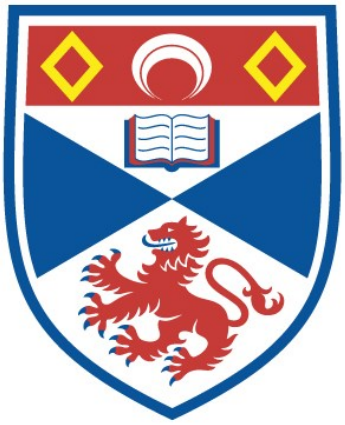
STUDIES ON GLUCOSE ISOMERASE FROM LACTOBACILLUS BREVIS

Maria Do Socorro Santos Ferreira

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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ABSTRACT

Studies on glucose isomerase from Lactobacillus brevis

Thesis presented by Maria do Socorro Santos Ferreira to the University of St. Andrews in application for the Degree of Doctor of Philosophy.

Biochemistry Department - June 1979

Glucose isomerase (E.C. 5.3.1.4) was extracted from Lactobacillus brevis N.CD.O 474 grown in xylose containing medium with a yield of cells (dry weight) of 2.3 - 3.3g.l^{-1} of medium and 300-310 glucose isomerase units. l^{-1}.

Several methods for releasing the intracellular enzyme were investigated and the specific activity recovery was highest with the heat autolysis method. The crude extract preparation was further purified by nucleic acid precipitation with $MnCl_2$, protein fractionation by ammonium sulphate and dialysis followed by chromatography on CM-cellulose, DEAE-cellulose and gel filtration on Sephadex G-200. A final purification of 24 fold was achieved with about 25% activity recovery in 4 purification steps as follows: enzyme extraction by heat autolyis , $MnCl_2$ treatment (nucleic acid precipitation), ammonium sulphate (2-3.6M pH 7.0) protein precipitation and CM-cellulose chromatography.

A mol. wt. of approximately 120,000 was calculated for the purified enzyme by gel filtration (Sephadex G-200) which dissociated in small subunits with mol wt. of 54,000-42,600 calculated by electrophoresis on 5% polyacrylamide - 3% SDS-8M urea.

The purified enzyme was immobilised with a PEI-derivative of nylon (polyethyleneimine) and the kinetic properties of both free and immobilised enzyme were investigated. Apparent Km values for the free purified enzyme were 7.4 x 10^{-3}M(D-xylose); 2.8M(D-glucose); 1.9M(D-fructose). The corresponding apparent V values were 0.45; 0.015 and 0.022 µmoles min^{-1}. µg $enzyme^{-1}$ respectively. Investigations were also carried out into several other possibilities of assaying glucose isomerase activity. Parameters for the coupled reaction assay system using sorbitol dehydrogenase -NADH were optimised.

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to the University of St. Andrews
in application for the degree of
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The University,
St. Andrews.

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CERTIFICATE

I hereby certify that Maria do Socorro Santos Ferreira has spent nine terms engaged in research work under my direction and that she has fulfilled the conditions of Ordinance General No. 12 and of the Resolution of the University Court 1967, No.1, and that she is qualified to submit the Accompanying thesis for the degree of Doctor of Philosophy.

DECLARATION

I hereby declare that this thesis is based on work carried out by me, that the thesis is of my own composition and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry, University of St. Andrews, under the direction of Dr. W.M. Ledingham.

ACADEMIC RECORD

I graduated with the degree of Bachelor of Pharmacy in 1967 and with the degree of Master of Science (Biochemistry) in 1973, both from the Federal University of Pernambuco, Recife, Brazil.

I matriculated as a research student in the Department of Biochemistry, University of St. Andrews, in October 1974.

ACKNOWLEDGEMENT

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I am also grateful to the British Council and to the Universidade Federal de Pernambuco, for financial support.

ABBREVIATIONS

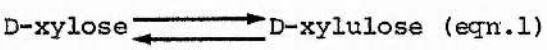
Bis	N,N^1 methylenebisacrylamide
TEMED	N,N,N^1,N^1-tetramethylenediamine
Tris	Tris-hydroxymethyl-aminomethane

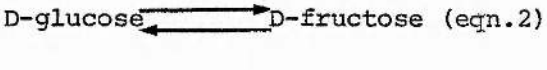
1. INTRODUCTION

1.1 General Introduction

Glucose isomerase entered the systematic enzyme nomenclature as xylose isomerase (D-xylose ketol isomerase) E.C. 5.3.1.5 because the enzyme discovered in cells of pseudomonas hydrophila (1) seemed to be specific for D-xylose.

Glucose isomerase is an intracellular enzyme that catalyses the reversible isomerisation reaction between:





Depending on the source, it can also catalyse the isomerization of D-ribose (2,3,4,5). Although the rate of isomerisation was much higher with D-xylose (eqn.1) than with D-glucose (eqn.2), the industrial importance of glucose isomerase is based on the isomerisation of D-glucose. The technological development of the production of a high fructose-glucose syrup (HFGS) was a consequence of an already established saccharification production of glucose syrup from potato or corn starch (6,7) and also as result of the world price of sucrose (7). In the United States, for example, corn starch is a plentiful and low cost raw material which made the production of fructose-glucose syrup highly competitive with the price of sucrose (7).

The hydrolysis of starch has been obtained in two steps:

1. Liquefaction step, in which the viscosity of starch has been reduced by partial hydrolysis with acid and α-amylase (8) or only by α-amylase action (9).

2. Saccharification step, catalysed by amyloglucosidase, a fungal enzyme (8,9,10,11).

Following the refining steps, the glucose syrup has been isomerised to about 42% fructose using glucose isomerase immobilised with insoluble supports (8,12,13,14,15) or fixed intracellularly (16,17,18). During isomerisation, the use of a series of reactors in parallel enabled a more efficient use of immobilised enzyme and allowed recharging of reactors as necessary with minimal disruption to production. Such a process has been used to produce upwards of 500,000 tonnes of a high fructose-glucose syrup (HFGS) commercially called Isomerose ^{R}100, in a computer-controlled production system (8,19).

Fig. 1.1 and Table 1.1.1 shows the industrial production and composition analysis of Isomerose ^{R}100, respectively.

Until recently, the industrial use of the chemical isomerisation of D-glucose in alkaline conditions, suffered the disadvantage of sugar degradation (6). However, it was found that this degradation could be substantially reduced by using a short period, high temperature isomerisation process (6). Table 1.1.2 shows the production of fructose and concomitant sugar degradation in both alkaline and enzymic isomerisations.

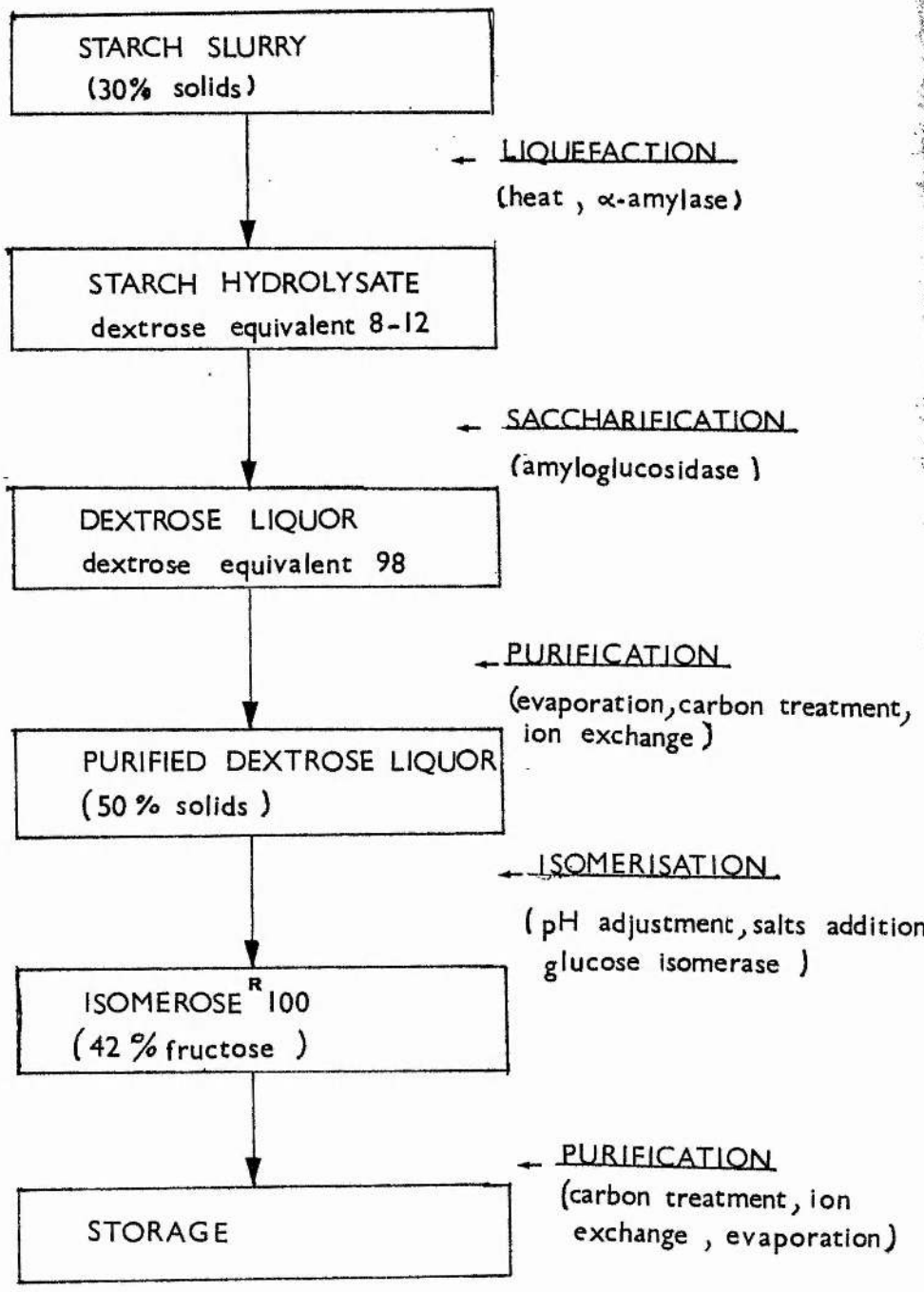


Fig. 1.1 High fructose glucose syrup process flow sheet.

TABLE 1.1.1 Composition of Isomerose ^{R}100 (a high fructose-glucose syrup) and Invert sugar.

Sugar	Composition (%)		Reference
Isomerose ^{R}100	glucose	50	
	fructose	42	
	Other saccharides	8	
	Ash (sulphated)	0.03	8
Invert sugar (from sucrose)	glucose	40	
	fructose	40	
	sucrose	20	20

Relative sweetness: Isomerose ^{R}100 ≡ 15% sucrose.

TABLE 1.1.2 Fructose production and sugar degradation (alkaline and enzymic isomerisation)

Isomerisation process	Reaction time range (min)	Reactor Type	Fructose production (%)	Sugar degradation (%)	Reference
NaOH (0.8 - 1.0%)		continuous			
w/v solution	5-75	flow	33-35	1.0-3.0	6
glucose isomerase					
immobilised	120-140	batch	42-45	1.0-1.4	8
		continuous			
		flow		0.4	8,12

1.2 Sources of glucose isomerase activity

The first reported source of glucose isomerase was Pseudomonas hydrophila grown in a xylose-containing medium. Subsequently the enzyme has been discovered in barley malt (21), wheat (22) and a very wide variety of microorganisms including many strains of Lactobacillus (23) and Streptomyces (8,16,24-38). Other microbial sources of glucose isomerase were reviewed by Hamilton et al (20) and Burke (39).

Table 1.2.1 shows some microbial sources of glucose isomerase and Table 1.2.2 shows production data for the main microorganisms producing glucose isomerase.

Most proteins displaying glucose isomerase activity are probably either glucose-6-phosphate isomerases (phosphoglucose isomerase) or xylose isomerases. Glucose isomerase induced in Lactobacillus brevis (23), Bacillus coagulans (45,46) and in several species of Streptomyces (20,24-38) exhibited D-xylose isomerase activity and the isomerisation of D-glucose showed no requirement for arsenic ions.

On the other hand, glucose isomerase induced in Pseudomonas hydrophila (1), Aerobacter cloacae (40), Aerobacter aerogenes (43) and Escherichia intermedia HN-500 (47) showed a requirement for arsenic ions for glucose isomerase activity.

Production of glucose isomerase varies with different microbial sources and growth media and much effort has gone into strain improvement through genetic and medium composition

TABLE 1.2.1 Microbial sources of glucose isomerase

Microorganism	Reference
Aerobacter cloacae HN-69	40,41,42
Aerobacter aerogenes HN-56	43
Actinoplanes missouriensis NRRL-B-3342	44
Bacillus coagulans HN-68	45,46
Escherichia intermedia HN-500	47
Lactobacillus brevis	23
" pentoaceticus	23
" fermenti	23
" mannitopoeus	23
" gayonii	23
" lycopersici	23
" bruchneri	23
Leuconostoc mesenteroides	23
Microbispora rosea nonnitritogenes	48
Pseudonocardia Sp.	49
Paracobacterium aerogenoides	50
Streptomyces albus	8,16,24-28
" flavovirens IFO 3197	29
" flavogriseus	30
" griseolus CL71	31
" olivaceus NRRL 3916	32
" olivochromogenes ATCC 21114	33
" phaeochromogenes	34,35,36
" wedmorensis ATCC 21175	32,37

TABLE 1.2.1 (contd) Microbial sources of glucose isomerase

Microorganism	Reference
Streptomyces venezuelae ATCC 21113	38
Streptosporangium album	51
Thermoactinomyces Sp.	49
Thermopolyspora Sp.	49
Thermomonospora Ap.	49

TABLE 1.2.2 Production data for the main microorganism producing glucose isomerase

Microorganism	Enzyme production				Yield (litre of medium)		Reference
	Inducer (%)	pH	°C	harvesting time	cells(g)	glucose isomerase units (μmoles.min^{-1})	
B. coagulans HN-68	xylose (2)	7.0	40	late log phase	4	86.17	45
L. brevis ATCC 8287 or IFO 3960	xylose (1) glucose (0.1)	5.5-6.0	37	" " "	-	20.72	4,56
L. brevis	xylose (2) glucose (0.5)	5.5	30	" " "	2.3-3.1	1353	53,54
S. albus	xylan (0.5) corn steep liquor (2)	7.0	30	" " "	10	2031	16,24
S. flavovirens IFO 3197	xylose (0.6) glycerol (0.5)	7.1	28	" " "	5.3	1070	29

manipulation. The treatment of active growing cultures with toxic agents such as ethyleneimine, hydrogen peroxide, 8-ethoxy-caffeine and radiation from u.v. light or radio-active isotopes has provided mutants with increased yield of glucose isomerase (20).

Improvement in the yield of cells has also been achieved by manipulation of the growth medium (39). Kent, (53,54) working with Lactobacillus brevis, reported a 3 to 4 fold increase in glucose isomerase yield as compared to that achieved by Yamanaka (52). Kent's increased yield however is likely to be an increased yield of cells rather than an increased level of enzyme per cell as his medium contained the same ingredients as Yamanaka's (apart from bacteriological peptone) but in much higher concentrations. A mutant from Streptomyces flavovirens with about 4 fold increase in the glucose isomerase yield was obtained by both improvement in the medium composition and screening (29).

In the majority of the microorganisms investigated, the induction of glucose isomerase activity required D-xylose as the carbon source. Nevertheless, most of the Streptomyces strains were able to utilise xylan-containing components because they synthesise the enzyme xylanase, which hydrolyses xylan to xylobiose. Xylobiose is a good inducer of glucose isomerase, at the concentration of 0.02% (16,28).

Among the xylan-containing materials, wheat bran and corn hulls were good replacements for D-xylose in those microorganisms

that have the enzyme xylanase (16). Streptomyces albus for example, give a yield of 2.78 glucose isomerase units. l^{-1} of medium containing wheat bran as compared with the yield of 2.36 glucose isomerase units. l^{-1} of a 1% D-xylose medium while streptomyces flavovirens TKK1 produced a very low yield of glucose isomerase in wheat bran and xylan medium (16,29).

Table 1.2.3 shows the effect of some xylan-containing material on the production of glucose isomerase by Streptomyces albus.

In Streptomyces phaeochromogenes the partial replacement of xylose by glucose or starch improved the production of glucose isomerase (35) while in Streptomyces flavovirens TKK1 the enzyme yield was repressed by glucose or starch and increased by the addition of glycerol (29). Mutants of Streptomyces olivochromogenes ATCC 21114 (57) and Arthrobacter NRRL-B-3725, 3726 and 3727 were able to produce glucose isomerase in the absence of xylose (58) and cobalt ions (57).

Organic nitrogen sources also effected the yield of glucose isomerase. Corn steep liquor, casein hydrolysate were effective nitrogen sources (24,29). The effect of the organic nitrogen source was evident with Actinoplanes missouriensis NRRL-B-3342 where the enzyme yield fell by a factor of 10 fold or more, depending on the source of the organic nitrogen (44). Bacillus coagulans HN-68 also had the glucose isomerase production affected by the nitrogen source (45).

TABLE 1.2.3 Effect of Xylose and Xylan-containing material on the induction of glucose isomerase in S. albus.

D-Xylose (%)	Xylan-containing material (%)		G.I. Yield U/litre of medium
1	-		2.36
-	Wheat bran	1	0.91
	"	2	1.84
	"	3	2.78
	"	4	2.05
-	Corn hulls	1	1.26
	"	2	1.99
	"	3	2.11
	"	4	0.51
-	Corn cobs	1	1.43
	"	2	1.93
	"	3	1.89
	"	4	0.72

Calculated from data on Tables 1 and 2 of reference 16.

1.3 Activators, Inhibitors and specificity of glucose isomerase

The addition of cobalt ions increased the thermal stability and activity of glucose isomerase from Streptomyces Sp. (5,16,18,28,34,59). The optimum concentration of cobalt and magnesium or manganese ions depended on the glucose isomerase source. The enzyme from S. phaeochromogenes had the maximum activity with 2×10^{-2}M magnesium ions. Cobalt ions had an activating effect only when the magnesium concentration was greater than 10^{-2}M. Tris buffer, at a concentration of 5×10^{-2}M, inhibited glucose isomerase activity about 10% while sodium arsenate (10^{-2}M) increased the activity about 10%. The optimum pH was between 9.0 - 9.5 and the equilibrium constant at 60°C was approximately 1.0 (34).

However, the enzyme from the mutant S. phaeochromogenes NRRL-B-3559 was not inhibited by Tris buffer and the optimum pH was about 9.0 with 5×10^{-2}M Tris buffer. This enzyme also required magnesium ions and a K_m value of 2.5×10^{-2}M was calculated for magnesium ions in presence of 0.8M D-glucose and 5×10^{-2}M Tris buffer pH 8.0 at 70°C. An increase of approximately 30% of the activity was achieved by the addition of 5×10^{-5}M cobalt ions (59).

Cobalt ions, at a concentration of 10^{-3}M, stimulated the formation of glucose isomerase during cell growth (24) and also acted as an effective protector of enzyme activity during its extraction (16,53) and purification involving heating steps from L. brevis (3,4,54) and Streptomyces (34).

Higher cobalt concentrations have an inhibitory effect on the enzyme from Streptomyces (14) increasing the K_m value for D-glucose (60). Both cobalt and magnesium ions were found in the structure of the purified glucose isomerase from S. albus (16,26). In the crystalline enzyme the contents were about 4.1 and 33 atoms of cobalt and magnesium respectively, per mole of enzyme. After filtration through Sephadex G-200, the contents dropped to about 1.4 and 0.3 atoms of cobalt and magnesium respectively, per mole of enzyme.

Glucose isomerase from S. albus showed no decrease in activity when held at pH's in the range 4.5 to 11 for 3 h at 20°C. The optimum pH for activity was 8.0 - 8.5 in phosphate buffer, and the optimum temperature for maximal catalytic activity was 80°C but this temperature corresponded with a fall of 20% of the original activity in 10 min. The equilibrium constant ([Fructose]/[glucose]) shifted from 0.74 to 1.30 as the temperature increased from 25°C to 70°C. The addition of borate at a molar ratio of 5:2 hexose:borate, induced an increase of 34-36% of fructose yield (27).

The enzyme from S. phaeochromogenes (24), S. phaeochromogenes NRRL-B-3559 (59) and S. albus (16,26) were specific for D-xylose and D-glucose. The Michaelis constant (K_m) for D-glucose was of the order of 10^{-1}M (see Table 1.3.1). No activity was detected with D-glucose-6-phosphate, D-mannose, D-galactose, L-arabinose and D-ribose. Cysteine, sodium

fluoride, sodium azide, sodium pyrophosphate, sodium arsenite, EDTA and dipyridyl at the concentration of 10^{-3}M did not inhibit the activity of glucose isomerase from S. phaeochromogenes (34).

The crystalline enzyme from Bacillus coagulans HN-68 (61) lost activity after treatment with EDTA at a concentration of 10^{-2}M. The addition of cobalt ions were essential to D-glucose and D-ribose isomerisation while manganese ions stimulated D-xylose isomerisation (2,61). Manganese (10^{-2} - 10^{-5}M), cobalt (10^{-3}M) and nickel (10^{-5}M) protected the enzyme against thermal inactivation (61). Cobalt ion at 10^{-2} and 10^{-3}M concentration inhibited the activity when added to reaction mixtures containing manganese, cupric, zinc or calcium ions at the same concentration (2).

In common with the enzyme from Streptomyces (16,18,28, 34,59), glucose isomerase from B. coagulans HN-68 preferentially utilises D-xylose as substrate (61). The apparent K_m values for D-xylose, D-glucose and D-ribose were 1.1 x 10^{-3}M, 0.9 x 10^{-1}M and 0.77 x 10^{-1}M respectively. The isomerisation of D-glucose was inhibited by D-xylose and D-ribose. All three isomerase activities (xylose, glucose, ribose) were competitively inhibited by tris (hydroxymethyl) aminomethane, D-xylitol, D-sorbitol and D-mannitol. The K_i values were 3 x 10^{-4}M, 2.5 x 10^{-3}M, 3 x 10^{-2}M and 7 x 10^{-2}M respectively (61).

An activation energy of 14,600 cal. mol^{-1} was found with D-xylose, D-glucose and D-ribose. The optimum pH in barbital

buffer was 8.0 - 8.5 for D-xylose isomerisation and 7.0 for D-glucose and D-ribose isomerisation. The three activities were stable in a pH range between 5.0 and 7.0 but complete inactivation occurred at pH's below 4.0. A loss of 20% of the initial activity was observed at pH 4.5 and 9.0 (61).

The enzyme from B. coagulans was inhibited by zinc, cupric and mercuric ions (45). Sulphydryl, oxidizing and chelating reagents at 10^{-3}M final concentration had little or no inhibitory effect on glucose isomerase from L. brevis (62). The enzyme was, however, inactivated after longer treatment with 10^{-3}M EDTA followed by dialysis against distilled water (62). The full activity was restored by the addition of manganese ions. Protection against thermal inactivation was achieved by the addition of manganese at 10^{-2}M or cobalt at 10^{-3}M concentration. Potassium chloride, bromide or sulphate ions were also effective in protecting against thermal inactivation (62). Manganese and cobalt ions also improved the production of glucose isomerase in L. brevis cells (52).

The enzyme from L. brevis was inhibited by high concentration of D-glucose (53, 54, 56) and by sorbitol and xylitol (54). The inhibition by xylitol was competitive with K_i values of 1.5×10^{-3}M and 4.5×10^{-3}M being calculated in presence of D-xylose and D-glucose as substrate (56).

Glucose isomerase preparations from B. coagulans HN-68 (61), L. brevis (4,53) were active on D-glucose, D-xylose and D-ribose while the enzyme from Streptomyces (16) had no activity on D-ribose. K_m values for D-glucose were higher

than for D-xylose for most of the glucose isomerase preparations (4,16,20,61), with the exception of the crude preparation from S. albus NRRL-5778 (5).

The partially purified glucose isomerase preparation from L-brevis (53,54) did not follow Michaelis kinetics and inhibition by substrate was apparent from above 1.67M D-glucose. K_m values for D-glucose of 0.52M (56), 0.92M (3) and 2.07 - 2.06M (54) were calculated in presence of saturating manganese and cobalt ions concentration. Table 1.3.1 shows apparent K_m values calculated for glucose isomerase from several microbial sources, indicating the glucose concentration range used, ion concentration, buffers, pH and degree of purity of the enzyme preparation. The equilibrium constant for glucose isomerisation was 1.6 and was independent of temperature in the range of 35°C - 60°C (54).

The glucose isomerase induced in Actinoplanes missouriensis NRRL-B-3342 was also able to isomerise D-ribose in addition to D-glucose and D-xylose. The maximal activity was achieved at pH 7.0 and with cobalt and magnesium ion concentrations of 3×10^{-4}M and 3×10^{-3}M respecitvely (63). Glucose isomerase from Arthrobacter NRRL-B-3726, 3727 and 3728 mutants did not require cobalt ions and was stable at 90°C (58).

Takasaki's reported a glucose isomerase which required NAD^+ and magnesium ions as cofactor. The enzyme was able to isomerise D-glucose and D-mannose to D-fructose and was inhibited by sulphydryl reagents and EDTA (50).

TABLE 1.3.1 The Michaelis constant (K_m app) of glucose isomerase from several microorganisms.

Microorganism	Enzyme Preparation	(Km(app.) (M)	Assay Parameters					pH	°C	Reference
			Glucose(M) [range]	Mg^{++}(M)	Mn^{++}(M)	Co^{++}(M)	Buffer(M)			
B. coagulans	purified (crystallised)	0.09	0.05-1.0	-	-	0.03	Barbital(0.02)	7.0	40	61
L. brevis	purified	0.52	0.05-1.0	-	0.01	0.01	Tris(0.05)	7.0	40	56
"	"	0.92	0.16-2.0	-	0.005	0.005	Maleate(0.025)	6.0	35	3
L. brevis NCDO 474	crude	0.80-1.6	0.13-2.0	-	0.01	0.001	Tris(0.02)	7.0	50	53
"	partially	0.96-1.42	0.10-2.0	-	"	"	"	"	"	55
	purified	2.07-2.66	0.10-2.0	-	"	"	"	"	"	54
Streptomyces sp.	partially									
	purified	0.14	0.028-0.2	0.09	-	-	Phosphate(0.05)	7.0	60	25
S. albus	purified (crystallised)	0.16	0.01-0.25	0.01	-	-	"	7.0	70	16,26
S. albus NRRL-5778	partially									
	purified	0.086	-	0.08	-	0.0004	Potassium phosphate(0.2)	7.2	70	5
S. phaeochromogenes	partially									
	purified	0.3	0.1-1.5	0.1	-	0.001	Ammonium(0.05)	9.0	60	34

1.4 Recent Purification Techniques

Table 1.4 shows the main steps that have been used to purify the glucose isomerase produced by several microorganisms. As glucose isomerase is an intracellular enzyme, the crude extract has been prepared by rupture of the cell wall followed by centrifugation of the cell debris.

A mixture of toluene and crystalline lysozyme was used to lyse the cell wall of Bacillus coagulans HN-68 at 20° for 20 h (2).

Grinding of L. brevis cells with alumina followed by the enzyme extraction with 0.02M Tris buffer (3,54,56) was less effective than heat autolysis at 40°C for 20 h (54).

However the temperature used was critical because higher temperatures resulted in fixation of the enzyme molecules within the cell (16). The release of G.I. from S. albus cells was accelerated by the addition of a cationic surface agent (16,26).

After purification of the crude extract from Bacillus coagulans HN-68 and S. albus (see Table 1.4) the enzyme preparation was homogenous on both ultracentrifugation and electrophoresis criteria (2,64). Approximately forty-five and ten fold purifications were achieved respectively with the preparation from B.coagulans (64) and S. albus (16). The activity recovery was about 46% and 2% respectively.

The enzyme from B. coagulans HN-68 was crystallized from ammonium sulphate solution and from acetone (64). In addition S. albus enzyme was crystallized from acetone (16) and L.brevis enzyme from ammonium sulphate solution (3,4).

The development of an affinity chromatography system with a Xylitol-Sepharose 4B derivative improved approximately ninefold the specific activity of a preparation of glucose isomerase contaminated with bovine serum albumin. The enzyme was eluted with 0.2M NaCl after the added bovine serum albumin had been washed through with buffer (65).

TABLE 1.4 Purification of G.I. from microbial sources

Microorganism	Purification Steps	Total Purification Fold	Activity Recovery (%)	Reference
B. coagulans HN-68	1. Crude extract 2. Mn treatment 3. $(NH_4)_2SO_4$ (0.0-0.5, pH 4.5-4.7) 4. DEAE-Sephadex a) pH 6.0 b) pH 8.6 5. Crystallisation	44.49	46.24	64
L. brevis	1. Crude extract 2. Mn treatment 3. $(NH_4)_2SO_4$ (0.45-0.95, pH 6.8-7.2) 4. Heat treatment 5. Acetone (20-40%) pH 5.0 6. DEAE-Sephadex pH 7.4 7. $(NH_4)_2SO_4$ (0.6-0.9) 8. Crystallisation	11.19	21.74	3
S. albus	1. Crude extract 2. Acetone (44-67%) pH 7.0 3. DEAE-cellulose pH 7.0 4. DEAE-Sephadex pH 7.0 5. Crystallisation 6. Sephadex G-200	10.10	2.04	16
S. griseolus CL 71	1. Crude extract 2. Acetone 3. DEAE-cellulose 4. Sephadex G-200	12.6	41.6	31
	1. Crude extract 2. Affinity chromatography	9.0	-	65

1.4.1 Molecular Weight and Evidence of Oligomeric Structure of Glucose Isomerase

Molecular weights from 157,000 to 165,000 was calculated from sedimentation (26,66) and diffusion measurements (26) for the enzyme from S. albus. Crystallographic data (66) and sedimentation analysis of the enzyme treated with 6M guanidine-HCl indicated that the molecule was composed of four identical or very similar subunits (66). There was no evidence of disulphide bridge interchains and the amino acid composition analysis indicated that cysteine was the limiting amino acid residue (66).

The molecular weight of the enzyme from B. coagulans HN-68 was calculated to be 175,000 or 160,000 by sedimentation-viscosity method and the gel filtration respectively (64). It was reduced to 49,000 after preincubation of the enzyme preparation with sodium dodecyl sulphate (SDS) at acidic pH or at pH 7.0 in the presence of manganese or cobalt ions (68). Treatment of the enzyme with 8M urea did not dissociate the subunits and the enzyme activity was completely recovered by dilution of the urea. However the inactivation was irreversible after treatment with 5.8M guanidine-HCl (68).

Also the enzyme preparation from L. brevis had a high molecular weight of 191,000-197,000 calculated from the sedimentation and sucrose gradient methods respectively (4). Table 1.4.1 summarises the molecular weights of glucose isomerases from microbial sources and methods employed in their determination.

TABLE 1.4.1 Molecular Weights (mol.wt.) of glucose isomerase from several microbial sources.

Microorganism	Purified enzyme preparation	Method	mol.wt.	Reference
S. albus	(untreated)	sedimentation	165,000	66
	treated with 6M guanidine-HCl	sedimentation	43,000	66
	S-carboxymethyl derivative	SDS-electrophoresis	40,000	66
S. albus	(untreated)	sedimentation/viscosity	157,000	26
	treated with 2-methyl-2,4 pentanediol	crystallography	39,000	67
B. coagulans	(untreated)	sedimentation	175,000	64
	(untreated)	viscosity	160,000	64
	treated with 0.05% SDS	SDS-electrophoresis	49,000	68
L. brevis	(untreated)	sedimentation	191,000	4
	(untreated)	sucrose gradient	197,000	4

1.4.2 Glucose Isomerase Mechanism of Action

The aldose-ketose isomerisation mechanism has been proposed to operate via an enediol intermediate and base catalysis (69,70). In the case of the enzyme phosphoglucose isomerase (EC 5.3.1.9) for example, it has been suggested that the protonated amino group of lysine catalyses the opening of the hexose ring structure which is isomerised via formation of an enediol intermediate with the non-protonated nitrogen of the imidazole group (71).

The enediol intermediate is also in evidence in isomerisation reactions catalysed by bacterial glucose isomerase (70,72,73). The enzyme has specificity for D-aldoses with 5 or 6 carbons and a cis configuration of hydroxyl groups at C_2 and C_4 (3).

The anomeric specificity of glucose isomerase from streptomyces sp. is for the α-D-anomer (72,73). An increase of approximately 3-fold was calculated for the K_m measured for an anomeric mixture of D-xylose (K_m = 10.4 x 10^{-3}M) as compared with that measured for α-D-xylose (K_m = 3.0 x 10^{-3}M) (73). The isomerisation of D-xylose proceeded via 1,2 proton transfer with no solvent exchange and the initial product had an α-configuration which was clearly indicated by nuclear magnetic resonance (72).

Table 1.4.2 illustrates the anomeric specificity of glucose isomerase as compared with other isomerases.

Phosphoglucose isomerase was also specific for the α-anomer (72,74) but the product of the isomerisation reaction was an equilibrium mixture of the α and β anomers (72). probably because of the additional presence of an anomerase in yeast phosphoglucose isomerase (74).

As with glucose isomerase, the rate of isomerisation of the β-anomer was much slower although studies with active-site labelled phosphoglucose isomerase indicated that both anomers reacted at the same enzyme site (75). It was suggested that torsion around C-C bonds of the β-anomer would bring both anomers to the same cis-enediol intermediate (75).

Studies on the role of metal ions in the isomerisation catalysed by glucose isomerase from B. coagulans suggested that cobalt and manganese ions were bound at different sites and that the binding of the substrate to the enzyme was independent of the metal ion (76). The interaction of manganese ions with the enzyme from L. brevis and Streptomyces sp., proceeded via a binary complex where the binding enzyme-manganese was very strong (77). However the ternary complex, enzyme-manganese-substrate or inhibitor had weak binding (77). As it has been observed that inhibition by high concentration of cobalt occurs (60) it was suggested that these weak binding sites may be inhibitory.

TABLE 1.4.2 Anomeric specificity of glucose isomerase as compared with other isomerases

Enzyme	Substrate anomeric specificity	Product	Reference
glucose isomerase (EC. 5.3.1.5)	α-D-Xylose	α-D-Xylulose	72
	α-D-Glucose	α-D-Fructure	73
	α-D-Xylose	α-D-Xylulose	
Phosphoglucose isomerase (EC. 5.3.1.9)	α-D-Fructose-6-P	equilibrium mixture α,β-D-Glucose	72,74
	α-D-Glucose-6-P	"	74
Arabinose isomerase (EC. 5.3.1.12)	β-L-Arabinose	β-L-xylulose	73

1.5 Immobilised enzymes

Enzymes have been immobilised through a variety of methods which can generally be classified as follows:

(a) Physical adsorption onto inert supports

(b) Ionic attraction between the enzyme and an anionic or cationic exchanger

(c) Entrapment inside a polymerised gel

(d) Cross-linking with a bifunctional reagent

(e) Microencapsulation

(f) Covalent binding

Evaluations of methods available for enzyme immobilisation on a variety of water-insoluble supports have been widely reviewed (78-85) including kinetic properties of the immobilised enzyme (78,79,81,82) and its potential applications in analysis (83,87), medicine (83,84,87), food technology (82,83,86) and pharmaceutical industry (82,83).

1.5.1 Immobilisation of glucose isomerase

Glucose isomerase has been immobilised through a variety of supports and methods which are summarised on Tables 1.5.1.1 and 1.5.1.2.

The choice of the immobilisation method and support depend very much on the enzyme properties and the purpose for which the enzyme is being immobilised. The adsorption method, for example, utilises mild conditions for the immobilisation process but has the disadvantage that the immobilised enzyme-complex might dissociate due to changes in the pH, temperature or ionic strength. However, a glucose isomerase from Streptomyces sp., adsorbed on DEAE-cellulose, has been used by the Clinton Corn Processing Co. (8,19) for the large scale production of a high fructose-glucose syrup available under the trade name of Isomerose ^{R}100.

Adsorption of whole cells on collagen membranes, followed by cross-linking with glutaraldehyde, was used to immobilise both Streptomyces and Bacillus sp. glucose isomerase (100). The intracellularly-immobilised glucose isomerase activities had, respectively, half-lives of 500 h and 1800 h. A complete process for the production of fructose from starch hydrolysate (using α-amylase and amyloglucosidase) was based on twenty-one column reactors packed with a collagen-whole cells (Bacillus sp.) complex (100). The reactor was replaced after three half-lives and a net return on investment of 16.2% was calculated.

TABLE 1.5.1.1 Whole cell immobilised glucose isomerase

Microorganism	Immobilisation method	Reference
Arthrobacter sp.	- flocculation by a polyelectrolyte	83
	- cells absorbed onto glass beads	83
Actinoplanes missouriensis	- cells entrapped in α-cellulose fibres	14,88
	- cell microencapsulation in gelatine and subsequent crosslinking with glutaraldehyde	12
Bacillus NRRL-B-5656	- cells entrapped with glutaraldehyde	89
Bacillus coagulans	- cells entrapped with glutaraldehyde	101
Lactobacillus brevis	- flocculation of chitosan	83
Streptomyces sp.	- adsorption onto DEAE-Sephadex	83
	-entrapment in polyacrylamide gel	83
	- cells crosslinked with diazotised benzidine	91
	- heat treatment to fix enzyme in cells	83
S. albus	- flocculation by cationic electrolyte	83
S. griseus	- adsorption to celite particles attached to anionic exchange resin	83
	- entrapment in polyacrylamide gel	83
S. olivaceus NRRL 3583	- crosslinking with glutaraldehyde	92
S. olivochromogenes	- cells absorbed on polystyrene beads	93

TABLE 1.5.1.1 (Contd)

Microorganism	Immobilisation method	Reference
S. phaeochromogenes	- heat fixation	15
	- heat fixation followed by glutaraldehyde immobilisation onto collagen membranes	93
	- crosslinking with diazotised 3,6 diaminoacridine	83
	- entrapment in polycarylamide gel	83
S. venezuelae	- entrapment with collagen membrane	94

TABLE 1.5.1.2 Cell-free immobilised glucose isomerase

glucose isomerase preparation	support	immobilisation method	% activity retained	reference
crude extract (Streptomyces)	- DEAE-cellulose	adsorption		8
crude extract (Streptomyces)	- porous alumina	adsorption	13-22*	13
crude extract (S. phaeochromogenes)	- chitin	crosslinking with glutaraldehyde	48	95
	- trimethylammonium resin	adsorption	91	96
	- polyacrylamide	entrapment	30	59
	- porous glass	covalent	35-50	97
	- phenol formaldehyde resin	adsorption/ covalent	28-36	98
	- collagen	electrodeposition/	Δ	99
crude extract (S. griseolus)	- cellulose	entrapment	-	31
crude extract (Streptomyces sp)	- porous glass	covalent	56	60
partially purified (L. brevis)	- microcrystalline cellulose	covalent	#	53,55.

* - calculated from data

Δ - authors reported a 12-fold enzyme activity increase

- authors reported a 30-fold enzyme activity increase

Cells from B.coagulans entrapped by glutaraldehyde were also used for the optimisation of parameters such as enzyme loading, reaction contact time, pH, temperature, oxygen, ions and syrup concentration for the large scale production of high fructose-glucose syrup (101).

Being an intracellular enzyme, glucose isomerase is too expensive to be used as a soluble enzyme for fructose-glucose syrup production and the search for new supports and immobilisation methods continues. Collagen has been widely used because of its hydrophilic properties and abundance of chemically available COOH groups (85,99,100). A purification effect was observed with the immobilisation of glucose oxidase, penicillin amidase and glucose isomerase by electrocodeposition on collagen membranes (99). A crude preparation of glucose isomerase was purified by 30 fold by electrocodeposition (99) and, as we can see from Fig. 1.5.1, in the case of glucose isomerase, electrocodeposition methods can facilitate enzyme purification and immobilisation (99).

Takasaki (102) developed a new method where the enzyme is immobilised with microbial cells as support. Amyloglucosidase from Aspergillus niger was immobilised by cross-linking with tolylene diisocyanate to cells of Streptomyces sp., Aspergillus niger and Rhizopus oryzae (102). As the highest activity was obtained with Streptomyces sp. as support, it has been used to immobilise β-amylase, invertase, glucose isomerase, trypsin and glucose oxidase-catalase. All of these enzymes were successfully bound and activity recovery assayed for glucose isomerase was

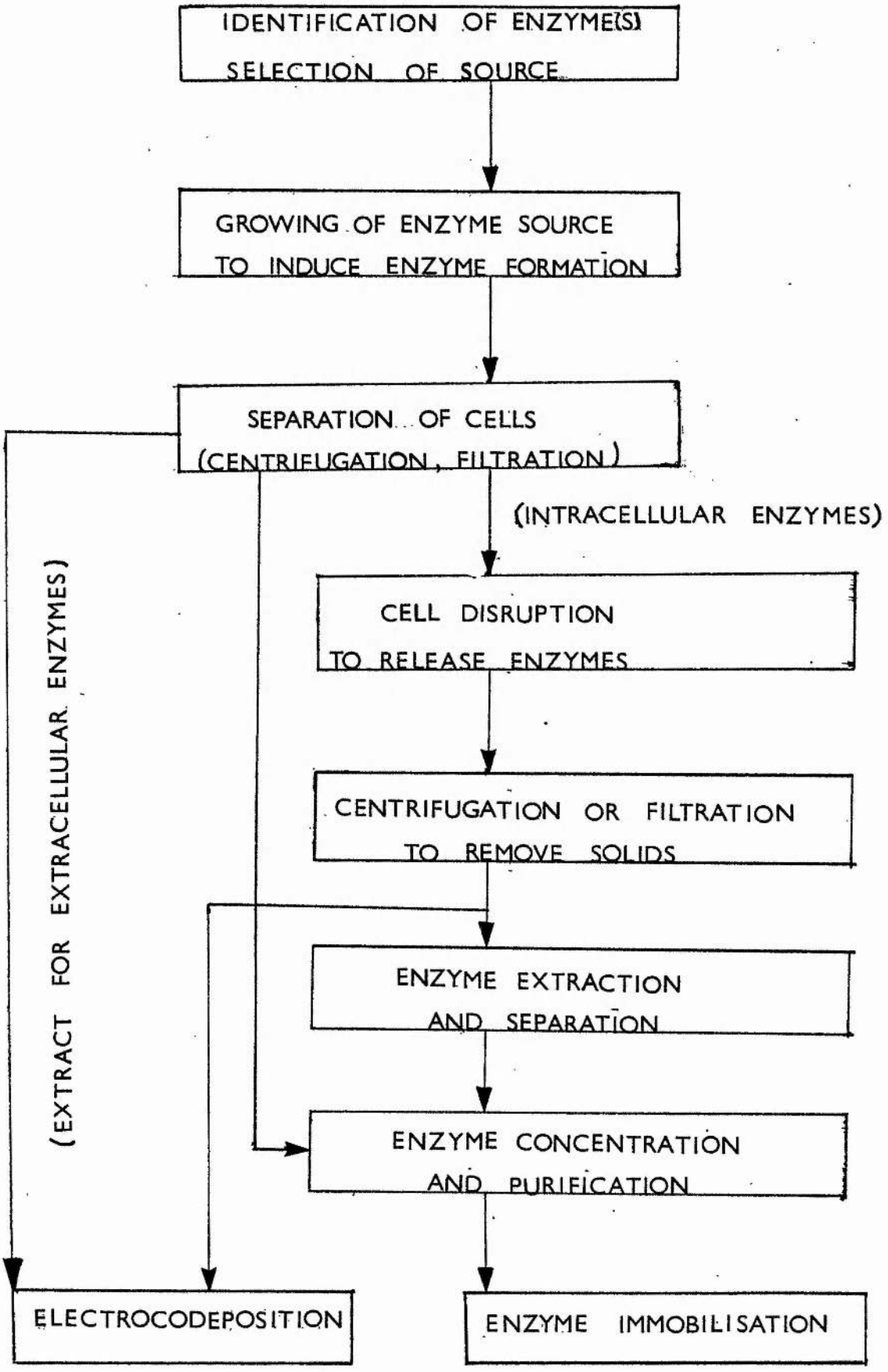


Fig. 1.5.1 Enzyme identification, preparation and immobilisation

about 40% of the initial activity (102).

Table 1.5.1.3 shows kinetic and thermodynamic data for immobilised glucose isomerase.

Whole cells of Actinoplanes missouriensis entrapped in α-cellulose fibres (14) showed the same optimal pH (7.5-8.0) and apparent K_m (0.83M for D-glucose) as that of the free glucose isomerase. The optimal temperature was in the range of 70-80°C as compared with a sharp optimum of 80°C for the free enzyme. Also the requirements for magnesium and cobalt ions were nearly the same for both enzyme preparations (14).

Glucose isomerase from Actinoplanes missouriensis immobilised with either α-cellulose (14) or gelatine (12) and glucose isomerase from S. griseolus CL 71 (immobilised in filamentous structure - nature unspecified) (31) and S. phaeochromogenes (immobilised in polyacrylamide) (59) showed inactivation in the presence of oxygen and calcium ions. In a batch reactor process, enzyme inactivation not found in continuous processes, has been reported (31,59) and might be due to by-product accumulation.

TABLE 1.5.1.3 Kinetic and thermodynamic data for immobilised glucose isomerase

Microorganism	Enzyme preparation	optimal conditions pH	optimal conditions °C	K_m (apparent) (M)	K_{eq}	E_a (Cal.mol^{-1})	Reference
	(A) Whole cells						
Actinoplanes missouriensis	- entrapped in α-cellulose	7.5-8.0	70-80	0.83	-	16200	14
S. phaeochromogenes	- heat treatment	7.0	70	0.24	1.35		15
	(B) cell-free enzyme						
L. brevis	- immobilised on microcrystalline cellulose via transition metal links	6.0					53,55
Streptomyces sp.	- covalently bound to porous glass	7.0	50	0.21	1.03		60

Apparent K_m values (for L. brevis enzyme) of 0.96M - 1.42M and 1.06M - 1.41M were calculated for the free and immobilised enzyme (55). Maximal velocity values of 287-334 mg. fructose formed $.h^{-1}.g^{-1}$ dry solid were calculated for the immobilised enzyme by different methods (55). The maximal activity was achieved with 1.67M D-glucose, decreasing with higher concentration. The ion requirements (Mn^{++}, Co^{++}) were unchanged after immobilisation but the optimal pH shifted from 6.5 - 7.0 (free enzyme) to 6.0 (immobilised enzyme) (55).

An apparent K_m value of 0.21M for D-glucose and 0.40M for D-fructose was reported for the immobilised enzyme from Streptomyces sp. (60). Maximal activity was 15.8 mg fructose formed $min^{-1}.ml^{-1}$ of glass enzyme (for D-glucose) and 30.5 mg glucose formed. $min.^{-1}ml^{-1}$ of glass enzyme (for D-fructose). Apparent K_m values for the enzyme from S. phaeochromogenes immobilised by adsorption on phenol-formaldehyde resin, increased from 0.59M to 2.35M according to the increase of the column flow rate (98). Also the enzyme stability was dependent on the flow rate. At low flow rates 60°C and pH 8.2 the adsorbed enzyme had a half-life of 36 days as compared with 40 days for the covalently bound enzyme (98). The activation energy decreased from 15,400 $cal.mol^{-1}$ to 11,900 $cal.mol^{-1}$ in accordance with the increase of flow rate.

Table 1.5.1.4 shows some operational data on the production of a fructose-glucose syrup by an immobilised glucose isomerase.

TABLE 1.5.1.4 Some operational data on the production of a high fructose-glucose syrup by immobilised glucose isomerase

Enzyme preparation	reactor type	half life (days)	Operating Temperature °C	fructose production (%)	Reference
(A) Whole cells					
Actinoplanes missouriensis					
- entrapped in α-cellulose fibres	column	45-42	60	20-40	14,88
- entrapped in gelatine	batch	18	65	42-46	12
S. olivochromogenes					
- immobilised with polystyrene beads	column	28		45	92
(B) Cell free enzyme					
Streptomyces sp.					
- covalently bound to porous glass	column	240	50	-	60
S. phaeochromogenes					
- covalently bound to porous glass	column	12-14	80	-	98
- bound to phenol/ formaldehyde resin	column	36-40	60	-	98

1.6 The aims of this work were as follows:

1. To seek a bacterial source of glucose isomerase and to establish a suitable extraction procedure for the enzyme.

2. To establish a rapid, accurate method for the assay of glucose or xylose isomerase activity.

3. To establish a purification procedure for the enzyme and to assess the degree of purity achieved.

4. To investigate the kinetic of the purified enzyme and evaluate the principal kinetic parameters.

5. To compare the kinetics of free and immobilised glucose isomerase.

2. MATERIALS

The following is a list of principal chemicals used with their suppliers. Full names and addresses are given at the end of this section.

Enzyme Source

Lactobacillus brevis, NCDO 474

Medium Components

D. Glucose	(Analar)	BDH
Magnesium sulphate	(Analar)	Fisons
Cobalt chloride	(Analar)	Fisons
Manganese chloride	(Analar)	M & B
Sodium acetate, anhydrous	(Analar)	M & B
Peptone bacteriological		Oxoid Ltd.
Yeast extract, powder		Oxoid Ltd.
Tomato juice agar, powder		Oxoid Ltd.
D (+) Xylose		Sigma

Enzyme Extraction and Fractionation

Ammonium sulphate	(Analar)	BDH
Protamine sulphate	(Grade II)	Sigma
Lysozyme	(Crystalline)	Sigma
Polyacrylic acid (25% solution, M.W. 230,000)		
Toluene		Sigma

Substrates

D (+) Glucose	(Analar)	BDH
D (+) Xylose		Sigma
β-D (+) Fructose		Sigma
β-Nicotinamide adenine dinucleotide, reduced from (β-NADH) disodium salt	Grade III	Sigma
β-Nicotinamide adenine dinucleotide (β-NAD^+)	Grade III	Sigma

Assay Components

(other than substrate)

Carbazole		BDH
Perchloric acid	(Analar)	BDH
Sulphuric acid	(Analar)	BDH
Folin & Ciocalteu's phenol reagent		BDH
L-cysteine hydrochloride		Sigma
o-dianisidine diHCl		Sigma
Albumin, bovine	(Fraction V)	Sigma
Catalase (from bovine liver)		Sigma
Glucose oxidase (from A. niger)	Type II	Sigma
Peroxidase (from horseradish)	Type I	Sigm a
Sorbitol dehydrogenase (from sheep liver)		Sigma

Chromatography Components and Proteins Used for Calibration

Apoferritin (from horse spleen)	(A-Grade)	Calbiochem
Sephadex G-200 (particle size: 10-40μ)	(Superfine)	Pharmacia

Blue dextran G-200		Pharmacia
Albumin, egg (ovalbumin)	(Grade V)	Sigma
Aldolase (from rabbit muscle)	(Grade I)	Sigma
Alcohol dehydrogenase		Sigma
α-Chymotrypsinogen A	(Type II)	Sigma
Cytochrome C (from horse heart)		Sigma
Lactic dehydrogenase (from rabbit muscle)	(Type II)	Sigma
DEAE-cellulose (DE 32)	Microgranular	Whatman
CM-cellulose (CM 52)	Microgranular	Whatman

Electrophoresis

Ammonium persulphate		BDH
EDTA	(Analar)	BDH
Urea	(Analar)	BDH
TEMED	(Analar)	BDH
Acrylamide	(Analar)	BDH
N-N' methylenebisacrylamide	(Analar)	BDH
Sodium lauryl sulphate		BDH
Bromophenol blue		BDH
Glycerol		BDH
Human albumin		Sigma
Albumin, egg (ovalbumin)	(Grade V)	Sigma
Myoglobin		Sigma
Phosphorylase a		Sigma
Coomassie Brilliant Blue R.250		

Immobilisation

Glutaraldehyde	(Grade II)	Sigma
Nylon beads		Gift from Dr. W.E.Hornby

Miscellaneous

Iodo-acetamide		BDH
Sodium dithionite		BDH
Sodium D-tartrate		BDH
Hydrochloric acid	(Analar)	Hopkin & Williams
Glutathione (reduced form)		Sigma
DL-dithiothreitol (Cleland's reagent; DTT)		Sigma
2-Mercaptoethanol	(Type I)	Sigma
Mercurous chloride		Sigma
Xylitol		Sigma
Glass distilled water		

All other chemicals used were of analar grade.

Suppliers addresses

1. BDH Chemicals Ltd., Poole, England.
2. Bio-Rad Laboratories Ltd., Bromley, Kent, England.
3. Boehringer Corporation Ltd., London.
4. Calbiochem, Los Angeles, California 90054, U.S.A.
5. Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire, England.
6. Hopkin & Williams, Chadwell Heath, Essex, England.
7. May and Baker (M & B) Ltd., Dagenham, Essex, England.
8. National Collection of Dairy Organism, Shinfield, Berkshire, England.
9. Oxoid Ltd., London, England.
10. Pharmacia Fine Chemicals, Uppsala, Sweden.
11. Sigma London Chemical Co. Ltd., Surrey, England.
12. Whatman Biochemicals Ltd., Kent, England.

3. METHODS

3.1 Culture of Lactobacillus brevis NCDO 474 for glucose isomerase production.

3.1.1 Stock cultures

Stock cultures of Lactobacillus brevis NCDO 474 were maintained at 4°C on tomato agar slants, in universal bottles, after growth at 30°C for 24 h.

3.1.2 Large scale growth of Lactobacillus brevis NCDO 474

Typically 10 l cultures of Lactobacillus brevis NCDO 474 were grown for glucose isomerase production.

3.1.2.1 Preparation of media

10 ml of tomato juice (without agar) at pH 6.0 was poured in a universal bottle and steam sterilised at 15 p.s.i. for 15 min.

100 ml, 1 litre and 10 l xylose-glucose media with the composition belowwere used in the scaling-up growth of Lactobacillus brevis.

Xylose-glucose medium:

A- Basic medium	% (w/v)
Peptone bacteriological	5
Yeast extract	3
Sodium acetate	1
$MnSO_4.7H_2O$	0.04

$MgSO_4.7H_2O$	0.01
$CoSO_4.6H_2O$	0.01

B - Sugar	% (w/v)
D (+) xylose	2
D (+) glucose	0.5

The basic medium was steam sterilised at 15 p.s.i. for 15 min. (100 ml, 1 litre) or 30 min. (10 l) and the sugars were sterilised by tyndallization at 100°C for 30 min. on five successive days and added aseptically to the basic medium just before inoculation.

3.1.2.2 Growth on 10 l scale

10 ml tomato juice medium was inoculated with a loopful of stock culture and grown at 30°C for 24 h without aeration. The whole culture was used to inoculate 100 ml of a xylose-glucose medium. The culture was grown at 30°C for 20 h without aeration and inoculated into 1 l fresh xylose-glucose medium.

After growth, the whole cutlure was transferred into a 16 l conical flask containing 10 l of xylose-glucose medium at pH 6.5 and 30°. Growth was followed by sampling the culture at suitable time intervals and measuring the absorbance of a 10 fold diluted sample at 600 nm in a SP-600 spectrophotometer (Unicam Instruments), 1 cm light path, against a distilled water blank.

3.1.2.3 Harvesting

Growth was allowed to proceed to late log phase (usually 16 - 18 h) and after withdrawing a 1 ml sample for dry weight estimation (see 3.1.3) the cells were harvested in a sharples turbine-driven continuous flow centrifuge, washed with cold distilled water, followed by 0.02M Tris-HCl buffer pH 7.0 at 4°C. The washed cells were harvested by centrifugation at 15,000 x g for 20 min, weighed and stored at -15°C.

3.1.3 Dry weight estimation

L. brevis cells grown at 30°C in 500 ml xylose and glucose medium (3.1.2) were harvested at 15,000 x g, after 20 h growth, washed two times with distilled water and suspended in distilled water.

Four samples of 1 ml were withdrawn from the homogeneous suspension and treated as follows:

Each of three 1 ml sample were transferred to previously weighed containers and dried at 80°C for 24 h followed by cooling in a dessicator.

The containers plus cells were weighed until constant weight was achieved. The average weight of the three 1 ml samples was taken as the cell dry weight contained in 1 ml sample.

A fourth 1 ml sample was diluted between 100 and 1000 fold with distilled water and after shaking well, each diluted

sample was transferred to a 1 cm light path glass cuvette and the absorbance at 600 nm was measured in an SP-600 spectrophotometer against a distilled water blank.

A plot was constructed relating the absorbance at 600 nm to the corresponding dry weight of the diluted sample (Fig. 3.1.3).

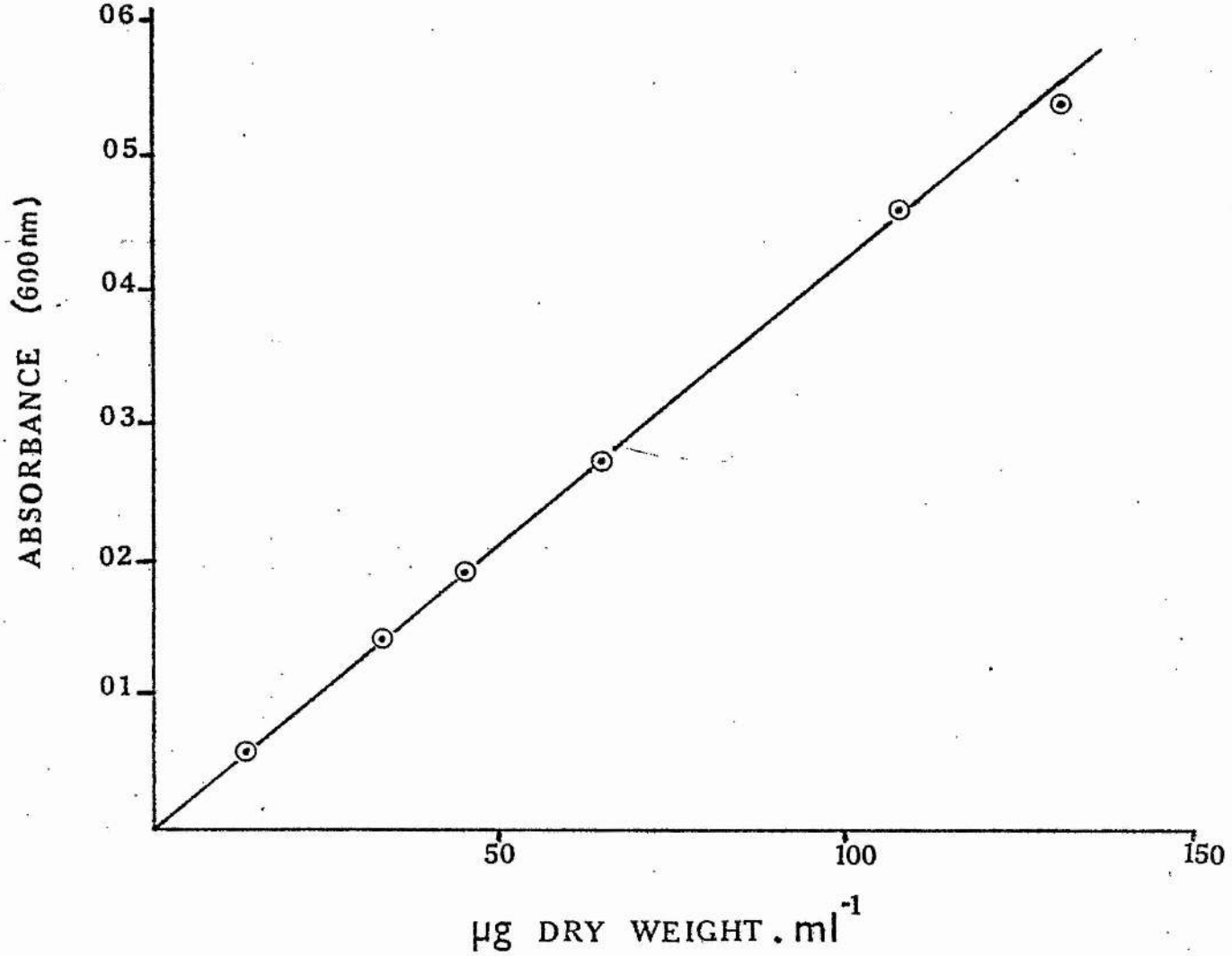
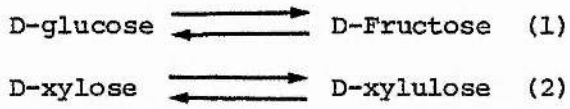


Fig. 3.1.3 Absorbance - dry weight calibration for Lactobacillus brevis cells.

3.2 Assay of glucose isomerase activity

Glucose isomerase catalyses the following reversible reactions



Based on the above reactions its activity was measured by the following techniques.

(a) Chemical assay of fructose production from glucose or xylulose production from xylose (3.2.1).

(b) Continuous spectrophotometric assay of fructose or xylulose production (eqn.1,2) using a coupled reaction system incorporating sorbitol dehydrogenase (SDH) and NADH (3.2.2).

(c) Discontinuous enzymatic assay of D-glucose (eqn.1) production from fructose using Lloyd and Whelan's method (3.2.3).

(d) Continuous D-glucose assay (eqn.4) using an oxygen electrode for measuring the rate of oxygen uptake from the reaction system D-fructose, glucose isomerase and glucose oxidase (4.5.2).

The unit of enzyme activity was defined as the amount of enzyme required to liberate 1μmol of product per minute under the assay conditions.

3.2.1 Chemical assay of fructose production from glucose or xylulose production from xylose.

In this chemical assay of fructose and xylulose the Dische & Borefreund method was used (3.2.1.2) in which coloured complexes are formed by the reaction of carbazole and cysteine with the acid-degraded products.

3.2.1.1 Glucose Isomerase assay using the Cysteine-Carbazole sulphuric acid method (3.2.1.2).

Glucose isomerase (activity between 0.1 and 2 units) was preincubated at 40°C for 10 min. in the presence of 10^{-2} M manganese chloride and 10^{-3} M cobaltous chloride (only when D-glucose or D-fructose was the substrate).

The reaction was started by the addition of either 2M D-fructose or 0.2M xylose made up in 0.02M Tris-HCl buffer pH 7.0.

The assay was stirred magnetically, aliquots of 0.1 ml being withdrawn at suitable intervals and added to test tubes each containing 1.9 ml 0.5M perchloric acid.

After suitable dilution, the amount of product formed was assayed by the cysteine-carbazole-sulphuric acid method (3.2.1.2).

3.2.1.2 Cysteine-Carbazole-Sulphuric acid method of Dische & Borefreund (103)

Reagents

Solution A: 1.5% (w/v) L-Cysteine-HCl solution made up in distilled water. This solution was freshly made and used on the same day.

Solution B: 75% (w/v) sulphuric acid solution.

Solution C: 0.12% (w/v) solution of carbazole made up in 99% ethanol.

Assay: Samples of 1 ml containing 5-50µg fructose (or xylulose) were added to 0.2 ml of 1.5% L-cysteine HCl solution.

After mixing well, 6 ml of a 75% sulphuric acid solution were added, mixed immediately, followed by the addition of 0.2 ml of 0.12% carbazole solution. The test tubes were shaken well, capped and left in a 40°C water bath for 30 min.

Absorbances were measured in a spectrophotometer (Unicam SP 600) at 520 nm (for xylulose estimation) or at 560 nm (for fructose estimation) against controls in which water replaced the sample and calibration plots were constructed (Fig. 3.2.1.1a and 3.2.1.1.b).

3.2.2 Continuous assay of fructose and xylulose production with the coupled system of sorbitol dehydrogenase and NADH.

Fructose or xylulose production by glucose isomerase can

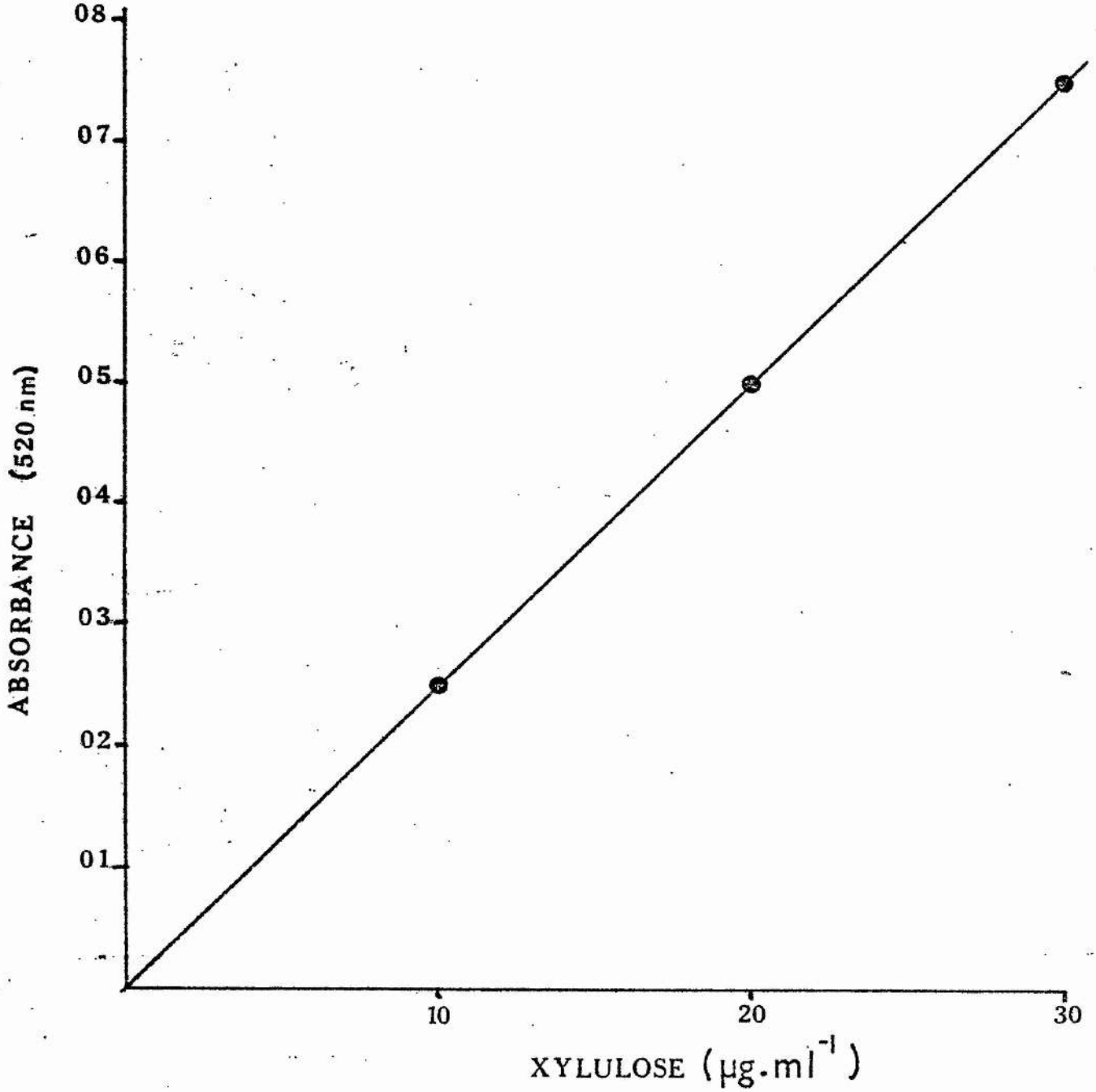


Fig. 3.2.1.1$_a$ Calibration plot for D-xylulose assay with cysteine-carbazole-sulphuric acid method.

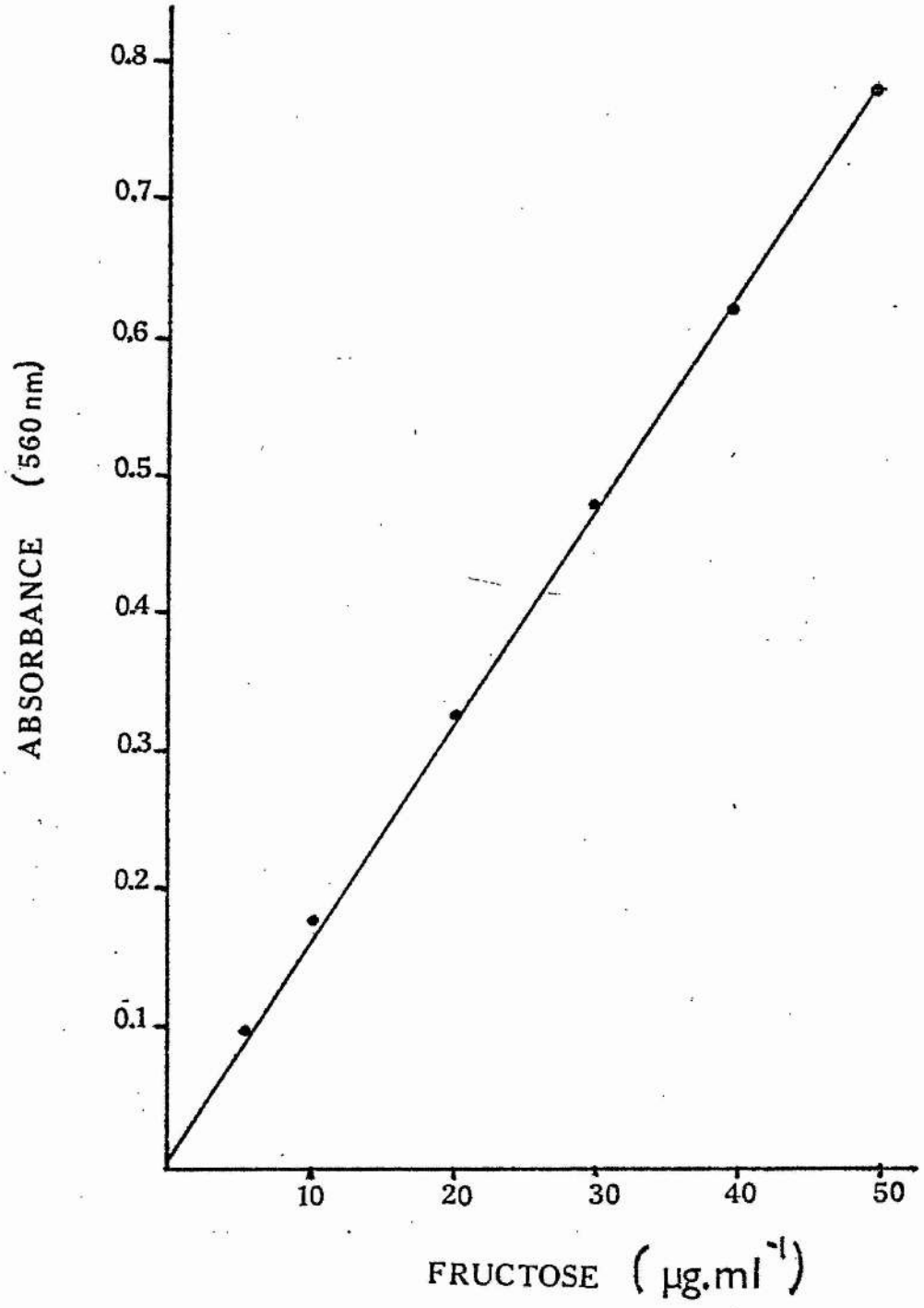


Fig. 3.2.1.1$_b$ Calibration plot for fructose assay with cysteine-carbazole-sulphuric acid method.

be estimated continuously by a coupled assay system including D-sorbitol dehydrogenase (L-iditol : NAD oxidoreductase, EC. 1.1.1.14) and NADH. The assay is based on the following reactions in which NADH oxidation is measured spectrophotometrically at 340 nm.

1. D-glucose $\underset{\text{glucose isomerase}}{\rightleftharpoons}$ D-fructose

$$\text{D-fructose} + \text{NADH} + \text{H}^+ \underset{\text{sorbitol dehydrogenase}}{\rightleftharpoons} \text{sorbitol} + \text{NAD}^+$$

2. D-xylose $\underset{\text{glucose isomerase}}{\rightleftharpoons}$ D-xylulose

$$\text{D-xylulose} + \text{NADH} + \text{H}^+ \underset{\text{sorbitol dehydrogenase}}{\rightleftharpoons} \text{xylitol} + \text{NAD}^+$$

Reagents

A: 0.02M Tris-HCl buffer pH 7.6

B: 0.2M D-xylose in 0.02M Tris-HCl buffer pH 7.6

C: 1M $MnCl_2$ solution made up in distilled water

D: 2.5×10^{-3} M β-NADH solution made up in 0.02M Tris-HCl buffer pH 7.6

E: sorbitol dehydrogenase (from sheep liver) 30 $U.ml^{-1}$ of 0.02M Tris-HCl buffer pH 7.6.

The following assay system was developed which gave a linear correlation between glucose isomerase activity and the rate of NADH oxidation.

Assay system (final concentration)

7.0×10^{-2}M D-xylose in 0.02M Tris-HCl buffer pH 7.6

3.0×10^{-2}M MnCl

2.5×10^{-4}M NADH

1.5U of sorbitol dehydrogenase (cuvette)

$0.27 \times 10^{-2} - 5.0 \times 10^{-2}$U of glucose isomerase (cuvette)

The volume was completed to 3 ml with 0.02M Tris-HCl buffer pH 7.6.

The system was pre-incubated at 40°C and the reaction was started by the addition of glucose isomerase. NADH oxidation was followed at 340 nm (Unicam SP 800 spectrophotometer/ AR 45 chart recorder).

Glucose isomerase activity was calculated on the basis that 1µmole of NADH oxidised corresponded to the reduction of 1µmole of xylulose by sorbitol dehydrogenase to xylitol.

Thus,

$$\mu\text{moles of xylulose min}^{-1} = \Delta A.\text{min} \times \frac{V}{\varepsilon\text{NADH} \times v}$$

where,

V = final volume

v = volume of glucose isomerase used in assay

εNADH (molar extinction coefficient of NADH disodium salt, Boehringer, grade II)

$= 6.7 \times 10^3$ l.mole^{-1}.cm^{-1} as experimentally determined.

The development of this coupled assay system is described in 4.5.1.

3.2.3 Discontinuous D-glucose assay by Lloyd & Whelan"s method (104)

D-glucose was assayed by the glucose oxidase/peroxidase method of Lloyd & Whelan (104) which is based on the following reactions:

$$\text{Glucose} \xrightarrow{\text{glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-dianisidine} \xrightarrow{\text{peroxidase}} H_2O + \text{oxidised o-dianisidine}$$

Reagents

Solution A: Tris-phosphate-glycerol buffer pH 7.0.

36.3g Tris and 50g $NaH_2PO_4.H_2O$ were dissolved in distilled water. 400 ml glycerol was added to this solution and the volume made up to 1ℓ with distilled water. The solution was adjusted to pH 7.0 by addition of solid $NaH_2PO_4.H_2O$.

Solution B: Glucose oxidase reagent 30 mg glucose oxidase 3 mg horseradish peroxidase and 50 mg o-dianisidine dihydrochloride were dissolved in 100 ml of Reagent A. The glucose oxidase reagent was stable at 4°C for at least two months.

Solution C: 5M HCl solution.

Assay: Samples of 1 ml containing 0-75µg glucose were added of 2 ml glucose oxidase reagent.

The tubes were capped, mixed well and left at 40°C for 30 min. The reaction was stopped by the addition of 4 ml 5M HCl. After mixing well, absorbances were measured in a spectrophotometer (Unicam SP 600) at 525 nm against a reagent blank.

A calibration plot was constructed (Fig. 3.2.3).

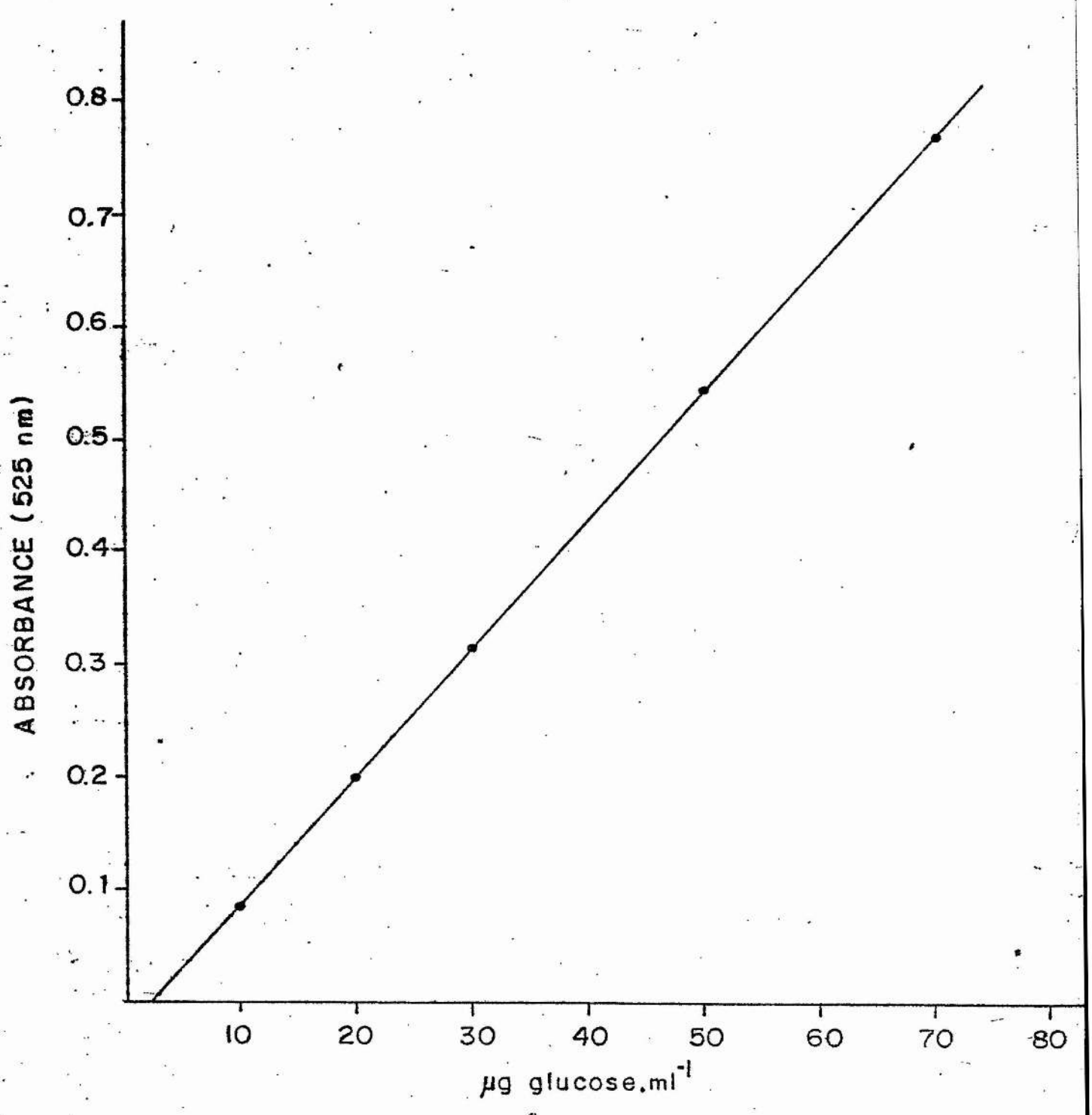


Fig. 3.2.3 Calibration plot for the glucose assay with Lloyd & Whelan method (3.2.3).

3.3 Protein determination methods

3.3.1 Alkaline copper solution methods

3.3.1.1 Folin-Phenol method of Lowry et al (105).

This method is a colorimetric one based on the reduction of the Folin reagent by the protein-copper complex.

The maximum colour results in a pH around 10. However, at this pH the Folin reagent is reactive only for a short time making immediate mixing imperative.

Reagents

Solution A: 2% (w/v) sodium carbonate in 0.1M NaOH.

Solution B: 1g sodium tartrate and 0.5 g cupric sulphate pentahydrate were dissolved in distilled water. After the addition of 10 ml 1M NaOH the final volume was made up to 100 ml with distilled water.

Solution C: 50 ml solution A + 1 ml solution B
This solution was freshly made.

Solution D: Folin reagent diluted to 1M with distilled water.

Assay: 1.8 ml protein solution (5-100µg) was added to 2 ml solution D, mixed well and left for 10 min at room temperature 0.2 ml solution C was then added, mixed thoroughly and left at room temperature for 30 min. The absorbance was measured in a spectrophotometer (Unicam SP 600) at 750 nm (samples containing 5-25µg protein) and at 500 nm (25-100µg protein range) against a control in which distilled water replaced the protein solution.

A calibration plot (3.3.1.1) was constructed with a standard solution (100µg.ml^{-1}) of lysozyme in distilled water.

3.3.1.2 Microbiuret method

This method (106) measures peptide bond concentrations chemically.

Reagents:

Solution A: (i) 173 g sodium citrate and 100 g sodium carbonate were dissolved in about 500 ml of warm distilled water.

(ii) 17.3 g copper sulphate pentahydrate was distilled in 100 ml distilled water and added to the first solution. The final volume was made up to 1 l with distilled water.

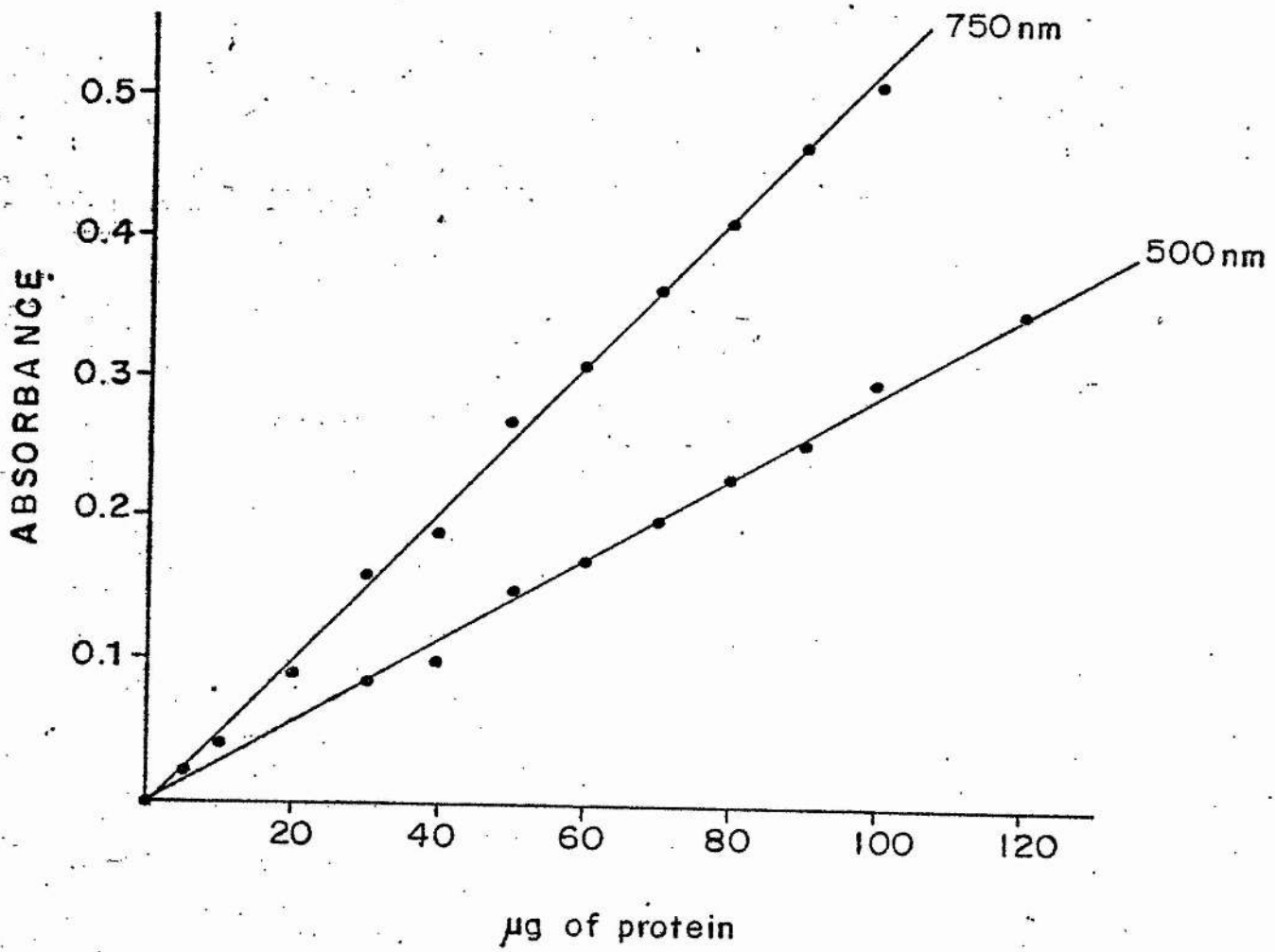


Fig. 3.3.1.1 Calibration plot for protein estimation by Lowry et al method (see 3.3.1.1) in standard solution (100µg.ml^{-1}) of lysozyme made up in distilled water was used.

Solution B: 6% (w/v) NaOH in distilled water.

Assay: 2 ml sample (0.1-4.0 mg protein) was added to 2 ml solution B, mixed, followed by the addition of 0.2 ml solution A. Absorbance was measured in a spectrophotometer (Unicam SP 600) at 330 nm and 540 nm against a reagent blank. A calibration plot (3.3.1.2) was constructed using lysozyme (2 $mg.ml^{-1}$) as standard.

3.3.2 Ultraviolet absorbance methods

3.3.2.1 Absorbance at 280 and 260 nm

Established by Warburg and Christian (107) this method measures the lysine and tryptophan concentration, although nucleic acids also adsorb at this wavelength.

Absorbances of protein solutions were measured at 280 and 260 nm in a spectrophotometer (Unicam SP 500) against a buffer blank.

The protein concentration was calculated from the formula of Kalckar (108).

$$mg\ protein.ml^{-1} = 1.45\ A_{280nm} - 0.74\ A_{260nm}$$

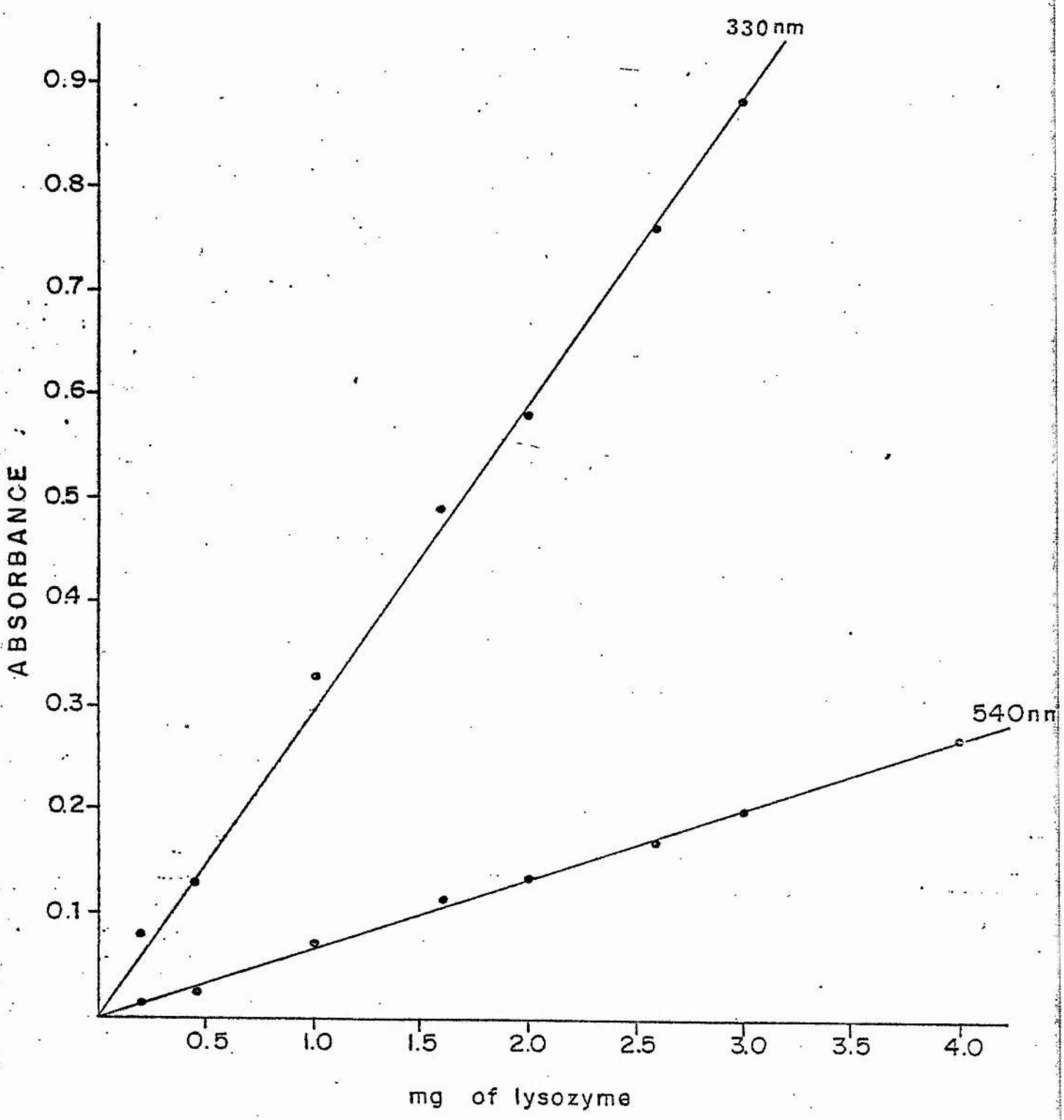


Fig. 3.3.1.2 Calibration plot for the assay of protein by the micro-biuret method. The calibration plot was constructed using lysozyme (2 $mg.ml^{-1}$) as standard.

3.3.2.2 Absorbance at 215 and 225nm

This method measures the peptide bond concentration (109). Since many other substances interfere at this wavelength, a calibration plot was constructed using lysozyme dissolved in 0.02M Tris-HCl buffer pH 7.0 as standard. From the calibration plot (Fig. 3.3.2.2) a factor of 121 was found and used as follows for the calculation of protein concentration.

$$\mu g \text{ protein.ml}^{-1} = A_{225} - A_{215} \times 121$$

3.4 Enzyme extraction and purification

3.4.1 Methods for the disruption of Lactobacillus brevis cells

3.4.1.1 Heat autolysis

The washed cells were suspended in 0.02M Tris-HCl buffer pH 7.0 at 4°C and frozen with either liquid nitrogen or with a solid CO_2/methanol mixture.

They were transferred to a water bath at 40°C and stirred gently for 16 h. The cells debris suspension were harvested by centrifugation at 1000 x g for 10 min, at 4°C. The supernatant was collected and assayed for glucose isomerase activity (3.2.1.1) and protein concentration (3.3.2.1).

3.4.1.2 Cell rupture using the Hughes Press

The cylinder and piston components of the Hughes Press were cooled to -30°C by immersion in a solid CO_2/methanol

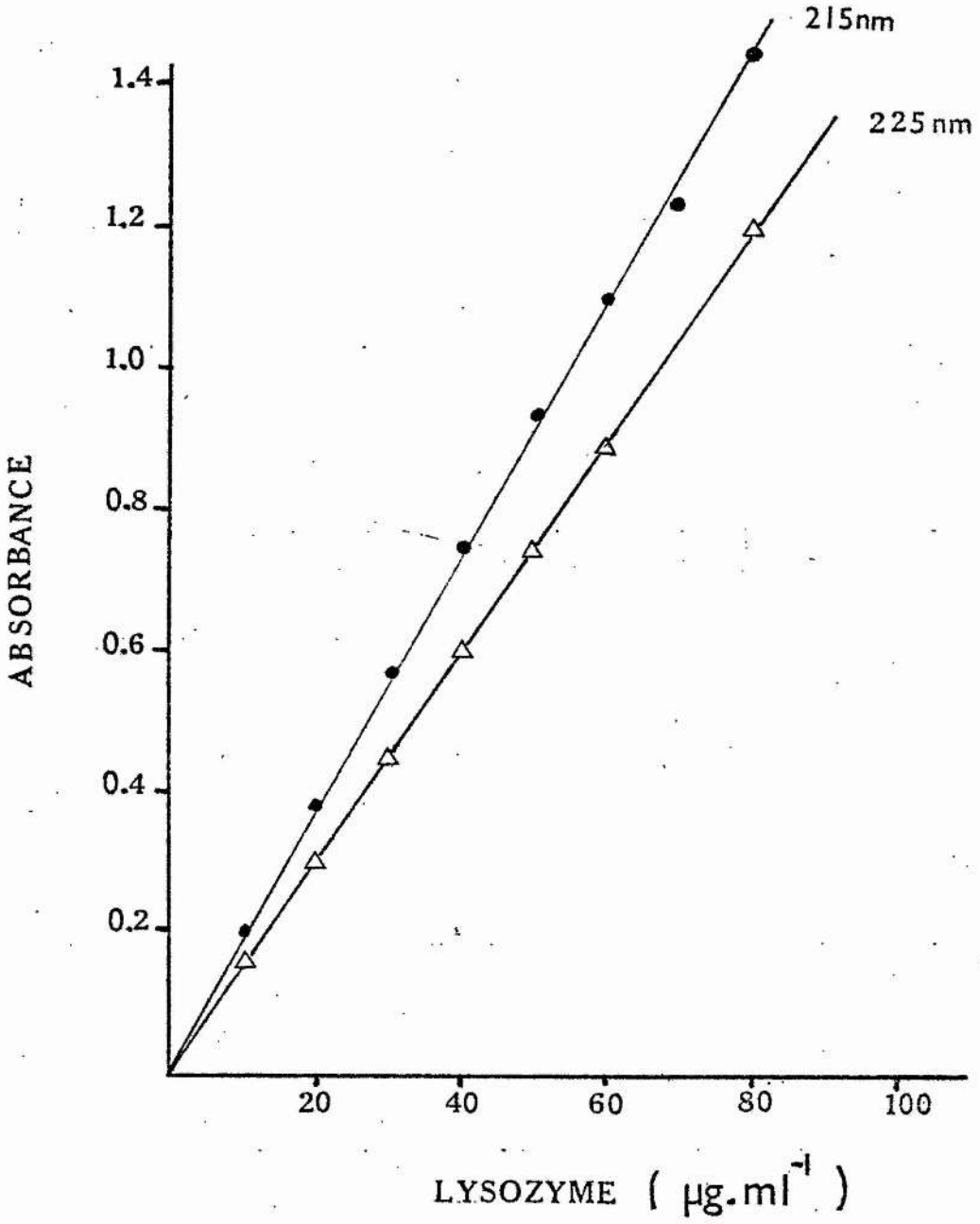


Fig. 3.3.2.2 Calibration plot for protein estimation by Wadell method. The standard was made up of lysozyme (100 $\mu g.ml^{-1}$) dissolved in 0.02M Tris-HCl buffer pH 7.0.

mixture. A thick suspension of washed cells in 0.02M Tris-HCl buffer, pH 7.0 at 4°C was added dropwise to a thermos containing liquid nitrogen to form frozen spheres of approximately 0.25 cm diameter. These were then packed into the press cylinder and the piston was fitted and driven home in a hydraulic press with a pressure of 5000 psi. The assembly was then taken apart and the broken cell paste recovered from the receiving compartment in the base of the cylinder component.

3.4.1.3 Cell rupture by lyzozyme treatment

60 mg of crystalline lysozyme was added to a suspension of washed cells (2.35 g) in 30 ml of 0.02M Tris-HCl buffer pH 7.0, and incubated with shaking at 34°C, overnight. After centrifugation (1000 x g, 10 min) the supernatant was assayed for glucose isomerase activity (3.2.1.1) and protein (3.3.2.1).

3.4.2 Precipitation of nucleic acids

1M Manganese chloride of 4°C was added dropwise with stirring to a crude extract to a final manganese chloride concentration of 5×10^{-2}M. The pH was then adjusted to 6.5 with 1M NaOH and the mixture was left undisturbed for 2 h at 4°C. The nucleic acid precipitate was removed by centrifugation (,51000 x g, 30 min) and discarded while the supernatant was assayed for glucose isomerase activity and protein (3.2,1.1, 3.3.2.1).

3.4.3 Protein fractionation

3.4.3.1 Precipitation with polyacrylic acid (110)

0.1% solutions of polyacrylic acid were prepared in 0.02M Tris-HCl pH 6.0; 0.1M acetate buffer pH 5.0, and 0.1M acetate buffer pH 4.5.

A volume of 0.1% polyacrylic acid was added to an equal volume of protein in the same pH-buffer system. After 10 min, the mixture was centrifugated (10,000 x g, 10 min), the supernatant was collected and assayed for glucose isomerase activity (3.2.1.1) and protein (3.3.2.1).

3.4.3.2 Precipitation with ammonium sulphate

Solid ammonium sulphate was added slowly, with stirring, to the manganese chloride treated fraction (3.4.2) to give a final concentration of 2M at 0°C (111). After centrifugation (10,000 x g, 30 min), the precipitated fraction was resuspended in 0.02M Tris-HCl buffer pH 7.0 and assayed for glucose isomerase activity (3.2.1.1) and protein (3.3.2.1).

The supernatant fraction was also collected and brought to 3.5M concentration with the addition of solid ammonium sulphate. The mixture was left overnight at 4°C. After centrifugation (10,000 x g, 30 min), the precipitate was collected, re-suspended in buffer and assayed for enzyme activity (3.2.1.1) and protein (3.3.2.1).

3.4.4 Cellulose-ion exchanger

DEAE-cellulose (DE 32) and CM-cellulose (CM 52) were pre-cycled equilibrated, degassed and packed in columns (Pharmacia SR 25/45) according to a technical information supplied by the manufacturers (112).

3.4.5 Gel filtration on Sephadex G-200

Sephadex G-200 superfine (particle size 10-40μ) was allowed to swell in 0.02M Tris-HCl buffer, pH 7.0 for 3 days. For removal of the fine particles, the slurry was made up of 150% of the wet settled volume of the Sephadex G-200 in buffer and poured into a glass cylinder and mixed by inversion.

The suspension was left settling for the amount of time necessary to give n = 1.3 (see eqn.1). Immediately the supernatant containing fine particles was removed by suction, leaving a final volume in the measuring cylinder equal to the wet settled gel volume +20%. Then the slurry was re-suspended in buffer making up again 150% of the wet settled volume of the gel. The process was repeated twice.

The time allowed for the gel to settle was calculated from the equation

$$t = nh \quad \text{(eqn.1)}$$

where,

t = time (min)

h = the total height of slurry in the measuring cylinder (cm)

n = a factor between 1.3 and 2.4.

Generally with n = 2.4 only the finest particles would be removed where an n value of 1.3 would give the desired flow.

The gel was degassed as described in (112) and poured into a 25 mm diameter x 500 mm column (Pharmacia, S.R 25/45). After all the slurry had been added the column top end was immediately attached and equilibration started with 0.1M NaCl solution in 0.02M Tris-HCl buffer pH 7.0 under an operating pressure of 14 cm H_2O.

The operating pressure was calculated as the vertical height between the free surface of the eluant and the column outlet. The flow rate at this operating pressure was 0.8 $ml.cm^{-2}.h^{-1}$ and the bed volume was 25 mm diameter x 370 mm. The column, the eluant and the fraction collected were refrigerated.

3.4.6 SDS-electrophoresis

It has been reported that the separation of proteins by polyacrylamide electrophoresis in the presence of the anionic detergent sodium dodecyl sulphate (SDS) is dependent on the molecular weights of their polypeptide chains.

Based on Shapiro et al (113), Weber & Osborn (114) established a SDS-acrylamide electrophoresis technique for the determination of the molecular weight of a wide variety of proteins with an accuracy of at least 10%. Except for

the acrylamide concentration we used the Weber & Osborn (114) technique as follows:

3.4.6.1 Preparation of gels

3.4.6.1.1 Acrylamide stock solution

Acrylamide 38.7 g

N-N' methylenebisacrylamide 2.66 g

were dissolved in distilled water to a final volume of 100 ml. This stock solution with approximately 40% (w/v) acrylamide concentration was stored at 4°C in a dark bottle.

3.4.6.1.2 Preparation of 5% acrylamide - 0.1% SDS and gel polimerysation

0.02 g ammonium persulphate was dissolved in 7.5 ml distilled water and to this were added 10 ml of gel buffer (3.4.6.2), 2.5 ml acrylamide stock solution (3.4.6.1.1) and 0.030 ml TEMED.

The mixture was mixed quickly and layered with a Pasteur pipette on the wall of each tube (5 mm diameter x 75 mm) to give a bed of approximately 60 mm depth. The tubes had been previously washed with chromic acid, distilled water, dried, the ends closed with two layers of parafilm.

Before the gel hardened a few drops of water were layered on top of the gel bed. After 20 min an interface could be seen indicating that the gel had solidified.

3.4.6.2 SDS-electrophoresis buffer

3.4.6.2.1 Gel buffer

0.5 g SDS (Analar)

0.38 g TEMED

120.00 g Urea (Analar)

30 ml 0.1M Na_2HPO_4

20 ml 0.1M NaH_2PO_4

The final volume was made up to 250 ml with distilled water. The pH was 7.0. This gel buffer was stored at 4° and used for gel preparation.

3.4.6.2.2 Chamber buffer

5 g of SDS were dissolved in 0.01M phosphate buffer pH 7.0 to a final volume of 5 l. This chamber buffer was used for dialysis of the samples and also in the chamber compartment of the electrophoresis apparatus.

3.4.6.3 Preparation of protein standards and samples

5 mg of phosporylase$_a$ (mol.wt = 92,500), human albumin (mol.wt = 68,000), egg albumin (mol.wt = 43,000) and myoglobin (mol.wt = 17,200) were each dissolved in 1 ml distilled water. Each solution was subsequently diluted 1:10 with chamber buffer

and to each solution was added 0.010 ml β-mercaptoethanol (s.g = 1.1168) to give 1% (w/v) final concentration of mercaptoethanol. The protein solutions were incubated at 37° for 2 h followed by overnight dialysis in chamber buffer. The standards were kept at a temperature of -15°.

Glucose isomerase samples were diluted with chamber buffer to give a protein concentration between 0.1-1.0 $mg.ml^{-1}$, treated with β-mercaptoethanol and dialysed against chamber buffer as described for the standards.

3.4.6.4 Sample application

In small test tubes cleaned in chromic acid, distilled water and dried, were mixed:

10µl of tracking dye (0.05% bromophenol blue in water)

1 drop of glycerol

10-50µl protein sample or standard

Before the application of the sample mixture, the parafilm caps were removed and the gel tops were rinsed with chamber buffer.

The samples were applied on the gels and overlaid with chamber buffer to fill the tube. The marked tubes were arranged on the chamber test tube rack, the chamber was filled with chamber buffer and electrophoresis was performed at a constant current of 2 mA per gel tube. Under these conditions, the tracking dye moved to the bottom of the 5% gel in

approximately 4 h.

After electrophoresis, the gels were removed from the tubes by inserting water with a syringe between the tube wall and the gel using a pipette bulb to exert pressure. The gel length (GL) and the tracking dye migration (BPP) were measured before the staining step.

3.4.6.5 Staining and destaining

Reagents: (a) Staining solution

1.25 g coomassie brilliant blue R-250

227 ml methanol

227 ml distilled water

46 ml glacial acetic acid

(b) Destaining solution

250 ml methanol

75 ml glacial acetic acid

Distilled water up to 1 l.

The gels were stained in a trough of staining solution for 20-30 min., rinsed with water and immersed in destaining solution for 12-36 h with 2-3 changes of solution. Subsequently each gel was scanned in a recording densitometer (Vitabron TLD-100) and the gel length after staining (GLS) and the migration of each band were measured (mm).

The mobility of each band was calculated using the equation

below:

$$\text{Mobility} = \frac{\text{Band}}{\text{GLS}} \times \frac{\text{GL}}{\text{BPP}}$$

where,

Band = distance (mm) of protein migration

GLS = gel length (mm) after staining

GL = gel length (mm) after electrophoresis

BPP = tracking dye migration (mm)

3.5 Immobilisation of glucose isomerase on a PEI-derivative of nylon (polyethyleneimine)

The immobilisation procedure utilised glutaraldehyde as cross-linking reagent and PEI-derivative of nylon (polyethyleneimine) as support (see Fig. 3.5) (115).

3.5.1 Preparation of the support

1 g of PEI-derivative of nylon (polyethyleneimine) supplied by DR. W.E. Hornby was added to 20 ml of 0.1M phosphate buffer pH 7.8 containing 5% glutaraldehyde. The mixture was stirred for 10 min at room temperature followed by thorough washings with 25 ml of 0.1M phosphate buffer pH 7.8. These washings were repeated 3-4 times with fast stirring (5 min).

3.5.2 Coupling procedure

Approximately 5 ml of an enzyme solution containing 2.0-8.0 mg of protein in 0.1M phosphate buffer pH 7.8 was coupled with the PEI-nylon-glutaraldehyde derivative prepared as described in (3.5.1). The coupling proceeded with stirring at 0°C for 2-3 h. Subsequently the suspension was filtered and the nylon-enzyme derivative was collected and washed with stirring for 20 min in 0.5M NaCl in 0.1M acetate buffer pH 6.0. Washing was repeated three times.

The immobilised enzyme was then washed with 0.1M acetate buffer pH 6.0 and stored as suspension in the same buffer

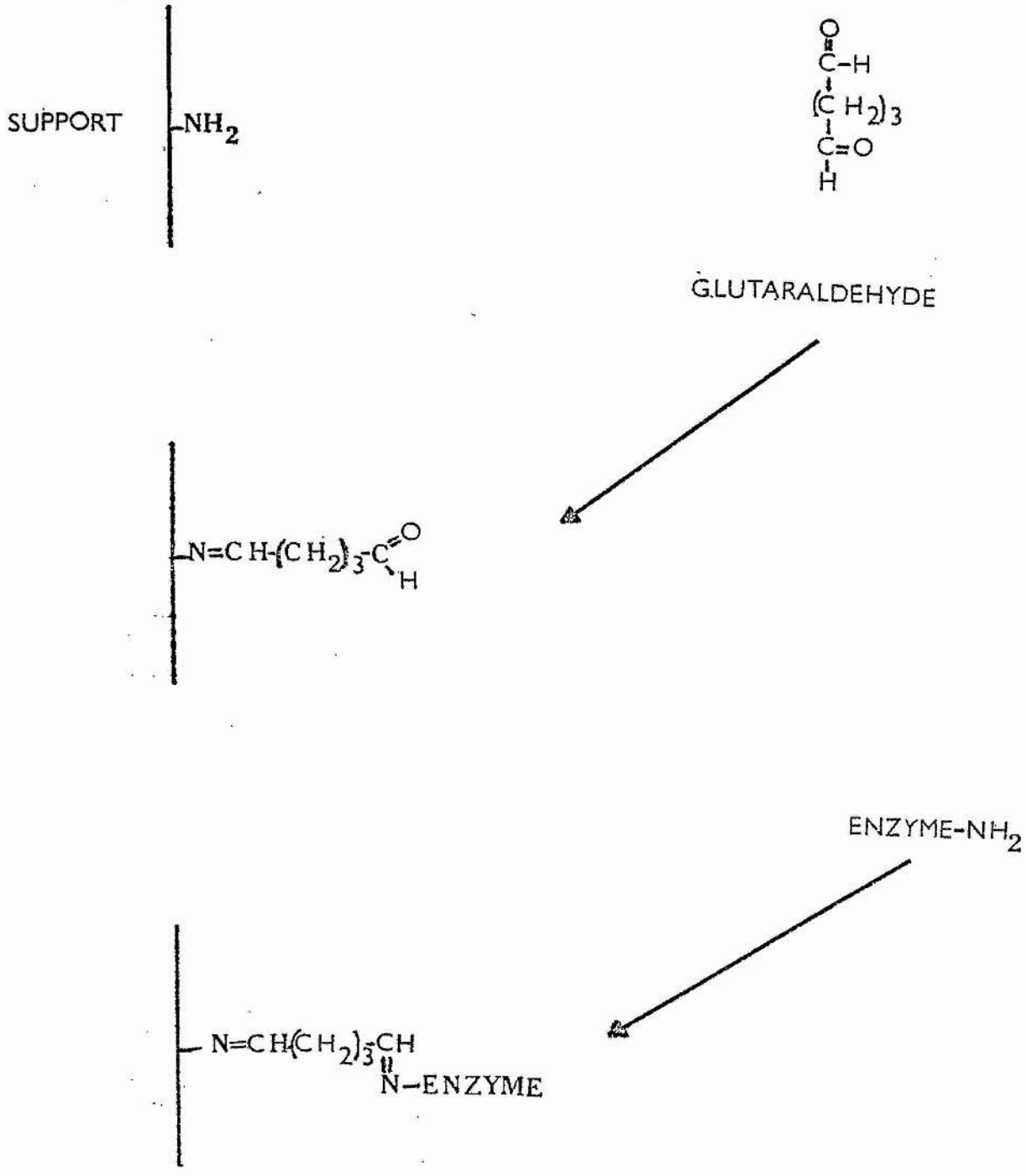


Fig. 3.5 Probable reaction steps in the immobilisation of glucose isomerase.

at 4°C.

3.5.3 Assay of nylon-immobilised glucose isomerase

Immobilised glucose isomerase was assayed in a stirred batch reactor at 40°C. The reaction system contained:

10 ml xylose (0.2M) in 0.02M Tris-HCl buffer pH 7.0

0.5 ml 2M $MnCl_2$

Reaction was started by the addition of approximately 6 g of immobilised derivative (equivalent to approximately 12 mg enzyme protein). 0.1 ml aliquots were withdrawn at appropriate time intervals and xylulose estimated as in (3.2.1.2).

3.5.4 Protein concentration determination

The amount of protein bounded was determined from the difference between the amount originally used and the residual unbound protein. The protein concentration in all aliquots was estimated by the Lowry method (105).

3.6 Supplementary method

3.6.1

Mohr's method (116) for chloride assay by titration with 0.1M silver nitrate and 2-5 drops of 5% potassium chromate solution was used.

A linear gradient apparatus was set up with a reservoir containing 800 ml of 1M HCl in 0.02M Tris HCl buffer pH 7.6 feeding a mixing chamber containing 0.02M Tris HCl buffer pH 7.6. Samples of 10 ml were collected and assayed for chloride. The concentration of chloride assayed in the sample was calculated assuming that

1 ml of 0.1M $AgNO_3$ ≡ 0.00745g KCl

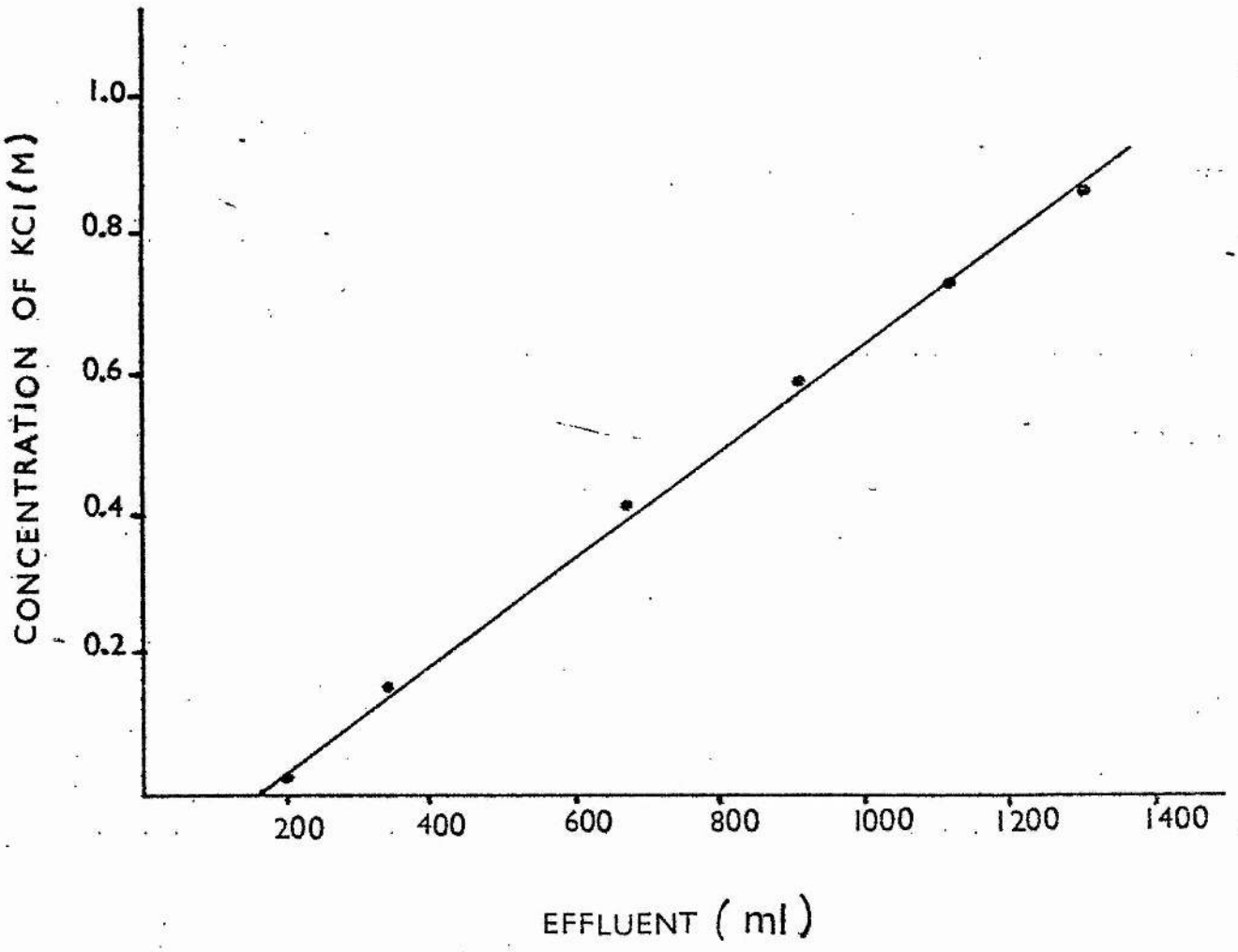


Fig. 3.6.1 Calibration plot for chloride estimation by Mohr's method (see 3.6.1 for details).

4. RESULTS AND DISCUSSION

4.1 Lactobacillus brevis growth and glucose isomerase production

Using an inoculum of 10% and medium composition and growth conditions as described in 3.1, the yield of cells (dry weight) was 2.3 - 3.3 $g.l^{-1}$ of medium and 300 - 310 glucose isomerase $units.l^{-1}$. Cell growth was followed by measuring the absorbance of a ten fold diluted sample, at 600 nm in a SP-600 spectrophotometer (Unicam Instruments Ltd.) with 1 cm light path against a distilled water blank.

200 ml aliquots of cell suspension were withdrawn at suitable time intervals during growth, harvested by centrifugation (1000 x g, 10 min), washed with cold distilled water, followed by 0.02M Tris-HCl buffer pH 7.0, 4°C, and submitted to heat autolysis as described in 3.4.1.1. Glucose isomerase activity was assayed by incubating the disrupted cell suspension in 1M glucose in 0.02M Tris-HCl buffer pH 7.0 and 10^{-3}M $CoCl_2$ and measuring fructose formation by the cysteine-carbazole-sulphuric acid method (3.2.1.2).

As compared with other microbial sources of glucose isomerase (29) the enzyme yield of 88 - 130 $units.g^{-1}$ of Lactobacillus brevis cells was low. Fig. 4.1 shows the relationship between the cell growth and enzyme production, which agrees with Yamanaka's (52) and Kent's (53) findings.

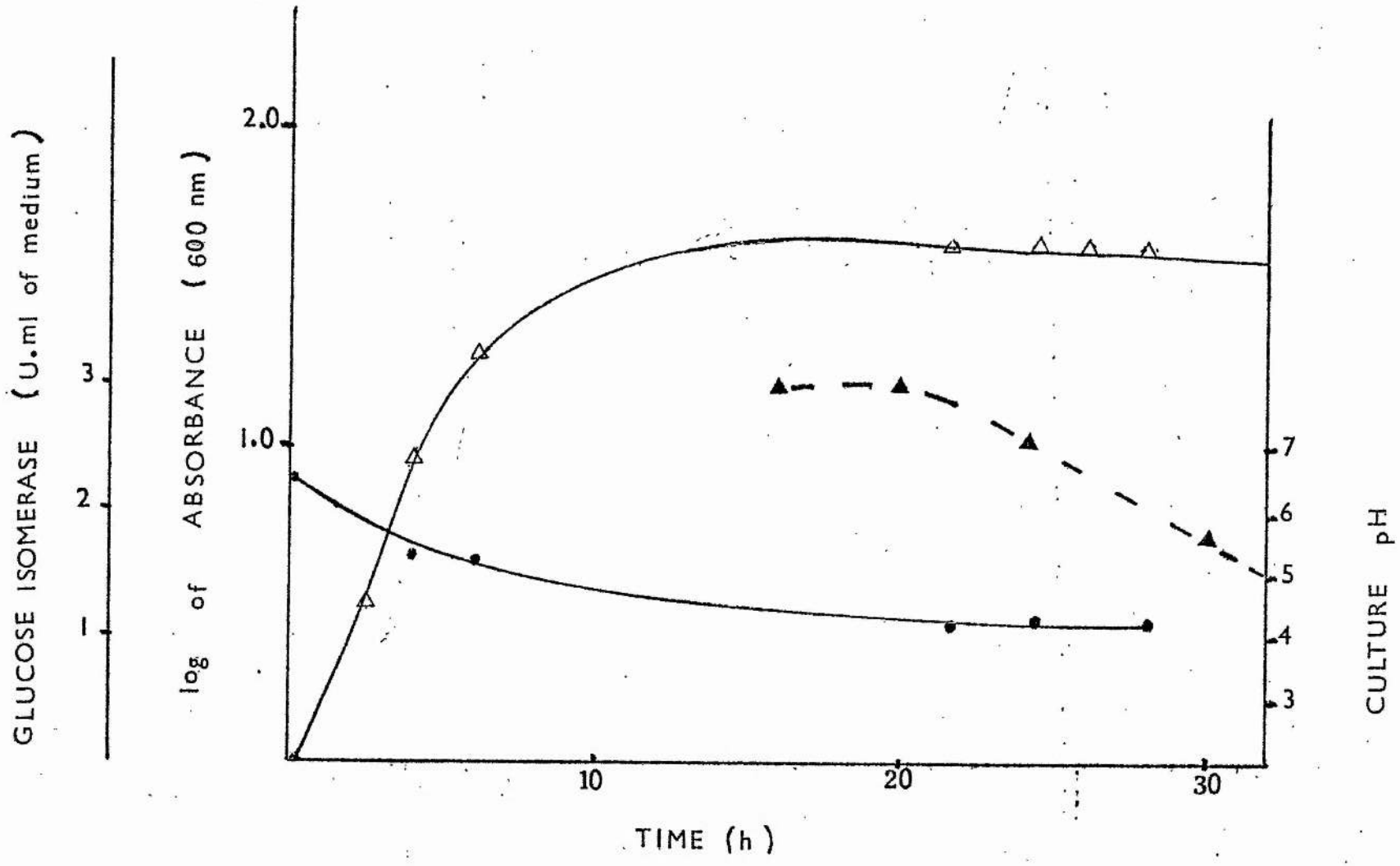


Fig. 4.1 Culture of Lactobacillus brevis NCDO 474 for production of glucose isomerase in xylose-glucose medium L.

logarithm of culture absorbance 600nm △—△

glucose isomerase activity (assayed with cysteine-carbazole-sulphuric acid method) ▲—▲

culture pH ●—●

4.2 Assessment of purification techniques

4.2.1 Cell disruption for enzyme release

Although cell disruption with the Hughes Press (3.4.1.2) resulted in greater protein release than the heat autolysis method (3.4.1.1), the specific activity released was higher with the later method (Table 4.2.1.1). The use of lysozyme to effect cell wall disruption was briefly investigated. However the cost of the crystalline enzyme and the amount necessary (see 3.4.1.3) were not found to justify the slight increase in specific activity (compared with the simple heat autolysis method).

TABLE 4.2.1.1 Comparison of cell disruption method (Lactobacillus brevis) and enzyme released

Cell disruption method	Enzyme activity released ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Specific activity $U.mg^{-1}$
Heat autolysis (40°C, 16 h)	1.93	2.44	0.79
Hughes Press	2.22	7.35	0.30

4.2.2 Nucleic acid and protein precipitation

Typically the precipitation of nucleic acid by $MnCl_2$ (see 3.4.2) gave a slight purification (Table 4.2.2.1).

Protein precipitation with polyacrylic acid was attempted at a variety of pHs (6.0, 5.0 and 4.5) (see Methods 3.4.3.1). At pH 4.5 about 40% of protein was precipitated with a glucose isomerase activity recovery of about 53% as compared with the initial activity of a crude extract control at pH 7.0 (Table 4.2.2.2).

Protein precipitation by the addition of solid ammonium sulphate (see 3.4.3.2) precipitated the bulk of protein with glucose isomerase activity at an ammonium sulphate concentration range of 2 - 3.6M at 0°C .

A combination of the $MnCl_2$ treatment and ammonium sulphate (2 - 3.6M) steps yielded about 19 fold purification with a total activity recovery of 68% (Table 4.2.2.3).

TABLE 4.2.2.1 Nucleic acid precipitation with $MnCl_2$

Fraction	Total protein (mg)	Total activity (units)	Specific activity ($U.mg^{-1}$)	Recovery %
Crude extract	4108	215	0.052	(100)
$MnCl_2$ treated	3400	200	0.059	93

TABLE 4.2.2.2 Glucose isomerase precipitation with polyacrylic acid

Fraction	pH	Specific Activity $U.mg^{-1}$	Recovery %	Purification fold
Crude extract	7.0	0.46	(100)	1
Protein precipitated	4.5	0.62	53.5	1.3

TABLE 4.2.2.3 Purification of glucose isomerase

Fraction	Total protein (mg)	Total Activity (units)	Specific Activity ($U.mg^{-1}$)	Recovery %	Purification fold
Crude extract	4108	214.6	0.050	(100)	1
$MnCl_2$ treated	3393	199.7	0.060	93	1.2
Ammonium sulphate					
0- 2 M	67.9	3.55	0.058		
2 - 3.6M	154.4	146.08	0.946	68.07	15.76

4.2.3 DEAE-cellulose chromatography

4.2.3.1 DEAE-cellulose chromatography by stepwise elution

40 ml of a partially purified glucose isomerase, containing 570 mg protein (specific activity 0.5 $U.mg^{-1}$) was applied onto a DEAE-cellulose (DE 32) column (2.5 cm diameter x 37 cm) which had been equilibrated with 0.02M Tris-HCl buffer pH 7.6. The elution (flow rate 1 $ml.min^{-1}$) was started with the same buffer (250 ml) followed by stepwise increasing concentration of KCl up to 0.5M in 0.02M Tris-HCl buffer pH 7.6. The chromatography was carried out at 4°C and the effluent was monitored at 258 nm on an LKB u.v. recorder and collected in 4 ml fractions.

Protein with glucose isomerase activity was split in two peaks (III, IV, Fig. 4.2.3.1) eluted with 0.2 - 0.3M KCl. The tubes with glucose isomerase activity were pooled (each peak separately) giving a specific activity of 1.3 $U.mg^{-1}$, with 2 fold purification in this step. The elution of glucose isomerase as two separate peaks had been previously reported (56) and could be explained by tight binding of the enzyme to DEAE-cellulose.

4.2.3.2 DEAE-cellulose chromatography by gradient elution

13 ml of a partially purified glucose isomerase, containing 182 mg protein (specific activity of 0.5 $U.mg^{-1}$) was applied onto a DEAE-cellulose bed (2.5 cm diameter x 37 cm) equilibrated as before, with 0.02M Tris-HCl buffer pH 7.6.

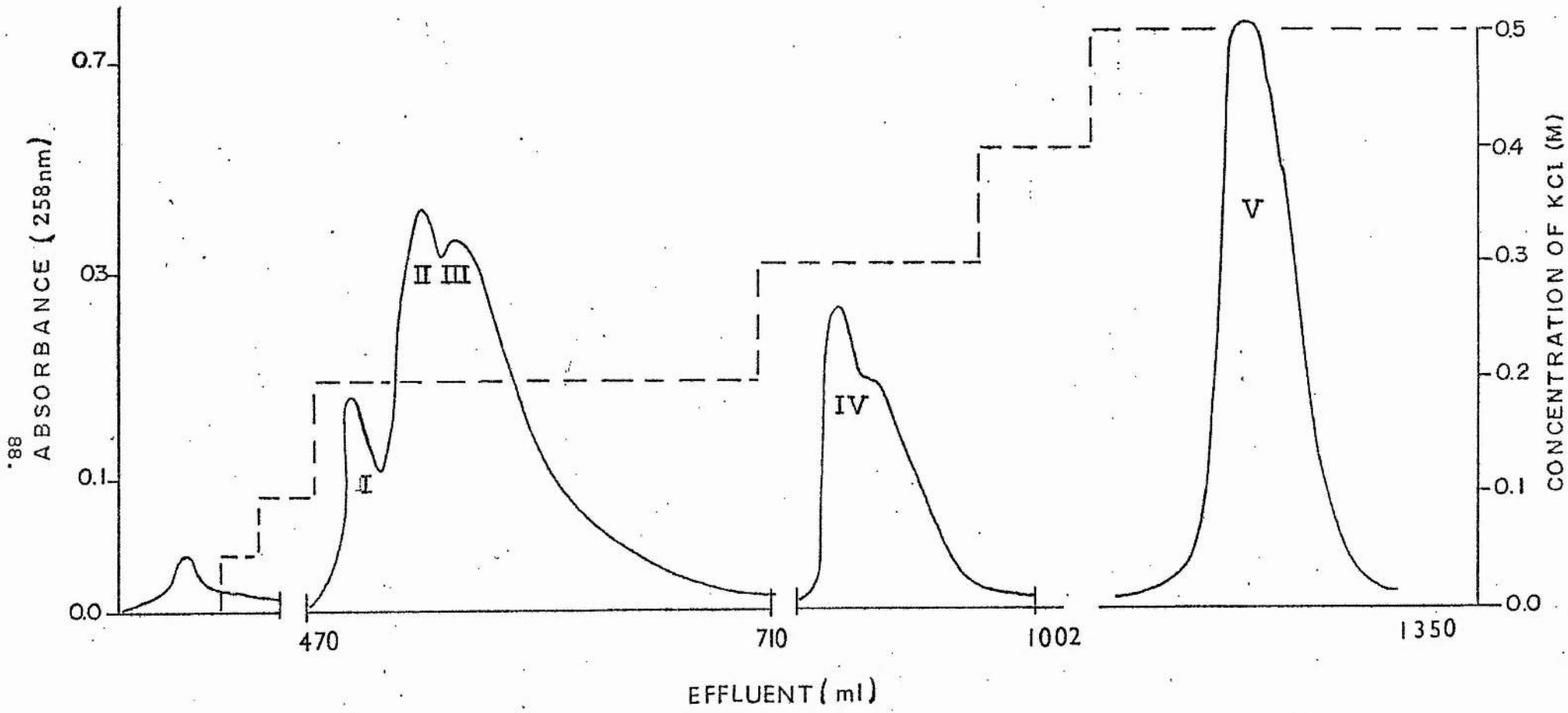


Fig. 4.2.3.1 Stepwise elution diagram of a partially purified glucose isomerase. 570 mg protein in 40 ml 0.02M Tris-HCl buffer pH 7.6 was applied onto a DEAE-cellulose bed (2.5 cm diameter x 35 cm), equilibrated with 0.02M Tris-HCl buffer pH 7.6. After 250 ml of equilibrating buffer had passed through the column a stepwise elution (0.05M-0.5M KCl in equilibrating buffer) was started. Fractions of 4 ml were collected at a flow rate of 1 $ml.min^{-1}$.

The elution was started with the equilibrating buffer (approximately 300 ml) followed by a linear gradient up to 0.6M KCl. A reservoir containing 800 ml of 1M KCl in 0.02M Tris HCl buffer pH 7.6 fed a mixing chamber containing 800 ml of 0.02M Tris HCl buffer pH 7.6. The mixing chamber was connected to the column and the effluent (flow rate 1 $ml.min^{-1}$) was monitored at 258 nm (as before) and collected in 3 ml samples. All operations were carried out at 4°C. Glucose isomerase activity was detected only in peak II.(Fig. 4.2.3.2) The specific activity was 1.8 $U.mg^{-1}$ which compared with the sample specific activity (0.5 $U.mg^{-1}$) gave about 3.6 fold purification.

Considering the strong binding of glucose isomerase to DEAE-cellulose (DE 32) an anion exchanger, it was decided to investigate CM-cellulose (CM 52), a cation exchanger. The experiments are described in the next section.

4.2.4 CM-cellulose chromatography

In small scale experiment, with CM-cellulose (CM-52) precycled (112), equilibrated with 0.02M Tris HCl buffer pH 7.0 and packed in a column with a bed volume of 2.5 cm diameter x 20 cm, it was found that protein with glucose isomerase activity would not bind to the CM-cellulose being the first peak eluted with the equilibrating buffer (Fig. 4.2.4).

Using a batch adsorption procedure, about 1.3 purification fold was achieved as follows. About 88 mg protein (in 8 ml

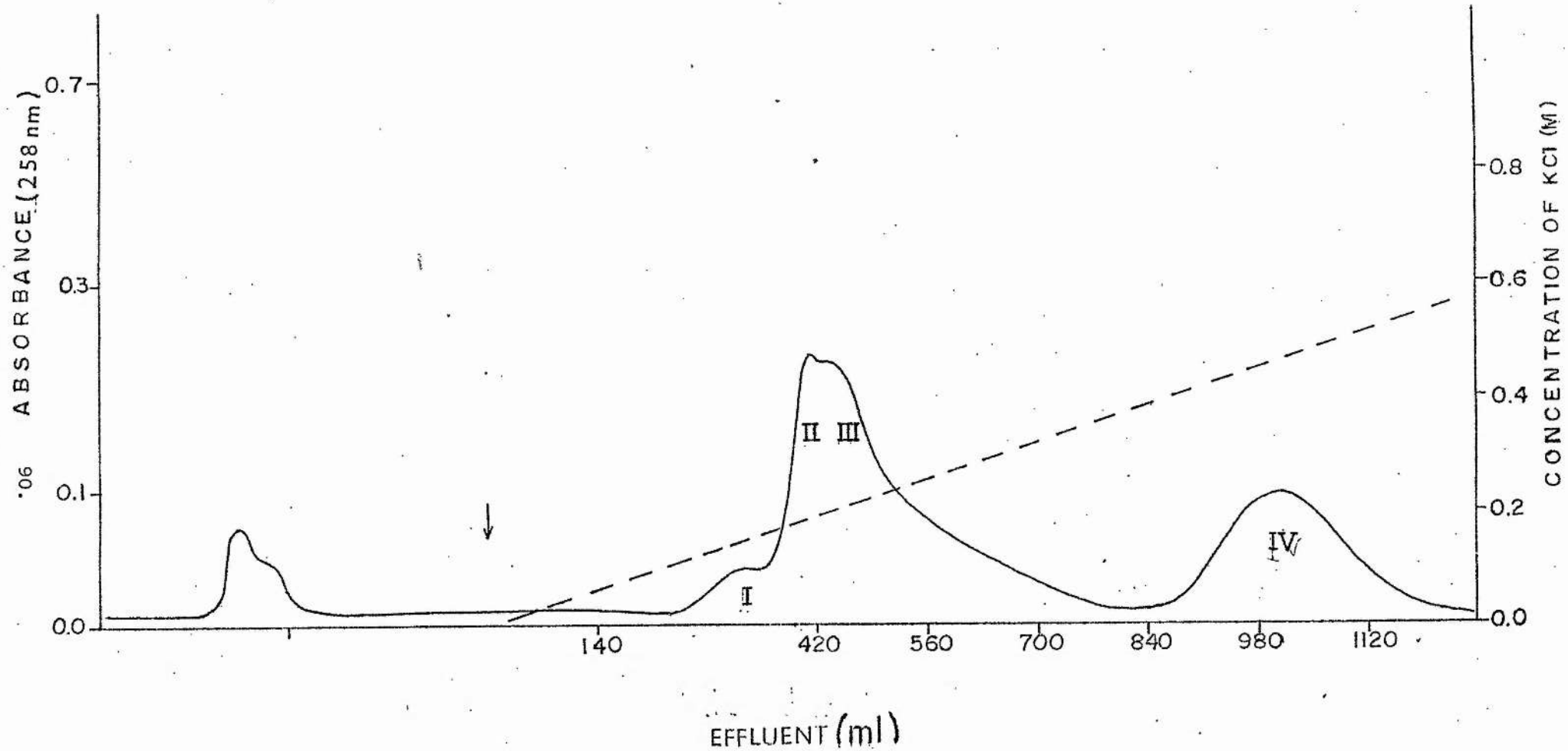


Fig. 4.2.3.2 Diagram of a DEAE-cellulose chromatography of a partially purified glucose isomerase by gradient elution. 182 mg protein in 13 ml 0.02M Tris-HCl buffer pH 7.6 was applied onto a DEAE-cellulose bed (2.5 cm diameter x 37 cm) equilibrated in the same buffer. After approx 300 ml of the equilibrating buffer had passed through the columm a linear gradient was applied, with a reservoir containing 800 ml 1 M KCl in 0.02M Tris-HCl buffer pH 7.6 feeding a mixing chamber containing 800 ml 0.02M Tris-HCl.

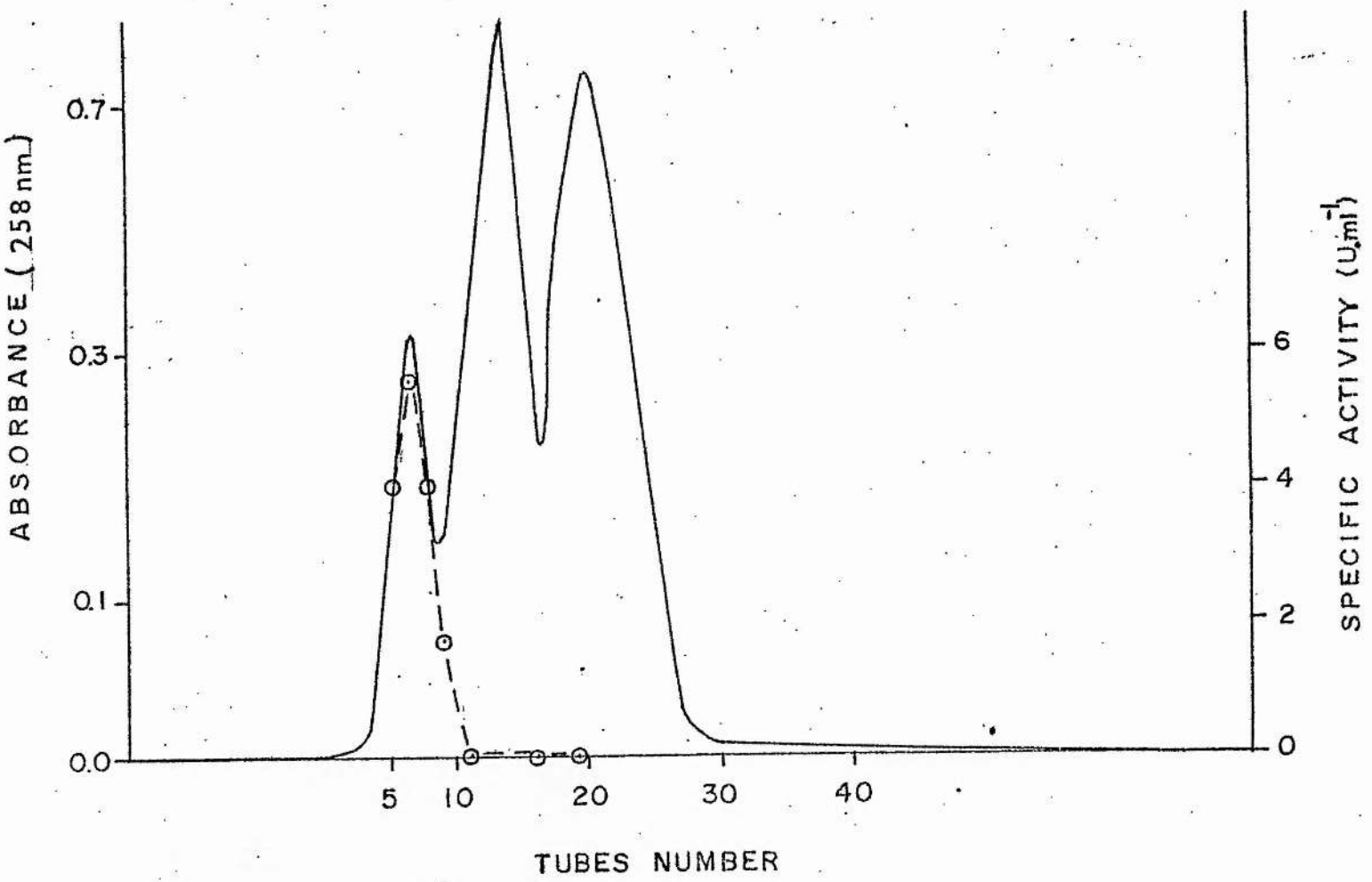


Fig. 4.2.4 Elution profile of glucose isomerase preparation (8 ml containing 88 mg protein in 0.02M Tris-HCl buffer pH 7.0) applied onto a CM-cellulose (CM-52) column (2.5 cm diameter x 20 cm) equilibrated in 0.02M Tris-HCl buffer pH 7.0. Elution was started with equilibrating buffer at a flow rate of 2 ml.min^{-1}. 6 ml fractions were collected.

of 0.02M Tris HCl buffer pH 7.0) was mixed with approximately 1 g (wet wt) of equilibrated CM-cellulose. After standing 1 h at 0°C, the mixture was centrifuged (1000 x g, 10 min at 4°C) and the supernatant assayed for glucose isomerase activity and protein. A specific activity of 1.8 $U.mg^{-1}$ as compared with the initial specific activity of 1.4 $U.mg^{-1}$ was calculated giving about 1.3 fold purification and 52.5% activity recovery in this step.

4.2.5 Discussion of the purification of glucose isomerase

An overall purification of glucose isomerase, from $MnCl_2$ treatment to batch CM-cellulose chromatography, gave about 24 fold purification with 24.7% total activity recovery (Table 4.2.5).

The ammonium sulphate step yielded an unusually high purification factor (18.9 fold) which was also achieved with the enzyme from B. coagulans (11 fold purification) although using different ammonium sulphate concentration bands (0 - 0.5) (64) Kent's results with the enzyme from Lactobacillus brevis (54) do not allow us to calculate the purification factor relative to this step. Although he reports 87.5% glucose isomerase activity recovery (with 0.60 - 0.85 ammonium sulphate saturation) the specific activity of this fraction (55.5 $U.mg^{-1}$) as compared with the subsequent fraction (0.86 - 0.95 ammonium sulphate saturation) does not suggest that a good protein fractionation was achieved. Kent's data lacks information for the specific

activity of his crude extract making comparison regarding purification factors impossible.

The chromatography with anion exchanger cellulose required prolonged elution (4.2.3) and the purification factor as compared with the previous step did not justify the inclusion of this step when, with a cation exchanger (CM-cellulose), a purification factors of 1.3 was achieved in less than 1 h. (4.2.4).

The purification factor achieved by DEAE-cellulose stepwise chromatography (2 fold) and DEAE-cellulose (linear gradient) chromagraphy (purification of 3.6 fold) as compared with the ammonium sulphate step is in the range achieved by other workers (20). In agreement with them it was found that glucose isomerase activity could not be separated from D-xylose isomerase activity (26,56,64).

Sephadex G-200 filtration of a partially purified glucose isomerase (CM-cellulose batch chromatographed) showed a single peak (Fig. 4.3) but with a slight increase in specific activity. This increase can probably be explained either as an inhibitor adsorption effect or the presence of contaminating protein(s) which co-chromatographed on CM-cellulose but separated on G-200.

A purification (after 4 steps) of 24-fold has been achieved. (Table 4.2.5) This compares favourably with Yamanaka's purification of glucose isomerase from a different strain

TABLE 4.2.5 Purification of glucose isomerase

Fraction	Total protein (mg)	Total Activity (units)	Specific Activity ($U.mg^{-1}$)	Recovery
Crude extract	4108	214.6	0.050	100
$MnCl_2$ treated	3393	199.7	0.060	93
Ammonium sulphate 2 - 3.6M pH 7.0	154.4	146.08	0.946	68.07
CM-cellulose (batch chromatography)	43.97	52.96	1.204	24.68
Total purification:	24 fold			
Homogeneity test:	gel filtration (Sephadex G-200, see 4.3)			

of Lactobacillus brevis which, employing 8 steps, achieved only an 11-fold purification. Total activity recovered in both purifications was low being the order of 20%.

From the present work, and other researchers reports (see Table 1.4.1) it appears that the purification of glucose isomerase presents difficulties and recoveries are low in comparison with what one generally considers reasonable (50 - 70%). These factors, allied to the intense biotechnological interest in glucose isomerase, have switched attention to the immobilised enzyme and, more recently, immobilised whole cells with glucose isomerase activity. The latter technology avoids completely the need to purify the enzyme.

4.3 Glucose isomerase homogeneity test and mol.wt. determination by gel filtration (Sephadex G-200)

Sephadex G-200 (prepared as described in 3.4.5) was used not only as a homogeneity test for the purified glucose isomerase (see Fig. 4.3) but also for its mol.wt. determination after calibration of the column by Andrews method (117).

The gel column was equilibrated with 0.1M NaCl in 0.02M Tris-HCl buffer pH 7.0 with a flow rate of 0.8 ml $cm^{-2}.h^{-1}$.

6 ml of purified glucose isomerase (25 mg protein in 0.02M Tris-HCl buffer pH 7.0 and 10^{-2}M $MnCl_2$) was applied onto a Sephadex G-200 column (2.5 cm diameter x 37 cm) and eluted with the equilibrating buffer.

A single peak (Fig. 4.3) was eluted with a Ve (elution volume) of 95.7 ml calculated from the position of the maximum of the peak in the elution diagram (118).

1.9 ml fractions were collected and glucose isomerase activity was assayed in the presence of 10^{-4}M Cleland's reagent, by the cysteine-carbazole-sulphuric acid method (3.2.1.2).

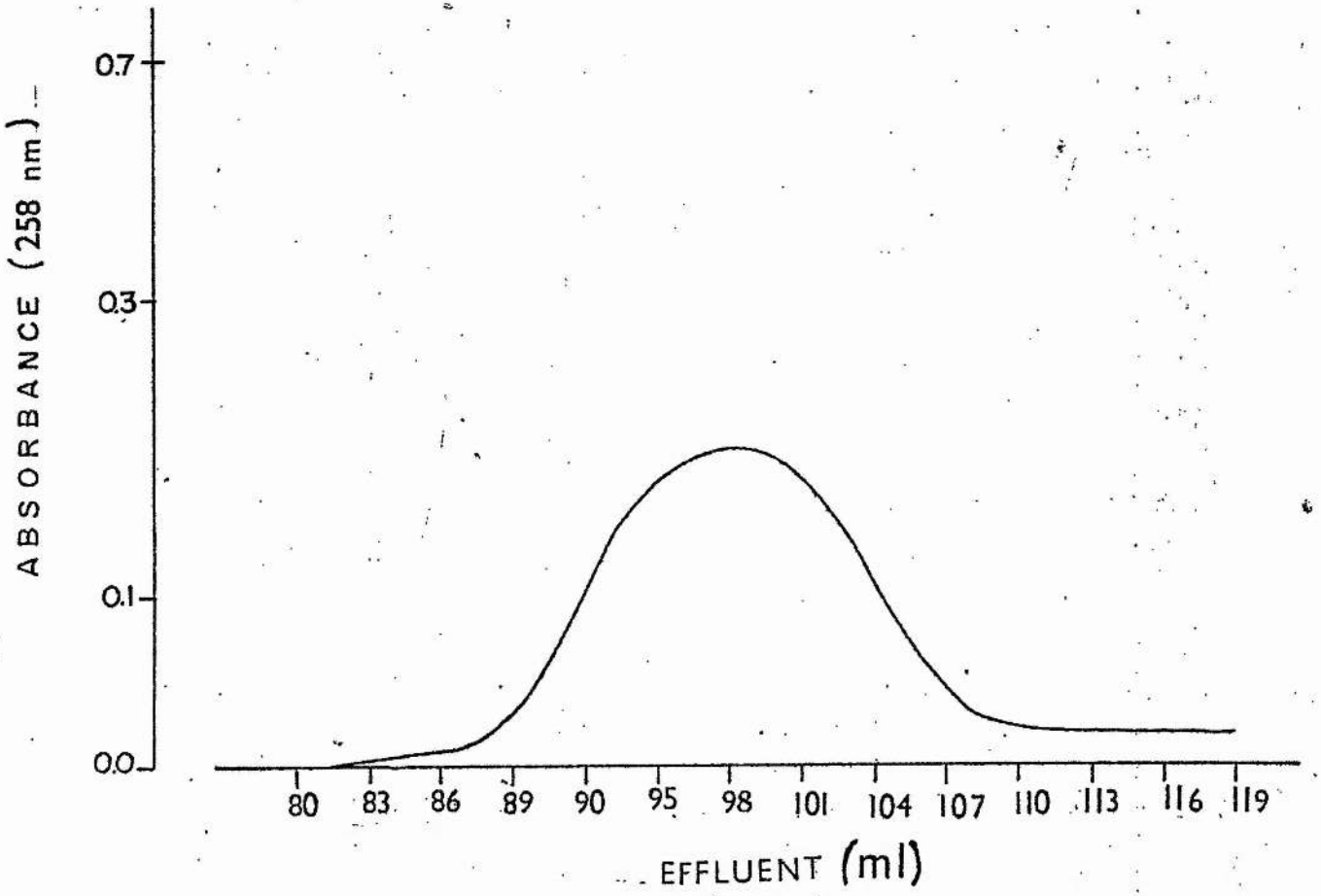


Fig. 4.3 Chromatography diagram on a Sephadex G-200 superfine column (2.5 cm diameter x 37 cm) of a purified glucose isomerase (CM.cellulose step). The sample (25 mg protein in 6 ml of 0.02M Tris-HCl buffer pH 7.0 containing 0.1M NaCl) was eluted with the same buffer at a flow rate of 0.8 $ml.cm^{-2}.h^{-1}$.

4.3.1. Calibration of the Sephadex G-200 column

The column with Sephadex G-200 equilibrated with 0.1M NaCl in 0.02M Tris-HCl buffer pH 7.0 was calibrated as follows: six protein standards were used cytochromo C (mol.wt. 12,400), ovalbumin (mol.wt. 44,000 - 46,000), alcohol dehydrogenase Mol.wt. 125,000 - 155,000), lactate dehydrogenase (mol.wt. 130,000 - 140,000), aldolase (mol.wt. 140,000 - 150,000) and apoferritin (mol.wt. 440,000 - 460,000). 10 mg of each protein was separately dissolved in the equilibrating buffer and developed with the same buffer.

After each run, the column was washed with the equilibrating buffer and used again.

A calibration plot was constructed by Andrews method (117) plotting Ve (elution volume, ml) against the log of the mol.wt. of each protein respectively (Fig. 4.3.1). Using this calibration, the mol.wt. of glucose isomerase from Lactobacillus brevis was calculated to be 120,000.

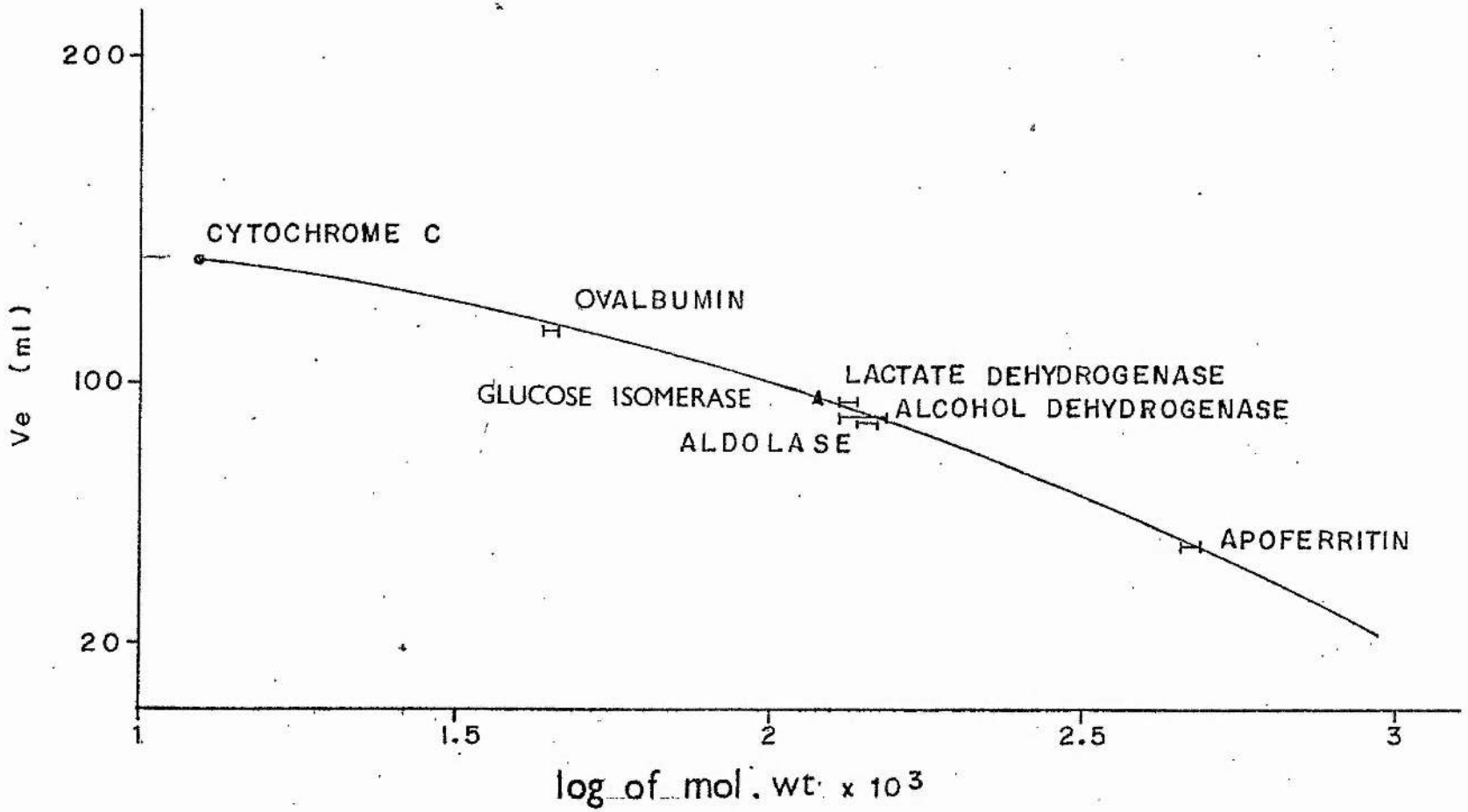


Fig. 4.3.1 Calibration plot for protein elution on a Sephadex G-200 column (2.50 cm diameter x 37 cm). Experimental details are given in 4.3.1. The lengths of bars indicate quoted ranges in mol.wt. (117).

4.4 Glucose isomerase electrophoresis

Electrophoresis of the purified glucose isomerase (homogenous on Sephadex G-200) on both 5% polyacrylamide - 0.1% SDS and 5% polyacrylamide 3% SDS-8M urea showed dissociation into subunits.

4.4.1 Glucose isomerase electrophoresis on 5% polyacrylamide gel - 0.1% SDS

20µl of the purified enzyme (treated with β-mercapto-ethanol, and dialysed against 0.1M sodium phosphate buffer, 0.1% SDS pH 7.0, as described in 3.4.6.3) was applied onto 5% polyacrylamide - 0.1% SDS gel tube (5mm diameter x 75 mm) and subjected to electrophoresis (see 3.4.6.4) with the apparatus chamber cooled by circulating water.

Protein standards (human albumin, egg albumin and myoglobin, 10µl of each) were also subjected to electrophoresis in the same conditions. A calibration plot was constructed (Fig. 4.4.1.1) by plotting the log of mol.wt. against the electrophoretic mobility of each protein standard (see equation in 3.6.5).

The densitometer record of the stained gel (coomassie blue) showed that glucose isomerase had dissociated into subunits, with a major band showing a mobility of 0.45, corresponding to a mol.wt. of 42,600. The same dissociation

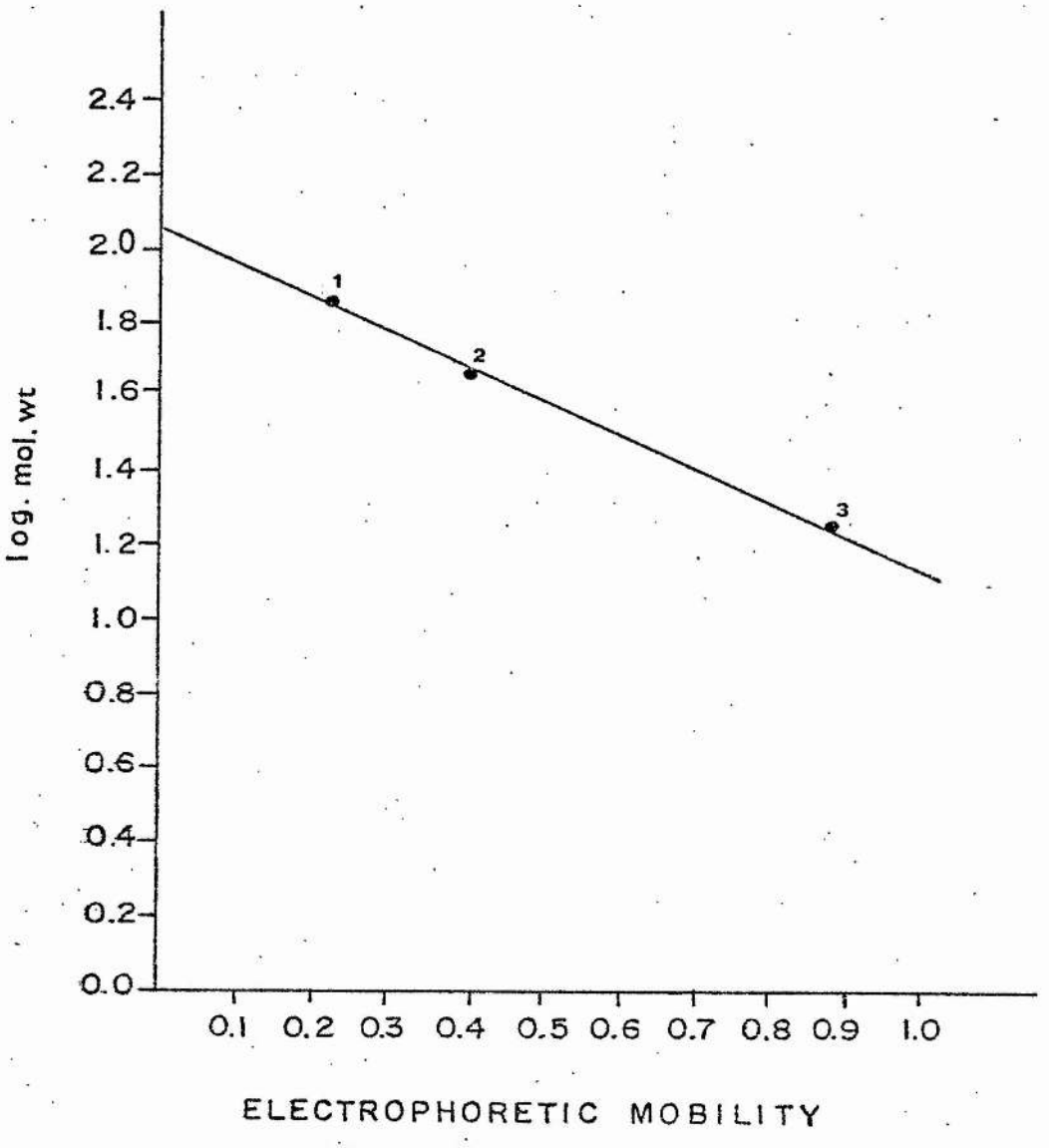


Fig. 4.4.1.1 Calibration plot for the determination of apparent mol.wt. of glucose isomerase subunits, by SDS-polyacrylamide gel electrophoresis. Mol.wt. of the polypeptide chain of the protein standards (114) 1. Human albumin (mol. wt. 68,000); 2. eggalbumin (mol.wt. 43,000); 3. myoglobin (mol.wt. 17,200).

occurred with a glucose isomerase sample which had been previously dialysed against 10^{-3}M EDTA (72 h, two changes) (see Fig. 4.4.1.2 and Fig. 4.4.1.3).

4.4.2 Glucose isomerase electrophoresis on 5% acrylamide gel - 3% SDS and 8M urea

A glucose isomerase sample was diluted 1:1 (v/v) with 8M urea, 3% SDS in 0.01M sodium phosphate buffer pH 7.1 and applied (40µl) onto 5% polyacrylamide - 3% SDS - 8M urea gel tube (5mm diameter x 75mm). Electrophoresis proceeded as with the previous samples.

Protein standards (phosphorylase a, (20µl) human albumin (10µl) egg albumin (10µl) and myoglobin (10µl)) were also subjected to electrophoresis under the same conditions and a calibration plot was constructed (Fig. 4.4.2.1).

The densitomer record of the stained gel also showed dissociation of the enzyme into subunits (mobility of 0.45) (Fig. 4.4.2.2). However the dissociation was not complete and a slower moving band (mobility = 0.21) was detected. From the calibration plot (Fig. 4.4.2.1) mol.wts. of 105,000 and 54,000 for the slow and fast moving bands were calculated.

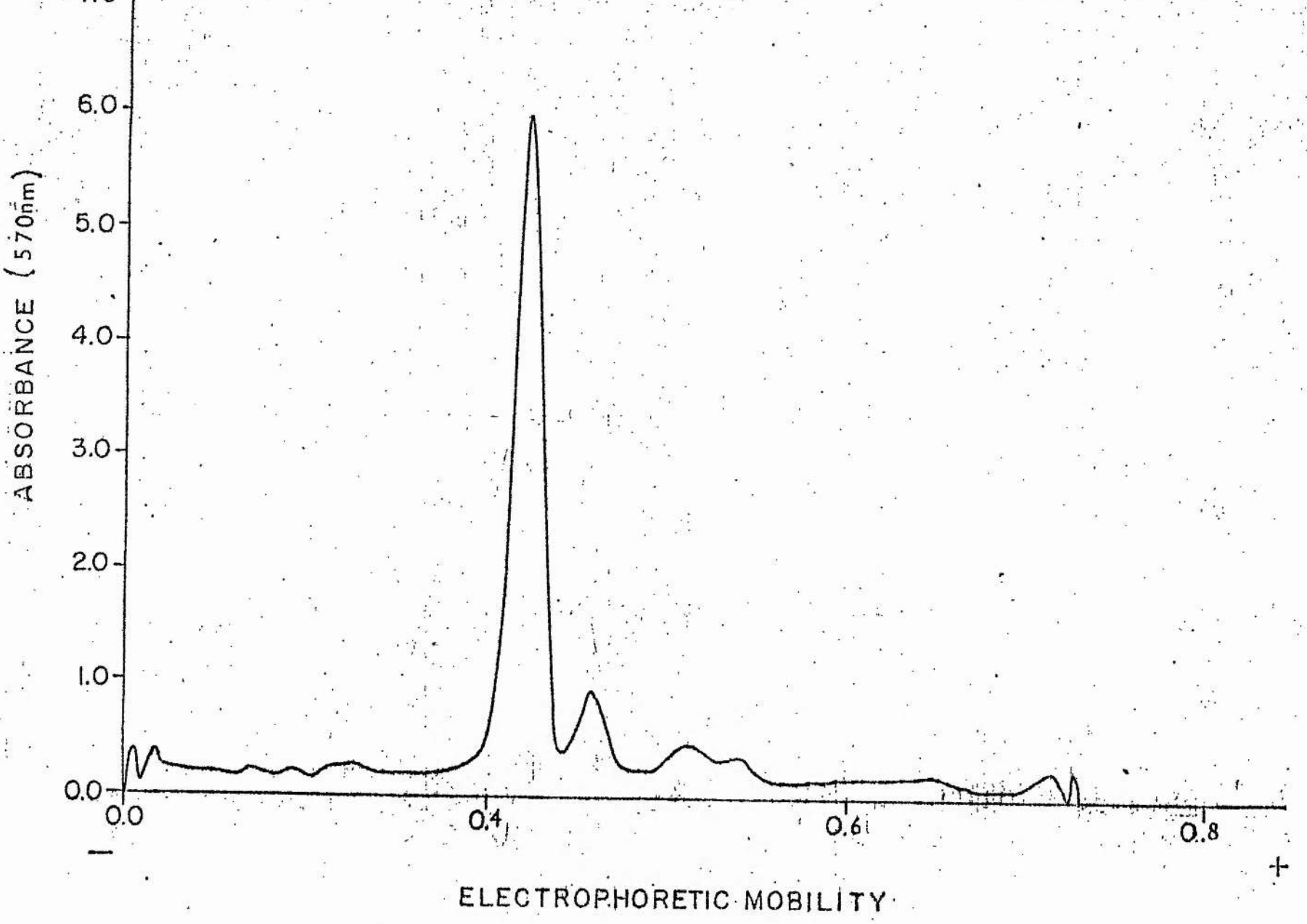


Fig. 4.4.1.2 Densitometer record after SDS-electrophoresis of glucose isomerase

Fig. 4.4.1.3 Photograph of the stained gel (coomassie blue) after SDS-acrylamide electrophoresis of glucose isomerase, treated with β-mercaptoethanol (gel 1) and EDTA (gel 2) (see 4.4.1 for experimental details).

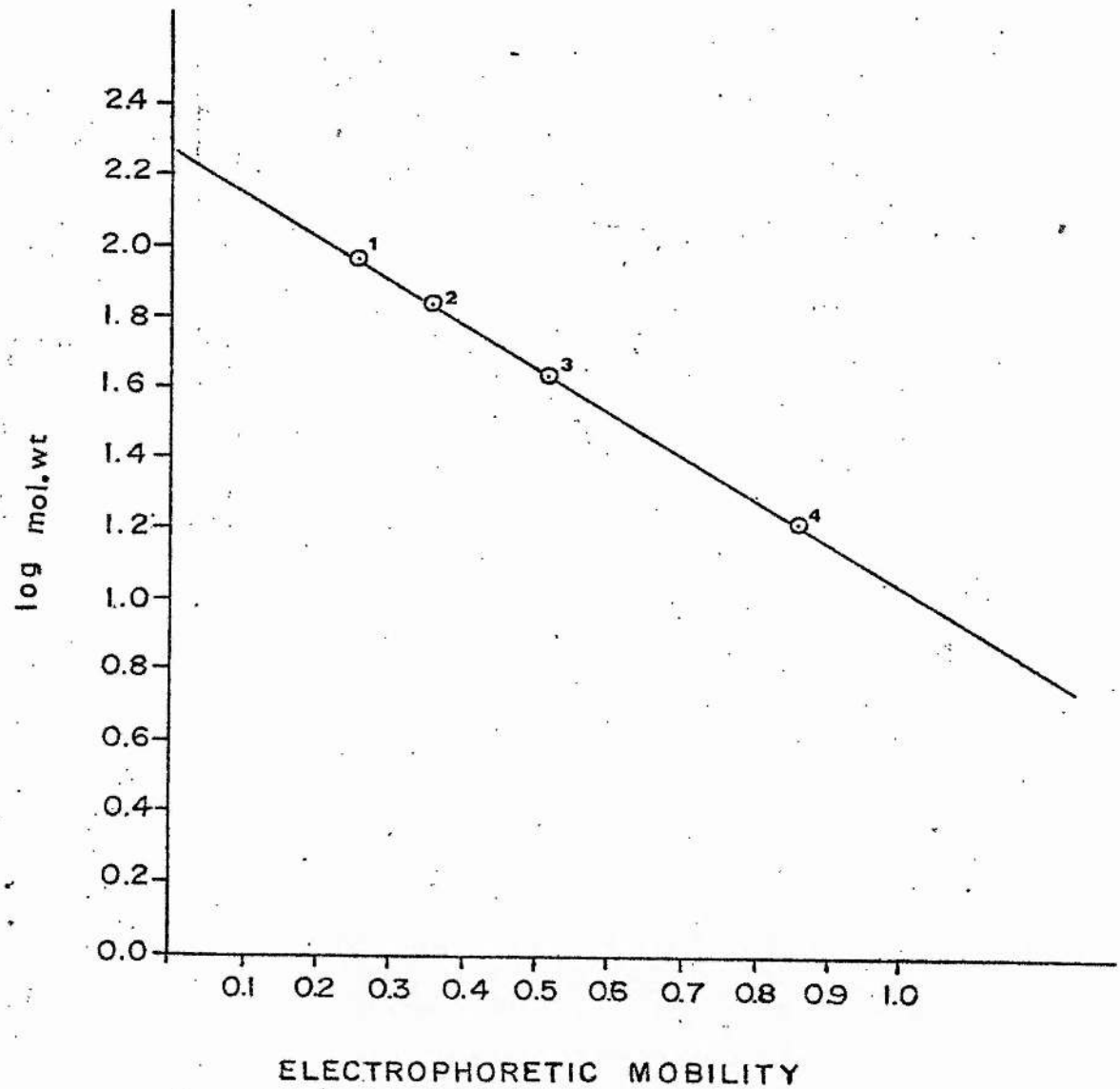


Fig. 4.4.2.1 Calibration plot for determination of the apparent mol.wt. of glucose isomerase subunits by electrophoresis in 5% acrylamide - 3% SDS and 8M urea. Mol.wt. of the polypeptide chain of the protein standards 1.phosphorylase a (mol.wt. 92,500) 2.human albumin(mo.wt.68,000) 3.egg albumin(mol.wt.43,000) 4.myoglobin(Mol.wt.17,200).

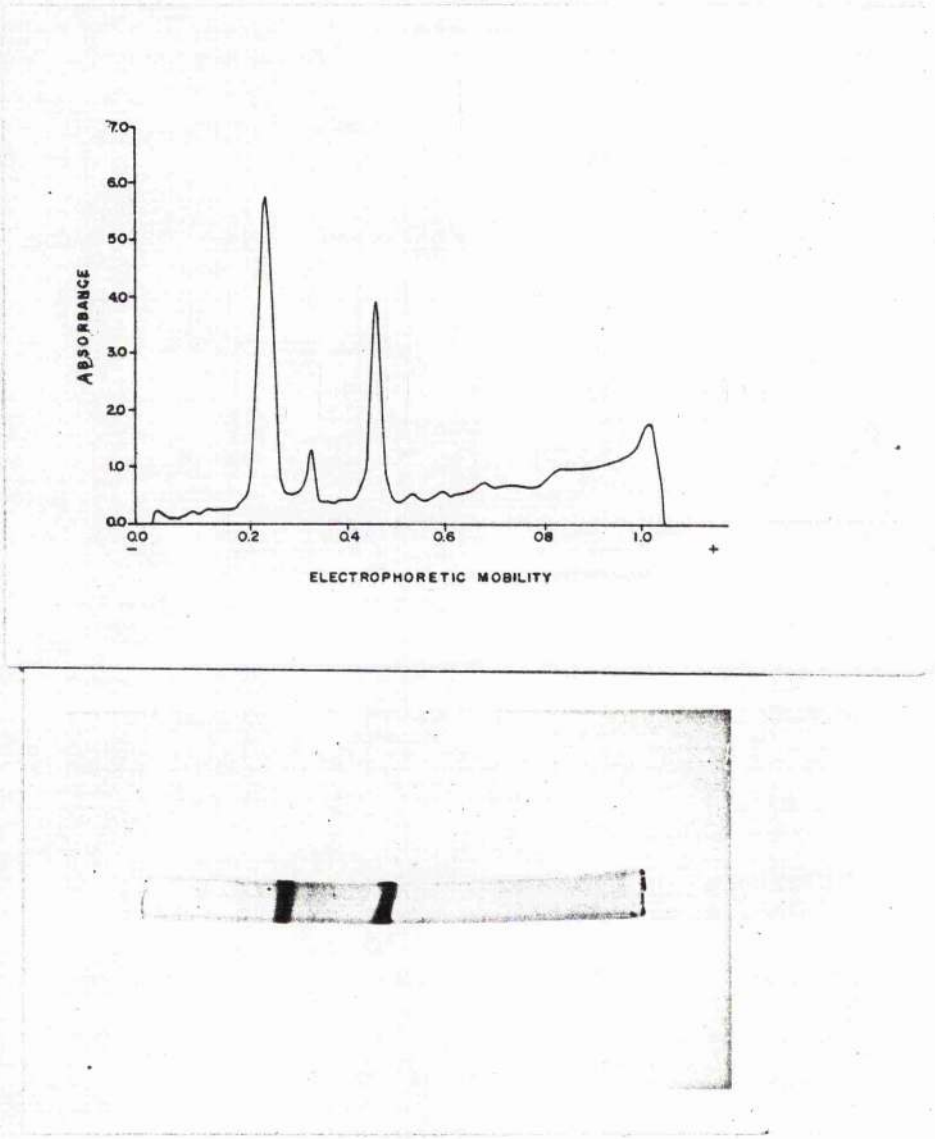


Fig. 4.4.2.2 Densitometer recorder obtained by scanning a 5 mm gel tube (5% polyacrylamide - 3% SDS - 8 M urea) onto which had been applied 40 µl of glucose isomerase in 8 M urea - 3% SDS - 0.01 M sodium phosphate buffer pH 7.1. Also is shown the photograhy of the stained gel (coomassie blue).

4.4.3 Discussion

The mol. wt. of glucose isomerase from Lactobacillus brevis calculated by gel filtration was about 120,000 compared with the mol. wt. values of 157,000 to 197,000 reported for glucose isomerase from B. coagulans, S. albus and other strain of Lactobacillus brevis (see Table 1.4.1).

Electrophoresis experiments (4.4.1 - 4.4.2) shown that glucose isomerase from Lactobacillus brevis consists of subunits, which is also in agreement with the findings reported for the enzyme from Streptomyces (66,67) and B. coagulans (68).

However, the dissociation behaviour of the enzyme from Lactobacillus brevis was quite different from what has been reported for the enzyme from B. coagulans (68). For example, glucose isomerase from B. coagulans did not dissociate with treatment with 8M urea and dissociated with SDS-polyacrylamide only when in presence of metal salts such as $CoCl_2$ or $MnCl_2$ (68). The subunit mol. wt. was estimated to be about 49,000. The role of metal salt in the enzyme dissociation by SDS, was explained assuming that in aqueous solution, without metal salt, the enzyme has a rigid structure in which SDS could not bring about any drastic change. Nevertheless, the enzyme from Lactobacillus brevis dissociated into subunits by either treatment with electrophoresis in presence of 8M urea (4.4.2) or with 0.1% SDS-5% polyacrylamide

electrophoresis (4.4.1) even after dialysis against EDTA which is a chelating reagent.

It was not clear from these experiments, which bonds were maintaining the enzyme structure. Perhaps the glucose isomerase native structure was maintained not only by H bonds but also by S-S linkages, since the subunit mol.wt. of 54,000 (calculated from electrophoresis in presence of 8M urea) decreased to 42,600 with the sample which had been reduced with β-mercaptoethanol and subjected to SDS-electrophoresis.

4.5 Development of continuous assay methods for glucose isomerase

4.5.1 Coupled assay system with sorbitol dehydrogenase and NADH

This assay (based on a coupled reaction with sorbitol dehydrogenase, described in 3.2.2) was investigated in order to optimise parameters and verify its applicability.

4.5.1.1 The effect of D-xylose concentration on glucose isomerase activity as measured by NADH oxidation.

Several concentrations of D-xylose (3.0×10^{-2}M - 20.0×10^{-2}M in 0.02M Tris-HCl buffer pH 7.6) were used in presence of $MnCl_2$ (3.0×10^{-2}M), β-NADH (2.5×10^{-4}M), sorbitol dehydrogenase (4.4 units/cuvette) in a final volume of 3 ml. The reaction (40°C) was started by the addition of glucose isomerase (10^{-2} units/cuvette).

It was found that the rate of the reaction catalysed by glucose isomerase (eqn.1)

$$\text{D-xylose} \rightleftharpoons \text{D-xylulose} \quad \text{(eqn.1)}$$

and measured by the oxidation of NADH by sorbital dehydrogenase (eqn.2)

$$\text{D-xylulose} + \text{NADH} + H^+ \rightleftharpoons \text{xylitol} + NAD^+ \quad \text{(eqn.2)}$$

was independent of D-xylose concentration above 6.6×10^{-2}M (see Fig. 4.5.1.1).

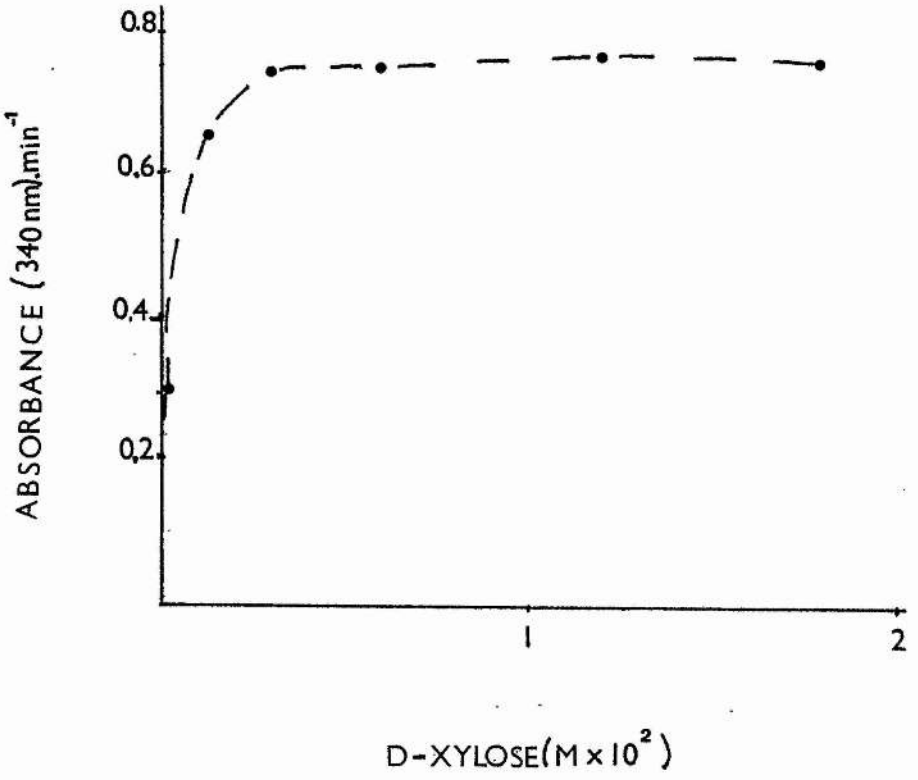


Fig. 4.5.1.1 The effect of D-xylose concentration on glicose isomerase activity as measured by NADH oxidation.

4.5.1.2 Effect of sorbitol dehydrogenase concentration on the rate measurement of glucose isomerase activity.

In order to economise on the use of Sorbitol dehydrogenase as a coupling enzyme, it was necessary to investigate the minimal sorbitol dehydrogenase concentration to avoid a rate-limiting coupled reaction.

Sorbitol dehydrogenase (0.4 - 6.6 units) were added to a 3 ml quartz cuvette (1 cm light path) containing D-xylose (6.6×10^{-2}M in 0.02M Tris-HCl buffer pH 7.6), $MnCl_2$ (3×10^{-2}M), β-NADH (2.5×10^{-4}M). The final volume was 3 ml (completed with 0.02M Tris-HCl buffer pH 7.6) and the reaction (40°C) was started by the addition of 1.52×10^{-2} glucose isomerase units.

With these assay conditions it was found that 1.5 units of sorbitol dehydrogenase was the minimal amount which could be used (Fig. 4.5.1.2).

4.5.1.3 Effect of glucose isomerase concentration upon the reaction rate measurement with sorbitol dehydrogenase - NADH

With D-xylose concentration constant ($6.6. \times 10^{-2}$M in 0.02M Tris-HCl buffer pH 7.6) and with 1.5U per cuvette of sorbitol dehydrogenase, the effect of glucose isomerase concentration on reaction rate was investigated. 1.35 to 13.5×10^{-2} units of glucose isomerase were added into a 3 ml cuvette containing D-xylose (6.6×10^{-2}M in 0.02M Tris-HCl buffer pH 7.6),

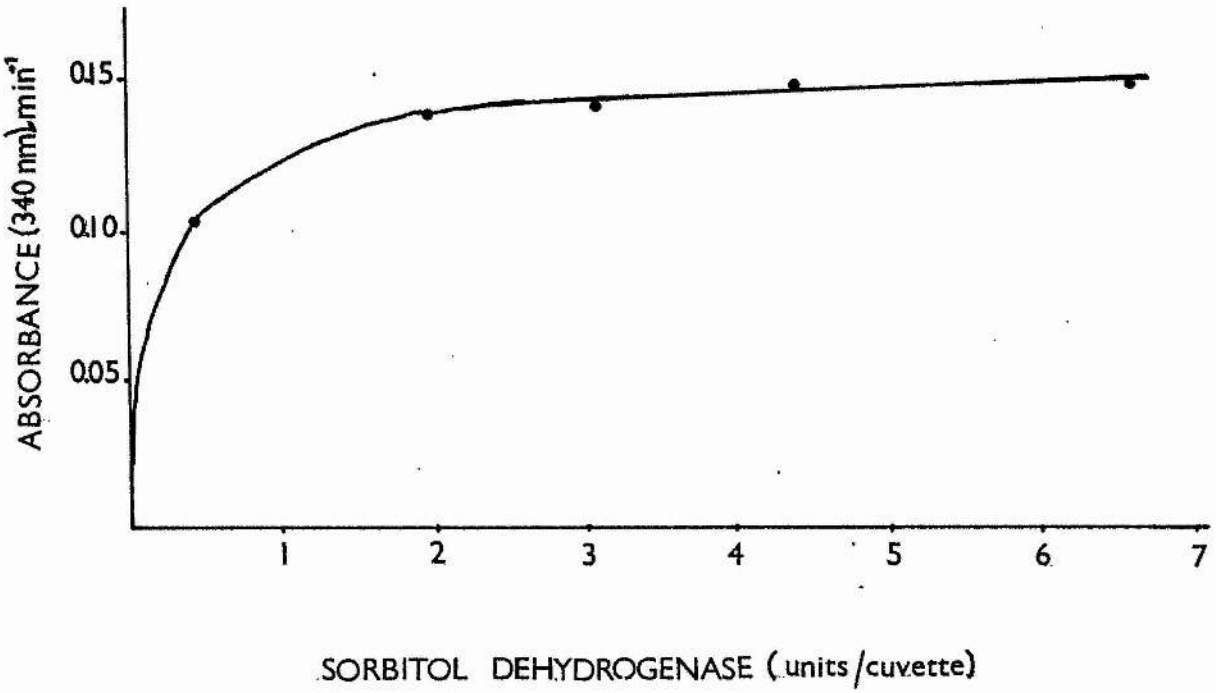


Fig. 4.5.1.2 Effect of sorbitol dehydrogenase concentration on the rate measurement of glucose isomerase activity.

$MnCl_2$ (3×10^{-2}M), β-NADH (2.5×10^{-4}M) and sorbitol dehydrogenase (1.5 units).

The reaction rate measurement was linear up to approximately 5×10^{-2} units of glucose isomerase (Fig. 4.5.1.3).

From these results, the parameters for assaying glucose isomerase activity by the coupled assay method (sorbitol dehydrogenase-NADH) was established as described in methods 3.2.2.

4.5.2 Glucose isomerase assay using an oxygen electrode system

Based on the following sequence of coupled reactions, a method for measuring the rate of glucose production was investigated.

D-Fructose $\underset{\text{glucose isomerase}}{\rightleftharpoons}$ α-D-glucose (eqn.1)

α-D-glucose $\xrightarrow[\text{mutarotase}]{}$ β-D-glucose (eqn.2)

β-D-glucose + O_2 $\xrightarrow[\text{glucose oxidase}]{}$ gluconic acid + H_2O_2 (eqn.3)

H_2O_2 $\xrightarrow[\text{catalase}]{}$ $H_2O + \frac{1}{2}O_2$ (eqn.4)

The reaction rate at 37°C was measured using a Clark oxygen electrode (YST biological oxygen monitor) to follow the oxygen uptake. For each mol of α-D-glucose produced by glucose isomerase (eqn.1) ½ mol O_2 was consumed (taking into account the eqn.4 since catalase is a contaminating activity of glucose oxidase).

For oxygen solubility a value of 193μmoles $O_2.l^{-1}$ (air-saturated buffer, 37°C) was used for calculation of rates. Thus

1 division of recorder paper

[full scale deflection = 100 divisions]

$$\equiv \frac{3 \text{ ml (assay volume)} \times 0.193\mu\text{moles } O_2.ml^{-1}}{100 \text{ (scale divisions)}}$$

= 5.79 nmoles

The calibration of the oxygen electrode was also done using the phenylhydrazine method (119) as follows:

3 ml of 1mM potassium ferricyanide (made up in 50mM acetate buffer pH 6.0) was poured into the electrode chamber, equilibrated at 37°C (circulating water) with magnetic stirring. Following assembly of the electrode/plunger into the chamber housing, 10-80μl of 10mM phenylhydrazine hydrochloride was added with a microsyringe.

Oxygen uptake (followed by chart recorder) was plotted against the phenylhydrazine concentration (Fig. 4.5.2.1)

3 ml of several D-fructose concentrations (0.05 - 2.0M in 20 mM acetate buffer pH 6.0) were added to the electrode chamber and saturated with air (37°C). Subsequently the oxygen electrode was fitted into the chamber and the reaction started by the addition of glucose isomerase (0.03 units in

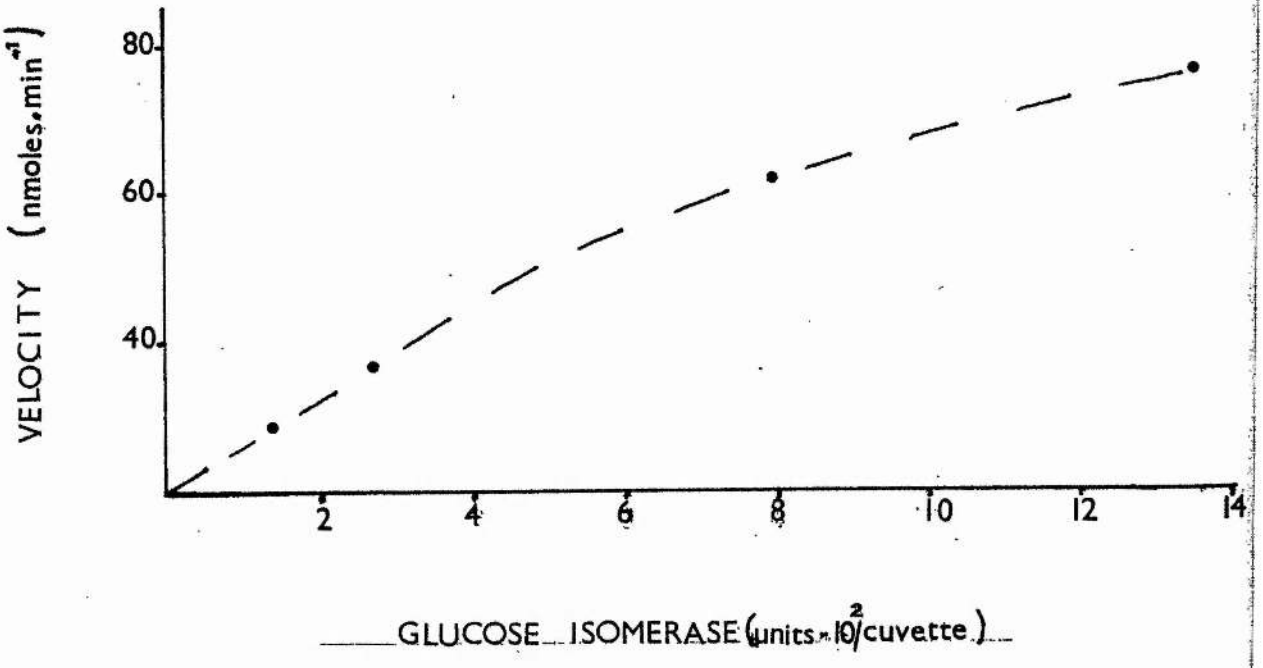


Fig. 4.5.1.3 Effect of glucose isomerase concentration upon the reaction rate measurement with sorbitol dehydrogenase - NADH

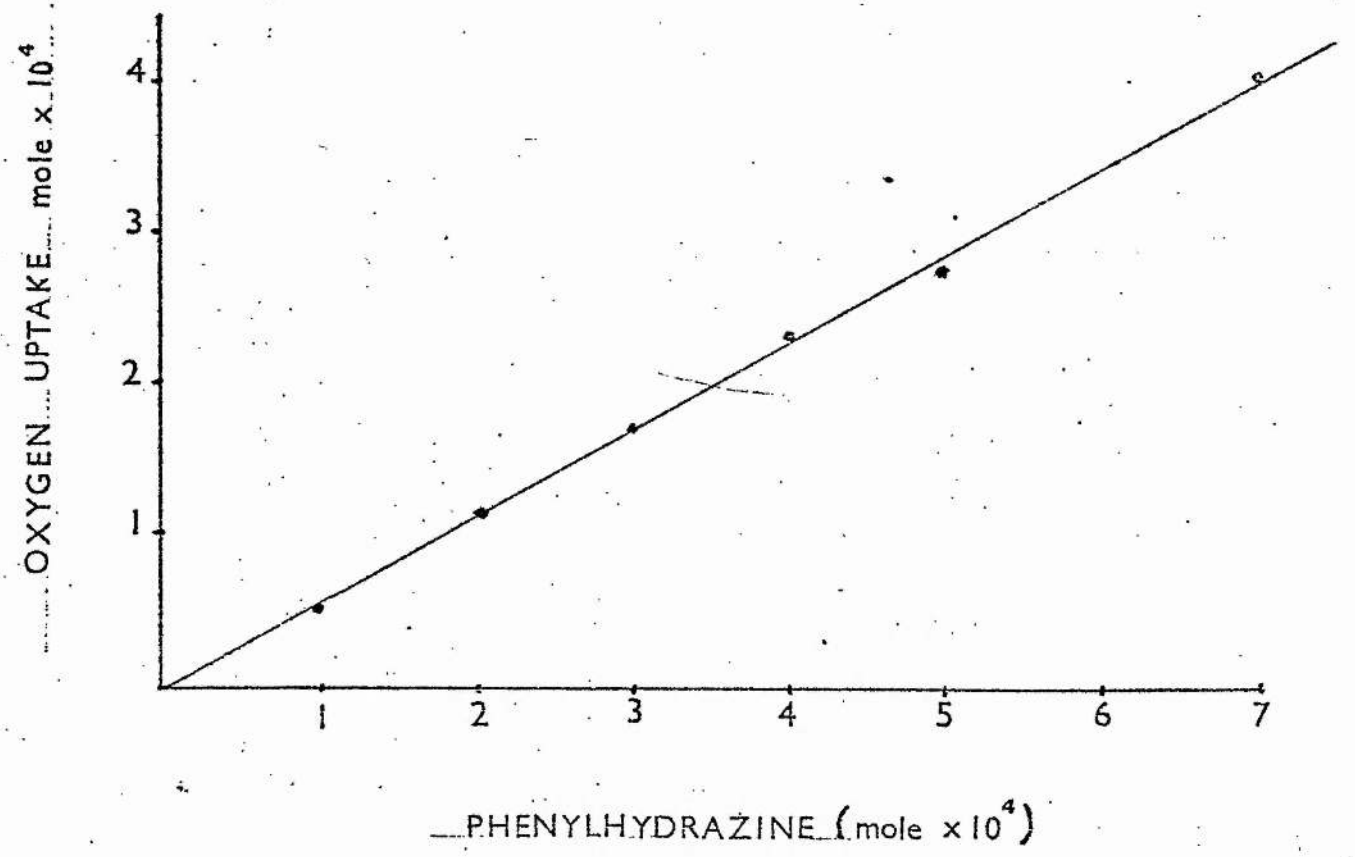


Fig. 4.5.2.1 Calibration plot for a Clark oxygen electrode by phenylhydrazine method (see 4.5.2).

10µl) and glucose oxidase (62 units in 100µl). A calibration plot relating % of oxygen consumption.min^{-1} against D-fructose concentration ($mol.l^{-1}$) (Fig. 4.5.2.2) showed that from above 1.5M fructose the reaction rate was independent of the D-fructose concentration.

With a reaction system of 3 ml 2M D-fructose (in 20mM acetate buffer pH 6.0), 39 units of glucose oxidase and a range of glucose isomerase concentrations (0.01 - 0.05 U) the % of oxygen uptake was linear with increasing enzyme concentration (Fig. 4.5.2.3).

Although the rate of oxygen uptake increased linearly with increasing glucose isomerase activity, this method of assay suffered severely from a "high background" rate (the oxidation of glucose present as a contaminant in fructose). The high concentration of fructose used in assay (2M), necessitated by the high K_m value of the glucose isomerase-fructose system, meant the presence, even in the purest grades of fructose, of sufficient contaminating glucose to give an appreciable rate of oxygen consumption. In fact, the only way to measure glucose isomerase activity with this system was to pre-incubate the system without glucose oxidase for some 30 mins and then add it and measure oxygen consumption - in effect a type of 'end point' assay.

These difficulties were further compounded by the discovery that glucose oxidase activity is inhibited by high concentrations of fructose (certainly by 2M). Because of the above problems

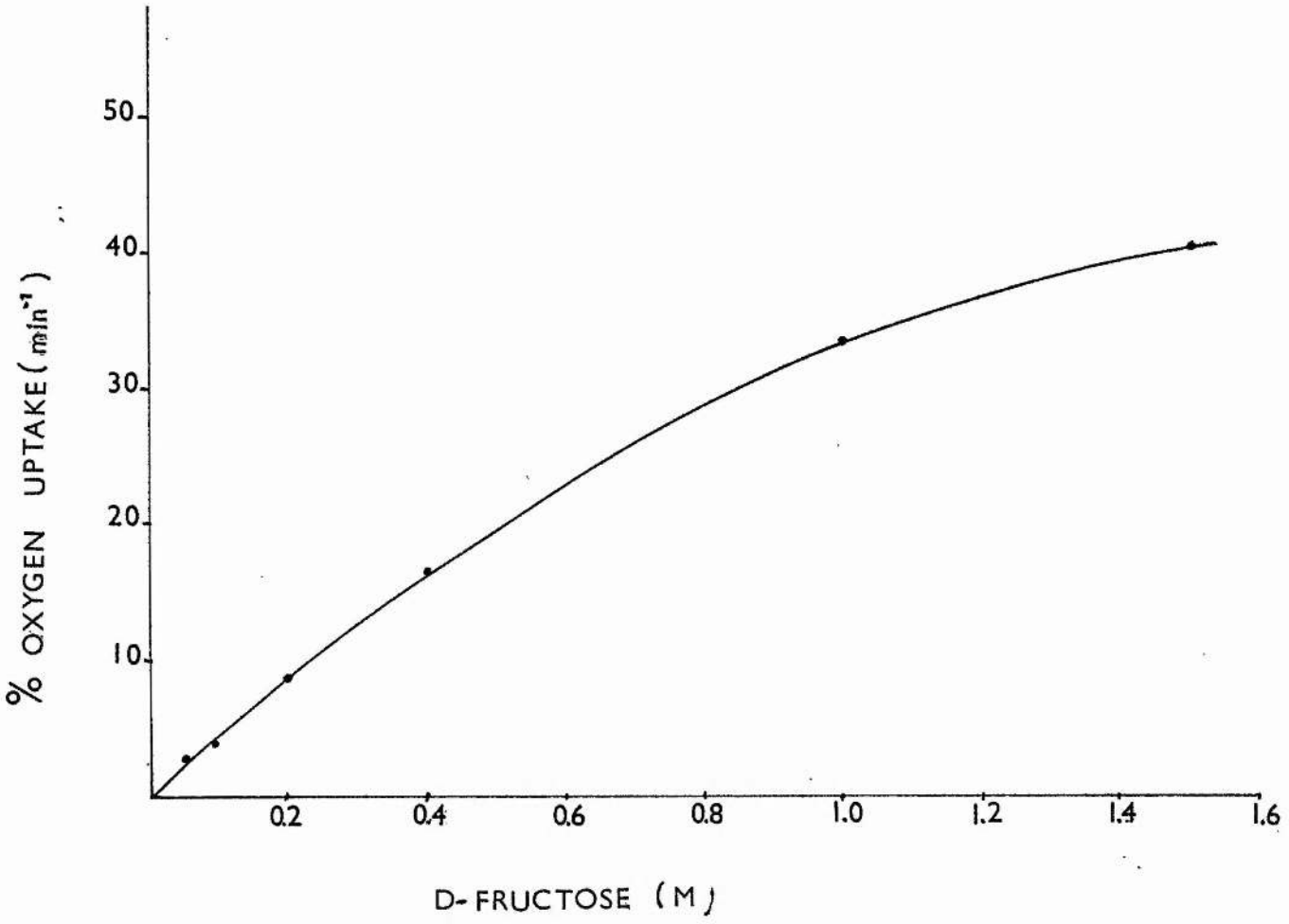


Fig. 4.5.2.2 Calibration plot for the rate of oxygen consumption ($\%.min^{-1}$) as a function of fructose concentration (0.05M - 1.6M, 3 ml in 20mM acetate buffer pH 6.0) in presence of 62 units glucose oxidase (100µl) and 0.03 units of glucose isomerase (30µl) at 37°C.

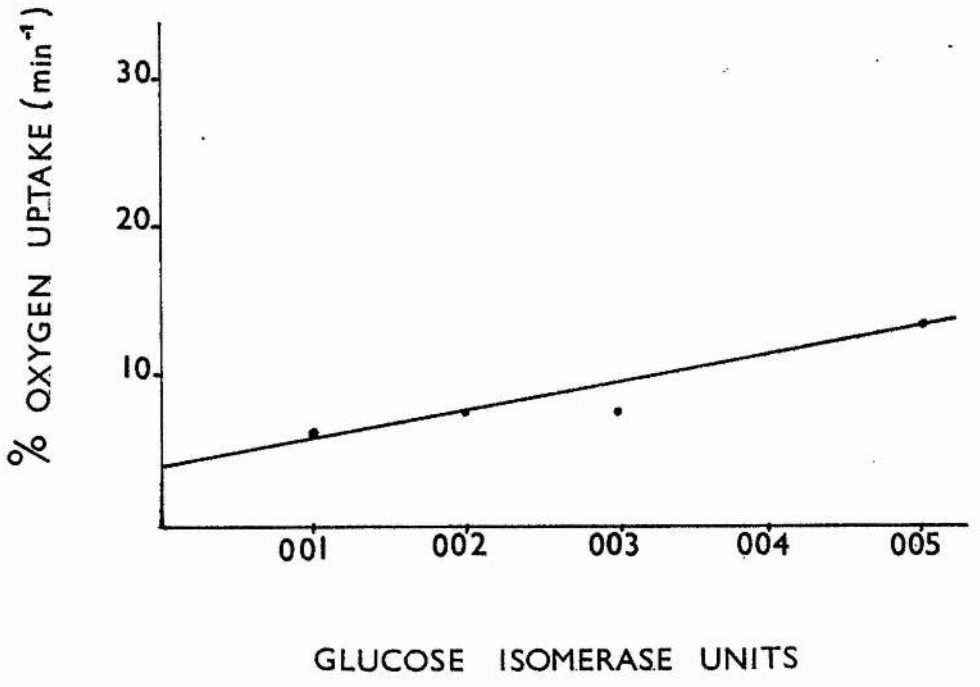


Fig. 4.5.2.3 Rate of oxygen consumption as function of glucose isomerase concentration in 2M fructose (3 ml) pH 6.0 at 37°C.

and the fact that the method was insensitive to low glucose isomerase activities further development of the oxygen electrode method was not attempted.

4.6 Kinetics studies on free and immobilised glucose isomerase.

4.6.1 Activity and protein assay of immobilised glucose isomerase.

Glucose isomerase was immobilised with PEI-derivative of nylon (see 3.5) and the amount of specific activity retained (3.5.3) and bound protein (3.5.4) are shown on Table 4.6.1.

TABLE 4.6.1 Immobilised glucose isomerase

Immobilised enzyme	Protein bound (mg.500 mg support)	Specific activity (U.mg bound enzyme)	% Specific activity retained
PEI-glucose isomerase	1.078	0.046	11.8

4.6.2 pH activity profile of free and immobilised glucose isomerase.

0.32 units of purified glucose isomerase (homogenous on Sephadex G-200) was incubated at 40°C with 10^{-2}M $MnCl_2$, and D-xylose made up in the following buffers:-

(i) 0.02M acetate buffer pH 4.1, 5.0;

(ii) 0.02M Tris-NaOH buffer pH 6.0, 6.4;

(iii) 0.02M Tris-Maleate buffer pH 7.0;

(iv) 0.02M Tris-HCl buffer pH 7.6, 8.0.

The activity was assayed by the cystein carbazole-sulphuric acid method and plotted as a percentage of the maximal initial activity against pH (Fig. 4.6.2).

The active profile of the immobilised enzyme was also calculated, using approximately 0.198U incubated in 0.2M D-xylose (5 ml) with 10^{-2}M $MnCl_2$ at several pH values (Fig. 4.6.2).

A sharp pH profile was achieved with both enzymes with the maximal activity at pH 7.0 (free enzyme) and pH 6.8 for the immobilised enzyme.

The immobilised enzyme showed a broader acid activity shoulder than the free enzyme.

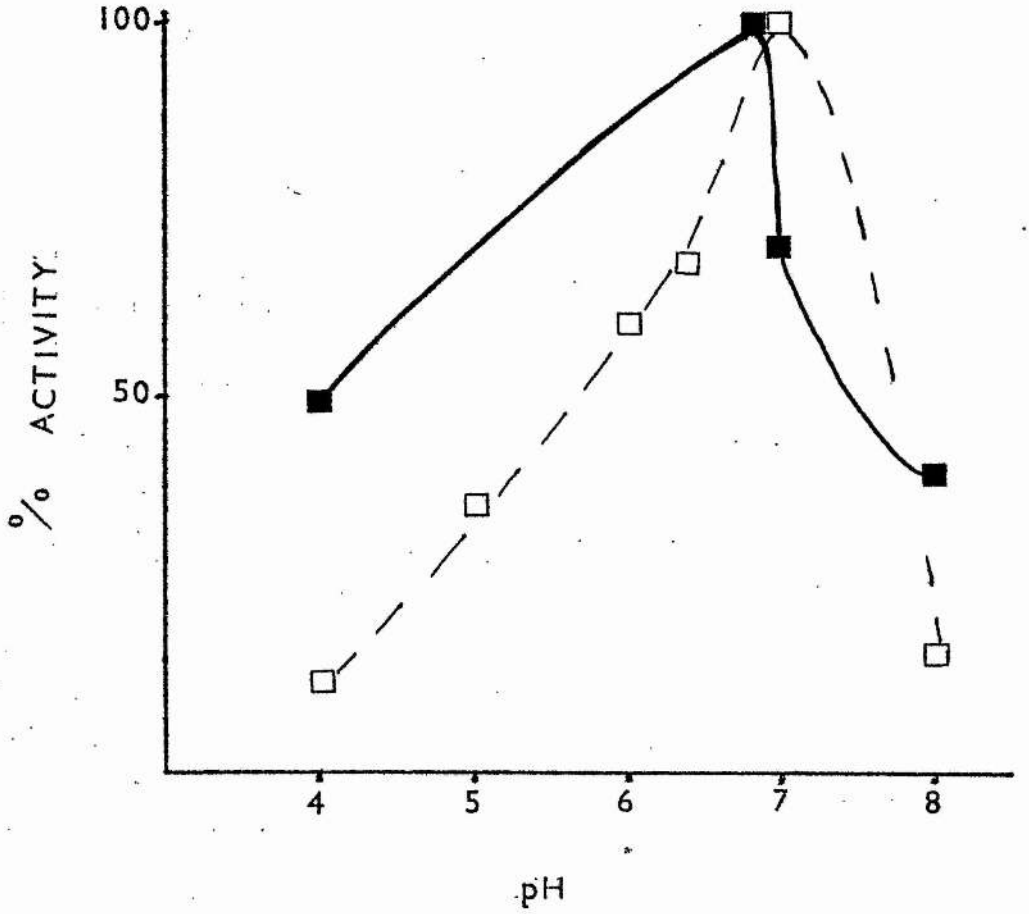


Fig. 4.6.2 pH activity profile

immobilised glucose isomerase ■-■

free glucose isomerase □-□

4.6.3 Thermal inactivation of free and immobilised glucose isomerase

Several aliquots of glucose isomerase (8 units) were preincubated in presence of 10^{-2}M $MnCl_2$ at a range of temperature (30°, 40°, 50°, 70° and 80°C) for 10 min, and immediately cooled in an ice bath. Subsequently each aliquot was incubated at 40°C with 0.2M xylose - 10^{-2}M $MnCl_2$ - in 0.02M Tris-HCl buffer pH 7.0 and the residual activity (%) calculated from the maximal initial velocity rate (≡ 100% activity).

The immobilised enzyme was subjected to the same treatment. The plot % activity against temperature (°C) shows that the enzyme from Lactobacillus brevis is stable up to 60°C and above this value total inactivation occurs with both free and immobilised enzyme (Fig. 4.6.3). Immobilisation did not increase the thermal stability of glucose isomerase.

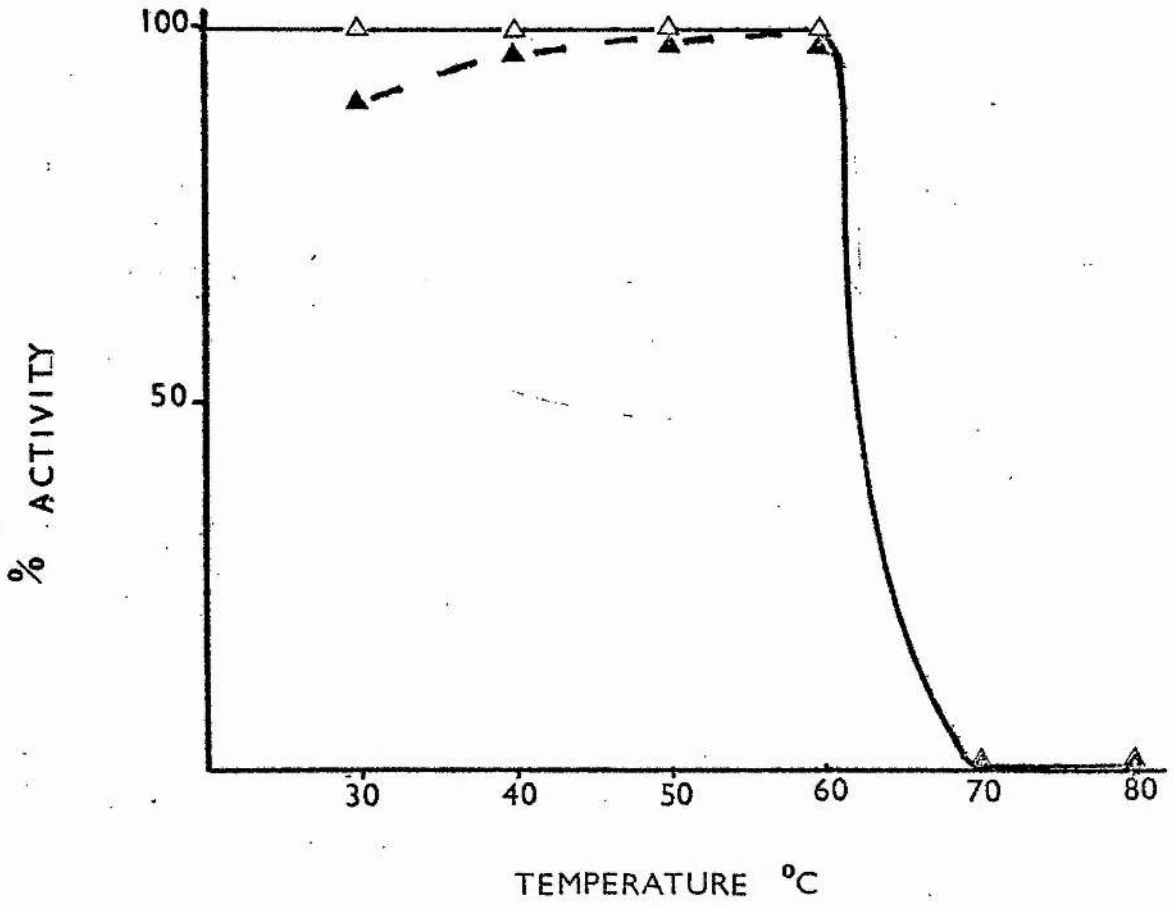


Fig. 4.6.3 Thermal inactivation profile of free glucose isomerase (△—△) and immobilised enzyme (▲—▲).

4.6.4 Temperature activity profile of free and immobilised glucose isomerase

Aliquots of glucose isomerase (0.8 units) were incubated at a range of temperatures (30°C 60°C) with 0.2M D-xylose 10^{-2}M $MnCl_2$ and 0.02M Tris-HCl buffer pH 7.0. The immobilised enzyme(0.55units) was incubated under the same conditions, and the activity assayed by the cysteine-carbazole-method (3.2.1.2).

The results plotted as initial activity (µmoles of product min^{-1}) against temperature (Fig. 4.6.4) shows a maximum activity at 50°C for the free enzyme as compared with a higher value (60°C) for the immobilised enzyme.

4.6.5 Investigation on the effect of mercuric ions on glucose isomerase activity

The effect of mercuric ions on glucose isomerase activity was investigated as follows: a range of $HgCl_2$ concentrations (0.2µg - 6µg per mg of enzyme) was added to purified glucose isomerase and incubated at 50°C for 1 h. Subsequently 0.2M D-xylose (containing 10^{-2}M $MnCl_2$ in 0.02M Tris-HCl pH 7.0 was added and glucose isomerase activity assayed. A control without mercuric ions was set up and its initial velocity (≡100% activity) was used for the calculation of the % of initial activity retained after incubation of the enzyme with increasing mercuric ion concentration.

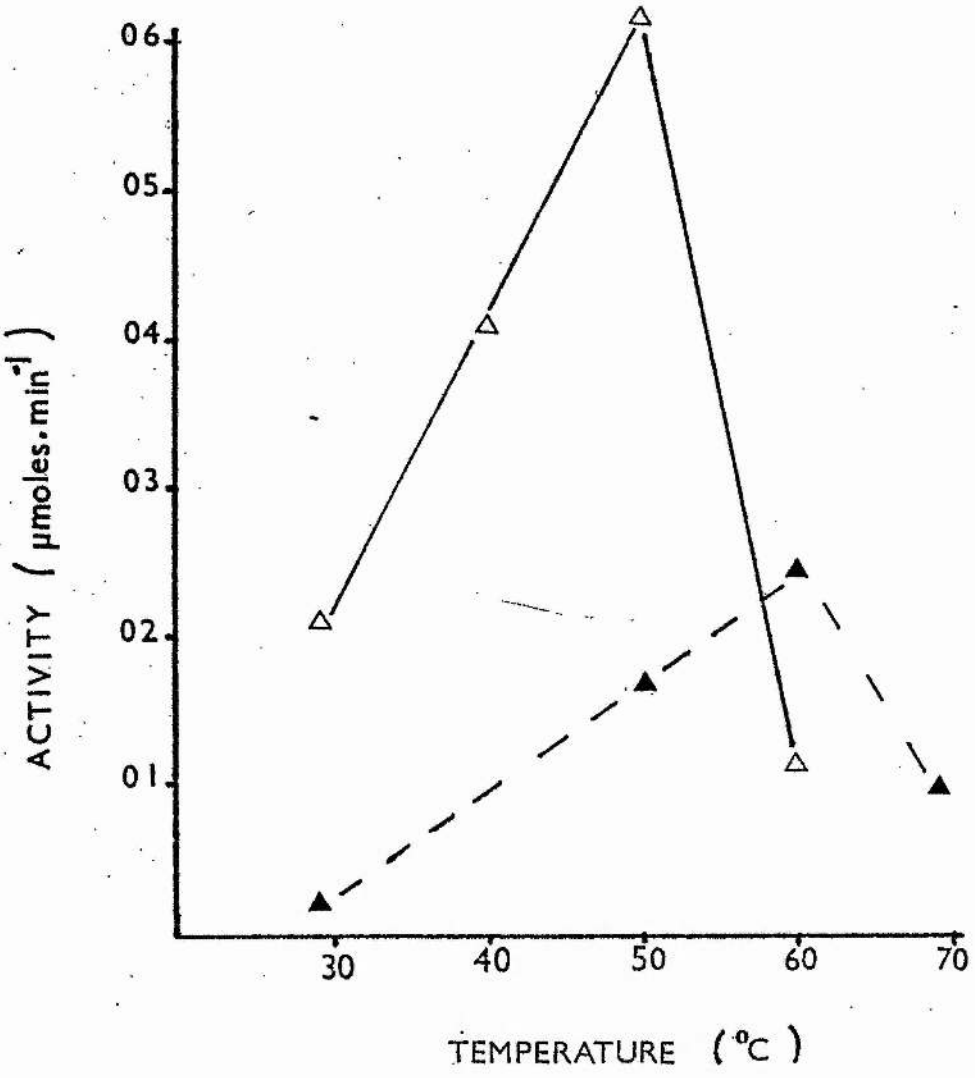


Fig. 4.6.4 Temperature activity profile

free glucose isomerase △—△

immobilised glucose isomerase ▲–▲

The plot % of activity against increasing mercuric ion concentration per mg of protein (Fig. 4.6.5) showed a decrease of about 20% of the initial activity, in the presence of 6µg mercuric ion per mg protein.

There are many ways in which mercuric ion can inhibit an enzyme. It can combine with single sulphydryl, imidazole, amino or carboxyl group or displace metals in metalloenzymes.

It has been reported that glucose isomerase from S. albus contains cobalt and magnesium ions in its structure, and 97% inhibition was detected with 0.001M $HgCl_2$. However monoiodoacetate and p-chloromercuribenzoate (SH group inhibitors) were not effective inhibitors of this enzyme (16).

4.6.6 Effect of substrate concentration on the activity of free and immobilised glucose isomerase.

4.6.6.1 Effect of D-xylose, D-glucose and D-fructose concentrations on the activity of free glucose isomerase.

Glucose isomerase (homogeneous on Sephadex G-200) was incubated with D-xylose, D-glucose and D-fructose as follows:

A range of D-xylose concentrations (0.33×10^{-2}M - 6.67×10^{-2}M in 0.02M Tris-HCl buffer pH 7.0 and 10^{-2}M $MnCl_2$) were incubated at 40°C with 10µg of glucose isomerase. The enzyme activity was measured by a continuous method (3.2.2) and

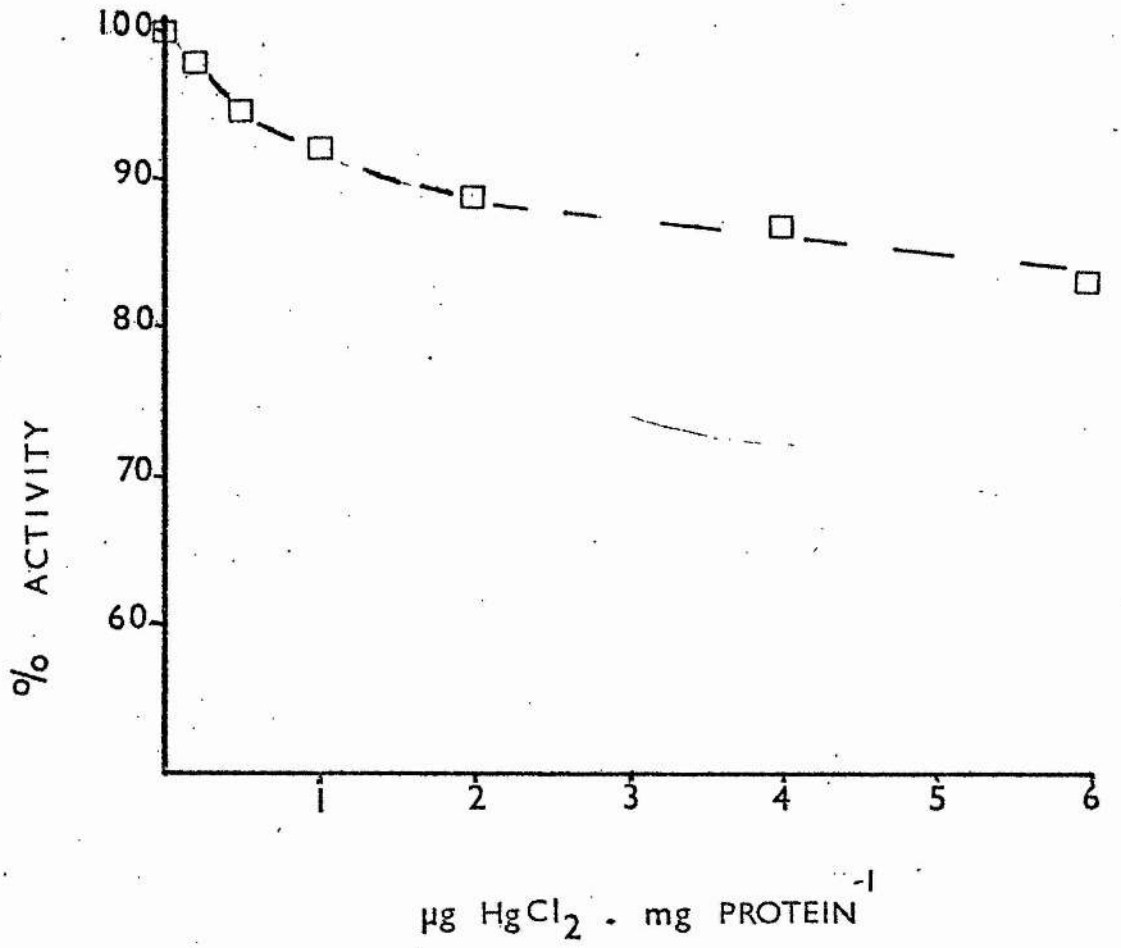


Fig. 4.6.5 Effect of mercuric ion on the activity of glucose isomerase

the initial velocity was calculated from the initial slope of a progress time reaction. Fig. 4.6.6.1.1 shows a S/v versus S plot. A K_m value of 7.4 ± 0.79 x 10^{-3}M and a V value of 4.46 ± 0.14μmoles.min^{-1} were calculated using a computer program (see section 6.0)

The effect of glucose concentration on the enzyme activity was estimated by incubation of 50μg of glucose isomerase with a range of glucose concentrations (0.2 - 2.5M in 0.02M Tris-HCl buffer pH 7.0 and 10^{-2}M $MnCl_2$, 10^{-3}M $CoCl_2$).

The enzyme initial velocity was measured as before and K_m (2.85 ± 0.089M) and V (0.75 ± 0.014μmoles.min^{-1}) values were calculated using a computer program.

Fig. 4.6.6.1.2 shows a S/v versus S plot for D-glucose isomerisation.

The effect of fructose concentration (0.5M - 1.33 in 0.02M Tris-HCl buffer pH 7.0 with 10^{-2} $MnCl_2$ and 10^{-3} $CoCl_2$) on glucose isomerase, at 40°C was estimated by Lloyd & Whelan method (3.2.3) Fig. 4.6.6.1.3 shows S/v versus S plot for D-fructose and Table 4.6.6 shows K_m and V values with standard errors, for glucose isomerase acting on D-xylose, D-glucose and D-fructose.

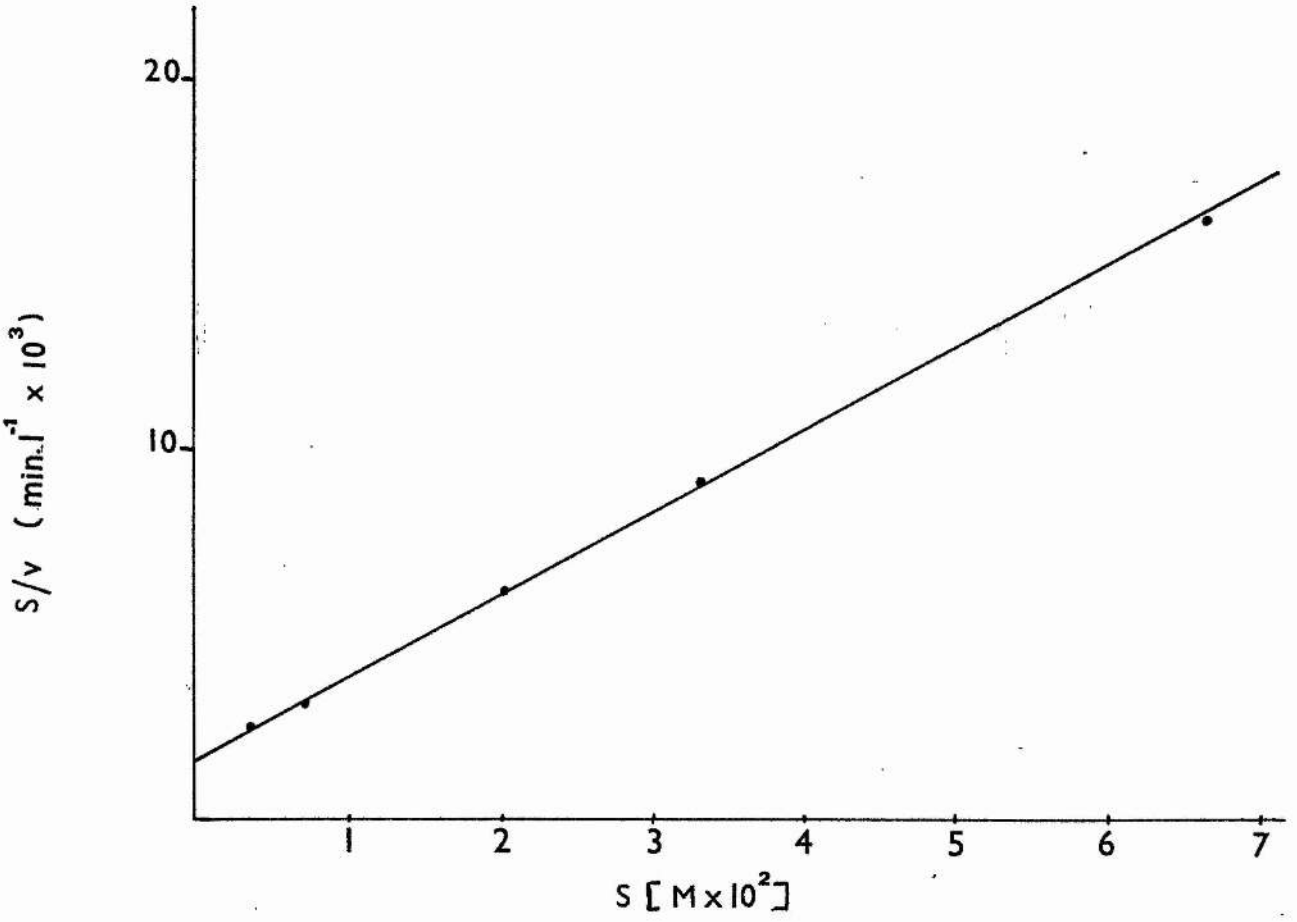


Fig. 4.6.6.1.1 S/v versus S plot for D-xylose isomerisation by glucose isomerase (10µg) at 40°C and pH 7.0.

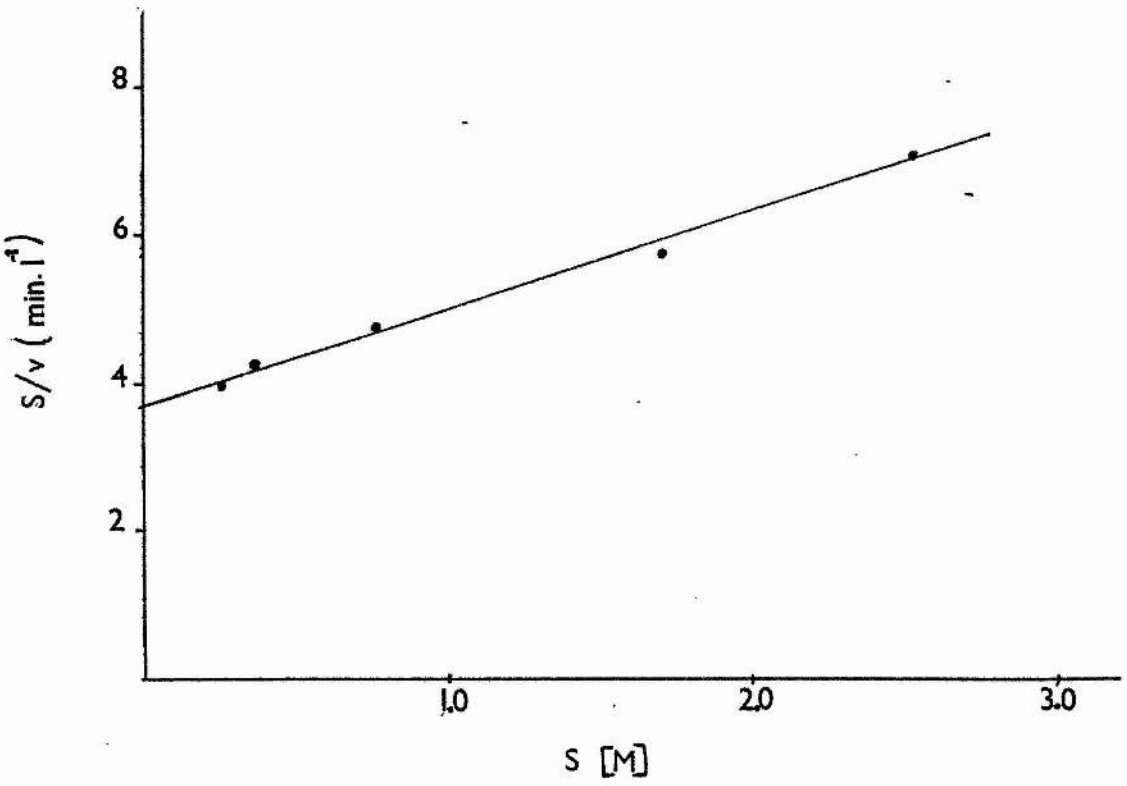


Fig. 4.6.6.1.2 S/v versus S plot for D-glucose isomerisation by glucose isomerase (50µg) at 40°C and pH 7.0.

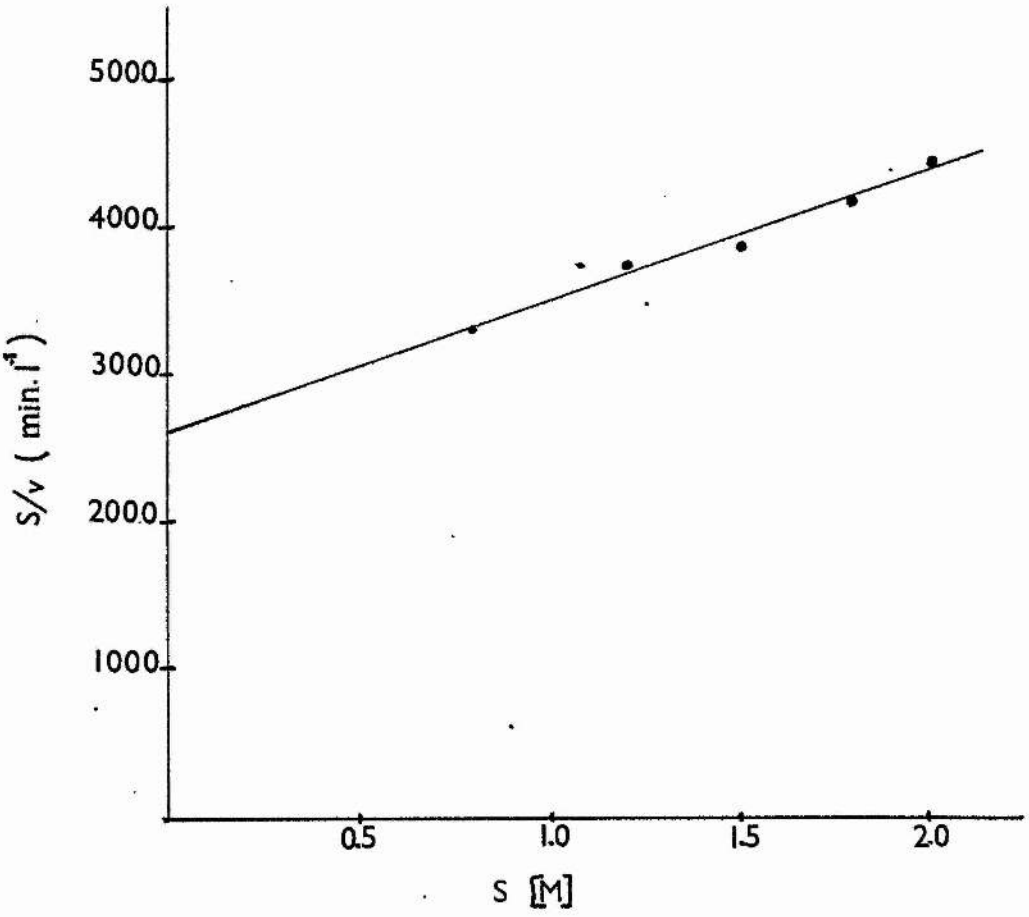


Fig. 4.6.6.1.3 S/v versus S plot for D-fructose isomerisation by glucose isomerase (50µg) at 40°C and pH 7.0.

Table 4.6.6 Apparent K_m and V values and standard errors

Substrate	App. K_m ± standard error (M)	V ± standard error ($\mu moles.min^{-1}.\mu g$ enzyme)
D-xylose	7.4 ± 0.8 (x 10^{-3})	0.446 ± 0.014
D-glucose	2.85 ± 0.089	0.015 ± 0.00028
D-fructose	1.89 ± 0.31	0.022 ± 0.0022

In agreement with other workers' findings (4, 16, 18, 20, 56, 61) glucose isomerase apparent K_m for D-glucose was higher than that for D-xylose. Using a continuous assay method the apparent K_m for D-xylose was in the range reported for the enzyme from β. coagulans ($K_m = 1.0 \times 10^{-3}M$) (61) and L. brevis ($K_m = 5 \times 10^{-3}M$) (3).

The apparent K_m value for D-glucose was in agreement with Kent (54) but was higher than the values reported by Yamanaka ($K_m = 0.92M$ (3), $K_m = 0.52M$ (56), with the enzyme from other L. brevis strain. It is worthy of note that Yamanaka used a colorimetric method for the enzyme activity assay and had a variation of nearly 57% between K_m values for the same enzyme substrate system (3, 56).

The interference of glucose on fructose assay by colorimetric methods and the need to use high glucose concentration for glucose isomerase assay, probably was the main source of error due to the high dilution necessary. With the colorimetric assay method we were able to calculate a K_m value of $1.27 \pm 0.69M$, with approximately 54% error which does not give much confidence in this method as compared with the continuous method.

It has been reported that the enzyme from L. brevis (56) is inhibited by xylitol ($K_i = 1.5 \times 10^{-3}M$) and sorbitol ($K_i = 4.5 \times 10^{-3}M$).

Another factor affecting K_m values could be the buffering

system used. Competitive inhibition by Tris-buffer has been reported (K_i = 3 x 10^{-4}M) with the enzyme from Bacillus coagulans (61) but as far as is known, noninhibition with Tris has been reported for the enzyme from Lactobacillus brevis - Tris buffer systems having been widely used with this enzyme (54,56).

The use of phosphate buffer does not help since at temperatures of 40° or more, it precipitates the manganese ions, essential for glucose isomerase activity.

Assuming that in the early stage of reaction the isomerisation catalysed by glucose isomerase, follows a reversible Michaelis-Menten mechanism

$$E + A \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EA \xrightarrow{k_{+2}} E + P \qquad \text{(eqn 1)}$$

the forward reaction has

$$V^f = k_{+2}\, Et \qquad \text{(eq 2)}$$

$$K_m^A = \frac{k_{-1} + k_{+2}}{k_{+1}} \qquad \text{(eq 3)}$$

and the reverse reaction has

$$V^R = k_{-1}\, Et \qquad \text{(eq 4)}$$

$$K_m^P = \frac{k_{-1} + k_{+2}}{k_{-2}} \qquad \text{(eq 5)}$$

Using these four equations (2 - 5) the Michaelis-Menten equation can be re-written as

$$v = \frac{\frac{V^f A}{K_m^A} - \frac{V^R P}{K^P{}_m}}{1 + \frac{A}{K_m^A} + \frac{P}{K_m^P}} \qquad \text{(eq 6)}$$

At equilibrium the net velocity must be zero,

$$\frac{V^f \, A \infty}{K_m^A} - \frac{V^R \, P \infty}{K_m^P} = 0$$

So,

$$\frac{V^f \, K_m^P}{V^R \, K_m^A} = \frac{P \infty}{A \infty} = K \qquad \text{(eq 7)}$$

Equation 7 (called Haldane relationship) gives the equilibrium constant (K) of the reaction.

Applying the kinetic values (app K_m and V) calculated for glucose isomerase acting on D-glucose and D-fructose (Table 4.6.6) an equilibrium constant of 0.45 was calculated using equation 7. This value was far below that which has been reported for L. brevis (Keq = 1.0).

This difference may well be connected with different assay procedures for the enzyme (colorimetric and sorbitol dehydrogenase-NADH) and possibly with enzyme inactivation occuring in solution of low activity.

4.6.6.2 Effect of D-fructose concentration on the activity of immobilised glucose isomerase

The measurement of the effect of substrate concentration on the activity of the immobilised enzyme was carried out using fructose as substrate (0.18M - 1.97M range in 0.02M Tris-HCl pH 7.0 buffer with 10^{-2}M $MnCl_2$ and 10^{-3}M $CoCl_2$) and approximately 6g of immobilised enzyme (12 mg protein).

The enzyme was assayed at suitable time intervals using Lloyd & Whelan method (3.2.3). Computer analysed results gave an apparent K_m = 0.513 ± 0.26M and V = 0.154 ± 0.041μmoles.min^{-1}.

5. SUMMARY

5.1 Glucose isomerase [EC 5.3.1.4] was extracted from Lactobacillus brevis N.C.D.O. 474 grown in a xylose containing medium, with a yield of cells (dry weight) of 2.3 - 3.3 $g.l^{-1}$ of medium and 300 - 310 glucose isomerase $units.l^{-1}$.

5.2 Several methods for releasing the intracellular enzyme were investigated and the crude extract preparation was further purified by nucleic acid precipitation with $MnCl_2$, protein fractionation by ammonium sulphate and dialysis followed by chromatography on CM-cellulose, DEAE-cellulose and Sephadex G-200. A final purification of 24 fold was achieved with about 25% recovery in 4 purification steps.

5.3 A mol.wt. of 120,000 was calculated for the purified enzyme by gel filtration (Sephadex G-200). Electrophoresis on 5% polyacrylamide - 0.1% SDS gel and 5% polyacrylamide - 3% SDS - 8M urea, showed dissociation of the enzyme into subunits with mol.wt. of 54,000 - 42,600.

5.4 Investigations on several possibilities of assaying the glucose isomerase activity was done using a coupled reaction system sorbitol dehydrogenase-NADH.

5.5 Immobilisation of the enzyme with PEI-derivative of nylon (polyethyleneimine) retained about 12% of the specific activity with 1.078 mg of protein bound per 500 mg of support.

5.6 The kinetic properties of both free and immobilised enzyme were investigated.

The optimum pH was nearly the same for both enzyme (7.0, free enzyme, 6.8 immobilised enzyme) and also thermal inactivation. The optimum temperature was 50°C for the free enzyme and 60°C for the immobilised enzyme. Apparent K_m and V values were calculated using D-glucose, D-fructose and D-xylose as substrate. The apparent K_m value for D-fructose was lower with the immobilised enzyme than for the free enzyme.

6. APPENDIX

Data Processing

A BASIC program, of which the listing is given below, was used to compute K_m and V values with standard errors.

PROGRAM

```
list

010 rem    THIS PROGRAM COMPUTES V MAX AND KM FROM
020 rem    SUBSTRATE/Vo DATA SETS.PROVISIONAL ESTIMATES
030 rem    ARE FIRST MADE BY LINEAR REGRESSION TO A
040 rem    LINEWEAVER-BURKE EQUATION.THESE ESTIMATES ARE THEN FITTED
050 rem    TO THE HYPERBOLIC MICHAELIS MENTEN FUNCTION USING A TAYLOR
060 rem    EXPANSION
070 read c,n
080 print " experiment no";c
090 a=b=g=d=e=v1=0
100 for i= 1 to n
110 read s(i),v(i)
120 v1=v1+v(i)**2
130 a=a+v(i)**3
140 b=b+v(i)**4
150 g=g+v(i)**3/s(i)
160 d=d+v(i)**4/s(i)
170 e=e+v(i)**4/s(i)**2
180 next i
190 t=a*e-g*d
200 k=(b*g-a*d)/t
210 v2=(b*e-d**2)/t
220 print "provisional estimate Km=";k
230 print " provisional estimate Vmax="; v2
240 a=b=c=d=e=0
250 for i=1 to n
260 f(i)=(v2*s(i))/(s(i)+k)
270 f1(i)=(-v2*s(i))/((s(i)+k)**2)
280 a=a+f(i)**2
290 c=c+f(i)*f1(i)
300 d=d+v(i)*f(i)
310 b=b+f1(i)**2
320 e=e+v(i)*f1(i)
330 next i
340 s1=(a*b)-c**2
350 b1=(b*d-c*e)/s1
360 b2=(a*e-c*d)/s1
370 v3=b1*v2
380 k1=k+b2/b1
390 print "fine estimate of v max = ";v3
400 print "fine estimate of Km =";k1
410 p2=(v1-b1*d-b2*e)/(n-2)
420 k2=(sqr(p2)/b1)*sqr(a/s1)
430 v4=v3*sqr(p2)*sqr(b/s1)
440 print " S.E. of Km is";k2
450 print " S.E of V max is";v4
460 end

*
```

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