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THE REGULATION OF SOME GLYCOLYTIC ENZYMES IN *STREPTOCOCCUS LACTIS*

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ABSTRACT

Certain aspects of the control of carbohydrate metabolism have been studied primarily in S. lactis C_{10}. The kinetic and regulatory properties of two enzymes, lactate dehydrogenase and pyruvate kinase were investigated in some detail whereas a third enzyme, 6-phosphogluconate dehydrogenase, was subjected to a preliminary investigation only. A brief investigation was made of the in vivo concentrations of some metabolites in exponentially growing cells in batch culture.

The S. lactis lactate dehydrogenase (LDH) was purified about 100 fold. The mobility pattern of the purified enzyme on polyacrylamide disc gel electrophoresis was a complex function of pH and ionic strength. From sodium dodecylsulphate-gel electrophoresis the LDH appeared to have a subunit molecular weight of 37,000. A tentative model indicating a pH dependent association/dissociation has been suggested on the basis of the gel results and heat stability studies. At acid and neutral pH values a tetrameric species is favoured. At alkaline pH values (pH 8.0) a dimeric species is favoured. The tetrameric protein is more stable to heat than the dimeric species. The purified LDH requires fructose-1,6-diphosphate (FDP) for catalytic activity at acid and neutral pHs. For pyruvate reduction, in the presence of FDP the pH optimum was 6.9 whereas in the absence of FDP only very low activity was found and the pH optimum was 8.0 to 8.2. The pH optimum for lactate oxidation in the presence or absence of FDP was 8.0 to 8.2 and the activation by FDP was very much less than the FDP activation of pyruvate reduction. The kinetics of lactate oxidation suggested that only the pyruvate reduction direction was significant in vivo.

A significant finding was the effect of different buffers on the FDP activation of LDH. The concentration of FDP required for 50% maximal activity was 0.002 mM when determined in triethanolamine/HCl buffer, 0.2 mM in tris/maleate buffer and 4.4 mM in phosphate buffer; a 2,000 fold difference depending on the choice of the assay buffer. At the pH optimum (pH 6.9) there appeared to be at least two FDP binding sites which interact with each other in a co-operative manner. The choice of buffer was shown to affect other properties of LDH, such as the pH effect on FDP binding, the heat stability of the enzyme at 55°C, the binding of NADH and pyruvate and the effect of the inhibitor, oxamate. Stopped-flow analysis of the LDH showed that a lag period was present at pH 6.9. This lag period could be eliminated by pre-incubation with FDP. No

such lag period was demonstrated at pH 8.2. It is suggested that this lag period is due to a conformational change in the tetrameric species induced by FDP. The properties of the S. lactis LDH, taking into account the buffer effects, have been discussed in terms of the carbohydrate metabolism and related to other FDP-activated streptococcal LDH's. A brief comparative study of the S. faecalis ATCC 8043 LDH was made. The two major findings were its insensitivity to phosphate inhibition and its activation by manganese ions.

Pyruvate kinase was purified to near homogeneity as determined by polyacrylamide gel electrophoresis, with and without SDS. With SDS, a subunit molecular weight of 60,750 was determined. From equilibrium sedimentation studies the molecular weight of the native protein is 235,000. The enzyme is therefore a tetrameric protein. The kinetic properties of the pyruvate kinase were more complex than those of LDH, for as well as requiring FDP as an activator, the enzyme had an essential requirement for both a monovalent and divalent cation. FDP under most conditions bound to the enzyme in a co-operative manner. Phosphoenolpyruvate (PEP), and to a lesser extent, ADP, showed co-operative binding to the enzyme only at unsaturating FDP concentrations. Both the monovalent and divalent cations showed co-operative binding to the enzyme in the presence of saturating FDP concentrations. The activation properties of the enzyme were considerably different when Mn^{++} was substituted for Mg^{++} as the divalent cation. Like LDH, the pyruvate kinase was also affected by the nature of the buffer components. Pyruvate kinase was inhibited by lower concentrations of phosphate than were required to inhibit LDH. In addition the pyruvate kinase activity was inhibited by high concentrations of Mg^{++} and ADP. The properties of the S. lactis pyruvate kinase have been discussed in relation to other pyruvate kinases and to carbohydrate metabolism in S. lactis.

The S. lactis 6-phosphogluconate dehydrogenase (6-PGDH) did not appear to be inhibited by FDP, nor did the enzyme from S. faecalis ATCC 8043. This is contrary to published findings by other workers. Because of the preliminary nature of this investigation, further work is required on the S. lactis 6-PGDH to establish whether or not its activity is regulated by FDP.

The in vivo concentration of several metabolites were determined in exponentially growing cells and related to the in vitro kinetic properties of the two enzymes, LDH and pyruvate kinase. The metabolites

studied were; FDP, PEP, triose phosphates, ADP, ATP, glucose-6-phosphate and pyruvate. The *in vivo* FDP concentration was at a sufficiently high level (12.7 to 14.9 mM) to fully activate the two enzymes as indicated by *in vitro* determinations under a number of different assay conditions. The *in vivo* studies have suggested further *in vitro* kinetic studies which may be useful to investigate to gain a fuller understanding of the regulation of carbohydrate metabolism in *S. lactis*.

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SECTION 1

GENERAL INTRODUCTION

1.1 General Characteristics of the Lactic Acid Bacteria.

The lactic acid bacteria are biochemically characterized by their main fermentation end product, lactic acid. They can be classified into two broad groups, i.e. either homofermentative or heterofermentative. The homofermentative or homolactic group is characterized by the formation of lactic acid as the only major product from glucose fermentation. The genera *Streptococcus*, *Diplococcus*, *Pediococcus*, and most members of the genus *Lactobacillus* belong to this group. The heterofermentative group, as well as having lactic acid as a fermentative end product, produce considerable amounts of CO_2, ethanol or acetic acid. Other fermentation end products may be formed and include glycerol, propionic acid and butyric acid. This group includes all members of the genus *Leuconostoc* and *Peptostreptococcus* and some members of the genus *Lactobacillus*. For reviews see Wood (1961) and Doelle (1969).

It is within the genus *Streptococcus*, that the important cheese starter organisms are found. This group, the lactic streptococci, consists of *Streptococcus lactis*, *Streptococcus cremoris* and variants. The biochemical property that makes the lactic streptococci particularly important in the manufacture of cheese is their ability for consistent and rapid production of lactic acid. They also have the ability to impart desirable flavour and texture to cheese.

1.2 The Main Pathways of Carbohydrate Catabolism in Lactic Acid Bacteria

a) Homofermentative organisms

The homofermentative organisms use the well known Embden-Meyerhof-Parnas (EMP) pathway to convert glucose into two molecules of pyruvate. The pyruvate is subsequently reduced by the enzyme lactate dehydrogenase (LDH) to form lactic acid. Hence the production of two molecules of lactate per molecule of glucose, by homofermentative organisms, is the same as that found in muscle glycolysis. In the majority of cases the streptococcal species form the L(+)-isomer of lactate. However other homofermentative species may form the D(-)-isomer or a combination of D(-)- and L(+)-lactate (Wood, 1961). The lactate dehydrogenase reaction, catalyzing lactate formation, is an important step as it is the means of replenishing NAD^+. A ready supply of NAD^+ is important for the continual functioning of the EMP pathway.

Gibbs et al. (1950) confirmed that Lactobacillus casei, a homofermentative bacterium, fermented glucose to lactic acid by the EMP glycolytic pathway. These workers used isotopic studies with specifically labelled glucose-^{14}C. The fermentation of glucose -1-^{14}C, under both anaerobic and aerobic conditions by L. casei lead to the label appearing almost exclusively in the methyl carbon of lactate. There was a 50% dilution of the specific activity in the methyl carbon compared with that of glucose-carbon 1. When glucose -3,4-^{14}C was fermented, carboxyl-labelled lactate was formed without dilution of the specific activity. The distribution of the label in the final product is thus consistent with the functioning of the EMP glycolytic pathway.

b) Heterofermentative organisms.

De Moss et al. (1951) found that the heterofermentative bacterium, Leuconostoc mesenteroides produced CO_2, ethanol and lactic acid in a constant ratio of 1:1:1 and that the EMP enzymes, aldolase and triose phosphate isomerase were absent. These findings are not consistent with the operation of the glycolytic pathway. Gunsalus and Gibbs (1952) showed that Leuconostoc mesenteroides gave product labelling that was also not consistent with the operation of the EMP glycolytic pathway. The fermentation of glucose-1-^{14}C by L. mesenteroides resulted in all of the isotope being found in CO_2 whereas in the homofermentative organism, Lactobacillus plantarum, about 97% of the isotope appeared in the methyl carbon of lactate. With glucose-3,4-^{14}C, L. mesenteroides also gave a different pattern from that in L. plantarum, as the isotope was contained in carbon 2 of the ethanol and the carboxyl carbon of the lactate.

Since in the heterofermentative bacteria, the carbon 1 of glucose yields CO_2, the hexose monophosphate (HMP) pathway was implicated. However a thiamine pyrophosphate-dependent enzyme has been purified from extracts of Lactobacillus plantarum (when growing on pentose - this species is homofermentative on glucose) which catalyzes the phosphorolytic cleavage of D-xylulose-5-phosphate to form acetyl phosphate and triose phosphate. The enzyme, called phosphoketolase (E.C.4.1.29) is specific for D-xylulose-5-phosphate (Heath et al., 1958). The phosphoketolase is distinct from the hexose monophosphate pathway enzyme, transketolase, which does not participate in the formation of acetyl phosphate. The phosphoketolase was also demonstrated in Leuconostoc mesenteroides by

Hurwitz (1958). The triose phosphate formed, glyceraldehyde-3-phosphate, yields lactic acid by the action of the EMP pathway enzymes and LDH. Acetylphosphate is dephosphorylated and either forms acetic acid or is reduced to ethanol via the intermediate formation of acetaldehyde. Under anaerobic conditions, complete reduction of acetylphosphate generally occurs. If oxygen is present, some of the acetylphosphate is converted to acetic acid and some to ethanol. Therefore theoretically, heterofermentative bacteria convert one molecule of glucose into one molecule each of CO_2, lactic acid and ethanol or acetic acid (Kandler, 1961). For reviews see Wood (1961), Marth (1962), Reiter and Moller-Madsen (1963) and Doelle (1969).

c) Other possible pathways.

The Entner-Doudoroff pathway (for reviews see Wood, 1961 and Doelle, 1969) has been well documented in pseudomonads. The two distinctive enzymes are phosphogluconate dehydratase (E.C.4.2.1.12) and 2-phospho-2-keto-3 deoxygluconate aldolase. These two enzymes are not found in the HMP, EMP or the phosphoketolase pathways. In S. faecalis (Sokatch and Gunsalus, 1957) gluconate can serve as an energy source. From both the fermentation balance and the tracer experiments it was suggested by Sokatch and Gunsalus that the complete conversion of gluconate to CO_2 and lactate occurred by a combination of the HMP, EMP and Entner-Doudoroff pathways. However, S. faecalis, a homofermentative bacterium, when grown on glucose, forms lactate more or less exclusively from the EMP pathway and LDH (Platt and Foster, 1958). Therefore the carbon source can alter the fermentation pathways. Reiter and Moller-Madsen (1963), from an observation by Kandler and Busse, suggest that S. lactis may be capable of fermenting glucose via the Entner-Doudoroff pathway. However, there is no further conclusive experimental evidence for this type of metabolism in S. lactis and the Embden-Meyerhof glycolytic pathway is undoubtedly the quantitatively significant pathway in S. lactis and other lactic streptococci.

As a result of the enzymatic studies of a large number of lactic acid bacteria, Buyze et al. (1957) have classified the lactic and bacteria into three fermentative types:

a) Obligate homofermenters, possessing fructose diphosphate aldolase but lacking glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

b) Obligate heterofermenters, possessing both the dehydrogenases mentioned in a) but lacking fructose diphosphate aldolase.

c) Facultative homofermenters, possessing all three enzymes but generally metabolizing carbohydrate by means of the Embden-Meyerhof pathway and LDH.

S. lactis appears to belong to the last group where the EMP pathway is the quantitatively significant pathway. As mentioned by Doelle (1969), organisms that generally grow on complex media (meat and yeast extract) use the EMP pathway for ATP generation. The EMP pathway does not however provide pentose sugars which are essential for nucleotide and nucleic acid biosynthesis. Pentose sugars arise from the HMP pathway of carbohydrate metabolism. The complete sequence of the HMP pathway enzymes and functioning of the HMP pathway does not appear from the literature to have been demonstrated in S. lactis. However, glucose-6-phosphate and 6-phosphogluconate dehydrogenases are present in S. lactis, so this species appears to have the potential to use the oxidative portion of the HMP pathway. The use of the incomplete HMP pathway by S. lactis could be sufficient to meet the biosynthetic requirements of the cell (NADPH and ribose-5-phosphate - if ribose phosphate isomerase is present).

1.3 Formation of Products other than Lactate by Streptococci.

As previously mentioned, the genus Streptococcus is classified as belonging to the homofermentative group, where lactic acid is the main product of glucose fermentation. Experimental evidence from a number of workers indicates that pyruvate is formed more or less exclusively from the EMP pathway and is subsequently reduced to lactate by the action of the NAD^+-dependent lactate dehydrogenase. However it is well documented that products other than lactate may result from fermentation, although these products are formed in small amounts only.

Friedemann (1939) found that in S. lactis the yield of lactic acid was 89%; low amounts of ethanol, acetate and formic acid, plus undetermined non-volatile acids were detected. In S. liquefaciens strain 815, a homofermentative organism, more than 90% of the glucose

fermented was converted to lactate (Gunsalus and Niven, 1942). However at pH 6.5 or higher, the combined yields of formic and acetic acid and ethanol may account for 25 to 40% of the sugar fermented. Platt and Foster (1958) made a quantitative study of the products by seven homofermentative lactic streptococci in their anaerobic fermentation of glucose. Lactate was the major product of glucose metabolism in fermentation with and without pH control. The by-products included ethanol, acetic acid, glycerol, diacetyl, acetoin, 2,3-butanediol and CO_2. In S. lactis, acetic acid, CO_2 ethanol and acetoin were detected, but no formic acid as in other reports. The S. lactis and S. cremoris strains were different from the five other species in that smaller amounts of by-products were formed at pH 7.0 than in fermentations allowed to become acid. Platt and Foster (1958) concluded from oxidation-reduction indices and carbon recoveries that the products found in small amounts, as well as the major product lactate, were formed from glucose. With $C^{14}O_2$, Platt and Foster showed that three species (includes S. lactis and S. cremoris) can fix CO_2. White and Sherman (1943) examined the effect of aeration on glucose fermentation by a range of streptococci. While little difference was observed between anaerobic and normal aerobic conditions, vigorous aeration markedly inhibited growth and resulted in an associated decrease in lactic acid production.

The presence of other minor fermentation products, by streptococci, indicates that the potential is present for fermentation of pyruvate by enzyme mechanisms other than lactate dehydrogenase, e.g. pyruvate-formate lyase, Lindmark et al. (1969).

1.4 Oxidative Phosphorylation in Lactic Acid Bacteria.

Lactic acid bacteria normally lack haematin enzymes in their electron transport system (Dolin, 1953). However evidence has accumulated to show that oxygen can be of benefit in the utilization of sugars and polyhydroxy alcohols by certain bacteria (Gunsalus and Sherman, 1943; Gunsalus and Umbreit, 1945 and Dolin, 1955). Gallin and Van Demark (1964) produced evidence for oxidative phosphorylation in Streptococcus faecalis and postulated that phosphorylation may be coupled to electron flow through an NADH/flavin system or during the oxidation of naphthoquinone. Later Smalley et al. (1968) used molar growth yields as evidence for oxidative phosphorylation with the same

organism, and calculated a P:O ratio approximating to 0.6. Whittenbury (1964) observed that many types of lactic acid bacteria synthesize catalase when provided with haematin and that a few strains also formed cytochromes of the a and b types. Whittenbury raised the possibility that some lactic acid bacteria might form a functional oxidative phosphorylation system. Later Bryan-Jones and Whittenbury (1969) presented evidence for the presence of a cytochrome electron transport system in haematin-grown S. faecalis which results in oxidative phosphorylation coupled to the oxidation of reduced pyridine nucleotide. The oxidative phosphorylation system was found in the membrane fraction, which contained a b_2 type cytochrome. However as mentioned by Bryan-Jones and Whittenbury (1969), though the presence of haematin stimulates S. faecalis to behave like an aerobe, it still performs inefficiently in that a functional Krebs cycle is lacking and the final products of glucose oxidation include lactate, acetate and acetoin. Ritchey and Seeley (1974) showed that functional cytochromes were found in the membrane fraction of Streptococcus faecalis var. zymogenes if grown aerobically with haematin. Inhibitors and uncouplers of oxidative phosphorylation verified the presence of cytochromes in the membrane fraction. Molar growth yields gave further evidence for oxidative phosphorylation. When the bacteria were grown without haematin only a flavin system of electron transport was present without additional ATP generation. The primary product during aerobic growth, with haematin, was acetate (92%) with small amounts of lactate, acetylmethylcarbinol and ethanol. This contrasts to anaerobic growth where 89% lactate was present with only small amounts of acetate, ethanol, acetylmethylcarbinol or 2,3-butanediol.

Sijpesteijn (1970) investigated the aerobic growth of S. lactis and Leuconostoc mesenteroides in a medium containing haematin and showed that the spectra of the a and b types of cytochromes was present. The S. lactis cells when grown in a glucose-basal-medium showed a poor capacity to take up oxygen and the major end product was lactic acid with only a small amount of acetic acid formed and a trace amount of acetoin. With a supplement of haemin (10 ppm) the O_2 uptake increased three fold and more acetic acid, acetoin and CO_2 were formed with an associated drop in lactic acid production. Sijpesteijn suggested that the cytochrome system was mainly responsible for the oxidation

of NADH with the NADH oxidase(s) playing only a minor role. The growth of *S. lactis* is stimulated by incorporation of haemin which may indicate that the cytochrome-mediated respiration gives rise to more ATP than the NADH-oxidase mediated respiration. However unlike *S. faecalis*, there is no direct evidence that cytochrome-mediated respiration in *S. lactis* is coupled to oxidative phosphorylation.

It is evident that under normal growth conditions *S. lactis* and other homofermentative bacteria, use pyruvate as the terminal hydrogen acceptor via the action of LDH. Though NADH oxidases are present, their function is not known, but they probably do not contribute to ATP production even under aerobic conditions. However if haemin is added to the growth medium, it is possible for at least some homofermentative bacteria, including *S. lactis*, to develop a functional cytochrome system which allows NAD^+ regeneration by an oxidative pathway and possibly an associated production of ATP. However, as yet, no completely functional Krebs cycle has been demonstrated under any circumstances for lactic acid bacteria. Therefore it can be concluded that the major energy production from carbohydrate metabolism is at the substrate level of phosphorylation of the Embden-Meyerhof-Parnas pathway.

1.5 Lactose and Galactose Metabolism.

McKay *et al.* (1969) studied the involvement of PEP in lactose utilization by group N *Streptococci*. Two *S. lactis* strains were studied (Strains 7962 and C_2F) and from the effect of sodium fluoride on the metabolism of lactose and o-nitrophenyl-β-D-galactopyranoside it was suggested that different mechanisms of lactose utilization exist in the two strains. The *S. lactis* C_2F, which had no β-galactosidase, was dependent on a PEP-dependent phosphotransferase system for lactose utilization, whereas in *S. lactis* 7962 the β-galactosidase was responsible for lactose utilization. McKay *et al.* (1970) by enzymatic and genetic analysis showed that the *S. lactis* C_2F hydrolysed o-nitrophenyl-β-D-galactopyranoside-6-phosphate (ONPG-6-P) and transported lactose by a PEP-dependent system which is similar to that found in *Staphylococcus aureus* (Simoni *et al.*, 1968). The enzyme β-D-phosphogalactoside galactohydrolase which hydrolyses lactose-phosphate to glucose and D-galactose-6-phosphate has been purified and studied by Johnson and McDonald (1974).

Bissett and Anderson (1973) showed that in Staphylococcus aureus, D-galactose-6-phosphate is metabolised further through the tagatose pathway: D-galactose-6-phosphate → D-tagatose-6-phosphate → D-tagatose-1,6-diphosphate → D-glyceraldehyde-3-phosphate + dihydroxyacetonephosphate. This differs from the usual pathway in which lactose is metabolised by β-galactosidase, to form D-glucose and D-galactose followed by the conversion of D-galactose to D-glucose-6-phosphate via the well known Leloir pathway (Maxwell et al., 1962): D-galactose → D-galactose-1-phosphate → UDP-galactose ⇌ UDP-glucose → D-glucose-1-phosphate → D-glucose-6-phosphate. Since the group N streptococci possess mainly phospho-β-galactosidase activity and little β-galactosidase activity (with only a few exceptions, see Vakil and Shahani, 1969 and McKay et al., 1970) a tagatose-6-phosphate pathway would be expected. Bissett and Anderson (1974) showed that of the four streptococci studied all possessed the enzymes for both the tagatose and the Leloir pathway. Other bacteria such as E. coli, S. aureus and Aerobacter aerogenes possess the enzymes of only one pathway. Hence streptococci have the enzymatic potential to use both pathways. However the relative participation of the two pathways is not known. Preferential use of the tagatose pathway would circumvent the opportunity for G-6-P to pass through at least the oxidative proportion of the HMP pathway. Also tagatose-1,6-diphosphate may be sufficiently different from FDP (an important control metabolite in many organisms) in its allosteric control of enzymes to effect a different cellular metabolism.

1.6 Metabolism of Non-Carbohydrate Compounds.

S. lactis C_{10} can grow on a number of carbohydrates, including glucose, galactose, lactose, mannose, salicin, fructose, maltose and dextrin, but not on sucrose, mannitol, arabinose, raffinose and sorbitol (Sandine et al., 1962). Apart from carbohydrate, arginine is the only substrate reported from which S. lactis can obtain energy (Barker, 1961).

Certain species of enterococci in the genus Streptococcus are able to metabolize pyruvate, citrate, malate, glycerol or gluconate alone as sources of energy for anaerobic growth (review by Deibel, 1964). London et al. (1970, 1971) showed that an inducible malic enzyme (decarboxylating) is present in strains of S. faecalis (and L. casei). The malic enzyme from both species was inhibited by the

glycolytic intermediates FDP and 3-phosphoglycerate. They report that other *Lactobacillus* and *Streptococcus* species (including *S. lactis*) which are unable to grow on malate, do not synthesize this inducible enzyme.

Streptococcus diacetilactis was unable to use citrate as a source for growth but the addition of citrate to a lactose-containing medium increased the specific growth rate by 35% (Harvey and Collins, 1963). Marth (1962) suggests that fermentation of citrate results in production of pyruvate without a simultaneous supply of reduced NAD^+, and hence products other than lactic acid are formed. The manner by which *S. diacetilactis* produces acetoin from citrate has been studied by Sandine *et al.* (1961). A citritase enzyme converts citrate into acetate and oxaloacetate, and an oxaloacetate decarboxylase catalyzes the production of pyruvate. Pyruvate is then converted to acetolactate and subsequently to acetoin.

Certain strains of lactic streptococci can also produce volatile carbonyl compounds and fatty acids from some amino-acids, although usually only in trace amounts (MacLeod and Morgan, 1958, Nakae and Elliott, 1965a and b).

1.7 Bacterial Regulatory Mechanisms: General Comments.

"It is evident that in any highly complex metabolic system, composed of interlocking and overlapping biosynthetic and degradative pathways, the regulation of certain strategic enzyme activities is more critical to the maintenance of fine balance between the various metabolic functions than is the regulation of others.", (Stadtman, 1966). The control of the strategic enzyme activities can be achieved in many ways (for reviews, see Sanwal, 1970; Stadtman, 1966; Stadtman, 1970 and Atkinson, 1970). The two basic controls are via regulation of enzyme activity and regulation of enzyme concentration. The regulation of enzyme concentration can be achieved in a number of ways: a) Substrate induction of enzyme synthesis, b) Catabolite repression, c) Feedback repression of enzyme synthesis, and d) Enzyme inactivation and degradation (Stadtman, 1970). Regulation of enzyme activity can also be achieved in a number of ways which include: a) Allosteric regulation of enzyme activity (Stadtman, 1966), b) Energy-linked controls (the energy charge of the adenylate system - Atkinson, 1968 and 1970) which may be of the allosteric type, c) Energy-dependent covalent modification of regulatory enzymes (*E. coli* - glutamine synthetase - Shapiro and Stadtman, 1967).

The allosteric regulation of enzyme activity is the most intensively studied area of regulation by control of enzyme activity. Allosteric enzymes show susceptibility to activation or inhibition by metabolites (modifiers or effectors) which may be quite distinct from the substrate or may be one or other of the substrate molecules. The original use of the term "allosteric" referred to situations in which the effector was structurally quite distinct from the substrate and bound at a distinct site (allosteric site). However, as Atkinson (1966) points out, "the word allosteric has since been employed in a variety of contexts, often with no vestige of the original definition in evidence".

Umbarger (1956) noted for E. coli threonine deaminase, that the substrate saturation function is sigmoidal rather than hyperbolic. Sigmoid kinetics indicate the existence of two or more interacting binding sites on the enzyme such that the binding of one molecule facilitates (or inhibits) the binding of a further molecule. If the co-operative binding interaction is between molecules of the same sort (whether these be substrate or distinct effector molecules) the interaction is termed homotropic. If the co-operative binding of a molecule is affected by a structurally distinct molecule then the interaction is termed heterotropic. The sigmoidal (or co-operative) velocity response is associated with most, but not all, allosteric enzymes (Stadtman, 1970).

Hill (1913) in studying the binding curve of oxygen to hemoglobin, developed a useful method of expressing the data, the Hill plot, which can be used as a diagnostic test for allosteric enzymes. In cases where there is co-operative interaction between binding sites the relationship between reaction velocity and effector concentration may be represented by the equation:

$$\log v/V_{max}-v = n_H \log M - \log K.$$

where n_H = Hill interaction coefficient
M = concentration of modifier (effector)
v = reaction velocity
V_{max} = maximum reaction velocity at optimum concentration of modifier (effector)
K = a constant

The Hill plot is a plot of $\log v/V_{max}-v$ versus $\log M$. The Hill interaction coefficient (n_H) obtained from the slope of the Hill plot,

indicates the minimum number of sites that are showing co-operative interaction. A Hill interaction coefficient (n_H) of greater than one indicates positive co-operativity, less than one indicates negative co-operativity and a n_H value equal to one indicates non-interacting sites (i.e. modifier binding shows hyperbolic kinetics). When the modifier is an inhibitor, the Hill interaction coefficient will be expressed as a negative slope (i.e. n_H = -numerical value). The concentration of modifier giving half maximum velocity, determined where $v/V_{max}-v = 1$ or $\log v/V_{max}-v = 0$, will be referred to as $M_{0.5V}$ in the present study except in cases (such as pyruvate kinase) where both substrate and effector show co-operativity. In these cases the particular substance will be specified, e.g. $PEP_{0.5V}$.

1.8 Some Aspects of Regulation of Carbohydrate Metabolism in Lactic Streptococci.

The regulation of carbohydrate metabolism in *E. coli* and related bacteria has been fairly thoroughly studied (Sanwal, 1970 and Kornberg, 1970). The carbohydrate and energy metabolism of homofermentative lactic acid bacteria differs in many respects from that of the enteric bacteria as described in Sections 1.1 to 1.6 (with special reference to *S. lactis*):

a) The EMP pathway is probably the only quantitatively significant pathway for formation of pyruvate from glucose.

b) Most of the pyruvate formed from glucose is converted to lactate. The potential exists for pyruvate to be converted to other products, but this potential is rarely realised to any appreciable extent at least in the lactic streptococci.

c) At least the oxidative enzymes of the HMP are present in *S. lactis* (while the remaining enzymes of the HMP are possibly absent).

d) There is no functional Kreb's cycle and, in the absence of haematin in the medium, no cytochrome system.

e) Lactose and galactose can be metabolised by two possible routes - the tagatose pathway and the Leloir pathway.

f) Phosphoenolpyruvate phosphotransferase is probably the main sugar transport system for all sugars including lactose.

g) Since no growth occurs on a casein hydrolysate-yeast extract medium without added carbohydrate, it would appear that gluconeogenesis cannot occur in these bacteria.

The above metabolic features of the lactic streptococci would involve distinctive regulatory features compared to other bacteria such as E. coli.

Because of the importance of the EMP pathway in these bacteria as the major energy production pathway by substrate level phosphorylation, this pathway must be controlled to meet the energetic and biosynthetic needs of the bacteria. Lactate dehydrogenase (LDH) in catalyzing the terminal step in glycolysis, fulfils the important function of NAD^+ regeneration. If NAD^+ regeneration is not effective then glycolysis cannot proceed beyond the formation of glyceraldehyde-3-phosphate and the nett result is no ATP production. Since the lactic streptococci normally possess no cytochrome system, no transhydrogenase activity and very little NADH oxidase activity, the only NAD^+ regeneration system is LDH. Therefore the regulation of LDH has the potential for controlling the overall activity of the EMP pathway.

Wolin (1964) reported that the L(+)-LDH's from several species of Streptococcus were specifically activated by FDP. Other glycolytic intermediates have no significant effect on activity. This activation appears to be present in only a few bacterial species other than streptococci. This FDP activation of LDH's is probably one way of ensuring a sufficient intracellular concentration of NAD^+. The FDP-activated LDH's will be reviewed more intensively in Section 2.1.

Another important aspect of the carbohydrate metabolism of lactic streptococci is the role of phosphoenolpyruvate (PEP). PEP can be converted to pyruvate by pyruvate kinase, one of the two EMP pathway reactions involved in ATP generation. PEP is also required by the PEP-phosphatransferase system. Control of PEP metabolism is therefore of considerable importance in streptococcal carbohydrate metabolism. Collins and Thomas (1974) showed recently that pyruvate kinase in lactic streptococci is activated by FDP. The role that pyruvate kinase may play is discussed more fully in Section 3.1.

Sanwal (1970) states that: "One of the enigmatic problems perhaps central to the understanding of carbohydrate metabolism is the nature of control system (or systems) which determines the distribution of glucose-6-phosphate into the pentose phosphate pathway on the one hand and the glycolytic pathway on the other". Since at least the oxidative enzymes of the HMP pathway are present in most streptococcal species, it is evident that some control must exist. Model and Rittenberg (1967) suggest for E. coli that the oxidative pathway is regulated by the availability of $NADP^+$. In S. faecalis Brown and Wittenberger (1971a) have shown that 6-phosphoglyconate dehydrogenase (6-PGDH) is specifically inhibited by FDP.

This aspect of metabolism of streptococci will be reviewed more intensively in Section 4.1.

In a number of S. mutans strains, that lack the oxidative portion of the HMP pathway and transhydrogenase activity, Brown and Wittenberger (1971b) showed that the enzyme, glyceraldehyde-3-phosphate exists as two separable isoenzymes. One of the enzymes is NAD^+-linked and the other is an $NADP^+$-linked enzyme. Brown and Wittenberger suggested that a physiological role for the $NADP^+$-linked enzyme is as a metabolic alternative to the oxidative portion of the HMP pathway in generation of NADPH.

At least three streptococcal enzymes appear to be regulated by FDP (LDH, pyruvate kinase and 6-PGDH). The enzymes, FDP aldolase and phosphofructokinase involved in the formation and degradation of FDP, and the tagatose pathway enzymes which bypasses FDP, all may play a role in control, by affecting the FDP level which in turn controls three important enzymes. The enzyme phosphofructokinase from Lactobacillus casei and Lactobacillus plantarum does not exhibit the sigmoidal kinetics (Doelle, 1972). While ADP, AMP and cyclic AMP inhibit the L. plantarum enzyme, the L. casei is not affected by these three nucleotides. Doelle concludes from the evidence that phosphofructokinase of these species is not an allosteric protein. This is in contrast to the allosteric properties of the phosphofructokinase from many mammalian and bacterial sources. Phosphofructokinase of lactic streptococci does not appear to have been investigated, but if the findings of Doelle apply to these organisms as well, then it is clear that the regulatory mechanisms in glycolysis are quite different from those operating in many other bacteria.

1.9 General Aims of the Present Investigation

S. lactis C_{10} was selected for the present study because of its importance in the New Zealand dairy industry as a cheese "starter" organism. While some information on regulatory mechanisms has been obtained for *S. faecalis* and for some cariogenic streptococci such as *S. mutans*, little information was available at the outset of this study on the lactic streptococci.

Three *S. lactis* C_{10} enzymes were chosen for study. They are lactate dehydrogenase, pyruvate kinase and 6-phosphogluconate dehydrogenase. A common feature of these three enzymes is the potential role of FDP in controlling their activities and as previously mentioned in Section 1.8, these enzymes may play important roles in the control of carbohydrate metabolism. To ascertain the possible role these three enzymes play in control, a detailed kinetic study of the enzymes was planned to investigate a large number of potential factors that may affect activity. To supplement the kinetic studies on the three enzymes an investigation of the *in vivo* levels of metabolites that affect their activities was also undertaken. By investigation of these three important enzymes it was hoped to arrive at a better understanding of their role in control of *S. lactis* carbohydrate metabolism.

SECTION 2

LACTATE DEHYDROGENASE

2.1 Introduction

Lactate dehydrogenase (LDH), (lactate : NAD^+ oxidoreductase) is widely distributed among living organisms. The most thoroughly studied is the LDH from higher animal tissues, but LDH's have also been studied in invertebrates, some plants, several fungi and many bacteria. The usual function is catalysis of the terminal step in glycolysis which serves to reoxidize the reduced NAD^+, generated in glycolysis under anaerobic conditions, or in those organisms lacking a mechanism for aerobic oxidation of NADH. Under appropriate conditions the enzyme may also catalyse lactate oxidation.

2.1.1 Mammalian L(+)-lactate Dehydrogenase

The L(+)-lactate dehydrogenase (L(+)-lactate : NAD^+ oxidoreductase, E.C. 1.1.1.27)(LDH) from higher animal tissues has been extensively studied (for comprehensive reviews see Schwert and Winer, 1963 and Everse and Kaplan, 1973). It is not relevant to the present study to review this work in detail but a brief summary of the main properties with particular emphasis on regulation is provided in the following paragraphs to serve as a basis for comparison with the streptococcal and other microbial LDH's.

In all higher animals, the product of the reduction of pyruvate is the L(+)-isomer of lactate and only the LDH's from certain invertebrates and some bacteria catalyze the formation of the D(-)-lactate isomer. All the animal L(+)-LDH's that have been purified to date have a molecular weight of approximately 140,000 and consist of four subunits (the invertebrate D(-)-LDH's, D(-)-lactate : NAD^+ oxidoreductase, E.C. 1.1.1.28, are exceptions which are dimeric and have molecular weights of about 70,000, e.g. horseshoe crab (Long and Kaplan, 1968)). Each of the four subunits of the L(+)-LDH's has a molecular weight of approximately 35,000. In mammalian LDH's there are two basic subunits (designated H and M) which can combine to give five different tetrameric isoenzymes, H_4, H_3M, H_2M_2, HM_3 and M_4 (Wilkinson, 1969). The H_4 form predominates in the heart tissue and the M_4 in muscle and these two forms of LDH have been most extensively studied. Though structurally similar there are physiologically significant differences between the H_4 and M_4 isoenzymes (Wilkinson, 1969), the most notable difference being in the sensitivity of the two forms to substrate inhibition (see next page).

Lactate dehydrogenase is thought to have an ordered, compulsory sequence of addition and release of substrates and products. Evidence for this comes from equilibrium isotope exchange experiments (Silverstein and Boyer, 1964) on bovine heart and rabbit muscle enzymes, from kinetic isotope effects for rabbit muscle L(+)-LDH (Thomson *et al.*, 1964) and from product inhibition studies (Zewe and Fromm, 1962). Fluorescence studies by several workers (Winer *et al.*, 1957 and Fromm, 1963) supported the proposed reaction scheme for LDH and gave evidence for binary and ternary complexes. All these lines of evidence indicate that the coenzyme NAD^+ (or NADH) must bind to the enzyme before binding of the substrate, lactate (or pyruvate).

The mammalian L(+)-LDH's are inhibited in the presence of high levels of substrates (Cahn *et al.*, 1962). This inhibition is observed in either direction and with both types of enzymes, although the inhibition is much more pronounced with the H-type LDH than with the M-type LDH.

The existence of abortive or dead-end ternary complexes (Arnold and Kaplan, 1974) has been extensively studied and evidence has been obtained in a number of laboratories that the phenomenon of pyruvate inhibition (Kaplan *et al.*, 1968) is related to the formation of such abortive ternary complexes. The metabolic control of H-type LDH by its own substrate and coenzyme via abortive ternary complexes is physiologically important as it restricts pyruvate reduction while retaining the capacity for lactate oxidation in the heart. Since the heart requires large amounts of ATP, metabolic control of H_4-LDH by pyruvate, ensures that pyruvate is metabolised by oxidation through the citric acid cycle with subsequent ATP production rather than being reduced to lactate. The kinetic properties of the H_4-LDH and M_4-LDH are such that under *in vivo* conditions, H_4-LDH favours lactate oxidation and M_4-LDH favours pyruvate reduction.

2.1.2 Bacterial Lactate Dehydrogenases

In contrast to the knowledge of mammalian LDH very much less is known about LDH's in bacteria even though this enzyme is of widespread occurrence. The properties of LDH's of bacteria appear to be more diverse than those of vertebrates. Bacterial LDH's are of two types, one NAD-linked and the other NAD-independent (usually flavine-linked).

The latter type may either be soluble or membrane bound. The membrane bound LDH's of some bacteria are thought to be of particular importance in the transport of metabolites across the bacterial membrane (Kaback and Milner, 1970). However the general functional significance of the NAD-independent LDH's in bacteria is still not clear (Stevenson and Holdsworth, 1973 and Doelle, 1971 c) and they will not be further discussed.

However, even if only the NAD-dependent LDH's are considered it is clear that many different types of enzyme exist in microorganisms, sometimes more than one type in the same organism.

NAD-dependent LDH's from the following bacteria have been purified and studied fairly fully.

Butyribacterium rettgeri	D(-)-LDH	Wittenberger and Haaf (1966) Wittenberger and Fulco (1967) Wittenberger (1968)
Escherichia coli	D(-)-LDH	Tarmy and Kaplan (1968 a and b)
Bacillus subtilis	L(+)-LDH	Yoshida and Freese (1965) Yoshida (1965)
Lactobacillus species	both D(-) and L(+)-LDH	Snoswell (1959) Snoswell (1961) Snoswell (1963) Dennis and Kaplan (1960) Dennis *et al.* (1965) Gasser *et al.* (1970)
Streptococcus species	L(+)- (rarely D)	see next section.

Other less extensive studies on bacterial LDH's include the LDH from *Staphylococcus aureus* (Stockland and San Clemente, 1969), *Acholeplasma laidlawii* (Neimark and Tung, 1973) and *Leuconostoc* (Garvie, 1969, Garland, 1973, and Doelle, 1971 a, b). A brief review of the properties of only the more fully studied bacterial LDH's will be given.

The D(-)-specific LDH from Butyribacterium rettgeri was studied by Wittenberger and Haaf (1966), Wittenberger and Fulco (1967) and Wittenberger (1968). The enzyme follows normal hyperbolic saturation kinetics with respect to its coenzyme, NADH, but unlike mammalian L(+)-LDH's and many bacterial D(-) and L(+)-LDH's it exhibits a sigmoidal kinetic response to increasing concentrations of pyruvate. Reduction of NAD^+ by lactate could not be demonstrated under a variety of conditions that favour lactate oxidation by mammalian L(+)-LDH's. The B. rettgeri D(-)-LDH is sensitive to inhibition by ATP, but unlike mammalian LDH's (Geyer, 1967) where ATP inhibition is competitive with respect to NADH, the inhibition was complex, with ATP being partially competitive with respect to NADH. The inhibition by ATP did not occur at low pyruvate concentrations. Since increasing pyruvate concentrations increase the apparent K_M for NADH, the authors suggest that ATP probably interacts with the B. rettgeri enzyme at a site separate and distinct from the coenzyme binding site.

Escherichia coli B D(-)-LDH has been studied by Tarmy and Kaplan (1968a and b). Like Butyribacterium rettgeri LDH, it shows sigmoidal kinetic response to increasing concentrations of pyruvate but differs in that ATP behaved as a simple competitive inhibitor with respect to NADH.

Yoshida and Freese (1965) and Yoshida (1965) purified and studied the L(+)-LDH from Bacillus subtilis. The enzyme had a molecular weight of 146,000 which is composed of four identical subunits. The B. subtilis LDH is similar to the heart-type enzyme rather than the muscle-type enzyme with respect to the following enzymic characteristics: the optimal pH for oxidation of L(+)-lactate was 7.2 whereas it was 6.0 for the reduction of pyruvate; the enzyme was inhibited by elevated concentrations (0.8 mM) of pyruvate; the enzyme was also rapidly inactivated by PCMB. The B. subtilis enzyme is different from the vertebrate LDH's in respect to activity with different NAD^+ analogues.

In Lactobacillus species, both D(-) and L(+)-LDH's occur. These enzymes have been purified from Lactobacillus plantarum and their kinetic properties studied and shown to be distinctive. Dennis and Kaplan (1960) separated and purified the D(-) and L(+)-LDH's from L. plantarum ATCC 8041 and demonstrated that the two LDH's are distinctly different molecules. The two LDH's show different specificity toward a range

of NAD analogues. The pH optima for D(-)-LDH and L(+)-LDH were respectively, 8.5 and 7.5. The two enzymes also showed differences in heat stability, oxalate inhibition, immunological properties and K_M values. The authors suggest that the two LDH's can couple together to form a reaction pathway for racemization of lactic acid. Mizushima et al. (1964) similarly separated and purified the D(-)- and L(+)-LDH's from Lactobacillus plantarum 11. These enzymes were distinguishable by their substrate specificity, heat stability and oxamate inhibition. The ATP was competitive with respect to NADH for both the D(-)- and L(+)-LDH's. ADP showed similar action and AMP showed no inhibitory effect at physiological concentrations. The above findings were confirmed in more detail by Snoswell (1959, 1961 and 1963) who showed that in addition to the distinctive D(-)- and L(+)-NAD dependent LDH's there were also D(-)- and L(+)-NAD-independent LDH's in the same strain of L. arabinosus (≡ L. plantarum). Gasser et al. (1970) purified and studied the properties of D(-)- and L(+)-NAD-dependent LDH's of different species of Lactobacillus but reported that ATP, ADP and AMP had no effect on the activity of D(-)- and L(+)-LDH's of most lactobacilli in contrast to the previous reports of inhibition by ATP and ADP on the D(-)- and L(+)-LDH's from L. plantarum 11 (Mizushima et al., 1964). The only exceptions were the L(+)-LDH's from two strains of L. acidophilus which did show some inhibition by ATP but this inhibition was regarded by the authors to be possibly due to fortuitous side effects (Gasser et al., 1970).

Another novel control of bacterial LDH's is that found in Leuconostoc mesenteroides by Doelle (1971 a and b). Malic acid if added to culture medium increased lactic acid production threefold with a reduction of the ratio of D(-)- to L(+)-lactic acid. Addition of malic acid apparently increased the synthesis of the two enzymes, but to different extents; the specific activity of L(+)-LDH increased 6.5 fold while that of D(-)-LDH increased 3.2 fold. Malate (and other carboxylic acids) stimulated the L(+)-LDH and inhibited the D(-)-LDH. The D(-)-LDH exhibited a different pattern of electrophoretic mobility depending on whether L. mesenteroides was grown in the presence or absence of malic acid, but no comparable effect was found for the L(+)-LDH. Garvie (1969) made a comparative study of the lactate dehydrogenases from strains of Leuconostoc. Of the 11 strains of genus Leuconostoc examined all had D(-)-NAD-dependent LDH's but no L(+)-LDH activity could be detected.

The widely differing properties of bacterial LDH's may well be related to the specific regulating mechanisms operating in each species but in very few cases has this relationship been explained. For example, the physiological significance of having both D(-) and L(+) specific NAD-dependent LDH's in one organism is not obvious, but the findings of Doelle on the differential regulation of these two enzymes in *Leuconostoc* by malate indicates that they may have distinct functions.

2.1.3 Fructose-1,6-Diphosphate-Activated L(+)-Lactate Dehydrogenases from Streptococci.

Wolin (1964) was the first to report the activation of streptococcal lactate dehydrogenases (L(+)-lactate : NAD^+ oxidoreductase, E.C. 1.1.1.27) (L(+)-LDH) by fructose-1,6-diphosphate (FDP). He found that LDH activity in a *Streptococcus bovis* strain was very low when assayed by the usual LDH assay procedure but activity was substantially increased if FDP was added to the assay system. Inorganic phosphate, fructose, fructose-1-phosphate, fructose-6-phosphate, glucose-6-phosphate, ribose-6-phosphate, mannitol, sorbitol, glyceraldehyde-3-phosphate, dehydroxyacetone-phosphate, glyceraldehyde, glycerol, adenosine tri-, di-, and monophosphate did not substitute for FDP. As well as activating the enzyme, FDP would also stabilise the activity. Wolin also showed that, in strains of other streptococcal species, the LDH's had a similar FDP requirement for activity, (*S. faecalis*, *S. thermophilus* and *S. agalactiae*). Another strain of *S. bovis* did not appear to require FDP for NADH oxidation and showed only slight stimulation of activity when FDP was added.

This requirement of LDH for FDP has subsequently been demonstrated for all other streptococcal LDH's that have been investigated. Outside of the genus *Streptococcus* this activation of LDH by FDP has only been reported to occur in *Bifidobacterium bifidus* (de Vries and Stouthamer, 1968), *Lactobacillus casei* L3 (de Vries *et al.*, 1970) and recently in the mycoplasma *Acholeplasma laidlawii* type A (Neimark and Tung, 1973) and in twenty-one *Staphylococcus* strains (Schleifer and Kocur, 1973).

The FDP activation appears to affect only the L(+)-LDH's. *Streptococcus lactis* strain 760 (Mou *et al.*, 1972) was the one exception in 32 strains of group N streptococci, selected for their ability to produce lactic acid rapidly in milk culture, that possessed a D(-) specific LDH as well as the usual L(+) specific LDH. The two

enzymes were isolated and purified and were found to differ in pH optima, pH and heat stability and only the L(+)-LDH was activated by FDP. A similar finding for *Streptococcus mutans* strain PK-1 was made by Yamada *et al*. (1970) where FDP appears to have an effect on L(+)-LDH but not on D(-)-LDH. Streptococcal species (with the exception of the examples mentioned above) generally produce only L(+)-lactic acid (Wood, 1961) and therefore they would be expected to possess only an LDH specific for the L(+)-lactate. Anders *et al*. (1970) surveyed the LDH activities of a large number of group N streptococci and showed that their LDH's were specific for the L(+)-isomer of lactate and were markedly activated by FDP. Most other workers, who have studied the FDP activated LDH, have confirmed that reduction of the NAD^+ was obtained with L(+)-lactate but not with D(-)-lactate. [In a subsequent section the stereospecificity of lactate oxidation for the LDH of the specific *S. lactis* strain (C_{10}) used in the present study is examined (2.4.6). The enzyme was shown to be an L(+)-LDH. The *S. lactis* C_{10} enzyme will therefore be referred to as an L(+)-LDH in subsequent pages.]

The streptococcal LDH's have been extensively studied by two groups of workers: a group working at the National Institute of Health in the U.S.A., Wittenberger and Angelo (1970), Wittenberger *et al*. (1971) and Brown and Wittenberger (1972), and a group working in Melbourne, Australia, Anders *et al*. (1970), Jago *et al*. (1971), Dynon *et al*. (1972), Jonas *et al*. (1972) and Mou *et al*. (1972). Their findings show that there are considerable differences between species in relation to the properties of the FDP activated LDH's.

Wittenberger and Angelo (1970) reported that the LDH purified 35-fold from *S. faecalis* required a FDP concentration of 0.045 mM for 50% maximum activity under standard assay conditions and had an absolute and specific requirement for FDP for catalytic activity. The activity of the enzyme was greater at pH 5.8 than it was at neutral or alkaline pH. The FDP lowered significantly the apparent K_M for both pyruvate and reduced nicotinamide adenine dinucleotide (NADH) but did not alter the V_{max}. In this study of the *S. faecalis* LDH all the kinetic properties were determined in 100 mM phosphate buffer. The enzyme was rendered heat labile by the presence of FDP or by high concentrations of phosphate buffer.

Jonas *et al*. (1972) studied the factors affecting the activity of the LDH partially purified (10-fold) from *S. cremoris* US3 and found

contrasting properties to that found for the S. faecalis LDH. In the absence of FDP slight activity did occur, but at a different pH optimum to that when FDP was present. Inorganic phosphate was reported by Anders et al. (1970) to inhibit the activation of group N streptococcal LDH's by FDP and this observation was extended by Jonas et al. (1972). An effect of inorganic phosphate on the LDH from S. faecalis was not reported (Wittenberger and Angelo, 1970). Increasing the FDP concentration lowered the K_M for both pyruvate and NADH for the S. cremoris LDH, but in contrast to the findings for the S. faecalis enzyme, FDP also increased the V_{max}. Another contrast with S. faecalis, was that FDP and inorganic phosphate gave protection against thermal inactivation of S. cremoris LDH. The FDP requirement for 50% maximum activity for S. cremoris LDH was approximately 0.005 mM, and was hence ten times lower than that for the S. faecalis LDH. All kinetic studies on the S. cremoris LDH (Jonas et al., 1972) were carried out in triethanolamine/HCl buffer, since it was shown that phosphate buffer increased the FDP requirement for maximal activity.

Brown and Wittenberger (1972) made a comparative study of the LDH's from a range of cariogenic streptococci. LDH's from some of the S. mutans strains studied by Brown and Wittenberger (1972) showed sigmoidal pyruvate saturation curves which contrasts to the hyperbolic pyruvate saturation curves for S. cremoris (Jonas et al., 1972) and S. faecalis (Wittenberger and Angelo, 1970) LDH's. The S. mutans NCTC 10449 LDH was studied in detail by Brown and Wittenberger (1972) and the FDP concentration for 50% maximum activity was 5.0 mM which is very different FDP requirement for the LDH's from S. faecalis and S. cremoris.

Table 2.1.3 summarizes the major differences between the streptococcal LDH's.

A few bacteria other than streptococci have FDP-activated LDH's and those which have been studied differ in a number of respects from the streptococcal LDH's. Acholeplasma laidlawii type A (Neimark and Tung, 1973) L(+)-LDH has a kinetic response to FDP that is apparently hyperbolic (but Hill plots were not shown) as opposed to the sigmoidal response characteristic of streptococcal species. Low activity was present in the absence of FDP at pH 6.0 to 7.2, but FDP was an absolute requirement in the region of pH 8.0 and caused an alkaline shift in the pH optimum. The K_M for FDP was very low at 2.4 μM and the pyruvate saturation curve in the absence of FDP displayed sigmoid

Table 2.1.3

Comparison of some properties of different streptococcal L(+)-LDH's

	Streptococcus mutans				*S. lactis*	*S. cremoris*	*S. faecalis*
Strain	NCTC 10449	21-typical	FA-1	SL-1	JR8-SM	US3	
Reference	1, 2	1, 2	1,2	1,2	1	3	4
$M_{0.5V}$, mM FDP	4.6 to 5.8	4.6 to 5.8	0.05 mM	4.6 to 5.8	0.038	0.005	0,045
Response to heat treatment — minus FDP	labile	labile	N.D.	labile	stable	labile	stable
Response to heat treatment — plus FDP	stable	stable	N.D.	stable	labile	stable	labile
Pyruvate saturation curve	sigmoidal	sigmoidal	sigmoidal	hyperbolic	N.D.	hyperbolic	hyperbolic
Pyruvate saturation curve — n_H	1.7	2.1 to 2.8	2.1 to 2.8	1.1 to 1.3	N.D.	N.D.	N.D.
$S_{0.5V}$ or K_M for pyruvate, mM pyruvate	5.2	2.2 to 3.5	2.2 to 3.5	0.8 to 1.4	N.D.	1.2	1.0
FDP affects -		N.D.	N.D.	N.D.	N.D.		
K_M for pyruvate,	affected					affected	affected
K_M for NADH	not affected					affected	affected
V_{max}	affected					affected	not affected

N.D. - Not determined

All kinetic data were determined in 100 mM phosphate buffer except the data for *S. cremoris* which were determined in triethanolamine/HCl buffer.

Reference: 1 (Wittenberger *et al.*, 1971); 2 (Brown and Wittenberger, 1972); 3 (Jonas *et al.*, 1972); 4 (Wittenberger and Angelo, 1970).

kinetic behaviour. Inorganic phosphate was slightly inhibitory. Also not reported for any streptococcal LDH's is the marked inhibition of the A. laidlawii LDH activity by high concentrations of NADH. The L(+)-LDH's from Bifidobacterium bifidus (de Vries and Stouthamer, 1968) and Lactobacillus casei (de Vries et al., 1970) require Mn^{++} in addition to FDP for activation and thus differ from both streptococcal and A. laidlawii FDP-activated L(+)-LDH's. However this Mn^{++} requirement is pH dependent, at least for Lactobacillus casei, as Mn^{++} is not required at pH 5.5 but enhances affinity for FDP at pH values above 5.8 (Holland and Pritchard, 1975).

At the outset of the investigation on the S. lactis C_{10} L(+)-LDH, the only intensive studies of streptococcal L(+)-LDH's were those of Wittenberger and Angelo (1970) on the kinetic properties of S. faecalis LDH and by Jago et al. (1971) on the physiochemical properties on the LDH of S. cremoris US3. So initially the aim of the present study was to make a detailed investigation of the kinetic properties of the LDH from S. lactis C_{10}. However with the publication of other data (Jonas et al., 1972; Wittenberger et al., 1971; and Brown and Wittenberger, 1972) which revealed differences between the streptococcal L(+)-LDH's, the investigation of the S. lactis C_{10} LDH changed its emphasis.

As the ultimate aim of studying the in vitro kinetic properties of the S. lactis LDH was to relate the properties to possible in vivo metabolic control, the cautionary note of Srere (1967) is important. Srere pointed out the potential hazards of extrapolation from observations in vitro on very dilute enzymes to conditions in vivo. The differences reported for the in vitro kinetic properties from streptococcal LDH's may be due to different in vitro assay procedures, but on the other hand the differences may be, in part, or entirely, a valid indication of the in vivo properties. If the latter is the case then it would appear that there are significant differences in the in vivo metabolic control between different streptococcal species. For example, the thousand-fold difference in the FDP concentration required to give half-maximum activity of LDH from different species, implies a very large difference in the in vivo FDP concentration.

In view of the apparent differences in the in vitro properties of the LDH's from different species of Streptococcus, an intensive

study of the kinetic properties of the LDH from *S. lactis* C_{10} was undertaken to help understand the significance of these differences and their relationship to metabolic control. One of the main lines of investigations was to study the effect of different assay conditions on the values for the various kinetic parameters (especially FDP). The buffer type, especially the effects of phosphate buffer, was investigated in detail as Jonas *et al.* (1972) showed that phosphate did effect the FDP activation of the *S. cremoris* LDH. The effect of phosphate was only investigated and reported by Jago *et al.* (1971), Jonas *et al.* (1972) and Anders *et al.* (1970) to a limited extent.

2.2 Materials and Methods

2.2.1 Organism

Streptococcus lactis C_{10} was obtained from the Dairy Research Institute, Palmerston North and was maintained at 0 to $4^{o}C$ on 1.5% agar slopes containing 30 g Trypticase (BBL) and 1 g yeast extract per litre of distilled water, subculturing at intervals of not more than one month.

2.2.2 Reagents

Substrates, coenzymes, substrate analogues and fructose-1, 6-diphosphate were obtained from the Sigma Chemical Company with the exception of sodium pyruvate which was obtained from Fluka (Buchs, Switzerland).

Yeast extract and Tryptone were obtained from Difco Laboratories and beef extract (Oxoid) from the Oxo Co. Ltd.

The DEAE-Protion used in the enzyme purification is an anion exchange cellulose developed by Tasman Vaccine Laboratories Ltd., New Zealand. It differs from the conventional DEAE-Cellulose in having a viscose regenerated cellulose base which enables a faster flow rate to be obtained.

The reagents, $3,3^{1}$-diaminodipropylamine and 1-cyclohexyl-3-(2-morpho-linoethyl)-carbodiimide metho-p-toluene sulphonate, used for preparing the oxamate affinity chromatography resin were supplied by the Chemical Procurement Laboratories Inc. and the Aldrich Chemical Company Inc., respectively. Bio-Gel A-15 m, 100-200 mesh, supplied by Bio-Rad Laboratories, California, U.S.A. was used as the agarose support for affinity chromatography.

The reagents, nitro-blue tetrazolium and phenazine methosulphate, used for activity staining of LDH activity were both supplied by the Sigma Chemical Company.

Streptomycin sulphate was supplied by Glaxo Laboratories Ltd., Palmerston North.

The reagents used for polyacrylamide disc gel electrophoresis were as follows: $N,N,N^{1}N^{1}$-tetramethylenediamine (TEMED) from Koch-Light Laboratories Ltd.; Ammonium persulphate from B.D.H. (Anala R); Acrylamide and N,N^{1}-methylenebisacrylamide both from Bio-Rad Laboratories (Electrophoresis grade).

2.2.3 Lactate Dehydrogenase Assay

Lactate dehydrogenase activity was estimated by measuring the rate of NADH oxidation at 340 nm. The standard assay mixture contained in a total volume of 3 cm^3 : 90 mM Tris/maleate/HCl buffer, pH 6.9; 0.167 mM NADH; 10 mM sodium pyruvate; 1 mM fructose-1, 6-diphosphate and 0.1 cm^3 of diluted enzyme. The enzyme was diluted in cold 0.005 M potassium dihydrogen phosphate/NaOH buffer (phosphate buffer) pH 7.0. Routine assays during enzyme purification were carried out at room temperature using a Unicam SP800 spectrophotometer. Kinetic studies were carried out at 25°C using a Beckman ACTA-3 spectrophotometer.

An absorbance change of 1.0 unit per minute is used throughout as the measure of enzyme activity unless otherwise stated.* Where comparison of V_{max} was made in a related series of kinetic studies then the term, units/cm^3, refers to the absorbance change per minute per cm^3 of the stock purified LDH used in that series of determinations. Although several different purified preparations were used in the course of the work, the specific activities were very similar.

The tetrasodium salt of FDP was used in all the assays reported. However in one series of kinetic determinations the tetracyclohexylammonium salt of FDP was used in the assays. The results obtained, not reported, were the same as if the tetrasodium salt of FDP was used.

2.2.4 Protein Determination

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard protein.

The method of estimating protein concentration by extinction at 260 nm and 280 nm according to the procedure given in Dawson et al. (1969) was used only for analysis of column fractions to obtain an approximate estimate of specific activity.

2.2.5 Preparation of Affinity Chromatography Resin

Biogel A-15 M was activated by treatment with cyanogen bromide (250 mg/cm^3 packed resin) and subsequently reacted with 3,3'-diaminodipropylamine according to the procedure of Cuatrecasas (1970). Only 20 cm^3 of packed agarose resin was used for preparing affinity resin at one time.

*See addendum at end of thesis

The terminal amino group was then condensed with oxalate via an amide bond to give an oxamate derivative by the following procedure. To each 20 cm^3 of packed resin, 14 cm^3 of 0.74 M oxalic acid was added and the pH adjusted to 4.7. Then 4 cm^3 of a solution containing 0.63 g of 1-cyclohexyl-3(2-morpho-linoethyl) carbodiimide metho-p-toluene sulphonate was added dropwise over 5 minutes, stirring the mixture continuously while the pH was maintained at 4.7. The mixture was then stirred gently at room temperature for 24 hours and finally washed with 5 litres of deionised distilled water. The resin was then ready for equilibration in the appropriate buffer.

2.2.6 Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gels consisting of 7% acrylamide and 0.18% N,N^1-methylene bisacrylamide (Bis) pH 8.9 were used. The separating gel formulation of Gabriel (1971) was used. The three 100 cm^3 stock solutions were prepared using deionised distilled water and stored in the dark at 0^oC. The stock solutions stored for up to one week, were used for gel preparations and the gels were used within two days of preparation.

The three stock solutions are (quantities given are per 100 cm^3 of solution):

(a) 1N HCl 48 cm^3
Tris 36.3 g
TEMED 0.23 cm^3 : resulting pH 8.9

(b) Acrylamide 28 g
Bis 0.735 g

(c) Ammonium persulphate 0.14 g

Working solutions of 1 part (a), 1 part (b) and 2 parts (c) were degassed separately in Thunberg tubes before mixing and adding mixture into clean glass tubes (5 mm internal diameter and 75 mm in length). Deionised distilled water was carefully layered on to the top of the gel mixture and then the gels were left to polymerize. No stacking gel was used, and the separating gels were pre-electrophoresed in the appropriate buffer for 2 hours at 3 mA/gel before using the gels for protein separation. The protein was layered on to the top of the gel surface as a 10% sucrose solution and in a volume no greater than 50 µl. Protein separation was

carried out at 2 mA per tube at room temperature until the bromophenol blue marker band had migrated to the end of the gel. The gels were removed from the glass tubes and the protein bands detected by staining the gels in 0.05% Amido black in 7% acetic acid for 1 hour and then destained overnight in 7% acetic acid.

2.2.7 Activity Staining of Gels for LDH Activity

The gels were stained to detect lactate oxidation as a means of assigning LDH activity to the appropriate protein stain in the following way. The gels were removed and washed in 0.1M triethanolamine/HCl buffer pH 7.9 for 5 minutes before being immersed in the activity staining mixture and placed in the dark at room temperature. The activity staining mixture contained 20 mM NAD^+; 166.7 mM DL lactate; 2.5 mM FDP; 2.5 mg nitro-blue tetrazolium and 1 mg phenazine methosulphate in 12 cm^3 of 100 mM triethanolamine/HCl buffer pH 7.9. When sufficient staining had occurred, the gels were removed and washed three times in 7% acetic acid before being stored in 7% acetic acid.

The gels were also stained to detect pyruvate reduction in the following way. The gels were removed and washed in 0.1 M tris/maleate buffer pH 6.9 for 5 minutes before being immersed in the reactant mixture and placed in the dark at room temperature. The reactant mixture contained 2.5 mM NADH, 10 mM sodium pyruvate and 2.5 mM FDP in 100 mM tris/maleate buffer pH 6.9. The gels were left in this mixture for half an hour, before being removed and quickly washed twice with distilled water. The washed gels were then added to a staining mixture containing 5 mg nitro-blue tetrazolium and 2 mg phenazine methosulphate in 8 cm^3 of 100 mM triethanolamine/HCl buffer pH 7.9. The stain was allowed to develop for 10 minutes in the dark before the gels were removed and washed three times in 7% acetic acid and then stored in 7% acetic acid solution in the dark. The rinsing to remove surplus reactant mixture had to be carefully done, as otherwise too much NADH was removed from the gel surface, which masked the clear zone that indicated the presence of LDH. The presence of LDH activity corresponded to the area of the gel where the NADH had been oxidised so that no colour stain was produced when the staining mixture was added to the washed gel. It was found that if washing was continued for longer than one minute then the clear zone corresponding to enzyme activity was less well defined.

2.2.8 Sodium Dodecyl Sulphate Polyacrylamide Disc Gel Electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide disc gel electrophoresis was carried out according to the general procedure of Weber and Osborn (1969) using 10% acrylamide gels. The modifications to the general procedure are described in the following paragraph.

The protein standards and the purified S. lactis C_{10} L(+)-LDH were treated in the following way. The protein samples were made up in 0.01 M sodium phosphate buffer pH 7.0 containing 1% SDS and 3% 2-mercaptoethanol. The protein solutions were then placed in a boiling water bath for 5 minutes, then cooled and applied to the gel according to the method of Weber and Osborn (1969). The gels were 14 cm long and were contained in glass tubes 16 cm long, 5 mm inside diameter. The gels were run at a constant current of 9 mA per gel for 8 hours. After removal the gels were stained with Coomassie brilliant blue (1.25 g Coomassie brilliant blue in 454 cm^3 of 50% methanol and 46 cm^3 of glacial acetic acid) at 37°C for 12 hours. The gels were then placed in a destaining solution (75 cm^3 acetic acid, 50 cm^3 methanol and 875 cm^3 distilled water) at 37°C on a shaker. At least 24 hours destaining was required before protein bands were clear enough to be measured.

2.2.9 Thermal Stability of Lactate Dehydrogenase

A purified enzyme sample containing 2.5 mg protein/cm^3 was diluted 1/10 in the appropriate buffer which had been pre-incubated for 10 minutes at 55°C. At appropriate time intervals 50 µl samples were withdrawn and diluted with 200 µl 0.02 M phosphate buffer pH 7.0 at 0°C. The diluted sample was assayed using the standard assay conditions within two minutes of being diluted. The various compounds tested for their effect on stability were standardised to the pH of the buffer in which the enzyme was to be incubated. The concentration of these compounds carried over from the incubated enzyme samples into the subsequent assay mixture was negligible compared with the concentrations present in the assay mixture and consequently did not affect the determinations of LDH activity.

2.3 Purification of Lactate Dehydrogenase

2.3.1 Growth and Harvest of *Streptococcus lactis* C_{10}

Streptococcus lactis C_{10} was grown at 30°C in a medium containing the following constituents per litre of distilled water: tryptone 30 g; yeast extract 10 g; lactose 30 g; KH_2PO_4 5 g; and beef extract 2 g (Jago *et al.*, 1971). The medium was sterilised by autoclaving at 15 lb per in^2 for 20 minutes. Cultures were grown in 20 litres of medium in a New Brunswick 50 litre Fermacell fermentor. The culture was stirred at an impellor speed of 150 rpm, but no additional aeration was provided. The pH was maintained between 6.0 and 6.5 by periodic addition of 2.5 M sodium hydroxide during fermentation.

The cells were harvested near the end of the logarithmic phase of growth by centrifugation at 5,500 g for 15 minutes at 0°C. The yield was 1.3 to 1.5 g wet weight of cells per 100 cm^3 of medium if grown as described. With no pH control the yield dropped to 0.6 g wet weight of cells per 100 cm^3 of medium. The harvested cells were washed three times in 0.005 M phosphate buffer pH 7.0 containing 1% NaCl. The cells were then stored frozen until required. The cells could be stored for a month with no resulting loss in L(+)-LDH activity in the cell free extract. Cells stored up to a month were used for preliminary work to establish a purification scheme. However, for the final large-scale purifications, cells were only stored frozen for up to two days before disrupting the cells for enzyme extraction.

2.3.2 Breakage of Cells

Cells were thawed and suspended in 0.005 M phosphate buffer pH 7.0 and disrupted by two passages through an Aminco French pressure cell at 5,500 lb per in.^{2}. Unbroken cells and cell debris were centrifuged down at 13,000 g for 15 minutes and the cell-free extract was dialysed against the same phosphate buffer for 15 hours at 4°C. All subsequent purification steps were carried out at 4°C.

2.3.3 Streptomycin Sulphate Treatment

Nucleic acids were precipitated from the dialysed cell-free extract by dropwise addition of streptomycin sulphate using 1.75 cm^3 of a 10% (w/v) solution for every 100 mg protein. The resulting

suspension was allowed to stand for 4 hours and the precipitate was removed by centrifugation at 13,000 g for 15 minutes.

Maximum nucleic acid precipitation (as determined by the A280/A260 ratio) was achieved when 150 mg streptomycin sulphate (1.5 cm^3 of a 10% solution) was added per 100 mg protein. If more than 200 mg streptomycin sulphate (2.0 cm^3 of a 10% solution) was added per 100 mg protein then the % recovery of LDH activity dropped rapidly with an associated drop in specific activity.

2.3.4 Ammonium Sulphate Precipitation

The supernatant after streptomycin sulphate treatment was dialysed against 0.005 M phosphate buffer pH 7.0 for 15 hours. Solid powdered ammonium sulphate was then added slowly to bring the solution to 50% saturation. After leaving for half an hour, the precipitate was removed by centrifugation at 13,000 g for 15 minutes. The concentration of the ammonium sulphate in the supernatant was then increased to 65% saturation. After three hours the precipitate was collected by centrifugation at 13,000 g for 15 minutes, redissolved in 300 cm^3 0.025 M citrate buffer pH 6.1 and then dialysed against the same citrate buffer for 24 hours.

2.3.5 DEAE-Protion Ion Exchange Chromatography

a) Development of a suitable Ion Exchange purification procedure

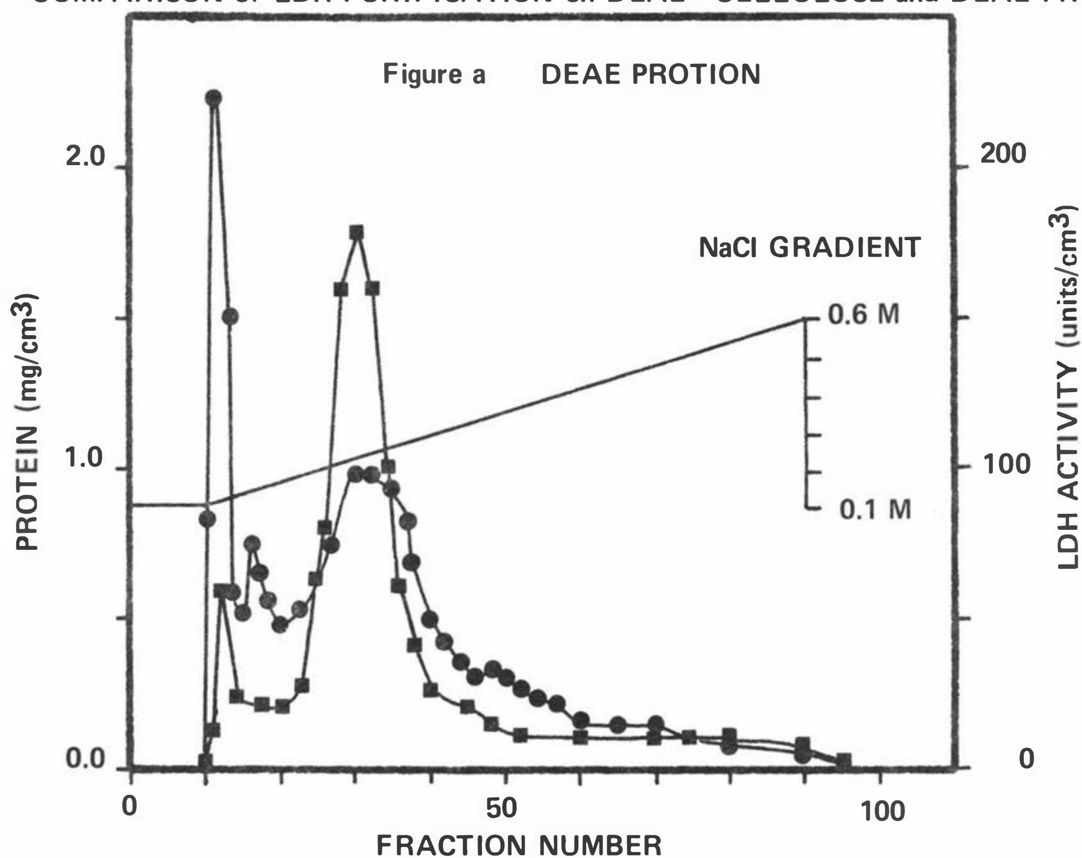
Since the enzyme activity was precipitated in buffer solutions at about pH 5.0 it was decided to use a DEAE type ion exchange resin in preference to a CM-type ion exchange resin.

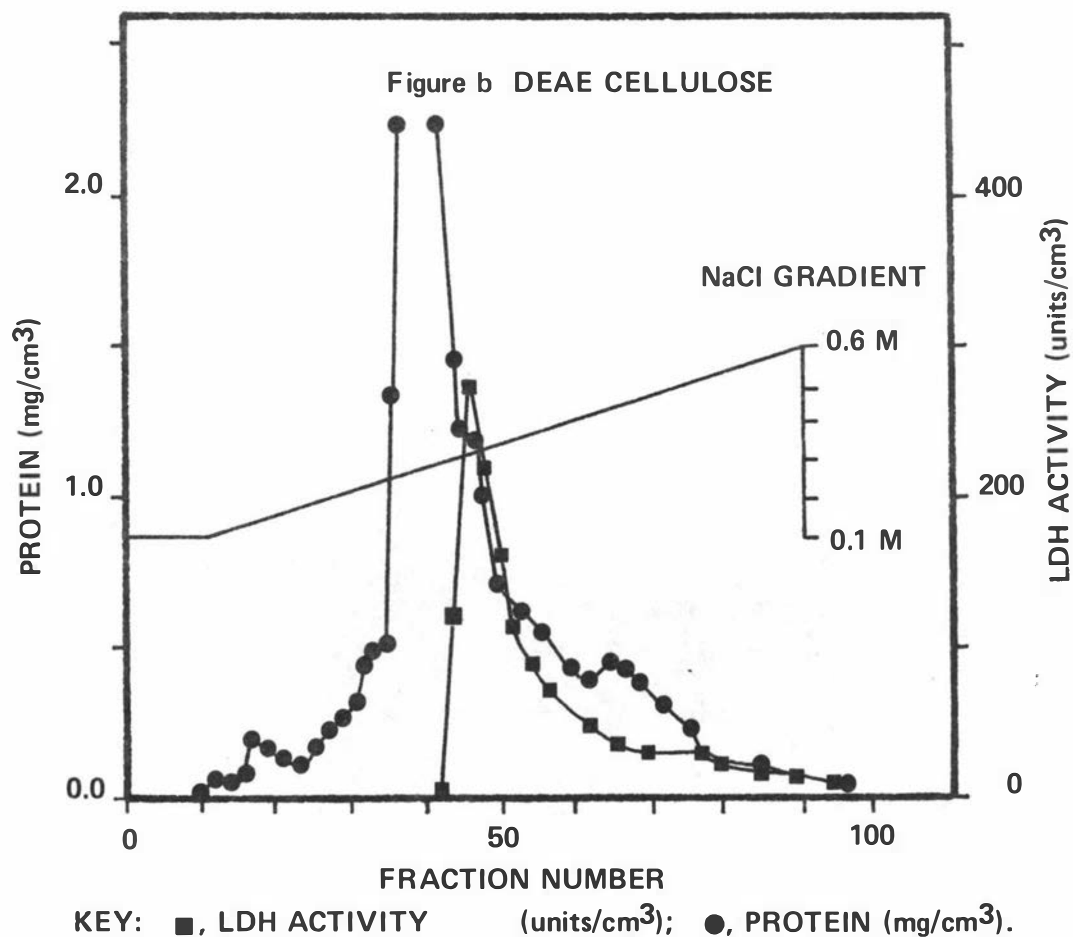
A comparison between DEAE-Cellulose (Whatman DE23) and DEAE-Protion was made using two glass columns of identical dimensions and packed with equal columns of the two resins. Identical conditions were used for both types of resin gradient runs.

Figures 2.3.5a and 2.3.5b show the differences in the protein and LDH activity elution profile between the two types of DEAE resins. The DEAE-Protion resin has a lower capacity for binding LDH as shown by the early peak of activity which did not bind to the resin. The main LDH activity peak on the DEAE-Protion resin was eluted earlier on the gradient, compared to the single L(+)-LDH activity peak eluted on the DEAE-Cellulose resin.

Figure 2.3.5: a and b

COMPARISON of LDH PURIFICATION on DEAE - CELLULOSE and DEAE PROTION





KEY: ■, LDH ACTIVITY (units/cm^3); ●, PROTEIN (mg/cm^3).

Although the DEAE-Cellulose has a higher capacity it was decided to try to develop a suitable purification step using DEAE-Protion for the following reasons:

1. DEAE-Protion was a newly developed resin and its potential for protein purification needed to be investigated.

2. The DEAE-Protion had a much better flow rate than DEAE-Cellulose. In the run just described the flow rate was 0.8 cm^3/minute and was the maximum for the DEAE-Cellulose resin under the described conditions. The DEAE-Protion, under the same conditions could have been run at a flow rate up to 10 cm^3/minute. Using a flow rate of 2 cm^3/minute a similar elution profile to the gradient run using a flow rate of 0.8 cm^3/minute was obtained, but increases in the flow rate above 2 cm^3/minute gave progressively less efficient separation. The faster flow rate allowed quicker preparation, equilibration and washing of DEAE-Protion resin compared to DEAE-Cellulose resin. This property is particularly advantageous when large columns are being used and also greatly reduces the packing problems which may be encountered by large DEAE-Cellulose columns.

3. Though the binding capacity of DEAE-Protion resin is less than that of DEAE-Cellulose, this can be easily compensated for by use of larger columns. The use of larger columns, although diluting the LDH activity, did not lower the % recovery of activity and in fact gave a better purification.

The tailing of the LDH activity peak, shown in the two figures (2.3.5a, b) did not appear to be due to a different LDH. The fractions, containing the tail of the activity peak were bulked, concentrated by ultrafiltration, dialysed in initial buffer and then rerun on the same column. The peak of activity was eluted in the same position on the gradient as the major peak of the first column run and had a similar tailing off, of activity.

Using DEAE-Protion different buffer gradients were investigated using phosphate and citrate buffer, under various conditions of pH and ionic strength. Gradients using tris/HCl or pyrophosphate buffers could not be tried as the LDH was unstable in these buffers. An LDH sample dialysed for 12 hours in 0.01 M tris/HCl buffer pH 7.5 retained only 10% of its activity and in 0.01 M pyrophosphate buffer pH 8.0 only 25% of the activity remained after 12 hours of dialysis. In 0.01 M

phosphate buffer (pH 6, 7 or 8) and citrate buffer (pH 6.0) activity was stable, even during prolonged dialysis (48 hours) and storage in diluted form (0.5 mg protein/cm^3) for up to one week. Citrate buffer was chosen in preference to phosphate since less tailing of the activity peak occurred in citrate.

From these trial gradients a stepwise elution of LDH from DEAE-Protion, using citrate buffer, was developed to give a good yield of fractions with high specific activity. The procedure finally adopted was as follows.

b) DEAE-Protion Purification of LDH

The redissolved precipitate from the 50-65% ammonium sulphate fraction after dialysis against 0.025 M citrate buffer pH 6.1 for 24 hours, was applied to a DEAE-Protion column (40 cm x 6 cm) pre-equilibrated with 0.025 M citrate buffer pH 6.1. The column was then eluted, at a rate of 3 cm^3/minute, with the same buffer until the absorbance of the eluate at 280 nm had dropped to zero. The LDH was eluted, at the same flow rate, with 0.05 M citrate buffer pH 6.1 and all fractions containing the enzyme at a specific activity greater than 300 units/mg protein were bulked. Solid powdered ammonium sulphate was added to bring the concentration of bulked fractions to 70% saturation. The precipitate was centrifuged down, redissolved in 0.05 M phosphate buffer pH 6.7 and dialysed against the same buffer for 24 hours.

2.3.6 Chromatography on an "Oxamate Affinity Resin".

a) Development of a suitable affinity chromatography procedure

O'Carra and Barry (1972) reported the use of affinity chromatography on a resin to which an oxamate group had been attached for characterization of pig heart lactate dehydrogenase (LDH), (H_4). LDH has a compulsory-ordered reaction mechanism (Thomson *et al.*, 1964; Silverstein and Boyer, 1964 and Fromm, 1963) in which the NADH coenzyme compulsorily binds first. O'Carra and Barry found that if NADH was included in the eluant, LDH was strongly retained by the oxamate affinity resin, but when NADH was omitted from the eluant, the LDH was eluted almost immediately. Hence oxamate, a pyruvate analogue, will only bind to LDH if NADH is bound first.

A trial run using an oxamate affinity resin (prepared as described in Section 2.2.5) to purify the S. lactis C_{10} LDH was designed on the assumption that this enzyme also has a compulsory-ordered mechanism and required both NADH and FDP for binding of oxamate. The result of this trial run can be seen in Figure 2.3.6. LDH did bind as shown by the initial high protein peak with no associated LDH activity peak. Eluant B would have been expected to elute off the LDH as NADH was omitted and pyruvate was present. However the LDH remained bound and was eluted off subsequently with eluant C, consisting of 0.2 M phosphate buffer pH 6.65.

Further trial runs confirmed that the LDH will bind to the resin in the absence of NADH and FDP to give the same elution profile as shown in Figure 2.3.6. The ionic strength of the first eluant (A) could be increased to 0.1 M, removing further contaminant proteins but leaving the L(+)-LDH bound. Subsequent elution with 0.15 M phosphate buffer eluted a small peak of contaminant protein before removing the LDH. Complete elution was achieved in 0.2 M phosphate buffer.

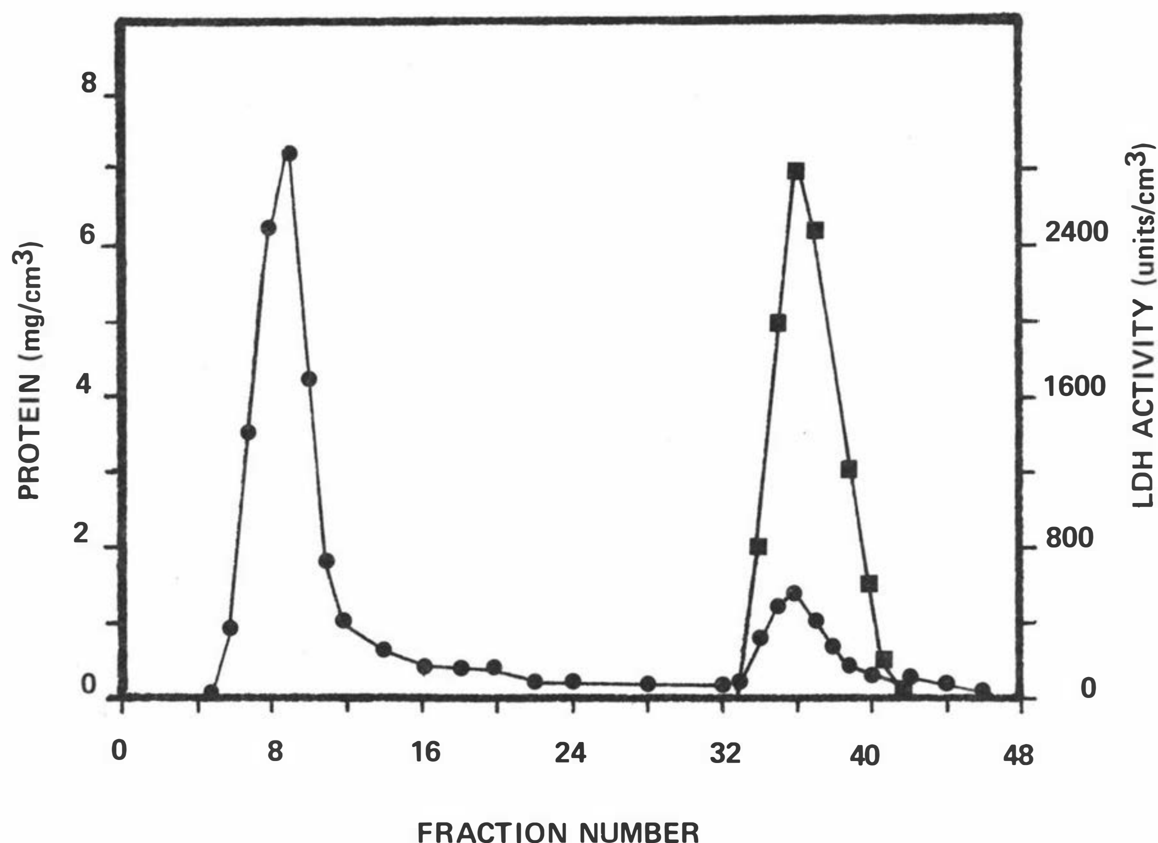
Although the LDH binds to the oxamate resin in the absence of FDP and NADH, kinetic studies of inhibition by oxamate, to be described later, have revealed a peculiar interaction between this compound and the LDH in phosphate buffer. The procedure described in the following paragraphs which relies solely on increasing ionic strength of the eluting buffer to remove the LDH, may not be a true affinity chromatography procedure dependent on specific binding of the LDH to oxamate at its substrate binding site. However since it gave a very good increase in specific activity the procedure was adopted in the final purification.

b) "Oxamate affinity chromatography" purification

The dialysed sample (in 0.05 M phosphate buffer pH 6.7) from the DEAE-Protion purification step, was applied to a 20 cm x 1 cm column of oxamate affinity resin in a total volume of 10 cm^3 containing not more than 100 mg of protein or 60,000 units of LDH activity. The column was pre-equilibrated with 0.05 M phosphate buffer pH 6.7 and was loaded with the sample at a rate of 0.3 cm^3/minute. After loading, the column was left standing for one hour before being eluted at a rate of 0.5 cm^3/minute with 0.1 M phosphate buffer pH 6.7. This step removed a

Figure 2.3.6

TRIAL "OXAMATE AFFINITY" CHROMATOGRAPHY OF LDH



Column Conditions: Column size — Length (10 cm); Diameter (1.0 cm). Pre-equilibration of resin was in 0.05 M phosphate buffer pH 6.65, and just prior to applying the sample, 20 cm^3 of 0.005 M NADH and 0.01 M FDP in 0.05 M phosphate buffer pH 6.65 were eluted through the column. Sample Applied — Protein (10 mg/cm^3) Activity (6400 $units/cm^3$); Volume (7 cm^3); Buffer (0.05M phosphate buffer pH 6.5 containing 0.005M NADH + 0.01M FDP). Sample Application — After the sample was loaded on to the resin at a flow rate of 0.1 cm^3/minute the column was turned off for one hour. Elution — three successive elutions were carried out at a flow rate of 0.3 cm^3/minute.

Eluant A — Fractions 0 to 16 - 0.05 M phosphate buffer + 0.01 M FDP + 0.005 M NADH pH 6.65.

Eluant B — Fractions 17 to 28 - 0.05 M phosphate buffer + 0.01 M FDP + 0.05 M Na Pyruvate pH 6.65.

Eluant C — Fractions 29 to 48 - 0.2 M phosphate buffer pH 6.65.

Column Fractions (Volume = 1.5 cm^3) were dialysed in 0.005 M phosphate buffer before protein was determined by Folins and the LDH activity assayed.

KEY: ■, LDH Activity ($units/cm^3$); ●, Protein (mg/cm^3).

considerable amount of contaminant protein while the LDH remained bound to the resin. LDH activity was removed from the resin by successive elution with 20 cm^3 of 0.15 M and 50 cm^3 of 0.2 M phosphate buffer pH 6.7. High specific activity lactate dehydrogenase fractions were pooled and concentrated by ultrafiltration.

The concentrated solution was finally dialysed against 0.01 M phosphate buffer pH 7.0 and frozen in small volumes.

The above purification procedures (2.3.1 to 2.3.6b) purified the S. lactis C_{10} LDH, overall by 100-fold as is summarised in Table 2.3. The results in Table 2.3 represent the purification from S. lactis C_{10} harvested from 20 litres of medium. The purification procedures were carried out on five separate occasions and in each case the purification scheme gave similar results to those outlined in Table 2.3. The specific activity of the cell-free extract obtained in each case was relatively constant and ranged from 23 to 27 units/mg and the final purified sample had a specific activity ranging from 2,290 to 3,200 units/mg. Thus in each case the enzyme was purified approximately 100-fold.

The major loss in activity was at the DEAE-Protion chromatography step. However only up to 30% of this lost activity was unaccounted for; the remaining activity was lost in discarding fractions of low specific activity.

Table 2.3

Summary of lactate dehydrogenase purification procedure

Treatment	Total activity units	Total protein mg	Specific activity units/mg	Purification factor	Per cent recovery
Cell free extract	9.1×10^5	32,400	26	-	-
Streptomycin sulphate supernatant	9.9×10^5	27,500	36	1.4	-
Ammonium sulphate 50-65% redissolved precipitate	8.0×10^5	7,300	110	4.3	92
DEAE Protion bulked high specific activity fractions	3.9×10^5	650	600	23.2	49
"Oxamate Affinity" chromatography	3.0×10^5	107	2,800	109.0	33

2.4 Properties of the Purified L(+)-Lactate Dehydrogenase

Unless specially mentioned all the work on the properties of L(+)-LDH was carried out using the purified enzyme (specific activity ranging from 2,290 to 3,200 units/mg). The frozen, purified, enzyme could be stored for up to three months without any loss in activity. After four months of storing frozen the enzyme had lost 5-10% of its activity and on storage for six months 20-30% of original activity was lost. An LDH sample stored for six months had the same K_M for pyruvate and NADH and the same FDP requirement as the enzyme immediately after purification. An LDH sample stored for two years had lost 60% of its activity although there was no change in the K_M for pyruvate and NADH or in FDP requirement. However it did not show the co-operativity in the binding of FDP which was characteristic of the enzyme stored for less than six months. All properties of the purified LDH described in the following sections (unless otherwise stated) were derived from samples frozen for less than three months.

2.4.1 Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis was carried out on the purified sample to determine the purity. Gels were run in several different buffers, and at different buffer pH values and ionic strengths. Under most conditions several protein bands were obtained and it has proved difficult to establish with certainty how far all of the minor bands represent modified forms of the enzyme or whether some are impurities.

When the gels were run in the tris/glycine buffer pH 8.3 as described by Gabriel (1971) and stained for protein they showed three major bands and two to three minor bands. Gels were stained to detect enzyme activity either in the direction of reduction of NAD by lactate or the oxidation of NADH by pyruvate as described in Section 2.2.7. When a gel was stained to detect lactate oxidation an activity band appeared corresponding to one of the three major bands but this band took 24 hours to develop. Gels run in 0.1 M tris/maleate buffer or 0.1 M phosphate buffer pH 8.0 and pH 7.0 gave a similar pattern of protein staining (Figure 2.4.1a gel 1). At pH 7.0 in both buffers the lactate oxidation activity stain took from four to six hours to develop and a second band of activity was distinguishable just above the main band of activity (gel 2). If the stain was allowed to develop for a

further four to six hours a third activity band appeared on top of the second band. However by this stage the background stain was too great to allow suitable photographs to be taken. A diffuse indistinct activity band also occurred close to the top of the gel not corresponding to any protein band. The pyruvate reduction activity stain showed in most cases a broad activity band covering the whole region of the three major protein bands. However when the gels (run in either buffer at pH 7.0) were stained for pyruvate reduction for short times (two to four minutes) a staining pattern was obtained that showed three bands of activity which corresponded to the three major protein stains. An unstained, diffuse zone was present near the top of the gel corresponding to the zone of lactate oxidation activity. At this pH (7.0 or greater) no protein band was detectable in this region. The contrast with the dark background stain in the rest of the gel 3 is not sufficient to reproduce these bands very clearly in the gel photograph - a line drawing accompanying the photograph (Figure 2.4.1b) shows the main features that would be distinguished in the gels. The indistinct protein bands of gel 1 in Figure 2.4.1a were often obtained particularly in tris buffers in comparison to the distinct protein stain such as that shown in gel 1 of Figure 2.4.1c which was characteristic of gels run in phosphate buffer.

At pH 6.0, in either 0.1 M tris/maleate or 0.1 M phosphate buffer, the protein and activity patterns were quite different from those at pH 7.0 or above. There was one slow moving protein corresponding to a band of high enzyme activity for both lactate oxidation and pyruvate reduction. This is shown in Figure 2.4.1c where gels 1, 2 and 3 were run in 0.1 M or 0.042 M tris/maleate buffer pH 6.0. The pattern is the same if gels were run in 0.1 M phosphate buffer pH 6.0. The lactate oxidation activity band took only five to ten minutes to develop compared to four hours or longer for the main band in gels run at pH 7.0. However if the molarity of either tris/maleate or phosphate buffer at pH 6.0 was dropped to a lower ionic strength the protein and activity pattern changed. The gel, shown in Figure 2.4.1d, was electrophoresed in 0.035 M tris/maleate buffer for the same time as the gels in Figure 2.4.1c. The slow moving protein has disappeared and is resolved into a broad protein band migrating considerably faster and two to three minor bands. The major band

does show activity on appropriate staining, but the activity stain for the lactate oxidation takes longer to develop (two hours).

Figure 2.4.1e shows the effect of electrophoresis of gels in low molarity (0.025 M) phosphate buffer pH 6.0. The protein band pattern of gel 1 is very similar to that obtained when gels are run at pH 7.0 or pH 8.0 in either tris/maleate or phosphate buffers. Gel 2 was run under the same conditions except that 15 μmoles of FDP was added with the 50 μg of protein layered on top of the gel prior to electrophoresis. In this case the bulk of the protein ran as a single highly active slow moving band. However the change of protein pattern with addition of FDP was not a specific effect of FDP since if the same amount of Na_2SO_4 or Fructose-6-Phosphate was added a similar effect on the protein pattern was obtained (gel 7). Gels 3 and 4 are the respective pyruvate reduction activity stains of gels 1 and 2, both showing two zones of activity. In gel 3 the top activity zone has no corresponding protein band in gel 1 and is probably indicative of the high specific activity of the slow migrating form. Gel 5 has FDP present during electrophoresis, but the pyruvate reduction activity stain was developed without FDP being present in the assay mixture. (Both gels 5 and 6 were left in the activity mixture twice as long (one hour) before staining as gels 3 and 4.) The resultant top activity band was much less noticeable and the lower activity band was very faint and does not appear in the photograph. Gel 6 had no FDP present during either running or development of activity; no activity bands were detectable. The effect of FDP on the activity stain development indicates that the activity being detected is due to LDH. This was further supported by the fact that if either substrate in the pyruvate reduction or lactate oxidation activity stain was left out then the activity stains either did not develop at all or developed at least four times as slowly compared to when both substrates were present.

It is not possible to draw any definite conclusions concerning the degree of purity of the enzyme from these studies. At pH 6.0 at appropriate ionic strength, a single band of protein and enzyme activity is obtained, but in view of this very slow migration of this band at this pH it may well consist of several unresolved components. Increasing the pH in an attempt to increase the

mobility of this band results in modification of the enzyme to a much faster running form but with considerably reduced activity. The multiple bands of protein and activity obtained at pH values of 7,0 and above probably represent different conformational and/or dissociated states of the enzyme rather than major impurities. The two fastest running bands obtained at these pH values may represent impurities since no activity bands corresponding to these ever appeared.

Attempts to improve the resolution of the slow running active form by using pH values intermediate between 6.0 and 7.0 or high ionic strengths at pH 7.0 did not clarify the situation any further. For example increasing the ionic strength to 0.25 M at pH 7.0 was tried. In contrast to the gel run in 0.1 M phosphate buffer where there are a number of medium migrating bands of protein and activity (gel 1, Figure 2.4.1e) gels run in 0.25 M phosphate pH 7.0 (Figure 2.4.1f) showed one main protein stain and associated activity stain appearing at the top of the gel. A gel run in 0.2 M tris/maleate buffer pH 7.0 did not give such a clear change compared to a gel run in 0.1 M tris/maleate buffer pH 7.0 as a broad protein stain migrating at an intermediate speed was demonstrated as shown in Figure 2.4.1g.

2.4.1.1 Sodium Dodecyl Sulphate Polyacrylamide Disc Gel Electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide disc gel electrophoresis was carried out as a further means of determining the purity of the *S. lactis* C_{10} LDH and to determine its subunit molecular weight. One major protein band and four minor bands were obtained (Figure 2.4.1.1a). The relative mobilities of the standard proteins and LDH were calculated by the method of Weber and Osborn (1969). By plotting the relative mobilities of the standard proteins against the log of their molecular weight, a standard curve was obtained from which the LDH molecular weight could be read off, knowing its relative mobility. Such a standard curve is shown in Figure 2.4.1.1b where the points for each of the standard proteins represent the average mobility of four separate runs. The average value obtained for the major protein for the *S. lactis* C_{10} LDH sample was 36,000 from 6 gel runs. The range for the *S. lactis* C_{10} LDH subunit molecular weight was from 34,000 to 37,000. This subunit molecular weight of 36,000 agrees fairly well with the subunit molecular weight of 37,000 reported for

Figure 2.4.1: a, b and c

Polyacrylamide Disc Gel Electrophoresis

Figure a

The gels were run in 0.1 M tris/maleate buffer pH 7.0. 40 µg of purified LDH was added to each gel. Gels are numbered from left to right.

Gel 1 Protein stain
Gel 2 Lactate oxidation activity stain
Gel 3 Pyruvate reduction activity stain

Figure b

Figure b shows a line drawing of the gels that are shown in Figure a. Gel 2 in this drawing shows a third activity band which appeared after a further 4 to 6 hours.incubation in the reaction mixture.

Figure c

The gels were run in 0.1M tris/maleate buffer pH 6.0. 40 µg of purified LDH was added to each gel. Gels are numbered from left to right.

Gel 1 Protein stain
Gel 2 Pyruvate reduction activity stain
Gel 3 Lactate oxidation activity stain

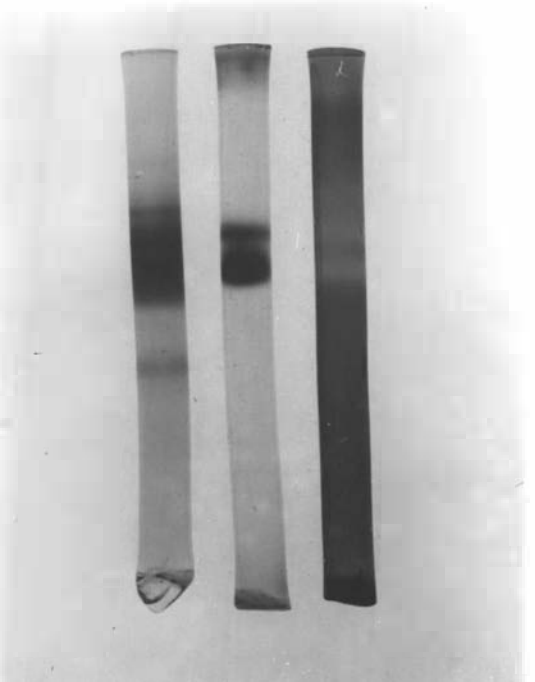


figure 2.4.1 b

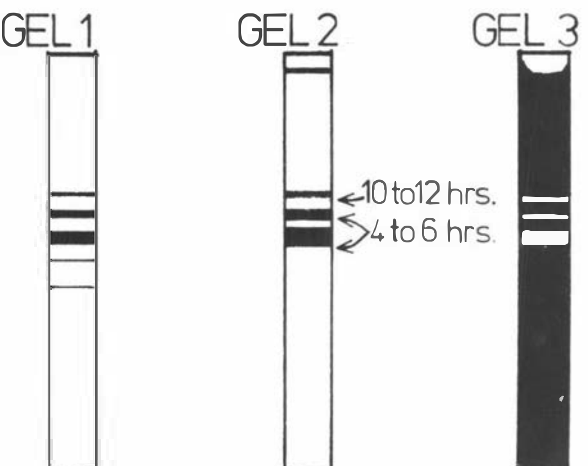


Figure 2.4.1: d and e

Polyacrylamide Disc Gel Electrophoresis

Figure d

The gel was run in 0.035 M tris/maleate buffer pH 6.0. 40 μg of purified LDH was added to the gel. The gel (1) is a protein stain.

Figure e

The gels were run in 0.025 M phosphate buffer pH 6.0. 50 μg of purified LDH was added to each gel. In addition to the protein added, gels 2, 3 and 5 had 15 μmoles of FDP present with the protein sample. Gel 7 in addition to the protein, had 15 μmoles of Na_2SO_4 added.

Gel 1 Protein stain

Gel 2 (FDP) Protein stain

Gel 3 (FDP) Pyruvate reduction activity stain

Gel 4 Pyruvate reduction activity stain

Gel 5 (FDP) Pyruvate reduction activity stain

Gel 6 Pyruvate reduction activity stain

Gel 7 (Na_2SO_4) Protein stain.

Note: Gels 5 and 6 had no FDP present in the pyruvate reduction activity mixture.

1234567
1

Figure 2.4.1: f and g

Polyacrylamide Disc Gel Electrophoresis

Figure f

The gels were run in 0.25 M phosphate buffer pH 7.0. 40 μg of purified LDH was added to each gel.

Gel 1 Protein stain

Gel 2 Pyruvate reduction activity stain

Figure g

The gels were run in 0.2 M tris/maleate buffer pH 7.0. 40 μg of purified LDH was added to each gel.

Gel 1 Pyruvate reduction activity stain

Gel 2 Protein stain

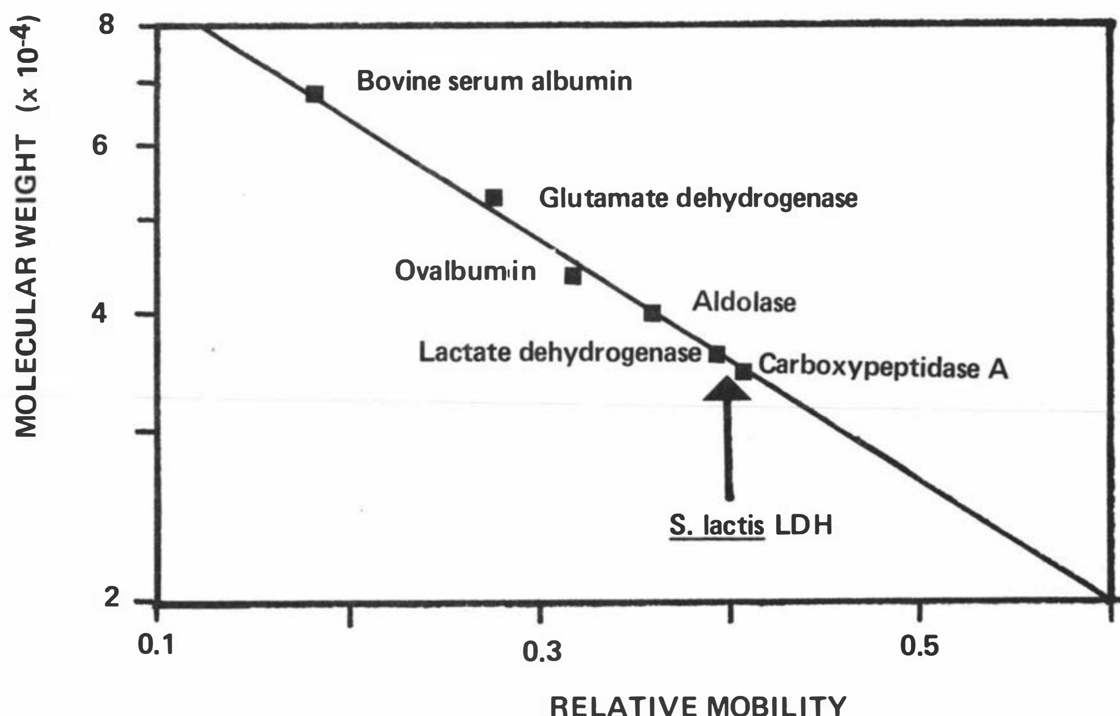
Figure 2.4.1.1 a

SDS Polyacrylamide Disc Gel Electrophoresis

40 μg of the purified LDH, denatured in SDS, was added to the gel shown. The gel was run under the conditions as described by Weber and Osborn (1969).

Figure 2.4.1.1.b

SDS POLYACRYLAMIDE DISC GEL ELECTROPHORESIS: STANDARD CURVE



The standard curve shown was obtained by plotting the relative mobilities (calculated by method of Weber and Osborn, 1969) of the standard proteins against log of their molecular weight. The point for each standard protein represents the average relative mobilities from four separate runs. The arrow indicates the average relative mobility of the S. lactis lactate dehydrogenase (LDH) (major protein stain on gel in Figure 2.4.1.1a) and this position corresponds to a molecular weight of 36,000. The standard proteins are: Bovine Serum Albumin (Fluka); Glutamate Dehydrogenase (Type I Bovine liver, Sigma); Ovalbumin (Sigma); Aldolase (Rabbit muscle, Sigma); Lactate Dehydrogenase (Rabbit muscle type II, Sigma); Carboxypeptidase A (Bovine pancreas, Sigma). The molecular weights of the standard proteins were obtained from Weber and Osborn (1969). 20 μg of each of the standard proteins was used.

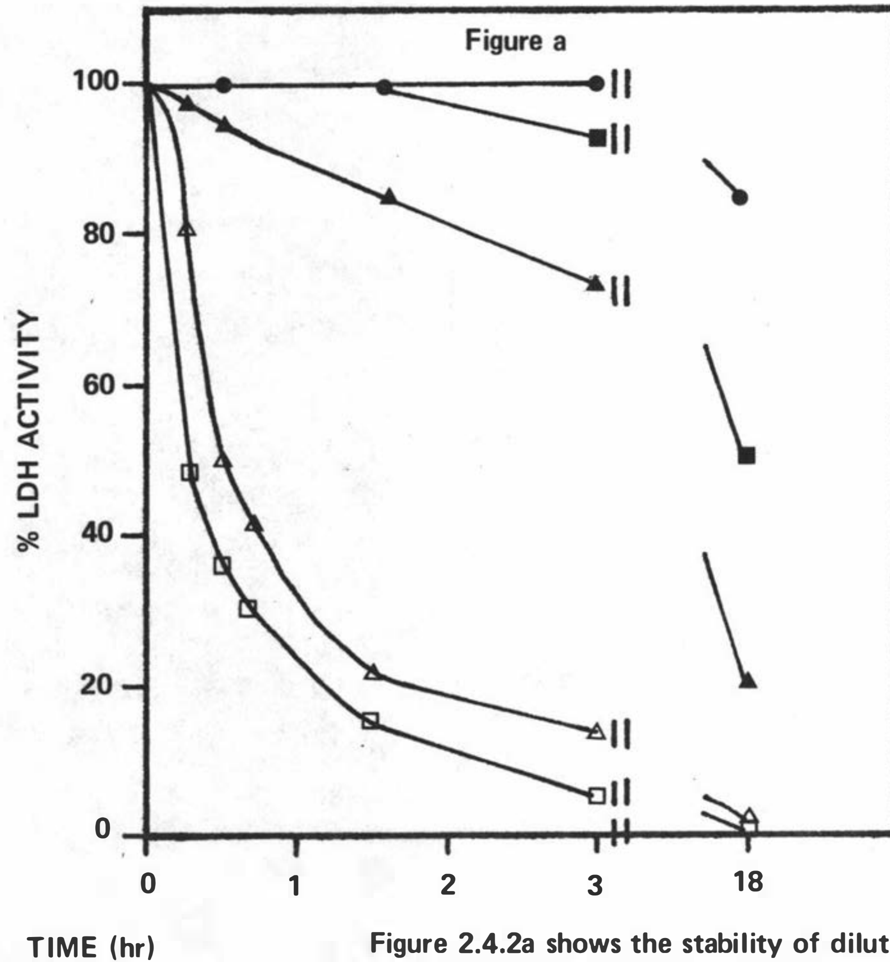
the L(+)-LDH from *S. cremoris* US3 by Dynon *et al.* (1972). The largest of the minor bands had a molecular weight of 70,000. Although this could be a dimer of the 35,000 subunit, it was not found by Dynon *et al.* (1972) and its persistence in the presence of reducing agents during SDS denaturation treatment indicates that it is more likely a contaminant protein.

From polyacrylamide disc gel electrophoresis, with and without SDS, the purified enzyme is clearly not completely homogenous. However the specific activity and the degree of purification of the *S. lactis* C_{10} L(+)-LDH is higher than that obtained by most previous workers studying streptococcal LDH's. Jago *et al.* (1971) studied the physicochemical properties of the LDH of *S. cremoris* US3 using a sample showing a single protein band on polyacrylamide disc gel electrophoresis. This sample was purified 80-fold compared to 100-fold purification for the *S. lactis* C_{10} LDH. However when Jonas *et al.* (1972) studied the kinetic properties of the LDH from *S. cremoris* US3, only a 10-fold purified sample was used. Brown and Wittenberger (1972) studied the LDH of *S. mutans* NCTC 10449 using a 137-fold purified preparation, and Wittenberger and Angelo (1970) studied the *S. faecalis* LDH using a 35-fold purified preparation.

2.4.2 Effect of pH on Activity of LDH

The effect of pH on lactate oxidation and pyruvate reduction, as catalysed by the *S. lactis* C_{10} LDH, was investigated in several different buffers and in the presence and absence of the activator, FDP. In order to ensure that the changes in activity with pH were not a consequence of enzyme inactivation over the assay period, the stability of the diluted ensyme at 25°C was examined at different pH values and in two different buffers. In Figure 2.4.2a it can be seen that the LDH becomes progressively unstable with increasing pH above 7.0 in 90 mM tris/maleate buffers. However, even at pH 8.5 (Figure 2.4.2b) no appreciable loss in activity occurs until after five minutes of incubation. Therefore changes in activity with pH described in this section are not due to enzyme inactivation during assay as the assay period was always less than three minutes. In triethanolamine buffer over its buffering range of pH 6.5 to 8.5 the enzyme showed the same pattern of stability as shown for tris/maleate buffer in Figures 2.4.2 a and b, although it appeared to be slightly less stable in the alkaline range.

Figure 2.4.2: a and b STABILITY OF LDH AT 25°C



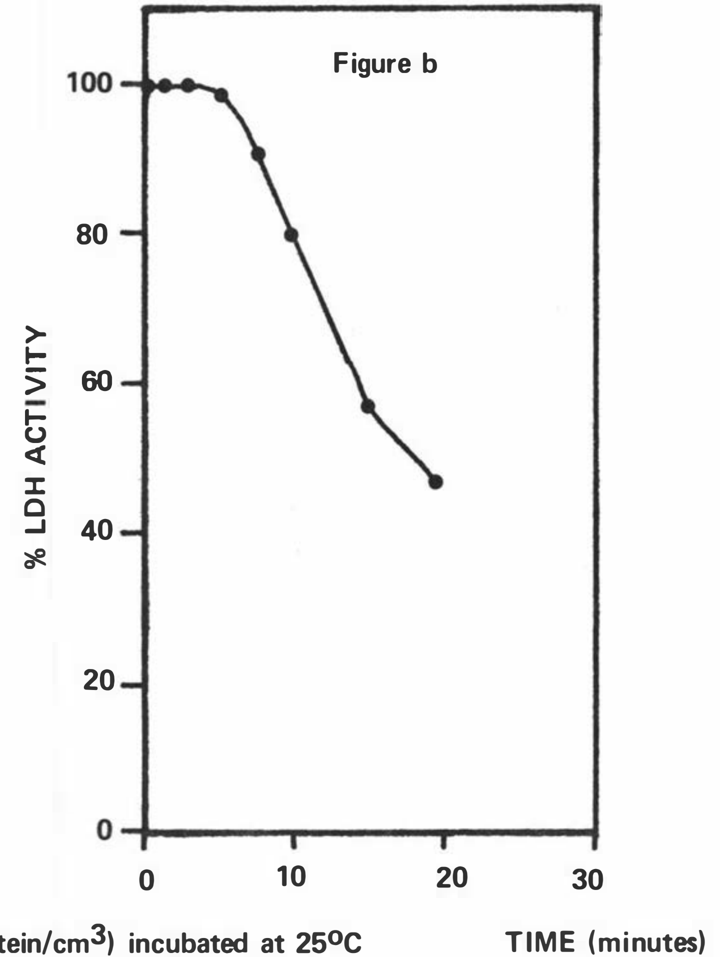


Figure 2.4.2a shows the stability of diluted LDH (2 [illegible]g protein/cm^3) incubated at 25°C for different times in 90 mM tris/maleate buffer pH 6.0, ● ; pH 7.0, ■ ; pH 7.5, ▲ ; pH 8.0, △ ; and pH 8.5, □ . Figure 2.4.2b shows the stability of diluted enzyme in 90 mM tris/maleate buffer pH 8.5 incubated at 25°C for shorter time intervals.

100% LDH activity is the activity of the sample prior to incubation at 25°C.

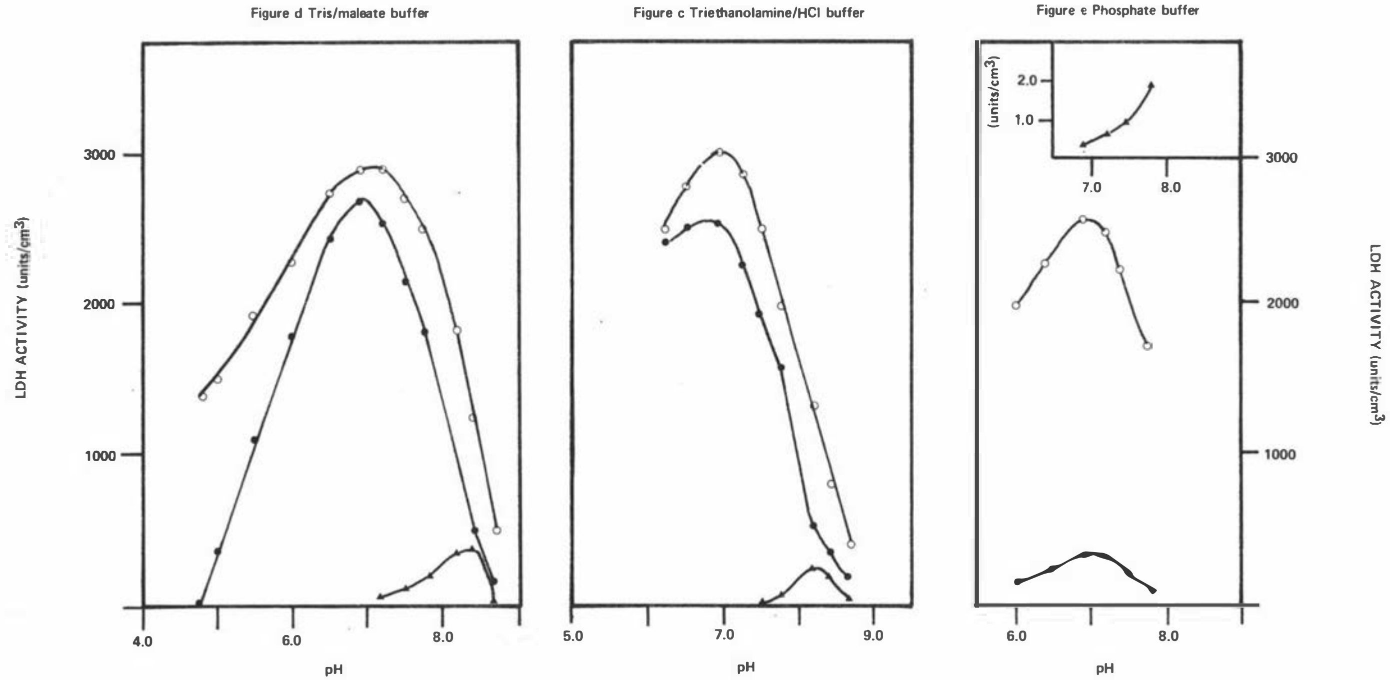
The effect of pH on the rate of pyruvate reduction was examined in 90 mM tris/maleate, triethanolamine/HCl, glycylglycine, imidazole and phosphate buffers at 0, 1 and 10 mM FDP under otherwise standard assay conditions. The four buffers, tris/maleate, triethanolamine/HCl, glycylglycine and imidazole, gave similar pH activity profiles. The three activity profiles in triethanolamine/HCl buffer in Figure 2.4.2c are very similar to the three curves of activity in tris/maleate buffer in Figure 2.4.2d. In both buffers, the activity at 10 mM FDP was only slightly higher than at 1 mM FDP and optimal activity occurred at pH 6.9 to 7.0 when FDP was present. However in the absence of FDP, the optimum pH shifted to pH 8.2 and the activity at this optimum pH was 60-fold less than optimal activity in the presence of FDP. In phosphate buffer (Figure 2.4.2e) the pH profile with 10 mM FDP was again very similar in shape to profiles in the other four buffers at 10 mM FDP. However with 1 mM FDP the activity was greatly reduced in phosphate buffer, to about 1/10 of that using 10 mM FDP, and activity was virtually undetectable in phosphate buffer in the absence of FDP.

The pH optimum for lactate oxidation was 8.0 to 8.2 for triethanolamine/HCl, tris/maleate and phosphate buffers, with and without FDP. Figure 2.4.2 f and g shows the pH activity profile for lactate oxidation in triethanolamine and phosphate buffer at 0, 1 and 10 mM FDP. Tris/maleate buffer gave the same profiles as triethanolamine/HCl buffer. As was the case for pyruvate reduction, the rate of lactate oxidation in triethanolamine/HCl buffer (Figure 2.4.2f) was only slightly less in 1 mM FDP than in 10 mM FDP. In phosphate buffer (Figure 2.4.2g) the activity in 1 mM FDP was only 50% of that in 10 mM FDP. With no FDP the rate of lactate oxidation in triethanolamine/HCl buffer dropped by half, while in phosphate buffer, at any pH value, there was no detectable activity.

The pH activity profile results obtained for the S. lactis C_{10} LDH are similar to those reported by Jonas et al. (1972) for S. cremoris US3, except that they obtained a much broader pH optimum for pyruvate reduction in the presence of FDP, extending from pH 7.5 down to pH 5.5, before activity fell off. The full pH profile for the S. cremoris US3 enzyme was only given for buffers containing phosphate (phosphate and phosphate/citrate buffers), but in triethanolamine buffer the partial pH activity profile for pyruvate reduction showed increasing activity down to pH 6.0. Hence the major difference between the LDH from

Figure 2.4.2: c, d and e

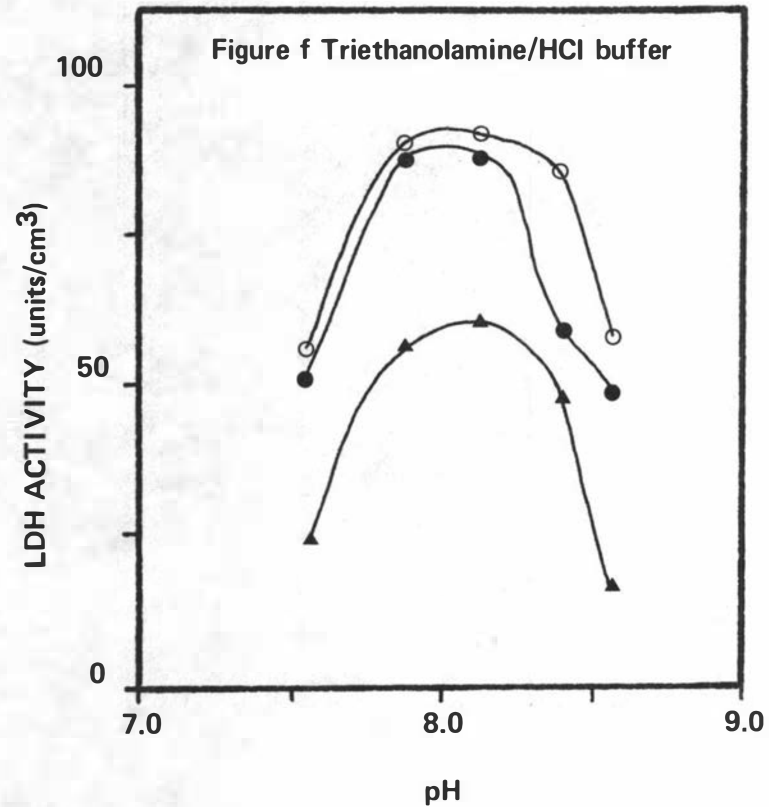
PYRUVATE REDUCTION pH PROFILES

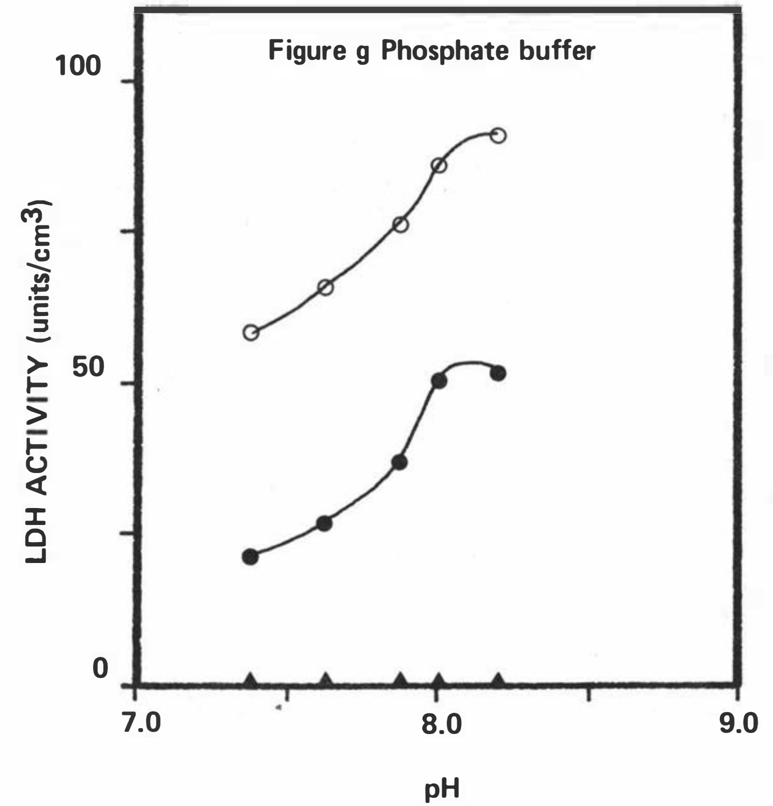


The effect of FDP on the pH optima curve for pyruvate reduction by S. lactis LDH was assayed in triethanolamine/HCl buffer (Figure c), tris/maleate buffer (Figure d) and in phosphate buffer (Figure e). The standard reaction mixture (total volume, 3 cm^3) for pyruvate reduction contained: 0.167 mM NADH; 10 mM Pyruvate and 90 mM buffer at pH values as indicated in the Figures. The three different FDP concentrations present in the reaction mixture were: ▲, no FDP; ●, 1 mM FDP; and o, 10 mM FDP.

Figure 2.4.2: f and g.

LACTATE OXIDATION pH PROFILES





The effect of FDP on the pH optima curve for lactate oxidation by S. lactis LDH was assayed in triethanolamine/HCl buffer (Figure f) and in phosphate buffer (Figure g). The standard reaction mixture (total volume, 3 cm^3) for lactate oxidation contained: 10 mM NAD^+; 33.3 mM L(+)–lactate and 90 mM buffer at pH values as indicated in the Figures. The three different FDP concentrations present in the reaction mixture were: ▲ , no FDP; ● , 1 mM FDP; and o, 10 mM FDP.

S. lactis C_{10} and S. cremoris was the acid pH optimum of the S. cremoris enzyme and the neutral pH optimum of the S. lactis C_{10} enzyme for pyruvate reduction.

pH versus activity profiles for the S. faecalis (Wittenberger and Angelo, 1970) and S. mutans (Brown and Wittenberger, 1972) LDH's were not included in the published studies on these two enzymes. However, from a study of the effect of pH on the FDP saturation curve it is clear that the LDH's from both these have a more acid pH optimum than that from S. lactis C_{10}. Thus for the S. mutans LDH maximum activity was greater at pH 5.5 than at 6.2 or 7.0 and for the S. faecalis LDH it was greater at pH 5.8 than at 6.5, 7.0 or 7.5. For both species, kinetic data were determined only in phosphate buffer (100 mM).

The kinetic studies reported in subsequent pages, were mostly carried out using tris/maleate buffer and the effect of phosphate on the activity of LDH was studied by comparison with activity in tris/maleate buffer. The pH optimum studies reported showed that tris/maleate and triethanolamine/HCl buffers at 0, 1 and 10 mM had the same effect on activity. However near the end of the experimental work on the LDH, it was found that the activity in tris/maleate is different from the activity in triethanolamine/HCl buffer when lower FDP concentrations are used. The differences in activity between triethanolamine/HCl and tris/maleate buffers will be reported with the appropriate kinetic results from studies of activity in phosphate and tris/maleate buffers.

2.4.3 Factors affecting the Fructose-1,6-Diphosphate Activation of *S. lactis* C_{10} L(+)-LDH

2.4.3.1 The effect of different buffers on FDP activation of L(+)-LDH

Large differences in $M_{0.5V}$ values (FDP requirement for half maximum activity) for FDP activation of the LDH's from various streptococci have been reported, as mentioned in the Introduction (2.1.3). The $M_{0.5V}$ values range from 5.0 mM for *S. mutans* (Brown and Wittenberger, 1972), 0.045 mM for *S. faecalis* (Wittenberger and Angelo, 1970) and down to approximately 0.005 mM for *S. cremoris* (Jonas *et al.*, 1972).

The work described in the previous section, on the effect of pH, showed that enzyme activity varied depending on the type of buffer used, particularly at low FDP concentrations. The effect of the three buffers (tris/maleate, triethanolamine/HCl and phosphate) on the $M_{0.5V}$ values for the *S. lactis* C_{10} LDH were determined at the pH optimum (6.9).

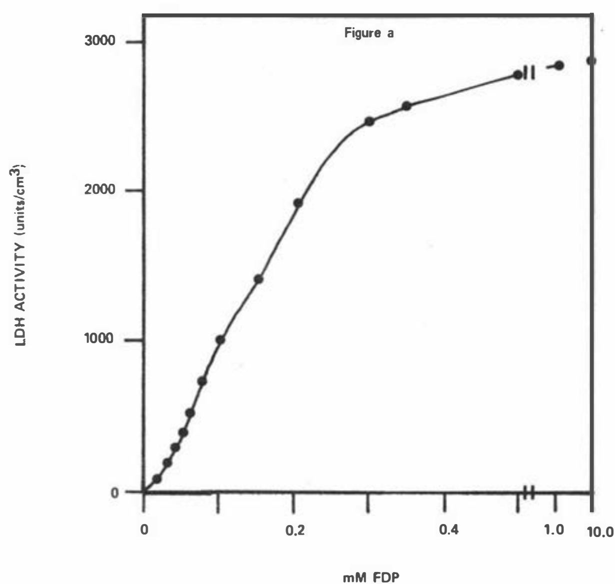
A plot of activity versus FDP concentration in tris/maleate buffer (Figure 2.4.3.1a) shows that the LDH activity was a sigmoidal function of FDP concentration. When the same data are plotted as a Hill plot (Figure 2.4.3.1b) (where log $v/V_{max}-v$ is plotted against log FDP concentration), the $M_{0.5V}$ value (where $v/V_{max}-v = 1$) is 0.2 mM FDP.

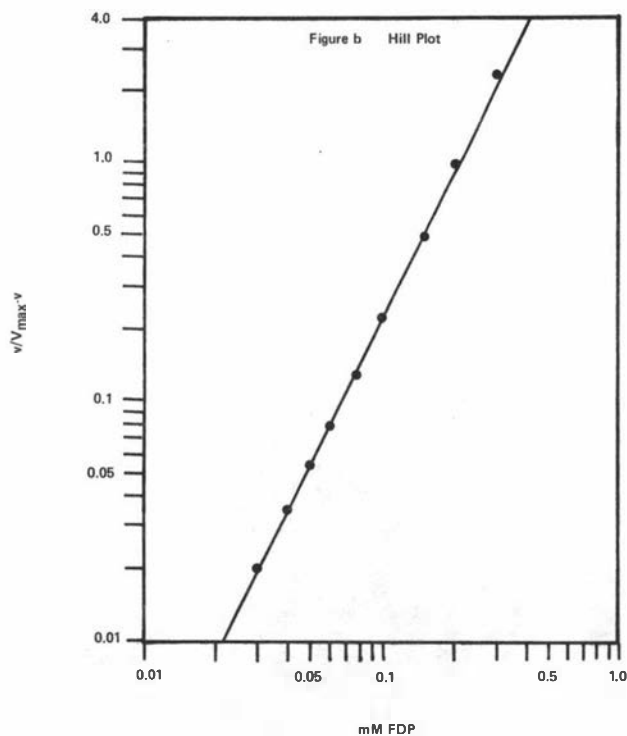
Hill plots of the data obtained in triethanolamine/HCl and phosphate buffer (using the same assay conditions as for tris/maleate), are compared with those in tris/maleate (Figure 2.4.3.1c). In phosphate buffer the $M_{0.5V}$ value is 4.4 mM FDP, whereas in triethanolamine/HCl buffer the $M_{0.5V}$ value is 0.002 mM FDP, i.e. a two thousand-fold difference. Jonas *et al.* (1972) showed that in phosphate buffer, the *S. cremoris* US3 LDH required a much higher concentration of FDP (10 mM) to obtain a reaction velocity equivalent to that obtained with 1 mM FDP in triethanolamine/HCl buffer. Though these workers did not study the effect of the two buffers over a range of FDP concentrations, it is evident that *S. cremoris* is at least qualitatively similar to *S. lactis* C_{10} in terms of relative activity in triethanolamine/HCl and phosphate buffers.

Thus the chemical composition of the buffer markedly effects the FDP activation of LDH in respect to the FDP concentration required for half maximal activation. The Hill interaction coefficient (n_H, i.e. the slope of the Hill plot) is relatively constant in the three buffers, being 2.1 in phosphate and tris/maleate buffers and 1.7 in triethanolamine/HCl buffer. This suggests that there are at least two interacting FDP binding sites in all three buffers.

Figure 2.4.3.1: a and b

EFFECT OF VARYING FDP CONCENTRATION ON ACTIVITY OF *S. lactis* LDH

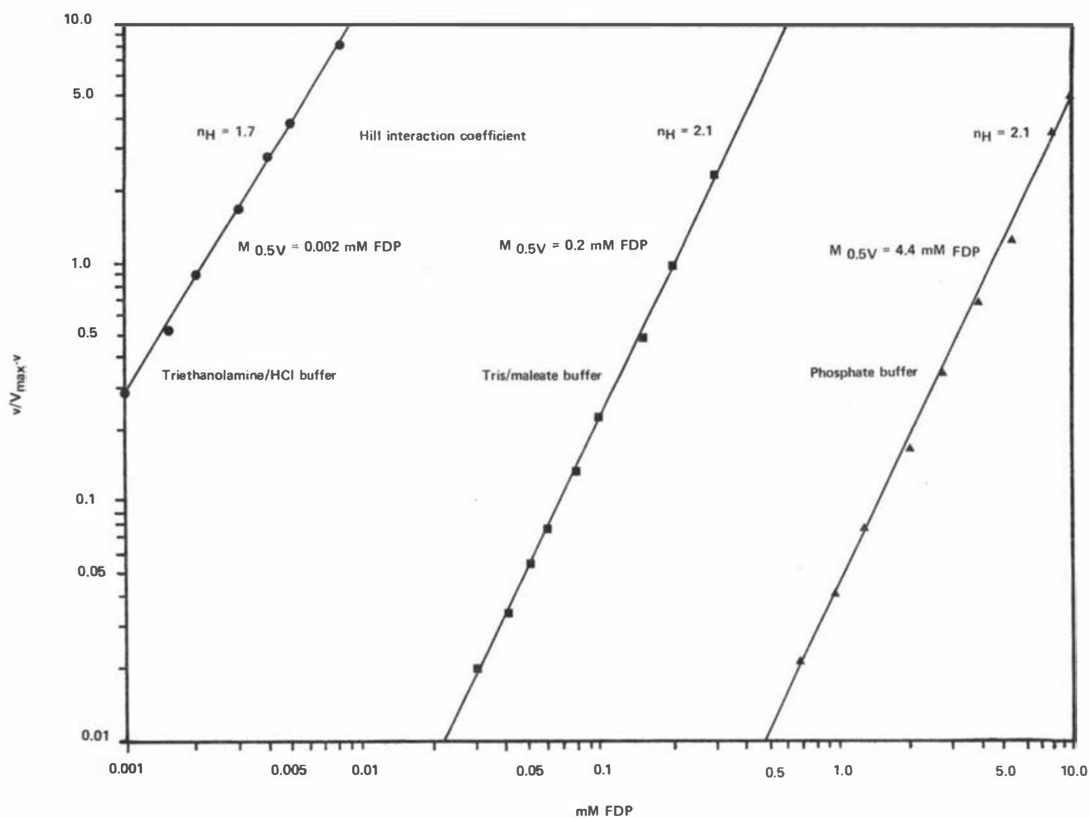




The effect of varying FDP concentration on the *S. lactis* LDH activity was studied. The reaction mixture contained (in a total volume of 3 cm^3): 90 mM tris/maleate buffer pH 6.9; 0.167 mM NADH; 10 mM pyruvate and 0.1 cm3 of diluted enzyme. The FDP concentration was varied as shown in the Figures. Figure a is a plot of LDH activity versus FDP concentration. Figure b is a Hill plot of the data from Figure a.

Figure 2.4.3.1 c

EFFECT OF THREE BUFFERS ON FDP ACTIVATION OF LDH



Hill plots (where log v/V_{max}-v is plotted against log FDP concentration) are shown for the FDP activation of LDH in three buffers: Triethanolamine/HCl buffer, ● ; tris/maleate buffer, ■ ; Phosphate buffer, ▲ . The reaction mixture contained in a total volume of 3 cm^3: 90 mM buffer, pH 6.9; 0.167 mM NADH; 10 mM pyruvate and 0.1 cm^3 of diluted enzyme. The FDP concentration was varied as shown in this Figure.

2.4.3.2 The effect of NADH and Pyruvate on FDP activation of L(+)-LDH

The influence of changes of NADH and pyruvate concentrations on FDP activation of LDH was investigated in 90 mM tris/maleate buffer pH 6.9. Brown and Wittenberger (1972) showed that for the S. mutans NCTC 10449 LDH, the $M_{0.5V}$ value for FDP decreased from 8.0 to 3.0 mM as the pyruvate concentration was increased from 5.0 to 20.0 mM. Pyruvate, did not, however, alter the Hill coefficient for FDP.

The results, summarised in Table 2.4.3.2, were obtained by measuring the activity at 12 different FDP concentrations for each different NADH and pyruvate concentration combination and then plotting the data as Hill plots. Changing the concentration of either substrate had only a small effect on both the Hill coefficient (n_H) and the $M_{0.5V}$ values. The n_H value (ranging from 1.7 to 2.1) is similar to that obtained by other workers for different strains of streptococci. A difference is seen between S. mutans NCTC 10449 (Brown and Wittenberger, 1972) and S. lactis C_{10} in that pyruvate concentration significantly effects the $M_{0.5V}$ value for the S. mutans enzyme but has little effect on the value for the S. lactis C_{10} LDH

2.4.3.3 The effect of pH on FDP activation of L(+)-LDH

Hill plots of data obtained by varying FDP concentrations at three different pH values in tris/maleate buffer are shown in Figure 2.4.3.3. Compared to the response at the optimum pH of 6.9, decreasing the pH to 5.9 has no significant effect on the interaction coefficient (2.1 to 1.9), but did increase significantly the $M_{0.5V}$ value from 0.2 mM to 0.5 mM FDP. At pH 8.0 on the other hand, the enzyme no longer showed a significant sigmoidal response to increasing FDP concentration (n_H = 1.2) and the $M_{0.5V}$ value was further decreased to 0.08 mM FDP.

These results differ considerably from the reported properties of the LDH's from S. mutans NCTC 10449 (Brown and Wittenberger, 1972) and S. faecalis (Wittenberger and Angelo, 1970). In both species, an increase in pH increased the $M_{0.5V}$ value, e.g. for S. faecalis, which showed the smallest change, the $M_{0.5V}$ value at pH 5.8 was approximately 0.03 mM FDP and increased to 0.06 mM FDP at pH 7.5. For S. mutans the $M_{0.5V}$ value at pH 5.5 was approximately 3.6 mM FDP and increased to 16 mM FDP at pH 7.0. For both species the n_H value was not affected significantly by varying the pH.

Table 2.4.3.2

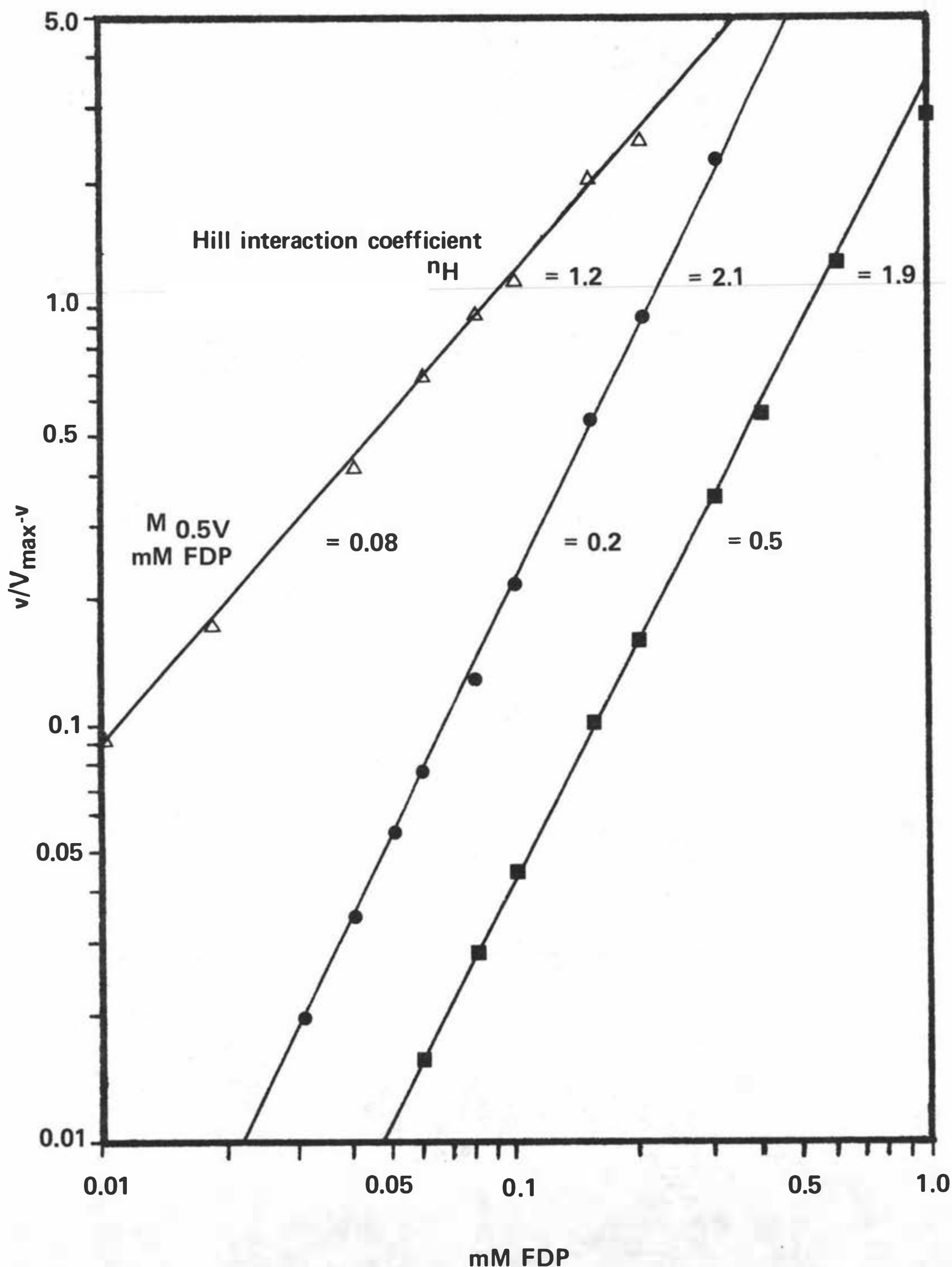
The effect of NADH and pyruvate on FDP activation of LDH

The activity at 12 different FDP concentrations, for each different NADH and pyruvate concentration combination, was measured in 90 mM tris/ maleate buffer pH 6.9. The appropriate V_{max} value obtained from double reciprocal plots was used to calculate the data for the Hill plot. The $M_{0.5V}$ values (FDP concentration required for half maximum activity) and the Hill interaction coefficient (n_H) values for FDP were obtained from the Hill plots (plot of log $v/V_{max}-v$ versus log FDP concentration) at different NADH and pyruvate concentrations.

		NADH 0.05 mM	NADH 0.1 mM	NADH 0.167 mM
Pyruvate 10 mM	V_{max}	1900 units/cm^3	2550 units/cm^3	2900 units/cm^3
	n_H	1.8	1.7	1.7
	$M_{0.5V}$	0.22 mM FDP	0.17 mM FDP	0.16 mM FDP
		Pyruvate 0.5 mM	**Pyruvate 2.0 mM**	**Pyruvate 10.0 mM**
NADH 0.167 mM	V_{max}	720 units/cm^3	1830 units/cm^3	2900 units/cm^3
	n_H	2.1	1.85	1.7
	$M_{0.5V}$	0.20 mM FDP	0.19 mM FDP	0.16 mM FDP

Figure 2.4.3.3

THE EFFECT OF pH ON FDP ACTIVATION OF LDH



Hill plots (log v/V_{max}-v versus log FDP concentration) of data obtained by varying FDP concentration at three different pH values in tris/maleate buffer are shown. V_{max} values were obtained from double reciprocal plots. The standard assay conditions were: 90 mM tris/maleate buffer; 10 mM pyruvate; 0.167 mM NADH and the FDP concentration was varied as shown in the figure. The three pH values are: △, pH 8.0; ●, pH 6.9; ■, pH 5.9.

Because the results obtained in the present study on the effect of pH on the $M_{0.5V}$ values for FDP were at variance with those of other workers, the effect of pH was examined in phosphate buffer and triethanolamine/HCl buffer. The results of the effect of pH on the $M_{0.5V}$ values and the n_H values obtained in the two buffers are tabulated in Table 2.4.3.3, along with the results obtained in tris/maleate buffer (Hill plots for tris/maleate buffer shown in Figure 2.4.3.3).

The results obtained, in triethanolamine/HCl buffer, on the effect of pH on FDP activation were different from those results in tris/maleate buffer. The results were, however, consistent with the *S. mutans* and *S. faecalis* findings in that an increase from pH 6.4 to 8.0 gave an increase in the $M_{0.5V}$ value (0.0013 to 0.09 mM FDP). The only major difference was at pH 8.0, where the Hill interaction coefficient for FDP was 1.0 over the entire FDP concentration range

In phosphate buffer, a third distinctive pattern of response to varying pH was obtained in that varying the pH from 5.65 - 8.00 had no significant effect on either the n_H or the $M_{0.5V}$ value. This result was unexpected since phosphate buffer was used in the studies on both the *S. faecalis* and *S. mutans* LDH's. The interacting effects of buffer ion type, pH and FDP concentration are clearly very complex and require more intensive study to resolve the mechanisms.

It is difficult to make a close quantitative comparison between the data obtained in the present study on the *S. lactis* C_{10} LDH and that of Jonas *et al.* (1972) for the LDH of *S. cremoris* US3. These workers used triethanolamine/HCl buffer and did study the effect of pH on the FDP requirement, but do not give $M_{0.5V}$ or n_H values. However, from the V versus FDP plots it is quite evident that the $M_{0.5V}$ value does increase as the pH increases from 6.0 to 8.0. This trend is similar to the pH effect in triethanolamine/HCl buffer on the FDP activation of *S. lactis* C_{10} LDH.

Table 2.4.3.3

The effect of pH and buffer components on FDP activation of LDH

The standard assay conditions are: 90 mM buffer, 10 mM pyruvate and 0.167 mM NADH. For each pH and buffer type the activity was measured at twelve appropriate FDP concentrations and the data were then plotted as a double reciprocal plot to obtain the V_{max} value. The V_{max} value obtained was used in plotting the respective Hill plot. Appropriate FDP concentrations were chosen for each pH and buffer type to give at least eight usable points to plot the respective Hill plot. The values tabulated below for tris/maleate buffer at the three pH values are obtained from the Hill plots shown in Figure 2.4.3.3a.

Tris/maleate buffer

pH	5.9	6.9	8.0
$M_{0.5V}$	0.5 mM FDP	0.2 mM FDP	0.08 mM FDP
n_H	1.9	2.1	1.2

Triethanolamine/HCl buffer

pH	6.4	6.9	8.0
$M_{0.5V}$	0.0013 mM FDP	0.0022 mM FDP	0.09 mM FDP
n_H	1.7	1.7	1.0

Phosphate (KH_2PO_4 NaOH) buffer

pH	5.65	6.9	8.0
$M_{0.5V}$	5.0 mM FDP	4.4 mM FDP	5.4 mM FDP
n_H	2.3	2.1	2.1

2.4.4 Effect of Varying Pyruvate and NADH on Enzyme Activity

2.4.4.1 Determinations of Michaelis constants for pyruvate and NADH for *S. lactis* C_{10} L(+)-LDH in tris/maleate buffer.

A concentration of 0.5 mM FDP was selected for determinations of the Michaelis constants (K_M) for NADH and pyruvate in 90 mM tris/maleate buffer, pH 6.9. Lineweaver-Burk double reciprocal plots, with NADH as the variable substrate and at 8 different pyruvate concentrations are shown in Figure 2.4.4.1 a and with pyruvate as the variable substrate at 6 different NADH concentrations in Figure 2.4.4.1 c. Both plots give a family of intersecting lines, indicating that the reaction mechanism is, as expected, sequential and not ping-pong. The point of intersection was on, or very close to, the 1/(substrate concentration) axis in both plots. The K_M for NADH was 0.08 mM, and the K_M for pyruvate was 1.5 mM.

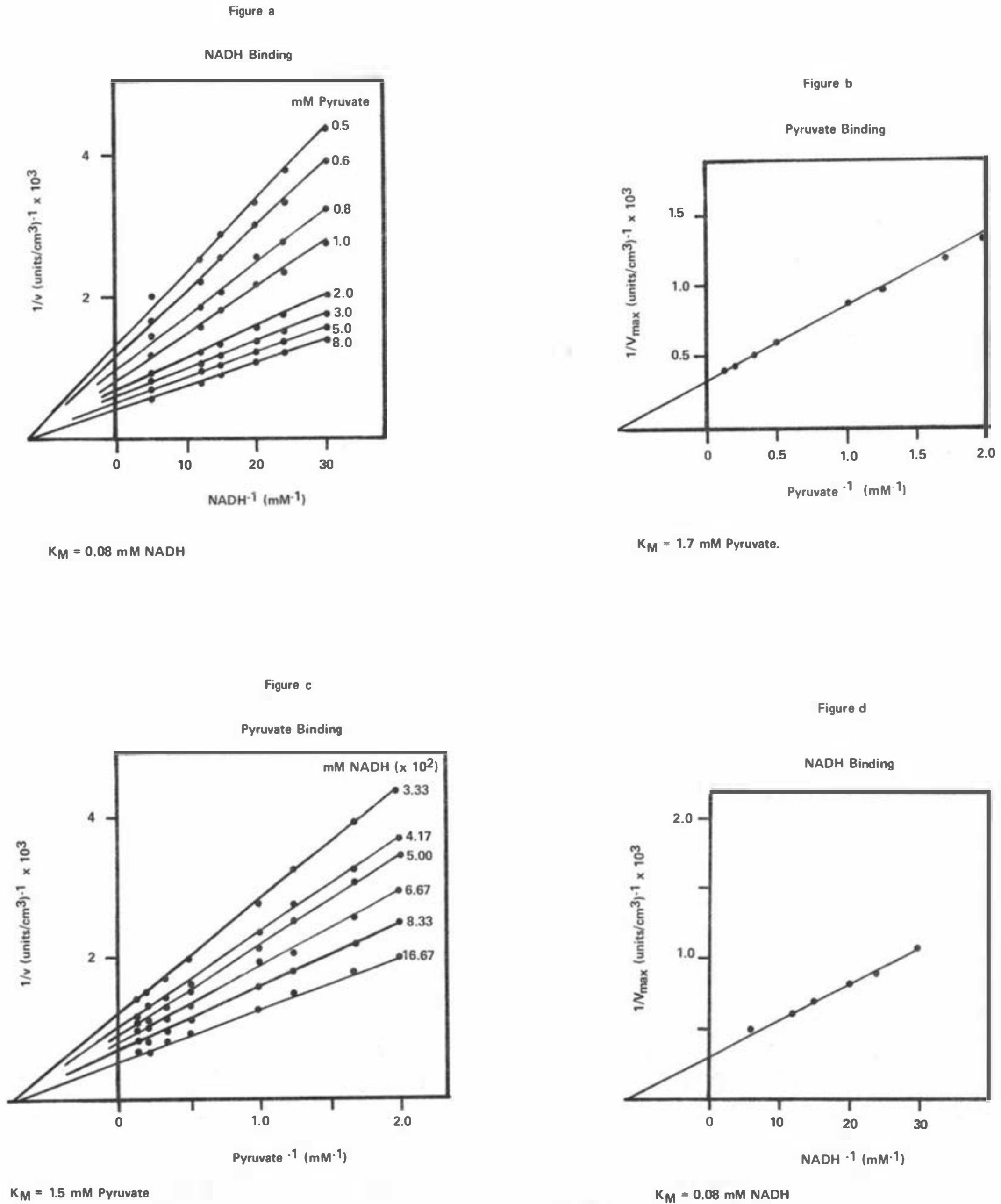
Secondary plots (Figures 2.4.4.1 b and d), obtained by plotting the Y intercepts of the previous two graphs (reciprocal of V_{max}) against the reciprocal of the respective substrate concentrations, gave K_M values for pyruvate of 1.7 mM and for NADH of 0.08 mM. These K_M values are in good agreement with the values obtained in the primary plots and are in reasonably good agreement with the K_M values obtained via similar 2° plots by Jonas *et al.* (1972) for *S. cremoris* US3 LDH, namely 1.15 mM for pyruvate and 0.044 mM for NADH at pH 6.0 (triethanolamine/HCl buffer) using 1.5 mM FDP.

The double reciprocal plots for both pyruvate and NADH (Figures 2.4.4.1 a and c) are quite linear over the range of concentrations used and if the data are plotted as Hill plots, no co-operative interaction is evident for either substrate as the Hill interaction coefficient is 1.0. There is thus no evidence of the non linearity of activity with respect to varying pyruvate concentration as is found with the L(+)-LDH's of certain strains of *S. mutans* (Brown and Wittenberger, 1972).

Figure 2.4.4.1 e shows that pyruvate will inhibit at concentrations greater than 20 mM in otherwise standard assay conditions. Although this inhibition effect has not been observed for other streptococcal LDH's it is consistent with the effect of high pyruvate concentrations on mammalian LDH's. However a significant difference exists between the pyruvate inhibition of the H_4 type mammalian LDH and that of the *S. lactis* C_{10} LDH in terms of possible *in vivo* control. For H_4 LDH

Figure 2.4.4.1: a, b, c and d

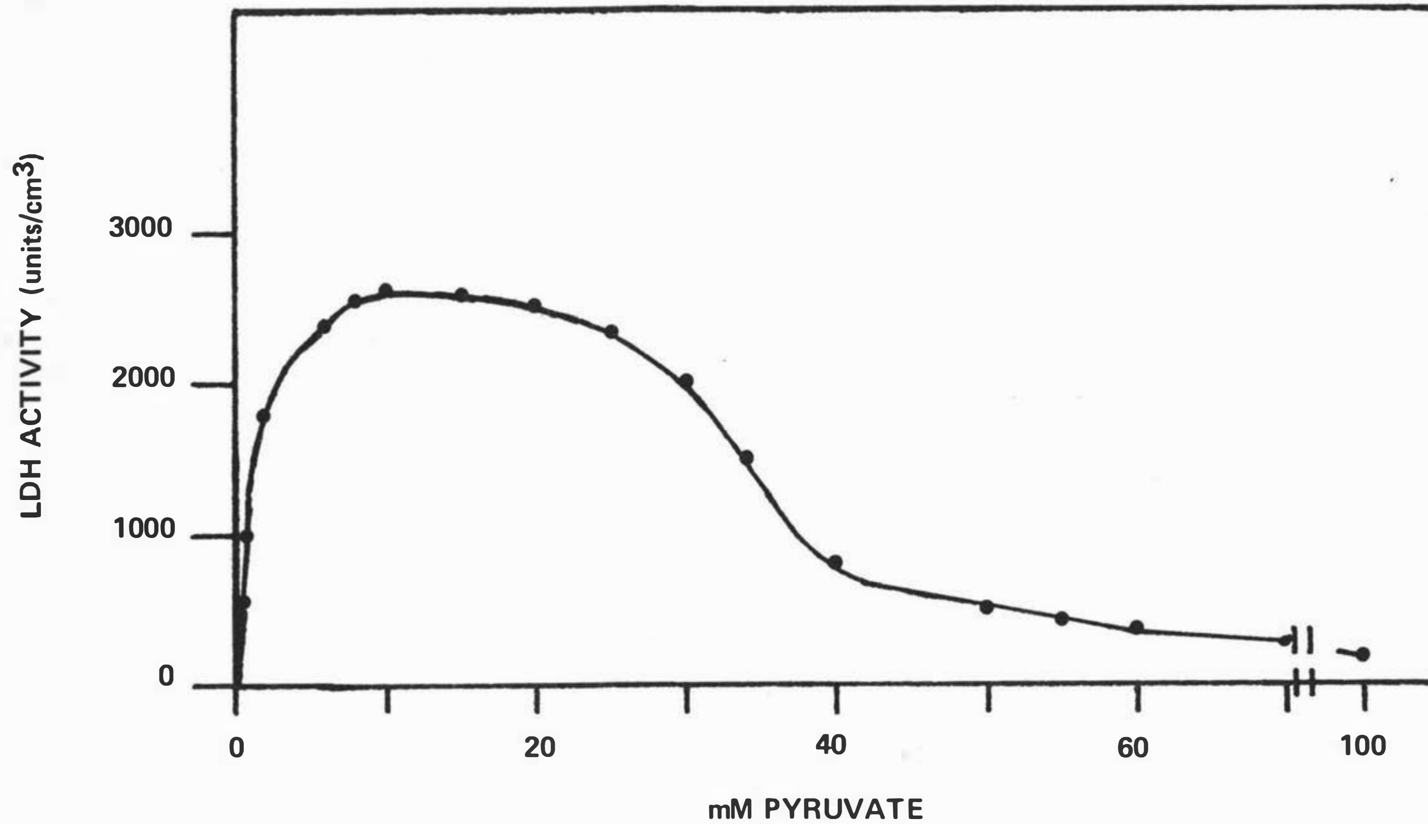
K_M VALUES FOR PYRUVATE AND NADH



The reaction mixture contained (in a total volume of 3 cm^3): 90 mM tris/maleate buffer pH 6.9; 0.5 mM FDP and 0.1 cm^3 of diluted enzyme. The NADH concentrations were varied as shown in Figure a, at 8 different pyruvate concentrations. Figure a (Lineweaver-Burk plot) is a plot of the reciprocal of the LDH activity versus the reciprocal of the NADH concentration. Figure b is a secondary plot of the data in Figure a where the reciprocal of the V_{max} values (determined from Figure a) are plotted against the reciprocal of the 8 respective pyruvate concentrations.
Figure c is a plot of the reciprocal of the LDH activity versus the reciprocal of the pyruvate concentration at six different NADH concentrations. Figure d is a secondary plot of the data in Figure c where the reciprocal of the V_{max} values (determined from Figure c) are plotted against the reciprocal of the 6 respective NADH concentrations.

Figure 2.4.4.1: e

PYRUVATE INHIBITION



The pyruvate concentration was varied as shown in the above figure, where LDH activity is plotted against pyruvate concentration. The standard assay conditions were: 90 mM tris/maleate buffer pH 6.9, 0.167 mM NADH and 1.0 mM FDP.

(Kaplan et al., 1968), pyruvate inhibits at concentrations greater than 1.0 mM whereas the pyruvate only inhibits the S. lactis LDH at pyruvate concentrations twenty times higher.

2.4.4.2 Determinations of Michaelis constants for pyruvate and NADH in different buffers

Kinetic data obtained by varying pyruvate and NADH in triethanolamine/HCl buffer are shown in Table 2.4.4.2 along with the data obtained at the same time using tris/maleate buffer. The K_M values for NADH and pyruvate determined in triethanolamine/HCl buffer at 1 mM FDP were the same as the K_M values determined in tris/maleate buffer under identical conditions. Reducing the FDP to an unsaturating concentration for the two buffers, i.e. to 0.001 mM FDP for triethanolamine/HCl buffer and 0.1 mM FDP for tris/maleate buffer, increased the K_M values for both substrates and decreased the V_{max} values, compared to those obtained at 1 mM concentrations of FDP. There appeared to be no sigmoid response to either substrate in triethanolamine/HCl buffer, as is the case also in tris/maleate buffer (Section 2.4.4.1).

The results, obtained from varying pyruvate in 90 mM phosphate buffer, at three FDP concentrations, are shown in Figure 2.4.4.2a and Table 2.4.4.2. The highest concentration of FDP used, 6.67 mM, was somewhat higher than the $M_{0.5V}$ value in phosphate buffer (4.4 mM FDP). The K_M for pyruvate at 6.67 mM FDP was 5.70 mM compared to a value of 2.0 to 2.5 mM pyruvate for a comparable FDP concentration in tris/maleate buffer (see following Table 2.4.5a). With decreasing FDP concentration, the K_M for pyruvate was increased and the V_{max} decreased, showing the same trend as found in triethanolamine/HCl and tris/maleate buffers.

However when NADH was varied in phosphate buffer, the activity showed a sigmoidal response to increasing NADH concentrations as shown in Figure 2.4.4.2b. The same data are plotted as a Hill plot in Figure 2.4.4.2c from which a Hill interaction coefficient (n_H) of 1.7 and a NADH concentration giving half maximum velocity ($NADH_{0.5V}$) of 0.14 mM were obtained. A sigmoidal response to NADH has not been reported in any other streptococcal LDH and did not occur in S. lactis C_{10} LDH assayed in tris/maleate and triethanolamine buffers.

This sigmoidal response of reaction velocity to NADH concentration, for S. lactis LDH was reproducible. The effect of NADH in phosphate buffer was tried on two separate LDH preparations and carried out in triplicate for each preparation, and in each case the response was the same as shown in Figure 2.4.4.2b and c.

Table 2.4.4.2

Influence of buffer composition on

K_M and V_{max} values for pyruvate and NADH

The K_M and V_{max} values for pyruvate and NADH under different assay conditions, were determined from Lineweaver-Burk plots. The different assay conditions refer to the buffer type and the different FDP concentrations as shown in the first two columns of the table. For all assays, 90 mM buffer pH 6.9 was used. For the assays where the pyruvate concentration was varied a constant concentration of 0.167 mM NADH was used and where the NADH concentration was varied a constant concentration of 10 mM pyruvate was used. The tabulated results for varying pyruvate concentrations in phosphate buffer at three different FDP concentrations were obtained from the plots shown in Figure 2.2.4.2a.

Varying Pyruvate concentration

Buffer type	FDP concentration (mM)	K_M for Pyruvate (mM)	V_{max} units/cm^3
Triethanolamine/HCl	1.0	1.25	500.0
Triethanolamine/HCl	0.001	1.70	340.0
Tris/maleate	1.0	1.25	500.0
Tris/maleate	0.1	3.30	220.0
Phosphate	6.67	5.70	560.0
Phosphate	3.33	11.40	400.0
Phosphate	1.33	44.40	200.0

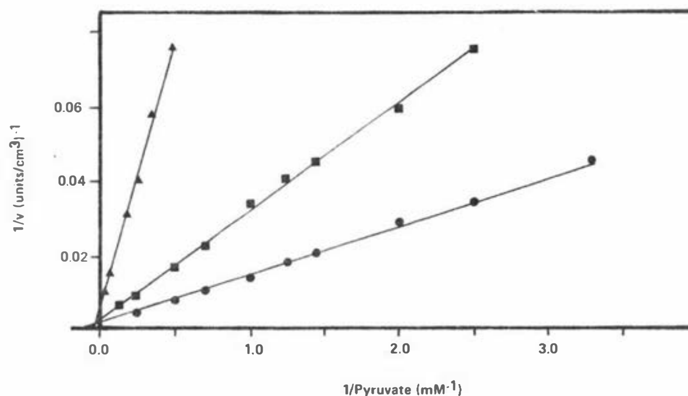
Varying NADH concentration

		K_M for NADH (mM)	
Triethanolamine/HCl	1.0	0.07	670.0
Triethanolamine/HCl	0.001	0.20	410.0
Tris/maleate	1.0	0.07	670.0
Tris/maleate	0.1	0.14	290.0

Figure 2.4.4.2: a, b and c

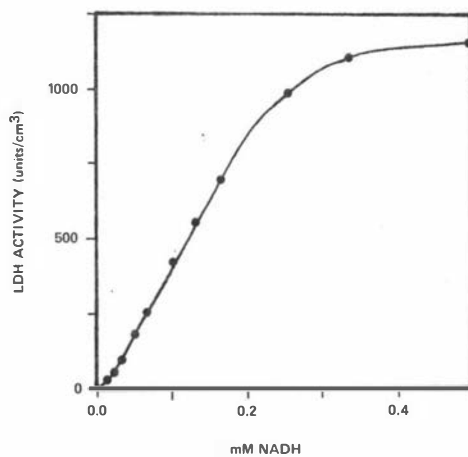
EFFECT OF PHOSPHATE BUFFER ON PYRUVATE AND NADH BINDING

Figure a Pyruvate Binding

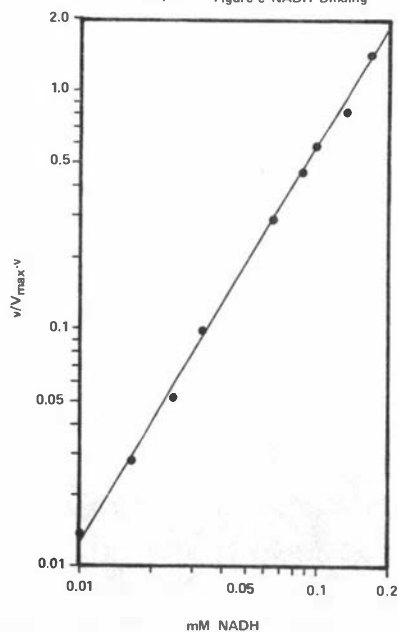


In Figure a the pyruvate binding in phosphate buffer is studied at three FDP concentrations: ●, 6.67 mM FDP; ■, 3.33 mM FDP; ▲, 1.33 mM FDP. The reaction mixture contained in a total volume of 3 cm^3: 90 mM phosphate buffer pH 6.9; 0.167 mM NADH and 0.1 cm^3 of diluted enzyme. The pyruvate concentrations are varied as shown in Figure a, where the reciprocal of the LDH activity is plotted against the reciprocal of the pyruvate concentration, for each different FDP concentration.

Figure b NADH Binding



Hill plot Figure c NADH Binding



In Figures b and c the sigmoidal binding of NADH to the S. lactis LDH is shown. Figure b is a plot of LDH activity versus NADH concentration. Figure c is a Hill plot of the same data in Figure b. The reaction mixture contained in a total volume of 3 cm^3: 90 mM phosphate buffer pH 6.9; 10 mM pyruvate; 10 mM FDP and 0.1 cm^3 of diluted enzyme. The NADH concentrations are varied as shown in Figures b and c. The V_{max} value used for calculating the Hill plot values was obtained by extrapolation of Lineweaver -Burk plots to the 1/v axis.

2.4.5 The Effect of Fructose-1,6-Diphosphate on Kinetic Parameters

In the previous section (2.4.4) it was shown that when the FDP concentration was varied, a lowering of FDP concentration increased K_M and lowered V_{max} for both substrates in all three buffer used. This apparent effect of FDP on the kinetic parameters was different from the results obtained by some other workers. Wittenberger and Angelo (1970) showed for S. faecalis that lowering the FDP concentration increased significantly the apparent K_M for both pyruvate and NADH without affecting V_{max}. Brown and Wittenberger (1972) found that lowering of the FDP concentration did not change the K_M for NADH but did lower the V_{max} for the LDH of S. mutans NCTC 10449. Therefore a more intensive investigation on the effect of FDP on the kinetic parameters of LDH from S. lactis C_{10} was carried out in 90 mM tris/maleate buffer pH 6.9.

The K_M for pyruvate and V_{max} were determined from Lineweaver-Burk double reciprocal plots at four different FDP concentrations. Two sets of determinations were made, each at a different NADH concentration. The results are shown in Table 2.4.5a. Table 2.4.5b summarizes the effect of FDP on NADH binding from a similar set of determinations.

Decreasing the FDP concentration increased the K_M for both pyruvate and NADH and substantially decreased the V_{max}. The effect of varying FDP on the K_M for NADH was virtually identical at the two pyruvate concentrations used. However the K_M for pyruvate increased from 4.9 to 10 mM at a low FDP concentration (0.05 mM) when the NADH concentration was decreased from 0.167 mM to 0.083 mM.

Although Jonas et al. (1972) demonstrated a similar effect of FDP in triethanolamine/HCl buffer on the kinetic parameters of the S. cremoris LDH, their comparison was made between activity determinations at pH 6.0 in the presence of 1.5 mM FDP and at pH 8.0 in the absence of FDP. Since high pH values may lead to a dissociation or conformational change of the enzyme (Jago et al., 1971) and consequently, perhaps, to a different mechanism, the change in kinetic parameters at pH 8.0 might not necessarily have been due to the absence of FDP. The results obtained for the S. lactis C_{10} LDH are quite different from those obtained by Wittenberger and Angelo (1970), for S. faecalis where V_{max} was unaffected by FDP and only the K_M for NADH and pyruvate was altered by varying the FDP concentration. The converse was reported by Brown and Wittenberger (1972) for the S. mutans enzyme, where the

K_M for NADH was unaffected and only V_{max} was altered by variation of FDP concentrations. The *S. mutans* LDH showed a sigmoidal response to varying pyruvate concentration and decreasing FDP concentrations increased the $S_{0.5V}$ value for pyruvate. Although this work on the *S. mutans* and *S. faecalis* LDH's was carried out in 100 mM phosphate buffer, the trend of the effect of FDP on the kinetic parameters for the *S. lactis* C_{10} LDH appeared to be the same in all three buffers (see results in Section 2.4.4.3).

There is thus an unresolved difference between the conclusions from the present study on the effect of FDP on kinetic parameters and those of Wittenberger's group on the LDH's from *S. mutans* and *S. faecalis*. The contrast with the *S. faecalis* enzyme is particularly striking where FDP concentration had no effect on V_{max} since in the enzyme from the other three species (*S. lactis* C_{10}, *S. cremoris* US3 and *S. mutans* NCTC 10449).studied V_{max} was markedly increased by increasing FDP concentration.

Table 2.4.5a

Effect of FDP on pyruvate binding

The effect of different FDP concentrations on pyruvate binding was studied at two different NADH concentrations.

For each combination of FDP and NADH concentration, the pyruvate concentration was varied and the activity was plotted against pyruvate concentration as a Lineweaver-Burk plot (double reciprocal plots). 90 mM tris/maleate buffer pH 6.9 was present in all assays and the FDP and NADH concentrations were as indicated in the table.

NADH concentration = 0.167 mM

FDP concentration (mM)	K_M Pyruvate (mM)	V_{max} units/cm^3
1.0	1.4	2840
0.5	1.6	2000
0.1	2.7	388
0.05	4.9	182

NADH concentration = 0.083 mM

FDP concentration (mM)	K_M Pyruvate (mM)	V_{max} units/cm^3
0.1	3.6	182
0.05	10.0	138

Table 2.4.5b

Effect of FDP on NADH binding

The effect of different FDP concentrations on NADH binding was studied at two different pyruvate concentrations.

For each combination of FDP and pyruvate concentration, the NADH concentration was varied and the activity was plotted against NADH concentration as a Lineweaver-Burk plot. 90 mM tris/maleate buffer pH 6.9 was present in all assays and the FDP and pyruvate concentrations were as indicated in the table.

Pyruvate concentration = 10 mM

FDP concentration (mM)	K_M NADH (mM)	V_{max} (units/cm^3)
10.0	0.05	4000
1.0	0.07	3640
0.5	0.07	3100
0.1	0.10	625
0.05	0.14	365

Pyruvate concentration = 1 mM

FDP concentration (mM)	K_M NADH (mM)	V_{max} (units/cm^3)
10.0	0.05	2500
1.0	0.06	2300
0.5	0.07	1940
0.1	0.10	263
0.05	0.14	172

2.4.6 Lactate Oxidation by *S. lactis* C_{10} L(+)-LDH.

The only work previously done on lactate oxidation by streptococcal LDH's is by Jonas *et al.* (1972) who studied the LDH from *S. cremoris* US3. These workers only studied the lactate oxidation in respect to pH optimum in triethanolamine/HCl and sodium phosphate buffer at three different FDP concentrations. The kinetics of lactate oxidation were studied to assess the potential significance of this reaction under *in vivo* conditions.

2.4.6.1 Determinations of K_M values for L(+)-Lactate and NAD^+

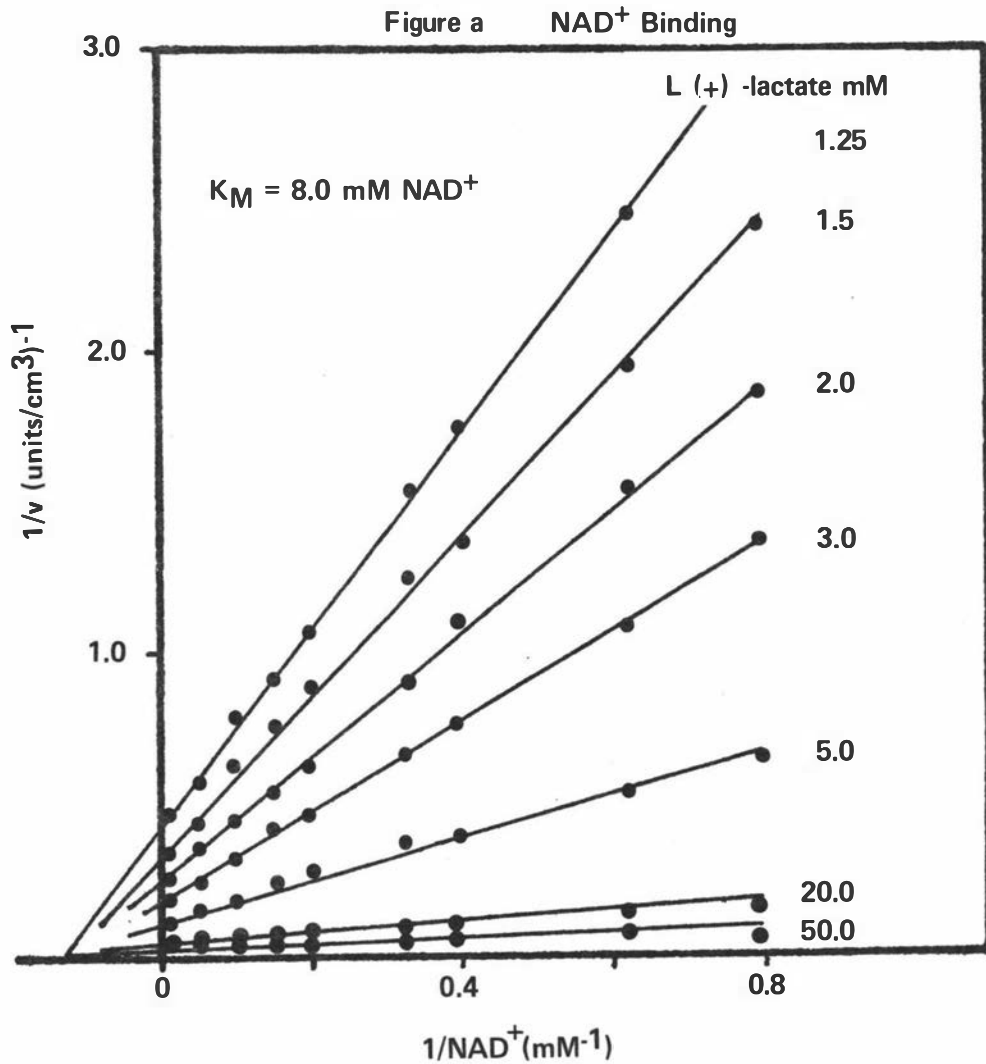
The *S. lactis* C_{10} LDH appeared to be specific for L(+)-lactate as no activity was found in the presence of D(-)-lactate. A small amount of activity was found at high D(-)-lactate concentrations (50 to 200 mM), but this activity could be entirely accounted for by the amount of contaminating L(+)-lactate stated to be present by the supplier of the D(-)-lactate (Sigma Chemical Company).

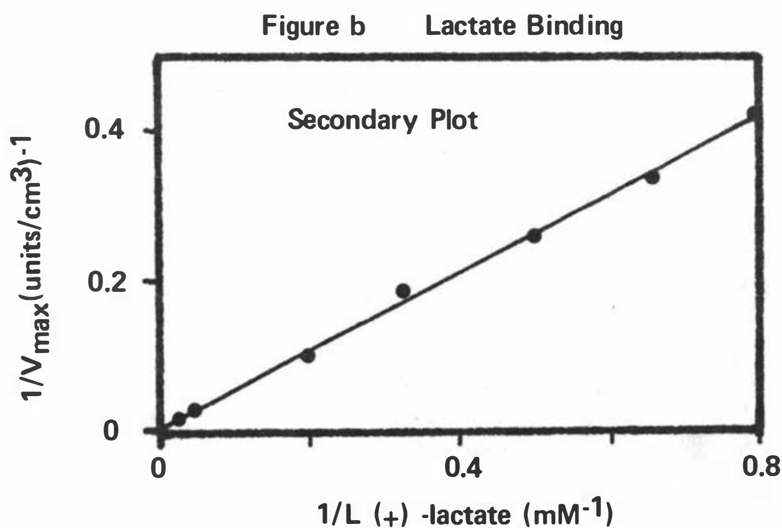
Michaelis constants for L(+)-lactate and for NAD^+ were determined in 90 mM triethanolamine/HCl buffer pH 7.9 in the presence and absence of FDP. Unlike pyruvate reduction, lactate oxidation has the same pH optimum with and without FDP (see Section 2.4.2).

Figure 2.4.6.1a shows that the K_M for NAD^+ is independent of L(+)-lactate concentration in the absence of FDP, as a family of intersecting lines is obtained, intersecting on the X axis to give a K_M of 8.0 mM NAD^+. When the V_{max} values obtained from the Y intercepts of the plots in Figures 2.4.6.1a were plotted as a 2° plot (plot of $1/V_{max}$ versus 1/mM L(+)-lactate), a straight line was obtained as shown in Figure 2.4.6.1b. The K_M for L(+)-lactate from this 2° plot could not be determined as the intercept of the plot was near the origin of the axes. The K_M for L(+)-lactate was estimated from a V versus S plot to be approximately 500 mM or greater, and at such high concentrations of L(+)-lactate, some inhibition probably occurs due to high ionic strength.

Data obtained in the presence of 1 mM FDP are shown in Figures 2.4.6.1 c and e. The two families of plots intersect on the X axis showing that the K_M values for NAD^+ and L(+)-lactate are both independent of the concentration of the other substrate. The respective 2° plots, shown in Figures 2.4.6.1 d and f give K_M values of 130 mM L(+)-lactate and 2.8 mM NAD^+ respectively. (The K_M values obtained from the 1° plots are 120 $\pm$ 10 mM L(+)-lactate and 2.4 $\pm$ 0.2 mM NAD^+.)

Figure 2.4.6.1: a and b K_M VALUES FOR L (+) - LACTATE AND NAD^+

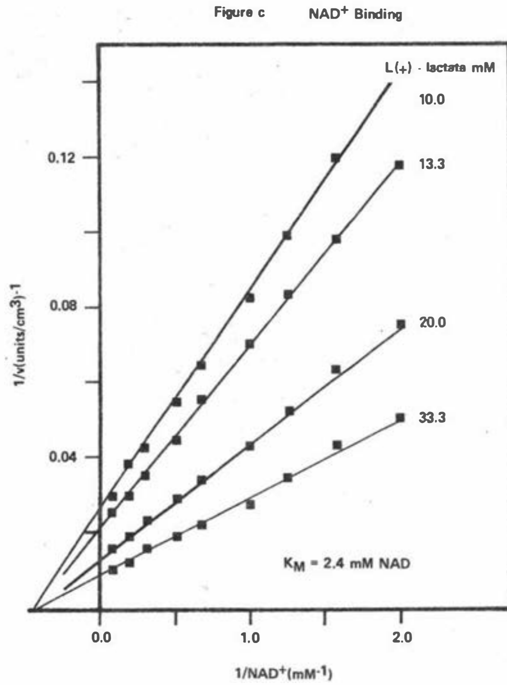


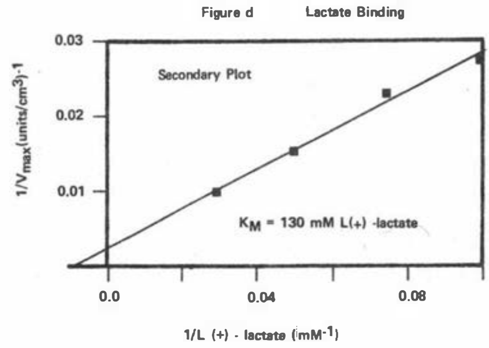


The K_M values for NAD^+ and L (+) -lactate for S. lactis LDH are determined in the absence of FDP. The reaction mixture contained (in a total volume of 3 cm^3) : 90 mM triethanolamine/HCl buffer pH 7.9 and 0.1 cm^3 of diluted enzyme. The NAD^+ concentrations are varied as shown in Figure a at seven different L (+) -lactate concentrations. Figure a (Lineweaver-Burk plot) is a plot of the reciprocal of the LDH activity versus the reciprocal of NAD^+ concentration. Figure b is a secondary plot of the data in Figure a, where the reciprocal of the V_{max} values (determined from Figure a) are plotted against the reciprocal of the seven respective L (+) -lactate concentrations.

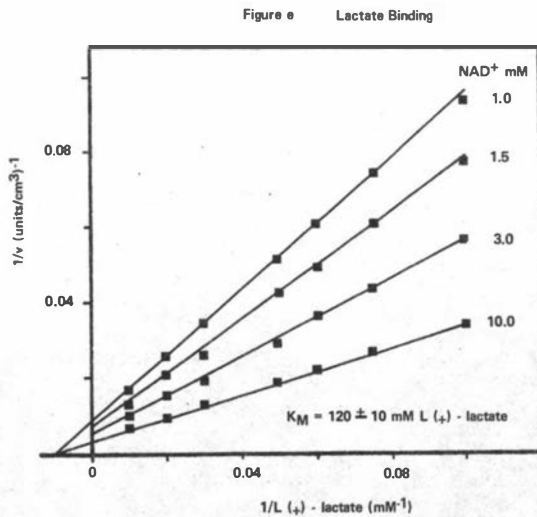
Figure 2.4.6.1: c, d, e and f

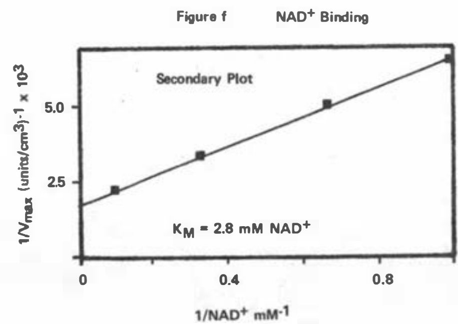
K_M VALUES FOR L (+) - LACTATE AND NAD^+





The K_M values for NAD^+ and L (+) - lactate for S. lactis LDH were determined in the presence of FDP. The reaction mixture contained in a total volume of 3 cm^3: 90 mM triethanolamine/HCl buffer, pH 7.9; 1 mM FDP and 0.1 cm^3 of diluted enzyme. The NAD^+ concentrations were varied as shown in Figure c at four different L (+) - lactate concentrations. Figure c (Lineweaver-Burk plot) is a plot of the reciprocal of the LDH activity versus the reciprocal of NAD^+ concentrations. Figure d is a secondary plot of the data in Figure c, where the reciprocal of the V_{max} values (determined from Figure c) are plotted against the reciprocal of the four respective L (+) - lactate concentrations.





The K_M values for NAD^+ and L (+) - lactate for S. lactis LDH are determined in the presence of FDP. The reaction mixture contained in the total volume of 3 cm^3 : 90 mM triethanolamine/HCl buffer, pH 7.9; 1 mM FDP and 0.1 cm^3 of diluted enzyme. The L (+) - lactate concentrations are varied as shown in Figure e at four different NAD^+ concentrations. Figure e (Lineweaver-Burk plot) is a plot of the reciprocal of the LDH activity versus the reciprocal of L (+) - lactate concentration. Figure f is a secondary plot of the data in Figure e, where the reciprocal of the V_{max} values (determined from Figure e) are plotted against the reciprocal of the four respective NAD^+ concentrations.

The effect of FDP on the kinetic parameters for lactate and NAD^+ is similar to the effect of FDP on pyruvate reduction, as in both reaction directions, the presence of FDP (or an increase of FDP concentration) increased the affinity for the respective substrates and increased V_{max}.

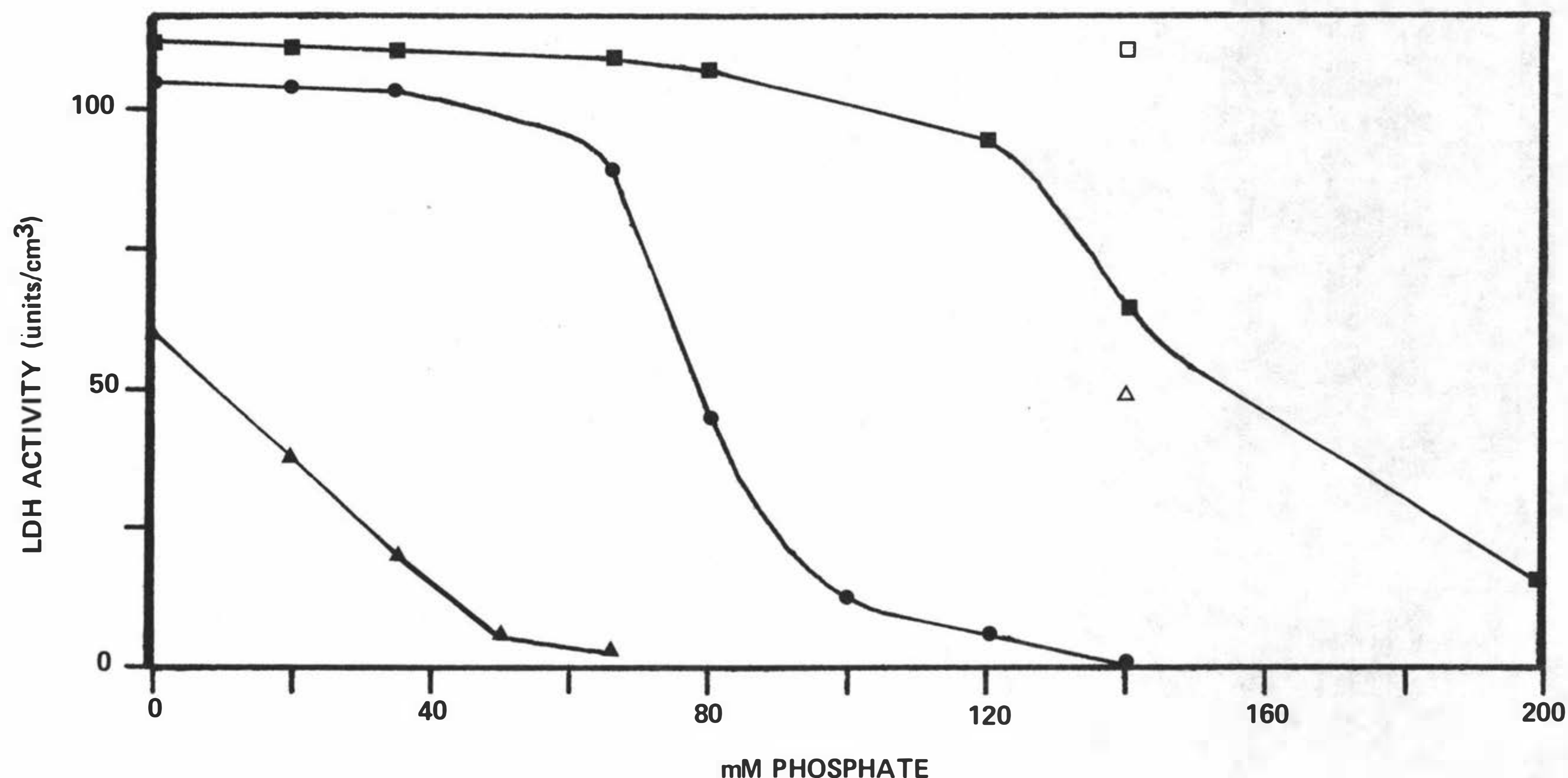
The K_M values for NAD^+ and L(+)-lactate in the presence of FDP, are very high compared to the K_M values for pyruvate and NADH at the same level of FDP. Thus the K_M for NAD^+ is 34 times greater than that for NADH (K_M = 2.4 mM NAD^+, K_M = 0.07 mM NADH) while the K_M for lactate is 87 times that for pyruvate (K_M = 130 mM L(+)-lactate, K_M = 1.5 mM pyruvate). Considering this difference and the fact that lactate oxidation has a pH optimum of 8.0 to 8.2 it is unlikely that the *S. lactis* C_{10} LDH catalyses lactate oxidation at any appreciable rate *in vivo*.

2.4.6.2 The effect of phosphate on lactate oxidation

In view of the fact that lactate oxidation is probably not a significant function of the L(+)-LDH of *S. lactis in vivo* an extensive comparison of the kinetic behaviour in different buffers has not been made. However, the effect of phosphate buffer (KH_2PO_4/NaOH) was briefly investigated.

Figure 2.4.6.2 shows the effect of increasing phosphate on lactate oxidation activity at 10 mM, 1 mM and no FDP. Phosphate clearly inhibits activity in the direction of lactate oxidation. FDP is shown to protect against phosphate inhibition as without FDP a concentration of 25 mM KH_2PO_4 gave 50% inhibition whereas in 10 mM FDP, 145 mM KH_2PO_4 is required to give 50% inhibition.

Figure 2.4.6.2 INHIBITION OF LACTATE OXIDATION BY PHOSPHATE



Phosphate inhibition of lactate oxidation was studied in 90 mM triethanolamine/HCl buffer pH 7.9 at constant conditions of 10 mM NAD^+ and 33.3 mM (L+) - lactate and at phosphate concentrations as shown in the above figure, where LDH activity is plotted against phosphate concentration. The phosphate (as KH_2PO_4) was adjusted to pH 7.9 with triethanolamine. The phosphate concentration was varied under three different conditions: ▲, 0.0 mM FDP; ●, 1 mM FDP; and ■, 10 mM FDP. Na_2SO_4 replaced phosphate at: △, 0.0 mM FDP; and □, 10 mM FDP.

2.4.7 Stopped-flow Analysis of Initial Reaction Rate

It was observed, in the course of determinations of steady state kinetic parameters, that when the reaction was started by addition of enzyme, as was the standard practice, a lag period often occurred before the steady rate of NADH oxidation was attained. This lag period was more pronounced at low FDP concentrations; no lag period was observed at saturating, or near saturating concentrations of FDP. If the enzyme was pre-incubated with low FDP concentrations then there was no lag period evident when the reaction was initiated with the missing substrate. If this lag period is due to a conformational change in the LDH, upon binding of the FDP to give a more active form of the enzyme, then a lag should still be observed at a saturating concentration of FDP when the reaction was started by addition of enzyme. Failure to observe such a lag at saturating FDP concentrations using the ordinary spectrophotometric assay procedure was probably due to the very short duration of the lag period under such conditions. The initial reaction rate was therefore observed by use of a stopped-flow apparatus.

Experiments were carried out using a Durrum-Gibson D110 stopped-flow spectrophotometer (Durram Instruments Corp., Palto Alto, California, U.S.A.) and a Hewlett-Packard model 141B storage oscilloscope. Reactions at 25°C were followed by measuring absorbance at 340 nm and oscilloscope traces were photographed on 35 mm film.

The data from the oscilloscope traces (Figures 2.4.7 a to d) were not used to obtain a quantitative measure of the lag period or of the steady state reaction rate. An optical cell with an 18 mm path length was used and when the cell was calibrated with known concentrations of NADH or potassium dichromate, the absorbance at high concentrations was considerably lower than the expected value. At the concentrations of NADH used to saturate the enzyme in the stopped-flow experiments, the absorbance was in the region where the relationship between concentration and absorbance was not linear. According to Cook and Jankow (1972) such a non-linear relationship could be due to stray light error which increases as a function of concentration. Cavalieri and Sable (1974), and Eyzaguirre (1974) have shown how stray light interference can lead to seriously erroneous interpretation of kinetic data obtained by spectrophotometric procedures in solutions of high absorbance. Therefore quantitative data obtained from stopped-flow experiments, at the high concentrations of NADH used, would be

invalidated by stray light errors. However, as different patterns of traces were observed, depending on the combination of the mixtures incubated in the two syringes, qualitative comparison of the traces can be made without being invalidated by stray light error.

Both curves 1 and 2 in photograph A show a distinct lag period before a constant rate of oxidation of NADH was observed. In this experiment the LDH was placed in one syringe and the substrates (NADH and pyruvate) and FDP in the other syringe prior to mixing. Thus the reaction was started by addition of enzyme to the other components as in the steady state kinetic studies. Photograph B shows that the same lag period was observed when enzyme and NADH were in one syringe and pyruvate and FDP in the other syringe prior to mixing. The same trace was obtained if pyruvate and enzyme were in one syringe and NADH and FDP were in the other syringe. Photographs C and D (note that the oscilloscope is set on different time and absorbance scales in D), obtained by incubation of FDP and enzyme in one syringe before mixing with the substrates in the other syringe, show that no distinct lag phase occurred before a constant rate of oxidation of NADH was observed (compare photographs A and B). Hence pre-incubation of FDP with enzyme abolishes the lag period.

The traces shown in photographs E to G were obtained at pH 8.2 instead of pH 6.9 (photographs A to D) and with a 10-fold increase in the enzyme concentration. The trace shown in photograph E was obtained when FDP was pre-incubated with enzyme before mixing with the substrates whereas photograph F shows the trace obtained when the enzyme was not pre-incubated with FDP before mixing. The rates observed in these two cases were very similar, and furthermore, both traces showed no significant lag period. The lack of a lag period, when the enzyme was not pre-incubated with FDP at pH 8.2 (photograph F) is in marked contrast to the result at pH 6.9 when a distinct lag period was obtained (photographs A and B). The trace shown in photograph G was obtained in the absence of FDP in either syringe. The rate observed without FDP appeared to be only slightly less than that observed with FDP present in the complete assay mixture. This contrasts to the situation at pH 6.9, where if FDP was absent from the assay mixture no reaction was observed using the stopped-flow apparatus. Photograph H is included to show that the reaction rate at pH 8.2, when studied using the same enzyme concentration as used at pH 6.9 (photographs A to D), is comparably much slower. Therefore the

relationship between reaction rate and pH in the presence and absence of FDP, as studied by the stopped-flow apparatus is the same as that studied by the ordinary spectrophotometric assay procedure (see Section 2.4.2).

The demonstration that the lag period could be eliminated by pre-incubation of the enzyme with its activator, FDP, at pH 6.9, was similar to the findings of Tarmy and Kaplan (1968b) studying the kinetics of *Escherichia coli* B D(-)-LDH. This enzyme is activated by its substrate pyruvate (no activation by FDP). They found, using a stopped-flow procedure that the lag period was eliminated only when the enzyme and pyruvate were pre-incubated together.

The lag period did not appear to vary as enzyme concentration was changed from 1.5 µg/cm^3 to 15 µg/cm^3. This was taken as evidence that the lag period was possibly due to a conformational change of the enzyme rather than a change in the state of aggregation of subunits. A lag period due to the time required for aggregation would be expected to be less with a higher enzyme concentration. However, for reasons stated earlier, conclusions based on detailed quantitative comparison of the lag period can only be tentative.

The traces shown in photographs A to H were reproducible in all trials carried out using the different incubation combinations. At least six trials were done using each type of incubation and all trials, when repeated in a separate experiment two months later, showed the same traces.

Figure 2.4.7a

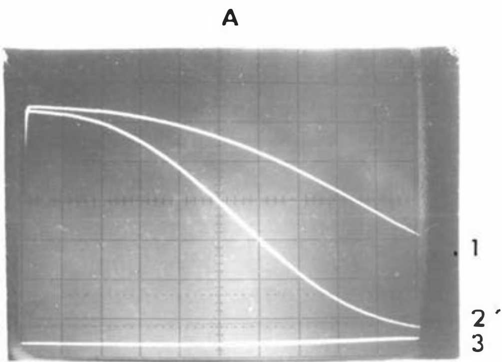
Stopped-flow analysis of initial reaction rate

Photographs A, B, C and D show oscilloscope traces from stopped-flow analysis of the initial reaction rate of oxidation of NADH by LDH under various conditions. The Y axis represents absorbance at 340 nm (absorbance units/division) and the X axis represents time (seconds/division). Contents of syringe A and syringe B were preincubated separately at 25°C for at least ten minutes before stopped-flow mixing occurred and the initial reaction rate was photographed from the oscilloscope trace. The final concentrations of substrate and modifier in the assay mixture are 8 mM pyruvate, 4 mM FDP and 0.2 mM NADH buffered in 100 mM tris/maleate buffer pH 6.9. The purified LDH was present in the reaction mixture at a final concentration of 1.5 μg protein/cm^3.

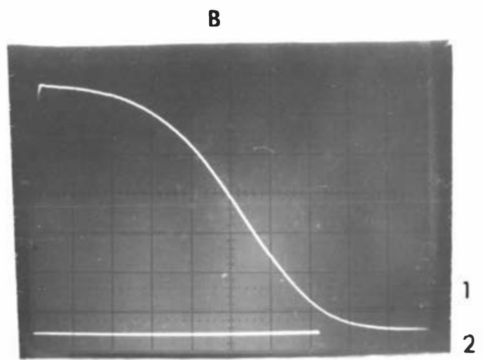
	Syringe A	Syringe B	Y axis	X axis
Photo A				
curve 1	Enzyme	Pyruvate,FDP,NADH	1 AU* /division	0.5 sec/division
curve 2	Enzyme	Pyruvate,FDP,NADH	1 AU/division	1.0 sec/division
curve 3	Trace of absorbance after reaction completed.			
Photo B				
curve 1	Enzyme,NADH	Pyruvate,FDP	1 AU/division	0.5 sec/division
curve 2	Trace of absorbance after reaction completed.			
Photo C	Enzyme,FDP	Pyruvate,NADH	1 AU/division	0.5 sec/division
Photo D	Enzyme,FDP	Pyruvate,NADH	0.2 AU/division	20 m sec/division

* AU = absorbance units

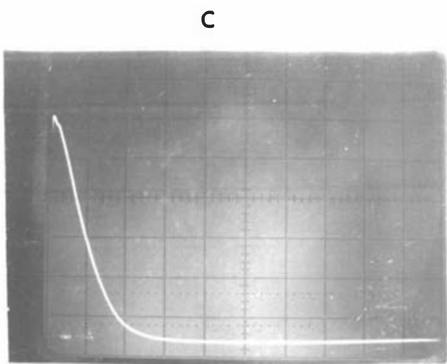
A



B



C



D

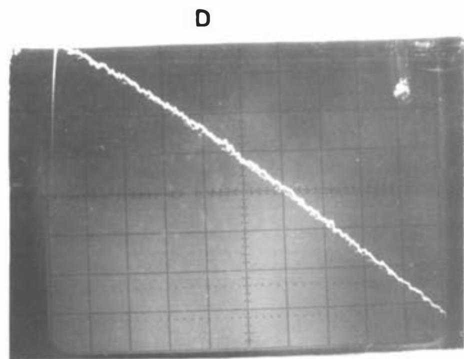


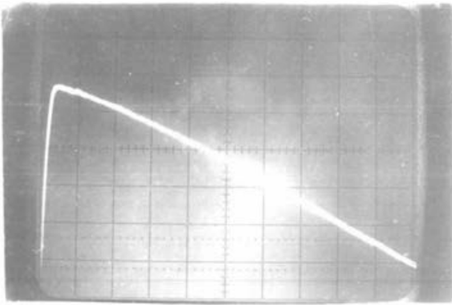
Figure 2.4.7b

Stopped-flow analysis of initial reaction rate

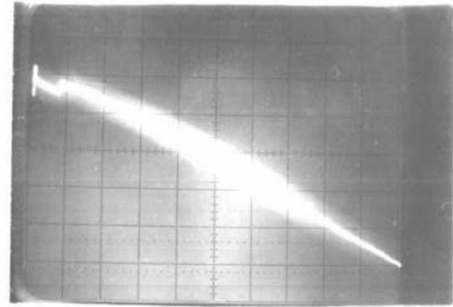
Conditions as for photographs A to D (Figure 2.4.7a) except that the reaction mixture was in 100 mM tris/maleate buffer pH 8.2. The purified LDH was present in the reaction mixture at a final concentration of 15 μg protein/cm^3 for photographs E, F and G and 1.5 μg protein/cm^3 for photograph H.

	Syringe A	Syringe B	Y axis	X axis
Photo E	Enzyme,FDP	NADH,Pyruvate	1 AU/division	0.2 sec/division
Photo F	Enzyme	NADH,Pyruvate,FDP	1 AU/division	0.2 sec/division
Photo G				
curve 1	Enzyme	NADH,Pyruvate	1 AU/division	0.2 sec/division
curve 2	Trace of absorbance after reaction completed.			
Photo H	Enzyme	NADH,Pyruvate,FDP	1 AU/division	0.5 sec/division

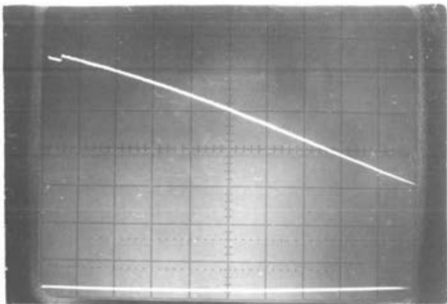
E



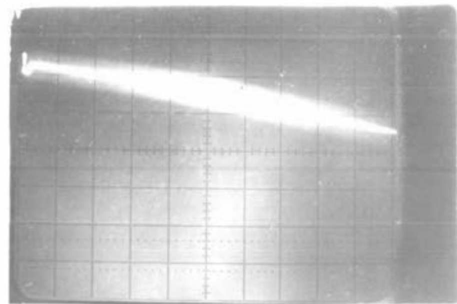
F



G



H



2.4.8 Effect of Inhibitors

The effects of inhibitors on streptococcal LDH activity have not been extensively studied. Brown and Wittenberger (1972) studied the affects of the pyruvate analogues α-ketobutyrate and oxamate on the S. mutans NCTC 10449 LDH, (which shows a sigmoidal response to pyruvate). They found that α-ketobutyrate stimulated LDH activity at limiting pyruvate concentrations and shifted the pyruvate saturation curve from sigmoidal to hyperbolic. Oxamate, on the other hand, inhibited the enyzme activity at all pyruvate concentrations and did not affect the sigmoidal nature of the pyruvate saturation curve. They also found that ATP was a potent inhibitor and ADP was almost as effective in inhibiting LDH activity. Jonas et al. (1972) showed that ATP inhibition of the S. cremoris LDH activity was competitive with respect to NADH.

A more detailed study on the effects of inhibitors on S. lactis C_{10} L(+)-LDH was carried out to provide a further basis for comparison with other streptococcal LDH's. Also some of the inhibitors such as ATP could be of significance in the in vivo regulation of LDH.

2.4.8.1 Inhibition with pyruvate as the varied substrate

The pyruvate analogues, oxamate, α-ketoglutarate, α-ketomalonate and α-ketobutyrate were first tested under standard assay conditions for their ability to act as alternative substrates to pyruvate for NADH oxidation. Only α-ketobutyrate served as a substrate (Figure 2.4.8.1a) with a K_M value of 18.7 mM, which is over ten times higher than that for pyruvate (1.4 mM). The V_{max} (500 units/cm^3) obtained with α-ketobutyrate was four times lower than that for pyruvate (2000 units/cm^3).

The inhibition of pyruvate reduction was examined at several different inhibitor concentrations and under standard conditions of 90 mM tris/maleate buffer, pH 6.9, 1 mM FDP and 0.167 mM NADH. The products of pyruvate reduction, NAD^+ and L(+)-lactate, were also tested for their ability to act as inhibitors. No inhibition by L(+)-lactate (at concentrations of 33.33, 66.67 and 100 mM) was detectable. All other compounds tested showed some degree of inhibition. The results for the other inhibitors are summarized in Figures 2.4.8.1 b, c, d and e.

NAD^+ showed non-competitive inhibition with respect to pyruvate with a K_I value of 2.1 mM (Figure 2.4.8.1b). α-ketomalonate

inhibition was also non-competitive but with a higher K_I value of 9.5 mM (Figure 2.4.8.1d). α-ketoglutarate (Figure 2.4.8.1c) showed competitive inhibition with respect to pyruvate with a K_I value of 3.0 mM. Oxamate (Figure 2.4.8.1e) was also a competitive inhibitor of pyruvate reduction, with a K_I value of 0.65 mM, which is about half of the K_M value for pyruvate (1.4 mM). The enzyme thus shows a very high affinity for oxamate.

2.4.8.2 Inhibition with NADH as the varied substrate

The adenine mono, di and trinucleotides may play a part in regulation of LDH as discussed in the introduction (2.1.2). Therefore ATP, ADP and AMP inhibition were studied in tris/maleate buffer pH 6.9, 10 mM pyruvate and 0.5 mM FDP by varying the NADH concentration. NAD^+ inhibition was also studied under the same conditions. The results are summarized in Figures 2.4.8.2 a, b, c and d. ATP and ADP were both competitive with respect to NADH and had the same K_I value of 2.4 mM (Figures 2.4.8.2a and b). AMP (Figure 2.4.8.2c) at concentrations of 10 and 20 mM showed an unusual effect. Although inhibiting over the range of NADH concentrations used, the Lineweaver-Burk plots apparently extrapolated (if a straight line fit is assumed) to give a V_{max} considerably higher than in the absence of AMP. Figure 2.4.8.2d shows that NAD^+ is a competitive inhibitor of NADH oxidation with a K_I value of 2.0 mM.

2.4.8.3 Inhibition of pyruvate reduction by phosphate

As mentioned in results in earlier sections, phosphate buffer effects the FDP requirement and the binding of NADH and pyruvate when compared to the same parameters determined in tris/maleate and triethanolamine/HCl buffer. Therefore phosphate inhibition of pyruvate reduction was studied in 90 mM tris/maleate buffer pH 6.9 at constant conditions of 10 mM pyruvate, 0.167 mM NADH and 1.0 mM FDP, by varying the phosphate concentration. Figure 2.4.8.3a is a plot of percentage of activity versus phosphate concentration and shows the sigmoidal nature of phosphate inhibition. A control in which Na_2SO_4 of equivalent concentration replaced phosphate shows that inhibition by phosphate could not be accounted for by high ionic concentration.

Figure 2.4.8.3b shows the same data plotted in the form of a Hill plot and gives a $I_{0.5V}$ value (phosphate concentration giving 50% inhibition) of 50 mM. The Hill interaction coefficient value (n_H) of minus 2.5 indicates that a high degree of interaction is occurring between phosphate binding sites. From the effect of

Figure 2.4.8.1: a, b, c and d

INHIBITION WITH PYRUVATE AS THE VARIED SUBSTRATE AND α–KETOBUTYRATE AS A SUBSTRATE

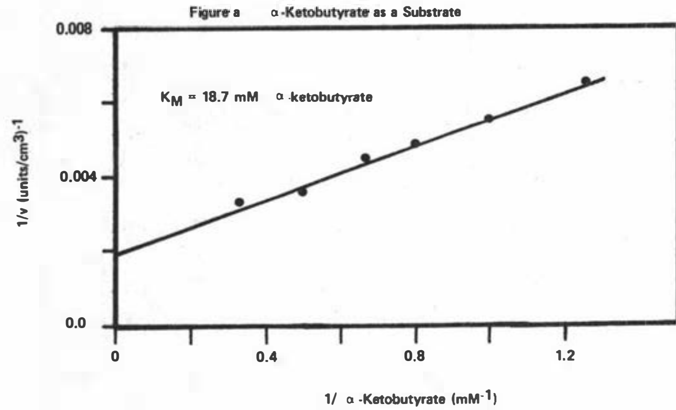


Figure a is a double reciprocal plot of LDH activity versus α-ketobutyrate concentration. The reaction mixture contained: 90 mM tris/maleate buffer pH 6.9; 1 mM FDP; 0.167 mM NADH; 0.1 cm^3 of diluted enzyme and α-ketobutyrate concentrations as indicated in Figure a.

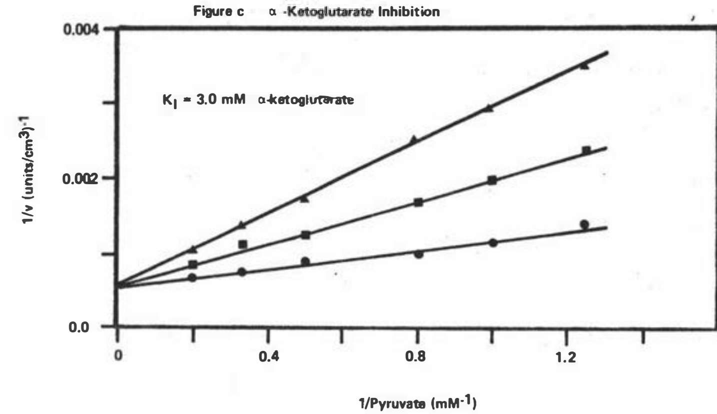


Figure c shows the inhibition effect of α-ketoglutarate on pyruvate binding. The reciprocal of LDH activity is plotted against the reciprocal of pyruvate concentration for each α-ketoglutarate (α-kg) concentration: ●, 0.0 mM α-kg; ■, 5 mM α-kg; ▲, 10 mM α-kg. The reaction mixture contained: 90 mM tris/maleate buffer pH 6.9; 1 mM FDP; 0.187 mM NADH; 0.1 cm^3 of diluted enzyme and pyruvate concentrations as indicated in Figure c.

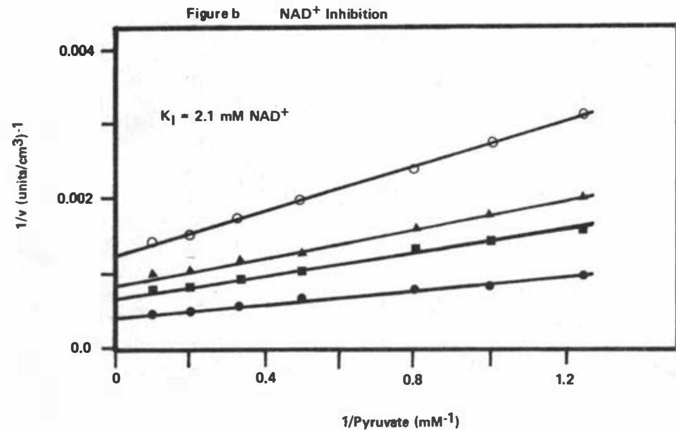


Figure b shows the inhibition effect of NAD$^+$ on pyruvate binding. The reciprocal of LDH activity is plotted against the reciprocal of pyruvate concentration for each NAD$^+$ concentration: ●, 0.0 mM NAD$^+$; ■, 1.0 mM NAD$^+$, ▲, 2.0 mM NAD$^+$; ○, 5.0 mM NAD$^+$. The reaction mixture contained: 90 mM tris/maleate buffer pH 6.0; 1 mM FDP; 0.167 mM NADH; 0.1 cm^3 of diluted enzyme and pyruvate concentrations as indicated in Figure b.

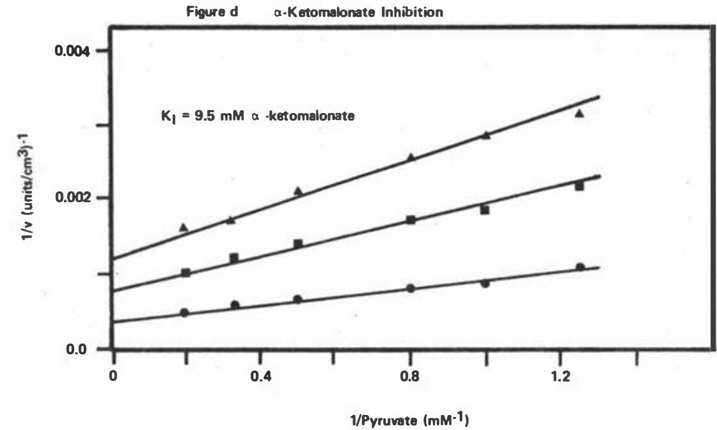
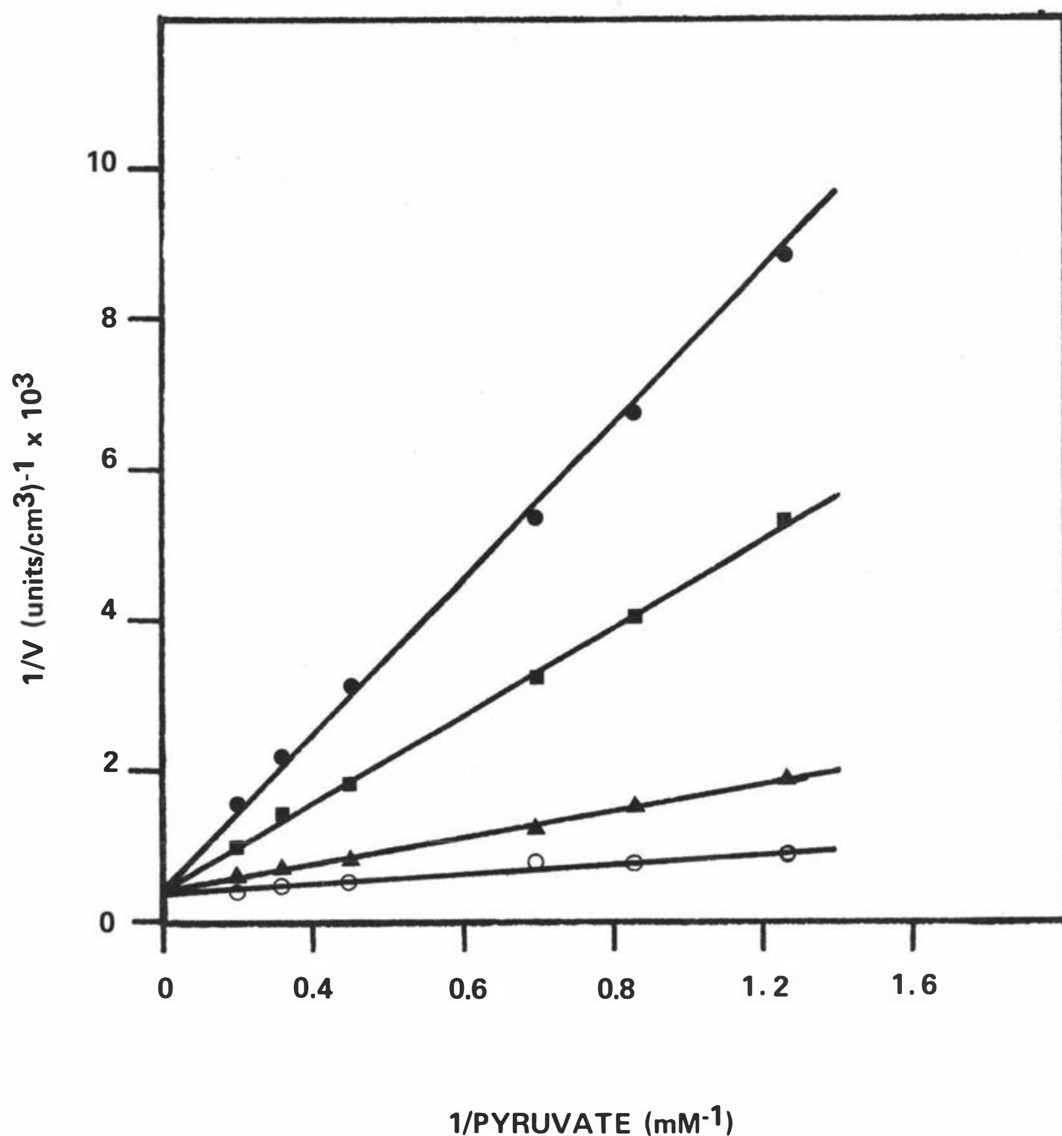


Figure d shows the inhibition effect of α-ketomalonate on pyruvate binding. The reciprocal of LDH activity is plotted against the reciprocal of pyruvate concentration for each α-ketomalonate (α-km) concentration: ●, 0.0 mM α-km; ■, 10 mM α-km; ▲, 20 mM α-km. The reaction mixture contained: 90 mM tris/maleate buffer pH 6.9; 1 mM FDP; 0.167 mM NADH; 0.1 cm^3 of diluted enzyme and pyruvate concentrations as indicated in Figure d.

Figure 2.4.8.1 e

INHIBITION WITH PYRUVATE AS THE VARIED SUBSTRATE



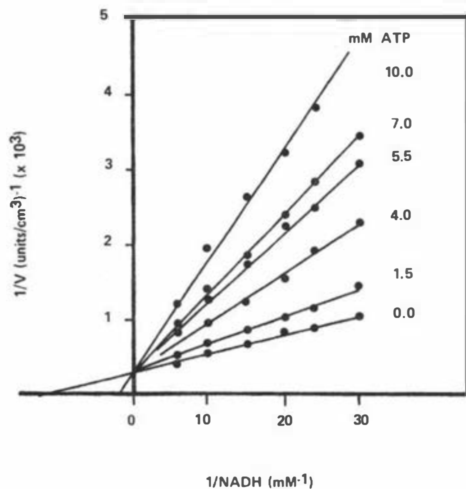
K_I = 0.65 mM Oxamate

In the above figure inhibition of pyruvate reduction by different oxamate concentrations is shown as double reciprocal plots (1/v versus 1/pyruvate concentration). The standard assay conditions are: 90 mM tris/maleate buffer pH 6.9, 1.0 mM and 0.167 mM NADH. The pyruvate concentrations were varied as shown in the figure. The oxamate concentrations present are: ●, 10 mM, ■, 5.0 mM; ▲, 1.0 mM; and 0.0 mM oxamate; ○,

Figure 2.4.8.2: a, b, c and d

INHIBITION WITH NADH AS THE VARIED SUBSTRATE

Figure a ATP INHIBITION



K_I = 2.4 mM ATP

Figure c AMP INHIBITION

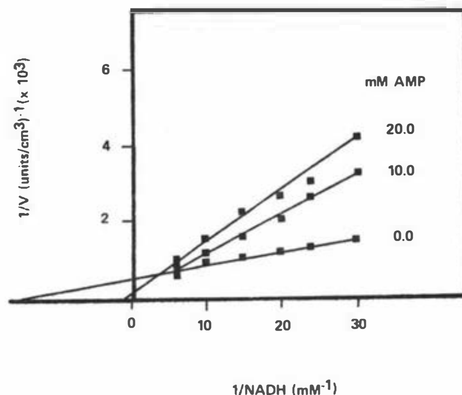
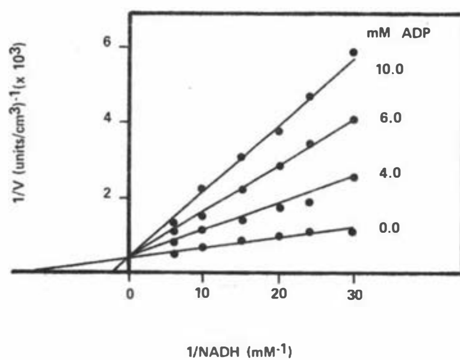
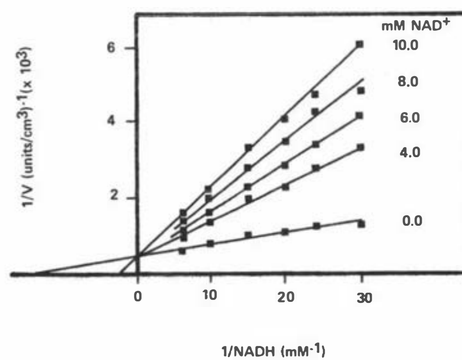


Figure b ADP INHIBITION



K_I = 2.4 mM ADP

Figure d NAD^+ INHIBITION

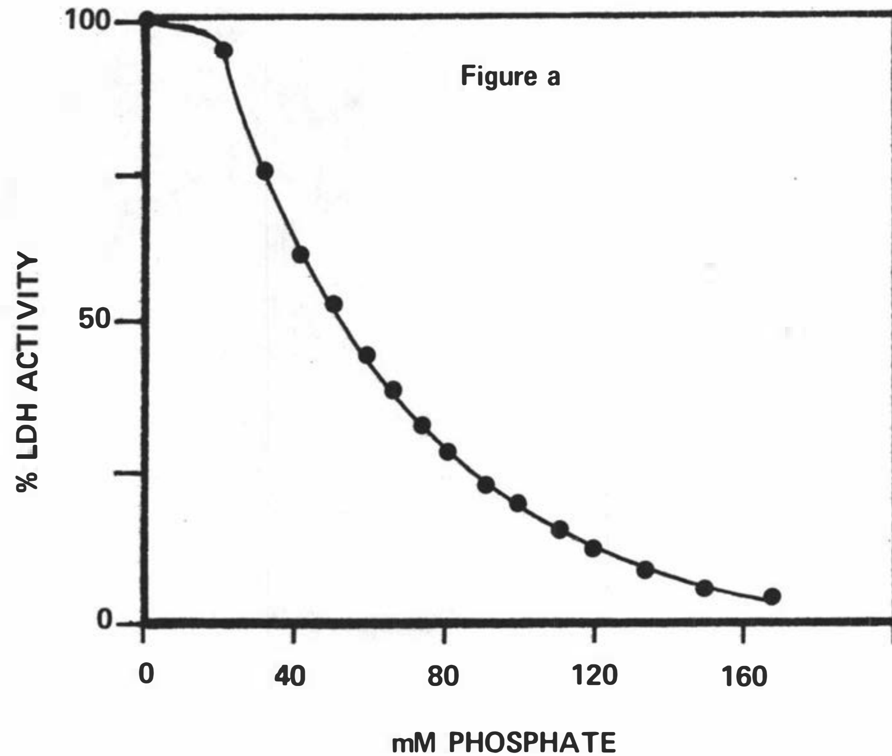


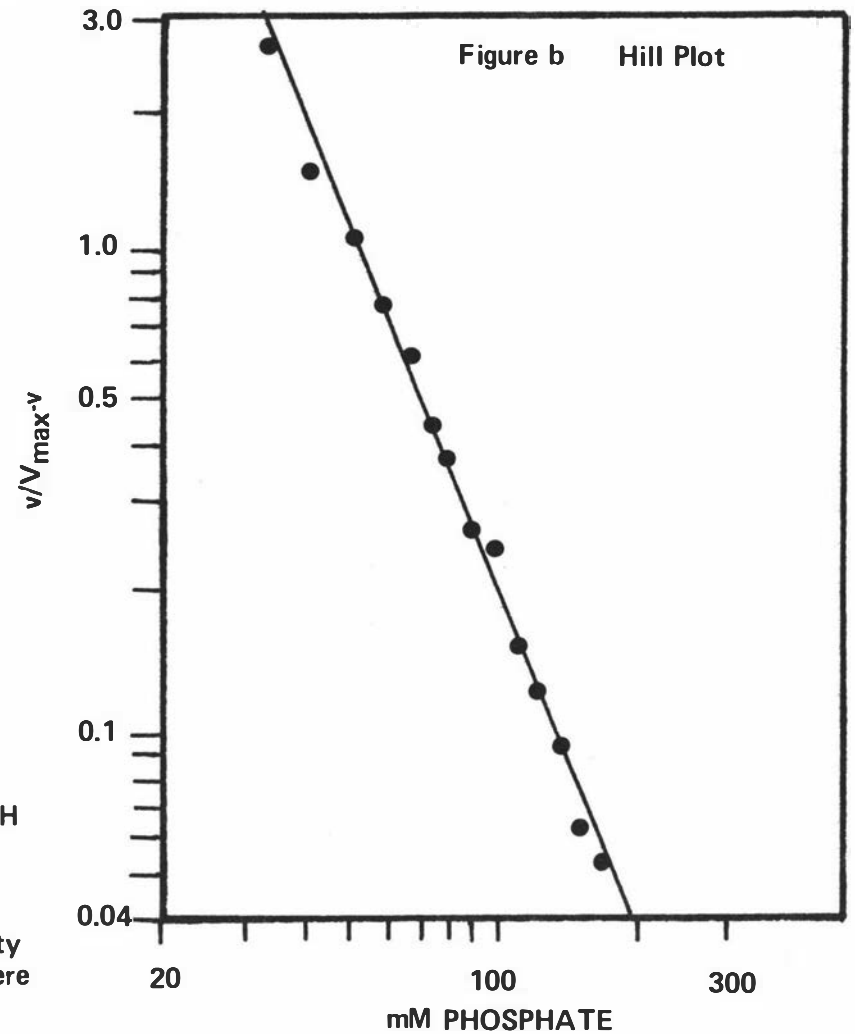
K_I = 2.0 mM NAD^+

The inhibitors of S. lactis LDH (ATP, ADP, AMP and NAD^+) were studied with NADH as the varied substrate and the kinetic data obtained are plotted as double reciprocal plots shown in Figures a, b, c and d. The reaction mixture for Figures a, b, c and d contained: 90 mM tris/maleate buffer pH 6.9; 1 mM FDP; 10 mM Pyruvate; 0.1 cm^3 of diluted enzyme, and NADH concentrations as indicated in the Figures. The inhibitor concentrations present in the reaction mixture are shown in the respective Figures.

Figure 2.4.8.3: a and b

INHIBITION OF PYRUVATE REDUCTION BY PHOSPHATE





Phosphate inhibition of pyruvate reduction was studied in 90 mM tris/maleate buffer pH 6.9 at constant conditions of 10 mM pyruvate, 0.167 mM NADH, 1.0 mM FDP and at phosphate concentrations as shown in the above figures. The phosphate (as $KH_2\,PO_4$) was adjusted to pH 6.9 with the tris/maleate buffer components. Figure a is a plot of % LDH activity versus phosphate concentration, where 100% LDH activity is the activity found in the absence of phosphate. Figure b is a Hill plot of the data of Figure a, where log v/V_{max}-v is plotted against log phosphate concentration.

phosphate on lactate oxidation (2.4.6.2) it might be expected that increasing the amount of FDP would increase the $I_{0.5V}$ value and this is supported from the pH optimum studies (2.4.2) where activity in 90 mM phosphate buffer at 10 mM FDP was the same as activity in 90 mM tris/maleate buffer at 1 mM FDP.

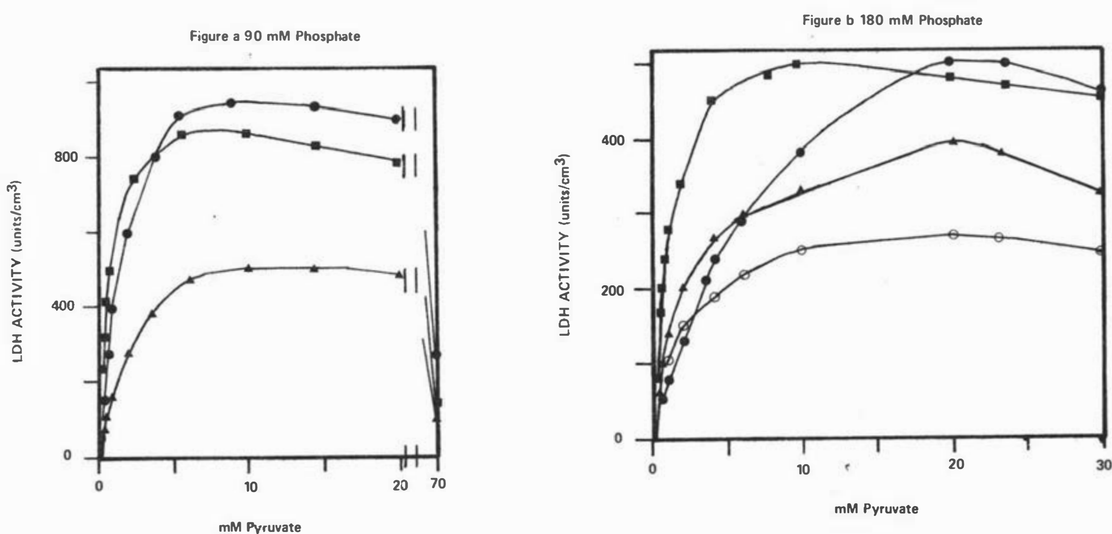
2.4.8.4 Effect of oxamate in phosphate buffer

Prior to the development of the affinity chromatography procedure in which oxamate was used as an LDH binding group, oxamate was tested for inhibition of L(+)-LDH in phosphate buffer pH 6.9. Assays were carried out in 90 mM phosphate buffer pH 6.9, with 5.0 mM FDP. In Figure 2.4.8.4a it can be seen that at low pyruvate concentrations the activity is considerably higher with 1 mM oxamate present, than without oxamate. Increasing the phosphate concentration to 180 mM in the assays further increased the activation by oxamate at low pyruvate concentrations as shown in Figure 2.4.8.4b. Even at 5 and 10 mM oxamate in 180 mM phosphate there is activation at low pyruvate concentrations. A double reciprocal plot of data from Figure 2.4.8.4b as shown in Figure 2.4.8.4c shows that oxamate increases the apparent affinity for pyruvate (K_M = 5.7 mM pyruvate in the absence of oxamate, K_M = 1.0 mM pyruvate in the presence of 1.0 mM oxamate).

The peculiar effects of oxamate in phosphate buffer are different from the effects of oxamate in tris/maleate buffer. In tris/maleate buffer oxamate was a simple competitive inhibitor with a high affinity for the enzyme (K_I value of 0.65 mM oxamate). The mode of action of oxamate activation in phosphate buffer is unclear. It is possible that oxamate is binding at a site, other than the pyruvate catalytic site, which is made available when phosphate is present, and activating the enzyme in perhaps an allosteric way, similar to FDP. Brown and Wittenberger (1972) showed for S. mutans NCTC 10449, that the LDH had at least two pyruvate binding sites. The presence of the two pyruvate sites was supported by the differential effect of the two pyruvate analogues, α-ketobutyrate and oxamate. Oxamate inhibited enzyme activity at all pyruvate concentrations, while α-ketobutyrate stimulated enzyme activity at limiting pyruvate concentrations. This work was done in phosphate buffer. The effect of α-ketobutyrate on the S. mutans enzyme is thus similar to the effect of oxamate on the S. lactis C_{10} LDH. However there is no evidence for a second pyruvate binding site on the S. lactis LDH (see Section 2.4.4) comparable to that found in the S. mutans NCTC 10449 enzyme.

Figure 2.4.8.4: a, b and c

PYRUVATE BINDING: EFFECT OF OXAMATE IN PHOSPHATE BUFFER



The effect of oxamate on pyruvate binding was studied at two phosphate buffer concentrations. In Figure a the phosphate buffer concentration was 90 mM. In Figure b 180 mM phosphate buffer was present in the reaction mixture. The reaction mixture contained (in a total volume of 3 cm^3): phosphate buffer pH 6.9; 5.0 mM FDP; 0.167 mM NADH and 0.1 cm^3 of diluted enzyme. The pyruvate concentration was varied as shown in the Figures. Both Figures a and b are plots of LDH activity versus pyruvate concentration at different oxamate concentrations: ●, no oxamate; ■, 1 mM Oxamate; ▲, 5 mM Oxamate; ○, 10 mM Oxamate.

Figure c Double Reciprocal Plot 180 mM Phosphate.

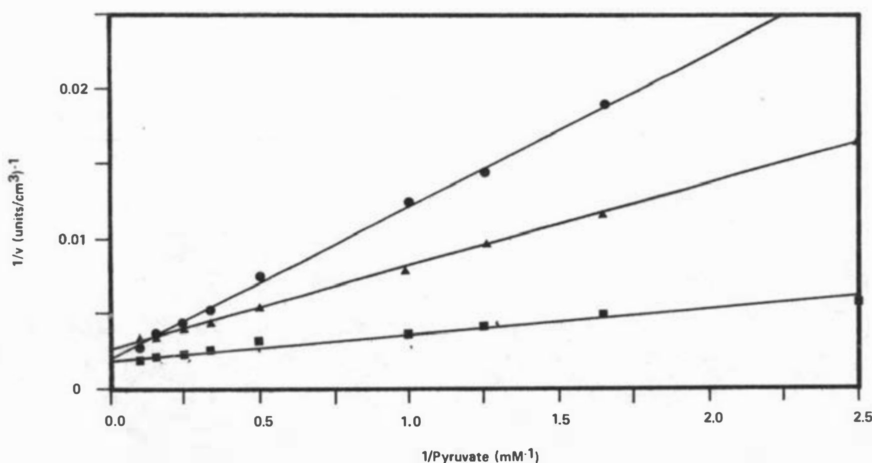


Figure c shows double reciprocal plots of the data from Figure b. From extrapolation of the plots: the K_M for pyruvate in the absence of Oxamate (●) is 5.7 mM pyruvate; in the presence of 1 mM Oxamate (■), the apparant K_M = 1.0 mM pyruvate; and in the presence of 5.0 mM Oxamate (▲), the apparant K_M = 2.4 mM pyruvate.

2.4.9 Factors Affecting the Stability of the S. lactis C_{10} LDH at 55°C.

Major differences have been reported in thermal stability between the LDH's from different streptococcal species. Wittenberger and Angelo (1970) showed that FDP increased the rate of inactivation of the LDH from S. faecalis while the LDH's from S. cremoris (Jonas et al., 1972) and S. mutans (Brown and Wittenberger, 1972) are protected by FDP against heat inactivation. The effect of various factors on the lability of the S. lactis C_{10} LDH was therefore studied at 55°C using the method described in Section 2.2.9.

Figure 2.4.9a shows the effect of differing ionic strengths of tris/maleate and phosphate buffer on stability of LDH at pH 7.0 At 0.1 M, phosphate buffer stabilizes the LDH more effectively than the same concentration of tris/maleate buffer, but the converse occurs at 0.05 M and 0.02 M. In both buffers a decrease in molarity decreases the stability of L(+)-LDH.

The effect of pH on stability of LDH in 0.1 M tris/maleate and in 0.1 M phosphate buffer is shown in Figure 2.4.9b. In both buffers, increasing the pH decreases the stability of the LDH to heat inactivation. Up to pH 7.0, the enzyme is more stable in phosphate buffer, but at pH 7.5 the enzyme is more stable in tris/maleate than in phosphate buffer.

Jonas et al. (1972) showed that the S. cremoris LDH, like the S. lactis enzyme, was more heat stable in phosphate buffer and that the higher the molarity of phosphate buffer the more stable the enzyme. However phosphate buffer was more effective than triethanolamine/HCl buffer in stabilizing the enzyme over the whole pH range and not just at pH values less than 7.0 as is found for the S. lactis C_{10} LDH.

Figure 2.4.9c shows the effect of FDP in stabilizing the LDH in 0.1 M tris/maleate and 0.1 M phosphate buffer pH 7.5. FDP appears to stabilize the LDH to a greater extent in tris/maleate buffer than in phosphate buffer. However, as the LDH is more unstable in phosphate buffer than in tris/maleate buffer at this pH in the absence of FDP (Figure 2.4.9c), then the relative degree of stabilization provided by FDP is really similar in the two buffers.

Figure 2.4.9d shows the effects of FDP, NADH and oxamate on stability of L(+)-LDH in 0.1 M tris/maleate buffer pH 8.0. FDP with oxamate or with NADH stabilizes the LDH to a slightly greater extent than does FDP alone. NADH plus oxamate stabilizes the enzyme to a greater extent than either substance alone but much less effectively

Figure 2.4.9: a, b, c and d.

FACTORS AFFECTING THE STABILITY OF LDH AT 55°C

The effects of ionic strength, pH, FDP, NADH and oxamate on LDH stability at 55°C, as shown in Figures a, b, c and d were studied in two buffers, using the method described in Section 2.2.9.

Figure a

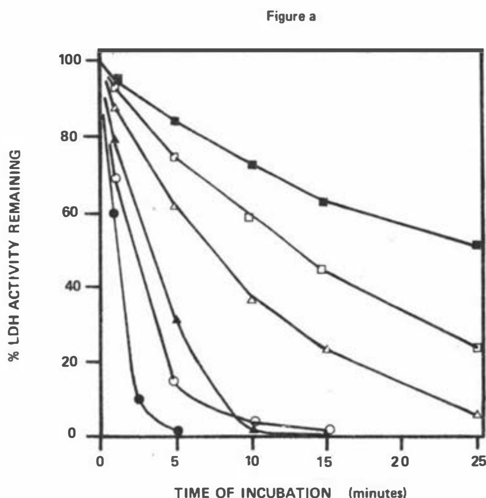


Figure a Effect of ionic strength on LDH stability in tris/maleate and phosphate buffers, pH 7.0. Ionic strengths of phosphate buffer: ■, 0.1 M; ▲, 0.05 M; and ●, 0.02 M. Ionic strengths of tris/maleate buffer: □, 0.1 M; △, 0.05 M; and ○, 0.02 M.

Figure b

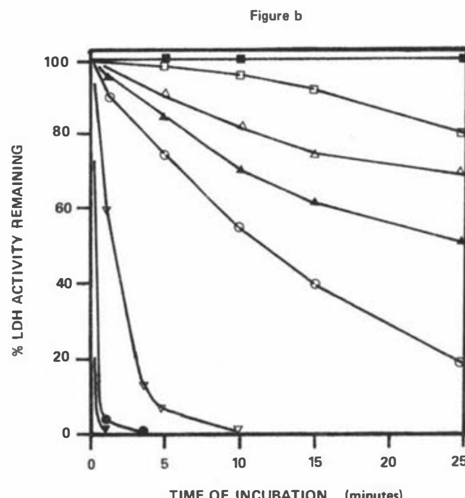


Figure b Effect of pH on LDH stability in 0.1 M tris/maleate and in 0.1 M phosphate buffer. pHs of phosphate buffer: ■, pH 6.0; ▲, pH 7.0; ●, pH 7.5; and ▼, pH 8.0. pHs of tris/maleate buffer: □, pH 5.5; △, pH 6.5; ○, pH 7.0; and ▽, pH 7.5.

Figure c

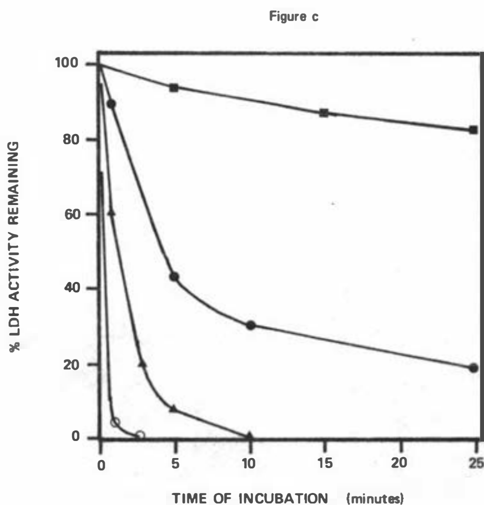


Figure c Effect of FDP on LDH stability in 0.1 M tris/maleate and 0.1 M phosphate buffer pH 7.5.

In phosphate buffer: ▲, 12 mM FDP; and ○, 0.0 mM FDP. In tris/maleate buffer: ■, 12 mM FDP; and ●, 0.0 mM FDP.

Figure d

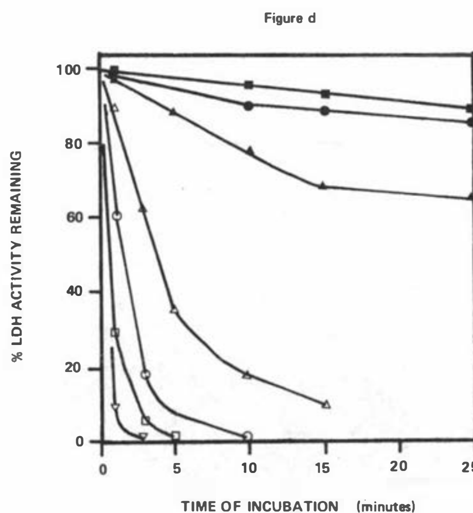


Figure d Effect of FDP, NADH and Oxamate on LDH stability in 0.1 M tris/maleate pH 8.0. Treatments are: ■, 12 mM FDP + 12 mM Oxamate; ●, 12 mM + 1 mM NADH; ▲, 12 mM FDP; △, 1 mM NADH + 12 mM Oxamate; o, 1 mM NADH; □, 12 mM Oxamate; and ▽, no factors present.

Figure 2.4.9: e and f

FACTORS AFFECTING THE STABILITY OF THE LDH AT 55°C

The effects of NADH, oxamate, pyruvate, and FDP on LDH stability at 55°C, as shown in Figures e and f were studied in two buffers, using the method described in Section 2.2.9.

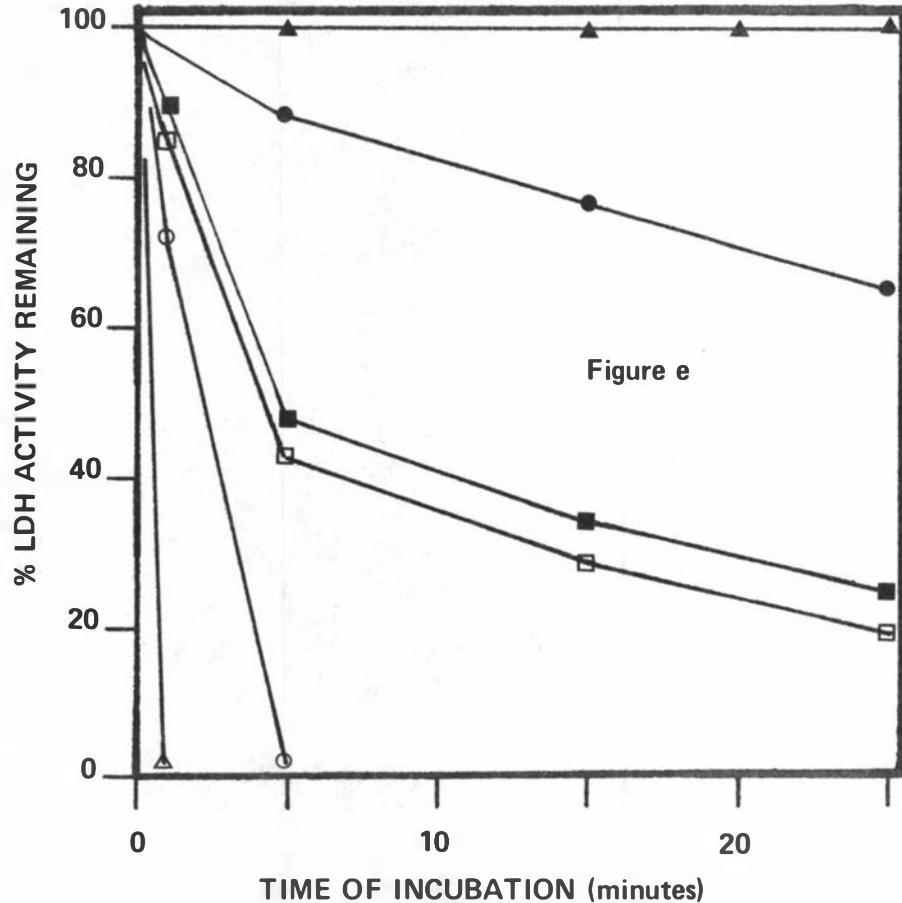


Figure e Effect of FDP, NADH, Pyruvate and Oxamate on LDH stability in 0.1M phosphate buffer pH 7.5. Treatments are:
▲, 1.0mM NADH + 24 mM Oxamate; ●, 24 mM Oxamate; ■, 24 mM FDP: □, 12 mM FDP: ○, 1.0 mM NADH: and △, 12 mM pyruvate or no pyruvate.

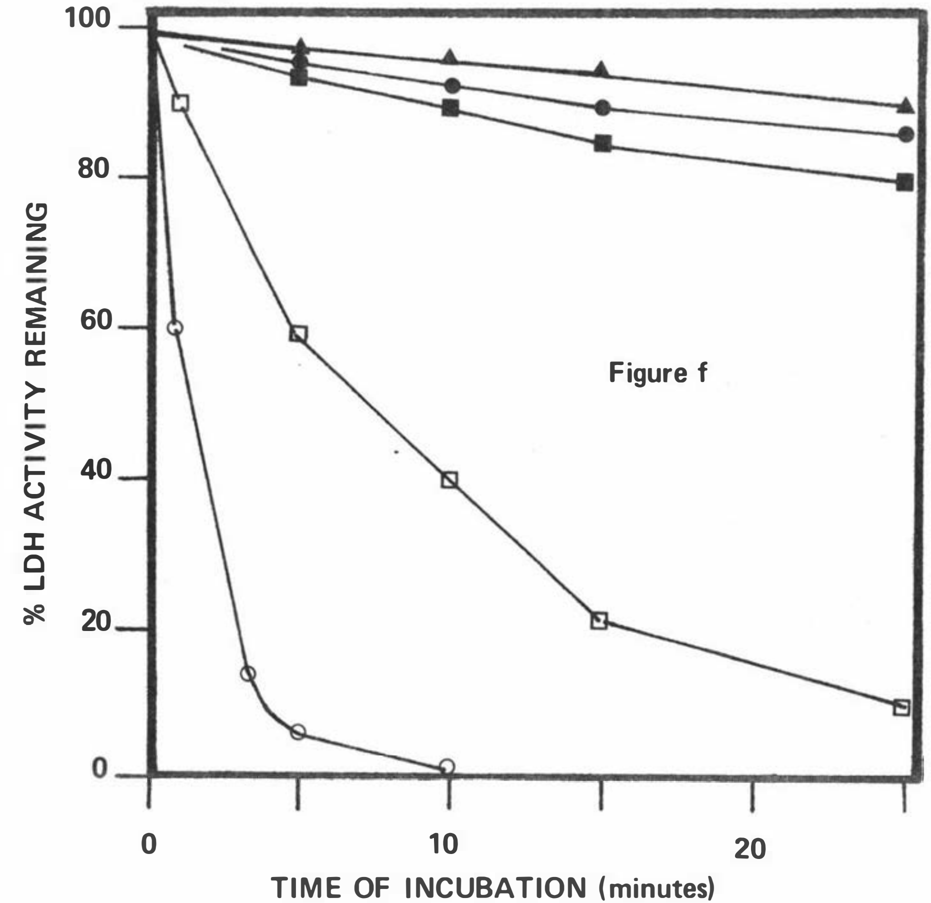


Figure f Effect of FDP, NADH, Pyruvate and Oxamate on LDH stability in 0.1 M tris/maleate buffer pH 7.5. Treatments are:
▲, 1.0 mM NADH + 24mM Oxamate; ●, 24 mM Oxamate; ■, 12 mM FDP; □, 1 mM NADH: and ○, 12 mM pyruvate or no pyruvate.

than FDP. Either NADH or oxamate by itself provides only a slight stabilization relative to a control of buffer alone.

A comparison made of the effect of additives on the stability properties of the LDH in phosphate and in tris/maleate buffer using a pH of 7.5 (Figures 2.4.9 e and f) revealed some significant differences between the two buffer systems. In tris/maleate buffer, NADH plus oxamate, oxamate alone or FDP all had a similar effect in that they all significantly stabilized LDH. In phosphate buffer, NADH plus oxamate stabilized the L(+)-LDH completely while oxamate alone stabilizes the LDH to a significantly lesser extent and FDP alone stabilizes the LDH to a lower extent still. NADH alone, in both buffers, stabilized the LDH to only a small extent while pyruvate did not increase the stability in either of the buffers.

When a comparison is made between stability of LDH in 0.1 M tris/maleate buffer pH 8.0 (Figure 2.4.9d) and in 0.1 M tris/maleate buffer pH 7.5 (Figure 2.4.9f), the major difference is that oxamate at pH 8.0 gives hardly any stabilization of LDH whereas at pH 7.5 oxamate is very effective at stabilizing the enzyme.

The stabilization by FDP and phosphate on *S. lactis* C_{10} LDH is similar to that found for the *S. cremoris* LDH (Jonas *et al.*, 1972) and the enzyme from both of these streptococci differs from that of *S. faecalis* (Wittenberger and Angelo, 1970) in that FDP and relatively high concentrations of phosphate buffer (200 to 600 mM) rendered the *S. faecalis* enzyme heat labile. Oxamate either alone or with NADH gave excellent protection of the *S. lactis* LDH at pH 7.5. This is different from the situation in *S. faecalis* (Wittenberger and Angelo, 1970) where oxamate with or without NADH afforded the enzyme no greater protection than did the coenzyme alone. Pyruvate exerted no protective effect for either *S. lactis* or *S. cremoris*.

The protective effect, on heat stability of *S. lactis* C_{10} LDH, by FDP, NADH and oxamate and combinations of the three could not be ascribed to increasing ionic strength alone, because Na_2SO_4 or NaCl at equivalent molarities and ionic strengths did not protect the enzyme to any appreciable extent.

2.5 Lactate Dehydrogenase from Streptococcus faecalis ATCC 8043. A comparison of some regulatory properties with those of S. lactis C_{10} LDH.

2.5.1 Introduction.

It was shown in Section 2.4.3 that the nature of the assay buffer markedly affects the $M_{0.5V}$ value for FDP. Thus for S. lactis C_{10} LDH, as reported in Section 2.4.3 of this thesis, the $M_{0.5V}$ value was 4.4 mM FDP in 90 mM phosphate buffer pH 6.9, 0.2 mM FDP in 90 mM tris/maleate buffer pH 6.9 and 0.0022 mM FDP in triethanolamine/HCl buffer pH 6.9. The high $M_{0.5V}$ value reported by Brown and Wittenberger (1972) for the FDP activation of the S. mutans 10449 LDH might be due to the use of phosphate buffer in kinetic studies. However Wittenberger and Angelo (1970) found that the S. faecalis LDH had a low $M_{0.5V}$ value (0.45 mM FDP) in 100 mM phosphate buffer. In an attempt to reconcile the widely differing $M_{0.5V}$ values reported in the literature it was considered desirable to carry out a limited amount of comparative work on the LDH of S. faecalis since the low $M_{0.5V}$ value in phosphate buffer suggests that this enzyme might be relatively insensitive to phosphate inhibition in contrast to the situation found in S. lactis.

In the course of investigating the FDP activation of the S. faecalis LDH in different buffers, the effect of Mn^{++} ions on the response to FDP was investigated and yielded some interesting results. The possibility that Mn^{++} ions might have some effect on the activity of S. faecalis LDH was suggested by the work of London et al. on the close similarity between certain enzymes of S. faecalis and Lactobacillus casei. The particular enzymes studied by London and co-workers were an inducible malic enzyme (London et al., 1970 and 1971) and the FDP-aldolase (London and Kline, 1973). Immunological methods showed a high degree of homology between the corresponding enzymes in the two species. Since the LDH of L. casei is unusual among Lactobacillus species in being activated by FDP, it seemed possible that the close similarity of enzymes between S. faecalis and L. casei might extend to the LDH as well. The LDH of L. casei is unusual, also, in that in addition to its activation by FDP it is activated by Mn^{++} over part of its pH range (de Vries et al., 1970; Holland and Pritchard, 1975). Accordingly the possible influence of Mn^{++} on the S. faecalis LDH and subsequently on the S. lactis LDH was investigated.

2.5.2 Partial Purification of LDH from *S. faecalis* ATCC 8043.

S. faecalis ATCC 8043 was obtained from the Microbiology Department, Massey University, and grown in a medium of 30 g Tryptone, 10 g Yeast extract, 15 g glucose, 2 g beef extract and 5 g KH_2PO_4 made up in one litre of distilled water. Glucose was autoclaved separately. The culture was grown in 600 cm^3 of medium at 30°C in a one litre flask without additional aeration. After $18\frac{1}{2}$ hours the cells were harvested by centrifugation at 5,500 g for 15 minutes at 0°C and washed three times in 0.005 M phosphate buffer pH 7.0 containing 1% NaCl. The washed cells were then suspended in 0.01 M phosphate buffer pH 7.0 and disrupted by three passages through an Aminco French pressure cell at 5,500 lbs per $in.^2$. Cell debris was centrifuged down at 13,000 g for 15 minutes and the cell-free extract was then dialysed against 0.01 M phosphate buffer pH 7.0 for 15 hours. Nucleic acid was then precipitated from solution by dropwise addition of streptomycin sulphate using 1.75 cm^3 of a 10% (w/v) solution for every 100 mg protein. The resulting suspension was allowed to stand for 4 hours and the precipitate was removed by centrifugation at 13,000 g for 15 minutes. The streptomycin sulphate supernatant was dialysed against 0.01 M phosphate buffer pH 7.0 for 15 hours before solid powdered ammonium sulphate was added to bring the solution to 50% saturation. After leaving for half an hour the precipitate was removed by centrifugation at 13,000 g for 15 minutes and then the concentration of ammonium sulphate was increased to 72% saturation. After 3 hours the precipitate was collected by centrifugation at 13,000 g for 15 minutes, redissolved in 0.01 M phosphate buffer pH 7.0 and then dialysed against the same buffer for 24 hours.

The partial purification of the *S. faecalis* LDH is summarized in Table 2.5.2. The 50-72% ammonium sulphate dialysed LDH sample, with a specific activity of 52.0 units/mg, was used in the kinetic studies described in the following sections.

Table 2.5.2

Summary of LDH preparation

	Protein (mg/cm^3)	Activity ($units/cm^3$)	Total Activity (units)	Specific Activity (units/mg)
Cell free extract	11.0	273	2480	24.8
Dialysed Streptomycin sulphate supernatant	6.5	220	2660	33.9
Dialysed, redissolved 50-72% $(NH_4)_2SO_4$ precipitate	6.0	314	1820	52.0

.... Protein was determined by the method of Lowry et al. (1951). The S. faecalis LDH activity was assayed under the same conditions and expressed in the same units used for the S. lactis C_{10} LDH as described in Section 2.2.3.

2.5.3 Properties of the S. faecalis LDH.

2.5.3.1 FDP activation of the S. faecalis LDH in relation to buffer composition.

The effect of varying FDP concentration on the S. faecalis LDH activity was studied in tris/maleate and phosphate buffer. In tris/maleate buffer the FDP effect was also studied in the presence of $MnCl_2$.

In Figure 2.5.3.1a, plots A and B show the activation effect of FDP on S. faecalis LDH activity in 90 mM tris/maleate and 90 mM phosphate buffers, pH 6.9 at 10 mM pyruvate and 0.167 mM NADH. The activity in phosphate buffer is higher for all concentrations of FDP than the corresponding activity in tris/maleate buffer. This is in direct contrast to the findings for S. lactis C_{10} LDH where activity is inhibited to a significant extent in phosphate buffer compared to tris/maleate buffer. The same data of plots A and B in Figure 2.5.3.1a are plotted as the Hill plots in Figure 2.5.3.1b. In tris/maleate buffer the $M_{0.5V}$ value is 0.01 mM FDP and the Hill interaction coefficient (n_H) is 1.7 compared to a $M_{0.5V}$ value of 0.008 mM FDP and a n_H value of 1.5 for phosphate buffer. In both buffers no activity was detected in the absence of FDP.

Figure 2.5.3.1: a and b

EFFECT OF VARYING FDP CONCENTRATION ON ACTIVITY OF S. faecalis LDH

Figure a

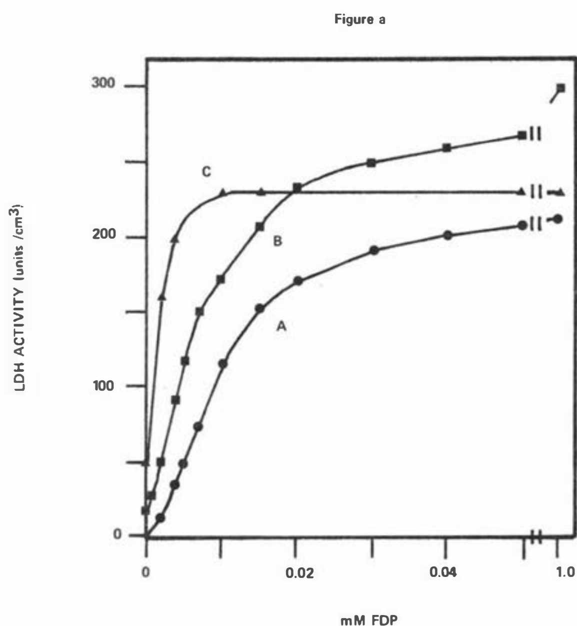
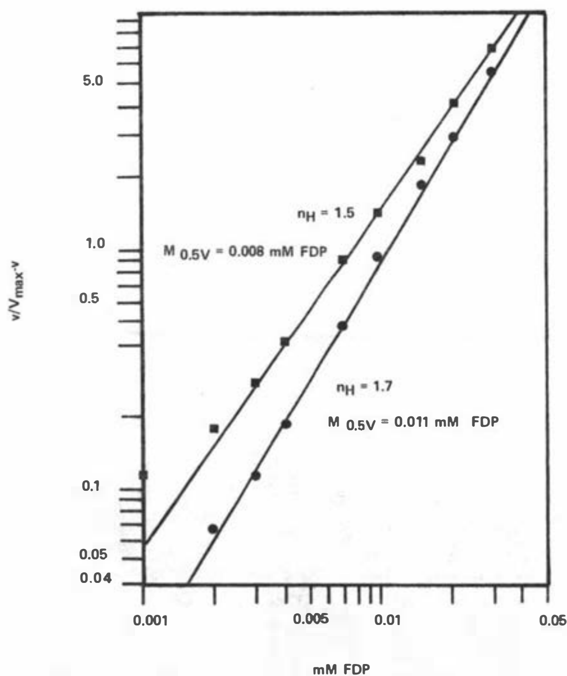
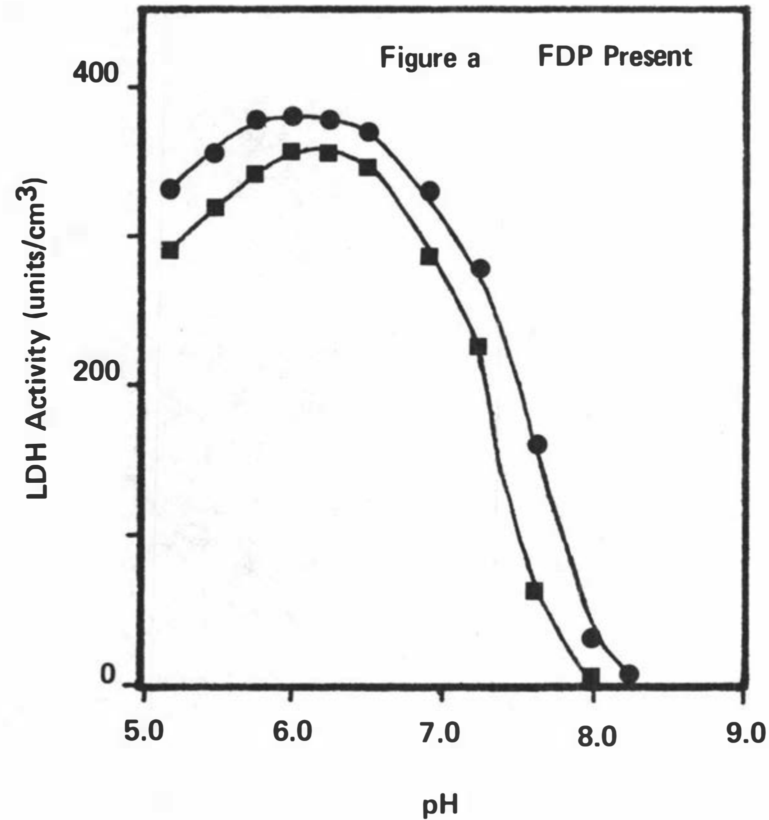


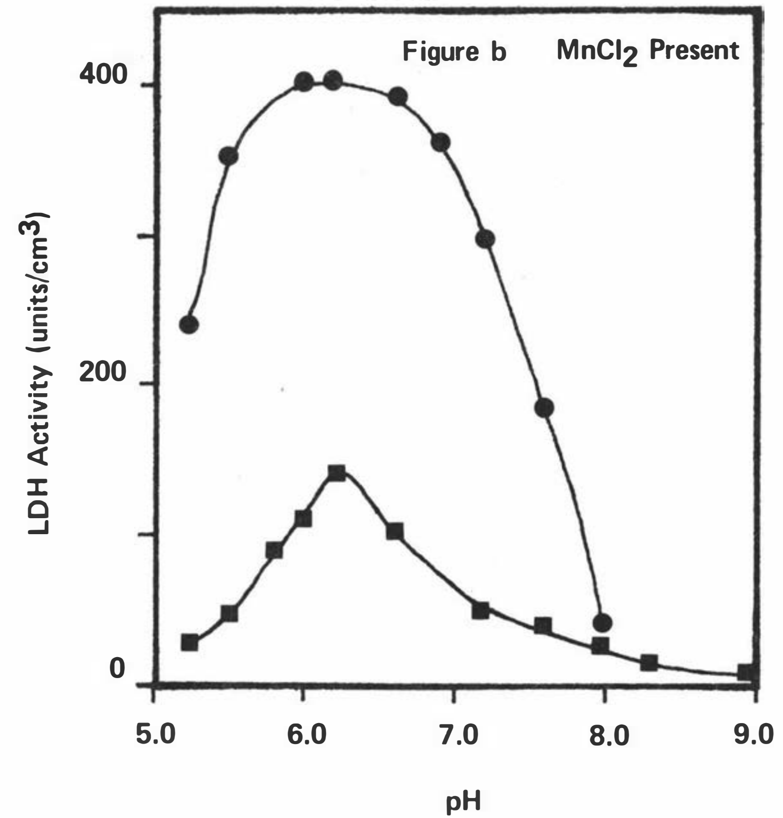
Figure b Hill plots



The effect of varying FDP concentration on the S. faecalis LDH activity was studied in (●) tris/maleate buffer (A) and in (■) phosphate buffer (B). The FDP concentration was also varied in tris/maleate buffer with 1 mM $MnCl_2$ present (C) (▲). The reaction mixture contained (in a total volume of 3 cm^3): 90 mM buffer pH 6.9; 0.167 mM NADH; 10 mM pyruvate and 0.1 cm^3 of diluted enzyme. The FDP concentration was varied as shown in the Figures. Figure a shows plots of LDH activity versus FDP concentration in three different conditions. Figure b shows Hill plots of the data from Figure a, but does not include the data obtained with 1 mM $MnCl_2$ present.

Figure 2.5.3.2: a and b pH OPTIMUM OF *S. faecalis* LDH





The pH optimum for *S. faecalis* LDH was determined in a reaction mixture containing (in a total volume of 3 cm^3): 90 mM tris/maleate buffer (pH varied as indicated in figures): 10 mM pyruvate; 0.167 mM NADH and 0.1 cm^3 of diluted enzyme. Figure a shows the pH optimum determined in the presence of two FDP concentrations: ●, 1.0 mM; ■, 0.2 mM FDP. Figure b shows the pH optimum determined without FDP present, but with two $MnCl_2$ concentrations present: ●, 10 mM $MnCl_2$; ■, 1.0 mM $MnCl_2$.

Plot C of Figure 2.5.3.1a shows that the presence of 1 mM $MnCl_2$ in tris/maleate buffer enhances the activation effect of FDP. The same enhancement by Mn^{++} occurred in phosphate buffer; with 0.0075 mM FDP activity was doubled by addition of 1 mM $MnCl_2$. However the Mn^{++} effect in phosphate buffer was not studied in detail because precipitation of manganese phosphate readily occurs and interferes with the assay. The activation of S. faecalis LDH by Mn^{++} has not been reported before so a further brief investigation was carried out.

2.5.3.2 Effect of Mn^{++} on activity and pH optimum of S. faecalis LDH

Although no S. faecalis LDH activity was detectable in the absence of FDP using the standard assay system, the enzyme was active in the absence of FDP if Mn^{++} was present. The pH optimum for the S. faecalis enzyme was determined in the presence of FDP as the enzyme activator, and in the presence of the alternative activator, Mn^{++}.

Figure 2.5.3.2a shows that the pH optimum in 90 mM tris/maleate buffer with 1.0 or 0.2 mM FDP, is pH 6.0 $\pm$ 0.1. This pH optimum is different from the value of pH 6.9 for the S. lactis LDH (Section 2.4.2).

In 90 mM tris/maleate buffer in the absence of FDP, but with $MnCl_2$ present at a concentration of 10 mM, the pH profile is similar to that in the presence of FDP with a pH optimum of pH 6.0 to 6.2 (Figure 2.5.3.2b). With 1 mM $MnCl_2$, the pH profile appears to be less broad with an optimum value of pH 6.2.

2.5.3.3 Effect of varying Mn^{++} concentration on the activity of S. faecalis LDH

$MnCl_2$ concentration was varied in 90 mM tris/maleate buffer pH 6.2, 10 mM pyruvate and 0.167 mM NADH in the absence of FDP. Figure 2.5.3.3a, a plot of activity versus $MnCl_2$ concentration, shows a sigmoidal response of activity to increasing Mn^{++} concentration. The same data are plotted as a Hill plot in Figure 2.5.3.3b. At high concentrations of Mn^{++} a Hill interaction coefficient (n_H) of 2.4 is obtained indicating interaction between Mn^{++} binding sites. It is interesting to note that the n_H value for Mn^{++} is greater than that obtained for FDP (1.5 to 1.7). The amount of $MnCl_2$ required to give half maximum activity ($MnCl_{2\ 0.5V}$) is 1.3 mM $MnCl_2$. The affinity for FDP in similar conditions is much higher (0.01 mM FDP).

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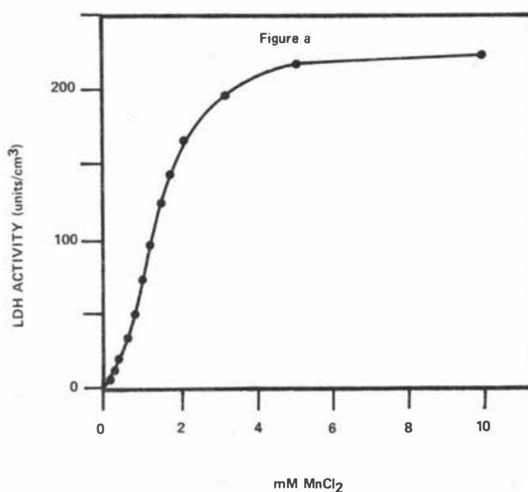
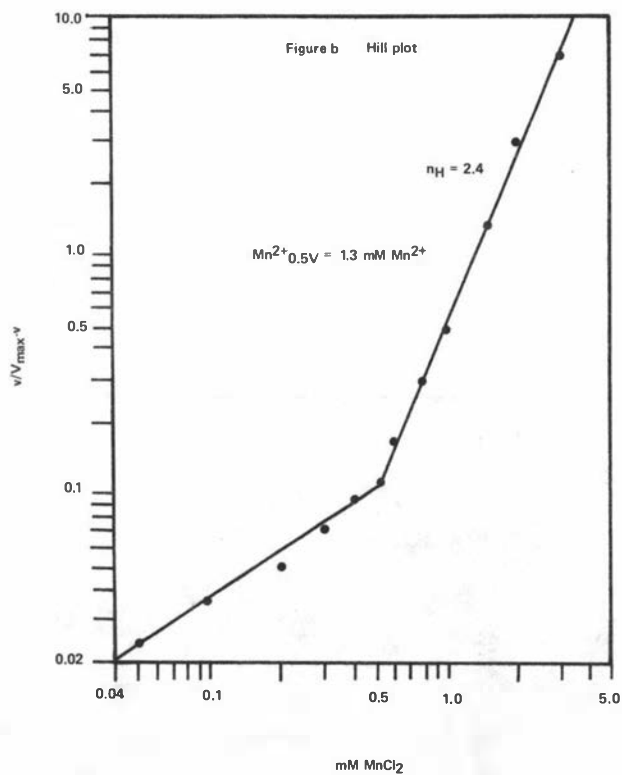
Figure 2.5.3.3: a and b

EFFECT OF VARYING $MnCl_2$ CONCENTRATION ON ACTIVITY OF *S. faecalis* LDH





The effect of varying $MnCl_2$ concentration on the *S. faecalis* LDH was studied. The reaction mixture contained (in a total volume of $3cm^3$): 90mM tris/maleate buffer pH 6.2; 0.167mM NADH; 10mM pyruvate and $0.1cm^3$ of diluted enzyme. The $MnCl_2$ concentration was varied as shown in the Figures. Figure a is a plot of LDH activity versus $MnCl_2$ concentration. Figure b is a Hill plot of the data from Figure a.

2.5.4 Response of S. lactis C_{10} LDH to Mn^{++}.

In view of $MnCl_2$ activation of the S. faecalis LDH, the effect of $MnCl_2$ on activity of the purified L(+)-LDH from S. lactis C_{10} was investigated. Using standard assay conditions of 10 mM pyruvate and 0.167 mM NADH and with either 90 mM tris/maleate or triethanolamine/HCl buffer pH 6.9 or pH 6.2, the Mn^{++} concentration was varied in the presence of 0.1, 1.0 and 10.0 mM FDP. The $MnCl_2$ concentration was also varied in the absence of FDP under otherwise standard assay conditions.

Under none of the above conditions could it be demonstrated that $MnCl_2$ affected the S. lactis C_{10} LDH activity in any way.

2.5.5 Discussion of Data obtained from the Brief Study of the S. faecalis LDH.

The study on the S. faecalis LDH has shown that at least one streptococcal LDH is not inhibited by phosphate. In fact, the S. faecalis LDH appeared to have slightly greater activity in phosphate buffer compared to the activity in tris/maleate buffer. Thus S. faecalis and S. lactis appear to differ markedly in their sensitivity to phosphate. This raises the possibility that phosphate inhibition has a physiological role in controlling the LDH of S. lactis, but not the LDH of S. faecalis.

Another major difference between the LDH's of S. lactis and of S. faecalis is in the response to Mn^{++}. Whereas this cation has no effect on the LDH of S. lactis, it apparently activates the S. faecalis LDH either in the presence or absence of FDP. Insufficient data have been obtained to discuss the mechanism of Mn^{++} activation and its relationship to that of FDP. The Mn^{++} activation of the S. faecalis enzyme indicates another point of similarity to the L. casei LDH in addition to the FDP activation. However the LDH's of the two species differ in at least two main features:

(1) Mn^{++} activation of the L. casei enzyme only occurs in the presence of FDP (de Vries et al., 1970). Mn^{++} merely enhances the binding of FDP over a restricted pH range (Holland and Pritchard, 1975).

(2) Phosphate inhibits the L. casei LDH even more strongly than the S. lactis LDH (Holland and Pritchard, 1975).

The S. faecalis enzyme appears to have an essential requirement for either FDP or Mn^{++} for activity. A significant difference between $MnCl_2$ and FDP activation of the S. faecalis LDH is that the activity at maximum activating concentrations of $MnCl_2$ is significantly less than if FDP is fully activating the enzyme as is shown in Figure 2.5.3.2a and b. Another difference is that FDP shows a higher affinity for the enzyme and a lower Hill interaction coefficient compared to Mn^{++}. These three differences between FDP and Mn^{++} activation suggest that the two different activators modify the enzyme in different ways. However the effect of Mn^{++} would need to be studied using a more highly purified enzyme before conclusions on its mechanism of action can be reached.

2.6 Discussion of the Results from Studies on the Properties of the *S. lactis* C_{10} LDH.

Discussion will be presented under the following three headings:

1. The FDP requirement of streptococcal LDH's in relation to the *in vivo* control of the activity of this enzyme.
2. A comparison of the *S. lactis* LDH properties with those of other streptococcal LDH's.
3. The interrelationship between the different properties of the *S. lactis* LDH.

Some comparison of the findings from the present study with those of other workers has already been made in the presentation of the results.

2.6.1 The FDP requirement of streptococcal LDH's.

One of the most significant properties in terms of relating *in vitro* activity to *in vivo* control is the FDP concentration required for half maximum activity ($M_{0.5V}$). This parameter is markedly influenced by the nature of buffer used in the *in vitro* assay. The $M_{0.5V}$ value ($FDP_{0.5V}$) at pH 6.9 was 4.4 mM FDP in phosphate buffer, 0.2 mM FDP in tris/maleate buffer and 0.002 mM FDP in triethanolamine/HCl buffer. Thus there is a 2000 fold difference between the two extreme values, depending on the buffer components. These results illustrate Srere's (1968) warning about the dangers of extrapolating from observations *in vitro*, to conditions *in vivo*.

While the above results may help to reconcile some of the widely differing values which have been reported for the $M_{0.5V}$ value of streptococcal LDH's, since different workers have used different buffers, the results also raise the very difficult question as to which buffer, if any, best represents *in vivo* conditions. A large number of different anions and cations will contribute to the internal buffering capacity of a cell and clearly no simple buffer system can hope to reproduce the *in vivo* situation. Tris/maleate was selected at an early stage of the present study on rather arbitrary grounds. Phosphate buffer, used by Wittenberger's group, was found in early studies on the *S. lactis* LDH to inhibit FDP binding, while triethanolamine/HCl used by Jago *et al.* did not have a sufficiently wide buffering range for the studies

envisaged. Tris/maleate did not appear in early studies to have the inhibiting effect shown by phosphate and suitably covered the pH range of the enzyme. In retrospect it was probably not the best choice since later studies showed that it does affect FDP binding and neither tris nor maleic acid are ideal buffering components from a physiological point of view (Good and Izawa, 1972). A much more thorough investigation of other possible buffers should be made before further work on the enzyme is carried out.

This question as to which buffer system best represents the *in vivo* conditions is a difficult one. The strong inhibitory effect of phosphate on FDP binding may be of *in vivo* significance. If a sufficient concentration of phosphate is present *in vivo* this may mean that the intracellular level of FDP required for activation must be considerably higher than the FDP $_{0.5V}$ value determined in the absence of phosphate would suggest.

The LDH's from *S. lactis* C_{10} and *S. faecalis* ATCC 8043 have been compared in respect to the effect of the buffer components on the FDP requirement. It was shown that a major difference between the *S. faecalis* and the *S. lactis* LDH is the relative insensitivity of the *S. faecalis* enzyme to phosphate inhibition (in fact phosphate slightly activates compared to tris/maleate buffer). The *S. lactis* C_{10} enzyme is strongly inhibited by phosphate. Therefore depending on the buffer system used in the assays, the *S. faecalis* and the *S. lactis* enzymes could have the same or different FDP requirements. Another, possibly physiologically significant, difference between the *S. faecalis* and *S. lactis* LDH's is that Mn^{++} has no effect on the *S. lactis* LDH whereas with the *S. faecalis* LDH, Mn^{++} apparently enhances the binding of FDP and even activates the enzyme in the absence of FDP.

The findings on the effect of buffer components on the FDP requirement of the *S. lactis* LDH may indicate that the high FDP requirement for the *S. mutans* NCTC 10449 LDH (FDP $_{0.5V}$ = 5 mM) studied in phosphate buffer (Brown and Wittenberger, 1972) is due to the use of phosphate buffer in the assays.

2.6.2 Comparison of the properties of the *S. lactis* C_{10} LDH with those of other streptococcal LDH's.

In addition to the FDP requirement, the LDH's from different streptococcal species differ in a number of other properties. The main differences are summarised in the following paragraphs.

A) pH optimum

The *S. lactis* C_{10} LDH has a pH optimum near neutrality (6.9) in the presence of FDP whereas LDH's from *S. faecalis* (Wittenberger and Angelo, 1970), *S. mutans* NCTC 10449 (Brown and Wittenberger, 1972) and *S. cremoris* US3 (Jonas *et al.*, 1972) all have a more acid pH optimum as does *S. faecalis* ATCC 8043 LDH studied in the present work with a pH optimum of 6.0 $\pm$ 0.1. In the *S. lactis* LDH the pH optimum was not affected by the nature of the buffer components.

B) Effect of FDP on kinetic parameters.

The effect of FDP on the kinetic parameters is similar in the LDH's from *S. cremoris* US3 (Jonas *et al.*, 1972) and *S. lactis* C_{10}. In both streptococcal LDH's, FDP effects both the V_{max} values and the binding of NADH and pyruvate. However in *S. mutans* (Brown and Wittenberger, 1972) FDP, though it effectsthe V_{max} and the binding of pyruvate, does not effect the K_M for NADH. Since Brown and Wittenberger used only three different FDP levels and these were all above the FDP $_{0.5V}$ value, it may be that the effect of the K_M for NADH was not detected by them. With *S. lactis* LDH the major effect of FDP is on the V_{max} and the effect of K_M for NADH and pyruvate is only small. However the *S. faecalis* LDH is markedly different in that the FDP apparently does not alter the V_{max} with either pyruvate or NADH as the variable substrate. FDP only alters the binding of pyruvate and NADH (Wittenberger and Angelo, 1970). This result was arrived at from the use of five different FDP levels spanning the FDP $_{0.5V}$ value and appears to be convincingly established. One can only conclude that FDP is activating the *S. faecalis* LDH in a somewhat different manner. In view of the many other features in which the *S. faecalis* LDH differs from that of other streptococcal LDH's it does appear to be a very distinctive enzyme.

C) Heat stability.

Another property in which the LDH from S. faecalis differs from that of other streptococcal species is in the response to heat treatment. For the S. lactis C_{10} LDH FDP stabilises the enzyme either in phosphate or tris/maleate buffers. The S. lactis LDH is therefore similar to a number of S. mutans strains (Brown and Wittenberger, 1972; Wittenberger et al., 1971) and S. cremoris US3 (Jonas et al., 1972), but different from S. faecalis (Wittenberger and Angelo, 1970). In the LDH from S. faecalis, the presence of FDP makes the enzyme labile to heat. In a comparative survey of a number of streptococcal strains (Wittenberger et al., 1971) the LDH from strain JR8-SM of S. lactis was found to be similar to S. faecalis, so in this respect the heat stability property appears to differ even between strains of a species.

D) Kinetic response to NADH and pyruvate.

In studying the S. lactis C_{10} LDH some properties as well as the FDP requirement were shown to be effected by the buffer components present in the assay system. In both tris/maleate and triethanolamine/HCl buffers the activity was a hyperbolic function of both NADH and pyruvate concentration. However in phosphate buffer, though activity was a hyperbolic function of pyruvate concentration, the response of activity to varying NADH concentration showed co-operative binding rather than Michaelis-Menten kinetics. It would be of interest to establish whether the findings of Brown and Wittenberger (1972) for the S. mutans NCTC 10449 LDH, where there appeared to be at least two pyruvate binding sites on the enzyme with some co-operative interaction between them, were due to the use by these workers of phosphate buffer.

E) Oxamate inhibition.

Brown and Wittenberger (1972) showed that oxamate inhibited activity at all pyruvate concentrations for the S. mutans enzyme. However when the S. lactis LDH was similarly studied in phosphate buffer, oxamate activated the enzyme at the lower pyruvate concentrations, and with an increase in the phosphate concentration the activation of oxamate was more marked.

F) Effect of pH on FDP binding.

The FDP binding for the *S. lactis* LDH was dependent on pH in two buffers, but in phosphate buffer the FDP binding was pH independent. The LDH's from *S. mutans* (Brown and Wittenberger, 1972) and *S. faecalis* (Wittenberger and Angelo, 1970) studied in phosphate buffer, showed a dependence on pH for FDP binding so these two LDH's differ from the *S. lactis* LDH studied in phosphate buffer. The *S. lactis* C_{10} LDH showed a different pattern of FDP binding in relation to pH between the two buffers, tris/maleate and triethanolamine/HCl buffer. Only the results in triethanolamine/HCl buffer were consistent with the pattern shown by the *S. mutans* and *S. faecalis* LDH, i.e. a decreasing affinity for FDP as the pH is raised.

Therefore of the four streptococcal LDH's studied in detail (*S. lactis*, *S. mutans*, *S. faecalis* and *S. cremoris*) there is sufficient evidence indicating that the LDH's are quite distinct from each other in their *in vitro* properties. The *S. lactis* C_{10} LDH is most similar to the *S. cremoris* LDH which is not surprising in view of the close taxonomic relationship betweenthese two species. Although the streptococcal LDH's show different *in vitro* properties it may be that the intracellular conditions are such that not all of the *in vitro* differences are physiologically significant in the *in vivo* situation. However it is possible that some of the differences in *in vitro* properties (especially pH optimum and FDP requirement and effect of phosphate on the FDP requirement) may well relate to differences in *in vivo* control features between the streptococcal LDH's.

2.6.3 The interrelationship between the properties of the *S. lactis* LDH.

The *S. lactis* LDH can be regarded as an allosteric enzyme with FDP (structurally dissimilar from the substrates, NADH and pyruvate) being the important allosteric effector. It follows from the discussion by Stadtman (1966) that the modulation of LDH activity is achieved through the binding of the metabolite effector (FDP) at specific regulatory sites that are distinct from the catalytic binding sites. As in most allosteric enzymes, the allosteric effector, FDP, yields sigmoidal curves (under most conditions) rather than hyperbolic saturation curves when LDH activity is plotted against the FDP concentration.

Monod *et al.* (1965) and Koshland *et al.* (1966) have proposed two basic models to mathematically treat and describe allosteric enzymes at the molecular level. In discussing the interrelationship between the properties of the *S. lactis* C_{10} LDH a model has been drawn (see Figure 2.6.3). However it is not intended that the model be considered either as a Monod *et al.* (1965) type or as a Koshland *et al.* (1966) type, but simply to summarise in part, some of the possible interrelationships of the *S. lactis* LDH properties.

With reference to the model shown in Figure 2.6.3 the interrelationships between the properties will be discussed under separate headings.

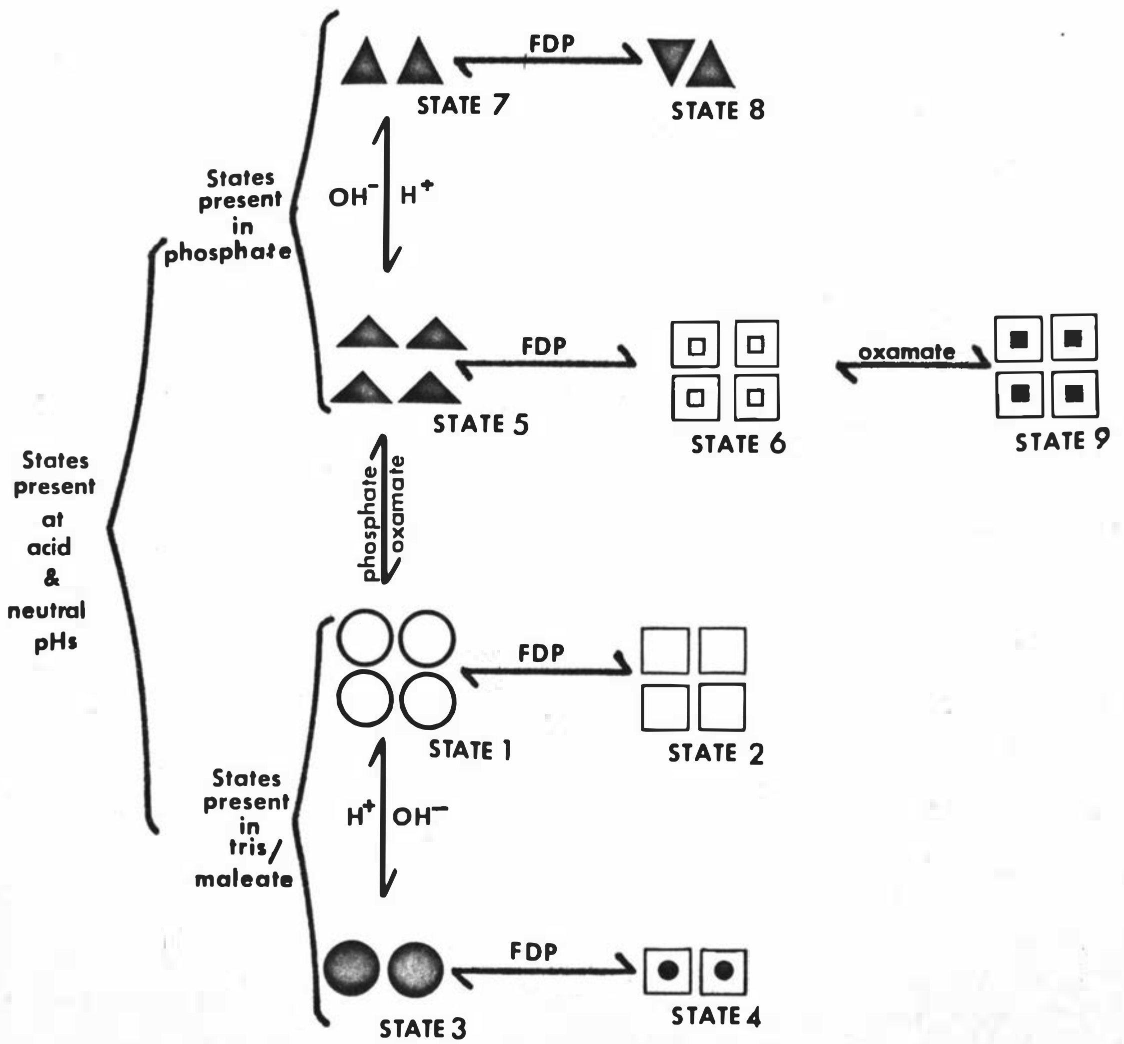
A) The *S. lactis* LDH can exist in different aggregated states.

From SDS polyacrylamide gel electrophoresis the *S. lactis* LDH sample showed a major protein band corresponding to a molecular weight of 36,000. The molecular weight of the native *S. lactis* LDH was not determined in a quantitative manner, because of the complexity of the behaviour of the active enzyme on polyacrylamide gel electrophoresis. However gel electrophoresis did appear to give a qualitative picture of the native molecular weight species of the *S. lactis* C_{10} LDH.

From gel electrophoresis of the LDH at acid pH (6.0) and at high ionic strength, a slow moving protein was observed whereas at alkaline pH values (above pH 7.0) only a number of faster moving proteins (three) with corresponding LDH activity were present. As gel electrophoresis separates proteins on the basis of a combination of charge and molecular weight of the protein, interpretation is difficult.

FIGURE 2·6·3

A MODEL OF S. LACTIS LDH



However the slow moving LDH species and the faster moving LDH species were considered to represent two different molecular weight forms rather than simply a change in mobility due to a charge increase on the protein as the pH was increased for the following reasons:

1) From polyacrylamide gel electrophoresis of the purified S. cremoris LDH Jago et al. (1971) showed that the relative mobilities were a linear function of pH. However in the study of the S. lactis LDH there appeared to be no intermediate forms between the slow migrating LDH activity and the faster migrating LDH activities when run at different pHs. Also at pH 6.0 the slow migrating LDH activity could be converted to the faster migrating LDH activities by simply lowering the buffer concentration (the reservoir buffer still showed buffering capacity after electrophoresis). Hence the S. lactis LDH, unlike the S. cremoris LDH, on gel electrophoresis would appear to migrate faster with an increase in pH due to deaggregation rather than simply just to an increase in charge.

2) From heat stability studies of the enzyme at 55°C, high ionic strength buffer and acid pH values increased the stability of the LDH. These same conditions favoured the slow migrating species which also developed a LDH activity stain very quickly in comparison to the activity stains of the faster migrating species present at low ionic strength buffers and at alkaline pH values. Therefore conditions that favour aggregation of the LDH will favour a stable form which, in turn, gives a corresponding quickly developing activity stain. Everse and Kaplan (1973) in their review on structure and function of LDH's state "At least for vertebrate LDH's, the evolution of the polymeric structure must be related to the fact that subunits are more stable in such a structure". This statement appears to be true also for the S. lactis C_{10} LDH. However in addition, in the S. lactis LDH, a polymeric structure is essential for the co-operative interaction of FDP with the enzyme.

3) Dynon et al. (1972) concluded from their investigations and the previous investigations of Jago et al. (1971) that the LDH from S. cremoris has a molecular weight of 140,000 and is a tetramer with identical subunits of molecular weight 35,000. Jago et al. (1971) found from ultracentrifuge studies on the S. cremoris LDH that an increase in pH, using triethanolamine/HCl buffer favoured a dissociated form of the enzyme (MWt 70,000) compared to the 140,000 form present in the pH range 5-7 and at pH 8.0 in phosphate buffer.

Considering these three points of evidence the aggregated form of the *S. lactis* LDH, tentatively called the tetrameric form (shown as state 1 in Figure 2.6.3) is the slow migrating form present in gel electrophoresis at acid pH and is relatively stable compared to the faster migrating LDH forms. The faster migrating LDH forms are collectively shown as state 3 in Figure 2.6.3. State 3 is shown as a dimeric protein, which is relatively unstable and possibly because of this instability, may exist in a number of forms. The existence of multiple forms (three) of LDH that migrate relatively fast may be due to charge or conformational differences rather than differences in their aggregation. However for simplicity and for want of further evidence, the dimeric protein is shown only as one form.

The evidence of the polyacrylamide gel work on the *S. lactis* LDH will have to be substantiated by more quantitative means such as ultra-centrifugation studies. Furthermore the conditions (pH and ionic strength of the buffer) that appear to alter the state of aggregation of the *S. lactis* LDH on gel electrophoresis may not necessarily affect the enzyme in a similar way when the kinetics and stability are studied in the absence of the gel medium. For example at pH 7.0 (the pH optimum of the LDH activity in presence of FDP) the evidence from gel electrophoresis indicated that the LDH was of the dimeric type (state 3). In inter-relating the kinetic properties at neutral pH values to the gel evidence indicating a dimeric typp, there are difficulties. Therefore it is reasoned that at pH 7.0 as well as pH 6.0 the enzyme is probably the tetrameric protein (state 1) under kinetic assay conditions, though not necessarily under gel electrophoresis conditions. It is probably reasonable to assume that the extreme conditions (acid or alkaline pH and high or low ionic strength) favour one of the two forms (aggregated - state 1 and deaggregated - state 3).

B) The effect of FDP on possible conformational changes.

The discussion under this heading refers to the properties of the enzyme in tris/maleate and triethanolamine/HCl buffers (not phosphate buffer - see under C). Although there were some significant differences between the kinetic properties in tris/maleate and triethanolamine/HCl buffers (e.g. FDP requirement and effect of pH on this property), for simplicity the LDH properties in these two buffers will be described in the model (Figure 2.6.3) as the same.

State 1 (Figure 2.6.3) of the LDH will show no pyruvate reduction or lactate oxidation activity in the absence of FDP at acid and neutral pH values. However at pH 8.0 state 3 is the predominate form of the enzyme, and it is this form that shows pyruvate reduction or lactate oxidation activity in the absence of FDP. This is supported from the kinetic findings on the S. lactis C_{10} LDH. At acid and neutral pH values no pyruvate reduction or lactate oxidation was found in the absence of FDP, whereas at alkaline pHs both lactate oxidation and pyruvate reduction activity was found in the absence of FDP.

With FDP present, the pyruvate reduction activity was significantly increased and the pH optimum shifted from an alkaline to a neutral pH (6.9). On the other hand, the pH optimum (8.0 - 8.2) for lactate oxidation activity was not altered by the presence of FDP. These findings can be related to the model in Figure 2.3.6. At neutral and acid pHs a conformation change occurs in the presence of FDP which favours the formation of state 2. State 2 of the LDH (tetrameric) is the activated form of the enzyme with a pH optimum for pyruvate reduction of 6.9.

At alkaline pH values FDP binds to the dimeric protein (state 3) and affects a possible conformational change to state 4. The conformation of the protein in state 4 is different from the FDP induced conformation of state 2, as well as different in the aggregation of subunits. This could then explain why FDP significantly activates the enzyme at acid and neutral pH values while only slightly activating at alkaline pH values.

In tris/maleate and triethanolamine buffers at acid and neutral pH values the FDP activation of the enzyme was sigmoidal (n_H = 1.6) indicating co-operative binding of FDP to the enzyme. In the same two buffers, at an alkaline pH, the FDP activation showed no co-operative binding to the enzyme (n_H = 1.0). These findings are consistent with the proposed model in Figure 2.6.3. In the deaggregated state 3 (dimeric), as is the possible species of the S. lactis LDH at alkaline pH, co-operative binding is less likely than in the aggregated enzyme of state 1.

FDP also afforded heat stability to the enzyme. With reference to the model, this can be interpreted as meaning that the conformations of states 2 and 4 are more stable to heat inactivation than the conformations of states 1 and 3 respectively.

From stopped-flow analysis, a lag period at pH 6.9 was demonstrated which could be eliminated by pre-incubation with FDP, but not with pyruvate or NADH. At pH 8.2, where activity is not so markedly affected by FDP, no lag period was demonstrated. The lag period

appeared to be due to a conformational change rather than a change in the state of aggregation. Therefore by addition of FDP to the enzyme, at acid and neutral pHs, the conformational change is sufficiently large that a lag period occurs in the interconversion of state 1 to state 2. On the other hand, the conformational change in going from state 3 to state 4, mediated by FDP, is smaller and possibly explains why FDP activation is not so great, why no co-operative binding occurs, and why no lag period was found at the alkaline pH values.

These conclusions are supported by work by Jago et al. (1971). These workers studied the S. cremoris LDH and concluded that FDP induced conformational changes in the structure of the enzyme, the unfolded form being more active.

C) The phosphate effect on the LDH.

One possible model that may explain the phosphate effect on the LDH activity is shown in Figure 2.3.6. At acid and neutral pHs, the phosphate binds to the enzyme in a co-operative manner and converts the enzyme into state 5. State 5 is similar to state 1 in that this form does not show pyruvate reduction or lactate oxidation activity in the absence of FDP and at alkaline pHs, dissociation occurs to give state 7, which does show pyruvate reduction activity (like state 3) but not lactate oxidation activity (unlike state 3) in the absence of FDP. However the conformations of states 5 and 7 are sufficiently different from the respective conformations of states 1 and 3 to give distinctive properties. First the conformation of state 5, in contrast to state 1, has a lower affinity for FDP, i.e. a higher FDP concentration is required to transform the enzyme from the inactive state 5, to the active state 6. The FDP activated state of the enzyme in phosphate (state 6) is visualised to be different from state 2 (FDP activated state in absence of phosphate) for the following reason: The enzyme in state 6, in contrast to state 2, shows sigmoidal kinetics with NADH as the varied substrate and an apparently higher K_M for pyruvate. Only in phosphate buffer did oxamate appear to activate the enzyme at low pyruvate concentrations. The different conformations in phosphate buffer could explain the differences in heat stability at 55°C between phosphate and tris/maleate buffers.

Oxamate could give an apparent activation by effecting a transition from state 5 to state 1. State 1, having a higher affinity for FDP, is

converted to state 2 which has a higher affinity for pyruvate. Alternatively a further conformation of LDH may be present in phosphate buffer, i.e. state 9, formed by oxamate counteracting the phosphate effect in some way and inducing a conformation state that has a higher affinity for pyruvate. This could explain why oxamate stabilizes the LDH to heat to a different extent in tris/maleate and phosphate buffers. Whether oxamate is binding at another site, other than the pyruvate catalytic site, as well, is not known. Pyruvate alone does not protect the enzyme from heat inactivation whereas oxamate does even in the absence of FDP and NADH. This suggests that oxamate does not bind to the pyruvate catalytic site but to a different site. This may explain why the "oxamate affinity" chromatography of S. lactis C_{10} was successful and why oxamate activated in phosphate since the oxamate site may be related to the binding site for phosphate. In phosphate buffer, at higher concentrations, oxamate probably binds to the pyruvate site since it inhibits the activity of LDH.

At pH 8.0, FDP binds in an apparently co-operative way in phosphate buffer, but not in the other two buffers. This co-operative binding to the state 7 form may be due to the FDP overcoming the phosphate effect as well as activating the enzyme.

2.6.4 Other factors that may regulate LDH activity.

Both NAD^+ and adenine nucleotides (ATP and ADP) inhibit the enzyme with quite low K_I values. The regulatory significance of such inhibitors in vivo is not obvious. Inhibition by NAD^+ (K_I 2.0 mM), which is competitive with respect to NADH, could ensure that a minimum pool size of NADH is maintained at a high NAD^+/NADH ratio as the reduced nucleotide may be required for some biosynthetic functions (although NADPH usually fulfils the biosynthetic function).

Since ADP and ATP inhibited the enzyme with similar K_I values, regulation by energy charge (Review by Atkinson, 1966) would seem unlikely. Though AMP inhibits activity over most of the NADH concentration range, this nucleotide did appear to activate the LDH at high NADH concentrations. However the interaction with other factors such as FDP, phosphate and pH would have to be further investigated before the significance of the nucleotide interaction could be understood.

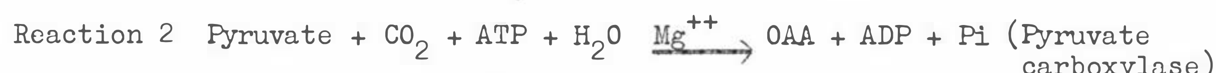
SECTION 3.

PYRUVATE KINASE

3.1 Introduction

Krebs and Kornberg (1957) proposed that the pacemaker enzymes of a metabolic pathway characteristically catalyse essentially irreversible reactions and have a low activity relative to other enzymes of the same pathway. The equilibrium of the reaction (Reaction 1) catalysed by pyruvate kinase (E.C.2.7.1.40) lies far to the side of pyruvate formation and is assumed to be practically irreversible under physiological conditions (Krebs and Kornberg, 1957; Utter, 1963). Pyruvate kinase therefore catalyses a strategic step in the glycolytic pathway (Krebs and Eggleston, 1965; Newsholme and Gevers, 1967). In organisms and tissues with the potential for gluconeogenesis, alternative reactions exist in order to bypass the irreversible step of glycolysis catalysed by pyruvate kinase. The two enzymes, pyruvate carboxylase (Reaction 2) and phosphoenolpyruvate carboxykinase (Reaction 3) result in pyruvate being converted back to phosphoenolpyruvate (PEP) via the intermediate formation of oxaloacetic acid (OAA).

Reaction 1 PEP + ADP $\xrightarrow{Mg^{++}\ K^{+}}$ Pyruvate + ATP (Pyruvate Kinase)


Reaction 2 Pyruvate + CO_2 + ATP + H_2O $\xrightarrow{Mg^{++}}$ OAA + ADP + Pi (Pyruvate carboxylase)

Reaction 3 OAA + GTP $\xrightarrow{Mg^{++}\ Mn^{++}}$ PEP + GDP + CO_2 (PEP - Carboxykinase)

The combined action of these three enzymes of glycolysis (Reaction 1) and gluconeogenesis (Reactions 2 and 3) would allow a "futile cycle" (Scrutton and Utter, 1968) to occur, resulting in the nett hydrolysis of nucleoside triphosphates, an energy-wasting process. Therefore in organisms where these three enzymes exist it is essential for the pyruvate kinase (as well as the other two enzymes) to be under physiological control so that no "futile cycle" is created and so that there is only a nett channelling of PEP either to an energy producing (glycolysis) or to an energy requiring (gluconeogenesis) pathway.

Pyruvate kinase would be expected to show different control properties depending on the role which this enzyme plays in metabolism. Thus the pyruvate kinase in an organism or tissue where only glycolysis can occur might be expected to have different properties from those of the enzyme found in a system where both glycolysis and gluconeogenesis can occur. Various groups of workers have studied the pyruvate kinases from different sources and have reported that there are distinctive

properties of the enzyme depending on its source. This diversity of the properties of different pyruvate kinases emphasises the importance of the physiological control function of this enzyme in relation to the particular pathways operating.

3.1.1 Mammalian Pyruvate Kinases

The most intensively studied mammalian pyruvate kinases are the two types of pyruvate kinase from rat liver.

Tanaka et al. (1965) studied the pyruvate kinase level in rat liver to elucidate the mechanism of control that prevents a "futile cycle" from operating and ensures that only the glycolytic or gluconeogenic pathway is operating. In crude extracts of liver two forms of the enzyme were identified by electrophoresis and immunological procedures and tentatively named type M and type L. The pyruvate kinase, type M, appears to be the same as the muscle type enzyme the characteristics of which have been described in detail by Boyer (1962). The type M is found in skeletal muscle, heart, liver and kidney and has similar properties in all these organs. The type L is found only in the liver and kidney. The level of the type L varies greatly under various physiological conditions whereas that of type M varies only slightly (Krebs and Eggleston, 1965; Tanaka et al., 1967; Weber et al., 1965; Yudkin and Krauss, 1967). For example in alloxan diabetic animals and those fed on a high protein diet or fasted for 48 hours, the level of type L pyruvate kinase was greatly decreased, and on subsequent insulin administration or administration of a normal diet the levels returned to normal.

A. Properties of rat liver L-type pyruvate kinase

Passeron et al. (1967) and Taylor and Bailey (1967) studied the rat liver pyruvate kinase (L-type) and found that it is activated by fructose-1,6-diphosphate (FDP) in vitro. The rabbit and rat muscle pyruvate kinase, on the other hand, was unaffected by FDP (Taylor and Bailey, 1967). The main kinetic characteristics of the activation of the rat liver pyruvate kinase (Taylor and Bailey, 1967) by FDP are homotropic co-operative interactions exhibited by both PEP and effector, FDP, and the abolition of the PEP co-operativity and the tenfold decrease in the apparent K_M for PEP at high concentrations of FDP. Taylor and Bailey (1967) suggested, in view of the high affinity of the liver enzyme for FDP combined with a low intracellular concentration of this metabolite

that the FDP stimulation constitutes a switching mechanism determining whether glycolysis or gluconeogenesis would predominate. This suggestion was further supported by Carminatti et al. (1968) who pointed out that FDP would control not only pyruvate kinase activity, but also the activity of the two other strategically related enzymes, fructose diphosphatase and phosphofructokinase.

Rozengurt et al. (1969) studied in detail the effect of pH on the allosteric behaviour of rat liver pyruvate kinase (type L). At pH values lower than 7, the enzyme obeys Michaelis-Menten kinetics with respect to both substrates (PEP and ADP) and cannot be activated by FDP. At pH values higher than 7.2 the enzyme has a sigmoidal response to PEP which is transformed into a normal hyperbolic relationship in the presence of FDP with the apparent affinity of the enzyme for PEP increasing. ATP, on the other hand, co-operatively inhibits the enzyme at low pH values, at pH 7.5 in the presence of 0.002 mM FDP or at high PEP concentrations. They concluded that small variations in the intracellular pH could be important in the regulation of pyruvate kinase activity by metabolites in vivo. Jimenez de Asua et al. (1970) showed that at pH 7.5 there is a sigmoidal velocity response of the rat liver pyruvate kinase (type L) with respect to K^+ or NH_4^+, but at saturating concentrations of PEP or FDP the velocity response is transformed into a normal hyperbolic relationship. The K^+ saturation curve follows a hyperbolic pattern at acid or very alkaline pH values. Jimenez de Asua et al. raised the possibility of modulation in vivo of pyruvate kinase activity by free K^+ level variations.

The importance of studying the interaction of substrates, activators and inhibitors over a wide concentration range before relating the in vitro properties to in vivo conditions is illustrated by the conflicting conclusions of Koster et al. (1972) and Van Berkel et al. (1974). Koster and Hulsmann (1970) reported that a number of phosphorylated hexoses and inorganic phosphate could activate the rat liver pyruvate kinase in a similar manner to FDP. Koster et al. (1972) subsequently showed that glucose-1,6-diphosphate has a similar effect to that of FDP. The concentration of FDP fluctuates in the liver during feeding and fasting while the GDP* concentration remains constant. The pyruvate kinase activity will therefore still be stimulated when the FDP concentration is low because the combined concentration of GDP and

* In this paragraph only GDP represents glucose-1,6-diphosphate.

the low FDP is still sufficient to fully activate the enzyme. Koster et al. concluded that the regulation of pyruvate kinase cannot be achieved by a fluctuating FDP concentration of the liver as previously suggested by Taylor and Bailey (1967). However, Van Berkel et al. (1974) studied the enzyme at physiological concentrations of substrates and modifiers, especially of PEP (Koster and Hulsmann, 1970 and Koster et al., 1972 used elevated PEP concentrations compared to the physiological level of PEP). They concluded that inorganic phosphate and phosphorylated hexoses, including glucose-1,6-diphosphate (but not FDP), at physiological concentrations and conditions would not be able to activate the pyruvate kinase. The only effective phosphorylated hexose activator in a physiological concentration range and under physiological conditions is FDP.

The interconversion of mammalian type L pyruvate kinase into two forms with different catalytic properties was first reported by Tanaka et al. (1967), who found that the rat liver enzyme, if incubated in KCl solution in the presence of EDTA, was completely desensitised towards activation by FDP. Other workers have also studied this phenomenon (Bailey et al., 1968 and Pogson 1968a), and have shown that the form insensitive to FDP also shows normal Michaelis-Menten kinetics with PEP. Susor and Rutter (1968) showed that the FDP insensitive form is also less sensitive to ATP inhibition. Preincubation with low concentrations of FDP (Llorente et al., 1970; Pogson, 1968a; Bailey et al., 1968; Susor and Rutter, 1968) and extraction and storage at low temperatures (0° - $20^{o}C$) (Llorente et al., 1970) are conditions that favour the form that is insensitive to FDP. Preincubation with citrate, ATP or between 25° and 37° (Pogson, 1968a) and isolation or preincubation in EDTA (Pogson, 1968a and b; Bailey et al., 1968) are conditions that favour the form that is sensitive to FDP. Van Berkel et al. (1973a) showed that the L-type pyruvate kinase from rat liver can exist in two forms with interconversion occurring by incubation of enzyme with oxidized or reduced 2-mercaptoethanol or glutathione. The oxidized enzyme has a decreased affinity for PEP and FDP. FDP is still able to activate the oxidized enzyme, but the concentration of FDP required for full activity is six times higher than the requirement for the reduced enzyme. Van Berkel et al. concluded that interconversion is by oxidation and reduction of the sulphydryl groups in the enzyme. The in vitro

oxidation and reduction of the two interconvertible forms of the L-type enzyme, though slow, may reflect a physiological regulation mechanism if an enzyme-catalysed interconversion exists *in vivo* as has been suggested for a number of enzymes, e.g. pyruvate-formate lyase from *Escherichia coli* (Knappe *et al.*, 1965) and xanthine oxidase from rat liver (Stirpe and Della Corte, 1970). Further support for the hypothesis of Van Berkel *et al.* (1973a) that -SH groups can play a role in the regulation of the pyruvate kinase activity *in vivo* comes from the work of the same group on human erythrocyte pyruvate kinase (Van Berkel *et al.*, 1973c). They showed that this enzyme can be converted into an oxidized form by incubation with oxidized glutathione. The oxidized enzyme showed a lower affinity for PEP and for FDP as did the enzyme from pyruvate kinase-deficient patients. It was therefore concluded that the human erythrocyte pyruvate kinase deficiency could be a consequence of an increased oxidized glutathione concentration in the red blood cells.

B. Rat liver M-type pyruvate kinase

The rat liver, M-type pyruvate kinase is immunologically indistinguishable from the muscle enzyme (Tanaka *et al.*, 1967) although it does differ in electrophoretic mobility (Susor and Rutter, 1968). Until recently it was uncertain whether these two pyruvate kinases had similar kinetic properties (Tanaka *et al.*, 1967). As recently as 1972 the rat liver M-type pyruvate kinase was described as a non-allosteric enzyme (Van Berkel *et al.*, 1972). These authors suggested that there are two kinds of glycolytic pathways in liver. One of these, proceeding by way of hexokinase and type M pyruvate kinase, was termed the basal pathway. The rate of the basal pathway would not be influenced by dietary and hormonal conditions and would meet the minimum demands of the cells. The regulatory pathway catalysed by glucokinase and type L pyruvate kinase would fluctuate in response to dietary and hormonal conditions and would meet the special demands of the cells. These workers based their conclusions on the basis of the M-type pyruvate kinase being a non-allosteric enzyme with no significant modifying ligands.

The same group of workers a year later (Van Berkel *et al.*, 1973b) published a paper on the allosteric kinetic properties of M-type pyruvate kinase from rat liver. They showed that the M-type pyruvate kinase from rat liver did show sigmoidal kinetics with respect to the substrate PEP

if the pH was above 7.25, the n_H value of PEP being 1.3 at pH 7.5 and 1.7 at pH 8.0. FDP was able to convert the PEP sigmoidal saturation curve to a hyperbolic relationship and stimulated activity at low PEP concentrations. Alanine acted as an allosteric inhibitor but this inhibition could be fully abolished by the addition of FDP. The alanine inhibition was also dependent on the pH and on PEP concentration. At pH 5.9 the alanine inhibition curve was sigmoidal with a n_H value of -2.0. At pH 5.9 full activity was completely restored with the addition of a sufficiently high concentration of FDP whereas at pH 7.5 if the alanine concentration is above 2.5 mM the FDP cannot restore activity.

Imamura, _et al._ (1972) also studied the M-type pyruvate kinase from rat liver, but, in contrast to Van Berkel _et al._ (1973b), found that FDP did not restore activity to the alanine inhibited enzyme. Van Berkel _et al._ (1973b) suggested that a possible explanation for this was that the M-type enzyme could exist in two forms which could be reversibly interconverted by sulphydryl reagents as had been shown for the L-type pyruvate kinase from rat liver (Van Berkel _et al._, 1973a). They showed that M-type enzyme if incubated overnight with oxidized glutathione, while having the same reaction velocity as the freshly prepared M-type enzyme, shows decreased affinity for PEP and the alanine inhibition is not affected by the addition of FDP. However, in contrast to the results obtained for the L-type from rat liver (Van Berkel _et al._, 1973a) the oxidation of the thiol groups of the M-type is not reversible. Van Berkel _et al._ (1973b) conclude that various authors have not detected the allosteric nature of the rat liver, M-type enzyme because they have studied the kinetics at pH 7.4 where there is very little allosteric interaction. Van Berkel _et al._ (1973b) state that "The metabolic implication of the possibility to regulate the M-type pyruvate kinase is not obvious".

It is evident from the studies described above and from others (e.g. Jimenez de Asua _et al._, 1971) that the kinetic properties of the enzymes have to be extensively studied under various combinations of conditions that could exist _in vivo_. The pH effect, the homotropic and heterotropic interrelationships between FDP, pH, PEP, metal ions and other controlling ligands (e.g. nucleotides and amino acids) all need to be studied. The possible modification of the enzyme by alteration of the thiol groups, as exemplified by the M- and L-types of rat liver pyruvate kinase, and the consequent change in kinetic properties illustrates Srere's (1968) warning of the dangers of extrapolating _in vitro_ data to _in vivo_ conditions.

C. Other mammalian pyruvate kinases

Different forms of pyruvate kinases also exist in adipose tissue (Pogson, 1968a and b), erythrocytes (Staal *et al.*, 1971) and kidney (Costa *et al.*, 1972). The adipose tissue isoenzymes are designated A and B forms. The A form is activated by FDP and shows co-operativity with respect to PEP and is thus similar to liver type L pyruvate kinase. The B form is generally similar to muscle (M) type. Both forms show some distinctive properties from the corresponding liver-enzymes (Pogson, 1968a and b). The isoenzymic pairs of erythrocytes and kidney cortex also show homologous properties to the two liver enzymes although the kidney type I pyruvate kinase shows some very distinctive features.

The cerebral pyruvate kinase (Nicholas and Bachelard, 1974) appears to be kinetically distinct from both M and L type.

3.1.2 Microbial Pyruvate Kinases

Pyruvate kinases have been purified and studied from a number of microbial sources. As with the mammalian pyruvate kinases the pyruvate kinases from different microorganisms differ from each other in ways which appear to be related to their physiological role in regulating carbohydrate metabolism.

Pyruvate kinases from the following microorganisms have been purified and studied in some detail:

Saccharomyces cerevisiae (and *S. carlsbergensis*)	Hess *et al.* (1966)
	Hess and Haeckel (1967)
	Haeckel *et al.* (1968)
	Hunsley and Suelter (1969)
	Macfarlane and Ainsworth (1972)
	Johannes and Hess (1973)
Escherichia coli	Maeba and Sanwal (1968)
	Malcovati and Kornberg (1969)
	Waygood and Sanwal (1971)
	Waygood and Sanwal (1972)
	Waygood and Sanwal (1974)
Bacillus licheniformis	Tuominen and Bernlohr (1971a and b)
Bacillus subtilis	Diesterhaft and Freese (1972)

Acetobacter xylinum	Benziman (1969)
Brevibacterium flavum	Ozaki and Shiio (1969)
Streptococcus faecalis	Wittenberger *et al.* (1973)
Streptococcus lactis ML_3	Collins and Thomas (1974)

The studies on yeast (*S. cerevisiae* and *S. carlsbergensis*), *E. coli* and *B. licheniformis* pyruvate kinases will be reviewed briefly as representing different types of physiological control. The *S. faecalis* and *S. lactis* ML_3 pyruvate kinases will be mentioned in the following section (3.1.3).

A. Yeast pyruvate kinase

Earlier studies (Hess *et al.*, 1966; Hess and Haeckel, 1967; Haeckel *et al.*, 1968) showed that the pyruvate kinase of *S. carlsbergensis* reacted allosterically to the addition of PEP, ATP, ADP and FDP. It was found that FDP transformed the sigmoidal saturation curve of the enzyme for PEP into a hyperbolic form. Similar findings are reported by Hunsley and Suelter (1969) on the *S. cerevisiae* enzyme, except that ADP exhibited no co-operative binding to the enzyme. ATP, CTP, GTP, UTP, ITP, AMP and $3^{1}5^{1}$AMP inhibited pyruvate kinase (Haeckel *et al.*, 1968) and the ATP inhibition was reported to be due to a negative heterotropic interaction with the binding of PEP. $NADP^{+}$, citrate and Ca^{++} have also been shown to inhibit the enzyme allosterically (Haeckel *et al.*, 1968). These workers have shown that low ATP (2.5 mM) may activate the enzyme with the activation being most pronounced at pH 6.0 and at a PEP:ADP ratio of 2.0.

In another yeast, *Candida utilis*, Gancedo *et al.* (1967) found that the pyruvate kinase, unlike that of *S. cerevisiae*, was not regulated by FDP. In this yeast, activity of the pyruvate kinase appears to be regulated by controlling enzyme synthesis. Pyruvate kinase activity is high when *C. utilis* is grown on glucose but markedly lower when grown ethanol (a gluconeogenic substrate). The same workers showed that the level of the FDP-activated pyruvate kinase of *S. cerevisiae* did not change in relation to the carbon source. Thus these two yeast species have adopted different types of control mechanisms to determine the balance between glycolytic and gluconeogenic activity.

C. Escherichia coli pyruvate kinase

Maeba and Sanwal (1968) studied the regulation of pyruvate kinase from E. coli B using a preparation partially purified by ammonium sulphate fractionation. They found that both FDP and AMP enhanced activity. These two positive effectors, however, affected differently the sigmoidal dependence of activity on PEP concentration. FDP changed the V_{max} but not the sigmoidal rate-concentration curves, whereas AMP changed the K_M for PEP and converted the sigmoidal plot into a hyperbolic plot. Subsequently Malcovati and Kornberg (1969) and Waygood and Sanwal (1971) showed that two types of pyruvate kinase are present in E. coli, one of which is activated by FDP and the other by AMP. Malcovati and Kornberg (1969) reported that the pyruvate kinase form I from E. coli K-12, which is activated by FDP is inducible whereas the other form of pyruvate kinase (II), which is not significantly affected by FDP, appears to be formed constitutively. The two forms of the enzyme were thought to represent different species of pyruvate kinase rather than different forms of the same enzyme which are interconvertible. The FDP-activated form was shown to be susceptible to a co-operative feedback inhibition by succinyl CoA and ATP (Waygood and Sanwal, 1972). Kornberg and Malcovati (1973) have found that the kinetic properties of pyruvate kinase in "permeabilized" E. coli were similar to the properties observed in vitro.

Waygood and Sanwal (1974) purified the FDP-activated E. coli pyruvate kinase and studied its regulatory properties. Of several nucleoside diphosphates which act as phosphate acceptors in the reaction, GDP, judging by its K_M value of 0.05 mM was the best substrate for the enzyme. They have suggested that GDP, rather than ADP, is the phosphate acceptor in vivo, at least for the FDP-activated E. coli enzyme. Similarly GTP is a more effective inhibitor than other nucleoside triphosphates although the degree of GTP inhibition depends on which nucleoside diphosphate is acting as the acceptor. ATP is a relatively poor inhibitor but with succinyl CoA (itself a poor inhibitor) a strong co-operative inhibition is obtained. On the other hand, GTP and succinyl CoA did not show the same increased co-operative inhibition.

The E. coli FDP-activated enzyme (Waygood and Sanwal, 1974) shows the distinctive property of not requiring K^+ for activity. Most other pyruvate kinases have an essential requirement for a monovalent cation. The only other reported exceptions to this are the pyruvate

kinases from Acetobacter xylinum (Benziman, 1969) and from Brevibacterium flavum (Ozaki and Shiio, 1969). Both Mg^{++} and Mn^{++} ions show homotropic co-operativity in binding to the enzyme. For both divalent cations the sigmoid plots become hyperbolic in the presence of 1 mM FDP. However with Mn^{++} as the divalent cation the V_{max} for the enzyme is approximately half of the value obtained when Mg^{++} is used. Under fully activated conditions the K_M for Mg^{++} is 0.4 mM and for Mn^{++} is 0.04 mM.

C. Bacillus licheniformis pyruvate kinase

The pyruvate kinase of the spore-forming bacterium B. licheniformis was purified and the kinetic properties studied by Tuominen and Bernlohr (1971a and b). They found that a single constitutively synthesised pyruvate kinase is present in B. licheniformis. They showed that a number of factors could modulate the activity of the purified pyruvate kinase of B. licheniformis. Pyruvate kinase appears to be regulated primarily by activation by AMP, ADP and PEP, and inhibition by ATP in the presence of Mg^{++}. Inorganic phosphate (Pi) and carbamyl phosphate inhibit activity. The authors state that inhibition of pyruvate kinase by carbamyl phosphate may not be physiologically significant. Regulation of the pyruvate kinase activity by in vivo concentrations of Pi may be significant with Pi serving as an indicator of NADH levels in glycolysing cells. FDP does not effect the enzyme's activity. The intracellular balance between Mg^{++} and Mn^{++} may be potentially important in the regulation of pyruvate kinase in B. licheniformis. With Mn^{++} present as the divalent cation, the K_M for PEP is seven-fold lower than in the presence of Mg^{++}. The sigmoid nature of ADP and PEP binding was increased by the presence of ATP in the presence of Mg^{++}. With Mg^{++} as the divalent cation, ATP inhibition was abolished by AMP and both ADP and PEP showed sigmoidal kinetics which were transformed to Michaelis-Menten kinetics if AMP was present. However with Mn^{++} present as the divalent cation, ATP did not alter the hyperbolic nature of the PEP saturation curve and the ATP inhibition was not relieved by AMP.

Other bacterial pyruvate kinases which are not apparently regulated by FDP include the pyruvate kinases from Acetobacter xylinum (Benziman, 1969) and from Brevibacterium flavum (Ozaki and Shiio, 1969). The Acetobacter xylinum enzyme appears to be regulated simply by ATP inhibition and PEP activation. Regulation of the Brevibacterium pyruvate

kinase is more complex with AMP acting as a positive allosteric activator and nucleoside triphosphates and several organic acids, citrate, isocitrate, malate and α-ketoglutarate acting as inhibitors.

3.1.3 Pyruvate Kinase of Streptococci

At the time that the present study of pyruvate kinase of *Streptococcus lactis* C_{10} was commenced the only information available on streptococcal pyruvate kinase was a personal communication from Mrs L.B. Collins of the New Zealand Dairy Research Institute that pyruvate kinase activity was detectable in extracts of *S. lactis* ML_3 only if FDP was included in the assay mixture. The D.R.I. group subsequently continued their studies of this enzyme and published a report on its properties (Collins and Thomas, 1974). Our attention was also drawn by Dr C.L. Wittenberger of the National Institute of Dental Research, Bethesda, to a preliminary report on the pyruvate kinases of *S. faecalis* presented to the 9th International Congress of Biochemistry.

Wittenberger *et al.* (1973) reported the presence of two forms of pyruvate kinase in *S. faecalis*, designated PK-I and PK-II. PK-I, with a molecular weight of 200,000, exhibits positive homotropic kinetics with its substrate, PEP (n_H = 2.9). This form of the enzyme has a low affinity for PEP (PEP $_{0.5V}$ = 9.0 mM) and is not activated by FDP (FDP is somewhat inhibitory). PK-I is slightly stimulated by AMP and is inhibited by ATP. PK-II, with a molecular weight of 100,000, is almost totally dependent upon FDP for activity, and exhibits weaker homotropic interactions with PEP (n_H = 1.6) than does PK-I. PK-II has a much higher affinity for PEP (PEP $_{0.5V}$ = 0.4 mM) and is unaffected by either AMP or ATP.

The work of Collins and Thomas (1974) on the FDP-activated pyruvate kinase of *S. lactis* ML_3 will be discussed in the relevant sections of the present study on *S. lactis* C_{10} pyruvate kinase.

The two main objectives in studying the *S. lactis* C_{10} pyruvate kinase were as follows:

1. To assess the importance of the FDP regulation of this enzyme, and to determine the effect of other factors that may regulate the enzyme, in the control of PEP utilisation in *S. lactis* C_{10}.

Probably no gluconeogenic pathway functions in S. lactis, so there is not the need to control the pyruvate kinase activity in respect to preventing a "futile cycle" from occurring. However PEP is required for the transport of carbohydrates into S. lactis by the PEP-phosphotransferase system, as shown by McKay et al. (1969 and 1970). Therefore the utilisation of PEP by pyruvate kinase needs to be regulated to ensure that adequate PEP is available for the PEP-phosphotransferase transport system.

2. To compare the FDP regulation of pyruvate kinase and LDH in the same strain of S. lactis.

The regulation of two consecutive and largely irreversible steps of a metabolic pathway by the same activator is a somewhat unusual situation. The metabolic significance of this is not immediately obvious. It is important to establish whether the concentration range of FDP over which the two enzymes are regulated is similar or different for the two enzymes. If the FDP requirement is similar then it is important to know if any other factors may differentially affect the activity of the two enzymes.

It is necessary to study the S. lactis C_{10} pyruvate kinase under a wide range of conditions and the interaction of as many factors as possible. This is evident from the work described in the previous section on LDH (Section 2), and from the review of work on pyruvate kinases of other organisms. Thus the FDP requirement of the S. lactis LDH was found to differ markedly depending on the buffer and pH used in the assay. Conflicting conclusions concerning the physiological role of a particular control mechanism may be drawn if the in vitro properties are studied under a limited range of conditions. This is exemplified by the opposing conclusions drawn by Koster et al. (1972) and Van Berkel et al. (1974) concerning the importance of FDP in regulating the L-type pyruvate kinase of mammalian liver.

Therefore in the present study of the S. lactis C_{10} pyruvate kinase an attempt has been made to investigate the interaction of substrates, activators, inhibitors and inorganic cations and anions on the enzyme's activity, as fully as possible.

3.2 Materials and Methods.

3.2.1 Organism

Streptococcus lactis C_{10} was maintained as described in Section 2.2.1.

3.2.2 Reagents

In view of the sensitivity of pyruvate kinase to a number of cations, anions and organic compounds, the source of the reagents is given in some detail. All nucleoside phosphates were stored desiccated as their dry salts in the freezer. For kinetic studies the solutions were made up in 0.1 M triethanolamine/HCl buffer pH 7.5 on the day of use, stored on ice, and only used for that day. Adenosine 5^1-Diphosphate (ADP), Guanosine 5^1-Diphosphate (GDP) and Adenosine 5^1-Triphosphate (ATP) were obtained as the disodium salts from the Sigma Chemical Company (Grade 1) and Adenosine 5^1-Monophosphate (AMP) was the disodium salt from Fluka.

Phosphoenolpyruvate (PEP) was the trisodium salt from Sigma. PEP was stored in the freezer as the dry salt in a desiccator. For kinetic studies solutions were made up in 0.1 M triethanolamine/HCl buffer pH 7.5 on the day of use, stored on ice, and only used for that day.

Nicotinamide Adenine Dinucleotide, Reduced Form (NADH) was the disodium salt from Sigma (Sigma grade, 98% pure). For kinetic studies, solutions were made up in 0.0025 M NaOH pH 8.5 on the day of use, stored on ice in the dark, and only used for that day.

D-Fructose-1,6-Diphosphate (FDP) was the tetrasodium salt from Sigma (Sigma grade, 98-100%). FDP was dissolved in 0.005 M triethanolamine/HCl buffer pH 6.8, stored on ice and used over a thirty hour period. In one series of kinetic determinations, the tetracyclohexylammonium salt of FDP (Sigma) was used and this will be clearly indicated.

The lactate dehydrogenase (LDH) (E.C.1.1.1.27) used in the pyruvate kinase assay system was the rabbit muscle type II, a suspension in 2.1 M $(NH_4)_2SO_4$ (10 mg protein/cm^3, 815 Sigma units/mg), obtained from Sigma.

The following monovalent and divalent metals were obtained from BDH as the Anala R grade: KCl, NH_4Cl, LiCl, $MgCl_2$, $MgSO_4$, $CoCl_2$, $CaCl_2$, $CuCl_2$, $BaCl_2$, $ZnCl_2$ and $MnCl_2$. NaCl was obtained from May and Baker ("AR").

Maleic acid was from Koch-Light Laboratories ("AR"), KH_2PO_4, imidazole and triethanolamine were from BDH (Anala R) and tris (hydroxymethyl) aminomethane was from Sigma (Trizma Base). Glycerol was obtained from BDH (Anala R) and 2-mercapto-ethanol from Fluka (98%).

For gel filtration, Bio-gel A 0.5 M, 100-200 mesh, was obtained from Bio-Rad Laboratories and for ion exchange chromatography DEAE-Cellulose, Whatman, DE-32 (Microgranular) was used.

3.2.3. Pyruvate Kinase Assay

Pyruvate kinase activity was estimated by measuring the rate of NADH oxidation at 340 nm. The standard assay mixture contained in a total volume of 3 cm^3: 80 mM triethanolamine/HCl buffer pH 7.5, 1 mM PEP, 3.3 mM ADP, 1 mM FDP, 13.33 mM KCl, 3.33 mM $MgCl_2$, 0.167 mM NADH, 20 µg dialysed LDH (20 Sigma units) and 0.1 cm^3 of diluted enzyme. The enzyme was diluted in 20% glycerol/deionised distilled water solution (v/v) at 0^oC. Routine assays during enzyme purification were carried out at room temperature using a Unicam SP 800 spectrophotometer. Kinetic studies were carried out at 25^oC using a Beckman ACTA-3 spectrophotemeter. In kinetic assays, particularly at low activator and substrate concentrations, when the reaction was started by addition of the pyruvate kinase sample, a lag period often occurred before a steady rate of NADH oxidation was attained. Unlike the study on LDH (Section 2.4.7) the lag period of pyruvate kinase was not investigated by stopped flow analysis. The pyruvate kinase activity was estimated from the steady rate of NADH oxidation.

An absorbance change of 1.0 unit per minute is used throughout as the measure of enzyme activity unless otherwise stated.* V_{max} values are expressed as units/cm^3 which refers to the OD change per minute that would be obtained using 1 cm^3 of the stock purified pyruvate kinase solution.

The stock LDH, used in the pyruvate kinase assay system, was a suspension in 2.1 M $(NH_4)_2SO_4$ (10 mg/cm^3, 815 Sigma units/mg). The day before use the stock LDH was diluted in 20% (v/v) glycerol in 0.1 M triethanolamine/HCl buffer pH 7.5 and dialysed against the same buffer for fifteen hours to remove the $(NH_4)_2SO_4$. The LDH was then diluted in the glycerol/triethanolamine buffer just prior to use. The twice diluted, dialysed LDH was used in the assay system for two days before a fresh diluted and dialysed LDH was prepared.

* See addendum at end of thesis

3.2.4 Protein Determinations

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard protein.

The method of estimating protein concentration by extinction at 260 nm and 280 nm according to the procedure given by Dawson *et al.* (1969) was used only for analysis of column fractions to obtain an approximate estimate of specific activity.

3.2.5 Polyacrylamide Disc Gel Electrophoresis

The polyacrylamide disc gel electrophoresis technique was the same as described for L(+)-LDH in Section 2.2.6 where gels (7% acrylamide, 0.18% Bisacrylamide, pH 8.9) were prepared according to the procedure of Gabriel (1971). The gels were run only in the tris/glycine buffer system of Gabriel (1971), (Tris, 3.0 g/glycine 14.4 g/litre deionised distilled water, pH 8.3).

3.2.6 Detection of Pyruvate Kinase Activity on Gels

The polyacrylamide gels were stained for pyruvate kinase activity in the following way. The gels were removed and washed in 0.1 M triethanolamine/HCl buffer pH 7.5 for five minutes before the gels were immersed in the activity mixture and placed in the dark at room temperature for sixty minutes. The activity mixture contained 2.5 mM NADH, 3 mM PEP, 10 mM ADP, 20 mM FDP and 20 μg Sigma LDH/cm^3 all in 100 mM triethanolamine/HCl buffer pH 7.5. The gels were removed from the activity mixture and quickly washed twice with distilled water. Washing was completed in half a minute and the washed gels were then immediately immersed in a staining mixture containing 5 mg nitro-blue tetrazolium and 2 mg phenazine methosulphate in 8 cm^3 of 100 mM triethanolamine/HCl buffer pH 7.9. The stain was allowed to develop for ten to twenty minutes in the dark and the gels were then removed and washed three times in 7% acetic acid before being stored in 7% acetic acid in the dark. The pyruvate kinase activity region showed as a clear zone against the dark background of reduced nitro-blue tetrazolium.

3.2.7 Sodium Dodecyl Sulphate Polyacrylamide Disc Gel Electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide disc gel electrophoresis was carried out according to the general procedure of Weber and Osborn (1969) using 10% acrylamide gels. The modifications to the general procedure have been described in Section 2.2.8.

3.3 Pyruvate Kinase Purification

3.3.1 Growth and Harvest of *Streptococcus lactis* C_{10}

Streptococcus lactis C_{10} was grown at 30°C in the medium of Jago *et al.* (1971) (see Section 2.3.1). Cultures were grown in 5L conical flasks containing 3 litres of medium without aeration. The pH was maintained between 6.0 and 6.5 by periodic addition of 2.5 M sodium hydroxide during growth.

The cells were harvested near the end of the logarithmic phase of growth by centrifugation at 5,500 g for fifteen minutes at 0°C and washed three times in 0.005 M phosphate buffer pH 7.0 containing 1% NaCl. The washed cells were stored frozen for no longer than sixteen hours before disruption for pyruvate kinase purification.

Unlike the L(+)-LDH, where the enzyme activity from cells frozen for varying periods up to one month showed hardly any decline, pyruvate kinase activity dropped by 50% after two weeks of storage of cells in the freezer. Storage of cells for even a few days in the freezer, while not altering activity, appeared to render the partially purified enzyme unstable and this trend became more noticeable with longer storage.

3.3.2 Breakage of Cells

Cells were thawed and suspended in 0.01 M phosphate buffer + 0.05% 2-mercaptoethanol (2-ME) pH 7.0 and disrupted by two passages through an Aminco French pressure cell at 5,500 lbs per in^2. Unbroken cells and cell debris were centrifuged down at 13,000 g for fifteen minutes at 4°C. All subsequent purification steps were carried out at 4°C.

3.3.3 Streptomycin Sulphate Treatment

Nucleic acids were precipitated from the cell-free extract by dropwise addition of streptomycin sulphate using 3.0 cm^3 of a 10% (w/v) solution for every 100 mg protein. The resulting suspension was allowed to stand for two hours before the precipitate was removed by centrifugation at 13,000 g for fifteen minutes.

3.3.4 Ammonium Sulphate Precipitation

The supernatant after streptomycin sulphate treatment was dialysed against 0.01 M phosphate buffer + 0.1% 2-ME pH 7.0 for fifteen hours. Solid powdered ammonium sulphate was then added slowly over half

an hour to bring the solution to 50% saturation and the resulting precipitate was then removed immediately by centrifugation at 13,000 g for fifteen minutes. The concentration of ammonium sulphate in the supernatant was then increased to 75% saturation over half an hour. The precipitate was collected immediately by centrifugation at 13,000 g for fifteen minutes to be redissolved and dialysed for twenty-four hours in 0.01 M phosphate buffer + 0.1 M KCl + 0.1% 2-ME pH 6.7.

In trial ammonium sulphate precipitations of pyruvate kinase it was found that the enzyme precipitated in the 50 to 70% ammonium sulphate fraction if left standing for two hours before centrifugation. However this led to 55% loss in activity and the pyruvate kinase appeared to be less stable during the two subsequent purification steps. Hence a 50 to 75% ammonium sulphate cut was used without any period of standing.

3.3.5 DEAE-Cellulose Ion Exchange Chromatography

A. Development of suitable Ion Exchange Purification procedure

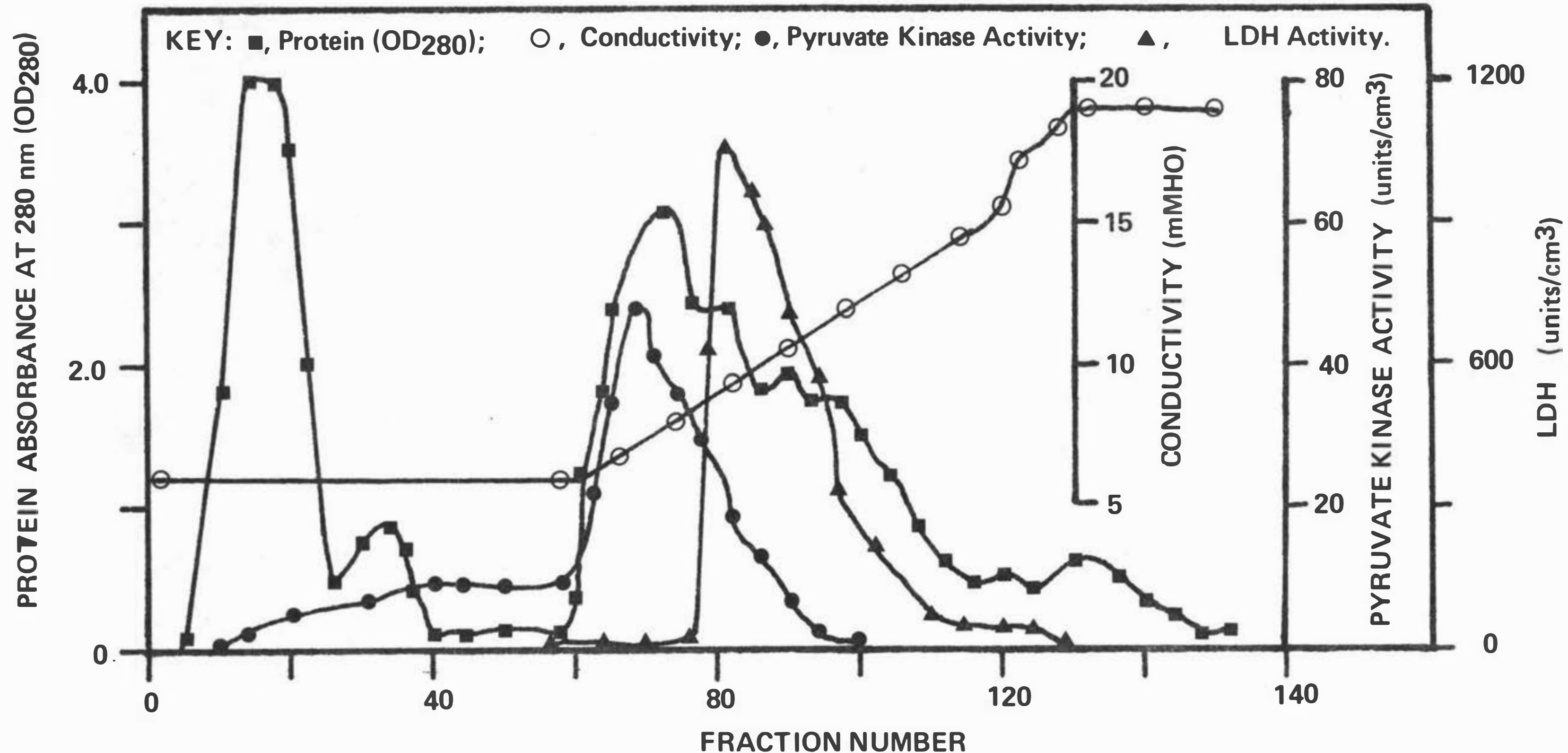
DEAE-Protion as used for L(+)-LDH purification (2.3.5) was tested for its suitability for pyruvate kinase purification. It was found that, to avoid overloading the resin, the pyruvate kinase had to be added to a relatively large volume of resin resulting in a low recovery of pyruvate kinase activity (over 40%). Microgranular DEAE-Cellulose (DE 32) was used in subsequent work since with this resin smaller volumes of resin could be used and dilution was not as great. The use of DEAE-Cellulose allowed greater recovery of activity.

Trial gradient runs on DEAE-Cellulose were carried out using a variety of conditions. Figure 3.3.5 is the gradient run from which a stepwise elution procedure for pyruvate kinase was developed. The pyruvate kinase was eluted earlier in the gradient than LDH but with the pyruvate kinase activity tailing into the LDH activity peak. A stepwise DEAE-Cellulose procedure was developed to give higher specific activity fractions free of LDH. The procedure finally adopted is as follows:

B. Stepwise DEAE-Cellulose Purification procedure for Pyruvate Kinase

The dialysed sample (in 0.01 M phosphate buffer + 0.1 M KCl + 0.1% 2-ME pH 6.7) from the ammonium sulphate 50 to 75% precipitation was applied to a DEAE-Cellulose column (Diameter 45 mm x Length 120 mm) pre-equilibrated in 0.01 M phosphate buffer + 0.1 M KCl +

Figure 3.3.5 DEAE-CELLULOSE – TRIAL GRADIENT RUN



COLUMN CONDITIONS: Column resin size – Length (15 cm); Diameter (3 cm). Pre-equilibration of resin (DE32) in 0.02 M phosphate buffer + 0.15 M KCl + 0.05% 2–ME pH 6.7 (pre-equilibration buffer). Sample Applied – Protein (15 mg/cm^3); LDH Activity (810 units/cm^3); Pyruvate Kinase Activity (80 units/mg); Volume (117 cm^3); Sample dialysed in pre-equilibration buffer. Gradient – After sample applied to column (flow rate, 0.9 cm^3/minute) the pre-equilibration buffer was used to eluate the column (until OD_{280} low) before gradient started (at fraction 46).
The gradient consisted of: Initial buffer, 220 cm^3 pre-equilibration buffer; Final buffer, 220 cm^3 of 0.02 M phosphate buffer + 0.5 M KCl + 0.05% 2–ME pH 6.7. Fraction size – Fractions 0 to 40 (12 cm^3); Fractions 41 to 150 (5.6 cm^3).

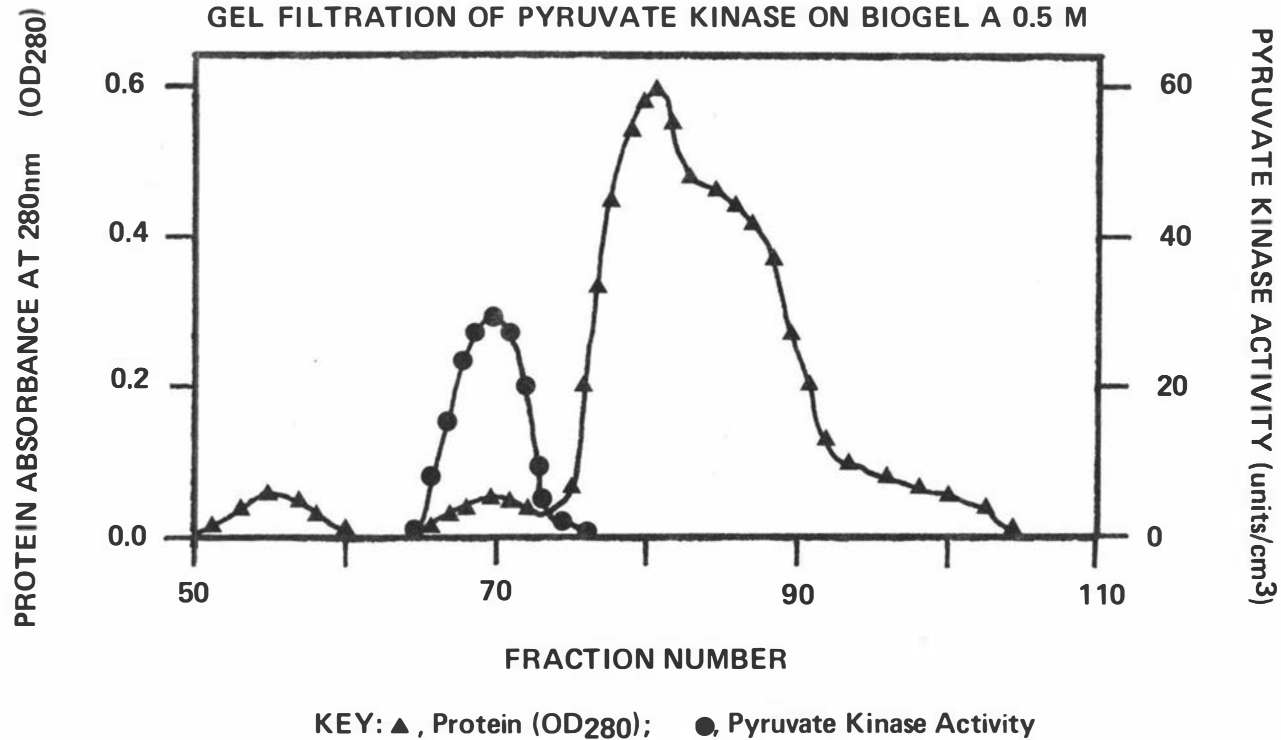
0.1% 2-ME pH 6.7. The column was then washed at a rate of 2 cm^3/minute with the same buffer until the absorbance of the eluant fractions at 280 nm had dropped to zero. No pyruvate kinase activity was found in these fractions. The pyruvate kinase was then eluted at the same flow rate with 0.02 M phosphate buffer + 0.15 M KCl + 0.1% 2-ME pH 6.6. All fractions containing the pyruvate kinase at a specific activity greater than 30 units/mg were bulked and concentrated by ultrafiltration using a Diaflo membrane (type XM-50). The concentrated pyruvate kinase was dialysed against 0.025 M phosphate buffer + 0.1% 2-ME pH 7.3 for fifteen hours.

3.3.6 Gel Filtration on Biogel A 0.5 M

An aliquot of dialysed concentrated sample (in 0.025 M phosphate buffer + 0.1% 2-ME pH 7.3) from the DEAE-Cellulose purification step was added to a 2.5 cm x 70 cm column of packed Biogel A 0.5 M (100-200 mesh) resin, pre-equilibrated in 0.025 M phosphate buffer + 0.1% 2-ME pH 7.3. In any one gel filtration run, no more than 80 mg total protein in a volume of 7 cm^3 was added to the column. The void volume of the column as calculated with glutamate dehydrogenase was 153.4 cm^3 and the total volume was 307 cm^3 as calculated from the elution peak of ammonium sulphate. In a total of five runs, including a sample that had previously been purified by gel filtration, the elution volume of the activity peak was 173 to 174 cm^3. Figure 3.3.6 shows a typical gel filtration purification of pyruvate kinase. The fractions containing pyruvate kinase with specific activity greater than 140 units/mg were bulked and concentrated to about 2 mg protein/cm^3 by ultrafiltration. The concentrate was added to an equal volume of deionised distilled water with 0.1% 2-ME and then quickly concentrated by further ultrafiltration to the previous volume. In this way the purified enzyme was obtained in 0.0125 M phosphate buffer pH 7.2 + 0.1% 2-ME as a 2 mg/protein/cm^3 solution. Only a small fraction of the activity was discarded and the bulk of the pyruvate kinase activity lost in gel filtration was due to deactivation.

The result of the above purification procedure (Sections 3.3.1 to 3.3.6) is summarised in Table 3.3 for a typical purification of pyruvate kinase. The enzyme was purified overall by 62 fold with a recovery of 29%. 55% of the activity lost in the DEAE-Cellulose purification step was a result of discarding fractions of low specific activity. The

Figure 3.3.6



COLUMN CONDITIONS: Column resin size – Length (70 cm); Diameter (2.5 cm); Resin (Biogel A 0.5 M) packed in a Pharmacia column.
Pre-equilibration of resin in 0.025 M phosphate buffer + 0.1% 2-ME pH 7.3 (pre-equilibration buffer). Sample Applied – Specific Activity (46 units/mg); Volume (6.8 cm^3, sample containing 50 mg total protein was dialysed in pre-equilibration buffer). Elution – Sample applied at a flow rate of 3 cm^3/20 minutes and then eluted with the pre-equilibration buffer at same flow-rate. Fraction size – 2 to 3 cm^3. Fractions 67 to 70 – concentrated and dialysed (Specific activity, 162 units/mg). Fractions 71 to 74 – concentrated and dialysed (Specific activity, 155 units/mg).

specific activity of cell free extracts from different batches of cells varied only slightly. The final purified sample did show some variation in specific activity for different preparations, due to differences in the degree of recovery. Where the ammonium sulphate purification step was a 50 to 70% cut, as mentioned in Section 3.3.4, the final specific activity was 83 units/mg. Two other purifications following the same procedure as for the preparation summarised in Table 3.3 gave a final specific activity of 135 and 155 units/mg.

Table 3.3

Summary of the purification of pyruvate kinase from *S. lactis* C_{10} harvested from 6L of medium

Purification step	Total activity standard assay units	Total protein mg	Specific activity units/mg	Total purification factor	Percent recovery
Cell free extract	12,800	5280	2.42	-	-
Streptomycin sulphate supernatant	13,100	5000	2.52	1.08	-
Ammonium sulphate 50-75% redissolved precipitate	8,700	1640	5.25	2.18	68
DEAE-Cellulose bulked high specific activity fractions	5,450	124	44.0	18.05	42
Gel filtration bulked high specific activity fractions	3,700	24.6	150.0	62.0	29

3.4 Studies on the Properties of the Purified Pyruvate Kinase

All studies on the properties of the pyruvate kinase, except where specifically mentioned, were carried out using the enzyme purified to a specific activity of between 135 to 155 units/mg. The concentrated purified enzyme from the gel filtration step was immediately diluted to a protein concentration of approximately 1.0 mg/cm^3. The diluent contained glycerol and $MgCl_2$ such that the enzyme was in a 0.005 M phosphate buffer pH 7.0 containing 50% glycerol (v/v) + 0.005 M $MgCl_2$ + 0.1% 2-ME. The enzyme was relatively stable in this buffer. The diluted enzyme solution was stored in 1 cm^3 samples either frozen or at 4°C. The samples stored at 4°C were used within two weeks. The activity over the two weeks remained constant. Samples stored frozen were all used within two months of storage. The activity did not decrease over the two months of storage and when thawed and stored at 4°C the activity was constant over a two week period. There was, however, some change in the co-operativity properties of the enzyme which will be described in more detail in later sections.

All kinetic assays used 0.1 cm^3 of the purified enzyme in the stabilizing buffer described above which was diluted at least by 1:20 with a 20% glycerol/deionised distilled water solution (v/v) at 0°C. The enzyme diluted in 20% glycerol was stable for ten hours but, when assayed 24-30 hours after initial dilution, activity had dropped by 20 to 30%. Therefore the diluted enzyme was not used for periods of longer than ten hours after dilution. The dilution of 1:20 ensured that the effects of phosphate ions, 2-ME or $MgCl_2$ on kinetic studies were negligible since the concentrations of these substances in the final assay system would be insignificant. It was shown that the glycerol introduced into the standard assay system by addition of the diluted enzyme did not affect activity since further addition of 0.3 cm^3 of a 50% glycerol/water solution to the assay mixture did not alter activity. For the different assay conditions, the purified pyruvate kinase appeared to be completely stable over the assay period (up to six minutes).

It was also established that the LDH present in the assay system was not rate-limiting. LDH was present in large excess in the standard assay system but under conditions of pH extremes (when determining the pH profile) or in the presence of inhibitors, which might affect the LDH as well as pyruvate kinase, it is possible that the LDH could become limiting. However, under such conditions it was shown that addition of a 2 fold excess of LDH did not alter the rate.

3.4.1 Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis was carried out on the purified pyruvate kinase sample (specific activity 155 units/mg). The gels were run in tris/glycine (3 g tris/14.4 g glycine/litre distilled water) buffer pH 8.3. In Figure 3.4.1 the photograph shows that the purified pyruvate kinase runs as one major protein band (gel 1) with only very faint minor bands present. Gel 2 shows that the clear band signifying pyruvate kinase activity corresponds to the major protein stain of gel 1. The activity stain was allowed to develop for sixty minutes in gel 2. If either PEP or ADP was omitted from the activity mixture no pyruvate kinase activity band was visible even after two hours of development. When FDP was left out from the activity mixture, the activity stain was only just visible if developed for sixty minutes but had the same intensity as with FDP present if the gel was allowed to remain in the activity mixture for 2½ hours. Hence the activity stain appears to be specific for pyruvate kinase.

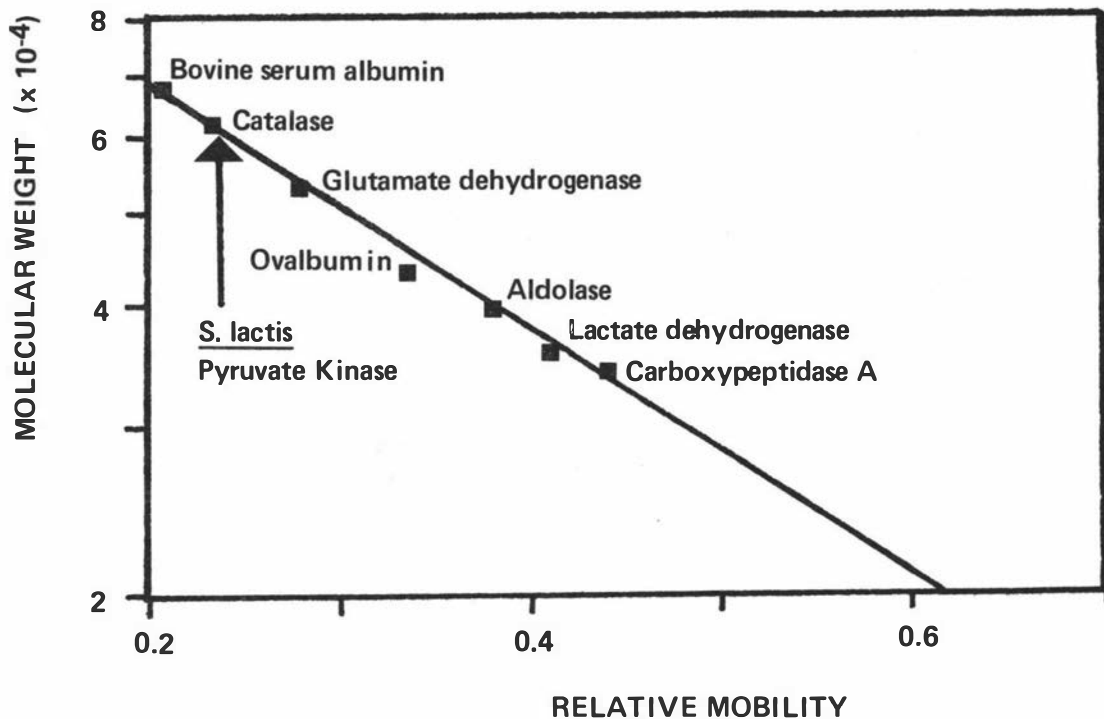
3.4.1.1 Sodium dodecyl sulphate polyacrylamide disc gel electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide disc gel electrophoresis was carried out on the same purified pyruvate kinase sample as a further means of demonstrating the purity of the pyruvate kinase and to determine its subunit molecular weight. Three separate runs were carried out, each with two SDS treated pyruvate kinase samples and a series of standard proteins (SDS treated) of known subunit molecular weight. The relative mobilities of the standard proteins and pyruvate kinase were calculated by the method of Weber and Osborn (1969). By plotting the relative mobilities of the standard proteins against the log of their molecular weight, a standard curve was obtained from which the pyruvate kinase molecular weight could be read off. Such a standard curve is shown in Figure 3.4.1.1a where the points for each of the standard proteins represent the average relative mobility from the three separate runs. From the relative mobilities of six gel samples the average value obtained for the pyruvate kinase subunit molecular weight was 60,750. Values from individual gels ranged from 59,000 to 62,000.

Figure 2.4.1.1b shows three SDS polyacrylamide gels run with 20, 30 and 50 μg (from left to right) of purified pyruvate kinase and then stained for protein. One major band of protein is evident and additional faint minor protein stains appear only when the gel is loaded with 50 μg of the purified pyruvate kinase.

Figure 3.4.1.1a

SDS POLYACRYLAMIDE DISC GEL ELECTROPHORESIS: STANDARD CURVE



The standard curve shown was obtained by plotting the relative mobilities (calculated by method of Weber and Osborn , 1969) of the standard proteins against log of their molecular weight. The point for each standard protein represents the average relative mobilities from three separate runs. The arrow indicates the average relative mobility of the S. lactis pyruvate kinase from 6 gel samples and this position corresponds to a molecular weight of 60, 750. The standard proteins are: Bovine Serum Albumin (Fluka); Catalase (Bovine liver, Sigma); Glutamate Dehydrogenase (Type I Bovine liver, Sigma); Ovalbumin (Sigma); Aldolase (Rabbit muscle, Sigma); Lactate Dehydrogenase (Rabbit muscle type II, Sigma); Carboxypeptidase A (Bovine pancreas, Sigma). The molecular weights of the standard proteins were obtained from Weber and Osborn (1969). 20 µg of each of the standard proteins was used.

Figure 3.4.1

Polyacrylamide Disc Gel Electrophoresis

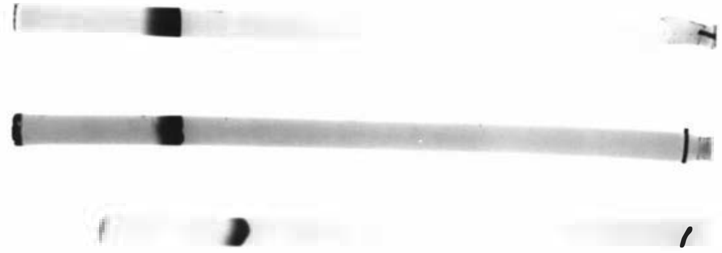
The gels were run in tris/glycine buffer pH 8.3. 40 μg of the purified pyruvate kinase sample was added to each gel. The gels are numbered from left to right.

Gel 1 Protein stain
Gel 2 Pyruvate kinase activity stain

Figure 3.4.1.1b

SDS Polyacrylamide Disc Gel Electrophoresis

The purified pyruvate kinase samples, denatured with SDS, were run in the conditions as described by Weber and Osborn (1969). The gels are numbered from left to right. The SDS treated samples were loaded on to the gels in the following concentrations: gel 1 (20 μg); gel 2 (30 μg) and gel 3 (50 μg).



Thus the evidence from polyacrylamide gel electrophoresis of both the active enzyme and the SDS treated enzyme indicates that the pyruvate kinase from S. lactis C_{10} has been purified to near homogeneity.

3.4.2 Molecular Weight Determination of Pyruvate Kinase by Equilibrium Sedimentation

The S. lactis C_{10} pyruvate kinase sample used for the molecular weight determination was a solution with a specific activity of 155 units/mg (1.35 mg protein/cm^3) in 25 mM phosphate buffer (KH_2PO_4/NaOH) + 0.05% 2-ME, pH 7.5. The Beckman Model E Analytical Ultracentrifuge with an A.n.D. Rotor was used. Dr J.W. Lyttleton of the Applied Biochemistry Division, D.S.I.R., Palmerston North, did the ultracentrifuge run on the S. lactis C_{10} pyruvate kinase sample. The "Long-Column Meniscus Depletion Sedimentation Equilibrium Technique" described by Chervenka (1970) was used where equilibrium is reached overnight. The fringe patterns were photographed on to glass plates. The developed plates were placed under a travelling microscope and sufficient readings were taken to allow a plot of log Δy versus r^2 to be made (symbols as in the formula of Chervenka, 1970).

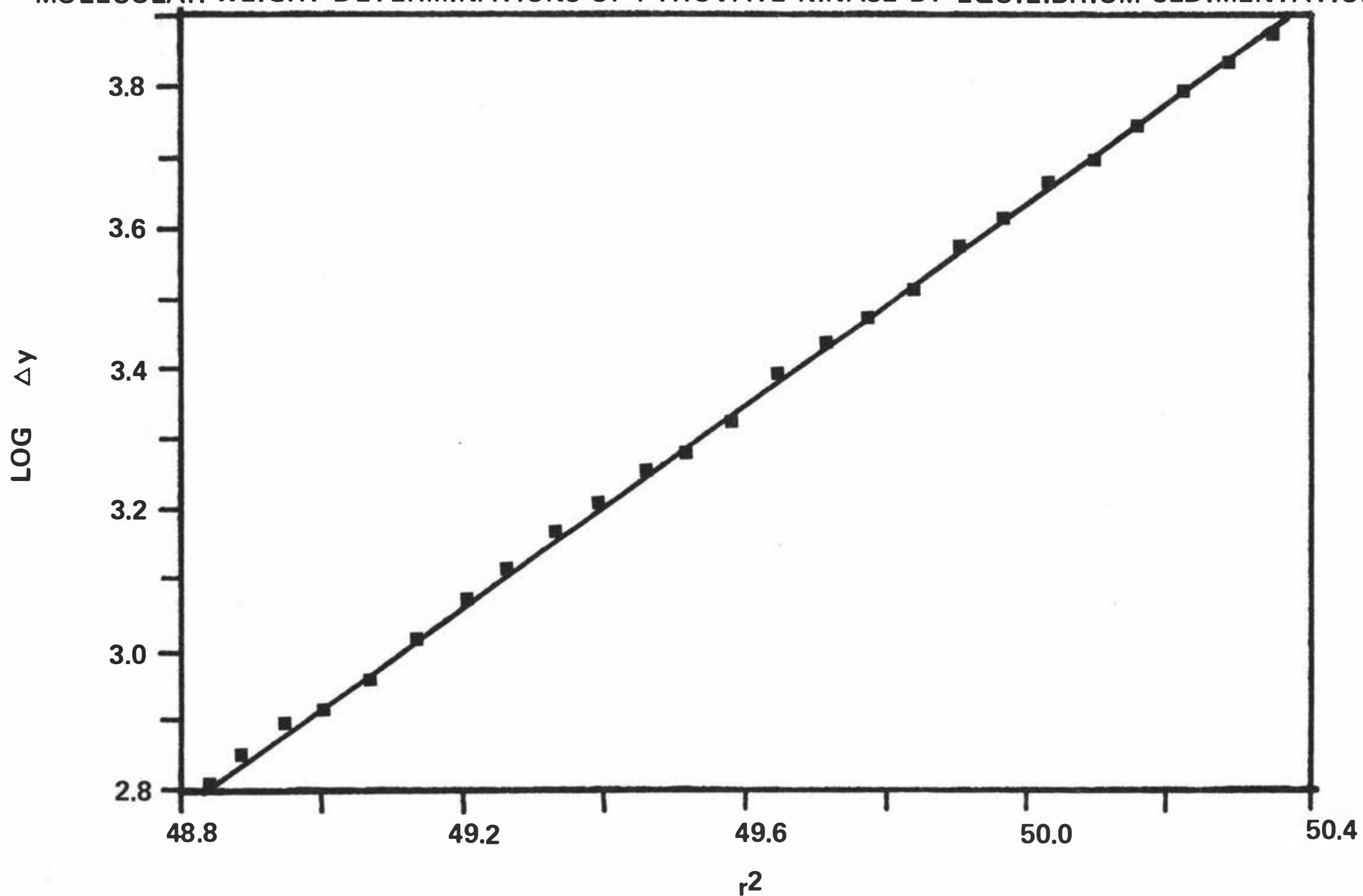
In Figure 3.4.2 a plot of log Δy versus r^2 is shown obtained from a pyruvate kinase run. The slope of this plot gives, using the formula of Chervenka, a molecular weight for pyruvate kinase of 235,140. The linearity of the log Δy versus r^2 plot is further indication of the purity of the pyruvate kinase sample.

Thus from equilibrium sedimentation studies of the purified S. lactis pyruvate kinase the molecular weight of the native protein is 235,000. The subunit molecular weight, as determined by SDS polyacrylamide disc gel electrophoresis is 60,750. Therefore these two results suggest that the pyruvate kinase from S. lactis C_{10} exists as a tetrameric enzyme of subunit molecular weight of 60,750. Similar tetrameric structures for pyruvate kinases from other sources have been reported.

The FDP-activated pyruvate kinase from E. coli (Waygood and Sanwal, 1974) has a molecular weight of 240,000 and a subunit molecular weight of 60,000. The molecular weights of the pyruvate kinase from rabbit muscle (Steinmetz and Deal, 1966) and rat liver (Tanaka et al., 1967) are similar to the E. coli enzyme. The yeast pyruvate kinase also exists as a tetrameric structure. Kuczenski and Suelter (1970) showed that the Saccharomyces cerevisiae pyruvate kinase had a molecular weight of 167,000 and a subunit

Figure 3.4.2

MOLECULAR WEIGHT DETERMINATIONS OF PYRUVATE KINASE BY EQUILIBRIUM SEDIMENTATION



A plot of log Δy versus r^2 is shown obtained from an Analytical Ultracentrifuge run on the purified pyruvate kinase sample (Specific activity 155 units/mg; 1.35 mg protein/ cm^3). Sample was in 25 mM phosphate buffer + 0.05% 2–ME pH 7.5. The molecular weight calculated (using the value of the slope from the plot) from the formula (Chervenka, 1970) was 235, 140.

molecular weight of 42,000. Bormann et al. (1974) reported for the yeast, *Saccharomyces carlsbergensis*, molecular weights of 190,000 and 48,000 for the native protein and the subunit of pyruvate kinase.

The pyruvate kinase-II from *S. faecalis* (Wittenberger et al., 1973), which is activated by FDP, was reported in a preliminary communication to have a molecular weight of 100,000. This is apparently a much smaller enzyme than the FDP-activated pyruvate kinase from *S. lactis* C_{10} (molecular weight of 235,000).

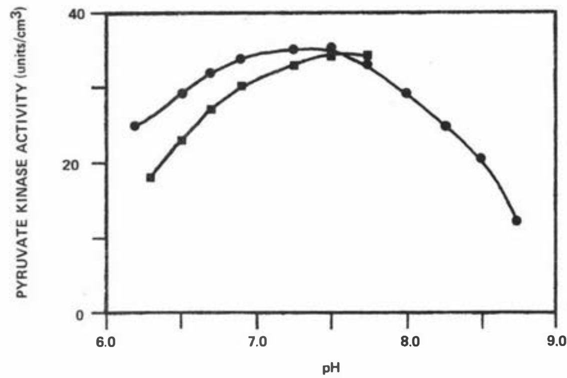
3.4.3 Effect of pH and Buffer Components on Pyruvate Kinase Activity

The activity of *S. lactis* C_{10} pyruvate kinase over a pH range in 80 mM triethanolamine/HCl, 80 mM tris/maleate, 80 mM imidazole/HCl and 80 mM phosphate buffer was investigated under otherwise standard assay conditions.

The optimum pH in triethanolamine/HCl buffer was 7.5 (Figure 3.4.3a). The activity in imidazole/HCl buffer was slightly less than in triethanolamine/HCl buffer particularly at pH values on the acid side of the pH optimum. The pyruvate kinases from *B. licheniformis* (Tuominen and Bernlohr, 1971b), *E. coli* (Maeba and Sanwal, 1968) and *S. lactis* ML_3 (Collins and Thomas, 1974) are reported to have a broad pH optimum in the region of 7.0 to 7.5, similar to that obtained for *S. lactis* C_{10}. The mammalian L type enzyme (Bailey et al., 1968) also has a similar pH optimum of 7.0 to 7.5, wherease the yeast enzyme (Hunsley and Suelter, 1969) has a pH optimum of 6.2.

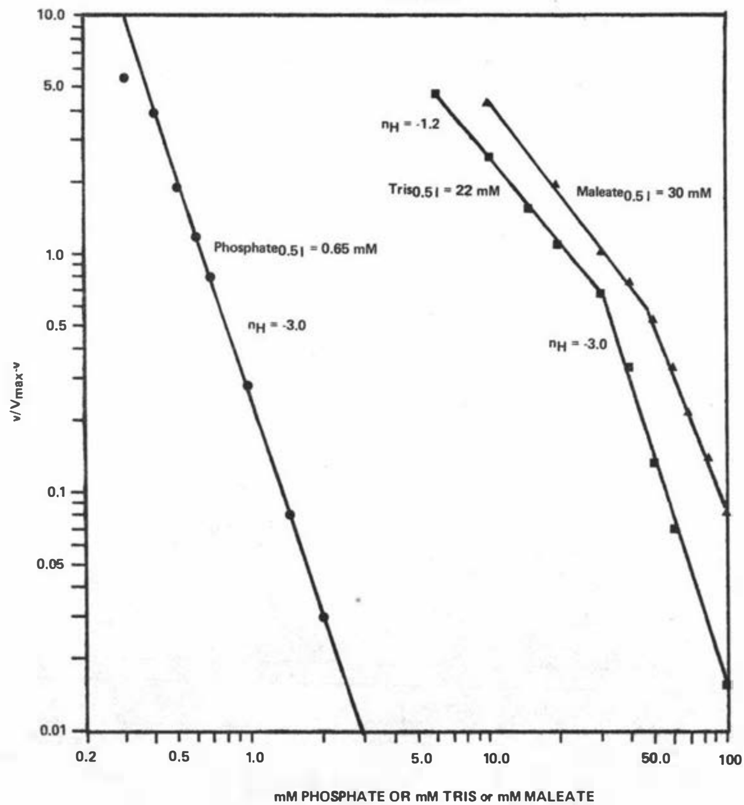
In 80 mM tris/maleate buffer the pyruvate kinase activity was greatly inhibited so the separate effects of maleic acid and tris on activity were investigated. Maleic acid or tris was added in different concentrations to the standard assay system (in triethanolamine/HCl buffer). Since inhibition by both compounds was a sigmoidal function of concentration, results are shown in Figure 3.4.3b as Hill plots. The Tris $_{0.5I}$ value (the concentration of tris required to give 50% inhibition of activity) is 22 mM and maleic acid $_{0.5I}$ is only slightly higher at 30 mM. Tris and maleic acid both give biphasic Hill plots. At higher concentrations the Hill interaction coefficient (n_H) of -3.0 indicates that both inhibitors are acting in a co-operative manner. The pyruvate kinases

Figure 3.4.3a EFFECT OF pH ON PYRUVATE KINASE ACTIVITY



The effect of pH on pyruvate kinase activity is shown in triethanolamine/HC1 buffer (●) and in imidazole/HCl buffer (■). The reaction mixture contained (in a total volume of 3 cm^3): 80 mM buffer; 1 mM PEP: 3.3 mM ADP: 1 mM FDP; 3.3 mM $MgCl_2$; 13.3 mM KCl and 0.1 cm^3 of diluted enzyme. The pHs of the two buffers used in the reaction mixture were varied as shown in the Figure.

Figure 3.4.3b HILL PLOTS SHOWING TRIS, MALEATE AND PHOSPHATE INHIBITION



The Hill plots (log v/V_{max}-v versus log inhibitor concentration) are shown for the inhibitors: Tris (■); Maleate (▲) and Phosphate (●). The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 1 mM PEP; 3.3 mM ADP: 1 mM FDP; 3.3 mM $MgCl_2$; 13.3 mM KCl and 0.1 cm^3 of diluted enzyme. The inhibitor concentrations were varied as shown in the Figure. The inhibitors were adjusted to pH 7.5 with the triethanolamine/HCl buffer components.

of B. licheniformis (Tuominen and Bernlohr, 1971b) and of rabbit muscle (Betts and Evans, 1968) have been reported to be slightly inhibited by tris but to a much lesser extent than found for the S. lactis C_{10} pyruvate kinase.

Pyruvate kinase activity was not detectable in 80 mM phosphate buffer. Inhibition by phosphate (KH_2PO_4) was studied by varying the phosphate concentration present in the standard assay system. The Hill plot of phosphate inhibition, in Figure 3.4.3b, shows that phosphate acts as a co-operative inhibitor (n_H = -3.0) over the whole range of phosphate concentrations tried and the concentration of phosphate giving 50% inhibition of activity is 0.65 mM, a value significantly lower than for tris or maleate. Inorganic phosphate was found to be a potent inhibitor of pyruvate kinase from S. lactis ML_3 (Collins and Thomas, 1974) and from B. licheniformis (Tuominen and Bernlohr, 1971b). On the other hand Flory et al. (1974) showed that the rat liver pyruvate kinase type L was not inhibited by 50 mM phosphate. Koster and Hulsmann (1970) studied the L-type pyruvate kinase from rat liver and Staal et al. (1971) studied the human erythrocyte enzyme. Both groups of workers found that inorganic phosphate and phosphorylated hexoses were allosteric activators of the two enzymes. This activation effect of inorganic phosphate on two mammalian pyruvate kinases (L-type) is in marked contrast to the inhibition of the S. lactis pyruvate kinase by phosphate.

All further properties of S. lactis C_{10} pyruvate kinase were studied in triethanolamine/HCl buffer pH 7.5, unless otherwise mentioned. Since the inhibition effect of phosphate could be of possible physiological significance it was further investigated as described in a later section.

3.4.4 FDP-Activation and the Effect of PEP, ADP and Mg^{++}/K^+ Concentrations on FDP-Activation.

One of the reasons for studying the S. lactis C_{10} pyruvate kinase was to investigate the FDP requirement of the enzyme and compare it to the FDP requirement of the L(+)-LDH from S. lactis C_{10} studied in Section 2 of this thesis. Therefore the FDP-activation of the pyruvate kinase was studied under a variety of conditions. The conditions included different PEP, ADP and K^+/Mg^{++} concentrations. That these factors can markedly influence the FDP-activation is evident from the literature.

For example, recent studies on the pyruvate kinase (L-type) from yeast (Johannes and Hess, 1973), erythrocytes (Garreau and Buc-Temkine, 1972) and rat liver (Van Berkel _et al_., 1974) have shown that the affinity of the enzyme for FDP is altered by an increase in the PEP concentration. Van Berkel _et al_. (1974) studied the rat liver pyruvate kinase at different PEP concentrations and at physiological concentrations of MgATP and alanine and concluded that FDP is the only effective activator _in vivo_. This conclusion was in direct contrast to that of Koster _et al_. (1972) who concluded that pyruvate kinase activity was not regulated _in vivo_ by FDP. This latter work was done at high PEP concentration and in the absence of MgATP and alanine. Therefore the importance of studying the kinetic parameters under a wide range of conditions is evident.

Throughout the work on _S. lactis_ C_{10} pyruvate kinase reported in this section the tetrasodium salt of FDP has been used. The importance of using this salt in preference to the tetracyclohexammonium salt is discussed fully in Appendix 3.4.1 at the end of the thesis.

3.4.4.1 The effect of PEP concentrations on FDP-activation

FDP concentration was varied at four different PEP concentrations under otherwise standard assay conditions (80 mM triethanolamine/HCl buffer pH 7.5, 3.3 mM ADP, 13.3 mM KCl and 3.3 mM $MgCl_2$). Figure 3.4.4.1a shows that the pyruvate kinase activity was a sigmoidal function of FDP concentration at the four PEP concentrations. Hill plots of the same data are shown in Figure 3.4.4.1b. The Hill interaction coefficient (n_H = 2.5 to 2.6) is not altered significantly by varying the PEP concentration. However decreasing the PEP concentration from 1.0 to 0.2 mM increases the FDP $_{0.5V}$ value from 0.16 to 0.29 mM. Pyruvate kinase had an absolute requirement for FDP for activity at the four PEP concentrations used.

3.4.4.2 The effect of ADP and Mg^{++}/K^{+} concentrations on FDP-activation

FDP was varied at four different ADP concentrations under otherwise standard assay conditions (13.3 mM KCl, 3.3 mM $MgCl_2$ and 1 mM PEP). The FDP concentration was also varied at three different ADP concentrations at an increased cation concentration, i.e. using 80 mM KCl rather than 13.3 mM and 8 mM $MgCl_2$ rather than 3.3 mM. The data were plotted as Hill plots and the results obtained from these are summarised in Table 3.4.4.2.

Figure 3.4.4.1: a and b
EFFECT OF PEP CONCENTRATION ON FDP ACTIVATION

Figure a v versus mM FDP

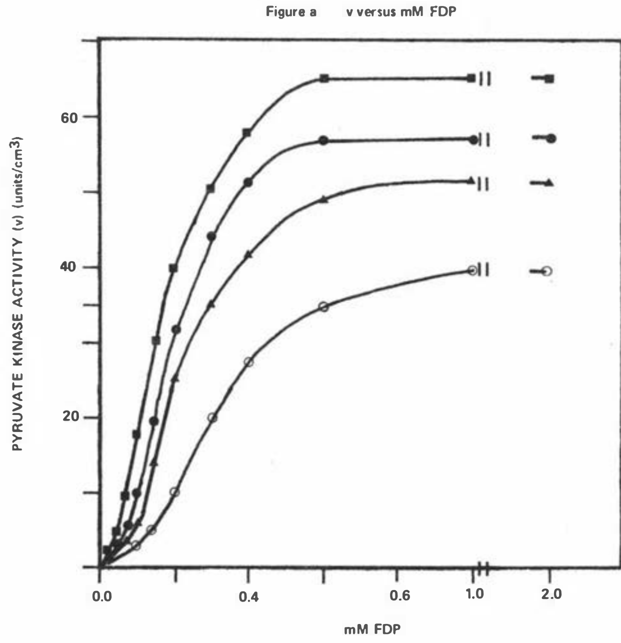
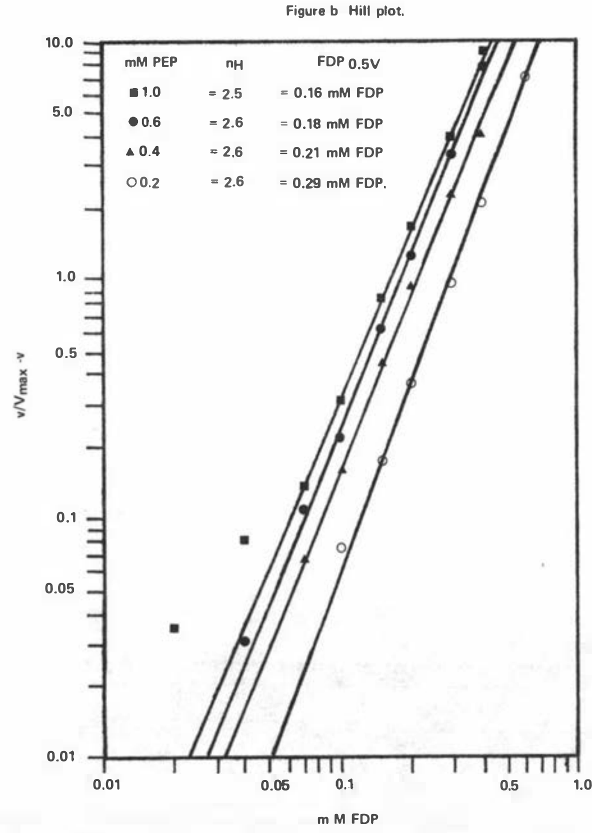


Figure b Hill plot.



The relationship between the pyruvate kinase activity (v, units/cm^3) of S. lactis C_{10} and FDP concentration (mM FDP), at four different concentrations of PEP, is shown in Figure a. Figure b is a Hill plot (log v/V $_{max}$ -v versus log FDP) of the data shown in Figure a. The V_{max} values used in the Hill plots were the respective activities at 1 mM FDP. The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanol-amine/HCl buffer pH 7.5; 3.3 mM ADP; 3.3 mM $MgCl_2$; 13.3 mM KCl and 0.1 cm^3 of diluted enzyme. The FDP concentrations were varied as shown in the Figures at four different PEP concentrations. The four different PEP concentrations are: ■, 1 mM PEP; ●, 0.6 mM PEP: ▲, 0.4 mM PEP; ○, 0.2 mM PEP.

Table 3.4.4.2

The effect of ADP and Mg^{++}/K^{+} concentrations on FDP activation

The FDP concentration was varied at four different ADP concentrations at otherwise standard assay conditions: 80 mM triethanolamine/HCl buffer pH 7.5; 13.3 mM KCl/3.3 mM $MgCl_2$; 1 mM PEP and 0.1 cm^3 diluted enzyme. The FDP concentration was also varied at three different ADP concentrations in the presence of an increased cation concentration of 80 mM KCl and 8 mM $MgCl_2$. The data were plotted as Hill plots and the results obtained from these are summarised below. The V_{max} values used in the Hill plots were obtained from the y intercepts of double reciprocal plots.

ADP concentration (mM)	V_{max} (units/cm^3)	Hill interaction coefficient (n_H)	$FDP_{0.5V}$ (mM)
		At 13.3 mM KCl/3.3 mM $MgCl_2$	
6.67	58.0	1.90	0.30
3.33	85.0	1.95	0.16
1.00	56.0	1.90	0.08
0.30	26.0	1.90	0.07
		At 80 mM KCl/8 mM $MgCl_2$	
6.67	140.0	1.85	0.13
1.00	130.0	1.85	0.12
0.30	72.0	1.85	0.10

The Hill interaction coefficient (n_H) for FDP-activation at all ADP concentrations and with the two different KCl/$MgCl_2$ concentrations had a constant value of 1.9 $\pm$ 0.05. This indicates that the co-operativity of FDP-activation is independent of ADP or KCl/$MgCl_2$ concentrations. However the n_H of 1.9 differs from the value reported in Section 3.4.4.1 where n_H was found to be 2.5 to 2.6. A possible explanation is that although the two sets of data were determined on the same enzyme sample, the data for the plots shown in Figure 3.4.4.1b were determined on freshly purified enzyme whereas the data given in Table 3.4.4.2 were determined on the same enzyme sample stored in the refrigerator for one week. The n_H for FDP-activation did not show any further significant decrease below 1.9 over longer periods of up to two months. This explanation was supported by results obtained from another pyruvate kinase preparation having a Hill interaction coefficient of 2.7 when a freshly purified sample was used, and ten days later when similar determinations were made the Hill interaction coefficient for FDP was 2.0.

At the low metal ion concentrations of 13.3 mM KCl/3.3 mM $MgCl_2$ the $FDP_{0.5V}$ values decreased with decreasing ADP concentrations (c.f. the opposite effect of PEP concentration on FDP activation). The high ADP concentration of 6.7 mM (where V_{max} = 58 units/cm^3) appeared to inhibit activity since V_{max} at 3.3 mM ADP was 85.0 units/cm^3. The use of higher K^+/Mg^{++} concentration abolished the large differences in the $FDP_{0.5V}$ values at the different ADP concentration although the same general trend was apparent. It should be noted that at the high metal ion concentration a very low level of activity was detectable in the absence of FDP.

The results obtained in the present study are in general agreement with the findings of Collins and Thomas (1974) for the pyruvate kinase of S. lactis ML_3 except for the effect of ADP on activity. Thus they found that decreasing PEP concentration increased the $FDP_{0.5V}$ value. They obtained a value for $FDP_{0.5V}$ of 0.07 mM FDP at 2 mM PEP which can be compared with the value of 0.13 mM FDP at 1 mM PEP obtained in the present study. However, they found that decreasing ADP concentration also increased the $FDP_{0.5V}$ value whereas in the present study the reverse trend was found, particularly at the low metal ion concentration. In view of these conflicting findings on the pyruvate kinases of closely related organisms the interaction of metal ion concentration and ADP concentration on pyruvate kinase activity was further investigated in later sections.

Comparison of results obtained in the present study with those obtained from the pyruvate kinases of other organisms reveals a significant difference in the dependence of FDP activation on PEP concentration. Both the FDP-activated pyruvate kinase of E. coli (Waygood and Sanwal, 1974) and the L-type pyruvate kinase of rat liver (Taylor and Bailey, 1967; Van Berkel et al., 1974) show a transition from a sigmoidal response to FDP at low PEP concentration to a hyperbolic response to FDP at high PEP concentration. The S. lactis C_{10} pyruvate kinase activity showed a sigmoidal response to FDP over the whole range of PEP concentrations used and the interaction coefficient did not change significantly over this range. Furthermore the E. coli pyruvate kinase and the mammalian enzymes (L-type) showed some activity in the absence of FDP while under comparable conditions with S. lactis C_{10} pyruvate kinase showed no activity in the absence of FDP except for very slight activity at the high metal ion concentration. The pyruvate kinase from S. lactis ML_3 studied by Collins and Thomas (1974) did show activity in the absence of FDP, but they used higher concentrations of PEP than were used in the present study and used the high metal ion concentration. In the absence of FDP the S. lactis ML_3 enzyme showed only slight activity at 2 mM PEP and increased in activity with increasing PEP concentrations, up to 8.0 mM PEP.

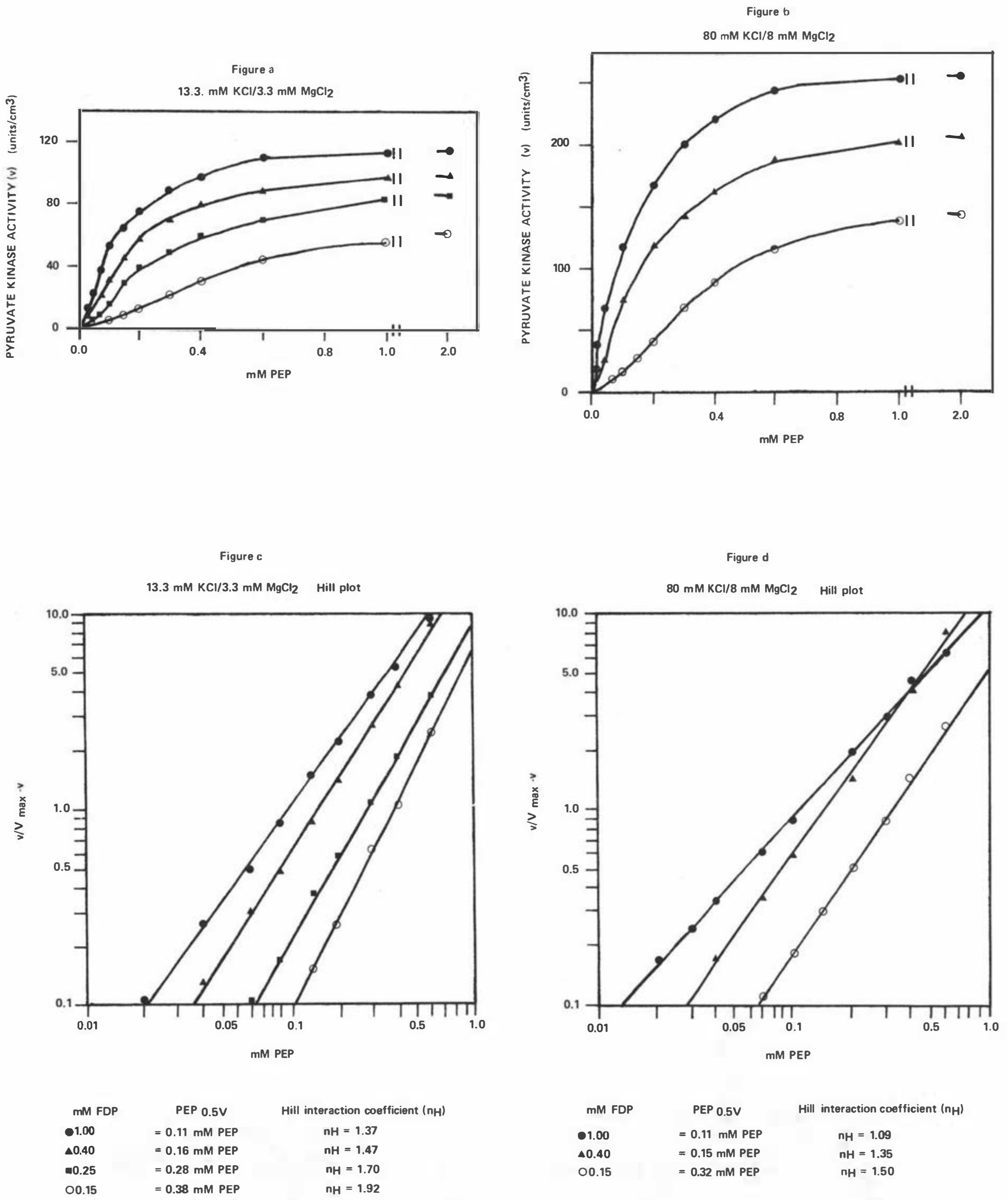
3.4.5 Response of Pyruvate Kinase to Varying PEP Concentrations

3.4.5.1 The effect of FDP and K^+/Mg^{++} concentration on the response to varying PEP concentration

Figures 3.4.5.1a, b, c and d show the relationship between pyruvate kinase activity and PEP concentration at different concentrations of FDP. The relationship was studied at two K^+/Mg^{++} concentrations. Activity showed a sigmoidal response to increasing PEP concentration although the extent of the sigmoidal character was dependent on the FDP concentration. The PEP requirement for half maximum activity (PEP $_{0.5V}$) was essentially the same at the two different K^+/Mg^{++} concentrations used. At both metal ion concentrations decreasing the FDP concentration from 1.0 to 0.15 mM increased both the PEP $_{0.5V}$ values and the n_H values and decreased the V_{max} values. The difference between the two metal ion concentrations was that the higher metal ion concentration increased the reaction

Figure 3.4.5.1:a, b, c and d

FACTORS AFFECTING PEP ACTIVATION



The relationship between the pyruvate kinase activity (v, units/cm^3) of S. lactis C_{10} and PEP concentration (mM PEP) with different concentrations of FDP and at two different metal ion concentrations, is shown in Figure a (13.3 mM KCl, 3.3 mM $MgCl_2$) and Figure b (80 mM KCl, 8 mM $MgCl_2$). Figures c and d are Hill plots (log v/V_{max} -v versus log PEP) of the data shown in Figure a and Figure b, respectively. The V_{max} values used in the Hill plots were obtained from the y intercepts of double reciprocal plots. The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 3.3 mM ADP and 0.1 cm^3 of diluted enzyme. The PEP concentrations were varied as shown in the Figures at different FDP concentrations and at two different metal ion concentrations. The different FDP concentrations are: ●, 1 mM FDP; ▲, 0.4 mM FDP; ■, 0.25 mM FDP; ○, 0.15 mM FDP.

velocity approximately 2 fold and suppressed the sigmoidal nature of the saturation curve to a certain extent.

The finding that FDP affects the nature of the PEP saturation curve, changing it to a progressively less sigmoidal form as the FDP concentration is raised appears to be a general trend for all FDP-activated pyruvate kinases. Thus the same trend has been found for S. lactis ML_3 pyruvate kinase (Collins and Thomas, 1974), E. coli pyruvate kinase (Waygood and Sanwal, 1974) and mammalian type-L pyruvate kinase (Carminatti et al., 1968; Rozengurt et al., 1969 and Irving and Williams, 1973).

Although the form of response to varying PEP is thus similar for pyruvate kinases (FDP-activated) from most organisms, the concentration of PEP required for half maximum velocity ($PEP_{0.5V}$) shows some difference from one organism to another. Results for other FDP-activated pyruvate kinases are tabulated below.

Source of pyruvate kinase	$PEP_{0.5V}$	Reference
Liver type-L		
no FDP	0.6 mM	Taylor and Bailey (1967)
Fully saturating FDP	0.06 mM	
E. coli		
no FDP	4 mM	Waygood and Sanwal (1974)
Fully saturating FDP	0.03 mM	
S. lactis ML_3		
no FDP	4 mM	Collins and Thomas (1974)
Fully saturating FDP	0.14 mM	

A very brief report on the FDP activated pyruvate kinase from S. faecalis (Wittenberger et al., 1973, available in abstract form only) gives a $PEP_{0.5V}$ value of 0.4 mM, presumably at saturating FDP.

The $PEP_{0.5V}$ value for the fully activated S. lactis C_{10} pyruvate kinase (0.11 mM) agrees fairly well with that obtained by Collins and Thomas (1974), (0.14 mM PEP). Since very little activity was detectable with the S. lactis C_{10} pyruvate kinase in the absence of FDP, a figure without FDP present was not obtained. At the lowest concentration of FDP used the $PEP_{0.5V}$ was 0.38 mM.

3.4.5.2 The effect of ADP and K^+/Mg^{++} concentration on PEP activation

The relationships between the activity of S. lactis C_{10} pyruvate kinase and PEP concentration at different concentrations of ADP, and at two different K^+/Mg^{++} concentrations, are summarised in Table 3.4.5.2.

The effect of ADP and K^+/Mg^{++} concentrations on PEP activation is similar to the effect of these compounds on FDP activation (see Section 3.4.4.2). At low metal ion concentrations, increasing ADP decreased the affinity for PEP (c.f. Collins and Thomas, 1974). Thus the PEP $_{0.5V}$ value dropped from 0.21 to 0.11 mM as the ADP concentration was decreased from 6.67 mM to 0.3 mM. The highest ADP concentration used (6.67 mM) inhibited activity at all PEP concentrations. Varying ADP had no effect on the Hill interaction coefficient for PEP binding which was 1.4 at all ADP levels.

In view of the difference between the effect of ADP on PEP binding in this work and in the study of Collins and Thomas (1974) on S. lactis ML_3 pyruvate kinase, the effect of ADP was investigated at the high metal ion concentration used by these workers.

In the presence of high metal ion concentration, again the highest ADP concentration (6.67 mM) used resulted in an increase in the PEP $_{0.5V}$ value (although it did not inhibit V_{max} as occurred at low metal ion concentrations). However over a lower ADP range (from 1.0 to 0.3 mM) the PEP $_{0.5V}$ value did increase with decreasing ADP as found by Collins and Thomas (1974). As in the presence of the low metal ion concentration, varying ADP does not affect the Hill interaction coefficient for PEP.

The enzyme did show slight sigmoidal behaviour in response to varying PEP at the low metal concentration ($n_H = 1.4$), but no sigmoidal behaviour was evident at the high metal concentration ($n_H = 1.0$). The sigmoidal nature of PEP binding was independent of the ADP concentration. In Section 3.4.5.1, the sigmoidal binding of PEP was shown to be markedly dependent on the FDP concentration and to a lesser extent on the metal ion concentration. Therefore it can be concluded that PEP will show positive co-operative binding to the pyruvate kinase of S. lactis if the FDP concentration is significantly lower than the saturating concentration and to a lesser extent, if the metal ion concentration is less than saturating. ADP concentration on the other hand does not effect the co-operativity of binding of PEP.

Table 3.4.5.2

The effect of ADP and Mg^{++}/K^{+} concentrations on PEP activation

The PEP concentration was varied at four different ADP concentrations at otherwise standard assay conditions: 80 mM triethanolamine/HCl buffer pH 7.5; 13.3 mM KCl/3.3 mM $MgCl_2$; 1 mM FDP and 0.1 cm^3 diluted enzyme. The PEP concentration was also varied at three different ADP concentrations in the presence of an increased cation concentration of 80 mM KCl and 8 mM $MgCl_2$. The data were plotted as Hill plots and the results obtained from these are summarised below. The V_{max} values used in the Hill plots were obtained from the y intercepts of double reciprocal plots.

ADP concentration (mM)	V_{max} (units/cm^3)	Hill interaction coefficient (n_H)	$PEP_{0.5V}$ (mM)
	At 13.3 mM KCl/3.3 mM $MgCl_2$		
6.67	96.0	1.4	0.21
3.33	110.0	1.4	0.14
1.00	95.0	1.4	0.11
0.30	60.0	1.4	0.11
	At 80 mM KCl/8 mM $MgCl_2$		
6.67	270.0	0.95	0.19
1.00	130.0	1.0	0.10
0.30	83.0	1.0	0.15

3.4.6 Response of Pyruvate Kinase to Varying ADP Concentration

An important consideration when studying the kinetic response of pyruvate kinase to varying ADP concentrations is whether ADP is binding as ADP Mg^-, ADP^{3-} or a combination of the two forms. Cleland (1967) has stated that the nucleoside di- and tri-phosphates always react in the form of their bivalent metal-ion complexes. This does not seem certain however, for Macfarlane and Ainsworth (1972) have concluded that free ADP alone is the nucleotide substrate of yeast pyruvate kinase. They showed that the reaction mechanism is an ordered Tri Bi mechanism (Cleland, 1963). The substrates bind in the order of PEP, ADP and Mg^{++} and direct phosphoryl transfer takes place in the quaternary complex with pyruvate being released before MgATP. Macfarlane and Ainsworth point out that such a reaction mechanism provides an understanding of the sequence of events at the active site. "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; Hamms _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 that cation 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 same workers, in studying the kinetics of the rabbit muscle pyruvate kinase (Ainsworth and Macfarlane, 1973), demonstrated a different kinetic mechanism, but, again concluded that ADP^{3-}, and not $MgADP^-$, was the nucleotide substrate. Their experimental results indicate that "... the reaction mechanism is equilibrium random-order in type and that the substrates and products are PEP, ADP, Mg^{++}, pyruvate and MgATP". On the other hand, Mildvan and Cohn (1966) also studying the rabbit muscle pyruvate kinase reaction but with Mn^{++} replacing Mg^{++} suggested that ADP binds to the enzyme in a combination of the two forms (i.e. ADP^{3-} and $MnADP^-$).

It is apparent that the form in which ADP binds is still uncertain with conflicting evidence even for pyruvate kinase from the same source. Consequently in the kinetic studies of pyruvate kinase form _S. lactis_ C_{10} the concentrations of the nucleotide will be expressed as simply ADP.

This use of the term "ADP" implies that the active species of the adenine dinucleotide is unknown and may be ADP^{3-}, $MgADP^{-}$ or a combination of the two. From the diverse and often conflicting results concerning the reaction mechanism, it is evident that only very intensive studies can establish the mechanism with any degree of certainty. Since the elucidation of the reaction mechanism was not one of the aims of the present study it was considered that the use of the term "ADP" was adequate.

3.4.6.1 The effect of FDP concentration on ADP binding to the *S. lactis* pyruvate kinase

Figure 3.4.6.1 shows the relationship between pyruvate kinase activity and ADP concentration at three different concentrations of FDP using otherwise standard assay conditions. The relationship is shown as Hill plots where log $v/V_{max}-v$ is plotted against log ADP.

The relationship between pyruvate kinase activity and ADP concentration changes from normal Michaelis-Menten kinetics to allosteric kinetics when the FDP concentration is sufficiently low; the n_H value for ADP binding is 1.0 at FDP concentrations of 1.0 and 0.25 mM, but at 0.1 mM FDP the n_H value increases to 1.5. Hence at low FDP concentrations, ADP can bind to the enzyme in a positive co-operative manner. This appears to be a distinctive property of the pyruvate kinase from *S. lactis*, as all other pyruvate kinases show normal Michaelis-Menten kinetics under all conditions, e.g. rat liver L-type (Carminatti *et al.*, 1968); *E. coli*, FDP activated enzyme (Waygood and Sanwal, 1974).

This co-operativity of ADP binding is however much weaker than the co-operativity of PEP binding at comparable FDP concentrations (c.f. Figures 3.4.5.1 and 3.4.6.1). For example at a FDP concentration of 0.25 mM the PEP binds to give a Hill interaction coefficient value of 1.7, whereas under similar conditions and at the same FDP concentration ADP binds in a non-allosteric manner as the Hill interaction coefficient is 1.0. Collins and Thomas (1974) found a similar relationship for the pyruvate kinase of *S. lactis* ML_3 in that the co-operative interaction of ADP with the enzyme was less than that of PEP at comparable levels of FDP concentrations.

As the concentration of FDP is decreased the ADP concentration required to give half maximum activity (ADP $_{0.5V}$) increases slightly from 1.3 mM (FDP = 1.0 mM) to 2.1 mM ADP (FDP = 0.1 mM), which is similar to the effect of FDP concentration on the PEP $_{0.5V}$ values (Section 3.4.5.1). ADP inhibition was evident at higher ADP concentrations at all three FDP levels. The V_{max} values used for calculating the Hill plot values were obtained by linear extrapolation of the Lineweaver-Burk plots to the 1/v axis.

3.4.6.2 The effect of PEP concentrations on ADP binding

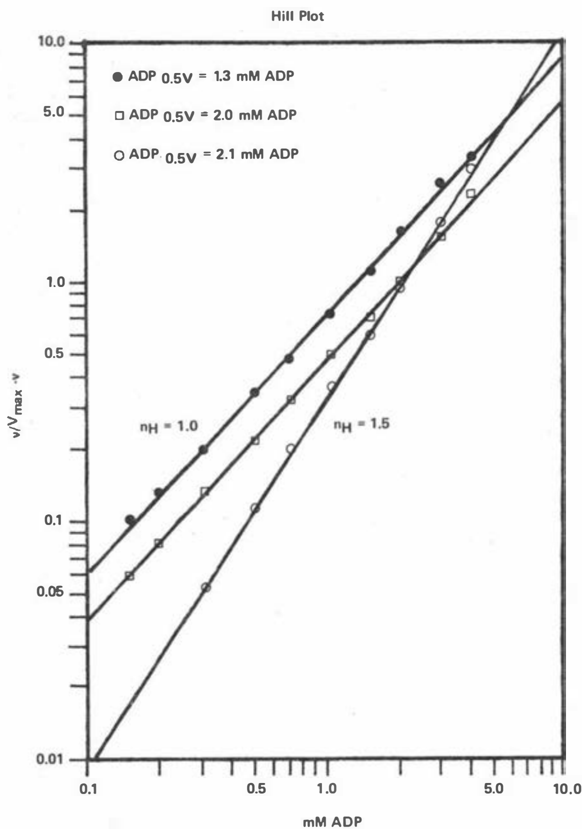
Figure 3.4.6.2a shows the relationship between the pyruvate kinase activity and ADP concentrations with five different PEP concentrations using otherwise standard assay conditions (FDP concentration for all determinations was 1.0 mM). The double reciprocal plots, at each PEP concentration, give a family of lines, intersecting on or near the x axis to give a K_M of 1.2 to 1.3 mM ADP. The secondary plot, a double reciprocal plot of the apparent V_{max}, obtained from the plots of Figure 3.4.6.2a, versus the respective PEP concentration, is shown in Figure 3.4.6.2b. The K_M for PEP obtained from this secondary plot is 0.13 mM PEP. At all concentrations of PEP used the reciprocal plots showed no indication of a sigmoidal relationship between reaction velocity and ADP concentrations. Hill plots, using the same data, all gave n_H values of 1.0 to 1.1.

Collins and Thomas (1974) found for the S. lactis ML_3 pyruvate kinase that the K_M values for PEP and ADP in the presence of 2 mM ADP were 0.17 and 1 mM, respectively; these values compare well with the values obtained for the S. lactis C_{10} pyruvate kinase (0.13 mM PEP and 1.2 to 1.3 mM ADP).

Pyruvate kinases from mammalian sources tend to have lower K_M values for ADP, e.g. 0.2 to 0.4 mM for rat liver, L-type pyruvate kinase (Carminatti et al., 1968). The pyruvate kinase from E. coli also has a lower K_M for ADP (0.24 mM) at saturating FDP.

Figure 3.4.6.1

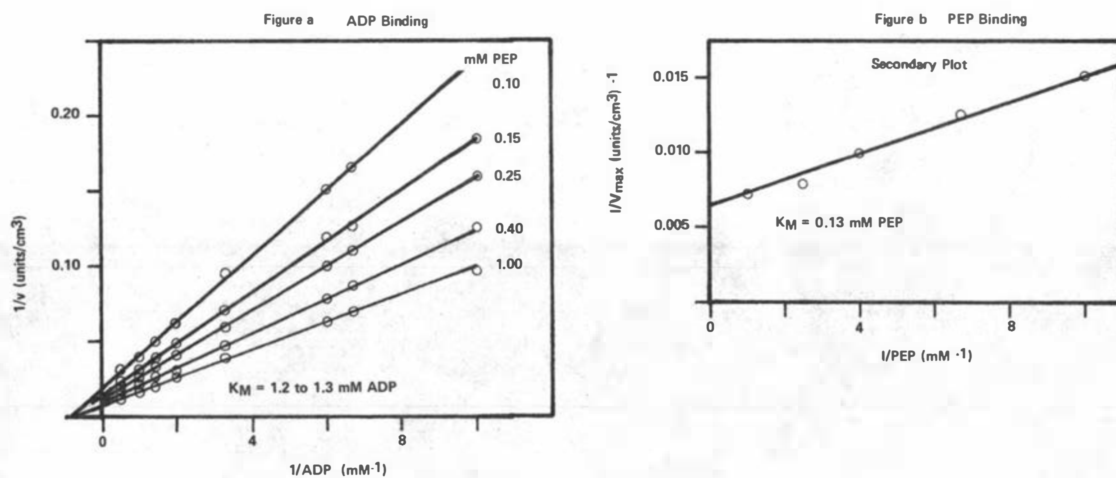
EFFECT OF FDP CONCENTRATION ON ADP ACTIVATION



The relationship between the pyruvate kinase activity (v) of S. lactis C_{10} and ADP concentration, at three different concentrations of FDP, is shown in the Figure as a Hill plot (log v/V_{max} -v versus log ADP). The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 1.0 mM PEP; 13.3 mM KCl; 3.3 mM $MgCl_2$ and 0.1 cm^3 of diluted enzyme. The ADP concentrations were varied as shown in the Figure at three different FDP concentrations. The three different FDP concentrations are: ●, 1 mM FDP; □, 0.25 mM FDP: ○, 0.1 mM FDP. The V_{max} values used for calculating the Hill plot values were obtained by linear extrapolation of the Lineweaver-Burk plots to the 1/v axis.

Figure 3.4.6.2: a and b

EFFECT OF PEP CONCENTRATION ON ADP BINDING



The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 1.0 mM FDP; 13.3 mM KCl; 3.3 mM $MgCl_2$ and 0.1 cm^3 diluted enzyme. The ADP concentrations were varied as shown in Figure a, at 5 different PEP concentrations. Figure a (Lineweaver-Burk plot) is a plot of the reciprocal of the pyruvate kinase activity (v, units/cm^3) versus the reciprocal of ADP concentration. Figure b is a secondary plot of the data in Figure a where the reciprocal of the V_{max} values (determined from Figure a) are plotted against the reciprocal of the 5 respective PEP concentrations.

3.4.7 The Effect of Guanosine 5^1-Diphosphate (GDP) on S. lactis C_{10} Pyruvate Kinase Activity

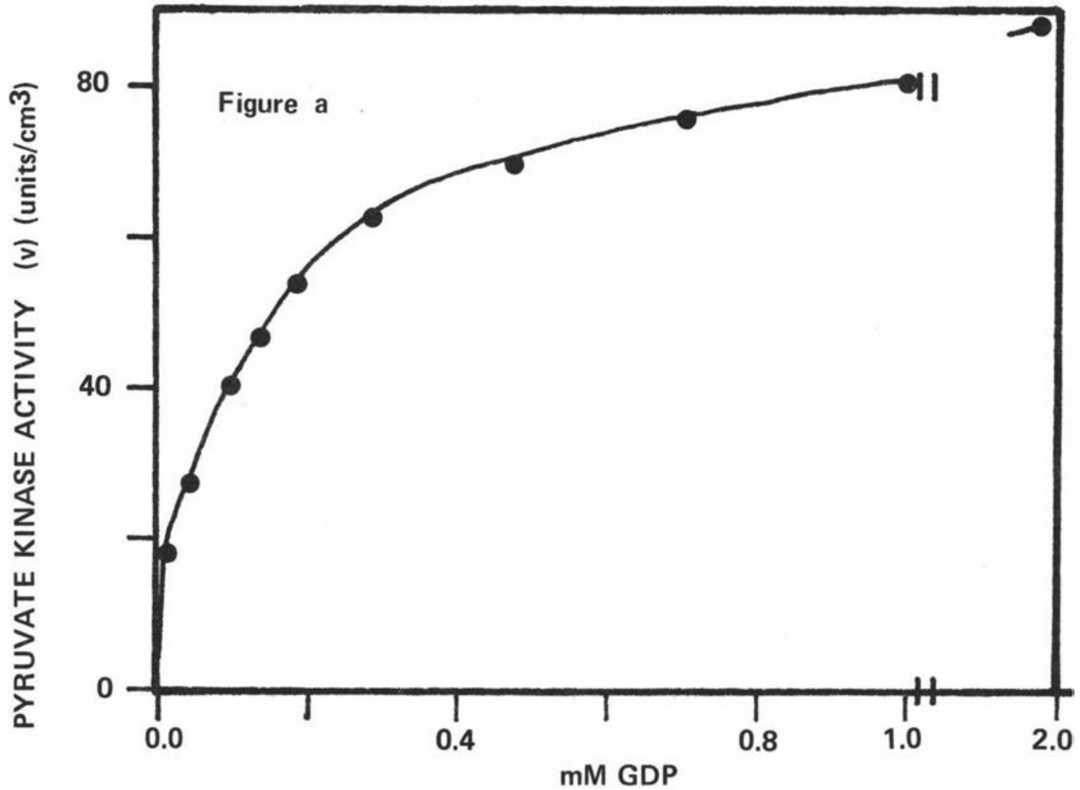
Waygood and Sanwal (1974) in studying the E. coli pyruvate kinase, activated by FDP, found that the nucleotides, ADP, GDP, UDP, IDP and CDP can act as phosphate acceptors in the reaction, and of these GDP had the lowest K_M value (0.05 mM) c.f. ADP with a K_M of 0.24 mM. Though GDP was the best phosphate acceptor, it did show substrate inhibition at levels higher than 0.04 to 0.05 mM. IDP and CDP also showed substrate inhibition but only at concentrations higher than 2 mM. The remaining nucleotides, ADP and UDP, gave no inhibition up to concentrations of 10 mM. In view of these findings for the E. coli enzyme it was decided to investigate the activity of the S. lactis C_{10} pyruvate kinase when GDP was used as the phosphate acceptor of the reaction.

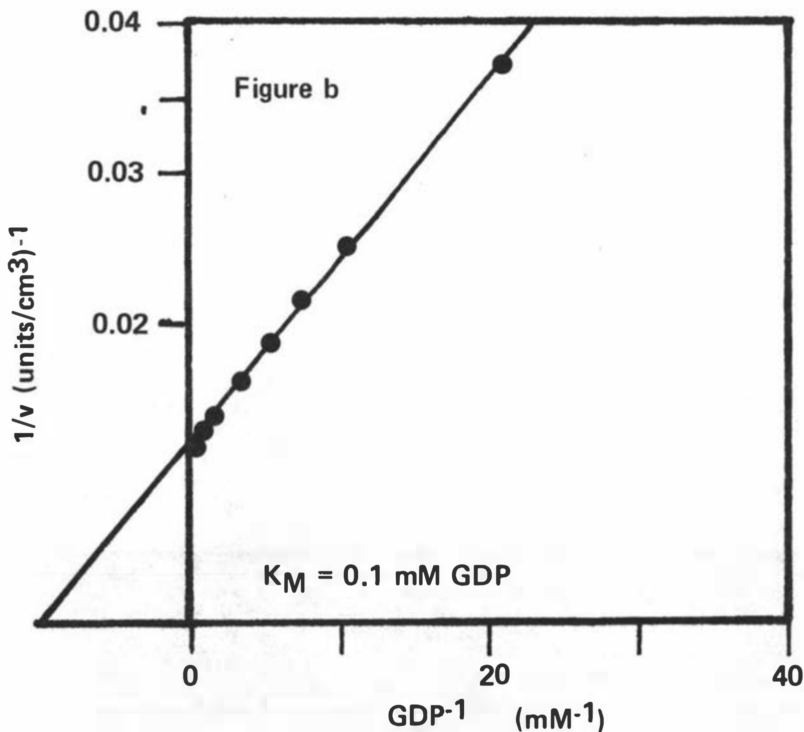
The GDP concentration was varied in the standard assay system (1 mM PEP, 1.0 mM FDP, 80 mM triethanolamine/HCl buffer pH 7.5 and 13.3 mM KCl/3.33 mM $MgCl_2$). Some activity was obtained in the absence of FDP but the activity with 1.0 mM GDP in the absence of FDP was only 5% of that obtained when 1 mM FDP was present. In Figure 3.4.7a and b the relationship between pyruvate kinase activity and GDP concentration is shown. From the Lineweaver-Burk plot the K_M for GDP is 0.1 mM, a value at least ten times lower than the K_M for ADP of 1.2 to 1.3 mM obtained in the same conditions. The V_{max} obtained with GDP as the nucleotide substrate was 83.0 units/cm^3 and is similar to the V_{max} value obtained from the double reciprocal plots when ADP is used as the nucleotide substrate under the same conditions (V_{max} = 90.0 units/cm^3). The difference is that with GDP as the nucleotide substrate, the V_{max} is slightly higher if estimated from a v versus s plot whereas with ADP the V_{max} obtained from a v versus s plot is about 25% lower than the extrapolated value obtained from a double reciprocal plot. Hence the E. coli and the S. lactis C_{10} pyruvate kinase differ in that the E. coli enzyme is inhibited by high GDP, and not ADP whereas the S. lactis C_{10} enzyme is inhibited by high ADP and not by GDP. Pyruvate kinases from both sources have a higher affinity for GDP than for ADP.

Clearly there is sufficient difference between GDP and ADP as the nucleotide substrate to warrant further investigation. Other kinetic properties, studied extensively with ADP, may be different if GDP is used as the nucleotide substrate.

Figure 3.4.7: a and b

EFFECT OF GDP ON PYRUVATE KINASE ACTIVITY





The relationship between the pyruvate kinase activity (v, units/cm^3) of S. lactis C_{10} and GDP concentration (mM GDP) is shown in Figure a. Figure b is a double reciprocal plot (lineweaver-Burk plot) (1/v versus 1/mM GDP) of the data shown in Figure a. The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 1 mM PEP; 1 mM FDP; 13.3 mM KCl; 3.3 mM $MgCl_2$ and 0.1 cm^3 of diluted enzyme. The GDP concentrations were varied as shown in the Figures.

3.4.8 The Effect of Monovalent Cations on Pyruvate Kinase Activity

Pyruvate kinase is one of the best known examples of enzymes which specifically need a monovalent cation for full activity (Evans and Sorger, 1966). However recent studies on the properties of the pyruvate kinase isolated from different sources have shown that this monovalent cation requirement is somewhat variable. Although K^+ is probably the main monovalent cation of physiological significance and Na^+ has usually only very weak activating properties (Kachmar and Boyer, 1953), the yeast pyruvate kinase was significantly activated by Na^+ in combination with FDP (Hunsley and Suelter, 1969). The pyruvate kinases from E. coli (Maeba and Sanwal, 1968) and Acetobacter xylinum (Benziman, 1969) are completely unaffected by K^+ or NH_4^+, but require Mg^{++} for activity. Hence considerable differences exist between the pyruvate kinases from different sources in respect to the monovalent metal ion requirement for enzyme activity.

3.4.8.1 The effect of monovalent cations on the S. lactis C_{10} pyruvate kinase activity

NH_4Cl, NaCl, LiCl and KCl were varied at different concentrations under otherwise standard assay conditions of 80 mM triethanolamine/HCl buffer pH 7.5, 1.0 mM FDP, 1.0 mM PEP, 3.3 mM ADP and 3.3 mM $MgCl_2$. The results are shown in Figure 3.4.8.1a and b. In Figure a the activity at unsaturating levels of NH_4Cl is slightly higher than the activity with the same KCl concentrations and V_{max} is similar for both K^+ and NH_4^+. In contrast, Figure b shows that Na^+ and Li^+ have only a weakly activating effect on the enzyme's activity, as the maximum activity obtained with Na^+ or Li^+ is less than 5% of that obtained with NH_4^+ or K^+ ions. Figure 3.4.8.1d represents the Hill plots of the data in Figure a where log $v/V_{max}-v$ is plotted against log K^+ and log NH_4^+. The two Hill plots for K^+ and NH_4^+ activation of pyruvate kinase activity indicate that there is a co-operative binding of the activating monovalent cations, K^+ and NH_4^+, as the Hill interaction coefficient (n_H) for both cations is 1.55 at least at higher concentrations. The affinity of the enzyme for the two activating monovalent cations is similar with the enzyme showing only slightly higher affinity for NH_4^+ ($NH_4^+{}_{0.5V}$ = 7.0 mM; $K^+{}_{0.5V}$ = 9.4 mM). At low concentrations of K^+ or NH_4^+, the n_H value drops to less than

unity (n_H = 0.68). At these low levels, the Na^+ introduced in the assay as the Na salts of PEP, ADP and FDP may be competing with K^+ or NH_4^+ ions.

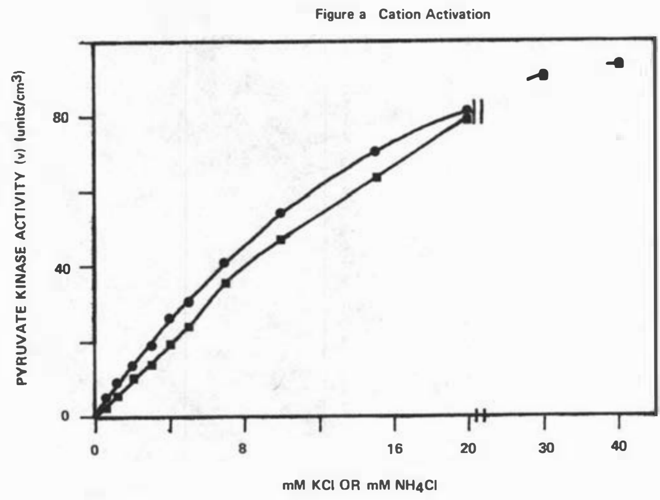
The inhibitory effect of Na^+ concentration is shown in Figure 3.4.8.1c, where all assays were carried out using standard conditions (13.33 mM KCl/ 3.33 mM $MgCl_2$), except that different concentrations of Na^+ were present. At concentrations of Na^+ greater than 30 mM (not including endogenous Na^+) inhibition of pyruvate kinase activity occurred, with 50% inhibition of activity occurring at 60 mM Na^+.

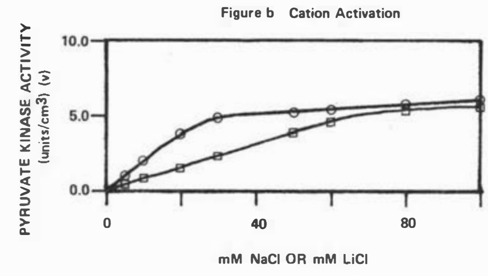
Therefore, like rabbit muscle pyruvate kinase (Kachmar and Boyer, 1953) and many other pyruvate kinase preparations (Boyer, 1962), the S. lactis enzyme requires an activating monovalent cation (either K^+ or NH_4^+) for activity. Like the muscle enzyme (Kachmar and Boyer, 1953) the S. lactis enzyme is only very weakly activated by Na^+.

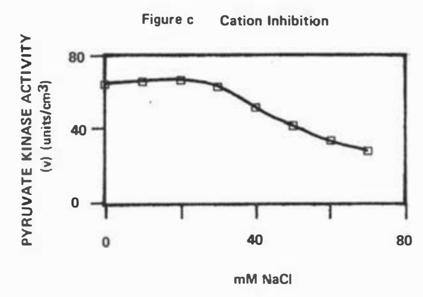
The sigmoidal response of the S. lactis pyruvate kinase to K^+ or NH_4^+ at saturating FDP levels appears to be a distinctive property of this enzyme. Hunsley and Suelter (1969) working on yeast pyruvate kinase and Jimenez de Asua et al. (1970) on the rat liver enzyme, found that although the monovalent cation activated the enzyme in a co-operative manner in the absence of FDP, at saturating levels of FDP the response was hyperbolic.

Figure 3.4.8.1: a, b, c and d

EFFECT OF MONOVALENT CATIONS ON ACTIVITY







The relationship between the pyruvate kinase activity (v, units/cm^3) of S. lactis C_{10} and activating concentrations of monovalent cations is shown in Figures a and b. In Figure a the monovalent cation salts are: ●, NH_4Cl; ■, KCl. In Figure b the monovalent cation salts are: ○, LiCl ; □, NaCl. The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 1 mM PEP; 3.3 mM ADP; 1 mM FDP; 3.3 mM $MgCl_2$ and 0.1 cm^3 of diluted enzyme. The monovalent cation concentrations were varied as shown in Figures a and b.
The relationship between the pyruvate kinase activity and inhibiting concentrations of the monovalent cation, NaCl, is shown in Figure c. The reaction mixture was the same as used in Figures a and b, except 13.3 mM KCl was present as well, and the NaCl concentration was varied as shown in Figure c.

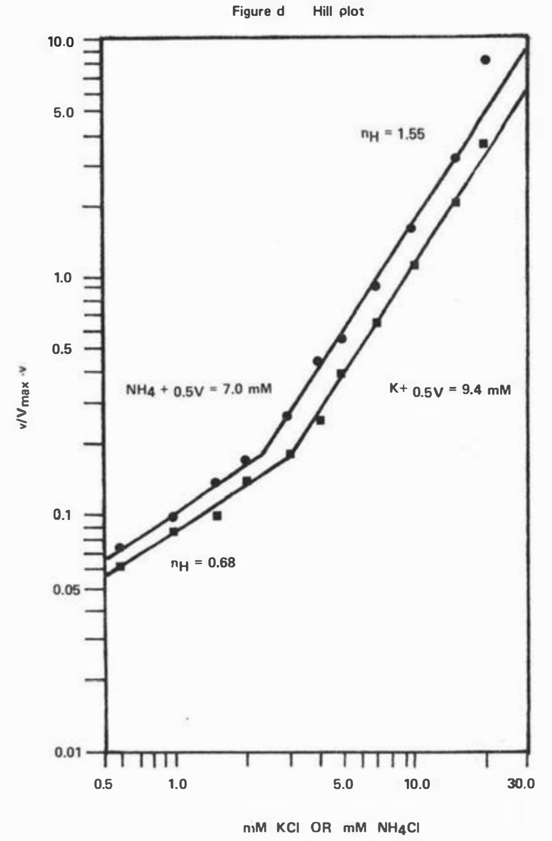


Figure d is a Hill plot (log $v/V_{max}{\cdot}v$ versus log monovalent cation) of the data shown in Figure a. KCl (■) and NH_4Cl (●) are the varying divalent cation salts. The V_{max} values used for calculating the Hill plot values were obtained by linear extrapolation of the Lineweaver-Burk plots to the 1/v axis.

3.4.9 The Effect of Divalent Cations on the Pyruvate Kinase Activity

All of the pyruvate kinases studied have an absolute requirement for a divalent cation (which will be referred to generally as M^{++} in cases where the particular cation is not specified). However the response of the pyruvate kinase to divalent cations is different depending on the source of the enzyme. The specificity of the divalent cation requirement and the relative effectiveness of different divalent cations differs considerably among the pyruvate kinases which have been studied. The enzyme may display sigmoidal or Michaelis-Menten kinetics with respect to varying concentrations of the divalent cations. Substrates and modifiers have differential effects on the divalent cation activation of the enzyme depending on the source of the enzyme.

The function of the divalent cation in relation to the nucleotide binding and the reaction mechanism of pyruvate kinase was discussed in Section 3.4.6. For reasons discussed in that section the divalent cation concentration will be expressed as a total concentration of M^{++} even though it may be binding to the enzyme as M ADP^- or M^{++} or a combination of the two.

3.4.9.1 The effect of divalent cations on the *S. lactis* pyruvate kinase activity

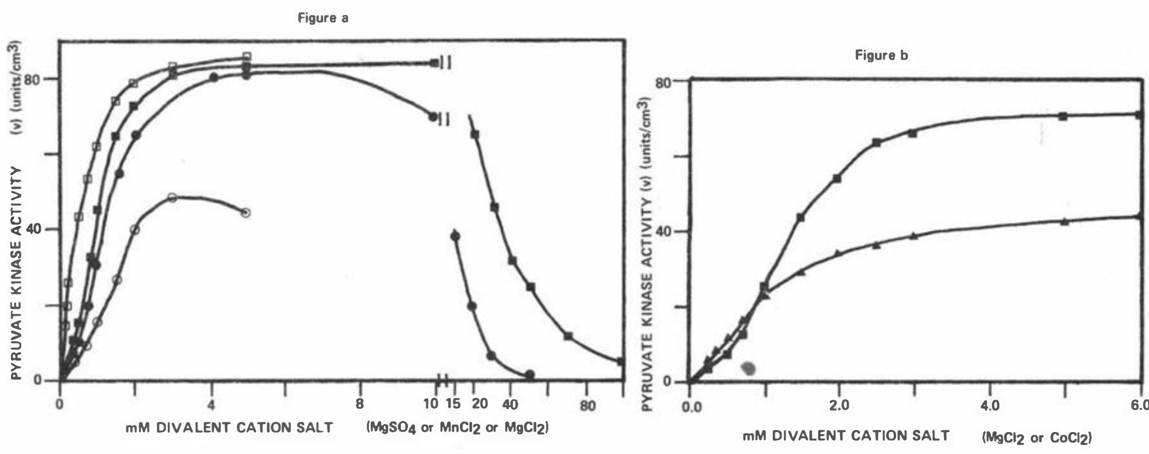
In Figure 3.4.9.1a the relationship between pyruvate kinase activity and divalent cation concentration is shown as a v versus M^{++} plot. $MgCl_2$ at all concentrations activates the enzyme to a slightly greater extent than equimolar concentrations of $MgSO_4$. More of a difference between the two salts of Mg^{++} is noticed at the high concentration (greater than 10 mM) where inhibition occurs at significantly lower concentrations of $MgSO_4$ than with $MgCl_2$. $MnCl_2$ is different from $MgCl_2$ and $MgSO_4$ in two respects. When a comparison is made between Mn^{++} and Mg^{++} with saturating FDP present (1 mM), then Mn^{++} maximally activates the enzyme at lower concentrations than does Mg^{++}. The second distinctive difference between Mg^{++} and Mn^{++} activation of the enzyme is that neither $MgCl_2$ or $MgSO_4$, with the conditions used in the assay system, could activate the enzyme in the absence of FDP, whereas $MnCl_2$ (shown in Figure 3.4.9.1a) can activate the enzyme in the absence of FDP. The maximum velocity achieved with saturating concentrations of Mn^{++}, in the absence of FDP, is about 60% of the maximum velocity achieved if FDP is present with saturating concentrations of $MnCl_2$, $MgSO_4$ or $MgCl_2$.

Co^{++} (as $CoCl_2$) also activates the enzyme (Figure 3.4.9.1b) but the V_{max} attained is significantly less than that obtained with $MgCl_2$ (note that a different enzyme preparation was used, hence the V_{max} value obtained with $MgCl_2$ is not comparable to that in Figure 3.4.9.1a). No activity was obtained with Co^{++} as the divalent ion when FDP was omitted from the reaction mixture.

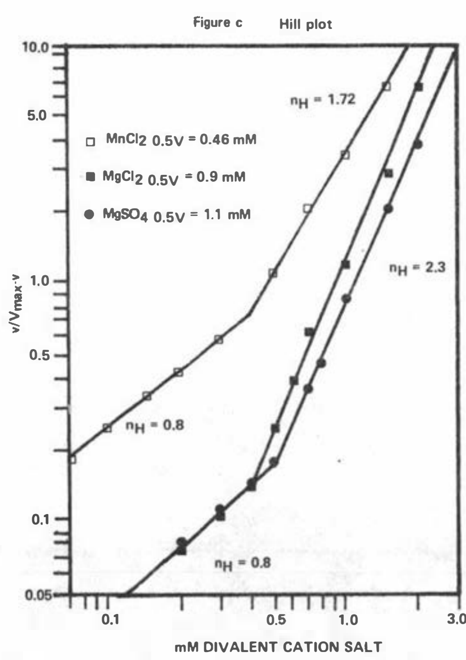
The data of Figure 3.4.9.1a are expressed as Hill plots in Figure 3.4.9.1c. Hill plots for both $MgSO_4$ and $MgCl_2$ give Hill interaction coefficient values (n_H) of 2.3 at Mg^{++} concentrations up to 2 mM, indicating that the binding of the divalent cation to the enzyme is co-operative when PEP, ADP and FDP concentrations are saturating. The divalent cation requirement to give half maximum activity ($M^{++}{}_{0.5V}$) for $MgCl_2$ and $MgSO_4$ is very similar with a range of 0.9 to 1.3 mM. With $MnCl_2$, the Hill plot was biphasic, but more markedly so than for $MgCl_2$ of $MgSO_4$. At the higher $MnCl_2$ concentrations Mn^{++} showed less co-operative interaction than Mg^{++} as n_H value was 1.72. The $Mn^{++}{}_{0.5V}$ value (0.46 mM) is less than half that for Mg^{++}. Data obtained for $MnCl_2$ in the absence of FDP do not give a linear Hill plot so the data is not shown. The data of 3.4.9.1b, expressed as Hill plots (Figure 3.4.9.1d) indicate weaker co-operative interaction for $CoCl_2$ (n_H = 1.62) but a similar $M^{++}{}_{0.5V}$ value when compared to $MgCl_2$ and $MgSO_4$. The n_H value with Co^{++} is similar to that of Mn^{++}.

With $MgCl_2$ and $MgSO_4$ at concentrations greater than 10 mM, enzyme activity was inhibited (Figure 3.4.9.1d). The high negative slope of the Hill plots shows that $MgCl_2$ and $MgSO_4$ are inhibiting in a co-operative manner, at higher divalent cation concentrations. $MgSO_4$ has a higher negative Hill interaction coefficient value of 3.94 (n_H = -) and a lower concentration to give half maximum inhibition ($MgSO_4{}_{0.5I}$ = 16 mM) compared to $MgCl_2$ with n_H = -2.50 and $MgCl_2{}_{0.5I}$ = 36 mM. The stronger inhibitory effect of sulphate is not surprising as Collins and Thomas (1974) reported that the S. lactis ML_3 was inhibited by SO_4^{2-}. Therefore with $MgSO_4$ at high concentrations there is a two-fold inhibitory effect, one being inhibition by elevated concentrations of Mg^{++} and the other inhibition effect being due to SO_4^{2-}. Sulphate inhibition did not appear to be significant at Mg^{++} concentrations where activation of the enzyme was incomplete as $MgCl_2$ and $MgSO_4$ activation of the enzyme was very similar as shown by their respective Hill plots in Figure 3.4.9.1c.

Figure 3.4.9.1: a, b, c and d
EFFECT OF DIVALENT CATIONS ON ACTIVITY



The relationship between the pyruvate kinase activity (v, units/cm^3) of S. lactis C_{10} and concentrations of divalent cation salts is shown in Figures a and b. In Figure a the divalent cation salts present are: □, $MnCl_2$; ■, $MgCl_2$; ●, $MgSO_4$; ○, $MnCl_2$ but with no FDP present in the reaction mixture. In Figure b the divalent cation salts present are: ■, $MgCl_2$; ▲, $CoCl_2$. The reaction mixtures for a and b contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 1 mM PEP; 3.3 mM ADP; 1 mM FDP (except as noted); 13.3 mM KCl and 0.1 cm^3 of diluted enzyme. The divalent cation salt concentrations were varied as shown in Figures a and b.



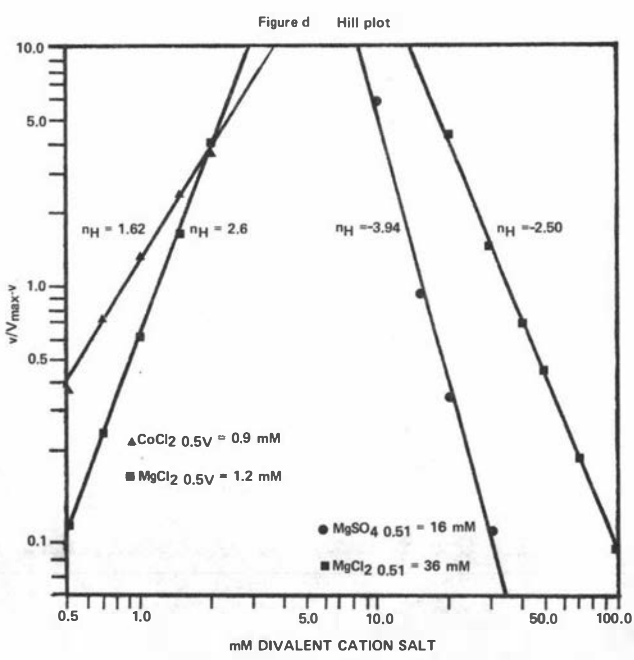


Figure c shows Hill plots (log v/V_{max}-v versus log divalent cation salt concentration) of the data from Figure a. The Hill plot obtained by varying the $MnCl_2$ in the absence of FDP has not been included. The V_{max} values used for calculating the Hill plot values were obtained by linear extrapolation of Lineweaver-Burk plots to the 1/v axis. The divalent cation salts are: □, $MnCl_2$; ■, $MgCl_2$; ●, $MgSO_4$.
Figure d shows Hill plots (log v/V_{max}-v versus log divalent cation salt concentration) of the data from Figure b. The divalent cation salts are: ■, $MgCl_2$; ▲, $CoCl_2$. Also shown in Figure d are Hill plots obtained from the data from Figure a, where the concentrations of $MgCl_2$ (■) and $MgSO_4$ (●) are inhibiting pyruvate kinase activity.

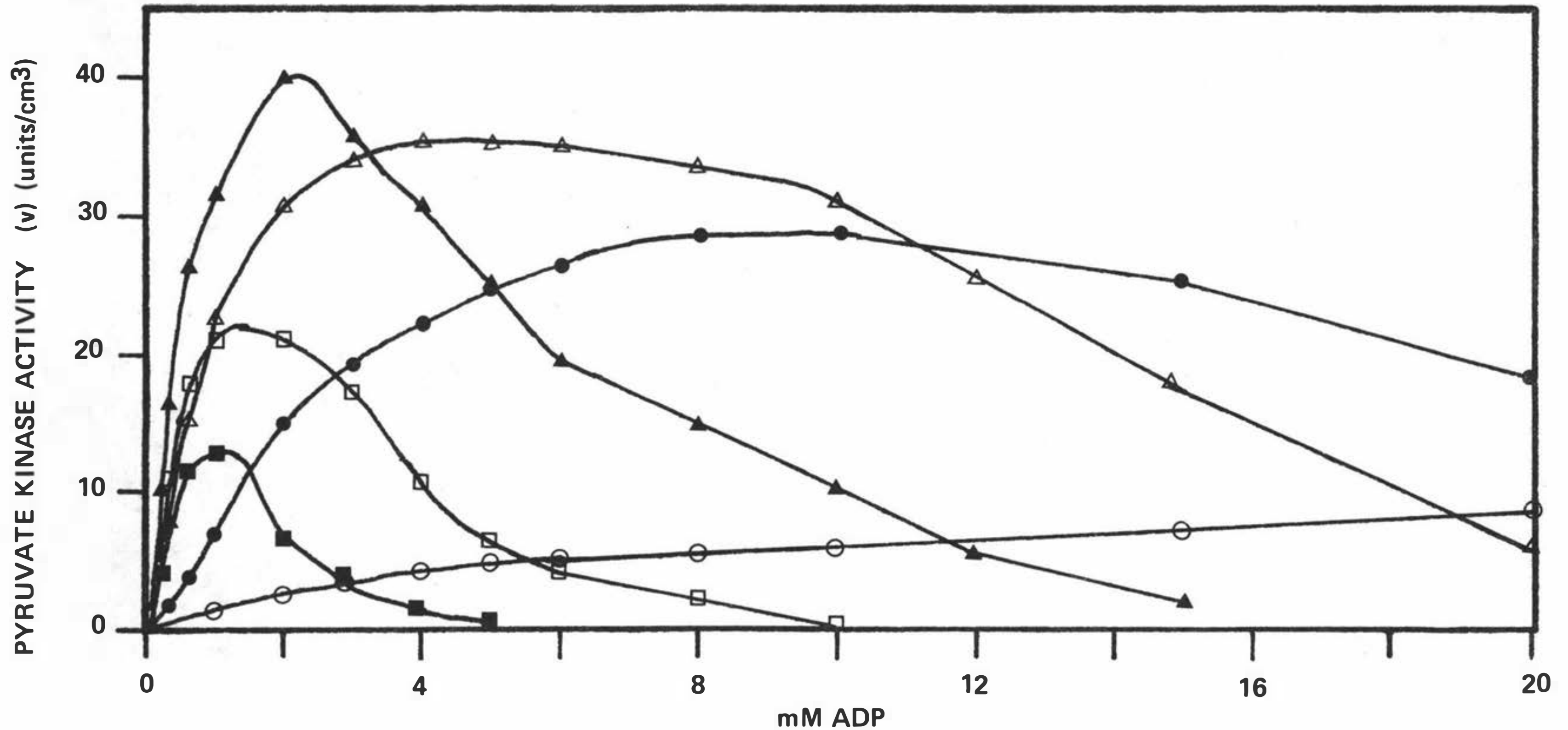
3.4.9.2 Effect of Mg^{++} concentration on ADP activation and inhibition of *S. lactis* pyruvate kinase

The relationship between ADP activation and inhibition of the *S. lactis* pyruvate kinase activity was investigated further by varying the ADP concentration at different concentrations of $MgSO_4$* and at a constant concentration of 30 mM KCl. In Figure 3.4.9.2 the relationship between reaction velocity and ADP concentrations at different concentrations of $MgSO_4$ can be seen. At the highest $MgSO_4$ concentration of 30 mM, ADP did not show any inhibition even at 20 mM ADP. However the V_{max} reached at this Mg^{++} concentration was very much less than at lower Mg^{++} concentrations. As the $MgSO_4$ concentration was decreased from 30 mM to 0.5 mM the inhibition of pyruvate kinase activity by high ADP became more evident and occurred at progressively lower ADP concentrations. The V_{max} reached was maximal at 4 mM Mg^{++}. Also the ADP concentration for half maximum activity ($ADP_{0.5V}$) decreases from 6.0 mM ADP at 30 mM $MgSO_4$ to 0.2 mM ADP at 0.5 mM $MgSO_4$.

The results of this experiment are consistent with the idea that the Mg^{++} and ADP^{3-} bind to the enzyme as a $MgADP^-$ complex. An excess of either free Mg^{++} or free ADP^{3-} results in inhibition in which free Mg^{++} or free ADP^{3-} competes with $MgADP^-$ for the binding sites. However the results could be interpreted as indicating that both ADP and Mg^{++} (either free or combined) can bind at sites other than the catalytic site which induce conformations with altered affinity for Mg^{++}, ADP^{3-} or $MgADP^-$ at the catalytic site.

* Footnote: It should be noted that $MgSO_4$ rather than $MgCl_2$ was used in this particular study. This experiment was carried out at an early stage of the pyruvate kinase study before the inhibiting effects of SO_4^{2-} were appreciated. Although there is a difference in response to $MgCl_2$ and $MgSO_4$ (as described above, see also Appendix 3.4.2), the use of sulphate does not negate the general conclusions that may be drawn from this experiment.

Figure 3.4.9.2 EFFECT OF $MgSO_4$ CONCENTRATION ON ADP ACTIVATION AND INHIBITION



The ADP activation and inhibition of the S. lactis C_{10} pyruvate kinase activity (v) was investigated at six different $MgSO_4$ concentrations. The relationship is shown as a v (units/cm^3) versus ADP concentration (mM) plot. The six different $MgSO_4$ concentrations are: ○, 30 mM $MgSO_4$; ●, 20 mM $MgSO_4$; △, 10 mM $MgSO_4$; ▲, 4 mM $MgSO_4$; □, 1 mM $MgSO_4$; ■, 0.5 mM $MgSO_4$. The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 1 mM PEP; 1 mM FDP: 30 mM KCl and 0.1 cm^3 of diluted enzyme. The ADP concentration was varied as shown in the Figure.

3.4.9.3 Inhibition by other divalent cations

The chloride salts of Cu^{++}, Zn^{++}, Ca^{++}, Ni^{++} and Ba^{++}, unlike Mg^{++}, Mn^{++} and Co^{++}, were found to be unable to activate the enzyme using the standard assay system (minus $MgCl_2$) at concentrations ranging from 0.1 to 20 mM. However if the standard assay system was used (13.3 mM KCl/3.3 mM $MgCl_2$) and the concentration of the chloride salts of Cu^{++}, Zn^{++}, Ca^{++}, Ni^{++} or Ba^{++} was progressively increased then inhibition occurred, different for each cation, as is shown in Figure 3.4.9.3a. Cu^{++} completely inhibited activity at a concentration of 0.1 mM, Zn^{++} was the next most potent inhibitor, followed by Ca^{++} then Ni^{++}, with Ba^{++} inhibiting to the least extent. Hill plots of the same data are shown in Figure 3.4.9.3b. For all four metals (Zn^{++}, Ca^{++}, Ni^{++} and Ba^{++}) the Hill interaction coefficient value is -1.55 to -1.60. The concentration of the divalent cation required for 50% inhibition of activity ($M^{++}{}_{0.5I}$) ranged from 0.65 mM $ZnCl_2$ to 3.2 mM $BaCl_2$. These values are low when compared with the respective values for $MgCl_2$ and $MgSO_4$ inhibition (16 mM $MgSO_4$ and 36 mM $MgCl_2$) shown previously in Figure 3.4.9.1d.

The inhibition by high concentrations of M^{++} could be accounted for if the M^{++} normally binds to the enzyme in the form of M ADP^- and possibly M PEP and M FDP complexes to some extent. At high concentrations of M^{++} the binding of the free M^{++} may be competing with the binding of the M-substrate and M-activator complexes. The evident multiplicity of binding sites for these complexes could account for the co-operativity of inhibitor binding. Alternatively the metal ions may inhibit by binding at sites other than those involved in the catalytic mechanism. These alternative sites may only become available when the enzyme is in the active conformation.

Either of these alternatives could apply to inhibition by divalent metal ions which do not activate, i.e. Zn^{++}, Ca^{++}, Ni^{++} and Ba^{++}, except that the binding sites for these metal ions have a relatively higher affinity than for inhibiting Mg^{++} concentrations. Zn^{++}, Ca^{++}, Ni^{++} and Ba^{++} may inhibit activity by binding only at the alternative sites (not catalytic) whereas Mg^{++} may inhibit both ways; by competing for the substrate (-complexes) at the catalytic sites and by binding at the alternative sites. This could explain the lower degree of co-operativity of binding of these non-activating ions (compared with Mg^{++} inhibition at high concentrations).

Figure 3.4.9.3: a and b
INHIBITION BY DIVALENT CATIONS

Figure a

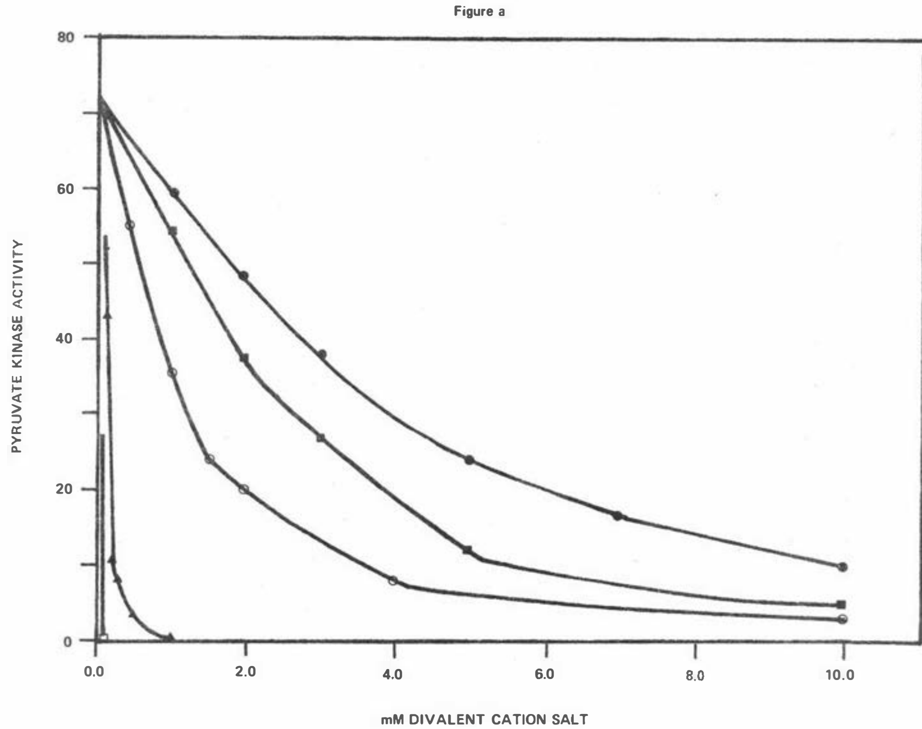
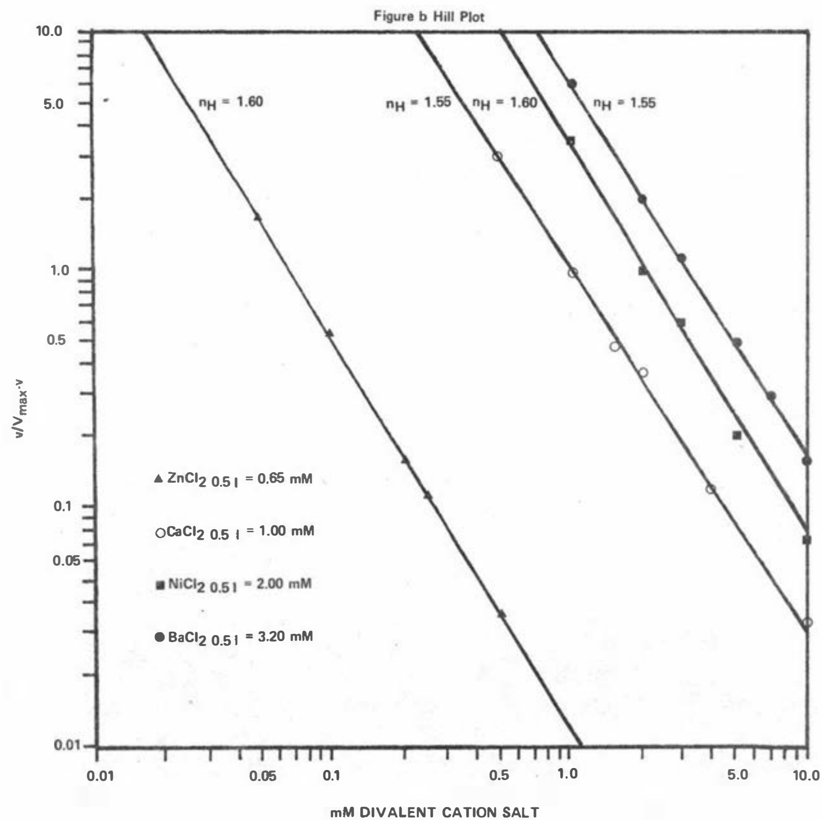


Figure b Hill Plot



The divalent cation inhibition of the *S. lactis* C_{10} pyruvate kinase activity was investigated. The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/ HCl buffer pH 7.5; 3.3 mM ADP; 1 mM PEP; 1 mM FDP; 13.3 mM KCl; 3.3 mM $MgCl_2$ and 0.1 cm^3 of diluted enzyme. The divalent cation salts (non activating) that were added to the reaction mixture, at concentrations as shown in the Figures, are: □, $CuCl_2$; ▲, $ZnCl_2$; ○, $CaCl_2$; ■, $NiCl_2$; ●, $BaCl_2$. Figure a is a plot of pyruvate kinase activity (v) versus mM Divalent cation salt.
Figure b shows the Hill plot (lov v/V_{max}-v versus log divalent cation salt concentration) of the data from Figure a. The V_{max} value used for calculating the Hill plots was the pyruvate kinase activity assayed in the absence of any inhibiting divalent cations.

3.4.9.4 Further investigation of Mn^{++} activation of the *S. lactis* pyruvate kinase

As shown in Section 3.4.9.1 the enzyme was markedly activated by Mn^{++} in the absence of FDP. Several workers have found that substitution of Mn^{++} for Mg^{++} enhances the binding of PEP (Tuominen and Bernlohr, 1971b; Mildvan and Cohn, 1966; Passeron and Terenzi, 1970; Waygood and Sanwal, 1974). With *E. coli* (Waygood and Sanwal, 1974) the enzyme shows sigmoidal activation by Mn^{++} in the absence of FDP, but this is also true of Mg^{++}. In *S. lactis* Mn^{++} and Mg^{++} show a qualitative difference in their ability to activate the enzyme in the absence of FDP. For this reason the effect of Mn^{++} was further investigated.

A. Comparison of FDP activation with Mn^{++} or Mg^{++} as the divalent cation

Using otherwise standard assay conditions the FDP concentration was varied in the presence of 3.0 mM $MgCl_2$ or 3.0 mM $MnCl_2$. The results are shown in Figure 3.4.9.4a. In the presence of Mn^{++} as the divalent cation FDP activates in a hyperbolic manner (n_H = 1.0) whereas with Mg^{++} FDP activates in a sigmoidal manner (n_H = 2.0). The FDP $_{0.5V}$ value with $MnCl_2$ present is 0.015 mM, a low value compared to 0.35 mM FDP with $MgCl_2$.

B. Response to varying PEP concentration with Mg^{++} or Mn^{++} as the divalent cation

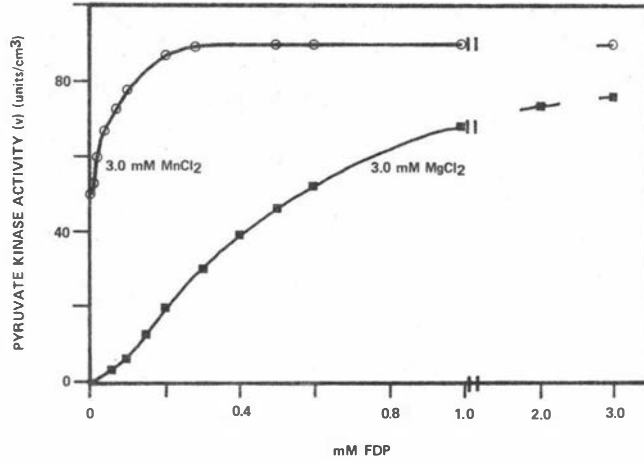
The Hill plots in Figure 3.4.9.4b were obtained by varying the PEP concentration, using the standard assay conditions, in the presence of 3.0 mM $MgCl_2$ or 3.0 mM $MnCl_2$. When Mn^{++} was used as the divalent cation the response to varying PEP was examined in both the presence (1 mM) and absence of FDP. The results obtained from the Hill plots in Figure 3.4.9.4b are summarised below.

Values from Hill plots shown in Figure

	n_H	PEP $_{0.5V}$ (mM)	V_{max} (units/cm^3)
3.0 mM $MgCl_2$ + 1.0 mM FDP	1.2	0.18	67.0
3.0 mM $MnCl_2$ + 1.0 mM FDP	1.8	0.015	66.0
3.0 mM $MnCl_2$ - no FDP	1.3	0.09	48.0

Figure 3.4.9.4 a

FDP ACTIVATION WITH Mn^{2+} OR Mg^{2+}

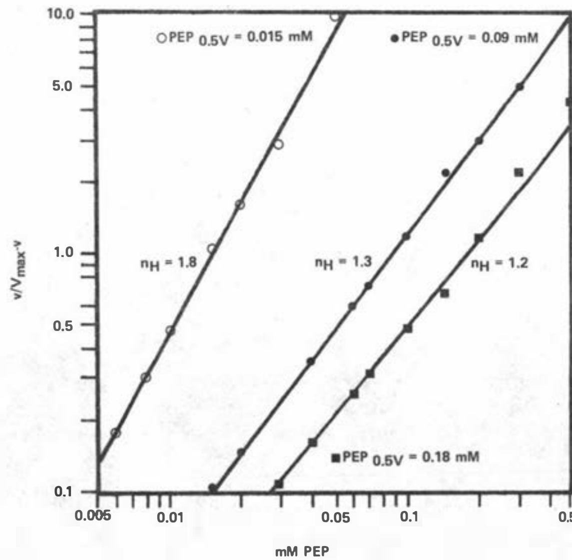


FDP Activation	From Hill Plots	
(with $MgCl_2$ present)	n_H = 1.0	FDP $_{0.5V}$ = 0.35 mM
(with $MnCl_2$ present)	n_H = 2.0	FDP $_{0.5V}$ = 0.015 mM

The relationship between the pyruvate kinase activity (v, units/cm^3) of S. lactis C_{10} and FDP concentration is shown in Figure a with 3.0 mM $MgCl_2$ (■) and 3.0 mM $MnCl_2$ (○) as the essential activating divalent cations. The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 1 mM PEP; 3.3 mM ADP; 13.3 mM KCl; 0.1 cm^3 of diluted enzyme and the FDP concentration varied as shown in the Figure, in the presence of 3.0 mM $MgCl_2$ or 3.0 mM $MnCl_2$.

Figure 3.4.9.4 b

PEP ACTIVATION WITH Mn^{2+} OR Mg^{2+}



The relationship between the pyruvate kinase activity (v, units/cm^3) of S. lactis C_{10} and PEP concentration is shown in Figure b at different divalent cation and FDP combinations. The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 3.3 mM ADP; 13.3 mM KCl; 0.1 cm^3 of diluted enzyme and the PEP concentration varied as shown in the Figure, in the presence of: ○, 3.0 mM $MnCl_2$ + 1 mM FDP; ●, 3.0 mM $MnCl_2$ with no FDP present; ■, 3.0 mM $MgCl_2$ + 1 mM FDP.

With FDP (1 mM) present in the assay system, replacement of Mg^{++} by Mn^{++} increases the Hill interaction coefficient from 1.2 to 1.8 and decreases the PEP $_{0.5V}$ value by at least ten-fold. With Mn^{++} as the activating divalent cation in the absence of FDP, the PEP $_{0.5V}$ value is 0.09 mM, which is half the value obtained with $MgCl_2$ in the presence of FDP. With FDP present the V_{max} with either Mn^{++} or Mg^{++} is essentially the same, whereas the V_{max} with Mn^{++} in the absence of FDP is significantly lower.

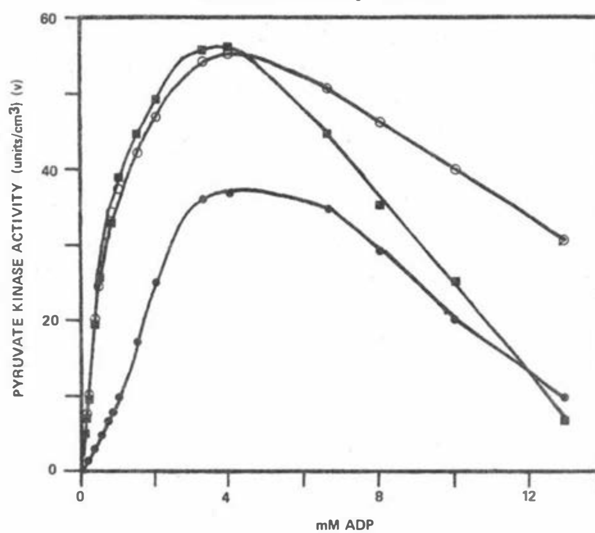
Saturating FDP concentrations (1 mM) were reported in Section 3.4.5.1 to reduce the allosteric response to varying of PEP (using $MgCl_2$ as the activating divalent cation). This can be seen in the Hill plot of Figure 3.4.9.4b. Here, PEP shows only weak, if any, homotrophic interaction with the enzyme (n_H = 1.2) where $MgCl_2$ is present with 1 mM FDP. Similarly with $MnCl_2$ present, but in the absence of FDP, PEP again shows only a weak homotrophic interaction with the enzyme (n_H = 1.3). This is not expected, as in Section 3.4.5.1, in the presence of $MgCl_2$ decreasing the FDP concentration increased the n_H value. However with saturating FDP present and Mn^{++} as the divalent cation the n_H value increases to 1.8. Thus when $MnCl_2$ is present with FDP, the FDP and $MnCl_2$ combination increases both the affinity and the homotrophic interaction of PEP with the enzyme. This, and the fact that PEP shows only a weak homotrophic interaction with the enzyme in the absence of FDP when Mn^{++} is present as the divalent cation, supports the idea that Mn^{++} and FDP are activating the enzyme in different ways.

C. Response to varying ADP concentration with Mg^{++} or Mn^{++} as the divalent cation

Figure 3.4.9.4c shows the reaction velocity as a function of ADP concentration using the same cation/FDP combinations as before, i.e. $MgCl_2$ with FDP, and $MnCl_2$ with and without FDP. For both divalent cations, maximum activity is achieved between 2 to 4 mM ADP with activity dropping off at higher ADP concentrations. $MnCl_2$ with FDP present, appears to suppress inhibition of the enzyme's activity by high concentrations of ADP to some extent. The enzyme with Mn^{++} but no FDP present in the assay system, has a lower V_{max} than for either Mg^{++} or Mn^{++}, both in the presence of FDP. The data from Figure 3.4.9.4c are plotted as Hill plots as shown in Figure 3.4.9.4d. (The V_{max} values

Figure 3.4.9.4 c

ADP BINDING WITH Mg^{2+} OR Mn^{2+}



The relationship between the pyruvate kinase activity (v, units/cm^3) of S. lactis C_{10} and ADP concentration is shown in Figure c with different divalent cation and FDP combinations. The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 1.0 mM PEP; 13.3 mM KCl; 0.1 cm^3 of diluted enzyme and the ADP concentration was varied as shown in the Figure, in the presence of: ○, 3.3 mM $MnCl_2$ + 1 mM FDP; ●, 3.3 mM $MnCl_2$ with no FDP present; ■, 3.3 mM $MgCl_2$ + 1 mM FDP.

Figure 3.4.9.4 d

HILL PLOTS FOR ADP BINDING WITH Mg^{2+} OR Mn^{2+}

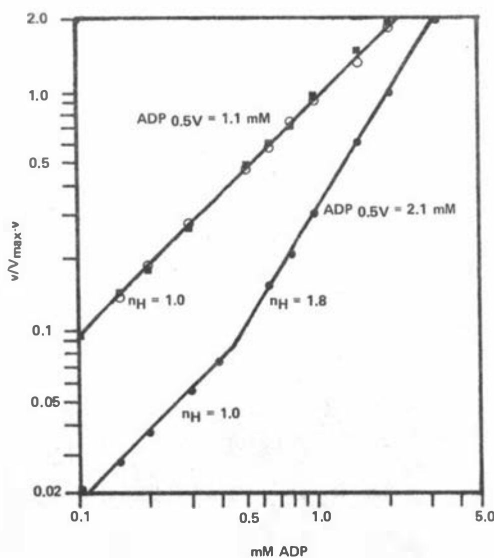


Figure d shows Hill Plots (log v/V_{max}-v versus log ADP concentration) of the data from Figure c where the ADP concentration was varied in the presence of: ○, 3.3 mM $MnCl_2$ + 1 mM FDP; ●, 3.3 mM $MnCl_2$ with no FDP present; ■, 3.3 mM $MgCl_2$ + 1 mM FDP. The V_{max} values used for calculating the Hill plot values were obtained by linear extrapolation of Lineweaver-Burk plots to the 1/v axis.

used were estimated by linear extrapolation of double reciprocal plots.) For $MnCl_2$ and $MgCl_2$ in the presence of FDP, the Hill plots were essentially identical. ADP shows no homotrophic interaction ($n_H = 1.0$) and the ADP concentration required for half maximum activity ($ADP_{0.5V}$) is 1.1 mM for both cations. When using $MnCl_2$ as the divalent cation in the absence of FDP, there is significant co-operative interaction of ADP with the enzyme ($n_H = 1.8$). This is probably simply a consequence of the absence of FDP since it was previously shown (Section 3.4.6.1) that decreasing the FDP concentration increases the co-operativity of ADP binding.

This replacement of Mg^{++} with Mn^{++} in the presence of FDP has quite distinctive effects on ADP and PEP binding. Neither the affinity ($ADP_{0.5V}$) nor the co-operative interaction of ADP with the enzyme is altered by using Mn^{++} in place of Mg^{++} whereas both the co-operativity of PEP binding and the affinity for PEP are significantly greater with Mn^{++} than they are with Mg^{++}.

The non-FDP activated pyruvate kinases from B. licheniformis (Tuominen and Bernlohr, 1971b) and rabbit muscle (Mildvan and Cohn, 1966) were both shown to have higher affinities for PEP, but not ADP, in the presence of Mn^{++} rather than in the presence of Mg^{++}. A similar effect of Mn^{++} has been reported for the FDP-activated pyruvate kinase from Mucor rouxii (Passeron and Terenzi, 1970). However in this case although Mn^{++} lead to a similar reduction in the apparent K_M for PEP, FDP did not activate the enzyme in the presence of Mn^{++}. With the S. lactis pyruvate kinase at saturating $MnCl_2$ concentrations, FDP does significantly activate the enzyme, though at considerably lower concentrations and in a hyperbolic rather than sigmoidal manner.

Tuominen and Bernlohr (1971b), in studying the properties of the B. licheniformis pyruvate kinase, concluded that Mn^{++} induces conformational changes in the enzyme that cannot be attained with any combination of ligands in the Mg-activated system. In S. lactis C_{10}, Mn^{++} with no FDP present (enzyme active), appears to induce conformational changes in the enzyme that cannot be attained in the Mg-activated system without FDP present (enzyme inactive). Also, like the B. licheniformis enzyme, the activated pyruvate kinase from S. lactis C_{10} possibly has a different conformational change with FDP and $MnCl_2$ present, than with FDP and $MgCl_2$ as is evident from the studies on PEP activation of the enzyme in the presence of the two divalent cations.

3.4.10 The Effect of pH on the Kinetic Properties of S. lactis Pyruvate Kinase

The kinetic data for the S. lactis C_{10} pyruvate kinase so far described have all been determined at pH 7.5 (the pH optimum of the enzyme). It is important to ascertain whether the kinetic properties of the pyruvate kinase are markedly different at other pHs. pH 6.4 and pH 8.75 were selected as they represented the pH values where the enzyme activity was very different from the pH optimum while still being within the buffering range of 80 mM triethanolamine/HCl buffer. The effect of the pH on PEP and FDP activation was studied with manganese and magnesium as the essential divalent cation.

A. Effect of pH on response to varying PEP concentration

The effects of pH on PEP activation are summarised in Table 3.4.10a. The affinity of pyruvate kinase for PEP, with Mg^{++} as the divalent cation, is optimal in alkaline pHs; the K_M is 0.13 mM PEP at pH 8.75, 0.18 mM at pH 7.5 and 0.4 mM PEP at pH 6.4. However when Mn^{++} is the divalent metal ion in the presence of FDP, the affinity of the enzyme for PEP decreases ten-fold at pH 8.75 compared with the affinity at pH 7.5 and 6.4. It is also interesting that the co-operativity of PEP binding seen at pH 6.4 and 7.5 in the present of Mn^{++} is no longer evident at pH 8.75.

When Mn^{++} alone is present (i.e. without FDP) the affinity of pyruvate kinase for PEP is much less dependent on pH (K_M values ranging from 0.13 mM to 0.2 mM PEP).

B. Effect of pH on FDP activation

The effects of pH on FDP activation of the S. lactis pyruvate kinase are summarised in Table 3.4.10b. With $MgCl_2$ as the divalent cation the FDP $_{0.5V}$ value is nearly the same at pH 6.4 and 7.5 (0.2 mM and 0.18 mM FDP, respectively), and increases to 0.45 mM at pH 8.75. The co-operative interaction of FDP binding is also affected by pH since at pH 6.4 and 7.5 the n_H value is 1.94 while at pH 8.75 n_H is 1.0. The FDP $_{0.5V}$ values are lower at all three pH values when Mn^{++} replaces Mg^{++}, but there is relatively a much greater increase in the FDP $_{0.5V}$ values from pH 7.5 to 8.75 with Mn^{++} as the divalent cation.

The effect of pH on the kinetic properties of the *S. lactis* pyruvate kinase appear from this brief study to be quite different from the results found by workers studying the mammalian pyruvate kinases. For both the liver type-L pyruvate kinase (Rozengurt *et al.* 1969) and the erythrocyte type-L pyruvate kinase (Staal *et al.*, 1971) the enzyme is not activated by FDP and shows only a Michaelis-Menten response to PEP below pH 7.2. Thus the *S. lactis* enzyme appears to maintain its allosteric properties at lower pH values than is the case for the mammalian pyruvate kinases.

The relationship between pH, FDP and PEP activation of the *S. lactis* pyruvate kinase, being different from mammalian pyruvate kinases, possibly indicates a different intracellular pH control for the *S. lactis* enzyme. $MnCl_2$ was shown to be effective in activating the *S. lactis* enzyme also at the two extremes of pH (6.4 and 8.75) with or without FDP present. Mn^{++} thus appears to have the potential for controlling the enzyme in vivo in a different manner to the FDP control.

Table 3.4.10a

The effect of pH on PEP activation of pyruvate kinase

The effect of pH on PEP activation of pyruvate kinase was studied in the presence of either 3.3 mM $MnCl_2$ (with or without 1 mM FDP) or 3.3 mM $MgCl_2$ (with 1 mM FDP) at standard assay conditions of: 80 mM triethanolamine/HCl buffer; 3.3 mM ADP and 13.3 mM KCl. The pH of the assay mixture was either the pH optimum (pH 7.5), pH 6.4 or pH 8.75. PEP was varied at each different condition, to give at least eight usable points to plot the respective Hill and Lineweaver-Burk plots. The Hill interaction coefficient values (n_H) obtained from Hill plots, and the K_M and V_{max} values obtained from Lineweaver-Burk plots, for varying the PEP concentration at the different conditions, are tabulated below.

Conditions			Lineweaver-Burk Plot		Hill Plot
pH	Divalent Cation (3.3 mM)	FDP (mM)	K_M (mM PEP)	V_{max} (units/cm^3)	n_H
7.5	$MgCl_2$	1.0	0.18	73.0	1.0
7.5	$MnCl_2$	1.0	0.02*	77.0	1.8
7.5	$MnCl_2$	0.0	0.20	35.0	1.0
6.4	$MgCl_2$	1.0	0.40	45.0	1.0
6.4	$MnCl_2$	1.0	0.02*	42.0	1.7
6.4	$MnCl_2$	0.0	0.20	22.0	1.0
8.75	$MgCl_2$	1.0	0.13	36.0	1.0
8.75	$MnCl_2$	1.0	0.13*	36.0	1.1
8.75	$MnCl_2$	0.0	0.13	18.0	1.0

* Values determined as $PEP_{0.5V}$ values from Hill plots.

Table 3.4.10b

The effect of pH on FDP activation of pyruvate kinase

The effect of pH on FDP activation of pyruvate kinase was studied in the presence of either 3.3 mM $MnCl_2$ or 3.3 mM $MgCl_2$ at standard assay conditions of: 80 mM triethanolamine/HCl buffer; 3.3 mM ADP; 1 mM PEP and 13.3 mM KCl. The pH of the assay mixture was either the pH optimum (pH 7.5), pH 6.4 or pH 8.75. FDP was varied at each different condition, to give at least eight usable points to plot the respective Hill and Lineweaver-Burk plots. The Hill interaction coefficient and FDP $_{0.5V}$ values obtained from Hill plots, and the V_{max} values obtained from Lineweaver-Burk plots for varying the FDP concentration at the different conditions, are tabulated below. The activity(v) obtained in the absence of FDP is also shown.

Conditions		Hill Plot		Lineweaver-Burk Plot	Activity (v)
pH	Divalent Cation (3.3 mM)	n_H	FDP $_{0.5V}$ mM FDP	V_{max} (units/cm^3)	(in absence of FDP)(units/cm^3)
7.5	$MgCl_2$	1.9	0.20	74.0	0.0
7.5	$MnCl_2$	1.0	0.02	74.0	30.0
6.4	$MgCl_2$	1.9	0.18	42.0	0.0
6.4	$MnCl_2$	1.0	0.0065	41.0	24.0
8.75	$MgCl_2$	1.0	0.45	30.0	0.0
8.75	$MnCl_2$	1.0	0.23	24.0	8.0

3.4.11 Effect of Phosphate on the Kinetic Properties of *S. lactis* Pyruvate Kinase

In the course of the investigation of varying pH on certain properties of pyruvate kinase, as described in the previous section, a study was made also with 1 mM phosphate present in triethanolamine/HCl buffer pH 7.5. The strong inhibitory effect of phosphate has already been described in an earlier section (3.4.3). The purpose of the determinations reported in Table 3.4.11 was to ascertain which particular kinetic properties of pyruvate kinase were sensitive to phosphate.

The presence of 1.0 mM phosphate at pH 7.5 with $MgCl_2$ as the divalent cation, increases the PEP $_{0.5V}$ value 25-fold (0.18 mM to 5.0 mM PEP) but does not change the V_{max} value or the n_H value.

When FDP activation is similarly studied in the presence of 1.0 mM phosphate (Table 3.4.11) the FDP $_{0.5V}$ value increased from 0.2 mM to 0.93 mM FDP. Though the n_H value did not change, the V_{max} value dropped from 74 units/cm^3 to 33 units/cm^3 when 1 mM phosphate was present. The drop in V_{max} probably indicates that the PEP is no longer at saturating concentrations at saturating FDP concentrations. Therefore phosphate effects both FDP and PEP activation, with PEP binding being more sensitive than FDP binding to phosphate inhibition.

Table 3.4.11

Effect of phosphate on the kinetic properties of *S. lactis* pyruvate kinase

Standard assay conditions were used: 80 mM triethanolamine/HCl buffer pH 7.5; 3.33 mM ADP; 13.33 mM KCl/3.33 mM $MgCl_2$; and either 1 mM PEP or 1 mM FDP. The effect of 1 mM phosphate on the response of the pyruvate kinase activity to varying PEP concentration and FDP concentration was investigated. The kinetic data, obtained from the respective double reciprocal plots and Hill plots, are shown below.

		Control	1 mM Phosphate
Varying PEP	PEP $_{0.5V}$	0.18 mM PEP	5.0 mM PEP
	V_{max}	73 units/cm^3	80 units/cm^3
	n_H	1.0	1.0
		Control	1 mM Phosphate
Varying FDP	FDP $_{0.5V}$	0.2 mM FDP	0.93 mM FDP
	V_{max}	74.0 units/cm^3	33.0 units/cm^3
	n_H	1.9	1.9

3.4.12 ATP Inhibition of Pyruvate Kinases

Pyruvate kinase from various sources has been shown to be markedly inhibited by ATP (Irving and Williams, 1973; Costa *et al.*, 1972; Staal *et al.*, 1971; Ainsworth and Macfarlane, 1973; Tuominen and Bernlohr, 1971b and Waygood and Sanwal, 1974). However the nature of the inhibitory effect appears to differ considerably from one enzyme to another and the different isoenzymes in any one tissue are usually affected by ATP in distinctive ways. Waygood and Sanwal (1974) found that GTP was a much more potent inhibitor of the *E. coli* pyruvate kinase than ATP.

Since ATP inhibition of pyruvate kinases may occur at physiological levels of ATP the effect of ATP on the *S. lactis* pyruvate kinase was investigated.

3.4.12.1 ATP inhibition of *S. lactis* pyruvate kinase

ATP concentration was varied using the standard assay conditions at three different FDP concentrations (0.5, 1.0 and 10.0 mM FDP). The relationship between pyruvate kinase activity and ATP concentration is shown in Figure 3.4.12.1a. ATP inhibited the enzyme at all three FDP concentrations. However this inhibition could be entirely due to chelation of Mg^{++} (Wood, 1968). Therefore ATP inhibition was studied at a higher concentration of cations (80 mM KCl/8 mM $MgCl_2$ instead of 13.3 mM KCl/3.3 mM $MgCl_2$ as used in the standard assays) as shown in Figure 3.4.12.1b. ATP inhibition still occurred at the higher cation concentration although the concentration of ATP required to cause 50% inhibition was increased somewhat. The data obtained at 1 mM FDP at the two cation concentrations are replotted as Hill plots in Figure 3.4.12c. At the low cation concentration the ATP is interacting with pyruvate kinase in a co-operative manner ($n_H = -3.0$) to inhibit activity. The concentration of ATP giving 50% inhibition of activity ($ATP_{0.5I}$) increases from 2.3 mM to 5.3 mM ATP as the cation concentration is increased. Inhibition of activity by ATP at the high cation concentration is not entirely due to chelation of Mg^{++} as activity with 80 mM KCl/8 mM $MgCl_2$ is identical to the activity assayed with 80 mM KCl/3 mM $MgCl_2$ present in the standard assay conditions. At the high cation concentration the Hill plot is biphasic with n_H values of -3 and -6. This biphasic response may be due to ATP inhibiting in more than one way. Inhibition by chelation of Mg^{++} will be important at high ATP concentrations. However

two other possible modes of inhibition exist. ATP may inhibit by binding as Mg ATP^{2-} at the ADP catalytic site or at an allosteric site independent of the ADP catalytic site.

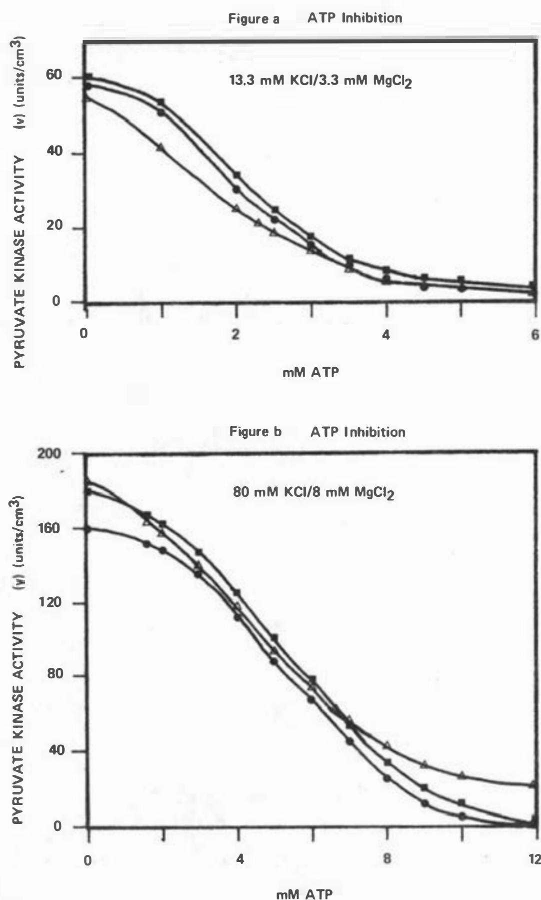
Hence S. lactis C_{10} pyruvate kinase is inhibited by ATP and this inhibition is not entirely due to chelation of Mg^{++}. Collins and Thomas (1974), studying the pyruvate kinase from S. lactis ML_3, reported that in the presence of 2 mM PEP, 5 mM ADP, 2 mM FDP and 80 mM KCl/ 8 mM $MgCl_2$ the addition of either ATP or AMP at concentrations up to 5 mM had no effect on the reaction rate of pyruvate kinase, but when FDP concentration was reduced ten-fold, 5 mM ATP caused 50% inhibition. Their results are therefore different from those reported above where ATP inhibition was found to be virtually independent of FDP concentration.

3.4.12.2 The effect of AMP on S. lactis pyruvate kinase

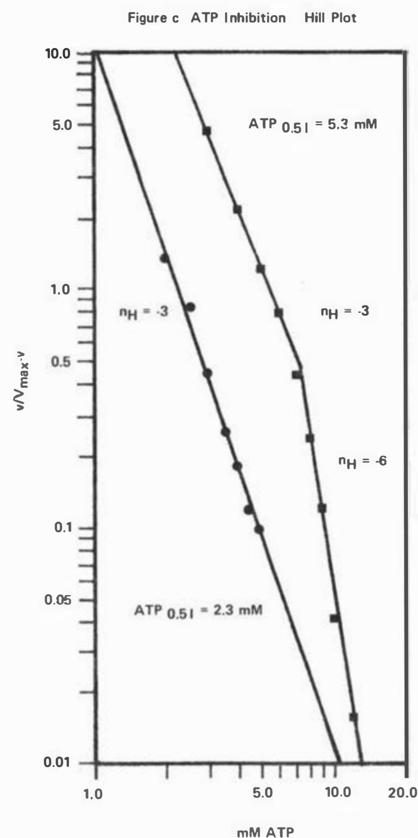
The effect of varying AMP concentration on the pyruvate kinase activity was studied at three different ADP concentrations under otherwise standard assay conditions of 13.33 mM KCl/3.33 mM $MgCl_2$, 1.0 mM PEP and 1.0 mM FDP. The data obtained were plotted as Hill plots (Figure 3.4.12.2). AMP inhibited activity in a co-operative manner. The strength of co-operative binding of AMP to the enzyme is dependent on the ADP concentration since the n_H value decreases from 1.85 to 1.3 as the ADP concentration was decreased from 2 mM to 0.25 mM. However the concentration of AMP giving 50% inhibition of activity appears to be relatively independent of ADP concentration with a value of 7.0 ± 0.5 mM AMP for the three ADP concentrations.

Thus unlike some other pyruvate kinases (Tuominen and Bernlohr, 1971b) the S. lactis pyruvate kinase is not activated by AMP. The pyruvate kinase from S. faecalis (PK-II) that is almost totally dependent upon FDP for activity is unaffected by either AMP or ATP (Wittenberger et al., 1973). The other pyruvate kinase from S. faecalis (PK-I) (not activated by FDP) studied by the same workers was activated by AMP and inhibited by ATP.

Figure 3.4.12.1: a, b and c ATP INHIBITION OF PYRUVATE KINASE

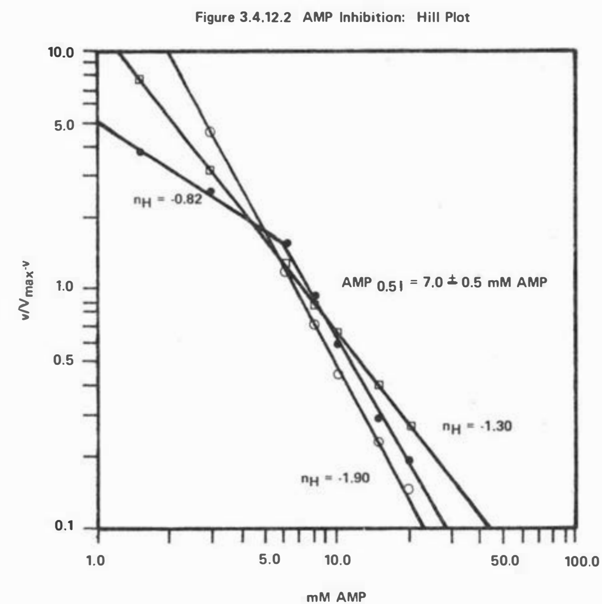


The ATP inhibition of pyruvate kinase activity (v, units/cm^3) of S. lactis C_{10} is shown in Figures a and b at different cation and FDP combinations. The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 1.0 mM PEP; 3.3 mM ADP; 0.1 cm^3 of diluted enzyme and ATP concentrations as shown in the Figures in the presence of: △, 10 mM FDP; ●, 1 mM FDP; ■, 0.5 mM FDP. In Figure a 13.3 mM KCl/3.3 mM $MgCl_2$ are present. In Figure b 80 mM KCl/8 mM $MgCl_2$ are present.



The data obtained from Figures a and b (at the two cation concentrations) in the presence of 1 mM FDP are replotted as Hill plots (log v/V_{max} -v versus log ATP concentration) in Figure c. The two V_{max} values used for calculating the Hill plots were the two respective pyruvate kinase activities assayed in the absence of ATP. The two cation concentrations are: ●, 13.3 mM KCl/3.3 mM $MgCl_2$; ■, 80 mM KCl/8 mM $MgCl_2$.

Figure 3.4.12.2 AMP INHIBITION OF PYRUVATE KINASE



The AMP inhibition of pyruvate kinase activity (v, units/cm^3) of S. lactis C_{10} is shown in Figure 3.4.12.2 as Hill plots at three different ADP concentrations. The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 1.0 mM PEP; 1.0 mM FDP; 13.3 mM KCl; 3.3 mM $MgCl_2$; 0.1 cm^3 of diluted enzyme and the AMP concentrations varied as shown in the Figure in the presence of: ●, 2 mM ADP; ○, 1 mM ADP; □, 0.25 mM ADP. The three V_{max} values used for calculating the Hill plots were the respective pyruvate kinase activities assayed in the absence of AMP.

3.5 Discussion of the Results from Studies of the S. lactis C_{10} Pyruvate Kinase.

In the introductory section (3.1), the pyruvate kinases from many sources were shown to have important allosteric control features indicating the importance of this enzyme in the regulation of carbohydrate metabolism. The results from the study of the S. lactis C_{10} pyruvate kinase have shown that its activity is also allosterically controlled by a number of factors. In its general properties the S. lactis C_{10} pyruvate kinase is not unlike FDP-activated pyruvate kinases from most other organisms such as the mammalian type L pyruvate kinase and the yeast and E. coli FDP-activated pyruvate kinases. The molecular weight (235,000) and subunit number (4) is similar to that of pyruvate kinases from other organisms and in this respect the S. lactis pyruvate kinase apparently differs from the much smaller FDP-activated pyruvate kinase from S. faecalis (Wittenberger et al., 1973). However in many of its detailed regulatory features the S. lactis C_{10} pyruvate kinase shows differences from the properties of other pyruvate kinases. Of course not all the factors that have been shown to control activity in vitro are necessarily important in determining the intracellular activity of the S. lactis pyruvate kinase.

3.5.1 Factors Controlling S. lactis C_{10} Pyruvate Kinase Activity

Three different sets of factors may be of importance in regulating activity in vivo. These are:

a) FDP and PEP
b) Nucleotides
c) inorganic cations and anions

a) FDP and PEP as regulators of pyruvate kinase activity

As with most other FDP-activated pyruvate kinases, FDP increased the affinity for the substrate PEP and changed the kinetic response to varying PEP concentration from a sigmoidal pattern at low FDP to a hyperbolic response at saturating FDP. The FDP $_{0.5V}$ values required for this activation were higher than for mammalian pyruvate kinases. As well as the heterotropic interaction between PEP and FDP, the activator, FDP, itself showed positive homotropic interaction with the enzyme, showing a high degree of co-operativity especially with the freshly isolated enzyme (n_H = 2.5 to 2.7). The allosteric activation of enzyme activity

by FDP was independent of PEP and ADP concentration. Even at pH 8.75, FDP did activate the enzyme, but in a hyperbolic manner rather than a sigmoidal activation. The S. lactis C_{10} pyruvate kinase showed virtually no activity when assayed in the absence of FDP. . The S. lactis ML_3 pyruvate kinase (Collins and Thomas, 1974) did show activity in the absence of FDP at high PEP and metal ion concentrations. The S. lactis C_{10} enzyme when assayed in the presence of saturating $MnCl_2$ concentrations showed significant activity in the absence of FDP, but activity further increased with addition of a saturating FDP concentration.

At unsaturating levels of FDP, PEP binds co-operatively to the enzyme. This homotropic activation by the substrate PEP may be of regulatory significance under such conditions. The other substrate, ADP, showed only very weak co-operativity in binding to the enzyme and this only occurred at very low FDP concentrations.

b) Regulation by nucleotides

Nucleoside diphosphates can influence pyruvate kinase activity both by their involvement as substrates and by acting as inhibitors at high concentrations. As mentioned earlier, ADP binding follows a hyperbolic saturation relationship except at very low FDP concentrations when the relationship is weakly sigmoidal in comparison to PEP and FDP. The affinity for GDP is significantly higher than for ADP, therefore GDP and possibly other di-nucleotides may play an important role in controlling the intracellular activity of the enzyme, and thus should be investigated further. Waygood and Sanwal (1974) found that the E. coli pyruvate kinase also showed a higher affinity for GDP in comparison to ADP.

At concentrations only slightly higher than those required to saturate the enzyme, ADP acts as an inhibitor. This finding along with the observed inhibition at high Mg^{++} concentrations could indicate that $MgADP^-$ is the substrate with free Mg^{++} and ADP^{3-} acting as inhibitors. However this might not necessarily be so as ADP and Mg^{++} could inhibit by binding to sites other than at the catalytic site. A detailed kinetic analysis of the reaction mechanism would be necessary to determine the nature of the inhibition by high ADP concentration.

ATP was shown to be a potent inhibitor ($ATP_{0.5I}$ = 2.3 to 5.3 mM) of the S. lactis C_{10} pyruvate kinase. The degree of inhibition was independent of the FDP concentration whereas the S. lactis ML_3 enzyme (Collins and Thomas, 1974) was only inhibited by ATP at unsaturating FDP concentrations. Therefore from in vitro evidence the ADP/ATP

ratio may play an important role in controlling the intracellular activity of pyruvate kinase. However, in considering ADP and ATP inhibition, two additional factors have to be considered. First is the finding of Carminatti et al. (1968) that the presence of Cu^{++} in ADP samples was responsible for the apparent ADP inhibition of rat liver type L pyruvate kinase activity. Cu^{++} was shown in the present study to be a potent inhibitor of the S. lactis pyruvate kinase. Cu^{++} completely inhibited activity at a concentration of 0.1 mM in the standard assay and therefore a 0.3% contamination of copper in a 5 mM ADP (ATP) solution would be more than sufficient to completely inhibit activity. A second consideration is that the S. lactis pyruvate kinase is very sensitive to phosphate inhibition (Phosphate $_{0.5I}$ = 0.65 mM). A 2% contamination of phosphate in a 5 mM ADP (ATP) solution would be sufficient to inhibit 50% of activity. Even though Sigma grades of these nucleotides were used the presence of Cu^{++} and phosphate should be tested before any conclusions on the importance of ADP and ATP inhibition in vivo can be drawn.

Unlike some other pyruvate kinases, for example, those from Brevibacterium flavum (Ozaki and Shiio, 1969) and Bacillus licheniformis (Tuominen and Bernlohr (1971b), no AMP activation of the S. lactis C_{10} enzyme was apparent. In fact AMP inhibited activity (AMP $_{0.5I}$ = 7.0 mM).

c) Regulation by inorganic cations and anions

The S. lactis C_{10} enzyme can be activated and inhibited by a number of monovalent and divalent cations. The enzyme has an essential requirement for activity for both a monovalent and divalent cation.

Of the four monovalent cations studied, NH_4^+ and K^+ allosterically activated the enzyme to a similar extent. Na^+ and Li^+ can only weakly activate and at higher concentrations inhibit the enzyme.

Only three of the divalent cations studied (Mg^{++}, n^{++} and Co^{++}) could activate the enzyme. Of these three, only with Mn^{++} as the divalent cation was significant activity detectable without FDP. Mg^{++}, in excess of saturating activating concentrations allosterically inhibited activity. Other divalent cations (Cu^{++}, Zn^{++}, Ca^{++}, Ni^{++} and Ba^{++}) though not activating did allosterically inhibit the enzyme's activity at low concentrations (compared to the concentration required for Mg^{++} inhibition) but with a n_H value considerably less than for Mg^{++} inhibition.

Both the divalent and monovalent activating cations allosterically activate the enzyme even in the presence of saturating FDP concentrations, an apparently unique feature among FDP activated pyruvate kinases. Higher cation concentrations than those used in the standard assay while not altering the n_H value for FDP binding did appear to decrease the n_H value for PEP binding to pyruvate kinase. The relationship between cation activators and other allosteric effectors (FDP, ADP and PEP) could be important in in vivo control and therefore should be more thoroughly investigated in future studies.

The activation of S. lactis C_{10} pyruvate kinase by Mn^{++} in the absence of FDP is very interesting. A similar apparent replacement of the essential requirement for FDP by Mn^{++} was found for the S. faecalis LDH (Section 2.5) although not for the S. lactis LDH. However the activation by Mn^{++} appears to be different from that of FDP, since the V_{max} in the presence of saturating $MnCl_2$ (no FDP) is lower than the V_{max} obtained in the presence of saturating $MgCl_2$ and FDP concentrations. Also the V_{max} in the presence of saturating $MnCl_2$ is increased by the addition of saturating amounts of FDP. The affinity for PEP in the presence of saturating amounts of FDP is much higher (ten times) if Mn^{++} is the divalent cation rather than Mg^{++}. Inhibition by high ADP is less marked when Mn^{++} rather than Mg^{++} is the divalent cation and the effect of pH on the kinetic properties of the enzyme is also different depending on whether Mn^{++} or Mg^{++} is the divalent cation. Such differences suggest that Mn^{++} has its own distinctive effect on enzyme conformation independent of the effect of FDP.

Even though the manner in which Mn^{++} activates the S. lactis enzyme is not known,the possible physiological significance of the manganese effect can be appreciated. The presence of Mn^{++} in the cell, at least from the in vitro kinetic evidence, could effectively modify the control of pyruvate kinase by FDP.

Inorganic anions as well as cations may have an important regulatory function. Of particular importance is the high sensitivity to phosphate ions (Phosphate $_{0.5I}$ = 0.65 mM) at concentrations that might well occur in vivo. Phosphate increases the PEP and FDP requirement of the enzyme and may imply that a high intracellular pyruvate kinase activity in the presence of phosphate can only be achieved by high in vivo FDP and PEP concentrations.

Maleate and sulphate anions and tris and cyclohexylammonium cations inhibit activity to different extents. However their inhibition effects are probably not important in relation to physiological control of the activity of the enzyme. The sensitivity of pyruvate kinase to a wide range of ions, including commonly used buffering ions such as tris and maleate, and ions that may be present in commercially supplied substrates such as the cyclohexylammonium ion highlight the care that must be exercised in extrapolating from *in vitro* kinetic studies to *in vivo* conditions.

The overall study of the *S. lactis* pyruvate kinase has shown that a large number of factors control its activity *in vitro*. The relevance of all these factors in relation to *in vivo* control of the enzyme is not known. A study of the relevant intracellular metabolite concentrations may be helpful in this respect. From this study on pyruvate kinase, it is evident that further investigation of the enzyme's properties is desirable to understand the role of the enzyme in metabolic control. With the complexity of factors affecting pyruvate kinase activity, a study of the intracellular metabolite concentrations may help in determining relevant *in vitro* properties to be further studied.

SECTION 4.

6-PHOSPHOGLUCONATE DEHYDROGENASE

4.1 Introduction

A brief investigation of the 6-phosphogluconate dehydrogenase (E.C.1.1.1.44) of S. lactis C_{10} was prompted by the observation of Brown and Wittenberger (1971a) that a partially purified preparation from S. faecalis was inhibited by FDP. FDP caused a decrease in V_{max} and an increase in the apparent K_M for 6-phosphogluconate and nicotinamide adenine dinucleotide phosphate ($NADP^+$). FDP did not however completely inhibit activity and the presence of 2-mercaptoethanol blocked the inhibition by FDP without effecting the catalytic activity. Brown and Wittenberger (1971a) also found that the 6-phosphogluconate dehydrogenase (6-PGDH) from other sources (e.g. Lactobacillus plantarum, E. coli, Saccharomyces cerevisiae, Candida utilis and guinea pig liver) were sensitive to inhibition by FDP. This suggested that inhibition of 6-phosphogluconate dehydrogenase by FDP may be a fairly general phenomenon.

From the above results Brown and Wittenberger (1971a) concluded that the regulation of the hexose-monophosphate pathway activity is effected in S. faecalis by FDP inhibition of 6-PGDH. Uncontrolled use of the oxidative portion of the hexose-monophosphate pathway is not desirable as this would lead to an imbalance in the proportion of biosynthetic intermediates such as pentose-phosphate and of NADPH, to the ATP produced by the glycolytic pathway. The regulatory role of FDP in S. faecalis is achieved by FDP inhibition of the hexose-monophosphate enzyme, 6-PGDH, on one hand and FDP activation of the glycolytic enzyme, LDH (Wittenberger and Angelo, 1970), on the other hand. Therefore the intracellular concentration of FDP can direct glucose carbon to proceed preferentially through the glycolytic pathway (at high in vivo FDP concentration) or through the hexose-monophosphate pathway (at low in vivo FDP concentration). With the demonstration that 6-phosphogluconate dehydrogenase activity was inhibited by FDP in crude extracts of several different organisms, Brown and Wittenberger (1971a) suggest that this regulatory mechanism may be of general significance and deserves further consideration.

Therefore the S. lactis C_{10} 6-phosphogluconate dehydrogenase was studied to ascertain the effect of FDP on its activity. The S. lactis C_{10} glucose-6-phosphate dehydrogenase (E.C.1.1.1.49) was also studied to discover whether FDP had any effect on its activity.

In view of the difficulty encountered in demonstrating the inhibition of S. lactis C_{10} 6-PGDH, the study was extended to a brief examination of the S. faecalis ATCC 8043 6-PGDH in an attempt to reproduce the results of Brown and Wittenberger (1971a).

4.2 Methods

4.2.1 6-Phosphogluconate Dehydrogenase Assay

6-PGDH activity was estimated by measuring the increase in absorbance at 340 nm resulting from the 6-phosphogluconate-dependent reduction of $NADP^+$. The standard assay system (for both the S. lactis C_{10} and S. faecalis ATCC 8043 enzymes) contained in a total volume of 3 cm^3 : 80 mM triethanolamine/HCl buffer pH 7.5; 2.0 mM disodium 6-phosphogluconate (Sigma Chemical Company, Sigma Grade); 1.0 mM $NADP^+$(Sigma Grade) and 0.1 cm^3 of enzyme sample. Reactions were usually initiated by addition of enzyme sample.

4.2.2 Glucose-6-Phosphate Dehydrogenase Assay

Glucose-6-phosphate dehydrogenase activity was measured by following the increase in absorbance at 340 nm resulting from the glucose-6-phosphate-dependent reduction of $NADP^+$. The standard assay system contained in a total volume of 3 cm^3 : 80 mM triethanolamine/HCl buffer pH 7.5; 2.0 mM monosodium D-glucose-6-phosphate (Sigma Grade); 1.0 mM $NADP^+$and 0.1 cm^3 of enzyme sample. Reactions were initiated by addition of enzyme sample.

Assays for both enzymes were carried out at 25°C using a Beckman ACTA-3 spectrophotometer.

An absorbance change of 1.0 unit per minute is used to express enzyme activity (for both enzymes). Protein concentration was determined by the method of Lowry et al. (1951). Specific activity is expressed as units per milligram of protein.

4.2.3 Partial Purification of the 6-Phosphogluconate Dehydrogenase and Glucose-6-Phosphate Dehydrogenase.

S. lactis C_{10} was maintained as described in Section 2.2.1. The growth and harvest of the S. lactis cultures was as described in Section 3.3.1. The harvested cells were stored frozen overnight before disruption of cells.

On the first partial purification of the two enzymes 2-mercaptoethanol (2-ME)was present in the buffers, whereas in a second purification 2-ME was omitted from the buffers. The following method has 2-ME present.

A. Breakage of cells. Cells were thawed and suspended in 0.01 M phosphate buffer + 0.05% 2-ME pH 7.0 and disrupted by two passages through an Aminco French pressure cell at 5,500 lbs per in^2. Unbroken cells

and cell debris were centrifuged down at 13,000 g for fifteen minutes at 4°C. All subsequent purification steps were carried out at 4°C.

B. Streptomycin sulphate treatment. Nucleic acids were precipitated from the cell free extract by dropwise addition of streptomycin sulphate using 3.0 cm^3 of a 10% (w/v) solution for every 100 mg protein. The resulting suspension was allowed to stand for two hours before the precipitate was removed by centrifugation at 13,000 g for fifteen minutes.

C. Ammonium sulphate precipitation. The supernatant after streptomycin sulphate treatment was dialysed against 0.01 M phosphate buffer + 0.1% 2-ME pH 7.0 for fifteen hours. Both enzymes were precipitated between 50 to 75% $(NH_4)_2SO_4$ saturation by the method as described in Section 3.3.4. The precipitate was redissolved in 0.01 M phsophate buffer + 0.1% 2-ME and dialysed against the same buffer for fifteen hours.

The partial purification of the S. lactis C_{10} 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase is shown in Table 4.2.3.

Table 4.2.3

Partial purification of the S. lactis C_{10} 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase.

Values obtained using the purification method in the absence of 2-mercaptoethanol are shown in brackets.

	Activity (units/cm^3)	Total Activity (units)	Specific Activity (units/mg protein)
Cell Free Extract			
6-phosphogluconate dehydrogenase	2.2 (2.4)	705 (710)	0.134 (0.130)
Glucose-6-phosphate dehydrogenase	5.4 (5.8)	1730 (1740)	0.330 (0.314)
Dialysed Streptomycin Sulphate Supernatant			
6-phosphogluconate dehydrogenase	1.7 (1.8)	790 (810)	0.162 (0.164)
Glucose-6-phosphate dehydrogenase	3.2 (3.6)	1520 (1620)	0.304 (0.330)
Redissolved Dialysed 50-75% $(NH_4)_2SO_4$ Precipitate			
6-phosphogluconate dehydrogenase	3.4 (4.2)	370 (390)	0.227 (0.237)
Glucose-6-phosphate dehydrogenase	9.6 (10.7)	1050 (995)	0.640 (0.600)

S. faecalis ATCC 8043 was grown on a glucose medium as described in Section 2.5.2. The partial purification of *S. faecalis* 6-phosphogluconate dehydrogenase simply involved treating a cell-free extract in 0.01 M phosphate buffer pH 7.0 with streptomycin sulphate as described in Section 2.5.2. No 2-ME was present in the buffers. The initial specific activity of the 6-PGDH in the *S. faecalis* crude extract was 0.156 units/mg and was only slightly increased by the streptomycin sulphate treatment to 0.171 units/mg.

4.3 Results

4.3.1 Studies on the Partially Purified 6-Phosphogluconate Dehydrogenase from *S. lactis* C_{10}.

The enzyme samples from the cell free extract, dialysed streptomycin sulphate supernatant and the redissolved dialysed $(NH_4)_2SO_4$ precipitate were all used in the study of the effect of FDP on the *S. lactis* 6-PGDH activity. The enzyme samples from the preparation where 2-ME was present in the buffers were all dialysed in 0.01 M phosphate buffer pH 7.0 for 24 hours prior to studying the FDP effect. Removal of 2-ME was necessary as Brown and Wittenberger (1971a) showed that the presence of 2-ME completely blocked the FDP inhibition of 6-PGDH from *S. faecalis*.

Using the standard assay conditions (2 mM 6-phosphogluconate and 1.0 mM $NADP^+$) with either the standard assay buffer (80 mM triethanolamine/HCl buffer pH 7.5), 80 mM tris/HCl buffer pH 7.5 or 80 mM phosphate buffer KH_2PO_4/NaOH pH 7.5, the activity of the samples was measured in the absence of FDP and then in the presence of 0.1, 1.0, 5.0 and 10 mM FDP. The 6-phosphogluconate and $NADP^+$ were decreased to 0.2 mM and 0.1 mM respectively, and the above assay procedures repeated. The enzyme samples prepared in both the presence and absence of 2-ME and from all three stages of the partial purification were used to study the effect of FDP on activity.

Under none of the above assay conditions, using any of the enzyme samples, did any of the FDP concentrations inhibit the *S. lactis* 6-PGDH activity. Enzyme activity was also unaffected by the choice of the assay buffer. If NAD^+ replaced $NADP^+$ as the coenzyme, then no activity was detected.

In the first attempt to show the FDP inhibition of the enzyme's activity the enzyme was purified in the presence of 2-ME. The enzyme samples were dialysed free of 2-ME before the FDP effect was studied.

However as there was no FDP inhibition of activity, it was possible that the purification in 2-ME had irreversibly altered the enzyme's sensitivity to FDP. Therefore the enzyme was purified in the absence of 2-ME, but the enzyme was still insensitive to FDP inhibition.

In a personal communication from Dr C.L. Wittenberger it was suggested that the order of addition of components of the assay mixture was important. The reaction when initiated with $NADP^+$ rather than enzyme, was more sensitive to FDP inhibition. Therefore FDP inhibition was also tested by adding $NADP^+$ last to initiate the S. lactis C_{10} 6-PGDH reaction. In this assay, the assay components were added to the curvette in the following order: buffer, 6-phosphogluconate, FDP and enzyme. The components are mixed and incubated at 25°C for three to five minutes. The reaction was then initiated by adding $NADP^+$. The two different $NADP^+$ and 6-phosphogluconate concentrations were used and the FDP was tested at concentrations of 0.1, 1.0, 5.0 and 10 mM FDP. Again, however, no inhibition by FDP was detected.

4.3.2 Studies on the 6-Phosphogluconate Dehydrogenase from S. faecalis ATCC 8043.

Because of the failure to demonstrate FDP inhibition of the S, lactis C_{10} 6-PGDH, attempts were made to repeat the observation of Brown and Wittenberger (1971a) on the 6-PGDH of S. faecalis MR (also strains 10Cl and N-55). Note that in this study a different S. faecalis strain is being used.

The samples from the cell-free extract and the dialysed streptomycin sulphate supernatant from S. faecalis were assayed for the effect of FDP on 6-PGDH activity. The same assay conditions were tried for the S. faecalis enzyme as were used in the study of the FDP effect of the S. lactis 6-PGDH activity.

At none of the assay conditions did any of the FDP concentrations inhibit the S. faecalis 6-PGDH activity. The enzyme's activity was also unaffected by the choice of the assay buffer.

4.3.3 Studies on the Partially Purified Glucose-6-Phosphate Dehydrogenase from S. lactis C_{10}.

The effect of FDP on the S. lactis C_{10} glucose-6-phosphate dehydrogenase activity was studied using the same conditions and the

same enzyme samples as described (Section 4.3.1) for studying 6-PGDH except that the substrate was glucose-6-phosphate at a concentration of 2.0 mM or 0.2 mM.

The glucose-6-phosphate dehydrogenase was not affected by FDP under any of the assay conditions.

4.4 Discussion

The 6-phosphogluconate dehydrogenase in relatively crude preparations from S. lactis C_{10} and from S. faecalis ATCC 8043 was shown to be insensitive to FDP inhibition under all of the assay conditions tried. This differs from the results of Brown and Wittenberger (1971a) where the enzyme from S. faecalis MR (also for two other S. faecalis species and from five other unrelated organisms) was specifically inhibited by FDP. While the discrepancy between the finding on the present study and those of Brown and Wittenberger (1971a) and later extensive studied by Bridges and Wittenberger (in press) have not been resolved, some possible reasons for the difference should be considered.

Bridges and Wittenberger (in press) state that the FDP inhibition of the S. faecalis MR (now designated as ATCC 27792) 6-phosphogluconate dehydrogenase is a complex phenomenon. Buffers such as imidazole, glycylglycine, Bicine and histidine markedly decrease the degree of inhibition by FDP. Brown and Wittenberger (1971a) showed that the presence of 2-mercaptoethanol prevents FDP from inhibiting the S. faecalis 6-PGDH. Removal of 2-ME by dialysis restores FDP sensitivity. If ethylenediaminetetra acetate (EDTA) was present in buffers, the enzyme became insensitive (or very much less sensitive) to FDP. However dialysis of free EDTA did not restore FDP activity.

In the present study, no EDTA was present at any stage of the purification or assay, the use of 2-ME was avoided in the second purification from S. lactis and was not present in the crude extract from S. faecalis. The same buffer (tris/HCl) as that used by Bridges and Wittenberger was one of the assay buffers used in the present study. None of these factors will therefore account for the difference between the findings of the present study and those of Bridges and Wittenberger. The latter workers have found that inhibition is dependent on protein concentration in crude extracts. The inability to demonstrate FDP inhibition in the present study with relatively crude preparations from both species must be re-investigated on more highly purified 6-phosphogluconate dehydrogenases.

The use of relatively crude preparations is open to the objection that other enzymes such as phosphatases or FDP aldolases may be present and hence could result in rapid removal of the FDP in the assay mixture. However, Brown and Wittenberger (1971a) found 60-80% inhibition of 6-PGDH in 10 mM FDP in crude cell-free extracts of S. faecalis and other species. No inhibition was found at 10 mM FDP in the present work on the S. lactis enzyme. Even if aldolase or phosphatases were present at a specific activity of 10 units/mg protein (i.e. 50 times higher than the 6-PGDH specific activity) it would take two minutes at least to decrease the FDP concentration from 10 mM to 1 mM. Since 1 mM FDP inhibited the partially purified 6-PGDH by 60% in the work of Brown and Wittenberger at least a transient inhibition by 10 mM FDP would have been detected in the present study. In the work with LDH and pyruvate kinase crude extracts pre-incubated with 1 mM FDP for two to three minutes before starting the reaction with pyruvate and PEP respectively, marked activation was found, indicating that FDP was not degraded to any significant extent in crude extracts.

Brown and Wittenberger (1971a) found that the S. faecalis MR 6-PGDH in partially purified extracts was not completely inhibited even by high concentrations of FDP. From this observation they suggested the possibility that two forms of the enzyme may exist, one being sensitive to FDP and the other being insensitive to this inhibitor. These could be interconvertible forms or genetically distinct isoenzymes. It would seem unlikely that one form of the 6-phosphogluconate dehydrogenase should be present in this study and the other form predominate in the study of Brown and Wittenberger (1971a).

It is possible that undefined differences in the conditions of preparation of the enzyme or bacterial growth between the present study and that of Brown and Wittenberger (1971a) may have resulted in the transformation of one form of the enzyme to another. In the present study the culture was grown without aeration but the medium was in contact with air whereas Brown and Wittenberger used strictly anaerobic conditions of growth. As described for pyruvate kinase (Section 3.1), both the type L and type M pyruvate kinases have been shown by a number of workers to exist in two interconvertible forms, each with distinctive kinetic properties, e.g. one form being sensitive to FDP activation and the other form being insensitive to FDP activation.

The medium used in the present study has 5 to 10 fold higher levels of sugars, tryptone and yeast extract than the medium in which Brown and Wittenberger grew their S. faecalis. If there exists regulated and unregulated isoenzymes (or interconvertible forms) of 6-PGDH then it is plausible that the regulated 6-PGDH might be repressed under conditions of high carbohydrate and nutrient availability.

S. faecalis MR also possesses a NAD^+-specific 6-phosphogluconate dehydrogenase which was present only when cells were grown on gluconate (not on glucose or lactose) as the carbon source (Brown and Wittenberger, 1972a). The NAD^+-linked enzyme was insensitive to FDP but was inhibited by ATP. This contrasts with the constitutive $NADP^+$-linked enzyme which is specifically inhibited by FDP but is insensitive to ATP. In this study, S. lactis C_{10} and S. faecalis ATCC 8043 were both grown on glucose or lactose media lacking gluconate so the inducible NAD^+-6-PGDH would not be present. This was confirmed for S. lactis C_{10} as the extracts showed no 6-PGDH activity if NAD^+ was the coenzyme.

Clearly this preliminary study has indicated that further work is required before the function of the S. lactis C_{10} and the S. faecalis ATCC 8043 6-phosphogluconate dehydrogenase in the control of carbohydrate metabolism can be discussed with any degree of certainty.

SECTION 5.

IN VIVO METABOLITE CONCENTRATIONS

5.1 Introduction.

In studying the properties of the S. lactis C_{10} LDH and pyruvate kinase, a number of findings have indicated that a study of the intracellular concentrations may help to solve some of the problems raised by the in vitro kinetic studies, for example the widely different FDP requirements for the LDH, depending on the buffer used in the assay. Comparison of in vivo concentrations of effectors with kinetic properties determined in vitro by other workers has proved useful in assessing the physiological significance of particular properties.

Taylor and Bailey (1967) found that the high affinity of the liver pyruvate kinase for FDP was matched by a low intracellular FDP concentration, and suggested that the FDP stimulation constitutes a switching mechanism from liver glycolysis to gluconeogenesis via a positive feed-forward effect. Van Berkel et al. (1974) showed that if the pyruvate kinase (L-type) from rat liver was studied at in vivo concentrations of PEP then the kinetic properties are such that the enzyme can be controlled by FDP, contrary to the conclusion of Koster et al. (1972), who studied the enzyme at elevated non-physiological PEP concentrations.

There appear to have been relatively few previous studies of the concentration of glycolytic intermediates in lactic acid bacteria. Mizushima and Kitahara (1964) studied the intracellular concentrations of glycolytic intermediates in glucose-metabolising, washed cells of Lactobacillus plantarum. They used the methods of Bartlett (1959a and b) to isolate the intermediates by ion exchange column chromatography and determined the intracellular concentrations by chemical methods. Fermentation was stopped by pouring the reaction mixture into ten volumes of boiling water. The concentrations of some of the glycolytic intermediates were found to be quite high: 17 mM FDP; 39 mM 3-phosphoglycerate and 5.5 mM PEP.

A quicker and more sensitive method was used by Lowry et al. (1971), who studied the effect of carbon and nitrogen sources on the level of intracellular concentrations in logarithmically growing E. coli. The medium was separated from the cells by rapid filtration and cell metabolism was immediately stopped by rapid freezing of the filter plus cells. $HClO_4$ was used to extract the metabolites and fluorometric-enzymatic analysis measured the concentrations. This general method of Lowry et al. (1971) was used for the measurement of intracellular metabolites in S. lactis C_{10}.

Collins and Thomas (1974) used the method of Lowry *et al.* (1971) to measure the intracellular metabolite concentrations (PEP, ADP and FDP) present in exponentially growing *S. lactis* ML_3 and compared the values to the *in vitro* kinetic properties of pyruvate kinase.

The study by Collins and Thomas (1974) was therefore used as a basis for the study of the intracellular metabolite concentrations present in *S. lactis* C_{10}. The *in vivo* concentrations of ATP, ADP, FDP, PEP and pyruvate measured in *S. lactis* C_{10} will be related to the relevant *in vitro* kinetic properties of LDH and pyruvate kinase studied in Sections 2 and 3 of this thesis.

The intracellular metabolite concentrations were measured at different stages of growth of *S. lactis* C_{10}.

The LDH of *S. lactis* C_{10} was shown to have different *in vitro* properties from the LDH of *S. faecalis* (Section 2.5 of this thesis and Wittenberger and Angelo, 1970). The metabolite concentrations were also determined in *S. faecalis* to see if any major concentration differences exist between the two species that may relate to their distinctive *in vitro* LDH properties. Data for *Lactobacillus casei* var. *rhamnosus* (ATCC 7469) are also reported in this section since this species has a FDP-activated LDH in which the FDP $_{0.5V}$ value varies widely depending on assay conditions (Holland and Pritchard, 1975). The work on *L. casei*, reported in this section of the thesis, was carried out by Dr G.G. Pritchard at the same time and with the same methods as for *S. lactis* and *S. faecalis*.

5.2 Materials and Methods.

5.2.1 Organisms.

Streptococcus lactis C_{10} was maintained as described in Section 2.2.1 and grown at 30°C in the medium of Jago *et al.* (1971) (see Section 2.3.1).

Streptococcus faecalis ATCC 8043 was grown on the same medium and under the same conditions as for *S. lactis* C_{10}.

Lactobacillus casei var. *rhamnosus* (ATCC 7469) was obtained from the National Collection of Industrial Bacteria, Torrey Research Station, Scotland. It was grown at 30°C in the medium of Gasser (1970).

5.2.2 Chemicals.

Aldolase (10 units/mg), α-glycerophosphate dehydrogenase-triose phosphate isomerase (rabbit muscle type II), lactate dehydrogenase (rabbit muscle type II, 960 units/mg), pyruvate kinase (390 units/mg), glucose-6-phosphate dehydrogenase (type XII from Torula Yeast, 420 units/mg, sulphate free) and hexokinase (type F-300, from Bakers Yeast, 200 units/mg, sulphate free) were all obtained from the Sigma Chemical Company. Adenosine 5^1-triphosphate (ATP), Phosphoenolpyruvate (PEP), Adenosine 5^1-Diphosphate (ADP), NADH, $NADP^+$ and imidazole (low fluorescence blank) were also obtained from Sigma. $MgCl_2$, KCl and K_2CO_3 were obtained from BDH (Anala R grade). Glucose and $HClO_4$ were obtained from Hopkins and Williams, and Riedel-De Haen, respectively.

5.2.3 Extraction of Intracellular Metabolites.

The cell samples were collected at the times indicated in Section 5.3.1 and the intracellular metabolites were extracted by the method of Lowry et al. (1971) as modified by Collins and Thomas (1974). The cells were collected from 15, 25 or 50 cm^3 of medium, depending on cell density, by rapid filtration on a 47 mm diameter membrane filter (Millipore Corp., 0.8 μm pore diameter). As soon as the liquid had been removed (30 to 60 seconds) the filter was placed on a stainless-steel block which was partially immersed in liquid nitrogen. The frozen filter was broken with forceps and put into a 15 cm^3 centrifuge tube containing 5 cm^3 of 0.6 M $HClO_4$ at 0°C. The tube and contents were thoroughly mixed and left for twenty minutes on ice. A calculated amount of K_2CO_3 was added to the tube contents to neutralize the $HClO_4$. The resulting $KClO_4$ and

filter remains were removed by centrifugation and the supernatant fluid (pH 6.4 to 6.8) for each extracted sample was divided into three equal volumes and stored frozen until it could be analysed as described in the next section.

5.2.4 Measurement of the Intracellular Metabolites.

The extracted metabolites were stored frozen. The next day aliquots of the samples were thawed and used for determination of the intracellular concentrations of triosephosphates and FDP. Samples stored frozen for two days were used for determination of the intracellular concentrations of PEP, ADP and pyruvate. Samples stored frozen for three days were used for determinations of the intracellular concentration of glucose-6-phosphate (G6P) and ATP. The concentration of all metabolites appeared to be constant for at least two further days if the extracts were refrozen and subsequently thawed and the measurements repeated. It was therefore assumed that the limited storage of the extracts did not affect the concentration of the metabolites to any significant extent.

All measurements of the intracellular metabolites were performed by fluorometric-enzymatic analysis of the extracts at 25°C with NADH indicator systems as described by Lowry et al. (1971). A Model 430 Spectrofluorometer from Turner Associates was used, set at an excitation wavelength of 350 nm and an emission wavelength of 468 nm. The sensitivity of the Spectrofluorometer was kept constant by standardization with a quinine sulphate/H_2SO_4 solution. Though 85 mM imidazole was present in the assay mixture, the samples still gave some fluorescence, so that for each extract a suitable blank had to be used. The slight quenching of NADH fluorescence by the assay enzymes was accounted for by appropriate blanks. As well as standardizing the fluorescence changes with different concentrations of NADH, standardization was also carried out with the appropriate assay systems using known concentrations of ATP, FDP and pyruvate. The fluorescence changes obtained from equivalent concentrations (i.e. taking into account that FDP is equivalent to 2 NADH) were the same for both methods.

A) Measurement of Triosephosphates and FDP.

FDP was measured by coupling aldolase, triosephosphate isomerase and α-glycerophosphate dehydrogenase reactions and measuring the decrease in fluorescence due to removal of NADH after completion of the reaction. Triosephosphates were measured in samples by omitting

aldolase from the reaction mixture. The presence of triosephosphates in the extracts was corrected for, in the determination of the FDP concentration.

The reaction mixture contained 85 mM imidazole/hydrochloride buffer pH 7.0, 5 μM NADH, 30 μg aldolase, 6 μg of triosephosphate isomerase-α-glycerophosphate dehydrogenase mixture and extract (up to 0.1 cm^3) in a final volume of 2.5 cm^3.

B) Measurement of PEP, ADP and Pyruvate.

PEP and ADP were measured by coupling pyruvate kinase and LDH reactions and measuring the decrease in fluorescence due to NADH after completion of the reaction. Pyruvate was measured in the extracts by omitting pyruvate kinase from the reaction mixture. The presence of pyruvate in the extracts was corrected for in the determinations of PEP and ADP concentrations.

The reaction mixture contained 85 mM imidazole/hydrochloride buffer pH 7.0, 5 μM NADH, 4 mM $MgCl_2$, 80 mM KCl, 20 μg of LDH, 8 μg of pyruvate kinase, either 200 μM ADP or 80 μM PEP (for PEP and ADP measurements, respectively) and extract sample (up to 0.1 cm^3) in a final volume of 2.5 cm^3.

C) Measurement of ATP and G6P.

ATP was measured by coupling hexokinase and glucose-6-phosphate dehydrogenase reactions and measuring the increase in fluorescence of NADPH after completion of the reaction. Glucose-6-phosphate (G6P) was measured in the extracts by omitting hexokinase from the reaction mixture. The presence of G6P in the extracts was corrected for in the calculations of ATP concentrations.

The reaction mixture contained 85 mM imidazole/hydrochloride buffer pH 7.0, 30 μM $NADP^+$, 5 mM $MgCl_2$, 200 μM glucose, 2.5 μg of glucose-6-phosphate dehydrogenase, 10 μg of hexokinase and extract sample (up to 0.1 cm^3) in the final volume of 2.5 cm^3.

In the measurement of most intracellular metabolites (PEP, FDP, ATP, G6P, Pyruvate and triosephosphates) reaction was complete within five to ten minutes. However in the measurement of intracellular ADP, the reaction was still not complete after ten minutes. The reaction rate for the assay mixture measuring intracellular ADP was biphasic.

The first phase of the decrease in fluorescence was fast, lasting between five to ten minutes, followed by a much slower decrease in fluorescence (five to ten fold slower) which reached a constant value after sixty to seventy minutes. Using standard ADP this slower rate of decrease in fluorescence was not noticed. The fast phase of the reaction was therefore taken to be the correct measure of the intracellular ADP concentration. The continued slow decrease after completion of the fast phase may have been due to nucleotides other than ADP also being measured. Both the concentration of ADP calculated from the fluorescence change after the fast fluorescence decrease and concentration calculated from the total fluorescence change after the slow fluorescence will be given.

5.2.5 Determination of Intracellular Volume.

The intracellular volume of the bacteria harvested from cultures was determined by two methods.

Method A. Cell samples were collected from 25 to 50 cm^3 of medium on a 47 mm diameter membrane filter (Oxoid, 0.45 μm pore diameter). Just prior to collecting the samples, 25 to 50 cm^3 of medium was run through the filter. The wet filter was quickly weighed and then used to collect the cells. The wet weight of cells from 25 to 50 cm^3 of medium could then be calculated. From a graph of wet weight of cells/25 cm^3 medium against time the wet weights of samples taken for metabolite estimation could be determined.

The intracellular volume was calculated from the wet weight of cells on the assumption that the density of the cells was 1.1 g/cm^3 (a mean value taken for estimates for a number of bacterial species) and that the ratio of protoplasm to total cell volume was 5/6. The ratio of protoplasm to total cell volume was estimated from electron micrographs of S. lactis ML_3 (Thomas, 1968). This calculation of intracellular volume did not take into account the interstitial water and therefore would lead to high values being calculated for the intracellular volume.

Method B. The second method takes into account the interstitial water. A conversion factor, derived by J. Thompson, and reported by Collins and Thomas (1974) was used to convert the dry weight of cells to their intracellular volume. The method used by Thompson was based on that of Black and Gerhardt (1962) in which the volume external to the

protoplast membrane was determined with ^{14}C sucrose (which does not penetrate the membrane) and total fluid volume with tritiated water. Thompson determined that 1 g (dry weight) of bacteria had a protoplasmic volume of 1.6 cm^3. He has found that this conversion factor is consistant for several streptococcal species, including *S. lactis* C_{10}. The use of this factor in determining the intracellular volume for *S. faecalis* ATCC 8043 and *L. casei* may be subject to some error.

5.2.6 Measurement of Manganese Content of Cells.

The manganese content of *S. lactis* C_{10} cells was determined as follows: One sample was collected from exponentially growing cultures (0.125 g wet weight of cells/25 cm^3 medium) and another sample from cultures near the end of the logarithmic phase of growth (0.24 g wet weight of cells/25 cm^3). For both samples, the cells were collected from 450 cm^3 of medium by centrifugation at 13,000 g for twenty minutes. The pellet of precipitated cells was quickly resuspended in 1% NaCl solution and reprecipitated by centrifugation at 13,000 g for ten minutes. This washing procedure was repeated once more before the cells were frozen. One week later the frozen cells were dried at 100°C, then ashed at 500°C. The ashed material was dissolved in 10 cm^3 of 0.1 M HCl and the manganese concentration was measured on an Atomic Absorption Spectrophotometer (Varian-Techtron) by Dr R. Brooks.

5.2.7 Determination of Reducing Sugar Present in Medium.

The amount of reducing sugar present in the medium at different stages of growth was determined. Cells from growing cultures were removed from the medium (20 cm^3) by centrifugation at 15,000 g for ten minutes. The medium was then frozen until used for determination of the reducing sugar.

The reducing sugar content in the cell-free medium was determined by the Somogyi-Nelson Method (Somogyi, 1952). To 1 cm^3 of suitably diluted sample, 1 cm^3 of Somogyi's copper reagent was added and the mixture placed in a boiling waterbath for ten minutes. The mixture was cooled and 1.0 cm^3 of Nelson's arsenomolybdate reagent was added, followed by 7 cm^3 of distilled water. The sample was mixed and absorbance read at 520 nm. A standard curve was constructed using known amounts of lactose as the reducing sugar.

5.3 Results and Discussion from Studies on the Intracellular Metabolite Concentrations.

5.3.1. Growth of S. lactis C_{10}

In studying the intracellular concentrations of metabolites in S. lactis C_{10}, two cultures were grown. The first culture was a trial run, so only four samples were taken to determine the intracellular concentrations. Two of these were from log phase and two when the culture had entered stationary phase. In the second experiment, eight samples were taken to determine the intracellular concentrations. It was intended to take samples covering a range of growth stages from mid-exponential phase to the stationary phase. However, before the last three samples from the second experiment were taken, the pH of the medium was inadvertently increased to pH 9.5 by excess addition of NaOH (which was being added to maintain the pH near 6.5). The growth of the S. lactis C_{10} in the second experiment is shown in Figure 5.3.1. In the same figure, the reducing sugar concentration is shown as a function of the S. lactis C_{10} growth. The jump in pH from 6.25 to 9.5, six hours after inoculation, caused a rapid cessation of bacterial growth and of reducing sugar utilization.

5.3.2 Intracellular Metabolite Concentrations from S. lactis C_{10}: First Experiment.

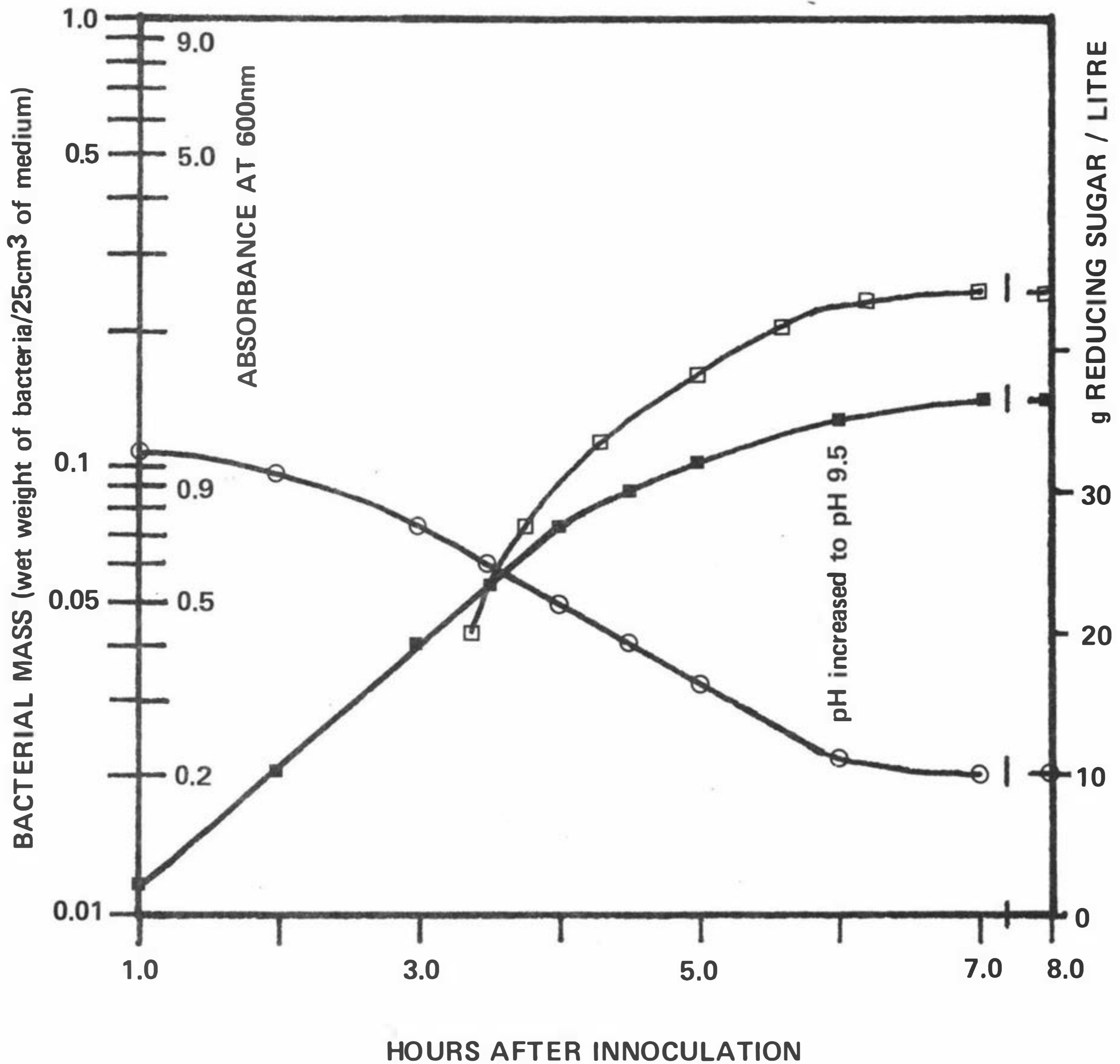
For the first experiment, two samples were taken from exponentially growing cultures and two samples were taken from the stationary phase at the end of growth. The intracellular concentrations of the metabolites (FDP, triosePO_4'S, G6P, ATP, Pyruvate, PEP and ADP) were determined in the four samples. Results are shown in Tables 5.3.2 a and b.

The intracellular concentrations, as estimated using intracellular volumes calculated by Method B, are 1.9 times higher than the concentrations estimated from the intracellular volumes calculated by Method A. This difference indicates that the error introduced in Method A by interstitial water is significant.

The two samples from the exponentially growing culture gave very similar metabolite concentrations. The two samples from the stationary phase also gave very similar concentrations. However some of the metabolites are present at different concentrations at the two different sampling times. The metabolites FDP, G6P and ATP appear to decrease

Figure 5.3.1

GROWTH OF S. lactis C10



The growth of Streptococcus lactis C10 in the routine medium is shown. The pH was increased from the normal pH of 6.25 + 0.25 to pH 9.5 with excess NaOH 6 hours after innoculation. The medium was innoculated with a log phase culture.

KEY: (□), bacterial mass (wet weight of bacteria/25cm^3 of medium).
(■), absorbance at 600nm.
(○), g reducing sugar/litre.

Table 5.3.2 a and b

The intracellular concentrations of metabolites

Table a

First experiment for *S. lactis* C_{10} where Method A of Section 5.2.5 was used to determine the intracellular volume.

Wet Weight of bacteria ($g/25\ cm^3$)	Intracellular concentration of metabolites (mM)							
	FDP	Triose PO_4S	G6P	ATP	Pyruvate	PEP	ADP*A	ADP*B
0.114	6.3	1.7	3.3	9.1	0.93	0.86	3.5	17.6
0.126	7.3	1.8	3.4	8.6	0.95	0.92	3.2	17.0
0.364	2.0	1.6	1.5	2.8	1.30	1.05	2.0	15.4
0.363	2.1	1.6	1.0	2.6	1.20	1.00	2.1	15.6

Table b

First experiment for *S. lactis* C_{10} where Method B of Section 5.2.5 was used to determine the intracellular volume.

0.114	12.0	3.2	6.3	17.2	1.7	1.6	6.6	33.0
0.126	13.8	3.4	6.5	16.3	1.8	1.7	6.0	32.0
0.364	3.8	3.0	2.8	5.1	2.5	2.0	3.9	28.0
0.363	4.0	3.0	1.9	4.9	2.3	1.9	4.0	29.5

*A The ADP concentration that is calculated only from the fluorescence change from the fast phase of the reaction.

*B The ADP concentration that is calculated from the total fluorescence change after the slow phase of the reaction was completed.

approximately three fold in concentration as the exponentially growing cultures enter stationary phase. These results indicate that it is important to investigate the intracellular metabolite concentration at different phases of growth of S. lactis C_{10}.

5.3.3 Intracellular Metabolite Concentrations from S. lactis C_{10}: Second Experiment.

In the second experiment, eight samples were taken from the S. lactis C_{10} culture. Five of these samples were taken at successive time intervals from exponentially growing cultures. The last three samples were taken late in exponential phase, after the medium pH had been increased to 9.5 (see Figure 5.3.1). The intracellular concentrations of metabolites in S. faecalis and L. casei (each from two samples), from exponentially growing cultures, were also determined. The results for the different metabolite concentrations present in S. lactis, S. faecalis and L. casei are shown in Table 5.3.3. The protoplast volume as calculated by Method B was used to estimate the intracellular concentrations.

The concentrations for the metabolites present in exponentially growing cells of S. lactis C_{10} are only slightly higher than the two values taken from samples at a similar growth phase in the first experiment.

The intracellular concentrations present in the three samples (see Table 5.3.3) taken from the S. lactis culture after the pH had been accidentally increased to 9.5, are substantially different from the concentrations determined from the five samples obtained from the exponentially growing cultures. The FDP concentration has decreased by ten fold, the G6P by four fold and the ATP concentration by eighteen fold. The pyruvate, PEP and ADP concentrations on the other hand, have increased at least two fold as the pH was increased to pH 9.5. The changes in the concentrations may be due, in part, to the culture being near the end of the exponential growth phase, but by far the most important reason for the change would be the alkaline pH stress. Since the pH increase is well above the limit that cells are likely to encounter under natural conditions, it would be unwise to draw conclusions on the physiological significance of the change induced. The lowering of the FDP concentration could result in increasing the PEP and pyruvate concentrations by changing the activity of the LDH and pyruvate kinase. The fact that the intracellular metabolites change their concentrations markedly with

Table 5.3.3

The intracellular concentration of metabolites in S. lactis, S. faecalis and L. casei.

This was the second experiment for S. lactis C_{10}. For all three species Method B of Section 5.2.5 was used to determine the intracellular volume.

Wet Weight of bacteria ($g/25\ cm^3$)	Intracellular concentration of metabolites (mM)								
	Mn^{++}	FDP	Triose PO_4S	G6P	ATP	Pyruvate	PEP	ADP*A	ADP*B
S. lactis									
0.055		14.9	4.5	9.6	19.8	2.6	1.9	7.6	41.5
0.109		14.9	4.0	5.7	18.0	1.9	1.7	7.8	44.0
0.118	0.50	13.2	3.2	6.4	20.8	3.2	1.9	7.7	43.0
0.155		13.2	2.5	8.3	18.9	3.4	2.3	7.9	45.0
0.189		12.7	2.3	4.2	11.7	2.5	1.7	7.7	43.0
0.241		1.8	1.3	1.3	1.3	11.4	5.3	15.5	75.0
0.240		1.1	1.3	0.94	0.94	11.9	5.7	15.7	77.0
0.240	0.26	1.0	1.3	0.94	1.3	12.3	5.7	15.3	75.0
S. faecalis									
0.021		4.7	2.7	4.2	4.9	24.0	7.6	8.5	47.0
0.023		4.3	2.3	4.0	5.2	26.0	8.5	8.5	47.0
L. casei									
0.071		22.0	3.2	6.4	14.2	6.2	3.6	9.4	55.0
0.098	50.0	11.7	3.2	5.7	12.9	3.8	3.6	8.9	49.0

*A The ADP concentration that is calculated only from the fluorescence change from the fast phase of the reaction.

*B The ADP concentration that is calculated from the total fluorescence change after the slow phase of the reaction was completed.

the alkaline pH stress does illustrate that further experiments using less extreme stresses and studying their subsequent effect on the intracellular metabolite concentrations might lead to a better understanding of streptococcal glycolytic control.

In comparing the intracellular metabolite concentration of the three species studied (results shown in Table 5.3.3) it must be borne in mind that the growth media for the three species are somewhat different, the stage of growth at which the samples were taken is considerably earlier for S. faecalis than for the other two species, and the factor used for calculating intracellular volume of S. lactis may be different for the other two species.

There is however quite close agreement between the concentrations of metabolites in S. lactis and those of L. casei except that pyruvate and PEP are higher in L. casei. On the other hand, the concentrations in the two early log phase samples from S. faecalis are quite different from the concentrations in the earliest samples of the S. lactis cultures. Notable differences are the lower FDP concentration in S. faecalis and also the lower ATP/ADP ratio and the much higher pyruvate and PEP concentrations. It is interesting to note that high pyruvate and PEP concentrations correlated with low FDP and ATP in the samples from S. faecalis. This trend was also evident in the last three samples from S. lactis where lactose uptake had presumably ceased.

The Mn^{++} concentrations of S. lactis and L. casei are very different. L. casei was grown in a medium containing 0.22 mM Mn^{++} whereas no Mn^{++} was added to the S. lactis medium. The high Mn^{++} concentration in L. casei is of interest in relation to the activation of the LDH in this species by Mn^{++} (de Vries et al., 1970; Holland and Pritchard, 1975). The concentration of Mn^{++} is calculated on the assumption that the Mn^{++} is distributed homogeneously throughout the cell volume which is unlikely to be the case. Even so the Mn^{++} concentration present in L. casei (see Table 5.3.3) is very high and may be sufficient to affect the LDH activity. Such a high in vivo Mn^{++} concentration implies an active transport system for Mn^{++}.

The LDH's from L. casei (Holland and Pritchard, 1975) and S. lactis (this thesis) are sensitive to phosphate whereas the S. faecalis LDH is insensitive to phosphate inhibition (this thesis). Both S. lactis and L. casei have apparently higher in vivo FDP concentrations than found in S. faecalis. This might imply a high

in vivo FDP concentration present in L. casei and S. lactis to overcome phosphate inhibition of the LDH. However the in vivo FDP concentration present in S. faecalis is still significantly higher than the in vitro FDP requirement for its activity. Because of this, the difference between the in vivo FDP concentration of S. faecalis and S. lactis is not necessarily related to the differential effect on LDH activity.

Collins and Thomas (1974) studied the intracellular concentration of metabolites in exponentially growing S. lactis ML_3 using virtually identical methods to those in the present study. The concentrations reported for S. lactis ML_3 are: FDP = 18.3 $\pm$ 1.8 mM; ADP = 2.4 $\pm$ 0.3 mM and PEP = 0.76 $\pm$ 0.15 mM. The values found in exponentially growing S. lactis C_{10} are shown in Table 5.3.3 where FDP = 12.7 to 14.9 mM; ADP = 7.6 to 7.9 mM and PEP = 1.7 to 2.3 mM. The FDP concentrations are of the same order but the PEP and ADP concentrations are considerably lower in S. lactis ML_3. A likely reason for the differences is the different media used. Collins and Thomas (1974) used a T5 medium as described by Thomas et al. (1974). The T5 medium contains 0.5% lactose compared to the 3% used for S. lactis C_{10} and the concentration of protein hydrolysates were also considerably lower in their medium.

Mizushima and Kitahara (1964) estimated the intracellular glycolytic concentrations in Lactobacillus plantarum by very different methods to those used for S. lactis C_{10} and S. lactis ML_3. Their estimate of the FDP (17 mM) and PEP (5.5 mM) were similar to the respective concentrations found in the two S. lactis species.

5.3.4 Relation of the Intracellular Metabolite Concentrations to the Kinetic Parameters of the S. lactis C_{10} LDH and Pyruvate Kinase

A. Lactate dehydrogenase. For S. lactis the appropriate intracellular metabolite concentrations present in an exponentially growing culture are shown along with the respective kinetic parameters for LDH in Table 5.3.4a. The in vivo FDP concentration is at a sufficiently high level to fully activate the enzyme in vitro even if the high FDP $_{0.5V}$ value found in phosphate buffer represents the in vivo affinity of the LDH for FDP. The in vivo FDP concentration was three times that required for half maximal activity (FDP $_{0.5V}$) determined in phosphate buffer and 6,000 times that required for half maximal activity determined

Table 5.3.4a

Relation of intracellular metabolite concentrations to the LDH kinetic parameters

	FDP (mM)	ATP (mM)	ADP (mM)	Pyruvate (mM)
Intracellular concentrations of metabolites determined from an exponentially growing *S. lactis* culture. (Values from Table 5.3.3) The variation of metabolite concentration is shown as determined from the first five samples.	12.7 to 14.9	11.7 to 20.8	7.6 to 7.9	1.9 to 3.4
Respective kinetic parameters for *S. lactis* LDH. (Values from Section 2.4) The standard assay conditions: 90 mM buffer pH 6.9, 0.167 mM NADH, 10 mM pyruvate and 1 mM FDP. A. Standard assay - phosphate buffer B. Standard assay - tris/maleate buffer C. Standard assay - triethanolamine/HCl buffer D. Standard assay - phosphate buffer except 6.67 mM FDP.	$FDP_{0.5V}$ (mM) 4.4[A] 0.2[B] 0.002[C]	K_I ATP (mM) 2.4[B]	K_I ADP (mM) 2.4[B]	K_M Pyruvate (mM) 1.25[B] 1.25[C] 5.7[D]

in triethanolamine/HCl buffer. In view of the fact that the LDH would appear to be fully activated it is somewhat surprising that there is a measurable pyruvate concentration. The *in vivo* pyruvate concentration is not much different from the K_M values for pyruvate. Since inhibition of *S. lactis* LDH (*in vitro*) occurred only at pyruvate concentrations greater than 20 mM, it is clear that pyruvate inhibition is of little physiological significance.

Although the FDP concentration is apparently more than adequate to fully activate the LDH even at a high *in vivo* phosphate concentration, it may be that other factors influence or modulate the FDP activity. Both the *in vivo* ATP and ADP concentrations are high enough to produce inhibition of LDH activity under *in vitro* assay conditions. It is possible that the high *in vivo* FDP concentration may overcome the nucleotide inhibition. However it would be necessary to study ATP and ADP inhibition at different FDP concentrations to establish whether these two components interact in determining LDH activity.

B. Pyruvate Kinase. In Table 5.3.4b the relationships between the appropriate intracellular metabolite concentrations are shown along with the respective kinetic parameters determined for pyruvate kinase. The concentration of FDP in exponentially growing cells is again sufficiently high to fully activate the enzyme on the basis of *in vitro* assay data. The enzyme assayed in the presence of 1 mM phosphate, has the highest FDP $_{0.5V}$ requirement (0.93 mM) which may be compared to *in vivo* FDP concentrations of 12.7 to 14.9 mM. The high *in vivo* FDP concentration may be due to the presence of a high *in vivo* phosphate concentration in which case high FDP concentration (Table 5.3.4b) could then overcome the phosphate inhibition of pyruvate kinase. This explanation is much more probable for pyruvate kinase than for LDH since concentrations of phosphate which inhibit pyruvate kinase (Phosphate $_{0.5I}$ = 0.7 mM) are quite likely to prevail *in vivo*. A concentration of phosphate sufficiently high to affect LDH activity (Phosphate $_{0.5I}$ = 50 mM) would completely inhibit pyruvate kinase activity.

The K_M value for ADP and the PEP $_{0.5V}$ values are lower than their respective intracellular concentrations except for the PEP $_{0.5V}$ value estimated in the presence of 1 mM phosphate and 1 mM FDP, which is 2.5 times higher than the *in vivo* concentration. The potential influence of phosphate in effecting pyruvate kinase activity is again apparent.

Table 5.3.4b

Relation of intracellular metabolite concentrations to the pyruvate kinase kinetic parameters

	Mn^{++} (mM)	FDP (mM)	ATP (mM)	ADP (mM)	PEP (mM)
Intracellular concentrations of metabolites determined from an exponentially growing *S. lactis* culture. (Values from Table 5.3.3) The variation of metabolite concentration is shown as determined from the first five samples, except the Mn^{++} concentration.	0.25 to 0.5	12.7 to 14.9	11.7 to 20.8	7.6 to 7.9	1.7 to 2.3
Respective kinetic parameters for *S. lactis* pyruvate kinase. (Values from Section 3.4)	$Mn^{++}_{0.5V}$ (mM)	$FDP_{0.5V}$ (mM)	$ATP_{0.5I}$ (mM)	K_M ADP (mM)	$PEP_{0.5V}$ (mM)
The standard assay conditions: 13.3 mM KCl, 3.3 mM	1.3[A]	0.16[A]	2.2[A]	1.3[A]	0.12[A]
$MgCl_2$, 80 mM triethanolamine/HCl buffer pH 7.5,		0.93[B]			5.0[B]
3.3 mM ADP, 1.0 mM FDP and 1.0 mM PEP.		0.02[C]			0.03[C]
A. Standard assay		0.18[D]			0.40[D]
B. Standard assay except 1 mM Phosphate present		0.45[E]			0.13[E]
C. Standard assay except Mg^{++} is replaced by Mn^{++}			5.5[F]		
D. Standard assay except pH 6.2	4.7[G]				
E. Standard assay except pH 8.75					
F. Standard assay except 80 mM K^+, 8 mM Mg^{++}					
G. Standard assay except no FDP present					

The in vivo ATP concentration is higher than the ATP $_{0.5I}$ value and may imply that ATP, as well as phosphate, may be partially inhibiting the pyruvate kinase. Even under conditions of very high carbohydrate availability (as was the case in the present study) some control of pyruvate kinase activity is essential to ensure that adequate PEP is available for the PEP-phosphotransferase system for continued carbohydrate uptake. The high steady state level of ATP generated during active glycolysis may serve to modulate the FDP activation of the pyruvate kinase as well as the LDH.

The in vivo Mn^{++} concentration was determined in S. lactis at two different stages of growth (see Table 5.3.3). The Mn^{++} concentration in a sample from the exponential phase (0.5 mM) was approximately double that of a sample (0.26 mM) from the culture that had been subjected to alkaline pH stress. The bacterial mass of the second sample was twice that of the first sample. It may be that all the medium Mn^{++} had been transported into the bacterial cells by the time the first sample was taken and the Mn^{++} concentration was subsequently diluted by cell growth. The Mn^{++} intracellular concentration (0.5 mM) present in S. lactis is probably not sufficiently high to effect the pyruvate kinase activity in vitro very markedly (see Table 5.3.4b). The Mn^{++} $_{0.5V}$ value with saturating FDP present is 1.3 mM Mn^{++}, twice the total in vivo level (the free Mn^{++} in the protoplastwill probably be a lot lower). However with a Mn^{++} supplement in the medium, the in vivo concentration could easily be sufficiently high to affect in vivo functioning of pyruvate kinase since Mn^{++} increases the affinity for both PEP and FDP. The effect of added Mn^{++} on the in vivo concentrations of glycolytic intermediates would be of considerable interest to investigate. A Mn^{++} supplemented medium for S. faecalis may beneficially effect growth as Mn^{++} appeared, from in vitro evidence, to activate the LDH. The in vivo concentration of pyruvate in the S. faecalis cells was high (24 to 26 mM), suggesting that the LDH was not fully active. A high in vivo concentration of Mn^{++} may activate the S. faecalis LDH with a subsequent drop in the pyruvate concentration.

Section 6

Summary of main conclusions and suggestions for further work.

The aim of the present study was to investigate the kinetic properties of three enzymes of potential importance in the regulation of carbohydrate metabolism and to relate these properties to the *in vivo* levels of substrates and effectors. Two enzymes, lactate dehydrogenase and pyruvate kinase, were investigated in some detail; the third enzyme, 6-phosphogluconate dehydrogenase, was subjected to a preliminary investigation only.

The *S. lactis* 6-phosphogluconate dehydrogenase (6-PGDH) did not appear to be inhibited by FDP. This contrasts to the findings of Brown and Wittenberger (1971a) who found that FDP inhibited the *S. faecalis* enzyme. In the present study, the 6-PGDH from *S. faecalis* (but a different strain from that used by Brown and Wittenberger) was also studied and like the *S. lactis* enzyme, no FDP inhibition could be demonstrated. Because of the preliminary nature of this investigation, further work is required to resolve the conflict between the findings of the present study and those of Brown and Wittenberger (1971a) and to establish what factors control the operation of the oxidative portion of the HMP pathway in *S. lactis*.

In the kinetic studies on the *S. lactis* LDH, FDP was shown to be an allosteric activator of the enzyme. An important finding from these studies on *S. lactis* LDH was the very large influence which the choice of buffer components has on the kinetic properties. Although phosphate buffer had the most striking effect on the *in vitro* properties, there were also differences between the properties in tris/maleate and triethanolamine/HCl buffers. Realization of this buffer effect is important not only in relating the *in vitro* properties of the enzyme to *in vivo* control but also in comparing the *S. lactis* C_{10} LDH with other streptococcal FDP-activated LDH's. The buffer effect raises the problem of deciding which *in vitro* properties best represent the properties of the enzyme that are important in its *in vivo* control. The *S. faecalis* ATCC 8043 LDH was studied briefly to compare it with the *S. lactis* enzyme. The major finding was its relative insensitivity to phosphate inhibition and effect of Mn^{++} in activating the enzyme.

The kinetics of the _S. lactis_ C_{10} pyruvate kinase were more complex than those of LDH, for as well as requiring FDP as an activator, pyruvate kinase also has an essential requirement for both a monovalent and divalent cation. Either Mn^{++} or Mg^{++} could function as the divalent cation but the properties were quite different depending on which cation was used. When Mn^{++} was the divalent cation the enzyme was no longer obligatarily dependent on FDP for activity, although FDP still functioned as an activator. The affinity for PEP was higher with Mn^{++}, compared to Mg^{++}. The _S. lactis_ pyruvate kinase activity, like LDH, was also affected by choice of the buffer components. In addition, its activity was affected by a number of anions and cations that are often present in laboratory reagents. The multitude of factors that affect the pyruvate kinase activity _in vitro_ makes the extrapolation to the _in vivo_ situation difficult.

The kinetic properties of the two enzymes, LDH and pyruvate kinase, have been related to the appropriate _in vivo_ metabolite concentrations determined in exponentially growing cells of _S. lactis_ C_{10}. The main finding from the present investigation was that the _in vivo_ FDP concentration is at a sufficiently high level to fully activate both pyruvate kinase and LDH when considered in relation to the characteristics of FDP dependence determined _in vitro_ under a number of different assay conditions. However for pyruvate kinase, the presence of relatively low intracellular phosphate levels (1 to 10 mM) may be sufficient to increase the FDP requirement, such that the _in vivo_ level of FDP could be below the limit required for activity. Since the concentration of phosphate required to inhibit FDP activation of the LDH is much higher than that for pyruvate kinase it is unlikely that the phosphate inhibition of LDH is of physiological significance. An _in vivo_ phosphate level that would sufficiently affect LDH would completely inhibit pyruvate kinase. It would clearly be useful to determine the _in vivo_ inorganic phosphate concentration.

Other metabolites, apart from FDP (and possibly phosphate), may be playing a role in the _in vivo_ control of the two enzymes. ATP has been shown to inhibit both LDH and pyruvate kinase _in vitro_ and the respective K_I and ATP $_{0.5I}$ values for both enzymes are less than the apparent _in vivo_ ATP concentration during exponential growth. This indicates that it would be profitable to investigate in more detail the interaction of ATP and FDP in the regulation of the two enzymes _in vitro_. Measurement of the NAD^+/NADH ratio would also shed light on the extent to which the LDH was fully activated _in vivo_.

By studying carbohydrate control by these two complementary methods (*in vivo* metabolite measurements and *in vitro* kinetic studies) a good understanding of the regulation of carbohydrate metabolism in *S. lactis* could be developed. Possible *in vitro* artifacts such as the buffer effect will be more easily recognised when related to *in vivo* conditions.

In the present study measurements of metabolite concentrations has been from samples taken from batch cultures in which there is a progressively changing medium, even in the exponential phase of growth. The use of continuous cultures would provide a steady-state culture of exponentially-growing cells and permit the growth rate to be regulated and the effect of nutritional conditions, such as carbohydrate concentration, to be determined. The *in vivo* metabolite levels under limiting carbohydrate concentration would be of particular interest in relation to the physiological significance of the control mechanisms investigated in this study. Such a study could indicate the factors which regulate *in vivo* FDP concentration and the limits between which the concentration of this metabolite varies.

The work described in this thesis has concentrated very largely on the two terminal reactions in the glycolytic pathway. Other aspects of carbohydrate metabolism of *S. lactis* that need to be investigated are:

1. Earlier reactions in the EMP pathway; particularly phosphofructokinase and also possibly FDP-aldolase and glyceraldehyde-3-phosphate dehydrogenase.

2. The relative importance of the tagatose and Leloir pathways of lactose and galactose metabolism and the regulation of enzymes of the tagatose pathway.

3. The existence, properties and regulation of other enzymes which may be involved in pyruvate metabolism.

APPENDIX: ABBREVIATIONS

ADP	adenosine 5^1-diphosphate
AMP	adenosine 5^1-monophosphate
ATP	adenosine 5^1-triphosphate
bis	N,N^1-methylenebisacrylamide
CDP	cytidine 5^1-diphosphate
CM	carboxymethyl
CoA	coenzyme A
CTP	cytidine 5^1-triphosphate
DEAE	diethylaminoethyl
EDTA	ethylenediamine-tetra-acetate
EMP	Embden-Meyerhof-Parnas
FDP	fructose-1,6-diphosphate
GDP	guanosine 5^1-diphosphate
G-6-P	glucose-6-phosphate
GTP	guanosine 5^1-triphosphate
HMP	hexose monophosphate
ITP	inosine 5^1-triphosphate
K_I	inhibitor constant
K_M	Michaelis-Menten constant
LDH	lactate dehydrogenase
$M_{0.5V}$	concentration of modifier giving half maximum velocity
2-ME	2-mercaptoethanol
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
n_H	Hill interaction coefficient
OAA	oxaloacetic acid
ONPG-6-P	ortho-nitrophenyl-β-D-galactopyranoside-6-phosphate
Pi	inorganic phosphate
PCMP	para-chloromercuricbenzoate
PEP	phosphoenolpyruvate
6-PGDH	6-phosphogluconate dehydrogenase
-SH-	sulphydryl group
TEMED	$N,N,N,^1N^1$-tetramethylenediamine
tris	tris (hydroxymethyl) aminomethane
UDP	uridine 5^1-diphosphate
UTP	uridine 5^1-triphosphate
v	velocity
V_{max}	maximum velocity.

Appendix 3.4 Pyruvate Kinase

A number of other observations were made on the properties of the *S. lactis* pyruvate kinase. In view of the complexity of the data already presented on this enzyme, these observations have been omitted from the main text of the pyruvate kinase section of the thesis. However, since they may be of practical value to other workers studying the enzyme, the observations have been briefly summarised in the form of two appendices.

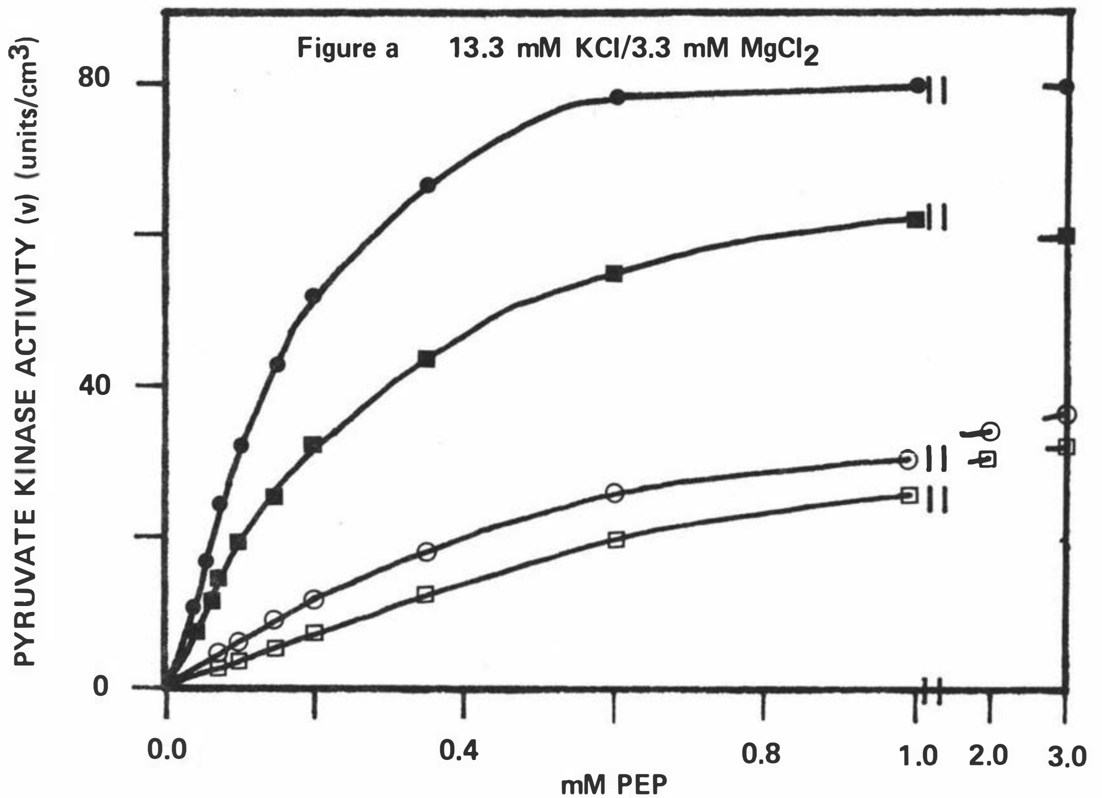
Appendix 3.4.1 The effect of two different FDP salts on pyruvate kinase activity.

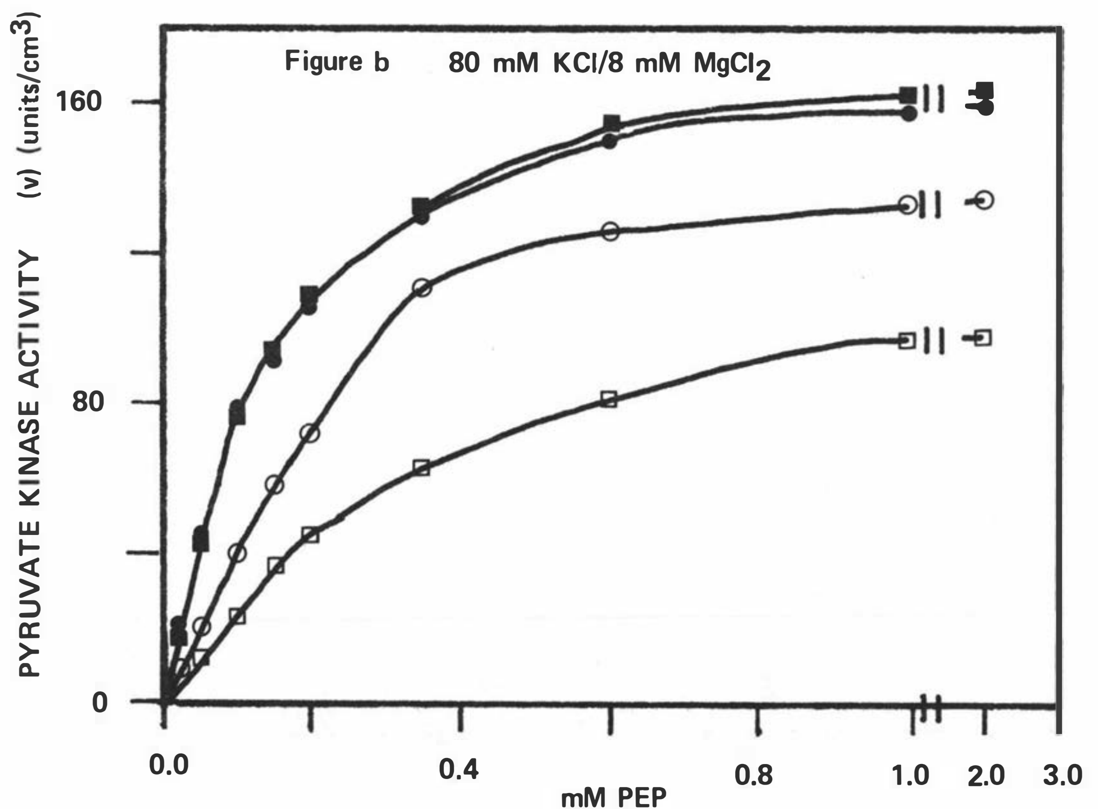
In the standard assay system, 1 mM (tetrasodium) FDP was present. If 10 mM FDP (tetrasodium) instead of 1 mM FDP was added to the standard assay system, then the activity dropped by 20%. As the tetrasodium salt of FDP was used in the assays it was possible that at 10 mM FDP, the 40 mM sodium ions introduced with the FDP could have produced the inhibition. Therefore the use of the tetracyclohexylammonium salt of FDP was investigated.

Figures A.3.4.1a and b show the effect of the two different salts of FDP (tetrasodium and tetracyclohexylammonium salt) both at concentrations of 1 and 10 mM FDP, on the PEP saturation curve. The standard metal ion concentrations of 13.3 mM KCl/3.3 mM $MgCl_2$ were present in the assays of Figure A.3.4.1a and the higher metal ion concentrations of 80 mM KCl/8 mM $MgCl_2$ were present in the assays of Figure A.3.4.1b. The data obtained were also plotted as double reciprocal and Hill plots but these are not shown.

Figure A.3.4.1a, where low metal ion concentrations are present, shows that the PEP saturation curve, at all PEP concentrations, is higher with 1.0 mM tetrasodium FDP than with 10 mM tetrasodium FDP and the respective PEP $_{0.5V}$ values are 0.14 mM (n_H = 1.3) and 0.2 mM (n_H = 1.1). The tetracyclohexylammonium FDP, at the two concentrations of 1 and 10 mM gives PEP saturation curves showing lower reaction velocities for all PEP concentrations than the same concentrations of tetrasodium FDP. The affinity for PEP is also less when the tetracyclohexylammonium FDP is used (PEP $_{0.5V}$ values at 1 mM FDP is 0.45 mM (n_H = 1.1) and at 10 mM FDP is 0.46 mM (n_H = 1.1)). However, activity with the tetracyclohexylammonium FDP is slightly higher at 10 mM than 1 mM FDP.

THE EFFECT OF TWO DIFFERENT FDP SALTS ON PYRUVATE KINASE





The relationship between the pyruvate kinase activity (v) (units/cm^3) of S. lactis C_{10} and PEP concentration is shown in Figures a and b, using two FDP salts each at two different concentrations. The tetrasodium FDP salt was present at: (■), 10 mM; (●), 1 mM. The tetracyclohexylammonium FDP salt was present at: (○), 10 mM; (□), 1 mM. The reaction mixture contained (in a total volume of 3 cm^3): 80 mM triethanolamine/HCl buffer pH 7.5; 3.3 mM ADP: 0.1 cm^3 of diluted enzyme and the PEP concentration varied as shown in the Figures, in the presence of 13.3 mM KCl/3.3 mM $MgCl_2$ (Figure a) and in the presence of 80 mM KCl/8 mM $MgCl_2$ (Figure b).

When high concentrations of KCl and $MgCl_2$ are present in the assay system, as shown in Figure A.3.4.1b, for the two tetrasodium concentrations the PEP saturating curves are similar, presumably indicating that the high K^+ has overcome the possible inhibition by Na^+. (This has been further dealt with in a previous section, Section 3.4.8, on the effect of monovalent cations). The high metal ion concentrations have also changed the form of PEP saturation curves at the two tetracyclohexylammonium FDP concentrations, as activity at 10 mM FDP is significantly greater than 1 mM FDP. At 10 mM tetracyclohexylammonium FDP, the activity at comparable PEP concentrations is only slightly less (1·5%) than at 1 and 10 mM tetrasodium FDP, whereas at the lower metal ion concentrations, activity, at a given PEP concentration of 10 mM tetracyclohexylammonium FDP, is significantly less (greater than 50%) than with 1 and 10 mM tetrasodium FDP. At the high metal ion concentrations, with tetrasodium FDP, the PEP $_{0.5V}$ values are 0.14 mM (at 1 mM FDP) and 0.13 mM (at 10 mM FDP). With tetracyclohexylammonium FDP their respective PEP $_{0.5V}$ values are 0.45 mM (1 mM FDP) and 0.21 mM (10 mM FDP).

The findings indicate that at high tetrasodium FDP concentrations and at low metal ion concentrations, the sodium ion concentration inhibits activity. Possibly more significant, is the finding that the cyclohexylammonium ion appears to inhibit the pyruvate kinase activity. High metal ion and FDP concentrations appear to eliminate this inhibition to a significant extent. There are no reports in the literature of the cyclohexylammonium ion inhibiting any other pyruvate kinase.

The tetrasodium salt of FDP was therefore used in routine and kinetic assays. At concentrations of up to 2 mM FDP, the amount of Na^+ present, in addition to that from ADP and PEP salts, was shown not to affect activity to any appreciable extent. In the standard assay system, only at Na^+ concentrations of 20 mM above the basal Na^+ concentration (introduced by the Na^+ salts of PEP, ADP and FDP) did inhibition of pyruvate kinase occur (see Section 3.4.8).

A point of interest to note in relation to use of the tetracyclohexylammonium salt, is the result obtained from the study of activation of the L-type rabbit liver pyruvate kinase by the tetracyclohexylammonium salt of FDP (Irving and Williams, 1973). These workers showed that inhibition of the stimulated reaction occurred with FDP

concentrations in excess of 0.5 mM. This inhibition effect was not investigated by Irving and Williams, but from the evidence obtained in the present study a possible reason for inhibition could be that the cyclohexylammonium ion is inhibiting the activity of the enzyme.

Appendix 3.4.2 The sulphate effect on the kinetic parameters of the *S. lactis* pyruvate kinase.

As reported in earlier sections, if $MgCl_2$ is replaced by $MgSO_4$ in the assay system, the inhibition of the enzyme by high concentrations of Mg^{++} and ADP appeared to be enhanced. The sulphate effect on the kinetic parameters of pyruvate kinase from *S. lactis* was further investigated using $MgSO_4$ as the divalent salt and varying the FDP, PEP and ADP concentrations in different combinations. The results of the kinetic studies, are similar to those results where $MgCl_2$ is the divalent cation salt, and so are summarised in table form to indicate the main differences in the kinetic properties between the chloride and sulphate salts of magnesium.

A. The effect of PEP and ADP on FDP activation.

Tables A.3.4.2a and b summarise the effect of PEP and ADP concentration on FDP activation of the enzyme in the presence of $MgSO_4$. The values obtained in the presence of $MgCl_2$ (see Section 3.4.4) are included in brackets. The main differences between the two magnesium salts are that the homotropic interaction of FDP with the enzyme is higher with the sulphate salt (n_H = 3.3, compared to n_H = 1.9 to 2.5 for $MgCl_2$) and the FDP $_{0.5V}$ values at the respective PEP and ADP concentrations are higher with the sulphate salt. The high homotropic interaction of FDP with the enzyme when the sulphate salt is present, showed a n_H value of 3.3 for the three weeks that the studies using $MgSO_4$ were carried out.

B. The effect of FDP and ADP on PEP activation

Tables A.3.4.2c and d summarise the effect of FDP and ADP on PEP activation in the presence of $MgSO_4$. The values obtained in the presence of $MgCl_2$ (see Section 3.4.5) are included in brackets. In the standard assays; the ADP and FDP concentrations were not the same for the two salts (6.67 mM ADP/3.33 mM ADP and 2 mM FDP/1 mM FDP for sulphate/chloride). However, this should not affect the general trend

of the differences. The main differences are that the homotropic interaction of PEP with the enzyme is higher with the sulphate salt and the PEP $_{0.5V}$ values are also higher with the sulphate salt.

C. The effect of FDP and PEP on ADP activation

Tables A.3.4.2e and f summarise the effect of FDP and PEP on ADP activation in the presence of $MgSO_4$. The values obtained in the presence of $MgCl_2$ (see Section 3.4.6) are included in brackets. In the standard assays the FDP concentration was not the same for the two salts (2 mM FDP/1 mM FDP for sulphate/chloride) but this should not affect the general trend. The presence of the sulphate salt increases the homotropic interaction of ADP with the enzyme in Table e at the low FDP concentrations in comparison with use of the chloride salt of magnesium. The difference in the ADP $_{0.5V}$ values between use of sulphate and the chloride magnesium salts is more complex. In Table e, with sulphate present decreasing the FDP concentrations results in a decrease in the ADP $_{0.5V}$ values, whereas with chloride present the ADP $_{0.5V}$ values increase. The ADP $_{0.5V}$ values in Table e are higher in the presence of the chloride salt rather than the sulphate salt, unlike the trend found for the PEP $_{0.5V}$ and FDP $_{0.5V}$ values. In Table f, the ADP $_{0.5V}$ values are unaffected by the PEP concentrations in the presence of the chloride salt in contrast to the ADP $_{0.5V}$ values increasing as the PEP concentration decreases in the presence of sulphate.

Sulphate, as studied by comparison of the kinetic data obtained from use of $MgCl_2$ and $MgSO_4$, has been shown to give greater potential to the enzyme for co-operative interaction with ADP, FDP and PEP to occur. The basic heterotropic relationships between ADP, PEP and FDP are not altered in the presence of sulphate. For both magnesium salts, FDP has the greatest potential to show co-operative kinetics, with PEP showing co-operative binding to the enzyme only at lower FDP concentrations. ADP has the least potential to co-operatively bind to the enzyme. The sulphate anion increases the PEP $_{0.5V}$ and FDP $_{0.5V}$ values and effects the ADP $_{0.5V}$ values in a more complex manner.

Tables A.3.4.2a and b

The effect of PEP and ADP on FDP activation: Sulphate effect

Table a. The FDP concentration was varied at four different PEP concentrations at standard assay conditions: 80 mM triethanolamine/HCl buffer pH 7.5; 13.33 mM KCl/3.33 mM $MgSO_4$ and 3.3 mM ADP.

Table b. The FDP concentration was also varied at five different ADP concentrations at standard assay conditions: 80 mM triethanolamine/HCl buffer pH 7.5; 13.33 mM KCl/3.33 mM $MgSO_4$ and 1.0 mM PEP.

The two parameters, Hill coefficient (n_H) and FDP $_{0.5V}$, obtained from Hill plots of data are tabulated below. The two parameters determined with the chloride salt (see Section 3.4.4), under otherwise similar assay conditions, are included in brackets.

Table a.

PEP (mM)	n_H	FDP $_{0.5V}$ (mM)
1.0	3.3 (2.5)	0.44 (0.16)
0.6	3.4 (2.6)	0.48 (0.18)
0.4	3.7 (2.6)	0.64 (0.21)
0.25	3.7 (2.6)	0.73 (0.29)

Table b.

ADP (mM)	n_H	FDP $_{0.5V}$ (mM)
6.67	3.2 (1.90)	0.52 (0.3)
3.33	3.2 (1.95)	0.45 (0.16)
2.00	3.1	0.21
1.00	3.2 (1.90)	0.26 (0.08)
0.60	3.3	0.25

Tables A.3.4.2c and d

The effect of FDP and ADP on PEP activation: Sulphate effect

Table c. The PEP concentration was varied at five different FDP concentrations at standard assay conditions: 80 mM triethanolamine/HCl buffer pH 7.5; 13.33 mM KCl/3.33 mM $MgSO_4$ and 6.67 mM ADP.

Table d. The PEP concentration was varied at five different ADP concentrations at standard assay conditions: 80 mM triethanolamine/HCl buffer pH 7.5; 13.33 mM KCl/3.33 mM $MgSO_4$ and 2 mM FDP.

The two parameters, Hill coefficient (n_H) and PEP $_{0.5V}$, obtained from Hill plots of data are tabulated below. The two parameters determined with the chloride salt (see Section 3.4.5) under otherwise similar assay conditions (except 3.33 mM ADP and 1 mM FDP present) are included in brackets.

Table c.

FDP (mM)	n_H	PEP $_{0.5V}$ (mM)
10.0	1.4	0.2
1.0	2.8 (1.37)	0.5 (0.11)
0.6	3.2 (1.47)	0.8 (0.16)
0.4	3.2 (1.70)	1.3 (0.28)
0.25	3.4 (1.92)	2.3 (0.38)

Table d.

ADP (mM)	n_H	PEP $_{0.5V}$ (mM)
6.67	1.8 (1.4)	0.30 (0.21)
3.33	1.8 (1.4)	0.17 (0.14)
1.33 (1.00)	1.9 (1.4)	0.14 (0.11)
0.60	1.6	0.20
0.30	1.7 (1.4)	0.24 (0.11)

Table A.3.4.2e and f

The effect of FDP and PEP on ADP activation: Sulphate effect

Table e. The ADP concentration was varied at three different FDP concentrations at standard assay conditions: 80 mM triethanolamine/HCl buffer pH 7.5; 13.33 mM KCl/3.33 mM $MgSO_4$ and 1 mM PEP.

Table f. The ADP concentration was varied at four different PEP concentrations at standard assay conditions: 80 mM triethanolamine/HCl buffer pH 7.5; 13.33 mM KCl/3.33 mM $MgSO_4$ and 2 mM FDP.

The two parameters, Hill coefficient (n_H) and ADP $_{0.5V}$, obtained from Hill plots of data are tabulated below. The two parameters determined with the chloride salt (see Section 3.4.6) under otherwise similar assay conditions (except 1 mM FDP present) are included in brackets.

Table e.

FDP (mM)	n_H	ADP $_{0.5V}$ (mM)
2.0 (1.0)	1.25 (1.0)	0.95 (1.3)
0.6 (0.25)	1.52 (1.0)	0.48 (2.0)
0.2 (0.1)	3.15 (1.5)	0.45 (2.1)

Table f.

PEP (mM)	n_H	ADP $_{0.5V}$ (mM)
1.0	1.25 (1.0)	1.0 (1.2)
0.6	1.02	1.8
0.4	1.07 (1.0)	2.4 (1.2)
0.25	1.05 (1.0)	3.0 (1.2)

REFERENCES

Ainsworth, S. and Macfarlane, N. (1973). A kinetic study of rabbit muscle pyruvate kinase. Biochem. J. 131, 223-236.

Anders, R.F., Jonas, H.A. and Jago, G.R. (1970). A survey of the lactate dehydrogenase activities in group N streptococci. Aust. J. Dairy Technol. 25, 73-76.

Arnold, L.J.Jr and Kaplan, N.O. (1974). The structure of the abortive diphosphopyridine nucleotide-pyruvate-lactate dehydrogenase ternary complex as determined by proton magnetic resonance analysis. J. Biol. Chem. 249, 652-655.

Atkinson, D.E. (1966). Regulation of enzyme activity. Annu. Rev. Biochem. 35, 85-124.

Atkinson, D.E. (1968). The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry 7, 4030-4034.

Atkinson, D.E. (1970). Enzymes as control elements in metabolic regulation. In The Enzymes (Student Edition) 1, 461-489. (Boyer, P.D. ed.) Academic Press, New York and London.

Bailey, E., Stirpe, F. and Taylor, C.B. (1968). Regulation of rat liver pyruvate kinase. The effect of preincubation, pH, copper ions, fructose, 16-diphosphate and dietary changes on enzyme activity. Biochem. J. 108, 427-436.

Barker, H.A. (1961). Fermentation of nitrogenous organic compounds. In The Bacteria 2, 151-207 (Gunsalus, I.C. and Stanier, R.Y. eds). Academic Press, New York and London.

Bartlett, G.R. (1959a). Human red cell glycolytic intermediates. J. Biol. Chem. 234, 449-458.

Bartlett, G.R. (1959b). Methods for the isolation of glycolytic intermediates by column chromatography with ion exchange resins. J. Biol. Chem. 234, 459-465.

Benziman, M. (1969). Factors affecting the activity of pyruvate kinase of Acetobacter xylinum. Biochem. J. 112, 631-636.

Betts, G.F. and Evans, H.J. (1968). The inhibition of univalent cation activated enzymes by tris (hydroxymethyl) aminomethane. Biochim. Biophys. Acta. 167, 193-196.

Bissett, D.L. and Anderson, R.L. (1973). Lactose and D-galactose metabolism in Staphylococcus aureus: pathway of D-galactose 6-phosphate degradation. Biochem. Biophys. Res. Commun. 52, 641-647.

Bissett, D.L. and Anderson, R.L. (1974). Lactose and D-galactose metabolism in group N streptococci: presence of enzymes for both the D-galactose-1-phosphate and D-tagatose 6-phosphate pathways. J. Bact. 117, 318-320.

Black, S.H. and Gerhardt, P. (1962). Permeability of bacterial spores. IV. Water content, uptake and distribution. J. Bact. 83, 960-967.

Bormann, L., Hess, B. and Zimmermann-Telschow, H. (1974). Mechanism of renaturation of pyruvate kinase of Saccharomyces carlsbergensis: activation by L-valine and magnesium and manganese ions. Proc. Nat. Acad. Sci. USA 71, 1525-1529.

Boyer, P.D. (1962). Pyruvate kinase. In The Enzymes 6, 95-113. (Boyer, P.D., Lardy, H. and Myrback, K. eds). Academic Press, New York and London.

Brown, A.T. and Wittenberger, C.L. (1971a). Mechanisms for regulating the distribution of glucose carbon between the Embden-Meyerhof and hexose-monophosphate pathways in Streptococcus faecalis. J. Bact. 106, 456-467.

Brown, A.T. and Wittenberger, C.L. (1971b). The occurrence of multiple glyceraldehyde-3-phosphate dehydrogenases in cariogenic streptococci. Biochem. Biophys. Res. Commun. 43, 217-224.

Brown, A.T. and Wittenberger, C.L. (1972). Fructose-1,6-diphosphate-dependent lactate dehydrogenase from a cariogenic Streptococcus: purification and regulatory properties. J. Bact. 110, 604-615.

Brown, A.T. and Wittenberger, C.L. (1972a). Induction and regulation of a nicotinamide adenine dinucleotide-specific 6-phosphogluconate dehydrogenase in Streptococcus faecalis. J. Bact. 109, 106-115.

Bryan-Jones, D.G. and Whittenbury, R. (1969). Haematin-dependent oxidative phosporylation in Streptococcus faecalis. J. Gen. Microbiol. 58, 247-260.

Buyze, G., van der Hamer, J.A. and de Haan, P.G. (1957). Correlation between hexose monophosphate shunt, glycolytic system and fermentation type in lactobacilli. Antonie van Leeuwenhoek 23, 345-350.

Cahn, R.D., Kaplan, N.O., Levine, L. and Zwilling, E. (1962). Nature and development of lactic dehydrogenases. The two major types of this enzyme form molecular hybrids which change in make up during development. Science 136, 962-969.

Carminatti, H., Jimenez de Asua, L., Recondo, E., Passeron, S. and Rozengurt, E. (1968). Some kinetic properties of liver pyruvate kinase (type L). J. Biol. Chem. 243, 3051-3056.

Cavalieri, R.L. and Sable, H.Z. (1974). Pitfalls in the study of steady state kinetics of enzymes: spurious inhibition patterns due to stray light errors. Anal. Biochem. 59, 122-128.

Chervenka, C.H. (1970). Long-column meniscus depletion sedimentation equilibrium technique for the analytical ultracentrifuge. Anal. Biochem. 34, 24-29.

Cleland, W.W. (1963). The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations. Biochim. Biophys. Acta 67, 104-137.

Cleland, W.W. (1967). Enzyme kinetics. Annu. Rev. Biochem. 36, 77-112.

Cohn, M. and Hughes, T.R.Jr (1960). Phosphorus magnetic resonance spectra of adenosine di- and triphosphates. I. Effect of pH. J. Biol. Chem. 235, 3250-3253.

Cohn, M. and Hughes, T.R.Jr (1962). Nuclear magnetic resonance spectra of adenosine di- and triphosphate. II. Effect of complexing with divalent metal ions. J. Biol. Chem. 237, 176-181.

Collins, L.B. and Thomas, T.D. (1974). Pyruvate kinase of Streptococcus lactis. J. Bact. 120, 52-58.

Cook, R.B. and Jankow, R. (1972). Effects of stray light in spectroscopy. J. Chem. Educ. 49, 405-408.

Costa, L., Jimenez de Asua, L., Rozengurt, E., Bade, E.G. and Carminatti, H. (1972). Allosteric properties of the isoenzymes of pyruvate kinase from rat kidney cortex. Biochim. Biophys. Acta 289, 128-136.

Cuatrecasas, P.C. (1970). Protein purification by affinity chromatography. Derivatizations of agarose and polyacrylamide beads. J. Biol. Chem. 245, 3059-3065.

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1969). (eds) Data for biochemical research. Second edition. Oxford at the Clarendon Press.

Deibel, R.H. (1964). The group D streptococci. Bact. Rev. 28, 330-366.

De Moss, R.D., Bard, R.C. and Gunsalus, I.C. (1951). The mechanism of the heterolactic fermentation: a new route of ethanol formation. J. Bact. 62, 499-511.

Dennis, D. and Kaplan, N.O. (1960). D- and L-lactic acid dehydrogenases in Lactobacillus plantarum. J. Biol. Chem. 235, 810-818.

Dennis, D., Reichlin, M. and Kaplan, N.O. (1965). Lactic acid racemization. Ann. N.Y. Acad. Sci. 119, 868-876.

Diesterhaft, M. and Freese, E. (1972). Pyruvate kinase of Bacillus subtilis. Biochim. Biophys. Acta 268, 373-380.

Doelle, H.W. (1969). Bacterial Metabolism. Academic Press, New York and London.

Doelle, H.W. (1971a). The influence of malate on lactate dehydrogenase of Leuconostoc mesenteroides. Proc. of the Australian Biochemical Society, 4, 52.

Doelle, H.W. (1971b). Influence of carboxylic acids on the stereospecific nicotinamide adenine dinucleotide-dependent and nicotinamide adenine dinucleotide-independent lactate dehydrogenases of Leuconostoc mesenteroides. J. Bact. 108, 1290-1295.

Doelle, H.W. (1971c). Nicotinamide adenine dinucleotide-dependent and nicotinamide adenine dinucleotide-independent lactate dehydrogenase in homofermentative and heterofermentative lactic acid bacteria. J. Bact. 108, 1284-1289.

Doelle, H.W. (1972). Kinetic characteristics of phosphofructokinase from Lactobacillus casei var. rhamnosus ATCC 7469 and Lactobacillus plantarum ATCC 14917. Biochim. Biophys. Acta 258, 404-410.

Dolin, M.I. (1953). The oxidation and peroxidation of reduced diphospho-pyridine nucleotide ($DPNH_2$) in extracts of Streptococcus faecalis 10C1. Archives Biochem. Biophys. 46, 483-485.

Dolin, M.I. (1955). The DPNH-oxidising enzymes of Streptococcus faecalis. II. The enzymes utilising oxygen, cytochrome C, peroxide and 2,6-dichlorophenol-indophenol or ferricyanide as oxidants. Archives Biochem. Biophys. 55, 415-435.

Dynon, M.K., Jago, G.R. and Davidson, B.E. (1972). The subunit structure of lactate dehydrogenase from Streptococcus cremoris US_3. Eur. J. Biochem. 30, 348-353.

Evans, H.J. and Sorger, G.J. (1966). Role of mineral elements with emphasis on the univalent cations. Annu. Rev. Plant Physiol. 17, 47-76.

Everse, J. and Kaplan, N.O. (1973). Lactate dehydrogenases: structure and function. In Advances in Enzymology 37, 61-133. (Meister, A. ed.) Interscience.

Eyzaguirre, J. (1974). Stray light interference in the spectrophotometric measurement of enzyme activity and determination of kinetic parameters. Biochem. Biophys. Res. Commun. 60, 35-41.

Flory, W., Peczon, B.D., Koeppe, R.E. and Spivey, H.O. (1974). Kinetic properties of rat liver pyruvate kinase at cellular concentrations of enzyme, substrates and modifiers. Biochem. J. 141, 127-131.

Friedemann, T.E. (1939). The carbohydrate metabolism of streptococci. J. Biol. Chem. 130, 757-761.

Fromm, H.J. (1963). Determination of dissociation constants of coenzymes and abortive ternary complexes with rabbit muscle lactate dehydrogenase from fluorescence measurements. J. Biol. Chem. 238, 2938-2944.

Gabriel, O. (1971). Analytical disc gel electrophoresis. In Methods in Enzymology 22, 565-578. (Jakoby, W.B. ed.) Academic Press, New York and London.

Gallin, J.I. and Van Demark, P.J. (1964). Evidence for oxidative phosphorylation in Streptococcus faecalis. Biochem. Biophys. Res. Commun. 17, 630-635.

Gancedo, J.M., Gancedo, C. and Sols, A. (1967). Regulation of the concentration or activity of pyruvate kinase in yeasts and its relationship to gluconeogenesis. Biochem. J. 102, 23c-25c.

Garland, R.C. (1973). Purification and properties of D(-)-lactate dehydrogenase from Leuconostoc mesenteroides. Archives Biochem. Biophys. 157, 36-43 (see Errata 158, 448).

Garreau, H. and Buc-Temkine, H. (1972). Allosteric activation of human erythrocyte pyruvate kinase by fructose-1,6-diphosphate: kinetic and equilibrium binding studies. Biochimie. 54, 1103-1107.

Garvie, E.I. (1969). Lactic dehydrogenases of strains of the genus Leuconostoc. J. Gen. Microbiol. 58, 85-94.

Gasser, F. (1970). Electrophoretic characterization of lactic dehydrogenases from the genus Lactobacillus. J. Gen. Microbiol. 62, 223-239.

Gasser, F., Doudoroff, M. and Contopoulos, R. (1970). Purification and properties of NAD-dependent lactic dehydrogenases of different species of Lactobacillus. J. Gen. Microbiol. 62, 241-250.

Geyer, H. (1967). Inhibition of lactic dehydrogenase by adenosine phosphates. Hoppe-Seyler's Z. Physiol. Chem. 348, 823-828.

Gibbs, M., Dumrose, R., Bennett, F.A. and Bubeck, M.R. (1950). On the mechanism of bacterial fermentation of glucose to lactic acid studied with C^{14}-glucose. J. Biol. Chem. 184, 545-549.

Good, N.E. and Izawa, S. (1972). Hydrogen ion buffers. In Methods in Enzymology 24B, 53-68. (San Pietro, A. ed.) Academic Press, New York and London.

Gunsalus, I.C. and Gibbs, M. (1952). The heterolactic fermentation. II. Position of C^{14} in the products of glucose dissimilation by Leuconostoc mesenteroides. J. Biol. Chem. 194, 871-875.

Gunsalus, I.C. and Niven, C.F.Jr (1942). The effect of pH on the lactic acid fermentation. J. Biol. Chem. 145, 131-136.

Gunsalus, I.C. and Sherman, J.M. (1943). Fermentation of glycerol by streptococci. J. Bact. 45, 155-162.

Gunsalus, I.C. and Umbreit, W.W. (1945). The oxidation of glycerol by *Streptoccus faecalis*. J. Bact. 49, 347-357.

Haeckel, R., Hess, B., Lauterborn, W. and Wuester, K.H. (1968). Purification and allosteric properties of yeast pyruvate kinase. Hoppe-Seyler's Z. Physiol. Chem. 349, 699-714.

Hammes, G.G., Maciel, G.E. and Waugh, J.S. (1961). The conformation of metal-adenosine triphosphate complexes in solution. J. Amer. Chem. Soc. 83, 2394-2395.

Harvey, R.J. and Collins, E.B. (1963). Roles of citrate and acetoin in the metabolism of *Streptococcus diacetilactis*. J. Bact. 86, 1301-1307.

Heath, E.C., Hurwitz, J., Horecker, B.L. and Ginsburg, J. (1958). Pentose fermentation by *Lactobacillus plantarum*. I. The cleavage of xylulose-5-phosphate by phosphoketolase. J. Biol. Chem. 231, 1009-1029.

Hess, B. and Haeckel, R. (1967). Interaction between potassium-, ammonium-fructose-1,6-diphosphate activation of yeast pyruvate kinase. Nature. 214, 848-849.

Hess, B., Haeckel, R. and Brand, K. (1966). FDP-activation of yeast pyruvate kinase. Biochem. Biophys. Res. Commun. 24, 824-831.

Hill, A.V. (1913). The combinations of haemoglobin with oxygen and with carbon monoxide. I. Biochem. J. 7, 471-480.

Holland, R. and Pritchard, G.G. (1975). Regulation of the L-lactate dehydrogenase from *Lactobacillus casei* by fructose-1,6-diphosphate and metal ions. J. Bact. 121, 777-784.

Hunsley, J.R. and Suelter, C.H. (1969). Yeast pyruvate kinase. II. Kinetic properties. J. Biol. Chem. 244, 4819-4822.

Hurwitz, J. (1958). Pentose phosphate cleavage by *Leuconostoc mesenteroides*. Biochim. Biophys. Acta 28, 599-602.

Imamura, K., Taniuchi, K. and Tanaka, T. (1972). Multimolecular forms of pyruvate kinase. J. Biochem. (Tokyo) 72, 1001-1015.

Irving, M.G. and Williams, J.F. (1973). Kinetic studies on the regulation of rabbit liver pyruvate kinase. Biochem. J. 131, 287-301.

Jago, G.R., Nichol, L.W., O'Dea, K. and Sawyer, W.H. (1971). Physico-chemical studies on the lactate dehydrogenase of Streptococcus cremoris US_3: the effects of modifiers. Biochim. Biophys. Acta 250, 271-285.

Jimenez de Asua, L., Rozengurt, E. and Carminatti, H. (1970). The kinetic properties of liver pyruvate kinase (Type L). III. Effect of monovalent cations on its allosteric behaviour. J. Biol. Chem. 245, 3901-3905.

Jimenez de Asua, L., Rozengurt, E., Devalle, J.J. and Carminatti, H. (1971). Some kinetic differences between the M isoenzymes of pyruvate kinase from liver and muscle. Biochim. Biophys. Acta 235, 326-334.

Johannes, K.-J. and Hess, B. (1973). Allosteric kinetics of pyruvate kinase of Saccharomyces carlsbergensis. J. Mol. Biol. 76, 181-205.

Johnson, K.G. and McDonald, I.J. (1974). β-D-Phosphogalactoside galactohydrolase from Streptococcus cremoris HP: purification and enzyme properties. J. Bact. 117, 667-674.

Jonas, H.A. Anders, R.F. and Jago, G.R. (1972). Factors affecting the activity of the lactate dehydrogenase of Streptococcus cremoris. J. Bact. 111, 397-403.

Kaback, H.R. and Milner, L.S. (1970). Relationship of a membrane bound D(-) lactic dehydrogenase to amino acid transport in isolated bacterial membrane preparations. Proc. Nat. Acad. Sci. USA 66, 1008-1015.

Kachmar, J.F. and Boyer, P.D. (1953). Kinetic analysis of enzyme reactions. II. The potassium activation and calcium inhibition of pyruvic phosphoferase. J. Biol. Chem. 200, 669-682.

Kandler, O. (1961). Stoffwechsel der Saurewecker-Organismen. Milchwissenschaft 16, 523-531.

Kaplan, N.O., Everse, J. and Admiraal, J. (1968). Significance of substrate inhibition of dehydrogenases. Ann. N.Y. Acad. Sci. 151, 400-412.

Knappe, J., Bohnert, E. and Bruemmer, W. (1965). S-Adenosyl-L-methionine, a component of the clastic dissimilation of pyruvate in Escherichia coli. Biochim. Biophys. Acta 107, 603-605.

Kornberg, H.L. (1970). The role and maintenance of the tricarboxylic acid cycle in Escherichia coli. In Biochemical Society Symposium: 30. British Biochemistry Past and Present. 155-171. (Goodwin, T.W. ed.) Academic Press, New York and London.

Kornberg, H.L. and Malcovati, M. (1973). Control in situ of the pyruvate kinase activity of Escherichia coli. FEBS Letters 32, 257-259.

Koshland, D.E.Jr, Nemethy, G. and Filmer, D. (1966). Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochemistry 5, 365-385.

Koster, J.F. and Hulsmann, W.C. (1970). The influence of inorganic phosphate and phosphorylated hexoses on the activity of pyruvate kinase. Archives Biochem. Biophys. 141, 98-101.

Koster, J.F., Slee, R.G., Staal, G.E.J. and Van Berkel, Th.J.C. (1972). The influence of glucose 1,6-diphosphate on the enzymatic activity of pyruvate kinase. Biochim. Biophys. Acta 258, 763-768.

Krebs, H.A. and Eggleston, L.V. (1965). The role of pyruvate kinase in the regulation of gluconeogenesis. Biochem. J. 94, 3c-4c

Krebs, H.A. and Kornberg, H.L. (1957). A survey of the energy transformations in living matter. Ergeb. Physiol. Biol. Chem. u. Exptl. Pharmakol. 49, 212-298.

Kuczenski, R.T. and Suelter, C.H. (1970). Yeast pyruvate kinase. Native and subunit molecular weight. Biochemistry 9, 2043-2047.

Lindmark, D.G., Paolella, P. and Wood, N.P. (1969). The pyruvate formate-lyase system of Streptococcus faecalis. I. Purification and properties of the formate-pyruvate exchange enzyme. J. Biol. Chem. 244, 3605-3612.

Llorente, P., Marco, R. and Sols, A. (1970). Regulation of liver pyruvate kinase and the phosphoenolpyruvate crossroads. Eur. J. Biochem. 13, 45-54.

London, J. and Kline, K. (1973). Aldolase of lactic acid bacteria: a case history in the use of an enzyme as an evolutionary marker. Bact. Rev. 37, 453-478.

London, J., Meyer, E. and Kulczyk, S. (1970). Allosteric control of a Lactobacillus malate dehydrogenase (decarboxylating) by two glycolytic intermediate products. Biochim. Biophys. Acta 212, 512-514.

London, J., Meyer, E.Y. and Kulczyk, S. (1971). Comparative biochemical and immunological study of malic enzyme from two species of lactic acid bacteria: Evolutionary Implications. J. Bact. 106, 126-137.

Long, L.G. and Kaplan, N.O. (1968). D-lactate specific pyridine nucleotide lactate dehydrogenases in animals. Science 162, 685-686.

Lowry, O.H., Carter, J., Ward, J.B. and Glaser, L. (1971). The effect of carbon and nitrogen on the level of metabolic intermediates in Escherichia coli. J. Biol. Chem. 246, 6511-6521.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Macfarlane, N. and Ainsworth, S. (1972). A kinetic study of Baker's-yeast pyruvate kinase activated by fructose 1,6-diphosphate. Biochem. J. 129, 1035-1047.

MacLeod, P. and Morgan, M.E. (1958). Differences in the ability of lactic streptococci to form aldehydes from certain amino-acids. J. Dairy Sci. 41, 908-913.

Maeba, P. and Sanwal, B.D. (1968). The regulation of pyruvate kinase of Escherichia coli by fructose diphosphate and adenylic acid. J. Biol. Chem. 243, 448-450.

Malcovati, M. and Kornberg, H.L. (1969). Two types of pyruvate kinase in Escherichia coli K12. Biochim. Biophys. Acta 178, 420-423.

Marth, E.H. (1962). III Certain aspects of starter culture metabolism. J. Dairy Sci. 45, 1271-1281.

Maxwell, E.S., Kurahashi, K. and Kalckar, H.M. (1962). Enzymes of the Leloir pathway. In Methods in Enzymology 5, 174-189. (Colowick, S.P. and Kaplan, N.O. eds) Academic Press Inc., New York.

McKay, L., Miller III, A., Sandine, W.E. and Elliker, P.R. (1970). Mechanisms of lactose utilization by lactic acid streptococci: enzymatic and genetic analyses. J. Bact. 102, 804-809.

McKay, L.L., Walter, L.A., Sandine, W.E. and Elliker, P.R. (1969). Involvement of phosphoenolpyruvate in lactose utilization by group N streptococci. J. Bact. 99, 603-610.

Mildvan, A.S. and Cohn, M. (1966). Kinetic and magnetic resonance studies of the pyruvate kinase reaction. II. Complexes of enzyme, metal, and substrates. J. Biol. Chem. 241, 1178-1193.

Mizushima, S., Hiyama, T. and Kitahara, K. (1964). Quantitative studies on glycolytic enzymes in *Lactobacillus plantarum*. III. Intracellular activities of reverse reaction of D- and L-lactate dehydrogenases during glucose fermentation. J. Gen. Appl. Microbiol. 10, 33-44.

Mizushima, S. and Kitahara, K. (1964). Quantitative studies on glycolytic enzymes in *Lactobacillus plantarum*. II. Intracellular concentrations of glycolytic intermediates in glucose-metabolizing washed cells. J. Bact. 87, 1429-1435.

Model, P. and Rittenberg, D. (1967). Measurement of the activity of the hexose monophosphate pathway of glucose metabolism with the use of ^{18}O glucose. Variations in its activity in *E. coli* with growth conditions. Biochemistry 6, 69-79.

Monod, J., Wyman, J. and Changeux, J.-P. (1965). On the nature of allosteric transitions: a plausible model. J. Mol. Biol. 12, 88-118.

Mou, L., Mulvena, D.P., Jonas, H.A. and Jago, G.R. (1972). Purification and properties of nicotinamide adenine dinucleotide-dependent D- and L-lactate dehydrogenases in a group N streptococcus. J. Bact. 111, 392-396.

Nakae, T. and Elliott, J.A. (1965a). Volatile fatty acids produced by some lactic acid bacteria. I. Factors influencing production of volatile fatty acids from casein hydrolysate. J. Dairy Sci. 48, 287-292.

Nakae, T. and Elliott, J.A. (1965b). Production of volatile fatty acids by some lactic acid bacteria. II. Selective formation of volatile fatty acids by degradation of amino-acids. J. Dairy Sci. 48, 293-299.

Neimark, H. and Tung, M.C. (1973). Properties of a fructose-1,6-diphosphate-activated lactate dehydrogenase from Acholeplasma laidlawii type A. J. Bact. 114, 1025-1033.

Newsholme, E.A. and Gevers, W.C. (1967). Control of glycolysis and gluconeogenesis in liver and kidney cortex. Vitamins and Hormones 25, 1-87.

Nicholas, P.C. and Bachelard, H.S. (1974). Kinetic properties of cerebral pyruvate kinase. Biochem. J. 141, 165-171.

O'Carra, P. and Barry, S. (1972). Affinity chromatography of lactate dehydrogenase. Model studies demonstrating the potential of the technique in the mechanistic investigation as well as in the purification of multi-substrate enzymes. FEBS Letters 21, 281-285.

Ozaki, H. and Shiio, I. (1969). Regulation of the TCA and glyoxylate cycles in Brevibacterium flavum. II. Regulation of phosphoenolpyruvate carboxylase and pyruvate kinase. J. Biochem. (Tokyo) 66, 297-311.

Passeron, S., Jimenez de Asua, L. and Carminatti, H. (1967). Fructose 1,6-diphosphate, a reactivator of Cu^{++}-inhibited pyruvate kinase from liver. Biochem. Biophys. Res. Commun. 27, 33-38.

Passeron, S. and Terenzi, H. (1970). Activation of pyruvate kinase of Mucor rouxii by manganese ions. FEBS Letters 6, 213-216.

Platt, T.B. and Foster, E.M. (1958). Products of glucose metabolism by homofermentative streptococci under anaerobic conditions. J. Bact. 75, 453-460.

Pogson, C.I. (1968a). Two interconvertible forms of pyruvate kinase in adipose tissue. Biochem. Biophys. Res. Commun. 30, 297-302.

Pogson, C.I. (1968b). Adipose-tissue pyruvate kinase. Properties and interconversion of two active forms. Biochem. J. 110, 67-77.

Reiter, B. and Moller-Madsen, A. (1963). Reviews of the progress of dairy science. Section B. Cheese and butter starters. J. Dairy Res. 30, 419-456.

Ritchey, T.W. and Seeley, H.W.Jun. (1974). Cytochromes in Streptococcus faecalis var zymogenes grown in a haematin-containing medium. J. Gen. Microbiol. 85, 220-228.

Rozengurt, E., Jimenez de Asua, L. and Carminatti, H. (1969). Some kinetic properties of liver pyruvate kinase (Type L). II. Effect of pH on its allosteric behaviour. J. Biol. Chem. 244, 3142-3147.

Sandine, W.E., Elliker, P.R. and Hays, H. (1962). Cultural studies on Streptococcus diacetilactis and other members of the lactic Streptococcus group. Canad. J. Microbiol. 8, 161-174.

Sandine, W.E., Seitz, E.W., Elliker, P.R. and Day, E.A. (1961). Studies on the mechanism of diacetyl synthesis by Streptococcus diacetilactis. J. Dairy Sci. 44, 1158-1159.

Sanwal, B.D. (1970). Allosteric controls of Amphibolic pathways in bacteria. Bact. Rev. 34, 20-39.

Schleifer, K.H. and Kocur, M. (1973). Classification of staphylococci based on chemical and biochemical properties. Archives Mikrobiol. 93, 65-85.

Schwert, G.W. and Winer, A.D. (1963). Lactate dehydrogenase. In The Enzymes 7, 127-148. (Boyer, P.D., Lardy, H. and Myrback, K. eds) Academic Press, New York and London.

Scrutton, M.C. and Utter, M.F. (1968). The regulation of glycolysis and gluconeogenesis in animal tissues. Annu. Rev. Biochem. 37, 249-302.

Shapiro, B.M. and Stadtman, E.R. (1967). Regulation of glutamine synthetase. IX. Reactivity of the sulphydryl groups of the enzyme from Escherichia coli. J. Biol. Chem. 242, 5069-5079.

Sijpesteijn, A.K. (1970). Induction of cytochrome formation and stimulation of oxidative dissimulation by hemin in Streptococcus lactis and Leuconostoc mesenteroides. Antonie van Leeuwenhoek 36, 335-348.

Silverstein, E. and Boyer, P.D. (1964). Equilibrium reaction rates and the mechanisms of bovine heart and rabbit muscle lactate dehydrogenase. J. Biol. Chem. 239, 3901-3914.

Simoni, R.D., Smith, M.F. and Roseman, S. (1968). Resolution of a staphylococcal phosphotransferase system into four protein components and its relation to sugar transport. Biochem. Biophys. Res. Commun. 31, 804-811.

Smalley, A.J., Jahrling, P. and Van Demark, P.J. (1968). Molar growth yields as evidence for oxidative phosphorylation in Streptococcus faecalis strain 10Cl. J. Bact. 96, 1595-1600.

Snoswell, A.M. (1959). Stereospecific diphosphopyridine nucleotide independent lactic dehydrogenases of Lactobacillus arabinosus 17·5. Biochim. Biophys. Acta 35, 574-575.

Snoswell, A.M. (1961). Lactic dehydrogenase of Lactobacillus arabinosus. Biochem. J. 79, 22P-23P.

Snoswell, A.M. (1963). Oxidized nicotinamide-adenine dinucleotide independent lactate dehydrogenases of Lactobacillus arabinosus 17·5. Biochim. Biophys. Acta 77, 7-19.

Sokatch, J.T. and Gunsalus, I.C. (1957). Aldonic acid metabolism. I. Pathway of carbon in an inducible gluconate fermentation by Streptococcus faecalis. J. Bact. 73, 452-460.

Somogyi, M. (1952). Notes on sugar determinations. J. Biol. Chem. 195, 19-23.

Srere, P.A. (1967). Enzyme concentrations in tissues. Science 158, 936-937.

Srere, P.A. (1968). Studies on purified citrate enzymes: metabolic interpretations. In Metabolic Roles of Citrate Proceedings. Biochemical Society Symposium, P11. (Goodwin, T.W. ed.) 27th Academic Press Inc., London.

Staal, G.E.J., Koster, J.F., Kamp, H., Van Milligen-Boersma, L. and Veeger, C. (1971). Human erythrocyte pyruvate kinase. Its purification and some properties. Biochim. Biophys. Acta 227, 86-96.

Stadtman, E.R. (1966). Allosteric regulation of enzyme activity. In Advances in Enzymology, 28, 41-154. (Nord, F.F. ed.) Interscience Publishers.

Stadtman, E.R. (1970). Mechanisms of enzyme regulation in metabolism. In The Enzymes (Student Edition) 1, 397-459. (Boyer, P.D. ed.) Academic Press, New York and London.

Steinmetz, M.A. and Deal, W.C.Jr (1966). Metabolic control and structure of glycolytic enzymes. III. Dissociation and subunit structure of rabbit muscle pyruvate kinase. Biochemistry 5, 1399-1405.

Stevenson, P.M. and Holdsworth, E.S. (1973). The distribution of NAD-independent lactic dehydrogenases amongst micro-organisms. J. Gen. Microbiol. 78, 83-88.

Stirpe, F. and Della Corte, E. (1970). The regulation of rat liver xanthine oxidase: conversion of type D (dehydrogenase) into type O (oxidase) by a thermolabile factor, and reversibility by dithioerythritol. Biochim. Biophys. Acta 212, 195-197.

Stockland, A.E. and San Clemente, C.L. (1969). Multiple forms of lactate dehydrogenase in Staphylococcus aureus. J. Bact. 100, 347-353.

Susor, W.A. and Rutter, W.J. (1968). Some distinctive properties of pyruvate kinase purified from rat liver. Biochem. Biophys. Res. Commun. 30, 14-20.

Tanaka, T., Harano, Y., Morimura, H. and Mori. R. (1965). Evidence for the presence of two types of pyruvate kinase in rat liver. Biochem. Biophys. Res. Commun. 21, 55- 60.

Tanaka, T., Harano, Y., Sue, F. and Morimura, H. (1967). Crystallization, characterization and metabolic regulation of two types of pyruvate kinase isolated from rat tissues. J. Biochem. (Tokyo) 62, 71-91.

Tarmy, E.M. and Kaplan, N.O. (1968a). Chemical characterization of D-lactate dehydrogenase from Escherichia coli B. J. Biol. Chem. 243, 2579-2586.

Tarmy, E.M. and Kaplan, N.O. (1968b). Kinetics of Escherichia coli B D-lactate dehydrogenase and evidence for pyruvate-controlled change in conformation. J. Biol. Chem. 243, 2587-2596.

Taylor, C.B. and Bailey, E. (1967). Activation of liver pyruvate kinase by fructose 1,6-diphosphate. Biochem. J. 102, 32c-33c.

Thomas, T.D. (1968). Metabolism of lactic acid bacteria. Ph.D. Thesis, Massey University.

Thomas, T.D., Jarvis, B.D.W. and Skipper, N.A. (1974). Localization of proteinase(s) near the cell surface of Streptococcus lactis. J. Bact. 118, 329-333.

Thomson, J.F., Darling, J.J. and Bordner, L.F. (1964). Isotope and solvent effects of deuterium on rabbit-muscle lactic dehydrogenase. Biochim. Biophys. Acta 85, 177-185.

Tuominen, F.W. and Bernlohr, R.W. (1971a). Pyruvate kinase of the spore-forming bacterium, Bacillus licheniformis. I. Purification, stability, regulation of synthesis, and evidence for multiple molecular states. J. Biol. Chem. 246, 1733-1745.

Tuominen, F.W. and Bernlohr, R.W. (1971b). Pyruvate kinase of the spore-forming bacterium, Bacillus licheniformis. II. Kinetic properties. J. Biol. Chem. 246, 1746-1755.

Umbarger, H.E. (1956). Evidence for a negative-feedback mechanism in the biosynthesis of isoleucine. Science 123, 848.

Utter, M.F. (1963). Pathways of phosphoenolpyruvate synthesis in glycogenesis. Iowa State J. Sci. 38, 97-113.

Vakil, J.R. and Shahani, K.M. (1969). Carbohydrate metabolism of lactic acid cultures. II. Different pathways of lactose metabolism of Streptococcus lactis and their sensitivity to antibiotics. J. Dairy Sci. 52, 162-168.

Van Berkel, Th.J.C., Koster, J.F. and Hulsmann, W.C. (1972). Distribution of L- and M-type pyruvate kinase between parenchymal and Kupffer cells of rat liver. Biochim. Biophys. Acta 276, 425-429.

Van Berkel, Th.J.C., Koster, J.F. and Hulsmann, W.C. (1973a). Two interconvertible forms of L-type pyruvate kinase from rat liver. Biochim. Biophys. Acta 293, 118-124.

Van Berkel, Th.J.C., Koster, J.F. and Hulsmann, W.C. (1973b). Some kinetic properties of the allosteric M-type pyruvate kinase from rat liver; influence of pH and the nature of amino acid inhibition. Biochim. Biophys. Acta 321, 171-180.

Van Berkel, Th.J.C., Koster, J.F., Kruyt, J.K. and Hulsmann, W.C. (1974). On the regulation and allosteric model of L-type pyruvate kinase from rat liver. Biochim. Biophys. Acta 370, 450-458.

Van Berkel, Th.J.C., Koster, J.F. and Staal, G.E.J. (1973c). On the molecular basis of pyruvate kinase deficiency. I. Primary defect or consequence of increased glutathione disulfide concentration. Biochim. Biophys. Acta 321, 496-502.

de Vries, W., Kapteijn, W.M.C., Van der Beek, E.G. and Stouthamer, A.H. (1970). Molar growth yields and fermentation balances of Lactobacillus casei L3 in batch cultures and in continuous cultures. J. Gen. Microbiol. 63, 333-345.

de Vries, W. and Stouthamer, A.H. (1968). Fermentation of glucose, lactose, galactose, mannitol and xylose by bifidobacteria. J. Bact. 96, 472-478.

Waygood, E.B. and Sanwal, B.D. (1971). Proc. Can. Fed. Biol. Soc. 14, 164. Reference in Waygood and Sanwal (1974).

Waygood, E.B. and Sanwal, B.D. (1972). Regulation of pyruvate kinases by succinyl coenzyme A. Biochem. Biophys. Res. Commun. 48, 402-407.

Waygood, E.B. and Sanwal, B.D. (1974). The control of pyruvate kinase of Escherichia coli. I. Physicochemical and regulatory properties of the enzyme activated by fructose 1,6-diphosphate. J. Biol. Chem. 249, 265-274.

Weber, K. and Osborn, M. (1969). The reliability of molecular weight determinations by dodecyl-sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244, 4406-4412.

Weber, G., Stamm, N.B. and Fisher, E.A. (1965). Insulin: inducer of pyruvate kinase. Science 149, 65-67.

White, J.C. and Sherman, J.M. (1943). The lactic acid fermentation of streptococci under aerobic conditions. J. Dairy Sci. 26, 371-374.

Whittenbury, R. (1964). Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. J. Gen. Microbiol. 35, 13-26.

Wilkinson, J.H. (1969). Lactate dehydrogenase isoenzymes. In Isoenzymes, 134-203. Chapman and Hall.

Winer, A.D., Novoa, W.B. and Schwert, G.W. (1957). Observations of new phenomena in the fluorescence spectrum of a diphosphopyridine nucleotide-linked dehydrogenase. J. Amer. Chem. Soc. 79, 6571-6572.

Wittenberger, C.L. (1968). Kinetic studies on the inhibition of a D(-)-specific lactate dehydrogenase by adenosine triphosphate. J. Biol. Chem. 243, 3067-3075.

Wittenberger, C.L. and Angelo, N. (1970). Purification and properties of a fructose-1,6-diphosphate-activated lactate dehydrogenase from Streptococcus faecalis. J. Bact. 101, 717-724.

Wittenberger, C.L. and Fulco, J.G. (1967). Purification and allosteric properties of a nicotinamide adenine dinucleotide-linked D(-)-specific lactate dehydrogenase from Butyribacterium rettgeri. J. Biol. Chem. 242, 2917-2924.

Wittenberger, C.L. and Haaf, A.S. (1966). Isolation of NAD-dependent and NAD-independent lactate dehydrogenases from extracts of Butyribacterium rettgeri. Biochim. Biophys. Acta 122, 393-405.

Wittenberger, C.L., Palumbo, M.P., Bridges, R.B. and Brown, A.T. (1971). Mechanisms for regulating the activity of constitutive glucose degradative pathways in Streptococcus faecalis. J. Dent. Res. 50, 1094-1103.

Wittenberger, C., Palumbo, M. and Klein, I. (1973). Two forms of pyruvate kinase from Streptococcus faecalis. In Ninth International Congress of Biochemistry Abstracts, 2e 15, P.62.

Wolin, M.J. (1964). Fructose-1,6-diphosphate requirement of streptococcal lactic dehydrogenases. Science 146, 775-777.

Wood, T. (1968). The inhibition of pyruvate kinase by ATP. Biochem. Biophys. Res. Commun. 31, 779-785.

Wood, W.A. (1961). Fermentation of carbohydrates and related compounds. In. The Bacteria 2, 59-149. (Gunsalus, I.C. and Stanier, R.Y. eds) Academic Press, New York and London.

Yamada, T., Hojo, S., Kobayashi, K., Asano, Y. and Araya, S. (1970). Studies on the carbohydrate metabolism of cariogenic Streptoccus mutans strain PK-1. Archives oral Biol. 15, 1205-1217.

Yoshida, A. (1965). Enzymic properties of lactate dehydrogenase of Bacillus subtilis. Biochim. Biophys. Acta 99, 66-77.

Yoshida, A. and Freese, E. (1965). Purification and chemical characterization of lactate dehydrogenase of Bacillus subtilis. Biochim. Biophys. Acta 99, 56-65.

Yudkin, J. and Krauss, R. (1967). Dietary starch, dietary sucrose and hepatic pyruvate kinase in rat. Nature 215, 75.

Zewe, V. and Fromm, H.J. (1962). Kinetic studies of rabbit muscle lactate dehydrogenase. J. Biol. Chem. 237, 1668-1675.