A COMPARATIVE STUDY OF PHOSPHOFRUCTOKINASE AND TAGATOSE 6-PHOSPHATE KINASE FROM *STREPTOCOCCUS LACTIS*

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ABSTRACT

In the lactic streptococci glucose is metabolised to lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway. Metabolism of lactose and galactose in these organisms involves participation of the D-tagatose 6-phosphate pathway in which galactose 6-phosphate is metabolised to triose phosphates via tagatose derivatives.

Phosphofructokinase (ATP : D-fructose 6-phosphate 1-phosphotransferase, E.C. 2.7.1.11) catalyses the ATP-dependent phosphorylation of fructose 6-phosphate in the EMP pathway. The analogous reaction in the tagatose 6-phosphate pathway, phosphorylation of tagatose 6-phosphate with ATP, is catalysed by a specific enzyme, tagatose 6-phosphate kinase. While phosphofructokinase (PFK) is known to be a major regulatory enzyme in carbohydrate metabolism in most organisms, little is known of the regulatory properties of tagatose 6-phosphate kinase (T6PK).

PFK and T6PK were purified from *Streptococcus lactis* C_{10} PFK was purified to homogeneity (364-fold purification) by affinity chromatography on Blue-dextran-Sepharose. Unlike PFK, T6PK did not bind to Blue-dextran-Sepharose : a 136-fold purification was achieved using ammonium sulphate fractionation, gel filtration, and ion exchange chromatography.

A study of some of the properties of PFK and T6PK from *S. lactis* C_{10} showed that these two enzymes are distinct proteins with different physical and kinetic characteristics.

S. lactis PFK is a tetramer (MW 145,000 daltons) of identical subunits of molecular weight 33,500 daltons. It therefore appears structurally similar to other bacterial PFKs.

T6PK from *S. lactis* has a molecular weight of approximately 114,000 daltons, a value similar to that of *Staphylococcus aureus* T6PK which is a dimer.

S. lactis PFK exhibited the co-operative binding of F6P and inhibition by high concentrations of ATP relative to F6P which is typical of most bacterial and mammalian PFKs. $F6P_{0.5}$ and K_m (MgATP) values were 0.28 mM and 0.18 mM respectively. ADP stimulated PFK activity, shifting the sigmoidal saturation curve to a more hyperbolic form, with a corresponding decrease in n_H. Ammonium and

potassium ions also activated PFK, while activity was inhibited by AMP, PEP, FBP, T6P and inorganic phosphate. In contrast to PFK, T6PK showed no co-operative binding of sugar phosphate substrate and was less sensitive than PFK to ATP inhibition. K_m values for T6P and MgATP were 0.16 mM and 0.4 mM respectively. Apart from ammonium and potassium ions, no activators of T6PK were found. Activity was inhibited by ADP, PEP, and FBP. PFK and T6PK could catalyse phosphorylation of both F6P and T6P although the enzymes showed a much greater affinity for their natural substrate. Maximum velocities attained were higher with the natural substrate than when the other sugar phosphate was used as substrate.

Both enzymes showed similar pH optima and divalent cation requirement.

Levels of PFK, T6PK, and Galactokinase (Gal K), enzymes of the Embden-Meyerhof-Parnas, Tagatose 6-phosphate, and Leloir pathways respectively, were measured in strains of *S. lactis*, *S. cremoris*, *S. diacetylactis* and *S. faecalis* grown on different sugars. Growth on lactose and galactose induced increased levels of T6PK and Gal K activity, galactose generally inducing higher levels of T6PK than lactose.

In most strains, addition of glucose to media containing lactose or galactose resulted in lowered activities of Gal K, comparable to those in glucose-grown cells. In contrast, T6PK activity was generally not suppressed by growth on glucose plus lactose, while in growth on glucose plus galactose, T6PK activity was approximately 50% of the activity in cells grown on glucose alone.

PFK activity was generally unaffected by the sugar in the growth medium.

In spite of changes in specific activities of PFK and T6PK throughout the growth period of *S. lactis*, the ratio of PFK : T6PK remained fairly constant.

The properties of *S. lactis* PFK and T6PK are compared to those of these enzymes in other bacteria, and the possible role of T6PK in regulation of carbohydrate metabolism in *S. lactis* is discussed.

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ABBREVIATIONS

A^{280}, A^{540}	absorbance at 280 nm and 540 nm
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
bisacrylamide	N, N'-methylene bisacrylamide
β-gal	β-D-galactosidase
β-P-gal	phospho-β-D-galactosidase
BPB	bromophenol blue
BSA	bovine serum albumin
cAMP	3', 5' cyclic AMP
CM	carboxymethyl
CTP, GTP, TTP, ITP, UTP	5'-triphosphates of cytosine, guanine, thymine, inosine, and uracil
DEAE	diethyl aminoethyl
DHAP	dihydroxy acetone phosphate
E. coli	*Escherichia coli*
ED	Entner-Doudoroff
EDTA	ethylene diamine-tetra acetic acid
EMP	Embden-Meyerhof-Parnas
F6P	fructose 6-phosphate
$F6P_{0.5}$	concentration of F6P giving half maximal velocity
FBP	fructose 1,6-bisphosphate
$\Delta G°$	change in free energy
G3P	glyceraldehyde 3-phosphate
G6P	glucose 6-phosphate
G6PDH	glucose 6-phosphate dehydrogenase
GBP	glucose 1,6-bisphosphate
Gal	galactose
Gal 6P	galactose 6-phosphate
Gal K	galactokinase

Glu	glucose
∝GPDH	∝-glycerophosphate dehydrogenase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMP	hexose monophosphate
K_i	inhibitor constant
K_m	Michaelis-Menten constant
Lac	lactose
LDH	lactate dehydrogenase
MES	2-(N-morpholino)ethanesulfonic acid
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
ND	not determined
n_H	Hill coefficient
ox	oxidised
PEG	polyethylene glycol
PEP	phospho-enol pyruvate
PFK	phosphofructokinase
PGA	phosphoglyceric acid
PGI	phospho glucose isomerase
P_i	inorganic phosphate
PK	pyruvate kinase
PMS	phenazine methosulphate
PP_i	inorganic pyrophosphate
PS	protamine sulphate
red	reduced
R5P	ribose 5-phosphate
R_F	relative mobility

$S_{0.5}$	concentration of substrate required to give half maximal velocity
SDS	sodium dodecyl sulphate
Sn	supernatant
T6P	tagatose 6-phosphate
T6PK	tagatose 6-phosphate kinase
TEMED	N, N, N', N' - tetramethylene diamine
TPI	triose phosphate isomerase
Tris	tris (hydroxymethyl) amino methane
Tris-glycerol buffer	50 mM Tris HCl containing 20% (v/v) glycerol, 5 mM EDTA, 5 mM $MgCl_2$, and 10 mM 2-mercapto ethanol
u	In all figures, u represents the Greek letter μ, the symbol for 'micro'
V_{max}, V_m	maximum velocity

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CHAPTER ONE

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 BACTERIAL CARBOHYDRATE METABOLISM : A GENERAL INTRODUCTION

The work described in this thesis is primarily concerned with the properties of phosphofructokinase (PFK) and tagatose 6-phosphate kinase (T6PK) in *Streptococcus lactis*, with particular reference to the role that these enzymes may play in the regulation of glucose, lactose, and galactose metabolism in this organism. Information currently available on the regulatory properties of these two enzymes is reviewed in the introductory sections of Chapters 2 and 3. This chapter presents a brief description of the pathways of carbohydrate metabolism, particularly the pathways of lactose and galactose catabolism, in bacteria, and also a review of our current knowledge of carbohydrate metabolism in lactic streptococci.

Carbohydrate metabolism in bacteria involves a number of metabolic pathways, several of which play a dual role catalysing synthetic or degradative pathways depending on the metabolic requirements of the cell. Such 'amphibolic' pathways operate, for example, in breakdown of carbohydrates to intermediates from which further reactions may proceed, with simultaneous production of ATP to sustain metabolism. The Embden-Meyerhof-Parnas (EMP) or glycolytic pathway, and the hexose monophosphate (HMP) pathway are the major pathways for production of pyruvate from glucose, and are common to most bacteria. Alternative pathways such as the Entner-Doudoroff (ED) and phosphoketolase pathways may operate in some organisms (De Ley, 1960; Cheldelin, 1961).

No single pathway provides all the requirements for growth. The EMP pathway produces ATP but not pentose phosphates which are necessary for nucleic acid synthesis, while the HMP and ED pathways synthesise pentoses but with reduced production of ATP. Most bacteria contain enzymes of several pathways and can metabolise glucose via alternative routes, or via a combination of pathways. Carbohydrates other than glucose are generally metabolised via the EMP pathway but specific pathways such as the Leloir pathway (Leloir, 1953) or the Tagatose 6-phosphate pathway of *Staphylococcus aureus*, the group N streptococci, and *Streptococcus mutans* (Bissett & Anderson, 1973;

Bissett & Anderson, 1974*a*; Hamilton & Lebtag, 1979) may be involved in conversion of these sugars to intermediates of the EMP pathway.

Because of the amphibolic nature of metabolic pathways such as the EMP and HMP pathways, the enzymes of these pathways require subtle regulation to ensure that the operation of the pathway fulfils the momentary demands of the cell. Bacteria employ fewer regulatory mechanisms than higher organisms; prokaryotes lack for example, hormonal regulation and the compartmentational control afforded to eukaryotes by intracellular organelles.

Regulation of metabolic pathways in bacteria may be achieved by controlling enzyme synthesis and degradation (substrate induction or catabolite repression of synthesis) or by modifying activities of specific enzymes in the pathway (Anderson & Wood, 1969). The regulatory enzymes in a pathway are generally allosterically controlled and are often situated at a branch point in the pathway, or catalyse the initial or terminal reactions in the sequence, e.g. PFK and pyruvate kinase (PK) of the EMP pathway. Allosteric regulation of these two enzymes is the major mechanism controlling the activity of the EMP pathway in *E. coli* (Dietzler *et al*, 1975). PFK activity is regulated by end-product inhibition by PEP, as well as by adenine nucleotide concentrations (Blangy *et al*, 1968) while PK is activated by FBP (Maeba and Sanwal, 1968). Determination of the intracellular concentrations of substrates and effectors has confirmed the significance of these regulatory mechanisms *in vivo*. Dietzler *et al* (1975) showed that the rate of glucose utilisation in *E. coli* is correlated with the intracellular levels of FBP, a result which may be explicable by the feed-forward activation of PK by FBP. FBP appears to be a key metabolite for feed-forward activation, regulating activities not only of pyruvate kinase, but also of PEP carboxylase (Sanwal and Maeba, 1966) and lactate dehydrogenase (Garvie, 1980) in many organisms. Negative feedback inhibition is also a common control mechanism in bacterial metabolism, e.g. PEP inhibition of PFK (Blangy *et al*, 1968), acetyl CoA inhibition of pyruvate dehydrogenase (Garland & Randle, 1964; Petit *et al*, 1975) and inhibition of PEP carboxylase by aspartate (Maeba & Sanwal, 1965; Izui *et al*, 1967). The concentration of nucleotide co-enzymes may also regulate metabolic pathways, e.g. the NADH concentration may regulate the tricarboxylic acid cycle in *E. coli* (Sanwal, 1969; Weitzman, 1966) and other Gram-negative bacteria.

In bacteria, as in higher organisms, enzyme regulation by ATP, ADP, AMP, and P_i provides a mechanism for controlling activity in response to the energy status of the cell. Theoretically AMP should provide the most sensitive control since levels of AMP show the greatest fluctuation in response to changes in ATP concentration (Sanwal, 1970). However Atkinson (1968, 1977) proposed that a ratio of nucleotides rather than the concentration of an individual nucleotide was an important regulatory mechanism, as activities of many enzymes are modulated by ADP, AMP and P_i, as well as ATP. The energy status of the cell was expressed in terms of the adenylate energy charge, i.e.

$$\frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$$

Atkinson postulates that this factor is responsible for the regulation of enzyme activity. Activities of ATP-regenerating enzymes have been shown to increase as the energy charge decreases, while activities of ATP-utilising enzymes decrease (Atkinson, 1969). The net effect of these changes in enzyme activity is to favour formation of ATP under conditions of high ADP, AMP and P_i, and low ATP, thus maintaining a constant energy charge.

Regulatory mechanisms must also exist to ensure that the rate of carbohydrate uptake is coupled to the rate of carbohydrate utilisation. Several mechanisms exist for transport of sugars into bacterial cells (Saier, 1977).

i Facilitated diffusion; translocation of a solute molecule along a concentration gradient mediated by a specific membrane protein, such as occurs in glycerol uptake by *E. coli* (Lin, 1976).

ii Active transport; protein-mediated accumulation of sugar against a concentration gradient. This system requires coupling to a source of free energy, via either ATP or the proton motive force (Harold, 1977).

iii Group translocation which involves translocation of a sugar molecule with simultaneous phosphorylation. The most common system for group translocation is the PEP-dependent phosphotransferase system (PEP PTS) which utilises PEP as a phosphate donor for phosphorylation during sugar transport (Kundig *et al*, 1964). Alternative phosphotransferase systems also exist which utilise other compounds such as inorganic phosphate, acyl phosphates and hexose phosphates as phosphate donors (Harold, 1966; Kamel and Anderson, 1967; Stevens-Clark *et al*, 1968). Bacteria may utilise one or more of these mechanisms in uptake of sugars. A brief mention will be made of the PEP PTS, as this system operates in lactose and galactose metabolism in lactic streptococci and may be one of the factors determining the choice of pathway for subsequent metabolism of the sugar.

A PEP-dependent PTS was first demonstrated in uptake of glucose by *E. coli* (Kundig *et al*, 1964) and has since been shown to be involved in uptake of several sugars in numerous bacteria (Roseman, 1969; Postma and Roseman, 1976; Saier, 1977). Transport via a PEP PTS involves simultaneous translocation and phosphorylation of the sugar, which appears within the cell phosphorylated at C_6. The following reactions outline the sequences involved in lactose transport via a PEP PTS.

$$\text{PEP + enzyme I} \xrightarrow{Mg^{2+}} \text{P - enzyme I + pyruvate}$$

$$\text{P - enzyme I + HPr} \longrightarrow \text{P - HPr + enzyme I}$$

$$\text{P - HPr + factor III}^{lac} \longrightarrow \text{P - factor III}^{lac} + \text{HPr}$$

$$\text{P - factor III}^{lac} + \text{lactose} \xrightarrow[\text{enzyme IIB}^{lac}]{Mg^{2+}} \text{lactose 6-phosphate + factor III}^{lac}$$

HPr and enzyme I are non specific proteins, synthesised constitutively. The sugar-specific proteins factor III and enzyme II are thought to be

induced by galactose 6-phosphate (Morse *et al*, 1968). Enzyme II is a membrane-bound complex, specific for one or a small number of sugars. This complex binds to the sugar and presumably acts as a permease (Saier, 1977).

The PEP PTS is dependent on a source of PEP which is generated by the EMP pathway. Thus the system is self-regulating as the transport system supplies carbohydrate for generation of PEP to enable further carbohydrate transport to proceed. However since PEP is also a substrate for other enzymes, e.g. PK and PEP carboxylase, regulation of these enzymes is important to ensure adequate partitioning of PEP between the various systems.

1.2 Lactose and Galactose Metabolism in Bacteria

Not all bacteria contain PEP-dependent phosphotransferases, and in those organisms which do, not all carbohydrates are transported via a PEP PTS. In *E. coli* a PEP PTS exists for transport of several sugars (Roseman, 1972) but both lactose and galactose are transported via permeases and are accumulated intracellularly in an unaltered form. Lactose is then cleaved to glucose and galactose by β-galactosidase, an enzyme which specifically cleaves the β1-4 linkage of lactose. The glucose moiety is presumably phosphorylated by hexokinase and ATP and metabolised via the EMP pathway. The galactose moiety of lactose, and galactose resulting from uptake of free galactose by the permease system is metabolised to glucose 6-phosphate via the Leloir pathway (Fig. 1.2.1) and the glucose 6-phosphate is subsequently converted to pyruvate via the EMP pathway. In *E. coli* the enzymes of the Leloir pathway, galactokinase, galactose 1-phosphate uridylyl transferase, and UDP-glucose 4-epimerase are induced by growth on lactose and on galactose (Bissett & Anderson, 1974*a*). In contrast to *E. coli*, *S. aureus* contains a PEP PTS for transport of both lactose and galactose (Hengstenberg *et al*, 1967, 1968; Simoni *et al*, 1973) and these sugars are phosphorylated at C_6 during transport into the cell. Lactose 6-phosphate is cleaved to glucose and galactose 6-phosphate by phospho-β-D-galactosidase, an enzyme distinct from the β-galactosidase found in *E. coli*. As in *E. coli*, the glucose moiety of lactose in *S. aureus* is metabolised via the EMP pathway.

The pathway by which galactose 6-phosphate is degraded in *S. aureus* was first elucidated in 1973 by Bissett and Anderson (1973). These

FIGURE 1.2.1

THE LELOIR PATHWAY

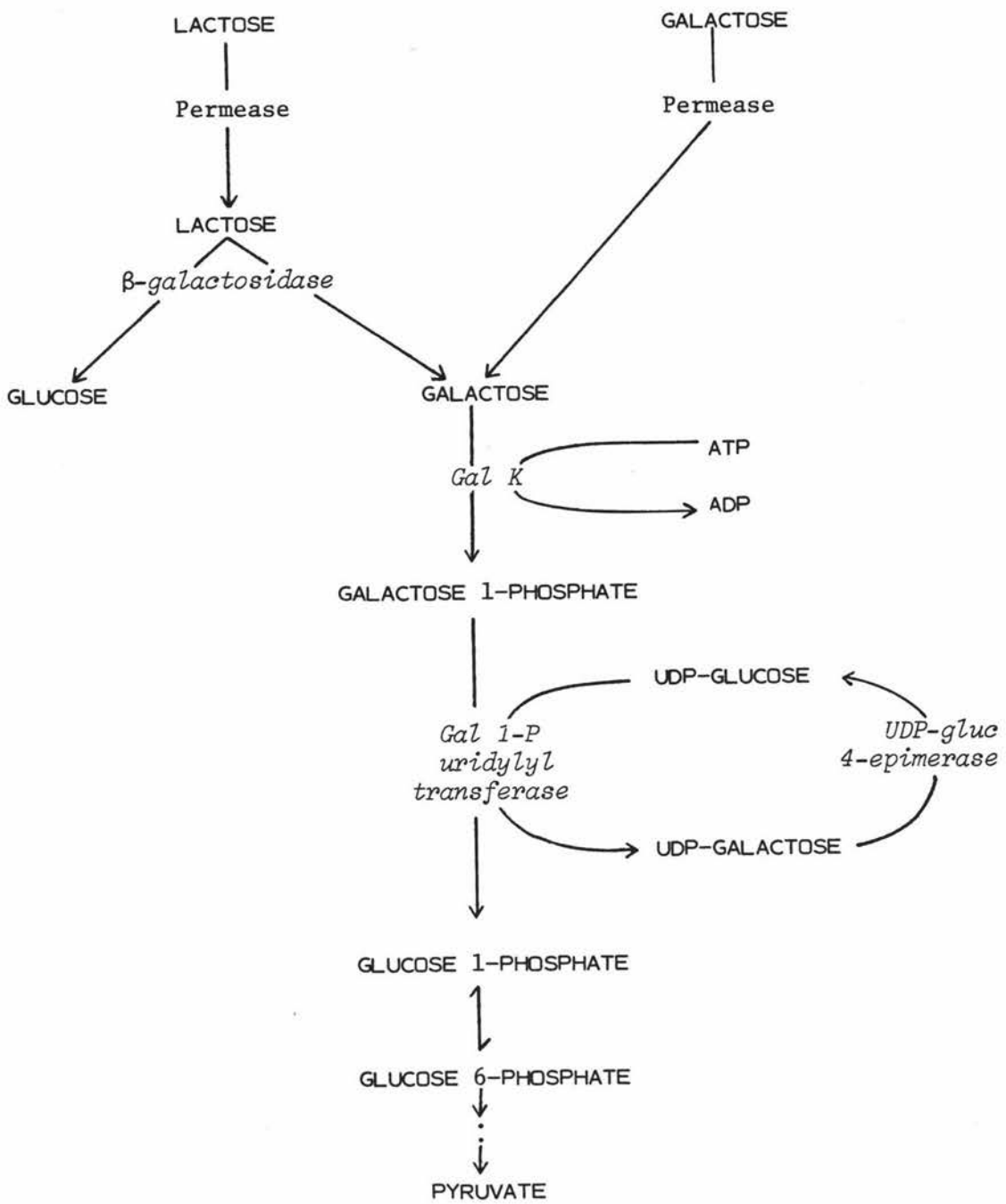
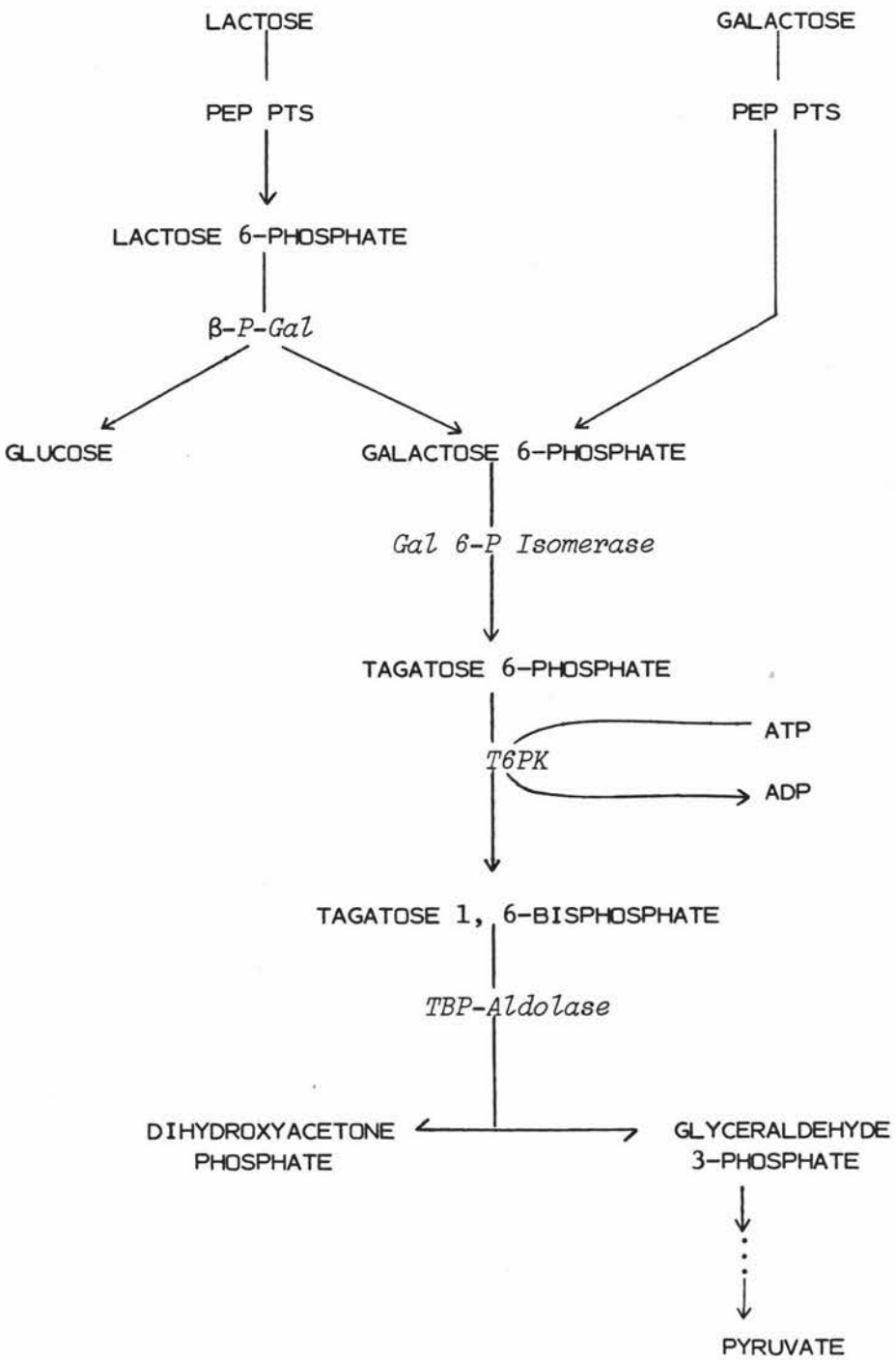


FIGURE 1.2.2

THE TAGATOSE 6-PHOSPHATE PATHWAY



authors demonstrated that galactose 6-phosphate is isomerised to tagatose 6-phosphate, which is phosphorylated by ATP. The resulting tagatose 1, 6-bisphosphate is cleaved to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate which are then metabolised via the EMP pathway (Fig. 1.2.2). The latter two reactions are analogous to those catalysed by PFK and FBP-aldolase in the EMP pathway. The enzymes of this 'Tagatose 6-phosphate pathway', like those of the Leloir pathway in *E. coli* are inducible by growth on lactose or galactose and are distinct from the corresponding enzymes of the EMP pathway (Bissett & Anderson, 1980*a*; Bissett & Anderson, 1980*b*). Bissett and Anderson were unable to detect the presence of the Leloir pathway enzymes in *S. aureus*. In addition, *S. aureus* mutants lacking in any one of the T6P pathway enzymes were unable to grow on lactose or galactose but could metabolise all other carbohydrates tested (Bissett & Anderson, 1974*b*). This evidence suggests that the T6P pathway is the only operative route for lactose and galactose metabolism in *S. aureus*.

The enzymes of the T6P pathway, galactose 6-phosphate isomerase, tagatose 6-phosphate kinase (T6PK) and TBP-aldolase were subsequently shown to be present in the group N Streptococci (*S. lactis, S. cremoris,* and *S. diacetylactis*) (Bissett & Anderson, 1974*a*), *S. mutans* (Hamilton & Lebtag, 1979), and also in several other Staphylococci (Schleifer *et al*, 1978).

1.3 Carbohydrate Metabolism in Lactic Streptococci

The Lactic streptococci (*S. lactis, S. cremoris* and *S. diacetylactis*) are members of the family of lactic acid bacteria, that comprises the genera *Streptococcus, Diplococcus, Pediococcus, Peptostreptococcus and Lactobacillus*.

The lactic acid bacteria can be classified as either homofermentative or heterofermentative according to their end products of glucose metabolism. As the terms imply, homofermentative bacteria normally ferment glucose mainly to lactic acid, although minor amounts of acetic acid and CO_2 are also produced. Heterofermentative organisms produce up to 50% lactic acid, plus considerable amounts of CO_2, ethanol, and acetic acid. The products of carbohydrate metabolism depend largely on the metabolic pathway involved in degradation of the sugar. Operation of the EMP pathway, the major pathway operating in homofermentative

metabolism, produces mainly lactate, whereas heterofermentative lactic acid bacteria may utilise other catabolic routes, e.g. the HMP, ED, or phosphoketolase pathways in place of, or in addition to, the EMP pathway. *Leuconostoc mesenteroides*, for example, lacks the enzymes FBP-aldolase and triose phosphate isomerase of the EMP pathway, and produces equal amounts of lactic acid, CO_2, and ethanol (De Moss *et al*, 1951). The results of labelling experiments with glucose 1-^{14}C implied an operative HMP pathway.

The lactic (or group N) streptococci metabolise glucose mainly via the EMP pathway. Of the enzymes of the HMP pathway, only glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are present, so the complete pathway does not operate (Lawrence *et al*, 1976). Lactic streptococci have a limited biosynthetic capability as they do not contain enzymes of the tricarboxylic acid cycle or a cytochrome system for electron transport under normal growth conditions (Lawrence *et al*, 1976). They therefore rely almost solely on the EMP pathway for generation of ATP, and of PEP for accumulation of carbohydrate via the PEP PTS. Some species however are able to synthesise ATP by metabolising arginine (Abdelal, 1979). Glucose and lactose metabolism by the lactic streptococci is normally homolactic. Although these bacteria contain enzymes capable of production of a variety of end products (Thomas *et al*, 1980), under normal conditions these activities are not expressed. However when carbohydrate becomes growth limiting, increased amounts of formate, acetate and ethanol are formed from glucose fermentation (Thomas *et al*, 1979).

In the lactic streptococci, as in *S. aureus*, lactose metabolism is usually initiated by phosphorylation at C_6 by the PEP PTS (Simoni *et al*, 1973; Thompson, 1979). The lactose 6-phosphate is cleaved by phospho-β-D-galactosidase, and the glucose and galactose 6-phosphate formed are metabolised via the EMP and T6P pathways respectively, as described for *S. aureus* in the previous section of this chapter. Most lactic streptococci were found to contain only very low levels of enzymes of both the Leloir and T6P pathways when grown on glucose (Bissett & Anderson, 1974*a*). Enzymes of the T6P pathway, and to a lesser extent the Leloir pathway were induced by growth on galactose or lactose. The T6P pathway enzymes appear partially constitutive in some strains of *S. lactis* (Bissett & Anderson 1974*a*), and *S. mutans* (Hamilton & Lebtag, 1979), as low levels are present in glucose-grown cells.

Unlike *E. coli* or *S. aureus* in which the enzymes of the T6P and Leloir pathways respectively are absent (Bissett & Anderson, 1974*a*), lactic streptococci contain the enzymes of both pathways when grown on lactose or galactose. They therefore have the potential to use either one or both of these pathways in lactose and galactose metabolism. *S. lactis* 7962, a strain which does not contain β-phosphogalactosidase (Molskness *et al*, 1973) does not utilise the T6P pathway for lactose metabolism, although the enzymes of this pathway are induced by growth on lactose and galactose. Lactose is cleaved by β-galactosidase to glucose and galactose, both of which are phosphorylated by ATP-dependent kinases (Kashket & Wilson, 1973, 1974).

While the metabolism of lactose appears to be similar in all lactic streptococci studied (except in *S. lactis* 7962) galactose metabolism is more variable. Galactose accumulation and metabolism in lactic streptococci may occur in two ways (Thompson, 1980):

i transport by a PEP PTS, followed by metabolism via the T6P pathway

ii uptake via an ATP-dependent permease system, with subsequent metabolism via the Leloir pathway.

The relative participation of these two pathways may vary in different strains and under different growth conditions (Thomas *et al*, 1980).

A question of interest is whether or not the end products of galactose metabolism are determined by the uptake system and/or the degradative pathway used. Utilisation of the T6P pathway bypasses G6P in the EMP pathway, the point at which the HMP and ED pathways depart. Therefore in organisms in which these latter routes are utilised, metabolism via the T6P pathway would tend to favour homolactic fermentation while metabolism via the Leloir pathway could promote either heterolactic or homolactic fermentation. However this would not apply to the lactic streptococci as these organisms do not utilise the HMP pathway. In the lactic streptococci formation of products other than lactate has been attributed to altered activity of lactate dehydrogenase and pyruvate-formate lyase, two enzymes involved in metabolism of pyruvate. During growth of *S. lactis* on galactose (heterolactic fermentation) the activity of pyruvate-formate lyase was higher, while activity of lactate dehydrogenase was lower than the corresponding activities in glucose-grown or lactose-grown

cells (homolactic fermentation) (Thomas *et al* 1980). This is possibly attributable to reduced intracellular levels of FBP and triose phosphates, effectors of the dehydrogenase and lyase activities respectively.

The metabolism of the glucose and galactose moieties of lactose via different pathways, the T6P and EMP pathways, presents an interesting regulatory problem. Neither galactose 6-phosphate nor glucose 6-phosphate accumulate intracellularly, both are metabolised simultaneously (Thompson *et al*, 1978). Obviously some regulatory mechanism operates, co-ordinating activities of the two pathways to ensure equal rates of metabolism of both glucose and galactose 6-phosphate. While the allosteric enzyme pyruvate kinase may control the flux through the EMP pathway by regulating the supply of PEP for the phosphotransferase system, there is no obvious mechanism by which this enzyme could co-ordinate activities of two separate pathways. However PK and LDH activities are modulated by both FBP and TBP, indicating some interaction of activity of the T6P pathway with the regulatory enzymes of the EMP pathway.

PFK is the obvious locus for regulation of the initial reactions of the EMP pathway in *S. lactis*. The allosteric control of PFK by intermediates of glycolysis and other metabolites and the regulatory role of this enzyme has been firmly established in mammalian systems (for reviews see Hofmann, 1976; Goldhammer and Paradies, 1979; Uyeda, 1979). There is also evidence that PFK fulfils a similar role in *E. coli* (Dietzler *et al*, 1975).

The regulatory characteristics of PFK from *S. lactis* are described in Chapter 2 of this thesis. In view of the similarities of the reactions catalysed by PFK and T6PK, it is possible that *S. lactis* T6PK may also be an allosterically controlled, regulatory enzyme. If so, one would expect T6PK to show similar kinetic properties to those of PFK in this organism. T6PK from *S. aureus* has been purified and characterised (Bissett and Anderson, 1980*a*), and did not appear to be subject to allosteric control. However no information is presently available on the properties of any of the T6P pathway enzymes in any other organism.

Another area of interest is the regulatory mechanism(s) involved in the sequential utilisation of sugars when cells are grown on mixtures of sugars. Most strains of *S. lactis* metabolise glucose and

lactose in preference to galactose (Lawrence *et al*, 1976; Thompson *et al*, 1978). When grown in a medium containing glucose, lactose and galactose, galactose-adapted cells of *S. lactis* ML_3 metabolise glucose and lactose simultaneously, while galactose is metabolised only when glucose and lactose supplies are exhausted. Addition of glucose or lactose to cells actively metabolising galactose inhibits galactose metabolism until lactose and glucose are metabolised (Thompson *et al*, 1978). Preferential utilisation of glucose has also been reported in other strains of *S. lactis* (Cords and McKay, 1974; Gilliland *et al*, 1972). Although the molecular controls responsible for sequential utilisation of sugars are relatively well understood in *E. coli* (Pastan and Adhya, 1976) it is not known whether similar mechanisms underlie this phenomenon in lactic streptococci.

1.4 AIMS OF THIS STUDY

Lactic streptococci are used widely in the dairy industry as cheese "starters". As the characteristics of cheeses depend partially on the fermentation products of the starter organisms, an understanding of the factors regulating their metabolic pathways is important in appreciating the potential of these organisms. *S. lactis* C_{10} was chosen for this study as previous studies on regulation of carbohydrate metabolism have been carried out in this strain. Although the regulatory properties of pyruvate kinase and lactate dehydrogenase have been studied in *S. lactis* C_{10} (Crow, 1975) there is little information available on the enzymes catalysing the initial reactions in the glycolytic pathway in this strain.

S. lactis utilises the tagatose 6-phosphate pathway for lactose and galactose metabolism. The enzymes of this pathway have not previously been studied in any organism other than *S. aureus* (Bissett & Anderson, 1974*a*, 1980*a*). As mentioned in the previous section, the activities of the T6P pathway and the EMP pathway must be co-ordinated during lactose metabolism so a comparison of the regulatory properties of homologous enzymes in the two pathways is of interest.

The major aim of this work was to purify PFK and T6PK from *S. lactis* C_{10} as far as possible and to characterise their major kinetic and regulatory properties. From such a study comparisons could then be made

i between T6PK from *S. lactis* and T6PK from *S. aureus*

ii between PFK from *S. lactis* and the PFKs from other bacteria

iii between PFK and T6PK from *S. lactis*.

To obtain information on the relative participation of the EMP, T6P, and Leloir pathways in metabolism of glucose, lactose, and galactose in lactic streptococci activities of PFK, T6PK and galactokinase (Gal K) were measured in cell-free extracts from strains of *S. lactis*, *S. cremoris*, and *S. diacetylactis* grown on different sugars. These activities were also measured in *S. faecalis*, a group D streptococcus. It was hoped that a study of PFK and T6PK in *S. lactis* would assist in assessing the possible role of these enzymes in carbohydrate metabolism via the T6P and EMP pathways, and contribute to our understanding of metabolic regulation in this organism.

CHAPTER TWO

PHOSPHOFRUCTOKINASE

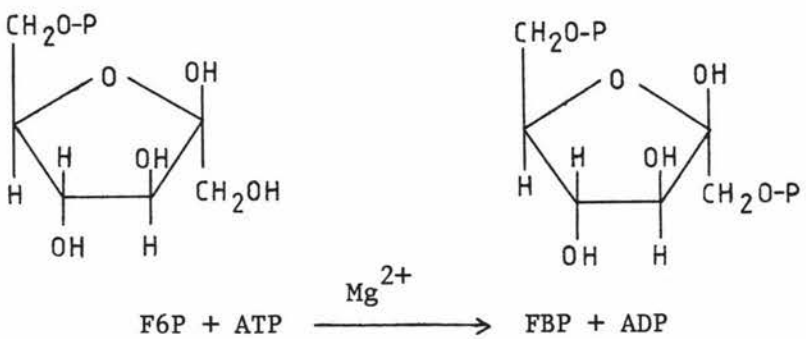
CHAPTER 2

PHOSPHOFRUCTOKINASE

2.1 INTRODUCTION

2.1.1 GENERAL CHARACTERISTICS OF PHOSPHOFRUCTOKINASE

Phosphofructokinase (ATP : D-fructose 6-phosphate 1-phosphotransferase, E.C.2.7.1.11) plays an important role in the regulation of carbohydrate metabolism, catalysing the ATP-dependent phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate.



Phosphofructokinase (PFK) has been studied from bacteria, plants, yeasts, and higher organisms (for reviews see Mansour, 1972; Ramaiah, 1975; Hofmann, 1976; Goldhammer & Paradies, 1979: Uyeda, 1979). The enzyme from most sources is allosterically controlled and shows co-operative binding of F6P. Activity is modulated by a number of effectors, many of which are intermediates in the glycolytic, or other metabolic pathways. The action of effectors depends on the source of the PFK.

Mammalian PFKs generally exhibit a more complex response to effectors than do the prokaryotic PFKs. Allosteric behaviour of bacterial PFKs is rather diverse, depending on the requirements of individual species. However three characteristics of PFK are conserved in mammalian, bacterial, and plant PFKs:

i The enzymes show a sigmoidal dependence on F6P concentration.

ii PFK activity responds to the energy requirements of the cell, increasing the rate of glycolysis when ATP is required.

iii Activity is subject to feedback inhibition by intermediates in the pathways of carbohydrate metabolism, namely citrate in eukaryotes and PEP in prokaryotes.

Enzyme response to allosteric effectors is a complex function consisting not only of interactions between enzyme and modifier, but also of interactions between modifier molecules (Kemp *et al*, 1976). Positive allosteric effectors increase the affinity of the enzyme for F6P, shifting the sigmoidal saturation curve towards a more hyperbolic form, while negative effectors decrease enzyme-substrate affinity. Most PFKs are inhibited by high concentrations of ATP, and this inhibition may be relieved in the presence of F6P, glucose bisphosphate, ADP, AMP, cAMP or P_i.

Although most PFKs are allosterically controlled, non-allosteric enzymes have been isolated from several bacteria (Baumann & Wright, 1968; Ferdinandus & Clark, 1969; Simon & Hofer, 1977, 1981; Babul, 1978). These enzymes have similar physical properties to the allosteric bacterial PFKs but do not show co-operative binding of F6P and are less sensitive to the action of metabolites.

PFK shows limited substrate specificity. Most PFKs are able to utilise other nucleoside triphosphates as phosphate donors, but not creatine phosphate, polyphosphates or PEP. Divalent cations are required for activity as the active ATP substrate is a MgATP complex (Paetkau & Lardy, 1967). Specificity for sugar phosphate depends on the source of the enzyme. Skeletal muscle PFK can phosphorylate tagatose 6-phosphate, fructose 1-phosphate, glucose 1-phosphate, sorbose 6-phosphate, psicose 6-phosphate, sedoheptulose 7-phosphate and fructose 6-sulphate in addition to F6P. PFK also shows anomeric specificity, rabbit muscle PFK phosphorylating the β-anomer of F6P, but not the α-anomer or the acyclic keto form (Fishbein *et al*, 1974; Wurster & Hess, 1974).

2.1.2 EUKARYOTIC PFK

PFK has been purified from numerous eukaryotic sources, from tissues such as skeletal muscle, heart muscle, liver, kidney, and erythrocytes, of rabbits, rats, humans, sheep, guinea pigs, chickens, pigs and oysters. The properties of these enzymes have been the

subject of several reviews (Mansour, 1972; Ramaiah, 1975; Hofmann, 1976; Goldhammer & Paradies, 1979; Uyeda, 1979). The properties of bacterial PFKs are less complex than those of PFK from higher organisms, thus many of the regulatory mechanisms operating in mammalian systems bear little relationship to those in bacteria. For this reason the characteristics of eukaryotic PFKs will be discussed only briefly.

Mammalian PFKs are proteins of molecular weight 3-5 x 10^5 daltons. The active form of the enzyme appears to be a tetramer consisting of identical subunits. However the proteins undergo aggregation, so multiple oligomeric forms of active PFK may exist (Paetkau & Lardy, 1967; Hofer, 1971; Aaronson & Frieden, 1972; Wenzel *et al*, 1972).

Most mammalian species contain at least two isozymes of PFK which are immunologically distinguishable and differ in their sensitivity to allosteric effectors (Meinhofer *et al*, 1972; Tanaka *et al*, 1971; Kurata *et al*, 1972). One isozyme is found in heart and skeletal muscle and a second enzyme occurs in liver and erythrocytes. Hybrid enzymes may also exist (Tsai & Kemp, 1972, 1973).

Most mammalian PFKs are allosteric enzymes, although under certain conditions co-operativity can be abolished and the enzyme displays Michaelis-Menten kinetics (Trivedi & Danforth, 1966). The major activators and inhibitors of mammalian PFK are listed in Table 2.1.1.

Yeast PFK

Yeast PFK differs from mammalian and bacterial PFKs in that the active enzyme appears to be an octamer rather than a tetramer (Kopperschlager *et al*, 1977; Pleitz *et al*, 1978). The yeast enzyme consists of two non-identical subunits α and β, differentiated by their antigenic properties (Herrmann *et al*, 1973). Subunit molecular weights ranging from 96,000 - 130,000 daltons have been determined and the molecular weight of the native enzyme ranges from 500,000 - 835,000 daltons depending on the purification procedure. Unlike mammalian PFK, yeast PFK does not undergo association and dissociation except at very low (<10 $\mu g\ ml^{-1}$) protein concentrations.

TABLE 2.1.1

ALLOSTERIC EFFECTORS OF MAMMALIAN PHOSPHOFRUCTOKINASE *

Activators**	Inhibitors
AMP	ATP
ADP	citrate
cAMP	3-phosphoglycerate
P_i	2-phosphoglycerate
NH_4^+	2, 3-bisphosphoglycerate
K^+	PEP
Fructose 2, 6-bisphosphate	phosphocreatine
Glucose 1, 6-bisphosphate	NADH
F6P	NADPH
FBP	Mg^{2+}
	Ca^{2+}

* Adapted from Tejwani (1978).

** Included in this list are compounds which do not directly activate PFK but may relieve inhibition caused by other effectors.

Yeast PFK shows co-operative binding of F6P and pH-dependent inhibition by ATP which is enhanced by P_i but overcome by NH_4^+ or ADP (Atzpodien & Bode, 1970). ADP however is inhibitory at non-inhibitory concentrations of ATP. PEP is a negative effector and AMP an activator.

2.1.3 PROKARYOTIC PFK

PFKs from various bacterial species show a remarkable similarity in physical and kinetic characteristics, in spite of the diversity of metabolic pathways employed. Most bacterial PFKs are proteins of MW approximately 140,000 daltons, and like the mammalian PFKs, consist of four identical subunits. Although most prokaryotic PFKs are allosterically controlled, non-allosteric enzymes have been isolated from the slime mould *Dictyostelium discoideum* (Baumann & Wright 1968), *E.coli* (Babul, 1978; Robinson & Fraenkel, 1978), *Arthrobacter crystallopoietes* (Ferdinandus & Clark 1969), *Streptococcus thermophilus* (Simon & Hofer, 1981) and also from some Lactobacillus species (Simon & Hofer, 1977).

While PFK from *L. acidophilus* is an allosteric enzyme (Simon & Hofer, 1977) exhibiting a sigmoidal dependence on F6P concentration, the enzymes from *L. caseii* and *L. plantarum* appear to be non-allosteric (Doelle, 1972; Simon & Hofer, 1977), showing Michaelis-Menten kinetics with respect to F6P. In spite of this kinetic difference both *L. acidophilus* and *L. plantarum* PFKs are structurally similar proteins. The native proteins (MW $\sim$ 154000) are tetramers of identical subunits, and are immunologically cross reactible. FBP activates *L. acidophilus* PFK, both increasing maximum velocity and decreasing the Hill co-efficient. Glucose bisphosphate, which activates PFK from several mammalian tissues (Hofer & Pette, 1968) did not affect activity. ADP and high concentrations of ATP were inhibitory while citrate, 3'5' cAMP and PEP had no effect on activity.

Two separate forms of PFK have been isolated from *E. coli* (Babul, 1978; Robinson & Fraenkel, 1978; Kotlarz & Buc, 1977), an allosterically controlled enzyme designated PFK I and a non-allosteric enzyme, PFK II. PFK I is specified by the pfk A gene, and PFK II was isolated from strains carrying a second gene pfk B (Morrissey & Fraenkel,1972; Fraenkel *et al*, 1973). PFK I is a tetramer of subunit molecular weight 34,000 daltons. A subunit molecular weight of 36,000

daltons for PFK II is reported (Babul, 1978) but the molecular weight of the native protein has been determined by separate workers as 69,000 daltons - a dimer (Kotlarz & Buc, 1977), and 150,000 daltons - a tetramer (Babul, 1978). No immunological cross reaction was detected between the two proteins. Initial experiments showed that PFK II, unlike PFK I did not show co-operative kinetics with respect to F6P, nor was it inhibited by PEP or activated by ADP. However PFK II has recently been shown to exhibit some regulatory features similar to PFK I (Kotlarz & Buc, 1981).

PFK II could utilise T6P as substrate whereas PFK I could not. *E. coli* mutants lacking PFK II were unable to grow on galactitol (Lengeler, 1977) so PFK II may function in galactitol metabolism in *E. coli*. *E. coli* has been shown to transport galactitol via a PEP-phosphotransferase system (Lengeler, 1975) and subsequent metabolism of the phosphorylated hexitol may proceed via T6P and TBP, as in *Klebsiella pneumoniae* (Markwell *et al*, 1976).

Different forms of PFK in *E. coli* and other Enterobacteraceae, depending on the O_2 tension of the medium, have also been reported (Doelle, 1974; Doelle & Hollywood, 1977). It was found that transition from anaerobic to aerobic conditions resulted in a change from a tetrameric ATP-sensitive form to a dimeric ATP-insensitive form. In addition the dimer may exist in two interconvertible forms, one active and the other inactive (Ewings & Doelle, 1976). Interconversion of these two forms may be due to a slow conformational change, or aggregation in the presence of positive effectors. However Babul *et al* (1977) found no differences between the PFKs isolated from aerobically and anaerobically grown cells of *E. coli*.

PFKs from the thermophilic microorganisms *Bacillus stearothermophilus* and *Thermus X1* have been purified (Cass & Stellwagen, 1975; Hengartner & Harris, 1975; Evans & Hudson, 1979). Despite the unusual thermostability of these proteins, both are typical of the tetrameric allosteric PFKs of bacteria; they are tetramers of identical polypeptide chains of molecular weight approximately 35,000 daltons. *B. stearothermophilus* PFK shows co-operative binding of F6P, and allosteric activation by ADP and inhibition by PEP.

In addition to the ATP-dependent fructose 6-phosphate kinase (PFK) common to most organisms, several bacterial species contain PFKs exhibiting specificity for substrates other than F6P or MgATP. A pyrophosphate-dependent PFK which catalyses the reversible reaction

$$F6P + PP_i \longleftrightarrow FBP + P_i$$

has been isolated from *Propionii bacterium shermanii* (O'Brien *et al*, 1975) and *Entamoeba histolytica* (Reeves *et al*, 1976). ATP-dependent PFK is present in these species at much lower concentrations than in other micro-organisms.

D-fructose 1-phosphate kinases distinct from fructose 6-phosphate kinases have also been isolated from *Aerobacter aerogenes* (Hanson & Anderson, 1966), *Bacteroides symbiosis* (Reeves *et al*, 1966), *Clostridium pasteurianum* (Hugo & Gottschalk, 1974*a*), *E. coli* (Fraenkel, 1968), *Rhodopseudomonas capsulata* (Conrad & Schlegel, 1974) and other saccharolytic clostridia (Hugo & Gottschalk, 1974*b*).

2.1.4 Plant Phosphofructokinases

PFKs from plants show regulatory properties similar to the PFKs from bacteria or higher organisms. Only partially pure preparations of PFK have so far been obtained from sources such as parsley and avocado (Lowry & Passoneau, 1964), carrot (Caldwell & Turner, 1979), brussels sprouts (Dennis & Coultate, 1967), spinach (Kelly & Latzko, 1975; 1977), pea seeds (Kelly & Turner, 1969), castor bean (Dennis & Green, 1975), and corn (Garrard & Humphreys, 1968). Plant PFKs are generally allosteric enzymes showing inhibition by ATP, ADP, citrate, 2-phosphoglycerate, 3-phosphoglycerate, and PEP. PEP inhibition is dependent on concentrations of ATP, F6P, Mg^{2+}, and monovalent cations. Kelly & Latzko (1977) obtained evidence for separate cytoplasmic and chloroplastic forms of PFK with different kinetic properties.

2.1.5 The Role of PFK in Regulation of Carbohydrate Metabolism

A role for PFK in the regulation of glycolysis in mammalian tissues has been clearly established. As well as modification of activity by allosteric effectors several other factors may also be involved in regulation of PFK activity : hormonal control of enzyme

synthesis (Beitner & Kalant, 1971; Weber, 1974), interconversion of active and inactive forms either by phosphorylation and dephosphorylation (Brand & Soling, 1975; Hussey *et al*, 1977) or by association and dissociation of protomers (Paetkau & Lardy, 1967: Hofer, 1971; Aaronson & Frieden, 1972; Leonard & Walker, 1972), and interaction of PFK with other macromolecules such as fructose bisphosphatase, a reversible inhibitor of PFK activity (Proffitt *et al*, 1976).

PFK is also the major enzyme responsible for the oscillations in concentrations of glycolytic intermediates noted in yeast, tumor cells, and beef heart (Hess & Boiteaux, 1971) and for the increased rate of glycolysis observed on transition from aerobic to anaerobic conditions i.e. the 'Pasteur Effect' (Ramaiah, 1975; Tejwani, 1978).

Control of bacterial PFK is, as far as is known, achieved largely via allosteric modulation of enzymes by metabolic intermediates, as micro-organisms appear to lack many of the control mechanisms such as hormonal control, association/dissociation dependent activation etc. which are commonly found in mammalian systems (Sanwal, 1970). Measurement of *in vivo* metabolite levels in *E. coli* (Lowry *et al*, 1971) revealed changes in concentrations under different growth conditions which supported the regulatory mechanisms proposed for PFK (Blangy *et al*, 1968). Gluconeogenesis was associated with lower levels of FBP and higher levels of PEP than in cells grown on glucose, conditions which would favour decreased activities of PFK, pyruvate kinase, and PEP carboxylase. In *E. coli*, as in mammalian tissues, gluconeogenesis involves fructose bisphosphatase activity so reciprocal allosteric control of PFK and FBPase is required to prevent substrate cycling.

Further evidence for the role of PFK in regulation of carbohydrate metabolism in *E. coli* was presented by Dietzler *et al*, (1975) who showed that the rate of glucose metabolism was directly proportional to the concentration of FBP in the cell, and was thus controlled by the activity of PFK. In plants too, PFK activity appears directly related to metabolic activity. The regulatory properties of PFK from brussels sprout have been shown to differ depending on the metabolic state of the tissue (Dennis & Coultate, 1967); the enzyme from immature tissue showing greater regulatory control than enzyme from mature leaves.

In the lactic streptococci nothing is known of the regulation of PFK. The absence of fructose bisphosphatase (Dr V.L. Crow, N.Z. Dairy Research Institute, Palmerston North, personal communication) and of a TCA cycle suggests that the sites and mechanisms of control of glycolysis in these organisms may be different to those in other bacteria. Regulation of the terminal reactions in the Embden-Meyerhof-Parnas pathway (those catalysed by PK and LDH) by precursor activation (Collins and Thomas, 1974: Crow & Pritchard, 1976), and the coupling of the rate of carbohydrate uptake to the availability of PEP (Thompson & Thomas, 1977) may provide sufficient regulation of glycolysis in these organisms. Thus regulation of PFK in lactic streptococci may be quite different to that in other organisms. However, as outlined in Chapter 1, the metabolism of the glucose and galactose moieties of lactose by two parallel but quite separate pathways converging at triose phosphates, suggests a requirement for co-ordination of activities of the enzymes of these two pathways. A characterisation of the regulatory properties of *S. lactis* PFK was therefore undertaken.

The following sections of this chapter describe some of the physical and kinetic properties of PFK purified from *S. lactis* C_{10}. In these sections the properties of *S. lactis* PFK are compared with those of other bacterial PFKs, and in Chapter 5 the similarities and differences between PFK and tagatose 6-phosphate kinase (T6PK) from *S. lactis* are described.

2.2 MATERIALS AND METHODS

2.2.1 REAGENTS

Peptone and yeast extract were obtained from Difco Laboratories, and beef extract from the Oxo Co. Ltd. All enzymes were purchased from the Sigma Chemical Company as ammonium sulphate suspensions, and stored at 4°C. Ammonium sulphate was removed before use, by dialysis.

Substrates, co-enzymes and effectors (Grade I) were obtained as the sodium salts from the Sigma Chemical Co. Solutions of these were made up in 50 mM Tris-HCl pH 7.5 and neutralised, if necessary, to pH 7.0 - 7.5 with HCl or NaOH. Solutions were stored frozen for up to one month in small volumes which were thawed when required, and generally discarded after use. Concentrations were checked periodically, wherever possible, by enzymic assay.

Sigma Chemical Co. also supplied the Tris, MES, HEPES and Tricine buffers, nitro blue tetrazolium, phenazine methosulphate, bovine serum albumin, Blue-dextran, Coomassie brilliant blue G250, and R250, and the molecular weight marker proteins used in SDS gel electrophoresis.

Sephacryl S200 and Sepharose 6B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and after use were washed and stored at 4°C in an aqueous solution of 0.02% (w/v) sodium azide. DEAE Sephadex (Sigma) and CM and DEAE celluloses (Whatman) were similarly stored after use.

Glycerol (98%) supplied by the National Dairy Association, N.Z., was used in preparation of buffers but this was replaced by 'Analar' glycerol (BDH) in the final dialysis of the purified enzyme.

Ultrafiltration membranes (PM30) were supplied by Amicon.

Aquacide was obtained from Calbiochem. $MgCl_2.6H_2O$ was obtained from BDH, and dried overnight at 100°C before use. The dried salt was weighed as anhydrous $MgCl_2$.

All other chemicals were obtained at the highest purity available from either Sigma Chemical Co., British Drug Houses, or Merck Chemical Co.

Dialysis tubing obtained from The Scientific Instrument Centre Ltd., London, was treated with ethanol, $NaHCO_3$ and EDTA before use

(McPhie, 1971) and stored at 4°C in 0.02% (w/v) sodium azide.

2.2.2 Culture and Maintenance of Bacteria

S. lactis C_{10} was obtained from the N.Z. Dairy Research Institute, Palmerston North. Cultures were maintained at 4°C on 1.5% nutrient agar slabs containing 2% (w/v) lactose. The organism was subcultured at intervals of 3-6 weeks. Cultures were checked regularly for purity by microscopic examination of Gram-stained cells.

2.2.3 Growth of Bacteria

S. lactis was cultured in 35 l batches in a Fermacell fermentor (New Brunswick Scientific Co.) in a medium containing the following constituents per litre of distilled water:

Lactose, 20 g: peptone, 10 g; yeast extract, 10 g; KH_2PO_4, 5 g; beef extract, 2 g; $MgSO_4$, 0.2 g; $MnCl_2$, 0.05 g.

The complete medium was sterilised at 121°C for one hour, cooled, and neutralised to pH 6.5 with sterile 2.5 M NaOH.

Bacterial growth was initiated by addition of 500 ml of a culture grown overnight on the same medium. The culture was stirred continuously at 300 r.p.m. and flushed with 95% N_2/5% CO_2 before inoculation and at intervals throughout the growth period. Temperature was maintained at 30°C and the pH was maintained at 6.4 $\pm$ 0.3 pH units by neutralisation of the lactic acid produced with sterile 2.5 M NaOH.

Cells were harvested in the late-log phase (after approx. 6 hr growth) by centrifugation at 11,000 g for 5 minutes at 0°C. The cells were washed twice by resuspension followed by centrifugation (5 mins, 11,000 g) in cold 50 mM Tris-HCl buffer, pH 7.5 and then were stored at -20°C in an airtight container until required.

S. lactis C_{10} grown and harvested under the conditions described, yielded 15-20 g cells (wet weight) per litre of medium. The maximum period of storage of frozen cells was five months; during this time no decrease in specific activity of PFK was noted. This is in contrast to *S. aureus* PFK which has been reported to be unstable

following freezing of whole cells (Bissett, 1975).

2.2.4 Preparation of Blue-dextran-Sepharose 6B 'Affinity' Resin

Blue-dextran was covalently coupled to cyanogen bromide-activated Sepharose 6B according to the procedure of Cuatrecasas (1970).

i Activation of Sepharose 6B

10 g (wet weight) of Sepharose 6B was washed with distilled water and resuspended in 10 ml distilled water. 3.5 g finely crushed CNBr was added with continual stirring, and crushed ice and 5 M NaOH added as required to maintain the temperature at 20 $\pm$ 5°C and pH at 11 $\pm$ 0.5 pH units. When the reaction was complete, as indicated by no further change of pH, excess ice was added, and the resin was washed with 500 ml cold 0.1 M $NaHCO_3$.

ii Coupling of blue-dextran to activated Sepharose 6B

The CNBr activated Sepharose 6B was immediately resuspended in 10 ml 0.4 M $Na_2CO_3/NaHCO_3$ pH 10.0 containing 0.25 g blue-dextran. The mixture was stirred by inversion for 48 hours at 25°C, then the resin was washed, first with 1 M KCl, then with distilled water until no further blue colour was washed from the resin. The blue-dextran-Sepharose was suspended in 50 ml 1 M ethanolamine at 25°C for one hour to block any unreacted groups, then washed with distilled water and stored at 4°C in 0.02% sodium azide. By measuring the absorbance of the wash at 280 nm, it was estimated there was 10-15 mg dye bound per g (wet weight) resin. The blue-dextran Sepharose was washed thoroughly with 3 M KCl before use, as some dye was released on prolonged storage.

2.2.5 Protein Determination

Protein concentrations were estimated by the Coomassie blue dye-binding method of Bradford (1976). Bovine serum albumin was used as a standard protein. Concentrations of albumin solutions were

determined spectrophotometrically using an extinction coefficient ($\mathcal{E}^{1\%}_{280}$) of 6.67.

2.2.6 PFK Assays

PFK activity was enzymically coupled to NADH oxidation as described below and the reaction monitored by following the decrease in absorbance at 340 nm on a Cecil spectrophotometer equipped with a Servoscribe chart recorder. Two assay systems were used.

i PFK activity was coupled to oxidation of NADH by ∝-glycerophosphate dehydrogenase (∝GPDH) via the enzymes aldolase and triose phosphate isomerase (TPI). This assay measures the formation of FBP (Figure 2.2.1) and phosphorylation of 1 µmol of F6P results in oxidation of 2 µmoles NADH. The assay mixture contained 50 mM Tris-HCl pH 7.5, 0.2% BSA, 10 mM 2-mercapto ethanol, 0.33 mM NADH, rabbit muscle aldolase (5 µl [1.8 units]/ml) and ∝GPDH-TPI mixture (5 µl [1.0 unit ∝GDH, 10 units TPI]/ml). Auxiliary enzymes were dialysed against 50 mM Tris-HCl pH 7.5 for 15-30 minutes before use to remove the ammonium sulphate in which they were stored.

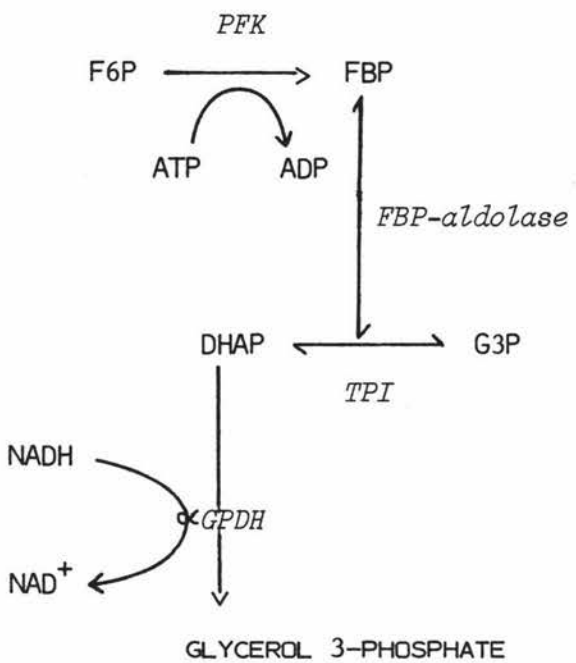
ii The second assay system used was less specific, measuring the rate of formation of ADP. PFK activity was coupled to NADH oxidation by lactate dehydrogenase (LDH) via pyruvate kinase (PK) (Figure 2.2.1). Phosphorylation of 1 µmole of F6P results in oxidation of 1 µmole NADH in this assay. The assay mixture contained the following:

50 mM Tris-HCl pH 7.5, 0.2% BSA, 10 mM 2-mercapto ethanol, 0.33 mM NADH, 2.0 mM PEP, PK (1 µl [1.9 units]/ml) and LDH (1.5 µl [5.2 units]/ml).

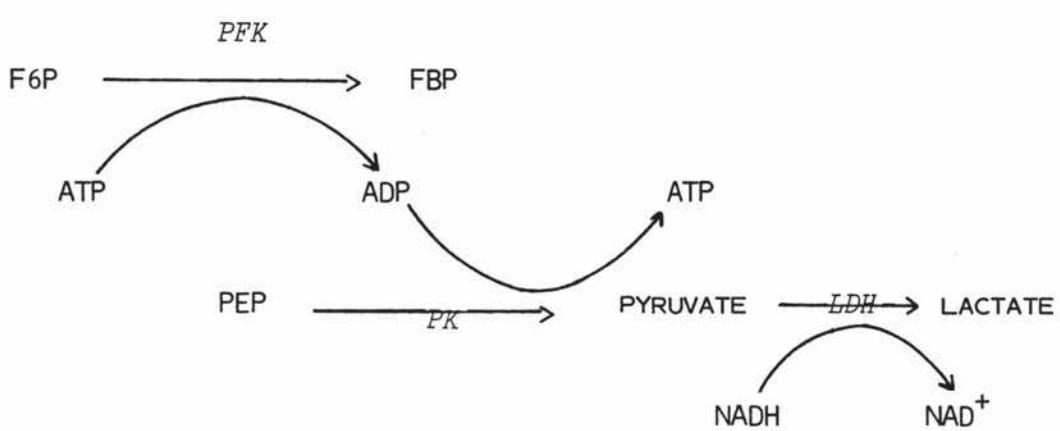
FIGURE 2.2.1

PFK ASSAYS

i 'ALDOLASE' ASSAY



ii 'PYRUVATE KINASE-LACTATE DEHYDROGENASE' ASSAY



Assays using either the 'aldolase' or the 'PK-LDH' assay systems were performed at 30 $\pm$ 1°C in a 1.0 ml capacity microcuvette by mixing 0.30 ml assay mixture, 50 µl of appropriately diluted MgATP and F6P solutions, 25-50 µl PFK, and buffer to a total volume of 0.50 ml. In assays using purified enzyme, the reaction was started by addition of F6P after incubation of enzyme with all other components of the assay mixture for 5 minutes at 30°C.

'Background' activity was measured either prior to addition of F6P or in a separate assay in which F6P was replaced by buffer. NADH oxidase activity was also measured in a separate assay containing 25-50 µl enzyme, 0.33 mM NADH, and buffer (total volume = 0.50 ml).

Assays using cell-free extracts were started by the addition of the extract to the reaction mixture, as a higher background activity was obtained than in assays using purified enzyme, particularly when using the PK-LDH system. (See Section 3.2.4 for discussion of this 'background' activity.)

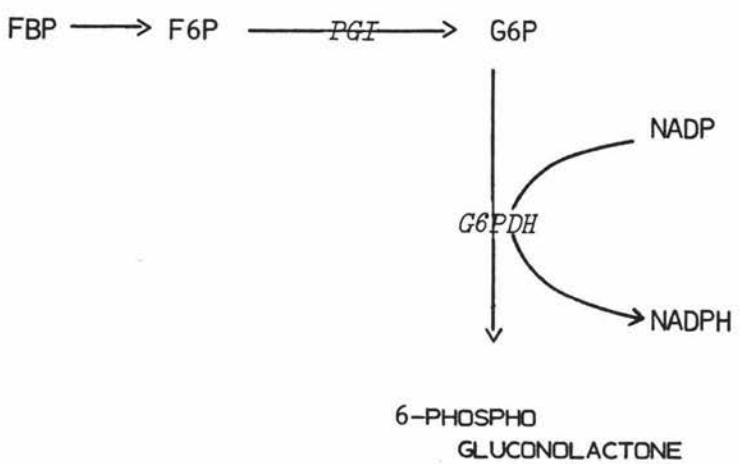
PFK activity is generally expressed as specific activity i.e. µmol F6P phosphorylated per minute per mg protein. In some cases activity is recorded in µmol F6P min^{-1} ml $enzyme^{-1}$. One enzyme unit is defined as the amount of enzyme catalysing phosphorylation of 1 µmol F6P per minute under the conditions of the assay.

iii Reversibility of PFK Activity

The assay system used to measure the formation of F6P from FBP by PFK contained 10 mM FBP, 2.0 mM ADP, 0.33 mM $NADP^+$ and excess phosphoglucose isomerase and glucose 6-phosphate dehydrogenase in 50 mM Tris-HCl buffer, pH 7.5 containing 0.2% BSA and 10 mM 2-mercapto ethanol. In this assay F6P is converted to glucose 6-phosphate by phosphoglucose isomerase and the reaction is monitored by following the reduction of $NADP^+$ as G6P is oxidised to 6-phospho gluconolactone (Figure 2.2.2). Reactions were carried out in a total volume of 0.50 ml, using 25-50 µl undiluted PFK (i.e. 20-40 times the amount used in assays in the forward direction).

FIGURE 2.2.2

ASSAY FOR FORMATION OF F6P
FROM FBP BY PFK



2.2.7 DETERMINATION OF KINETIC PARAMETERS

Inhibition patterns and K_m values were calculated from double reciprocal plots of $\frac{1}{V}$ against $\frac{1}{[\text{substrate}]}$ (Lineweaver & Burk, 1934). When V_{max} was not evident from a plot of reaction velocity vs substrate concentration, it was estimated by plotting the reciprocal of the velocity at a given substrate concentration against the reciprocal of the velocity at half that concentration (Endrenyi *et al*, 1975). Such a graph allows calculation of V_{max} and the Hill coefficient n_H, from the slope and intercept of the lines with the vertical axis. However care was required in using this method for determining V_{max} as plots were often not linear, and small errors in determining the slope or intercept resulted in a large error in the calculated V_{max}.

Hill coefficients were also determined from plots of $\text{Log}_{(V_m - V)}^{V}$ vs Log substrate concentration, as were substrate concentrations required for half maximal velocity ($S_{0.5}$). When graphs were linear, a least squares fit was obtained using non-weighted linear regression analysis.

All results are the average of duplicate assays.

2.2.8 GEL ELECTROPHORESIS

i Discontinuous Gel electrophoresis : Non Denaturing conditions

7.5% polyacrylamide gels (7.5% acrylamide, 0.18% bisacrylamide) pH 8.9 were prepared according to the method outlined by Maurer (1971). Separation gel solutions were mixed and deaerated under vacuum, then polymerised in 9 cm tubes of 0.5 cm internal diameter by addition of ammonium persulphate to a final concentration of 0.07%. When solidified, the separation gel was overlaid with 0.5 - 1 cm of stacking gel solution (2.5% acrylamide, 0.6% bis-acrylamide, 20% sucrose, 0.05% TEMED, 0.07% ammonium persulphate in Tris-HCl buffer, pH 6.8). A level gel surface was achieved by overlaying the gel solution with water during polymerisation.

Electrophoresis was performed at room temperature, or at 4°C when gels were required for activity staining. Protein samples were dialysed against Tris-glycine electrode buffer (5 mM Tris, 0.04 M glycine) containing 10 mM 2-mercapto ethanol, 10% glycerol and approximately 0.01% bromophenol blue. 5-100 µl solution was applied to each gel. Gels were electrophoresed at 1.5 mA per gel until the dye-front entered the separation gel, then at 3 mA per gel for 2-3 hours, until the dye-front approached the end of the gel.

Protein was stained for 1-2 hours in a solution of Coomassie brilliant blue R250 (20 mg ml^{-1}) in a mixture of methanol:acetic acid: water (2:1:7). The background was destained in the same solvent until protein bands were clearly visible. Protein was also stained in the solution of Coomassie blue G250 in ethanol and phosphoric acid used for protein determinations (Bradford, 1976). This stain did not require destaining and enabled protein bands to be visualised in 10-30 minutes after staining.

Mobility of the protein was measured relative to mobility of bromophenol blue and expressed as R_F.

$$R_F = \frac{\text{distance migrated by protein (cm)}}{\text{distance migrated by bromophenol blue (cm)}}$$

The dimensions of the gel were not affected initially by the staining procedure, but gels increased in size on prolonged storage in destaining solution, so all measurements of R_F were made as soon as protein bands became clearly visible.

ii SDS Gel Electrophoresis

SDS gel electrophoresis was performed on 15% polyacrylamide gels (15% (w/v) acrylamide, 0.09% (w/v) bisacrylamide) containing 0.1% SDS. Two systems were used.

a) A continuous system in which gels were prepared according to the method of Weber and Osborne (1969) except that Tris-glycine buffer, pH 8.9, (6 g Tris, 28.8 g glycine per litre) was used as both the gel buffer and the electrode buffer.

b) A discontinuous gel system which was identical to that described for native gel electrophoresis except that both separation and spacer gels contained 0.1% SDS. This system generally gave sharper protein bands than the continuous gel system, and was used when protein samples greater than 25 µl were to be electrophoresed.

Cylindrical gels, 0.5 cm x 9 cm were polymerised by addition of ammonium persulphate after overlaying the gel with water. Protein samples were dialysed against the Tris-glycine electrode buffer containing 10 mM 2-mercapto ethanol then made to 1% SDS by addition of a 10% (w/v) SDS solution. One drop of glycerol and of an 0.1% (w/v) aqueous solution of bromophenol blue was added, and the protein was denatured by heating for two minutes on a boiling waterbath. Dilute protein solutions were concentrated at 4°C before heating with SDS by surrounding a dialysis tube containing the sample with Aquacide II until sufficient solvent had been extracted.

5-50 µl protein was applied to each gel and electrophoresed at a constant current of 1 mA/gel for 10-15 minutes, then at 3 mA/gel until the dye band approached the end of the gel. Dyefronts were marked with wire and the protein bands stained for 1-2 hrs in a solution of Coomassie brilliant blue R250 (20 mg ml^{-1}) in methanol: acetic acid:water (2:1:7). Gels were destained in the same solvent. Mobility of the protein was measured as previously described.

2.2.9 Molecular Weight Determination

i Gel Filtration

The molecular weight of native PFK was determined by gel filtration on a Sephacryl S200 column. A column of S200 (100 cm x 2.5 cm) was equilibrated at 4°C with 50 mM Tris-HCl buffer pH 7.5, containing 20% (v/v) glycerol, 10 mM 2-mercapto ethanol and 0.2 M KCl. PFK plus a mixture of the proteins listed in Table 2.2.1 were applied to the column in a volume of less than 4 ml. The column was eluted at a flow rate of 10 ml hr^{-1} and 1-2 ml fractions collected. Solvent volumes included in, and excluded from the

TABLE 2.2.1

PROTEINS USED FOR CALIBRATION OF SEPHACRYL S200 COLUMN FOR MOLECULAR WEIGHT DETERMINATION

Protein †	Source	Molecular Weight * (daltons)
Pyruvate Kinase	Rabbit muscle	237,000
Fumarase	Pig heart	194,000
Aldolase	Rabbit muscle	150,000
Glucose 6-phosphate dehydrogenase	Yeast	128,000
Hexokinase	Yeast	99,000
Albumin	Bovine serum	68,000

† All proteins were purchased from the Sigma Chemical Co. as ammonium sulphate suspensions or lyophilised powders.

* Molecular weights were taken from the 'Enzyme Handbook' (Barman, 1969).

gel were estimated by chromatographing a mixture of blue-dextran (MW $\sim$ 2 x 10^6 daltons) and glycine under identical conditions in a separate experiment. Fractions containing glycine were identified by reaction with ninhydrin reagent (1% (w/v) ninhydrin in acetone : 1.5% cadmium acetate, 17:3).

Standard proteins, with the exception of BSA and fumarase were identified by the spectrophotometric assays at 340 nm outlined in Appendix 1. Fumarase was identified by following the absorbance increase at 220 nm as malate was converted to fumarate. BSA was located by absorbance at 280 nm.

ii SDS Gel Electrophoresis : Protein Subunit Molecular Weight Determination

Protein subunit molecular weight was determined by SDS gel electrophoresis, and comparison of the R_F of the protein with R_F values of proteins of known molecular weight. The following proteins (subunit molecular weights are listed in parentheses) were used as standards:

Bovine serum albumin (68,000), ovalbumin (45,000), pepsin (35,000), trypsinogen (24,000), β-lactoglobulin (18,400) and lysozyme (14,300).

The molecular weights listed are those quoted by the manufacturers (Sigma Chemical Co.). PFK and the marker proteins were electrophoresed on a single gel to minimise errors introduced by variation between gels.

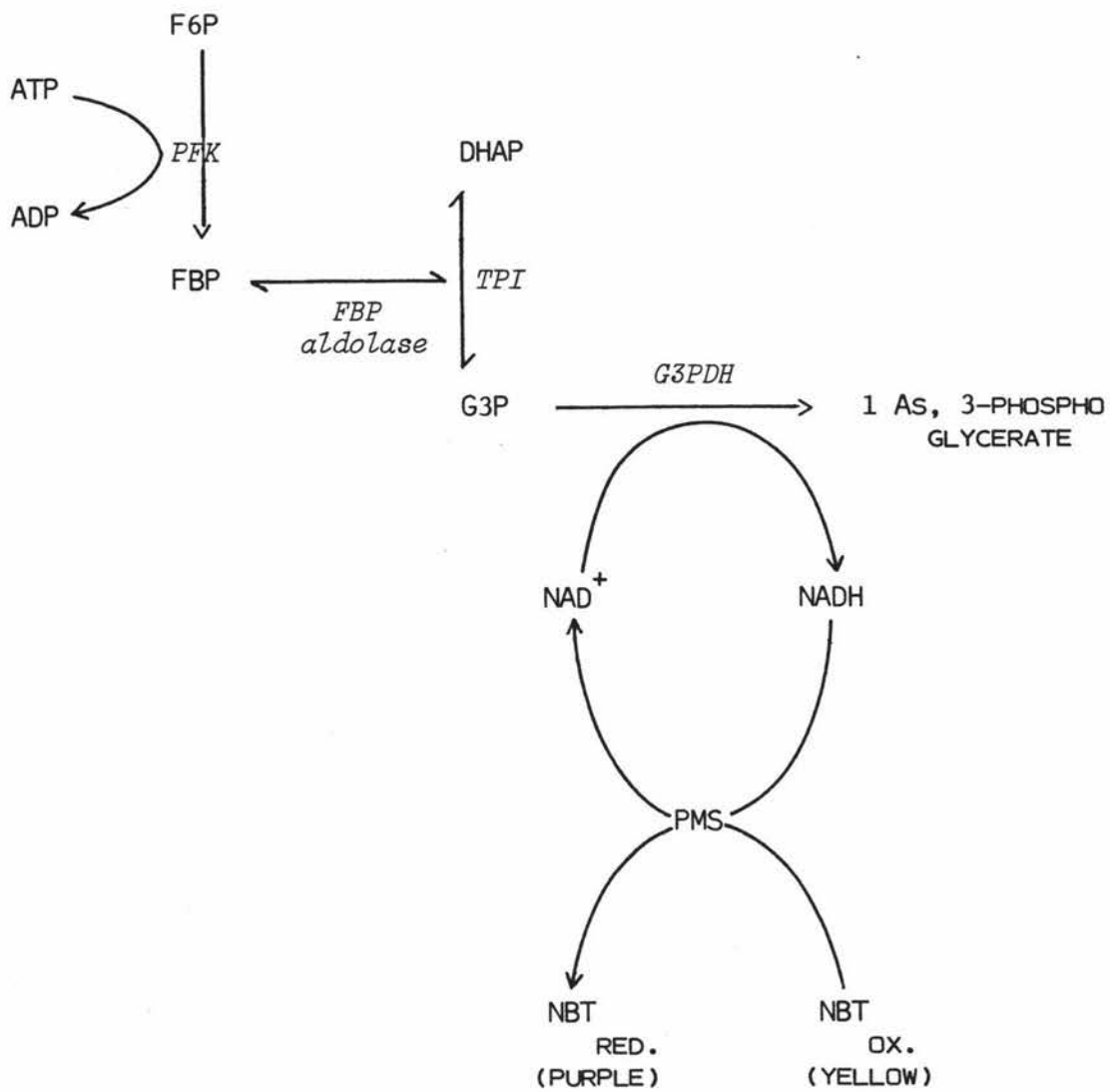
2.2.10 Location of PFK Activity on Polyacrylamide Gels

i Activity Stain

The reactions involved in coupling PFK activity to dye reduction are summarised in Figure 2.2.3. Polyacrylamide gels electrophoresed at 4°C under non denaturing conditions were stained for PFK activity in 50 mM Tris-HCl pH 7.5 containing 5.0 mM F6P, 2.0 mM ATP, 5.0 mM arsenate, 2.0 mM NAD^+,

FIGURE 2.2.3

STAIN FOR PFK ACTIVITY ON POLYACRYLAMIDE GELS



2.0 mg ml^{-1} nitroblue tetrazolium 0.025 mg ml^{-1} phenazine methosulphate, FBP-aldolase [10 µl (3.6 units) ml^{-1}], triose phosphate isomerase [10 µl (14 units) ml^{-1}], and glyceraldehyde 3-phosphate dehydrogenase [25 µl (30 units) ml^{-1}].

Gels were stained in the dark for 30-90 minutes then washed and stored in 7% acetic acid. PFK activity, indicated by NAD^+ and nitro blue tetrazolium reduction stained as purple brown bands when F6P was included in the staining mixture, but not in a control stain in which F6P was omitted.

ii Assay of Gel Slices

8 cm polyacrylamide gels were sliced into 2.0 mm sections, and each slice incubated in the aldolase assay mixture described in Section 2.2.7. Gel slices were macerated in a mixture containing 0.45 ml assay mixture, 50 µl 20 mM MgATP and 50 µl 20 mM F6P. After incubation with occasional stirring for 15 minutes at 30°C, the macerated gel was removed by suction filtration through glass wool and the absorbance of the assay mixture was measured. A decrease in absorbance indicated NADH oxidation and hence PFK activity.

iii Gel Scanning

Gels stained for protein with Coomassie blue were scanned at 580 nm on an Isco Model 1310 Gel Scanning apparatus (Instrumentation Specialities Co.)

2.2.11 PREPARATION OF PFK FROM *S. lactis* C_{10}

i Breakage of Cells

Frozen cells of *S. lactis* C_{10} were thawed and resuspended in 2 volumes (w/v) of 50 mM Tris-HCl pH 7.5 containing 20% (v/v) glycerol, 5 mM $MgCl_2$, 5 mM EDTA, and 10 mM 2-mercapto ethanol (referred to below as 'Tris-glycerol' buffer).

Cells were disrupted by two passages through a French Pressure Cell (American Instrument Co. Inc., Washington D.C., U.S.A.) at 5,500 psi, (38 MPa), and cellular debris and unbroken cells were removed by centrifugation at 27,000 g for 15 minutes. All centrifugation and subsequent purification steps were performed at 0-4°C.

ii Removal of Nucleic Acids : Protamine Sulphate Treatment

To the cell-free extract, 1 mg protamine sulphate was added per 15 mg protein by dropwise addition of a freshly prepared aqueous solution of protamine sulphate (20 mg ml^{-1}). The mixture was stirred gently for 15 minutes then centrifuged for 15 minutes at 27,000 g.

iii Ammonium Sulphate precipitation

Solid ammonium sulphate (24.2 g/100 ml) was slowly added to the supernatant from the previous step. The mixture was stirred for 15 minutes, then the insoluble protein removed by centrifugation for 15 minutes at 27,000 g, and a further 20.2 g ammonium sulphate added per 100 ml solution. After centrifuging, the precipitated protein was redissolved in a minimum volume of the Tris-glycerol buffer and dialysed for 12 hours against two changes of the same buffer.

iv Blue-dextran-Sepharose Affinity Chromatography

Protein solution (approximately 100 ml containing 300-400 mg protein) was applied to a 10 cm x 1 cm column of blue-dextran Sepharose 6B equilibrated with the 'Tris-glycerol' buffer described. The column was washed with buffer until no further protein was eluted, as indicated by the absorbance at 280 nm of the eluate. PFK was eluted at a flow rate of 24 ml hr^{-1} with 100 ml Tris-glycerol buffer containing a linear gradient of KCl from 0-2.0 M. Fractions containing PFK activity greater than 1.0 unit mg^{-1} were combined and the

KCl was removed by dialysis. These dialysed fractions were applied to a second 10 cm x 1 cm blue-dextran-Sepharose column and the column washed with buffer as before. PFK was then eluted with 100 ml Tris-glycerol buffer containing a linear gradient of 0-20 mM ATP (pH 7.5). Fractions containing PFK activity greater than 25 units mg^{-1} were combined and concentrated in dialysis tubing by extraction of the solvent with Aquacide II.

The purified PFK was dialysed against 2-3 changes of Tris-glycerol buffer. The final dialysis buffer contained no Mg^{2+} or EDTA and the glycerol content was increased to 50% Analar glycerol. The purified enzyme was stored at -20°C in 50 mM Tris-HCl pH 7.5, 50% glycerol and 10 mM 2-mercapto ethanol.

The results of a typical PFK preparation are summarised in Table 2.2.2. The purification procedure used was reproducible; enzyme from successive preparations was purified 300-400 fold with a yield of approximately 1.0 mg PFK per gram of frozen cells. The specific activity of the purified PFK was 120-180 enzyme units per mg protein, a value comparable to the specific activities of purified PFKs from mammalian skeletal muscle from several species, (Uyeda, 1979) *C. pasteurianum* (Uyeda & Kurooka, 1970) and *E. coli* (Griffin *et al*, 1967; Babul, 1978). The specific activity of PFK in the cell-free extract ranged from 0.2 - 0.8 units per mg and was independent of the number of passages through the French press. However up to seven passages were required before no further protein was released from the cells (Figure 2.2.4). As approximately 70% of the activity was released in the first two passages this was considered sufficient breakage of cells.

The blue-dextran-Sepharose 'affinity' column proved a rapid and efficient method of purifying PFK from *S. lactis*. Similar resins have been used in purification of PFK from yeast (Kopperschlager *et al*, 1971), *E. coli* (Ewings & Doelle, 1976; Thornburgh *et al*, 1978), Lactobacilli (Kawai & Eguchi, 1977; Simon & Hofer, 1977), and human erythrocytes (Cottreau *et al*, 1979). Attempts to bind PFK from other mammalian sources to blue-dextran have been unsuccessful (Ramadoss *et al*, 1976). *S. lactis* PFK was quantitatively recovered from the blue-dextran-Sepharose column; losses in activity resulted

TABLE 2.2.2

PURIFICATION OF *S. lactis* PHOSPHOFRUCTOKINASE

	Volume (ml)	Total Activity (units)	Protein (mg)	Specific Activity (Units mg^{-1})	Purification	Recovery (%)
Cell-free extract	86	900	2045	0.44	1.0	100
Protamine Sulphate Treatment	88	864	1878	0.46	1.1	96
Ammonium Sulphate precipitation	25	620	900	0.69	1.6	68
Blue-dextran-Sepharose: KCl gradient	30	514	4.8	107	243	57
Blue-dextran-Sepharose: ATP gradient	16	485	3.0	160	364	54

TABLE 2.2.2 shows the results of a typical preparation of PFK from ~ 40 g frozen cells. Purification steps were carried out as described in the text.

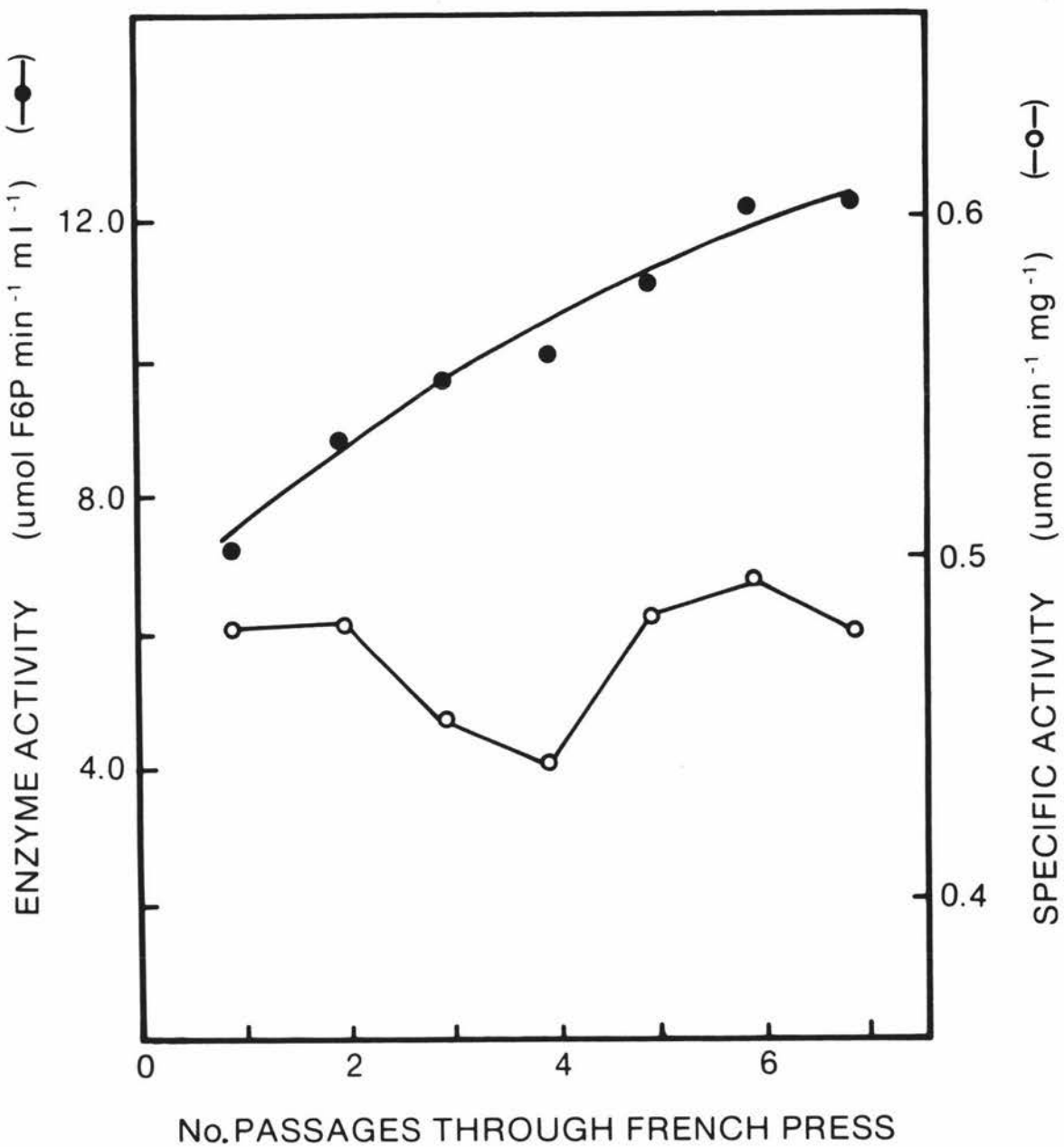


FIGURE 2.2.4 Breakage of cells of *S. lactis* C_{10} in the French Pressure Cell. PFK activity was assayed in cell-free extracts obtained after centrifugation of a cell suspension which was passed through a French Pressure Cell up to 7 times.

The cell-free extract was diluted 1:40 in Tris-glycerol buffer for assay.

Symbols: ● - enzyme activity (µmol F6P per minute per ml cell-free extract).

O - specific activity (µmol F6P per minute per mg protein).

Column dimensions : 10 cm x 1 cm.

Temperature : 0 - 4°C.

Equilibration buffer : 50 mM Tris-HCl pH 7.5 containing 20% (v/v) glycerol, 5 mM $MgCl_2$, 5 mM EDTA, and 10 mM 2-mercapto ethanol.

Elution buffer : 100 ml linear gradient, 0 - 2.0 M KCl in the above buffer.

Flow rate : 24 ml hr^{-1}.

Sample : Ammonium sulphate precipitate, dialysed.

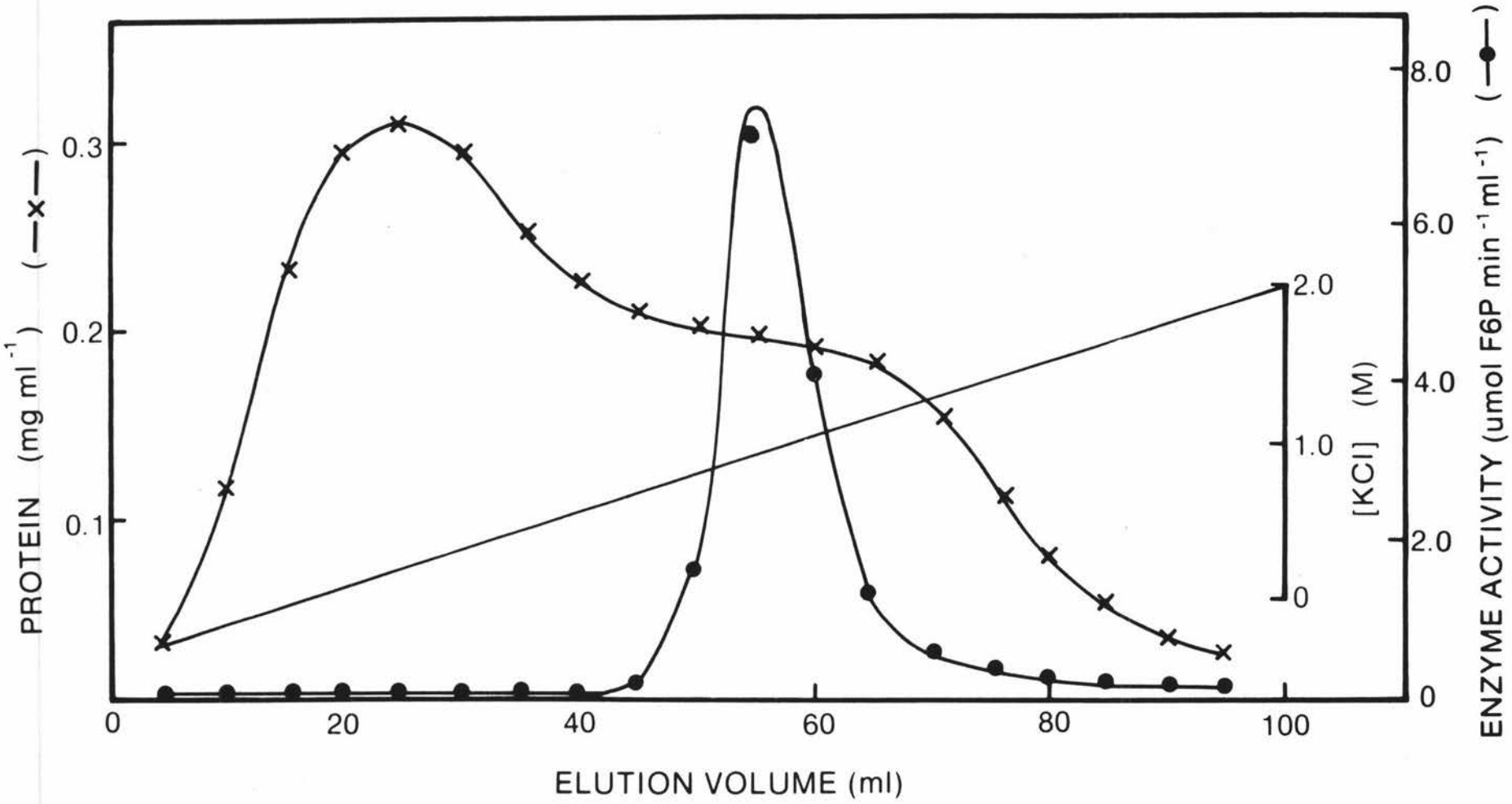


FIGURE 2.2.5 KCl Gradient Elution of PFK from Blue-dextran-Sepharose.

Column dimensions : 10 cm x 1 cm.

Temperature : 0 - 4°C.

Equilibration buffer : 50 mM Tris-HCl pH 7.5 containing 20% (v/v) glycerol, 5 mM $MgCl_2$, 5 mM EDTA, and 10 mM 2-mercapto ethanol.

Elution buffer : 100 ml linear gradient, 0 - 20 mM ATP in the above buffer.

Flow rate : 24 ml hr^{-1}.

Sample : Combined PFK fractions (dialysed) after KCl gradient elution from Blue-dextran-Sepharose.

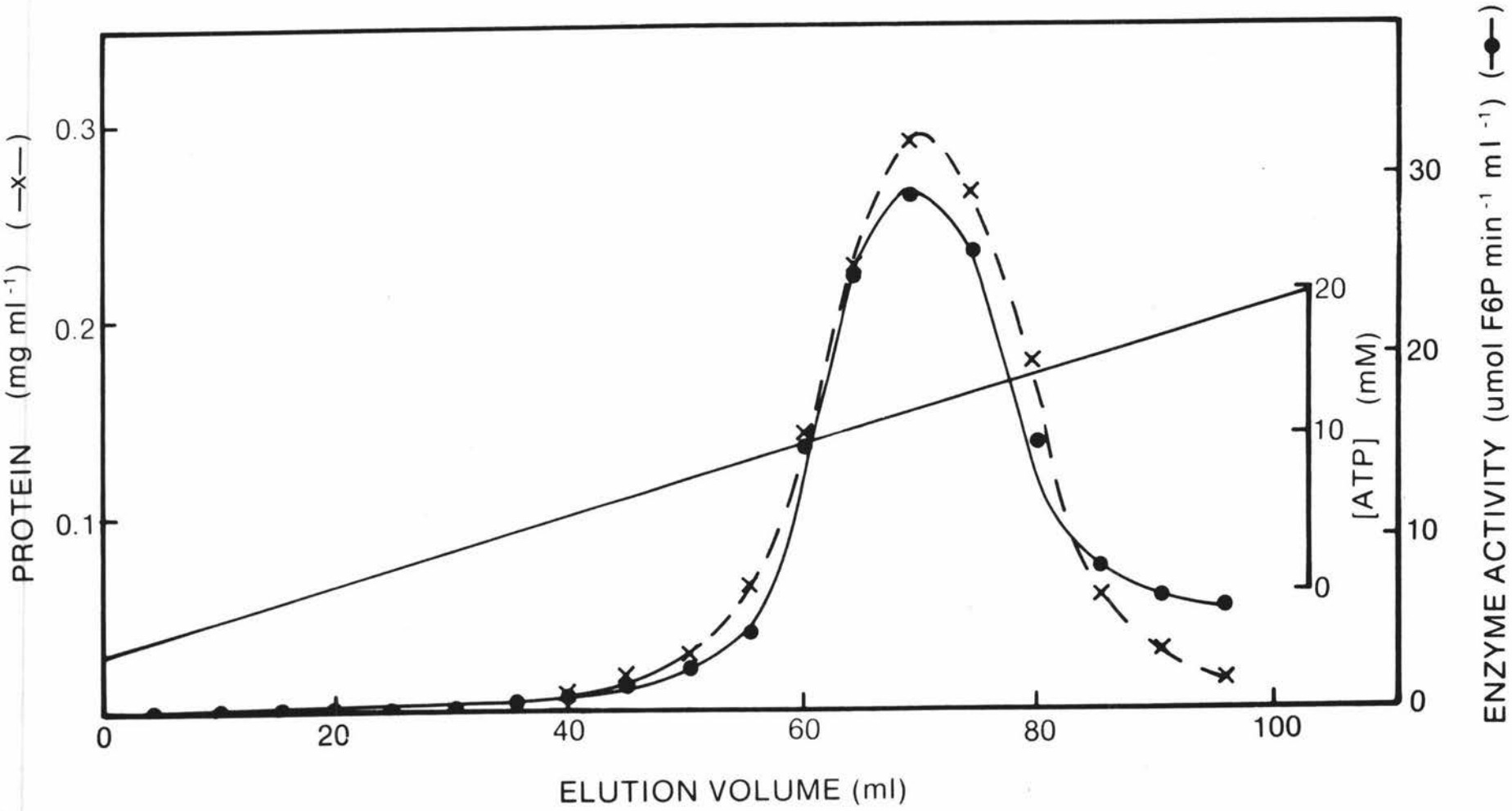


FIGURE 2.2.6 ATP Gradient Elution of PFK from Blue-dextran-Sepharose.

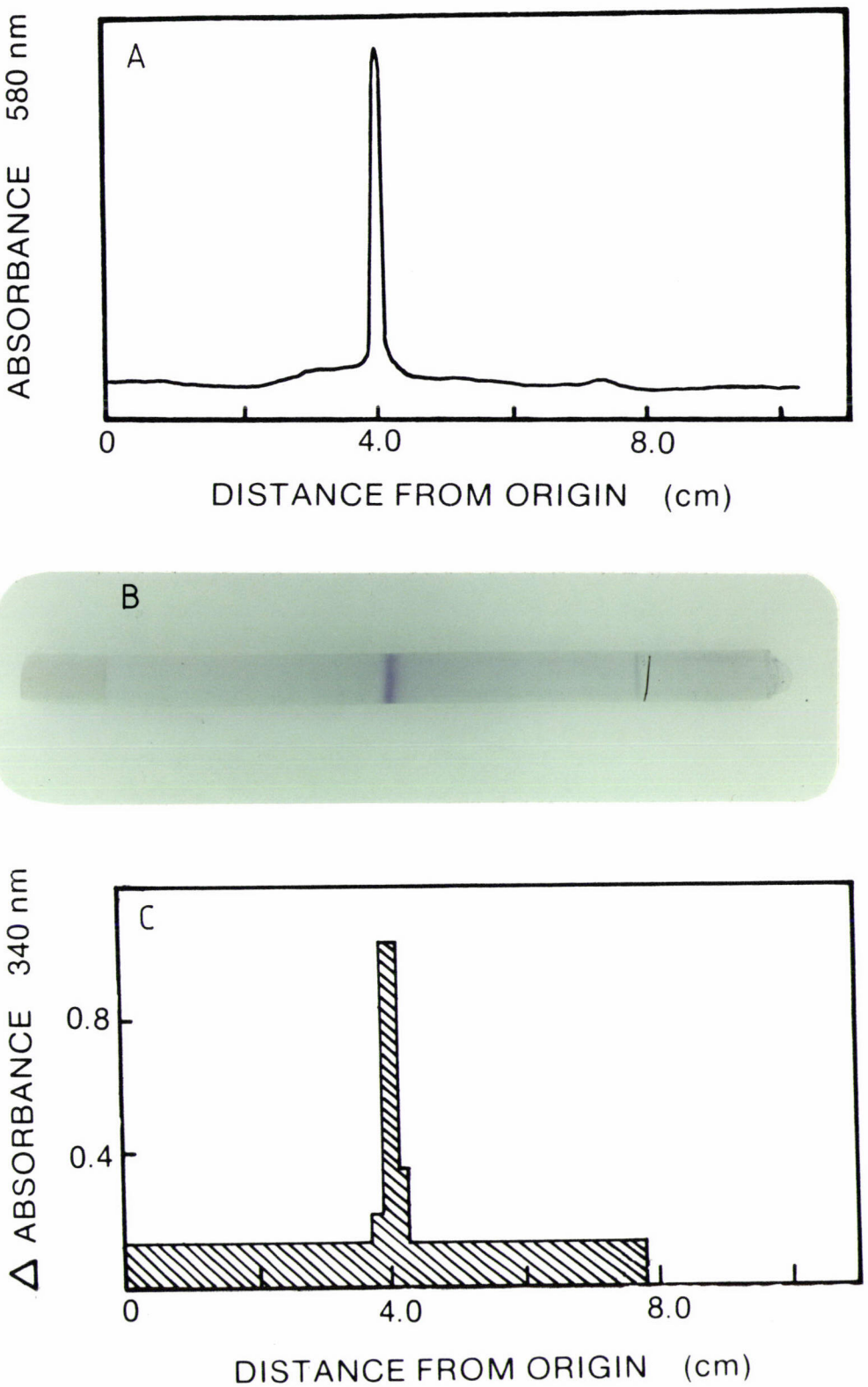


FIGURE 2.2.7 Location of PFK Activity on Polyacrylamide Gels. 25 µl purified PFK was electrophoresed on 7.5% polyacrylamide gels as described in Section 2.2.8. The protein band stained with Coomassie Blue is shown in 'B'.

Figure A shows the scan of an identical gel stained specifically for PFK activity (Section 2.2.10).

Figure C shows the result of incubating individual gel slices in the PFK assay mixture.

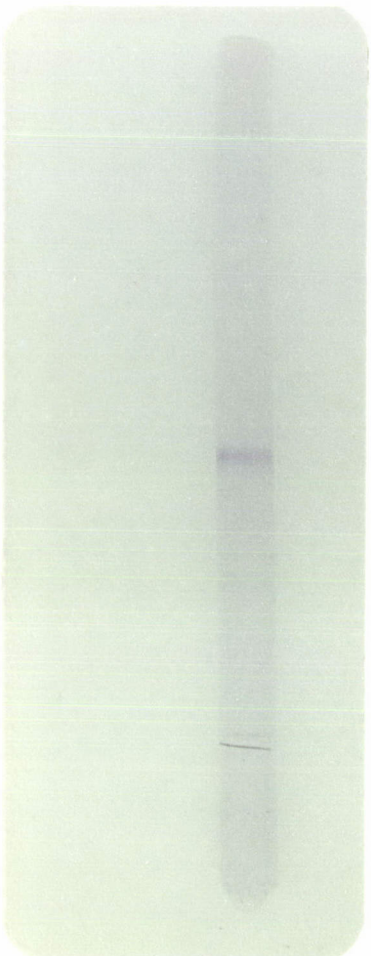


PLATE I:

S. lactis phosphofructokinase : gel electrophoresis of the undenatured protein.

2.2.12 CRYSTALLISATION OF PFK

Buffers for crystallisation of PFK were made up as follows: 25 mM Na_2HPO_4/NaH_2PO_4 pH 7.0, 5 mM $MgCl_2$, 5 mM EDTA, 10 mM 2-mercapto ethanol, and 11.0, 14.0, 17.0, or 20.5 g ammonium sulphate per 100 ml buffer. A second series of buffers was made up as above, with the addition of 2.0 mM F6P and 2.0 mM ATP. Purified PFK was dialysed for 12 hours against two changes of 25 mM sodium phosphate buffer containing 10 mM 2-mercapto ethanol, then centrifuged for 30 minutes at 27,000 g, and 4°C. 50 µl enzyme was then dialysed at 4°C against each of the eight buffers and checked periodically for formation of crystals. Dialysis tubes containing sodium phosphate buffer instead of enzyme were used as controls.

Crystals of *S. lactis* PFK are shown in Plate II.

2.2.13 OPTIMISATION OF ASSAY CONDITIONS

Coupled assay systems may present problems in assaying allosteric enzymes since components essential for the coupling enzymes in the assay mixture may activate or inhibit the enzyme being assayed. In order to optimise the assay conditions for PFK, a brief survey of the effects of various components of the assay mixture on PFK activity was performed.

i Effect of Ionic Strength

Varying the concentration of Tris-HCl buffer pH 7.5 between 20 mM and 200 mM had little effect on PFK activity (Figure 2.2.8). Activity was lowered by 36% and 22% at 0 mM (where buffer was replaced by distilled water) and 10 mM buffer respectively. All subsequent assays were performed in 50 mM Tris-HCl buffer at pH 7.5. No attempt was made to correct for the slight changes in ionic strength of the assay mixture when different concentrations of effectors were added or when concentrations of substrates were altered.

ii Effect of NADH concentration

NADH, an essential component of both the PK-LDH and

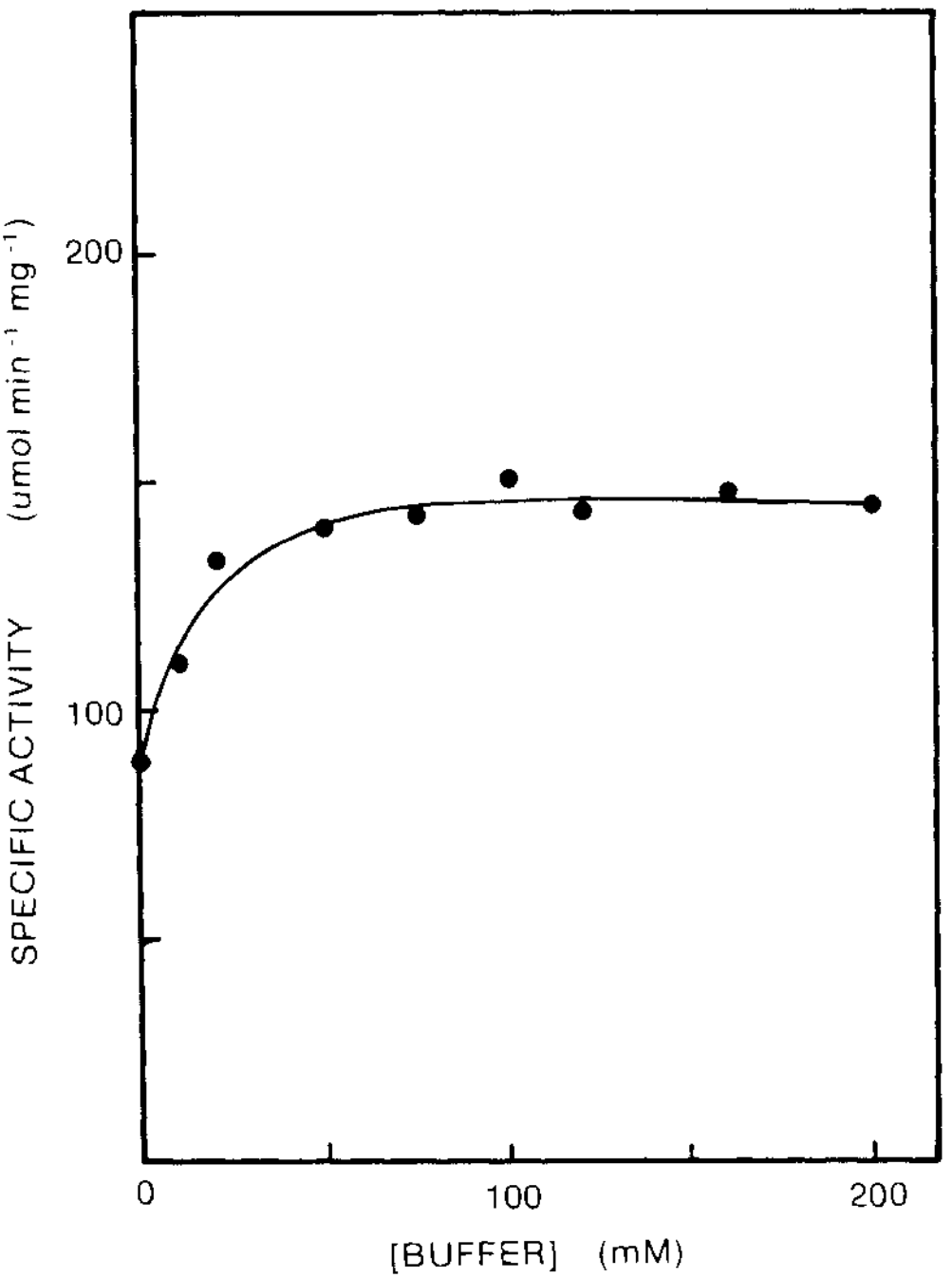


FIGURE 2.2.8 The Effect of Ionic Strength on PFK Activity. Purified PFK (0.21 µg per assay) was assyed at increasing concentrations of Tris-HCl, pH 7.5, using the aldolase assay system. All assays were at saturating concentrations (2.0 mM) of both MgATP and F6P.

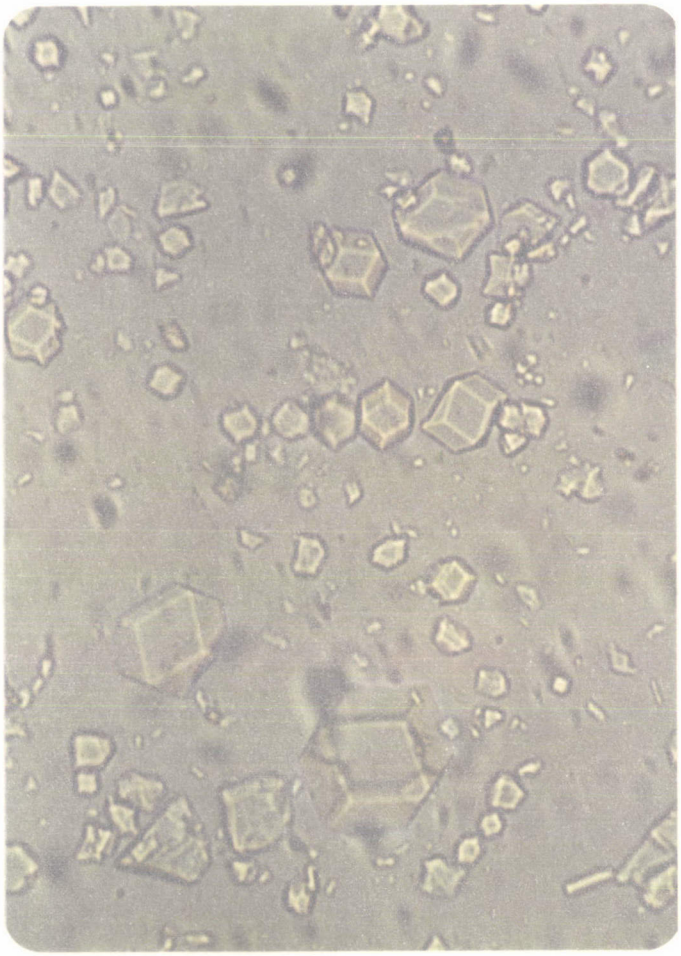


PLATE II:

Crystalline *S. lactis* phosphofructokinase.

aldolase assay systems has been reported to inhibit PFK activity (Brock, 1969). A study of the effect of NADH concentration on PFK activity was therefore conducted.

The concentration of NADH that can be used in the assay is limited by the absorbance at 340 nm of the solution. Figure 2.2.9A shows that the optimum NADH concentrations range from 0.12 - 0.15 mM, above which NADH becomes slightly inhibitory. An NADH concentration of 0.33 mM was used in all subsequent assays; the inhibition produced by this concentration is negligible (Figure 2.2.9A).

The PFK assays also exhibited a lag period which was dependent on both the NADH concentration (Figure 2.2.9B) and the enzyme concentration in the assay. The lag period was not reduced by pre-incubation of the enzyme in the assay mixture in the absence of F6P prior to initiating the reaction. A similar lag was noted in rabbit muscle PFK using an indicator-coupled assay (Hood & Holloway, 1976), thus the lag does not appear to be due to the coupling enzymes in the assay. Hood & Holloway propose that the lag represents the time taken for accumulation of FBP, an activator of rabbit muscle PFK, in the initial stages of the reaction. However as FBP is an inhibitor, not an activator of *S. lactis* PFK, and the aldolase assay system results in a rapid removal of FBP, this proposal is not sufficient to explain the observed lag in the *S. lactis* PFK activity.

The PK-LDH assay system is not ideal because of the initially high concentrations of PEP, an inhibitor of PFK. One could expect an increase in PFK activity due to a decrease in PEP concentration as the assay proceeds. Similarly, in the aldolase assay system, accumulation of ADP (an activator of PFK) as the assay proceeds may also cause an apparent increase in activity. However as both assay systems gave comparable activities when assayed at saturating levels of MgATP and F6P, and the substrate saturation profiles were identical regardless of which assay system was used, the change in concentrations of PEP and ADP in the assay mixtures appears to have little effect on PFK activity.

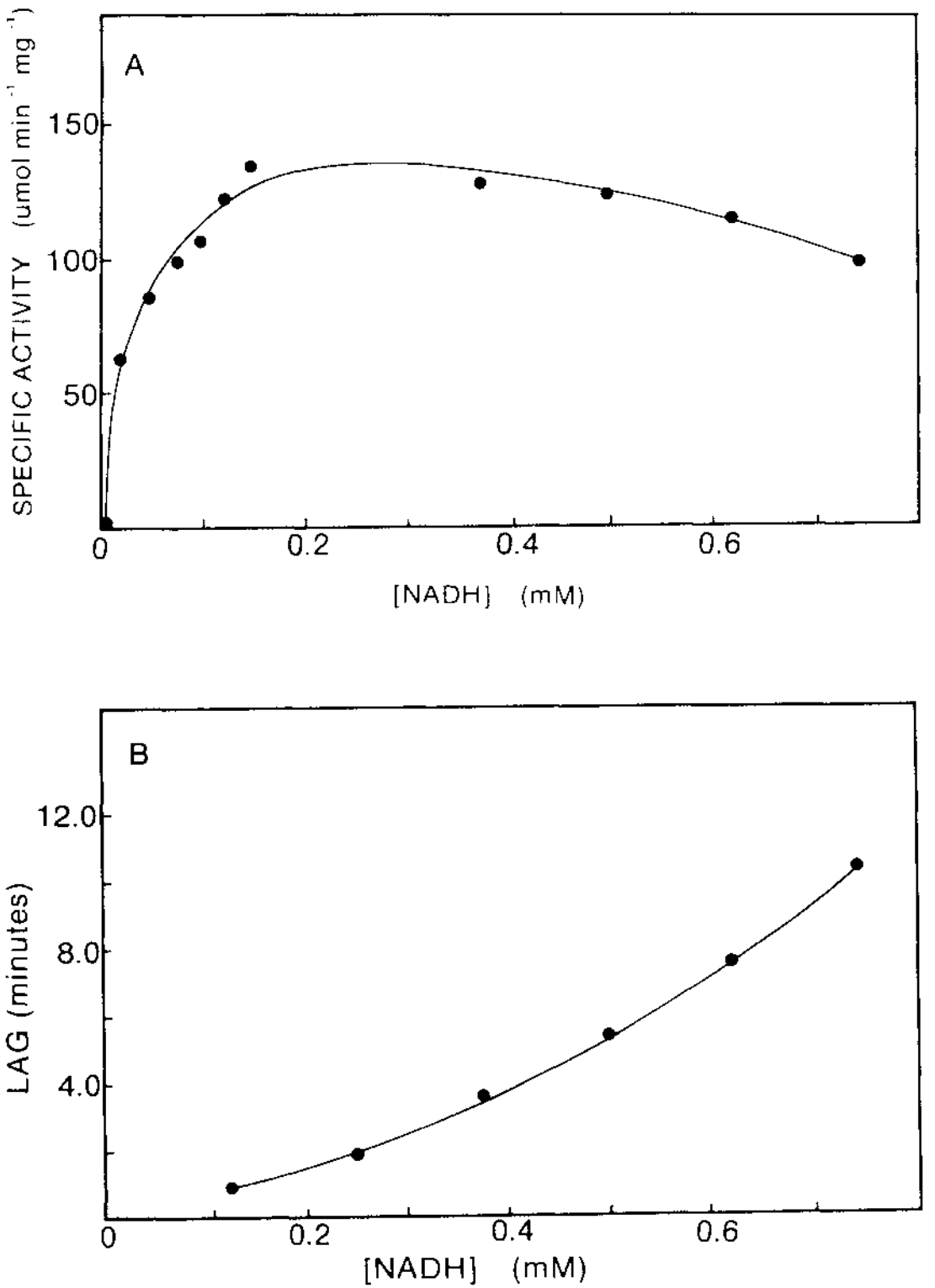


FIGURE 2.2.9 The Effect of NADH on PFK Activity. Using the aldolase assay system, and 0.21 µg purified PFK per assay, 0.15 - 0.3 mM NADH was required for optimal PFK activity at saturating (2.0 mM) substrate concentrations (Figure A). Increasing concentrations of NADH caused increased lag periods in the assay (Figure B).

2.3 PROPERTIES OF THE PURIFIED PHOSPHOFRUCTOKINASE : RESULTS AND DISCUSSION

2.3.1 DEPENDENCE OF ACTIVITY ON PROTEIN CONCENTRATION

Figures 2.3.1A and B show the relationship between observed enzyme activity and amount of protein in the assay. The relationship was linear over the range of protein concentrations studied in both the cell-free extract (Figure 2.3.1B) assayed using the aldolase-linked assay system, and the purified enzyme (Figure 2.3.1A) assayed using both the aldolase and the PK-LDH assay systems. This linear relationship suggests there is no association-dependent activation of *S. lactis* PFK at protein concentrations below 0.8 μg ml^{-1}. Human erythrocyte PFK which undergoes activation on conversion of dimers to multimers exhibits a sigmoidal dependence on protein concentration (Wenzel *et al* 1976).

2.3.2 STABILITY OF THE PURIFIED PFK

The effect on the stability of PFK of a number of compounds was investigated in order to determine which buffer was most suitable for enzyme preparation and under what conditions the purified enzyme was most stable. Results of this survey are shown in Figures 2.3.2 and 2.3.3.

The concentration of PFK in each buffer was approximately 0.015 mg ml^{-1}. PFK lost least activity when stored at 4°C in phosphate or Tris buffers, pH 7.5, containing 20% (v/v) glycerol. Addition of 10 mM dithiothreitol or 2.0 mM F6P to the Tris buffer enhanced stability. Long-term stability appeared to be dependent on the presence of glycerol in the buffer; removal of glycerol resulted in complete loss of activity in less than 21 days. 20% (v/v) glycerol enhanced stability to a greater extent than 5% (v/v) and 10% (v/v) glycerol (results not shown). Stability was further enhanced by raising the glycerol content to 50% (v/v).

PFK stored in Tris-HCl buffer pH 7.5 containing 20% (v/v) glycerol was no more stable at -20°C than at 4°C and addition of 2.0 mM ATP, 2.0 M KCl, 2.0 mM citrate and 40% (w/v) ammonium sulphate did not increase stability. In Tris-HCl buffer the enzyme was more stable at pH 7.5 than at pH 6.0 or pH 8.0.

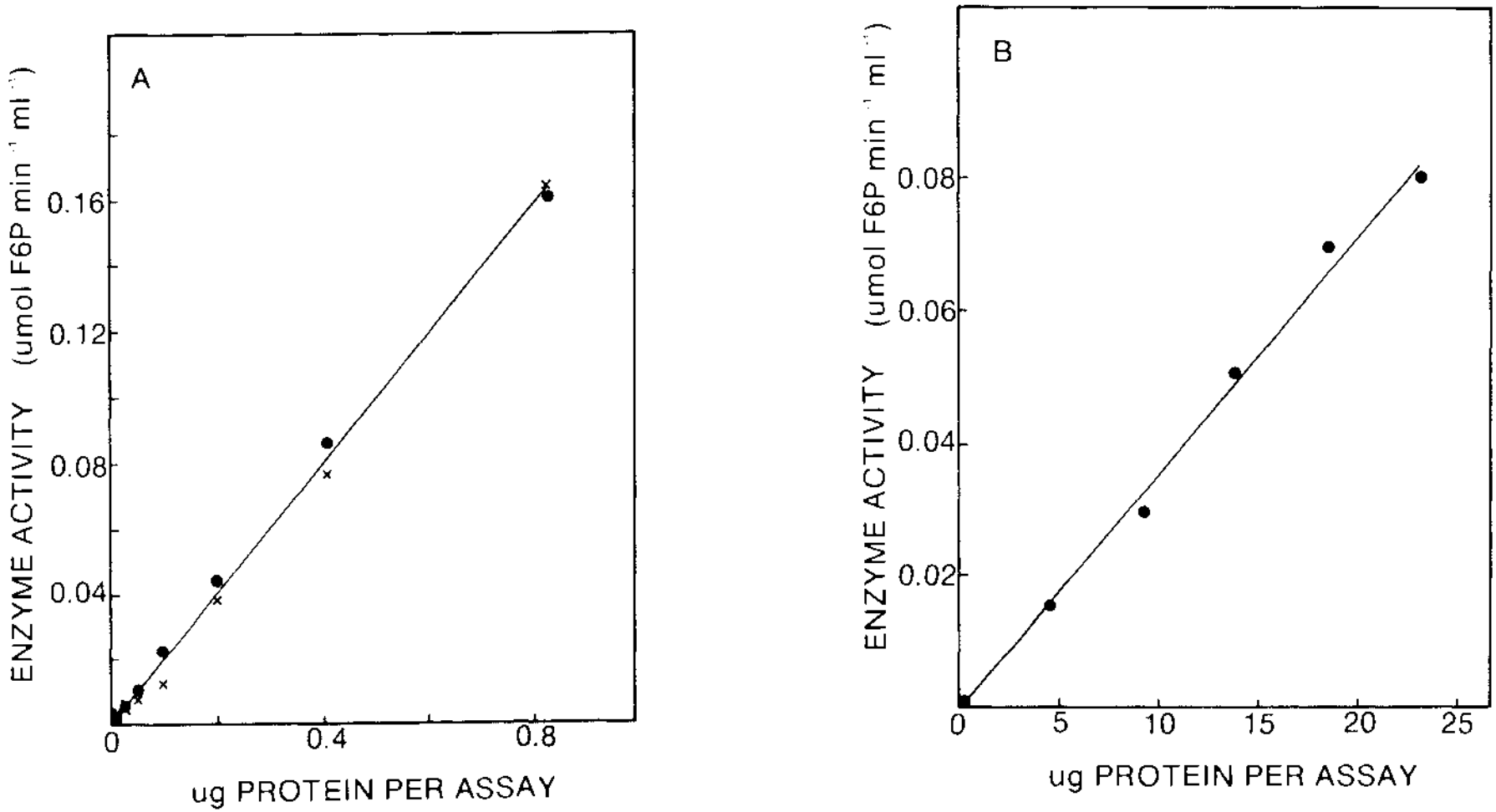


FIGURE 2.3.1 The Dependence of PFK Activity on Protein Concentration. PFK was assayed at varying protein concentrations using both the pyruvate kinase - lactate dehydrogenase assay (×) and the aldolase assay (●) as described in Section 2.2.6. Assays were performed at saturating concentrations of substrates (2.0 mM MgATP, 2.0 mM F6P) using both purified PFK (Figure A) and a freshly prepared cell-free extract (Figure B).

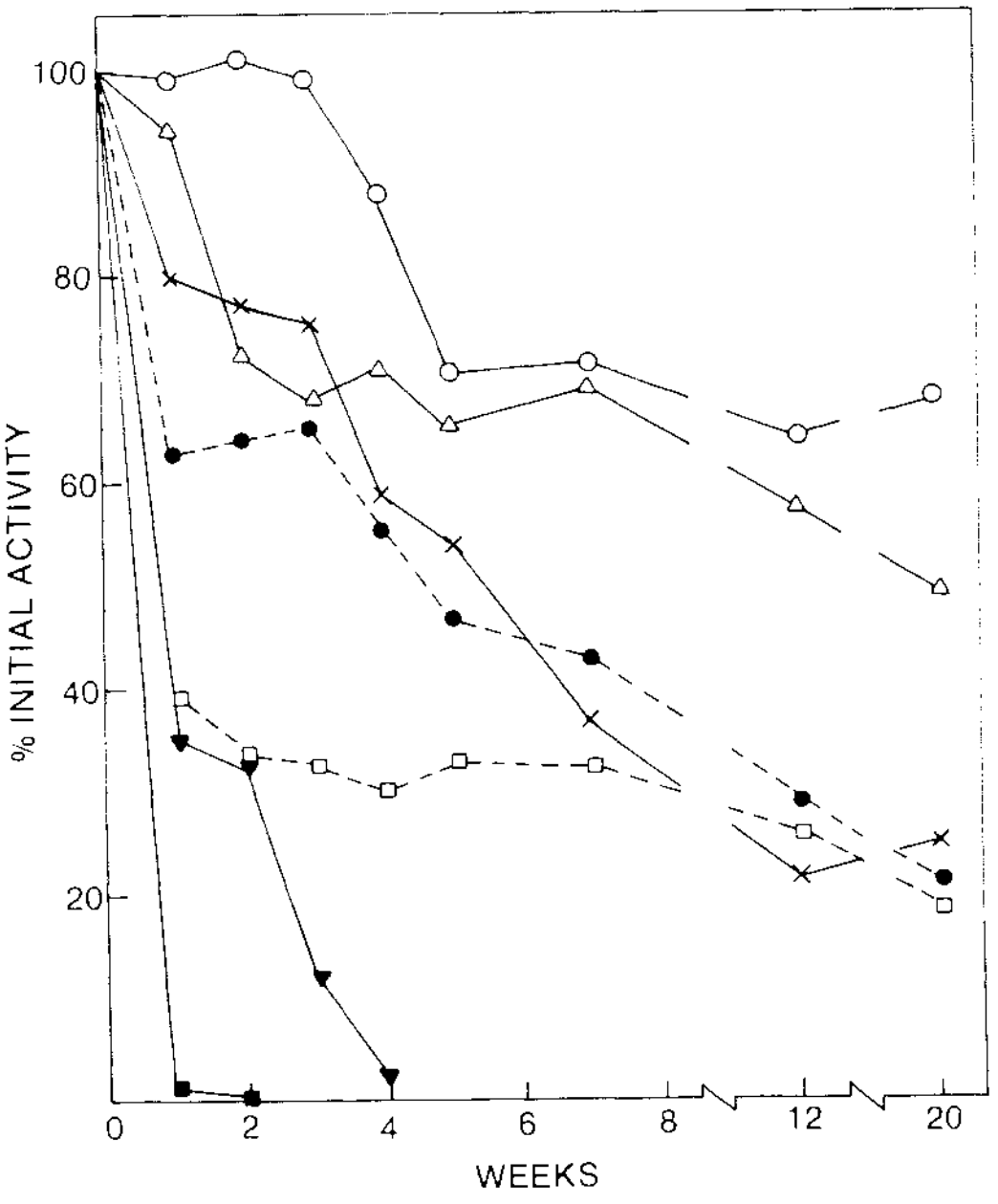


FIGURE 2.3.2 The Effect of Buffer Composition on the Stability of Purified PFK. This figure shows the decline in activity, over 20 weeks of purified PFK (15 μg ml^{-1}) stored at 4°C in the following buffers. Each buffer was at a concentration of 50 mM, and contained 20% (v/v) glycerol. ▼-Tris-HCl, pH 6.0; ● -Tris-HCl, pH 7.5; □-Tris-HCl, pH 8.0; △-phosphate buffer, pH 7.0. PFK activity was also measured in 50 mM Tris-HCl, pH 7.5, containing no glycerol (■); and after storage at -20°C in 50 mM Tris-HCl, pH 7.5 containing 20% (v/v) (×) and 50% (v/v) (O) glycerol.

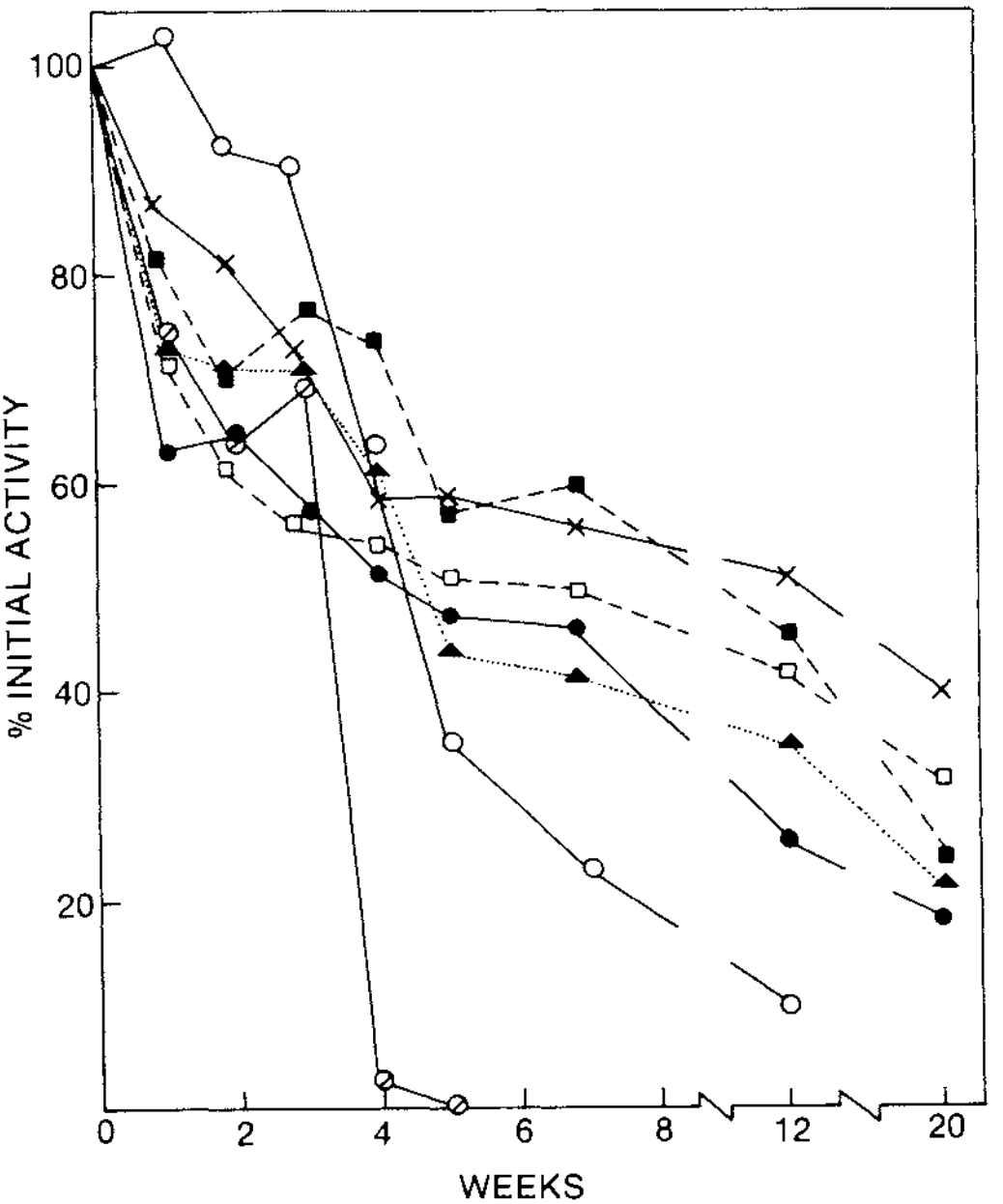


FIGURE 2.3.3 Compounds affecting the stability of PFK.
Purified PFK (15 µg ml) was stored at 0°C in 50 mM Tris-HCl buffer, pH 7.5, containing 20% (v/v) glycerol and the following compounds at the concentrations specified: ● - Tris-HCl, pH 7.5, 20% (v/v) glycerol only; □ - 2 mM citrate; ▲ - 10 mM dithiothreitol; ○ - 2 M KCl; ■ - 2 mM ATP; × - 2 mM F6P; ⊘ - 40% ammonium sulphate.

Stability was also dependent on protein concentration. Although this effect was not investigated quantitatively it was repeatedly noted that enzyme diluted 5-20 fold lost activity more rapidly than the undiluted enzyme.

Although PFK appeared to be more stable in phosphate buffer than in Tris buffer it was decided to use Tris buffer in enzyme preparation as phosphate is known to be an allosteric effector of several PFKs (see review by Ramaiah, 1975). For the same reason addition of F6P to the buffer was avoided.

PFK prepared for kinetic studies was stored at a concentration of 0.15 - 0.35 mg ml^{-1} in Tris-HCl buffer pH 7.5 containing 10 mM 2-mercapto ethanol and 50% (v/v) 'Analar' glycerol. The enzyme was stored at -20°C to minimise microbial growth and under these conditions 80-90% activity remained after 6 months storage. The concentration of purified enzyme was 10-20 fold higher than that used in determining the effects of compounds on the stability of PFK. This possibly explains why the stability of the stored enzyme was greater than that of the enzyme used in the stability experiments reported in this section.

2.3.3 Effect of pH on PFK Activity

Figure 2.3.4A shows the pH profiles for PFK in several buffers, each at a concentration of 50 mM. The optimum pH for activity lies between 7.4 and 7.8 in Tris bis propane, imidazole, Tris-HCl and Tricine-KOH buffers. The maximal activities are similar in the two Tris buffers, but are increased by 25% and 54% respectively in Tricine buffer and imidazole buffer. In phosphate buffer both the maximum velocity (110 µmol F6P min^{-1} mg^{-1}) and the pH optimum (pH 6.9) are lowered.

To eliminate any effects of buffer composition on enzyme activity the pH optimum was also determined in a mixture of buffers, 40 mM each of MES, Tricine, and HEPES (Figure 2.3.4B). A pH optimum of 7.5 was obtained, but activity was fairly constant over the pH range 6.8 - 8.5. PFK activity appeared more sensitive to acid than to alkaline pH, activity decreasing more rapidly when pH was lowered below pH 6.8 than when pH was raised above pH 8.5. PFK was active at pH values as low as pH 4.8, below which the enzyme precipitated.

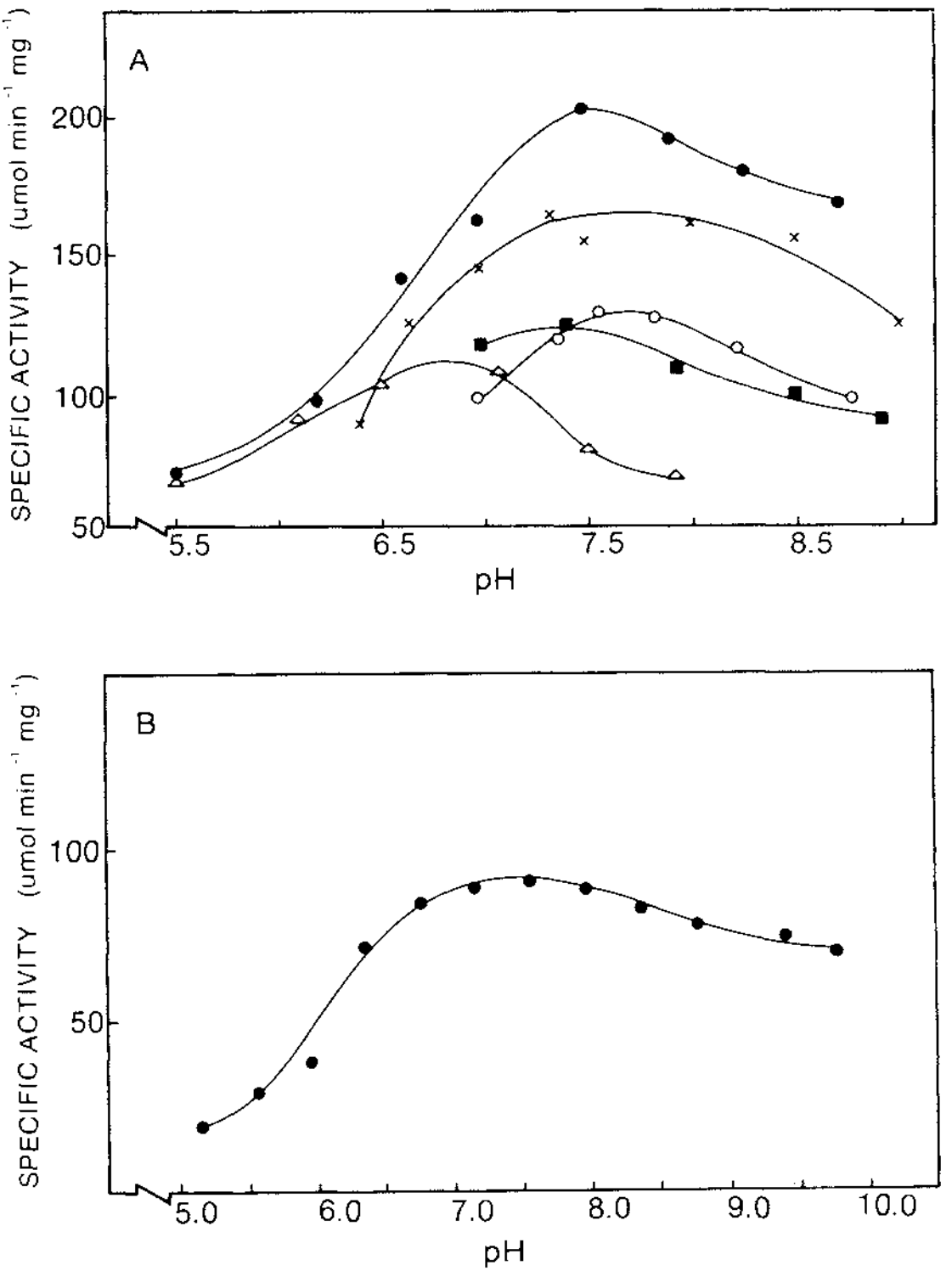


FIGURE 2.3.4 The Effect of pH on PFK Activity. Purified PFK (0.21 µg per assay) was assayed using the aldolase assay system described in Section 2.2.6, except that the Tris-HCl buffer in the assay mixture was replaced with distilled water, and each assay contained 50 µl of the appropriate buffer (0.5 M) in addition to the assay components listed. Assays were performed at the pH indicated.

Figure A shows the pH profiles for PFK in the following buffers:
○ - Tris-HCl; △ - phosphate; ● - Imidazole;
× - Tricine-KOH; ■ - Tris bis propane.

In Figure B, the buffer used was a mixture of MES, Tricine and HEPES, (0.4 M of each buffer).

Activity of PFK was also measured at two extreme pH values (pH 5.5 and pH 9.4) after pre-incubation of the enzyme at this pH for up to 10 minutes. No change in activity was observed after 0, 1, 2, 5 and 10 minutes pre-incubation. As all assays were completed in less than 10 minutes the pH effect was not due to inactivation of the enzyme during the course of the assay.

2.3.4 Molecular Weight Determination

The molecular weight of non-denatured PFK was determined by gel filtration on Sephacryl S200, by comparing the elution volume of PFK with that of proteins of known molecular weight. Figure 2.3.5 shows the linear relationship between the log of the molecular weight of a protein, and its elution volume. On applying purified PFK to the Sephacryl column, activity was eluted in an elution volume of 218 ml which, from Figure 2.3.5, suggests a molecular weight of 145,000 daltons for the native enzyme.

To ascertain that the molecular weight of PFK was not altered by the purification procedure the gel filtration experiment was repeated using cell-free extract instead of purified enzyme. A molecular weight of 152,000 daltons was obtained, in good agreement with the value obtained for the purified enzyme.

The subunit molecular weight of PFK was determined by SDS polyacrylamide gel electrophoresis after denaturation of the enzyme by heating in the presence of 1% (w/v) SDS and 10 mM 2-mercapto ethanol. A single protein band was obtained (Plate III) with a relative mobility of 0.59. By comparing this value with the relative mobilities of proteins of known molecular weight electrophoresed under identical conditions (Figure 2.3.6), a subunit molecular weight of 33,500 $\pm$ 3,500 daltons was obtained. *S. lactis* PFK therefore appears to be a tetramer of identical subunits.

2.3.5 Effect of ATP Concentration on PFK Activity

Most kinases require a divalent metal cation (generally Mg^{2+}) for activity as the active form of ATP is a MgATP complex. However both free ATP and free Mg^{2+} may also affect enzyme activity. The actual concentrations of Mg^{2+}, ATP and MgATP in solution depend on the initial concentrations of Mg^{2+} and ATP (Cornish-Bowden, 1976).

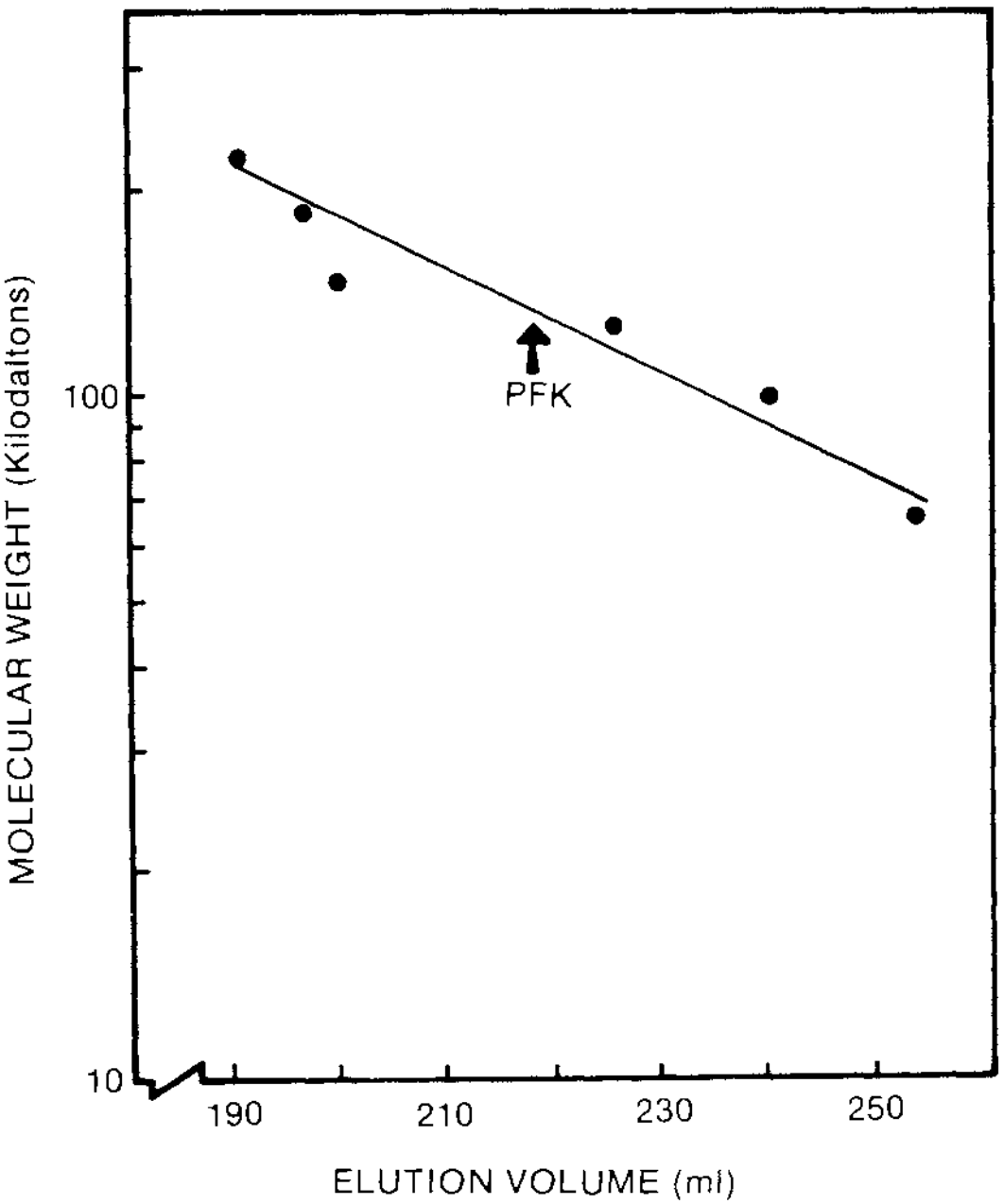


FIGURE 2.3.5 Molecular Weight Determination of PFK by Gel Filtration on Sephacryl S200. The molecular weight of PFK was determined by Gel filtration on Sephacryl S200 as described in Section 2.2.9. Standard molecular weight proteins, in decreasing order of size, are as follows: pyruvate kinase; fumarase; aldolase; glucose 6-phosphate dehydrogenase; hexokinase; bovine serum albumin. The arrow marks the position of PFK on the standard curve, indicating a molecular weight of 145,000 daltons.

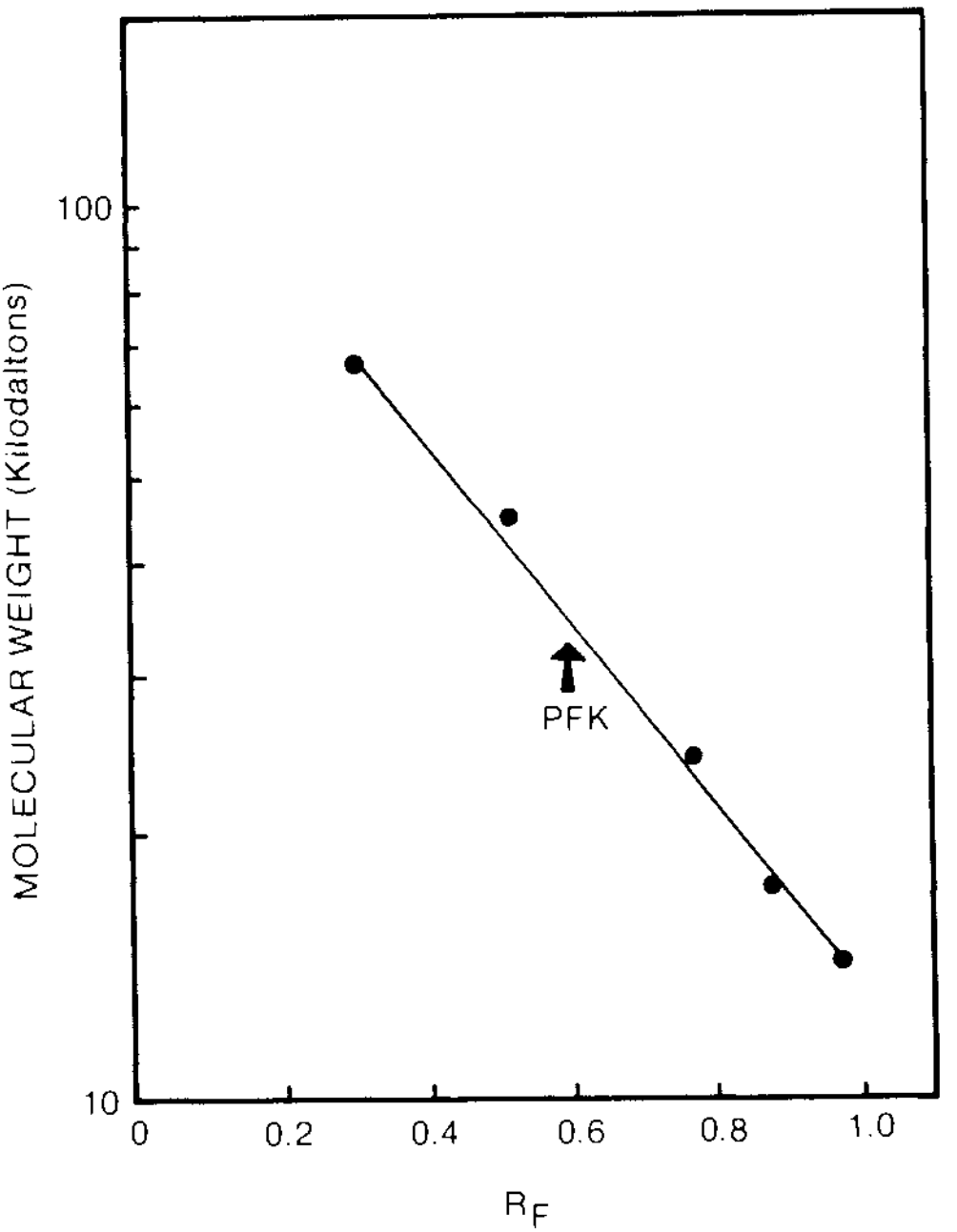


FIGURE 2.3.6 SDS Gel Electrophoresis of PFK. PFK and proteins of known molecular weight were denatured and subjected to electrophoresis as described in Section 2.2.9. Standard proteins are: lysozyme (MW 14,300), ß-lactoglobulin (MW 18,400), trypsinogen (MW 24,000), ovalbumin (MW 45,000), and bovine serum albumin (MW 66,000). The relative position of PFK on the standard curve is denoted by the arrow.

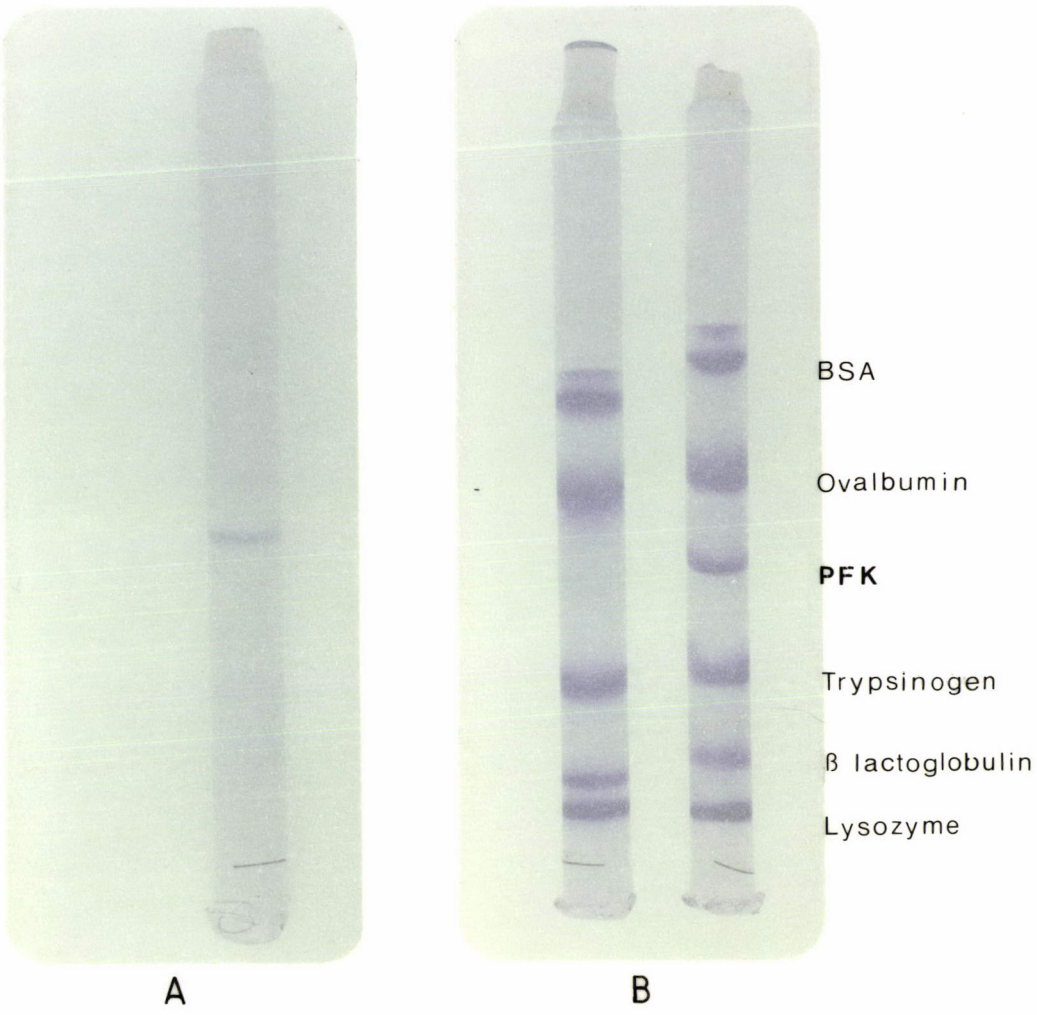


PLATE III:

S. lactis phosphofructokinase : SDS gel electrophoresis of purified PFK (A) and of PFK and standard molecular weight proteins (B).

Because of this different results can be obtained in a study of the effects of ATP on activity, depending on whether the Mg^{2+} concentration is held constant, at a fixed excess above ATP concentration, or at a fixed ratio to ATP. When interpreting the effects of either component it is important to know the actual concentrations of the three species Mg^{2+}, ATP, and MgATP in solution. The method used for calculating the concentrations of these species, and the manner in which MgATP concentration varies depending on the concentrations of Mg^{2+} and ATP are shown in Appendix 2. Although an over simplification, (the method used did not acknowledge the presence of other possible MgATP intermediates such as ATP^{4-}, $HATP^{3-}$, $MgHATP^{-}$, Mg_2ATP) the calculated concentrations of MgATP agree well with those circulated by Storer and Cornish-Bowden (1976) after considering all possible intermediates.

In this section the effects of varying ATP concentrations were studied with the Mg^{2+} concentration held constant. The effects of varying Mg^{2+} concentrations at a constant concentration of ATP, and of varying MgATP concentrations are described in following sections.

No PFK activity was detected in the absence of ATP. At a constant Mg^{2+} concentration of 1.0 mM, maximum activity was obtained at 0.8 - 1.0 mM ATP at a saturating F6P concentration (2.0 mM), and at 0.2 - 0.6 mM ATP at a non-saturating F6P concentration (0.2 mM) (Figure 2.3.7). Maximum activity at 2.0 mM F6P was only 20% of the activity at optimum Mg^{2+} and ATP concentrations (see Section 2.3.6). This reduced activity is probably due to Mg^{2+} limiting activity, as subsequent experiments (Figures 2.3.8 and 2.3.13) showed that a Mg:ATP ratio of $\sim$ 5:1 was required for maximum activity. Increasing concentrations of ATP inhibited activity. This inhibition could be overcome by increasing the F6P concentration. 10 mM ATP resulted in 100% inhibition at 0.2 mM F6P, but only 31% inhibition at 2.0 mM F6P.

2.3.6 Effect of Mg^{2+} Concentration on PFK Activity

PFK activity in the absence of Mg^{2+} was less than 5% of the activity at optimal Mg^{2+} concentrations. An absolute requirement for Mg^{2+} could not be demonstrated in spite of pre-treatment of the enzyme with EDTA. The activity in the absence of Mg^{2+} is possibly due to residual cations in the components of the assay mixture or to tightly

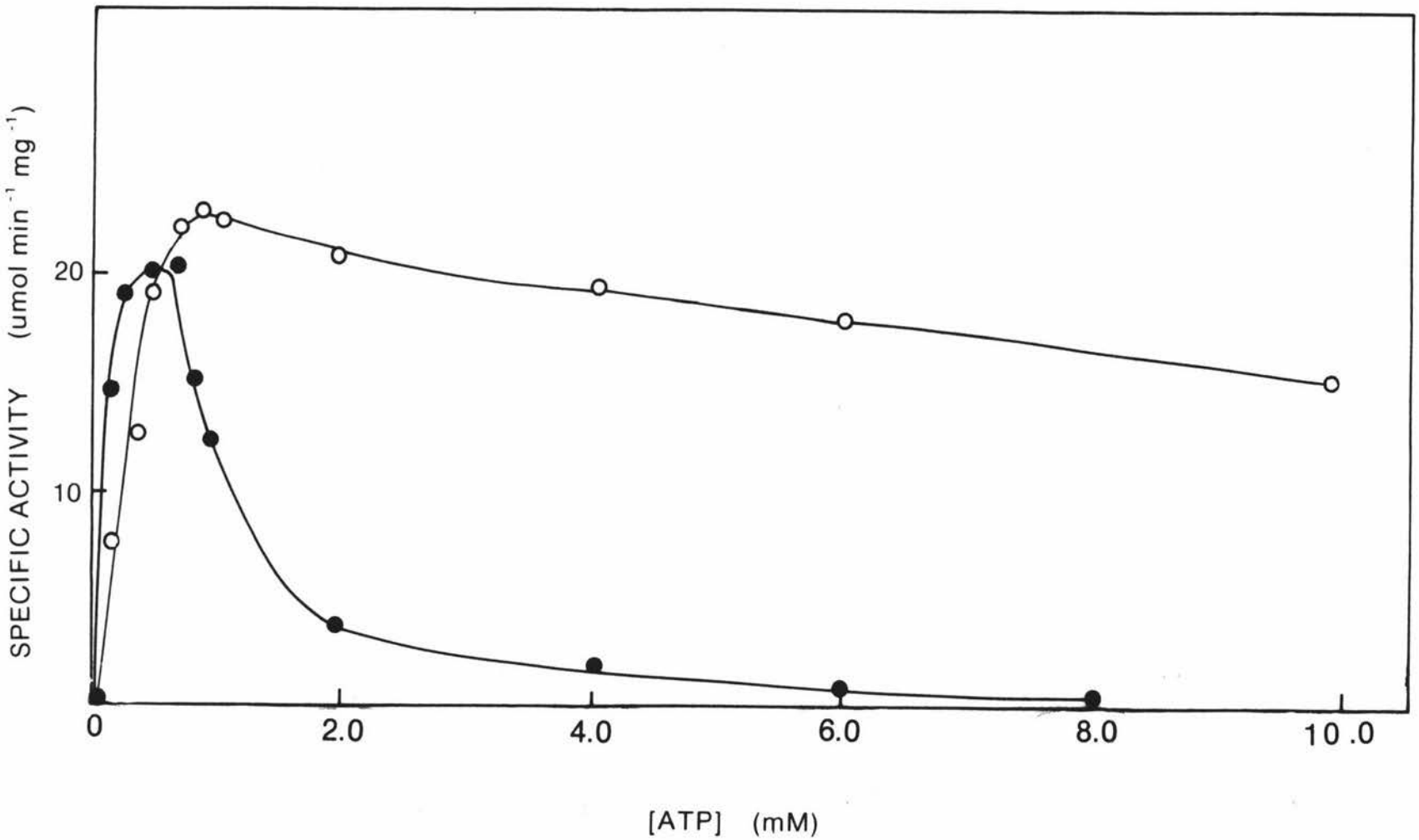


FIGURE 2.3.7 The Effect of ATP Concentration on PFK Activity. The effect of ATP concentration on activity of purified PFK was determined at a constant Mg^{2+} concentration of 1.0 mM, at 2.0 mM F6P (O) and at 0.2 mM F6P (●). The protein concentration in assays was 0.21 µg (O) or 0.42 µg (●).

bound Mg^{2+} on the purified enzyme.

At a constant ATP concentration of 1.0 mM the optimum concentration of Mg^{2+} was 5-10 mM at both saturating (2.0 mM) and non-saturating (0.2 mM) concentrations of F6P (Figure 2.3.8). Free Mg^{2+} inhibited PFK activity at concentrations greater than 10 mM. Addition of extra aldolase, TPI or αGPDH to the assay once maximal velocity had been reached did not affect the rate, confirming that inhibition by Mg^{2+} was due to inhibition of PFK activity not to a reduction of activity of the auxiliary enzymes in the assay. Inhibition by Mg^{2+}, in contrast to inhibition by free ATP (Section 2.3.5) or MgATP (Section 2.3.8) appeared to be independent of F6P concentration.

2.3.7 SPECIFICITY OF DIVALENT CATION REQUIREMENT

The ability of other divalent cations to replace Mg^{2+} as a co-factor was investigated by assaying PFK at constant F6P and ATP concentrations of 2.0 mM and 1.0 mM respectively in the absence of Mg^{2+} ions. PFK was first treated with 10 mM EDTA (1 hr, 4°C), which was removed by gel filtration on Sephadex G-25 before the enzyme was assayed.

The results of increasing cation concentrations are shown in Figure 2.3.9. The cation requirement could be partially fulfilled by Mn^{2+} or Co^{2+}, optimum concentrations of these cations giving 80% and 55% respectively of the maximum activity with Mg^{2+}. Addition of Zn^{2+} at concentrations less than 1.0 mM gave activities comparable to those obtained with similar concentrations of Co^{2+}, but at higher concentrations of Zn^{2+} the hydroxide precipitated from solution. No significant activity was found with Ca^{2+}, Ni^{2+} or Cu^{2+}. The K_m concentrations calculated from the double reciprocal plots in Figure 2.3.10 were 0.67 mM, 0.28 mM, and 0.88 mM for Mg^{2+}, Mn^{2+} and Co^{2+} respectively.

In the absence of any divalent cation PFK showed a low activity (6 μmol F6P min^{-1} mg^{-1}; 4.5% of the activity at optimum cation concentration). This rate was not affected by increasing the F6P concentration from 0.2 - 2.0 mM suggesting that the metal ion was the rate-limiting factor in the assay. This activity could be abolished by including EDTA in the assay mixture, but not by pre-treatment of the enzyme with EDTA.

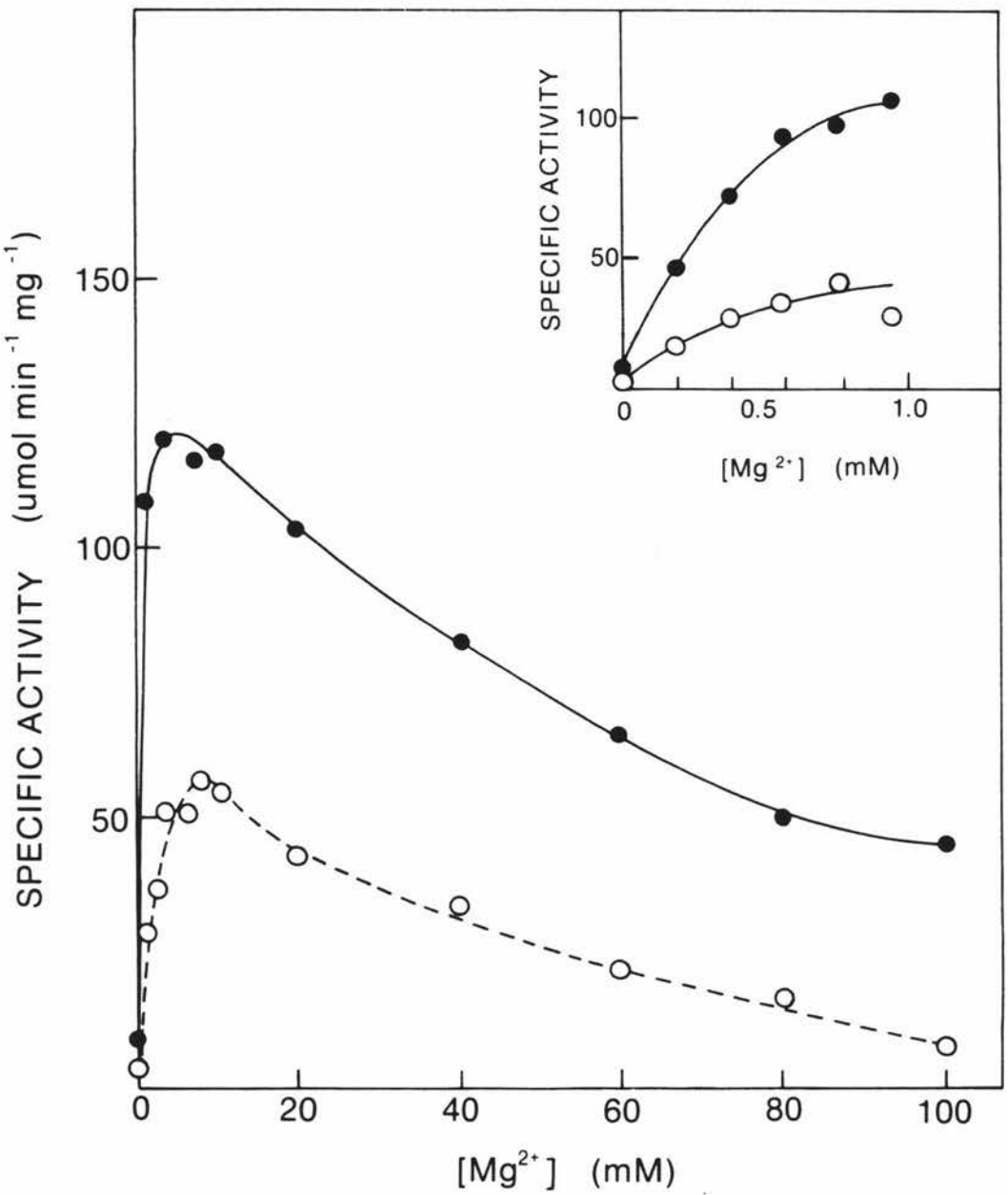


FIGURE 2.3.8 The Effect of Mg^{2+} Concentration on PFK Activity. The effect of Mg^{2+} concentration on PFK activity was determined at a constant ATP concentration (1.0 mM) and at 2.0 mM (●) and 0.2 mM (○) F6P. Each assay contained 0.42 µg protein.

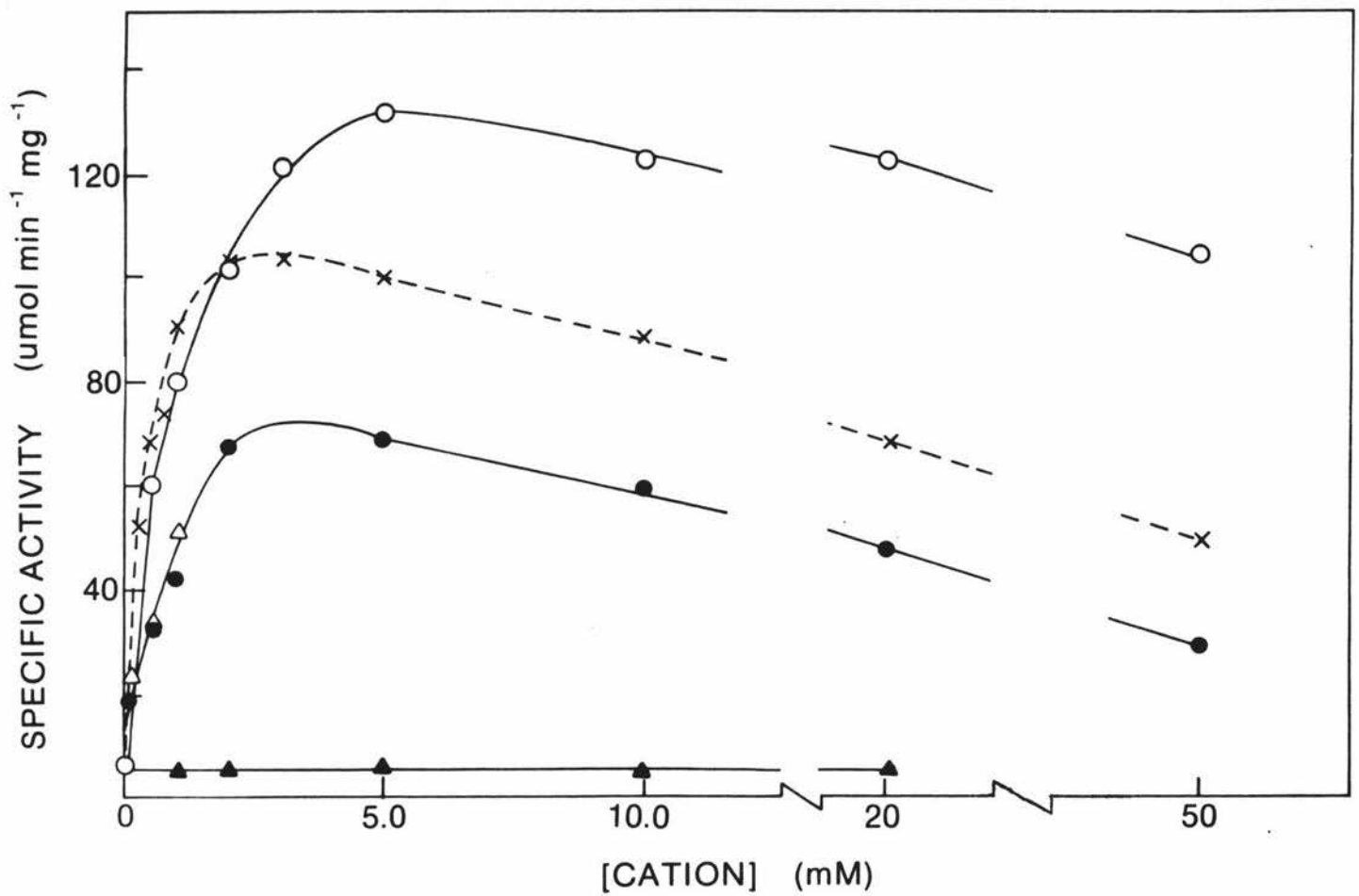


FIGURE 2.3.9 PFK:Divalent Cation Specificity. PFK was assayed at 2.0 mM ATP, 2.0 mM F6P and 0 - 50 mM concentrations of Mg^{2+} (O); Mn^{2+} (×); Co^{2+} (●); Zn^{2+} (△); and Ca^{2+}, Cu^{2+}, and Ni^{2+} (▲).

Each assay contained 0.2 - 0.4 µg protein.

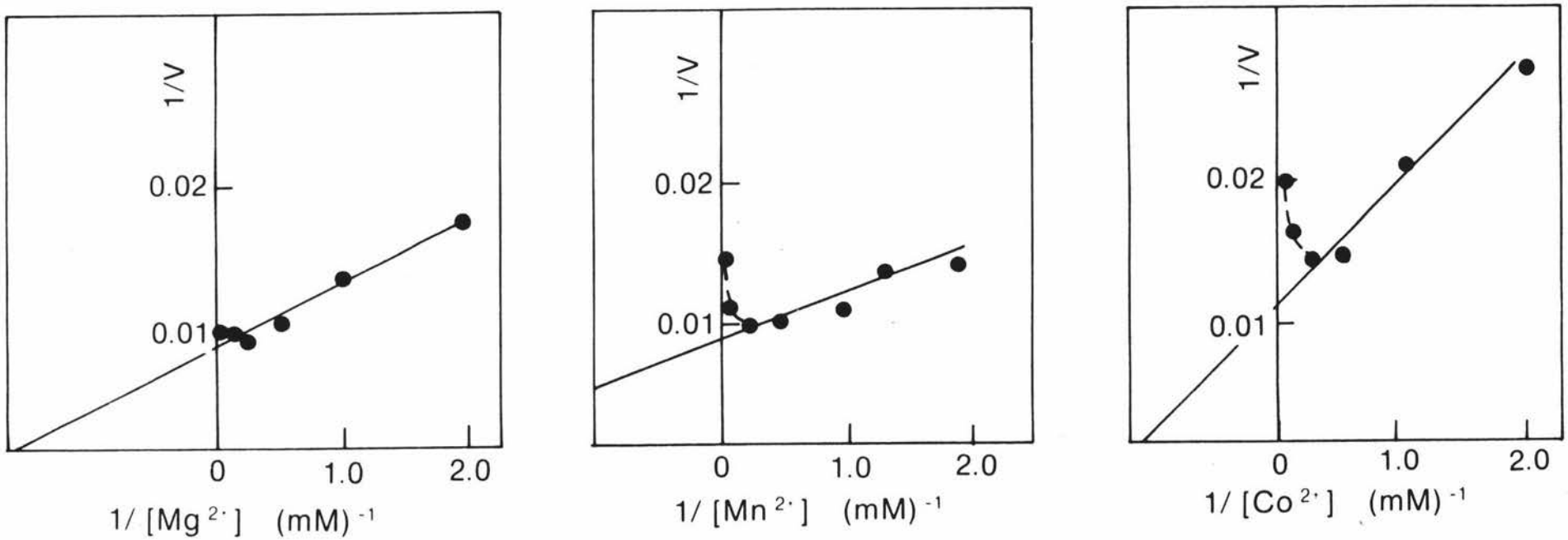


FIGURE 2.3.10 Lineweaver-Burk Plots for determination of Cation-Binding Constants.
Data from Figure 2.3.9 were plotted as double reciprocal plots. The reciprocal of the velocity (1/V) is expressed as $(\mu mol\ min^{-1}\ mg^{-1})^{-1}$.
K_m values for cations are: Mg^{2+} - 0.67 mM, Mn^{2+} - 0.28 mM, Co^{2+} - 0.88 mM.

2.3.8 DEPENDENCE OF PFK ACTIVITY ON MgATP CONCENTRATION

The dependence of PFK activity on MgATP concentration is shown in Figure 2.3.11 at F6P concentrations ranging from 0.1 mM - 1.0 mM. All MgATP solutions were made up with Mg^{2+} present in a 5 mM excess over ATP, conditions under which more than 98% of the ATP is complexed with Mg^{2+} (see Appendix 2) PFK exhibited a hyperbolic response to increasing MgATP concentration; this is reflected in Hill coefficients approximating 1.0 (Table 2.3.1). Increasing the F6P concentration did not markedly alter the value of the Hill coefficient, but as expected the maximum velocity was proportional to F6P concentration. The concentration of MgATP required for half saturation (K_m (MgATP)) obtained was 0.12 - 0.18 mM in successive experiments. The K_m (MgATP) appeared to increase with increasing concentrations of F6P, although as the maximum velocity is uncertain because of ATP inhibition, the increase in K_m (MgATP) could be an artefact.

MgATP is inhibitory at concentrations which are high relative to F6P concentration. Inhibition can be overcome by increasing the F6P concentration, thus MgATP concentrations less than or equal to 2 mM are not inhibitory at 2 mM F6P, but MgATP becomes inhibitory at increasingly lower concentrations as the F6P concentration is lowered. MgATP inhibition results in the characteristic upward curvature of a double reciprocal plot (data not shown).

Figure 2.3.13 shows the effect of varying ratios of Mg^{2+}:ATP on the MgATP saturation profile. In agreement with the results shown in Figure 2.3.8 maximum activity was obtained with Mg^{2+}:ATP ratios of 5:1 - 10:1. Each ratio of Mg^{2+}:ATP produced a hyperbolic curve but the maximum velocities attained decreased with decreasing Mg^{2+}:ATP ratios. No co-operative binding of MgATP was evident, as was found for T6PK (Section 3.3.8) when Mg^{2+} and ATP concentrations were varied in a constant ratio (Figure 2.3.12).

Inhibition of PFK by MgATP is a characteristic of all bacterial PFKs which have been studied with the exception of the enzymes from *Arthrobacter crystallopoietes* (Ferdinandus & Clarke, 1969), *Lactobacillus casei* and *Lactobacillus plantarum* (Doelle, 1972; Simon & Hofer, 1977) *Flavobacterium thermophilum* (Yoshida *et al*, 1972) and *Dictyostelium discoideum* (Kono & Uyeda, 1974). These enzymes are all non-allosteric enzymes which show Michaelis-Menten saturation behaviour even at high ATP and low F6P concentrations.

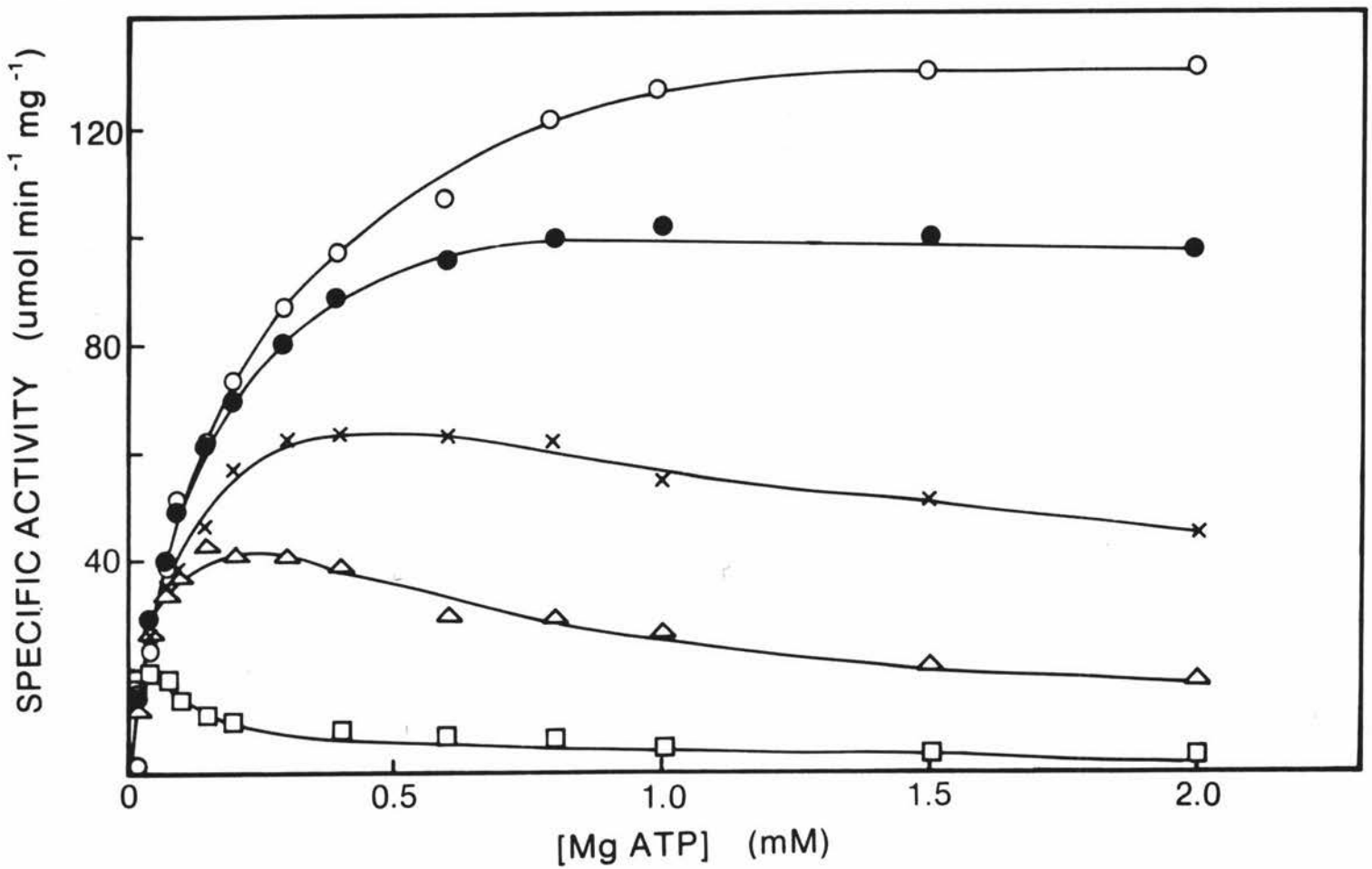


FIGURE 2.3.11 The Effect of MgATP Concentration on PFK Activity. Purified PFK (0.2 or 0.4 µg protein per assay) was assayed at varying MgATP concentrations and at the following F6P concentrations:

○ - 1.0 mM; ● - 0.5 mM; × - 0.3 mM; △ - 0.2 mM; □ - 0.1 mM.

Mg^{2+} ions were present in a 0.5 mM excess over ATP concentration in all assays.

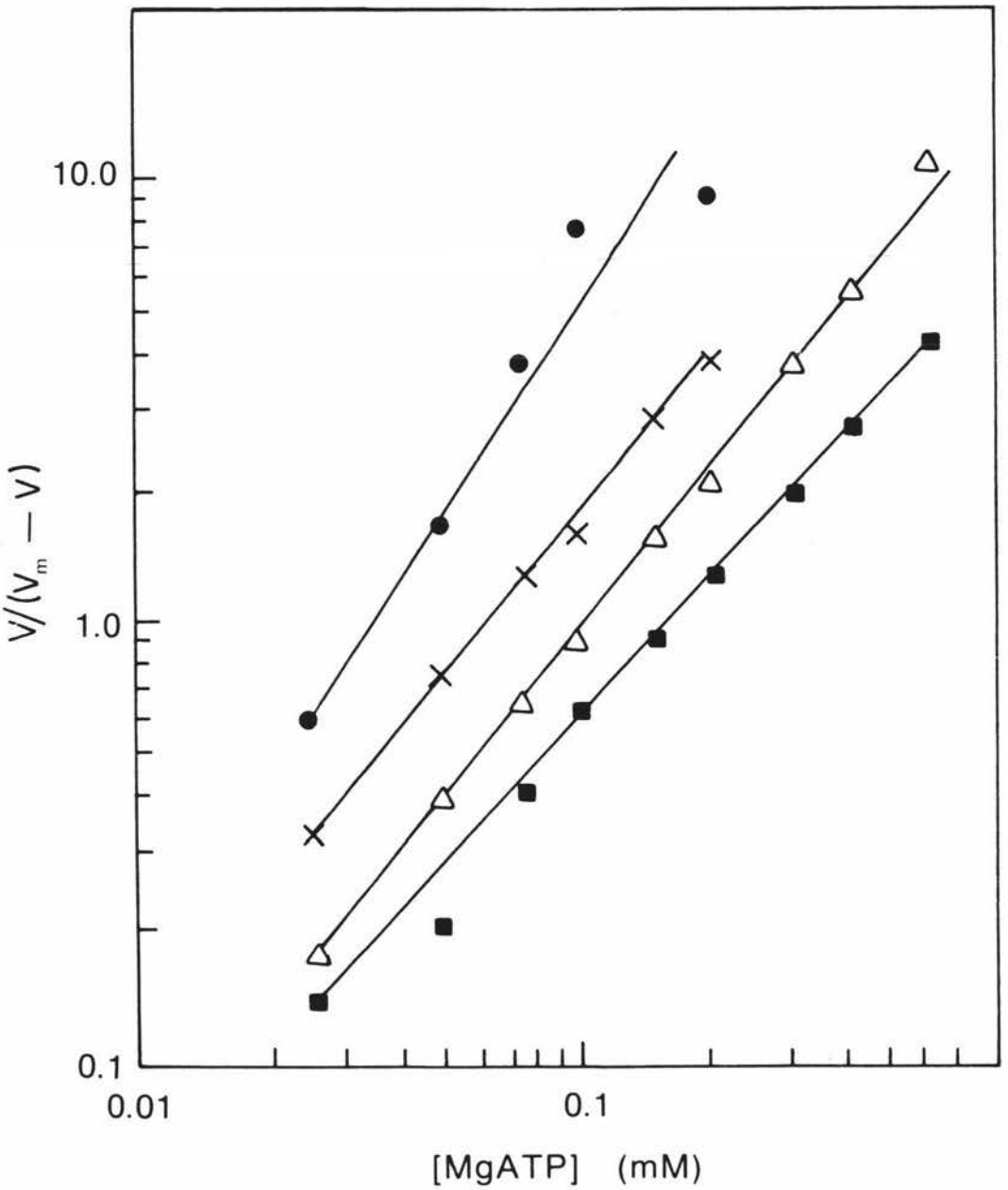


FIGURE 2.3.12 Hill Plots : The Effect of MgATP Concentration on PFK Activity. $V/(V_m - V)$ was determined using V_m values calculated from Endrenyi plots (data not shown).

■ - 1.0 mM F6P; △ - 0.5 mM F6P;
× - 0.3 mM F6P: ● - 0.2 mM F6P.

n_H values of 1.0 - 1.6 were obtained at all F6P concentrations, while increasing concentrations of F6P caused a corresponding increase in $MgATP_{0.5}$.

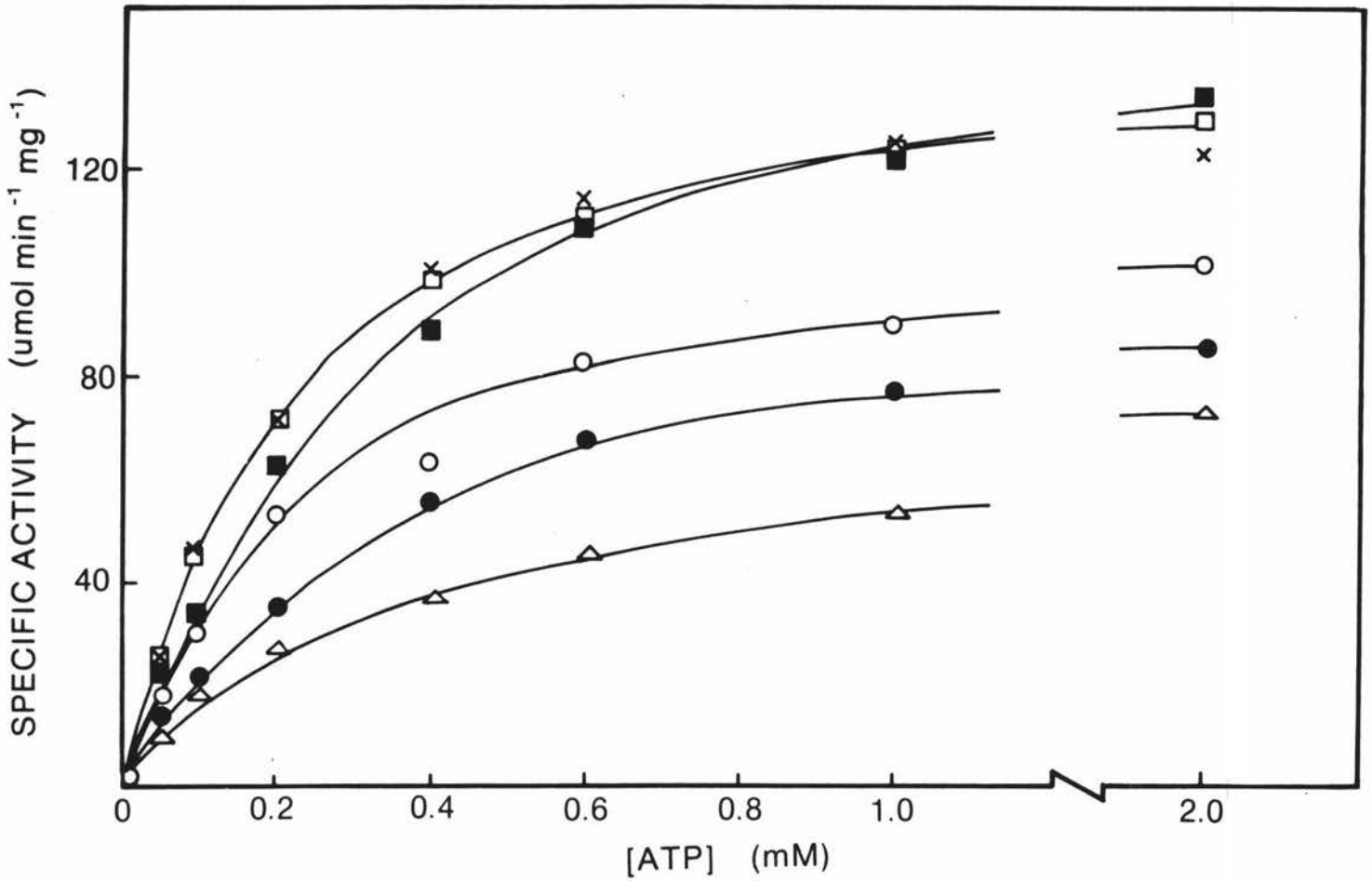


FIGURE 2.3.13 The Effect of the Mg^{2+}:ATP Ratio on the MgATP Saturation Profile. Assays were performed at 2.0 mM F6P and at 0 - 2.0 mM ATP. ATP solutions were made up with a constant ratio of Mg^{2+}:ATP and diluted to give the desired ATP concentrations.

Mg^{2+}:ATP ratios: △ - 0.25:1; ● - 0.5:1; ○ - 1:1; ■ - 2:1; □ - 5:1; × - 10:1.

Each assay contained 0.21 or 0.42 µg protein.

TABLE 2.3.1

DEPENDENCE OF V_{MAX}, n_H AND K_m (MgATP) ON F6P CONCENTRATION

[F6P] mM	V_{max} µmol F6P min^{-1} mg^{-1}	n_H	K_m (MgATP) mM
0.1	19	ND	~0.01*
0.2	43	1.55	0.04
0.3	63	1.23	0.06
0.5	103	1.23	0.11
1.0	132	1.08	0.16

n_H and K_m (MgATP) were calculated from Hill plots (data not shown).

ND = not determined.

* Not determined graphically, but estimated from Figure 2.3.11.

The observed inhibition of PFK activity may be due to inhibition by Mg^{2+}, ATP or a MgATP complex, or a combination of these. MgATP rather than free ATP inhibits yeast PFK (Mavis and Stellwagen, 1970) and Blangy *et al* (1968) showed that ATP inhibition of *E. coli* PFK could be abolished by maintaining a 10:1 ratio of MgATP, suggesting that free ATP rather than free Mg^{2+} or MgATP was inhibitory. Free ATP is also a greater inhibitor of muscle and erythrocyte PFK than is MgATP or free Mg^{2+} (Paetkau & Lardy, 1967; Otto *et al*, 1974). Although *S. lactis* PFK appears to be inhibited by free Mg^{2+} and ATP (increasing concentrations of either Mg^{2+} or ATP when the other compound is maintained at a constant level produces inhibition (Sections 2.3.5 and 2.3.6)) the MgATP complex is also inhibitory. The inhibition observed in Figure 2.3.11 was achieved with Mg^{2+} present in the assay in a 0.5 mM excess over ATP concentration, conditions under which approximately 95% of the ATP is complexed, thus the free Mg^{2+} and free ATP concentrations are very low.

Different mechanisms appear to be involved in Mg^{2+} and ATP inhibition : inhibition by free ATP and MgATP can be overcome by increasing the F6P concentration whereas inhibition by Mg^{2+} appears to be independent of F6P concentration.

2.3.9 Dependence of PFK Activity on F6P Concentration

PFK shows co-operative binding of F6P, resulting in a sigmoidal dependence of activity on the concentration of F6P. Figure 2.3.14 shows the saturation profiles for F6P at concentrations of MgATP ranging from 0.05 - 1.0 mM, when Mg^{2+} was maintained at a concentration of 0.5 mM above the ATP concentration. In contrast to the MgATP saturation profiles (Figure 2.3.11) no inhibition was observed with increasing substrate (F6P) concentration, even at concentrations of MgATP as low as 0.05 mM. The overlapping of the curves at low F6P concentrations is a consequence of the substrate inhibition by MgATP (Figure 2.3.11).

At saturating concentrations of ATP (0.5 - 1.0 mM) maximum activity was attained at approximately 1 mM F6P. The concentration of F6P required for enzyme saturation was dependent on MgATP concentration, the $F6P_{0.5}$ value increasing with increasing MgATP concentration (Table 2.3.2). The sigmoidal response to F6P

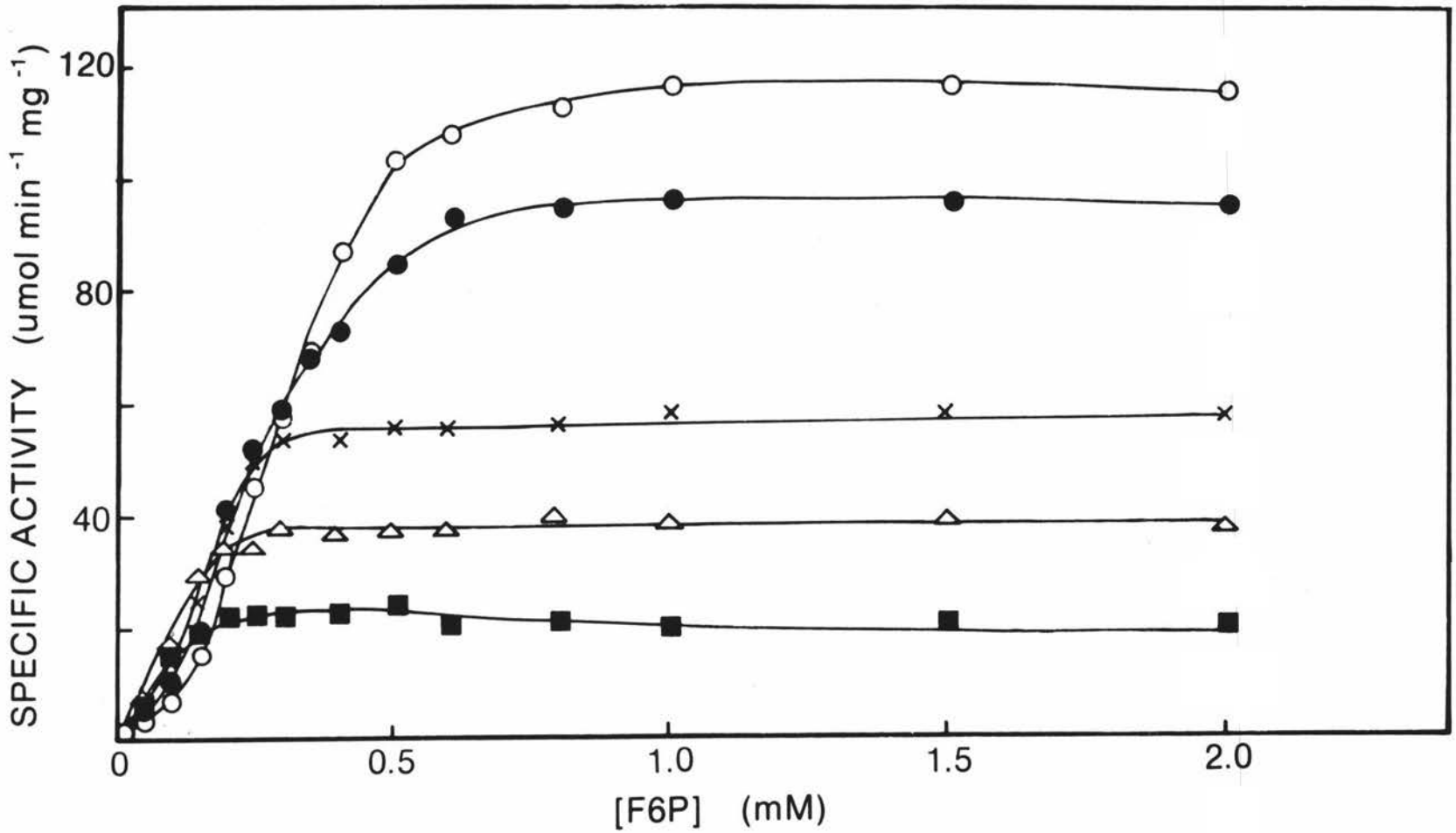


FIGURE 2.3.14 The Effect of F6P Concentration on PFK Activity. Assays were performed at constant MgATP concentrations, using 0.21 µg purified PFK per assay. F6P concentrations ranged from 0 - 2.0 mM as indicated.

MgATP concentrations were as follows: ○ - 1.0 mM; ● - 0.5 mM; × - 0.2 mM; △ - 0.1 mM; and ■ - 0.05 mM.

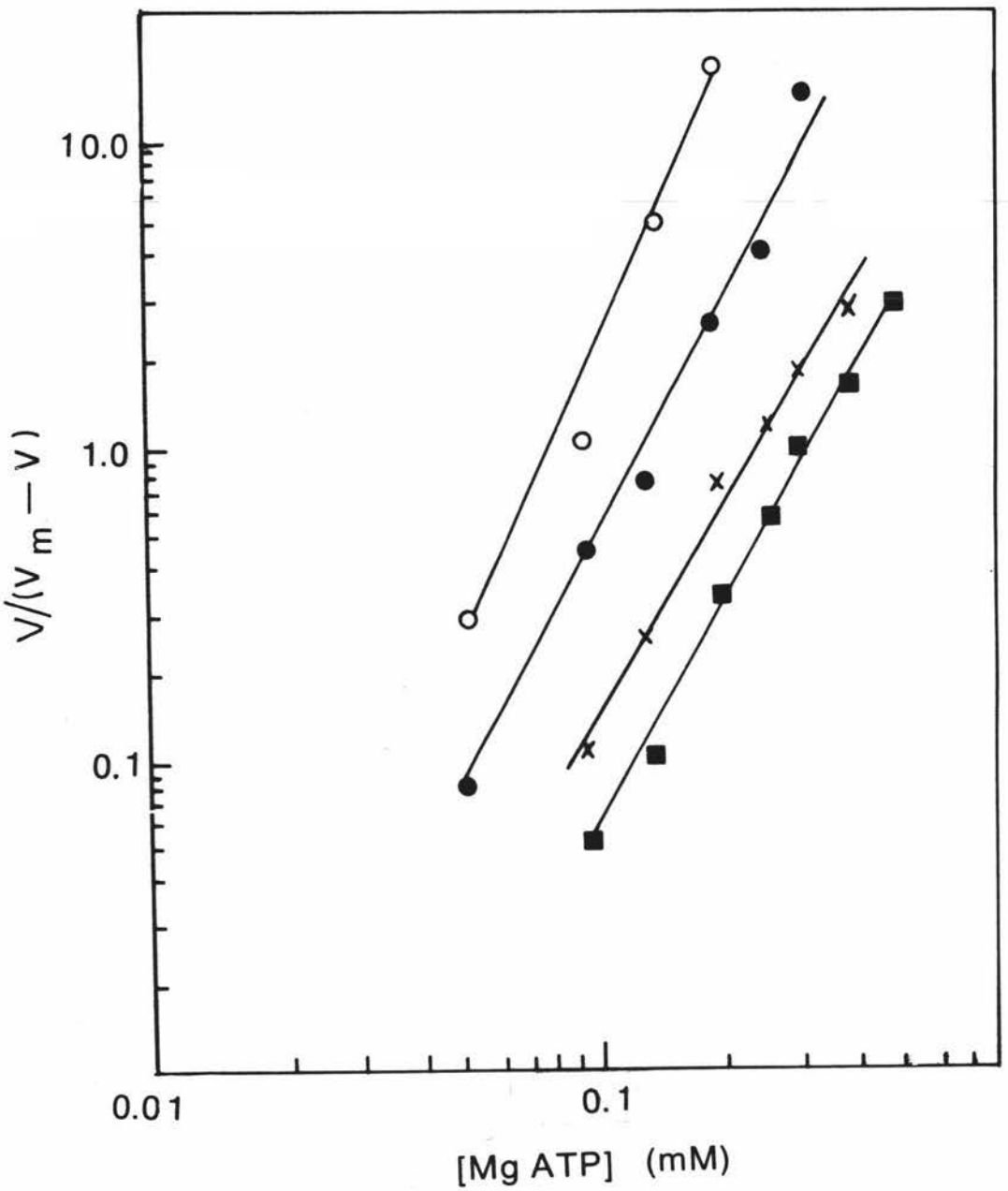


FIGURE 2.3.15 Hill plots : data obtained at constant MgATP and varying F6P concentrations. n_H and $F6P_{0.5}$ values calculated are listed in Table 2.3.2.

MgATP concentrations: ○ - 0.1 mM; ● - 0.2 mM; × - 0.5 mM; ■ - 1.0 mM.

TABLE 2.3.2

DEPENDENCE OF V_{MAX}, n_H AND $F6P_{0.5}$ ON MgATP CONCENTRATION

[MgATP] mM	V_{max} µmol F6P min^{-1} mg^{-1}	n_H	$F6P_{0.5}$ mM
0.10	36	2.78	0.08
0.20	56	2.93	0.16
0.50	95	2.50	0.25
1.0	116	2.56	0.32

n_H and $F6P_{0.5}$ were calculated from the Hill plots (Figure 2.3.15), and the V_{max} obtained directly from the graphs of specific activity vs [F6P].

concentration is reflected in the Hill coefficient of approximately 3.0. This was not altered markedly by changing the MgATP concentration, whereas the $F6P_{0.5}$ values and maximum velocities were dependent on the MgATP concentration (Table 2.3.2, Figure 2.3.15).

The co-operative binding of F6P shown by *S. lactis* PFK is typical of all allosteric PFKs studied. As with PFK from other sources the affinity of *S. lactis* PFK for F6P could be altered by varying the concentrations of effectors (see Sections 2.3.12 - 2.3.20) or the pH (this section.)

To explain the co-operative binding of F6P to *E. coli* PFK, Blangy *et al* (1968) proposed two different conformational states of enzyme, R and T, each with different affinities for F6P and allosteric effectors. Effectors of *E. coli* PFK altered the affinity of the enzyme for its substrate without affecting the co-operativity of F6P binding. A similar model was proposed for yeast PFK (Hoffmann, 1976) to explain the action of effectors in modifying the sigmoidal F6P binding curve, as in this case effectors also produced a simultaneous change in n_H.

X-ray crystallographic studies on *Bacillus stearothermophilus* PFK (Evans and Hudson, 1969; Evans *et al*, 1981) confirm the above hypotheses, showing that the *B. stearothermophilus* enzyme exists in two forms depending on the presence and absence of P_i or F6P.

The F6P saturation profile shown in Figure 2.3.14 gives a $F6P_{0.5}$ value of 0.29 mM. However in subsequent experiments, two different values of $F6P_{0.5}$ were obtained, one 0.25 - 0.29 mM, and one 0.55 - 0.65 mM. Reasons for this variation were not clear. The "high K_m" form of the enzyme ($F6P_{0.5} \sim 0.65$ mM) appeared to be related to ageing of the enzyme. PFK which exhibited a low $F6P_{0.5}$ of 0.28 mM had an $F6P_{0.5}$ of 0.55 mM after three months storage at -20°C and at a concentration of 0.3 mg/ml. The "high K_m" form was also found in enzyme from preparations in which protein concentration was low during purification. Several small preparations of enzyme (from only 5 g frozen cells) consistently gave an $F6P_{0.5}$ of 0.5 - 0.65 mM even when assayed within five hours of preparation. The $F6P_{0.5}$ was not altered by freezing the cells; preparations of PFK from freshly grown cells of *S. lactis*, or from cells frozen for one day and three months showed similar $F6P_{0.5}$ values. Nor was the

$F6P_{0.5}$ dependent on the stage of growth at which the cells were harvested. PFK from cells harvested in mid-log, late-log, and stationary phases, and approximately 24 hours after reaching stationary phase had similar $F6P_{0.5}$ values of approximately 0.6 mM. The two forms of enzyme differed only in the concentration of F6P required to saturate the enzyme; they otherwise exhibited identical kinetic characteristics with respect to maximum velocity, inhibition by T6P, and activation by ADP and NH_4^+.

The apparent variation in $F6P_{0.5}$ raises the question of to what extent the parameters measured *in vitro* are a true reflection of the enzyme activity *in vivo*. To establish that the purification procedure was not significantly altering the enzyme's affinity for substrates the K_m (MgATP) and $F6P_{0.5}$ values were determined in freshly prepared cell-free extracts. The results are shown in Figures 2.3.16 A and B. The K_m (MgATP) determined was 0.3 mM, higher than the value (0.16 - 0.18 mM) determined with purified enzyme. However several other enzymes in the extract may also bind MgATP, thus effectively lowering the concentration in the assay.

The $F6P_{0.5}$ value determined using the cell-free extract was 0.65 mM. However the net change in absorbance when each assay was allowed to run to completion was only 30-70% (depending on F6P concentration) of the expected change in absorbance based on the initial concentration of F6P in the assay. This suggests that there is at least one other F6P-utilising enzyme in the cell free extract, either with a higher affinity for F6P than PFK, or present in much higher concentrations. This was confirmed by incubating cell-free extract plus F6P in the absence of MgATP prior to initiating the reaction by addition of MgATP. Only NADH oxidation due to NADH oxidase activity was noted during incubation in the absence of MgATP, but the PFK activity decreased with increasing length of pre-incubation. This could be explained by the action of a phosphatase on F6P. *S. lactis* C_{10} contains at least three different phosphatases which hydrolyse ATP (see Chapter 3); these enzymes may also hydrolyse other phosphorylated glycolytic intermediates. The existence of a phosphatase hydrolysing phosphorylated sugar analogues has also been demonstrated in *S. lactis* (Thompson and Saier, 1981).

The shape of the F6P saturation curve for *S. lactis* PFK can be altered by varying concentrations of several metabolites, as shown

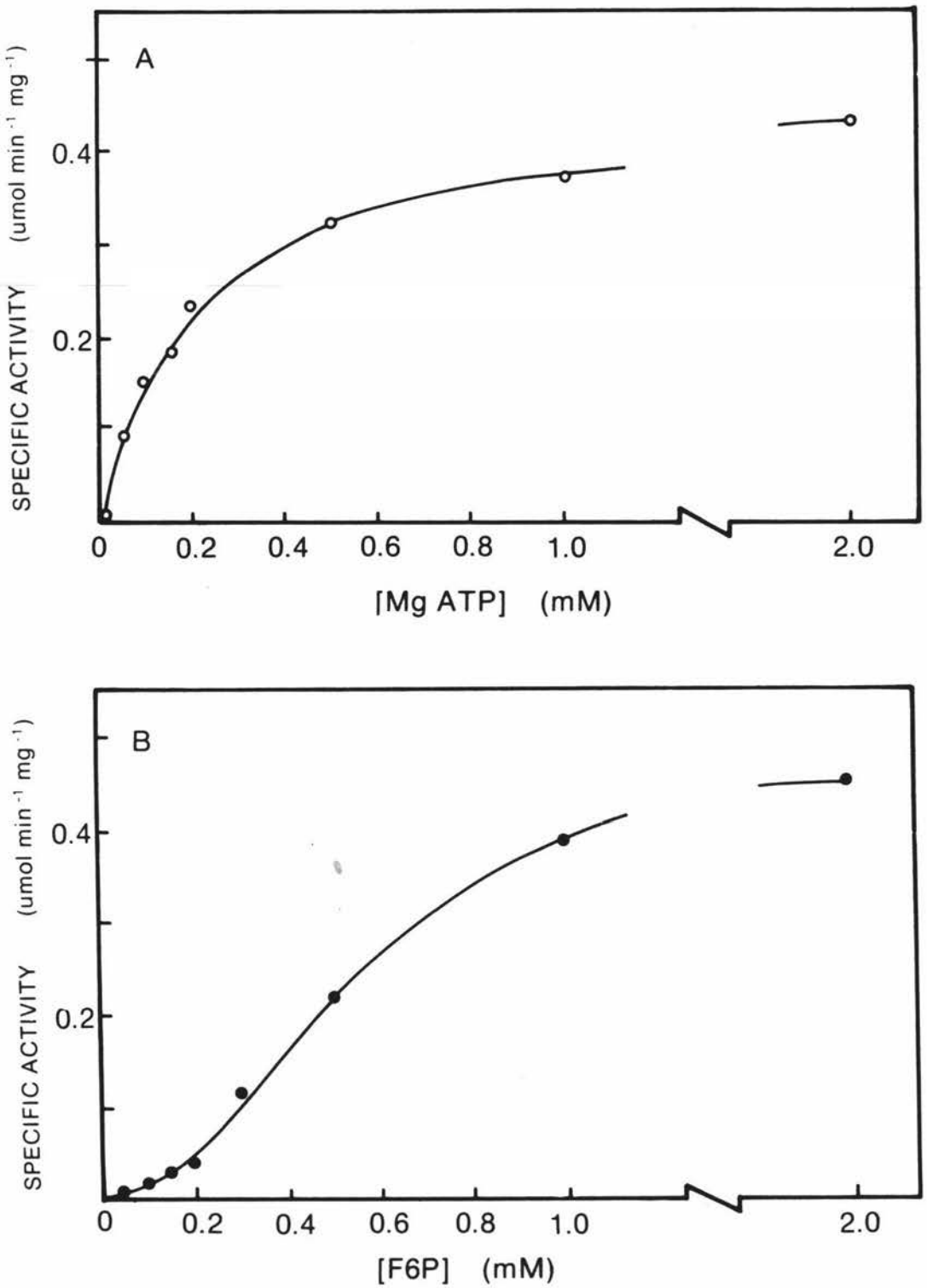


FIGURE 2.3.16 Substrate saturation profiles of PFK assayed in cell-free extract. This figure shows the dependence of PFK activity on MgATP (Figure A) and F6P (Figure B) when the enzyme is assayed in a freshly prepared cell-free extract using the aldolase assay system.

In Figure A, F6P concentration was maintained constant (2.0 mM) : In Figure B the MgATP concentration was 2.0 mM.

Each assay contained 15 µg protein.

in subsequent sections of this chapter (Sections 2.3.11 - 2.3.20). The effect of pH on F6P binding was also studied (Figure 2.3.17). Assays were performed using the aldolase assay system described in Section 2.2.6, except that the Tris-HCl buffer in the assay mixture was replaced by 50 mM Imidazole buffer, pH 6.5, 7.5, or 8.5.

A sigmoidal saturation curve was obtained at all three pH values. The Hill coefficient was lower at pH 8.0 (n_H = 2.8) than at pH 6.5 or 7.5 (n_H = 3.5 and 3.8 respectively). The $F6P_{0.5}$ was not affected by increasing pH. Co-operativity of binding of F6P to mammalian PFK and to *L. acidophilus* PFK is also pH dependent. Unlike *S. lactis* PFK, *L. acidophilus* PFK showed greater co-operativity at pH 8.5 than at pH 6.0 (Simon & Hofer, 1977). Co-operativity of binding to mammalian and yeast PFK can be abolished by increasing and decreasing pH respectively (Trivedi & Danforth, 1966; Kopperschlager *et al*, 1968).

2.3.10 REVERSIBILITY OF PFK ACTIVITY

PFK was assayed for activity in the reverse direction (F6P formation from FBP) by coupling F6P production to $NADP^+$ reduction by glucose 6-phosphate dehydrogenase via phosphoglucose isomerase.

No formation of F6P from FBP could be detected, thus the reaction catalysed by PFK appears to function only in the forward direction. This is not surprising as the PFK-catalysed reaction is generally found to be irreversible, the forward reaction (F6P phosphorylation) being favoured by a large negative $\Delta G°$. Only the pyrophosphate-dependent PFKs as in *Propionibacterium shermanii* (O'Brien *et al*, 1975) and *Entamoeba histolytica* (Reeves *et al*, 1974) catalyse readily reversible reactions. ATP-dependent PFKs may catalyse a slow formation of F6P *in vitro* (Lorenson & Mansour, 1968; Uyeda, 1970; Babul, 1978) but the reverse reaction *in vivo* is catalysed by fructose bisphosphatase.

2.3.11 EFFECTORS OF PFK ACTIVITY

In most systems studied, PFK activity has been shown to be regulated by a number of metabolic intermediates. In a search for possible allosteric effectors of *S. lactis* PFK, PFK activity was measured in the presence and absence of various compounds at

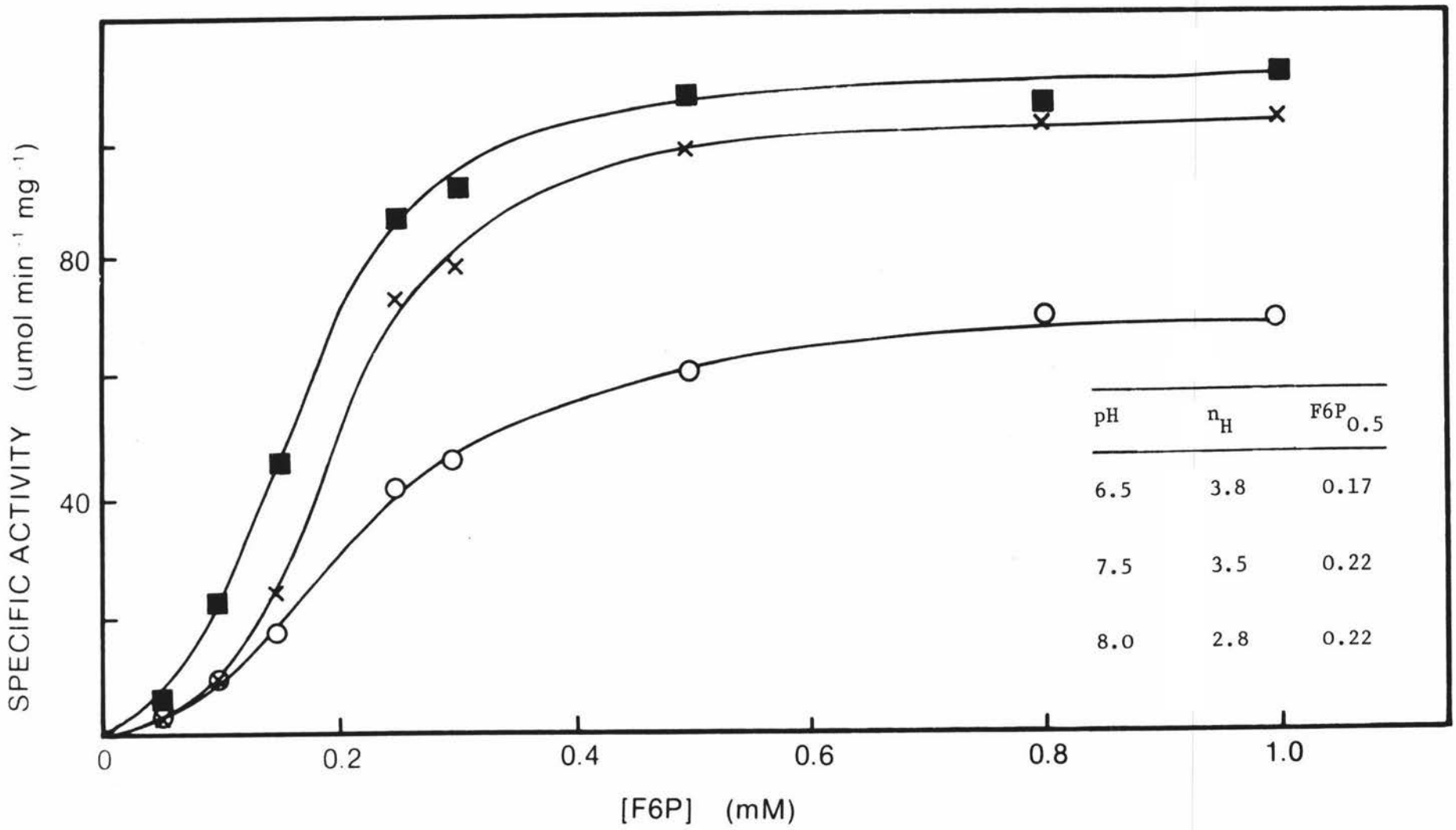


FIGURE 2.3.17 The Effect of pH on the F6P Saturation Profile. Assays were performed at saturating (2.0 mM) MgATP, using 0.2 µg purified PFK per assay. The assay buffer was **Imidazole, pH 6.5** (O); pH 7.5 (×); or pH 8.0 (■). n_H and $F6P_{0.5}$ values were calculated from a Hill plot (data not shown).

concentrations ranging from 0.1 mM to 25 mM. Purified PFK was incubated at 30°C in the presence of the effector for approximately 5 minutes, then the reaction was started by the addition of F6P.

Effectors of enzyme activity may act by altering the affinity of the enzyme for a substrate or by changing the V_{max}. To determine whether the K_m, V_{max}, or both these parameters were affected by the presence of effectors, each compound was tested using the following combinations of substrate concentrations:

i 2.0 mM MgATP, 2.0 mM F6P; conditions under which PFK is saturated with both substrates

ii a non-saturating MgATP concentration (0.1 mM), and saturating F6P concentration (2.0 mM)

iii a saturating MgATP concentration (2.0 mM) and non-saturating F6P concentration (0.15 mM).

The non-saturating substrate concentrations were approximately half the K_m concentration for each substrate.

The results of this survey are shown in Table 2.3.3 in which activity in the presence of various concentrations of effector is expressed as a percentage of the activity in the control assay (no effector). Effectors of *S. lactis* PFK generally resulted in a change in $S_{0.5}$, with little change in n_H. Their mode of action on *S. lactis* PFK therefore appears to be similar to that for the *E. coli* enzyme (Blangy *et al*, 1968). However unlike *E. coli* PFK, both ADP (an activator) and T6P (an inhibitor (see Section 2.3.13)) caused a change in both n_H and $S_{0.5}$.

In general the activatory or inhibitory effect of compounds was more pronounced at non-saturating levels of either F6P or MgATP than at saturating levels of both substrates. The compounds tested could be roughly grouped into two classes:

i those which produced a more marked effect at non-saturating F6P than at non-saturating MgATP concentrations. These compounds included FBP, G6P, glucose, ADP, PEP and AMP.

TABLE 2.3.3

THE EFFECT OF VARIOUS METABOLITES ON PFK ACTIVITY

Effector	[Substrate] (mM)		[Effector] (mM)				
	F6P	MgATP	0.1	1.0	5.0	10.0	25.0
NH_4^+	2	2	92	100	-	111	115
	0.15	2	109	109	-	114	115
	2	0.1	124	222	-	604	756
K^+	2	2	108	93	-	103	99
	0.15	2	104	106	-	115	116
	2	0.1	-	109	-	141	155
ADP	2	2	104	97	-	66	39
	0.15	2	264	645	-	381	148
	2	0.1	117	88	-	7	-
Citrate	2	2	106	106	-	82	64
	0.15	2	115	99	-	42	24
	2	0.1	98	50	-	22	-
PEP	2	2	93	85	86	69	-
	0.15	2	114	59	15	6	-
	2	0.1	100	100	74	56	-
FBP	2	2	102	100	-	104	96
	0.15	2	82	78	-	65	50
	2	0.1	78	66	-	59	57
AMP	2	2	98	96	-	50	4
	0.15	2	82	35	-	0	0
	2	0.1	106	80	-	50	25
G6P	2	2	100	96	97	89	-
	0.15	2	100	87	50	30	-
	2	0.1	95	100	88	87	-
PP_i	2	2	95	98	59	23	-
	0.15	2	99	77	17	7	-
	2	0.1	80	32	7	3	-
P_i	2	2	106	105	-	103	87
	0.15	2	100	91	-	46	40
	2	0.1	97	91	-	87	75
Glucose	2	2	103	97	-	103	97
	0.15	2	99	92	-	111	128
	2	0.1	98	95	-	91	88
R5P	2	2	102	94	-	72	-
	0.15	2	95	84	-	109	-
	2	0.1	96	80	-	57	-

PFK was assayed in the presence and absence of the metabolites listed (0.1 - 25 mM concentrations). All assays were performed using the aldolase assay system except assays in which FBP was included; the PK-LDH assay system was used in these assays.

Figures = % control activity.

ii Those compounds such as NH_4^+ and K^+ which exerted a greater effect at non-saturating concentrations of ATP.

Not surprisingly intermediates of the TCA cycle and pentose-phosphate pathway had little effect on PFK activity; these pathways do not operate in *S. lactis*.

Atkinson & Walton (1965) postulated that increased enzyme activity in the presence of an activator was usually a result of increased affinity for substrate, therefore the extent of activation decreased as the substrate concentration became saturating. Similarly the action of allosteric inhibitors can be explained by assuming a decreased affinity for substrate, a change which would also produce maximal effect on activity at low substrate concentrations. In agreement with this postulate, all compounds which affected *S. lactis* PFK activity caused a change in $S_{0.5}$.

The apparent inhibition by citrate and PP_i was shown to be due to complexing of Mg^{2+} since inhibition could be overcome by addition of extra Mg^{2+}.

Of the compounds studied, ADP, AMP, PEP, FBP, NH_4^+, and P_i produced an effect on PFK activity at concentrations which could be of physiological significance. These effectors were selected for further study.

2.3.12 The Effect of ADP on PFK Activity

The effect of ADP on the binding of both MgATP and F6P to PFK was investigated. Figure 2.3.18 shows the effect of ADP on PFK activity at a saturating (2.0 mM) MgATP concentration and increasing concentrations of F6P. ADP stimulated activity at F6P concentrations less than or slightly greater than the $F6P_{0.5}$ concentration, but was inhibitory at saturating F6P concentrations. Maximal activation was obtained with 0.1 mM ADP. Higher concentrations of ADP (1 - 5 mM) were also activatory but the extent of activation decreased as the ADP concentration increased. Inhibition of PFK activity by ADP at higher concentrations of F6P (>0.6 - 1.0 mM) was probably due to inhibition of MgATP binding by ADP so that at these concentrations of F6P, the MgATP concentration becomes rate limiting.

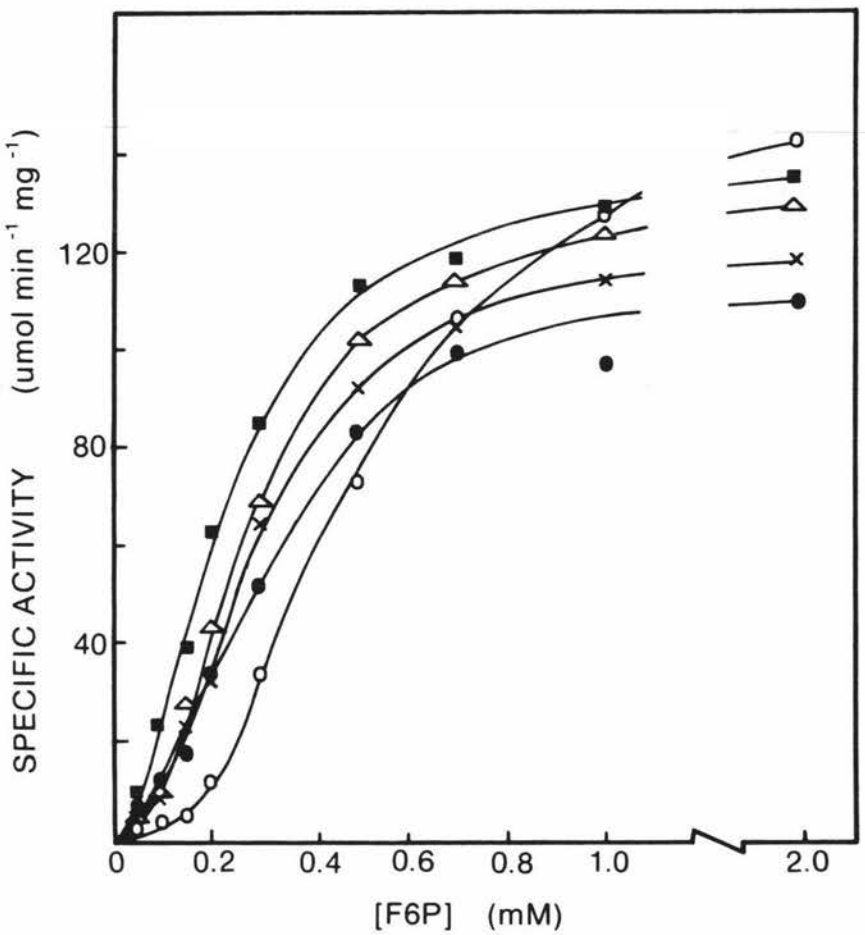


FIGURE 2.3.18 The Effect of ADP on PFK Activity at Different F6P Concentrations. Enzyme concentration: 0.2 - 0.4 µg per assay. F6P concentration ranged from 0 - 2.0 mM as indicated and MgATP was maintained at 2.0 mM.

ADP concentrations: O - Control (no ADP);
■ - 0.1 mM; △ - 1.0 mM; × - 2.0 mM;
● - 5.0 mM.

Maximum specific activities (V_{max}) were calculated from an Endrenyi plot (Endrenyi *et al*, 1975) and these values (Table 2.3.4) agreed well with the maximum velocities estimated from Figure 2.3.18. The values determined by the Endrenyi method were used in calculation of n_H and $F6P_{0.5}$ values from a Hill plot (Figure 2.3.19).

ADP is an activator of mammalian PFK (Passoneau & Lowry, 1964) and an inhibitor of plant PFK (Dennis and Coultate, 1967). Early studies on yeast PFK implied no effect of ADP on activity (Vinuela *et al*, 1964; Lindell and Stellwagen, 1968) but it was later shown that ADP stimulates yeast PFK activity in the presence of P_i or at inhibitory concentrations of ATP (Atzpodien & Bode, 1970). A similar enhancement of ADP activation in the presence of P_i was noted with mammalian PFK (Passoneau & Lowry, 1963).

ADP activation of *S. lactis* PFK is similar to the activation of *E. coli* PFK by AMP (Atkinson & Walton, 1965) i.e. ADP stimulates activity at low concentrations of F6P by increasing the affinity for F6P, but the V_{max} in the presence of ADP is lower than in the absence of ADP. AMP was an inhibitor, not an activator of *S. lactis* PFK.

Ewings and Doelle (1976) postulated that *E. coli* PFK activity may depend on the ratio of ADP:F6P. This may also be the case in *S. lactis*. Low concentrations of both ADP and F6P resulted in maximal activation. However the magnitude of the stimulation decreased as the F6P concentration was increased so that at saturating F6P concentrations (1 - 2 mM) all concentrations of ADP (0.1 - 5 mM) were inhibitory. This inhibitory effect of ADP at higher F6P concentrations may be explained by direct competition with MgATP, since the enzyme shows a high affinity for MgADP (K_m (MgADP) = 0.015 mM). However such competition would produce inhibition which was independent of F6P concentration.

ADP probably also binds at an allosteric site distinct from the catalytic site. Muscle PFK contains at least three binding sites for substrates and effectors per protomer (MW 90,000 daltons) (Kemp and Krebs, 1967; Lowry & Passoneau, 1966; Lorenson & Mansour, 1969). Effectors which act as competitive inhibitors bind to both catalytic and allosteric sites.

In spite of their smaller size, bacterial PFKs may also contain multiple binding sites. Structural studies of *B. stearothermophilus*

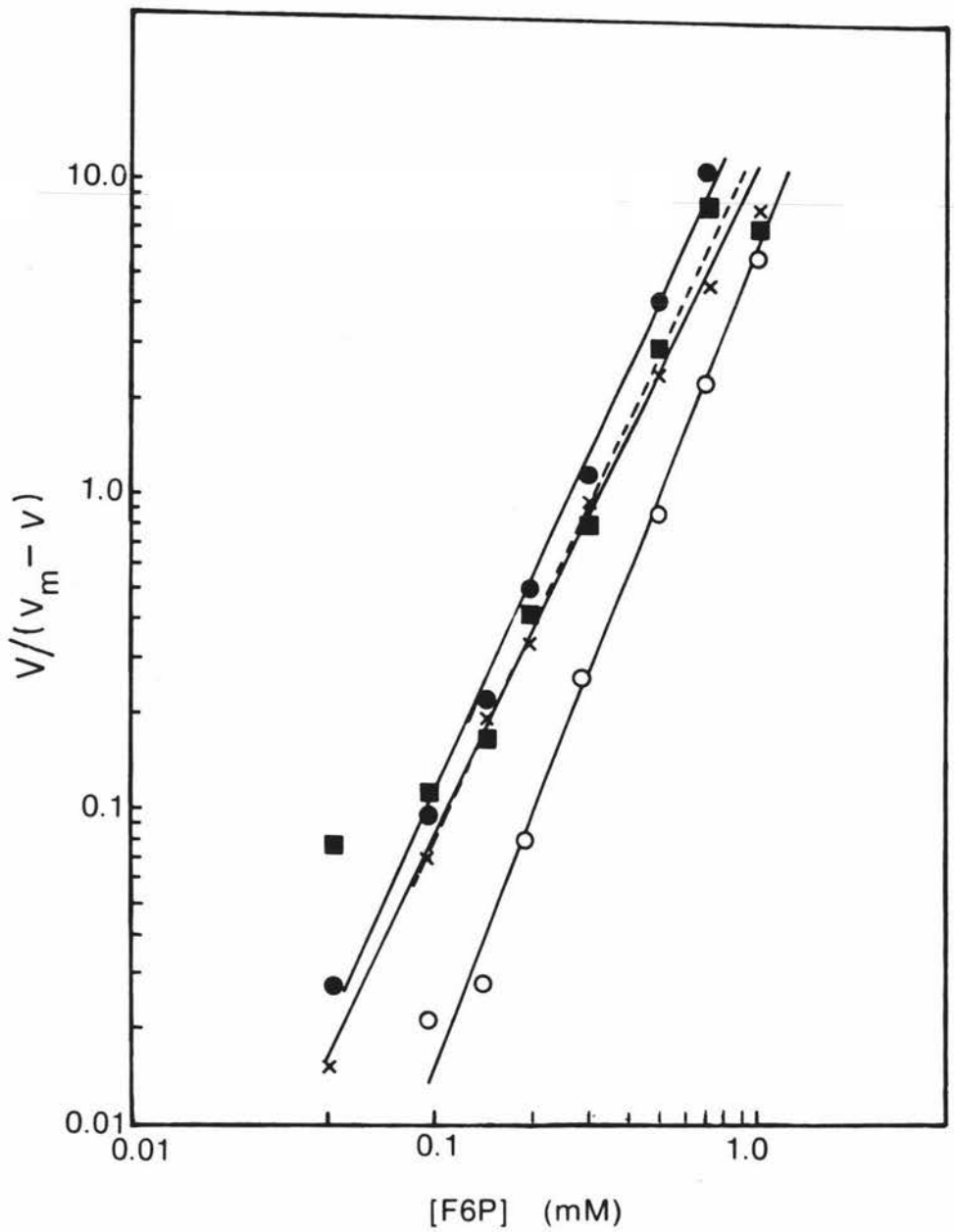


FIGURE 2.3.19 Hill Plot of the effect of ADP on PFK Activity. The effects of 0.1 mM (■), 2.0 mM (×), and 5.0 mM (O) ADP on PFK activity were studied. Hill coefficients and $F6P_{0.5}$ values determined from this graph are listed in Table 2.3.5.

The control (no ADP) is shown by the closed circles (●).

TABLE 2.3.4

THE EFFECT OF ADP ON V_{MAX}, n_H AND $F6P_{0.5}$ VALUES

[ADP] (mM)	V_{max} †	n_H i	n_H ii	$F6P_{0.5}$
0	149	2.47	2.70	0.50
0.1	142	2.53	2.82	0.24
1.0	125	2.41	2.95	0.28
2.0	127	2.06	2.13	0.32
5.0	111	1.60	2.30	0.30

† calculated from Endrenyi plot.

i " " " "

ii calculated from Hill plot.

PFK revealed two catalytic sites and one effector site per subunit (Evans & Hudson, 1969; Evans *et al*, 1981). ADP and Mg^{2+} or Mn^{2+}, and P_i were able to bind at both catalytic and effector sites.

Figure 2.3.20A shows the effect of ADP on binding of MgATP to *S. lactis* PFK. Increasing concentrations of ADP inhibited activity resulting in an increase in K_m (MgATP) (Table 2.3.5). Inhibition could be overcome by increasing the MgATP concentration, thus V_{max} was not affected by ADP. This is indicative of competitive inhibition of MgATP binding by ADP. Inhibition may be due either to competition for a single binding site on the enzyme, or to competition for Mg^{2+}. The double reciprocal plot (Figure 2.3.20B) also indicates competitive inhibition.

The effect of increasing ADP concentrations was studied at 2.0 mM MgATP and 0.25 mM F6P, an F6P concentration at which ADP stimulated activity. Increasing ADP concentration resulted in a hyperbolic saturation curve, reaching maximum velocity at approximately 0.2 mM ADP (Figure 2.3.21A). A K_m MgADP of 0.015 mM was calculated from the double reciprocal plot in Figure 2.3.21B.

2.3.13 The Effect of T6P on PFK Activity

The effect of T6P on PFK activity with F6P is shown in Figure 2.3.22. In the absence of T6P, PFK exhibits a sigmoidal dependence on F6P concentration. Addition of T6P (0.2 mM - 2.0 mM) to the assay results in a shift towards a more hyperbolic curve, and the double reciprocal plot (Figure 2.3.23) indicates competitive inhibition of PFK by T6P. In assays containing 0.2 mM and 0.5 mM T6P, the double reciprocal plots are linear only at higher concentrations of F6P as the activity vs concentration curves still exhibit some sigmoidicity.

A plot of $\frac{1}{V}$ vs [T6P] (Figure 2.3.24B) shows a pattern consistent with competitive inhibition and gives a K_i for T6P of 1.75 mM. Table 2.3.6 lists the Hill coefficients and $F6P_{0.5}$ values obtained at different inhibitor (T6P) concentrations.

2.3.14 Phosphorylation of T6P by PFK

Mammalian PFK can phosphorylate T6P (the epimer at C_4 of F6P) (Totton & Lardy, 1949). However unlike mammalian species the group N streptococci possess a T6P kinase in addition to PFK (see Chapters

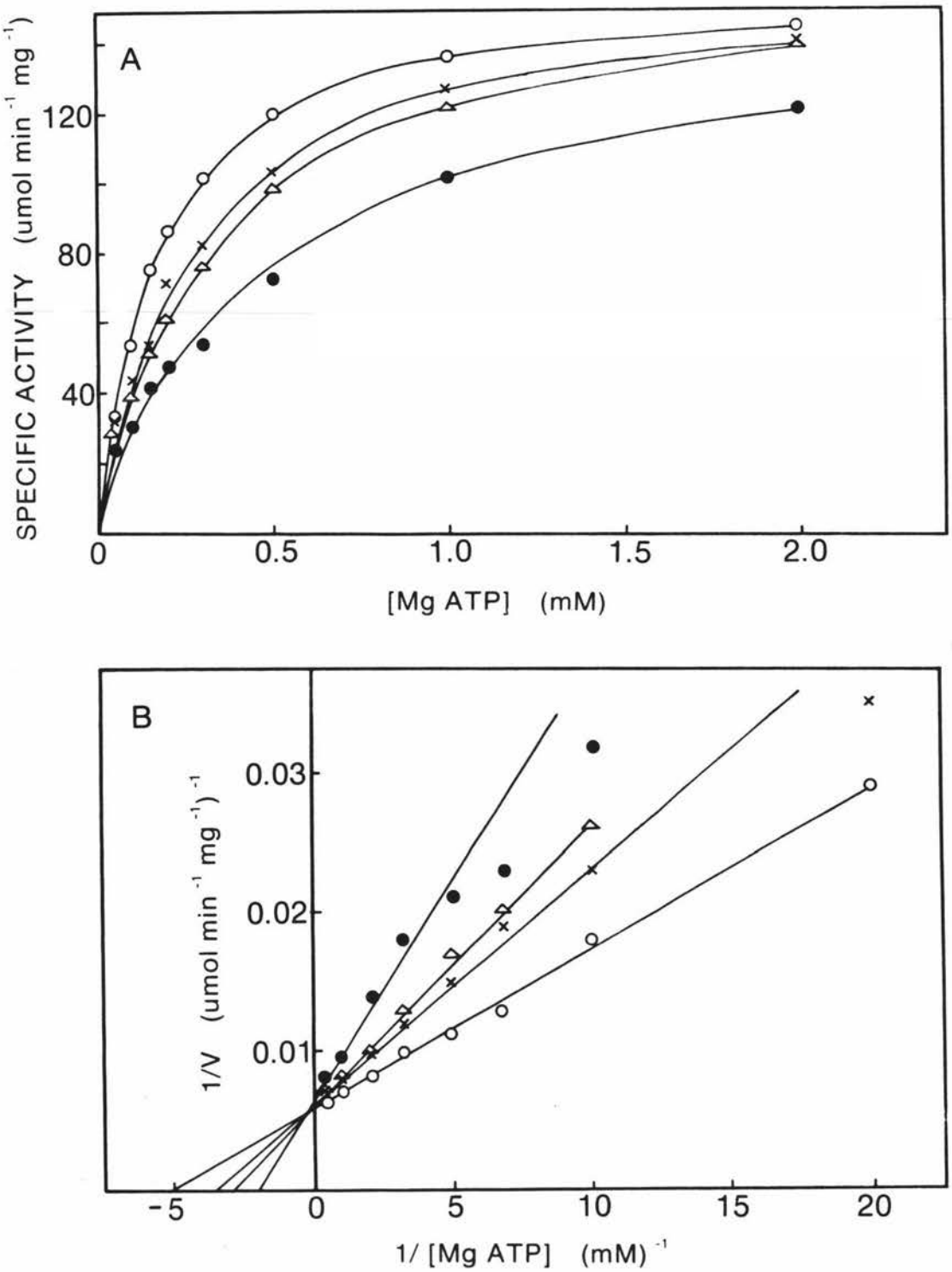


FIGURE 2.3.20 The Effect of ADP on PFK Activity at Different ATP Concentrations. Enzyme concentration: 0.2 - 0.4 µg per assay.

MgATP concentration ranged from 0 - 2.0 mM as indicated and F6P was maintained at 2.0 mM.

ADP concentrations: 0 mM (Control) - O ; 1 mM - × ;
2 mM - △ ; 5 mM - ● .

Data from Figure A are plotted as a double reciprocal plot in Figure B.

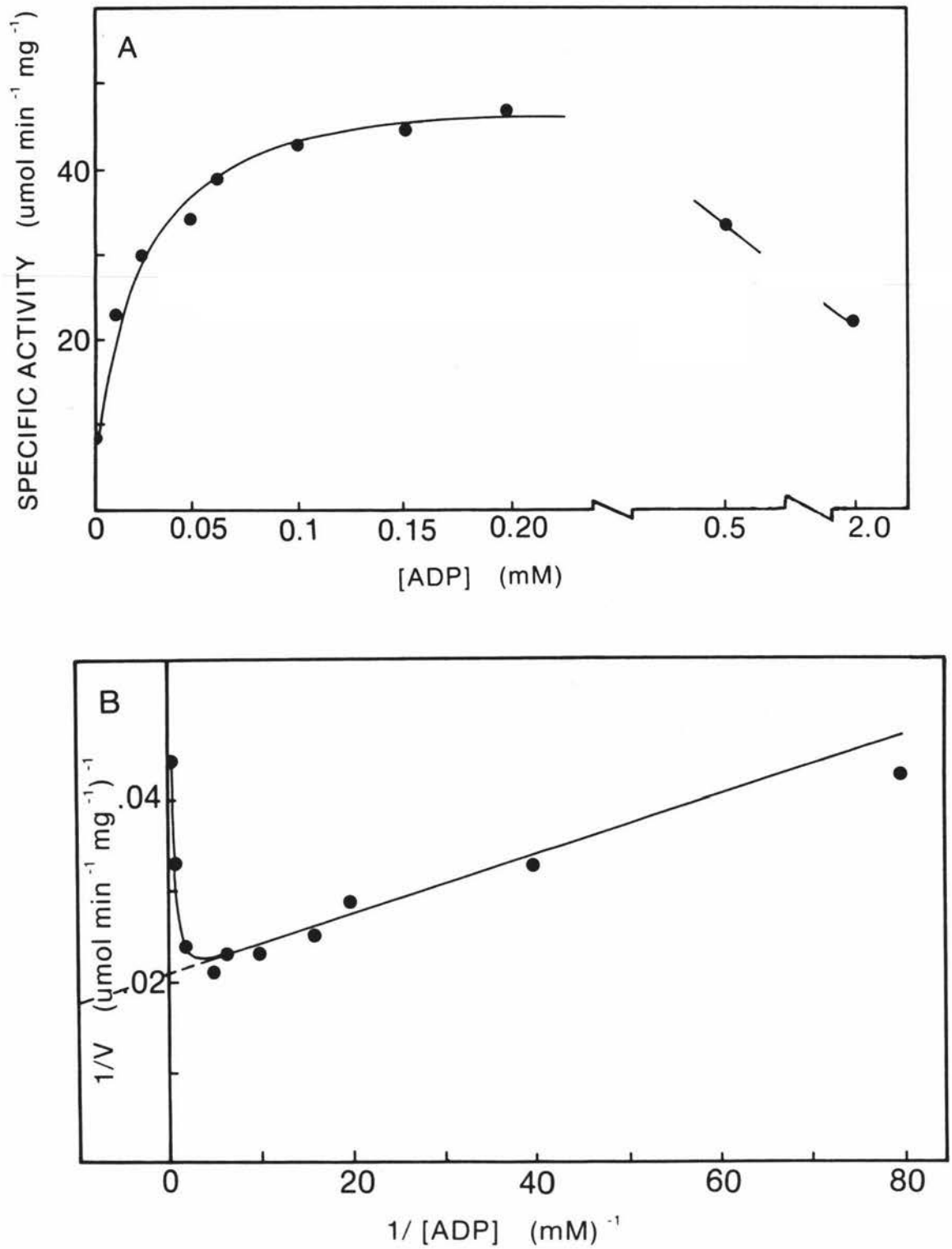


FIGURE 2.3.21 The Effect of Increasing Concentrations of ADP on PFK Activity. PFK (0.21 µg/assay) was assayed at 2.0 mM MgATP and 0.15 mM F6P, an F6P concentration at which ADP stimulated activity (see Figure 2.3.18).

Figure A shows the effect of increasing concentrations of ADP from 0 - 5.0 mM.

From the double reciprocal plot in Figure B, a K_i (ADP) of approximately 0.02 mM was calculated.

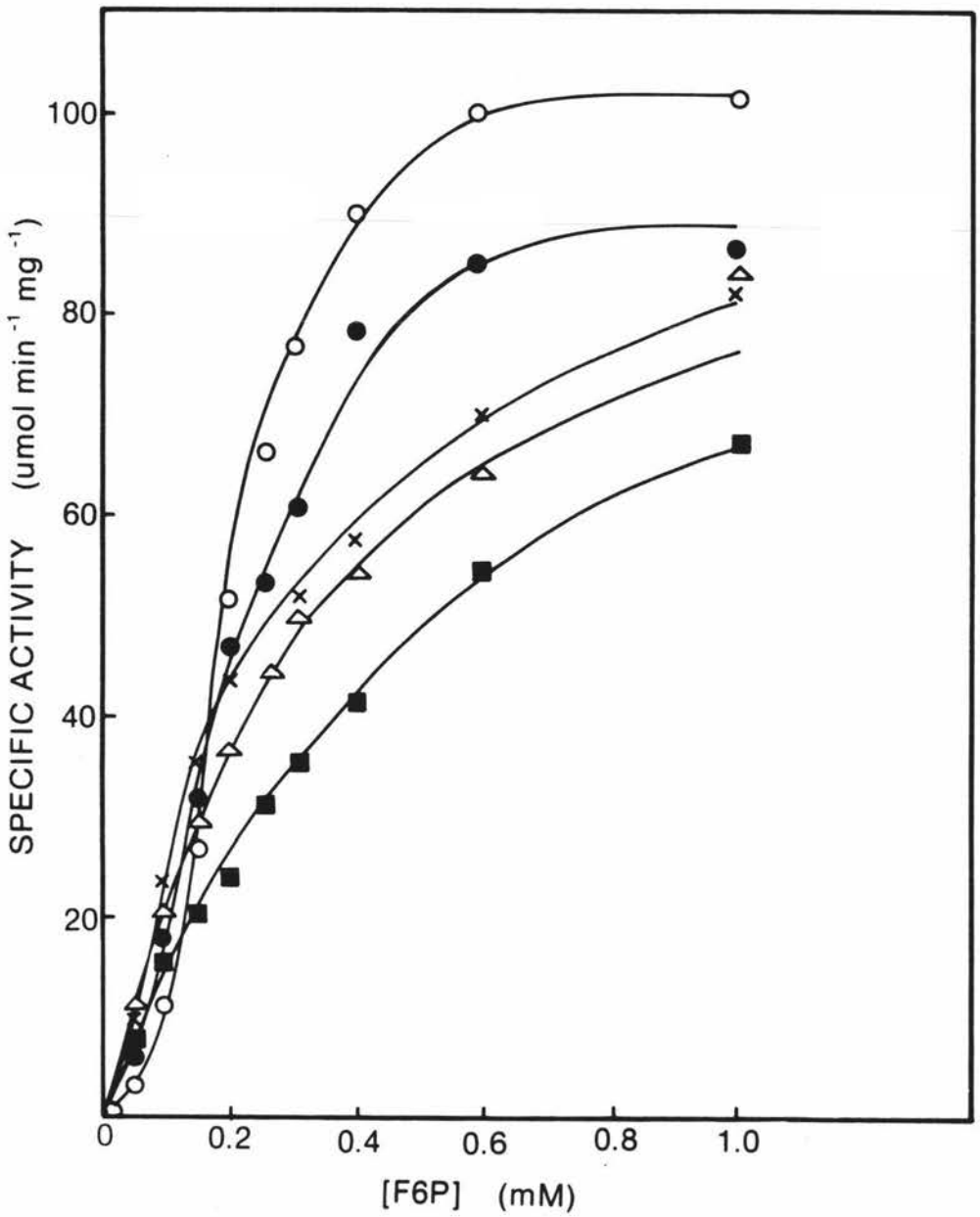


FIGURE 2.3.22 The Effect of T6P on PFK Activity. PFK was assayed for activity with F6P (0 - 1.0 mM as indicated) in the presence of increasing concentrations of T6P. MgATP concentration = 2.0 mM in all assays. Enzyme concentration = 0.21 µg per assay.

T6P concentrations: 0 (Control) - ○ ; 0.2 mM - ● ; 0.5 mM - × ; 1.0 mM - △ ; 2.0 mM - ■ .

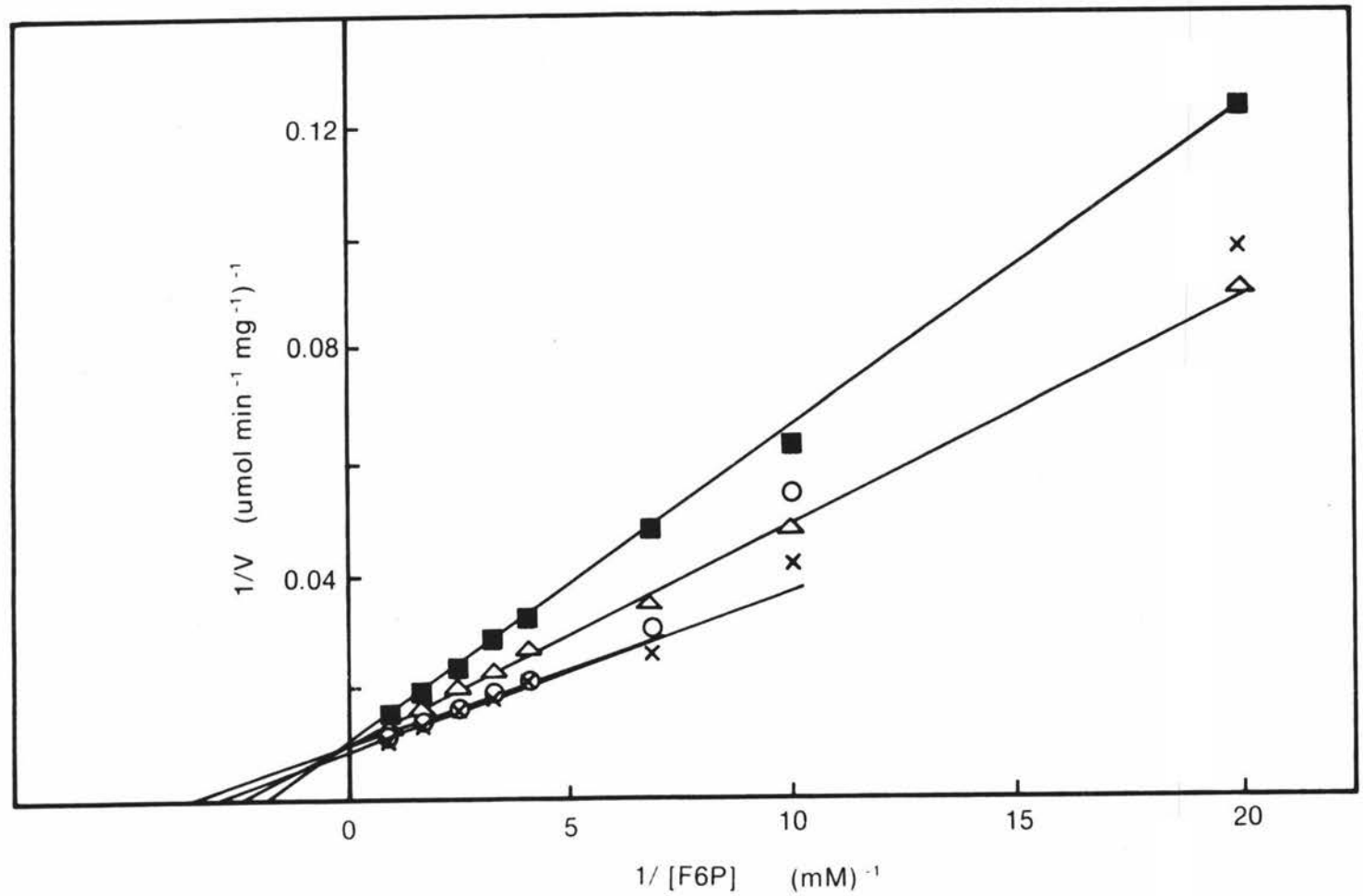


FIGURE 2.3.23 Lineweaver-Burk Plot : The Effect of T6P on PFK Activity. This graph shows the data from Figure 2.3.22 plotted as a double reciprocal plot.

T6P concentrations: ○ - 0 mM; × - 0.5 mM; △ - 1.0 mM; ■ - 2.0 mM :

Intersection of the lines on the ordinate suggests competitive inhibition of PFK activity by T6P.

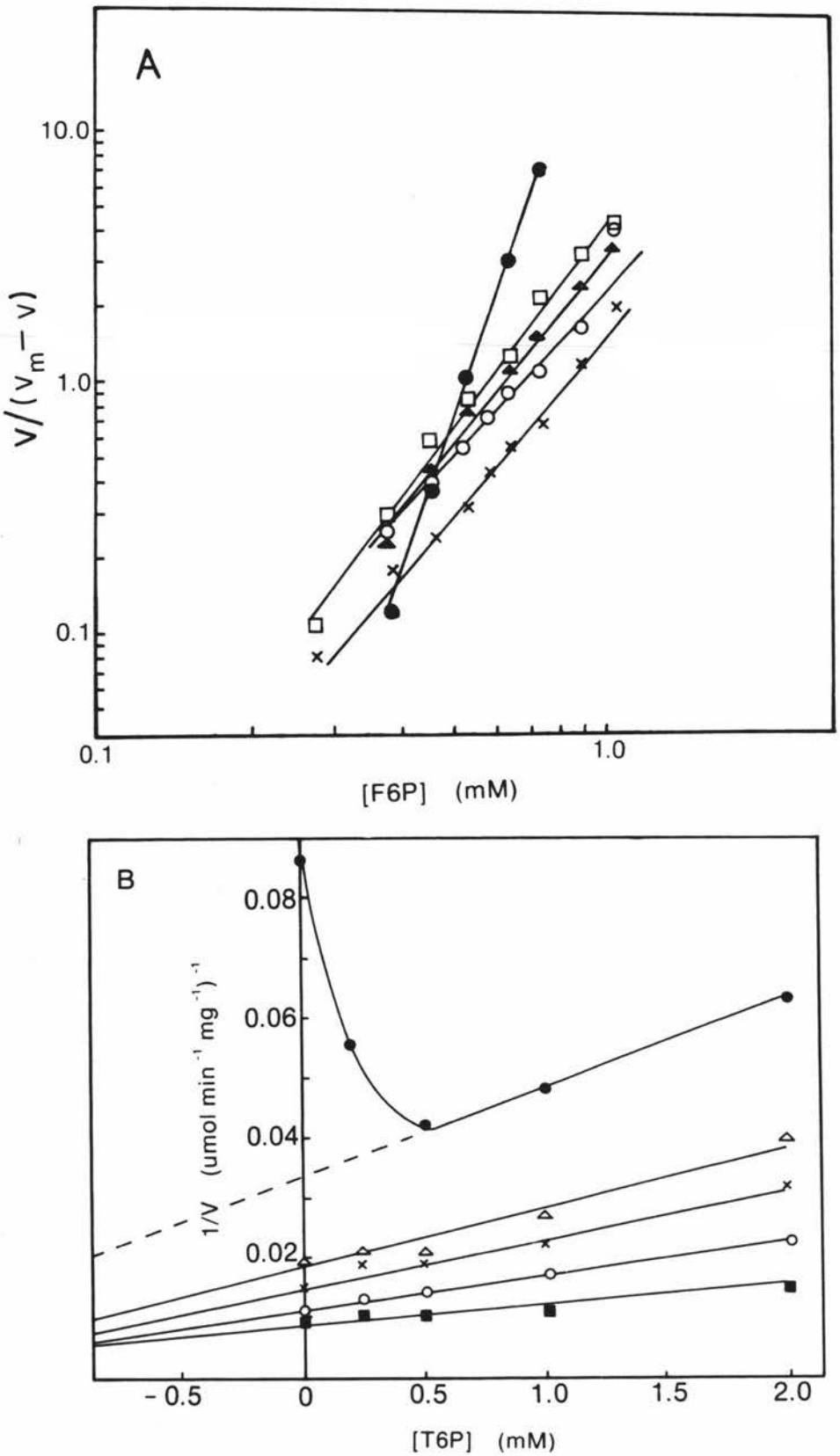


FIGURE 2.3.24 The Effect of T6P on PFK Activity. Figure A shows the Hill plots of data from Figure 2.3.22.

0 T6P - ● ; 0.2 mM T6P - □ ; 0.5 mM T6P - ▲ ; 1.0 mM T6P - ○ ; 2.0 mM T6P - × .

A K_i for T6P of 1.75 mM was determined by plotting $\frac{1}{V}$ vs [T6P] (Figure B).

The lines represent F6P concentrations of 0.10 mM (●); 0.20 mM (△); 0.25 mM (×); 0.40 mM (○); and 0.8 mM (■) F6P.

TABLE 2.3.5

THE EFFECT OF ADP ON n_H AND K_m (MgATP)

[ADP] (mM)	n_H	K_m(MgATP)(mM)
0	1.24	0.16
1.0	1.20	0.22
2.0	1.10	0.27
5.0	0.86	0.43

n_H and K_m (MgATP) values were calculated from a Hill Plot of the data in Figure 2.3.20A (data not shown). A V_{max} of 152 µmol min^{-1} mg, calculated from the double reciprocal plot in Figure 2.3.20B was used in all calculations.

TABLE 2.3.6

THE EFFECT OF T6P ON n_H AND $F6P_{0.5}$

[T6P] (mM)	n_H	$F6P_{0.5}$ (mM)
0	2.90	0.21
0.2	1.74	0.23
0.5	1.38	0.25
1.0	1.19	0.31
2.0	1.14	0.51

n_H and $F6P_{0.5}$ values were calculated from a Hill plot (Figure 2.3.24A). A V_{max} of 104 µmol F6P min^{-1} mg^{-1} was used in all calculations.

1 and 3) so it was of interest to determine whether the purified *S. lactis* PFK also showed any activity towards T6P.

Both F6P and T6P were phosphorylated by purified *S. lactis* PFK. As with F6P, PFK showed a sigmoidal dependence on T6P concentration (Figure 2.3.25). However the maximum specific activity (22 µmol T6P min^{-1} mg^{-1}) was only 16% of the maximum activity obtained with F6P as substrate (160 µmol F6P min^{-1} mg^{-1}). The Hill coefficient was 2.69, compared with a value of 3.0 - 3.7 obtained with F6P as substrate, and the $T6P_{0.5}$ (2.82 mM), (Figure 2.3.25, inset) is approximately 10-fold higher than the $F6P_{0.5}$ (0.25 mM).

Phosphorylation of T6P by *S. lactis* PFK is not unexpected as competitive inhibition of PFK activity by T6P (Section 2.3.13) suggests that both substrates bind at the same site. PFKs generally do not show absolute specificity for sugar phosphate substrate; the enzymes from most higher organisms are able to phosphorylate a variety of sugar phosphates. *S. lactis* PFK appears to be similar in this respect as both F6P and T6P were phosphorylated.

Few bacterial PFKs have been assayed for their ability to phosphorylate T6P, partly because of the relatively recent discovery of the participation of T6P in microbial carbohydrate metabolism and because of the unavailability of T6P commercially. The non-allosteric PFK of *E. coli* has been shown to phosphorylate T6P (Babul, 1978) at a rate (20% of the maximum rate of F6P phosphorylation) comparable to the rate of activity of *S. lactis* PFK with T6P (16% of the maximum rate of F6P phosphorylation). However the non-allosteric enzyme of *E. coli* is immunologically distinct from the allosteric PFK in the same organism, and exhibits different kinetic properties to the latter enzyme. In most respects *S. lactis* PFK appears to be more closely related to the allosteric PFK of *E. coli*.

2.3.15 The Effect of PEP on PFK Activity

The effect of PEP on the binding of F6P to PFK was studied (Figure 2.3.26). PEP inhibited PFK activity at concentrations greater than 1.0 mM, increasing concentrations of PEP shifting the sigmoidal saturation curve to the right. This shift resulted in an increase in $F6P_{0.5}$, but little change in n_H (Table 2.3.7). PEP exhibited a greater inhibitory effect at non-saturating F6P concentrations than at non-saturating MgATP concentrations (Table 2.3.3), 10 mM PEP

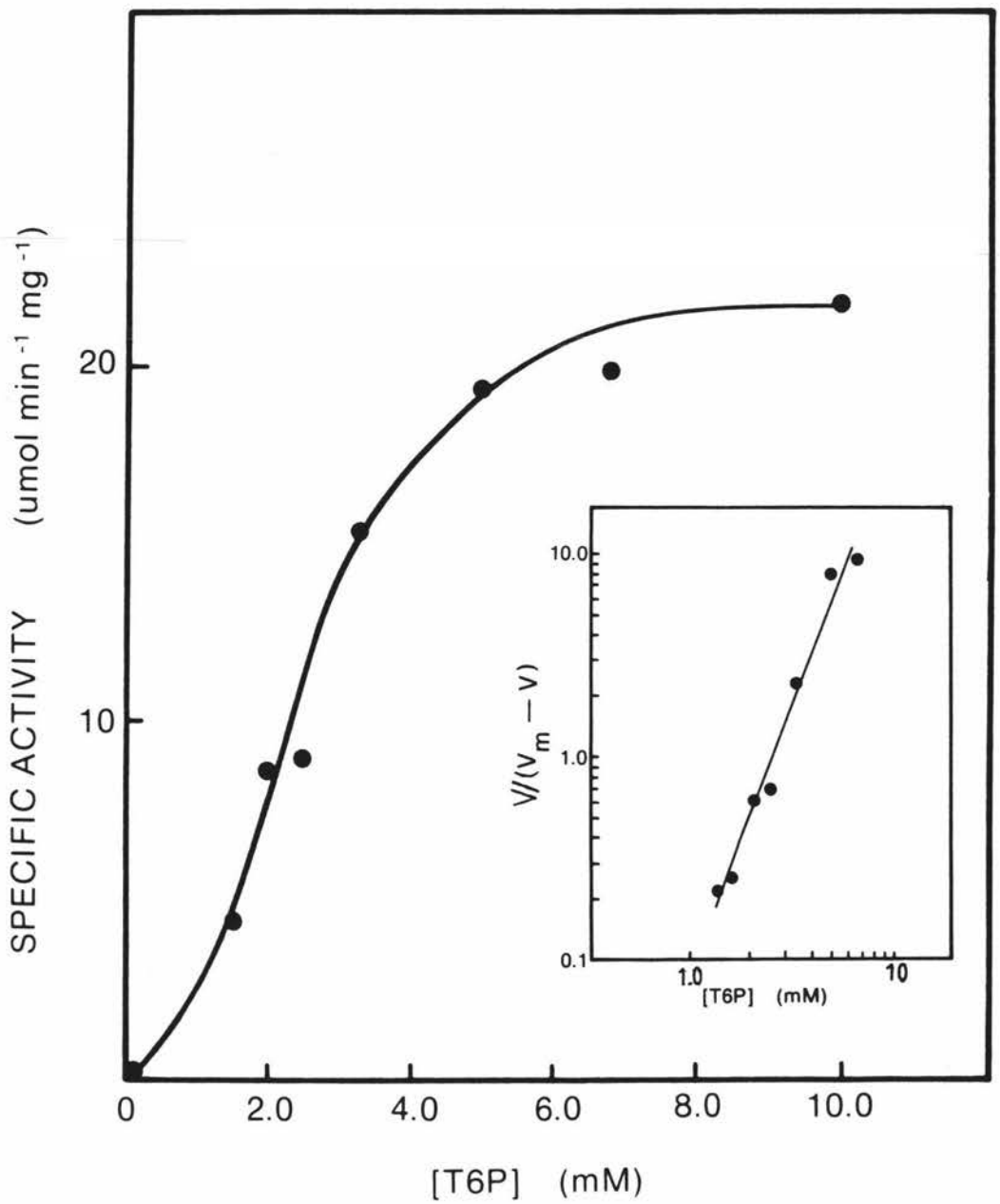


FIGURE 2.3.25 Phosphorylation of T6P by PFK. PFK was assayed at 2.0 mM MgATP and 0 - 1.0 mM T6P using the TBP aldolase assay system described for T6PK in Chapter 3.

Enzyme concentration: 4.2 µg per assay.

A Hill plot of the data (inset) indicates a n_H of 2.69, and a $T6P_{0.5}$ of 2.82 mM.

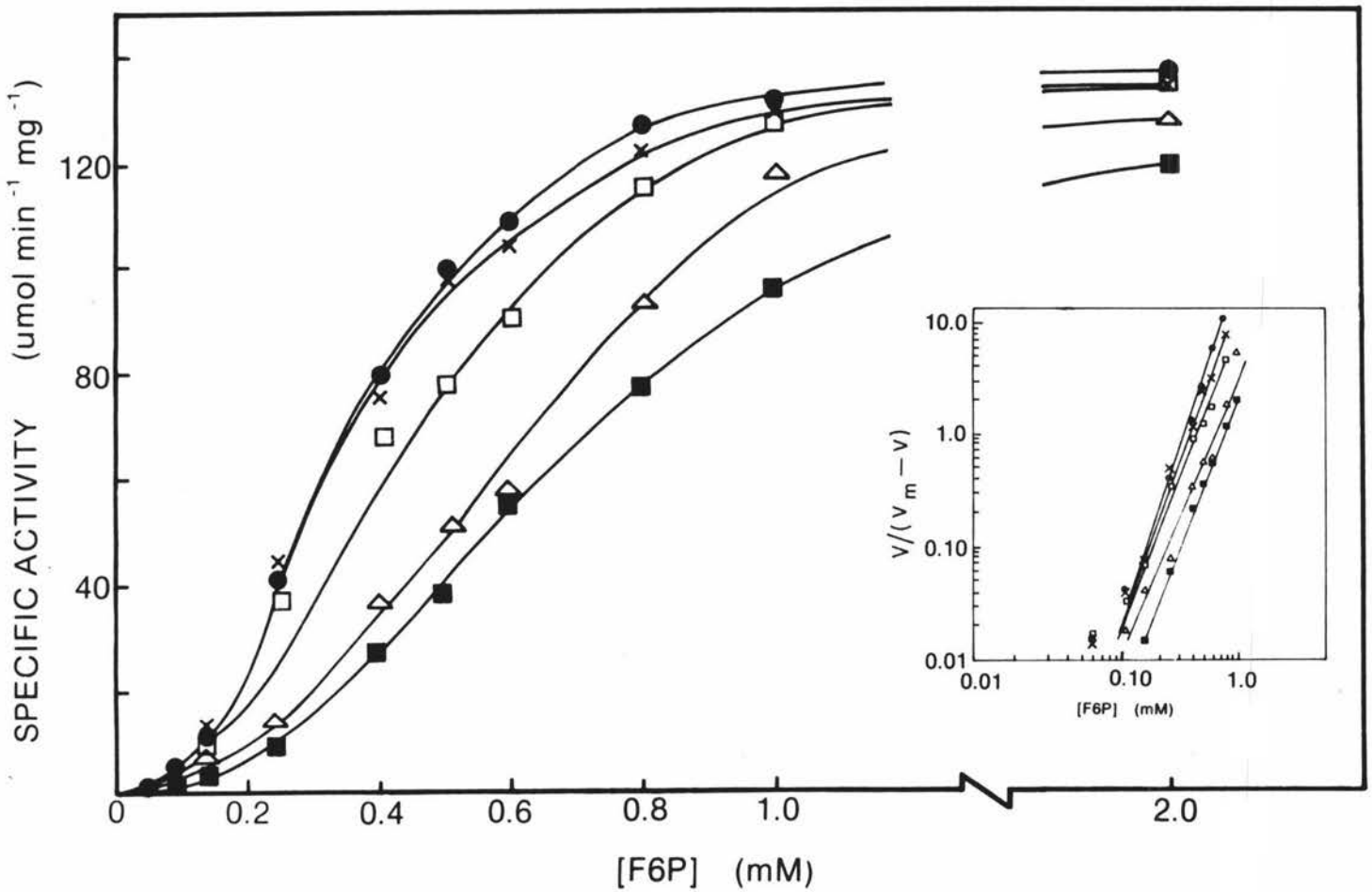


FIGURE 2.3.26 The Effect of PEP on PFK Activity. Increasing concentrations of PEP resulted in a shift of the sigmoidal F6P saturation curve to the right. All assays contained 0.21 µg purified PFK and 2.0 mM MgATP.

The inset figure shows the effect of PEP on the Hill plots.

PEP concentrations: ● - 0 mM; × - 0.5 mM; □ - 1.0 mM; △ - 2.5 mM; and ■ - 5.0 mM.

TABLE 2.3.7

THE EFFECT OF PEP CONCENTRATION ON THE $F6P_{0.5}$ AND HILL COEFFICIENT

[PEP] (mM)	n_H	$F6P_{0.5}$ (mM)
0	2.98	0.34
0.5	2.60	0.37
1.0	2.28	0.42
2.5	2.31	0.60
5.0	2.42	0.72

n_H and $F6P_{0.5}$ were calculated from Hill plots (Figure 2.3.26 Inset). A V_{max} value of 136 µmol F6P min^{-1} mg^{-1} was used in all calculations.

causing 94% inhibition at non-saturating F6P (0.15 mM) but only 44% inhibition at non-saturating MgATP (0.1 mM).

PEP in bacterial cells has been postulated to play a similar role to that of citrate in mammalian systems i.e. PEP is the product of glycolysis which is largely responsible for feedback inhibition of PFK in order to regulate flux through the EMP pathway (Boiteaux & Hess, 1981).

The PEP concentration in actively glycolysing cells of *S. lactis* is approximately 3.0 mM (Thompson, 1978). However on depletion of carbohydrate the concentration of PEP increases 4-5 fold (Thompson, 1978; Thompson & Thomas, 1977). *S. lactis* PFK is not significantly inhibited by PEP concentrations less than 2.5 mM, thus in growing cells PFK activity would be unaffected by the concentration of PEP in the cell. The lower PEP inhibition at low MgATP concentrations (Table 2.3.3) is also expected since when MgATP is low, PFK activity would be expected to be maximal to favour synthesis of ATP by glycolysis.

Although an increase in PEP concentration may have little effect on PFK activity at high concentrations of F6P, in starved cells (when F6P concentrations would be low) the intracellular concentration of PEP ($\sim$ 13 mM) would effectively inhibit PFK activity (5 mM PEP is sufficient to cause 79% - 100% inhibition at F6P concentrations below the $F6P_{0.5}$ value).

PEP did not affect the co-operativity of binding of F6P to *S. lactis* PFK; Hill coefficients of 2.28 - 2.98 were obtained with 0 - 5 mM concentrations of PEP. In contrast PEP abolishes the co-operative binding of F6P to *E. coli* PFK, and introduces co-operativity of binding in *Flavobacterium thermophilum* (Yoshida, 1972) and pea seed PFK (Kelly & Turner, 1969).

2.3.16 The Effect of AMP on PFK Activity

AMP inhibited *S. lactis* PFK in a similar manner to PEP (Section 2.3.15) and FBP (Section 2.3.17). Increasing concentrations of AMP shifted the sigmoidal F6P binding curve to the right (Figure 2.3.27) resulting in an increase in $F6P_{0.5}$ but little change in n_H (Table 2.3.8).

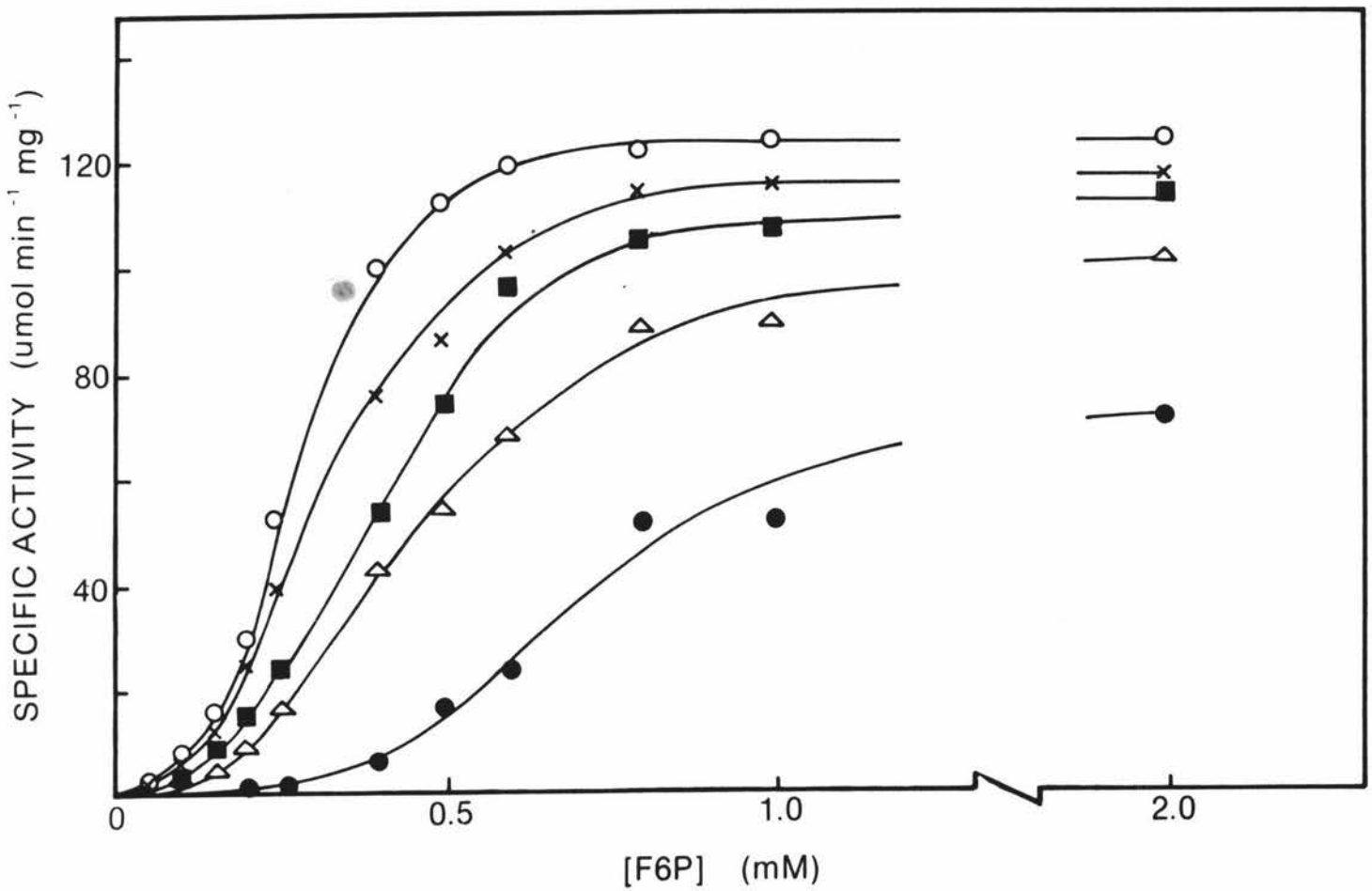


FIGURE 2.3.27 The Effect of AMP on PFK Activity. Inhibition of PFK by AMP is shown in this figure. PFK was assayed at 2.0 mM MgATP and at 0 - 2.0 mM F6P.

AMP concentrations: 0 mM - ○ ; 1.0 mM - × ; 2.5 mM - ■ ; 5.0 mM - △ ; 10.0 mM - ● .

Enzyme concentration: 0.2 - 0.4 µg per assay.

TABLE 2.3.8

THE EFFECT OF AMP ON $F6P_{0.5}$ AND HILL COEFFICIENT

[AMP] (mM)	$F6P_{0.5}$ (mM)	n_H
0	0.26	3.80
1.0	0.33	2.74
2.5	0.40	2.86
5.0	0.52	2.58
10.0	0.92	3.20

$F6P_{0.5}$ and n_H values were calculated from Hill plots of the data shown in Figure 2.3.27. A V_{max} value of 124 µmol F6P min^{-1} mg^{-1} was used in all calculations.

AMP has a variable effect on bacterial PFKs. The enzymes from *S. aureus, Clostridium perifringens, Aerobacter aerogenes, Lactobacillus plantarum*, and plants, are inhibited by AMP (Lowry & Passoneau, 1964; Dennis & Coultate, 1967; Kelly & Turner, 1969; Sapico & Anderson, 1969; Doelle, 1972). Activities of PFKs from *E. coli* and *Lactobacillus casei*, however, are not affected (Blangy *et al*, 1968; Doelle, 1972). Dennis and Coultate (1967) suggested that inhibition by AMP (and ADP) may not be significant *in vivo*, as although both compounds inhibited PFK *in vitro*, PFK was also strongly activated by P_i which would be produced simultaneously with ADP and AMP.

2.3.17 The Effect of FBP on PFK Activity

FBP inhibits PFK activity, 5 - 10 mM concentrations causing up to 90% inhibition depending on the F6P and MgATP concentrations. Low concentrations ($\leq$2 mM) cause little inhibition. As the intracellular concentration of FBP may be as high as 25 mM (Thomas *et al*, 1979) the partial inhibition by 5 - 10 mM FBP may be significant *in vivo*.

FBP lowers the maximum velocity of the PFK reaction (Figures 2.3.28A and 2.3.29A) and shifts the sigmoidal saturation curve to the right. The $F6P_{0.5}$ is increased slightly but n_H is not greatly affected (Table 2.3.9). Non competitive inhibition is indicated by the intersection on the abscissa of the lines in the double reciprocal plot in Figure 2.3.29B.

Recent work has shown that fructose 2, 6-bisphosphate is also an activator of PFK in mammalian tissues (Claus & Schlumpf, 1981; Furuya & Uyeda, 1981; Pilkis *et al*, 1981). This compound is a more potent activator of PFK than fructose 1, 6-bisphosphate (Pilkis *et al*, 1981) but as yet its origin and its role in the regulation of PFK is unknown.

2.3.18 The Effect of Monovalent Cations on PFK Activity

The effects of monovalent cations on PFK activity are shown in Figures 2.3.30A and B at saturating concentrations of F6P (2.0 mM) and at saturating (2.0 mM) and non-saturating (0.1 mM) concentrations of MgATP respectively. (All ions tested showed a similar effect at both saturating and non-saturating F6P concentrations.) Na^+ had

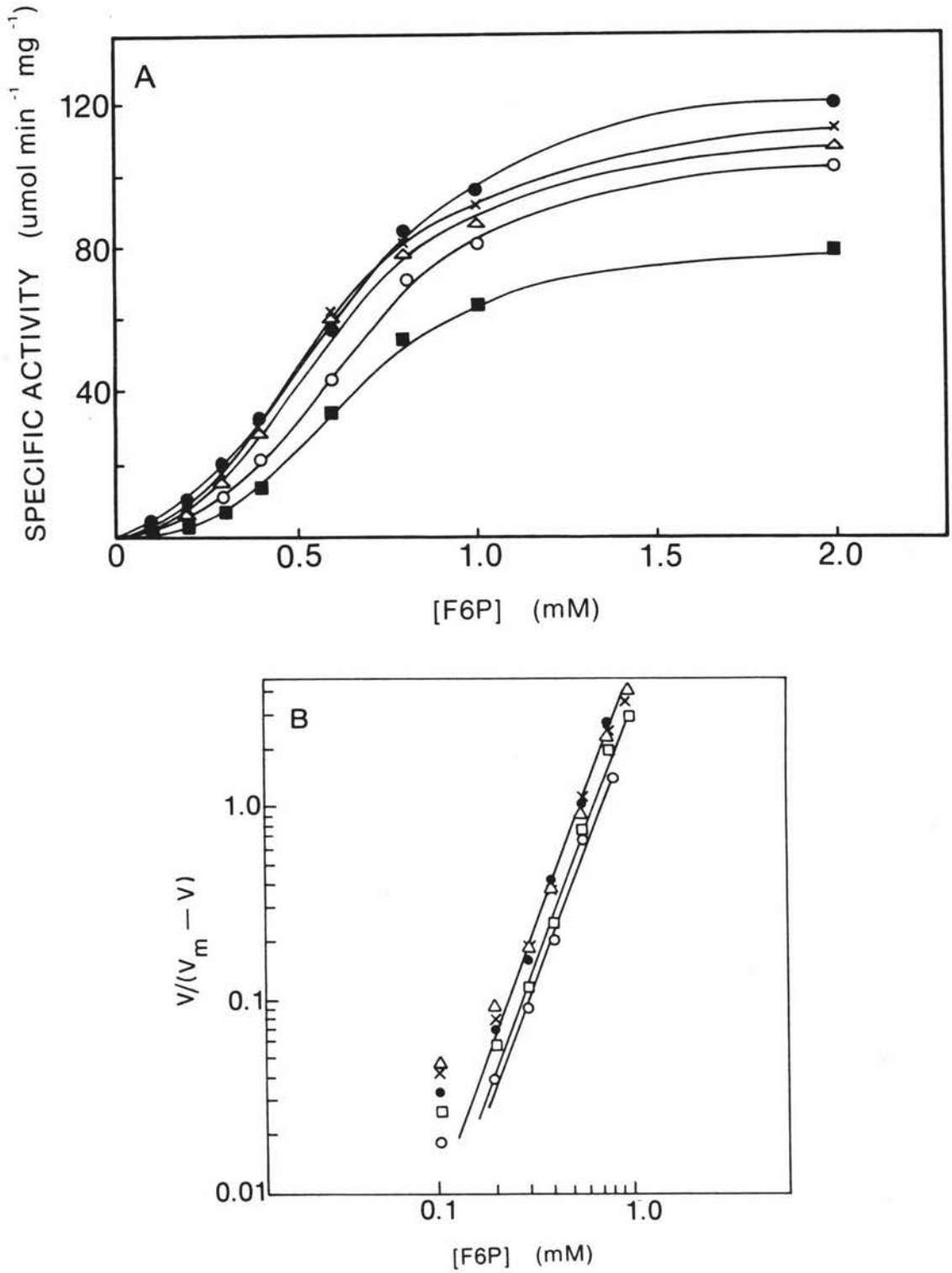


FIGURE 2.3.28 The Effect of FBP on PFK Activity at Different F6P Concentrations. The effect of FBP on PFK activity was studied at 2.0 mM MgATP and 0 - 2.0 mM F6P (Figure A).

FBP concentrations: 0 mM (Control) - ● ; 1.0 mM - × ; 2.0 mM - △ ; 5.0 mM - ○ ; 10.0 mM - ■ .

Figure B shows the Hill plots used in calculating the n_H and $F6P_{0.5}$ values listed in Table 2.3.9.

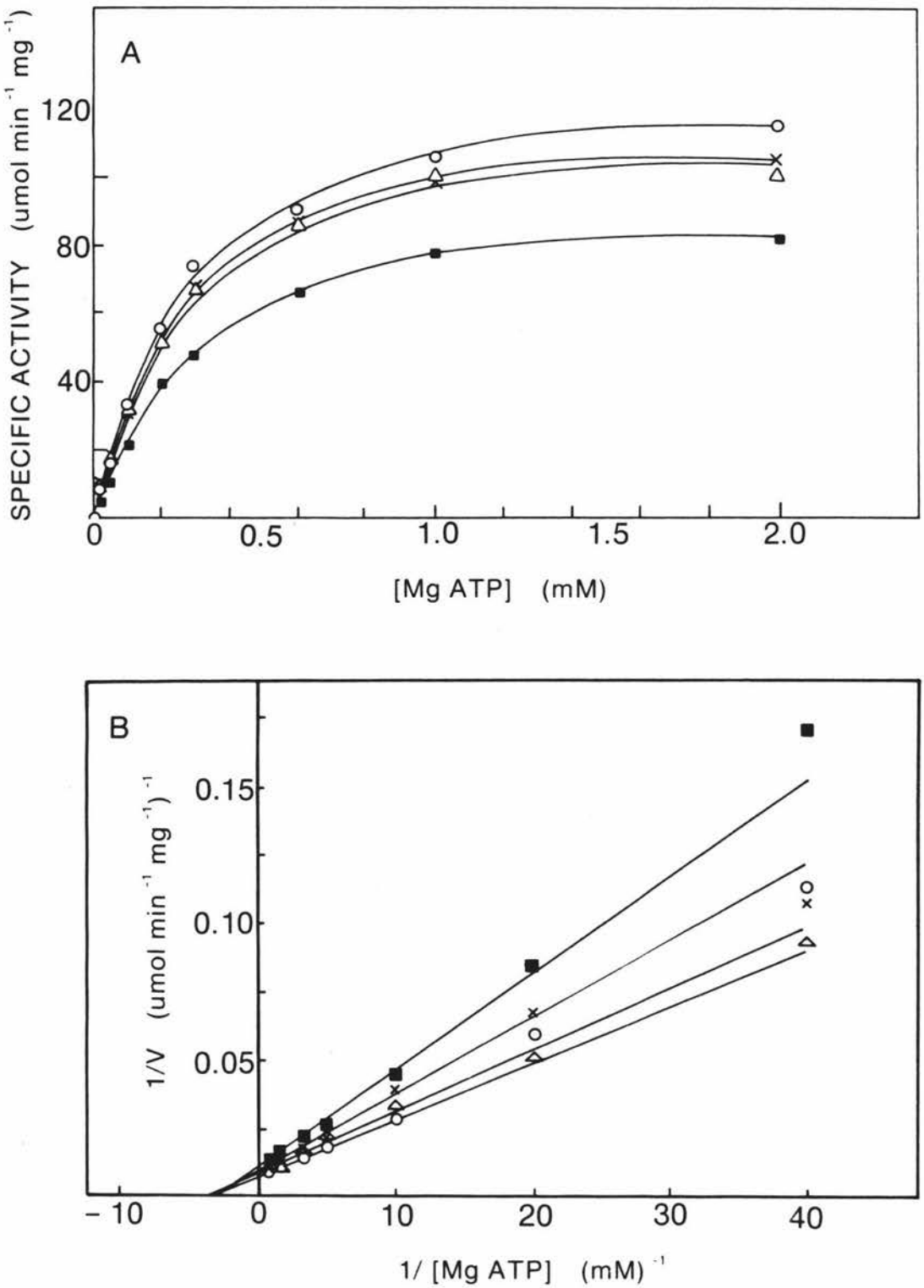


FIGURE 2.3.29 The Effect of FBP on PFK Activity at Different MgATP Concentrations. PFK assays were performed using 0.2 or 0.4 µg protein per assay, at 2.0 mM F6P and 0 - 2.0 mM MgATP as indicated, and with increasing concentrations of FBP in the assay.

Symbols: 0 mM FBP (Control) - O ; 1.0 mM FBP - △ ;
2.0 mM FBP - × ; 10.0 mM FBP - ■ .

Data from Figure A is plotted as a double reciprocal plot in Figure B, which indicates non-competitive inhibition of PFK activity by FBP.

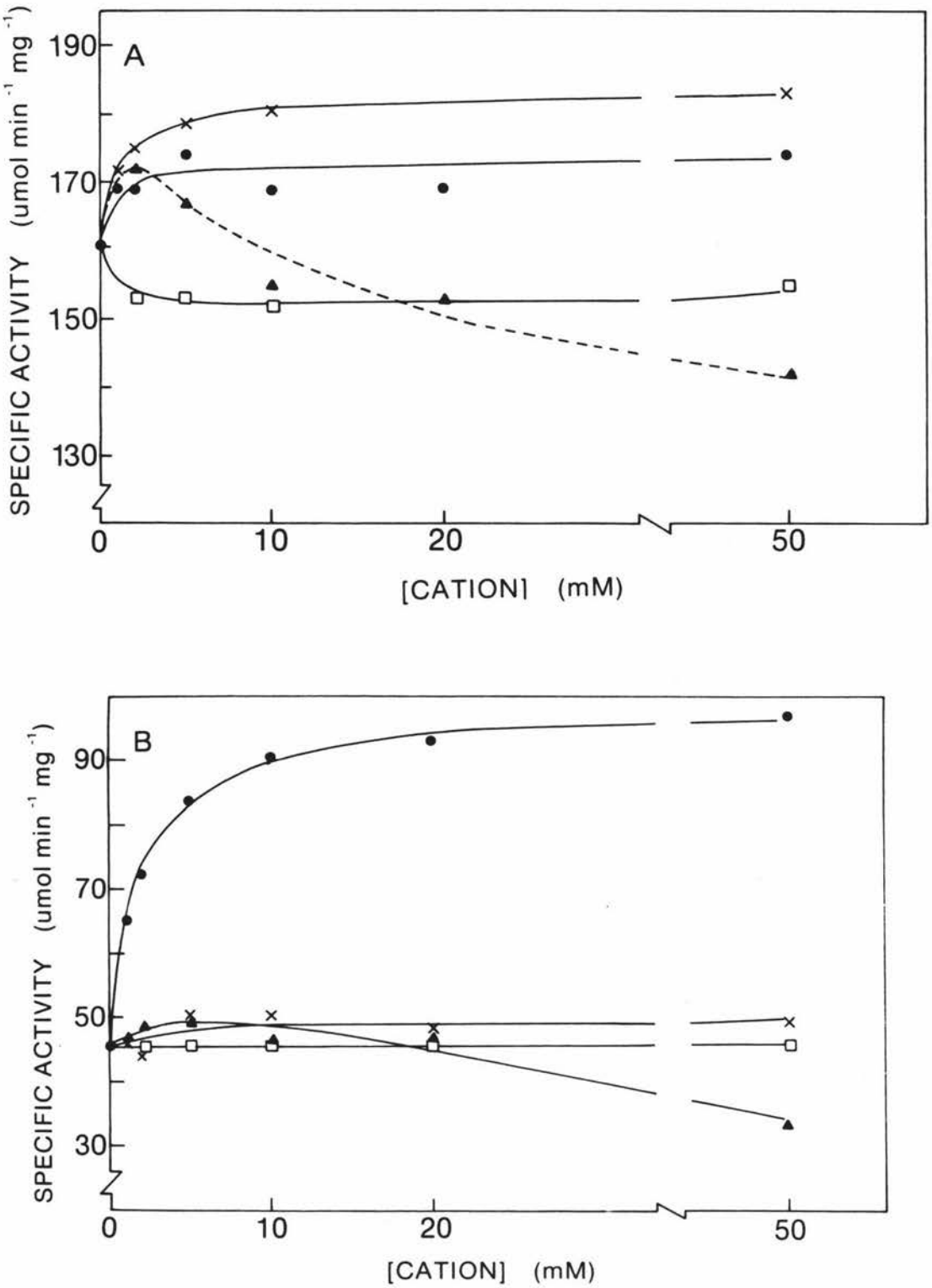


FIGURE 2.3.30 The Effect of Monovalent Cations on PFK Activity. The effect of Monovalent Cations on PFK activity is shown at saturating F6P (2.0 mM) with saturating (2.0 mM) ATP (Figure A) and non-saturating (0.2 mM) ATP (Figure B) concentrations.

Symbols: ● - NH_4^+; × - K^+; □ - Na^+; ▲ - Li^+.

TABLE 2.3.9

THE EFFECT OF FBP ON $F6P_{0.5}$ AND HILL COEFFICIENT

[FBP] (mM)	$F6P_{0.5}$ (mM)	n_H
0	0.58	2.56
1	0.58	2.56
2	0.56	2.62
5	0.65	2.77
10	0.66	2.86

$F6P_{0.5}$ and n_H values were determined from a Hill plot of the data in Figure 2.3.28A (Figure 2.3.28B). The V_{max} value used was the activity (µmol F6P min^{-1} mg^{-1}) at 2.0 mM F6P for each concentration of FBP.

little effect at either MgATP concentration. Li^+ stimulated activity slightly ($\leqslant$10% activation) at concentrations less than 5 mM, above which Li^+ became inhibitory, causing 25% and 12% inhibition at 0.1 mM and 2.0 mM MgATP respectively.

Both NH_4^+ and K^+ enhanced PFK activity. Activation by NH_4^+ was most marked at non-saturating concentrations of MgATP, whereas K^+ did not affect activity at the concentration of MgATP used in this experiment. At saturating concentrations of MgATP, stimulation of activity by K^+ was greater than by NH_4^+. The K_m (NH_4^+) determined from the double reciprocal plot in Figure 2.3.31 was 0.53 mM. The double reciprocal plot for K^+ was not linear but the small increases in activity with correspondingly large increases in K^+ concentration suggest a low K_m value for K^+.

Monovalent cations affect activity of many enzymes (Suelter, 1974) and in general NH_4^+, K^+, Rb^+ and Cs^+ are activators, while Na^+ and Li^+ are inhibitors, as was found for *S. lactis* PFK. Unlike Mg^{2+}, monovalent cations are thought to act by forming enzyme-cation rather than substrate-cation complexes.

Stellwagen & Thompson (1979) have shown that K^+ is necessary for substrate-binding co-operativity in *Thermus* PFK, and NH_4^+ is an absolute requirement for PFK activity in *Dictyostelium discoideum* (Baumann and Wright, 1968) and *Clostridium perifringens* (Uyeda & Kurooka, 1970). However neither the activity nor the co-operativity of *S. lactis* PFK appeared to be absolutely dependent on monovalent cations.

2.3.19 The Effect of P_i on PFK Activity

P_i inhibition of *S. lactis* PFK activity was suggested when it was noted that specific activities of PFK in 50 mM phosphate buffer were consistently lower than in other buffers. Preliminary experiments showed that inhibition by P_i was greater at non-saturating F6P concentrations than at non-saturating MgATP concentrations or at saturating concentrations of both substrates. The effect of P_i on the F6P binding curve was therefore studied (Figure 2.3.32) by including sodium phosphate buffer in the assay described in Section 2.2.6.

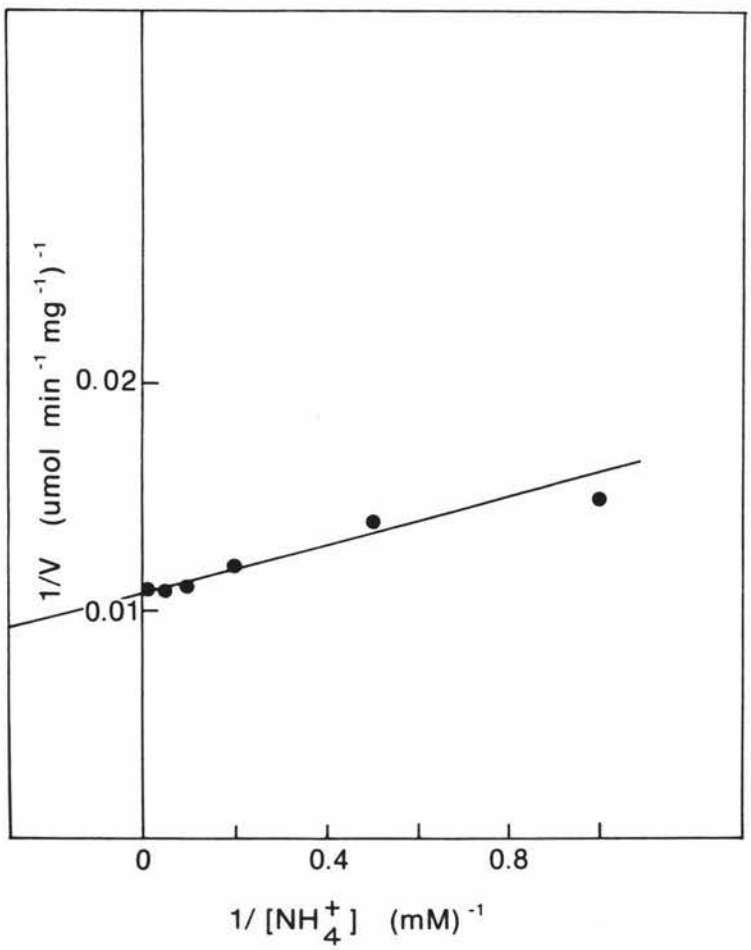


FIGURE 2.3.31 Lineweaver-Burk Plot for Determination of K_m (NH_4^+). Assays were performed at a non-saturating MgATP concentration (0.1 mM) and at 2.0 mM F6P, using 0.2 µg protein per assay. Extrapolation of the curve to the abscissa gives a K_m (NH_4^+) of 0.53 mM.

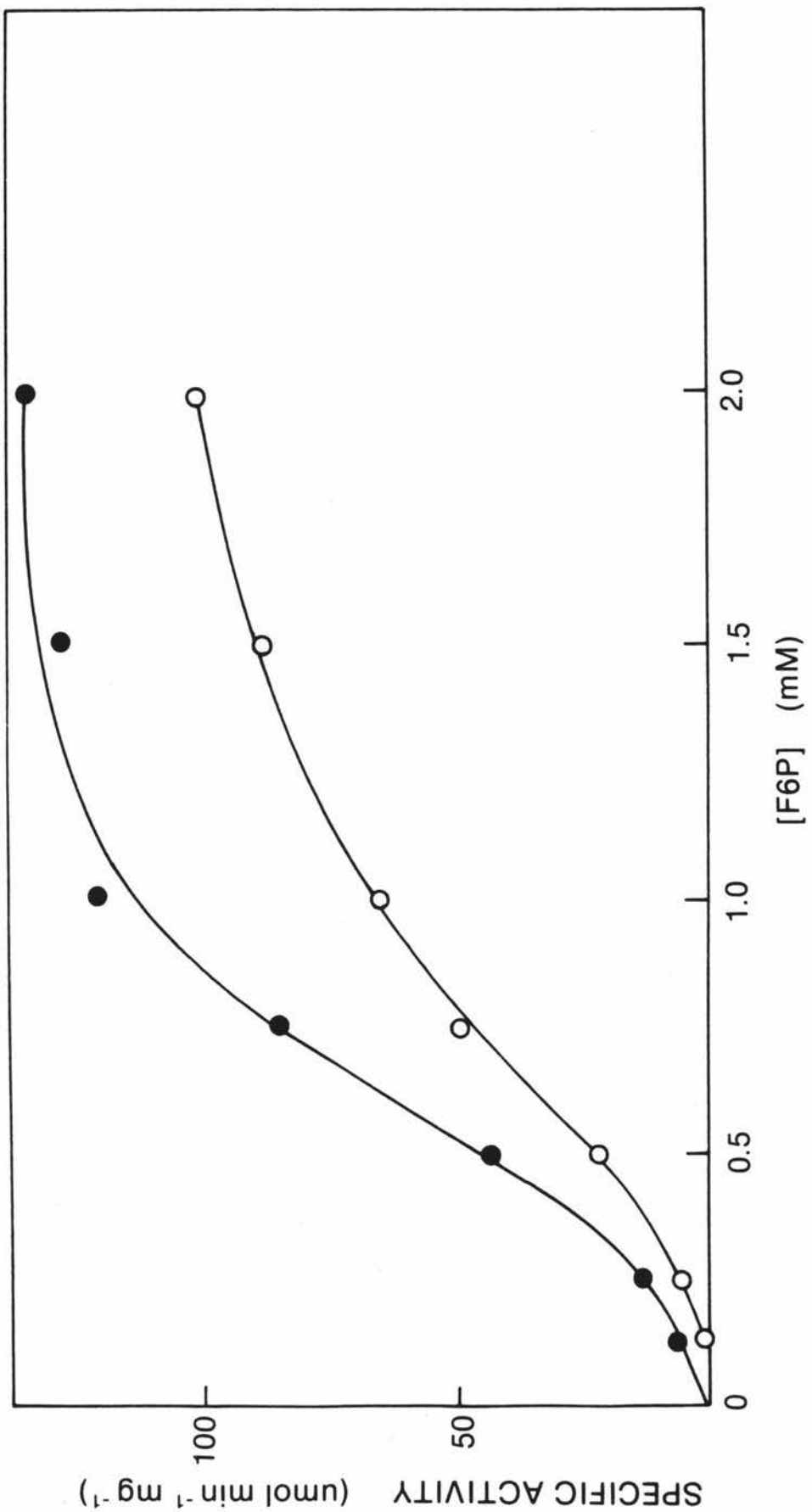


FIGURE 2.3.32 The Effect of P_i on PFK Activity. Assays were performed at 2.0 mM MgATP and 0 - 2.0 mM F6P in Tris-HCl buffer (as described in Section 2.2.6). 20 mM P_i inhibited activity, increasing the $F6P_{0.5}$, but causing little change in the Hill coefficient (data not shown).

Curves show PFK activity in the absence of P_i (●), and at 20 mM P_i (○).

20 mM P_i increased the $F6P_{0.5}$ from 0.65 - 0.88 but did not affect the co-operativity of binding; Hill coefficients calculated using the specific activities at 2.0 mM F6P as V_{max} were 3.0 in the absence of P_i and 2.7 at 20 mM P_i. The observation that P_i is more inhibitory at non-saturating F6P concentrations than at non-saturating MgATP concentrations is unexpected, as P_i is a product of PFK activity and could therefore be expected to competitively inhibit MgATP binding. Evans & Hudson (1979) showed two binding sites for P_i on *B. stearothermophilus* PFK, the F6P-binding catalytic site and an allosteric site. The effect of P_i on F6P binding is probably due to binding of P_i at either site.

A K_i for P_i of 4.8 mM was determined (Figure 2.3.33B). As the K_m (MgATP) is 0.18 mM, approximately 20-fold lower than the K_i for P_i, this difference in affinities for the two compounds may explain the lower inhibition at non-saturating levels of MgATP than at non-saturating levels of F6P.

The effects of increasing P_i concentrations at 2.0 mM MgATP and 0.4 mM and 2.0 mM F6P are shown in Figure 2.3.33A. 5 - 10 mM P_i had little effect on activity, while concentrations of P_i above 10 mM were inhibitory at both saturating and non-saturating F6P concentrations. 40 mM P_i resulted in 20% inhibition at saturating concentrations of MgATP and F6P. This is consistent with the activity in 50 mM P_i buffer (Figure 2.3.4A) in which activity was 25% lower than activity in Tris buffer. The observed inhibition is unlikely to be due to the Na^+ added in the phosphate buffer, as sodium ions had little effect on activity at the MgATP and F6P concentrations used.

The effects of P_i on PFK activity appear to depend on the presence of other metabolites. Most mammalian PFKs show activation or relief of inhibition by P_i (Passoneau and Lowry, 1962; Uyeda and Racker, 1965; Wu, 1966). Inhibition of PFK activity by P_i has been reported in yeast (Atzpodien and Bode, 1970), *E. coli* (Atkinson & Walton, 1965; Blangy *et al*, 1968; Lowry & Passoneau, 1964), plants (Dennis & Coultate, 1967; Kelly & Turner 1969; Kelly & Latzko, 1977) and *Clostridium perifringens* (Lowry & Passoneau, 1964). Atzpodien & Bode (1970) showed that P_i was a competitive inhibitor (with respect to F6P) of yeast PFK, suggesting competition for a single binding site, or binding of P_i to an allosteric site which affected only the F6P catalytic site. P_i was shown to abolish

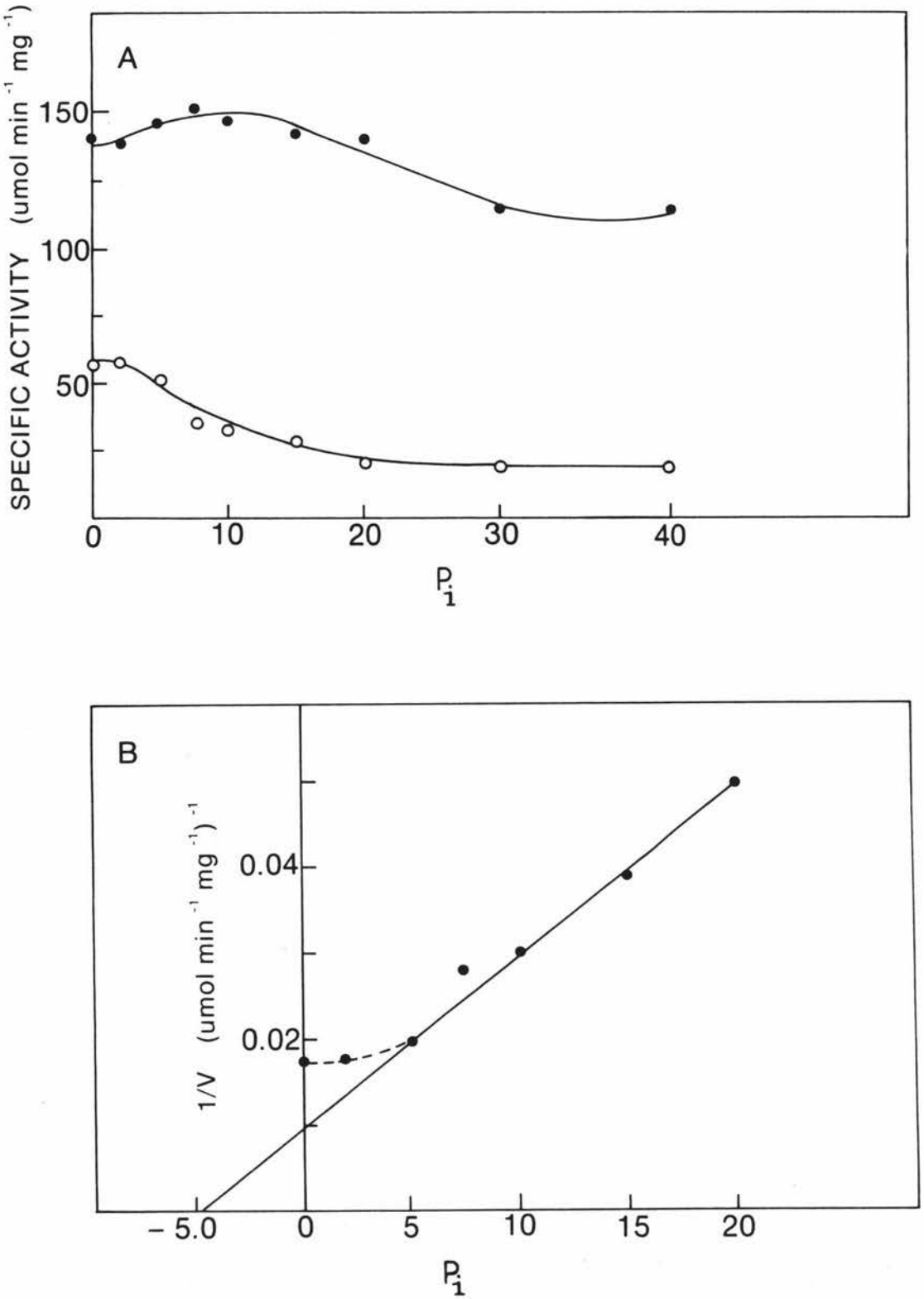


FIGURE 2.3.33 The effects of P_i on PFK activity at saturating and non-saturating MgATP concentrations. The effect of increasing phosphate concentrations on PFK activity is shown at 2.0 mM MgATP, and at 2.0 mM (●) and 0.4 mM (○) F6P (Figure A). Tris HCl buffer was used in all assays. Enzyme concentration = 0.42 µg/assay.

The graph plotted in Figure B gives a K_i for phosphate ions of approximately 4.8 mM.

co-operativity of F6P binding. However Banuelos *et al* (1977) found that P_i activated yeast PFK and activation was enhanced in the presence of AMP and ADP. The activity of yeast PFK in the absence of P_i was insufficient to account for the observed rate of glucose utilisation. Recent work by Mason *et al* showed that P_i levels increased dramatically in starved cells of *S. lactis* 7962, and these authors suggested that P_i may play an important role in the regulation of glycolysis in *S. lactis* by regulating activities of pyruvate kinase and lactate dehydrogenase.

2.3.20 The Effect of NH_4^+ and FBP on PFK Activity in the Presence of other Inhibitors

Studying the effect of a single metabolite on PFK activity *in vitro* can only provide an over-simplified concept of enzyme regulation as enzyme activity *in vivo* may be the result of combined action of several metabolites. In addition effector compounds may require a particular 'environment' such as high or low concentrations of another metabolite to modify activity. To investigate this possibility the effects of NH_4^+, an activator and FBP, an inhibitor of *S. lactis* PFK were studied in the presence of PEP, AMP and P_i, three compounds which inhibit PFK activity to determine

i whether or not inhibition by PEP, AMP and P_i is relieved in the presence of NH_4^+ or FBP

ii whether the effects of NH_4^+ and FBP are modified by the effects of other metabolites.

The results are shown in Tables 2.3.10 and 2.3.11.

Addition of NH_4^+ resulted in a three fold increase in activity at a non-saturating (0.05 mM) MgATP concentration, but little change in activity at a non-saturating (0.15 mM) F6P concentration, in agreement with results of previous experiments (see Figures 2.3.30A and B, and Table 2.3.3). In the absence of NH_4^+ PEP, AMP and P_i inhibited activity at non-saturating concentrations of both MgATP and F6P, while ADP activated PFK at non-saturating F6P concentrations but was inhibitory at non-saturating MgATP concentrations. In the presence of NH_4^+, the % inhibition by AMP, P_i and ADP at 0.05 mM MgATP was

TABLE 2.3.10

EFFECT OF FBP ON PFK ACTIVITY IN THE PRESENCE OF AMP, P_i AND PEP

	- FBP		+ FBP	
	Specific Activity $\mu mol\ min^{-1}\ mg^{-1}$	% Control Activity	Specific Activity $\mu mol\ min^{-1}\ mg^{-1}$	% Control Activity
i 0.05 mM MgATP, 1.0 mM F6P				
Control	16.7	100	11.2	100
10 mM AMP	13.9	84	9.8	88
25 mM P_i	12.9	77	9.1	82
10 mM PEP	14.2	85	9.9	88
ii 1.0 mM MgATP, 0.15 mM F6P				
Control	9.8	100	0.99	100
10 mM AMP	7.2	74	3.2	322
25 mM P_i	6.6	67	3.5	356
10 mM PEP	3.1	31	2.9	288

TABLE 2.3.11

EFFECT OF NH_4^+ ON PFK ACTIVITY IN THE PRESENCE OF PEP, AMP, ADP AND P_i

		- NH_4^+		+ NH_4^+	
		Specific Activity μmol min^{-1} mg^{-1}	% Control Activity	Specific Activity μmol min^{-1} mg^{-1}	% Control Activity
i	0.05 mM MgATP, 1.0 mM F6P				
	Control	16.2	100	49.8	100
	10 mM PEP	13.8	85	49.5	99
	10 mM AMP	13.8	85	44.9	90
	10 mM ADP	9.7	60	29.9	60
	25 mM P_i	9.8	61	29.4	59
ii	1.0 mM MgATP, 0.15 mM F6P				
	Control	11.2	100	13.0	100
	10 mM PEP	8.1	72	10.1	78
	10 mM AMP	1.5	13	1.6	12
	10 mM ADP	64.9	580	165.2	1271
	25 mM P_i	2.3	20	2.5	19

comparable to the inhibition in the absence of NH_4^+, although the absolute specific activity was higher. This suggests that although NH_4^+ activates PFK, it does not relieve inhibition by these components. However PEP inhibition was decreased very slightly in the presence of NH_4^+. At non-saturating F6P (0.15 mM), NH_4^+ did not relieve inhibition by PEP, AMP or P_i, but increased by approximately two fold the activation by ADP.

FBP inhibited PFK activity at non-saturating MgATP (0.05 mM) and F6P (0.15 mM) concentrations by 33% and 90% respectively. At non-saturating MgATP concentrations FBP did not affect the inhibition by AMP, P_i or PEP. However at non-saturating concentrations of F6P, and in the presence of AMP, P_i and PEP, FBP increased PFK activity to approximately three times the activity in the control (+ FBP), although activities remained less than the activity in the absence of FBP. This suggests that inhibition by FBP, AMP, P_i and PEP is relieved by a combination of FBP with the latter three metabolites.

The number of apparent contradictions in the literature regarding effects of metabolites on PFK activity emphasises the complexity of the allosteric control of PFK. It is likely that no single metabolite is the major effector of PFK, but that PFK activity is a result of the combined action of numerous metabolites, the concentrations of which reflect the momentary metabolic requirements of the cell.

2.3.21 SUMMARY

PFK from *S. lactis* C_{10} exhibits a number of kinetic features typical of the allosteric PFKs of mammalian and other bacterial species. It therefore appears to have the potential to act as a regulatory enzyme in carbohydrate metabolism in *S. lactis*.

However although different enzymes may exhibit similar properties *in vitro*, activity *in vivo* is dependent on the intracellular concentrations of substrates and effectors. These may differ between species depending on the activities of other enzymes in the pathway. Thus to compare *in vitro* kinetic data of enzymes from different organisms, or to relate such data to enzyme regulation *in vivo*, requires a knowledge of both the metabolites affecting enzyme activity and the intracellular concentrations of these metabolites.

A discussion of the kinetic properties of PFK in relation to the regulation of carbohydrate metabolism in *S. lactis* will be presented in Chapter 5.

CHAPTER THREE

TAGATOSE 6-PHOSPHATE KINASE

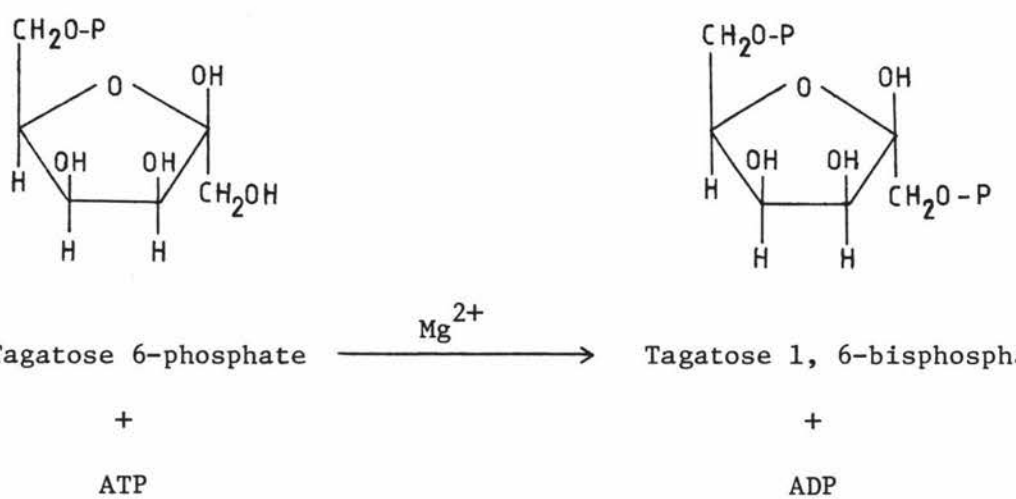
CHAPTER 3

TAGATOSE 6-PHOSPHATE KINASE

3.1 INTRODUCTION

Tagatose 6-phosphate kinase (T6PK) is an inducible enzyme which functions in the metabolism of lactose and galactose in bacteria including *S. aureus* (Bissett & Anderson, 1973), the group N streptococci (Bissett & Anderson, 1974*a*) and *S. mutans* (Hamilton & Lebtag, 1979). As described in Chapter 1, metabolism of galactose 6-phosphate derived from either lactose or galactose in these bacteria involves isomerisation to tagatose 6-phosphate and phosphorylation to tagatose 1, 6-bisphosphate, followed by cleavage to triose phosphates. The latter two reactions parallel the initial reactions of the Embden-Meyerhof-Parnas pathway. Tagatose 6-phosphate kinase catalyses the ATP-dependent phosphorylation of D-tagatose 6-phosphate in this parallel pathway, a reaction analogous to that catalysed by phosphofructokinase in the Embden-Meyerhof-Parnas pathway.

Reaction 1



The substrates of PFK and T6PK, D-Fructose 6-phosphate and D-Tagatose 6-phosphate are epimers, differing only in the configuration of the hydroxyl group at carbon 4.

T6PK has been purified, and some of its physical and kinetic properties studied, only from *S. aureus* (Bissett, 1975; Bissett & Anderson, 1980a). The *S. aureus* enzyme is a dimer of subunit molecular weight 52,000 daltons. Under the assay conditions used by Bissett and Anderson *S. aureus* T6PK appears to be a non-allosteric enzyme quite distinct from the PFK also present in the same organism. *S. aureus* T6PK exhibited non-co-operative binding of both its substrates, MgATP and D-tagatose 6-phosphate, and was not inhibited by high concentrations of ATP. Citrate, ADP, AMP, and P_i (all at 2mM concentrations) had no effect on activity. The enzyme was specific for D-T6P; D-F6P could also be phosphorylated but the K_m for the latter substrate (150mM) was 10,000-fold higher than the K_m for D-T6P (16 μM). F6P was a competitive inhibitor with respect to T6P. Fructose 1-phosphate, sorbose 1-phosphate, galactose 6-phosphate, glucose 6-phosphate and mannose 6-phosphate were not phosphorylated.

T6PK showed less specificity for the phosphate donor than for the sugar phosphate substrate. CTP, UTP, ITP, GTP, ATP and TTP, but not PP_i, PEP, acetyl phosphate, creatine phosphate or carbamyl phosphate were utilised.

As for other kinases, divalent cations were required for activity. Mg^{2+} could be replaced by Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Ca^{2+} and Zn^{2+} giving maximum activities ranging from 2%-54% of the activity with Mg^{2+}. Activity was also affected by monovalent cations; K^+, NH_4^+, Rb^+ and Cs^+ stimulating activity, while Na^+ and Li^+ were inhibitory.

The preceding paragraph summarises the information currently available on the kinetic and physical properties of *S. aureus* T6PK. As the enzyme has so far only been purified from a single source, it was of interest to study T6PK in other bacterial species.

The purification and some of the properties of T6PK from *S. lactis* C_{10} are presented in the following sections of this chapter.

3.2 METHODS

3.2.1 INTRODUCTION

Many of the methods used in the study of T6PK were also used in the study of PFK and have been described in Chapter 2. The methods outlined in this section are those which required modification for use with T6PK or were not applied to PFK and have not been described in the previous chapter.

3.2.2 PREPARATION OF TAGATOSE 6-PHOSPHATE

The barium salt of D-tagatose 6-phosphate was synthesised from D-galacturonic acid by Dr C.H. Moore, following the procedures used by Bissett (1975), and Thomas (Dr T.D. Thomas, N.Z. Dairy Research Institute, personal communication). Although D-tagatose is commercially available D-galacturonic acid was preferred as the starting material as commercial preparations of D-tagatose may contain contaminating sugars. The synthesis is summarised below and in Figure 3.2.1.

D-galacturonic acid (I) was isomerised to the calcium salt of D-tagaturonic acid (II) by stirring in the presence of CaO. Hydroxyl groups were blocked by methylation with ethereal diazomethane and the methylated derivative (3,4:5,6 di-o-isopropylidene 5-keto galactofuronic acid) (III) was reduced with $LiAlH_4$ to form 1,2:3,4 di-o-isopropylidene D-tagatose (IV). A phosphate group was introduced at carbon 6 by reacting the blocked tagatose (IV) with diphenylchloro phosphonate (V) followed by reduction with H_2 and a PtO_2 catalyst (VI). Isopropylidene blocking groups were removed by heating in pentane, and then a solution of barium hydroxide was added to form the barium salt of D-tagatose 6-phosphate (VI).

The barium salt of T6P was converted to the sodium salt as required by ion exchange chromatography on a Dowex 50X8 cation exchange resin, H^+ form (Sigma Chemical Co.). Dowex 50X8 was washed with 0.1 M HCl, then with distilled water until the wash was neutral. 2 ml of an aqueous solution of the barium salt of T6P (0.1 g/ml) was washed through a column containing 2 g resin, and the eluate titrated to pH 7.0 with 1 M NaOH, then freeze dried. The sodium salt of T6P was stored dessicated at -20°C.

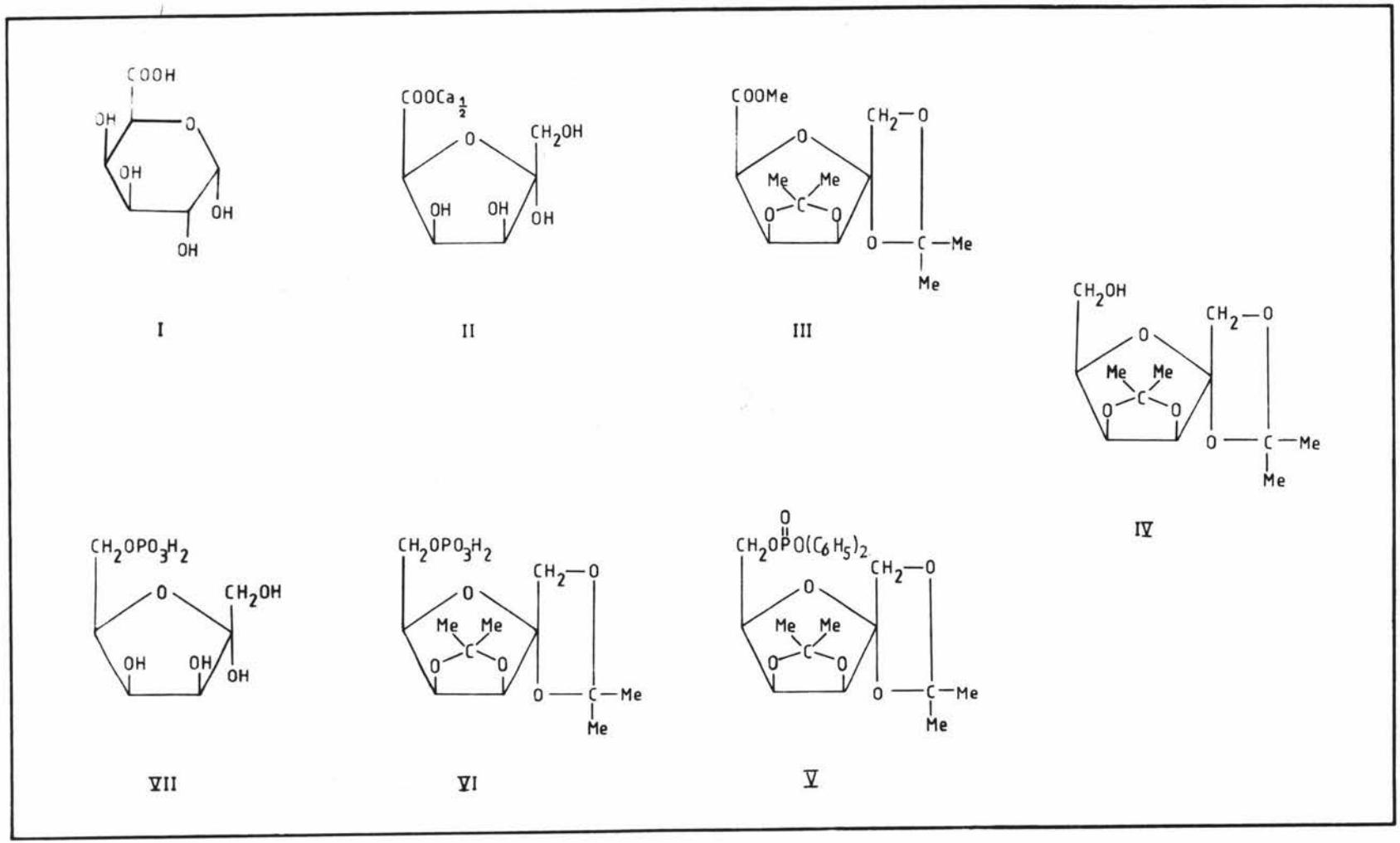


FIGURE 3.2.1 Synthesis of D-Tagatose 6-phosphate. This diagram summarises the steps involved in the synthesis of D-tagatose 6-phosphate from galacturonic acid, as described in the text (Section 3.2.2).

3.2.3 Characterisation of the T6P Preparation

i Enzymatic determination of purity

0.025 µmoles of the sodium salt of T6P (assuming the sample was 100% pure) was phosphorylated by purified *S. lactis* T6PK and the reaction allowed to proceed to completion. Activity was coupled to NADH oxidation via the aldolase-linked assay system described in Section 3.2.6 and the reaction monitored by following the decrease in absorbance at 340 nm. Purity of T6P was calculated by comparing the observed change in absorbance with the theoretical absorbance change, assuming 2 µmol NADH oxidised per mole of T6P phosphorylated. A molar extinction coefficient for NADH of 6.22×10^3 (moles l^{-1})$^{-1}$ cm was used. The T6PK used in the assay had been assayed during purification with T6P obtained from Dr T. Thomas of the N.Z. Dairy Research Institute, Palmerston North.

The net changes in absorbance due to formation of NAD^+, after allowing repeated reactions to proceed to completion were 0.480, 0.558, 0.500, 0.535 and 0.550 units. Comparing these values with the expected change in absorbance of 0.622 units, an average purity for T6P of 84% was obtained.

To determine the water content of the preparation, 10 mg of the sodium salt of T6P was dried by incubation at 100°C for 30 minutes in a tared container. The dried sample was transferred to a dessicator and then re-weighed. From the difference in weights of the hydrated and dried T6P, a water content of 13.5% was calculated. Thus the T6P preparation appears to be approximately 97.5% T6P.

ii Thin Layer Chromatography

T6P was identified by ascending chromatography on polyethyleneimine impregnated polyamide sheets. Approximately 20 nmoles of T6P was applied as a single spot to a thin layer plate. 20 nmoles of each of glucose 6-phosphate, galactose 6-phosphate, fructose 6-phosphate, fructose 1, 6-bisphosphate and tagatose 6-phosphate (obtained from N.Z. Dairy Research Institute) were applied as standards. The plate was developed for approximately three hours in distilled water, dried, then developed in a 1:1 mixture of 0.5 M LiCl - 2 M formic acid until the solvent front reached a height of

12-15 cm. Phosphorylated sugars were visualised by dipping the plate in a solution containing 0.1 g $FeCl_3.6H_2O$, 7 g sulphosalicylic acid, 25 ml ethanol and 75 ml distilled water. Phosphorylated compounds appeared as white fluorescent spots which faded rapidly but reappeared after 24-48 hours.

A single compound was revealed after staining for phosphorylated sugars as described. No contaminating sugar phosphates were apparent. The R_F value of the compound was 0.43, while the R_F values of glucose 6-phosphate, galactose 6-phosphate, fructose 6-phosphate, fructose 1, 6-bisphosphate and of a sample of T6P prepared by Dr T.D. Thomas of the Dairy Research Institute, Palmerston North, were 0.46, 0.48, 0.39, 0.21, and 0.42 respectively. Thus the T6P preparation appears to be authentic T6P.

iii Assay with PFK

Assays were performed using 2.0 mM T6P and the aldolase assay described for PFK in Section 2.2.6. The T6P preparation, assayed with PFK using rabbit muscle aldolase gave only 0.5 - 1.5% of the activity obtained using T6PK and TBP-specific aldolase in the assay. On the assumption that muscle aldolase is specific for FBP (Thomas, 1975), these results indicate a slight trace of F6P present in the T6P preparation. However rabbit muscle aldolase may also be capable of a slow cleavage of TBP (Tung *et al*, 1954).

3.2.4 T6PK Assays

T6PK was assayed by coupling activity to NADH oxidation by either α-glycero phosphate dehydrogenase or lactate dehydrogenase (Figure 3.2.2), and activity was monitored by following the decrease in absorbance at 340 nm on a Cecil spectrophotometer equipped with a Servoscribe chart recorder. Assays were performed at 30°C, in a total volume of 0.5 ml, and the reaction initiated by addition of T6P or T6PK to the reaction mixture.

i Pyruvate kinase - Lactate dehydrogenase linked Assay

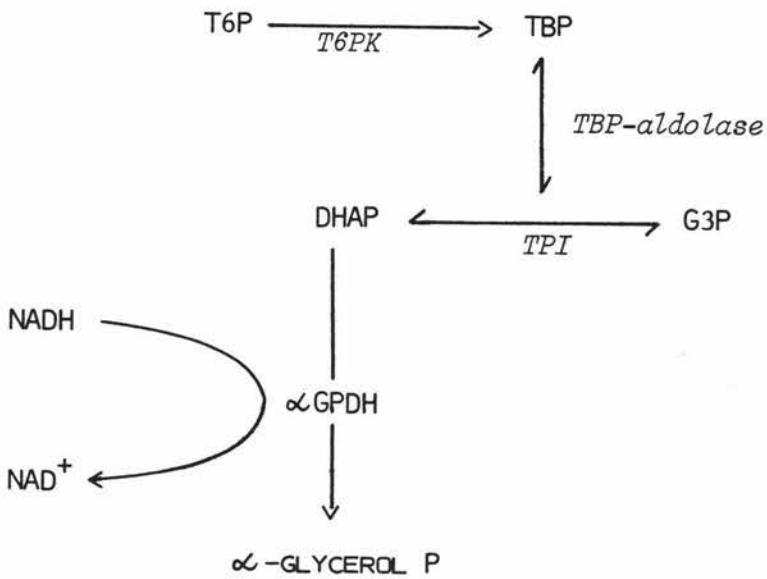
The stock assay mixture contained the following components in 50 mM Tris-HCl buffer, pH 7.5 :

0.2% BSA, 10 mM 2-mercapto ethanol, 0.33 mM NADH,
2.0 mM PEP, 100 mM KCl, LDH (1.5 µl (5.2 units)/ml)
PK (1 µl (1.9 units)/ml).

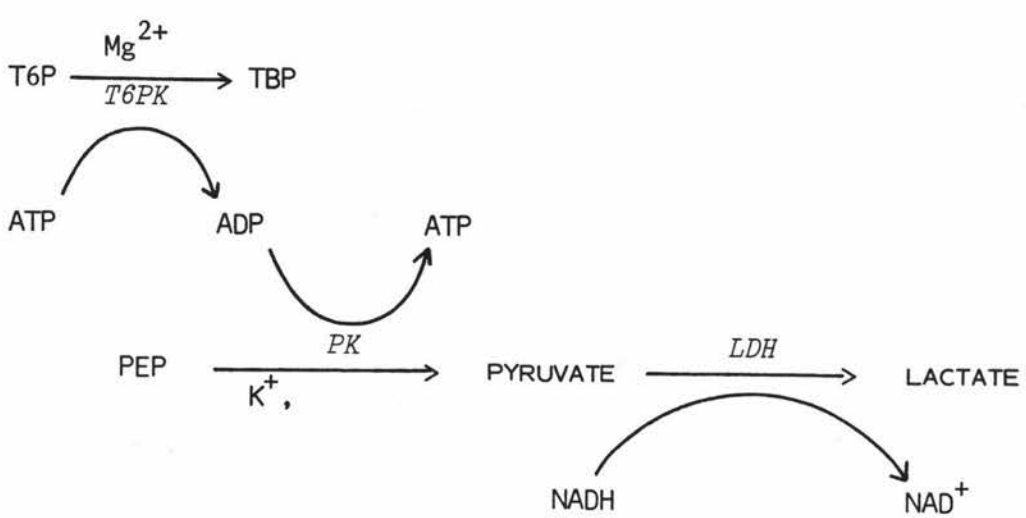
FIGURE 3.2.2

ASSAY SYSTEMS USED FOR T6PK ASSAYS

i TBP-ALDOLASE ASSAY



ii PYRUVATE KINASE-LACTATE DEHYDROGENASE ASSAY



Each assay contained 0.3 ml of assay mixture, 0.05 ml of each of MgATP and T6P diluted to give the desired concentrations, 0.025-0.1 ml enzyme, and buffer to a total volume of 0.5 ml. Background activity was measured in a separate assay in which T6P was replaced by buffer.

ii Aldolase-linked assay

The assay mixture contained 50 mM Tris-HCl pH 7.5, 0.2% BSA, 10 mM 2-mercapto ethanol, 0.33 mM NADH, and α-glycero phosphate/triose phosphate isomerase mixture [5 µl, (1.0 unit αGPDH, 10 units TPI/ml)]. Each assay contained 0.3 ml assay mixture plus 5-10 µl (15-30 µg) of TBP-aldolase from *S. cremoris*, 0.05 ml of T6P and MgATP solutions, and enzyme and buffer to a total assay volume of 0.5 ml. Background activity was measured prior to addition of T6P.

The TBP-aldolase was obtained from Dr V.L. Crow, N.Z. Dairy Research Institute, Palmerston North. The specific activity of this enzyme with TBP as substrate was $\sim$20 µmol min^{-1} mg^{-1}. FBP was also cleaved but the enzyme showed a greater affinity for TBP and the maximum velocity obtained with FBP as substrate was lower than that obtained with TBP as substrate. The TBP-aldolase appeared homogeneous: a single protein band was obtained on SDS polyacrylamide gel electrophoresis.

Estimation of T6PK activity in cell-free extracts was complicated by a high 'background' activity when the PK-LDH assay was used. Assays containing ATP but no T6P resulted in activity which was 2-4 fold greater than the T6PK activity in cell-free extracts and which persisted throughout the initial steps of the purification scheme. The 'background' activity using the aldolase assay system was only 10-20% of the T6PK activity and was due to the NADH oxidase activity which was measured in a separate assay. The difficulty in separating T6PK from the background activity prompted a brief investigation of the latter activity. A similar result was noted in *S. aureus* (Bissett, 1975); activity in cell-free extracts in the absence of T6P was 63% of the activity with T6P present. Table 3.2.1 lists activities measured in a cell-free extract from *S. lactis* (after passage through a Sephadex G25 column to remove endogenous substrate). NADH oxidase activity was measured in a separate assay containing only buffer,

TABLE 3.2.1

ACTIVITY OBSERVED IN T6PK ASSAYS IN WHICH VARIOUS COMPONENTS OF THE ASSAY MIXTURE WERE OMITTED

	PK-LDH-linked assays	Aldolase linked assays
NADH oxidase *	0.09	0.095
Complete assay mixture	1.65	0.655
- T6P	1.30	0.086
- ATP	0.105	0.090
- aldolase	-	0.215
- PEP	0.105	-
- PK	0.105	-
- LDH	0.126	-

* NADH oxidase was measured in an assay containing only buffer, NADH and cell-free extract.

The figures shown are the activities (μmol NADH min^{-1} ml^{-1}) in a cell-free extract from *S. lactis* C_{10} after passage through a G25 column to remove endogenous substrate.

0.33 mM NADH, and cell-free extract. This activity was dependent on the concentration of NADH used, and was easily separated from T6PK activity by gel filtration. Omission of ATP, PEP or auxiliary enzymes from the PK-LDH linked assays resulted in a rate of NADH oxidation (approximately 0.10 µmol NADH min^{-1} ml^{-1}) which could be accounted for by NADH oxidase activity. With ATP, but no T6P present a rate of 1.3 µmol NADH min^{-1} ml^{-1} was observed. Partial activity was observed in the absence of aldolase, suggesting appreciable endogenous levels of TBP aldolase [FBP aldolase does not cleave TBP (Dr V.L. Crow, N.Z. Dairy Research Institute, Palmerston North - personal communication)].

The observation that ATP-dependent activity was observed in the PK-LDH assay system but not in the aldolase assay system implied the presence of an ATPase or a non-specific phosphatase hydrolysing ATP, since the former assay system measures the rate of formation of ADP from ATP. Activity staining for phosphatase activity on polyacrylamide gels revealed two major bands of activity with relative mobilities (R_F) of 0.38 and 0.75 and a minor band of R_F = 0.21. A study of these enzymes was not pursued. A recent paper by Thompson and Saier (1981) implicates involvement of phosphatase activity in dephosphorylation and subsequent expulsion of sugars from *S. lactis*. The characterisation of these phosphatases and determination of their role in carbohydrate metabolism may prove an interesting area for further research.

Part of the background rate appeared due to adenylate kinase activity resulting from an AMP contaminant in the ATP used. Addition of AMP stimulated background activity but gave no activity in the absence of ATP. In the partially purified T6PK the background activity curve appeared to be biphasic, the initial rate being stimulated by AMP while the secondary rate was not. This is consistent with there being more than one activity contributing to the background rate.

'Background' activity in purified T6PK preparations was noticeable only in assays in which the PK-LDH assay system was used. This background activity was less than 0.01 units per mg protein, i.e. less than 2% of the T6PK activity at the lowest concentration of T6P assayed (0.025 mM). The background rate was measured and subtracted from all assays and was sufficiently low to be unlikely to affect the initial concentrations of ATP in the assays.

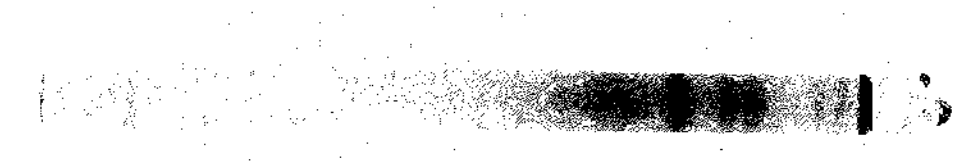
3.2.5 Location of T6PK Activity on Polyacrylamide Gels

Attempts to achieve a specific activity stain for T6PK by coupling T6PK activity to reduction of nitroblue tetrazolium (as described for PFK in Section 2.2.10) were unsuccessful; no stained bands became visible before the background colour developed. A 'negative stain' also proved unsuccessful. Gels were soaked for 30 minutes at 4°C in a 2 mM solution of NADH, then rinsed with distilled water and transferred to the PK-LDH assay mixture described in Section 3.2.4, except that NADH was omitted from the mixture. After standing for 45 minutes at 30°C gels were rinsed and incubated at room temperature in an aqueous solution containing 2 mg ml^{-1} nitroblue tetrazolium and 0.025 mg ml^{-1} phenazine methosulphate. With this stain, bands of T6PK activity should appear as clear bands on a dark purple-brown background. Repeated attempts using the latter stain showed a single broad band of R_F 0.7-0.85 in both control (lacking T6P) and sample gels. Although the band in the control gel was narrower than that on the sample gel (identical samples were electrophoresed in both gels), no conclusive results could be drawn. The activity staining on the control gel was possibly due to adenylate kinase or ATPase present in the T6PKpreparation, and the band visible on the sample gel was probably due to activity of both T6P and adenylate kinase or ATPase running in a single region of the gel.

Because of the inability to obtain an unequivocal localisation of T6PK by gel staining an alternative procedure was adopted. The entire gel was cut into 2 mm slices, and each slice was assayed for T6PK activity after maceration of individual gel slices for 30 minutes in aldolase assay mixture previously described (Section 3.2.4). A decrease in absorbance indicated the area of T6PK activity on the gel (see Figure 3.2.3).

3.2.6 Purification of T6PK

Frozen cells of *S. lactis* C_{10}, grown and harvested as described in Chapter 2 were disrupted by passage twice through a French pressure cell at 5500 p.s.i. (38 MPa) in 50 mM Tris-HCl buffer pH 7.5, containing 5 mM $MgCl_2$, 5 mM EDTA, 10 mM 2-mercapto ethanol and 20% (v/v) glycerol. (Tris-glycerol buffer). The cell-free extract was treated with protamine sulphate and ammonium sulphate exactly as described in the PFK purification procedure described in Chapter 2.



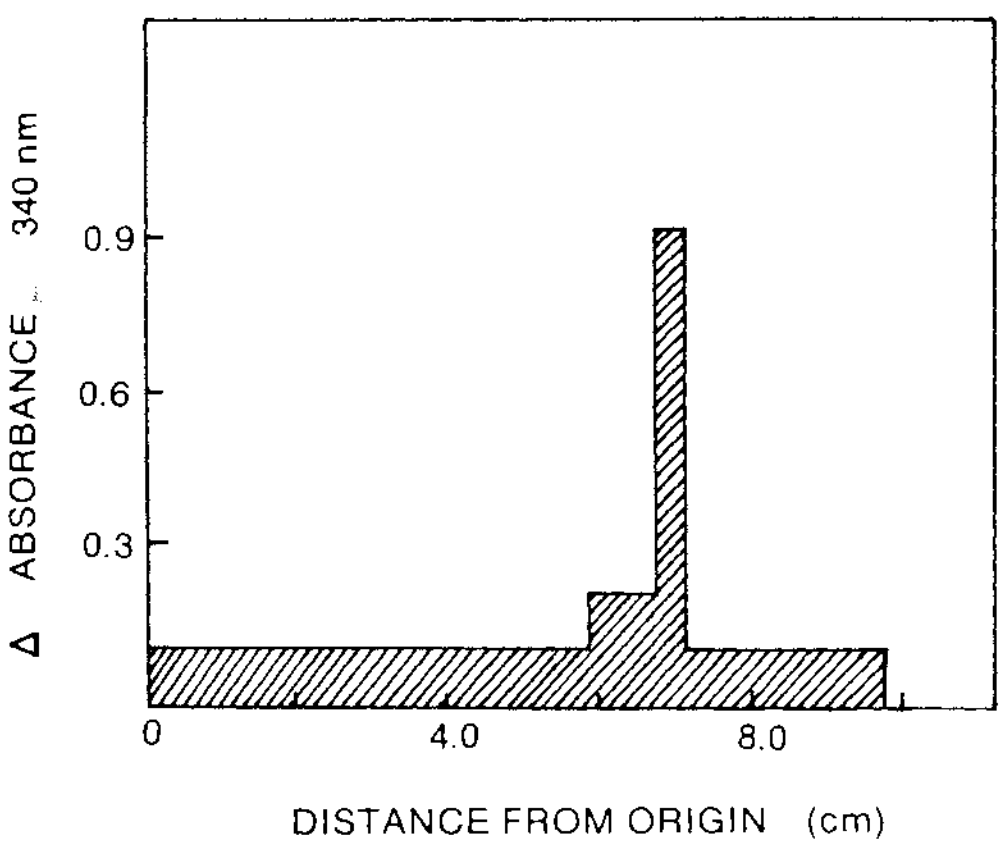


FIGURE 3.2.3 Location of T6PK Activity on Polyacrylamide Gels. An entire polyacrylamide gel (unstained) was sliced and incubated in the T6PK assay mixture as described in the text. T6PK activity indicated by the decrease in absorbance after 30 minutes corresponded to the major protein band after staining with Coomassie Blue as shown in the photo.

i Blue-dextran-Sepharose 'affinity' chromatography

Unlike PFK, T6PK did not bind to blue-dextran Sepharose-6B. 18 ml protein solution (approximately 400 mg protein) was applied to a 10 cm x 1 cm blue-dextran-Sepharose column equilibrated with Tris-glycerol buffer. The column was washed with buffer at a flow rate of 20-24 ml hr^{-1} and 6-8 ml fractions were collected until no further protein was eluted from the column as indicated by absorbance at 280 nm. The fractions containing T6PK of specific activity greater than 0.2 µmol min^{-1} mg^{-1} were pooled and concentrated to 3-6 ml by ultrafiltration through a PM 30 membrane in a Diaflo ultrafiltration apparatus.

ii Gel filtration on Sephacryl S200

The concentrated T6PK fractions (<6 ml) were loaded onto a 250 cm x 2.5 cm column of Sephacryl S200 which had been equilibrated in Tris-glycerol buffer containing 0.2 M KCl. The column was eluted at 4°C with the above buffer at a flow rate of 15 ml hr^{-1}. 5 ml fractions were collected. Fractions of specific activity greater than 0.75 µmol min^{-1} mg^{-1} were pooled, concentrated by ultrafiltration and applied to a second Sephacryl S200 column (250 cm x 2.5 cm). Protein was eluted using the same conditions described for the previous column. Fractions containing T6PK activity ($\geq$2.0 µmol min^{-1} mg^{-1}) were combined and dialysed for 24 hours at 4°C against 20 mM potassium phosphate buffer, pH 6.5, containing 20% glycerol and 10 mM 2-mercapto ethanol.

iii Ion exchange chromatography

DEAE Sephadex A25 was swollen for 1-2 days in 0.5 M potassium phosphate buffer pH 6.5, then washed in a Buchner funnel with 20 mM potassium phosphate buffer pH 6.5, containing 20% (v/v) glycerol and 10 mM 2-mercapto ethanol (Phosphate-glycerol buffer). The resin was packed into an 8 cm x 1.6 cm column, and washed with the 20 mM phosphate-glycerol buffer until the pH and conductivity of the eluate equalled that of the washing buffer. Dialysed T6PK fractions were loaded onto the DEAE column, at 4°C with a flow rate of approximately 20 ml hr^{-1}. Protein bound to the column was eluted with a linear gradient of 150 ml of 0 - 0.5 M KCl in phosphate-glycerol buffer. T6PK fractions were concentrated at 4°C in dialysis tubing on Aquacide II,

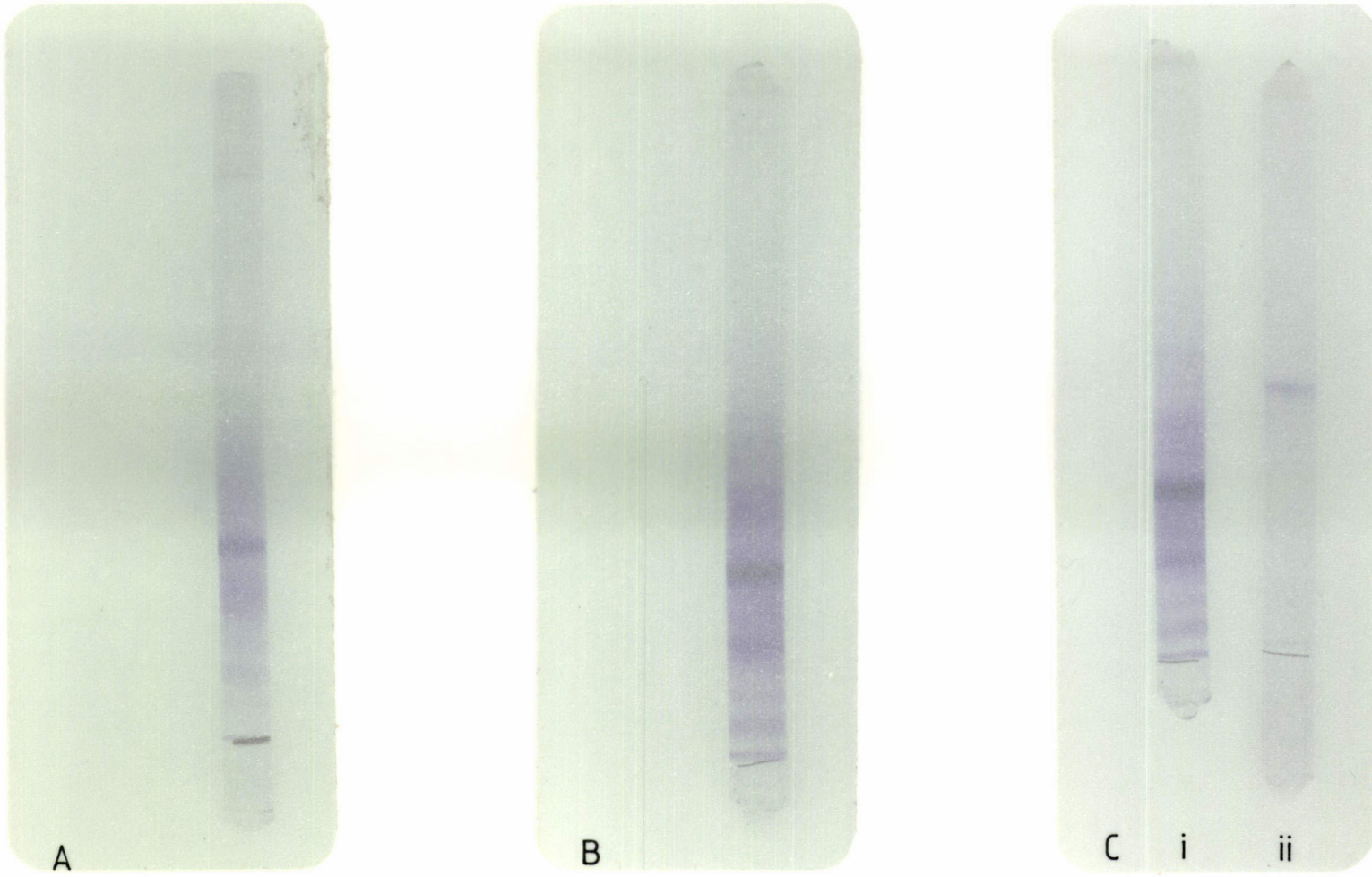


PLATE IV:

S. lactis tagatose 6-phosphate kinase : gel electrophoresis of two separate preparations of T6PK (A, B) and of T6PK (Ci) and PFK (Cii) from *S. lactis*.

dialysed for 24 hours against three changes of Tris-glycerol buffer, then finally dialysed against Tris-glycerol buffer with the glycerol content increased to 50% (v/v) Analar glycerol. The purified T6PK was stored at -20°C.

The purification procedure described above, and summarised in Table 3.2.2 gave a 136-fold purification of T6PK, with an 8% yield. Electrophoresis of the purified enzyme under non-denaturing conditions revealed one major protein band with several other proteins present in lesser amounts (Plate IV). The major protein band was shown to be T6PK by demonstrating that this protein band corresponded to the area of T6PK activity on an unstained gel electrophoresed under identical conditions. The zone of T6PK activity detected by slicing a gel and assaying individual gel slices (see Sections 2.2.6 and 3.2.4) corresponded to a band of R_F = 0.74 which agreed well with the R_F of the major stained protein band (Figure 3.2.3).

The purification scheme used was not ideal as it did not yield a homogeneous preparation of T6PK. However this purification was chosen only after an extensive investigation of alternative methods. Because of the lack of an effective 'affinity' column, a combination of several steps was required. Only a 2-5 fold purification could be achieved in any single step, and even to attain this often involved discarding appreciable amounts of enzyme in fractions of low specific activity. However the major aim of the preparation was to obtain T6PK completely free of PFK activity, and this was achieved through 'affinity' binding of PFK to the blue-dextran-Sepharose column.

Figures 3.2.4 and 3.2.5 show the elution profiles of protein eluted from two successive Sephacryl S200 columns. The initial run did not give a very extensive purification but enabled much of the protein to be discarded, thus enabling better resolution to be achieved in the second passage through the column. Ion exchange chromatography on DEAE Sephadex (Figure 3.2.6) resulted in a further 3-fold purification. However the yield of enzyme was low (<25%) and the specific activity of the purified enzyme after elution from DEAE Sephadex varied from 2.5 - 15 units mg protein^{-1}. The decrease in specific activity which sometimes occurred after the DEAE Sephadex step (the specific activity of the enzyme after gel filtration was approximately 5 units mg protein^{-1}) suggests that T6PK is denatured on passage through the ion exchange column. However the T6PK eluted from the

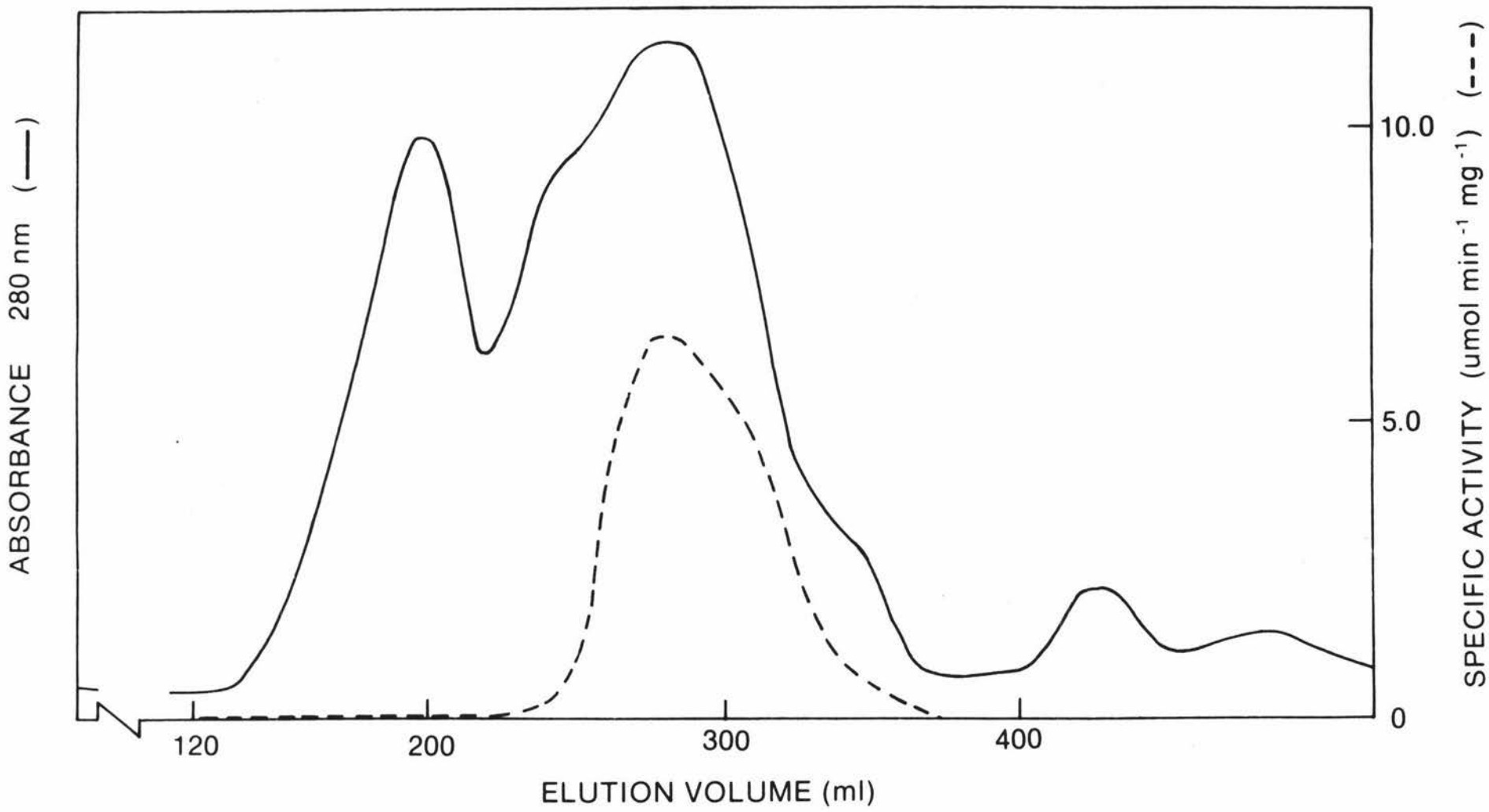


FIGURE 3.2.4 Elution profile of T6PK on Sephacryl S200 : First separation.

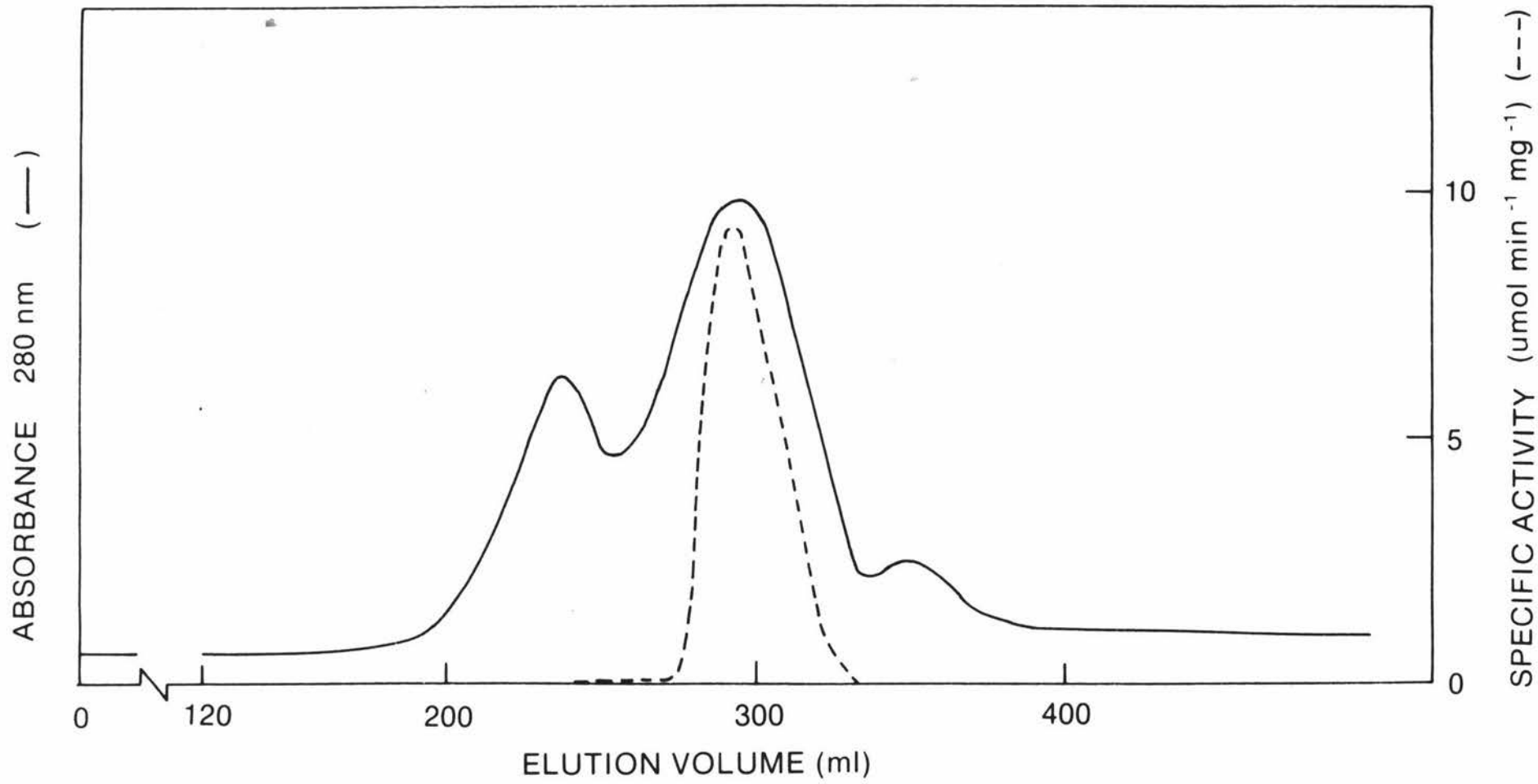


FIGURE 3.2.5 Elution profile of T6PK on Sephacryl S200 : Second separation.

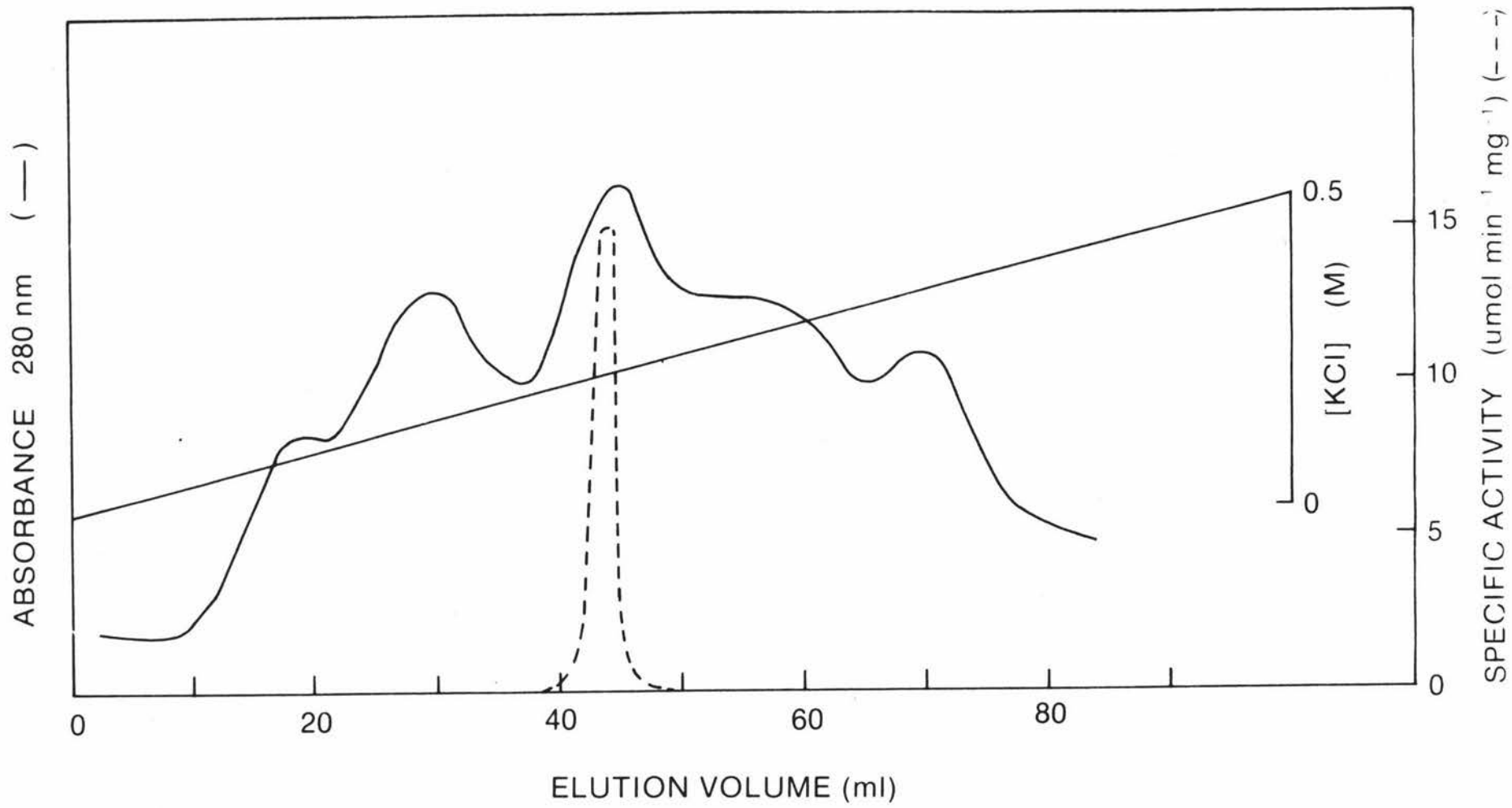


FIGURE 3.2.6 Ion-exchange chromatography of T6PK on DEAE-Sephadex.

TABLE 3.2.2

PURIFICATION OF T6PK FROM *S. lactis* C_{10}

	Vol (ml)	Total Activity (units)	Total protein (mg)	Specific Activity (µmol T6P) (min^{-1} mg^{-1})	Purification	Recovery %
Cell free extract	75	287	2610	0.11	1.0	100
PS Sn	75	266	2047	0.13	1.2	93
70% Ammonium sulphate ppt	18	207	690	0.3	2.7	72
Blue column	28	175	389	0.45	4.1	61
S200 (1)	50	138	123	1.12	10.2	48
S200 (2)	20	100	21.3	4.71	42.8	35
DEAE Sephadex	12	24	1.6	15 *	136	8.5

* Specific activity of T6PK eluted from DEAE Sephadex varied from 2.5 - 15 units mg^{-1}.

Table 3.2.2 shows the results of a typical preparation of T6PK from ~40 g frozen cells of *S. lactis* C_{10}, as described in the text.

DEAE column had an electrophoretic mobility identical to that of the enzyme recovered from the gel filtration columns and the enzyme from both stages of the preparation appeared to have identical kinetic properties.

The purified T6PK contained no measurable NADH oxidase, lactate dehydrogenase, pyruvate kinase, FBP-aldolase, TBP-aldolase, PFK, hexokinase, glucose 6-phosphate dehydrogenase or FBPase activities. Some preparations contained a low level of adenylate kinase activity (5% of the T6PK activity at 2 mM MgATP and 0.1 mM T6P) which was corrected for in calculating the specific activity of T6PK.

As T6PK has previously been purified only from *S. aureus* there is little information available on purification procedures for this enzyme, so a brief mention will be made of the purification steps which proved unsuccessful with *S. lactis* T6PK. *S. lactis* T6PK had different properties to the *S. aureus* enzyme, so the procedure used by Bissett (Bissett, 1975; Bissett & Anderson, 1980α) for purification of the *S. aureus* enzyme was unsuitable. In contrast to *S. aureus*, in which PFK activity was reduced after freezing cells for 1 week, both PFK and T6PK of *S. lactis* were stable to freezing in whole cells for periods of up to 3 months. Bissett also reported 30% losses in activity after protamine sulphate treatment and inconsistent recoveries after ammonium sulphate fractionation; neither of which were found with the *S. lactis* enzyme. Although most of the protein in the *S. lactis* cell-free extract co-precipitated with T6PK during ammonium sulphate fractionation, this was a convenient method of concentrating the enzyme and enabled a single ammonium sulphate fraction to be used for both PFK and T6PK preparations. Precipitation of T6PK with polyethylene glycol (PEG) was attempted, but the enzyme remained soluble at 24% PEG. Because of the viscosity of the solution subsequent removal of PEG on DEAE Sephadex or Sephacryl S200 gave extremely poor resolution. Bissett (1975) achieved approximately a 3.5 fold purification with hydroxylapatite. However T6PK from *S. lactis* appeared to bind irreversibly to hydroxylapatite and was not eluted with 1 M phosphate buffer or Tris-glycerol buffer containing up to 2 M KCl. Addition of bentonite to the cell-free extract resulted in a two-fold purification, however most of the protein removed by bentonite treatment was also removed by ion exchange chromatography on DEAE Sephadex, so inclusion of bentonite treatment in the purification was of no advantage. Heat treatment at 45°C and

58°C was not effective. No purification was achieved at 45°C, and up to 70% of activity was lost by heating to 58°C for one minute. Acid precipitation was also ineffective as little protein precipitated above pH 5.2 while below this pH activity decreased and could not be recovered by resuspension of the precipitated protein.

T6PK did not bind to blue-dextran-Sepharose or to 5' AMP agarose, both of which quantitatively bound PFK. Binding of T6PK to blue-dextran-Sepharose was attempted in 50 mM Tris-HCl and phosphate buffers, pH 7.5, and in Tris-HCl buffers ranging from pH 6.0-8.0, with ionic strengths as low as 10 mM. Binding was also attempted in the presence and absence of 10 mM Mg^{2+} and 0.1 mM T6P. None of these conditions promoted binding of T6PK to the column. However passage of the protein extract through the blue-dextran-Sepharose column did retard the T6PK slightly, T6PK eluting after the initial protein was washed from the column rather than being uniformly distributed throughout the protein peak. Alteration of the column dimensions, flow rate, and size of the sample loaded were investigated in an attempt to achieve a better purification by increasing the retardation of T6PK by the column. However no conditions were found to improve the separation of T6PK from the remaining protein.

Blue-dextran is reported to bind specifically to those proteins containing a secondary structure known as the 'dinucleotide fold' (Stellwagen *et al*, 1975; Thompson *et al*, 1975). This structure forms the NAD^{+} binding site of many dehydrogenases (Adams *et al*, 1970; Hill *et al*, 1972; Buehner *et al*, 1973) and the ATP binding site of some kinases (Schulz *et al*, 1974). Blue-dextran is a competitive inhibitor with respect to nucleotide cofactors (Wilson, 1976). However not all proteins requiring nucleotide cofactors bind to blue-dextran, e.g. hexokinase, an ATP-utilising enzyme is not bound, so T6PK is not unusual in this respect. Cibacron blue, the chromophoric group of blue-dextran has been reported to be less selective in binding (Wilson, 1976) and to bind to PFK more tightly than does blue-dextran (Bohme, 1972). Cibacron blue was coupled to Sepharose 6B using the same procedure used to couple blue-dextran, (Section 2.2.4) but this resin also showed no affinity for T6PK.

The limited availability of T6P did not allow synthesis of a T6P-affinity column. F6P was bound to Sepharose 6B after activation of the resin with benzoquinone (Porath & Axen, 1976) but this column

had only a low affinity for PFK and did not bind T6PK at all.

T6PK did not bind to phosphocellulose and CM-cellulose at pH values of above 5.0, in either phosphate or Tris buffers. Below pH 4.8 the protein precipitated so cation exchange resins could not be used. T6PK bound to DEAE-substituted resins at pH's of above 6.0 but lowering the pH at which the enzyme was bound and eluted did not markedly increase the purification factor. DEAE-Sephadex gave better resolution than DEAE-Cellulose, but both columns resulted in low recoveries and variable specific activity of the enzyme. In contrast, DEAE resins gave good purification of the *S. aureus* enzyme. However DEAE Sephadex did eliminate several major protein bands from the band pattern obtained on gel electrophoresis.

'Hydrophobic' chromatography on alkyl-substituted agarose gels was also attempted. 50-75% T6PK bound to agarose gels containing hydrophobic 'arms' of 4-10 carbon units, however only 10-15% activity was recovered on elution with KCl or ammonium sulphate.

3.3 PROPERTIES OF THE PARTIALLY PURIFIED T6PK : RESULTS AND DISCUSSION

3.3.1 DEPENDENCE OF T6PK ACTIVITY ON PROTEIN CONCENTRATION

T6PK activity was measured as a function of the amount of protein in the assay. With both cell-free extract and the partially purified enzyme there was a linear relationship between the protein concentration and activity (Figures 3.3.1A and 3.3.1B).

In the course of this investigation it was originally found that T6PK activity measured by the aldolase-linked assay was only about 50% of the activity measured by the PK-LDH assay. A search for possible reasons for this discrepancy revealed that the lower activity obtained using the aldolase assay was due to the lower levels of activating monovalent cations in this assay; in the PK-LDH assay KCl was included at a concentration of 100 mM as it is required for PK activity. Addition of 100 mM KCl to the aldolase-linked assay increased the activity to a rate equal to that obtained in the PK-LDH assay (Figure 3.3.1B).

A consequence of this finding is that the concentration of K^+ in the assay mixture may affect the kinetic properties of the enzyme, and activation by K^+ in the PK-LDH assay system may mask any activatory or inhibitory effects of other compounds. For this reason, assays (wherever possible) were performed using the aldolase-linked assay system. K^+ was not included in the assay mixture. The only factor limiting the use of this assay system was the availability of sufficient TBP-aldolase. Except where otherwise specified, the results described in the following sections were obtained using the aldolase assay. A study of the K^+ activation of T6PK is presented later (Section 3.3.16).

3.3.2 STABILITY OF PURIFIED T6PK

Figure 3.3.2 records the activity of partially purified T6PK (approximately 0.06 mg ml^{-1}) stored in different buffers at 4°C over 15 weeks. In Tris-HCl buffer containing 20% (v/v) glycerol, 5 mM $MgCl_2$, 5 mM EDTA and 10 mM 2-mercapto ethanol (Tris-glycerol buffer) the enzyme lost 97% and 90% activity at pH 7.0 and 8.0 respectively over 5-10 weeks. T6PK was more stable at pH 6.0, 66%

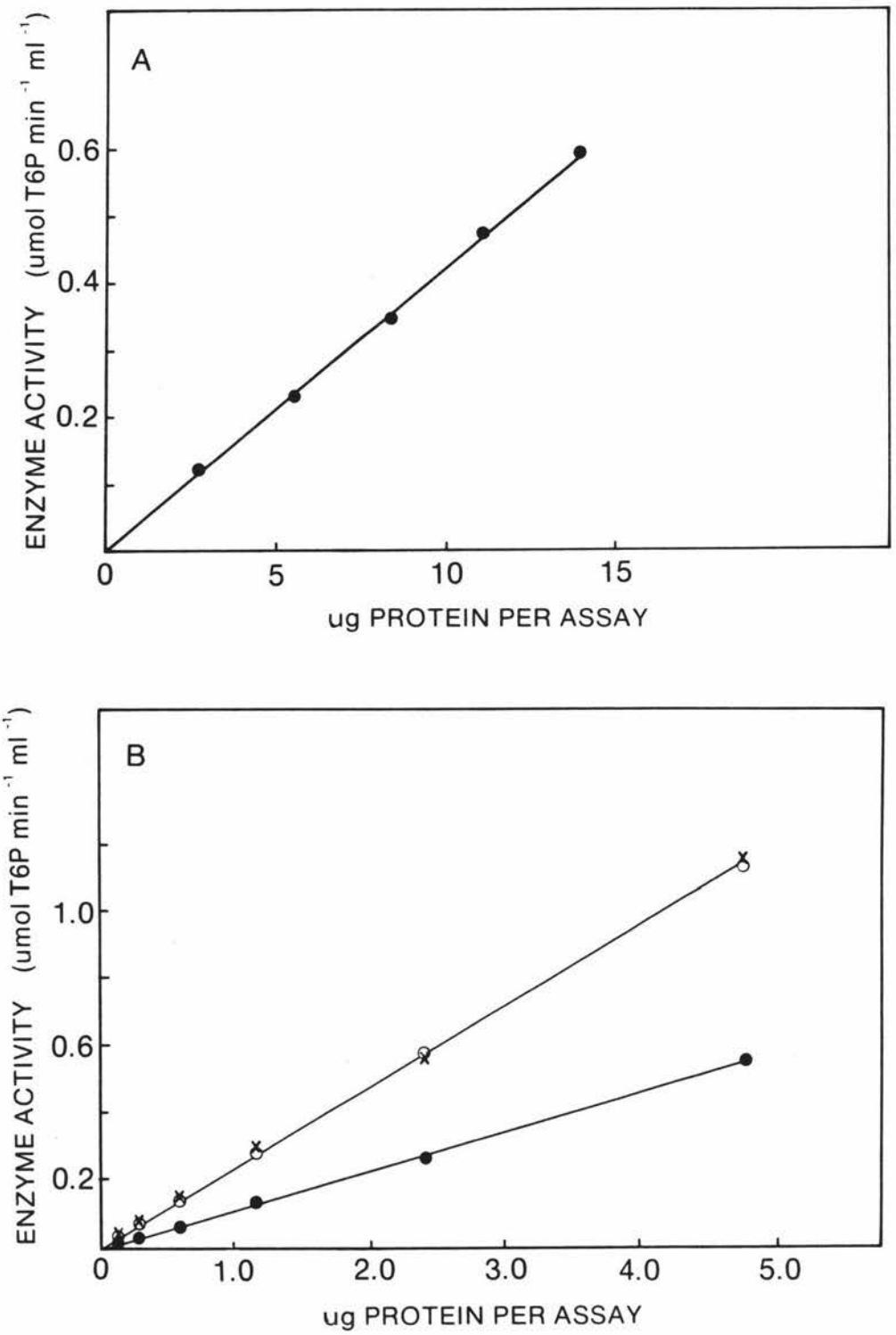


FIGURE 3.3.1 Dependence of T6PK Activity on Protein Concentration. T6PK was assayed at varying protein concentrations using both the pyruvate kinase-lactate dehydrogenase assay (O) and the aldolase assay (●) as described in Section 3.2.4. Assays were performed at 2.0 mM MgATP and 0.5 mM T6P, using both purified T6PK (Figure A) and a freshly prepared cell-free extract (Figure B). On addition of 100 mM KCl to the aldolase assay system (×) the observed T6PK activity equalled that measured by the PK-LDH assay.

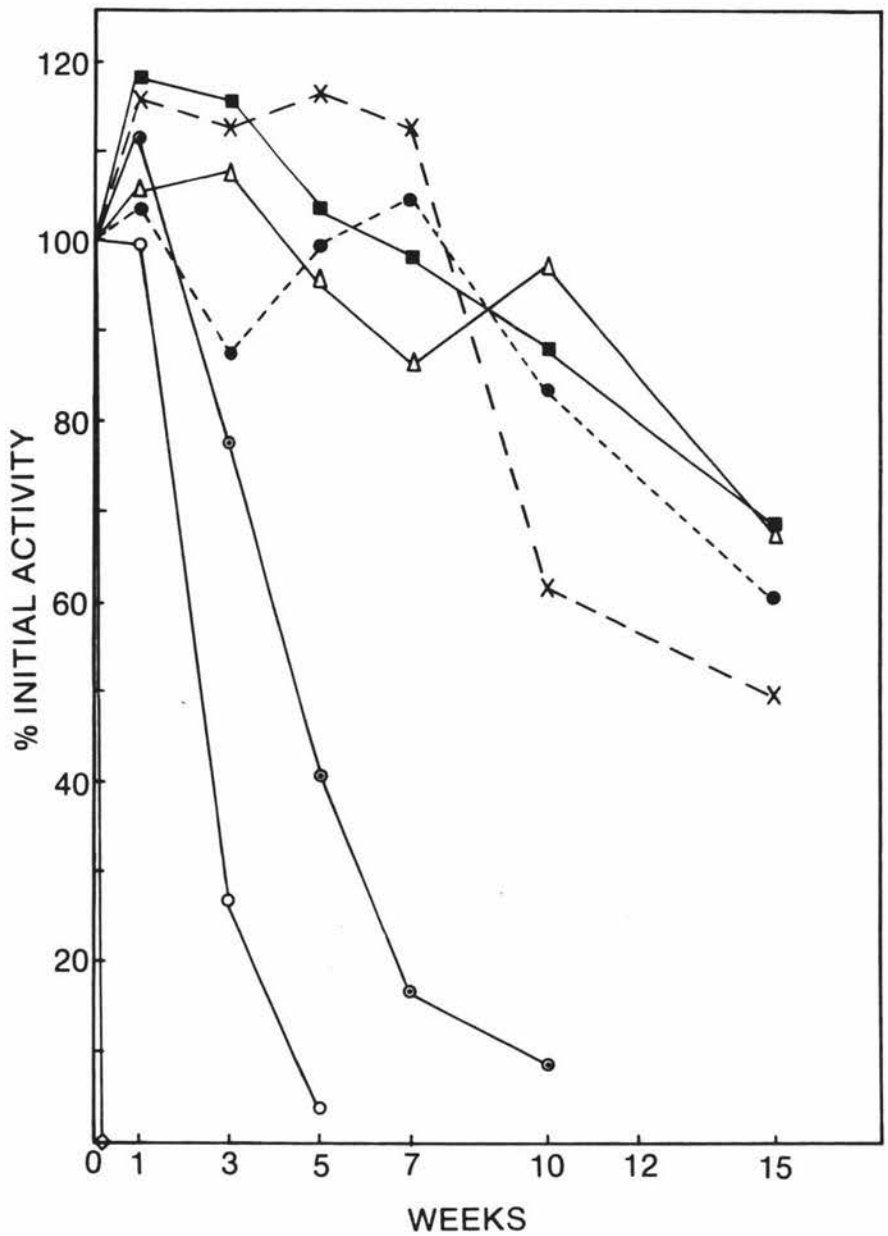


FIGURE 3.3.2 Stability of Purified T6PK. The effect of buffer composition on the stability of purified T6PK is shown in the above figure. Enzyme was stored at 4°C at a protein concentration of 0.13 mg ml^{-1} in the following buffers (each 50 mM containing 20% v/v glycerol): ●-Tris-HCl pH 6.0; ⊙-Tris-HCl pH 7.5; ○-Tris-HCl pH 8.0; ×-potassium phosphate buffer, pH 7.0. Activity was also measured after storage in 50 mM Tris-HCl, pH 7.5 containing 50% glycerol at -20°C, (△); in 50 mM Tris-HCl pH 7.5 buffer (4°C) without glycerol present (◇); and in 50 mM Tris-HCl pH 7.5 containing 5% (w/v) polyethylene glycol (■), at 4°C.
(PK/LDH assay).

activity remaining after 15 weeks. Removal of glycerol from the buffer, by passage through a 10 ml column of Sephadex G25 resulted in a very rapid inactivation of T6PK. The eluate from the column was turbid, and contained all the protein applied to the column but none of the T6PK activity. Phosphate ions enhanced stability; T6PK was more stable at pH 7.0 in phosphate-glycerol buffer than in Tris-glycerol buffer. Replacing the glycerol content of the Tris-glycerol buffer by 5% (w/v) PEG increased the stability of the enzyme, as did storage at -20°C after raising the glycerol content of the buffer to 50%. Enzyme stored in buffer containing 1 mM ADP, AMP, ATP, F6P, FBP, or T6P, or 2 M KCl was no more stable than enzyme stored in Tris-glycerol buffer alone (data not shown).

A notable feature of the purified enzyme is the dependence of activity on glycerol. *S. lactis* PFK was also less stable in the absence of glycerol (Section 2.3.2) but did not show the complete dependence shown by T6PK. Glycerol has been previously used as a stabiliser in purification of microbial enzymes (Smart, 1980) but the mechanism by which glycerol enhances enzyme stability is not known.

Although purified T6PK lost activity almost immediately on removal of glycerol, activity in cell-free extracts did not appear to be dependent on glycerol, possibly because of the high protein concentration, or the presence of other stabilising factors in these extracts. Activities of T6PK in cell-free extracts prepared in the presence and absence of glycerol were similar, although the long-term stabilities of the two were not investigated. T6PK was most stable at pH 6.0, although at this pH activity was only 50-75% of the activity at pH 7.0-8.0.

3.3.3 The Effect of pH on T6PK Activity

The optimum pH for T6PK activity was 7.8-8.2 in Tris-HCl, Tricine-KOH and HEPES-KOH buffers (Figure 3.3.3A). The specific activity of the enzyme (approximately 15 enzyme units mg^{-1}) was was similar in all three buffers. In a mixture of Tricine, MES and HEPES buffers (40 mM in each buffer) enzyme activity remained fairly constant over a pH range of 6.8-8.0, decreasing at pH's less than 6.0 or greater than 8.0 (Figure 3.3.3B).

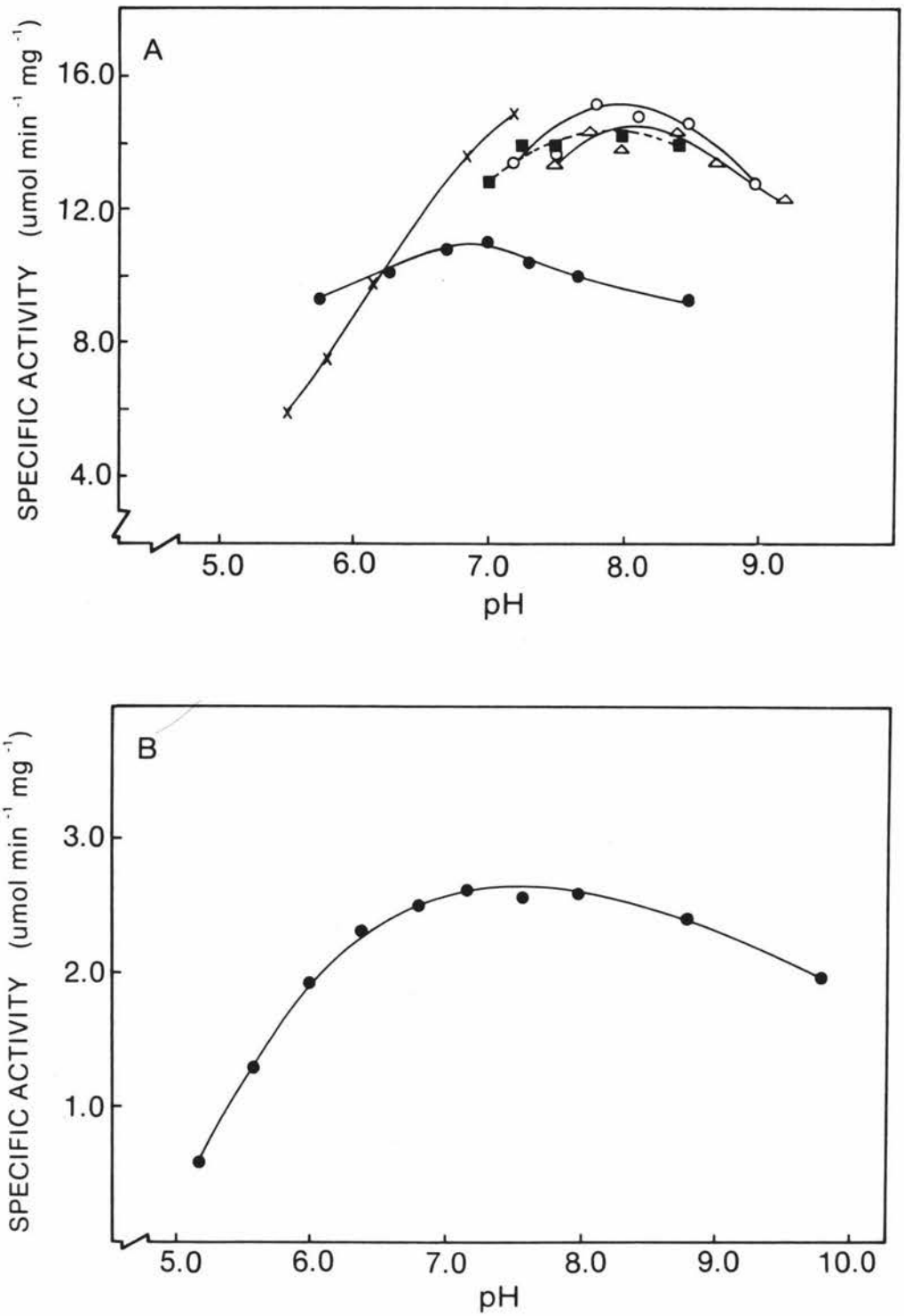


FIGURE 3.3.3 The Effect of pH on PFK Activity. Purified T6PK (5.0 µg per assay) was assayed in Tris-HCl (○), MES-KOH (×), Tricine-KOH (△), HEPES-KOH (■), and potassium phosphate (●) buffers. Each assay contained 50 mM buffer of the pH indicated.

In Figure B the assay buffer used was a mixture of MES, Tricine and HEPES (40 mM of each buffer).
(PK/LDH assay).

The pH profile obtained for T6PK is similar to those obtained for *S. lactis* PFK (see Chapter 2) and *S. aureus* T6PK (Bissett, 1975; Bissett & Anderson, 1980*a*) which show maximum activities at pH 7.5 and 8.0-8.5 respectively. Phosphate buffer decreases both the pH optimum and the maximum activity of T6PK. Similar effects were found for the T6PK from *S. aureus* (Bissett & Anderson, 1980*a*) and for PFK from *S. lactis* (Section 2.3.3).

3.3.4 MOLECULAR WEIGHT DETERMINATION

The molecular weight of purified T6PK, as determined by gel filtration on Sephacryl S200 (see Section 2.2.9) was estimated as 128,000 and 115,000 daltons in two separate determinations (Figure 3.3.4). To ensure that this was not a dissociated form of the enzyme, the molecular weight of T6PK in a cell-free extract was determined. Elution of T6PK after passage of the cell-free extract through the Sephacryl column indicated a molecular weight of 98,500 daltons, a value comparable to the molecular weight obtained for the purified enzyme. *S. lactis* T6PK therefore appears to be similar in size to the *S. aureus* enzyme which has a molecular weight of 104,000 daltons, and is composed of two subunits, each 52,000 daltons (Bissett & Anderson, 1980*a*). A subunit molecular weight for *S. lactis* T6PK could not be obtained since the enzyme was not homogeneous. Although there was only one major protein band on electrophoresis of the native protein (Plate IV) several protein bands of approximately equal intensity were obtained on SDS gel electrophoresis of the T6PK preparation, none of which could be positively identified as a T6PK subunit. Because of the similarity in size of the undenatured T6PKs from *S. lactis* and *S. aureus*, it is possible that *S. lactis* T6PK, like the *S. aureus* enzyme is a dimer of identical subunits. However the possibility of smaller or non identical subunits cannot be discarded.

PFK and T6PK from *S. lactis* were sufficiently different in size to allow complete separation of the two enzymes by passage through a Sephacryl S200 column. It is obvious, therefore, that these two enzymes are quite distinct both physically and kinetically (see Chapter 5 for a comparison of the kinetic properties of *S. lactis* PFK and T6PK.

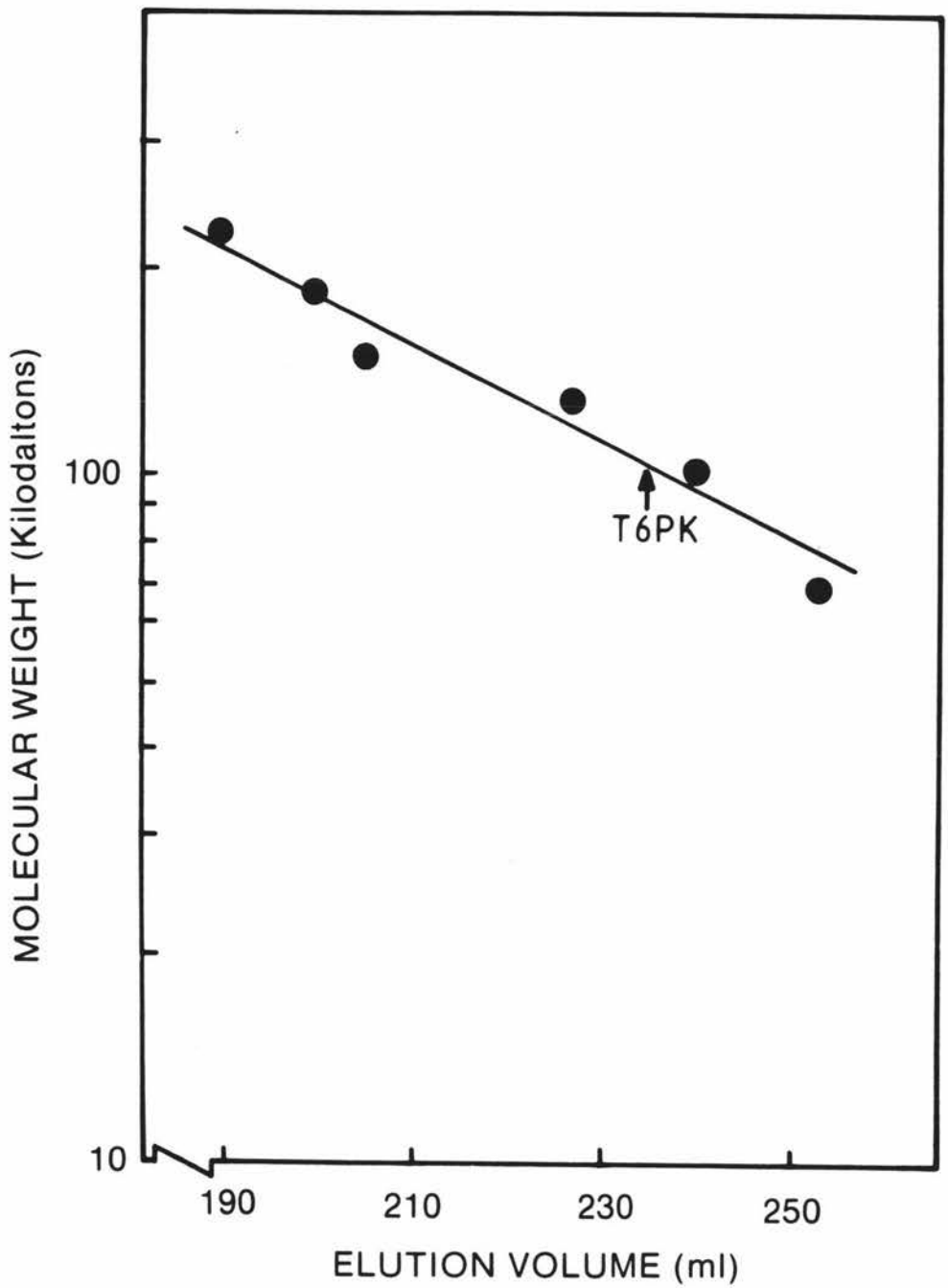


FIGURE 3.3.4 Molecular weight determination of T6PK by Gel Filtration on Sephacryl S200. Purified T6PK was chromatographed on Sephacryl S200 and the elution volume compared with those of the standard proteins listed in Table 2.2.1. A molecular weight of 115,000 daltons for native T6PK is indicated in this figure.

3.3.5 The Effect of ATP Concentration on T6PK Activity

As noted in Chapter 2 (Sections 5 and 6) activity of an enzyme requiring both Mg^{2+} and ATP for activity can be affected not only by the concentration of MgATP (the active complex) but also by the concentrations of free ATP and Mg^{2+}. The concentration of ATP is also dependent on the initial concentrations of Mg^{2+} and ATP (see Appendix 2).

At a constant Mg^{2+} concentration of 1.0 mM, T6PK showed maximum activity at 0.8 mM ATP at 0.5 mM T6P and at 0.6-0.8 mM ATP at 0.1 mM T6P (Figure 3.3.5). Since it was subsequently shown that a Mg^{2+} concentration of 5-10 mM was required for maximum activity at 1.0 mM ATP (i.e. a Mg^{2+}:ATP ratio of 5:1 - 10:1) (Figure 3.3.7), maximum activity at 1.0 mM Mg^{2+} was expected to be achieved at an ATP concentration of 0.1-0.2 mM. Thus the ATP concentration giving maximum activity in the experiment shown in Figure 3.3.5 is unexpectedly high. However the specific activity of T6PK was only 40% of the activity obtained at optimal Mg^{2+} concentrations which suggests that Mg^{2+} was limiting activity. The maximum specific activity of 1.1 µmol T6P min^{-1} mg^{-1} at 0.5 M T6P compares well with results from a subsequent experiment (Figure 3.3.12) in which a specific activity of 1.25 µmol T6P min^{-1} mg^{-1} was obtained when a constant Mg^{2+}:ATP ratio of 1:1 was maintained at saturating concentrations of T6P.

At a constant Mg^{2+} concentration of 1.0 mM, ATP concentrations greater than 0.8 mM were inhibitory. A double reciprocal plot (Figure 3.3.6) shows the upward curve towards the ordinate which is typical of substrate inhibition, and the K_m value for ATP was estimated as 0.33 mM at 0.5 mM and 0.1 mM T6P.

Although higher concentrations of ATP were inhibitory when the Mg^{2+} concentration was maintained at 1.0 mM, when Mg^{2+} was present in a 0.5 mM excess over ATP concentration no inhibition of T6PK by ATP was observed. Figure 3.3.10 shows that no ATP inhibition is apparent at 2.0 mM MgATP even at T6P concentrations as low as 0.1 mM. However with a Mg^{2+} concentration of 1.0 mM, 2.0 mM ATP resulted in 40-50% inhibition at both 0.1 mM and 0.5 mM T6P (Figure 3.3.5).

In contrast to this, *S. lactis* PFK was inhibited by high concentrations of MgATP relative to F6P concentration even when Mg^{2+} was present in excess. The concentration of MgATP required to

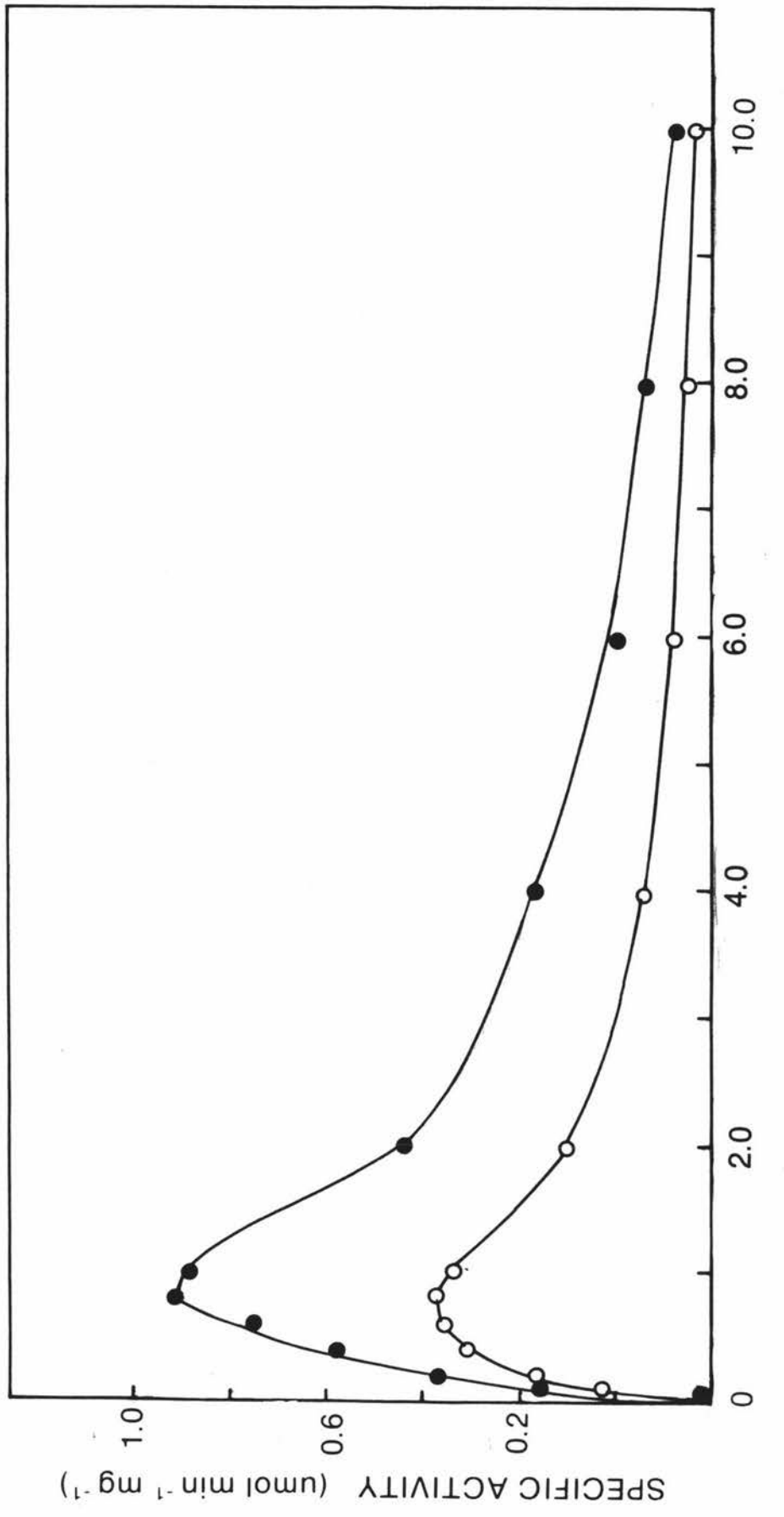


FIGURE 3.3.5 The Effect of ATP Concentration on T6PK Activity. Purified T6PK (2.5 - 5 µg per assay) was assayed at varying ATP concentrations and at 0.1 mM (O) and 0.5 mM (●) T6P, using the aldolase assay system described in Section 3.2.4. The Mg^{2+} concentration was maintained at 1.0 mM in all assays.

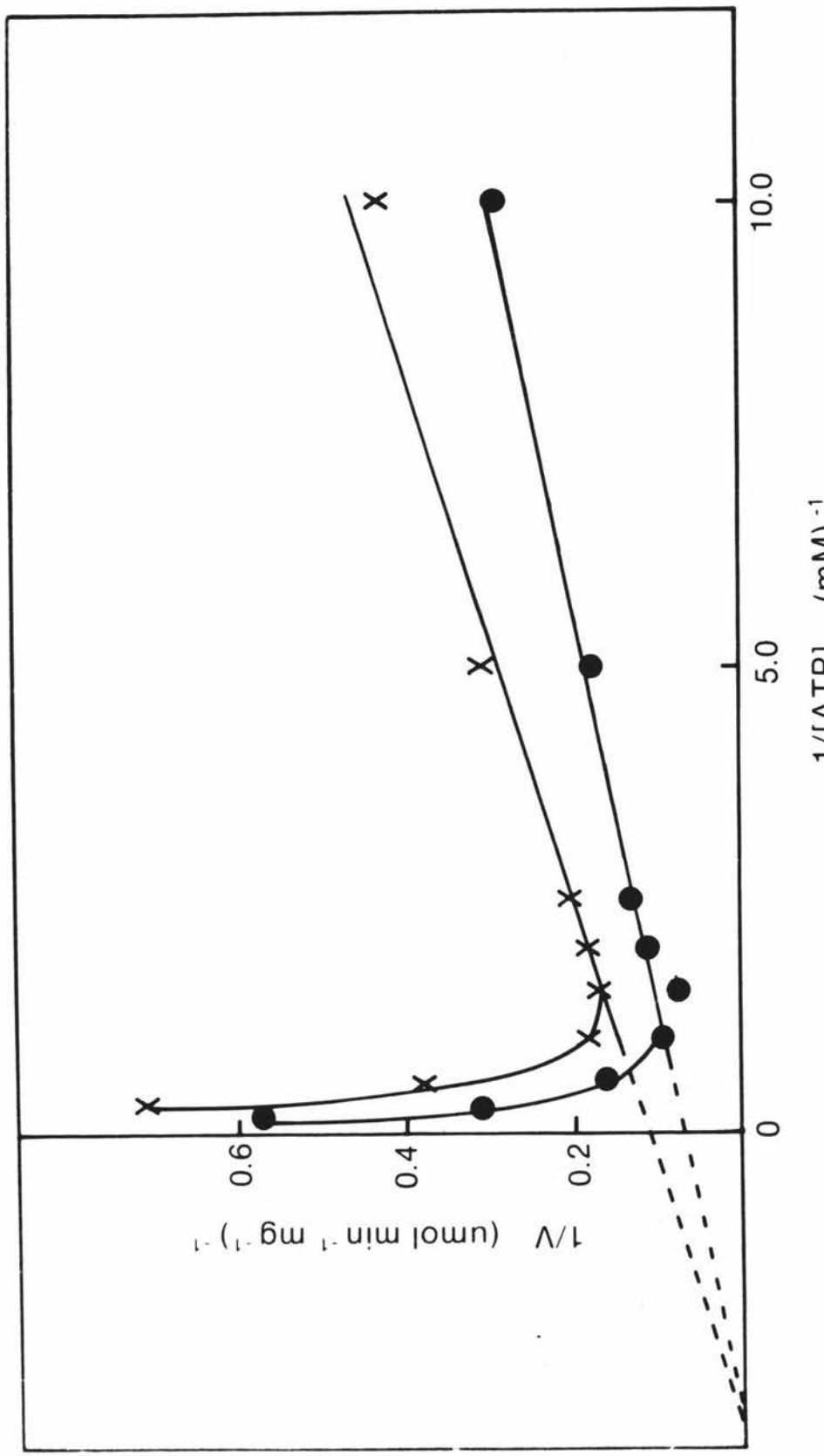


FIGURE 3.3.6 Lineweaver-Burk Plot : The Effect of ATP on T6PK Activity. Data from Figure 3.3.5 are plotted on this double reciprocal plot. The upward curvature of the lines reflects inhibition by increasing concentrations of ATP. Assays were performed using 2.5 - 5.0 µg purified T6PK, at a constant Mg^{2+} concentration of 1.0 mM and at 0.1 mM (×) and 0.5 mM (●) T6P.

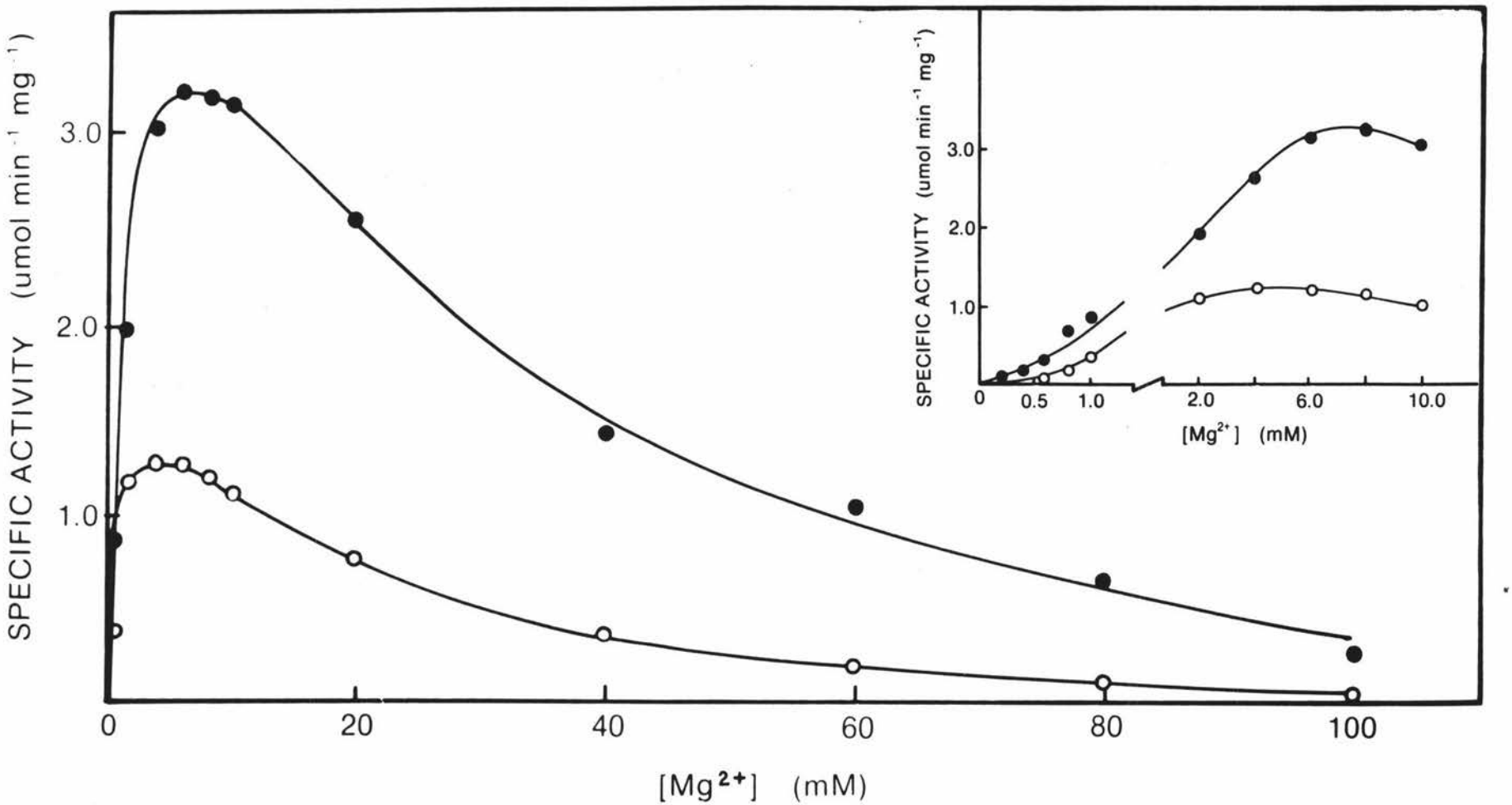


FIGURE 3.3.7 The Effect of Mg^{2+} Concentration on T6PK Activity. Purified T6PK (2.5 - 5 µg per assay) was assayed at varying Mg^{2+} concentrations and at 0.1 mM (O) or 0.5 mM (●) T6P, using the aldolase assay system described in Section 3.2.4.

The ATP concentration was maintained at 1.0 mM in all assays.

The inset clearly shows the co-operativity of Mg^{2+} binding at both 0.1 mM and 0.5 mM T6P.

produce inhibition of PFK was dependent on the F6P concentration, and inhibition could be overcome by increasing the F6P concentration. *S. lactis* T6PK therefore appears to show similar properties to the *S. aureus* enzyme which also does not exhibit T6P-dependent inhibition by MgATP.

3.3.6 The Effect of Mg^{2+} Concentration on T6PK Activity

T6PK was shown to require a divalent cation for activity. Of several divalent cations tested (see Section 3.3.7) Mg^{2+} was the most effective activator of T6PK. T6PK treated with 10 mM EDTA showed no activity in the absence of cation, hence divalent cations are essential for activity.

At a constant ATP concentration of 1.0 mM, T6PK required 5 mM Mg^{2+} for maximal activity at 0.1 mM T6P, and 7.5 mM Mg^{2+} at 0.5 mM T6P (Figure 3.3.7). Activity was inhibited at higher concentrations of Mg^{2+}. The observed inhibition of *S. lactis* T6PK by Mg^{2+} was not due to inhibition of the aldolase, α-glycero phosphate dehydrogenase, or triose phosphate isomerase in the assay mixture since addition of extra auxiliary enzymes to the inhibited reaction produced no increase in activity.

Binding of Mg^{2+} to T6PK is co-operative as shown by the sigmoidal curve in the inset to Figure 3.3.7. A Hill plot (Figure 3.3.8) indicates a Hill coefficient of 2.25, reflecting the co-operative binding, and a $Mg^{2+}_{0.5}$ of 1.7 mM. Similar values (n_H = 2.32, $Mg^{2+}_{0.5}$ = 1.4 mM) were obtained with a constant ATP concentration of 2.0 mM.

3.3.7 Specificity of Divalent Cation Requirement

Although optimum activity was found with Mg^{2+} as the cation cofactor, Co^{2+}, Mn^{2+}, Cu^{2+} and Ca^{2+} could also be used, optimum concentrations of these ions giving 64%, 28%, 19% and 3% respectively of the maximum activity obtained with Mg^{2+} as cofactor (Figure 3.3.9). No activity was found in the presence of Zn^{2+} or Ni^{2+}. Similar results have been reported for *S. aureus* T6PK (Bissett & Anderson, 1980*a*). Activities of *S. aureus* T6PK in the presence of various divalent cations are compared with the results obtained for *S. lactis* in Table 3.3.1.

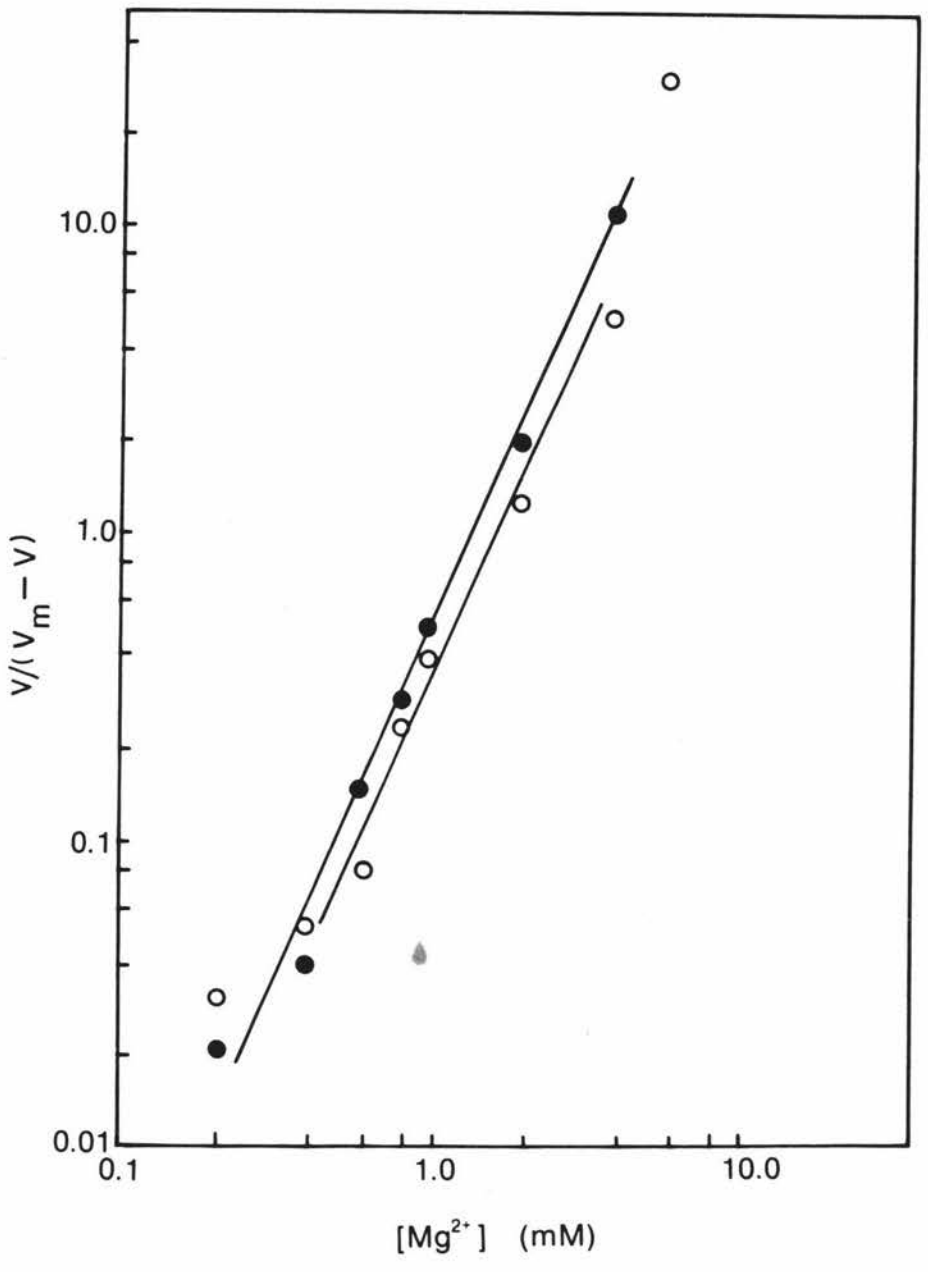


FIGURE 3.3.8 Hill plot of data obtained at constant ATP and varying Mg^{2+} concentrations. The two lines show the results of assays at 0.1 mM (O) and 0.5 mM (●) T6P, each at 1.0 mM ATP.

$Mg^{2+}_{0.5}$ values of 1.4 mM and 1.7 mM, and n_H values of 2.32 and 2.25 were obtained at 0.1 mM and 0.5 mM T6P respectively.

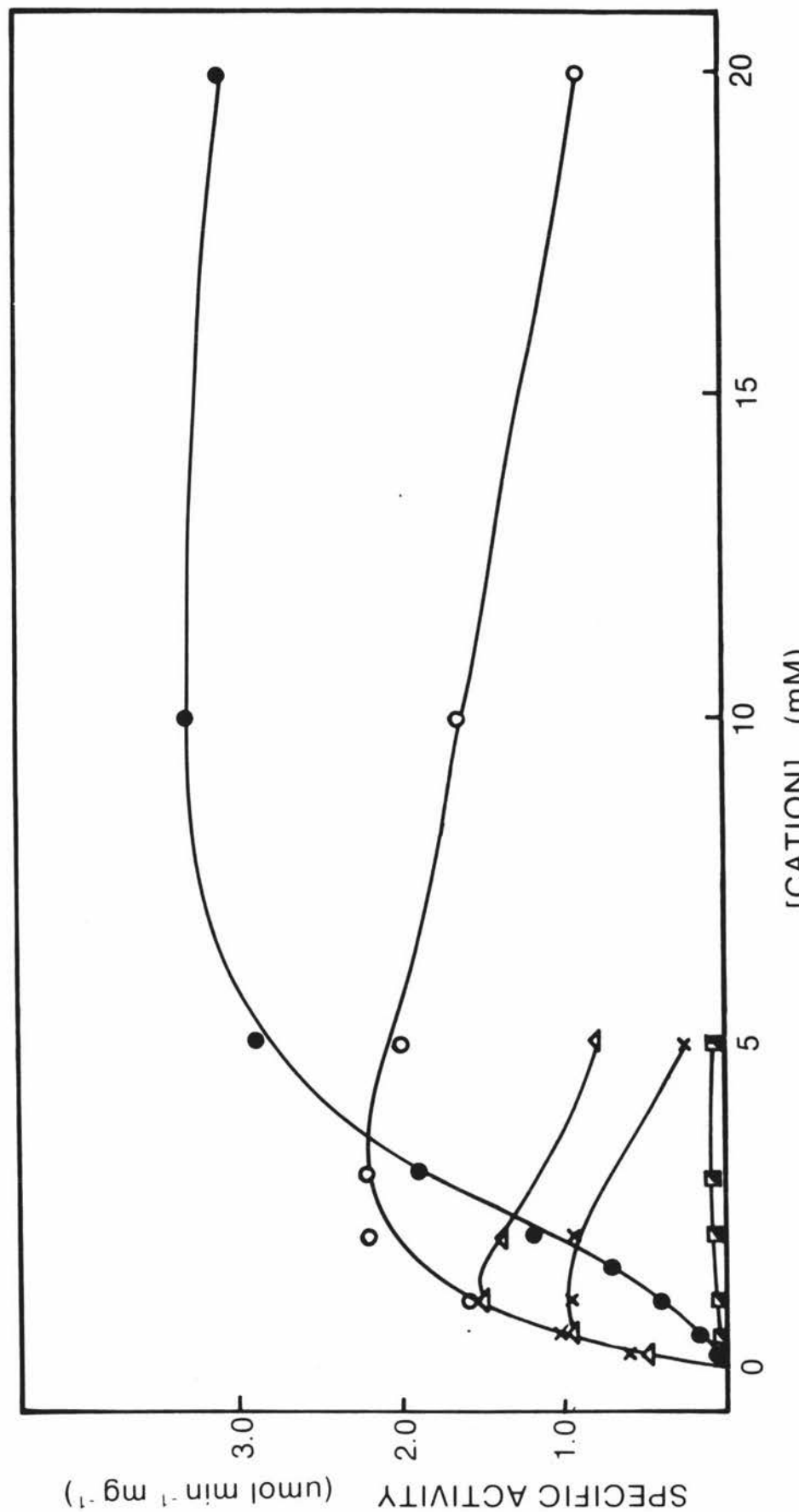


FIGURE 3.3.9 The Effect of Divalent Cations on T6PK Activity. T6PK was assayed at 2.0 mM ATP and 1.0 mM T6P in the presence of 0 - 20 mM concentrations of Mg^{2+} (●); Co^{2+} (○); Mn^{2+} (△); Cu^{2+} (×); Ca^{2+} (□); and Ni^{2+} and Zn^{2+} (◪).

Each assay contained 2.5 µg purified enzyme.

TABLE 3.3.1

DIVALENT CATION REQUIREMENTS OF T6PK FROM *S. lactis* AND *S. aureus*

Cofactor	% of Maximum activity with Mg^{2+}	
	S. lactis	*S. aureus**
Mg^{2+}	100	100
Co^{2+}	64	54
Mn^{2+}	28	27
Ni^{2+}	0	15
Cd^{2+}	ND	8
Ca^{2+}	3	3
Zn^{2+}	0	2
Fe^{2+}	-	0
Ba^{2+}	-	0
Cu^{2+}	19	0
0	0	0

* Taken from Bissett & Anderson, 1980*a*.

Assays were performed at saturating concentrations of ATP and F6P. ND denotes not assayed. Maximum activities could not be determined with Fe^{2+} and Ba^{2+} as the hydroxides precipitated from solution.

Only Mg^{2+} produced a sigmoidal binding curve. At saturating T6P and ATP concentrations the $Mg^{2+}_{0.5}$ value calculated from a Hill plot (data not shown) was 2.8 mM, compared to values of 1.4 mM and 1.7 mM determined previously (Figure 3.3.8). The Hill coefficient of 2.83 reflects the co-operativity of Mg^{2+} binding. Mn^{2+}, Co^{2+} and Cu^{2+} all exhibit lower K_m values ($\ll$1 mM) than that for Mg^{2+}, but maximum velocities with these ions are lower than that obtained with Mg^{2+}. This could be due to a difference in the equilibrium constants of formation of the ion-ATP complex which may result in different concentrations of the active ion-ATP complex depending on the cation used, even although the initial concentrations of metal ions and ATP are constant.

Higher concentrations of all cations were inhibitory. Cu^{2+} and Mn^{2+} inhibited activity at concentrations greater than 2 mM, while concentrations of Co^{2+} and Mg^{2+} greater than 5 mM and 10 mM respectively were required for inhibition.

3.3.8 Dependence of Activity on MgATP Concentration

Figure 3.3.10 shows the relationship between T6PK activity and MgATP concentration. When the Mg^{2+} concentration exceeded the ATP concentration by 0.5 mM the MgATP saturation curve was hyperbolic. A K_m (MgATP) of 0.4 mM was determined from the double reciprocal plot in Figure 3.3.11.

Conditions under which the MgATP binding curve appeared sigmoidal were found, but the Hill coefficient was low (<2.0) and the apparent sigmoidicity of the curve was a function of the Mg^{2+} and ATP concentrations used in the assay. Figure 3.3.12 shows that if the concentrations of Mg^{2+} and ATP are maintained at a constant ratio of 1:1 or 2:1 the saturation curves appear sigmoidal. Although the curve tends toward a more hyperbolic form when the Mg:ATP ratio is increased, the Hill coefficients are not markedly affected; n_H values of 1.2 - 1.8 were obtained with Mg^{2+}:ATP ratios of 1:1 - 10:1.

The sigmoidicity observed at low Mg^{2+}:ATP ratios (2:1 or less) may be an artefact due to limiting Mg^{2+} concentrations. Experiments described in Section 3.3.6 in which the ATP concentration was held constant and the Mg^{2+} concentration varied showed that at 1.0 mM ATP a concentration of Mg^{2+} of at least 5.0 mM is required for maximum

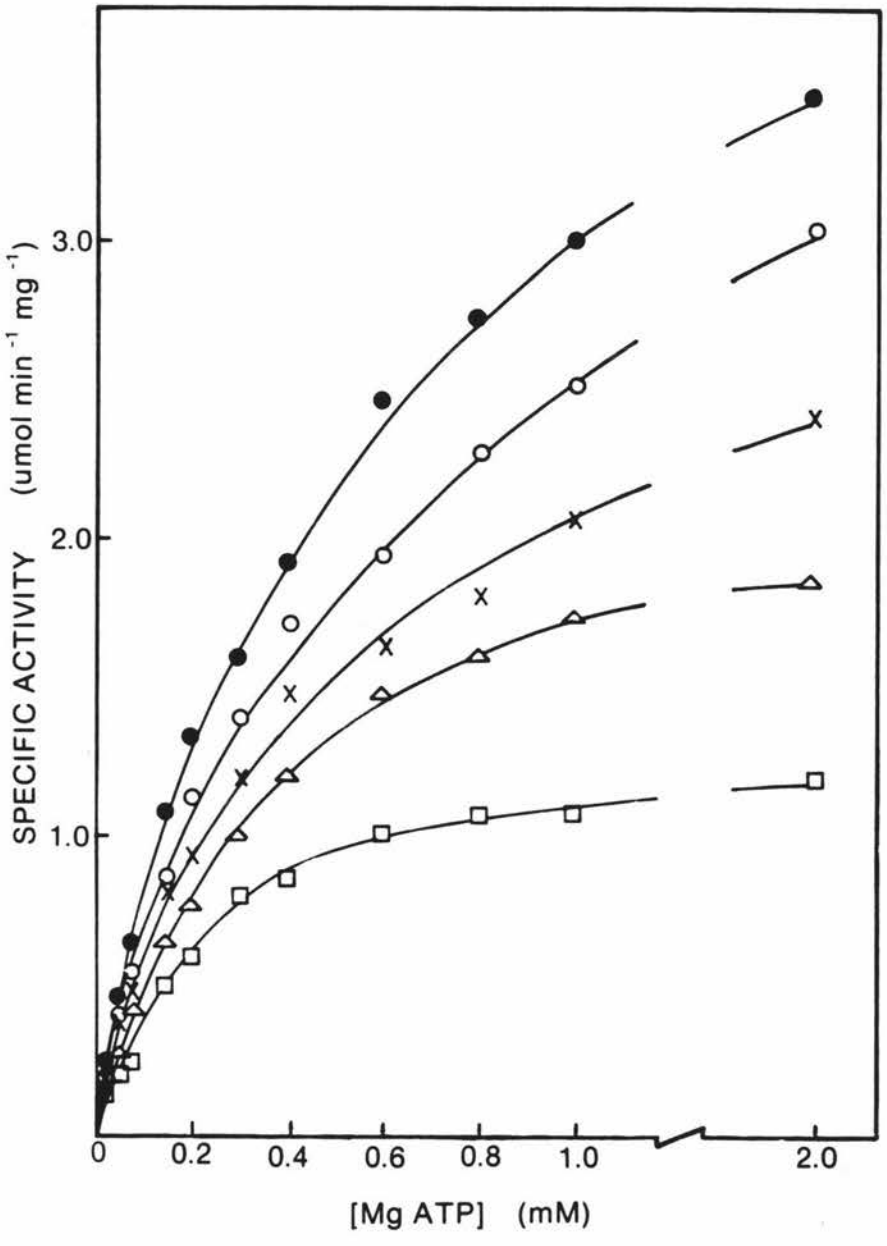


FIGURE 3.3.10 The Effect of MgATP Concentration on T6PK Activity. Purified T6PK (2.5 - 5 µg) was assayed at a constant T6P concentration and varying MgATP concentrations. Mg^{2+} was maintained in a 0.5 mM excess over ATP in all assays. T6P concentrations in each assay were: ● - 1.0 mM; ○ - 0.5 mM; × - 0.3 mM; △ - 0.2 mM; □ - 0.1 mM.

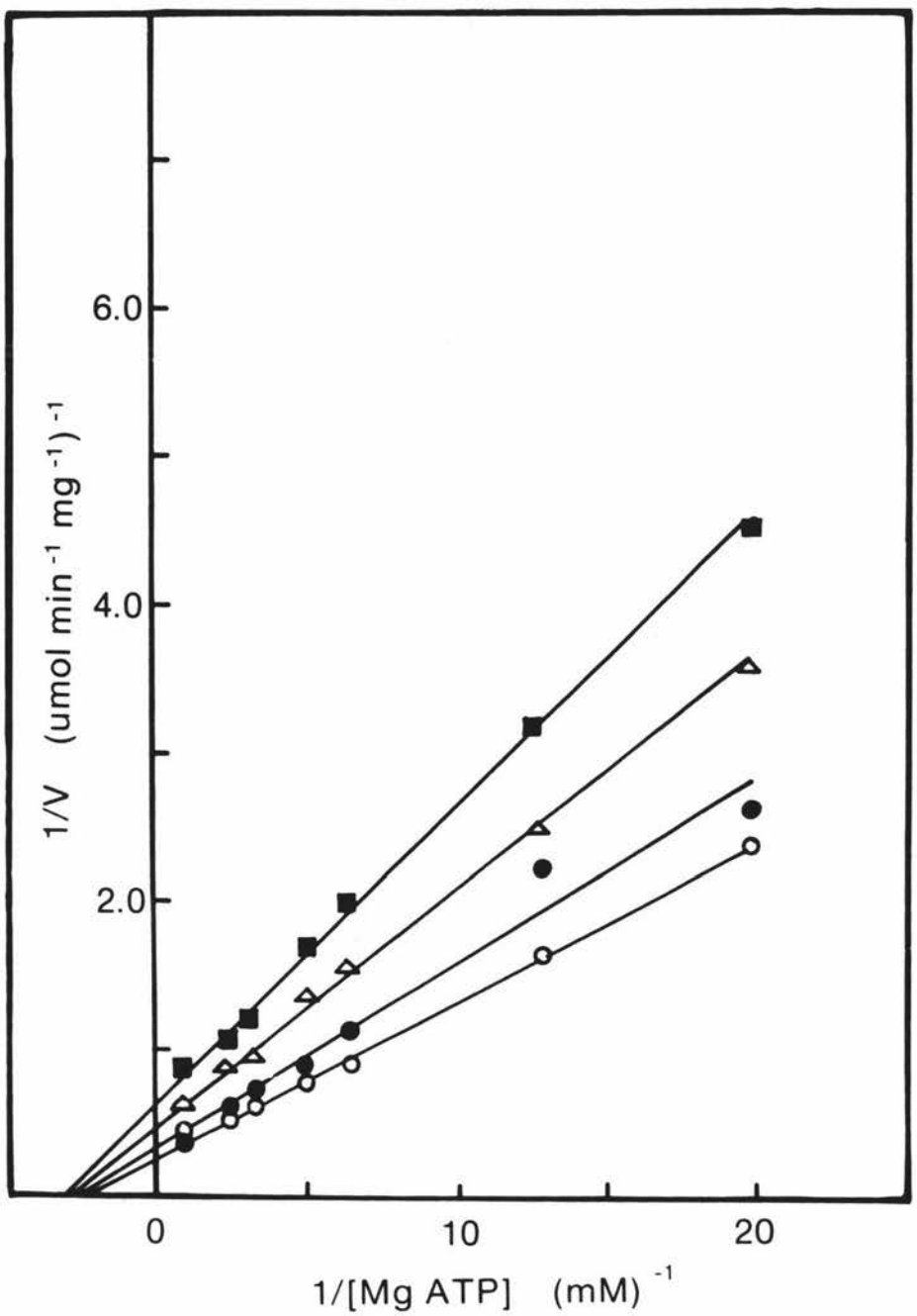


FIGURE 3.3.11 Lineweaver-Burk Plot : Determination of K_m (MgATP).
Data from Figure 3.3.10 are plotted on this double reciprocal plot. A K_m (MgATP) of approximately 0.4 mM was calculated from this figure.

Data from assays at 0.1 mM (■); 0.2 mM (△); 0.5 mM (●); and 1.0 mM (○) T6P are shown.

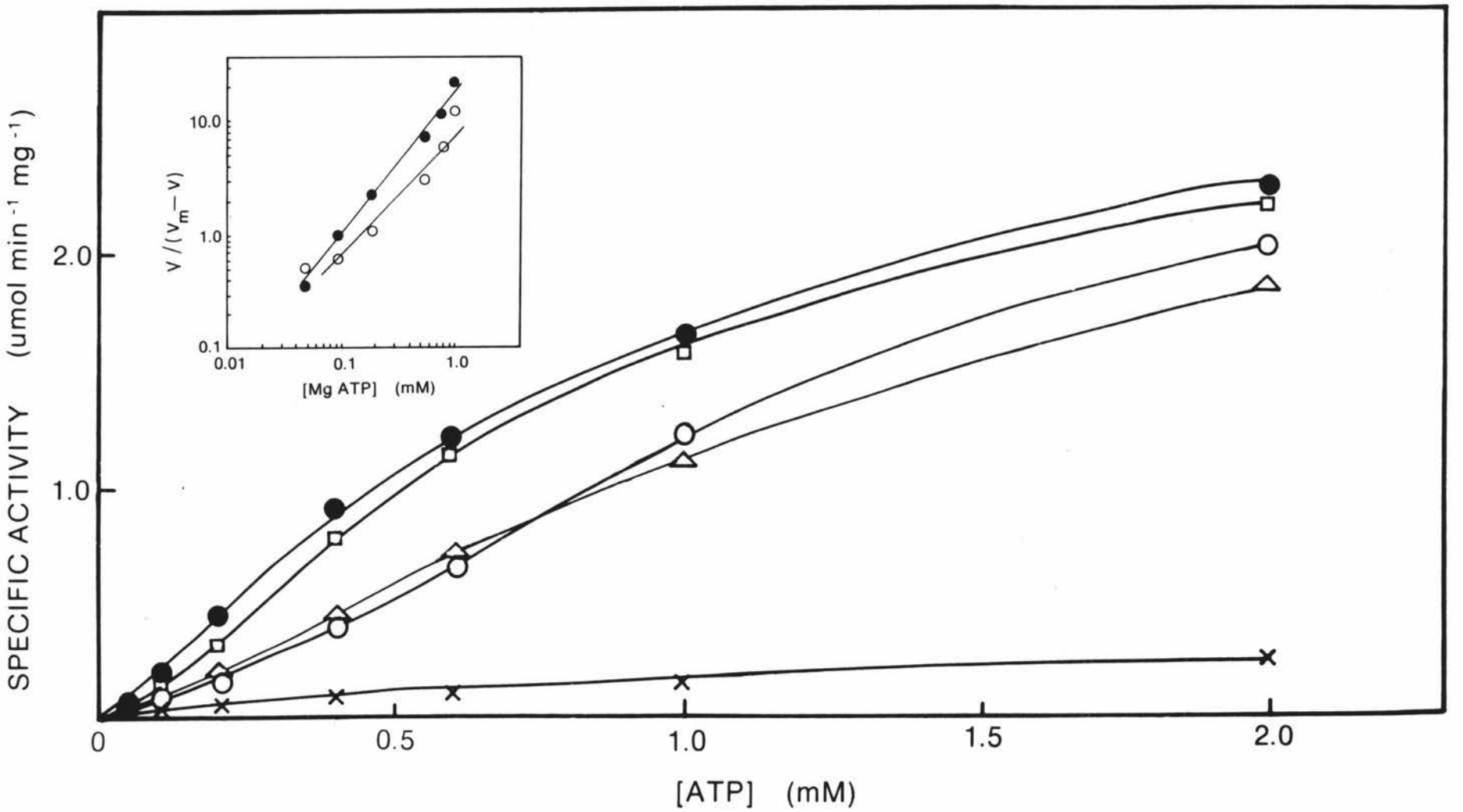


FIGURE 3.3.12 The Effect of Mg^{2+}:ATP ratios on the MgATP Saturation Profile. Purified T6PK was assayed at 1.0 mM T6P and varying concentrations of ATP, with Mg^{2+}:ATP ratios of 10:1 (●); 5:1 (□); 2:1 (△); 1:1 (○); and 0.5:1 (×).

Each assay contained 2.5 or 5 µg protein.

The Hill plot (inset) shows the curves obtained with Mg^{2+}:ATP ratios of 10:1 (●) and 2:1 (○).

activity (Figure 3.3.7). Hence ratios of Mg^{2+}:ATP of less than 5:1 may not be sufficient to produce maximum activity.

S. lactis T6PK therefore appears to be similar to the *S. aureus* enzyme in showing Michaelis-Menten kinetics with respect to both its substrates. No inhibition was apparent at high ratios of MgATP : sugar phosphate as was observed with PFK in *S. lactis*.

3.3.9 Dependence of Activity on T6P Concentration

In contrast to *S. lactis* PFK, T6PK exhibited no co-operative binding of T6P. The hyperbolic dependence on T6P concentration (Figure 3.3.13) was not due to accumulation of ADP in the assay (ADP shifts the F6P binding curve of PFK from a sigmoidal to a hyperbolic form) as a hyperbolic curve was also obtained when the PK-LDH assay system (in which ADP is not accumulated) was used. Also, in subsequent experiments ADP was shown to inhibit T6PK activity. The K_m (T6P) determined from the double reciprocal plot in Figure 3.3.14 is 0.16 mM. The K_m (T6P) was independent of the concentration of MgATP.

3.3.10 Effectors of T6PK Activity

A range of nucleotides, glycolytic intermediates and organic acids were tested for their effect on T6PK activity. In an initial survey assays were performed in the presence of 2.0 mM concentrations of each effector, at saturating T6P (1.0 mM) and non-saturating MgATP (0.2 mM) concentrations, and at non-saturating T6P (0.10 mM) and saturating MgATP (2.0 mM) concentrations. Effects on the K_m value for either substrate would thus be detected. The PK-LDH assay system was used in this survey.

In a second survey, the effects of a limited range of compounds was tested over a range of concentrations from 1-20 mM using the aldolase assay system.

Few compounds were found to affect T6PK activity. Apart from NH_4^+ and K^+ no activators of *S. lactis* T6PK were found. T6PK activity was stimulated 2-fold by K^+ (see Section 3.3.16). As previously mentioned (Section 3.3.1) this stimulation resulted in an apparent discrepancy in the two assay systems used. T6PK activity was approximately 2-fold higher in the PK-LDH assay (which contained

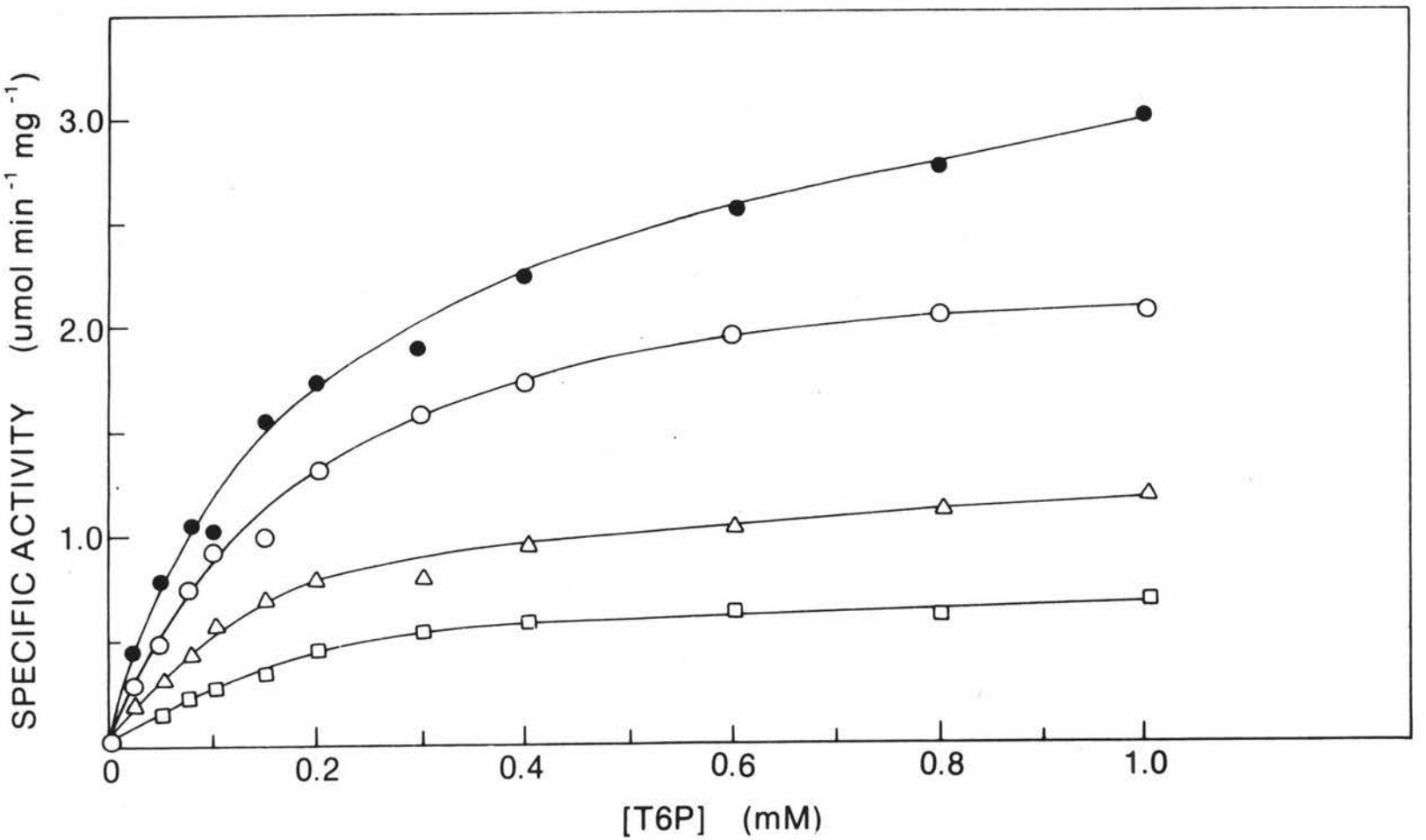


FIGURE 3.3.13 The Effect of T6P Concentration on T6PK Activity. Purified T6PK (2.5 µg per assay) was assayed using the aldolase assay system described in Section 3.2.4. The T6P concentration was varied between 0 and 1.0 mM as shown, and MgATP concentrations were kept constant at 1.0 mM (●); 0.5 mM (○); 0.2 mM (△); and 0.1 mM (□). A 0.5 mM excess of Mg^{2+} over ATP concentration was maintained.

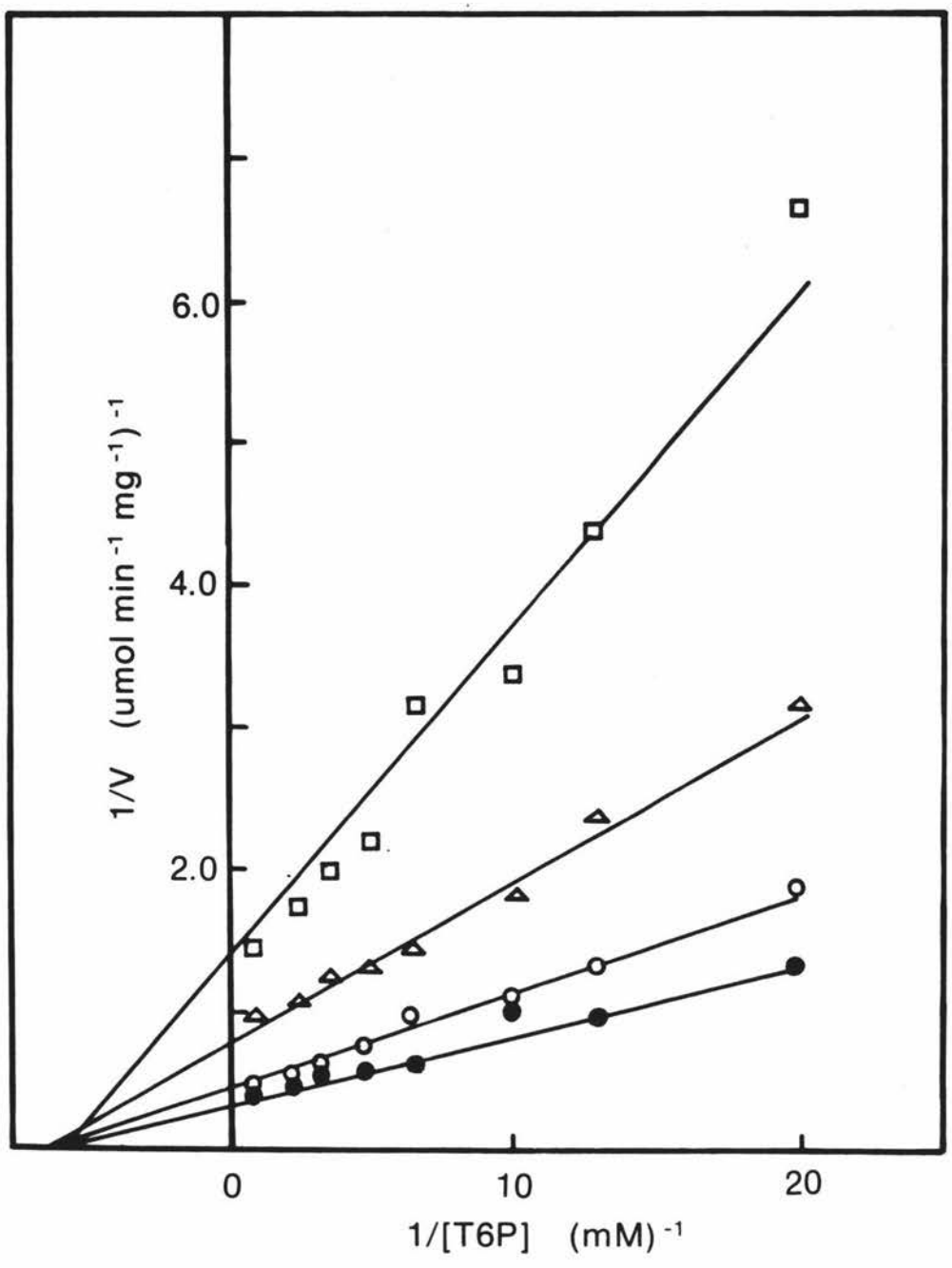


FIGURE 3.3.14 Lineweaver-Burk plot for the determination of K_m (T6P). The data shown (from Figure 3.3.13) indicate a K_m (T6P) of 0.18 mM. Each assay contained 2.5 - 5.0 µg protein and 0 - 1.0 mM T6P. Results shown on this graph are data from assays containing 0.1 mM (□); 0.2 mM (△); 0.5 mM (○); and 1.0 mM (●) MgATP.

100 mM KCl to activate the pyruvate kinase in the assay) than in the TBP-aldolase assay system in which KCl was absent.

Of the metabolites tested (Tables 3.3.2 and 3.3.3) only citrate, ADP and PEP showed any inhibitory effect on T6PK activity at 2.0 mM concentrations, while FBP inhibited activity only at higher concentrations (5-20 mM). Inhibition by citrate appeared to be solely due to Mg^{2+} complexing as activity could be restored to the level in the absence of citrate by addition of extra Mg^{2+}. This emphasises the importance of checking the dependence of apparent inhibitory effects on reaction conditions, especially when cofactors are required, or in coupled assay systems in which the activity of the auxiliary enzymes as well as the enzyme being assayed may be affected.

Inhibition by ADP, PEP, and FBP was generally more pronounced at non-saturating levels of MgATP than at non-saturating levels of sugar phosphate, a feature also noted with PFK from *S. lactis*.

The effects of ADP, PEP, F6P, and FBP were examined further (Sections 3.3.11 - 3.3.16).

3.3.11 The Effect of ADP on T6PK Activity

ADP was an inhibitor of T6PK activity (Figures 3.3.15A and 3.3.16A). Double reciprocal plots indicated competitive inhibition with respect to MgATP (Figure 3.3.16B) in which the K_m but not the V_{max} was affected, and non-competitive inhibition with respect to T6P (Figure 3.3.15B). This suggests that ADP either competes with ATP for Mg^{2+} or for a single binding site or binds at an allosteric site, the binding to which affects the affinity of the enzyme for ATP but not for T6P. K_i values for ADP calculated from plots of $\frac{1}{V}$ vs [ADP] (Figures 3.3.17A and B) are 0.75 mM (with varying MgATP concentrations) and 3.35 mM (with varying T6P concentrations). This difference in K_i (ADP) values may be due to the different concentrations of MgATP in the two sets of assays. With respect to the inhibition of T6PK by ADP, the *S. lactis* enzyme differs from *S. aureus* T6PK in that the activity of the latter enzyme was not affected by 2 mM ADP (Bissett & Anderson, 1980*a*). *S. lactis* PFK, in contrast to T6PK from the same organism, is strongly activated by ADP, concentrations as low as 0.1 mM causing a marked decrease in $F6P_{0.5}$.

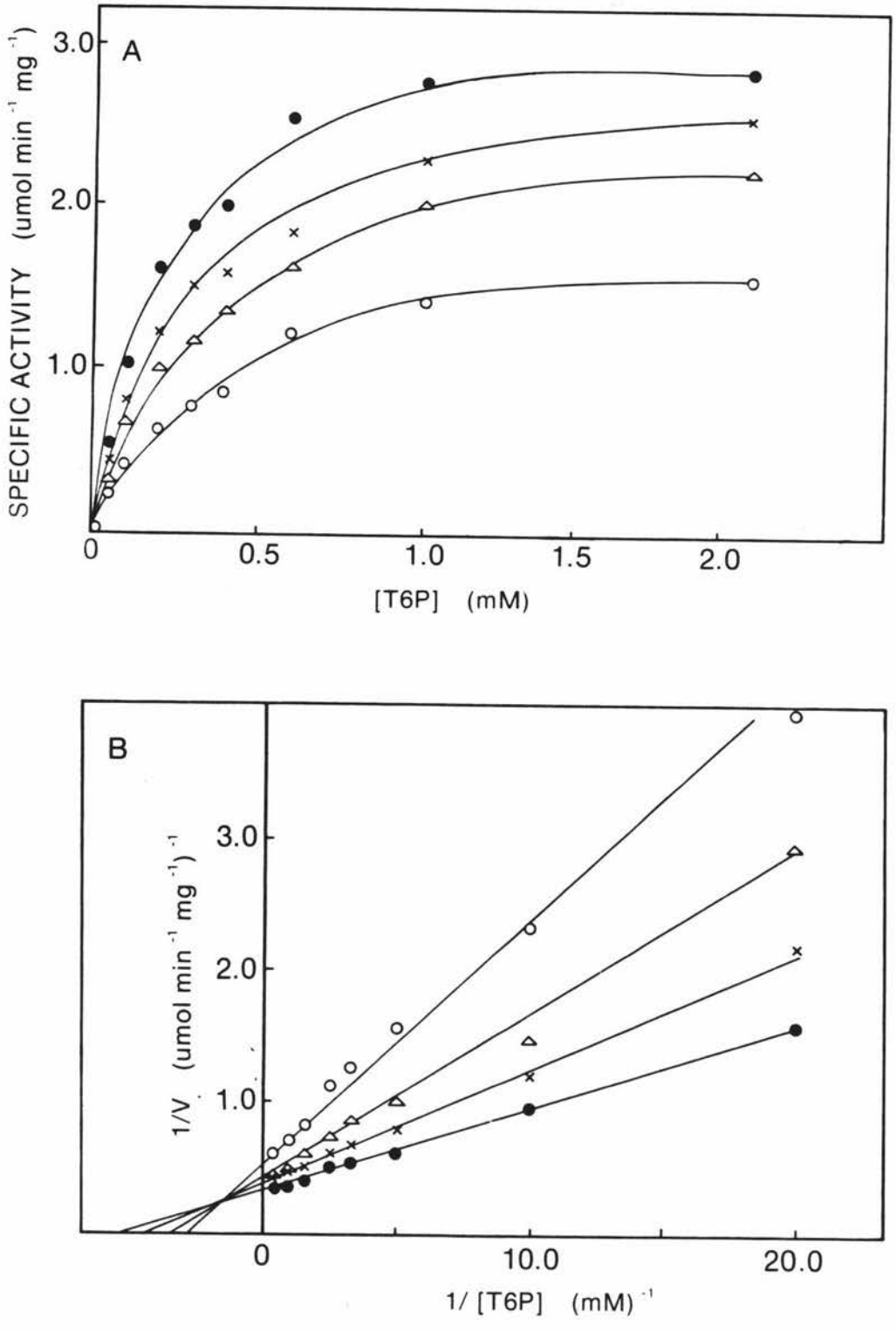


FIGURE 3.3.15 The Effect of ADP on T6PK Activity at Different T6P Concentrations. Inhibition of T6PK activity by ADP is shown in Figure A. Purified T6PK (2.5 - 5 µg per assay) was assayed at 2.0 mM MgATP and at 0 - 2.0 mM T6P, and with ADP concentrations of: ● - 0 mM; × - 1.0 mM; △ - 2.0 mM; ○ - 5.0 mM.

Data from Figure A are plotted as a double reciprocal plot in Figure B, which indicates non-competitive inhibition by ADP with respect to T6P.

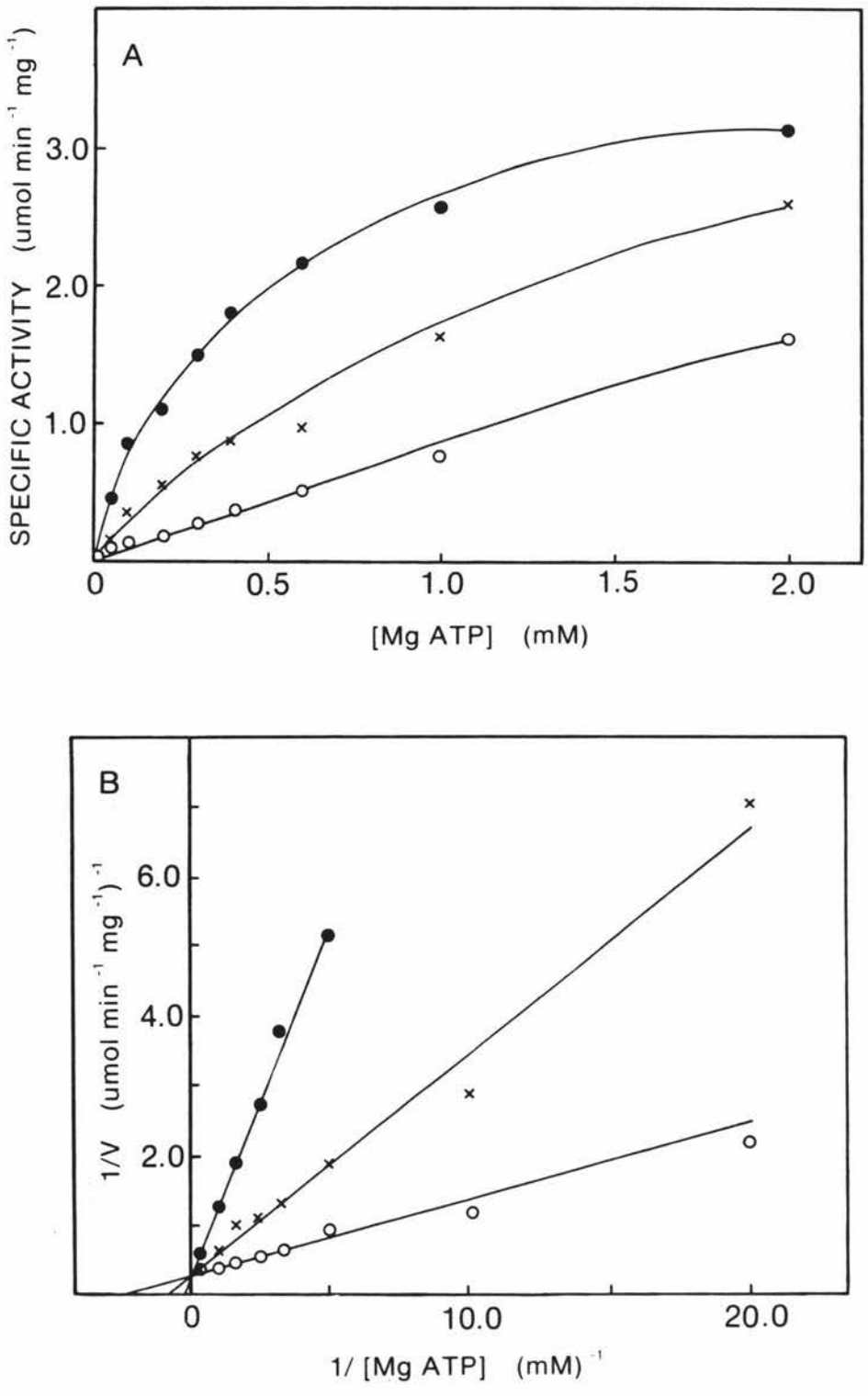


FIGURE 3.3.16 The Effect of ADP on T6PK Activity at Different MgATP Concentrations. Purified T6PK (2.5 - 5 µg per assay) was assayed at 1.0 mM T6P and at 0 - 2.0 mM MgATP concentrations, at ADP concentrations of: ● - 0 mM; × - 1.0 mM; O - 5.0 mM (Figure A). Data from Figure A are plotted as a double reciprocal plot in Figure B. The intersection of the lines on the ordinate indicates competitive inhibition by ADP with respect to MgATP.

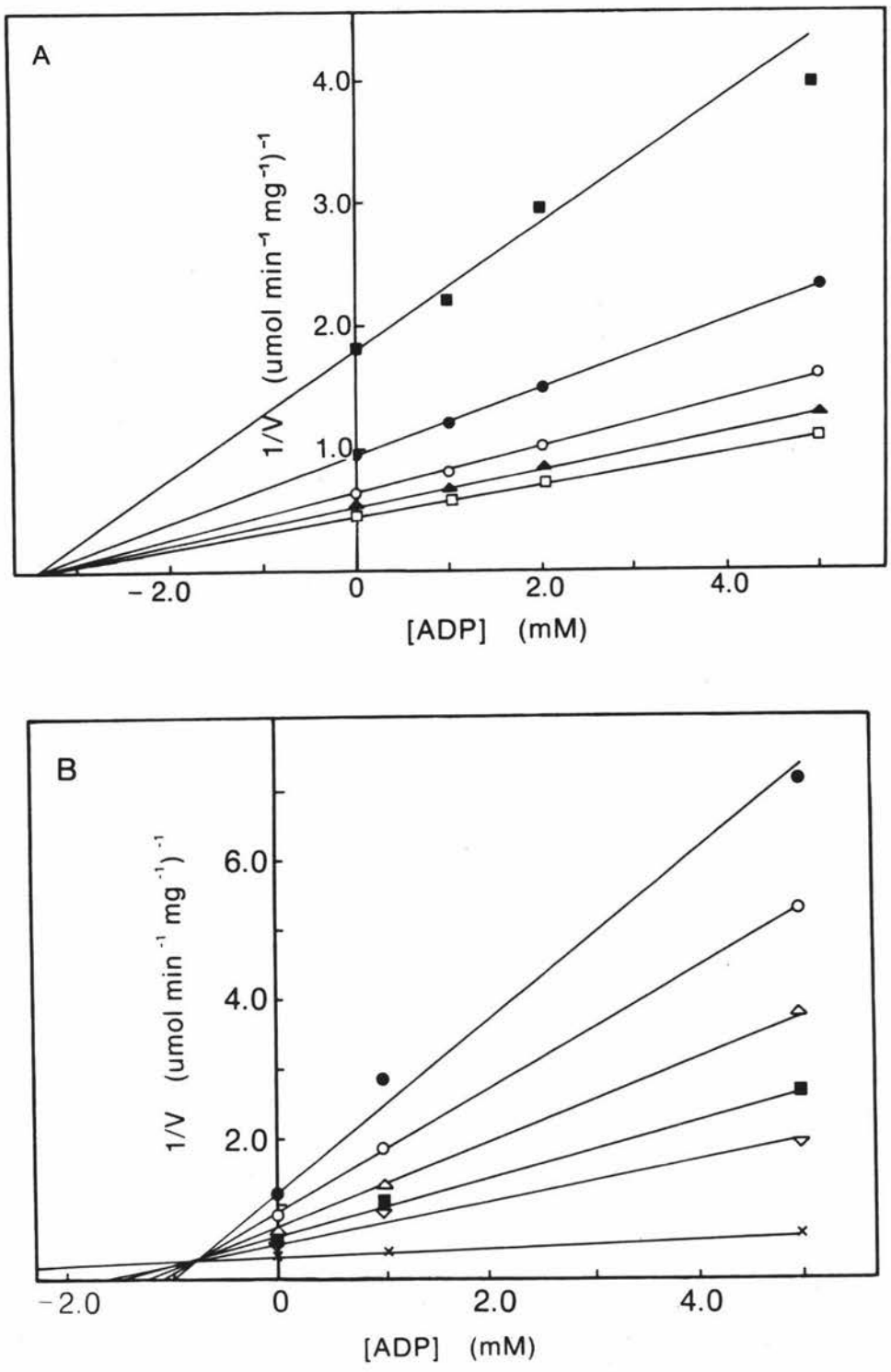


FIGURE 3.3.17 $\frac{1}{V}$ vs [ADP] plots.

In Figure A, MgATPconcentration was 2.0 mM in all assays, and the T6P concentrations shown on the graph are ■ - 0.1 mM; ● - 0.2 mM; ○ - 0.3 mM; △ - 0.4 mM; × - 0.6 mM. A K_i (ADP) of 3.35 mM is indicated by the intersection of the lines.

In Figure B, T6P concentration was maintained at 1.0 mM while the MgATPconcentration was varied ● - 0.05 mM ○ - 0.1 mM △ - 0.2 mM ■ - 0.3 mM ▽ - 0.4 mM × - 1.0 mM These data indicate a K_i (ADP) of 0.75 mM.

TABLE 3.3.2

THE EFFECT OF VARIOUS METABOLITES ON T6PK ACTIVITY

Effector	Substrate Concentration	
	1.0 mM T6P 0.2 mM MgATP	0.1 mM T6P 2.0 mM MgATP
0	100	100
AMP	104	98
ADP	39	70
PEP	83	83
F6P	98	92
Gal 6P	94	96
Glu 6P	97	102
Glucose	90	102
Ribose 5P	90	91
Acetate	84	98
Lactate	100	106
Citrate*	39	69
3 PGA	104	102

* Inhibition could be overcome by adding excess Mg^{2+}.

Each effector was tested at a concentration of 2.0 mM in the presence of the MgATP and T6P concentrations shown.

Figures listed are the % of the activity observed in the absence of any effector.

TABLE 3.3.3

THE EFFECT OF VARIOUS CONCENTRATIONS OF METABOLITES ON T6PK ACTIVITY

Effector	Substrate Concentrations (mM)		Effector Concentration (mM)				
	T6P	MgATP	1.0	2.5	5.0	10.0	20.0
Glu 6P	2.0	2.0	96	97	89	-	-
	0.1	2.0	108	97	97	-	-
	1.0	0.2	92	91	89	-	-
Gal 6P	2.0	2.0	99	92	94	-	-
	0.1	2.0	108	100	96	-	-
	1.0	0.2	84	81	75	-	-
FBP	2.0	2.0	-	-	89	88	72
	0.1	2.0	-	-	95	79	40
	1.0	0.2	-	-	66	59	37
Lactose	2.0	2.0	107	-	107	104	-
	0.1	2.0	96	-	98	93	-
	1.0	0.2	99	-	97	95	-
Ribose 5P	2.0	2.0	109	-	114	99	-
	0.1	2.0	109	-	109	82	-
	1.0	0.2	100	-	100	95	-

Each effector was tested over the range of concentrations shown and at the MgATP and T6P concentrations indicated.

Figures listed are the % of the activity observed in the absence of any effector.

3.3.12 The Effect of F6P on T6PK Activity

The intracellular concentration of F6P in *S. lactis* is approximately 0.26 mM (Thompson, 1978, 1979). Although at this concentration, F6P had no effect on T6PK activity the effect of F6P concentrations up to 20 mM was investigated to determine the nature of binding of F6P to T6PK. Figure 3.3.18A shows the effects of 5-20 mM F6P on T6PK assayed at 2.0 mM ATP and increasing T6P concentrations. F6P concentrations less than 5 mM had little effect on activity. 10 mM and 20 mM F6P produced 20-35% inhibition at most T6P concentrations. However this inhibition is not likely to be significant *in vivo* as F6P does not accumulate to these levels in the cell. The scatter in points of the double reciprocal plot (Figure 3.3.18B) does not indicate a clear inhibition pattern. However the maximum velocity reached appears to decrease with increasing inhibitor (F6P) concentration, a feature typical of non-competitive inhibition. As T6PK is able to phosphorylate F6P, (Section 3.3.13) F6P must bind at the catalytic site, hence competitive inhibition between F6P and T6P is expected. However the enzyme may contain an alternative binding site to which F6P binds in the presence of T6P, due to the lower affinity of T6PK for F6P.

3.3.13 Phosphorylation of F6P by T6PK

T6PK was assayed for activity with F6P as substrate using the FBP-aldolase linked assay described in Section 2.2.6. The maximum specific activity of the enzyme calculated from the intercept on the ordinate of the double reciprocal plot (Figure 3.3.20A) was 0.33 µmol min^{-1} mg^{-1}, approximately 10-fold lower than the specific activity obtained with T6P as substrate. T6PKexhibited a hyperbolic dependence on F6P concentration (Figure 3.3.19) as was the case with T6P. A Hill plot (Figure 3.3.20B) gave a Hill coefficient of 1.03 and a K_m F6P value of 42 mM. The K_m (F6P) determined from a double reciprocal plot was approximately 40 mM, hence the affinity of T6PK for F6P is approximately 250-fold less than the affinity of the enzyme for T6P. (K_m (T6P) = 0.16 mM).

T6PK from *S. aureus* exhibits an even greater difference in affinities for F6P and T6P, the respective K_m values being 150 mM and 16 µM (Bissett & Anderson, 1980*a*). However unlike the *S. lactis* enzyme, *S. aureus* T6PK attained equal velocities at saturating

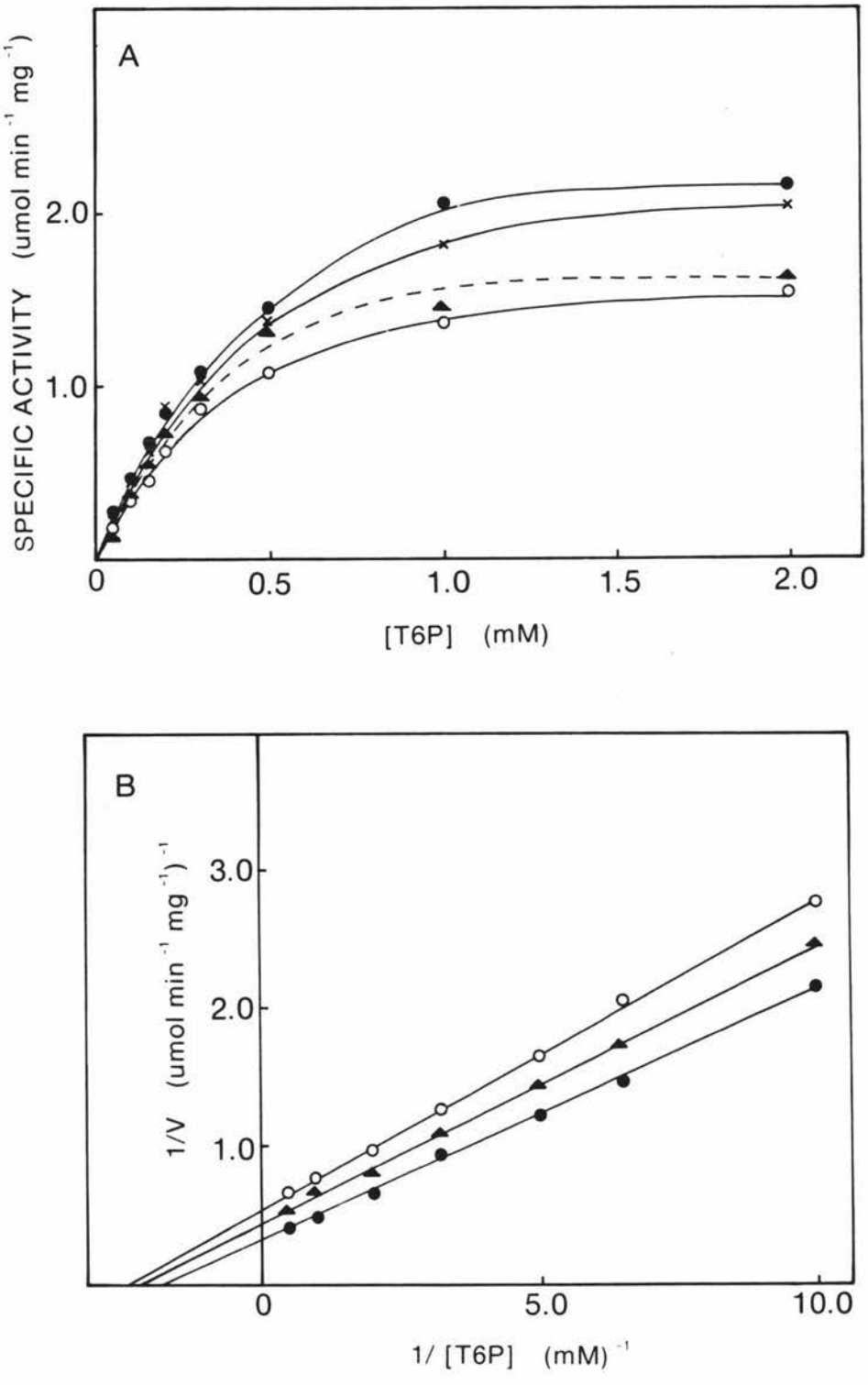


FIGURE 3.3.18 The Effect of F6P on T6PK Activity at Different T6P Concentrations. Purified T6PK (2.5 - 5 µg per assay) was assayed at 2.0 mM MgATP and at 0 - 2.0 mM T6P in assays which contained 0 mM (●); 5 mM (×); 10 mM (▲); and 20 mM (O) F6P. Inhibition by F6P is shown in Figure A.

Figure B shows the data from assays containing 0 mM (●); 10 mM (▲); and 20 mM (O) ADP plotted as a double reciprocal plot.

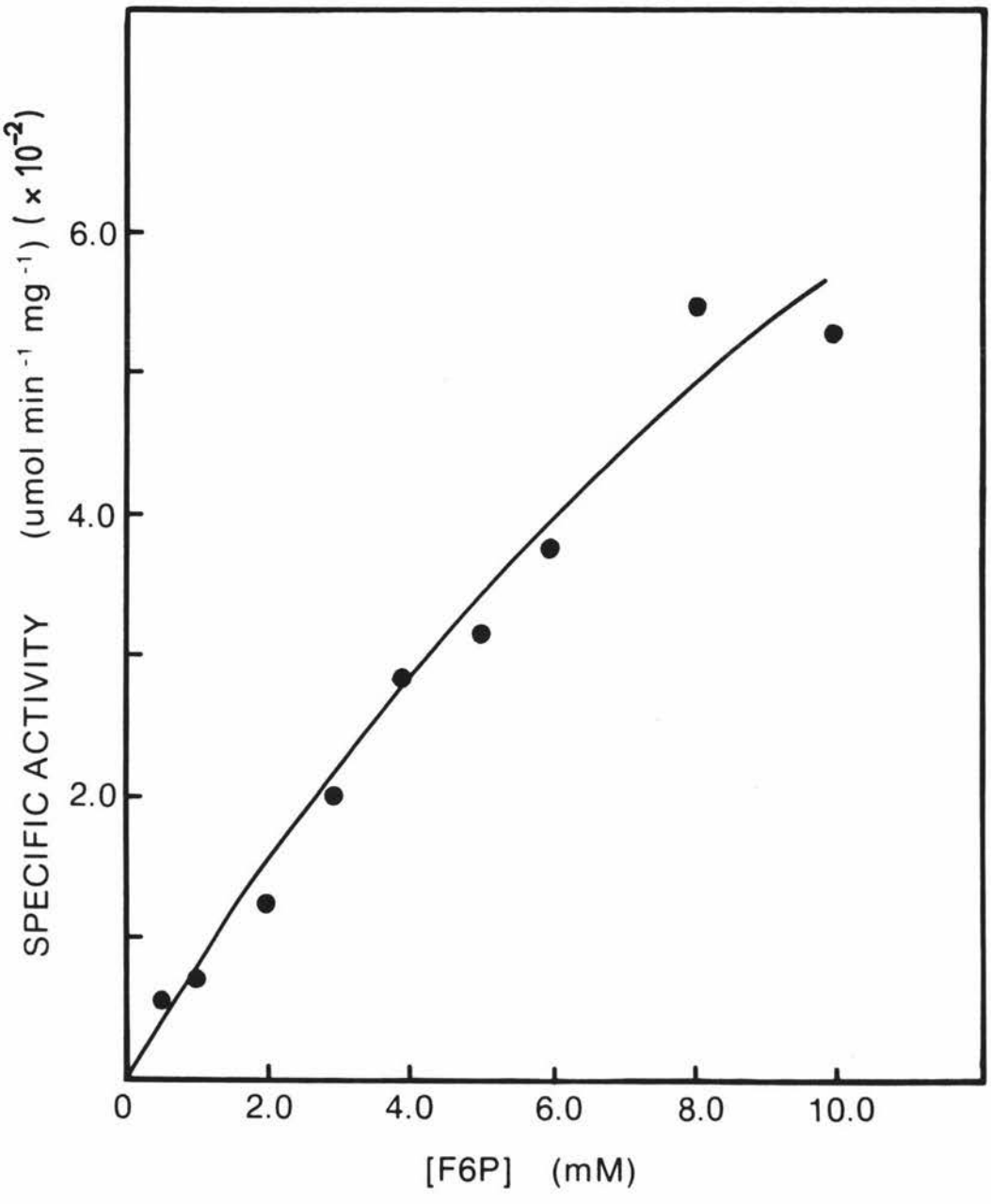


FIGURE 3.3.19 Phosphorylation of F6P by T6PK. T6PK (12 µg per assay) was assayed using the aldolase assay system described for PFK (Section 2.2.6) at 2.0 mM MgATP and at 0 - 10.0 mM F6P concentrations.

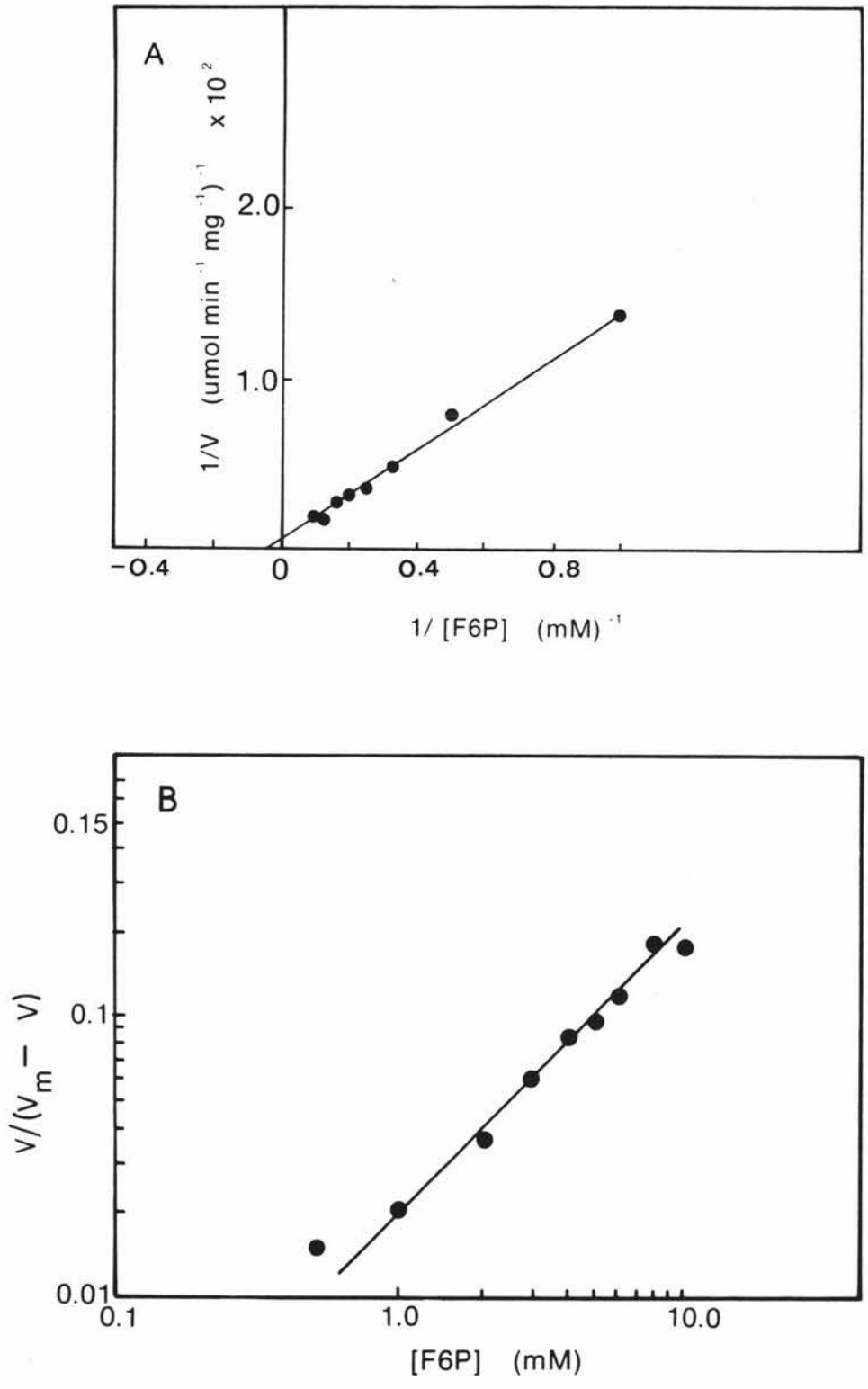


FIGURE 3.3.20 Phosphorylation of F6P by T6PK. Figure A shows a Lineweaver-Burk plot of the data in Figure 3.3.19. The K_m (F6P) calculated from this graph is 42 mM. In Figure B the data are plotted as a Hill plot which indicates an $F6P_{0.5}$ of 42 mM and a Hill coefficient (n_H) of 1.03.

concentrations of F6P and T6P. The large difference in K_m values implies that if T6PK is operative, T6P is utilised solely by T6PK and not by PFK in *S. lactis* and *S. aureus*.

A 'PFK' catalysing phosphorylation of T6P has been reported in *E. coli* (Babul, 1978), although the enzymes of the T6P pathway have not been demonstrated in this organism. Structural studies have shown the enzyme catalysing T6P phosphorylation (PFK II) to be similar in size and subunit composition to PFK (PFK I) from the same organism although the kinetic properties of PFK II are similar in many respects to those of the T6PKs of *S. lactis* and *S. aureus*. The enzyme appeared non-allosteric; it showed a hyperbolic dependence on both substrates and activity was not affected by ADP or PEP although FBP was an inhibitor. However the enzyme exhibited a much lower K_m for F6P (0.013 mM) than for T6P (~6.0 mM) and T6P was phosphorylated at only 20% of the rate of F6P phosphorylation so this enzyme must be considered to be a PFK rather than a T6PK.

3.3.14 The Effect of PEP on T6PK Activity

S. lactis T6PK was inhibited by PEP (Figure 3.3.21). Inhibition was non-competitive with respect to T6P, as shown by the double reciprocal plot in Figure 3.3.22A. A K_i for PEP of ~10 mM was estimated (Figure 3.3.22B). 5 mM PEP resulted in 25-35% inhibition depending on T6P concentration.

As the intracellular concentrations of PEP increase from 3-11 mM in carbohydrate-limited cells of *S. lactis* (Thompson, 1978), PEP inhibition of T6PK activity would decrease the flux through the T6P pathway during carbohydrate limitation. The results in Table 3.3.2 indicate that similar magnitudes of PEP inhibition are obtained at non-saturating concentrations of MgATP to those obtained at non-saturating concentrations of T6P.

3.3.15 The Effect of FBP on T6PK Activity

FBP inhibited T6PK activity at saturating concentrations of both MgATP and T6P as well as at non-saturating concentrations of either substrate. 10 mM FBP resulted in 33% inhibition at saturating levels of substrates (Figure 3.3.23). Inhibition was non-competitive with respect to T6P (Figure 3.3.24A) and the K_i calculated from the plot in Figure 3.3.24B was approximately 13 mM.

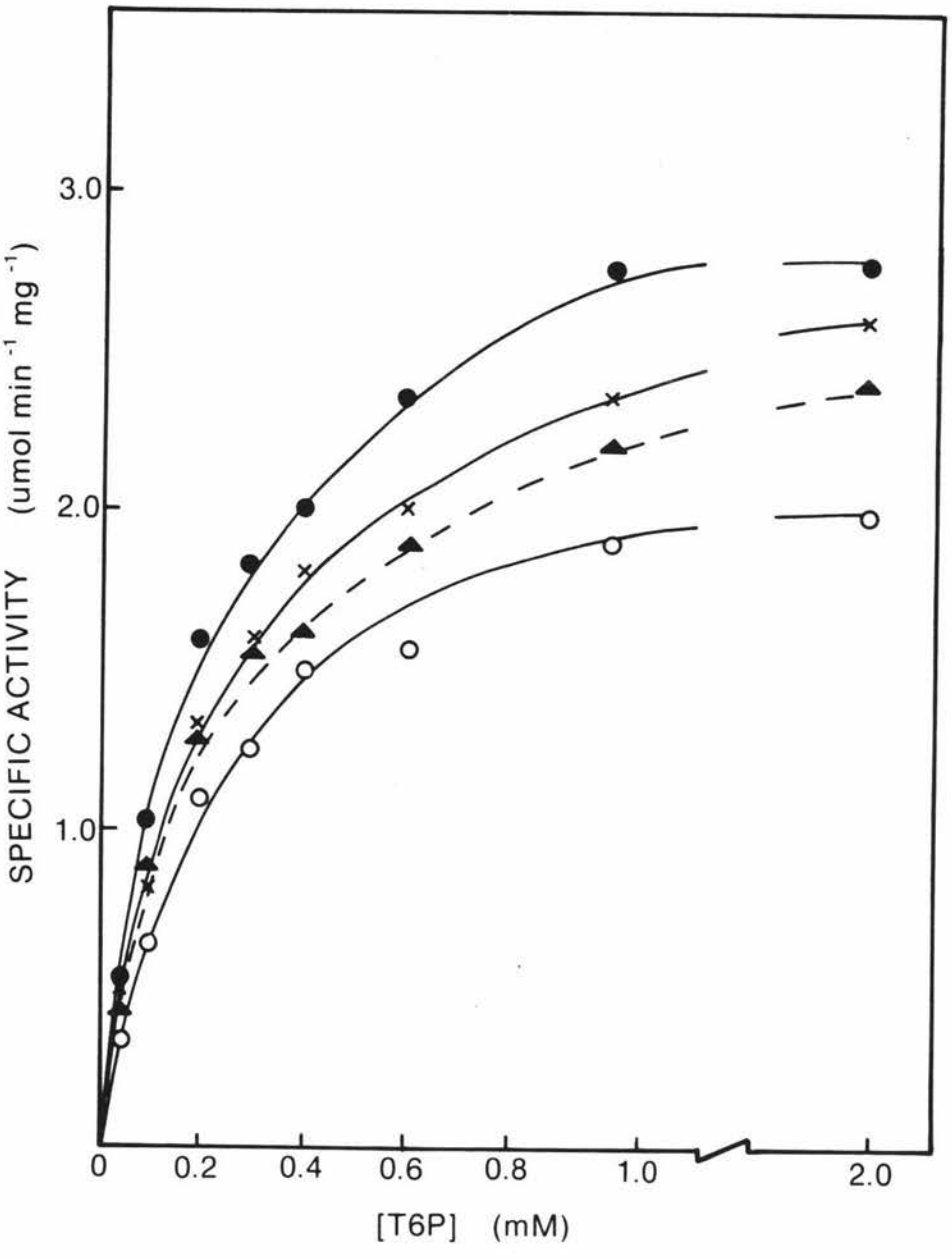


FIGURE 3.3.21 The Effect of PEP on T6PK Activity. This figure shows the effect of 1.0 mM (×); 2.0 mM (▲); and 5.0 mM (O) PEP on T6PK activity. Activity in the absence of PEP is shown by the closed circles (●). All assays were performed at 2.0 mM MgATP and 0-2 mM T6P, using 2.5 - 5 µg purified T6PK per assay.

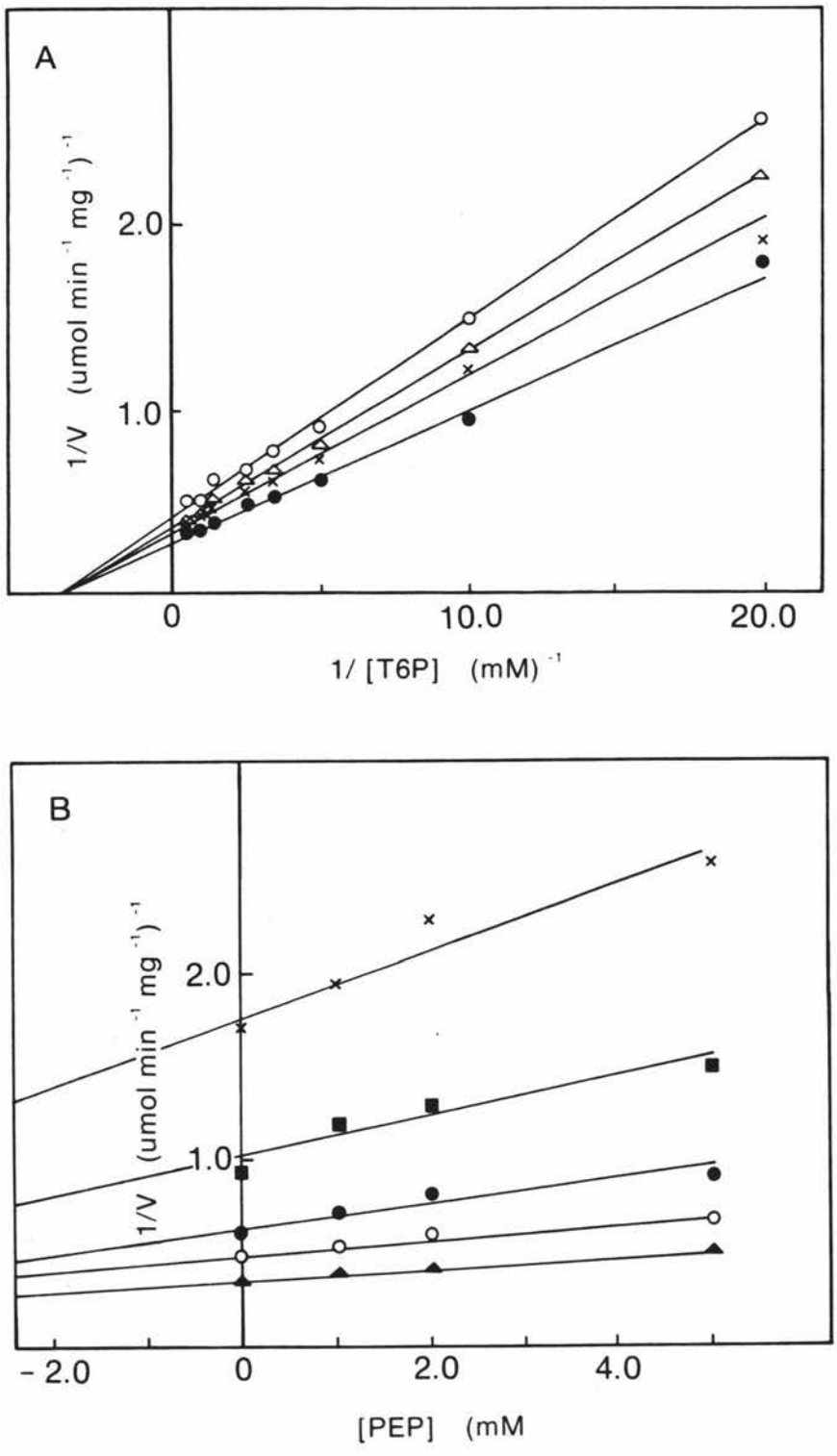


FIGURE 3.3.22 The Effect of PEP on T6PK Activity. The double reciprocal plot (Figure A) indicates non-competitive inhibition (with respect to T6P) by PEP. PEP concentrations in the assays are: ● - 0 mM; × - 1.0 mM; △ - 2.0 mM; and ○ - 5.0 mM.

In Figure B, $\frac{1}{V}$ is plotted against [PEP] at 0.05 mM (×); 0.1 mM (■); 0.2 mM (●); 0.4 mM (○) and 1.0 mM (▲) T6P concentrations. A K_i (PEP) of approximately 10 mM is indicated by the point of intersection of the lines.

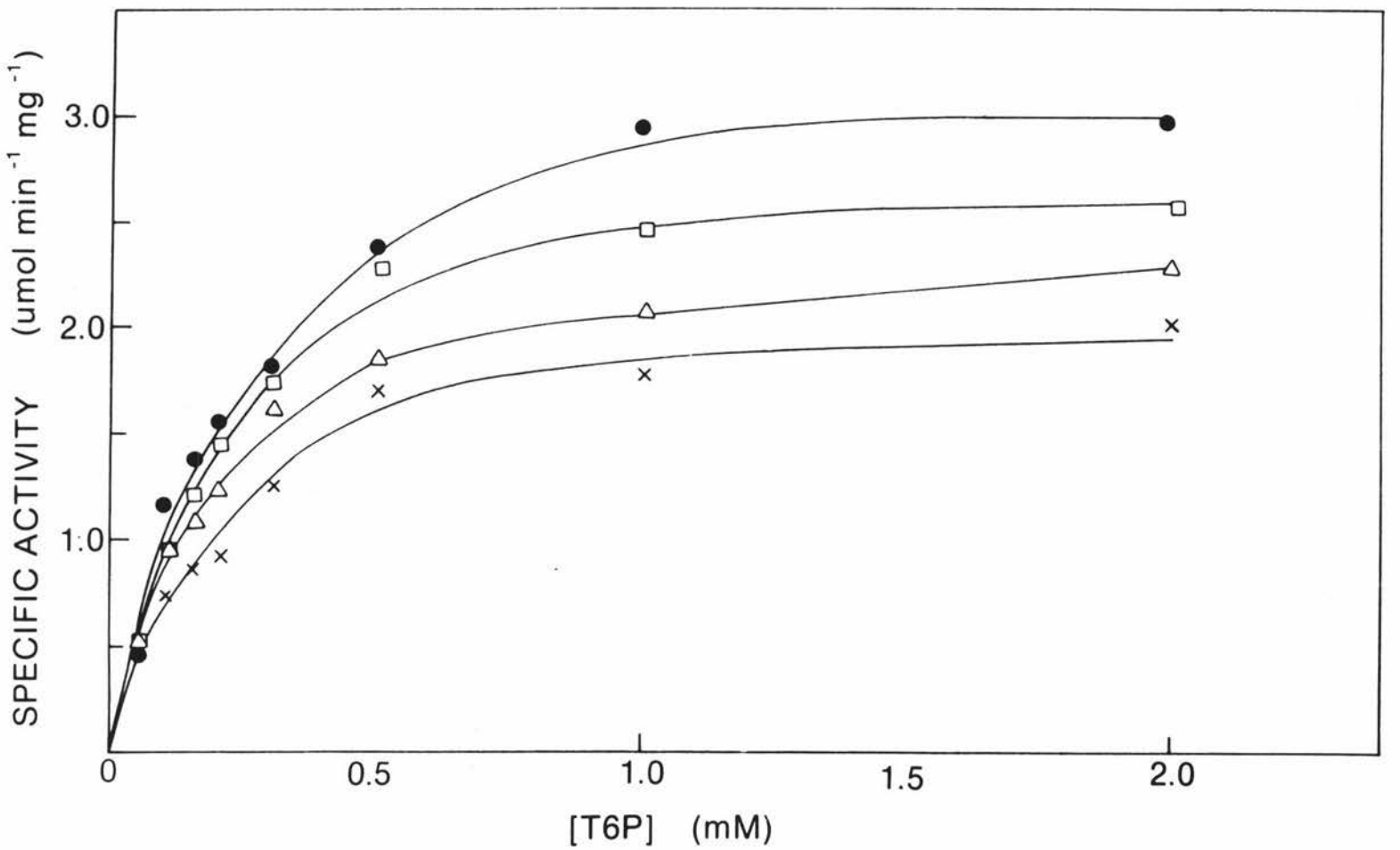


FIGURE 3.3.23 The Effect of FBP on T6PK Activity. T6PK was assayed using the pyruvate kinase - lactate dehydrogenase assay described in Section 3.2.4 and 2.5 - 5 µg protein per assay. Assays contained 2.0 mM MgATP, 0 - 2.0 mM T6P as indicated, and 0 mM (●); 2 mM (□); 5 mM (△); or 10 mM (×) FBP.

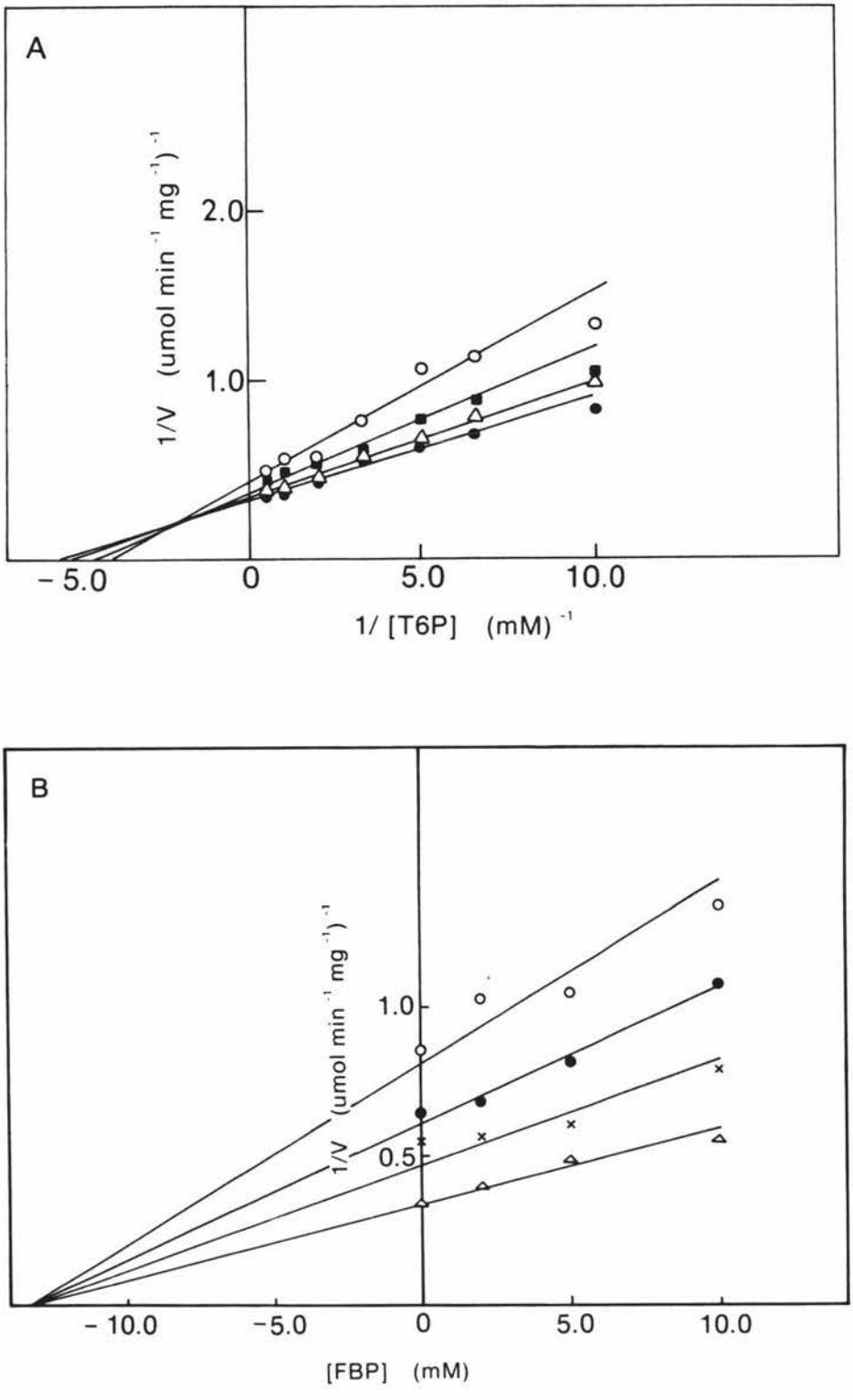


FIGURE 3.3.24 The Effect of FBP on T6PK Activity. Inhibition of T6PK activity by FBP is shown by the double reciprocal plot in Figure A. FBP concentrations: ○ - 0 mM; △ - 2.0 mM; ■ - 5.0 mM; ○ - 10.0 mM. The point of intersection of the lines indicates non-competitive inhibition with respect to T6P.

The K_i (FBP) determined from Figure B is approximately 13 mM. T6P concentrations: ○ - 0.1 mM; ● - 0.2 mM; × - 0.3 mM; △ - 1.0 mM.

Since concentrations of FBP in actively glycolysing cells of *S. lactis* are in the range of 15-25 mM (Thompson, 1978; Thomas *et al*, 1979) it is possible that T6PK activity is partly inhibited *in vivo* by FBP.

3.3.16 The Effect of Monovalent Cations on T6PK Activity

Figure 3.3.25 shows the effect of monovalent cations on T6PK activity at saturating (2.0 mM) T6P and non-saturating (0.2 mM) MgATP concentrations. NH_4^+ and K^+ stimulated activity resulting in up to a 2-fold increase in activity, while Na^+ and Li^+ were inhibitory. 20 mM concentrations of Na^+ and Li^+ gave 20% and 73% inhibition respectively. Similar effects of all ions were found at non-saturating levels of T6P and at saturating levels of both substrates. However in the latter case the effects were less marked.

S. aureus T6PK also exhibited inhibition by Na^+ and Li^+, while NH_4^+, Rb^+, K^+ and Cs^+ (in order of decreasing effectiveness) were activators (Bissett & Anderson, 1980*a*). K^+ and NH_4^+ are also activators of *S. lactis* PFK (Section 2.3.18) as well as of PFK from numerous bacterial and mammalian sources (Vinuela *et al*, 1963; Lowry & Passoneau, 1964; Uyeda & Kurooka, 1970; Doelle, 1972).

The effect of increasing concentrations of K^+ on T6PK activity is shown in Figure 3.3.26A. Activation showed a hyperbolic dependence on K^+ concentration. Maximum activation (approximately 2-fold) was attained at 10-20 mM K^+, but unlike divalent cations, excess cation (concentrations up to 100 mM) did not inhibit activity. The K_m (K^+) calculated from a double reciprocal plot (Figure 3.3.26B) was 1.35 mM.

3.3.17 Summary

The preceding sections of this chapter have summarised some of the kinetic properties of T6PK from *S. lactis* C_{10}. The results of this study have shown that *S. lactis* T6PK appears similar in many respects to the T6PK from *S. aureus*. Both enzymes are proteins of similar size (MW $\sim$ 100,000 daltons) and both appear to be non-allosteric enzymes, in contrast to the PFKs from *S. lactis*, *S. aureus* and most other bacteria. A comparison of the kinetic properties of T6PK and PFK from *S. lactis* C_{10}, and the possible significance of these results to the roles of T6PK and PFK in the regulation of carbohydrate metabolism in *S. lactis* will be discussed in Chapter 5.

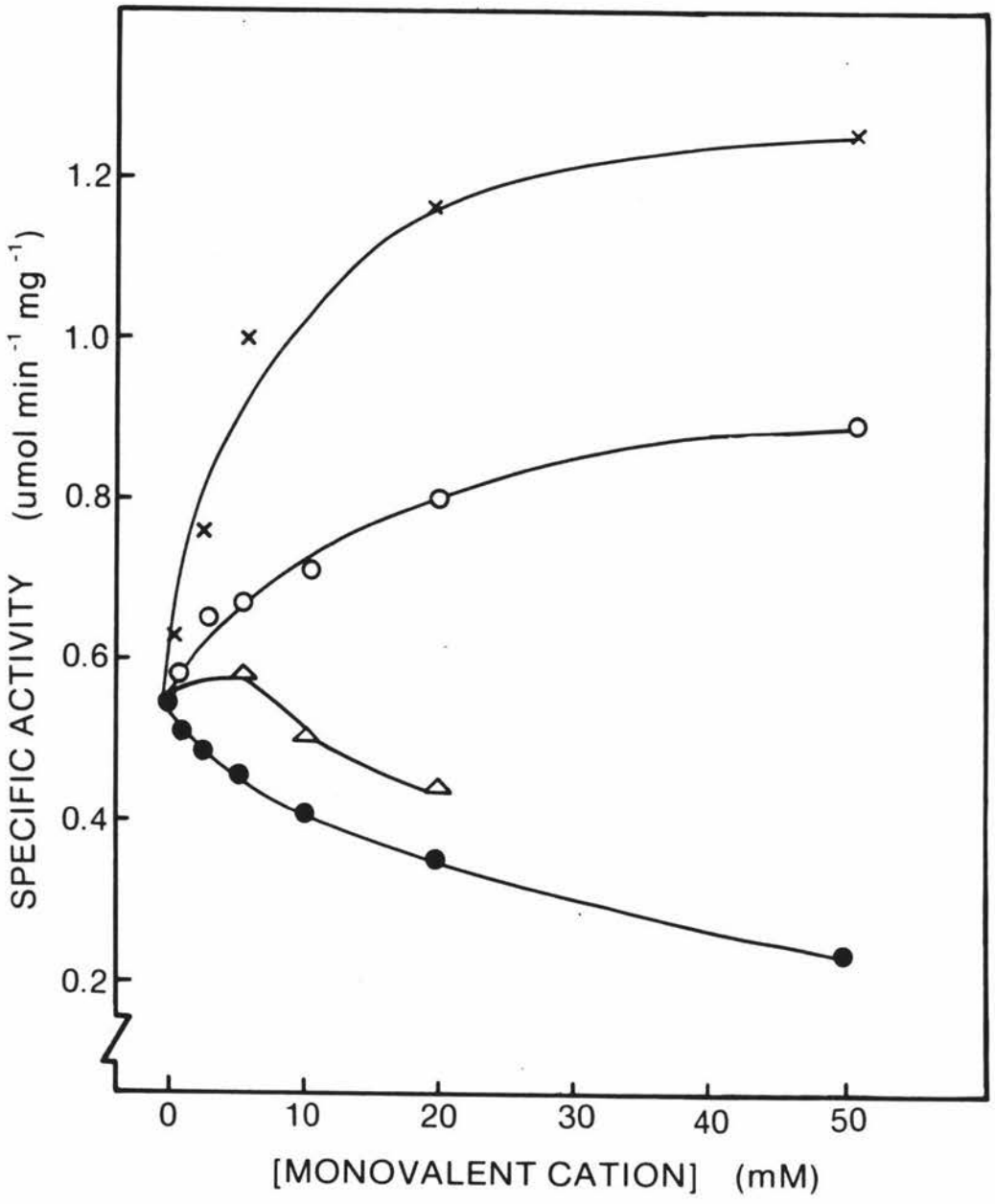


FIGURE 3.3.25 The Effect of Monovalent Cations on T6PK Activity. Purified T6PK (2.5 µg per assay) was assayed at 0.2 mM MgATP and 2.0 mM T6P, and at 0 - 50 mM concentrations of K^+ (×); NH_4^+ (○); Na^+ (△); and Li^+ (●). All monovalent cations were added as solutions of the chloride salts.

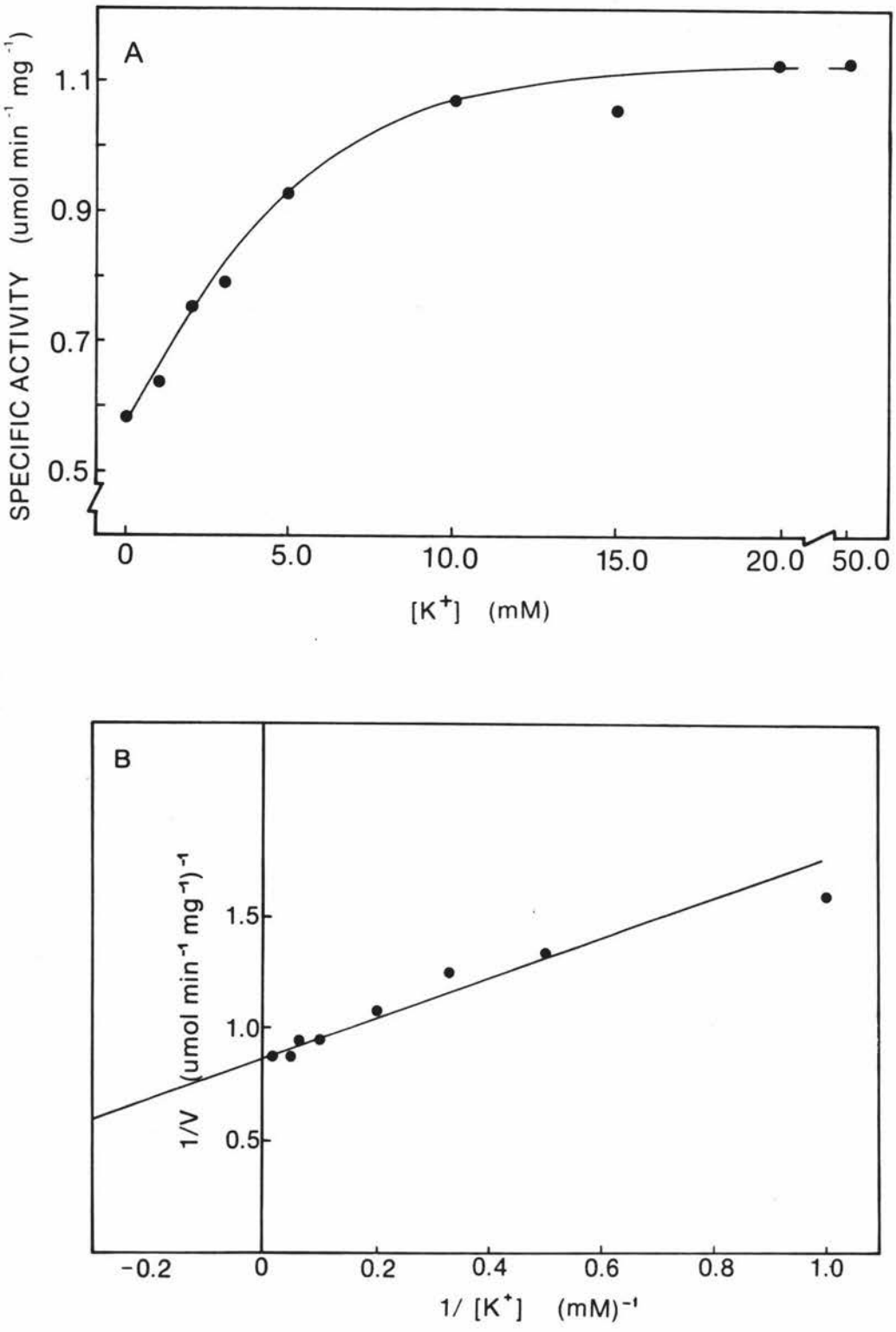


FIGURE 3.3.26 The Effect of K^+ on T6PK Activity. T6PK was assayed using the aldolase assay system described in Section 3.2.4 in the presence of KCl. Each assay contained 5.0 µg protein.

Figure A shows the increase in T6PK activity observed with increasing KCl concentration.

In Figure B, the double reciprocal plot indicates a K_m (K^+) of 1.35 mM.

CHAPTER FOUR

THE EFFECT OF CARBOHYDRATE COMPOSITION OF MEDIA ON ACTIVITIES OF PFK, T6PK, AND GALACTOKINASE IN LACTIC STREPTOCOCCI

CHAPTER 4

THE EFFECT OF CARBOHYDRATE COMPOSITION OF MEDIA ON ACTIVITIES OF PFK, T6PK, AND GALACTOKINASE IN LACTIC STREPTOCOCCI.

4.1 INTRODUCTION

The group N streptococci (*S. lactis*, *S. cremoris*, and *S. diacetylactis*), and *S. mutans* have been shown to contain the enzymic potential to utilise either the T6P or the Leloir pathways in the metabolism of galactose (Bissett & Anderson, 1974*a*; Hamilton & Lebtag, 1979). In contrast, other organisms such as *E. coli* use only the Leloir pathway (Bissett & Anderson, 1974*a*) and in *S. aureus* galactose is metabolised solely by the T6P pathway (Bissett & Anderson, 1974*a*).

The relative importance of these two pathways of galactose metabolism in lactic streptococci varies according to the strain and growth conditions (Thomas *et al*, 1980). While some strains of *S. cremoris* appear to use mainly (perhaps only) the T6P pathway, the Leloir pathway appears to be the predominant pathway in *S. lactis* grown at low galactose concentrations. Thomas *et al* proposed that the proportions of galactose metabolised through the two pathways could be determined by the transport system involved in sugar uptake. Strains utilising the high affinity galactose permease system utilised mainly the Leloir pathway for galactose metabolism, while in strains in which the low affinity galactose PTS operated, the T6P pathway was predominant.

The pathways used in various strains may also depend on the extent to which the enzymes of the T6P pathway are induced. The enzymic complement of *S. lactis* has been shown to vary depending on the nature of the carbohydrate on which the cells are grown (Bissett & Anderson, 1974*a*). Levels of T6P pathway enzymes were low in glucose-grown cells and were induced by growth on galactose or lactose. A similar induction of the T6P pathway enzymes occurs in *S. mutans* (Hamilton & Lebtag, 1979).

S. lactis has also been shown to exhibit diauxic growth when grown in a medium containing a mixture of glucose, galactose and lactose (Lawrence *et al*, 1976). In most strains glucose and lactose are used in preference to galactose, and addition of glucose to cells

growing exponentially on galactose inhibits utilisation of galactose until all the glucose is metabolised. This effect may be due to either 'catabolite repression' - repression of synthesis of the enzymes of the pathway involved in galactose metabolism, or 'catabolite inhibition' - regulation of enzyme activities by glucose.

The experiments described in this section were undertaken for several reasons:

i To reinvestigate the findings of Bissett & Anderson (1974*a*) on the inducibility of T6PK in *S. lactis* by lactose and galactose, and to extend the survey to a wider range of strains of *S. lactis* and *S. cremoris*. Two strains known to possess unusual metabolic features were included in this study. *S. lactis* 7962 lacks phospho-β-D-galactosidase and apparently uses the Leloir pathway for both lactose and galactose metabolism (Citti *et al*, 1965). *S. lactis* ML_8 displays homofermentative metabolism of galactose in contrast to most other strains which are heterofermentative (Thomas *et al* 1980). It is possible that the unusual characteristics of these two strains are correlated with differences in relative levels of the Leloir, EMP and T6P pathway enzymes.

ii To compare the relative enzyme levels in cultures grown on mixtures of sugars (glucose plus lactose, glucose plus galactose, and galactose plus lactose) in an attempt to determine whether the diauxic growth observed (Moustafa & Collins, 1968; Lawrence *et al*, 1976) is due to repression of synthesis of the T6P pathway enzymes by glucose or to some other control mechanism.

In an attempt to answer these questions the levels of T6PK, PFK and galactokinase (Gal K), enzymes representative of the T6P, EMP, and Leloir pathways, respectively, were measured in several strains of group N streptococci, and in one strain of *S. faecalis*, a group D streptococcus, which had been grown on glucose, lactose, and galactose, and various mixtures of these sugars.

4.2 METHODS

4.2.1 MAINTENANCE OF ORGANISMS

S. lactis strains C_{10}, ML_8, ML_3 and 7962, *S. cremoris* strains HP, E_8, AM_2, and 134, and *S. diacetylactis* DRC_1 were obtained from the culture collection of the N.Z. Dairy Research Institute, Palmerston North. *S. faecalis* 8034 was from the Department of Microbiology & Genetics, Massey University. All organisms were maintained on nutrient agar plates containing 2% (w/v) lactose, stored at 4°C. Cultures were subcultured at intervals of 4-6 weeks, and were checked regularly for purity by microscopic examination of Gram-stained cells.

Cultures for inoculation were grown overnight in the medium described in Chapter 2 except that the lactose was replaced by 0.5% (w/v) of the sugar on which the cells were to be grown.

4.2.2 CULTURE OF BACTERIA

For comparison of enzyme activities in different strains, batch cultures of bacteria were grown in 100 ml of a medium containing the following constituents per litre of distilled water: Carbohydrate, 5-20 g; peptone, 10 g; yeast extract, 10 g; beef extract, 2 g; KH_2PO_4, 5 g; $MnCl_2$, 0.05 g; and $MgSO_4$, 0.2 g. The medium was sterilised prior to the addition of the carbohydrate by autoclaving at 15 psi for 10 minutes. Carbohydrate solutions, sterilised separately, were added after sterilisation of the other components of the medium. Each flask was inoculated with 1-2 ml of a culture which had been grown overnight, and the culture was grown at 30°C for 12-15 hrs, with no agitation or neutralisation.

Larger batches of selected strains were grown in 3 litres of the same medium in a CC 1500 fermentor (L.H. Engineering, Stoke Poges, Bucks, England). The medium was flushed continually throughout growth with a 95% N_2/5% CO_2 mixture passed through a heated copper coil to remove traces of O_2, and the culture was stirred at 300 r.p.m. pH was maintained at 6.4 $\pm$ 0.2 pH units by addition of sterile 2.5 M NaOH.

Bacterial growth was monitored by periodic determination of the absorbance at 540 nm of a suitably diluted sample from the culture. Absorbance was linearly related to cell mass over the range measured (Fig. 4.2.1). Cells were harvested and washed as previously described (section 2.2.3) at various stages throughout the growth curve.

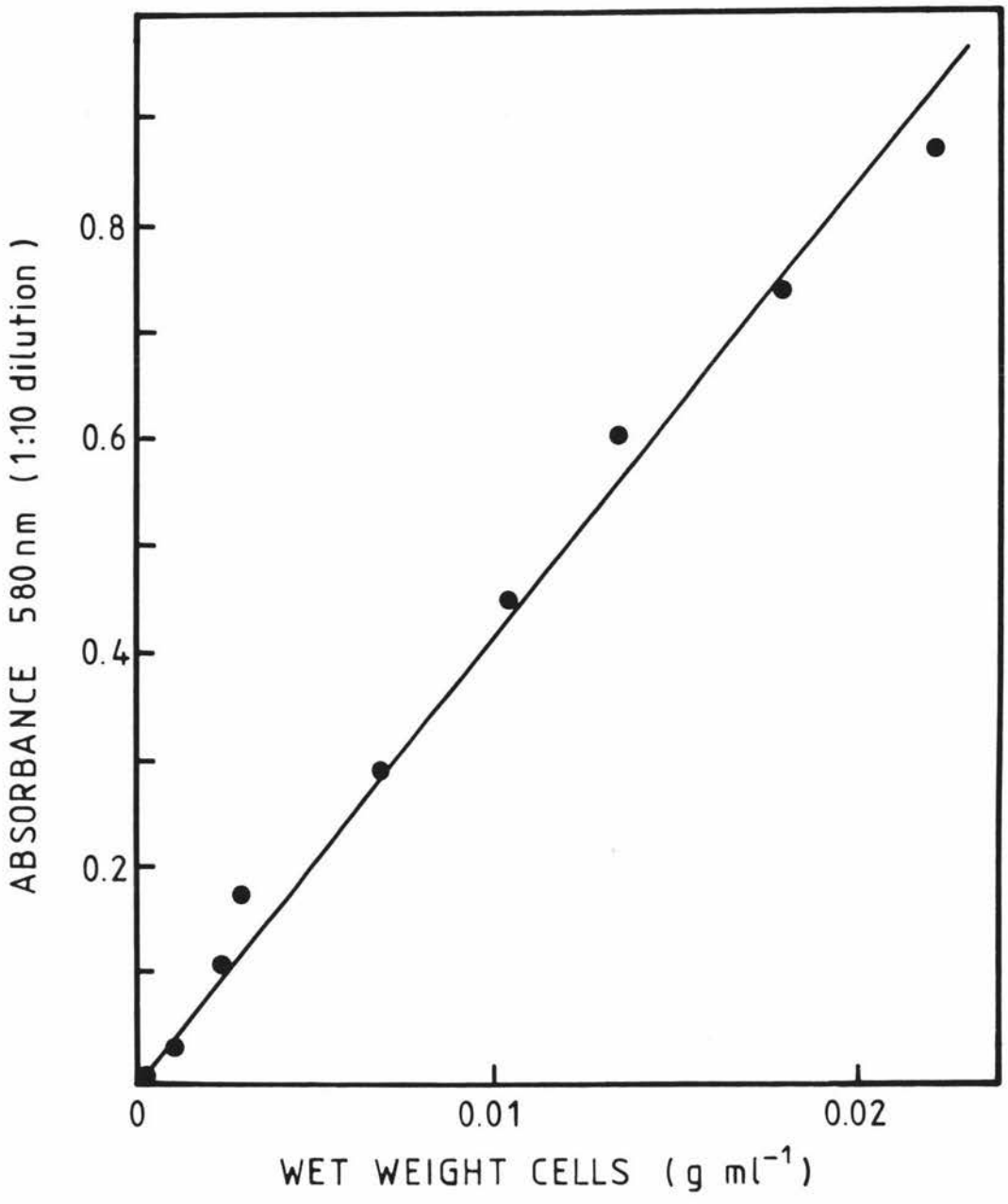


FIGURE 4.2.1 Relationship of Optical Density to Cell Mass. *S. lactis* C_{10} was grown to stationary phase in 3 litres of medium in a fermentor. At various stages throughout the growth curve samples of cells were harvested by centrifugation, washed, and weighed. Optical density was measured at 580 nm on a Bausch and Lomb 'Spectronic 20' Spectrophotometer.

4.2.3 Preparation of Cell-free Extracts

Bacterial cells were harvested by centrifugation at 10,000 g for 10 minutes. The cell-free medium (supernatant) was immediately frozen at -20°C until required for reducing-sugar determinations. Harvested cells were frozen until required. Cells were washed and cell-free extracts prepared as described in Chapter 2. All cell-free extracts were prepared within two days of harvesting the cells, and all assays were performed within 6 hours of disrupting the cells. Cell free extracts were diluted, if necessary, in the Tris-glycerol buffer in which the extract was prepared.

4.2.4 Enzyme Assays

Assays for PFK and T6PK have been described in previous chapters (see Sections 2.2.6 and 3.2.4). The aldolase-linked assay system was used for both enzymes to minimise 'background' activity. NADH oxidase was measured in a separate assay (Section 2.2.6) and this activity taken into account in calculating PFK and T6PK activities. Saturating levels (2 mM) of ATP, T6P and F6P were used in all assays unless otherwise stated. Other components of the assay system were present at the concentrations specified in the standard procedure (Sections 2.2.6 and 3.2.4). K^+ ions which activate T6PK (see Chapter 3) were not added to the aldolase assay for T6PK, so activity *in vivo* may be higher than the activity measured, if intracellular K^+ is saturating. Galactokinase activity was measured using the PK-LDH linked assay system described in Chapter 2, except that 5 mM galactose was used as substrate. Activities of PFK, T6PK and Gal K were not affected by freezing whole cells prior to preparation of cell-free extracts. 'Background' activity was measured in a separate assay containing MgATP but no galactose.

4.2.5 Reducing-Sugar Determination

The reducing-sugar content of the medium was determined by the Nelson-Somogyi method (Somogyi, 1952) after removal of bacterial cells by centrifugation.

0.5 ml of suitably diluted sample was heated with 1.0 ml alkaline copper reagent on a boiling water bath for 20 minutes. The mixture was cooled and 1 ml arsenomolybdate reagent added. Absorbance of the solution at 540 nm was determined after dilution to 10 ml with distilled water, and the reducing-sugar content was calculated from a standard curve prepared with the same sugar.

4.3 Results and Discussion

4.3.1 Comparison of Enzyme Levels in some Lactic Streptococci

Table 4.3.1 shows the specific activities of PFK, T6PK, and galactokinase (Gal K) in several strains of *S. lactis* and *S. cremoris*, in *S. diacetylactis* and in *S. faecalis*. Activities were measured in cell-free extracts prepared from cells grown to late-log phase (12-15 hr growth) in 100 ml medium containing 2% (w/v) glucose, lactose, or galactose.

In all strains the levels of Gal K were low when cells were grown on glucose. Growth on galactose or lactose induced increased levels of Gal K activity, galactose usually causing a more marked increase in activity than lactose. In all *S. cremoris* and *S. lactis* strains (except *S. lactis* 7962) the activity of Gal K was lower than the activities of PFK and T6PK in both lactose-grown and galactose-grown cells.

Much higher specific activities of Gal K were found in cells of *S. lactis* 7962 grown on lactose or galactose. Activity of Gal K was higher than the activity of T6PK, suggesting that the Leloir pathway is a major route for lactose and galactose metabolism in this organism. This is in agreement with earlier studies on *S. lactis* 7962 which showed that this strain lacks a PEP PTS for both lactose and galactose transport (McKay *et al*, 1969; Kashket & Wilson, 1972, 1974) and also lacks a phospho-β-D-galactosidase (Citti *et al*, 1965), and would therefore be unable to metabolise lactose via the T6P pathway. Growth of *S. faecalis* 8043 on lactose and galactose, and growth of *S. diacetylactis* DRC_1 on galactose also led to high levels of Gal K which suggests that in these organisms the Leloir pathway may be the major pathway utilised in metabolism of these sugars.

PFK activities did not vary greatly between strains, and were similar in cells grown on glucose, galactose or lactose. PFK activity was generally greater than T6PK activity except in *S. lactis* strains C_{10} and ML_3 which contained higher levels of T6PK than of PFK when grown on galactose. However T6PK activity *in vivo* may be greater than the activity measured using the aldolase assay system as K^+ may be limiting activity in the assay (see Chapter 3). Increased levels of T6PK activity were induced by growth on lactose and galactose, with galactose inducing higher levels of activity than lactose in most

TABLE 4.3.1

SPECIFIC ACTIVITIES OF PFK, T6PK AND GAL K IN SEVERAL STRAINS OF STREPTOCOCCI

Organism	Strain	Carbohydrate in Growth Medium	Specific activity µmol substrate min^{-1} mg^{-1} PFK	T6PK	Gal K
S. lactis	C_{10}	glu	1.72 ± 0.40	0.41 ± 0.05	0.02 ± 0.02
		lac	1.25 ± 0.15	0.60 ± 0.15	0.15 ± 0.07
		gal	0.78 ± 0.12	1.12 ± 0.18	0.27 ± 0.12
"	ML_8	glu	1.46 ± 0.01	0.45 ± 0.20	<0.01
		lac	1.46 ± 0.05	0.65 ± 0.15	0.03 ± 0.01
		gal	1.09 ± 0.05	1.06 ± 0.01	0.36 ± 0.08
"	ML_3	glu	1.75 ± 0.34	0.20 ± 0.08	0.12 ± 0.05
		lac	1.80 ± 0.21	0.40 ± 0.12	0.26 ± 0.05
		gal	0.60 ± 0.18	0.90 ± 0.14	0.70 ± 0.10
"	7962	glu	0.87 ± 0.03	0.17 ± 0.11	0.15 ± 0.09
		lac	1.62 ± 0.27	0.48 ± 0.13	1.86 ± 0.32
		gal	0.98 ± 0.08	0.44 ± 0.05	2.50 ± 0.50
S. cremoris	HP	glu	1.23 ± 0.07	0.07 ± 0.0	0.07 ± 0.04
		lac	1.12 ± 0.04	0.32 ± 0.02	0.14 ± 0.03
		gal	1.24 ± 0.02	0.85 ± 0.04	0.08 ± 0.03
"	AM_2	glu	1.06 ± 0.28	0.14 ± 0.04	<0.01
		lac	1.63 ± 0.17	0.26 ± 0.09	0.07 ± 0.01
		gal	1.62 ± 0.24	0.36 ± 0.05	0.15 ± 0.02
"	E_8	glu	1.31 ± 0.14	0.23 ± 0.02	0.15 ± 0.02
		lac	1.57 ± 0.22	0.46 ± 0.05	<0.01
		gal	1.54 ± 0.16	0.74 ± 0.11	0.02 ± 0.02
"	134	glu	1.43 ± 0.06	0.20 ± 0.01	<0.01
		lac	1.98 ± 0.12	0.31 ± 0.01	0.09 ± 0.02
		gal	1.79 ± 0.03	0.62 ± 0.04	0.09 ± 0.03
S. diacetylactis	DRC_1	glu	1.34 ± 0.08	0.15 ± 0.01	0.13 ± 0.06
		lac	1.60 ± 0.14	0.40 ± 0.05	<0.01
		gal	1.95 ± 0.04	0.55 ± 0.05	1.34 ± 0.09
S. faecalis	8043	glu	1.50 ± 0.01	0.29 ± 0.02	0.37 ± 0.17
		lac	1.05 ± 0.03	0.73 ± 0.01	3.2 ± 0.31
		gal	1.34 ± 0.03	0.72 ± 0.06	1.87 ± 0.06

Enzyme activities were measured in cell-free extracts prepared as described in Section 4.2.2. Activities are expressed in µmol substrate min^{-1} mg^{-1} and are the averages ± standard deviations of 2-3 separate determinations.

strains. Activities of T6PK were similar in galactose-grown and lactose-grown cells of *S. lactis* 7962, *S. cremoris* AM_2, *S. diacetylactis* DRC_1 and *S. faecalis* 8043.

Contrary to expectation, significant levels of T6PK activity were detected in glucose-grown cells of all strains. Activity in cells grown on glucose ranged from 8-40% of the activity in lactose-grown cells. However this apparent T6PK activity may be due partly to phosphorylation of T6P by PFK in the cell-free extract so the magnitude of actual T6PK activity is uncertain. To minimise any phosphorylation of T6P by PFK, the T6P concentration in the assay was lowered from 2.0 mM to 0.2 mM. Since the K_m(T6P) of PFK is 2.8 mM, lowering the T6P concentration to 0.2 mM would be expected to cause approximately a 10-fold reduction in the observed reaction rate if activity was due to PFK, but not if activity was *bona fide* T6PK activity (K_m(T6P) of T6PK = 0.18 mM). Activity in assays performed at 0.2 mM T6P was 65% of the activity at 2.0 mM T6P. Thus the activity in glucose-grown cells appears to be largely due to T6PK. This activity did not appear due to an F6P contaminant in the T6P, as the net change in absorbance after the reaction (at 0.2 mM T6P) was allowed to proceed to completion was that expected assuming complete utilisation of the substrate.

The relative PFK and T6PK levels shown in Table 4.3.1 show a similar pattern to the relative FBP aldolase and TBP aldolase activities (Table 4.3.2) determined by Dr V.L. Crow of the Dairy Research Institute, Palmerston North. Ratios of TBP aldolase : FBP aldolase are approximately 1:10, 1:5, and 1:2 respectively in glucose, lactose, and galactose grown cells, compared with ratios of T6PK : PFK of approximately 1:10, 1:3, and 1:1. TBP aldolase, like T6PK, was induced to higher levels by growth on galactose than by growth on lactose, except in *S. lactis* strains ML_3, ML_8, and 7962 in which lactose and galactose were equally effective in inducing TBP aldolase activity. While *S. lactis* strains ML_8, ML_3, and 7962 contained little or no TBP aldolase activity when grown on glucose, no glucose-grown strains were found to completely lack T6PK activity.

4.3.2 Enzyme levels in Cells grown on a Mixture of Sugars

Lactose and galactose have been shown to induce T6PK synthesis in lactic streptococci (Bissett & Anderson, 1974*a*) and these results are confirmed by the data presented in the previous section. *S. lactis* is also known to utilise glucose and lactose in preference to galactose

TABLE 4.3.2

ALDOLASE LEVELS IN STRAINS OF *S. lactis* AND *S. cremoris*(1)

Organism	Strain	Sugar in Growth medium	TBP(2) aldolase	FBP(2) aldolase
S. cremoris	E_8, AM_2	Glu	0.2	1.9
		Lac	0.5	2.2
		Gal	1.3	2.4
S. cremoris	HP, C_{13}, ML_1	Glu	0.3	2.8
		Lac	0.6	2.6
		Gal	1.5	2.5
S. lactis	ML_3, ML_8, 7962	Glu	0-0.002	1.6
		Lac	0.9	1.9
		Gal	0.8	2.1
S. lactis	C_{10}, H_1	Glu	0.2	1.5
		Lac	0.4	1.7
		Gal	1.1	1.8

(1) TBP-aldolase and FBP-aldolase activities were determined by Dr V.L. Crow, N.Z. Dairy Research Institute, Palmerston North.

(2) Specific activities, μmol sugar min^{-1} mg^{-1}.

(Lawrence *et al*, 1976), an effect which could be explained by repression by glucose of the enzymes involved in galactose utilisation.

To determine whether or not glucose affects synthesis of enzymes of the T6P and Leloir pathways, levels of T6PK, Gal K and PFK were measured in cells grown on mixtures of sugars. Cells were grown at 30°C for 12-15 hours in batch cultures in 100 ml medium containing 1% (w/v) of each sugar. In a medium containing 1% (w/v) sugar enough acid is produced during fermentation to inhibit bacterial growth before exhaustion of the carbohydrate supply. The non-growing cells (pH inhibited) continue to metabolise carbohydrate but do not actively synthesise protein (Turner & Thomas, 1975). Thus with a mixture of two different carbohydrates (each 1% (w/v)) in the medium the enzymes present in the cell should be only those active in metabolism of the sugar(s) initially metabolised.

Table 4.3.3 lists activities of enzymes in cells grown on glucose plus galactose, glucose plus lactose, and galactose plus lactose. A comparison of these results with enzyme activities in cells grown on a single sugar (Table 4.3.1) shows that glucose may cause repression of both T6PK and Gal K.

Of the lactic streptococci, only *S. cremoris* HP contains a significant level of Gal K when grown on a medium containing glucose. In all other strains the addition of glucose to either lactose or galactose results in a level of Gal K comparable to that in glucose-grown cells. This is the case even in *S. lactis* 7962, a strain in which Gal K activities are high when grown on galactose or lactose alone, (Table 4.3.1) and where the Leloir pathway is probably the sole pathway of galactose metabolism (Lawrence *et al*, 1976). Thus it appears that glucose would be used in preference to galactose or lactose in this strain, in agreement with published data (Lawrence *et al*, 1976). Gal K activities in *S. faecalis* 8043 show a similar response to those in *S. lactis* 7962 except that activity is still reasonably high in cells grown on glucose plus lactose.

In contrast, T6PK activities were generally not suppressed by growth on glucose plus lactose; activities in all strains except *S. cremoris* HP and *S. lactis* 7962 were comparable to activities in cells grown on lactose alone. However T6PK activity was generally lower in cells grown on glucose plus galactose than in galactose-grown cells (except in *S. lactis* 7962 and *S. cremoris* AM_2). This repression

TABLE 4.3.3

SPECIFIC ACTIVITIES OF PFK, T6PK AND GAL K IN SEVERAL STRAINS OF STREPTOCOCCI GROWN ON A MIXTURE OF SUGARS

Organism	Strain	Carbohydrate in Growth Medium	Specific activity μmol substrate min^{-1} mg^{-1} PFK	T6PK	Gal K
S. lactis	C_{10}	lac + gal	1.18 ± 0.01	0.83 ± 0.07	<0.01
		glu + lac	1.28 ± 0.07	0.69 ± 0.11	0.02 ± 0.02
		glu + gal	1.54 ± 0.66	0.75 ± 0.18	0.04 ± 0.03
"	ML_8	lac + gal	1.84 ± 0.08	1.07 ± 0.14	0.05 ± 0.01
		glu + lac	1.65 ± 0.10	0.92 ± 0.03	0.02 ± 0.01
		glu + gal	1.35 ± 0.12	0.72 ± 0.04	<0.01
"	7962	lac + gal	0.97 ± 0.03	0.74 ± 0.06	1.96 ± 0.12
		glu + lac	0.45 ± 0.05	0.18 ± 0.01	0.09 ± 0.02
		glu + gal	0.89 ± 0.06	0.44 ± 0.01	0.07 ± 0.02
S. cremoris	AM_2	lac + gal	1.30 ± 0.04	0.69 ± 0.02	<0.01
		glu + lac	1.30 ± 0.08	0.44 ± 0.03	<0.01
		glu + gal	1.23 ± 0.10	0.61 ± 0.01	<0.01
"	HP	lac + gal	1.13 ± 0.07	0.23 ± 0.01	0.30 ± 0.01
		glu + lac	1.08 ± 0.06	0.16 ± 0.02	0.60 ± 0.01
		glu + gal	0.97 ± 0.02	0.07 ± 0.01	0.05 ± 0.02
S. faecalis	8043	lac + gal	1.46 ± 0.01	0.71 ± 0.04	2.83 ± 0.28
		glu + lac	2.84 ± 0.07	0.64 ± 0.01	0.67 ± 0.05
		glu + gal	1.26 ± 0.06	0.27 ± 0.01	0.10 ± 0.02

Cells were grown in 100 ml batch cultures containing 1% (w/v) of each sugar. Enzyme activities were measured in cell-free extracts prepared as described in Section 4.2.2. Activities are expressed in μmol substrate min^{-1} mg^{-1} and are the averages ± standard deviations of 3 separate determinations.

of T6PK activity by growth of cells on glucose plus galactose, but not on glucose plus lactose suggests that glucose and lactose are utilised in preference to galactose in most strains. This order of sequential metabolism has been demonstrated in *S. lactis* ML_3 (Lawrence *et al*, 1976) and it appears that the *S. cremoris*, *S. diacetylactis* and *S. faecalis* strains used in this survey may metabolise glucose, lactose, and galactose in a similar fashion.

PFK activity in most strains was not significantly affected by the mixture of sugars in the medium. This is not surprising as PFK is a constitutive enzyme in these organisms and activities did not vary greatly between cells grown on glucose, lactose, or galactose.

4.3.3 Changes in Enzyme Levels during Growth of *S. lactis* C_{10} and *S. cremoris* AM_2.

To determine the change in PFK, T6PK and Gal K levels during the growth of *S. lactis* and *S. cremoris*, cultures were grown in 3 l batches at pH 6.5 and harvested at various stages throughout the growth curve. *S. lactis* C_{10} and *S. cremoris* AM_2 were selected as representative strains of the two species of lactic streptococci. Starter cultures used for inoculation were grown on the sugar on which the batch cultures were to be grown. Ideally inocula should be grown on glucose, as this sugar does not induce the enzymes of the T6P or Leloir pathways. However growth on glucose can cause impairment of ability to metabolise lactose (Le Blanc *et al*, 1979; Anderson & McKay, 1977) so for this reason glucose-grown cells were not used to inoculate media containing lactose or galactose.

S. lactis C_{10} and *S. cremoris* AM_2 grew on glucose, lactose, and galactose media (containing an initial carbohydrate concentration of 2% (w/v)) with the doubling times listed in Table 4.3.4. *S. cremoris* grew more slowly than *S. lactis* (except on galactose) and exhibited a longer lag phase (5-12 hrs depending on the size of the inoculum). *S. lactis* grew equally rapidly on glucose and lactose, but more slowly on galactose, whereas *S. cremoris* grew with equal rates on all three sugars.

Figures 4.3.1 and 4.3.2 show the variation in specific activities of PFK, T6PK, and Gal K throughout the growth curves of *S. lactis* C_{10} and *S. cremoris* AM_2 grown on lactose. In *S. cremoris* both PFK and T6PK activity reached a maximum level as cells approached the late log

TABLE 4.3.4

DOUBLING TIMES OF *S. lactis* C_{10} AND *S. cremoris* AM_2 GROWN ON DIFFERENT SUGARS

Organism	Carbohydrate in Medium	Doubling time (mins)
S. lactis C_{10}	glu	39
	lac	42
	gal	65
S. cremoris AM_2	glu	68
	lac	60
	gal	68

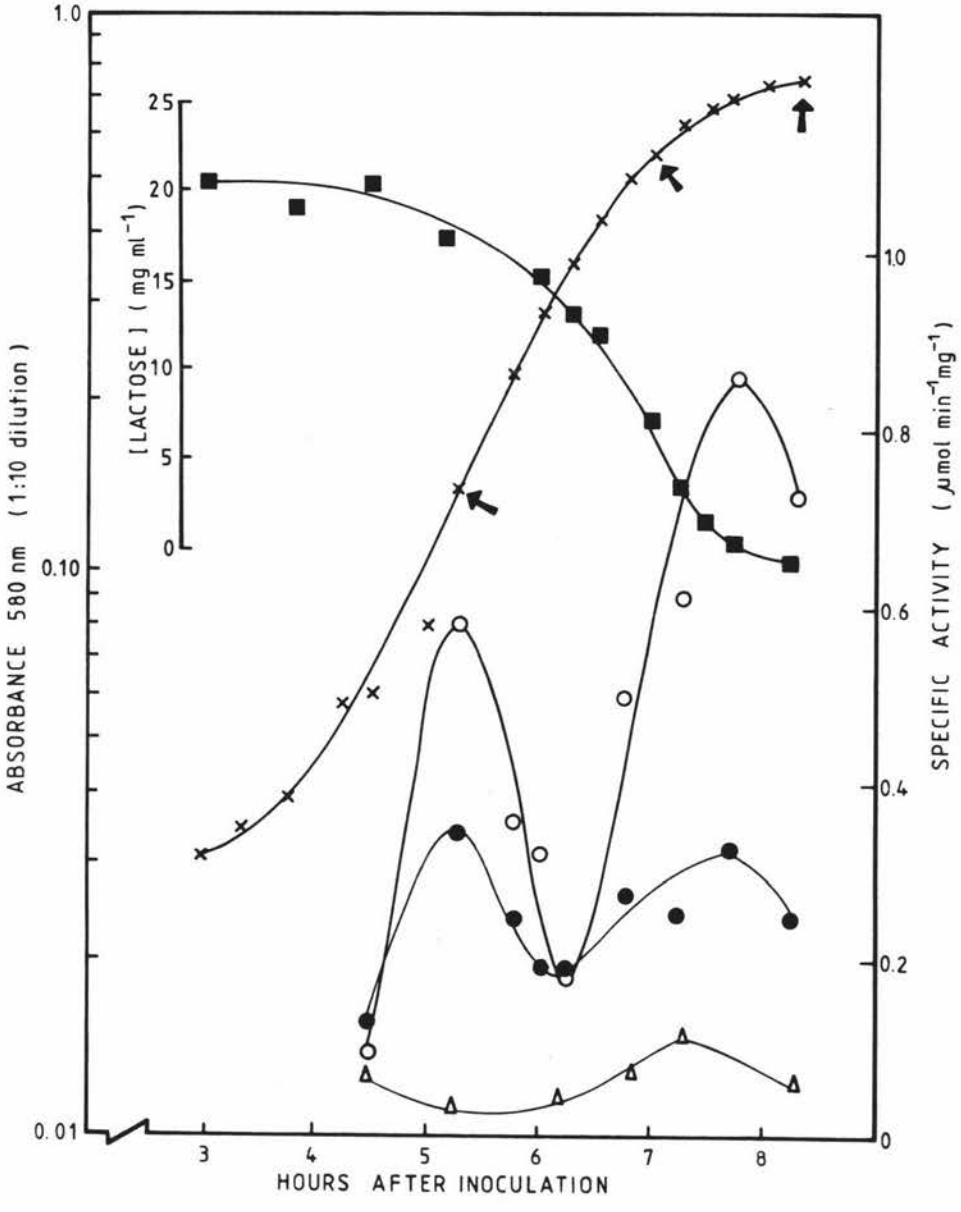


FIGURE 4.3.1 Activities of PFK, T6PK, and Galactokinase, *S. lactis* C_{10} grown on lactose. Activities of PFK (○), T6PK (●), and galactokinase (△) were measured at various stages throughout the growth curve. Cell growth (×) was monitored by measuring the absorbance at 580 nm of suitably diluted culture samples. Lactose remaining in the medium (■) was measured colorimetrically. The arrows indicate the stages of the growth curve at which cells were harvested for purification of PFK (see Section 4.3.3).

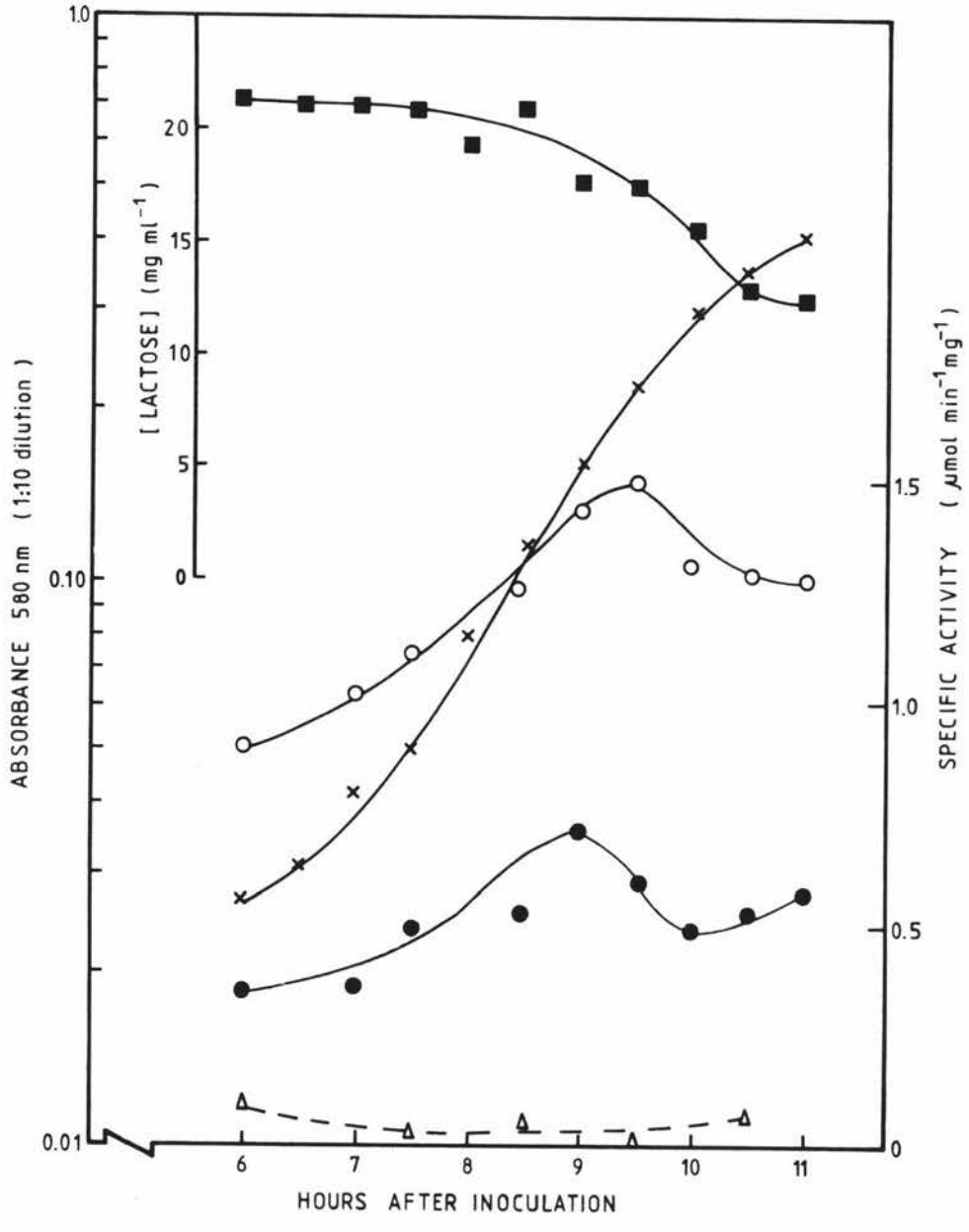


FIGURE 4.3.2 Activities of PFK, T6PK, and Galactokinase in *S. cremoris* AM2 grown on lactose. Activities of PFK (O), T6PK (●) and Gal K (△) were measured at various stages throughout the growth curve. Gell growth (×) was monitored by measuring the absorbance at 580 nm of suitably diluted culture samples. Lactose remaining in the medium (■) was determined colorimetrically.

phase of growth and activity declined as cells entered stationary phase. The ratio of PFK to T6PK remained fairly constant throughout the growth curve. Growth of *S. cremoris* on glucose and galactose produced similar curves. On glucose the ratio of PFK to T6PK was approximately 3:1, while on galactose PFK and T6PK activities were approximately equal (PFK : T6PK = 0.9 : 1) (Table 4.3.5). The average ratios of PFK : T6PK during growth on different sugars follow a similar pattern to the ratios of FBP aldolase : TBP aldolase, as shown in Table 4.3.5. Ratios of both PFK : T6PK and FBP aldolase : TBP aldolase were lowest in galactose-grown cells in both *S. lactis* and *S. cremoris*.

During growth of *S. lactis* C_{10} on lactose two peaks of enzyme activity were observed, one in mid-log phase and a second peak in late-log phase (Fig. 4.3.1). This occurred with both PFK and T6PK activity and possibly also with Gal K activity. Lactate dehydrogenase also showed two peaks of activity, although LDH activity was very low as the enzyme was not assayed under optimum conditions. Fluctuation of PFK specific activity was greater than that of T6PK resulting in ratios of T6PK : PFK ranging from 0.35 : 1 at peak specific activities to approximately 1:1 at minimum specific activity. Growth of *S. lactis* C_{10} on glucose produced similar double peaked specific activity profiles for both PFK and T6PK, but only a single peak corresponding to the second peak of activity was noted in growth on galactose (Data not shown).

The reason for the two peaks of specific activity in *S. lactis* C_{10} is not known. Two separate batch cultures of *S. lactis* C_{10} were grown on lactose and each gave the same two-peaked specific activity profiles. In both experiments and in growth on both lactose and glucose the activity minima corresponded to the period of most rapid carbohydrate breakdown, and the second peak of activity corresponded to a decrease in the rate of utilisation of carbohydrate. One would expect maximum enzyme activity to coincide with the period of rapid growth and carbohydrate utilisation as in *S. cremoris*, but this was not observed in *S. lactis* C_{10}. No contaminant organisms were found on microscopic examination of Gram-stained cells from the cultures so the dual peaks are not due to growth of a mixed culture.

To ascertain whether the two peaks represented the same enzyme, or separate induction of two different enzymes, PFK was prepared according to the procedure described in Chapter 2 from cells harvested

TABLE 4.3.5

RELATIVE ACTIVITIES OF PFK AND T6PK, AND FBP ALDOLASE AND TBP ALDOLASE IN LACTIC STREPTOCOCCI GROWN ON DIFFERENT SUGARS

Organism	Strain	Carbohydrate in Growth Medium	Ratio $\frac{\text{PFK}}{\text{T6PK}}$ *	Ratio $\frac{\text{FBP aldolase}}{\text{TBP aldolase}}$ †
S. lactis	C_{10}	glucose	4.8	7.5
		galactose	0.6	1.6
		lactose	1.4	4.2
S. cremoris	AM_2	glucose	2.6	9.5
		galactose	0.9	1.9
		lactose	2.6	4.3

* Values are the average of ratios calculated at 10 points throughout the growth curve.

† FBP-aldolase and TBP-aldolase activities were measured by Dr V.L. Crow, N.Z. Dairy Research Institute, Palmerston North.

in mid-log, late-log and stationary phases as indicated by the arrows on Figure 4.3.1. No differences between the three enzyme preparations were found. Enzyme from all three stages in the growth cycle had identical R_F values on polyacrylamide gel electrophoresis and exhibited a sigmoidal dependence on F6P. K_m(MgATP) and $F6P_{0.5}$ values determined were similar in all three enzyme preparations. Thus PFK activity appears to be due to the same enzyme at all stages of the growth curve.

4.3.4 CONCLUSIONS

Of the organisms in which the enzymes of the T6P pathway have been demonstrated, only the group N streptococci and *S. mutans* have the potential to use either the T6P pathway or the Leloir pathway in metabolism of lactose and galactose (Bissett & Anderson, 1974*a*; Hamilton & Lebtag, 1979). *Staphylococcus aureus, S. epidermis* and *S. hominis* have also been shown to contain the T6P pathway enzymes (Bissett & Anderson, 1973; Bissett & Anderson, 1974*a*; Schleifer *et al*, 1978) but these organisms lack the enzymes of the Leloir pathway under all growth conditions.

Table 4.3.1 shows the induction of T6PK and Gal K by growth on lactose or galactose in strains of *S. lactis, S. cremoris, S. diacetylactis,* and *S. faecalis*. In most strains both T6PK and Gal K were induced to higher levels by growth on galactose than by growth on lactose. A similar induction pattern for T6PK is found in *S. aureus* (Bissett & Anderson, 1974*a*). In contrast, two of the three strains of *S. mutans* studied by Hamilton and Lebtag (1979) contained higher levels of T6PK in lactose-grown cells than in galactose-grown cells, although in most strains Gal K was induced to higher levels by growth on galactose than by growth on lactose.

T6PK activity was also detected in glucose-grown cells in all the strains used in this study. Apparent T6PK activity was present in glucose-grown cells at levels of 8-40% of the induced level of activity, depending on the strain. Thus T6PK appears to be partially constitutive in these organisms. However part of this activity may be due to phosphorylation of T6P by PFK. Partially constitutive T6PK has been reported in some strains of *S. mutans* (Hamilton & Lebtag, 1979) and low levels of T6PK activity were detected in glucose-grown cells of *S. cremoris* and *S. diacetylactis* (Bissett & Anderson, 1974*a*). Although

the specific activities of T6PK found by these workers were generally lower than the specific activities found in the strains used in this study, as in *S. lactis* C_{10} the T6PK activity in glucose-grown cells ranged from 10-40% of the induced activity.

Gal K activity was induced by growth on lactose and galactose in all strains except *S. cremoris* E_8, and in lactose-grown cells of *S. diacetylactis*. The absence of Gal K in lactose-grown cells of *S. diacetylactis* was also noted by Bissett and Anderson (1974*a*).

The detection of T6PK activity in *S. faecalis* 8043 is in contrast to the results of Schleifer *et al* (1978) who found no T6PK activity in *S. faecalis* strains DSM 20371 and DSM 20380. This difference could be due to strain variation : Schleifer *et al* also showed that several species of staphylococci contain T6PK although other species do not. The possibility that the apparent T6PK activity in *S. faecalis* 8043 was due to phosphorylation of T6P by PFK was not eliminated. Although no T6PK activity was detected in the *S. faecalis* strains studied by Schleifer *et al*, at least one strain of *S. faecalis* transports lactose via a PEP-PTS and has been shown to contain a phospho-β-D-galactosidase but no β-D-galactosidase (Heller and Roschenthaler, 1978). As the occurrence of the T6P pathway enzymes appears so far to be correlated with the presence of phospho-β-D-galactosidase it is likely that T6PK also exists in this strain of *S. faecalis*.

High levels of T6PK in the absence of Gal K or vice versa suggests operation of either the T6P or Leloir pathways. In most strains specific activities of T6PK were higher than specific activities of Gal K so the T6P pathway, rather than the Leloir pathway appears to be operative. However the presence of both T6PK and Gal K in some strains, notably *S. lactis* strains ML_3 and 7962, *S. diacetylactis* DRC_1, and *S. faecalis* 8043, suggests that both pathways operate or that there is some other regulatory mechanism determining the flux through the pathways, such as uptake of sugar via the permease or PEP phosphotransferase systems.

As the importance of enzyme levels in determining which pathway is used is not clear, the relative participation of the T6P and the Leloir pathways is unknown. Detection of enzymes of a particular pathway *in vitro* is not necessarily an indication of operation of that pathway *in vivo* as enzyme activity is dependent on the intracellular

environment. *S. lactis* 7962 is one example of this. Lactose-grown cells contain T6PK but the T6P pathway is probably not utilised in lactose metabolism as this strain transports lactose via an ATP-dependent permease and lacks the phospho-β-D-galactosidase necessary for cleavage of lactose-phosphate. However induction of T6PK by galactose, and the presence of galactose 6-phosphate in galactose-grown cells of *S. lactis* 7962 (Thomas *et al*, 1980) is strong evidence for operation of the T6P pathway in galactose metabolism although presence of a PEP phosphotransferase system for galactose has not been demonstrated in this strain (Kashket & Wilson, 1972).

Measurement of intracellular intermediates may provide a better indication of operation of a pathway than measurement of enzyme activities, although absence of a detectable pool of an intermediate may be due to rapid turnover rather than non-operation of the pathway. However the data on enzyme activities produced in this study agree well with the conclusions drawn by Thomas *et al* (1980) from studies of intracellular concentrations of sugar phosphates. These workers measured intracellular levels of galactose 1-phosphate and galactose 6-phosphate in several strains of *S. lactis* and *S. cremoris* to determine the relative participation of the Leloir and T6P pathways in galactose metabolism. All strains of *S. lactis* studied contained significant levels of both galactose 1-phosphate and galactose 6-phosphate under the conditions assayed. Thomas *et al* suggest that in *S. lactis* the Leloir pathway is the predominant route of galactose metabolism at low galactose concentrations, while the T6P pathway is significant at high concentrations of galactose. In *S. cremoris* the T6P pathway appears to be the more important (if not the only) route.

The results of this study have not provided any explanation for the unusual metabolic behaviour of *S. lactis* ML_8, the only strain which shows homofermentative metabolism of galactose. The relative amounts of pyruvate converted to lactate and to other end-products (formate, acetate and ethanol) may be determined by the levels of FBP and TBP which act as activators of lactate dehydrogenase (Thomas *et al* 1980). Therefore a possible explanation of the homolactic fermentation of *S. lactis* ML_8 could be that this strain may possess unusually high activities of PFK or T6PK, the enzymes catalysing the formation of these activators. However this is clearly not so. Activities of

T6PK and Gal K in *S. lactis* ML_8 are comparable to the activities in other strains. PFK activity, although slightly higher in galactose-grown cells of *S. lactis* ML_8 than in other strains, is comparable to the activity in *S. cremoris* and these organisms do not display this unusual homolactic fermentation of galactose.

Thompson *et al* (1978) showed that addition of glucose or lactose to *S. lactis* ML_3 growing exponentially on galactose, inhibited galactose metabolism, and concluded that this was due to catabolite inhibition, not catabolite repression as the enzymes necessary for galactose metabolism were already induced. T6PK activity in cells grown on glucose plus galactose was generally lower than activity in cells grown on galactose alone. Thus it appears that in the strains tested, diauxic growth is partially due to suppression by glucose of the enzymes required for galactose metabolism (catabolite repression). Catabolite inhibition by glucose may also be involved although glucose was not found to inhibit T6PK activity. In contrast, glucose does not appear to repress synthesis of T6PK in most strains grown on a mixture of glucose plus lactose. The presence of T6PK in cells grown on a mixture of glucose plus lactose suggests that both glucose and lactose may be metabolised simultaneously, assuming that transport of lactose occurs via the PEP phosphotransferase system.

The results in Figures 4.3.1 and 4.3.2 show that although the specific activities of T6PK and PFK in *S. lactis* and *S. cremoris* vary at different stages of the growth curves, changes in activity of PFK are accompanied by a corresponding change in T6PK activity so that the ratio of T6PK : PFK remained between 0.35 : 1 and 1 : 1. This may simply reflect the changes in total protein synthesis in the cell, or may indicate a requirement for co-ordination of PFK and T6PK activities for efficient carbohydrate metabolism. The reasons for the variation in specific activities of the two enzymes throughout the growth curve is not clear, but this is an interesting observation which warrants further study.

CHAPTER 5

CONCLUSIONS

CHAPTER 5

CONCLUSIONS

5.1 COMPARISON OF PFK AND T6PK

Characterisation of purified PFK and T6PK from *S. lactis* has clearly shown that in this organism, as in *S. aureus*, these are two distinct enzymes with markedly different physical and kinetic properties. T6PK is a smaller protein than PFK in both *S. lactis* and *S. aureus*. While PFK from both sources is a tetramer of subunit molecular weight approximately 33,500 daltons, the molecular weight of both T6PKs is approximately 100,000 daltons, and the *S. aureus* enzyme has been shown to be a dimer (Bissett and Anderson, 1979). *S. lactis* T6PK could be completely separated from PFK by gel filtration on Sephacryl S200 or by passage through a column of blue dextran-Sepharose, an 'affinity' resin which bound PFK but not T6PK (Section 2.2.11).

S. lactis PFK is an allosteric enzyme which shows co-operative binding of F6P and inhibition by high concentrations of ATP relative to F6P, properties which are common to most bacterial and mammalian PFKs. PFK activity in *S. lactis* is also regulated by various metabolites including monovalent cations, ADP, PEP, P_i, T6P, and FBP. A comparison of the regulatory properties of PFK from various sources including *S. lactis* shows that while there is considerable variation between species with respect to the specific regulation by individual effectors, two regulatory features are conserved in most bacterial, plant, and mammalian PFKs : regulation of activity by the energy status of the cell, and feedback inhibition of activity by intermediates in the pathways of carbohydrate metabolism. Citrate, the major metabolite responsible for feedback inhibition of mammalian PFK has little effect on *S. lactis* PFK. However in *S. lactis*, as in other bacteria, PFK activity is inhibited by PEP. The energy status of the cell is largely reflected by the levels of ATP, ADP, and AMP. In eukaryotes, PFK activity is generally inhibited by high concentrations of ATP, and activated by AMP and ADP. These metabolites exert a similar effect on PFK in several bacteria; in *S. lactis* PFK activity was inhibited by both AMP and high concentrations of ATP (relative to F6P concentration) and activated by ADP.

In contrast to PFK, T6PK appears to be less subject to allosteric control in both *S. lactis* and *S. aureus*. T6PK exhibited no sigmoidal dependence on T6P concentration, and was not as sensitive as PFK to inhibition by high concentrations of ATP relative to T6P concentration.

Both PFK and T6PK from *S. lactis* showed similar pH optima (pH 7.5 - 8.0) and divalent cation requirement. Neither enzyme had an absolute requirement for monovalent cations, but both were activated by K^+ and NH_4^+ and inhibited by Na^+ and Li^+. Both PFK and T6PK were able to phosphorylate F6P and T6P, but the maximum velocity obtained with the natural substrate was much higher than when the other sugar phosphate was used as substrate. Phosphorylation of T6P by PFK, and of F6P by T6PK is probably not significant *in vivo* as the $T6P_{0.5}$ of PFK is approximately 10-fold higher than the $F6P_{0.5}$, and 18-fold greater than the K_m (T6P) of T6PK. Similarly the K_m (F6P) of T6PK (42 mM) is over 250-fold greater than the K_m (T6P). The differences in affinities of PFK and T6PK for the sugar phosphates suggests that F6P and T6P are phosphorylated *in vivo* almost solely by PFK and T6PK respectively.

5.2 THE ROLE OF PFK AND T6PK IN REGULATION OF CARBOHYDRATE METABOLISM IN *S. lactis*

The T6P pathway requires regulation for two reasons:

i As outlined in Chapter 1 there must be some co-ordination of activities of the enzymes of the T6P pathway and the corresponding enzymes in the EMP pathway, since metabolism of lactose results in simultaneous metabolism of both glucose and galactose 6-phosphate moieties, without accumulation of either hexose (Thompson *et al*, 1978).

ii Some regulatory mechanism might be expected to co-ordinate the activity of the enzymes of the T6P pathway with that of pyruvate kinase and lactate dehydrogenase which are the major regulatory enzymes of the EMP pathway in lactic streptococci. Such co-ordination would be particularly important in galactose metabolism in organisms such as *S. cremoris* strains E_8 and HP in which the T6P pathway is the major route of entry of carbohydrate into the EMP pathway (Thomas *et al*, 1980). Both pyruvate kinase and lactate dehydrogenase from lactic streptococci are known to be activated by both FBP and TBP (Thomas, 1975; 1976; Crow & Pritchard, 1977), thus providing a link between PFK and T6PK activities (which could affect pool sizes of FBP and TBP) and activities of pyruvate kinase and lactate dehydrogenase. Furthermore, the results of this study have demonstrated that both PFK and T6PK activities can be regulated by ADP and PEP, substrates of pyruvate kinase.

PEP has a similar inhibitory effect on both PFK and T6PK. Accumulation of PEP could occur if activities of any of the enzymes involved in its metabolism were inhibited, an instance in which it would be advantageous to control flux through the EMP pathway at an earlier point in the pathway. PEP also accumulates in carbohydrate-starved cells in which PEP is not being utilised for uptake of sugars

via the PEP-PTS (Thompson & Thomas, 1977); under these conditions both PFK and T6PK activity would be suppressed.

ADP affected the activities of *S. lactis* PFK and T6PK in different ways. Whereas ADP was an activator of PFK, 0.1 - 5.0 mM ADP resulting in a shift of the sigmoidal F6P saturation curve towards a more hyperbolic form, ADP (1.0 - 5.0 mM) inhibited T6PK activity. ADP inhibition of T6PK activity was competitive with respect to ATP, and non-competitive with respect to T6P. The regulatory implications of this reciprocal effect on the two enzymes are not clear. ADP activation of PFK activity, combined with inhibition by high concentrations of ATP, is a mechanism by which activity of PFK could be regulated according to the energy status of the cell. A similar effect is achieved in mammalian systems by AMP activation and ATP inhibition of PFK activity. The low concentrations of MgADP required for activation of PFK reflect the sensitivity of the response of PFK to the cellular energy requirement.

Activation of PFK by ADP is consistent with the position of PFK in the glycolytic pathway, an ATP-generating sequence. PFK, although an ATP-utilising enzyme does not respond as expected to changes in the adenylate energy charge (Atkinson, 1969). Activity is increased, not decreased, as the ATP concentration decreases, due to activation by ADP. T6PK however shows a 'normal' response for an ATP-utilising enzyme, in that its activity is decreased as ATP supplies are depleted. This suggests that in growth on lactose T6PK activity is less important than PFK activity in maintaining ATP supplies.

The inhibition of T6PK by ADP would present interesting regulatory problems in organisms such as *S. cremoris* strains HP and E_8 grown on galactose, where the T6P pathway is believed to be the predominant pathway for galactose metabolism (Thomas *et al*, 1980). These strains may possibly contain a T6PK with different regulatory properties to those of T6PK from *S. lactis* C_{10}; one could expect an enzyme which was activated by ADP in a similar manner to *S. lactis* PFK. However the inhibition of T6PK by ADP observed *in vitro* may not be physiologically significant. The concentration of ADP required to produce significant inhibition of T6PK was 1-5 mM. Intracellular concentrations of ADP in *S. lactis* of 2.4 mM (Collins & Thomas, 1974) and 7.8 mM (Crow, 1975) have been reported in strains ML_3 and C_{10}

respectively. Under these conditions T6PK would be inhibited and PFK activated, even though the magnitude of ADP activation decreases with increasing ADP concentration (Section 2.3.12) (The optimum concentration of ADP for PFK activation is 0.1 mM). However as both PFK and T6PK are also inhibited by AMP, the ratio of nucleotides (i.e. adenylate energy charge) rather than concentrations of an individual nucleotide may be the factor controlling activity. In addition, ADP levels may vary between strains depending on the particular metabolic pathways used.

Although T6PK lacks the co-operative binding of substrate exhibited by PFK, and appears to be less sensitive than PFK to modulation of activity by most glycolytic intermediates this does not mean that T6PK is merely an unregulated enzyme. As Hess and Boiteaux (1980) have noted, although most of the glycolytic enzymes exhibit Michaelis-Menten kinetics "multiple functions of substrate, cofactor and inhibitor concentrations definitely modulate the activities of these enzymes as well".

By analogy with the corresponding enzymes of the EMP pathway, T6PK would appear the most likely point of control of the T6P pathway. However it is possible that allosteric regulation of T6PK may not be required, T6PK activity being controlled primarily by the availability of substrate. Co-ordination of fluxes through the T6P and EMP pathways may be achieved by allosteric regulation of PFK activity in response to intermediates of the T6P pathway. A comparison of the effects of F6P and T6P on PFK and on T6PK suggest that this may be so.

T6P (0.2-2.0 mM) was a competitive inhibitor of PFK, lessening the co-operativity of binding of F6P. In contrast, T6PK was inhibited only by 10-20 mM F6P and there was little effect on T6PK activity at lower concentrations of F6P. As the physiological concentration of F6P in actively glycolysing cells of *S. lactis* is approximately 0.26 mM (Thompson, 1978) the activity of T6PK *in vivo* is unlikely to be inhibited by F6P. This suggests that T6PK activity would be largely unaffected by minor fluctuations in sugar phosphate concentration while small increases in the intracellular concentration of T6P could result in inhibition of PFK activity.

Simultaneous metabolism of the galactose 6-phosphate and glucose moieties of lactose could also be achieved by regulating substrate

availability. Cleavage of lactose by phospho-β-D-galactosidase ensures an equal rate of substrate supply to both the EMP and T6P pathways. This enzyme, situated at the branch point of the two pathways is ideally located to function as a regulatory enzyme. Thus if an intermediate of either pathway acts as a feedback inhibitor of phospho-β-D-galactosidase the carbohydrate supply to both pathways is restricted. Galactose 6-phosphate, but not glucose, glucose 6-phosphate, or galactose has been shown to inhibit phospho-β-D-galactosidase from *S. cremoris* HP (Johnson & McDonald, 1974) while the *S. mutans* enzyme is inhibited by PEP and ATP in addition to galactose 6-phosphate and glucose 6-phosphate (Calmes & Brown, 1979). A similar inhibition in *S. lactis* could be sufficient to co-ordinate the activities of the T6P and EMP pathways.

Although bacterial PFKs exhibit similar kinetic properties *in vitro* to mammalian PFKs, the importance of allosteric control of PFK in regulation of carbohydrate metabolism is less well defined than in mammalian systems. However PFK plays an important role in regulation of carbohydrate metabolism in *E. coli* as shown by Deitzler *et al*, (1974) who demonstrated that during exponential growth of *E. coli* the rate of glucose utilisation was directly proportional to the intracellular concentration of FBP, which is dependent on activity of PFK.

Many bacterial species contain only a non-allosteric form of PFK (see Section 2.1), while *E. coli* contains both a non-allosteric and an allosteric enzyme (Babul, 1978; Robinson & Fraenkel, 1978). Robinson and Fraenkel (1978) have postulated that the co-operative binding of F6P to PFK observed *in vitro* may not be significant *in vivo* as physiological metabolite concentrations, in particular concentrations of PEP and ADP may be such that the PFK exhibits Michaelis-Menten kinetics. Studies on *E. coli* mutants which contained different levels of the allosteric and non-allosteric forms of PFK showed no large differences in growth rates or yields, providing one of the enzymes was present in sufficient amounts.

However the non-allosteric nature of PFK II has been questioned by Kotlarz and Buc (1981) who showed that PFK II presented some regulatory properties *in vitro*. High concentrations of ATP inhibited PFK II : this inhibition could be relieved by increasing F6P concentrations as has been found with PFK I (Blangy *et al*, 1968). ATP also induced formation of a tetrameric enzyme thus suggesting the presence of a second (allosteric) ATP-binding site on the PFK II protomer.

One might expect a less rigid control of PFK activity in *S. lactis* as this organism lacks a TCA cycle and does not carry out gluconeogenesis. The lack of FBPase in *S. lactis* (Dr V.L. Crow, N.Z. Dairy Research Institute, Palmerston North, personal communication) circumvents the need for regulation of PFK activity to prevent dissimilation of ATP by 'futile cycling'.

Induction of the enzymes of the T6P and Leloir pathways (Chapter 4) by growth on lactose or galactose constitutes a 'coarse' control mechanism, providing the organism with the enzymic potential to utilise either one or both pathways. However there is clearly some other regulatory mechanism controlling the flux through these two pathways. Although the levels of T6PK and Gal K are comparable in most strains of *S. lactis* and *S. cremoris* studied, it is known that the T6P and Leloir pathways are used to different extents in different strains (Thomas *et al* 1980). Demonstration of an enzymic potential does not establish that a particular pathway is operative *in vivo*. Growth of *S. lactis* 7962 on lactose or galactose induces activity of T6PK, but as this strain lacks a PEP PTS for lactose and galactose transport (Kashket and Wilson, 1972) and the phospho-β-D-galactosidase necessary for cleavage of lactose 6-phosphate (Citti *et al*, 1965) the T6P pathway is not utilised in lactose or galactose metabolism in this strain.

In considering the regulatory properties of purified PFK and T6PK *in vitro*, two further questions arise:

i To what extent do the parameters measured *in vitro* reflect the activities of the enzymes *in vivo* i.e. do the properties of the enzyme alter as a result of purification?

ii What is the physiological significance of the *in vitro* regulatory properties?

To determine the relevance of *in vitro* kinetic studies to regulation *in vivo* Banuelos *et al* (1977) and Sols *et al* (1973) measured PFK and pyruvate kinase activities in cell-free extracts and permeabilised cells of yeast. Comparable activities were found, thus it appears justifiable to relate kinetic parameters obtained *in vitro* to *in vivo* situations. Calculation of metabolic fluxes based on kinetic

constants of several glycolytic enzymes obtained *in vitro* yields results which correlate well with the observed metabolic rate (Barwell & Hess, 1972).

As enzyme activities are complex functions of substrate and product concentrations as well as other controlling factors, e.g. co-factor availability, pH etc., a more accurate estimation of activity might be obtained by conducting *in vitro* experiments at substrate and effector concentrations as near as possible to physiological concentrations. However this too, has its limitations as the number of metabolites which may modulate activity is large, and intracellular metabolite concentrations fluctuate.

One method of detecting any change in properties of an enzyme during purification is to compare the regulatory properties of purified enzyme with those of the enzyme in crude extracts. This requires a specific assay system: the PK-LDH assay system used initially in the study of T6PK (because of unavailability of TBP-specific aldolase) was unsuitable for measuring activity in the cell-free extract because of the high 'background' activity observed due to activities of other ADP-producing enzymes. However the K_m (MgATP) values determined for T6PK and PFK using the specific aldolase assay systems described in Sections 2.2.6 and 3.2.4 showed reasonable agreement between values determined using the purified enzyme and those obtained with the cell-free extract.

Although the difference between values of $F6P_{0.5}$ for PFK obtained in cell-free extract ($F6P_{0.5}$ = 0.65 mM) and values obtained in initial experiments using purified PFK ($F6P_{0.5}$ = 0.28 mM) could be explained by the presence of other enzymes in the extract with a high affinity for F6P (as appeared to be the case in *S. lactis*), some preparations of purified enzyme also showed a $F6P_{0.5}$ of approximately 0.65 mM, suggesting that some change in the enzyme occurred during purification which led to a lower affinity for the substrate. However this change in $F6P_{0.5}$ did not appear to alter the other physical or kinetic properties of the enzyme.

To determine the physiological significance of the *in vitro* kinetic properties of *S. lactis* PFK and T6PK, further experimentation is required. This study has shown that *S. lactis* PFK is similar both physically and kinetically to many other bacterial PFKs which is

perhaps surprising in view of the diversity of the metabolic pathways employed in carbohydrate metabolism. *E. coli*, for example, which operates a TCA cycle and undergoes gluconeogenesis could be expected to exhibit different enzymic regulation to *S. lactis* which lacks both pathways. *S. lactis* T6PK is similar to the *S. aureus* enzyme, but whether this similarity extends to other species is unknown. A comparative study of the regulatory properties of PFK and T6PK (and other glycolytic enzymes) in organisms known to differ in certain biochemical or physiological characteristics should reveal whether or not these enzymes play a key role in determining the physiology of the organism. Several systems invite further study:

i Organisms such as *S. mutans* which contain a non-allosteric form of PFK (Dr V.L. Crow, N.Z. Dairy Research Institute, Palmerston North - personal communication) in addition to T6PK.

ii Strains of *S. lactis* and *S. cremoris* which contain enzymes of both the T6P and Leloir pathways, but which utilise these two pathways to different extents (Thomas *et al*, 1980). A study of the enzymes of these two pathways should show whether this is due to differences in properties of the enzymes or whether operation of these pathways is controlled by other means e.g. by the sugar uptake system utilised.

iii Strains such as *S. lactis* ML_3 and ML_8 which display different physiological characteristics depending on their growth conditions (Thomas *et al* 1979). The unusual homolactic galactose metabolism displayed by *S. lactis* ML_8 (Thomas *et al*, 1980) appears to be closely correlated to increased FBP levels in the cell. This was not due to increased specific activity of PFK in *S. lactis* ML_8 (see Chapter 4 of this thesis) thus a study of PFK in this organism may reveal kinetic differences which could account for the characteristic behaviour of this strain.

iv *S. lactis* mutants deficient in T6PK and TBP-aldolase. A comparison of these with wild-type organisms may provide insight into the role of the T6P pathway in carbohydrate metabolism in lactic streptococci.

Determination of the intracellular concentrations of metabolites is essential in order to fully understand the regulation of an enzyme *in vivo*. A study of the changes in the steady-state concentrations of substrates and effectors of PFK and T6PK under different growth conditions may suggest corresponding changes in enzyme activities. Thus correlation between the ADP and F6P concentrations at different growth rates or sugar concentrations might indicate whether ADP activation of PFK was of significance *in vivo*.

There is little information available on the intracellular levels of the T6P pathway intermediates, however the availability of methods for purifying T6PK (see Chapter 3 of this thesis) and TBP-aldolase (Dr V.L. Crow, N.Z. Dairy Research Institute, Palmerston North, personal communication) facilitates the assays of these intermediates. It would be of interest to monitor T6P and TBP levels in *S. lactis* in response to changes in lactose and galactose concentrations in the medium to determine whether the concentrations of these intermediates fluctuate in a similar manner to the levels of F6P and FBP in *S. lactis* when the glucose concentration of the medium is varied (Thomas *et al*, 1979).

This study has provided some physical and kinetic data on PFK and T6PK from *S. lactis* C_{10}. These data allow limited speculation on the operation of these two enzymes *in vivo*. However to provide definitive answers as to the role of PFK and T6PK in the regulation of carbohydrate metabolism in *S. lactis* would require a detailed study of several other enzymes of the EMP, T6P, and Leloir pathways, and of the intracellular concentrations of metabolic intermediates. In view of the multiple mechanisms which may operate simultaneously in co-operation with other mechanisms to control enzyme activity (Boiteaux and Hess, 1981) it is obvious that allosteric regulation of any enzyme cannot be attributed to a single metabolite. Nor can regulation of a pathway be considered in terms of a single enzyme activity. Hence *in vitro* studies can only provide possible mechanisms which may contribute towards regulation.

However this investigation has demonstrated that PFK and T6PK from *S. lactis* are two distinct proteins with different properties and has provided data which allow comparison of these two enzymes with the corresponding enzymes from other organisms. It has shown that both T6PK and PFK activities can be modulated by a number of glycolytic intermediates. Hence although T6PK appears less subject to allosteric regulation than PFK there are possible mechanisms for the co-ordination of PFK and T6PK activities and for control of the EMP pathway via the reactions catalysed by PFK and T6PK.

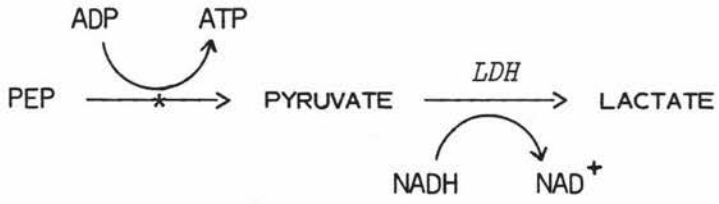
APPENDICES

APPENDIX 1

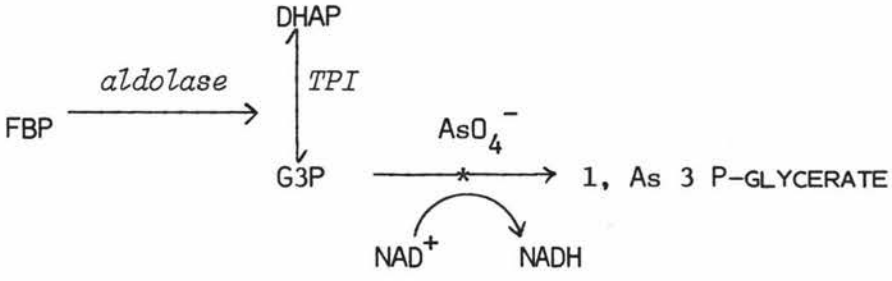
DETECTION OF ENZYMES USED AS MOLECULAR WEIGHT STANDARDS IN GEL FILTRATION

Pyruvate kinase, glyceraldehyde 3-phosphate dehydrogenase, hexokinase and aldolase were assayed spectrophotometrically at 340 nm by coupling activity to oxidation or reduction of nicotinamide cofactors. The assay systems used are summarised below:

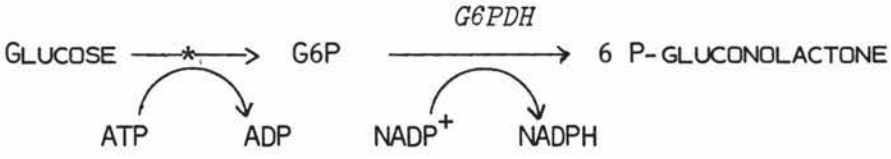
i Pyruvate kinase



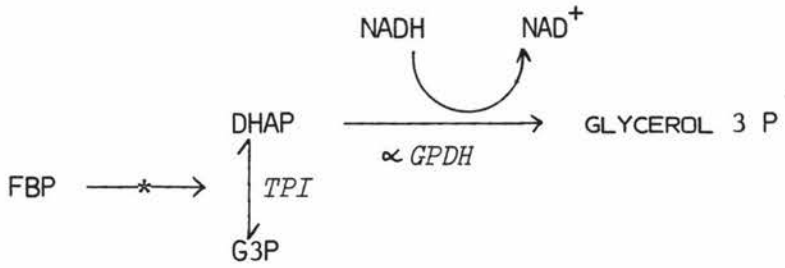
ii Glyceraldehyde 3-phosphate dehydrogenase



iii Hexokinase



iv Aldolase



The reaction catalysed by the enzyme being assayed is marked with an asterisk (*). Coupling enzymes are labelled in italics. Reaction mixtures (0.5 ml total volume) contained 2.0 mM substrate(s), 0.33 mM NADH or $NAD(P)^+$, and excess auxiliary enzymes.

APPENDIX 2

CALCULATION OF MgATP CONCENTRATION

An approximate MgATP concentration was calculated by solving the equation:

$$K_{eq} = \frac{[MgATP]_{eq}}{[Mg^{2+}]_{eq}\,[ATP]_{eq}}$$

Equilibrium concentrations of Mg^{2+} and ATP were assumed to be equal to the initial concentration minus the amounts complexed

$$\text{i.e.} \quad [Mg^{2+}]_{eq} = [Mg^{2+}]_{i} - [MgATP]_{eq}$$

$$[ATP]_{eq} = [ATP]_{i} - [MgATP]_{eq}$$

where subscripts 'eq' and 'i' denote the equilibrium concentration and the initial concentration of the species respectively. An equilibrium constant of 20,000 M^{-1} for the MgATP complex was used (O'Sullivan & Perrin, 1964; Mavis & Stellwagen, 1970).

Table 2.3.12 shows the calculated concentrations of MgATP when Mg^{2+} and ATP concentrations are varied in different ways. When Mg^{2+} and ATP concentrations are varied in a constant ratio the amount of ATP complexed is dependent on the ATP concentration and differs greatly at different Mg^{2+}:ATP ratios. Thus this method of varying Mg^{2+} and ATP concentrations is unsuitable. Maintaining Mg^{2+} at a constant concentration or at a constant excess over the ATP concentration is preferable as under these conditions the MgATP concentration approximates the initial ATP concentration.

Unless otherwise stated the Mg^{2+} concentration in all MgATP solutions exceeded the ATP concentration by 5 mM, so that after dilution in the assay an excess of 0.5 mM Mg^{2+} over ATP concentration was maintained.

TABLE 2.3.12

CALCULATION OF MgATP CONCENTRATIONS

[ATP] (mM)	Constant $[Mg^{2+}]$		Constant Ratio Mg^{2+} : ATP			Constant excess Mg^{2+} > ATP		
	1 mM	2 mM	1:1	2:1	5:1	0.5 mM	2 mM	5 mM
0.025	.024	.024	.006	.010	.024	.022	.024	.025
0.05	.047	.049	.019	.029	.040	.044	.049	.050
0.10	.094	.097	.050	.071	.089	.090	.098	.099
0.20	.188	.195	.122	.164	.188	.189	.195	.198
0.50	.456	.484	.365	.458	.488	.472	.488	.495
1.0	.800	.954	.800	.954	.988	.945	.975	.990
2.0	.954	1.70	1.71	1.95	1.99	1.89	1.95	1.98

Figures shown are the calculated concentrations of MgATP at the given concentrations of Mg^{2+} and ATP.

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