ISOLATION, PURIFICATION AND PROPERTIES OF A MAMMALIAN GLUCOSIDASE AND A BACTERIAL FRUCTOSIDASE

BY

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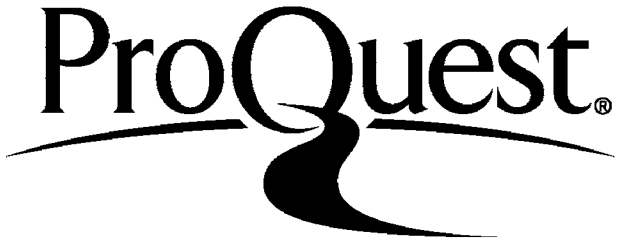

TO MY PARENTS

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ABSTRACT

Isolation, Purification and Properties of a Mammalian Glucosidase and a Bacterial Fructosidase

BY KEITH WILLIAM DAWES

PART 1

The enzyme acid α-D-glucosidase has been isolated from three batches of pig's liver, and purified by molecular gel filtration. Disc gel electrophoresis of the purified enzyme established homogeneity. An isoenzyme of acid α-D-glucosidase, purified to homogeneity, was isolated from one batch.

Sodium dodecyl sulphate electrophoresis indicated that the enzyme existed in subunits with a molecular weight of 126,000, 101,500, and 87,500 for each.

A study of the enzymic properties of the enzyme was carried out. The enzyme liberated D-glucose in a exo-manner from cibrachon blue amylose. The enzymic hydrolysis of phenyl α-D-glucopyranoside yielded the α-anomer of D-glucose, as confirmed by nmr spectroscopy. A series of rate equations were derived and applied to describe the reaction. The enzyme hydrolysed a variety of α-linked glucosyl oligosaccharides, glycosides and polysaccharides. Maltose was the preferred substrate with a K_m of 4.0 to 4.4 mM. Glycogen was completely hydrolysed to D-glucose.

A comparison of the properties of the two isoenzymes showed that they both had similar pH optima, thermal stabilities, and subunit structures. The isoenzymes could be separated by cellulose acetate electrophoresis. One isoenzyme had a diminished affinity for glycogen and dextran T_{10}.

PART 2

The extracellular levanase of *Streptococcus salivarius* strain '51' was isolated from a culture grown on a levan containing medium, gel filtration, ion-exchange and affinity chromatography were used in an attempt to purify the enzyme to homogeneity. A partially purified enzyme preparation was used to study the properties of the enzyme. Heat denaturation studies indicated that two enzymes were present in the preparation, one of which was thermally unstable. Both enzymes had the ability to hydrolyse both levan and inulin. The preparation was also able to hydrolyse raffinose, sucrose, inulin- and levan-oligosaccharides and methyl β-D-fructofuranoside.

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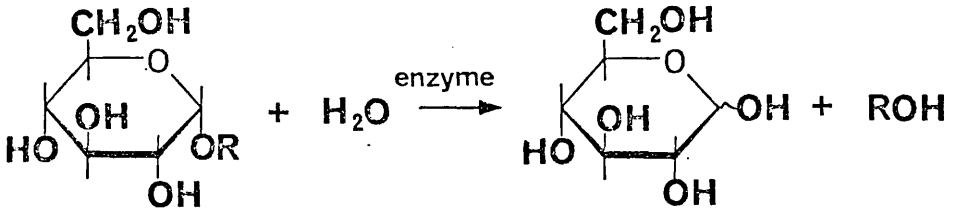
Chapter I Part I

Acid α-D-glucosidase of Swine liver

I-1. Introduction

1.A. Classification of Acid α-D-glucosidase

The enzyme α-D-glucosidase catalyses the following reaction:



Its catalogue number according to "The Enzyme Handbook"[1] is 3.2.1.20. However, in the early literature enzyme preparations which have catalysed the above reaction have been given a variety of names which are often based on the substrates they hydrolysed. Some examples are given in Table 1.

Table 1 Variation in the naming of α-D-glucosidase

Name	Reason for Naming
Glucoamylase	Property of producing solely glucose from amylose
γ-Amylase	γ-amylolysis of glycogen
Maltase	Hydrolysis of maltose
Dextranase	Hydrolysis of dextrans
α-Glucosidase	Stresses broad specificity of action

This variation clearly resulted from the lack of the detailed knowledge of the substrate specificity of the enzyme. This is amplified by the

definition given by Gottschalk[2] in 1950. He defined an α-D-glucosidase as an enzyme which cleaved the α-D-glucosidic linkage in oligosaccharides and in α-D-glucosides. It was therefore popular to term the enzyme as a maltase because of its ability to hydrolyse 4-O-α-D-glucopyranosyl-α-D-glucopyranose (maltose). At the present time it is acknowledged that the enzyme is also able to hydrolyse polysaccharides made up of α-D-glucopyranose residues.

The α-D-glucosidases can be divided into two groups depending on the pH at which maximum enzymic activity is exerted. Acid α-D-glucosidases show maximum activity at pH 4 to 5, neutral α-D-glucosidases at pH 6 to 7.

The enzyme under investigation is acid α-D-glucosidase (E.C. 3.2.1.20) from swine liver and is referred to as acid α-D-glucosidase.

1.B. Mammalian Acid α-D-Glucosidases

Before 1950 most of the α-D-glucosidases were either isolated from bacteral, fungal, or plant sources. They were isolated from these sources because partial purification was easier to carry out. This arose because contamination from glycoside hydrolases was small in comparison to those present in animal sources. It was not until the late 1950's and early 60's, when new biochemical techniques such as ion-exchange chromatography, molecular exclusion chromatography, and electrophoretic techniques were developed, that separation of contaminating enzymes could be achieved. Towards the middle and late 60's therefore α-D-glucosidases were being purified, some to homogeneity.

Gottschalk[2] has reviewed the α-D-glucosidases up to 1950. From his definition of a glucosidase given in Section 1.A. he named these enzymes capable of hydrolysing maltose, maltases.

Maltases, as defined by Gottschalk[2] were first shown to be present in mammals by Emil Fischer.[3] Later, maltase was found in the liver of rat, cat, rabbit, pig[4] and skeletal muscle.[5] It was also shown to be present in pig kidney.[6] These enzymes were not characterised as neutral or acid glucosidases. The broad specificity of action of α-D-glucosidases was not known at this time.

Up to 1960 very few α-D-glucosidases were extracted from mammalian sources. The majority of those extracted were mostly neutral α-D-glucosidases from intestinal mucosa. Dahlqvist[7] using heat inactivation and ion-exchange chromatography separated three different enzymes from intestinal mucosa. He characterised them as being a maltase, trehalase, and a isomaltase. An enzyme isolated from the spleen of rat, rabbit, and dog which had the property of hydrolysing dextrans was described by Rozenfeld.[8,9] Maximum enzyme activity was obtained at pH 4.8. The enzyme was deactivated at a pH greater than 7. Although not classified as an acid α-D-glucosidase but as a dextran 1,6-glucosidase it was confirmed later that the enzyme was an acid α-D-glucosidase.[10] A maltase isolated from equine serum[11] was classified as a neutral α-D-glucosidase. Although it showed no activity towards dextran it did have activity against 6-O-α-D-glucopyranosyl-α-D-glucopyranose (isomaltose), starch, and glycogen. The first clear differentiation between the location of neutral and acid α-D-glucosidase within the cell was made by Hers et al.[12] Using preparative centrifugation it was shown that in rat liver cells acid maltase was located in the lysosomes with the marker reference enzyme acid phosphatase. Neutral α-D-glucosidase was located in the microsomes with the marker reference enzyme glucose-6-phosphatase.

The same author, later, using the same technique isolated rat liver lysosomes and studied some of the properties of the acid α-D-glucosidase.[13] The Michaelis constant (K_m) defined as the substrate concentration at which the initial velocity equals half of the maximum velocity, was found for maltose to be 4mM. Inhibition studies using 3-O-α-D-glucopyranosyl-α-D-fructofuranose (turanose) showed that the maltase activity of the acid α-D-glucosidase could be inhibited completely at a concentration of 50 mM, the inhibition was non-competitive.[14] Shibko used the same procedure as Hers to isolate rat kidney lysosomes containing acid α-D-glucosidase. Only 10% of the total maltase activity was associated with the acid α-D-glucosidase in the lysosomes. Two protein fractions from perfused rabbit liver were found to contain α-amylase and γ-amylase.[15] Maximum activity for γ-amylase was found between pH 4.8 to 5.0. In neutral media the enzyme was deactivated. It was also stable to heating up to 55°C. Glucose was liberated when the enzyme was incubated with glycogen. γ-Amylase also showed slight dextranase activity.

The first authors who reported a detailed study of the kinetic properties of an acid α-D-glucosidase were Torres and Olavarria.[16] The neutral and acid α-D-glucosidases were isolated from dogs liver but in very poor yields. Of the total maltase activity in the crude extract a final yield of 3.13% and 2.56% was accomplished for the netural and acid α-D-glucosidases, respectively. The enzyme had a high affinity for maltose, as a substrate, as shown by a Michaelis constant of 4 to 5 mM. In comparison with other substrates the enzyme exhibited a higher affinity for maltose than most of the other substrates as shown in Table 2.

Table 2. $V^{\blacktriangle}_{max.}$ values for the acid α-D-glucosidase of dog's liver

Substrate	Linkage	$V^{+}_{max.}$
Kojibiose°	α-(1 → 2)	38
Nigerose*	α-(1 → 3)	120
Maltose	α-(1 → 4)	100
Isomaltose	α-(1 → 6)	4
Phenyl α-D-gluco-pyranoside		24
Glycogen	α-(1 → 4) α-(1 → 6)	17.4

+ Velocities expressed as percentage of glucosidic bonds hydrolysed relative to maltose.

▲ $V_{max.}$ is defined as the maximum initial velocity of the reaction under the given conditions and is the limiting value that the initial velocity approaches as the substrate concentration approaches infinity.

° 2-O-α-D-glucopyranosyl- α-D-glucopyranose

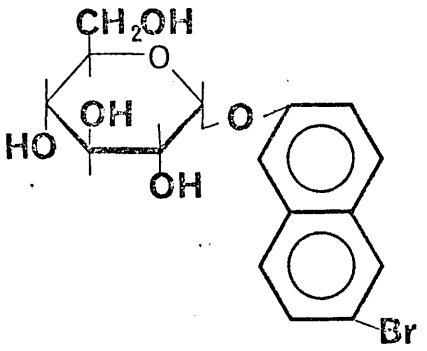
* 3-O-α-D-glucopyranosyl- α-D-glucopyranose

Both enzymes were able to transfer D-glucose from a donor to an acceptor molecule and hence showed glucosyl transferase activity. The neutral α-D-glucosidase seemed to be more active. Using maltose as a donor of glucose, the riboflavinyl glycoside was synthesised when riboflavin was used as the acceptor molecule. D-Mannose, as well as D-galactose and D-fructose also acted as acceptors. A series of oligosaccharides containing these sugars were found.

Auricchio et al.[17] utilised the dextranase activity of acid α-D-glucosidase to separate the neutral and acid enzymes on the dextran gel, Sephadex G-100. The acid α-D-glucosidase from rat liver and human kidney was purified 700 fold using this method. Both acid α-D-glucosidases exhibited maximum activity, using glycogen and maltose as substrates, at the same pH. Later, by incorporating a further step using gel filtration, the same workers[18] purified the rat liver enzyme to homogeneity. Only 7% of the total maltase activity was isolated as acid α-D-glucosidase. The enzyme was very active, having 5520 maltase units per mg of protein. The human and rat enzymes gave a Michaelis constant for maltose, of 13.6 and 4.7 mM, respectively. Both enzymes were inhibited by erythritol and 2-amino-2-(hydroxyl methyl) propane-1,3-diol (tris), the rat liver enzyme being inhibited competitively. In 1969 the same workers[19] were the first to study the kinetic and physico-chemical properties of the homogeneous acid α-D-glucosidase from cattle liver. A very high yield of 43% of the total maltase activity was obtained. The enzyme had a very high specific activity of 34,000 maltase units per mg of protein. The enzyme was demonstrated to have both maltase and glucoamylase activity. Erythritol, tris, and turanose all inhibited the maltase activity of the enzyme competitively. The Michaelis constant of 10 mM for maltose was comparable to the values obtained for the enzyme from other sources. In later publications,[20,21] it was shown that the enzyme was capable of transglucosidase activity. Using either D-glucose or maltose as donors D-glucose became incorporated into glycogen. This is in agreement with the results of Torres et al.[16] who found that the same activity was demonstrated

by the dog's liver acid α-D-glucosidase. D-Glucose was found to inhibit the hydrolysis of 6-bromo-2-naphthyl α-D-glucopyranoside (I). Ultracentrifuge studies in guanidine-HCl indicated that the enzyme

I



existed in subunits of molecular weight 25,000, held together by non-covalent bonds. D-Glucose was also found to competitively inhibit the maltase activity of the enzyme.

Acid α-D-glucosidase has been extracted from human pancreas[22] and purified. It was found to degrade maltose and starch to the extent of 100% and 40%, respectively, at pH 4.4. Iodoacetate, potassium cyanide, and mercuric chloride were found to be inhibitory.

The first crystallisation of an acid α-D-glucosidase was achieved by Fujimori[23] and co-workers. When acetone was added to make a 20% v/v solution, crystals of the enzyme were obtained. During the purification procedure of the bovine spleen enzyme the maltase and glucoamylase activities differed after each step, indicating that both activities were not exerted by the same enzyme. The pH at which maximum maltase and glucoamylase activities were demonstrated was pH 4.4 and 4.8, respectively. Also, the heat sensitivities of the two differed. Turanose was found to inhibit both activities. However, they were affected differently by increasing concentration of inhibitor. It was concluded that the enzyme existed in more than one form. The properties of each differed in such a way as to discriminate maltase and glucoamylase activity.

The rat liver enzyme has been fully investigated by Brown et al.[24] A reasonable yield of 13% of the total maltase activity was obtained, resulting in a 1300 fold purification of the enzyme. A very high specific activity of 15,460 units per mg protein was shown by the purified enzyme. Although homogeneity was established by equilibrium centrifugation, on disc gel electrophoresis, the enzyme separated into two protein bands. The enzyme was found to exhibit both maltase and isomaltase activity. However, maximum activity was obtained at two different pH values, pH 3.7 and 4.2 for the maltase and isomaltase activities, respectively. The enzyme showed cation activation, potassium chloride stimulating glycogen breakdown. At pH 4.0 the enzyme was found to have transglucosylase activity. When incubated with maltose and glucose, oligosaccharides were formed. Glycogen acted as an acceptor of glucose. The weight average molecular weight of the purified enzyme was found to be 114,000. A later publication dealt with the kinetic properties of the enzyme.[25] Maltose, maltotriose, maltotetrose, and maltopentose all exhibited substrate inhibition at concentrations between 5 to 10 m$\underline{\underline{M}}$. Glycogen did not exhibit any substrate inhibition but the enzyme was activated by potassium chloride. The Michaelis constant for glycogen varied with different concentrations of potassium chloride. At a concentration of 4.5 m$\underline{\underline{M}}$ the Michaelis constant was 0.7 m$\underline{\underline{M}}$ on increasing the concentration to 115 m$\underline{\underline{M}}$., the Michaelis constant became 6.5 m$\underline{\underline{M}}$. The Michaelis constant for maltose and isomaltose was 3.8 m$\underline{\underline{M}}$ and 32.9 m$\underline{\underline{M}}$, respectively. Isomaltose did not exhibit any substrate inhibition.

Palmer[26,27] isolated and purified the rabbit muscle acid α-$\underline{\underline{D}}$-glucosidase and studied the kinetic properties of the enzyme. The yield was 22% of the total initial maltase activity. After a 3500 fold

purification the enzyme exhibited a specific activity of 6167 units/mg protein. Maximum activity for the maltase and glucoamylase activities was shown at pH 4.5 and 5.1, respectively. Activation of enzyme activity by an inorganic salt was observed as shown previously by Brown et al.[24] However, this activation was shown by sodium chloride instead of potassium chloride. Table 3 shows a comparison of the Michaelis Constants and the relative maximum velocities ($V_{max.}$) shown for a series of substrates for the rabbit muscle α-D-glucosidase.

Table 3 K_m and relative $V_{max.}$ values for the rabbit muscle acid α-D-glucosidase

Substrate	K_m (mM)	$V_{max.}$+ (maltose)
Maltose	3.7	1
Maltotriose	1.8	1.4
Maltotetrose	3.2	1.6
Maltopentose	3.7	1.3
Maltohexose	3.4	1.2
p-Nitrophenyl α-Maltoside	1.2	1.4
Methyl α-Maltoside	4.2	0.8
Rabbit liver glycogen	8.3	0.5
Shellfish glycogen	4.0	0.3
Panose°	20	0.1

+$V_{max.}$ expressed as μmoles/glucose liberated per mg protein\ min

°O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→4)-α-D-glucopyranose

The enzyme had the greatest affinity for maltotriose and p-nitrophenyl α-maltoside. Pronounced substrate inhibition was encountered with the malto-oligosaccharides at concentrations greater than 5 mM. This was also true of the maltosides but not for glycogen. The cation activation tended to increase the Michaelis constant and maximum velocity values. The enzyme was able to degrade completely the glycogen phosphorylase limit dextrin and the shellfish glycogen β-amylase limit dextrin. Thus, the enzyme showed α-(1→4) and α-(1→6) glucohydrolase activity. Transglucosylase activity was demonstrated by the enzyme. On incubation of [^{14}C]-maltose, labelled in the non-reducing glucosyl unit, with pullulan or glycogen, [^{14}C]-glucosyl units became incorporated into the polysaccharides. Replacement of [^{14}C]-maltose with D-[^{14}C]-glucose, [^{14}C]-maltose labelled exclusively in C-1 of the reducing glucosyl unit, lead to no incorporation of radioactivity into glycogen. It was concluded that the reaction was a transglucosylation with transfer of the non-reducing glucosyl moiety of maltose into the polysaccharide. These results were in agreement with those found for the enzyme from other sources.[16,24]

Two acid α-D-glucosidases have been purified from monkey liver and spleen.[28] A 1200 fold purification was obtained for the liver enzyme and a 200 fold purification for the spleen enzyme. Maximum glucoamylase and maltase activity was exhibited by both enzymes at pH 4.8, indicating that both activities were exerted by the same enzyme. Shown below in Table 4 are some of the kinetic properties of the two enzymes.

Table 4 Kinetic properties of the spleen and liver acid α-D-glucosidases of monkey

Source	Substrate			
	Starch		Maltose	
	K_m Mg/cm^3	$V_{max.}$ +	K_m mM	$V_{max.}$ +
Liver	8.3	3.1	19	5.3
Spleen	9.0	7.5	10	6.0

+ $V_{max.}$ expressed as μmoles glucose liberated/min/mg protein

Both enzymes showed a similar Michaelis constant for starch, but the spleen enzyme had a greater affinity for maltose, with a smaller Michaelis constant. No activity was detected against dextran, isomaltose, and some limit dextrins. The authors suggested that this was due to the same number of enzyme units being used in all the incubations. Therefore, no consideration was given to the fact that the enzyme has a lower activity for these substrates. Both tris and turanose were found to inhibit the enzyme.

Iwanowski[29] purified and crystallised the enzyme from horse skeletal muscle. The enzyme was purified 10 fold. Homogeneity was established as the enzyme migrated as one protein band on disc gel electrophoresis. Transglucosylase activity was observed. Under the conditions of incubation with maltose, transglucosylation was not observed. However, on incubation with a substituted maltoside not only were products of hydrolysis formed but maltotrioside and its derivatives. Later, the same worker[30] described the 1800 fold

purification of the acid α-D-glucosidase from calf heart muscle. It was homogeneous by disc gel electrophoresis. The enzyme was found to be thermally stable up to 40°C, after which activity decreased. Both glycogen and amylopectin were degraded by the enzyme, the Michaelis constant being 0.49 mg/cm^3 and 0.71 mg/cm^3, respectively. In agreement with other authors the enzyme was activated by either sodium or potassium ions. Both tris, and α-D-glucopyranosyl α-D-glucopyranoside (trehalose) were found to inhibit the glucoamylase activity.

The acid α-D-glucosidase from pig's spleen has been isolated and purified.[31] The enzyme migrated as a single band on disc gel electrophoresis. A series of kinetic properties of the enzyme were determined as shown in Table 5.

Table 5 Kinetic parameters of the Pig's spleen acid α-D-glucosidase

Oligosaccharide	Linkage	K_m (mM)	$\frac{V}{V_{max.}}$ +(maltose)
Maltose	1→4	5.8	1.00
Nigerose	1→3	7.3	0.73
Kojibiose	1→2	2.7	0.16
Isomaltose	1→6	29.0	0.07
Isomaltotriose	1→6	53.0	0.13
Isomaltotetrose	1→6	47.0	0.12

+ units of $V_{max.}$ not given by authors.

The enzyme had a greater affinity for maltose than for other substrates. The relatively low Michaelis constant for kojibiose was reported to be due to greater substrate-enzyme binding. In contrast to other acid α-D-glucosidases which have shown inhibition by oligosaccharide substrates,

no inhibition was shown by the substrates listed in Table 5. As it was established that the enzyme was capable of degrading all α-linkages some incubations were carried out with dextrans. Several dextrans containing α-(1→3) linkages were nearly completely degraded whereas those containing α-(1→2) bonds were only partially degraded. The molecular weight of the enzyme determined by gel filtration was 106,000. Both urea and trehalose were found to inhibit the maltase and glucoamylase activity of the enzyme.

An α-D-glucosidase from pig's liver[32] has been purified to homogeneity. After a nine step purification the enzyme had a high specific activity of 22,000 units/mg protein. A poor yield of 0.74% of the total maltase activity in the crude extract was obtained. The properties of the enzyme were consistent with it being a neutral α-D-glucosidase. Maximum maltase activity was found at pH 6.0. Although the enzyme hydrolysed most substrates shown to be hydrolysed by acid α-D-glucosidases no activity was shown towards dextran, isomaltose, or phenyl α-D-glucopyranoside. These results coupled with the high transglucosylase activity of the enzyme, which is reported to be associated with the neutral α-D-glucosidase, suggested that this enzyme was a neutral α-D-glucosidase. Several acid α-D-glucosidases have been isolated from human tissues and been purified. Interest has increased in the human enzyme due to its importance in Pompe's disease which is discussed in Section 1.E. Hers and co-workers[33] purified the human placental acid α-D-glucosidase over 10,000 fold isolating an enzyme with a specific activity of 7300 units/mg protein. Although maltose is known to cause substrate inhibition in other acid α-D-glucosidases no inhibition was found for the human placental enzyme.

Both sodium and potassium ions were found to be stimulating as has been shown for the rat liver[24] and rabbit muscle[26] enzymes. The enzyme was found to have transglucosylase activity transferring D-glucose from maltose to glycogen. There was a slight difference in the pH at which maximum activity of the transglucosylation occurred compared to the maltase activity of the enzyme. Inhibition studies showed that maltose, turanose, and D-glucose competitively inhibited the hydrolysis of 4-methylumbelliferyl α-D-glucopyranoside. When glycogen was the substrate, turanose acted as a non-competitive inhibitor.

Brown *et al.*[24] have purified the human liver acid α-D-glucosidase and determined some of the kinetic properties of the enzyme. After a 2000 fold purification the enzyme exhibited a very high specific activity of 30,000 units/mg protein. Although the enzyme gave a single band on disc gel electrophoresis several isoenzymes were shown to be present after isoelectric focussing. No substrate inhibition by malto-oligosaccharides was detected up to a concentration of 100 mM. The Michaelis constant for maltose was 9 mM and that for isomaltose 33 mM. A later publication by Koster and Slee[35] also described the purification of the human liver enzyme. In good agreement with Drown *et al.*[34] the Michaelis constant for maltose was found to be 10 mM and that for glycogen 50 mg/cm^3. The use of serum albumin to stabilise acid α-D-glucosidase was mentioned by DeBarsy and co-workers.[33] However, Koster found that serum albumin could activate the human liver enzyme. Evidence was given from the results of an Arrhenius plot. With maltose as substrate a straight line was obtained with an activation energy of 3.58 KJ/Mole. With glycogen, however, a break point was seen whether serum albumin was present or not, and the two

lines were parallel. If the activation by albumin was simply due to protection of the enzyme against denaturation, no parallel lines would have been expected. Further evidence to suggest that albumin activated the enzyme came from the pH shift for the maximum activity shown for the hydrolysis of glycogen. With no albumin present, maximum activity was exhibited at pH 5. On addition of albumin maximum activity was found at pH 4.5. Inhibitor studies showed that both maltose and glycogen inhibited the hydrolysis of 4-methylumbelliferyl α-D-glucopyranoside. Table 6 shows some of the properties of these α-D-glucosidases which have been purified.

1.C. Use of Inhibitors to probe the Active Site

Inhibitors of enzyme activity are of great importance in probing the active site. Several detailed studies have been carried out with acid α-D-glucosidases from various sources to determine if more than one active site was present.

Hers[12] used turanose to inhibit the maltase activity of the rat liver enzyme and found it to be non-competitive, indicating that the inhibitor did not block the active site on the enzyme for maltose. Bruni and co-workers[18] found that both tris and erythritol inhibited the maltase activity of the rat liver, and human kidney acid α-D-glucosidase, erythritol being a competitive inhibitor. All three inhibitors were found to inhibit the cattle liver enzyme competitively,[21] in contrast to the results of Hers. The inhibitor constants, K_i, (dissociation constant of the enzyme-inhibitor complex) were 27, 5.0, and 1.4 mM for erythritol, tris, and turanose, respectively. Thus all three inhibitors were found to block the active site.

Table 7 shows a comparison of the Michaelis and Inhibitor Constants found for the rat liver acid α-D-glucosidase by Brown et al.[25] The different Michaelis Constants for glycogen

Table 6 Some Properties of the Mammalian acid α-D-glucosidases which have been purified

Source and reference	Specific Activity*	Activity shown towards:	Molecular Weight	Inhibited by:	K_m Maltose (mM)	Transglycosylase Activity
Dog's liver[16]	145.5	α-(1→4)			4.5	Yes
		α-(1→3)				
		α-(1→2)				
		α-(1→6)				
		α-D-glucosides				
Rat liver[18]	5520	α-(1→4)	83,000	erythritol tris	4.7	
[24]	15,360	α-(1→4)	114,000	substrate inhibition	3.8	Yes
		α-(1→6)		turanose		
Cattle liver[19]	34,000	α-(1→4)				
		α-(1→6)	107,000	erythritol tris		
		α-D-glucosides	(subunit structure)	turanose		
				methyl α-D-glucoside	10	Yes
Rabbit muscle[26]	6167	α-(1→4)		trehalose		
		α-(1→6)-glucosides		substrate inhibition		
				methyl α-D-glucoside		Yes
				turanose, polyols	3.7	

Table 6 (continued)

Source and reference	Specific Activity*	Activity shown towards:	Molecular Weight	Inhibited by:	K_m Maltose (mM)	Transglycosylase Activity
Human placenta [33]	7300	α-(1→4) α-D-glucosides		turanose maltose glucose	11	Yes
[37]		α-(1→4) α-(1→6) α-D-glucosides	103,000	maltose	7-8	
Human liver [34]	30,000	α-(1→4) α-(1→6) α-D-glucosides	100,000		9	
[35]	132,000	α-(1→4) α-(1→6) α-D-glucosides		glycogen maltose	10	
Pig spleen [31]		α-(1→4) α-(1→6) α-(1→3) α-(1→2)	106,000	urea, trehalose	5.8	

* Specific Activity is expressed as nmoles maltose hydrolysed/min/mg protein.

Table 7 Kinetic Values for the inhibition of the rat liver acid α-D-glucosidase

Substrate	K_m (mM)	Inhibitor	K_i (mM)	Type of Inhibition
Maltose	3.4	glycogen	8.6	mixed
Glycogen	1.7	maltose	0.6	competitive
Maltose	3.5	isomaltose	61	competitive
Isomaltose	32.9			
Maltose		turanose	2.8	mixed
Glycogen	6.4	turanose	7.9	mixed
Isomaltose		turanose		non-competitive

were due to the stimulatory effect of potassium ions. A similar value for the inhibitor and Michaelis constant signifies a competitive type of inhibition. The inhibitor constant, for glycogen inhibition of maltose activity was 8 times its Michaelis constant found under the same conditions. Also, maltose inhibition of glucoamylase activity gave a inhibitor constant of 0.6 mM which was 1/6th of the Michaelis constant for maltose. Therefore, it was not possible to decide whether a fully competitive or mixed type of inhibition was shown. Isomaltose gave a competitive type of inhibition of maltase activity, suggesting a mutually accessible site. Most of the data was consistent with the hypothesis that the enzyme had at least two catalytically active binding sites. One of the sites binding disaccharide substrates and perhaps oligosaccharides. The other site seemed to bind polysaccharide substrates. Because of the mixed nature of the turanose inhibition it was also proposed that an inhibitor site may be present within the enzyme molecule. Turanose had no affinity for the glycogen binding site.

Inhibition studies on the cattle liver enzyme[21] indicated the presence of more than one catalytic binding site. Turanose, methyl α-D-glucopyranoside, and trehalose were found to competitively inhibit the hydrolysis of both isomaltose and maltose. Isomaltose was also found to competitively inhibit the maltase activity of the enzyme, suggesting the same catalytic centre for each. The inhibitor constant for each inhibitor are very similar when compared to either inhibition of maltase or isomaltase activity. The inhibitor constant for isomaltose inhibition of maltase activity was very similar to its Michaelis constant as shown in Table 8.

Table 8 Kinetic parameters for the Inhibition of the Cattle liver acid α-D-glucosidase

Substrate	K_m (mM)	Competitive inhibitor	K_i (mM)
Maltose	10	glucose	4.3
Isomaltose	140	turanose	2.6
Isomaltose	160	Me α-Glu *	200
Isomaltose	160	trehalose	290
Maltose	10	isomaltose	100
Maltose	10	glycogen	41×10^3
Maltose	10	Me α-Glu *	140
Glycogen	32×10^3	Me α-Glu *	17
Maltose	10	trehalose	360
Glycogen	33×10^3	trehalose	14

* methyl α-D-glucopyranoside

Glycogen was found to competitively inhibit the maltase activity of the enzyme with an inhibitor constant similar to the Michaelis constant for glycogen. This suggested a single catalytic centre for

maltose and glycogen differing from the results of Jeffreys et al.[25] Turanose was found to competitively inhibit the maltase activity but gave a mixed type of inhibition for the glucoamylase activity of the enzyme. The data suggested that the maltose and glycogen binding sites are similar, but not identical, and that the maltose active centre may help in binding glycogen but other additional binding sites are required.

Palmer[27] showed that the rabbit muscle enzyme was competitively inhibited by trehalose, methyl α-D-glucopyranoside, β-D-fructofuranosyl α-D-glucopyranoside (sucrose), O-α-D-glucopyranosyl-(1→3)-β-D-fructofuranosyl α-D-glucopyranoside (melezitose) when glycogen was used as the substrate. This was in agreement with the results of Rozenfeld and Belenki.[36] Turanose gave a mixed type of inhibition of the maltase and glucoamylase activities of the enzyme in agreement with the results of Jeffrey.[25] The glucoamylase and maltase activity of the enzyme was competitively inhibited by D-glucono-1,5-lactone, erythritol and tris acting as inhibitors of the maltase and glucoamylase activity of the enzyme. In accord with the results of Bruni[18] tris inhibition was competitive. Palmer concluded that at least two but possibly three catalytic binding sites could be present. One centre to bind oligosaccharides and one to bind polysaccharides. The third site possibly had a role in the transglucosylase activity of the enzyme.

Similar results to those already mentioned have been found for the rat liver and human placental acid α-D-glucosidases.[33] The hydrolysis of 4-methylumbelliferyl α-D-glucopyranoside was competitively inhibited by maltose, glucose, and turanose. Turanose inhibited the glucoamylase activity of both enzymes non-competitively, thus suggesting

the presence of two catalytic centres in each enzyme. Table 9 shows some similar results by a different author[37] for the human placental enzyme.

Table 9 A Comparison of some of the kinetic data for the human placental acid α-D-glucosidase by two different workers

Substrate	K_m (mM)	Inhibitor	K_i (mM)
	ref.33		
Mu-glucoside^{o}	1.1	turanose	3
Mu-glucoside^{o}	1.1	maltose	15.8
Mu-glucoside^{o}	1.1	glucose	11.5
maltose	11		
glycogen	2.0		
	ref.37		
Mu-glucoside^{o}	1.78$^{\Delta}$	maltose	10.2$^{\Delta}$
Mu-glucoside^{o}	1.46^{+}	maltose	9.5^{+}
maltose	8.3$^{\Delta}$		
maltose	7.0^{+}		

^{o} 4-methylumbelliferyl α-D-glucopyranoside

$^{\Delta}$ isoenzyme-1

$^{+}$ isoenzyme-2

The results of Swallow[37] are repeated in the table because the results were determined for two isoenzymes. As is seen the inhibitor constant for maltose inhibition is very similar for both enzymes, the inhibitor constant for maltose being a similar value to its Michaelis constant. In contrast to the results shown in Table 9 the human liver enzyme[38] was inhibited non-competitively by maltose

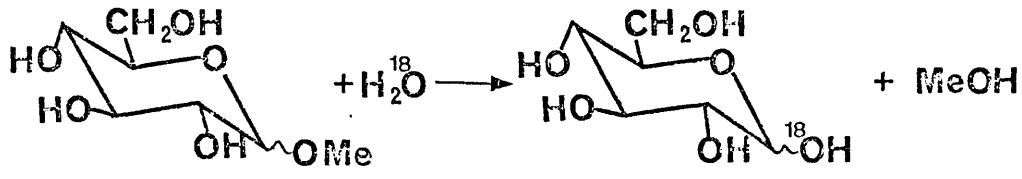
(K_i 25 mM, K_m 10 mM). Glycogen was shown to competitively inhibit the hydrolysis of 4-methylumbelliferyl α-D-glucopyranoside (K_i = 45 mg/cm^3, K_m = 50 mg/cm^3).

All the inhibition studies so far carried out indicate the presence of more than one active centre in acid α-D-glucosidases. One site binding the small molecular weight substrates including those with different α-linkages and the second site binding polysaccharide substrates.

1.D. Mechanism of action of glycoside hydrolases

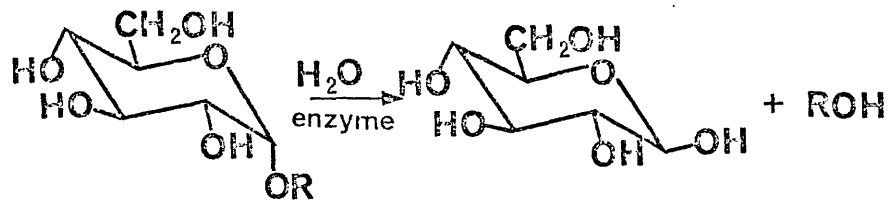
It is still not understood by what mechanism glycosidases exert their action. Most mechanisms are therefore based on hypothesis in which certain reaction mechanisms, based on chemical data, are adapted to their use within enzyme chemistry.

Oxygen-18 tracer experiments have shown that in a large number of glycosidase-catalysed hydrolyses bond fission occurs between carbon-1 and the glycosidic oxygen. α-Glucosidase from brewers yeast has been shown to catalyse the following reaction[39]:

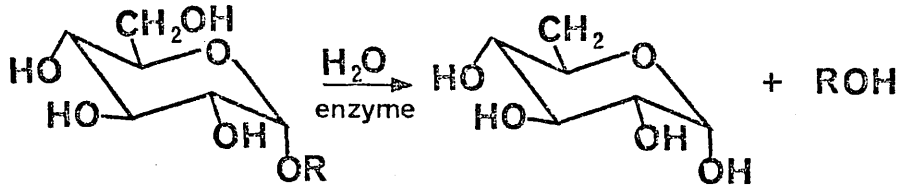


These reactions are formally nucleophilic substitutions at saturated carbon (C-1) of the glycosides. Glycosidases may be divided into two classes according to whether the hydrolysis reactions which they catalyse, proceed with inversion (I) or retention of configuration (II).

I. Inversion of Configuration



II. Retention of Configuration



The table below shows a series of glycosidases which either catalyse a reaction by inversion or retention.

Table 10 Stereochemistry of some enzymic hydrolyses of glycosides

Enzyme	Retention or Inversion of the anomeric configuration of the initial product
β-amylase[40]	Inversion
glucoamylase[41]	"
α-D-glucosidase[42]	Retention
β-D-glucosidase[43]	"
β-D-galactosidase[44]	"
lysozyme[45]	"
pullulanase[46]	"

The determination of the configuration of the released product, until recently, was carried out by polarimetry.[47] A more accurate method was developed by Parrish and Reese,[48] who showed that the

different anomers released during enzymic action could be analysed by separating trimethylsilyl derivatives by gas-liquid chromatography. More recently, however, nuclear magnetic resonance (nmr) has been successfully applied to discriminate the anomeric protons of the released sugar.[42] Two main types of mechanism have been invoked to explain retention of configuration.[49] In the first the reaction proceeds involving either a nucleophilic participation by a group in the substrate or by a group in the enzyme. The latter process would yield a glucosyl-enzyme of inverted configuration, which on reaction with water or another hydroxylic compound, would yield a product with the same configuration as the starting material. Wallenfels[50] and Koshland[51] have named this a Double-displacement mechanism. Fig. 1 shows a representation of this mechanism. In the second mechanism after the substrate becomes attached to the enzyme, alternatively, it is proposed that a proton is transferred from a group on the enzyme to the glycosidic oxygen. This favours the formation of a oxy-carbocation, which is formed when the C-1 oxygen bond breaks. The intermediate oxy-carbocation then electrostatically interacts with a negatively charged group on the enzyme.[52] This has been shown to lead to an intermediate of lower energy.[53] The incoming attacking nucleophile therefore, can only position itself opposite the negatively charged group.

This mechanism is shown diagrammatically in Fig. 2, applied to the action of α-D-glucosidase. Once the oxy-carbocation is formed it interacts with the carboxylate group on the enzyme. This sterically hinders the incoming nucleophile from attacking from any other direction than from the side opposite the carboxylate group, resulting in the formation of the α-anomer. This mechanism has

Fig. 1

Double-displacement reaction proposed by Wallenfels and Koshland

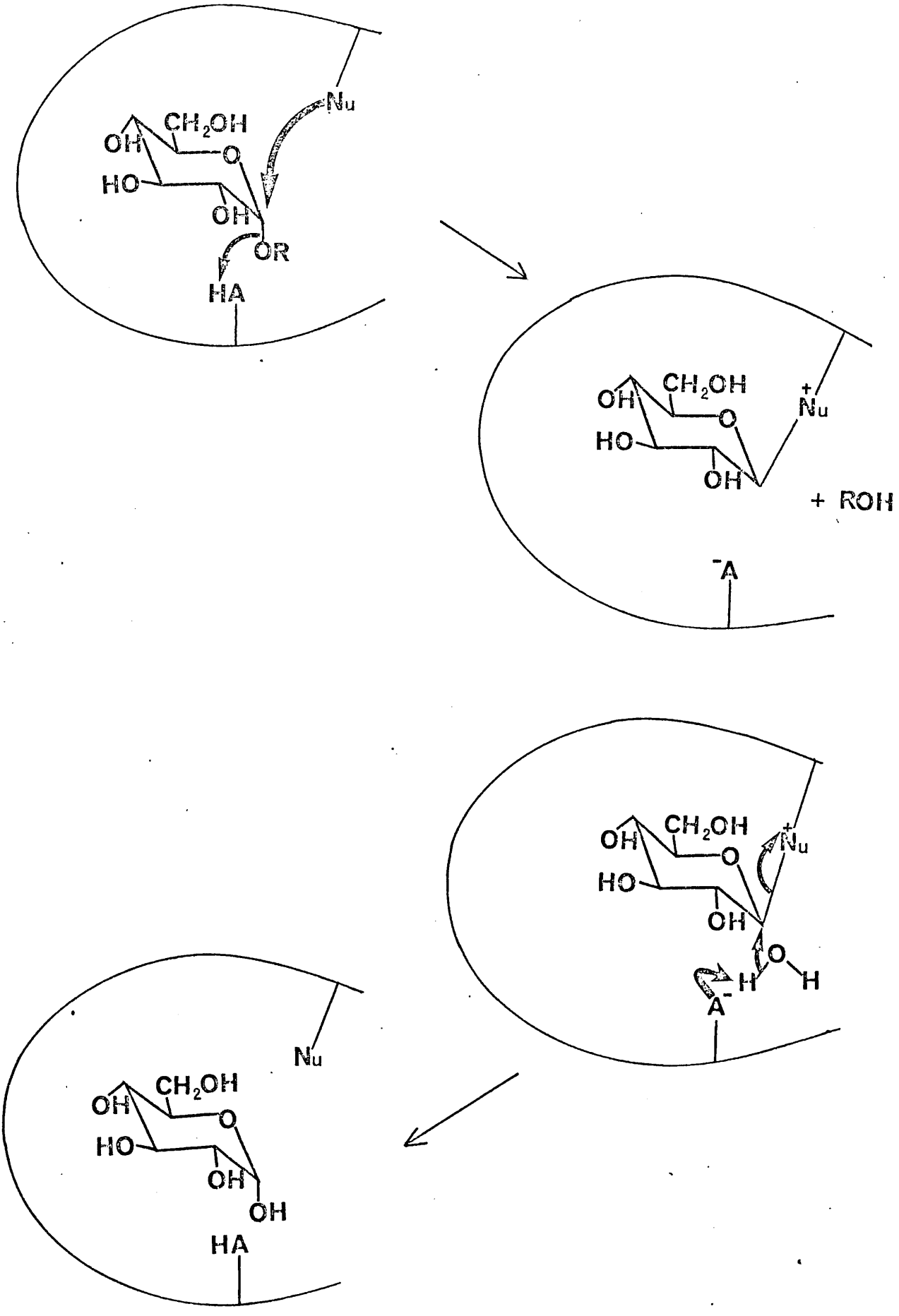
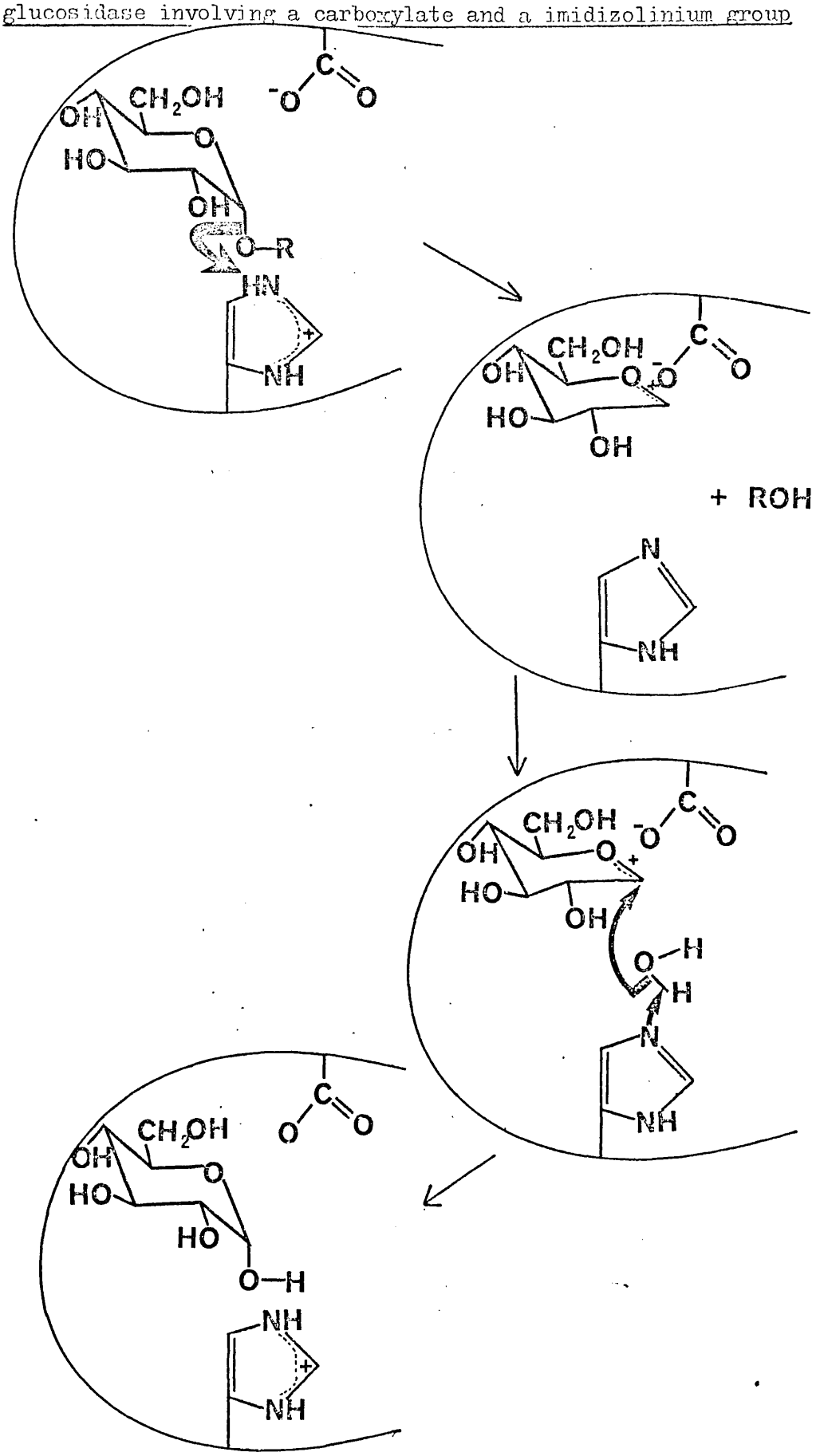


Fig. 2

Proposed mechanism for the hydrolysis of an α-D-glucoside by α-D-glucosidase involving a carboxylate and a imidizolinium group



been proposed for the action α-amylase[54] and lysozyme.[55] Most of the evidence to suggest such a mechanism was given when maximum velocity ($V_{max.}$) values were found to depend on pH for the action of α-amylase, this being interpreted as indicating that carboxylate and imidizolinium act as catalytic groups.[54] The Double-displacement reaction is really two substitution nucleophilic bimolecular (SN_2) reactions. The other proposed mechanism being a substitution nucleophilic unimolecular (SN_1) solvolysis reaction.

The covalent enzyme-glucosyl intermediate, proposed as a product of the mechanism suggested by Wallenfels and Koshland has been successfully isolated. The method used was that of Mitchell et al.[56] in which phenol was used to trap the phosphoryl enzyme intermediates of succinyl CoA synthetase,[56] phosphoglycerate mutase,[57] and of glucose-6-phosphatase.[57] More recently Butler et al.[59] have employed the same technique to isolate the enzyme-glucosyl intermediate of the brewers yeast α-D-glucosidase.

A rapid flow technique was employed in which phenol was used to terminate the reaction and to trap the product of the hydrolysis of methyl α-D-[^{14}C]-glucopyranoside by methyl α-D-glucosidase. After extracting the denatured enzyme radioactivity was found in the protein. It was proposed that a covalent linkage was involved because continued washing of the denatured protein failed to remove the radioactivity. To confirm that the glucose was not mechanically trapped in the denatured enzyme a preparation of the enzyme-glucosyl intermediate was subjected to trypsin digestion. The radioactivity was retained by the tryptic peptide. Most of the radioactivity was recovered as D-glucose after the denatured enzyme was treated with hydrochloric

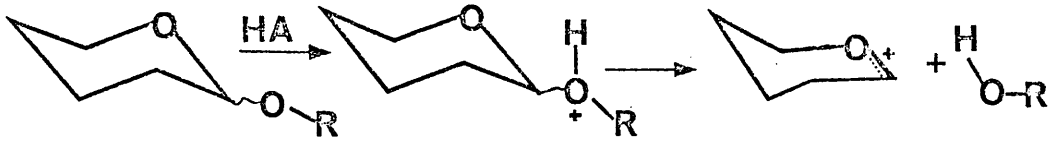
acid. A similar covalent enzyme-glucosyl intermediate has also been reported for sweet almond β-D-glucosidase.[60]

Hall et al.[61] investigated the influence of structure on the hydrolysis of some substituted phenyl α-D-glucosides by α-D-glucosidase from brewers yeast. They found that the affinity of the substrate for the enzyme was greater when the phenyl group carried electron-attracting substituents. However, only slight variations in the rate constant for the breakdown of the enzyme substrate intermediate existed. Capon[49] suggested that the small variations in the rate constant could arise from the reaction proceeding via a glucosyl-enzyme intermediate.

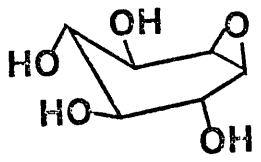
The type of functional group available at the active site of an enzyme was not known until recently. With the advent of the analysis of the structure of lysozyme a fresh approach has begun. Selective active site inhibitors have become a useful group of compounds which are important in probing the functional groups associated with the active site.

Tris has been known for some time to be a good inhibitor of α- and β-D-glucosidase. Studies made with various hydroxylated alkyl amines have indicated that the inhibitory power of tris may be due to the combined influence of the amino and the polyhydroxy groups present. Axelrod and Lai[62] reasoned that a compound possessing the essential configuration of a specific glycone as well as the amino group might act as effective inhibitors of glycosidase action. Glycosylamines were found to meet these specifications. D-Glucosylamine was found to competitively inhibit both maltase and α-D-glucosidase from yeast. It was suggested by the authors that

glycosylamines exhibit a strong affinity for the glycosidases because they are bound not only at the site that determines the glycone specificity but also through their glycosidic amine at the acidic site present in the catalytic centre. The mechanism proposed was that based on the mechanism suggested by Phillips[55] for lysozyme in which a carboxylic acid group in the active centre invokes general acid catalysis to yield a glycosyl oxy-carbocation, as shown below:

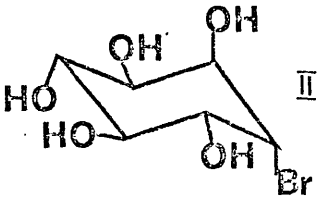


Legler[63,64,65] has applied active site directed inhibitors to identify functional groups at the active site and therefore deduce a mechanism. To label the active site he used epoxides that were structural analogues of substrates. Conduritol B epoxide (I) was used to inactivate yeast α-D-glucosidase. Previously Legler had

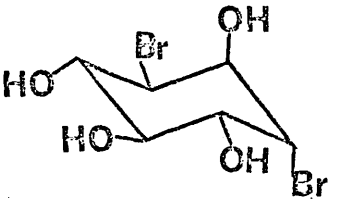


I

shown that β-D-glucosidase could be inactivated by 6-bromocyclohexanepentol (II) and 4,6-dibromocyclohexanetetrol (III) but the

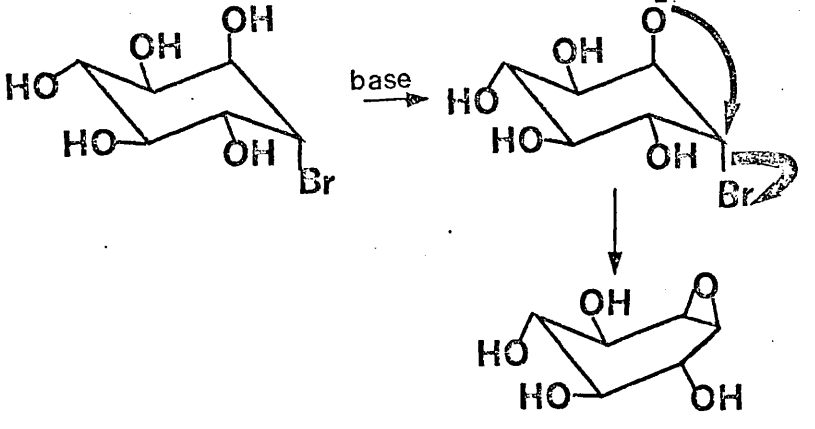


II



III

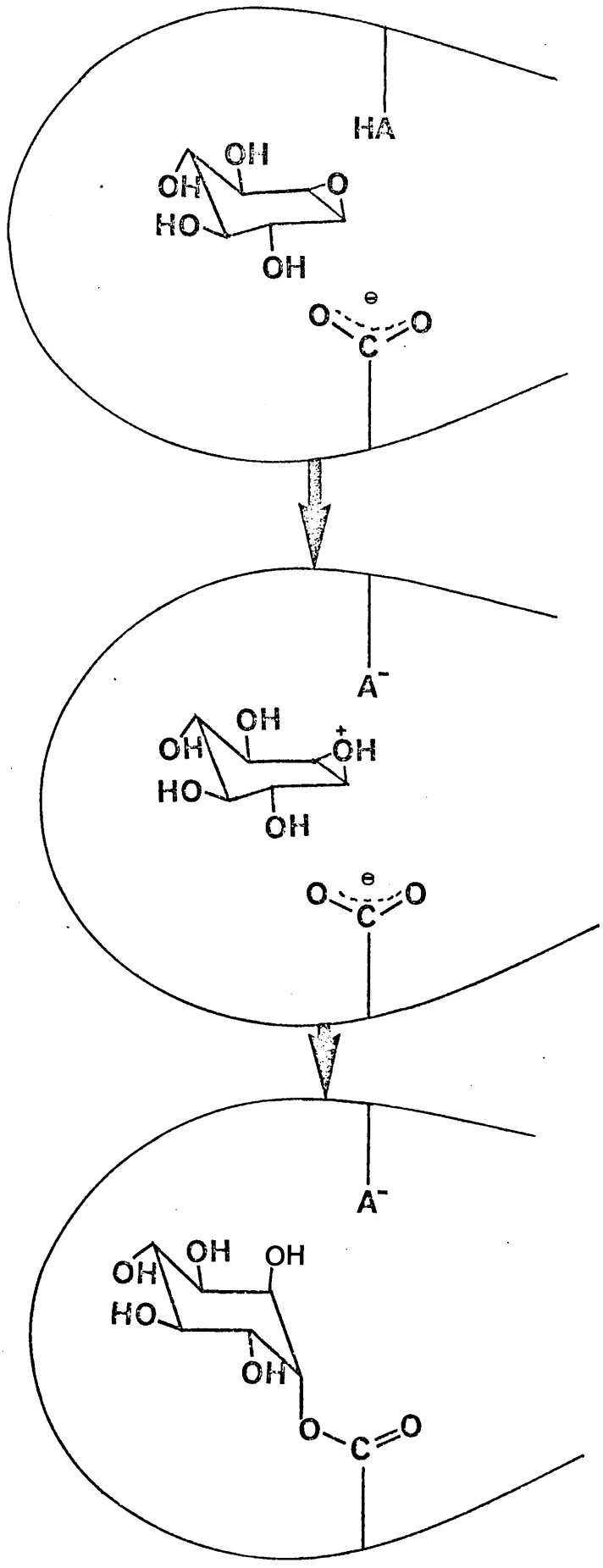
active inhibitor was conduritol B epoxide (I), presumably formed as shown below:

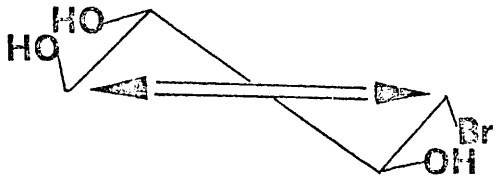


It was suggested that the acid at the active site protonates the epoxide oxygen, and forms a species that will alkylate a nucleophile in a suitable position at the active site. This mechanism is shown in Fig. 3. A much higher rate of deactivation was shown by 6-bromo-3,4,5-trihydroxy cyclohexene (IV) than for conduritol B epoxide.

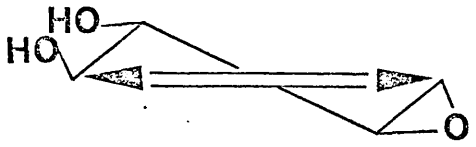
Fig. 3

Proposed mechanism for the action of β-glucosidase on conduritol B epoxide



IV 

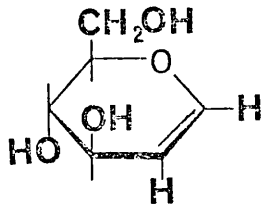
The pH dependence of the inactivation rate was bell-shaped with a maximum at pH 6.1, indicating that an acid and a base were involved in the reaction. Legler suggested that if the basic group was alkylated it would be difficult to assign a role to the acid group. However, the acid could play a catalytic role if the bromo conduritol was converted to the epoxide by a basic group on the enzyme and then acts as an alkylating agent after protonation by the acid. To test this hypothesis the epoxide (V) was used to see if it was an effective inhibitor as the bromoconduritol. The

V 

epoxide was found to be nearly as effective as the bromoconduritol but the pH dependence of the inactivation rate was sigmoidal, indicating the participation of an acid of $pK_a = 6.8$. Amino acid analysis and the determination of histidine showed that bromo-conduritol alkylates a histidine residue. One sulphydryl group was also found located at the active site but it was improbable that it took a part in the catalytic process. It was concluded that an acid and a base participated in the mechanism operating at the active site. The former was thought to be a carboxylate ion and the latter a imidazolium or N-terminal ammonium ion.

A study of the action of α and β-D-glucosidase on 1,2-dideoxy-D-arabino hex-1-enopyranose (D-glucal, VI) has been carried out by Hehre and co-workers.[66] It was the first

VI



demonstration of the capacity of glycosidases to create anomeric configuration de novo, in that α- and β-D-glucosidase were shown to exert their different specificities on a common substrate that lacks α- and β-anomeric configuration. NMR spectroscopy was used to study the reaction in which the labile hydrogen atoms of the enzyme had been exchanged for deuterium. Incubation was carried out with D_2O and deuterium exchanged D-glucal. The action of yeast α-D-glucosidase on D-glucal resulted in the product being 2-deoxy-α-D-[2(a)-^{2}H] glucose (VII) with characteristic resonances for the proton at H_1 and the proton at $H_{2(e)}$ and the absence of the proton signal at the $H_{2(a)}$ position, associated with the β-glucosidase incubation.

VII

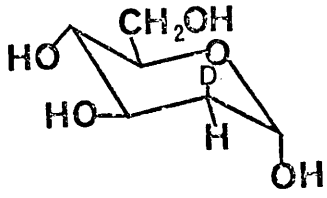
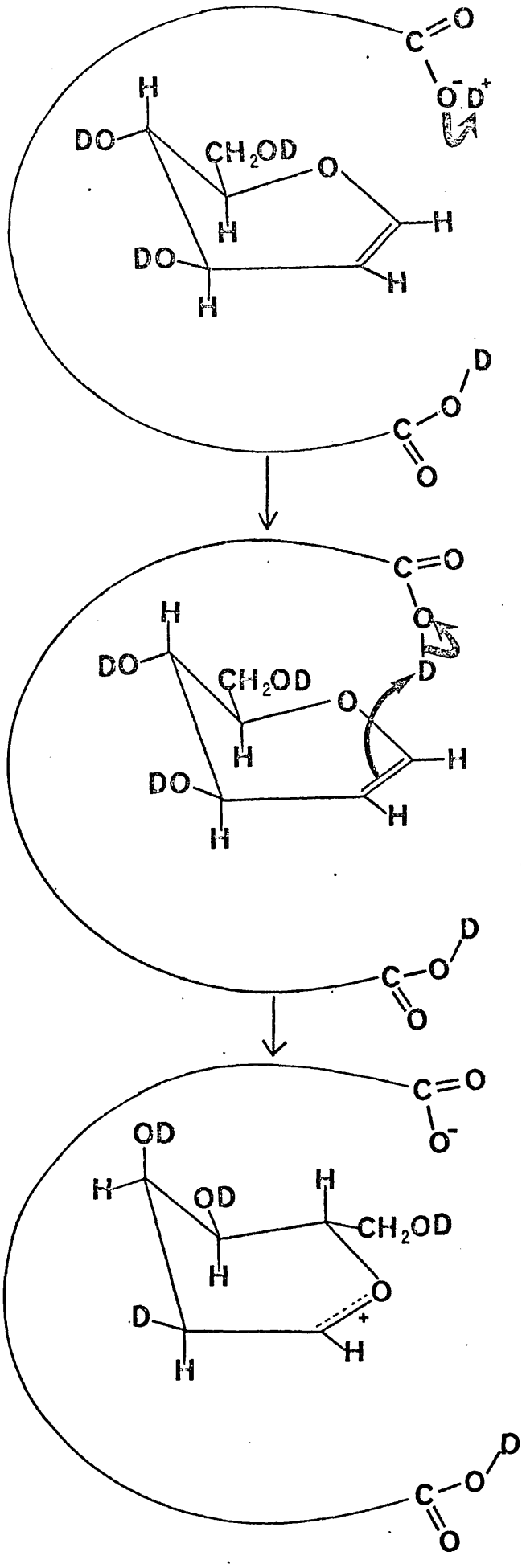


Fig. 4

Proposed mechanism for the hydration of D-glucal by yeast α-D-glucosidase



The incorporation of deuterium in the axial position at C-2 in the product indicated that the deuteration of the double bond occurred from above this plane as shown in Fig. 4. To account for the mechanism the authors based their results on those of Phillips[55] who showed the presence of two carboxyl groups at the active site of lysozyme. Hehre and co-workers suggested that one carboxyl group acts as a general acid, protonating the double bond, whilst the second carboxyl group acts as a nucleophilic base. Whether D-glucal or an aryl or alkyl glucoside is taken as a substrate a glycosyl oxy-carbocation is formed. The evidence being taken from its observed action on D-glucal and the limited information available of the type and arrangement of the catalytic groups at the active centre.

Active site inhibitor studies have shown more clearly the type of functional group occurring at active sites, but only a limited number have been used. Until sequence studies are undertaken to isolate labelled residues and characterise them no reliable information concerning the catalytic groups at the active site will be available.

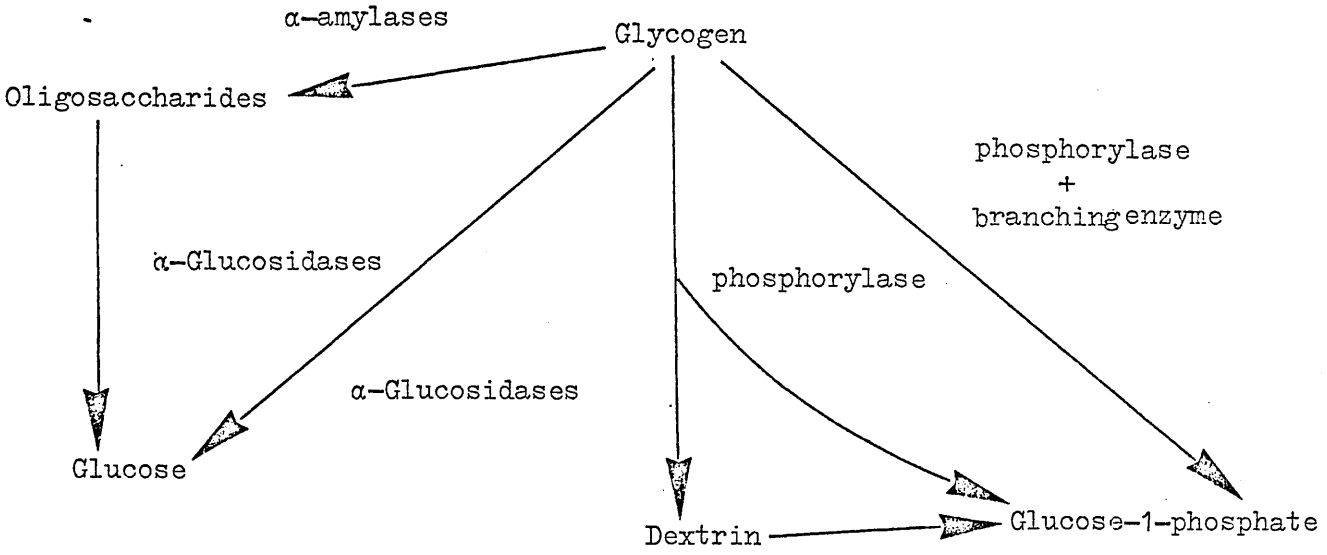


Fig. 5 In vivo breakdown of glycogen

1.E. The role of Acid α-D-Glucosidase in Mammalian metabolism and its Significance in Type II Glycogenesis

It is now known that glycogen breakdown in animal tissues is able to occur not only by phosphorolysis, or α-amylolysis, producing glucose-1-phosphate or oligosaccharides, but also by the enzymic hydrolysis of the terminal glycosidic linkage of the polysaccharide, as illustrated in Fig. 5. Rozenfeld[67] proposed this new pathway and named it γ-amylolysis from the enzyme responsible, γ-amylase, or acid α-D-glucosidase. The role of the enzyme in the metabolism of glycogen within animal tissues still remains obscure. However, its importance cannot be denied.

Hormones play an important role in the regulation of the action of enzymes participating in the synthesis and degradation of glycogen. The effects of adrenalin, insulin, and glucocorticoids upon phosphorylase and glycogen synthetase have been reviewed fully.[68,69,70] But it is of interest to review the effects of hormones in the activity of α-D-glucosidase because of the relative obscurity of this enzyme. Hormonal studies have tended to yield inconclusive results, because of the lack of study or all of the enzymes associated with glycogen degradation. Workers tended to carry on research on one or perhaps two enzymes rather than examining them all.

Rozenfeld[71] put forward the proposal that an increase in the activity of this enzyme might lead to an increased blood sugar level arising from the degradation of glycogen. However, the activity of acid α-D-glucosidase in rabbits with alloxan diabetes varied in the same range as for control animals.[72] The same was found for alloxan mice.[73]

A different picture was seen when hyperglycemia was induced by injections of adrenaline. Under the influence of adrenaline, the activity of acid α-D-glucosidase was increased in the liver and skeletal muscle of rabbits.[72] The same was found for induced hyperglycemia in rat.[74] The increase in the activity was so fast that even after one minute increased activity was detected, indicating that the hormone activates the enzyme.

Other hormones have been used to try and stimulate the enzyme but with little success. Insulin had no effect on the acid and neutral α-D-glucosidase from rabbit liver,[72] and glucagon had no effect on the acid and neutral enzymes from rat liver or on heart muscle, and skeletal muscle enzymes.[75] Glucagon has been found not to stimulate amylo α-(1→6) glucosidase, and that limit dextrins could not be isolated suggesting no change in the structure of glycogen. However, limit dextrins have been isolated from rat liver[74] and dog liver[76] after stimulation with adrenaline, indicating that both phosphorylase and acid glucosidase are involved in maintaining the structure of liver glycogen.

Assay for the enzymes participating in carbohydrate metabolism from a person with a condition similar to hypothyroidism, showed that all of the enzymes in the muscle were of normal activity, except acid α-D-glucosidase.[77] After prolonged treatment with thyroxine for three months the muscle acid α-D-glucosidase activity was normal 6 months after treatment. Koster[78] found that thyroxine treated rats possessed a more active liver and leucocyte acid maltase, than control rats. These results suggest some correlation between thyroid function and the acid α-D-glucosidase activity of some tissues.

Bourne et al.[79] showed that rats injected with cortisone showed an increased activity of acid α-D-glucosidase in the liver. A rise in the enzyme activity was also seen in liver slices, isolated lysosomes, and skin fibroblast cultures. A similar effect has been shown in rabbit liver.[80]

It is known that the enzymic degradation of glycogen arises mainly through the activity of phosphorylase and the debranching system associated with it. Phosphorylase activity can be controlled by hormones and the similar effect of adrenalin on acid α-D-glucosidase goes some way in explaining that the enzyme must exercise some control on the metabolism of glycogen.

The importance of acid α-D-glucosidase within the liver is demonstrated by its absence in people suffering from Type II Glycogen Storage Disease (Pompe's disease). Hers[81,82] showed that in humans suffering from Pompe's disease there was a lack of acid-maltase activity in the liver, heart, and skeletal muscle. Children born with this disease show a characteristic feature of having increased glycogen deposits within the tissues, the effects of which usually cause death within the first year.

A detailed account of the pathological effects of this disease and associated symptoms are well reviewed elsewhere.[69,83,84] The purification and properties of some acid α-D-glucosidases related to the problem of this disease will only be reviewed.

It is now clear that the neutral glucosidase is present in the tissues of patients suffering from Pompe's disease. Hers reported the lack of this enzyme in human liver.[69] Brown[85] et al. however, although confirming the lack of the acid α-D-glucosidase in tissues of

patients suffering from this disease, found that neutral glucosidase was present in the microsomal fraction of tissues subjected to differential centrifugation. This enzyme was found to have no activity towards glycogen, but readily hydrolysed maltose, and maltotriose.

A highly purified acid α-D-glucosidase has been extracted both from rat liver and human placenta, and the properties of both have been studied.[33] One purpose for having pure enzymes is to raise antibodies by injecting rabbits with the enzyme and harvesting them. Thus, one way of investigating this disease is to inhibit the enzyme *in vivo*. With both the rat liver and the human placenta enzymes the conversion of glycogen to glucose and transglucosylation from maltose to glycogen could be 90 to 100% inhibited, whereas, the hydrolysis of small molecular weight substrates was not effected. Some cross reactivity between species was noted. The human enzyme antibodies were found to inhibit the rat or mouse glucosidase, but the reverse was not true. No reactive protein could be demonstrated from a patient with Pompe's disease in as much that no precipitation reaction occurred between antibodies for the human enzyme and a liver homogenate of a patient with the disease. The incomplete cross reactivity with specific antibodies, and the different kinetic properties of the two enzymes, lead to the proposal that important differences exist in enzyme structure between species.

Similar antibody studies have been carried out using human liver enzyme.[38] Again, glycogen degradation was inhibited by antibodies in heart, liver and skeletal muscle extracts, maltose was still degraded. Studies on the enzyme from kidney showed a more heat stable enzyme to be present. When the same experiment was done on a kidney extract from a person with Pompe's disease, using maltose as a substrate, it was

more heat stable than when it was measured with glycogen. It is known that patients with Pompe's disease have appreciable acid α-D-glucosidase activity within the kidney.[86,87] Antibody studies on a control kidney and a kidney from a patient with Pompe's disease gave different results.[38] The activity of the enzyme in the control kidney was inhibited, whilst in the patient kidney, no inhibition was observed. No explanation is as yet forthcoming as to why the kidney has acid α-D-glucosidase activity in patients with Pompe's disease but it would seem that some different properties do exist between the kidney, muscle, liver and heart acid α-D-glucosidases.

Most antibody studies have confirmed the original work of Hers,[81,82] but as yet no one has proposed the role of isoenzymes which exist in most tissues of acid α-D-glucosidase. Brown and coworkers[34] showed that several isoenzymes were present when isoelectric focussing of the purified human liver enzyme was carried out. The enzyme gave one band on disc gel electrophoresis. The enzyme was found to be a glycoprotein. These workers were also able to produce an induced form of glycogen storage disease in skin fibroblasts. Cultures of fibroblasts grown in the presence of trehalose, an inhibitor of acid α-D-glucosidase, showed an increase in their glycogen content. The turn over of [^{14}C]-glycogen within fibroblasts treated with trehalose and fibroblasts from a patient suffering from Pompe's disease was distinctly lower than for the control.

The studies so far reviewed confirm the proposal that the lack of acid α-D-glucosidase leads to a build up of glycogen within the tissues. Interest, however, seems to be growing in the direction of the properties of the different acid α-D-glucosidases within different tissues and their isoenzymes.

In 1973 Dreyfus and Alexandre[88] showed that acidic and neutral glucosidase activities could be separated by cellulose acetate electrophoresis, using 4-methylumbelliferyl α-D-glucopyranoside as a substrate. In normal extracts from human tissues two spots could be distinguished, depending on the pH of the incubation, with substrate. The neutral glucosidase not being active at pH 4.0 did not show up. However, when the kidney extract was incubated with the substrate after electrophoresis, an extra spot was developed, indicating the presence of an extra isoenzyme. Electrophoresis of a muscle extract from a patient with Pompe's disease showed a lack of the normal acid α-D-glucosidase, but another spot was distinguished with a low mobility at acid pH. Another extract of fibroblasts from a patient with a milder type of Pompe's disease showed a spot more mobile than the acid glucosidase at pH 4. This study therefore was the first to demonstrate two variants of α-D-glucosidase deficiency.

Some conflicting evidence however for distinguishing isoenzymes by cellulose acetate electrophoresis has come from some studies by Swallow and co-workers.[37] They were able to distinguish a number of isoenzymes by starch gel electrophoresis. Thus, acid α-D-glucosidase was shown to be polymorphic by using this technique. However, they could not be separated by agarose or cellulose acetate electrophoresis. Three different phenotypes were detected in a population census and designated as follows. The slow moving enzymically active band was α-glucosidase 1, a faster moving enzymically active band α-glucosidase 2, and a third type containing both bands α-glucosidase α-1, (Fig. 6). Neither of the two acid α-D-glucosidases were present in the fibroblasts of a patient with Pompe's disease, though, the neutral enzymes were present as normal.

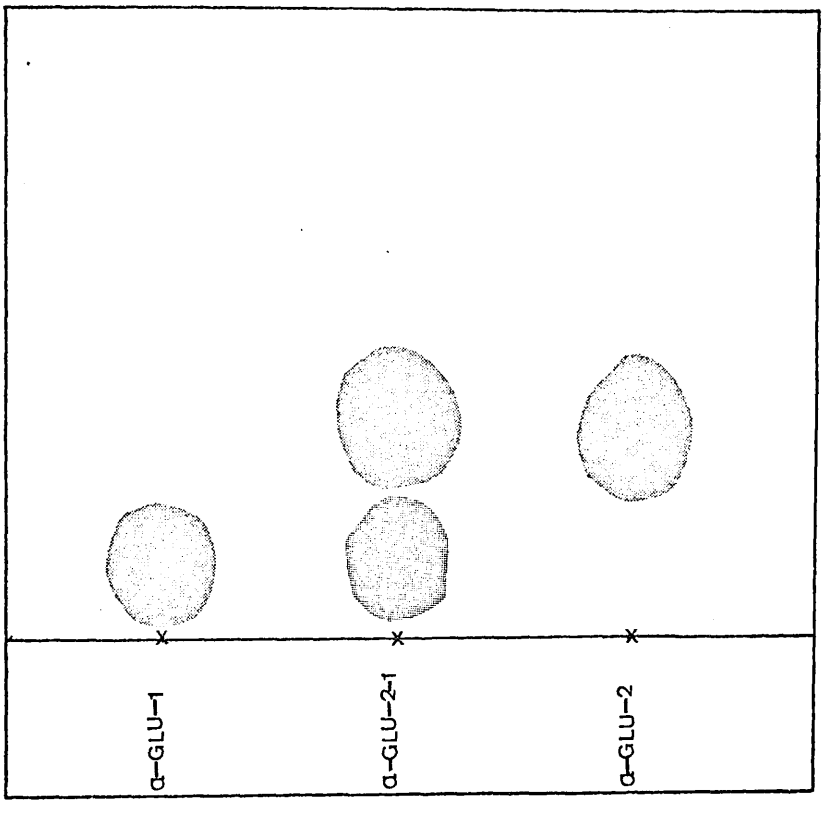


Fig.6

Starch electrophoretograms of placental extracts containing α-D-glucosidase

On Sephadex chromatography the type α-glucosidase 1 was found to be retarded by the dextran gel but the type α-glucosidase 2 was eluted within the volume of the column. This suggests diminished binding or a lower activity of the α-glucosidase 2 type for polysaccharides. The possibility of the type 2 being functionally defective and associated with a pathological state was dismissed. The high incidence of this genotype within the population, and the actual progress of the child who had the type 2 in not showing any condition associated with Pompe's disease, seemed sufficient evidence against this suggestion.

Other aspects of the disease not reviewed including treatment and the genetic implications are discussed in detail in several reviews.[89,90]

I.2. Results and Discussion

2.A. Isolation and Purification of the enzyme

2.A.a. General aspects of purification

As is the history of enzyme purification only recently have the techniques been available for the purification of this enzyme. One of the main obstacles which had to be overcome, before the enzyme could be purified, was the presence of similar acting enzymes including amylases, maltases, and neutral α-D-glucosidases. Two main techniques have been successfully applied to this problem, ion-exchange and gel filtration. Both, either used separately or together have become the main tools for purifying acid α-D-glucosidase.

The origin of acid α-D-glucosidase, in the lysosomes within the cell, was discovered by Hers et al.[12] using preparative high speed centrifugation. He showed that the enzyme was attached to the lysosomes in rat liver, and later managed to separate the neutral and acid maltase activities. Using the same technique Shibko et al.[14] isolated lysosomes from rat kidney, and found that this fraction contained only 10% of the total α-D-glucosidase activity. From the results of both these authors it became fairly clear that any extraction of this enzyme would contain some neutral glucosidase.

One of the first workers to use a chemical method to try and separate α-D-glucosidase and α-amylase was Rozenfeld.[91] She found that a suspension of potato starch in ethanol specifically adsorbed α-amylase under conditions excluding the adsorption of α-glucosidase; unfortunately the extent of purification was rather low. A modification of this method was developed by Iwanowski and Gandrya[92] for obtaining acid

α-D-glucosidase from rabbit muscle, involving the repeated freezing and thawing of the sample. The preparations obtained in this way were ten times more active than those isolated previously.

The first chromatographic technique used for separating neutral and acid α-D-glucosidases was ion exchange. Dahlqvist,[93] using DEAE-cellulose, managed to separate an extract of swine intestinal mucosa into three enzymically active peaks, one of which was thought to be an acid α-D-glucosidase. Separation of two maltase enzymes from dog liver was achieved by Torres and Olavarria.[16] Using a calcium phosphate gel, and a DEAE-cellulose column, two distinct glucosidase activities were separated. The first was enzymically active between pH 3 and 6 (acid α-D-glucosidase) and the second between pH 4 to 7.5 (neutral α-D-glucosidase), a yield of only 5% was obtained. Lukomskaya[94] separated two maltases from rabbit liver and swine kidney by using DEAE-cellulose. The acid maltase showed maximum activity at pH 4.8, whilst the neutral maltase showed maximum activity at pH 7.0. One of the first acid α-D-glucosidases to be purified to a high degree was the enzyme from bovine spleen.[23] It was purified by ion exchange on DEAE-Sephadex A-50 followed by a phosphocellulose column, a 2400 fold purification was obtained and a crystalline product obtained. Good preliminary purification has been obtained after primary fractionation in organic solvents by Iwanowski.[29] Using isopropanol, ethanol, and acetone, final purification of the enzyme from horse skeletal muscle was achieved by using a DEAE-cellulose column. The same worker[30] later obtained a homogeneous enzyme from calf heart muscle. Again, fractionation by use of organic solvents was carried out, followed by

purification on a phosphocellulose column; final purification was obtained by gel-permeation chromatography on Biogel P-150. Both extractions gave a yield of approximately 20%.

By far the most adaptable method for the purification of acid α-D-glucosidase was the method developed by Auricchio and co-workers.[95,96,17,18] The isolation and characterisation of the enzyme from various sources was based on the following phenomenon. Elution of proteins through columns packed with dextran gel (Sephadex) may not be a function of their molecular weights only, some retardation of the protein on the column may occur. Taking into account that acid α-D-glucosidase's attack dextrans as shown by Rozenfeld,[91] it was suggested that the enzyme could be retarded on dextran gels due to the α-(1→6) linkages acting as a substrate for the enzyme. Thus, it was shown by Auricchio *et al.* that acid α-D-glucosidase is eluted after the bulk of the contaminating proteins had been removed. Early papers by this group of workers described the purification of rat liver,[17] and human kidney,[18] acid α-D-glucosidases. A homogeneous preparation was obtained for the rat liver enzyme using Sephadex G-100. Later, purification of the cattle liver enzyme to a homogeneous state was described,[19] giving a 5000 fold purification after gel filtration chromatography on Sephadex G-100 and Biogel P-60. A yield of 43% was obtained.

Using the technique developed by Auricchio the enzyme has been purified to a homogeneous state from various sources, by different workers.

Palmer[26] extracted the enzyme from rabbit muscle and after only one purification step involving a chromatographic technique (separation on Sephadex G-100), obtained an enzyme giving a single band on disc-gel

electrophoresis. This method has been repeated by Rozenfeld and co-workers on the isolation of the enzyme from pig's spleen[31] and human liver.[97] Both preparations gave one band on disc gel electrophoresis. A 10,000 fold purification has been described for the rat and mice liver, and the human placenta enzyme,[33] after a one step purification on Sephadex G-100. Although no mention is made about the homogeneity of the preparation such a large increase in purification indicates the preparation of a highly purified enzyme.

Several workers have included ion-exchange with Sephadex chromatography, but purity has not been established. Jeffrey et al.[24] included DEAE-cellulose ion-exchange after purification on Sephadex G-100. Although a highly purified enzyme from rat liver was prepared it was not electrophoretically homogeneous. The same method was used by Seetharam and co-workers[28] to purify the monkey liver and spleen enzyme, with a 1200 and 2000 fold purification, respectively. Adlung[22] separated amylase from α-D-glucosidase by DEAE-cellulose ion-exchange, and the further purification of the human pancreas enzyme was carried out on Sephadex G-100. Koster et al.[38] has also repeated this method in purifying the human enzyme from various organs.

A successful modification of the Auricchio method has been developed by Belenki and Rozenfeld.[98] The disadvantage of the previous method is the requirement of very large elution volumes. By using competitive inhibitors (methyl α-D-glucopyranoside, or α-trehalose) rapid elution of the enzyme could be achieved in a small elution volume. This method has resulted in the purification of a homogeneous enzyme for the pig spleen,[31] and human liver[97] and α-D-glucosidase.

It would seem rather obvious why affinity chromatographic techniques have not been used extensively for the purification of the enzyme. Separation of neutral maltase from acid maltase would remain a problem if the technique had to rely on substrate-enzyme interaction, for separation. Not only acid α-D-glucosidase but other acid hydrolases have been found to be glycoproteins.[99] Beutler et al.[100] using a con-A sepharose column to purify an extract of crude human placenta, but when elution was performed several acid hydrolases were eluted together. It was therefore suggested that affinity techniques should be used as a primary purification step and individual hydrolases should be separated later on.

Table II gives a list of mammalian acid α-D-glucosidases which have been extracted and purified by varying techniques.

In order to study the properties of swine liver acid α-D-glucosidase it was necessary to purify the enzyme to homogeneity. The procedure adopted was that of Auricchio and co-workers[19] employing the use of gel permeation chromatography. Later, slight modifications were used to increase the efficiency of the technique.

Table 11 Acid α-D-glucosidases isolated from Mammalian sources

Author and reference	Source	Method of purification	Criteria of purity	Purification (fold)	Yield (%)
Auricchio[19]	Cattle liver	Sephadex G-100 Biogel P-60	Single band disc gel electrophoresis and on ultracentrifugation	5,000	43
Fujimori[23]	Cattle spleen	DEAE-Sephadex A-50 Phosphocellulose	(Crystallised)	2,400	8
Palmer[26]	Rabbit muscle	Sephadex G-100	Single band disc gel electrophoresis	3,500	22
Seetharam[28]	Monkey liver Spleen	DEAE-Sephadex Sephadex G-100		1,200 2,000	
T. de Barsy[33]	a Rat liver b Mice liver c Human placenta	Sephadex G-100		10,000	a 20 b 30
Rozenfeld[31]	Pig's spleen	Sephadex G-100	Single band disc gel electrophoresis	2,000	43
Auricchio[17]	Rat liver	Sephadex G-100		700	
Torres[16]	Dog liver	Calcium phosphate DEAE-cellulose		35	3
Sica[18]	Rat liver Human kidney	Sephadex G-100	Single band on diso gel electrophoresis	6,000 400	7 19

Table 11 (continued)

Author and reference	Source	Method of purification	Criteria of purity	Purification (fold)	Yield (%)
Dahlqvist[101]	Human jejunum	Sephadex G-200 TEAE-cellulose DEAE-cellulose			
Adlung[22]	Human pancreas	DEAE-cellulose Sephadex G-100			
Jeffrey[24]	Rat liver	Sephadex G-100 DEAE-cellulose	No, two bands on disc gel electrophoresis	1,300	12
Rozenfeld[97]	Human liver	Sephadex G-100	Single band disc gel electrophoresis and on ultracentrifugation		
Iwanowski[29]	Horse skeletal muscle	Solvent fractionation DEAE-cellulose	Single band disc gel electrophoresis	1,800	22
Iwanowski[30]	Calf heart	Solvent fractionation Phosphocellulose Biogel P-150	Single band disc gel electrophoresis	1,800	18

2.A.b. Preliminary Purification of the enzyme

Preliminary purification of any enzyme is fundamental in the achievement of total purification of a protein. These basic steps individually constitute an important objective which if not carried out, significantly alter the ordered process of purification.

Homogenation was the first step involved in preliminary purification, which resulted in the soluble proteins being extracted into the supernatant, the procedure is described in Experiment 1.

Table 12 gives a comparison of the activity figures for three separate batches of the pig liver at this stage.

Table 12. Activity figures for the crude extract of acid α-D-glucosidase for batch 1, 2 and 3

	Crude Extract			
	Volume (cm^3)	Total enzyme° units	Total protein (mg)	Specific+ activity
Batch 1	7500	1.6×10^6	3.8×10^5	4.2
2	12300	2.157×10^6	4.059×10^5	5.3
3	7300	2.24×10^6	1.42×10^5	15.7

+ specific activity is the number of nmoles maltose hydrolysed per minute per mg protein.

° One unit of enzyme activity is the hydrolysis of 1 nmole of maltose per minute.

To gain an accurate measurement of the glucosidase activity present in the extract, the high activity of the enzyme blank had to be taken into account. This was due to the presence of glycogen in the supernatant.

In order to facilitate the removal of glycogen, autolysis, i.e. self digestion was allowed to proceed overnight at 37°C, as described in Experiment 2. The enzymes responsible for glycogen metabolism therefore converted the stored glycogen to D-glucose. As expected the increase in specific activity after this procedure was very small as shown in Table 13.

Table 13 Purification figures after autolysis for α-D-glucosidase for batch 1, 2 and 3.

	Autolysis				
	Volume (cm^3)	Total° activity units	Total protein (mg)	Specific+ activity	Yield %
Batch 1	3420	1.5×10^6	1.4×10^5	10.7	94
2	12020	1.933×10^6	3.245×10^5	5.95	89.6
3	7000	1.79×10^6	1.05×10^5	17.0	79.9

+ Specific activity is the number nmoles maltose hydrolysed per minute per mg protein.

° One unit of enzyme activity is the hydrolysis of 1nmole of maltose per minute.

The variable yields obtained seem likely to evolve from the stabilities of other acting glucosidases under heat treatment, this is demonstrated later by the similar yields obtained on final purification.

Fractionation with ammonium sulphate was the final preliminary purification step carried out. The solution was made 30% w.r.t. ammonium sulphate and the α-D-glucosidase was found to precipitate between 30-60% saturation with salt. Experimental details are given in Experiment 3.

Table 14 Purification figures for α-D-glucosidase after ammonium sulphate precipitation for batch 1,2 or 3

	Ammonium sulphate precipitation					
	Volume (cm^3)	Total° activity units	Total protein (mg)	Specific+ activity	Yield %	Increase in purity
Batch 1	520	8.7×10^5	4.0×10^4	22	54	x5.
2	2180	1.43×10^6	1.13×10^5	13	66	x2.4
3	1570	1.460×10^6	5.02×10^4	29.3	65.3	x2.

+ Specific activity is the number of nmoles of maltose hydrolysed per minute per mg protein.

° One unit of enzyme activity is the hydrolysis of 1nmole of maltose per minute.

A 5-fold purification for the first batch was obtained at the cost of only a 54% yield. This suggests that not all of the glucosidase has been precipitated. For batch 2 and 3 a yield of 65% was obtained. However, there was some difference in the specific activity of the preparations.

Summarising, after the three preliminary purification steps a yield of approximately 65% and a 2-fold purification would be expected.

2.A.c. Separation of high molecular weight impurities by gel filtration

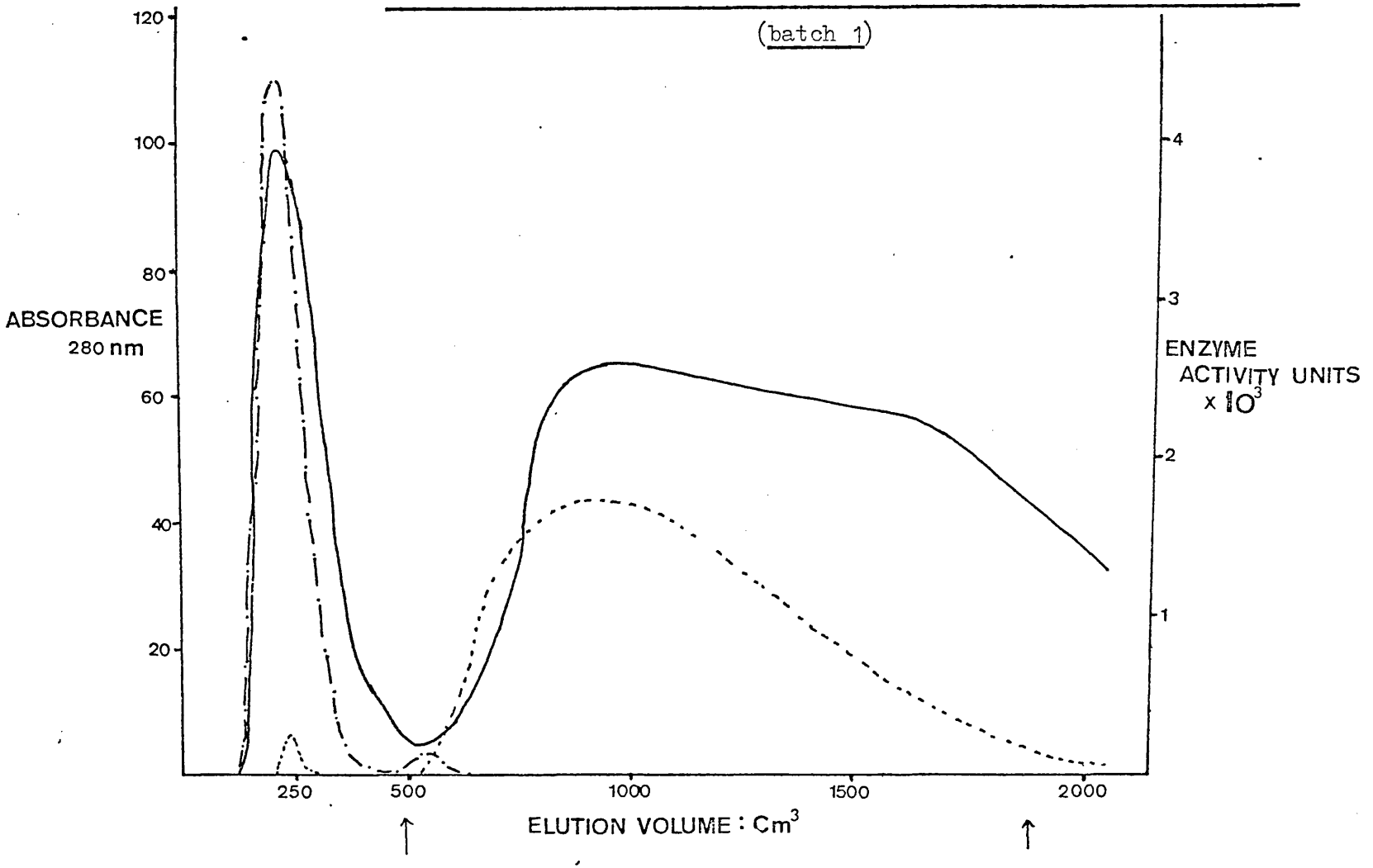
Sephadex G-100 chromatography

In order to remove proteins of a higher molecular weight, than the acid α-D-glucosidase, the material from batch 1, subjected previously to preliminary purification, was loaded onto a column containing Sephadex G-100 as described in Experiment 4. Fig. 7 shows an elution diagram of the maltase and dextranase activity and of absorbance at 280 nm of the fractions eluted from a Sephadex G-100 column (490 cm^3 total volume). The column was loaded with 40% (100 cm^3) of the material from the first batch as not to overload the column; the remaining material was chromatographed later. The bulk of the protein was eluted in a volume corresponding to the free volume of the column.

Two large maltase peaks were observed, one eluted with the bulk of the protein, the other was eluted over a wide volume range. According to Bruni et al.[19] the first peak corresponds to a neutral maltase, where the second peak is an acid maltase; known as acid α-D-glucosidase. Two peaks were obtained for the dextranase activity of the eluted fractions. The first, which was small, was eluted with the neutral maltase, while the second was a broad peak, showing retardation on the dextran gel, which coincided with the second maltase peak. The fractions containing the bulk of the maltase and dextranase activity were pooled as indicated in Fig. 7. After concentrating the material by ultrafiltration a 20-fold purification with a yield of 12% was obtained. The high activity of the neutral α-D-glucosidase goes some way to explain the low yield of the acid α-D-glucosidase.

Fig. 7

Elution profile of pig liver acid α-D-glucosidase on Sephadex G-100



A) Eluant: 25 mM NaCl containing 1 mM EDTA; pH brought to 6.7 with 2 M NaOH

B) One unit of enzyme activity will liberate 1 nmole of glucose under the condtions described in the Experimental (IV.A. 11).

C) ——— Maltase activity.

D) .—.—. Absorbance (280 nm).

E) - - - - - - - - Dextranase activity.

Further purification was carried out by several workers because of the presence of low molecular weight contaminating proteins. The elution profile (Fig. 7) shows that when the fractions were pooled, some inactive low molecular protein was pooled together with the acid α-D-glucosidase. It was therefore thought desirable to remove this protein using a lower molecular weight fractionating gel filtration media.

2.A.d. Separation of low molecular weight impurities by gel filtration

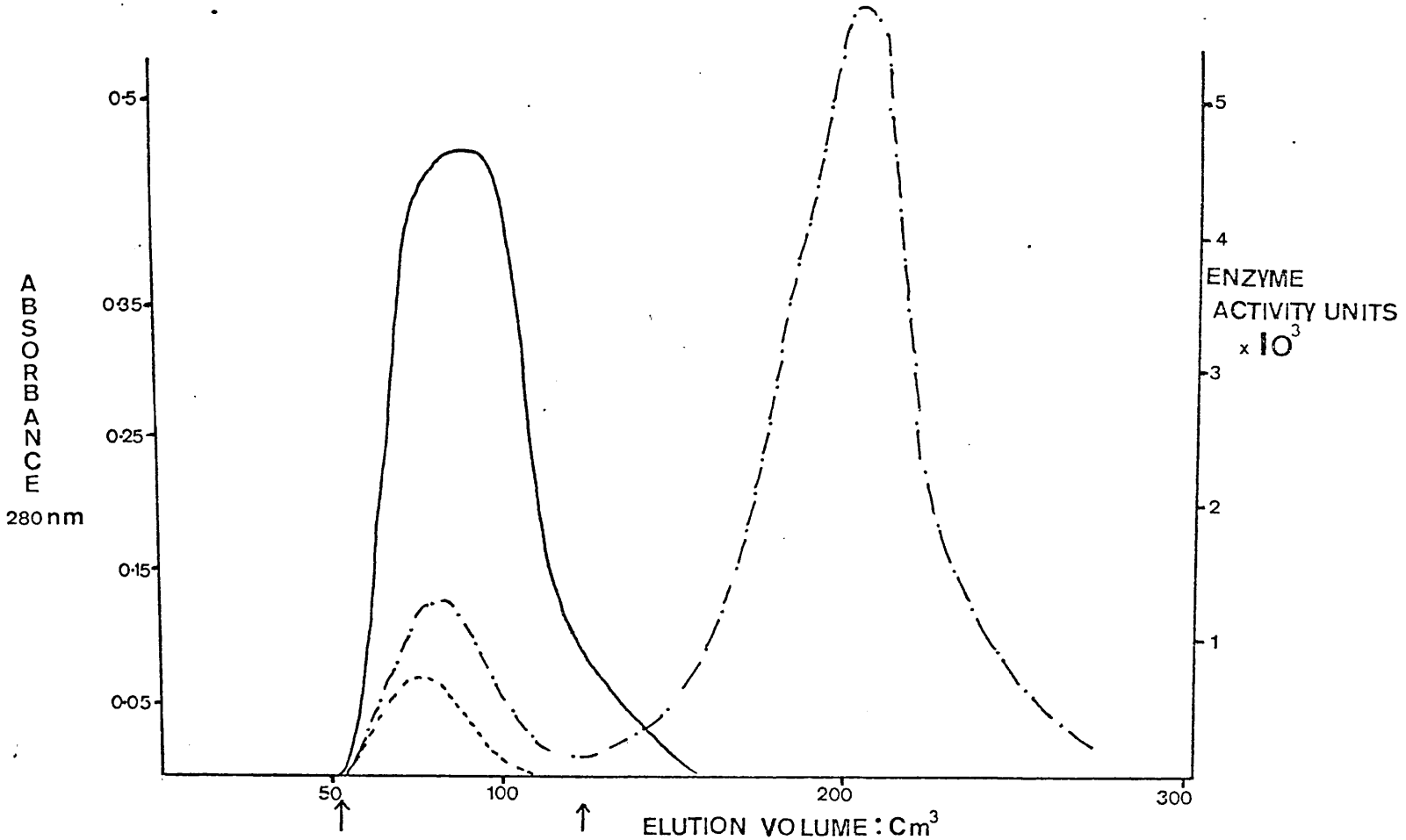
Biogel P-60 chromatography

The concentrated enzyme preparation from the previous step was loaded onto a Biogel P-60 column (Experiment 5), and an elution profile obtained as shown in Fig. 8. The bulk of the protein (88%) was eluted in a volume corresponding to the column volume. Both the maltase and dextranase activities were eluted together, in the free volume of the column. Glucose was detected in the fractions corresponding to the column volume before incubation with any substrate. This liberated glucose could have only have arisen from the preceding step, and thus confirms that the glucosidase enzymically attacks the Sephadex gel, releasing glucose as the product.

To remove any high molecular weight impurities remaining, the enzyme was again placed on a Sephadex G-100 column.

Fig. 8.

Elution profile of pig liver acid α-D-glucosidase on Biogel P-60 (batch 1)



A) Eluant: 25 mM NaCl containing 1 mM EDTA; pH brought to 6.7 with 2 M NaOH.

B) One unit of enzyme activity will liberate 1 nmole of glucose under the conditions described in the Experimental (IV.A. 1 1).

C) Fractions between arrows were pooled.

D) ——— Maltase activity.

E) ·—·—· Absorbance (280 nm).

F) ·-------- Dextranase activity.

2.A.e. Final Purification of the enzyme to homogeneity by gel filtration

Sephadex G-100 Chromatography

Final purification was carried out on Sephadex G-100 so that once again we could take advantage of the retardation of the enzyme due to its affinity for the dextran gel. The procedure is described in Experiment 6. The material from the previous step was applied to a Sephadex G-100 column, and an elution profile obtained as shown in Fig. 9. Nearly all of the maltase activity was eluted over a large volume range starting at a volume greater than the volume of the column. Separation therefore was achieved, the enzyme thus being eluted away from the contaminating proteins. A 2-fold purification was achieved giving a final yield of 10.5% as shown in Table 15. Overall a 1500-fold purification resulted from the full purification procedure giving a specific activity of 6444 units per mg of protein.

Table 15 Purification Table for α-D-glucosidase from Batch 1

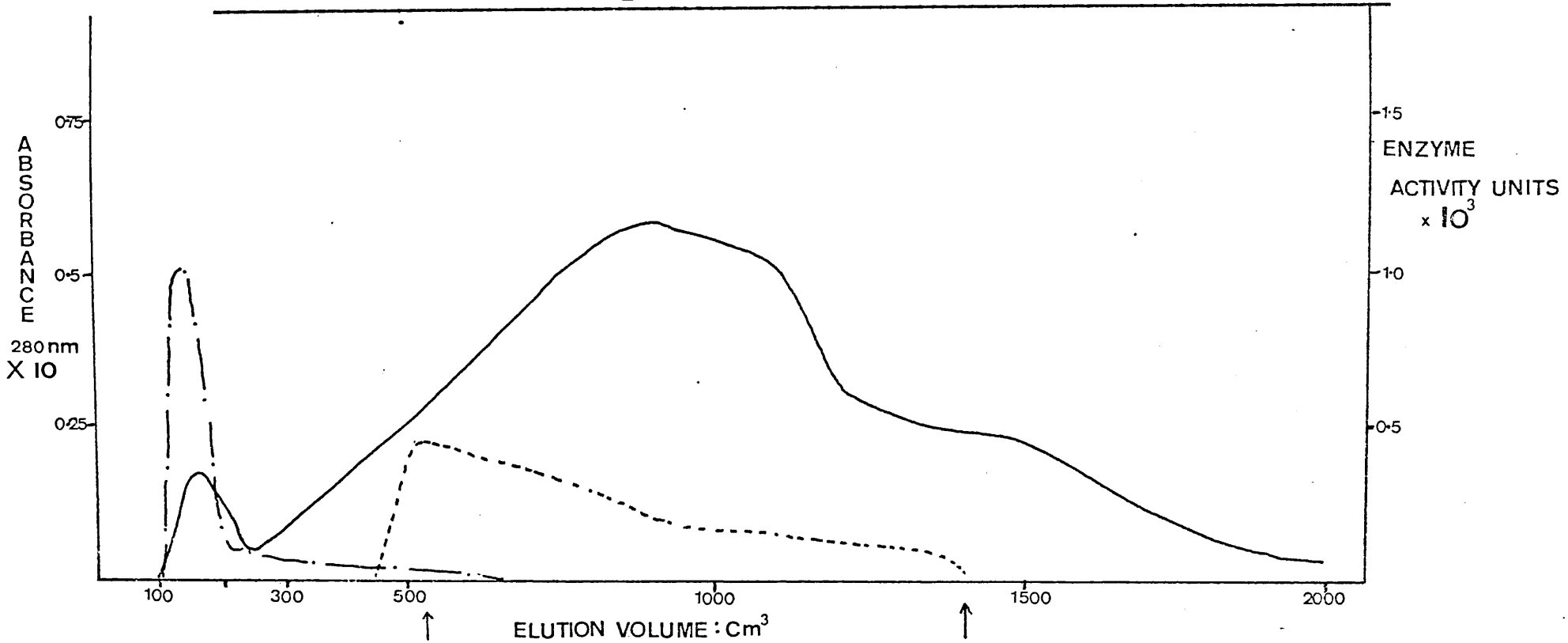
Step	Volume (cm^3)	Total⊕ activity units	Total protein (mg)	Specific+ activity	Yield %
Crude extract	7500	1.6×10^6	3.8×10^5	4.2	100
Autolysis	3420	1.5×10^6	1.4×10^5	10.7	94
Ammonium sulphate fractionation	520	8.7×10^5	4.0×10^4	22	54
Sephadex G-100 chromatography	35	7.68×10^4	18.24×10^1	421	12
Biogel P-60 chromatography	17	7.2×10^4	20.4	3529	11
Sephadex G-100 chromatography	12.5	6.96×10^4	10.8	6444	10.5

+ Specific activity is the number of nmoles maltose hydrolysed per minute per mg protein.

⊕ One unit of enzyme activity is the hydrolysis of 1nmole of maltose per minute.

Fig. 9

Elution profile of pig liver α-D-glucosidase on the second Sephadex G-100 column (batch 1).



A) Eluant: 25 mM NaCl containing 1 mM EDTA; pH brought to 6.7 with 2 M NaOH.

B) One unit of enzyme activity will liberate 1nmole of glucose under the conditions described in the Experimental (IV.A. 11).

C) Fractions between arrows were pooled.

D) --------- Dextranase activity.

E) ———— Maltase activity.

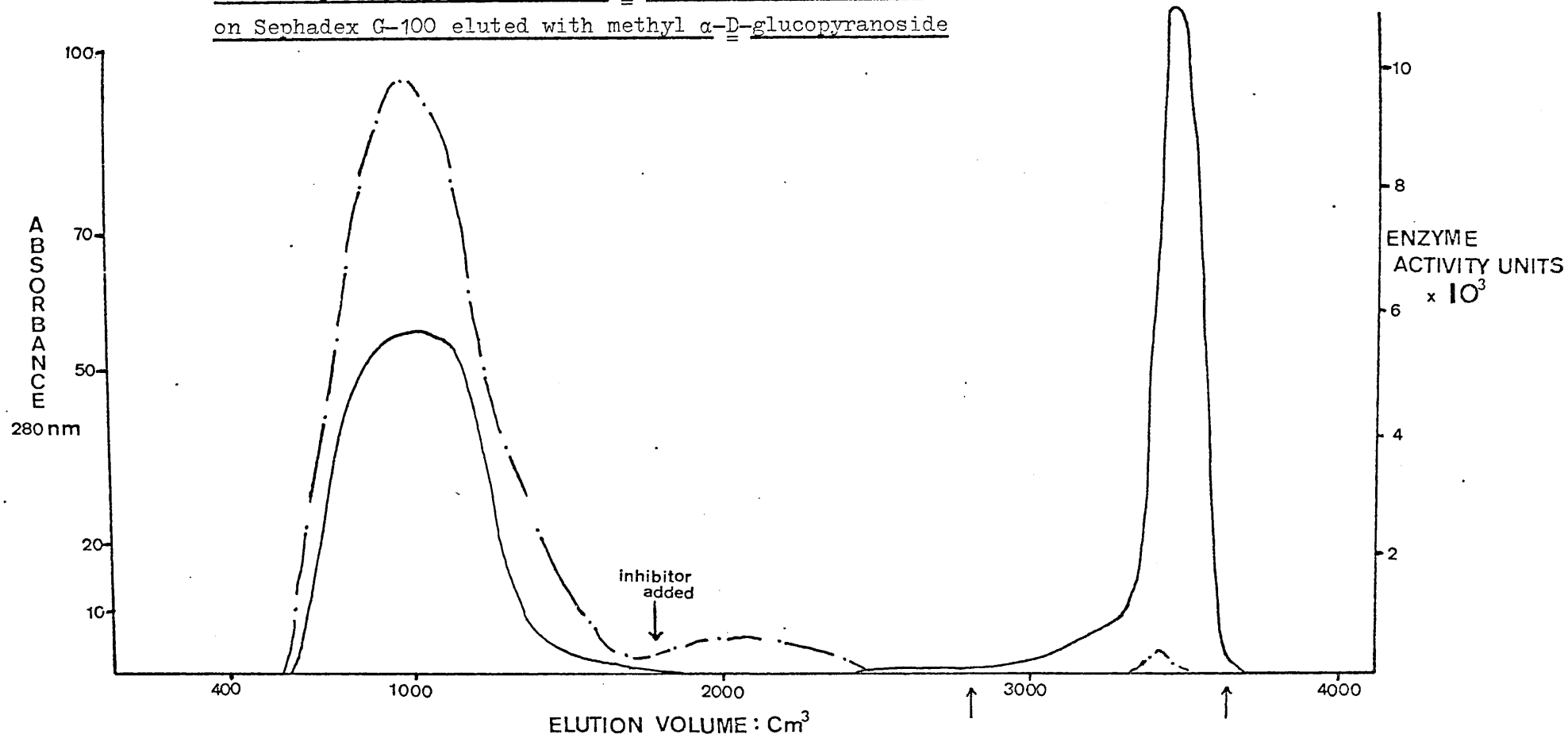
F) ·——·—· Absorbance (280 nm).

2.A.f. An attempt to purify the enzyme to homogeneity by the use of one gel filtration column eluted with a competitive inhibitor of acid α-D-glucosidase

A modification of the Bruni method for purifying α-D-glucosidase has been successfully applied by Belenki and Rozenfeld.[98] By using a competitive inhibitor of the enzyme either methyl α-D-glucopyranose or α-trehalose certain problems associated with the elution of the enzyme on Sephadex have been overcome. Application of the inhibitor at the moment the enzyme was bound to the dextran gel lead to a dissociation of the enzyme-substrate complex, and thus, the rapid elution of the enzyme in a small elution volume. The advantage of this method is two fold. As all other contaminating proteins are eluted within a column volume the enzyme is eluted in such a volume as to be completely separated from these proteins. Secondly, the elution volume is decreased so that a more concentrated enzyme is obtained, and the enzyme is detected by its absorbance at 280 nm. Using this method Rozenfeld has been able to purify both the pig's spleen[31] and human liver[97] acid α-D-glucosidases, after only one purification step involving gel filtration on Sephadex G-100. Fig. 10 shows an elution profile of the enzyme on Sephadex G-100 eluted with methyl α-D-glucopyranoside. The sample (450 cm^3) from the second batch after preliminary purification (Table 14), was loaded on the column (volume 1640 cm^3), and eluted with buffer, as described in Experiment 7. After approximately one column volume had passed the methyl α-D-glucopyranoside in buffer (1% w/v) was used to elute the enzyme. Two peaks showing maltase activity were apparent, the greatest part of the activity being in the first protein peak eluted close to the free volume of the column, containing most of the protein. The second peak,

Fig. 10

Elution profile of pig liver α-D-glucosidase (batch 2) on Sephadex G-100 eluted with methyl α-D-glucopyranoside



A) Eluant: 25 mM NaCl containing 1 mM EDTA; pH brought to 6.7 with 2 M NaOH, until an elution volume of ca. 1800 cm^3

B) After an elution volume of 1800 cm^3 eluant: methyl α-D-glucopyranoside (1% w/v) in 25 mM NaCl containing 1 mM EDTA pH brought to 6.7 with 2 M NaOH.

C) Fractions between arrows were pooled.

D) ——— Maltase activity.

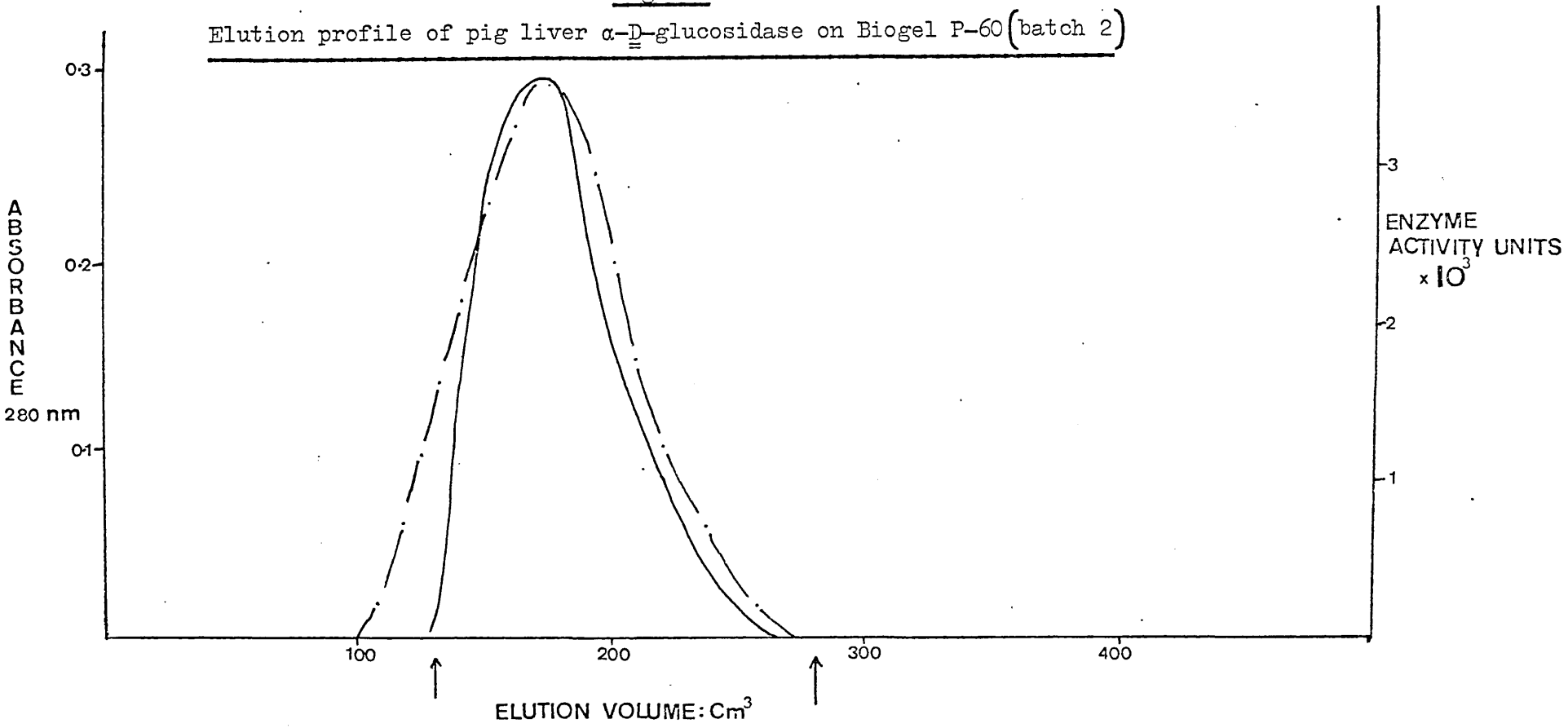
E) ·—·—· Absorbance (280 nm).

F) One unit of enzyme activity will liberate 1nmole of glucose under the conditions described in the Experimental (IV.A. 11).

corresponding to acid α-D-glucosidase, was eluted in a small sample volume (400 cm^3). A 300-fold purification was achieved (Table 16) for this one step purification procedure. In comparison with the purification of the enzyme from batch 1, when only buffer was used to elute the Sephadex column, only a 19-fold purification was achieved. The lower specific activity (3850 units/mg protein) compared with the final activity of the preparation from batch 1 (6444 units/mg protein) suggested that perhaps the enzyme was not homogeneous. In order to clarify this situation, disc gel electrophoresis was performed on the concentrated, diafiltrated, acid α-D-glucosidase. Electrophoresis was carried out as described in Experiment 8, at pH 4.3 and 8.5. After staining the gels, (pH 8.5) four bands were visible, one major and three minor bands, (pH 4.3), three bands were visible one major and two minor bands, indicating that the preparation was not homogeneous. The presence of more than one protein indicated that there could be more than one active α-D-glucosidase present in the extract. It has already been shown that some dextranase activity is associated with the maltase eluted in the free volume of the column. Therefore, the possibility of some residual neutral glucosidase being retarded upon the dextran gel is not easily dismissed. Although not thought very applicable, it would seem unrealistic not to suggest that the separation of the low molecular weight impurities, from the glucosidase, may not have been achieved completely. Rozenfeld, who used the inhibitor to purify the enzyme from pig spleen[31] did not find any dextranase activity associated with the bulk of the protein, eluted in the free volume of the column. This therefore indicates the possibility of the presence of more than one active enzyme as previously considered.

Fig. 11

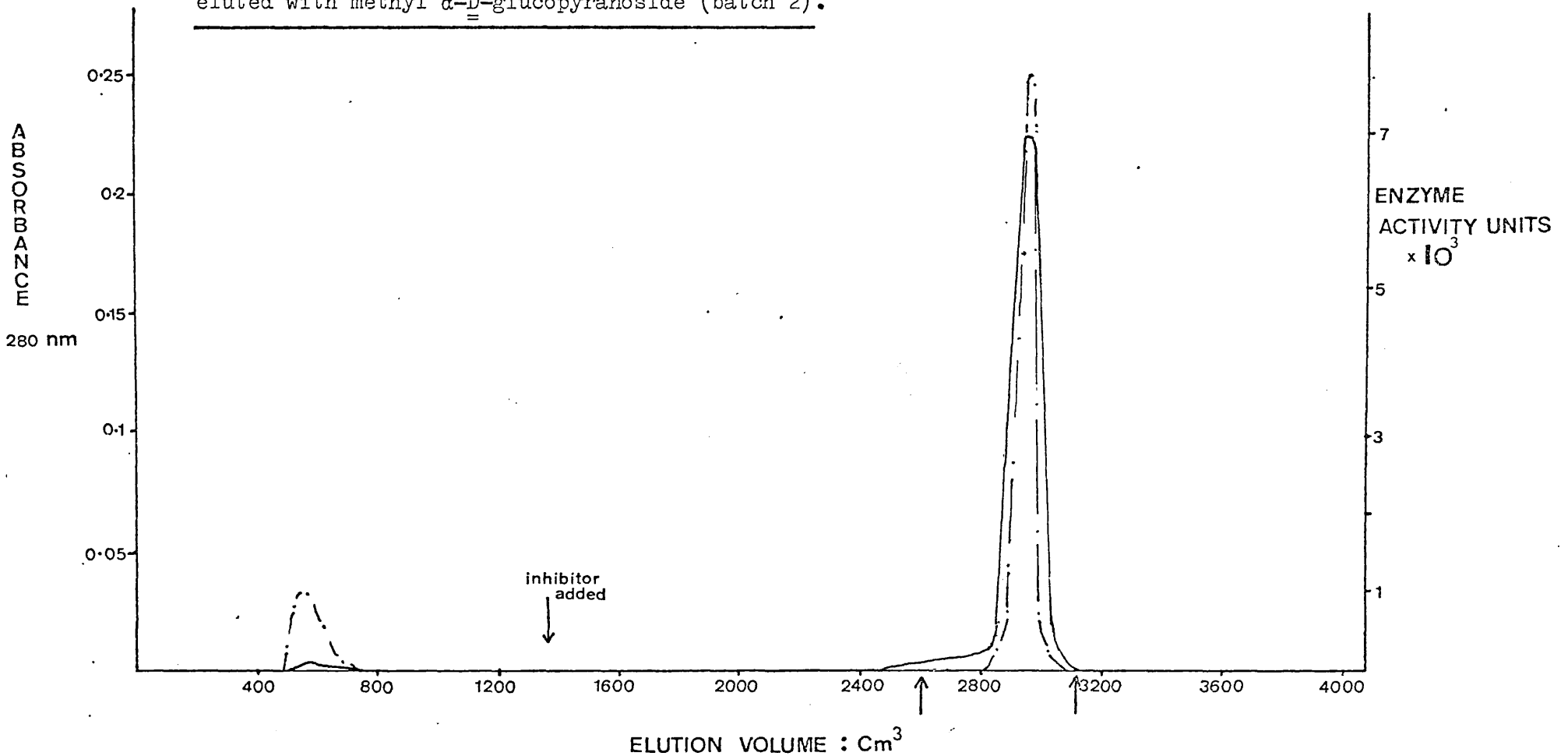
Elution profile of pig liver α-D-glucosidase on Biogel P-60 (batch 2)



A) Fractions between arrows were pooled.
B) Eluant: 25 mM NaCl containing 1 mM EDTA; pH brought to 6.7 with 2 M NaOH.
C) One unit of enzyme activity will liberate 1 nmole glucose under the conditions described in the Experimental (IV.A.11).
D) ——— Maltase activity.
E) .——.— Absorbance (280 nm).

Fig.12

Elution profile of pig liver α-D-glucosidase on the second Sephadex G-100 column eluted with methyl α-D-glucopyranoside (batch 2).



A) Eluant: 25 mM NaCl containing 1 mM EDTA; pH brought to 6.7 with 2 M NaOH until an elution volume of 1380 cm^3.

B) After an elution volume of 1380 cm^3 eluant: methyl α-D-glucopyranoside (1% w/v) in 25 mM NaCl containing 1 mM EDTA; pH brought to 6.7 with 2 M NaOH.

C) Fractions between arrows were pooled.

D) ——— Maltase activity.

E) ·———·— Absorbance (280 nm).

F) One unit of enzyme activity will liberate 1 nmole of glucose under the conditions described in the Experimental (IV.A. 11).

In order for a homogeneous enzyme to be prepared the procedure adopted previously for the removal of low and high molecular weight proteins had to be repeated. The material from batch 2, previously purified on Sephadex G-100 eluted with methyl α-D-glucopyranoside (Fig. 10), was loaded on a Biogel P-60 column as described in Experiment 9. An elution profile as shown in Fig. 11 was obtained. Absorbance measurements at 280 nm of the eluted fractions showed no appreciable separation of any proteins. This was evident by a very small increase in the specific activity of the glucosidase (Table 16). It would seem, therefore, that very little of the contaminating proteins present in the extract are of low molecular weight. It is still unclear, however, whether the small increase in specific activity arises from the increased separation obtained by using the inhibitor to elute the Sephadex column, or that, the loss of these proteins was facilitated by the use of a higher molecular weight cut off ultrafiltration membrance. Disc gel electrophoresis of a sample of enzyme gave two protein bands on staining, one major, one minor, indicating the loss of some protein.

As a final purification step the material from batch 2, previously purified on Biogel P-60 (Fig. 11) was loaded onto a Sephadex G-100 column as described in Experiment 9. Fig. 12 shows the elution profile of the acid α-D-glucosidase preparation on Sephadex G-100. Once again the enzyme was eluted from the column with methyl α-D-glucopyranoside in buffer (1% w/v) as shown in Fig. 12. Approximately 40% of the protein was eluted in the free volume of the column giving rise to a 1.5-fold increase in specific activity.

Table 16 Purification Table for α-D-glucosidase from Batch 2

Step	Vol. (cm^3)	Total[o] activity units	Total protein (mg)	Specific[+] activity	Yield %
Crude extract	12300	2.157×10^6	4.059×10^5	5.3	
Autolysate	12020	1.933×10^6	3.245×10^5	5.95	89.6
$(NH_4)_2SO_4$ Precipitation	2180	1.43×10^6	1.13×10^5	13	66
Sephadex G-100	68	2.88×10^5	74.8	3850	13.35
Biogel P-60 chromatography	49	2.29×10^5	56.35	4063	10.62
Sephadex G-100 chromatography	29	2.119×10^5	34.8	6089	10

+ Specific activity is the number of nmoles maltose hydrolysed per minute per mg of protein.

o One unit of activity is the hydrolysis of 1nmole of maltose per minute

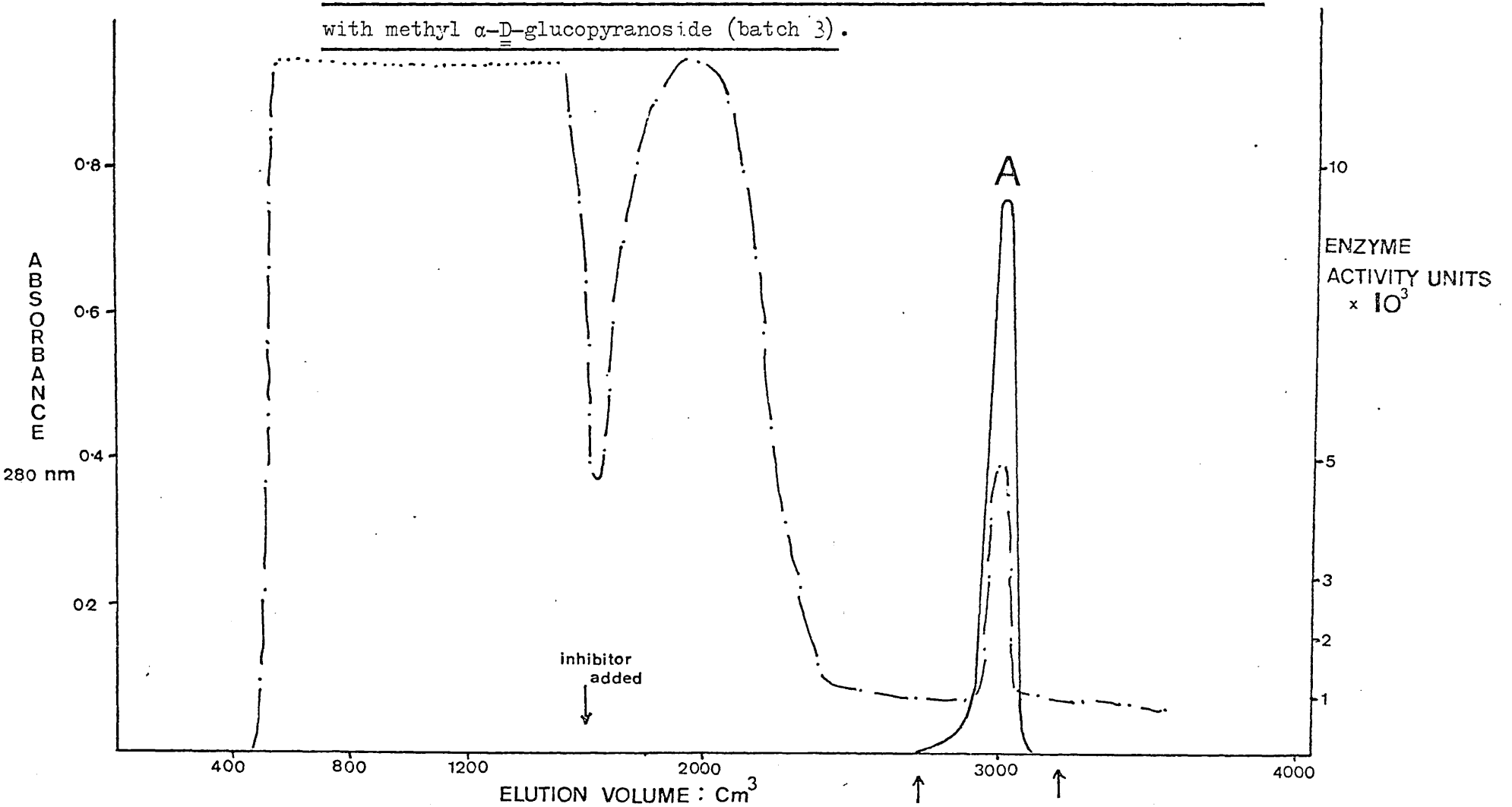
A final yield of 10% (Table 16) compared favourably with the 10.5% yield obtained for the extraction of the enzyme from batch 1. The yields so far obtained suggest that only 10% of the maltase activity in swine liver is due to the action of acid α-D-glucosidase.

2.A.g. Separation and isolation of an isoenzyme of acid α-D-glucosidase

It is well documented that acid α-D-glucosidase extracted from mammalian sources may be present in multiple forms, or isoenzymes.[34,89] Evidence from authors suggests that they may

Fig. 13

Elution profile of acid α-D-glucosidase eluted from a Sephadex G-100 column with methyl α-D-glucopyranoside (batch 3).



A) Eluant: 25 mM NaCl containing 1 mM EDTA; pH brought to 6.7 with 2 M NaOH with an elution volume of 1560 cm^3.

B) After an elution volume of 1560 cm^3 eluant: methyl α-D-glucopyranoside (1% w/v) in 25 mM NaCl containing 1 mM EDTA; pH brought to 6.7 with 2 M NaOH.

C) Fractions between arrows were pooled.

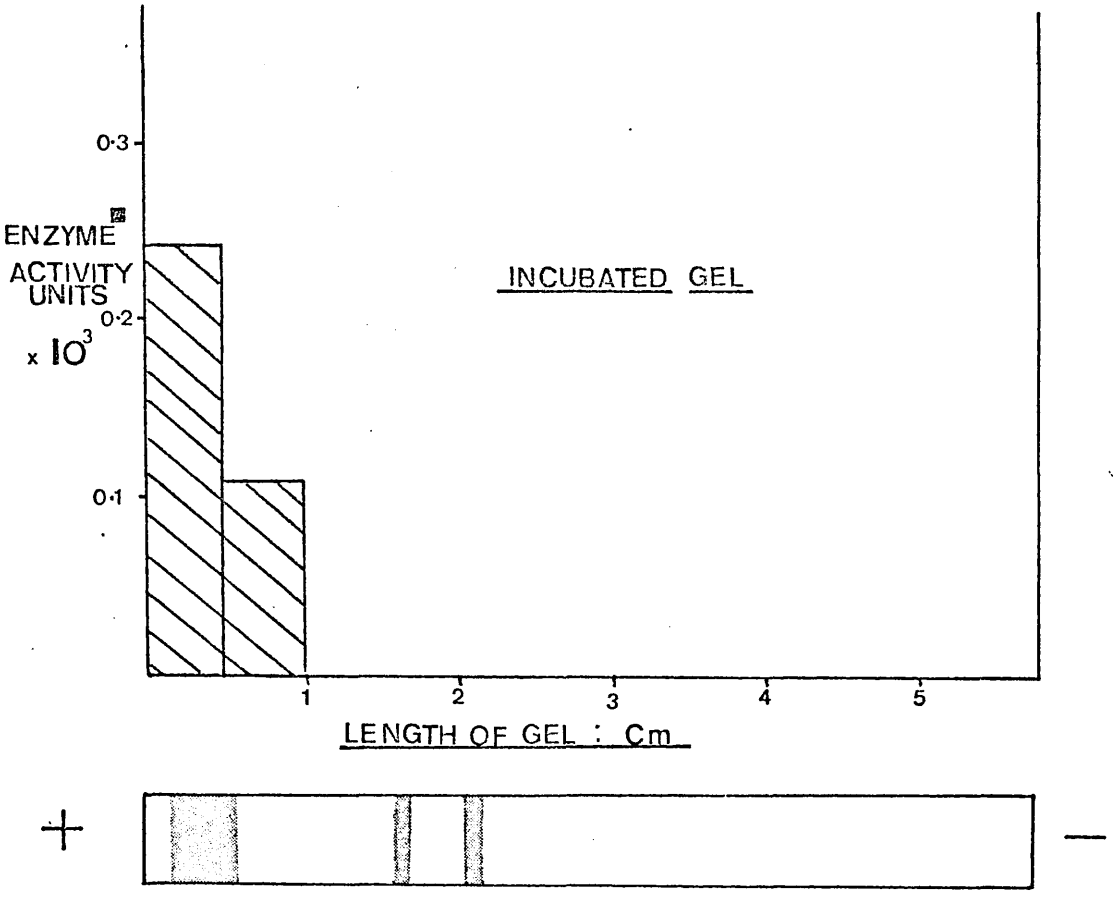
D) ——— Maltase activity.

E) —·——·— Absorbance (280 nm)

F) One unit of enzyme activity will liberate 1 nmole of glucose under the conditions described in the Experimental (IV.A.11).

Fig. 14

Disc gel electrophoresis of peak A (Fig. 13)



STAINED GEL

■ One unit of enzyme activity will liberate 1nmole of glucose under the conditions described in Experiment 10.b.

have different physical and enzymic properties.[37] Some initial evidence to suggest that such an enzyme was present was available from the early purification results. The specific activity of the extract during preliminary purification was found to be greater than usually expected indicating the presence of an "extra" enzyme. After preliminary purification the α-D-glucosidase extract from batch 3 (Table 14) was loaded onto a Sephadex G-100 column as described in Experiment 10a. Fig. 13 shows the elution profile. The enzyme was eluted with methyl α-D-glucopyranoside (1% w/v) in buffer as indicated in Fig. 13. A 300-fold purification (Table 17) was achieved after only a one step purification on Sephadex G-100. The specific activity (8600 units) was the highest obtained at any stage so far. This was also coupled with the largest yield obtained at this stage of 23.4% (Table 17). If we are to assume that the neutral maltase was separated from the acid α-D-glucosidase, this leads us to speculate the presence of another very active enzyme not seen before in any other extractions. It was decided to try and separate, if possible, the two glucosidase activities by disc gel electrophoresis. After running samples at pH 4.3 and 8.5, and staining as described in Experiment 10b, three bands were visible, one major band and two minor bands. Instead of staining all of the gels, run at pH 4.3, one gel was sliced into pieces (0.5 cm) and incubated with the substrate p-nitrophenyl α-D-glucopyranoside. After assaying for glucose only one glucosidase active band was found corresponding to the major stained protein band as shown in Fig. 14. An attempt at incubating the gels run at pH 8.5 was unsuccessful due to the decrease in activity of the enzyme at that pH. It was not possible

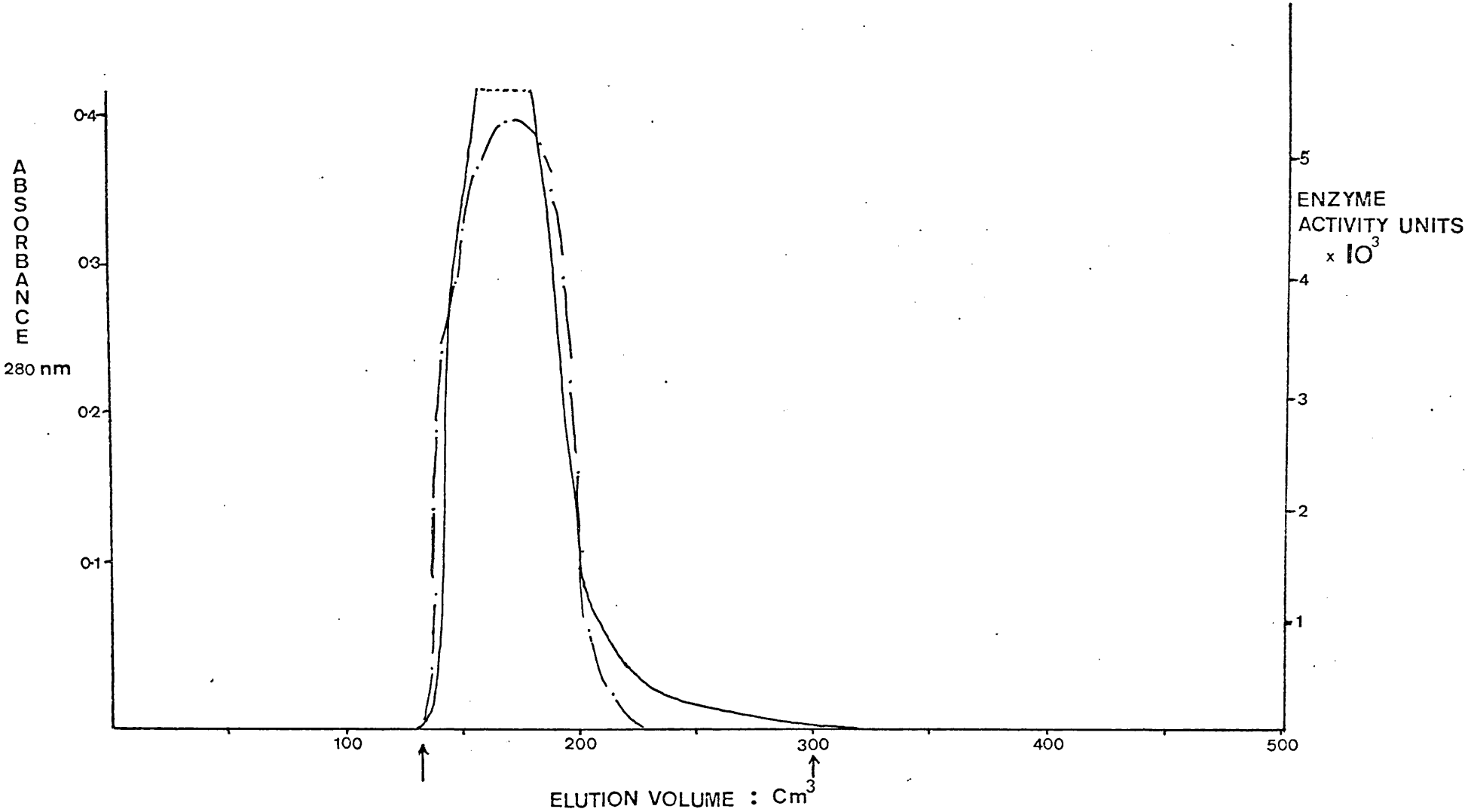
therefore to distinguish more than one active protein band by disc gel electrophoresis. As it was shown by disc gel electrophoresis that more than one protein was present in the α-D-glucosidase extract, further purification would have to be carried out. The purification procedure therefore adopted previously for batch 1 and 2 would have to be followed.

The material from batch 3 previously purified on Sephadex G-100 was loaded onto a Biogel P-60 column as described in Experiment 10c. An elution profile as shown in Fig. 15 was obtained. Absorbance measurements, at 280 nm, of the eluted fractions indicated that the only protein eluted from the column was the acid α-D-glucosidase. This indicated that no separation of small molecular weight proteins was achieved. This was further substantiated by the low increase in specific activity after this stage (Table 17).

Further purification of the enzyme was carried out on Sephadex G-100 as described in Experiment 10d. The column was freshly packed into a small column to reduce the length of time required for chromatography. The elution profile is shown in Fig. 16. The enzyme was eluted with methyl α-D-glucopyranoside as shown in Fig. 16. A comparison with previous elution profiles (Figs. 9 and 12), shows one major difference. Previously the protein eluted in the free volume of the column showed only minor maltase activity and consequently was not thought of importance. However, on this occasion this peak, as shown in Fig. 16, showed a considerable increase in maltase activity. Both maltase peaks were pooled separately and concentrated as indicated in Fig. 16. No increase in the specific activity of the acid α-D-glucosidase was achieved, after purification on Sephadex G-100 (Table 17). To account for this result it was concluded that an

Fig. 15

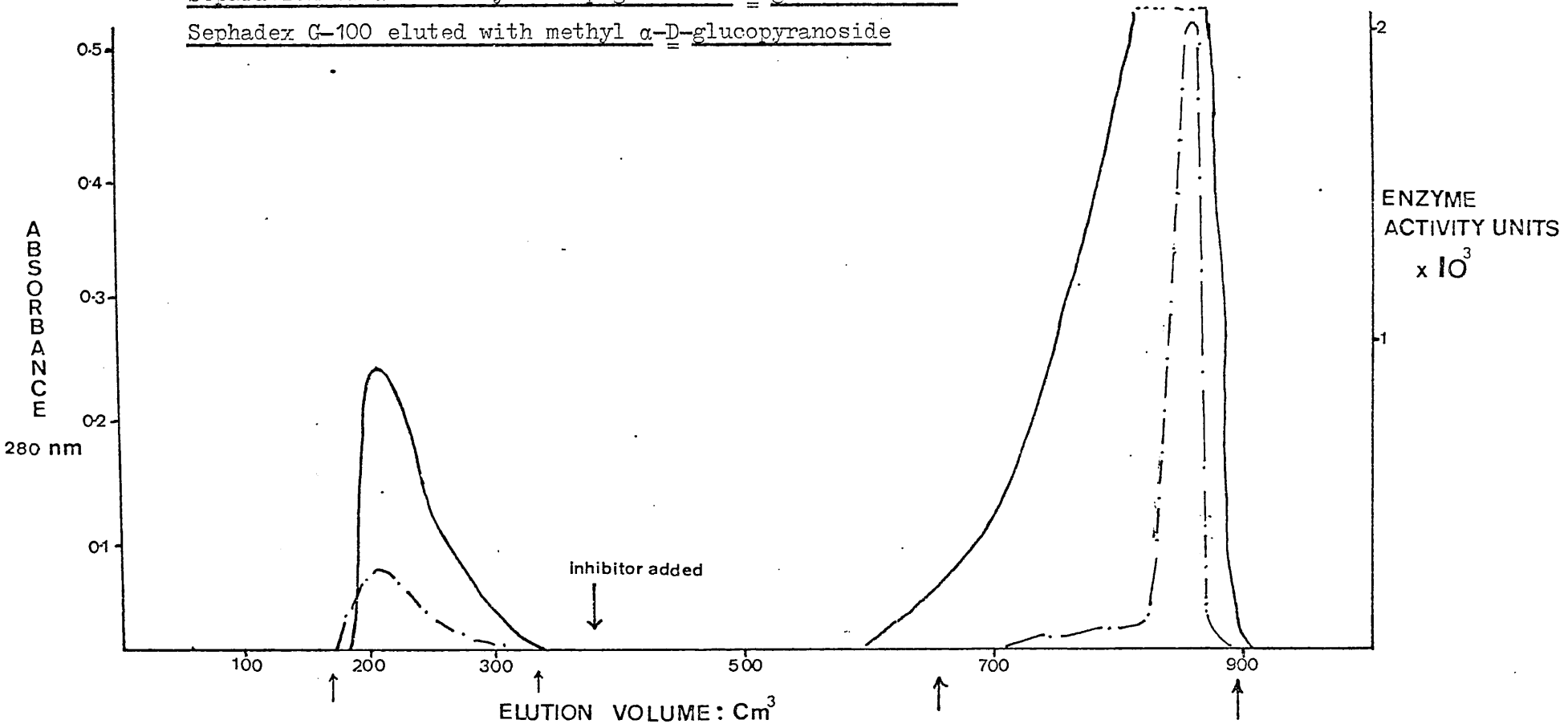
Elution profile of acid α-D-glucosidase from pig liver on Biogel P-60 (batch 3)



A) Fractions between arrows were pooled.
B) Eluant: 25 mM NaCl containing 1 mM EDTA; pH brought to 6.7 with 2 M NaOH.
C) ——— Maltase Activity.
D) —·—·— Absorbance.
E) One unit of enzyme activity will liberate 1 nmole of glucose under the conditions described in Experimental (IV.A.11).

Fig. 16

Separation of an isoenzyme of pig liver α-D-glucosidase on Sephadex G-100 eluted with methyl α-D-glucopyranoside



A) Fractions between arrows were pooled
B) Eluant: 25 mM NaCl containing 1 mM EDTA; pH brought to 6.7 with 2M NaOH, until an elution of 380 cm^3.
C) After an elution volume of 380 cm^3, eluant: methyl α-D-glucopyranoside (1% w/v) in 25 mM NaCl containing 1 mM EDTA; pH brought to 6.7 with 2 M NaOH.
D) ——— Maltase activity.
E) .—.—.— Absorbance (280 nm)
F) One unit of enzyme activity will liberate 1 nmole of glucose under the conditions described in Experimental (IV.A.11).

Table 17 Purification Table for acid α-D-glucosidase from Batch 3

Step	Volume (cm^3)	Total[o] activity units	Total protein (mg)	Specific[+] activity	Yield (%)
Crude extract	7300	2.24×10^6	1.42×10^5	15.7	100
Autolysis	7000	1.79×10^6	1.05×10^5	17	79.9
$(NH_4)_2SO_4$	1570	1.469×10^6	5.02×10^4	29.3	65.3
Sephadex G-100 chromatography	60	5.26×10^5	60	8600	23.48
Biogel P-60 chromatography	30	3.618×10^5	40.5	8933	16.15
Sephadex G-100 chromatography	25	2.119×10^5	24	8829	9.45

+ Specific activity is the number of nmoles of maltose hydrolysed per minute per mg of protein.

o One unit of enzyme activity will hydrolyse 1 nmole of maltose per minute.

additional glucosidase had been separated from the acid α-D-glucosidase. Table 18 gives the purification figures for both enzymes. The second protein peak containing the majority of the maltase activity was termed isoenzyme I, and the first protein peak containing only minor maltase activity isoenzyme II.

Table 18 Purification values for two maltase active enzymes extracted from Batch 3 after final purification on Sephadex G-100

Isoenzyme	Volume (cm^3)	Total[o] activity units	Total protein (mg)	Specific[+] activity	Yield %
Isoenzyme I	25	2.119×10^5	24	8829	9.45
Isoenzyme II	26	9.198×10^3	3.9	2358	0.41

[o] One unit of enzyme activity is the hydrolysis of 1nmole of maltose per minute.

[+] Specific activity is the number of nmoles maltose hydrolysed per minute per mg protein.

Several of the results shown in the table do not correlate with those expected. There is an unexpected decrease in the number of activity units recovered for isoenzyme I. The percentage protein recovered as isoenzyme I (59%) is in good agreement with that recovered for batch 1 (53%), and batch 2 (62%), this therefore does not lend any evidence to the suggestion of the loss of enzyme. Therefore, it would seem reasonable to suggest that a significant lowering of activity occurs when the two isoenzymes are separated. Some evidence however exists to disprove this theory. If the protein contents of both isoenzymes are added together the total does not equal that put onto the column. No evidence exists for the elution of any other protein than that of the two enzymes. A comparison of the areas of the two glucosidase peaks gives a ratio of approximately 5:1 of isoenzyme I to isoenzyme II. These figures correlate quite well with the protein figures for the two enzymes. Concluding, it would seem reasonable to suggest that the activity of acid α-D-glucosidase (isoenzyme I), may be effected by the presence or absence of isoenzyme II.

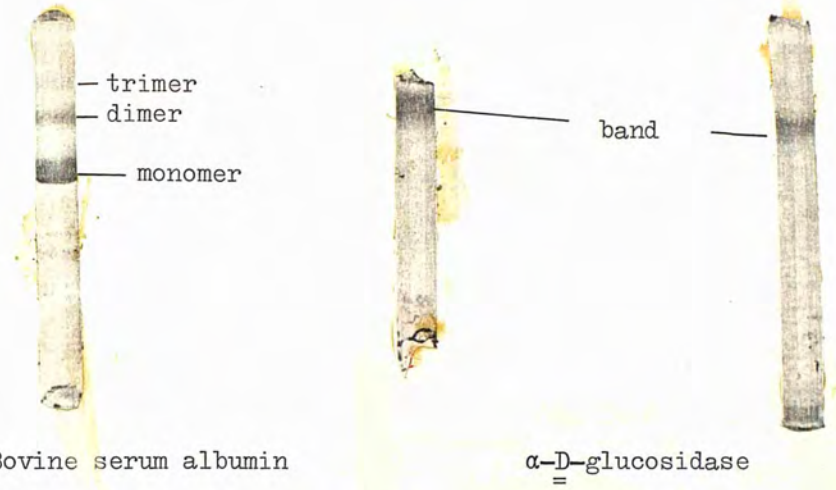
Although there are many acid α-D-glucosidases from a variety of sources which have been purified and characterised little or no literature is available concerning the purification of the isoenzymes of acid α-glucosidases. Fujimori et al.[102] managed to separate two isoenzymes of the acid α-D-glucosidase from bovine spleen by chromatography on Sephadex G-200. It was suggested by this author that the separation of the isoenzymes was caused by the low activity of one isoenzyme for the dextran gel. This phenomenon was applied by Swallow and co-workers[37] to separate glucosidase isoenzymes from extracts of human organs. It has always been possible, however, to separate isoenzymes by isoelectric focussing as reported by Brown et al.[34] Although it seems the work of Fujimori and Swallow has been repeated here some interesting factors arise concerning Sephadex separation of isoenzymes. Both these workers separated isoenzymesof acid α-D-glucosidase by a one stage purification on Sephadex. One isoenzyme therefore, having a diminished affinity for the dextran gel, was eluted with the neutral α-D-glucosidase. Because both isoenzymes were separated after purification on the second Sephadex column, there must be a reason why both enzymes were not fully separated on the first Sephadex column. This suggests, that either incomplete separation occurred on the first Sephadex column, or, if in fact separation was complete then isoenzyme II must be a product of isoenzyme I. Whatever is the reason sufficient evidence is available to show that both isoenzymes must be very closely related. Further evidence concerning the incomplete separation of the isoenzymes upon dextran gel chromatography is given in section 2.C.b.

2.A.h. Homogeneity of the Glucosidase preparation

Homogeneity was established when a single protein band was stained after electrophoresis on polyacrylamide gel. Disc gel electrophoresis was performed on each glucosidase extract from batch 1, 2 and 3 which had been purified as described in this section. Electrophoresis was performed as described in Experiment 11 at pH 8.5 and 4.3. Each purified enzyme extract gave one protein band on staining (Fig. 17 a,b). The enzyme isolated and termed isoenzyme II also gave one band after disc gel electrophoresis (Fig. 17b).

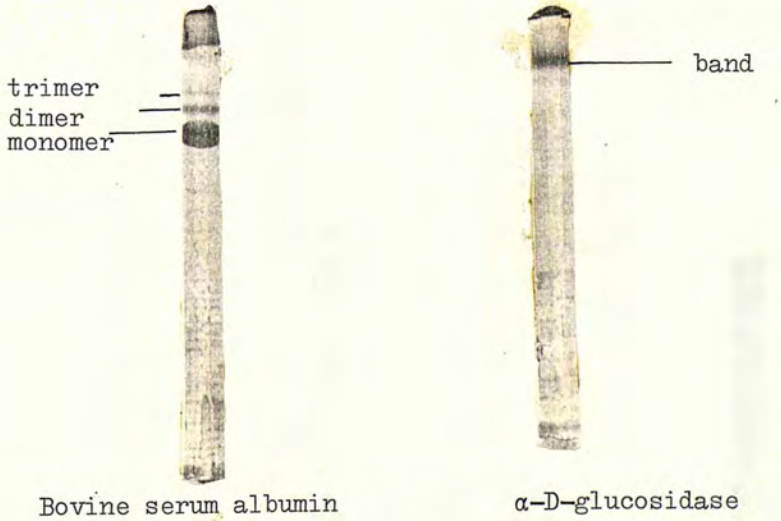
Fig. 17a

Disc gel electrophoresis of α-D-glucosidase from batch 2



Bovine serum albumin α-D-glucosidase

pH 8·5

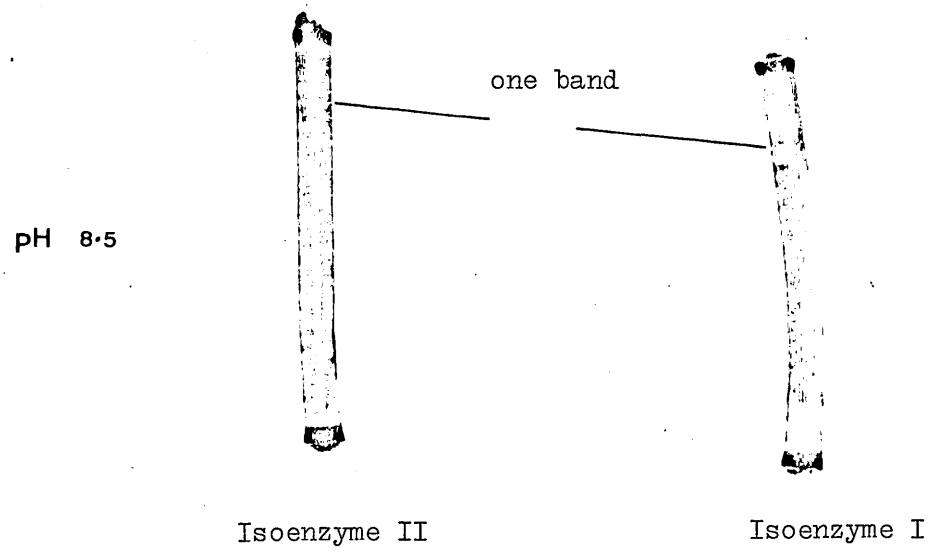


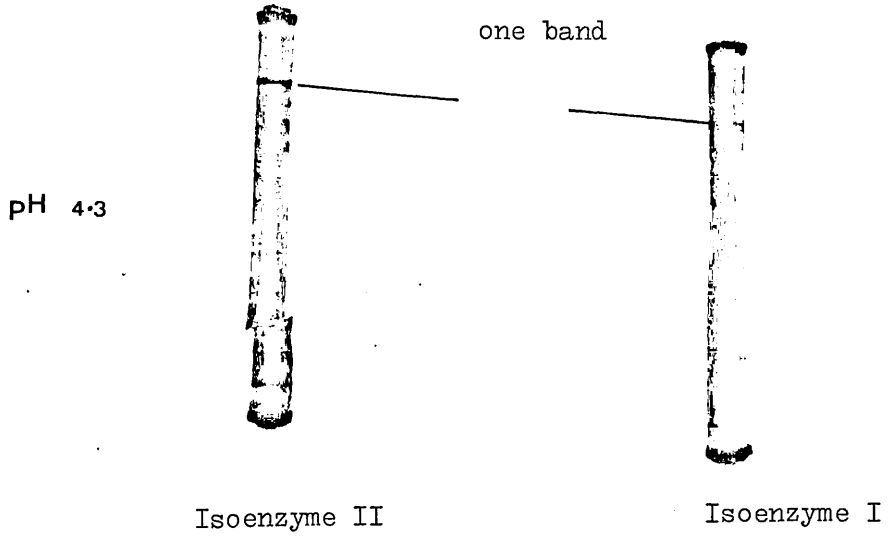
Bovine serum albumin α-D-glucosidase

pH 4·3

Fig. 17b

Disc gel electrophoresis of acid α-D-glucosidase (Isoenzyme I and II) from batch 3





I.2.B. Properties of Isoenzyme I

a. General Aspects

The following section deals with the enzymic and some physico-chemical properties of the enzyme. Preliminary experiments were carried out in order that the enzyme could be classified, firstly by its action pattern, and then by its specificity. No thorough investigation of the enzymes physico-chemical properties was undertaken because of the small amounts of protein finally extracted. The experiments performed give a clear indication of the kinetic properties of the enzyme. One or two experiments although discussed in detail in this section will be discussed further in chapter I.2.C in relation to some of the results obtained for isoenzyme II.

Isoenzyme I relates to the enzyme that was selectively absorbed by the dextran gel - Sephadex G-100, that is, the enzyme normally isolated and termed acid α-D-glucosidase showing the expected properties associated with it.

b. Subunit structure and molecular weight of the purified enzyme

Sodium dodecyl sulphate [(S.D.S.) $CH_3(CH_2)_{10}CH_2OSo_3Na$] has the ability to dissociate protein aggregates into their constituent polypeptide chains. It minimizes the native charge differences so that all proteins migrate as anions as the result of complex formation.[103,104] Protein samples prepared with S.D.S. and 2-mercaptoethanol undergo complete denaturation and reduction of the disulphide bonds.[105] It is well reported that the mobility of these proteins upon electrophoresis is a function of their molecular weights.[106]

The method itself is both easy and quick to carry out.[107,108]

From the results obtained using this method we will be able to determine whether the enzyme existed as a protein aggregate being built up of different subunits, and the molecular weights of these subunits. The determination of the molecular weights of unknown proteins is carried out by comparing the mobilities of standards, of known molecular weight, with that of the unknown.

The gels were prepared and electrophoresis was performed as described in Experiment 12. A series of protein standards were also run at the same time. The mobilities, relative to bromophenol blue (BPB) of the standards were determined as shown:

$$\text{Mobility}_{\text{BPB}} = \frac{\text{distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before staining}}{\text{distance of migration of bromophenol blue}}$$

Table 19 gives the mobilities of the standards:

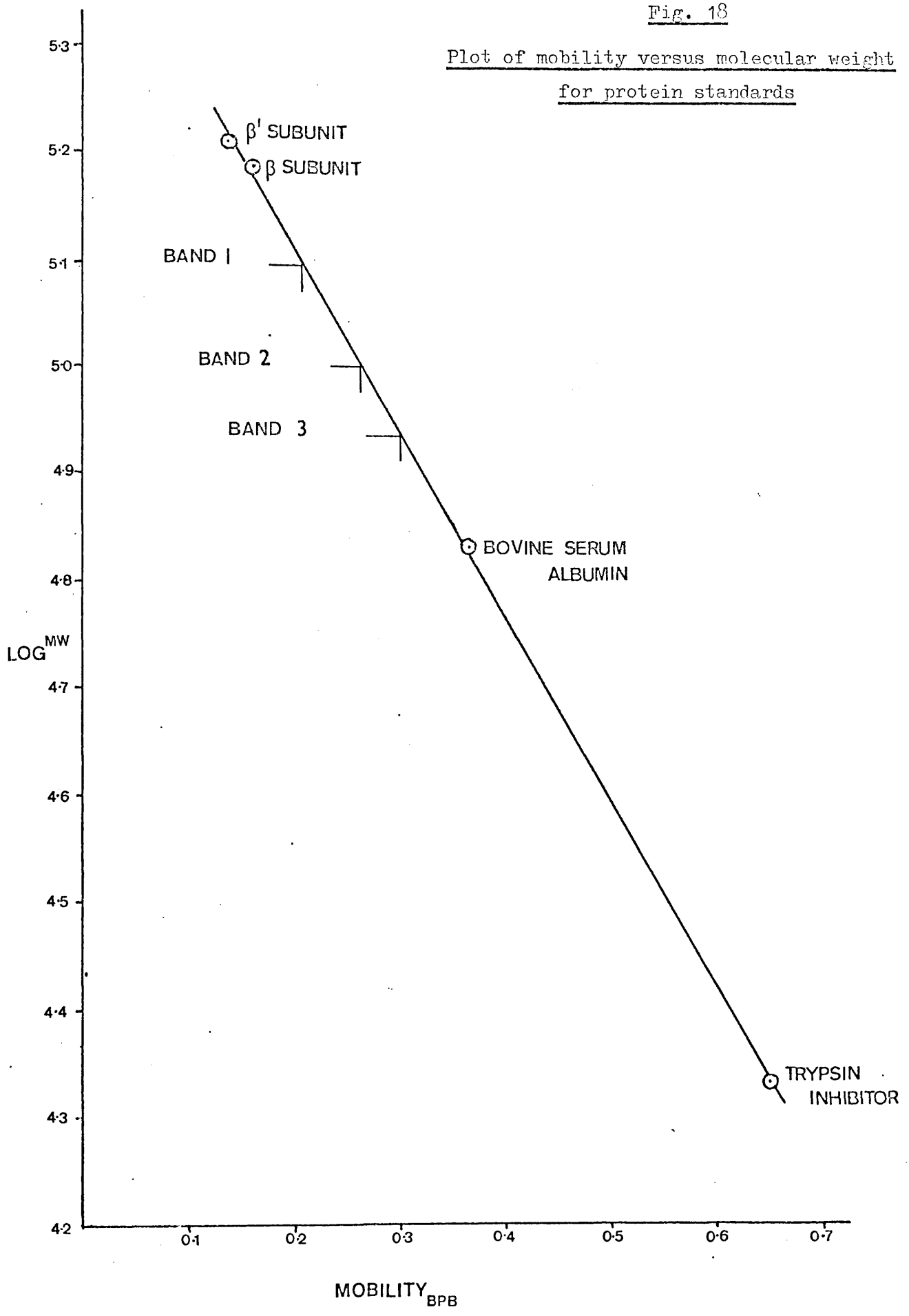
Table 19 Mobilities of protein standards derived by SDS electrophoresis

Protein standard	Molecular weight	Log molecular weight	Mobility BPB
Trypsin inhibitor from soya bean	21,500	4.3324	0.65
Bovine serum albumin	68,000	4.8325	0.365
RNA polymerase from E. Coli.			
β-subunit	155,000	5.1903	0.16
B'-subunit	165,000	5.2174	0.14

A plot of Log molecular weight versus mobility is shown in Fig. 18.

Fig. 18

Plot of mobility versus molecular weight for protein standards



Acid α-D-glucosidase gave four bands on staining, two major and two minor bands (Fig. 19a). The molecular weights of these bands were derived from Fig. 18, by finding the molecular weight corresponding to the mobility of each stained band. As the mobilities of the two minor bands were very close and the bands hard to discriminate a mean value is given.

Table 20 Molecular weights of the bands obtained by S.D.S. electrophoresis of acid α-D-glucosidase

	Band 1 (major)		Band 2 (major)		Band 3 + 4 (minor) (mean)	
	Mobility (BPB)	Molecular weight	Mobility (BPB)	Molecular weight	Mobility (BPB)	Molecular weight
Gel 1	0.2075	125,000	0.264	101,000	0.301	87,000
Gel 2	0.199	127,000	0.259	102,000	0.298	88,000

The purified enzyme, although only one band is seen on disc gel electrophoresis, gives four bands on S.D.S. electrophoresis (Fig. 19). This suggests the presence of perhaps more than one protein. However, one can also suggest that the enzyme is made up of several subunits. Rosenfeld and Belenki[97] found that when S.D.S. electrophoresis was carried out on the human liver enzyme three bands were visible on staining. On disc gel electrophoresis run at two pH's one single protein band was visible. They therefore suggested that the enzyme consisted of three structurally different subunits. The swine acid α-D-glucosidase gave two major bands with an average molecular weight of 126,000 and 101,500, for band 1 and band 2, respectively.

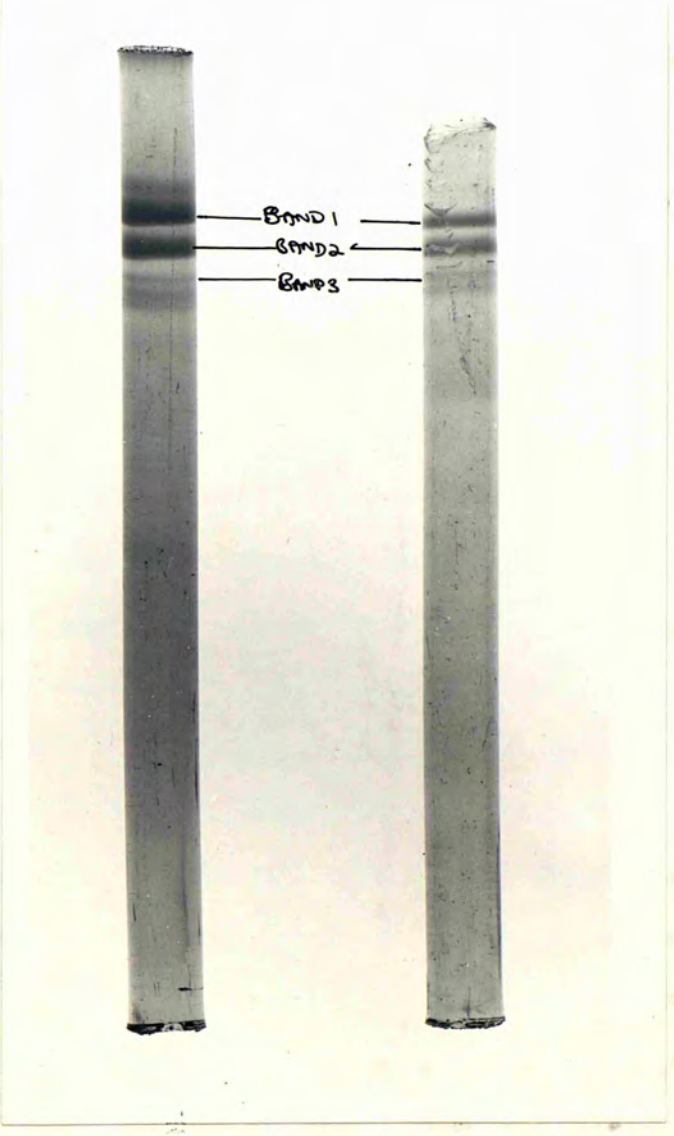


Fig. 19 a,b

*Photograph of a stained sodium dodecyl sulphate electrophoresis gel of acid α-D-glucosidase (Isoenzyme I) and Isoenzyme II

*The gels were restained before photography.

It is impossible from these results to give an accurate molecular weight for the enzyme, even though a deviation of ±10% can exist. Known values for the enzyme range from 100,000[20,24,25] to 117,000, depending on the source, and the pig spleen enzyme has been found to have a molecular weight of 106,000.[31] It would be unreasonable to expect a large deviation from the figure recorded for the spleen enzyme. The presence of some isoenzyme II although a real possibility could not in fact have given rise to such differences in the molecular weight of the two major bands because of the very small differences in molecular weights of isoenzymes. It has been reported that glycoproteins containing more than 10% carbohydrate on S.D.S. electrophoresis give results not consistent with their true molecular weights.[103] As it seems likely that acid α-D-glucosidase is a glycoprotein this could account for the rather high values obtained for its molecular weight. However, no figures are available concerning the percentage carbohydrate within the enzyme molecule.

The results obtained therefore suggest a subunit structure existing for the pig's liver enzyme with a molecular weight in the range quoted for the enzyme from other sources (Table 6, chapter I.B).

c. Mode of Action

I Exo-nature of the acid α-glucosidase

A method for detecting an endo acting enzyme in the presence of an exoenzyme was devised by Marshall using Cibachron Blue F3GA-amylose.[109] This substrate is substituted with a dye molecule on some of the primary hydroxyl groups. It is postulated that one dye molecule is attached every 7-10 maltose units in the amylose molecule.[110] The action of an exo acting enzyme on Cibachron Blue F3GA-amylose results in the release of D-glucose

Fig. 20

Determination of coloured dye products released by the action of α-amylase and α-D-glucosidase from Cibachron B Amylose

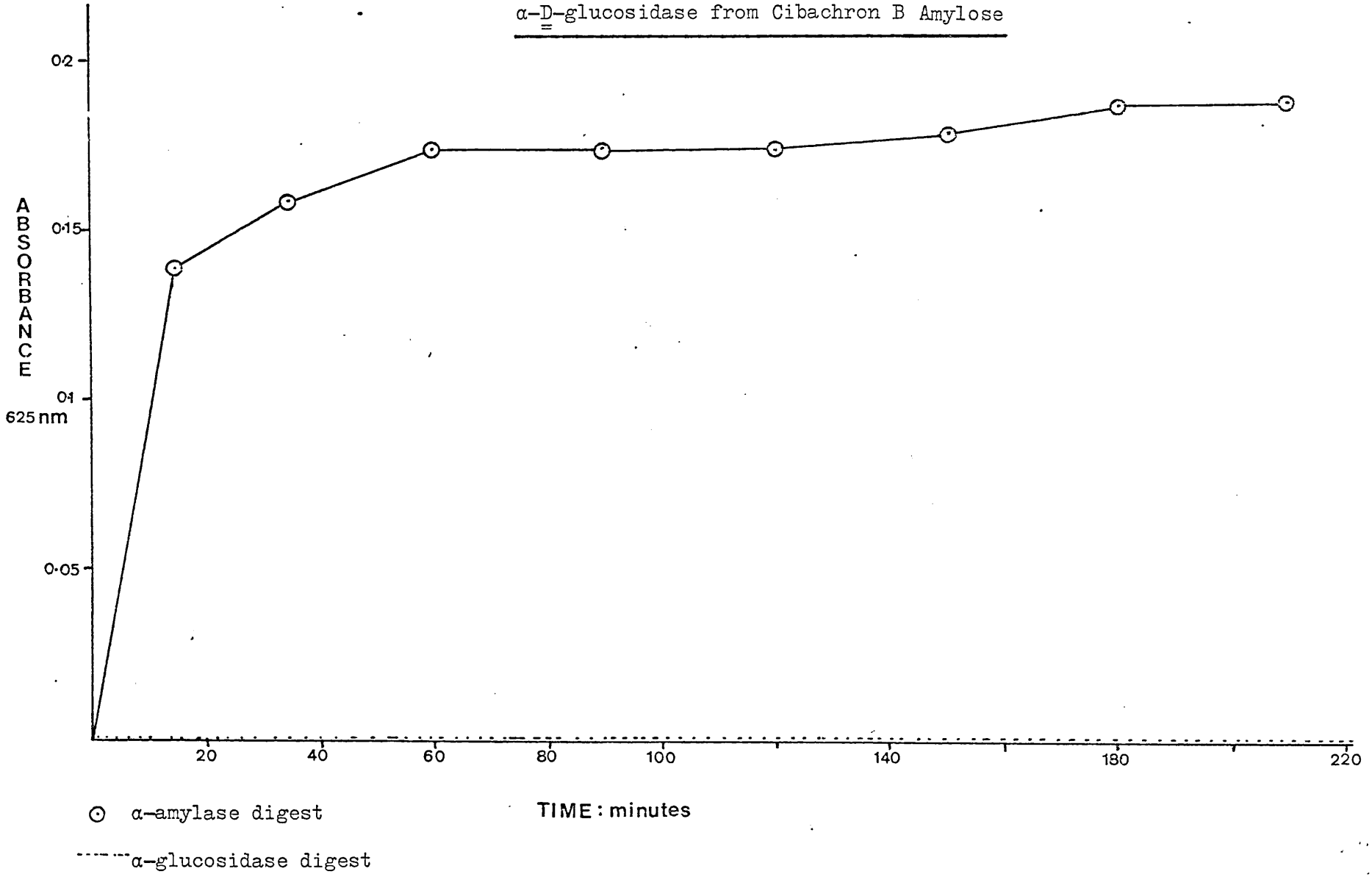
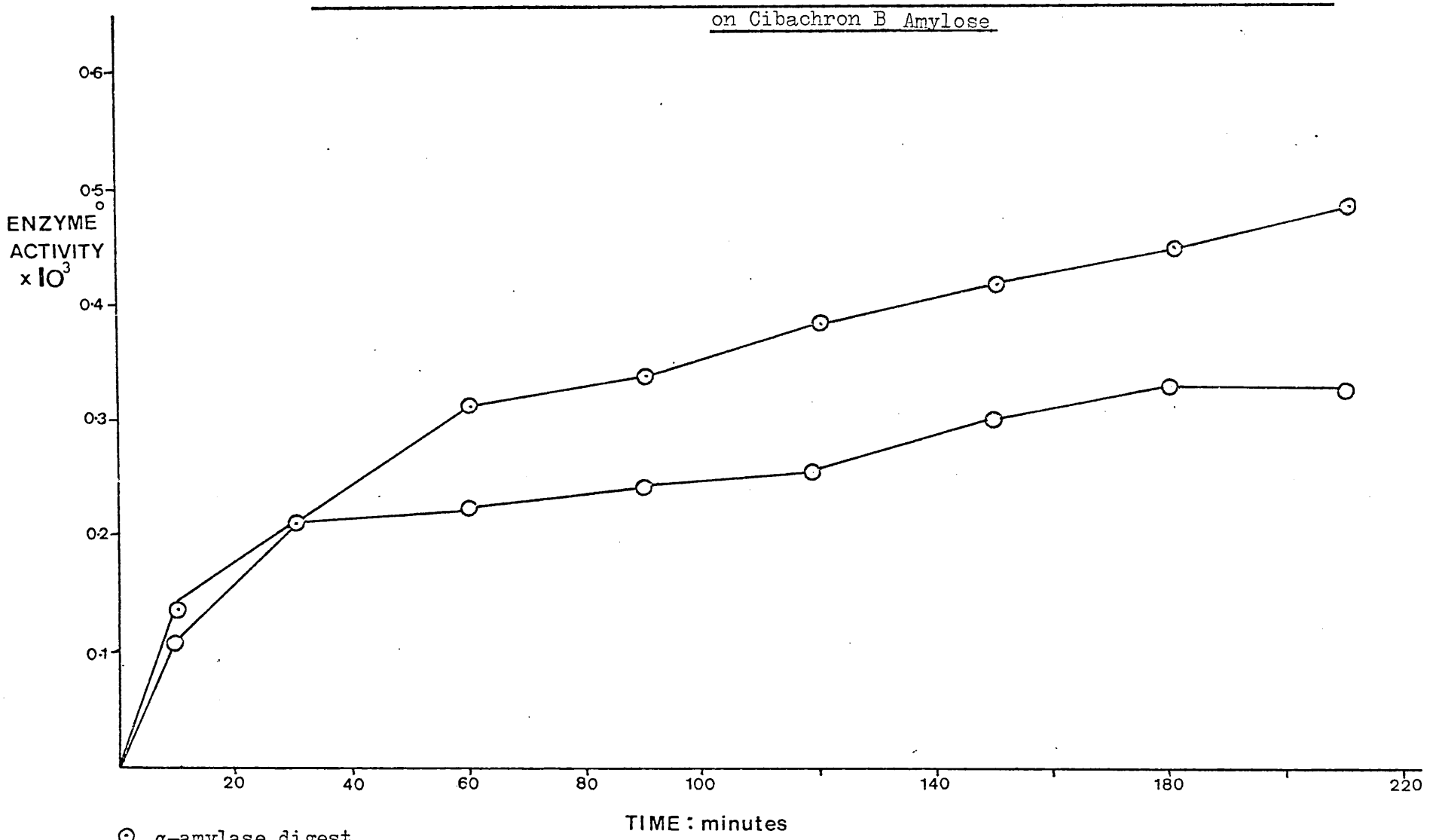


Fig. 21

Comparison of the glucose liberated by the action of α-amylase and α-D-glucosidase on Cibachron B Amylose



⊙ α-amylase digest

○ α-glucosidase digest

° Expressed as nmoles glucose liberated.

from the non-reducing end of the molecule but on reaching a glucose unit substituted with a dye molecule, enzymic action ceases.[109] An endo acting enzyme however randomly cleaves the dyed amylose to release oligosaccharides which are soluble in water, are coloured, and thus can be measured spectrophotometrically.

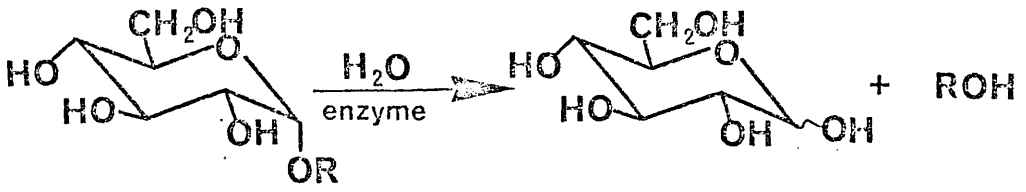
The method was carried out as described in Experiment 13. From Fig. 20 it can be seen that acid α-D-glucosidase did not release any colour from Cibachron Blue F3GA-amylose on incubation over a period of 240 minutes. The endo acting enzyme α-amylase, from B. subtilis released some coloured products under similar conditions, except the pH of the reaction was kept at pH 6. The amount of D-glucose released during the incubation is given in Fig. 21. For the incubation of α-D-glucosidase with Cibachron blue-amylose, some tailing off of its activity indicating that the enzyme had reached a point where its action was blocked. The endo acting α-amylase, however, showed hardly any deviation from its initial activity.

There is a limitation associated with this method. The amount of Cibachron blue-amylose in the incubation has got to be quite high because the Cibachron blue-amylose is actually insoluble in water and therefore the solution is heterogeneous.

Summarising the enzyme gave results consistent with it being an exo-acting enzyme.

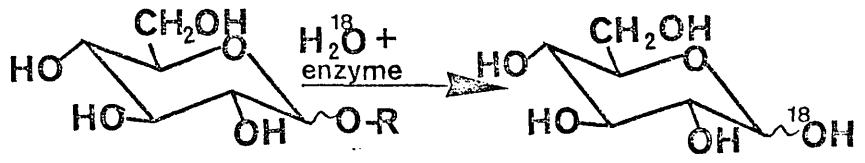
II The Configuration of the product released during enzymic hydrolysis

Enzymic hydrolysis by acid α-D-glucosidase involves the cleavage of the glycosidic bond with the subsequent release of D-glucose (1).

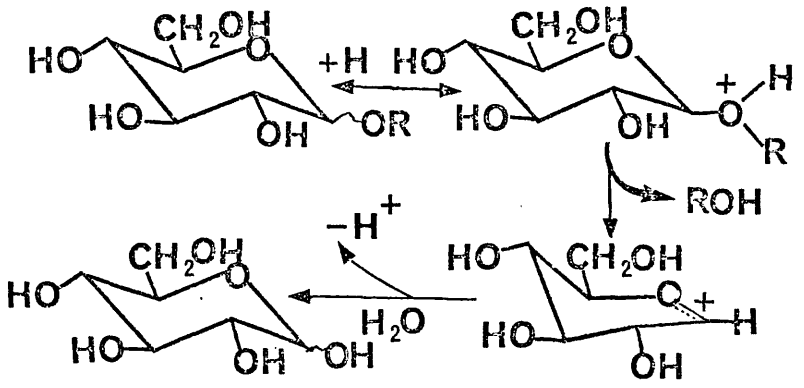


(1)

This reaction is known to involve the cleavage of the C-1 oxygen bond in a number of glycosidase catalysed reactions (2).[39,111] They therefore resemble acid catalysed hydrolyses of simple alkyl and aryl glucosides in which the C-1 oxygen bond is cleaved (3).[112]

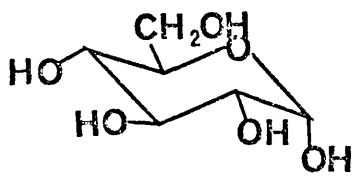


(2)

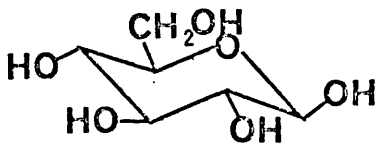


(3)

The enzymic reaction shown in (2) can result in two different products, either α- or β-D-glucopyranose (I,II).

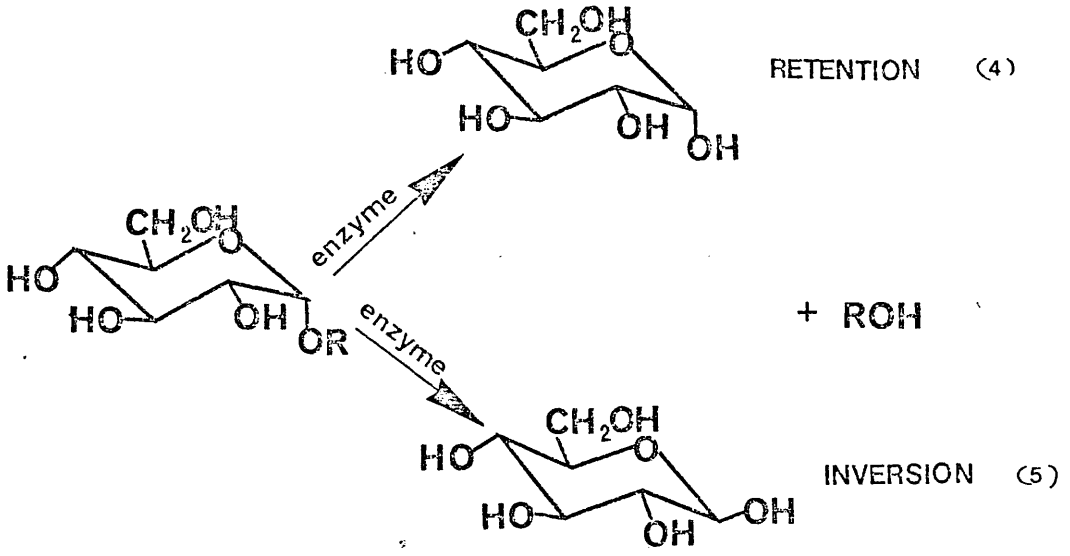


I

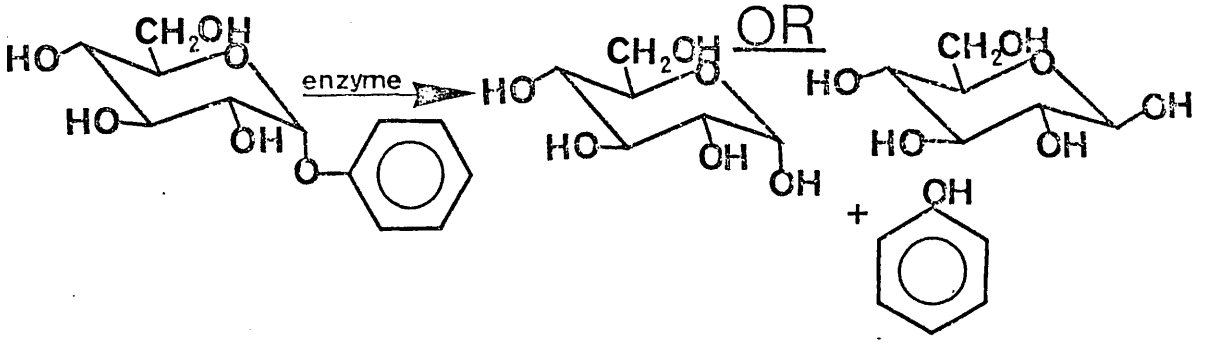


II

Glycosidases which catalyse the cleavage of the C-1 oxygen bond can be divided into two classes, as to whether the configuration of the hydroxyl group on C-1 is retained (4) or inverted (5).[49]



Thus, the reaction of acid α-D-glucosidase with phenyl α-D-glucopyranoside can result in the product either being α-D-glucopyranose or β-D-glucopyranose.



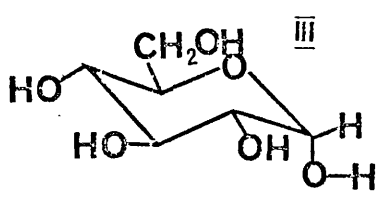
One of the earliest methods for determining the configuration of the released product, was by measuring the change in optical rotation with time (mutarotation). This involved taking readings from a polarimeter, which contained the enzyme and substrate in the polarimeter tube.

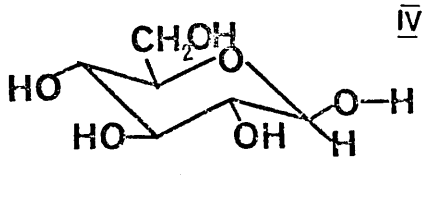
Armstrong[47] was the first worker to use this method. He found that when maltase acted on methyl α-D-glucopyranoside there was a marked fall in rotatory power. This indicated that α-D-glucose was liberated and mutarotation proceeded downwards towards the β-D-glucose. He also found the same result when emulsin acted on methyl β-D-glucopyranoside and for the action of invertase on sucrose. Armstrong implied that these glycosidases acted on substrates having the same anomeric configuration as that found in the product. It was only later, when Kuhn[113] showed that the action of β-amylase on starch resulted in inversion of configuration that Armstrong's theory was disproved. A number of glycosidases have been classified by the use of this method, which until recently was the only method available. Generally only qualitative data were abstracted from these results, using polarimetry. However, several workers have derived quantitative data to study the kinetics of the mutarotation reaction.

Koshland *et al.*[114] studied the action of β-amylase on maltotetrose and amylodextrin. The incubation was found to give a rise in rotary power, indicating that β-maltose was produced. The mutarotation reaction was found to follow first order kinetics, typical of simple mutarotation processes. Ono *et al.*[115] studied the hydrolysis of phenyl α-D-maltoside by glucoamylase and found that the glucose released was in the β-form. A method was described for determining the amounts of substrate and products, when the rate of enzymic reaction was comparable to or slower than that of mutarotation.

Gas liquid chromatography has been used to separate the trimethylsilyl derivatives of the sugars released during enzymic hydrolysis.[48] After enzymic hydrolysis had proceeded for some time, the reaction was stopped by freeze drying the sample. The trimethylsilyl derivatives were then synthesised and separated by G.L.C. The ratio of the two anomers was found from peak areas. From the ratio of the two anomers it was shown that α-D-glucosidase and β-D-glucosidase show retention of configuration, and glucoamylase shows inversion of configuration.

Another approach has been used by Perlin et al.[42] to differentiate between the anomers released during enzymic hydrolysis by glycosidases. Proton magnetic resonance (pmr) spectroscopy was used to detect the α- or β-anomer released. As the chemical shifts are characteristic of the different anomeric protons the presence of one anomer or the other could be seen directly. When α-D-glucosidase was incubated with phenyl α-D-glucopyranoside the signal for the α-H1(e)(III; δ 5.7 ppm) proton could be seen after three minutes. After seventeen minutes

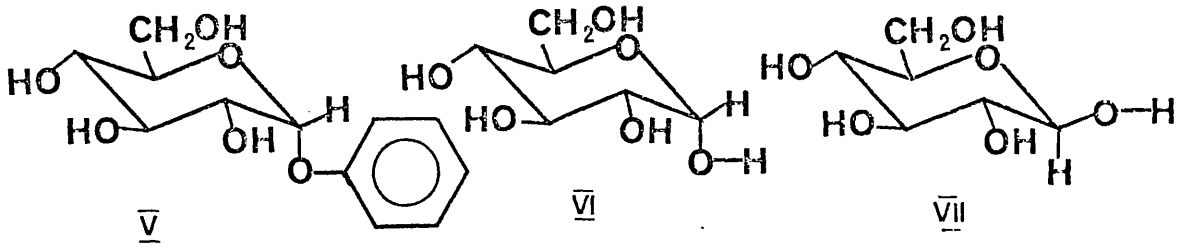




the signal for the β-H1(a)(IV, δ 5.1 ppm) proton could be seen, as a result of mutarotation of the α-D-glucose. Thus, it was concluded that the reaction proceeded with retention of configuration.

A series of glycosidases were characterised in this respect by Perlin and co-workers.[42] The results obtained by Perlin were qualitative. That is, he classified an enzyme by visually monitoring the production of the α-anomer and β-anomer. No attempt was made at integrating the peaks under investigation, to gain quantitative measurements of the substrate concentration, and of the amount of the liberated α- and β-anomers present with time.

To determine whether the reaction of acid α-D-glucosidase with phenyl α-D-glucopyranoside proceeded with retention or inversion of configuration at the anomeric centre, the method used by Perlin was adopted. In order to investigate the reaction in greater detail with respect to the mechanism involved, it was decided to attempt to integrate the proton signals corresponding to the H1(e)(V), H1(e)(VI), H1(a)(VII), of the substrate (phenyl α-D-glucopyranoside),



V VI VII

α-D-glucopyranose and β-D-glucopyranose, respectively.

Before an attempt at monitoring the reaction between acid α-D-glucosidase and phenyl α-D-glucopyranoside was carried out an attempt was made at monitoring the reaction between α-D-glucosidase from yeast, and phenyl α-D-glucopyranoside. The experimental details

Fig. 22

60 MHz ^{1}H pmr partial spectrum of phenyl α-D-glucopyranoside

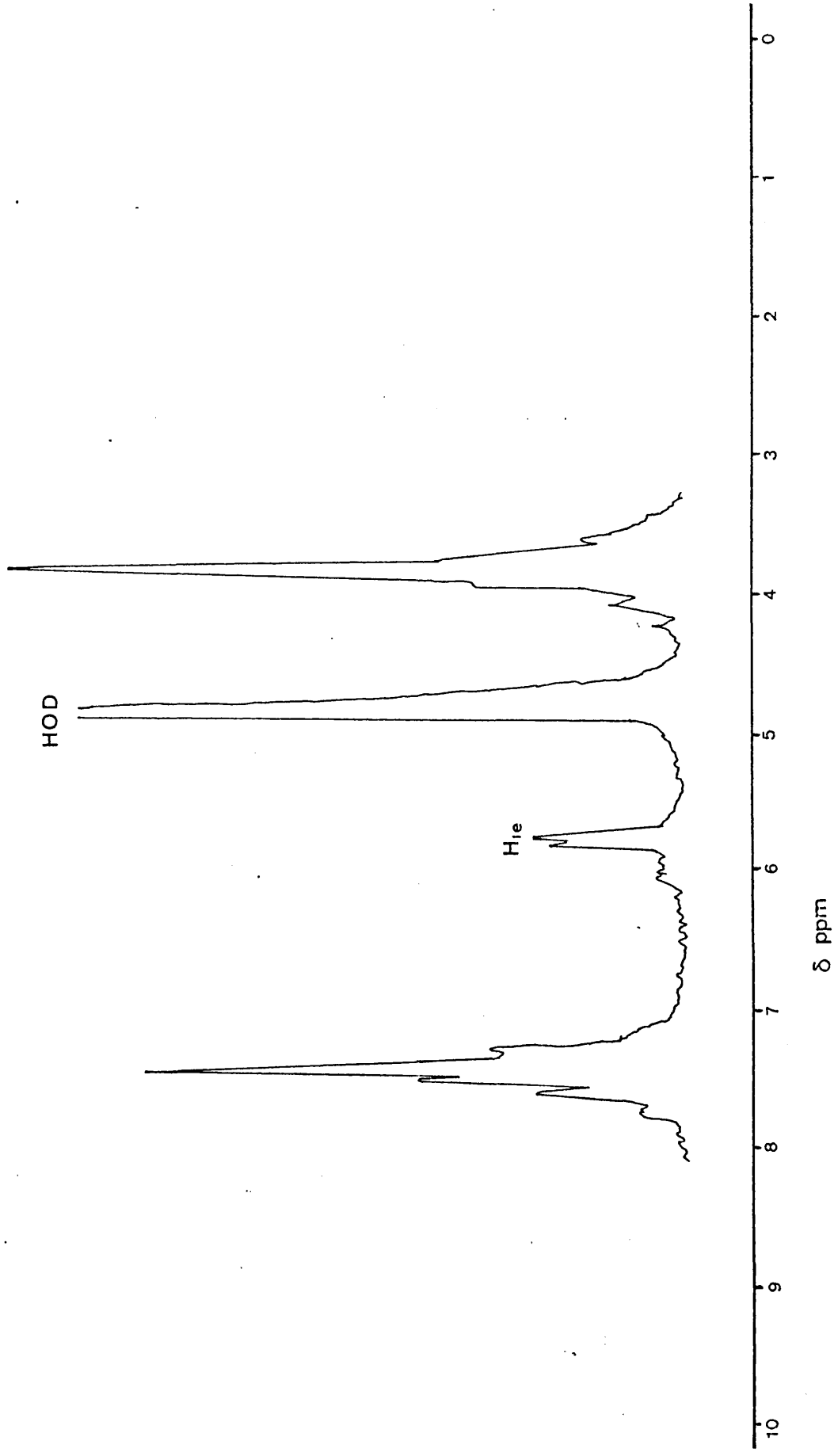
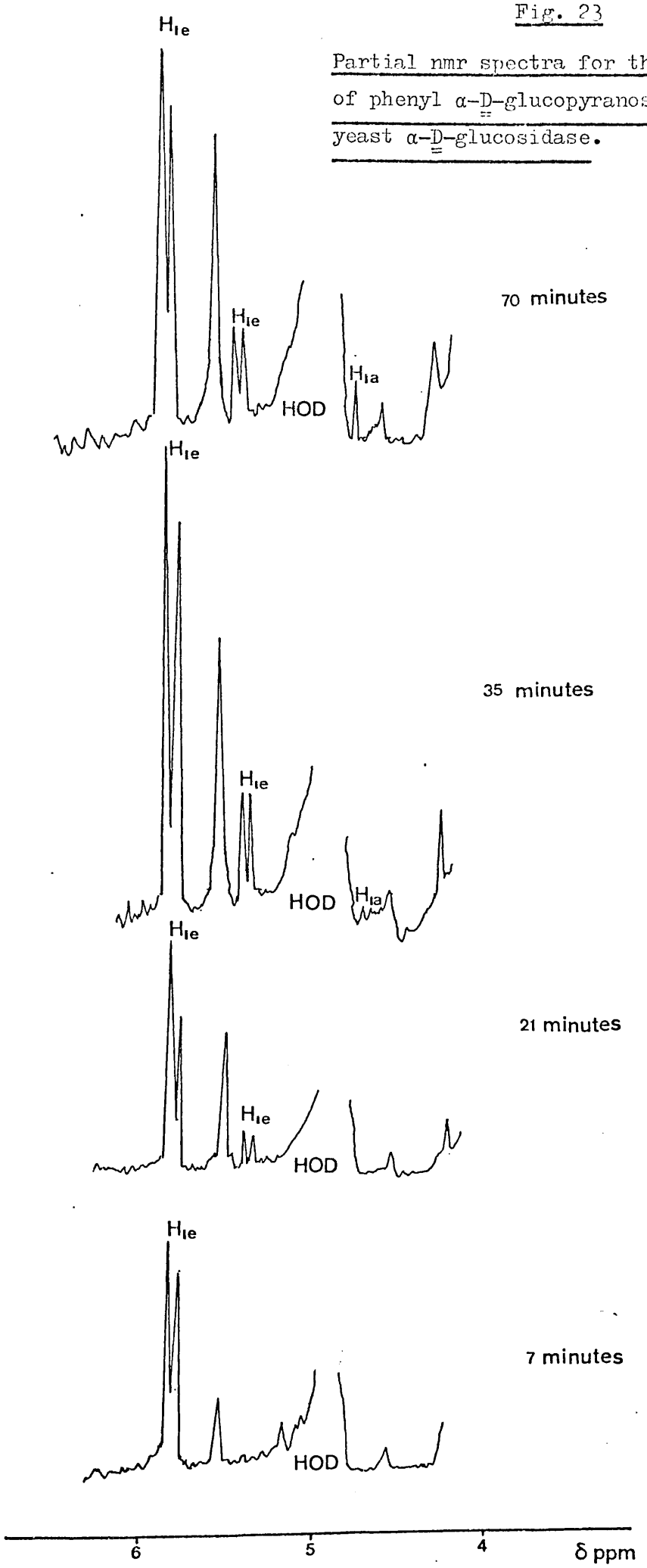


Fig. 23

Partial nmr spectra for the incubation of phenyl α-D-glucopyranoside with yeast α-D-glucosidase.



are given in Experiment 14. A low resolution instrument was used to see if the signals associated with the three sugars could firstly be resolved and integrated. Fig. 22 shows the 60 MHz spectrum of phenyl α-D-glucopyranoside. The H1(e) proton signal is shown at δ 5.8 ppm. Fig. 23 shows the spectra obtained for the incubation of yeast α-D-glucosidase with substrate. The signal seen at δ 5.4 ppm after seven minutes was due to the H1(e) proton of the α-D-glucopyranose. The signal due to the HOD was found to overlap with the H1(a) proton signal for the β-D-glucopyranose, but this was overcome by increasing the spin of the nmr tube. After thirty-four minutes the H1(a) signal was detected for the β-D-glucopyranose arising from the mutarotation of the α-D-glucose (δ 4.8 ppm). Correspondingly, the signal for the H1(e) proton of the phenyl α-D-glucopyranoside became weaker with time. No attempt was made at integrating the peaks being monitored.

When the method was repeated using acid α-D-glucosidase very similar spectra were obtained. However, no H1(a) signal was detected for the β-D-glucopyranose because of the overlap of the HOD peak. This peak could not be moved sufficiently up or down field to prevent the overlap of these two signals. After eighty minutes no signal due to the H1(e) proton of the glycoside was distinguishable indicating that complete hydrolysis of the substrate had occurred. It was impossible to state whether the reaction catalysed by acid α-D-glucosidase had resulted in retention or inversion of configuration at C-1. The reaction catalysed by yeast α-D-glucosidase however, proceeds with retention of configuration, as shown.

Fig. 24

220 MHz ^{1}H pmr partial spectrum of phenyl α-D-glucopyranoside

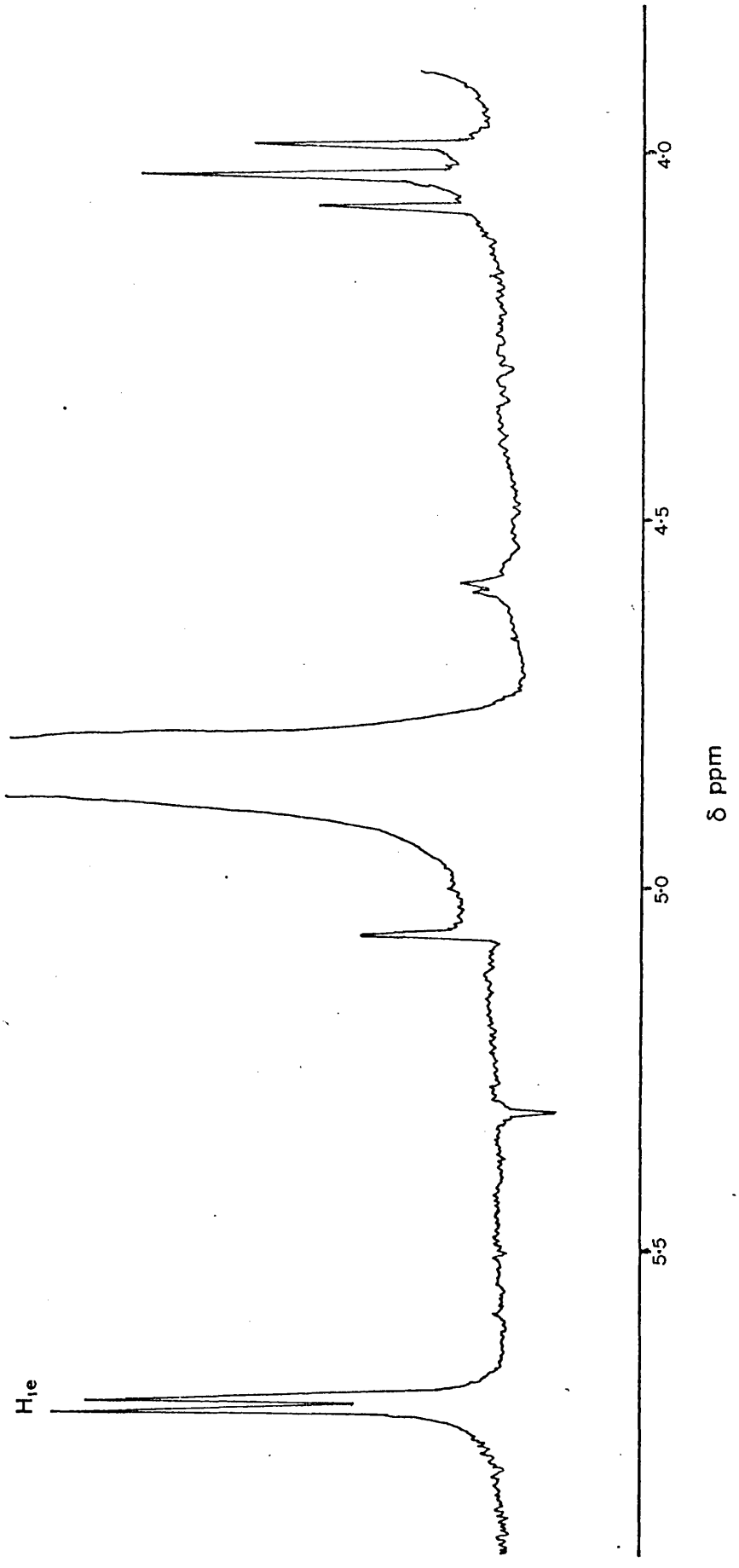


Fig. 25

220 MHz ^{1}H pmr partial spectrum of D-glucose

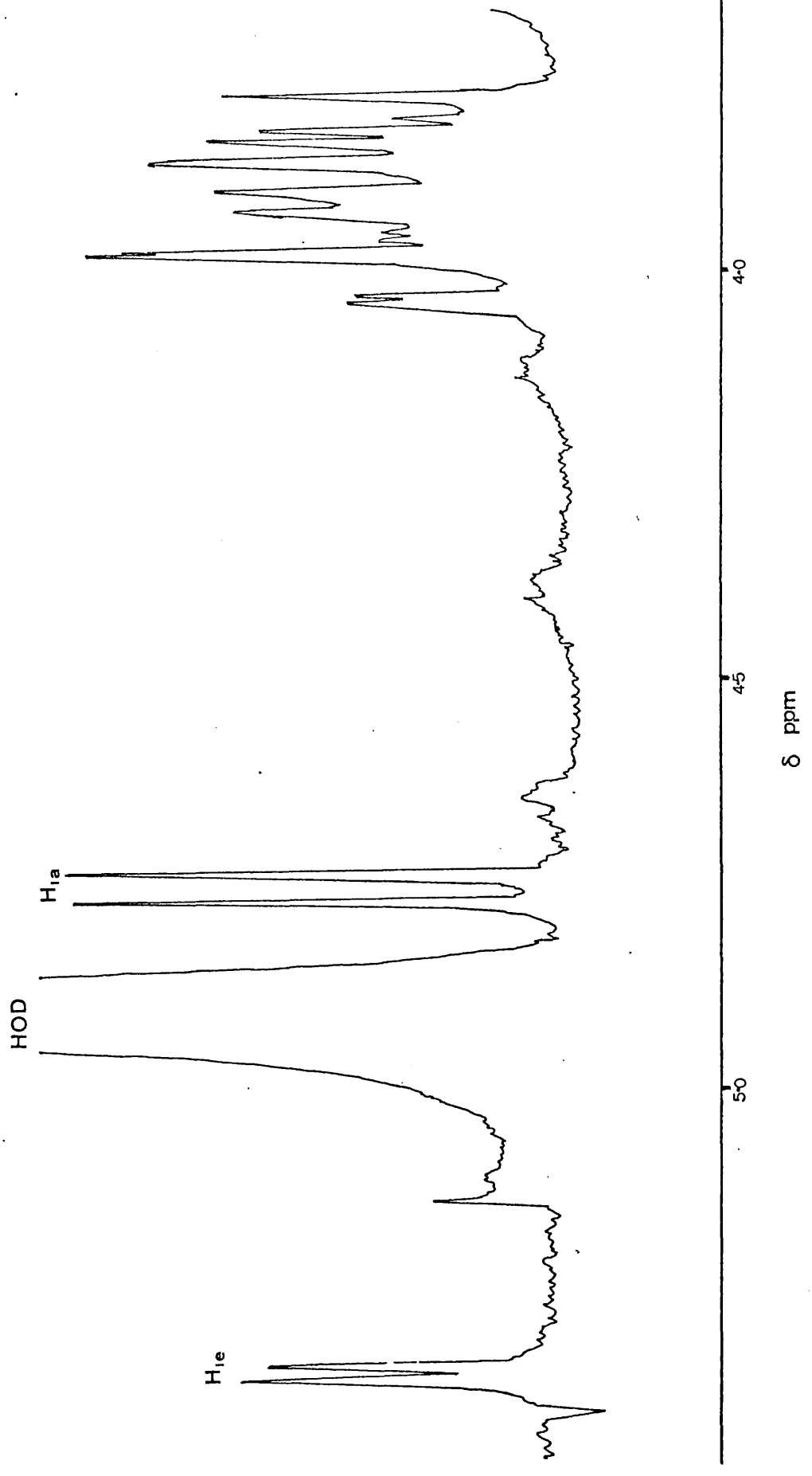
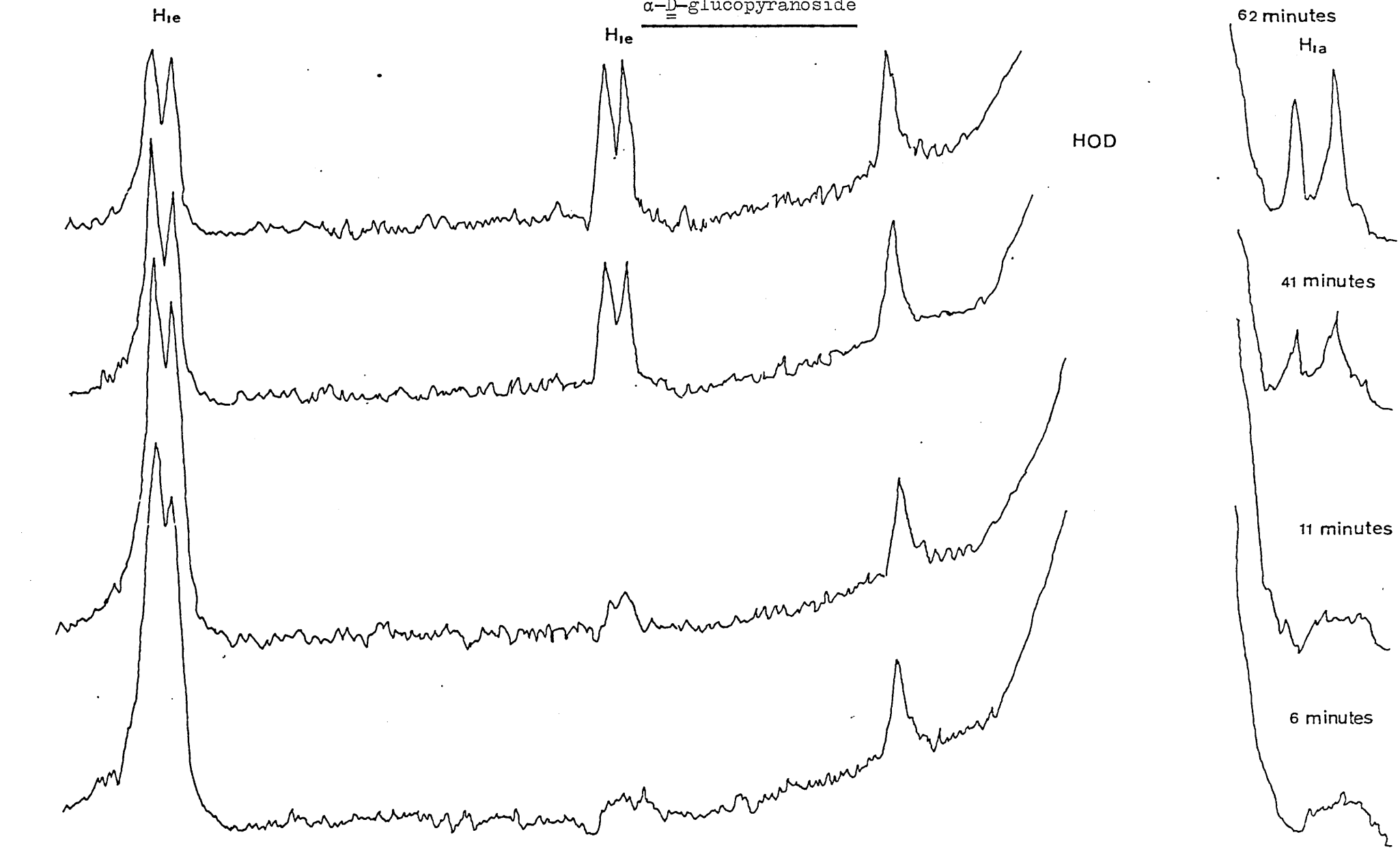
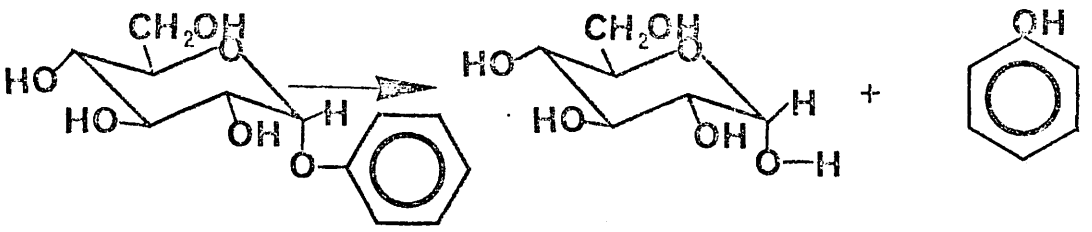


Fig. 26 Partial 220 MHz nmr spectra for the incubation of acid α-D-glucosidase with phenyl α-D-glucopyranoside





yeast α-D-glucosidase

In order to resolve the signals from the sugars under investigation it was thought necessary to use a spectrometer with a higher resolving capacity. Once resolved these peaks might be integrated. Experimental details are given in Experiment 14.

Figs 24 and 25 show a 220 MHz spectrum of phenyl α-D-glucopyranoside and D-glucose, respectively. Because the temperature could be controlled and an expanded scale used, all three proton peaks due either to the H1(e), or the H1(a) protons, could be resolved as doublets. The proton signal at δ 5.70 ppm in Fig. 24 was due to the H1(e) proton of the phenyl α-D-glucopyranoside. The proton signal at δ 5.34 ppm in Fig. 25 was due to the H1(e) of the α-D-glucopyranose and the signal at δ4.75 ppm due to the H1(a) of the β-D-glucopyranose. It was found that all three signals could be integrated successfully. Fig. 26 shows the spectra for the incubation of phenyl α-D-glucopyranoside with acid α-D-glucosidase with time. The H1(e) signal for α-D-glucopyranose (δ 5.34 ppm) was visible after six minutes from the start of the incubation. After eleven minutes the H1(a) signal for the β-D-glucopyranose (δ 4.74 ppm) was visible. Table 21 gives the values in mg of the concentration of substrate and α and β-D-glucose at various times after the start of the incubation. These values were obtained from standard spectra gaining the integrals of the relevant peaks, and thus correlating integrals of the standards with those obtained during the enzyme reaction. Where appropriate the concentration values for the α- and β-D-glucose

Table 21 Concentration of substrate, α-D-glucose, and β-D-glucose determined from NMR spectra for the enzyme reaction

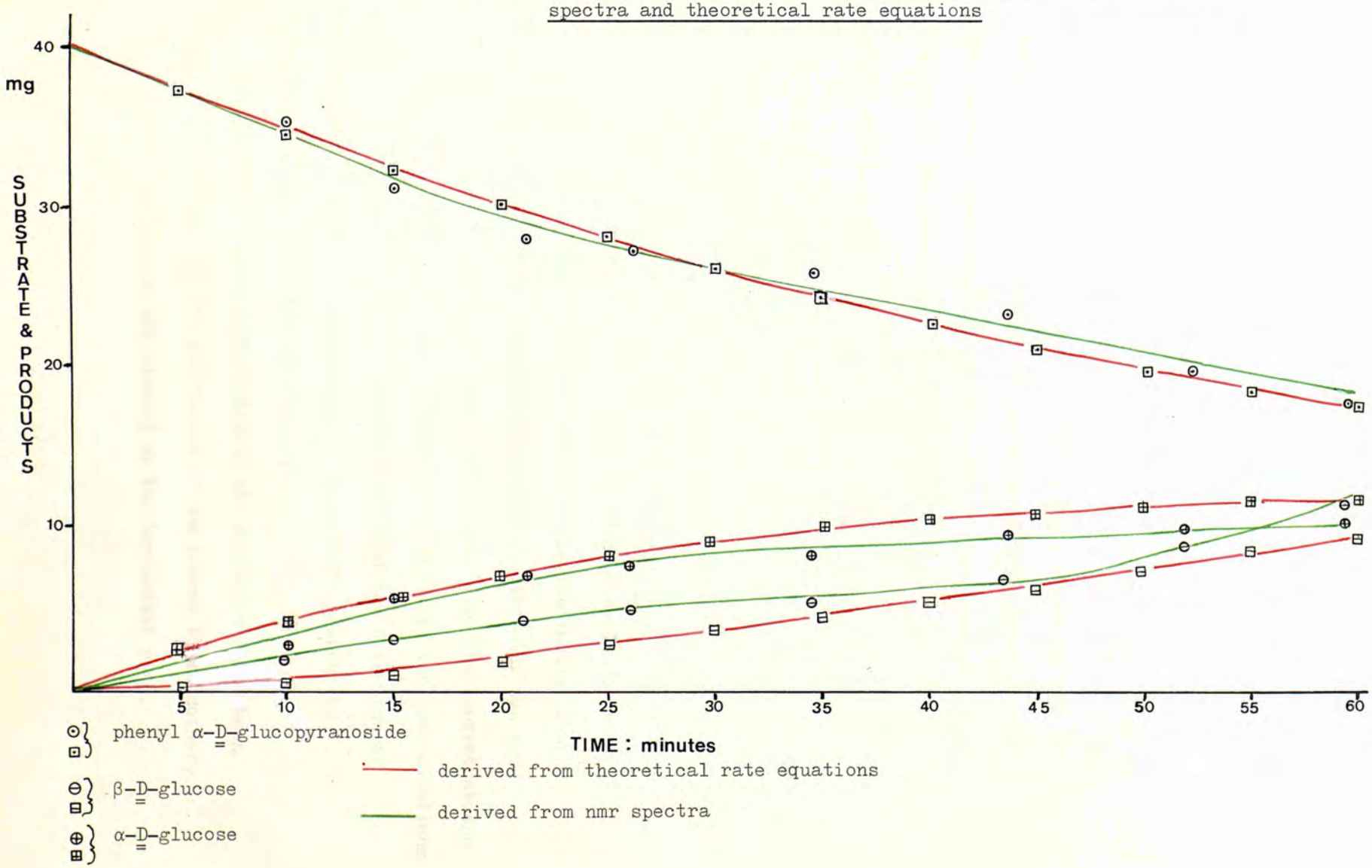
Time (Mins.)	Amount of substrate and products in mg					
	Not adjusted			Adjusted*		
	Substrate	α-D-glucose	β-D-glucose	Substrate	α-D-glucose	β-D-glucose
0	40 mg	0	0	40 mg	0	0
10	31.57	2.42	1.28	35.75	2.74	1.51
15	28.63	4.9	2.56	31.55	5.872	2.84
21	24.42	6.06	3.93	28.38	7.04	4.57
26	23.15	6.36	4.08	27.56	7.57	4.85
34.5	21.47	6.96	4.24	26.28	8.52	5.19
43.5	17.26	6.96	5.15	23.5	9.48	7.02
52	15.57	7.57	6.96	20.69	10.05	9.24
59.5	13.47	7.57	8.78	18.06	10.15	11.77
68.5	13.0	7.57	9.09	17.53	10.2	12.25
77	11.36	6.96	9.39	16.39	10.04	13.55
92.5	10.52	6.66	11.2	14.82	9.38	15.78
104	10.1	6.66	11.8	14.41	9.32	16.52
120	9.68	6.66	12.12	13.75	9.46	17.22
135	9.26	6.06	13.03	13.06	8.55	18.38
145	8.84	6.06	13.3	12.53	8.3	18.86

* These figures were adjusted so that:

[α-D-glucose]+[β-D-glucose]+[phenyl α-D-glucopyranoside] = 40 mg.

Fig. 27

Plot of concentration of substrate and products versus time derived from nmr spectra and theoretical rate equations



and for the glucoside were adjusted, to equal the concentration of starting product, where:

$$[\alpha\text{-}\underline{\underline{D}}\text{-glucose}]_t + [\beta\text{-}\underline{\underline{D}}\text{-glucose}]_t + [\text{phenyl } \alpha\text{-}\underline{\underline{D}}\text{-glucopyranoside}]_t = 40 \text{ mg}$$

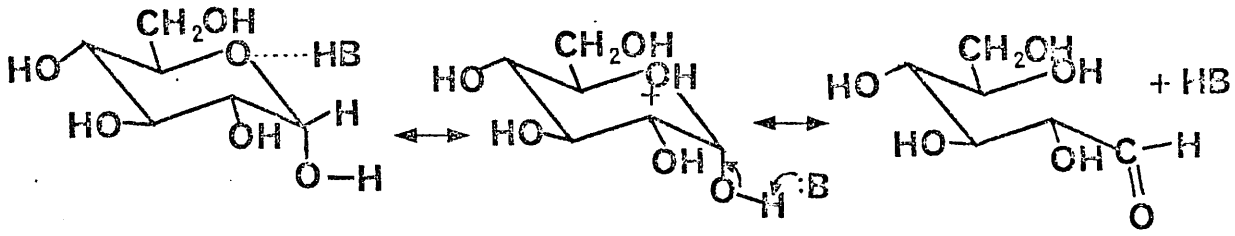
A plot of the concentrations of substrate and product against time is shown in Fig. 27. Initially it can be seen that the product of the reaction was α-$\underline{\underline{D}}$-glucose and later due to the mutarotation of the α-anomer to the β-anomer, after fifty five minutes:

$$[\alpha\text{-}\underline{\underline{D}}\text{-glucose}] = [\beta\text{-}\underline{\underline{D}}\text{-glucose}]$$

After this time the rate of formation of α-$\underline{\underline{D}}$-glucose is less than the formation of β-$\underline{\underline{D}}$-glucose.

Although after six minutes the signal for the H1(e) proton of the α-$\underline{\underline{D}}$-glucopyranose was visible, no figures for the concentration of the α or β-$\underline{\underline{D}}$-glucose were obtainable. Thus, no evidence is available regarding the mechanism exerted during the initial stages of the incubation when the sensitivity of the spectrometer was too low to detect the different anomers. The figures for the concentrations of substrate and products with time (Table 21) describe the concentrations of each component in solution, and we assume that the direction of mutarotation is from the α-anomer to the β-anomer. Hence, the β-$\underline{\underline{D}}$-glucose arises from the α-$\underline{\underline{D}}$-glucose.

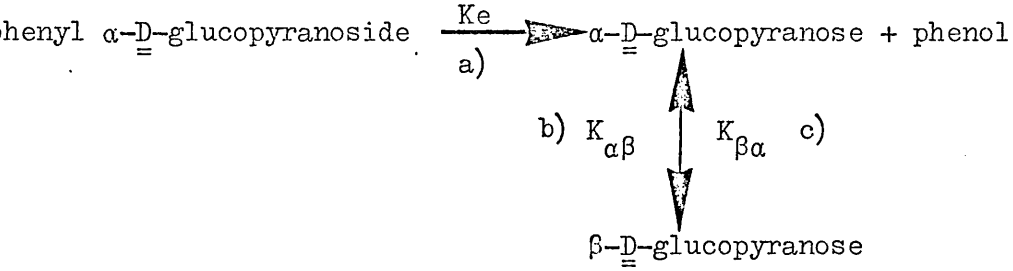
Mutarotation is acid-base catalysed and mechanism A has been suggested to account for the production of one anomer from another. This involves the opening and closing of the hemiacetal ring.



Mechanism A

Temperature and pH have an effect upon the rate of mutarotation.[116] As the reaction of acid-D-glucosidase with phenyl α-D-glucopyranoside was carried out in acid media at 31°C it would be expected that mutarotation would occur quite rapidly. It was suggested that if a series of rate equations could be derived to account for the mutarotation reaction a comparison could be made between the results obtained, using the rate equations, and these found experimentally.

Given:



the decay of substrate into α- and β-D-glucose is proportional to the amount of substrate. Therefore, the differential equation describing the process should be:

1 $$\frac{dS}{dt} = - KeS$$

where: Ke = rate constant for reaction a)
t = time
S = substrate concentration

The rate of change of α-D-glucose on the other hand is proportional to the amount of substrate and β-D-glucose, as well as the amount of α-D-glucose itself, hence:

2 $\frac{d\alpha}{dt} = KeS + K_{\beta\alpha}\beta - K_{\alpha\beta}\alpha$

where: α = amount of α-D-glucose

β = amount of β-D-glucose

$K_{\alpha\beta}$ = rate constant for reaction b)

$K_{\beta\alpha}$ = rate constant for reaction c)

Finally the rate of change of β-D-glucose is proportional only to the amount of α-D-glucose and β-D-glucose, namely

3 $$\frac{d\beta}{dt} = K_{\alpha\beta}\alpha - K_{\beta\alpha}\beta$$

With the aid of equations 1 , 2 , and 3 it is seen that the coefficients K_e, $K_{\alpha\beta}$, and $K_{\beta\alpha}$ describe the reactions completely. Summing the above three equations gives:

$$\frac{d}{dt}(S + \alpha + \beta) = 0$$

with the solution.

4 $$S_{(t)} + \alpha_{(t)} + \beta_{(t)} = S_o$$

where S_o is the amount of substrate at the beginning of the reaction.

The first equation is solved giving:

5 $$\boxed{S_{(t)} = S_o e^{-K_e t}}$$

Since we have already two solutions, 4 and 5 , it is enough to solve one of the equations 2 or 3 to complete the solution. For instance, with the aid of solution 4 and 5 , equation 2 becomes:

$$\frac{d\beta}{dt} + V\beta = K_{\alpha\beta}S_o - K_{\alpha\beta}S_o e^{-K_e t}$$

where $V = K_{\alpha\beta} + K_{\alpha\beta}$.

This is an inhomogeneous 1st order equation and the solution is the sum of the homogeneous part $\beta_H(t)$:

$$\beta_H(t) = Ae^{\lambda t}$$

and the inhomogeneous part $\beta_I(t)$

$$\beta_I(t) = B + Ce^{-K_e t}$$

where A, B, C and λ are constants. Substituting these into the equation for β gives:

$$\lambda = -V$$

$$B = \frac{K_{\alpha\beta} S_o}{V}$$

$$C = -\frac{K_{\alpha\beta} S_o}{V - K_e}$$

The constant A can be easily determined from the initial value of:

$$\beta(o) = 0$$

giving

$$A = S_o \frac{K_e K_{\alpha\beta}}{V(V-K_e)}$$

thus the complete solution for β is:

$$6 \quad \boxed{\beta_{(t)} = S_o \left[\frac{K_{\alpha\beta}}{V} - \frac{K_{\alpha\beta}}{V-K_e} e^{-K_e t} + \frac{K_e K_{\alpha\beta}}{V(V-K_e)} e^{-Vt} \right]}$$

Fig.28

Plot of $\log \frac{a}{a-x}$ versus time for the enzyme reaction

where a = conc. of substrate at t = 0
x = conc. of substrate at time t.

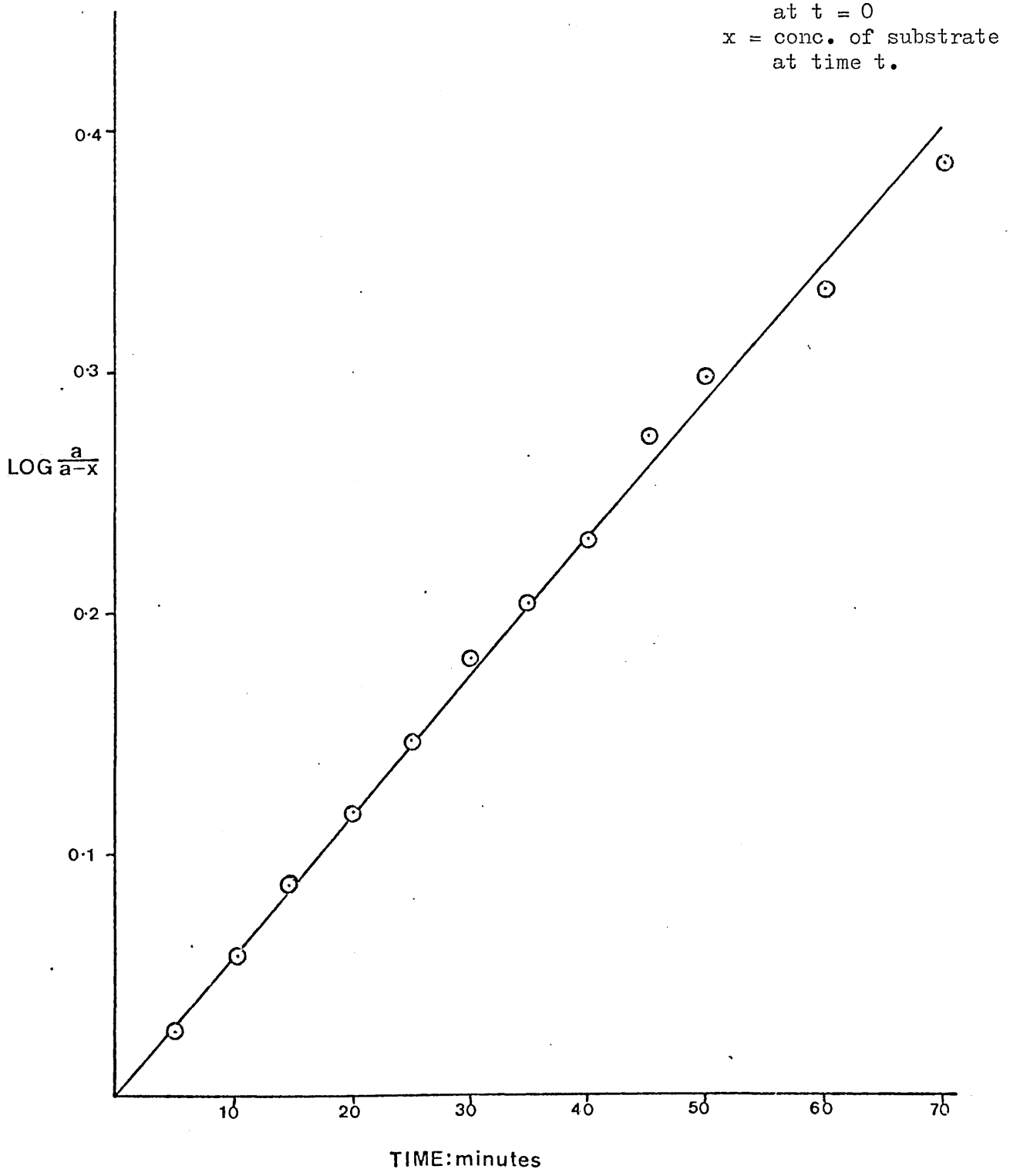


Fig. 29 Calculation of K_e $K_{\alpha\beta}$ $K_{\beta\alpha}$

Calculation of the rate constant K_e for the enzyme reaction

From Fig. 28 the slope of the straight line

$$\text{slope} = \frac{K_e}{2.303} \qquad \text{slope} = \frac{0.2309}{40} = 5.77 \times 10^{-3}$$

for the enzyme reaction: $K_e = (5.77 \times 10^{-3}) \times 2.303$

$$\boxed{K_e = 13.3 \times 10^{3}\ \text{min}^{-1}}$$

Calculation of the rate constants ($K_{\alpha\beta}$, $K_{\beta\alpha}$) for the mutarotation reaction

$$\alpha \underset{K_{\beta\alpha}}{\overset{K_{\alpha\beta}}{\rightleftharpoons}} \beta \quad \text{reversible 1st order reaction}$$

1 equilibrium constant $K_{\equiv} = \dfrac{[\beta]_{\equiv}}{[\alpha]_{\equiv}} = 1.777$

2 rate constant $K = K_{\alpha\beta} + K_{\beta\alpha}$

K = slope x 2.303

from Fig. 30 slope $= \dfrac{0.36}{20} = 0.018$

$K = 0.018 \times 2.303 = 0.041454$

from 1 as $K_{\alpha\beta} = 1.77\ K_{\beta\alpha}$

3 Therefore $K = 1.77\ K_{\beta\alpha} + K_{\beta\alpha}$

$$0.041454 = 2.77\ K_{\beta\alpha}$$

$$\boxed{K_{\beta\alpha} = 14.9 \times 10^{-3}\ \text{min}^{-1}}$$

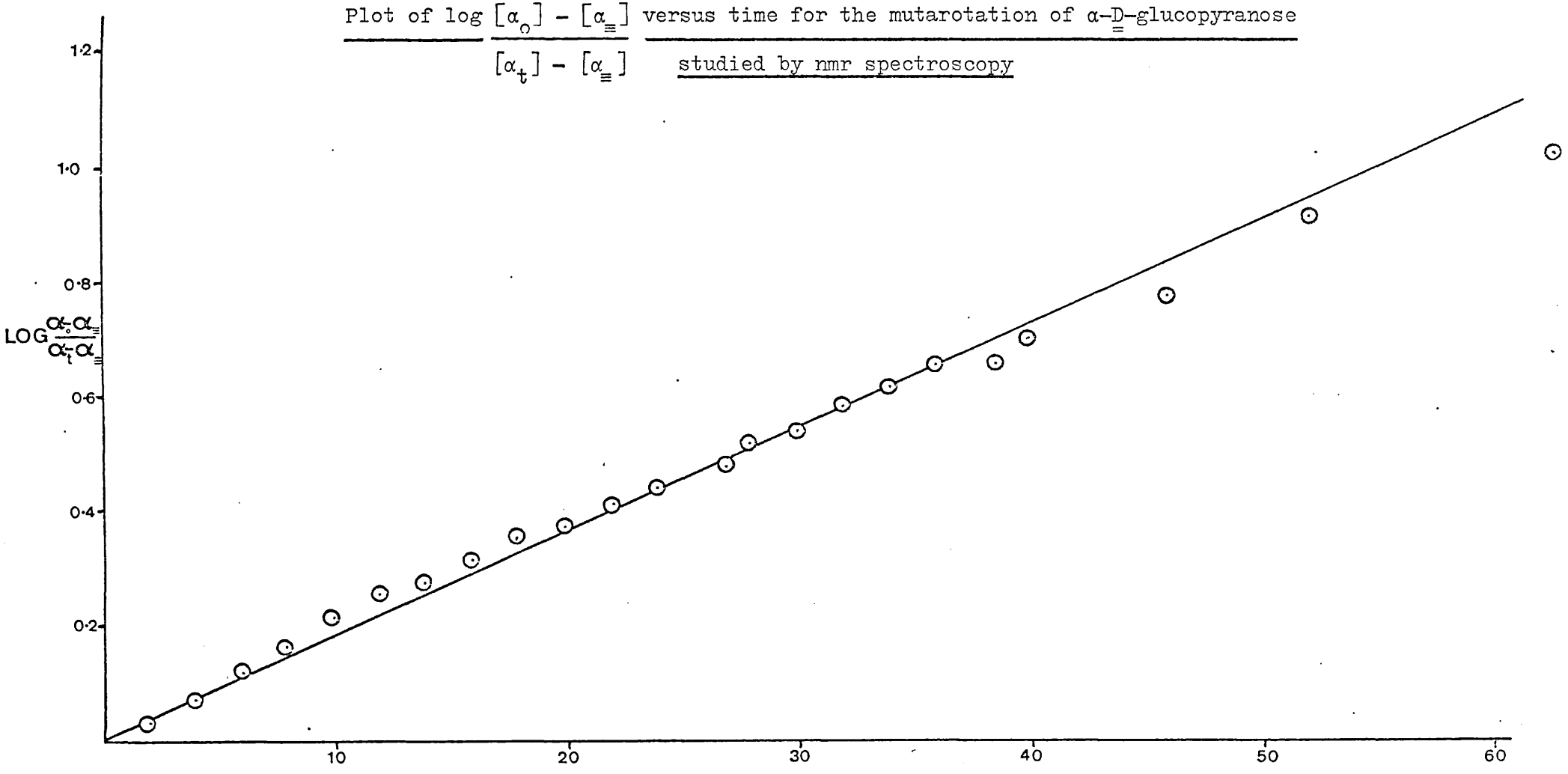
Substituting into 2

$$0.041454 = 14.9 \times 10^{-3} + K_{\alpha\beta}$$

$$\boxed{K_{\alpha\beta} = 26.4 \times 10^{-3}\ \text{min}^{-1}}$$

Fig. 30

Plot of $\log \frac{[\alpha_o] - [\alpha_\infty]}{[\alpha_t] - [\alpha_\infty]}$ versus time for the mutarotation of α-D-glucopyranose studied by nmr spectroscopy



α_o = conc. of α-D-glucose at t = 0.

α_t = conc. of α-D-glucose at time t

α_∞ = conc. of α-D-glucose at equilibrium

Finally with the aid of the above solution and equations 4 and 5 the solution for α is:

7 ◈
$$\alpha(t) = S_o \frac{K_{\beta\alpha}}{V} + \frac{-K_{\beta\alpha} + K_e e^{-K_e t}}{V - K_e} - \frac{K_e K_{\alpha\beta}}{V(V-K_e)} e^{-Vt}$$

Thus by using equations 5, 6 and 7 the amounts of substrate, α-and β-D-glucose with time can be calculated.

Fig. 28 shows the plot of log $\frac{a}{a-x}$ versus time for the enzyme reaction. The straight line indicated that the reaction was 1st order. The rate constant (K_e) was calculated (Fig. 29) from the slope of the curve. To determine the rate constants ($K_{\alpha\beta}$, $K_{\beta\alpha}$) for the mutarotation reaction the reaction was studied under the same conditions present for the enzyme reaction. Experimental details are given in Experiment 14. A plot of log $\frac{[\alpha_o]-[\alpha_\infty]}{[\alpha_t]-[\alpha_\infty]}$ versus time is shown in Fig. 30. A straight line curve indicated a 1st order reaction. The rate constants ($K_{\alpha\beta}$, $K_{\beta\alpha}$) were calculated (Fig. 29) from the slope of the straight line. Using the theoretically derived equations, the concentration of substrate, α- and β-D-glucose with time, was found. A plot is shown in Fig. 27 of the concentration of each versus time. If a comparison is made of both sets of curves one set derived experimentally the other theoretically. The curves show a good resemblance. Values for the concentration of α-D-glucose and β-D-glucose derived theoretically for the initial times that integration was not possible are shown in Table 22. The ratio of $\frac{\alpha}{\beta}$ shows that the

◈ These equations were derived by Dr E. Detyna.

Table 22 Concentration of products with time derived from rate equations

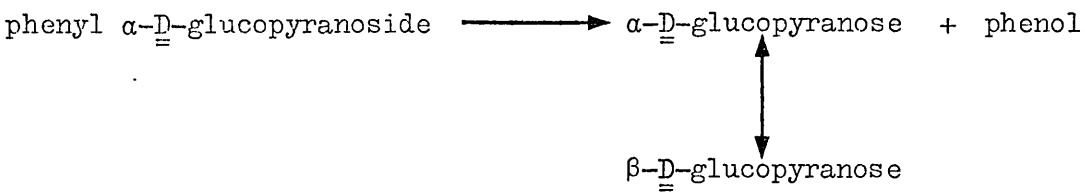
Time	α-anomer mg	β-anomer mg	ratio $\frac{\alpha}{\beta}$
12 secs.	0.10597	0.000280	378
24 "	0.211120	0.001115	192
36 "	0.31542	0.002501	126
48 "	0.41891	0.004429	95
60 "	0.52158	0.006896	77
2 mins	1.02288	0.027090	38
3 mins	1.5047	0.05986	25
4 mins	1.96785	0.10453	19
5 mins	2.41304	0.160437	15

α-anomer is always present at a far higher concentration than the β-anomer.

In order to definitely state that the concentrations of substrate and products arise purely through the enzymic hydrolysis of the substrate, and through the mutarotation of the product, consideration has to be given to other reactions which may alter the concentrations of the substrate and products. Although transglycosylase activity has been associated with the properties of the enzyme from other sources no information is available in the literature which suggests

that phenol may be an acceptor and glucose a donor. Most transglycosylase studies have utilised maltose as the donor which on hydrolysis donates a glucosyl residue to an acceptor.[16,27] Incubation of glucose with acid α-D-glucosidase did not result in the formation of any oligosaccharides. Confirming previous results by other workers that glucose does not act as an acceptor. It is unlikely that the enzyme would exert such activity at high substrate concentrations. If this activity was being exerted by the enzyme to any significant extent one would not expect both sets of curves to show such a similarity (Fig. 27). This would be expected as transglycosylase activity was not considered in the reaction pathway used to derive the rate equations.

As the enzyme reaction was carried out in acid media it was necessary to discover whether any acid hydrolysis of the phenyl α-D-glucopyranoside occurred. Using the same conditions as used during the enzyme hydrolysis no α- or β-D-glucose was detected, using nmr spectroscopy over a period of five hours. It was thus concluded that the concentration of substrate and products were obtained purely through the reactions shown:



The results obtained in this section confirm that acid α-D-glucosidase catalyses the hydrolysis of phenyl α-D-glucopyranoside with retention of configuration i.e. anomeric configuration is retained.

Investigations into the mechanism of action of the glycosidases is at a very early stage. Up to the present time with insufficient knowledge concerning the groups present at the active site and the structure of the intermediates involved in the reactions, few conclusions can be made.

Two mechanisms have been proposed for glycosidase action in which the anomeric configuration of the substrate is retained in the product. Firstly the general acid catalysis mechanism proposed by Phillips[55] (Fig. 2) and secondly the double displacement reaction proposed by Koshland[117] (Fig. 1). Both are discussed in detail in chapter I.1.D.

The general acid catalysis reaction is thought to proceed via an oxy-carbocation. Evidence to confirm this mechanism has come from inhibitor[118,119] and kinetic isotope[120] experiments both of which have suggested that an oxy-carbocation may be formed.

Evidence to suggest a double displacement mechanism has come from kinetic[121] and active site inhibitor[122,123] studies, and from the isolation of a glucosylenzyme intermediate,[59] which is thought to play an important part in the mechanism.

III Effect of pH on the rate of enzyme hydrolysis

The enzyme was incubated with substrate between pH 4.0 to 8.0 as described in Experiment 15.

The effect of pH on the maltase and dextranase activity of the purified enzyme is shown in Fig. 31. The enzyme was active over a wide range but showed maximum dextranase and maltase activity between 4.0 to 5.2, thus indicating that both activities are a property of one enzyme which prefer on acid environment for maximal activity.

All subsequent enzymic reactions carried out with the purified enzyme were buffered at pH 4.6. Other mammalian acid α-D-glucosidases show a similar effect of pH on their activity.[19,26]

IV Thermal Stability of the enzyme

In order to acknowledge the range of temperature that the enzyme is capable of working over the stability of the enzyme at various temperatures was investigated. The enzyme was preincubated for a fixed time, cooled and incubated with substrate as detailed in Experiment 16. Fig. 32 shows the thermal stability of the maltase and dextranase activities of the purified enzyme. Both activities were stable up to 45°C and then progressive deactivation occurred until complete deactivation at 70°C. These results confirmed the stability of the enzyme at the chosen temperature of incubation of 37°C.

V pH Stability of the enzyme

The enzyme was preincubated in acetate and phosphate buffers as detailed in Experiment 17. Fig. 33 shows the effect of preincubation in buffer of different pH, on the maltase activity of the purified enzyme. The enzyme was found to be stable up to pH 6.8 and then it was progressively deactivated until it was nearly completely deactivated at pH 8.0.

Experiments C III, IV, V have shown that the enzyme exerts both maltase and dextranase activity and that both activities are shown to have similar stabilities. From these results it is clear that no neutral glucosidase is present in the extract.

Fig. 31

Effect of pH on the rate of enzymic hydrolysis

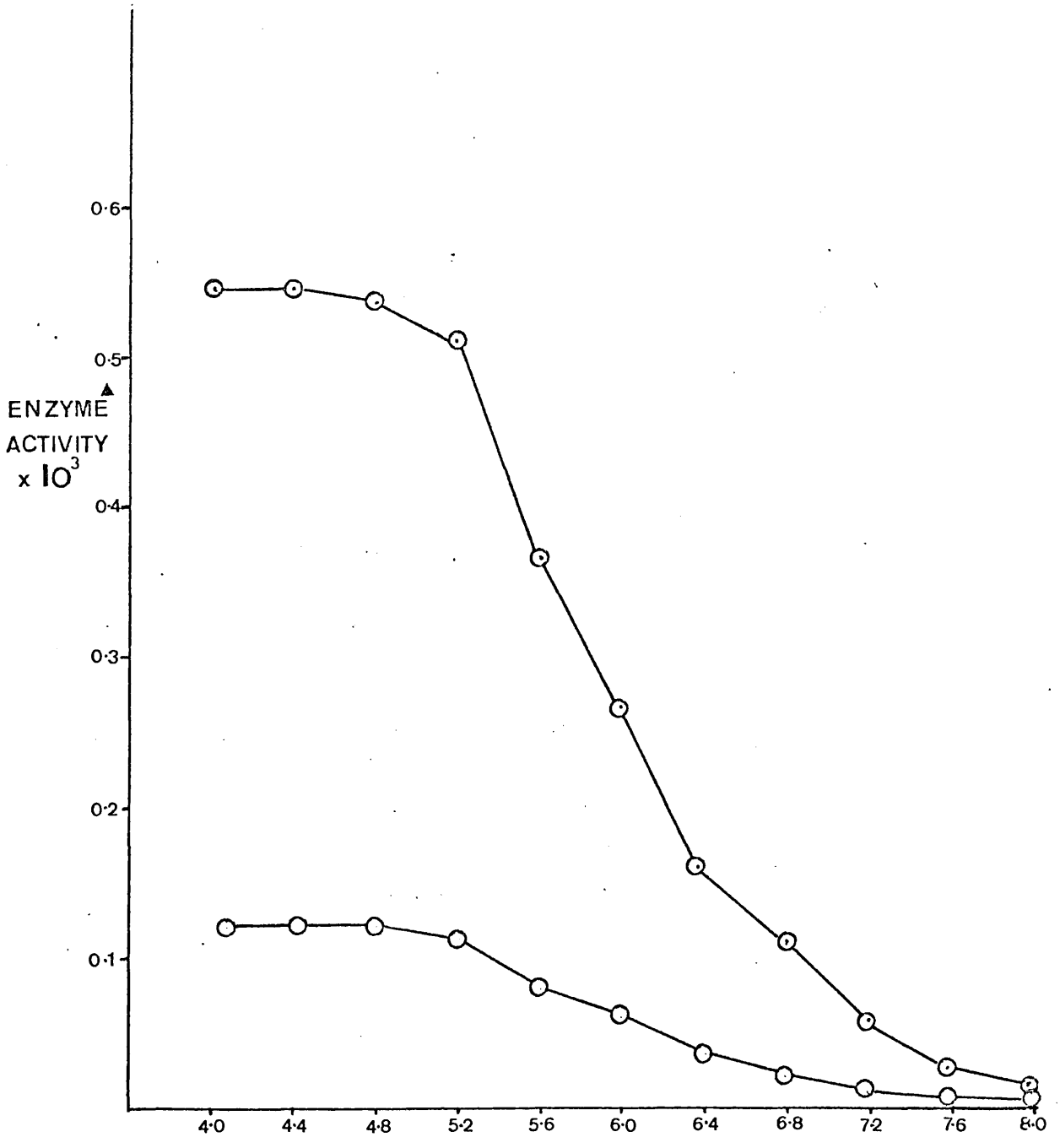
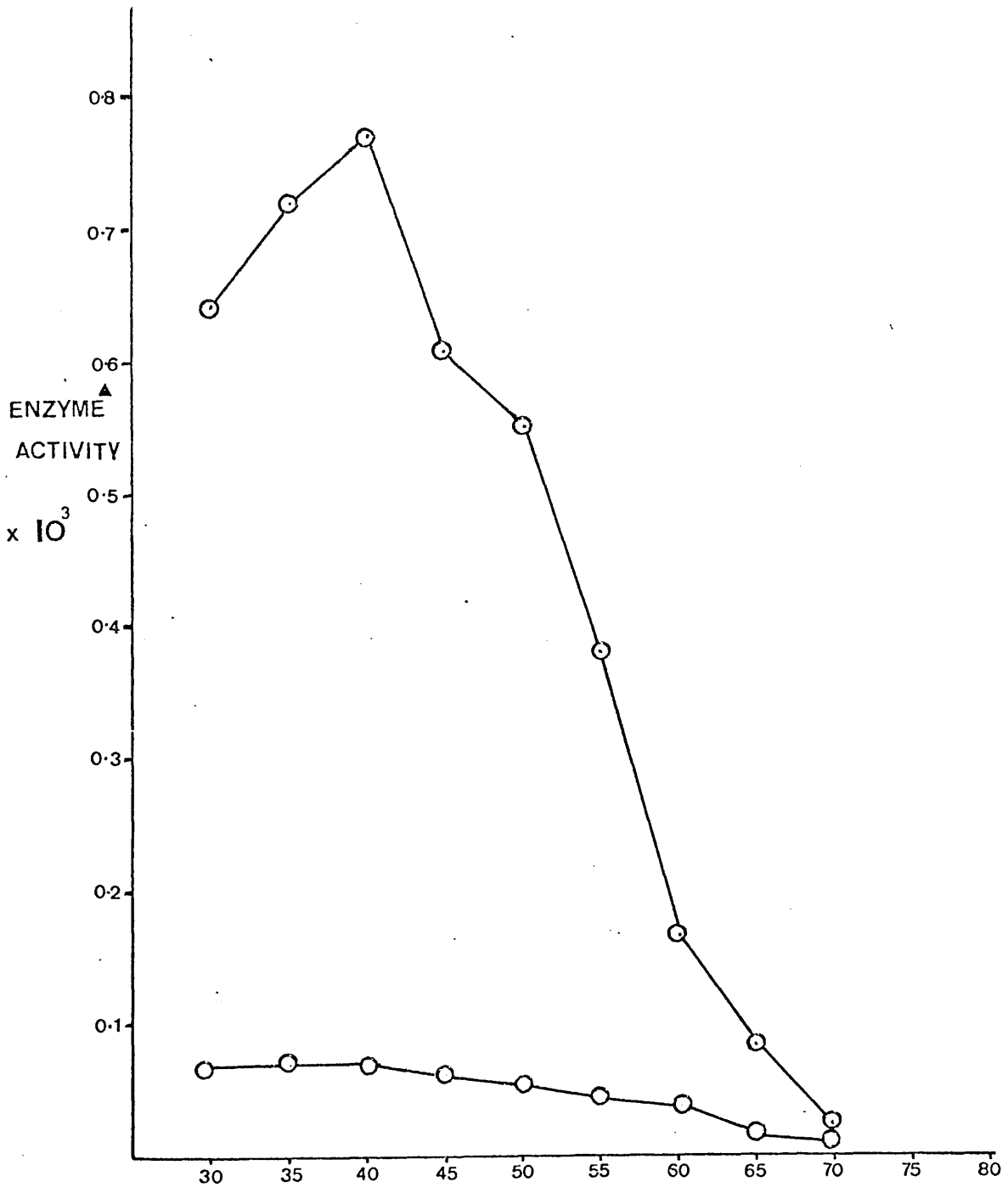


Fig. 32

Effect of temperature on the stability of the maltase and dextranase activity of acid α-D-glucosidase



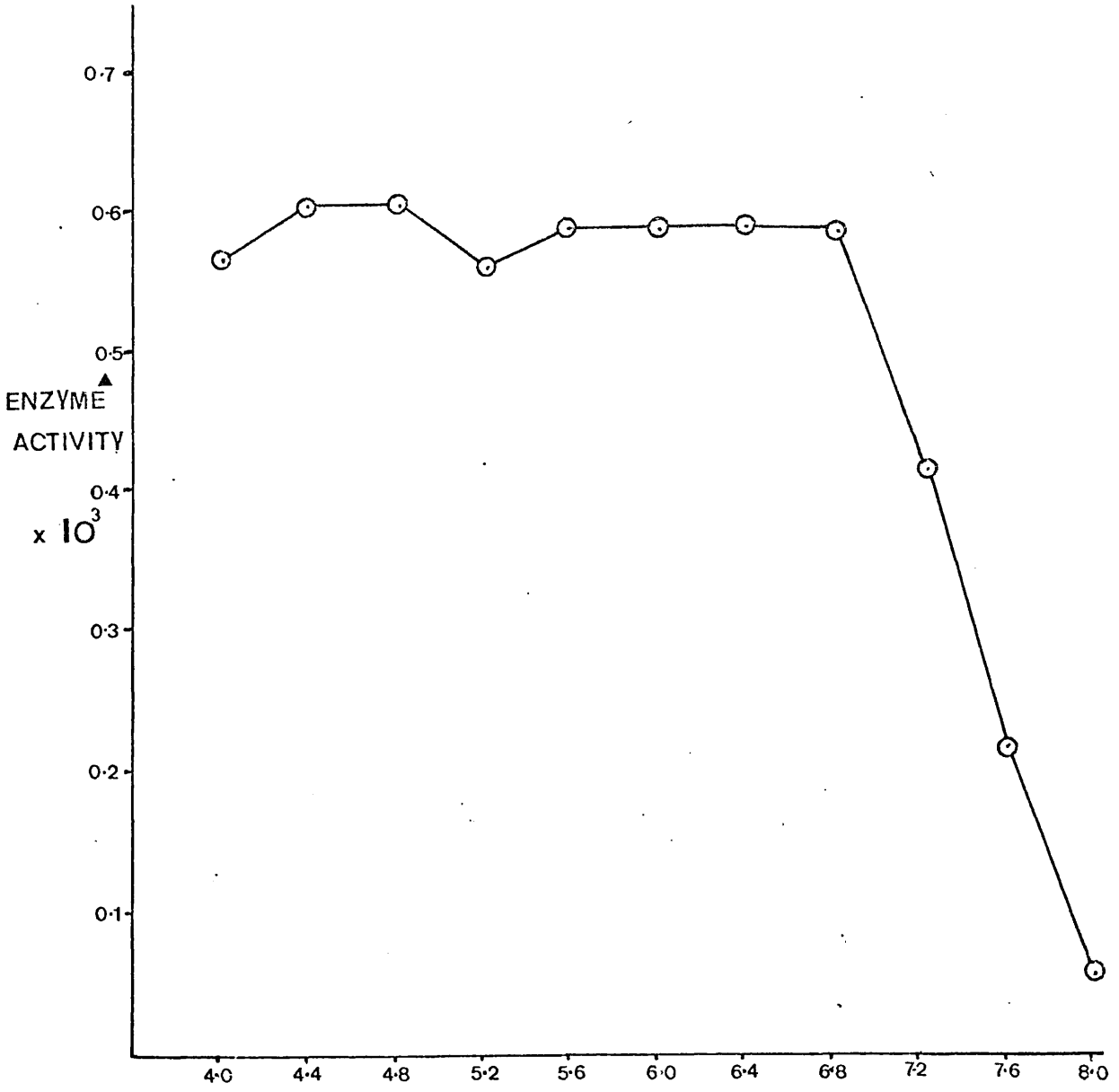
⊙ Maltose digest

O Dextran digest

▲ Expressed as nmoles glucose liberated.

Fig. 33

Effect of pH on the stability of the enzyme



▲ Expressed as nmoles glucose liberated.

Storage of the purified enzyme at 4°C in acetate buffer for several months did not lead to any great decrease in the catalytic activity of the enzyme. The enzyme was also found to be stable to freeze drying over short periods of time as there was no decrease in the maltase activity of a freeze-dried sample. The pH and thermal stability results demonstrated are in good agreement with those found for the bovine spleen[102] and pig spleen enzymes.[31]

VI Action on Glycosides and Disaccharides to show its broad specificity

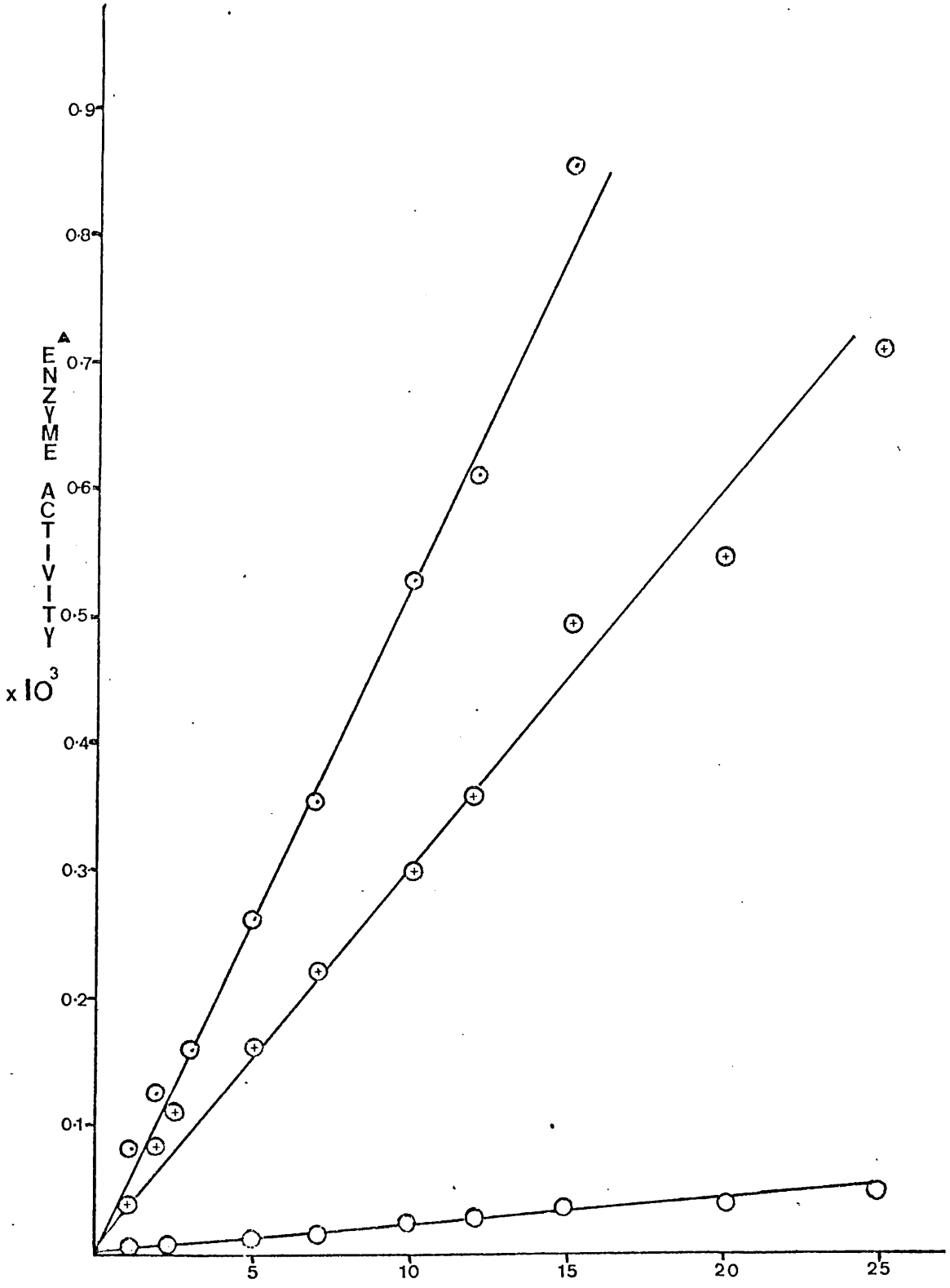
An investigation by other workers on the enzymic properties of the enzyme from other sources has shown that not only is it able to hydrolyse α-(1→4) linkages in disaccharides but also α-(1→2) α-(1→3) and α-(1→6) linkages.[20,31,16] Thus, the enzyme has been designated as having a broad specificity. This property is further enhanced by its ability to hydrolyse α-D-glucosides to produce D-glucose and the corresponding alcohol. In order to determine whether the acid α-D-glucosidase from pig's liver had similar properties to those shown by acid α-D-glucosidases from other sources, an investigation was carried out to determine whether the enzyme was able to hydrolyse a series of α-link glucosyl disaccharides and α-D-glucosides.

All the substrates required were readily available except isomalto-oligosaccharides which were prepared as described in Experiment 18. The incubations were carried out as described in Experiment 19.

Fig. 34 shows an activity time plot for several α-glycosides. The relative rates are given in Table 23.

Fig. 34

Activity versus time for the incubation of acid α-D-glucosidase with α-glycosides



⊙ p-nitrophenyl α-D-glucopyranoside digest

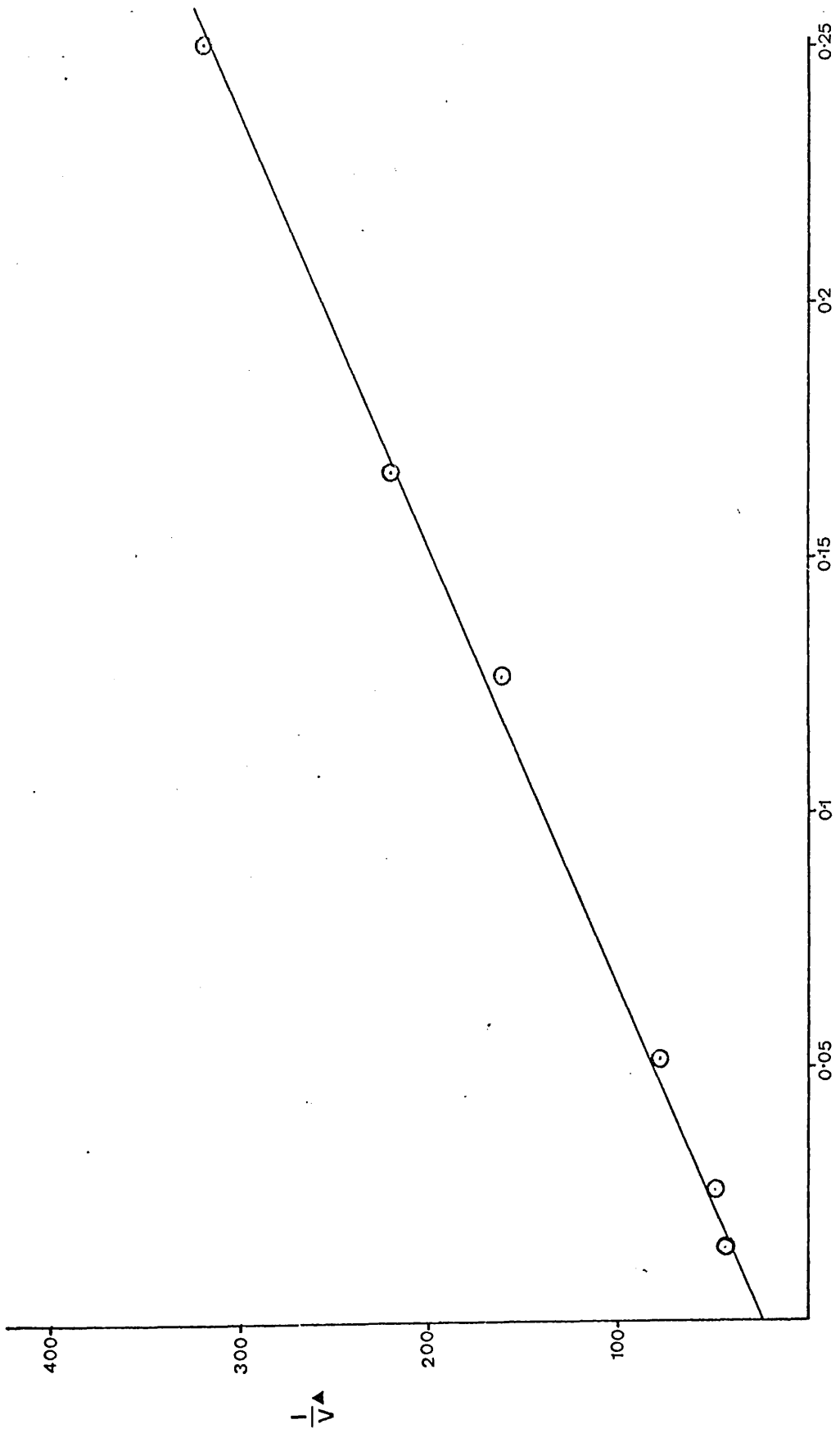
⊕ phenyl α-D-glucopyranoside digest

○ methyl α-D-glucopyranoside digest

▲ Expressed as nmoles glucose liberated.

Fig. 35

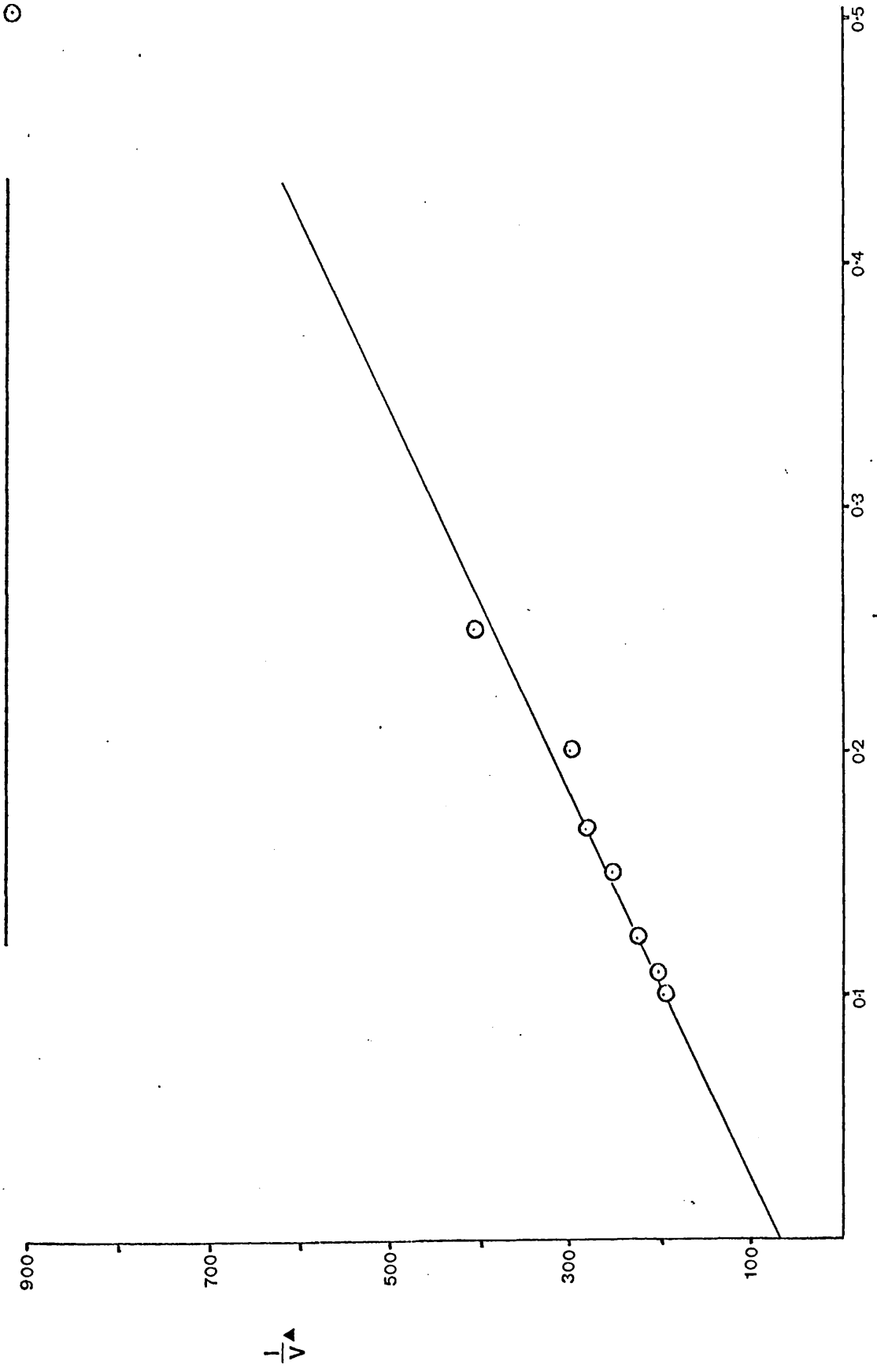
Lineweaver-Burke plot for Kojibiose



▲ Velocity expressed as mmoles glucose liberated per minute per mg protein.

Fig. 36

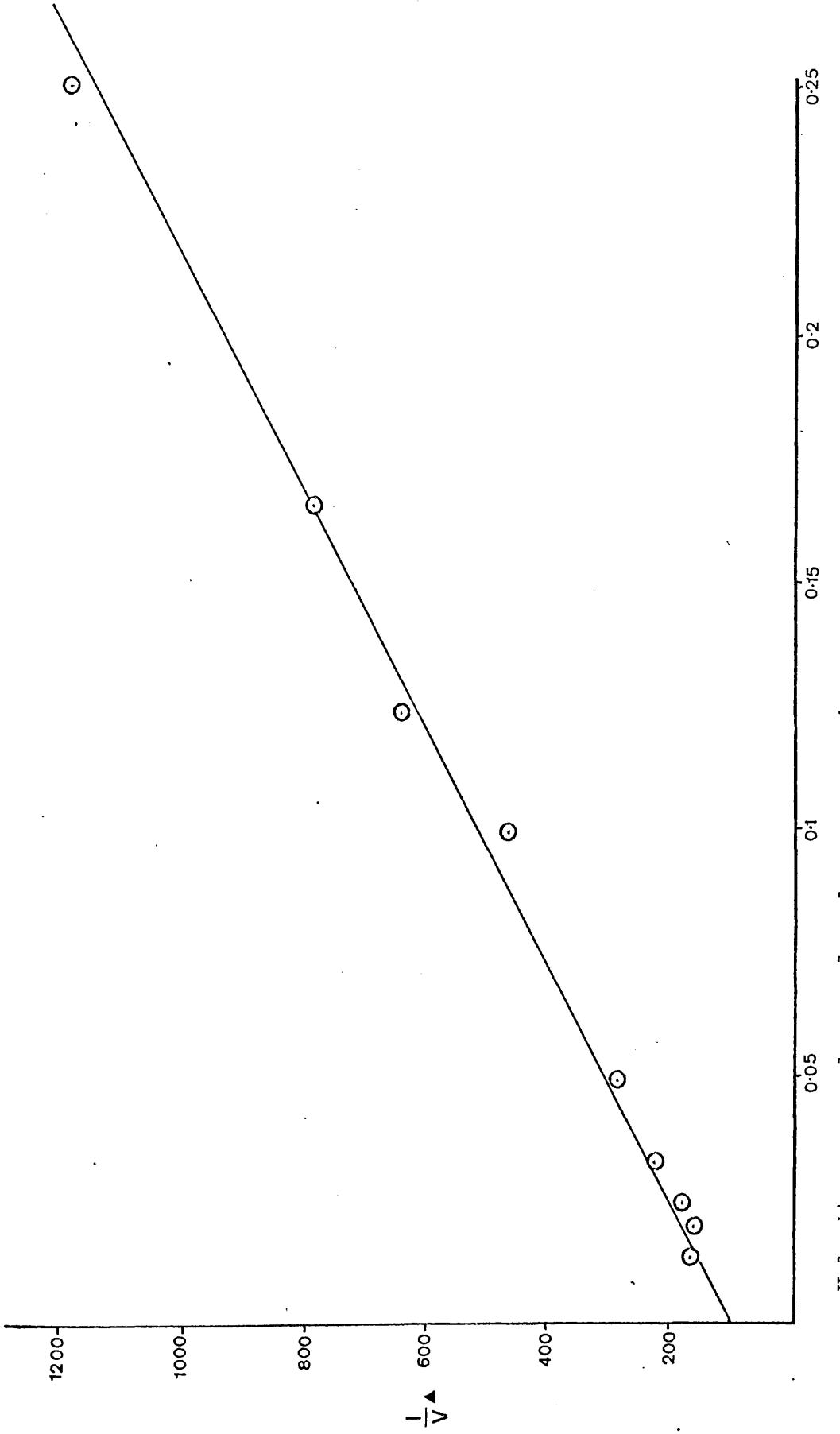
Lineweaver-Burke plot for p-nitrophenyl α-D-glucopyranoside



▲ Velocity expressed as mmoles glucose liberated per minute per mg protein.

Fig. 37

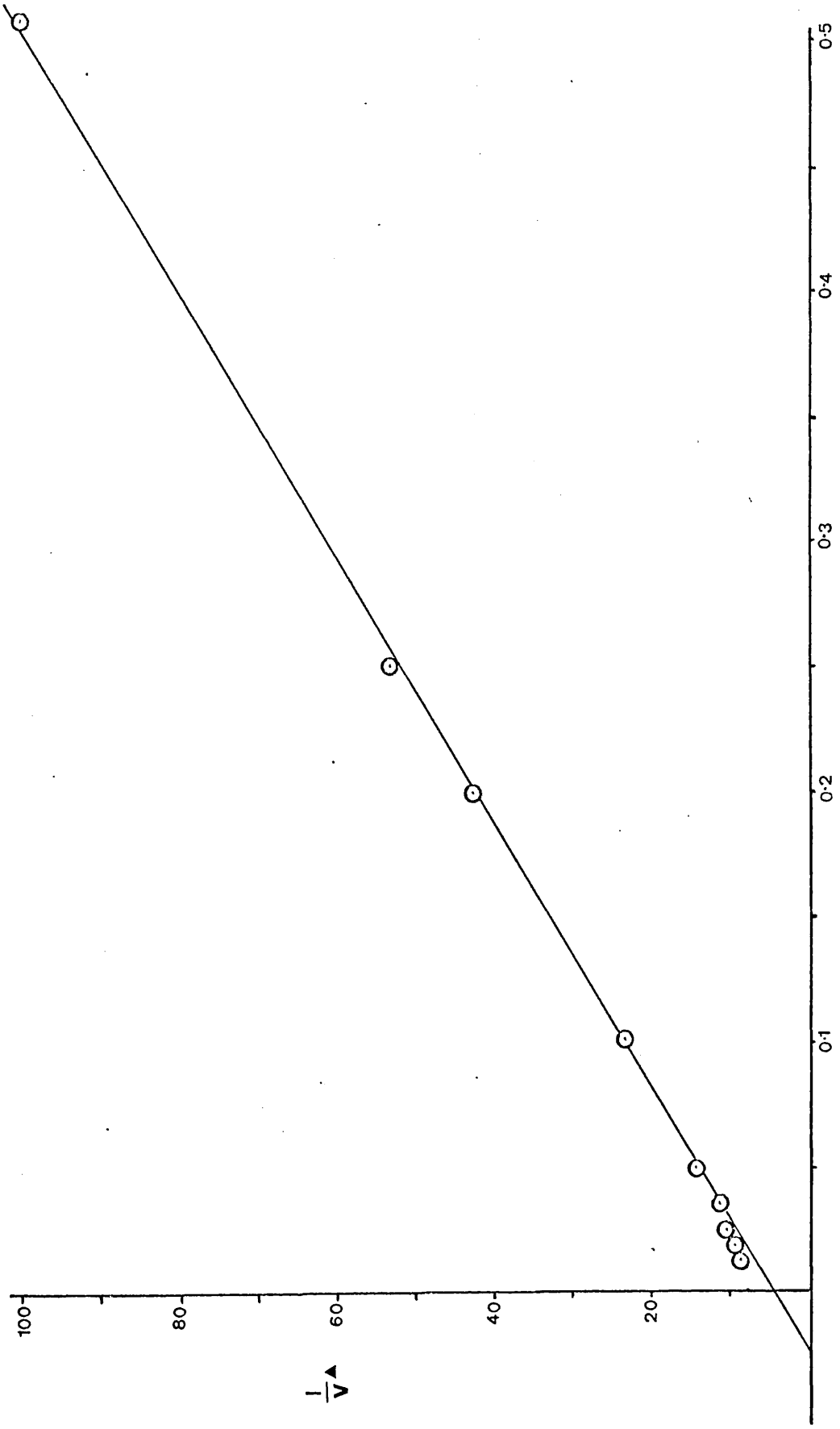
Lineweaver-Burke plot for isomaltose



▲ Velocity expressed as mmoles glucose liberated per minute per mg protein.

Fig. 38

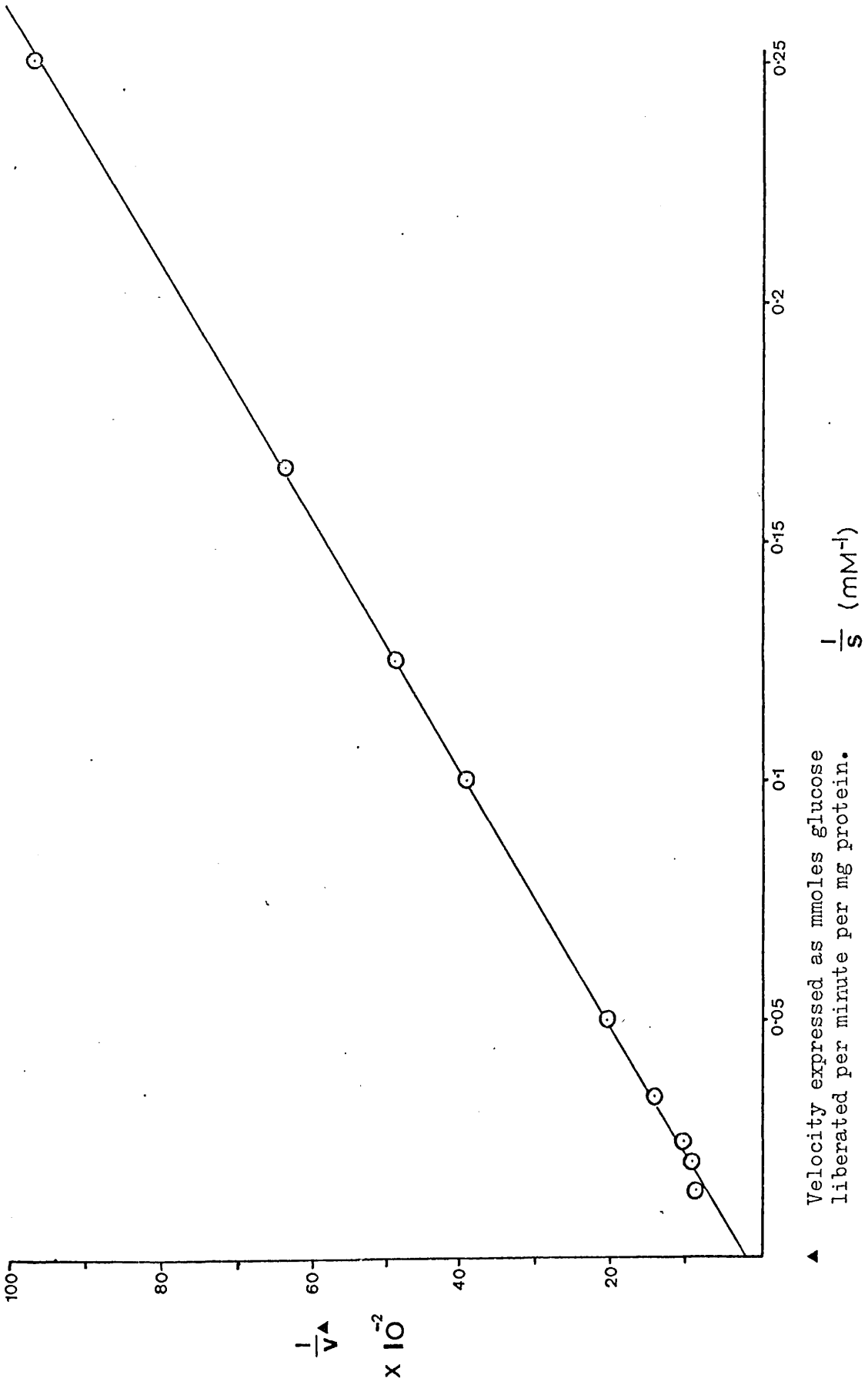
Lineweaver-Burke plot for maltose



▲ Velocity expressed as mmoles glucose liberated per minute per mg protein.

Fig. 39

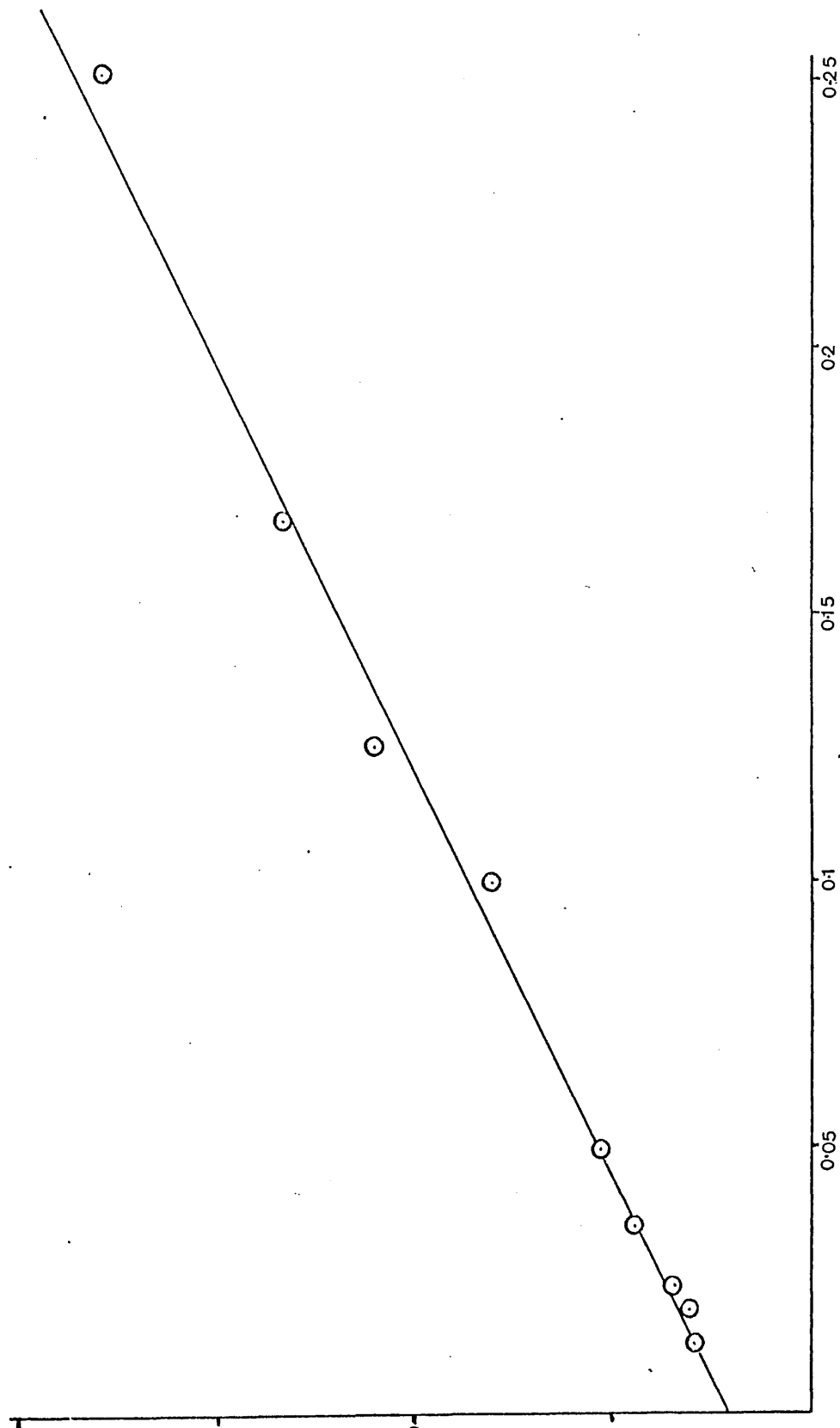
Lineweaver-Burke plot for methyl α-D-glucopyranoside



▲ Velocity expressed as mmoles glucose liberated per minute per mg protein.

Fig. 40

Lineweaver-Burke plot for phenyl α-D-glucopyranoside



▲ Velocity expressed as mmoles glucose liberated per minute per mg protein.

Table 23 Relative rates for the hydrolysis of α-glucosidases by α-D-glucosides

Substrate	Relative rate
p-nitrophenyl α-D-glucopyranoside	1
phenyl α-D-glucopyranoside	0.56
methyl α-D-glucopyranoside	0.056

The enzyme was found to be least active towards the methyl α-D-glucopyranoside than the phenyl and finally the p-nitrophenyl α-D-glucopyranoside

As shown by Hall et al.[61] the presence of an electron withdrawing group in the phenyl ring seems to facilitate a higher reaction rate than when the phenyl glycoside is used. In common with other mammalian acid α-D-glucosidases the enzyme was thus demonstrated to have the ability to hydrolyse the α-linkage in α-D-glucopyranosides.

To study the kinetic properties in more detail it was decided to determine the velocity constants ($V_{max.}$) for the enzyme on a series of disaccharides and glycosides. Details are given in Experiment 20. The Lineweaver-Burke plots are shown in Figs. 35 to 40, $V_{max.}$ was determined from the intercept on the y-axis where the intercept = $\frac{1}{V_{max.}}$ the results are shown in Table 24. The Michaelis constant (K_m) for maltose was also determined and found to be 4.0 to 4.4 mM. This figure lies within a range of values for maltose determined for other acid α-D-glucosidases. Rozenfeld[31] found the Michaelis constant for maltose

Fig. 41

Eadie plot showing deviation from linearity at high substrate concentrations of maltose

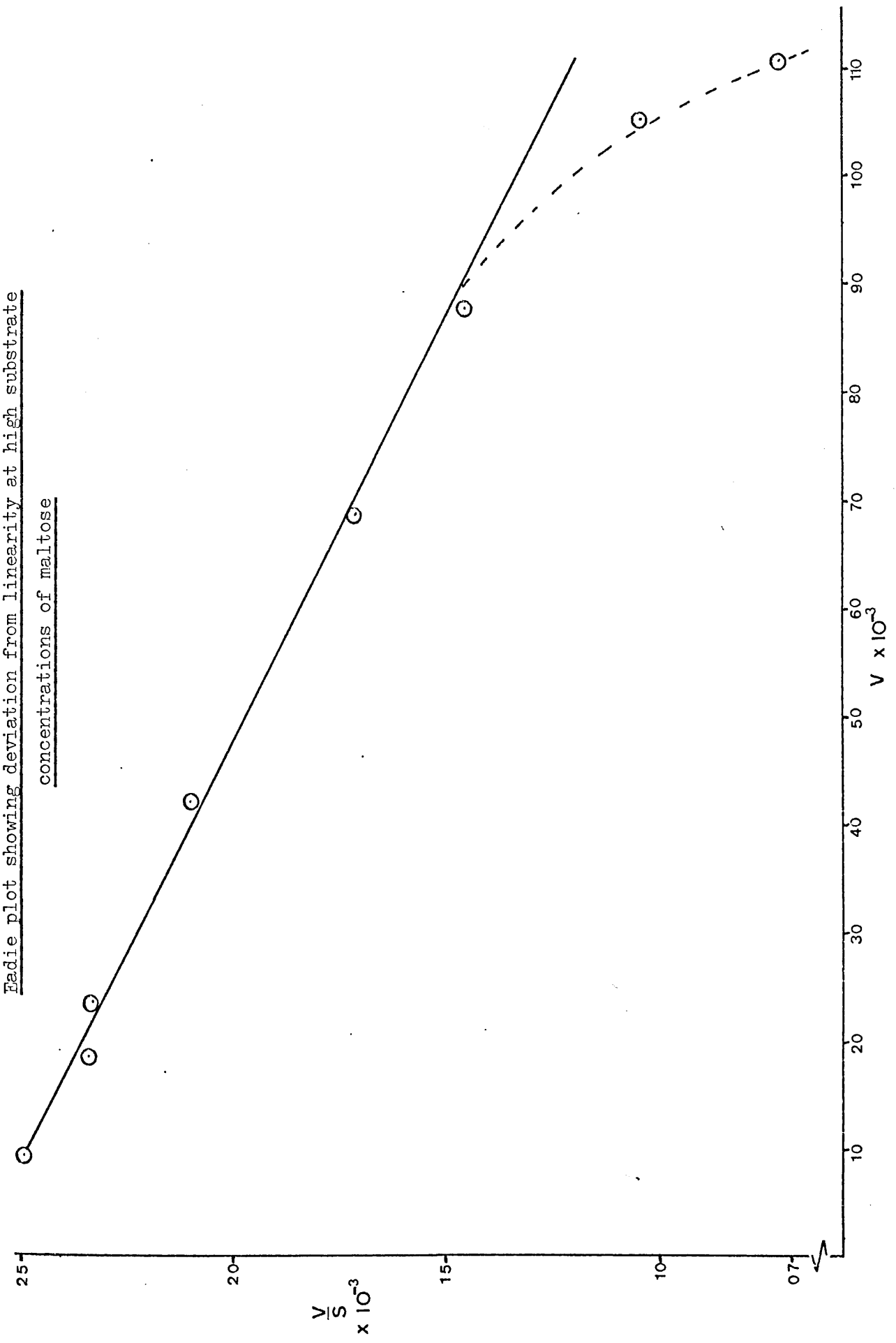


Table 24 Relative velocity constants determined for a series of disaccharides and α-glycosides

Substrate	Linkage	$V^{*}_{substrate} / V_{max.\ maltose}$
Maltose	α 1→4	1
Isomaltose	α 1→6	0.05
Kojibiose	α 1→2	0.18
Methyl α-D-glucoside	α	0.03
Phenyl α-D-glucoside	α	0.115
p-Nitrophenyl α-D-glucoside	α	0.133

* ($V_{max.}$ expressed as mmoles Glucose/liberated min/per mg protein).

of the spleen enzyme (5.8 mM) which is close to that found for the liver enzyme. From the values obtained for the maximum velocity constants the enzyme was found to be a lot more active towards the α(1→4) bond in maltose than for other substrates containing other types of α-linkage. The very small velocity constant for isomaltose and methyl α-D-glucopyranoside indicate the low affinity the enzyme has for these substrates. With maltose as substrate the Lineweaver-Burke plot deviates from linearity at high substrate concentrations. This is seen more clearly in Fig. 41 where an Eadie plot magnifies the departure from linearity. It is likely that this is caused by substrate inhibition which has been reported previously for acid α-D-glucosidases.[24,26] The other substrates listed in Table 24 did not show any substrate inhibition at high substrate concentrations.

VII Dextranase properties of the enzyme

In Section C.VI it was demonstrated that the enzyme had a broad specificity towards disaccharides and α-D-glucosides. It was decided to investigate the properties of the enzyme towards polysaccharides. Firstly some of the dextranase properties were investigated because of the varied type of linkage found in dextrans. The main chain is made up of α-(1→6) linked glucosyl units with α-(1→3) or α-(1→2) side branches. The dextran used to study the properties of acid α-D-glucosidase (Experiment 21) comprised of mainly α-(1→6) linked glucosyl units (95%) with 5% α-(1→3) linked glucosyl units as branch points. Most of the side chains are more than one glucose unit long.[124]

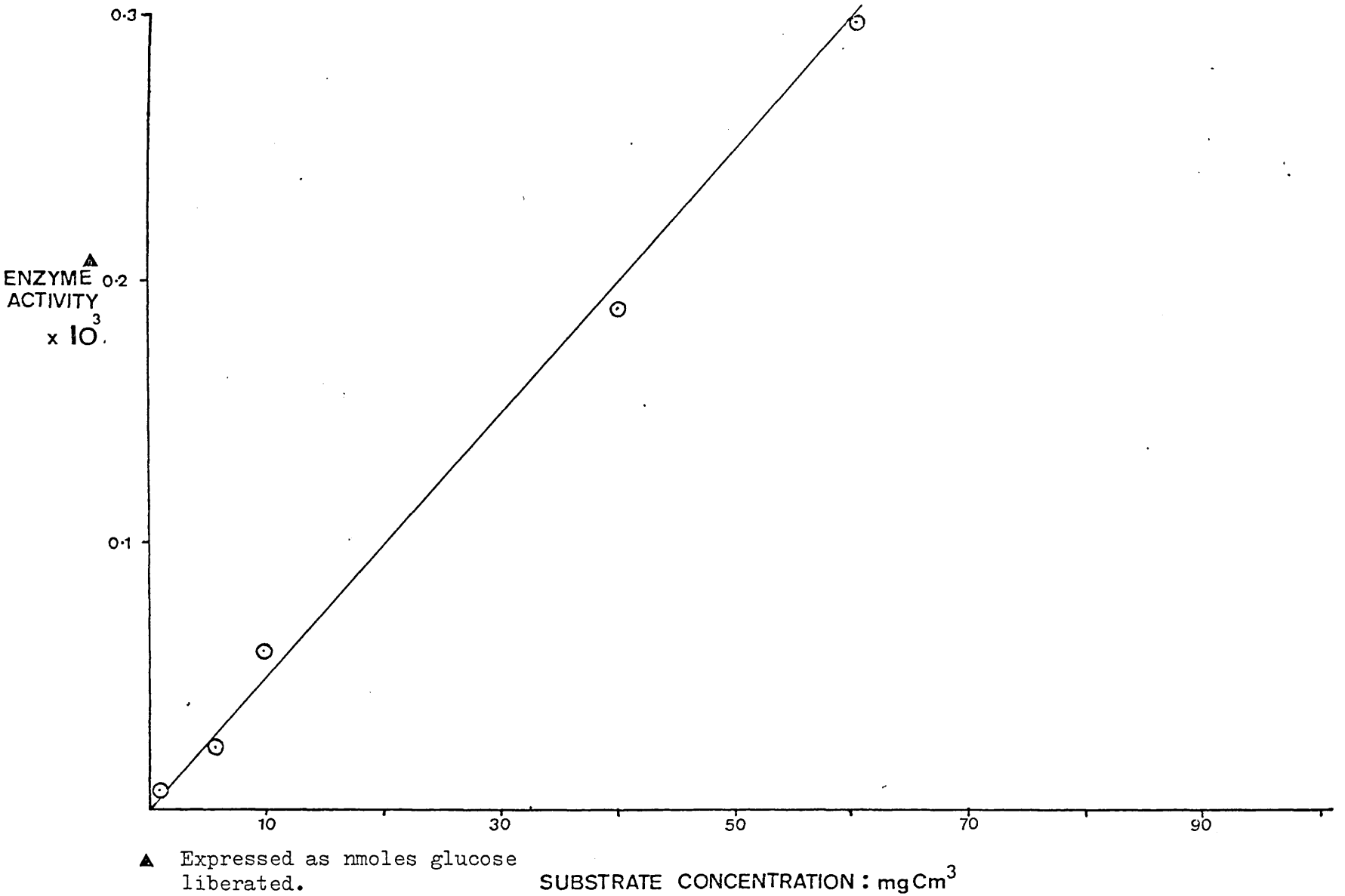
Fig. 42 shows the dependence of dextranase activity on substrate concentration. At the concentration of enzyme taken for the experiment the activity of the enzyme was proportional to the substrate concentration. It was impossible to saturate the enzyme even at a substrate concentration of 150 mg/cm^3. At higher substrate concentrations the solutions became very viscous.

The effect of enzyme concentration on activity was similar (Fig. 43). At the concentration of dextran T_{40} used activity was proportional to the enzyme concentration. Substrate was not limiting even up to very high enzyme concentrations.

Finally under the conditions selected the amount of glucose liberated was proportional to the length of time of the incubation (Fig. 44).

Fig. 42

Effect of substrate concentration on the dextranase activity of α-D-glucosidase



▲ Expressed as nmoles glucose liberated.

Fig. 43

Effect of Enzyme concentration on the dextranase activity of α-D-glucosidase

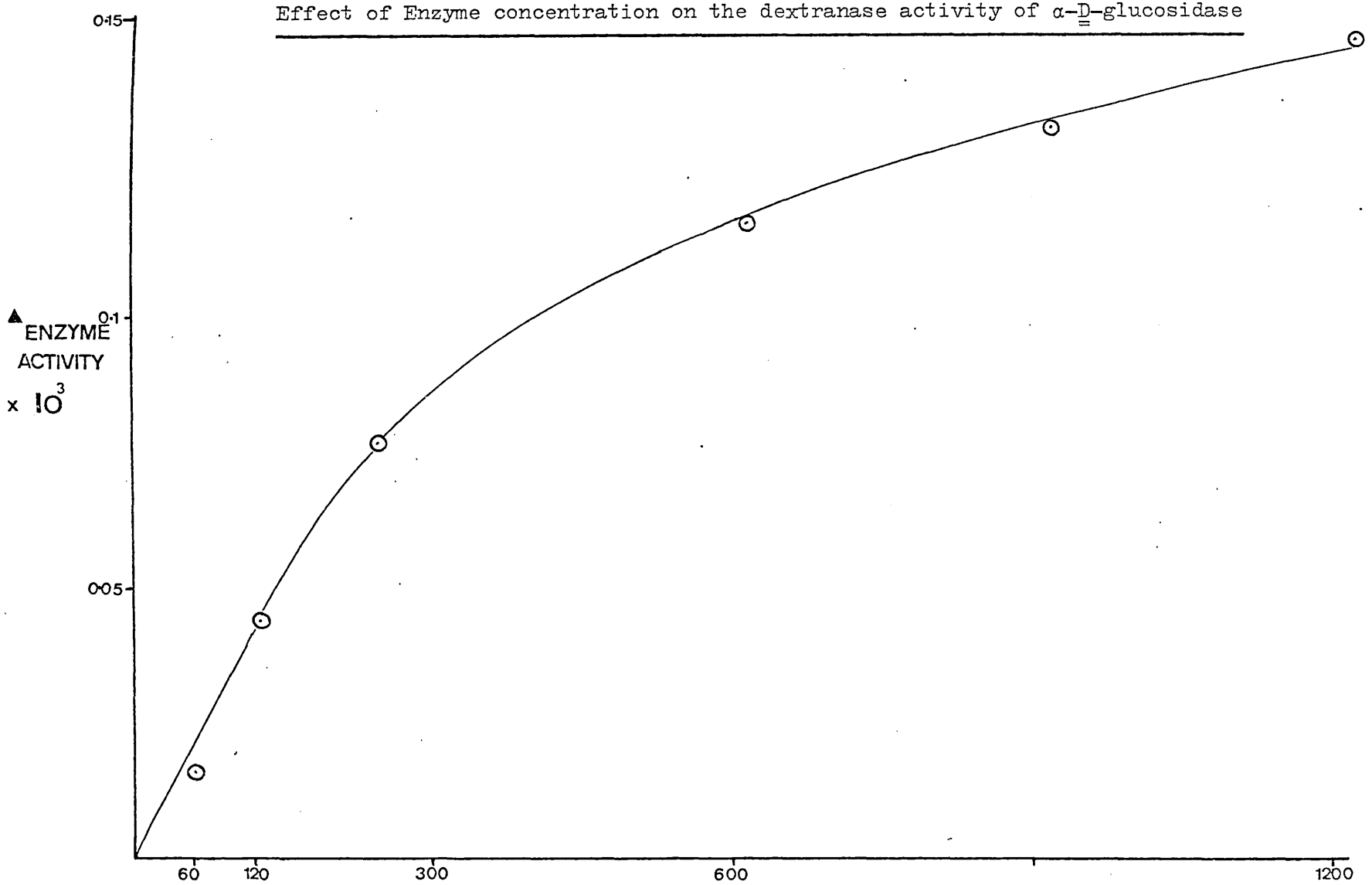
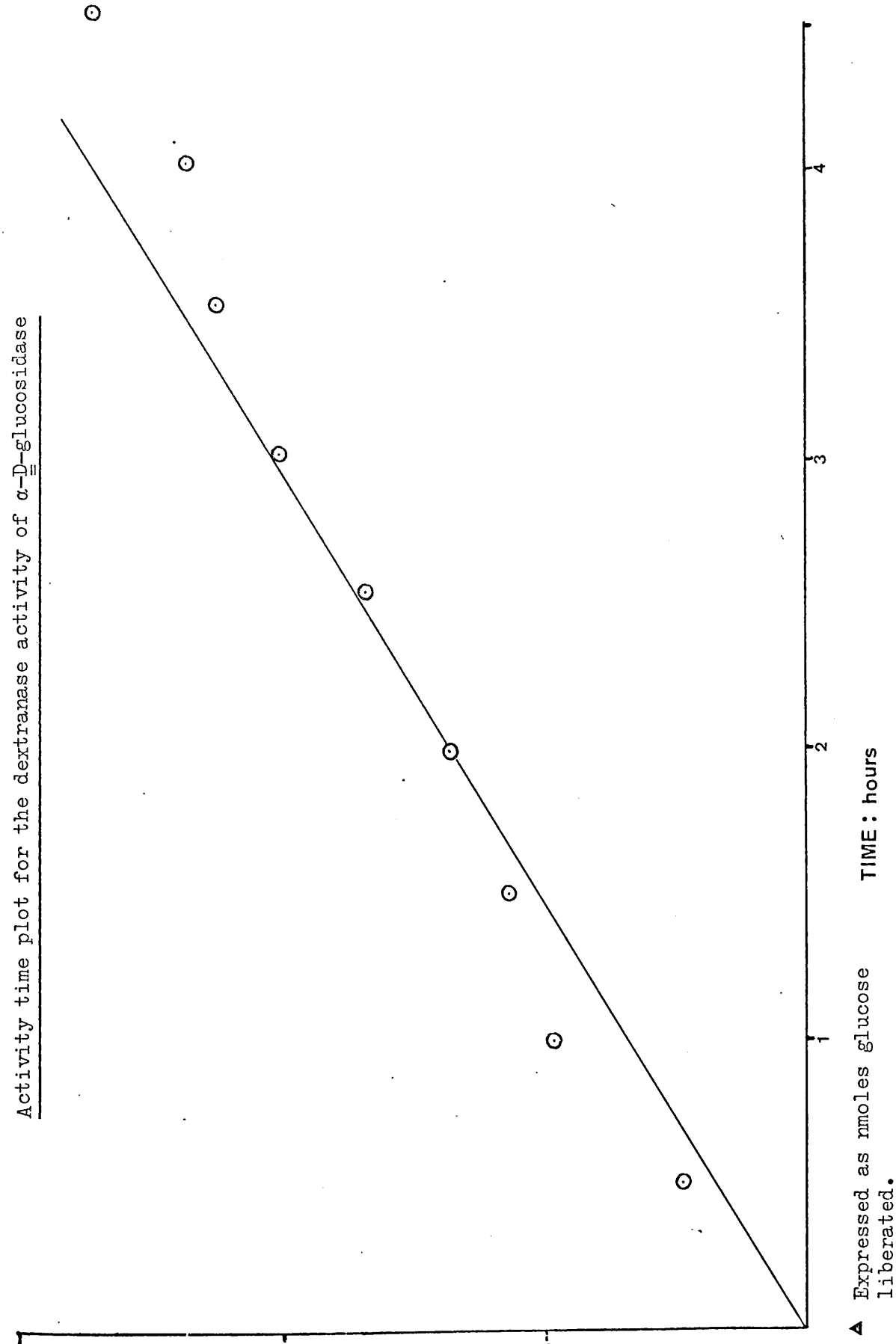


Fig. 44

Activity time plot for the dextranase activity of α-D-glucosidase



▲ Expressed as nmoles glucose liberated.

VIII Studies on the Degradation of linear and branched Polysaccharides

It is of interest to know whether acid and D-glucosidase is able to degrade branched polysaccharides completely. Both Jeffreys[25] and Fujimori[102] have shown that two different acid α-D-glucosidases are able to degrade glycogen branch points. A β-amylase and phosphorylase limit dextrin have both been degraded by the rat enzyme proving the ability of the enzyme to hydrolyse α-(1→6) branch points.[25] One can assume to a certain extent that because α-D-glucosidase is able to hydrolyse a variety of different α-linkages in disaccharides, the same must be true for polysaccharides. However, the lack of branched oligosaccharide substrates, structurally analogous to the branch points in polysaccharides, has hindered an investigation into the ability of the enzyme to hydrolyse branch points. The evidence that exists for the variation in activity of the enzyme towards differently linked disaccharides has an application in the relative activity of the enzyme towards differently linked polysaccharides. However, certain other factors have to be considered to relate the structure of the polysaccharide to the enzymes activity. If a substrate is highly branched and therefore has an abundance of non-reducing ends, as long as the substrate is saturated with enzyme, each end group would be hydrolysed towards the branch point. Therefore, the activity of the enzyme differs between branch and unbranched polysaccharides and between these containing different α-linked glucosyl units. Whelan[105] however, has suggested that in a highly branched structure perhaps exo-acting enzymes in general are sterically hindered from attacking all non-reducing end groups. Product inhibition has been found for several acid α-D-glucosidases[25,21] although generally

insufficient evidence exists to generalise that glucose may inhibit the polysaccharase activity of the enzyme. In Table 25 are listed the different polysaccharide substrates taken to investigate the polysaccharase activity of the enzyme. The procedure is described in Experiment 22. Figs 45 and 46 show the percentage degradation of each substrate.

A comparison of the rate of D-glucose release from amylopectin and glycogen, by the action of acid α-D-glucosidase, shows that the rates are quite similar. This indicates that both substrates are structurally similar. Hence, any difference in the rate of D-glucose released would be accounted by the difference in the average chain length and the degree of multiple branching in both polysaccharides.

Table 25 Structure of a series of polysaccharides used to study the polysaccharase activity of the enzyme

Name	Linear or branched	Type of Linkage	Branch Linkage
Glycogen	Branched	α 1→4 α 1→6	α 1→ 6
Amylopectin	Branched	α 1→4 α 1→6	α 1→ 6
Amylose	Linear	α 1→4	-
Dextran			
T_{10}	Branched	α 1→6 α 1→3	α 1→3
T_{40}	Branched	α 1→6 α 1→3	α 1→3
B512	Branched	α 1→3 α 1→6 α 1→4	α 1→3
B1299S	Branched	α 1→6 α 1→2 α 1→3	α 1→2

The shape of the graphs for these two highly branched polysaccharides give a good indication of the pattern of attack of the enzyme. Firstly, all of the non-reducing ends being attacked towards the branch points, then the branch points, and then the inner chains. Thus, as the number of non-reducing ends decreases during enzymic hydrolysis the rate of D-glucose release diminishes. As the rate of D-glucose release slows down after three hours it can be assumed that the majority of the branch linkages have been hydrolysed. Thus, at this point only the inner chains would remain un-degraded. The percentage of D-glucosyl units in the inner chains compared with the average chain length is approximately 24% for glycogen,[126] which compares well with the 22% percentage of glycogen not degraded after three hours. For amylopectin the figure is 29.5%[126] compared with 20% of amylopectin not degraded after this time.

The relative rates for the degradation of glycogen, amylopectin and amylose are shown in Table 26.

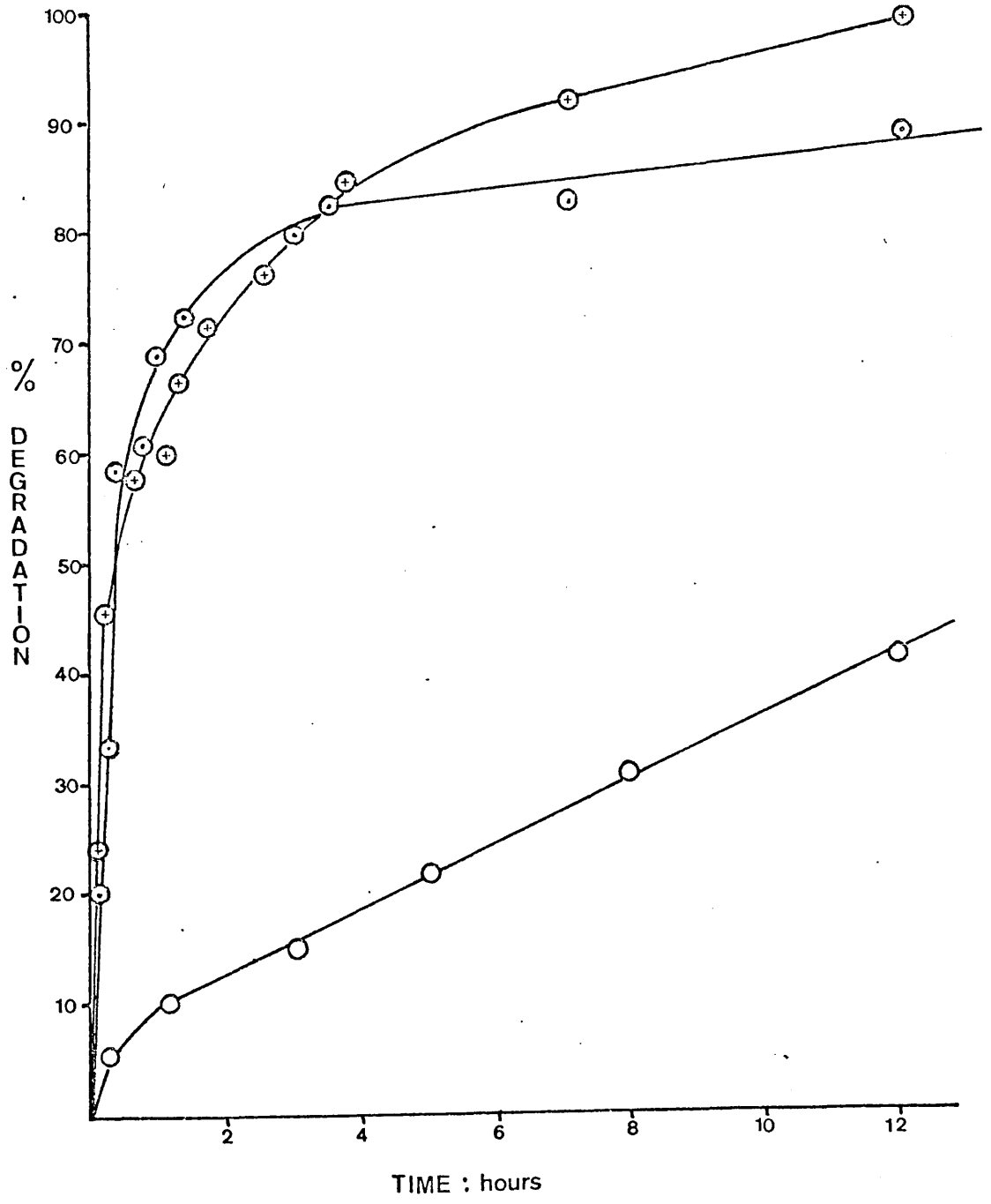
Table 26 Relative Rates for polysaccharides containing α-(1→4) linkages.

	Relative Rate	
	30 mins	3 hrs
Amylopectin	100	100
Glycogen	88	98
Amylose	13.9	20.78

As can be seen the rate increases for glycogen and for amylose, in comparison to that for amylopectin. After thirteen hours glycogen

Fig. 45

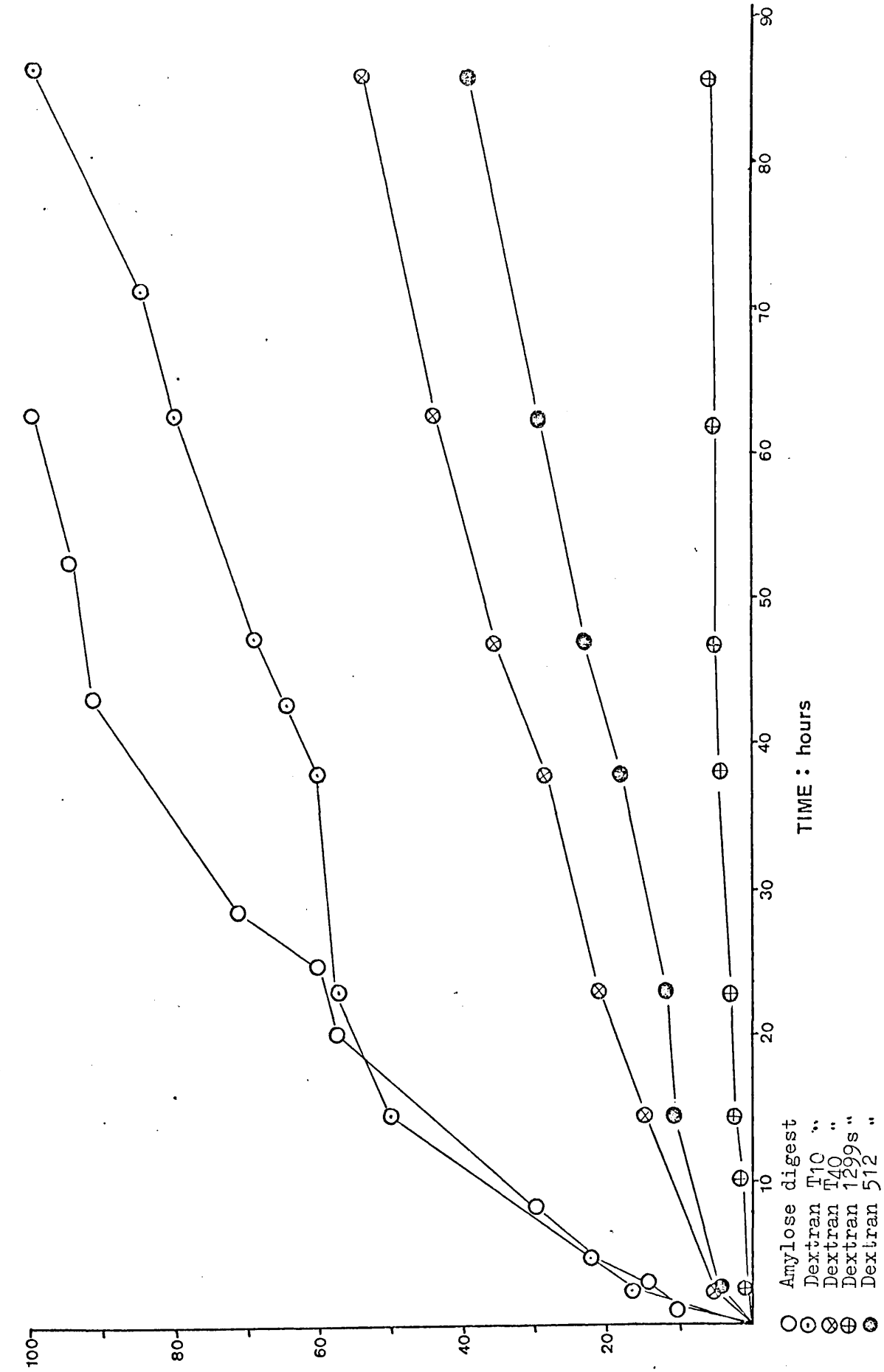
% Degradation studies on amylopectin amylose and glycogen



⊙ amylopectin digest
⊕ glycogen digest
○ amylose digest

Fig. 46

Degradation studies on a series of polysaccharides



was degraded to the extent of 100% whilst amylopectin was degraded 93%.

Table 27 shows the relative rates for the degradation of the dextrans hydrolysed by the acid α-D-glucosidase.

Table 27 Relative Rates for differently linked polysaccharides

Substrate	Relative Rate	
	20 hours	40 hours
Amylose	100	100
Dextran:		
T_{10}	96	71.59
T_{40}	32.75	34
B512	20.6	22.72
B1299S	2.24	2.84

Initially the rate for the degradation of dextran T_{10} was very close to that of amylose, but decreased later on. When we compare this result with those obtained for the other three dextrans, where the rate of D-glucose release remains fairly constant, it is evident that the branch linkages (if any) in dextran T_{10} have been hydrolysed. In the other dextrans the rate of D-glucose release suggests that not all, if any, branch points have been hydrolysed. As dextran T_{10} is an acid hydrolysed product from a high molecular weight dextran it was thought desirable to confirm that the sample did contain some branch points.

A sample of dextran T_{10} was methylated, hydrolysed, reduced, and acetylated as described in Experiment 23, to give the corresponding

O-acetyl-O-methyl-glucitols. The methylated sugars were then separated by gas-liquid chromatography. The di, tri and tetra-O-methyl glucitols were found to be present suggesting that branch linkages may be present in dextran T_{10}. Unfortunately, the expected ratio of $\frac{\text{tetra}}{\text{tri}} \simeq 1$ was not found indicating that perhaps complete methylation had not occurred. In Fig. 46 the percentage degradation versus time is shown for amylose and a series of dextrans. In Table 28 the percentage degradation of each polysaccharide is noted.

Table 28 Percentage degradation of various differently linked polysaccharides

	% degradation	
	63 hours	86 hours
Amylose	100	100
Dextran:		
T_{10}	81	100
T_{40}	44	54
B512	30	40
B1299S	5	5

Both amylose and dextran T_{10} were completely degraded to D-glucose. As no Michaelis constants (K_m) or maximum velocity values ($V_{max.}$) were determined for the hydrolysis of the various polysaccharides by acid α-D-glucosidase, the relative rates will be used to compare the activity of the enzyme towards differently linked substrates.

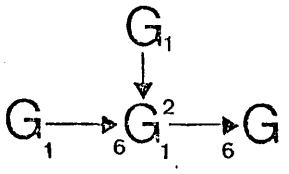
The enzyme was most active towards glycogen and amylopectin. Both are highly branched polysaccharides containing mainly α-(1→4)linked

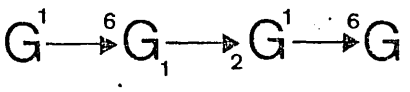
glucosyl units and α-(1→6) branch points. Of the disaccharide substrates maltose, α-(1→4) linked, was found to be the preferred substrate. Thus, the affinity of the enzyme for α-(1→4) links is shown both in oligosaccharide and in polysaccharide degradation studies. It it useful to note that *in vivo* glycogen is reported to be the main substrate of the enzyme.[89] From the percentage degradation studies shown in Fig. 45 it would seem reasonable to suggest that amylopectin as well as glycogen could be degraded fully to D-glucose. Product inhibition by D-glucose was not involved in any of the polysaccharide incubations. As glycogen, dextran T_{10}, and amylose, were degraded to such a great extent there is no reason to suppose that with time the other substrates would be degraded to the same degree. One assumes that these substrates are all degraded at the same active site on the enzyme. It is acknowledged that at least two active sites are present in the enzyme. One binding small substrates such as oligosaccharides, and the other binding larger substrates such as polysaccharides. We can correlate the similar rates for amylose and dextran T_{40} by suggesting that the affinity of the enzyme for the linear α-(1→4) glucosyl units is matched by the increase in the number of non-reducing groups open for attack by the enzyme. This counteracts the decreased affinity of the enzyme for α-(1→6) linkages.

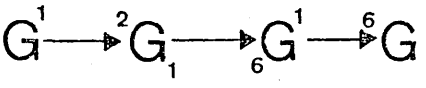
All the dextran polysaccharides used in the incubations except B-1299S dextran came from the same bacterial synthesising species - *L. mesenteroides* B512, and therefore their structures were very similar. The major difference was that of molecular weight although a slight variation in the degree of branching may exist. This is exemplified

when one looks at the relative rates observed for these dextrans. The B1299S dextran is highly branched and contains α-(1→2) linked branch points, a considerable amount of α-(1→3) linked glucose is present, as well as the α-(1→6) linkages in the main chains. The low activity of the enzyme towards this substrate may arise from the non-uniformity of its structure. This could give rise to some steric effects where by the enzyme is unable to effectively hydrolyse the non-reducing end groups. It was unfortunate however that nigeran, a polysaccharide linked linearly α-(1→3) and α-(1→4) although available as a substrate could not be dissolved into solution either by dissolution in alkali and neutralisation with acid, or by shaking and ultrasonification. Determination by the phenyl sulphuric method of the amount of carbohydrate in solution gave a low figure, equivalent to 100 μg of glucose per cm^3.

As has already been mentioned the use of model compounds to study the action of the enzyme is the only way in which some relationship may be determined between structure and activity of the enzyme. We have no idea at all of the action of the enzyme at branch points and the mechanism involved. Recently however, it has been reported that the tetrasaccharide I was found to be resistent to the attack of pig spleen acid α-D-glucosidase.[127] Tetrasaccharides II and III, were found to be hydrolysed by this enzyme. The authors therefore suggested that dextrans containing α-(1→2) linkages may be immune to attack by acid α-D-glucosidase.

I 

II 

III 

I = O-α-D-glucopyranosyl-(1→6)-[O-α-D-glucopyranosyl-
-(1→2)]-O-α-D-glucopyranosyl-(1→6)-α-D-glucopyranose.

II = O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-
-(1→2)-O-α-D-glucopyranosyl-(1→6)-α-D-glucopyranose.

III = O-α-D-glucopyranosyl-(1→2)-O-α-D-glucopyranosyl-
-(1→6)-O-α-D-glucopyranosyl-(1→6)-α-D-glucopyranose.

I.2 C. Properties of Isoenzyme II

a) General Aspects

The term isoenzyme or isozyme was first proposed by Market and Møller[128] for multiple molecular forms of an enzyme involved in the same catalytic reaction. Isoenzymes although catalysing the same reaction do have different properties, such as, their electrophetic mobilities, enzyme kinetics, or molecular structures.[129] Multiple forms of glycosidases are often found in the lysosomes. Several lysosomal diseases are associated with the isoenzyme levels within the lysosomes.[130,131] A lack of one of the three β-galactosidases in liver lysosomes leads to a lysosomal disease.[132] The same applies in Taysachs disease where one of the two β-N-acetyl-hexosamindases is missing.[133] No correlation however, has been found yet between the isoenzyme levels of acid α-D-glucosidase and Pompe's disease. This arises from the little work which has been done concerning the separation and investigation of the properties of the isoenzymes of acid α-D-glucosidase. Only one worker so far has carried out a detailed study on the properties of the isoenzymes of α-D-glucosidase.[37]

b) The relationship between Isoenzyme I and Isoenzyme II

The isolation of isoenzyme II has already been described in chapter I.2.A(g). Homogeneity was established, as the enzyme gave a single band when disc gel electrophoresis was carried out on the isolated enzyme (Fig.17b).

It was suggested that isoenzyme II may in actual fact be generated from isoenzyme I by some unknown mechanism. Thus, with time, more isoenzyme II may be generated from isoenzyme I. In order to investigate if this in fact occurred, several experiments were undertaken

to determine if with ageing the isoenzyme I preparation contained some isoenzyme II.

Dreyfus and Alexandre[88] have shown that it is possible to separate the isoenzymes of α-D-glucosidase by cellulose acetate electrophoresis. To see if it was possible to separate both isoenzymes by this method electrophoresis was carried out as described in Experiment 24. Electrophoresis was performed on isoenzyme I, isolated as described in chapter I.2.A.(g), and isoenzyme II two weeks after both isoenzymes were initially isolated from one another. Both enzymes were detected by incubating the electrophoretogram with 4-methylumbelliferyl α-D-glucopyranoside and then placing the electrophoretogram under a u.v. lamp. Any enzymic activity present is indicated by the appearance of a fluorescent spot. The results are shown in Fig. 47. The mobilities of both enzymes differed quite considerably. Isoenzyme I had a greater mobility than isoenzyme II. However, what is of interest is that a very faint fluorescent spot was detected for isoenzyme I, equivalent to the mobility of isoenzyme II, thus indicating that some isoenzyme II may be present in the isoenzyme I extract.

The extract of isoenzyme I was left to age at 4°C for two months. After this period a sample of isoenzyme I was loaded onto a column packed with Sephadex G-100 as described in Experiment 25, to see if any more isoenzyme II could be isolated. The elution profile is shown in Fig. 48. Unfortunately, the maltase activity of the eluted fractions was not determined, but as can be seen, absorbance measurements at 280 nm indicated that a protein with an elution volume

Fig. 47

Cellulose acetate electrophoretogram of Isoenzyme I and Isoenzyme II

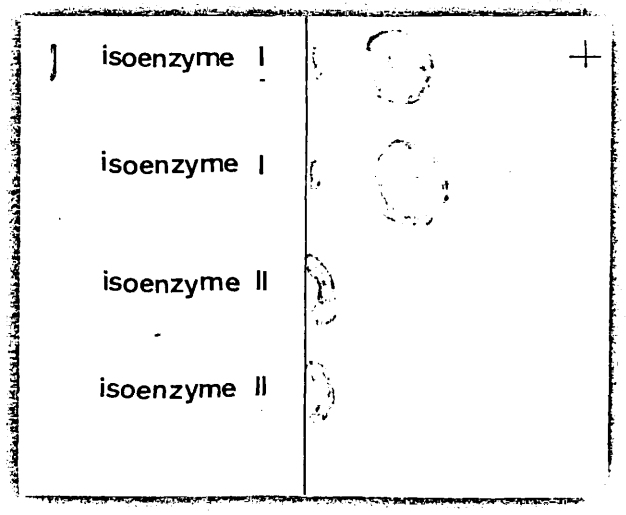
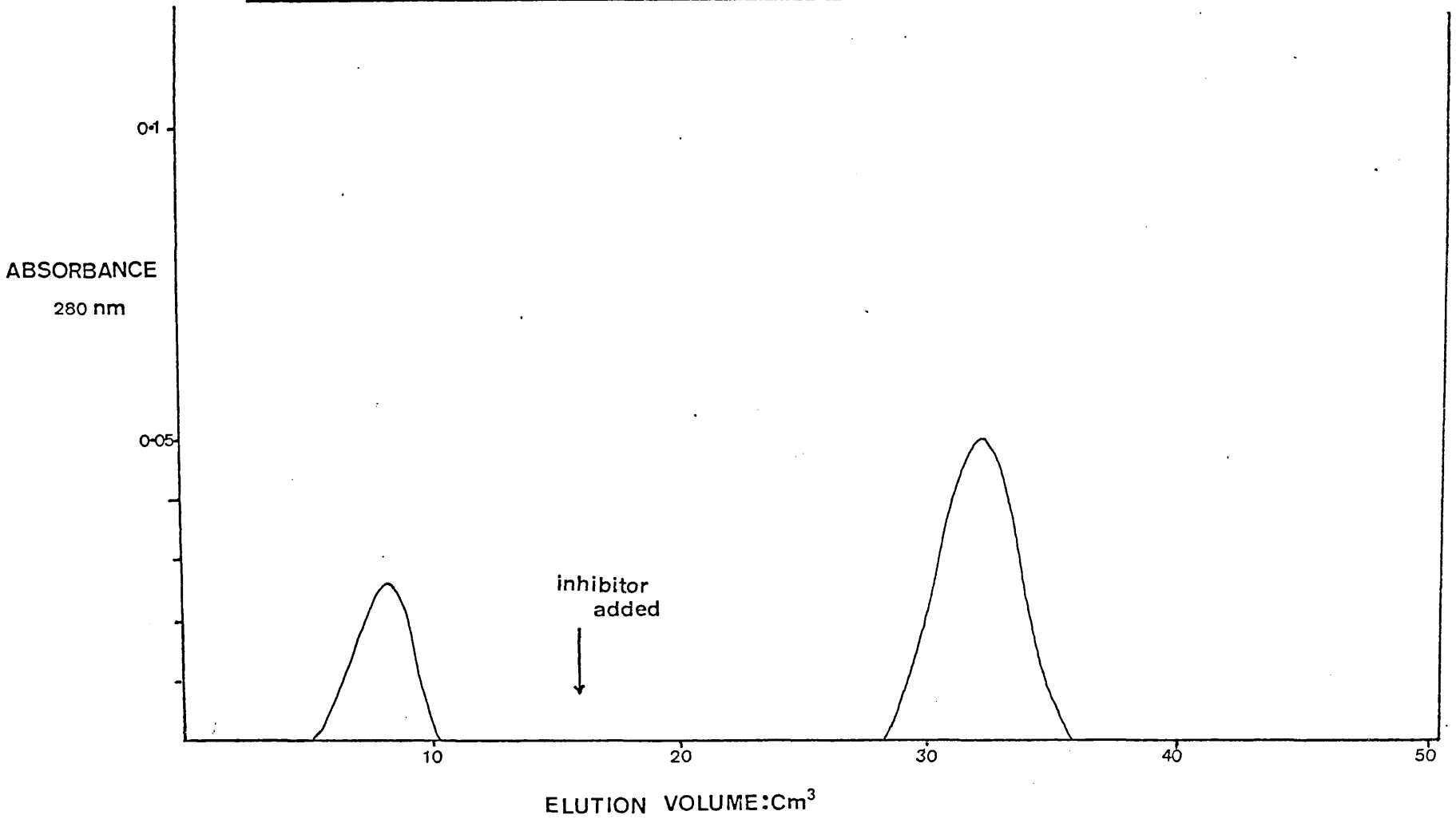


Fig. 48

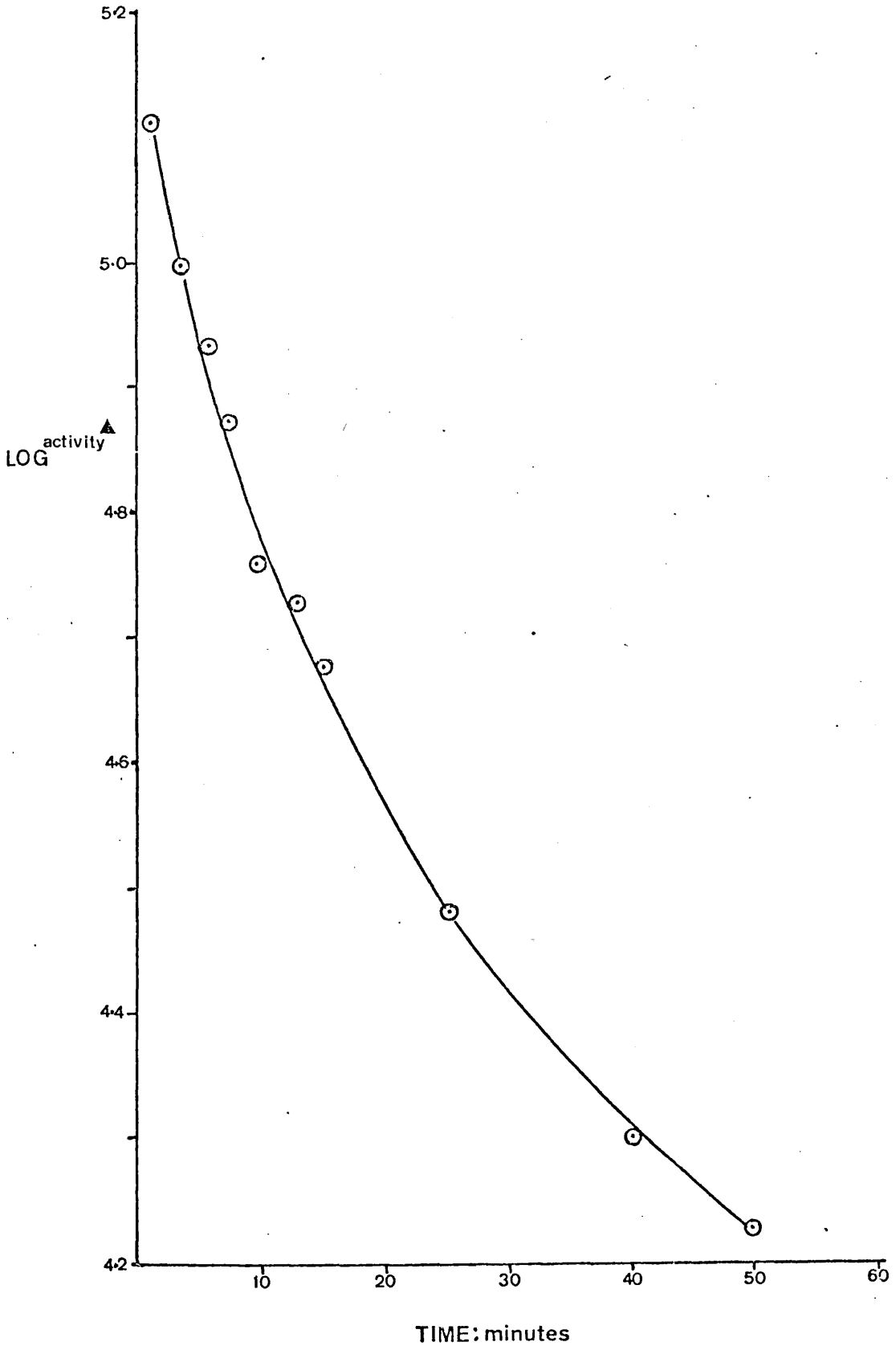
Elution profile of an aged sample of Isoenzyme I on Sephadex G-100



a) Eluant: 50 m$\underline{\underline{M}}$ acetate buffer pH 4.6. b) After fraction 32 eluant: methyl α-$\underline{\underline{D}}$-glucopyranoside (1% w/v) in 50 m$\underline{\underline{M}}$ acetate buffer pH 4.6.

Fig. 49

Heat denaturation of the maltase activity of Isoenzyme I



▲ Expressed as nmoles glucose liberated per minute.

corresponding to isoenzyme II was eluted from the column. In order to clarify the situation more fully an investigation of the thermal stability of the aged isoenzyme I extract was undertaken. Incubation was carried out at 65°C, as described in Experiment 26. Fig. 49 shows a plot of log activity versus time for the incubation of isoenzyme I with maltose. As can be seen a curve was produced as distinct from a single straight line. This indicates perhaps the presence of two enzymes having very similar enzymic as well as denaturation properties. On repeating the experiment under the same conditions the same results were incurred.

On the evidence given in this section so far, the suggestion that isoenzyme II can be generated from isoenzyme I is not easily dismissed. However, it is not possible to state conclusively that isoenzyme II was not present in the isoenzyme I extract from the time of separation during the extraction procedure. To obtain conclusive evidence, experiments similar to those described in this section should have been carried out on isoenzyme I immediately after the separation of the isoenzymes on Sephadex G-100, and after ageing of the isoenzyme I extract. Swallow et al.[37] characterised three phenotypes (appearance of a characteristic due to genetic inheritance) in humans having different isoenzymes. The isoenzymes were found to have different mobilities on starch electrophoresis. Type 1 had a slow moving isoenzyme (α-glucosidase I), type 2 having a fast moving isoenzyme (α-glucosidase II) and type 2-1 having both isoenzymes. When an attempt was made to see whether α-glucosidase I could generate α-glucosidase II and vice versa, under the conditions used, no generation or loss of isoenzymes occurred. It is worth

considering however that the ageing experiments were only carried out over four days.

To conclude a closer examination of the *in vitro* relationship between both isoenzymes would be necessary before any conclusive proposals could be made regarding the generation of one isoenzyme from the other.

c) The molecular weight and subunit structure of isoenzyme II

In order to compare the molecular weight and subunit structure of each isoenzyme, the same method was used as described for isoenzyme I. Sodium dodecyl sulphate electrophoresis was carried out on a sample of isoenzyme II as described in Experiment 27. After staining two very strong bands were visible with two very faint bands showing a greater mobility, (Fig. 19b). The staining pattern for isoenzyme II was identical to that obtained for isoenzyme I, (Fig. 19a). The molecular weights of the stained bands were derived as described in chapter I.2.B(b) and are shown in Table 29. A mean value for the molecular weight of the two minor bands is given as they were found hard to visibly segregate.

Table 29 Molecular weights of the bands obtained by S.D.S. electrophoresis of Isoenzyme II

	Band 1 (major)		Band 2 (major)		Band 3 (minor)	
	Mobility BPB	Molecular weight	Mobility BPB	Molecular weight	Mobility BPB	Molecular weight
Gel 1	0.1866	136,000	0.2281	116,000	0.2695	98,000
Gel 2	0.1862	137,000	0.2254	117,000	0.2647	100,000

A comparison of the molecular weights obtained for the stained bands of both isoenzymes (Table 20,29) shows that the values for isoenzyme II show an increase of approximately 10,000 over the corresponding bands for isoenzyme I. This is within the 10% deviation applicable to the determination of molecular weights. Of more interest is the identical pattern of bands obtained for both isoenzymes. One can therefore assume that the subunit structure of both enzymes is similar. In discussing the results obtained for isoenzyme I [chapter I.2.B.(b)] it was suggested that one of the major bands could be due to the presence of isoenzyme II within the isoenzyme I extract. If this was so one would have to conclude that some isoenzyme I was present in the isoenzyme II extract for the same electrophoretic pattern to have been obtained for both isoenzymes. As this seems unlikely, it is therefore concluded that both isoenzymes have very similar molecular weights and subunit structures. Further evidence to confirm this theory comes from the results obtained during cellulose acetate

electrophoresis, described earlier in this section. The isoenzyme I extract used during cellulose acetate and S.D.S. electrophoresis had aged for the same period of time. As it was possible that a small amount of isoenzyme II may have been present in the isoenzyme I extract, and the same electrophoretic pattern was obtained for both isoenzymes, it would seem therefore that the isoenzymes cannot be separated by S.D.S. electrophoresis.

To evaluate further the similar properties of both isoenzymes an investigation of the enzymic properties of isoenzyme II was carried out.

d) Effect of pH on the rate of enzyme hydrolysis

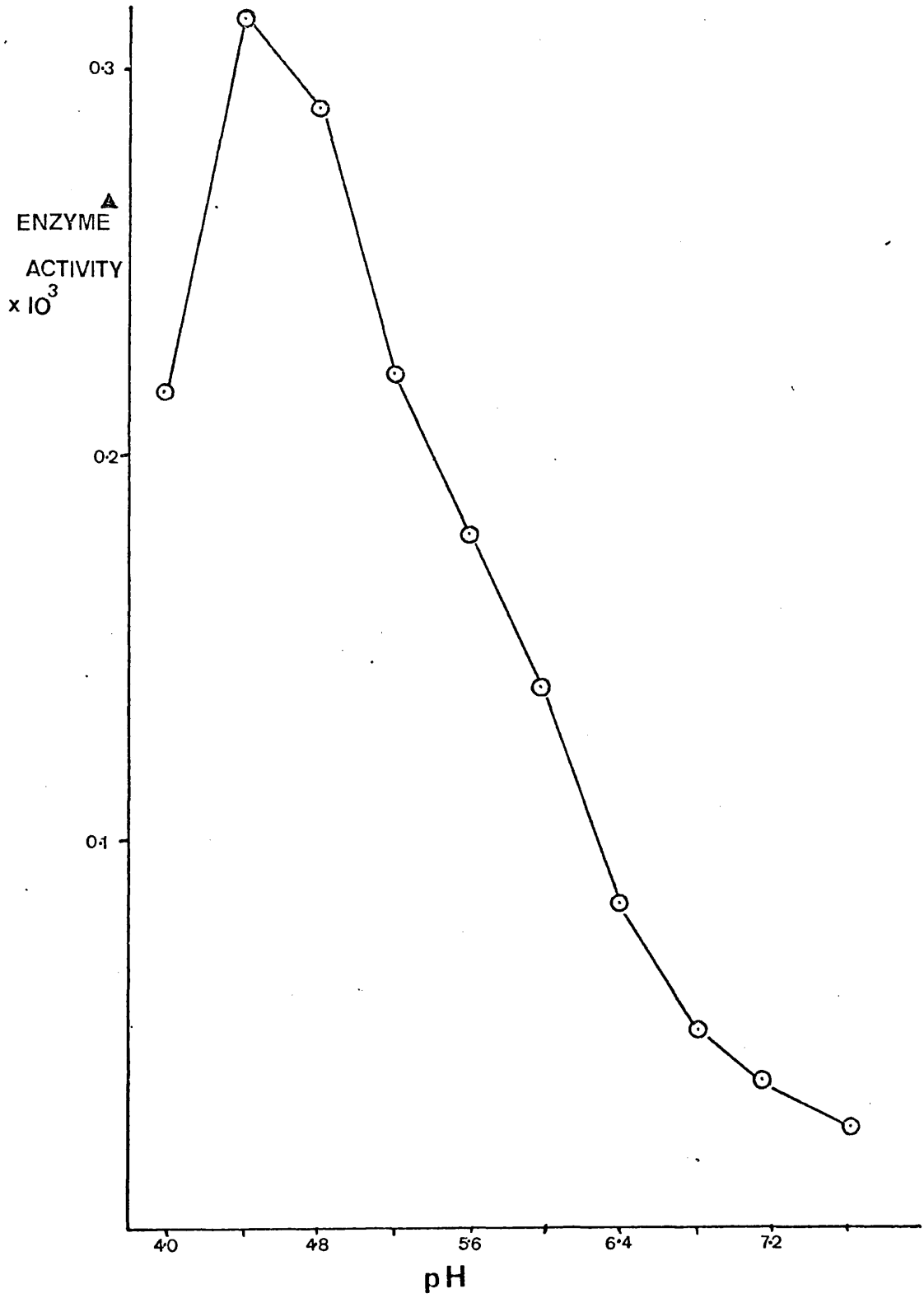
The effect of pH on the maltase activity of isoenzyme II is shown in Fig. 50. The procedure was carried out as described in Experiment 28. Maximum activity was exerted at approximately pH 4.4, with the activity of the enzyme decreasing with increasing pH. Only a small amount of activity was shown by the enzyme at pH 7.6. A comparison with the results obtained for isoenzyme I, (Fig. 31) indicates that both enzymes show a similar effect of pH on their enzymic activity. In any further enzymic experiments isoenzyme II, was buffered at pH 4.6.

e) Thermal Stability of isoenzyme II

The procedure was carried out as described in Experiment 29. Fig. 51 shows the effect of temperature on the maltase activity of isoenzyme II. The maltase activity was stable up to approximately 45°C and then progressive deactivation occurred, until only slight activity was exerted at temperatures $>65^{\circ}C$.

Fig. 50

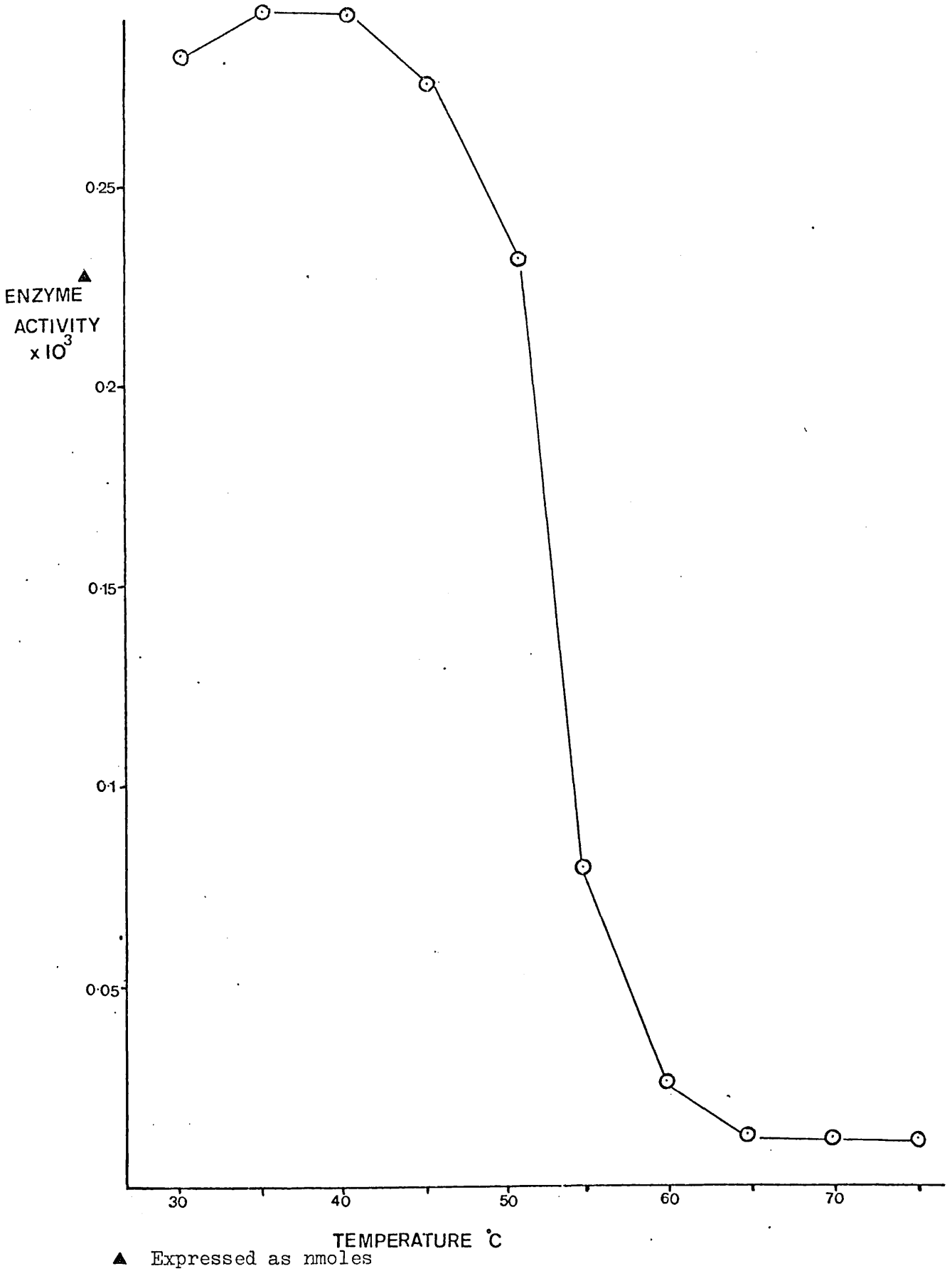
Effect of pH on the rate of maltose hydrolysis by Isoenzyme II



▲ Expressed as nmoles glucose liberated.

Fig. 51

Effect of temperature on the stability of the maltase activity of Isoenzyme II



▲ Expressed as nmoles glucose liberated.

Similarly, isoenzyme I, (Fig. 32) showed a similar effect of temperature on its maltase activity.

f) Activity of isoenzyme II towards maltose, dextran T_{10} and glycogen

Several authors have shown that an isoenzyme of α-D-glucosidase is able to be separated from α-D-glucosidase by its decreased affinity for dextran[37,102] (Sephadex). This suggests that the polysaccharase activity of isoenzyme II may be extremely small when compared to isoenzyme I.

Incubations were carried out as described in Experiment 30. Unfortunately, it was impossible to investigate the activity of isoenzyme II towards a full range of substrates because of the small amount of enzyme isolated. Figs. 52 and 53 show a comparison of the dextranase and glucoamylase activities of the enzymes. Relative rates could not be determined because of the different experimental conditions under which the incubations were carried out. However, the specific activities are shown in Table 30.

Table 30 Specific activities of isoenzyme I and II shown towards maltose, glycogen and dextran T_{10}

	Specific Activity	
	Isoenzyme I	Isoenzyme II
maltose	8829*	2358*
dextran T_{10}	106Δ	7.76Δ
glycogen	4166Δ	237Δ

* n moles substrate hydrolysed/min/mg protein

Δ n moles glucose liberated/min/mg protein.

Fig. 52

Comparison of the glucoamylase activity of Isoenzyme I and Isoenzyme II

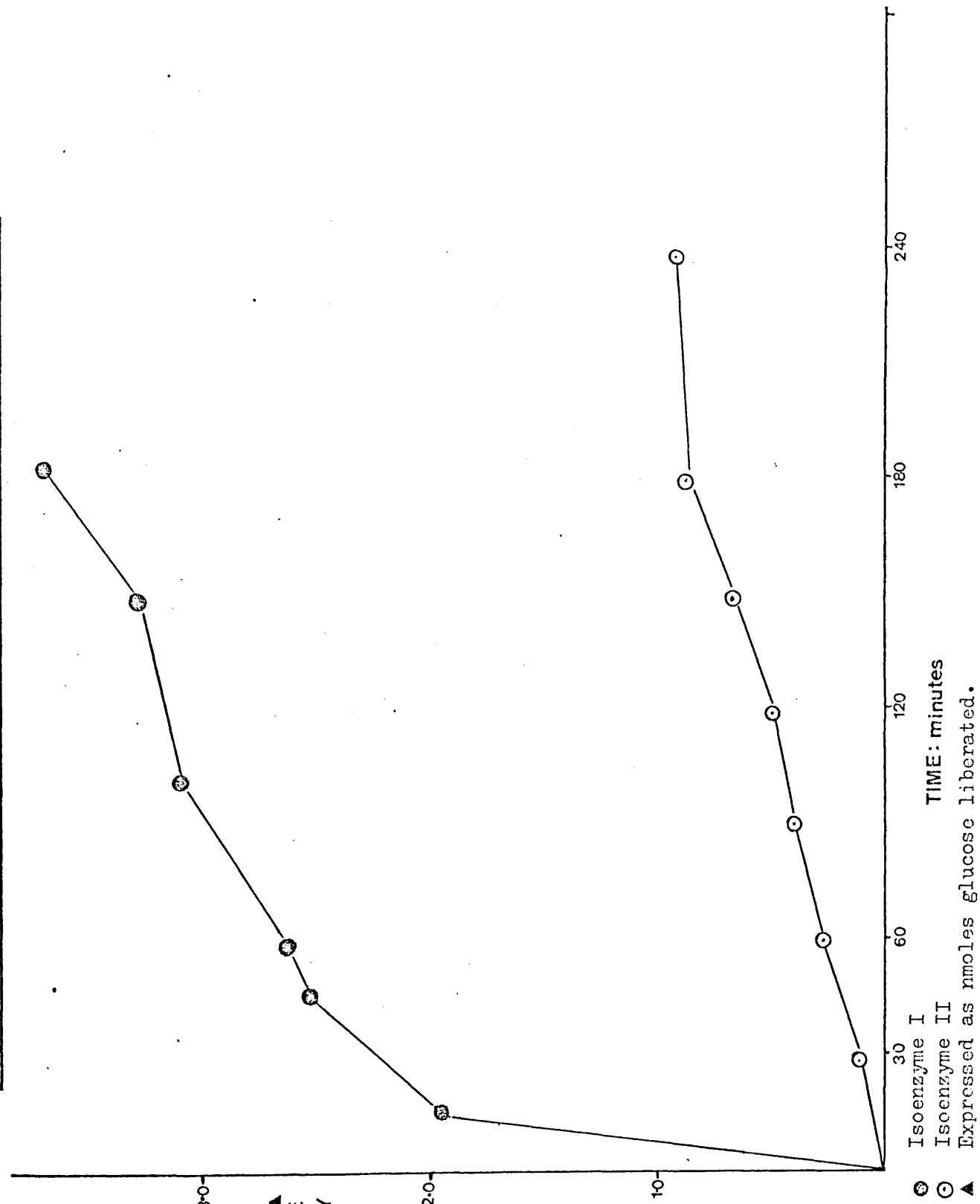
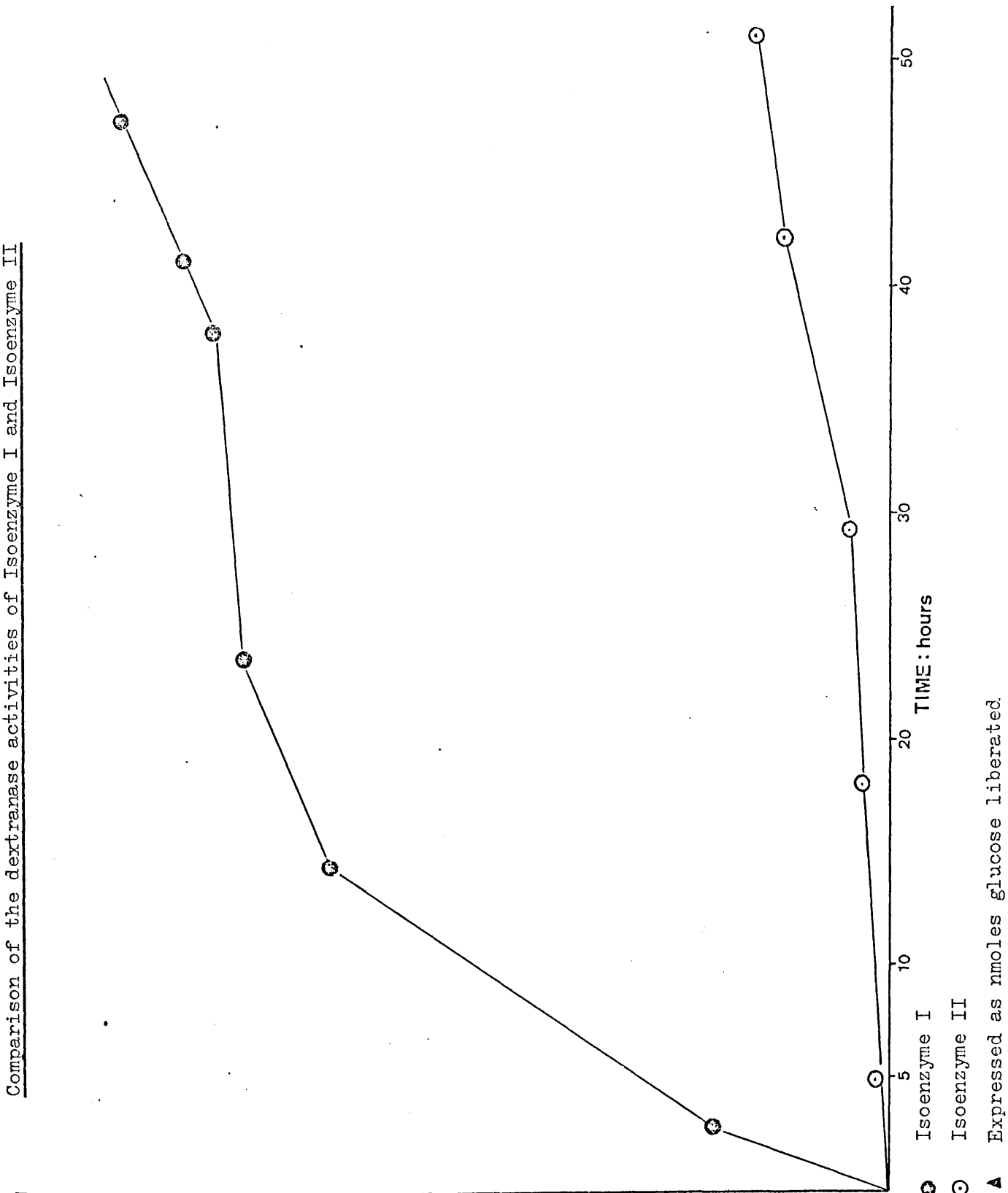


Fig. 53

Comparison of the dextranase activities of Isoenzyme I and Isoenzyme II



Isoenzyme II showed a decrease in activity towards maltose when compared with the activity of isoenzyme I. A large decrease in activity was shown by isoenzyme II towards dextran T_{10} and glycogen when compared with isoenzyme I. There seems to be, therefore, a much stronger affinity for maltose than for dextran or glycogen, shown by isoenzyme II. This would account for the fact that isoenzyme II is able to be separated from isoenzyme I by Sephadex chromatography, as isoenzyme II shows only slight activity towards dextran T_{10}.

On the evidence given in this section it is difficult to fully state the origin of isoenzyme II, one could suggest the possible existence of this isoenzyme because of some alteration of the structure of isoenzyme I, but the reason for such a phenomena occurring remains unknown. For both isoenzymes to have such a similar subunit structure and yet show such a marked difference in mobility on cellulose acetate, demonstrates how such a small variation in structure gives rise to such dissimilar properties. Although very similar denaturation curves were produced for both isoenzymes the marked decrease in activity towards dextran T_{10} and glycogen for isoenzyme II signifies considerably the different roles each enzyme must play *in vivo*. Whether one can assume that the active site is blocked on isoenzyme II for this activity to be diminished, resulting from a change in structure, would depend on further studies. From the studies carried out it would seem that isoenzyme II is equivalent to α-glucosidase 2 which has been investigated by Swallow and co-workers[37] from human placenta.

Chapter II Part II

Levanase of Streptococcus salivarius strain '51'

II-1. Introduction

1.A. Naming of the enzymes associated with levan synthesis and degradation

The property of some oral Streptococci of utilising β-D-fructofuranosyl α-D-glucopyranoside (sucrose) to produce a polyfructan levan, is initiated by the enzyme levansucrase. This enzyme catalyses the cleavage of a sucrose molecule, the transfer of the fructose released to an acceptor molecule, with the subsequent release of D-glucose. Levan sucrase [(2→6) β-D-fructan: D-glucose-6-fructosyl transferase E.C. 2.4.1.10.], thus catalyses the following reaction:

$$\text{sucrose} + [(2\rightarrow 6)\text{-}\beta\text{-D-fructosyl}]_{n-1} \leftrightarrows [(2\rightarrow 6)\text{-}\beta\text{-D-fructosyl}]_{n} + \text{D-glucose}$$

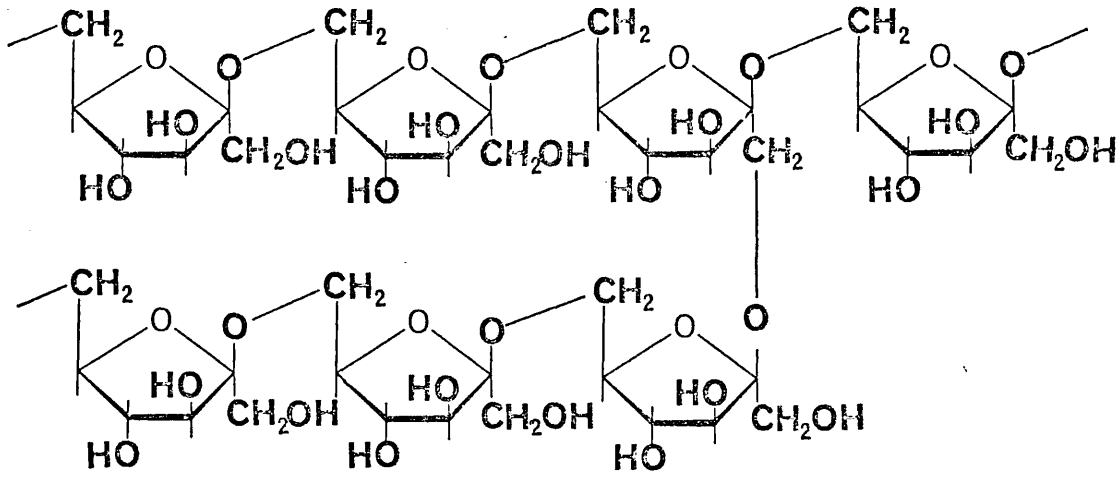
Recently, an enzyme has been found which is able to cleave sucrose and related fructans with the subsequent release of D-fructose. Several names have been given to this enzyme such as sucrase, invertase, levanase depending on the properties investigated. This enzyme(s) is thought to be β-fructofurancsidase, [β-D-fructofuranoside fructohydrolase E.C. 3.2.1.26], and catalyses the following reaction:

A β-D-fructofuranoside + H_2O = an alcohol + D-fructose

1.B. Definition of the term levan

Levan is a homopolymer consisting of D-fructofuranose units joined by β-(2→6) bands. The main linkage is the β-(2→6)

type but branching occurs through β-(2→1) as shown below:



1.C. Brief Survey of the Bacterial fructans and their role in dental caries

In nature, levans are not the only polyfructans to be found. A polysaccharide termed 'inulin' is frequently found in some higher plants such as Compositae[134] and is thought probable that it is used as a food reserve. This group are unbranched polysaccharides comprising β-(2→1) fructosyl linkages, and having molecular weights of approximately 5000.[135,136] Other types of polyfructan have been found in plants.[137]

It is well established that certain bacteria originating in the mouth are able to synthesise extracellular polysaccharides and induce dental caries. This is the loss of tooth substance resulting in cavitation.[138] Because of the warmth and moisture of the mouth an

ideal environment is provided in which bacteria may grow.

Early workers believed that Lactobacilli[139] were the bacteria responsible for dental caries since they produced acids which could be held responsible for dental caries. Miller[140] was the first to recognise that several types of bacteria were found associated with dental caries and that one of which was Streptococci. The minor role of the Lactobacilli compared to the Stretptococci in dental caries was amplified by the work of Winkler and Backer-Dirks.[141] They found that although the Lactobacilli secrete lactic acid onto the surface of the teeth the production of this acid was a lot slower than that of the Streptococci. They also found that Lactobacilli were actually quite rare when compared with the number of Streptococci present within the mouth.

The first real evidence that bacteria play an essential role in dental caries was shown by Orland et al.[142] who showed that dental caries could be induced in rats after infection with Enterococcus. Fitzgerald,[143] later showed that a strain of Streptococcus was able to induce dental caries in rats as well.

It is now accepted that the presence of plaque results in the formation of dental caries.[144] The constituents of plaque are mainly bacteria and carbohydrates which can account for as much as 70% and 10% dry weight, respectively.[144] The cariogenic role of the Streptococci is now understood.

Several cariogenic Streptococci have been shown to produce large amounts of extracellular polysaccharide. One of the main oral bacteria found to produce large amounts of polysaccharide was Streptococcus mutans.[145,146] McDougall[147] was able to show that

a fructan was present in dental plaque, after isolating it from an aqueous extract. Later, Critchley[148] found glucans and fructans to be present in extracts from dental plaque.

Although it was known that the Streptococcci were capable of synthesising extracellular levans,[147] dextrans,[149] or both[150] it was not known whether levan or dextran or both were responsible for dental caries. However, Gibbons and Banghart[151] showed that cariogenic Streptococci were able to produce large amounts of dextran. They found that the dextran was bound to the surface of the teeth which suggested that dextran was the main polysaccharide responsible for dental caries. Later reports substantiated this claim.[152,153,154] Streptococci found to produce extracellular levan were found on the tongue and throat[155,156] and could not be linked directly with cariogenicity.

Since it is questionable whether levan has any function in dental caries, the role of levan is under discussion. A possible role of levan can be deduced from recent studies which indicated that it may provide the necessary carbon source for growth, as Streptococci were found to utilise it to a greater extent than other carbohydrates.[151,157] This fact was supported from the results shown by DaCosta et al.[158] and Manly et al.[159] who both managed to induce plaque Streptococci to produce levan hydrolases. Thus, although not directly associated with cariogenicity levans may serve indirectly in caries formation.

The relationship between oral bacteria and dental plaque has been summarised in several review articles.[160,161,162]

Although it has been known for some time that certain bacteria are able to synthesise polysaccharides, when grown on a carbohydrate enriched medium, interest in the bacteria which are able to synthesise levan began when it was realised how important some strains of Streptococci were in the formation of dental caries. Since this period levan has been found to be produced by a variety of bacteria from species of Aerobacter,[163] Bacillicus,[163] Streptococcus,[156] Pseudomonas[163] and Azotobacter,[164] amongst many others.

Proof of the structure of levans has come from methylation studies on the isolated polysaccharides. On methylation levan was found to give 1,3,4,6-tetra-O-methyl-D-fructose, 1,3,4-tri-O-methyl-D-fructose and 3,4-di-O-methyl-D-fructose, indicating that the units were linked through C-2, C-6 and C-1.[165,166] The structure of the levan synthesised by the strain of bacteria under investigation in this thesis has been characterised fully by Marshall and co-workers.[167] They showed that the fructosyl units were in the furanose form and linked together by β-(2$\rightarrow$6) linkages with branch points of β-(2$\rightarrow$1) linkages. Studies on the degree of branching of various levans by methylation studies have given values between 9-12%,[168,169] corresponding to an average chain length of 7 - 12 units.

The molecular weight of the levans, determined in the analytical ultracentrifuge,[170,171,172] have both found to range from 10^6 to 10^7. Higher[173] values have been determined but it is suggested that this was caused by association of the levan molecules during sedimentation in the ultracentrifuge.

Viscosity measurements on different levans haveshown that the molecule exists in a compact and symmetrical state.[172] Examination of a levan molecule by electron microscopy revealed the spherical or ellipsoidal nature that would be expected of a branched structure.[172]

1.D. Levansucrase

The enzyme responsible for levan synthesis has been under investigation for a considerable period of time. This may seem unusual as the enzyme has only been purified within the last 10 years, but, because of its bacterial origin the isolation procedure has been easy to carry out. Preparations of this enzyme have been easily obtained because in certain bacteria it is secreted into the growth medium and therefore, fairly pure cell free extracts have been prepared. As long as contaminating enzymes such as levanases, sucrases, were deemed not to be present the enzymic properties of levansucrase could be investigated.

Hestrin and co-workers[174] were the first to term the enzyme 'levansucrase' and obtain a cell free extract by selective diffusion through on agar gel. They also found that a cell free extract of the enzyme from Aerobacter levanicum catalysed the formation of levan from sucrose.[175] This enzyme was found to be constitutive and endocellular and not inducible as found for the enzyme from B. subtilis.[174] In a series of articles on the enzyme from A. levanicum the properties were studied in detail.[176,177,178] It was found to be able to produce levan from sucrose and O-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl β-D-fructofuranoside (raffinose) but not from D-glucose, D-fructose, α-D-glucopyranosyl α-D-glucopyranoside (trehalose), 4-O-α-D-glucopyranosyl-α-D-glucopyranose (maltose), 4-O-β-D-galactopyranosyl-

α-D-glucopyranose (lactose) methyl β-D-fructofuranoside, inulin, and some sugar phosphate esters. The substrate specificity of the enzyme was very high. D-Glucose was found to competitively inhibit the synthesis of levan from sucrose. However, other sugars such as D-galactose, D-xylose, L-arabinose, maltose and lactose but not D-mannose, D-fructose, or D-glucosamine, was found to inhibit. It was therefore proposed that the configuration at C-2 of a reducing sugar was the major factor in contribution to inhibitory power. No primers for levan synthesis could be isolated or the reversibility of the levansucrase reaction be demonstrated. Nor could it be proved that levansucrase comprises two enzymes; invertase and polymerases. Levansucrase was therefore defined as an enzyme which catalysed the following transformations;

$$(n+m)\ \text{sucrose} + (n)\ H_2O \longrightarrow (n+m)\ \text{glucose} + (n)\ \text{fructose} + [\text{levan}]$$

$$(n+m)\ \text{raffinose} + (n)\ H_2O \longrightarrow (n+m)\ \text{melibiose}^{*} + (n)\ \text{fructose} + [\text{levan}]$$

* (6-O-α-D-galactopyranosyl-α-D-glucopyranose), where n and m moles of substrate are converted into levan and aldose, and into fructose and aldose respectively, where [levan] represents the levan formed from m fructosidic residues of substrate.

Hehre[179] also described the preparation of a cell free levansucrase from Streptococcus salivarius which synthesised levan from sucrose and raffinose.

In search for a primer molecule, responsible for the initiation of the synthesis of levan, Kohanyi and Dedonder[180] showed that oligo-saccharides were present at intermediate stages in the synthesis, by

extracts of levansucrase from B. subtilis. Hestrin[181] also ran paper chromatograms of the reaction mixture of Aerobacter levansucrase with sucrose, which revealed D-glucose, D-fructose, non-migratory levan, and in addition a small amount of an oligosaccharide corresponding roughly to O-β-D-fructofuranosyl-(2 → 6)-O-β-D-fructofuranosyl-(2 → 6)-β-D-fructofuranose (levantriose). Later, it was suggested that the process whereby the aglycone of β-D-fructofuranosyl aldosides is transferred reversibly to the anomeric carbon position of an aldose may give an insight into the mechanism of levan production.[182] The enzyme from Aerobacter levanicum was therefore able to catalyse the following reaction:

Melibiose + sucrose ⇆ Glucose + raffinose

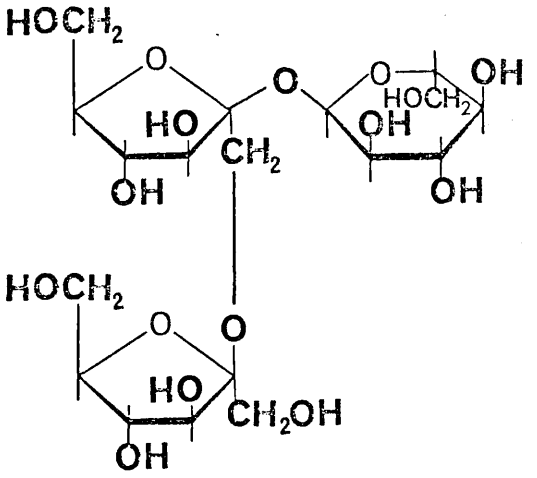
The series of aldoses were also able to be converted to corresponding aldosyl-β-D-fructofuranosides on reaction with raffinose or sucrose in the presence of levansucrase. Subsequently two reactions were proposed for the mechanism in which the first reaction was reversible and the second irreversible as follows:

$$fr\sim R + enz \leftrightarrows fr\sim enz + R \qquad (1)$$

$$fr\sim enz + fr_n \longrightarrow fr_{n+1} + enz \qquad (2)$$

Equation 1 shows the transfer of the fructofuranosyl group (fr) from a donor molecule (fr~R) to the enzyme (enz) with release of aldose (R), and equation 2 shows the reaction in which a levan chain (fr_n) of n-anhydrofructose residues is increased to n+1 residues. Hestrin found that all saccharides containing a fructosyl-aldosyl group with the fructosyl as the end group and the C-1, C-2 and C-6 of the fructosyl

part unsubstituted have substrate properties. In a later publication[178] he was also able to confirm the results of previous workers who isolated a series of fructo-oligosaccharides when levansucrase was incubated with sucrose. The major oligosaccharide product was O- β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl α-D-glucopyranoside (1-Kestose, I) and traces of O- β-D-fructofuranosyl-(2→6)- β-D-fructofuranosyl α-D-glucopyranoside(6-Kestose), were also found.



I

A further paper[183] was published by this group of workers dealing with the synthesis of aldosyl fructosides by levansucrase from A. levanicum. Ebert and Schenk [184] reported that levansucrase from the same source catalysed three reactions: a polyreaction, a transfer reaction and a hydrolysis reaction. They proposed a different mechanism than that proposed by Hestrin. It was assumed by Hestrin that the formation of levan by levansucrase proceeded by a stepwise reaction: free enzyme reacting with sucrose forming an enzyme-fructosyl complex, which then reacted with a primer to form levan (a), with sucrose or aldose to form oligosaccharide (b) and with water to

give the inversion products (c). This is shown below:

a) $fr\text{-}R + P \longrightarrow fr_n - P + (n)R$

b) $v(q-1)\ fr\text{-}R + (v)\ fr\text{-}R \longrightarrow (v)\ fr_q\text{-}R + v(q-1)R$

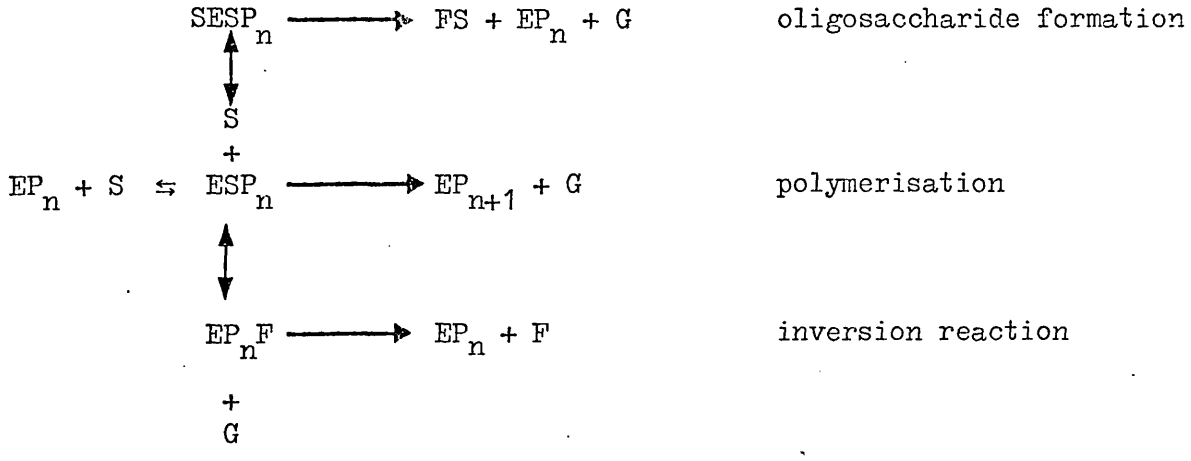
c) $fr\text{-}R + (m)H_2O \longrightarrow (m)$ fructose + $m(R)$

where P = primer molecule

Overall:

$fr\text{-}R + (m)H_2O + P \longrightarrow (m)$ fructose + $(v)fr_q - R + fr_n - P + (s-v)R$

For the suggested polymerisation reaction no primer was ever detected, and the initiation reaction proposed by Hestrin with two molecules of an enzyme intermediate, was excluded by Ebert. Instead he proposed a reaction mechanism in which the levan formation follows an insertion-type growth. The oligosaccharide formation is a transfer reaction of the fructose from the substrate, to the acceptor, and the inversion reaction is an hydrolysis reaction via a further intermediate. The Scheme is shown below:



where P_n denotes a levan molecule and S, F, and G denote sucrose, fructose, and glucose, respectively. Ebert based his results on kinetic data determined for the three reactions.

Dedonder and co-workers[185] used the levansucrase from B. subtilis to investigate levan synthesis. The D-glucose liberated during the reaction of levansucrase with sucrose was found to be in the α-form, indicating that the glucose is liberated with retention of configuration. Levansucrase transferred the fructosyl residue onto monosaccharides, disaccharides, methanol and glycerol and even transferred fructose from sucrose onto glucuronic acid. This indicated that the fructosyl acceptor specificity was poor. Because of the broad specificity of the enzyme the authors suggested three active sites on the enzyme molecule. A site for sucrose fixation, subdivided into a ketosyl and an aldosyl site and a site for the levan acceptor. In a further publication Dedonder[186] confirmed that no primer existed for levan synthesis. Sucrose was found to be the first acceptor for levan synthesis and 1-kestose was the first product synthesised. Further products were formed by the addition of (2→6) linked fructosyl residues to 1-ketose. Both D-glucose and D-fructose at high concentration were found to initiate levan synthesis. To account for these results neither the "insertion" type mechanism or the "multimolecular" mechanism could be applied, instead the authors suggested a "multi" attack mechanism demonstrated for β-amylase. Fructosyl residues are successively transferred to the "free" end of the levan chain which is accommodated at the non-specific acceptor site. Eventually distortion becomes too great and the levan molecule dissociates from the enzyme molecule.

The reader is recommended to read several reviews[181,184] which discuss the mechanism for levan formation by levansucrase in more detail.

Studies on the properties of purified levansucrases have been scarce. Since the importance of dextrans in the formation of dental caries has been confirmed, a far greater study has been carried out on dextransucrases rather than levansucrases. Hehre[187] investigated the properties of a cell free levansucrase as mentioned previously. Studies on the levansucrases of Bacilli, Streptococci and Aerobacter showed that these levansucrases utilised sucrose and raffinose and had Michaelis constants for sucrose in the range 20 to 60 mM. None of the following respiratory or glycolytic inhibitors : cyanide, fluoride, iodoacetate, failed to suppress levan formation. The enzyme was shown to have maximum activity between pH 5.0 to 5.8.

A pure enzyme preparation has been obtained from B. subtilis.[188] After precipitation with ethanol and salting out with ammonium sulphate, followed by chromatography on hydroxylapatite, the enzyme gave a single symmetrical peak on ultracentrifugation. A molecular weight of 40,000 was obtained by analytical ultracentrifugation, compared with a value of 22,000 determined for the enzyme from A. levanicum. Maximum sucrase activity was obtained between pH 5.8 to 6.0. The Michaelis constant (Km) for sucrose of 20 mM, was similar to the value obtained by Hehre, for several levansucrases from different bacterial sources. 2-Amino-2(hydroxymethyl) propane-1,3,diol (tris) was found to be a very potent inhibitor, and several reducing agents were found to suppress the activity of the enzyme.

Carlsson,[189] in 1970, purified the levansucrase from the cariogenic bacterium Streptococcus mutans. Purification of the enzyme was achieved in two steps by adsorption on hydroxylapatite followed by isoelectric focussing. A final yield of 10% was obtained. The ultimate purity of

Table 31 Properties of the purified levansucrases

Reference	Source	Purification procedure	Yield %	Molecular weight	Inhibitors	pH at maximum activity	K_m sucrose (mM)
188	*B. subtilis* BS5	EtOH ppt, $(NH_4)_2 SO_4$ hydroxylapatite	15	40,000	Tris, reducing agents	5.8 - 6.0	20-50
190	*B. subtilis* Marburg QB13	As previous	28	40.000	-	6.0	27
189	*S. mutans* JC2	Hydroxylapatite, isoelectric focussing x 2	10	-	Heavy metal ions	6.0	-
191	*S. salivarius* SS2	Biogel A-15m " P-60 DEAE-cellulose	60	34,500	EDTA reversible	6.0	17
192	*S. mutans* FA-1	Biogel P-60 DEAE-cellulose Biogel A-15m	2	220,000	Heavy metal ions	6.0	-
193	*Actinomyces viscosus* T-14v	DEAE-cellulose, hydroxy-apatite. Agarose A-0.5m hydroxylapatite	5	220,000	Lactose cellobiose tris, reducing agents.	6	12

the enzyme was not proved. Maximum sucrase activity was shown at pH 6.0, and the isoelectric point was found to be pH 4.2. Although tris and sodium fluoride were found not to inhibit the activity of the enzyme, ethylenediaminetetracetic acid (EDTA) reduced the overall activity of the enzyme by 75%. Most divalent cations however were found to restore the activity. The properties of this streptococcal levansucrase were similar to those described previously for levansucrase from A. levanicum and B. subtilis.[188]

A summary of the levansucrases which have been purified is given in Table 31.

1.E. β-Fructosidases

Most of the bacterial fructosidases which have been isolated have been classified as β-fructofuranosidases, commonly known as invertases. Another common name also used is that of sucrases. The term levanase has been used to name an enzyme responsible for the enzymic degradation of the bacterial polysaccharide, levan.

These bacterial enzymes have been found to be both constitutive and inducible, depending on the strain. The location of these enzymes differs from strain to strain, some are cell associated intracellular, or extracellular, secreted out of the bacterial cell.

Invertases have been extensively studied over a long period of time principally from fungal and mould sources. Several reviews have been published but bacterial invertases have not been covered because of a lack of scientific interest shown in them. However, in 1950, Newburg and Mandl[194] mention in a review article that invertases had been found to be present in certain strains of bacteria. Included are the bacteria that synthesise acetic and lactic acids, (Acetobacter and Lactobacilli)

but not all strains. The amounts found in Escherichia coli varied but was uniform in Thermobacterium mobile and sulphur bacteria.[195] It was demonstrated in some pathogenic bacteria (Vibrio cholerae, Streptococci and Pneumococci).

Two enzymes capable of degrading levan but not classified as β-D-fructofuranosidase have been extracted from bacteria. Hestrin and co-workers have successfully isolated extracts of the same enzyme from different bacterial sources. Hestrin and Goldburn[196] isolated a levan degrading enzyme from a strain of Azotobacter and also from a strain of Bacillus and termed it levanpolyase. Both cultures contained levan as the carbon source. When a cell-free extract of the enzyme was incubated with levan the reducing power was found to correspond to 18% of the total determined as D-fructose in the levan. By adding more enzyme a mixture of oligolevans were produced consisting of 20% levantriose and levantetrose and 80% higher members of the levan series, no fructose was produced. It was concluded that levanpolyase either hydrolyses branch linkages only or it acted as an endo enzyme cleaving interfructosidic linkages in suitably long chains and only if the linkage was several units from the chain terminals. A similar acting enzyme has also been isolated from Aerobacter levanicum strain 7.[181]

Fuchs[163] carried out an extensive investigation into the bacterial breakdown of levans. He found that strains of Pseudomonas, Azotobacter, Aerobacter, Serratia, Bacillus, and Clostridium degraded levan and thus showed that the property of hydrolysing levan is widespread amongst bacteria. Two types of enzyme were distinguishable. Type one produced only D-fructose as the product indicating the gradual breakdown of levan,

the enzyme acting in an exo fashion. Type two produced a homologue series of oligosaccharides containing β-(2→6) linked fructosyl units, when incubated with levan. This enzyme type differed from strain to strain in that sometimes D-fructose was produced. Type two was equivalent to the levan polyase of Hestrin. Type one is most probably a β-fructofuranosidase.

A levan hydrolysing enzyme which cleaves the β-(2→6) fructofuranosidic linkages has been isolated from Pseudomonas.[197] The organism was grown on levan as the carbon source. The enzyme was extracellular being secreted into the growth medium. The enzyme was therefore easily isolated by precipitating the enzyme with ammonium sulphate. On incubation of the enzyme with levan the products of hydrolysis were identified as levanbiose and a limit levan. No D-fructose or oligosaccharide products containing a β-(2→1) linkage were isolated thus indicating that the enzyme was unable to cleave the β-(2→1) linkage at branch points. It was proposed that the enzyme acts on a levan molecule by sequential degradation liberating levanbiose from the non-reducing ends similar to the action of β-amylase on glycogen or amylopectin.

Sucrose can be degraded by a variety of bacterial enzymes (Fig. 54). Both levansucrase and dextransucrase are able to hydrolyse sucrose and catalyse the transfer reaction to produce levan and dextran, respectively.

Three enzymes, namely, α-glucosidase, sucrose phosphorylase, and β-D-fructofuranosidase are able to hydrolyse sucrose with the subsequent release of D-fructose and D-glucose, or glucose-1-phosphate in the case of sucrose phosphorylase.

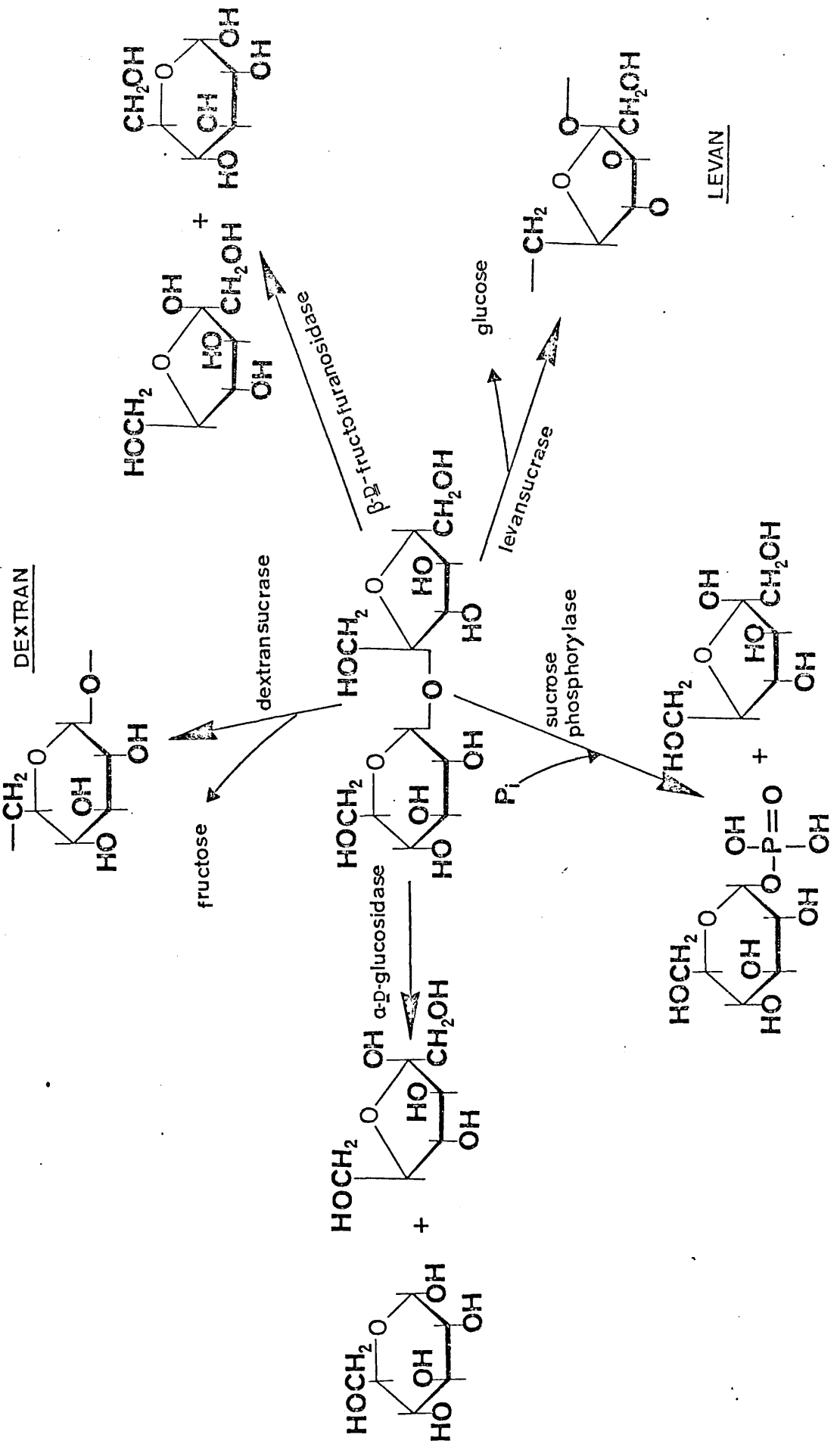


Fig. 54 Different pathways of Sucrose degradation catalysed by bacterial enzymes

Table 32 List of β-D-fructofuranosidases isolated from different bacteria

Strain and references	Inducible	Constitutive	Location of enzyme		Carbon source
			Intracellular	extracellular	
B. subtilis [200] 168	√	—	√	—	Glycerol and sucrose or sucrose
B. subtilis QB [200] 13	√	—	—	√	Sucrose or glucose glucose
S. mutans [211] GS5	—	√	—	√	Glucose
S. mutans [201] KI-R	—	√	√	—	Glucose
S. sanguis [201]	√	—	√	—	Glucose or sucrose
S. mutans [205] SL-1	√	—	√	—	Sucrose or glucose
S. mutans [206] LM7	?	?	—	√	Glucose or sucrose
S. salivarius [206]	?	?	—	√	
S. mutans [212] HS-6	—	√	—	√	Glucose

The first worker who was able to isolate and purify a β-fructofuranosidase from a bacterial source, was Negoro.[198] Purification was carried out by salting out with ammonium sulphate and fractionation with ethanol. A crystallised enzyme was prepared which had a 4×10^4 higher activity than that in the crude filtrate. The enzyme, from Bacillus subtilis, was classified as a β-D-fructofuranosidase.

Most of the bacterial invertases which have been investigated have been from cariogenic Streptococci. Those which have been isolated are listed in Table 32, with details of whether the synthesis is inducible or not, and the location of the enzyme. Usually when the location of the enzyme was intracellular some invertase activity was found to be extracellular and vice versa. Not all of the invertases isolated from bacterial sources have been purified, those which have, are listed in Table 33.

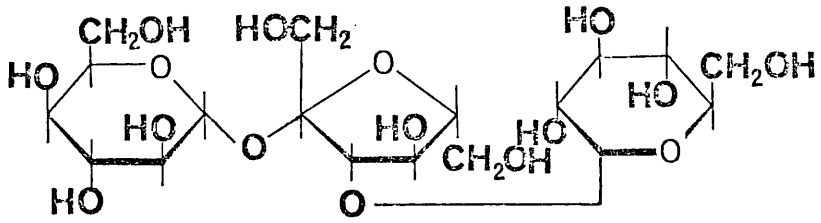
Following the work of Negoro other workers were able to induce sucrase activity in B. subtilis strain 160.[199] When lactate was substituted for glycerol or glucose as the carbon source it was found that increased levels of sucrose hydrolysis occurred. However, regardless of the medium used the activity, which was induced, proved to be quite unstable. Of the various compounds used to try and stabilise this activity EDTA was found to have an appreciable effect on sucrase activity. It stabilised extracts for a short period of time.

Dedonder and co-workers[200] were the first to separate the two enzymes responsible for sucrase activity, namely levansucrase and invertase. Using a hydroxylapatite column, two sucrase active fractions were isolated. The properties of fraction I, which are

Table 33 Properties of the purified bacterial β-D-fructofuranosidases

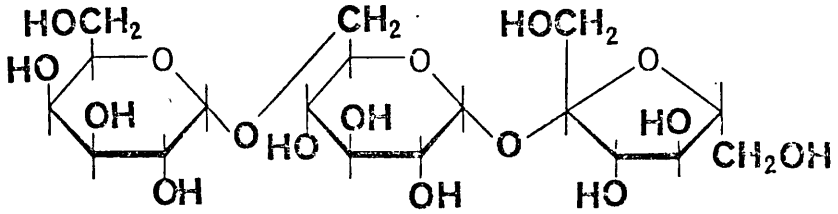
Strain and reference	Purification method	Criteria of purity	pH at maximum activity	Molecular weight	K_m (Sucrose) (mM)	Inhibitors
B. subtilis[200] QB13	Hydroxylapatite	-	6.2	40,000	40	Sulphydryl group reagents.
S. mutans[211] GS5	Biogel A-15 DEAE-Sephadex	-	7.0	47,000	140	Sulphydryl reagents Fructose
S. mutans[205] SL1	DEAE - Cellulose	-	5.5 - 6.2	48,000	35	
S. mutans[212] HS-6	Sepharose 6B DEAE - Cellulose hydroxylapatite	disc-gel electro-phoresis	5.2	160,000	20	
S. mutans[207] SL-1	Sephadex G-100	-	6.2	48,000	-	
Oral Streptococci[209]	Isoelectric focussing (pI = 4.4)	-	6-7	-	7	

typical of all the bacterial invertases isolated, showed conclusively that the sucrase was indeed an invertase. It did not hydrolyse O-α-D-glucopyranosyl-(1→3)-β-D-fructofuranosyl α-D-glucopyranoside II (melezitose)



II

or a sucrose derivative with a substituted fructofuranosyl residue. However, it did hydrolyse raffinose (III) to give melibiose and D-fructose.



III

McCabe et al.[201] further studied the catalytic properties of the invertase from S. mutans strain KI-R. The enzyme hydrolysed sucrose and raffinose in which both have a β-D-fructofuranosyl linkage available for hydrolysis. In contrast, the enzyme had no action on melezitose a trisaccharide in which the fructosyl residue of the sucrose moiety is linked to another second glucosyl residue and hence is not available for invertase action. In addition 3-O-α-D-glucopyranosyl-α-D-fructofuranose(turanose), trehalose, and

the methyl α- and β-glucopyranosides could not be hydrolysed indicating that trehalase and glucosidase activities were absent.

It is possible that contaminating enzymes may be present together with invertase in which case results not compatible with the hydrolytic action of one enzyme may arise. Sucrose phosphorylase has been proposed as one enzyme which may be present.[202] This enzyme catalyses the following reaction:

$$\text{Sucrose} + P_i \leftrightarrows \alpha\text{-D-Glucopyranosyl phosphate} + \text{D-fructose}$$

If both sucrose phosphorylase and invertase were present together in an incubation containing sucrose it would be expected that the concentration of D-fructose would be greater than D-glucose. Another enzyme which has been detected is invertase extracts is amylomaltase.[203] A series of oligosaccharides are produced when this enzyme acts on maltose together with D-glucose.

It has been proposed[204] that in S. mutans strain BS5 a sucrose permease system may occur for the transport of sucrose across the bacterial membrane. Gibbons used sodium fluoride to inhibit the glycolytic pathway of growing cells and then compared the invertase activities of some cells which had been treated with toluene, with others which had not been treated. He found that those treated with toluene exhibited a 10 times greater activity of sucrase.. A comparison of those cells grown on D-glucose or sucrose showed that the toluene treated cells grown on D-glucose exhibited sucrase activity comparable to sucrose grown cells. The same transport mechanism was also proposed later by Tanzer et al.[205] because of the intracellular location of the invertase from S. mutans strain SL-1.

A study of seven different strains of Streptococci has shown that all were able to show considerable invertase activity.[206] Considerable extracellular invertase activity was found in five Streptococcus mutans strains and one Streptococcus salivarius strain. The invertase was characterised as a β-D-fructofuranosidase by its substrate specificity as described previously. In all of the cases studied the extra- and intracellular invertases were always present but their levels varied depending on the carbohydrate in the medium. A comparison of the electrophoretic behaviour of the intra- and extracellular invertases from S. mutans strain SL-1 showed that they had identical migration rates, indicating a close similarity.[207] Chassy[208] found rather different results when he compared the properties of the intra- and extracellular invertases of S. mutans strain LM-7. The extracellular invertase had a molecular weight of 500,000 and a Michaelis constant (K_m) for sucrose of 7 mM, whilst the molecular weight for the intracellular enzyme was 40,000 with a Michaelis constant of 94 mM. Both the pH at maximum activity and isoelectric points differed.

Instead of isolating the enzyme from bacterial cultures Dahlqvist and Birkhed[209] isolated and characterised the enzyme from human saliva. The relative low value of 7 mM for the Michaelis constant (K_m) indicated the high affinity of the enzyme for sucrose. Many characteristics of the sucrose activity found during the study indicated the presence of more than one enzyme. The kinetics of the heat inactivation indicated the presence of more than one sucrase component. No mention, however, was made as to the origin of the other sucrase activity whether it was due to dextransucrase, levansucrase or perhaps

another β-D-fructosidase. Birkhed[210], later managed to show that three different sucrase activities were detectable and separated them on Sephadex G-150. Only one "sucrase" did not produce any polysaccharide from sucrose and he deduced that this enzyme was an invertase. The other two he later classified as dextransucrase and levansucrase, after analysing the polysaccharides they produced.

Some variation does exist for some properties of the bacterial invertases investigated. The Michaelis constant for sucrose varies considerably between 7 - 140 mM (Table 33), the highest affinity for this substrate being shown by the invertase isolated from human saliva. Values for the molecular weight of the bacterial invertases seem to be in the range of approximately 40,000, although much higher values have been found. These higher values seem to be indicative of either aggregation of protein subunits or binding of carbohydrate to the enzyme molecule, suggesting some may in fact be glycoproteins.

The ability of bacterial invertases to catalyse a transfer reaction is not substantiated at the present time. An unknown product of a reaction between S. mutans LM-7 invertase[206] and [U-^{14}C] sucrose has been primarily identified as possibly being a kestose. Thus a transfructosylation reaction may be possible, as it is known that a transfructosylation reaction iscatalysed by yeast invertase.[213]

Although the bacterial invertases have been fully characterised as β-D-fructofuranosidases little information is available regarding the ability of these enzymes to degrade inulin or levan. Hestrin and co-workers[214] stated the following ... "The ability of the yeast invertase to hydrolyse inulins and levans probably varies depending on the polymerisation degree; whereas the short chained members of these polymer homologous series are readily hydrolysed by yeast invertase concentrates, the action on the corresponding native

high polymers is extremely slow or negligible". The criteria for β-D-fructofuranosidase action is that a substrate most have a terminal unsubstituted β-D-fructofuranosyl unit in which case there should be no reason why this enzyme should not attack polysaccharide substrates.

Purified invertase preparations from both brewer's yeast and baker's yeast[215] showed inulase activity. However, it was suggested that the activity could be due to a specific inulase. Takahashi et al.[216] however, showed that some different invertases did hydrolyse inulin. Invertase from Lactobacillus plantanum, Saccharomyces fragilis, and Aspergillus awamori hydrolysed sucrose, inulin and raffinose. All three substrates were found to be competitive inhibitors of one another indicating the presence of only one invertase. A commercial preparation of invertase did not hydrolyse inulin. Marshall[217] has shown that a commercial preparation of invertase from yeast is able to hydrolyse inulin and levan.

The evidence available to clarify whether a levanase is in fact a β-D-fructofuranosidase (invertase) is scarce. As stated previously several workers have isolated an enzyme termed a levanase but little or no investigation of the properties has been carried out. A β-D-fructosidase has been isolated from the yeast Saccharomyces fragilis[218] and termed inulinase. The yeast was grown on a medium containing inulin as the carbon source. The enzyme was able to hydrolyse inulin, sucrose, raffinose, and bacterial levans and hence it attacked β-(2→1) and β-(2→6) linkages. The enzyme was found to differ from invertase by its increased activity towards inulin or levans. Maximum sucrase activity was obtained at pH 4.3 whilst maximum inulinase activity took place at pH 5.1.

Da Costa et al.[158] induced the formation of a fructan hydrolase by a Streptococcal oral strain grown on levan. Maximum levanase activity was observed at pH 6.0. It was relatively heat stable, no loss of enzyme activity was observed after a 60 minute incubation at 40°C. It was found that the enzyme cleaved terminal fructosyl units from the levan molecule. The levanase was found to be able to hydrolyse inulin, also enzyme preparations from an inulin broth culture also hydrolysed levan. Thus, the enzyme was found capable of hydrolysing β-(2→6) and β-(2→1) linkages. The authors suggested there was either more than one fructan hydrolase present or a non-specific fructan hydrolase similar to that produced by Saccharomyces fragilis.[218]

Mesner[219] prepared an inducible levanase from Odontomyces viscosus. The enzyme had maximum activity in the pH range 5.4 - 6.6. It was inactivated completely after dialysis against 10 mM ethylenediaminetetracetic acid (EDTA). Activity was restored on addition of calcium ions and partially restored by manganese or magnesium ions.

A partially purified levanase has been isolated from a mutant Bacillus subtilis strain 168.[220] The enzyme was found to be extracellular and constitutive. Purification was carried out on a hydroxylaptite column, after adsorption on the column the enzyme was eluted with phosphate buffer. Maximum levanase activity was obtained at a pH of 5.5. The enzyme was capable of hydrolysing sucrose, inulin and levan with Michaelis constants of 50 mM, 5 mM, 0.25 mM, respectively. After determining the properties of the levanase the

authors classified the enzyme as a β-D-fructofuranosidase.

Several authors have suggested that the enzyme may have a function relating to the survival of the bacteria.[158,220] It is reported that after carbon source depletion the enzyme would be used to provide an alternative carbon source through the degradation of levan.

2. Results and Discussion

II.2.A. Isolation and purification of Streptococcus salivarius Strain '51' Levanase

a) General aspects of purification

Up to the present time no information is available in the literature concerning the purification of this enzyme. Therefore, the following section describes the various techniques which have been utilised by the author to try and purify the enzyme. No order is used but each technique is discussed on its merit. The final enzyme preparation used in Chapter II.2.B. to investigate the properties of the bacterial levanase was purified as described in Section 2.A.(h). It should be borne in mind that several batches of the enzyme were prepared for use in this section. Also, as a technique is described it will not be described again if used in another isolation batch, only the degree of purfication will be given.

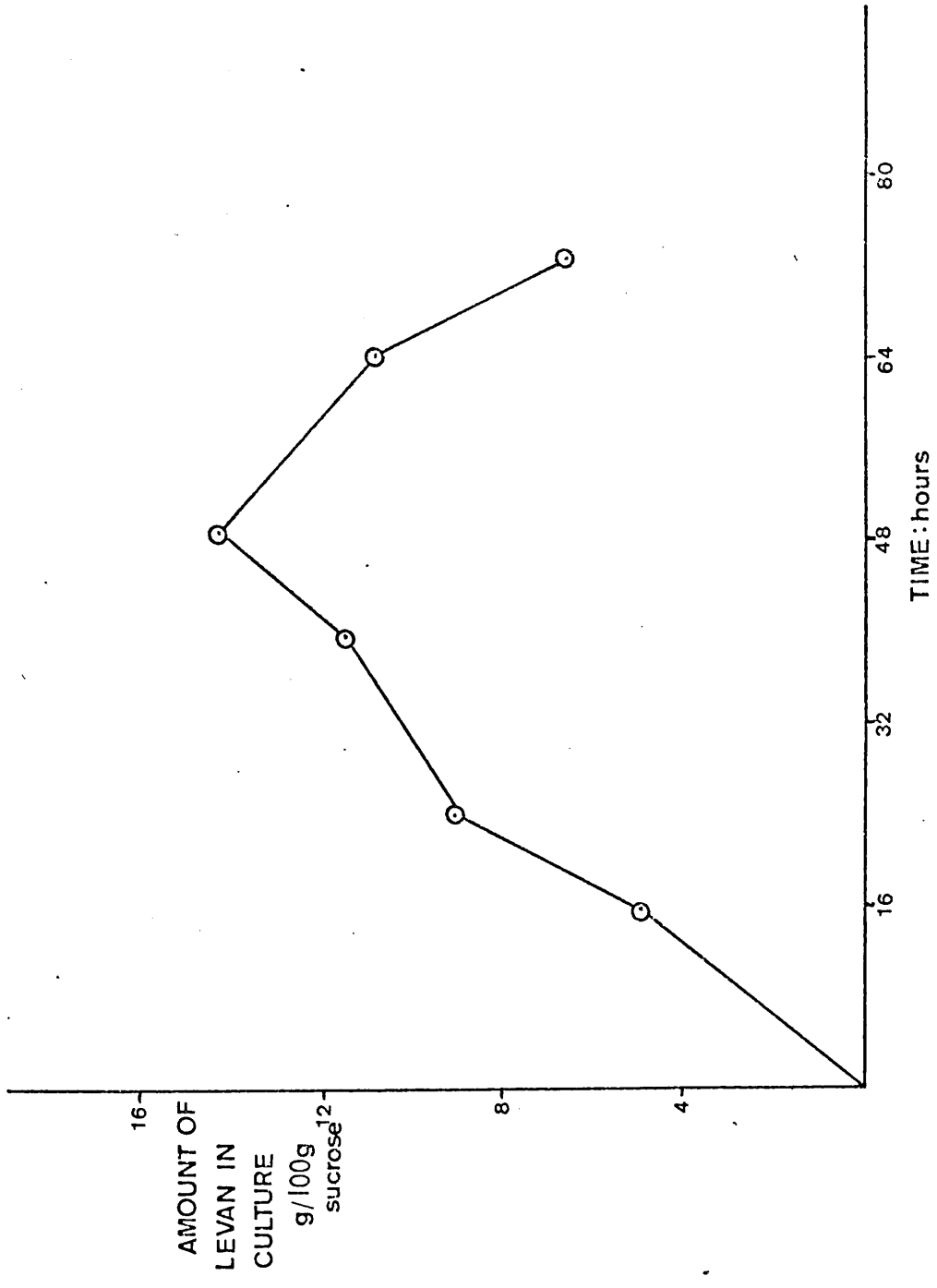
b) Preparation of the enzyme

In order to induce the bacteria to produce levanase sufficient polysaccharide had to be prepared. This was carried out by growing the bacteria on a medium containing sucrose as described in Experiment 31.II.

To determine the optimum time at which maximum yields of levan could be isolated, the amount of levan synthesised over a 72 hour period was found. The procedure is described in Experiment 31.IIa. The results are shown in Fig. 55. Maximum levan production occurred after 48 hours after the start of the incubation of the culture.

Fig. 55

Polysaccharide concentration in culture of S. salivarius, strain 51



After this time the amount of levan in the medium decreased indicating that the polysaccharide was being enzymically degraded. These results are in agreement with those found by Marshall.[217]

Large scale cultures for the preparation of between 70 - 100 g of levan were carried out as described in Experiment 31.IIb. Yields of levan were ca. 16 g per 100 g of sucrose.

The preparation of the culture medium and the experimental details for the production of the levanase are described in Experiment 31.III. Cultures were maintained at 37°C for 48 hours as suggested by Marshall.[217] Because of the large volumes involved in the preparation of the enzyme no activity or protein determinations were made on the culture medium. The presence of reducing sugars would also hinder any activity determinations.

c) Preliminary Purification of the enzyme preparation

To be able to isolate the enzyme from the culture medium it was decided to salt out the protein with ammonium sulphate. The procedure is described in Experiment 31.III. Although Marshall[217] precipitated the enzyme from 0 to 60% w.r.t. ammonium sulphate, this result could not be repeated. On addition of ammonium sulphate to 70% saturation the enzyme was precipitated, further saturation to 80% yielded more protein with a small amount of levanase activity. Further cultures were therefore salted out to 80% saturation with ammonium sulphate. An average of 28 enzyme units per litre of culture media with a specific activity of 1.7 units per mg protein was obtained.

d) The partial purification of the enzyme by gel-permeation chromatography using Sephadex G-200.

To remove contaminating proteins of differing molecular weight than the enzyme, the levanase preparation was subjected to Sephadex G-200 chromatography as described in Experiment 32.

An elution profile is shown in Fig. 56. The enzyme was eluted in a volume corresponding approximately to the void volume of the column. Less than a two fold purification was obtained for the enzyme preparation as shown in Table 34.

Table 34 Purification figures for S. salivarius levanase after purification on Sephadex G-200

Stage	Volume (cm^3)	Total° activity units	Total protein (mg)	Specific* activity	Yield %
Ammonium sulphate precipitation	28	192	98	1.95	-
Sephadex G-200 chromatography	15.9	132	55.6	2.37	69

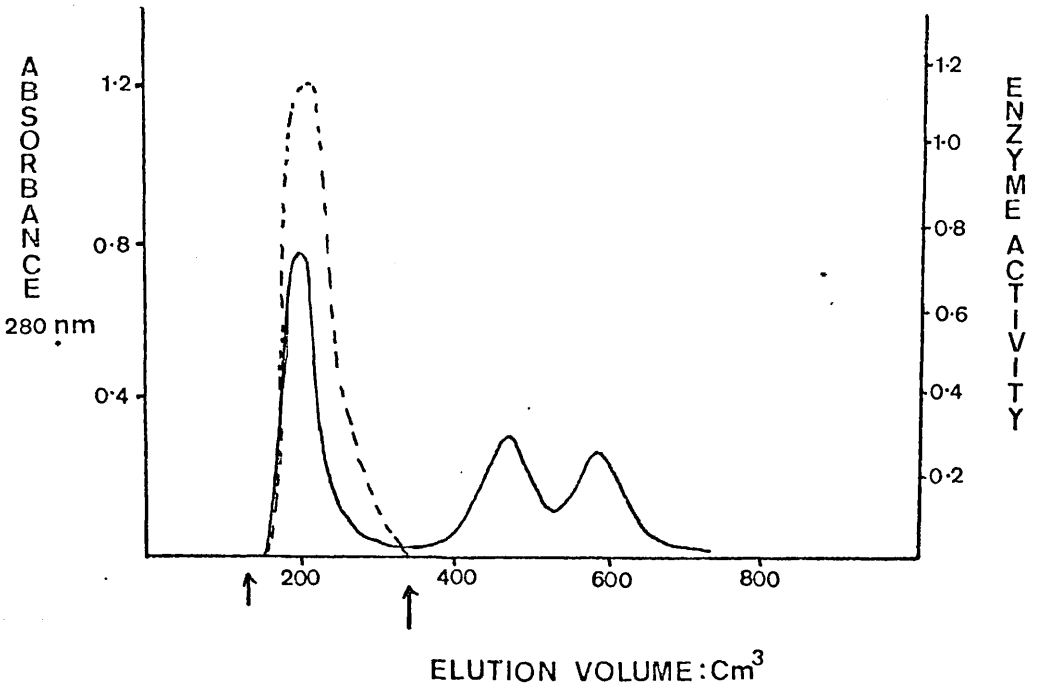
° One unit of activity is the liberation of 1 µmole fructose per minute.

* Expressed as the number of enzyme units per mg protein.

Disc gel electrophoresis of the partially purified enzyme was carried out as described in Experiment 33. Several protein bands were visible after staining the gel (Fig. 57). From the elution profile of the levanase preparation on Sephadex G-200 (Fig. 56) it is shown that the enzyme must have a molecular weight greater than

Fig. 56

Elution profile for S. salivarius levanase on Sephadex G-200



A) Eluant: 100mM phosphate buffer pH 6.6.

B) Fractions between arrows were pooled.

C) Enzyme activity is expressed as number of μmoles fructose liberated per minute per cm^3 eluate solution.

D) --------- levanase activity.

E) ——————absorbance (280 nm).

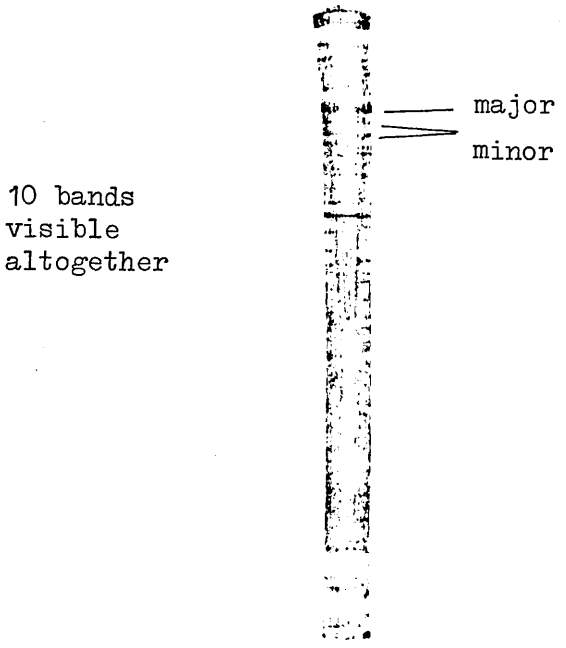


Fig. 58

S.D.S. Electrophoresis of Sephadex G-200 purified levanase

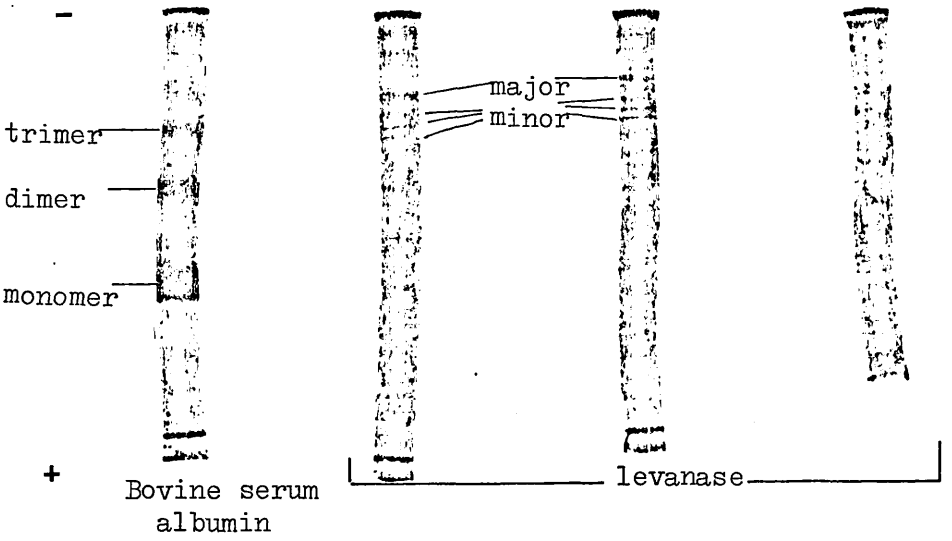


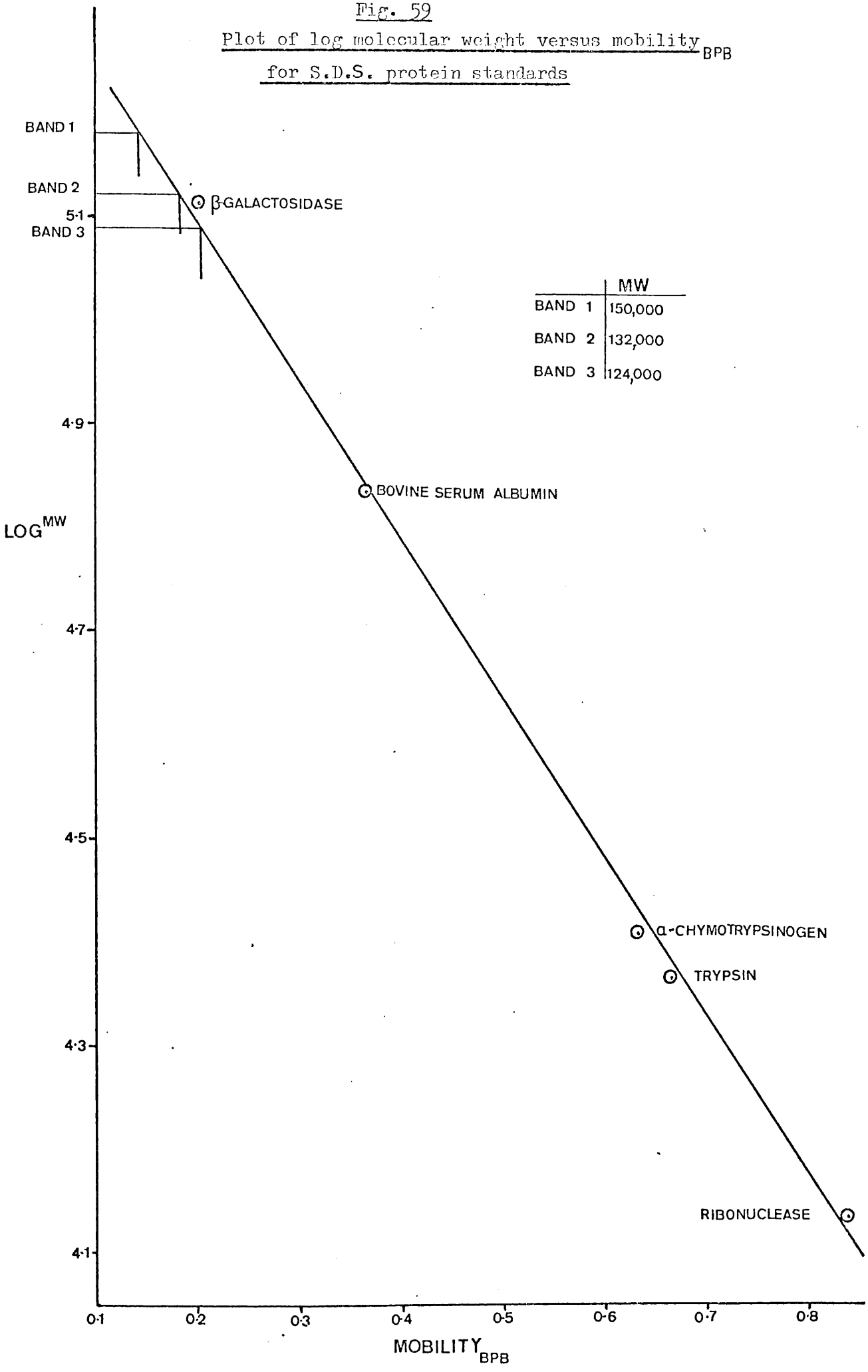
Fig. 57

Disc gel electrophoresis of Sephadex G-200 purified levanase

Fig. 59

Plot of log molecular weight versus mobility $_{BPB}$

for S.D.S. protein standards



the effective fractionation range of the gel. Also, the contaminating proteins that are present, as indicated by disc gel electrophoresis, must have a molecular weight above this value. To gain some ideas as to the molecular dimensions of the proteins present in the partially purified extract, S.D.S. electrophoresis was performed as described in Experiment 34. A gel stained after electrophoresis is shown in Fig. 58. A total of ten bands were visible. The molecular weights of the major, and two minor bands slightly more mobile than the major band, gave values of ca. 150,000, 132,000 and 124,000, respectively (Fig. 59). The results suggest the breakdown of protein aggregates as these values are less than what is expected. It would seem therefore that it may not be possible to separate all the contaminating proteins by fractionation on a gel permeation column. It was therefore considered appropriate to consider other techniques for separating contaminating proteins from the levanase preparation.

e) <u>The instability of the enzyme to purification by Ion-exchange chromatography</u>

The use of this technique for separating proteins by charge is well documented.[221] Proteins are good electrolytes and hence depending on the charge exerted at a known pH they can be bound to either an anion or cation exchanger.

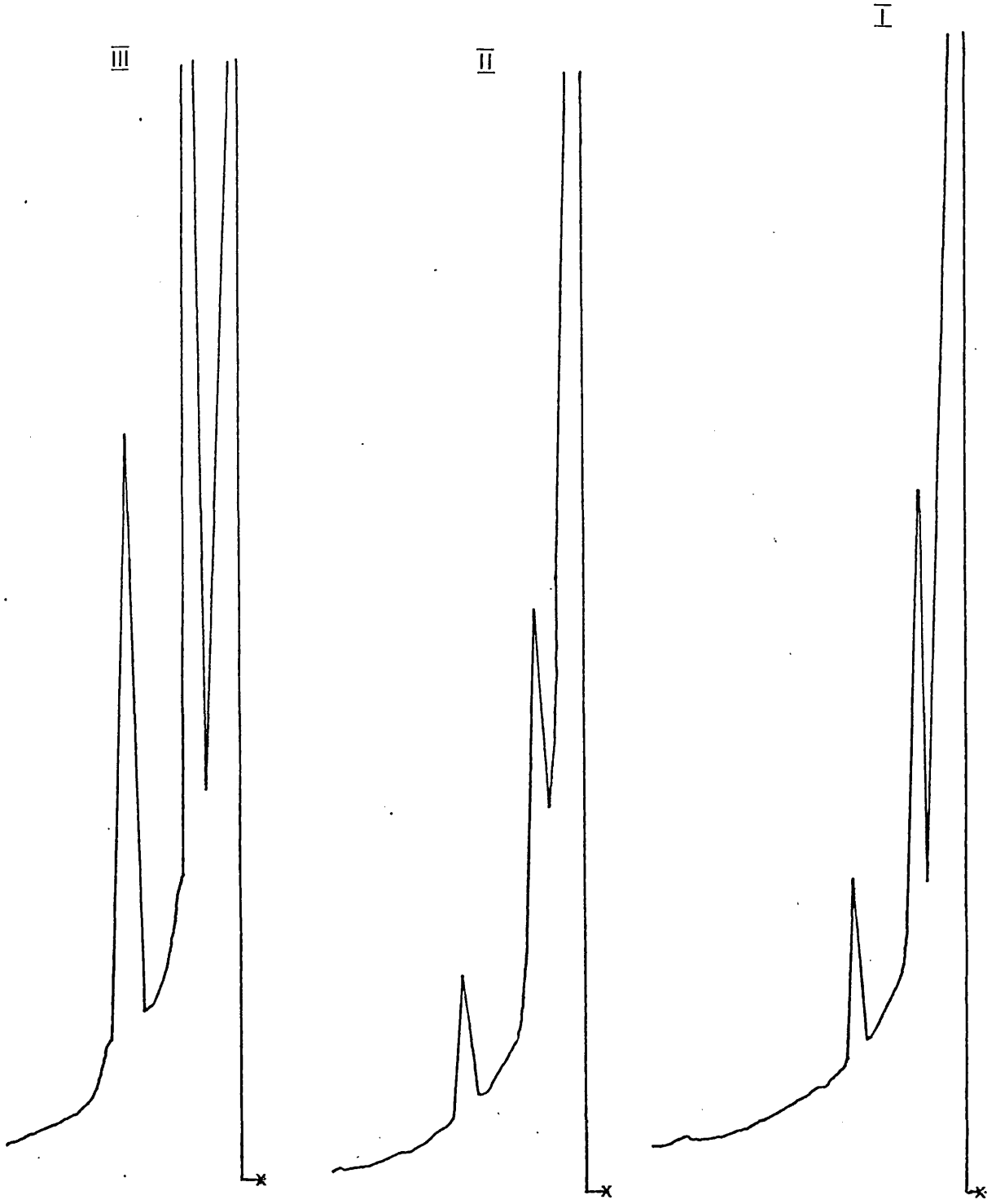
Preliminary experiments were carried out on a test tube scale to find the right type of exchanger which at a certain pH would bind the enzyme, (Experiment 35). This involved taking a small amount of either carboxy-methyl-cellulose (CM) or diethylaminoethyl-

cellulose (DEAE) in a test tube, buffered at a known pH, and binding the enzyme to it. Activity determinations were carried out on the supernatant and compared to an enzyme blank to determine whether any enzyme had been bound. The results indicated that CM-cellulose at pH 4 would bind the enzyme.

A column packed with CM-cellulose was prepared as described in Experiment 36. The enzyme previously purified on Sephadex G-200 was placed on the column, after being dialysed against the starting buffer. Elution was performed using a stepwise sodium chloride gradient. No levanase activity was detected in any of the fractions collected. Fractions containing protein were pooled into four separate samples, concentrated, and dialysed against the buffer used during the isolation of the enzyme, to determine whether activity was hindered by such a low pH. No activity was detected in the concentrated fractions. It was therefore decided to pool and concentrate all four fractions to see if activity could be regenerated in this fashion. Again after standing overnight no activity was detected. As a cation exchanger was used to purify the enzyme it was thought possible that if metal ions were required by the enzyme for activity they could have been removed during the passage of the enzyme through the ion-exchanger. Incubations were then carried out in the presence of magnesium, calcium, and manganese ions and the products analysed by G.L.C. This method of analysis was used because of the interference of these metal ions with the Nelson reducing test. The results are shown in Fig. 60. Fructose was shown to be present in the incubation containing calcium ions but it was also shown to be present in the blank.

Fig. 60

G.L.C. of trimethylsilyl derivities of fructose



I Incubation contained: 1) Ca^{2+} 2) Enzyme 3) Levan

II Incubation contained: 1) Ca^{2+} 2) Levan

III Fructose blank

Unfortunately, insoluble phosphates were precipitated because of the presence of phosphate buffer. The presence of fructose in the blank suggests that a shift in the pH of the incubation occurred when insoluble phosphates were precipitated, resulting in acid hydrolysis of the levan. This was confirmed later when the pH of a blank containing calcium ions and phosphate buffer was found to be approximately 5.5. It was concluded that irreversible denaturation of the enzyme had occurred during the purification procedure due to the low pH of the buffer.

Before any other chromatographic techniques were employed to purify the enzyme it seemed appropriate to investigate whether EDTA had any inhibitory effect upon the levanase activity of the enzyme. The procedure is described in Experiment 37 and the results are given in Table 35. No appreciable loss of activity was found up to a final concentration of 10 mM EDTA.

Table 35 Effect of EDTA on levanase activity

Sample	Concentration of EDTA 1 mM	5 mM	10 mM
A. Control	100%	100%	100%
B. % activity remaining	100	100	96

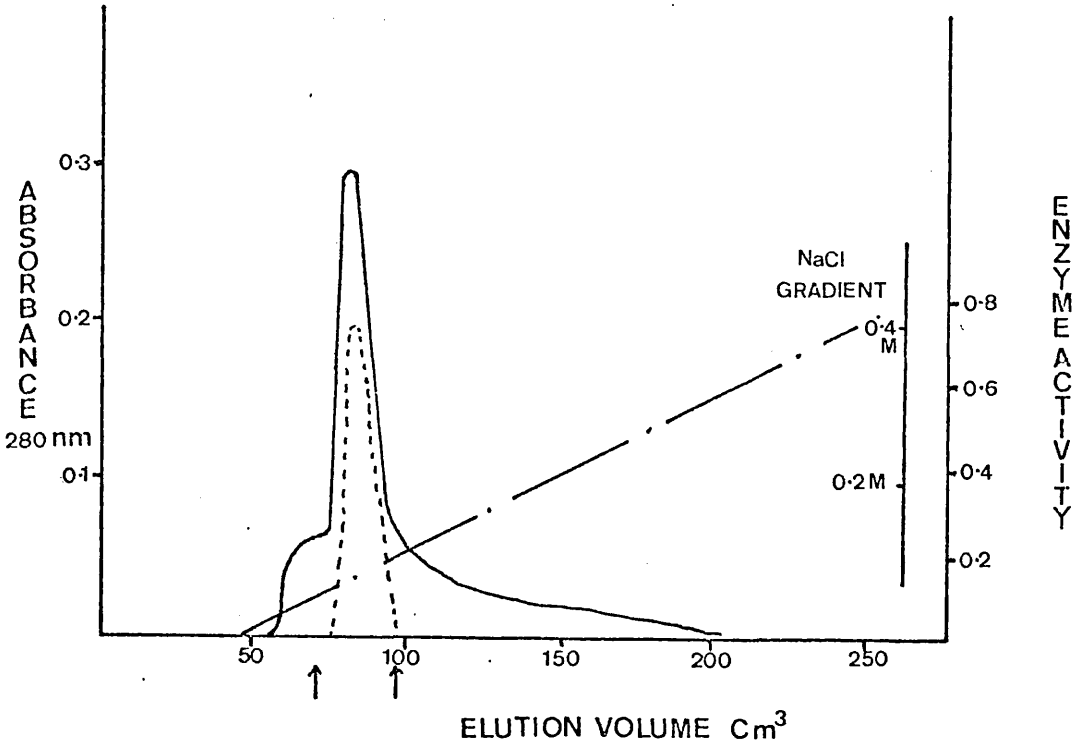
These results amplified the suggestion that the enzyme was deactivated through pH denaturation rather than cation loss upon ion-exchange chromatography at pH 4. Although denaturation was found to take place at pH 4 on CM-cellulose it was not known whether it would be possible to bind the enzyme to DEAE-cellulose

under more favourable conditions. Under the conditions chosen preliminary experiments on a test tube scale indicated that the enzyme could be bound to DEAE-cellulose at pH 7.5. In order to confirm that the enzyme would be eluted in an active form increasing concentrations of sodium chloride were used to elute the enzyme. Levanase activity determinations on each sodium chloride fraction indicated that the enzyme could be eluted in an active form between 0.1 to 0.3M sodium chloride.

A column of DEAE-cellulose was prepared as described in Methods III.G, and chromatography was carried out as described in Experiment 38. A 6 cm^3 sample containing 49 levanase units was applied to the column and elution performed initially with tris/HCl buffer and then with a linear sodium chloride gradient. The elution profile is shown in Fig. 61. The enzyme was eluted by sodium chloride at an approximate concentration of 0.1M. Activity and protein determinations on the concentrated enzyme showed that only 15% of the activity was recovered and the degree of purification of the enzyme was very low as shown in Table 36. Disc gel electrophoresis, carried out as described in Experiment 39 on the enzyme yielded three major and five minor bands, on staining. The low regain of activity was thought to have arisen from the loss of protein during concentration of the enzyme by ultrafiltration. On using a new ultrafilter and re-ultrafiltrating the effluent little activity was found in this concentrate. The possibility of protein still being bound to the exchanger was overlooked at this stage because of the high concentration of sodium chloride used to elute the column. It was decided to repeat the method using a different

Fig. 61

Elution profile for S. salivarius levanase on DEAE-cellulose buffered with tris/HCl at pH 7.5



A) Fractions between arrows pooled

B) Eluant: 100mM tris/HCl pH 7.5

C) After elution volume of 50 cm^3 a continuous NaCl gradient started.——.

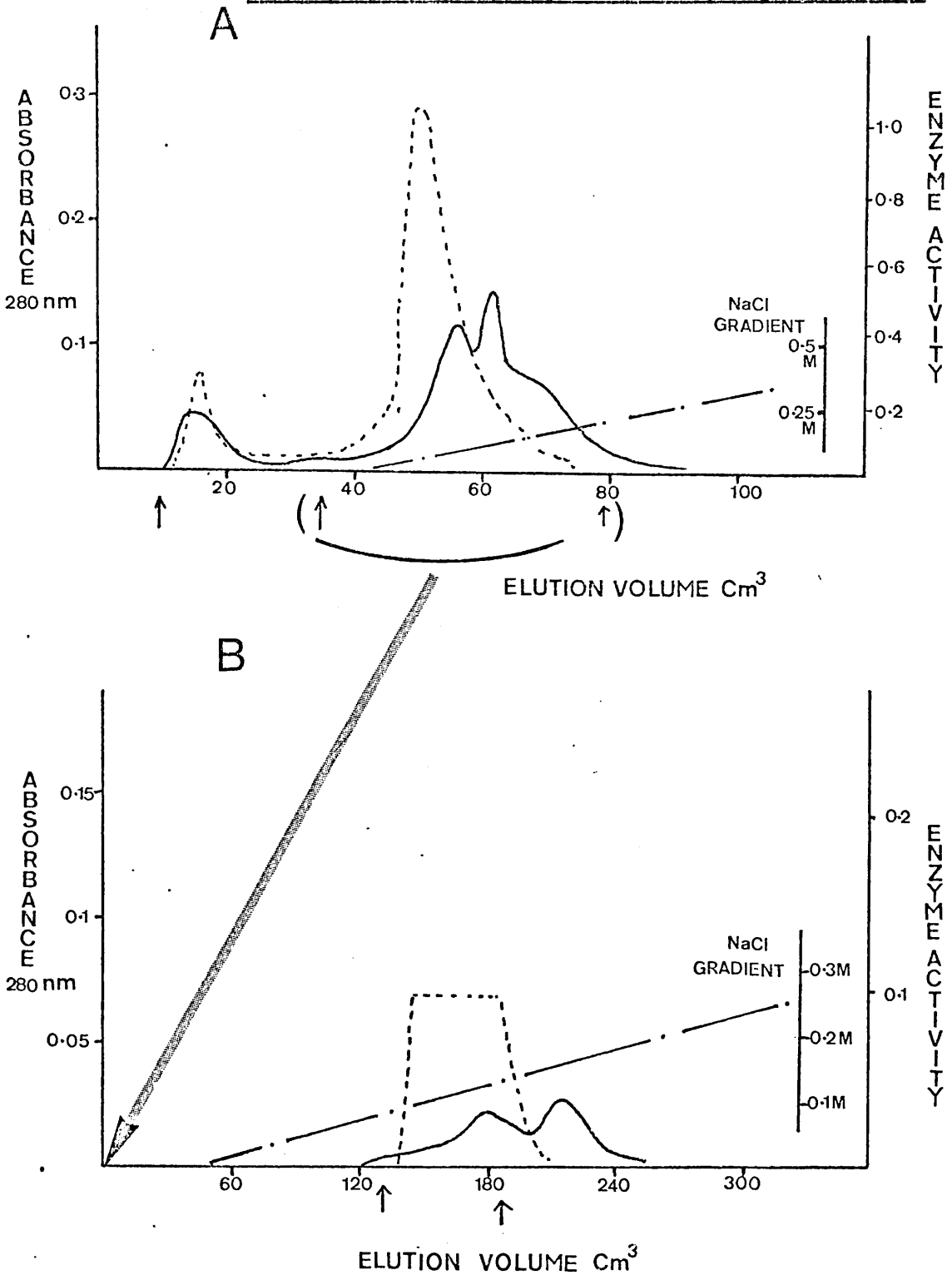
D) Enzyme activity is expressed as the number of µmoles fructose liberated per minute per cm^3 eluate solution

E) —— Absorbance (280 nm)

F) ------ Levanase activity.

Fig. 62

Elution profile for S. salivarius levanase on DEAE-cellulose buffered at pH 7.5 with phosphate buffer



A) Eluant: 100 mM phosphate buffer pH 7.5

B) Fractions between arrows pooled

C) —— Absorbance (280 nm)

D) ------ Levanase activity

E) Enzyme activity expressed as μmoles fructose liberated per minute per cm^3 eluate solution

F) . —— . —— NaCl gradient.

buffer. Instead of tris/HCl which was found to interfere with the protein determinations, phosphate buffer pH 7.5 was used to elute the column as described in Experiment 40. A 5 cm^3 sample containing 41 levanase units was applied to the column. The elution profile is shown in Fig. 62A. Some levanase activity was found in the protein eluted straight through the column. The greater portion of the activity was eluted at a sodium chloride concentration of approximately 0.1M. The presence of a contaminating protein peak very close to the levanase peak resulted in an attempt at re-chromatographing the concentrated fractions containing activity as previously described. However, in order to try and separate these two peaks the sodium chloride gradient was not as steep as previously used. The elution profile is shown in Fig. 62B. Both peaks could be separated using a less steep gradient. The concentrated enzyme had a three fold increase in specific activity. As previously found a low yield was obtained with only 10% of the activity originally applied to the column being recovered. Absorbance readings at 280 nm of the eluted fractions showed that no protein was eluted between 0.2M and 0.5M. It was decided that rather than carry out an investigation into why such a low yield was obtained that the conditions used during ion-exchange would be changed. A phosphate buffer pH 7.0 was decided upon because the pH was close to the pH at which maximum levanase activity was shown by the enzyme. The results of the purification of the enzyme, using this buffer, upon DEAE-cellulose is shown in Table 36. A fifteen fold increase in specific activity was obtained with a 50% recovery of the amount of enzyme applied to the column. (Unfortunately no elution profile

Table 36 Purification Table for S. salivarius levanase

	Volume (cm^3)	Total° Activity units	Total protein (mg)	Specific+ activity	Yield %
Ammonium sulphate precipitation	28	192	98	1.959	-
Sephadex G-200	15.9	132	55.6	2.37	69
DEAE-Cellulose Tris/HCl pH 7.5					
Applied	6	49	20.98	2.37	-
Recovered		7.6	2.8	2.71	15.5Δ
DEAE-Cellulose phosphate pH 7.5					
Applied	5	41	17	2.37	-
Recovered		4.15	0.5	8.2	10Δ
DEAE-Cellulose phosphate pH 7.0					
Applied	3	24	10.49	2.37	-
Recovered		12.8	0.374	34.2	52Δ

° One unit of enzyme activity is the liberation of 1 µmole fructose per minute.

+ Specific activity is the number of enzyme units per mg protein.

Δ The percentage yields for the recovery of the enzyme from the ion-exchange columns is a percentage of the amount applied.

Fig. 63

Disc gel electrophoresis of S. salivarius levanase purified by DEAE cellulose and Sephadex G-200

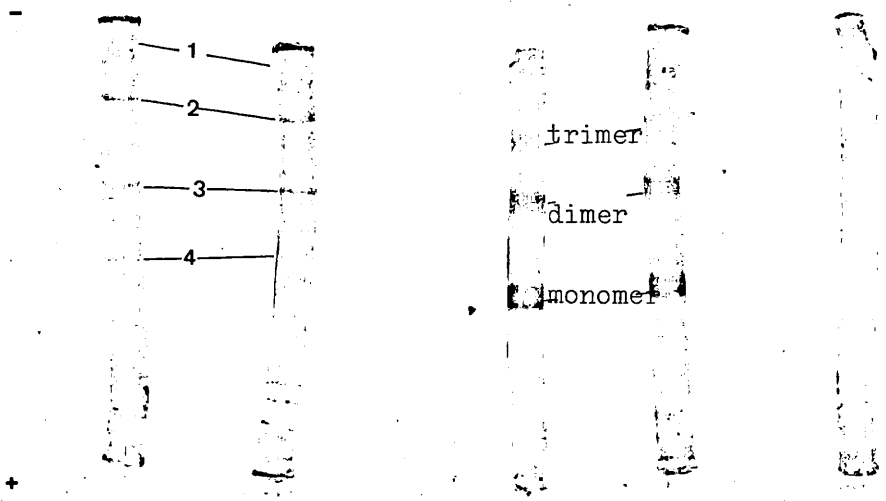
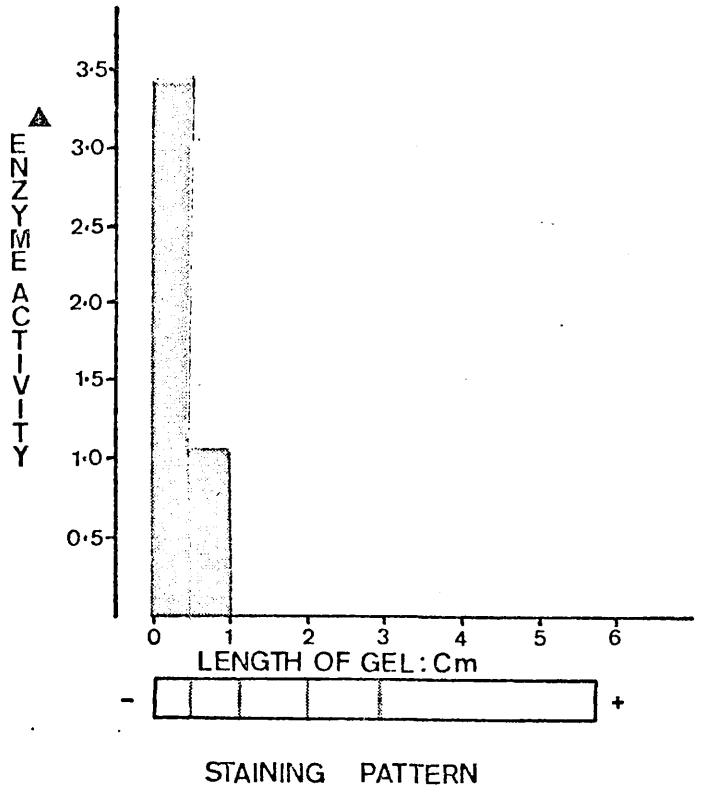
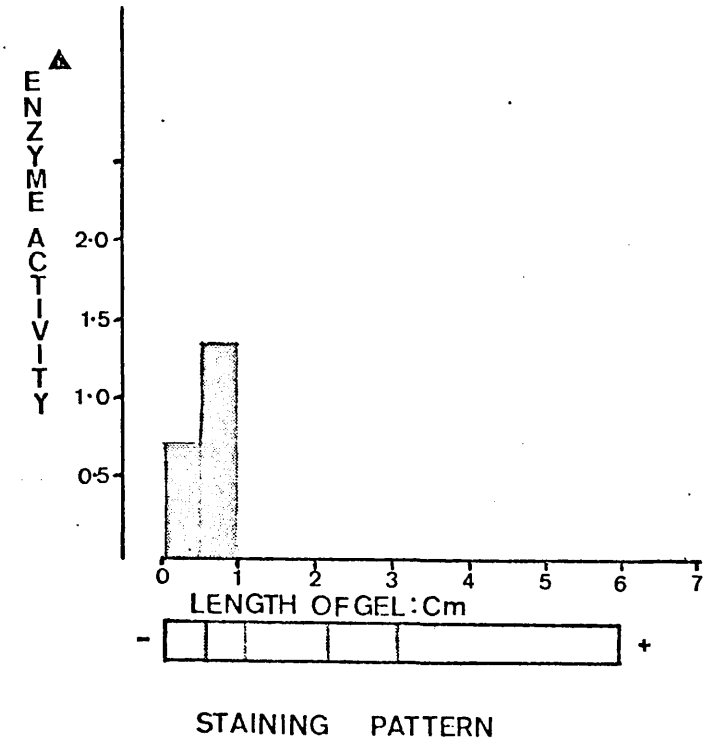


Fig. 64

Disc gel electrophoresis of the levanase preparation purified by DEAE-cellulose; levanase activity of the separated proteins





▲ Expressed as μmoles fructose liberated per incubation

can be shown due to the column running dry at the point where elution of enzyme activity was nearly complete). The concentration of sodium chloride at the point where the enzyme was eluted was approximately 0.1M. Although not enough protein was obtained for full characterisation of the enzyme at this stage enough protein was obtained to carry out disc gel electrophoresis of the enzyme, as described in Experiment 42. Four protein bands were visible on staining, one major and three minor bands (Fig. 63). Two gels which were not stained were cut into 0.5 cm pieces and incubated overnight with levan. Enzyme activity was found to be due to one band which corresponded to the band which was less mobile than the other proteins, this is shown in Fig. 64. Although the enzyme was purified to quite a high degree three other contaminating proteins still remained.

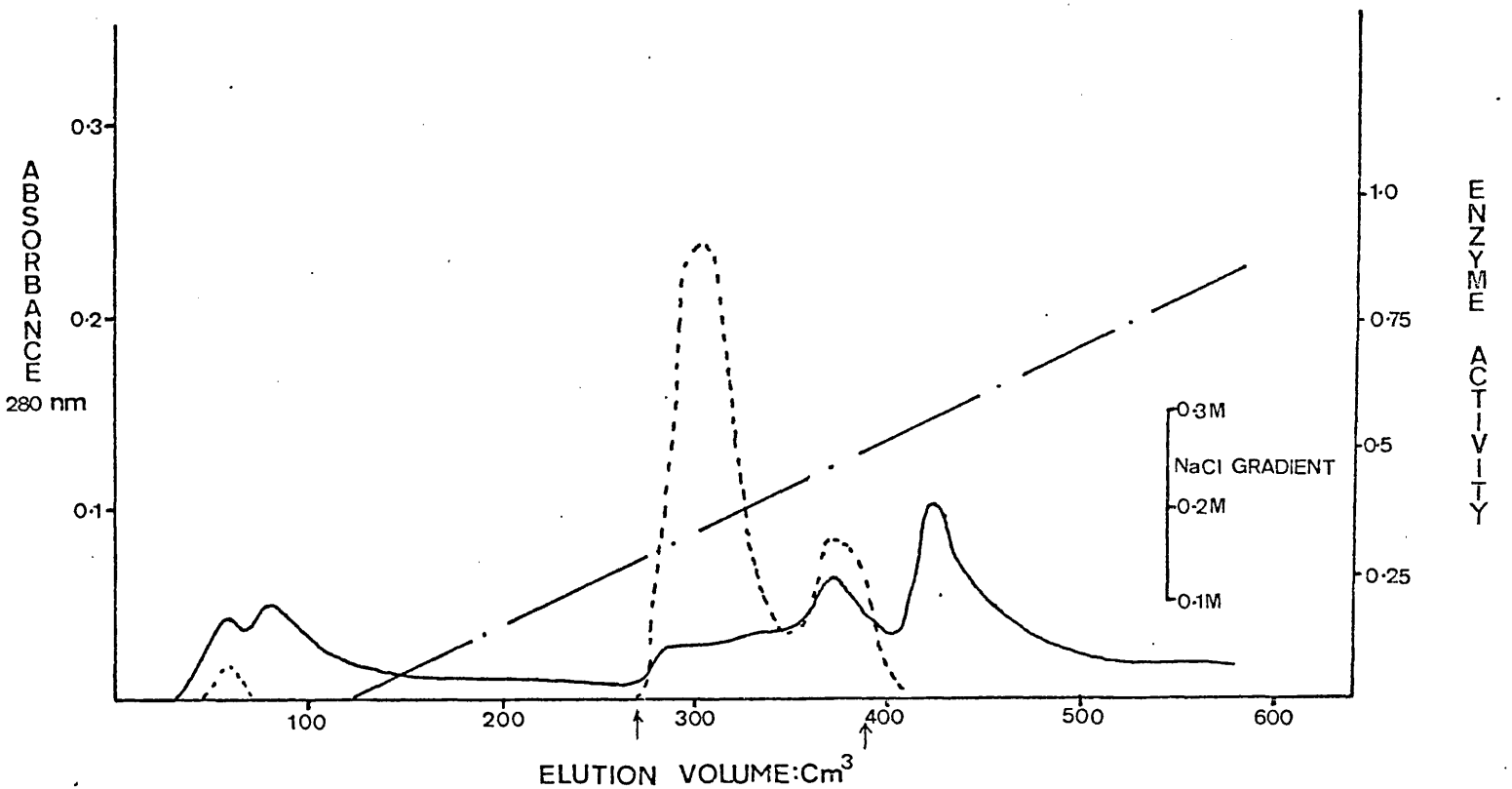
As it was now possible to gain higher yields from an ion-exchange column it was therefore thought reasonable that by increasing the number of enzyme units applied the yield of enzyme would be sufficient to continue the purification procedure.

The column was packed as described in Methods (III.G) and chromatography carried out as described in Experiment 43a.

Two levanase active proteins were separated on DEAE-cellulose by using a buffer of a lower ionic strength. The pH of the phosphate buffer was 6.6. Only preliminary purification of the enzyme preparation was carried out before 140 enzyme units were applied, in two lots, to the ion-exchange column. A typical elution profile is given in Fig. 65. Levanase activity was detected in some fractions soon after the major levanase peak was eluted. The separation of this peak from the main levanase peak and also the increased concentration of sodium chloride required to elute the enzyme(s), was assumed to have occurred because of the lower ionic

Fig. 65

Before gel permeation chromatography

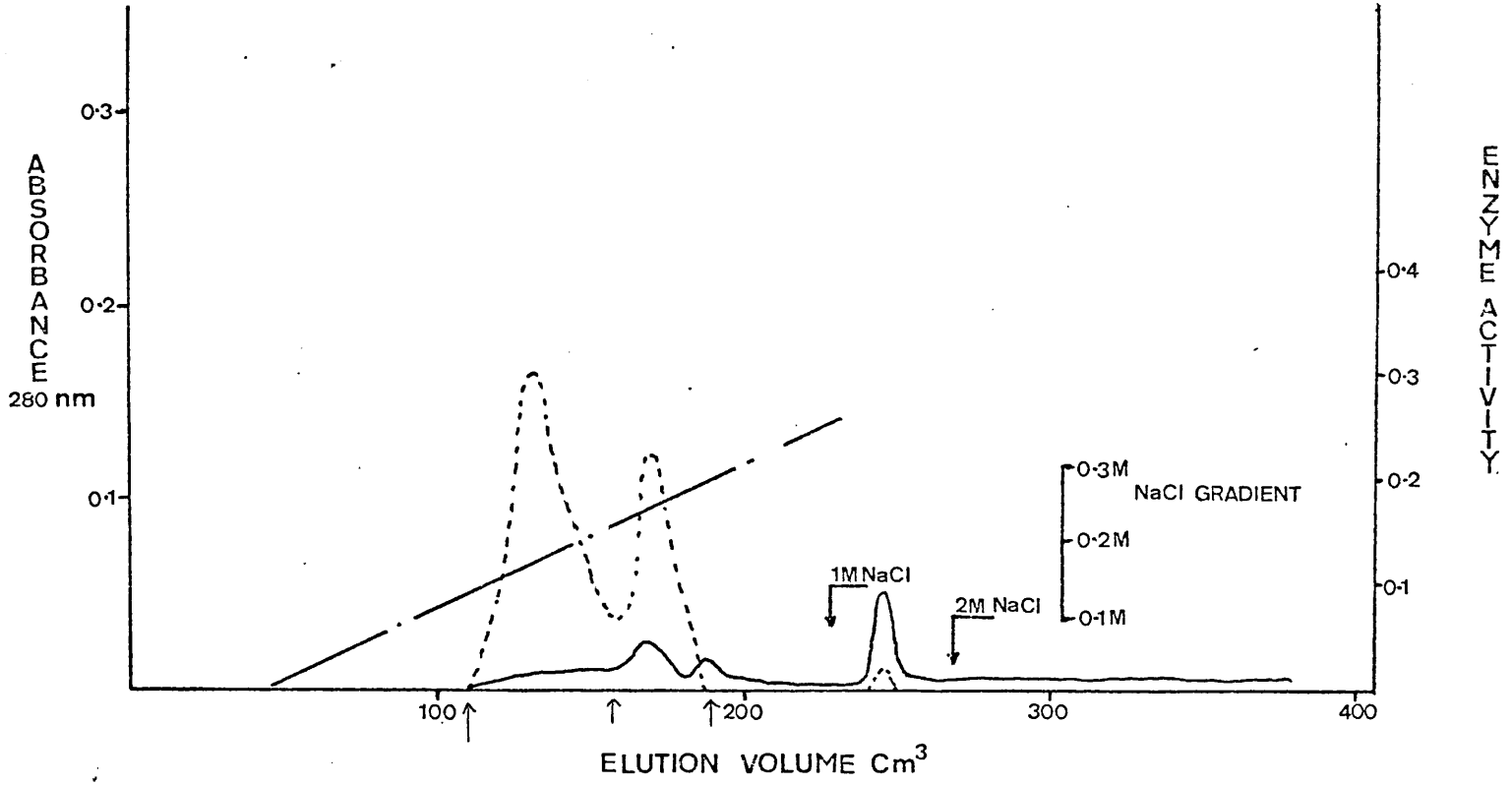


Purification of S. salivarius levanase on DEAE-cellulose buffered with 50 mM phosphate pH 6.6

A) Enzyme activity is expressed as μmoles fructose liberated per minute per cm^3 eluate solution.
B) Eluant: 50 mM phosphate buffer pH 6.6.
C) ——— Absorbance (280 nm)
D) - - - Levanase activity
E) ·——· NaCl gradient
F) Fractions between arrows pooled.

Fig. 66

After gel permeation chromatography



Elution profile of S. salivarius levanase on DEAE-cellulose buffered with phosphate pH 6.6

A) Enzyme activity is expressed as μmoles fructose liberated per minute per cm^3 eluate solution.

B) Eluant: 50 mM phosphate buffer pH 6.6.

C) ——— Absorbance (280 nm).

D) - - - - - - Levanase activity.

E) ·——·— NaCl gradient.

F) Fractions between arrows pooled.

strength of the buffer, resulting in increased binding of the proteins to the ion-exchanger. Both levanase peaks were pooled together giving a yield of 39% of the total activity applied to the columns (Table 37). The increase in specific activity was low, only a 2.5 times increase being gained. To check if the low yields of enzyme was due to enzyme still being bound to the ion-exchanger increased concentrations of sodium chloride were used to elute the ion-exchanger. Levanase activity equivalent to approximately one unit was found in the concentrated effluent from eluting the column with 0.5M and 1.0M sodium chloride. No activity was detected in the effluent using 2.0M sodium chloride. Further purification of this extract was carried out as described in Section 2.A(f).

A similar elution profile was obtained for an extract of the enzyme previously purified on Ultrogel AcA.22 as shown in Fig. 66 (Experiment 43b). The ionic strength of the starting buffer (phosphate pH 6.6 50 mM) was the same as used previously. Two levanase peaks were detected and pooled separately. Elution with sodium chloride was continued up to a concentration of 5.0M. A small amount of activity was detected in some fractions after elution of the column with 1.0M sodium chloride. The major levanase peak gave a yield of 10.6% and a 3.5 fold increase in purification. The minor levanase peak gave a yield of 2.3%, no degree of purification was achieved. Overall only 17% of the total activity applied to the column was recovered (Table 38).

Evidence to suggest that the enzyme undergoes progressive deactivation upon purification by ion-exchange came from purification studies carried out on DEAE-cellulose, run with 50 mM phosphate buffer,

Table 38 Purification Table for S. salivarius levanase

Step	Volume (cm^3)	Total° Activity units	Total protein (mg)	Specific+ activity	Yield %
Ammonium sulphate precipitation	9	65.7	41.5	1.59	-
Ultrogel AcA.22	8.4	49.4	4.7	10.5	75
DEAE-Cellulose phosphate pH 6.6					
Peak 1	1.5	7.0	0.1995	35	10.6
Peak 2	1.8	1.5	0.45	3.3	2.3

Table 37 Purification Table for S. salivarius levanase

	Volume (cm^3)	Total° Activity units	Total protein (mg)	Specific+ activity	Yield %
Ammonium sulphate precipitation	29	140	90.9	1.54	
DEAE-Cellulose phosphate pH 6.6	12.3	54.6	13.5	4.05	39
Ultrogel AcA.22	13	4.47	1.56	2.8	3

° One unit of activity is the liberation of 1 μmole fructose per minute.

+ Specific activity is the number of units per mg protein

and on Ultrogel AcA.22. As already described in this section, when ion-exchange was carried out before gel filtration the yield of enzyme was reasonable enough to carry on purification by gel filtration. After gel filtration on Ultrogel AcA.22 the yield of enzyme was only 8% of the total applied from the previous step. A decrease in specific activity occurred which suggested denaturation or deactivation of the enzyme. This is shown in Table 37. In order to determine whether separation of a component required for activity was achieved during gel filtration all the other fractions eluted from the column were added to the enzyme. No increase in enzyme activity was noted. Fractions eluted from the ion-exchange column were also added with no increase in activity. It was thus concluded that deactivation of the enzyme was irreversible. This claim was further substantiated when gel filtration on Ultrogel AcA.22 was carried out before ion-exchange. After this stage a yield of 75% was achieved. On further purification on DEAE-cellulose using 50 mM phosphate buffer pH 6.6 a low yield of 14% was obtained. The specific activity of the enzyme preparation was high as shown in Table 38. After storage of this preparation at $4^{\circ}C$ for two weeks the enzyme was found to have lost its activity. From the results described it is clear that substantial deactivation of the enzyme occurs upon purification on DEAE-cellulose. The poor yields obtained using this purification technique suggested a large amount of activity was still bound to the exchanger, however, little activity was detected when high concentrations of sodium chloride were used to elute the column. From a comparison of the absorbance measurements for th eluted fractions from the ion exchange columns it would seem possible

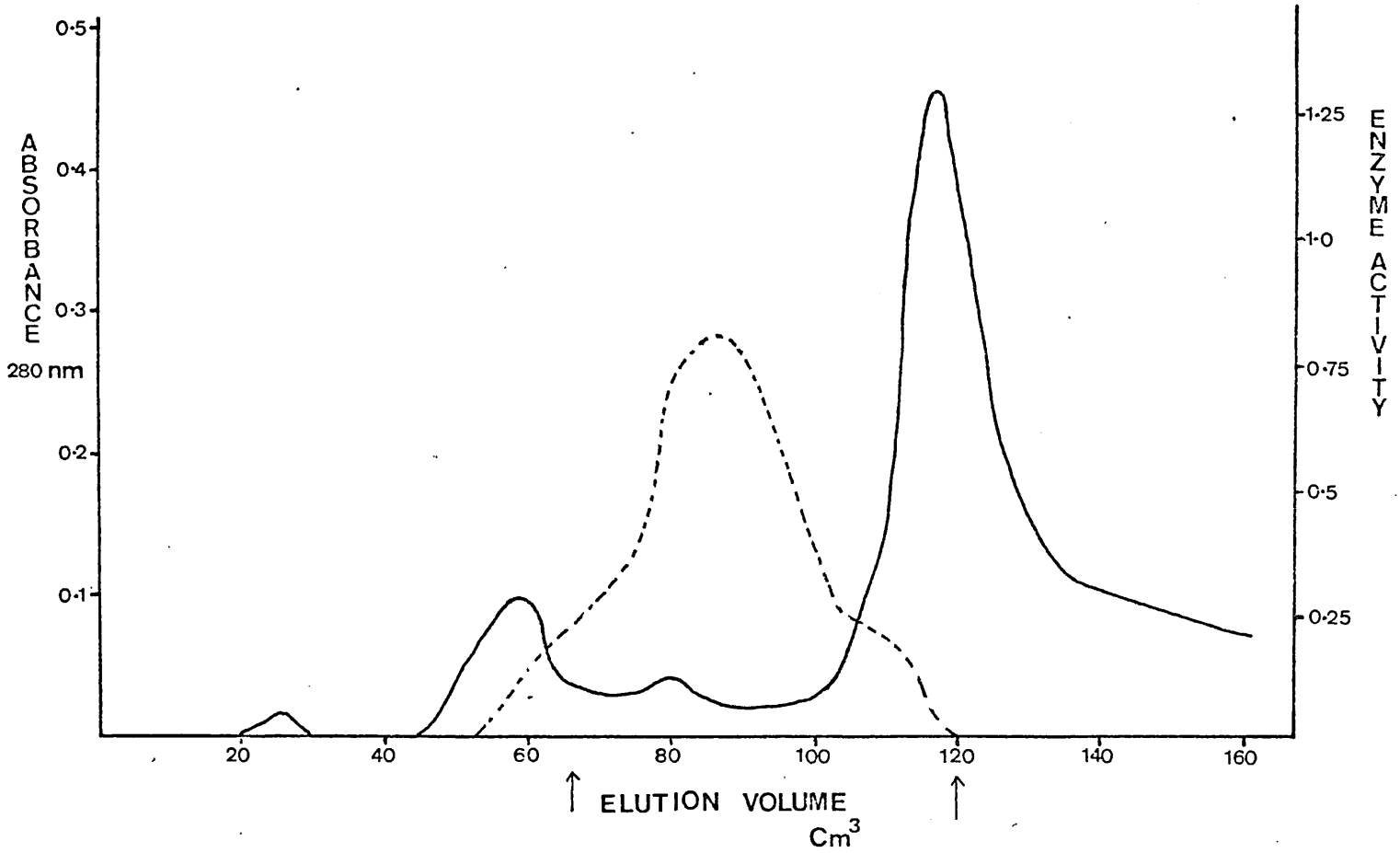
that these measurements do not correlate with the amount of protein applied to the column. This suggests that protein was still bound to the column. As it has been shown that progressive deactivation of the enzyme takes place, it could be possible that the enzyme is in fact already partially deactivated when eluted from the ion-exchange column. What reasons exist therefore for the poor yields and the progressive deactivation of the enzyme?. Perhaps under the conditions in which ion-exchange was carried out strong ionic effects upon the column resulted in a breakdown in the structure of the enzyme. The possibility of the enzyme requiring an activator should not be overlooked. Without the presence of this activator enzymic activity might be severely inhibited. Whatever reason may in the future be forthcoming such varied yields and degrees of purification were obtained that other techniques therefore had to be found to purify the enzyme.

f) Purification of the enzyme by gel permeation chromatography on Ultrogel AcA. 22

Elution profiles for the enzyme upon Sephadex G-200 showed that the majority of contaminating proteins were in a molecular weight range above the effective range of fractionation of the gel filtration media. The degree of purification of the enzyme upon Sephadex was low. Therefore, a gel filtration media was chosen that had a fractionation range which was capable of separating proteins of a molecular weight which normally would have to be separated firstly on Sephadex and then on Sepharose. Ultrogel AcA. 22 was the

Fig. 67

Elution profile of S.salivarius Levanase on Ultrogel AcA.22



A) Fractions between arrows were pooled.

B) Eluant: 100 mM phosphate buffer pH 6.6.

C) Enzyme activity as expressed as μmoles fructose liberated per minute per cm^3 eluate solution.

D) —— Absorbance 280 nm.

E) ----- Levanase activity.

selected packing material having a fractionation range between 100,000 to 1,200,000. The procedure was carried out as described in Experiment 44.a.

Fig. 67 shows the elution profile of the enzyme on Ultrogel. The enzyme had only been preliminary purified by ammonium sulphate precipitation. The total number of enzyme units applied was 65.7. Absorbance readings at 280 nm showed that good separation of proteins was achieved. However, the enzyme was eluted over a wide elution volume which resulted in some contaminating proteins being pooled with the enzyme. The greater portion of the protein was eluted in an elution volume corresponding to the column volume. A good yield of enzyme was obtained with 75% of the amount of the enzyme being applied, being recovered. A 6.6 fold increase in purification of the preparation was obtained. The results are shown in Table 39.

Table 39 Purification values for the purification of S. salivarius levanase on Ultrogel AcA.22

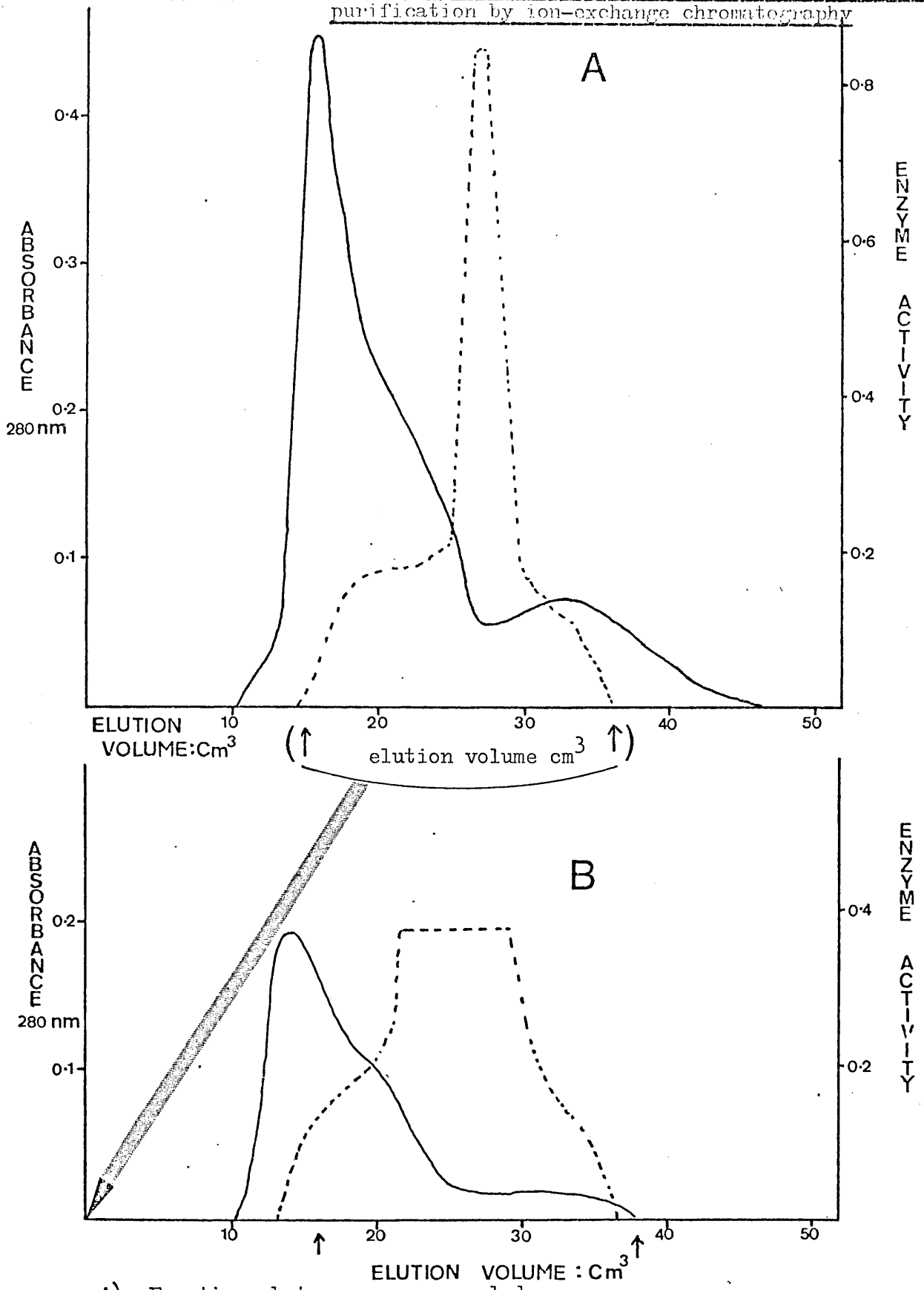
Stage	Volume (cm^3)	Total° Activity units	Total protein (mg)	Specific+ activity	Yield %
$(NH_4)_2SO_4$ saturator	9	41.5	65.7	1.59	-
Ultrogel AcA.22	8.4	4.7	49.4	10.5	75

° One unit of activity is the liberation of 1 µmole fructose per minute.

+ Specific activity expressed as the number of units per mg protein.

Fig. 68

Elution profile for S. salivarius levanase on Ultrogel AcA.22 after purification by ion-exchange chromatography



A) Fractions between arrows pooled.
B) Eluant: 100 mM phosphate buffer pH 6.6.
C) Enzyme activity expressed as μmoles fructose liberated per minute per cm^3 eluate solution.
D) ——— Absorbance (280 nm).
E) - - - - - Levanase activity.

Further purification of this preparation was undertaken on DEAE-cellulose using 50 mM phosphate buffer pH 6.6 as discussed in Section 2.A(e).

The elution profile of a sample of the enzyme previously purified by DEAE-cellulose ion exchange is shown in Fig. 68. After pooling the fractions containing levanase activity, the enzyme was again applied to the same column containing Ultrogel AcA.22. Separation of proteins upon the first column was not as good as obtained for the ammonium sulphate preparation previously described. Hence, the levanase preparation was passed down the column again. The elution profile for the extract on the first column (Fig. 68A) shows that not all of the levanase activity is associated with one enzyme. The molecular weights of the enzymes are similar as indicated by the similar elution profiles. A total of 54 enzyme units were applied to the column of which only 4.5 units were recovered. A decrease in specific activity of the extract indicated deactivation of the enzyme preparation. The reasons for this are discussed in the previous section. It is assumed that previous purification of the extract on DEAE-cellulose resulted in deactivation of the preparation. A comparison of the elution profiles shown in Figs. 67 and 68 where 65 and 54 enzyme units were applied respectively, shows that the levanase activity profiles are dissimilar therefore suggesting that deactivation or denaturation of the levanase extract had occurred before it was placed on the column.

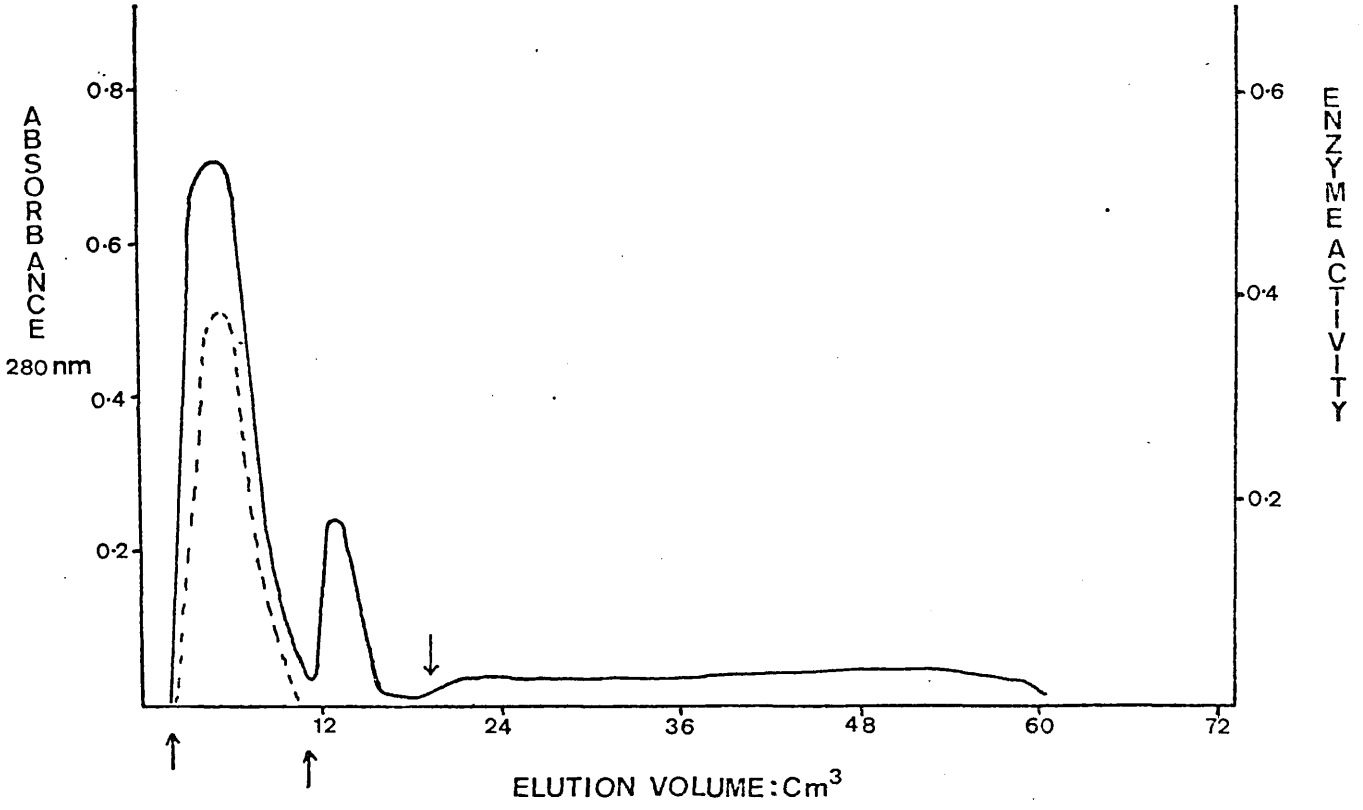
g) Attempt at the purification of the enzyme by affinity chromatography

By coupling an enzyme to a solid support and exploiting its biological specificity it is possible to achieve a degree of purification greater than could be expected using less specific techniques such as ion-exchange or gel permeation chromatography. Enzymes can be bound by a variety of methods utilising such as antigen-antibody, enzyme-inhibitor, and enzyme-substrate interactions. Once the enzyme is bound to a selected affinity media the main obstacle is the ability of the enzyme to undergo elution and again be gained in its uncoupled form.

The first type of packing material to be investigated was concanavalin A bound Sepharose 4B. This material is specific for binding glycoproteins containing α-D-mannosyl, α-D-glucosyl and sterically related residues. Previous to the attempt at coupling the enzyme to Con.A-Sepharose it was not known whether the enzyme was a glycoprotein or not. A small column was packed and equilibrated as described in Methods section (III.J). Fig. 69 shows the elution profile of the enzyme upon con.A-Sepharose. The enzyme was previously purified on Sephadex G-200. A total of 26 levanase units were applied to the column. Initial elution was performed with 100 mM phosphate buffer (pH 6.6) containing 500 mM sodium chloride. After 20 cm^3 of buffer was passed 0.5 M methyl α-D-glucopyranoside in the same buffer was used to elute the column. As can be seen from the elution profile levanase activity was only detected in one protein peak which was eluted straight through the column. On pooling the fractions containing levanase activity a total of 14 levanase units were recovered with a decreased specific activity indicating that

Fig. 69

Purification of S. salivarius levanase on Con A-Sepharose



A) Eluant: 100 mM phosphate buffer pH 6.6.

B) ——— Absorbance (280 nm).

C) ------ Levanase activity.

D) Fractions between arrows pooled.

E) After fraction No.30 eluant: 0.5M methyl α-D-glucopyranoside in A).

F) Enzyme activity expressed as μmoles fructose liberated per minute per cm^3 eluate solution.

some enzyme was bound to the column. The purification figures are given in Table 40.

Table 40 Purification values for the unbound levanase of Con.A-Sepharose

Stage	Volume (cm^3)	Total[o] activity units	Total protein (mg)	Specific[+] activity
Sephadex G-200	6	26.3	5.1	5.1
Con.A-Sepharose	8.4	14	3.94	3.8

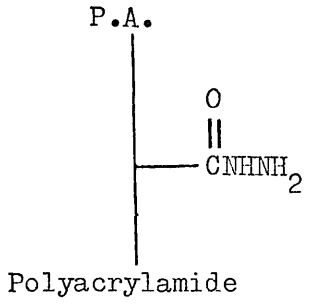
[o] One unit of activity is the liberation of 1 μmole fructose per minute

[+] Number of enzyme units per mg protein

Although elution was continued with methyl α-D-glucopyranoside for some time no levanase activity was detected in any of the fractions collected. It was therefore assumed that the enzyme was so strongly attached to the Con.A-Sepharose that it could not be eluted with methyl α-D-glucopyranoside. Alternative methods of elution were proposed, one included the use of oligosaccharides containing β-(2→6) fructofuranose units as substrates to elute the enzyme, but the amount available would have only made up a solution of low concentration, perhaps not enough to elute the enzyme.

As only 46% of the amount of enzyme activity was coupled and problems were found in eluting the enzyme an alternative affinity technique was chosen which would hopefully couple all of the activity applied to it. In this case utilisation was made of an enzyme-substrate interaction for coupling the enzyme.

A hydrazide derivative of Biogel P-300 was prepared as described in "Methods" (III.I.I.). The structure is shown as follows:



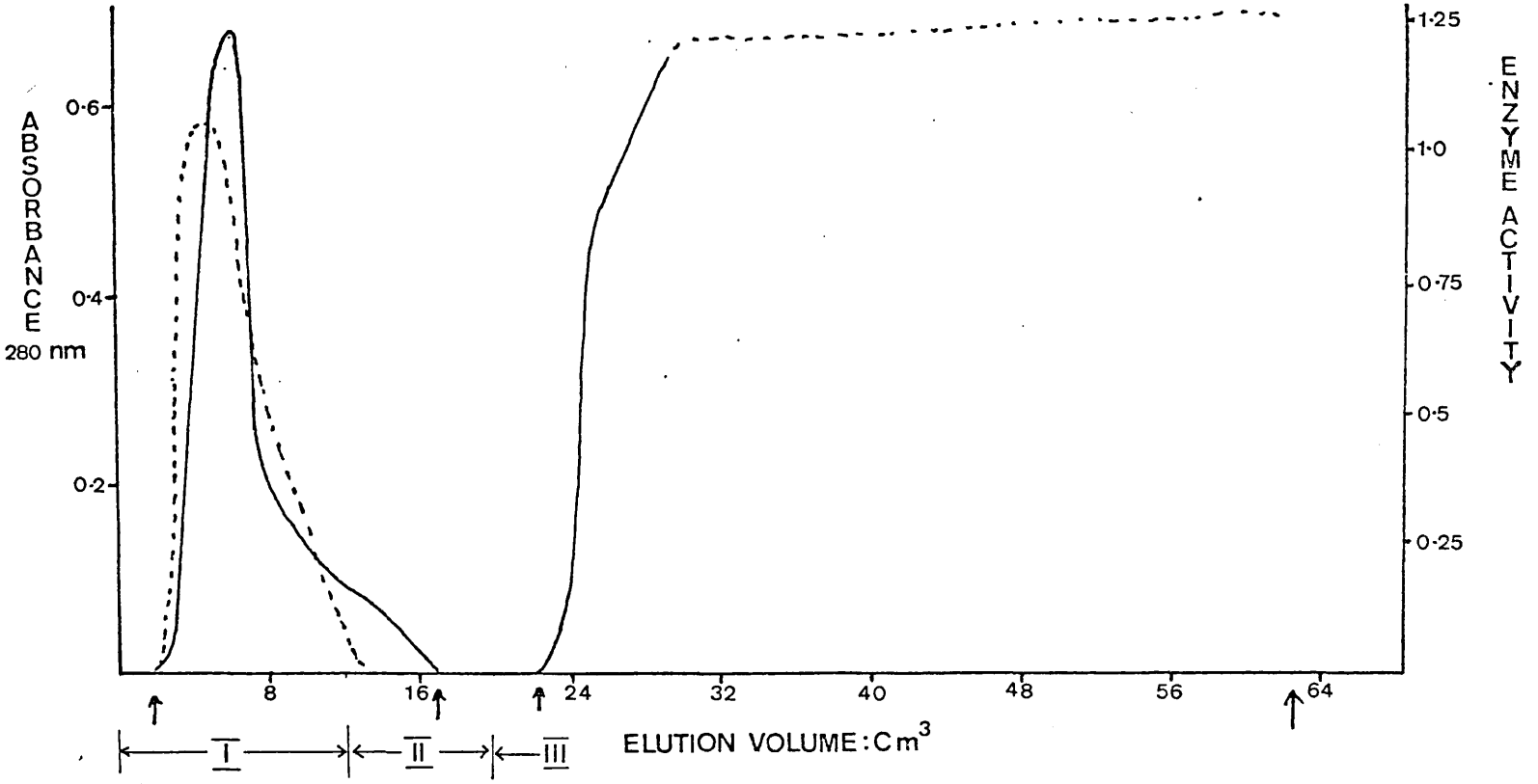
Hamazaki and Hotta[222] coupled lactose to the hydrazide Biogel P-300 derivative and adsorbed β-galactosidase from wheat-germ to the bound ligand. The free enzyme was eluted using the substrate 4-O-β-D-galactopyranosyl-D-glucitol. It is reported that the linkage between lactose and polyacrylamide-hydrazide is that of a hydrazone.

It was decided to try and couple β-(2→6) fructosyl containing oligosaccharides to the hydrazide derivative so that the enzyme could be adsorbed. The procedure is described in "Methods" (III.I.II.). Partial acid hydrolysis of the polysaccharide, levan, gave the required β-linked oligosaccharides. Paper chromatography of the hydrolysate indicated that oligosaccharides had been prepared. In order to save time no separation of these sugars was undertaken. The concentrated hydrolysate was therefore coupled to the hydrazide Biogel P-300 gel directly. To confirm that coupling had taken place a portion of the material was subjected to a phenol sulphuric test which indicated that some sugar was coupled.

Several small columns were run packed with the affinity gel and an attempt at coupling the enzyme was carried out. Fig. 70 shows an elution profile for 7.3 levanase units applied to the affinity column. As can be seen a large amount of enzyme was found to pass through the affinity column without becoming attached. The amount

Fig. 70

Elution profile for S. salivarius levanase on hydrazide Biogel P-300



A) Eluant I: 10 mM phosphate buffer pH 6.6.
II: " " " " containing 100 mM NaCl.
III: " " " " containing levan hydrolysate.

B) Fractions between arrows were pooled.

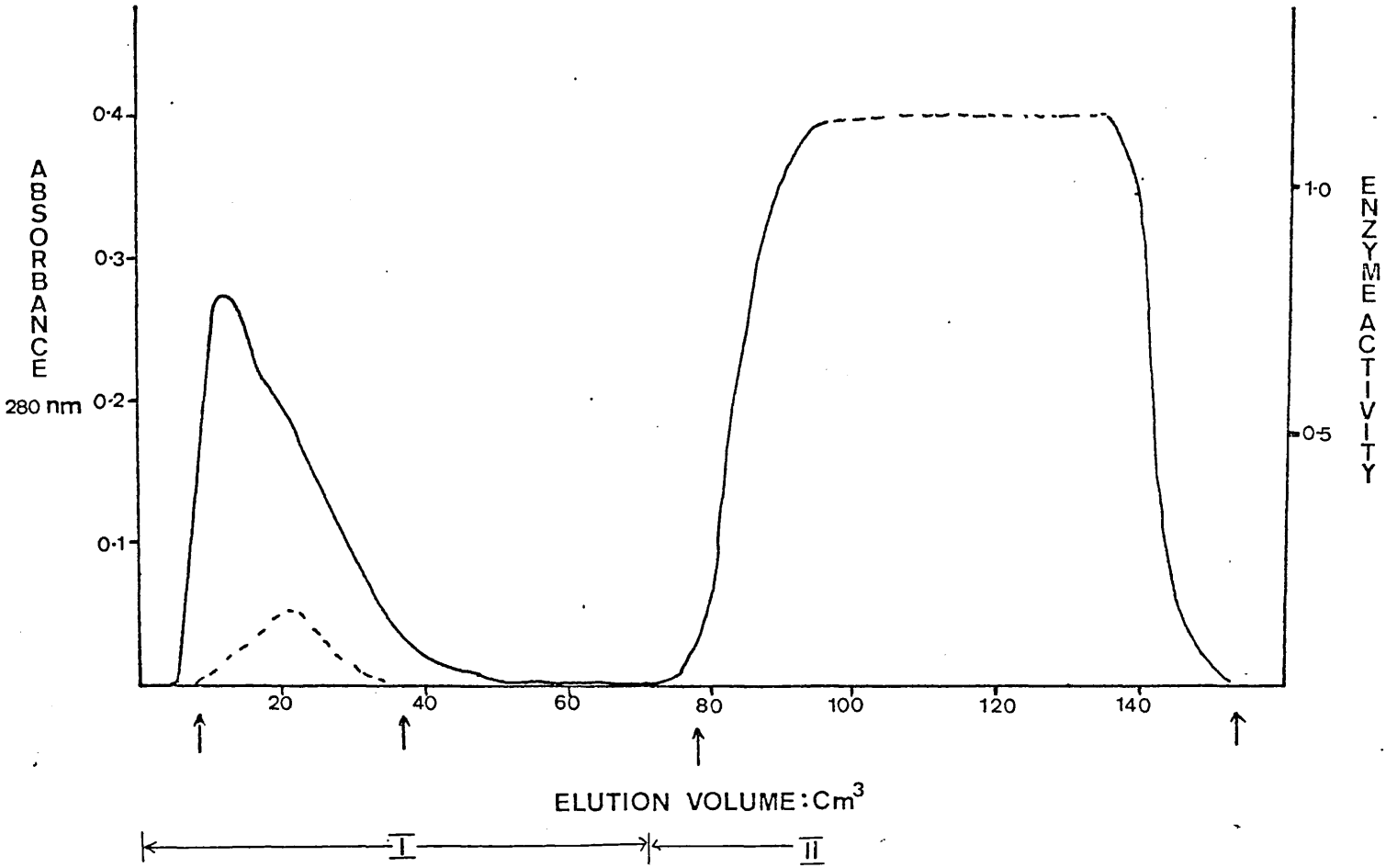
C) —— Absorbance (280 nm).

D) ------ Levanase activity.

E) Enzyme activity is expressed as μmoles fructose liberated per minute per cm^3 eluate solution.

Fig. 71

Elution profile for S. salivarius levanase on Biogel P-300 affinity column



A) Eluant I: 10 mM phosphate buffer pH 6.6.
II: 10 mM phosphate buffer pH 6.6 containing levan hydrolysate.

B) - - - - - - - Levanase Acitivity

C) ——————— Absorbance (280 nm).

D) Fractions between arrows pooled.

E) Enzyme activity is expressed as μmoles fructose liberated per minute per cm^3 eluate solution.

of enzyme not being adsorbed was 5.1 units. On elution with the levan hydrolysate, which absorbed at 280 nm, 0.8 levanase units were regained. The small percentage of enzyme adsorbed was thought to have arisen because of the presence of fructose in the hydrolysate. Fructose was present in such a concentration as to have become coupled to the Biogel to a greater extent than any of the substrates. Marshall[217] suggested that rotary evaporation of solutions of levan oligosaccharides resulted in the degradation of these sugars to give fructose, thus indicating that they were thermally labile. The concentration of the acid hydrolysate by rotary evaporation was discontinued.

A further attempt was made at coupling a levan hydrolysate in which all the small molecular weight sugars were removed by diafiltration. A preliminary attempt was carried out with just 4 levanase units being applied to the column. Less than 0.5 of a unit was not adsorbed. On elution with the levan hydrolysate no activity was detected in the pooled fractions. As a large percentage of the enzyme was adsorbed onto the affinity column it was decided to apply more enzyme. A total of 14.6 units were applied. An elution profile is shown in Fig. 71. It was found that 0.96 units were not adsorbed to the column. Only 1.49 units were recovered after eluting the column with the levan hydrolysate. No protein determination was done on the recovered enzyme because of the small amount expected.

Although the percentage recovery of the enzyme from the hydrazide column was very low, it is suggested that this limitation could be overcome. The lack of the pure substrates for coupling and eluting the hydrazide column goes some way to explain the low recovery.

The author is confident that if enough levanbiose or inulinbiose was available a far larger amount of enzyme would be recovered. It is considered that rather than a loss of enzyme occurring during elution that the enzyme is still adsorbed to the column. The possibility of coupling a substrate, which is available commercially is fairly large quantities, and in a pure form, should not be overlooked. It would certainly overcome some of the problems described, in producing substrates in large enough quantities. As it is probable that the enzyme is able to attack β-fructosyl links; sucrose would be an ideal substrate for coupling to an affinity column. Unfortunately, sucrose cannot form a hydrazone so another method of coupling would be required. Epoxy-activated Sepharose 6B couples sugars by way of an ether linkage through the hydroxyl groups; it may well be possible therefore to couple sucrose this way. Elution could be performed with fairly concentrated solutions of sucrose. Although no purification details are available for the methods used, absorbance measurements at 280 nm do suggest that a very high degree of purification can be achieved using affinity techniques.

h) Removal of small and large molecular weight proteins by gel permeation chromatography on Biogel A-5m and by use of a molecular filtration membrane

The purification techniques which have been used previously do not justify their use because of the poor yields that were recovered. The degree of purification obtained either by use of ion-exchange or affinity chromatography is high but at the cost of

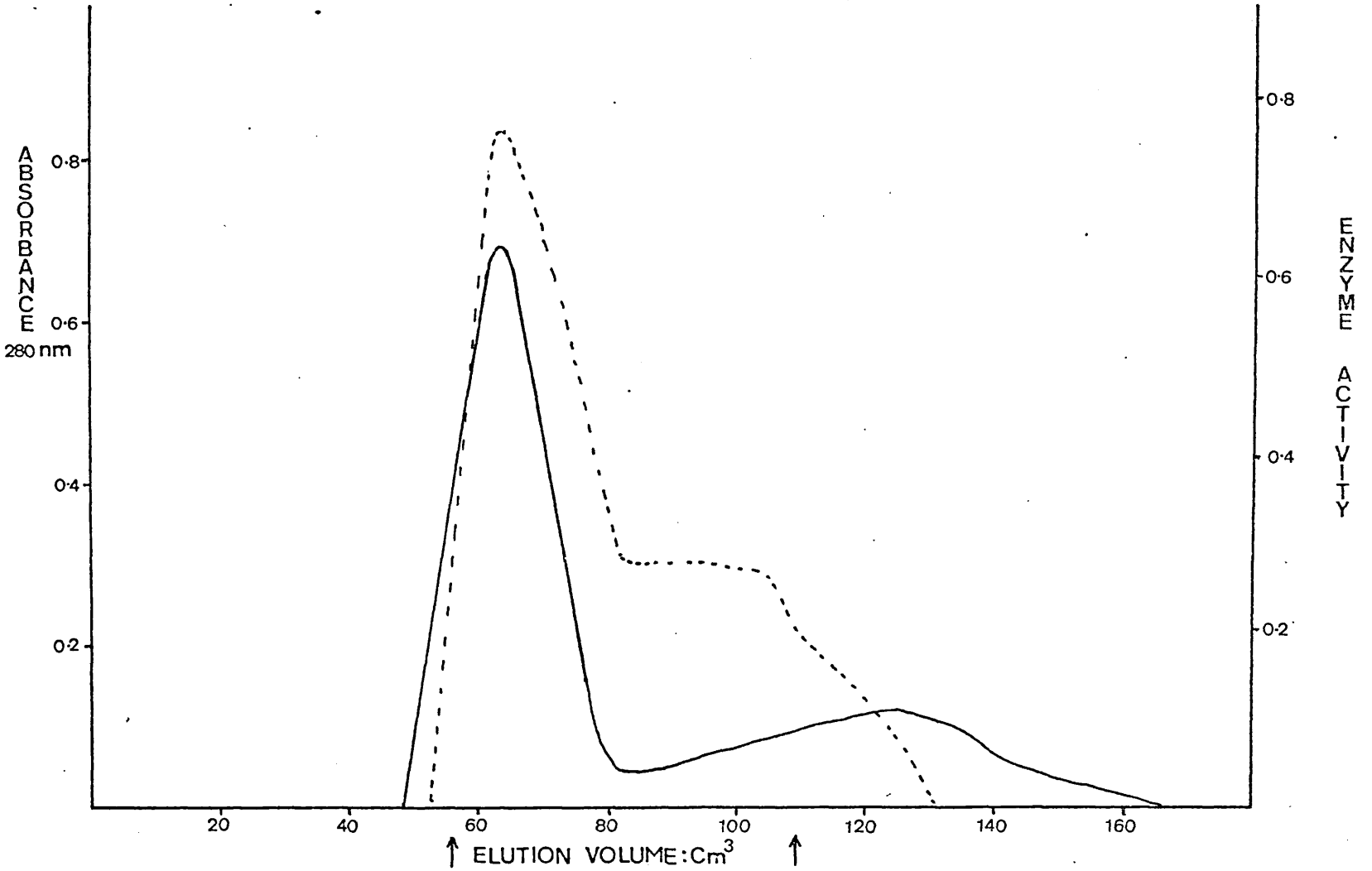
poor yields. It was therefore decided that the levanase preparation would be purified as much as possible by molecular exclusion techniques. A study of the properties of the levanase preparation would then be undertaken.

After saturation with ammonium sulphate this batch was to have been purified on Biogel P-60 (exclusion limit 60,000). As previous experiments have shown that the enzyme(s) have a molecular weight above 100,000 it was contemplated that if the extract was passed through a Xm-100 membrane (exclusion limit 100,000) some time could be saved. Thus, prior to separation on Biogel A-5m low molecular weight proteins were removed in this fashion.

Fig. 72 shows the elution profile of the levanase preparation on Biogel A-5m. A total of 125 levanase units were applied to the column, as described in Experiment 47. A similar elution profile was obtained to that of the levanase preparation on Ultrogel AcA.22 (Fig. 67). The small molecular weight proteins however were present in a small proportion compared with the amount eluted in the column volume of the Ultrogel column. On concentrating the fractions containing levanase activity 82 units were recovered giving a yield of 71%. Evidence for the existence of more than one enzyme having levanase activity is given by the elution profile which shows partial separation of two active peaks. This confirms the results already found for the preparation; whereby other techniques have shown there to be more than one levanase active enzyme present.

Fig. 72

Elution profile for S. salivarius levanase on Biogel A-5m



A) Eluant: 100 mM phosphate buffer pH 6.6.

B) Fractions between arrows pooled.

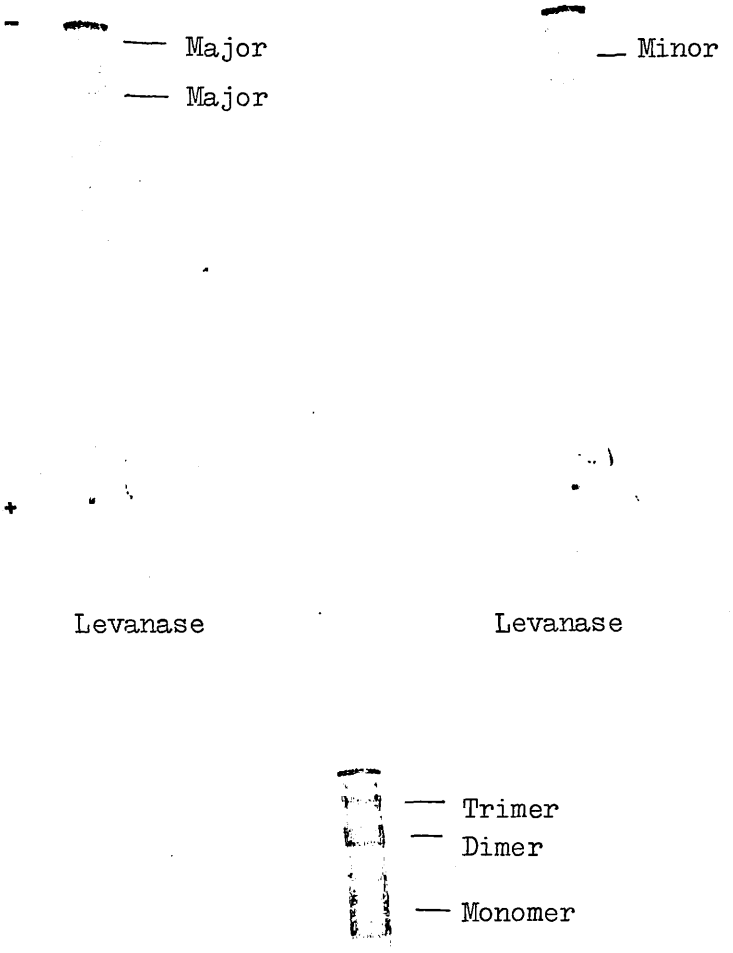
C) ——— Absorbance (280 nm).

D) ----- Levanase activity.

E) Enzyme activity is expressed as μmoles fructose liberated per minute per cm^3 eluate solution.

Fig. 73

Disc gel electrophoresis of S. salivarius levanase purified by chromatography on Biogel A-5M



The purification table is given below:

Table 41 Purification values for the levanase extract on Biogel A-5m

Stage	Volume (cm^3)	Total° activity units	Total protein (mg)	Specific* activity	Yield %
$(NH_4)_2SO_4$ saturator	20.5	125.2	98.4	1.27	-
Diaflo Xm-100 Biogel A-5m	7.7	88.9	11.08	8.03	71

° One unit of activity is the liberation of 1 μmole fructose per minute.
* Specific activity is the number of units per mg protein.

Disc gel electrophoresis of the partially purified enzyme was carried out as described in Experiment 48. Three bands were visible on staining. Two major bands and one minor band as shown in Fig. 73. The partially purified levanase preparation obtained was used for the investigation into the properties of the enzyme as described in Chapter II.2.B. A technique not previously used in the attempted purification of the enzyme was the last to be utilised and is described in the following Section.

i) Use of adsorption chromatography on hydroxylapatite for the purification of S. salivarius levanase

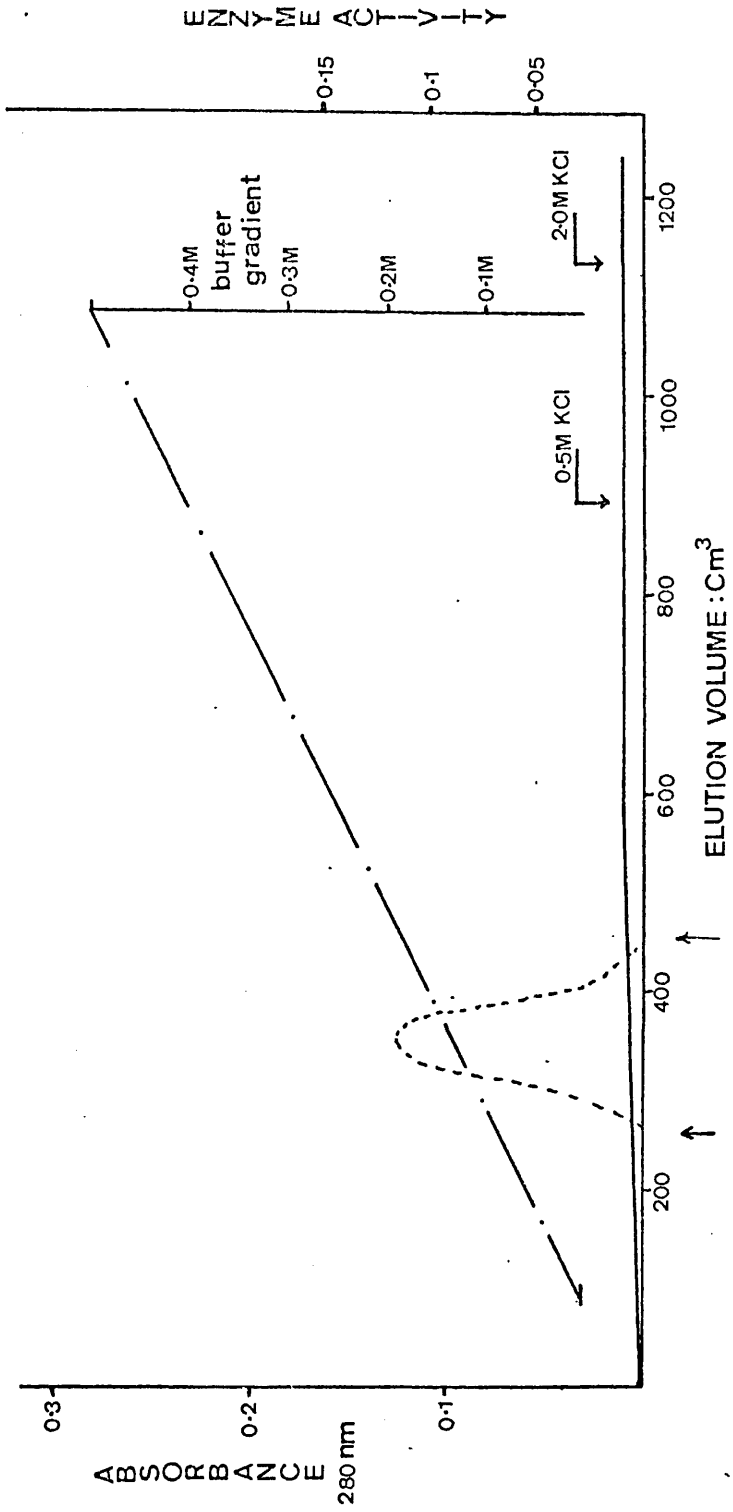
Several authors have utilised a calcium phosphate gel to purify levansucrases[189] and some invertases.[200,220] Separation of proteins is based on adsorption, elution being performed by an increasing buffer concentration. Hydroxylapatite, a form of calcium phosphate, is the usual packing material.

A column was prepared as described in "Methods" (III.H). To facilitate the flow of the column the hydroxylapatite was mixed with cellulose powder. After primary equilibration with a very dilute buffer to minimise any adsorption of buffer, 37 levanase units equilibrated with starting buffer, were applied to the column. Elution was performed by use of a linear buffer gradient, as described in Experiment 49. The elution profile is shown in Fig. 74. Absorbance measurements at 280 nm of the eluted fractions showed that only a very small amount of protein was eluted from the column.

Only one levanase active peak was eluted from the column. Only 2 levanase units were recovered after concentrating these fractions. These results, indicating a very poor recovery of enzyme, seem similar to the results obtained for the enzyme purified by ion-exchange. The possibility of regaining activity by addition of the other eluted fractions from the column was suggested. However, on addition of all the other fractions collected no increase in activity was observed. The specific activity of the pooled fractions was 3.1, 50% lower than the specific activity of the applied extract. A total of 5 mg of protein was applied to the column of which only 0.42 mg was recovered. It would seem therefore that the greater portion of the protein was still adsorbed to the hydroxylapatite. The final concentration of the eluting buffer of 0.5 M is normally sufficient to elute all bound proteins. Because of the insolubility of disodium hydrogen phosphate above 0.5 M this was the final concentration of the eluting buffer. Potassium chloride in phosphate buffer was therefore used to elute the column. To conclude it would seem that protein is still adsorbed to the hydroxylapatite even after elution with 0.5 M buffer and potassium chloride. A deactivating

Fig. 74

Elution profile for S. salivarius levanase on Hydroxylapatite



A) ·——·—— Buffer gradient.
B) ---------- Levanase activity.
C) ——————— Absorbance (280 nm).
D) Eluant: phosphate buffer, pH 6.6.
E) Fractions between arrows pooled.
F) Enzyme activity is expressed as μmoles fructose liberated per minute per cm^3 eluate solution.

effect which occurred on ion-exchange cannot be dismissed for the results obtained on hydroxylapatite as the only levanase eluted from the column was found to have a very low activity.

Summary

The results from this chapter indicate very clearly that separation techniques based on molecular size are useful in the purification of the enzyme. However, techniques involving separation based on charge should be avoided. In order for the enzyme to be purified to homogeneity an understanding of the behaviour of the enzyme on ion-exchange would seem the first objective to be carried out, although quite a thorough investigation was carried out by the author. Two schemes can be proposed for purifying the enzyme to homogeneity. Both, involve firstly preliminary purification with ammonium sulphate followed by gel permeation chromatography either on Ultrogel AcA.22 or Biogel A-5m. Scheme I would involve an affinity technique as described in Section 2.A(g). Advantage should be taken of substrates which are available commercially in a pure form, i.e. sucrose. Indeed, better use of this substrate could be adapted to the application of Con.A-Sepharose for purification of the extract. Scheme II involves isoelectric focussing. Although it has already been stated that techniques involving charge should be avoided, but, not only may relatively pure enzymes be prepared using this method, but some information may be forthcoming as to an understanding of the properties of the preparation on ion-exchange.

2. Results and Discussion

II.2.B Properties of S. salivarius strain '51' Levanase

The following section describes the work which was carried out to investigate the enzymic properties of the levanase preparation. The preparation was partially purified as described in section 2.A(h). From the results described in this section an attempt will be made to systematically name the enzyme.

a) Determination of the pH at maximum levanase activity

The levanase activity of the preparation was determined at different pH as described in Experiment 50. Fig. 75 shows the pH profile for the variation of levanase activity with pH. The preparation was found to be active over a wide pH range with maximum levanase activity at pH 6.6. This value is in agreement with the value determined by Marshall.[217] The property of the enzyme of having maximum levanase activity at approximately neutral pH shows that it would be well adapted to the environment found in the mouth.

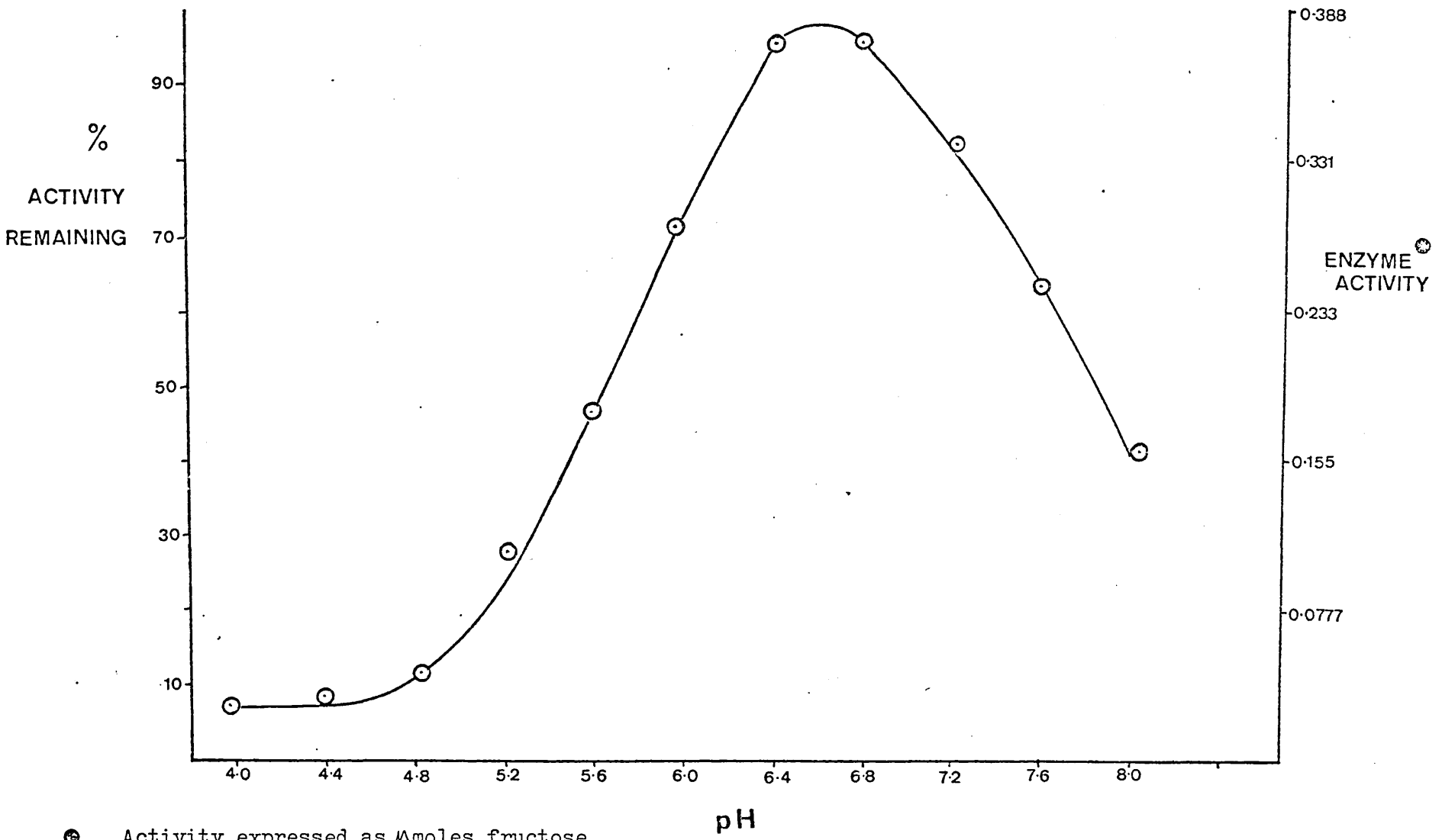
b) Effect of variation of the reaction temperature on the enzyme activity

The procedure is described in Experiment 51. The enzyme reaction was found to proceed at different temperature values for levanase and inulinase activity. Fig. 76 shows the effect of temperature on both reactions.

Levanase activity was found to be most active up to 30°C and fell 50% at approximately 37°C. Inulinase activity was found to be stable up to 35°C and fell 50% at approximately 43°C. These results suggest, as both activities are not equally affected, that two

Fig. 75

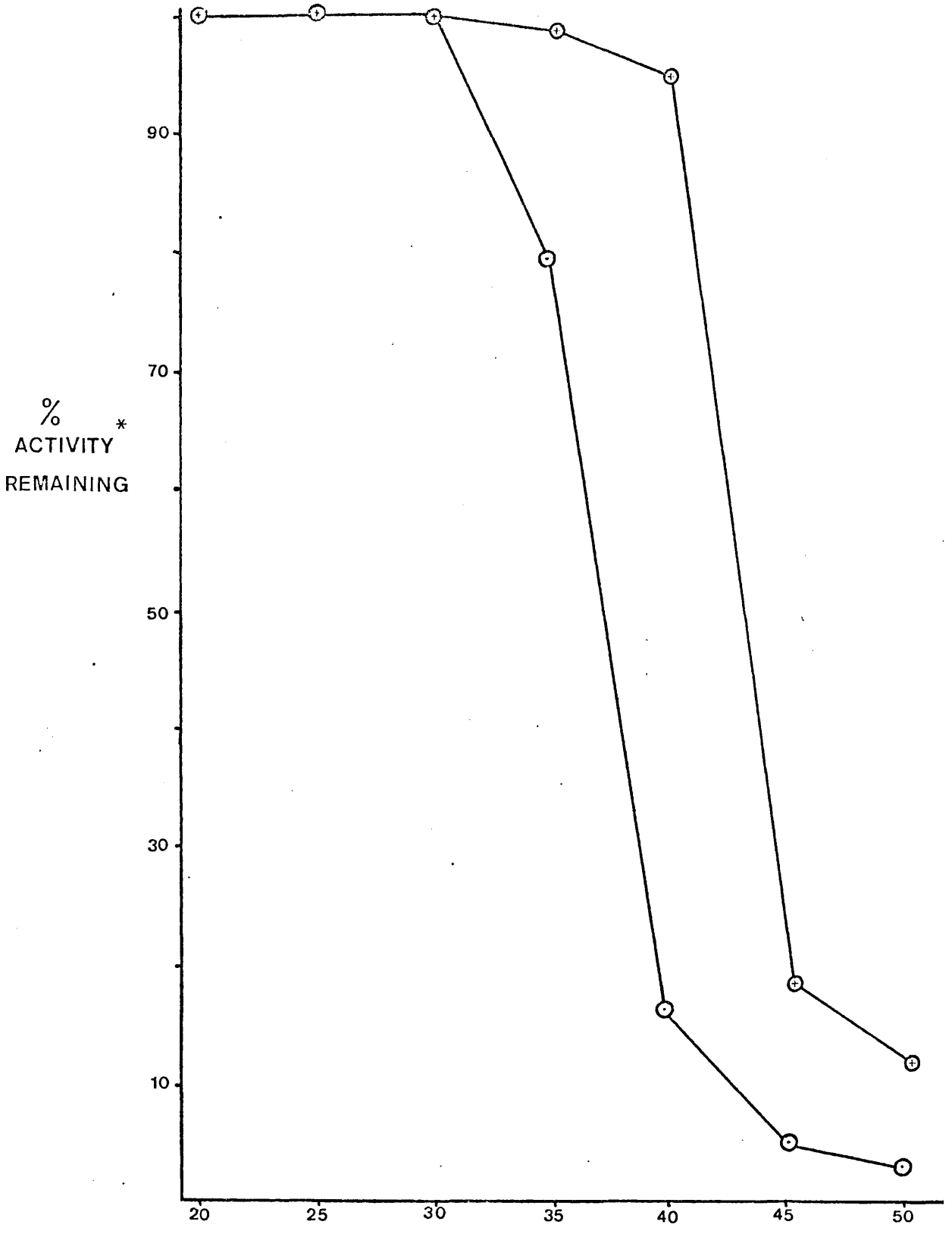
pH optimum for maximum levanase activity



❁ Activity expressed as μmoles fructose liberated under the conditions described in Experiment 50.

Fig. 76

Effect of temperature on enzyme activity



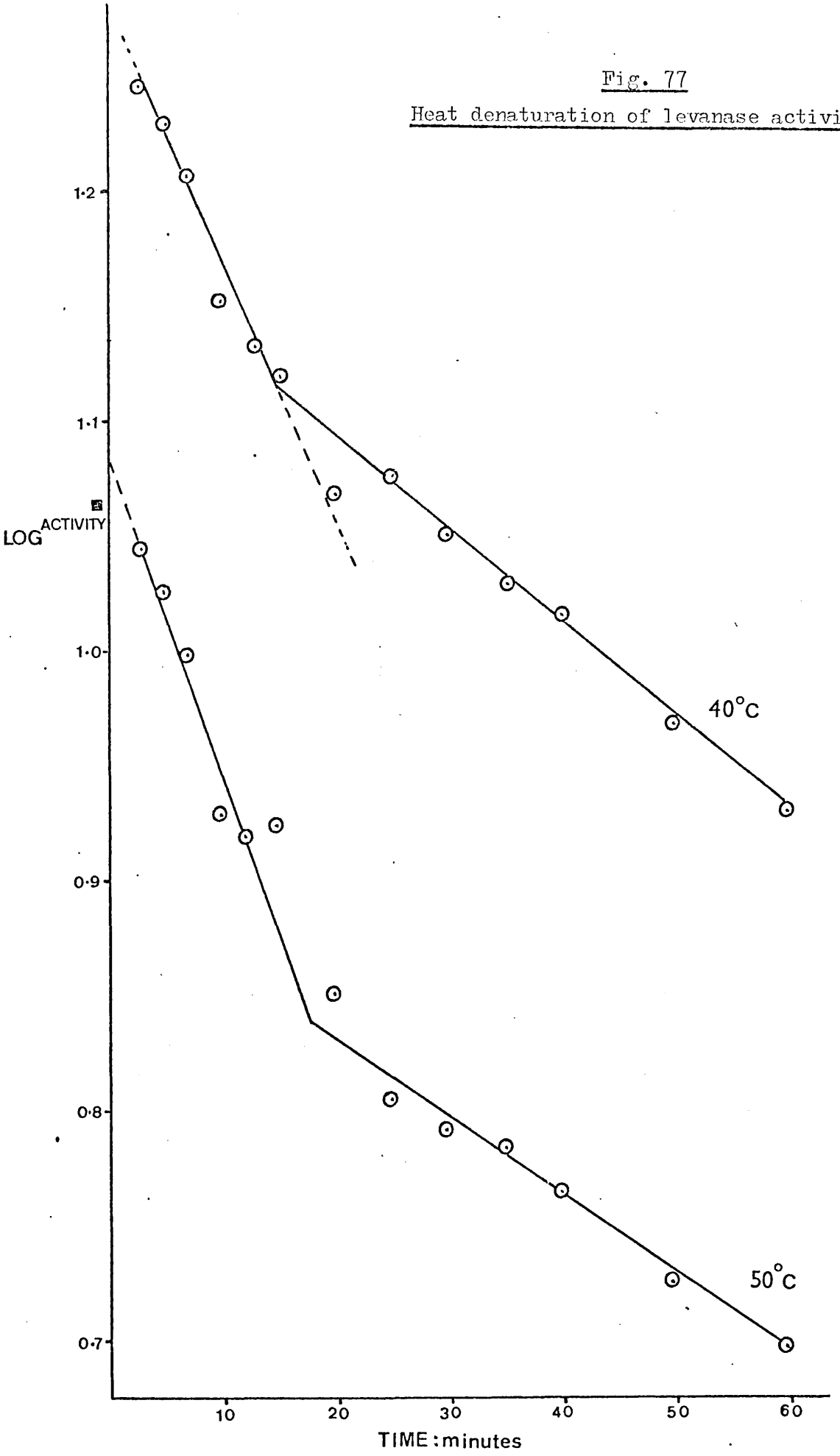
⊙ levan digest

⊙ inulin digest

* Activity expressed as μmoles fructose liberated under the conditions described in Experiment 51.

Fig. 77

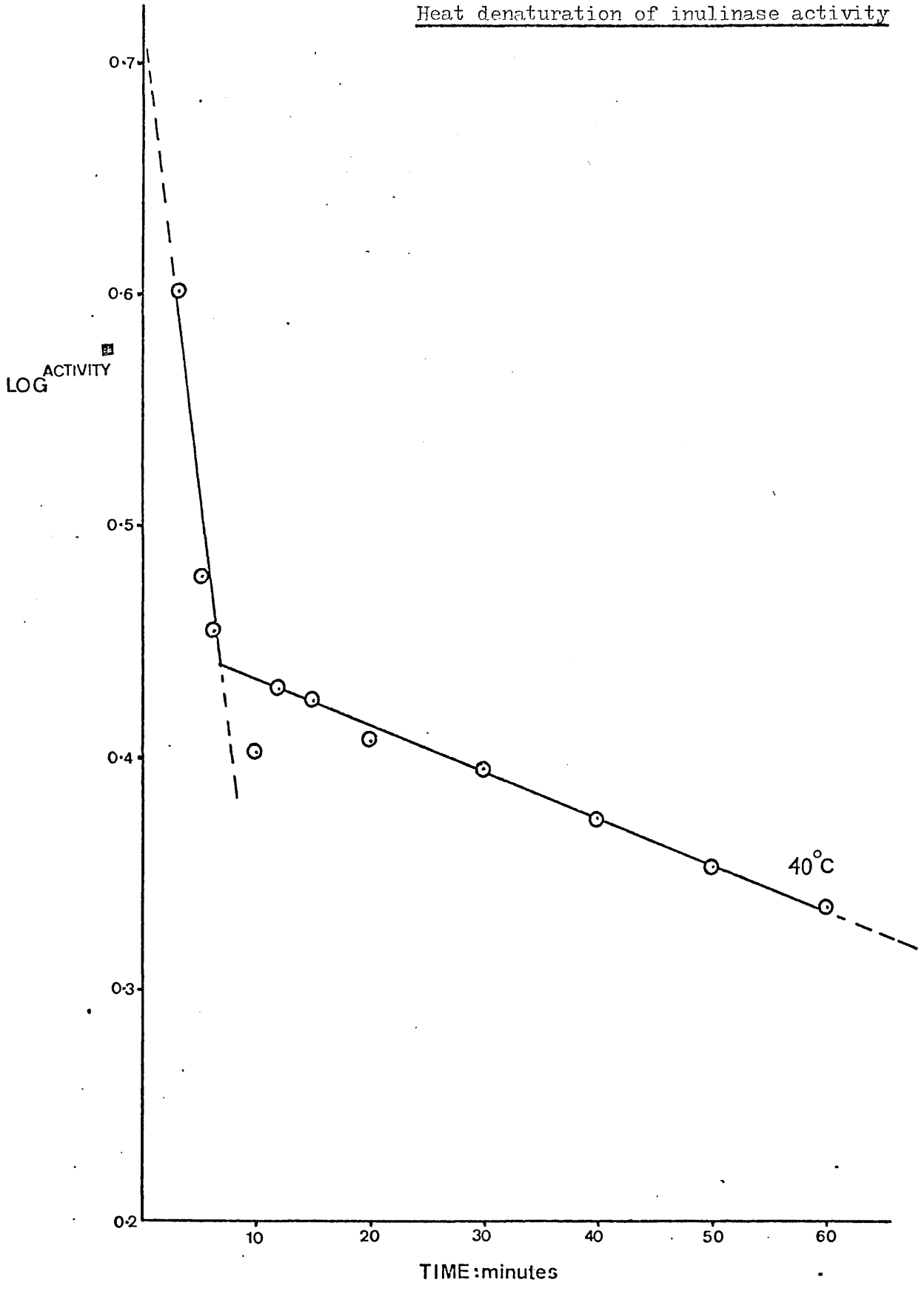
Heat denaturation of levanase activity



■ Activity expressed as μmoles fructose liberated per minute.

Fig. 78

Heat denaturation of inulinase activity



■ Activity expressed as μmoles fructose liberated per minute

distinct enzymes may be present in the extract showing different thermal stabilities. To clarify this situation heat deactivation studies were carried out.

c) Heat inactivation of the levanase and inulinase activity

The effect of heat inactivation on the levanase and inulinase activity of the preparation was carried out as described in Experiment 52. The results of the heat inactivation of the levanase activity is shown in Fig. 77. About 36% of the levanase activity persisted after heat inactivation for 60 min. at 40°C and 26% after 60 min. at 50°C. Because the inactivation of the levanase activity occurs at two different rates, indicated by the two straight lines it suggests the presence of two enzymes in the preparation, both capable of hydrolysing levan. The heat inactivation of the inulinase activity is shown in Fig. 78. Because of two different rates of deactivation it is suggested that two separate inulinase activities exist. At 40°C, after 60 minutes approximately 40% of the inulinase activity remained. Inulinase activity was completely inactivated after incubation at 50°C for several minutes. It is likely that two enzymes are present both of which are able to degrade inulin and levan. As it has been shown that one enzyme was heat labile the temperature of incubation was decreased from 37°C to 30°C for future incubations.

d) Levanase properties of the partially purified preparation

Some of the levanase properties of the preparation were determined as described in Experiment 53. The results are shown in Figs. 79, 80, 81. The activity of the preparation was proportional to

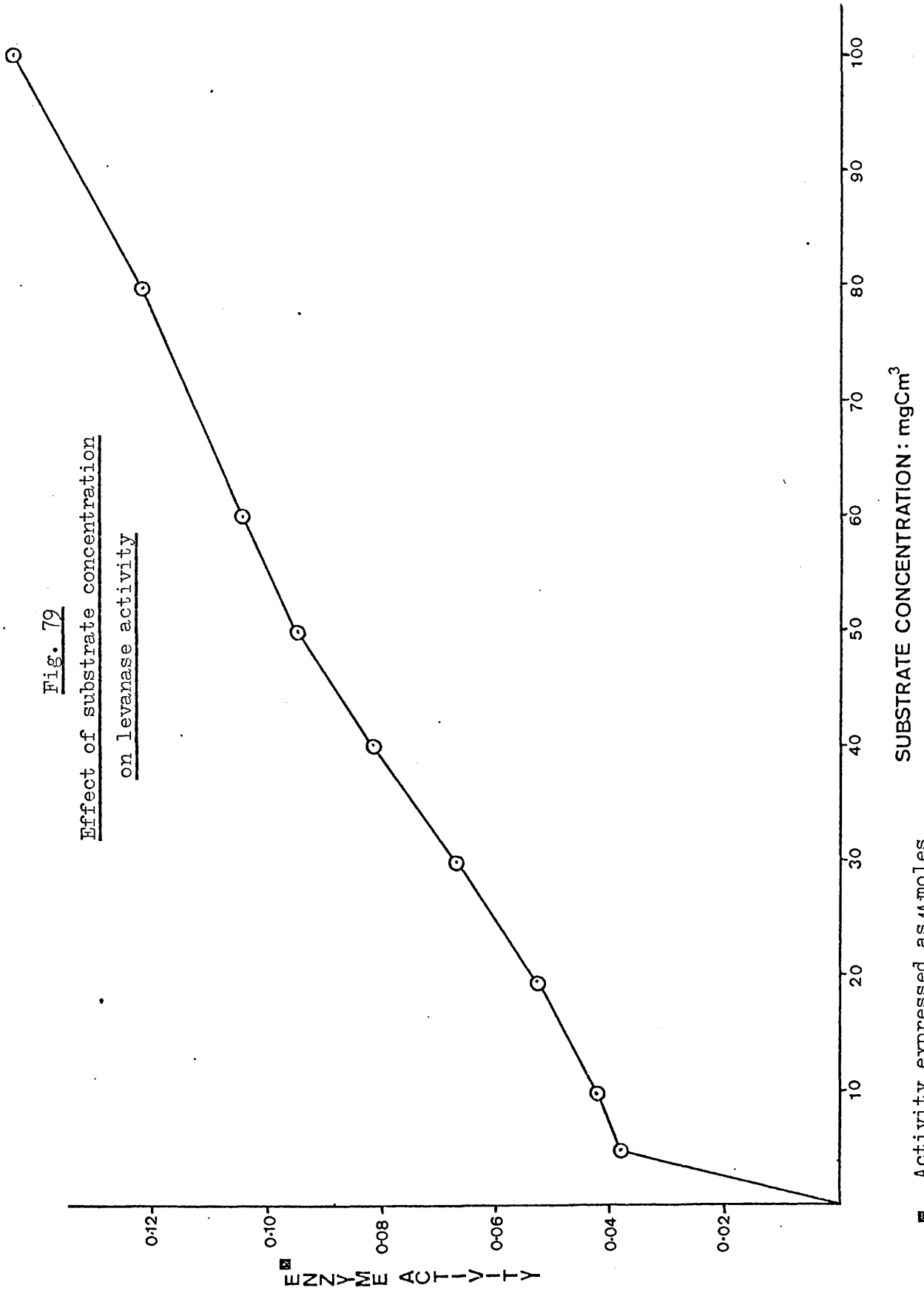


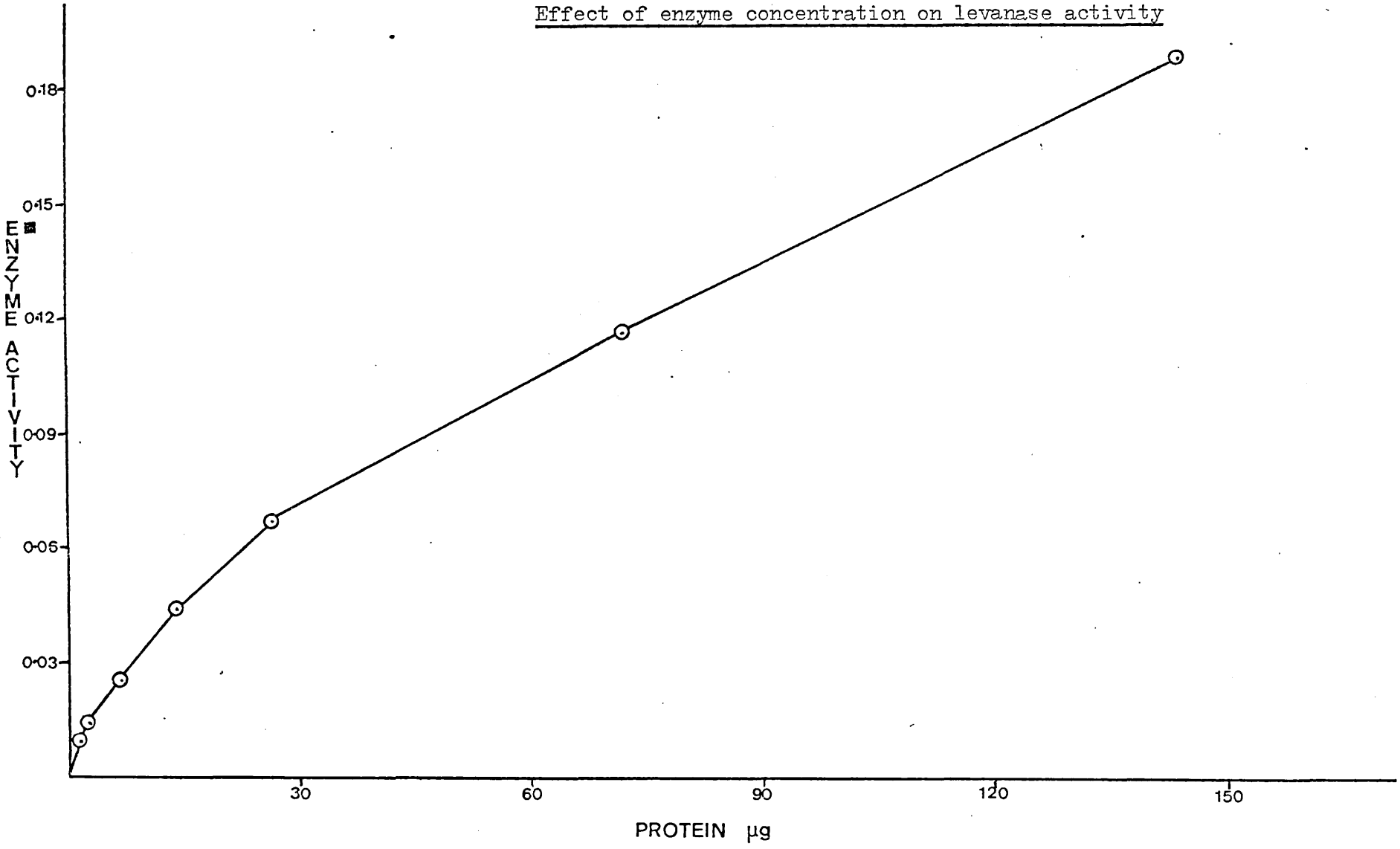
Fig. 79

Effect of substrate concentration on levanase activity

■ Activity expressed as μmoles fructose liberated per min.

Fig. 80

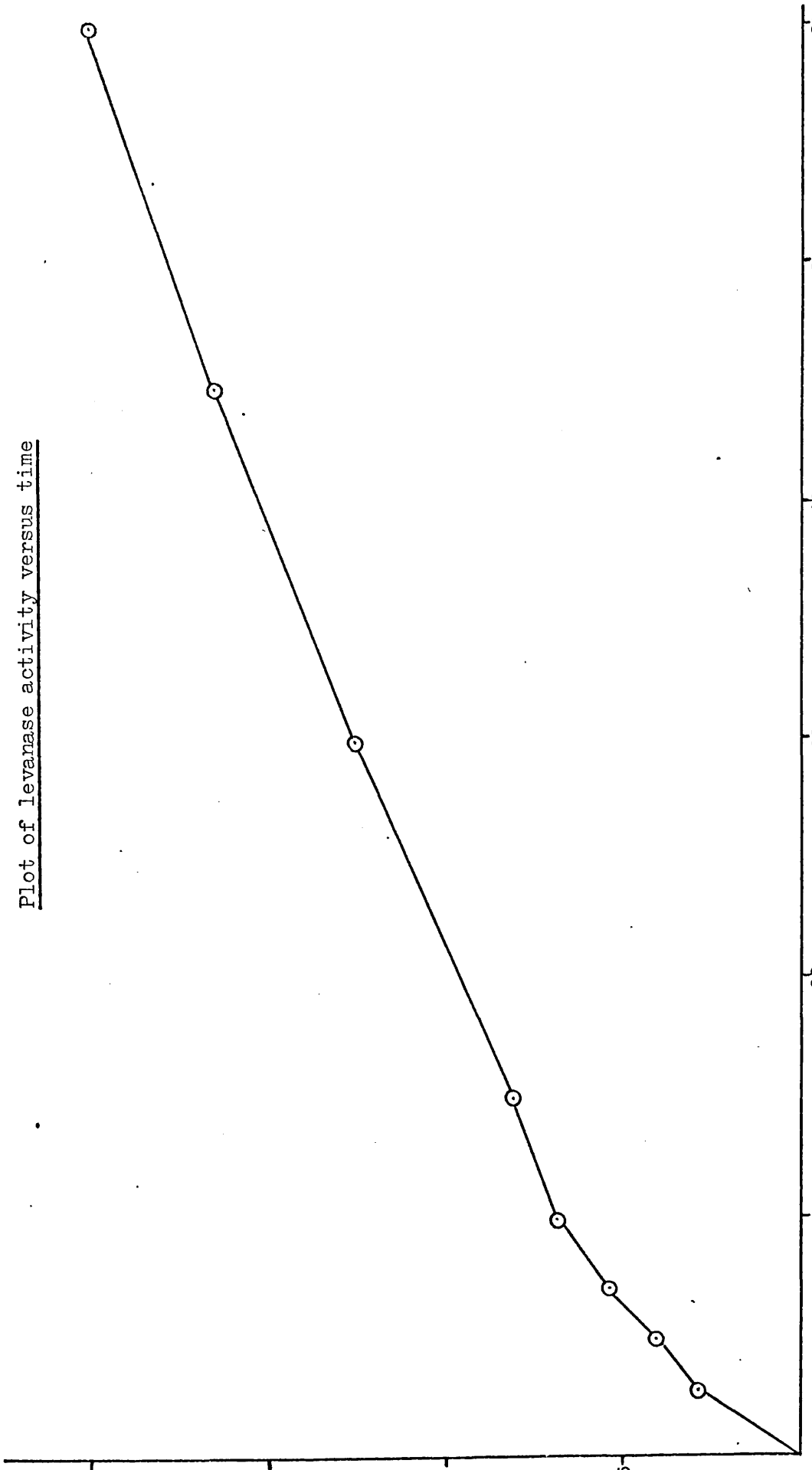
Effect of enzyme concentration on levanase activity



■ Activity expressed as µmoles fructose liberated per minute.

Fig. 81

Plot of levanase activity versus time



■ Activity expressed as μmoles fructose liberated under the conditions described in Experiment 53c.

the substrate concentration. It was not possible to saturate the enzyme(s) even up to a concentration of 100 mg/cm^3. Fig. 80 shows that at the substrate concentration taken the amount of reducing sugar produced was proportional to the amount of enzyme present. Under the conditions chosen for determining levanase activity the amount of reducing sugar produced was proportional with time.

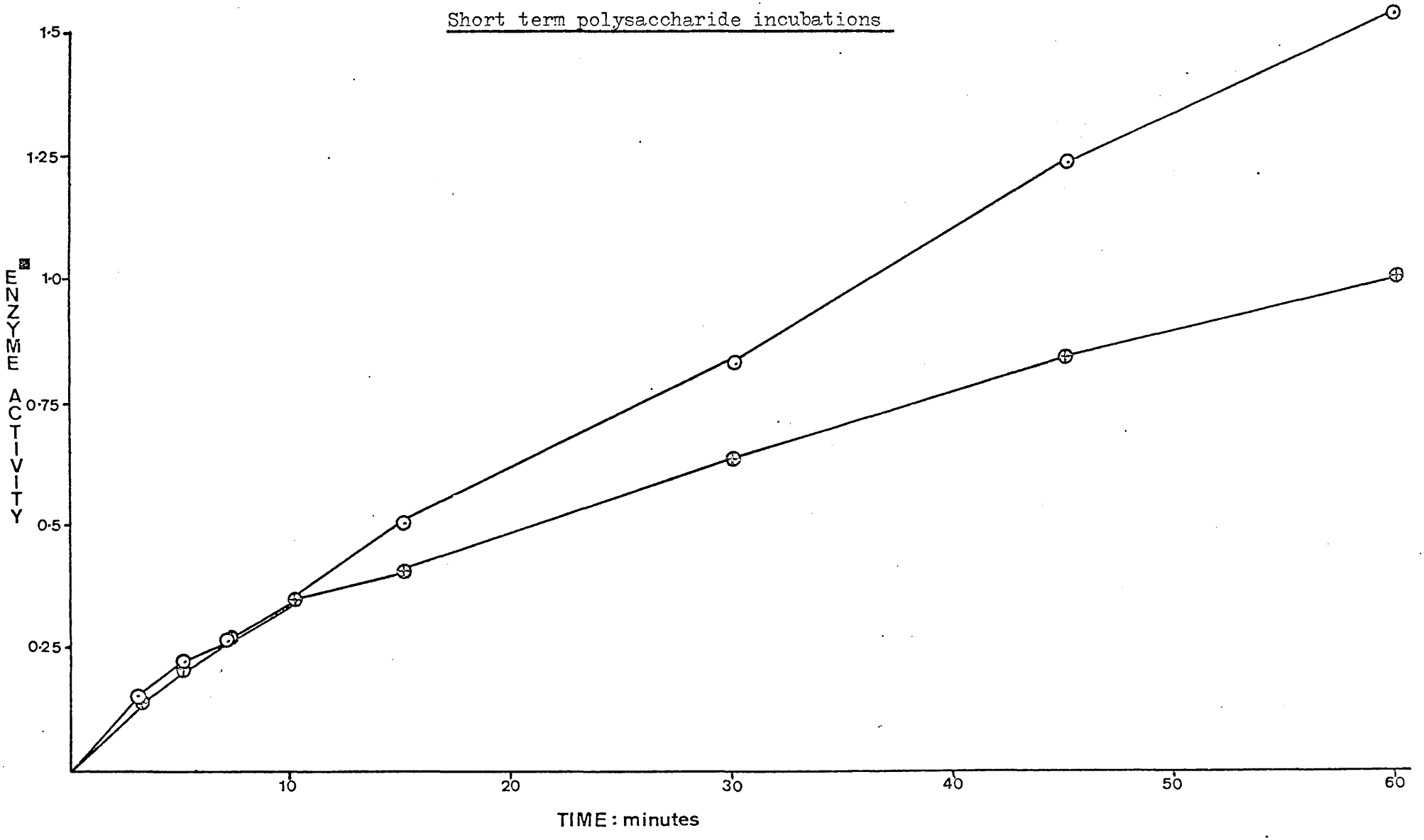
e) Activity of the preparation towards β-(2→1) and β-(2→6) links in polysaccharides

It has already been shown in Section **2.c.** that the preparation has both inulinase and levanase activity and is therefore able to degrade β-(2→1) and β-(2→6) linkages. Heat inactivation studies on both the inulinase and levanase activity of the preparation, indicated that no specific enzyme is present, which selectively hydrolyses β-(2→1) branch linkages. Short and long term incubations were carried out as described in Experiment 54. Fig. 82 shows the short term incubation of the enzyme preparation with levan and inulin. Both incubations were carried out under different conditions. As the enzyme preparation was found to be less active towards inulin the enzyme concentration was increased, therefore relative rates will not be given. The activity time plots shown in Fig. 82 show that the preparation has both inulinase and levanase activity. That is, the enzyme preparation is capable of cleaving β-(2→1) fructosyl linkages and β-(2→6) fructosyl linkages.

The percentage degradation of the two polysaccharides levan, and inulin by the enzyme preparation is shown in Fig. 83. After a period of 28 hours the levan was hydrolysed completely to fructose.

Fig. 82

Short term polysaccharide incubations



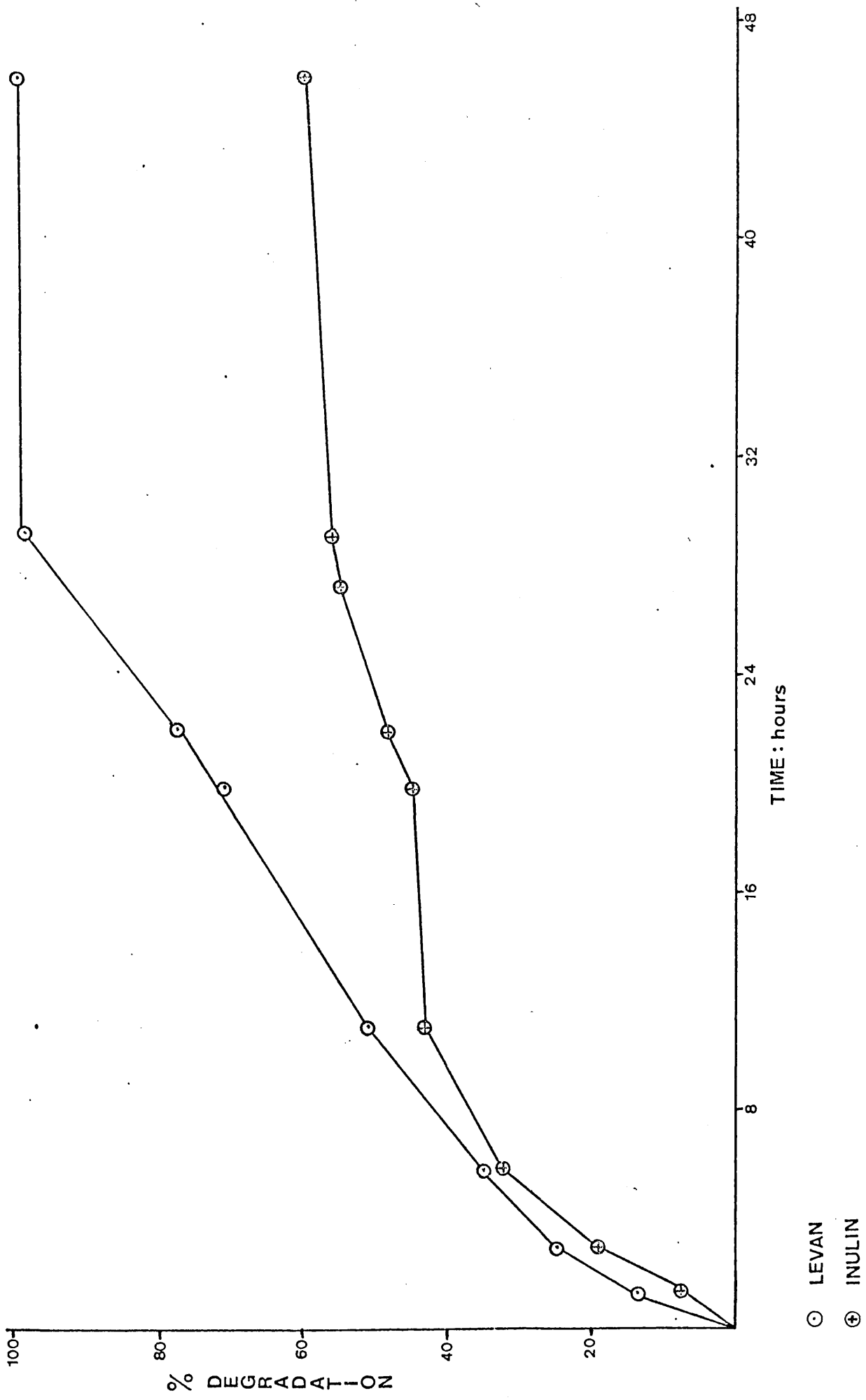
⊙ levan digest

⊕ inulin digest

▣ expressed as μmoles fructose liberated under the conditions described in Experiment 54.

Fig. 83

Percentage degradation of levan and inulin by the levanase preparation



A paper chromatogram spotted with samples of the 3,6 and 10 hr incubation was run with a fructose standard. Fructose was found to be the only sugar liberated. No fructose containing levan oligosaccharides were detected (Fig. 89b). The levanase extract was therefore able to completely degrade levan, hydrolysing β-(2→6) and β-(2→1) fructosyl linkages with the subsequent release of D-fructose. In comparison, after 28 hours the inulin was degraded to the extent of 56%. After 28 hours the figure was only slightly increased to 60%. A previous incubation of inulin with the enzyme preparation gave a higher degradation figure of 75% after 10 hours indicating that the enzyme may have lost some activity when these incubations were carried out. A paper chromatogram of the 3,6 and 10 hour incubation (Fig. 89a) showed that fructose was the only product of the enzymic hydrolysis of inulin. The possibility of product inhibition in the inulin digest was ruled out when later studies described in section II.B(h) indicated that D-fructose was not inhibitory.

As D-fructose was the sole product of the enzymic hydrolysis of both inulin and levan the enzymic hydrolysis of these substrates proceeds by the splitting off of D-fructose units. This confirms the result obtained by Marshall[217] that the enzyme(s) present attack(s) its substrates in an exo-fashion.

f) Hydrolysis of oligosaccharides containing a terminal β-linked fructosyl unit

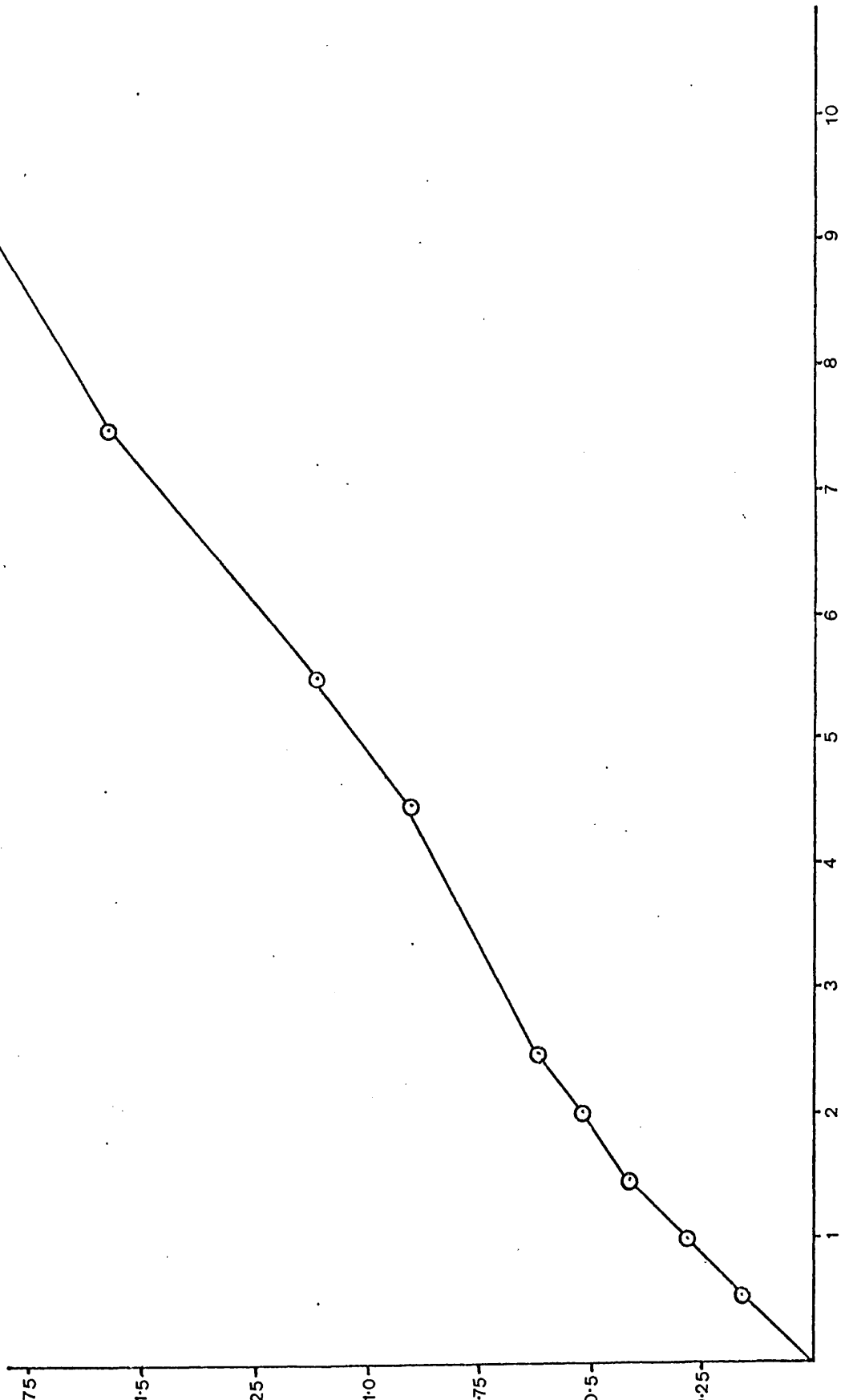
The invertases isolated from strains of Streptococci have all had the property of hydrolysing β-fructosyl units from certain oligosaccharides.[201,211,212] In addition to sucrose being

hydrolysed raffinose and methyl β-D-fructofuranoside are also attacked releasing D-fructose. Melibiose, turanose, trehalose amongst many others are not hydrolysed. It was therefore interesting to see whether the levanase preparation would be able to hydrolyse oligosaccharides containing a terminal β-linked fructosyl unit. Both sucrose and raffinose were chosen as substrates. Incubations were carried out as described in Experiment 55. Fig. 84 shows the activity versus time plot when raffinose was incubated with the enzyme preparation. It is proposed that the terminal β-fructosylunit is hydrolysed to give melibiose and D-fructose as the products of the reaction. As melibiose is a reducing sugar the amount of D-fructose released was determined by dividing the total reducing sugar by the number of reducing equivalents liberated e.g. $\frac{\text{total reducing sugar}}{2}$. Paper chromatograms were obtained from the $1\frac{1}{2}$, $5\frac{1}{4}$ and $10\frac{2}{3}$ hours incubations. Standards applied to the chromatogram were fructose, glucose, galactose and raffinose. The results are shown in Fig. 88a,b. Some raffinose, as expected, still remained after $10\frac{2}{3}$ hours. The products of the enzymic hydrolysis of raffinose were fructose and a sugar which had a greater mobility than raffinose but not as great as the monosaccharides. This was assumed to be melibiose.

Fig. 85 shows the number of μmoles fructose liberated when sucrose was incubated with the enzyme preparation. It is proposed that glucose and fructose are the sole products of the reaction. The amount of fructose liberated was determined by dividing the total reducing sugar liberated by two. A paper chromatogram was obtained from the $\frac{1}{2}$, 1 and 2 hour incubations. The results are shown in Fig. 89c. The products, both of which had agreater mobility than sucrose, were assumed to be glucose and fructose.

Fig. 84

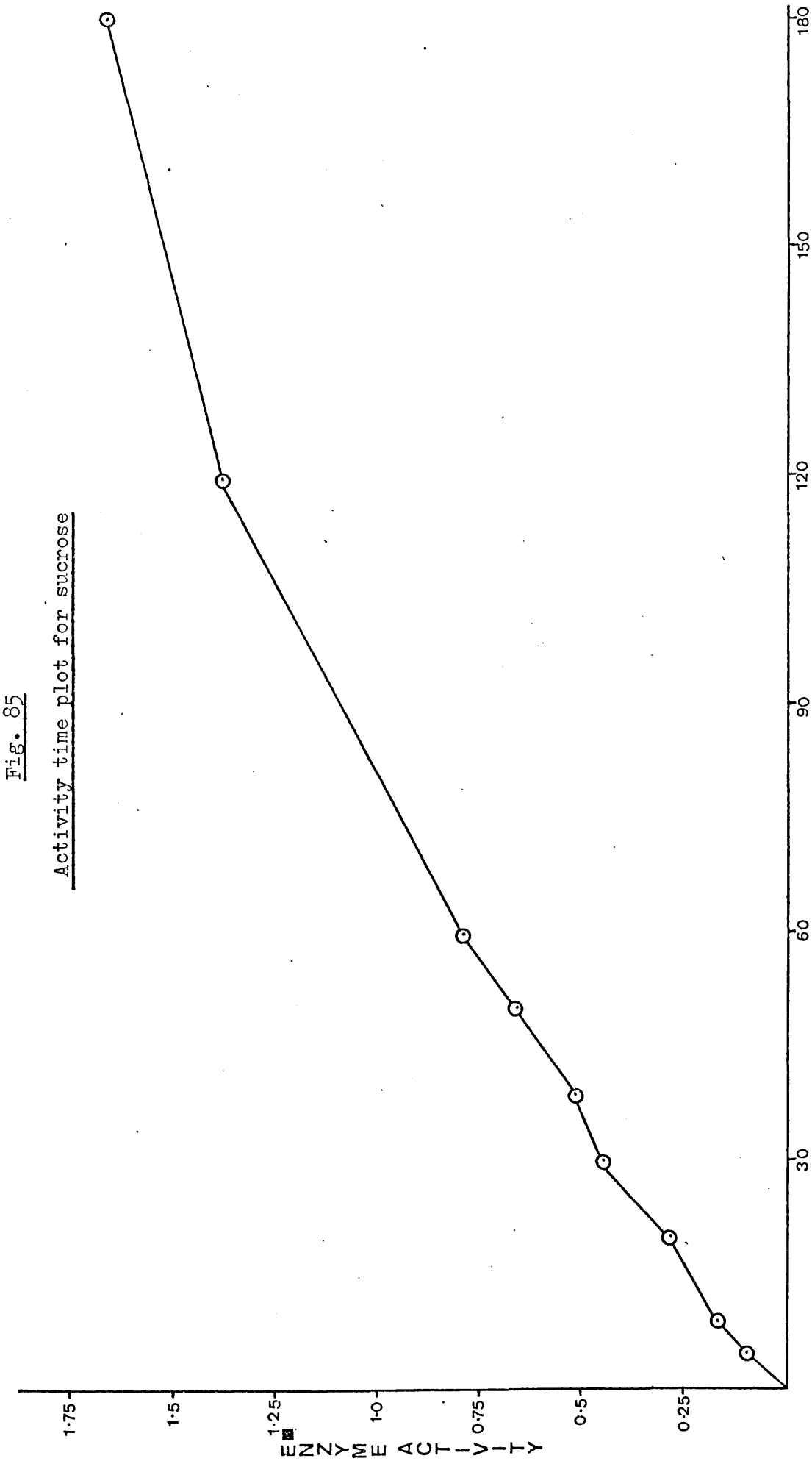
Activity time plot for Raffinose



■ Expressed as µmoles fructose liberated under the conditions described in Experiment 55.

Fig. 85

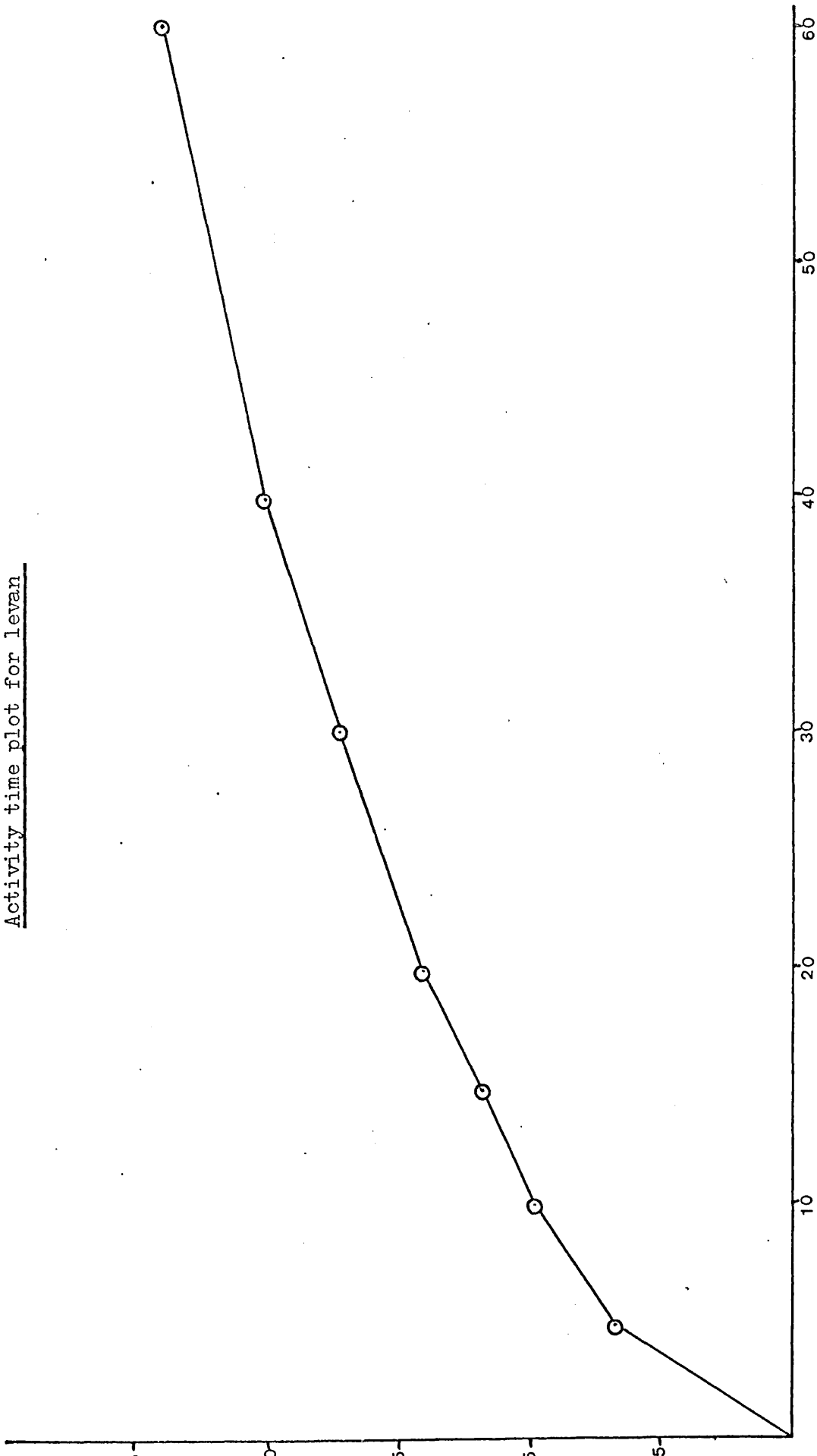
Activity time plot for sucrose



■ Expressed as μmoles fructose liberated under the conditions described in Experiment 55.

Fig. 86

Activity time plot for levan



■ Expressed as μmoles fructose liberated under the conditions described in Experiment 55.

It is of interest to note that no oligosaccharides were detected in the digests with raffinose and sucrose, indicating the lack of levan-sucrase within the enzyme preparation. In Table 42 the relative rates of the enzymic hydrolysis of levan, raffinose and sucrose are given. The activity versus time plot for levan is shown in Fig. 86. This incubation was carried out under the same conditions as for the other two incubations.

Table 42 Relative rates for the hydrolysis of levan, sucrose and raffinose by the levanase preparation

Substrate	Structure	Products	Relative rate°	
			18 mins.	1 hour
Levan	β-(2→1) fructosyl, branch links β-(2→6) fructosyl linear links.	D-fructose	100	100
Raffinose	O-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl β-D-fructofuranoside	Melibiose + D-fructose	10	22
Sucrose	β-D-fructofuranosyl α-D-glucopyranoside	D-glucose + D-fructose	37	63

° Activity of the enzyme preparation shown towards levan in comparison with the substrates listed after the stated time.

The enzyme preparation shows a much higher activity towards levan than towards either sucrose or raffinose. These results show that the enzyme preparation is also able to hydrolyse terminal β-fructosyl units in oligosaccharides containing monosaccharides other than fructose. These results are consistent with the known properties of a β-D-fructofuranosidase.

g) <u>The coupling of an enzymic method for the determination of glucose and fructose with the levanase system: activity of the preparation towards β-linked fructosyl-oligosaccharides and other substrates</u>

Although it has been shown in section II.B that the polysaccharide substrates are the most active substrates for the enzyme preparation it was of interest to investigate the activity of the enzyme(s) towards inulin-oligosaccharides and levan-oligosaccharides. A problem associated with the use of these substrates was that they were reducing sugars and hence interferred with the determination of D-fructose liberated by enzymic action. An enzymic method described by Sturgeon[223] was therefore adapted for use. The following series of enzymic reactions occur by this method:

a) D-fructose + ATP $\xrightarrow{\text{Hexokinase}}$ D-fructose 6-P + ADP

D-fructose 6-P $\rightleftharpoons$ D-glucose 6-P (phosphoglucoisomerase)

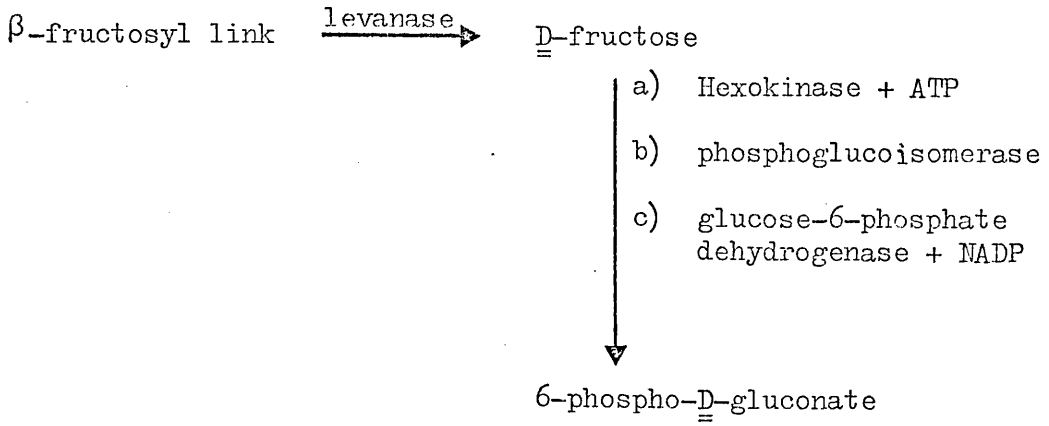
b) D-glucose + ATP $\xrightarrow{\text{Hexokinase}}$ D-glucose 6-P + ADP

D-glucose 6-P = glucose-6-phosphate

c) D-glucose 6-P + NADP $\xrightarrow{\text{dehydrogenase}}$ 6-phospho-D-gluconate + NADPH + H^+

The measured change in extinction at OD_{340} of the reduced nicotinamide adenine dinucleotide (NADPH) is used directly in the calculation of the number of μmoles glucose or fructose present. The procedure is described in 'Methods' III.M. Originally it was proposed to incubate the enzyme(s) and substrate over a period of time, stop the reaction and determine the amount of D-fructose liberated. However, this method would have involved vast amounts of enzymes and therefore was deemed too costly, and time consuming. It was proposed therefore, to try and

couple both enzyme systems together as shown:



The D-fructose liberated by the action of the levanase could therefore be determined with time. A problem associated with the coupling of both systems was the pH of the incubation. The pH for the enzymic determination of fructose was pH 7.6 and the levanase system was buffered at pH 6.6. Here, a compromise was achieved by buffering at pH 7.0 so that both enzyme systems were still active. It is apparent that by buffering at this pH some loss of enzymic activity would occur as the pH at maximum levanase activity was found to be pH 6.6 - 6.8. The procedure is described in Experiment 56.

The relative rates for the substrates investigated, to determine if the levanase preparation could hydrolyse the β-fructosyl links are shown in Table 43. The activity time curves are shown in Fig. 87.

Table 43 Relative rates for a series of oligosaccharides hydrolysed by S. salivarius levanase.

Substrate	Structure	Products of levanase action	Relative rate*
Levan	n-fructosyl units	D-fructose + (n-1) fructosyl units	100
Methyl β-D-fructo-furanoside		MeOH + D-fructose	28

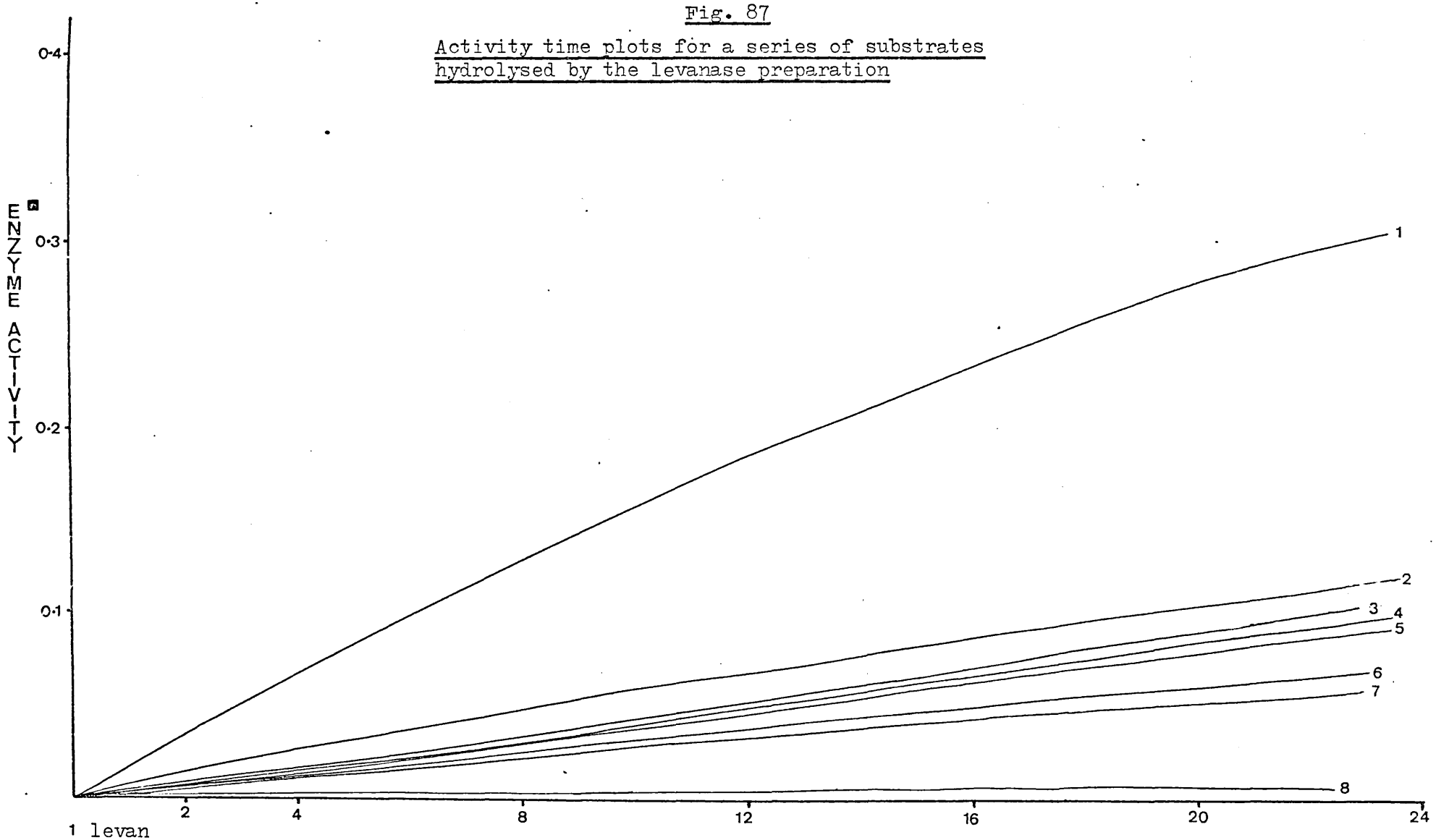
Table 43 (continued)

Substrate	Structure	Products of levanase action	Relative rate*
Sucrose	β-D-fructofuranosyl α-D-glucopyranoside	D-glucose + D-fructose	27
Inulinbiose	1-O-β-D-fructofuranosyl-β-D-fructofuranose	2(D-fructose)	38
Inulinitriose	O-β-D-fructofuranosyl-(2→1)-O-β-D-fructofuranosyl-(2→1)-β-D-fructofuranose.	3(D-fructose)	18
Raffinose	O-α-D-galactopyranosyl (1→6)-α-D-glucopyranosyl β-D-fructofuranoside	Melibiose + D-fructose	3
Levanbiose	6-O-β-D-fructofuranosyl-β-D-fructofuranose	2(D-fructose)	22
Levantriose	O-β-D-fructofuranosyl (2→6)O-β-D-fructofuranosyl-(2→6)-β-D-fructofuranose.	3(D-fructose)	25
p-Nitrophenyl β-D-glucopyranoside			0
Cellobiose	4-O-β-D-glucopyranosyl-β-D-glucopyranose		0
Melibiose	6-O-α-D-galactopyranosyl-α-D-glucopyranose		0

* The activity of the enzyme preparation towards the substrates listed compared with levan after 10 minutes.

Fig. 87

Activity time plots for a series of substrates hydrolysed by the levanase preparation



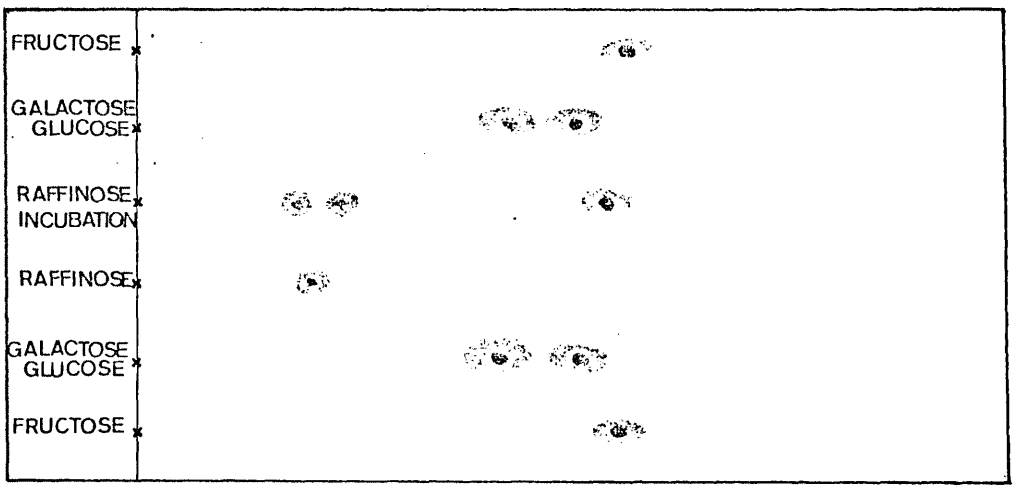
1 levan
2 inulinbiose
3 methyl β-D-fructofuranoside
4 sucrose
5 levantriose
6 levanbiose
7 inulintriose
8 raffinose

■ Expressed as μ moles fructose liberated under the conditions described in Experiment 56.

The levanase preparation was able to hydrolyse any substrate containing a terminal β-fructosyl unit. A greater activity was shown by the enzyme(s) towards levan in comparison with the other substrates. Inulinbiose was hydrolysed at a greater rate than the other di- and trisaccharides. The enzyme preparation showed only slight activity towards raffinose in comparison with the other substrates. Substrates containing a β-(2→1) or a β-(2→6) fructosyl linkage were also hydrolysed. The inability of the enzyme preparation to hydrolyse cellobiose, melibiose or p-nitrophenyl β-D-glucopyranoside indicated the lack of β-glucosidase or α-galactosidase within the preparation. Although it is acknowledged that no levansucrase is present in the preparation the presence of another sucrose degrading enzyme e.g. sucrose phosphorylase[202] cannot be ruled out. Also, the possibility of an invertase,[201,211,212] which are known to be produced by the Streptococci, being present within the preparation cannot be overlooked. The results shown in Fig. 87 are in agreement with those of Kunst et al.[220] who showed that the levanase from B. subtilis was able to hydrolyse sucrose, inulin and levan.

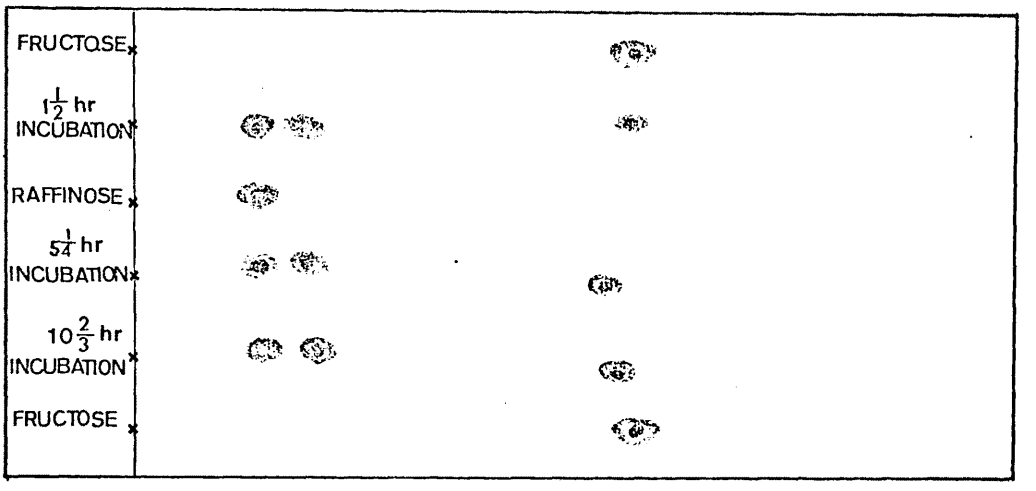
Marshall[217] found that a levanase preparation from the same source failed to hydrolyse inulin and methyl β-D-fructofuranoside, which is not in accord with the results found by the author. On comparing the experimental conditions in which the incubations were carried out, by Marshall and the author, it was found that large differences in the number of enzyme units present in the incubations existed. The author used for the incubation with inulin, an enzyme preparation, the number of enzyme units of which was larger by a factor

A



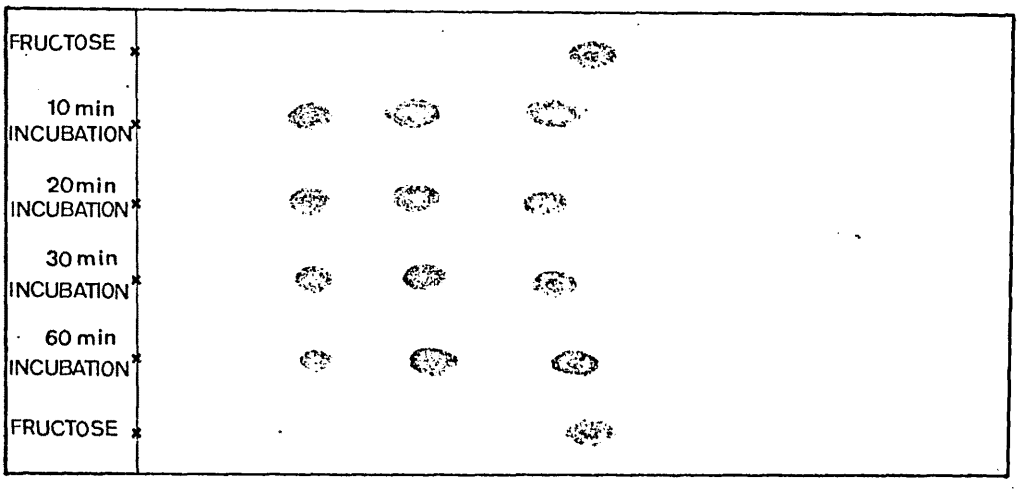
Raffinose incubation

B



Raffinose incubation

C

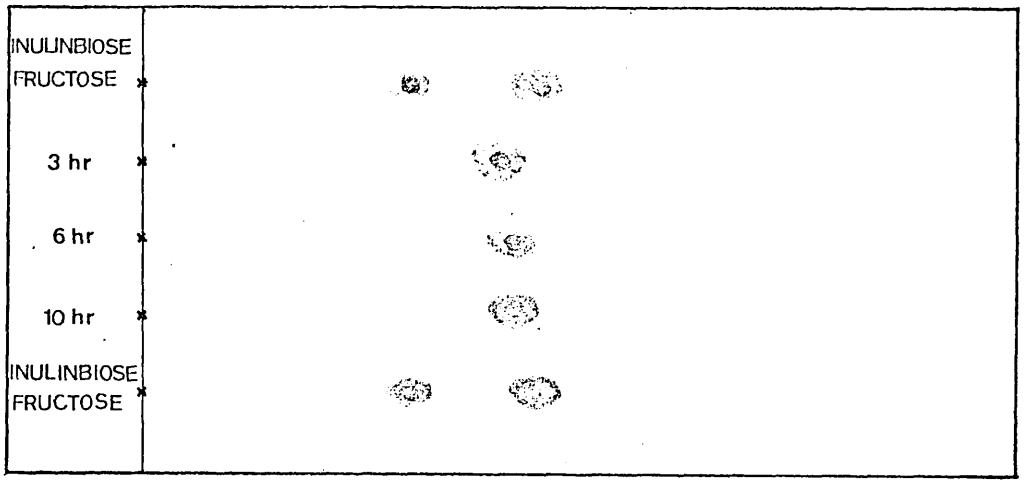


Inulintriose incubation

Fig. 88

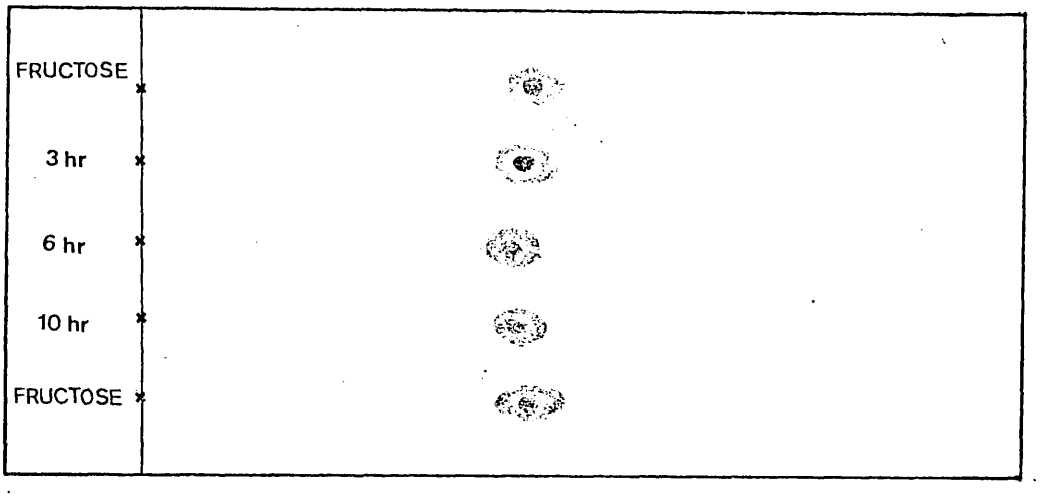
Paper chromatograms of enzymically degraded substrates

A



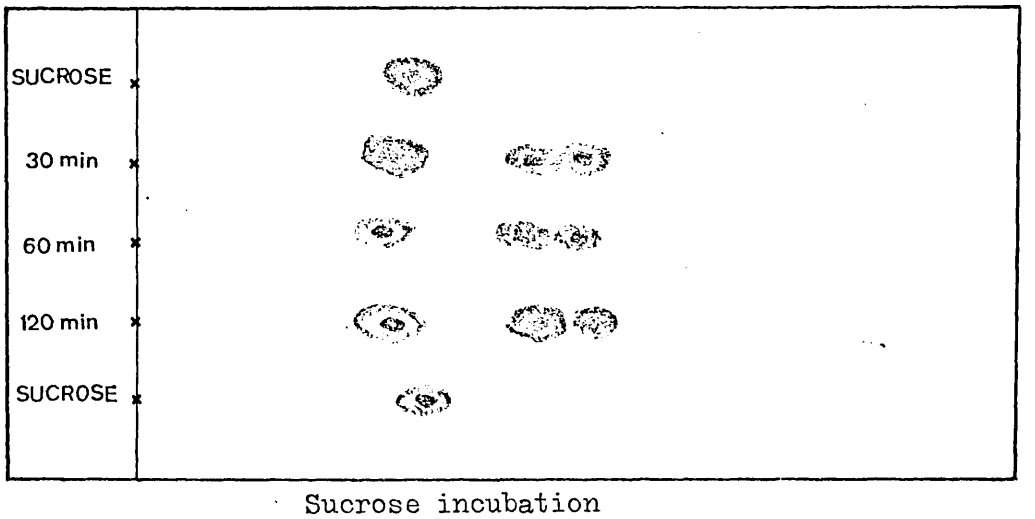
Inulin incubation

B



Levan incubation

C



Sucrose incubation

Fig. 89

Paper chromatograms of enzymically degraded substrates

of 50. The methyl β -D-fructofuranoside incubation also contained a 2.5 times greater number of enzyme units compared with that taken by Marshall. No large deviation in the substrate concentration was noted between those taken by the author and Marshall. It is unlikely that such a small deviation in the number of enzyme units in the methyl β-D-fructofuranoside incubation would have given rise to the inactivity of the enzyme towards this substrate, as found by Marshall. No information is available in the literature regarding the action of a levanase on methyl β-D-fructofuranoside.

Marshall[217] also found that the levanase preparation failed to hydrolyse inulintriose but hydrolysed inulinbiose and the β-(2→1) linked series of oligosaccharides up to DP7. The preparation obtained by the author was found to hydrolyse inulintriose when the D-fructose liberated was determined using the coupled enzyme system described in this section. In order to confirm this result an incubation was carried out for two fixed times (Experiment 57) and the D-fructose liberated determined using the procedure described in 'Methods' (III.M). After 30 and 60 minutes the amount of D-fructose liberated was 15 μg and 295 μg, respectively. A paper chromatogram of the 10, 20, 30 and 60 minute incubation with a fructose standard show that fructose, inulintriose, and a sugar with a mobility between inulintriose and fructose, presumably inulinbiose to be present, (Fig. 88c). To ensure that the sample was in fact a triose the degree of polymerisation was determined as described in Experiment 59. The results are shown in Table 44.

Table 44 Degree of polymerisation of an oligosaccharide thought to be Inulintriose

Sample	DP determined	
1	2.6	2.8
2	3.0	

To conclude, the levanase preparation obtained by the author was able to hydrolyse inulintriose in contrast to the results of Marshall.[217] If a comparison is made between the relative rates determined using the two different methods for measuring the amount of D-fructose liberated, they are found to deviate considerably, as shown in Table 45.

Table 45 Comparison of relative rates derived by two different methods for determining liberated fructose

Substrate	Relative Rates	
	A^{o}	B^{+}
Levan	100	100
Sucrose	63	27
Inulintriose	41.7	18

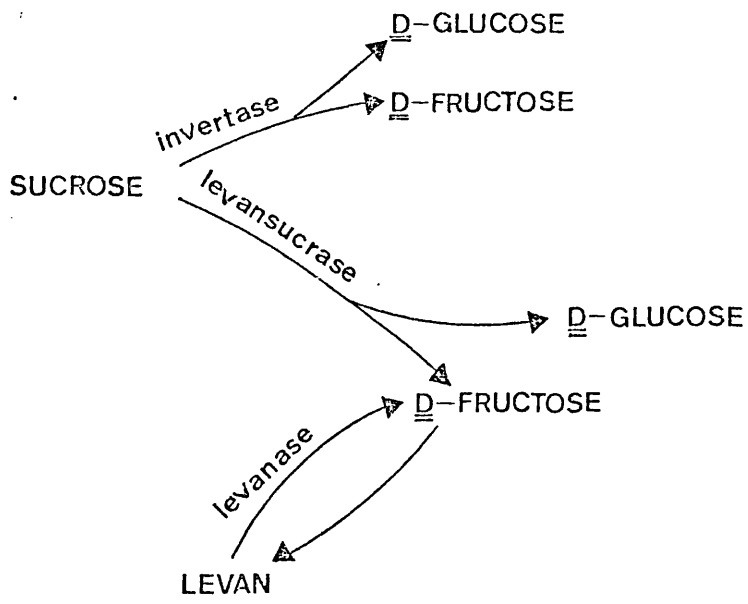
^{o} fructose determined by Nelson reducing test

$^{+}$ fructose determined by coupled enzyme system

To account for these results it is suggested that the difference in the pH at which the incubations were carried out gave rise to the different

relative rates. Because two enzymes were present in the preparation it is assumed that the change in pH may have a greater effect on one enzyme than the other. If this deactivation effect was more effective against one enzyme which showed a higher activity towards oligosaccharide substrates than the other enzyme, one would expect the rate at which these substrates are enzymically hydrolysed to decrease.

There would seem enough evidence available to link quite closely the properties of the levanase with that of a β-D-fructofuranosidase. The specificity of the levanase preparation would seem to indicate its preference for large molecular weight substrates. It is known that the levanase from Bacillus subtilis[220] has a broad specificity hydrolysing inulin, sucrose, and levan. A β-D-fructofuranosidase isolated from Lactobacillus plantanium[216] was found to hydrolyse sucrose, inulin and raffinose. As it is proposed that the levanase is an enzyme important in the degradation of levan to yield fructose for energy requirements, the survival of the bacteria could depend on the broad specificity of action of its enzymes. Indeed three of the enzymes responsible for sucrose metabolism seem to show very similar enzymic properties as shown:



Their relationship is based on their ability to hydrolyse the β-fructosyl linkage. It is proposed that bacterial invertases are able to degrade the small molecular weight sugars for immediate energy requirements. The presence of a large concentration of sucrose or raffinose would undoubtedly activate levansucrase to synthesise storable carbohydrate in the form of levan. The lack of immediate energy yielding sugars initiates the production of the bacterial levanase to degrade the storage polysaccharide. Under the extreme conditions that some bacteria live the adaption of its enzymes would seems a logical step for survival. This would involve a broad specificity of action for most of the enzymes named which seems to be the case as shown in Table 46.

Table 46 Comparison of some of the enzymic properties of three enzymes responsible for sucrose metabolism

Substrate	ENZYME Levansucrase[189,220]	Invertase[201,211,212,220]	Levanase[220]
Sucrose	✓	✓	✓
Levan	Only very slowly	?	✓
Inulin	X	?	✓
Raffinose	✓	✓	✓

✓ = shows activity

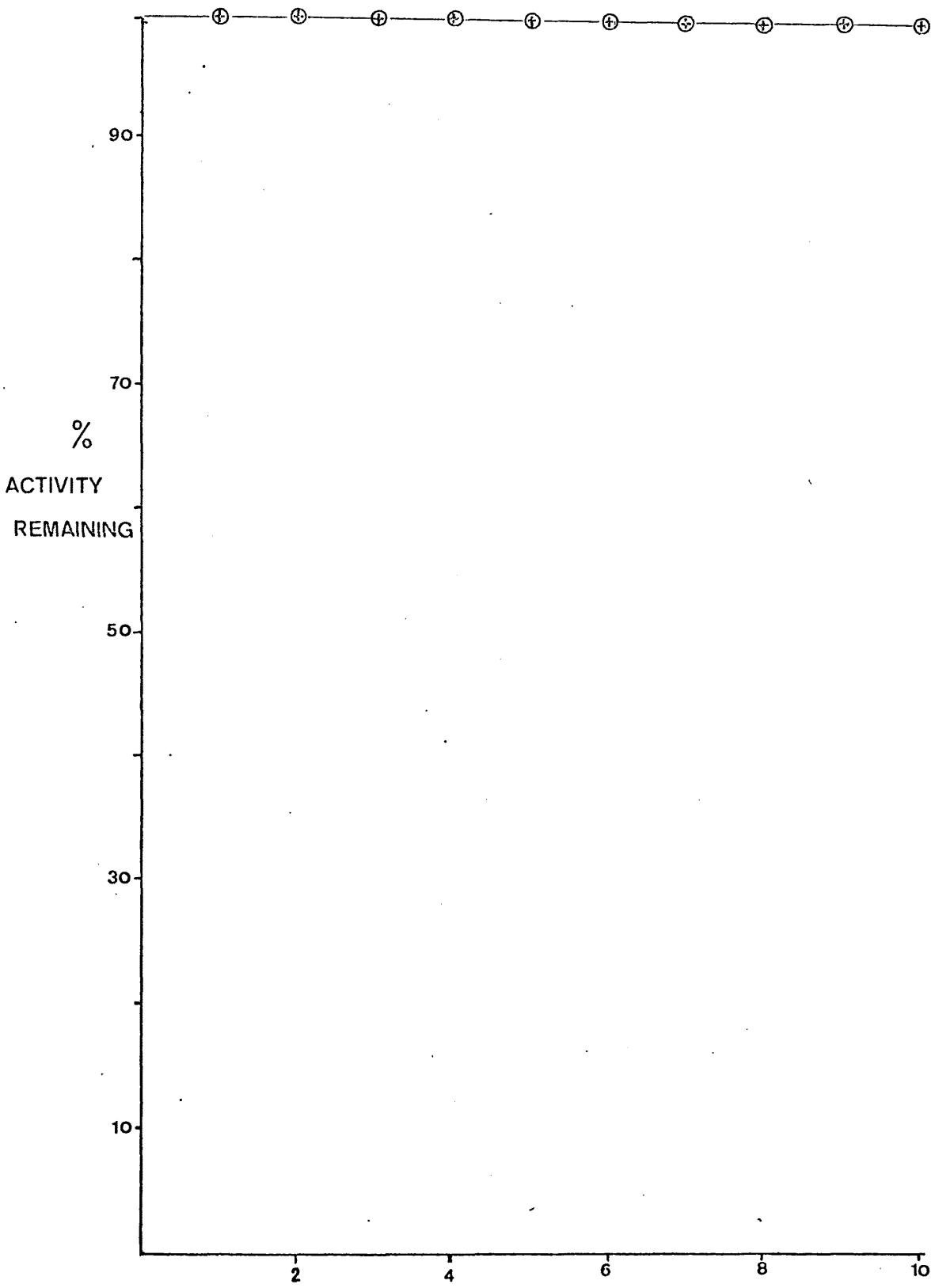
X = no activity

? = variable results

h) Inhibition studies on the inulinase and levanase properties of the enzyme

Very little information is available concerning inhibition studies on levanase preparations. Mesner showed that the levanase from Odontomyces viscosus[219] could be deactivated by EDTA, but its activity could be restored by addition of calcium ions. As already described in section II.2.A(e), EDTA had no inhibitory effect on the levanase preparation. If any controls existed for the functioning of the levanase during the degradation of levan it would likely involve product inhibition. It was therefore decided to investigate the effect of D-fructose on the levanase and inulinase properties of the enzyme(s).

Fig. 90



O Inulin Digest

⊕ Levan Digest

Inability of D-fructose to Inhibit the levanase and inulinase activities.

The maximum fructose concentration was equivalent to the concentration of substrate taken in all previous incubations. Fig. 90 shows the percentage inhibition by fructose of the inulinase and levanase activity of the enzyme preparation. No inhibition of both activities was shown up to a final concentration of 10 m$\underline{\underline{M}}$. An increase in the levanase and inulinase activity was observed at increasing concentrations of $\underline{\underline{D}}$-fructose. This would therefore suggest that fructose stimulated the activity of the enzyme(s). However, a concentration effect due to the high fructose concentration in comparison to the $\underline{\underline{D}}$-fructose liberated from enzymic action, may have given rise to the higher activity. Longer incubation times, which would have increased the amount of fructose liberated by enzymic action, would have clarified the reason for the increased fructose concentration. The possibility of there being any levan-sucrase activity being present could be ruled out as the amount of $\underline{\underline{D}}$-fructose found did not vary from the amount expected.

Chapter III General Methods

III.A Common procedures

III.A.I Evaporation was carried out under reduced pressure with a "Buchi" rotary film evaporator.

III.A.II Water - deionised water was used unless otherwise stated.

III.A.III Lyophilisation - (freeze-drying) was carried out on a "Chem Lab" freeze-drier.

III.A.IV Dialysis - was performed with visking tubing, initially rendered free of glycerol by boiling twice with distilled water, and then with deionised water.

III.A.V Ultraviolet and visible absorbances were measured using a 'Pye Unicam' SP500 spectrometer or a bench top "Eel" 197 spectra spectrometer with a flow through cell.

III.A.VI Centrifugation was carried out with a "Beckman" J21 centrifuge using a JA10 or JA20 rotor.

III.A.VII Enzyme concentration was carried out using a "Amicon" model 202 or 52 concentration cell with the stated membrane.

III.A.VIII Substrate purity - all substrates were checked for purity by paper chromatography in solvent 1 or 2.

III.A.IX Reagents - all chemicals were of "Analar" grade unless otherwise stated.

III.B. Paper chromatographic methods

III.B.I Chromatography solvents

Solvent 1.

n-butanol	:	40 parts by volume
ethanol	:	11
water	:	19

Solvent 2.

n-butanol : 6 parts by volume

pyridine : 4

water : 3

III.B.II Staining reagent for paper chromatography[224]

a) Silver nitrate

Stock solution : saturated silver nitrate

Dip solution : 5 cm^3 stock solution in acetone (1 litre). Precipitate redissolved with water.

b) Sodium hydroxide

Stock solution : 500 cm^3 water + 250 g sodium hydroxide

Dip solution : 960 cm^3 ethanol + 40 cm^3 stock solution

c) 10% aqueous sodium thiosulphate.

III.B.III Paper chromatography (qualitative)

Paper chromatography was carried out using Whatman No. 1 paper by descending solvent method. After development the papers were air dried and stained using sodium hydroxide - silver nitrate reagents.

III.B.IV Paper chromatography (quantitative)

Preparative chromatography was performed using Whatman No.17 and No. 3 papers. The papers were prewashed with water and allowed to dry. No.17 papers were fitted with a wick of No.3 and the mixture to be chromatographed streaked along the origin. After development and air drying, three strips 2 cm wide were cut from the two edges and centre of the paper, and stained.

Having detected the separated components of the mixture the paper was then cut into strips and eluted with water followed by freeze-drying.

III.C. Paper electrophoresis

Paper electrophoresis was carried out using Whatman No.3 paper on a 'Shandon' High Voltage electrophoresis instrument. The standard was D-glucose and the non-moving marker tetra-O-methyl-D-glucose. Development was carried out by applying 1200V corresponding to a current of 60 mA for 2 hours. After air drying staining was carried out with sodium hydroxide - silver nitrate.

III.D. Terms used in chromatography

a) Terms used in paper chromatography

$$R_x = \frac{\text{distance moved by component from base line}}{\text{distance moved by standard x from base line}}$$

b) Terms used in paper electrophoresis

$$M_x = \frac{\text{distance from non-moving marker by a component}}{\text{distance from non-moving marker by a standard x}}$$

In sodium borate electrolyte the standard was glucose and the non-moving marker tetra-O-methyl-D-glucose.

c) Terms used in gas-liquid chromatography

$$T_x = \frac{\text{time taken for the component to pass through column}}{\text{time taken for standard x to pass through column}}$$

For g.l.c. of methylated alditol acetates the standard was 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.

III.E. Buffers

All buffers were made up according to the tables in "Methods in Enzymology".[225]

i) Phosphate[226]

The following stock solutions were made up:

A : 0.2 M solution of sodium dihydrogen phosphate (27.8 g in 100 cm^3).

B : 0.2 M solution of disodium hydrogen phosphate heptahydrate (53.65 g in 100 cm^3).

x cm^3 A + y cm^3 B to 100 cm^3; diluted for the molarity required.

ii) Acetate[227]

Stock solutions:-

A : 0.2 M solution of acetic acid (11.55 cm^3 in 1000 cm^3).

B : 0.2 M sodium acetate (16.4 g in 1000 cm^3).

x cm^3 A + y cm^3 B to 100 cm^3; diluted for molarity required.

iii) Tris/HCl

Stock solutions:-

A : 0.2 M tris (24.2 g in 1000 cm^3)

B : 0.2 M hydrochloric acid.

50 cm^3 A + x cm^3 B to 200 cm^3; diluted for molarity required.

III.F. Gel filtration chromatograhy

III.F.I Preparation of gel filtration media

Sephadex (Pharmacia) and Biogel P(Bio-rad) were obtained as powders. Swelling was carried out by adding water and heating on a boiling water bath. The length of time that the suspension was heated was that recommended by the manufacturers. After heating, the water was decanted off and several aliquots of the eluting buffer were added.

Both Ultrogel AcA.22 (L.K.B.) and Biogel A-5m (Bio-rad) were obtained already in a swollen state.

III.F.II. Packing the column

Any gel filtration media to be packed was first deaerated by means of a water pump until cessation of bubbles . The column to be packed was first filled with eluant, until the dead space was taken up, and then the gel slurry was poured down the edge of the column, until full. A funnel was then fixed to the top of the column, filled with gel, and then the column allowed to flow at a rate dictated by the working pressure. When very slow flow rates were required a perstaltic pump was used for packing the column. Equilibration was carried out by letting the column flow for at least two column volumes.

III.F.III. Determination of the void volume (V_o) and the total volume (V_t) of the column packed with gel filtration media

Blue dextran [2 mg/cm^3, Pharmacia] and glucose (4 mg/cm^3) were dissolved in elution buffer and applied to the column. After washing through the samples the column was eluted with buffer at the desired flow rate, and fractions collected. Blue dextran was detected by taking absorbance readings at 600 nm of the eluted fractions. Glucose was determined by means of the phenol sulphuric method (III.M.II). From the elution volumes (V_e) of the samples V_o and V_t could be found where:-

V_e dextran blue = V_o (Interstitial volume between the gel granules)

V_e glucose = V_t (Total volume of the packed column)

III.G Ion Exchange chromatography

III.G.I. Preparation of Ion-exchange packing material

Both carboxymethyl cellulose (CM-52, Whatman Ltd.) and diethylaminoethyl cellulose (DE-52, Whatman Ltd.) were obtained in a pre-swollen state. All buffer solutions were made up from carbon dioxide free water and kept free of carbon dioxide.

DEAE-cellulose was degassed by placing the ion-exchanger in the acid component of the buffer and a vacuum applied by means of a water pump. The basic component was then added to bring the buffer to the desired pH. No degassing of CM-cellulose was required. Equilibration was carried out by adding buffer to the ion-exchanger, decanting, and repeating the procedure until the filtrate had the required pH. Fines were removed by pouring the exchanger into a measuring cylinder and allowing the exchanger to settle for a specific time.

III.G.II Packing of the column

The column was packed by the same method as in III.F.II, using a funnel. Buffer was allowed to flow at a flow rate of at least 45 $cm^3/hr/cm^2$ of the cross-sectional area of the column until the bed height was constant.

III.H. Hydroxylapatite chromatography

The hydroxylapatite was prepared by the method of Massey[228] as described in "Methods in Enzymology".[229]

Hydroxylapatite (Type 1, Sigma) was obtained as a suspension in buffer. To increase the flow of the column hydroxylapatite was mixed with cellulose powder as follows:-

100 cm^3 of 10% w/v cellulose (Whatman, CF11) was placed in a 250 cm^3 beaker, 50 cm^3 of hydroxylapatite (30 mg/cm^3) was added with stirring and the suspension stirred for 20 minutes. After standing, and allowing the contents to settle the media was washed twice with water, finally centrifuged, and suspended in 5 m$\underline{M}$ phosphate buffer pH 6.6.

The column (3.3 x 16 cm) was packed as described in III.F.II. However, flow was not started until the hydroxylapatite settled under gravity. After the bed was stable two column volumes of buffer (CO_2 free) were passed through at a flow of 21 cm^3/hr.

III.I Affinity chromatography on a modified Biogel P-300

III.I.I. Preparation of the hydrazide derivative of Biogel P-300

The method used was that of Inman[230] et al. Approximately 1 g of Biogel P-300 (Bio-rad) was swelled overnight in water. Hydrazine hydrate (B.D.H.) sufficient to give a final concentration of 6$\underline{M}$ was preincubated at 47-50°C. The gel was placed in a thermostatically controlled water bath at 47-50°C and the hydrazine hydrate added slowly, with stirring, and left overnight at 47-50°C with stirring. The derivatised gel was filtered off on a porcelain Buchner funnel and washed with water. To check that the excess hydrazine had been removed KI/I_2 solution was added to the filtrate until the solution remained coloured.

III.I.II Coupling of levan oligosaccharides to the derivatised Biogel P-300

Levan-oligosaccharides were prepared by hydrolysis of the S. salivarius levan.[217] 10 g of levan was heated

on a water bath at 70°C with 5 mM oxalic acid for 1½ hours. The excess acid was then neutralised by the addition of calcium carbonate which was removed by centrifugation. The supernatant was deionised by shaking with Amberlite IR120 (H^+) resin, followed by filtration, and then with Amberlite IR 45 (OH^-), followed by filtration. Concentration was carried out in a ultrafiltration cell fitted with a PM10 membrane (Amicon) which facilitated the removal of low molecular weight sugars. The concentrated hydrolysate was then freeze-dried. Paper chromatography in solvent 1 was carried out to check that oligosaccharides were produced. In certain experiments concentration was carried out by rotary evaporation followed by freeze-drying. Marshall,[217] however, suggested that the levan oligosaccharides may be heat labile, hence, rotary evaporation was not used in later experiments. Fructose always remained a major product of hydrolysis which in initial experiments was not removed by ultrafiltration.

Coupling of the oligosaccharides with the hydrazide derivative of Biogel P-300 was carried out as described by Hamazaki[222] *et al.* As no molarity values could be determined for the oligosaccharides the freeze-dried preparation was dissolved in approximately 40-50 cm^3 of water. All glassware was siliconised with dimethyldichlorosilane (B.D.H.) before use. The hydrazide Biogel P-300 was suspended in the oligosaccharide solution, and heated on a boiling water bath for 1 hour. The preparation was then left to stand overnight. Free ligand was removed by washing the gel exhaustively with 50 mM phosphate buffer pH 6.6. Phenyl sulphuric test was then carried out on a small sample of gel to check that some sugar was bound. Not all of the hydrazide derivative of Biogel P-300 was coupled at one time. The coupled gel

was packed into a glass syringe (10 cm^3) and the column equilibrated with 10 mM phosphate buffer pH 6.6.

III.J. Concanavalin A - Sepharose chromatography

Con A-Sepharose was obtained from Pharmacia Ltd. as a suspension. The packing material was packed into a glass syringe (10 cm^3) and pre-equilibrated with 10^{-3}M $MnCl_2$, $MgCl_2$, $CaCl_2$, and washed with 0.1M phosphate buffer pH 6.6, containing 0.5 M NaCl, prior to use.

III.K. Charcoal - celite chromatography[231]

An equal volume of B.D.H. activated charcoal and Lights No.545 celite were mixed, treated with 4 volumes of concentrated hydrochloric acid, and allowed to stand for 24 hours. The mixture was then washed with tap water and poured onto a large Buchner funnel. Washing was continued with tap water until the filtrate was neutral. Two volumes of absolute ethanol was then added and the mixture left to stand for 24 hours. A final washing was then given with distilled water.

A glass column (90 x 7.5 cm) was prepared for packing. Glass wool (5 cm) was placed at the bottom of the column followed by Whatman CF11 cellulose (5 cm), added as a slurry. This was followed by a layer of Celite (5 cm^3; previously washed in acid), also added as a slurry. The column was then packed under gravity and allowed to run throughout the packing procedure. After packing down approximately 70 cm the column was closed with a 3 cm layer of washed Celite followed by a 3 cm layer of Whatman CF 11 cellulose. A filter paper disc was then placed on the column surface to hold it firm. The column was eluted with distilled water for 5 days before use.

III.L. Gas Liquid chromatography (G.L.C.)

A Pye 104 dual column chromatograph equipped with flame ionisation and glass columns (9' x 0.25") was used. Nitrogen was employed as the carrier gas at a flow rate of approximately 40 cm^3 min^{-1}. The column was packed with the following material:

a) OV-225 - 3% of cyanopropylmethyl-phenyl methylsilicone coated on 80-100 mesh Gas-chrome Q.

Retention times and peak areas were measured by a Hewlett Packard 3370B/71B integrator.

A Pye 104 chromatograph was also used when GLC/mass spectrometry was carried out. Helium was the carrier gas and the chromatograph was coupled to a VG Micromass 12F mass spectrometer with a total ion monitor detector system. For EI spectra the 'ion source' was operated at 200°C, 70 eV and 20 μA target current under a pressure of 10^{-6} torr.

III.M. Analytical techniques

III.M.I. Determination of glucose[232]

Glucose was determined by means of the glucose oxidase reagent. The following reagents were made up:

Solution A: tris (36.3 g) and sodium dihydrogen phosphate monohydrate (50 g) were dissolved in water, 400 cm^3 of glycerol was added and water to 1 litre, the pH was adjusted to 7 with sodium dihydrogen phosphate monohydrate.

Solution B: glucose oxidase (Boehringer 30 mg); horseradish peroxidase (Boehringer, 3 mg); o-dianisdine dihydrochloride (Sigma, 10 mg) dissolved in 1000 cm^3 of A.

To 1 cm^3 of test solution containing 0–100 μg glucose 2 cm^3 of solution B was added. After mixing, the solutions were incubated at 37°C for 30 minutes, 4 cm^3 of 5M hydrochloric was then added and the solutions mixed well. Absorbance readings were then determined at 525 nm against a water blank. Unknowns were diluted appropriately and their absorbance compared to a standard graph.

III.M.II. Carbohydrate content – Phenol sulphuric method[233]

Stock solution; 40% phenol in water. To a 1 cm^3 solution containing 0 – 100 μg of sugar 1 cm^3 of phenol solution was added followed, after mixing, by 5 cm^3 of concentrated sulphuric acid. After allowing the tubes to cool absorbance readings were then taken at 490 nm against a water blank. The amount in μg of unknown samples was found by a comparison of their absorbance reading with that of a standard curve.

III.M.III. Reducing sugar determination

The method is as described by Nelson.[234] The following stock solutions were made up:

Solution A: anhydrous sodium carbonate (25 g), sodium potassium tartrate (25 g), sodium bicarbonate (20 g) and anhydrous sodium sulphate(200 g) dissolved in 800 cm^3 of water and made up to 1000 cm^3.

Solution B: Copper sulphate pentahydrate (30 g) was dissolved in 200 cm^3 of water, containing 4 drops of concentrated sulphuric acid.

Solution C: ammonium molybdate tetrahydrate (25 g) was dissolved in 450 cm^3 water to which 21 cm^3 of concentrated sulphuric acid had been added. Sodium arsenate heptahydrate (3 g) was dissolved in 25 cm^3 of water and added slowly to the above solution. This solution was then made up to 500 cm^3 and warmed for 30 minutes in a water bath at 30°C.

Solution D: 1 cm^3 of solution B was added to 25 cm^3 of solution A.

To a 1 cm^3 solution containing 0 - 300 μg of reducing sugar, 1 cm^3 of solution D was added and the contents boiled for 10 minutes. After cooling for 5 minutes in cold water, 1 cm^3 of solution C was added and the contents shaken until the evolution of carbon dioxide had ceased. Water (22 cm^3) was added and the contents mixed and allowed to stand for 20 minutes. Absorbances were then read at 520 nm against a reagent blank. The concentration of reducing sugar was determined by comparison with a standard curve for glucose.

III.M.IV. Protein content

The method is that described by Lowry et al.[235]

Solution A: 2% sodium carbonate in 0.1M sodium hydroxide.

Solution B: 0.5% copper sulphate pentahydrate in 1% sodium or potassium tartrate.

Solution C: 50 cm^3 of solution A with 1 cm^3 of solution B. Discarded after one day.

Solution D: Folin-ciocalteu reagent (BDH).

To a 1 cm^3 solution containing 0 - 100 μg of protein 5 cm^3 of solution C was added. After shaking the contents, and allowing to stand for

10 minutes, 0.5 cm^3 of solution D was added and the contents mixed. After standing for 30 minutes the absorbance was read at 750 nm. The protein concentration was determined by comparison with a standard curve using bovine serum albumin (Sigma).

III.M.V. Enzymic determination of D-glucose and D-fructose.[223]

The following reagents were prepared:

Reagent A: 50 mM triethanolamine pH 7.6, containing 70 mM magnesium chloride and 1 mM mercaptoethanol.

Reagent B: 17 mM adenosine triphosphate [(ATP), BDH].

Reagent C: 11 mM nicotinamide adenine dinucleotide phosphate [(NADP), Sigma].

Reagent D: hexokinase [(Sigma, 465 units/mg, 3 mg/cm^3) or BDH, 40 units/mg].

Reagent E: glucose 6-phosphate dehydrogenase (Sigma, 240 units mg, 0.6 mg/cm^3).

Reagent F: phosphoglucose isomerase (Sigma, 435 units/mg, 0.8 mg/cm^3).

Into a 1 cm^3 silica cell, 2.7 cm^3 of reagent A, 0.1 cm^3 reagent B, 0.1 cm^3 reagent C, 0.01 cm^3 reagent D and 0.1 cm^3 of a solution containing D-glucose, D-fructose containing up to a total of 0.3 μmoles of sugar, was added. The initial absorbance was determined on a "Pye Unicam" SP1800 scanning spectrometer at 340 nm. Reagent E (0.02 cm^3) was then added to the cell and the absorbance followed until constant. The extinction change was used in the calculation of the amount of D-glucose in the sample. Reagent F (0.02 cm^3) was then added and the absorbance followed until constant. This extinction change was used in the calculation of the amount of D-fructose in the sample.

The amount of sugar in μmoles in the cell was given by:

$$\frac{A \times V}{E \times d}$$

A = absorbance change

V = volume wihin the cell

E = 6.22 $cm^2/\mu M$

d = light path in cm.

III.N. Electrophoretic techniques

III.N.I. Disc gel electrophoresis

This was carried out at low pH as described by Reisfeld,[236] or at high pH as described by Davis,[237] using a "Shandon" small scale electrophoresis apparatus.

Precibore tubes were first cleaned in chromic acid, followed by "Decon 90" (BDH), rinsed with tap water, finally rinsed with distilled water, and dried. Stock solutions were made up and stored in amber glass bottles and kept in a refrigerator except the persulphate solutions which were made up fresh. Deaerated deionised water was used in the making up of all solutions.

The following stock solutions were made up:

A Tris buffers	pH 8.5	pH 4.3
a_1 small pore	Tris (36.3 g)	1M potassium hydroxide (48.0 cm^3)
	1M hydrochloric acid (48.0 cm^3)	glacial acetic acid (17.2 cm^3)
	temed (0.46 cm^3) (N,N,N',N'-Tetramethyl-ethylene diamine BDH)	temed (4.0 cm^3)
	Water to 100 cm^3	Water to 100 cm^3
a_2 large pore	Tris (5.7 g)	1M potassium hydroxide (48.0 cm^3)
	1N phosphoric acid (25.6 cm^3)	glacial acetic acid (2.87 cm^3)
	Water to 100 cm^3	temed (0.46 cm^3)
		Water to 100 cm^3

B Acrylamide solutions

	pH 8.5	pH 4.3
b_1 small pore	acrylamide [BDH, 30.0g]	acrylamide (30.0 g)
	bis (0.8 g)	bis (0.8 g)
	[(N,N' methylene bis acrylamide), Eastman Organic]	Water to 100 cm^3
	potassium ferricyanide (0.015 g)	
	Water to 100 cm^3	
b_2 large pore	acrylamide (10.0 g)	Same as for pH 8.5
	bis (2.5 g)	
	Water to 100 cm^3	

C Initiators

	pH 8.5	pH 4.3
c_1 small pore	ammonium persulphate (0.004 g)	ammonium persulphate (0.28 g)
	Water to 100 cm^3	Water to 100 cm^3
c_2 large pore	riboflavin (0.004 g)	Same as for pH 8.5
	Water to 100 cm^3	

Reservoir Buffer	pH 8.5	pH 4.3
	glycine (28.8 g)	β-alanine (31.2 g)
	tris (6.0 g)	glacial acetic acid (8.0 cm^3)
	Water to 1000 cm^3	Water to 1000 cm^3

Stain

a) Coomassie blue (0.2 g, Lamb, London)
glacial acetic acid 7.0 cm^3
Water to 100 cm^3

destain glacial acetic acid 7.0 cm^3
Water to 100 cm^3

b) Coomassie blue 1.25 g
50% methanol 454 cm^3
glacial acetic acid 46 cm^3

destain glacial acetic acid 75 cm^3
methanol 50 cm^3
Water to 1000 cm^3

The following working solutions were made up in the following proportions by volume:

		pH 8.5	pH 4.3
Small pore gel	a_1	1	1
	b_1	2	2
	c_1	4	3
	H_2O	1	2
Large pore gel	a_2	1	1
	b_2	2	2
	c_2	1	1
	H_2O	4	4

The small pore solution was degassed on a water pump for 10 minutes. Each running tube was then filled with small pore gel to within 1.5 cm from the top of the tube, and layered carefully with 0.5 cm^3 of water. After polymerisation was complete, as shown by a clear interface, the water was removed. Degassed large pore solution was then added to within 0.5 cm off the top of the tube and again water layered on top. Polymerisation was initiated by the use of a fluorescent lamp and was complete within 30 minutes.

Samples of protein to be used were first dialysed against deionised water to remove buffer and freeze dried. Reservoir buffer was then added to make a concentration of 1 mg/cm^3, several drops of glycerol were then added. Samples (50 - 100 μl) containing 50 - 100 μg protein were layered on top of the large pore gel and

buffer layered on the sample to give an interface. The tubes were then inserted into the apparatus and the electrode buffer added to the upper (250 cm^3) and lower (300 cm^3) reservoirs. At alkaline pH a few drops of bromophenol blue (0.001%) were added to the upper reservoir as a tracking dye. Electrophoresis was carried out at constant current, 4 to 5 mA/tube, for approximately 45 minutes. To give a good indication as to whether good separation had occurred bovine serum albumin was run as a standard.

Gels were removed from the glass tubes by gently inserting a needle, connected to a syringe filled with water or buffer, between the glass wall and the gel. Whilst rotating the tube the syringe was discharged loosening the gel from the glass tube. A pipette teat was used to remove the gel from the tube. Staining was carried out overnight with coomassie blue and then destained until the bands were visible. If gels were to be incubated with substrate 0.5 cm. Slices were cut along the length of the gel, cut into smaller pieces, and placed in enzyme buffer. Incubation was then carried out with substrate. Activity was determined by analytical techniques as mentioned in specific experiments. A comparison was made between a stained gel and an incubated gel to discriminate which protein band(s) had enzymic activity.

III.N.II. Sodium Dodecyl Sulphate gel electrophoresis

The method described here is that of Shapiro[106] et al. modified by Weber and Osborn.[107]

The protein was heated in a boiling water bath for 5 minutes in 10 mM sodium phosphate buffer pH 7.0, containing 1% sodium dodecyl sulphate [(S.D.S.), BDH] and 1% 2-mercaptoethanol. The protein

concentration was approximately 1 mg/cm^3 and the ratio of SDS to protein was never less than 3:1.

Preparation of gels

The following solutions were made up as follows:

gel buffer sodium dihydrogen phosphate dihydrate (8.22 g)
disodium hydrogen phosphate dodecohydrate (51.56 g)
S.D.S. (2.0 g)
Water to 1000 cm^3.

acrylamide solution acrylamide (10.0 g)
bis (0.3 g)
Water to 100 cm^3.

The tubes were cleaned as described previously in a) gel buffer (15 cm^3) and acrylamide solution (13.5 cm^3) were mixed and deaerated by use of a water pump. Temed (0.045 cm^3) and ammonium persulphate (10 mg/cm^3, 1.5 cm^3, freshly made up) were then added with mixing. The tubes were filled to within 1 cm of the top and water carefully layered on top. Polymerisation was usually complete within 30 minutes when an interface could be seen, the water was then removed.

For each gel the following were mixed together and placed on top of the gel:

3 µl bromophenol blue (0.05%)
1 drop glycerol
5 µl mercaptoethanol
50 µl gel buffer
50 µg protein solution

Buffer was then carefully layered on top to give an interface and the tubes filled. The reservoirs were filled with buffer and the positive electrode attacked to the lower compartment. Electrophoresis was then carried out at constant current (8 mA/tube) until the tracking dye was approximately 1 cm from the end of the tube. The apparatus used was that described in a). Removal of the gels, staining, and destaining, was as described for disc gel electrophoresis.

Molecular weight determinations of proteins using S.D.S. is described later in Experiments 12 and 34.

III.N.III. Cellulose Acetate electrophoresis

Electrophoresis was performed on a "Shandon" model U77 electrophoresis apparatus using cellulose acetate (Celagram, Shandon) membrane filters. Samples were applied by use of an applicator. The reservoirs were filled with buffer and wicks attached to both electrodes. The cellulose acetate filter was then placed over the wicks and the lid placed over the apparatus. Electrophoresis was then performed at a constant current of 0.5 mA per cm of paper for 2 - 3 hours.

Chapter IV Experimental

During enzyme assays where appropriate either enzyme or substrate blanks were taken. All substrates were subjected to paper chromatography for the determination of purity.

IV.A. Experiments relating to chapter I.2.A.

IV.A.I. General techniques

All operations were carried out at 4°C unless otherwise stated. The pig liver was obtained from the local abattoir. The livers, kept on ice, were used within 2 hours after the animals had been killed. In extracting the tissue 100 mM acetate buffer, pH 5.0, containing 1 mM EDTA was used. During separation on columns containing Sephadex or Biogel, 25 mM sodium chloride containing 1 mM EDTA, the pH of the solution was brought to 6.7 with 2 M sodium hydroxide, (solution A)was used to elute the columns. During gel permeation chromatography fractions were collected using a LKB fraction collector and the absorbance measured at 280 nm automatically, and recorded using a LKB Autoanalyser.

IV.A.II. Enzyme assays and units of activity

The enzyme activity was assayed by measuring the rate of formation of glucose from maltose or dextran T_{40}. One cm^3 of the incubation contained 1.8 mg of maltose or 1 mg of dextran T_{40} together with 100 mM acetate buffer, pH 4.6. The maltase assay was carried out by incubation for 15 minutes and the dextranase assay for 1 hour. The reaction was stopped by immersing the enzyme incubation in boiling water for 1 minute. Glucose and soluble protein was determined as described in III.M.I. and III.M.IV. The incubation of samples from the fractions obtained for the purification of the

ammonium sulphate saturator on Sephadex G-100, with substrate, were terminated by adding sodium hydroxide and zinc sulphate (final concentration 60 mM, and 30 mM, respectively. Protein was then removed by centrifugation before liberated glucose was determined. After each isolation step the number of enzyme units and the specific activity of the preparation was determined.

A unit of maltase activity was defined as the amount of enzyme that catalyses the hydrolysis of 1 nmole of maltose per minute. A unit of dextranase activity was defined as the amount which catalyses the liberation of 1 nmole of glucose per minute. Specific activity is defined as the number of enzyme units per mg of protein.

Experiment 1 Homogenation of the pig liver

For the three extractions described in chapter I.2.A.(b) the following weights of tissue were taken:

Batch 1 2.5 kg
" 2 5.0 kg
" 3 3.6 kg.

The livers, freed from fat and connective tissue, were cut into small pieces and placed in a Waring blender. An equal volume of acetate buffer (IV.A.I.) was added and the tissue homogenised at maximum speed for 3 minutes. One more volume of acetate buffer was added to the homogenate and it was left overnight for extraction.

Experiment 2 Autolysis of the glucosidase extract

The homogenate was centrifuged using a JA10 rotor at 4000 g for 30 minutes. The solid collected was discarded. Autolysis was

then carried out at 37°C overnight, the extract being layered with toluene. The glucose liberated through autolysis was removed later, through precipitation of protein and finally through diafiltration, as described in the next experiment.

Experiment 3 Ammonium sulphate precipitation

The extract from Experiment 2 was centrifuged at 4000 g for 30 minutes and the precipitate discarded. Ammonium sulphate (170 g/l) was added to the extract slowly with stirring to give a 30% saturated solution. The enzyme preparation was left overnight. Centrifugation was then carried out as described previously. The precipitate was discarded. More ammonium sulphate (180 g/l) was then added with stirring to give a 60% saturated solution. The final percentage saturation was corrected for temperature.[238] After standing for 12 hours the enzyme solution was again centrifuged as described previously. The precipitate obtained was diafiltrated using a Millipore PSAC membrane, with the sodium chloride - EDTA solution described in IV.A.I. The effluent obtained from the ultrafiltration cell was always, in this experiment and in subsequent experiments, checked for maltase activity and absorbance at 280 nm to make sure that no leakage of the membrane had occurred. Finally the extract was concentrated before placing on a Sephadex G-100 column.

The degree of purification of α-D-glucosidase from all three batches after Experiments 1, 2 and 3 is given in Tables 12, 13 and 14.

Experiment 4 Purification of α-D-glucosidase upon Sephadex G-100

Sephadex G-100 was prepared and packed into a column (2.6 x 100 cm) as described in III.F. The column had a total volume (V_t) of 490 cm^3 and a void volume (V_o) of 130 cm^3.

The material from Experiment 3 (batch 1) was concentrated to 210 cm^3. So as not to overload the column a 100 cm^3 fraction was applied, the remainder was applied later on. Flow was kept constant at 30 cm^3/hr by means of a peristaltic pump, 10 cm^3 fractions were collected. Elution was performed with solution A(IV.A.I.). The maltase and dextranase activity of each fraction was determined (IV.A.II.). The elution profile is shown in Fig. 7. Fractions 49-187 were pooled and concentrated by ultrafiltration using a PSAC membrane filter (Millipore).

Experiment 5 Removal of low molecular weight impurities by Biogel P-60

Biogel P-60 (Bio-rad) was prepared and packed into a column (2.6 x 40 cm) as described in III.F. The column had a total volume (V_t) of 180 cm^3 and a void volume (V_o) of 53 cm^3.

The concentrated extract from Experiment 4 (35 cm^3) was applied to the column. Elution was performed with solution A (IV.A.I.) at a constant flow rate of 5 cm^3/hr. and 5 cm^3 fractions were collected. The dextranase and maltase activity of each fraction was determined (IV.A.II). The elution profile is shown in Fig. 8. Fractions 10 - 24 were pooled and concentrated by ultrafiltration.

Experiment 6 Final purification of the enzyme upon Sephadex G-100

The column used was the same as described in Experiment 4.

The enzyme solution from Experiment 5 (16 cm^3, batch 1) was applied to the column and the elution procedure described in Experiment 4 carried out. The maltase and dextranase activity of each fraction was determined (IV.A.II.). The elution profile is shown in Fig.9. Fractions 54 - 140 were pooled and concentrated and diafiltrated against 50 mM acetate buffer pH 4.6. The degree of purification of acid α-D-glucosidase after all of the stages is shown in Table 15.

Experiment 7 Elution of the α-D-glucosidase from the Sephadex column by methyl α-D-glucopyranoside

The Sephadex G-100 (Pharmacia) was prepared and packed into a column (5.0 x 100 cm) as described in III.F. The column had a total volume (V_t) of 1640 cm^3 and a void volume (V_o) of 600 cm^3. The enzyme solution (batch 2), previous to being placed on the column, was preliminary purified as described in Experiments 1, 2 and 3. The degree of purification of the enzyme after these stages is shown in Tables 12, 13 and 14. The enzyme was concentrated by ultrafiltration (PM-10, Amicon) to 700 cm^3 of which 450 cm^3 was placed on the column, the remainder was applied later on. Fractions (20 cm^3) were collected at a flow rate of 40 cm^3/hr. Elution was performed with solution A (IV.A.I.). After fraction 90, 1% w/v methyl α-D-glucopyranoside in solution A was applied. The maltase activity of each fraction was determined (IV.A.II.). The elution profile is shown in Fig. 10. Fractions 140 - 180 were pooled and concentrated by ultrafiltration (PM-10, Amicon). Fractions 140 - 160 were pooled and concentrated after the rest of the enzyme solution was chromatographed. Both solutions were then pooled and diafiltrated against solution A (IV.A.I.)

Experiment 8 Disc gel electrophoresis of α-D-glucosidase after purification on Sephadex G-100 only

The preparation and running of the gels is described in III.N.I. Enzyme (110 μg) was applied to each polyacrylamide gel. Electrophoresis was carried out at pH 4.3 and 8.5.

Staining was carried out with stain a). The results are described in Chapter I.2.A.(f).

Experiment 9 Further purification of α-D-glucosidase eluted from the Sephadex G-100 column with methyl α-D-glucopyranoside.

Biogel P-60 (Bio-rad) was prepared and packed into a column (2.6 x 100 cm) as described in III.F. The column had a total volume (V_t) of 460 cm^3 and a void volume (V_o) of 125 cm^3.

Enzyme solution (6.7 cm^3, from Experiment 7) was applied and elution carried out with solution A (IV.A.I.) at a flow rate of 5 cm^3/hr., 5 cm^3 fractions were collected. The maltase activity of each fraction was determined (IV.A.II.). The elution profile is shown in Fig. 11. Fractions 26-58 were pooled and concentrated (PM-10, Amicon).

Disc gel electrophoresis of a sample of the enzyme after purification on Biogel P-60 was carried out as described in III.N.I. Enzyme (110 μg) was applied to each gel and electrophoresis carried out at 4 mA/tube for 45 minutes. Staining was carried out with stain a). The results are discussed in chapter I.2.A.(f).

Sephadex G-100 chromatography was carried out using the column described in Experiment 7. Enzyme solution (47 cm^3) was applied to the column and elution carried out with solution A (IV.A.I.), at a flow rate of 40 cm^3/hr, 20 cm^3 fractions were collected. After fraction

69, 1% w/v methyl α-D-glucopyranoside in solution A was applied. The maltase activity of each fraction was determined (IV.A.II.). The elution profile is shown in Fig. 12. Fractions 130 - 156 were pooled and concentrated by ultrafiltration (PM-10, Amicon). Table 16 gives the degree of purification of α-D-glucosidase after all stages.

Experiment 10 · Separation and isolation of an isoenzyme of α-D-glucosidase

Preliminary purification of batch 3 was carried out as described in Experiments 1, 2 and 3. Purification figures are given in Tables 12, 13 and 14.

a) Sephadex G-100 chromatography

The column described in Experiment 7 was used.

Enzyme solution (400 cm^3) was applied to the column and elution carried out with solution A (IV.A.I.). Fractions (20 cm^3) were collected at a flow rate of 40 cm^3/hr. After fraction 78, 1% w/v methyl α-D-glucopyranoside in soltuion A was applied. The maltase activity of each fraction was determined (IV.A.II.). The elution profile is shown in Fig. 13. Fractions 135 - 160 were pooled and concentrated by ultrafiltration (PM-10, Amicon).

b) Disc-gel electrophoresis of α-D-glucosidase from Experiment 10a

The gels were prepared as described in III.N.I. Enzyme (100 μg) was applied to each polyacrylamide gel and electrophoresis carried out at 4 mA/tube for 50 minutes at pH 4.3 and 8.5. Staining was carried out with stain a. Bromophenol blue was not added to the reservoir if the gels were to be incubated with substrate. After electrophoresis the length of the gel was measured and the gel cut into 0.5 cm pieces. After being finely cut into smaller pieces,

p-nitrophenyl α-D-glucopyranoside (1 cm^3, 2 mg/cm^3 in 50 mM acetate buffer pH 4.6), was added and the solutions incubated at 37°C for 2 hours. The liberated glucose was determined (III.M.I.). The results are shown diagrammatically in Fig. 14.

c) Biogel P-60 chromatography

From a total of 60 cm^3 of enzyme solution, concentrated after Sephadex chromatography (Experiment 10a), 49 cm^3 was applied to the Biogel. The column described in Experiment 9 was used. The flow rate was maintained at 5 cm^3/hr and 5 cm^3 fractions were collected. Elution was carried out with solution A (IV.A.I.). The maltase activity of each fraction was determined (IV.A.II.). The results are shown graphically in Fig. 15. Fractions 26 to 60 were pooled and concentrated by ultrafiltration (PM-10, Amicon).

d) Sephadex G-100 chromatography

A column (2.6 x 95 cm) of Sephadex G-100 was prepared which had a void volume (V_o) of 160 cm^3 and a total volume (V_t) of 500 cm^3. A 29 cm^3 sample was applied to the column and elution performed with solution A (IV.A.I.). Fractions (5.5 cm^3) were collected at a flow rate of 11 cm^3/hr. After fraction 70, 1% (w/v) methyl α-D-glucopyranoside in solution A was used as eluant. The maltase activity of each fraction was determined (IV.A.II.). The results are shown graphically in Fig. 16. Fractions 32 to 60 and 120 to 160 were pooled separately and concentrated. The final purification figures for α-D-glucosidase from batch 3 are given in Table 17.

Experiment 11 Homogeneity of the purified acid α-D-glucosidase preparation

Disc gel electrophoresis was carried out on the finally purified enzyme at two pH values as described in III.N.I. The following amounts of protein were applied to the gels:

Batch 1 (Experiment 6) 72 μg per gel

Batch 2 (Experiment 9) 120 μg per gel

Batch 3 (Experiment 10) 97 μg per gel

Isoenzyme II 30 μg per gel

Staining was carried out with stain a). The results are shown in Fig. 17 a,b.

IV.B. Experiments relating to Chapter I.2.B.

Experiment 12 Subunit structure and molecular weight of the purified enzyme

S.D.S. electrophoresis was carried out as described in III.N.II. Extracted enzyme samples were dialysed prior to electrophoresis, to remove buffer. Approximately 30 - 50 μg of protein was applied to each gel.

The following protein standards were run at the same time as the enzyme samples:

1) Trypsin inhibitor from soybean (Sigma; MW 21,500)

2) Bovine serum albumin (Sigma; MW 68,000)

3) RNA-polymerase from E. Coli (Sigma; MW 165,000, 155,000)

After staining the mobilities of each standard protein relative to bromophenol blue were found from the following expression:

$$\text{mobility}_{BPB} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

A plot of mobility versus log molecular weight gave a straight line from which the molecular weights of the unknown bands could be found (Table 20). These results are shown graphically in Fig. 18.A photograph of a stained gel is shown in Fig. 19a.

Experiment 13 Incubation of acid α-D-glucosidase with Cibachron F3GA-amylase[109]

Solutions of α-amylase (B. subtilis, Calbiochem, 120 units in 50 mM phosphate buffer pH 6 (1.2 cm^3), cibachron blue amylose (10 mg in 50 mM phosphate buffer pH 6 (10.8 cm^3), were kept at 37°C for 5 minutes and then mixed. Fractions (1 cm^3) were transferred at regular and measured time intervals into a solution of tris (0.5 M, 1.5 cm^3). After removing the insoluble blue-amylose by centrifugation the absorbance (625 nm) of the supernatant was determined. Glucose was determined (III.M.I.) on the same aliquots.

A similar incubation was prepared containing acid α-D-glucosidase (Experiment 9, 1700 maltase units), cibachron blue amylose (100 mg) in 100 mM acetate buffer pH 4.6 (12 cm^3). Incubation was carried out at 37°C and the procedure followed as described before.

The results are shown graphically in Figs. 20 and 21.

Experiment 14 Determination of the configuration of the product released during glucosidase action by ^1H nmr spectroscopy

a) At 60 MHz

Phenyl α-D-glucopyranoside (Koch-Light, 40 mg), and yeast α-glucosidase (4.1 u/mg, 1.6 mg, Sigma) were dissolved separately

in deuterium oxide at room temperature and the solutions mixed (final volume, 0.5 cm^3) in a 20 cm nmr tube. ^1H nmr spectra were recorded at regular intervals using a Varian EM 360 spectrometer operating at 60 MHz. Chemical shifts were determined relative to external tetramethylsilane. The results are shown in Fig. 23. A spectrum of either equilibrated D-glucose (20 mg) or phenyl α-D-glucopyranoside (40 mg), Fig. 22, in deuterium oxide (0.5 cm^3) was obtained as described previously.

For the experiment with acid α-D-glucosidase, a buffer was prepared by dissolving glacial acetic acid (7.74 mg) and sodium acetate (10.09 mg) in deuterium oxide (5 cm^3) to give a 25 mM (final concentration) solution. The incubation contained the following:

a) phenyl α-D-glucopyranoside (40 mg)

b) 25 mM acetate buffer (0.8 cm^3, equivalent to pH 4.6).

c) freeze-dried α-D-glucosidase (2.92×10^4 maltase units, Experiment 9).

Spectra were recorded as described previously. In order to discount the appearance of D-glucose through non-enzymic hydrolysis, a blank was prepared containing phenyl α-D-glucopyranoside (40 mg) in 25 mM acetate buffer (0.5 cm^3, pD = 5) dissolved in deuterium oxide. Spectra were recorded over the same length of time as the enzyme incubations.

b) At 220 MHz

Spectra were recorded on a Varian HR 220 spectrometer at the Physico-chemical measurements Unit, Harwell. Chemical shifts were determined relative to external tetramethylsilane. The temperature probe reading was 31°C.

A solution was prepared containing freeze-dried acid α-D-glucosidase (Experiment 9, 2.92 x 10^4 maltase units) and phenyl α-D-glucopyranoside (40 mg) in 25 mM acetate buffer, pD = 5, in D_2O (0.5 cm^3) spectra were taken over a period of 4 hours. Integrals of the peaks at 5.34, 4.74 and 5.7 ppm from tms were taken directly after each spectrum was recorded. The results are shown in Fig. 26.

The mutarotation of α-D-glucose was observed over the same time period as that used for the enzyme reaction. The sample contained α-D-glucose (40 mg, Sigma) and 0.5 cm^3 of D_2O containing 25 mM acetate buffer pD 5. Spectra and integrals were taken at regular intervals. The results are shown graphically in Fig. 30.

A spectrum of D-glucose (40 mg) and phenyl α-D-glucopyranoside (40 mg) in 0.5 cm^3 D_2O containing 25 mM acetate buffer pD5 was also recorded.

The results are shown in Figs. 24 and 25.

Experiment 15 Effect of pH on the rate of enzyme hydrolysis

A series of acetate and phosphate buffer solutions of constant concentration (50 mM) but varying pH (4.0 - 8.0) and containing maltose (2 mg/cm^3) or dextran T_{40} (Pharmacia, 10 mg/cm^3) were prepared. For the determination of maltase activity maltose solutions (0.9 cm^3) were mixed with enzyme solution (0.1 cm^3, containing 14.6 maltase units, Experiment 9) and incubated at 37°C for 15 minutes. For the determination of dextranase activity dextran solutions (0.1 cm^3) were mixed with enzyme solution (0.9 cm^3, containing 657 maltase units) and incubated at 37°C for 1½ hours. The reactions were stopped by placing the tubes in a boiling water bath for 1 minute. Glucose was then determined (III.M.I.). The results are shown graphically in Fig. 31.

Experiment 16 Thermal stability of the enzyme

a) Maltase activity

Tubes containing enzyme solution (0.1 cm^3, containing 14.6 maltase units each, Experiment 9) were incubated at different temperatures ranging from 25 to 75°C for 15 minutes. After being rapidly cooled in an ice bath 0.9 cm^3 of maltose (2 mg/cm^3 in 50 mM acetate buffer pH 4.6) was added to the tubes which were then incubated for 15 minutes at 37°C. The reaction was terminated by heating the tubes in a boiling water bath for 1 minute. Glucose was determined in each of the solutions as described in III.M.I. The results are shown graphically in Fig. 32.

b) Dextranase activity

The same procedure was followed as in a) except that 657 maltase units of enzyme in 0.9 cm^3 were taken and incubated for 1 hour. Dextran T_{40} (0.1 cm^3, 10 mg/cm^3) was added and the tubes incubated for 1½ hours at 37°C before measurement of the glucose produced. The results are shown graphically in Fig. 32.

Experiment 17 pH stability of the enzyme

A series of enzyme solutions (0.1 cm^3, containing 14.6 maltase units, Experiment 9) were made up in 50 mM acetate or phosphate buffers of different pH (4.0 - 8.0). The solutions were preincubated for 1 hour at 37°C. Maltose (0.9 cm^3, 2 mg/cm^3) was then added and the enzyme incubated at 37°C for 15 minutes. The reaction was stopped by heating at 100°C and the glucose determined as described in III.M.I. The results are shown graphically in Fig. 33.

Experiment 18 Preparation of isomalto-oligosaccharides

The method used for the acid hydrolysis of dextran T_{40} was that of Sidebotham.[239] Dextran T_{40} (35 g) was treated with 700 cm^3 of 1$\underline{N}$ sulphuric acid for $1\frac{1}{4}$ hours at 100°C. The hydrolysate was neutralised with solid barium carbonate and the insoluble salt removed by centrifugation. The supernatant was filtered (Whatman No. 42 paper) and deionised by adding successively Amberlite IR 120(H^+) and Amberlite 400 (OH^-). Rotary evaporation was then carried out at 40°C. The hydrolysate was subjected to paper chromatography in Solvent 2. The chromatogram indicated there to be oligosaccharides present.

A charcoal celite column (70 x 7.5 cm) was prepared as described in III.K. The syrup (33 g) was taken up in a minimum quantity of water and placed on the column. The column was eluted progressively with increasing percentages of aqueous ethanol and fractions (35 cm^3) were collected and monitored chromatographically using solvent 2. Details of the separation are recorded in Table 47.

All syrups were refractionated by paper chromatography in solvent 2 as described in III.B.IV. The separated sugars were filtered and freeze-dried.

Table 48 shows the chromatographic properties of each oligosaccharide.

Table 47 Separation of oligosaccharides from partially acid hydrolysed dextran T_{40}

Eluant	Fractions (35 cm^3)	Component	Chromatographing as: in solvent 2
Distilled water	99 - 139	1	Glucose
6% aqueous ethanol	315 - 364	2	Isomaltose
16% aqueous ethanol	384 - 504	3	Isomaltotriose
↓	504 - 514	3	Isomaltotriose
		4	Isomaltotetrose
	514 - 524	4	Isomaltotetrose
		5	Isomaltopentose
↓	524 - 579	5	Isomaltopentose
25% aqueous ethanol		6	Isomaltohexose
		7	Isomaltoheptose

Table 48 Chromatographic properties of a series of oligosaccharides isolated from an acid hydrolysate of dextran T_{40}

Component	Yield (g)	Chromatography solvent (2) (Rg)	Electrophoresis borate buffer pH 10 (Mg)
Glucose	-	1.0	1.0
Isomaltose	1.3	0.53	0.69
Isomaltotriose	0.5	0.27	0.54
Isomaltotetrose	0.45	0.16	0.48
Isomaltopentose	0.37	0.08	0.43
Isomaltohexose	0.23	0.04	-
Isomaltoheptose	0.34	0.02	-

Experiment 19 Incubation of acid α-D-glucosidase with α-glucosides

Solutions were prepared containing 2 mg/cm^3 of glucoside (methyl α-D-glucopyranoside, Koch-Light) (p-nitrophenyl α-D-glucopyranoside, Koch-Light) or (phenyl α-D-glucopyranoside, Cambrian Chemicals) in 50 mM acetate buffer pH 4.6 (10.8 cm^3) and enzyme solution (1.2 cm^3, 1752 maltase units, Experiment 9) in 50 mM acetate buffer pH 4.6.

Both solutions were preincubated at 37°C for 5 minutes before mixing. At regular intervals up to 30 minutes 1 cm^3 samples were withdrawn and the enzyme reaction stopped by immersing the solution in a boiling water bath. Glucose was determined as described in III.M.I. Phenol released during enzymic hydrolysis was found to interfere with the glucose oxidase reagent. To account for this a standard curve was obtained using solutions containing calculated amounts of phenol. The results are shown graphically in Fig. 34.

Experiment 20 Determination of Michaelis constants (K_m) and Maximum velocity values ($V_{max.}$) for acid α-D-glucosidase

Maltose substrate

A series of maltose solutions of concentration 4 to 200 mM were prepared in 50 mM acetate buffer pH 4.6. Enzyme (0.1 cm^3, 146 maltase units, Experiment 9) and substrate solutions were preincubated at 37°C for 5 minutes, mixed and incubated for 15 minutes. The reaction was stopped by heating at 100°C and the glucose liberated determined as described in III.M.I. The results are shown graphically in Fig. 38.

$V_{max.}$ for methyl and phenyl α-D-glucopyranoside isomaltose and kojibiose substrates

Methyl α-D-glucopyranoside was obtained from Koch-Light and phenyl α-D-glucopyranoside from Cambrian Chemicals, the disaccharides were from the departmental collection.

The substrate (8 to 150 mM) in 50 mM acetate buffer pH 4.6 (0.9 cm^3) was incubated at 37°C with enzyme solution (0.1 cm^3, 730 maltase units, Experiment 9) for 15 minutes. The results are shown graphically in Figs. 39, 40, 37 and 35.

$V_{max.}$ for p-nitrophenyl α-D-glucopyranoside substrate

The substrate (Koch-Light, 4 - 20 mM in pH 4.6 acetate buffer, 0.9 cm^3) was incubated at 37°C with enzyme solution (0.1 cm^3, 730 maltase units, Experiment 9) for 15 minutes. The results are shown graphically in Fig. 36.

Experiment 21 Dextranase activity of acid α-D-glucosidase

Dependence on substrate concentration

A range of dextran T_{40} solutions (1 - 200 mg/cm^3 in 50 mM acetate buffer 4.6) were prepared. Enzyme solution (0.9 cm^3, 657 maltase units, Experiment 9) and substrate solution (0.1 cm^3) were preincubated for 5 minutes and then mixed. After incubation for 1 hour at 37°C the reaction was stopped and the liberated glucose determined (III.M.I.). The results are shown graphically in Fig. 42.

Dependence on enzyme concentration

A series of enzyme solutions containing from 6.5 to 6575 maltase units in 0.9 cm^3 of pH 4.6 acetate buffer were prepared and kept at 37°C for 5 minutes. Dextran T_{40} (10 mg/cm^3) in 50 mM acetate buffer pH 4.6 was prepared and also preincubated at 37°C for 5 minutes. Enzyme (0.9 cm^3) and dextran (0.1 cm^3) solutions were mixed and incubated at 37°C for 1 hour. The reaction was stopped and the glucose liberated determined as described in III.M.I. The results are shown graphically in Fig. 43.

Dependence of activity on time

To 10.8 cm^3 of dextran T_{40} solution (10 mg/cm^3) preincubated at 37°C, 0.9 cm^3 of enzyme solution containing 657 maltase units was added, and incubation carried out at 37°C. At regular intervals 1 cm^3 fractions were withdrawn, the reaction stopped, and the liberated glucose determined as described previously. The results are shown graphically in Fig. 44.

Experiment 22 Degradation of polysaccharides by acid α-D-glucosidase

Leuconostoc mesenteroides B-512 dextran and *L. mesenteroides* B-1299S dextran were obtained from the departmental collection. Amylopectin and rabbit liver glycogen were obtained from BDH and amylose V from Tunnel Refineries, London. Both dextran T_{40} and T_{10} were obtained from Pharmacia.

Solutions (6 cm^3) containing substrate 2 mg/cm^3 in 50 mM acetate buffer pH 4.6, 4.8 cm^3) and enzyme (10.1 x 10^3 maltase units, Experiment 10) were incubated at 37°C. Samples were withdrawn, heated at 100°C for 1 minute and analysed for glucose (III.M.I.). The results are shown graphically in Figs. 45 and 46.

Experiment 23 Methylation analysis of amylopectin degraded by acid α-D-glucosidase

A solution (18 cm^3) was prepared containing amylopectin (BDH, 12.5 mg/cm^3 in 50 mM acetate buffer pH 4.6, 14.4 cm^3) and enzyme (30.5 x 10^3 maltase units, Experiment 10, in buffer, 3.6 cm^3) and incubated at 37°C. Aliquots varying in volume were removed over an 18 hour period and heated at 100°C for 1 minute. Fractions (0.1 cm^3) were used to determine the amount of glucose liberated. The remainder was dialysed exhaustively and then freeze-dried.

Methylation

The following reagents and solvents were required:

a) dimethyl sulphoxide (dmso) was distilled under reduced pressure from calcium hydride and stored over 4Å molecular sieve.

b) iodomethane (methyl iodide) was distilled at atmospheric pressure and stored over dry silver oxide in the dark at 4°C.

c) dimethylsulphinyl carbanion was prepared by the method of Hakamori[240] and was donated by Dr. R. Foyle.

The freeze-dried degraded amylopectin (~5 mg) was placed into a 1 oz McCartney bottle, containing a small teflon-covered magnetic stirrer. Dimethylsulphoxide (2 cm^3) was added, and the bottle flushed with nitrogen prior to the cap being screwed on. The bottle was warmed to 40°C to ensure dissolution of the degraded polysaccharide. Carbanion solution (1 cm^3) was added from a syringe and the mixture stirred overnight. The bottle was then cooled in ice-water and methyl iodide (0.1 cm^3), added by syringe. The mixture was stirred for 10 hours. Further carbanion

(1 cm^3) was added and the mixture stirred overnight. After cooling in ice-water an excess of methyl iodide (0.5 cm^3) was added, and the mixture stirred for a further 10 hours. The resulting solution was poured into water (25 cm^3) and dialysed against deionised water until only the solid methylated polysaccharide remained. The mixture was then concentrated to dryness by rotary evaporation.

Hydrolysis

The sample was dissolved in 90% formic acid and solid carbon dioxide added to give an inert atmosphere. The flask was sealed and heated for 7 hours at 100°C. After this time water (15 cm^3) was added to the hydrolysate and the flask heated for a further two hours at 100°C. The solution was concentrated to dryness and the formic acid removed by codistillation with methanol.

Reduction

To the solid obtained from the previous step, water was added (2 cm^3), followed by sodium borohydride (10 mg). The mixture was maintained at room temperature overnight and neutralised with IR.120(H^+) resin. The resin was removed by filtration and the filtrate concentrated to dryness. Finally the solid was thrice codistilled with methanol and concentrated to dryness.

Acetylation

The partially methylated alditols obtained previously were treated with dry pyridine (1 cm^3), and acetic anhydride (1 cm^3) and heated for 10 minutes at 100°C under anhydrous conditions. After dilution with water the sample was concentrated to dryness. The sample was dissolved in dry chloroform and analysed by G.L.C. or G.C.-M.S. using column a).

Samples (5 mg) each of undegraded amylopectin or dextran T_{10} were also subjected to the same methylation analysis procedure. The results are presented and discussed in Appendix 1.

IV.C. Experiments relating to chapter I.2.C.

Experiment 24 Cellulose acetate electrophoresis of the isoenzymes of acid α-D-glucosidase

Electrophoresis was carried out as described in "General Methods" (III.IV.III.). Because of the lower activity of isoenzyme II the enzyme was concentrated before application to the cellulose acetate. The reservoir buffer was 10 mM phosphate buffer pH 7.5. Enzyme activity was detected by placing a filter paper saturated with methylumbelliferyl α-D-glucopyranoside (Koch-Light, 0.2 mg/cm^3) in 50 mM acetate buffer pH 4.6 over the electrophoretogram. The plate was incubated at 37°C for 15 minutes. Activity was detected by placing the cellulose acetate paper under an ultra-violet lamp. The results are shown diagrammatically in Fig. 47.

Experiment 25 Re-chromatography of Isoenzyme I on Sephadex G-100

The packing material was prepared and packed into a column (0.9 x 30 cm) as described in "General Methods" (III.F.). The column had a total volume (V_t) of 17 cm^3 and a void volume (V_o) of 4 cm^3. A 1 cm^3 sample of acid α-D-glucosidase (isoenzyme I) was applied to the column and eluted with 50 mM acetate buffer pH 4.6. The flow rate was 8 cm^3/hr and 0.5 cm^3 fractions were collected. The elution procedure was the same as that described in Experiment 7. The absorbance of the fractions was determined at 280 nm; the elution profile is shown in Fig. 48.

Experiment 26 Heat deactivation studies on isoenzyme I

To 10.8 cm^3 of maltose solution (2 mg/cm^3) in 50 mM acetate buffer pH 4.6) preincubated at 65°C for 10 minutes, 1.2 cm^3 of enzyme solution (Experiment 10, 254 maltase units) was added. At regular intervals over a 60 minute period 1 cm^3 fractions were withdrawn, heated for 1 minute at 100°C and the liberated glucose determined (III.M.I.). The results are shown graphically in Fig. 49.

Experiment 27 Subunit structure and molecular weight of isoenzyme II

The procedure was the same as that described in Experiment 12. Approximately 30 µg of protein was applied to the polyacrylamide gel. A photograph of the stained gel is shown in Fig. 19b.

Experiment 28 Effect of pH on the rate of enzyme hydrolysis

The procedure was the same as that described in Experiment 15. However, the enzyme solution (0.1 cm^3) contained 7.07 maltase units (Experiment 10). The results are shown graphically in Fig. 50.

Experiment 29 Thermal stability of isoenzyme II

The method was the same as that described in Experiment 16. The enzyme solution contained 7.07 maltase units. The results are shown graphically in Fig. 51.

Experiment 30 Incubation of isoenzyme II with glycogen and dextran T_{40}

The procedure was the same as that described in Experiment 22. However, the incubation contained 3.0 cm^3 substrate solution (2 mg/cm^3), and 3.0 cm^3 enzyme solution (Experiment 10, 528 maltase units). The results are shown graphically in Figs. 52 and 53.

IV.D. Experiments relating to chapter II.?.A.

a) General techniques

All operations were carried out at 4°C unless otherwise stated. Streptococcus salivarius strain '51' was obtained from the Departmental collection. In extracting the enzyme 100 mM phosphate buffer pH 6.6 was used. During separations on gel filtration media the same buffer was used to elute the columns. All other chromatographic columns were eluted with the buffer stated in the appropriate experiment. All chromatographic separations were monitored at 280 nm using a LKB autoanalyser and fractions collected using a LKB fraction collector. Reducing sugar and protein was determined as described in III.M.

b) Enzyme assay and units of activity

The enzyme activity was assayed by measuring the rate of formation of reducing sugar from S. salivarius strain '51' levan. Each incubation (1 cm^3), contained 1.8 mg of levan in 100 mM phosphate buffer pH 6.6. Incubation was carried out at 37°C for 30 minutes, and the reaction was stopped by immersing the sample in a boiling waterbath for 1 minute.

After each isolation step the levanase activity and specific activity of the preparation was determined.

A unit of levanase activity was defined as the amount of enzyme which liberated 1 μmole of fructose per minute under the conditions stated. Specific activity was defined as the number of levanase units per mg of protein.

Experiment 31 Preparation of Streptococcus salivarius strain '51' levanase

I. Subculturing of Streptococcal strains

A growth medium was prepared which contained brain heart infusion concentrate (Oxoid, 5 tablets), thioglycollate medium (Difco, 2.4 g), D-glucose (0.5 g) made up to 100 cm^3 with distilled water. Streptococcus salivarius strain '51' was subcultured every month. Calcium carbonate (~0.4 g) and growth medium (10 cm^3) were placed into a 1 oz. McCartney bottle and the tops screwed on. Sterilisation was then carried out at 15 p.s.i. for 15 minutes. After cooling at room temperature a 1 cm^3 suspension of cells was introduced into the bottle by means of a sterile disposable syringe. The culture was maintained at 37°C for 24 hours and then stored at 4°C ready for use.

II. Preparation of S. salivarius levan[156]

A medium was prepared which contained typtone (Difco, 1% w/v), sucrose (8%, w/v), dipotassium hydrogen phosphate (0.2%, w/v), made up with distilled water.

IIa) Determination of optimum culture time

To each of six McCartney bottles calcium carbonate (~0.4 g), and growth medium (15 cm^3) was added, the tops screwed on, and sterilisation carried out at 15 p.s.i. for 15 minutes. Culture medium from I was diluted 1 in 10 with sterilised tryphone-sucrose medium, and 0.6 cm^3 of this diluted sample added to the cooled bottles by means of a sterilised disposable syringe. The bottles were then incubated at 37°C between 6 and 72 hours.

The culture fluid was centrifuged in a JA 10 rotor at 5000 g for 20 minutes. Ethanol was then added to the supernatant to a concentration of 70% v/v. The precipitated polysaccharide was centrifuged at 705 g for 20 minutes and re-dissolved in water. Ethanol was again added to a concentration of 70% v/v and the procedure described carried out twice more before the polysaccharide was dialysed against water for 48 hours and finally freeze-dried. The weight of polysaccharide isolated from each bottle was determined. The results are shown graphically in Fig. 55.

IIb) Large scale preparation of polysaccharide

Usually 5 - 8 litres of medium was prepared and 2 - $2\frac{1}{2}$ litres placed in 5 litre conical flasks together with 40 - 60 g of calcium carbonate. Cotton wool bungs surrounding a Pasteur pipette were used to prevent contamination. Sterilisation was carried out as described in IIa. After cooling, 7 - 10 cm^3 of a freshly prepared suspension of bacterial cells was delivered via the Pasteur pipette, into the flask, using a sterile disposable syrings. The mouth of the flask and the cotton wool bung were flamed off being replacement. Incubation was then carried out at 37°C for 40 hours. The synthesised polysaccharide was isolated as described in IIa. The initial centrifugation was carried out at 2740 g and from then on at 980 g.

III. Preparation of S. salivarius levanase

A culture medium was prepared which contained tryptone (Difco, 1% w/v) yeast extract (Oxoid, 0.5%w/v), dipotassium hydrogen phosphate (0.3%, w/v), levan from IIb (1.0%, w/v) and D-glucose (0.1%, w/v).

Between 4 and 7 litres of medium was usually prepared at one time. Calcium carbonate (10g/per litre of medium) was added and the sterilisation procedure described in IIb carried out. A freshly cultured S. salivarius strain '51' suspension (5 cm^3/per litre medium above) was then added and contamination precautions carried out as described in IIb. The culture was maintained for 48 hours at 37°C. Centrifugation was then carried out at 2740 g for 35 minutes at 4°C. Ammonium sulphate was added to the supernatant slowly with stirring to achieve a final concentration of 40% saturation. After being left for 12 hours at 4°C centrifugation was carried out at 2740 g for 35 minutes. The precipitate was discarded. More ammonium sulphate was then added to a final concentration of 80% saturation and the levanase preparation left overnight. The precipitated protein was collected by centrifugation at 2740 g for 35 minutes at 4°C. The supernatant was discarded and the protein dissolved in 100 mM phosphate buffer pH 6.6 and diafiltrated using a PM-10 (Amicon) membrane.

Experiment 32 Sephadex G-200 chromatography

Sephadex G-200 was prepared and packed into a column (2.6 x 100 cm) as described in section III.F. The column had a total volume (V_t) of 520 cm^3 and a void volume (V_o) of 186 cm^3.

Enzyme (28 cm^3) from Experiment 31 III was applied to the column and elution performed with phosphate buffer (IV.D.a). Fractions (3 cm^3) were collected at a flow rate of 6 cm^3/hr. The levanase activity of each fraction was determined (IV.D.b). The elution profile is shown in Fig. 56. Those fractions containing levanase activity were pooled and concentrated by ultrafiltration. Table 34 shows the purification figures.

Experiment 33 Disc gel electrophoresis of the levanase preparation after purification on Sephadex G-200

The preparation and running of the gels is described in 'General Methods' (III.N.).

A 78 µg protein sample was applied to each gel and electrophoresis was carried out at pH 8.5 for 45 minutes at 4mA/tube. Staining was carried out with stain b). The results are shown in Fig. 57.

Experiment 34 S.D.S. electrophoresis of the levanase preparation after purification on Sephadex G-200

The preparation and running of the gels is described in Section III.N.

The following proteins of known molecular weight were run:

	Molecular weight	Mobility
β-galactosidase (E. Coli, gift from Dr Dey)	130,000	0.19
Bovine serum albumin (Sigma)	68,000	0.3618
α-Chymotrypsinogen (Bovine pancreas, Sigma)	25,700	0.6328
Trypsin (Bovine pancreas, Sigma)	23,300	0.0623
Ribonuclease (Bovine pancreas, Sigma)	13,700	0.8388
Levanase	-	0.141 0.202 0.1819

For the protein standards 50 µg of protein was applied and for the levanase sample 78 µg was applied. Staining was carried out with stain b.

The determination of mobilities and of molecular weights of unknown bands is described in Experiment 12. The results are shown graphically in Fig. 59. A stained gel is shown in Fig. 58.

Experiment 35 Preliminary investigation to find the right type of ion-exchanger to bind S. salivarius levanase

To each of two tubes either CM-cellulose (Whatman CM-52, 0.05 g) or DEAE-cellulose (Whatman DE-52, 0.05 g) was added. To the tube containing CM-cellulose 100 m$\underline{\underline{M}}$ acetate buffer pH 4 (30 cm^3) was added, to the other tube 100 m$\underline{\underline{M}}$ tris/HCl buffer pH 8.0 (30 cm^3). After decanting the excess buffer, equilibration was achieved by adding at least two more aliquots of buffer. After allowing the ion-exchanger to sediment the supernatant was removed to give a total volume of 5 cm^3. Enzyme solution, previously dialysed against water to remove excess buffer, was added and the tubes shaken. A comparison was then made of the enzymic activity of each supernatant with that of an enzyme blank.

The same procedure was carried out with tris/HCl buffer (100 m$\underline{\underline{M}}$ pH 7.5) and DEAE-cellulose, but sodium chloride solutions (0.01 - 0.1$\underline{\underline{M}}$) were used to elute any bound enzyme. The activity of the released enzyme was then compared to the total activity added to the ion-exchanger.

Experiment 36 Separation of S. salivarius levanase on CM-cellulose

CM-Cellulose (Whatman CM-52) was prepared and packed as described in section III.G and equilibrated with 100 m$\underline{\underline{M}}$ acetate buffer pH 4.0. Enzyme solution (50 levanase units) from Experiment 32 was dialysed against water and freeze-dried. Acetate buffer

($2\ cm^3$) was then added and the sample applied to the column. Initially elution was performed with acetate buffer at a flow rate of 50 cm^3/hr, and 2.5 cm^3 fractions were collected. After fraction 40 elution was performed with a stepwise sodium chloride gradient (0.1 - 2M) in acetate buffer. The levanase activity of the eluted fractions was determined (IV.D.b).

As no enzyme activity was detected, fractions were pooled, concentrated, and exhaustively diafiltrated using a PM-10 (Amicon) membrane against 100 mM phosphate buffer pH 6.6. The activity of the pooled fractions was determined.

In order to determine whether metal ions had been removed the following salts were made up to a 1M solution;

1) calcium chloride dihydrate,
2) magnesium sulphate hexahydrate,
3) manganese sulphate.

To $0.1\ cm^3$ of the concentrated protein solution $0.1\ cm^3$ of salt solution was added and the solution incubated for 5 minutes at 37°C. Levan ($2\ mg/cm^3$, $0.8\ cm^3$) in water was added and incubation carried out at 37°C for 2 hours. The reaction was stopped by placing the incubation in a boiling water bath for one minute.

Any liberated fructose was determined by analysis of the trimethylsilyl derivative[241] by GLC using column a) at 150°C.

The incubation mixture was concentrated to dryness and pyridine ($1\ cm^3$) added. The solid dissolved on warming. The reagents were warmed before being added to the pyridine solution. Hexamethylsilazane ($0.2\ cm^3$) was added and the solution warmed then trimethylsilylchloride ($0.1\ cm^3$) was then added and the mixture was once again warmed.

After centrifuging the mixture the supernatant was injected directly onto the G.L.C. column. The chromatograms are shown in Fig. 60.

Experiment 37 Effect of ethylenediaminetetraacetic acid (EDTA) on levanase activity

To 0.1 cm^3 of levanase solution obtained in Experiment 31 III (0.736 levanase units), 0.1 cm^3 of EDTA solution (1 - 10 mM final concentration) was added and incubation carried out at 37°C for 10 minutes. Levan (2 mg/cm^3, 0.8 cm^3) was added and incubation carried out at 37°C for 30 minutes. The reducing sugar liberated was determined (III.M.III.) and compared to the value obtained for an enzyme blank containing no EDTA. The results are tabulated in Table 35.

Experiment 38 Separation of S. salivarius levanase or DEAE-cellulose using tris/HCl buffer pH 7.5

DEAE-cellulose (Whatman DE-52) was prepared and packed into a column (1.3 x 19 cm) as described in section III.G. The column was fully equilibrated with 100 mM tris/HCl buffer pH 7.5.

A 6 cm^3 sample of enzyme from Experiment 32 was applied to the column and elution carried out with the tris/HCl buffer. A flow rate of 30 cm^3/hr was used and 2 cm^3 fractions were collected. After fraction 25 a continuous linear gradient of sodium chloride (0 - 2 M) dissolved in buffer (500 cm^3 in each vessel), was applied. The levanase activity of each fraction was determined (IV.D.b). The elution profile is shown in Fig. 61. The fractions containing activity were pooled and concentrated. Diafiltration was then carried out against 100 mM phosphate buffer pH 6.6. The degree of purification is shown in Table 36.

Experiment 39 Disc gel electrophoresis of the levanase purified on DEAE-cellulose using tris/HCl buffer pH 7.5

The gels were prepared and run as described in Section III.N.

A 100 µg sample was applied to each polyacrylamide gel. Electrophoresis was carried out at pH 8.5 for 35 minutes at 5 mA/tube. Staining was carried out with stain a).

Experiment 40 Purification of the levanase on DEAE-cellulose using phosphate buffer pH 7.5

DEAE-cellulose (Whatman DE-52) was prepared and packed into a column (1.3 x 20 cm) as described in III.G. The column was equilibrated fully with 100 mM phosphate buffer pH 7.5.

A 5 cm^3 sample of enzyme obtained from Experiment 32 was applied to the column and elution performed with phosphate buffer. Two cm^3 fractions were collected at a flow rate of 30 cm^3/hr. After fraction 21 elution was performed with a continuous gradient of sodium chloride (0 - 2M) in phosphate buffer (200 cm^3 in each vessel). The levanase activity of each fraction was determined (IV.D.L.). The elution profile is shown in Fig. 62A. Fractions 6 - 18 and 18 - 40 were pooled separately and concentrated. Sodium chloride was removed by diafiltration.

The pooled fractions 18 - 40 were applied to a column (1.6 x 30 cm) packed with DEAE-cellulose equilibrated with phosphate buffer. Fractions (3 cm^3) were collected at a flow rate of 45 cm^3/hr. Elution was performed with phosphate buffer until fraction 18 when a less steep sodium chloride gradient (0 - 1 M) in phosphate buffer (500 cm^3 in each vessel) was used to elute the column. The levanase activity of each fraction corresponding to the elution volume of the enzyme was determined. The elution profile is shown in Fig. 62B.

Fractions 46 - 61 were pooled and concentrated and diafiltrated against 100 mM phosphate buffer pH 6.6. The degree of purification is shown in Table 36.

Experiment 41 Purification of the levanase on DEAE-cellulose using phosphate buffer pH 7.0

DEAE-cellulose (Whatman DE-52) was prepared and packed into a column (1.7 x 35 cm) as described in III.G.

A 3 cm^3 sample of enzyme obtained from Experiment 32 was applied to the column and elution carried out with 100 mM phosphate buffer pH 7.0. Fractions (3 cm^3) were collected at a flow rate of 45 cm^3/hr. After fraction 48 a continuous sodium chloride gradient (0 - 1 M) in phosphate buffer pH 7.0 (500 cm^3 in each vessel) was applied. The levanase activity of each fraction was determined (IV.D.b). Fractions 80 - 88 were pooled concentrated and diafiltrated against 100 mM phosphate buffer pH 6.6.

Experiment 42 Disc gel electrophoresis of the levanase purified on DEAE-cellulose using phosphate buffer pH 7.0

The gels were prepared as described in section III.N. To each polyacrylamide gel 50 µg of dialysed, freeze-dried enzyme from Experiment 41 was added with running buffer (pH 8.5) and glycerol. Electrophoresis was carried out at 4mA/tube for 50 minutes using glycine buffer, pH 8.5, in the reservoirs. Staining was carried out with stain b). A stained gel is shown in Fig. 63.

Gels which were incubated with substrate were prepared as described in III.N. Sliced gels were incubated overnight with 1 cm^3 of levan solution (2 mg/cm^3) in 100 mM phosphate buffer pH 6.6.

The reducing sugar liberated was determined (III.M.III.). The results are shown graphically in Fig. 64.

Experiment 43 Purification of S. salivarius levanase on DEAE-cellulose using 50 mM phosphate buffer pH 6.6.

a) Before gel-filtration chromatography

The ion-exchange cellulose (Whatman DE-52) was prepared and packed into a column (1.6 x 35 cm) as described in III.G.

A 15 cm^3 enzyme sample obtained from Experiment 31 III was applied to the column. Elution was performed with 50 mM phosphate buffer pH 6.6. Fractions (2 cm^3) were collected at a flow rate of 40 cm^3/hr. After fraction 61 a continuous linear sodium chloride gradient (0 - 1 M) in phosphate buffer (500 cm^3 in each vessel) was applied. A stepwise sodium chloride gradient (0.5 - 2 M) was applied after fraction 260. The levanase activity of each fraction was determined (IV.D.b). The elution profile is shown in Fig. 65. Those fractions containing activity were pooled and concentrated. The degree of purification is shown in Table 37.

b) After gel filtration chromatography

DEAE-cellulose (Whatman DE-52) was prepared and packed into a column (1.3 x 18 cm) as described in III.G.

The enzyme previously purified on Ultrogel AcA.22 from Experiment 44a was concentrated and placed on the column. Elution was carried out with 50 mM phosphate buffer pH 6.6. Fractions (1.1 cm^3) were collected at a flow rate of 11 cm^3/hr. After fraction 38 elution was performed with a continuous sodium chloride gradient (0 - 1 M) in phosphate buffer (250 cm^3 in each vessel). A stepwise gradient was used after fraction 209 with 1 M sodium chloride, and

after fraction 240, 2 M sodium chloride and finally after fraction 295 with 5 M sodium chloride. The levanase activity of each fraction was determined (IV.D.b). The elution profile is shown in Fig. 66.

Experiment 44 Purification of S. salivarius levanase on Ultrogel AcA.22

a) Before ion-exchange chromatography by DEAE-cellulose

Ultrogel AcA.22 (LKB) was prepared and packed on a column (1.5 x 90 cm) as described in III.F. The column had a total volume (V_t) of 140 cm^3 and a void volume (V_o) of 23 cm^3.

A solution of enzyme (2 cm^3, from Experiment 31.III) was applied to the column and elution carried out with 100 mM phosphate buffer pH 6.6. Fractions (1.2 cm^3) were collected at a flow rate of 2.4 cm^3/hr. The levanase activity of each fraction was determined (IV.D.b). The elution profile is shown in Fig. 67. Fractions 54 - 100 were pooled and concentrated.

b) After ion-exchange chromatography by DEAE-cellulose

Ultrogel AcA.22 (LKB) was prepared and packed in a column (0.9 x 60 cm) as described in section III.F. The column had a total volume (V_t) of 36 cm^3 and a void volume (V_o) of 6.4 cm^3.

A solution of enzyme (1.5 cm^3, from Experiment 43a) was applied to the column. Elution was carried out with 100 mM phosphate buffer pH 6.6. Fractions (0.4 cm^3) were collected at a flow rate of 1.6 cm^3/hr. The levanase activity of the fractions was determined. Fractions 36 - 90 were pooled and concentrated and rechromatographed on the same column, using the same conditions. The levanase activity of each fraction was determined (IV.D.b). Fractions 40 - 90 were pooled and concentrated.

Experiment 45 Attempted binding of the levanase to concanavalin-A Sepharose

A small syringe (5 cm^3) was used as a column and packed with concanavalin A-Sepharose (Pharmacia) as described in section III.J.

Enzyme solution (6 cm^3, from Experiment 32) was applied to the column. Fractions (0.66 cm^3) were collected at a flow rate of 5 cm^3/hr. Initially elution was performed with 100 mM phosphate buffer pH 6.6, but after fraction 30 elution was performed with 0.5 M methyl α-D-glucopyranoside in phosphate buffer. The levanase activity of each fraction was determined (IV.D.b). The elution profile is shown in Fig. 69. Fractions 4 - 17 were pooled and concentrated. Purification figures are given in Table 40.

Experiment 46 Binding of the levanase to a hydrazide derivative of Biogel P-300

The preparation of the hydrazide derivative of Biogel P-300 and the binding of the levan-oligosaccharides is described in 'Methods' III.I

a) Column 1 - crude hydrolysate bound to Biogel P-300. The derivatised Biogel P-300 was packed into a 10 cm^3 syringe and washed with 10 mM phosphate buffer pH 6.6 (elution buffer).

Enzyme solution (1 cm^3, from Experiment 31 III) was applied to the column and the column eluted with buffer. Fractions (1 cm^3) were collected at a flow rate of 2 cm^3/hr. After fraction 12, 0.1 M sodium chloride in elution buffer was used to elute the column. After fraction 20 further elution was carried out with a solution of levan-oligosaccharides (8.76 g/100 cm^3 of elution buffer). The levanase activity of fractions 0 - 22 was determined (IV.D.b).

The elution profile is shown in Fig. 70. Fractions 2 - 17 were pooled and concentrated. Fractions 22 - 60 were pooled and diafiltrated against 100 mM phosphate buffer pH 6.6 using a XM-100 (Amicon) membrane.

b) Column 2 - diafiltrated levan hydrolysate bound to Biogel P-300. The derivatised Biogel P-300 was placed in a 20 cm^3 glass syringe and equilibrated with 10 mM phosphate buffer pH 6.6, (elution buffer).

Enzyme solution (2 cm^3, from Experiment 31 III) was applied to the column and elution performed with elution buffer. Fractions (1.3 cm^3) were collected at a flow rate of 5.2 cm^3/hr. The oligosaccharide solution (2.4 g/50 cm^3 of elution buffer) was used as eluant after fraction 55. The levanase activity of fractions 0 - 56 was determined (IV.D.b). The elution profile is shown in Fig. 71. Fractions 7 - 26 were pooled and concentrated. Fractions 56 - 124 were pooled, concentrated, and diafiltrated as described in a) using a XM-100 (Amicon) membrane.

Experiment 47 Purification of S. salivarius levanase on Biogel A-5M

The preparation and packing of the column is described in III.F. The column measured 1.5 x 86 cm and had a total volume (V_t) of 135 cm^3 and a void volume (V_o) of 45 cm^3.

Enzyme solution (125 levanase units from Experiment 31 III) was exhaustively diafiltrated against 100 mM phosphate buffer pH 6.6 using a XM-100 (Amicon) membrane. This solution after concentrating ($\sim$5 cm^3) was applied to the column. Elution was performed with 100 mM phosphate buffer pH 6.6 at a constant flow rate of 19 cm^3/hr and 10 minute fractions were collected. The levanase activity of

each fraction was determined (IV.D.b). The elution profile is shown in Fig. 72. Fractions 17 - 34 were pooled and concentrated. Purification figures are given in Table 41.

Experiment 48 Disc gel electrophoresis of the enzyme preparation purified on Biogel A-5M

The gels were prepared as described in III.N. Enzyme solution (0.4 cm^3, from Experiment 47) was exhaustively dialysed against distilled water to remove buffer ions. After freeze-drying approximately 75 μg of protein was applied to each of the gels. Electrophoresis was then carried out at pH 8.5 for 45 minutes at 4 mA per tube. Staining was carried out with stain b. The results are shown in Fig. 73.

Experiment 49 Purification of S. salivarius levanase on hydroxylapatite

Hydroxylapatite (Sigma, type 1) was mixed with cellulose powder and packed into a column (3.3 x 16 cm) as described in III.H.

Enzyme solution (3.5 cm^3, from Experiment 47) was diafiltrated against 5.0 m$\underline{M}$ phosphate buffer pH 6.6 and concentrated to 5 cm^3. The sample was applied to the column and elution carried out with 5.0 m$\underline{M}$ phosphate buffer pH 6.6 at a flow rate of 21 cm^3/hr. Ten minute fractions being collected. After fraction 25 a linear phosphate buffer gradient (5 - 500 m$\underline{M}$, 500 cm^3 in each vessel) was used to elute the column. After fraction 260, 0.5 $\underline{M}$ buffer was used to elute the column and after fraction 290, 2$\underline{M}$ potassium chloride in 0.5 $\underline{M}$ buffer. The levanase activity of each fraction was determined. The elution profile is shown in Fig. 74. Initially fractions 76 - 130 were concentrated using a PM-10 (Amicon) membrane. Later, all fractions

were pooled, concentrated, and diafiltrated against 100 mM phosphate buffer pH 6.6.

IV.E. Experiments relating to chapter II.2.B

A strict correlation between the number of enzyme units present in an incubation and the activity present may not be shown. This is because of a loss of enzyme activity with storage time. Thus, the number of enzyme units added is taken from the calculation of the number present at the time of isolation and purification.

Experiment 50 Effect of pH on the rate of hydrolysis of levan by S. salivarius levanase

Levan (2 mg/cm^3) was made up in a series of 100 mM phosphate and acetate buffers of pH 4.0 - 8.0. Into a series of test tubes 0.9 cm^3 of each solution was pipetted and incubated at 37°C for 5 minutes. Enzyme solution (0.1 cm^3, 0.029 levanase units, from Experiment 47) previously preincubated at 37°C was added to each tube and incubation carried out for 15 minutes. The reaction was stopped by immersing the tubes in a boiling water bath for 1 minute. The liberated fructose was determined by means of the Nelson reducing test (III.M.III). The results are shown in Fig. 75.

Experiment 51 Thermal stability of S. salivarius levanase

A series of test tubes containing 0.1 cm^3 of enzyme solution from Experiment 47 (0.0577 levanase units) were incubated for 15 minutes at temperatures ranging from 20 to 60°C. After being rapidly cooled in iced water, 0.9 cm^3 of levan solution (2 mg/cm^3) in 100 mM phosphate buffer pH 6.6, preincubated at 37°C, was added to the tubes, and incubation carried out for 15 minutes. The reaction

was terminated and the fructose liberated determined (III.M.III.).

For the inulinase incubations the same concentration of substrate (Inulin) was taken but 0.288 levanase units were used. Incubation was carried out at 30°C for 15 minutes. The results are shown graphically in Fig. 76.

Experiment 52 Heat inactivation of the levanase and Inulinase activity of the enzyme preparation

Solutions containing levan (12.6 cm^3, 2 mg/cm^3 in 100 mM phosphate buffer pH 6.6), and levanase (1.4 cm^3, 0.84 levanase units, from Experiment 47) were prepared. After preincubating the levan solution at 50°C for 10 minutes both solutions were mixed and incubated at 50°C. At regular intervals over a 60 minute period 1 cm^3 aliquots were removed heated for 1 minute at 100°C and the liberated fructose determined (III.M.III.). The method was repeated at 40°C.

The inulinase incubation contained the same concentration of substrate (Inulin) as described for the levanase incubation. A total of 2.7 levanase units were used and incubation carried out at 40°C. The results are shown graphically in Figs. 77 and 78.

Experiment 53 Levanase properties of the enzyme preparation

a) Dependence of activity on substrate concentration

A range of levan solutions (5 - 100 mg/cm^3 in 100 mM phosphate buffer pH 6.6) were prepared. Enzyme solution (0.05 cm^3, 0.05 levanase units from Experiment 47) and substrate solution (0.45 cm^3) were preincubated at 30°C for 5 minutes and then mixed.

After incubation for 15 minutes at 30°C the reaction was stopped and the liberated fructose determined (III.M.III.). The results are shown graphically in Fig. 79.

b) Dependence of activity on enzyme concentration

A series of enzyme solutions containing from 0.00577 to 0.577 levanase units (from Experiment 47) in 0.05 cm^3 of 100 m$\underline{\underline{M}}$ phosphate buffer pH 6.6 were prepared and kept at 30°C for 5 minutes. Levan solution (0.45 cm^3, 2 mg/cm^3 in 100 m$\underline{\underline{M}}$ phosphate buffer pH 6.6) was prepared and preincubated at 30°C for 5 minutes. Enzyme (0.05 cm^3) and substrate (0.45 cm^3) solutions were mixed and incubated at 30°C for 15 minutes. The reaction was stopped and the liberated fructose determined (III.M.III.). The results are shown graphically in Fig. 80.

c) Variation of levanase activity on time

To 14.4 cm^3 of levan solution (2 mg/cm^3 in 100 m$\underline{\underline{M}}$ phosphate buffer pH 6.6) preincubated at 30°C for 5 minutes, 1.6 cm^3 of enzyme solution (0.92 levanase units from Experiment 47) was added and incubation carried out at 30°C. At regular intervals over a 60 minute period 1 cm^3 aliquots were removed the reaction stopped and the liberated fructose determined (III.M.III.). The results are shown graphically in Fig. 81.

Experiment 54 Degradation of inulin and levan by S. salivarius levanase

a) Short term incubations

Both levan and inulin were from the departmental collection. Enzyme solution, 1.6 cm^3 (0.92 levanase units, levan incubation, and 3.08 levanase units, inulin incubation) and substrate solution,

inulin or levan (14.4 cm^3, 2 mg/cm^3 in 100 mM phosphate buffer pH 6.6), were preincubated at 30°C for 5 minutes. The solutions were mixed and the procedure described in Experiment 53c) carried out. The results are shown graphically in Fig. 82.

b) Long term incubations

Enzyme solution (1.2 cm^3, 1.39 levanase units for levan incubation, 13.8 levanase units for inulin incubation from Experiment 47) and substrate solution, inulin or levan (10.8 cm^3, 2 mg/cm^3 in 100 mM phosphate buffer pH 6.6) were preincubated at 30°C for 5 minutes. The solutions were mixed and the procedure described in a) carried out over a 48 hour period. The results are shown graphically in Fig. 83.

Aliquots which were to be spotted on a paper chromatogram were first deionised with Biodermineralite resin (BDH) by shaking for 1 hour. The resin was removed by filtration and the filtrate freeze-dried. After adding a few drops of water the filtrates were subjected to paper chromatography in solvent 1. The chromatograms are shown diagrammatically in Fig. 89.

Experiment 55 Hydrolysis of oligosaccharides containing a terminal β-linked fructosyl unit

Sucrose was obtained from BDH (Analar) and raffinose from the departmental collection.

Sucrose solution (10.8 cm^3, 2 mg/cm^3 of sucrose, raffinose or levan in 100 mM phosphate buffer pH 6.6) and enzyme solution (1.2 cm^3 1.39 levanase units from Experiment 47) were preincubated at 30°C for 5 minutes. After mixing the solutions were incubated at 30°C. Aliquots were removed over a 10 hour period for the raffinose incubation, and 3 hours for the sucrose and levan incubations. The procedure

described in Experiment 53 c) was followed. The results are shown graphically in Figs. 84 and 85.

Fractions were prepared for paper chromatography as described in Experiment 54b). The chromatograms are shown diagrammatically in Figs. 88 and 89.

Experiment 56 Continuous monitoring of the β-fructosidase activity of S. salivarius levanase

The inulin- and levan-oligosaccharides, methyl β-D-fructofuranosidase and melibiose were from the departmental collection. Celliobiose was from BDH and p-nitrophenyl β-D-glucopyranoside from Sigma.

The reagents and enzymes were made up as stated in Section III.M.V, however, reagent A contained 50 mM tris/HCl buffer pH 7.0 and not triethanolamine. The silica cell contained the following: reagent A (2.6 cm^3), reagent B (0.1 cm^3), reagent C (0.1 cm^3), reagent D (0.01 cm^3), substrate (0.19 cm^3, in deionised water) and reagent E (0.02 cm^3). The absorbance was monitored on a "Pye Unicam" SP 1800 scanning spectrometer at 340 nm.

The contents of the cell were mixed and placed in the spectrometer until a steady base line was obtained. Reagent F (0.02 cm^3) was then added with mixing and the cell placed in the spectrometer until a steady base line was obtained. The cell was usually left in the spectrometer for five minutes to make sure that no D-fructose was present as a contaminant in the substrate solution. Levanase solution

(10 μl, 0.1155 levanase units from Experiment 47) was then added, with mixing, and the cell placed quickly back in the spectrometer. The reaction was then monitored at 340 nm for 30 minutes, at a temperature of 30°C.

The extinction value at pH 7.0 was determined using fructose standards and thus the amount of D-fructose liberated could be determined as described in III.M.V.

The activity versus time plots are shown in Fig. 87.

Experiment 57 Incubation of Inulintriose with levanase

Enzyme solution (0.6 cm^3, 0.69 levanase units from Experiment 47) and substrate solution (5.4 cm^3, 2 mg/cm^3 in 100 mM phosphate buffer pH 6.6) were prepared and preincubated at 30°C for 5 minutes. After mixing the solutions and incubating at 30°C, aliquots (1 cm^3) were removed over a 60 minute period and the reaction stopped. The liberated fructose was determined (III.M.V.). Samples were prepared for paper chromatography as described in Experiment 54 b). The chromatogram is shown diagrammatically in Fig. 88.

Experiment 58 Fructose inhibition studies

A series of enzyme solutions (0.1 cm^3, 0.1155 levanase units for the levan incubation, 1.155 levanase units for the inulin incubation) were prepared. Fructose solution (4 mg/cm^3, 0 - 0.45 cm^3) and phosphate buffer (100 mM, pH 6.6) was added to give a final volume of 0.55 cm^3. The solutions were then preincubated at 30°C for 10 minutes. Substrate solution (0.45 cm^3, 4 mg/cm^3 levan or inulin in 100 mM phosphate buffer pH 6.6) was then added and the solutions incubated at 30°C for 30 minutes. The reaction was stopped and the fructose determined (III.M.III.). The results are shown graphically in Fig. 90.

Experiment 59 The degree of polymerisation of an oligosaccharide[242]

Two solutions were prepared:

2% w/v potassium borohydride (0.5 cm^3) and the oligosaccharide (30 - 60 μg) in water (0.5 cm^3);

2% w/v potassium borohydride (0.5 cm^3) and the same weight of oligosaccharide in 1M-sulphuric acid (0.5 cm^3).

The tubes were left to stand overnight. The carbohydrate content of each tube was determined by the phenol sulphuric method (III.M.II.). The degree of polymerisation was calculated from the following equation:

$$D.P. = \frac{\text{Absorbance at 487 nm of unchanged oligosaccharide}}{\text{Absorbance at 487 nm of reduced oligosaccharide}}$$

The results are tabulated in Table 44.

BIBLIOGRAPHY

1 "The Enzyme Handbook"
p.576 (Springer Verlag, 1969).

2 A.Gottschalk in "The Enzymes"
Vol.1, p.551 (Academic Press, 1950).

3 E. Fischer,
Ber., 27, 2985 (1894).

4 G.E. Glock,
Biochem. J., 30, 2313 (1936).

5 A.N. Petrova,
Biokhimiya., 12, 209 (1947).

6 E. Laborde, I.H. Fiszerman, D. Fiszerman-Garber,
Bull. Soc. Pharmacol., 40, 65 (1933).

7 A. Dahlqvist,
Acta. Chem. Scand., 14, 172 (1960).

8 E.L. Rozenfeld,
Biokhimiya., 21, 77 (1956).

9 E.L. Rozenfeld,
Clin. Chim. Acta., 2, 105 (1957).

10 E.L. Rozenfeld, I.S. Lukomskaya, N.K. Rudakova, A.I. Shubina,
Biokhimiya., 24, 965 (1959).

11 I. Lieberman and W.H. Eto,
J. Biol. Chem., 228, 899 (1957).

12 H.G. Hers, D.G. Thines-Sempeux, N. Lejeune,
Biochem. J., 84, 28p (1962).

13 Ibid.,
86, 16 (1963).

14 S. Shibko and A.L. Tappel,
Biochem. J., 95, 731 (1965).

15 E.L. Rozenfeld and I.A. Popova,
Bull. Soc. Chim. Biol., 44, 129 (1962).

16 H. Torres and J. Olavarria,
J. Biol. Chem., 239, 2427 (1964).

17 F. Auricchio and C.B. Bruni,
Biochem. J., 105, 35 (1967).

18 C.B. Bruni, F. Auricchio, V. Sica,
Biochem. J., 108, 161 (1968).

19 C.B. Bruni, F. Auricchio, I. Covelli,
J. Biol. Chem., 244, 4735 (1969).

20 V. Sica, C.B. Bruni, F. Aurrichio, I. Covelli,
Biochim. Biophys. Acta., 212, 470 (1970).

21 V. Sica, A. Siani, C.B. Bruni, F. Aurrichio,
Biochim. Biophys. Acta., 242, 422 (1971).

22 A. Adlung,
Z. Gastroenterol., 7, 304 (1969).

23 K. Fujimori, S. Hizukuri, Z. Nikuni,
Biochem. Biophys. Res. Commun., 32, 811 (1969).

24 P.L. Jeffrey, D.H. Brown, B.I. Brown,
Biochemistry., 9, 1403 (1970).

25 Ibid.
9, 1416 (1970).

26 T.N. Palmer,
Biochem. J., 124, 701 (1971).

27 Ibid.,
124, 713 (1971).

28 B. Seetharam and N. Swaminathan,
Biochem. J., 117, 939 (1970).

29 H. Iwanowski,
Arch. Immunol. Ther. Exp., 20, 745 (1972).

30 Ibid.,
21, 615 (1973).

31 M.E. Preobrazhenskaya, A.L. Minakova, E.L. Rozenfeld.,
Carbohydr. Res., 38, 267 (1974).

32 K. Uchida and Y. Suzuki,
Agri. Biol. Chem. (Japan), 38, 195 (1974).

33 T. De Barsy, P. Jacquemin, P. Devos, H.G. Hers,
Eur. J. Biochem., 31, 156 (1972).

34 B.I. Brown, A.K. Murrey, D.H. Brown,
Amer. Chem. Soc. Symp. No. 15, Physiological effects of food carbohydrates p.223 (1975).

35 J.F. Koster and R.G. Slee,
Biochim. Biophys. Acta., 482, 89 (1977).

36 E.L. Rozenfeld and D.M. Belenki,
Bull. Soc. Chim. Biol., 50, 1305 (1968).

37 D.M. Swallow, G. Corney, H. Harris.
Ann. Hum. Genet. (Lond), 38, 391 (1975).

38 J.K. Koster, R.G. Slee, J.M. van der Klei-van Moorsel, P.J.G.M. Rietra, C.J. Lucas, Clin. Chim. Acta., 68, 49 (1976).

39 C.A. Burton, T.A. Lewis, D.R. Llewellyn, H. Tristam, C.A. Vernon, Nature., 174, 560 (1954).

40 G.G. Freeman and R.H. Hopkins, Biochem. J., 30, 451 (1936).

41 C.E. Weil, R.J. Burch, J.W. van Dyk, Cereal. Chem., 31, 150 (1954).

42 D.E. Eveleigh and A.S. Perlin, Carbohydr. Res., 10, 87 (1969).

43 J.E.G. Barnett, W.T.S. Jarvis, K.A. Munday, Biochem. J., 103, 699 (1967).

44 K. Wallenfels and O.P. Malhotra, Enzymes., 4, 409 (1960).

45 J.A. Rupley, Proc. Roy. Soc (Lond)., B167, 416 (1967).

46 K. Wallenfels and I.R. Rached, Biochem. Z., 344, 524 (1966).

47 E.F. Armstrong, J. Chem. Soc., 83, 1305 (1903).

48 F.W. Parrish and E.T. Reese, Carbohydr. Res., 3, 424 (1967).

49 B. Capon, Chem. Rev., 69, 433 (1969).

62 B. Axelrod and H-Ya L. Lai,
Biochem. Biophys Res. Commun., 54, 463 (1973).

63 G. Legler,
Acta. Microbiol. Acad. Sci. Hung., 22, 403 (1975).

64 G. Legler and W. Lotz,
Hoppe-Seylers, Z. Physiol. Chem., 354, 243 (1973).

65 G. Legler,
Mol. Cell. Biochem., 2, 31 (1973).

66 E.J. Hehre, D.S. Genghof, H. Sternlicht,
Biochemistry., 16, 1780 (1977).

67 E.L. Rozenfeld,
Dokl. Akad. Nauk. SSSR., 122, 1298 (1959).

68 D.G. Walker in
"Carbohydrate Metabolism and its disorders", p.465 (Academic Press, 1968).

69 H.G. Hers in
"Control of Glycogen Metabolism", p.354 (Churchill, 1964).

70 E.H. Fischer, L.M.G. Heilmeyer, R.H. Harschke,
Curr. Top. Cell. Regul., 4, 211 (1971).

71 E.L. Rozenfeld,
Pathol. Biol., 23, 71 (1975).

72 E.L. Rozenfeld and I.A. Popova,
Vop. Med. Khim., 8, 465 (1962).

73 J. Lundqvist,
Horm. Met. Res., 4, 151 (1972).

74 E.L. Rozenfeld, I.A. Popova, V.S. Orlova,
Biokhimiya., 35, 356 (1970).

75 E.L. Rozenfeld and I.A. Popova,
Biochimie., 53, 939 (1971).

76 T. Palmer and B. Ryman,
FEBS Letters., 18, 277 (1971).

77 L. Hurwitz, D. McCormick, J. Allen,
Lancet., 11, 67 (1970).

78 J.F. Koster,
Lancet., 11, 1187 (1970).

79 E.J. Bourne, K. Clarke, J. Pridham, J. Rowe,
Biochem. J., 121, 663 (1971).

80 E.L. Rozenfeld and A.S. Sayenko,
Biokhimiya., 28, 552 (1963).

81 H.G. Hers,
Chem. Weekbl., 57, 437 (1961).

82 H.G. Hers,
Biochem. J., 86, 11 (1963).

83 K. Kahana, C. Telem, K. Steinitz, M. Soloman,
J. Pediat., 65, 243 (1964).

84 M. Dinesoy, H. Dinesoy, A. Kessler, M. Jackson, J. Sidbury,
J. Pediat., 67, 728 (1965).

85 D. Brown and B. Illingworth-Brown,
Biochim. Biophys. Acta., 110, 124 (1965).

86 K. Steinitz and A. Ruthenberg,
Isr. J. Med. Sci., 3, 411 (1967).

87 I.S. Salafsky and H. Nadler,
J. Lab. Clin. Med., 81, 450 (1973).

88 J.C. Dreyfus, and Y. Alexandre,
Biochem. Biophys. Res. Commun., 48, 914 (1972).

89 H.G. Hers and T. DeBarsy, in
"Lysosome and Storage Diseases", p.197 (Academic Press, 1973).

90 R.J. Desnick, S.R. Thorpe, M.B. Fiddler,
Physiol Rev., 56, 57 (1976).

91 E.L. Rozenfeld in
"Control of Glycogen Metabolism", p.176 (Churchill, 1964).

92 H. Iwankowski and E. Gandrya,
Nauk. Wyzs. Szkol. Roln. we. Wrocl., 24, 105 (1968).

93 A. Dahlqvist,
Acta. Chem. Scand., 13, 1817,(1959).

94 I.S. Lukomskaya,
Dokl. Acad. Nauk. SSSR., 157, 716 (1964).

95 F. Auricchio and V. Sica,
J. Chromat., 28, 26 (1967).

96 F. Auricchio and G. Semenza,
Biochim. Biophy. Acta., 65 173 (1962).

97 D.M. Belenki and E.L. Rozenfeld,
Clin. Chim. Acta., 60, 397 (1975).

98 D.M. Belenki and E.L. Rozenfeld,
Dokl. Akad. Nauk. SSSR., 199, 708 (1971).

99 A. Goldstone, P. Konecny, H. Koenig,
FEBS Letters., 13, 68 (1971).

100 E. Beutler, E. Guinto, W. Kuhl,
J. Lab. Clin. Med., 85, 672 (1975).

101 A. Dahlqvist and U. Telenius,
Biochem. J., 111, 139 (1969).

102 K. Fujimori, T. Fukui, Z. Nikuni,
Agr. Biol. Chem. (Tokyo), 36, 483 (1972).

103 K. Weber, J.R. Pringle, M. Osborn,
Methods in Enzymology, Vol. XXVI, p.3 (1972).

104 Ibid.,
Vol. XXVIII, p.54 (1973).

105 F.H. White,
Ibid. Vol. XXV, p.387 (1972).

106 A.L. Shapiro, E. Vinuela, J. Maizel,
Biochem. Biophys. Res. Commun., 28, 815 (1967).

107 K. Weber,
J. Biol. Chem., 244, 4406 (1969).

108 J. Svasti and B. Panijpan,
J. Chem. Ed., 54, 560 (1977).

109 J.J. Marshall,
Anal. Biochem., 37, 466 (1970).

110 B. Klein, J.A. Foreman, R.L. Searcy,
Anal. Biochem., 31, 413 (1969).

111 J.G. Fleetwood and H. Weigel,
Nature., 196, 984 (1962).

112 B. Capon,
Chem. Review., 69, 415 (1969).

113 R. Kuhn,

Ann. Chem., 443, 1 (1925).

114 J.A. Thoma and D.E. Koshland,

J. Biol. Chem., 235, 2511 (1960).

115 S. Ono, K. Hirani, Z. Hamauzu,

J. Biochem. (Tokyo), 57, 34 (1965).

116 F.J. Bates in

"Polarimetry Saccharimetry and the Sugars", National Bureau of Standards C440.

117 D.E. Koshland,

Biol. Rev. Cambridge. Phil. Soc., 28, 416 (1953).

118 C.F. Blake, L.N. Johnson, G.A. Mair, A.T. North, D.C. Phillips, V.R. Sarma,

Proc. Roy. Soc. (Lond.), B167, 378 (1967).

119 J. Conchie, A.J. Hay, I. Strachan, G.A. Levvy,

Biochem. J., 102, 929 (1967).

120 F.W. Dahlqvist, T. Rand-Meir, M.A. Raftery,

Biochemistry., 8, 4214 (1969).

121 R.L. Nath and H.N. Rydon,

Biochem. J., 57, 1 (1954).

122 E.T. Reese and F.W. Parrish,

Carbohydr. Res., 18, 381 (1971).

123 G. Legler,

Hoppe-Seylers Z. Physiol. Chem., 345, 197 (1966).

124 R.L. Sidebotham, in

"Adv. Carbohydr. Chem. Biochem., 30, 371 (1974).

125 Z. Gunja-Smith, J.J. Marshall, C. Mercier, E.E. Smith, W.J. Whelan, FEBS Letters., 12, 101 (1970).

126 J.J. Marshall, in "Adv. Carbohydr. Chem. Biochem.", 30, 325 (1974).

127 M.E. Preobrazhenskaya and A.L. Minkova, Dokl. Acad. Nauk. SSSR., 232, 240 (1977).

128 C.L. Market and F. Møller, Proc. Nat. Acad. Sci. USA., 45, 753 (1959).

129 S. Sato and T. Sugimura, in "Methods in Cancer Research", Vol. 12 p.257 (Academic Press, 1976).

130 O. Touster, Mol. Cell. Biochem., 2, 169 (1973).

131 D. Robinson, Enzyme., 181, 114 (1974).

132 E. Beutler and W. Kuhl, Nature., 239, 207 (1972).

133 Y.Z. Frohwein and S. Gatt, Biochim. Biophys. Acta., 128, 216 (1966).

134 J.S.D. Bacon and J. Edelman, Biochem. J., 48, 114 (1951).

135 H.D.K. Drew and W.N. Haworth, J. Chem. Soc., 2690 (1928).

136 W.N. Haworth and A. Leamer, J. Chem. Soc., 619 (1928).

137 H.H. Schlubach and O.K. Sinh,
Ann. Chem., 544, 101 (1940).

138 J.A. Cole,
Biochem. Soc. Trans., 5, 1232 (1977).

139 T.H. Grenby,
Chem. Br., 7, 276 (1971).

140 R.J. Gibbons, in
"The Arts and Science of Dental Caries Research", p.77
(Academic Press, 1968).

141 K. Winkler, and O.B. Backer-Dirks,
Int. Dent. J., 8, 561 (1958).

142 F.J. Orland, J.R. Blayney, R.W. Harrison, J.A. Reneers,
P.C. Trexler, M. Wagner, H.A. Gordon, T.D. Luckey,
J. Dent. Res., 33, 147 (1954).

143 R.J. Fitzgerald, H.V. Jordon, H.R. Stanley,
J. Dent. Res., 39, 925 (1960).

144 S.A. Leach,
Ala. J. Med. Sci., 5, 247 (1968).

145 J. Carlsson,
Odontol. Revy., 18, 55 (1967).

146 J. Carlsson,
Odontol. Revy., 19, 137 (1968).

147 W.A. McDougall,
Aust. Dent. J., 9 1 (1964).

148 P. Critchley, C.A. Saxton, A.B. Lolendo,
Caries Res., 2, 115 (1968).

149 H.J. Koepsell, H.M. Tsuchiya, N.N. Hellman, A. Kazenko,
C.A. Hoffmann, E.S. Sharpe, R.W. Jackson,
J. Biol. Chem., 200, 793 (1953).

150 R.J. Gibbon and M. Nygaard,
Arch. Oral. Biol., 13, 1249 (1968).

151 R.J. Gibbons and S. Banghart,
Arch. Oral. Biol., 12, 11 (1967).

152 K.G. Konig and B. Guggenheim,
Adv. Oral. Biol., 3, 217 (1968).

153 R.J. Gibbons,
J. Dent. Res., 47, 926 (1968).

154 E. Newburn, R. Lacy, T.M. Christie,
Arch. Oral. Biol., 16, 863 (1971).

155 E.J. Hehre,
Science., 93, 237 (1941).

156 C.F. Niven, K.L. Smiley, J.M. Sherman,
J. Bacteriol., 41, 479 (1941).

157 J. van Houte and H.M. Jansen,
Arch. Oral. Biol., 13, 827 (1968).

158 T. DaCosta and R.J. Gibbons,
Arch. Oral. Biol., 13, 609 (1968).

159 R.S. Manly and D.T. Richardson,
J. Dent. Res., 47, 1080 (1968).

160 K.K. Makinen,
Int. Dent. J., 22, 363 (1972).

161 R.J. Gibbons and J. van Houte,
Ann. Rev. Microbiol., 29, 19 (1975).

162 R.S. Levine,
Br. Dent. J., 140, 9 (1976).

163 A. Fuchs,
Thesis, (University of Leiden, Holland, 1959).

164 H.H. Schlubach and J. Berndt,
Ann. Chem., 677, 176 (1964).

165 D.S. Feingold,
J. Polym. Sci., 23, 783 (1957).

166 S. Hestrin,
Proc. Intern. Congr. Biochem. 4th 1958, p.18.

167 R.A. Hancock, K. Marshall, H. Weigel,
Carbohydr. Res., 49, 351 (1976).

168 S.W. Challinor, W.N. Haworth, E.L. Hirst,
J. Chem. Soc., 676 (1934).

169 D.J. Bell and R. Dedonder,
J. Chem. Soc., 2866 (1954).

170 J. Carlsson,
Caries Res., 4, 113 (1970).

171 K.H. Ebert and G. Shenk,
Ber. Bunsenges, Phys. Chem., 68, 765 (1964).

172 E. Newbrun and S. Baker,
Carbohydr. Res., 6 165 (1968).

173 H. Stricker,
Dissertation, Technische Hochschule, Munchen, (1963).

174 S. Hestrin, S. Avineri-Shapiro, M. Aschner,
Nature., 149, 527 (1942).

175 S. Hestrin, S. Avineri-Shapiro, M. Aschner,
Biochem. J., 37, 450 (1943).

176 S. Hestrin and S. Avineri-Shapiro,
Biochem. J., 38, 2 (1944).

177 S. Hestrin, D.S. Feingold, G. Avigad,
Biochem. J., 64, 340 (1956).

178 Ibid,
Biochem. J., 64, 351 (1956).

179 E.J. Hehre,
Proc. Soc. Exp. Biol. Med., 58, 219 (1945).

180 G. Kohanyi and R. Dedonder,
C.R. Acad. Sci. Paris, 233, 1142 (1951).

181 S. Hestrin,
6th Inter. Congr. Microbiol. Symp., p.63 (1953).

182 S. Hestrin, D.S. Feingold, G. Avigad,
J. Am. Chem. Soc., 77, 6710 (1955).

183 S. Hestrin and G. Avigad,
Biochem. J., 69, 388 (1958).

184 K.H. Ebert and G. Schenk,
Adv. Enzymol., 30, 179 (1968).

185 G. Rapoport and R. Dedonder,
Bull. Soc. Chim. Biol., 48, 1311 (1966).

186 G. Rapoport, R. Dionne, E. Toulouse, R. Dedonder,
Bull. Soc. Chim. Biol., 48, 1323 (1966).

187 E.J. Hehre,
Methods in Enzymology, Vol. I, p.297 (1951).

188 R. Dedonder,
Methods in Enzymology, Vol. VIII, p.500 (1966).

189 J. Carlsson,
Caries. Res., 4, 97 (1970).

190 M. Pascal and R. Dedonder,
Carbohydr. Res., 24, 365 (1972).

191 E.J. Whitaker and J.R. Edwards,
Archs. Oral. Biol., 21, 565 (1976).

192 W.R. Scales,
Ph.D. Thesis (Villanova University, USA, 1973).

193 M.J. Pabst,
Infect. Immun., 15, 518 (1977).

194 C. Newberg and T. Mandl, in
"The Enzymes" 1st Ed. Vol. 1, p.527 (Academic Press, 1950).

195 S. Forssman,
Biochem. Z., 264, 231 (1933).

196 S. Hestrin and J. Goldblum,
Nature., 172, 1046 (1953).

197 G. Avigad and R. Zelikson,
Bull. Res. Counc. Isr., Sect. A, 253 (1963).

198 H. Negoro,
Nippon. Nogei Kagaku. Kaishi., 31, 153 (1957).

199 L.S. Prestidge and J. Spizizen,
J. Gen. Microbiol., 59, 285 (1969).

200 M. Pascal, J.A. Lepesant, R. Dedonder, K. Kunst ,
Biochimie., 53, 1059 (1971).

201 M.M. McCabe, E.E. Smith, R.A. Cowman,
Arch. Oral. Biol., 18, 525 (1973).

202 T. Yamada, S. Hojo, K. Kobayashi, Y. Asano, S. Araya,
Arch. Oral. Biol., 15, 1205 (1970).

203 T.M. Palmer, B.E. Ryman, W.J. Whelan,
FEBS Letters., 1, 1 (1968).

204 R.J. Gibbons,
Caries Res., 6, 122 (1972).

205 J.M. Tanzer, A.T. Brown, M.F. McInerney
J. Bacteriol., 116, 192 (1973).

206 B.M. Chassy, R.M. Bielawski, J.R. Beall, E.V. Porter,
M.J. Krichevsky, J.A. Donkersloot, Life. Sci., 15, 1173 (1974).

207 R.M. Osborne, B. Lambert, A.H. Roush,
Experimentia., 31, 1399 (1975).

208 E.V. Palumbo and B.M. Chassey,
Fed. Proc. Fed. Am. Soc. Exp. Biol., 33, 1315 (1974) (Abstract).

209 D. Birkhead and A. Dalqvist,
Odont. Revy., 26, 109 (1975).

210 D. Birkhead,
Odont. Revy., 26, 185 (1975).

211 H.K. Kuramitsu,
J. Bacteriol., 115, 1003 (1973).

212 K. Fukui, Y. Fukui, T. Moriyama,
J. Bacteriol., 118, 796 (1974).

213 J.O. Lampen, in
"The Enzymes" 3rd Ed. Vol. V, p.291 (Academic Press 1970).

214 S. Hestrin, D.S. Feingold, M. Schramm,
Methods in Enzymology, Vol. I p.256.

215 M. Adams, N.K. Richtmeyer, C.S. Hudson,
J. Am. Chem. Soc., 65, 1369 (1943).

216 M. Takahashi and S. Soutome,
Utsunomiya, Daigaku, Nogakubu. Gakujutsu. Hokoka., 9, 95 (1975).

217 K. Marshall,
Ph.D. Thesis, (University of London, 1977).

218 H.E. Snyder and H.J. Phaff,
J. Microbiol. Serol., 26, 433 (1960).

219 Z. Mesner,
J. Dent. Res., 50, 670 (1971) (Abstract).

220 F. Kunst. M. Steinmetz, J.A. Lepesant, R. Dedonder,
Biochimie., 59, 287 (1977).

221 A Guide to Ion-exchange chromatography, Pharmacia Ltd., 1972.

222 H. Hamazaki and K. Hotta,
FEBS Letters., 76, 299 (1977).

223 R. Sturgeon,
Adv. Carbohydr. Chem. (to be published).

224 W.E. Trevelyn, D.P. Proctor, D.S. Harrison,
Nature., 166, 444 (1950).

225 G. Gomori,
Methods in Enzymology, Vol. 1 p.138.

226 S.P.L. Sorensen,
Biochem. Z., 21, 131 (1909).

227 G.S. Walpole,
J. Chem. Soc., 105, 2501 (1914).

228 V. Massey,
Biochim. Biophys. Acta., 37, 310 (1960).

229 M. Koike and M. Hamada,
Methods in Enzymology, Vol. XXII, p.339.

230 J.K. Inman and H.M. Dintzis,
Biochemistry., 8, 4074 (1969).

231 R.L. Whistler and D.F. Durso,
J. Am. Chem. Soc., 73, 677 (1950).

232 J.B. Lloyd and W.J. Whelan,
Anal. Biochem., 30, 467 (1969).

233 M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith,
Anal. Chem., 28, 350 (1956).

234 N. Nelson,
J. Biol. Chem., 153, 375 (1944).

235 O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem., 193, 265 (1951).

236 D.E. Williams and R.A. Reisfeld, Ann. N.Y. Acad. Sci., 121, 373 (1964).

237 B.J. Davis, Ann. N.Y. Acad. Sci., 121, 404 (1964).

238 F. Di Jeso, J. Biol. Chem., 243, 2022 (1968).

239 R.L. Sidebotham, Ph.D. Thesis (University of London, 1970).

240 S. Hakamori, J. Biochem.(Tokyo), 55, 205 (1964).

241 C.C. Sweeley, R. Bentley, M. Makita, W.W.Wells, J. Am. Chem. Soc., 85, 2497 (1963).

242 T.E. Timell, SV. Papperstidn., 63, 668 (1960).

Appendix I

An attempted study to show the mode of action of acid α-D-glucosidase at branch points in amylopectin

Enzymes capable of hydrolysing carbohydrate substrates have become increasingly important in determining the structure of polysaccharides. Many types of carbohydrases have been purified and a detailed investigation of their properties carried out. Moreover, those enzymes capable of hydrolysing amylopectin and glycogen have been investigated more fully than any other type of enzymes. These include the 'exo' type enzymes, phosphorylase, glucoamylase, α-D-glucosidase, the 'endo' type enzymes, α-amylase and the debranching enzymes, pullulanase, isoamylase.

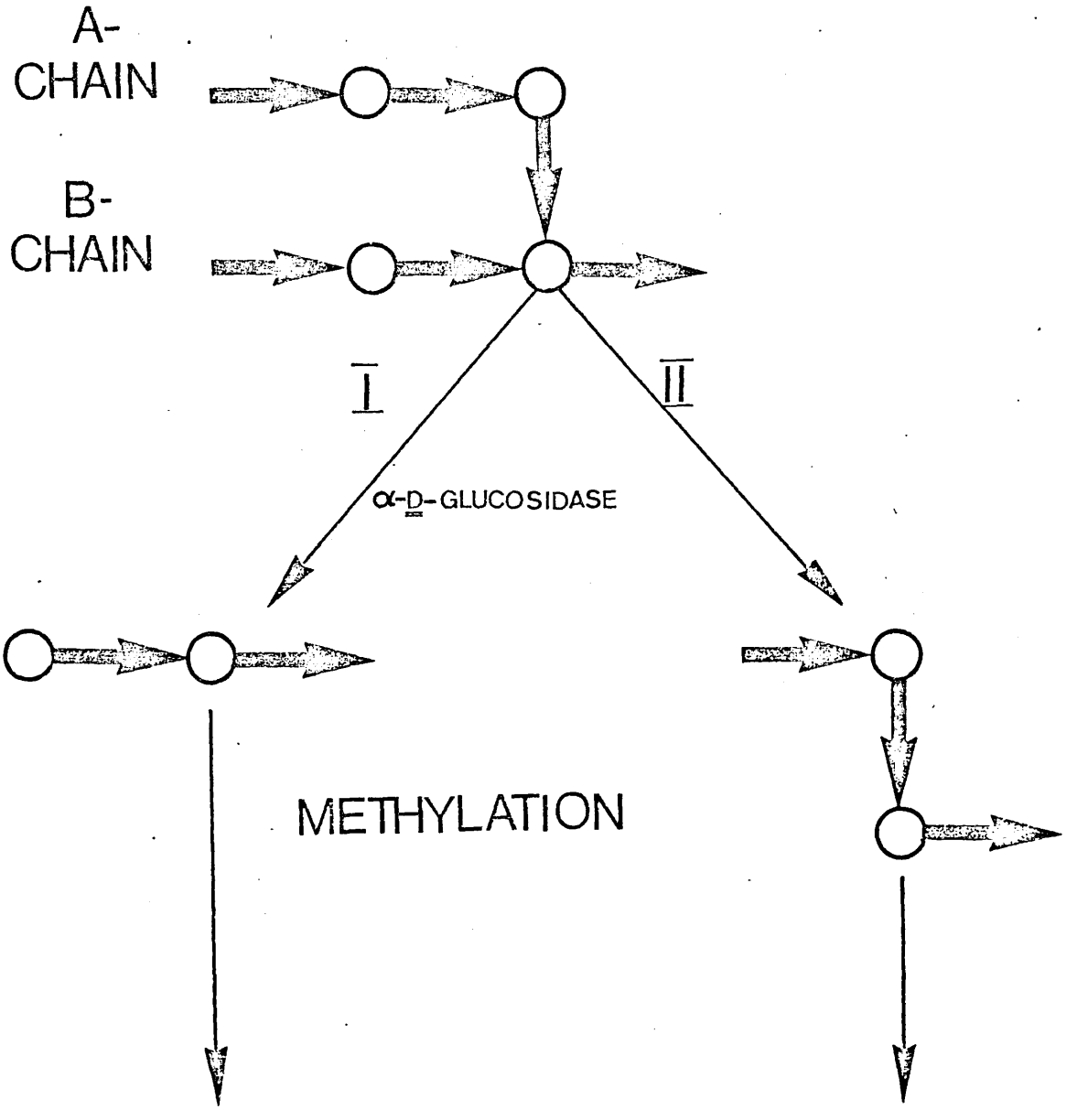
From the results discussed in Section 2-B, describing the properties of α-D-glucosidase from pig's liver, it has been shown that the enzyme shows a broad substrate specificity. That is, it is capable of hydrolysing the majority of α-linked glucosyl units in a variety of substrates. Therefore, in substrates such as amylopectin or glycogen they can be fully hydrolysed to D-glucose. Although α-D-glucosidase lacked the specificity of action of α- or β-amylase, it was decided to see in what manner the enzyme could be applied to the structural elucidation of the polysaccharide, amylopectin.

In both the branched polysaccharides amylopectin, and glycogen, three different types of chain are linked together. The A-chains are glycosidically linked to C-6 of glucose units in adjacent chains. The B-chains to which the A-chains are attached and the C-chain which carries the reducing group. The enzyme, α-D-glucosidase is an 'exo' type enzyme and thus liberates D-glucose from the non-reducing ends.

Fig. 1A

Linkage analysis of Amylopectin degraded by

α-D-Glucosidase



2,3,6 tri-O-methyl glucitol at all times

2,3,4-tri-O-methyl glucitol

It will, therefore, hydrolyse the A and B-chains from the non-reducing ends towards the branch points. Depending on the average number of glucosyl units in the A and B-chains, from the non-reducing end to the first branching unit, the enzyme must cease hydrolysing at or near the branch point if the B-chain is shorter than the A-chain (Fig. 1A). If not, then D-glucose would not be the sole product of the reaction. Alternatively, if the A-chains are shorter than the B-chains the enzyme would not need to cease hydrolysing the branch points for D-glucose to be the sole product. To investigate whether the enzyme did cease hydrolysing the B-chains at the branch point it was proposed to carry out a linkage analysis by methylation on the enzyme degraded polysaccharide. If indeed this occurred, in addition to the 2,3-di, 2,3,6-tri- and 2,3,4,6-tetra-O-methyl-glucitols expected from a normal linkage analysis of amylopectin, the 2,3,4-tri-O-methyl glucitol would be produced which could be separated from the other alditol acetates by G.L.C. and characterised by mass spectroscopy.

The procedure involved removing aliquots from an incubation containing α-D-glucosidase and amylopectin over a period of time, stopping the reaction, determining the amount of D-glucose liberated, and performing a linkage analysis on the degraded amylopectin. As the length of time that the incubation was carried out increased so greater volumes were removed. After dialysis had been performed to remove buffer ions and D-glucose, the degraded amylopectin was subjected to linkage analysis as follows:

a) methylation
b) hydrolysis
c) reduction
d) acetylation

Fig. 1B

% Degradation of Amylopectin

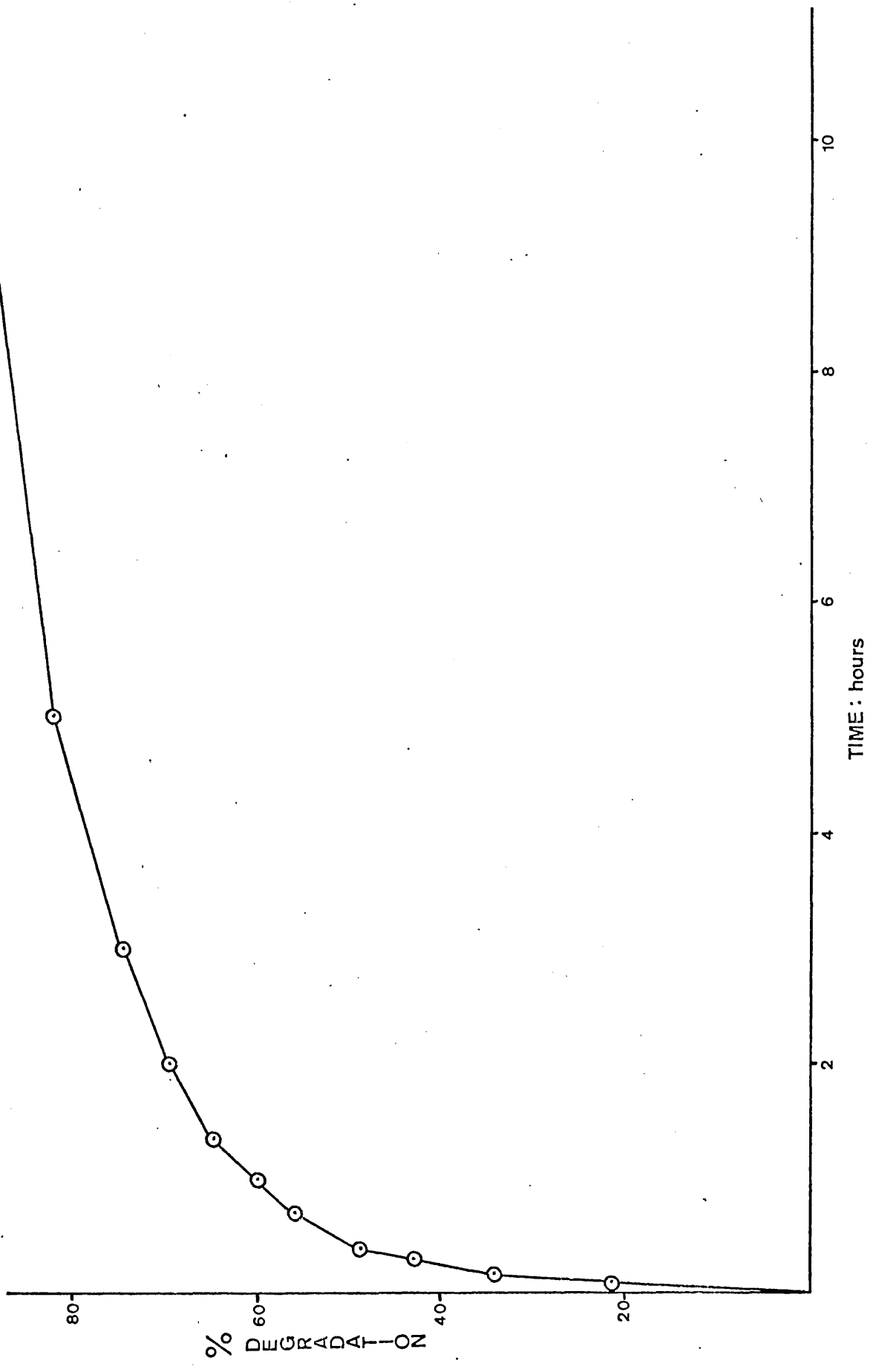
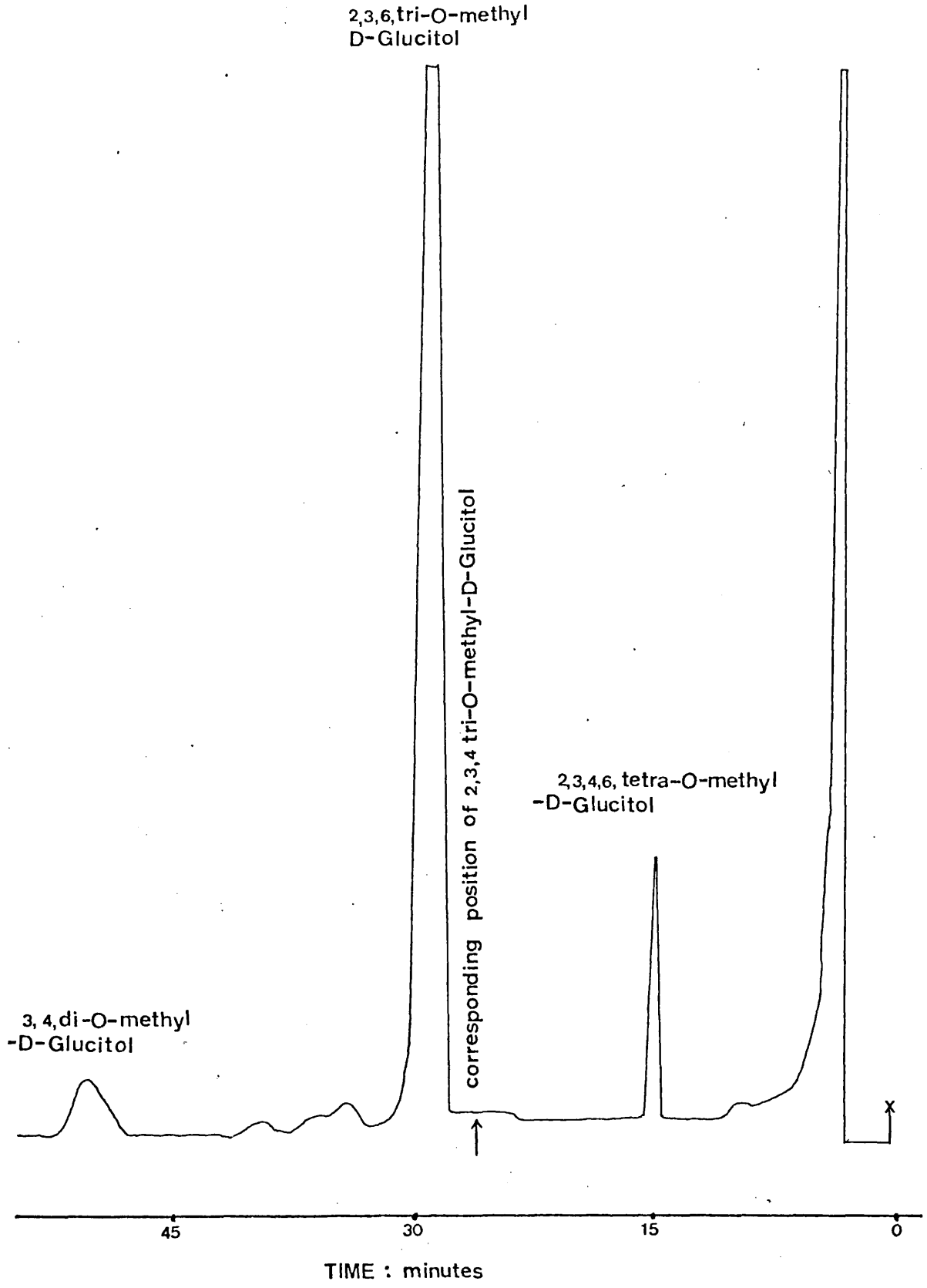


Fig. 1C

Separation of the alditol acetates derived from the linkage analysis of amylopectin



The procedure is described in Experiment 23. Fig. 1B shows the percentage degradation of amylopectin with time. All samples which were removed over a 20 hour period were subjected to G.L.C. using column a. and the relative areas of the di- tri- and tetra-O-glucitols determined. G.L.C.-mass spectrometry was carried out on six samples removed at time intervals between 4 to 40 minutes. The retention time for the 2,3,4-tri-O-methyl glucitol was found from the literature. A typical G.L.C. trace is shown in Fig. 1C indicating the position where mass spectroscopy was carried out approximating to the retention time of the 2,3,4 tri-O-methylglucitol. Fragmentation patterns of the alditol acetates are well documented and will not be discussed here.

The relative peak areas of the di-, tri- and tetra-O-methyl-glucitols, both of the blank undergraded amylopectin and the degraded samples were found to vary considerably. Although the average chain length (the ratio of tetra-O-methyl glucitol compared with the total of di- tri- and tetra-O-methyl glucose) of the blank sample was found to give a reasonable value of 23 glucosyl units, the end/branch ratio for all of the samples was found to vary considerably. This,therefore suggested that the methylation of the samples had not gone to completion. Little, or no 2,3,4 tri-O-methyl-glucitol was detected by mass spectroscopy in any of the degraded amylopectin samples.

These results were disappointing. It was therefore impossible from these results to gain any information regarding the action of the enzyme towards branch points. There is no reason to suppose that had methylation gone to completion that the objective of the experiment would have been fulfilled.

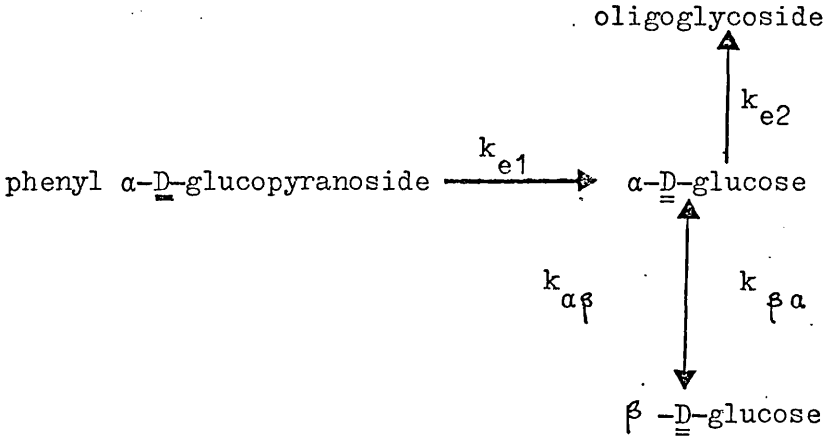
Appendix II

Further studies of the configuration of the product released during enzymic hydrolysis

The enzyme acid α-D-glucosidase has been found by various authors to catalyse a transglycosylation reaction.[16,20,26,29,33] That is, the transfer of D-glucose from a donor to an acceptor molecule. It was therefore considered that the enzyme might be capable of transferring D-glucose to phenyl α-D-glucopyranoside to produce an oligoglycoside.

During the study of the enzymic hydrolysis of phenyl α-D-glucopyranoside by nmr spectroscopy attention was drawn to the figures in Table 21. These concentration figures were derived from nmr spectra. The concentration of α-D-glucose and β-D-glucose after an incubation time of ∧80 minutes suggested that the β-anomer was present in a far greater concentration than expected from a mutarotated solution $\left(\frac{\beta \equiv}{\alpha \equiv} = 1.77\right)$. This therefore suggested that perhaps the α-anomer was being utilised in a transglycosylation reaction with phenyl α-D-glucopyranoside, catalysed by α-D-glucosidase. The following scheme was proposed:

A



Therefore this can be described by a mathematical model using the following equations:

a_1 $$\frac{ds}{dt} = -k_{e1}s$$

a_2 $$\frac{d\alpha}{dt} = k_{e1}s + k_{\beta\alpha}\beta - k_{\alpha\beta}\alpha - k_{e2}\alpha$$

a_3 $$\frac{d}{dt} = -k_{\beta\alpha}\beta + k_{\alpha\beta}\alpha.$$

a_4 $$\frac{dp}{dt} = k_{e2}\alpha$$

where s, α, β and p are

[substrate] (phenyl α-D-glycopyranoside),

[α-D-glucose], [β-D-glucose], and

[oligoglycoside], respectively.

These equations can be solved with the initial conditions

$$s(o) = s_o$$

$$\alpha(o) = \beta(o) = p(o) = 0$$

a_5 giving $\alpha_{(t)} = Ae^{\lambda_1 t} + Be^{\lambda_2 t} + Ce^{-k_{e1}t}$

a_6 $$(t) = -A\frac{(1+k_{e2})}{\lambda_1}e^{\lambda_1 t} - B\frac{(1+k_{e2})}{\lambda_2}e^{\lambda_2 t} - [s_o + C\frac{(1-k_{e2})}{k_{e1}}]e^{-k_{e1}t}$$

a_7 $$p_{(t)} = A\frac{k_{e2}}{\lambda_1}e^{\lambda_1 t} + \frac{k_{e2}}{\lambda_2}Be^{\lambda_2 t} - \frac{k_{e2}}{k_{e1}}Ce^{-k_{e1}t} + s_o$$

a_8 $$s_{(t)} = s_o e^{-k_{e1}t}$$

a_9 where $\lambda_{1,2} = -\frac{v}{2} \pm \sqrt{\frac{v^2}{4} - k_{e2}\, k_{\beta\alpha}}$

$$v = k_{\alpha\beta} + k_{\beta\alpha} + k_{e2}$$

a_{10} $$A = s_o \frac{\lambda_1 \lambda_2}{k_{e2}(\lambda_1 - \lambda_2)} - C\lambda_1 \frac{1 + \lambda_2/k_{e1}}{\lambda_1 - \lambda_2}$$

a_{11} $$B = s_o \frac{\lambda_1 \lambda_2}{k_{e2}(\lambda_2 - \lambda_1)} - C\lambda_2 \frac{1 + \lambda_1/k_{e1}}{\lambda_2 - \lambda_1}$$

a_{12} $$C = s_o \frac{k_{e1}(k_{\beta\alpha} - k_{e1})}{k_{e1}(k_{e1} - v) + k_{e2}\, k_{\beta\alpha}}$$

It can be noted that for $k_{e2} = 0$ this solution reverts to the previous model (Chapter I.2.B (C.II.) i.e. $p_{(t)} = 0$ and $\alpha_{(t)}$ $\beta_{(t)}$ $s_{(t)}$ are as in equations 5,6,7 (Chapter I.2.B(CII.). The rate constant k_{e2} has been established by making the least square fit to the unadjusted experimental results (Table 21) giving $k_{e2} = 16.5 \times 10^{-3}$ mn^{-1} as can be seen in Fig. 2A.

Fig. 2B shows the concentrations of the various species in solution, as found from the rate equations. As can be seen the concentration of α and β-glucose over a $2\frac{1}{2}$ hour period is very similar to those determined experimentally. This, therefore suggests that oligosaccharide formation does take place. This pathway becomes more involved in the overall reaction pathway in the later stages of the incubation.
An attempt has also been made to establish if the substrate is hydrolysed into β as well as α-D-glucose as shown;

Fig. 2A

Determination of K_{e2} by least square fit.

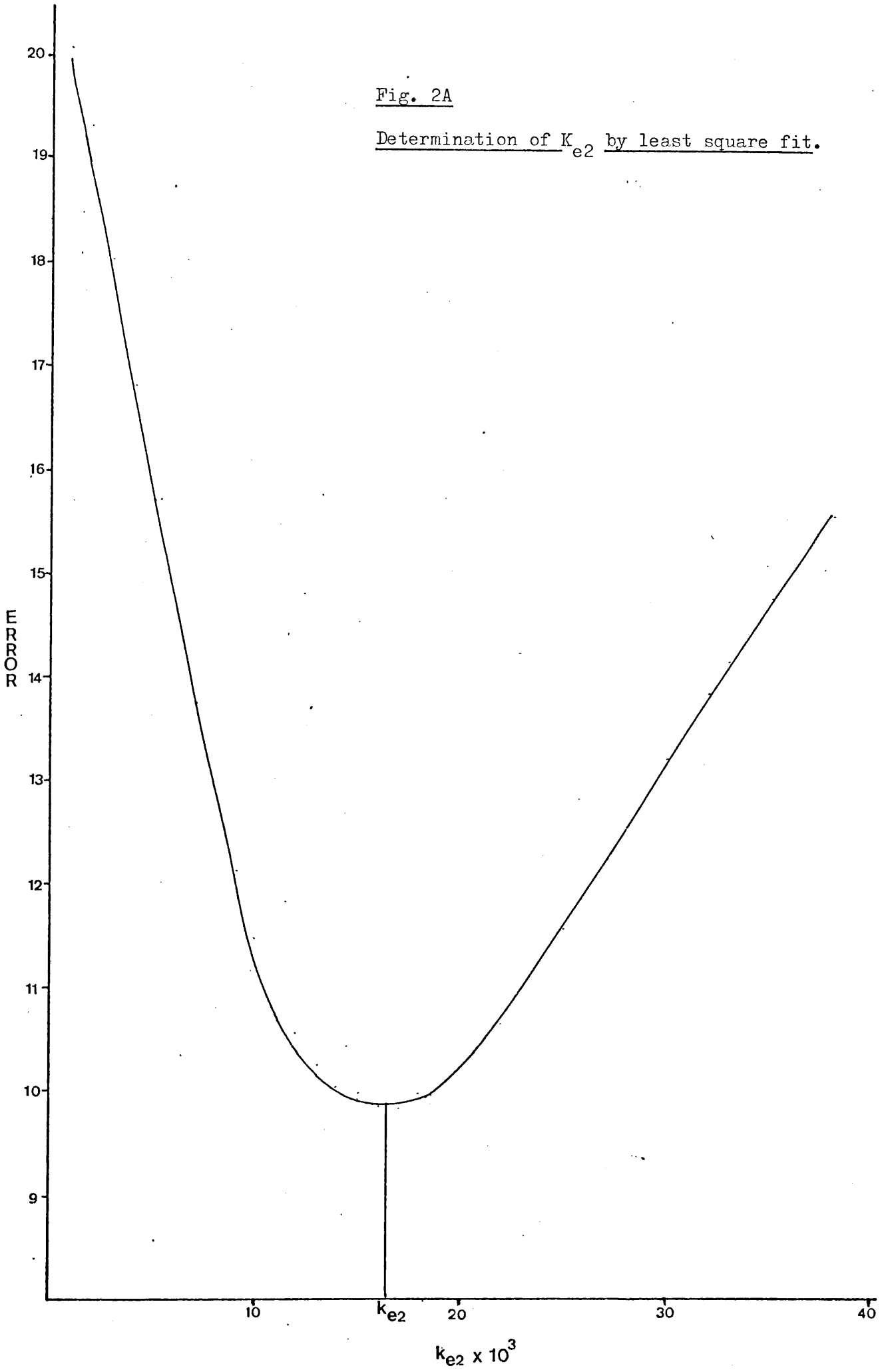
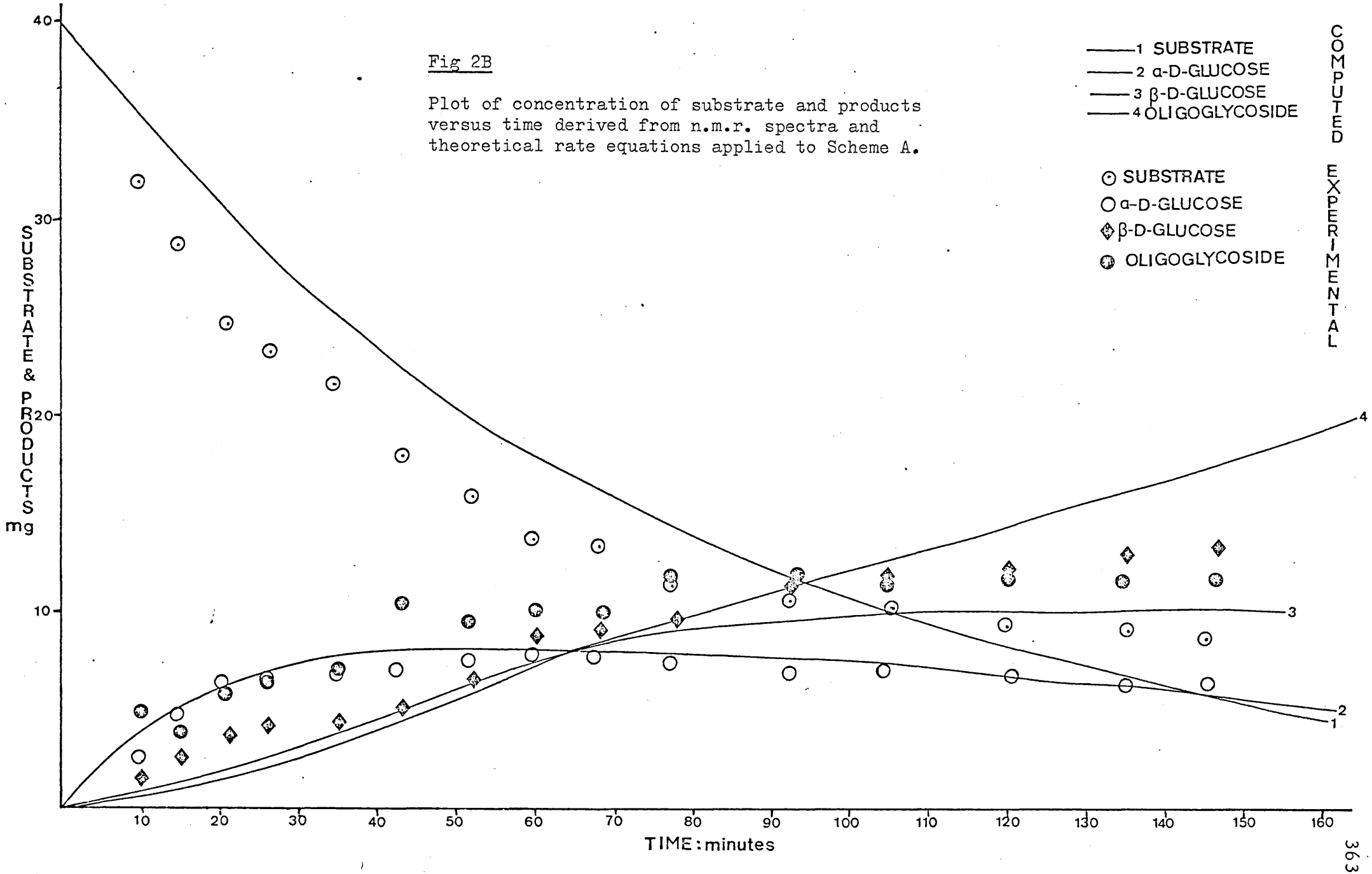
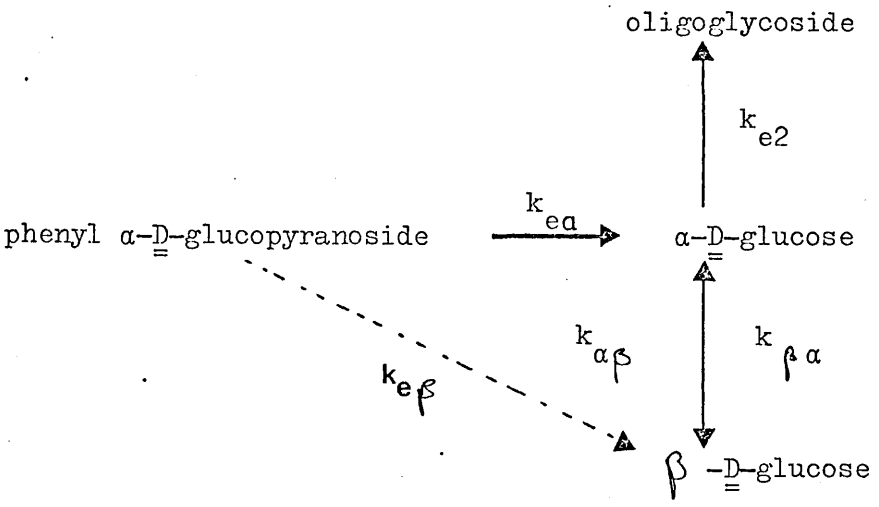


Fig 2B

Plot of concentration of substrate and products versus time derived from n.m.r. spectra and theoretical rate equations applied to Scheme A.



B



where of course $k_{e\alpha} + k_{e\beta} = k_{e1}$

This added reaction does not change equations a_1 and a_4 but equations a_2 and a_3 are modified as follows:

$$\frac{d\alpha}{dt} = k_{e\alpha} s + k_{\beta\alpha} \beta - k_{\alpha\beta} \alpha - k_{e2} \alpha$$

$$\frac{d}{dt} = k_e s - k_{\beta\alpha} \beta + k_{\alpha\beta} \alpha$$

The solution of these modified equations can be equally easily obtained giving the same general behaviour as in $a_5 \longrightarrow a_8$, but now the constants A, B and C are changed

$$C \text{ is now } = s_o \quad \frac{k_{e1}(k_{\beta\alpha} - k_{e\alpha})}{k_{e1}(k_{e1} - v) + k_{e2} k_{\beta\alpha}}$$

and A and B are now calculated with this new constant C. By varying $k_{e\alpha}$ between $0 \longrightarrow k_{e1}$ we can find $k_{e\alpha}$ by least square fit to the experimental results (Table 21). It can be seen (Table 2A) that the minimum error occurs at $k_{e\alpha}$ within 10% of k_{e1}. Therefore, it is safe to assume that the reaction; substrate into β-D-glucose is insignificant compared with the other pathways.

Table 2A

Least errors for $k_{e\alpha}$ and $k_{e\beta}$ proposed in Scheme B

	Error	$k_{e\alpha}$	$k_{e\beta}$
1	18.5	1.33	11.97
2	17.05	2.66	10.64
3	15.61	3.99	9.31
4	14.17	5.32	7.98
5	12.70	6.65	6.65
6	11.32	7.98	5.32
7	10.12	9.31	3.99
8	9.18	10.64	2.66
9	9.12	11.97	1.33
10	9.83	13.3	0.00