THE ROLE OF PYRUVATE KINASE IN THE REGULATION OF GLYCOLYSIS AND GLUCONEOGENESIS IN *PROPIONIBACTERIUM SHERMANII*

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John Beresford SMART

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ABSTRACT

Pyruvate kinase catalyses the main irreversible reaction of glycolysis in Propionbacterium shermanii since the ATP-dependent phosphofructokinase is largely replaced by a pyrophosphate-dependent phosphofructokinase catalysing a reversible reaction. Measurement of activity of several glycolytic enzymes in glucose-, glycerol- and lactate-grown cells showed that pyruvate kinase activity is much higher than that of pyruvate, orthophosphate dikinase which catalyses the reversible interconversion of PEP and pyruvate.

This poses problems in cells grown on lactate where gluconeogenesis must operate to supply hexoses and pentoses for biosynthesis. The regulatory properties of this enzyme were accordingly studied.

The partially purified (140-fold) pyruvate kinase displayed sigmoidal kinetics for both of its substrates, phosphoenolpyruvate (PEP) and ADP. At pH 7.5 the interaction coefficient (n_H) for PEP saturation in the absence of effectors was in the range 1.9-2.5 while for ADP saturation it was 1.7-2.1. The pyruvate kinase was shown to be activated by glucose 6-phosphate (G6P) at non-saturating (0.5 mM) PEP concentrations but other glycolytic and hexose monophosphate pathway intermediates and AMP were without effect. Half-maximal activation was obtained at 1 mM G6P. The presence of G6P decreased both the $PEP_{0.5V}$ and $ADP_{0.5V}$ values and the Hill interaction coefficient for each substrate. The enzyme was strongly inhibited by ATP and inorganic phosphate (P_i) at all PEP concentrations. At non-saturating (0.5 mM) PEP half-maximal inhibition was obtained at 1.8 mM ATP and 1.4 mM P_i. The inhibition of both P_i and ATP could be largely overcome by G6P. The G6P activation and other regulatory properties of the enzyme were pH dependent.

On the basis of this *in vitro* study it was suggested that the activity of pyruvate kinase *in vivo* is determined by the balance between activators and inhibitors such that it is inhibited by ATP and P_i during gluconeogenesis while, during glycolysis, the inhibition is relieved by G6P. Such a mechanism requires that the G6P concentration should be sufficiently high when cultures are grown on glucose or glycerol but not on lactate.

To verify this proposed mechanism the *in vivo* concentrations of a number of selected glycolytic intermediates were measured in *P.shermanii* growing under a range of nutritional conditions using batch and continuous cultures. The pyruvate kinase activator, G6P, was maintained at levels of 1-2 mM in lactate-grown cells but, when growing in the presence of high levels of glucose or glycerol, G6P was present at levels between 5 and 16 mM in the cell.

F6P and FBP were always present at levels below 1.0 mM and 0.2 mM respectively in lactate- and glucose-grown cells but attained significantly higher levels on glycerol-grown cells. At high concentrations of glucose where high G6P levels were found, F6P was present at concentrations much lower than would be expected from the equilibrium constant of the phosphoglucoisomerase; this was not the case when high G6P levels were attained by growth on glycerol. The reason for this is not known.

Levels of the inhibitor of pyruvate kinase activity, ATP, were maintained in the range 1-2 mM under all the conditions studied. At the *in vivo* levels of the substrates PEP and ADP this level of ATP would not by itself maintain pyruvate kinase in an inactive state in the presence of the 1-2 mM G6P shown to be present in lactate-grown cells. The other inhibitor, P_i, must also be important but the *in vivo* levels of this effector were not determined.

A reinvestigation of the pyruvate kinase at the *in vivo* levels of substrates (PEP and ADP) and effectors (G6P and ATP) found in the cell indicated that, at levels above 10 mM P_i, concentrations of 5-15 mM G6P (as found in cells growing on glucose and glycerol) were required to activate the enzyme while concentrations of 1-2 mM G6P (as found in lactate-grown cells) were not able to overcome the inhibition by ATP and P_i. A concentration of 10 mM P_i in the cell does not seem unreasonable and at this concentration the proposed mechanism for control of the pyruvate kinase *in vivo* would be able to operate.

During this investigation data were also collected on growth yields and carbon balances in both batch and continuous cultures during growth on the three substrates lactate, glucose and glycerol.

Succinate was found to account for up to 26% of the products in glucose cultures but was only present at negligible levels in lactate cultures. Accumulation of succinate in the glucose cultures was highest in the later stages of growth of batch cultures and in carbon-limited continuous cultures when the G6P level was low. This suggests that regulation of pyruvate kinase by G6P may also serve to determine the relative flux via the PEP:carboxytransphosphorylase and pyruvate kinase enzymes - the carboxytransphosphorylase functioning to provide PP_i for the PP_i-dependent phosphofructokinase in glycolysis.

It is also proposed that G6P may be involved in partitioning glucose metabolism via glycolysis and the hexose monophosphate pathway - high internal G6P concentrations favouring flow through the hexose monophosphate pathway and thus bypassing the PP_i-dependent phosphofructokinase - however, this is highly speculative.

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ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
Bistrispropane	2 bis[tris (hydroxymethyl) methylamino]-propane
CDP	cytidine 5'-diphosphate
CM-	carboxymethyl-
CoA	coenzyme A
DE-, DEAE-	diethylaminoethyl-
DHAP	dihydroxyacetone phosphate
DNase	deoxyribonuclease
ED	Entner-Doudoroff
EDTA	ethylenediamine tetra-acetic acid
EMP	Embden—Meyerhof-Parnas
FBP	fructose 1,6-bisphosphate
F6P	fructose 6-phosphate
Ga 3-P, Glyceraldehyde 3-P	glyceraldehyde 3-phosphate
GDP	guanosine 5'-diphosphate
glycerol 3-P	glycerol 3-phosphate, α-glycerophosphate
G6P	glucose 6-phosphate
GTP	guanosine 5'-triphosphate
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid
HMP	hexose monophosphate
IDP	inosine 5'-diphosphate
MES	2[N-morpholino]ethane sulphonic acid
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced

NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
n_H	Hill interaction coefficient
OD_{540}	optical density at 540 nm
OAA	oxaloacetic acid
PEP	phosphoenolpyruvate
6-PG	6-phosphogluconate
3-PGA	3-phosphoglyceric acid
P_i	inorganic phosphate
PP_i	inorganic pyrophosphate
ribose 5-P	ribose 5-phosphate
ribulose 5-P	ribulose 5-phosphate
Tricine	N-tris (hydroxymethyl) methylglycine
Tris	tris (hydroxymethyl) aminomethane
UDP	uridine 5'-diphosphate
V_{max}	maximum velocity

E.coli	Escherichia coli
N.crassa	Neurospora crassa
S.lactis	Streptococcus lactis

Chapter 1

GENERAL INTRODUCTION

1.1 REGULATION OF CARBOHYDRATE METABOLISM IN BACTERIA

Bacteria employ a number of different metabolic pathways for the breakdown of carbohydrates to the common intermediate pyruvate, from which further catabolic or synthetic reactions proceed (Doelle, 1969; Gottschalk, 1978). In addition to the Embden - Meyerhof - Parnas (EMP) pathway which is the major pathway in many bacteria, the hexose monophosphate (HMP) pathway or pentose phosphate pathway is also commonly found. Other pathways of more restricted distribution are the phosphoketolase pathway of heterolactic lactic acid bacteria and bifidobacteria and the Entner - Doudoroff (ED) pathway in pseudomonads. In many bacteria the degradation of glucose may involve a combination of two or more of these pathways. Catabolism of sugars other than glucose may involve other pathways such as the tagatose pathway for galactose catabolism in staphylococci and streptococci (Bissett and Anderson, 1974).

These pathways frequently serve a dual role, functioning both as catabolic pathways for ATP production and as sources of precursors such as pentose phosphates or glycerol 3-phosphate for biosynthesis of cell constituents.

Bacteria growing on 3- and 4-carbon compounds (glycerol, pyruvate, lactate, succinate, malate etc.) must be able to use these compounds not only as an energy source but as carbon sources for carbohydrate synthesis. Carbohydrate synthesis from simple organic compounds (gluconeogenesis) necessitates reactions to bypass the irreversible steps of glycolysis (phosphofructokinase and pyruvate kinase). Thus, for example, in E.coli PEP:carboxykinase and NADP-specific malic enzyme, during growth on

4-carbon compounds, and PEP:synthetase, during growth on 3-carbon compounds, are able to bypass the irreversible pyruvate kinase reaction. In other bacteria synthesis of PEP from pyruvate may be accomplished via the enzymes pyruvate carboxylase and PEP:carboxykinase which convert pyruvate to PEP via the intermediate OAA. In organisms utilising the full glycolytic pathway the phosphofructokinase reaction is bypassed during gluconeogenesis by the enzyme fructose bisphosphatase. Other reactions of the glycolytic pathway are reversible and are used for either glycolysis or gluconeogenesis depending on the growth substrate.

The fact that the pathways of carbohydrate metabolism in bacteria fulfil both anabolic and catabolic functions (i.e. they are what Sanwal (1970) refers to as amphibolic pathways) requires the existence of control mechanisms to regulate the flow of metabolites into either the biosynthetic or energy-generating pathways. Regulation of these pathways can be either by control of enzyme synthesis or degradation in the cell or by control of enzyme activity. Bacteria have evolved a wide range of mechanisms for the control of the activity of enzymes at key points in the metabolic pathways. Both end-product inhibition and precursor or 'feed-forward' activation mechanisms are widespread in bacteria. The particular types of control mechanisms found are usually related to the specific metabolic pathways operating in any particular organism.

Amphibolic pathways are involved in both the generation of energy (ATP) and its utilisation via the biosynthetic channels and are thus found to be sensitive to regulation by a number of the common intermediates of energy metabolism e.g. AMP, ADP, ATP, PP_i and P_i. Chapman and Atkinson (1977) consider that the influence of the energy status of the

cell in the regulation of metabolism is mediated by way of the 'energy charge' which they define as the following ratio of adenine nucleotide concentrations: (ATP + ½ADP)/(ATP + ADP + AMP). Thus enzymes that participate in ATP-generating sequences are inhibited by increasing values of the energy charge while those that participate in ATP-utilising sequences show the reverse response.

Reduced coenzymes are also frequently utilised as control signals for the regulation of diverging or converging pathways (Sanwal, 1970). The NADH level may serve as an indicator of the rate of glycolysis in enteric bacteria and has been found to inhibit reactions which function in the opposite direction to glycolysis such as those of gluconeogenesis. The HMP pathway is generally considered to be regulated by the availability of oxidised NADP and in E.coli it has been found that NADH may control the NADPH generating pathways of the cell (Sanwal, 1970).

Enzymes situated at key positions in metabolic pathways, such as at a branchpoint, are ideally sited to control the flow of metabolites through those pathways. Such enzymes are frequently found to display non-Michaelis-Menten kinetics and are susceptible to allosteric control by their substrates, products or other effectors.

1.1.1 Role of Pyruvate Kinase in Regulating Carbohydrate Metabolism in Bacteria

As mentioned earlier, pyruvate is a common intermediate in the metabolism of carbohydrates in most bacteria. The final reaction in the pathway by which pyruvate is formed from glucose is catalysed by the enzyme pyruvate kinase (I)

$$\text{PEP} + \text{ADP} \xrightarrow{Mg^{++}, K^{+}} \text{Pyruvate} + \text{ATP} \qquad \text{(I)}$$

Both the substrate, PEP, and product, pyruvate, of this reaction are substrates for a number of other reactions in bacteria and consequently the pyruvate kinase has been found to display cooperative saturation curves for PEP and ADP and to be subject to a wide range of allosteric controls depending on the particular metabolic pathways of the organism.

The major emphasis of this thesis is on the role of pyruvate kinase in controlling the pathways of glycolysis and gluconeogenesis in Propionibacterium shermanii. The role of this enzyme in regulating the pathways of carbohydrate metabolism in a number of other bacteria, where it has been well studied, will be dealt with in this section.

Escherichia coli

The major pathway of glucose catabolism in E.coli is the EMP pathway, although an active HMP pathway is also present. Phosphofructokinase has been established as the rate-limiting enzyme of glycolysis (Scrutton and Utter, 1968) and is controlled in E.coli both through responses to the energy-charge and by end-product inhibition by PEP (Sanwal, 1970). The reverse reaction in gluconeogenesis, from FBP to F6P, is catalysed by fructose bisphosphatase. The phosphofructokinase and fructose bisphosphatase are subject to reciprocal allosteric modulation by changes in the energy charge such that phosphofructokinase is inhibited by high values (i.e. high ATP) while fructose bisphosphatase shows the reverse response (being inhibited by AMP) and thus energy wasteful 'futile cycling' is prevented (Sanwal, 1970).

The second major point of control of glycolysis in E.coli is at pyruvate kinase where two forms of the enzyme have been identified (Malcovati and Kornberg, 1969). From their molecular weights, chemical

and allosteric properties it was concluded that the two pyruvate kinases were distinct non-interconvertible enzymes which have been labelled type I and type II pyruvate kinase. The two forms appear to co-exist, although in different proportions, under most physiological conditions and consequently the total pyruvate kinase activity of the cell results from the contribution of both forms of the enzyme (Waygood and Sanwal, 1974; Waygood *et al*, 1975; Valentini *et al*, 1979).

Kinetic properties of the two enzymes differ, although both show cooperative saturation curves for the substrate PEP. The type I enzyme is allosterically activated by FBP (which abolishes the cooperativity of the PEP saturation) and inhibited by succinyl CoA and GTP. GDP is a better phosphate acceptor than ADP for the type I pyruvate kinase *in vitro* and the inhibition by GTP is strongest when GDP is the nucleotide phosphate acceptor rather than ADP. These facts suggest that GDP may function as the phosphate acceptor *in vivo* (Waygood and Sanwal, 1974; Waygood *et al*, 1976).

The type II pyruvate kinase is activated by a number of nucleotide monophosphates (AMP, GMP etc.) and sugar monophosphates (ribose 5-P, ribulose 5-P, G6P etc.), but not FBP. Ribose 5-P is the most potent activator and abolishes the cooperativity of the PEP and ADP saturation curves. ATP, succinyl CoA and P_i are all powerful inhibitors of the type II enzyme. Succinyl CoA inhibition is competitive with respect to both substrates, ADP and PEP. P_i inhibition can be completely overcome by ribose 5-P and thus P_i must bind at a site distinct from the substrate binding site. ATP inhibition can only be partially overcome by ribose 5-P and apparently functions both as a product and as an allosteric inhibitor (Waygood *et al*, 1975; Somani *et al*, 1977).

Kotlarz et al (1975) found a correlation between the conditions favouring derepression of a particular isoenzyme of pyruvate kinase in E.coli and the nature of the allosteric signal which regulates its enzymic activity. Thus the biosynthesis of the type I enzyme, which is activated by a specific metabolite of the glycolytic pathway (FBP), is strongly dependent on the nature of the carbon source (being induced by growth on glucose). On the other hand the amount of the type II enzyme, which is regulated by AMP, depends on whether growth occurs under aerobic or anaerobic conditions (being favoured by anaerobiosis). Waygood et al (1975) suggest that as the type II enzyme is activated by ribose 5-P and phosphorylated intermediates of the HMP pathway it is likely to function under aerobic conditions. However Somani et al (1977) felt that while the physiological significance of the strong activation by intermediates of the HMP pathway could be accepted for growth in aerobic conditions it appeared unlikely to be important in anaerobic conditions when the type II enzyme is maximally derepressed. Under such conditions they suggest that the regulation of the type II pyruvate kinase by AMP would be of greater significance, as suggested by Kotlarz et al (1975).

In E.coli PEP may be converted either to pyruvate via the pyruvate kinase or to OAA via a PEP carboxylase (Kornberg, 1970). Both of these enzymes are subject to precursor activation by FBP but while the pyruvate kinase is inhibited by GTP the PEP carboxylase is activated by it. Waygood and Sanwal (1974) suggest this modulation, by GTP, of the two enzymes may be a mechanism which regulates the distribution of PEP into different channels according to the energy state of the cell.

Streptococcus lactis

The homofermentative lactic acid bacteria also convert glucose to

pyruvate by the glycolytic pathway. The pyruvate is then reduced to lactate although under certain circumstances other products are formed (e.g. ethanol, acetate and formate).

While the enzymes G6P dehydrogenase and phosphogluconate dehydrogenase are present in S.lactis the HMP pathway is not complete and possibly serves mainly for the generation of NADPH and ribose 5-P. Other features of these bacteria are their apparent inability to carry out gluconeogenesis and oxidative phosphorylation and their lack of a tricarboxylic acid cycle. They thus rely almost solely on glycolysis for their ATP production. Consequently the mechanisms for regulation of glycolysis in lactic streptococci would be expected to differ from those in bacteria such as E.coli (Lawrence et al, 1976).

The glycolytic pathway in S.lactis appears to be regulated mainly by 'feed-forward' activation of the enzymes catalysing the terminal steps, pyruvate kinase and lactate dehydrogenase (Collins and Thomas, 1974; Crow, 1975; Crow and Pritchard, 1976, 1977; Thompson and Thomas, 1977; Thomas et al, 1979). Pyruvate kinase displays sigmoidal kinetics for both of the substrates PEP and ADP, is inhibited by ATP and P_i, and is activated by FBP and a large number of other sugar phosphates (Thomas, 1976). In vivo data on the levels of the activators and substrate (PEP) of the pyruvate kinase in growing and starved cells of S.lactis have confirmed its key role in the regulation of glycolysis. Thus during growth cellular FBP levels are very high (about 20 mM) and they are rapidly depleted during starvation while opposite changes are observed in the levels of PEP (Thompson and Thomas, 1977). FBP levels are considerably higher than all other glycolytic intermediates in cells metabolising glucose although it has not yet been established as the only

physiologically important activator of this pyruvate kinase. The data suggest that the intracellular PEP level is controlled by the level of one or a number of the activators of the pyruvate kinase thus providing a coupling between the transport of a sugar into the cell via the PEP-phosphotransferase system and its subsequent metabolism.

FBP is also an important activator of lactate dehydrogenase in S.lactis. This activator may be involved in controlling the flow of metabolites into either homolactic (via the lactate dehydrogenase) or heterolactic (via alternative routes for pyruvate metabolism) fermentation (Thomas et al, 1979).

Pseudomonas citronellolis

P.citronellolis has been shown to metabolise glucose mainly via the ED pathway with the HMP pathway only playing a minor role, while the key enzymes of glycolysis, phosphofructokinase and FBP aldolase, are absent. This organism, like S.lactis, has only one pyruvate kinase and it has been clearly demonstrated to be a regulatory enzyme showing strong positive cooperativity with respect to the substrate PEP and being subject to allosteric modulation of activity (Chuang and Utter, 1979). There appear to be two activation sites on the enzyme, one for 2-keto-3-deoxy-6-phosphogluconate(KDPG) and another for a number of compounds including ribose 5-P, AMP and F6P. A strong synergism exists between the two sites. The activators abolish the cooperativity of the PEP saturation curve and increase the affinity of the enzyme for PEP. Both GTP and ATP inhibit the enzyme by lowering its affinity for PEP. However this inhibition can be largely overcome by KDPG.

Since KDPG is an intermediate of the ED pathway its stimulation of the pyruvate kinase may play a 'feed-forward' role in the regulation of

this pathway *in vivo* in a manner analogous to the role of FBP in the glycolytic pathway in *E.coli*. The physiological significance of ribose 5-P activation however, is less apparent. It may function *in vivo* as a synergistic stimulator of the KDPG activation or it may be important during growth on gluconeogenic substrates. However the mechanism of synthesis of ribose 5-P from gluconeogenic precursors is not clear in *P.citronellolis* and the measurement of ribose 5-P concentration in the cell is required to assess its role *in vivo*.

ATP and GTP inhibit the G6P dehydrogenase of *P.aeruginosa* (Lessie and Neidhardt, 1967). This suggests that nucleotide triphosphates could exercise concerted control of the *Pseudomonas* pyruvate kinase both by directly inhibiting its activity and by inhibiting the activity of the G6P dehydrogenase and thus lowering the level of its activator KDPG (Chuang and Utter, 1979).

Other Bacteria

Alcaligenes eutrophus H 16 is able to metabolise fructose via the ED pathway, the enzymes of glycolysis being used only for gluconeogenesis during growth on 2-, 3- and 4-carbon carboxylic acids. In this bacterium the pyruvate kinase displays cooperative kinetics with the substrate PEP and is allosterically regulated by P_i (inhibitor) and AMP, ribose 5-P and G6P (activators) while ATP inhibits competitively with PEP (Wilke and Schlegel, 1975).

These authors suggest that the activation of the pyruvate kinase by ribose 5-P and G6P provides a plausible 'feed-forward' activation of the ED pathway. However, *in vivo* data on adenine nucleotide levels suggest that the inhibition caused by ATP may be more physiologically important.

The extreme thermophile, Thermus thermophilus, contains the enzymes of both the EMP and gluconeogenic pathways. Of these, phosphofructokinase, fructose bisphosphatase and pyruvate kinase have been established as regulatory, allosteric enzymes (Yoshizaki and Imahori, 1979 a and b). The phosphofructokinase is activated by ADP and inhibited by PEP while the fructose bisphosphatase is activated by PEP and inhibited by AMP. The pyruvate kinase is activated by G6P and F6P and inhibited by ATP. The in vitro data provide a mechanism for "coupled regulation" of the glycolytic/gluconeogenic pathways in T.thermophilus and this has been confirmed by in vivo measurement of the key metabolites. The adenine nucleotides appear to function as a secondary control signal with the levels of PEP, F6P and G6P being the main signals. Thus PEP, a substrate of pyruvate kinase, exerts a reciprocal control on phosphofructokinase and fructose bisphosphatase while F6P, and/or G6P, acts as a 'feed-forward' activator of the pyruvate kinase. Pyruvate kinase appears to determine mainly the rate of glycolysis, while phosphofructokinase and fructose bisphosphatase determine both the direction of flow and the rate of glycolysis/ gluconeogenesis (Yoshizaki and Imahori, 1979 a and b).

The pyruvate kinases of the facultative phototrophic bacteria Rhodopseudomonas sphaeroides and Rhodopseudomonas capsulata are activated by AMP and sugar monophosphates (ribose 5-P, G6P and F6P), again suggesting a relationship between the activation of this enzyme and the type of hexose degradation operating, in this case the ED pathway (Schedel et al, 1975; Klemme, 1974). R.capsulata pyruvate kinase is inhibited by ATP and fumarate while the R.sphaeroides enzyme is inhibited by ATP, P_i, succinate and fumarate. Inhibition by

succinate and fumarate is a logical regulatory device for organisms which, like Rhodopseudomonas spp., grow preferentially with dicarboxylic acids as carbon sources.

Summary

Pyruvate kinase has been found to be a key enzyme in controlling the pathways of carbohydrate metabolism in bacteria. It is invariably regulated by responses to the energy status of the cell, as reflected either by ATP (GTP) inhibition or by AMP activation, as would be expected for an enzyme playing an important role in the energy metabolism of the cell. It catalyses an irreversible reaction in vivo and is thus unique to the glycolytic pathway. Conversion of pyruvate to PEP in gluconeogenesis must be accomplished by a different route. The substrate, PEP, is frequently the common intermediate of diverging metabolic pathways. Pyruvate kinase is thus ideally situated to regulate both the rate and direction of carbohydrate metabolism. Indeed, the wide range of effectors of the enzyme found in different bacteria, the nature of which is related to the particular pathways of carbon metabolism, confirms its importance in this role.

1.2 CARBOHYDRATE METABOLISM IN PROPIONIC ACID BACTERIA

The propionibacteria are able to ferment a wide variety of carbohydrates, polyols and organic acids to propionate, acetate, CO_2 and succinate, the relative proportions of which vary according to substrate and growth conditions. The following account of metabolism in propionic acid bacteria will be restricted to a consideration of the pathways and

enzymes involved in the metabolism of glucose, glycerol and lactate. Most published work has dealt with the metabolism of one or other of these compounds and these three substrates have been used in the studies described in this thesis.

The theoretical stoichiometry for the fermentation of each of these three substrates is as follows (Wood, 1961).

$$3/2 \text{ glucose} \longrightarrow 2 \text{ propionate} + \text{acetic acid} + CO_2 + H_2O$$

$$3 \text{ lactate} \longrightarrow 2 \text{ propionate} + \text{acetic acid} + CO_2 + H_2O$$

$$\text{glycerol} \longrightarrow \text{propionate} + H_2O$$

However, these stoichiometries are seldom obtained in practice (Wood and Werkman, 1936; Van Niel, 1928). While the ratio of CO_2 to acetate is usually 1:1, the ratio of propionate to acetate has been found to be as high as 5:1 and succinate is frequently found to accumulate. On glucose, succinate has been found to make up from 8-26% of the products formed (Hettinga and Reinbold, 1972b).

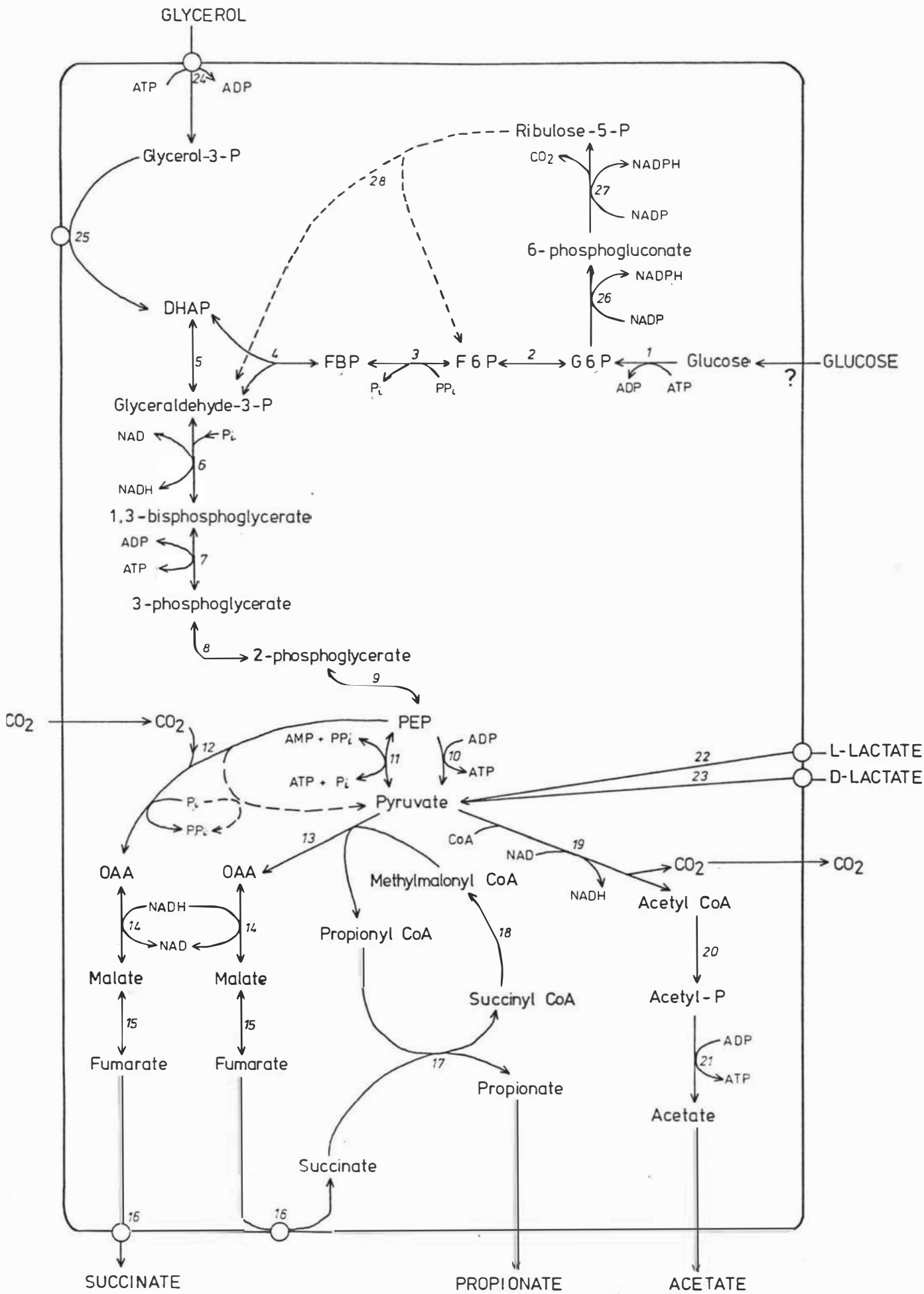
Figure 1.2 summarises the current knowledge of the metabolic pathways involved in the dissimilation of lactate, glucose or glycerol by the propionic acid bacteria. All the enzymes shown have been either directly demonstrated or deduced from experimental evidence.

Catabolism of pyruvate, the common intermediate of glucose, lactate or glycerol dissimilation, to propionate, acetate and CO_2 occurs via a sequence of reactions elucidated largely by Wood and co-workers (Allen et al, 1964). The biotin-dependent enzyme, transcarboxylase, catalyses the transfer of a carboxyl group from methylmalonyl-CoA to pyruvate yielding OAA and propionyl-CoA. The OAA is reduced to malate which is, in turn, converted to fumarate. The fumarate is then reduced to succinate. This reaction is catalysed by a membrane-bound fumarate-

1. Hexokinase
2. Phosphoglucoisomerase
3. PP_i- dependent phosphofructokinase
4. Aldolase
5. Triosephosphate isomerase
6. Ga 3-P dehydrogenase
7. Phosphoglycerate kinase
8. Phosphoglycerate mutase
9. Enolase
10. Pyruvate kinase
11. Pyruvate, orthophosphate dikinase
12. PEP : carboxytransphosphorylase
13. Transcarboxylase
14. Malate dehydrogenase
15. Fumarase
16. Succinate dehydrogenase (Fumarate reductase)
17. CoA transferase
18. Methyl malonyl isomerase and methyl malonyl racemase
19. Pyruvate dehydrogenase
20. Phosphotransacetylase
21. Acetyl kinase
22. L-lactate dehydrogenase
23. D-lactate dehydrogenase
24. Glycerol kinase
25. Glycerophosphate dehydrogenase
26. G6P dehydrogenase
27. Phosphogluconate dehydrogenase
28. Hexose monophosphate pathway

Figure 1·2

CARBOHYDRATE METABOLISM IN *P. SHERMANII*



reductase enabling fumarate to act as a terminal electron acceptor of reducing equivalents from various donors (de Vries et al, 1973). CoA is transferred from the propionyl-CoA to succinate to form succinyl-CoA and propionate. The succinyl-CoA is then converted to methylmalonyl-CoA by the corrinoid enzyme methylmalonyl-CoA mutase. A methylmalonyl-CoA racemase converting the (R) to the (S)-isomer completes the cycle. Allen et al (1964) also demonstrated the presence of two of the enzymes responsible for the production of acetate in propionibacteria, the phosphotransacetylase and acetyl kinase, and postulated the presence of a pyruvate dehydrogenase, the first enzyme in the pathway to acetate. However, Castberg and Morris (1978) were unable to demonstrate the presence of the classical three-enzyme pyruvate dehydrogenase in P.shermanii. A study of this reaction would be of interest as it would require regulation during growth on glycerol where acetate is not a product of the fermentation. When succinate is produced as an end-product the propionate cycle is interrupted and the enzyme PEP: carboxytransphosphorylase (Siu et al, 1961) converts PEP to OAA which is then converted to fumarate and reduced to succinate.

Catabolism of glucose to pyruvate is considered to occur mainly via the EMP pathway although, as described below, the conversion of F6P to FBP is catalysed by an enzyme quite different from the usual phosphofructokinase. Evidence for the operation of the glycolytic pathway in propionibacteria has been provided by the isolation of several of the intermediates and enzymes of glycolysis and by the major distribution pattern of isotope label in various intermediates which is consistent with such a pathway (Wood et al, 1955; Allen et al, 1964; Hettinga and Reinbold, 1972b).

However, evidence has accumulated which is difficult to reconcile

with the operation of a single route for hexose degradation (Wood, 1961; Hettinga and Reinbold, 1972b). For example, glucose degradation is relatively insensitive to glycolytic inhibitors such as fluoride, although interpretation of this data is made difficult by the complicated effects of fluoride on cell metabolism. Also, the isotope distribution data do not completely conform to any one pathway of carbohydrate dissimilation.

Vandemark and Fukui (1956) found enzymes of both the glycolytic and HMP pathways (including transketolase and transaldolase) to be present in cell-free preparations of Propionibacterium pentosaceum supporting the isotopic evidence for dissimilation of hexose by routes other than conventional glycolysis. Stjernholm and Flanders (1962) came to similar conclusions from studying the metabolism of ^{14}C-labelled ribose and gluconate in cell-free extracts of P.shermanii.

Glycerol is also actively metabolised by propionibacteria. Its conversion to DHAP requires a glycerol kinase and glycerol phosphate dehydrogenase. The presence of glycerol kinase activity has been demonstrated in membrane preparations of Propionibacterium freudenreichii by de Vries et al (1973) although its association with the membrane was not studied further. Glycerol phosphate dehydrogenase has been partially purified and its properties reported (Sone and Kitsutani, 1972; Sone, 1973). It is a membrane-bound, NAD-independent dehydrogenase transferring reducing equivalents via an electron transport pathway to fumarate (de Vries et al, 1973). Wood and Werkman (1936) demonstrated a CO_2 requirement for growth of P.shermanii on glycerol and later showed that the amount of succinate formed is equivalent to the net uptake of CO_2 (Wood and Werkman, 1940). The enzymic basis for this is discussed below.

The ability to ferment lactate anaerobically is one of the distinctive features of the propionibacteria and is responsible for their value in the ripening, flavour development and eye formation in Swiss cheese manufacture. Both stereoisomers of lactate can be used by propionibacteria (de Vries *et al*, 1972). A NAD-independent lactate dehydrogenase was described by Molinari and Lara (1960) but there have not been any reports of purified D- and L-specific forms of this enzyme. However, it is clear from studies in our laboratory (Asmundson and Pritchard, personal communication) that two separate membrane-bound enzymes differing in stability and inhibitor sensitivity are involved.

This investigation is concerned primarily with the regulation of the pathways of glycolysis and gluconeogenesis in *P.shermanii*. These pathways contain a number of unusual enzymes in this bacterium.

O'Brien *et al* (1975) reported that, while the enzymes ATP:phosphofructokinase and fructose bisphosphatase are present in crude extracts of *P.shermanii*, their activities are very much lower than those of other glycolytic enzymes. However, these workers also demonstrated the presence of a reversible pyrophosphate-dependent phosphofructokinase (reaction I) at comparatively high levels of activity.

$$F6P + PP_i \rightleftharpoons FBP + P_i \qquad (I)$$

They suggest that this enzyme could fulfil the role of both phosphofructokinase in glycolysis and fructose bisphosphatase in gluconeogenesis. The PP_i-dependent phosphofructokinase is present at levels 6-fold higher than the ATP:phosphofructokinase and 15-20 fold higher than the fructose bisphosphatase in crude extracts of *P.shermanii* (Wood *et al*, 1977). The enzyme has a high affinity for PP_i (K_m 0.069 mM) and is freely

reversible with the FBP-forming reaction having a slightly higher V_{max} *in vitro* (Wood *et al*, 1977). O'Brien *et al* (1975) did not find any allosteric effectors of the PP_i-dependent phosphofructokinase and they suggest that, if there are no specific controls of the activity of this enzyme, the flux through FBP would depend on the kinetic properties of the enzyme and the intracellular concentrations of the substrates and products of the reaction. Previous to its discovery in *P.shermanii* the PP_i-dependent phosphofructokinase had only been reported in *Entamoeba histolytica* (Reeves, 1974) but it has subsequently been reported in *Bacteroides fragilis* (Macy et al, 1978) and in pineapple leaves (Carnal and Black, 1979).

In higher organisms and in many bacteria the interconversion of F6P and FBP is the principle point for independent control of glycolysis and gluconeogenesis via reciprocal allosteric modulation of the two separate, virtually irreversible reactions catalysed by ATP-dependent phosphofructokinase and fructose bisphosphatase. In the absence of independent glycolytic and gluconeogenic reactions for interconversion of F6P and FBP in propionibacteria the major control point determining the relative glycolytic and gluconeogenic capacities of the organism is therefore likely to be that between PEP and pyruvate.

Evans and Wood (1968) discovered a pyruvate, orthophosphate dikinase in *P.shermanii* which catalyses reaction II.

$$PEP + AMP + PP_i \rightleftharpoons \text{pyruvate} + ATP + P_i \qquad \text{(II)}$$

Like the PP_i-dependent phosphofructokinase the pyruvate, orthophosphate dikinase is also PP_i dependent. It is interesting to note that it shows the same unusual distribution as the PP_i-dependent phosphofructokinase having been reported in *Entamoeba histolytica* (Reeves, 1968), *Bacteroides*

symbiosus (Reeves et al, 1968) and higher plants possessing Crassulacean acid metabolism (Kluge and Osmond, 1971) and C_4 photosynthesis (Hatch and Slack, 1968). While the equilibrium constant for the pyruvate, orthophosphate dikinase reaction is near unity, Wood et al (1977) consider that its main function is to catalyse the synthesis of PEP from pyruvate during gluconeogenesis when growing on substrates such as lactate. The enzymes pyruvate carboxylase and PEP:carboxykinase, which fulfil this gluconeogenic function in higher organisms, are absent in propionibacteria. However, in contrast to the situation with the PP_i-dependent phosphofructokinase, where a single enzyme probably functions in both glycolysis and gluconeogenesis, the conversion of PEP to pyruvate can be catalysed by a pyruvate kinase which is also present at high levels of activity in propionibacteria (Wood et al, 1977). The properties of the pyruvate kinase have not been previously reported and its presence in lactate-grown cells clearly presents the possibility of a futile cycle in conjunction with the dikinase. The recognition of this problem formed the starting point for the present investigation.

PEP is also the substrate of another unusual enzyme found in this bacterium, the PEP:carboxytransphosphorylase, first reported by Siu and Wood (1962). This enzyme catalyses reaction III and is the enzyme responsible for the uptake of CO_2 by propionibacteria.

$$PEP + P_i + CO_2 \rightleftharpoons OAA + PP_i \qquad (III)$$

Wood et al (1977) consider that the carboxytransphosphorylase serves an anaplerotic function in these organisms and is required only to provide the OAA that is converted to the 4-carbon dicarboxylic acid, succinate, that accumulates as an end-product and the OAA that is used for anabolic purposes such as synthesis of aspartate and glutamate. It is not involved in the propionate cycle of propionic acid bacteria.

For every mole of PEP carboxylated to OAA via the carboxytransphosphorylase, however, the cell is deprived of a mole of ATP that would have been derived from the pyruvate kinase reaction. Clearly the carboxytransphosphorylase requires regulation *in vivo*. O'Brien and Wood (1974) demonstrated such a possible control mediated by ligand-induced subunit interactions of the enzyme. Thus OAA, fumarate and malate (but not succinate or aspartate) are able to induce dimerisation of the tetrameric enzyme with a concomitant decrease in activity. This provided a possible means for control of the pathway leading to succinate production and may be important in determining the relative proportions of PEP metabolised through the diverging pathways.

In addition to the above reactions known to involve PEP, another possible drain on the PEP pool may be the coupled uptake of glucose to form G6P by a membrane-bound PEP:phosphotransferase system analogous to that found in a wide range of bacteria possessing the EMP pathway of glucose metabolism (Romano *et al*, 1970). However, as yet there is no experimental evidence supporting the existence of such a system.

As described above there are three enzymes which utilise PP_i as a cofactor in the carbohydrate metabolism of *P.shermanii*; the PP_i-dependent phosphofructokinase, pyruvate, orthophosphate dikinase and PEP:carboxytransphosphorylase (reactions I, II and III respectively). Of these three Wood *et al* (1977) consider the PP_i-phosphofructokinase to be the only one involved in the utilisation of PP_i since this enzyme probably accounts for a significant portion of the flux through FBP during glycolysis. Thus there must be an equally significant production of PP_i as an energy source when fermenting glucose. This, however, would not be the case for growth on glycerol or lactate where the PP_i-dependent

phosphofructokinase is not required to operate in the glycolytic direction.

Although, during growth, there is an extensive production of PP_i via a wide range of anabolic reactions, a definite, major catabolic source of PP_i, which would be required for the PP_i-phosphofructokinase reaction, is not known (Wood, 1977). Pyruvate, orthophosphate dikinase could provide a significant source of PP_i during growth on lactate but this would not be the case in the fermentation of glucose. PEP:carboxytransphosphorylase would generate PP_i, by reaction III, but only to the extent that succinate accumulates as an end-product. However, Wood (1977) suggests that PP_i could be formed from PEP by the carboxytransphosphorylase catalysing reaction IV, which occurs at very low concentrations of CO_2.

$$PEP + P_i \longrightarrow \text{pyruvate} + PP_i \qquad \text{(IV)}$$

Such a reaction might in part replace the pyruvate kinase reaction to generate PP_i instead of ATP.

Wood *et al* (1977) also speculate that PP_i rather than ATP may be generated by the electron-transfer-linked phosphorylation thus providing a catabolic source of PP_i. A membrane-bound anaerobic electron-transfer system using fumarate as electron acceptor has been demonstrated in propionic acid bacteria (de Vries *et al*, 1973) and there is indirect evidence that electron transfer is coupled to phosphorylation. The demonstration of an energy-linked membrane-bound pyrophosphatase as in photosynthetic bacteria (Baltscheffsky and von Stedingk, 1966) has not yet been established in propionic acid bacteria. Propionic acid bacteria, like most other bacteria, contain inorganic pyrophosphatase (Wood, 1977). Inorganic pyrophosphatases are generally considered to contribute to a

thermodynamically favourable steady-state for those biosynthetic reactions which generate PP_i, by hydrolysing the PP_i to P_i.

In the presence of an active pyrophosphatase the level of PP_i in the cell would be expected to approach zero. However, Klemme (1976) reports on several recent papers showing that the intracellular concentration of PP_i is in the range of 0.1 to 1.0 mM i.e. at levels comparable to those of other intermediates of energy metabolism. He suggests that the PP_i-dependent transphosphorylases found in P.shermanii can effectively compete with the hydrolytic inorganic pyrophosphatases (which may be regulated in vivo) for the common substrate, PP_i.

It can be seen then, that both the carbon and energy metabolism of the propionibacteria contain features unique to the genus which would require specific control mechanisms to regulate the direction and rate of flow of metabolism.

1.3 AIMS OF THIS INVESTIGATION

The major aim of this study was to determine possible regulatory mechanisms for the control of the opposing pathways of glycolysis and gluconeogenesis in P.shermanii. These bacteria metabolise glucose to pyruvate via a glycolytic pathway in which the ATP-dependent phosphofructokinase is largely replaced by a PP_i-dependent phosphofructokinase, catalysing a freely reversible reaction. This same enzyme apparently functions during gluconeogenesis from lactate, which is readily fermented by propionibacteria. The existence of this distinctive, enzyme-catalysed reaction for the interconversion of F6P and FBP shifts the necessity for control of these pathways to the reactions involved in the interconversion of PEP and pyruvate where

independent enzyme-catalysed reactions exist. In addition to the need for regulation of glycolysis and gluconeogenesis, the substrate and product of the pyruvate kinase reaction, PEP and pyruvate, are common intermediates in a number of diverging metabolic pathways. These considerations suggested that pyruvate kinase is a likely site for regulation in propionibacteria as it is in other organisms (Section 1.1.1).

One possible mechanism of regulation would be by control of enzyme synthesis - the distinctive glycolytic or gluconeogenic enzymes may be induced or repressed under appropriate conditions. This was tested by measurement of enzyme levels under different nutritional conditions. This particularly concerned the enzymes of PEP metabolism; PEP:carboxy-transphosphorylase, pyruvate, orthophosphate dikinase and pyruvate kinase (Chapter 3). Having established that pyruvate kinase must be controlled by allosteric modulation rather than by an induction/repression mechanism a detailed study of the enzyme was undertaken to establish its regulatory properties *in vitro* (Chapter 4).

A second important aim of the investigation was to determine the *in vivo* concentrations of key metabolites in *P.shermanii* in order to relate the *in vitro* data on enzyme regulation to the *in vivo* situation. *In vivo* metabolite concentrations were determined in both batch and continuous cultures of *P.shermanii* growing on three different carbon sources - lactate, glucose and glycerol (Chapters 5 and 6). This provided a wide range of nutritional conditions for the bacteria in which the different metabolic pathways of the cell would operate with differing relative activities. Such variations of the cellular metabolism would provide a good means for studying the *in vivo* importance of

regulatory mechanisms.

Finally, both the *in vitro* data on enzyme regulation and *in vivo* data on metabolite levels were re-evaluated to identify areas for further study. Some very preliminary investigations of these areas were undertaken (Chapter 7) with a view to opening up other lines of approach to the study of metabolic regulation in *P.shermanii*.

Chapter 2

MATERIALS AND METHODS

2.1 INTRODUCTION

This chapter describes the general procedures and analytical methods which were used routinely during this investigation and therefore apply to more than one of the following chapters. The description of methods or procedures which apply to specific parts of the investigation are contained in the relevant chapters.

2.2 MATERIALS

Biochemicals were obtained from the Sigma Chemical Company, U.S.A. Except where otherwise specified, ADP, guanosine 5'-diphosphate, NADH, AMP, ATP and F6P were the disodium salts, PEP and 6-phosphogluconate were the trisodium salts, FBP was the tetrasodium salt and G6P and NADP were the monosodium salts.

The buffer components, N-tris(hydroxymethyl)methylglycine (Tricine), tris(hydroxymethyl) amino methane (Tris), 1,3-bis[tris (hydroxymethyl)methylamino]-propane (Bis-tris propane), 2[N-morpholino] ethane sulphonic acid (MES), and N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid (HEPES) were all obtained from Sigma.

G6P dehydrogenase (bakers yeast), lactic dehydrogenase (rabbit muscle), pyruvate kinase (rabbit muscle), myokinase (rabbit muscle), α-glycerophosphate dehydrogenase/triosephosphate isomerase (rabbit muscle), phosphoglucoisomerase (yeast), hexokinase (yeast), aldolase (rabbit muscle), and malate dehydrogenase (pig heart) were obtained from Sigma.

Lactate dehydrogenase used in Chapter 4 was a highly purified preparation from pig muscle (500 units/mg protein) free from any detectable pyruvate kinase.

Sodium D/L lactate (60% solution) was obtained from British Drug Houses Ltd., U.K.. Lactate used for the chromatographic standard was the lithium salt obtained from Sigma.

Sephacryl S200 was obtained from Pharmacia and DEAE-Sephadex A-25-120 was obtained from Sigma.

Coomassie Brilliant Blue G-250 used in protein assays was obtained from Sigma.

Low fluorescence Grade III Imidazole used in the fluorometric analyses was obtained from Sigma.

Pyruvic acid (sodium salt) was obtained from Merck (Germany).

L-isomers of cysteine/HCl, proline, asparagine, methionine, isoleucine, serine, tryptophan, glutamine, lysine/HCl, tyrosine and histidine were obtained from Sigma. The L-isomers of the remaining amino acids and glycine, were obtained from British Drug Houses Ltd.

The radiochemicals D-[U-^{14}C]glucose (333 mCi/mmol), [1-^{14}C]glycerol (56 mCi/mmol), DL-[1-^{14}C]lactic acid, sodium salt (51 mCi/mmol) and carrier-free [^{32}P]NaH_2PO_4 were obtained from The Radiochemical Centre, Amersham.

All other reagents and inorganic salts were obtained as the Analar grade from British Drug Houses Ltd.

2.3 ORGANISM AND MAINTENANCE

Propionibacterium shermanii ATCC 9614 was obtained from the Department of Food Science and Nutrition, University of Minnesota, and was maintained in broth culture containing 10 g/l casein

hydrolysate, 10 g/l yeast extract, 20 g/l 60% sodium D/L lactate, 0.25 g/l KH_2PO_4, 0.20 g/l $MgSO_4.7H_2O$ and 0.05 g/l $MnCl_2.4H_2O$. Stock cultures grown for 24-30 h at 30°C were maintained at 4°C and subcultured at intervals of not more than one month.

Cultures were regularly checked for purity by microscopic examination of Gram-stained cells and by plating out on agar medium (using medium described above with 2% Davis agar). P.shermanii does not grow on agar plates when incubated aerobically and so this procedure was used for detecting aerobic contaminants. Anaerobic contaminants were detected by incubating the plates anaerobically using an anaerobic jar (Gaspak; Becton, Dickinson and Co., Maryland, U.S.A.).

2.4 DETERMINATION OF THE RELATIONSHIP BETWEEN OPTICAL DENSITY AND DRY WEIGHT OF CELL SUSPENSIONS

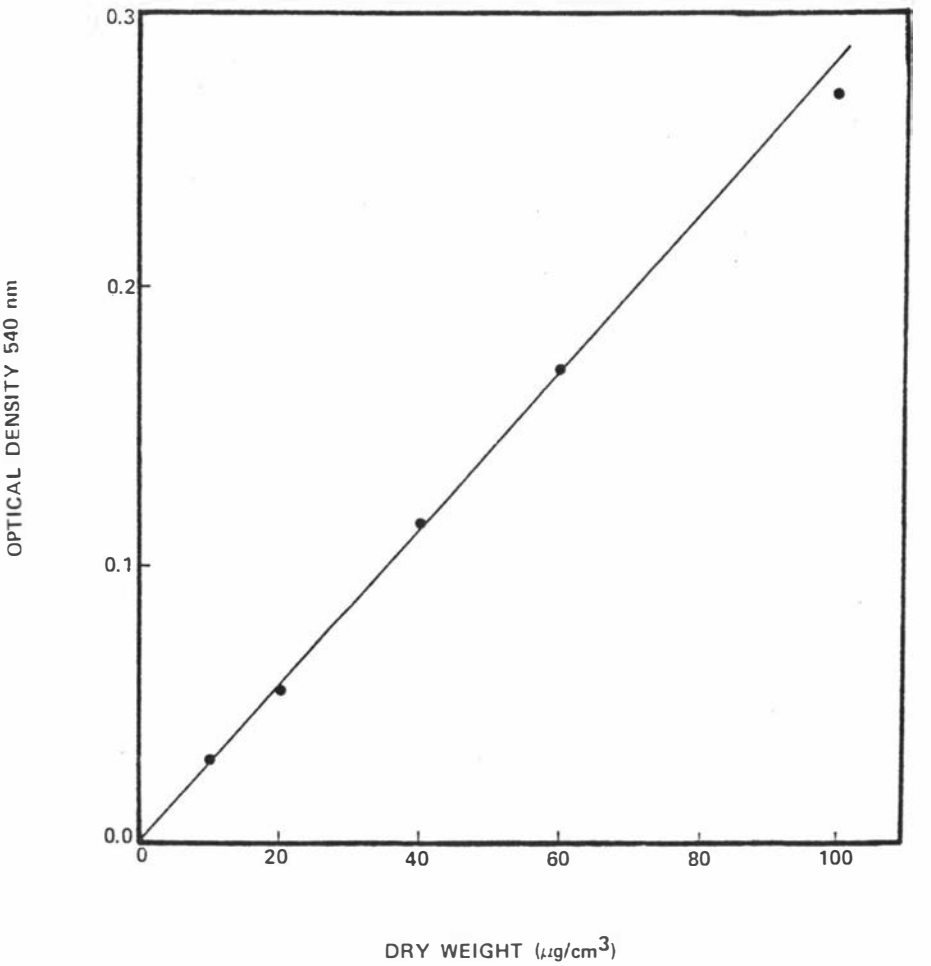
Bacterial cell density was determined by measurement of turbidity at 540 nm in a Bausch and Lomb Spectronic 20 colorimeter. Cultures were diluted with distilled water so that the absorbance measured was within the range 0.05-0.30.

The relationship between optical density at 540 nm and dry weight was determined by the membrane filter method (Cook et al, 1976) using a cell suspension of 40 mg wet weight/cm^3 which had been prepared from the centrifuged pellet (10000 g, 10 min) of cells harvested in the mid-logarithmic phase of growth on a lactate defined medium (Section 3.2).

Dry weights were measured by suction filtering samples of this cell suspension (in triplicate) onto 0.8 μm Millipore filters which were then dried to a constant weight in an oven at 60°C.

Figure 2.4

RELATIONSHIP BETWEEN DRY WEIGHT AND OPTICAL DENSITY



The figure shows the relationship between dry weight and optical density for *P.shermanii* as determined by the membrane filter method (Section 2.4).

Further portions of the cell suspension were diluted and assayed turbidimetrically at 540 nm. The relationship between dry weight and OD_{540nm} is given in Figure 2.4. (OD_{540nm} of 1.0 = 1.4 mg wet weight/cm^3 = 0.35 mg dry weight/cm^3)

2.5 ESTIMATION OF SUBSTRATES AND PRODUCTS IN THE GROWTH MEDIUM

Samples were taken from cultures as indicated in the relevant sections, the cells removed by centrifugation (27000 g, 5 min), and the clear supernatants stored frozen (-20°C).

2.5.1 Glycerol

Glycerol was determined colorimetrically according to the method of Neish (1952).

2.5.2 Lactate and Succinate

Lactate and succinate were determined as their methyl esters by gas-liquid chromatography using a 10% EGSS-X (ethylene succinate-methylsilicone copolymer) on 100-200 mesh Chromosorb Q column in a Packard Model 831 Gas Chromatograph. The methyl esters were prepared directly from the samples of culture medium essentially according to the method of Siegel *et al* (1977). The column was run isothermally at 130°C with flow rates of 33 cm^3/min for N_2 and 400 cm^3/min for air and 35 cm^3/min for H_2 for the flame ionisation detector. Under these conditions lactate, pyruvate and succinate were the only organic acid esters detected in eluates from the column.

Levels of lactate and succinate in media supernatants were determined by comparison with an internal standard of fumarate added

to the media sample before preparation of the methyl esters. Calibration curves relating peak heights to known concentrations of pure standard were prepared for succinate and lactate.

2.5.3 Acetate and Propionate

Acetate and propionate were determined directly by gas - liquid chromatography using a 150 x 0.4 cm coiled glass column packed with 20% FFAP (Carbowax 20 M-2 nitroterephthalic acid complex) on Anakrom Q (60-70 mesh) in a Varian Aerograph Series 2700. The column was operated isothermally at 149°C with flow rates of 27 cm^3/min for N_2 and 210 cm^3/min for air and 25 cm^3/min for H_2 for the flame ionisation detector. Sodium butyrate was added to the samples as an internal standard. Samples were acidified to 1 M H_3PO_4 using 5 M H_3PO_4 immediately prior to injection and relative proportions of the compounds determined by measurement of peak heights. Actual concentrations of propionate and acetate were determined from calibration curves prepared by chromatography of a known mixture of the acids (Kirk et al, 1971).

2.5.4 Glucose

Glucose was measured enzymatically essentially according to the method of Slein et al (1950). This is based on the fluorometric estimation of the NADPH produced in a system containing hexokinase, G6P dehydrogenase and NADP. The system used was the same as that described for the estimation of ATP in Section 2.8 except that the 2 mM glucose in the assay mixture for ATP determination was replaced with 1 mM ATP and aliquots of the medium containing between 0.2 and

10 nmol glucose were added. The reaction was initiated by addition of 10 µg hexokinase. The amount of glucose present in the sample was estimated from the increase in fluorescence which was then related to glucose content by measuring the subsequent increase in fluorescence caused by the addition of standard amounts of glucose.

2.6 PROTEIN ESTIMATION

Protein concentration was determined either by the method of Lowry et al (1951) or the Coomassie Brilliant Blue protein-dye binding method of Bradford (1976). The binding assay was extensively used throughout the early stages of the investigation because of its convenience. Good agreement was obtained initially between the values found by the dye binding assay and those using the Folin-Ciocalteu reagent. However, it was later found that different batches of the Coomassie Brilliant Blue G-250 used in the binding assay varied in quality and gave results significantly different from those obtained by the Lowry method.

For each assay protein concentration was calculated from a standard curve constructed using bovine serum albumin. The particular assay method used for each determination will be specified at the relevant place.

2.7 MEASUREMENT OF ENZYME ACTIVITIES

Enzyme activities were determined by spectrophotometric determination of NAD(P)H production or consumption at 340 nm either directly or by enzymatic coupling to NAD(P)H producing or consuming reactions.

Activities were determined at 30°C in a Unicam SP 1800 Spectrophotometer in a total volume of 1.0 cm^3 which contained 0.1 cm^3 of a suitably diluted enzyme extract in reaction mixtures containing substrates, cofactors, coupling enzymes and buffers at the final concentrations indicated below.

In all of these assays the enzyme sample was incubated in the reaction mixture, minus the metabolite underlined, for a few minutes to obtain any background rate due to interfering enzyme activities before starting the reaction by addition of the underlined metabolite.

Enzyme activities were estimated from the rate of disappearance of NADH or appearance of NADPH using the molar extinction coefficient for these compounds at 340 nm of 6.2×10^3.

Enzymic activity was measured in enzyme units, one enzyme unit being the amount of enzyme that converts one µmole of substrate per minute.

Pyruvate Kinase, (E.C. 2.7.1.40): 2 mM ADP, 0.25 mM NADH, 20 mM $MgCl_2$, 5 mM PEP, 3 units lactate dehydrogenase, in 0.1 M Tricine/NaOH buffer (pH 7.5).

Pyruvate, Orthophosphate Dikinase, (E.C. 2.7.9.1): 6 mM PP_i, 6 mM AMP, 0.25 mM NADH, 24 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 2.5 mM PEP, 3 units lactate dehydrogenase, in 0.1 M Tricine/NaOH buffer (pH 7.5).

Phosphoenolpyruvate:Carboxytransphosphorylase, (E.C. 4.1.1.3): 10 mM K_2HPO_4, 30 mM $KHCO_3$, 12 mM $MgCl_2$, 0.1 mM $CoCl_2$, 0.25 mM NADH, 2.5 mM PEP. The reaction mixture (minus PEP) was brought to pH 6.5

by bubbling with CO_2 for 15 min. The assay requires malate dehydrogenase as a coupling enzyme. This is present in excess in crude extracts of P.shermanii so addition of commercial malate dehydrogenase was unnecessary. When assaying the enzyme in more purified preparations 3 units of commercial malate dehydrogenase was added to the assay mixture.

Pyrophosphate-dependent Phosphofructokinase:

(a) For measurement in the direction of FBP production: 1.5 mM F6P, 0.25 mM NADH, 20 mM $MgCl_2$, 1 mM PP_i, 2 units aldolase, 0.83 units triosephosphate isomerase/glycerophosphate dehydrogenase, in 0.1 M Tricine/NaOH buffer (pH 7.5).

(b) For measurement in the direction of F6P production: 0.25 mM NADP, 10 mM $MgCl_2$, 1 mM P_i, 2 mM FBP, 2 units phosphoglucoisomerase, 2.7 units G6P dehydrogenase, in 0.1 M Tricine/NaOH buffer (pH 7.5). This assay would include any activity due to fructose 1,6-bisphosphatase in the extract.

Glucose 6-phosphate Dehydrogenase, (E.C. 1.1.1.49): 0.25 mM NADP, 10 mM $MgCl_2$, 2 mM G6P, in 0.1 M Tricine/NaOH buffer (pH 7.5).

Phosphogluconate Dehydrogenase, (E.C. 1.1.1.44): 0.25 mM NADP 10 mM $MnCl_2$, 2 mM 6-PG, in 0.1 M Tricine/NaOH buffer (pH 7.5).

Hexokinase, (E.C. 2.7.1.1.): 2 mM ATP, 10 mM $MgCl_2$, 0.25 mM NADP, 2 mM glucose, 2.7 units G6P dehydrogenase, in 0.1 M Tricine/NaOH buffer (pH 7.5).

Aldolase, (E.C. 4.1.2.13): 0.25 mM NADH, 2 mM FBP, 0.83 units triosephosphate isomerase/glycerophosphate dehydrogenase, in

0.1 M Tricine/NaOH buffer (pH 7.5).

Malate Dehydrogenase, (E.C. 1.1.1.37): 0.25 mM NADH, 0.375 mM OAA, in 0.1 M Tricine/NaOH buffer (pH 7.5).

Phosphoglucoisomerase, (E.C. 5.3.1.9): 10 mM $MgCl_2$, 0.25 mM NADP, 2 mM F6P, 2.7 units G6P dehydrogenase, in 0.1 M Tricine/NaOH buffer (pH 7.5).

Adenylate Kinase, (E.C. 2.7.4.3): 2 mM PEP, 2 mM AMP, 10 mM $MgCl_2$, 0.25 mM NADH, 2 mM ATP, 2 units pyruvate kinase, 3 units lactate dehydrogenase, in 0.1 M Tricine/NaOH buffer (pH 7.5).

NADH-oxidase: 0.25 mM NADH, in 0.1 M Tricine/NaOH buffer (pH 7.5).

2.8 DETERMINATION OF GLYCOLYTIC METABOLITES BY ENZYMIC/ FLUOROMETRIC TECHNIQUES

Glycolytic intermediates were determined using enzyme systems coupled to reactions producing or consuming NAD(P)H which could be followed fluorometrically. The assays were derived from methods described by Lowry et al (1971), Maitra and Estabrook (1964), and Thompson and Thomas (1977).

Assays were performed at 25°C in an Aminco SPF 500 Spectrofluorometer with extinction and emission wavelengths set at 340 nm (0.5 nm bandpass) and 460 nm (4 nm bandpass) respectively. The fluorometer was used in the ratio mode at a range setting of 10; the range vernier was set to give a full scale deflection equivalent to 2.5 nmol NADH.

The glycolytic intermediates were assayed by 3 distinct

enzyme-coupled systems. All assays were carried out in a total volume of 2.5 cm^3 in 50 mM Imidazole/HCl buffer (pH 7.1) using reaction mixtures containing components at the final concentrations given in the following description.

A. Estimation of G6P, F6P and ATP: 10 µM NADP, 10 mM KCl, 2 mM $MgCl_2$, 2 mM glucose, 100-250 µl extract.

G6P in the extract was determined first by addition of 10 µg G6P dehydrogenase. After allowing sufficient time (2-3 min) for this reaction to go to completion, F6P was determined by addition of 4 µg phosphoglucoisomerase. Finally after a further 2-3 min ATP was estimated by addition of 10 µg hexokinase (3-6 min).

B. Estimation of Triose phosphates (DHAP and Ga 3-P) and FBP: 5 µM NADH, 10 mM KCl, 2 mM $MgCl_2$, 100-250 µl extract.

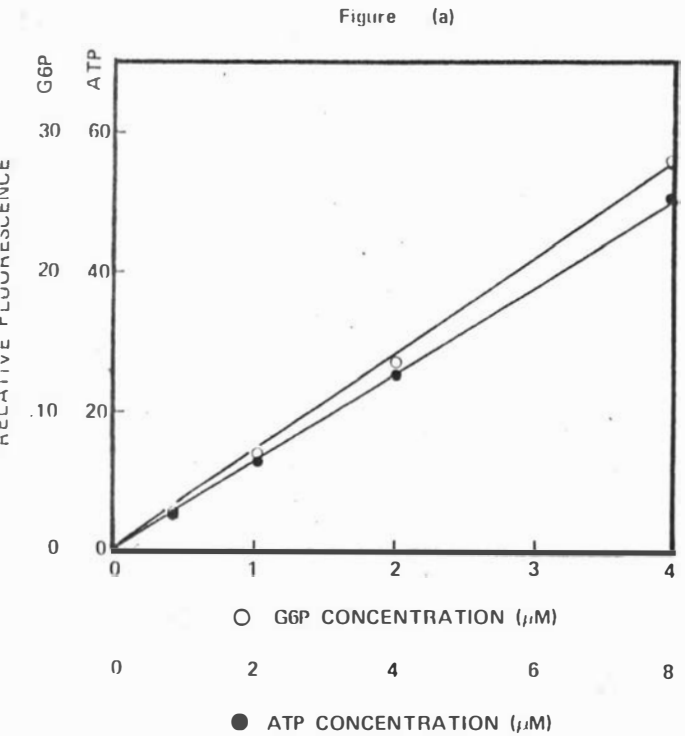
The triose phosphates in the extract were determined by addition of 15 µg triosephosphate isomerase/glycerophosphate dehydrogenase after which (3-5 min) FBP was determined by addition of 10 µg aldolase (3-5 min).

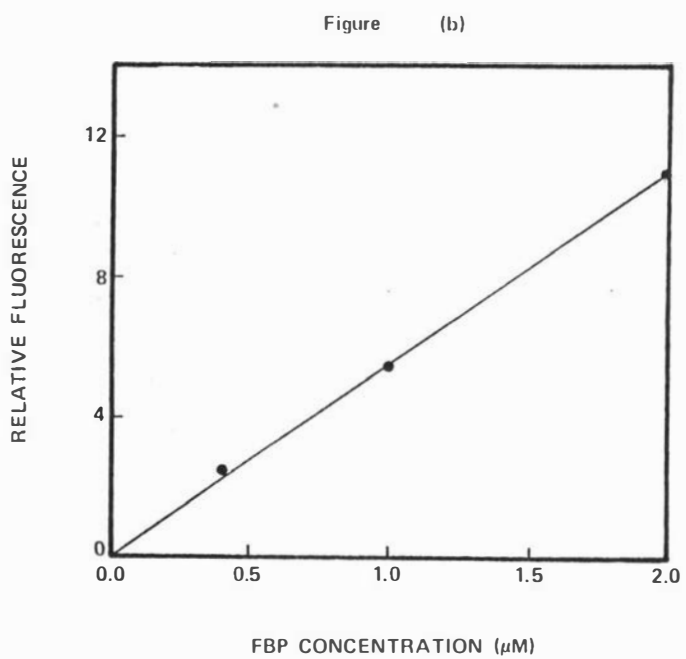
C. Estimation of Pyruvate, PEP and ADP: 5 µM NADH, 40 mM KCl, 2 mM $MgCl_2$, and either 1 mM ADP (for assay of PEP) or 1 mM PEP (for assay of ADP), 25-250 µl extract.

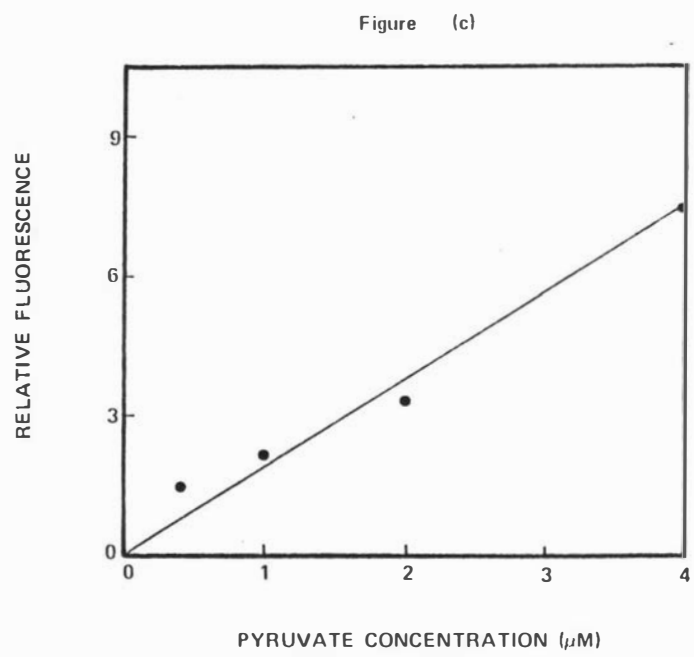
Pyruvate in the extract was determined by addition of 2 µg lactate dehydrogenase after which (3-6 min) either PEP or ADP was estimated by addition of 10 µg pyruvate kinase (3-6, 5-10 min respectively).

Figure 2.8

FLUOROMETRIC ANALYSIS OF METABOLITES







Figures (a), (b) and (c) show the standard relationships for the fluorometric analyses of metabolites for the three enzyme-coupled systems (A, B and C) described in Section 2.8 Reaction conditions as specified in Section 2.8 with levels of standard metabolites as indicated.

In all cases the native fluorescence due to the enzymes added was determined separately and allowance made for this in estimating the concentrations of the various metabolites in the particular extract. This correction was usually negligible except in the case of triosephosphate isomerase/glycerophosphate dehydrogenase enzyme mixture which often had a greater fluorescence than that caused by the triose phosphates in the extract.

The response of each assay system outlined above was found to be linear with increasing amounts of the respective metabolites over the range used (Figure 2.8). Thus the amounts of particular metabolites in an extract could be directly determined by comparison of the fluorescence change caused by the metabolite in the extract with the change caused by a known amount of that metabolite added into the same assay mixture after the first reaction was complete.

2.9 ESTIMATION OF CELL WATER VOLUME

In order to express the levels of metabolites found by fluorometry as concentrations in the cell it is necessary to have a value for the volume of intracellular water contained in a known weight of cell material. This has been determined for a number of bacterial species using the solute dilution method (Black and Gerhardt, 1962; Collins and Thomas, 1974; Cook *et al*, 1976). In this method the intracellular water is determined by the difference between total water content and extracellular water content for a known weight of cell material. The total water content is determined gravimetrically by drying a known weight of dense cell suspension; the extracellular water is determined by measuring the dilution of a

known volume of solution of an isotopically labelled, non-permeant solute.

Using this method the following cell water values have been reported in the literature:

Alcaligenes eutrophus	1.4 µl/mg dry weight	Cook et al (1976)
Acinetobacter calcoaceticus	1.7 µl/mg dry weight	Cook and Fewson (1972)
Staphylococcus aureus	1.5 µl/mg dry weight	Mitchell and Moyle (1956)
Streptococcus lactis	1.6 µl/mg dry weight	Collins and Thomas (1974)
Streptococcus lactis 7962	1.5 µl/mg dry weight	Kashket and Wilson (1973)
Streptococcus faecalis	1.3 µl/mg dry weight	Harold and Spitz (1975)
Escherichia coli	2.4 µl/mg dry weight	Alemohammad and Knowles (1974)

There is a good agreement between the values apart from that obtained for E.coli which is significantly higher.

Attempts were made to estimate the cell water volume of P.shermanii using ^{14}C-sucrose as the solute. The reported inability of P.shermanii to grow on sucrose (Hettinga and Reinbold, 1972a) was confirmed. The experiment was performed on lactate- and glucose-grown cells with extensive duplication. Highly reproducible results were achieved but in all cases the values obtained for cell water were very close to zero. Shortening the time for diffusion of the sucrose into the accessible water volume did not alter the result. It seems that although sucrose was not metabolised it can rapidly penetrate the cell membrane.

In view of the close agreement between the published values

for cell water content for other bacteria it was felt that the expense involved in searching for some other isotopically labelled non-permeant solute was not justified. Since an exact value for cell water volume for P.shermanii has not been obtained the metabolite levels found in this investigation have been reported on a cell dry weight basis. However in order to estimate the corresponding intracellular concentration of a metabolite (for comparison with the concentrations of substrates and effectors producing certain effects on an enzyme in vitro) a value of 1.6 µl cell water per mg dry weight was taken as an average of the reported values.

Chapter 3

GROWTH CHARACTERISTICS OF PROPIONIBACTERIUM SHERMANII ON THREE DIFFERENT CARBON SOURCES

3.1 INTRODUCTION

As pointed out in defining the aims of this investigation (Section 1.3) considerable importance was attached to studying the metabolite and enzyme levels of *P.shermanii* cultures when grown on different substrates. In order to ensure that the nature and quantity of the substrate supplied was known with certainty and to avoid possible effects due to the presence of small quantities of carbohydrates, organic acids etc. in complex media, a fully defined medium was developed for use in this investigation.

This chapter describes the growth characteristics, formation of end products and specific activities of a range of enzymes involved in carbohydrate metabolism in *P.shermanii* ATCC 9614 when grown on fully defined media containing lactate, glucose or glycerol as the carbon source. (Note: Lactate, glucose and glycerol are referred to loosely as carbon sources throughout this work. This is not intended to imply that they constitute the sole carbon source, since, most of the carbon is, of course, supplied by the amino acids in the medium).

3.2 COMPOSITION OF THE DEFINED MEDIUM

The medium was the same as that used by de Vries *et al* (1973) except that the casamino acids were replaced with a fully defined mixture of amino acids. When lactate was used as the carbon source a growth rate comparable to that on a complex medium was obtained by supplying only 8 amino acids but the full complement of 20 amino acids was required to obtain a comparable growth rate when using glucose or glycerol as the carbon source.

The fully defined medium contained the following per litre:

Mineral Salts: $(NH_4)_2SO_4$, 1.5 g; KH_2PO_4, 3g; Na_2HPO_4, 2.6g; $MgSO_4.7H_2O$, 0.16g; $MnCl_2.4H_2O$, 0.003g; $Co(NO_3)_2$, 0.01 g;

$FeSO_4.7H_2O$, 0.008g.

Nitrogen Bases: adenine, guanine, uracil and xanthine, all 0.005g.

Vitamins: thiamine HCl, 0.001g; p-amino benzoate, 0.0001g; nicotinic acid, 0.001g; Ca-pantothenate, 0.001g; pyridoxine, 0.002g; biotin, 0.0007g; flavin mononucleotide, 0.001g.

Amino Acids: L-cysteine, 0.86g; L-histidine, 0.75g; L-valine, 0.62g; L-arginine, 0.82g; L-lysine, 0.85g; DL-aspartic acid, 1.25g; L-tyrosine, 0.74g; L-glutamic acid, 3.0g and the L-isomers of methionine, proline, threonine, serine, alanine, asparagine, glutamine, isoleucine, leucine, phenylalanine and tryptophan and glycine, all at 0.2g.

Carbon Source:

either	Na D/L lactate (60% syrup)	20g
or	glycerol	4g
or	glucose	2g

The relative amounts of the three carbon sources were based on the molar growth yields given by de Vries *et al* (1973) and give approximately equal yields of cell dry weight per litre of culture.

3.3 GROWTH CONDITIONS

P.shermanii cultures were grown in 2.5 litres of the defined medium, containing the carbon source indicated, in a 3 litre CC 1500 fermenter (L.H. Engineering Co., U.K.). The fermenter was maintained at 30°C and kept anaerobic by continuous sparging with 95% N_2,

5% CO_2 gas mixture while stirring at 350 rev/min. Cultures were maintained at a pH of 6.7 ± 0.1 by automatic pH control using 2 M NaOH.

The medium was inoculated in all cases with 25 cm^3 of a 24-30 h broth culture growing on lactate defined medium. In all cases the organism was subcultured directly from a lactate complex broth medium to the lactate defined medium used to inoculate the fermenter.

Cultures were checked regularly for purity both optically by Gram staining and by plating out (Section 2.3).

Samples of medium were taken for measurement of optical density and then centrifuged to obtain a cell-free supernatant which was stored frozen (-15^{o}C) for later substrate and end-product analysis.

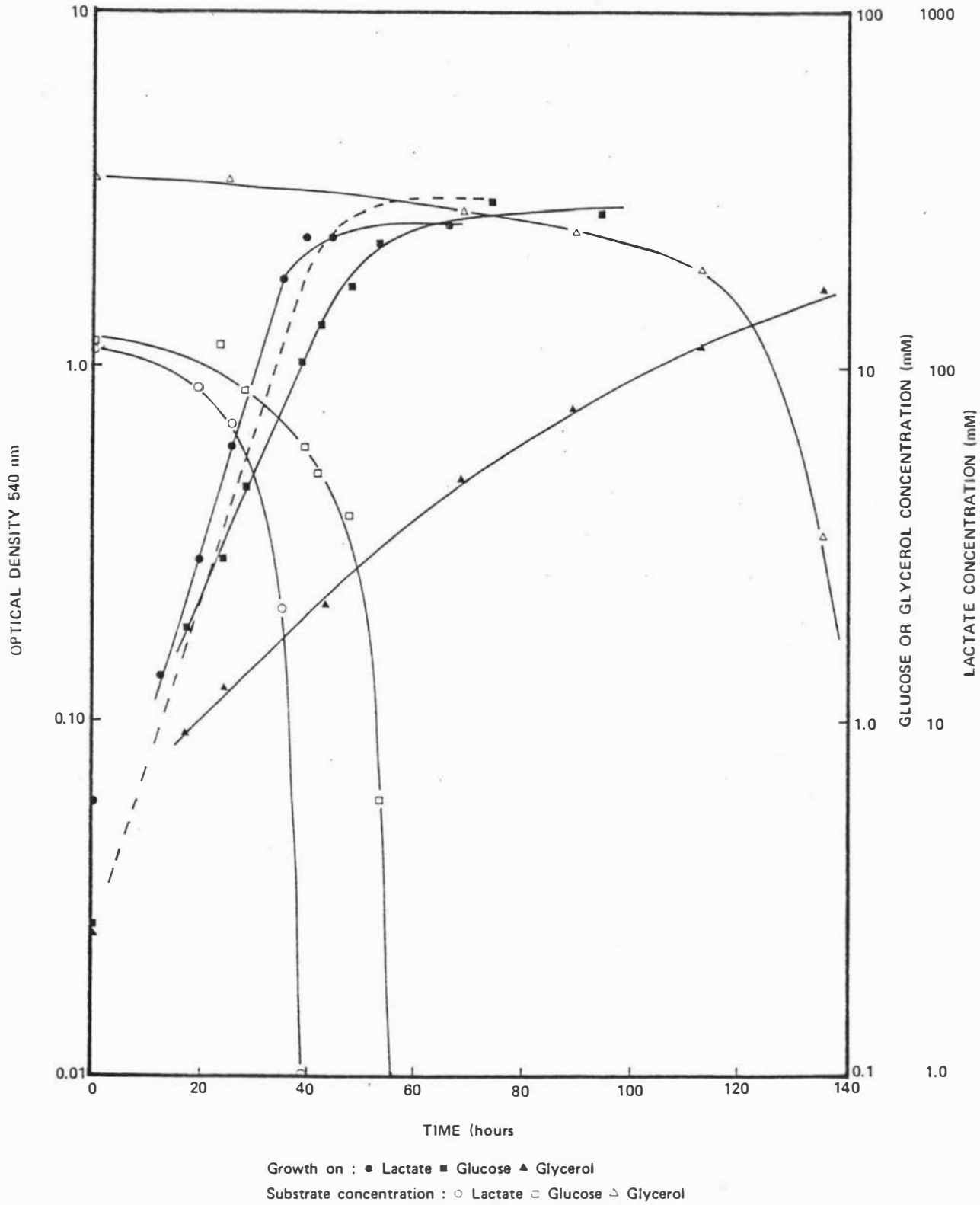
At the end of growth the cells were harvested, centrifuged, resuspended in 40 cm^3 10 mM Tricine/NaOH buffer (pH 7.5) containing 10% glycerol, recentrifuged and stored as a frozen pellet for subsequent measurement of enzyme activities.

3.4 GROWTH OF P.SHERMANII ON THE DEFINED MEDIUM

Typical curves for the growth of P.shermanii in the defined medium using the three substrates lactate, glucose and glycerol are shown in Figure 3.4. Growth was fastest on the lactate defined medium with a maximum growth rate of μ = 0.125 h^{-1} (generation time 5.5 h) compared to 0.069 h^{-1} on glucose and an initial rate of 0.043 h^{-1} on glycerol. The growth rate on the lactate defined medium

Figure 3.4

GROWTH OF *P.SHERMANII* IN THE DEFINED MEDIUM ON LACTATE, GLUCOSE AND GLYCEROL



The figure shows the growth and substrate utilisation of *P.shermanii* on the defined medium with either lactate, glucose or glycerol as the carbon source as described in Section 3.4. The dotted line shows the growth of a culture on glycerol defined medium with 2 g/1 acetate added (from Section 5.3.2.2.).

was almost identical to that obtained on a lactate complex broth (containing yeast extract and casamino acids).

Propionate, acetate and succinate were the main products found as well as occasional trace amounts of pyruvate. The relative proportions of the fermentation end-products differed according to the carbon source (Table 3.4).

Table 3.4

Carbon Source	Fermentation End-Product		
	Propionate	Acetate	Succinate
Lactate	55%	41%	4%
Glucose	43%	38%	19%
Glycerol	80%	0%	20%

The log growth curves for cultures growing on lactate and glucose were normally linear over the log phase of growth and growth rates were regularly reproducible. However when cultures were grown on glycerol defined medium widely varying growth curves resulted. In some cases the log growth curve on glycerol was linear over the early stages of substrate utilisation but in all cases became non-linear well before all the available glycerol was used. This suggested that the defined medium contained an inadequate amount of some essential nutrient for growth on glycerol which became the growth limiting factor. Two other observations supported this conclusion. Firstly, while P.shermanii could be subcultured

on the lactate defined medium indefinitely, subcultures on glycerol failed to grow after one transfer away from the lactate complex medium. Secondly, attempts to obtain a continuous culture of P.shermanii on glycerol defined media resulted in wash-outs of the culture when shifting from the batch phase of growth into the continuous phase, even at low dilution rates.

On reflection it was realised that the growth limiting nutrient was most likely to be acetate which is an end-product of growth on lactate and glucose but not of growth on glycerol (see Table 3.4). Addition of acetate (2 g/l) to the defined medium did indeed enable linear growth curves (as shown by dotted line in Figure 3.4) and continuous culture steady-states (Section 5.3.3)to be achieved on glycerol.

This requirement for acetate on the glycerol defined medium had been masked in earlier studies by the use of lactate broth cultures to inoculate all cultures of P.shermanii including those on glycerol defined medium. These inocula contained sufficient acetate to allow an initial high growth rate of P.shermanii on the glycerol defined medium but lead to the wide variability in subsequent growth rates.

At a later stage of the research programme it was noted that cultures growing on glycerol or glucose defined media were very sensitive to inhibition by traces of oxygen. Accordingly the N_2/CO_2 gas stream was passed through a heated copper coil to remove the last traces of oxygen. This resulted in improved growth rates

on glycerol and glucose media comparable to those obtained on lactate media (see Table 5.3.2.2).

3.5 ENZYME LEVELS IN BATCH CULTURES OF P.SHERMANII

Frozen pellets of the cells grown on the three carbon sources (lactate, glucose and glycerol) were thawed, resuspended in 10 mM Tricine/NaOH buffer (pH 7.5) containing 10% glycerol to make a thick slurry and disrupted by two passages through a French pressure cell (38000 kN/m^2). The resultant suspension was centrifuged at 27000 g for 15 min at 4^oC and the supernatant further centrifuged at 225000 g_{max} to remove fine particulate matter with a high NADH-oxidase activity.

The supernatant was kept at 4^oC and assayed for the enzyme activities according to the procedures described in Section 2.7. The results of these assays are shown in Table 3.5.

A range of enzymes known to catalyse reactions of glycolysis, gluconeogenesis and the pentose phosphate pathway (Section 1.2) were assayed. All enzymes determined were found to be constitutive at least on the three carbon sources used and only relatively small differences in specific activities between cultures grown on the different carbon sources were found. The most significant differences were induced by growth on glycerol. Glycerol-grown cells had an increased level of pyruvate kinase and PEP-carboxytransphosphorylase and lower levels of pyruvate, orthophosphate dikinase, hexokinase and aldolase compared to cells from glucose- and lactate - containing media. The only other difference of possible significance is the much lower level of G6P dehydrogenase on lactate-grown cells

Table 3.5 Enzyme levels in batch cultures of P.shermanii.[a,b]

Enzyme	Carbon Source		
	Lactate	Glucose	Glycerol[c]
Pyruvate kinase	0.34 ± 0.05	0.33 ± 0.02	0.72 ± 0.09
Pyruvate, orthophosphate dikinase	0.078 ± 0.023	0.078 ± 0.014	0.022 ± 0.001
PEP : carboxytransphosphorylase	0.081 ± 0.013	0.100 ± 0.014	0.205 ± 0.025
Hexokinase	0.041 ± 0.004	0.058 ± 0.013	0.012 ± 0.005
PP_i-dependent phosphofructokinase			
(a) FBP formation	0.093 ± 0.031	0.053 ± 0.010	0.069 ± 0.032
(b) F6P formation	0.069 ± 0.017	0.058 ± 0.017	0.050 ± 0.009
Aldolase	0.084 ± 0.025	0.119 ± 0.040	0.042 ± 0.014
G6P-dehydrogenase	0.0037 ± 0.0015	0.028 ± 0.010	0.021 ± 0.016
6-Phosphogluconate dehydrogenase	0.065 ± 0.011	o.074 ± 0.007	0.080 ± 0.026
Malate dehydrogenase	64 ± 11	61 ± 16	50 ± 28

[a] Enzyme activity is expressed as units/mg and values are the mean from at least three separate determinations ± standard error.

[b] Protein concentration was estimated by the method of Bradford (1976).

[c] Glycerol cultures were grown on the defined medium (Section 3.3) without any acetate added.

than on glycerol- or glucose-grown cells.

3.6 DISCUSSION

A fully defined medium has been described which was able to support good growth of P.shermanii on the three substrates lactate, glucose and glycerol. The end-products propionate, acetate and succinate were produced in varying proportions in the three fermentations indicating differences in the activity of particular metabolic pathways discussed in the introduction (Section 1.2).

Specific activities of a number of enzymes, representative of these pathways, measured in batch cultures did not change greatly on the different substrates (Table 3.5). This suggests that alteration in enzyme levels is unlikely to be the major factor controlling metabolic flow through these pathways and that allosteric modulation of the activity of key enzymes at branch points is probably of greater importance.

As was discussed in the introduction (Section 1.2) the most probable site of regulation of the opposing pathways of glycolysis and gluconeogenesis is at the PEP to pyruvate step. Here the glycolytic reaction is catalysed by pyruvate kinase while the reversible reaction catalysed by the enzyme pyruvate, orthophosphate dikinase functions in the direction of gluconeogenesis. From the data in Table 3.5 it can be seen that although the levels of these enzymes do change with the different growth substrates the pyruvate kinase was present at considerably higher specific activity on all three substrates than the pyruvate, orthophosphate dikinase. Clearly

the pyruvate kinase must be regulated *in vivo* to allow gluconeogenesis to occur when growing on lactate.

This prompted a thorough study of the pyruvate kinase which is described in the following chapter.

Chapter 4

PYRUVATE KINASE

4.1 INTRODUCTION

The chief function of pyruvate kinase (E.C. 2.7.1.40) *in vivo* is the transfer of the phosphoryl group from phosphoenolpyruvate to a nucleotide acceptor (I).

$$PEP + ADP \xrightarrow{Mg^{++},\ K^{+}} Pyruvate + ATP \qquad (I)$$

Pyruvate kinase has been well established as a key regulatory enzyme in glycolysis, catalysing a virtually irreversible reaction, thus maintaining the metabolic flux in one direction. (Seubert and Schoner, 1971). As discussed in Section 1.1, in tissues capable of gluconeogenesis several different reaction pathways exist to bypass this irreversible step of glycolysis. The conversion of pyruvate to PEP is usually accomplished by the two enzymes pyruvate carboxylase and PEP-carboxykinase via the intermediate oxaloacetic acid. In *Acetobacter xylinum*, *Acetobacter acettii*, *P.shermanii* and the C_4 plants (Wood *et al*, 1977) the enzyme pyruvate, orthophosphate dikinase functions in the direction of PEP formation. An enzyme very similar to the dikinase, PEP-synthetase, was found in *E.coli* by Cooper and Kornberg (1965).

It can be seen that pyruvate kinase is ideally situated to play a major role in regulating the direction of flux of metabolism, depending on the needs of the tissues or cells. It is not surprising then, that pyruvate kinases from different sources have been found to be subject to a large range of different controls.

4.1.1 Mammalian Pyruvate Kinases

In mammalian tissues and organs several electrophoretically and

immunologically distinguishable iso-enzymic species with distinct kinetic properties have been described. There are three basic types of non-interconvertible pyruvate kinase isoenzymes designated L, M and K (or M_2 or A). Type L is the major species present in liver, type M the major isoenzyme in muscle, brain, heart and leucocytes, and type K the main form in kidney cortex cells, regenerating liver, and bone marrow (Ibsen and Trippet, 1973; Ibsen, 1977).

The L, M and K type isoenzymes possess different kinetic and regulatory properties although the distinctions are not clear cut. Generally the type L and type K pyruvate kinase isoenzymes are typical regulatory enzymes showing a sigmoidal response to the substrate, PEP, and the monovalent cation, K^+. These isoenzymes are frequently inhibited by amino acids (particularly alanine and phenylalanine) and ATP and are strongly activated by FBP which abolishes the sigmoidicity of the K^+ and PEP saturation curves. The type M_1 isoenzyme typically shows an hyperbolic dependence on PEP concentration and was assumed not to possess regulatory properties. However recent evidence suggests there may be situations where this form of pyruvate kinase departs from Michaelis-Menten type kinetics (Irving and Williams, 1973; Ainsworth and MacFarlane, 1973; Phillips and Ainsworth, 1977; Ibsen and Trippet, 1973).

4.1.2 Bacterial Pyruvate Kinases

The amphibolic pathways of glycolysis and gluconeogenesis fulfil both biosynthetic and energy-generating functions in bacteria. To

ensure a correct, co-ordinated flow of carbon skeletons into the biosynthetic channels and energy generating pathways, bacteria have evolved control mechanisms which are unique to the enzymes of carbohydrate metabolism (Sanwal, 1970).

Thus it is not surprising that a key regulatory enzyme such as pyruvate kinase has been found to have differing properties, when isolated from different bacteria, in ways which appear to be related to its physiological role in regulating carbohydrate metabolism. Pyruvate kinases from a large number of bacteria have been purified and studied and while they show great diversity in their individual control mechanisms there does appear to be an overall pattern applicable to the regulation of pyruvate kinases in bacteria. A fuller description of the role of pyruvate kinases in bacterial metabolism is presented in Section 1.1.1.

Most bacterial pyruvate kinases are typical regulatory enzymes showing co-operative substrate kinetics and being subject to control by allosteric effectors.

All of the enzymes studied require Mg^{++} for activity and some also require a monovalent cation, usually K^{+}. A wide range of compounds have been reported to effect the activity of various bacterial pyruvate kinases giving a greater diversity of control mechanisms than in mammalian systems.

Generally bacterial pyruvate kinases are activated by two classes of compounds, phosphorylated sugars and nucleotide monophosphates. The phosphorylated sugars provide a 'feed-forward' activation for the catabolic pathway of which they are a precursor or an intermediate, while the nucleotide monophosphate activation relates the activity

of the pyruvate kinase to the energy status of the cell.

The catabolic pathways from different carbohydrate substrates to pyruvate are much more diverse in bacteria than in mammals where the glycolytic pathway is of paramount importance. FBP as the 'feed-forward' activator of pyruvate kinase in mammals is not found so invariably in bacteria. FBP is often supplemented or in some cases replaced by one or a number of other sugar phosphates as the 'feed-forward' activator of bacterial pyruvate kinases.

As pointed out by Schedel et al (1975) the inhibitors of bacterial pyruvate kinases fall into three main groups: nucleotide triphosphates, inorganic phosphate and tricarboxylic acid cycle intermediates.

ATP (or GTP) is a very common inhibitor of bacterial pyruvate kinases, as it is of the mammalian enzyme, and like AMP activation relates the control of the enzyme to the energy status of the cell. Thus under conditions of high energy charge (ATP concentration high relative to AMP concentration) pyruvate kinase activity is inhibited as is the catabolic pathway of which it is a part, and the biosynthetic pathways are thus correspondingly promoted.

Inhibition of pyruvate kinase by tricarboxylic acid cycle intermediates is often found in those bacteria which grow preferentially on dicarboxylic acids as the carbon source (e.g. Rhodopseudomonas spp.). It may also be important in adjusting the activity of the enzyme to the demands on tricarboxylic acid intermediates as biosynthetic precursors.

P_i is just as widespread and as potent an inhibitor of bacterial pyruvate kinases as is ATP. However the way in which it fits into the

Table 4.1.2 Summary of kinetic properties of some bacterial pyruvate kinases.

Species and Reference	Cation Requirement	Substrate Kinetics	Effectors and Comments
Alcaligenes eutrophus H16 Wilke and Schlegel (1975)	Requires Mg^{++}(K_m 0.85 mM) but not K^+ or Na^+	K_m for PEP 0.12mM, n_H 0.96. K_m for ADP 0.145mM	Activated by ribose 5-P, G6P and AMP. Inhibited by ATP and P_i. P_i inhibition relieved by activators.
Escherichia coli Type I enzyme Waygood and Sanwal (1974) Waygood et al (1976)	Requires Mg^{++} and K^+ (K_m 1.8mM)	Sigmoidal response to PEP (n_H 3.0). GDP best phosphate acceptor (K_m 0.05mM)	Activated by FBP which abolishes cooperativity of PEP binding. Inhibited by succinyl CoA and GTP.
Escherichia coli Type II enzyme Waygood et al (1975) Maeba and Sanwal (1969)	Requires Mg^{++} but not K^+	Sigmoidal response to both PEP and ADP at low substrate levels	Activated by a wide range of compounds, notably AMP, GMP, ribose 5-P, G6P but not FBP. Inhibited by succinyl CoA, ATP and P_i. ATP and P_i inhibition overcome by activators.
Streptococcus lactis Crow and Pritchard (1976) Collins and Thomas (1974) Thomas (1976)	Requires Mg^{++} and K^+	Sigmoidal response to PEP and ADP (at low PEP)	Activated by FBP which abolishes cooperativity of PEP binding. Inhibited by ATP, P_i. Also activated by a further 20 compounds from the glycolytic and tagatose 6-phosphate pathways.
Pseudomonas citronellolis Chuang and Utter (1979)	Requires Mg^{++}(K_m 6mM) but not K^+	Sigmoidal response to PEP (n_H 2.3)	Activated by ribose 5-P, F6P, KDPG, AMP and others. Activators abolish cooperativity of PEP binding. Synergistic activation by KDPG and ribose 5-P (or F6P). Inhibited by GTP and ATP, relieved by KDPG.
Thermus thermophilus Yoshizaki and Imahori (1979)	Requires Mg^{++} but not K^+	Sigmoidal response to PEP and ADP. $PEP_{0.5V}$, 2.1mM; $ADP_{0.5V}$, 1.4mM	Activated by a number of phosphorylated carbon compounds, especially G6P and F6P which both abolish cooperativity of PEP binding. Inhibited by ATP, P_i, glycerate 3-P and glycerate 2-P.
Rhodopseudomonas sphaeroides Schedel et al (1975)	Requires Mg^{++}(K_m 3mM) but not K^+	Sigmoidal response to PEP dependent on pH. Hyperbolic response to ADP (K_m 0.12mM)	Activated by AMP, G6P and ribose 5-P which abolish cooperativity of PEP binding. Inhibited by ATP, P_i, succinate, fumarate, citrate and malate.

regulatory mechanisms of these bacteria is not clear.

Table 4.1.2 summarises the major kinetic and regulatory properties of a number of bacterial pyruvate kinases reported in the literature while their relevance *in vivo* has been more fully discussed in Section 1.1.1.

4.1.3 *P.shermanii* Pyruvate Kinase

At the time of this investigation no detailed study had been carried out on the pyruvate kinase of *P.shermanii* although the enzyme had been reported to be present in crude extracts of the bacterium at a level sufficient to account for the known rates of glycolysis (Wood *et al*, 1977; Allen *et al*, 1964).

4.2 PYRUVATE KINASE PURIFICATION

4.2.1 Growth and Harvest of Propionibacterium shermanii

P.shermanii was grown at 30°C in the complex medium described in Section 2.3 using 4 g of glycerol per litre of medium as the fermentable carbon source (in place of lactate). *P.shermanii* cells grown on this substrate had been found to have higher levels of pyruvate kinase than those grown on lactate or glucose (Section 3.5). Cultures were grown in a 50 l New Brunswick fermenter containing 20 l of medium, at 30°C and with sparging by a 95% N_2, 5% CO_2 gas mixture to maintain anaerobic conditions. The pH was held between pH 6.0 and 6.5 by periodic addition of 2.5 M NaOH during growth.

The culture was inoculated with 1 l of a rapidly growing broth culture in the same medium. The cells were harvested in the late

logarithmic phase of growth i.e. after about 20 h growth, where the pyruvate kinase activity was highest. Cells were sedimented by centrifugation at 10000 g for 10 min at 4°C and washed twice in 10 mM Tricine/NaOH buffer (pH 7.5) containing 10% glycerol. The washed cells were stored frozen at -20°C until required for pyruvate kinase purification.

Figure 4.2.1 shows the growth curve for a typical culture in the fermenter indicating how the specific activity of pyruvate kinase changes during growth.

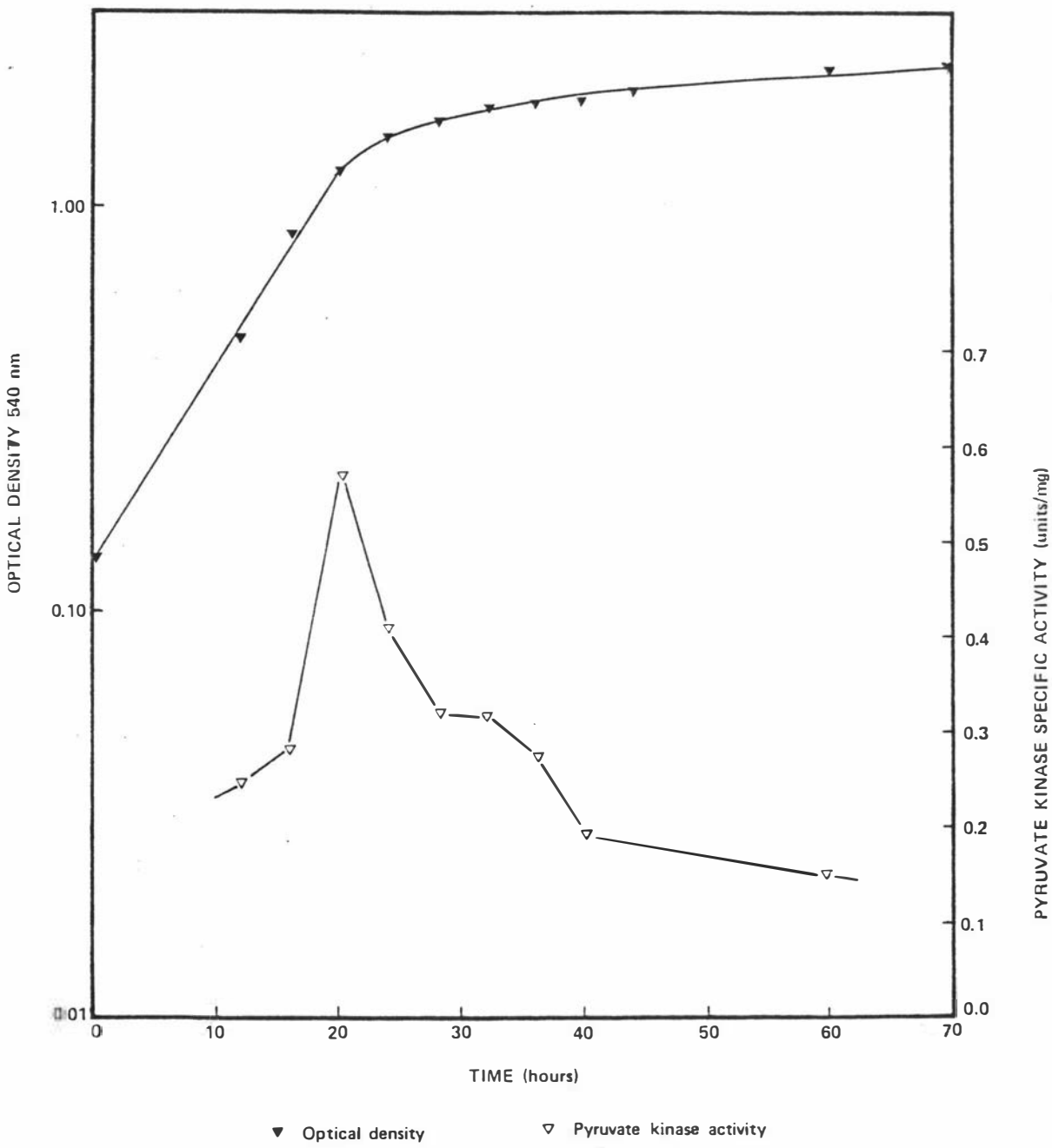
4.2.2 Breakage of Cells and Preparation of Cell-Free Extract

Cells were thawed and suspended in 10 mM Tricine/NaOH buffer (pH 7.5) + 20% glycerol to make a thick slurry (40 g frozen cells made up to a slurry of 70 cm^3), and disrupted by two passages through an Aminco French pressure cell at 38000 kN/m^2. The suspension was made up to twice its volume with 10 mM Tricine/NaOH buffer (pH 7.5) + 20% glycerol and 0.5 mg deoxyribonuclease I added to every 40 cm^3 of suspension. After standing 1 h the unbroken cells and cell debris were removed by centrifugation at 27000 g for 1 h at 4°C. The resulting pellet was packed into a Hughes press and cooled to -25°C before being disrupted for a second time. A second breakage of the cells in a Hughes press at least doubled the yield of cell protein compared to that obtained by breakage in the French press alone.

The broken cells from the Hughes press were made into a thin slurry with 10 mM Tricine/NaOH buffer (pH 7.5) + 20% glycerol and treated with deoxyribonuclease as for the French press breakage.

Figure 4.2.1

SPECIFIC ACTIVITY OF PYRUVATE KINASE DURING GROWTH OF *P.SHERMANII*



The figure shows the growth of *P.shermanii* on the complex medium as described in Section 4.2.1. The specific activity of pyruvate kinase during growth (determined on culture samples taken at regular intervals) is shown as justification for the harvesting time used for the preparation of this enzyme.

After centrifugation (27000 g for 1 h at 4^{o}C) this supernatant was added to the supernatant from the French press and centrifuged again at 370000 x g_{max} for 2 h in a 60 Ti ultracentrifuge rotor to give a cell-free supernatant. Ultracentrifugation was necessary for removal of fine particulate matter with a high NADH oxidase activity.

4.2.3 Streptomycin Sulphate Treatment

The DNase treatment following cell breakage only partially degrades the DNA, reducing the viscosity so that clean separation of particulate matter from the homogenate can be obtained on centrifugation. Polynucleotides were precipitated from the cell-free extract by dropwise addition of streptomycin sulphate using 2.0 cm^3 of a 10% (w/v) solution for every 100 mg of protein. The resulting suspension was allowed to stand for 1 h before the precipitate was removed by centrifugation at 27000 g for 15 min. This and all subsequent steps were carried out at 4^{o}C.

4.2.4 Ammonium Sulphate Precipitation

The supernatant after streptomycin sulphate treatment was dialysed against 10 mM Tris/HCl buffer (pH 7.5) + 10% glycerol for 2 h. Powdered ammonium sulphate was then added slowly to bring the solution to 40% saturation. The resulting suspension was allowed to stand for 30 min before the precipitate was removed by centrifugation at 27000 g for 15 min. The concentration of ammonium sulphate in the supernatant was then increased to 60% saturation. After standing for 1 h the precipitate was collected by centrifugation at 27000 g for 15 min, redissolved in 10 mM Tris/HCl buffer (pH 7.5) + 10% glycerol and dialysed for 2 h against the same buffer.

4.2.5 DEAE- Sephadex Ion Exchange Chromatography

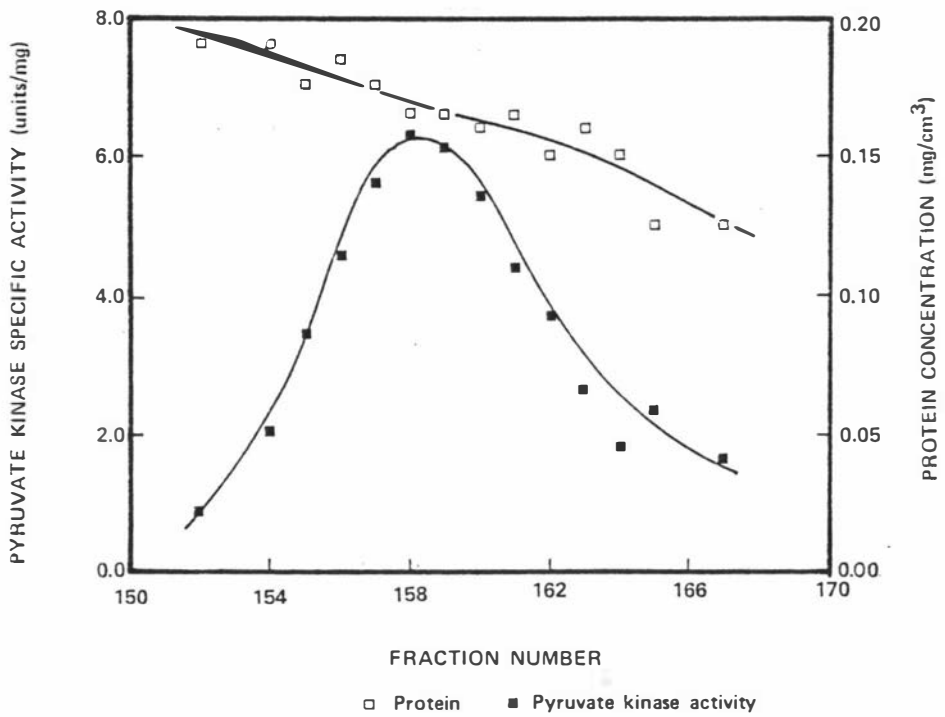
The dialysed sample from the ammonium sulphate fractionation (with a conductivity of less than 5 mmho) was applied to a DEAE - Sephadex A-25 column (20 cm x 4.5 cm) equilibrated in 10 mM Tris/HCl buffer (pH 7.5) + 0.1 M KCl + 20% glycerol, and washed with 100 cm^3 of the equilibration buffer. A considerable amount of protein as monitored by absorbance at 280 nm, was washed directly through the column by this procedure, but was completely free of any pyruvate kinase activity. The pyruvate kinase was subsequently eluted with a salt gradient of 0.1 M KCl to 0.4 M KCl (11 x 11) in the buffer (10 mM Tris/HCl (pH 7.5) + 20% glycerol) at a flow rate of 1.5 cm^3/min. Figure 4.2.5 shows the pyruvate kinase elution from a typical DEAE-Sephadex ion exchange purification. All fractions (10 cm^3 each) containing pyruvate kinase at a specific activity greater than 3.0 units/mg were pooled and concentrated by ultrafiltration using a Diaflo membrane XM-50 (Millipore Corporation, U.S.A.).

4.2.6 Gel Filtration on Sephacryl S200

The concentrated sample from the DEAE- Sephadex purification was precipitated by addition of solid ammonium sulphate to 70% saturation. After standing for 1 h the precipitate was collected by centrifugation at 27000 g for 30 min, then redissolved in a minimum volume (less than 3 cm^3) of 10 mM Tricine/NaOH buffer (pH 7.5) + 20% glycerol + 0.2 M KCl. This was then applied to the top of a Sephacryl S200 gel filtration column (90 cm x 2.5 cm) equilibrated in the same buffer. The column was developed in this buffer at a flow rate of 6.0 cm^3 per hour, collecting 3.0 cm^3 fractions. Pyruvate kinase elution from a typical gel filtration purification on Sephacryl S200 is shown in

Figure 4.2.5

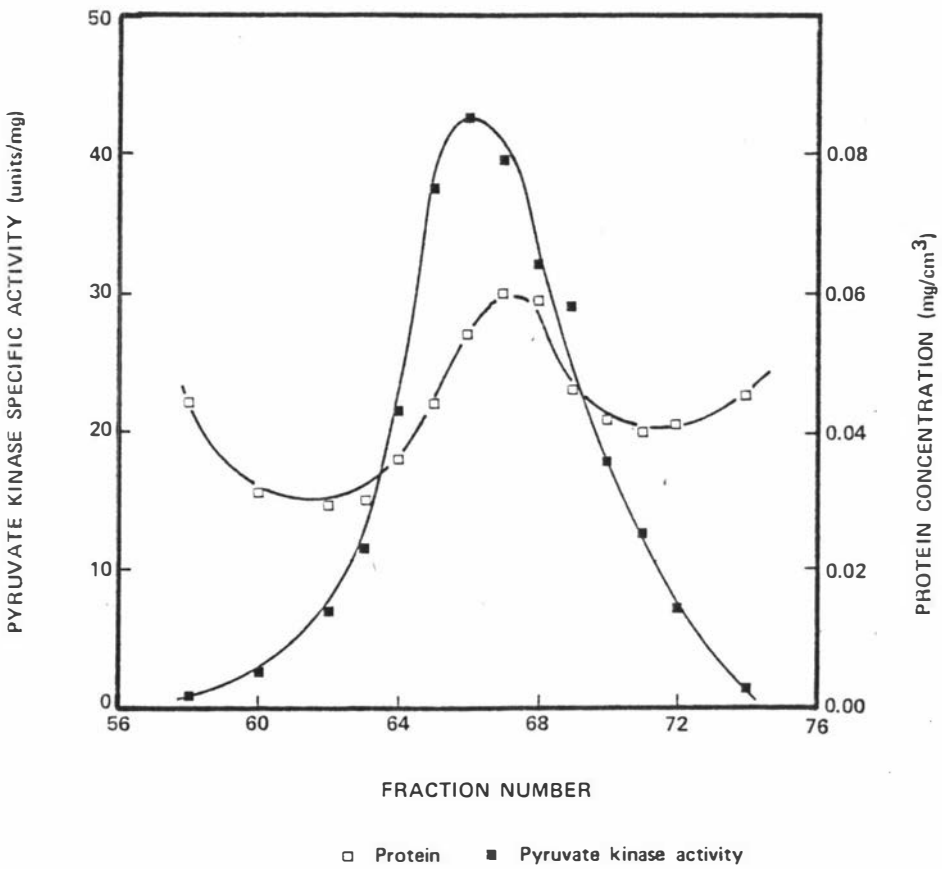
DEAE–SEPHADEX ION EXCHANGE CHROMATOGRAPHY



Column Conditions : *Column resin size*– 20 cm x 4.5 cm. *Equilibration of resin*– in 0.01 M Tris/HCl buffer (pH 7.5 + 20% glycerol + 0.1 M KCl. *Sample applied*– protein, 265 mg; pyruvate kinase activity, 0.83 units/mg; volume, 35 cm^3; sample dialysed against equilibration buffer. *Gradient*– after sample applied to column the equilibration buffer was used to elute the column before gradient was started. The gradient consisted of : initial buffer, 1 ℓ equilibration buffer ; final buffer, 1 ℓ 0.01 M Tris/HCl buffer (pH 7.5) + 20% glycerol + 0.4 M KCl. *Fraction size*– 10 cm^3/fraction.

Figure 4.2.6

SEPHACRYL S 200 GEL FILTRATION



Column Conditions : *Column resin size-* 90 cm x 2.5 cm. *Equilibration of resin-* in 0.01 M Tricine/NaOH buffer (pH 7.5) + 20% glycerol + 0.2 M KCl. *Sample applied-* protein, 12.4 mg; pyruvate kinase activity, 4.2 units/mg; volume, 3.0 cm^3. *Elution-* sample eluted with the equilibration buffer at a flow rate of 6 cm^3/h. *Fraction size-* 2.8 cm^3/fraction.

Figure 4.2.6. The fractions containing pyruvate kinase at a specific activity greater than 30 units/mg were pooled and dialysed against 10 mM Tricine/NaOH buffer (pH 7.5) containing 20% glycerol for 2 h with two changes of buffer, before being stored frozen at -20^{o}C in 2.0 cm^{3} aliquots.

The result of the above purification procedure (Sections 4.2.1 to 4.2.6) is summarised in Table 4.2 for a typical purification of pyruvate kinase from 40 g of wet, packed weight of glycerol grown P.shermanii cells. The enzyme was purified overall by 140 fold with a recovery of 9%.

Examination of the partially purified preparation by polyacrylamide gel electrophoresis (Davis, 1964) showed, in addition to the main pyruvate kinase band (identified by a specific activity stain, Crow (1975)), the presence of one major contaminating protein band and several minor bands. However the preparation was free from any detectable fructose bisphosphatase, pyrophosphate-dependent phosphofructokinase, pyruvate, orthophosphate dikinase, PEP:carboxytransphosphorylase, G6P dehydrogenase, G6P isomerase, NADH oxidase and adenylate kinase as assayed by the methods described in Section 2.7.

4.2.7 Discussion of the Purification Scheme

A considerable period of time was spent on the development of this purification scheme so some of the problems encountered during this phase of the investigation are described briefly below.

The preparation of crude extract and the streptomycin sulphate precipitation of nucleic acids were straightforward steps. The procedure described gave reliable and repeatable results.

Table 4.2 Purification of pyruvate kinase from 40g wet packed weight of P.shermanii cells.

Purification step	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Purification Factor	Percentage Recovery
Cell free extract	340	970	0.35	1.00	100
Streptomycin sulphate supernatant after dialysis	300	808	0.37	1.06	90
40-60% ammonium sulphate fraction after dialysis	220	265	0.83	2.38	65
DEAE Sephadex concentrated high specific activity fractions	52	12.4	4.20	12	15
Sephacryl S-200 pooled high specific activity fractions - after dialysis	30	0.62	49.20	140	9

Ammonium sulphate fractionation was also achieved without problems, although recoveries at this step varied from 70% to 100% in different preparations. However as the purification obtained at this stage was only of the order of 2 fold the possibility of replacing it by the use of the water-soluble non-ionic polymer polyethylene glycol 6000 (PEG) was investigated. While purification factors of 4-5 fold could be achieved by taking an 18% to 28% PEG fraction the advantages of this were countered by the length of time required to precipitate the pyruvate kinase from the 28% PEG solution (up to 2 days) and recoveries of only 50% to 70%.

Various ion exchange chromatography procedures were examined before the scheme outlined in Section 4.2.5 was decided upon. Initial studies were centred around the cellulose - based ion exchangers produced by Whatman. Both an anion exchanger, DEAE-cellulose (DE-23), and a cation exchanger, CM-cellulose (CM-23), were investigated. The pyruvate kinase bound completely to DE-23 at pH 7.5 but only low recoveries were obtained on elution by salt gradients. Attempts were made to stabilise the enzyme on the column with 20% glycerol (see Section 4.3.4), however total recoveries were still much less than 50%. Resolution on DE-23 columns was also poor giving only 3-4 fold purification of the enzyme.

Low pH values (pH 5.0-5.5) were required for the pyruvate kinase to bind to the cation exchanger CM-23, with resultant low recoveries (less than 10%) on elution of the enzyme by high salt concentrations. Similar problems were encountered with the use of phosphocellulose (Whatman PII); low pH values were required for binding the enzyme which resulted in very low recoveries.

The Sephadex based ion exchangers produced by Sigma, DEAE-Sephadex A-25 and CM-Sephadex C-25, were then investigated. DEAE-Sephadex gave marked improvement on the cellulose anion-exchanger, DE-23, for the purification of pyruvate kinase. By washing the DEAE-Sephadex column with 0.1 M KCl after loading but before elution with a KCl gradient a large portion of protein which otherwise "tailed" through the salt gradient was removed. This procedure allowed purifications of up to 10-fold when the enzyme was eluted by a 0.1 M to 0.4 M KCl gradient. The total recovery was still low however, being consistently around 50%, and no means of improving on this was found. A high salt wash (1 M KCl) eluted no further pyruvate kinase containing fractions.

The CM-Sephadex gave similar results to the CM-23 and P II ion-exchange resins.

The development of a suitable gel filtration step was also more difficult than anticipated. The agarose-based gel filtration medium, Biogel A 0.5 m, was investigated first and gave poor recoveries. Attempts to "reactivate" any inactive enzyme by addition of a number of metabolites were unsuccessful, and running the column in high salt concentrations (0.2 M KCl) and 20% glycerol gave only slightly better results (recoveries of less than 10%). The acrylamide/agarose medium, Ultrogel AcA34 was tried and gave similar results to the Biogel A 0.5 m column.

Sephadex G200 gave much improved results with recoveries of about 50% and 3-5 fold purification. However flow rates were very slow which may have contributed to the loss of activity.

Finally Sephacryl S200, a cross-linked dextran/acrylamide rigid gel, was tried. Its good flow and column packing properties made it

a convenient gel filtration medium to use. Recoveries of up to 90% were repeatedly obtained with good resolution, giving approximately a further 10-fold purification of the pyruvate kinase after the DEAE-Sephadex step.

Attempts were made to develop an affinity chromatography step in the purification scheme, but with no success. Affinity adsorption chromatography was tried using both 5' AMP-Sepharose and Blue-Sepharose 6B but the pyruvate kinase did not bind to these columns. Affinity elution chromatography was investigated using CM-Sephadex, however the low pH required to bind pyruvate kinase to the column (pH 5.5) and the inability to find any compound which could specifically elute the enzyme prevented any progress being made.

The purification scheme described in Sections 4.2.1 to 4.2.6 gives up to 140 fold purification of pyruvate kinase. The use of 10% - 20% glycerol throughout the procedure improved recoveries considerably as enzyme stability was very poor in the absence of glycerol (Section 4.3.4).

4.3 PROPERTIES OF THE PARTIALLY PURIFIED PYRUVATE KINASE FROM P.SHERMANII

4.3.1 Pyruvate Kinase Assay

Pyruvate kinase activity was estimated by measuring the rate of NADH oxidation in a coupled assay with excess lactate dehydrogenase as described in Section 2.7. The enzyme was diluted in cold ($4^{o}C$) 10 mM Tricine/NaOH buffer (pH 7.5) containing 20% glycerol. Routine assays during enzyme purification were carried out at room temperature

using a Beckman ACTA-3 spectrophotometer. Kinetic studies were performed at 30°C using a Unicam SP 1800 spectrophotometer. All protein determinations in this chapter were carried out by the method of Bradford (1976) as described in Section 2.6.

Under the standard assay conditions a lag phase occurred before a linear reaction velocity was attained. This could be overcome by incubation of the enzyme plus reaction mixture at 30°C, minus the PEP, for 10 min. Using this system linear initial rates were obtained upon starting the reaction with PEP.

4.3.2 Treatment of Kinetic Data

Initial rates were obtained using the assay procedure just described and plotted as initial velocity (v) versus substrate concentration graphs. To obtain a measure of the cooperativity of the binding of the substrates the coefficient (n_H) was evaluated from the slopes of the respective Hill plots. Application of the Hill plot requires the assumption of a value for V_{max} or saturation binding. Estimation of V_{max} often involves substantial uncertainty since double reciprocal plots of data obtained from cooperative binding systems are non-linear.

Endrenyi *et al* (1975) investigated procedures for the evaluation of the Hill coefficient which did not require prior estimation of the asymptotic velocity or binding. This involves using plots composed from data points which are derived from pairs of observations (υ and ω); the concentration ratio in all pairs is maintained at a fixed, constant value (α). Thus υ equals the reaction velocity at substrate concentration c and ω equals the reaction velocity at substrate concentration α c. Each data-pair is related by an

Figure 4.3.2

TREATMENT OF KINETIC DATA

Figure (a) PEP Saturation Curve

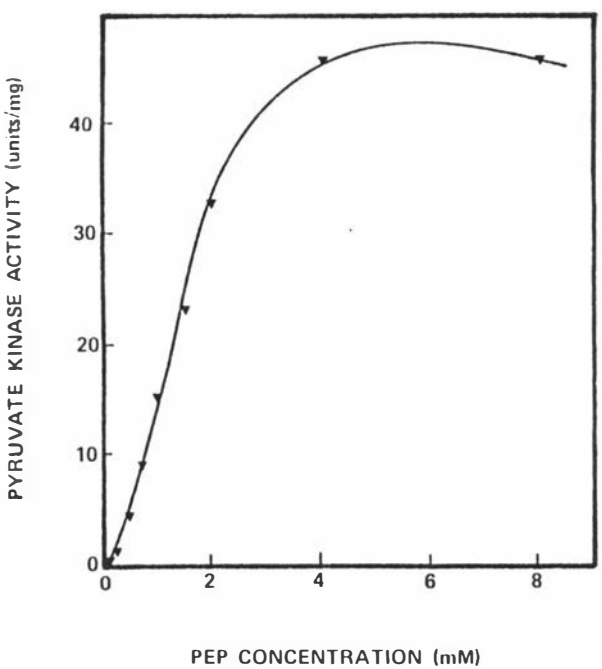


Figure (b) Endrenyi Plot

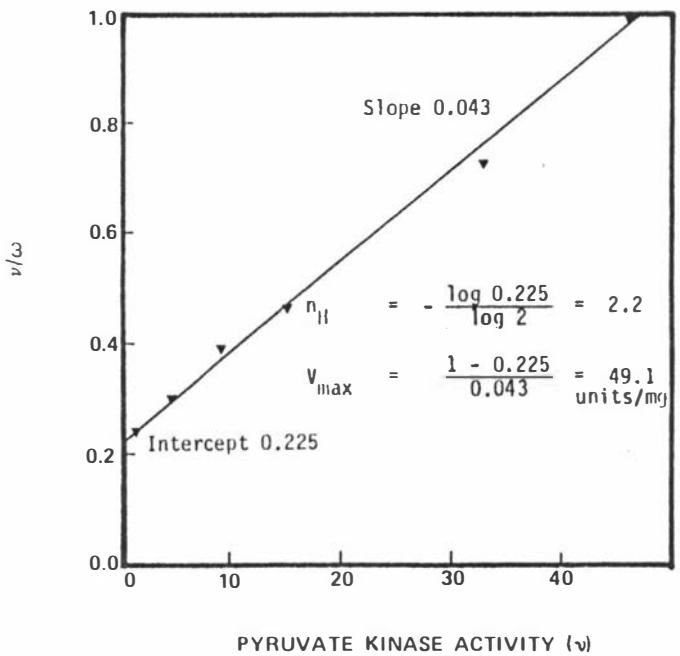


Figure (c) Hill Plot

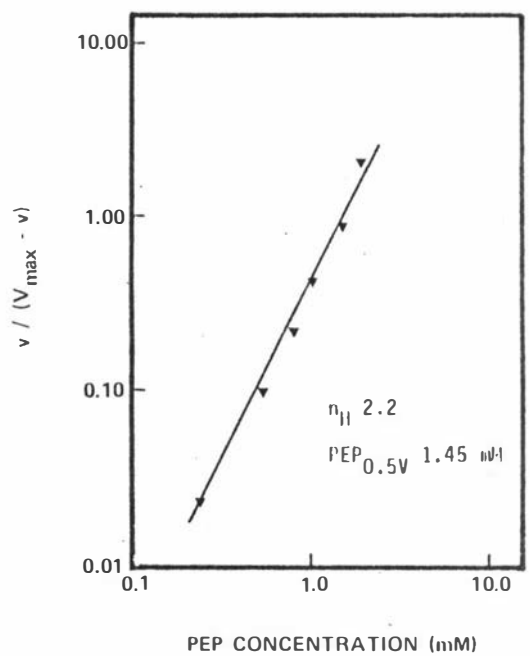


Figure (a) shows the relationship between pyruvate kinase activity and PEP concentration under the conditions specified in Section 4.3.1. but at 1.0 mM ADP instead of 2.0 mM ADP. Figure (b) shows a linearisation of this curve by the method of Endrenyi *et al* (1975) using data pairs related by the constant $\alpha = 2$. The value for V_{max} derived from the Endrenyi plot was then used in the evaluation of the Hill equation as shown in figure (c) to obtain the Hill number (n_H). See Section 4.3.2 for a fuller discussion of procedure.

hyperbolic relation: $\omega_i = D\upsilon_i/(E + \upsilon_i)$ (where $E = V_{max}(\alpha^n - 1)$ and $D = \alpha^n E$ and are both constants and $n = n_H$), which can be linearised by using the plots applied for the Michaelis-Menten equation. In this thesis the plot υ/ω versus υ was used. The values for the Hill coefficient (n_H) and V_{max} can be evaluated directly from these graphs. Thus in the υ/ω versus υ plot n_H = - log Vertical Intercept/log α and V_{max} = 1 - Vertical Intercept/Slope.

In Section 4.3 the data were collected in concentration pairs related by the constant $\alpha = 2$ and values for n_H and V_{max} evaluated as described above. This value for V_{max} was then used in the Hill equation and the original data plotted a second time as a Hill plot from which a second estimation of n_H was obtained, as well as a value for the apparent binding constant $k_{0.5V}$. Figure 4.3.2 gives a graphical example of the procedure used for the relationship between velocity and PEP concentration at 1.0 mM ADP (from Table 4.3.8(a)).

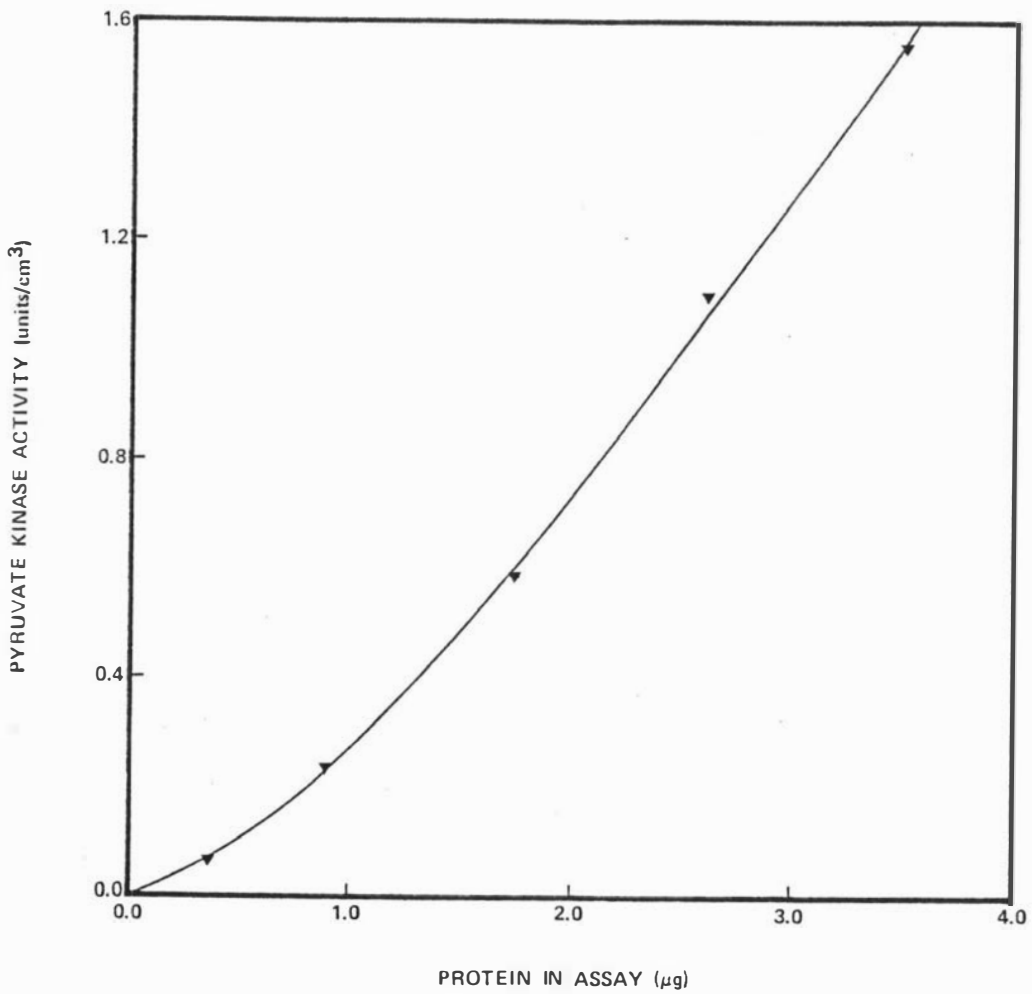
In cases where the data fitted an hyperbolic relationship the parameters V_{max} and K_m were evaluated by the direct linear plot method of Eisenthal and Cornish-Bowden (1974). Values obtained by this method agreed closely with those determined from Lineweaver-Burk plots of the same data.

4.3.3 Effect of Enzyme Concentration on Activity

The relationship between the enzyme concentration and activity of the P.shermanii pyruvate kinase was investigated over the range 0.35 µg to 3.50 µg protein per assay. The assays were carried out under otherwise standard conditions (Section 4.3.1) at 30°C and

Figure 4.3.3

EFFECT OF ENZYME CONCENTRATION ON ACTIVITY



The relationship between pyruvate kinase concentration and its activity was determined over the range 0.35 to 3.5 μg protein per assay. Reaction conditions as specified in Section 4.3.1.

were performed in duplicate. Figure 4.3.3 shows that the relationship was not linear. While reasons for this were not investigated, all assays in any one experiment were carried out using the same amount of enzyme to ensure that changes in rate of reaction were not due to concentration-dependent changes in enzyme activity. Initial rates of assays at low substrate concentrations were determined by increasing the absorbance scale expansion of the spectrophotometer used. This was found to be satisfactory for the data collected in this investigation. Most experiments described in this section were carried out using enzyme concentrations close to 2 µg per assay.

4.3.4 Studies on the Stability of Pyruvate Kinase

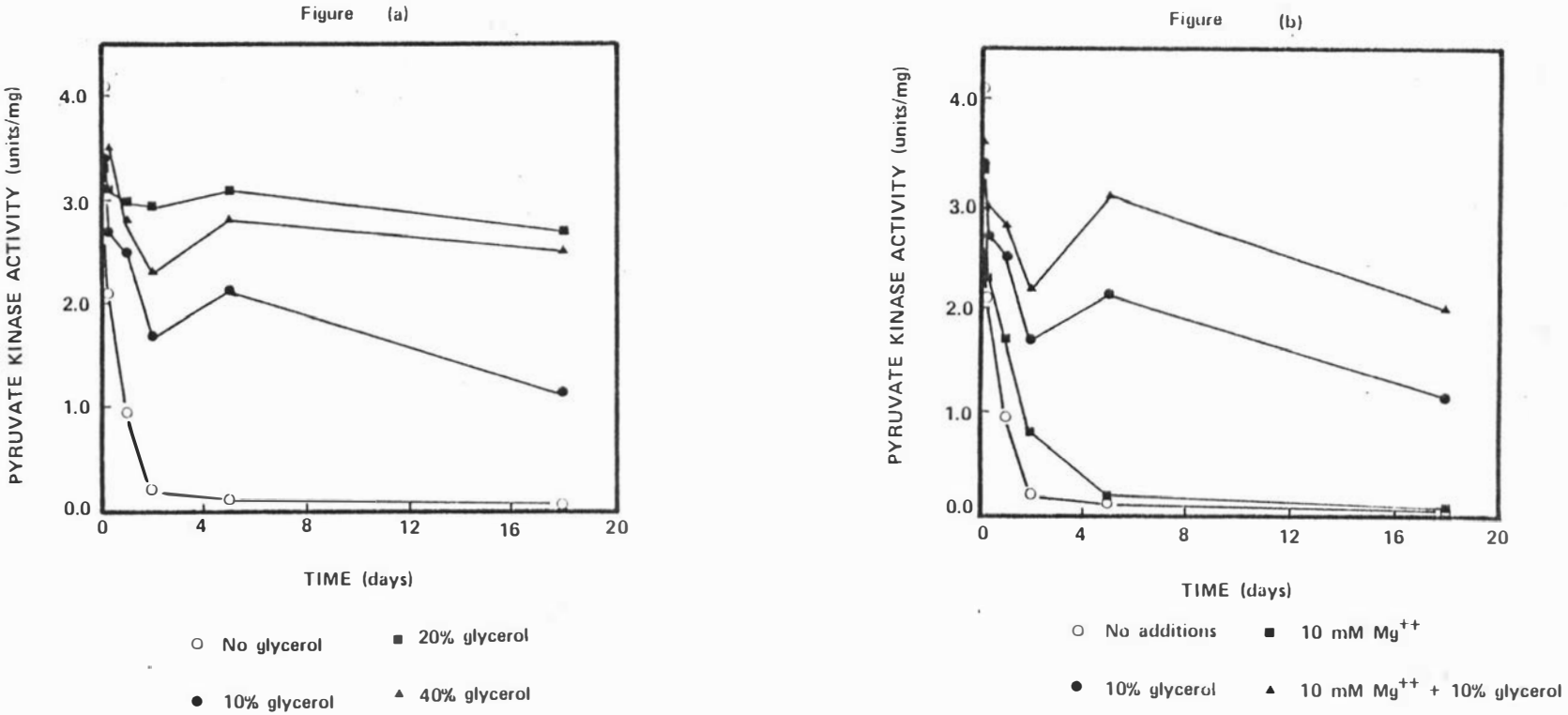
The pyruvate kinase lost over 95% of its activity within 2 days when stored in 0.1 M Tris/HCl buffer (pH 7.5) at 4°C. Glycerol, Mg^{++}, 2-mercaptoethanol and EDTA were investigated as possible stabilisers of the enzyme. Of these, only glycerol and Mg^{++} were found to have any stabilising effect and were therefore investigated further. Figure 4.3.4 shows the stabilising effects of glycerol and Mg^{++} on the pyruvate kinase over a period of 18 days when left to stand in 0.1 M Tris/HCl buffer (pH 7.5) at 4°C. Figure 4.3.4(a) shows that 20% glycerol gave optimum stabilisation of the pyruvate kinase while in Figure 4.3.4(b) it can be seen that, by itself, Mg^{++} had only a minor effect on stability of the enzyme although it appeared to enhance the stability afforded by glycerol.

4.3.5 pH Profile of Pyruvate Kinase

The activity of P.shermanii pyruvate kinase was studied over

Figure 4.3.4

STABILITY OF PYRUVATE KINASE



The enzyme was incubated at 0-4°C in the presence of either glycerol or $MgCl_2$, as indicated. The activity was assayed at measured intervals. Figure (a) shows the effect of increasing glycerol concentration on the stability of pyruvate kinase. Figure (b) shows the effect of 10 mM Mg^{++} and 10% glycerol on pyruvate kinase stability. Reaction conditions as specified in Section 4.3.1. Enzyme concentration : 21 μg protein per assay.

the pH range 5.50 to 9.75 using 5 different buffers: Bistrispropane/HCl, Tris/HCl, HEPES/NaOH, MES/NaOH and Tricine/NaOH. The buffers were used at 0.1 M concentration and were adjusted to the appropriate pH with either NaOH or HCl. The standard assay described in Section 4.3.1 was used with the 0.1 M Tricine/NaOH buffer (pH 7.5) being replaced by one of the above buffers as indicated. The pH of each assay mixture was measured after reaction and this value used as the pH of the assay. Figure 4.3.5 shows the pH profiles obtained. In most buffers the enzyme is active over a wide pH range with a broad optimum around pH 7.5. The activity in the buffers Bistrispropane, HEPES, MES and Tricine is similar within the range pH 7.2 to 7.6. However the pH profile in Tris buffer was markedly different, having an optimum at pH 8.5 while activity at pH 7.5 was less than 50% of the maximum activity.

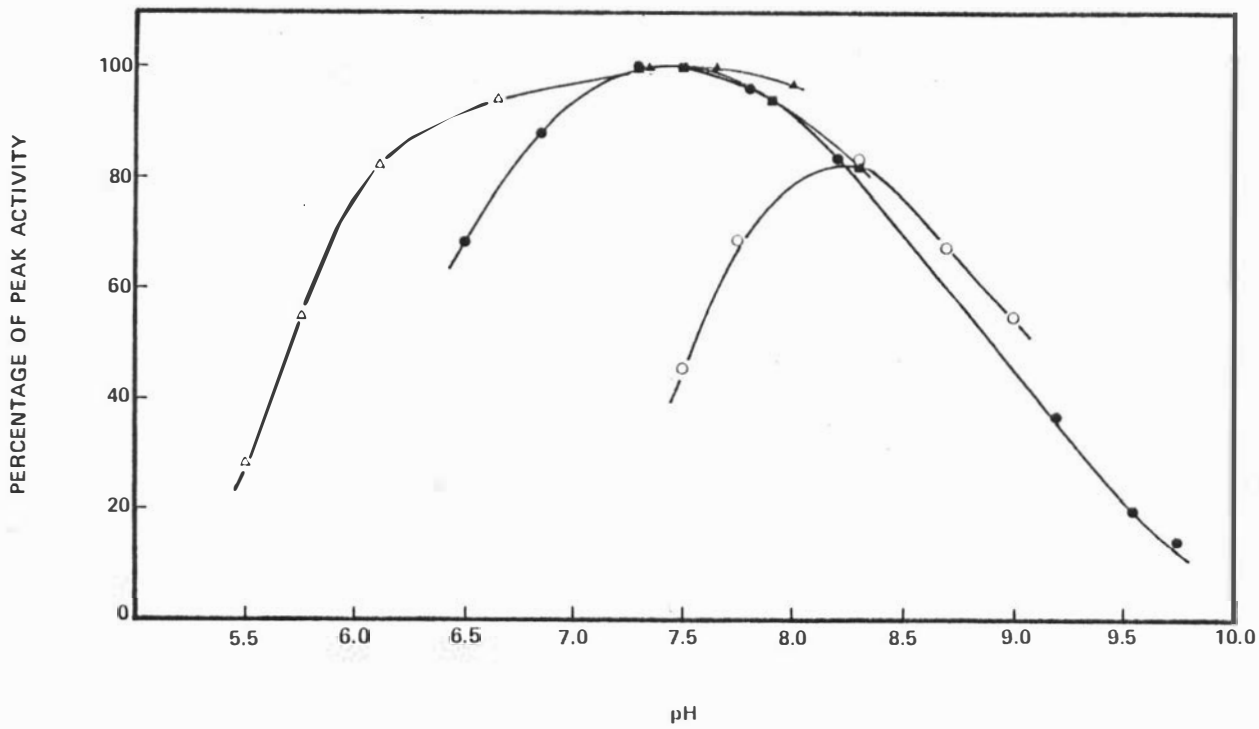
4.3.6 Monovalent Cation Requirement of Pyruvate Kinase

Most pyruvate kinases studied have an absolute monovalent cation requirement for activity, and it often affects the allosteric properties of the enzyme. However the pyruvate kinases from Rhodopseudomonas sphaeroides (Schedel et al, 1975); Acetobacter xylinum (Benziman, 1969); and Escherichia coli type II (Waygood et al, 1975) have all been reported to lack any requirement for a monovalent cation.

Under standard assay conditions the P.shermanii pyruvate kinase appeared to be unaffected by the addition of monovalent cations. To investigate this further the P.shermanii enzyme was assayed using the Tris salts of ADP and NADH, the tricyclohexylamino salt of PEP, and 100 mM Bistrispropane/HCl buffer (pH 7.5) as the assay buffer (in place of Tricine/NaOH). The pyruvate kinase sample and the

Figure 4.3.5

pH PROFILE OF PYRUVATE KINASE



● Bistrispropane/HCl ○ Tris/HCl ▲ HEPES/NaOH △ MES/NaOH ■ Tricine/NaOH

The pH profile of *P.shermanii* pyruvate kinase was determined as described in the text. Reaction conditions as specified in Section 4.3.1 except that the reaction buffer was replaced by one of the buffers shown and the assay performed at the pH indicated.

Enzyme concentration : 2 μg protein/assay.

lactate dehydrogenase used in the assay were extensively dialysed against 10 mM Bistrispropane/HCl buffer (pH 7.5) to remove any monovalent cations from that source. Thus apart from any cation contaminants in the reagents, the assay system was essentially free from inorganic monovalent cations. Using this system addition of the following monovalent cations: K^+, NH_4^+, Na^+, Li^+ and Cs^+ (all at 40 mM) had no effect (either positive or negative) on pyruvate kinase activity suggesting that this enzyme has no requirement for a monovalent cation.

4.3.7 Effectors of Pyruvate Kinase Activity

Pyruvate kinases from different sources are influenced by a large number of widely different effectors ranging from sugar mono- and di- phosphates, nucleotide mono-, di- and tri- phosphates and amino acids to a few inorganic ions.

Table 4.3.7 shows the results of the effects of 30 different metabolites on the activity of P.shermanii pyruvate kinase. Metabolites were included in the standard assay at the concentrations shown and assayed under both saturating (5.0 mM) and non-saturating (0.5 mM) concentrations of PEP (see Section 4.3.8).

At 5.0 mM PEP the pyruvate kinase activity was not activated by any of the 30 metabolites investigated, but at 0.5 mM PEP the presence of G6P at 2.0 mM caused over 100% activation of the enzyme. No other metabolite appeared to activate the enzyme significantly at 0.5 mM PEP.

A number of the compounds inhibited the enzyme activity under both saturating and non-saturating conditions of PEP. Inorganic phosphate (P_i), pyrophosphate (PP_i), ATP and acetyl phosphate

Table 4.3.7 Effect of metabolites on pyruvate kinase activity[a].

Additions		Activity at 5.0 mM PEP (units/mg)	Activity at 0.5 mM PEP (units/mg)
(a) 9.7 µg protein per assay			
No additions		15.6	3.15
		13.0	4.58
		13.7	2.66
G6P	0.1 mM	14.5	4.82
	0.5 mM	14.0	7.40
	2.0 mM	11.4	7.73
FBP	0.5 mM	13.5	3.33
	2.0 mM	13.7	2.00
F6P	0.5 mM	13.0	3.16
	2.0 mM	13.5	3.57
Glycerol-3-phosphate	0.5 mM	12.2	2.59
	2.0 mM	13.5	1.91
Ga 3-P	0.5 mM	13.2	3.74
	2.0 mM	13.5	2.66
6-phosphogluconate	0.5 mM	13.0	3.66
	2.0 mM	12.2	1.83
Ribose-5-phosphate	0.5 mM	12.6	2.33
	2.0 mM	12.7	2.82
ATP	0.1 mM	13.7	3.57
	0.5 mM	13.7	2.82
	2.0 mM	10.5	0.67
AMP	0.5 mM	13.0	2.10
	2.0 mM	13.0	3.74
	5.0 mM	11.5	3.96
PP_i	0.5 mM	13.2	3.16
	2.0 mM	9.5	1.58

Table 4.3.7 continued

Additions		Activity at 5.0 mM PEP (units/mg)	Activity at 0.5 mM PEP (units/mg)
P_i	0.5 mM	12.7	2.76
	2.0 mM	7.7	1.33
(b) 9.7 µg protein per assay			
No additions		15.5	4.41
		13.7	2.87
GMP	0.5 mM	16.1	4.24
	2.0 mM	-	3.36
GTP	0.5 mM	13.0	3.53
	2.0 mM	8.1	2.01
GDP	0.5 mM	14.3	4.09
	2.0 mM	10.0	3.16
L-lactate	3.0 mM	16.0	4.82
D-lactate	3.0 mM	16.0	3.00
Fumarate	3.0 mM	15.5	4.16
Succinate	3.0 mM	15.5	2.16
Malate	3.0 mM	14.5	4.32
Citrate	2.0 mM	14.2	2.91
Acetyl CoA	0.1 mM	15.5	4.49
	1.0 mM	14.5	4.82
Acetyl phosphate	0.5 mM	14.5	3.07
	2.0 mM	9.2	0.60
$NADP^+$	0.5 mM	14.2	3.62
	2.0 mM	-	1.65
(c) 4.0 µg protein per assay			
No additions		15.6	5.64
		19.8	5.70

Table 4.3.7 continued

Additions		Activity at 5.0 mM PEP (units/mg)	Activity at 0.5 mM PEP (units/mg)
Glucose-1-phosphate	2.0 mM	16.9	6.25
Alanine	2.0 mM	18.9	6.04
Valine	2.0 mM	19.3	6.66
Phenylalanine	2.0 mM	16.0	6.16

[a] Reaction conditions as specified in Section 4.3.1. Parts (a), (b) and (c) carried out on separate days.

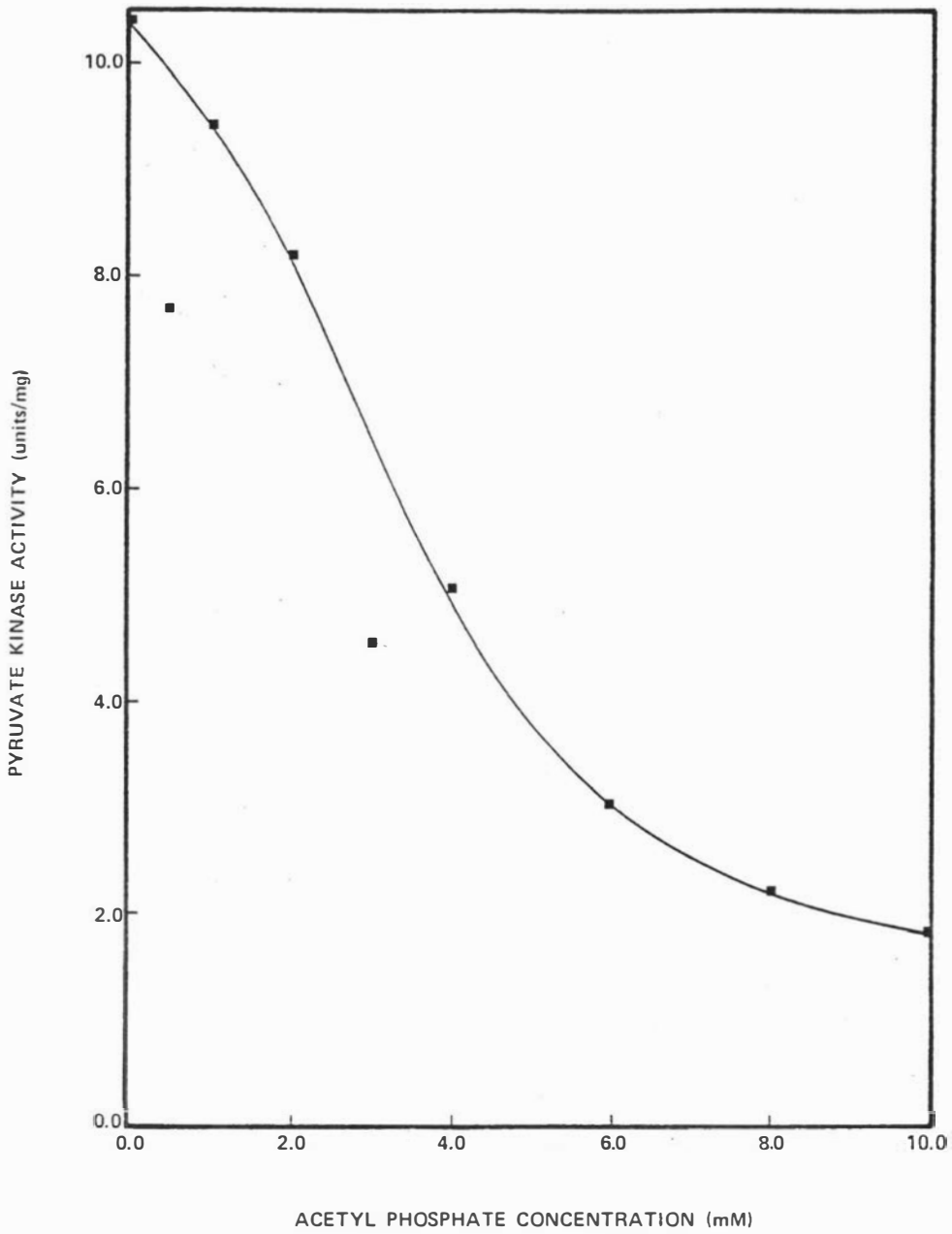
appeared to be the most significant inhibitors. Of these four compounds, little further work was done on the effects of PP_i or acetyl phosphate. The low solubility of the Mg-PP_i complex prevented assays being carried out above 2 mM PP_i. Acetyl phosphate is an unstable compound, rapidly breaking down to acetate and P_i. The apparent inhibitory effect of acetyl phosphate is probably caused by the P_i released on decomposition. Evidence for this emerged when the effect of increasing acetyl phosphate concentration on pyruvate kinase activity was being studied (Figure 4.3.7).

The acetyl phosphate was freshly prepared and all assays completed within 40 min. Assays containing 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 mM acetyl phosphate were performed in that order following which the assays containing 0.5 and 3.0 mM acetyl phosphate were carried out. As shown in Figure 4.3.7 the values for pyruvate kinase activity in these last two assays lay well below the curve produced by the earlier set of results showing that the inhibitory effect of the acetyl phosphate had increased significantly with time. All other assay conditions being equal these results were interpreted as showing that acetyl phosphate was in fact dissociating very rapidly and the apparent inhibition was due to the release of inorganic phosphate. The inhibitory effects of P_i and ATP are described in Sections 4.3.14 and 4.3.15.

An important feature of the data in Table 4.3.7 is the lack of activation of P.shermanii pyruvate kinase by either FBP or AMP, two common activators of pyruvate kinases from other sources. The effect of these two metabolites was investigated on a number of other occasions and although AMP at high concentrations (greater

Figure 4.3.7

EFFECT OF ACETYL PHOSPHATE CONCENTRATION ON PYRUVATE KINASE ACTIVITY



The figure shows the effect of increasing acetyl phosphate concentration on pyruvate kinase activity, as described in the text. Reaction conditions as specified in Section 4.3.1. Enzyme concentration : 2.1 μg protein per assay.

than 5 mM) could partially reverse inhibition of the enzyme activity by P_i at no stage were either AMP or FBP found to activate the enzyme.

4.3.8 Relationship Between Pyruvate Kinase Activity and Varying PEP Concentration

4.3.8.1 Effect of ADP concentration on the PEP saturation curve

Most pyruvate kinases studied from both eukaryotic (Seubert and Schoner, 1971; Van Berkel and Koster, 1973; Ibsen and Trippet, 1973; Costa et al, 1972; Kapoor, 1975; and Zink, 1977) and prokaryotic sources (Waygood and Sanwal, 1974; Waygood et al, 1975; Benziman, 1969; Collins and Thomas, 1974; Liao and Atkinson, 1971; and Schedel et al, 1975) show a sigmoidal response to increasing PEP concentration.

The homotropic cooperativity of PEP binding was reduced by increasing ADP concentrations for the yeast pyruvate kinase (Seubert and Schoner, 1971) although it was not completely abolished at up to 10 mM ADP. Similarly in the AMP-activated enzyme from E.coli (Waygood et al, 1975) the cooperative binding of PEP found at non-saturating concentrations of ADP became completely hyperbolic at an ADP concentration above 1.33 mM, at pH 6.3. However, in S.lactis (Crow, 1975) changing the ADP concentration from 0.3 mM to 6.67 mM had no effect on the cooperativity of PEP binding.

The relationship between P.shermanii pyruvate kinase activity and PEP concentration at 5 different ADP concentrations is shown in Figure 4.3.8(a) and summarised in Table 4.3.8(a). The Hill coefficient remained within the range 2.2 to 2.5 for the range of

Figure 4.3.8

EFFECT OF ADP ON THE RELATIONSHIP BETWEEN ACTIVITY AND PEP CONCENTRATION

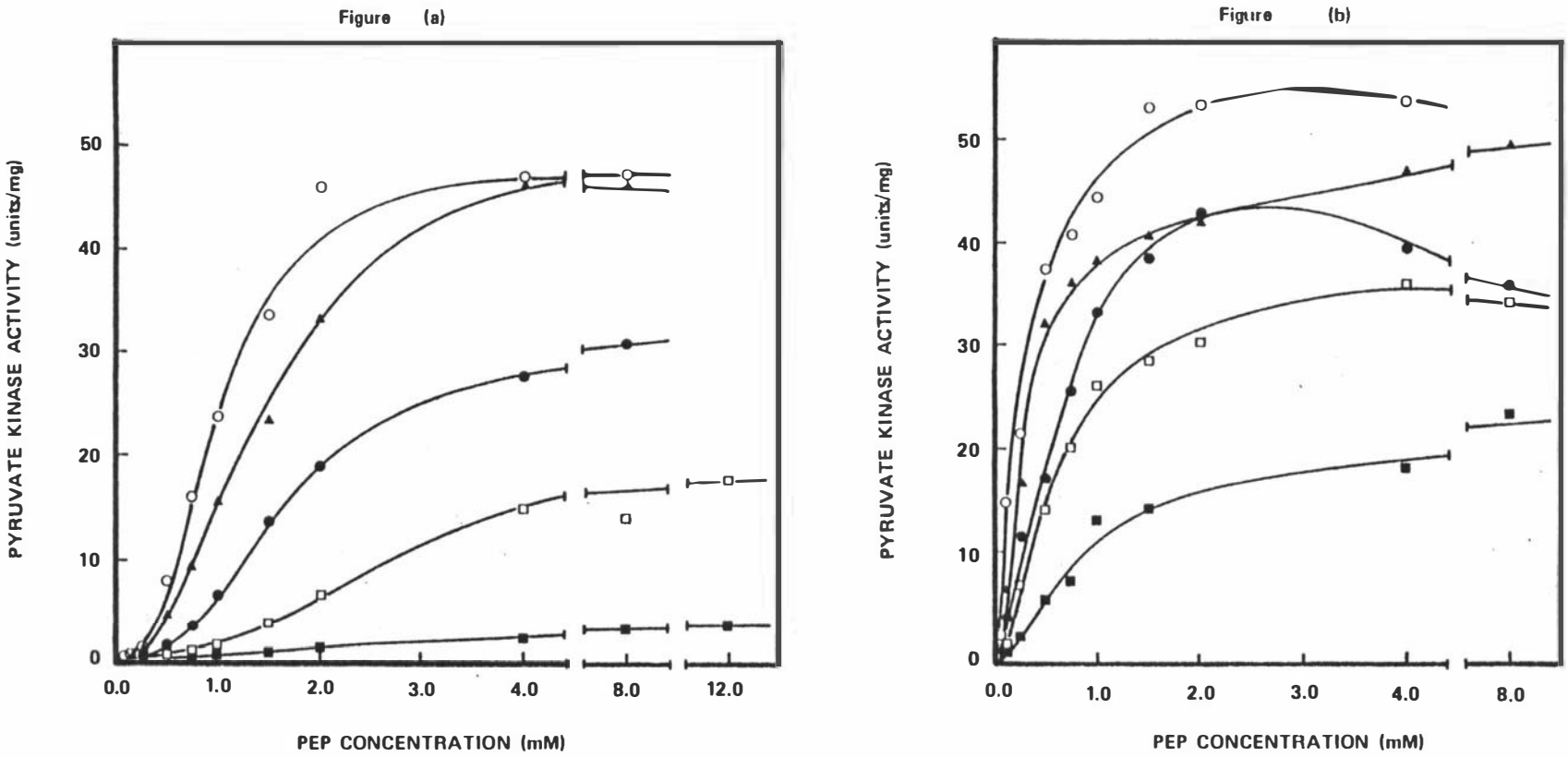


Figure (a) shows the relationship between pyruvate kinase activity and PEP concentration at 5 different concentrations of ADP. Figure (b) shows the same relationship in the presence of 2.0 mM G6P. Reaction conditions as specified in Section 4.3.1. at the concentrations of PEP shown and the following concentrations of ADP : ○ 2.0 mM ; ▲ 1.0 mM ; ● 0.5 mM ; □ 0.25 ; ■ 0.1 mM.
Enzyme concentration : 2.2 μg protein per assay.

Table 4.3.8 Effect of ADP on the relationship between activity and PEP concentration[a].

(a) No G6P					(b) In the presence of 2.0 mM G6P				
ADP Concentration (mM)	Interaction Coefficient n_H[b]	Interaction Coefficient n_H[c]	$PEP_{0.5V}$[b] (mM)	V_{max}[c] (units/mg)	ADP Concentration (mM)	Interaction Coefficient n_H[b]	Interaction Coefficient n_H[c]	$PEP_{0.5V}$[b] (mM)	V_{max}[c] (units/mg)
2.00	2.4	2.3	1.05	51.9	2.00	1.6	1.5	0.350	55.1
1.00	2.2	2.2	1.45	49.1	1.00	1.8	1.9	0.345	45.0
0.50	2.5	2.4	1.70	31.7	0.50	1.9	2.0	0.620	45.0
0.25	2.5	2.7	2.45	18.0	0.25	2.0	1.9	0.595	34.9
0.10	1.9	1.8	2.40	3.8	0.10	1.6	1.8	0.940	21.0

[a] Reaction conditions as specified in Section 4.3.1. Enzyme concentration: 2.2 μg protein per assay.

[b] Determined graphically from Hill plots using V_{max} values determined by the method of Endrenyi et al (1975).

[c] Determined directly from slope and intercept of Endrenyi plot.

ADP concentrations from 0.25 mM to 2.0 mM but dropped slightly to 1.9 at 0.10 mM ADP. Lowering the ADP concentration increased the $PEP_{0.5V}$ values from 1.05 mM PEP at 2.0 mM ADP to 2.40 mM PEP at 0.10 mM ADP.

Secondary plots of the data in Table 4.3.8(a) according to the method of Endrenyi et al (1975) gave a true V_{max} (Section 4.3.9.1) of 54.1 units/mg and an $ADP_{0.5V}$ value (from the Hill plot) of 0.36 mM ADP.

4.3.8.2 Effect of 2.0 mM G6P on the PEP saturation curve

Glucose-6-phosphate was found to be the major heterotropic activator of pyruvate kinase in P.shermanii (Section 4.3.7). The relationship between PEP concentration and activity at 5 different ADP concentrations (as for Section 4.3.8.1 above) was repeated in the presence of 2.0 mM G6P. The results are shown in Figure 4.3.8(b) and summarised in Table 4.3.8(b).

Comparing the data in Tables 4.3.8(a) and (b) it can be seen that the presence of G6P decreased the cooperativity of the PEP binding, lowering the Hill coefficient from a mean value of 2.35 to 1.85 in the presence of 2.0 mM G6P. At this concentration (2.0 mM), G6P appeared to have little effect on the V_{max} value of the PEP binding curves at high ADP concentrations ($\geq$1.0 mM ADP). Later data showed however that high concentrations of G6P lowered the V_{max} value of the reaction at saturating PEP and ADP concentrations. At low ADP concentrations however, the V_{max} was significantly higher in the presence of 2.0 mM G6P. G6P had a major effect on the binding of the substrate PEP. The $PEP_{0.5V}$ values at all ADP concentrations used were significantly lowered by the 2.0 mM G6P.

4.3.9 The Relationship Between Pyruvate Kinase Activity and Varying ADP Concentration

Pyruvate kinases show a varied response to ADP activation with some enzymes showing a sigmoidal relationship while others have typical Michaelis-Menten kinetics. The pyruvate kinases from rabbit liver (type L) (Irving and Williams, 1973); rat liver (type L), rat muscle (type M_1) and rat kidney cortex (type M_2) (Ibsen and Trippet, 1973); and Acetobacter xylinum (Benziman, 1969) all give an hyperbolic response for ADP binding while the pyruvate kinases from E.coli (type I)(Waygood and Sanwal, 1974); S.lactis (Crow, 1975) and yeast (Seubert and Schoner, 1971) all display positive homotropic cooperativity for ADP.

The E.coli (type II) enzyme (Waygood et al, 1975) shows positive cooperativity for ADP at low PEP concentrations but becomes hyperbolic at PEP concentrations greater than 1 mM and at pH's above 7.5. Similarly rabbit muscle (type M_1) pyruvate kinase (Phillips and Ainsworth, 1977) gives a sigmoidal response to ADP at low Mg^{++} concentrations which is abolished above 1 mM Mg^{++}.

4.3.9.1 Effect of PEP concentration on the ADP saturation curve

The relationship between P.shermanii pyruvate kinase activity and ADP concentration at 5 different PEP concentrations is shown in Figure 4.3.9(a) and summarised in Table 4.3.9(a). ADP showed positive cooperativity which remained constant over the range of PEP concentrations used (0.25 to 3.0 mM) with a Hill coefficient of around 1.90. The $ADP_{0.5V}$ values increased from 0.34 mM ADP at 3.0 mM PEP to 0.70 mM ADP at 0.25 mM PEP.

Figure 4.3.9

EFFECT OF PEP ON THE RELATIONSHIP BETWEEN ACTIVITY AND ADP CONCENTRATION

Figure (a)

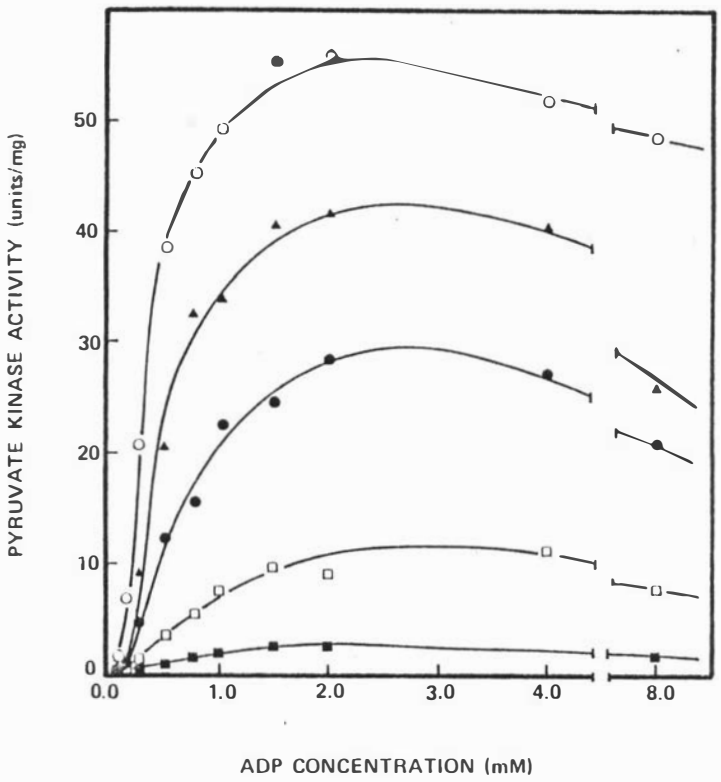


Figure (b)

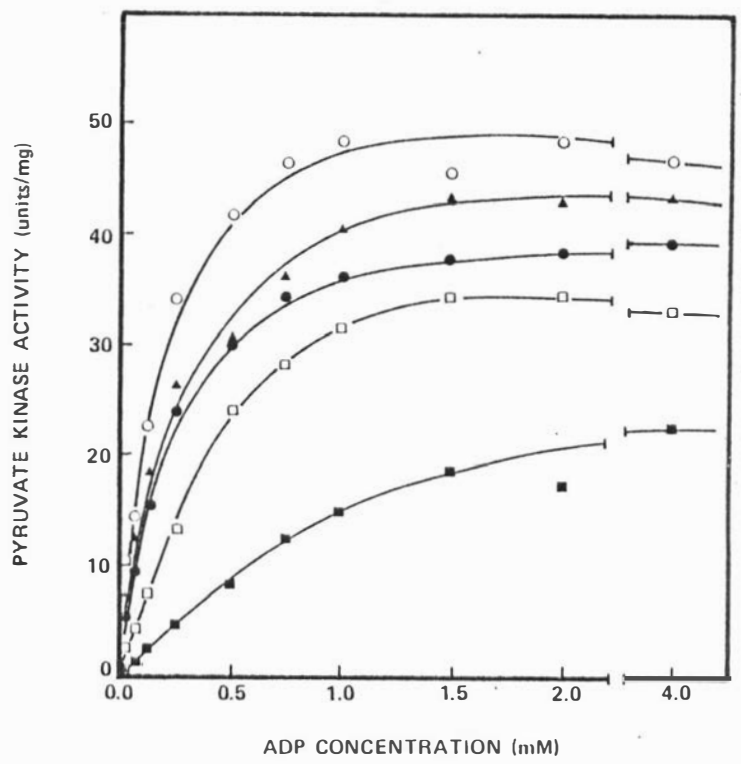


Figure (a) shows the relationship between pyruvate kinase activity and ADP concentration at 5 different concentrations of PEP. Figure (b) shows the same relationship in the presence of 2.0 mM G6P. Reaction conditions as specified in Section 4.3.1. at the concentrations of ADP shown and the following concentrations of PEP : ○ 3.0 mM ; ▲ 1.5 mM ; ● 1.0 mM ; □ 0.5 mM ; ■ 0.25 mM.
Enzyme concentration : 2.2 μg protein per assay.

Table 4.3.9 Effect of PEP on the relationship between activity and ADP concentration[a].

(a) No G6P					(b) In the presence of 2.0 mM G6P				
PEP Concentration (mM)	Interaction Coefficient n_H[b]	Interaction Coefficient n_H[c]	$ADP_{0.5V}$[b] (mM)	V_{max}[c] (units/mg)	PEP Concentration (mM)	n_H[d]	$ADP_{0.5V}$[d] (mM)	K_m[e] (mM)	V_{max}[e] (units/mg)
3.00	1.9	1.9	0.34	56.0	3.00	1.1	0.16	0.16	54.0
1.50	2.1	2.0	0.49	43.9	1.50	1.0	0.21	0.21	49.4
1.00	1.8	1.9	0.62	30.6	1.00	1.0	0.22	0.23	44.2
0.50	1.7	1.5	1.05	14.9	0.50	1.1	0.70	0.71	53.0
0.25	2.0	2.0	0.70	2.8	0.25	0.9	1.70	2.30	46.9

[a] Reaction conditions as specified in Section 4.3.1. Enzyme concentration: 2.2 μg protein per assay.

[b] Estimated graphically from Hill plots using V_{max} values determined by the method of Endrenyi *et al* (1975).

[c] Determined directly from slope and intercept of Endrenyi plot.

[d] Estimated graphically from Hill plots using V_{max} values determined by the method of Eisenthal and Cornish-Bowden (1974).

[e] Determined by the method of Eisenthal and Cornish-Bowden (1974).

Secondary plots of the data in Table 4.3.9(a) according to the method of Endrenyi et al (1975) gave a true V_{max} (Section 4.3.8.1) of 62.4 units/mg and a $PEP_{0.5V}$ value (from a Hill plot) of 1.0 mM PEP.

4.3.9.2 The effect of 2.0 mM G6P on the ADP saturation curve

The relationship between pyruvate kinase activity and ADP concentration at 5 different PEP concentrations (as in Section 4.3.9.1 above) was repeated in the presence of 2.0 mM G6P. The results are shown in Figure 4.3.9(b) and summarised in Table 4.3.9(b). Comparing the data in Tables 4.3.9(a) and (b) it can be seen that G6P abolished the cooperativity of ADP binding, lowering the Hill coefficient from 1.90 to 1.00. Similar to its effect on PEP binding, G6P had little effect on the V_{max} under saturating PEP concentrations (3.0 mM). However as the PEP concentration was decreased, the presence of 2.0 mM G6P enhanced the V_{max} considerably over that found in its absence. G6P also had a marked effect on the $ADP_{0.5V}$ values. At PEP concentrations above 1.0 mM the presence of 2.0 mM G6P significantly lowered the $ADP_{0.5V}$ value. However at 0.25 mM PEP the $ADP_{0.5V}$ value was increased over 3 fold by the 2.0 mM G6P.

4.3.10 Effect of GDP on Pyruvate Kinase Activity

Waygood and Sanwal (1974) studying the type I pyruvate kinase of E.coli found that GDP, UDP, IDP and CDP could replace ADP as the phosphate group acceptor. GDP was in fact by far the best phosphate acceptor for the enzyme with a K_m of 0.05 mM compared to 0.24 mM for ADP and a V_{max} of 110 units/mg compared to 55 units/mg

for ADP. Unlike ADP, GDP showed substrate inhibition above 0.05 mM. Despite this Waygood and Sanwal (1974) suggested the E.coli type I pyruvate kinase was perhaps specific for GDP as the phosphate acceptor in vivo.

Crow (1975) found that the S.lactis pyruvate kinase was also able to use GDP as the nucleotide phosphate acceptor instead of ADP. In this case GDP gave a comparable V_{max} to that obtained with ADP as nucleotide substrate and, as for the E.coli case, the GDP had a much lower K_m (0.1 mM GDP) than the ADP (1.2 mM ADP). Unlike the E.coli situation, in S.lactis the pyruvate kinase was inhibited by high ADP concentrations and not by high GDP concentrations.

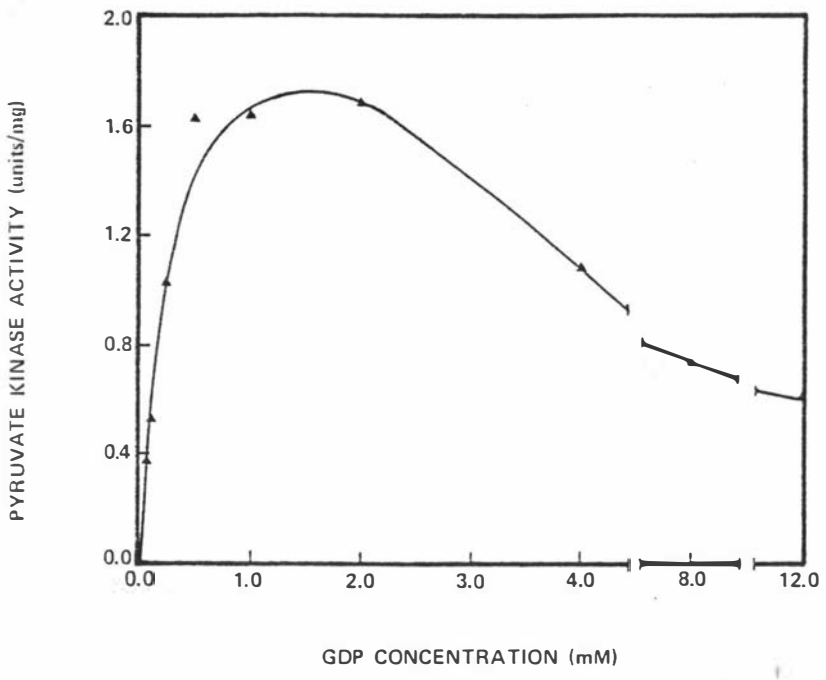
In the light of these experiments it was decided to investigate the effect of GDP on the activity of the P.shermanii pyruvate kinase. The relationship between pyruvate kinase activity and GDP concentration is shown in Figure 4.3.10. As for the E.coli type I enzyme the GDP showed substrate inhibition although at a higher concentration (2.0 mM GDP in P.shermanii compared with 0.05 mM GDP in E.coli). The K_m for GDP was about 0.2 mM compared with 0.34 mM for ADP under similar conditions, however the V_{max} obtained using GDP as phosphate acceptor was less than 10% of that for the reaction using ADP and thus it would seem unlikely that GDP is the nucleotide phosphate acceptor for the P.shermanii pyruvate kinase in vivo.

4.3.11 Effect of Mg^{++} on Pyruvate Kinase Activity

All pyruvate kinases so far studied have an absolute requirement for a divalent cation, generally Mg^{++} although this can often be replaced by other divalent cations which also activate the reaction.

Figure 4.3.10

EFFECT OF GDP CONCENTRATION ON PYRUVATE KINASE ACTIVITY



The relationship between pyruvate kinase activity and GDP concentration was determined in the absence of ADP. Reaction conditions as specified in Section 4.3.1. with GDP substituted for ADP as indicated. Enzyme concentration : 4.5 μg protein per assay.

Depending on the source the enzyme may display sigmoidal or Michaelis-Menten kinetics with respect to varying concentrations of Mg^{++}; substrates and modifiers may have different effects on its activation.

4.3.11.1 Effect of Mg^{++} on pyruvate kinase activity of P.shermanii

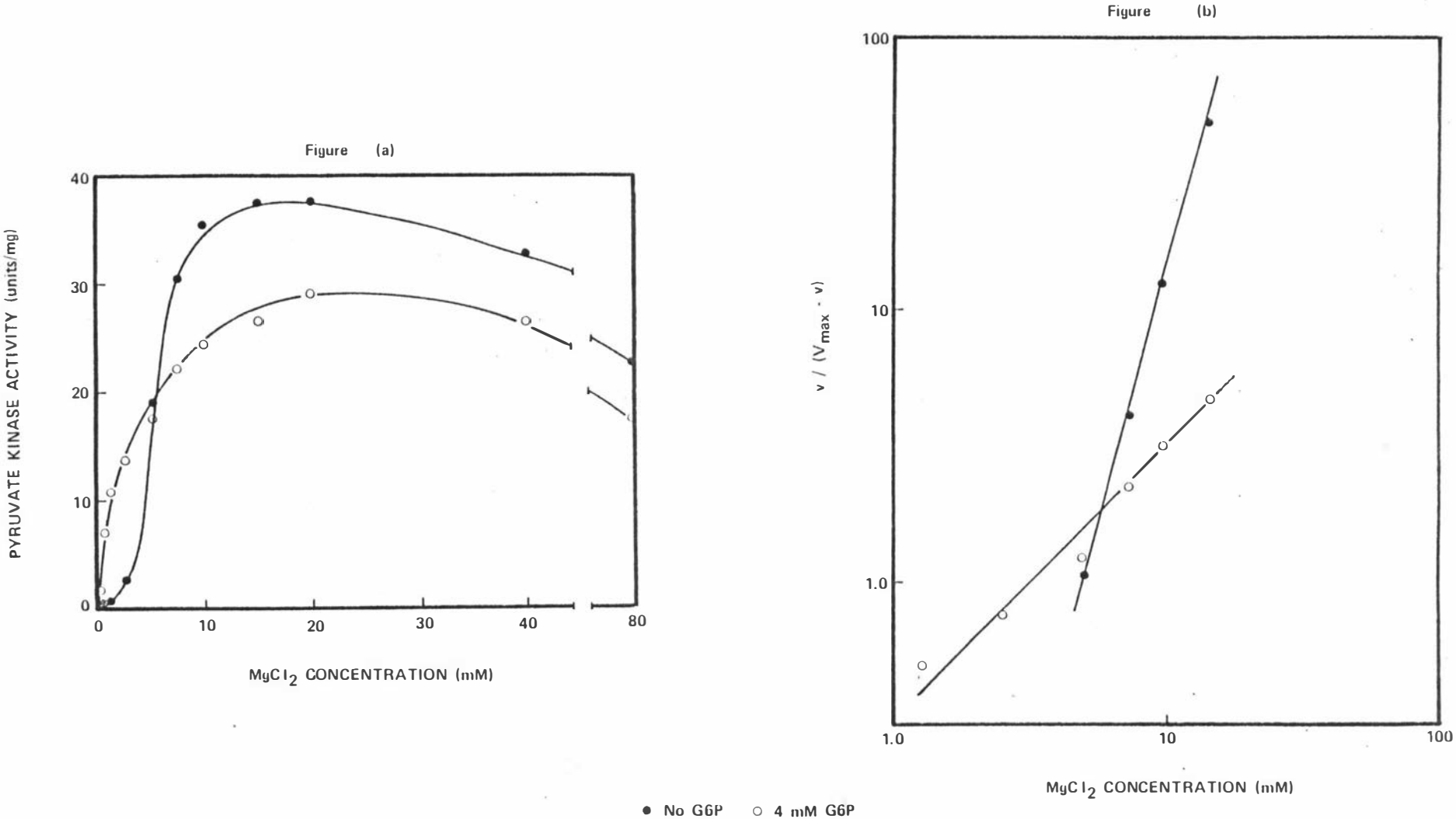
The relationship between P.shermanii pyruvate kinase and Mg^{++} concentration is shown in Figure 4.3.11 as a rate versus Mg^{++} concentration graph with its corresponding Hill plot. The Hill coefficient of 3.66 for Mg^{++} is very high and suggests that the interaction with Mg^{++} is highly cooperative. The $Mg_{0.5V}$ value of 5.0 mM Mg^{++} obtained from the Hill plot is also large suggesting that a high concentration of Mg^{++} is required to saturate the enzyme. This is borne out by the fact that 20 mM Mg^{++} is required for maximum activation of the enzyme.

4.3.11.2 Effect of G6P on Mg^{++} activation of pyruvate kinase

Figure 4.3.11 also shows the relationship between pyruvate kinase activity and Mg^{++} concentration in the presence of 4 mM G6P. The most striking effect of G6P on the activation of pyruvate kinase by Mg^{++} is the complete abolition of its cooperativity (lowering the Hill number from 3.66 to 0.90). Consistent with the earlier kinetic data G6P did not increase the V_{max} of the reaction, in fact in the presence of 4 mM G6P the V_{max} was only 32.1 units/mg compared to 38.4 units/mg in its absence. However G6P lowered the $Mg_{0.5V}$ value from 5.0 mM Mg^{++} to 3.0 mM Mg^{++}.

Figure 4.3.11

EFFECT OF Mg^{++} CONCENTRATION ON PYRUVATE KINASE ACTIVITY



The relationship between pyruvate kinase activity and $MgCl_2$ concentration both in the presence and absence of G6P is shown in Figure (a). The corresponding Hill plots are shown in Figure (b). Reaction conditions as specified in Section 4.3.1. with $MgCl_2$ as indicated. Enzyme concentration : 2.0 μg protein per assay.

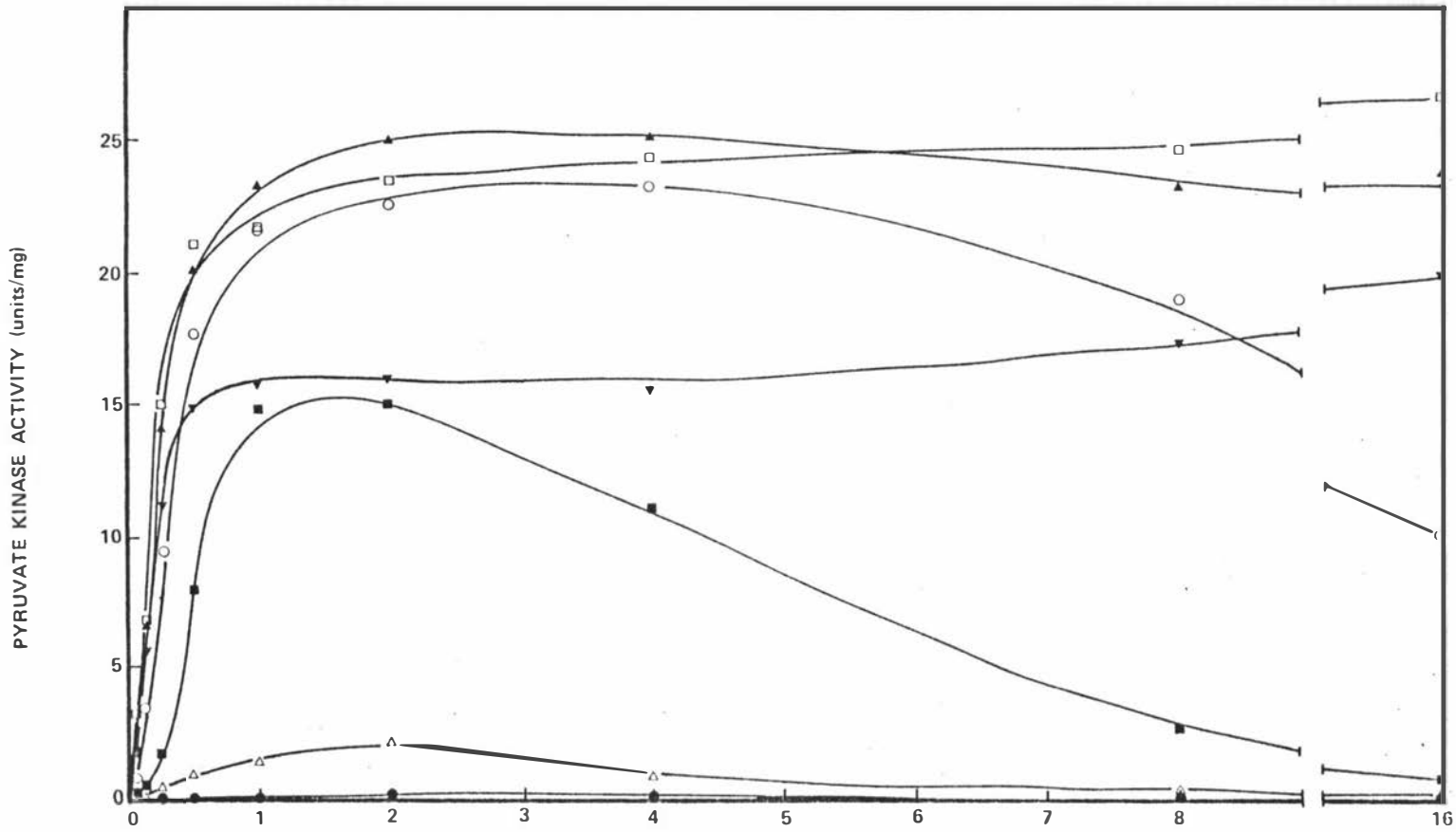
4.3.11.3 Interaction between Mg^{++} and ADP

It appears that for different pyruvate kinases the nucleotide phosphate acceptor ADP may participate in the reaction either as free ADP^{3-}, ADP Mg^{-} or a combination of both. There is considerable evidence indicating that the adenosine nucleotides (ADP and ATP) are involved in the reaction as their magnesium salts, with the bivalent cations forming a bridge between the enzyme and ADP (Melchior, 1965; Mildvan and Cohn, 1965). However, Phillips and Ainsworth (1977) studying the rabbit muscle pyruvate kinase concluded from their observations that "... it is both necessary and sufficient to suppose that the true substrates of the activated enzyme are Mg^{++} and the Mg^{++}-free phosphoenolpyruvate and ADP species." Similarly, Macfarlane and Ainsworth (1972) concluded that the free ADP alone is the nucleotide substrate of yeast pyruvate kinase and they add "In solution Mg^{++} is bound between the α- and β- phosphate groups of ADP and the β- and γ- groups of ATP (Cohn and Hughes, 1960, 1962; Hammes et al, 1961). Thus if MgADP were to be a substrate there would be some difficulty in explaining the shift in position of Mg^{++} during reaction. However, the separate binding of Mg^{++} readily suggests that it bridges the phosphate group of phosphoenolpyruvate and the terminal phosphate group of ADP, assisting the phosphorylation of the latter, and ultimately being eliminated bound between the β- and γ- phosphate groups of ATP."

The elucidation of the reaction mechanism of the P.shermanii pyruvate kinase would involve very intensive kinetic studies and this was not one of the aims of this work. Thus no attempt was made to determine whether the active species of the adenine dinucleotide was ADP^{3-} or $ADPMg^{-}$ or a combination of the two, and instead the

Figure 4.3.11.3

EFFECT OF Mg^{++} CONCENTRATION ON THE RELATIONSHIP BETWEEN PYRUVATE KINASE ACTIVITY AND ADP CONCENTRATION



The figure shows the relationship between pyruvate kinase activity and ADP concentration at 7 different $MgCl_2$ concentrations.

Reaction conditions as specified in Section 4.3.1 at the following $MgCl_2$ concentrations : ● 0.5 mM; △ 2.0 mM; ■ 5.0 mM; ○ 10 mM; ▲ 20 mM; □ 40 mM and ▼ 80 mM. Enzyme concentration : 4.0 µg protein per assay.

concentration of the nucleotide was expressed simply as ADP, meaning the total ADP in the assay.

To determine the concentrations of ADP and Mg^{++} required to give maximum activation of the pyruvate kinase the relationship between activity and ADP concentration was followed at 7 different Mg^{++} concentrations and is shown in Figure 4.3.11.3. From this figure it was concluded that 20 mM Mg^{++} and 2.0 mM ADP were the optimum concentrations for the P.shermanii enzyme and these were used as the standard conditions for Mg^{++} and ADP throughout this study.

Figure 4.3.11.3 also shows that high ADP concentrations inhibit the reaction and this is enhanced at non-saturating Mg^{++} concentrations.

Secondary plots of the data in Figure 4.3.11.3 give a $Mg_{0.5V}$ value of 4.5 mM Mg^{++} and an interaction coefficient of 3.45 which compare favourably with the data presented in Section 4.3.11.1. A secondary plot of V_{max} versus Mg^{++} concentration shows that high concentrations of Mg^{++} inhibit the reaction.

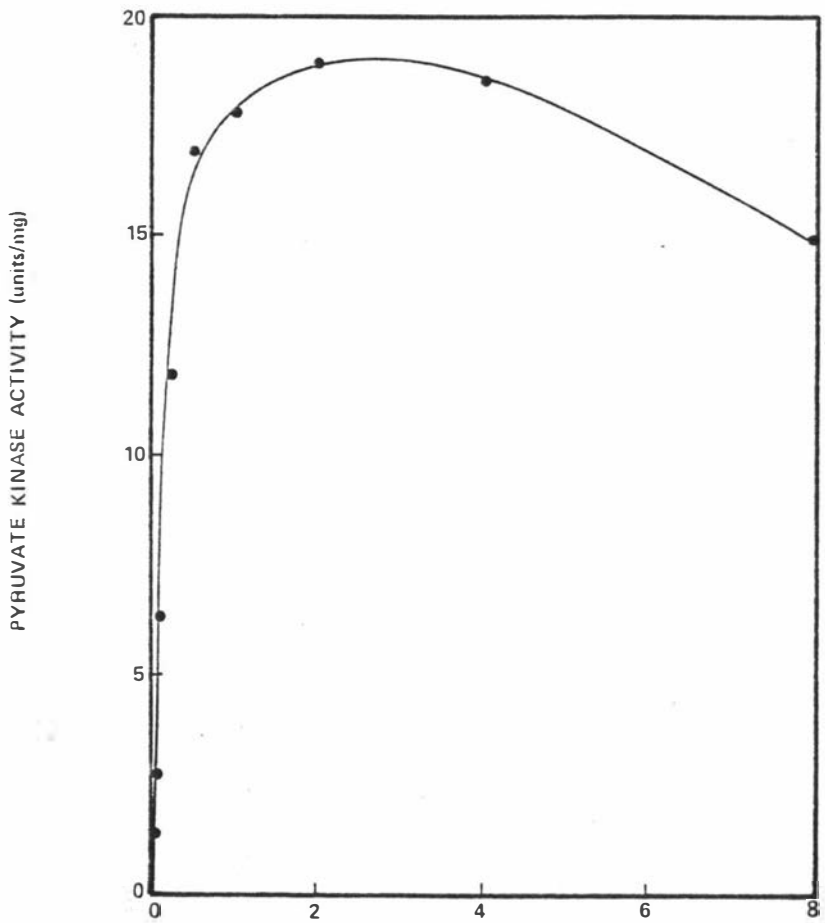
4.3.12 Effect of Mn^{++} on the PEP Saturation Curve of Pyruvate Kinase

Mn^{++} can frequently replace Mg^{++} as the divalent cation in pyruvate kinase reactions and several workers have found that it enhances the binding of PEP (Tuominen and Bernhlor, 1971; Waygood and Sanwal, 1974; Crow, 1975).

The relationship between P.shermanii pyruvate kinase and PEP concentration was investigated in the presence of 20 mM Mn^{++} instead of 20 mM Mg^{++} and is shown in Figure 4.3.12. The interaction coefficient for the relationship was 1.66 and the $PEP_{0.5V}$ value was 0.19 mM PEP, both of which are significantly lower than for the

Figure 4.3.12

EFFECT OF $MnCl_2$ CONCENTRATION ON THE PEP SATURATION CURVE



The figure shows the relationship between pyruvate kinase activity and PEP concentration in the presence of $MnCl_2$. Reaction conditions as specified in Section 4.3.1. except that the $MgCl_2$ was replaced with 20 mM $MnCl_2$. Enzyme concentration : 2.0 µg protein per assay.

relationship when using Mg^{++} as the divalent cation. Thus it appears that Mn^{++} lowers the cooperativity of the rate versus PEP relationship as well as increasing the binding of PEP. The V_{max} from Figure 4.3.12 was 19.2 units/mg which was only half the value obtained using Mg^{++} as the divalent cation.

4.3.13 Effect of Varying G6P Concentration on Pyruvate Kinase Activity

The effect of increasing G6P concentration on the activity of P.shermanii pyruvate kinase was followed under both saturating and non-saturating concentrations of PEP (5.0 mM and 0.5 mM) (Figure 4.3.13). At 5.0 mM PEP addition of G6P caused a slight but distinct inhibition of activity (about 10% inhibition at 2.0 mM G6P), while at 0.5 mM PEP the addition of G6P markedly activated the rate (6-fold activation at 6.0 mM G6P).

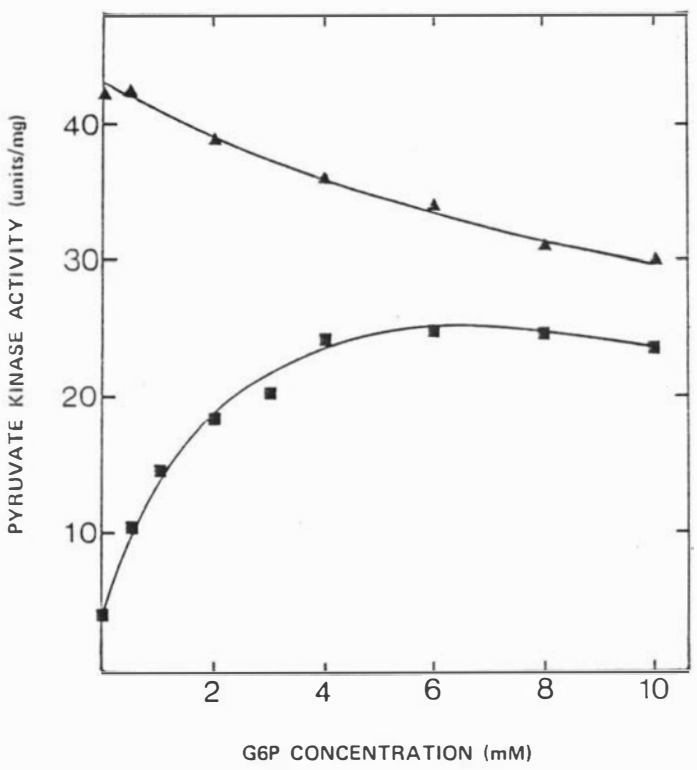
When graphed as an Hill plot the data in Figure 4.3.13 yield an interaction coefficient of 1.0 for the G6P activation (at 0.5 mM PEP). The $G6P_{0.5V}$ value obtained was 0.7 mM G6P.

4.3.14 Effect of Varying ATP Concentration on Pyruvate Kinase Activity

The effect of increasing ATP concentrations on the enzyme activity was determined at 3 different PEP concentrations (Figure 4.3.14). ATP was a potent inhibitor of the pyruvate kinase at all PEP concentrations causing almost total inhibition of the standard assay (5.0 mM PEP) at 10 mM ATP.

Figure 4.3.13

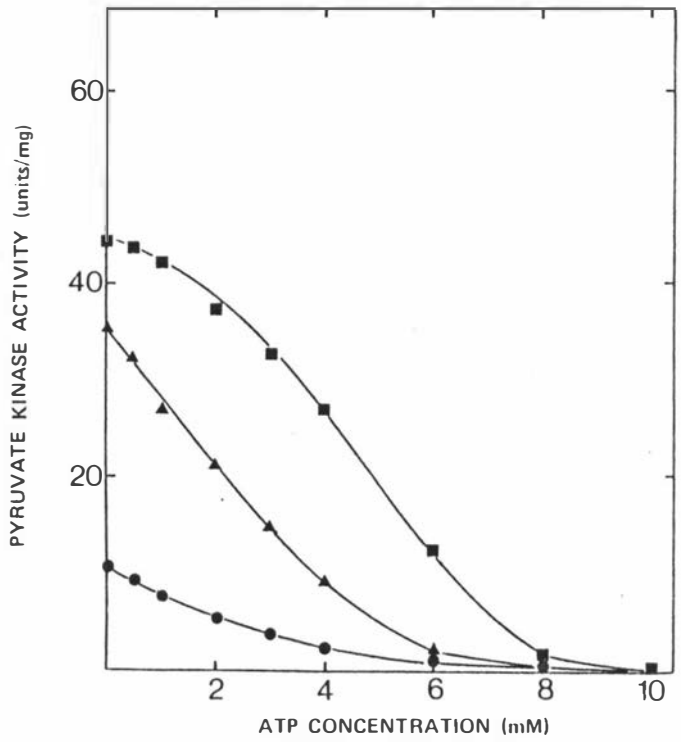
EFFECT OF G6P CONCENTRATION ON PYRUVATE KINASE ACTIVITY



The figure shows the effect of increasing G6P concentration on pyruvate kinase activity under both saturating and non-saturating concentrations of PEP. Reaction conditions as specified in Section 4.3.1. with G6P concentration as indicated and either ▲ 5.0 mM PEP or ■ 0.5 mM PEP. Enzyme concentration : 1.5 μg protein per assay.

Figure 4.3.14

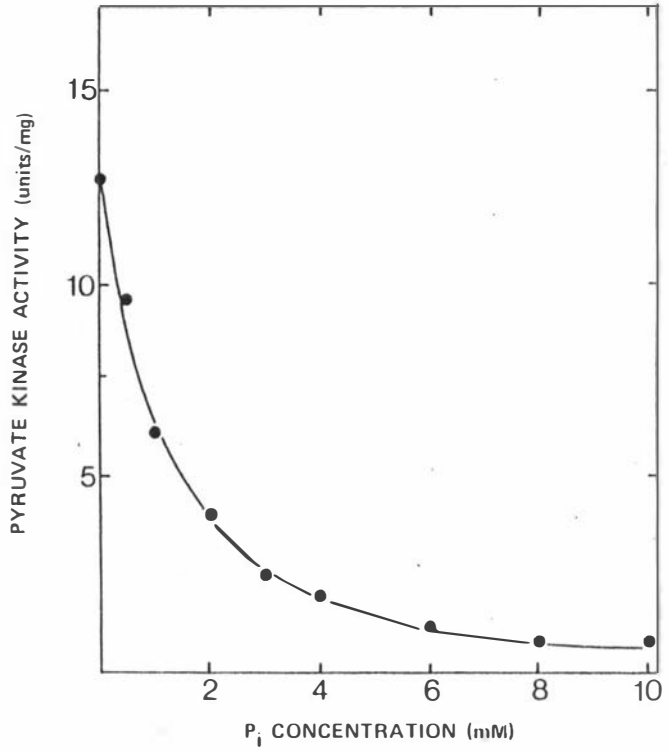
EFFECT OF ATP CONCENTRATION ON PYUVATE KINASE ACTIVITY



The figure shows the effect of increasing ATP concentration on pyruvate kinase activity at 3 different PEP concentrations. Reaction conditions as specified in Section 4.3.1. with ATP concentration as indicated and ■ 5.0 mM PEP, ▲ 1.25 mM PEP, or ● 0.5 mM PEP. Enzyme concentration : 2.1 μg protein per assay.

Figure 4.3.15

EFFECT OF P_i CONCENTRATION ON PYRUVATE KINASE ACTIVITY



The figure shows the effect of increasing P_i concentration on pyruvate kinase activity at 0.5 mM PEP. Reaction conditions as specified in Section 4.3.1. with P_i concentration as indicated and 0.5 mM PEP. Enzyme concentration : 2.1 μg protein per assay.

Table 4.3.14 Effect of ATP concentration on pyruvate kinase activity[a].

PEP Concentration (mM)	Interaction Coefficients[b] $n_H(1)$	$n_H(2)$	$ATP_{0.5V}$[b] (mM)
5.00	-1.8	-7.0	6.2
1.25	-1.5	-4.5	2.5
0.50	-1.1	-2.9	1.8

[a] Reaction conditions as specified in Section 4.3.1 with PEP concentration as indicated. Enzyme concentration: 2.1 µg protein per assay.

[b] Estimated graphically from Hill plots using the activity obtained at zero ATP concentration as the V_{max}.

Table 4.3.14 shows the parameters obtained from Hill plots of the experiment described. At all PEP concentrations the Hill plots were biphasic with the slope becoming much steeper at higher ATP concentrations. This suggests that there may be different mechanisms involved in ATP inhibition at the higher concentrations. It may be acting at an allosteric binding site, by the chelation of Mg^{++}, by competition with ADP for its substrate binding site, or a combination of these possibilities. The chelation of Mg^{++} is most likely to be important at high ATP concentrations.

Table 4.3.14 shows that the binding of ATP is affected by the PEP concentration. The $ATP_{0.5V}$ values decrease from 6.2 mM ATP at 5.0 mM PEP to 1.8 mM ATP at 0.5 mM PEP suggesting that the ATP binding is stronger at non-saturating levels of substrate.

4.3.15 Effect of Varying P_i Concentration on Pyruvate Kinase Activity

The effect of increasing P_i concentration on the enzyme activity was studied at 0.5 mM PEP and is shown in Figure 4.3.15. Like ATP, P_i was a potent inhibitor of the P.shermanii pyruvate kinase and under these conditions gave a $P_{i_{0.5V}}$ value of 1.4 mM P_i. The interaction coefficient for P_i was -1.5 and was not biphasic as was the case with ATP.

4.3.16 Interaction Between G6P and ATP or P_i in the Relationship Between Pyruvate Kinase Activity and PEP Concentration

Having determined what appear to be the major effectors of the P.shermanii enzyme a study was made of the interaction between the activator G6P and each of the two inhibitors, ATP and P_i, in their

effect on the relationship between pyruvate kinase activity and PEP concentration (which was described in detail in Section 4.3.8).

The effect of G6P (4.0 mM)and ATP (6.0 mM) on the relationship between activity and PEP concentration is shown in Figure 4.3.16(a) and the effect of G6P (4.0 mM)and P_i (6.0 mM) on the relationship in Figure 4.3.16(b). The data from these 2 graphs are summarised in Table 4.3.16. G6P, ATP and P_i all lowered the cooperativity of the PEP binding although none of them completely abolished it. The three effectors similarly all caused a decrease in the V_{max} (as determined by the method of Endrenyi et al (1975)) P_i being the most effective, lowering it from 52.4 units/mg to 7.3 units/mg.

The most significant effects of G6P, ATP and P_i however were on the $PEP_{0.5V}$ values. G6P (4.0 mM) markedly lowered the $PEP_{0.5V}$ from 0.75 mM PEP to 0.17 mM PEP; ATP (6.0 mM) increased it to 2.45 mM PEP while P_i (6.0 mM) also increased it to 1.85 mM PEP. Thus G6P activates by enhancing the binding of PEP while the inhibitors, ATP and P_i, weaken it. 4.0 mM G6P was able to reverse the inhibition by both ATP and P_i, lowering the $PEP_{0.5V}$ values to 0.34 mM PEP and 0.46 mM PEP in the presence of 6.0 mM ATP and 6.0 mM P_i respectively.

The reversal of ATP and P_i inhibition by the activator G6P may be a key feature of the in vivo regulation of P.shermanii pyruvate kinase. Thus physiological situations promoting a high level of G6P in the cell could cause a reactivation of the enzyme depressed by ATP and/or P_i inhibition.

4.3.17 Effect of pH on the Kinetic and Allosteric Properties of Pyruvate Kinase

The allosteric properties of a number of pyruvate kinases

Figure 4.3.16

INTERACTION BETWEEN G6P AND ATP OR P_i ON THE PEP SATURATION CURVE

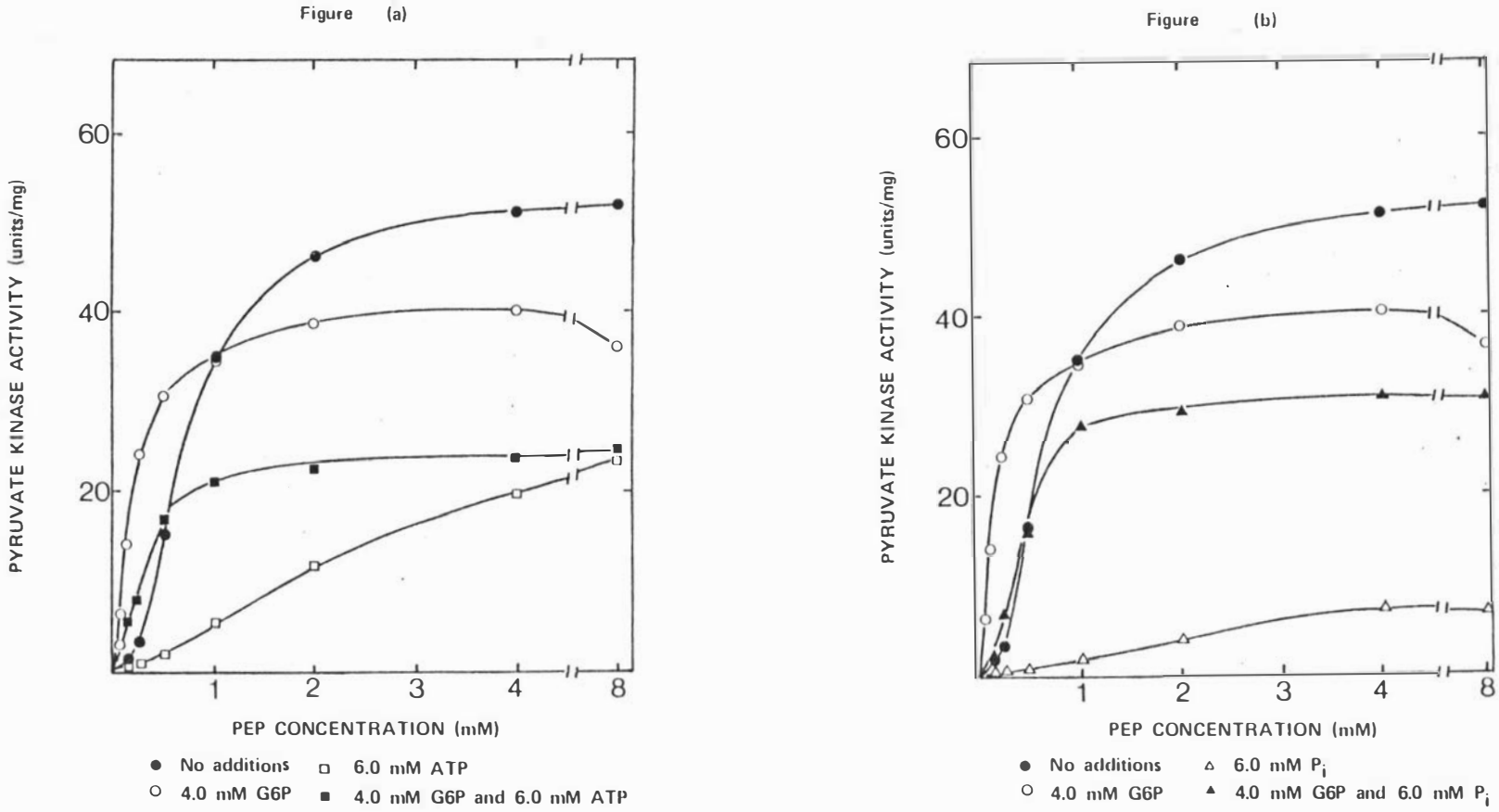


Figure (a) shows the effect of G6P and ATP on the relationship between pyruvate kinase activity and PEP concentration, while figure (b) shows the effect of G6P and P_i on the same relationship. Reaction conditions as specified in Section 4.3.1. with PEP concentrations and additions as indicated. Enzyme concentration : 2.1 μg protein per assay.

Table 4.3.16 The effects of G6P, ATP and P_i on the relationship between pyruvate kinase activity and PEP concentration[a].

Additions	n_H [b]	$PEP_{0.5V}$ [b] (mM)	V_{max} [c] (units/mg)
No additions	2.3	0.75	52.4
4.0 mM G6P	1.7	0.17	38.6
6.0 mM ATP	1.6	2.45	28.0
4.0 mM G6P + 6.0 mM ATP	1.2	0.34	25.1
6.0 mM P_i	1.9	1.85	7.3
4.0 mM G6P + 6.0 mM P_i	1.9	0.46	30.9

[a] Reaction conditions as specified in Section 4.3.1. Enzyme concentration: 2.1 µg protein per assay.

[b] Estimated graphically from Hill plots using V_{max} values determined by the method of Endrenyi et al (1975).

[c] Determined directly from slope and intercept of Endrenyi plot.

have been found to be dependent on pH. Rabbit liver (type L) pyruvate kinase (Irving and Williams, 1973) obeys Michaelis-Menten kinetics with respect to PEP below pH 7.0 and is largely unaffected by FBP. However as the pH is raised the kinetic response to varying PEP concentrations becomes increasingly sigmoidal and FBP activation is more pronounced. The pH of the assay buffer is also important in determining the allosteric properties of the rabbit muscle (type M) pyruvate kinase (Phillips and Ainsworth, 1977), cooperativity of substrate binding again increasing with pH.

The involvement of pH in determining the allosteric properties of pyruvate kinases is also widespread in bacterial and fungal enzymes, being important in E.coli (type II) (Waygood et al, 1975), N.crassa (Kapoor and Tronsgard, 1972), yeast (Seubert and Schoner, 1971) and S.lactis (Crow, 1975) pyruvate kinases.

4.3.17.1 Effect of pH on the PEP saturation curve

All of the kinetic data described in this chapter were obtained at pH 7.5 in 0.1 M Tricine/NaOH buffer. It was decided to determine whether there was any significant change in the allosteric properties of the P.shermanii pyruvate kinase over the pH range 6.0 to 7.5. This was accomplished by studying the relationship between activity and PEP concentration at pH 6.0, 6.5, 7.0 and 7.5 in 0.1 M Bistrispropane/HCl buffer which conveniently covers this pH range. Table 4.3.17.1 summarises the results of this experiment. Although the results confirm that pH 7.5 was the optimum pH in terms of the V_{max} the cooperativity of the relationship was at its strongest (n_H 2.56) at pH 7.0, as was the PEP binding ($PEP_{0.5V}$ 0.85 mM).

Table 4.3.17.1 The effect of pH on the relationship between pyruvate kinase activity and PEP concentration[a].

pH	n_H [b]	$PEP_{0.5V}$ [b] (mM)	V_{max} [c] (units/mg)
6.0	2.0	1.45	24.7
6.5	2.3	1.18	29.2
7.0	2.6	0.85	33.9
7.5	2.1	0.93	36.6

[a] Reaction conditions as specified in Section 4.3.1 except that the assay buffer was replaced by 0.1 M MES/NaOH buffer at the pH values shown. Enzyme concentration: 2.0 µg protein per assay.

[b] Estimated graphically from Hill plots using V_{max} values determined by the method of Endrenyi et al (1975).

[c] Determined directly from slope and intercept of Endrenyi plot.

While the effect of pH on the interaction with PEP may not be as great as in some other pyruvate kinases it may have a distinct influence on the control of the P.shermanii enzyme via some other component of its regulatory system. For example the effect of pH may be more profound on the binding of ADP and Mg^{++} or through interacting with the positive and negative effectors of the enzyme.

4.3.17.2 Effect of G6P and ATP on the PEP saturation curve at pH 6.0

To investigate further the possible importance of pH on the allosteric properties of the P.shermanii enzyme the effect of 2.0 mM G6P and 5.0 mM ATP on the relationship between activity and PEP concentration was studied at pH 6.0 in 0.1 M MES/NaOH buffer (compare with data in Section 4.3.16 at pH 7.5). Table 4.3.17.2 shows the kinetic parameters obtained for the interactions described at pH 6.0. The effect of G6P (2.0 mM) on the PEP binding was much stronger than at pH 7.5 where it only lowered the Hill number from 2.33 to 1.74 (Table 4.3.16). At pH 6.0, 2.0 mM G6P was sufficient to completely abolish the cooperativity of the PEP binding, lowering the Hill number from 2.03 to 0.98. Its effect on the $PEP_{0.5V}$ value, however, was not as marked as it was at pH 7.5.

ATP also had a more pronounced effect on the relationship at pH 6.0 than it did at pH 7.5. At pH 6.0, 5.0 mM ATP caused a greater decrease of the interaction coefficient to 1.26 but had its main effect on the $PEP_{0.5V}$ value which was increased to 5.0 mM PEP compared with 2.45 mM PEP at pH 7.5 (Table 4.3.16).

It would appear that the in vivo pH is likely to be of importance in the regulation of the pyruvate kinase through its interaction with

Table 4.3.17.2 The effect of G6P and ATP on the relationship between pyruvate kinase activity and PEP concentration at pH 6.0[a].

Additions	n_H [b]	$PEP_{0.5V}$ [b] (mM)	V_{max} [c] (units/mg)
No additions	2.0	0.80	26.6
2.0 mM G6P	1.0	0.35	23.4
2.0 mM ATP	1.3	5.00	19.1

[a] Reaction conditions as specified in Section 4.3.1 except that the assay buffer was replaced by 0.1 M MES/NaOH buffer (pH 6.0). Enzyme concentration: 4.8 µg protein per assay.

[b] Estimated graphically from Hill plots using V_{max} values determined by the method of Endrenyi et al (1975).

[c] Determined directly from slope and intercept of Endrenyi plot.

the positive and negative effectors of the reaction.

4.3.17.3 Relationship between pyruvate kinase activity and ADP concentration at pH 6.0

The effect of pH on the relationship between activity and ADP concentration was determined at pH 6.0 in 0.1 M MES/NaOH buffer, at 3.0 mM PEP. A Hill plot of the results gave an interaction coefficient of 1.18 and an $ADP_{0.5V}$ value of 0.22 mM ADP. This is quite different from the situation at pH 7.5 (Table 4.3.9(a)) where the corresponding parameters are 1.9 (for n_H) and 0.34 mM ADP, again stressing the significance of pH in determining the allosteric properties of the enzyme.

4.3.17.4 Effect of G6P on the pH profile of pyruvate kinase

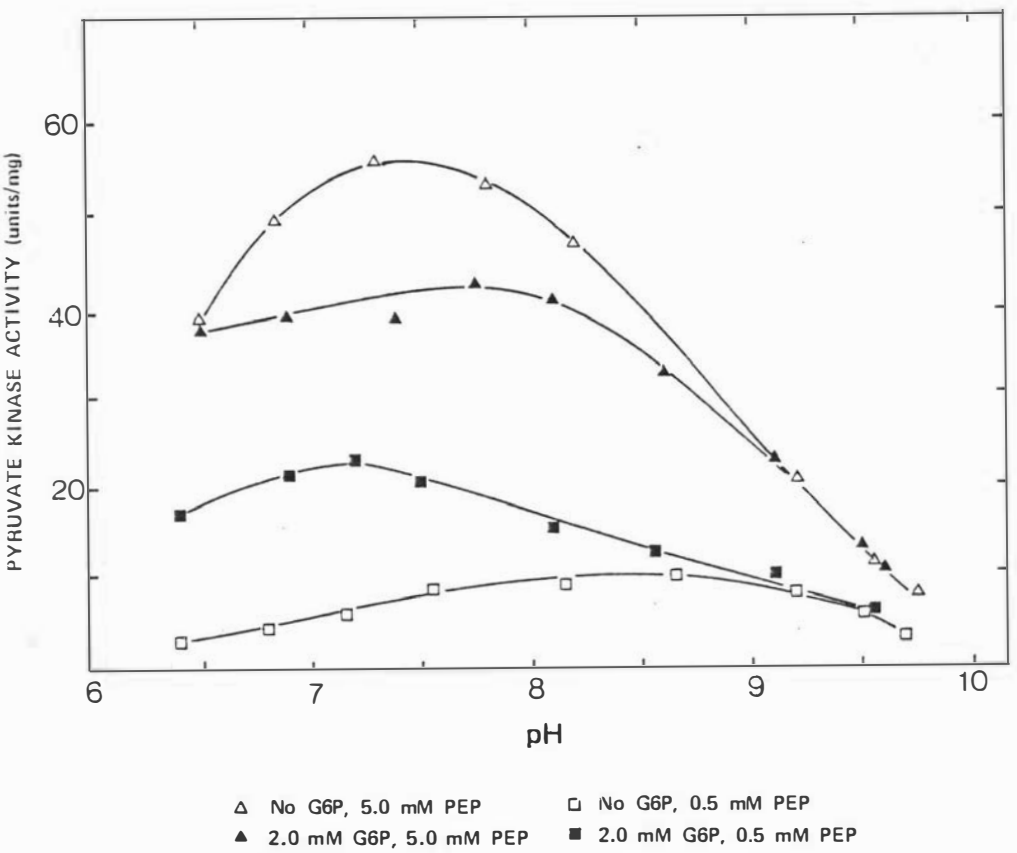
The effect of 2.0 mM G6P on the pH profile of P.shermanii pyruvate kinase was studied over the range pH 6.5 to 9.5 in 0.1 M Bistrispropane/HCl buffer. The system was the same as that used in Section 4.3.5 with the pH of the assay mixtures being measured after reaction.

The profile was determined at both saturating (5.0 mM) and non-saturating (0.5 mM) PEP concentrations in the presence and absence of 2.0 mM G6P (Figure 4.3.17.4). At 5.0 mM PEP the pH optimum in the absence of G6P was pH 7.3 compared with pH 8.5 at 0.5 mM PEP. In the presence of G6P however, the optimum shifted from pH 7.8 to 7.2 on lowering the PEP concentration.

Both the inhibition of activity at saturating PEP and the activation at non-saturating PEP (0.5 mM PEP) as described in Section 4.3.13 were confirmed by this experiment and it can be seen

Figure 4.3.17.4

EFFECT OF G6P ON THE pH PROFILE OF PYRUVATE KINASE



The figure shows the effect of G6P on the pH profile of pyruvate kinase at both saturating and non-saturating concentrations of PEP. Reaction conditions as specified in Section 4.3.1. except that the reaction buffer was replaced with 0.1 M Bistrispropane/HCl buffer at the pH shown and with PEP and G6P concentrations as indicated. Enzyme concentration : 2.0 μg protein per assay.

that both were pH dependent. At pH 6.4, 2.0 mM G6P caused 5-6 fold activation of the rate at 0.5 mM PEP but only 1-2 fold at pH 9.6.

4.3.18 Some Kinetic Data on the Pyruvate Kinase in a Crude Extract from P.shermanii Cells

To ensure that the kinetic and allosteric properties of the partially purified pyruvate kinase as described were not an artifact of the purification process, a brief study was made of some of the kinetic properties of the enzyme in a crude extract.

The crude extract was obtained as described in the purification scheme including an ultracentrifuge spin of 2 hours at 370000 g_{max}. The extract was almost free of NADH-oxidase activity which was a problem only when determining activities at very low substrate concentrations. In these cases the NADH-oxidase activity was subtracted from the "pyruvate kinase" activity as assayed to give the "real" pyruvate kinase activity.

The relationships studied were those between activity and both PEP and ADP concentrations. The effect of G6P on the PEP relationship was also determined. Table 4.3.18 lists the parameters obtained from these experiments and shows that the data from enzyme in the crude extract were very similar to those for the partially purified enzyme. Although the Hill numbers are slightly lower in the crude extract for both the PEP and ADP interactions the pattern of cooperativity and the effect of G6P is the same in both the partially purified enzyme and the enzyme in a crude extract.

Table 4.3.18 Kinetic data on pyruvate kinase in a crude extract of P.shermanii.

(a) Activity versus PEP concentration

Additions	n_H [b]	$PEP_{0.5V}$ [b] (mM)	V_{max} [c] (units/mg)
No additions	2.0	0.74	0.56
2.0 mM G6P	1.5	0.25	0.49

(b) Activity versus ADP concentration

Additions	n_H [b]	$PEP_{0.5V}$ [b] (mM)	V_{max} [c] (units/mg)
No additions	1.3	0.30	0.60

[a] Reaction conditions as specified in Section 4.3.1. Enzyme concentration: 270 µg protein per assay.

[b] Estimated graphically from Hill plots using V_{max} values determined by the method of Endrenyi et al (1975).

[c] Determined directly from slope and intercept of Endrenyi plot.

4.4 DISCUSSION

The P.shermanii pyruvate kinase, unlike the mammalian and yeast pyruvate kinases does not require monovalent cations for activity. Hess and Haeckel (1967) reported that the activation of the yeast pyruvate kinase by K^+ and NH_4^+ ions was affected by FBP which changed the rate versus ion concentration relationship from a sigmoidal to a hyperbolic one. However, a number of bacterial pyruvate kinases share with the P.shermanii enzyme the lack of a monovalent cation requirement. Benziman (1969) pointed out that the Acetobacter xylinum pyruvate kinase not only was not activated by FBP but also had no requirement for any monovalent cations. It is interesting to note that the pyruvate kinases from Rhodopseudomonas sphaeroides (Schedel et al, 1975), Pseudomonas citronellolis (Chuang and Utter, 1979) and the type II E.coli enzyme (Waygood et al, 1975), all of which have no monovalent cation requirement are also not activated by FBP. The type I pyruvate kinase from E.coli however, which is activated by FBP, does have a monovalent cation requirement for activity (Waygood et al, 1976). The P.shermanii pyruvate kinase provides a further example of the correlation between the absence of FBP activation and the absence of a monovalent cation requirement.

Like all other pyruvate kinases studied the P.shermanii enzyme has an absolute requirement for a divalent cation, Mg^{++} being preferred to Mn^{++}. Under standard assay conditions, with the PEP and ADP concentrations kept constant, the response of enzyme activity to increasing Mg^{++} concentration was highly cooperative, having a Hill coefficient of 3.7.

Both of the substrates PEP and ADP showed homotropic cooperativity which was largely unaffected by the concentration of the other substrate. Generally speaking it is unusual for more than one substrate

of a regulatory enzyme to show sigmoidal binding characteristics. Sanwal (1970) has pointed out that for an allosteric enzyme which utilises a nucleotide (e.g. ADP) as the second substrate it is usually the non-nucleotide substrate which is the "target substrate" i.e. which shows cooperative binding. However pyruvate kinases would appear to provide an exception to this generalisation as there are a number which, like the P.shermanii enzyme, show sigmoidal kinetics for both of the substrates PEP and ADP (Waygood and Sanwal, 1974; Crow and Pritchard, 1976; Seubert and Schoner, 1971). In most of these cases, however, the ADP binding is only cooperative over a limited range of experimental conditions (see Section 4.3.9). While the sigmoidal nature of the ADP saturation curve for the P.shermanii enzyme is much reduced by lowering the pH to 6.0 and the addition of the activator G6P, it is nonetheless cooperative over a wide range of conditions. Thus the P.shermanii pyruvate kinase is an example of a regulatory enzyme with a very high degree of cooperativity of binding of its substrates. The property of cooperativity has the advantage that the affinity of the substrate for the enzyme can be decreased or increased sharply over a narrow, threshold concentration range. This characteristic makes possible a sensitive control system and it appears that the P.shermanii pyruvate kinase is well suited to such control.

The particular compounds which can function as allosteric effectors of the P.shermanii pyruvate kinase, namely G6P, ATP and P_i are commonly found to fulfil this role among bacterial pyruvate kinases. The specificity of G6P as the only activator is however, unusual. Most bacterial pyruvate kinases are activated by a number of different sugar phosphates and/or nucleotide monophosphates (see Table 4.1.2). Streptococcus mutans pyruvate kinase is solely activated by G6P but,

unlike the P.shermanii enzyme, has an absolute requirement for G6P for activity (Yamada and Carlsson, 1975).

G6P is a typical 'K' type allosteric activator (nomenclature of Monod et al, 1965), causing a change from a sigmoidal kinetic response to a more hyperbolic type for the substrates PEP, ADP and Mg^{++}. It activates not by increasing the maximum rate of the reaction but by increasing the binding of the enzyme for its substrates (lowering the $K_{0.5V}$ values). Considering the high degree of cooperativity shown by the substrates of the P.shermanii pyruvate kinase this ability of G6P to lower their $K_{0.5V}$ values would provide a plausible mechanism for regulating the activity of the pyruvate kinase in vivo.

As mentioned, ATP has been found to inhibit most pyruvate kinases and this could be important in relating the control of the enzyme to the energy charge of the cell. Atkinson (1977) considers that the energy charge (ratio of ATP + ½ADP/ATP + ADP + AMP) of the cell is one of the major regulatory inputs into many metabolic pathways, both catabolic and biosynthetic. This is due to the widespread role of the adenine nucleotides as common intermediates of these pathways. As one of the prime functions of glycolysis is to provide ATP for biosynthesis and growth it would be expected that a regulatory, glycolytic enzyme like pyruvate kinase would display a negative response to increasing energy charge.

The role of inorganic phosphate as an inhibitor of a number of bacterial pyruvate kinases is not clear since there is very little information on the factors controlling the in vivo level of this metabolite. Tuominen and Bernlohr (1971) suggest that P_i may serve as an indicator of NADH levels in glycolysing cells. P_i is a substrate

of the glyceraldehyde 3-phosphate dehydrogenase reaction which has been shown to be the rate-limiting step in glycolysis under certain conditions (Lowry et al, 1964). Under conditions of high ATP and NADH, the levels of P_i and triose phosphate might be expected to increase relative to that of 1,3-diphosphoglycerate. They reason that the P_i level in cells might be expected to decrease when the NADH level falls and a new steady-state ratio of triose phosphate to 1,3-diphosphoglycerate is established.

If the P_i level does change significantly in vivo under different metabolic conditions the inhibition of pyruvate kinase by P_i could have a physiological role. Unlike most bacteria the gluconeogenic reaction from pyruvate to PEP is catalysed in P.shermanii by pyruvate, orthophosphate dikinase which uses P_i as a substrate in the formation of PEP. Thus with P_i as a substrate of the gluconeogenic pathway its use as an inhibitor of the glycolytic enzyme pyruvate kinase is physiologically plausible. PP_i caused a slight inhibition of pyruvate kinase activity at a concentration of 2 mM, however its effect could not be investigated at higher concentrations than this for technical reasons (Section 4.3.7). The possible physiological significance of this inhibition is not clear.

An important feature of the allosteric control of the P.shermanii pyruvate kinase is the ability of G6P to overcome the inhibition caused by ATP and P_i at low substrate concentrations. This is accomplished by lowering the $PEP_{0.5V}$ (and possibly $ADP_{0.5V}$) value and thus in the presence of the inhibitors (which raise the $PEP_{0.5V}$ value) the activation caused by G6P is enhanced even further.

It is clear from studies on other pyruvate kinases that pH frequently has a profound effect on the allosteric and regulatory

properties of the enzyme. This was also true for the P.shermanii enzyme. Not only was the degree of cooperativity of the saturation curves for PEP and ADP sensitive to pH (with the Hill number for the PEP saturation greatest at pH 7.0) but the effect of G6P and ATP on the $PEP_{0.5V}$ values was also strongly influenced by pH. G6P completely abolished the cooperativity of the activity versus PEP concentration curve at pH 6.0 (unlike the situation at pH 7.5) and the pH dependence of the G6P activation could be clearly seen by its effect on the pH profile. The pH then may play an important role in the allosteric properties displayed by the P.shermanii pyruvate kinase *in vivo*.

It is clear from the data outlined in this chapter that the regulatory properties of the pyruvate kinase from P.shermanii are consistent with the general properties found for this enzyme in other bacteria (see Section 4.1.2), in that it requires Mg^{++} for activity, displays sigmoidal saturation curves for its substrates, is activated by a sugar phosphate and is inhibited by a nucleotide triphosphate and P_i. However, the specific regulatory properties peculiar to the P.shermanii pyruvate kinase are apparently closely related to the particular biochemical features of this organism.

In P.shermanii G6P appears to act as the "feed-forward" activator of glycolysis and is also well placed to influence the flow of metabolites through the HMP pathway which may operate in P.shermanii (Section 1.2). The concentrations of ATP and P_i required for significant inhibition of the pyruvate kinase activity (1.8 mM ATP and 1.4 mM P_i giving 50% inhibition at 0.5 mM PEP) are comparable to those reported for other bacterial pyruvate kinases (Schedel *et al*, 1975; Ng and Hamilton, 1975) and, at least in the case of ATP, within the range of concentrations found *in vivo* (Cook *et al*, 1976; Chapman and Atkinson, 1977). Inhibition

by ATP and possibly P_i would provide a mechanism by which the pyruvate kinase could be regulated *in vivo* as demonstrated by Wilke and Schlegel (1975) for *Alcaligenes eutrophus*. However when supplied with a glycolytic substrate such as glucose the increased level of G6P would overcome the inhibition by ATP and/or P_i and permit active glycolysis.

The measurement of substrate and effector concentrations *in vivo* is required to substantiate this proposed mechanism for control of pyruvate kinase in *P.shermanii*. This is the subject of the following chapters.

Chapter 5

IN VIVO LEVELS OF METABOLITES IN P.SHERMANII GROWING UNDER DIFFERENT NUTRITIONAL CONDITIONS

5.1 INTRODUCTION

The data presented in the previous chapter provide a plausible mechanism whereby the activity of pyruvate kinase may be controlled so that it would be active under conditions where rapid glycolysis is required (growth on glucose or glycerol) but inactive during growth on substrates such as lactate where carbohydrate synthesis must prevail.

Much of the rest of this thesis is directed towards a study of the levels of metabolites in P.shermanii cells under a variety of conditions in an endeavour to assess the significance of this mechanism in controlling pyruvate kinase activity in vivo. The first part of this chapter describes some preliminary work on resting cell suspensions while subsequent parts deal with metabolite levels in growing cells in batch and continuous cultures.

5.2 COMPARISON OF METABOLITE LEVELS IN RESTING CELL SUSPENSIONS USING ^{14}C-LABELLED CARBON SOURCES

The use of washed resting cell suspensions as a system for studying metabolite levels in P.shermanii was originally investigated for two reasons.

Firstly, the metabolism of a particular carbon source can be studied under fully defined conditions without complications due to cell growth. Furthermore comparisons can be made of the metabolism of a particular carbon source by cells grown on different carbon sources. For example, by comparing the metabolism of glucose by lactate, glucose and glycerol grown cells it should be possible to assess the importance of differences in enzyme levels and uptake systems that may be induced by growth on a particular carbon source.

The second consideration is that there are practical advantages in using washed cell suspensions. Much denser suspensions of cells can be obtained, facilitating the determination of metabolites present at low concentrations. Also very rapid "quenching" of metabolism is possible thus minimising changes in metabolite concentrations which might occur between sampling and killing of cells (these considerations will be dealt with in Section 5.3.1).

A good example of the use of washed cell suspensions to study *in vivo* metabolite levels in relation to the control of pyruvate kinase is provided by the work of Thompson and Thomas (1977) on *Streptococcus lactis*.

However a major assumption in using washed cell suspensions in metabolic studies is that the procedure of removing cells from a growth medium and resuspending them in a simple medium lacking many of the components required for growth does not greatly distort the "normal" metabolism under study. In an organism such as *P.shermanii*, which is quite sensitive to the presence of oxygen, washing and resuspension may bring about irreversible damage to the cell. Schwartz *et al* (1976) studied the effect of aeration on resting cell suspensions of *P.shermanii* and found that the cells remained viable when subjected to oxygen, however propionate production was diminished upon returning the suspension to anaerobic conditions. Thus while the resting cell suspensions were able to ferment glucose to propionate and acetate under anaerobic conditions Schwartz *et al* (1976) could not exclude the possibility that the cells may have been injured by the aerobic washing procedure.

A considerable amount of time was spent in attempting to assess the ability of resting cells of *P.shermanii* to metabolise glucose, glycerol

and lactate. It was found that while all three carbon sources were metabolised with the production of the normal end-products, the rates of metabolism were slower than those in growing cell cultures and the time course curves of substrate utilisation more complex.

Consequently only some preliminary studies on the metabolism of ^{14}C-labelled substrates by resting cells are included in this thesis. This work has been included since it gives strong semiquantitative confirmation of the results presented in later sections on *in vivo* metabolite levels.

In this study suspensions of cells grown on the appropriate carbon source were resuspended in a buffered solution of the same carbon source labelled with ^{14}C. After 2 min the ^{14}C-labelled intermediates were extracted, separated by thin-layer chromatography and the intermediates detected by radioscanning and autoradiography. The 2 min sampling time was arrived at on the basis of earlier time-course studies which showed rapid substrate utilisation for 2 min followed by a much slower rate. This procedure gives a profile of the relative pool sizes of the major intermediates formed during utilisation of the different carbon substrates and assisted in the selection of those intermediates to be determined by subsequent fluorometric analysis.

5.2.1 Preparation of Resting Cell Suspensions

P.shermanii was grown under standard conditions (Section 3.3) in 2.5. l of the defined medium containing lactate, glucose or glycerol as the carbon source. Cells were harvested at the mid-logarithmic phase of growth by centrifugation at 10000 g (10 min, room temperature), resuspended in 10 mM Tricine/NaOH buffer (pH 7.5) which had been degassed, and centrifuged again at 10000 g (5 min, room temperature). The pellet

was again resuspended to a concentration of 10 mg (wet weight)/cm^3 in the degassed 10 mM Tricine/NaOH buffer (pH 7.5) at 30°C and bubbled through with 95% N_2, 5% CO_2 gas mixture to maintain anaerobic conditions.

5.2.2 Labelling and Separation of Glycolytic Intermediates

An aliquot (5.0 cm^3) of each suspension was placed in a 25 cm^3 conical flask (still under 95% N_2, 5% CO_2) for 5 min on a shaking water bath at 30°C. Then [1-^{14}C]lactate (specific activity 0.4 μCi/μmol; 5 mM lactate), or [U-^{14}C]glucose (specific activity 0.8 μCi/μmol; 2.5 mM glucose), or [1-^{14}C]glycerol (specific activity 0.8 μCi/μmol; 2.5 mM glycerol) was injected into the appropriate cell suspension, i.e. ^{14}C-lactate was used for cells grown on lactate, and so on.

After 2 min further shaking (30°C, under 95% N_2, 5% CO_2) the entire contents of each flask were rapidly filtered through a 0.8 μm Millipore filter and the filter quickly placed into 5.0 cm^3 cold 10% trichloroacetic acid solution for 30 min. Extracts were then prepared according to the method of Thompson (1978).

Trichloroacetic acid was extracted by 5 x 5.0 cm^3 washes with water-saturated ether. The extracts were neutralised with solid $KHCO_3$, frozen and freeze-dried.

After freeze-drying the extracts were taken up in 200 μl distilled, deionised water for analysis by thin-layer chromatography on pre-coated polyethyleneimine-cellulose plates (5 x 20 cm) (Macherey-Nagel and Co., Germany). Separation was carried out by the two solvent system of Thompson (1978). Extracts (10 μl) were applied as a thin streak in a line 2 cm from the bottom of the plate. The plate was developed in distilled, deionised water to within 1 cm of the top. After thorough drying the plate was developed in the second solvent (0.5 M LiCl : 2M

formic acid (1:1)) to about 3-4 cm from the first solvent front.

After drying, the radioactive bands were detected initially by scanning on a Packard Series 7200 Radiochromatogram Scanner and more accurately by autoradiography on Kodirex Xray film with 7 days exposure. Identification of radioactive bands was made by co-chromatography using a wide range of standard marker compounds.

One or more of the following markers (using 5-10 nmol of each) were applied and developed as for the extracts: G6P, 6-PG, F6P, ribose 5-P, Ga 3-P, DHAP, 3-PGA, FBP, PEP and glycerol 3-P. The marker compounds were detected after chromatography by dipping the dried layer into a solution containing 0.1 g $FeCl_3 . 6H_2O$, 7 g 5-sulphosalicylic acid, 25 cm^3 water and ethanol to a final volume of 100 cm^3. Phosphorylated compounds appeared as white fluorescent spots against a purple background which faded in about 10 min but which reappeared to assume their original intensity after 24-48 h and were then stable for several weeks.

Autoradiographs and radioscans of extracts prepared with [1-^{14}C]lactate, [U-^{14}C]glucose and [1-^{14}C]glycerol are shown in Figure 5.2.2. The residual ^{14}C-glucose or ^{14}C-glycerol in the extracts was washed to the top of the plate by the first solvent and thus did not interfere with the labelled intermediates. However, in the case of lactate the residual ^{14}C-lactate band was very close to the G6P/F6P band. Most of the residual lactate could be removed by washing the cells on the Millipore filter with a portion (3 cm^3) of cold buffer. By comparing a chromatogram of this extract (with no residual ^{14}C-lactate) and the one shown in Figure 5.2.2 for the ^{14}C-lactate extract the radioactive lactate band could be identified.

Figure 5.2.2

Autoradiographs of ^{14}C-labelled Intermediates

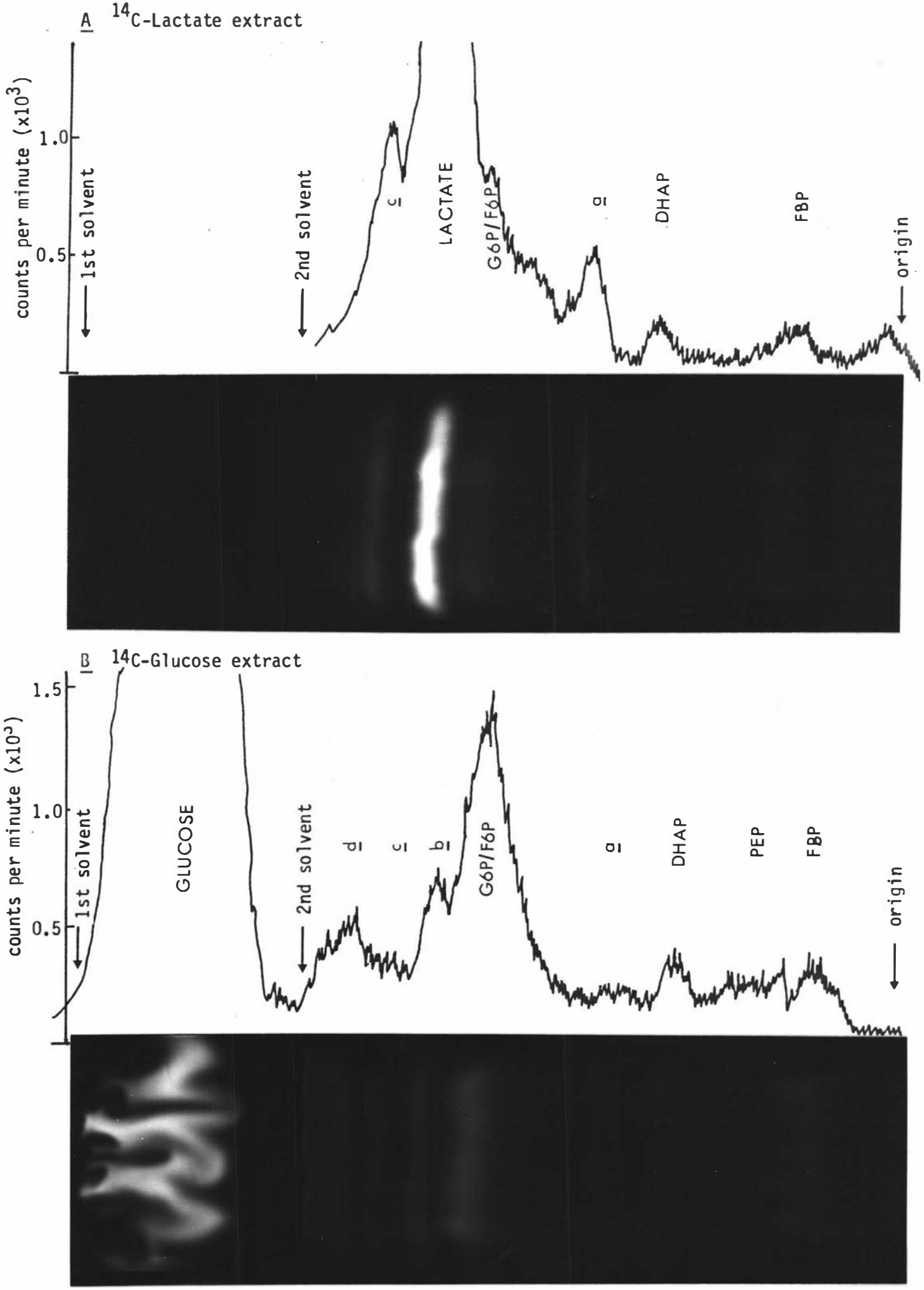
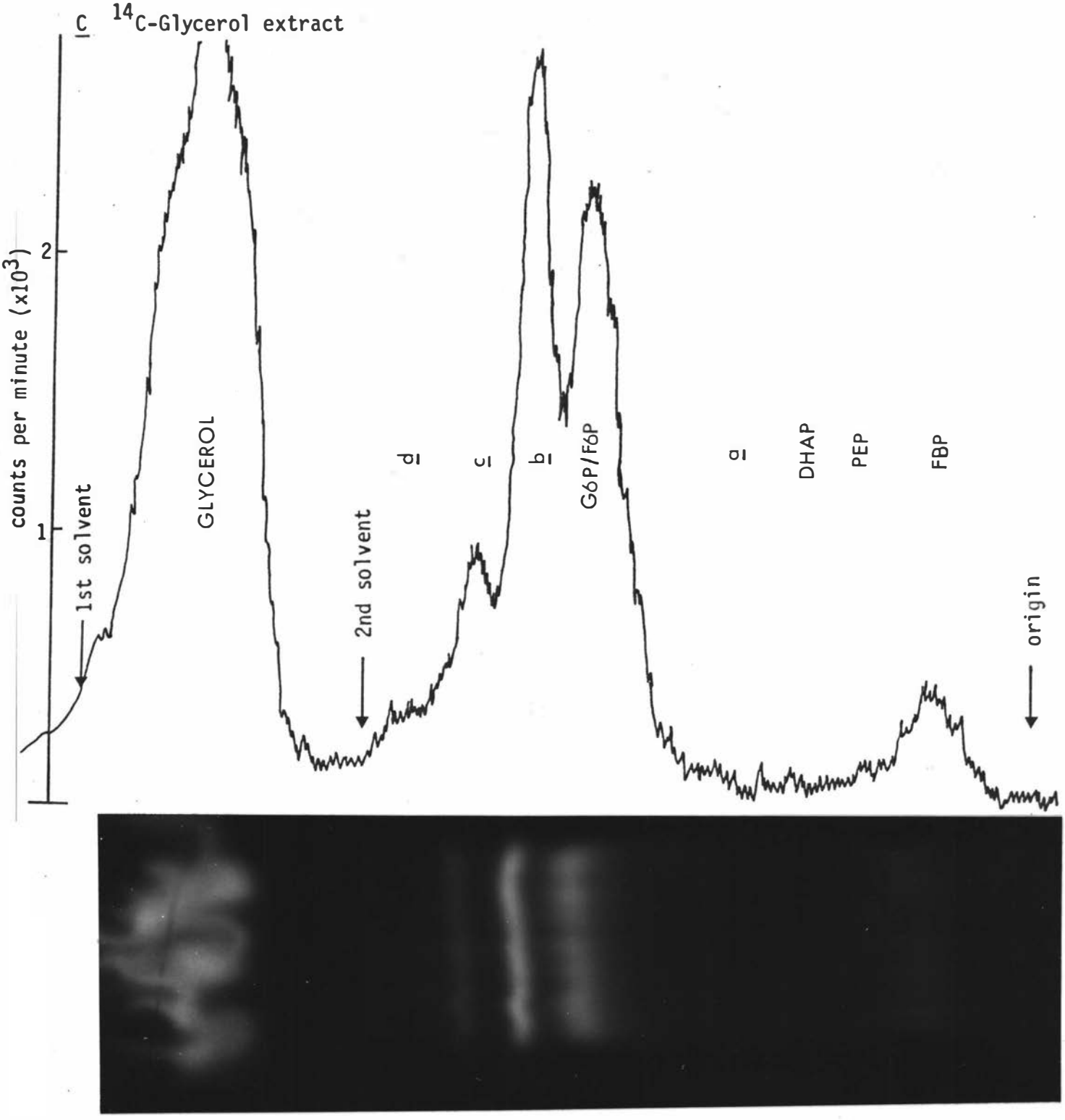


Figure 5.2.2 continued



The figure shows the autoradiographs and radioscans obtained from thin-layer chromatography plates of ^{14}C-labelled intermediates prepared as described in Section 5.2.2. Figures A, B and C represent the chromatographs obtained from extracts labelled with ^{14}C-lactate, ^{14}C-glucose and ^{14}C-glycerol respectively.

The following R_f values were obtained for the markers used: Ga 3-P, 0.90; α-glycerol 3-P, 0.78; G6P, 0.68; F6P, 0.68; ribose 5-P, 0.55; 6-PG, 0.52; DHAP, 0.53; 3-PGA, 0.43; PEP, 0.39; FBP, 0.24. However, the chromatographic procedure was very sensitive to the presence of salts in the labelled extracts which caused large shifts in the R_f values obtained for the markers. Thus identification of radioactive bands was accomplished by running the markers concurrently with the labelled extracts. Radioactive bands identified in the extracts were FBP, which appeared as a faint band in all three extracts but was more prominent in the glycerol extract, traces of PEP and DHAP and, most clearly, a G6P/F6P band (these two compounds were not clearly separated by this procedure).

The main difference between the three autoradiographs was that the G6P/F6P band was much more intense in the glucose and glycerol extracts than in the lactate extract (although its identification here was often made difficult by the labelled lactate band). Several unidentified bands were also present. The band labelled a in Figure 5.2.2 was always present, its position and sharpness being strongly affected by the presence of salts. Bands b, c and d all ran faster than the G6P/F6P markers and had R_f values similar to those of α-glycerophosphate and glyceraldehyde 3-P but none of the bands could be positively identified as either of these by co-chromatography. In the extract from cells metabolising glycerol band b in particular was a major band with a level of radioactivity comparable to that of the G6P/F6P band.

5.3 IN VIVO CONCENTRATIONS OF METABOLITES IN BATCH AND CONTINUOUS CULTURES OF P.SHERMANII

The measurement of metabolite concentrations *in vivo* can provide evidence for the operation of control mechanisms which have been

postulated from *in vitro* studies on isolated enzymes. This is especially so in bacteria where the uniformity of cell type, minimum of compartmentation and the ease with which the cell environment can be controlled offer a simpler system than is the case with differentiated eukaryotic tissues. Methods are now available for the measurement of a wide range of metabolites in bacteria (Lowry *et al*, 1971; Cook *et al*, 1976).

In this section the *in vivo* levels of various metabolites determined during growth of *P.shermanii* on the three susbstrates lactate, glucose and glycerol are reported. The particular metabolites determined were selected on the basis of the data from ^{14}C labelling of intermediates (Section 5.2.2) and from a knowledge of the compounds affecting the activity of pyruvate kinase *in vitro* (Chapter 4) as well as the availability of suitable assay methods. The levels of G6P, F6P, FBP, triose phosphates, PEP, pyruvate, ADP and ATP were determined in both batch and continuous cultures of *P.shermanii*.

In addition to measuring metabolite levels, data were also collected on growth yields, substrate utilisation, product formation and enzyme levels in an attempt to gain some insight into the relative importance of the different metabolic pathways during growth on the three substrates.

This study was carried out using the fully defined medium (Section 3.2) for both batch and continuous cultures. Batch cultures enable the metabolism of the organism to be studied at its maximum growth rate (in mid-logarithmic phase) in the presence of a relatively high level of the carbon source. However, comparison of metabolic rates, enzyme activities and metabolite levels between cultures grown on different carbon sources is complicated by the fact that the maximum growth rates differ on the different carbon sources (Section 3.4).

To overcome this problem the growth of P.shermanii was also studied in continuous cultures on the three substrates. Continuous cultures allow the bacteria to be grown at constant predetermined submaximal rates in which the environment does not change with time and metabolism on different carbon sources can be compared under the same conditions.

5.3.1 Comparison of Extraction Procedures for Analysis of Glycolytic Intermediates

Measurement of internal levels of metabolites in bacteria by enzymatic/fluorometric techniques involves determination of very small amounts of these compounds. In cultures of P.shermanii used in this investigation cell concentrations were usually less than 1.0 mg dry weight/cm^3. In order to obtain a sufficient concentration of cells to enable accurate estimation of the metabolite levels an extraction procedure is required which concentrates the cells at the same time as it quenches cellular reactions and disrupts the cells. This can be conveniently accomplished by filtering a relatively large volume of the culture medium through a fine filter which retains the cells and is then rapidly placed into a smaller volume of the extraction solution. However this procedure involves a delay period of 10-15 sec (while the filter is being transferred to the extraction solution) during which time the cells are not surrounded by culture medium. Thus they are in a temporarily starved situation which may affect the levels of cellular metabolites if their rate of turnover is rapid.

By using washed cell suspensions, containing high cell concentrations, methods for the direct quenching of reactions and disruption of cells can be employed which have no built-in delay period. This can be achieved either by direct injection of perchloric acid into the cell suspension or by the rapid suction of a measured volume of the suspension

into a glass syringe containing the required amount of perchloric acid.

These two types of extraction procedures were compared using a resting cell suspension of P.shermanii. Also the effect of the delay period between sampling and killing on the levels of metabolites was investigated by deliberately increasing the delay period for the filtration method of extraction on cells from a growing culture of P.shermanii.

Cells were grown to mid-logarithmic phase (OD_{540} 1.13) under standard conditions (Section 3.3) using lactate defined medium. To compare the effect of increasing extraction time on metabolite levels a volume of culture containing 17.5 μg dry weight of cells was removed from the fermenter, rapidly filtered through a 0.8 μm Millipore filter and the filter placed at measured time intervals into a beaker containing 5.0 cm^3 0.9 M perchloric acid at room temperature. This was then placed on ice for 20 min before neutralising the perchloric acid by addition of 0.23 g K_2CO_3. The extract was clarified by centrifugation and stored on ice for analysis.

For comparison of the two extraction procedures a resting cell suspension was prepared as in Section 5.2.1 except that the cells were resuspended to a final concentration of 17.5 μg dry weight/5.0 cm^3 in 10 mM Tricine/NaOH buffer (pH 7.5) (degassed), containing 40 mM lactate. Two duplicate flasks of cell suspension were prepared. The contents of one flask were extracted by the filtration method just described (with a 15 sec delay) while the other was extracted by direct injection of concentrated perchloric acid to a final concentration of 0.9 M, to represent a zero sampling time. Preparation of the extract was as for the filtration method.

Table 5.3.1 Effect of extraction procedures on *in vivo* metabolite levels[a].

Extraction Procedure	Metabolite Concentration[b]	
	G6P	ATP
Growing Cells		
Filtration + 15 sec	2.1	2.4
Filtration + 30 sec	2.1	2.1
Filtration + 60 sec	2.6	2.1
Resting Cells		
Filtration + 15 sec	3.5	1.45
Direct injection	4.3	2.1

[a] A comparison between the two extraction procedures and different delay periods before killing the cells was made as described in Section 5.3.1.

[b] G6P and ATP were estimated as described in Section 2.8. Metabolite concentrations are expressed as nmol/µg dry weight of cells.

In both experiments G6P and ATP levels were determined fluorometrically. Results from these two experiments are shown in Table 5.3.1. Increasing the delay period in the filtration procedure from 15 to 60 seconds had very little effect on the levels of both G6P and ATP in the cell extracts. However, comparison of the two extraction procedures using the washed cell suspension showed that, when extracted by the direct injection of acid, the levels of both G6P and ATP found were somewhat higher than when extracted by the filtration method with a 15 sec delay.

Dietzler *et al* (1979) also compared these two types of extraction procedure for the measurement of metabolites in *E.coli* and analysed the extracts for ATP. In their case the cellular levels of ATP obtained by the two methods agreed well for a number of different nutritional conditions.

The filtration method for extraction of cellular metabolites was the procedure used routinely during this investigation due to its convenience for use with growing cultures. However, the above results suggest that values obtained with this procedure may possibly represent slightly underestimated values of the *in vivo* levels.

5.3.2 Batch Culture Studies

5.3.2.1 Preliminary investigation of in vivo concentrations of metabolites

In the course of developing assay systems for the measurement of the internal concentrations of metabolites a preliminary comparison was made of metabolite levels in *P.shermanii* grown on three different carbon sources.

300 cm^3 of the defined medium (Section 3.2) was sterilised in 500 cm^3 conical flasks, maintained at 30°C in a waterbath and continuously sparged with 95% N_2, 5% CO_2 gas mixture. The three flasks were each inoculated with 25 cm^3 of a culture in lactate defined medium. Samples for measurement of metabolites were removed in the mid-logarithmic phase, rapidly filtered (0.8 μm Millipore filter) and extracted in 0.9 M perchloric acid (Section 5.3.1).

A set of data from this type of experiment is shown in Table 5.3.2.1. Several different experiments of this type were carried out and the results were generally in close agreement except for the levels of G6P, which varied greatly in extracts from glucose- and glycerol-grown cells, and of pyruvate which varied greatly in extracts from lactate-grown cells. Generally, however, the level of G6P was much higher in cells grown on glucose or glycerol than it was in lactate-grown cells while the opposite was the case for pyruvate levels.

5.3.2.2 Detailed study of metabolite levels on batch cultures of P.shermanii

A much more detailed study of batch cultures of P.shermanii grown in a fermenter was then carried out. Data were collected on substrate utilisation and product formation in addition to metabolite levels. The culture conditions have been described earlier (Sections 3.2 and 3.3). Two subsequent improvements, the addition of acetate to the glycerol defined medium and the use of a fully deoxygenated N_2/CO_2 gas mixture enabled relatively similar maximum growth rates to be obtained on defined medium containing lactate, glucose and glycerol as carbon source (see Section 3.4).

The results from one such set of experiments are shown in Figure

Table 5.3.2.1 *In vivo* concentration of metabolites in batch cultures.[a]

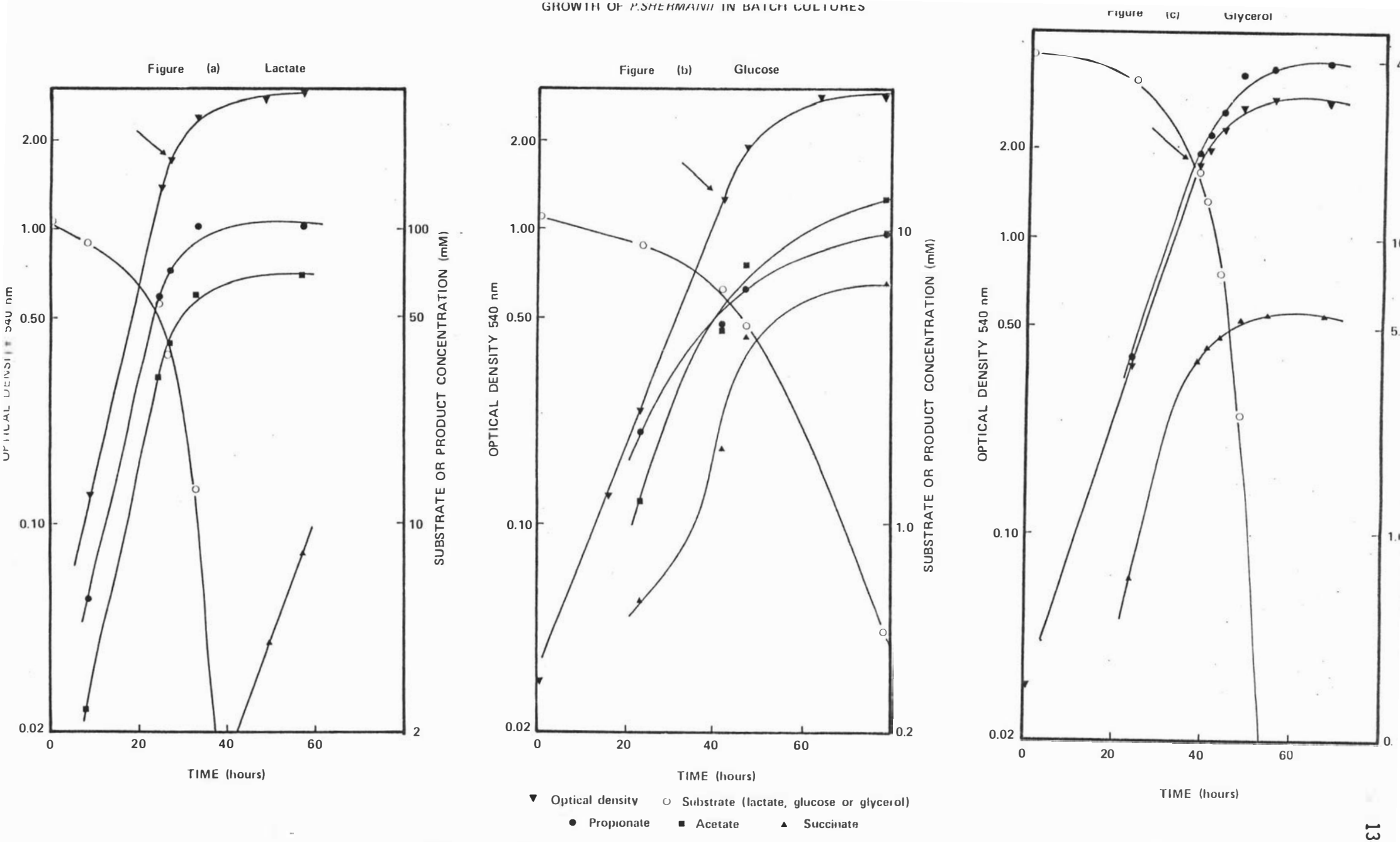
Metabolite[b]	Carbon Source		
	Lactate	Glucose	Glycerol
G6P	3.04 (1.9)	25.6 (16.0)	9.40 (5.9)
F6P	0.64 (0.4)	1.90 (1.2)	1.10 (0.7)
FBP	<0.16 (<0.1)	0.24 (0.15)	0.64 (0.4)
Triose-P	<0.16 (<0.1)	<0.16 (<0.1)	<0.16 (<0.1)
PEP	<1.60 (<1.0)	<0.64 (<0.4)	<0.64 (<0.4)
Pyruvate	150 (94)	5.80 (3.6)	7.80 (4.9)
ADP	<1.60 (<1.0)	2.20 (1.4)	1.80 (1.1)
ATP	1.80 (1.1)	3.50 (2.2)	1.80 (1.1)

a The concentration of metabolites were determined in extracts from batch cultures growing on lactate, glucose and glycerol as described in Section 5.3.2.1.

b Metabolites were determined as described in Section 2.8. Metabolite concentrations are expressed as nmol/µg dry weight of cells. The values in brackets represent their concentration in the cell in mmol/l assuming a value of 1.6 µl cell water per mg dry weight (Section 2.9).

5.3.2.2 and Table 5.3.2.2. As found in Section 3.4 growth was fastest on the lactate defined medium. High carbon recovery values (130-150%) for the lactate-grown culture were found on four separate occasions. No satisfactory explanation for this has been found. One possibility is that some of the propionate and acetate may have been derived from amino acids in the medium as well as from the lactate. Quantitative analysis of the residual amino acids in the medium at the time of sampling (using a Beckman Amino acid Analyser) showed considerable utilisation of aspartic acid (which was present at quite high concentrations in the initial medium) and of other amino acids (present at much lower concentrations). Even assuming complete fermentation of these to propionate and/or acetate the high recovery cannot be fully accounted for. Since the molar growth yield is almost identical to that found by Bauchop and Elsden (1960) and by de Vries et al (1973) for propionibacteria growing on defined media it is unlikely that the lactate estimations are in error. The possibility of net CO_2 fixation (equivalent to the succinate produced) has been taken into account in calculating carbon recoveries. Finally carbon recoveries close to 100% were found in continuous culture experiments with lactate as the limiting substrate. The high carbon recoveries in batch cultures on lactate remain an unsolved problem. Carbon recoveries when growing on glucose or glycerol defined media were always much nearer to 100%.

Molar growth yields differed considerably for the different carbon sources. The values obtained for the glycerol and lactate cultures (24 and 7.5 respectively) agree well with values reported by de Vries et al (1973) for the closely related species P.freudenreichii growing on the same substrates in a semi-defined medium (24.1 for glycerol and 8.1 for lactate). However, the molar growth yield of 94 for growth



The figures show the growth, substrate utilisation and product formation of *P.shermanii* in batch cultures on the defined medium. Figure (a) shows the growth on lactate, figure (b) the growth on glucose and figure (c) the growth on glycerol. In each figure the arrow indicates the time of sampling for measurement of substrates, products and metabolites as listed in Table 5.3.2.2.

a Batch cultures of P.shermanii on the defined medium with either lactate, glucose or glycerol as carbon source were prepared as described in Section 5.3.2.2. All data in this table were obtained from samples taken at the times indicated in Figure 5.3.2.2.

b Acetate was present at a level of 2 g/l in the glycerol culture and its concentration did not deviate from this during growth.

c Carbon recoveries were calculated assuming one mole of CO_2 is fixed for each mole of succinate produced and one mole of CO_2 is released for each mole of acetate produced.

d Metabolites were determined as described in Section 2.8. Metabolite concentrations are expressed as nmol/µg dry weight. The values in brackets represent their concentration in the cell, assuming a value of 1.6 µl cell water per mg dry weight (Section 2.9), in mmol/l. Values given are the average of two separate determinations which agreed $\pm$ 10%.

N.D. Not determined. Accurate estimation of PEP and ADP levels were not possible in the presence of high levels of pyruvate.

Table 5.3.2.2 Batch cultures of P.shermanii on lactate, glucose and glycerol defined media.[a]

Carbon Source	Lactate	Glucose	Glycerol
Initial Substrate (mM)	120	11	42
Residual Substrate at Sampling (mM)	41	6.3	16.8
Maximum Growth Rate (μ)	0.125 h^{-1}	0.092 h^{-1}	0.11 h^{-1}
Products at Time of Sampling (mM)			
Propionate	72	4.7	19
Acetate	42	4.6	-[b]
Succinate	1	1.8	3.8
Carbon Recovery[c]	140%	111%	94%
Cell Dry Weight (g/l)	0.60	0.44	0.61
Molar Growth Yield (g/mol)	7.6	94	24
In vivo Levels of Metabolites[d]			
G6P	2.65 (1.65)	16.2 (10.1)	13.75 (8.6)
F6P	0.72 (0.45)	0.85 (0.53)	4.0 (2.5)
FBP	<0.16 (<0.1)	<0.16 (<0.1)	0.98 (0.61)
Triose-P	0.37 (0.23)	0.45 (0.28)	0.44 (0.27)
PEP	N.D.	1.00 (0.61)	<0.16 (<0.1)
Pyruvate	109 (68)	4.2 (2.6)	6.3 (4.0)
ADP	N.D.	2.4 (1.55)	1.45 (0.90)
ATP	2.2 (1.4)	2.1 (1.3)	1.7 (1.05)

on glucose found here was much higher than the corresponding value found by de Vries et al (1973) for P.freudenreichii (65 g dry weight/ mol glucose). The high molar growth yield on glucose was also found in continuous cultures (Table 5.3.3.1).

The proportions of fermentation end-products also differed for the different carbon sources. For cultures grown on lactate the proportions of products at mid-logarithmic phase of growth were 63% propionate, 36% acetate and 1% succinate, on glucose the proportions were 42% propionate, 41% acetate and 17% succinate and on glycerol, 83% propionate and 17% succinate (compare with Table 3.4). Thus a considerably higher proportion of succinate was produced during growth on glucose and glycerol than on lactate.

Internal concentrations of the metabolites found in these cultures were similar to values found in the preliminary investigation (Section 5.3.2.1). G6P was much higher in cells grown on glucose and glycerol than in lactate-grown cells, while the reverse was the case for the pyruvate levels.

There were also distinct differences in the concentrations of several of the other metabolites determined, depending on the carbon source. In glycerol-grown cells both F6P and FBP were present at higher levels than in cells grown on glucose or lactate. Another difference was in the level of PEP which was present at the 1.0 nmol/µg level in glucose-grown cells but was less than 0.16 nmol/µg in glycerol-grown cells. However the level of PEP was very difficult to determine accurately when pyruvate was present (as were the ADP levels) so the significance that can be attached to these values is doubtful. In the lactate cultures the level of pyruvate was too high to enable any reasonable determination of the much lower levels of ADP or PEP.

5.3.3 Continuous Cultures

Continuous cultures of P.shermanii were grown in the 3 l fermenter using the same media, conditions and controls as described for batch cultures (see Sections 3.3 and 5.3.2.2). The culture volume was maintained at 2.25 l. The inlet was connected aseptically to a reservoir for fresh medium and the outlet port to a 30 l sterile vessel to collect the overflow culture. During continuous flow the fresh medium was pumped into the fermenter using a peristaltic pump and displaced an equal volume out the overflow port.

The medium was inoculated with 25 cm^3 of a starter culture grown on lactate defined medium and the culture allowed to grow to late logarithmic phase before the continuous flow was started. A period of continuous flow equal to five times the mean residence time or turnover time (i.e. approximately seven generation times) was allowed for establishment of the steady-state before samples were taken for measurement of internal metabolite concentrations, enzyme activities and substrate/product carbon balances.

Regular monitoring of the OD_{540} and substrate and product concentrations in the medium were carried out throughout the experiments to ensure that the steady-states had been attained.

5.3.3.1 Carbon balances during continuous growth on lactate, glucose and glycerol defined media

Continuous cultures were set up on the three carbon sources as described and steady-states achieved at two different dilution rates for each substrate. The low dilution rate chosen (0.04 h^{-1}) was well below the maximum growth rate on all three carbon sources. At this

dilution rate growth would be carbon-limited and a comparison of metabolite and enzyme levels between cultures on the three different carbon sources at the same growth rate could be made. The values for the higher dilution rate were set as close as possible to the maximum growth rate in batch culture but sufficiently below the wash-out rate so that a steady-state could be achieved. The object of this was to try to obtain a substrate-excess state so that metabolite and enzyme levels for a given carbon source could be compared under both substrate-limited and substrate-excess conditions. Because the maximum growth rates on glucose and glycerol were lower than on lactate the values selected for the higher dilution rate were not the same for each carbon source.

Results from these experiments are shown in this and the following two sections. In addition to the experiment described a preliminary continuous culture experiment was carried out on each carbon source and data obtained were consistent with those described here.

Table 5.3.3.1 shows the carbon balances and growth yields for these experiments. At the low dilution rate (0.04 h^{-1}) only negligible amounts of each substrate remained in the medium as expected. For the lactate and glycerol cultures at the high dilution rates significant levels of the substrates remained in the medium indicating that the dilution rates chosen were indeed close to the maximum for these substrates. However there was very little glucose remaining in the medium for the glucose culture at the high dilution rate (0.09 h^{-1}) suggesting that the maximum growth rate in continuous culture on this substrate could be higher than the 0.092 h^{-1} attained in batch culture (Table 5.3.2.2).

Table 5.3.3.1 Carbon balances in *P.shermanii* continuous cultures.[a]

Carbon Source	Lactate		Glucose		Glycerol	
Dilution Rate (h^{-1})	0.04	0.11	0.04	0.09	0.04	0.09
Initial Substrate (mM)	120	120	11	11	42	42
Residual Substrate (mM)	-	27	0.14	0.11	0.1	8.2
Products (mM)						
Propionate	71	53	7.5	7.0	41.5	26
Acetate	48	30	8.7	10.9	-[b]	-[b]
Succinate	5.0	1.5	5.5	6.2	5.9	4.6
Carbon Recovery[c] (%)	103	91	99	109	111	92
Cell Dry Weight (g/l)	1.1	0.71	1.1	0.90	1.23	0.89
Molar Growth Yield (g/mol)	9.2	7.7	99	81	29.2	26

a Continuous cultures were prepared on the defined medium with each of the three carbon sources as described in Section 5.3.3.1. Five complete turnovers of the fermenter volume were allowed at each dilution rate before sampling of the steady-state culture.

b Acetate was present at 2 g/l in the glycerol culture and its concentration did not deviate from this at any stage.

c Carbon recoveries were calculated on the same basis as those in Table 5.3.2.2.

The proportions of end-products formed in the lactate-grown culture were 57% propionate, 39% acetate and 4% succinate at the low dilution rate and 63% propionate, 36% acetate and 1% succinate at the high dilution rate. These were very similar to the proportions found in the mid-logarithmic phase of growth for batch cultures on lactate (Table 5.3.2.2).

For the glucose-grown culture the values were 34% propionate, 40% acetate and 25% succinate at the low dilution rate and 29% propionate, 45% acetate and 26% succinate at the high dilution rate. Here there was a significant difference from the batch culture values (Table 5.3.2.2). The proportion of propionate produced fell from 42% in the batch culture (mid-logarithmic phase) to 34% and 29% at the low and high dilution rates respectively in the continuous culture. This decrease in the proportion of propionate was largely balanced by a corresponding increase in succinate production from 17% in the batch culture to 26% in the continuous culture. Acetate production accounted for 40% of the products in the batch and low dilution rate continuous culture but increased slightly to 45% in the high dilution rate culture.

In the glycerol continuous culture the proportions of products were 88% propionate and 12% succinate at the low dilution rate and 85% propionate, 15% succinate at the high dilution rate, i.e. similar to the values obtained in the glycerol batch culture (Table 5.3.2.2). At no stage did the level of acetate rise above that supplied in the defined medium.

Carbon recovery values ranged from 91-111% for the different steady-states on the three carbon sources. This differed from the batch

cultures where recovery values as high as 140% were found for growth on lactate (Table 5.3.2.2). No explanation for this difference was found.

The relative molar growth yields on the different carbon sources were similar to those found for the batch cultures (Table 5.3.2.2) and to values reported in the literature (de Vries et al, 1973) except that the values obtained for growth on glucose were very high.

One surprising feature of the molar growth yield value is that for all three substrates the value is lower at the faster growth rate, whereas the reverse would be expected (Pirt, 1965). An investigation of this anomaly was not attempted since the molar growth yield values were not being used to indicate the operation of particular metabolic pathways. The values obtained at the high growth rate are close to those reported by de Vries et al (1973) so the high apparent value found at the low growth rate may be due to a high contribution from storage material or extracellular polysaccharide to the dry weight.

5.3.3.2 In vivo concentrations of metabolites in continuous cultures

Duplicate samples were removed from the continuous cultures at the two different steady-states described in Section 5.3.3.1 and cell-free extracts were prepared by the filtration method (Section 5.3.1) for measurement of metabolite levels.

These extracts were analysed (within 8 hours) for glycolytic intermediates according to the procedures described in Section 2.8 and the results are shown in Table 5.3.3.2.

Table 5.3.3.2 *In vivo* levels of metabolites in continuous cultures[a].

Carbon Source	Low dilution rate steady-states			High dilution rate steady-states		
	Lactate	Glucose	Glycerol	Lactate	Glucose	Glycerol
Dilution Rate (h^{-1})	0.04	0.04	0.04	0.11	0.09	0.09
Residual Substrate (mM)	0.50	0.14	0.10	27	0.11	8.2
Metabolites[b]						
G6P	1.95 (1.2)	3.15 (2.0)	4.30 (2.7)	1.70 (1.05)	3.30 (2.05)	12.1 (7.55)
F6P	0.50 (0.31)	0.92 (0.60)	0.95 (0.60)	0.61 (0.38)	0.91 (0.57)	2.65 (1.65)
FBP	< 0.16 (<0.1)	0.29 (0.18)	0.27 (0.17)	0.29 (0.18)	0.20 (0.13)	1.25 (0.81)
Triose-P	0.39 (0.24)	0.55 (0.34)	< 0.16 (<0.1)	0.36 (0.22)	0.48 (0.30)	0.94 (0.59)
PEP	N.D.	1.40 (0.85)	2.10 (1.3)	N.D.	2.20 (1.40)	< 0.20 (<0.12)
Pyruvate	9.80 (6.1)	2.35 (1.5)	5.35 (3.35)	41 (25.5)	2.40 (1.50)	8.45 (4.50)
ADP	N.D.	1.50 (0.90)	1.10 (0.70)	N.D.	2.80 (1.75)	1.60 (1.00)
ATP	1.70 (1.1)	2.35 (1.5)	2.20 (1.4)	1.55 (0.98)	1.65 (1.02)	1.80 (1.10)

a Continuous cultures were prepared as for Table 5.3.3.1.

b Metabolites were determined as described in Section 2.8. Metabolite concentrations are expressed as nmol/µg dry weight. The values in brackets represent their concentration in the cell in mmol/l assuming a value of 1.6 µl cell water per mg dry weight (Section 2.9). Values given are the average of two separate determinations which agreed ± 10%.

N.D. Not determined (see Table 5.3.2.2).

At the low dilution rate differences in the metabolite levels between the cultures grown on the three carbon sources (Table 5.3.3.2(a)) were only minor. G6P varied from 1.95 nmol/µg on lactate to 4.3 nmol/µg on glycerol while differences in the levels of F6P, ATP, triose phosphates, FBP, ADP and PEP were all relatively small. Pyruvate was present at 9.8 nmol/µg in the lactate-grown cells but was only 2.35 and 5.35 nmol/µg in the glucose- and glycerol-grown cells respectively.

At the high dilution rates (Table 5.3.3.2(b)) differences between the metabolite levels were more pronounced. G6P was present at levels similar to those in the slow-growing cultures in both lactate- and glucose-grown cells but in glycerol-grown cells the level of G6P (12 nmol/µg) at the high growth rate was much higher than that (4.3 nmol/µg) at the low growth rate. Levels of F6P, FBP and the triose phosphates were also elevated in the glycerol-grown cells at the high dilution rate compared to both the lactate and glucose cultures. The level of PEP, however, was much lower in the glycerol-grown cells than in the glucose-grown cells. Pyruvate was considerably higher in the lactate-grown cells than in either the glycerol- or glucose-grown cells while ATP and ADP (where measured) were present at fairly similar levels for all three growth substrates.

Comparison of metabolite levels for each carbon source at the two different dilution rates suggests that the increased growth rate *per se* has very little effect on metabolite levels. For example the metabolite levels in the lactate- and glucose-grown cells are very similar at the two dilution rates (except for pyruvate which was much higher in the lactate-grown cells at the high dilution rate).

The level of substrate in the medium appears to be of much greater

significance in determining the metabolite levels. In the case of the glycerol culture where the growth rate had been increased to a point where considerable residual substrate was present in the medium the levels of G6P, F6P, FBP and the triose phosphates were all significantly higher than in the carbon-limited culture at low growth rate. In the lactate-grown culture, pyruvate is the only metabolite significantly increased under substrate excess conditions. In both of these cases the levels of metabolites approximate to those found in batch cultures (Table 5.3.2.2) at mid-logarithmic phase with high external substrate concentrations. With the glucose culture growth was apparently still glucose-limited at the high growth rate (i.e. there was virtually no residual substrate) and consequently G6P was present at much lower levels than in the batch culture with a high residual glucose concentration in the medium.

5.3.3.3 Specific activities of selected enzymes in continuous cultures.

After sampling of the continuous culture steady-state levels for analysis of substrates, products and metabolites further culture samples were removed for measurement of enzyme specific activities according to the following procedure.

A sample of culture (about 200 cm^3) was removed, rapidly centrifuged at 10000 g for 10 min at 4^oC and stored frozen. The frozen pellet was thawed, resuspended in a minimum volume of 10 mM Tricine/NaOH buffer (pH 7.5) containing 10% glycerol and disrupted by two passages through an Aminco French pressure cell (38000 kN/m^2). Cell debris was removed by centrifugation at 27000 g for 15 min (4^oC) and the supernatant further centrifuged at 225000 g_{max} for 2 h (4^oC)

to remove fine particulate material. The supernatant (25 cm^3) was dialysed for two successive 2 h periods against 2 l volumes of 10 mM Tricine/NaOH buffer (pH 7.5) containing 20% glycerol. This crude extract was stored frozen overnight for analysis the following day.

The selected enzymes were assayed using 100 µl of the crude extract (or a suitable dilution of it) according to the procedures in Section 2.7. The results are shown in Table 5.3.3.3.

The values obtained here cannot be compared directly with those listed in Table 3.5 since the crude extracts obtained from the continuous cultures were dialysed and stored overnight before analysis. Results in Table 3.5 were obtained by analysis of crude extracts on the same day as breakage of the cells and without any dialysis of the extract, so the values could be affected by the presence of any effectors of the enzymes assayed in the crude extract. Also the specific activity values presented in Table 3.5 were based on the Bradford protein assay while those in Table 5.3.3.3 were based on the Folin method and this may have caused some differences (see Section 2.6).

However, the relative values of the enzyme levels found here from cells grown on the three carbon sources are very similar to those reported in Section 3.5. Thus, except for the G6P dehydrogenase value, the enzyme levels in the lactate- and glucose-grown cells at both dilution rates were very similar. However in the glycerol-grown cells there were marked differences as was found in Section 3.5. Glycerol-grown cells had an increased level of pyruvate kinase, PEP:carboxytransphosphorylase and malate dehydrogenase and slightly lower levels of pyruvate, orthophosphate dikinase compared to cells from lactate and glucose cultures.

Inorganic pyrophosphatase activity was determined in crude extracts

Table 5.3.3.3 Enzyme levels in continuous cultures[a].

Carbon Source	Lactate		Glucose		Glycerol[b]
Dilution Rate (h^{-1})	0.04	0.11	0.04	0.09	0.04
Enzymes[c]					
Pyruvate kinase	0.20	0.14	0.15	0.13	0.36
PEP:carboxytransphosphorylase	0.065	0.048	0.069	0.061	0.20
Pyruvate, orthophosphate dikinase	0.083	0.041	0.078	0.051	0.046
PP_i-dependent phosphofructokinase					
FBP formation	0.12	0.092	0.15	0.14	0.20
Aldolase	0.34	0.27	0.40	0.37	0.44
G6P dehydrogenase	0.009	0.006	0.013	0.01	0.007
Malate dehydrogenase	42	29	33	21	155
Inorganic pyrophosphatase[d]	0.25		0.21		0.09

a Continuous cultures were prepared as for Table 5.3.3.1. Samples for analysis of enzyme activity were prepared as described in Section 5.3.3.3.

b Enzyme activities were not determined at the high dilution rate for the glycerol culture due to an error in sample collection.

c Enzyme activity was determined as described in Section 2.7 and expressed as units/mg. Protein concentration was estimated by the method of Lowry et al (1951).

d Inorganic pyrophosphatase activity was determined on a crude extract prepared from cells in the overflow carboy of each culture by the procedure outlined in Section 5.3.3.3.

prepared from cells in the overflow vessel of the three cultures according to the method of Mansurova et al (1977). As found by Wood (1977), the enzyme is present in P.shermanii and appears to be constitutive in cells grown on the three carbon sources lactate, glucose and glycerol.

5.4 DISCUSSION

The primary objective of the experiments described in this chapter was to obtain information on the in vivo levels of those metabolites which are involved as substrates or effectors of pyruvate kinase in order to assess the validity of the model proposed for the regulation of this enzyme in Chapter 4. However, in the course of these experiments, determinations of substrate and product concentrations, enzyme activities and levels of some other intermediary metabolites were also included to obtain a more general metabolic description of P.shermanii when grown under different nutritional conditions.

The following paragraphs summarise the main findings that emerged from these experiments. A fuller discussion of the significance and interpretation of these results will be postponed until Chapter 8.

Metabolite Levels

In Table 5.4 the data on the levels of G6P, ATP, PEP and ADP (effectors and substrates of pyruvate kinase) from both batch and continuous cultures are brought together (from Tables 5.3.2.1, 5.3.2.2 and 5.3.3.2).

It proved difficult to obtain an accurate measurement of the levels of the substrates PEP and ADP under those conditions where high levels of pyruvate were present. ADP levels were mostly between 1-2 mM and did not differ greatly on the different carbon sources. At high substrate

Table 5.4 Summary of data on *in vivo* levels of metabolites in *P.shermanii*.[a]

	Metabolite Levels			
	G6P	ATP	PEP	ADP
Lactate				
Continuous culture				
(a) Slow growth rate (μ = 0.04 h^{-1}) Low external lactate (0.5 mM)	1.2	1.1	N.D.	N.D.
(b) Fast growth rate (μ = 0.11 h^{-1}) High external lactate (27 mM)	1.05	1.0	N.D.	N.D.
Batch culture				
Fast growth rate High external lactate	1.65,1.9	1.4,1.1	1.0	1.0
Glucose				
Continuous culture				
(a) Slow growth rate (μ = 0.04 h^{-1}) Low external glucose (0.14 mM)	2.0	1.5	0.85	0.90
(b) Fast growth rate (μ = 0.09 h^{-1}) Low external glucose (0.11 mM)	2.05	1.0	1.40	1.75
Batch Culture				
Fast growth rate High external glucose	16,10.1	2.2,1.3	0.40,0.61	1.40,1.55
Glycerol				
Continuous culture				
(a) Slow growth rate (μ = 0.04 h^{-1}) Low external glycerol (0.1 mM)	2.7	1.4	1.3	0.70
(b) Fast growth rate (μ = 0.09 h^{-1}) High external glycerol (8.2 mM)	7.6	1.1	0.12	1.0
Batch culture				
Fast growth rate High external glycerol	5.9,8.6	1.1,1.05	0.40, 0.16	1.1,0.90

levels the concentration of PEP was always less than 1 mM (and mostly less than 0.5 mM), i.e. below the $PEP_{0.5V}$ value of 1 mM for pyruvate kinase (Section 4.3.9.1). However at low glucose or glycerol concentrations the PEP level (which could be determined relatively accurately under these conditions) varied from 0.85 - 1.4 mM.

The most pronounced changes were in the level of the activator G6P. During growth on lactate the level of this metabolite was maintained at between 1-2 mM regardless of growth rate and concentration of external lactate. However, during growth on glucose or glycerol at high substrate concentrations the level of G6P was much higher (from 6-16 mM) while at low substrate concentrations it was only 2-3 mM. These fluorometric estimations of G6P levels are confirmed by the radiochemical data obtained from washed cell suspensions.

The levels found for the inhibitor ATP are nearly all in the range 1.0-1.5 mM, except for one high value obtained during batch growth on glucose.

The significance of these in vivo levels in relation to the control of pyruvate kinase will be considered in Chapter 8. However it is clear even from a superficial examination of the data that the high G6P levels prevailing at high glucose and glycerol concentrations would result in high pyruvate kinase activity and consequently, active glycolysis. Interpretation of two other aspects of the data however, is less clear.

Firstly, would the levels of effectors found in cells during gluconeogenesis on lactate be sufficient to inhibit pyruvate kinase activity and so prevent substrate cycling? This cannot be answered without information on the level of the other main effector of pyruvate kinase activity, P_i. Several multi-enzyme fluorometric assays have been reported

for the determination of very small quantities of P_i but were not used in this investigation due to their complexity, cost and difficult interpretation. Cellular disruption and extraction procedures (usually involving strongly acid conditions) would release bound and organic phosphate from the cell into the cell extract. This would elevate the levels of P_i subsequently determined in the extract and make estimation of the free P_i in the cell impossible. Other approaches to this problem will be considered in later chapters.

Secondly, in the continuous culture experiment, the high dilution rate glucose culture is presumably actively metabolising glucose via the glycolytic pathway yet the level of G6P is similar to that found at the low dilution rate and in the lactate continuous culture. A high rate of glycolysis is thus not necessarily dependent on a high G6P level. The G6P level was elevated only in cells growing in the presence of a high concentration of external glucose. Chapter 6 will present further information on the relationship between substrate concentration and G6P levels.

In addition to the metabolites considered above two other correlations between metabolite levels and carbon sources emerged from these experiments. There is apparently a close relationship between external lactate concentration and cellular pyruvate level. The results of a further investigation of this relationship will be presented in the next chapter. The other correlation concerns the levels of F6P and FBP. These two metabolites are found at levels below 1 mM for F6P and below 0.2 mM for FBP except in cells growing on glycerol where, under conditions of high external substrate concentration they are both present at significantly higher levels.

Attempts were made to determine the level of PP_i in the cell under the different growth conditions by the method of Drake et al (1979).

However, although indications were that the PP_i level in the cells was of the order of 1 mM, reliable results could not be obtained due to the instability of the adenylyl sulphate (which is required in the assay) on transportation from the United States to New Zealand. Klemme (1976) reports that the intracellular level of PP_i in a number of microbes is in the range 0.1-1.0 mM.

End-products of Fermentation

Propionate, acetate and succinate were the major end-products of the fermentation on the carbon sources lactate, glucose and glycerol when growing on the fully defined medium used in this investigation. The ratio of propionate to acetate during growth on lactate was 1.5-1.8 while on glucose it was 0.6-1.0 (Tables 5.3.2.2 and 5.3.3.1). These values, especially on glucose, were lower than those found for propionibacteria growing in complex medium (Hettinga and Reinbold, 1972b). de Vries *et al* (1973) obtained values of 2.0 and 2.1 for the propionate:acetate ratio during growth of *P.freudenreichii* on lactate and glucose in their synthetic medium. The low values obtained in this investigation can be partially explained by the 95% N_2, 5% CO_2 gas mixture used to keep the cultures anaerobic. The 5% CO_2 was included to enable growth of *P.shermanii* on glycerol as pointed out by Wood and Werkman (1936) and, to maintain standard conditions, it was also used during growth on the other two substrates. Under these conditions the yield of succinate is increased (Wood, 1961) via the CO_2-fixing carboxytransphosphorylase reaction (O'Brien and Wood, 1974). Thus the use of 5% CO_2 to sparge the cultures appears to have caused a shift in the flow of metabolites, during growth on glucose in particular, away from propionate production to succinate production causing the low propionate:acetate ratios found. During growth

on glycerol, acetate was not produced, as has been found by other workers (Hettinga and Reinbold, 1972b), and again a significant proportion of succinate was produced (Tables 5.3.2.2 and 5.3.3.1). The significance of succinate production in relation to the regulation of pyruvate kinase will be considered in Chapter 8.

Enzyme Activities

Regulation of carbohydrate metabolism by induction or repression of enzyme synthesis does not appear to be of major importance in P.shermanii since the levels of almost all the enzymes determined are very similar under glycolytic (growth on glucose) and gluconeogenic (growth on lactate) conditions. The approximately 2-fold increase in the level of pyruvate kinase and the approximately 3-fold increase in the level of carboxytransphosphorylase found in glycerol-grown cells do, however, appear to be consistently reproducible findings. The somewhat lower level of the pyruvate, orthophosphate dikinase on glycerol is also consistent and has been reported by Wood et al (1977).

Inorganic pyrophosphatase was present at similar levels in cells grown on all three carbon sources. The presence of this enzyme raises important problems in connection with the maintenance of adequate PP_i levels in the cells to sustain the activity of the PP_i-dependent phosphofructokinase. As Wood (1977) and Klemme (1976) point out the activity of pyrophosphatase must be regulated in vivo.

Chapter 6

THE ROLE OF G6P IN THE REGULATION OF METABOLISM OF P.SHERMANII

6.1 INTRODUCTION

The results described in the two previous chapters suggest that the level of G6P in the cell is an important regulator of the activity of pyruvate kinase *in vivo*. G6P was shown to be a specific activator of the purified enzyme and its concentration in the cell is markedly affected by the nature and concentration of the carbon source supplied, being elevated under those conditions where active glycolysis would be expected to be occurring.

In an attempt to relate the data on *in vivo* levels of G6P more closely to the *in vitro* data on the effect of G6P on pyruvate kinase activity two further investigations were carried out. Firstly, the cellular concentration of G6P was determined at various times throughout growth of batch cultures on glucose and glycerol in order to define more precisely the range of concentrations of G6P found in the cell and its relationship to external substrate concentration. Using this information and the data on levels of substrates and other effectors described in Chapter 5 a brief investigation of the regulation of pyruvate kinase using a partially purified preparation was carried out. The results of these two studies are described in this chapter.

6.2 RELATIONSHIPS BETWEEN EXTERNAL CARBON SOURCE CONCENTRATION AND G6P OR PYRUVATE LEVELS

6.2.1 Relationship between External Glucose Concentration and Internal G6P Concentration

Samples were taken throughout the log and early stationary phases

of growth from a batch culture of P.shermanii grown on the glucose defined medium as described in Section 5.3.2.2. Measurements were made of both the internal G6P concentration and the external glucose concentration.

Data from this experiment are shown in Table 6.2.1 while the relationship between the concentrations of external glucose and internal G6P is shown directly in Figure 6.2. As the glucose concentration in the medium declined so did the internal G6P concentration, but in a biphasic manner. At an external glucose concentration of 6.4 mM the internal G6P level was 24 nmol/µg, but when the glucose concentration had dropped to 0.71 mM G6P was still maintained at a relatively high level of 11.8 n mol/µg. However, as the external glucose concentration dropped further to 0.51 mM the internal G6P concentration dropped abruptly to 3.0 nmol/µg. This change occurred, as the culture was entering stationary phase, over a relatively short interval of time (4.5 h). As the culture continued into stationary phase the G6P level showed only a comparatively small decrease to 2.2 nmol/µg after a further 21 hours.

Thus there seems to be a certain threshold level of external glucose (about 0.5 - 1.0 mM) above which the internal G6P concentration is at a high level (greater than 11 nmol/µg) and below which it is maintained at about 2-3 nmol/µg.

6.2.2 Relationship between External Glycerol Concentration and Internal G6P Concentration

G6P concentration was similarly determined and related to external substrate concentration in a culture grown on the defined medium with glycerol as the carbon source. For some samples F6P was also determined.

Data from this experiment are shown in Table 6.2.2 while the relationship between external glycerol and internal G6P is shown directly

Table 6.2.1 Relationship between external glucose concentration and internal G6P concentration.[a]

Time (h) (After inoculation)	OD_{540}	External Glucose (mM)	Internal G6P (nmol/µg)
30.0	1.07	6.4	24.0 (15.0)
31.0	1.24	4.3	15.0 (9.4)
33.5	1.50	3.2	15.8 (9.9)
36.0	1.87	2.3	13.9 (8.7)
39.0	2.32	0.71	11.8 (7.4)
43.5	2.33	0.51	3.0 (1.9)
48.0	2.35	0.26	2.7 (1.7)
64.75	2.20	0.16	2.2 (1.4)

[a] A batch culture on glucose defined medium was prepared as described in Section 6.2.1 and samples for measurement of external glucose concentration and internal G6P concentration taken at the times indicated. The concentration of G6P is expressed as nmol/µg dry weight. The values in brackets represent its concentration in the cell as mmol/l assuming a value of 1.6 µl cell water/mg dry weight (Section 2.9).

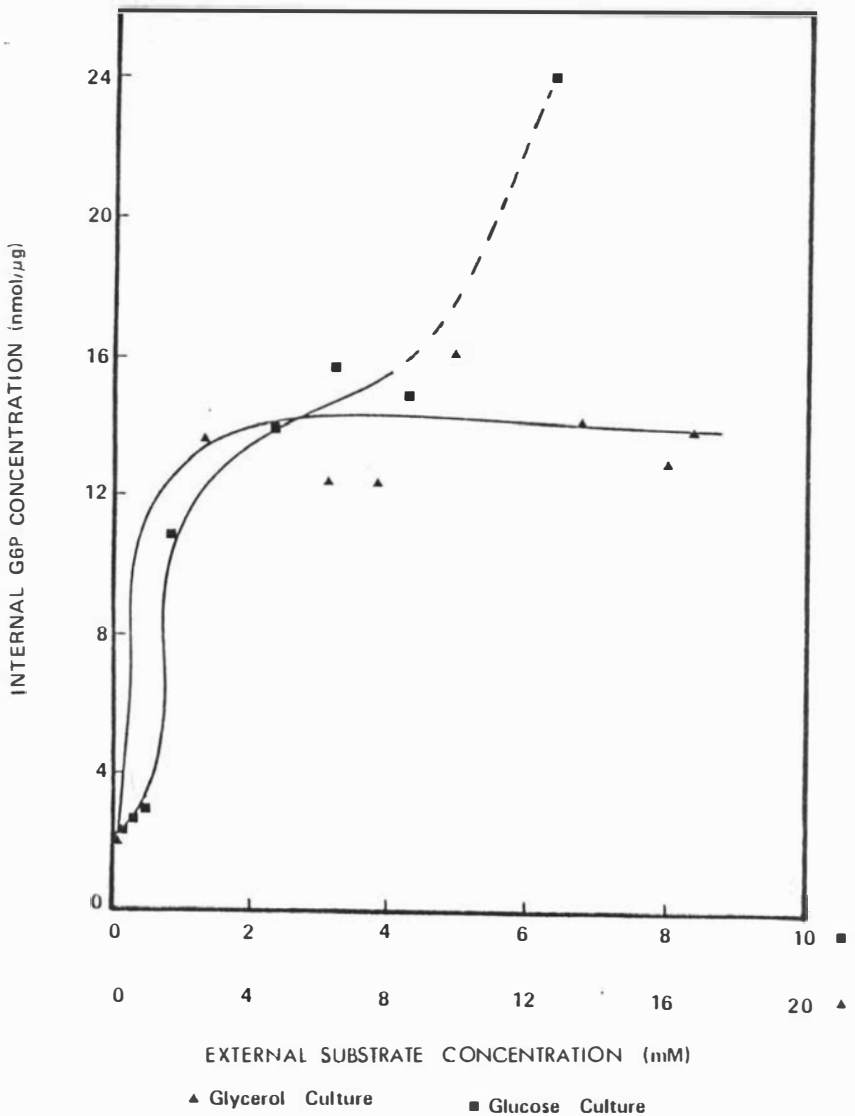
Table 6.2.2 Relationship between external glycerol concentration and internal G6P and F6P concentrations.[a]

Time (h) (after inoculation)	OD_{540}	External Glycerol (mM)	Internal G6P (nmol/μg)	Internal F6P (nmol/μg)
38.5	1.75	16.8	13.9 (8.7)	4.0 (2.5)
39.5	1.90	16.0	13.0 (8.1)	
40.5	2.00	13.5	14.7 (9.2)	
42.0	2.13	9.8	16.3 (10.2)	4.2 (2.6)
43.5	2.30	7.6	12.5 (7.8)	
45.5	2.45	6.2	12.5 (7.8)	
48.0	2.80	2.5	13.6 (8.5)	
50.5	2.90	<0.1	2.1 (1.3)	0.61 (0.38)
54.5	2.90	<0.1	1.8 (1.1)	
67.0	2.80	<0.1	1.9 (1.2)	0.42 (0.26)

[a] A batch culture on glycerol defined medium was prepared as described in Section 6.2.2 and samples for measurement of external glycerol concentration and internal G6P and F6P concentrations taken at the times indicated. The concentrations of G6P and F6P are expressed as nmol/μg dry weight. The values in brackets represent their concentrations in the cell as mmol/l assuming a value of 1.6 μl cell water/mg dry weight (Section 2.9).

Figure 6.2

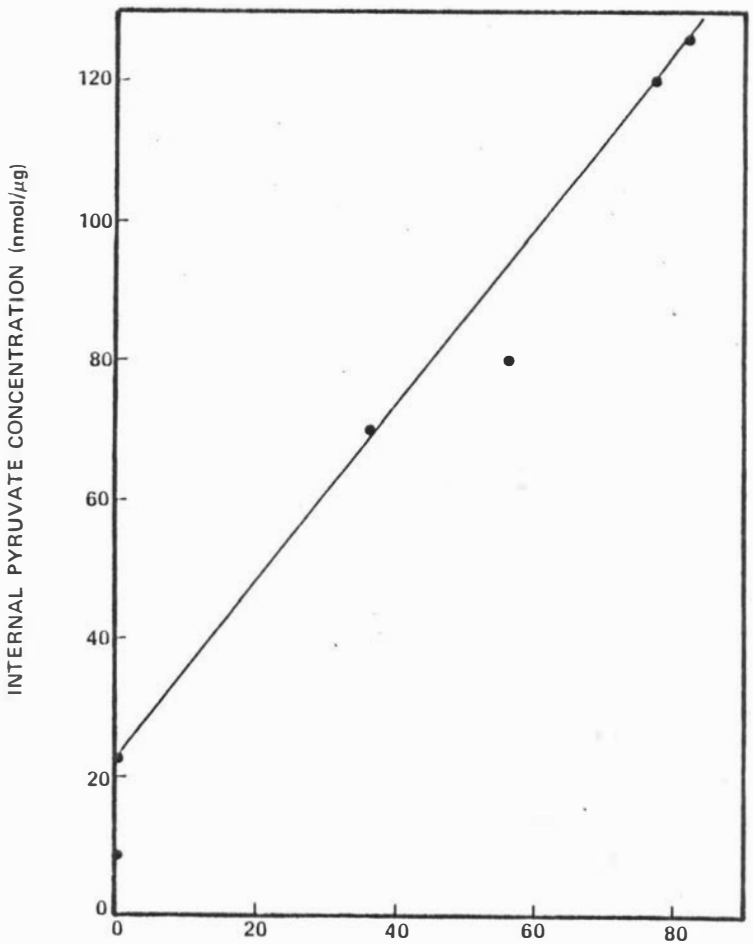
RELATIONSHIP BETWEEN INTERNAL G6P CONCENTRATION AND EXTERNAL GLUCOSE OR GLYCEROL CONCENTRATION



The figure shows the relationship between internal G6P concentration and external glucose or glycerol concentration in batch cultures on the defined medium as described in Sections 6.2.1. and 6.2.2.

Figure 6.2.3

RELATIONSHIP BETWEEN INTERNAL PYRUVATE CONCENTRATION AND EXTERNAL LACTATE CONCENTRATION



The figure shows the relationship between internal pyruvate concentration and external lactate concentration in a batch culture on the defined medium as described in Section 6.2.3.

in Figure 6.2. Again the relationship was not a simple linear increase in internal G6P concentration with increasing external carbon source. At glycerol concentrations in the medium of above 2 mM, the internal G6P concentration was maintained approximately between 12.5 and 16.3 nmol/µg while at very low external glycerol levels the internal G6P concentration was about 2.0 nmol/µg.

Again a very abrupt drop in internal G6P level occurred over a relatively short space of time (2.5 h), as the culture was entering stationary phase. From the few F6P analyses done the levels of this metabolite appeared to behave in a similar manner to G6P, although it is not known whether an abrupt drop in the concentration of this metabolite occurred as the culture entered stationary phase.

6.2.3 Relationship between External Lactate Concentration and Internal Pyruvate Concentration

In the continuous culture experiments using lactate as the carbon source it was found that the G6P level did not increase when external lactate concentration was increased at the higher dilution rate. However intracellular pyruvate concentration showed a large increase at high external lactate concentration (Section 5.3.3.3). Cellular pyruvate levels were therefore measured through a lactate-grown batch culture.

The results from this experiment are summarised in Table 6.2.3 and the relationship between external lactate and internal pyruvate is shown directly in Figure 6.2.3. In this case the relationship was linear with decreasing external lactate being accompanied by decreasing internal pyruvate although there was still a significant level of pyruvate in the cell when all the lactate was utilised. This residual pyruvate gradually

Table 6.2.3 Relationship between external lactate concentration and internal pyruvate concentration.[a]

Time (h) (after inoculation)	OD_{540}	External Lactate (mM)	Internal Pyruvate (nmol/µg)
25.5	0.80	82	126 (78.8)
28.0	1.05	77	120 (75)
30.75	1.40	56	80 (50)
34.0	1.90	36	70.4 (44)
47.0	2.55	<0.5	23 (14)
51.5	2.50	<0.5	9 (5.6)

[a] A batch culture on lactate defined medium was prepared as described in Section 6.2.3 and samples for measurement of external lactate concentration and internal pyruvate concentration taken at the times indicated. The concentration of pyruvate is expressed as nmol/µg dry weight. The values in brackets represent its concentration in the cell as mmol/l assuming a value of 1.6 µl cell water/mg dry weight (Section 2.9).

diminished with time as the culture entered the stationary phase.

6.3 REINVESTIGATION OF THE REGULATION OF PYRUVATE KINASE ACTIVITY AT IN VIVO CONCENTRATIONS OF SUBSTRATES AND EFFECTORS

The levels of ADP, PEP, G6P and ATP have been measured under a number of different metabolic conditions (Chapter 5) in an attempt to confirm the proposed mechanism for control of the enzyme pyruvate kinase *in vivo*. These levels of the substrates and effectors were used in the investigation described below. As mentioned earlier (Section 5.4) the level of P_i (an inhibitor of pyruvate kinase) in the cell has not been determined in this investigation. The effect of G6P on pyruvate kinase activity was therefore studied over a range of P_i concentrations.

6.3.1 Partial Purification of Pyruvate Kinase

For this study a simple purification scheme was used essentially to remove any small molecules in the extract (such as G6P and P_i) which may interfere with the activity of pyruvate kinase.

Glycerol-grown (defined medium) *P.shermanii* cells which had been stored frozen were thawed and resuspended to a thick slurry in 10 mM Tricine/NaOH buffer (pH 7.5) containing 10% glycerol. The cells were disrupted by two passages through a French pressure cell (38000 kN/m^2) and the debris removed by centrifugation at 27000 g at 4°C for 30 min. The supernatant was centrifuged for a further 2 h at 225000 g_{max} at 4°C to remove fine particulate matter with a high NADH-oxidase activity. This supernatant was fractionated with ammonium sulphate (as described in Section 4.2.4) and the 40-60% fraction was resuspended in 4.0 cm^3 of 10 mM Tricine/NaOH buffer (pH 7.5) containing 20% glycerol and 0.2 M KCl. This solution was applied to the top of a Sephacryl S200 gel filtration column (90 cm x 2.5 cm) equilibrated in the same

Table 6.3.1 Partial purification of pyruvate kinase.[a]

Fraction	Volume (cm^3)	Protein Concentration (mg/cm^3)	Total Activity (units)	Specific Activity (units/mg)	Purification (fold)
Crude Extract	22	14.2	72	0.25	1.0
0-40% Ammonium Sulphate fraction	2.8	25.0	29	0.42	1.7
40-60% Ammonium Sulphate fraction	4.0	27.5	39	0.35	1.4
S200 Gel Filtration pooled high specific activity fractions	16.0	1.4	26	1.14	4.6

[a] Pyruvate kinase was partially purified by the scheme outlined in Section 6.3.1 from 12 g wet packed weight of glycerol-grown P.shermanii cells. Protein concentration was estimated by the method of Lowry et al (1951).

buffer. The column was developed in the equilibration buffer at 8 cm^3/h, collecting 4 cm^3 fractions. Fractions containing pyruvate kinase at an activity greater than 1.5 units/cm^3 were pooled and dialysed for two successive 2 h periods against 2 l volumes of 10 mM Tricine/NaOH buffer (pH 7.5) containing 20% glycerol before being stored frozen at -20°C. The purification for pyruvate kinase using 12 g wet packed weight of cells is shown in Table 6.3.1.

6.3.2 Studies on the Partially Purified Pyruvate Kinase

Pyruvate kinase assays were performed as specified in Section 4.3.1 at 30°C in an SP 1800 recording spectrophotometer. Each assay contained 100 µl (0.14 mg protein) of the partially purified pyruvate kinase solution. On the basis of the data on the *in vivo* metabolite concentrations as summarised in Table 5.4 the concentrations of the substrates, PEP and ADP, were set at 0.5 mM and 1.0 mM respectively. ATP was added at a final concentration of 1.0 mM since this compound was found to be present in cells at about this level under all the conditions studied. A range of G6P concentrations from 1.0-15.0 mM was used, while the level of P_i was also varied over a wide range, from 0-12 mM.

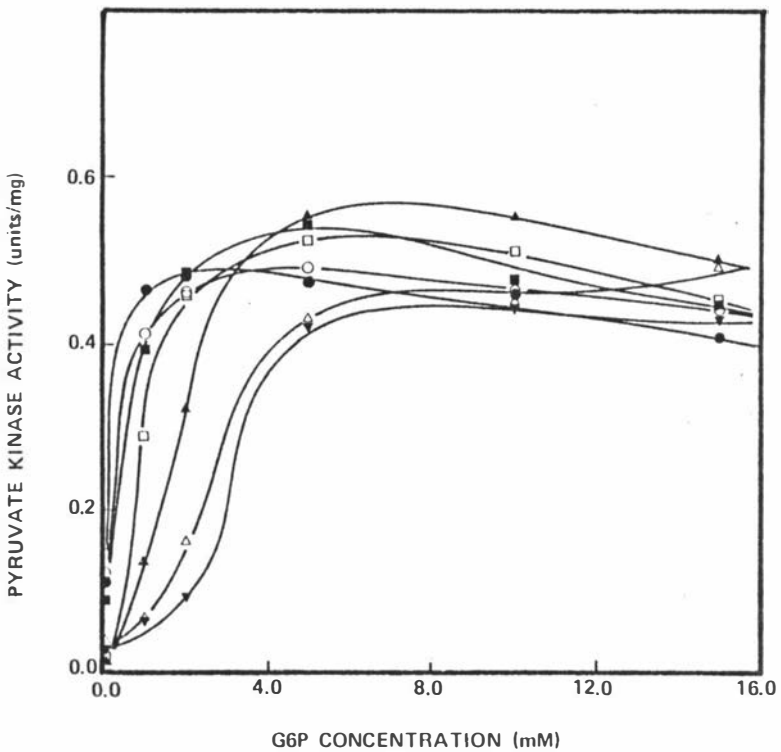
The results from this experiment are shown in Figure 6.3.2 where the same data are plotted in two different ways to illustrate both the effect of varying G6P concentration and the effect of varying P_i concentration. Table 6.3.2 lists the Hill coefficients for the relationship between pyruvate kinase activity and increasing G6P concentration at the different P_i concentrations. In the absence of any P_i in the assay the Hill coefficient was 0.9, similar to that reported in Section 4.3.13 for the relationship in the absence of any inhibitors, but as the P_i concentration in the assay was increased so did the Hill

Figure 6.3.2

PYUVATE KINASE ACTIVITY AT *IN VIVO* CONCENTRATIONS OF SUBSTRATES AND EFFECTORS

Figure (a)

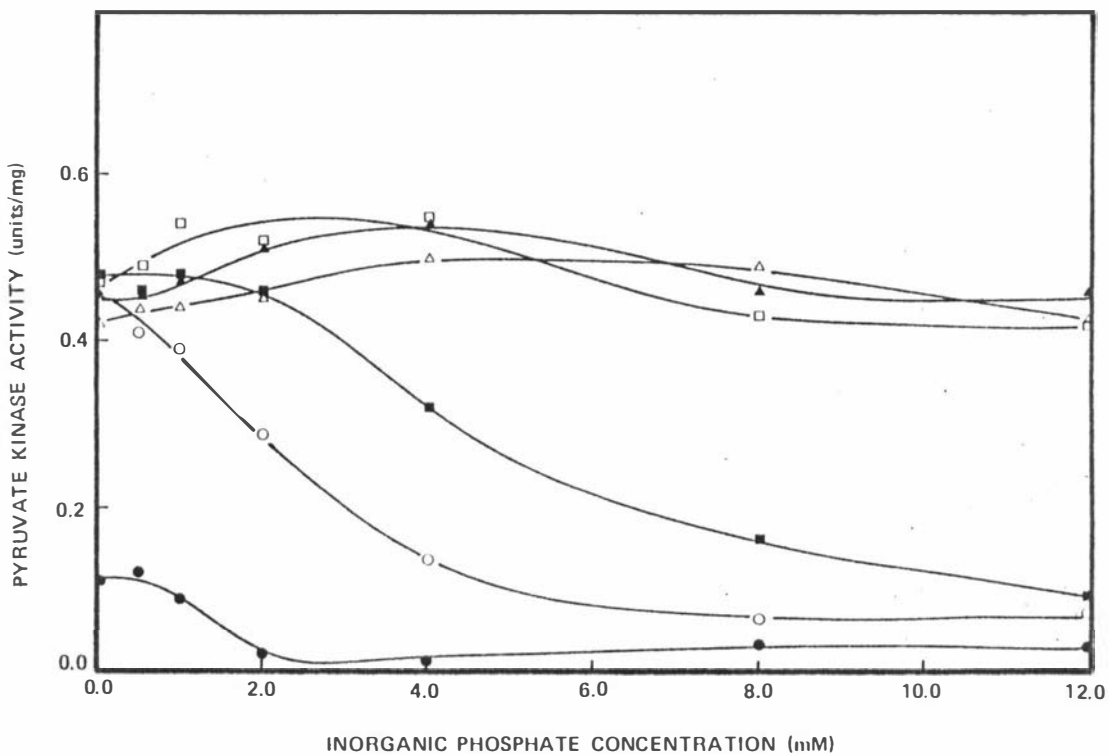
Pyruate Kinase Activity versus G6P Concentration



P_i concentration : ● 0.0 mM; ○ 0.5 mM; ■ 1.0 mM; □ 2.0 mM; ▲ 4.0 mM; △ 8.0 mM; ▼ 12.0 mM.

Figure (b)

Pyuvate Kinase Activity versus P_i Concentration



G6P concentration : ● 0.0 mM; ○ 1.0 mM; ■ 2.0 mM; □ 5.0 mM; ▲ 10.0 mM; △ 15.0 mM.

Pyruvate kinase activity was measured at the *in vivo* level of substrates and ATP and in the presence of varying concentrations of G6P and P_i. Figure (a) shows the relationship between activity and G6P concentration at different P_i levels while figure (b) shows the same data plotted as a function of P_i concentration. Reaction conditions as specified in Section 4.3.1 but with 1.0 mM ADP and 0.5 mM PEP. Reactions were performed in 1.0 mM ATP and in the presence of G6P and P_i at the concentrations shown.
Enzyme concentration : 0.14 mg protein per assay.

Table 6.3.2 Effect of inorganic phosphate on the cooperativity of G6P activation of pyruvate kinase.[a]

Inorganic Phosphate Concentration (mM)	Interaction Coefficient (n_H)
0.0	0.9
0.5	1.4
1.0	2.0
2.0	2.1
4.0	2.0
8.0	2.4
12.0	3.9

[a] The relationship between pyruvate kinase activity and G6P concentration at 7 different P_i concentrations was determined as described in Section 6.3.2 and is shown in Figure 6.3.2. Interaction coefficients for the relationship were determined by the method of Endrenyi et al (1975).

number increase until in the presence of 12 mM P_i it was 3.9, indicating a highly cooperative system.

Figure 6.3.2 (b) plots the same data as in Figure 6.3.2 (a) to emphasise the importance of P_i in controlling the activity of pyruvate kinase. At low concentrations of P_i (1-2 mM) even low concentrations of G6P (1-2 mM) readily overcame the inhibition of pyruvate kinase activity caused by both the P_i and the 1 mM ATP in the assay. However at higher levels of P_i (8-12 mM) concentrations of 5-15 mM G6P were required to reactivate the pyruvate kinase activity as 1-2 mM G6P had little effect. At these high levels of P_i the difference between the ability of 2 mM G6P and 5 mM G6P to reactivate the pyruvate kinase was quite dramatic indicating further the highly cooperative nature of the relationship between activity and G6P concentration under these conditions.

6.4 DISCUSSION

The concentration of G6P in P.shermanii cells growing on either glucose or glycerol was maintained at two distinct levels depending on the concentration of the carbon source in the medium. When the external concentration of either glucose or glycerol was relatively high (conditions which favour active glycolysis) the level of G6P in the cells was maintained above 7 mM (Tables 6.2.1 and 6.2.2). However as the cultures entered stationary phase and the external concentration of glucose or glycerol fell to a low level the level of G6P dropped abruptly to concentrations between 1 and 2 mM in the cell.

It can be seen from Figure 6.3.2 that provided the P_i concentration in the cell is above 10 mM a change in the G6P concentration from 2 mM to 5 mM will have a marked effect on the activity of pyruvate kinase because of the high degree of cooperativity of the binding of G6P to the enzyme

at this concentration of phosphate.

It is significant to note that the change in G6P level required to reactivate the pyruvate kinase under *in vivo* conditions (i.e. from 2 to 5 mM G6P) corresponds very closely with the difference in G6P levels found in rapidly glycolysing cells compared to that in cells not actively glycolysing (i.e. from 2 to 7 mM G6P). Thus it appears that the ability of the cell to activate pyruvate kinase and thus glycolysis through the effector G6P coincides directly with the change in the cell from a situation where active glycolysis is not required to a situation where it is. This provides strong *in vivo* confirmation of the validity of the proposed model for the control of glycolysis outlined in Section 4.4.

CHAPTER 7

SOME ADDITIONAL ASPECTS OF METABOLIC REGULATION IN P.SHERMANII

7.1 INTRODUCTION

This chapter describes the results of three quite different investigations of a very preliminary nature. They arose out of a consideration of some of the questions raised in earlier chapters. The results of these three studies are not particularly conclusive and would require much more thorough investigation before they can be clearly interpreted but they have been included here because they suggest possible methods of approach to problems of interest.

7.2 THE EFFECT OF G6P CONCENTRATION ON G6P DEHYDROGENASE ACTIVITY

The results of studies on G6P levels in batch and continuous cultures described in Chapter 5 present an apparent paradox. Both the batch culture and the continuous culture at the high dilution rate on the glucose-containing medium have similar growth rates and so presumably would require similar rates of glycolysis. However, G6P concentration which, it has been suggested, controls the activity of pyruvate kinase is very different in the two cultures, being at an 'activating' level in the batch culture but at a probably 'non-activating' level in the continuous culture. From the results described in Chapter 6, the level of G6P was shown to be related to the external glucose concentration and not necessarily to the rate of growth. As will be discussed in the final chapter, the role of the G6P regulation of pyruvate kinase in glucose-utilising cultures may be to control the relative proportions of PEP used for ATP production (via pyruvate kinase) and PP_i production (via the PEP:carboxytransphosphorylase). This line of reasoning lead to the possibility that the level of G6P in the cell might also determine the relative proportions of glucose metabolised by the EMP pathway (which requires a source of PP_i)

and the HMP pathway, which bypasses the PP_i-requiring phosphofructokinase. Such a control could be mediated by the nature of the G6P dependence of the enzyme G6P dehydrogenase which catalyses the first step of the HMP pathway. A study of this dependence was carried out on a crude extract from P.shermanii.

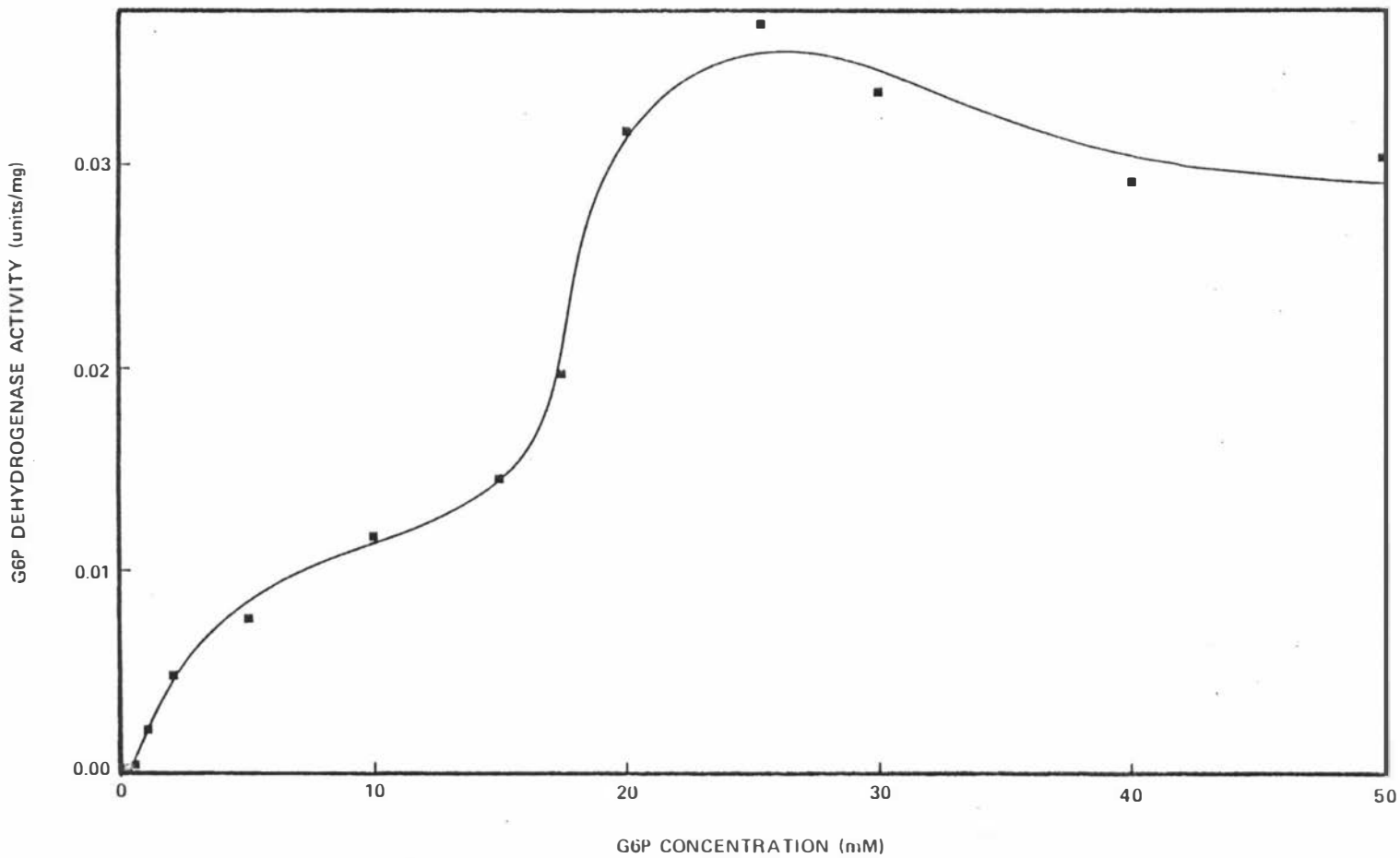
Glycerol-grown cells which had been stored frozen were thawed, resuspended to a thick slurry in 10 mM Tricine/NaOH buffer (pH 7.5) containing 20% glycerol and broken by two passages through a French pressure cell (38000 kN/m^2). Cell debris was removed by centrifugation (27000g, 1 h, 4°C) and the supernatant dialysed for 20 h against 2 l of 10 mM Tricine/NaOH buffer (pH 7.5) containing 20% glycerol and then stored at -20°C.

This crude cell-free extract was assayed for G6P dehydrogenase activity essentially as described in Section 2.7 except that the G6P concentration was varied as indicated and the reaction was initiated by addition of NADP instead of G6P. The crude extract (1.25 mg protein per assay) was incubated in the reaction mixture for 10-15 min at 30°C before starting the reaction with NADP.

The relationship between enzyme activity and G6P concentration for the G6P dehydrogenase in the crude extract is shown in Figure 7.2. The relationship was highly complex, showing an abrupt increase in activity between 15 and 20 mM G6P. This was a reproducible feature but it is difficult to provide any adequate interpretation without further study. Interpretation of the data may be confused by the presence of 6-phosphogluconate dehydrogenase in the crude extract which would be able to use the 6-phosphogluconate, produced by the G6P dehydrogenase reaction, as a substrate. NADPH production would therefore be due to the combined activity of these two different enzymes. However, even

Figure 7.2

RELATIONSHIP BETWEEN GLUCOSE 6-PHOSPHATE DEHYDROGENASE ACTIVITY AND G6P CONCENTRATION



Reaction conditions as specified in Section 7.2 Enzyme concentration : 1.25 mg protein per assay.

taking this into account the very complex shape of the activity versus G6P concentration curve would still not be expected. It is possible that more than one G6P dehydrogenase may be present. However, this study was carried out at the end of the present investigation and time did not permit a more thorough investigation.

Whatever mechanisms are operating in the utilisation of G6P, as measured by the production of NADPH, it appears that the cell extract may have the ability to use the G6P much more rapidly when that compound is present at very high levels (above 15-20 mM). Concentrations of up to 16 mM G6P (Table 5.3.2.1) have been found in the cell during growth on glucose and this value may be higher in cells growing in a higher concentration of external glucose.

This very preliminary experiment suggests a possible means by which G6P concentration may influence the flow of metabolites through the diverging HMP and glycolytic pathways during growth on glucose.

7.3 THE EFFECT OF CARBON SOURCE ON THE UPTAKE OF PHOSPHATE

The level of P_i in the cell is an important factor in the proposed model for the regulation of pyruvate kinase presented in earlier chapters. For reasons outlined in Section 5.4 the level of P_i in the cell was not measured and this remains one of the major uncertainties in establishing the validity of the regulatory mechanism suggested. From the results described in Chapter 6 it is clear that a concentration of P_i greater than 10 mM is required to ensure a sufficient level of inhibition of pyruvate kinase under gluconeogenic conditions. While the proposed model does not require that the level of free P_i itself should vary according to the nature of the growth substrate this would be an additional

possible mechanism whereby control might be exercised. A high P_i concentration under gluconeogenic conditions would not only increase the degree of inhibition of pyruvate kinase but would also assist in maintaining favourable conditions for the gluconeogenic activity of pyruvate, orthophosphate dikinase and PP_i-dependent phosphofructokinase, both of which require P_i as a substrate when proceeding in this direction.

A consideration of the way in which the P_i level might be altered according to the nature of the carbon source suggested the possibility of 'catabolite repression' or 'catabolite inhibition' of the phosphate uptake system by substrates of glycolysis.

The rate of uptake of phosphate in P.shermanii was followed using the radioisotope ^{32}P injected into resting cell suspensions of equal cell density in which both the differences due to the carbon source the cells were grown on and the carbon substrate they were fermenting could be studied independently.

Resting cell suspensions were prepared as described in Section 5.2.1 from cells grown on lactate, glucose or glycerol defined media. Cells were suspended at a concentration of 7 mg (wet weight)/cm^3 in 10 mM Tricine/NaOH buffer (pH 7.5) containing 1 mM NaH_2PO_4. Aliquots (5.0 cm^3) of each cell suspension (from lactate, glucose or glycerol-grown cells) were pipetted into each of three 50 cm^3 conical flasks and to these either lactate (40 mM), glucose (10 mM) or glycerol (20 mM) was added. This gave a total of nine flasks which were maintained under 95% N_2, 5% CO_2 at 30°C on a shaking water bath until required. To each flask 100 µl carrier-free ^{32}P (7.6×10^5 cpm) was added, after which 500 µl aliquots were removed at regular time intervals (up to 15 min), rapidly filtered through a 0.8 µm Millipore filter (prewashed with 0.1 M NaH_2PO_4)

and washed with 2 x 3.0 cm^3 amounts of 10 mM Tricine/NaOH buffer (pH 7.5) containing 1 mM NaH_2PO_4. The filters were placed in counting vials, 10 cm^3 distilled water added and radioactivity determined using a Beckman LS 8000 liquid scintillation counter (Cerenkov radiation - Hariland and Bieber (1970)).

The results from two separate experiments are shown in Table 7.3. The rates were calculated from the slopes of the best-fit lines of plots of radioactivity versus time. In cases where the relationship was not linear initial slopes were drawn over at least the first three points.

There were some differences in the rate of uptake of the inorganic phosphate both between cells grown on the different carbon substrates and between cells utilising the different substrates. The rates of ^{32}P uptake by cells grown on lactate and glucose were much higher than the rates obtained from cells grown on glycerol. For the cells grown on glycerol or lactate those utilising lactate had a higher ^{32}P uptake rate than those using glucose and glycerol while for cells grown on glucose there was little difference in the ^{32}P uptake rate when using the three substrates.

It would be premature to draw conclusions from the results of these experiments even though the data from the two entirely separate experiments are very consistent. While there are no really large differences in the rate of P_i uptake between cells grown on or metabolising different substrates, the finding that the cells grown on or metabolising lactate had the highest rate of uptake in both experiments could provide a mechanism by which such cells could sustain a higher pool size of P_i than those growing on glycerol and glucose.

Table 7.3 Effect of carbon source on phosphate uptake in *P.shermanii*.[a]

Growth Substrate[b]	Carbon source being metabolised[c]	Phosphate Uptake[d] (cpm ^{32}P/mg dry wt/min)
Glycerol	Glycerol	60 ; 75
	Glucose	35
	Lactate	86 ; 186
Glucose	Glycerol	220 ; 205
	Glucose	200 ; 205
	Lactate	210 ; 210
Lactate	Glycerol	190 ; 205
	Glucose	260 ; 242
	Lactate	475 ; 320

a The rate of ^{32}P - NaH_2PO_4 uptake was determined in resting cell suspensions of *P.shermanii* as described in Section 7.3.

b The cells were grown in the defined medium containing the carbon source indicated and harvested in the mid-logarithmic phase of growth.

c The cells were washed and resuspended in the buffer described in Section 7.3 with either glycerol, glucose or lactate as the carbon source.

d The two figures given represent the results from two entirely independent experiments.

7.4 GROWTH AND METABOLISM OF P.SHERMANII ON A MIXED GLUCOSE/LACTATE DEFINED MEDIUM

In a short paper by Lee et al (1974) it was reported that P.shermanii showed, what the authors referred to as a diauxic growth pattern when supplied simultaneously with both glucose and lactate i.e. lactate was utilised first and only after exhaustion of the lactate was glucose utilised. This finding was of interest in itself since it represents a reversal of the commonly found diauxic patterns in which glucose represses the utilisation of other compounds. The only other well documented case is the citrate-glucose diauxie in Pseudomonas aeruginosa (Hamilton and Dawes, 1959). Moreover, such a finding is difficult to interpret in the light of the findings of the present study.

It has been shown earlier that levels of enzymes of glucose catabolism are very similar in lactate- and glucose-grown cultures so there is no apparent repression of the enzymes by lactate. Furthermore, the 'feed-forward' activation mechanism proposed for the regulation of pyruvate kinase would not provide any basis for understanding lactate inhibition of glucose utilisation unless lactate inhibited the glucose-uptake process. Accordingly it was decided to re-investigate the finding of Lee et al (1974).

A batch culture was prepared under standard conditions (Section 5.3.2.2) in the defined medium containing 1 g/l glucose and 10 g/l Na D/L lactate. Samples were taken throughout the logarithmic and early stationary phases of growth for measurement of glucose and lactate in the medium. G6P and pyruvate levels were also determined in cells during the mixed culture as potential indicators of the state of metabolism in the cell. Table 7.4 and Figure 7.4 describe the results of this experiment.

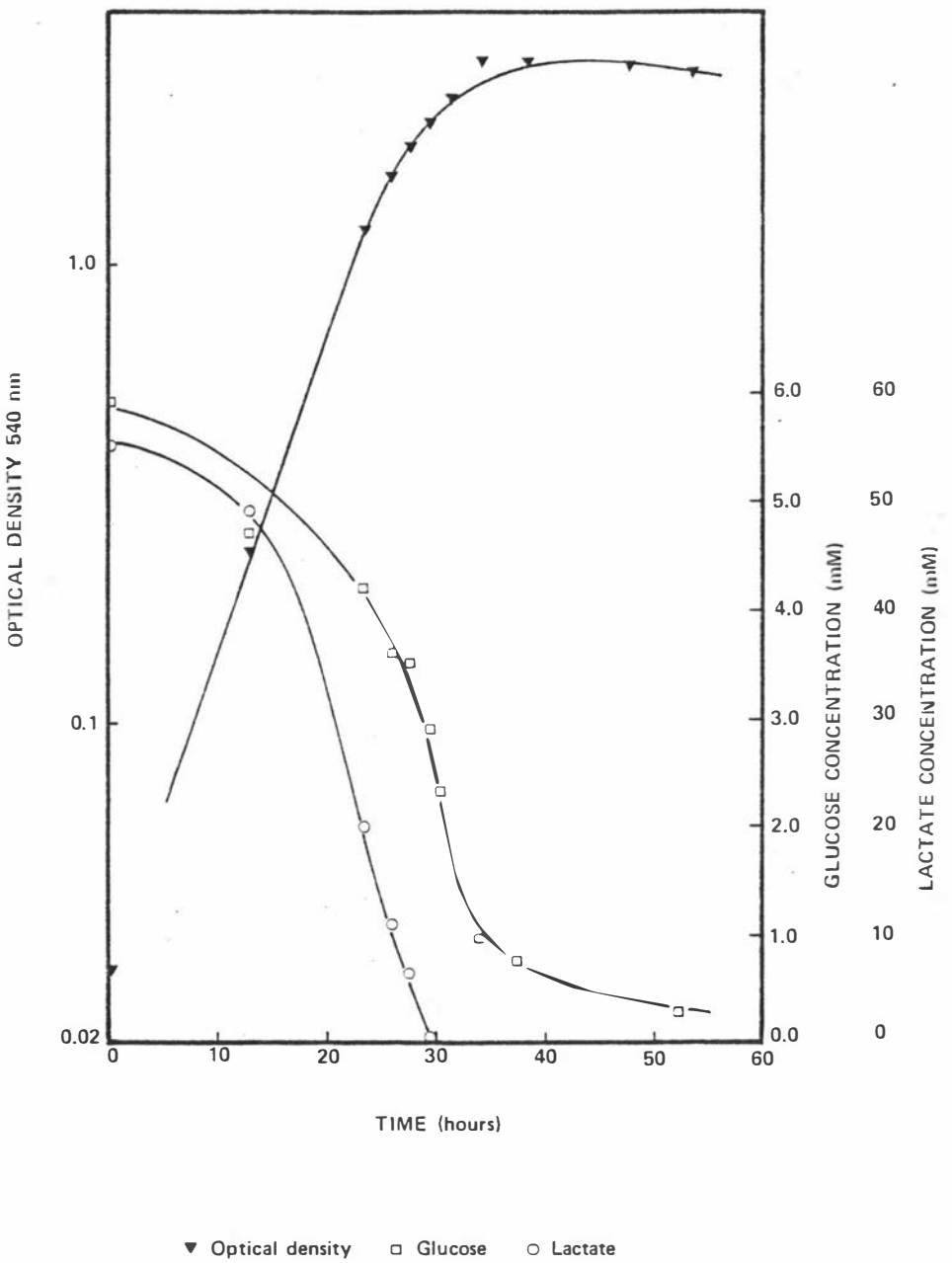
Table 7.4 Growth of P.shermanii on mixed glucose/lactate defined medium.[a]

Time (h)	OD_{540}	External Glucose (mM)	Internal G6P (nmol/μg)	Internal Pyruvate (nmol/μg)	External Lactate (mM)
zero	0.03	5.9			55
13.25	0.25	4.7			49
23.0	1.20	4.2	25	67	20
25.25	1.60	3.6	10.7		11
26.65	1.78	3.5	10.2	78	6.5
28.5	2.12	2.9	11	43	0.5
30.5	2.42	2.4	12	27	-
33.5	2.80	0.95	3.5	17	-
37.5	2.80	0.75			-
47.0	2.80		3.2	8.2	-
52.5	2.70	0.27	1.8	8.6	-

[a] A mixed glucose/lactate batch culture of P.shermanii was prepared as described in Section 7.4 and samples taken for measurement of external glucose and lactate concentrations and internal G6P and pyruvate concentrations at the times indicated. The concentrations of G6P and pyruvate are expressed as nmol/μg dry weight.

Figure 7.4

GROWTH OF *P.SHERMANII* ON MIXED LACTATE/GLUCOSE MEDIUM



The figure shows the growth and substrate utilisation of *P.shermanii* in mixed lactate/glucose defined medium as described in Section 7.4

There was no strongly marked diauxie in the growth curve and although the lactate was consumed faster than the glucose there was significant utilisation of the glucose over the first part of growth in the presence of high levels of lactate. The maximum growth rate obtained was slightly higher (μ = 0.16 h^{-1}) in this mixed culture on lactate and glucose than in the corresponding batch cultures on each individual substrate (Section 5.3.2.2). The cellular level of G6P was high throughout the growth curve, even in the presence of high levels of lactate, until the external concentration of glucose fell below 1 mM. Thus it is clear that lactate does not inhibit glucose uptake. These results do not, therefore, substantiate the finding of Lee *et al* (1974).

Chapter 8

GENERAL DISCUSSION

8.1 REGULATION OF PYRUVATE KINASE DURING GLYCOLYSIS AND GLUCONEOGENESIS

The primary aim of this investigation of the regulation of pyruvate kinase in P.shermanii was to find a possible mechanism whereby its activity might be controlled so that it was operative during glycolysis (growth on glucose or glycerol) but inhibited during gluconeogenesis (growth on lactate). The need for regulation is clearly evident from the results of the study of the relative activities of some of the enzymes of carbohydrate metabolism (Section 3.6). This study showed that pyruvate kinase was always present at levels greater than the pyruvate, orthophosphate dikinase whether the cultures were grown on lactate, glucose or glycerol media. Furthermore, the interconversion of PEP and pyruvate is possibly the only point at which the pathways of glycolysis and gluconeogenesis can be controlled independently since the more usual regulatory mechanism controlling the interconversion of F6P and FBP is not operative in P.shermanii.

In order to discover a possible regulatory mechanism a detailed study of the properties of the partially purified pyruvate kinase was undertaken. It was shown to exhibit highly cooperative substrate kinetics and to be allosterically activated by G6P and strongly inhibited by ATP and P_i. The most significant finding was that inhibition by both ATP and P_i could be largely overcome by G6P if present at a sufficiently high concentration. These in vitro data suggest that the activity of the pyruvate kinase may be regulated during growth on lactate by the combined inhibitory effects of ATP and P_i but when supplied with a glycolytic substrate such as glucose the increased level of G6P would overcome the inhibition caused by ATP and/or P_i and permit active glycolysis.

Determination of the concentrations of the effectors and substrates of pyruvate kinase in P.shermanii growing under a wide range of nutritional conditions suggest that G6P is present at sufficiently high levels to be able to activate pyruvate kinase during growth on glucose or glycerol (Section 5.4). However, during growth on lactate the level of G6P in the cell remains in the range 1-2 mM which, while much lower than the levels found in cells growing on glucose or glycerol, would be high enough to cause significant activation of the pyruvate kinase in the presence of the 1.0-1.5 mM ATP found in the cells. The cellular concentration of the other major inhibitor of pyruvate kinase activity, P_i, has not been determined but clearly, for the proposed mechanism for the control of pyruvate kinase to work in vivo, its concentration must be sufficiently high to cause significant inhibition of activity under the conditions found in cells growing on lactate (i.e. 1.0 mM PEP and ADP, 1.0-1.5 mM ATP and 1.0-2.0 mM G6P (Table 5.4)). In the presence of high glucose or glycerol concentrations however, the internal P_i concentration must not be so high as to inhibit the pyruvate kinase activity under the conditions found in those cells (i.e. 0.6 mM PEP, 1.0-1.5 mM ADP, 1.0-2.0 mM ATP and 5-16 mM G6P).

For this model to be valid either the increased level of G6P found in the glucose- and glycerol-grown cells compared to that in lactate-grown cells must be able to activate pyruvate kinase at the concentration of P_i in the cell, or the pool of free P_i in the cell must change during growth on the different carbon sources. A brief, preliminary investigation of the rate of uptake of ^{32}P by washed, resting cells of P.shermanii does give some support to the possibility that the pool of free P_i may be maintained at a higher level in cells growing on lactate compared to

those on glucose or glycerol (Section 7.3). However, the data presented in Section 6.3.2 on pyruvate kinase activity at *in vivo* levels of substrates and effectors suggest that the former possibility is most likely. Thus, at concentrations above 10 mM P_i, and at the levels of PEP, ADP and ATP found *in vivo*, concentrations of 1-2 mM G6P (as found in cells growing on lactate) were unable to activate the pyruvate kinase while concentrations of 5-15 mM G6P (as found in cells growing in the presence of high levels of glucose or glycerol) were readily able to overcome the inhibition caused by P_i.

It should be noted that the precise *in vivo* concentrations of metabolites are not known since a cell water volume was not determined for *P.shermanii* (Section 2.9) but the concentrations reported would not be significantly different even if the actual cell water volume lies at the extremes of the range of values reported for other bacteria.

The high levels of G6P found in *P.shermanii* during growth on glucose have not been found in other bacteria where this metabolite has been measured. Under conditions favouring glycolysis G6P concentrations of up to 1.1 mM have been reported for *E.coli* (Dietzler *et al*, 1979), 1.6 mM for *Streptococcus lactis* (Thompson, 1978), 1.75 mM for *Alcaligenes eutrophus* H 16 (Cook *et al*, 1976) and 1.25 mM for *Thermus thermophilus* (Yoshizaki and Imahori, 1979b). In these organisms however, G6P is not the sole 'feed-forward' activator of pyruvate kinase as is apparently the case in *P.shermanii* (Section 1.1.1).

In *E.coli* the type I pyruvate kinase is activated by FBP while the type II enzyme is activated by a wide range of compounds including G6P, but ribose 5-P and AMP are probably the most significant activators (Waygood and Sanwal, 1974; Waygood *et al*, 1975). In this organism the *in vivo* levels of FBP are reported to be as high as 3.2 mM when growing on

glucose (Dietzler et al, 1975a). These workers propose a mechanism for the control of its carbohydrate metabolism through fluctuations in the levels of both FBP and G6P, caused by the influence of the energy charge on phosphofructokinase.

In Streptococcus lactis the pyruvate kinase is also activated by a wide range of compounds. However in this bacterium FBP appears to be the primary regulatory signal in promoting glycolysis as its level in the cell has been reported to be as high as 20-25 mM when metabolising glucose but falls to less than 0.1 mM on starvation (Thompson and Thomas, 1977).

Yoshizaki and Imahori (1979b) have reported that the levels of FBP, F6P and G6P are all elevated in Thermus thermophilus during growth on a glucose-containing medium compared to a gluconeogenic medium while levels of PEP undergo opposite changes. They consider however that the levels of PEP and F6P only are the main regulatory signals in this organism providing for a mechanism of "coupled regulation" of glycolysis and gluconeogenesis as outlined in Section 1.1.1.

In P.shermanii G6P is the only glycolytic intermediate, of those determined (apart from pyruvate), found at levels above 1 mM during growth on glucose. The levels of FBP found in P.shermanii are less than 0.2 mM during growth on glucose. This is in marked contrast to the concentrations of FBP found in E.coli, Streptococcus lactis and Thermus thermophilus mentioned above. This difference in the metabolite profile for P.shermanii compared to that in other bacteria may be related to the replacement of the irreversible ATP-dependent phosphofructokinase found in those bacteria (which may provide a thermodynamic situation favouring the accumulation of FBP) by the freely-reversible PP_i-dependent phosphofructokinase in P.shermanii (which would allow an equilibrium to be established

between the intermediates FBP, F6P and G6P).

As the phosphoglucoisomerase catalysed interconversion of G6P and F6P is freely reversible these two intermediates would be expected to co-vary in the cell according to the equilibrium constant for the reaction which is 0.45 (Rolleston and Newsholme, 1967). However, the expected mass-action ratio of G6P:F6P of 2.2:1 has not always been found *in vivo*. Rolleston and Newsholme (1967) found ratios of greater than 5:1 in cerebral cortex slices as did Yoshizaki and Imahori (1979b) in *Thermus thermophilus* while Thompson and Thomas (1977) found ratios of G6P:F6P as high as 6.5:1 in *Streptococcus lactis* cells metabolising glucose. In this investigation the ratios of G6P:F6P found in *P.shermanii* growing on either lactate or glycerol under all the conditions studied and also in the continuous cultures on glucose are in the range 2.8:1 to 8:1 and most are about 4:1, similar to the ratios reported in other organisms (Tables 5.3.2.1, 5.3.2.2 and 5.3.3.2). However in batch cultures on glucose, where the external glucose concentration is high, the G6P:F6P ratio ranges from 13:1 (Table 5.3.2.1) to 19:1 (Table 5.3.2.2) which is much higher than expected (a similar value of 18:1 was found for this ratio in another batch culture on glucose, which has not been reported in this thesis).

This very large shift from the equilibrium ratio found under certain conditions is difficult to explain unless the phosphoglucoisomerase in *P.shermanii* possesses some unusual kinetic properties. It appears not to be related to the high G6P levels found in the glucose batch cultures since in the glycerol batch cultures where the G6P is also high the G6P:F6P ratio is much lower. Nor is it likely that two separate pools of G6P exist as there is no cytological basis for compartmentation in prokaryotes and in any event it is unlikely that compartmentation would be different in glucose- and glycerol-grown cells. The data seem to suggest that the

'forward' reaction (i.e. G6P ⟶ F6P) catalysed by the phosphoglucoisomerase may somehow be inhibited at high external glucose concentrations. The possible significance of this point will be discussed later. Clearly, both the G6P:F6P mass-action ratios *in vivo* and the phosphoglucoisomerase warrant further examination to determine the validity or otherwise of this possibility.

Levels of glycolytic intermediates, apart from pyruvate, in *P.shermanii* when growing on lactate are generally low and do not vary significantly with changes in the growth rate or carbon source concentration. However pyruvate attained exceptionally high concentrations on lactate and varied according to the concentration of lactate in the medium (Section 6.2.3). The significance of this finding was not investigated and it is possible that pyruvate may function as an important regulatory 'signal' in carbohydrate metabolism during growth on lactate.

The concentration of ATP found in *P.shermanii* remained constant within the range 1.0-2.2 mM over a wide range of nutritional conditions. This value is similar to those reported for ATP in other bacteria. Levels of 1.5-2.5 mM ATP have been found in *E.coli* (Lowry *et al*, 1971; Dietzler *et al*, 1975a and b), 2.8 mM in *Thermus thermophilus* (Yoshizaki and Imahori, 1979a) and 2.6-4.2 mM in *Alcaligenes eutrophus* H 16 (Cook *et al* 1976; Wilke and Schlegel, 1975).

ATP appears to be a 'secondary signal' in regulating pyruvate kinase activity in *P.shermanii* providing a constant inhibitory action upon which the activating effect of G6P is imposed as a 'primary regulatory signal'. The role of the other major effector of pyruvate kinase activity, inorganic phosphate, cannot be resolved as its level in the cell is not known. The data presented in Section 6.3.2 on pyruvate kinase activity suggest that a constant level of at least 10 mM P_i in the cell would allow for a control

of the pyruvate kinase by varying the G6P concentration within the range of values found *in vivo*. However, changes in the levels of P_i under different nutritional conditions cannot be ruled out as an alternative means of control. There is little information available on the levels of free P_i in bacteria due to difficulties in its measurement and the interpretation of results. Harold and Spitz (1975) reported that *Streptococcus faecalis* cells attained an internal concentration of 75 mM P_i on a medium containing 10 mM P_i so the value of 10-15 mM P_i required for the operation of the mechanism of regulation of pyruvate kinase in *P.shermanii* is not unreasonably high. The ability of G6P to overcome inhibition at concentrations of P_i greater than 12 mM was not investigated. This would be of interest.

In summary, the regulatory properties of pyruvate kinase in *P.shermanii*, while conforming to the general properties of bacterial pyruvate kinases, are specifically related to the key features of carbohydrate metabolism in this organism.

8.2 FURTHER POSSIBLE ROLES OF G6P AS A REGULATOR OF CARBOHYDRATE METABOLISM IN P.SHERMANII

The regulation of pyruvate kinase has so far been considered solely from the point of view of the need to control the opposing pathways of glycolysis and gluconeogenesis and the proposed model does seem to provide a plausible mechanism for this.

However, the model is not entirely consistent with some of the results presented. In particular, the results of one experiment are difficult to reconcile with the proposal that elevated G6P levels (in excess of 5 mM) are essential for active glycolysis. In the high dilution rate continuous culture on glucose (Section 5.3.3.2) the growth rate was very similar to

the maximum obtained in batch cultures on glucose and presumably, therefore, the rate of glycolysis was also similar. Yet the level of G6P in these cells was similar to that found in the low dilution rate glucose continuous culture rather than that found in the glucose batch cultures. In fact, the G6P level was similar to that found in cells growing on lactate, where the pyruvate kinase should be strongly inhibited. Thus it seems that a high rate of glycolysis is not necessarily dependent on a high G6P concentration in the cell.

This apparent anomaly suggests that a considerable portion of the glycolytic flux under these conditions (of low G6P concentration) may proceed via the pyruvate, orthophosphate dikinase, which can also function in the direction PEP to pyruvate (Evans and Wood, 1968), rather than via the pyruvate kinase. The exact extent to which the dikinase would need to carry the glycolytic flux from PEP to pyruvate would depend upon the precise concentration of free inorganic phosphate in P.shermanii when growing on glucose. If the level of P_i in these cells is lower than that in cells growing on lactate then the concentrations of 2.0 and 2.05 mM G6P found in the glucose continuous cultures (Table 5.3.2.2) may well cause some activation of the pyruvate kinase whereas the 1.05-1.9 mM G6P found in the various lactate cultures would presumably not be sufficient to overcome the inhibition at the P_i concentration present in these cells. Under these conditions of low internal G6P concentration it may be more likely that both the pyruvate, orthophosphate dikinase and pyruvate kinase enzymes contribute to the glycolytic flux at this level.

If the dikinase (plus the residual activity of the 'inhibited' pyruvate kinase) can function at a sufficient rate to sustain growth at near its maximum rate on a glucose medium then what is the significance

of the proposed mechanism for the control of the pyruvate kinase?

It is possible that the regulation of pyruvate kinase may have another function - to regulate the relative amounts of PEP metabolised via the pyruvate kinase (which generates ATP) and the PEP:carboxytransphosphorylase (which generates PP_i).

Metabolism of PEP via the carboxytransphosphorylase results in the accumulation of succinate since the transcarboxylase reaction, essential for propionate formation, is bypassed. It is the carboxytransphosphorylase reaction which leads to CO_2 fixation in propionibacteria and it was this discovery by Wood and Werkman in the 1930's that lead to the recognition of the importance of heterotrophic CO_2 fixation in living organisms. Subsequent workers (e.g. Wood and Werkman, 1940; Johns, 1951) described the relationship between CO_2 concentration and succinate accumulation although at that time the succinate so formed was considered to be an intermediate in propionate formation. It is now clear from the later work by Wood and co-workers that succinate and propionate are alternative products of PEP metabolism depending on whether this proceeds via the carboxytransphosphorylase or via pyruvate and the transcarboxylase.

The production of succinate was measured in the experiments described in Chapter 5 and was shown to differ considerably according to the growth substrate. On lactate it amounts to only 1-4% of the products, on glucose up to 26% and on glycerol usually just less than 20% (Tables 3.4, 5.3.2.2 and 5.3.3.1). Since the production of succinate by P.shermanii is important as an indicator of the flow of metabolites from PEP through the PEP:carboxytransphosphorylase reaction this shift towards much greater succinate production on glucose (and glycerol) has important implications

for the carbohydrate metabolism of P.shermanii.

As described in Section 1.2 the glycolytic reaction from F6P to FBP in propionibacteria is catalysed by a PP_i-dependent phosphofructokinase which utilises the high standard free energy change of hydrolysis of PP_i instead of ATP. During active glycolysis this requires a major catabolic source of PP_i. As pointed out in Section 1.2, such a source of PP_i would not be required during growth on lactate or glycerol but only on glucose. If, as suggested above, the pyruvate, orthophosphate dikinase also functions in glycolysis and accounts for a portion of the flux from PEP to pyruvate when the internal G6P concentration is low, it would also require a catabolic source of PP_i during growth on glucose.

The so-called 'pyruvate reaction' catalysed by the PEP:carboxytransphorylase (Section 1.2) is not likely to provide a source of PP_i under the growth conditions used in this investigation as the cultures were continuously sparged with a 95% N_2, 5% CO_2 gas mixture and thus the CO_2 level in the cell would probably prevent the 'pyruvate reaction' proceeding (Wood, 1977).

The high succinate levels in glucose cultures of P.shermanii (up to 26% of products) however, would suggest that the carboxytransphosphorylase does indeed provide one major source of PP_i via the normal reaction to OAA. In the high dilution rate continuous culture on glucose the steady-state level of succinate produced was 6.2 mM which would provide 0.56 moles of PP_i (from the carboxytransphosphorylase reaction) per mole of glucose metabolised. However, if glucose metabolism is proceeding from F6P to FBP entirely via the PP_i-dependent phosphofructokinase and also if the pyruvate, orthophosphate dikinase is solely responsible for the flux from PEP to pyruvate under these conditions this molar ratio would only provide about 21% of the PP_i required. The actual figure may well be much higher

than this though, since, as described above, the pyruvate kinase may remain partially active under these conditions and still carry a portion of the flux from PEP to pyruvate. Also, the HMP pathway, which operates at least to some extent during glucose metabolism (Section 1.2) may carry a portion of the flow of metabolites from G6P to triose phosphate, thus at least in part bypassing the PP_i-dependent phosphofructokinase reaction.

Similar calculations on the amount of PP_i produced cannot be performed for the batch cultures as the concentrations of end-products determined are not steady-state values as in continuous cultures. However it can be seen qualitatively, from the glucose batch culture shown in Figure 5.3.2.3(b), that the proportion of succinate produced increases during growth - representing 14% of the products early in the logarithmic phase of growth and increasing to 24% late in the logarithmic phase. This is consistent with the falling G6P levels in the cell resulting in a greater diversion of PEP via the carboxytransphosphorylase reaction as the glucose concentration drops. This leaves the problem of how the cell obtains sufficient PP_i during the early logarithmic phase when the G6P concentration is high and the succinate levels are low. This problem will be considered below.

The high proportion of succinate (up to 20%) produced in glycerol cultures of P.shermanii suggest that a considerable proportion of this substrate is also directed into PP_i production by the carboxytransphosphorylase reaction. The fact that CO_2 is required for growth on glycerol would tend to support this conclusion. However, the reason for the production of PP_i is not as clear for growth on glycerol as it is for growth on glucose as the PP_i-dependent phosphofructokinase is not required to operate in the direction of PP_i consumption during the utilisation of glycerol.

The pyruvate, orthophosphate dikinase may also function in the glycolytic direction during growth on glycerol and to this extent PPi would be required. However, when growing in the presence of high levels of glycerol, where the internal G6P concentration is also high, the pyruvate kinase would be expected to be fully active. Under these conditions there is no obvious need for PP_i production and yet significant levels of succinate are still produced.

The low proportion of succinate (and thus PP_i) produced during growth on lactate (1-4%) is consistent with the lack of any PP_i utilising enzyme involved in the metabolism of this substrate and further confirms the apparent correlation between succinate production and PP_i utilisation found during growth on glucose (and glycerol). It would be of interest to study the factors which specifically restrict the diversion of PEP destined for gluconeogenesis from being metabolised via the PEP:carboxytransphosphorylase during growth on lactate. O'Brien and Wood (1974) described a possible control of the carboxytransphosphorylase by ligand-induced subunit interactions as described in Section 1.2. Thus OAA (a product of the reaction), fumarate and malate could inhibit activity by inducing dimerisation. The effect of pyruvate on this enzyme was apparently not determined. However, as it can be a product of the reaction in certain circumstances (Wood, 1977), it may also be able to induce dimerisation. As the levels of pyruvate found were exceptionally high during growth on lactate this could present a plausible mechanism whereby PEP could be prevented from being diverted into succinate production during gluconeogenesis on this substrate. This requires further study.

A further role of G6P in regulating carbohydrate metabolism in P.shermanii may be to control the relative flux via glycolysis and the HMP pathway. A preliminary study of the relationship between G6P

concentration and G6P dehydrogenase activity (Section 7.2) suggests that activity increases up to 20 mM G6P although in a very complex fashion. Thus at high internal G6P concentrations, as in glucose batch cultures during the logarithmic phase of growth, there may be a considerable flow of glucose metabolism via the HMP pathway which bypasses at least one PP_i requiring step of glycolysis. Diversion into the HMP pathway may be further enhanced if the phosphoglucoisomerase reaction, in the direction G6P→F6P, is indeed inhibited at high glucose concentrations as suggested earlier although such a possibility is highly speculative. If a considerable portion of glucose is metabolised to triose via the HMP pathway the problem raised earlier regarding PP_i production and utilisation in glucose batch culture can be explained as follows.

During the early stages of batch culture, when the external glucose and internal G6P concentrations are both high, a significant portion of glucose catabolism is via the HMP pathway to triose and thence via the pyruvate kinase (which would be fully active) to pyruvate and on to propionate and acetate. In this situation the requirement for PP_i for the PP_i-dependent phosphofructokinase and pyruvate, orthophosphate dikinase reactions would be low. The production of PP_i is also low here as there is little flow via the carboxytransphosphorylase since the pyruvate kinase is active. In late logarithmic phase as the external glucose concentration falls so does the internal G6P concentration and the pyruvate kinase would be less active as a result. Under these conditions more PEP would be diverted to succinate production via the carboxytransphosphorylase giving sufficient PP_i to allow a greater proportion of glucose metabolism to occur via glycolysis instead of the HMP pathway.

In conclusion there appear to be three possible roles of G6P as a regulator of carbohydrate metabolism in P.shermanii:

1. Control of the relative rates of PEP metabolism through pyruvate kinase during glycolysis and gluconeogenesis.
2. Control of the relative rates of PEP metabolism via the pyruvate kinase and PEP:carboxytransphosphorylase reactions.
3. Control of the relative metabolic flux into the glycolytic and HMP pathways.

8.3 AREAS FOR FURTHER STUDY

Enzyme Regulation

While the kinetic and regulatory properties of a number of enzymes of carbohydrate metabolism in P.shermanii have now been determined, a further study of several other enzymes would be of interest. Apart from the pyruvate kinase described in this investigation the PEP:carboxytransphosphorylase has also been shown to possess regulatory properties (O'Brien and Wood, 1974). O'Brien et al, (1975) studied the PP_i-dependent phosphofructokinase but were unable to find any allosteric effectors of this enzyme. The reaction mechanism of the pyruvate, orthophosphate dikinase has also been investigated in some detail (Milner and Wood, 1976). However, in view of the strategic position of this enzyme in metabolism and the possibility that it functions both in glycolysis and gluconeogenesis a further study is warranted to determine possible regulatory properties of the enzyme.

The preliminary study of the G6P dehydrogenase described in Section 7.2 clearly indicates the need for a much more detailed investigation of this enzyme. It has been found to be a regulated enzyme in other bacteria

and as it catalyses the first reaction of the HMP pathway it is ideally situated to control the flow of metabolites into that pathway.

Phosphoglucoisomerase catalyses the first reaction in glycolysis after the HMP/glycolytic branchpoint and the unusually high G6P:F6P ratios found in glucose batch cultures have suggested the need for further study of this enzyme.

Allen et al (1964) postulated the presence of a pyruvate dehydrogenase in P.shermanii. However, Castberg and Morris (1978) were unable to demonstrate the presence of the three-enzyme pyruvate dehydrogenase complex found in other microorganisms. The characterisation of this enzyme and a study of its regulation is required to understand the control of acetate formation in propionibacteria.

Carbohydrate Metabolism

On the basis of observations made in the course of this study some areas which deserve a much fuller and more systematic investigation include:

(a) The significance of succinate formation

Succinate accounts for up to 26% of the products during growth on glucose and it has been suggested that formation of this product via the carboxytransphosphorylase may provide at least some of the PP_i required for glycolysis. This in turn may be related, either directly or indirectly, to the relative flux through glycolysis and the HMP pathway by control of G6P levels. Since the amount of succinate produced is affected by the level of CO_2 provided in the medium (Wood and Werkman, 1940) a systematic study of product balances and the relative activity of glycolysis and the HMP pathway at different CO_2 concentrations could provide useful information on the significance of the carboxytransphosphorylase reaction

in controlling carbohydrate metabolism.

(b) The mechanism of glucose uptake.

The relationship between external glucose concentration and internal G6P concentration suggests that glucose uptake may involve more than one uptake system. This relationship needs to be defined more carefully especially in the early stages of growth where G6P levels in excess of 15 mM may occur. It is not known whether the PEP:phosphotransferase system operates in propionibacteria and this requires investigation.

(c) The metabolism of glycerol

The metabolism of glycerol has not been as fully investigated as that of glucose or lactate and the present study has left a number of unanswered questions. The specific activities of both pyruvate kinase and PEP:carboxytransphosphorylase are consistently higher in cells grown on glycerol compared to either glucose or lactate while levels of the pyruvate, orthophosphate dikinase are lower. The reason for the differences in these enzyme levels between the glycerol- and glucose-grown cells is not clear.

There is significant production of succinate during growth on glycerol. The reason for this is not obvious since PP_i is not required for glycolysis on glycerol. This may be related to the requirement for CO_2 for growth on glycerol.

Perhaps the most interesting aspect of glycerol metabolism is that acetate is not produced in the fermentation. The mechanism by which acetate production is shut off is not known and requires further study. The metabolite profile obtained from ^{14}C-labelling of glycolytic intermediates

indicated a major band in the region of glycerol 3-phosphate in the glycerol extract (Section 5.2.2). Although this was not confirmed it would not be unexpected as glycerol 3-phosphate is the first intermediate of glycerol metabolism in the cell. This metabolite might conceivably be involved in inhibiting the production of acetate by affecting one of the enzymes in the pathway to acetate.

(d) Significance of high levels of pyruvate in cells grown on lactate.

The exceptionally high levels of pyruvate found in P.shermanii growing on lactate were not explained and require further study to determine any role they may play in controlling metabolism during growth on this substrate. One possibility that has been suggested already is that pyruvate may be able to inhibit the carboxytransphosphorylase reaction thus preventing the accumulation of succinate during gluconeogenesis from lactate.

Energy Metabolism

Atkinson (1977) considers that the energy charge of living cells remains constant over a broad range of nutritional conditions providing a constant input into the regulation of cellular metabolism. This is consistent with the finding of fairly constant levels of ATP and ADP in extracts of P.shermanii over a wide range of conditions.

However, the in vivo levels of two other intermediates of energy metabolism (P_i and PP_i) probably attain a greater significance in P.shermanii than in other bacteria due to the unique metabolic features of this organism (Section 1.2).

Levels of P_i have not been determined in P.shermanii for reasons discussed elsewhere and while preliminary attempts were made to measure

the levels of PP_i, and indicated the presence of detectable levels, the values obtained are not reliable. The *in vivo* concentrations of these metabolites remain to be determined.

The metabolism of P_i and PP_i in *P.shermanii* is likely to be complex and its regulation may be important in the overall regulation of carbohydrate metabolism. Wood *et al* (1977) suggest PP_i may be generated by electron-transfer-linked phosphorylation to fumarate, while the presence of polyphosphate may also be involved in P_i/PP_i metabolism. As reported by Wood (1977), and confirmed in this study, *P.shermanii* contains an inorganic pyrophosphatase which presumably must be controlled *in vivo*. These are likely to be fruitful areas for further study.

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