GLUCOSE ISOMERASE FROM AN *ARTHROBACTER* SPECIES

by

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Abstract

C.A. Smith Glucose Isomerase from an Arthrobacter Species

This dissertation describes the investigation of possible means of increasing the yield of glucose isomerase from a species of Arthrobacter. Glucose isomerase catalyses the interconversion of D-glucose and D-fructose, although its natural substrate is D-xylose. The enzyme is of importance in the production of high fructose syrups for use as sweeteners in the food industry.

The possibility of manipulating the normal pathways of glucose metabolism by mutation to make the enzymic conversion of glucose to fructose an essential step in glucose metabolism was explored. Since the activity of the enzyme towards glucose is low under physiological conditions, it would then be rate-limiting for growth on glucose. This would enable the selection of mutants producing either elevated levels of the wild type enzyme or an enzyme of increased specificity for glucose, by virtue of their faster growth in glucose-limited chemostat culture. Evidence was obtained that this approach was not likely to be successful in the case of Arthrobacter. No activity able to phosphorylate intracellular fructose could be detected.

The enzyme was purified from a strain constitutive for its synthesis and was found already to account for at least ten percent of the soluble cell protein.

The activity of the purified enzyme towards various potential substrates was determined, to evaluate the possibility of using gratuitous substrates other than glucose to select for mutants with elevated levels of the isomerase. The use of D-lyxose for this purpose was investigated.

Another possible method of selecting for mutants super-producing the glucose isomerase would be the use of a non-metabolisable inhibitor to render the activity of the enzyme rate-limiting for utilisation of its natural substrate, xylose. Xylitol was shown to be a powerful inhibitor of the purified enzyme and the effect of competitive inhibitors in vivo was investigated.

Preface

It gives me great pleasure to express my thanks to:

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Abbreviations and Symbols

Most of the abbreviations used in this dissertation are standard; however, attention is drawn to the following:

A_x	Absorbance at a wavelength of x nm
ADP	Adenosine 5'-diphosphate
A.T.C.C.	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
bis-acrylamide	NN'-Methylenebisacrylamide
C.A.S.E.	Co-operative Awards in Science and Engineering
CH	Casein hydrolysate
cpm	Counts per minute
Cys	Cysteine
DPI	2,6-Dichlorophenol indophenol
dpm	Disintegrations per minute
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease I
ED Path	Entner-Doudoroff pathway
EDTA	Ethylenediamine tetraacetic acid
EMB	Eosin/Methylene Blue
EMP Path	Embden-Meyerhof-Parnas pathway
F6P	Fructose-6-phosphate
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
HMP Path	Hexose monophosphate pathway
[i]	Inhibitor concentration
I.C.I.	Imperial Chemical Industries
K^{eq}	Equilibrium constant
K_i	Inhibitor constant
K_m	Michaelis constant
NAD^+/NADH	Oxidised/reduced nicotinamide adenine dinucleotide
$NADP^+$/NADPH	Nicotinamide adenine dinucleotide phosphate
NG	N-Methyl-N'-nitro-N-nitrosoguanidine
PEP	Phosphoenolpyruvate
PFK	Phosphofructokinase
6PG	6-Phosphogluconate
6PGDH	6-Phosphogluconate dehydrogenase

PGI	Phosphoglucose isomerase
PGM	Phosphoglucomutase
pI	Isoelectric point
PMSF	Phenylmethane sulphonyl fluoride
PTS	Phosphotransferase system
RNA	Ribonucleic acid
RNase	Ribonuclease A
rpm	Revolutions per minute
[s]	Substrate concentration
SDS	Sodium dodecyl sulphate
S.R.C.	Science Research Council
TEMED	Tetramethylethylenediamine
Tricine	N-Tris(hydroxymethyl)-methylglycine
Tris	Tris(hydroxymethyl)aminoethane
TYE	Tryptone/yeast extract
UV light	Ultra violet light
v	Initial velocity of reaction
V	Maximum specific forward velocity of reaction
xg	Acceleration relative to that due to gravity

Phenotypic Symbols:

Fru^+	able to utilise D-fructose
Glc^+	able to utilise D-glucose
$Glnt^+$	able to utilise D-gluconate
Leu	requiring leucine for growth
Lyx^+	able to utilise D-lyxose
Mal^+	able to utilise maltose
Met	requiring methionine for growth
Rif^r	resistant to rifampicin
Str^r	resistant to streptomycin
Trp	requiring tryptophan for growth
Xyl^+	able to utilise D-xylose

Genotypic Symbols:

glk	lacking glucokinase
pfk	lacking phosphofructokinase
pgi	lacking phosphoglucose isomerase
xyl^c	constitutive for enzymes of D-xylose catabolism
$xylI^-$	lacking D-xylose isomerase
$xylK^-$	lacking D-xylulokinase (of the D-xylose path)
zwf	lacking glucose-6-phosphate dehydrogenase

Contents

List of Figures

List of Tables

List of Photographs

Chapter I

Introduction

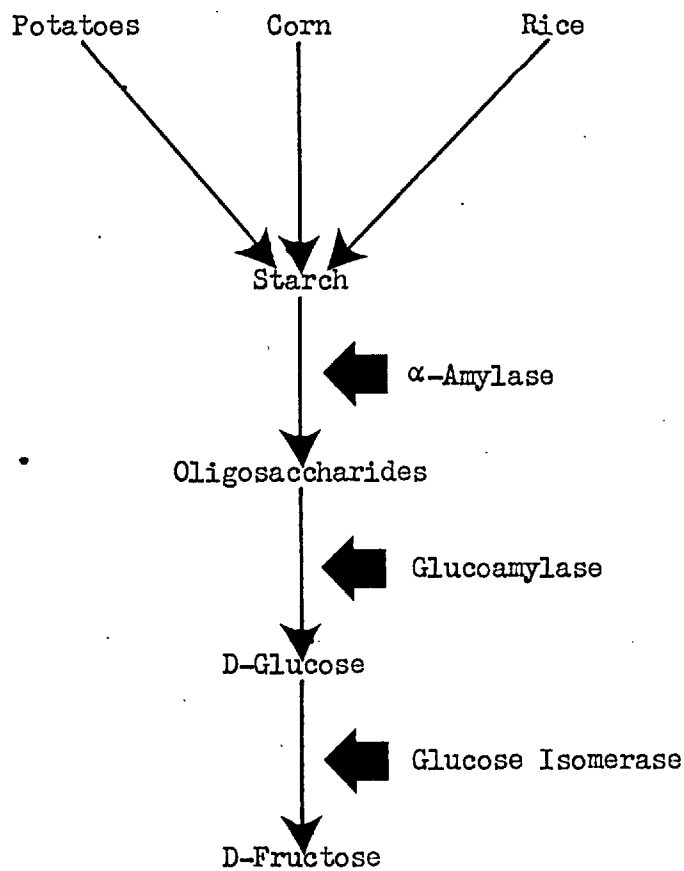
Sweeteners

Mankind has always sought sweet tasting substances to add to his food and drink. In ancient times, honey was the principal substance used as a sweetener, but it has been replaced as a food additive in our own society by sucrose, derived either from sugar-cane (grown in tropical climates) or from sugar-beet (which may be grown in more temperate climates). More recently, artificial sweeteners, which are, weight for weight, many times sweeter than sucrose have been available, such as sodium cyclamate and sodium saccharin, which are respectively 30-fold and 500-fold as sweet as sucrose (van der Wel et al., 1972). Although both of these compounds have been widely used as non-nutritive sweeteners, both are now considered to be possible health hazards, and it is likely that they will be replaced by new natural sweeteners of plant origin. These include glycosides such as glycyrrhizin (30-fold), naringin dihydrochalcone (300-fold) and stevioside (300-fold), and glycoproteins such as the thaumatins (1 600-fold) and monellin (2 000-fold as sweet as sucrose; van der Wel et al., 1972). However, despite these recent additions to the range of sweet substances available, sucrose and 'invert sucrose' continue to fill the major part of the demand for bulk sweeteners.

'Invert sucrose' is an equimolar mixture of the sugars glucose and fructose, prepared by the enzymic hydrolysis of sucrose. Fructose is slightly sweeter than sucrose, and the inversion of sucrose therefore leads to an increase in its sweetness; fructose also enhances the effectiveness of fruit flavourings. In addition invert sucrose has a greater osmotic strength than the equivalent concentration of sucrose,

Figure 1a

Production of Glucose and Fructose from Starch



and does not readily crystallise when concentrated, instead forming a syrup. These properties make invert sucrose particularly useful in the manufacture of confectionery, soft drinks and jams.

However, due to current economic trends, the price of sucrose has risen rapidly in recent years, and is expected to continue to increase.

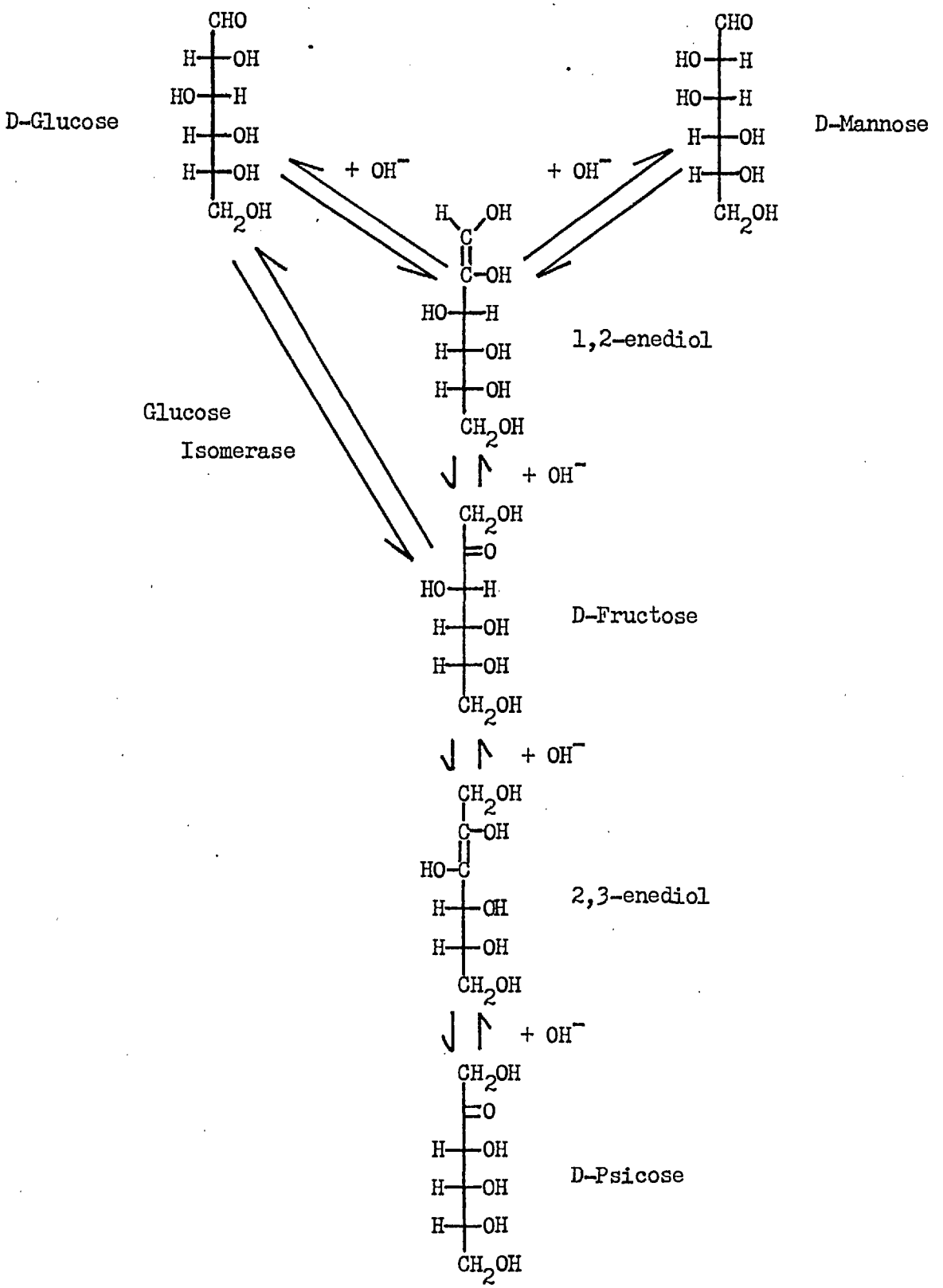
Isomerose

Glucose may be prepared comparatively cheaply, by the enzymic hydrolysis of starch, which may be derived from potatoes, corn or rice, but is of little use as a sweetener. However, since the aldohexose glucose is an isomer of fructose, the latter being the homologous ketohexose, it is possible to interconvert the two sugars. An equilibrium mixture of the two isomers contains nearly equal concentrations of each, and is therefore very similar to invert sucrose. Mixtures of glucose and fructose prepared by the isomerisation of glucose derived from starch (Figure 1a) are known as 'high-fructose syrups' or 'isomerose'. In practice the isomerisation is not allowed to proceed to equilibrium, and the product typically contains about 40% fructose and 60% glucose. Since isomerose may be prepared more cheaply than sucrose, and provides a suitable substitute for either sucrose or invert sucrose in many applications, it has assumed considerable commercial significance in both the U.S.A. and Japan. However, within the E.E.C., the market for isomerose has been artificially restricted by the imposition of a special levy, in order to protect the sugar-beet industry.

Although it is possible to use alkali to catalyse the isomerisation of glucose to fructose, this process is not specific, leading also to the epimerisation of D-glucose to D-mannose and of D-fructose to the unnatural sugar D-psicose , which is environmentally undesir-

Figure 1b

Isomerisation of D-Glucose to D-Fructose



Re-drawn from Geyer (1974).

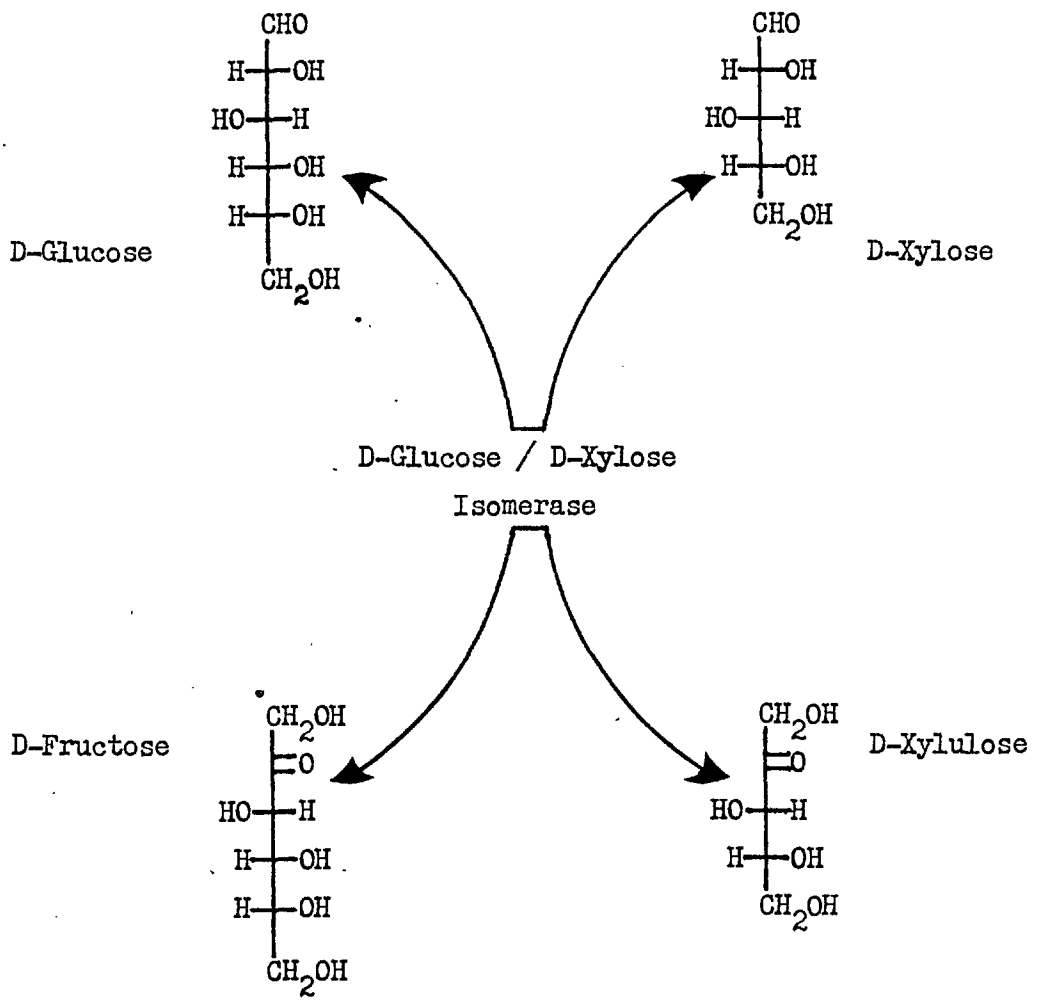
able (Figure 1b). It is therefore preferable to make use of the specificity offered by enzymic catalysis, and to carry out the isomerisation under conditions which minimise these side reactions. An enzyme which carries out this isomerisation may be described as a D-glucose ketol-isomerase (EC 5.3.1.18) or glucose isomerase.

Glucose Isomerase

The direct interconversion of glucose and fructose is not known to occur during the normal metabolism of either sugar by any organism. However, enzymic activities capable of carrying out this isomerisation have been found in a great variety of microorganisms (Vaheri & Kauppinen, 1977). These activities are of two types. Firstly those whose natural substrate is glucose-6-phosphate (phosphoglucose isomerase; EC 5.3.1.9), such as that found in Escherichia intermedia (Natke, 1966). Glucose isomerising enzymes of this type, which require arsenate as a cofactor (presumably to replace the phosphate group of the natural substrate), are not of commercial interest and are not discussed again in this thesis. The second, more widely described, type of glucose isomerising activity is attributable to D-xylose isomerase (EC 5.3.1.5), which normally interconverts D-xylose and D-xylulose. Figure 1c shows the configurations of D-xylose, D-glucose, D-xylulose and D-fructose; D-glucose and D-xylose possess the same configurations at the C_2, C_3 and C_4 positions. In contrast to the first type, these glucose isomerising enzymes require a divalent metal ion as cofactor (Mg^{2+}, Mn^{2+} or Co^{2+}). In most cases the enzyme is only present after growth of the organism in the presence of xylose or xylan, at least in wild type isolates. The activity of these enzymes is greater towards D-xylose than towards D-glucose; although the exact figures vary for the enzymes from different species, the Michaelis constant for D-glucose is usually

Figure 1c

D-Glucose and D-Xylose as Substrates for Glucose Isomerase



from 10-fold to 100-fold that for D-xylose. Although D-xylose isomerase has been demonstrated in many species, the only microorganisms which produce glucose isomerase activities which were of commercial interest at the time that this work was started were members of the genera Actinoplanes, Arthrobacter, Bacillus, Lactobacillus and Streptomyces (Vaheri & Kauppinen, 1977).

The isomerisation reaction is typically carried out by passing glucose syrup through a column packed with a suitable preparation of the isomerase, which may consist of whole cells in pelletised form, or of immobilised purified or partially purified enzyme. The syrup is usually adjusted to a pH value of from 7.0 to 8.0, and temperatures from 60°C to 70°C are commonly used, both because of the increase in the rate of the reaction and because of the decrease in the viscosity of concentrated syrups obtained by increasing the temperature. The use of higher temperatures leads to unwanted side reactions. Because the temperatures used are high, by biological standards, only those glucose isomerising enzymes which are thermostable are suitable for industrial use. Surprisingly, the glucose isomerase enzymes produced by many mesophilic species are quite thermostable. Because of the high glucose concentrations used (e.g. 50% w/v = 2.8M), the high values of the Michaelis constant for glucose (90 to 300mM) for these enzymes are unlikely to limit the rate of isomerisation, and it would therefore be of little value to lower these values. However, it would clearly be desirable to increase the yield of the enzyme obtained (i.e. the specific activity of the whole cells or of crude lysates), particularly in those cases where whole cells are used in the isomerisation process.

The classical approach to the problem of strain improvement would be to isolate many clones from a mutagenically treated culture,

to assay each for glucose isomerase activity and then to subject the clone which showed the greatest activity to further mutagenesis; however, this approach is both laborious and time consuming. Any method which provided a positive selection for clones possessing increased levels of glucose isomerase activity would greatly facilitate strain improvement, since it would enable the screening of many more clones than could be tested by individual assay. The work described in this thesis represents an attempt to apply techniques developed during experimental studies on the evolution of metabolic pathways, which were carried out for purely scientific reasons, to a problem of commercial relevance, the selection of strains with significantly improved yields of glucose isomerase activity.

Experimental Evolution of Metabolic Pathways

Theories of evolution attempt to account for observed biological diversity in terms of series of specific mutations which have confered on their possessor a selective advantage over its ancestors under a particular set of environmental conditions, and have therefore become fixed, leading eventually to the formation of new species. At the molecular level, such mutations, which result in 'fitter' phenotypes, must result from the alteration of existing structural genes, the creation of new structural genes or changes in the control of the expression of structural genes. At the level of higher organisms, theories are necessarily speculative, since they must be based on the morphological and biochemical similarities and differences between present day species and on an imperfect fossil record whose evidence is almost exclusively morphological. However, in the case of microorganisms, which have relatively short generation times, an experimental approach has been possible. By subjecting an organism to a

particular environmental pressure it is possible to observe the types of mutation resulting in increased evolutionary fitness which actually arise and are fixed, providing a partial test of some aspects of the theories which attempt to account for microbial evolution at the level of molecular biology. The provision of a novel carbon source, for which an organism has no pre-existing catabolic pathway is a convenient form of environmental pressure which has been used widely for this purpose. Clarke (1978) has recently reviewed this field. When a microorganism is confronted with a novel substrate which is related structurally to some compound which is a normal metabolic intermediate, it requires three functions in order to be able to utilise the new compound as a growth substrate:

i) the ability to take up the new compound,

ii) an enzymic activity capable of converting the novel substrate into a normal metabolite (or a series of such activities),

iii) the ability to express the above activities in the presence of the novel substrate.

It is often found that an enzyme which normally acts on some compound which is structurally related also shows activity towards the novel compound, but is normally synthesised only during growth in the presence of the substrate of the catabolic pathway in which its natural substrate is an intermediate. Thus, when a microorganism aquires the ability to utilise a novel substrate, the initial step is often a control mutation, leading either to a change in the specificity of induction or, more commonly, to the constitutive expression of the gene for an enzyme already able to act on the novel substrate (St Martin & Mortlock, 1977). If the activity of the enzyme towards the novel substrate is low, the growth of such initial mutants is slow and the activity of the enzyme rate-limiting. Further mutations leading to more rapid utilisation of the novel substrate can then be of three

different types:

i) mutations which lead to the accumulation of higher intracellular levels of the substrate,

ii) mutations in the structural gene for the enzyme which lead to an increased activity towards the novel substrate,

iii) mutations which result in the presence of increased amounts of the enzyme.

Selection of Fitter Mutants in Continuous Culture

The chemostat has proved to be a powerful tool for the selection of mutants successively better adapted to the use of a novel substrate. A constant volume of culture is continuously supplemented with fresh medium, excess culture being overflowed at a steady rate. Subject to certain limitations, a steady state is established, with the growth rate of the organism equal to the dilution rate at which the chemostat is run. However, if a mutant arises which can grow faster than its parent, the steady state is disturbed and the mutant is likely to displace its ancestors and take over the population. The occurrence of such a take over event leads to an increase in the culture biomass and a decrease in the concentration of the substrate in the effluent from the chemostat. For a review see Harder et al. (1977).

Evolution of K. aerogenes to Growth on Xylitol

A system of this type which has been investigated in this group is the evolution of Klebsiella aerogenes to growth on xylitol (Rigby et al., 1975; Hartley et al., 1976). Wild type K. aerogenes does not utilise the pentitol xylitol, although it is able to catabolise both ribitol and D-arabitol by means of activities encoded in operons induced in the presence of each of these pentitols. Each operon

encodes both a dehydrogenase, which converts the pentitol to the corresponding ketopentose, and a pentulokinase activity. The ribitol dehydrogenase shows slight activity towards xylitol, and mutants constitutive for its synthesis are able to grow slowly on xylitol. The xylitol is oxidised to D-xylulose, which may then be phosphorylated either by the D-xylulokinase of the D-arabitol operon or by that of the D-xylose operon (Charnetzky & Mortlock, 1974). Whereas K_m, the Michaelis constant, of the dehydrogenase for ribitol is 1mM, that for xylitol is nearly 1M (Burleigh et al., 1974), and the activity of the enzyme is therefore rate-limiting for xylitol utilisation. Continuous culture experiments have led to the isolation of two classes of mutants better adapted to growth on xylitol. Some of the mutants isolated synthesize an altered dehydrogenase with a lowered Michaelis constant for xylitol, but the most frequent response to selective pressure for faster growth on xylitol has been the synthesis of elevated levels of the wild type ribitol dehydrogenase ('super-production'). No mutants with altered xylitol uptake properties have been detected amongst the faster growing evolvants obtained. Increases in ribitol dehydrogenase specific activity of 15-fold have been obtained, resulting in enzyme levels representing 17% of the total soluble cell protein in the super-producing strain (Rigby et al., 1975 ~~1974~~).

Evolution of a New Catabolic Pathway for Glucose

Since all the glucose-isomerising enzymes which have been described have high Michaelis constants for glucose, the results of the work on xylitol utilisation described above suggested the possibility that mutants possessing substantially increased levels of the isomerase might be obtained by applying selective pressure for the evolution of a new catabolic pathway for glucose involving the isomerase activity.

Most microorganisms possess efficient pathways for the catabolism of glucose, and it was therefore envisaged that it would be necessary to block the normal pathway(s) by mutation before attempting to construct a novel path involving the isomerase. Such mutants would need to be essentially non-revertable (e.g. deletions), since otherwise revertants would be expected to outgrow any initial evolvants possessing only the new pathway. Phenotypic revertants of such a mutant which had regained the ability to utilise glucose would be expected to synthesise the isomerase in the presence of glucose, either constitutively or due to a change in the specificity of induction. The growth of such initial evolvants in glucose-limited continuous culture would then be expected to lead to the selection of super-producing mutants. Since the activity of the isomerase would still be rate-limiting for glucose utilisation in such strains, it was expected that genetic instability would not be a problem, since glucose could be used as carbon source during the large scale cultivation of the super-producing strains for the commercial production of the isomerase. Assuming that the normal xylose-induced level of the isomerase was about 1% of the total soluble cell protein, in common with those of other inducable enzymes which catalyse the initial steps in the catabolism of other substrates (see Table 7e) it seemed probable that a 20-fold increase in specific activity would be possible, based on the results of Rigby et al. (1975 ~~1974~~) for ribitol dehydrogenase.

Choice of Microorganism

For ease of experimental manipulation, strain construction and mutant isolation, it would obviously have been advantageous to have used a microorganism which is well characterised and for which well established genetic techniques are available, such as Escherichia coli.

However, the D-xylose isomerase of E. coli shows little or no activity towards D-glucose (David & Weismeyer, 1970). Because this work was supported by an S.R.C. C.A.S.E. award with I.C.I. Agricultural Division, the organism chosen was a species of Arthrobacter which was already being used by I.C.I. for the commercial production of glucose isomerase. The isomerase produced by this species is sufficiently thermostable to be used at 60°C for long periods, and requires Mg^{2+} as its cofactor, rather than Mn^{2+} or Co^{2+}, which minimises the treatment needed to reduce the concentration of the cation in the product and in the plant effluent to an acceptable level. In addition, this species has properties which make it suitable for the prepartion of a pelletised product for use in enzyme reactor columns in the production of isomerose in which the enzyme remains entrapped in whole cells. The use of the glucose isomerase from this organism is covered by patent (Reynolds, 1973), and five strains are deposited with the A.T.C.C., designated Arthrobacter nov. sp. NRRL B3724 to B3728.

Unfortunately the definition of the genus Arthrobacter is at present mainly morphological (Buchanan & Gibbons, 1974), and the taxon appears to be 'artificial', since it includes species which possess fundamentally different pathways for the metabolism of glucose (Zagallo & Wang, 1962). This made the application of published results for other species to the strains used without experimental confirmation impossible. The plan initially pursued therefore involved the development of conditions suitable for the generation and isolation of mutants (Chapter IV), the use of these to obtain mutants unable to utilise glucose (Chapter V) and the characterisation of the lesions present in these mutants together with the investigation of the pathways for glucose catabolism operating in the wild type strain (Chapter VI).

Chapter II

Materials and Methods

"The method employed I would gladly explain,
While I have it so clear in my head,
If I had but the time and you but the brain -
But much yet remains to be said."

'The Hunting of the Snark' by Lewis Carroll, Fit the Fifth.

Materials

Eosin, Methylene Blue and TEMED (tetramethylethylenediamine) were obtained from Aldrich Chemical Co., Gillingham, Dorset.

Amino acids, casein hydrolysate, dithiothreitol, Bromophenol Blue, Tricine, D-ribose, D-arabinose, L-arabinose, and acrylamide and NN'-methylenebisacrylamide (both specially purified for electrophoresis) were from BDH Ltd., Poole, Dorset.

Ampicillin was obtained from Beecham Veterinary Products, Crawley , Sussex as 'Penbritin'.

Spectinomycin from Upjohn Ltd., Crawley, Sussex as 'Trobitin'.

D-glucose, sucrose and Folin-Ciocalteu reagent were from Fisons Ltd., Loughborough, Leics.

Pentitols, D-fructose (low in D-glucose), D-xylose and D-sorbitol were from Cambrian Chemicals Ltd., Croydon.

Tryptone, yeast extract and 'Bacto' agar from Difco Labs., West Molesey, Surrey.

'Purified' agar and 'No. 3' agar from Oxoid Ltd., Basingstoke, Hants.

Ultragel AcA34 from LKB, South Croydon, Surrey.

Sephadex G200, Sephadex G150 and Blue Dextran 2000 were from Pharmacia, Hounslow, Middlesex.

DEAE-Sepharose CL-6B was the gift of Dr D. Byrom of I.C.I.

PPO (2,5-diphenyloxazole) was from Koch-Light Ltd., Colnbrook, Bucks.

[U-^{14}C]-D-xylose (specific activity 90mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks.

Purified ribitol dehydrogenase was available at Imperial College, and partially purified D-arabitol dehydrogenase was the gift of Dr M.S. Neuberger of Imperial College.

All other antibiotics and sugars, sugar analogues, sugar phosphates, NG (N-methyl-N'-nitro-N-nitrosoguanidine), PMSF (phenylmethane sulphonylfluoride), POPOP (1,4-bis-(2-(4-methyl-5-phenyloxazolyl))-benzene), triphenyl tetrazolium chloride, Coomassie Brilliant Blue R250, lysozyme chloride, bovine serum albumin, ovalbumin, d-biotin, enzymes used in coupled assays or as molecular weight markers and all other biochemicals were from Sigma (London) Ltd., Poole, Dorset.

Inorganic chemicals (AR grade) and solvents were obtained from several suppliers.

Bacterial Strains

Details of the sources, relevant phenotypes and (where known) genotypes of the *Arthrobacter* strains used in this work are given in Table 2a. All are derived from isolates deposited by Reynolds Tobacco with the A.T.C.C. (Reynolds, 1973). Although assigned to the genus *Arthrobacter*, these strains have not been definitively assigned to a species; however, they resemble most closely *A. globiformis* as described by Buchanan and Gibbons (1974), (Dr D. Byrom, personal com-

Table 2a

List of Arthrobacter Strains

Strain	Source	Phenotype	Genotype (if known)
B3724	A.T.C.C.	isomerase inducible	*xyl*$^+$
SA401	? see page 228	Xyl$^-$ Frosty	*xylI*$^-$ *xylK*$^+$
B3725	A.T.C.C.	isomerase inducible	*xyl*$^+$
B3726	A.T.C.C.	constitutive	*xyl*^{c}
B3727	A.T.C.C.	constitutive	*xyl*^{c}
B3728	A.T.C.C.	constitutive	*xyl*^{c}
SA4000	Spont. from B3724	Str^r	
SA4010	Spont. from SA401	Rif^r Xyl$^-$ Frosty	
SA402	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^+$	*glk*
SA403	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^-$	
SA404	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^+$	
SA405	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^-$	
SA406	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^-$	
SA407	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^+$	
SA408	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^+$	
SA409	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^+$	*glk*
SA410	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^-$	
SA411	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^-$	
SA412	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^-$	
SA413	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^-$	
SA414	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^-$	
SA415	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^-$	
SA416	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^-$	
SA417	NG from B3724	Fru$^+$ Glc$^-$ Glnt$^-$	

Table 2a

(continued)

Strain	Source	Phenotype	Genotype (if known)
SA418	Nitrous acid from B3724	Met	? point mutation
SA419	Nitrous acid from B3724	Leu	? point mutation
SA420	Nitrous acid from B3724	Trp	? deletion
SA421	Nitrous acid from B3724	Trp	? deletion
SA422	Nitrous acid from B3724	Trp	? deletion
SA423	Nitrous acid from B3724	Trp	? deletion
SA424	Nitrous acid from B3724	Trp	? deletion
SA425	Nitrous acid from B3724	Trp	? point mutation
15/123	I.C.I. UV & NG from B3728		elevated isomerase level, altered isomerase
37/70	I.C.I. NG from 15/123 (?)		elevated isomerase level, wild type isomerase

Key:

Spont.	spontaneous
NG	mutagenesis with N-methyl-N'-nitro-N-nitroso-guanidine
UV	mutagenesis with ultra violet light

The phenotypic and genotypic symbols used are explained on page 5, and again, where relevant, in the main text of this dissertation.

munication). Of the five strains deposited by Reynolds Tobacco, two, B3724 and B3725 are inducible for the isomerase, whilst B3726, B3727 and B3728 are constitutive. It is believed that the other strains are derived from strain B3724. Strains B3724 to B3728 each exist as two sub-strains or varieties, which differ in their colonial morphology, appearing either 'smooth' or 'frosty' when viewed at an obtuse angle to the source of illumination. Apart from this difference in appearence and differences in foaming properties during large scale culture, the 'smooth' and 'frosty' varieties of each strain are indistinguishable; each variety gives rise to mutants of the other morphology at a detectable frequency. Apart from strain SA401 and its derivative SA4010, all the strains used in the work described in this thesis were of the 'smooth' variety.

Strains 15/123 and 37/70 were isolated from strain B3728 by I.C.I., following repeated mutagenesis. Both strains are auxotrophic; details of their isolation are given in Chapter IX.

The E. coli K12 strain NC100 is described in Neuberger et al. (1979), whilst HB101/pRD351 is described in Neuberger and Hartley (1979).

General Techniques

Scintillation Counting

An Intertechnique SL-30 Liquid Scintillation Spectrometer was used, employing the pre-set window for ^{14}C counting. The scintillation fluid used both for samples on glass-fibre filters and for small aqueous samples contained:

5g/l PPO (2,5-diphenyloxazole)

250mg/l POPOP (1,4-bis(2-(5-phenyloxazolyl))-benzene)

660ml/l toluene (Koch-Light puriss. AR grade)

330ml/l Triton X100 (Fisons scintillation grade)

Samples for counting were placed in disposable plastic inserts with 4ml scintillation fluid and stored overnight at 4°C in the dark before counting in plastic scintillation vials. The overnight storage of the samples allowed the decay of photoluminescence, which can cause considerable problems with this scintillation mixture.

Spectrophotometry

All enzyme assays coupled to changes in optical absorbance were carried out in a Gilford 252 spectrophotometer, fitted to a Unicam SP500 monochromator. The temperature of the cuvette compartment was controlled by circulating water from a Grant SE15 water bath. Quartz cuvettes of 1cm light path were used. Rates of change of absorbance were determined using a Gilford 600 chart recorder or one of several other comparable recorders.

Constant absorbances were determined either with the Gilford 252 or with a Pye-Unicam SP800 split-beam scanning spectrophotometer, modified by the addition of a digital millivolt-meter connected across the 10mV output provided for slave recorder operation, providing a direct readout of the absorbance.

When necessary, the wavelength calibration of the Gilford 252 was checked as follows. The deuterium lamp was switched on and allowed to warm up for three hours. The wavelength dial was set to about 486nm and the slit width adjusted to give an absorbance reading of about two units, with no cuvette in the light path. The wavelength control was then altered to locate the minimum in absorbance reading corresponding to the strong ^{2}H emission line at 486.1nm.

Microscopy

A Leitz-Wetzlar Ortholux microscope, fitted with Zernicke phase contrast equipment was used.

Conductivity Determination

The conductivities of buffers were determined at room temperature using a Radiometer Conductivity Meter CDM 3 fitted with a type CDC 314 cell.

Determination of pH

The pH values of solutions were routinely determined using a Radiometer type PHM 26 pH meter, fitted with a GK 2302 combined electrode and reference cell.

Polyacrylamide Gel Electrophoresis

The conditions used were based on the system described by Laemmli (1970). Polyacrylamide slab gels of 1.5mm thickness were used for the electrophoretic separation of proteins, both in the presence of SDS (sodium dodecyl sulphate) and under native conditions. The resolving gels were approximately 15cm x 15cm, and were prepared by the polymerisation of 7% w/v acrylamide (for native electrophoresis) or 10% w/v acrylamide (for electrophoresis in the presence of SDS). The samples to be subjected to electrophoresis were applied in wells formed in a low percentage acrylamide stacking gel, at a pH value less than that of the resolving gel.

Resolving Gel Preparation

Gels were prepared by polymerising a mixture of acrylamide and bis-acrylamide (NN'-methylenebisacrylamide) by the addition of ammonium persulphate and the catalyst TEMED (tetramethylethylenediamine). A stock solution containing 30% w/v acrylamide and 0.8% w/v bis-acrylamide was stored in the dark at 4°C. Gels were prepared by mixing:

7% (Native)	10% (SDS)	
25ml	25ml	750mM-Tris.HCl pH 8.8
11.7ml	16.5ml	acrylamide/bis-acrylamide
12.2ml	6.9ml	distilled water
60µl	60µl	TEMED
-	500µl	10% w/v SDS
1ml	1ml	1% w/v ammonium persulphate (freshly prepared)

The buffer, acrylamide/bis-acrylamide and water were mixed and thoroughly degassed before the addition of the TEMED, SDS (if required) and ammonium persulphate. The mixture was rapidly poured between two glass plates, separated by 1.5mm Perspex spacers (sealed with melted soft paraffin wax), and was over-layed wtih butan-1-ol to exclude air. The polymerisation was complete after about an hour.

Stacking Gel Preparation

Native	SDS	
3.4ml	3.4ml	750mM-Tris.HCl pH 6.5
2ml	2ml	acrylamide/bis-acrylamide
12.4ml	12.4ml	distilled water
20µl	20µl	TEMED
-	200µl	10% SDS
2ml	2ml	1% w/v ammonium persulphate

The components of the stacking gel were mixed and degassed in the order described for the resolving gel. The top of the polymerised resolving gel was washed free of butan-1-ol with distilled water and the stacking gel mixture was poured on. A Perspex 'comb' with serations of the same thickness as the spacers was introduced between the glass plates, to form wells in the stacking gel. The wells had

a depth of 1.5cm and a width of 8mm, and the depth of stacking gel left between the well bottoms and the resolving gel was from 1cm to 1.5cm. The polymerisation of the stacking gel was complete in about fifteen minutes.

Sample Preparation

Samples for electrophoresis in the presence of SDS were dried in vacuo in Durham tubes, dissolved in 50µl SDS sample buffer and heated to 105°C for fifteen minutes (after covering with polythene film) to ensure complete denaturation. For electrophoresis under native conditions, samples in volumes up to 30µl were mixed directly with 40µl native sample buffer. The sample buffers, which were kept at 4°C in sealed vials, contained per 10ml:

Native	SDS	
200µl	200µl	750mM-Tris.HCl pH 6.5
-	1ml	10% w/v SDS
-	1ml	2-mercaptoethanol
5ml	5ml	50% v/v glycerol
10mg	10mg	Bromophenol Blue
4.8ml	2.8ml	distilled water

Using sample wells of 8mm width, suitable loadings of protein per well were found to be 100µg to 200µg of crude cell extracts or 1µg to 10µg of any single protein species.

Running the Gel

After the polymerisation of the stacking gel, the comb and the bottom spacer were removed and the gel was fitted to a gel tower. The upper and lower compartments were filled with electrode buffer, which contained 3g/l Tris base, 1.45g/l glycine and (for SDS gels) 1g/l SDS. Native and SDS electrode buffers were prepared by dilution

of a ten-fold concentrated stock solution. The samples were introduced into the wells in the stacking gel using a 50µl Hamilton microsyringe. A Shandon Southern SAE 2761 power-pack was used, and the connections to the electrodes were made through a Perspex cover, which shrouded both buffer tanks. Both native and SDS gels were run at room temperature, for from 16 to 20 hours, at a current of 8mA, until the dye front reached the bottom of the gel.

Staining for Protein

Gels which were to be stained for protein were fixed by soaking in 25% w/v trichloroacetic acid for 15 minutes, washed for 10 minutes by soaking in 10% v/v acetic acid and incubated in staining solution for from 40 to 60 minutes at 60°C. The staining solution was prepared by mixing equal volumes of stock solutions of 20% v/v aqueous acetic acid and 0.6% w/v Coomassie Brilliant Blue R250 in methanol. The background was destained overnight with several changes of a 1:2:7 (by volumes) glacial acetic acid: methanol:water mixture. Stained gels were photographed by transmitted fluorescent light, using a green filter (Kodak No. 57). Stained gels were stored in 10% v/v acetic acid.

Staining for Isomerase Activity

The activity stain used was based on that described by Yamanaka (1975); this makes use of the reaction between a ketose and triphenyl tetrazolium chloride under alkaline conditions. It could in principle be used to stain for activity on any of the aldose substrates of the isomerase; however, D-xylose was chosen, because it is the substrate towards which the enzyme shows the greatest activity, and therefore gives the greatest sensitivity of staining. Activity staining of native gels was carried out immediately after electrophoresis. The

gel was incubated at 37°C for ten minutes in 250ml of prewarmed 100mM-Tricine.NaOH/30mM-$MgCl_2$ pH 8.0, containing 50mM-D-xylose. The gel was washed with distilled water to remove excess substrate and immediately incubated in the dark with a 1mg/ml solution of triphenyl tetrazolium chloride in 1M-NaOH for about two minutes. Red bands were formed in the region of any active material. The reaction was then terminated by soaking the gel in 2M-HCl, and the stained gel was stored in 10% v/v acetic acid. Activity stained gels were photographed under the same conditions as gels stained for protein.

Microbiological Techniques

Sterility

Media and solutions for use in microbiological work were sterilised by autoclaving at 123°C, unless otherwise stated. Volumes up to 400ml were autoclaved for 20 minutes, and larger volumes (i.e. 1.5 litres in 4 litre flasks) for 60 minutes. Solutions of sugars were normally autoclaved at low pH values, obtained by the addition of dilute phosphoric acid. Solutions of materials unsuitable for autoclaving were sterilised by filtration through Millipore 0.22µm filters.

Glassware, pipettes, wooden cocktail sticks and velvet pads for replica-plating were autoclaved for 60 minutes and dried *in vacuo* for 20 minutes. Pipettes were sterilised in metal canisters, cocktail sticks in glass beakers covered with aluminium foil and velvet squares in packets made from Sterilin Autoclavable Disposal Bags. Polycarbonate centrifuge tubes and Millipore filters were autoclaved for 20 minutes, without a drying cycle.

Liquid Media

LB
10g/l tryptone
5g/l yeast extract
5g/l NaCl

The pH was adjusted to 7.4 with NaOH

Medium A
5.8g/l Na_2HPO_4
3.0g/l KH_2PO_4
0.5g/l NaCl
1.0g/l NH_4Cl
5.5g/l tryptone
1.5g/l yeast extract

After autoclaving 1ml/l sterile 1M-$MgSO_4$ and 10ml/l sterile 50% w/v D-glucose were added. The pH of the medium is 7.0.

Fermenter Medium
6.0g/l $(NH_4)_2HPO_4$
2.0g/l KH_2PO_4
12.0g/l corn steep liquor
57.8g/l glucose monohydrate
1.2g/l $MgSO_4.7H_2O$
0.1g/l Silicone RD anti-foam

This medium was used for 400 litre batch cultures in the Imperial College Pilot Plant. The phosphates and corn steep liquor were dissolved in mains water and sterilised by steam injection at pH 6.0. The glucose and $MgSO_4$ were autoclaved at pH 4.0 and added to the sterile medium. The pH was then adjusted to a value of 7.0, and was maintained at this value throughout incubation, by the injection of ammonia. Yields of about 25g/l dry weight of cells were obtained with this medium.

Trace Elements Concentrate

500mg/l $FeSO_4 \cdot 7H_2O$
70mg/l $CuSO_4 \cdot 5H_2O$
45mg/l $MnCl_2 \cdot 4H_2O$
50mg/l $ZnSO_4 \cdot 7H_2O$
1.32g/l $CaCl_2 \cdot 6H_2O$
100mg/l $CoCl_2 \cdot 6H_2O$
7mg/l H_3BO_3
100mg/l $Na_2MoO_4 \cdot 2H_2O$

Sufficient H_2SO_4 was added to keep the pH of the solution below a value of 1.6.

MM Medium

6.0g/l $(NH_4)_2HPO_4$
2.0g/l KH_2PO_4
0.3g/l $MgSO_4 \cdot 7H_2O$
0.65g/l K_2SO_4
2ml/l trace elements concentrate
5ml/l 100µg/ml d-biotin

The phosphates and sulphates were prepared as two twenty-fold concentrates and diluted as needed. The carbon source and d-biotin were added as sterile solutions after autoclaving. The pH is 7.0.

MM' Medium

2.72g/l KH_2PO_4
5.68g/l Na_2HPO_4
6.6g/l $(NH_4)_2SO_4$
0.3g/l $MgSO_4 \cdot 7H_2O$
2ml/l trace elements concentrate
5ml/l 100µg/ml d-biotin

This medium was essentially equivalent to MM medium. The phosphates and sulphates were prepared as two twenty-fold concentrates and diluted as needed. The carbon source and d-biotin were added as sterile solutions after autoclaving. The pH of the medium is 7.0.

Solid Media

Sterilin disposable Petri dishes of 9cm diameter were used.

M9 Plates

5.8g/l Na_2HPO_4
3.0g/l KH_2PO_4
0.5g/l NaCl
1.0g/l NH_4Cl
1ml/l 1M-$MgSO_4$
2ml/l trace elements solution
5ml/l 100µg/ml d-biotin
15g/l agar

The agar, $MgSO_4$, trace elements and d-biotin were autoclaved together in 90% of the final volume of the medium, and cooled to 60°C before the addition of the other salts as a freshly autoclaved ten-fold concentrate. The carbon source was either autoclaved with the agar or added as a sterile concentrate. Unless otherwise stated all carbon sources were added to a concentration of 0.2% w/v, except for sodium gluconate, which was added to 0.25% w/v to allow for the weight of sodium present. During early experiments Difco 'Bacto' agar was used in the preparation of minimal plates; however, it was later found that the Arthrobacter strains used were capable of significant growth on plates prepared with this agar even in the absence of any added carbon source. Since this background growth interfered with growth tests, Oxoid 'Purified' agar, which supports much less background growth was used instead thereafter.

L-Amino acids were added when required to a concentration of 25µg/ml as sterile solutions at 10mg/ml, either before pouring plates or by spreading with a sterile glass rod on individual solidified plates. The pH of M9 plates is 7.0.

TYE Plates

10g/l tryptone
5g/l yeast extract
8g/l NaCl
20g/l agar

The pH was checked, and if necessary adjusted to a value of 6.5 with NaOH before autoclaving. Difco 'Bacto' agar or Oxoid agar 'No. 3' was used to solidify rich plates.

EMB Glucose Plates

10g/l tryptone
10g/l D-glucose
2g/l K_2HPO_4
400mg/l Eosin Yellow
65mg/l Methylene Blue
15g/l agar

The Eosin and Methylene Blue were autoclaved separately as a 100-fold concentrate and added to the agar before pouring the plates. The glucose was autoclaved with the agar, tryptone and K_2HPO_4.

Antibiotic and Glucose Analogue Plates

Ampicillin was added to the autoclaved agar to 20µg/ml or 100µg/ml as a 20mg/ml solution, sterilised by Millipore filtration

Spectinomycin was added to a concentration of 100µg/ml as a 20mg/ml solution, sterilised by filtration.

Streptomycin sulphate was added to the sterile agar to 100µg/ml as a 20mg/ml solution, sterilised either by filtration or by autoclaving; solutions of streptomycin sulphate develop a brown coloration on autoclaving, but remain active.

Rifampicin was added to the autoclaved agar to 50µg/ml as a 50mg/ml solution in methanol.

Nalidixic acid was dissolved in dilute NaOH at 2mg/ml and

added to the agar before autoclaving, to a concentration of 100µg/ml.

2-Deoxy-D-glucose was added to the agar before autoclaving, to concentrations of 5mM, 10mM or 20mM.

5-Thio-D-glucose was sterilised by Millipore filtration and spread on solidified plates to give final concentrations of 5mM or 10mM.

Culture Conditions

Cultures of Arthrobacter strains in liquid media were routinely grown either in 500ml conical flasks containing 100ml medium or in tubes (15.5cm x 2.5cm diameter) containing up to 15ml medium. Both were plugged with non-absorbent cotton wool, and were incubated at 30°C on a rotary shaking table at 200 rpm with a radius of gyration of 4.5cm. Cultures of E. coli strains were grown under similar conditions at 37°C.

Cultures of Arthrobacter strains on solid media were incubated at 32°C; when prolonged incubation was required the plates were wrapped with Parafilm, to retard drying. Plate cultures of E. coli strains were incubated at 37°C.

Plating Techniques

Suspensions of cells were diluted in sterile 100mM-KH_2PO_4.KOH pH 7.0 in steps of 1/100 and 1/10. 0.1ml portions of appropriate dilutions were spread on dry plates, using a glass rod sterilised in ethanol.

Viable Cell Counts The culture or suspension was plated on TYE agar at suitable dilutions to give from 50 to 500 colonies per plate. For overnight cultures in rich media and saturated cultures in minimal media, dilutions of 10^{-6} and 10^{-7} were suitable.

Replica Plating Master plates were prepared, either by spreading a suitable dilution of a culture to obtain about 250 colonies

per plate, or by gridding up to 100 individual colonies on a plate using sterile wooden cocktail sticks. After growing up, the master plate was pressed lightly onto a sterile velvet square (15cm x 15cm) which had been stretched over a wooden former of 8cm diameter and was held in place by a metal retaining ring. Up to five replicas were prepared by pressing fresh plates (which had to be both flat and dry) onto the velvet. The final replica was made either on the same medium as the master plate or on TYE agar. Since all colonies on the master plate should have formed replicas on this plate, it provided a control to ensure that sufficient cells from each master colony had been transfered onto the velvet.

Antibiotic Resistance Markers

Antibiotic resistance markers were used to enable the unequivocal identification of mutants derived from strains B3724 (*xyl*$^+$) and SA401 (*xylI*$^-$*xylK*$^+$).

Antibiotics for which single step chromosomal mutations conferring high level resistance are found include nalidixic acid, spectinomycin, streptomycin and rifampicin. As described in Chapter IV, all the Arthrobacter strains used in this work were resistant to nalidixic acid. Strains B3724, B3728 and SA401 all showed some background growth in addition to resistant colonies when streaked on TYE plates containing 100µg/ml spectinomycin. However, streptomycin sulphate (100µg/ml) and rifampicin (50µg/ml) both gave clear backgrounds with all three strains. Further tests showed that strain B3724 was unable to form colonies on TYE plates containing 10µg/ml of either antibiotic.

Spontaneous streptomycin resistant (Str^r) and rifampicin resistant (Rif^r) mutants were isolated as follows. 10ml of an overnight culture in LB was spun down and the supernatant discarded.

The pellet was resuspended by vortexing (without the addition of any liquid) and spread on a TYE plate supplemented with the appropriate antibiotic. Resistant colonies were streaked on fresh antibiotic plates, and single colonies were isolated and retested for the phenotype of the original strain before storing one clone as the antibiotic resistant derivative strain. Mutants resistant to 100µg/ml streptomycin sulphate arose spontaneously at a frequency of about 10^{-9} and mutants resistant to 50µg/ml rifampicin at about 10^{-8}.

SA4000 is a Str^r derivative of B3724, and SA4010 a Rif^r derivative of SA401 (Table 2a).

Strain Storage

Strains of Arthrobacter were stored in two ways; in 40% v/v glycerol at -18°C and on minimal plates at 4°C. Single colonies of strains for storage in glycerol were used to inoculate either LB medium or MM + 2% w/v casein hydrolysate. 2ml portions of the saturated culture were added to bijou bottles containing 2ml sterile 80% v/v glycerol, and mixed thoroughly before storage at -18°C in the dark. Cell suspensions stored in this manner remained viable for at least 2½ years. Strains stored in the dark at 4°C on minimal plates, wrapped with Parafilm to retard drying, remained viable for up to one year. Ensign (1970) has reported that members of the genus Arthrobacter show the ability to withstand long periods of nutrient starvation.

Attempts to store Arthrobacter strains for long periods in 'stabs' were not successful, nor were attempts to store them on slants of TYE agar in sealed bottles. In both cases there was rapid loss of viability, both at room temperature and at 4°C. The reason for the poor survival of Arthrobacter strains in sealed containers may lie in the fact that they are strict aerobes, whilst the enteric bacteria, which may be successfully stored in this manner, are facultatively

anaerobic and therefore capable of basal metabolism under these conditions.

Culture Scatterings

The turbidities (apparent absorbances due to light scattering) of cell suspensions were determined after dilution 1:1 with Cell Fixation Buffer, which contained:

6.8g/l KH_2PO_4

8.5g/l Na_2HPO_4

50ml/l 40% w/v formaldehyde (= 36% v/v)

Turbidities were determined at a wavelength of 550nm against buffer blanks, using 4ml of the diluted cell suspension. The Pye-Unicam SP800 spectrophotometer was used, and the cuvettes were placed in the holder closer to the photocell. Since Arthrobacter strains undergo large changes in cell size and shape during batch culture (Buchanan & Gibbons, 1974), culture turbidities were only useful for estimating the cell densities of exponentially growing cultures, which contained cells of uniform size and shape. Separate standard curves of turbidity plotted against cell density were prepared for cultures in LB medium and in MM + glucose.

Culture Dry Weight Determination

Pyrex tubes (10cm x 12mm diameter) were soaked in chromic acid, washed with distilled water and dried at 105°C. After cooling in a desicator the tubes were weighed with a Mettler H72 balance. Duplicate 5ml samples of culture were pipetted into pre-weighed tubes, which were placed inside 15ml polypropylene centrifuge tubes and spun at 3 000 rpm in an MSE Super Minor bench centrifuge for 15 minutes. The pellet was resuspended and washed twice with 5ml portions of 10mM-KH_2PO_4.KOH pH 7.0 (washing with this dilute buffer in place of distilled water

gave firmer pellets, without leading to any detectable increase in the dry weight values obtained), and the washed pellets were dried at 105°C for 24 hours. After cooling in a desicator the tubes were weighed and the increment in weight due to the cell pellet in each was calculated.

Determination of Total Cell Protein

A 10ml sample of culture was spun down, the cells were washed with 100mM-KH_2PO_4.KOH pH 7.0 and then resuspended in a suitable final volume with distilled water. The protein concentration of the washed cell suspension was then determined by the biuret method described below. For the Arthrobacter strains used, the protein content was typically about 50% of the total dry weight of the cells.

Protein-Chemical Techniques

Protein Estimation

Lowry Assay Whenever possible a modified Lowry assay (Miller, ~~1972~~ 1959) was used, because of its sensitivity. Stock solutions were prepared, containing:

1% w/v $CuSO_4.7H_2O$

2% w/v potassium tartarate

10% w/v Na_2CO_3 in 0.5M-NaOH

The stock solutions were mixed 1:1:20 just before use, and 1ml of the mixture was added to a 1ml sample containing from 20µg to 200µg protein. After standing for ten minutes at room temperature, 3ml of a 1:11 dilution of Folin-Ciocalteu Phenol Reagent was added and the sample mixed thoroughly by vortexing. After a further 30 minutes at room temperature the A_{560} of the solution was determined against a reagent blank. Standard curves were prepared for each set of assays,

using a standard solution of bovine serum albumin.

Biuret Assay The Lowry assay is only suitable for soluble samples; furthermore, it is subject to severe interference from the reducing agent 2-mercaptoethanol. Therefore the biuret method, which is less sensitive, but more specific, was used to determine the protein content of whole cells and of cell-free extracts containing 2-mercaptoethanol. The method used was based on that described by Herbert et al. (1971). 1ml 3M-NaOH was added to 2ml sample (containing from 1mg to 16mg protein) in a 12.5cm x 12mm diameter Pyrex tube, which was then placed in a boiling water bath for 15 minutes. After cooling, 1ml 2.5% w/v $CuSO_4.7H_2O$ was added, and the tube was immediately vortexed thoroughly to break up the gel-like precipitate. After ten minutes, 1.5ml of the sample was transfered to a disposable plastic centrifuge tube (1.5ml capacity) and the precipitate was removed by a two minute spin in a Beckman Microfuge B bench top centrifuge. The supernatant was decanted directly into a 1ml cuvette and the A_{555} determined against a reagent blank. A standard curve was constructed using a solution of bovine serum albumin. Tris buffer gives a considerable A_{555} with the biuret reagents; for low concentrations of Tris (10mM) correction for this absorbance was made by adding an equal concentration of Tris to the blank.

Column Techniques

The flow rates of columns were controlled with an LKB 12000 Varioperpex peristaltic pump. The UV absorbance of the effluent was monitored with an LKB Uvicord II system and fractions were collected using an LKB 7000 Ultrorac system, fitted with a drop counter.

Anion Exchange Chromatography The anion exchange resin DEAE-Sepharose CL-6B was used. This is supplied in pre-swollen form,

and does not require pre-cycling. However, before packing columns the resin was equilibrated to the pH and ionic strength of the loading buffer by washing until the pH of the buffer used remained constant. Columns were packed at high flow rates, as recommended by the manufacturers of the resin, and were washed overnight with the loading buffer before the application of the sample.

After the elution of the isomerase, columns were washed with several volumes of buffer containing 500mM-NaCl. The resin from the top of each column, containing strongly bound material, was discarded, and the remainder was stored at 4°C after the addition of sodium azide to 0.02% w/v.

Gel Filtration The filtration media Sephadex G200 and Sephadex G150 (coarse grade) were prepared by swelling the calculated weight of the dry solid for several days at room temperature, in the buffer to be used for equilibration of the column. The swollen gel was degassed and was packed under natural flow, using a head of 12cm (for G200) or 20cm (for G150). Columns were equilibrated by pumping through buffer at a flow rate higher than that used during chromatography. Ascending flow was used, in order to reduce the danger of compaction of the gel. Ultragel AcA34, which is supplied pre-swollen, was packed by pumping at the flow rate recommended by the manufacturers and was equilibrated with two column volumes of buffer. Since AcA34 is more rigid than G200, descending flow was used. Columns were loaded by pumping the sample directly into the gel. When using ascending flow the sample was chased with 10ml of buffer supplemented with NaCl to increase its density. This reduced trailing of the sample during loading.

Ultrafiltration Protein samples were concentrated by ultrafiltration. Amicon pressure cells were used, with Amicon PM30 memb-

ranes, and were operated at a pressure of 70 p.s.i., using nitrogen gas to apply the pressure.

Assays for Gel Filtration Markers:

Blue Dextran Blue Dextran 2000 was assayed by its absorption at 640nm. For a pure solution of Blue Dextran the ratio A_{280}/A_{640} is 4.3.

The assays for enzymes used as molecular weight markers were carried out at 30°C and were all coupled to changes in optical absorbance.

Pyruvate Kinase (rabbit muscle; M. Wt. 237 000)

The assay mixture contained:

50mM-Tris.HCl pH 7.5

75mM-KCl

10mM-$MgCl_2$

0.2mM-ADP

1mM-phosphoenolpyruvate

0.33mM-NADH (Sigma grade III)

20U/ml L-lactate dehydrogenase (Sigma type XI)

The assay was started by addition of the sample, and the initial rate of decrease in A_{340} was determined.

Fumarase (pig heart; M. Wt. 204 000)

The assay mixture was prepared by dissolving L-malic acid in 100mM potassium phosphate buffer to a concentration of 50mM and re-adjusting the pH to a value of 7.6 with NaOH. The formation of fumarate was followed by monitoring the increase in A_{240}.

Lactate Dehydrogenase (rabbit muscle; M. Wt. 142 000)

The initial rate of decrease in A_{340} was determined, after the addition of the sample to an assay mix containing:

60mM-KH_2PO_4.KOH pH 7.0

0.33mM-NADH

30mM-sodium pyruvate

Ribitol Dehydrogenase (K. aerogenes; M. Wt. 108 000)

The assay mixture contained:

100mM-KH_2PO_4.KOH pH 7.0

0.83mM-NAD^+ (sodium salt)

50mM-ribitol

The initial rate of increase in A_{340} was determined after adding the sample.

Alkaline Phosphatase (E. coli; M. Wt. 86 000)

The artificial substrate paranitrophenyl phosphate was used ('Sigma 104' phosphatase substrate), at a concentration of 1mM in 200mM-Tricine.NaOH pH 8.0. The reaction was followed by the rate of increase in A_{410}.

Performic Acid Oxidation

The conditions used for the oxidation of purified glucose isomerase with performic acid were based on those described by Hirs (1956).

Performic acid was prepared by adding 50µl hydrogen peroxide solution (100 volume) to 1ml formic acid (98% w/v) and incubating the mixture for 2.5 hours at room temperature. Salt free glucose isomerase (1.1mg) was dissolved in 84µl of a 1:5 (by volumes) mixture of methanol and formic acid. The solution was cooled to - 10°C, 70µl of the performic acid was added and the mixture was incubated for 2.5 hours in a propan-2-ol/solid carbon dioxide bath at from -5°C to -15°C. The oxidised protein was freeze-dried, re-dissolved in formic acid and stored frozen at -18°C.

Acid Hydrolysis

Samples of salt-free protein for hydrolysis were dissolved in formic acid. Portions were dried down under vacuum in glass tubes (52mm x 13mm diameter), which had been washed with chromic acid or roasted to destroy any organic contaminants. 50µl of 6M-HCl containing 200µM-L-norleucine and a trace of phenol (one crystal of phenol per 25ml) was added to each tube. The top of each tube was heated in a methane/oxygen flame and then drawn out to form a thin neck of about 2mm diameter. Each tube was attatched to a pump and evacuated for 90 seconds before passing the neck through the flame to seal the tube. The sealed tubes were incubated for suitable periods (from 14 to 86 hours) at 106°C and cooled to room temperature. A line was scribed round each tube near to the top. After passing the tops of the tubes through a cool flame (i.e. with the oxygen supply off) to drive the hydrolysate to the bottom, each tube was opened by touching the scribe mark to the side of a very hot flame, causing a crack to proagate round the tube following the line. The rims of the opened tubes were rounded off in the flame, and the hydrolysates were dried down under vacuum. The dried hydrolysates were stored at -18°C until they were dissolved in the appropriate buffer for the amino acid analyser used.

Assay of Arthrobacter Enzymes

Preparation of Arthrobacter Cell-Free Extracts

Attempts to prepare Arthrobacter cell lysates by sonication were not successful. The cells from 40ml of an overnight culture in LB medium were resuspended in 5ml buffer and placed in a 10ml vial on ice. The cells were subjected to sonication for a total of ten minutes, using a Dawe Soniprobe Type 7530A, on setting 6, tuned to

give maximum amplitude. Sonication was in one minute bursts, separated by two minute cooling periods. No change in the appearance of the cells under phase contrast microscopy was observed following the sonication. Under the same conditions, sonication for a total of five minutes is sufficient to achieve complete disruption of E. coli or K. aerogenes cells. Similar results were obtained using an MSE sonic disintegrator.

Very poor lysis of Arthrobacter cells was also obtained using a Manton-Gaulin homogeniser; four passes at a pressure of 10 000 p.s.i. gave only a 20% cell breakage (Dr D. Herbert, personal communication).

Therefore lysozyme rupture was used to prepare cell-free extracts for enzyme assays and electrophoresis. The cells from 40ml or 80ml of culture were washed in 30ml of 10mM-Tris.HCl/10mM-EDTA pH 8.0, resuspended in 5ml of this buffer and transfered to a 9ml polycarbonate centrifuge bottle. The thiol reducing reagent 2-mercaptoethanol was added to a concentration of 10mM, and the serine protease inhibitor PMSF (phenylmethane sulphonyl fluoride) to a concentration of 100μM as a 100mM stock solution in propan-2-ol. Lysozyme chloride was added to a concentration of 1mg/ml as a 50mg/ml stock solution, and the cell suspension was incubated at 30°C for from 45 to 60 minutes (unless the extract was to be assayed for glucokinase, in which case lysis was allowed to proceed at room temperature for from 60 to 90 minutes, as incubation at higher temperatures led to a decrease in the activity observed). The viscosity of the gel formed by lysis was reduced by the addition of 100μl 1M-$MgCl_2$ followed by 10μl of a solution of DNase and RNase at a concentration of 500μg/ml each. After incubation for a further 20 minutes, 'cell ghosts', debris and any unruptured cells were pelleted by centrifugation at 48 000 xg in the SS-34 head of a Sorvall RC5 centrifuge for 15 minutes, at from 0°C to 10°C. The resulting cell supernatants were kept on ice and assayed as rapidly

as possible. When the cell debris resulting from lysozyme treatment of Arthrobacter cells was examined by phase contrast microscopy, it was found that many 'cell ghosts' were present; these retained the rod shape of unlysed cells, but had very little contrast and were readily distinguished from whole cells. It should be noted that the Arthrobacter strains used in the work described in this thesis appear to possess very thick cell walls when viewed by electron microscopy following staining and sectioning (Dr D. Herbert, personal communication).

In the calculation of specific activities no allowance was made for the contribution made by lysozyme to the total protein concentration of the cell-free extracts, since it was not certain whether a significant fraction of that added was pelleted with the cell debris or whether the major part remained in the supernatant. The contribution may have been substantial, since the protein concentrations of the extracts were in the range 2mg/ml to 12mg/ml. However, in the work described in Chapter VI the relative values of the specific activities of the different extracts assayed were of greater importance then the absolute values.

All the assays used to measure the levels of intracellular enzymes in cell-free extracts were carried out at 25°C in Tris buffer of pH 7.5. The assays were coupled either to the reduction of $NADP^+$ or to the oxidation of NADH, and were followed spectrophotometrically by monitoring the change in absorbance at 340nm. Incubations were carried out in volumes of 1ml in quartz cuvettes of 1cm light path. Under these conditions a change in A_{340} of 6.22 units corresponded to the oxidation or reduction of 1umol of the coenzyme. One unit of activity was the amount required to obtain a rate of 1μmol product formed per minute.

Glucokinase The assay used was based on that described by Anderson and Kamel (1966). Incubations contained, in addition to cell-free extract:

60mM-Tris.HCl pH 7.5

6mM-$MgCl_2$

3mM-ATP

0.6mM-$NADP^+$

0.5U/ml glucose-6-phosphate dehydrogenase (G6PDH; from Torula Yeast)

12mM-D-glucose

The cell extract (up to 100µl) was pre-incubated with the whole assay mixture, apart from the substrate, and the low background rate of increase in A_{340} observed was subtracted from the rate obtained after the addition of the glucose. The rate of increase in A_{340} observed remained constant for nearly one absorbance unit, and was proportional to the volume of cell-free extract used.

Fructokinase The assay used was the same as that for glucokinase, except that 0.5U/ml phosphoglucose isomerase (PGI; from Yeast) was added to the assay mixture and D-fructose was substituted for D-glucose. The ability of this assay to detect any fructose-6-phosphate formed was checked by adding a sample of authentic fructose-6-phosphate (F6P) to the assay mixture.

Glucose-6-phosphate Dehydrogenase (G6PDH) Incubations contained, in addition to cell-free extract:

60mM-Tris.HCl pH 7.5

6mM-$MgCl_2$

0.6mM-$NADP^+$

0.5mM-glucose-6-phosphate (G6P)

The reaction was started by adding the G6P, after pre-incubating the cell extract with the rest of the assay mix. The rate of increase in A_{340} observed decreased with time. Increasing the substrate conc-

entration and/or the concentration of the coenzyme did not result in a linear rate of increase in A_{340} with time; therefore inhibition of the enzyme by its product, 6-phosphogluconate, seems the most probable explanation for the decrease in rate observed. The initial rate was used to calculate the activity of the enzyme.

Phosphoglucose Isomerase (PGI) This enzyme was assayed in the direction of the formation of G6P. The incubations contained:

60mM-Tris.HCl pH 7.5

0.6mM-$NADP^+$

0.5U/ml G6PDH

12mM-fructose-6-phosphate (F6P)

On the addition of the F6P to the rest of the assay mixture a burst of $NADP^+$ reduction which lasted for about three minutes was observed. The size of this burst corresponded to contamination of the F6P used (Sigma grade I) with G6P to about 0.5%. The assay was therefore started by adding the cell extract to pre-incubated assay mixture.

Phosphoglucomutase The incubations contained:

60mM-Tris.HCl pH 7.5

6mM-$MgCl_2$

0.6mM-$NADP^+$

0.5U/ml G6PDH

12mM-glucose-1-phosphate (G1P)

The assay was started by the addition of the G1P after pre-incubating the cell-free extract with the rest of the assay mixture.

Glucose Dehydrogenase (EC 1.1.1.47) Incubations contained:

100mM-Tris.HCl pH 7.5

0.3mM-NAD^+ or $NADP^+$

30mM-D-glucose

This assay was carried out at 30°C. The cell extract was pre-inucbated with the buffer and coenzyme, and the reaction was started by the addition of the glucose. The assay was based on that described by Avigad et al. (1968).

Glucose Oxidase (EC 1.1.3.4) The assay used was based on that described by Ng and Dawes (1973), and was carried out at 30°C. Incubations (total volume 3ml) contained:

33mM-KH_2PO_4.KOH pH 6.6

15mM-D-glucose

0.5mM-KCN

33µg/ml dichlorophenolindophenol (DPI)

The reaction was started by the addition of the glucose, and the decrease in A_{600} was monitored.

Phosphofructokinase (PFK) The incubations contained:

60mM-Tris.HCl pH 7.5

10mM-$MgCl_2$

0.33mM-NADH

3mM-ATP

0.5U/ml aldolase (from rabbit muscle)

0.5U/ml glyceraldehyde phosphate dehydrogenase, containing excess triose phosphate isomerase (Sigma type III).

2.5mM-fructose-6-phosphate

The cell extract was pre-incubated with the complete assay mix, apart from the F6P, and the slit width of the spectrophotometer was adjusted to give an A_{340} reading of 2.0, before the addition of the substrate. The initial rate of reaction was determined and corrected by the subtraction of the low background rate of NADH oxidation observed before the addition of the substrate. It should be noted that each molecule of fructose-1,6-diphosphate formed gave rise to two mol-

ecules of triose phosphate. The rate of NADH oxidation was therefore twice the rate of the kinase reaction.

Fructose-1-phosphate Kinase (F1PK) The same conditions were used as for the assay of phosphofructokinase, except that the F6P was replaced by F1P at a concentration of 1.5mM.

Gluconate-6-phosphate Dehydrogenase Incubations contained:

60mM-Tris.HCl pH 7.5

10mM-$MgCl_2$

0.3mM-$NADP^+$

0.5mM-6-phosphogluconate (6PG)

The cell extract was pre-incubated with the assay mixture, and the reaction was started by the addition of the 6PG. The rate of increase in A_{340} observed fell slowly, and was not restored by the addition of extra substrate. The initial rates were used to calculate the activities of the extracts assayed.

Gluconate Kinase The assay system used for gluconate kinase was based on that described by Fraenkel and Levisohn (1967) and relied on endogenous gluconate-6-phosphate dehydrogenase activity for coupling. In order to ensure that sufficient dehydrogenase activity was present, 25µl or 50µl of a cell-free extract prepared from strain B3724 after growth on glucose was added to each incubation and the rate redetermined. This extract contained 0.6U/ml of the dehydrogenase, but no gluconate kinase activity. Incubations contained:

60mM-Tris.HCl pH 7.5

10mM-$MgCl_2$

3mM-ATP

0.3mM-$NADP^+$

2mM-potassium gluconate

The cell extract was pre-incubated with the assay mixture, and

the assay was started by the addition of the substrate.

Assay of D-xylulokinase

The method used for the assay of D-xylulokinase was based on that described by Lee and Bendet (1967) for the assay of L-ribulokinase. [^{14}C]-D-xylulose was generated *in situ* by the action of excess D-xylose isomerase on [^{14}C]-D-xylose; [^{14}C]-D-xylulose-5-phosphate formed from this by the kinase was precipitated as the barium salt, collected on glass-fibre filters and determined by scintillation counting. The activity of the kinase was calculated from the rate of conversion of the radioactive material present into barium-precipitable form.

Incubations contained: 100µl cell-free extract
100µl purified isomerase (0.4mg protein)
800µl assay mixture

The incubations were carried out at 30°C. At ten minute intervals 100µl samples were withdrawn from each incubation and added to 50µl 200mM-glucose-1-phosphate (to act as carrier for the xylulose-5-phosphate) in a disposable plastic tube (7cm x 1cm diameter). 100µl 1M-$BaCl_2$ was added to each tube, followed by 1ml ethanol, and the tubes were then cooled on ice. The precipitate from each tube was collected on a 2.1cm glass-fibre disc (Whatman GF/C), by washing the tube with 3 x 2ml 80% v/v ethanol, pre-cooled to 0°C. After washing with 2 x 5ml ice cold 80% ethanol each filter was dried under an infra-red lamp and placed in a scintillation vial insert for counting. The assay mixture contained:

50mM-KH_2PO_4.KOH pH 7.8
33mM-magnesium acetate
1mM-EDTA
8mM-ATP
10mM-KF

2mM-sodium azide

2mM-dithiothreitol

340µg/ml chloramphenicol

500µg/ml streptomycin sulphate

0.5mM-D-xylose

0.1µCi/ml [^{14}C]-D-xylose

Assay of PEP:Sugar Phosphotransferase Systems

Phosphoenolpyruvate (PEP) phosphotransferase systems for the concomitant uptake and phosphorylation of sugars were assayed essentially as described by Kornberg and Reeves (1972), using cells treated with toluene to render them permeable. The formation of pyruvate from phosphoenolpyruvate in the presence of a substrate for the system was coupled to the oxidation of NADH, using lactate dehydrogenase.

Cells grown under suitable conditions were spun down, washed in 100mM-KH_2PO_4.KOH/5mM-$MgCl_2$ pH 7.5 and resuspended in this buffer at a density of about 1mg/ml dry weight. The cell suspension was chilled on ice, and 1% by volume of a 1:9 (v:v) mixture of toluene and ethanol was added slowly, while the suspension was being vortexed. The vortexing was continued for one minute after the addition of the toluene. The toluene treated cells were kept on ice.

Assays were carried out at 30°C in volumes of 1ml, and contained, in addition to suitable volumes of the cell suspension:

93mM-KH_2PO_4.KOH pH 8.0

4.6mM-$MgCl_2$

165µM-NADH

1mM-PEP

2U/ml L-lactate dehydrogenase (rabbit muscle)

5mM substrate

The cell suspension was pre-incubated with the assay mixture, apart from the substrate. The spectrophotometer was adjusted to give an A_{340} reading of about 2 units, and the background rate of NADH oxidation was determined. The reaction was started by adding the substrate, and the rate of decrease in A_{340} obtained was corrected by subtraction of the background rate. One unit of phosphotransferase activity will catalyse the substrate dependent formation of pyruvate from PEP at a rate of 1μmol per minute.

Pentitol Dehydrogenase Assays

D-arabitol dehydrogenase and ribitol dehydrogenase were assayed spectrophotometrically, by following the rate of increase in A_{340} due to the reduction of NAD^+. The assays were performed at 28°C in a volume of 1ml, in cuvettes of 1cm light path. Incubations contained:

100mM-KH_2PO_4.KOH pH 7.0

0.83mM-NAD^+

50mM-D-arabitol or ribitol

Cuvettes containing the assay mixture were pre-incubated at 28°C, and the assays were started by the addition of the enzyme sample with a Hamilton microsyringe. One unit of either dehydrogenase is that amount which converts pentitol to ketopentose at a rate of one micromole per minute under the conditions of the assay. This corresponds to a rate of increase in A_{340} of 6.22 units per minute.

Chapter III

Assays for Isomerase Activity

The Arthrobacter glucose isomerase, in common with D-glucose isomerising enzymes from members of other bacterial genera, shows a wide substrate specificity (Chapter VIII). This makes it possible to use a variety of substrates for the assay of the enzyme, including D-glucose, D-fructose, D-xylose, D-ribose and L-arabinose. However, the activity which is of commercial interest is the inter-conversion of glucose and fructose at 60°C, whilst the only activity which is normally of physiological importance to the bacterium is the conversion of D-xylose to D-xylulose at 30°C.

This chapter describes four different assay systems for the isomerase. Two are discontinuous assays, in which either the rate of formation of fructose from glucose (the standard assay used by I.C.I.) or the rate of formation of glucose from fructose (used as the standard assay in the work described in this thesis) is measured. In both cases the assay is carried out at 60°C. The other two are continuous spectrophotometric assays, carried out at 30°C, which use pentitol dehydrogenases to couple the generation of ketopentose from either D-xylose or D-ribose to the oxidation of NADH. The coupled assay for D-xylose isomerase activity was used to identify and compare active fractions during the enzyme purification work described in Chapters VII and IX. The D-ribose isomerase assay was not used for the quantitation of the isomerase, but is described here because it formed the basis for the investigation of the kinetics of the isomerase towards D-ribose (Chapter VII). Finally the cysteine/carbazole assay used in the study of the L-arabinose isomerase activity is described.

Assay as D-Glucose Isomerase

The standard assay conditions used by I.C.I. Agricultural Division for the quantitation of the isomerase are 1M-D-glucose as substrate in 100mM-Tricine.NaOH/30mM-$MgCl_2$ pH 8.0 at 60°C. A discontinuous assay system is used. For the assay of whole cells or other suitable samples, 3ml portions of buffer/substrate concentrate are cooled on ice, before the addition of the samples for assay (0.5ml). The tubes are then transfered to a water bath at 60°C for 40 minutes and returned to the ice bath for ten minutes, before the addition of 1.5ml 1M-HCl to each. After a low speed centrifugation the supernatants are assayed for fructose as described below. The values obtained from blank incubations, containing enzyme samples which have been inactivated by boiling, are subtracted to obtain the amount of fructose formed in each incubation due to the action of the isomerase. The unit of glucose isomerase activity used is that amount which will catalyse the formation of one gram of fructose per minute from glucose, under the conditions described. This corresponds to the formation of 5 550 micromoles of fructose per minute.

Determination of Fructose

The principle source of difficulty with this assay system is the need for the determination of fructose in the presence of a large (50-fold) excess of glucose, for which there is no suitable enzymic method at present available. Although several chemical methods for the determination of fructose have been published, all of these depend on the formation of a substituted furfural from fructose in the presence of a strong acid, followed by the reaction of the furfural with some compound to form a suitable coloured derivative. Unfortunately this reaction is not totally specific to the keto-sugar; all the assays

therefore depend on the use of conditions under which the ketose reacts more rapidly than the aldose. The relative rates of reaction for glucose and fructose are critically dependent both on the concentration of acid used and on the temperature at which the reaction is allowed to proceed.

The method used by I.C.I. is the Seliwanoff reaction, in which resorcinol is used and a purple derivative is formed from fructose (Seliwanoff, 1887; Roe, 1934; Foreman et al., 1973). The problem of ensuring that each set of samples and standards receive identical treatment is overcome by the use of an Autoanalyser system. The samples are diluted automatically, before mixing with 10M-HCl and a solution of resorcinol in ethanol (1.428g/l) in the ratio 1:2:2 by volumes. The mixture is passed through a 40 minute delay coil in a 70°C bath, cooled and passed through a 1.5cm light path cell, in which the A_{480} is determined. The standards used contain from 2mg/ml to 12mg/ml D-fructose. Under these conditions the absorbance reading obtained from unit concentration of fructose is about 150-fold that due to unit concentration of glucose. Attempts to use a manual version of this method did not appear promising, since the results obtained for replicate standard samples of known concentration were not sufficiently reproducible. The main difficulty lay in ensuring that the incubation conditions were identical for each member of a series of samples.

Several of the other published methods for the assay of fructose were evaluated for the determination of fructose in the presence of excess glucose.

The 'improved' resorcinol method of Roe et al. (1949), in which thiourea and acetic acid are added to the reaction in addition to the resorcinol, has a greater sensitivity than the original method, but a decreased specificity. A ratio of 1:80 was obtained for the absob-

ances due to unit concentrations of glucose and fructose.

In the method of Dische and Devi (1960) cysteine is added to the sample, followed by strong sulphuric acid. Although the mixture is allowed to stand at room temperature, it becomes hot due to the latent heat of mixing. Production of the yellow colour (A_{max} 414nm) from fructose was complete after two hours, and the absorbance then remained constant for at least three hours. This system therefore appeared suitable for the batch assay of samples for fructose. However, when glucose was used in place of fructose, the absorbance continued to increase for at least five hours. At the time when the absorbance due to fructose first reached its maximum value, that due to an equal concentration of glucose had already reached 1/83 of this value, and was increasing too rapidly for a batch assay system to have been feasible for the determination of fructose in the presence of excess glucose.

The method of Dische and Borenfreund (1951) is similar to the above, except that carbazole is added to the assay after the addition of the acid. This results in the development of a much more intense colour, which in the case of fructose has an absorbance maximum at a wavelength of 558nm. The reaction, which continues for several days, is not allowed to go to completion, but the absorbance of each assay incubation is read after a fixed period of time. The use of this system for the determination of fructose in the presence of glucose was investigated, incubating the assay mixtures either at 37°C or at room temperature. In both cases the discrimination between glucose and fructose obtained was no better than that obtained with the cysteine method, although the sensitivity was greater. This method was, however, used to quantitate L-ribulose during the investigation of the kinetic properties of the enzyme as an L-arabinose isomerase, as des-

cribed in Chapter VIII. The details of the conditions used are given in the last section of this chapter.

Pogell (1954) described a system for the determination of fructose using skatole (3-methylindole) and strong HCl. Carvalho and Pogell (1957) described a modification of this procedure, in which the coloured product formed is extracted into chloroform; after this extraction the colour (A_{max} 508nm) is stable for many hours. (It should be noted that the products of the resorcinol and cysteine/carbazole reactions cannot be extracted into either chloroform or carbontetrachloride.) The one hour incubation described in the earlier paper was used, together with the extraction procedure described in the later paper, because the shorter incubation gives better discrimination between glucose and fructose, whilst the increased sensitivity of the later method was not needed. Samples (1ml) containing from 50µg/ml to 600µg/ml fructose were cooled on ice and mixed with 8ml of ice-cold 10M-HCl and 1ml of ice-cold 0.04% w/v skatole in ethanol. After incubation for one hour at 37°C, the tubes were cooled on ice and the coloured product extracted into 10ml chloroform. The A_{508} of each chloroform layer was determined against a reagent blank. Under these conditions a linear relation between fructose concentration and absorbance was obtained, and the values obtained for replicate samples were much more reproducible than those obtained using the other assays described. Moreover, the ratio between the absorbances which were obtained with unit concentrations of glucose and fructose was approximately 1:250. This system therefore appeared to be the most suitable of those tested for the manual determination of fructose in the presence of glucose after glucose isomerase assays, apart from the health hazard involved in the routine use of large amounts of chloroform.

However, at this point it was decided that glucose isomerase activity would be determined in the reverse direction (i.e. the formation of glucose from fructose) as a routine assay. When it was essential to determine fructose in the presence of glucose (e.g. for the kinetic studies described in Chapter VIII), the samples were taken to Billingham and assayed with the Autoanalyser system.

Assay as D-Fructose Isomerase

The assay of the isomerase in the direction of formation of glucose from fructose had the advantage that it was possible to make use of the specificity of the enzyme glucose oxidase to determine the amount of glucose formed under mild conditions. As a result of the availability of this method for the determination of glucose, the assay of the isomerase in the 'backward' direction was both more precise and less lengthy than the 'forward' assay described above. The conditions used for the assay were the same as those of the standard glucose isomerase assay, apart from the substitution of D-fructose for D-glucose in the assay mixture. Incubations contained 1M-D-fructose in 100mM-Tricine.NaOH/30mM-$MgCl_2$ pH 8.0 at 60°C.

The buffer/substrate concentrate was prepared by dissolving 20.9g Tricine and 7.1g $MgCl_2.6H_2O$ in 700ml distilled water and adjusting the pH to a value of 8.5 at room temperature, by the addition of 1M-NaOH. This resulted in a pH value of 8.0 at 60°C. D-Fructose (210g) was added and the solution made up to 1 litre. The D-fructose used was from Cambrian Chemicals Ltd. (catalogue No. F204) and contained less than 0.01% D-glucose, as determined by the glucose oxidase method. The buffer/substrate concentrate was stored frozen, to prevent the growth of mould.

Cultures for assay were spun down in a bench centrifuge, and

the cells were washed once in 100mM-KH_2PO_4.KOH pH 7.0 and resuspended in distilled water (at a concentration of about 5mg/ml dry weight, in the case of cells of normal activity). Replicate 0.5ml portions of the washed cell suspension, or of other material to be assayed, were placed in glass tubes (12cm x 13mm diameter) and cooled in an ice/water bath, before the addition to each of 3ml ice-cold buffer/substrate concentrate. Blank tubes, containing either boiled enzyme samples or distilled water, were also prepared, to enable the results obtained to be corrected for the non-enzymic formation of glucose. The concentrations of glucose formed in these two types of blank incubation did not differ significantly. The tubes were transfered to a 60°C water bath and incubated without shaking. The standard length of incubation used was 40 minutes; however, longer incubations were also used, and the amount of glucose formed was proportional to the length of the incubation for at least two hours under the conditions used. The rack containing the tubes was then returned to an ice/water bath for 10 minutes, approximately compensating for the time taken for the tubes to reach 60°C at the start of the incubation, before the addition to each tube of 1.5ml of 15% w/v trichloroacetic acid to precipitate the whole cells/protein present. About 1.5ml from each tube was then transfered to a disposable centrifuge tube (1.5ml capacity) and spun for one minute in a Beckman Microfuge B bench top centrifuge. The supernatants were diluted 1 in 25 by adding 200µl to 4.8ml 100mM potassium phosphate buffer pH 7.0 (or in the case of very active samples 1 in 50 or 1 in 100). The use of buffer for this dilution ensured that the pH of the glucose assay system was not altered by the addition of the sample.

Determination of D-Glucose

The concentration of glucose present in the diluted supernatants

was determined using the reagents of a commercial system intended for the determination of blood glucose levels. The system used was from Boehringer ("Test-Combination Glucose; GOD-Perid Method"); this kit contains a stabilised standard solution containing 91µg/ml D-glucose (reagent 1) and vials of dry buffer/enzyme/chromogen mixture, which are dissolved in distilled water to prepare reagent 2. Both reagents were stored at 4°C in dark glass bottles. For medical use the dry reagent is marked with an expiry date, and the solution is assigned an expiry date six weeks after its preparation; however, for laboratory use both of these dates may be exceeded without the observation of any increase in the blank reading or any alteration in the standard curve obtained. The green colour formed in the presence of glucose has an absorbance maximum at a wavelength of 418nm.

200µl samples of the diluted supernatants were added to 3ml portions of the Boehringer reagent 2 and incubated for 35 minutes in a 30°C water bath. The A_{418} of each incubation was read against a blank prepared using water in place of the sample. For small numbers of samples, all the incubations were performed together in a single batch, since the colour, once developed, remains stable for about 20 minutes. When larger numbers of samples were to be assayed (e.g. during the kinetic studies described in Chapter VIII), individual incubations were started at timed intervals and the absorbance of each was determined after 35 minutes. A standard curve was prepared for each set of determinations, using suitable volumes of the reagent 1 and distilled water in place of the sample. The relation obtained between concentration and absorbance was linear. The concentration of glucose in the undiluted supernatants was calculated, and used to deduce the activities of the samples assayed for fructose isomerase activity.

The unit of fructose isomerase activity is that amount which will form one gram of glucose from fructose per minute under the conditions of the assay. This unit is not quite the same as the unit used by I.C.I.; comparison of the results obtained at Imperial College using the fructose isomerase assay with those obtained at Billingham for the same freeze-dried material using the glucose isomerase assay gave a mean ratio of:

$$\frac{\text{Glucose Isomerase Activity}}{\text{Fructose Isomerase Activity}} = 1.11$$

The values of the Michaelis constants (K_m) and maximum specific forward velocities (V) for glucose and fructose presented in Chapter VIII (Table 8a) predict a ratio of 1.03, which is in reasonable agreement with the experimental value.

Unless otherwise stated, all glucose isomerase activities given in this thesis were determined by the fructose isomerase assay, and have not been corrected for the difference between the units of this assay and those of the glucose isomerase assay. The specific activities of whole cells were calculated relative to protein concentration as determined by the biuret method (Chapter II), not relative to dry weight.

Note on the Assay of the Isomerase in Whole Cells

The glucose isomerase assay is routinely performed on whole cells, as described above. The following experiment was performed to check the validity of this method of determining the enzyme levels present; in addition the experiment demonstrated that the isomerase has considerable activity as a glucose/fructose isomerase at physiological temperatures.

Cells of strain B3728 (which is constitutive for the isomerase)

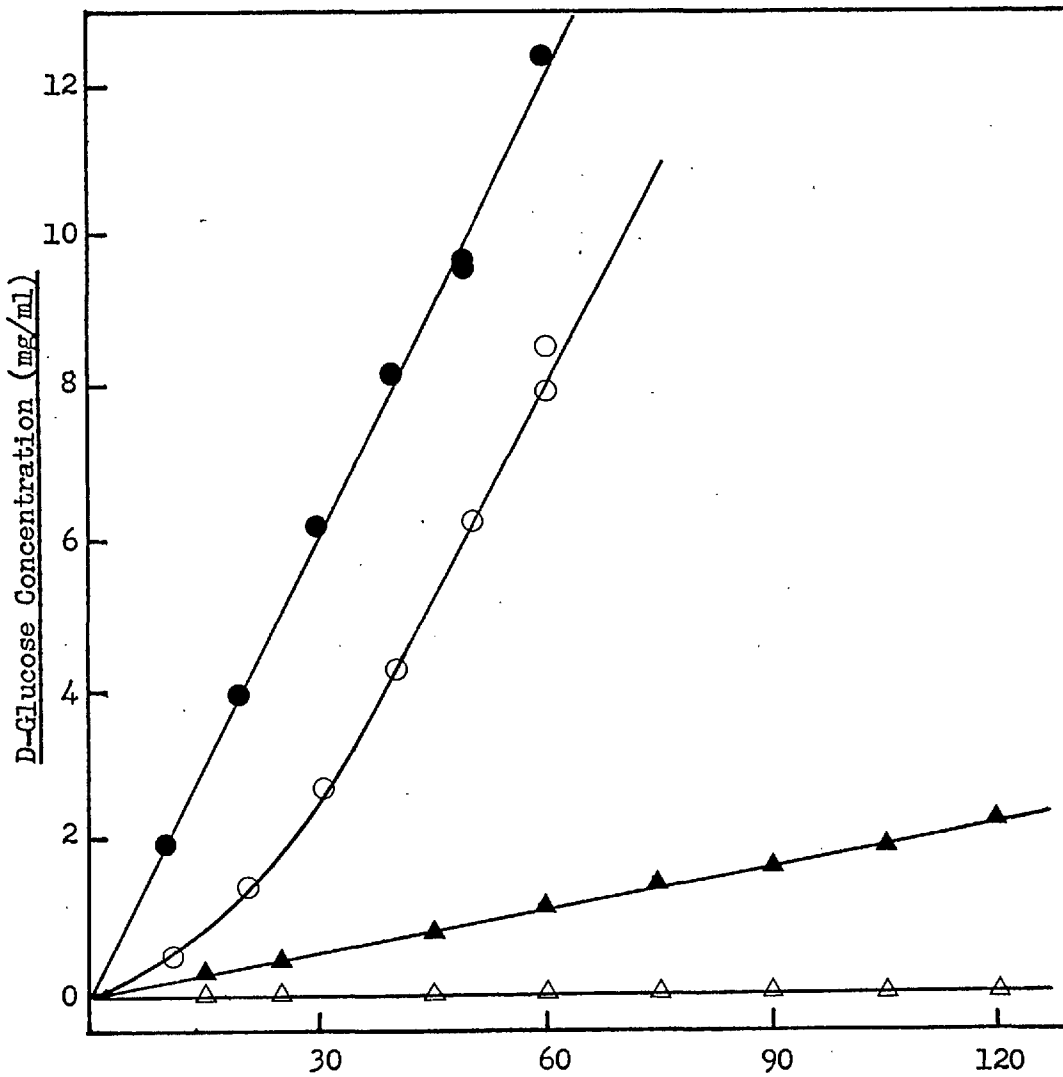
were washed and resuspended in 10mM-Tris.HCl/10mM-EDTA pH 8.0 at a protein concentration of 10.6mg/ml. Half of this cell suspension was treated with lysozyme, followed by Mg^{2+}, DNase and RNase, as described in Chapter II. The cell debris was not removed from the lysate. Sodium arsenate was added to the remainder of the cell suspension to a concentration of 20mM, together with $MgCl_2$ and distilled water to give the same final volume and Mg^{2+} concentration as those of the lysate.

Tubes containing 6ml each of the standard buffer/substrate concentrate used for the assay of fructose isomerase activity were cooled in an ice/water bath. A 1ml portion of whole cell suspension, lysate or distilled water was added to each tube. One set of tubes was incubated at 60°C, and another at 30°C. Because of the possibility that at 30°C any glucose formed might be metabolised, despite the addition of the arsenate, an additional tube containing buffer/substrate and whole cell suspension was supplemented with glucose to a concentration of about 3.5mg/ml and incubated at 30°C. At suitable intervals 700µl samples were removed from the incubations and added to 300µl portions of 15% w/v trichloroacetic acid in 1.5ml 'microfuge' tubes to quench the reaction. After the removal of the precipitate by centrifugation, the supernatants were assayed for glucose as described above. Figure 3a presents the results obtained.

At 60°C the rates of formation of glucose observed in the presence of whole cells and cell lysate were similar, although there was a lag before the full activity of the whole cell suspension was expressed. This lag may have corresponded to a need for the cell membrane to be disrupted by incubation at 60°C before the enzyme became available to extra-cellular substrate. Since work performed by I.C.I. has shown that there is no tendency for the enzyme to escape from the

Figure 3a

Isomerase Activity of Whole and Lysed Cells at 60°C and 30°C



No catabolism of glucose was detected in the control tube incubated at 30°C with whole cells.

cells, even on prolonged incubation at 60°C, it appears possible that the cell wall (which is very thick) forms a 'sack' after the disruption of the membrane, which permits free diffusion of small molecules, whilst remaining impermeable to intracellular enzymes.

At 30°C the lysed cell preparation showed considerable fructose isomerase activity, and hence presumably also possessed significant glucose isomerase activity. The rate of enzymic glucose formation was about 1/10 that at 60°C. This contrasts with the results reported by Reynolds (1973), which suggest that the enzyme has very little activity as glucose isomerase below 40°C. This demonstration that the enzyme possesses significant glucose isomerase activity at physiological temperatures was essential for the feasibility of the evolution of a new pathway for glucose catabolism involving the isomerase. In contrast, no enzymic conversion of fructose to glucose was observed at 30°C in the presence of whole cells, whilst there was no significant disappearence of glucose from the incubation which had been supplemented with this sugar (Figure 3a). These results suggested that the isomerase was not available to extracellular substrates under physiological conditions, lending weight to the theory that the isomerase was an intracellular enzyme.

Coupled Isomerase Assays

The use of a continuous spectrophotometric assay offers considerable advantages over a discontinuous assay system, particularly in applications where it is desirable to obtain the results for individual samples rapidly (e.g. when identifying active fractions during enzyme purification). None of the substrates of the isomerase is chromogenic, nor is it apparent how an artificial chromogenic substrate could be devised. It was therefore necessary to use a coupled assay

system to obtain a change in absorbance linked to the action of the enzyme. Coupled assays were developed for both the D-xylose and the D-ribose isomerising activities of the enzyme, using pentitol dehydrogenases as coupling enzymes. Fortuitously, both D-arabitol dehydrogenase (which interconverts D-arabitol and D-xylulose) and ribitol dehydrogenase (which interconverts ribitol and D-ribulose) were readily available at Imperial College when this work was performed.

In theory the glucose isomerase reaction could be coupled in an analogous manner, using either D-mannitol dehydrogenase or D-sorbitol dehydrogenase. Although a dehydrogenase suitable for use at 60°C would be required, it is probable that a D-sorbitol dehydrogenase of sufficient thermostability could be prepared from Bacillus stearothermophilus strain NCA 1503. This strain is capable of growth on D-sorbitol as sole carbon source at 60°C, and it is known that the growth of Bacillus subtilis on sorbitol involves the induction of a D-sorbitol dehydrogenase (Horowitz & Kaplan, ~~1963~~ 1964). However, this possibility was not pursued, because although a coupled assay would have been useful in the kinetic studies with the purified enzyme described in Chapter VIII, it would have been of no use for the assay of whole cell suspensions or solid material.

D-Xylose Isomerase Assay

The use of D-arabitol dehydrogenase in a coupled assay for D-xylose isomerase was first reported by Yamanaka (1969b); however, the method has not found widespread use, presumably due to the difficulty of preparing D-arabitol dehydrogenase in sufficiently pure form from wild type inducible strains. Recent work in this deparment has involved the construction of E. coli strains which synthesise high levels of this enzyme constitutively, by virtue of a high copy number plasmid (pRD351) which carries the structural gene for the dehydrogenase

but not the repressor gene, of the K. aerogenes D-arabitol operon (Neuberger & Hartley, 1979). Crude cell supernatants prepared from such strains have sufficiently high specific activities to be used to couple the isomerase assay without further purification.

The D-xylose isomerase assay was carried out at 30°C in a volume of 1ml in quartz cuvettes of 1cm light path. Incubations contained:

100mM-Tricine.NaOH pH 8.0

30mM-$MgCl_2$

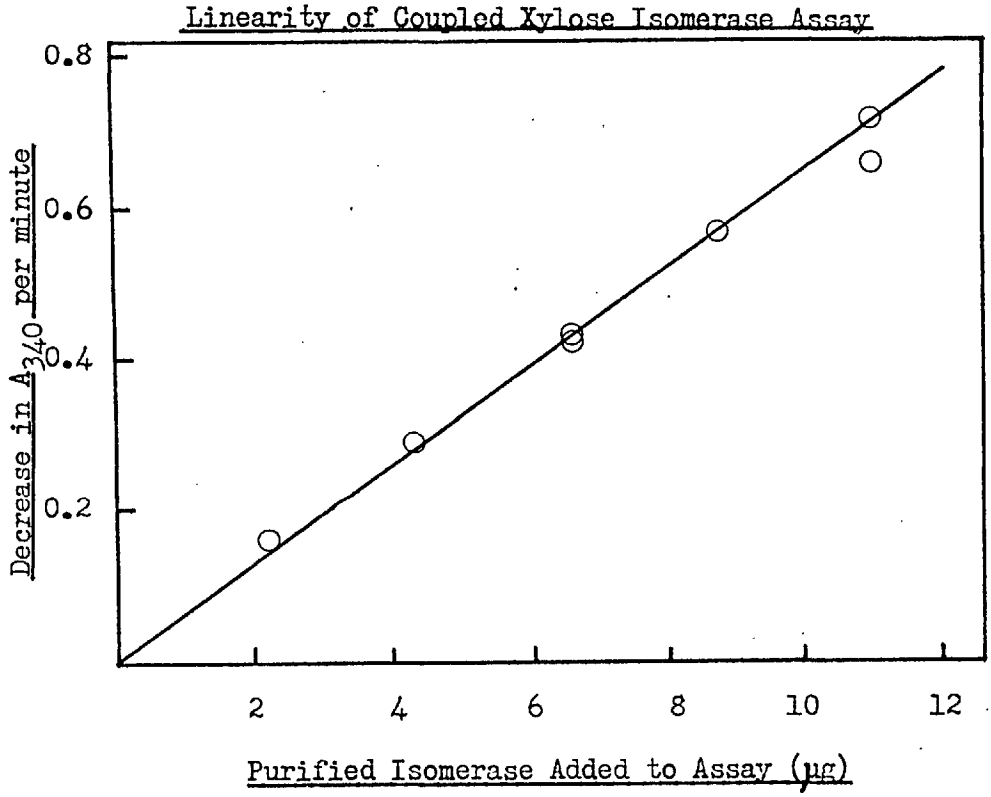
50mM-D-xylose

0.33mM-NADH

0.5U/ml D-arabitol dehydrogenase

The assay was carried out as follows. A solution of NADH was prepared freshly, by dissolving the solid disodium salt (Sigma grade III) in 200mM-Tricine.NaOH pH 8.0 at 2.56mg/ml, giving a concentration of 3.3mM, and was kept on ice. 100µl of NADH solution and 800µl of pre-warmed buffer/substrate concentrate, containing 37.5mM-$MgCl_2$ and 62.5mM-D-xylose in 100mM-Tricine.NaOH pH 8.0, were added to a cuvette. The correct volume of arabitol dehydrogenase solution was added with a Hamilton microsyringe, followed by any water needed to give the correct final volume, and the assay mixture was pre-incubated at 30°C. The spectrophotometer was adjusted to give an A_{340} reading of 2.0 with the assay mixture in the light path and any background rate of NADH oxidation was determined. The background rate was usually negligible. The assay was started by adding the sample, containing up to 0.1 units of D-xylose isomerase activity. On addition of the isomerase the rate of decrease in A_{340} increased for a time before reaching a steady value, which then remained constant until the NADH became depleted at an A_{340} of about 0.3. The steady state rate was taken as corresponding to the rate of the isomerisation reaction. One unit of D-xylose isomerase

Figure 3b



Incubations contained 0.5U D-arabitol dehydrogenase, in a volume of 1ml 100mM-Tricine.NaOH buffer pH 8.0 containing 30mM-$MgCl_2$, 50mM-D-Xylose and 0.33mM-NADH. Volumes of 10, 20, 30, 40 and 50µl of a solution of purified glucose isomerase from strain B3728 at a concentration of 220µg/ml were added to assays.

activity is that amount which will form D-xylulose from D-xylose at a rate of one micromole per minute under the conditions of the assay. This rate corresponds to a rate of decrease in A_{340} of 6.22 units per minute. Figure 3b shows that the rate observed was proportional to the amount of isomerase added to the assay over the range of isomerase concentrations used.

The D-arabitol dehydrogenase used to couple the isomerase assay was itself assayed as described in Chapter II. It should be noted that the dehydrogenase assay was carried out at 28°C and pH 7.0, in the direction of formation of D-xylulose from D-arabitol, whereas in the coupled assay system the dehydrogenase was used to reduce xylulose to arabitol at 30°C and pH 8.0. It was not, therefore, possible to calculate the ratio of the activities of the isomerase and dehydrogenase in the assay system. However, the activity of D-arabitol dehydrogenase is greater at 30°C and pH 8.0 than at 28°C and pH 7.0.

Cell free extracts containing D-arabitol dehydrogenase were prepared as follows. E. coli strain HB101/pRD351 was grown up in MM' + 2% casein hydrolysate, supplemented with ampicillin to a concentration of 25µg/ml. (Since plasmid pRD351 carries an ampicillin resistance determinant the inclusion of the antibiotic in the medium provided a counter selection against cells which had lost the plasmid during the early stages of growth.) The cells were harvested by centrifugation, washed with 100mM-KH_2PO_4.KOH pH 7.5 and resuspended in 5ml of the same buffer, supplemented with 2-mercaptoethanol to a concentration of 10mM. The cells from 400ml culture were resuspended in a volume of 5ml. After the addition of NAD^+ to 1mM and PMSF to 0.1mM, the cells were ruptured by sonication for a total of five minutes, under the conditions described in Chapter II under the preparation of Arthrobacter cell-free extracts. The cell debris was removed by centrifugation at 48 000 xg for 30 minutes at 0°C to 10°C in the

SS-34 rotor of a Sorvall RC5 centrifuge, and the supernatant was then subjected to a second centrifugation at the same speed for a further thirty minutes. The final supernatant had a D-arabitol dehydrogenase specific activity of about 40U/ml, and was kept on ice until used.

During most of the experiments involving D-xylose isomerase activity described in Chapters VII, VIII and IX partially purified D-arabitol dehydrogenase was used in place of crude cell-free extracts. This partially purified material was the gift of Dr M.S. Neuberger and was derived from side fractions obtained during the purification of the enzyme (Neuberger et al., 1979). However, as the D-arabitol dehydrogenase is not very stable, it would be more convenient to use freshly prepared crude cell supernatants than to purify the enzyme specifically for use in the assay. The dehydrogenase is stable for short periods at 4°C in the presence of thiol reducing reagents such as dithiothreitol. Freezing solutions of the D-arabitol dehydrogenase leads to loss of activity, but the enzyme may be stored for longer periods at -18°C, after the addition of glycerol to 50% v/v to prevent freezing. However, glycerol is an inhibitor of the isomerase, and must therefore be removed by extensive dialysis before preparations stored in the presence of glycerol may be used to couple the D-xylose isomerase assay.

D-Ribose Isomerase Assay

D-Ribose isomerase activity was assayed in a manner analogous to that used for D-xylose isomerase activity. Incubations contained:

100mM-Tricine.NaOH pH 8.0

30mM-$MgCl_2$

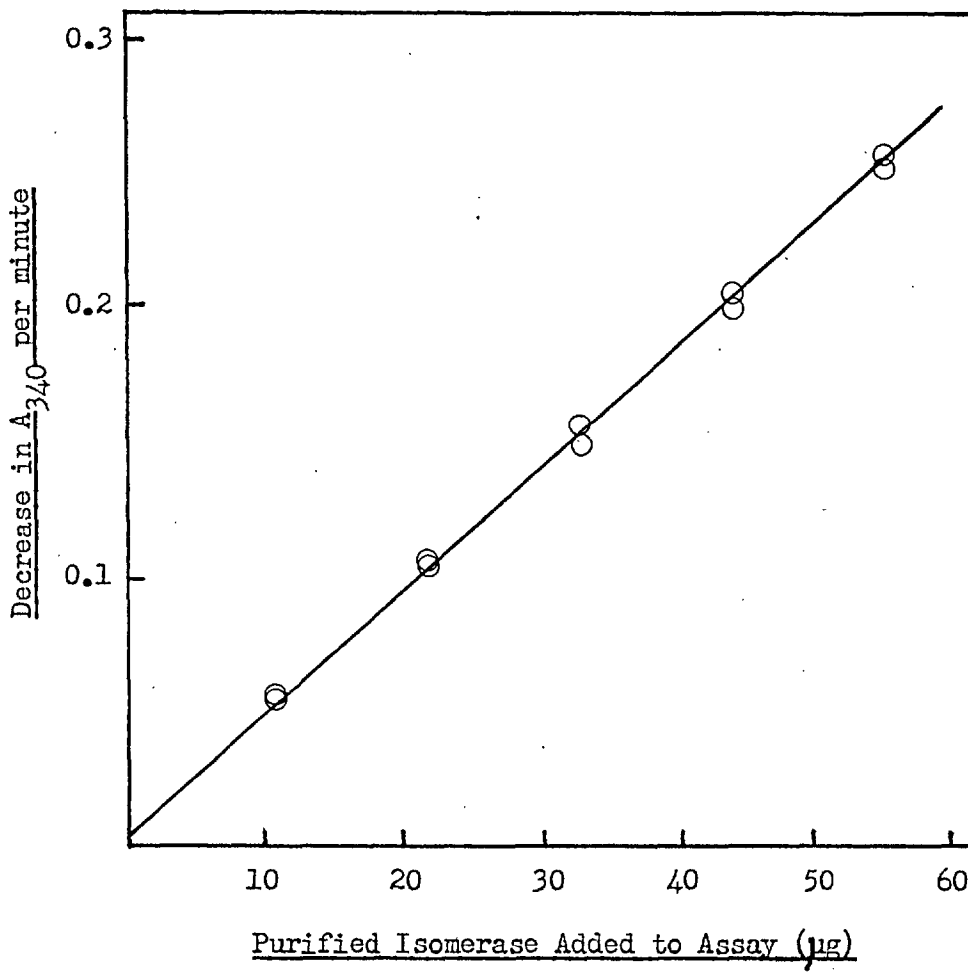
200mM-D-Ribose

0.33mM-NADH

0.6U/ml ribitol dehydrogenase

Figure 3c

Linearity of Coupled Ribose Isomerase Assay



Incubations contained 0.6U ribitol dehydrogenase, in a volume of 1ml 100mM-Tricine.NaOH buffer pH 8.0 containing 30mM-$MgCl_2$, 200mM-D-Ribose and 0.33mM-NADH. Volumes of 10, 20, 30, 40 and 50µl of a solution of the purified glucose isomerase from strain B3728 containing 2.2mg/ml protein were added to duplicate incubations.

The assay was carried out as described for the D-xylose isomerase assay. When the dehydrogenase was added to the rest of the assay mixture, a burst of NADH oxidation lasting for about three minutes was observed. The magnitude of this burst was proportional to the concentration of D-ribose used and corresponded to the reduction of about 0.04% of the pentose present. It appeared probable that the D-ribose used (BDH; Prod. No. 38058) was contaminated with 0.04% of D-ribulose. The burst of NADH oxidation was allowed to go to completion before the addition of the isomerase to start the assay.

Figure 3c shows that the activity observed in this coupled assay was proportional to the amount of isomerase added.

The assay used to determine the activity of ribitol dehydrogenase is described in Chapter II. Purified ribitol dehydrogenase from K. aerogenes, previously prepared in this group as described by Taylor et al. (1974), was used to couple the isomerase assay. Unlike D-arabitol dehydrogenase, ribitol dehydrogenase may be stored frozen with little loss in activity. However, several strains (both of K. aerogenes and of E. coli) exist which super-produce ribitol dehydrogenase to levels sufficient for the use of crude cell supernatants to couple this D-ribose isomerase assay (Neuberger & Hartley, 1979).

The D-ribose isomerase assay was used only during the kinetic studies described in Chapter VIII, because the D-xylose isomerase assay, using the normal physiological substrate of the isomerase, provided a more sensitive continuous spectrophotometric assay for routine use during purification work.

Cysteine/Carbazole Assay for Ketopentose

The assay used to quantitate L-ribulose formed during the kinetic studies on the L-arabinose isomerase activity of the purified

Arthrobacter isomerase was based on that described by Dische and Borenfreund (1951). The reagents used were:

1.5% w/v cysteine hydrochloride (freshly prepared),

H_2SO_4 (prepared by mixing 95ml distilled water and 225ml concentrated H_2SO_4 and cooling to 20°C)

and 0.12% w/v carbazole in ethanol

To the sample (0.5ml) were added 0.1ml cysteine hydrochloride, 3ml H_2SO_4 and 0.1ml carbazole. After thorough mixing, each assay was incubated for 60 minutes at room temperature, before the determination of the A_{540} of the mixture against a reagent blank.

Chapter IV

Conditions for the Generation and Isolation of Mutants

This chapter describes the development of suitable conditions for the treatment of Arthrobacter strain B3724 with the mutagen N-methyl-N'-nitro-N-nitrosoguanidine and for the antibiotic enrichment of mutagenically treated cultures for mutants unable to grow under specific conditions, using ampicillin. In addition the results of attempts to obtain mutants unable to catabolise glucose by the isolation strains resistant to inhibition by analogues of glucose are presented, and an experiment to demonstrate that deletion mutants of strain B3724 can be induced by treatment with nitrous acid is described.

Choice of Mutagen

It was decided that N-methyl-N'-nitro-N-nitrosoguanidine (NG) would be used initially, as high mutation frequencies can be obtained using this mutagen. This was considered to be desirable, since it appeared possible that no single losion would lead to a Glc^- Fru^+ phenotype, as multiple paths for glucose uptake, phosphorylation and entry into central metabolism are known to exist in those prokaryotic organisms which have been extensively investigated (Curtis & Epstein, 1975; Fraenkel & Vinopal, 1973). Furthermore, under conditions which result in only a low degree of killing, a high proportion of all the cells surviving treatment with NG are mutants (Adelberg et al., 1965). This is a considerable advantage in the absence of a positive selecton for the desired mutant. However, the use of NG has the disadvantage that the lesions induced are primarily G.C to A.T base transitions,

as has been shown both for yeast (Prakash & Sherman, 1973) and for E. coli (Coulondre & Miller, 1977); such base substitution mutations revert at relatively high frequencies. It was considered that it would be necessary to obtain mutants which did not revert at any detectable frequency, such as deletion mutants, in order for the evolution of a new pathway of glucose metabolism involving the glucose isomerase activity to be possible, since otherwise revertants would be expected readily to outgrow initial evolvants. It was therefore envisaged that mutagenesis with nitrous acid or UV light, both of which give lower absolute mutation rates than NG, but give rise to a relatively large proportion of deletions (Schwartz & Beckwith, 1969), would be necessary once it had been demonstrated that mutants with the Glc^- Fru^+ phenotype could be obtained. Point mutations generated with NG would allow the determination of the genotype(s) necessary for this phenotype and the identification of any other phenotypic traits of such mutants, besides their inability to utilise glucose, which might enable a more powerful screening for Glc^- Fru^+ mutants to be devised.

Conditions for NG Mutagenesis of Strain B3724

The procedure described by Miller (1972) for NG mutagenesis of E. coli K12 provided a basis from which to develop a method suitable for use with Arthrobacter strain B3724. It is necessary to determine the correct conditions to obtain a kill of about 50% for any strain used, since higher kills result in a large proportion of the surviving cells carrying unwanted auxotrophies, making the isolation of desired mutants more difficult (Adelberg et al., 1965). This may be done by varying either the time of exposure to the mutagen or the concentration used.

Cells in exponential growth phase in LB were harvested by centrifugation at a density of 4×10^8/ml, washed twice and resuspended in 0.1M citrate buffer pH 5.5. Aliquots were incubated for 30 minutes at 30°C with NG at concentrations from 25µg/ml to 175µg/ml. The cells were then washed twice with phosphate buffer pH 7.0 and the viable cell count was determined. The recovery of viable cells from control incubations to which no NG had been added was less than 25%. It was observed that after incubation in citrate buffer the cells gave rise to colonies of very variable size on TYE plates. This suggested that poor viability rather than physical loss of the cells was the cause of the low recovery observed.

After incubation in acetate buffer pH 4.6 or in a buffer of pH 6.0 containing 50mM-Tris/50mM-maleate, even poorer recoveries of viable cells were obtained than with citrate buffer; colonies of variable sizes were again formed. However, incubation in 0.1M-potassium phosphate buffer pH 7.0 lead to no loss of viability and colonies of uniform size were obtained.

Although buffers of low pH enhance the mutagenic effect of NG towards E. coli (Adelberg et al., 1965), and phosphate ions may catalyse the decomposition of NG (McCalla et al., 1968), it was decided that phosphate buffer of pH 7.0 would be used, to avoid further delay while other buffers were tested. A killing curve for strain B3724 was constructed, using the conditions described above, except that citrate buffer was replaced by phosphate. A concentration of about 75µg/ml NG was required to obtain a kill of 50%.

Antibiotic Enrichment

"A thousand shall fall beside thee, and ten thousand at thy right hand: but it shall not come nigh thee."

Psalm 91 (A.V.)

When isolating mutants with such phenotypes as antibiotic or bacteriophage resistance, or the ability to utilise a new carbon source, positive selection of the required cells is possible, using conditions under which only the mutants are able to form colonies. This makes it possible to screen very large numbers of cells on a single plate. However, in the case of the loss of ability to utilise a carbon source, such selection is not possible, unless the mutation results in resistance to an antimetabolite as a secondary phenotype. Instead, all the cells screened must be allowed to form individual colonies. The desired mutants are then selected either by the colour of the colonies after growth on an indicator medium or after treatment with an indicator stain, or by their failure to form replica colonies on a selective medium. In the absence of known Glc^- mutants of B3724 with which to test indicator media, it was necessary to use replica plating in the crucial experiment of isolating Glc^- Fru^+ mutants. This technique restricts the number of clones which can be screened on each master plate to about 250 (using Petri dishes of 9cm diameter). It was therefore necessary to enrich the mutagenised culture for cells of the desired phenotype before attempting mutant isolation.

Antibiotic enrichment provides a method of enrichment for cells unable to grow in a particular medium. An antibiotic which kills only growing cells is added to a culture in the selective medium. After incubation the surviving cells are recovered by centrifugation or filtration and washed to remove the antibiotic before plating to select mutants or growing up in a non-selective medium ready for a

further cycle of enrichment. Suitable antibiotics include penicillin G (Davis, 1948; Lederberg & Zinder, 1948), ampicillin (Molholt, 1967) and D-cycloserine (Curtiss et al., 1965), which act by blocking cell wall synthesis, leading to autolysis of growing cells (Gale et al., 1972). Nalidixic acid, which acts at the level of DNA replication (Hoffman et al., 1970) and 8-azaguanine, which is incorporated into RNA (Mangalo & Wachsman, 1962), have also been used.

Antibiotic Enrichment Conditions for Strain B3724

Preliminary experiments showed that neither nalidixic acid nor 8-azaguanine was suitable for use with strain B3724. The growth of B3724 on TYE plates containing nalidixic acid at a concentration of 100µg/ml was as rapid as on unsupplemented TYE plates. When 8-azaguanine was added to cultures of B3724 growing in MM + 0.5% (w/v) glucose to concentrations of up to 200µM, no loss of viability was detected after incubation for either five or sixteen hours, although growth was slower than in the absence of the antimetabolite. This contrasts with the results obtained by Wachsman and Mangalo (1962) with Bacillus megaterium, using incubations of two and five hours with 100µM-8-azaguanine to enrich for auxotrophic mutants.

When antibiotics which lead to cell lysis are used for auxotroph enrichment, lysis products can enable mutant cells to grow, which leads to a loss of the specificity of killing (Rossi & Berg, 1971). It is therefore usual to carry out the enrichment with low initial cell densities and to use as short an incubation as is consistant with a satisfactory degree of killing (99.9% or higher). However, this problem is likely to be less severe when selecting mutants unable to utilise a carbon source than when enriching for auxotrophs.

The effect of various concentrations of penicillin G, ampicillin

Table 4a Effect of Cell Wall Antibiotics on Strain B3724

Antibiotic	Concentration	Incubation Time (Hours)	Initial Density (Viable Cells/ml$\times 10^{-8}$)	Killing (Percentage)	Generations in Control Incubation
Penicillin G	2.5×10^{2}U/ml	3	3	70	1.7
"	5×10^{2}U/ml	3	3	70	"
"	2.5×10^{3}U/ml	3	3	50	"
"	5×10^{3}U/ml	3	1.3	95	1.8
"	10^{4}U/ml	3	1.3	85	"
"	10^{5}U/ml	4	1	95	3.0
Ampicillin	20µg/ml	4	1.3	80	3.4
"	40µg/ml	4	1.3	90	"
"	200µg/ml	4	1.1	95	3.2
"	"	4	1.2	92	2.6
"	2mg/ml	4	2.1	95	"
D-Cycloserine	2mM	5	0.55	*	3.4
"	20mM	4	1.2	42	2.6
"	200mM	4	2.1	96	"
Ampicillin + D-Cycloserine	100µg/ml + 10mM	4	1.2	75	2.6

* No decrease in viable cell count, but bacteriostatic effect at this concentration.

and D-cycloserine on cultures of strain B3724 was investigated. Aerobic incubations of three to five hours were used, in MM + 0.5% glucose, with initial cell densities of about 10^8/ml. After incubation the surviving cells were recovered by centrifugation at a relative acceleration of 27 000 xg in a Sorvall RC5, to minimise physical loss. The cells were washed twice in 0.1M phosphate buffer pH 7.0 and plated on TYE to determine the viable count. Table 4a summarises the results of these experiments.

Since ampicillin and D-cycloserine block cell wall synthesis at different sites (Gale et al., 1972), it was hoped that a combination of the two antibiotics might prove much more effective than either alone. However, no improvement over the killing obtained with high concentrations of ampicillin alone was observed (Table 4a).

In view of the apparent resistance of strain B3724 to these antibiotics acting on cell wall synthesis, it was decided to check that satisfactory kills were obtained with an E. coli K12 strain (NC100) using the concentrations recommended by Miller (1972). To facilitate comparison with the results obtained with Arthrobacter, a three hour incubation at 30°C was used instead of a shorter incubation at 37°C. The cell density before the addition of the antibiotics was 1.1 x 10^8/ml and during the incubation 2.2 generations occurred in a control to which no antibiotic was added. The results obtained were:

Antibiotic	Concentration	Kill
Penicillin G	10^4U/ml	99.98%
Ampicillin	20µg/ml	99.98%
D-Cycloserine	2mM	99.96%

This confirmed that with E. coli good kills could be obtained with all three antibiotics, using an incubation of the same length as that used in the experiments with strain B3724, and with a similar

generation time in the absence of the antibiotic.

Strain B3724 was tested at MRE Porton for sensitivity to a variety of antibiotics. In the plate tests used bacitracin (8U) and cephaloridine (5μg) both produced larger zones of inhibition than ampicillin (10μg) (Dr A. Atkinson, personal communication). Both of these antibiotics act on cell wall biosynthesis; cephaloridine at the same site(s) as the penicillins, and bacitracin by blocking recycling of the C_{55} isoprenoid carrier lipid (Gale et al., 1972). Both should therefore kill only growing cells and should be suitable for use in antibiotic enrichment, provided that it is possible to remove them from non-growing cells by washing. However, the use of these antibiotics was not investigated, as suitable conditions for the use of ampicillin were found.

Saier et al. (1976) enriched cultures of Salmonella typhimurium for mutants unable to catabolise various sugars using a twenty-four hour incubation with penicillin (100U/ml). Long incubations (10 to 24 hours) were tested for strain B3724, using ampicillin at concentrations of 20μg/ml and 200μg/ml. Incubations of about eighteen hours gave kills of about 99.8%, although considerable variations in the degree of killing obtained under identical conditions were observed. Under these conditions the culture in control incubations without antibiotic reached stationary phase, after nearly six generations.

Having found conditions under which a sufficiently high kill was obtained to make enrichment possible, it was considered desirable to check that there was selective killing of growing cells, in view of the length of the incubation used. Ideally this would have been done by adding a known number of mutant cells to a culture of the wild type and determining the kill for each strain in a single incubation; this would have shown whether cross-feeding by lysis products was a

significant problem under the conditions used. However, since no suitable mutant was available at this stage for such a test, the following method was used to test the specificity of killing.

Two flasks of MM, one containing 0.5% (w/v) glucose, the other without any carbon source, were inoculated to a cell density of about 10^8/ml from an overnight culture of B3724 in MM + glucose. Each was shaken at 30°C until the turbidity of the cell suspension in MM alone remained constant (2.5 hours) indicating that cell division had ceased. The viable cell count of each was then determined, and 10ml aliquots of each were incubated for a further 21 hours with and without the addition of ampicillin to a concentration of 20µg/ml. After pelleting the cells at 27 000 xg and washing twice with phosphate buffer, the viable cell count of each incubation was again determined. The following results were obtained:

Medium	Initial Cell Density	Ampicillin Incubation	Control
MM + glucose	2.8×10^8/ml	99.8% kill	5.3 generations
MM alone	2.5×10^8/ml	40% kill	40% kill

The survival of the cells incubated without any substrate was satisfactory and no extra loss of viability occurred in the presence of ampicillin. At this point it was considered that a satisfactory enrichment procedure had been found, and the isolation of mutants of Glc^- Fru^+ phenotype was attempted, as described in the next chapter.

Resistance to Glucose Analogues

In *E. coli* and *S. typhimurium*, both of which possess PEP:sugar phosphotransferase systems for glucose uptake, it is observed that glucose represses the utilisation of fructose. Analogues of glucose such as 2-deoxyglucose, 3-deoxy-3-fluoroglucose and 5-thioglucose, which retain the hydroxyl group at the C_6 position, are also taken up

and phosphorylated by this system, although they cannot be further catabolised. Such analogues also prevent the utilisation of fructose. This has provided a powerful method for the isolation of mutants of these organisms defective in glucose transport/phosphorylation (Kornberg & Smith, 1972). Arthrobacter globiformis does not use a phosphotransferase system for glucose uptake (Romano et al., 1970), a result since confirmed for strain B3724 (Chapter VI). However, it was considered that the use of glucose analogues to select Glc^- mutants was worth investigating, since the formation of phosphorylated glucose analogues which could not be further metabolised might still have led to inhibition of growth. The availability of a positive selection for Glc^- mutants would have removed the necessity for enrichment of mutagenised cultures before mutant isolation, resulting in a considerable saving in time.

M9 plates containing 5mM-fructose with or without 10mM-2-deoxyglucose were inoculated with Arthrobacter strains SA4000, SA402 and SA409. SA4000 is a streptomycin resistant derivative of B3724, and has a wild type Glc^+ phenotype, whilst strains SA402 and SA409 are Glc^- mutants isolated by replica plating following ampicillin enrichment and known to lack glucokinase activity (Chapters V & VI). Strain SA4000 showed severe inhibition of growth in the presence of 2-deoxyglucose, whilst the growth of the Glc^- mutants was not affected. Similar inhibition of the growth of strain SA4000 was observed using 5-thioglucose in place of 2-deoxyglucose. However, when 25 independently isolated spontaneous mutants of strain SA4000, selected for resistance to inhibition by 2-deoxyglucose were tested for growth on M9 glucose plates, all proved to have retained a Glc^+ phenotype. It therefore appeared that this method was not likely to provide a powerful positive screening for Glc^- mutants, and further experiments were not performed to determine the nature of these mutants.

Nitrous Acid Mutagenesis

Nitrous acid mutagenesis has been widely used for the induction of deletion mutants. Both base substitutions and deletions are generated. In the case of E. coli about 6% of the lesions induced with nitrous acid are deletions (Schwartz & Beckwith, 1969). The ability of this mutagen to induce deletion mutations in Arthrobacter strain B3724 was investigated by screening amino acid auxotrophs isolated after nitrous acid treatment for non-reverting mutants. Since many more enzymes are involved in the elaboration of amino acids than in the peripheral stages of glucose catabolism, it was expected that amino acid auxotrophs would be generated with higher frequency than Glc^{-} Fru^{+} mutants.

Nitrous acid mutagenesis was carried out essentially as described by Miller (1972). Since the mutagenesis must be performed at a low pH value, and poor viability had been observed previously after the incubation of Arthrobacter cells in buffers of low pH value, a control was performed in which the nitrous acid was omitted. After incubation in buffer alone a survival of 20% was observed, whilst after incubation with nitrous acid the survival was reduced to 0.0015%. Such high percentage kills are expected during nitrous acid mutagenesis. A 20ml portion of a saturated culture of B3724 in LB was spun down and the cells were washed in 10ml of 100mM sodium acetate buffer pH 4.6 and again pelleted. The pellet was resuspended in 0.5ml 'HNO_2', freshly prepared by dissolving 345mg $NaNO_2$ in 100ml of the acetate buffer and sterilised by Millipore filtration. After incubation at 30°C for ten minutes, the mutagenesis was stopped by the addition of 15ml 0.1M phosphate buffer pH 7.0 and the cells were pelleted. The mutagenised cells were resuspended in LB medium and grown up overnight. The culture was then subjected to two rounds of ampicillin enrichment for mutants requiring methionine, proline, arginine, histidine, leucine,

isoleucine or tryptophan.

The ampicillin enrichments were carried out essentially as described above. Each incubation was for twenty hours, with 200µg/ml ampicillin, in MM + 0.5% glucose supplemented with 200µg/ml each of alanine, aspartate, glutamine and cysteine, to ensure rapid growth. After each round of enrichment the survivors were grown up in MM + glucose supplemented with 20µg/ml of each of the amino acids for which auxotrophs were sought. Kills of 99.98% and 98% were obtained in the first and second rounds of enrichment, as determined by plating on TYE.

Auxotrophs were detected by replica plating from master plates of M9 glucose supplemented with 800µg/ml casein hydrolysate and 20µg/ml tryptophan onto unsupplemented M9 glucose plates. A total of 3 500 colonies were screened and 41 auxotrophic clones were detected. These were further characterised by replica plating onto M9 glucose plates supplemented with methionine, proline, arginine, histidine, leucine, isoleucine or tryptophan to a concentration of 20µg/ml. Two clones required leucine (Leu phenotype), three methionine (Met) and 27 tryptophan (Trp); the requirements of the remainder were not satisfied by any of the single amino acids tested. Five of the Trp clones showed no sign of reversion when streaked heavily on unsupplemented M9 glucose plates, whilst the remainder and the Met and Leu clones all gave rise to revertant colonies. After repurification, a Met clone was stored as strain SA418, a Leu clone as SA419, the non-reverting Trp clones as SA420 to SA424 and one of the Trp clones which showed reversion as SA425.

Strains SA418, SA419 and SA425 all show reversion frequencies of about 10^{-8}, as determined by plating on M9 glucose, and are presumed to be point mutants. No Trp^+ revertants of strains SA420 to SA424 have been detected. It should be noted that it is possible that

these five strains are in fact separate isolates derived from a single mutational event. In the case of strain SA420 no spontaneous Trp^+ revertants were present in 5×10^{11} cells screened; nor were any induced when a crystal of the mutagen NG was added to the centre of an M9 glucose plate spread with 3×10^{10} cells. It appears probable that SA420 to SA424 are deletion mutants, although either insertion mutants or multiple point mutants could also have shown very low reversion frequencies. It was therefore concluded that nitrous acid mutagenesis would probably be a suitable method for inducing non-reverting Glc^- mutants of strain B3724.

Chapter V

Isolation and Phenotypic Characterisation of Mutants SA402 to SA417

This chapter describes the isolation of Glc^- Fru^+ point mutants induced by the mutagen NG. Three mutageneses were performed using different concentrations of the mutagen, and the mutants were isolated by replica plating, after two rounds of ampicillin enrichment. Growth tests showed that these mutants belonged to two phenotypic classes. One class grew normally on gluconate, whilst the second class was unable to utilise gluconate as a carbon source and showed severely inhibited growth on rich media (TYE) supplemented with glucose.

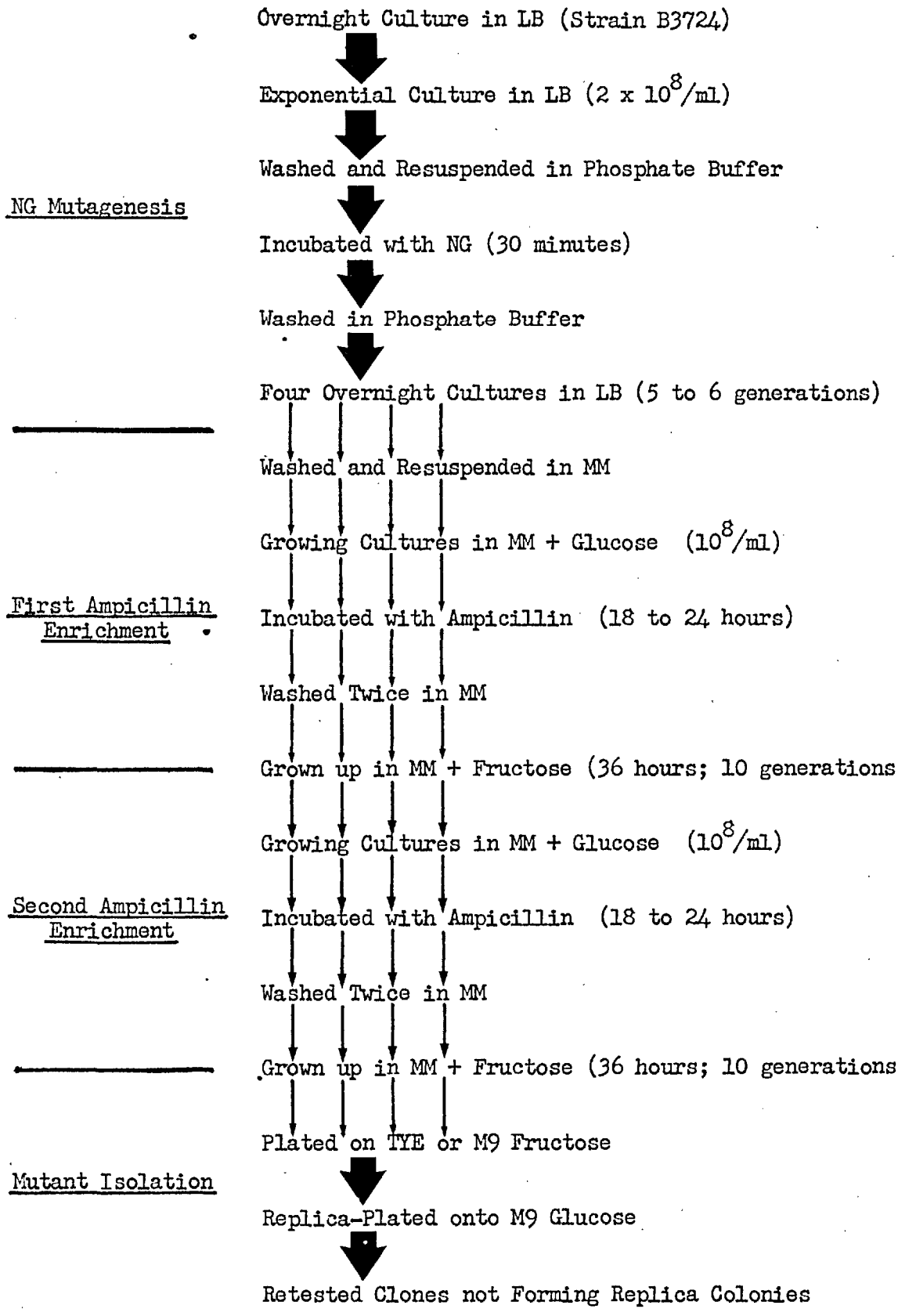
Figure 5a is a flow sheet for the induction and isolation of the mutants and Table 5a contains details of the mutageneses and ampicillin enrichments.

Mutagenesis

A single colony of strain B3724 was used to inoculate an overnight culture in LB medium. The saturated culture was used to inoculate a fresh flask of LB, which was incubated until the culture scattering indicated that a cell density of about 2×10^8/ml had been reached. A 20ml portion of this exponentially growing culture was spun down and the cells were washed twice and resuspended in phosphate buffer (100mM-KH_2PO_4.KOH, pH 7.0). The cell suspension was then titred on TYE plates to determine the viable cell count. NG was added to the desired concentration as a freshly prepared solution at 2mg/ml, and the cell suspension was incubated at 30°C for 30 minutes, without shaking. The cells were again pelleted by centrifugation,

Figure 5a

Flow Sheet for the Isolation of Glc^- Fru^+ Mutants



washed twice in phosphate buffer and resuspended in LB medium. The viable cell count of this mutagenised cell suspension was determined, to enable the kill to be calculated. The LB suspension was then divided into four separate cultures, which were grown up overnight. The division of the mutagenised cell suspension was intended to ensure the isolation of mutants derived from at least four indepentdent mutational events.

A concentration of 75µg/ml NG was used in the first mutagenesis, as this was expected to give a kill of about 50% (Chapter IV). However, only a single Glc^- Fru^+ clone was isolated after this mutagenesis. Therefore the concentration of the mutagen was increased to 100µg/ml and 200µg/ml in the two subsequent treatments, to increase the frequency of mutation.

Ampicillin Enrichment

Each overnight culture was spun down and the cells were washed twice in phosphate buffer and resuspended in MM. A 1ml portion of each cell suspension was used to inoculate a shake flask containing 100ml prewarmed MM + 0.5% (w/v) glucose and the cultures were incubated at 30°C until a culture scattering corresponding to a cell density of about 10^8/ml was reached. One culture was then titred on TYE plates. A 10ml portion of each culture was shaken with ampicillin at 30°C for from 18 to 24 hours. At intervals the incubations were vortexed, to resuspend aggregated material. The surviving cells were recovered by centrifugation at 48 000 xg for 30 minutes in the SS-34 rotor of a Sorvall RC5, washed twice in MM and resuspended in MM + 0.5% (w/v) fructose. The suspensions were titred on TYE plates to enable the kill to be calculated, and then incubated with shaking at 30°C for from 36 to 48 hours. The saturated cultures from the first round of

Table 5a

Details of NG Mutageneses and Ampicillin Enrichments

NG Mutagenesis.	1st.	2nd.	3rd.
NG concentration	75µg/ml	100µg/ml	200µg/ml
Kill	36%	80%	85%
First Enrichment			
Incubation Time	23.5 hours	24 hours	24 hours
Ampicillin Concentration	20µg/ml	40µg/ml	40µg/ml
Kill	98%	98%	98%
Second Enrichment			
Incubation Time	18.5 hours	20 hours	20 hours
Ampicillin Concentration	20µg/ml	160µg/ml	160µg/ml
Kill	98%	96%	99%
Isolation of Mutants			
Enrichments Screened	1	2	2
Clones Screened	7 000	4 500 4 500	4 500 4 000
Glc^- Fru^+ Clones	1	31 56	42 25
Percentage Glc^- Fru^+ in Enriched Culture	(0.014%)	0.7% 1.2%	0.9% 0.6%

enrichment were used to inoculate fresh flasks of MM + glucose for the second round. After the second round of enrichment the saturated cultures were spread to form the master plates for mutant isolation.

A concentration of 20µg/ml ampicillin was used in both cycles of enrichment of the cultures derived from the first mutagenesis. During the second cycle of ampicillin treatment, two of the incubations grew up overnight and a third began to show rapid growth after about 16 hours. These cultures were plated on TYE supplemented with ampicillin at a concentration of 20µg/ml, but no colonies were observed after prolonged incubation. However, it is possible that mutants possessing a weak lactamase activity were able to survive in the enrichment incubations for long enough to destroy all the ampicillin added, but were killed by the same initial concentration of the antibiotic on TYE plates because of the faster growth possible on this rich medium. These three cultures were discarded. The problem of the unwanted selection of antibiotic resistant mutants during enrichment procedures for non-growing cells can be reduced either by the use of different antibiotics for successive cycles of enrichment (e.g. of ampicillin followed by D-cycloserine) or by the use initially of a low, and subsequently of a higher, concentration of a single antibiotic. The latter method was adopted and the first round enrichments following the second and third mutageneses were carried out using an ampicillin concentration of 40µg/ml, whilst for the second rounds a concentration of 160µg/ml was used.

Kills of from 96% to 99% were observed during the ampicillin enrichments; these were considerably lower than the kills obtained under similar conditions using cultures which had not been subjected to mutagenesis (Chapter IV). However, many of the cells present at the start of the first round ampicillin incubations will have been

auxotrophs unable to grow in minimal media, but able to form colonies on TYE plates. The true percentage kills of wild type cells in the first round treatments will therefore have been higher than the values calculated from the viable cell counts presented in Table 5a. The growing up of the survivors from the first round enrichments through about ten generations in MM + fructose will have selected against such auxotrophs, although up to 0.1% of the population present at the start of the second round of enrichment could still have been auxotrophs.

None of the cultures in MM + fructose inoculated with cells recovered after ampicillin treatment showed any sign of growth after incubation overnight, and in each case the cells present had formed an aggregate. However, after from 36 to 48 hours all but two had reached saturation. Presumably a long lag phase was required for cell wall repair before growth and division were possible. Two of the cultures derived from the second round enrichment after the third mutagenesis (200µg/ml NG) failed to show any growth after three days, although both gave viable cell counts of about 5×10^6/ml on TYE plates. This suggested that only auxotrophic mutants unable to grow in MM + fructose were present in these incubations, although it is surprising that so many auxotrophs should be present at this stage.

Isolation of Glc^- Fru^+ Mutants

The single enriched culture derived from the first mutagenesis was spread on both TYE and M9 fructose plates, at a dilution giving from 200 to 250 colonies per 9cm diameter plate. Approximately 4 500 clones were replica plated onto M9 glucose plates from TYE and 2 500 from M9 fructose plates. A total of 22 clones failed to form replica colonies and were retested by streaking on both M9 glucose and M9 fructose plates. Seventeen clones proved to be Glc^+ Fru^+, four did

not grow on either M9 glucose or M9 fructose plates and one had the desired phenotype, Glc^- Fru^+. A single colony of this clone was isolated, retested and stored as strain SA402.

Two enriched cultures derived from the 100µg/ml NG mutagenesis and two from the 200µg/ml mutagenesis were spread on M9 fructose plates at a suitable dilution, grown up and replica plated onto M9 glucose plates. About 5 000 clones derived from each of the four enriched cultures were screened in this manner. Those clones which failed to form replica colonies were gridded on fresh M9 fructose plates, grown up and again replica plated onto M9 glucose. Of a total of 190 clones retested in this way, a few proved to be Glc^+; most showed only very poor growth on M9 glucose plates.

At this point the agar used to solidify minimal plates was changed from Difco 'Bacto Agar' to Oxoid 'Purified Agar'. This change was made because some background growth was obtained with strain B3724 on M9 plates prepared with 'Bacto Agar' without any added carbon/energy source; this problem was much less severe when 'Purified Agar' was used.

Fifty of the Glc^- Fru^+ clones were retested by streaking on M9 plates with various carbon sources, prepared using 'Purified Agar'. Mutant SA402 and the wild type strain, B3724, were also streaked out for comparison. Five clones failed to grow on M9 fructose plates, and were discarded. These strains presumably required some vitamin or other trace substance which was present in 'Bacto Agar' but absent from 'Purified Agar'. The remaining clones all showed good growth on M9 fructose plates and very poor growth, with isolated revertant colonies, on M9 glucose plates.

Screening for Temperature Sensitive Mutants

In order to determine whether any of the Glc^- Fru^+ mutants had

a temperature sensitive phenotype, duplicate sets of M9 glucose plates were incubated at the highest and lowest temperatures practicable. One set of plates was incubated at 32°C, whilst the other was left at room temperature (20°C to 25°C). None of the clones tested showed better growth at room temperature than at 32°C.

Utilisation of Xylose

Strain SA402 and the 45 Glc$^-$ Fru$^+$ clones derived from the second and third mutageneses all grew well on M9 xylose plates (Xyl$^+$ phenotype). This confirmed that these strains had not lost the ability to produce the isomerase, which was essential if they were to be used to test the feasibility of developing a new pathway for glucose catabolism as described in the Introduction.

Utilisation of Maltose

Strain B3724 readily utilises maltose as sole carbon/energy source. It was considered possible that some of the Glc$^-$ mutants would be able to utilise maltose (Mal$^+$), whilst others would have a Glc$^-$ Mal$^-$ phenotype. If strain B3724 possessed separate uptake systems for glucose and maltose, mutants deficient in glucose transport would be expected to show a Glc$^-$ Mal$^+$ phenotype. If intracellular maltose were split hydrolytically, forming two molecules of free glucose, mutants deficient in glucokinase would be Glc$^-$ Mal$^-$. However, maltose might alternatively be split to form glucose-1-phosphate and glucose, either directly or indirectly via the action of an amylomaltase and a phosphorylase acting on the polymeric material formed (Weismeyer & Cohn, 1960; Gottschalk, 1979). In this case mutants defective in glucose phosphorylation would probably be able to grow on maltose, using phospho-

glucomutase to convert glucose-1-phosphate to glucose-6-phosphate, provided that accumulation of non-metabolisable free glucose did not lead to inhibition of growth.

Both SA402 and the other 45 Glc$^-$ clones tested showed a similar degree of background growth and a similar reversion frequency on M9 glucose and M9 maltose plates. This result would be consistent with the presence of an extracellular maltase activity, or with an uptake system capable of transporting both glucose and maltose, followed by an intracellular maltase. However, insufficient mutants were screened for any firm conclusions to be drawn concerning the pathway of maltose catabolism in the wild type organism.

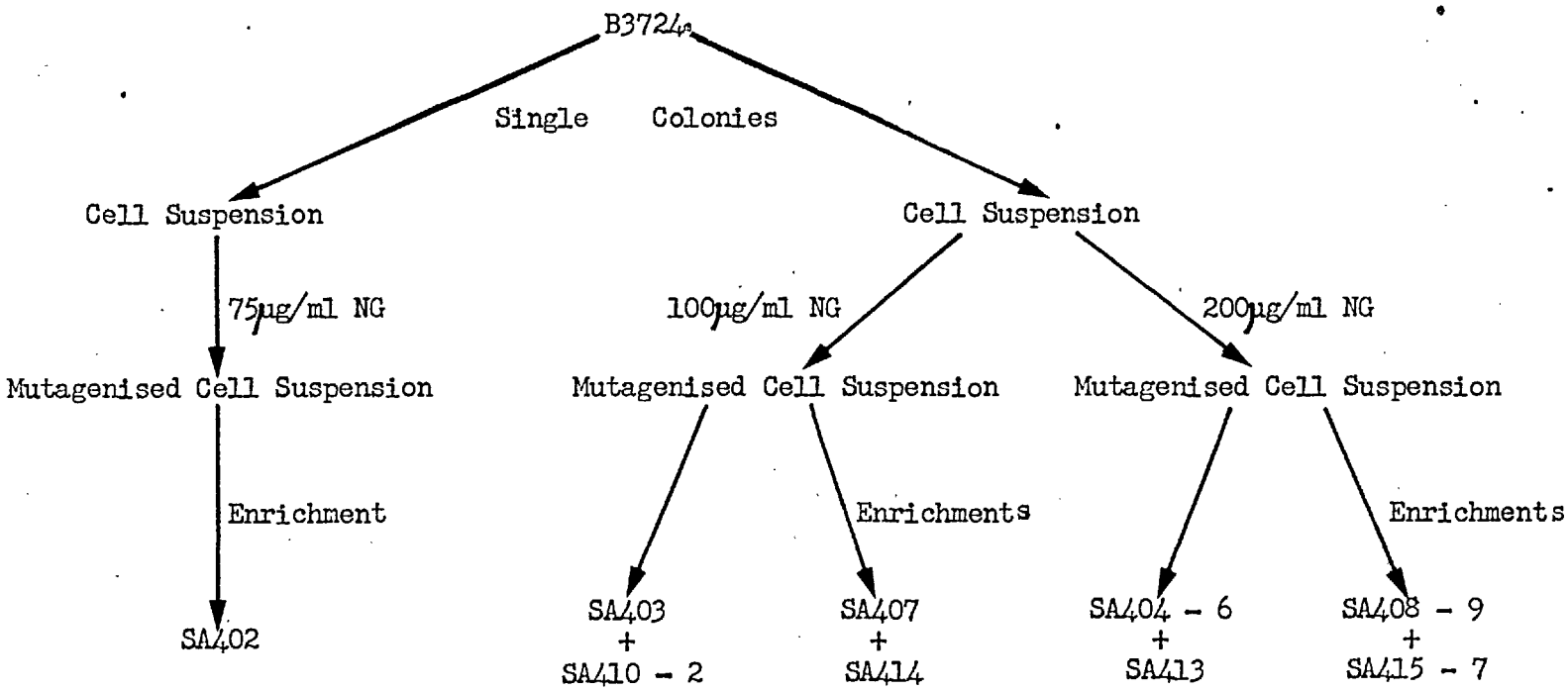
Selection of Strains SA403 to SA417

Figure 5b summarises the isolation of the Glc$^-$ Fru$^+$ mutants SA402 to SA417. The isolation of strain SA402 has been described above. Seven clones derived from the second and third mutageneses were selected for their low background growth on M9 glucose plates, as judged after incubation for three days. After the isolation and retesting of single colonies these clones were stored as strains SA403 to SA409. Since they were isolated from four separately enriched cultures (Figure 5b), these strains represent at least four independent mutational events. However, it is possible that SA408 and SA409, which were derived from a single enriched culture are two isolates of the same mutant strain; similarly any two, or all three, of SA404, SA405 and SA406 might be derived from the same mutational event.

Subsequently eight more clones, which had initially shown some background growth on M9 glucose plates, but which showed none after incubation for a further six weeks at room temperature, were selected and stored as strains SA410 to SA417. Figure 5b shows from which

Figure 5b

Isolation of Strains SA402 to SA417



enriched culture each of these strains was isolated.

Utilisation of Glucose-6-phosphate

It was hoped that it would be possible to use ability to grow on plates containing glucose-6-phosphate as sole carbon/energy source as a phenotypic test to distinguish between mutants unable to form glucose-6-phosphate and mutants blocked at some later stage in glucose metabolism. However, the wild type, strain B3724, proved to be able to grow only exceedingly slowly on M9 plates containing 5mM glucose-6-phosphate. Presumably Arthrobacter, or at least the Reynolds strains used in this work, lack an uptake system for glucose-6-phosphate analogous to the inducible hexose phosphate transport system found in E. coli (Winkler, 1966).

It might have been possible to isolate mutants of strain B3724 able to utilise glucose-6-phosphate. However, it would have been necessary to perform experiments to determine whether glucose-6-phosphate was taken up intact or dephosphorylated extracellularly by such mutants, since strains catabolising glucose-6-phosphate by the latter route would have been of no use in the characterisation of lesions in glucose metabolism. Furthermore, since there are no proven techniques for obtaining recombination between strains of Arthrobacter, it would have been necessary to repeat the isolation of Glc^- Fru^+ mutants using such a mutant as the starting strain. It was therefore considered that it would be easier to characterise mutants unable to form glucose-6-phosphate biochemically, by means of enzyme assays, as described in Chapter VI, than to perform genetic manipulations to make a phenotypic test possible.

Growth on EMB Glucose Plates

Eosin/Methylene Blue (EMB) plates have been widely used in the isolation of mutants unable to utilise various carbohydrates. EMB plates contain, in addition to the two dyes, both peptone and the carbohydrate to be tested. Growth on peptone alone does not normally lead to acidification of the environment, whilst the utilisation of carbohydrates as carbon/energy source generally does lead to acidification, due either to the excretion of acids as products of fermentation or to the uptake of ammonia as nitrogen source. Acid-producing strains form dark colonies with a green sheen, whilst colonies which do not produce acid remain pale on EMB plates. The growth of B3724 and three of the Glc^- mutants, SA402, SA407 and SA409 on EMB glucose plates was tested to determine whether the use of these plates would provide a suitable means of detecting Glc^- mutants after any further mutageneses.

Although when streaked on EMB glucose plates strain B3724 gave the expected dark colonies, whilst the three mutants each formed pale pink colonies, growth on these plates was very slow. Furthermore, when EMB glucose plates were spread with dilutions of cultures of B3724 and SA402, no colonies were observed after incubation for several days, although normal titres were obtained on TYE plates spread with the same cell suspensions. It therefore appears probable that one or both of the dyes used is toxic to Arthrobacter cells. Since no further isolation of Glc^- mutants was in fact undertaken, the use of other types of indicator plate was not investigated.

Utilisation of Gluconate

Strain B3724 readily utilises gluconate as sole carbon/energy

source. When mutants SA402 to SA417 were tested for ability to use gluconate, SA402, SA404, SA407 and SA409 all showed growth similar to that of the wild type strain ($Glnt^+$ phenotype) on M9 gluconate plates. The remaining strains all showed very poor growth on gluconate ($Glnt^-$), and gave rise to revertants showing approximately normal growth. Since mutants of Glc^- $Glnt^-$ phenotype were derived from four separate enriched cultures, it seemed more probable that a single lesion was responsible for the failure of these mutants to grow on both carbon sources than that several independent double mutants had been isolated. Five of the Glc^- $Glnt^-$ mutants, SA411, SA412, SA414, SA415 and SA417, were investigated further. Revertants to either a Glc^+ or a $Glnt^+$ phenotype were selected on M9 plates and tested for growth on both carbon sources by replica plating. The results obtained, together with the estimated reversion frequency of each strain to either phenotype, are presented in Table 5b.

In each case the frequency of reversion to a Glc^+ phenotype was greater than that to $Glnt^+$ by at least tenfold. Moreover, out of 226 Glc^+ revertants tested, only ten proved to be $Glnt^+$, whilst all 98 $Glnt^+$ revertants tested were also Glc^+. This suggested that a single lesion was indeed responsible for both the Glc^- and $Glnt^-$ phenotypes of these mutants, and that whilst further mutation could lead to a recovery of the ability to utilise glucose, only reversion of the locus containing the original lesion led to a recovery of the $Glnt^+$ phenotype. This hypothesis was further supported by the fact that whereas $Glnt^+$ revertants selected from these strains had a wild type Glc^+ phenotype, those Glc^+ revertants which were $Glnt^-$ grew less rapidly on M9 glucose plates than the wild type, and gave rise to further revertants of nearly wild type growth rate.

Table 5b

Reversion of Glc^- $Glnt^-$ Mutants

Strain	Reversion to Glc^+				Reversion to $Glnt^+$			
	Frequency	Total Tested	$Glnt^+$	$Glnt^-$	Frequency	Total Tested	Glc^+	Glc^-
SA411	4×10^{-6}	46	0	46	4×10^{-7}	14	14	0
SA412	1×10^{-5}	46	1	45	1×10^{-8}	27	27	0
SA414	3×10^{-6}	47	1	46	1×10^{-7}	24	24	0
SA415	2×10^{-6}	45	3	42	7×10^{-8}	10	10	0
SA417	2×10^{-6}	42	5	37	-	23	23	0

The frequencies of reversion of the mutants to growth on glucose (Glc^+) and gluconate ($Glnt^+$) were estimated by plating dilutions of cultures of each strain in MM' + fructose on M9 glucose, M9 fructose and M9 gluconate plates.

Glucose Toxicity

In order to prepare cell-free extracts suitable for the assay of enzymes involved in glucose catabolism, as described in Chapter VI, attempts were made to grow various Glc^- mutants in the presence of glucose, using MM' + 2% (w/v) casein hydrolysate supplemented with glucose to 0.2% (w/v). It was found that, whereas strains SA402 and SA409 (Glc^- $Glnt^+$) grew equally well on casein hydrolysate alone or in the presence of glucose, the growth of strains SA411 and SA417 (Glc^- $Glnt^-$) was severely inhibited by the addition of glucose. The ability of all the Glc^- strains to grow in the presence of glucose was tested, by streaking on TYE plates and TYE plates which had been supplemented with glucose to 0.2% (w/v). Strains SA402 to SA407 and SA409 showed no inhibition of growth by glucose, whilst strains SA408 and SA410 to SA417 all showed inhibition, although the inhibition only became apparent after prolonged incubation. After incubation for 24 hours, the growth of those strains which were inhibited by glucose did not appear different from that of the wild type on TYE + glucose plates; however, after incubation for from two to three days, faster growing colonies were visible against a background of weak confluent growth.

The finding that the Glc^- $Glnt^-$ class of mutants were inhibited by glucose suggested that they might accumulate some intermediate which could not be further metabolised, leading to glucose toxicity. This is discussed further in the next chapter.

Chapter VI

Hexose Metabolism and the Lesions of Glc^- Mutants

The work described in this chapter was undertaken with two aims: the elucidation of the normal pathways of glucose and fructose metabolism in Arthrobacter strain B3724, and the characterisation of the enzyme deficiencies leading to the phenotypes of the Glc^- Fru^+ mutants described in Chapter V.

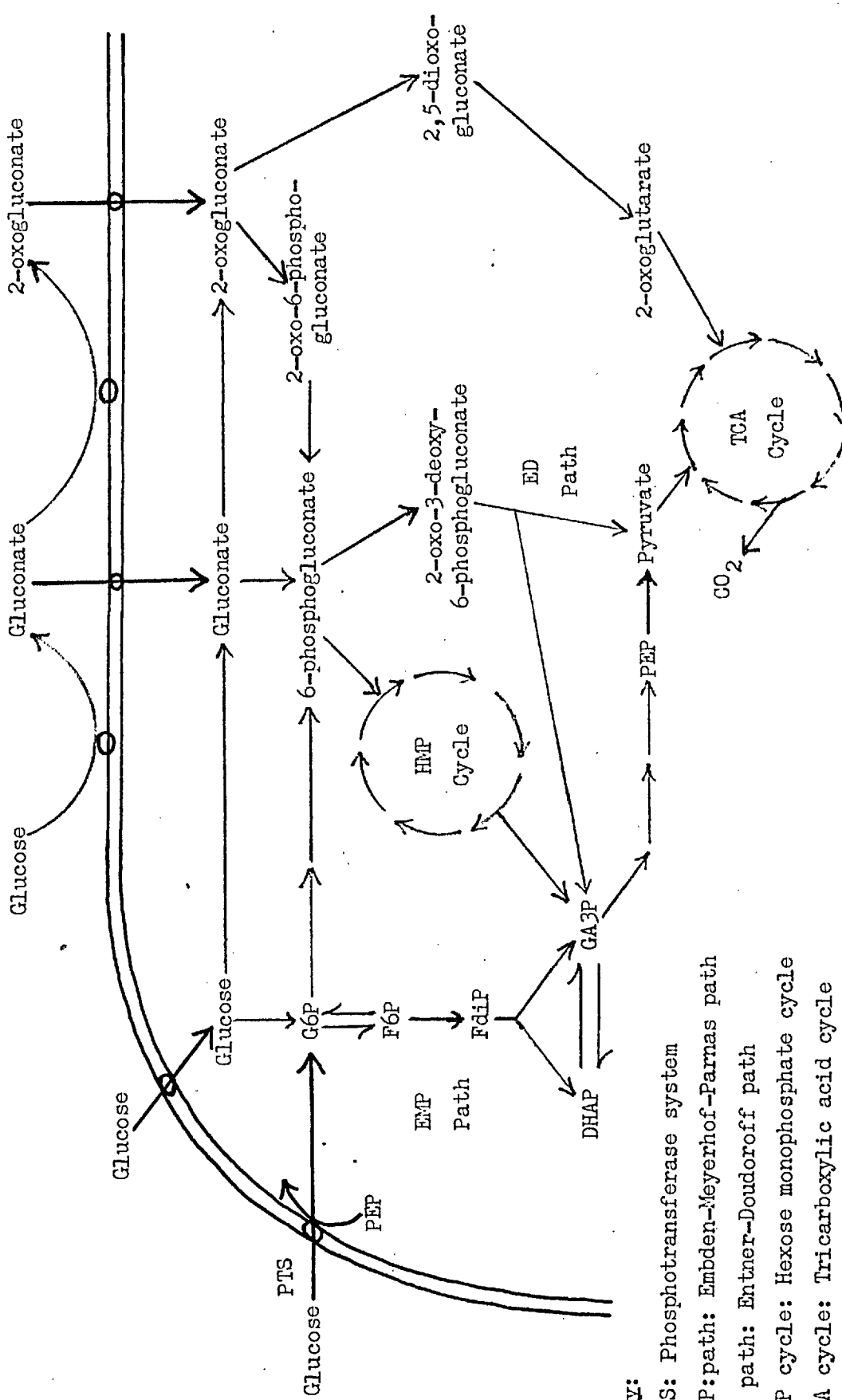
A knowledge of the normal peripheral stages in the catabolism of glucose and fructose would enable an assessment to be made of the feasibility of obtaining mutants suitable for use in the evolution of a new pathway for glucose utilisation involving the glucose isomerase activity. It is believed that the Arthrobacter D-xylose isomerase, which is responsible for the glucose isomerase activity, has an intracellular location, in common with other bacterial pentose isomerases. Supportive evidence for the location of the enzyme within the cellular permeability barrier, rather than at some periplasmic site, was provided by the observation that at physiological temperatures the enzyme is not available to extracellular substrate in whole cell suspensions (Chapter III). In order for a strain to be a suitable tool for the application of selective pressure to the isomerase by glucose limited continuous culture, it would require the following properties:

i) the ability to transport free glucose into the cell, and probably the ability to accumulate free intracellular glucose by an active transport system,

ii) an inability to catabolise glucose by other pathways,

iii) an inability to phosphorylate glucose (since the accumulation of non-catabolisable phosphorylated intermediates would otherwise be

Figure 6a Pathways of Glucose Metabolism



Key:

PTS: Phosphotransferase system

EMP:path: Embden-Meyerhof-Parnas path

ED path: Entner-Doudoroff path

HMP cycle: Hexose monophosphate cycle

TCA cycle: Tricarboxylic acid cycle

G6P and F6P: glucose- and fructose-6-phosphate

PEP: phosphoenolpyruvate; DHAP: dihydroxyacetone phosphate

GA3P: glyceraldehyde-3-phosphate; FdiP: fructose-1,6-diphosphate

likely to lead to inhibition of growth in the presence of glucose), and iv) the ability to metabolise intracellular fructose formed from glucose by the isomerase.

Figure 6a summarises pathways for glucose uptake and entry into central metabolism which have been described in various prokaryotic organisms.

Glucose Transport

Romano et al. (1970) surveyed a wide range of bacterial species for the occurrence of phosphoenolpyruvate:sugar phosphotransferase systems (PTS) for the concomitant uptake and phosphorylation of glucose. The method which they used involved the incubation of cell lysates with [^{14}C]-2-deoxyglucose and phosphoenolpyruvate (PEP), followed by determination of the fraction of the radioactivity present which was incorporated into phosphorylated intermediates. They found that a PTS for glucose uptake was generally absent from the strictly aerobic species tested, including Arthrobacter globiformis. A. globiformis is the species which the Reynolds strains B3724 to B3728 most closely resemble (Dr D. Byrom, personal communication).

Krulwich and Ensign (1969) investigated glucose uptake by Arthrobacter crystallopoietes, a strain which is probably a subjective synonym for A. globiformis (Buchanan & Gibbons, 1974). They found that there was an inducible system for energy dependent glucose uptake, in addition to a constitutive passive system for glucose exit. Krulwich and his co-workers have also published the results of a number of studies on sugar uptake by a strain designated Arthrobacter pyridinolis. However, this strain is unable to utilise most sugars, including glucose, as sole carbon/energy source, which is in marked contrast to the wide metabolic capabilities of typical members of the

Table 6a

PEP:Glucose Phosphotransferase Activities of E. coli and Arthrobacter

Organism	PEP:glucose Phosphotransferase		Glucokinase
	Protein in Incubation (mg)	Analogue Phosphorylation	Specific Activity
E. coli K *	14.3	69%	0.021
E. coli K	13.9	95%	0.017
A. globiformis *	10.0	0.9%	0.038
Strain B3725	9.2	0.9%	0.070

* Results from Romano et al. (1970).

The percentage analogue phosphorylation indicates the amount of radioactivity associated with phosphorylated material, expressed as a percentage of the total radioactivity recovered. The specific activity of glucokinase is expressed as μmol G6P formed/min per mg total protein.

The phosphotransferase assay was performed as described by Romano et al. (1970), except that [^{14}C]-α-methylglucoside was used in place of [^{14}C]-2-deoxyglucose and, for strain B3725, lysozyme rupture was used in place of freezing and thawing to decryptify the activities and the two assays were performed at 30°C instead of 37°C. The cells were grown in medium A, supplemented with 0.5% (w/v) glucose. Decryptified cell suspension containing about 10mg protein was incubated for one hour in 3ml assay mix, containing 1.67mM [U-^{14}C]-α-methylglucoside (specific activity 5 x 10^4 dpm/μmol), 1.5mM-phosphoenolpyruvate and 3mM-$MgCl_2$ in 30mM-KH_2PO_4.KOH pH 7.0. After removal of the cells, 0.5ml of the supernatant was applied to a column of DE-Acidite FF (Permutit; 100 to 200 mesh; bed volume 2ml). The column was washed with 12 x 0.5ml water to remove free α-methylglucoside and α-methylglucoside-6-phosphate was then eluted with 12 x 0.5ml 500mM-sodium formate/200mM-formic acid. The radioactivity in each fraction was determined and corrected for quenching by Mr W. Routlidge at the laboratories of I.C.I. Agricultural Division, Billingham.

genus Arthrobacter. It is therefore uncertain to what extent their findings with A. pyridinolis are likely to be applicable to other species.

No glucose dependent PTS activity was detected in cells of strain B3724 grown on glucose as sole carbon/energy source, using the coupled assay for toluene treated cells described in Chapter II. (The detection of PTS uptake systems for other substrates using this assay, described later in this chapter, confirmed that this method of assay is generally applicable to Arthrobacter.) In addition, strain B3725, which is closely related to strain B3724, was tested for glucose linked PTS activity by a method essentially the same as that used by Romano et al. (1970). In Table 6a the results obtained for B3725 are compared both with the results of Romano et al. for A. globiformis and with results obtained for E. coli, an organism known to use a PTS for glucose uptake (Saier, 1977). The results for the two Arthrobacter strains were essentially identical, suggesting the absence of a PTS for glucose uptake in both. It could be argued that a negative result in this assay is not meaningful, due to the possibility either of the destruction of the phosphotransferase system during the decryptification treatment or of further metabolism of the phosphorylated glucose analogue, leading eventually to non-phosphorylated intermediates, despite the use of supposedly non-metabolisable analogues. Moreover, the use of this assay involves the implicit assumption that 2-deoxyglucose and α-methylglucoside are substrates for all PEP:glucose phosphotransferase systems. However, other results presented by Romano et al. (1970) argue against these interpretations. They demonstrated that the uptake of [^{14}C]-2-deoxyglucose by whole cells of A. globiformis was inhibited in a competitive manner by 6-deoxyglucose, which lacks the hydroxyl group normally phosphorylated by PEP:glucose

phosphotransferase systems. In contrast, the uptake of 2-deoxy-glucose was not inhibited significantly by 6-deoxyglucose in either E. coli or S. typhimurium, organisms in which a PTS for glucose uptake has been thoroughly established, both by enzyme assays and by genetic evidence (Saier, 1977), and which show PTS activity in the assay described above.

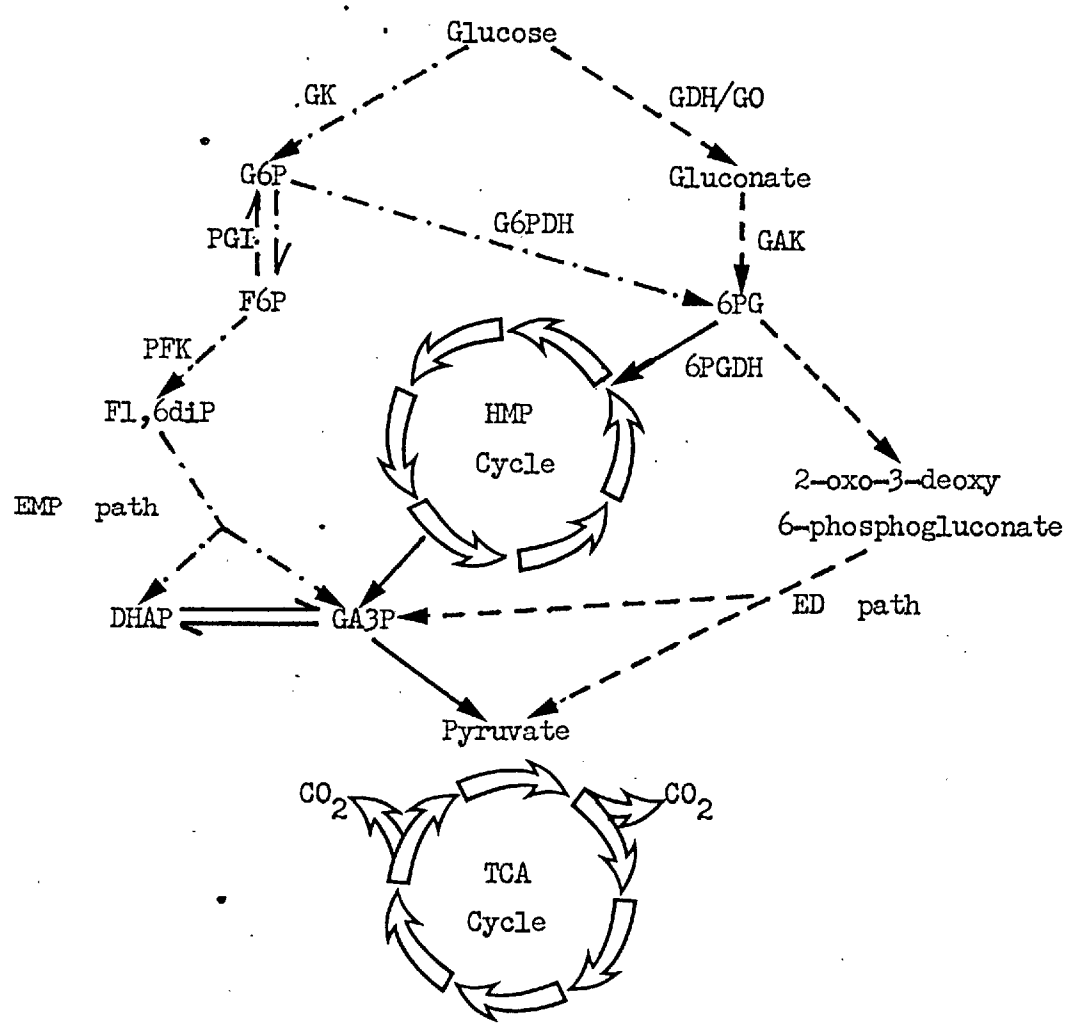
It is therefore considered that the Reynolds Arthrobacter strains do not use a PTS uptake system for glucose. Although it has not been confirmed experimentally, it is probable that these strains possess an energy dependent system for glucose accumulation similar to that of A. crystallopoietes.

Metabolism of Intracellular Glucose

Morris (1960) made a thorough study of the metabolic pathways of A. globiformis. He studied glucose metabolism both by analysis of the enzymic activities present in cells grown on various substrates and by tracing the fate of isotopic label incorporated at specific positions into glucose molecules. He found that the same level of glucokinase activity was present in cells grown on glucose or gluconate, whilst gluconate kinase was detectable only following growth in the presence of gluconate. He concluded that glucose was not metabolised by direct oxidation to gluconate, but by phosphorylation to glucose-6-phosphate. Isotopic tracer studies showed that, after phosphorylation, about 65% of the glucose was converted to pyruvate by means of the Embden-Meyerhof-Parnas (EMP) pathway and the remainder by the hexose monophosphate (HMP) pathway, the Entner-Doudoroff (ED) path not being operational during growth on glucose.

Zagallo and Wang (1962) compared the catabolism of glucose and gluconate by five species of Arthrobacter, by studying the fate of

Figure 6b: Pathways of Glucose Catabolism in Arthrobacter



Key:

— · — · — · Pathways functioning in group one Arthrobacter species

— — — — Pathways functioning in group two Arthrobacter species

——— Pathways common to species in both groups

GK	Glucokinase (EC 2.7.1.2)
GDH	Glucose dehydrogenase (EC 1.1.1.47)
GO	Glucose oxidase (EC 1.1.3.4)
PGI	Phosphoglucose isomerase (EC 5.3.1.9)
PFK	Phosphofructokinase (EC 2.7.1.11)
G6PDH	Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
GAK	Gluconate kinase (EC 2.7.1.12)
6PGDH	Decarboxylating 6-phosphogluconate dehydrogenase (EC 1.1.1.44)
EMP path	Embden-Meyerhof-Parnas pathway
ED path	Entner-Doudoroff pathway
HMP cycle	Hexose monophosphate cycle
TCA cycle	Tricarboxylic acid cycle

^{14}C derived from substrate molecules labelled at specific positions. They were able to divide the five species into two groups on the basis of the pathways of glucose metabolism operating in each. Figure 6b shows in outline the pathways by which glucose is catabolised in each group.

The first group included A. globiformis and A. ureafaciens, which do not oxidise glucose directly to gluconate, but phosphorylate it and then catabolise it mainly by the EMP path and, to a lesser extent, by the HMP pathway. According to Krulwich and Ensign (1969), A. crystallopoietes belongs to this group, as would be expected if it is indeed a sub-species of A. globiformis.

The second group, including A. simplex, A. pascens and A. atrocyaneus, showed nearly identical patterns of isotope distribution from glucose and from gluconate. The EMP pathway is not active in this group and glucose is oxidised directly to gluconate, which is phosphorylated and catabolised by the ED and HMP paths. This pattern of glucose metabolism resembles that shown by members of the genus Pseudomonas. The mechanism and site of glucose oxidation in these species of Arthrobacter is not known. Pseudomonas aeruginosa possesses a membrane-bound 'glucose dehydrogenase' system, which acts on extracellular (or periplasmic) glucose. The gluconate formed may then either enter the cell and be phosphorylated, or undergo further oxidation to formogluconate before uptake and phosphorylation (Ng & Dawes, 1973; Roberts et al., 1973; Whiting et al., 1976). This membrane-bound 'glucose dehydrogenase' can be coupled to 2,6-dichlorophenolindophenol (DPI) and is probably in fact a flavoprotein glucose oxidase (EC 1.1.3.4). However, Acetomonas (Gluconobacter) and Acetobacter species possess intracellular NADP-linked glucose dehydrogenase activities (EC 1.1.1.47), in addition to flavoprotein

activities which can be coupled to DPI (Doelle, 1975). Both of these mechanisms of glucose oxidation are included in Figure 6a.

The finding that these members of the genus Arthrobacter can be divided into two classes, which use fundamentally different catabolic pathways for a substrate so ubiquitous as glucose, emphasises the breadth of the taxon and its 'artificial' nature, already mentioned in the Introduction to this thesis. It appears probable that the genus, which is at present defined mainly on morphological grounds (Buchanan & Gibbons, 1974), corresponds to at least two 'natural' taxa of generic level.

The metabolism of glucose in strain B3724 was investigated by determining the specific activities of various enzymes in cell-free extracts prepared by lysozyme rupture of cells grown in MM to which various carbon sources had been added. The cells used were in late exponential or early stationary phase; therefore significance should only be ascribed to large differences in specific activities between cells grown on different substrates.

In Arthrobacter strains belonging to the first group defined by Zagallo and Wang (1962), the initial step in glucose catabolism is the phosphorylation of glucose by glucokinase to form glucose-6-phosphate. In strains belonging to their second group, 6-phosphogluconate, formed by the action of gluconate kinase, is the first phosphorylated intermediate in the catabolism of glucose. In order to determine to which of these groups the Reynolds strains belong, the specific activities of these two kinases were determined in cell-free extracts prepared from B3724 cells grown on a variety of substrates. The results obtained are presented in Table 6b.

Glucokinase activity was present at a basal level after growth on all the carbon sources tested, but showed an eight to ten-fold

Table 6b

Glucokinase and Gluconate Kinase Activities of Strain B3724

Carbon Source (w/v)	Glucokinase (mU/mg)	Gluconate Kinase (mU/mg)
2% casein hydrolysate	5 - 11	0
0.5% glucose	82 - 99	0
0.5% sodium gluconate	9 - 17	52 - 109
0.5% maltose	79 - 125	NT
0.5% sucrose	81 - 86	0
0.5% fructose	10 - 25	NT
0.5% xylose	11 - 12	NT

The figures given represent the minimum and maximum values obtained for separately prepared cell-free extracts. The specific activities are expressed as nmol product formed/minute per mg soluble protein.

'0' indicates that no activity was detected (i.e. activity less than 0.5mU/mg).

'NT' signifies that the extracts were not tested for activity.

The assays used and the method of preparation of the cell-free extracts are described in Chapter II.

increase in specific activity following growth on glucose, maltose or sucrose. In contrast, gluconate kinase activity was present at a detectable level only after growth in the presence of gluconate. It was therefore concluded that strain B3724 belongs to the first class of Zagallo and Wang, typified by A. globiformis, in which the direct oxidation of glucose to gluconate does not occur. The observation that higher levels of glucokinase are induced by growth on glucose contrasts with the situation found in A. globiformis, where Morris (1960) found similar levels of glucokinase activity after growth on glucose or gluconate.

Two paths are available for the further metabolism of glucose-6-phosphate formed by glucokinase (Figure 6a). Reversible conversion to fructose-6-phosphate may be followed by a second phosphorylation, forming fructose-1,6-diphosphate, which is then catabolised to pyruvate by the Embden-Meyerhof-Parnas (EMP) pathway; alternatively, glucose-6-phosphate may be oxidised to 6-phosphogluconate, which may then be catabolised by either of the hexose monophosphate (HMP) and Entner-Doudoroff (ED) pathways. The enzymes required for the first alternative are phosphoglucose isomerase and phosphofructokinase, whilst the second requires glucose-6-phosphate dehydrogenase and a hydrolase, which catalyses the opening of the lactone formed by the dehydrogenase. Table 6c contains results obtained for the specific activities of the isomerase, kinase and dehydrogenase in cell-free extracts of strain B3724 prepared after growth on various substrates.

All three activities were present in extracts of cells grown on glucose, suggesting that both paths for the catabolism of glucose-6-phosphate are functional in strain B3724. Both Morris (1960) and Zagallo & Wang (1962) found that the HMP path makes a smaller contribution than the EMP path to the overall rate of glucose catabolism in

Table 6c

G6PDH, PGI, PFK and PGM Activities of Strain B3724

Carbon Source (w/v)	G6PDH (mU/mg)	PGI (mU/mg)	PFK (mU/mg)	PGM (mU/mg)
2% casein hydrolysate	0 - 0.3	9 - 21	76	12 - 50
0.5% glucose	6	66	90 - 99	68 - 88
0.5% fructose	5	103	385	94 - 103
0.5% sucrose	NT	NT	148 - 238	97
0.6% sodium gluconate	8	75	NT	108
0.5% xylose	0.5 - 2	NT	NT	73

G6PDH: glucose-6-phosphate dehydrogenase

PGI: phosphoglucose isomerase

PFK: phosphofructokinase

PGM: phosphoglucomutase

Where two values are given, they represent the minimum and maximum specific activities observed for separately prepared cell-free extracts.

'NT' signifies that the extracts were not tested for activity.

The details of the assays used and of the preparation of the cell-free extracts are given in Chapter II.

The specific activities are expressed as nmol product formed/minute per mg soluble protein.

species of Arthrobacter belonging to the first group of the latter authors, whilst the ED pathway does not function during the utilisation of glucose. The observed specific activity of glucose-6-phosphate dehydrogenase in extracts of strain B3724 was much lower than that of either phosphoglucose isomerase or phosphofructokinase; but it is unlikely that the specific activities obtained in the assays were an accurate indication of the relative activities of these enzymes in vivo. It is not, therefore, possible to draw any conclusions from these results concerning the relative rates of glucose-6-phosphate catabolism by the two pathways. In order to determine the relative usage of the HMP and EMP paths, and to confirm that the ED path plays no part in the metabolism of glucose by strain B3724, it would have been necessary to perform radiorespirometric studies similar to those of Zagallo and Wang (1962). Such work would only have been justified if study of the metabolism of Arthrobacter had been the primary objective of the work described in this thesis.

The Lesions of Glc^- Fru^+ Mutants

As described in Chapter V, it was possible to divide the Glc^- Fru^+ mutants that were obtained into two classes, on phenotypic grounds. Two mutants from each phenotypic class, isolated after separate mutagenic treatments, were chosen for further investigation. SA402 and SA409 belong to the class of mutants which although unable to utilise glucose as sole carbon/energy source are not inhibited by the presence of glucose during growth on other substrates. SA411 and SA417 belong to the second class, the members of which are not able to use either glucose or gluconate as carbon/energy source, and are inhibited by glucose during growth on rich media.

As a first step towards identifying the lesions responsible for

Table 6d

Glucokinase, PGI, PFK and G6PDH Activities of Glc⁻ Mutants

Strain	Carbon Source (w/v)	Glucokinase (mU/mg)	PGI (mU/mg)	PFK (mU/mg)
B3724	2% CH + 0.2% glucose	30 - 68	65	NT
SA402	2% CH + 0.2% glucose	0	20	NT
SA409	2% CH + 0.2% glucose	0 - 0.5 *	30 - 44	NT
B3724	2% casein hydrolysate	5 - 11	9 - 21	76
SA402	2% casein hydrolysate	0	NT	44
SA409	2% casein hydrolysate	0	18	70 - 86
SA411	2% casein hydrolysate	7 - 8	11 - 30	75
SA417	2% casein hydrolysate	5	14	89

Strain	Carbon Source (w/v)	G6PDH (mU/mg)
B3724	0.5% fructose	5
SA402	0.5% fructose	9 - 10
SA409	0.5% fructose	5
SA411	0.5% fructose	3
SA417	0.5% fructose	6

* This amount of activity is only marginally significantly above background.

PGI: phosphoglucose isomerase

PFK: phosphofructokinase

G6PDH: glucose-6-phosphate dehydrogenase

CH: casein hydrolysate

'NT' signifies that the extracts were not tested for activity.

The specific activities are expressed in terms of nmol product formed/minute per mg soluble protein. Details of the assays used and of the preparation of the cell-free extracts are given in Chapter II.

the phenotypes of these mutants, cell-free extracts prepared after growth on suitable substrates were assayed for the enzymes responsible for the initial stages of glucose metabolism in the wild type strain. The results obtained are presented in Table 6d. All four mutants showed essentially normal levels of phosphoglucose isomerase, phosphofructokinase and glucose-6-phosphate dehydrogenase (although strain SA402 consistently gave slightly higher levels of glucose-6-phosphate dehydrogenase activity than did either B3724 or the other mutants).

Strains SA402 and SA409 did not possess significant levels of glucokinase activity, whilst both SA411 and SA417 possessed the same basal level as strain B3724. It is presumed that the failure of strains SA402 and SA409 to synthesise any active glucokinase is responsible for their failure to utilise glucose (i.e. that their glk genotype is responsible for their Glc^- phenotype). The inability of these glk mutants, which possess normal levels of phosphoglucose isomerase (pgi^+), phosphofructokinase (pfk^+) and glucose-6-phosphate dehydrogenase (zwf^+), to utilise glucose provided further evidence that the wild type strain, B3724, does not posses a PEP:glucose phosphotransferase system.

Mutants SA411 and SA417 appeared to synthesise all four enzymes to approximately wild type levels. Although the assays used might not have detected mutants producing enzymes of decreased substrate affinity ('K_m mutants'), which could have greatly reduced activities under in vivo conditions, it is not probable that such mutations would result in a total Glc^- phenotype. Similarly, mutants defective in glucose uptake would be expected to show at least some degree of 'leakiness' at high glucose concentrations, rather than the inhibition by glucose exhibited by strains SA411 and SA417. Mutants which are inhibited by compounds which can be utilised by the parental strain

Table 6e

Gluconate Kinase and 6-phosphogluconate Dehydrogenase Activities

Strain	Carbon Source (w/v)	6PGDH (mU/mg)	GAK (mU/mg)
B3724	0.6% sodium gluconate	250	105
SA402	0.6% sodium gluconate	285	105 - 125
SA409	0.6% sodium gluconate	340 - 360	135
B3724	2% CH + 0.25% sodium gluconate	130	8 - 9
SA411	2% CH + 0.25% sodium gluconate	9 - 11 *	-
SA417	2% CH + 0.25% sodium gluconate	7 - 9 *	-
B3724	2% casein hydrolysate	37	0
SA402	2% casein hydrolysate	45	0
SA409	2% casein hydrolysate	45 - 50	0
SA411	2% casein hydrolysate	3	NT
SA417	2% casein hydrolysate	2	NT
B3724	0.5% glucose	140 - 180	0
B3724	2% CH + 0.2% glucose	122	0
SA402	2% CH + 0.2% glucose	45	6 - 7
SA409	2% CH + 0.2% glucose	60 - 80	6 - 7

6PGDH: 6-phosphogluconate dehydrogenase (EC 1.1.1.43/44)

GAK: gluconate kinase CH: casein hydrolysate

'NT' signifies that the extract was not tested for activity.

* These figures represent rates obtained without the addition of substrate to the assay; the addition of 6-phosphogluconate did not lead to any increase in the rate observed. All other extracts gave no background rate before the addition of the substrate.

Where two figures are given, these represent the minimum and maximum specific activities obtained for separately prepared extracts. Details of the assays and of the preparation of the cell-free extracts are given in Chapter II. The specific activities are expressed as nmol product formed/minute per mg soluble protein.

are often found to be blocked in the catabolism of some phosphorylated intermediate, which therefore accumulates to toxic levels in the cell. It was considered improbable that strains SA411 and SA417 were blocked in the metabolism of fructose-1,6-diphosphate, since they have a Fru^+ phenotype. Moreover, the fact that they are unable to utilise gluconate ($Glnt^-$) and that $Glnt^+$ revertants were found also to be Glc^+ (Chapter V) implied that they carried a lesion in some step common to the catabolism of both glucose and gluconate. It therefore seemed probable that these mutants were blocked in the catabolism of 6-phosphogluconate. Table 6e presents the results of assays for gluconate kinase and 6-phosphogluconate dehydrogenase activities in strain B3724 and the four mutants.

The apparent levels of 6-phosphogluconate dehydrogenase activity in the cell-free extracts prepared from strains SA411 and SA417 were less than 10% of those observed in extracts of strain B3724, although in the case of both mutants some increase in activity was still observed following growth in the presence of gluconate. As described in the legend to Table 6e, the dehydrogenase activity present in the extracts of these strains prepared from cells grown in the presence of gluconate appeared already to be saturated by endogenous substrate. This was investigated further, by adding 100µl of the cell extract from strain SA411 grown in the presence of gluconate to a cuvette containing assay mixture and cell extract from strain B3724 grown on gluconate. This resulted in the same rate of $NADP^+$ reduction as that observed following the addition of authentic 6-phosphogluconate to a similar cuvette. This result strongly suggested that SA411 and SA417 do indeed accumulate intracellular 6-phosphogluconate to substantial levels when grown in the presence of gluconate. The presence of this endogenous substrate made it impossible to assay these cell-free

extracts for gluconate kinase activity using the coupled assay system.

There are several possible explanations for the reduced level of 6-phosphogluconate dehydrogenase activity observed in the mutants SA411 and SA417. These strains might synthesise normal amounts of a mutant enzyme of reduced specific activity due to a structural gene mutation, or reduced amounts of the normal enzyme due to a control mutation (e.g. a 'down-promoter' or attenuator mutation). In either of these cases it is necessary to postulate the isolation of two mutants with very similar residual activities following separate mutagenic treatments. This would appear a priori to be an improbable coincidence, since structural gene mutations resulting in a completely inactive gene product and control mutations totally preventing gene expression must surely arise at a much greater frequency than mutations which result in any particular level of residual activity. However, it is possible to envisage that mutations resulting in total 6-phosphogluconate dehydrogenase deficiency might be lethal, due to the accumulation of 6-phosphogluconate to toxic levels during growth on all substrates; it might then be the case that only mutants with a narrow range of residual dehydrogenase activities would both be viable and exhibit a Glc$^-$ phenotype. It should be noted that the growth of strains SA411 and SA417 on fructose is slower than that of B3724 and the mutants SA402 and SA409 (glk), and that faster growing revertants may be detected on M9 fructose plates. This suggests that some accumulation of 6-phosphogluconate occurrs during growth on fructose, presumably due to the action of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, which lends support to the suggestion that mutations leading to the complete abolition of 6-phosphogluconate dehydrogenase activity would result in a Fru$^-$ phenotype, even if they were not lethal.

An alternative explanation would be to postulate that the wild type strain possesses two different enzymes with 6-phosphogluconate dehydrogenase activity, and that one of these, which accounts for 90% of the total activity present in strain B3724 is entirely absent or inactive in mutants SA411 and SA417. For example, in addition to the normal decarboxylating enzyme forming ribulose-5-phosphate (EC 1.1.1.44), strain B3724 might posses a low level of 6-phosphogluconate-2-dehydrogenase activity (EC 1.1.1.43), forming 2-oxo-6-phosphogluconate. This would explain the existence of similar levels of activity in the two mutants examined.

Whichever (if either) of the above explanations is correct, the accumulation of intracellular 6-phosphogluconate to high levels due to a greatly reduced 6-phosphogluconate dehydrogenase activity provides a reason both for the inability of these strains to utilise glucose or gluconate and for the inhibition of their growth in the presence of glucose. The observation that all Glnt$^+$ revertants isolated from SA411 and SA417 (and from SA412, SA414 and SA415; Chapter V) were also Glc$^+$ is readily explained. Reversion to the synthesis of nearly normal levels of dehydrogenase activity is presumably the only way in which these mutants can regain the ability to utilise gluconate; such reversion would necessarily result also in the recovery of the ability to utilise glucose. It is also possible to suggest an explanation for the greater frequency with which Glc$^+$ revertants arose from these strains than Glnt$^+$ revertants, and for the fact that the majority of such Glc$^+$ revertants remained Glnt$^-$. Mutations resulting in the loss of glucose-6-phosphate dehydrogenase activity would be expected to arise more frequently than mutations restoring the activity of 6-phosphogluconate dehydrogenase. Such double mutants would have lost the ability to accumulate 6-phosphogluconate in the presence of glucose,

and would therefore be able to utilise glucose by means of the EMP pathway (Figure 6b). Such mutants would still be unable to utilise gluconate, and would therefore have the observed Glc^+ $Glnt^-$ phenotype.

Table 6c also includes results for the Glc^- $Glnt^+$ mutants SA402 and SA409, already shown to be glucokinase deficient. As expected, these strains possessed essentially wild type specific activities of 6-phosphogluconate dehydrogenase and gluconate kinase after growth in the presence of gluconate. However, unlike strain B3724, both of these mutants reproducibly possessed low levels of gluconate kinase activity after growth on casein hydrolysate supplemented with glucose, although no activity was detected after growth on casein hydrolysate alone. It therefore appears that these mutants are able to carry out a limited amount of direct oxidation of glucose to gluconate, despite the fact that this does not normally occur in the wild type organism. It is probable that in the absence of glucokinase activity these strains accumulate much higher intracellular glucose concentrations than the parental strain B3724; such accumulation might permit the operation in these mutants of some intracellular enzyme with a K_m value too high to permit significant activity under normal conditions. Cell-free extracts of strains SA402 and SA409 grown in the presence of glucose were assayed both for NAD/NADP-linked glucose dehydrogenase activity (EC 1.1.1.47) and for DPI-linked glucose oxidase activity (EC 1.1.3.4), as described in Methods. Neither activity was detected in either strain; however, the level of glucose oxidising activity present in these strains must be very low, since they have the ability to metabolise intracellular gluconate yet do not utilise glucose at a sufficient rate to show a Glc^+ phenotype.

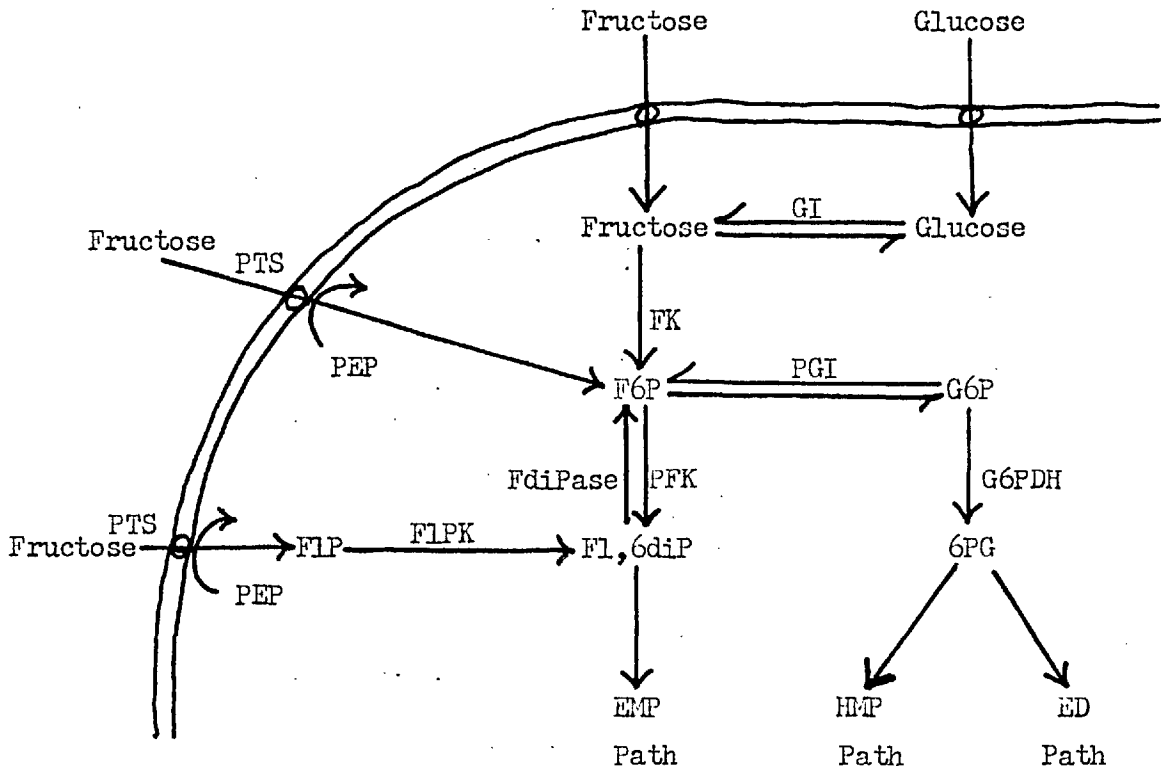
The existence of this activity suggested that mutational 'rescue' of a latent pathway for glucose metabolism by oxidation to gluconate

might be possible, either through increased synthesis of the enzyme responsible for oxidation or by changes in its kinetic parameters leading to a higher activity *in vivo*. Competition from mutants using this pathway could have interfered seriously with attempts to select evolvents with a novel pathway for glucose catabolism involving the glucose isomerase activity. This problem could, however, have been circumvented by using a double mutant, both deficient in glucokinase and blocked in gluconate metabolism, as the starting strain for such experiments. The most suitable lesion in gluconate catabolism for this purpose would have been lack of gluconate kinase, since this peripheral enzyme is normally synthesised only during growth in the presence of gluconate, and its absence could therefore have no effect on the metabolism of other compounds. Because of the results described in the next section, the isolation of such double mutants was not attempted.

Metabolism of Fructose by Strain B3724

Figure 6c summarises pathways by which fructose can be taken up and enter central metabolic pathways in various bacterial species. The Figure also shows the proposed novel pathway for glucose utilisation by means of the isomerase activity. It can be seen that some activity able to phosphorylate intracellular fructose is essential to this scheme. Table 6c shows that cell-free extracts prepared from B3724 after growth on fructose or sucrose possessed higher levels of phosphofructokinase than did those prepared from cells grown on casein hydrolysate; moreover, no fructose-1-phosphate kinase activity (or activity able to convert fructose-1-phosphate to fructose-6-phosphate) was detected in either cell free extract. This strongly suggested that fructose-6-phosphate is the first phosphorylated inter-

Figure 6c Pathways of Fructose Metabolism



Key:

PTS: Phosphotransferase system

EMP path: Embden-Meyerhof-Parnas path

HMP path: Hexose monophosphate path

ED path: Entner-Doudoroff path

PEP: phosphoenolpyruvate; F6P: fructose-6-phosphate

F1P: fructose-1-phosphate; G6P: glucose-6-phosphate

F1,6diP: fructose-1,6-diphosphate; 6PG: 6-phosphogluconate

GI: Glucose isomerase (EC 5.3.1.18)

FK: Fructokinase (EC 2.7.1.4)

PGI: Phosphoglucose isomerase (EC 5.3.1.9)

PFK: Phosphofructokinase (EC 2.7.1.11)

F1PK: Fructose-1-phosphate kinase (EC 2.7.1.56)

G6PDH: Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

FdiPase: Fructose-1,6-diphosphate 1-phosphatase (EC 3.1.3.11)

The dehydrogenation of fructose to 5-oxofructose found in the genera Acetomonas and Acetobacter is not included, since the product is not further metabolised (Englard et al., 1965; Avigad et al., 1966).

mediate involved in the catabolism of fructose in the Reynolds strains of Arthrobacter.

No fructokinase activity (i.e. less than 0.5mU/mg protein) was detected in cell-free extracts of strain B3724 grown on either fructose or sucrose, using the coupled assay system described in Methods. Other compounds which might be metabolised in Arthrobacter via free intracellular fructose were therefore tested for the ability to induce fructokinase activity. Two such compounds are D-mannitol and D-sorbitol, the two hexitols corresponding to D-fructose. Each of these polyols can be catabolised by either of two paths, one involving the formation of fructose by a direct dehydrogenation, the other involving phosphorylation followed by dehydrogenation to form fructose-6-phosphate (Doelle, 1975). Strain B3724 failed to utilise D-sorbitol, but readily grew on D-mannitol as sole carbon/energy source. However, neither fructokinase nor mannitol dehydrogenase activity was detected in extracts of strain B3724 prepared after growth on mannitol. It was concluded that mannitol was metabolised via mannitol-1-phosphate, formed either by a kinase or by a PEP:mannitol phosphotransferase.

Krulwich et al. (1973) reported that mutants of A. pyridinolis which were defective in the normal PTS for fructose uptake and phosphorylation were still able to utilise fructose, when grown in the presence of malate. Exogenous malate was required to permit the operation of a secondary system for the uptake of fructose, linked to the concomitant uptake and oxidation of malate. They were able to demonstrate the presence of low levels of fructokinase activity in this species, with a specific activity of about 4mU/mg protein in wild type cells grown on fructose as sole carbon source and about 10mU/mg in cells grown on fructose supplemented with malate. However, no fructokinase activity was detected in extracts of strain B3724 grown

Table 6f

PEP:Sugar Phosphotransferase Activities in Strain B3724

Carbon Source (w/v)	Substrate Tested (5mM)	PTS Activity (mU/mg)
2% casein hydrolysate	fructose	0
0.5% fructose	fructose	20 - 52
0.5% sucrose	fructose	31 - 37
0.5% glucose	fructose	0
0.6% sodium gluconate	fructose	0
0.5% glucose	glucose	0
0.5% fructose	glucose	0
0.5% sucrose	glucose	0
0.5% sucrose	sucrose	0 - 2
0.6% sodium gluconate	gluconate	0
0.5% mannitol	mannitol	11 - 22

One unit of PTS activity will form pyruvate from phosphoenolpyruvate in the presence of substrate at a rate of 1 μmol/minute.

The specific activities are expressed in terms of the total cell protein present.

Details of the assay used and of the toluene treatment of cells are given in Chapter II. The assay relied on the detection of the substrate dependent generation of pyruvate from phosphoenolpyruvate, using lactate dehydrogenase to couple pyruvate formation to oxidation of NADH. The background rates of NADH oxidation observed in the absence of added substrate were subtracted from the rates obtained after the addition of the substrate. These background rates accounted for about 50% of the total rates observed after the addition of the substrate to active cells. In contrast to the findings of Kornberg and Reeves (1972) for E. coli cells, in which the PTS activity remained "stable and reproducible for many hours" after decryptification, the activity observed with toluene treated Arthrobacter cells stored on ice continued to increase for at least two hours after treatment. The results should therefore be treated as qualitative only.

in MM' + fructose supplemented with malate (using 50mM-fructose + 15mM-L-malate, as used by Krulwich et al., 1973). It therefore appears unlikely that strain B3724 possesses such a system for the uptake of free fructose.

Table 6f presents the results obtained when toluene treated cells of strain B3724, grown on various substrates, were assayed for PTS activities using the coupled assay described in Methods. The absence of a PEP:glucose phosphotransferase system has been discussed above. Growth on mannitol induced a mannitol PTS, whilst a fructose PTS was induced by growth on either fructose or sucrose, suggesting that B3724 possesses an extracellular (or periplasmic) invertase activity. Since elevated levels of phosphofructokinase, but no fructose-1-phosphate kinase were detected in cells grown on fructose or sucrose (Table 6c), the fructose PTS present in strain B3724 must presumably form fructose-6-phosphate. This differs from the results obtained for A. pyridinolis by Sobel and Krulwich (1973). In this organism the normal pathway for fructose metabolism involves a PEP: fructose phosphotransferase system which forms fructose-1-phosphate, followed by the action of a fructose-1-phosphate kinase. The fructose-1,6-diphosphate formed may then either enter the EMP pathway or be converted to fructose-6-phosphate by an inducible fructose-1,6-diphosphatase activity (Figure 6c).

From the results described above it appeared that free intracellular fructose is not a normal metabolic intermediate in Arthrobacter strain B3724. This raised severe doubts concerning the ability of mutant strains blocked in the normal paths of glucose metabolism to utilise any free fructose formed from glucose in the cytoplasm by the isomerase activity. The original reason for the attempt to develop a new pathway for glucose catabolism involving

the isomerisation of glucose to fructose was to enable the isolation of mutants possessing an elevated level of glucose isomerase activity. In order for selective pressure placed on the whole organism by carbon limited chemostat culture to result in selective pressure on the isomerase, it was essential that the activity of this enzyme should be rate-limiting for the catabolism of glucose by the new pathway. It was therefore necessary that the starting strain should possess an efficient system for the phosphorylation of intracellular fructose. Passive exit of free fructose from the cell followed by re-uptake mediated by the fructose PTS would be very unlikely to provide a sufficiently rapid system, and would probably require the accumulation of a high concentration of intracellular free fructose, leading to product inhibition of the isomerase reaction.

Published evidence concerning the ability of PEP:sugar phosphotransferase systems to phosphorylate intracellular substrates is conflicting. However, even in those cases where this ability probably is present, the rate of such phosphorylation is very low (Postma & Roseman, 1976). It is therefore very improbable that the PEP:fructose phosphotransferase system of strain B3724 would be capable of sufficiently rapid phosphorylation of intracellular fructose to play any useful role in a novel pathway for glucose utilisation.

It therefore appeared unlikely that it would be possible to make the activity of the isomerase rate-limiting for glucose catabolism, as envisaged in the original scheme. The possibility that the isomerase might instead be made rate-limiting for the utilisation of fructose was considered. If this were possible, it would enable selective pressure to be applied to the isomerase by fructose limited continuous culture, providing an alternative method for the isolation of mutants producing elevated levels of the isomerase. A strain in

which the activity of the isomerase was rate-limiting for fructose utilisation would require the following properties (cf. the list of properties required to render the isomerase rate-limiting for glucose utilisation, page 110):

i) the ability to accumulate free intracellular fructose, or at least to allow rapid passive entry of free fructose,

ii) inability to metabolise fructose by any other route,

iii) inability to phosphorylate fructose,

iv) the ability to catabolise any intracellular glucose formed from fructose by the isomerase.

Strain B3724 possesses the ability to catabolise intracellular glucose (iv) and lacks the ability to phosphorylate intracellular fructose; a mutant deficient in the fructose specific PTS would therefore, presumably, be able neither to metabolise (ii) nor to phosphorylate (iii)fructose. However, the lack of both fructokinase and fructose-1-phosphate kinase activities suggests that strain B3724 is not normally capable of the uptake of free fructose (i). It might have been possible to select mutants capable of transporting fructose by means of some system normally involved in the uptake of some other substrate; however, it would first have been necessary to isolate a mutant lacking fructose PTS activity. Since this would have involved a considerable amount of work and might have served only to demonstrate the impossibility of the scheme, this was not pursued.

Instead the isomerase was purified and its activity towards various potential substrates was investigated, in order to assess the feasibility of using a gratuitous substrate other than glucose or fructose to select mutants synthesising elevated levels of the enzyme (Chapters VII & VIII).

Chapter VII

Purification of Glucose Isomerase from Strain B3728

This chapter describes the purification of Arthrobacter glucose isomerase and the determination of its native and sub-unit molecular weights, isoelectric point and amino acid composition. Strain B3728 was chosen as the starting material for the purification. This xyl^c strain synthesises the isomerase constitutively to a specific activity about twice that which is present in the wild type strain B3724 when it is fully induced (Chapter II). The enzyme was released from the cells by lysozyme rupture, and purified by ion exchange chromatography on DEAE-Sepharose followed by gel filtration on Sephadex G200. At this stage the enzyme was essentially pure. It was found that the isomerase represented a much larger fraction of the total soluble protein present in cells of strain B3728 than had originally been anticipated.

Buffer

Unless otherwise specified, the buffer used during the purification of the isomerase was 50mM-Tris.HCl/10mM-$MgCl_2$, adjusted to pH 7.5 at 4^oC. Phenylmethane sulphonyl fluoride (PMSF), which inactivates serine proteases, was routinely added to buffers immediately before use to a final concentration of 0.1mM, using a 100mM solution in propan-2-ol. Buffers were not routinely degassed, nor was 2-mercaptoethanol added, since the isomerase was known to be very stable in the absence of thiol reducing reagents, whilst Mg^{2+} was included as it enhances isomerase stability (Mr E. Corcoran, personal communication).

Assays

Protein concentrations were determined by the Lowry method, except in the case of the whole cell suspension, for which the biuret method was used (Chapter II). Two methods of assaying the isomerase were used during the work described in this chapter. The coupled xylose isomerase assay was used to locate the enzyme after elution from columns and to compare the activity of different fractions, on account of its rapidity. However, the activity of the pooled material at each stage was determined by measuring the rate of isomerisation of fructose to glucose at 60°C. Both of these assays are described in Chapter III.

Cell Lysis

Cells from a 300 litre batch culture of strain B3728, grown in Fermenter Medium at the Imperial College Pilot Plant, were harvested using a Westphalia continuous flow centrifuge and stored frozen.

A 1kg portion of the crude cell paste was thawed into 1 litre of 50mM-Tris.HCl/10mM-EDTA pH 7.5 and the cells were pelleted by centrifugation at 27 000 xg for 30 minutes. 614g of the resulting washed cell paste was resuspended in 50mM-Tris.HCl/10mM-EDTA pH 7.5 (total volume 1840ml), lysozyme chloride (614mg) was added and the suspension was incubated at 37°C with shaking for two hours. To the gel resulting from the lysozyme treatment were added 60ml 1M-$MgCl_2$ and 20mg DNase. After shaking for a further 30 minutes at 37°C, the cell debris was removed by three sequential centrifugations at 4°C, for 30 minutes each at 27 000 xg, yielding 1100ml supernatant.

All further steps were performed at 4°C.

A streptomycin sulphate precipitation step was used in pre-

liminary experiments; however, following DNase treatment very little highly polymerised nucleic acid remained, and this step was therefore omitted.

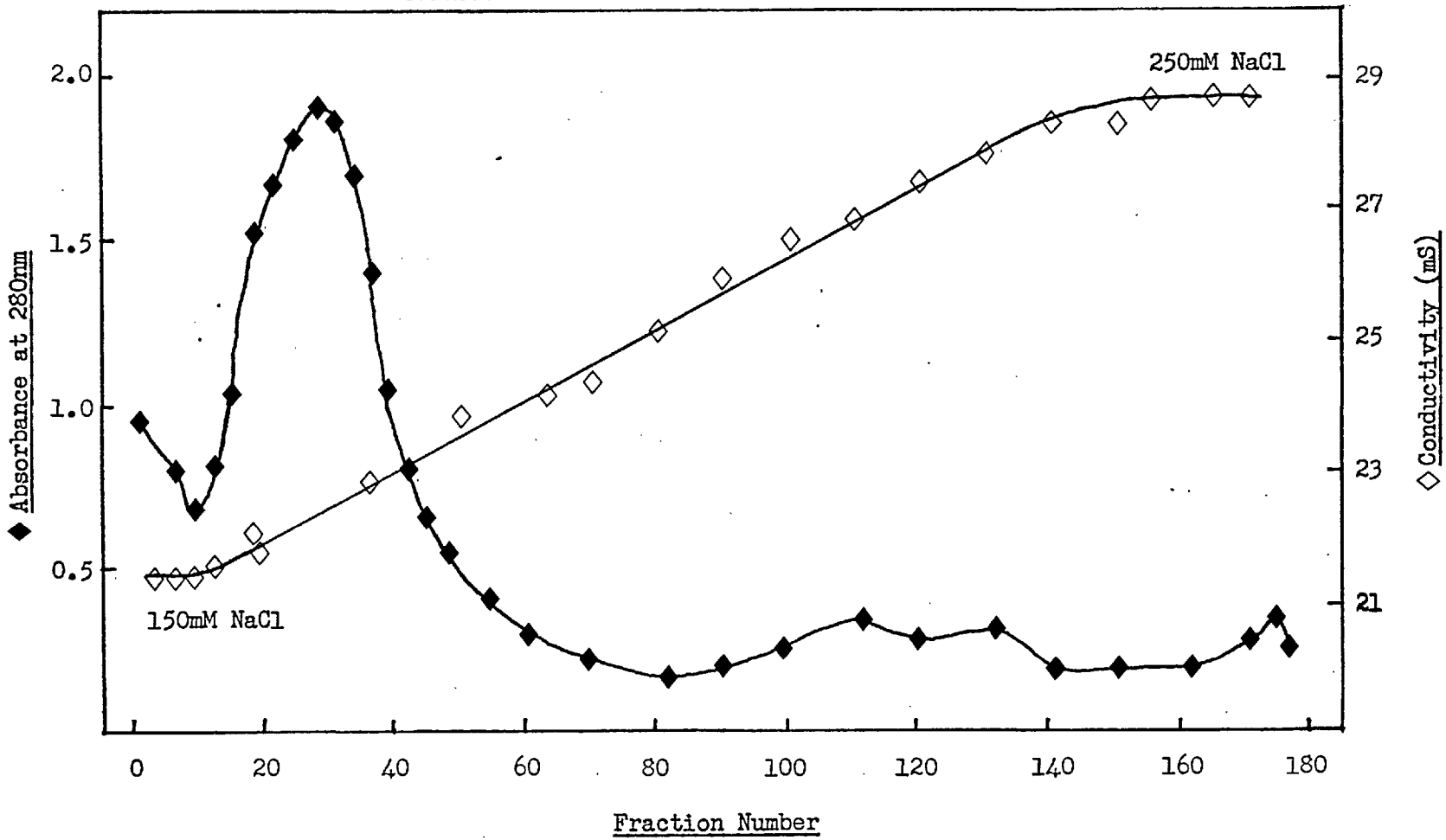
Anion Exchange Chromatography

The Arthrobacter glucose isomerase was known to bind tightly to the anion exchange resin DEAE-cellulose (Mr E. Corcoran, personal communication). Preliminary experiments confirmed that it also bound tightly to DEAE-Sepharose, a resin with better mechanical properties that DEAE-cellulose. A concentration of about 200mM-NaCl in buffer was required to elute the enzyme, whilst almost all of the protein present in the lysis supernatant either did not bind to the resin or was eluted at much lower salt concentrations, and nucleic acid did not begin to elute until a concentration of greater than 250mM-NaCl.

After diafiltration overnight with 3.5 litres of buffer/50mM-NaCl, using an Amicon PM30 membrane, the lysis supernatant was passed down a DEAE-Sepharose column of 5cm diameter and 420ml bed volume. This column probably had a considerable excess in binding capacity over that needed. The column was washed with one volume of the loading buffer, followed by four volumes of buffer/150mM-NaCl. The UV monitor of the column effluent returned to its base-line after two column volumes of this wash. The enzyme was eluted by a three litre linear gradient from 150mM to 250mM-NaCl in buffer; it was expected that the isomerase would be eluted near the middle of this gradient. The gradient was developed at a flow rate of 200ml/hour and 20ml fractions were collected. The elution profile from this column is shown in Figure 7a. Unexpectedly, the enzyme was eluted at the start of the gradient, at about 160mM-NaCl; this may have led

Figure 7a

Elution Profile of Glucose Isomerase from Column



to the loss of some active material. The early elution may have been caused by a difference between batches of the resin, or by an unintentional difference in the pH or concentration of the buffers used. In any further preparations a longer gradient, starting at a lower salt concentration would be preferable; such a gradient was used in the preparation of the mutant enzyme from strain 15/123 as described in Chapter IX. The active fractions were examined by SDS PAGE and the purest were pooled and concentrated to a volume of 29ml by ultrafiltration, using an Amicon PM30 membrane.

Gel Filtration

The concentrated pooled material was passed up a Sephadex G200 column (82cm x 5cm diameter) equilibrated with buffer/100mM-NaCl, at a flow rate of 35ml/hour. The effluent was collected in 10ml fractions, and samples of those containing xylose isomerase activity were examined by SDS PAGE. This showed that the major protein species present had been resolved from a contaminant of higher sub-unit molecular weight. However, a pattern of minor bands of lower molecular weight, whose intensities appeared to remain constant relative to that of the main band, was present in all fractions. Native PAGE showed only a single band at loadings similar to those used for the SDS gels. It therefore appeared probable that the minor bands observed after electrophoresis in the presence of SDS were fragments of the enzyme derived from nicked sub-units. Such sub-units would remain associated except under denaturing conditions, and would therefore be almost impossible to resolve from the intact enzyme without destroying its activity. The fractions from the column were combined to give two pools, the purest material and side fractions (of the same specific activity, but containing a small amount of the higher molecular weight

contaminant). Each pool was concentrated by ultrafiltration, as described above.

The gel filtration medium Ultrogel AcA34 has a similar fractionation range to Sephadex G200, but possesses a more rigid matrix. Higher flow rates are therefore possible without compaction of the column or loss of resolution. Ultrogel AcA34 was used in place of Sephadex G200 during the purification of the mutant isomorase from strain 15/123 (Chapter IX), and would be the gel filtration medium of choice in any further preparations of the wild type enzyme.

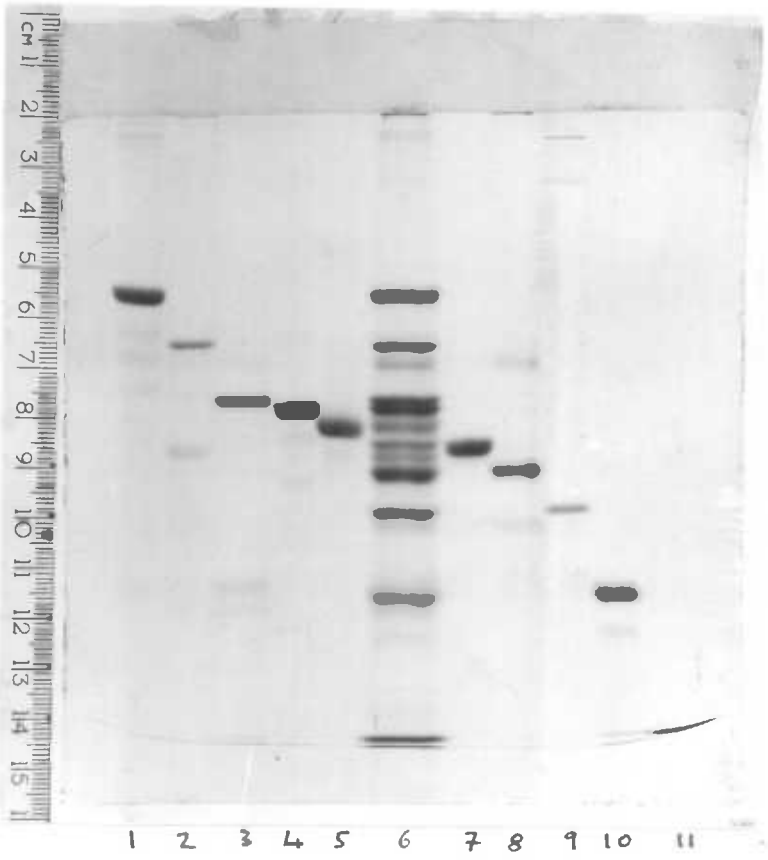
At this stage it was considered that the enzyme was sufficiently pure for kinetic and protein chemical studies. Half of the purest material was desalted, by dialysis against 50mM-ammonium acetate/0.1mM-PMSF (four changes of 100 volumes each) followed by glass distilled deionised water (two changes), freeze-dried and stored at -18°C. Most of the remaining material was used in the studies described in the next chapter. Some was stored at 4°C for rapid use, whilst the rest was stored at -18°C after the addition of glycerol to 50% v/v. Before the material stored in glycerol was used for kinetic studies it was dialysed against several changes of buffer/50mM-NaCl, as glycerol was found to be an inhibitor of the isomerase.

Further Purification

If it had been desired to remove trace contaminants from the isomerase preparation, for example in order to prepare material of suitable purity for use in the raising of antibodies to the enzyme, a further stage of purification would have been needed. The use of chromatography on hydroxyapatite for this purpose was investigated. A small column of hydroxyapatite (2ml bed volume) was equilibrated with 10mM-KH_2PO_4.KOH buffer pH 7.0, and a sample of the purified

Photograph 7a

Sub-unit Molecular Weight of Glucose Isomerase

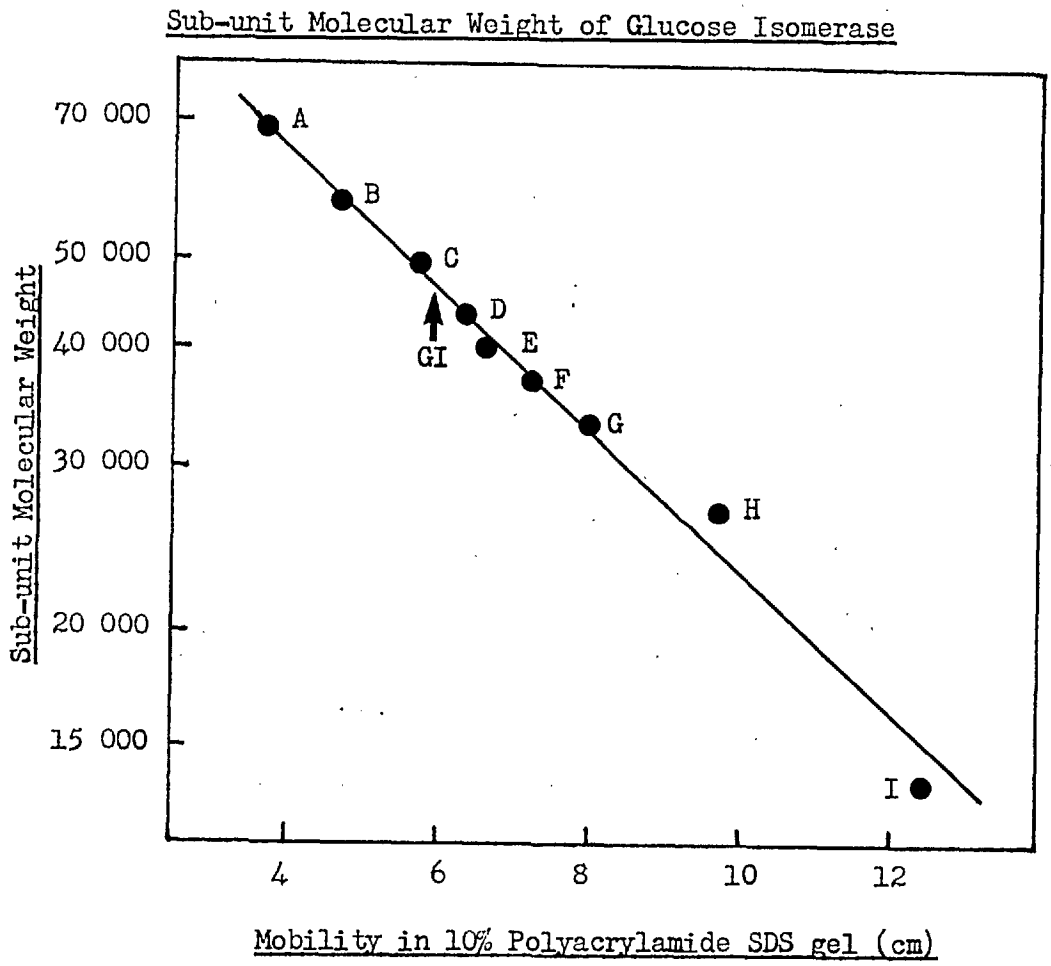


10% polyacrylamide SDS gel stained for protein.

Track	Sample
1	Bovine serum albumin
2	Pyruvate kinase
3	Fumarase
4	Glucose Isomerase
5	Ovalbumin
6	Mixture of markers
7	Aldolase
8	D-amino acid oxidase
9	L-lactate dehydrogenase
10	Ribitol dehydrogenase
11	Lysozyme

Approximately 5µg of each marker protein was used.

Figure 7b



Marker		Sub-unit M. Wt.
A	Bovine Serum Albumin	68 000
B	Pyruvate Kinase (rabbit muscle)	57 000
C	Fumarase (pig heart)	49 000
D	Ovalbumin	43 250
E	Aldolase (rabbit muscle)	40 000
F	D-Amino Acid Oxidase	37 000
G	L-Lactate Dehydrogenase (rabbit muscle)	35 500
H	Ribitol Dehydrogenase	27 000
I	Lysozyme	14 000

The gel is shown in Photograph 7a. The mobility of the pure glucose isomerase corresponded to a molecular weight of 47 000.

isomerase, dialysed against three changes of this buffer, was passed down the column. The column was then washed successively with five volumes each of 10mM, 25mM and 50mM phosphate buffer. About 80% of the activity bound to the column was eluted by the 25mM phosphate wash. Although the binding of the isomerase to hydroxyapatite at pH 7.0 was therefore rather weak, it was sufficient for chromatography on this medium to have provided a useful final purification stage if required.

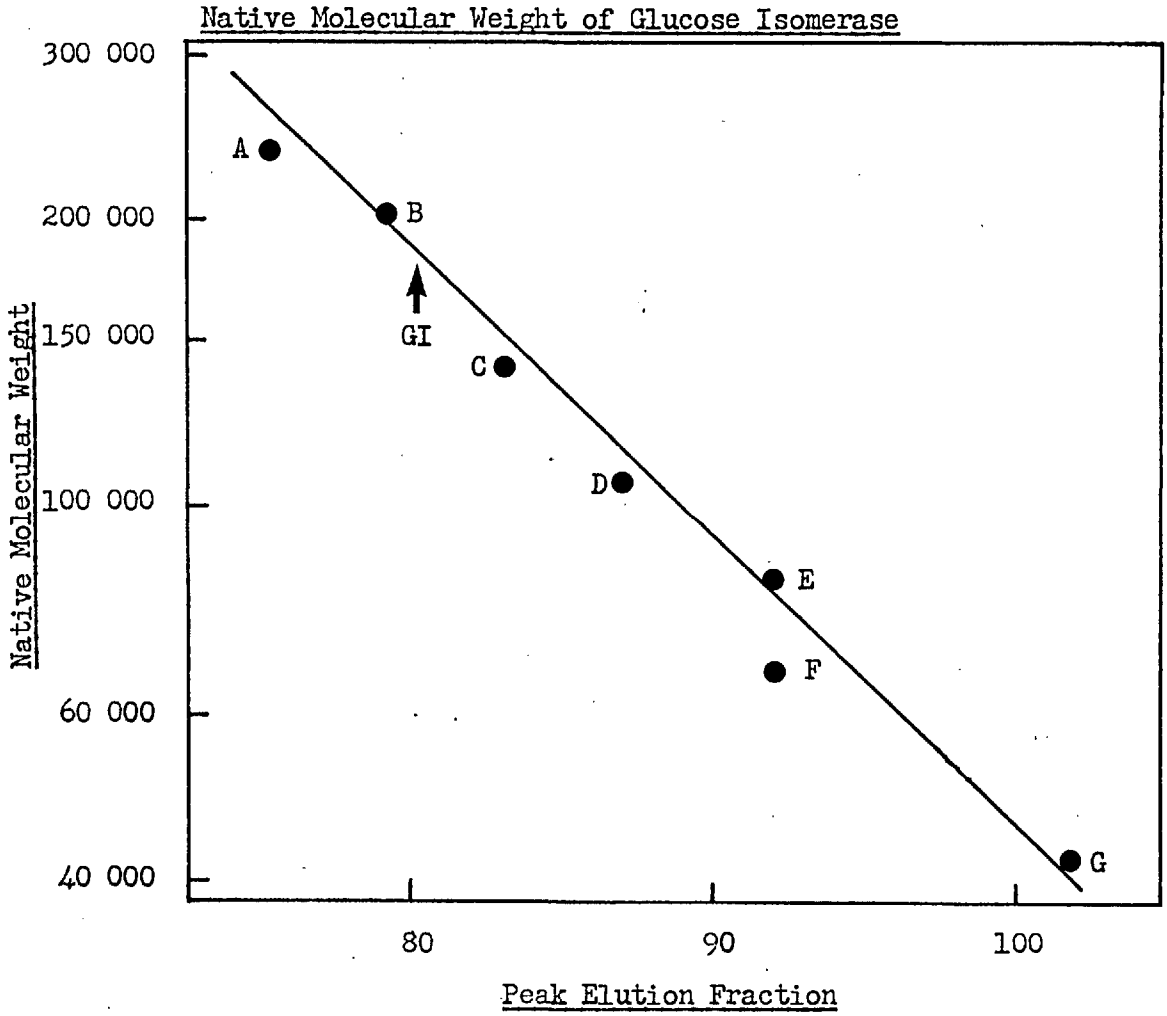
Sub-unit Molecular Weight

The sub-unit molecular weight of the Arthrobacter glucose isomerase was determined from its electrophoretic mobility relative to markers of known molecular weight during SDS PAGE (Weber & Osborn, 1975). A value of 47 000 was obtained from three gels. One gel, and the corresponding plot of log(molecular weight) against mobility are shown in Photograph 7a and Figure 7b.

Native Molecular Weight

The native molecular weight of the isomerase was estimated by gel filtration (Andrews, 1965). A Sephadex G150 column was used, of diameter 3.2cm and length 80cm. Fractions of 4ml were collected, at an ascending flow rate of 8ml/hour. Bovine serum albumin and ovalbumin were located by their absorbance at 280nm, and their identities confirmed by SDS PAGE; the other markers used were identified by the specific assays described in Chapter II. The isomerase was identified by the coupled xylose isomerase assay. Two runs were performed and the relative positions of the elution peaks for each of the markers did not vary by more than one fraction between runs.

Figure 7c



Marker		Native Mol. Wt.
A	Pyruvate Kinase (rabbit muscle)	237 000
B	Fumarase (pig heart)	204 000
C	Lactate Dehydrogenase (rabbit muscle)	142 000
D	Ribitol Dehydrogenase	108 000
E	Alkaline Phosphatase (E. coli)	86 000
F	Bovine Serum Albumin	68 000
G	Ovalbumin	43 250

Gel filtration was performed as described in the text. The peak elution position of the isomerase corresponded to a native molecular weight of 185 000 ± 5 000. The assays used to detect tho markers are described in Chapter II.

For the first run the column was equilibrated and eluted with buffer/ 50mM-NaCl, whilst for the second the concentration of NaCl was raised to 100mM. Figure 7c shows a plot of log(molecular weight) against peak elution fraction for the second run.

A value of 185 000 ± 5 000 was obtained for the native molecular weight of the isomerase. This corresponds to a tetrameric structure of 4 x 47 000 (predicted molecular weight 188 000).

The partition coefficient of glucose isomerase between the liquid and gel phases (K_{av}) was calculated to be 0.12. This is a rather low value, indicating that a gel with a higher exclusion limit, such as Sephadex G200 or Ultrogel AcA34, would have been a more suitable choice of filtration medium for this experiment. However, the linearity of the plot obtained using Sephadex G150 was satisfactory.

Isoelectric Point

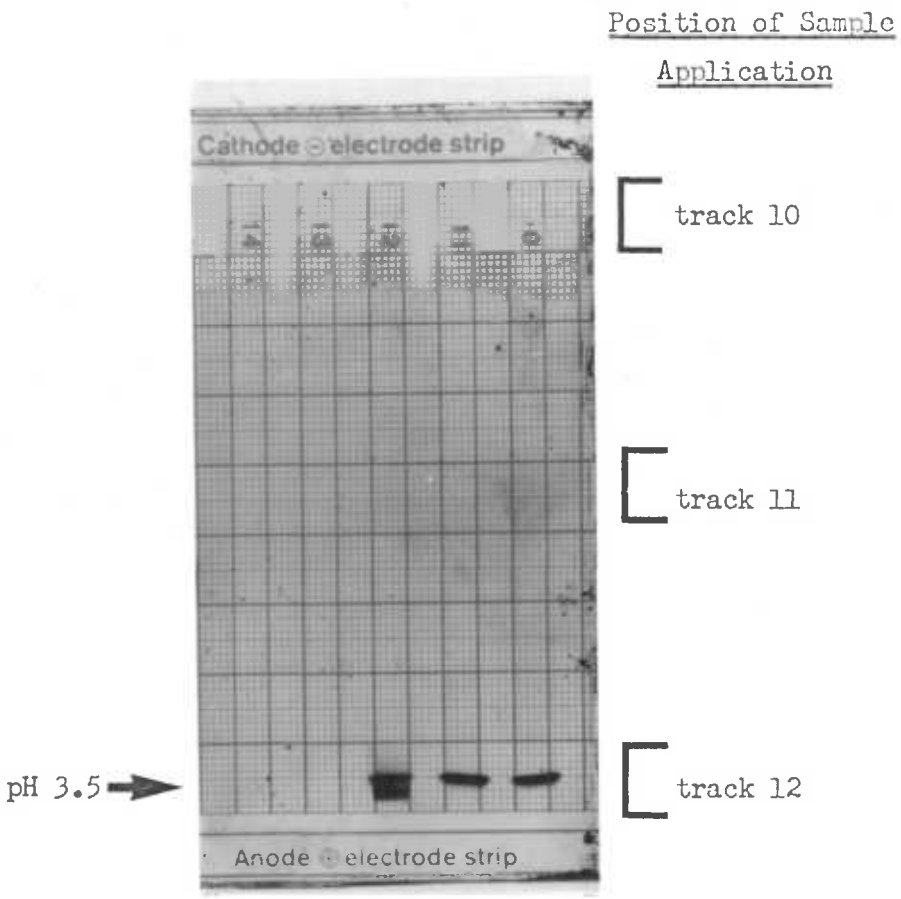
The isoelectric point of the enzyme was estimated by electrofocusing PAGE, using the LKB Ampholine system. Two experiments were performed and in each a sharp band was formed at a position corresponding to a pI between pH 3 and pH 4. Greater precision was not possible, since the position of the band was close to the anodic edge of the gel, where the pH gradient was both steep and non-linear. One gel and the corresponding pH profile are shown in Photograph 7b and Figure 7d. The low pI of the Arthrobacter glucose isomerase is consistent with the tightness with which it binds to DEAE-resins.

Amino Acid Composition

Tryptophan and tyrosine were determined spectrophotometrically, by the method recommended by Beaven and Holiday (1952). A sample of

Photograph 7b

Isoelectric Focusing of Glucose Isomerase

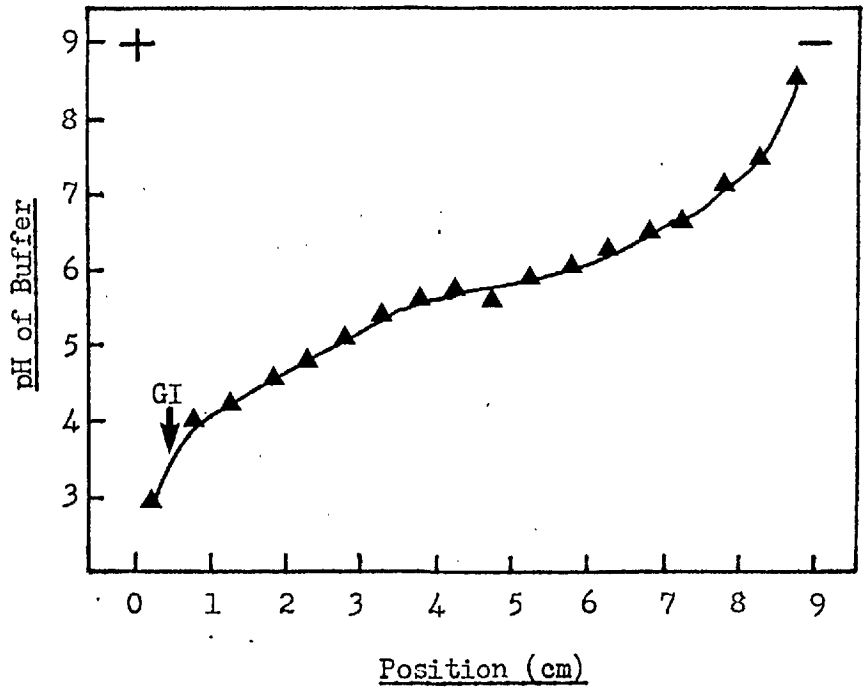


Each sample contained 10µg of purified glucose isomerase. The gel was fixed and stained using a mix prepared by adding 22.5g sulphosalicylic acid and 75g trichloroacetic acid to a mixture of 465ml distilled water and 225ml methanol containing 750mg Coomassie Brilliant Blue R-250. The background was destained for four days with several changes of a 1:3:8 mixture by volumes of acetic acid, ethanol and distilled water.

The double band in track 12 is probably an artefact caused by the application of the sample at a position too close to the pI of the isomerase, resulting in the precipitation of part of the protein during the formation of the pH gradient. Figure 7d shows the profile of the pH gradient obtained.

Figure 7d

Isoelectric Focusing of Glucose Isomerase



Isoelectric focusing was carried out for four hours at a maximum power of 10W and a maximum potential difference of 1000V. The temperature of the cooling water was 11° to 12°C. The pH gradient was determined by removing a 15mm strip from the gel immediately after focusing and soaking 5mm slices in 3ml volumes of degassed distilled water for two hours in sealed vials. The pH of the eluted buffer was then determined at 11°C with an EIL pH meter 7020, freshly calibrated using standard buffer solutions. The position of the band due to the isomerase corresponded to an isoelectric point between pH 3.0 and pH 4.0.

the freeze-dried desalted protein was redessicated over P_2O_5 for one week, weighed and dissolved in 100mM-NaOH at a concentration of 1mg/ml. The wavelength calibration of a Gilford 252 spectrophotometer was checked as described in Chapter II, and the $A_{294.4}$ and $A_{280.0}$ of the solution were determined against 100mM-NaOH. The values determined were: $A_{294.4} = 0.595$ and $A_{280.0} = 0.705$. For a light path of 1cm the molar concentrations of tyrosine and tryptophan residues (M_{Tyr} and M_{Trp}) are given by:

$$M_{Tyr} = (0.592 \times A_{294.4} - 0.263 \times A_{280.0}) \times 10^{-3} M$$

$$M_{Trp} = (0.263 \times A_{280.0} - 0.170 \times A_{294.4}) \times 10^{-3} M$$

The values obtained were:

$M_{Tyr} = 167 \mu M$ $\quad$ $M_{Trp} = 85.5 \mu M$ $\quad$ $M_{Tyr}/M_{Trp} = 1.96$

Taking the molecular weight of the Arthrobacter glucose isomerase sub-unit as 47 000, this corresponds to 7.85 tyrosine residues and 4.0 tryptophan residues per polypeptide chain.

Triplicate samples (24µg) of the desalted protein were acid hydrolysed for periods of 14, 38, 52 and 76 hours, as described in Chapter II. The amino acid compositions of the resulting hydrolysates were determined with a Durrum D500 amino acid analyser by Mr K. Edwards, at the Laboratory of Molecular Biology, Cambridge. The values for threonine, serine and histidine were extrapolated to zero hydrolysis time to correct for their experimental decay, whilst those for valine, leucine and isoleucine were extrapolated to infinite hydrolysis time. Values for methionine were estimated as the sum of methionine and methionine-sulphone, and were then extrapolated to zero hydrolysis time. For the other amino acids the values obtained for all four hydrolysis times were averaged.

In order to investigate the cysteine/half-cystine content of

Table 7a

Amino Acid Composition of Arthrobacter Glucose Isomerase

Residue	% Weight	Residues per Sub-unit
Aspartate/Asparagine	12.2	50.0
Glutamate / Glutamine	13.8	50.3
Serine	3.9	21.0
Threonine	4.9	23.0
Glycine	5.1	42.1
Alanine	9.2	60.9
Valine	3.2	15.4
Leucine	10.4	43.3
Isoleucine	4.1	17.0
Proline	3.7	17.7
Methionine	2.4	8.7
Cysteine	-	(<0.15)
Phenylalanine	6.8	22.7
Tyrosine	2.3	6.7 (7.9)*
Tryptophan	1.4	3.4 (4.0)*
Histidine	4.0	13.7
Lysine	6.5	23.9
Arginine	6.0	18.1
Amido N	-	20 - 30

A sub-unit molecular weight of 47 000 was assumed in the calculation of the number of residues of each amino acid per sub-unit of the enzyme.

The estimate of the number of amido groups present was based on the amount of ammonia recovered following acid hydrolysis, as determined on the Rank-Hilger Chromaspek, and should therefore be treated as very approximate.

* The values for tyrosine and tryptophan given in the main column are based on the amount of tyrosine recovered following acid hydrolysis and the tyrosine/tryptophan ratio obtained spectrophotometrically. The alternative figures (bracketed) are the values obtained spectrophotometrically.

the enzyme, a sample of the freeze-dried desalted protein was oxidised with performic acid, as described in Chapter II. No cysteic acid was detected after the acid hydrolysis of 84µg samples of the oxidised protein for periods of 16 and 40 hours, using a Rank-Hilger Chromaspek amino acid analyser at Imperial College. This placed an upper limit on the cysteic acid content of the oxidised material of about 0.15 residues per sub-unit. The amount of methionine detected in the hydrolysates of oxidised samples was less than 4% of that present in unoxidised samples, confirming that the performic acid treatment had been effective. The lack of cysteine residues in the Arthrobacter glucose isomerase explains why, unlike most cytoplasmic enzymes, it is not more stable in the presence of thiol reducing agents.

The percentage amino acid composition and the estimated number of each residue per sub-unit of the glucose isomerase are presented in Table 7a.

Comparison of Glucose Isomerase Molecules from Various Species

Table 7b contains details of the molecular weights, probable sub-unit structures and cysteine contents of glucose isomerising enzymes purified from species belonging to four bacterial genera. The physiological substrate of each of the enzymes listed is D-xylose.

The tetrameric structure of the Arthrobacter enzyme is shared by the enzymes from Lactobacillus brevis, Streptomyces albus and, probably, by that from Bacillus coagulans. Because, in each case, sub-units of a single molecular weight were detected under denaturing conditions, it is most probable that each enzyme consists of a single sub-unit type. However, this has only been proven in the case of the enzyme from S. albus, for which the number of peptides isolated after

Table 7b

Properties of Glucose Isomerases from Various Species

Species	Native Mol. Wt.	Sub-unit Mol. Wt.	Sub-unit Structure	Cys Content	References
Arthrobacter Strain B3728	180 000 - 190 000	47 000	Tetrameric	0	This work
Bacillus coagulans	160 000 - 175 000	49 000	Tetrameric	0	Danno, 1970a Danno, 1973
Bacillus stearothermophilus	127 000	130 000	Monomeric	0	Suekane et al., 1978
Lactobacillus brevis	191 000 - 197 000	-	-	0	Yamanaka, 1968a,b
Lactobacillus xylosus	183 000	45 000	Tetrameric	-	Yamanaka & Takahara, 1977
Streptomyces albus	157 000 - 187 000	40 000 - 43 000	Tetrameric	4	Hogue-Angeletti, 1975 Takasaki et al., 1969a,b
Streptomyces olivochromogenes	115 000 - 117 000	56 000	Dimeric	0	Suekane et al., 1978

tryptic digestion implies a unique polypeptide chain of the molecular weight of the sub-unit (Hogue-Angeletti, 1975).

The only isomerase reported to be monomeric is that from the thermophile Bacillus stearothermophilus. It should be noted that the native molecular weight of this enzyme was determined at 15°C and 20°C; therefore the possibility that the enzyme is multimeric at the normal growth temperature of the organism (50°C to 60°C), but dissociates at lower temperatures cannot be excluded. Cases of such dissociation are well known, and are usually considered to result from the effect of temperature on the entropic contribution to the free energy of association due to 'hydrophobic' interactions (Lee & Muench, 1969; Fersht, 1977). Nevertheless, it is of interest to note that the enzyme from B. stearothermophilus shows a molecular weight between two and three times that of the sub-unit of the isomerase from any of the other species, including that from the closely related organism B. coagulans. This suggests that the structural gene for the isomerase in B. stearothermophilus may have undergone either fusion with some other coding sequence, or tandem duplication followed by self-fusion, at a recent time in evolutionary history. Such an event could have led both to the abolition of association (either by the removal or obstruction of the sites necessary for sub-unit interaction) and to the removal of any requirement for activation of the enzyme by sub-unit association (by stabilising the catalytic portion of the hybrid polypeptide in the conformation normally found in the multimeric form of the enzyme).

Comparison of the amino acid compositions of iso-functional enzymes is not usually a useful criterion for judgement of their evolutionary relatedness. Residues which are exposed at the surface of a protein may frequently be altered without causing gross changes in the overall conformation of the molecule; likewise residues may be

inserted or deleted at positions where they do not interfere with regions of secondary structure. Although not strictly iso-functional, the serine proteases provide an example of a group of functionally related enzymes, which display strongly conserved features in their tertiary structures, yet have widely divergent amino acid compositions, due to changes of the types described above (Hartley, ~~1973~~ 1974). However, it is of interest to note that, of the six bacterial glucose isomerase enzymes for which amino acid compositions are available, five lack cysteine residues (Table 7b). In the case of the sixth enzyme, that from S. albus, the presence of a single cysteine residue per sub-unit has been established by the identification of a unique carboxymethyl-cysteine containing peptide following the tryptic digestion of the carboxymethylated protein (Hogue-Angeletti, 1975). Slein (1955) has suggested that the D-xylose isomerase of Pasturella pestis contains cysteine, since it is reversibly inhibited by parachloromercuribenzoate; however, he did not report whether his enzyme preparation showed any glucose isomerising activity. It is not clear whether the absence of cysteine from most of these D-xylose/D-glucose isomerase enzymes is of any significance.

Yield and Purification of Glucose Isomerase

Table 7c summarises the course of the purification of the Arthrobacter isomerase, and the SDS-polyacrylamide gel shown in Photograph 7c illustrates the purification achieved after each stage. A total of 870mg of purified material was obtained, representing a yield of 36% of the activity in the starting material. Photograph 7d shows a polyacrylamide gel run under native conditions, stained both for activity and for protein, demonstrating that the active species co-migrates with the major protein band in the purified enzyme

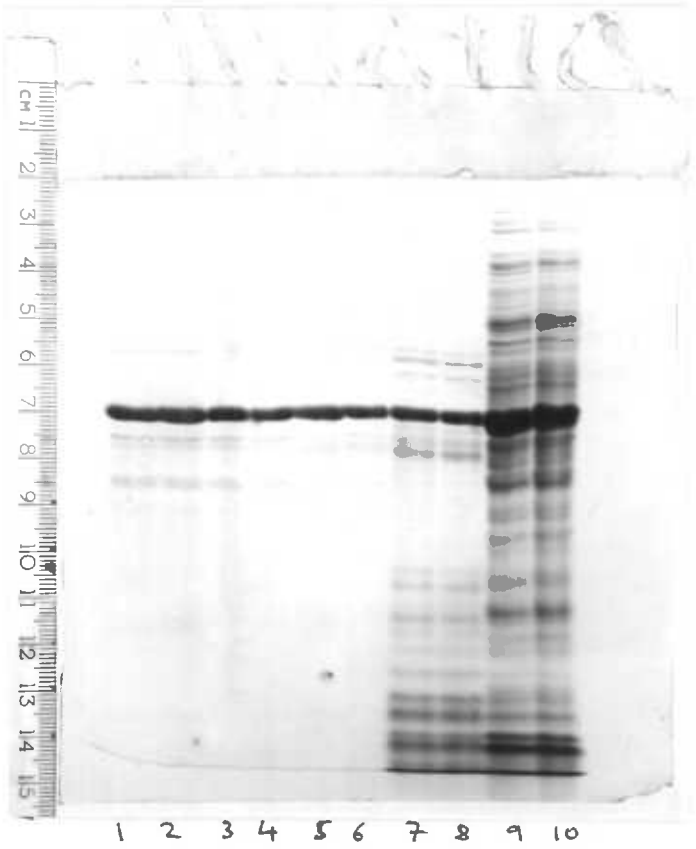
Table 7c

Purification and Yield of Glucose Isomerase

Stage	Volume (ml)	Activity (mU/ml)	Total Activity (mU)	Protein (mg/ml)	Specific Activity (μU/mg)	Yield (%) Step	Yield (%) Total	Purification Step	Purification Total
Washed Cell Suspension	1840	5.24	9630	25	210	100	100	1	1
Lysis Supernatant	1100	6.07	6680	9.0	675	69	69	3.2	3.2
DEAE-Sepharose Pool (concentrated)	29	140	4050	45.2	3100	61	42	4.6	14
Main Sephadex G200 Pool (concentrated)	29	86	2500	22	4000	62			
						85	36	1.3	19
Sephadex G200 Side Pool (concentrated)	28	33	920	8.2	4000	23			

Photograph 7c

Purification of Glucose Isomerase

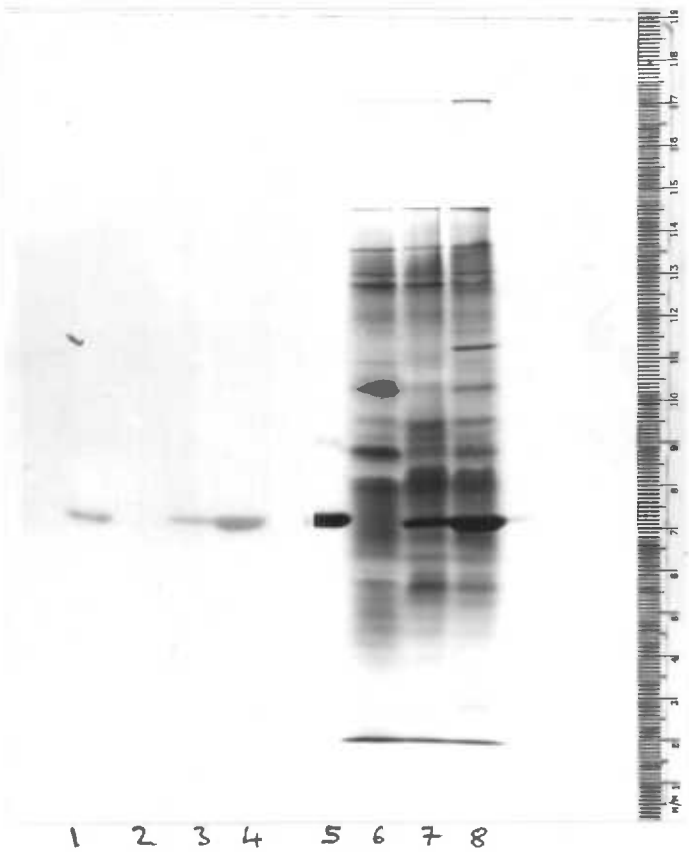


10% acrylamide gel stained for protein.

Tracks	Sample
1 & 2	Pooled material from DEAE-Sepharose.
3 & 4	Pooled Sephadex G200 side-fractions.
5 & 6	Main pool from Sephadex G200.
7 & 8	Lysis supernatant from preparation.
9 & 10	Strain B3728 cell supernatant prepared as described in Chapter II.

Photograph 7d

Induction and Purification of Glucose Isomerase



7.5% polyacrylamide gel run under native conditions
Tracks 1 to 4 have been stained for isomerase activity, whilst tracks 5 to 8 have been stained for protein.

Tracks	Sample
1 & 5	Purified glucose isomerase from B3728.
2 & 6	Cell-free extract of strain B3724.
3 & 7	Cell-free extract of induced B3724.
4 & 8	Cell-free extract of strain B3728.

preparation. In addition, this gel shows that the active species in crude cell-free extracts both of strain B3728 and of strain B3724 (when induced for isomerase synthesis) also co-migrate with the purified enzyme. This provides circumstantial evidence that no substantial alteration of the enzyme took place during the purification.

Unexpectedly, the band corresponding to the isomerase proved to be the most abundant protein species in the crude cell-free extracts of strain B3728 and of xylose-induced strain B3724. Comparison of the intensities of the Coomassie Blue staining of the bands due to the isomerase in the cell-free extract of strain B3728 and in the purified enzyme preparation suggested that at least 5% of the total protein in the cell-free extract was glucose isomerase. The specific activity of the purified isomerase was about twenty-fold that of the whole cells from which it was prepared (Table 7c); therefore, assuming that no significant inactivation of the enzyme occurred during the purification, the isomerase would have accounted for 5% of the total cell protein of strain B3728, and for as much as 17% of the protein in the lysis supernatant. However, comparison of the specific activities of typical cell-free extracts with that of the purified enzyme suggests that the isomerase normally accounts for about 10% of the soluble protein in strain B3728 and about 5% of that in cells of strain B3724 fully induced for synthesis of the isomerase. The gel shown in Photograph 7c confirms that the isomerase constituted a larger proportion of the total protein present in the lysis supernatant than in a cell-free extract prepared by the method described in Chapter II. Presumably the absence of 2-mercaptoethanol from the buffer and the higher temperature of the lysozyme treatment used during the isomerase purification led to the precipitation, and subsequent removal, of proteins which would have remained in the supernatant during the preparation of normal cell-free extracts for enzyme assays.

Table 7d

Glucose Isomerase Levels in Various Species

Species	Level (%)	Reference
Arthrobacter Strain B3724	5	This work
Arthrobacter Strain B3728	10	This work
Bacillus coagulans	1.5	Danno et al., 1967
Bacillus stearothermophilus	2	Suekane et al., 1978
Lactobacillus brevis	8.5	Yamanaka, 1968 (a)
Lactobacillus xylosus	3	Yamanaka & Takahara, 1977
Streptomyces albus	10	Takasaki et al., 1969a
Streptomyces olivochromogenes	14	Suekane et al., 1978

The figures given represent the percentage of the total soluble protein in cell lysates contributed by the isomerase. All the strains were induced for isomerase expression by growth in the presence of xylose, except for strain B3728, which is constitutive. The figures for Arthrobacter are based on the specific activities of typical cell extracts and of the purified enzyme from strain B3728. For the other species the values are derived from purification tables, assuming that no inactivation occurred during the purification. It should be noted that the cell lysates were prepared by several different methods; it is therefore possible that the relative levels of the enzyme present in the different species have been distorted by differential release of the isomerase in some of the procedures used.

Table 7e

Levels of Various Inducible Catabolic Enzymes

Species	Enzyme	Level (%)	Reference
Escherichia coli	L-Arabinose Isomerase	0.7	Lee, 1978
Klebsiella aerogenes	L-Arabinose Isomerase	1.2	Yamanaka & Wood, 1966
Lactobacillus plantarum	L-Rhamnose Isomerase	0.4	Domagk & Zech, 1966
Klebsiella aerogenes	D-Lyxose Isomerase	0.9	Anderson & Allison, 1965
Klebsiella aerogenes	D-Arabinose Isomerase	3.7	Yamanaka & Izumori, 1975
Klebsiella aerogenes	Ribitol Dehydrogenase	0.6	Fossit & Wood, 1966
Klebsiella aerogenes	D-Arabitol Dehydrogenase	0.6	Neuberger et al., 1979
Propionibacterium pentosaceum	D-Arabinose Kinase	0.9	Volk, 1966

The figures given represent the percentage contribution of each enzyme to the total soluble protein in lysates of cells induced for the expression of the appropriate catabolic pathway. Most of the figures have been calculated from purification tables, assuming no inactivation.

Glucose Isomerase Levels

Table 7d contains estimates of the percentage contribution made by the glucose isomerase to the total soluble protein in cell lysates of species belonging to four bacterial genera, each induced for isomerase synthesis by growth in the presence of D-xylose or a D-xylan. Table 7e contains similar information for a number of other inducible enzymes, each of which (in common with D-xylose isomerase) catalyses the first intracellular step in the catabolism of a carbohydrate carbon/energy source. It is unfortunate that no species occurrs in both tables and that only one genus is represented in each; however, it is apparent that the levels of the D-xylose/D-glucose isomerase present in most of the species listed in Table 7d are considerably higher than those found for most of the other enzymes with similar catabolic roles listed in Table 7e. It should be noted that the species listed in Table 7d were all selected for their relatively high levels of glucose isomerase activity; it is therefore possible that these species possess abnormally high levels of the D-xylose isomerase protein. No figures are available for the levels of D-xylose isomerase present in those organisms (such as *E. coli*; David & Weismeyer, 1970) which possess isomerases which show little activity as D-glucose isomerases.

It was originally expected that the isomerase would account for between 0.5% and 2% of the soluble protein the 'simple' constitutive strain B3728 and that it would be possible to increase this level to about 20% in super-producing strains, as discussed in Chapter I. The effect of the unexpectedly high level of the isomerase already present in strain B3728 on the likelihood of obtaining strains producing still higher levels of sufficient genetic stability to be of use in the production of the isomerase is discussed in Chapter XI.

Chapter VIII

Enzymic Properties of the Glucose Isomerase from Strain B3728

The effects of pH and temperature on the activity of the purified enzyme were investigated. Kinetic constants were determined at 30°C for the isomerisation of the physiological substrate of the enzyme, D-xylose, and at both 30°C and 60°C for the interconversion of D-glucose and D-fructose, the substrate/product pair of commercial importance. The isomerase also showed activity at 30°C towards the aldopentoses D-ribose, L-arabinose, D-lyxose and L-lyxose. The possibility of using any of these substrates to select mutants synthesising elevated levels of the isomerase is discussed in Chapter X; kinetic constants were determined for the isomerisation of D-ribose, L-arabinose and D-lyxose.

The purified isomerase showed an absolute requirement for a divalent cation for activity towards D-xylose. The greatest activity was obtained in the presence of Mg^{2+}, although Mn^{2+} and Co^{2+} also activated the isomerase. The apparent binding constant for Mg^{2+} was determined.

The inhibition of the isomerase by polyol substrate analogues was also investigated. Xylitol proved to be an extremely potent competitive inhibitor of xylose isomerase activity.

Dependence of Isomerase Activity on pH

The effect of pH on the activity of the isomerase at 60°C was investigated, using 1M-D-fructose as substrate, in 100mM-Tricine.NaOH/ 30mM-$MgCl_2$. A range of pH values from 5.0 to 9.5 was used; at higher pH values Mg^{2+} is not soluble in Tricine buffer. The pH of the

buffer was adjusted at 60°C, using an EIL pH Meter 7020, which was standardised with phosphate buffer pH 6.97 at 60°C. Since Tricine has very little buffering capacity at the lower pH values used, the pH of each incubation was checked at the end of the experiment; no variations in pH value were detected. For each pH value three incubations containing 54µg of purified glucose isomerase in 3.5ml buffer/substrate were used, together with one blank incubation containing no isomerase. After incubation for 20, 40 and 60 minutes at 60°C, 700µl samples were removed and added to 300µl aliquots of 15% (w/v) trichloroacetic acid to quench the reaction. The acidified samples were then treated as described for the normal assay of the enzyme using fructose as substrate in Chapter III. The rate of isomerisation at each pH was determined from plots of glucose concentration against time, and the non-enzymic rates, obtained from the blank incubations, were subtracted from the rates observed in the presence of the isomerase to obtain the enzymic rates of isomerisation. At the pH values used no inactivation of the isomerase was observed during the course of the incubations.

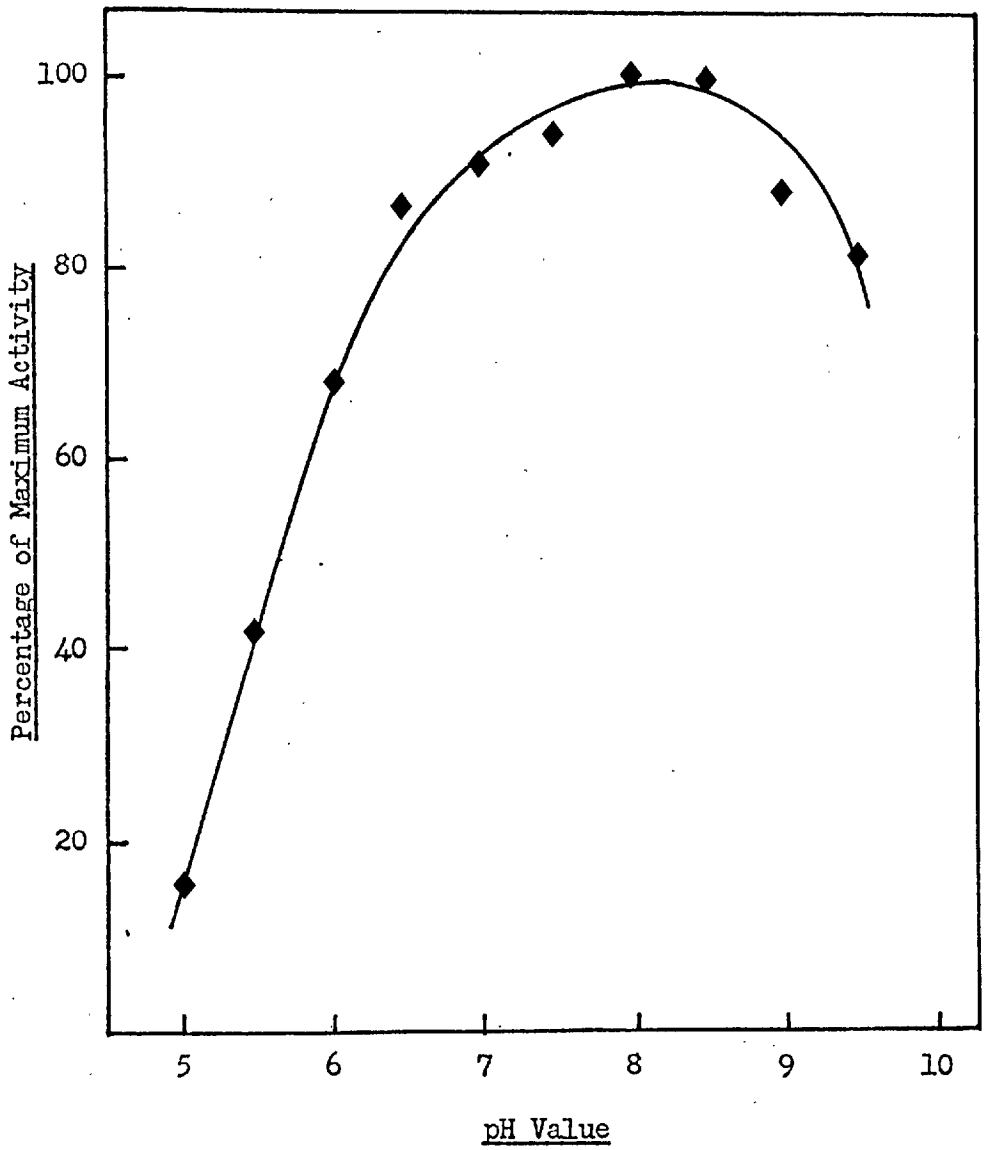
The results are presented in Figure 8a. The pH optimum for the isomerisation of D-fructose by the Arthrobacter enzyme was pH 8.0, which is similar to the optimal pH value previously reported for the isomerisation of D-glucose (Reynolds, 1973).

Effect of Temperature on Isomerase Activity

The effect of temperature on the rate of enzymic isomerisation of D-fructose was investigated. Tricine buffers show considerable variation in pH with temperature, whilst phosphate buffers show little change (the pH of 100mM-Tricine buffer decreaces by about 0.5 units when the temperature is raised from 30°C to 60°C, whilst that of

Figure 8a

pH Dependence of Isomerase Activity



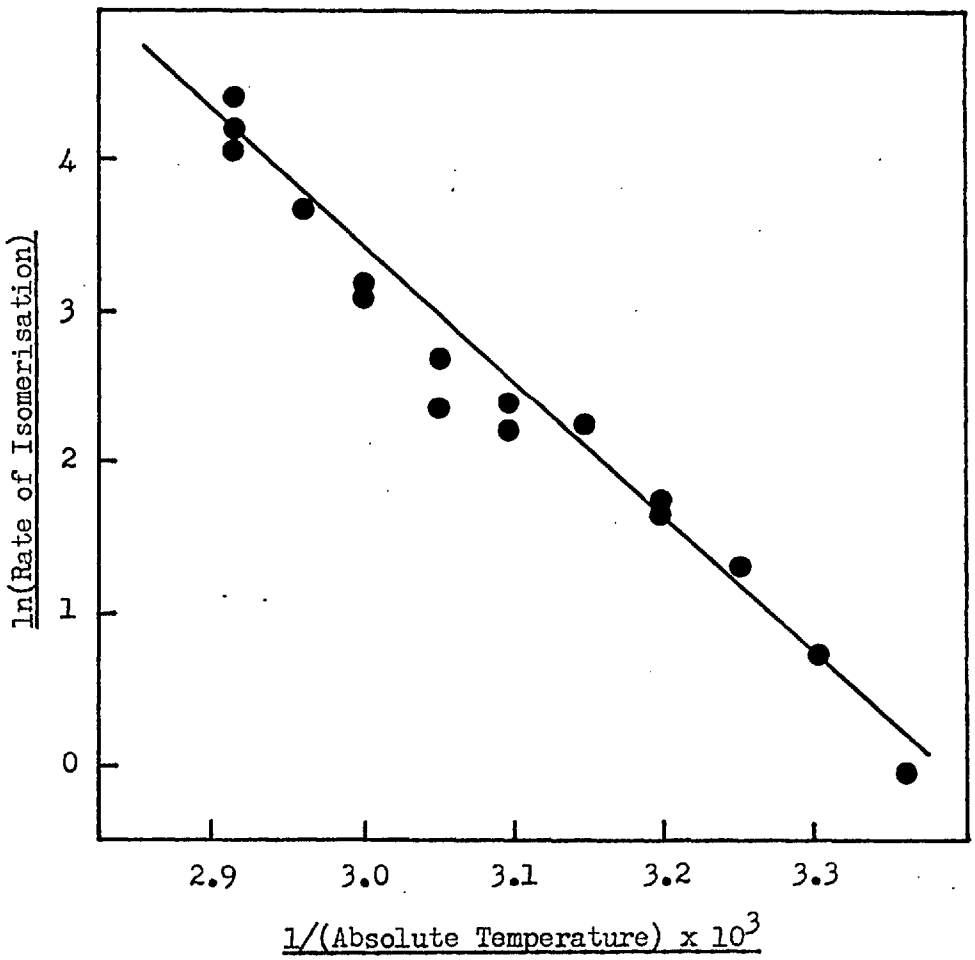
The activity of the isomerase was determined at 60°C, using 1M-D-fructose as substrate, in 100mM-Tricine.NaOH, containing 30mM-$MgCl_2$. After subtraction of the non-enzymic rate of isomerisation at each pH value, the rate was expressed as a percentage of the rate obtained at pH 8.0, the optimum pH value.

100mM-phosphate buffer falls by only 0.03 units); it was therefore decided that phosphate buffer of pH 7.0 would be used in this investigation. Incubations contained, in addition to 1M-D-fructose and suitable amounts of purifed enzyme, 100mM-KH_2PO_4.KOH/10mM-$MgCl_2$/10mM-NaCl, pH 7.0 (at 25°C). Three incubations containing isomerase and one blank incubation were used at each temperature. After incubation for 20, 40 and 60 minutes 700µl samples were withdrawn, added to 300µl aliquots of 15% (w/v) trichloroacetic acid and assayed for glucose as described in Chapter III. The range of temperatures tested was from 25°C to 85°C. Below 70°C the rate of isomerisation was constant during the experiment, whilst above this temperature the increments in glucose concentration for successive 20 minute periods decreased, indicating that inactivation of the purified enzyme was occurring during the incubation.

Figure 8b shows an Arrhenius Plot of the dependence of the rate of isomerisation on temperature from 25°C to 70°C. Over this range of temperature the non-enzymic rate of isomerisation observed in the blank incubations was negligible. The gradient of the Arrhenius Plot corresponds to an activation energy of about 75kJ/mol for the isomerisation of D-fructose to D-glucose by the Arthrobacter enzyme. Danno (1970b) has reported that the isomerase from Bacillus coagulans shows an activation energy of 61kJ/mol for the isomerisation of the three aldose substrates D-xylose, D-ribose and D-glucose; however, it is not possible meaningfully to compare the activation energies for the forward and back reactions of the two different glucose isomerase enzymes.

Figure 8b

Arrhenius Plot for Enzymic Isomerisation of Fructose



$$\text{Activation Energy} = \frac{d\ \ln(\text{rate})}{d\ (1/T)} \times R$$

$$\frac{d\ \ln(\text{rate})}{d\ (1/T)} = 9 \times 10^3\ K; \quad R = 8.315\ JK^{-1}mol^{-1}$$

$$\text{Activation Energy} = 75\ kJmol^{-1}$$

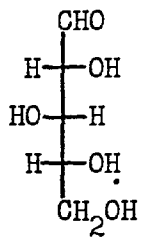
Substrate Specificity and Kinetic Constants

It was decided that kinetic studies with the purified isomerase from strain B3728 would be carried out at pH 8.0, which is the optimum pH for the reaction, as well as being the pH used in the normal assay of the enzyme. Because of the width of the activity peak (Figure 8a) the activity of the isomerase at this pH is close enough to that under physiological conditions for the results obtained to be useful in the evaluation of substrates as potential carbon sources for mutant selection. Michaelis constants (K_m) and maximum specific forward velocities (V) were determined for the isomerisation by the enzyme of the hexoses D-glucose and D-fructose at both 30°C and 60°C, and of the aldopentoses D-xylose, D-ribose, L-arabinose and D-lyxose at 30°C, in 100mM-Tricine.NaOH/30mM-$MgCl_2$, pH 8.0. Under these conditions no enzymic isomerisation of D-arabinose, L-xylose or L-rhamnose was detected, whilst results for L-lyxose suggested slight activity. The remaining aldopentose, L-ribose, was not tested for isomerisation, since its configuration at the C_2 position suggested that it was not likely to be a substrate for the enzyme, and it is too expensive to be used as a carbon source. The configurations of these sugars are given in Figure 8c.

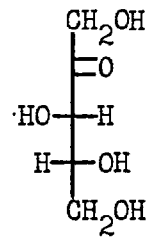
The kinetic parameters K_m and V were extracted from the results by plotting [s]/v against [s], where v is the initial rate of isomerisation at a particular concentration [s] of the substrate. The intercept on the axis of [s] is $-K_m$ and the gradient is 1/V (Hofstee, 1952). This method gives equal statistical weight to points corresponding to all values of [s], the independent variable, and is therefore more satisfactory than the Lineweaver-Burk double reciprocal plot, which gives excessive weighting to those experimental points which correspond to the lowest values of [s] and v, and are therefore likely

Figure 8c

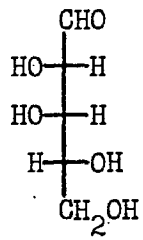
Configurations of Sugars



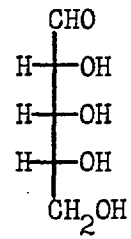
D-Xylose



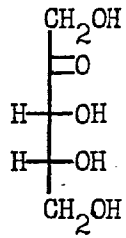
D-Xylulose



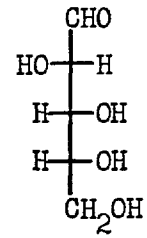
D-Lyxose



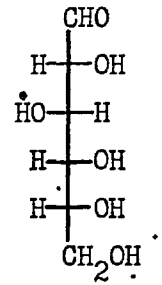
D-Ribose



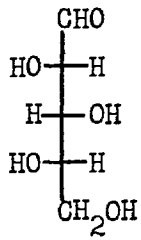
D-Ribulose



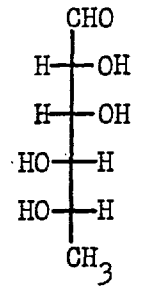
D-Arabinose



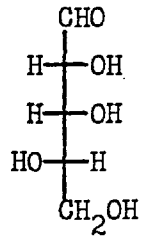
D-Glucose



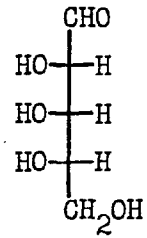
L-Xylose



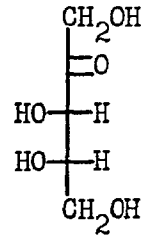
L-Rhamnose



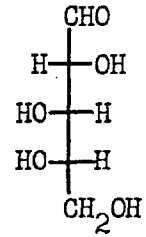
L-Lyxose



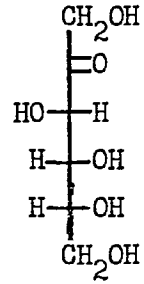
L-Ribose



L-Ribulose



L-Arabinose



D-Fructose

Table 8a

Kinetic Parameters for Purified Glucose Isomerase from Strain B3728

Substrate	K_m (mM)	V (μmol/min per mg)	V (mg/min per mg)	V/K_m (ml/min per mg)
D-Xylose (30°C)	3.3	8.9	-	2.7
L-Arabinose (30°C)	280	1.8	-	6.7×10^{-3}
D-Ribose (30°C)	190	0.71	-	3.7×10^{-3}
D-Lyxose (30°C)	86	0.086	-	1.0×10^{-3}
D-Glucose (30°C)	225	3.2	0.57	1.4×10^{-2}
D-Fructose (30°C)	170	2.3	0.41	-
D-Glucose (60°C)	210	27.4	4.93	-
D-Fructose (60°C)	250	28.2	5.08	-

to be the least accurate. Linear regression was used to obtain the best estimates of the slope and intercept for each plot. Figures 8d to 8k show plots for each substrate, and Table 8a presents the values obtained for the kinetic constants.

For D-glucose, D-fructose and L-arabinose discontinuous assays were used. Samples containing various concentrations of substrate in 100mM-Tricine.NaOH/30mM-$MgCl_2$, pH 8.0, and suitable amounts of the purified enzyme were incubated at 30°C or 60°C. For D-glucose and D-fructose, enzyme concentrations of 330µg/ml (30°C) and 31µg/ml (60°C) were used, whilst a concentration of 430µg/ml was used for L-arabinose at 30°C. At intervals of ten or fifteen minutes 700µl samples were withdrawn from the incubations and added to 300µl aliquots of 15% (w/v) trichloroacetic acid to stop the reaction. After centrifugation to remove the precipitated protein, the supernatants were assayed for the isomerisation product. Initial rates were determined from graphs of product concentration against time. Triplicate determinations of the rate were made for each concentration of D-glucose and D-fructose, and duplicate determinations for each concentration of L-arabinose, using separate incubations. In addition a blank incubation, containing no isomerase, was carried out at each concentration of each substrate; the non-enzymic rates of isomerisation determined from the blanks were subtracted from the rates obtained in the presence of the enzyme. Figures 8d to 8h present the plots obtained for these substrates.

Glucose concentration was determined as described in Chapter III, whilst fructose concentration was determined at Billingham by Mr T. Ogden, using the automated version of the resorcinol method. L-ribulose, the isomerisation product of L-arabinose was quantitated by the cysteine/carbazole method described in Chapter III. Since no L-ribulose was available with which to calibrate this assay, the value

Figure 8d: [s]/v against [s] Plot for D-Glucose at 60°C

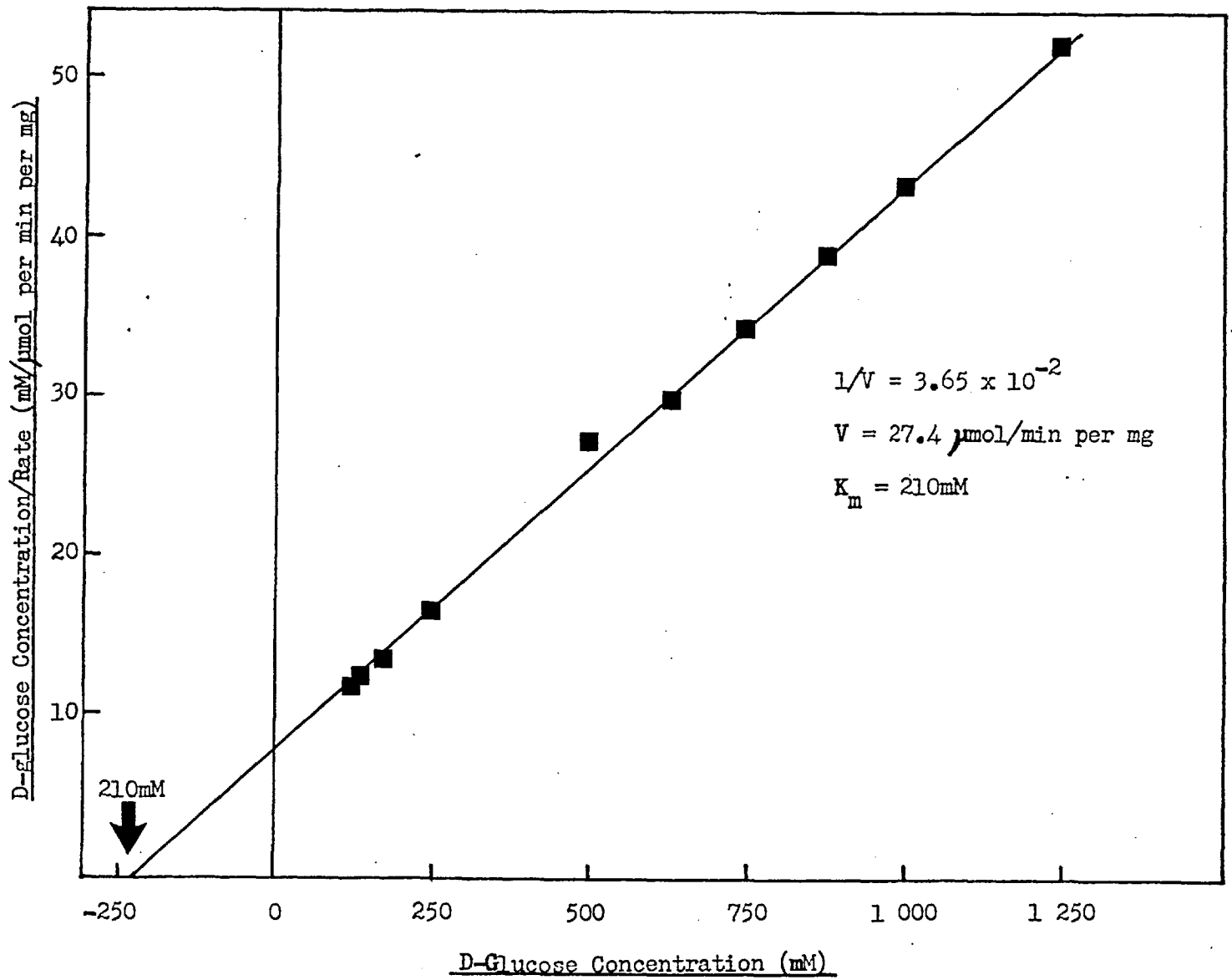


Figure 8e; [s]/v against [s] Plot for D-Fructose at 60°C

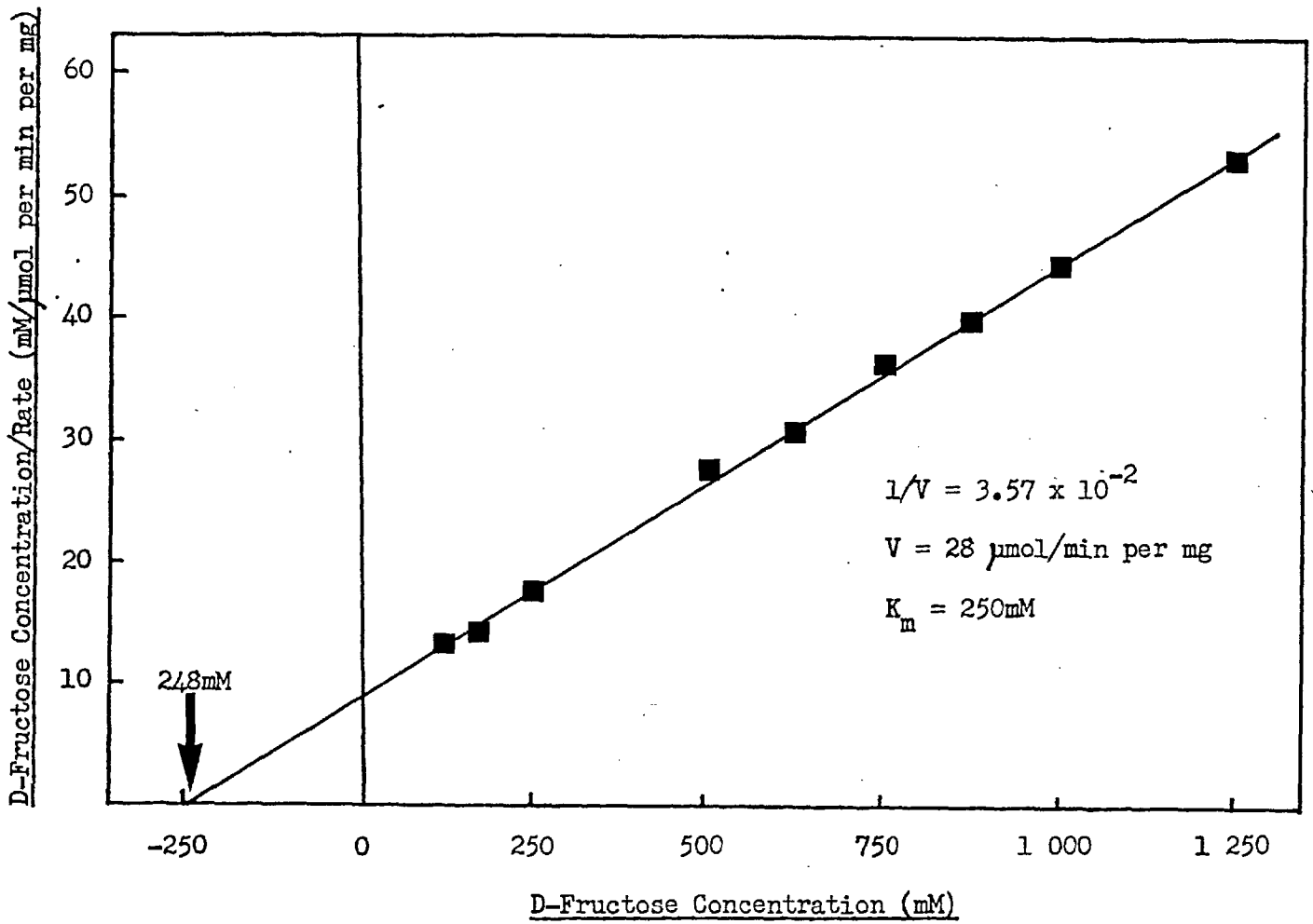


Figure 8f: [S]/v against [S] Plot for D-Glucose at 30°C

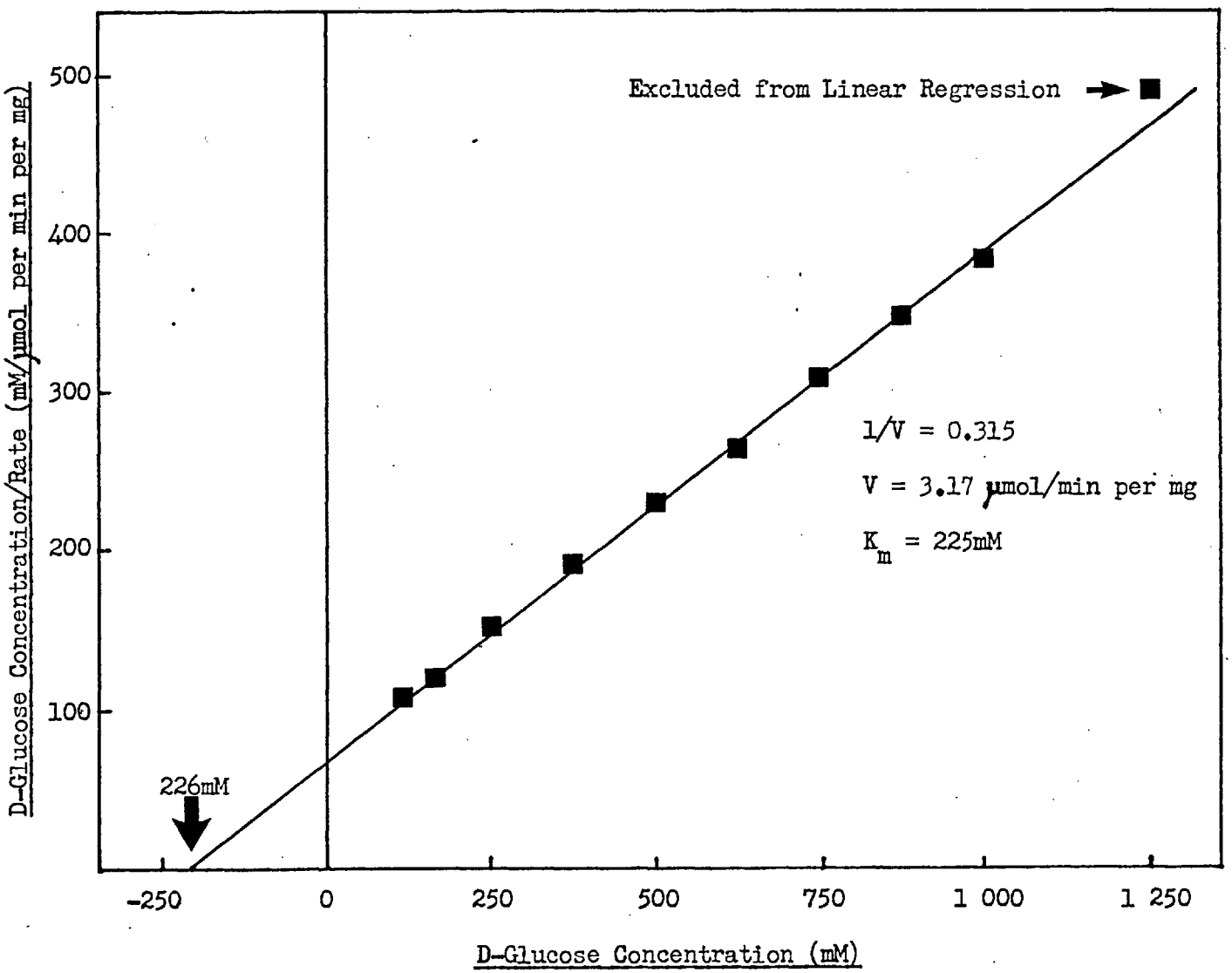


Figure 8g; [s]/v against [s] Plot for D-Fructose at 30°C

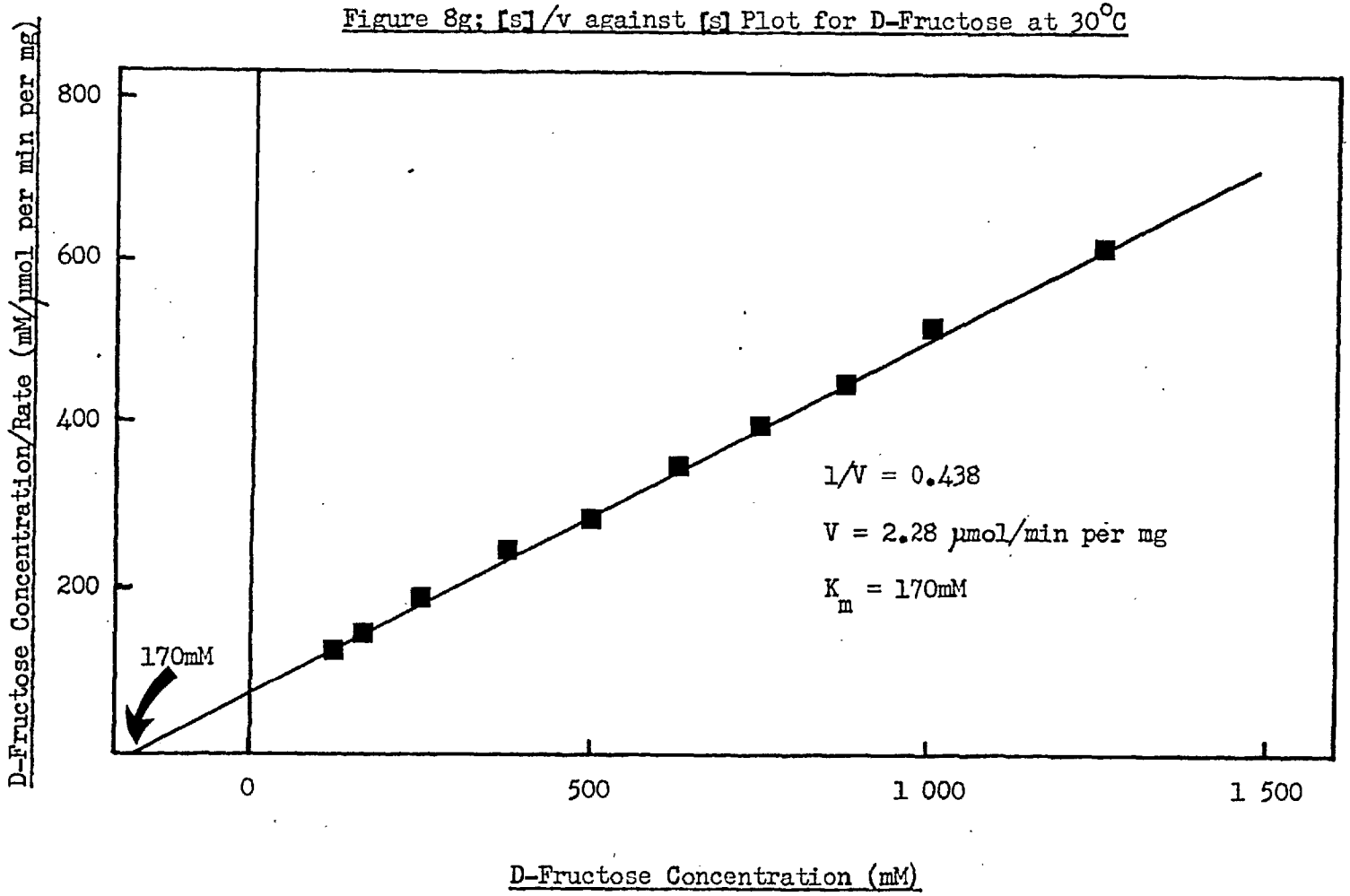
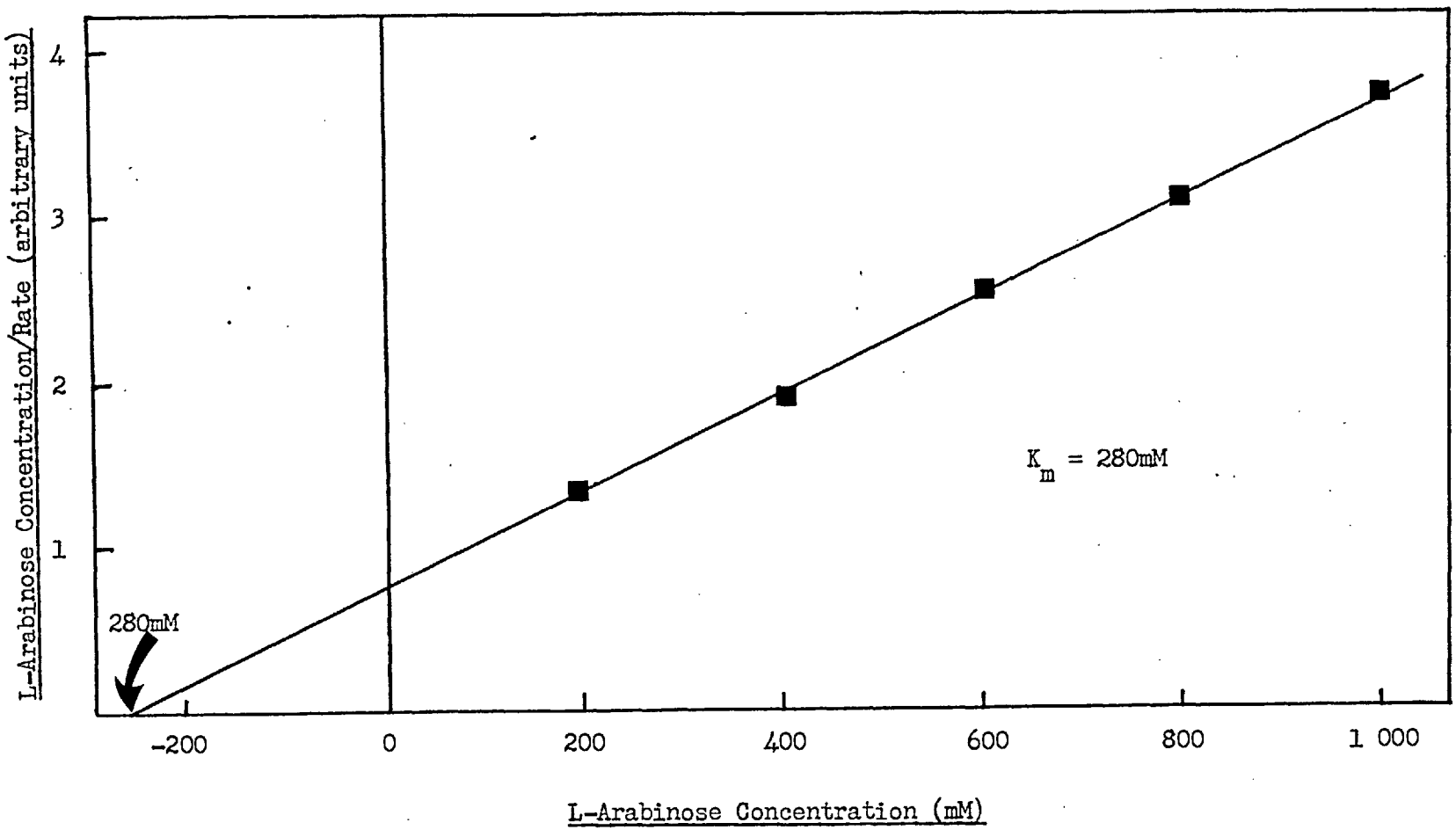


Figure 8h; [s]/v against [s] Plot for L-Arabinose at 30°C



N.B. [s]/v is expressed in arbitrary units, for reasons given in the text.

of V for L-arabinose was estimated by a method based on the assumption that equal amounts of ribulose and xylulose give the same absorbance reading under the assay conditions used. A known amount of the purified isomerase was incubated with 1M-D-xylose under the same conditions as were used for L-arabinose. Samples were removed at suitable times, added to trichloroacetic acid, and assayed by the cyteine/carbazole method in parallel with those from the incubations containing L-arabinose. The results were used to calculate the ratio of the values of V for L-arabinose and D-xylose, and hence to deduce a value of V for L-arabinose, using the value obtained for D-xylose using the coupled assay system as described below. This method was clearly liable to considerable experimental error, in addition to the uncertainty of the assumption on which it was based; the value of V for L-arabinose given in Table 8a should therefore be treated as only an approximate estimate.

In order to test L-xylose, L-lyxose and L-rhamnose as substrates, purified isomerase (310µg/ml) was incubated with L-xylose (1M), L-rhamnose (1M) or L-lyxose (750mM) in 100mM-Tricine.NaOH/30mM-$MgCl_2$ pH 8.0 at 30°C. Samples were withdrawn from each incubation at ten minute intervals, treated as described above, and assayed for ketose by the cysteine/carbazole method. The rate of non-enzymic isomerisation of each sugar was determined from a blank incubation. No enzymic isomerisation of L-xylose or L-rhamnose was detected after incubation for 60 minutes (i.e. less than 0.02µmol ketose formed/min per mg enzyme). The results obtained for L-lyxose suggested that the enzyme has slight activity towards this substrate (estimated to be about 0.05µmol L-xylulose formed/minute per mg enzyme at 750mM-L-lyxose; cf. 8.9µmol D-xylulose formed/minute per mg enzyme from a similar concentration of D-xylose). Since L-lyxose has the same

configuration at the C_2 position as the substrates D-xylose, D-ribose, L-arabinose and D-glucose (Figure 8c), it is probable that the enzyme is indeed able to catalyse the isomerisation of L-lyxose to L-xylulose. The fact that L-lyxose differs in configuration from the physiological substrate of the isomerase, D-xylose, at both the C_3 and C_4 positions is consistant with the finding that it is a very poor substrate for the enzyme. The possibility of using this substrate as a carbon source for the selection of mutants with elevated levels of the isomerase is discussed in Chapter X.

For D-xylose a continuous spectrophotometric assay was used, in which the formation of D-xylulose was coupled to the oxidation of NADH by means of D-arabitol dehydrogenase, whilst for D-ribose ribitol dehydrogenase was used to couple D-ribulose formation to NADH oxidation. Both assays were performed as described in Chapter III, except that the concentration of the substrate was varied. Three determinations of the rate were made for each substrate concentration. The results obtained are presented in Figures 8i to 8k. Figure 8j shows a direct plot of v against [s] for D-xylose; at high substrate concentrations the observed rate of isomerisation exceeded V, the predicted maximum forward velocity, which was obtained from the data for low concentration of D-xylose (Figure 8i) assuming that Michaelis-Menten kinetics were obeyed. This finding could be a trivial consequence of the use of a coupled assay system. In order to obtain any given rate of reaction from the coupling enzyme (in this case D-arabitol dehydrogenase) it is necessary for a certain finite concentration of its substrate (in this case D-xylulose) to be present. Since the substrate of the coupling enzyme is the product of the reaction under study, the observed rate of the coupled assay system will not be the true initial rate obtained in the absence of the product, but will be

Figure 8i; [s]/v against [s] Plot for D-Xylose at 30°C

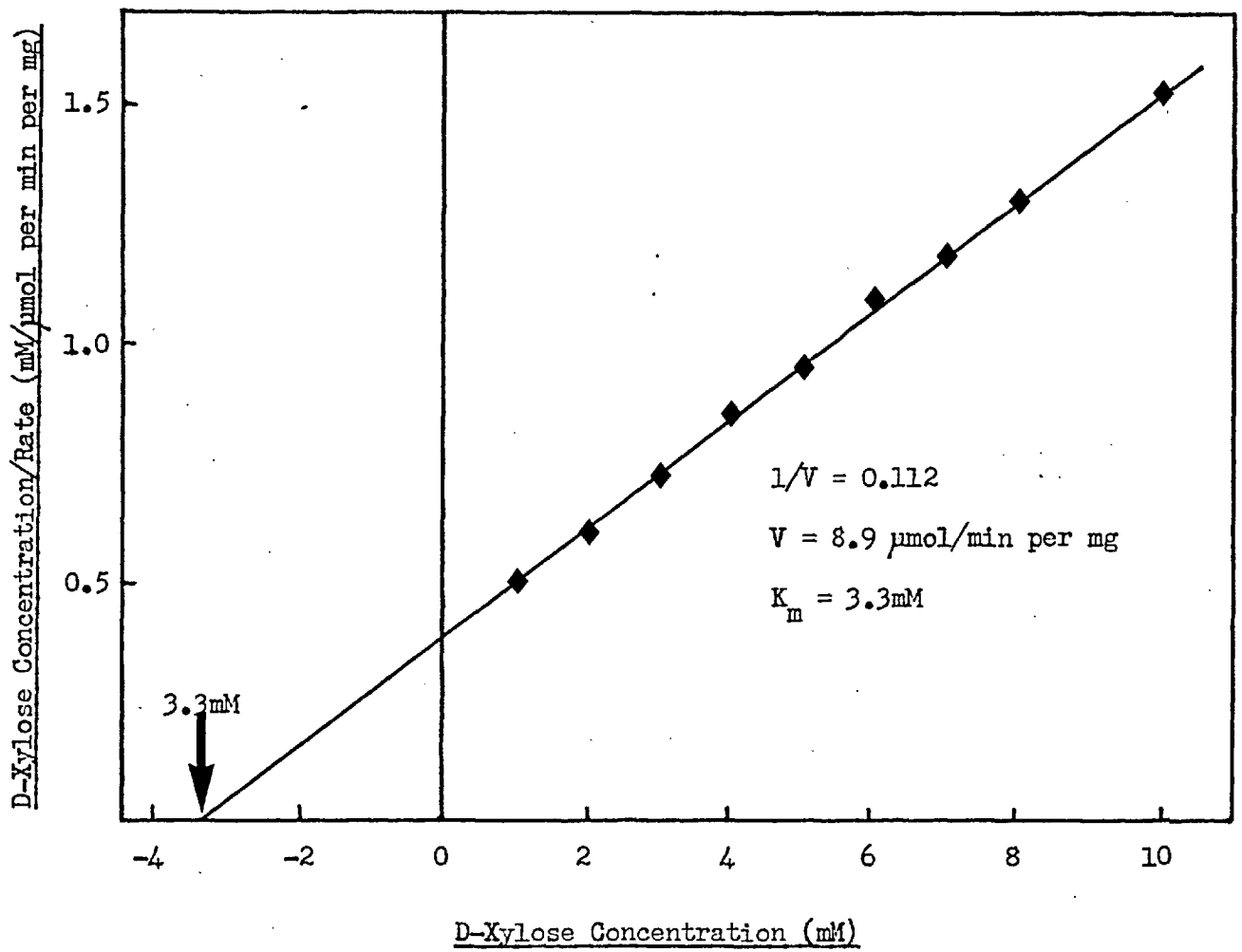


Figure 8j; Direct Plot of Rate against D-Xylose Concentration

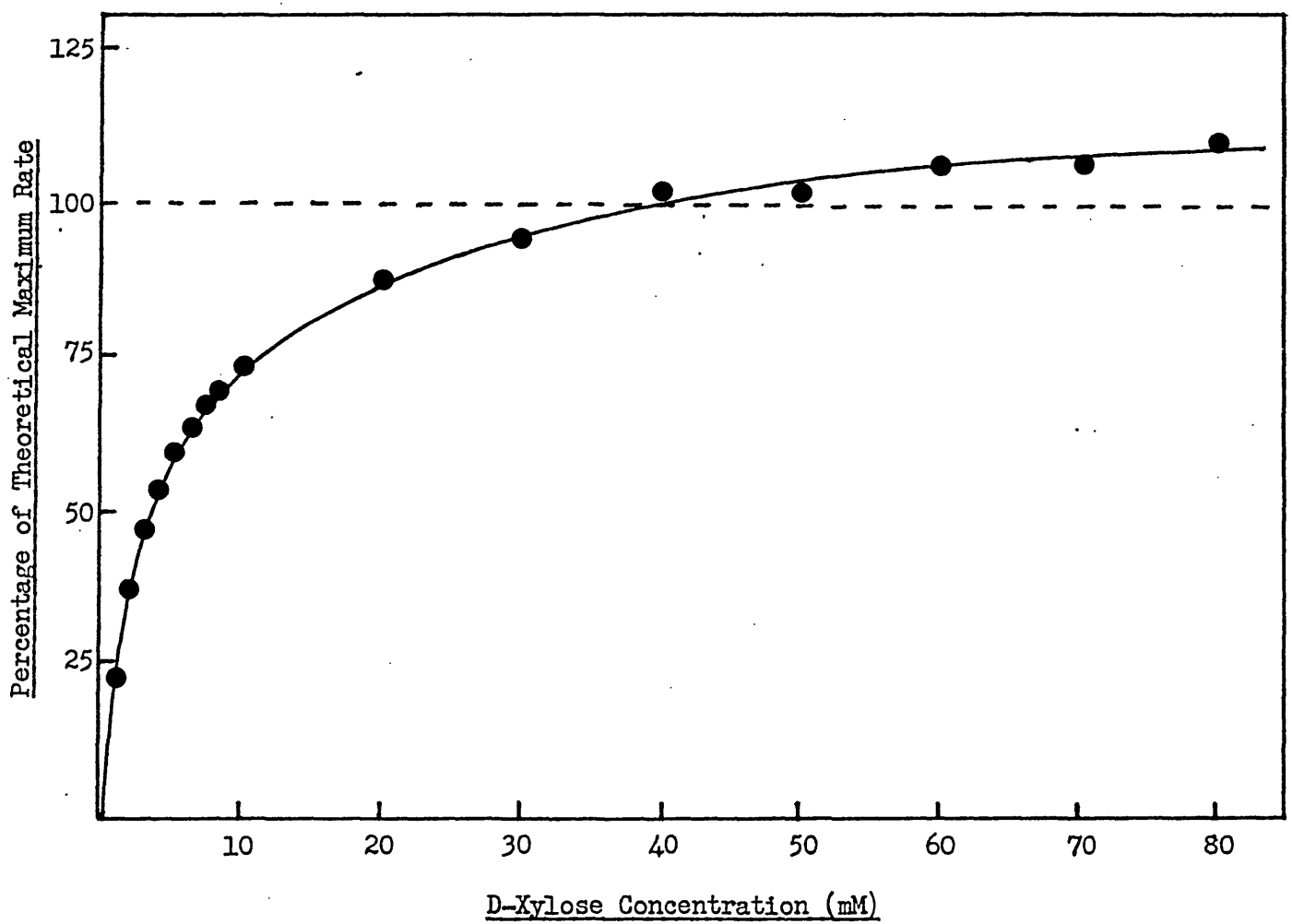
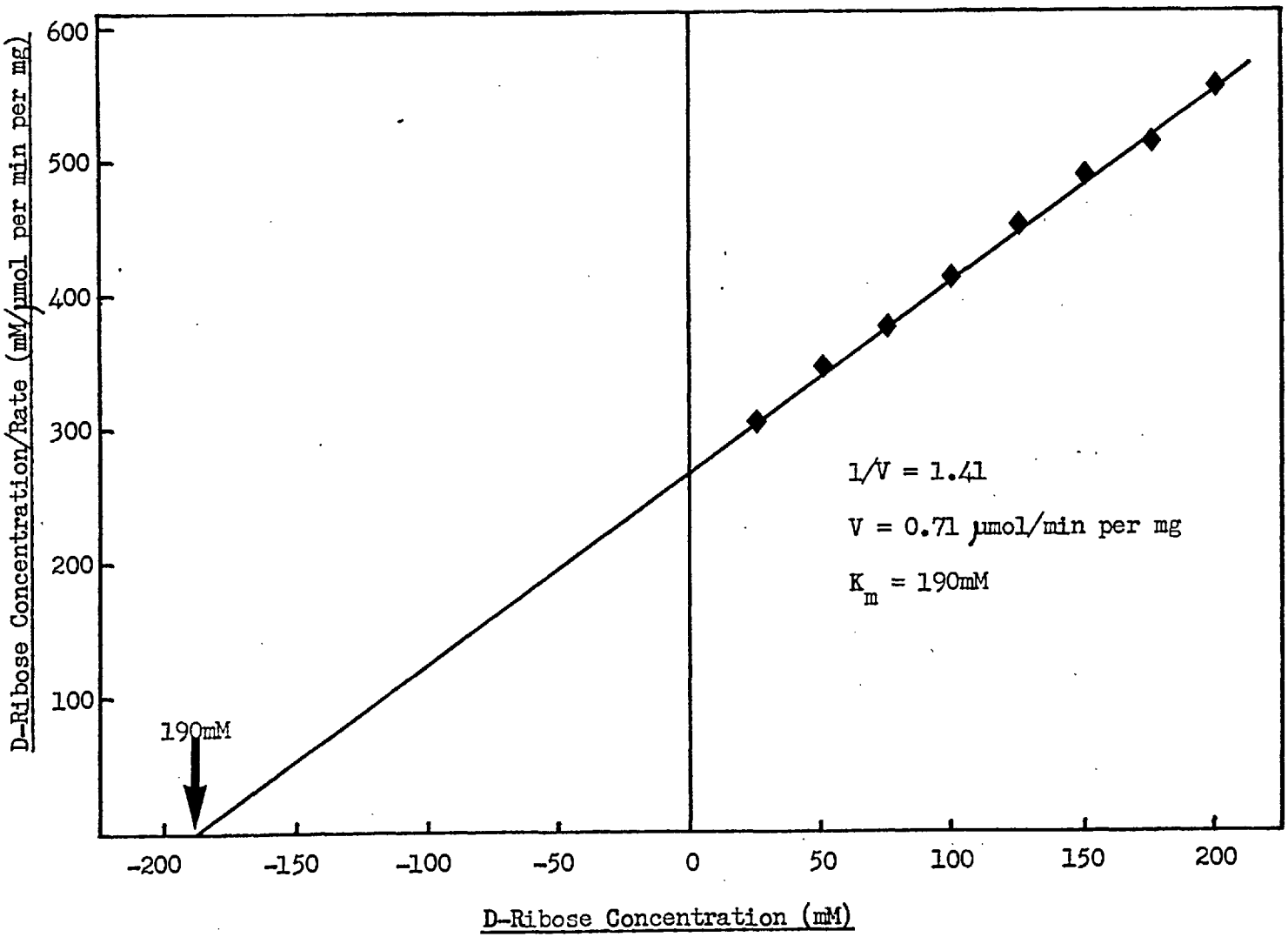


Figure 8k; [s] /v against [s] Plot for D-Ribose at 30°C



a lower rate corresponding to a finite product concentration. Since a large excess of the coupling enzyme is used, the concentration of its substrate needed will be low, and will be approximately proportional to the observed rate of the coupled reaction. The difference between this observed rate and the true initial rate of reaction will therefore be greatest at the lowest concentration of the substrate used and will decrease at higher substrate concentrations, as the ratio of product to substrate necessary for a steady state rate falls. Without a knowledge of the kinetic parameters of D-arabitol dehydrogenase for the reduction of D-xylulose, and of the effect of excess D-xylose on this reaction, it is not possible to assess the magnitude of this effect in the case of the coupled D-xylose isomerase assay.

Another possible explanation for the rate of isomerisation observed at high D-xylose concentrations would be that the isomerase displays positive cooperativity. In this case, the binding of the substrate to one sub-unit of a tetramer would facilitate subsequent binding of substrate molecules to the remaining sub-units, by means of a change in conformation. However, cooperative effects have not been reported in the case of other purified D-xylose/D-glucose isomerase enzymes.

Since the C_2 epimers of D-xylose (D-lyxose) and D-ribose (D-arabinose) correspond to the same pair of ketopentoses, D-xylulose and D-ribulose, it was possible to use the coupled assay systems described above to determine whether either was a substrate for the purified isomerase. No activity was detected towards 400mM-D-arabinose (i.e. less than 0.002μmol D-ribulose formed/minute per mg isomerase). However, in the presence of 400mM-D-lyxose a significant rate of generation of D-xylulose was observed (0.07μmol/minute per mg enzyme used).

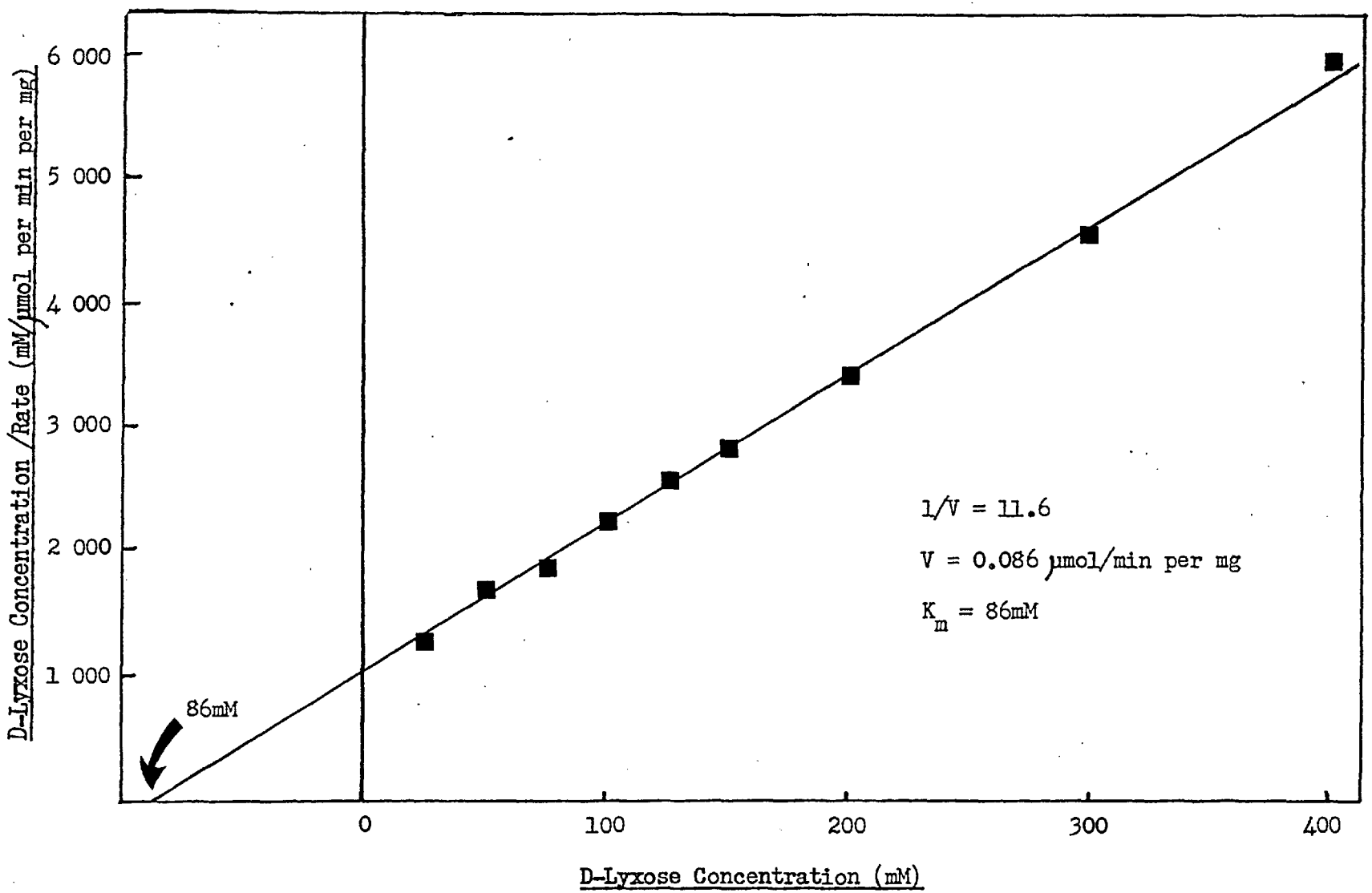
D-Lyxose as a Substrate for the Isomerase

Since D-lyxose differs in its configuration at the C_2 position from D-xylose and the other known substrates of the isomerase (Figure 8c), and it is at the C_2 position that the hydroxyl group is replaced by a carbonyl group during isomerisation, it would appear a priori improbable on structural grounds that the enzyme would show activity towards D-lyxose. Several workers (e.g. Yamanaka, 1968; Anderson & Allison, 1965; Parker, 1978) have indeed reported that D-lyxose and D-xylose isomerising enzymes from various bacterial species show no cross specificity. However, it should be noted that the assays used by these workers were not so sensitive as the coupled assay used in this work, and might therefore have failed to detect levels of activity similar to that observed for the Arthrobacter enzyme with D-lyxose.

The ketopentose corresponding to D-lyxose is D-xylulose, which is a normal intermediate in the catabolism of D-xylose. It therefore appeared that, if D-lyxose is indeed a poor substrate for the isomerase, it would provide an ideal selective carbon source for the isolation of strains constitutively producing elevated levels of the enzyme. The kinetics of the apparent isomerisation of D-lyxose were therefore investigated. Because the activity observed was very low it was necessary both to use high enzyme concentrations (550µg/ml) and to follow the rate of change of absorbance for up to 45 minutes; therefore only one determination of the rate was made at each concentration of D-lyxose. The results obtained obeyed Michaelis-Menten kinetics, and are presented in Figure 81.

The possibility remained that the generation of D-xylulose observed using commercially obtained D-lyxose as substrate was in fact due to contamination with trace amounts of D-xylose. However, the argument given below suggests that contamination with D-xylose could

Figure 81: [s]/v against [s] Plot for D-Lyxose at 30°C



not account for the observed extent of the reaction.

If the results observed in the presence of the commercial D-lyxose were due to the isomerisation of D-xylose, it is reasonable to suppose that the D-lyxose present would have acted as a competitive inhibitor, since it is a structural analogue of D-xylose. Such competitive inhibition by D-lyxose has been demonstrated in the case of the D-xylose isomerase of Lactobacillus brevis (Yamanaka, 1969). When an enzyme catalysing a single substrate reaction is subjected to competitive inhibition, the normal Michaelis-Menten rate equation is replaced by:

$$v = \frac{V}{1 + \frac{K_m}{[s]} + \frac{[i]K_m}{[s]K_i}}$$

Where v is the initial forward velocity of the reaction at a substrate concentration [s], in the presence of inhibitor at a concentration [i], and V, K_m and K_i are the maximum forward velocity, Michaelis constant and inhibitor constant (Dixon & Webb, 1964). Since the ratio of D-lyxose to D-xylose in the preparation of D-lyxose used was constant, we may substitute $R = \frac{[i]}{[s]}$ and obtain:

$$v = \frac{V}{1 + \frac{K_m R}{K_i} + \frac{K_m R}{[i]}} = \frac{V \Big/ \left(1 + \frac{K_m R}{K_i}\right)}{1 + \frac{K_m R \Big/ \left(1 + \frac{K_m R}{K_i}\right)}{[i]}}$$

If [s] is much less than [i], [i] is effectively equal to the total concentration of the commercial D-lyxose preparation used. The rate equation has the form of a normal Michaelis-Menten equation, where the apparent values of V and K_m for D-lyxose are given by:

$$V^{lyx}_{app} = \frac{V^{xyl}}{1 + \frac{K_m^{xyl} \cdot R}{K_i}} \qquad K^{lyx}_{m,app} = \frac{K_m^{xyl} \cdot R}{1 + \frac{K_m^{xyl} \cdot R}{K_i}}$$

Substitution of the values of V^{lyx}_{app}, V^{xyl}, $K^{lyx}_{m,app}$ and K^{xyl}_{m} from Table 8a into these simultaneous equations yields a value of 2,700 for R.

Therefore, if the apparent isomerisation of D-lyxose resulted from the isomerisation of D-xylose, only 1/2,700 of the commercial D-lyxose preparation should have been convertable to D-xylulose by the action of the isomerase. In order to test this prediction, two 10ml samples containing 100mM-D-lyxose in 100mM-Tricine.NaOH/30mM-$MgCl_2$ pH 8.0, one containing 7mg of the purified isomerase, the other with no addition, were incubated at 30°C for 36 hours. The concentration of D-xylulose in each incubation was then determined by adding samples to cuvettes containing NADH and D-arabitol dehydrogenase and measuring the total change in A_{340} which occurred. No D-xylulose was detected in the blank incubation, whilst the change in absorbance observed when samples from the incubation containing the isomerase were assayed corresponded to a D-xylulose concentration of about 80μM, indicating that the isomerisation of about 1/25 of the aldopentose present in the incubation had occurred. This was inconsistant with the predicted maximum of 1/2,700 based on the assumption that D-xylose was responsible for the activity observed.

It was therefore concluded that D-lyxose is indeed a substrate for the Arthrobacter D-xylose isomerase. Evidence is presented in Chapter X that the activity of this enzyme towards D-lyxose permits mutants constitutive for its synthesis to utilise this sugar as sole carbon/energy source.

It is possible to separate D-xylose and D-lyxose by ion exchange chromatography in the presence of borate (Yamanaka, ~~1968~~ 1968a). If time had permitted, this method would have been used to demonstrate the purity of the commercial D-lyxose preparation.

Discussion of Substrate Specificity and Kinetic Parameters

The substrate specificity of the purified isomerase from Arthrobacter strain B3728 was similar to those of other purified D-glucose isomerising enzymes, several of which are reported to isomerise D-ribose and L-arabinose in addition to D-xylose and D-glucose. The unexpected activity of the Arthrobacter enzyme towards D-lyxose, the epimer of D-xylose at the C_2 position, has been discussed above. Unfortunately the published kinetic parameters for the enzymes from other species were determined under a variety of conditions of temperature and pH, rendering comparison with those obtained for the Arthrobacter enzyme difficult. However, it is of interest to note that the value of K_m obtained for D-glucose at 60°C and pH 8.0 (210mM) is lower than those obtained under similar conditions for other purified isomerase enzymes, except those from Bacillus coagulans (90mM at 50°C and pH 7.0; Danno et al., 1967) and Streptomyces albus (160mM at 70°C and pH 7.0; Takasaki et al., 1969). Because of the different temperatures involved, comparison of the values of V reported for these enzymes would not be meaningful.

The value of K_m is not normally equal to the dissociation constant of the substrate-enzyme complex; however, it will generally reflect the strength of substrate binding (Fersht, 1977). It is therefore of interest to discuss briefly the values of K_m and V for the purified Arthrobacter isomerase in terms of the stereochemical configurations of the various substrates concerned. In order of increasing K_m (weaker binding) at 30°C, the aldose substrates of the isomerase are: D-xylose, D-lyxose, D-ribose, D-glucose and L-arabinose (Table 8a; Figure 8c). Considered naively, this order suggests that the configuration of the substrate at the C_2 position is least, that at the C_3 position second and that at the C_4 position most important

for the specificity of substrate recognition and binding, whilst the effect of the addition of a hydroxymethyl group to the C_5 position on the binding of the substrate is intermediate between the effects of epimerisation at the C_3 and C_4 positions.

However, it must be pointed out that these aldo-sugars exist in solution as mixtures of the α and β-pyranose, α and β-furanose and open chain forms; there is considerable variation in the fraction present in each form at equilibrium. At neutral pH a solution of ribose contains significant amounts of all five species, with about 30% in the open chain form, whilst the other aldopentoses and glucose are present mainly in the pyranose ring forms (Cantor & Peniston, 1940; Pigman & Horton, 1972). In so far as K_m reflects substrate binding, it reflects the dissociation constant expressed in terms of the total substrate concentration, rather than in terms of the concentration of the particular species which binds to the enzyme. Therefore it is not really valid to attempt to interpret the order of the K_m values for different substrates in terms of their configurations without making allowance for the effect of differences in the fraction of each present in each form.

This problem does not apply to the interpretation of the order of the values of V obtained for different substrates, since these reflect the rate of isomerisation once the substrate is already bound to the enzyme. The relative values of V for the various substrates of the Arthrobacter isomerase, in decreasing order are: D-xylose (100), D-glucose (36), L-arabinose (21), D-ribose (8) and D-lyxose (1). This indicates that substitution at the C_5 position, remote from the end of the molecule at which isomerisation takes place, has relatively little effect on the rate of reaction, whilst epimerisation at the C_4, C_3 or C_2 position has an increasingly large effect (Figure 8c).

As has been discussed above, it is surprising that the enzyme shows any activity towards D-lyxose. It may be shown by thermodynamic arguments that an enzyme which is well adapted to its substrate should have maximised V/K_m, since this minimises the activation energy for the enzymic reaction (Fersht, 1977). Table 8a lists the value of V/K_m for each of the aldose substrates of the isomerase. The order of the values is the same as that of the values of V, although the difference between D-xylose, the physiological substrate of the enzyme, and the other 'gratuitous' substrates is emphasised.

The following equation relates the equilibrium constant for a one substrate reaction catalysed by an enzyme which is not subject to allosteric regulation by either the substrate or the product and the kinetic parameters for the forward and back reactions:

$$K^{eq} = \frac{V^f . K_m^b}{V^b . K_m^f}$$

Where K^{eq} is the equilibrium constant, K_m^f and K_m^b are the Michaelis constants for the forward and back reactions, and V^f and V^b the maximum velocities of the forward and back reactions (Dixon & Webb, 1964). Substitution of the values of V and K_m for D-glucose and D-fructose determined for the Arthrobacter enzyme into this equation (treating the formation of D-fructose as the forward reaction) yields values of the equilibrium constant of 1.03 (at 30°C) and 1.14 (at 60°C), both at pH 8.0. These values correspond to equilibrium mixtures containing 51% and 53% D-fructose, respectively. Takasaki et al. (1969) reported a value of 1.15 for the equilibrium constant at 60°C and pH 7.0, determined by allowing the isomerisation to reach equilibrium and measuring the amount of ketose present. The precision with which this result agrees with the value derived from the kinetic parameters obtained for the Arthrobacter enzyme is fortuitous; nevertheless, the fact that the results are in general

agreement suggests that the values of K_m obtained for D-glucose and D-fructose are not seriously at fault. Unfortunately the equation does not provide a check of the values obtained for V, since partial inactivation of the enzyme would not have altered the ratio V^f/V^b.

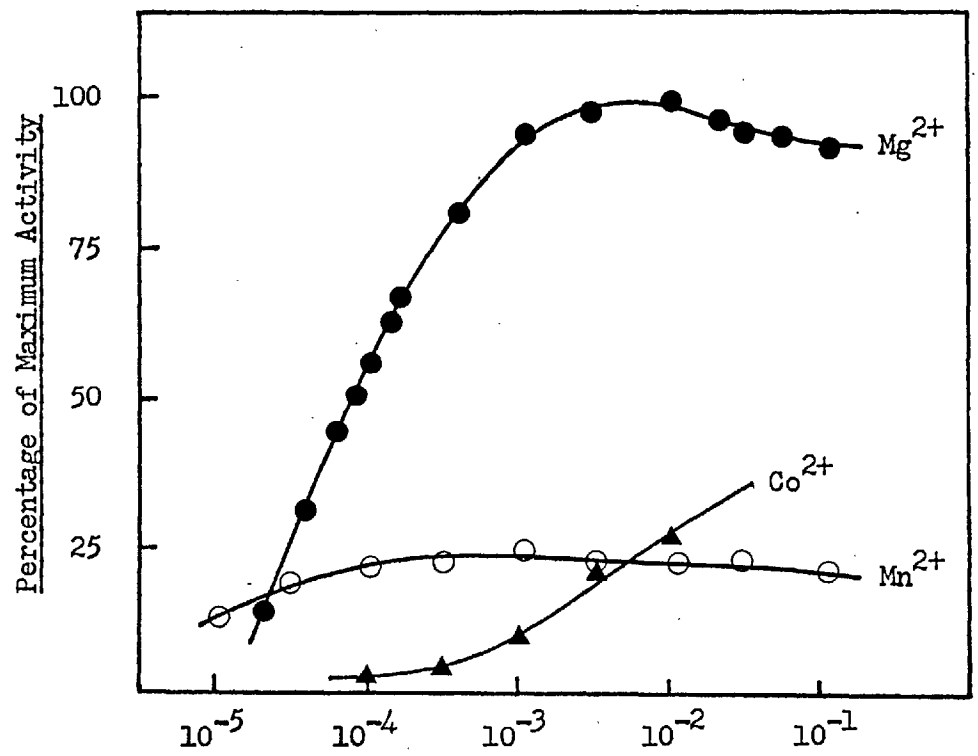
Divalent Metal Ion Requirement

All known D-xylose/D-glucose isomerase enzymes require either Mg^{2+}, Mn^{2+} or Co^{2+} as a cofactor for maximal activity. In the case of most of those isomerases which have been purified the requirement for a divalent cation is absolute, although in the case of the enzyme from Lactobacillus xylosus some activity apparently remains after dialysis against EDTA to remove divalent metal ions (Yamanaka & Takahara, 1977). In the case of the enzymes from Bacillus coagulans (Danno, 1971) and Streptomyces albus (Takasaki et al., 1969), the binding of a single divalent cation (Co^{2+}) to each sub-unit of the isomerase has been demonstrated. The role of the metal ion in the functioning of the isomerase is not clear. In the case of the enzyme from Lactobacillus brevis the metal ion (Mn^{2+}) is reported to be involved in the binding of the substrate (Yamanaka, 1969). However, in the case of the enzyme from Bacillus coagulans substrate and metal ion binding appear to be independent (Danno, 1971). This isomerase requires different metal ion cofactors for the optimal isomerisation of D-xylose (Mn^{2+}) and D-glucose or D-ribose (Co^{2+}) (Danno, 1970b; Danno 1971). Such variation in substrate specificity with metal ion cofactor has not been reported for the enzyme from any other species.

The Arthrobacter glucose isomerase is known to require Mg^{2+} for the optimal isomerisation of D-glucose (Reynolds, 1973). The use of an isomerase which requires Mg^{2+} rather than Co^{2+} is an advantage in the commercial production of high fructose syrups, since it

Figure 8m

Dependence of Isomerase Activity on Divalent Cation Concentration



The activity was determined using D-xylose as the substrate, in 100mM-Tricine.NaOH buffer. In the case of Mg^{2+} and Mn^{2+} the pH was 8.0; for Co^{2+} pH 7.0 was used, and the rate was expressed relative to the maximum rate obtained with Mg^{2+} at the same pH value.

Table 8b; Competition between Divalent Cations

	Activity
1mM-Mg^{2+} + 1mM-Ca^{2+}	28
10mM-Mg^{2+} + 1mM-Ca^{2+}	86
50mM-Mg^{2+} + 1mM-Ca^{2+}	91
1mM-Mg^{2+} + 10mM-Ca^{2+}	5
10mM-Mg^{2+} + 10mM-Ca^{2+}	22
50mM-Mg^{2+} + 10mM-Ca^{2+}	53
10mM-Mn^{2+} + 10mM-Ca^{2+}	16

	Activity
1mM-Mg^{2+} alone	91
10mM-Mg^{2+} alone	100
50mM-Mg^{2+} alone	91
10mM-Mn^{2+} alone	23
1mM-Mg^{2+} + 10mM-Mn^{2+}	25
10mM-Mg^{2+} + 10mM-Mn^{2+}	51
50mM-Mg^{2+} + 10mM-Mn^{2+}	72

avoids the need for costly treatments to reduce the concentration of the toxic Co^{2+} ion to an acceptable level both in the product and in plant effluent.

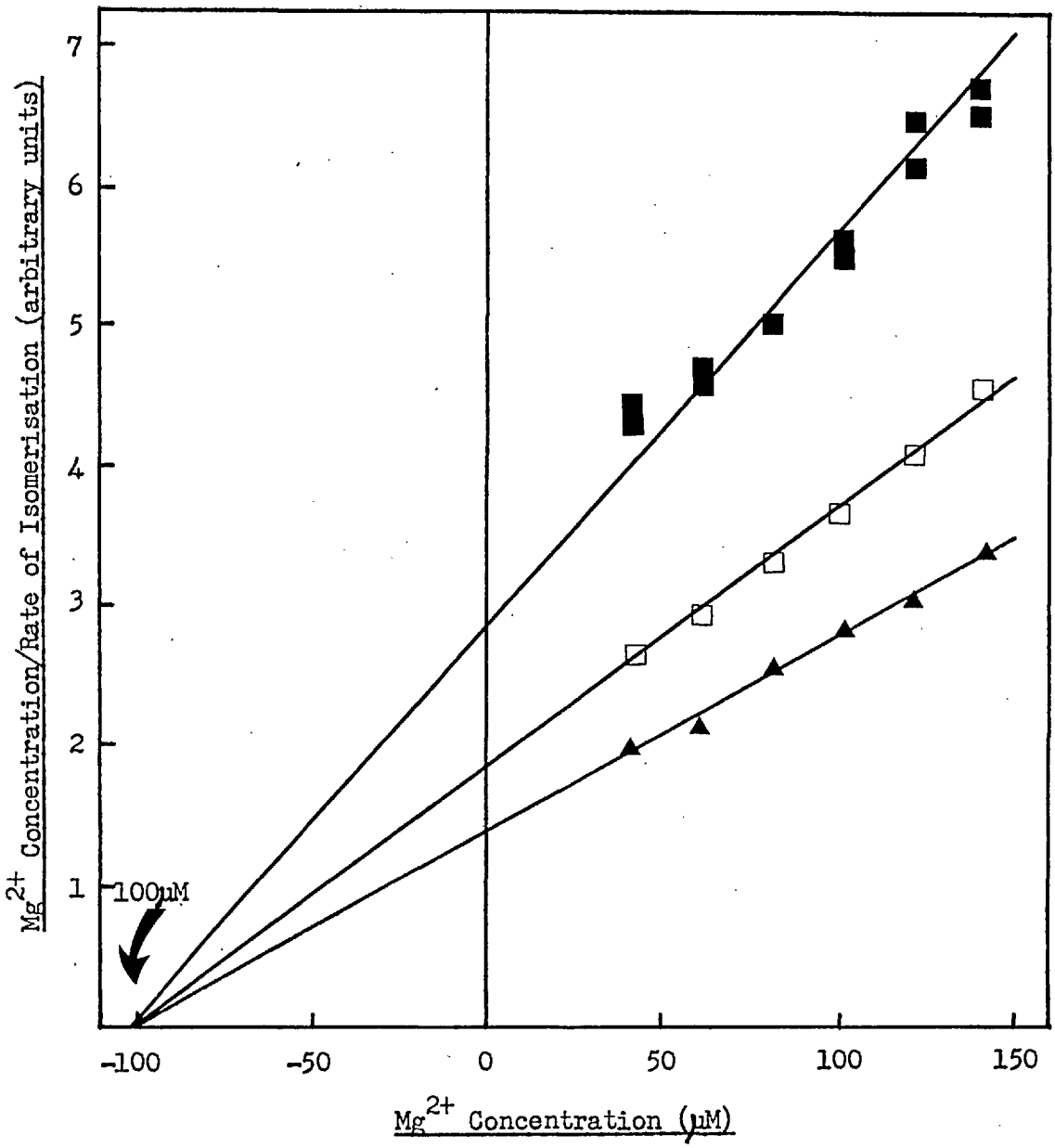
In order to test the metal ion requirement of the Arthrobacter isomerase, a sample of the purified enzyme was dialysed against 50mM-Tris.HCl/100mM-NaCl/10mM-EDTA/0.1mM-PMSF pH 7.5 (three changes of 100 volumes each) to remove the Mg^{2+} present and then against 50mM-Tris.HCl/100mM-NaCl/0.1mM-PMSF pH 7.5. Samples of the EDTA-treated enzyme were incubated at 30°C with various concentrations of divalent cations and 10mM-D-xylose, in 100mM-Tricine.NaOH pH 8.0 (except in the case of Co^{2+}, which is not soluble in degassed Tricine buffer of pH 8.0, for which Tricine buffer of pH 7.0 was used). The activity of the enzyme was determined using the spectrophotometric assay system described previously for the assay of D-xylose isomerase (Chapter III).

At 30°C the enzyme showed an absolute requirement for a divalent cation for activity towards D-xylose. Mg^{2+} gave the greatest activation, $MgCl_2$ and $MgSO_4$ being equally effective. The greatest activity was obtained with a conentration of 10mM-Mg^{2+}. Mn^{2+} and Co^{2+} were also able to activate the isomerase, but did not result in an activity as great as that achieved with Mg^{2+}. Figure 8m shows the dependence of D-xylose isomerase activity on metal ion concentration for all three cations. The dissociation constant for Mg^{2+} was estimated from plots of $v/[Mg^{2+}]$ against $[Mg^{2+}]$ in the presence of 2.5mM, 5mM and 10mM-D-xylose (Figure 8n); a value of 100μM was obtained.

The isomerase was not activated by Ca^{2+}, Ba^{2+}, Zn^{2+} or Cu^{2+} at a concentration of 10mM, whilst Fe^{2+}, Ni^{2+} and Sn^{2+} were not soluble at this concentration in the buffer used. Controls showed that the failure to observe activity in the presence of these ions was not due to inhibition of the D-arabitol dehydrogenase used to couple the assay.

Figure 8n

Kinetics of Mg^{2+} Concentration Dependence of Activity



The intercept corresponds to a dissociation constant for Mg^{2+} of about 100μM.

■ 2.5mM-D-xylose substrate

□ 5.0mM-D-xylose substrate

▲ 10 mM-D-xylose substrate

The presence of Ca^{2+} inhibited activation of the isomerase by either Mg^{2+} or Mn^{2+} (Table 8b); moreover, the activation of the enzyme by these two ions was not additive, and a lower rate was observed in the presence of 10mM-Mg^{2+} + 10mM-Mn^{2+} than in the presence of 10mM-Mg^{2+} alone. This suggests that these three metal ions compete for a single binding site on the enzyme, although the results could also be explained by mutually exclusive binding at separate sites, as has been proposed for the cations which activate the enzyme from Bacillus coagulans for the isomerisation of different substrates (Danno, 1971).

Inhibitors of Glucose Isomerase

The effect of xylitol and D-sorbitol as inhibitors of the D-xylose isomerase activity of the enzyme at 30°C was investigated. Since these polyols have the configurations corresponding to the substrates D-xylose and D-glucose (Figure 8o), they were expected to act as competitive inhibitors of the isomerase.

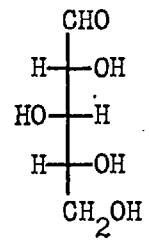
The results obtained are presented both as Dixon Plots (Figures 8p & 8q) and as Linweaver-Burk Plots (Figures 8r & 8s). The Dixon Plots were used to obtain the inhibitor constants (K_i) for xylitol and D-sorbitol. In a Dixon Plot 1/v is plotted against [i] for various values of [s], where v is the initial rate of reaction, [i] the concentration of the inhibitor and [s] the concentration of the substrate. The projection on the axis of [i] of the point of intersection of the lines obtained for various values of [s] is equal to $-K_i$ (Dixon, 1953).

The value of K_i for xylitol was about 0.3mM, whilst that for D-sorbitol was 6.5mM.

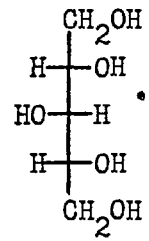
The form of the Lineweaver-Burk Plots obtained for various values of [i] is diagnostic of the type of inhibition occurring. In the case of strictly competitive inhibition, binding of the inhibitor

Figure 8o

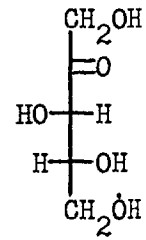
Configurations of Polyols



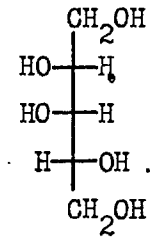
D-Xylose



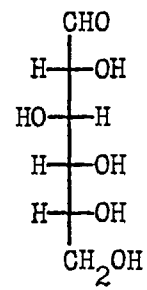
Xylitol



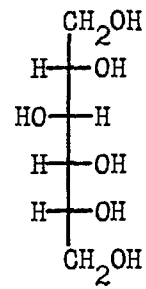
D-Xylulose



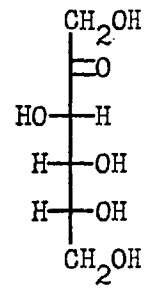
D-Arabitol



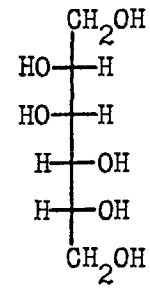
D-Glucose



D-Sorbitol



D-Fructose



D-Mannitol

Figure 8p; Dixon Plot for Xylitol Inhibition of D-Xylose Isomerase Activity

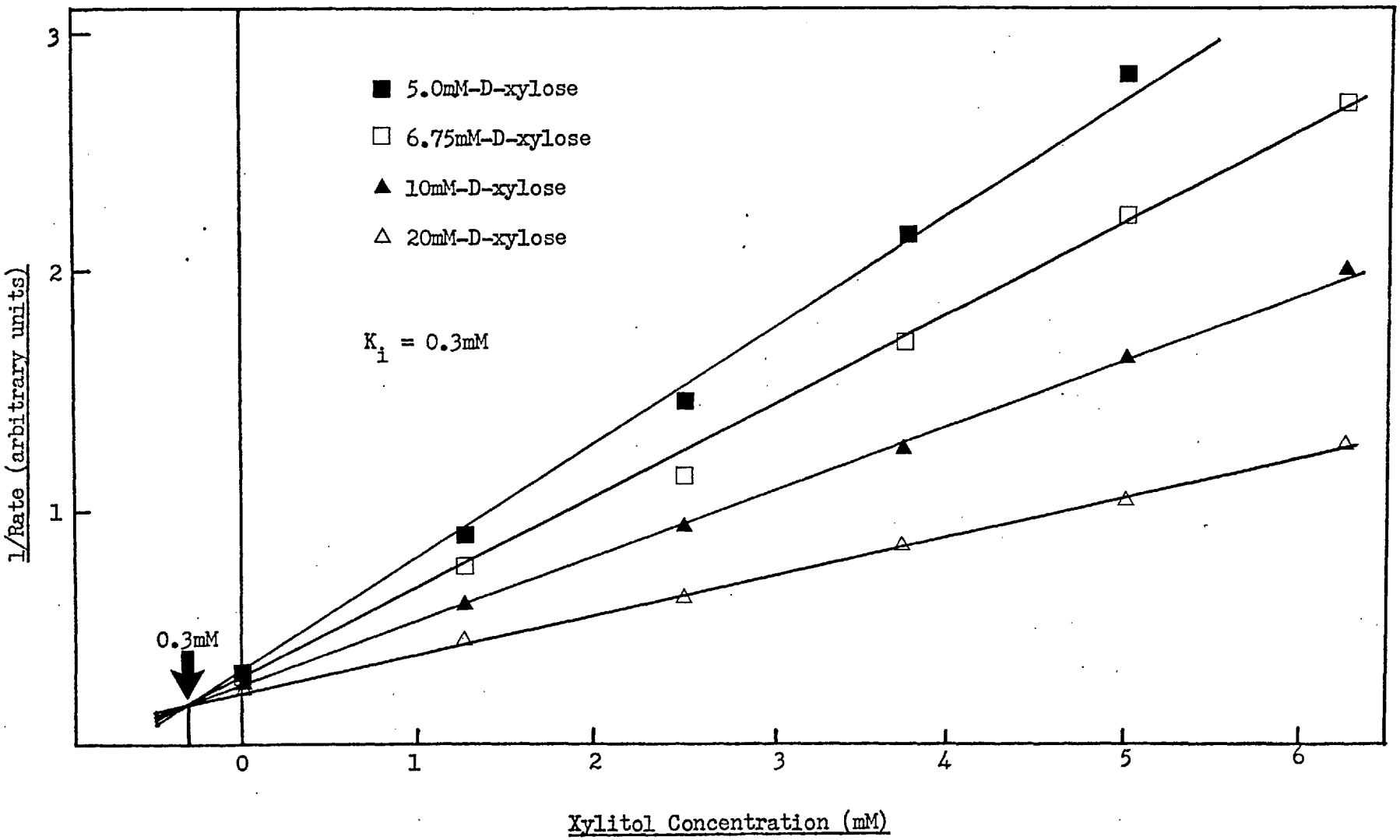


Figure 8q; Dixon Plot for D-Sorbitol Inhibition of D-Xylose Isomerase Activity

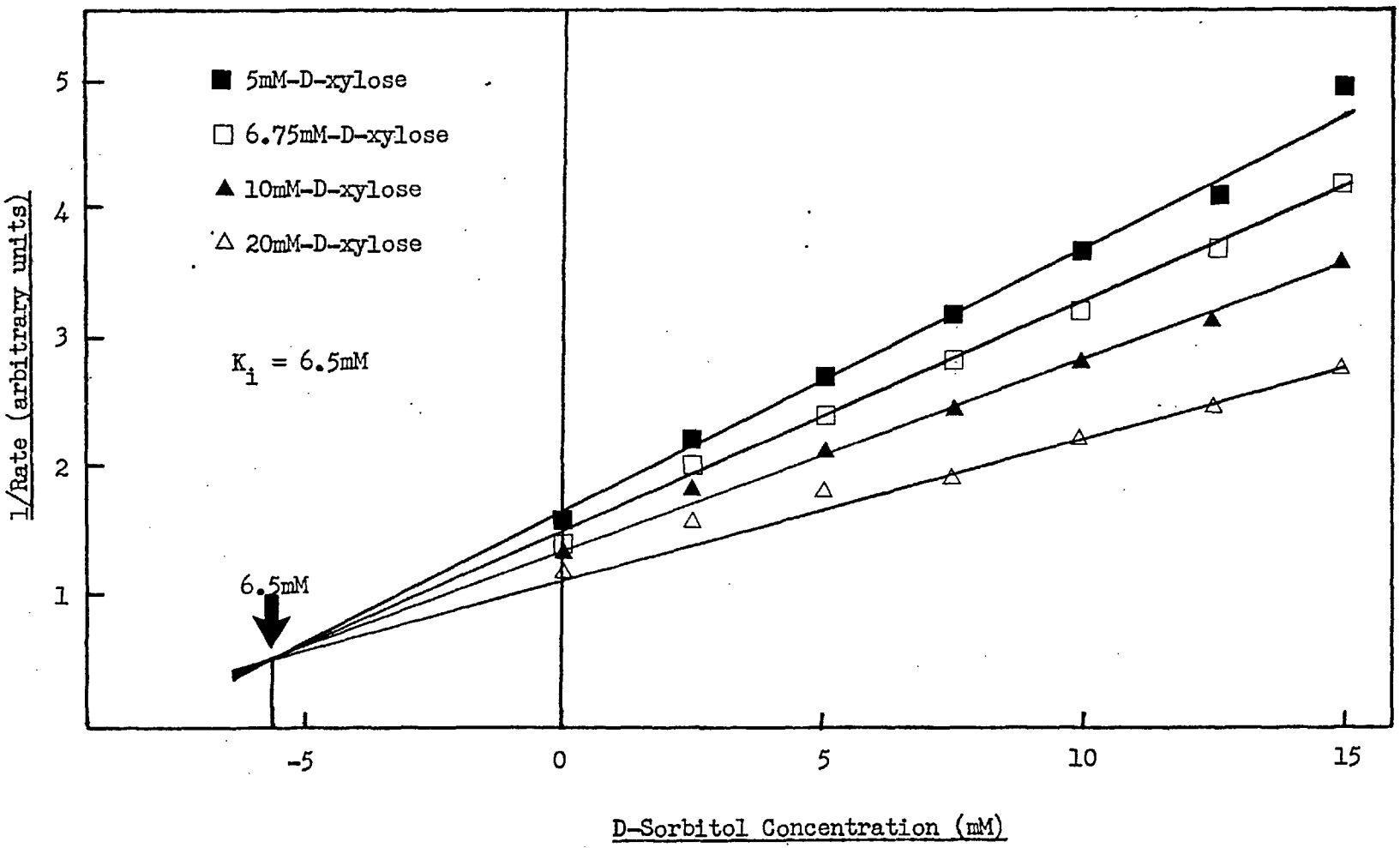


Figure 8r; Lineweaver-Burk Plots for D-Xylose Isomerase Activity in the Presence of Xylitol

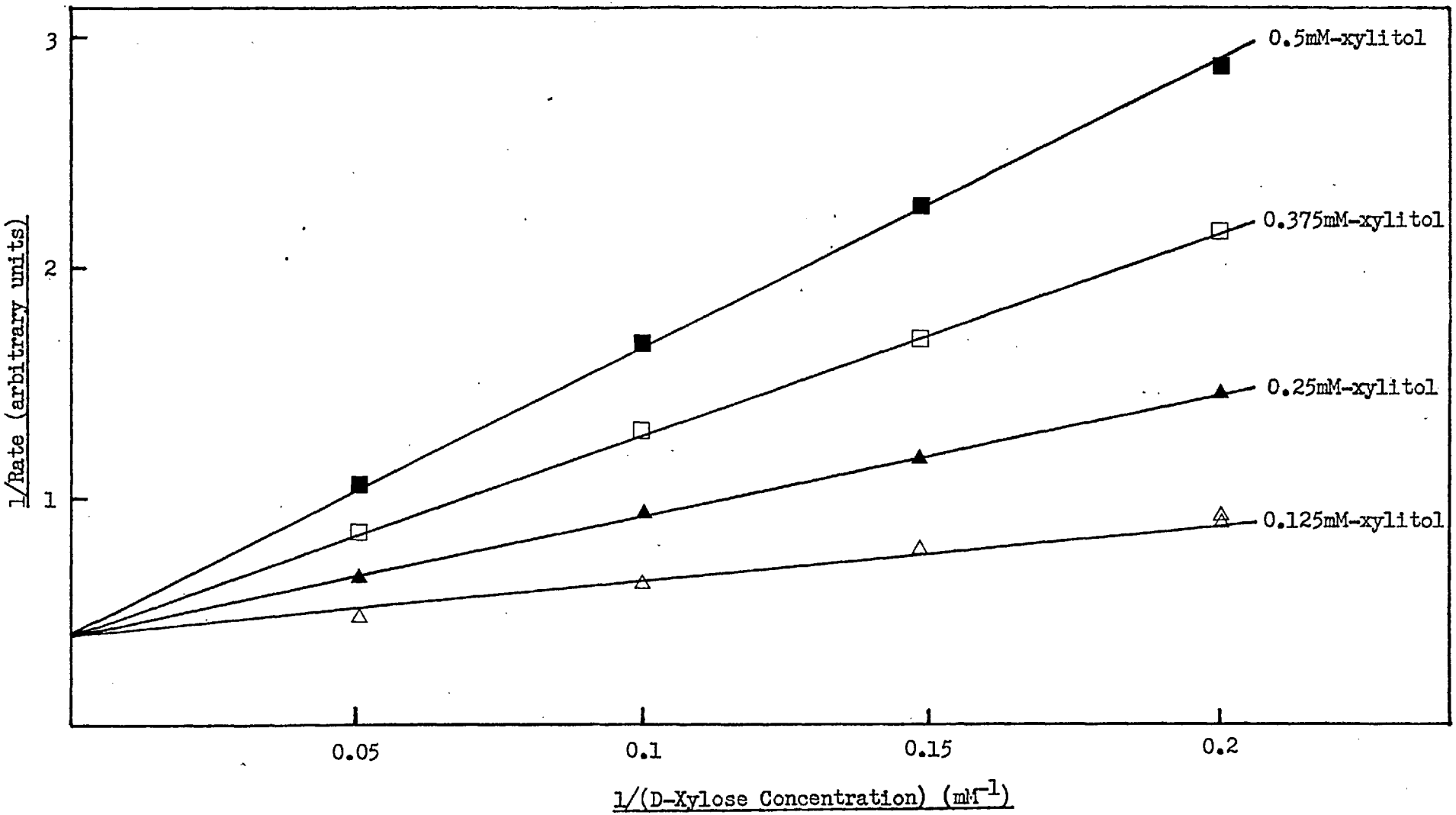
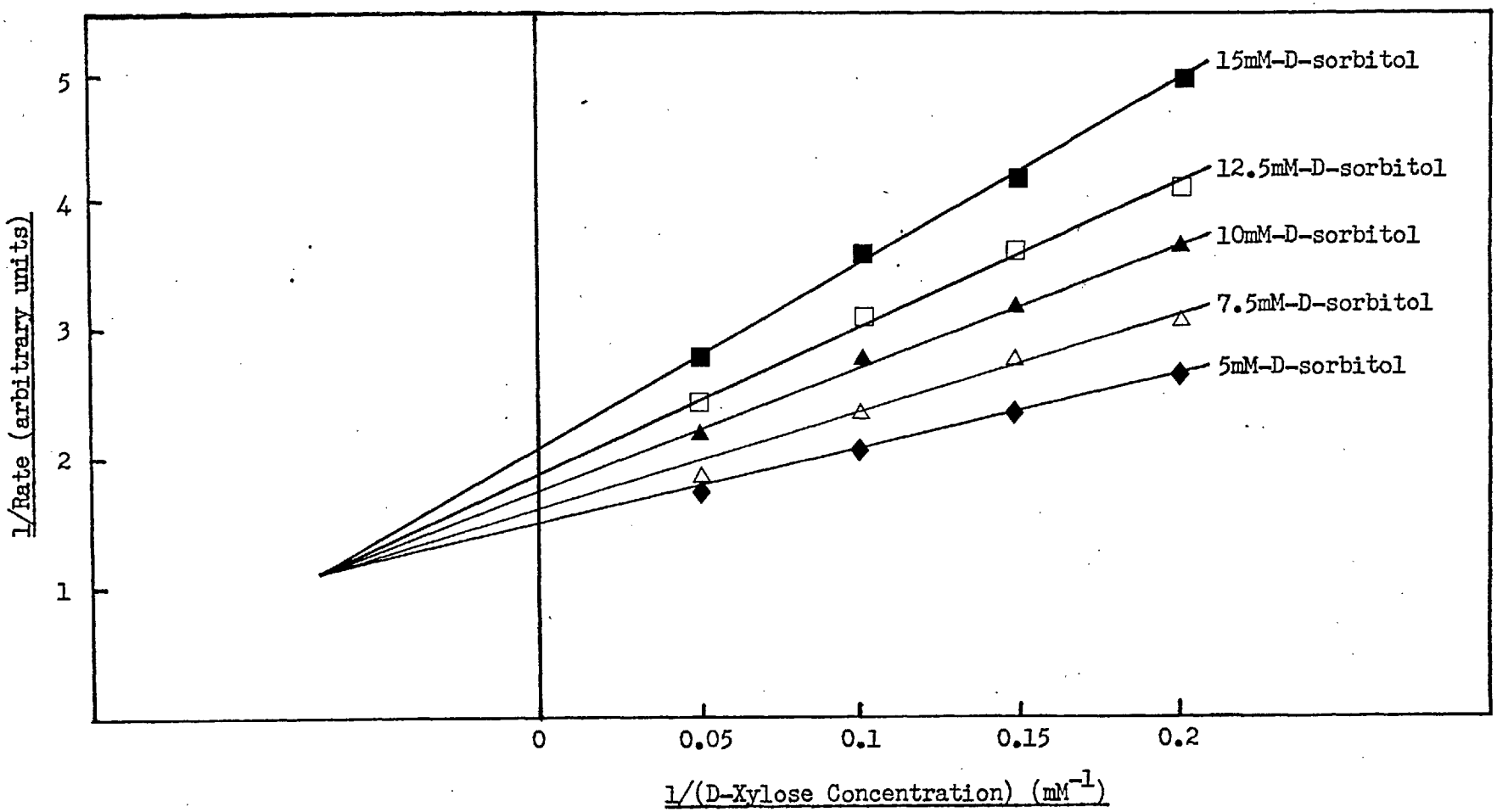


Figure 8s; Lineweaver-Burk Plots for D-Xylose Isomerase Activity in the Presence of D-Sorbitol



and substrate are mutually exclusive. This results in an increase in the apparent value of the Michaelis constant in the presence of the inhibitor, without any change in the maximum forward velocity of the reaction. In this case K_i is the dissociation constant of the enzyme-inhibitor complex (Dixon & Webb, 1964), and the velocity of the reaction is given by:

$$v = \frac{V}{1 + \frac{K_m}{[s]}\left(1 + \frac{[i]}{K_i}\right)}$$

The lines corresponding to different values of [i] all intersect on the axis of 1/v. Figure 8r shows that xylitol is a simple competitive inhibitor of the isomerase. In contrast, Figure 8s shows that D-sorbitol is a 'mixed' inhibitor; both K_m and V are altered in the presence of the inhibitor, and K_i does not have a simple physical interpretation.

A possible explanation of the different effects of xylitol and D-sorbitol may lie in the fact that, whilst the former is a structural analogue of D-xylose, the latter is an analogue of D-glucose. If different conformational states are required for an isomerase sub-unit to bind D-xylose or xylitol and D-glucose or D-sorbitol, the binding of either D-xylose or xylitol to one sub-unit of a tetramer would have the same effect on the environment of the remaining sub-units, whilst the binding of D-sorbitol would result in the other sub-units being subjected to a different environment. Thus binding of D-sorbitol by one sub-unit of the isomerase could result in changes in the catalytic properties of the remaining sub-units, leading to the 'mixed' inhibition observed. If this model were correct, the use of D-glucose in place of D-xylose as substrate would render D-sorbitol a simple competitive inhibitor and xylitol a 'mixed' inhibitor. This prediction has not been tested. It should, however, be noted that in the case of the isomerase from Bacillus coagulans, the three inhibitors xylitol,

D-sorbitol and D-mannitol all show mixed inhibition and each shows the same inhibitor constant against each of the three substrates D-xylose, D-glucose and D-ribose. This suggests that, at least in the case of this enzyme, all three substrates bind to the same conformational state of the enzyme (Danno, 1970b).

The very low K_i observed for xylitol (0.5mM; *cf* K_m 3.3mM for D-xylose) suggested that it might be possible to use this inhibitor *in vivo* to render the activity of the isomerase rate limiting for growth on its natural substrate, D-xylose. The results of attempts to use this method to select strains producing elevated levels of the isomerase are described in Chapter X.

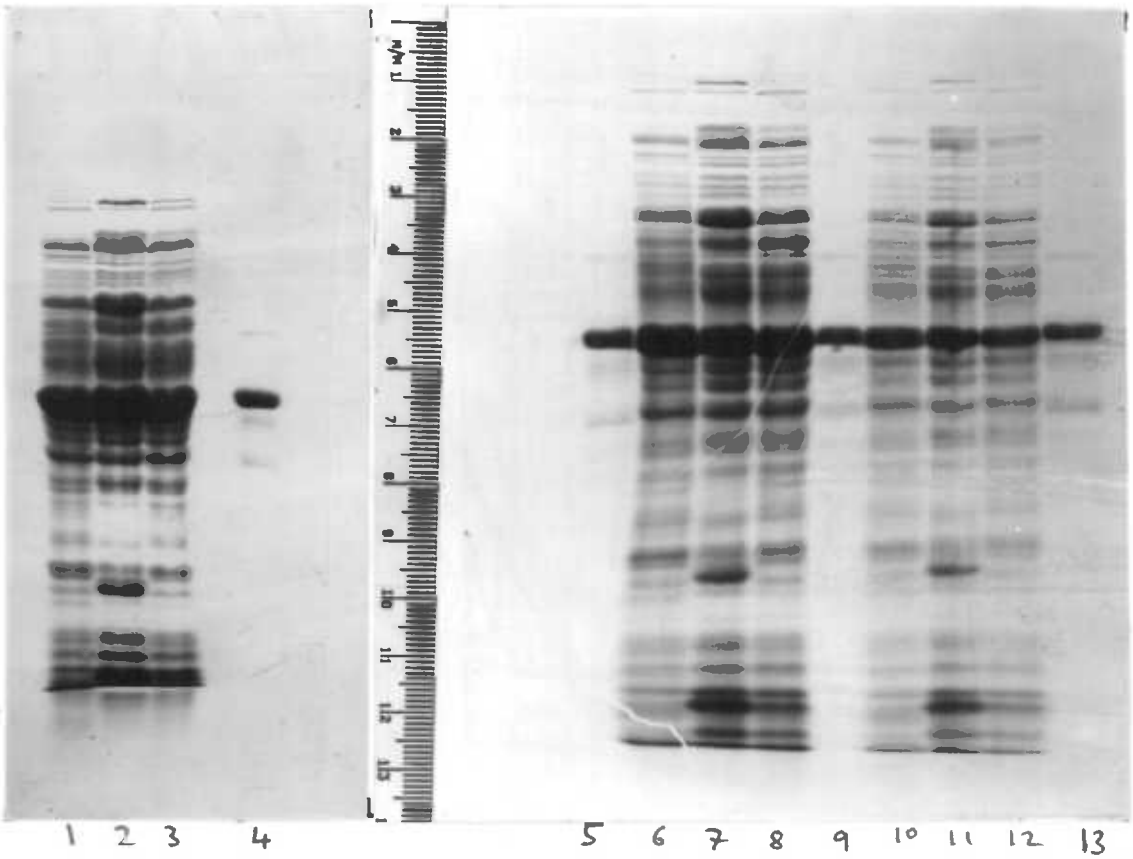
Chapter IX

Electrophoretically Altered Glucose Isomerase from Strain 15/123

The main objective of the work described in this thesis was the development of a method for the direct selection of mutants producing substantially increased levels of glucose isomerase activity. While this work was in progress, a more conventional programme for strain improvement was being pursued by I.C.I. at Trafford Park. Strain B3728 was subjected to repeated rounds of mutagenesis. After each mutagenic treatment a large number of clones was isolated and each was tested for glucose isomerase activity. The clone which showed the highest specific activity was then subjected to further mutagenic treatment. After three cycles of UV light mutagenesis followed by four cycles of N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis, strain 15/123 was isolated. This strain has a glucose isomerase specific activity about 1.5-fold that of strain B3728. After a further seven cycles of mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine, a strain designated 37/70 was isolated from strain 15/123. This strain has a specific activity about twice that of the starting strain, B3728. Neither 15/123 nor 37/70 is prototrophic, and neither gives rise to prototrophic revertants at a detectable rate (i.e. the frequency of reversion is less than 10^{-11}). In addition, strain 37/70 shows a growth rate in rich medium (LB) considerably lower than that of strain B3728. It is therefore probable that these strains have multiple auxotrophies as the result of secondary, non-selected, mutations accumulated during the repeated mutagenic treatments to which they have been subjected. Such additional lesions are

Photographs 9a & 9b

Cell-free Extracts of Strains B3728, 37/70 and 15/123



10% polyacrylamide SDS gels stained for protein.

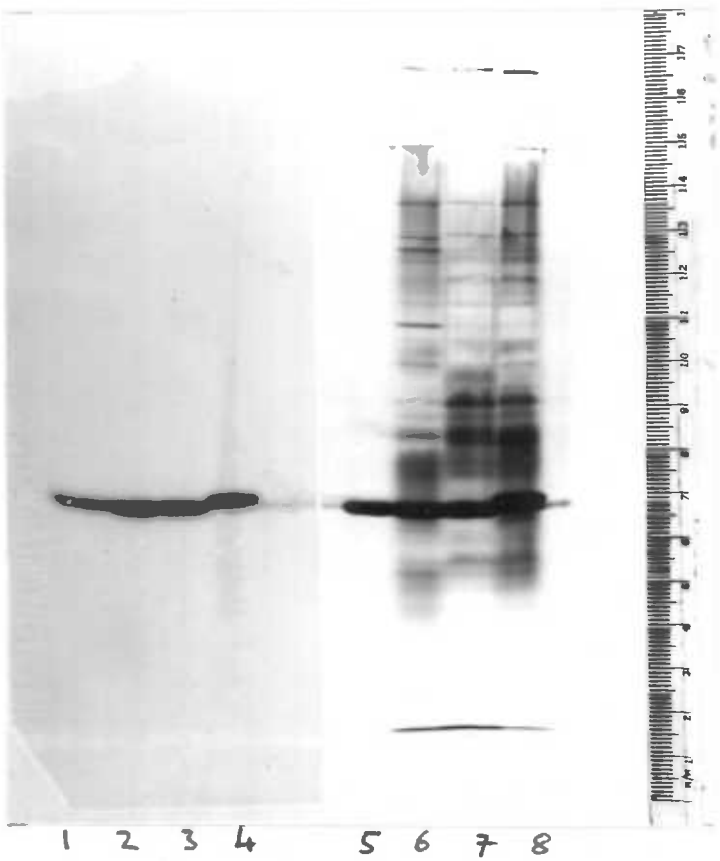
Track	Sample
1	200µg protein; strain B3728 cell-free extract.
2	200µg protein; strain 37/70 cell-free extract.
3	200µg protein; strain 15/123 cell-free extract.
4	6µg protein; purified isomerase from B3728.
5	4µg protein; purified isomerase from B3728.
6	50µg protein; strain B3728 cell-free extract.
7	50µg protein; strain 37/70 cell-free extract.
8	50µg protein; strain 15/123 cell-free extract.
9	4µg protein; purified isomerase from B3728.
10	25µg protein; strain B3728 cell-free extract.
11	25µg protein; strain 37/70 cell-free extract.
12	25µg protein; strain 15/123 cell-free extract.
13	4µg protein; purified isomerase from B3728.

commonly found following severe mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (Guerola et al., 1971).

The enhanced glucose isomerase activity of these strains could have been achieved either by an increase in the amount of enzyme present or by the production of an altered enzyme showing increased activity towards glucose. It was therefore considered to be of interest to examine cell-free extracts of these strains electrophoretically, to look for evidence either of increased levels of the enzyme or of the presence of an altered isomerase protein. Photographs 9a and 9b show SDS polyacrylamide gels of extracts of strains B3728, 15/123 and 37/70, together with purified glucose isomerase from strain B3728. In the presence of SDS the major protein species in each cell-free extract co-migrated with the purified isomerase sample. It is not clear from these gels whether the isomerase represents an increased percentage of the total soluble protein in either strain 15/123 or strain 37/70; densitometry would have been of little use as a method of quantitation because, in order to avoid overloading the gels with the isomerase, it was necessary to use loadings of the cell-free extracts at which variations in the backgroud staining of the gel would have led to severe errors in the estimation of the amount of material present in the remaining bands. It is noteworthy that the cell-free extract from strain 37/70 gave rise to an extra major band on electrophoresis; this band could correspond to D-xylulokinase, which is known to be synthesised constitutively by strains B3728 and 15/123 (Chapter X), although if this were the case it is surprising that the band is not also visible in the extract of strain 15/123. However, since it is known that strain 37/70 carries other mutations in addition to those resulting in increased glucose isomerase activity, it appears more probable that the extra band results from the constitutive syn-

Photograph 9c

Cell-free Extracts of Strains B3728, 37/70 and 15/123



7.5% polyacrylamide gel, run under native conditions. Tracks 1 to 4 were stained for isomerase activity, whilst tracks 5 to 8 were stained for protein.

Tracks	Sample
1 & 5	Purified isomerase from B3728 (10µg protein).
2 & 6	Cell-free extract of strain B3728 (200µg).
3 & 7	Cell-free extract of strain 37/70 (200µg).
4 & 8	Cell-free extract of strain 15/123 (200µg).

N.B. the decreased mobility of the isomerase produced by strain 15/123.

thesis of some protein which is unrelated to D-xylose metabolism.

Cell-free extracts of strains B3728, 15/123 and 37/70, together with purified glucose isomerase from strain B3728, were also subjected to polyacrylamide gel electrophoresis under native conditions, and the gel was stained both for isomerase activity and for protein (Photograph 9c). It can be seen that, whilst the major protein species in the extracts of strains B3728 and 37/70 co-migrated with the sample of the purified isomerase, that in the extract of strain 15/123 migrated with a significantly lower mobility. The activity stain confirmed that the major protein species in each extract corresponded to the enzymically active material. Since the isomerase bands in the cell-free extracts of all three strains co-migrated in the presence of SDS, it appeared probable that the altered mobility of the enzyme from strain 15/123 under native conditions was due to an amino acid substitution resulting in a decrease in the net negative charge of the protein at pH 8.8. Strain 37/70 is said to have been derived from strain 15/123 by further mutagenesis; if this is indeed the case a further mutation or mutations must have restored the net charge of the isomerase to that of the enzyme from strain B3728. This could be achieved either by a back mutation reverting the original amino acid substitution, or by mutation at another site or sites introducing a compensating charge change.

Thus it was clear that strain 15/123 produces a mutant glucose isomerase enzyme. It therefore appeared possible that the increased glucose isomerase activity of this strain might result from the synthesis of an enzyme with altered kinetic properties, rather than of elevated levels of the enzyme. It was therefore decided to purify enough glucose isomerase from strain 15/123 to permit a comparison of its kinetic properties with those of the enzyme previously purified from strain B3728.

Purification of Glucose Isomerase from Strain 15/123

The buffer used during the purification of the isomerase was 50mM-Tris.HCl/10mM-$MgCl_2$ pH 7.5, unless otherwise specified, and PMSF was added to a concentration of 0.1mM.

A single colony of strain 15/123 was used to inoculate a 200ml culture in medium A, which was grown up overnight at 30°C. Six four litre flasks, each containing 1.5 litres of medium A, were inoculated with 20ml each of the overnight culture and incubated for 24 hours at 30°C on an orbital shaker table. The cells were then harvested by centrifugation, washed with 50mM-Tris.HCl/10mM-EDTA pH 7.5, and again pelleted.

The washed cell paste (32g) was resuspended in 200ml 50mM-Tris. HCl/10mM-EDTA pH 7.5, and 38mg lysozyme chloride was added, followed by PMSF to a concentration of 0.1mM. After incubation for 3.5 hours at 37°C, 6ml 1M-$MgCl_2$ and 4mg DNase were added to the lysate, resulting in a rapid decrease in viscosity. Because of the small scale of the preparation, it was possible to remove the cell debris by centrifugation at 48 000 xg for 30 minutes in the SS-34 rotor of a Sorvall RC5 centrifuge; this resulted in a firmer pellet than that obtained from the lysate during the purification of the wild type enzyme from strain B3728, leading to an improved recovery of the supernatant.

The supernatant was diluted with one volume of distilled water, to decrease the ionic strength, and then loaded directly onto a column of DEAE-Sepharose (bed volume 140ml, diameter 43mm), which had been equilibrated with buffer/50mM-NaCl. The replacement of the diafiltration step used in the purification of the wild type enzyme described in Chapter VII by a dilution led to a considerable saving in time, but was possible only because of the small scale of the preparation. The column was washed overnight with 1 litre buffer/50mM-NaCl. A linear

Figure 9a

Elution Profile of 15/123 Glucose Isomerase from DEAE-Sepharose

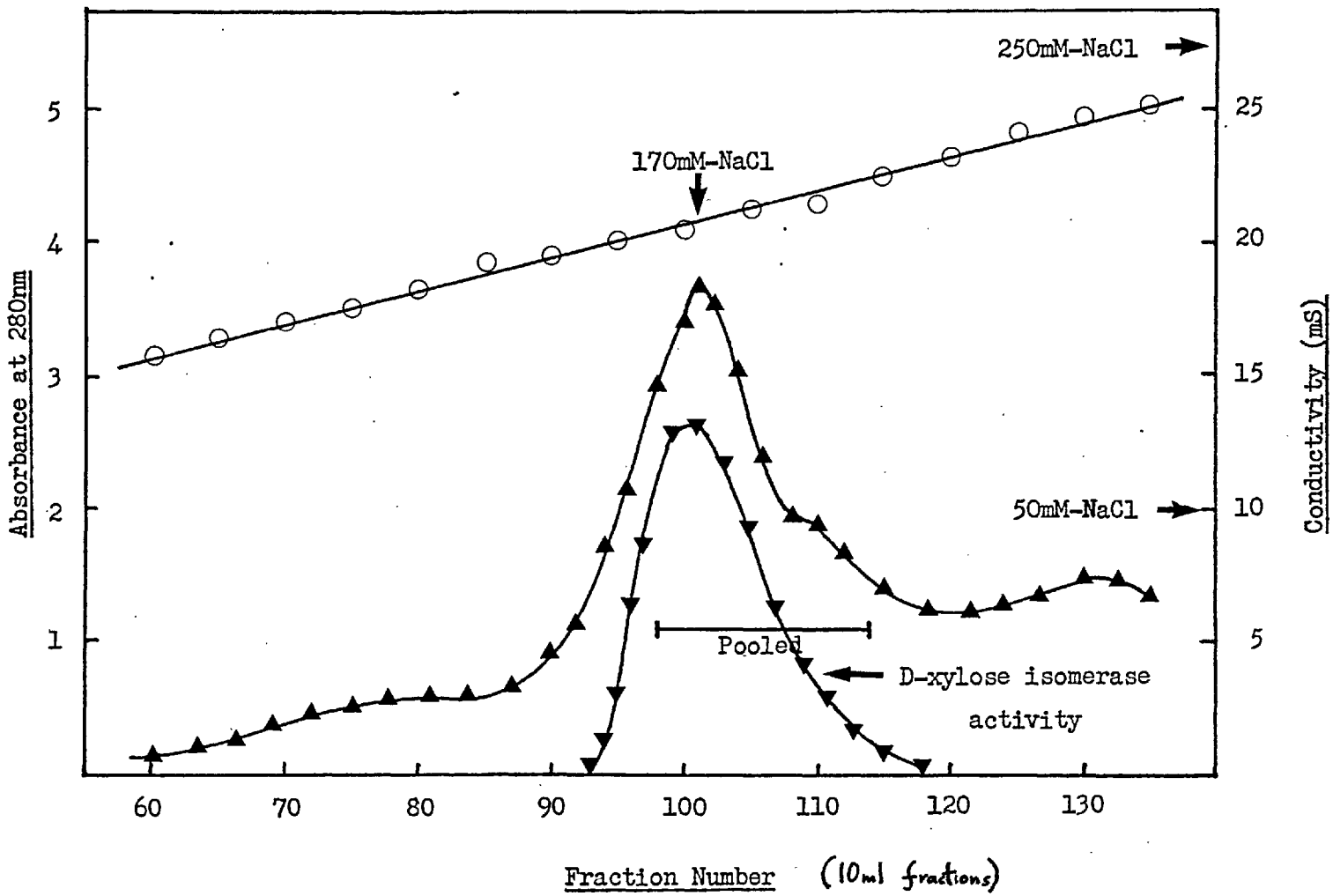
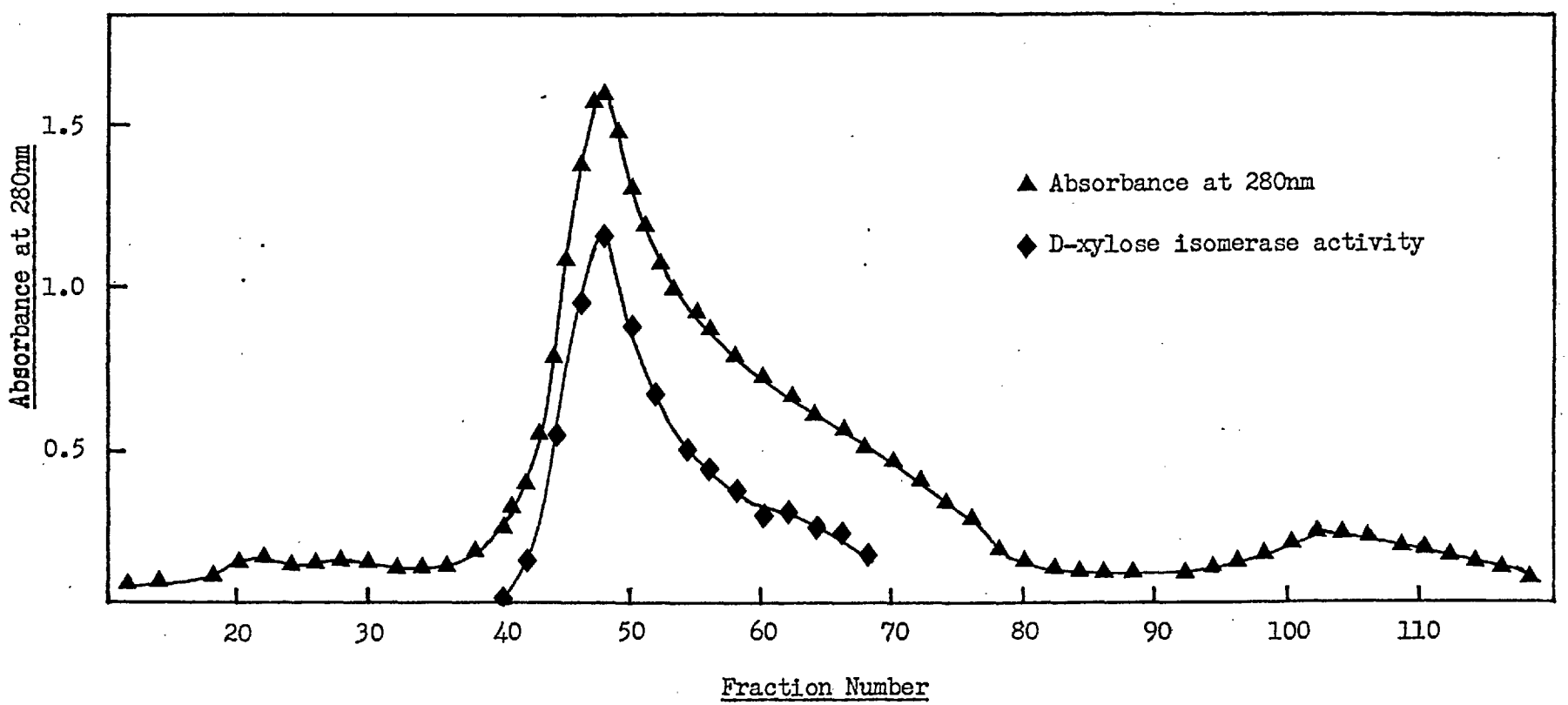


Figure 9b; Elution Profile of 15/123 Glucose Isomerase from Gel Filtration in AcA34

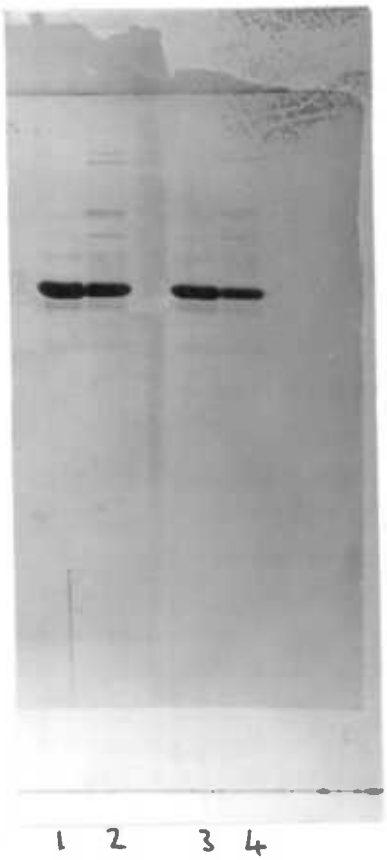


gradient from 50mM to 250mM-NaCl in buffer (total volume 1.2 litre) was applied to the column at a flow rate of 100ml/hour. Figure 9a shows the elution profile from this column. The fractions were pooled as indicated in the Figure, and concentrated to a volume of 19ml by ultrafiltration, using an Amicon PM30 membrane.

The concentrated pool, which contained 300mg protein, was then passed down an Ultragel AcA34 gel filtration column (79cm x 5cm diameter; bed volume 1550ml) at a flow rate of 80ml/hour, and the effluent was collected in 10ml fractions. For reasons unrealated to this work, the gel filtration step was performed at room temperature, and the buffer used for the equilibration and elution of the column was 100mM-KH_2PO_4.KOH/0.5mM-EDTA/5mM-$MgCl_2$ pH 7.0, to which 2-mercaptoethanol (10mM) and PMSF (0.1mM) had been added after degassing. Figure 9b shows the elution profile from this column. Samples of the active fractions were examined by SDS polyacrylamide gel electrophoresis, to determine their purity. As in the preparation of the wild type enzyme from strain B3728 (Chapter VII), the fractions were combined and concentrated to give two pools, the purest material (77mg) and the side fractions containing trace amounts of impurities of higher sub-unit molecular weight (100mg). The SDS gel shown in Photograph 9d illustrates the purity of these pools, whilst the native gel shown in Photograph 9e demonstrates the difference in mobility of the enzymes purified from strains B3728 and 15/123. The purest material was desalted and freeze-dried, as described in Chapter VII. Part of the side-pool material was used for the kinetic studies described below, and the remainder was stored at -18°C, after the addition of glycerol to 50% v/v.

Photograph 9d

Purified Glucose Isomerase from Strain 15/123

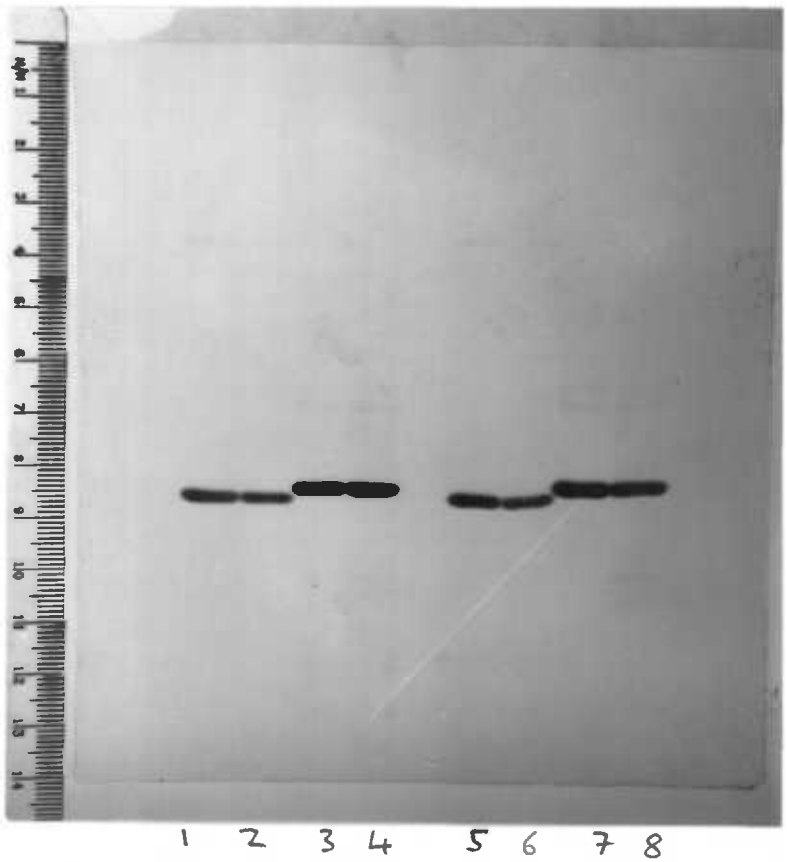


10% polyacrylamide SDS gel stained for protein.

Track	Sample
1	Main pool from AcA34 (10µg).
2	Side-fraction pool from AcA34 (8µg).
3	Main pool from AcA34 (5µg).
4	Side-fraction pool from AcA34 (4µg).

Photograph 9c

Comparison of Mobilities of Purified Glucose Isomerases



7.5% polyacrylamide gel, run under native conditions and stained for protein.

Tracks	Sample
1 & 5	B3728 glucose isomerase (8µg).
2 & 6	B3728 glucose isomerase (4µg).
3 & 7	15/123 main pool from AcA34 (10µg).
4 & 8	15/123 side pool from AcA34 (8µg).

Comparison of the Purified Isomerases from Strains B3728 and 15/123

The numbers of tryptophan and tyrosine residues per sub-unit present in the purified enzyme from strain 15/123 were estimated spectrophotometrically, as described for the wild type enzyme in Chapter VII. The absorbances of a solution of the salt-free enzyme at a concentration of 1mg/ml in 0.1M-NaOH were: $A_{294.4} = 0.613$ and $A_{280.0} = 0.727$. These figures correspond to 8.05 tyrosine and 4.08 tryptophan residues per sub-unit, giving a Tyr/Trp ratio of 1.97. As was expected, these values are the same as those obtained for the wild type enzyme, to the accuracy of the determination. The amino acid composition of the mutant enzyme was not investigated further, as it was not expected that a single amino acid substitution would result in a detectable change in the percentage amino acid composition of the whole protein.

Michaelis constants (K_m) and maximum specific forward velocities (V) were determined for the isomerisation of D-xylose (at 30°C) and D-fructose (at 60°C) by the purified mutant enzyme, as described in Chapter VIII for the wild type enzyme. The plots obtained are shown in Figures 9c and 9d; Table 9a contains the kinetic constants obtained for the purified enzymes from both strains. For each substrate the values of K_m obtained for the two enzymes did not differ significantly, whilst the value obtained for V was lower than that obtained for the wild type isomerase. The difference in the values obtained for V may have reflected a real decrease in the activity of the enzyme produced by strain 15/123, but is more likely to have resulted from partial inactivation of the mutant enzyme during purification. These results demonstrated that the increased glucose isomerase activity of strain 15/123 is not due to the synthesis of an isomerase with altered kinetic properties. This strain must therefore carry, in addition to

Figure 9c: [s]/v against [s] Plot for D-Xylose at 30°C

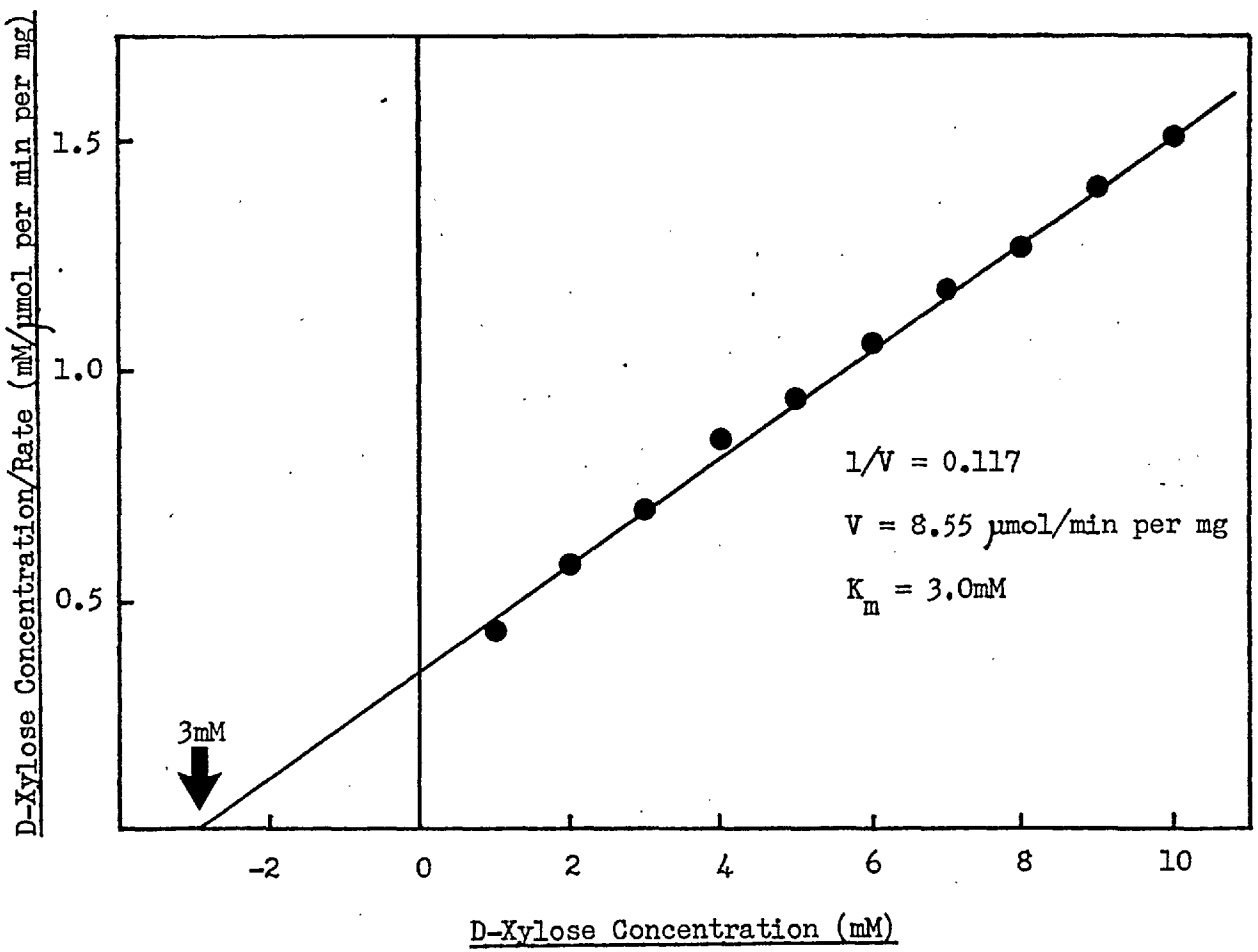


Figure 9d; [s]/v against [s] Plot for D-Fructose at 60°C

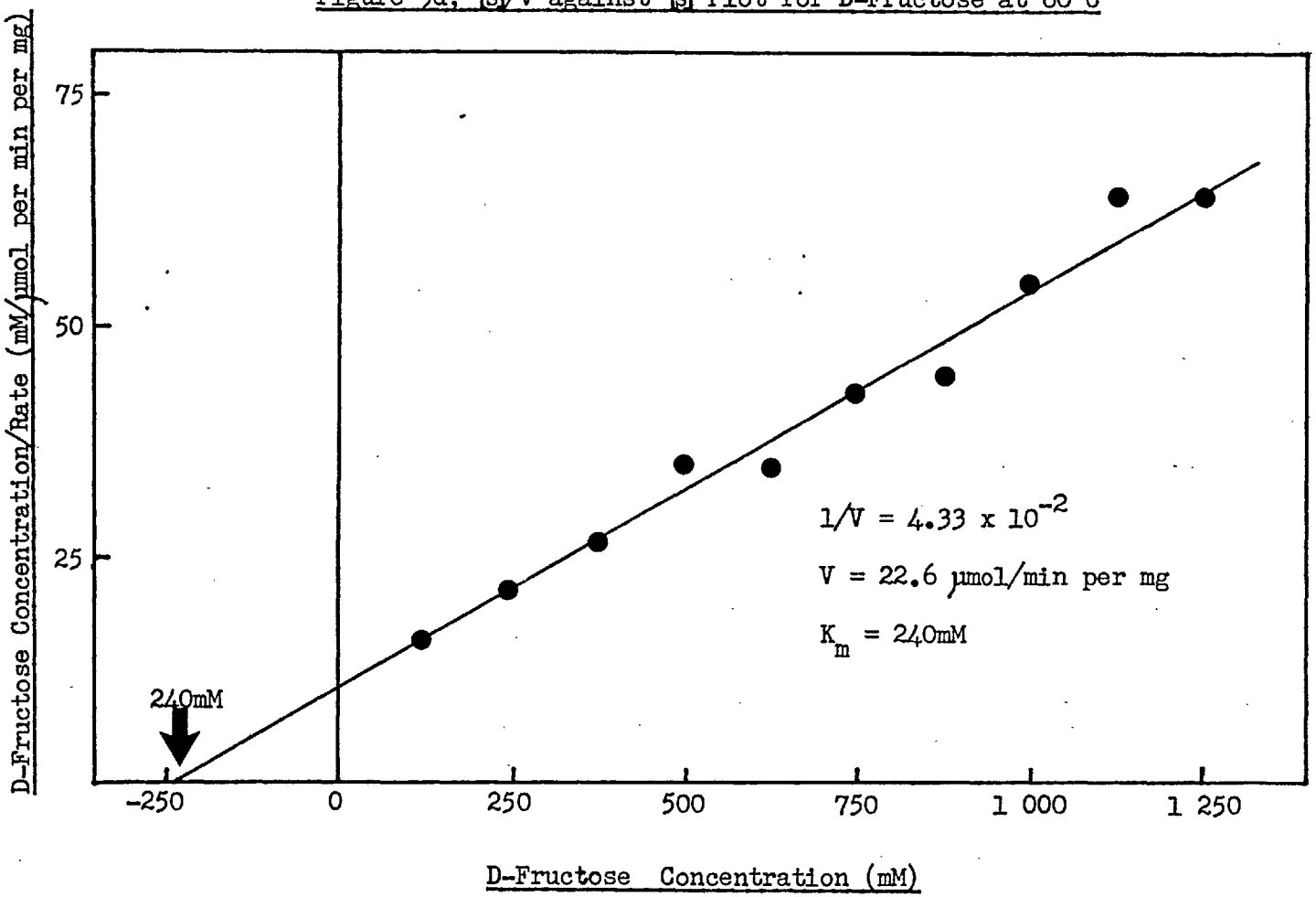


Table 9a

Comparison of Kinetic Parameters for Purified Isomerases from Strains B3728 and 15/123

Substrate	Parameter	B3728	15/123
D-Xylose (30°C)	K_m (mM)	3.3	3.0
D-Fructose (60°C)	K_m (mM)	250	240
D-Xylose (30°C)	V (µmol/min per mg)	8.9	8.55
D-Fructose (60°C)	V (µmol/min per mg)	28	22.6

a mutation in the structural gene for the isomerase, some other mutation(s) which lead to the synthesis of higher levels of the protein. If in strain B3724, believed to be the wild type strain from which B3728 was isolated, synthesis of the D-xylose isomerase is under the negative control of a repressor/inducer system analogous to that of the lac operon of E. coli (as appears probable from results described in Chapter X), it is reasonable to assume that the constitutive strain B3728 produces no functional repressor. If this is the case, any further mutations leading to increased expression of the isomerase must be closely linked to the structural gene. In view of the known ability of the mutagen N-methyl-N'-nitro-N-nitrosoguanidine to induce closely linked multiple mutations (Guerola et al., 1971), it is therefore not very surprising (in retrospect) that strain 15/123 should carry in the structural gene for the isomerase a mutation which is not involved in the increased glucose isomerase activity for which the strain was selected. Since neither the activity nor the subunit structure of the isomerase appears to be altered by the mutation which leads to the decreased electrophoretic mobility of the enzyme from strain 15/123, nor is the enzyme rendered unstable, it appears that the mutation can have little effect on the evolutionary fitness either of the isomerase or of the whole organism. Presumably the fixation of such 'neutral' (or nearly neutral) mutations due to their fortuitous linkage to control mutations conferring a selective advantage on their possessors has played a part in the divergence of strutural gene sequence during bacterial speciation.

Chapter X

Strain SA401 and the Utilisation of D-Lyxose and Xylitol

Chapter VIII describes the investigation of the substrate and inhibitor specificities of the purified isomerase from Arthrobacter strain B3728. This chapter describes attempts to use the knowledge of these specificities to devise new schemes for the isolation of mutant strains possessing elevated levels of glucose isomerase activity to replace the original proposal involving the evolution of a novel pathway for glucose utilisation, which appeared likely to prove impossible in the light of the results described in Chapter VI.

The selection of mutants able to grow slowly on media containing D-lyxose (a very poor substrate for the Arthrobacter isomerase) as sole carbon/energy source proved to be a powerful method for the isolation of strains constitutive for the synthesis of the isomerase from the inducible strain B3724. Experiments with strain SA401 (inducible for D-xylulokinase activity, but lacking D-xylose isomerase activity) provided evidence that the activity of the isomerase, in addition to that of D-xylulokinase, was essential for the utilisation of D-lyxose, confirming that the side specificity of the D-xylose isomerase was responsible for the conversion of D-lyxose to D-xylulose in vivo. The isolation of strains capable of more rapid growth on D-lyxose appeared likely to provide a method of selecting mutants with higher levels of the enzyme, but so far no such mutants have been isolated.

Attempts were also made to use xylitol, a competitive inhibitor of the purified enzyme, to render the activity of the isomerase rate-limiting for the catabolism of D-xylose, its normal physiological

substrate. These led to the discovery that the Arthrobacter strains used were capable of slow metabolism of xylitol. Results obtained with strain SA401 suggested that D-xylose was an intermediate in the catabolism of this pentitol.

Investigations of the D-xylulokinase activity present in the strains SA401, B3724, B3728 and 15/123 led to conclusions concerning the probable arrangement and control of the genes coding for the D-xylose isomerase and D-xylulokinase activities in the Arthrobacter strain B3724, believed to be the parental strain from which the other strains were derived.

Choice of Substrate for a new Catabolic Pathway

The results described in Chapter VIII showed that the aldopentoses D-ribose, L-arabinose, D-lyxose and, probably, L-lyxose are all poor substrates for the Arthrobacter D-xylose isomerase. The possibility of using each of these four gratuitous substrates as a selective carbon source which would be utilised at a rate dependent on the activity of the isomerase present, enabling the selection of strains possessing elevated levels of the isomerase, was considered. The results of growth tests on various carbon sources for strains B3724 (inducible for the isomerase), B3728 (constitutive for the isomerase) and SA401 (lacking D-xylose isomerase activity; see below) are presented in Table 10a. It is clear that these strains of Arthrobacter possess efficient pathways for the utilisation of D-ribose and L-arabinose which do not depend on the activity of the D-xylose isomerase. The use of either of these sugars was therefore less attractive than that of D-lyxose or L-lyxose, since it would have been necessary to block the exisiting catabolic pathway by a non-reverting mutation or mutations before attempting to make use of either.

Table 10a

Growth Tests with Strains B3724, B3728 and SA401

Substrate	Strain B3724 (*xyl*$^+$)	B3728 (*xyl*^{c})	SA401 (*xylI*$^-$ *xylK*$^+$)
D-xylose	+++	+++	-
D-ribose	+++	+++	+++
L-arabinose	+++	+++	+++
L-lyxose	-	-	-
L-xylose	-	-	-
D-arabinose	-	-	-
D-lyxose	-	+	-
L-rhamnose	+++	+++	+++
D-sorbitol	-	-	-
D-mannitol	+++	+++	+++
xylitol	(+)	(+)	-
D-arabitol	+++	+++	+++
ribitol	-	-	-
L-arabitol	-	-	-

Key: +++ Good growth

\+ Growth after prolonged incubation

(+) Slight growth after long incubation

\- Growth not detected

Growth tests were performed by streaking the strains on M9 plates containing the relevant carbon source at a concentration of 0.2% (w/v). The plates were incubated at 32°C for up to three weeks, after wrapping to retard drying.

However, it is of interest to compare the merits of using either of the two with the use of D-glucose as originally proposed.

In order to make the evolution of a novel pathway of D-ribose catabolism involving isomerisation to D-ribulose possible, a D-ribulokinase activity permitting the further metabolism of the ketopentose would be essential. Although the normal pathway of D-ribose metabolism found in prokaryotes involves direct phosphorylation of the aldose and therefore does not involve the generation of free ketopentose, catabolic pathways for ribitol and D-arabinose which do involve the phosphorylation of free D-ribulose occur in some bacterial species (Doelle, 1975). However, strain B3724 does not utilise either of these compounds (Table 10a); it is therefore improbable that it possesses any D-ribulokinase activity. D-Ribose therefore seemed to be unsuitable as the substrate for a novel catabolic pathway involving the D-xylose isomerase, for reasons analogous to those for which the attempt to use D-glucose for this purpose was abandoned (Chapter VI).

In the case of L-arabinose, two fundamentally different catabolic pathways are found in different groups of bacterial species. In the enteric bacteria, Bacilli and Lactobacilli, an isomerase converts L-arabinose to L-ribulose, which is then phosphorylated by an L-ribulokinase, whilst in the pseudomonads the aldose is dehydrogenated to form the lactone of L-arabinoate (Doelle, 1975). Since the strains of Arthrobacter used in the work described in this thesis do not belong to the group which resemble the pseudomonads in their catabolism of D-glucose (Chapter VI), it seems likely that they utilise L-arabinose by the former pathway. If this is indeed the case, a mutant of strain B3724 lacking the normal L-arabinose isomerase but retaining the L-ribulokinase activity (and the ability to take up L-arabinose) would be able to regain the ability to utilise L-arabinose

by a further mutation leading to constitutive synthesis of the D-xylose isomerase. Since the K_m of this enzyme for L-arabinose is very high (280mM), it is certain that its activity would then be rate limiting for growth on L-arabinose; therefore strains synthesising elevated levels of the isomerase would be expected to provide one class of mutants showing an increased growth rate on this substrate. Thus it seemed that L-arabinose was potentially a more suitable substrate than either D-glucose or D-ribose for the development of a novel catabolic pathway involving the isomerase. However, before L-arabinose could have been used for this purpose, a considerable amount of work would have been required, both to confirm that the normal pathway for the catabolism of L-arabinose by the Reynolds strains of Arthrobacter was as postulated, and to isolate a suitable non-reverting mutant lacking L-arabinose isomerase activity. This approach was not pursued, both because sufficient time was not available, and because the use of D-lyxose (which did not require such lengthy preliminary work) appeared to be a more promising method.

Neither D-lyxose nor L-lyxose supports the growth of strain B3724 (Table 10a). The isomerisation product of L-lyxose is L-xylulose; an L-xylulokinase activity would therefore be necessary to enable strains constitutive for the isomerase to utilise L-lyxose. Besides L-lyxose, L-xylose and L-arabitol are the only two compounds which are usually catabolised by pathways involving L-xylulose as an intermediate (Doelle, 1975). Since neither compound is utilised by strain B3724, it is unlikely that L-xylulose can be metabolised by this strain. As expected, strain B3728, although constitutive for the isomerase, did not show any ability to utilise L-xylulose; it was therefore concluded that L-lyxose would not be a suitable carbon source for the selection of mutants with high levels of isomerase activity.

Utilisation of D-Lyxose

As discussed in Chapter VIII, because D-lyxose is the epimer of D-xylose at the C_2 position, each has the same isomerisation product, D-xylulose. It therefore appeared probable that strain B3728, which is constitutive for the synthesis both of the isomerase and of D-xylulokinase (as shown by the kinase assays presented later in this chapter), would be able utilise D-lyxose, provided that the uptake of this sugar was possible. Strain B3728 did indeed show the ability to grow in the presence of D-lyxose as sole carbon source, albeit at a very low rate (Table 10a). Using M9 plates containing 0.2% w/v D-lyxose, incubation for two weeks was required to obtain colonies of a size comparable to those formed in two days on M9 with 0.2% D-xylose; this large difference in growth rate was consistent with the difference in the kinetic parameters of the purified isomerase for these two sugars (V for D-lyxose 1/100 that for D-xylose and K_m for D-lyxose 25-fold that for D-xylose; Chapter VIII).

In contrast strain B3724 did not show the ability to grow on D-lyxose. In order to determine whether D-lyxose was able to induce the D-xylose isomerase, as might have been the case if D-xylulose were the actual inducer, strain B3724 was grown in MM' + 2% w/v casein hydrolysate supplemented with 0.5% w/v of either D-lyxose or D-xylose. The saturated cultures were tested for glucose isomerase activity using the qualitative assay described in Chapter III. Activity was observed only in the case of the culture grown in the presence of D-xylose; it was therefore concluded that D-lyxose was not able to induce the synthesis of the isomerase.

The isolation of mutants of B3724 able to utilise D-lyxose (Lyx^+) was then undertaken, since it was expected that at least some of the mutants with this phenotype would prove to be constitutive for the

isomerase. In order to provide an unequivocal means of distinguishing between putative constitutive mutants of strain B3724 obtained in this manner and strain B3728, a spontaneous streptomycin resistant (Str^r) mutant of strain B3724 was isolated and designated strain SA4000 (Chapter II). Washed cell suspensions of strain SA4000 were spread at various dilutions on M9 plates containing 0.2% D-lyxose as sole carbon source. Crystals of the mutagen N-methyl-N'-nitro-N-nitrosoguanidine were added to the centres of some of the plates spread with SA4000, to enhance the frequency of mutation. The plates were wrapped to retard drying, and incubated at 32°C for three weeks, after which the colonies formed were picked and repurified by growth on fresh M9 D-lyxose plates, before testing for streptomycin resistance and constitutive isomerase synthesis. Of 33 spontaneous Lyx^+ clones isolated, all were both Str^r and isomerase constitutive, whilst of 27 Lyx^+ clones (all Str^r) isolated from plates to which mutagen had been added, 25 were constitutive for the isomerase, the other two giving negative results in the qualitative assay used. It therefore appeared that mutation to constitutive synthesis of the D-xylose isomerase is by far the most frequent means by which strain B3724 can aquire the ability to utilise D-lyxose.

At this point it is pertinent to describe the results which have been published concerning the isolation of mutants able to utilise D-lyxose from other bacterial species which do not normally catabolise this sugar. Stevens and Wu (1976) reported the isolation of a mutant of E. coli K12 which had aquired the ability to grow on D-lyxose. This strain synthesised constitutively a novel isomerase, which could convert D-lyxose to D-xylulose, but showed a lower K_m for the conversion of D-mannose to D-fructose, although not normally involved in the catabolism of D-mannose by the parent strain. In the presence of this constitutive isomerase activity D-lyxose was able to induce the D-xylose

uptake, D-xylose isomerase and D-xylulokinase activities normally synthesised only in the presence of D-xylose. It was demonstrated that the D-xylose uptake system was able to transport D-lyxose, although less efficiently than D-xylose. These results suggested the possibility that the activity of the Arthrobacter D-xylose isomerase towards D-lyxose might not be responsible for the growth of strain B3728 and the mutants isolated from strain B3724; if these strains were also constitutive for the D-xylose uptake system, it might be the case that the uptake of D-lyxose allowed its catabolism by some pathway involving an enzyme or enzymes not involved in D-xylose metabolism. However, the results of experiments with strain SA401 described later in this chapter argue against this interpretation.

Sanchez and Quinto (1975) reported the selection of mutants of Streptomyces phaeochromogenes constitutive for D-xylose isomerase by means of their ability to grow on D-lyxose. However, Parker (1978) was unable to repeat their results; of 9 Lyx^+ mutants isolated after mutagenesis, none was constitutive for D-xylose isomerase (nor had any aquired the ability to synthesise the isomerase in the presence of D-lyxose).

It would appear from these results for members of the genera Escherichia and Streptomyces that the isolation of mutants able to utilise D-lyxose is not likely to provide a general method for obtaining strains constitutive for the enzymes of D-xylose catabolism; but the method does appear to be suitable in the special case of the Arthrobacter strains used in the work described in this thesis.

The next stage in the development of a novel pathway for D-lyxose catabolism in Arthrobacter would clearly have been the isolation of mutants showing faster growth on D-lyxose, and the determination of the D-xylose/D-glucose isomerase specific activities of such mutants.

The isolation of mutants with enhanced growth rates on D-lyxose could have been attempted in two ways: either by screening on D-lyxose plates for clones forming larger colonies (by plating out a culture enriched for such mutants by growth in minimal medium with D-lyxose as sole carbon source) or by continuous culture on D-lyxose as limiting nutrient. However, time did not permit more than a preliminary attempt to isolate such mutants on M9 D-lyxose plates, which was not successful. If this project were pursued further, the isolation of such mutants and their characterisation, together with the determination of the isomerase specific activities of a range of the first stage Lyx^+ mutants would be given very high priority.

Use of Inhibitors

Xylitol, a potent competitive inhibitor of the purified Arthrobacter D-xylose isomerase (K_i 0.3mM cf. K_m for D-xylose of 3.3mM; Chapter VIII), appeared from preliminary growth tests not to be utilised by strain B3724. It was therefore considered worthwhile to investigate the feasibility of using xylitol to inhibit the isomerase in vivo, rendering its activity rate limiting for the utilisation of D-xylose, its natural substrate. If this were possible, mutants possessing elevated levels of the isomerase would be expected to show resistance to inhibition of D-xylose utilisation by xylitol, providing an alternative to the methods described above for the isolation of such mutants involving the catabolism of novel substrates. Clearly the success of such a scheme would require the uptake of xylitol, either active or passive, to allow inhibition of the intracellular isomerase. However, if such uptake were not possible, inhibition of D-xylose utilisation might still occur if xylitol acted as an inhibitor of the D-xylose uptake system. In this case it might still be possible

to obtain mutants possessing elevated levels of isomerase activity by isolating clones resistant to inhibition by xylitol, if the synthesis of the D-xylose uptake and isomerase activities are under coordinate control.

Both strain B3724 (inducible) and strain B3728 (constitutive) showed slower growth on M9 plates containing 0.2% w/v D-xylose + 0.2% w/v xylitol than on plates containing xylose alone. However, slow growth of both strains was observed on control plates containing 0.2% w/v xylitol as sole carbon source, and in both cases colonies showing faster growth arose. Xylitol therefore appeared unsuitable for use as an in vivo inhibitor of the isomerase. Results obtained with strain SA401, described below, suggested that D-xylose is an intermediate in the catabolism of xylitol by these strains of Arthrobacter.

D-sorbitol was also found to inhibit the isomerase, although less effectively than xylitol (K_i 6.5mM; Chapter VIII). Strain B3724 showed no ability to utilise D-sorbitol, or to give rise to mutants able to do so. However, the use of D-sorbitol as an inhibitor of D-xylose utilisation was not investigated, both because of lack of time and because the use of D-lyxose appeared to be a more promising technique for the isolation of strains with elevated levels of isomerase activity.

D-Xylulokinase Activity: Strain SA401 - a $xylI^-$ $xylK^+$ Mutant

Strain SA401 is unable to utilise D-xylose (Xyl^- phenotype) and does not synthesise D-xylose isomerase when grown in the presence of D-xylose (no activity is present and the major band corresponding to the isomerase observed in cell-free extracts of strain B3724 induced for the isomerase after polyacrylamide gel electrophoresis in the presence of SDS is absent in cell-free extracts of strain SA401).

Strain SA401 was isolated fortuitously from a culture of strain B3724, inoculated from an ampoule of freeze-dried culture supplied by the A.T.C.C., on account of its 'frosty' colonial morphology (see strain list, Chapter II). Apart from its 'frosty' morphology and its inability to utilise D-xylose, strain SA401 is indistinguishable from the 'frosty' varient of strain B3724, from which it is believed to be derived. Strain SA4010, a spontaneous rifampicin resistant (Rif^r) mutant of strain SA410, gave rise to Xyl^+ Rif^r revertants at a frequency of about 3×10^{-10}. Such revertants were inducible for the isomerase activity.

Strain SA401 does not show any growth on xylitol or D-lyxose as sole carbon/energy source. The isolation of mutants of strain SA4010 able to utilise xylitol (Xtl^+) or D-lyxose (Lyx^+) was attempted. Xtl^+ mutants arose at a frequency similar to Xyl^+ revertants, and of 19 Xtl^+ Rif^r isolates tested all were also Xyl^+, whilst of 49 Xyl^+Rif^r isolates all were found to be Xtl^+. It was concluded that one or more of the activities involved in D-xylose catabolism must also be essential for the utilisation of xylitol. No Lyx^+ clones were isolated from strain SA4010 either spontaneously (10^{12} cells screened) or after mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine.

It was therefore considered to be of interest to determine whether strain SA401 was able to synthesise D-xylulokinase, since if it lacked only the isomerase, its inability to give rise to Lyx^+ clones at a frequency compatable with a single step mutation would confirm that the activity of the latter was essential for D-lyxose utilisation. Table 10b presents the results obtained when cell-free extracts of strains SA401, B3724, B3728 and 15/123 were assayed for D-xylulokinase and glucose isomerase activities. Strain B3724 is inducible for both the isomerase (*xylI*$^+$) and the kinase (*xylK*$^+$) activities, whilst strain

Table 10b

D-Xylulokinase Activities of Various Strains

Strain		Protein (mg/ml)	DXK Activity (cpmpm)	DXK SA (cpmpm/µg)	XI SA (µU/mg)
SA401		2.75	-	-	-
SA401	(induced)	3.2	17.8	5.5	-
B3724		5.4	-	-	-
B3724	(induced)	7.05	34.6	5.05	30
B3728		7.0	129	18.5	260
15/123		5.55	111	20	300

Strains were grown in MM' + 2% casein hydrolysate; in order to induce strains SA401 and B3724, the medium was supplemented with 0.2% D-xylose. Cell-free extracts were prepared as described in Chapter II, except that 50mM phosphate buffer pH 7.0 was used in place of Tris.

D-Xylulokinase activity was determined as described in Chapter II and D-xylose isomerase was quantitated as fructose isomerase as described in Chapter III. The activity of the kinase is expressed as the rate of incorporation of radioactivity into barium precipitable material (cpm per minute).

Strain SA401, which lacks D-xylose isomerase activity, clearly remains inducible for D-xylulokinase activity.

SA401 is inducible for the kinase activity only (*xylI*$^-$ *xylK*$^+$). Strains B3728 and 15/123 are constitutive for both activities (*xyl*^{c}); strain 15/123 possesses higher levels of both activities than strain B3728.

These results suggest that the D-xylose isomerase and D-xylulokinase activities are under coordinate control, and the fact that the levels of both are increased in strain 15/123 is (weak) evidence that the structural genes for both activities may form a single transcriptional unit. Since *xyl*^{c} mutants (selected for their Lyx$^+$ phenotype) arose spontaneously from strain SA4000 at a frequency of between 10^{-8} and 10^{-9}, it appears probable that the expression of the *xyl* genes is under the negative control of a repressor/inducer system analogous to that of the *E. coli lac* operon. In the case of such a control system any mutation which either results in the synthesis of no repressor or of an inactive repressor leads to constitutive expression of the genes. Thus the control of the *xyl* genes in *Arthrobacter* strain B3724 appears to differ from that in *Salmonella typhimurium*, in which the *xyl* genes have recently been shown to be under the positive control of a regulatory gene (Shamana & Sanderson, 1979a,b). Mutations mapping in this control locus which prevented the induction of the D-xylose isomerase, D-xylulokinase and D-xylose uptake activities were readily obtained, whilst no mutants constitutive for any of these three activities were reported. Merodiploid analysis showed that the wild type regulatory gene allele was trans-dominant. This system of gene control resembles that of the *E. coli ara* operon (Lee, 1978). Constitutive mutants of such positively controlled activator/inducer systems arise at lower frequencies than constitutive mutants of negatively controlled operons, because they result only from specific alterations in the activator protein which render its action independent of the inducer or from mutations in the operator region which render transcription independent

of the function of the activator protein.

In view of the fact that strain SA401 is unable to utilise xylitol, although it possesses D-xylulokinase activity, and that mutants able to do so have regained the ability to synthesise the isomerase, it is tempting to speculate that xylitol is metabolised via the formation of D-xylose, rather than by the direct formation of D-xylulose. Similarly the inability of the strain to give rise to single step mutants able to utilise D-lyxose argues strongly in favour of the postulated role of the D-xylose isomerase in the catabolism of D-lyxose. Unfortunately, however, time did not permit the investigation of the D-xylose uptake system of the Reynolds Arthrobacter strains; therefore the possibility remains that strain SA401 lacks D-xylose uptake activity as well as isomerase acitivity. Thus it is not possible to draw unequivocal conclusions from the results obtained with this strain, since it could be argued that lack of ability to take up D-lyxose or xylitol is responsible for the effects attributed to lack of the isomerase.

In summary, it appears probable that the genes coding for the D-xylose isomerase and D-xylulokinase activities form an operon in the Reynolds Arthrobacter strains, which is under negative control in strain B3724. The ability of mutants constitutive for D-xylose isomerase to utilise the novel carbon source D-lyxose provides a powerful screening for the isolation of such strains. The very low growth rate of such mutants on D-lyxose, together with the probability that the activity of the isomerase is rate limiting for D-lyxose utilisation, suggest that the selection of mutants showing faster growth on D-lyxose would be likely to lead to the isolation of mutants producing elevated levels of the isomerase activity.

Chapter XI

Summary and Discussion

"My poor client's fate now depends on your votes."
Here the speaker sat down in his place,
And directed the Judge to refer to his notes
And briefly to sum up the case.

But the Judge said he never had summed up before;
So the Snark undertook it instead,
And summed it so well that it came to far more
Than the Witnesses ever had said!

'The Hunting of the Snark' by Lewis Carroll; Fit the Sixth.

The basic aim of the work described in this thesis was to find a means of selecting mutants of an Arthrobacter strain which possess elevated levels of glucose isomerase activity. As described in Chapter I, the enzyme responsible for this activity is D-xylose isomerase, which normally plays no part in glucose metabolism and, in the wild type strain (B3724), is synthesised only in response to the presence of D-xylose in the growth medium. The method initially adopted involved blocking the normal pathway(s) of glucose metabolism by mutation, in such a manner that the conversion of glucose to fructose by the isomerase became an essential step in the utilisation of glucose by a novel catabolic pathway. Because of the high Michaelis constant of this enzyme for D-glucose, it was predicted that the activity of the isomerase would be rate-limiting for the growth on glucose of any strain possessing only this novel pathway for glucose catabolism.

The situation would then have been analogous to that found for the utilisation of xylitol by strains of Klebsiella aerogenes, where the conversion of xylitol to D-xylulose, catalysed by ribitol dehydro-

genase, is the rate-limiting step (Rigby et al., ~~1974~~ 1975). In such a situation selective pressure applied to the whole organism by selection for faster growth on the substrate of the novel pathway acts on the rate-limiting enzyme, leading to the isolation of mutants which either produce an altered enzyme of increased specificity for the novel substrate, or produce elevated levels of the wild type enzyme ('super-producers'). Carbon-limited continuous culture provides a convenient method for the application of such selective pressure, and in the case of xylitol utilisation by K. aerogenes has led to the isolation both of families of super-producing mutants possessing successively higher levels of the dehydrogenase and of mutants which synthesise altered enzymes with decreased values of K_m for xylitol (Hartley et al., 1976).

It was hoped, therefore, that it would prove possible to obtain Arthrobacter mutants which super-produced the glucose isomerase in an analogous manner, by glucose-limited continuous culture of mutants using the novel pathway involving the isomerase activity for growth on glucose. However, the analogy between the cases of the Arthrobacter glucose isomerase and the K. aerogenes xylitol dehydrogenase activities was not complete. Whereas the utilisation of xylitol by K. aerogenes was chosen as a model system for the experimental study of enzyme evolution for purely scientific reasons, the reasons for attempting to super-produce the Arthrobacter glucose isomerase were essentially commercial. Advantages of the former system over the latter included:

i) K. aerogenes possesses no normal pathway for the metabolism of the unusual pentitol xylitol, whereas the Arthrobacter strains used already possessed efficient pathways for the catabolism of the ubiquitous metabolite glucose.

ii) The general metabolic pathways of K. aerogenes were already well known, and it was possible to predict that any D-xylulose formed by the action of the dehydrogenase on xylitol could be further metabolised, since D-xylulose is a normal intermediate in the catabolism of both D-xylose and D-arabitol by this species. In contrast, little was known about the metabolism of the Arthrobacter strains used for this work, and it was therefore necessary to investigate the normal pathways of glucose and fructose catabolism in order to be able to decide whether the development of a novel pathway for glucose utilisation involving the isomerase was feasible.

iii) Since the ribitol dehydrogenase and xylitol dehydrogenase reactions are linked to the reduction of NAD^+, a simple spectrophotometric assay was possible, whilst in the case of the isomerase it was necessary to use either a discontinuous assay or a coupled assay system.

iv) Because K. aerogenes had already been extensively studied, suitable conditions for mutagenesis and enrichment were already known and, moreover, the existance of generalised transducing phage for this species made genetic analysis of mutants possible. In contrast, it was necessary to develop conditions suitable for the mutagenesis and antibiotic enrichment of the Arthrobacter strains used; furthermore no system for genetic analysis or recombination was available for use with these strains.

Chapters IV and V described the development of conditions for the mutagenesis and ampicillin enrichment of strain B3724, and the isolation and phenotypic characterisation of Glc^- mutants using these techniques. Chapter VI described the investigation both of the normal pathways of glucose and fructose catabolism in strain B3724, and of the lesions responsible for the phenotypes of representative Glc^- mutants. The results obtained suggested strongly that strain B3724 possesses no

enzymic activity capable of phosphorylating intracellular fructose; it was therefore concluded that it was very improbable that it would be possible to carry out the original scheme for the development of a novel catabolic pathway for glucose involving the glucose isomerase activity. In retrospect it is tempting to suggest that this scheme was over-ambitious, since it involved the manipulation of the metabolic pathways of an essentially unknown microorganism, and could therefore have proved to be impossible for any of a number of reasons.

Since the use of glucose as a carbon source for the selection of mutants possessing elevated levels of the isomerase did not appear to be feasible, consideration was given to the possibility of using some other poor substrate of the isomerase for this purpose. In order to identify potentially suitable gratuitous substrates, it was necessary to purify the Arthrobacter glucose isomerase and investigate its specificity. Chapter VII described the purification and physical characterisation of the isomerase from the constitutive strain B3728, whilst Chapter VIII described the investigation of the substrate specificity and kinetic properties of the purified enzyme. It was found that the enzyme could isomerise the aldoses D-ribose, L-arabinose, L-lyxose and (unexpectedly) D-lyxose, in addition to D-xylose and D-glucose. Xylitol was found to be a potent competitive inhibitor of the isomerase, suggesting that the use of this compound in vivo to render the activity of the isomerase rate-limiting for the utilisation of its natural substrate, D-xylose, might provide an alternative method for the selection of mutants producing higher levels of the isomerase. As described in Chapter X, D-ribose, L-arabinose and L-lyxose proved to be unsuitable as substrates for novel catabolic pathways involving the D-xylose isomerase, whilst attempts to use xylitol as an inhibitor of the isomerase in vivo were abandoned when it was discovered that

the strains of Arthrobacter used have the ability to catabolise this pentitol at a low rate.

However, D-lyxose appeared to be an ideal substrate for catabolism by a novel pathway, both because strain B3724 (inducible for D-xylose isomerase) did not utilise this pentose and because its isomerisation product, D-xylulose, was known to be a normal metabolic intermediate in the catabolism of D-xylose by this strain. Mutants isolated from strain B3724 for the ability to utilise D-lyxose were found to be constitutive for the synthesis of the isomerase. Such mutants grew very slowly on media containing D-lyxose as sole carbon source. Unfortunately, time did not permit the isolation of further mutants showing faster growth on D-lyxose. However, in view of the kinetic parameters of the isomerase for D-lyxose (V 1/100 that for D-xylose and K_m 84mM), it is almost certain that it is the activity of the isomerase which limits the growth rate of constitutive strains on D-lyxose. It therefore seems very probable that mutants possessing elevated levels of this enzyme would be found amongst mutants showing faster growth on D-lyxose. Such work would clearly be worth pursuing further, either by the isolation of mutants producing larger colonies on solid media containing D-lyxose as sole carbon source, or by the use of D-lyxose-limited continuous culture.

The work described in Chapter IX resulted from the observation that strain 15/123, a mutant with an increased level of glucose isomerase isolated by I.C.I. in a classical strain improvement programme, possessed an isomerase of altered elecrophoretic mobility. It therefore appeared possible that this strain produced an isomerase with an improved specificity for D-glucose; however, the kinetic properties of the enzyme purified from this strain were not significantly different from those of the wild type enzyme.

Stability of Super-Producing Strains

Since it appears probable that Arthrobacter strains which super-produce the glucose isomerase could be obtained by the selection of mutants capable of rapid growth on D-lyxose, it is relevant to discuss the likelihood that such strains would be suitable for use in the commercial production of the isomerase.

As was discussed in Chapter X, it is probable that the expression of the structural genes of the catabolic pathway for D-xylose is under negative control in the inducible strain B3724. It is also likely that the constitutive strain B3728 lacks active repressor protein, since mutations resulting in the abolition of repressor activity would be expected to arise much more frequently than operator mutations resulting in a completely constitutive phenotype, both from a theoretical consideration of the expected lengths of DNA available for mutation in the repressor and operator loci, and from experimental evidence available for the lac operon of E. coli (Davis & Jacob, 1968). There are two types of mechanism by which mutants synthesising higher levels of the isomerase could arise from such a constitutive strain; firstly by mutations resulting in an increase in the rate at which the protein was synthesised from a single copy of the structural gene, and secondly mutations resulting in an increased gene dosage for the isomerase structural gene. Possible mutations resulting in an increased synthesis of polypeptide from a single structural gene include both mutations leading to an increased rate of transcription of the gene, such as 'up-promotor mutations', mutations which abolish or reduce the efficiency either of transcription attenuator sites or of sites involved in natural polarity or mutations abolishing any requirement for activation by cyclic AMP and the catabolite gene activator protein, and mutations leading to an increased rate of translation of

the messenger RNA, such as improvements in the ribosome binding site (Miller & Reznikoff, 1978). Mutations of this type are relatively stable, but can lead only to limited increases in the level of protein synthesis. It is probable that the mutation(s) which are responsible for the increased glucose isomerase activities of strains 15/123 and 37/70 (Chapter IX) are of this type.

The second type of mechanism by which strains producing elevated levels of the enzyme could arise involves an increase in gene dosage for the structural gene; this could be achieved either by the incorporation of the gene into an episome, or by an increase in the number of copies of the gene present in the main bacterial chromosome. In either case the increase in gene dosage is not limited to duplication, and very high levels of super-production are therefore possible. For example, a 28-fold amplification of D-arabitol dehydrogenase synthesis has been achieved in E. coli by the incorporation of the structural gene into a high copy number plasmid, resulting in a strain in which the dehydrogenase represents 19% of the total soluble cell protein (Neuberger et al., 1979), whilst increased chromosomal gene dosage has been implicated in a 15-fold amplification of ribitol dehydrogenase synthesis obtained in Klebsiella aerogenes, which resulted in the dehydrogenase accounting for 17% of the soluble cell protein of the super-producing strain (Rigby et al., ~~1974~~ 1975). However, in both of the examples described above, the super-producing strains were genetically unstable. In the case of gene amplification by incorporation into a plasmid, loss of the plasmid provides a mechanism for the loss of gene copies, whilst although certain forms of chromosomal gene duplications should be stable (e.g. invert duplications and direct duplications separated by unique copies of genes essential for the survival of the organism), gene duplications isolated experimentally by the selection

of faster growing derivatives under conditions where a given gene product is rate-limiting for growth have generally been found to be unstable (Horiuchi et al., 1963; Rigby et al., 1975; Straus & Hoffmann, 1975). It is probable that the gene duplications obtained in these cases are direct and tandem (i.e. the repeated sequencies are in the same orientaion on the chromosome and are not separated by any non-repeated material); the loss of the extra gene copies present by homologous recombination is therefore possible.

So long as it is possible to grow such gene-amplified strains under conditions where the activity of the super-produced enzyme is rate-limiting for growth, the formation of segregants with lower activities is not a problem, since the segregants grow more slowly than the super-producing strain. However, when such strains are cultured under conditions where the activity of the super-produced enzyme is unnecessary for growth, it is generally found that segregants which synthesise lower levels of the enzyme show a higher growth rate than the super-producer and rapidly take over the population. Andrews and Hegeman (1976) showed that the constitutive synthesis by E. coli strains of from 0.6% to 3.5% of their total cellular protein as unnecessary enzymes of lactose catabolism led to a reduction in their growth rates in continuous culture on glycerol of from 2.1% to 2.6%, compared to that of an otherwise isogenic lac repressed strain. Results obtained in this group have shown that when a strain of K. aerogenes which synthesises 17% of its total soluble protein as ribitol dehydrogenase was grown in continuous culture on inositol, take over of the population by a segregant synthesising haploid levels of the enzyme was complete within 150 generations, whilst when the same strain was grown on glucose, which causes severe catabolite repression of the dehydrogenase, no decrease in the specific activity of the population was detected after 1 100 generations (Dr J.M. Dothie, unpublished work).

These results demonstrate clearly that the synthesis of unnecessary protein can confer a strong selective disadvantage under conditions of continuous culture.

It would obviously be impossible to use D-lyxose as a carbon source for the culture of a glucose isomerase super-producing strain of Arthrobacter on a scale suitable for the commercial production of the isomerase. It would therefore appear probable from the results described above that any attempt to use such super-producing strains would merely result in the selection of revertants synthesising lower levels of the isomerase. However, in strain B3728 the isomerase, which is of no known use to the organsim in the absence of D-xylose or D-lyxose, already accounts for 5% of the total cell protein or 10% of the soluble protein present (Chapter VII). It would therefore be expected from the results of Andrews and Hegeman (1976), described above, that this strain would be at a selective disadvantage with respect to mutants synthesising less or none of the isomerase, when grown in continuous culture under conditions of carbon limitation. In the U.K. I.C.I. Agricultural Division grow strain B3728 in continuous culture under conditions of carbon limitation for the commercial production of glucose isomerase. Cultures containing 10^{21} cells have been grown through 430 generations without observing any decrease in the specific activity of the culture (Dr K.A. Powell, personal communication). It is improbable that mutants which do not synthesise the isomerase arise spontaneously at a frequency less than 10^{-10}; it therefore appears that protein synthesis cannot be rate-limiting for growth under the conditions used for the culture of strain B3728, since otherwise such mutants would be expected to take over the population in the fermenter. It should be noted that in the experiments with E. coli and K. aerogenes described above continuous culture was carried out using minimal media, and the organisms were therefore ob-

liged to elaborate the amino acids required for protein synthesis from ammonia, sulphate ions and the carbon source provided, whereas the medium used for the continuous culture of strain B3728 contained corn steep liquor as a source of amino acids, in addition to glucose as carbon/energy source. However, since in the case of E. coli the energy input required for the synthesis of the amino acids makes a contribution of only about 7% to the total energy cost of protein synthesis during growth on minimal media (Gottschalk, 1979), it does not seem probable that the addition of amino acids to the medium could lead to a sufficient reduction in the burden caused by the synthesis of unnecessary protein to account for the observed stability of strain B3728 in continuous culture.

Whatever the true explanation for the difference between the results obtained for Arthrobacter and those obtained for E. coli and K. aerogenes may be, it is clear that the synthesis of 5% of its total cell protein as an unnecessary enzyme does not confer a selective disadvantage on strain B3728 under the conditions of continuous culture used for the production of the isomerase. It therefore appears at least possible that gene-duplicated strains producing higher levels of the isomerase would also prove to be sufficiently stable in continuous culture to be of use in the commercial production of glucose isomerase by this method. In any case, even if such super-producing strains proved to be unstable in continuous culture, it might still prove possible to use them in the commercial production of the enzyme by a batch culture process. In such a process the inoculum is grown through only a limited number of generations before harvesting; thus any segregants with higher growth rates and lower isomerase activities which arose during culture would only be able to make a relatively small contribution to the final cell population. A primary inoculum could be grown up on D-lyxose, to ensure the initial purity of the

strain. In the U.S.A. I.C.I. routinely use batch culture for the commercial production of glucose isomerase.

In view of the fact that the isomerase already accounts for such a large fraction of the total cell protein present in strain B3728, it is improbable that an increase in specific activity of more than a few-fold could be obtained, rather than the 20-fold envisaged at the start of the project (Chapter I), even given the powerful positive selection for mutants possessing elevated levels of the isomerase provided by the use of D-lyxose. Mutants which show up to a two-fold increase in glucose isomerase specific activity have already been obtained by I.C.I., using a more conventional approach to strain improvement involving the assay of many random isolates following mutagenesis (Chapter IX). The use of D-lyxose as a carbon source for the direct selection of mutants which possess elevated levels of the isomerase offers a less laborious approach to strain improvement and, since the selection is carried out on minimal media, avoids the isolation of mutants which carry unwanted auxotrophies in addition to mutations of the desired type.

"'Tis a pitiful tale," said the Bellman, whose face
Had grown longer at every word;
"But, now that you've stated the whole of your case,
More debate would be simply absurd."

'The Hunting of the Snark' by Lewis Carroll; Fit the Fourth.

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