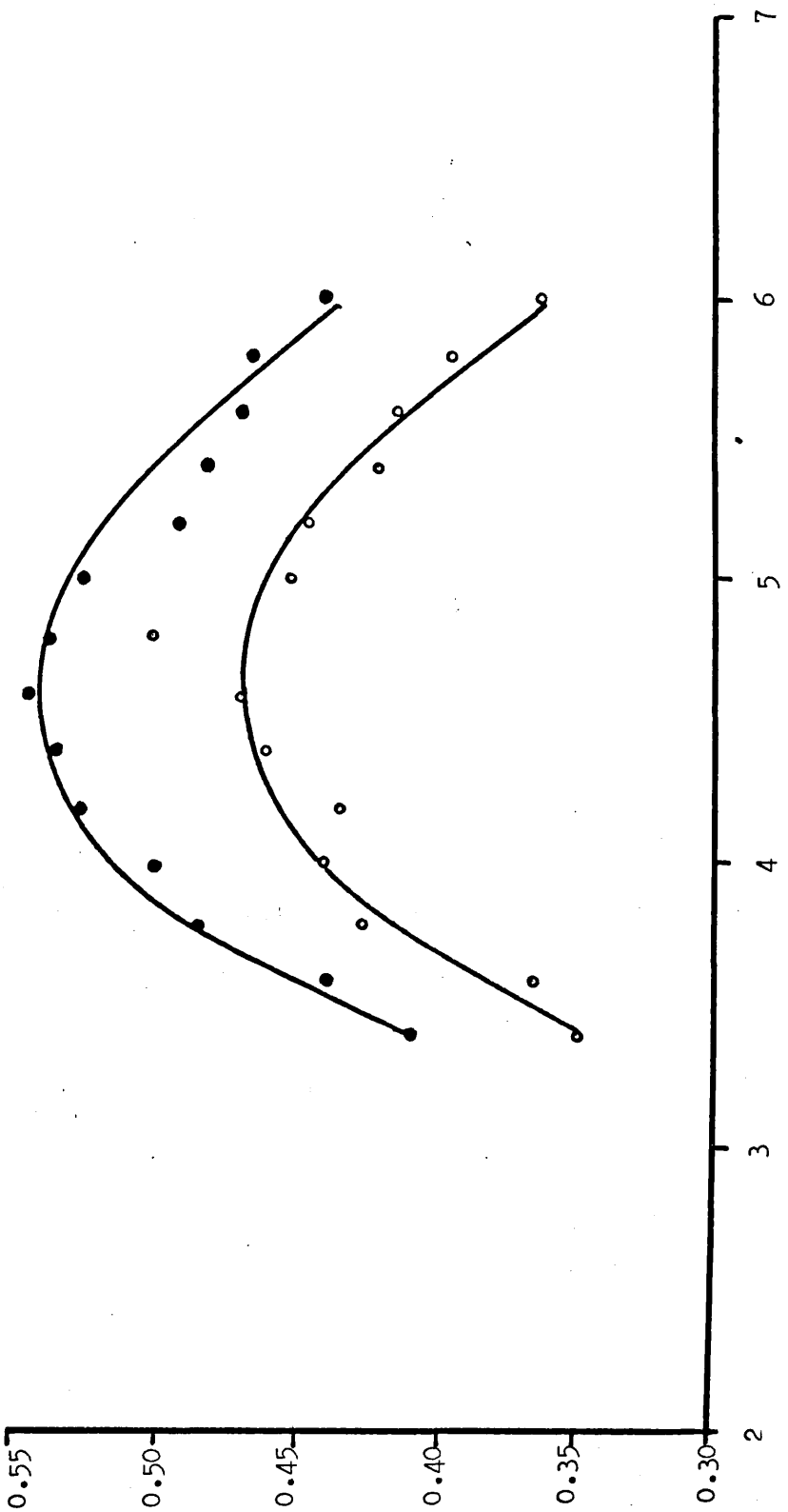


FIGURE 5.10 pH optima for action of glucoamylase I (•) and glucoamylase II (○) on wheat amylopectin.

reversion, rather than simply aiming for maximum glucose concentration regardless of any subsequent reversion which appears to be the current industrial practice. It should also be emphasised that reversion is a kinetically slow process compared to hydrolysis, and higher degrees of conversion may be achieved by stopping the conversion process before the attainment of equilibrium.

It is clear from this discussion that there is a great need for further reliable quantitative data on the free energies of hydrolysis of glycosidic linkages and on the compositions of reversion product mixtures. The effects of using different enzymes and enzyme mixtures for catalysing the reversion process also requires further investigation.

5.5.1 The pH Optimum for Glucoamylase Activity

The optimum pH values for glucoamylase activity which have been reported in the literature are given in TABLE 5.13.

5.5.2 Results and discussion

The pH optimum was determined for glucoamylase I and II in this work, using 1% w/v wheat amylopectin as the enzyme substrate, and found to be 4.6 and 4.7 respectively. The results are shown graphically in FIGURE 5.10 and are in agreement with those found by other workers which are given in TABLE 5.13. A test was also carried out at pH 9.0 but no glucoamylase activity was detected.

5.6 Experimental

5.6.1 The activity of Glucoamylases I and II towards dextran

Samples of "Sephadex" G-100 and G-200 (1.0 g) were prepared as described in CHAPTER 3, 3.3.2, of this Thesis, and the gels washed with citrate buffer(0.05M, pH 5.0). Samples of glucoamylase I and II (5 mg) were weighed and each made up to 5 cm^3 with buffer. Approximately 0.15 g of "Sephadex" G-100 gel was placed in a beaker (5 cm^3) and glucoamylase I (1.0 cm^3) added. The procedure was repeated for glucoamylase II, and with "Sephadex" G-200. Blanks were made by replacing the enzyme solutions with buffer. The samples were incubated at 25°C for 24 hours, with occasional stirring. Aliquots (0.5 cm^3) were withdrawn after this time and tested for the presence of D-glucose using a standardised glucose oxidase reagent.[41] The experiment was repeated with a soluble "Glaxo" dextran* at 1% w/v concentration. Aliquots were withdrawn after 1, 6, 12, 24, 48, and 72 hours and paper chromatograms run as described in 5.6.5 using D-glucose and isomaltose* as standards.

5.6.2 The activity of Glucoamylases I and II towards Cibachron Blue F3GA-amylose

Samples of Cibachron Blue F3GA- amyloseℵ (200 mg) were weighed into four stoppered Pyrex 6" x 1" boiling tubes. Acetate buffer 0.1M, pH 4.5 (200 cm^3) was prepared[132] and boiled prior to use.

* "Glaxo" soluble dextran, and isomaltose were kindly donated by Dr H. Weigel of Royal Holloway College.

ℵ Cibachron Blue F3GA-amylose was kindly donated by Dr J.J. Marshall of the Department of Biochemistry, University of Miami.

Glucoamylase I and II (1 mg) were made up to 1.0 cm^3 with buffer. Digests were made up as shown in TABLE 5.14 and incubated at 25°C on a mechanical shaker.

TABLE 5.14

Digest compositions used for Cibachron Blue F3GA-amylose experiment

ENZYME	VOLUME OF ENZYME CORRESPONDING TO 6.2 INTERNATIONAL UNITS cm^3	VOLUME OF ACTIVITY BUFFER. cm^3
GLUCOAMYLASE I	0.41	9.59
GLUCOAMYLASE II	0.36	9.64
CRUDE DIALYSED GLUCOAMYLASE	0.17	9.83
BLANK	0.00	10.00

Aliquots (1.0 cm^3) were withdrawn after 0, 25, 85, 115, 175, 235, 295, and 355 minutes and added to TRIS buffer (2.0 cm^3) 0.5M, pH 10.25. The optical densities of these samples were measured at 625 nm using a Pye Unicam SP 500 spectrometer. Aliquots (20 μ dm^3) were also withdrawn after 25, 115 and 355 minutes and analysed for D-glucose using a standardised glucose oxidase reagent,[41] measured against a blank taken after 5 minutes. The results are shown in FIGURE 5.1 and 5.2.

5.6.3 The Specific Activity of Glucoamylases I and II towards wheat amylopectin

Samples of glucoamylases I and II (1.0 mg) were weighed and made up to 25 cm^3 with citrate buffer 0.05M, pH 5.0. A 2% w/v

wheat amylopectin solution was made up in the same buffer. Duplicate digests were made up using the following procedure. 2% w/v Wheat amylopectin (1.0 cm^3), citrate buffer (0.95 cm^3) and enzyme (0.05 cm^3) were reacted in Pyrex 6" x 5/8" test tubes for 15 minutes at 25°C and enzyme reaction terminated by heating to 100°C for 5 minutes in a boiling water bath. After cooling, 1.0 cm^3 was withdrawn and analysed for D-glucose, using a standardised glucose oxidase reagent.[41] A sample containing buffer (0.05 cm^3) to replace the enzyme was used as a blank. The results are given in TABLE 5.1.

5.6.4 The Specific Activity of Glucoamylases I and II towards maltose

The same glucoamylase stock solutions used in 5.6.3 were also employed in this experiment A 2% w/v maltose* solution was made up in citrate buffer[133] (0.05M, pH 4.6). Duplicate digests were made up and treated as described in 5.6.3 except that maltose replaced wheat amylopectin. The results are given in TABLE 5.1.

5.6.5 Test of maltose homogeniety using paper chromatography

Paper chromatography was carried out using Whatman No.1 paper with a butanol-pyridine-water (6:4:3 v/v/v) solvent system. Standards of D-glucose and isomaltose were applied on either side of the maltose* test sample. After chromatography for 24 hours at 20°C, the papers were air dried and reducing sugars located by a silver

* "Merck" - specially prepared for Biochemistry.

nitrate - sodium hydroxide dip reagent described by Trevelyan et al.[134] No isomaltose or glucose was detected in the maltose sample.

5.6.6 Conversion of wheat amylopectin to glucose

1,000 cm^3, 0.05M citrate buffer pH 5.0 was prepared[133] using deionised water and was boiled prior to use. 5.055 mg wheat amylopectin was made up to 5.0 cm^3 with this buffer. Samples of glucoamylases I and II (1.0 mg) were weighed and each made up to 1.0 cm^3 with buffer. Digests were made up as shown in TABLE 5.15 and incubated at 25°C in a water bath.

TABLE 5.15

Digest compositions for starch conversion by glucoamylases

TYPE OF GLUCOAMYLASE	VOLUME OF SUBSTRATE cm^3	VOLUME OF GLUCOAMYLASE SOLUTION cm^3	VOLUME OF BUFFER cm^3
GLUCOAMYLASE I	0.20	0.20	1.60
GLUCOAMYLASE II	0.20	0.20	1.60
CRUDE DIALYSED GLUCOAMYLASE *	0.20	0.10	1.70
BLANK	0.20	0.00	1.80

* 700×10^{-6} g

Aliquots (200 μ dm^3) were withdrawn after 5, 21, 40, 60, 120, 240, and 480 minutes and enzyme activity terminated by heating in a boiling water bath for 5 minutes. The D-glucose produced was assayed using the glucose oxidase reagent,[41] which had been previously standardised against D-glucose.

A phenol sulphuric acid assay as described by Dubois et al.[71] was carried out on the wheat amylopectin solution (0.1 cm^3) and on a D-glucose solution to produce a standard graph.

The experiment was repeated in the presence of α-amylase using the following procedure:

Crystalline bacterial α-amylase (5 mg) from B. subtilis[+] was made up to 100 cm^3 with buffer. Digests were made up as shown in TABLE 5.16 and incubated at 25°C in a water bath. Aliquots were withdrawn and analysed as previously described.

TABLE 5.16

Digest compositions for starch conversion by glucoamylase in the presence of α-amylase

TYPE OF GLUCOAMYLASE	VOLUME OF SUBSTRATE cm^3	VOLUME OF α-AMYLASE SOLUTION cm^3	VOLUME OF BUFFER cm^3	VOLUME OF GLUCOAMYLASE SOLUTION cm^3
GLUCOAMYLASE I	0.20	0.66	0.94	0.20
GLUCOAMYLASE II	0.20	0.66	0.94	0.20
CRUDE DIALYSED GLUCOAMYLASE *	0.20	0.66	1.04	0.10
BLANK	0.20	0.00	1.80	0.00

* 700×10^{-6} g

The results are given in TABLES 5.4 and 5.5, and in FIGURES 5.3 and 5.4.

+ Calbiochem

5.6.7 The reversion catalysed by glucoamylase from 40% w/v D-glucose solution

1,000 cm^3, 0.05M citrate buffer pH 5.0 was prepared[133] using deionised water and boiled prior to use. D-glucose* (10 g) was made up to 25 cm^3 with this buffer. Samples of glucoamylases I and II (1.15 mg) and (0.82 mg) respectively were weighed and made up to 1.0 cm^3 with buffer. Glucoamylase (Agidex 3,000) (1 cm^3) was dialysed against this buffer as described in CHAPTER 2, 2.4.1 of this Thesis. Duplicate digests were made up as shown in TABLE 5.17 and incubated at 25°C.

TABLE 5.17

Digest composition used for reversion catalysed by glucoamylases

ENZYME	VOLUME OF ENZYME CORRESPONDING TO 70 $\mu g/cm^3$. cm^3	VOLUME OF D-GLUCOSE SOLUTION cm^3
GLUCOAMYLASE I	0.14	2.0
GLUCOAMYLASE II	0.17	2.0
CRUDE DIALYSED GLUCOAMYLASE	0.01	2.0
BLANK	0.00	2.0

Aliquots (0.1 cm^3) were withdrawn after 0.33, 2.83, and 23 hours, and made up to 25 cm^3 with buffer. Then aliquots (0.02 cm^3) were withdrawn and made up to 1.0 cm^3 for glucose assay using the glucose oxidase procedure described by Lloyd *et al.*[41] The optical densities of the solutions were measured at 525 nm using a Pye Unicam SP 500

* B.D.H. Micro-analytical grade reagent.

spectrometer against a blank of citrate buffer, and the percentage reversion calculated relative to the D-glucose blank taken at the same time. The results are given in TABLE 5.7.

5.6.8 The reversion from D-glucose catalysed by purified glucoamylases doped with α-amylase from B. subtilis

Crystalline bacterial α-amylase (5 mg) from B. subtilis* was weighed and made up to 50 cm^3 with buffer as used in previous experiment. D-glucose (5.61 mg) was weighed and made up to 5 cm^3 with buffer. Samples of glucoamylases I and II (1.0 mg) were weighed and each made up to 1.0 cm^3 with buffer. Digests and blanks were made up as shown in TABLE 5.18 and 5.19 respectively, and incubated at 25°C in a water bath. Aliquots (200 μ dm^3) were withdrawn after 1.0, 24.5 and 45.0 hours and enzyme activity terminated by heating in a boiling water bath for 5 minutes. The D-glucose content was assayed using the glucose oxidase reagent,[41] which had been previously standardised against D-glucose. Each aliquot was measured against an appropriate blank comprising the glucoamylase or α-amylase/glucoamylase mixture withdrawn at the same time intervals as reaction digests.

* Calbiochem

TABLE 5.18

Digest compositions for reversion by purified and α-amylase doped glucoamylases

VOLUME OF α-AMYLASE cm^3	VOLUME OF GLUCOAMYLASE I cm^3	VOLUME OF GLUCOAMYLASE II cm^3	VOLUME OF D-GLUCOSE cm^3	VOLUME OF BUFFER cm^3
0.33	0.20	0.00	0.20	1.27
0.33	0.00	0.20	0.20	1.27
0.00	0.20	0.00	0.20	1.60
0.00	0.00	0.20	0.20	1.60

From the optical density of glucose oxidase assay a percentage loss of D-glucose was calculated for both the purified, and doped glucoamylases, at each time of sampling and the results are given in TABLE 5.8 (averaged over 45 hours).

TABLE 5.19

Composition of blanks used for reversion by purified and α-amylase doped glucoamylases

VOLUME OF α-AMYLASE cm^3	VOLUME OF GLUCOAMYLASE I cm^3	VOLUME OF GLUCOAMYLASE II cm^3	VOLUME OF BUFFER cm^3
0.33	0.20	0.00	1.47
0.33	0.00	0.20	1.47
0.00	0.20	0.00	1.80
0.00	0.00	0.20	1.80

5.6.9 pH Optima of glucoamylases I and II

Samples of glucoamylases I and II (1.0 mg) were weighed and made up to 25 cm^3 with deionised water. Citrate buffers[133] were made up in increments of 0.2 pH units between pH 3.4 to pH 6.0, together with an ammonium chloride/ammonia buffer at pH 9.0. Wheat amylopectin (1.11 g) was made up to 50 cm^3 with deionised water. Duplicate digests at each pH were made up as shown in TABLE 5.20, with a blank being made from pH 4.8 buffer to replace the enzyme.

TABLE 5.20

Digest compositions used for pH optimum of glucoamylases I and II

VOLUME OF GLUCOAMYLASE cm^3	VOLUME OF BUFFER cm^3	VOLUME OF WHEAT AMYLOPECTIN SOLUTION cm^3
0.05	1.05	0.90

After 15 minutes incubation at 25°C enzyme activity was terminated by heating digests in a boiling water bath for 5 minutes. After cooling aliquots (1.0 cm^3) were withdrawn and analysed for D-glucose using a standardised glucose oxidase reagent.[41] The results are shown in FIGURE 5.10.

References

1. RUTTLOFF, H., FRIESE, R., KUPKE, G., TAUFEL, A., Z. allg. Mikrobiol., 9, 39, 1969.

2. RUTTLOFF, H., Ann. Technol. Agric., 21, 287, 1972.

3. HAMAUZU, Z., HIROMI, K., ONO, S., J. Biochem., 57, 39, 1965.

4. LINEBACK, D.R., RUSSELL, I.J., RASMUSSEN, C., Arch. Biochem. Biophys., 134, 539, 1969.

5. ABDULLAH, M., FLEMING, I.D., TAYLOR, P.M., WHELAN, W.J., Biochem. J., 89, 35p, 1963.

6. RYZHAKOVA, V.G., Dokl. Akad. Nauk, S.S.R., 204, 737, 1972.

7. KAWAMURA, S., WATANABE, T., MATSUDA, K., Tohoku. J. Agr. Res., 20, 137, 1969.

8. KAWAMURA, S., WATANABE, T., MATSUDA, K., Tohoku. J. Agr. Res., 20, 143, 1969.

9. KAWAMURA, S., WATANABE, T., MATSUDA, K., Tohoku. J. Agr. Res., 21, 170, 1970.

10. TSUJISAKA, Y., FUKUMOTO, J., Sci. Indust., 30, 398, 1956.

11. BARKER, S.A., BOURNE, E.J., FLEETWOOD, J.G., J. Chem. Soc., 4865, 1957.

12. TAKAHASHI, K., YOSHIKAWA, Y., HIROMI, K., ONO, S., J. Biochem., 58, 251, 1965.

13. PAZUR, J.H., ANDO, T., J. Biol. Chem., 235, 297, 1960.

14. FLEMING, I.D., STONE, B.A., Biochem. J., 97, 13p, 1965.

15. SUETSUGU, N., HIROOKA, E., YASUI, H., HIROMI, K., ONO, S., J. Biochem., 73, 1223, 1973.

16. ONO, S., HIROMI, K., HAMAUZU, Z., J. Biochem., 57, 34, 1965.

17. NAGAO, A., Thesis for Bachelor Degree, University of Osaka Perfecture, 1963.

18. PAZUR, J.H., KLEPPE, K., J. Biol. Chem., 237, 1002, 1962.

19. HIROMI, K., Biochem. Biophys. Res. Commun., 40, 1, 1970.

20. WATANABE, K., FUKIMBARA, T., J. Ferm. Technol., 44, 25, 1966.

21. THIVEND, P., MERCIER, C., GUILBOT, A., Methods in carbohyd. chem. Vol. 5., 100, 1972.

22. GREENWOOD, C.T., MILNE, E.A., Adv. Carbohyd. Chem., 23, 358, 1968.

23. DENAULT, L.J., UNDERKOFLER, L.A., Cereal Chem., 40, 618, 1963.

24. MARSHALL, J.J., WHELAN, W.J., F.E.B.S. Letters, 9, 85, 1970.

25. FLEETWOOD, J.G., WEIGEL, H., Nature, 196, 984, 1962.

26. WEILL, C.E., BURCH, R.J., VAN DYK, J.W., Cereal Chem., 31, 150, 1954.

27. EVELEIGH, D.E., PERLIN, A.S., Carbohyd. Res., 10, 87, 1969.

28. KUJAWSKI, M., ZAJAC, A., Die Starke, 26, 93, 1974.

29. MARSHALL, J.J., Anal. Biochem., 37, 466, 1970.

30. KLEIN, B., FOREMAN, J.A., SEARCY, R.L., Anal. Biochem., 31, 412, 1969.

31. EWEN, L.M., Clinica. Chimica Acta., 47, 233, 1973.

32. TROTMAN, E.R., In "Dyeing and chemical technology of textile fibres", p.489, 1964. Griffin and Co. Ltd., London.

33. RINDERKNECHT, H., WILDING, P., HAVERBACK, B.J., Experientia, 23, 805, 1967.

34. SMITH, E.E., DRUMMOND, G.S., MARSHALL, J.J., WHELAN, W.J., Fed. Proc., Fed. Amer. Soc. Exp. Biol., 29, 930, 1970.

35. MARSHALL, J.J., WHELAN, W.J., Anal. Biochem., 43, 316, 1971.

36. Report of the Commission on Enzymes of the International Union of Biochemistry. (1961). Pergamon Press Ltd.

37. ROBYT, J.F., WHELAN, W.J., In "Starch and its derivatives". (RADLEY, J.A., Ed.) p.430, 1968. Chapman and Hall, London.

38. FUKUMOTO, J., cited In "Starch and its derivatives". (RADLEY, J.A., Ed.) p.502, 1968. Chapman and Hall, London.

39. PAZUR, J.H., ANDO, T., J. Biol. Chem., 234, 1966, 1959.

40. SMILEY, K.L., HENSLEY, D.E., SMILEY, M.J., GASDORF, H.J., Arch. Biochem. Biophys., 144, 694, 1971.

41. LLOYD, J.B., WHELAN, W.J., Anal. Biochem., 30, 467, 1969.

42. ONO, S., HIROMI, K., ZINBO, M., J. Biol. Chem., 55, 315, 1964.

43. ONO, S., HIROMI, K., KAWAI, M., 7th Int. Congr. Biochem. Abstracts 4, 768, 1967.

44. BENDETSKII, K.M., Mol. Biol., 2, 267, 1968.

45. BENDETSKII, K.M., YAROVENKO, V.L., LUK'YANOVA, L.N., Biokhimiya, 36, 525, 1971.

46. ALLEN, W.G., SPRADLIN, J.E., Brewers Digest, 49, 48, 1974.

47. HIROMI, K., ONO, S., 7th Int. Congr. Biochem. Abstracts 4, 768, 1967.

48. HIMOMI, K., TAKAHASHI, K., HAMAUZU, Z., ONO, S., J. Biol. Chem., 59, 469, 1966.

49. ONO, S., HIROMI, K., ZIMBO, M., cited In "Starch and its derivatives"., (RADLEY J.A. ed.) p.502, 1968. Chapman and Hall, London.

50. PHILLIPS, L.L., CALDWELL, M.L., J. Am. Chem. Soc., 73, 3563, 1951.

51. OKAZAKI, H., Arch. Biochem. Biophys., 63, 322, 1956.

52. MEYER, K.H., Experientia, 8, 405, 1952.

53. MEYER, K.H., BERNFELD, P., Helv. Chem. Acta., 25, 404, 1942.

54. CORI, G.T., LARNER, J., J. Biol. Chem., 188, 17, 1951.

55. FUKUMOTO, J., TSUJISAKA, Y., Kagaku. to Kogyo., 28, 285, 1954.

56. FUKUMOTO, J., TSUJISAKA, Y., Kagaku. to Kogyo., 30, 130, 1956.

57. DOBROLINSKAYA, G.M., RODZEVICH, Y.I., Fermentnaya i Spirit. Prom., 30, 6, 1964.

58. HIROMI, K., HAMAUZU, Z., TAKAHASHI, K., ONO, S., J. Biochem., 59, 411, 1966.

59. TSUJISAKA, Y., FUKUMOTO, J., YAMAMOTO, T., Nature, 181, 770, 1958.

60. PEAT, S., TURVEY, J.R., EVANS, J.M., J. Chem. Soc., 3223, 1959.

61. FLEMING, I.D., In "Starch and its derivatives"., (RADLEY, J.A., Ed.) p. 498, 1968. Chapman and Hall, London.

62. BAILEY, R.W., MACRAE, J.C., F.E.B.S. Letters, 31, 203, 1973.

63. SHETTY, R.M., LINEBACK, D.R., SEIB, P.A., Cereal Chem., 51, 364, 1974.

64. MACRAE, J.C., ARMSTRONG, D.G., J. Sci. Food, Agr., 19, 578, 1968.

65. MANNERS, D.J., Biochem. J., 123, 1p, 1971.

66. EVERS, A.D., GONG, B.M., PYRBUS, J.N., Die Starke, 23, 16, 1971.

67. ROBYT, J.F., WHELAN, W.J., In "Starch and its derivatives", (RADLEY, J.A., Ed.) p.474, 1968. Chapman and Hall, London.

68. TOYAMA, T., HIZUKURI, S., NIKUNI, Z., J. Soc. Starch. Technol., 13, 69, 1966.

69. HATTORI, Y., TAKEUCHI, I., Agr. Biol. Chem., 25, 895, 1961.

70. LEACH, H.W., SCHOCH, T.J., Cereal Chem., 38, 34, 1961.

71. DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS, P.A., SMITH, F., Anal. Chem., 28, 350, 1956.

72. FRENCH, D., KNAPP, D.W., J. Biol. Chem., 187, 463, 1950.

73. FUKUMOTO, J., TSUJISAKA, Y., Sci. Indust., 28, 92, 1954.

74. UEDA, S., Bull. Agr. Chem. Soc. Japan, 20, 148, 1956.

75. KITAHARA, K., KURUSHIMA, M., J. Ferm. Technol., 27, 254, 1949.

76. CORMAN, J., LANGLYKKE, A.F., Cereal Chem., 25, 190, 1948.

77. KERR, R.W., CLEVELAND, F.C., KATZBECK, W.J., J. Am. Chem. Soc., 73, 3916, 1951.

78. OKAZAKI, H., Symp. on Enzyme Chem. Japan., 9, 43, 1954.

79. TSUJISAKA, Y., FUKUMOTO, J., YAMAMOTO, T., Nature, 181, 94, 1958.

80. HAYASHIDA, S., Bull. Agr. Chem. Soc. Japan, 21, 386, 1957.

81. FUKUMOTO, J., TSUKISAKA, Y., ARAKI, M., MATSUMOTO, E., Kagaku. to.Kogyo., 35, 412, 1961. Chem. Abs., 57, 14168f.

82. HATTORI, Y., TAKEUCHI, I., Agr. Biol. Chem., 26, 316, 1962.

83. PEAT, S., PIRT, S.J., WHELAN, W.J., J. Chem. Soc., 705, 1952.

84. CUNNINGHAM, W.L., MANNERS, D.J., WRIGHT, A., FLEMING, I.D., J. Chem. Soc., 2602, 1960.

85. RYZHAKOVA, V.G., FENIKSOVA, R.V., Biokhimiya., 37, 1019, 1972.

86. BARKER, S.A., FLEETWOOD, J.G., J. Chem. Soc., 4857, 1957.

87. WATANABE, K., FUKIMBARA, T., J. Ferm. Technol., 43, 690, 1965.

88. WATANABE, K., FUKIMBARA, T., J. Ferm. Technol., 44, 392, 1966.

89. ROSENFELD, E.L., LUKOMSKAYA, I.S., POPOVA, I.A., Enzymologia, 30, 1, 1966.

90. LINEBACK, D.R., BAUMANN, W.E., Carbohyd. Res., 14, 341, 1970.

91. IWANOWSKI, H., Arch. Immunol. Ther. Exp., 21, 615, 1973.

92. SEETHARAM, B., SWAMINATHAN, N., RADHAKRISHNAN, A.N., Biochem. J., 117, 939, 1970.

93. McGEENEY, K.F., O'DONNELL, D., Biochem. J., 125, 115p, 1971.

94. DURMISHIDZE, S.V., KVESITADZE, G.I., KOKONASHVILI, G.N., NURAUVISZE, N.N., Soobschch. Akad. Nauk. Gruz. S.S.R., 69, 461, 1973.

95. JOLLEY, M.E., Ph. D. Thesis, University of Birmingham, 1972.

96. PAZUR, J.H., KNULL, H.R., CEPURE, A., Carbohyd. Res., 20, 83, 1971.

97. TSUBOI, A., YAMASAKI, Y., SUZUKI, Y., Agr. Biol. Chem., 38, 543, 1974.

98. FUKUI, T., NIKUNI, Z., Agr. Biol. Chem., 33, 884, 1969.

99. RODZEVICH, B.I., DOBROLINSKAYA, G.M., Fermentnaya i Spirit. Prom., 32, 5, 1966.

100. HIROMI, K., TAKAHASHI, K., HAMAUZU, Z., ONO, S., J. Biol. Chem., 59, 469, 1966.

101. PARK, K.Y., DE-LIMA, D.C., Rev. Brasil. Tecnol., 3, 67, 1972 Chem. Abs., 78, 54844g.

102. VAMOS, L., ROSE, P., Die Starke, 25, 195, 1973.

103. MAHER, G.G., Die Starke, 20, 228, 1968.

104. FENOXOVA, R.V., RHYZHAKOVA, V.G., Mikrobiologija, 39, 974, 1970.

105. LINEBACK, D.R., GEOGI, C.E., DOTY, R.L., J. Gen. Appl. Microbiol., 12, 27, 1966.

106. KOL'TSOVA, E.V., SADOVA, A.I., Pricl. Biochim. Microbiol., 6, 48, 1970.

107. WATANABE, T., KAWAMURA, S., SASAKI, H., MATSUDA, K., Die Starke, 21, 44, 1969.

108. PAZUR, J.H., OKADA, S., Carbohyd. Res., 4, 371, 1967.

109. RHYZHAKOVA, V.G., Pricl. Biochim. Microbiol., 6, 407, 1970.

110. WATANABE, T.., KAWAMURA, S., SASAKI, H., MATSUDA, K., Die Starke, 21, 18, 1969.

111. UNDERKOFLER, L.A., DENAULT, L.J., HOU, E.F., Die Starke, 17, 179, 1965.

112. RHODSEVICH, V.I., DOBROLINSKAYA, G.M., Fermentnaya i Spirit. Prom. 4, 8, 1965.

113. PAZUR, J.H., ANDO, T., Arch. Biochem. Biophys., 93, 43, 1961.

114. ROSE, P., VAMOS, L., Szeszipar, 21, 3, 1973.

115. HEHRE, E.J., OKADA, G., GENGHOF, D.S., Arch. Biochem. Biophys., 135, 75, 1969.

116. FUKUMOTO, J., TSUJISAKA, Y., KIMOTO, K., Papers for the 14th Symposium on Enzyme Chemistry (Koso Kagaku Shinpojiumu) FUKUOKA, p.13, 1962.

117. WATANABE, T., KAWAMURA, S., SUGAWARA, K., MATSUDA, K., Nippon. Nogei. Kagaku. Kaishi., 40, 401, 1966.

118. MARSHALL, J.J., Wallerstein Labs. Commun., 35, 49, 1972.

119. SMILEY, K., CADMUS, M.C., HENSLEY, D.E., LAGODA, A.A., Appl. Microbiol., 12, 455, 1964.

120. FENIXOVA, R.V., RHYZHAKOVA, V.G., Pricl. Biochim. Microbiol., 4, 270, 1968.

121. PALMER, T.N., Biochem. J., 124, 713, 1971.

122. LUKOMSKAYA, I.S., Enzymologia, 24, 327, 1962.

123. TORRES, H.N., OLAVARRIA, J.M., J. Biol. Chem., 239, 2427, 1964.

124. JEFFREY, P.J., BROWN, D.H., BROWN, B.I., Biochemistry, 9, 1403, 1970.

125. HIRS, H.G., Biochem. J., 86, 11, 1963.

126. YAMASAKI, Y., MIYAKE, T., SUZUKI, Y., Agr. Biol. Chem., 37, 131, 1973.

127. YAMASAKI, Y., MIYAKE, T., SUZUKI, Y., Agr. Biol. Chem., 37, 251, 1973.

128. HEHRE, E.J., GENGHOF, D.S., Arch. Biochem. Biophys., 142, 382, 1971.

129. HUGHES, R.C., SMITH, E.E., WHELAN, W.J., (Unpublished results) cited by ROBYT, J.E., WHELAN, W.J., In "Starch and its Derivatives", (RADLEY, J.A., Ed.) p.428. Chapman and Hall. London.

130. BURTON, K., KREBS, H.A., Biochem. J., 54, 94, 1953.

131. KALCKAR, H.M., cited in "The Mechanism of Enzyme Action", McELROY, W.D., GLASS, p.675-739, 1954.

132. GOMORI, G., In "Methods in Enzymology" Vol. 1., (COLOWICK, S.P., KAPLAN, N.O., Ed.) p.138-146, Academic Press, N.Y. 1955.

133. Biochemists Handbook, (LONG, C., Ed.) p.28-41, Spon. E & F.N., London, 1961.

134. TREVELYAN, W.E., PROCTER, D.R., HARRISON, J.S., Nature, 166, 444, 1950.

CHAPTER 6

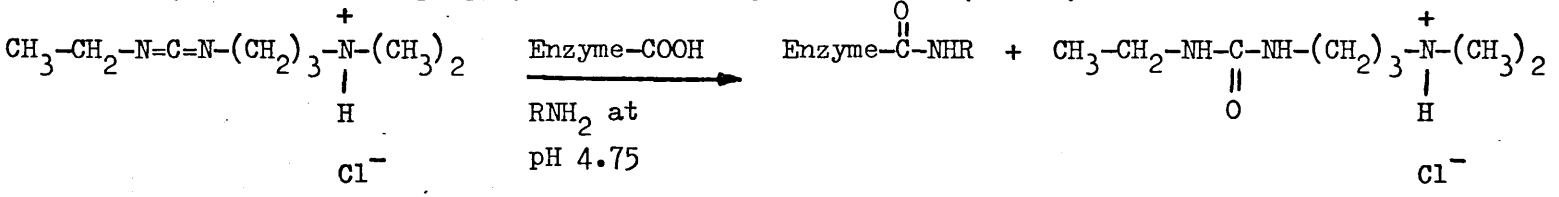
CHEMICAL MODIFICATION OF GLUCOAMYLASES I AND II

6.1 Introduction

In CHAPTER 1 of this Thesis five methods used for the study of hydrolase enzyme active sites were described in detail. Many of the methods have also been applied to other enzymes and proteins and the reader is referred to the excellent reviews which have been written on the modification of particular functional groups by various reagents.[50, 89-94] The chemical modification of enzymes has been reported by Hartley[67] to be the main way in which groups important to catalytic activity or specificity have been discovered and in addition to yielding information in these fields, the chemically modified proteins may undergo an alteration in their properties and stabilisation and protection from proteolytic digestion. The latter discovery may prove useful in using enzymes for therapeutic purposes.[94] In this Thesis the chemical modification of enzymes and proteins has been mainly concerned with the modification of their carboxyl groups and a list of reagents used for this purpose together with the relevant reactions are given in TABLE 6.1. Some of the procedures used and the results obtained will be discussed. Some enzymes which have either been shown or suggested to contain carboxyl residues in their active sites are given in TABLE 6.2.

TABLE 6.1 Reagents used for the chemical modification of carboxyl groups in proteins.

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



1-cyclohexyl-3-(2-morpholinyl-4 ethyl) carbodiimide metho-p-toluene sulphonate (C.M.C.)[32]

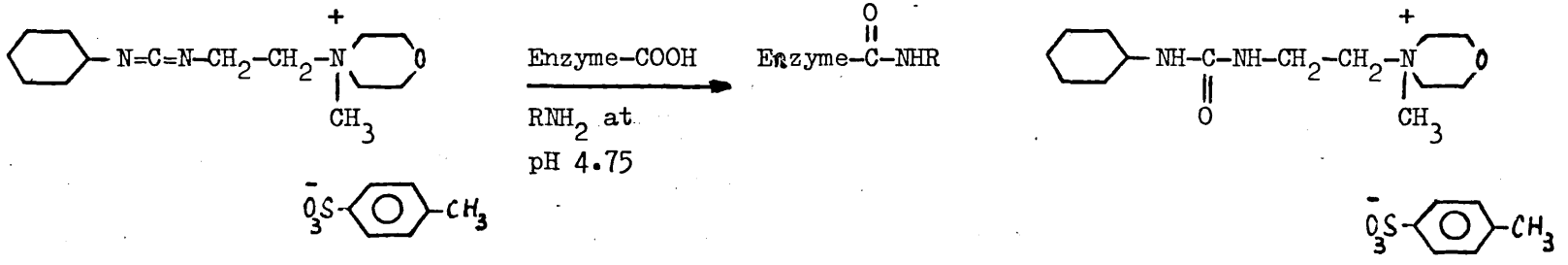
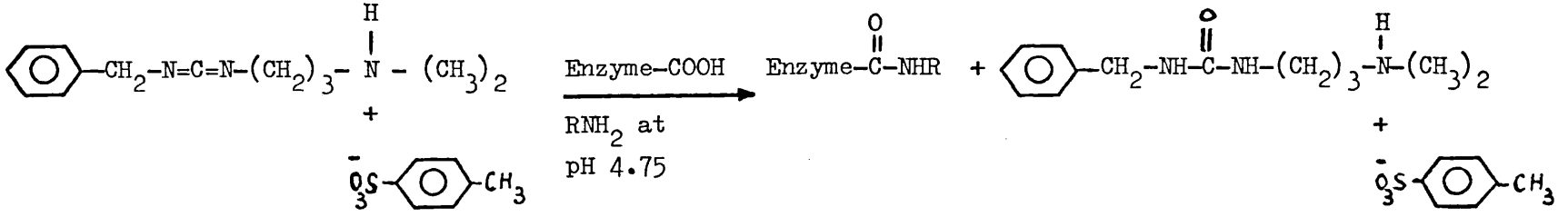
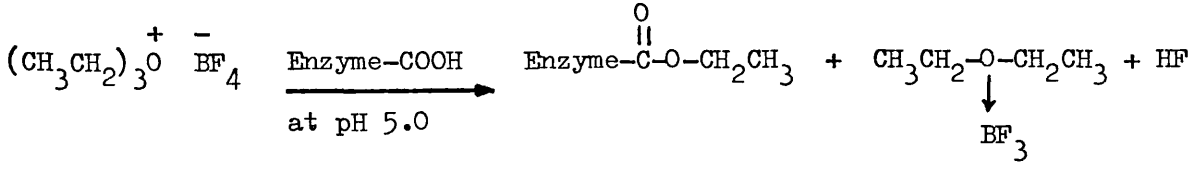


TABLE 6.1 (continued)

1-benzyl-3-(3-dimethylaminopropyl) carbodiimide p-toluene sulphonate (B.D.C.)



Triethyloxonium fluoroborate



N-alkyl-5-phenylisoxazolium fluoroborate

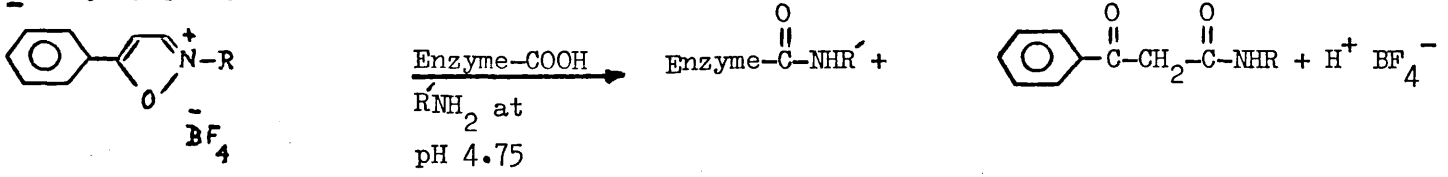
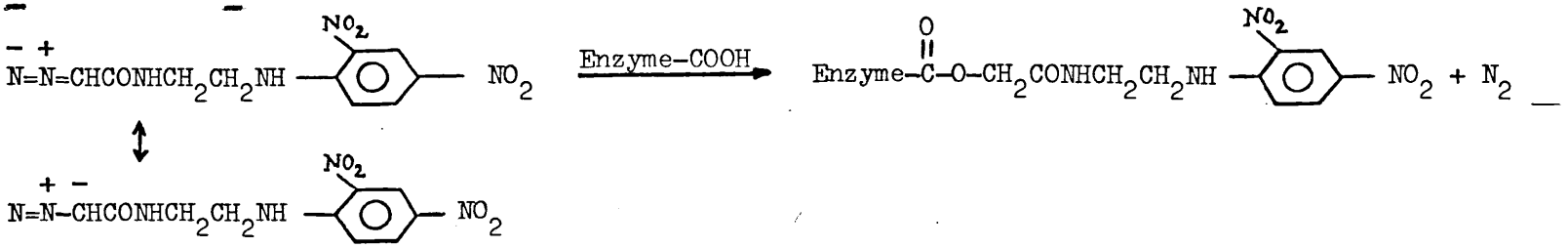
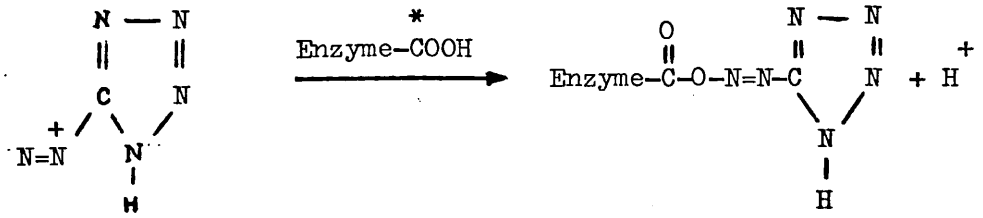


TABLE 6.1 (continued)

N-diazoacetyl-N'-2,4-dinitrophenylethylenediamine



5-diazo-1H-tetrazole



prepared in situ

* Reaction uncertain.[30]

TABLE 6.2

Table of enzymes which probably contain a carboxyl residue in the active site

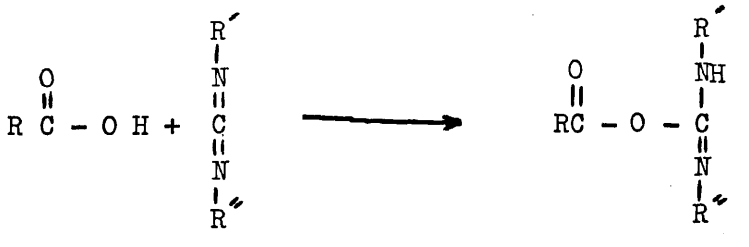
ENZYME	EVIDENCE	REFERENCE
Lysozyme	X-ray analysis, titration	17,22-25,27
	labelling and sequencing.	42
Porcine Pancreatic α-amylase	pH-activity	35
Glucoamylase	labelling	36,37
Carboxypeptidase A	sequencing	12,96,97
" B	sequencing	61,95
L-Glutamate Dehydrogenase	labelling	31
Ribonuclease	labelling	17,18,53
Trypsin	labelling	13-15
Lactose synthetase	labelling	60
Chymotrypsin	labelling	32,33
Subtilisin	labelling	29
Pepsin	labelling	1-11,26
Deoxy-ribonuclease A	labelling	16
Glycogen phosphorylase	labelling	30
Acid proteinase	labelling	77
Pancreatic lipase	labelling	28
β-D-glucosidase	labelling	19-21

From the methods of chemical modification of carboxyl groups described in CHAPTER 1 of this Thesis, it may appear that the most direct method of active site labelling is that carried out using a quasi-substrate. This type of labelling has been carried out on α-D-[38] and β-D-glucosidase[25,39-41] and on β-D-galactosidase[42] using inositol epoxides. Affinity labelling has been used to study the amino acid side chains, either directly involved, or in the vicinity of, the active sitesof β-D-galactosidase,[43] cellulase,[42] hexokinase,[44] lysozyme,[42] and β-D-glucosidase.[42] In general although there are obvious exceptions, both the methods referred to have been used to covalently label the active sites of enzymes whose substrates have a low molecular weight. When the normal enzyme substrate has a high molecular weight, the active site has been investigated either using a non-specific reagent such as triethyloxonium fluoroborate, or the differential labelling technique employing the carbodiimide coupling procedure first devised by Sheehan et al[56] and later developed by other workers.[17,57] The essential factor in this technique is that the reagent is specific, can be easily analysed and forms a stable covalent bond with the enzyme. Developments made in the sequencing of peptides using the technique originally devised by Edman[69] whereby the free amino terminal residue of the peptide is chemically modified and identified with the remainder of the peptide being recovered has now been automated.[70] The subject has been reviewed by Perini.[68] Cromwell et al.[71] have described a method for the determination of the carboxyl termini of proteins using ammonium thiocyanate and acetic anhydride with direct identification of the thiohydantoins It was reported that the process could be repeated over two or three cycles

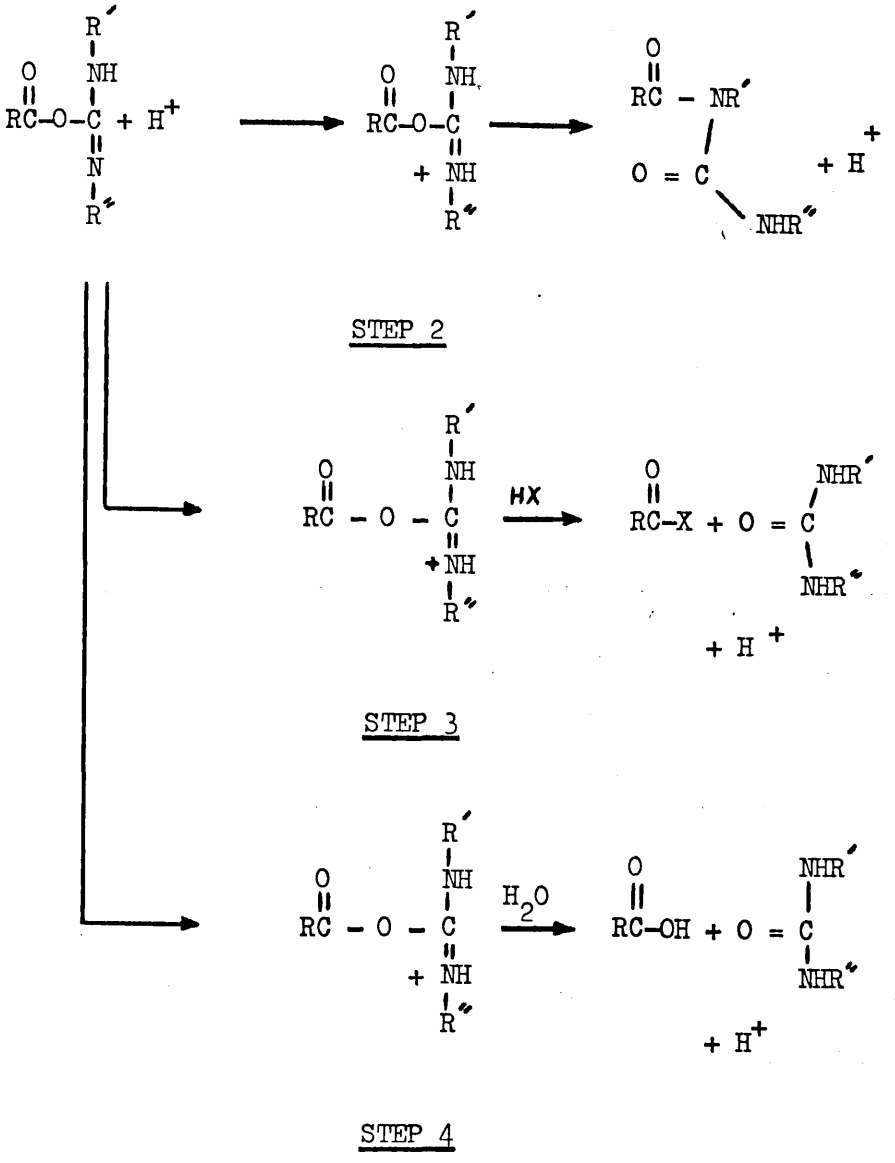
with two large peptides from insulin but attempts to use the method sequentially with ribonuclease A, lysozyme, glucagon, and aspartate transcarbamylase were not successful.

Woodward et al.[72-74] have developed a technique in which the chemical modification of carboxyl groups has been applied to give a simple and practical method of peptide synthesis.

The scheme proposed by Khorana[75] for the chemical modification of a carboxyl group with a water soluble carbodiimide is shown in SCHEME 6.1. The initial step is the reaction between the carboxyl group and the carbodiimide to form an O-acylisourea. This may then rearrange to form the N-acylurea, shown in the second step or react with a nucleophile to form the modified carboxylic acid, shown in the third step. If the O-acylisourea reacts with water, shown in the fourth step, the carboxylic acid is regenerated. It can be seen therefore that the reaction of water causes only minor inconvenience since it regenerates the carboxyl group which can react again with carbodiimide.



STEP 1



SCHEME 6.1 Proposed mechanism[75] for carboxyl group modification by a water soluble carbodiimide.

In experiments carried out on acetic acid in the absence of added nucleophile, very little N-acrylurea was detected and it was concluded that hydrolysis (Step 4) was much more rapid than the rearrangement (Step 2). It has also been shown by Hoare et al.,[17] that phenolic hydroxyl groups undergo modification in a similar way to carboxyl groups when reacted with nucleophiles in the presence of a carbodiimide.

This is a disadvantage to some protein modifications because tyrosine residues as well as carboxyl residues may undergo reaction. The former, however, can be regenerated by reaction with hydroxylamine.[57] This technique has been applied to regenerate the tyrosine residues in bovine trypsin,[15] chymotrypsin and chymotrypsinogen,[33] after their chemical modification. However, when α-lactalbumin[60] was chemically modified with aminomethanesulphonic acid in contrast to using glycinamide, hydroxylamine treatment did not give a satisfactory recovery of tyrosine. Also in the chemical modification of the carboxyl groups in ribonuclease and α-chymotrypsin carried out respectively by Wilchek et al.[18] and Banks et al.,[32] trysosine residues were reported not to undergo chemical modification with the carbodiimide coupling reagent. The chemical modification of tyrosine residues in α-amylase have been investigated by Ohnishi et al.[88]

The chemical modification of the majority of the enzymes given in TABLE 6.2 is now discussed in more detail.

Lysozyme

Parsons et al.[24] identified aspartic acid residue[52] as being critical to the catalytic activity of this enzyme after chemical modification using triethyloxonium fluoroborate

$$\text{lysozyme-COOH} + \text{Et}_3\text{O}^+\ \text{BF}_4^- \longrightarrow \text{lysozyme-COOEt} + \text{Et}_2\overset{+}{\text{O}}\ \overset{-}{\text{B}}\text{F}_3 + \text{HF}$$

The ethyl ester derivative was separated by ion-exchange chromatography on Bio-Rex 70 using a sodium phosphate gradient. It was found that although chitotriose could be bound to this derivative, it was devoid

of catalytic activity, and upon enzymatic digestion yielded β-ethyl aspartic acid from residue 52. Rand-Meir et al.[45] have used synthetic substrates to study binding and catalysis by lysozyme and have also applied secondary α-deuterium kinetic isotope effects to studies of catalysis by this enzyme.[46] Lin et al.,[47] modified the carboxyl groups present in chicken lysozyme using either 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (E.D.C.) or 1-benzyl-3-(3-dimethylaminopropyl) carbodiimide p-toluenesulphonate (B.D.C.) in the presence of nucleophiles such as aminomethanesulphonic acid, glycinamide or glycine methyl ester at pH 4.75, 25°C. It was found that eight carboxyl residues were modified per molecule of protein after 400 minutes reaction. In the quantitative modification and estimation of carboxyl groups in proteins described by Hoare et al.[17], lysozyme was reacted with a nucleophile in the presence of a water soluble carbodiimide. The kinetics of the reaction indicated that there were limits to the variation of carbodiimide and nucleophile when quantitative modification was desired but that a wide variety of reagents could be used if quantitative reaction was not essential. A variation in the structure of the carbodiimide could affect the carboxyl groups activated as there may be positions in the protein for example in which an aryl- or substituted arylcarbodiimide could not react whereas an ethyl derivative could, and variation in the charge, size, chemical and spectral properties of the nucleophile could alter the type of modification at a specific carboxyl group. It was reported that the modification reaction proceeded equally well in high concentrations of urea (7.5 M) or guanidine hydrochloride (5.0 M) and in the case of lysozyme, all the carboxyl groups were

modified during a 280 minute and 30 minute reaction period in the presence of each respective reagent. Fraenkel-Conrat et al.,[48] successfully esterified the carboxyl groups in lysozyme with methanol and hydrochloric acid, and in a later study by Kramer et al.,[49] using a similar acid catalysed esterification with tritiated methanol, the reaction was followed by incorporation of ^{3}H into the enzyme. From this work, the half lives were calculated for each carboxyl group undergoing modification. In a study of the sulphanilation of lysozyme by a carbodiimide reaction carried out by Kramer et al.,[25] it was shown that 90% of the carboxyl groups present were modified using 1.2M nucleophile in the presence of E.D.C. at pH 5.0. It was shown that on decreasing the concentration of nucleophile to 0.05M, Glu-35 and Asp-101 were most reactive while Glu-7, Asp-18 and Asp-66 were least. A change of nucleophile concentration altered carboxyl reactivity and addition of inhibitor reduced the reactivity of Asp-101 and Glu-35. Side reactions were reported to be unimportant. Wang et al.,[27] have carried out a chemical modification of carboxyl groups of lysozyme in a non aqueous liquid ammonia phase using 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-p-toluenesulphonate (C.M.C.) as the coupling reagent. 70% of the initial catalytic activity remained after reaction although six carboxyl groups had been amidized. When C.M.C. was replaced by Woodwards Reagent (N-ethyl-5-(m-phenyl-sulphonic acid)-isoxazolium chloride), 75% of the initial catalytic activity remained and three carboxyl groups were reported to have been modified.

Pepsin

The determination of the number of catalytically essential carboxyl groups in pepsin has been carried out by Paterson et al.[11] using trimethyloxonium fluoroborate labelled with ^{14}C, at pH 5.0. The loss of catalytic activity which resulted from enzyme modification was ascribed to the esterification of carboxyl groups. The relationship between the number of methyl groups incorporated and the remaining catalytic activity provided evidence that at least two carboxyl groups were essential to the activity of pepsin. The variation of enzyme activity with the number of methyl groups incorporated was treated according to the method described by Tsou Chen-Lu.[51] For n equally reactive groups i of which are catalytically essential, the fraction of activity remaining is given by $a = x^i$ where x is the fraction of groups modified. It is possible to find i by plotting $a^{1/i}$ against x. Matyash et al.[26] carried out a modification of the carboxyl groups in pepsin, using N-2,4 dinitrophenyl-1,6-diaminohexane in the presence of C.M.C. at pH 5.5. 40-50% loss of initial catalytic activity occurred after 1 mole of nucleophile had been incorporated, but three carboxyl groups were finally modified. Using affinity labelling, Chen et al.,[52] showed that it was possible to modify active site carboxyl groups and remove peptidase activity using an epoxide or an azide but without affecting the sulphite esterase activity of pepsin.

Trypsin

Bodlaender et al.,[13] and Feinstein et al.,[14] have studied the modification of essential carboxylic acid side chains of trypsin with izoxazolium salts. Both the structure of the reagent and the

pH of the modification reaction were important in achieving a selective modification. N-Methyl-5-phenylisoxazolium fluoroborate and N-ethyl-5-phenylisoxazolium fluoroborate at pH 3.8 resulted in the most specific reaction producing nearly complete inactivation by the modification of two to three carboxyl groups.

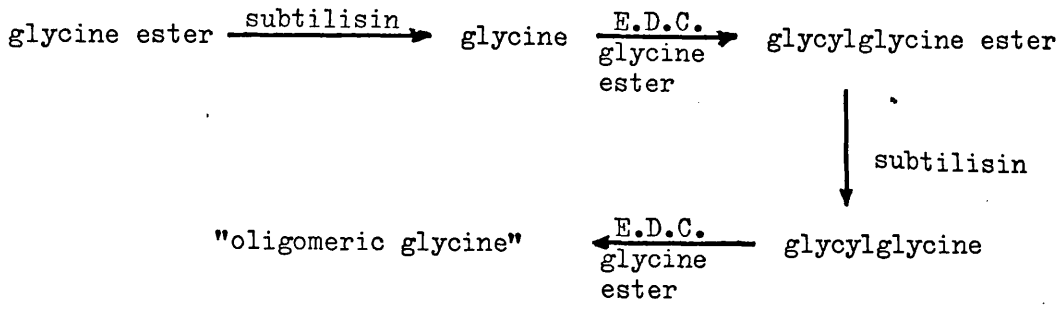
The modification could be largely prevented by the use of benzamide as a competitive inhibitor. The enol esters produced by the activation with isoxazolium salts were treated with glycine ethyl ester and O-methylhydroxylamine forming the corresponding amides. This second reaction did not affect the degree of inactivation. N-Methylhydroxylamine on the other hand, although fully displacing the activating reagent, was not incorporated and restored tryptic activity. Hydroxylamine also quantitatively displaced the reagent with some reactivation, the extent of which varied with reaction time and degree of initial modification. Lossen degradation of the trypsin hydroxamic acid derivative indicated modification of aspartic and glutamic acid residues. Eyl et al.,[15] modified bovine trypsin with glycinamide in the presence of E.D.C. Benzamidine was found to act as a competitive inhibitor of the modification reaction thus protecting certain essential carboxyl groups from reacting with the nucleophile. The inhibitor was removed from partially modified trypsin by dialysis, and the enzyme subjected to a further modification reaction using ^{14}C labelled glycinamide. The radio-labelled trypsin derivative was then degraded by 1-chloro-3-tosylamido-4-phenyl-2-butanone-treated trypsin and thermolysin and the

labelled peptides isolated and identified by their amino acid composition. The primary site of labelling was found to be Asp-177. Some radioactivity was also associated with Asp-182. In 8M urea, Asp-90 was modified, suggesting that in its native state, this amino acid residue is buried deeply within the trypsin molecule. 99% of the initial activity of trypsin was lost after modification with glycinamide and the results were reported to be highly reproducible with this nucleophile. When glycine methyl ester was used to replace glycinamide, satisfactory results were not obtained and this was attributed to a spontaneous hydrolysis of its ester linkage under the conditions used for the modification thus generating new carboxyl groups.

Subtilisin type Novo

Adams[29] has reported the coupling of glycinamide and glycine methyl ester to subtilisin type Novo using E.D.C. X-ray crystallography has shown that sixteen carboxyl groups are at or near the surface of the enzyme, however, examination of enzyme preparations obtained under a variety of reaction conditions suggested that between six and eight carboxyl groups were capable of reaction in the native enzyme. Very similar amounts of glycinamide were incorporated into both native and denatured subtilisin, demonstrating that covalent cross-linking between the carboxyl and other nucleophilic residues in the enzyme were not responsible for the low incorporation in the case of the native enzyme. Modification accompanied by loss in activity was less pronounced when the substrate was a small synthetic ester. Some difficulties, due to the production of an insoluble glycine

derivative were encountered when glycine methyl ester was coupled to the enzyme. This precipitate was probably oligomeric glycine formed by the reaction of free glycine, resulting from the rather facile enzymatic hydrolysis of the ester. The mechanism of formation of "oligomeric glycine" may be:



Chymotrypsin and Chymotrypsinogen

It has been shown by Carraway et al.[33] that the carboxyl modification of chymotrypsin with glycine methyl ester in the presence of E.D.C. showed a modification of about 13 of the 16 carboxyl groups of the enzyme, with no further modification even after prolonged (150 minutes) exposure to the reagent. It would appear that the majority of the carboxyl groups are exposed, while two or three are partially or completely buried within the three dimensional enzyme structure. When the modification was carried out in the presence of 7.5M urea, all of the carboxyl groups underwent esterification and the chymotrypsin thus obtained was catalytically inactive, whereas that prepared in the absence of urea was active. A similar treatment of chymotrypsinogen produced parallel results. In the absence of urea, 11 of the 14 carboxyl groups were modified suggesting that the same three carboxyls were buried in the zymogen and the active enzyme,

and that 11 groups were exposed in both proteins. Apparently the two new carboxyl groups produced during the activation were on the surface of the protein. The partially modified zymogen showed a more rapid activation than the native unmodified zymogen. The fully modified protein could not be activated.

Banks et al.[32] showed that at pH 7.0, 25°C, α-chymotrypsin could be completely inactivated by C.M.C. This inactivation was attributed to the reaction between C.M.C. and a single aminoacyl residue on the enzyme. At pH 5.0 the reaction between C.M.C. and α-chymotrypsin resulted in a partially active enzyme although this was not investigated further. From the kinetics of inactivation it was discovered that C.M.C. interacted with the substrate-binding site of the enzyme before the inactivation reaction occurred.

Ribonuclease

This enzyme was sequenced by Smyth et al.,[53] and in addition to the study of the carboxyl group modification of lysozyme carried out by Hoare et al.,[17] these workers also investigated the carboxyl group modification of ribonuclease in the presence of 7.5M urea. The reaction using glycine methyl ester in the presence of B.D.C. effected a 96% incorporation of the nucleophile into the number of free carboxylic acid residues as determined by sequence studies. Wilchek et al.[18] modified bovine pancreatic ribonuclease by binding glycine or alanylglycine to the carboxyl functions of the enzyme, making use of the phthalimidomethyl group for the reversible blocking of the glycine or dipeptide carboxyl groups by N-phthalimidomethyl ester formation and using B.D.C. for the coupling reaction. It was found that all 11 carboxyl groups of ribonuclease reacted with

glycine, while only 8 reacted with the dipeptide. In each case the electrical charges due to the carboxylate ions were not removed although their steric positions were displaced. Riehm et al.,[54] treated ribonuclease with C.M.C. and isolated from the reaction product five chromatographically distinguishable components, each of which possessed enzyme activity although this was lower than that of the native enzyme. Gibson et al.[55] have carried out a chemical modification of ribonuclease A using serine methyl ester or glycine ethyl ester in the presence of E.D.C. The modification was carried out as part of a procedure for locating the amidated and non-amidated residues in an automated sequence analysis of proteins. It was found that all 11 carboxyl groups of ribonuclease A reacted with glycine ethyl ester whereas only 7 reacted with serine methyl ester. As the coupling reaction had been carried out in the presence of 6M guanadine hydrochloride, it was unlikely that specific carboxyl groups would remain completely uncoupled. It was concluded that the lower incorporation observed in the case of serine methyl ester may have resulted from the amino acid residues being in the amide form. When taurine was used as the nucleophile in the chemical modification at pH 4.75, the degree of coupling was unsatisfactory. An improved coupling was obtained when the reaction was carried out at pH 7.0 but the resulting protein product gave low yields in the sequenator suggesting that it may have been partly blocked at the amino terminus. Poulos et al.,[16] studied the involvement of serine and carboxyl groups in the activity of bovine pancreatic deoxyribonuclease A. It was found that greater than 90% loss of activity was achieved within 20 minutes when E.D.C. was reacted with

the enzyme and this resulted in the modification of between 18 and 20 carboxyl groups. When the reaction was carried out in the presence of 0.1M calcium chloride, between 11 and 12 carboxyl groups were modified with a 46% loss of activity. It was concluded therefore that Ca^{2+} ions protected between 6 to 8 carboxyl groups.

L-Glutamate Dehydrogenase

Swaisgood et al.,[31] have studied the effect of carboxyl group modification on some of the enzyme properties of L-glutamate dehydrogenase from bovine liver. Enzyme modification was carried out using glycine methyl ester in the presence of E.D.C. By properly selecting the reaction conditions, the number of carboxyl groups modified per polypeptide chain could be varied from 2 to 3 to nearly complete modification. In the native form 30 to 40 fewer carboxyl groups reacted with E.D.C. than under conditions favouring unfolding and subunit dissociation. A comparison of the number of groups reacting with the theoretical number available[58,59] shows that essentially all groups are reactive under the latter conditions.

Lactose Synthetase

Lin[60] modified the carboxyl groups in α-lactalbumin, one of the two component proteins of bovine lactose synthetase, using glycinamide in the presence of E.D.C. At pH 4.75 the treatment caused rapid inactivation of lactose synthetase activity with 20 carboxyl groups modified within 400 minutes. An homologous reaction on hen egg-white lysozyme, which resembles α-lactalbumin in amino acid composition and sequence, led to modification of 8 out of 11 carboxyl groups with a loss of the cell-wall lytic activity. When the reactions were carried out using ^{14}C labelled glycinamide, in 4M

guanidine hydrochloride, an extra 0.6 and 2.1 residues of the nucleophile were incorporated for α-lactalbumin and lysozyme respectively. The major ^{14}C labels were located in residues 63-79 in α-lactalbumin and in residues 34-35, 6-13, and 62-68 in lysozyme. α-Lactalbumin was also reacted with aminomethanesulphonic acid in the presence of E.D.C. The carboxyl group modification was almost quantitative with this nucleophile and when the modified protein was subjected to further modification treatment with glycinamide in 4M guanidine hydrochloride, only 0.4 residue of glycine per molecule of protein was incorporated. The aminomethanesulphonic acid modified α-lactalbumin was found to be functionally inactive.

Glycogen Phosphorylase

Avromovic-Zikic *et al.*,[30] have shown that phosphorylase is rapidly inactivated by 5-diazo-1H-tetrazole or by C.M.C. in the presence of glycine ethyl ester. In both cases incorporation of approximately 1 mole of reagent per mole of monomer resulted in complete inactivation. Consideration of the conditions for inactivation and the known specificity of the C.M.C. reagent suggested that the inactivation of the enzyme was brought about by a modification of a carboxyl group.

Pancreatic Lipase

Dufour *et al.*,[28] modified 14 out of 52 carboxyl groups present in this enzyme using glycine ethyl ester in the presence of E.D.C. When norleucine methyl ester was used in the presence of C.M.C., 5 out of 52 carboxyl groups were modified, illustrating that the degree of modification was dependent on the nucleophile and

carbodiimide employed. In both cases the enzyme activity was suppressed but the active site was titratable with diethyl p-nitrophenyl phosphate.

Acid Protease

Lin et al.,[77] have showed that an aspartic acid residue at the active site of Rhodotorula glutinis acid protease is specifically inactivated by N-diazoacetyl-N'-2,4-dinitrophenylethylenediamine.

Peptic hydrolysis and analysis showed a residue attached to the β-carboxyl group of aspartic acid in a sequence Ile-Ala-Asp.

Bovine Carboxypeptidase B

The active centre of this enzyme was studied by Kimmel et al.,[61] using ^{14}C labelled α-N-bromoacetyl-D-arginine. The product was hydrolysed with pepsin and the radio-labelled fraction obtained by gel-permeation chromatography on "Sephadex" G-25 and ion exchange chromatography on CM cellulose. Various analyses suggested a sequence of a 12 unit oligopeptide containing a glutamic acid residue.

Insulin

This protein was chemically modified with glycine methyl ester in the presence of B.D.C. by Hoare et al.[17] It was found that all six carboxyl groups were modified within 1 hour when the reaction was carried out in 7.5M urea at pH 4.75, 25°C.

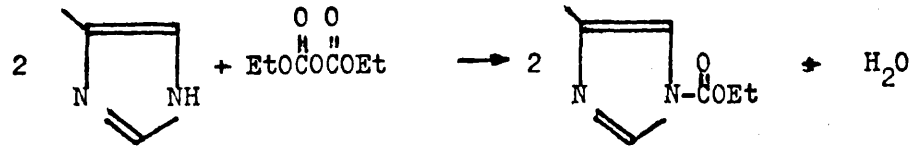
Myoglobin

When sperm-whale myoglobin was modified by Atassi et al.,[62-64] using glycine methyl ester or histidine methyl ester in the presence of a carbodiimide it was found that Glu-83 and Glu-85 underwent

chemical modification, and the resulting myoglobin was unfolded. The modified myoglobin was shown to be homogeneous on polyacrylamide gel electrophoresis. The histidine methyl ester modified myoglobin reacted well with antisera of native myoglobin and it was concluded that Glu-83 and Glu-85 were not located in an antigenic reactive region in myoglobin. When Glu-83 and Glu-85 were reduced with diborane, no conformation change was observed in the protein and no change in reactivity with antisera was detected although its relative mobility on starch gel electrophoresis was reduced to 0.9 of the native protein.

Porcine Pancreatic α-Amylase

It has been implied by Wakim et al.,[35] that α-amylase has a carboxylate anion and imidazolium cation at its catalytic centre. The role of the histidyl residues in α-amylase has been investigated by Elodi[34] using diethylpyrocarbonate. This reagent converts hystidyl side chains to carbethoxy-histidine. When four of the eight



histidines of the native α-amylase reacted with diethyl-pyrocarbonate, no gross structural conformational change occurred in the enzyme, however, when only 2 moles of carboxy-histidine per mole of enzyme were present, amylase activity was almost completely removed. The modified enzyme was still able to bind substrate analogues and it was therefore apparent that the catalytic histidyl residues and the substrate binding side chains were located relatively far from each other.

Nine pure peptides obtained from two forms of porcine pancreatic amylase after treatment with cyanogen bromide have been isolated and characterised by Cozzone et al.[87] The location of 4 disulphide bridges and 2 free SH groups in the peptides were determined.

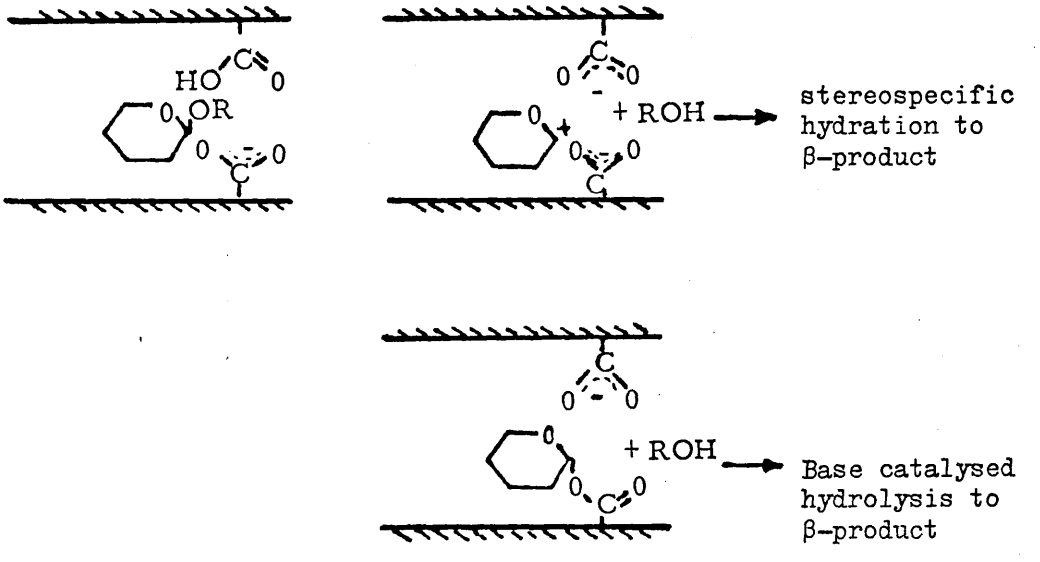
Glucoamylase I from Aspergillus niger (Agidex 3,000)

Gray et al.[65] and Jolley[66] have reported the inactivation of glucoamylase I using glycine methyl ester hydrochloride in the presence of E.D.C. It was found that an initial 20% loss of enzyme activity occurred rapidly, followed by a slower first order loss. When the reaction was carried out in the presence of maltose, the rapid initial loss of activity still occurred but the subsequent process was almost completely suppressed. The rapid initial loss in activity was attributed to a modification of carboxyl groups, other than those present in the active site, by a non-specific process. By carrying out amino acid analyses on both partially modified glucoamylase I resulting from the reaction in which the active site was protected with maltose, and that of the fully modified glucoamylase, it was concluded that probably 2 or 3 carboxylic acid groups were present in the enzyme active site.

The possible roles of active site carboxyl groups in the enzymic catalysis of glycosyl transfer reactions have been most thoroughly investigated and discussed in the case of lysozyme[106,107] and have been considered for a number of other glycoside hydrolases.[108]

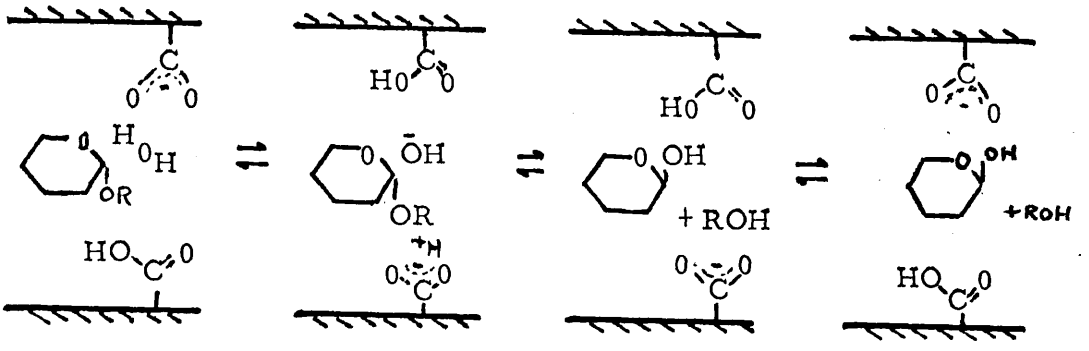
The precise mechanism of enzyme catalysed hydrolysis involving the amino acid side chain carboxyl groups must depend upon whether

the reaction proceeds with inversion or retention of configuration at C1 of the glycosyl residue transferred. In the case where the anomeric configuration is retained it has been suggested by Koshland[98] that this may result from two consecutive inversions or alternatively a transient glycosyl carbonium ion could be generated as advanced by Mayer and Larner,[99] and be stabilised electrostatically by an enzyme carboxyl group situated at the side remote from that of the departing aglycone as proposed by Blake et al.[100] for lysozyme. The latter proposed mechanism is shown in SCHEME 6.2.

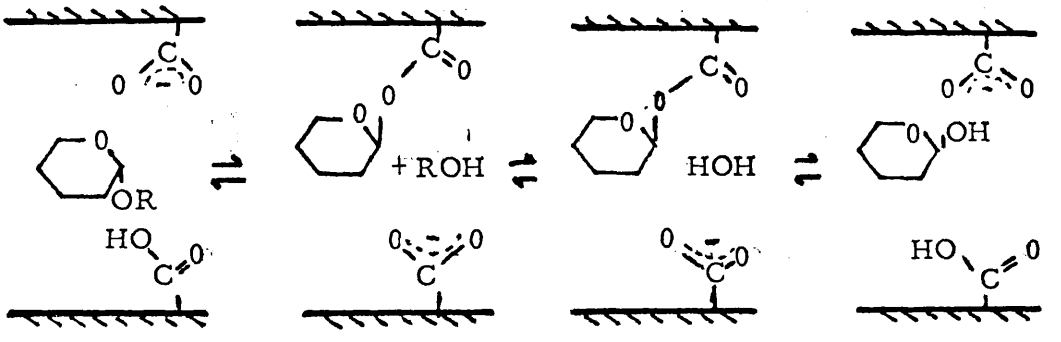


SCHEME 6.2 Proposed mechanism[100] for enzyme hydrolysis proceeding with retention of anomeric configuration of product.

For enzymes like glucoamylase which cause an inversion of the anomeric configuration either an intermediate is not proposed and the role of the side chain carboxyl groups of the enzyme are general acid and or general base:



or if an intermediate is proposed[39] it must break down via acyl oxygen fission:



In this work, differential labelling has been carried out on glucoamylases I and II using a radio-labelled nucleophile in the presence of a water soluble carbodiimide. In this way it was hoped to incorporate the radio-label into the enzyme active sites which had been protected with maltose during the initial non-specific modification of catalytically non-essential groups with unlabelled nucleophile. The radio-labelled glucoamylases I and II were digested by trypsin and the radio-labelled peptides isolated. The number of tyrosine residues in glucoamylase I undergoing chemical modification with taurine in the presence of E.D.C. has been estimated.

6.2 Results and Discussion

6.2.1 The chemical modification of glucoamylase I with glycine ethyl ester hydrochloride in the presence of E.D.C.

Glycine ethyl ester hydrochloride was chosen as a nucleophile for the chemical modification of glucoamylase in this work because it was commercially readily available radio-labelled with ^{14}C, and also to provide an alternative nucleophile to glycine methyl ester hydrochloride used by Gray et al.,[65] and Jolley[66] in a similar modification of this enzyme discussed earlier.

Glucoamylase I (350 µg) was reacted with E.D.C. (75 mM), and glycine ethyl ester hydrochloride (0.7 M) in the absence and presence of maltose (0.67 M) at pH 4.75, 25°C. Samples were withdrawn from the reaction mixture and enzyme activity measured by the amount of D-glucose liberated from 1% w/v wheat amylopectin using the glucose oxidase assay procedure described by Lloyd et al.[76] To correct for D-glucose produced from maltose during the modification reaction a control experiment was carried out in which E.D.C. was absent and samples withdrawn and incubated with wheat amylopectin at the same time intervals as those from the experiment in which E.D.C. was present. The percentage glucoamylase activity remaining at each time interval during modification was calculated. The results are shown in TABLES 6.3 and 6.4 and in FIGURE 6.1.

FIGURE 6.1 Activity of glucoamylase I during modification with glycine ethyl ester hydrochloride and E.D.C. in the presence (•) and absence of maltose (o).

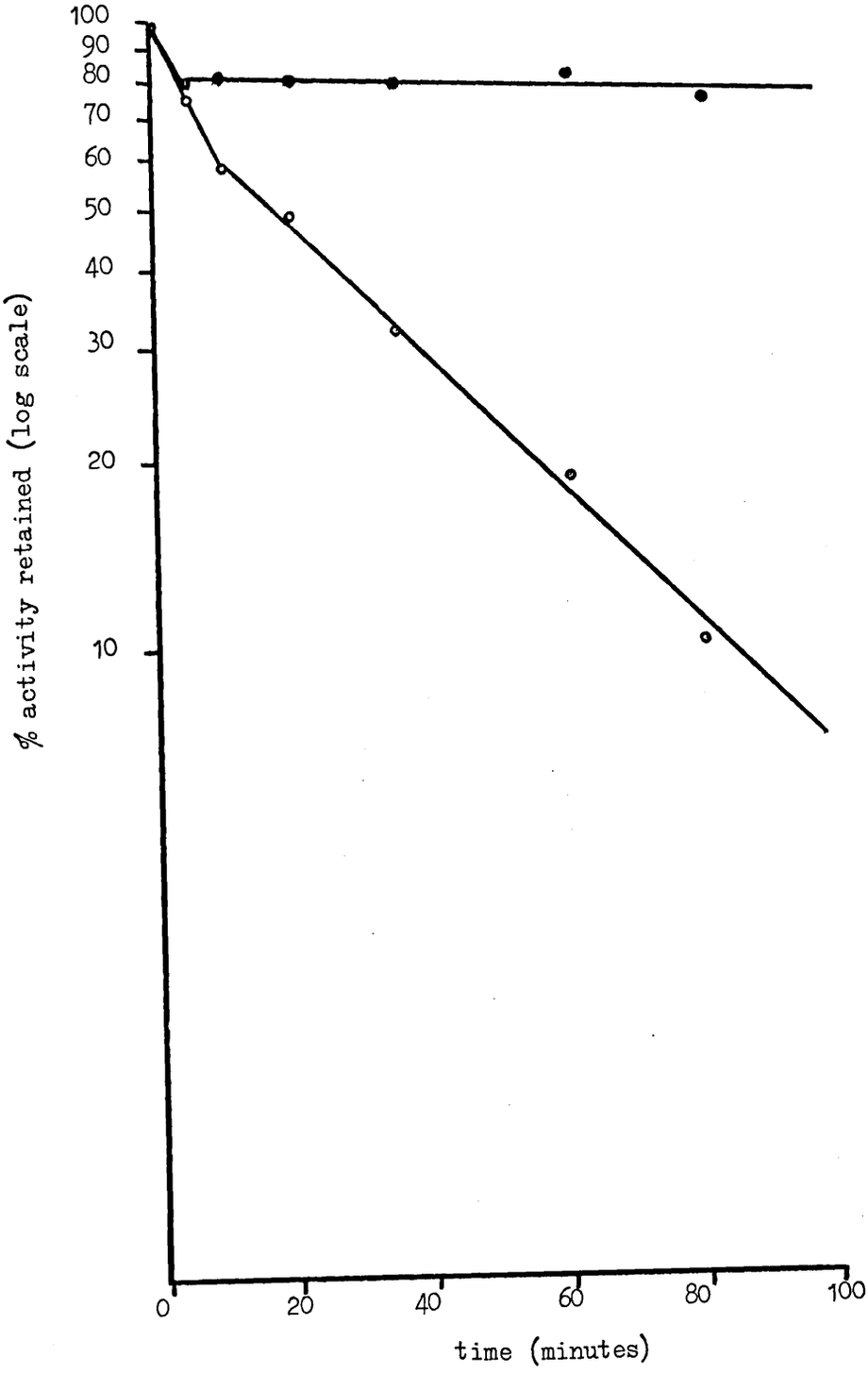


TABLE 6.3

Activity of glucoamylase I during modification with glycine ethyl ester hydrochloride and E.D.C. at pH 4.75.

time (minutes)	optical density at 525 nm from glucose oxidase assay	percentage glucoamylase activity remaining
0	0.370	100%
5	0.280	75.7
10	0.220	59.5
20	0.182	49.2
35	0.120	32.4
60	0.071	19.2
80	0.039	10.5

TABLE 6.4

Activity of glucoamylase I during modification with glycine ethyl ester hydrochloride in the presence of maltose in the presence and absence of E.D.C.

E.D.C. present		E.D.C. absent		
time (minutes)	optical density at 525 nm from glucose oxidase assay	time (minutes)	optical density at 525 nm from glucose oxidase assay	percentage glucoamylase activity remaining
1	0.310	1	0.318	97.6
5	0.295	5	0.366	80.6
10	0.322	10	0.389	82.8
20	0.364	20	0.449	81.1
35	0.438	35	0.539	81.3
60	0.580	60	0.690	84.1
80	0.660	80	0.841	78.6

It can be seen from FIGURE 6.1 that the inactivation of glucoamylase I on treatment with glycine ethyl ester hydrochloride and E.D.C. appears to be a two step process since the points lie on two straight lines From the slope of each line the pseudo first order rate constant for the loss of glucoamylase activity is initially 0.050 min^{-1} followed by 0.024 min^{-1} at 25°C. Extrapolation of the second line corresponding to a slower reaction gives an intercept of approximately 78% indicating that about 22% of the activity is rapidly lost while the remainder is lost more slowly by a pseudo first order process. In the reaction carried out in the presence of maltose it can be seen that the rapid initial loss of activity still occurs but the subsequent process is almost completely suppressed.

The pseudo first order rate constant for the partial deactivation is 0.045 min^{-1} (c.f. 0.050 min^{-1} for the initial deactivation in the absence of maltose). Maltose does not therefore prevent the initial loss of glucoamylase activity which probably results from a non-specific modification of the enzyme at regions other than the active site.

After chemical modification of glucoamylase with glycine ethyl ester hydrochloride, carried out in the absence of maltose, a precipitate formed in the reaction vessel. The precipitate may have been modified glucoamylase I which was rendered insoluble by a loss of its ionisable groups on esterification. Except for the precipitation, the results of deactivation of glucoamylase I using glycine ethyl ester hydrochloride are in close agreement to those reported by Gray *et al.*[65] and Jolley[66] for glycine methyl ester hydrochloride.

6.2.2 The chemical modification of glucoamylase I with glycine methyl ester hydrochloride in the presence of E.D.C.

In an attempt to overcome the problem of precipitation of glucoamylase after chemical modification in the absence of maltose, observed in 6.2.1, a similar experiment to that described was carried out in which glycine ethyl ester hydrochloride was replaced with glycine methyl ester hydrochloride.

This nucleophile had been used by Gray *et al.*[65] and Jolley[66] to chemically modify glucoamylase I and no precipitation was mentioned in their reports.

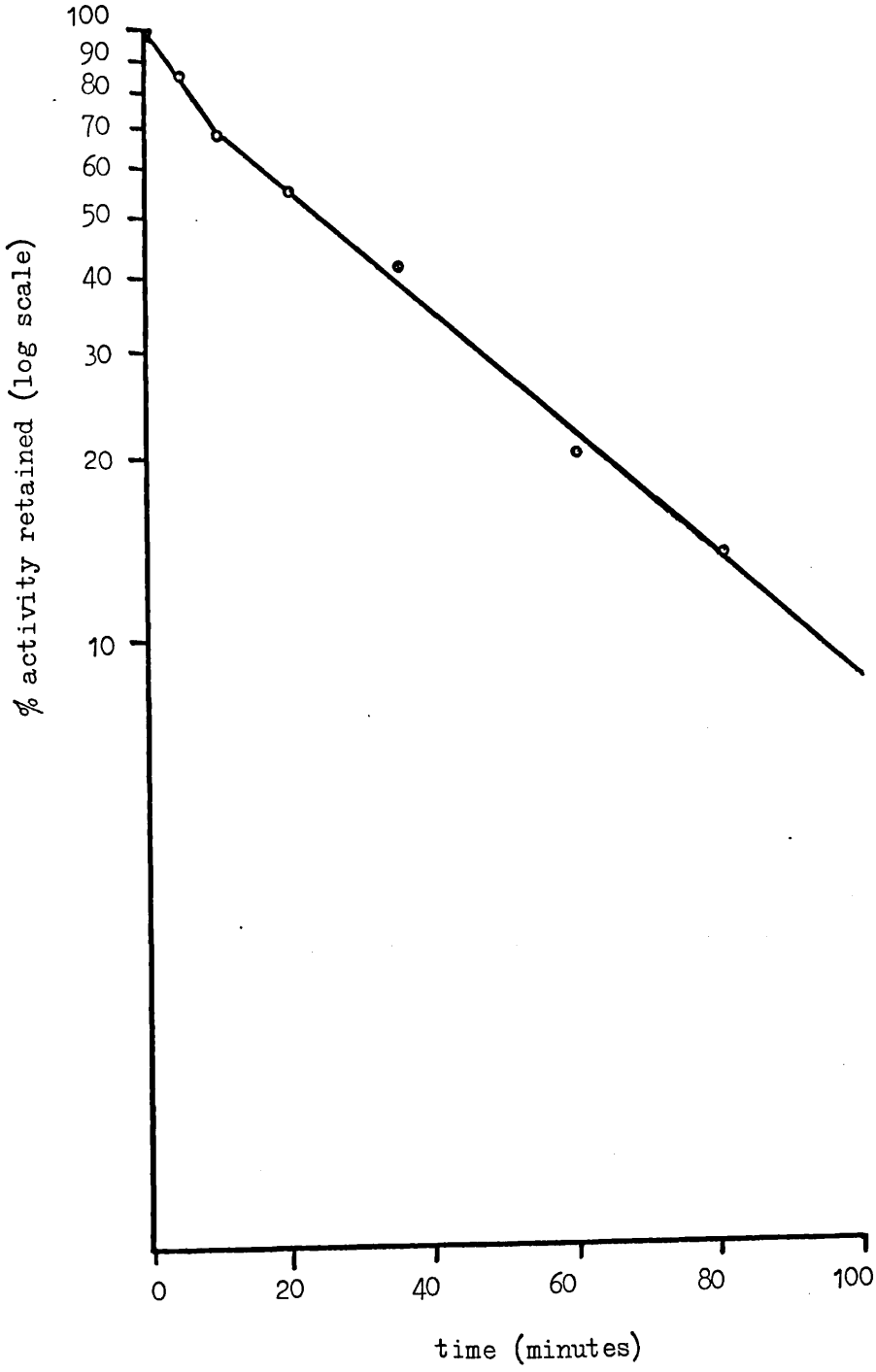
Glucoamylase I (11.4 mg) was reacted with E.D.C. (75 mM) and glycine methyl ester hydrochloride (0.7 M) in the absence of maltose at pH 4.75, 25°C. Samples were withdrawn from the reaction mixture, and enzyme activity tests carried out. The results are shown in TABLE 6.5 and FIGURE 6.2.

TABLE 6.5

Activity of glucoamylase I during modification with glycine methyl ester hydrochloride and E.D.C. at pH 4.75.

time(minutes)	optical density at 525 nm from glucose oxidase assay	percentage glucoamylase activity remaining
0	0.6080	100
5	0.5250	86.4
10	0.4150	68.3
20	0.3375	55.5
35	0.2554	42.0
60	0.1251	20.5
80	0.0851	14.0

FIGURE 6.2 Activity of glucoamylase I during modification with glycine methyl ester hydrochloride and E.D.C. in the absence of maltose



From the slope of each line the pseudo first order rate constant for the loss of glucoamylase activity is initially 0.039 min^{-1} followed by 0.023 min^{-1} at 25°C.

The enzyme inactivation is very similar to that reported by Gray *et al.*[65] and Jolley,[66] however, a precipitate formed after the 80 minute reaction period and this was presumed to be insoluble modified glucoamylase as before. Because of the problem of this precipitation, glycine methyl ester hydrochloride was considered to be unsatisfactory as a nucleophile for the chemical modification of glucoamylase in this work.

6.2.3 The chemical modification of glucoamylases I and II with taurine in the presence of E.D.C.

The use of taurine as a nucleophile in the chemical modification of carboxyl groups had first been suggested by Hoare *et al.*[17] This nucleophile has the advantage that it contains a sulphonic acid group which remains after the carboxyl group undergoing modification has been esterified.

$$\text{Enzyme–COOH} + H_2N(CH_2)_2SO_3H \xrightarrow[25^{\circ}C]{\text{E.D.C. pH 4.75}} \text{Enzyme–}\overset{O}{\overset{\|}{C}}\text{–NH}(CH_2)_2SO_3^- + H^+$$

Although the sulphonic acid group is displaced from the position of the carboxyl group after chemical modification, it offers an alternative ionisable group for solvation, and therefore may help to keep the chemically modified material in solution. Another advantage in the use of taurine for the chemical modification of carboxyl groups in proteins is that it is commercially readily available labelled with ^{14}C, and can be used to identify particular carboxylic acid groups

through peptide chromatography and mapping. It is also easily analysed on an amino acid analyser.

Glucoamylase I (5.7 mg) was reacted with E.D.C. (75 mM) and taurine (0.8M) in the absence of maltose, at pH 4.75, 25°C. Samples were withdrawn from the reaction mixture and activity tests carried out. The results are shown in TABLE 6.6 and FIGURE 6.3.

TABLE 6.6

Activity of glucoamylase I during modification with taurine and E.D.C. at pH 4.75.

time (minutes)	optical density at 525 nm from glucose oxidase assay	percentage glucoamylase activity remaining
0	0.5500	100
2.5	0.4010	72.9
5	0.2755	50.0
10	0.1385	25.2
15	0.0885	16.1
20	0.0540	9.8
35	0.0280	5.1
60	0.0200	3.6

After reaction the solution was left to stand at pH 4.75, 25°C for 7.5 hours. No precipitation was observed over this period.

The experiment was repeated in the presence of maltose (0.67M) and the results shown in TABLE 6.7 and FIGURE 6.3.

FIGURE 6.3 Activity of glucoamylase I during modification with taurine and E.D.C. in the presence (•) and absence of maltose (○)

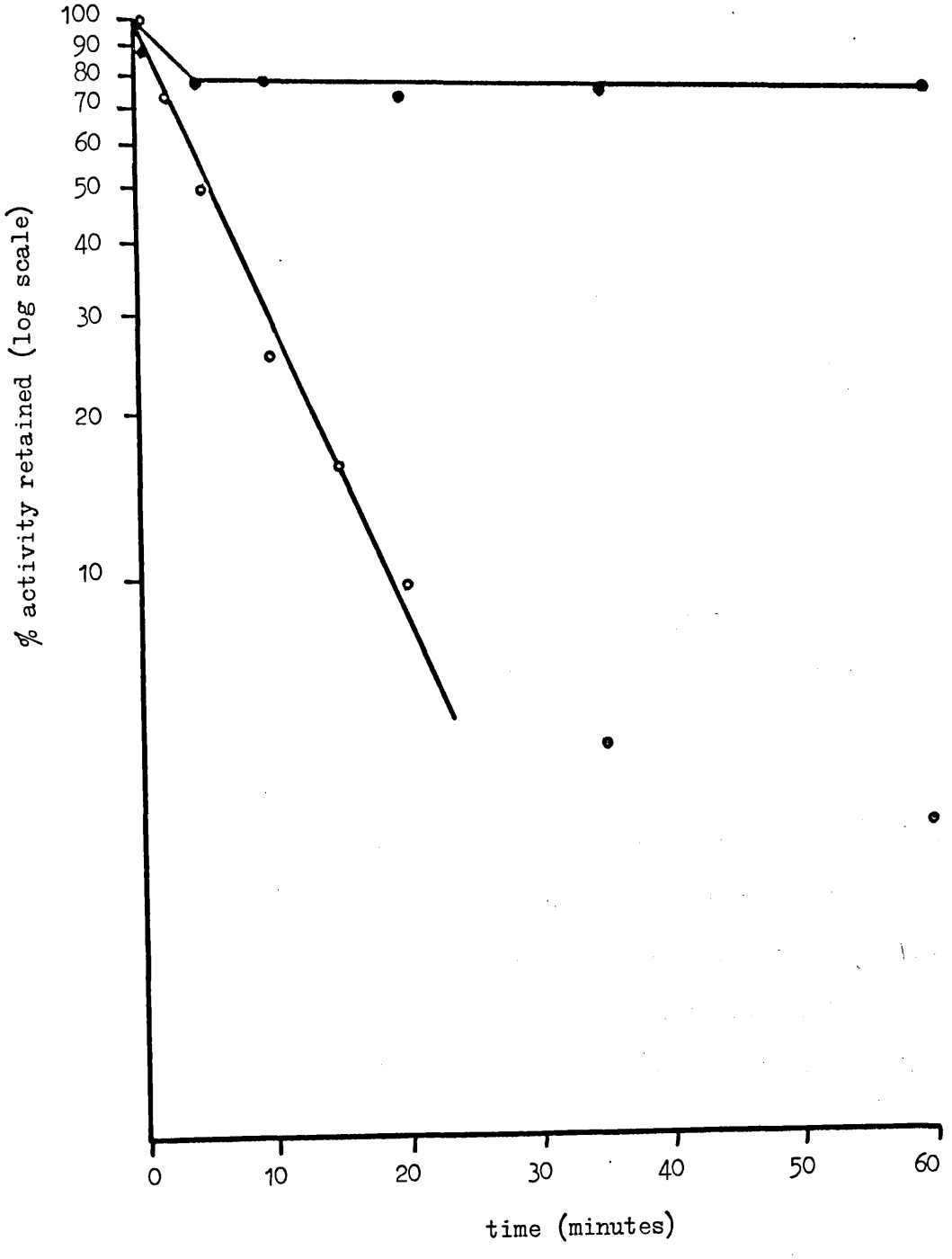


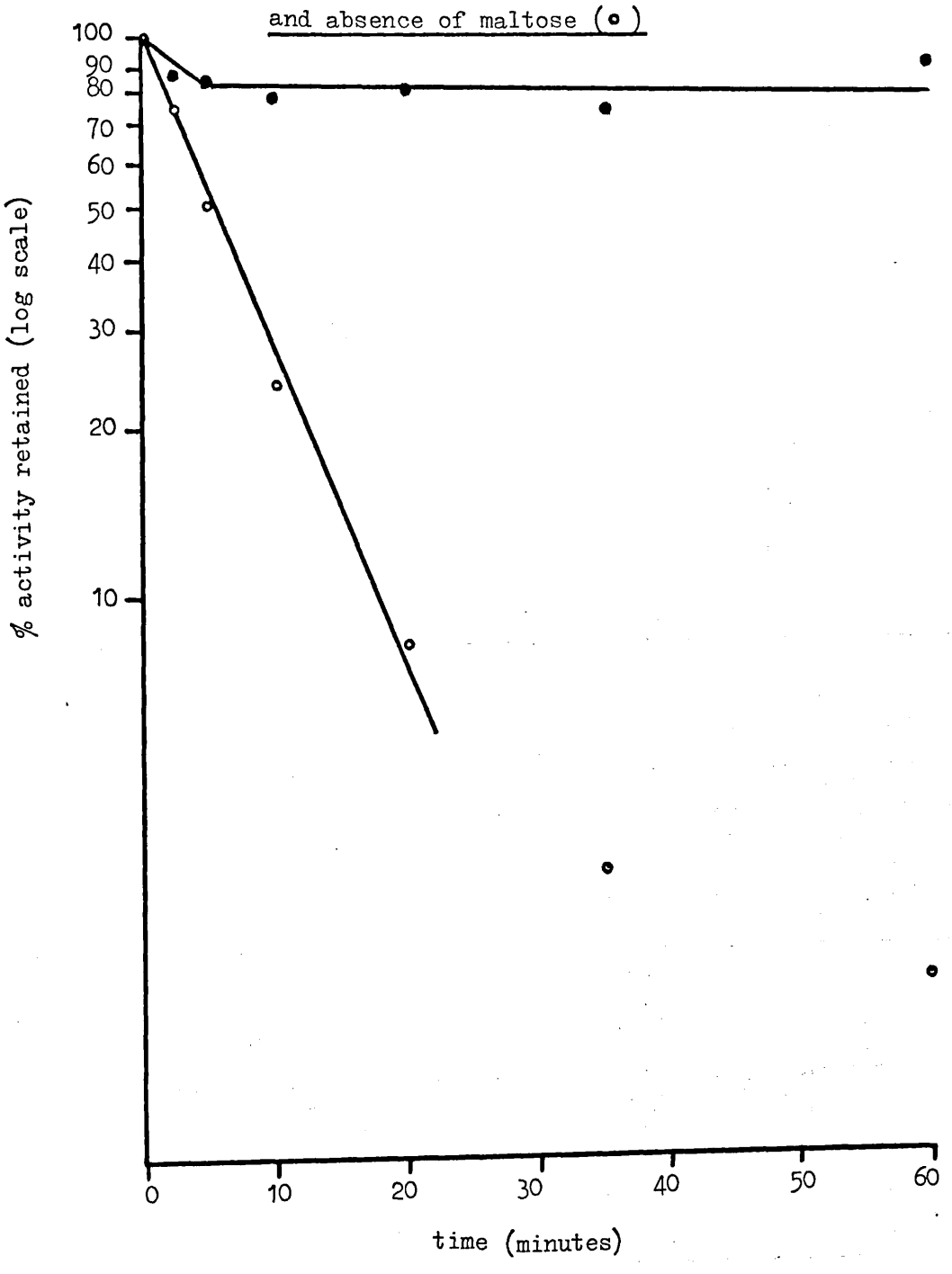
TABLE 6.7

Activity of glucoamylase I during modification with taurine in the presence of maltose and in the presence and absence of E.D.C.

E.D.C. present		E.D.C. absent		percentage glucoamylase activity remaining
time (minutes)	optical density at 525 nm from glucose oxidase assay	time (minutes)	optical density at 525 nm from glucose oxidase assay	
2	0.3320	2	0.3700	89.7
5	0.3420	5	0.4410	77.6
10	0.3565	10	0.4500	79.2
20	0.3780	20	0.5125	73.8
35	0.4400	35	0.5850	75.2
60	0.5300	60	0.7000	75.7
80	0.6050	80	0.8300	72.9

It can be seen from FIGURE 6.3 that the inactivation of glucoamylase I on treatment with taurine and E.D.C. appears to be a first order process in which 90% of the enzyme activity is lost after 20 minutes reaction. The pseudo first order rate constant for the deactivation is 0.124 min^{-1}. When the chemical modification was carried out in the presence of 0.67M maltose, a 20% loss of glucoamylase activity occurred rapidly with a similar pseudo first order rate constant to that observed in 6.2.1. No significant decrease in enzyme activity occurred over a further 80 minute reaction period.

FIGURE 6.4 Activity of glucoamylase II during modification with taurine and E.D.C. in the presence (•) and absence of maltose (o)



The experiments were repeated using glucoamylase II and the results are shown in TABLES 6.8 and 6.9 and FIGURE 6.4.

TABLE 6.8

Activity of glucoamylase II during modification with taurine and E.D.C. at pH 4.75

time (minutes)	optical density at 525 nm from glucose oxidase assay	percentage glucoamylase activity remaining
0	0.7000	100
2.5	0.5310	75.9
5	0.3600	51.4
10	0.1710	24.4
20	0.0590	8.4
35	0.0160	2.3
60	0.0080	1.1
80	0.0050	0.7

TABLE 6.9

Activity of glucoamylase II during modification with taurine in the presence of maltose and in the presence and absence of E.D.C.

E.D.C. present		E.D.C. absent		normalised percentage glucoamylase activity remaining
time (minutes)	optical density at 525 nm from glucose oxidase assay	time (minutes)	optical density at 525 nm from glucose oxidase assay	
1	0.3250	1	0.2790	100
2.5	0.3200	2.5	0.3140	87.5
5	0.3400	5	0.3400	85.8
10	0.3500	10	0.3800	79.1
20	0.4500	20	0.4620	83.6
35	0.4900	35	0.5570	75.5
60	0.7300	60	0.6725	93.2*

* high result may have occurred from an error in sampling.

The pseudo first order rate constants for the enzyme deactivation carried out in the presence and absence of 0.67M maltose are 0.045 min^{-1} and 0.132 min^{-1} respectively.

TABLE 6.10

The pseudo first order rate constants for the deactivation of glucoamylase by various nucleophiles in the presence and absence of 0.67M maltose.

glucoamylase	0.67M maltose present	nucleophile and molarity	pseudo first order rate constant min^{-1}
glucoamylase I	no	glycine methyl ester hydrochloride 0.7M	initially 0.039 secondly 0.023
	no	glycine ethyl ester hydrochloride 0.7M	initially 0.050 secondly 0.024
	yes		initially 0.045
	no	taurine 0.8M	0.124
	yes		initially 0.045
glucoamylase II	no		0.132
	yes		initially 0.045

Taurine effects the deactivation of glucoamylases I and II by a single step process while glycine methyl ester and glycine ethyl ester deactivations proceed by a two step process. While the rate constants for the partial loss of glucoamylase activity in the presence of 0.67M maltose are the same for each nucleophile, in the absence of maltose taurine effects a more rapid deactivation than either glycine methyl ester or glycine ethyl ester.

TABLE 6.11

Literature pK_a values of compounds used in the chemical modification of glucoamylases.

compound	pK_a at 25°C	Reference
glycine methyl ester	7.59	101
glycine ethyl ester	7.64	102
taurine	9.06	103

From TABLE 6.11 it can be seen that taurine is the strongest base used for the chemical modification of glucoamylase although at pH 4.75 all would be protonated. As taurine facilitates deactivation of glucoamylases I and II by a single step process it apparently does not discriminate between groups on the surface of the enzymes and within their active sites. In the presence of maltose only groups remote from the enzyme active site are chemically modified and this would appear to be independent of the nucleophile employed.

In order to test the reproducibility of the chemical modification of glucoamylases I and II using taurine as a nucleophile, and to obtain samples of chemically modified enzymes in which the amount of taurine incorporated could be determined quantitatively by amino acid analysis on the protein hydrolyzates, experiments were carried out as previously described and the chemical modification quenched after 60 minutes by addition of glacial acetic acid to 75 mM. The reaction mixture was then passed through a "Sephadex" G-100 gel-permeation chromatography column equilibrated with 0.01M ammonium

bicarbonate solution. After the optical density of each fraction had been measured at 280 nm, tubes containing protein were pooled and freeze-dried. The results of the enzyme activity both in the presence and absence of maltose were similar to those observed in the previous experiments, and it was therefore concluded that the chemical modification of glucoamylases I and II with taurine was reproducible.

Because the active sites of glucoamylases I and II could be protected with maltose when the enzyme was chemically modified with taurine, and the enzyme did not precipitate when reacted with this nucleophile in the absence of maltose, it was decided to use taurine as the nucleophile for the chemical modification of glucoamylases I and II for the active site investigation carried out in this work.

In order to determine whether some carboxyl groups essential for glucoamylase activity reacted preferentially with E.D.C. and taurine, an experiment was carried out in which the amount of E.D.C. in the modification reaction corresponded approximately to three molar equivalents of enzyme. This figure was taken because it had been reported by Gray et al.[65] that probably 2 or 3 carboxyl groups were involved in the active site of glucoamylase I.

Glucoamylase I (5.7 mg, 0.07 μM) was reacted with E.D.C. (0.214 μM) and taurine (0.8M) in the absence of maltose at pH 4.75, 25°C. Samples were withdrawn from the reaction mixture and activity tests carried out. The results are given in TABLE 6.12 and FIGURE 6.5. After 80 minutes reaction, the solution was passed through a "Sephadex" G-100 gel-permeation column and fractions containing protein were pooled and freeze-dried.

FIGURE 6.5 Activity of glucoamylase I during modification with taurine and low concentration (0.214 μM) E.D.C.

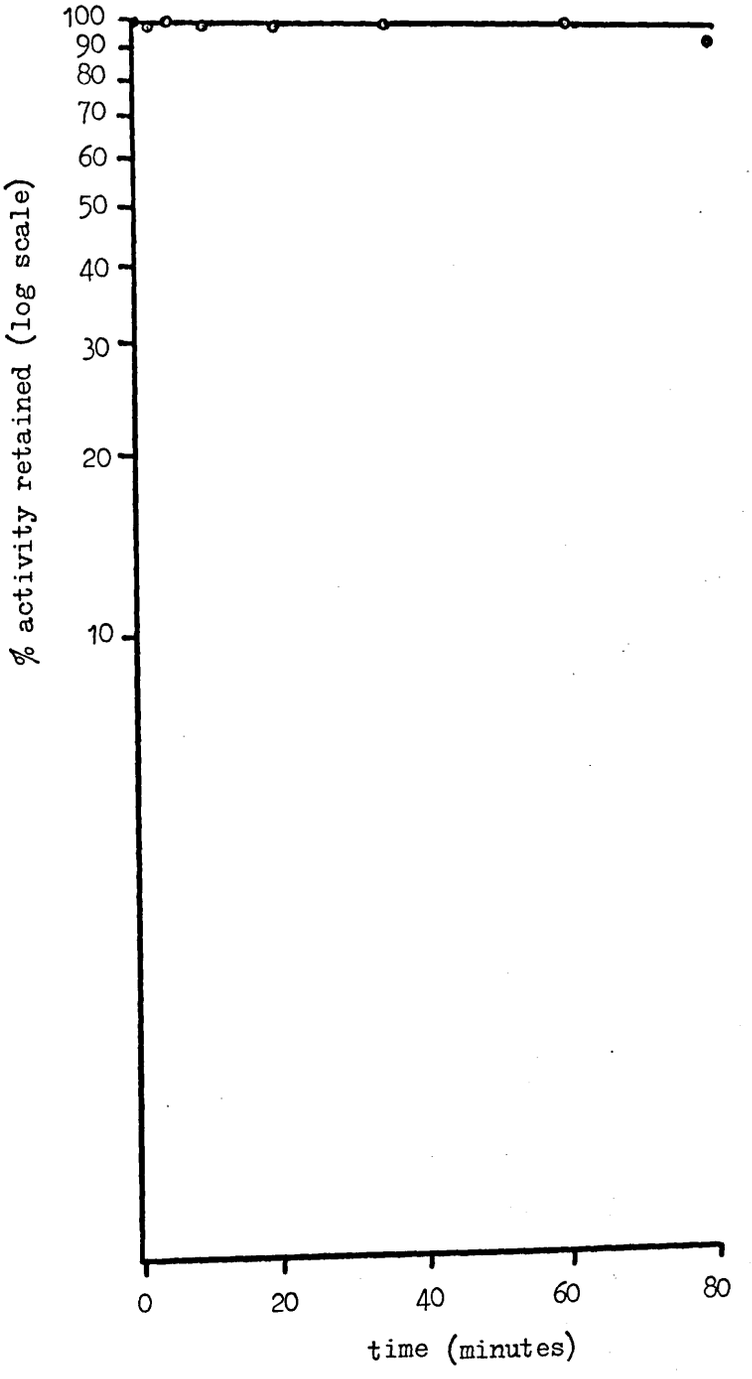


TABLE 6.12

Activity of glucoamylase I during modification with taurine and low concentration (0.214 μM) E.D.C.

time (minutes)	optical density at 525 nm from glucose oxidase assay	percentage glucoamylase activity remaining
0	0.7320	100
2.5	0.7280	99.4
5	0.7325	100
10	0.7310	99.8
20	0.7300	99.7
35	0.7290	99.6
60	0.7320	100
80	0.6850	93.6

From these results it was concluded that glucoamylase I did not possess carboxyl groups in the active site which exhibited an enhanced reactivity toward chemical modification with taurine in the presence of a low concentration of E.D.C.

In order to determine whether more carboxyl residues were susceptible to chemical modification when the enzyme was denatured glucoamylase I (5.7 mg) was reacted with E.D.C. (75 mM) and taurine (08. M) in the presence of guanidine hydrochloride (5.0 M) at pH 4.75, 25°C. Samples were withdrawn from the reaction mixture and activity tests carried out. The results are shown in TABLE 6.13. After 80 minutes the reaction was quenched by the addition

of glacial acetic acid to 75 mM, and the reaction mixture passed through a "Sephadex" G-100 gel-permeation chromatography column as previously described. Fractions containing protein were pooled and freeze-dried.

Because the deactivation of glucoamylase I in this experiment represented both a chemical modification and a denaturation the pseudo first order rate constants for enzyme inactivation were not determined but the sample was used to measure the total number of groups modifiable. The results are given in TABLE 6.14.

TABLE 6.13

Activity of glucoamylase I during modification with taurine and E.D.C. in the presence of 5.0M guanidine hydrochloride.

time (minutes)	optical density at 525 nm from glucose oxidase assay	percentage glucoamylase activity remaining
0	0.3400	100
5	0.2585	76.0
10	0.2015	59.3
20	0.1440	42.4
35	0.0920	27.1
60	0.0400	11.8
80	0.0400	11.8

The reason for 11.8% glucoamylase activity remaining after 60 minutes reaction under denaturing conditions is probably due to the very low optical density measurement recorded.

Each freeze-dried chemically modified protein sample was hydrolysed with 6M hydrochloric acid at 110°C for 16 hours. After hydrolysis the samples were evaporated to dryness and made up to a standard volume with buffer prior to amino acid analysis. From the amino acid analyses the amounts of valine and taurine present in each sample was determined. From the former amino acid the amounts of glucoamylases I and II could be calculated from the number of residues per 100 g protein determined in CHAPTER 4, TABLE 4.3 of this Thesis, and hence the number of moles of taurine incorporated per mole of enzyme evaluated. The results are given in TABLE 6.14.

TABLE 6.14

Amounts of taurine incorporated in glucoamylases I and II after chemical modification.

glucoamylase	maltose present	guanidine hydro-chloride present	amount of gluco-amylase present from amino acid analysis μM	amount of taurine present from amino acid analysis μM	moles of taurine incorpora-ted per mole of enzyme
glucoamylase I	no	no	0.0007513	0.01623	21.60
	no	yes	0.0006471	0.02917	45.07
	yes	no	0.0020660	0.03687	17.84
glucoamylase I low E.D.C. concentration	no	no	not determined	0.0	0.0
glucoamylase II	no	no	0.0022030	0.05234	23.76*
	yes	no	0.0011850	0.02898	24.45

* results subject to error see text.

From these results it can be seen that a further 4 moles of taurine are incorporated into glucoamylase I when the enzyme is chemically modified in the absence of maltose. This suggests that there are four groups on the enzyme which are capable of undergoing chemical modification but in the presence of maltose (substrate) are unable to do so. These groups are therefore probably involved in the active site of glucoamylase I. When the chemical modification of glucoamylase I was carried out under denaturing conditions (i.e. in 5M guanidine hydrochloride) a further 23 moles of taurine were incorporated into the enzyme. These probably resulted from the chemical modification of groups normally in hydrophobic regions of the enzyme and although not directly involved in the active site may be responsible for maintaining the three dimensional structure of glucoamylase I. However, the number of moles of taurine incorporated into carboxyl groups of glucoamylase I under these conditions represent approximately half the number expected from the sum of aspartic and glutamic acid residues from amino acid analysis carried out in CHAPTER 4 of this Thesis. The conclusions to be drawn from this are either that the chemical modification of carboxyl groups present in glucoamylase I by taurine were far from complete even under denaturing conditions, or perhaps more reasonably that a number of the aspartic and glutamic acid residues existed as asparagine and glutamine in the enxyme and therefore did not undergo chemical modification. The amount of taurine incorporated into glucoamylase I both in the presence (17.8 moles) and absence (21.6 moles) of maltose are lower than the corresponding values of 36 and 38 moles reported by Gray et al.[65]

using glycine methyl ester as a nucleophile, however, their values were based on a protein molecular weight of 61,000. When glucoamylase I was reacted with a low concentration of E.D.C., no taurine was detected in the enzyme hydrolyzate. This agrees with the results given in TABLE 6.12 and FIGURE 6.5 in which effectively no loss in glucoamylase activity was observed during the reaction period. In cases where taurine has been incorporated into glucoamylase, a decrease in enzyme activity has been observed.

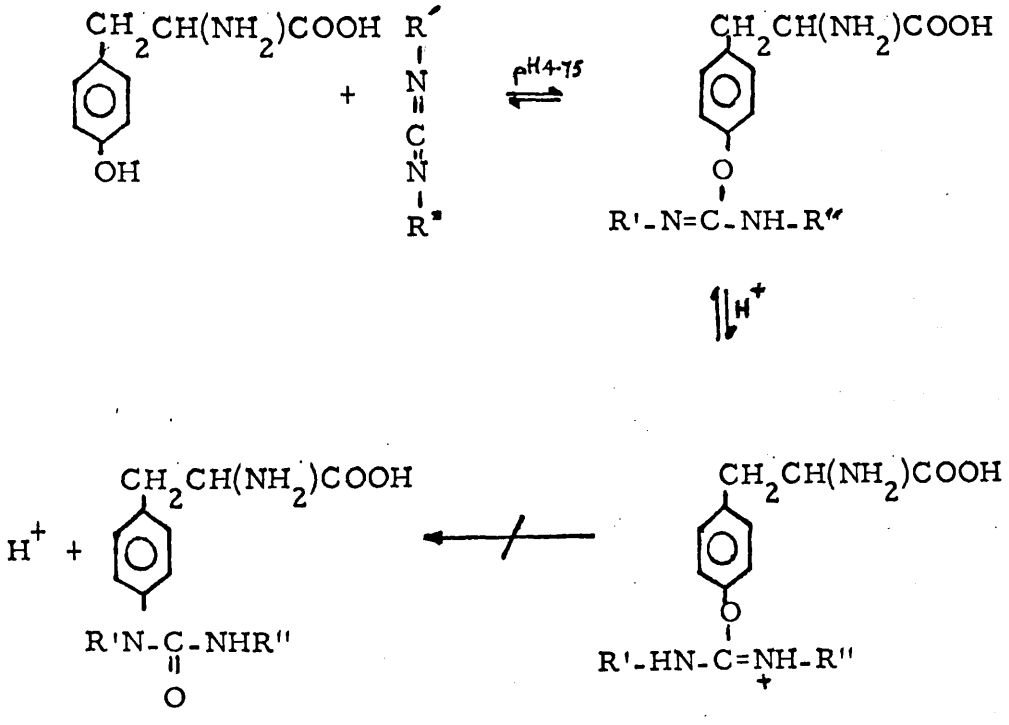
While the results obtained for the chemical modification of glucoamylase II in the presence of maltose show a slightly greater number of moles of taurine incorporated per mole of enzyme than in glucoamylase I, the amount of taurine incorporated in glucoamylase II in the absence of maltose is smaller, and this clearly cannot be correct. During the acid hydrolysis of glucoamylase II which had been chemically modified with taurine in the absence of maltose, the ampoule had cracked allowing the hydrochloric acid to escape. This effectively made a comparison of the results impossible.

Because glucoamylase II incorporated a greater amount of taurine than glucoamylase I when reacted in the presence of maltose, it is reasonable to suppose that the former enzyme has a greater number of groups accessible for chemical modification than the latter enzyme.

6.2.4 The regeneration of tyrosine residues in chemically modified glucoamylase I

Carraway *et al.*[57] have established that phenolic groups present in tyrosine residues in proteins undergo a similar chemical modification to carboxyl groups when reacted with a carbodiimide in

the presence of a nucleophile. The proposed mechanism[75] for the chemical modification of phenolic groups shown in SCHEME 6.3 is similar to that given in SCHEME 6.1 except that in the case of tyrosine, which is only weakly acidic, the product has been shown to be an O-aryl isourea and that the rearrangement to the N-aryl urea only occurs in the case of strongly acidic phenols.[81-83]



SCHEME 6.3 Proposed mechanism[75] for the chemical modification of tyrosine by a carbodiimide.

The chemical modification of tyrosine may be reversed by reaction with hydroxylamine thus regenerating the free tyrosine phenolic groups in the protein.

To determine the number of tyrosine groups reacting when glucoamylase I was chemically modified with taurine and E.D.C. in the absence of maltose as described in section 6.2.3, a sample of chemically modified glucoamylase I (19.2 mg) was prepared and a portion treated with hydroxylamine. After reactants had been removed from the modified enzyme samples by gel-permeation chromatography on "Bio-Gel" P-60, the protein samples were acid hydrolysed and subjected to amino acid analysis. The results showed that from 15.7 moles of taurine incorporated into glucoamylase I only 9.4 moles remained after hydroxylamine treatment. Hence 6.3 moles of tyrosine had been regenerated from chemically modified glucoamylase I using this procedure. These results however may be subject to error because the amount of taurine incorporated into glucoamylase I is lower than the value obtained (21.6 moles) in an earlier experiment. (See TABLE 6.14). By comparing the decrease in glucoamylase activity shown in TABLE 6.15 and FIGURE 6.6 with that shown in TABLE 6.6 and FIGURE 6.3 it can be seen that the extent of enzyme inactivation in the former experiment was slightly greater and this may have accounted for the larger amount of taurine incorporated in the enzyme. This observation tends to agree with the report by Gibson *et al.*[55] that the degree of coupling of taurine in the chemical modification of proteins at pH 4.75 was unsatisfactory however it does not agree with earlier work in which taurine gave reproducible results.

FIGURE 6.6 Activity of glucoamylase I during modification with taurine and E.D.C. in the absence of maltose (sample used to determine extent of tyrosine modification).

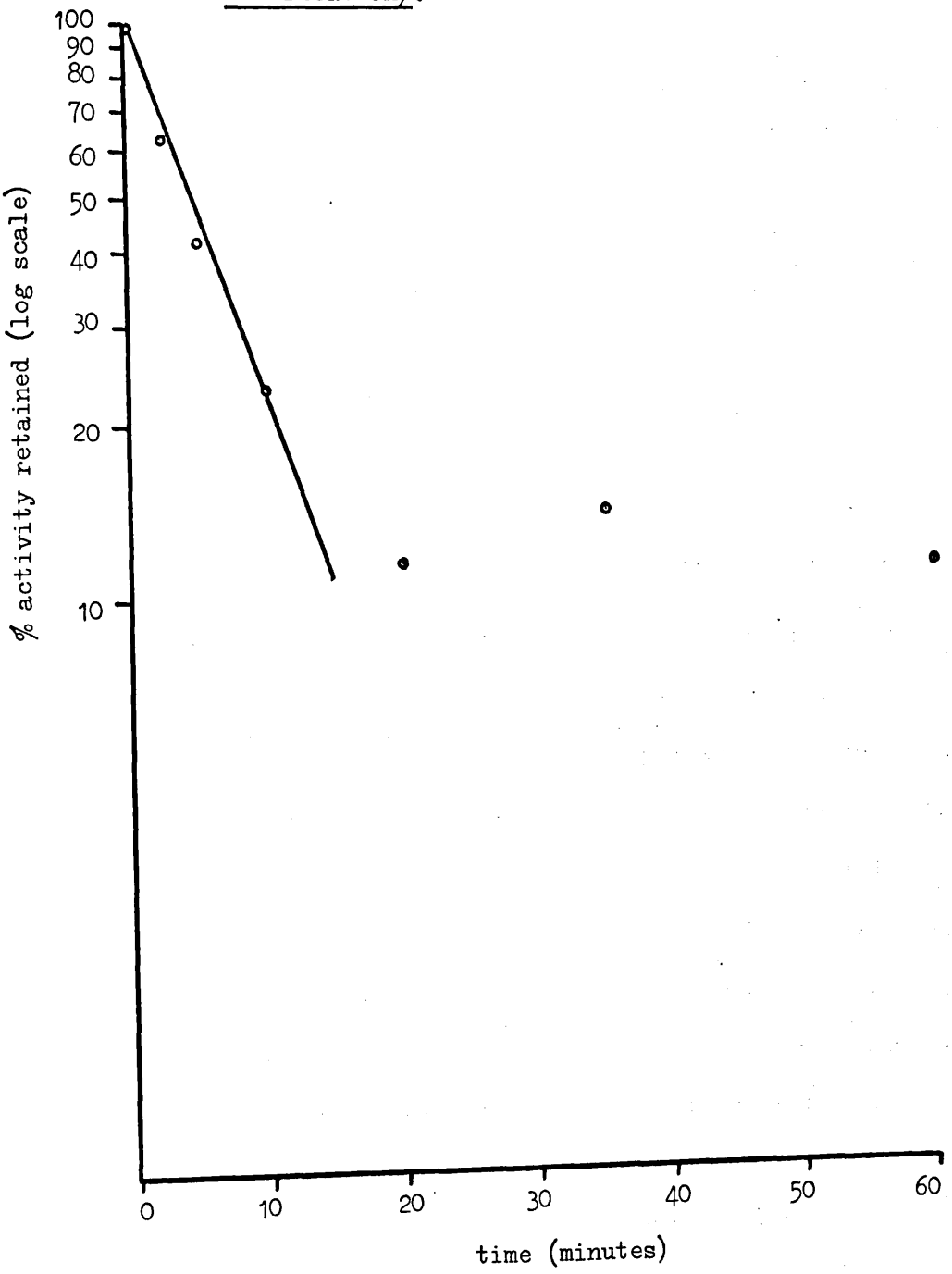


TABLE 6.15

Activity of glucoamylase I during modification with taurine and E.D.C. at pH 4.75. (Sample used to determine the extent of tyrosine modification).

time (minutes)	optical density at 525 nm from glucose oxidase assay	percentage glucoamylase activity remaining
0	0.3375	100
2.6	0.2510	63.8
5	0.1600	42.4
10	0.0790	23.4
20	0.0400	11.9
35	0.0480	14.2
60	0.0390	11.6

It was also observed that the recovery of amino acids in the hydroxylamine treated protein were only 75% of those from the untreated protein even though almost equal weights of each were taken for acid hydrolysis. This is not explained, but if a correction were to be made to increase the recovery of amino acids in the treated protein this would have the effect of reducing the difference in the amount of taurine between the treated and untreated protein. The amount of glucoamylase present in each sample is based on glutamic acid and leucine amino acid residues. It was not possible to use valine as in 6.2.3 because this peak overlapped with methionine in this analysis.

This work was carried out with the assistance of Mr D Fitzpatrick and Mr D L Fortune as an undergraduate research procject.

6.2.5 The partial chemical modification of glucoamylases I and II with taurine to obtain samples for subsequent reaction with ^{14}C radio-labelled taurine

In order to carry out an investigation into the number of chemically reactive groups in the active site region of glucoamylases I and II without relying on complete acid hydrolysis and amino acid analysis of the modified enzymes, which leads to subtraction of two large numbers to obtain the answer, experiments were carried out using ^{14}C radio-labelled taurine and the amount of radioactivity incorporated in each enzyme measured.

Glucoamylase I (20 mg) was reacted with E.D.C. (75 mM) and taurine (0.8 M) in the presence of maltose (0.67M) at pH 4.75, 25°C.

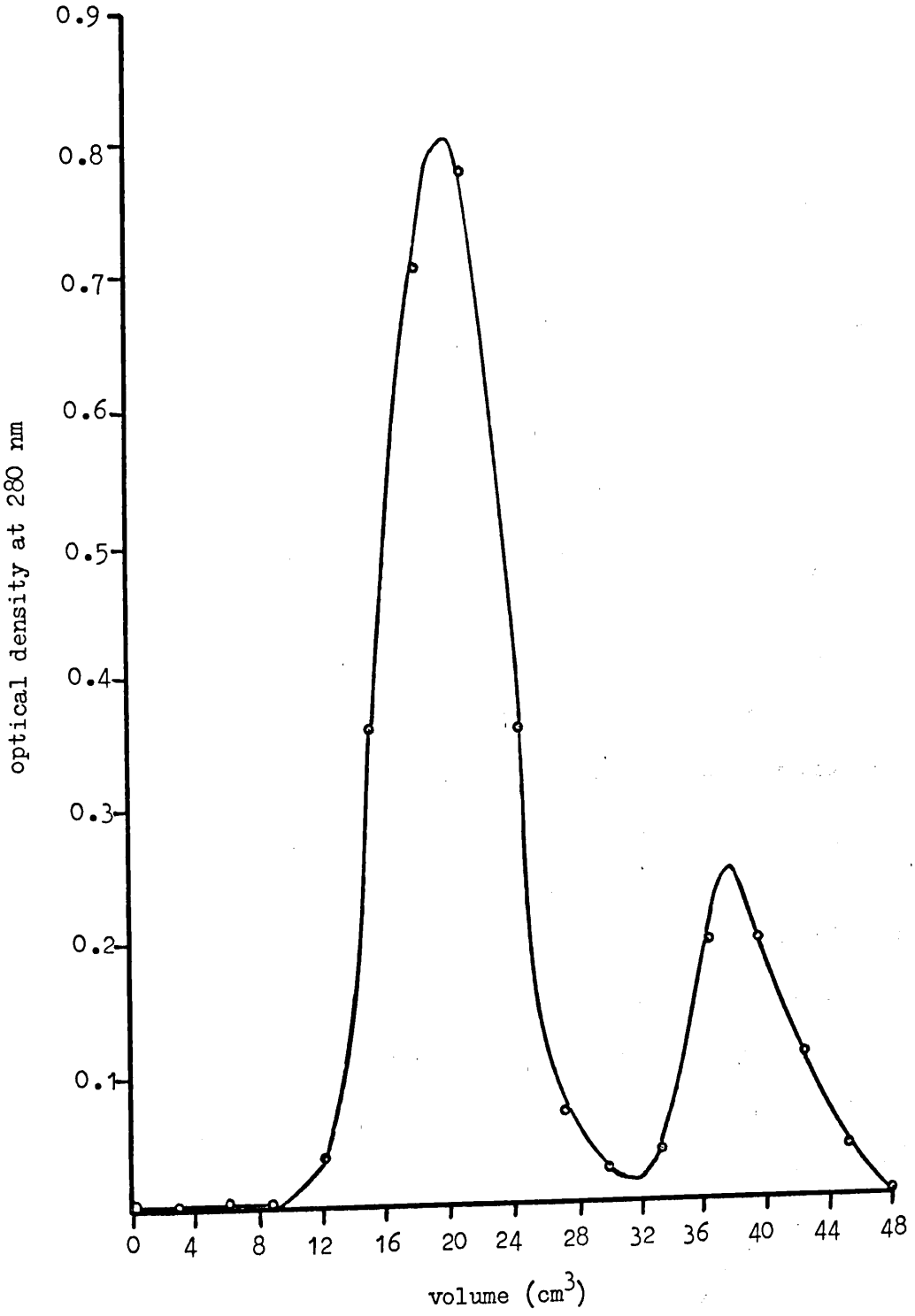
Samples were withdrawn from the reaction mixture and activity tests carried out. The results are given in TABLE 6.16.

TABLE 6.16

Activity of glucoamylase I during modification with taurine and E.D.C. in the presence of maltose

time (minutes)	optical density at 525 nm from glucose oxidase assay
0	0.3870
2	0.3940
60	0.5270

FIGURE 6.7 Chromatographic separation of partially modified glucoamylase I from taurine, maltose and E.D.C. on "Sephadex" G-100



After 60 minutes, the chemical modification was quenched by the addition of glacial acetic acid to 75 mM and the reaction mixture passed through a "Sephadex" G-100 gel-permeation chromatography column as previously described. The separation is shown in FIGURE 6.7. Fractions containing protein (as shown by optical density at 280 nm) were pooled and freeze-dried. The sample was then dissolved in 0.01 M ammonium bicarbonate solution pH 8.1 and re-applied to the gel-permeation column. Fractions containing protein were pooled and freeze-dried and the chromatographic procedure repeated. After the partially modified glucoamylase I had been passed through the gel column three times, samples were withdrawn from the column fractions immediately following the protein and tested for the presence of maltose using the phenol sulphuric acid assay procedure described by Dubois et al.[79] The absence of colour in this test indicated that the maltose used to protect the active site of the enzyme had been completely removed from the enzyme by the gel-permeation chromatography and the partially modified glucoamylase I was freeze-dried and stored desiccated at room temperature prior to reaction with radiolabelled taurine.

A sample of glucoamylase II was chemically modified and purified in a similar way to that described for glucoamylase I and the result of the enzyme activity in the presence of maltose shown in TABLE 6.17.

TABLE 6.17

Activity of glucoamylase II during modification with taurine and E.D.C. in the presence of maltose

time (minutes)	optical density at 525 nm from glucose oxidase assay
0	0.3250
2	0.3310
60	0.5720

6.2.6 The chemical modification of glucoamylase I with ^{14}C radio-labelled taurine in the presence of E.D.C.

The desiccated freeze-dried partially modified glucoamylase I (12.14 mg) was weighed directly into the reaction vial using a beam balance accurate to five places of decimals. ^{14}C Radio-labelled taurine (3 mg, 50 μCi) and unlabelled taurine (0.9975 g) were also weighed into the reaction vial and dissolved in deionised water. E.D.C. (75 mM) was added to the solution and the pH adjusted to 4.75 with 1M hydrochloric acid. Samples were withdrawn from the reaction mixture and activity tests carried out. The results are shown in TABLE 6.18. After 40 minutes reaction a further 75 mMoles of E.D.C. were added to the reaction solution to ensure an effective modification of the groups previously protected by maltose.

TABLE 6.18

Activity of partially modified glucoamylase I during modification with ^{14}C radio-labelled taurine in the presence of E.D.C. at pH 4.75.

time (minutes)	optical density at 525 nm from glucose oxidase assay
2	0.4000
60	0.0500

After 2 hours reaction the mixture was passed through a "Sephadex" G-100 gel-permeation chromatography column and fractions containing protein pooled and freeze-dried. All the column fractions immediately following the protein peak were pooled and rotary evaporated to dryness prior to recrystallisation of radio-labelled taurine. To remove all traces of taurine which were not

covalently bonded to the chemically modified glucoamylase I, the chromatographic procedure was repeated four times. After the fifth column pass, fractions immediately following the protein peak were assayed for radioactivity by liquid scintillation counting (discussed in section 6.3.12) and as no radioactivity was detected, it was concluded that traces of non-covalently bound taurine had been removed from chemically modified glucoamylase I.

While the results given in TABLE 6.16 show that the partially modified glucoamylase I retained activity after 60 minutes reaction, the results given in TABLE 6.18 show that 87.5% of this activity was lost in the chemical modification of the enzyme with ^{14}C radio-labelled taurine in the absence of maltose over the same period of time.

From the amount of radio-labelled taurine incorporated into the partially modified glucoamylase I it was calculated (section 6.3.14) that a further 6 moles of this nucleophile were incorporated into the enzyme in the absence of maltose. In view of the precautions taken to ensure complete removal of maltose from the enzyme active-site prior to reaction with radio-labelled taurine, and the subsequent removal of non-covalently bound radio-labelled nucleophile from the enzyme after reaction, it was considered that the value for the number of moles of nucleophile incorporated into the enzyme in the absence of maltose was more reliable than the value of 4 obtained in section 6.2.3.

6.2.7 Recrystallisation of ^{14}C radio-labelled taurine

^{14}C Radio-labelled taurine recovered from the reaction mixture described in section 6.2.7, was purified by recrystallisation

described in section 6.3.11. The recrystallised material ran as a single spot on T.L.C. and had a melting point coincident with that reported for taurine in the literature.[80]

6.2.8 The chemical modification of glucoamylase II with ^{14}C radio-labelled taurine in the presence of E.D.C.

Desiccated freeze-dried partially modified glucoamylase II (5.65 mg) was weighed directly into the reaction vial as described for glucoamylase I. ^{14}C Radio-labelled recrystallised taurine (0.4645 g) was also weighed into the reaction vial and dissolved in deionised water. E.D.C. (75 mM) was added to the solution and the pH adjusted to 4.75 with 1M hydrochloric acid. Samples were withdrawn from the reaction mixture and activity tests carried out. The results are shown in TABLE 6.19. After 40 minutes reaction a further 75 mM of E.D.C. was added to the reaction solution as discussed for glucoamylase I.

TABLE 6.19

Activity of partially modified glucoamylase II during modification with ^{14}C radio-labelled taurine in the presence of E.D.C. at pH 4.75.

time (minutes)	optical density at 525 nm from glucose oxidase assay
2	0.1750
120	0.0200

After 2 hours reaction the mixture was passed through a "Bio-Gel" P-60 gel-permeation chromatography column and fractions containing protein pooled and freeze-dried. All the column fractions immediately following the protein peak were pooled and rotary evaporated to dryness to recover radio-labelled taurine. To remove non-covalently bound taurine from the chemically modified glucoamylase II, the chromatographic procedure was repeated four times as in the case of modified glucoamylase I.

While the results given in TABLE 6.17 show that the partially modified glucoamylase II retained activity after 60 minutes reaction, the results given in TABLE 6.19 show that 88.6% of this activity was lost in the chemical modification of the enzyme with ^{14}C radio-labelled taurine in the absence of maltose. It was also apparent from the optical density value measured after 2 minutes in TABLE 6.19 that some glucoamylase activity had been lost during the purification procedure employed to remove maltose from the partially purified glucoamylase II. This loss in glucoamylase activity was not observed in the case of glucoamylase I. It was concluded that partially modified glucoamylase II is less stable than the corresponding glucoamylase I although the reason for this is not known.

From the amount of radio-labelled taurine incorporated into the partially modified glucoamylase II it was calculated (section 6.3.15) that a further 5 moles of this nucleophile were incorporated into the enzyme in the absence of maltose.

6.2.9 The digestion of radio-labelled glucoamylase I by D.C.C. treated trypsin

^{14}C Radio-labelled glucoamylase I (5 mg), prepared in section 6.2.6, was oxidised for 9 hours with performic acid and

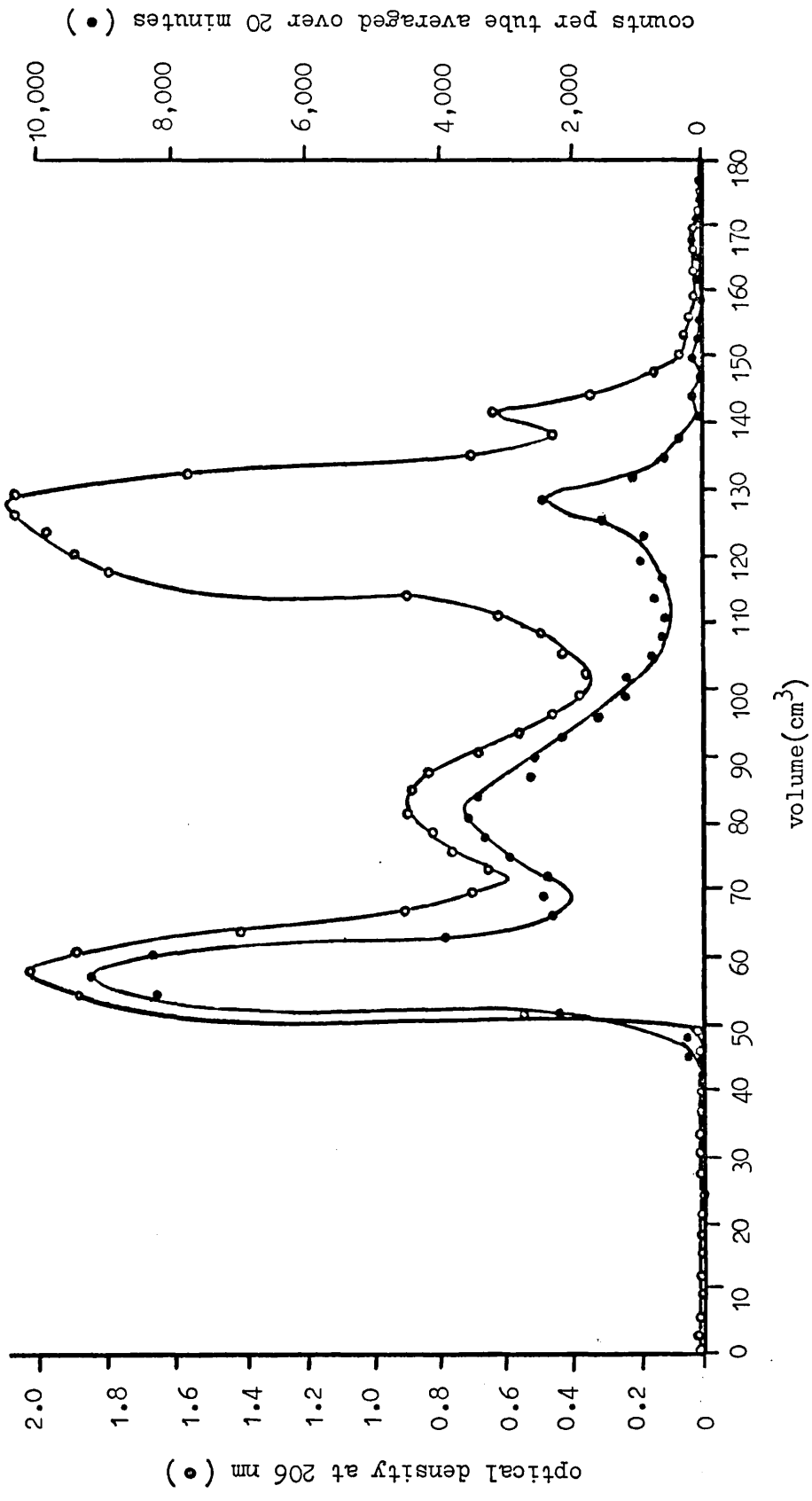
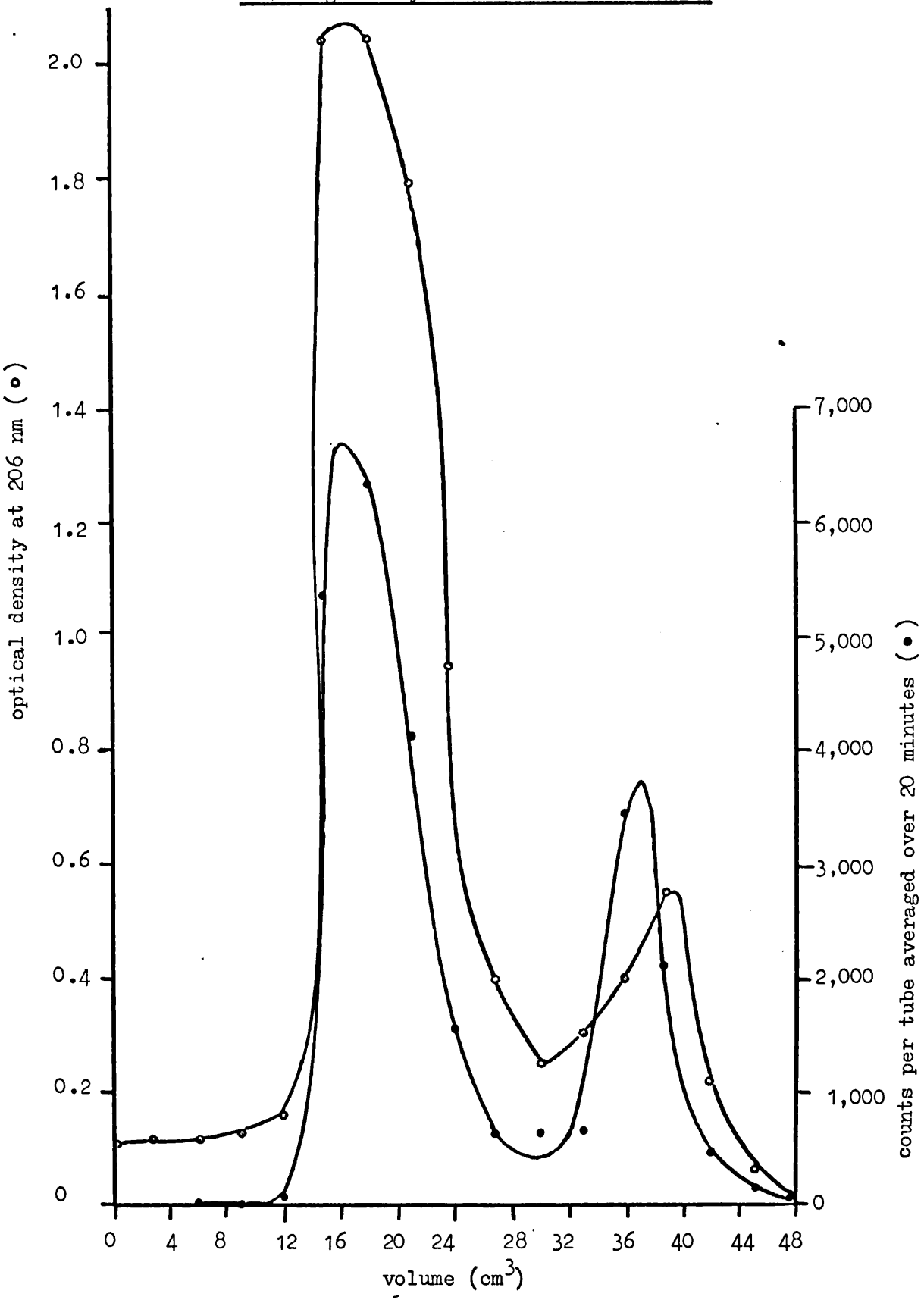


FIGURE 6.8 Chromatography of tryptic digest of radio-labelled glucoamylase I on "Bio-Gel" P-4

FIGURE 6.9 Chromatography of first peak eluted from "Bio-Gel" P-4 of glucoamylase I on "Bio-Gel" P-60.



freeze-dried. The sample was dissolved in deionised water (400 cm^3) and freeze-dried to remove all traces of performic acid. The sample was then hydrolysed for 24 hours at 25°C with D.C.C. treated trypsin (1 mg) and dissolved in 0.1M sodium hydroxide solution in the presence of 0.001M Ca^{2+} ions. After hydrolysis the sample was passed through a "Bio-Gel" P-4 gel-permeation chromatography column equilibrated with 0.01M ammonium bicarbonate solution. After the optical density of each fraction had been measured at 206 nm, samples were withdrawn from each tube, and dried on a filter disc. The amount of radio-activity present on each disc was determined by liquid scintillation counting over eight 20 minute periods for each vial. After correcting the figures obtained for background contribution and instrument efficiency, an average value in d.p.m. was obtained for each disc and hence the radioactivity in each fraction determined. The results are shown in FIGURE 6.8. Fractions comprising the first peak eluted from the column were pooled and reduced to 2 cm^3 volume by rotary evaporation. This solution was passed through a "Bio-Gel" P-60 gel-permeation chromatography column equilibrated with 0.01M ammonium bircarbonate solution. The optical densities and radio-activity of the column fractions were measured and the results shown in FIGURE 6.9. It can be seen that the single peak eluted from the first column was resolved into two by the second. The first of these peaks probably contained some undegraded radio-labelled glucoamylase I.

FIGURE 6.10 Chromatography of tryptic digest of radio-labelled glucoamylase II on"Bio-Gel" P-4

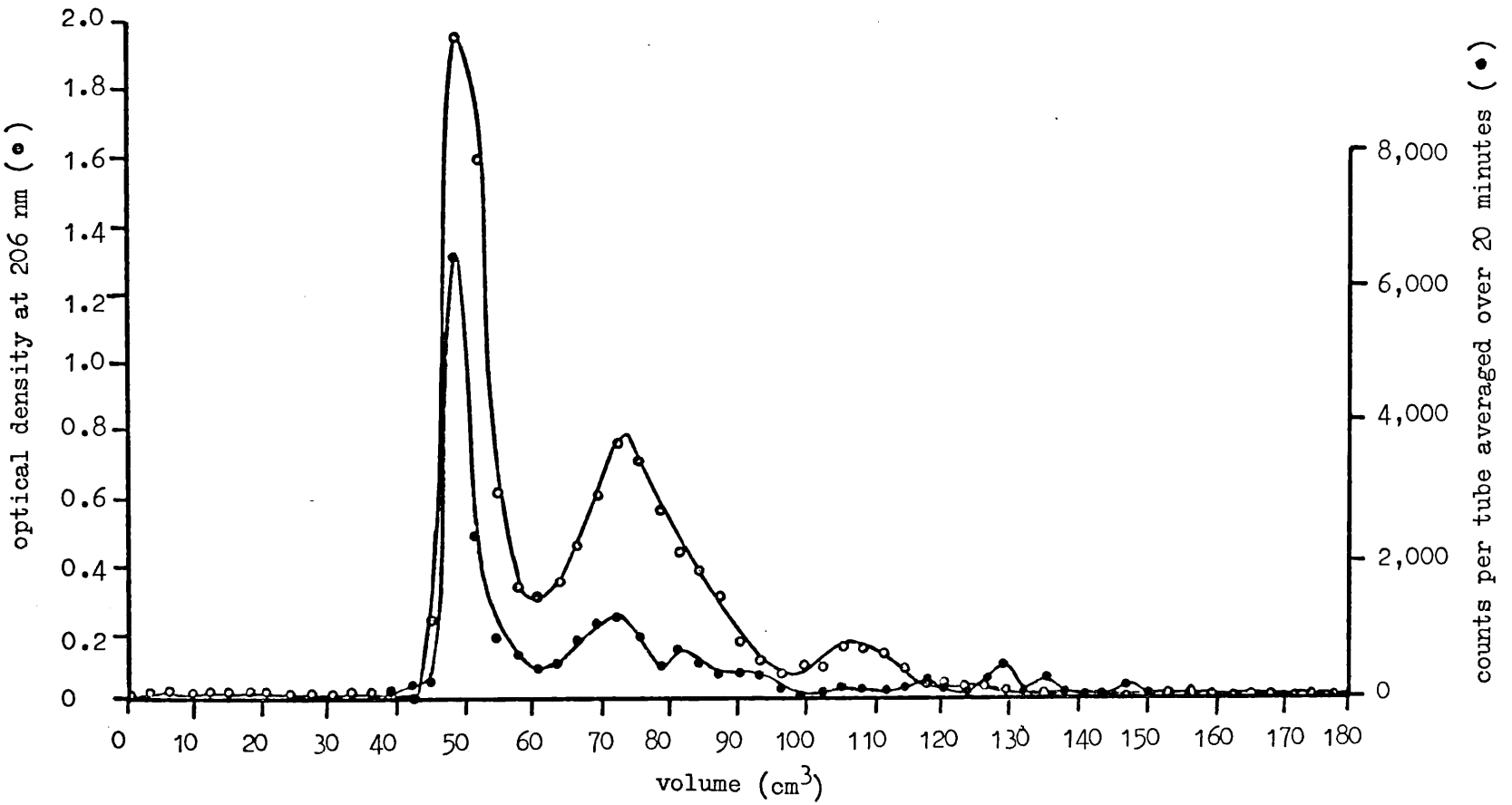
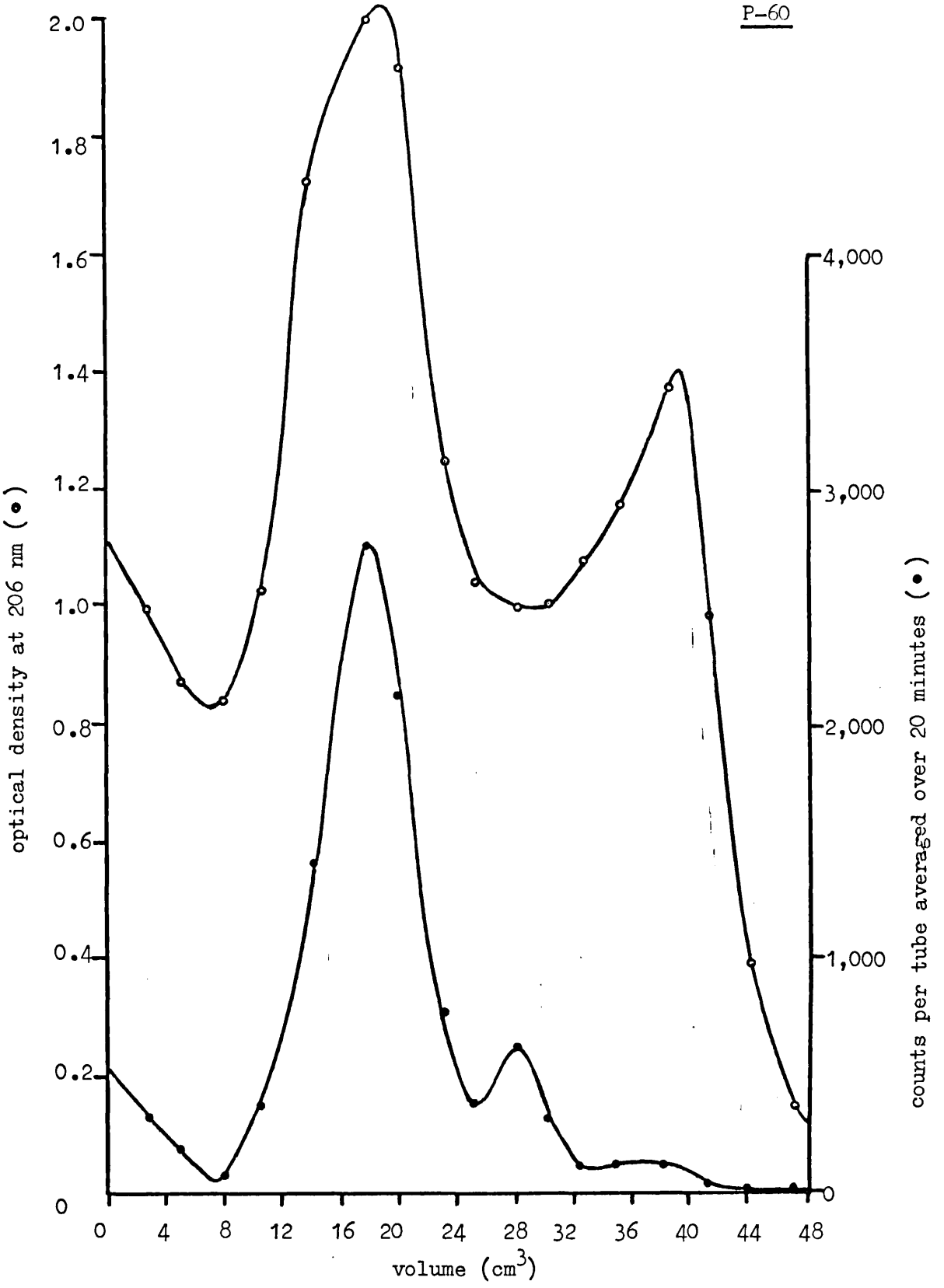


FIGURE 6.11 Chromatography of first peak eluted from "Bio-Gel" P-4 of glucoamylase II on "Bio-Gel" P-60



6.2.10 The digestion of radio-labelled glucoamylase II by D.C.C. treated trypsin

^{14}C Radio-labelled glucoamylase II (3 mg), prepared in section 6.2.8, was oxidised for 9 hours with performic acid and freeze-dried. The sample was dissolved in deionised water (400 cm^3) and freeze-dried to remove all traces of performic acid. The sample was then hydrolysed for 24 hours at 25°C with D.C.C. treated trypsin (0.75 mg) dissolved in 0.1M sodium hydroxide in the presence of 0.001M Ca^{2+} ions. The chromatographic procedures described in section 6.2.9 for glucoamylase I were repeated for this sample and the results shown in FIGURES 6.10 and 6.11.

From FIGURES 6.8 to 6.11 it can be seen that extent of degradation of chemically modified glucoamylases I and II was far from complete, the undegraded protein being eluted in the void volume on both columns. It may be that chemically modified glucoamylases are more resistant to proteolysis than the native enzymes. Although the scale of the proteolytic digestion of chemically modified glucoamylase II was smaller than glucoamylase I, by comparing FIGURES 6.8 and 6.10 it appears that the extent of digestion of glucoamylase II is lower than glucoamylase I.

6.2.11 Peptide mapping of radio-labelled glucoamylases I and II

Fractions comprising the peaks obtained from column chromatography of the radio-labelled peptides from tryptic digestion of chemically modified radio-labelled glucoamylases I and II were pooled separately giving eight solutions, four from each enzyme.

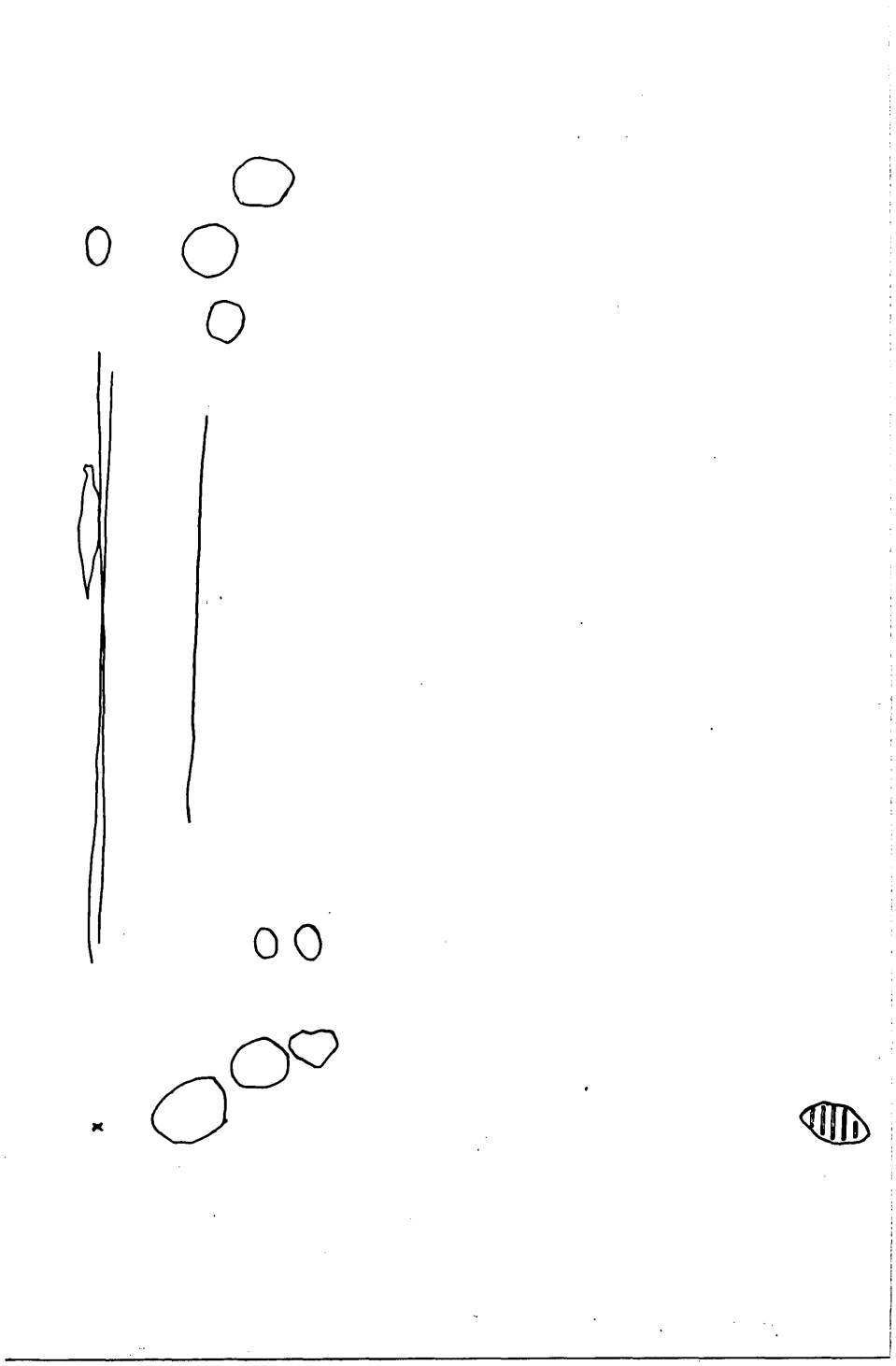


Plate 6.1 Map of peptides from glucoamylase I after chromatography on Bio-Gel P-60, fractions eluted between 12 and 27 cm^3. X = application point, shaded spot = congo red marker.

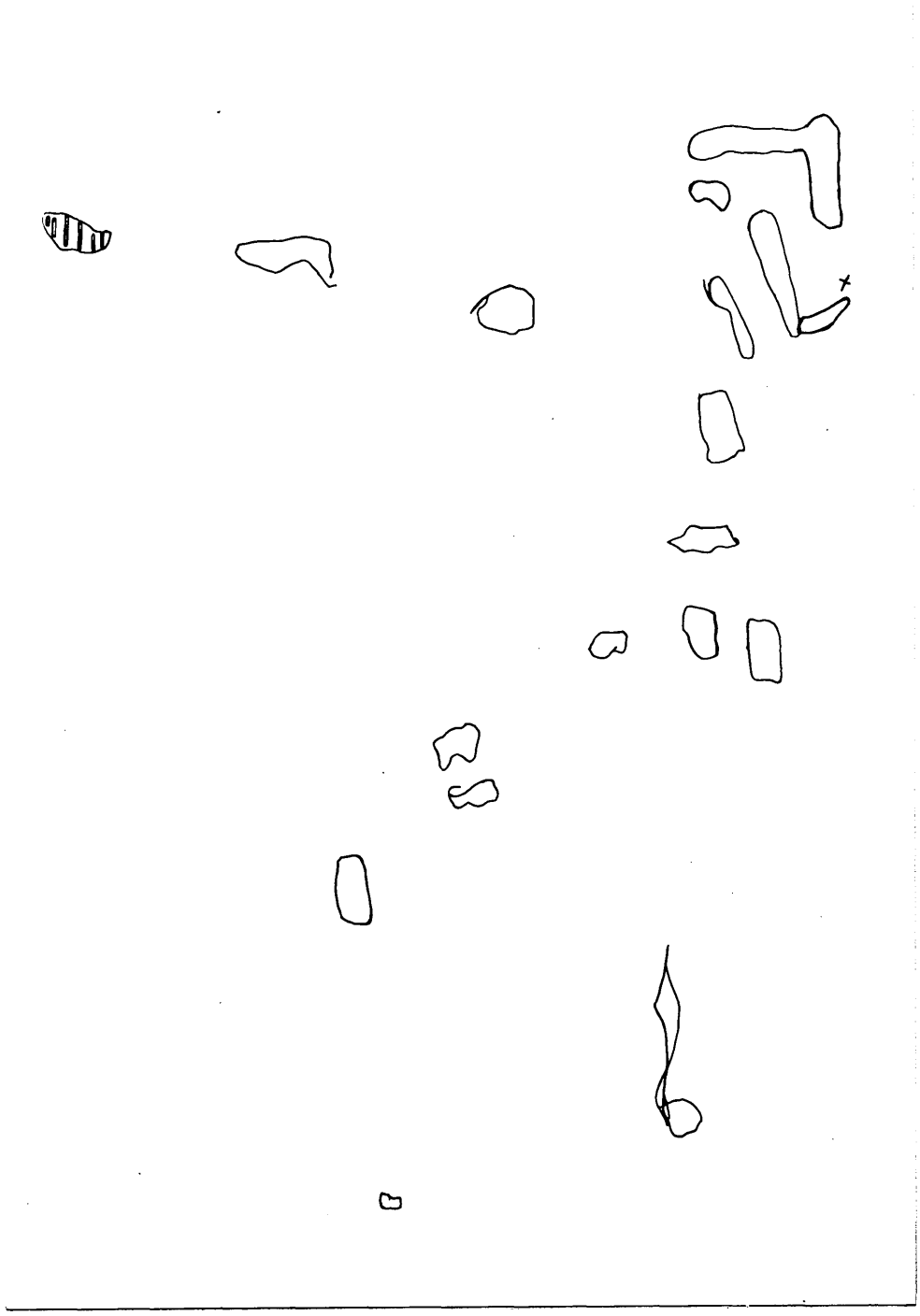


Plate 6.2 Map of peptides from glucoamylase II after chromatography on Bio-Gel P-60, fractions eluted between 8 and 25 cm^3.
X = application point, shaded spot = congo red marker.

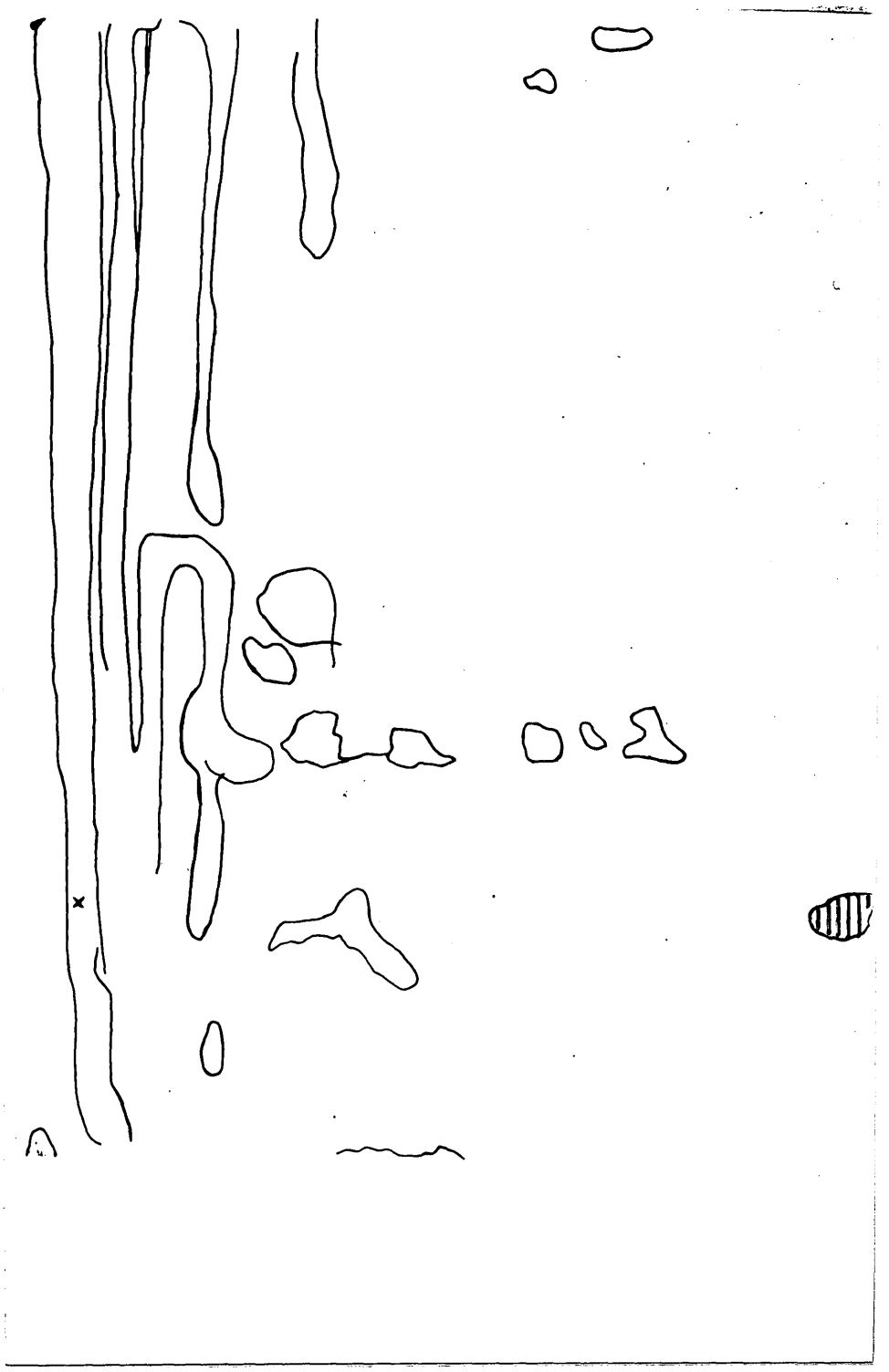


Plate 6.3 Map of peptides from glucoamylase I after chromatography on Bio-Gel P-60, fractions eluted between 33 and 45 cm^3. X = application point, shaded spot = congo red marker.

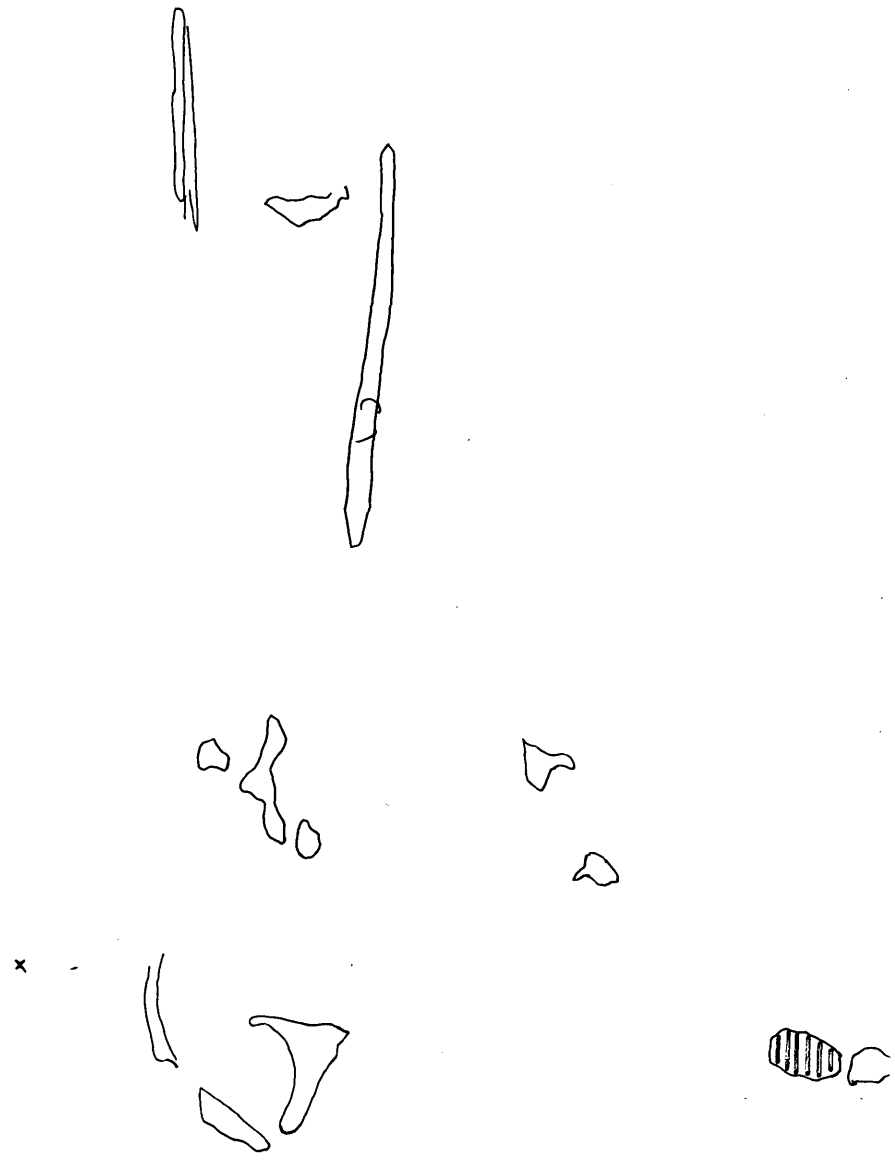


Plate 6.4 Map of peptides from glucoamylase II after chromatography on Bio-Gel P-60, fractions eluted between 28 and 41 cm^3.
X = application point, shaded spot = congo red marker.

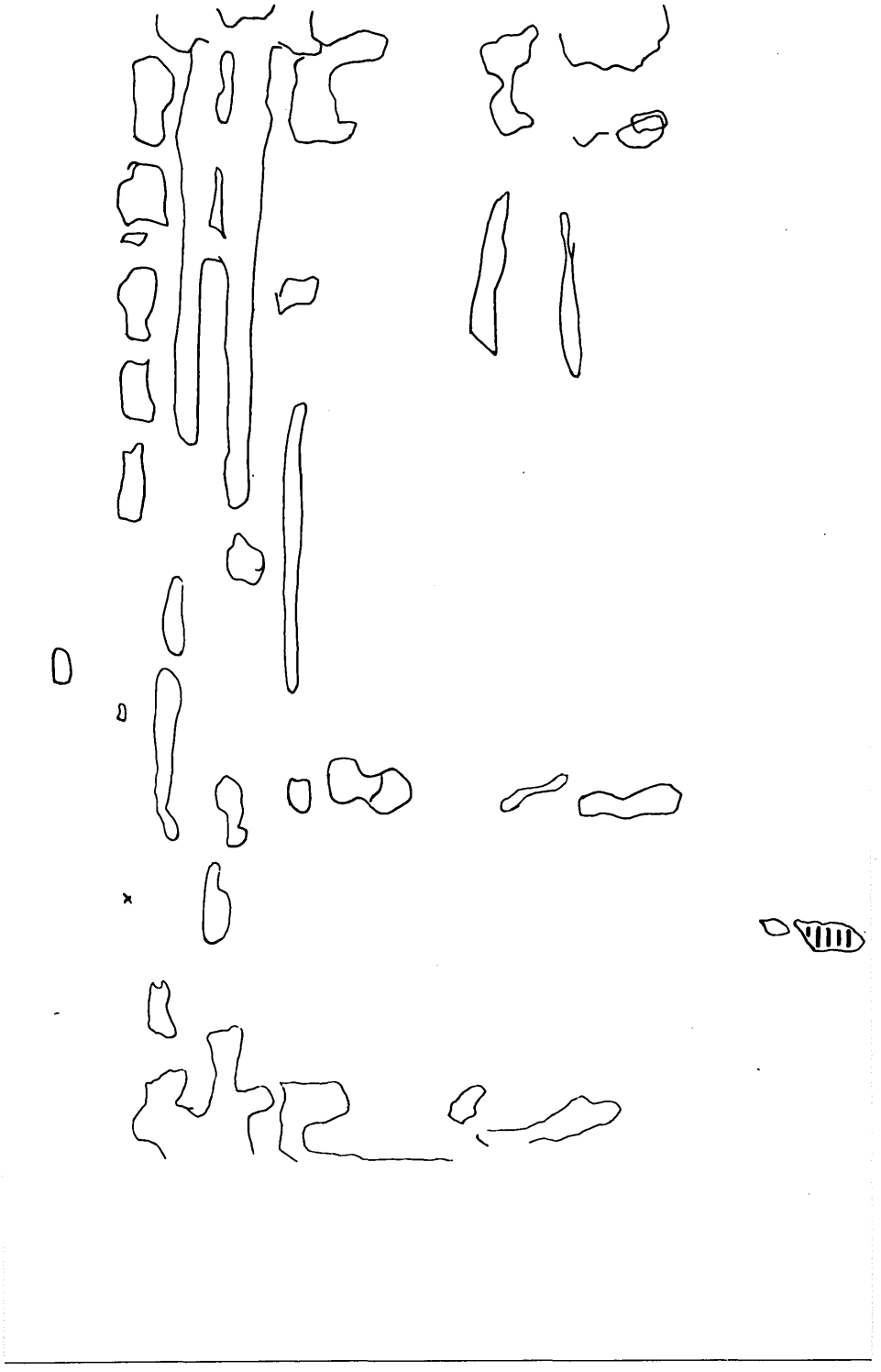


Plate 6.5 Map of peptides from glucoamylase I after chromatography on Bio-Gel P-4, fractions eluted between 72 and 111 cm^3. X = application point, shaded spot = congo red marker.

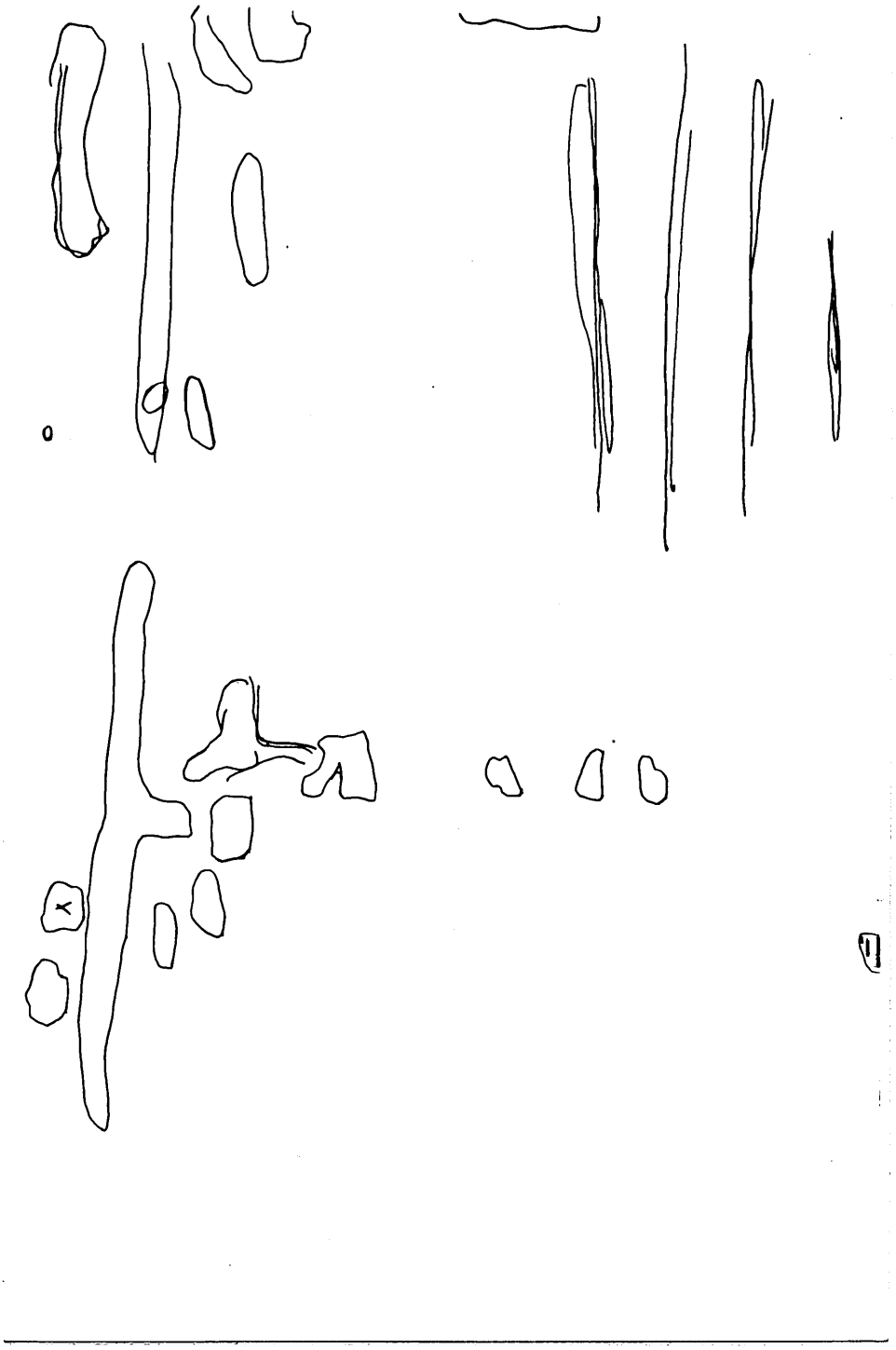


Plate 6.6 Map of peptides from glucoamylase II after chromatography on Bio-Gel P-4, fractions eluted between 63 and 96 cm^3.
X = application point, shaded spot = congo red marker.

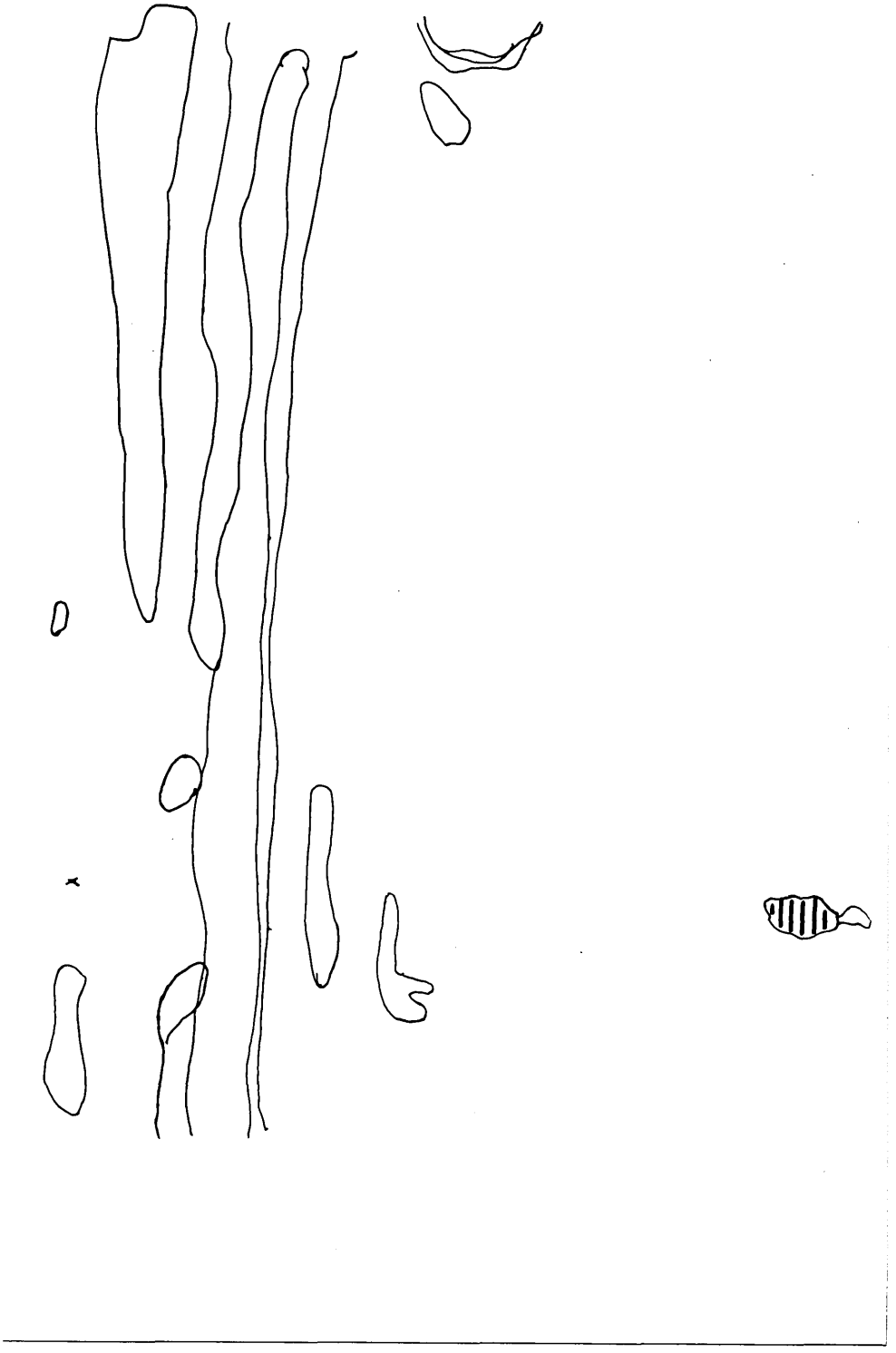


Plate 6.7 Map of peptides from glucoamylase I after chromatography on Bio-Gel P-4, fractions eluted between 114 and 147 cm^3.

X = application point, shaded spot = congo red marker.

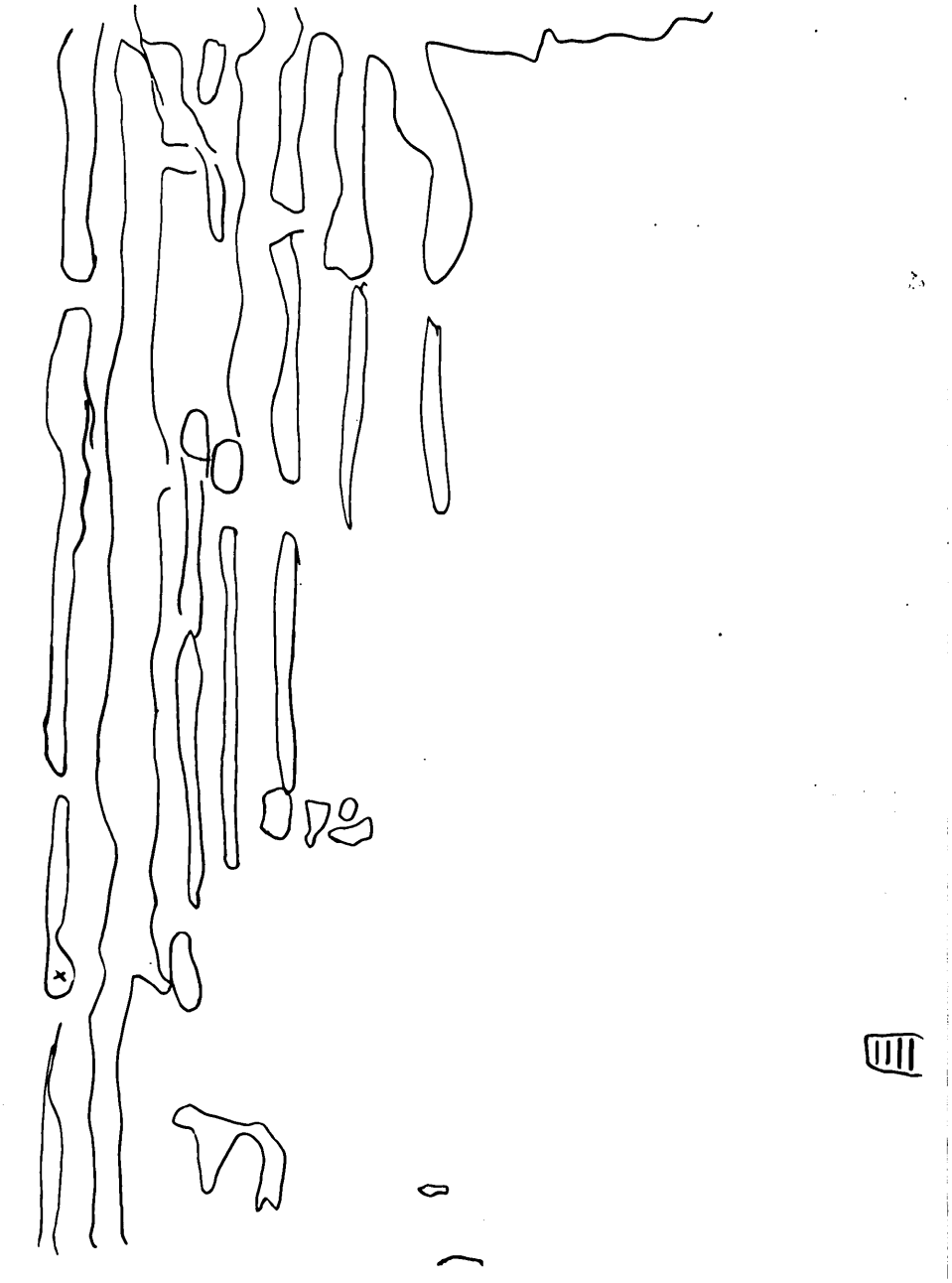


Plate 6.8 Map of peptides from glucoamylase II after chromatography on Bio-Gel P-4, fractions eluted between 99 and 150 cm^3.
X = application point, shaded spot = congo red marker.

These solutions were then rotary evaporated to dryness. Each sample was dissolved in 0.1M hydrochloric acid and spotted on Whatman 3M papers 43 x 45 cm prior to electrophoresis in pyridine, acetic acid, water (1 : 6 :89 v/v/v) at 2,500 volts, 60 mA for 1 hour. After air drying, the papers were subjected to descending chromatography, at right angles to the direction of electrophoresis, in pyridine, isoamyl alcohol, water (30:30:35 v/v/v)[84] using congo red as a marking dye. When this dye had migrated to within 5 cm of the end of the chromatographic papers (approximately 14 hours), they were removed, air dried, and sprayed with 1% ninhydrin dissolved in 96% ethanol. The papers were developed by heating in an oven at 80°C for 10 minutes. The results are shown on PLATES 6.1 to 6.8. From the PLATES it can be seen that considerable degradation of the chemically modified glucoamylases had occurred during proteolysis with D.C.C. treated trypsin. Trypsin is a highly specific enzyme which cleaves peptide bonds involving carbonyl groups of the basic amino acids lysine and arginine. These amino acids become C-terminal in the peptides produced. Theoretically one should obtain one more peptide than the total number of lysine and arginine residues unless one of these is already C-terminal when the total number of peptides produced equals the sum of these residues. From the amino acid analyses of glucoamylases I and II carried out in CHAPTER 4 of this Thesis the sum of lysine and arginine residues are 23.6 and 27.2 for each respective enzyme. Because of its high specificity trypsin is widely used for fingerprinting, e.g. it revealed that the replacement of glutamic acid with valine in haemoglobin was responsible for causing sickle cell anaemia. By comparing corresponding pairs of peptide

maps e.g. PLATE 6.1 and 6.2, it can be seen that some similarities are shown but the distinct differences in the patterns and numbers of spots must reflect differences in primary structures of glucoamylases I and II. As glucoamylase I contains a lower number of lysine and arginine residues than glucoamylase II it would be expected that the former enzyme would give rise to fewer spots on peptide mapping after proteolytic digestion by trypsin. This was not observed in this work, however, the lower concentration of glucoamylase II used throughout the experiment may account for the deficiency in the number of spots, the concentration of the peptides being too low to be detected by ninhydrin.

An attempt to obtain radioautographs from the peptide maps and hence locate peptides from the enzyme active site proved unsuccessful presumably because of the dilute nature of the radioactive material dispersed over the surface of the chromatograms, and hence no peptides originating from the neighbourhood of the active site of glucoamylases I and II were characterised.

6.2.12 General conclusions

It has been shown that glucoamylases I and II can be chemically modified at pH 4.75, 25°C by taurine using the carbodiimide coupling procedure described by Hoare et al.[17] Taurine has an advantage over nucleophiles such as glycine methyl ester and glycine ethyl ester because the chemically modified protein exhibits a greater degree of stability which is due possibly to the presence of its ionisable sulphonic acid group, and it is easily analysed using an amino acid analyser. The rate of deactivation of glucoamylases I and II is much greater with taurine than with glycine

esters due possibly to its greater basicity. Glucoamylases I and II can be chemically modified at carboxyl groups with a variety of groups with the same high level of retention of activity (80%) by protecting the active site region of the enzymes with maltose. The active site of glucoamylases I and II appear to contain between 5-6 modifiable groups. This would appear to be reasonable since Hiromi et al.[104,105] postulated a binding cleft for 7 sugar residues based on kinetics of degradation of oligosaccharides. Out of 45 modifiable groups in the denatured enzyme approximately half are modifiable in the native state. This was considered to be reasonable because all the potentially modifiable groups cannot be readily accessible on the enzyme surface, where one expects to find hydrophilic groups. Glucoamylase I and II appear to be different on the basis of analysis of tryptic digests by gel-permeation chromatography and peptide mapping.

6.3 Experimental

6.3.1 The chemical modification of glucoamylase I with glycine ethyl ester hydrochloride and E.D.C.

The following stock solutions were made up. E.D.C.* (0.2876 g) dissolved in deionised water (1 cm^3). . Glycine ethyl ester hydrochloride (1.6284 g) dissolved in deionised water (5 cm^3). Glucoamylase I (3.5 mg) dissolved in deionised water (1 cm^3). The reaction mixture was made up from the stock solutions as shown in TABLE 6.20, glucoamylase I being added last.

* Cyclo chemical Company.

The reaction was carried out in a 2" x 1" flat bottomed sample tube which contained a magnetic stirrer and a pH micro electrode. After addition of reactants, the pH was adjusted and maintained at 4.75 throughout the reaction period by addition of 1M hydrochloric acid.

TABLE 6.20

Reaction composition for the chemical modification of glucoamylase I using glycine ethyl ester hydrochloride in the absence of maltose.

deionised water cm^3	E.D.C. cm^3	glycine ethyl ester hydrochloride cm^3	glucoamylase I cm^3
6.4	0.5	3.0	0.1

Aliquots (0.05 cm^3) were withdrawn after 0, 5, 10, 20, 35, 60, and 80 minutes reaction and glucoamylase activity measured on 0.05 cm^3 sample using 1% w/v wheat amylopectin as described in CHAPTER 2, 2.4.3 of this Thesis. The results are shown in TABLE 6.3 and FIGURE 6.1.

Using similar stock solutions to those described and glucose free maltose* (2.6824 g) dissolved in deionised water (5 cm^3) separate digests were made up as shown in TABLE 6.21. Aliquots were withdrawn for activity measurements as before and the results shown in TABLE 6.4 and FIGURE 6.1.

* Merck - Specially prepared for biochemistry

TABLE 6.21

Digest composition for the chemical modification of glucoamylase I using glycine ethyl ester hydrochloride and maltose in the presence and absence of E.D.C.

deionised water cm^3	E.D.C. cm^3	glycine ethyl ester hydrochloride cm^3	maltose cm^3	glucoamylase I cm^3
2.4	0.0	3.0	4.5	0.1
1.9	0.5	3.0	4.5	0.1

6.3.2 The chemical modification of glucoamylase I with glycine methyl ester hydrochloride and E.D.C.

The following stock solutions were made up. E.D.C. (0.2876 g) dissolved in deionised water (1 cm^3). Glycine methyl ester hydrochloride (1.4653 g) dissolved in deionised water (5 cm^3). Glucoamylase I (11.4 mg) dissolved in deionised water (1 cm^3). The reaction mixture was made up from the stock solutions as shown in TABLE 6.22, glucoamylase I being added last.

TABLE 6.22

Reaction composition for the chemical modification of glucoamylase I using glycine methyl ester hydrochloride in the absence of maltose

deionised water cm^3	E.D.C. cm^3	glycine methyl ester hydrochloride cm^3	glucoamylase I cm^3
5.5	0.5	3.0	1.0

The reaction was carried out as described in section 6.3.1 and aliquots (0.1 cm^3) withdrawn after 0, 5, 10, 20, 35, 60 and 80

minutes reaction. After dilution with 0.2M acetate buffer[78] pH 4.8 (1.4 cm^3), glucoamylase activity was measured on a 0.1 cm^3 sample using 1% w/v wheat amylopectin. The results are shown in TABLE 6.5 and FIGURE 6.2.

6.3.3 The chemical modification of glucoamylase I with taurine and E.D.C.

The following stock solutions were made up. E.D.C. (0.2876 g) dissolved in deionised water (1 cm^3). Taurine (1.177 g) dissolved in deionised water (10 cm^3). Glucoamylase I (5.7 mg) dissolved in deionised water (1 cm^3). The reaction mixture was made up from the stock solutions as shown in TABLE 6.23, glucoamylase I being added last.

TABLE 6.23

Reaction composition for the chemical modification of glucoamylase I using taurine in the absence of maltose

E.D.C. cm^3	taurine cm^3	glucoamylase I cm^3
0.5	8.5	1.0

The reaction was carried out as described in section 6.3.1 and aliquots (0.1 cm^3) withdrawn after 0, 2.5, 5, 10, 15, 20, 35 and 60 minutes reaction. After dilution with 0.2M acetate buffer[78], pH 4.8 (1.4 cm^3), glucoamylase activity was measured on a 0.1 cm^3 sample using 1% w/v wheat amylopectin. The results are shown in TABLE 6.6 and FIGURE 6.3. In a later experiment to obtain a sample of chemically modified glucoamylase I for amino acid analysis, the reaction was quenched after 60 minutes by addition of glacialacetic

acid (0.04 cm^3) and the reaction mixture passed through a "Pharmacia" type K9/60, 0.9 x 60 cm, 38 cm^3 capacity glass column, packed with "Sephadex" G-100 gel equilibrated with 0.01M ammonium bicarbonate solution, pH 8.1. Fractions (3.0 cm^3) were collected and their optical densities (280 nm) recorded automatically using a Pye Unicam SP 1,800 spectrometer. Fractions comprising protein were pooled and freeze-dried.

Using stock solutions similar to those described and glucose free maltose (2.6824 g) dissolved in taurine stock solution (5 cm^3), separate digests were made up as shown in TABLE 6.24.

TABLE 6.24

Digest composition for the chemical modification of glucoamylase I with taurine and maltose in the presence and absence of E.D.C.

E.D.C. cm^3	taurine cm^3	maltose in taurine cm^3	glucoamylase I cm^3	deionised water cm^3
0.5	4.0	4.5	1.0	0.0
0.0	4.0	4.5	1.0	0.5

Aliquots were withdrawn as before and activity measured using 0.05 cm^3 samples. The results are shown in TABLE 6.7 and FIGURE 6.3. The sample prepared for amino acid analysis was quenched by the addition of glacial acetic acid (0.04 cm^3) and passed through a "Sephadex" G-100 gel-permeation column as previously described and the protein fractions isolated and freeze-dried.

6.3.4 The chemical modification of glucoamylase I with taurine and low concentration of E.D.C.

The experiment was carried out essentially as described in section 6.3.1 using similar stock solutions to those described in section 6.3.3 except for E.D.C. which was made up by dissolving 9.1 mg in deionised water (100 cm^3). The reaction mixture was made up as shown in table 6.25, glucoamylase I being added last.

TABLE 6.25

Reaction composition for the chemical modification of glucoamylase I using taurine and low concentration of E.D.C.

E.D.C. cm^3	taurine cm^3	glucoamylase I cm^3
0.5	8.5	1.0

Aliquots were withdrawn as before and activity measured using 0.1 cm^3 samples. The results are shown in TABLE 6.12 and FIGURE 6.5. After 80 minutes the reaction mixture was passed through a "Sephadex" G-100 gel-permeation column as previously described and the protein fractions isolated and freeze-dried.

6.3.5 The chemical modification of glucoamylase I with taurine and E.D.C. in the presence of 5M guanidine hydrochloride

The experiment was carried out essentially as described in section 6.3.1 using similar stock solutions to those described in section 6.3.3 and guanidine hydrochloride (5.6194 g) dissolved in taurine stock solution (10 cm^3). The reaction mixture was made up as shown in TABLE 6.26, glucoamylase I being added last.

TABLE 6.26

Digest composition for the chemical modification of glucoamylase I with taurine in the presence of 5M guanidine hydrochloride

E.D.C. cm^3	guanidine hydrochloride in taurine cm^3	glucoamylase I cm^3
0.5	8.5	1.0

Aliquots were withdrawn for activity measurements as before and the results shown in TABLE 6.13. After 80 minutes reaction glacial acetic acid (0.04 cm^3) was added and the mixture passed through a "Sephadex" G-100 gel-permeation column. Fractions containing protein were pooled and freeze-dried.

6.3.6 The chemical modification of glucoamylase II with taurine and E.D.C.

The experimental procedure employed was a repetition of that described in section 6.3.3. The results of the experiment are shown in TABLES 6.8 and 6.9 and FIGURE 6.4.

6.3.7 Amino acid analysis of chemically modified glucoamylases I and II

Freeze-dried samples of glucoamylases I and II prepared as described were placed separately into six labelled "Pyrex" glass ampoules 6" x 5/8". 6M Hydrochloric acid (2.0 cm^3) was added to each ampoule. Each sample was de-gassed and the ampoules filled with oxygen free nitrogen prior to sealing. The samples were then placed in an oven at 110°C for 16 hours. After hydrolysis, the ampoules were

cooled and the contents washed into separate pear shaped flasks ($25\ cm^3$) with deionised water ($5\ cm^3$). The samples were evaporated to dryness on a Buchi rotary evaporator. Deionised water ($5\ cm^3$) was added to each sample flask and rotary evaporated to dryness. The process was repeated until the samples had no smell of acid vapour. Amino acid analysis of each sample was carried out using a "Jeol" auto-analyser. The amount of taurine and valine in each sample were measured relative to standards, the latter being used to determine the amount of glucoamylase present in the hydrolyzate. The results are shown in TABLE 6.14.

6.3.8 The chemical modification of glucoamylase I with ^{14}C radio-labelled taurine in the presence of E.D.C.

Glucoamylase I (20 mg) was chemically modified with taurine and E.D.C. in the presence of maltose as described in section 6.3.3 and the results shown in TABLE 6.16. After 60 minutes reaction, glacial acetic acid ($0.04\ cm^3$) was added and the mixture passed through a "Sephadex" G-100 gel-permeation column. The separation is shown in FIGURE 6.7. Fractions ($3\ cm^3$) containing protein were pooled and freeze-dried and the chromatography procedure repeated three times. On the last occasion, samples ($1\ cm^3$) were withdrawn from the column fractions immediately following the protein and tested for the presence of maltose using the phenol sulphuric acid procedure described by Dubois et al.[79] No colour was detected in this test.

Desiccated freeze-dried partially modified glucoamylase I (12.14 mg) prepared as described was weighed directly into the reaction vial using a beam balance accurate to five places of decimals. ^{14}C

Radio-labelled taurine (3 mg, 50 μ Ci) and unlabelled taurine (0.9975 g) were also weighed into the reaction vial. After the reactants had dissolved in deionised water (9.5 cm^3), E.D.C. (75 mM) was added to the solution and the pH adjusted to 4.75 with 1M hydrochloric acid. Aliquots were withdrawn for activity measurements after 2 and 60 minutes reaction time and the results shown in TABLE 6.18. After 40 minutes reaction further E.D.C. (75 mM) was added to the reaction mixture. After 2 hours reaction the mixture was passed through a "Sephadex" G-100 gel-permeation column. Fractions containing protein were pooled and freeze-dried and the chromatography procedure repeated four times. Fractions immediately following the protein peak after each column pass were pooled and rotary evaporated to dryness. ^{14}C Radio-labelled taurine was recrystallised from this material as described in section 6.3.11. After the fifth column pass, fractions immediately following the protein peak were assayed for radioactivity by withdrawing samples (250 μdm^3) from each tube and applying them to filter discs prior to liquid scintillating counting described in section 6.3.12.

6.3.9 The chemical modification of glucoamylase II with ^{14}C radio-labelled taurine in the presence of E.D.C.

Glucoamylase II (20 mg) was chemically modified with taurine and E.D.C. in the presence of maltose as described in section 6.3.3 and the results shown in TABLE 6.17. The partially modified glucoamylase II was then purified by column chromatography as described for glucoamylase I in 6.3.8.

Desiccated freeze-dried partially modified glucoamylase II (5.65 mg) prepared as described was weighed into the reaction vial using a beam balance accurate to five places of decimals. ^{14}C Recrystallised radio-labelled taurine (0.4645 g) was also weighed into the reaction vial. The reactants were dissolved in deionised water (3.75 cm^3) and E.D.C. (75 mM) added. The pH was adjusted to 4.75 with 1M hydrochloric acid. Aliquots were withdrawn for activity measurements after 2 and 120 minutes reaction time and the results shown in TABLE 6.19. After 40 minutes reaction further E.D.C. (75 mM) was added to the reaction mixture. After 2 hours reaction the mixture was passed through a "Bio-Gel" P-60 gel-permeation column. The fractions collected were treated as described in section 6.3.8. The chromatography procedure was repeated four times.

6.3.10 Regeneration of tyrosine residues in chemically modified glucoamylase I

Glucoamylase I (19.2 mg) was chemically modified with taurine as described in section 6.3.3, and the enzyme activity of 0.05 cm^3 samples after dilution shown in TABLE 6.15 and FIGURE 6.6. The chemically modified glucoamylase was purified by repeated gel-permeation chromatography on "Bio-Gel" P-60 and freeze-dried. The modified enzyme was then divided into two samples (7.8 mg) and (7.4 mg) the latter being used as a control. The first sample was dissolved in acetate buffer (0.5 cm^3) and reacted with 0.57M hydroxylamine (4.5 cm^3) for 5 hours at pH 7, 23.5°C. After reaction the mixture was passed through a "Bio-Gel" P-60 gel-permeation column and protein fractions isolated and freeze-dried. The chromatographic

procedure was repeated three times. The control sample was also passed through the column an equal number of times. The freeze-dried samples were then subjected to amino acid analysis as described in section 6.3.7. The results are given in section 6.2.4.

6.3.11 Recrystallisation of ^{14}C radio-labelled taurine

The solid obtained from the rotary evaporation of pooled fractions obtained after the chemically modified glucoamylase I had been eluted from the gel-permeation column, were dissolved in a minimum volume of dionised water at 60°C. The solution was filtered through a fluted filter paper, allowed to cool to room temperature and placed in a refrigerator overnight. The crystals obtained were washed with ice cold deionised water and collected on a No.3 micro sintered glass funnel. After dissolving the crystals in a minimum volume of deionised water, the crystallisation process was repeated and the resulting crystals dried and stored in a vacuum desiccator. A melting point was taken and found to agree exactly with the value for taurine reported in the literature i.e. 328°C.[80] The recrystallised taurine ran as a single spot on a silica gel T.L.C. plate using chloroform, methanol, 17% ammonium hydroxide, (2:2:1 v/v/v) as solvent. The plate was developed by spraying with a solution of 0.1% ninhydrin in 96% ethanol and heating at 60°C for 5 minutes.

6.3.12 Liquid Scintillation Counting

A practical review course in liquid scintillation counting by Price[85] was found useful in this work.

Two counting channels of a Tracerlab Coru Matic 100a liquid scintillation counter were optimised to count ^{14}C by obtaining instrument settings which gave a maximum value of (efficiency)^2/

background count using manufacturers ^{14}C standards. Scintillation cocktail was made up as shown in TABLE 6.27, the recipe being taken from the instrument manufacturers instruction manual.

TABLE 6.27

Scintillation cocktail used in this work

reagent	quantity
* toluene	1,000 cm^3
* 2,5-diphenyloxazole	4 mg
* 1,4-bis-(5-phenyloxazol-2-yl)benzene	50 mg

* Hopkin and Williams - scintillation grade chemicals.

Aliquots (10 cm^3) were dispensed into glass counting vials fitted with aluminium foil seal screw caps. Each vial was counted over four separate 20 minute periods and the results averaged to obtain a background count for each vial. To determine a value of average count efficiency of the instrument, various ^{14}C fructose standards were made up, dried on filter discs and placed inside scintillation vials of known background count. The vials containing filter discs were counted for eight 20 minute periods and the average results obtained after background count subtraction are given in TABLE 6.28.

TABLE 6.28

Scintillation counting of ^{14}C radio-labelled fructose standards ($1\ \mu dm^3$ ^{14}C fructose = 2.2×10^5 d.p.m.).

volume ^{14}C labelled fructose μdm^3	volume deionised water μdm^3	dilution	theoretical d.p.m. per 5 μdm sample	counts observed averaged over eight repeats	percentage count efficiency
5	20	5	220,000	113,157.09	51.44
5	45	10	110,000	59,100.51	53.73
5	95	20	55,000	26,572.51	48.31
5	495	100	11,000	5,364.49	48.77

From these results the instrument counting efficiency was found to be 50.56%.

A sample of ^{14}C radio-labelled taurine (1.09 mg) was weighed out using a beam balance accurate to five places of decimals and dissolved in deionised water (0.1 cm^3) and absorbed on a filter disc. The container was washed a further five times with aliquots of deionised water (0.1 cm^3) and each washing applied to the filter disc. After drying in warm air the disc was placed in a scintillation vial of known background count and the vial counted over six 1 minute intervals. An average count was obtained and after correction for instrument efficiency, the value in d.p.m. for 1 mg of ^{14}C radio-labelled taurine shown in TABLE 6.29. The process was repeated using desiccated freeze-dried samples of chemically modified radio-labelled glucoamylase I (0.95 mg) and glucoamylase II (0.90 mg). The vials were counted over thirty six 20 minute intervals. The results are shown in TABLE 6.29.

TABLE 6.29

Table of data obtained from scintillation counting of ^{14}C radio-labelled taurine and chemically modified radio-labelled glucoamylases I and II.

sample	time of count (minutes)	number of times counted	average count value minus background	average count value after efficiency correction	d.p.m./mg
recrystallised ^{14}C taurine	1.0	6	64,207.16	126,552.31	116,103.04
radio-labelled glucoamylase I	20.0	36	9,950.33	19,612.10	1,032.22
radio-labelled glucoamylase II	20.0	36	8,132.43	16,029.02	890.50

Radioactivity present in the chromatography fractions obtained after digestion of radio-labelled glucoamylases I and II with D.C.C. treated trypsin was determined by absorbing samples (0.25 cm^3) withdrawn from each tube on filter discs. Scintillating counting was carried out over eight 20 minute periods for each sample. The results are shown in FIGURES 6.8 to 6.11.

6.3.13 Calculation of specific activity of diluted ^{14}C radio-labelled taurine used for the chemical modification of partially modified glucoamylases I and II

Known from experiment 1 mg diluted taurine has 116,103.04 d.p.m.

$1\ \mu Ci = 2.2 \times 10^6$ d.p.m.

$\therefore \quad 1$ d.p.m. $= 1/2.2 \times 10^6\ \mu Ci$

$$116,103.04 \text{ d.p.m.} = \frac{116,103.04}{2.2 \times 10^6}\ \mu Ci$$

$$= 0.05277\ \mu Ci$$

1mM taurine = 125.1 mg = $125.1 \times 0.05277\ \mu Ci = 6.602\ \mu Ci$

Specific activity of diluted taurine in experiment = 6.602×10^{-3} mCi/mM

6.3.14 Calculation of amount of ^{14}C radio-labelled taurine incorporated into partially modified glucoamylase I

Weight of enzyme reacted = 12.14 mg

Molecular weight of enzyme = 80.000

Let **x** be the number of moles of radio-labelled taurine incorporated per mole of enzyme.

$$1,000 \times \frac{12.14}{1,000} \times \frac{1}{80,000} \times \frac{x}{1} = \text{mMoles radio-labelled taurine incorporated in 12.14 mg enzyme}$$

$$6.602 \times 10^{-3} \times \frac{12.14}{1,000} \times \frac{x}{80,000} \times 1,000 \text{ mCi}$$

$$= 9.8948x \times 10^{-7} \text{ mCi incorporated in 12.14 mg.}$$

Known from experiment 1 mg radio-labelled glucoamylase I has 1032.22 d.p.m.

$\therefore$ 12.14 mg = 12531.10 d.p.m.

$\therefore$ $9.8948x \times 10^{-7} \times 10^{3} \times 2.2 \times 10^{6} = 12531.10$

$$x = \frac{12531.10}{2.2 \times 10^{6} \times 10^{3} \times 10^{-7} \times 9.8948} = 5.76 \text{ moles taurine incorporated}$$

To nearest integer = 6

6.3.15 Calculation of amount of ^{14}C radio-labelled taurine incorporated into partially modified glucoamylase II

Weight of enzyme reacted = 5.65 mg

Molecular weight of enzyme = 80,000

Let y be the number of moles of radio-labelled taurine incorporated per mole of enzyme.

$1{,}000 \times \frac{5.65}{1{,}000} \times \frac{1}{80{,}000} \times y$ = mMoles radio-labelled taurine incorporated in 5.65 mg enzyme.

$6.602 \times 10^{-3} \times \frac{5.65}{1{,}000} \times \frac{y}{80{,}000} \times 1{,}000$ mCi.

$= 4.605y \times 10^{-7}$ mCi incorporated in 5.65 mg

Known from experiment 1 mg radio-labelled glucoamylase has 890.50 d.p.m.

$\therefore$ 5.65 mg = 5,031.33 d.p.m.

$\therefore$ $4.605\ y \times 10^{-7} \times 10^{3} \times 2.2 \times 10^{6} = 5{,}031.33$

$$y = \frac{5{,}031.33}{4.605 \times 10^{-7} \times 10^{3} \times 2.2 \times 10^{6}} = 4.97 \text{ moles of taurine incorporated}$$

To nearest integer = 5

6.3.16 The digestion of radio-labelled glucoamylases I and II by D.C.C. treated trypsin

^{14}C Radio-labelled glucoamylase I (5 mg) and glucoamylase II (3 mg) were reacted for 9 hours at 0°C with performic acid reagent (2 cm^3) prepared as described by Hirs.[86] The samples were freeze-dried and redissolved in deionised water (400 cm^3). The solutions were then freeze-dried. Each sample was dissolved in 0.1M sodium hydroxide (5 cm^3) which contained calcium chloride (0.001M). The samples were hydrolysed with D.C.C. trypsin (1 mg for glucoamylase I, 0.75 mg for glucoamylase II) for 24 hours at 25°C. After hydrolysis, the samples were passed separately through a "Pharmacia" type K15/90, 1.5 x 90 cm, 154 cm^3 capacity glass column, packed with "Bio-Gel" P-4 gel equilibrated with 0.01M ammonium bicarbonate solution, pH 8.1. Fractions (3 cm^3) were collected and their optical densities (206 nm) recorded automatically using a Pye Unicam SP1,800 spectrometer. The results obtained for glucoamylases I and II are shown in FIGURE 6.8 and 6.10 respectively. Fractions occurring between 48 to 66, 72 to 111, and 114 to 147 cm^3 for glucoamylase I and between 42 to 60, 63 to 96 and 99 to 150 cm^3 for glucoamylase II were pooled separately and the latter two in each case rotary evaporated to dryness. The first pooled fractions were reduced to 2 cm^3 volume by rotary evaporation and each passed through a "Pharmacia" type K9/60, 0.9 x 60 cm, 38 cm^3 capacity glass column, packed with "Bio-Gel" P-60 gel equilibrated with 0.01M ammonium bicarbonate solution, pH 8.1. Fractions (3 cm^3) were collected and their optical densities (206 nm) recorded automatically as previously described and the results shown in FIGURES 6.9 and 6.11. Fractions occurring between 12 to 27 and 33 to 45 cm^3 for glucoamylase I and between 8 to 25 and 28 to 41 cm^3 for glucoamylase II were pooled and evaporated to dryness.

6.3.17 Peptide mapping of radio-labelled glucoamylases I and II

The dried peptide samples obtained from the column chromatography described in section 6.3.16 were dissolved in 0.1M hydrochloric acid (0.5 cm^3) and spotted on Whatman 3M chromatography papers 43 x 45 cm prior to electrophoresis in pyridine, acetic acid, water (1 : 6: 30 v/v/v) at 2,500 V, 60 mA for 1 hour using a Shandon horizontal bed high voltage electrophoresis apparatus. After air drying the papers were spotted with congo red marker dye and subjected to descending chromatography at right angles to the direction of electrophoresis in pyridine, isoamyl alcohol, water (30:30:35 v/v/v) as described by Baglioni.[84] When the dye had migrated to within 5 cm of the end of the chromatography papers, they were removed, air dried and sprayed with 1% ninhydrin dissolved in 96% ethanol. The papers were developed by heating in an oven at 80°C for 10 minutes. The results are shown on PLATES 6.1 to 6.8.

References

1. DELPIERRE, G.R., FRUTON, J.S., Proc. Natl. Acad. Sci. U.S., 54, 1161, 1965.

2. DELPIERRE, G.R., FRUTON, J.S., Proc. Natl. Acad. Sci. U.S., 56, 1817, 1966.

3. RAJAGOPALAN, T.G., STEIN, W.H., MOORE, S., J. Biol. Chem., 241, 4295, 1966.

4. ONG, E.B. PERLMANN, G.E., Nature, 215, 1492, 1967.

5. KOZLOV, L.V., GINODMAN, L.M., OREKHOVICH, V.N., Biokhimiya, 32, 1011, 1967.

6. ERLANGER, B.F., VRATSANOS, S.M., WASSERMANN, N., COOPER, A.G., Biochem. Biophys. Res. Commun., 28, 203, 1967.

7. STEPANOV, V.M., VAGANOVA, T.I., Biochem. Biophys. Res. Commun., 31, 825, 1968.

8. FRY, K.T., KIM, O., SPONA, J., HAMILTON, G.A., Biochem. Biophys. Res. Commun., 30, 489, 1968.

9. BADLEY, R.A., TEALE, F.W.J., Biochem. J., 108, 15p, 1968.

10. LUNDBLAD, R.L., STEIN, W.H., J. Biol. Chem., 244, 154, 1969.

11. PATERSON, A.K., KNOWLES, J.R., Eur. J. Biochem., 31, 510, 1972.

12. LIPSCOMB, W.N., HARTSUCK, J.A., REEKE, G.N., Jnr., QUIOCHO, F.A., BETHGE, P.H., LUDWIG, M.L., STEITZ, T.A., MUIRHEAD, H., COPPOLA, J.C., Brookhaven Symp. Biol., 21, 24, 1969.

13. BODLAENDER, P., FEINSTEIN, G., SHAW, E., Biochemistry, 8, 4941, 1969.

14. FEINSTEIN, G., BODLAENDER, P., SHAW, E., Biochemistry, 8, 4949, 1969.

15. EYL, A.W., Jnr., INAGAMI, T., J. Biol. Chem., 246, 738, 1971.

16. POULOS, T.L., PRICE, P.A., J. Biol. Chem., 249, 1453, 1974.

17. HOARE, D.G., KOSHLAND, D.E., Jnr., J. Biol. Chem., 242, 2447, 1967.

18. WILCHEK, M., FRENSDORFF, A., SELA, M., Biochemistry, 6, 247, 1967.

19. HOFSTEE, B.H.J., J. Am. Chem. Soc., 80, 3966, 1958.

20. LEGLER, G., HASNAIN, S.N., Z. Physiol. Chem., 351, 25, 1970.

21. LEGLER, G., GILLER, H., Z. Physiol. Chem., 351, 741, 1970.

22. PHILLIPS, D.C., Proc. Natl. Acad. Sci. U.S., 57, 484, 1967.

23. PARSONS, S.M., JAO, L., DAHLQVIST, F.W., BORDERS, C.L., Jnr., GROFF, T., RACS, J., RAFTERY, M.A., Biochemistry, 8, 700, 1969.

24. PARSONS, S.M., RAFTERY, M.A., Biochemistry, 8, 4199, 1969.

25. KRANMER, K.J., RUPLEY, J.A., Arch. Biochem. Biophys., 156, 414 1973.

26. MATYASH, L.F., OGLOBLINA, O.G., STEPANOV, V.M., Eur. J. Biochem., 35, 540, 1973.

27. WANG, R., OZAKI, T., HAYASHI, K., FUNATZU, M., Kyushu Daigaku Nogakubu Gakugei Zasshi., 27, 1, 1972. Chem. Abs., 78, 13328y.

28. DUFOUR, C., SEMERIVA, M., DESNUELLE, P., Biochim. Biophys. Acta., 327, 101, 1973.

29. ADAMS, K.R., C.R. Trav. Lab. Carlsberg., 28, 481, 1972.

30. AVRAMOVIC-ZIKIC, O., BREIDENBACH, W.C., MADSEN, N.B., Can. J. Biochem., 52, 146, 1974.

31. SWAISGOOD, H., NATAKE, M., J. Biochem., 74, 77, 1973.

32. BANKS, T.E., BLOSSEY, B.K., SHAFER, J.A., J. Biol. Chem. 244, 6323, 1969.

33. CARRAWAY, K.L., SPOERL, P., KOSHLAND, D.E., Jnr., J. Mol. Biol., 42, 133, 1969.

34. ELODI, P., Acta. Biochim. Biophys. Acad. Sci. Hung., 7, 241, 1972.

35. WAKIM, J., ROBINSON, M., THOMA , J.A., Carbohyd. Res., 10, 487, 1969.

36. GRAY, C.J., JOLLEY, M.E., F.E.B.S. Letters, 29, 197, 1973.

37. JOLLEY, M.E., Ph. D. Thesis University of Birmingham, 1972.

38. BARNETT, J.E.G., MERCIER, D., GERO, S.D., F.E.B.S. Letters, 16, 37, 1971.

39. LEGLER, G., Z. Physiol. Chem., 349, 767, 1968.

40. LEGLER, G., Biochim. Biophys. Acta., 151, 728, 1968.

41. LEGLER, G., Z. Physiol. Chem., 345, 197, 1966.

42. LEGLER, G., Molecular and Cellular Biochemistry, 2, 31, 1973.

43. YARIV, J., WILSON, K.J., HILDESHEIM, J., BLUMBERG, S., F.E.B.S. Letters 15, 24, 1972.

44. BESSELL, E.M., THOMAS, P., WESTWOOD, J., Chem. Biol. Interactions, 7, 327, 1973.

45. RAND-MEIR, T., DAHLQUIST, F.W., RAFTERY, M.A., Biochemistry, 8, 4206, 1969.

46. RAND-MEIR, T., DAHLQUIST, F.W., RAFTERY, M.A., Biochemistry, 8, 4214, 1969.

47. LIN, T.Y., KOSHLAND, D.E., Jnr., J. Biol. Chem., 244, 505, 1969.

48. FRAENKEL-CONRAT, H., OLCOTT, H.S., J. Biol. Chem., 161, 259,1945.

49. KRAMER, K.J., RUPLEY, J.A., Arch. Biochem. Biophys., 158, 566, 1973.

50. STARK, G.R., Adv. in Protein Chem., 24, 261, 1970.

51. TSOU CHEN-LU, Scientia Sinica, 11, 1535, 1962.

52. CHEN, H.J., KAISER, E.T., J. Am. Chem. Soc., 96, 625, 1974.

53. SMYTH, D.G., STEIN, W.H., MOORE, S., J. Biol. Chem., 238, 227, 1963.

54. RIEHM, J.P., SCHERAGA, H.A., Biochemistry, 5, 99, 1966.

55. GIBSON, D., ANDERSON, P.J., Biochem. Biophys. Res. Commun., 49, 453, 1972.

56. SHEEHAN, J.C., HLAVKA, J.J., J. Org. Chem., 21, 439, 1956.

57. CARRAWAY, K.L., KOSHLAND, D.E., Jr., Biochim. Biophys. Acta., 160, 272, 1968.

58. SMITH, E.L., LANDON, M., PIZKIEWIEZ, D., BRATTIN, W.J., LANGLEY, T.J., MELAMED, M.D., Proc. Natl. Acad. Sci. U.S., 67, 724, 1970.

59. LANGLEY, T.J., SMITH, E.L., J. Biol. Chem., 246, 3789, 1971.

60. LIN, T.Y., Biochemistry, 9, 984, 1970.

61. KIMMEL, M.T., PLUMMER, T.H., Jr., J. Biol. Chem., 247, 7864, 1972.

62. ATASSI, M.Z., SINGHAL, R.P., J. Biol. Chem., 247, 5980, 1972.

63. ATASSI, M.Z., SINGHAL, R.P., Immunochemistry, 9, 1057, 1972.

64. ATASSI, M.Z., PERLSTEIN, M.T., Biochemistry, 11, 3984, 1972.

65. GRAY, C.J., JOLLEY, M.E., F.E.B.S. Letters, 29, 197, 1973.

66. JOLLEY, M.E., Ph. D. Thesis, University of Birmingham, 1972.

67. HARTLEY, B.S., Biochem. J., 119, 805, 1970.

68. PERINI, F., Intra-Science Chemistry Reports, 5, 381, 1971.

69. EDMAN, P., Acta. Chem. Scand., 4, 283, 1950.

70. EDMAN, P., BEGG, G., Eur. J. Biochem., 1, 80, 1967.

71. CROMWELL, L.D., STARK, G.R., Biochemistry, 8, 4735, 1969.

72. WOODWARD, R.B. OLOFSON, R.A., J. Am. Chem. Soc., 83, 1007, 1961.

73. WOODWARD, R.B., OLOFSON, R.A., MAYER, H., J. Am. Chem. Soc., 83, 1010, 1961.

74. WOODWARD, R.B. OLOFSON, R.A., MAYER, H., Tetrahedron Suppl., 8, 321, 1966.

75. KHORANA, H.G., Chem. Rev., 53, 145, 1953.

76. LLOYD, J.B., WHELAN, W.J., Anal. Biochem., 30, 467, 1969.

77. LIU, C.L., HATANO, H., F.E.B.S. Letters, 42, 352, 1974.

78. GOMORI, G., In "Methods in Enzymology", Vol. 1., (COLOWICK, S.P., KAPLAN, N.O., Eds.) p.138-146, Academic Press, N.Y., 1955.

79. DUBOIS, M., GILLES, K.A., HAMILTON, J.K, REBERS, P.A., SMITH, F., Anal. Chem., 28, 350, 1956.

80. "Dictionary of Organic Compounds" p.2946. 1965.
E and F.N. Spon.

81. KURZER, F., DOURAGHI-ZACEH, K., Chem. Rev., 67, 107, 1967.

82. BUSCH, M., BHUME, G., PUNGS, E., J. Prakt. Chem., 79, 513, 1909.

83. VOWINKEL, E., Chem. Ber., 96, 1702, 1963.

84. BAGLIONI, C., Biochim . Biophys. Acta., 48, 392, 1961.

85. PRICE, L.W., "Practical Course in liquid scintillation Counting" Parts 1 to 5. Laboratory Practice, January to June 1973.

86. HIRS, C.H.W., In "Methods in Enzymology" Vol XI. (COLOWICK, S.P., KAPLAN, N.O., Eds) pp.197-199. Academic Press, New York, 1967.

87. COZZONE, P., PASERO, L., BEAUPOIL, B., MARCHIS-MOUREN, G., Biochimie, 53, 957, 1971.

88. OHNISHI, M., TOSHIHIKO, S., HIROMI, K., J. Biochem., 76, 7, 1974.

89. THOMAS, J.O., In 'Chemical modification of Proteins'Companion Biochem., (BULL, A.T., Ed.), p.87, Longman Group, London,1974.

90. MEANS, G.E., FEENEY, R.E., 'Chemical modification of Proteins', Holden-Day, San-Francisco, 1971.

91. Methods in Enzymology, (HIRS, C.H.W., Ed.), Vol. 11, Academic Press, New York, 1967.

92. Methods in Enzymology, (HIRS, C.H.W., Ed.), Vol. 25, Academic Press, New York, 1972.

93. SINGER, S.J., Adv. Prot. Chem., 22,

94. WOLD, F., Birth Defects, 9, 46, 1973.

95. PLUMMER, T.H., Jr., J. Biol. Chem., 246, 2930, 1971.

96. BRADSHAW, R.A., ERICSSON, L.H., WALSH, K.A., NEURATH, H., Proc. Nat. Acad. Sci. U.S.A., 63, 1389, 1969.

97. HASS, G.M., NEURATH, H., Biochemistry, 10, 3535, 1971.

98. KOSHLAND, D.E., Jr., Biol. Rev., 28, 416, 1953.

99. MAYER, F.C., LARNER, J., J. Amer. Chem. Soc., 81, 188, 1959.

100. BLAKE, C.C.F., JOHNSON, L.N., MAIR, G.A., NORTH, A.C.T., PHILLIPS, D.C., SARMA, V.R., Proc. Roy. Soc. Ser. B., 167, 378, 1967.

101. EDSALL, J.T., BLANCHARD, M.H., J. Amer. Chem. Soc., 55, 2337, 1933.

102. EMERSON, O.H., KIRK, P.L., J. Biol. Chem., 87, 597, 1930.

103. KING, E.J., J. Amer. Chem. Soc., 75, 2204, 1953.

104. HIROMI, K., Biochem. Biophys. Res. Commun., 40, 1, 1970.

105. HIROMI, K., NITTA, Y., NUMATA, C., ONO, S., Biochim. Biophys. Acta., 302, 362, 1973.

106. "Lysozyme", E.F. OSSERMAN, R.E. CANFIELD and S. BEYCHOK eds., Academic Press, New York, 1974.

107. DUNN, B.M., BRUICE, T.C., Adv. Enzymology, 37, 1, 1973.

108. "The Enzymes", Third Edition, BOYER, P.D., ed., Academic Press, New York, Vol. V, 1971, Vol. VII, 1972.

CHAPTER 7

SOME SUGGESTIONS FOR FUTURE WORK ON GLUCOAMYLASES FROM *Aspergillus niger*

The two forms of glucoamylase obtained in this work could be examined by the isoelectric focussing technique to determine whether any microheterogeneity exists within the purified enzymes. As crystals of glucoamylase have already been obtained from *Endomyces* species IFO 0111[1], *Mucor rouxianus*[2] and *Aspergillus awamori*[3], further investigations of enzyme structure could be carried out on purified glucoamylases from *Aspergillus niger* with priority being given to an X-ray analysis on suitable crystals provided these could be obtained from this enzyme source. Because of the commercial significance of the reversion reaction catalysed by glucoamylases, this reaction could be studied in greater detail with particular emphasis on the time dependence, and on the effect of α-amylase on the reaction. However, before an exact theoretical treatment of reversion could be carried out, more reliable thermodynamic data (e.g. ΔG_{hyd}) than that used in this work would be required.

In a recent paper by Gasdorf *et al.*[4] it was reported that the action pattern of glucoamylase isozymes from black *Aspergillus* differed in their rate of hydrolysis of glycogen. Glucoamylase I was reported to readily hydrolyse this substrate, whereas glucoamylase II showed very little hydrolytic activity. In view of this report it would be interesting to establish whether the purified glucoamylases prepared in this work exhibited similar properties

toward glycogen and carry out kinetic work on simple substrates.

In a review of the chemical modification of enzymes by Wold,[5] it was pointed out that some enzymes could be modified with a high retention of catalytic activity by using the active site protection principle. In some cases the modified enzymes showed an increased stability toward proteolytic attack. It may be possible to use the principle of chemical modification with protection to investigate the preparation of immobilised enzymes of high activity or the therapetuic application of chemically modified enzymes. Inthe latter case with glucoamylase as a possible treatment for Type II glycogenosis[6], the anomeric configuration of enzyme product may be important. It would be necessary to determine this for each enzyme form especially since it has been reported by Marshall[7] that mammalian glucoamylase (γ-amylase) gives products with an α-configuration while that of fungal origin gives the β form.

References

1. FUKUI, T., NIKUNI, Z., Agr. Bio. Chem., 33, 884, 1969.

2. TSUBOI, A., YAMASAKI, Y., SUZUKI, Y., Agr. Biol. Chem., 38, 543, 1974.

3. RODZEVICH, B.I., DOBROLINSKAYA, G.M., Ferm. Spirt Prom., 32, 5, 1966.

4. GASDORF, H.J., ATTHASAMPUNNA, P., DAN, V., HENSLEY, D.E., SMILEY, K.L., Carbohyd. Res., 42, 147, 1975.

5. WOLD, F., Birth Defects, 9, 46, 1973.

6. HUG, G., SCHUBERT, W.K., J. Cell. Biol., 35, C1, 1967.

7. MARSHALL, J.J. Wallerstein Labs. Commun., 35, 49, 1972.

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