SOME ASPECTS OF GLUCONEOGENESIS IN *VEILLONELLA PARVULA* M_4

by

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_ In memory of my mother
吳戴傑文

_ Dedicated to my father
吳多泰

my wife
吳許慧華

my daughter
吳愛恆

my mother-in-law
許麥惠玲

_ for their unfailing help
and forbearance.

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ABSTRACT

The anaerobic oral microbe, Veillonella parvula M_4, unable to metabolize carbohydrates but utilizing short chain acids, such as lactate and pyruvate for energy, was examined for its ability to carry out gluconeogenesis. As an initial step, pure cultures of V. parvula M_4 were incubated in tryptone-yeast extract broth containing lactate-U-^{14}C in an atmosphere of pure nitrogen at 37 C and the cells harvested in the late exponential phase. The distribution of label in cellular and extracellular material, as well as metabolic end-products, was determined. Significant lactate-^{14}C carbon appeared in two extracellular lipopolysaccharide fractions, LPS_1 (1.4%) and LPS_2 (22.0%), while minor quantities were found in cellular components such as amino acids (0.6%) and DNA material (0.6%); the greatest fraction on the ^{14}C was present in the catabolic end-products: propionate, acetate and CO_2 (68.9%). The LPS_1 fraction contained only glucose and galactose while the LPS_2 material, originally bound to a protein fraction, consisted of glucose, glucosamine, galactose, galactosamine and ribose. Labelled lactate carbon was also observed in the cellular amino acids: arginine, aspartate, glutamate, alanine, serine, as well as in an unknown ninhydrin positive spot. These observations demonstrated that gluconeogenesis had occurred in V. parvula M_4 during growth in the lactate medium.

The second phase of the project undertook to obtain information on the enzymes involved in the initial reactions of gluconeogenesis i.e., from pyruvate to P-enolpyruvate. Presumptive evidence was obtained for the

presence of pyruvate kinase, PEP synthetase, PEP carboxykinase (ATP), PEP carboxylase, enolase, ATP-dependent pyruvate carboxylase, NADP- and NAD-specific malic enzymes, and an unusual ATP-independent pyruvate carboxylase.

The presence of a pyruvate kinase in a non-glycolytic micro-organism such as V. parvula M_4 was surprising and prompted the isolation and purification of the enzyme. The enzyme was purified 126-fold by protamine sulphate and ammonium sulphate precipitation (10 - 30%), and by Sephadex G-200 and Sephadex G-100 column chromatography; the enzyme showed one band on disc gel electrophoresis. The purified pyruvate kinase was free of contaminating enzymes.

The purified pyruvate kinase had an optimum pH of 7.0 and exhibited sigmoid kinetics with PEP, ADP and Mg^{2+}, with apparent Michaelis constants of 1.2, 3.5 and 6.0 mM, respectively. Hill plots gave 'n' values of 4 for PEP and 2 for both ADP and Mg^{2+} ions. Under optimal conditions, the enzyme activity increased linearly with increasing temperature from 15 to 40 C and tended to level off between 40 and 50 C. The dritical temperature for the enzyme was at 32 C and the experimental equilibrium constant was 828 in the direction of pyruvate formation.

The V. parvula enzyme was inhibited non-competitively by ATP (K_i = 3.4 mM) and this inhibition could not be completely reversed by increasing concentrations of Mg^{2+} ions up 12 mM. Competitive inhibition was also observed with 3-P-glycerate, 2,3 diphosphoglycerate and malate; the K_i values were 4.4, 7.5 and 5.5 mM, respectively. The enzyme was activated by glucose-6-P, fructose-6-P, fructose-1,6-P_2, dihydroxy-acetone-P, and AMP; the Hill coefficients were 2.1, 2.5, 1.8, 1.8 and 0.9,

respectively. The activation constants for glucose-6-P, fructose-1,6-P_2 and AMP were 5.3, 0.3 and 1.1 mM, respectively, with the V_{max} for the enzyme increased as follows: AMP>fructose 1,-6P_2>dihydroxyacetone-P> glucose-6-P>fructose-6-P. The enzyme was not affected by glucose, glyceraldehyde-3-P, 2-P-glycerate, lactate, malate, fumarate, succinate and cyclic AMP. The results indicate that the V. parvula pyruvate kinase plays a central role in the control of gluconeogenesis by regulating the concentration of PEP.

The ability of dialyzed and charcoal-treated crude extracts of V. parvula M_4 to catalyze the formation of oxaloacetate from pyruvate and CO_2 in the absence of ATP initiated attempts to purify this ATP-independent 'pyruvate carboxylase' (IPC) activity from the organism. Subsequently, the enzyme was purified following treatment with protamine sulphate and activated charcoal, and by ammonium sulphate precipitation (30 - 50%). Following further purification by Sephadex G-100 and CM-cellulose column chromatography, the ATP-independent activity was purified 135-fold and was essentially homogeneous except for very minor contamination by malate dehydrogenase. The enzyme had a pH optimum of 7.0 and required only pyruvate, HCO_3^- and Mg^{2+} for oxaloacetate formation. The rate curves for these compounds were hyperbolic and produced K_m values of 3.3, 1.74 and 1.3 mM for pyruvate, HCO_3^- and Mg^{2+}, respectively. The Keq of the purified IPC enzyme was 2.13 in favour of oxaloacetate formation.

The IPC enzyme was competitively inhibited by ATP (K_i = 4.75 mM), as well as by increasing concentrations of arsenate, AMP and cyclic AMP. On the other hand, the ATP-independent activity was activated by aspartate

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(K_a = 33 mM) and produced a change in the Hill coefficient from 0.9 to 2.0 at pyruvate concentrations above 9.0 mM. The role of the ATP-independent 'pyruvate carboxylase' in catabolism and in gluconeogenesis in V. parvula M_4 is discussed.

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CHAPTER 1

INTRODUCTION

The main theme of the investigations outlined in this thesis concern the process of gluconeogenesis in the obligate anaerobe, Veillonella parvula M_4, originally isolated from human saliva. This organism and other members of the genus, Veillonella, constitute a significant fraction (11-15%) of the microflora of the oral cavity and are unique since they cannot metabolize carbohydrates; these organisms rather convert short chain acids, such as lactate and pyruvate, to propionate, acetate, carbon dioxide and hydrogen for energy. Mergenhagen and coworkers have also shown that the growth of Veillonella species on lactate also results in the formation of a serologically specific lipopolysaccharide, which contains glucosamine, galactosamine, galactose, glucose and fucose. Since the cells were grown in an undefined medium composed of tryptone, yeast extract and lactate, it was not known whether these large molecules arose from the lactate carbon source or from the other components of the medium. The possibility that gluconeogenesis proceeded from lactate was questioned because, although Veillonella species contain various enzymes of the glycolytic pathway, recent work by Rogosa, and by Michaud and Delwiche, has indicated that this scheme in these bacteria is truncated.

Thus, the first part of this investigation (Chapter 3) set out to determine whether cells of V. parvula M_4, growing on lactate, could form complex lipopolysaccharide material and, if so, whether lactate carbon

was distributed in this material. Since it was subsequently shown that lactate carbon formed a part of the lipopolysaccharide complexes in V. parvula M_4, it was clear that some form of reverse glycolysis was possible. This general demonstration of gluconeogenesis then led to a general survey of the initial reactions in this process, i.e., those involving the formation of P-enolpyruvate from pyruvate (Chapter 4). The studies in Chapters 3 and 4 constitute the first section of this thesis.

The second section presents detailed information on the properties and characteristics of two enzymes detected in the preliminary survey in Chapter 4: pyruvate kinase (Chapter 5) and an ATP-independent 'pyruvate carboxylase' (Chapter 6). Pyruvate kinase was selected for further study because its presence in the non-glycolytic veillonella has been questioned, and because it regulates the availability of P-enolpyruvate for gluconeogenesis. Interest in the second enzyme arose from the observation that dialyzed and charcoal-treated crude extracts of V. parvula M_4 formed oxaloacetate from pyruvate and carbon dioxide in the absence of an energy source. This ATP-independent 'pyruvate carboxylase' forms part of the 'indirect pathway' of P-enolpyruvate formation from pyruvate.

CHAPTER 2

GENERAL LITERATURE REVIEW

Gluconeogenesis is a biosynthetic process whereby non-carbohydrate precursors, such as lactic and amino acids, are converted to carbohydrates generally represented by glucose. This ability to form glucose is of considerable physiological importance to the living organism since glucose represents a primary source of energy for cell maintenance as well as the necessary building blocks for the biosynthesis of essential cell components. Since gluconeogenesis is a universal process, occurring in plant, animal and microbial cells, it is impossible to divide the knowledge gained on this subject into respective biological kingdoms since the findings from all sources are complementary. The main concern of this introduction is not to give a complete account, which is in itself an impossible task, but rather to give a brief history of gluconeogenic processes in animal and bacterial cells.

A. The origin of sugars.

Speculation concerning the origin of sugars in nature has developed concurrently with the growth of knowledge of their chemistry. Butlerow (1861a,b) was the first to demonstrate that mild alkalis would

convert formaldehyde to a mixture of monosaccharides of unknown composition, which he called 'formose' or 'methose'. Baeyer (1870) later proposed the controversial 'formaldehyde theory' in the belief that this aldehyde was polymerized into carbohydrate. He suggested that in plants, formaldehyde was formed from carbon dioxide and water in the presence of sunlight. The formaldehyde, in turn, resulted in the formation of carbohydrates. Similar findings were reported by Loew in 1886. In the period 1880-90, the general concensus of opinion favored the theory, although views differed as to the chemistry of the process (Fischer and Passmore, 1889; Fischer, 1890a,b,c). More recently, proponents of the formaldehyde theory received encouragement when Hough and Jones (1951) and Mariani and Torraca (1953) showed that the 'formose' solution produced by Butlerow (1861a,b) and Loew (1886) was a complex mixture containing glyceraldehyde, trioses, tetroses, pentoses and hexoses. However, Rabinowitch (1945) disputed the suggestion that formaldehyde itself was a precursor of carbohydrates in plants since it had never been found in plant tissue despite an extensive search for it. The earlier isotopic studies of Ruben, Kaman and Hassid (1940) supported this contention.

Clearly, the introduction of the formaldehyde theory signalled the start of a long program of research on the chemical synthesis of hexoses from trioses in alkaline solutions (Schmitz, 1913; Fischer and Baer, 1936; Meyerhof and Schulz, 1936; Berl and Feazel, 1951). These studies set the stage for the suggestion that hexoses could be synthesized enzymatically from trioses. In fact, studies by Utkin (1949, 1950, 1955) demonstrated that hexoses arose biologically by the condensation of

'glycerose' with dihydroxyacetone. This was the first indication that gluconeogenesis could occur in nature.

A vast amount of information has been derived from investigations with microbial and muscle extracts, two dissimilar systems with similar biosynthetic processes. Although D-glucose is converted to ethanol and carbon dioxide by yeast, and to lactate acid by muscle during contraction, both processes depend on a single metabolic scheme: glycolysis. Of particular importance is the fact that this glycolysis is, for the most part, reversible, providing a means of glucose biosynthesis from small molecules.

B. Early studies in animal systems.

Metabolic studies carried out with fasted, phlorizinized, depancreatized and other types of experimentally diabetic animals have yielded a great deal of information concerning the kinds of cellular substances which are 'sugar formers'. However, the process of hexose biosynthesis was not elucidated until the various end-products of carbohydrate catabolism had been determined.

In 1899, Morishima proved that d-lactate was present in normal liver, blood, muscle, kidney and the gastro-intestinal wall following the addition of sugar. However, at that time it was not known whether the lactic acid produced arose from the sugar administered or from the degradation of protein.

When comparing the phenomena of tetanus and rigor mortis in frog, Werther (1890) noted that both processes were accompanied by a reduction in muscle glycogen and an increase in blood lactic acid suggesting the

formation of lactic acid from glycogen. However, Araki (1891), opposed this conclusion and indicated that lactic acid (as well as sugar) appeared in the urine under conditions where the tissues were oxygen starved through the action of carbon monoxide, morphins, curarin, amyl nitrite, hydrocyanic acid, strychnin or veratrin. However, subsequent work by Embden (1905) clearly demonstrated that carbohydrate metabolism by animal tissues resulted in the formation of lactic acid. For example, high concentrations of lactic acid were found in blood percolated through liver rich in glycogen, or in blood rich in sugar percolated through glycogen-free liver. Little lactic acid was observed in blood containing low levels of sugar. Earlier, Stokalasa, Jelinek and Cerny (1902) had isolated cell-free extracts from muscle and other organs which were able to convert dextrose to lactic acid. These results supported the notion that lactic acid formation in animal tissues resulted from the metabolism of carbohydrate.

Asher and Jackson (1901), by ligating the coeliac, superior and inferior mesenteric, phrenic and renal arteries, cut off the blood supply to the intestine, liver, spleen, pancreas, and kidneys of a dog. Under these conditions the lactic acid concentration in the blood increased. Since the blood lactic acid concentration did not increase upon the ingestion of carbohydrates, the authors concluded that the lactic acid was derived from the metabolism of protein. This hypothesis was supported by the data of Neuberg and Langstein (1903), who isolated glycogen in the liver and lactic acid in the urine of a rabbit following the ingestion of alanine, and by von Noorden and Embden (1906), who demonstrated the production of lactic acid from leucine. Thus, by 1906, it was clear that

lactic acid formation was the result of both carbohydrate and protein metabolism (Mandel and Lusk, 1906).

Probably the most important observation from these studies was that provided by Neuberg and Langstein (1903), who first demonstrated that the process of gluconeogenesis could occur in animal liver. This postulation was confirmed by the experiments of Amalagia and Embden (1905), which showed that the ingestion of d-alanine by a dog with pancreatic diabetes increased the sugar output in the urine significantly. Embden and coworkers (1906) expanded their observations by showing that the administration of glycerol and alanine to depancreatized dogs resulted in an increase in glucose elimination. Ringer and Lusk (1906), and Hockendorf (1909) supported Embden's hypothesis and opened a new field of carbohydrate metabolism: The Chemistry of Gluconeogenesis.

Until 1912, it was generally concluded that for a substance to be gluconeogenic, it must contain an alcohol, aldehyde or a ketone group. Ringer (1912), however, disproved this suggestion by showing the propionic acid administered to a phlorphizinized dog could be quantitatively converted into glucose. Ringer's findings have been supported in recent times by Weideman and Krebs (1969), Anand and Black (1970) and Ruderman et al (1971). Thus, it is now apparent that the non-carbohydrate precursors required for the process of gluconeogenesis in animals can be either a carboxylic acid, an amino acid, or a fatty acid.

C. Gluconeogenesis in microorganisms.

The study of gluconeogenesis in microorganisms has received much less attention than the process in mammalian tissue. The reason

for this comparative lack of information may be due to one or all of the following: the complex composition of many bacterial growth media, the lack of rigid compartmentalization within the bacterial cell, and the complex nature of the intracellular and extracellular polysaccharides produced by most microorganisms. All of these factors made it difficult to discern clearly one biosynthetic process from another. Despite these problems, however, bacteria possess many advantages as a tool for the study of metabolism: (a) They can be grown on simple organic compounds which provide the precursors for many biosynthetic reactions. (b) The relative ease of genetic manipulation and mutant selection has made it possible to identify the various reactions concerned with energy metabolism in bacteria, e.g. the Embden-Meyerhof-Parnas glycolytic pathway (Elsden and Peel, 1958), the Entner-Doudoroff pathway (McGee and Doudoroff, 1954; Entner and Doudoroff, 1952) and the pentose cycle (Sokatch and Gunsalus, 1957; Nutting and Carson, 1952; Neish and Simpson, 1954) and others.

Initially, the study of gluconeogenesis in microorganisms required the formulation of the Embden-Meyerhof-Parnas glycolytic pathway (Gunsalus, Horecker and Wood, 1955; Burk, 1939; Elsden, 1952; Wood and Weiss, 1958) as the preliminary step. However, two other discoveries were also of major importance to this research: (a) the elucidation of carbon dioxide fixation processes and (b) the observed reversible operation of the tricarboxylic acid cycle (TCA cycle). Carbon dioxide fixation was first observed by Wood and Werkman (1935,1936) following fermentation studies with the propionibacteria. Since pyruvate was known to be present in these bacteria (Wood, Stone and Werkman, 1937), it was

proposed that pyruvate condensed with carbon dioxide to produce oxaloacetate (Wood-Werkman reaction).

The TCA cycle, originally proposed by Krebs and Johnson (1937), was considered by Lynen and Neciullah (1939), Lynen (1942,1943), Weinhouse and Millington (1947) and Martin and Lynen (1950) and others, to function solely as the main pathway of acetate oxidation in yeast cells. However, Krebs, Gurin and Eggleston (1952), with the information of the Wood-Werkman reaction at hand, proposed that the TCA cycle could not only provide energy via the oxidation of acetate, but also the carbon skeletons for various cellular constituents. This suggestion was supported by tracer studies with Escherichia coli (Cutinelli et al, 1951a; Roberts et al, 1955), Torulopsis utilis (Cutinelli et al, 1951b; Roberts et al, 1955), Rhodospirillum rubrum (Cutinelli et al, 1951c) and Neurospora crassa (Andersson-Kotto et al, 1954; Roberts et al, 1955). As a result of these investigations, microbial gluconeogenesis was postulated to proceed from pyruvate via the Wood-Werkman reaction, the TCA cycle and the reversal of the glycolytic pathway (Friedberg and Marshall, 1954). Of these three reactions, the most important one was the reversal of the glycolytic pathway, which was originally considered to be solely catabolic in function. The dual role of the glycolytic scheme prompted the introduction of the term 'amphibolic' by Davis (1961) to denote those enzymes or reactions which fulfill equally an anabolic and catabolic function. However, this new classification is not entirely free of ambiguities, e.g. it is not clear whether the pentose phosphate pathway is amphibolic or anabolic in nature. The term 'anaplerotic' was recently proposed by Kornberg (1965) to denote any reaction, such as the Wood-

Werkman reaction, which is used to replenish the dicarboxylic acids in the TCA cycle.

D. Pathway of gluconeogenesis.

Any plausible scheme for carbohydrate (glucose) biogenesis must depend on the free energy of the system in question. In order to minimize the length of this general introduction, the scope of the free energy discussion with regard to gluconeogenesis will be limited to that process converting lactate or pyruvate to glucose, bearing in mind that such a process can also include the amino acids and fatty acids (Fig. 2.1).

In discussing energy transformation in living matter, Krebs and Kornberg (1957) have stated that carbohydrate synthesis from lactate is, in essence, the reversal of one of the following two reactions:

$$\text{glycogen} + 3\ \text{ADP} + 3\text{HPO}_4^- + 3\ \text{H}^+ \longrightarrow 2\ \text{lactate} + 3\ \text{ATP} + 3\ \text{H}_2\text{O} \quad (2.1)$$

$$\text{glucose} + 2\ \text{ADP} + 2\ \text{HPO}_4^- \longrightarrow 2\ \text{lactate} + 2\ \text{ATP} + \text{H}_2\text{O} \quad (2.2)$$

where the ΔG for equation (2.1) is -22.2 Kcal and that for equation (2.2) is -26.9 Kcal.

The conversion of adenosine diphosphate (ADP) to high concentrations of adenosine triphosphate (ATP) by oxidative phosphorylation was taken to be the required driving force for both processes. The reversal of these two schemes, however, is not possible since three important steps of the glycolysis are not freely reversible.

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose-6-P} + \text{ADP} + \text{H}^+ \quad (2.3)$$

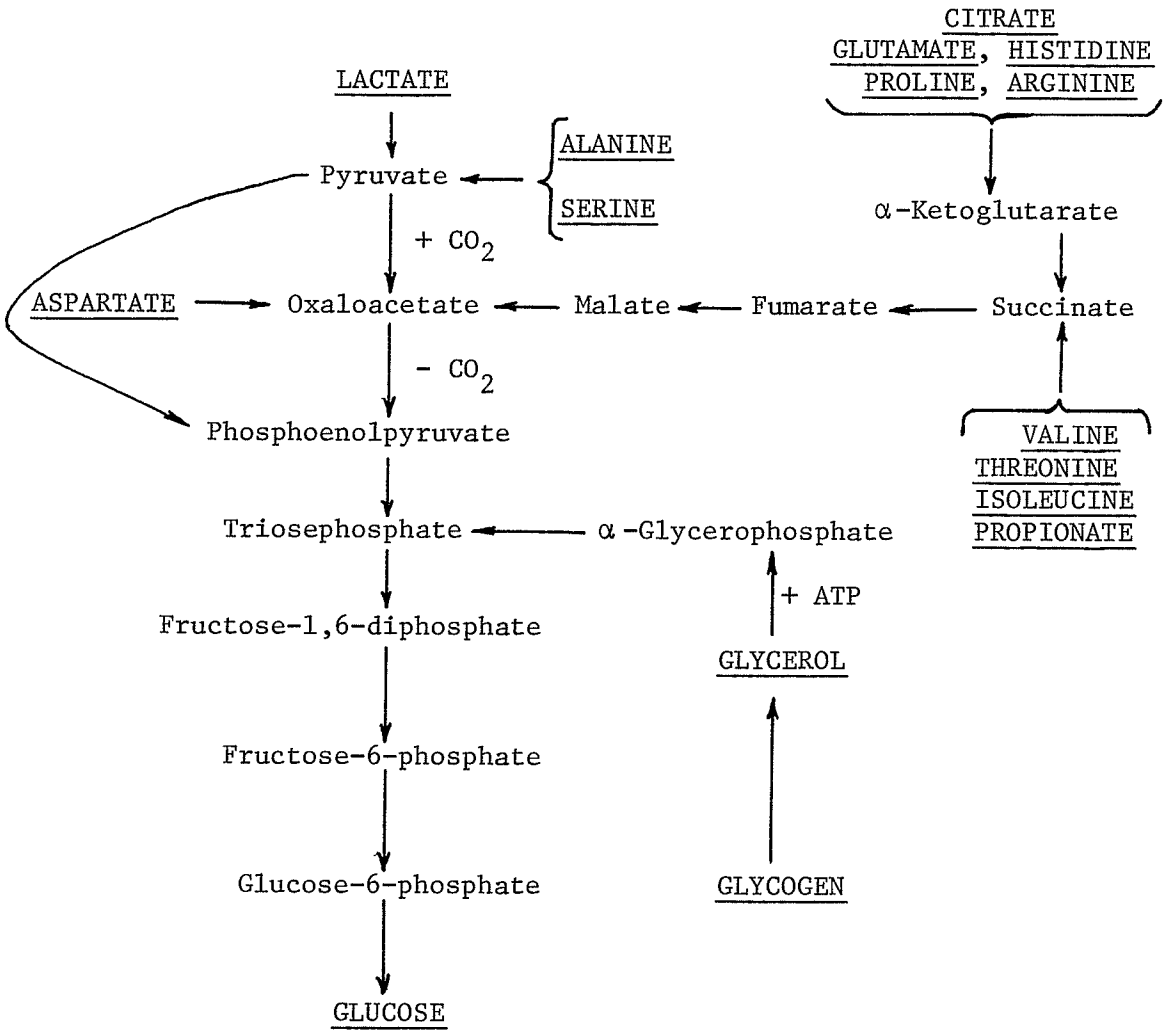


Fig. 2.1. Possible pathways of gluconeogenesis in microorganisms.

$$\text{Fructose-6-P} + \text{ATP} \longrightarrow \text{Fructose-1,6-P}_2 + \text{ADP} + \text{H}^+ \qquad (2.4)$$

$$\text{P-enolpyruvate} + \text{ADP} + \text{H}^+ \longrightarrow \text{Pyruvate} + \text{ATP} \qquad (2.5)$$

Reaction (2.3) is catalyzed by hexokinase (Meyerhof, 1927; Crane and Sols, 1953), reaction (2.4) by phosphofructokinase (Harden and Young, 1909; Ostern, Guthke and Terzakowec, 1936) and reaction (2.5) by pyruvate kinase (Kubowitz, 1944; Bucher and Pfleiderer, 1955). These three enzymatic reactions form an energy barrier against reversal since they are endergonic (when proceeding from right to left) by about 5.6 Kcalories (Krebs and Kornberg, 1957; Burton and Krebs, 1953; Burton, 1955). Since reaction (2.5) must occur twice, most of the total energy of 26.9 Kcalories (equation 2.2) required to synthesize one molecule of glucose from two molecules of lactate must be overcome by four individual reactions. The concentrations of reactants which favor such reversal can be calculated from the following:

$$\Delta G = \Delta G^{o} + RT\ln \frac{\text{Product of concentrations of end-products}}{\text{Product of concentration of starting material}}$$

From this equation, the free energy involved in converting glucose to glucose-6-P is + 5.1 (Burton and Krebs, 1953; Burton, 1955), while that for the fructose reaction is + 4.2 (Burton and Krebs, 1953; Burton, 1955) and that for pyruvate to P-enolpyruvate is + 6.1 Kcalories (Burton, 1955). Krebs and Kornberg (1957) calculated that a high ATP/ADP ratio was needed for the reversal of reaction (2.5) (about 10^6 if the other reactants were present at equal concentrations) whilst a low ratio was needed for the reversal of reactions (2.3) and (2.4) (about 10^{-6} if the other reactants

were present at equal concentrations). The two conditions (high and low ATP/ADP ratios) can hardly exist side by side in a cell, particularly in microorganisms. Therefore, a simple reversal of glycolysis was discounted as a mechanism of net synthesis. Evidence was then presented to indicate that the pathway of carbohydrate synthesis from lactate used those reactions of glycolysis which were readily reversible, but included special reactions which circumvented the energy barriers at reactions (2.3 to 2.5).

In the case of reactions (2.3) and (2.4), the barrier can be overcome if the phosphate group of the hexose ester is removed by hydrolysis rather than by its transfer to ADP. Thus, the reversal of reaction (2.3) is, in effect, replaced by reaction (2.6):

$$\text{Glucose-6-P} + H_2O \longrightarrow \text{Glucose} + H_3PO_4^- \qquad (2.6)$$

and the reversal of reaction (2.4) by:

$$\text{Fructose-1,6-P}_2 + H_2O \longrightarrow \text{Fructose-6-P} + H_3PO_4^- \qquad (2.7)$$

The two enzymes required for these reactions do, in fact, occur in gluconeogenic animal tissues and in microorganisms, e.g. glucose-6-phosphatase (equation 2.6) (Fantl and Rome, 1945; Hers and DeDuve, 1950; Swanson, 1950; Beaufays et al, 1954; Cori and Cori, 1952) and fructose-1,6-diphosphatase (equation 2.7) (Gomori, 1943; Hers and Kusaka, 1953; Hers, Beaufays and DeDuve, 1953; Pogell and McGilvery, 1954).

The energy barrier presented by the need to form P-enolpyruvate from pyruvate (equation 2.5) is circumvented by a more complex mechanism, which will be discussed in later chapters of this thesis since it forms one of the main concerns of the present investigation.

SECTION 1.

The research in this section was designed to answer two questions:

(1) Does gluconeogenesis occur in *V. parvula* M_4 during growth in lactate-broth? (Chapter 3).

(2) If gluconeogenesis does occur, what enzymes are involved in the initial states of gluconeogenesis from lactate (pyruvate) to P-enolpyruvate? (Chapter 4).

CHAPTER 3

GLUCONEOGENESIS IN VEILLONELLA PARVULA M_4

I. INTRODUCTION

The production of extracellular polysaccharide by Pneumococcus sp was first observed by Pasteur (1881) and Sternberg (1881) during their investigation of the etiology of pneumonia. However, some years passed before the relationship of this extracellular polysaccharide to the disease was recognized (Fraenkel, 1886a,b; Weichselbaum, 1886a,b). This relationship initiated the interest of investigators in the polysaccharides synthesized by microorganisms, the development of which can be divided into two main categories: (a) synthesis from carbohydrate precursors and (b) synthesis from non-carbohydrate substrates. Since the latter category is the main interest of this thesis, only brief reference will be made to the first mode of synthesis. Furthermore, the discussion will refer to both extra- and intracellular polysaccharides, since no distinction was made between these two types in the early literature.

A. Polysaccharide synthesis from carbohydrates.

In 1899, Cremer reported that the cell-free extracts of yeast were capable of synthesizing a transient compound with the properties of glycogen during the fermentation of glucose. Since then several investigators have reported the formation of polysaccharide material by

sterile filtrates obtained from various species of spore-forming bacilli. Beijerinck in 1910, observed the formation of a 'slime' layer on a sucrose-agar medium, which was catalyzed by an enzyme present in a filtered preparation of Bacillus mesentericus: he proposed the name 'viscosaccharase' for the enzyme. This 'viscosaccharase' was later isolated and purified by Peat, Schluchterer and Stacey (1938). The 'slime' was also identified as a fructose polymer (levan) appearing in the form of a coacervate ('emulsion ersheinung') (Beijerinck, 1912; Harrison, Tarr and Hibbert, 1930). Similar results were obtained with cell-free preparations from other bacteria such as Oerskov's milk bacillus (Dienes, 1935), Aerobacter (Aschner et al, 1942), Leuconostoc mesenteroides (Hehre and Sugg, 1942) and Acetobacter riscosum and Acet. capsulatum (Hehre and Hamilton, 1951). Many reports are also available on polysaccharide synthesis from carbohydrates with growing or washed bacterial cells, e.g. E. coli (Dawson and Happold, 1943; Dagley and Dawes, 1949), Neisseria perflava (Carlson and Hehre, 1949; Hehre and Hamilton, 1948), Betacoccus arabinosaceus (Barker et al, 1955), Streptococcus bovis (Bailey, 1949) and Azotobacter vinelandii (Kaufman, 1961). The study of polysaccharide synthesis in Pneumococcus sp has also contributed considerable information to the field of microbial genetics (Griffith, 1928; Avery, MacLeod and McCarty, 1944).

B. Polysaccharide synthesis via gluconeogenesis.

In 1922, Furth and Lieben found that one-half of the lactic acid utilized in an aerated yeast suspension was converted to a "hydrolysis-resistant carbohydrate". The following year Lieben (1923) reported that pyruvate

was both degraded to carbon dioxide and assimilated with the formation of "body substance". This observation was confirmed and extended by Smedley-Maclean and Hoffert (1926), and Hoffert (1926), who reported that both the fat and carbohydrate content of yeast cells increased during the oxidation of pyruvate, lactate, acetate, or ethanol. In addition, Stephenson and Whetham (1923), and Lineweaver (1933) showed that Bacillus subtilis and Azotobacter sp. metabolized either glucose or lactic acid as the sole source of carbon.

The study of carbon assimilation was considerably advanced through the investigations of Barker (1936) on the oxidation of a number of simple organic compounds by Prototheca zopfi (a colorless alga). On the basis of his data, Barker concluded that the assimilation of glycerol, ethyl alcohol, glucose, as well as, acetic, lactic, propionic, n-butyric, isobutyric and valeric acids proceeded in two experimentally distinct states. The first, which was called the primary process of assimilation, consisted of the oxidative conversion of the substrate into a carbohydrate, which was stored in the cells as glycogen. The second state was the degradation of this stored carbohydrate. Pickett and Clifton (1943) supported this conclusion in studies with Staphylococcus albus, Micrococcus luteus, Aerobacter aerogenes, Aero. clocae, Proteus vulgaris, Eberthella typhosa, Salmonella schottmiileri, Shigella dysenteriae, Serratia marcescens and Pseudomonas aeroginosa. Gluconeogenesis in other bacteria has been studied by Tarr and Hibbert (1931), Giesberger (1936), Clifton and Logan (1938), Ruben et al (1939) and Winzler (1940).

C. Extracellular polysaccharides of the Veillonella.

To-date, most of the information on microbial extracellular polysaccharide synthesis from non-carbohydrate precursors has been derived from studies with aerobic and facultative bacteria. Relatively little information is available concerning the process in the obligate anaerobes, which is undoubtedly due, in part, to the difficulty in the isolation and maintenance of these bacteria. Furthermore, many of the studies with the polysaccharides from the anaerobes has been concerned mainly with their immunological properties.

The human oral cavity is a ready source of anaerobic bacteria and contains large quantities of members of the genus, Veillonella. Species of this genus were first isolated from the mouth by Veillon and Zuber in 1898, while investigating the pathological role of certain obligate anaerobes. Since this time, the taxonomic position of members of the Veillonella has undergone a number of reviews and reclassifications (Ng, 1968). A complete revision of the classification of the Veillonella was undertaken by Rogosa in 1965.

The metabolism of Veillonella sp has been studied by Foubert and Douglas (1948), Delwiche (1948), Langford, Farber and Pelczar (1950), Johns (1951a,b), Whiteley and Douglas (1951), McCormick, Ordal, Whiteley (1962a,b), Rogosa and Bishop (1964a,b), Michaud and Delwiche (1970), and Ng and Hamilton (1971). In many cases, the results of these studies appear under the older nomenclature of Foubert and Douglas (1948) e.g. Micrococcus lactilyticus. These studies were mainly concerned with the degradation of lactate by the various species and the anabolic processes were largely ignored. However, the possibility that extracellular

polysaccharide could be synthesized from lactate in the Veillonella was suggested from the immunological studies of Mergenhagen (1960), and Rogosa, Hampp and Mackintosh (1961). With the exception of V. alcalescens, a lipopolysaccharide could be extracted with phenol and water from all of the oral strains of Veillonella following growth on sodium lactate (Mergenhagen and Varah (1963)). At the time, there was no reasonable explanation for the lack of lipopolysaccharide synthesis by V. alcalescens, except that variations in the amount of endotoxin that could be isolated from other gram-negative bacteria had been reported (Michael and Landy, 1961). As a result of this, most of the investigations on the extracellular polysaccharides of the veillonella were carried out with V. parvula or strains V2, V3 and V4 (Mergenhagen, 1960; Mergenhagen, Hampp and Scherp, 1960; Mergenhagen, Hampp and Scherp, 1961; Mergenhagen, Zipkin and Verah, 1962). Mergenhagen (1965) analysed the extracellular 'slime' produced by his strain of V. parvula and observed that the water-soluble lipopolysaccharide fraction (LPS_1), after deproteinization, contained 52% carbohydrate and 24% bound lipid. Further analysis revealed that the carbohydrate moiety was composed solely of the monosaccharides: glucose and galactose. The somatic lipopolysaccharide (LPS), on the other hand, contained less carbohydrate (18%) and more lipid (52%); analysis showed that the carbohydrate complex was composed of glucosamine, galactosamine, glucose and an unknown component similar to the methyl pentose, fucose.

The study of gluconeogenesis in species of the veillonella has been hindered by the lack of a defined medium for the growth of these bacteria. This has made it difficult to ascertain which compound in the

medium is acting as sources of carbon. The fastidious nature of these obligate anaerobes has undoubtedly hindered the development of such a media. This problem can be overcome by the use of radioactive tracers. In the present chapter, gluconeogenesis by cells of V. parvula M_4 will be demonstrated by determining the distribution of radioactive carbon from uniformally labelled sodium lactate-^{14}C into complex lipopolysaccharide and other material.

II. METHODS

A. Isolation and growth of cells.

A strain of V. parvula, designated M_4, was isolated anaerobically from human saliva (Ng, 1968; Ng and Hamilton, 1971) with the aid of antibiotics (Rogosa, 1965; Rogosa et al, 1958). Rogosa's 1% lactate medium (Rogosa, 1964) was used throughout and contained the following: tryptone, 10g; yeast extract, 5 g; Tween 80, 1 ml: KH_2PO_4, 6.0 g; sodium lactate (42.5%), 24 ml; sodium thioglycollate 0.75 g per litre of deionized water. The viability and purity of all stock cultures was checked at monthly intervals by the staining procedures and routine taxonomic tests described by Rogosa (1964) and Rogosa and Bishop (1964a,b). The Veillonella strain isolated was classified according to the scheme proposed by Rogosa (1965).

V. parvula M_4 has the following characteristics: (Ng, 1968, Ng and Hamilton, 1971); obligate anaerobe; small coccoid (0.4 μ dia.); unable to

ferment carbohydrates; growth in tryptone-yeast extract (TYE) broth with lactate pyruvate, oxaloacetate, malate, fumarate and succinate (Table 3.1); produced H_2S and nitrite from nitrate; was indole-, gelatin-, starch- and catalase- negative and exhibited β-hemolysis on blood-lactate agar plates. Putrescine and cadaverine were not required for growth in semi-defined basal medium containing 1% lactate. The basal medium employed was that proposed by Rogosa and Bishop (1964a) without hypoxanthine, uracil and putrescine.

B. Experimental procedure.

In order to demonstrate that the carbon atom comprising sodium lactate actually participated in the formation of the cellular and extracellular components of V. parvula M_4, cells were grown in the presence of uniformally labelled sodium lactate-^{14}C and the distribution of the ^{14}C in the system determined. Since the total recovery of the labelled carbon was desired, the experiment was designed to isolate all metabolic end-products, including the catabolic end-products: propionate, acetate, carbon dioxide and hydrogen (Ng and Hamilton, 1971). In order to collect the CO_2 evolved during anaerobic growth, cells were grown in a flask fitted with a 3-hole rubber stopper. The medium was sparged with high purity N_2 gas through one hole, while a second hole contained a gas outlet tube. The latter tube was connected to two gas dispersion tubes connected in sequence each containing 25 mls of 6 N NaOH to absorb the carbon dioxide. The third hole was a sample port through which samples were removed by sterile syringe (the samples were used to measure growth and for lactate analysis). Growth was followed turbidometrically in a

TABLE 3.1

Growth characteristics of Veillonella parvula M_4 in tryptone-yeast extract broth with various carbon sources

Substrate (0.2%)	Average generation time (min)	k[a]	Yield[b]
Lactate	88	0.49	0.053
Pyruvate	88	0.49	0.052
Malate	167	0.26	0.022
Oxaloacetate	174	0.25	0.013
Fumarate	174	0.25	0.014
Succinate	228	0.19	0.007

[a] Growth rate constant.

[b] Cell dry weight (g)/g of substrate.

Klett-Summerson colorimeter containing a red filter (640 to 700 nm).

Cells of V. parvula M_4 were grown in 2 litres of Rogosa's lactate (TYE-lactate) broth which had been equilibrated at 37 C and sparged with N_2 to remove unlabelled carbon dioxide before inoculation. Prior to this, filter-sterilized radioactive lactate-U-^{14}C was added asceptically to a final concentration of 1%. Following inoculation with an overnight culture (0.5%), the media was again sparged for 15 min and then the gas supply shut off until the late-exponential phase of growth. At this point, the culture was sparged for the third time with N_2 for 15 min and then the growth flask disconnected from the gassing tubes and the cell suspension rapidly chilled. The cells were then sedimented by centrifugation at 40,000 g for 30 min at 4 C. The cell fraction obtained following this centrifugation step was designated P_1, while the supernatant was identified as S_1 (Fig. 3.2).

C. Analyses.

a) Lactic acid. Samples (0.5 ml) were treated with 0.1 ml cold 25% $ZnSO_4$ to stop the reaction and then neutralized with NaOH. The cell-free supernatant, obtained after centrifugation at 30,000 g for 15 min, was assayed enzymatically for lactic acid by the method of Cohen and Noell (1960).

b) Carbon dioxide. The sodim hydroxide solutions in the gas dispersion tubes were combined and 50-200 µl samples counted in vials containing 6 ml of methanol and 10 ml of a scintillation solution composed of 1,4-di (2,5-phenyloxazolyl)-benzene (POPOP), 50 mg, and 2.6 di-phenyloxazole (PPO), 4 g in one litre of toluene. The radioactivity was measured in a

three-channel Nuclear Chicago Liquid Scintillation counter and the counts corrected to 100% efficiency either by the channels-ratio method or by internal standardization.

c) Acidic end-products and LPS_2 fraction. To a sample of the cell-free supernatant (S_1), obtained following growth, was added two volumes of 95% ethanol to precipitate polysaccharide material. The mixture was kept at 4 C for 4 hours to allow complete precipitation and the precipitate (P_2) collected by centrifugation at 30,000 g for 15 min (Fig. 3.2). This viscous material was washed twice with ethanol and the washings pooled with the original supernatant (S_2).

The S_2 supernatant was then treated with an equal volume of 15% butanol-chloroform to extract the metabolic acids. The phases were separated by centrifugation (10,000 g - 15 min) and the water phase (WP) recovered and repeatedly washed 3 times with the butanol-chloroform (BC) solution. The pooled BC phase was reduced in volume by flash evaporation and counted by the liquid scintillation procedure. The radioactivity in this fraction represented the lactate carbon present in the end-products: acetate and propionate (Ng and Hamilton, 1971).

The water phase (WP) obtained during the butanol-chloroform extraction was evaporated to dryness and then dissolved in 15 ml of phosphate buffer (pH 6.5, 50 mM). The resultant solution, however, contained a precipitate which was soluble in ether-ethanol (1:3) suggesting it was composed of lipid material. The radioactivity of the precipitate and the buffer-soluble material (LPS_2 fraction) was determined by liquid scintillation counting. Since the ^{14}C content of the precipitate (ether-ethanol-soluble) fraction was negligible, it was discarded. The

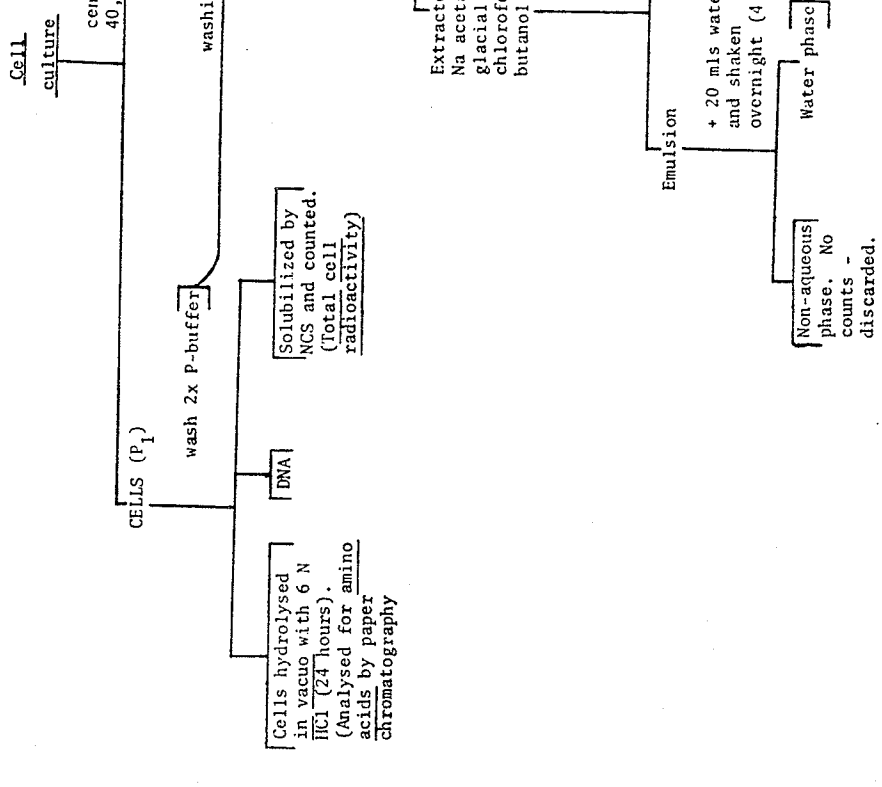


Fig. 3.2. Protocol for the isolation of various cellular and metabolic products following the growth of V. parvula M_4 in Rogosa's medium containing lactate-U-^{14}C.

LPS_2 material, on the other hand, contained considerable radioactivity and was subjected to further analysis.

d) Extracellular polysaccharides and lipids. The lipopolysaccharide complexes from the growth medium were isolated according to the method of Mergenhagen (1965). The viscous sediment (P_2) obtained from the ethanolic precipitation of the cell-free growth medium (S_1) was dissolved in a minimal amount of deionized water and then divided into two portions. One portion (20 mg anthrone-positive material) was solubilized with 1 ml NCS solubilizer (Nuclear Chicago Solubilizer) and counted. The remaining portion (70 mg anthrone-positive material) was extracted with sodium acetate, 2.0 g; glacial acetic acid, 0.2 ml; chloroform, 4.0 ml; n-butanol, 0.8 ml and 20 mls of water. The mixture was shaked in a sealed tube for 2 hours in 4 C and then centrifuged at 10,000 g for 30 min at the same temperature. The water phase was withdrawn and stored at -10 C under nitrogen. To the remaining emulsion was added 20 ml of distilled water and the solution again shaken in a sealed tube overnight at 4 C. Following centrifugation, the water phase from both extractions were pooled and lyophilized; this water-soluble fraction will be referred to as the LPS_1 fraction.

e) Cells. The cells obtained in the initial centrifugation (Fig. 3.2) were washed twice with phosphate buffer, centrifuged at 30,000 g for 15 min and then resuspended in 15 ml of buffer before being divided into three equal portions. The first portion (5 ml) was employed for DNA isolation. Using Marmur's method (1961), the DNA strands were isolated at the interphase between the water-ethyl alcohol (2:1) and chloroform-isoamyl alcohol (24:1) solution. The second 5 ml portion was hydrolysed

in vacuo with 6 N HCl for 24 hours at 121 C in an autoclave. The HCl was removed by evaporation and the total radioactive content of the hydrolyzed sample determined. Portions of this hydrolyzate were subjected to two dimensional paper chromatography on Whatman No. 1 with tert. butanol-methy ethyl ketone-conc. NH_4OH-water (200:200:60:100) as the primary solvent system and sec-butanol-formic acid-water (400:60:100) as the secondary solvent (Wyatt, Loughhead and Wyatt, 1956). The radioactive amino acids were detected by radiography employing X-ray film. The third 5 ml portion was hydrolysed overnight at 37 C with an equal volume of NCS solubilizer and the radioactivity measured.

Materials

The lactate-^{14}C was purchased from the Radiochemical Center, Amersham, England.

III. RESULTS

A. Total distribution of lactate carbon.

Table 3.2 summarizes the total distribution of ^{14}C into the various cellular and metabolic fractions resulting from the growth of V. parvula M_4 in Rogosa's medium containing lactate-U-^{14}C. The catabolic end-products, propionate, acetate and carbon dioxide contained 68.9% of the total lactate-^{14}C metabolized; the remaining 24.6% was distributed among the products of biosynthesis. Of this latter radioactivity, the lipopolysaccharide (LPS_2) fraction contained 22% of the total, 16% of which

TABLE 3.2

Distribution of ^{14}C in biosynthetic and metabolic end-products following growth of *V. parvula* M_4 in Rogosa's medium with lactate-U-^{14}C.

	Percentage[a]	
Catabolic products		
Propionate + acetate	60.5	
Carbon dioxide	8.4	
	68.9	
Biosynthetic products		
DNA	0.6	
Cellular amino acids	0.6	
Extracellular polysaccharide (LPS_1)	1.4	
Lipopolysaccharide (LPS_2)	22.0	
Carbohydrate		(16.0)
Lipid		(6.0)
	24.6	
Recovery	93.5	

[a] Per cent of total lactate-U-^{14}C added to the growth medium (1.5×10^8 cpm/litre).

was found in carbohydrate material and 6% in a lipid fraction. The remaining biosynthetic label (3.2%) was distributed in DNA (0.6%), cellular amino acids (0.6%) and the extracellular polysaccharide (LPS_1) fraction (1.4%). Since the LPS_1 and LPS_2 fractions appeared to contain complex molecules, an effort was made to determine the compounds comprising these molecules and the corresponding distribution of radioactivity.

B. Analysis of the LPS_1 fraction.

Mergenhagen (1965) observed that the LPS_1 fraction isolated from his strain of V. parvula by alcohol precipitation contained only glucose and galactose. The V. parvula M_4 LPS_1 material was subjected to thin layer chromatography on silical gel G using the butanol-pyridine-water (9:5:8) solvent system employed by Mergenhagen (1965) (Fig. 3.3). The spots on the TLC plates were located by spraying with silver nitrate reagent (Smith, 1960) and the radioactivity of these spots determined by scanning duplicate and undeveloped plates in a Packard radiochromatogram scanner. While the resultant plate showed four spots after spraying with the silver nitrate reagent, only two spots remained (solid lines) after drying at 37 C. Standards, run under the same conditions, indicated that these two radioactive spots corresponded to galactose and glucose, thus confirming the earlier work by Mergenhagen (1965).

C. Analysis of the LPS_2 fraction.

The absorption spectrum and anthrone analysis of the V. parvula M_4 LPS_2 fraction suggested that it was composed of protein and polysaccharide material. In order to test for the association of the carbohydrate and

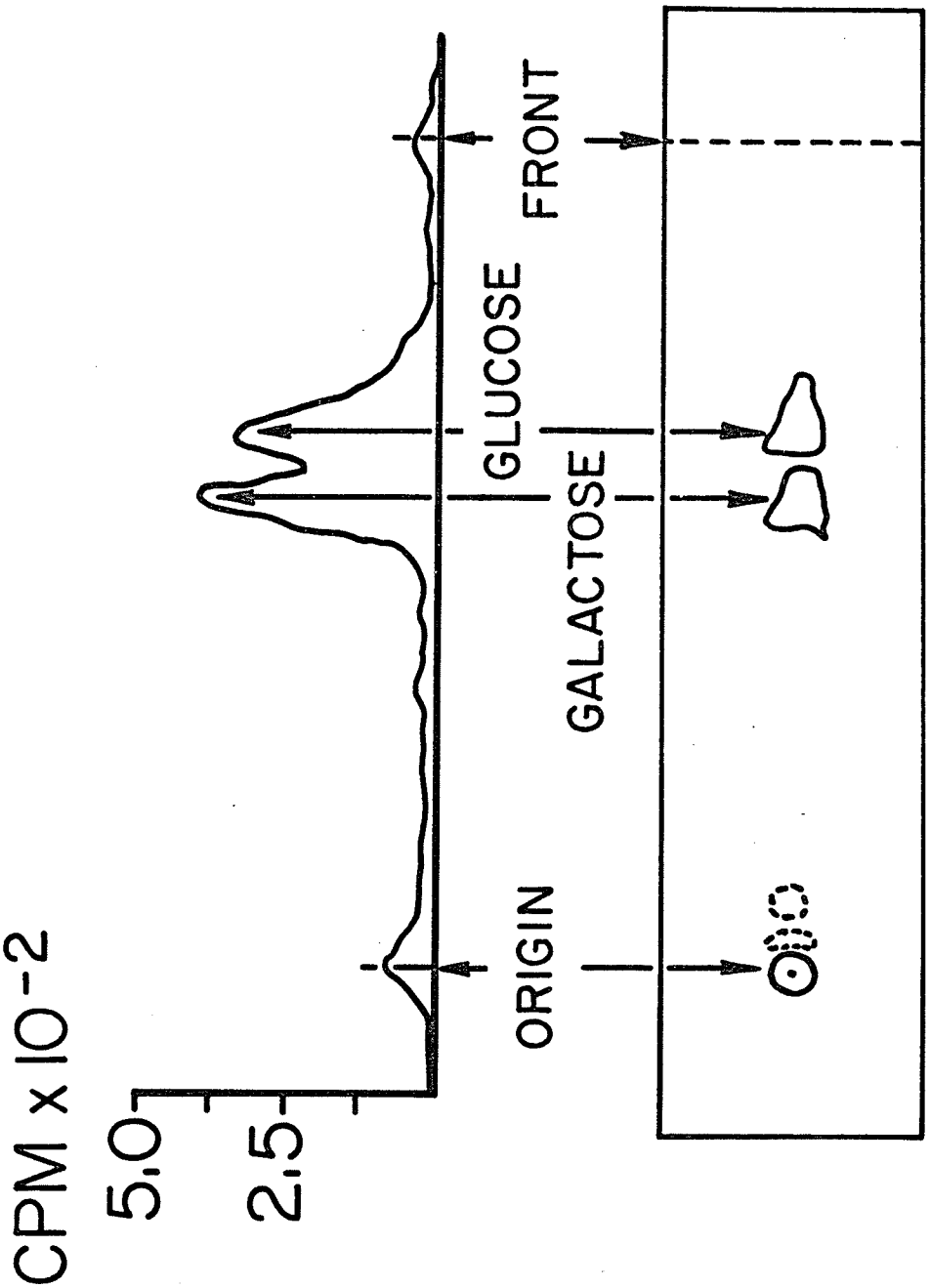


Fig. 3.3. Thin layer chromatogram and radioactive scan profile of the LPS_1 fraction isolated from V. parvula M_4.

protein moieties, the LPS_2 fraction was subjected to G-50 Sephadex column (2.5 x 45 cm) chromatography eluting with phosphate buffer (pH 6.5, 50 mM). Fig. 3.4 shows that two separate peaks of 280 nm absorbing material were obtained following this chromatography with only one of these peaks (peak I - fractions 14-24) contained radioactivity. Peak I was anthrone positive and eluted from the column after the void volume suggesting that the molecular weight of the material in this fraction was less than 30,000. The radioactive fractions in peak I from G-50 Sephadex were pooled and subjected to TLC analysis after the conversion of the anthrone-positive fraction to free sugars according to the method of Gallai-Hatchard and Gray (1966). Seven spots (other than the origin) appeared after spraying with silver nitrate and only five remained after drying at 37 C (Fig. 3.5). While two spots were not identified, the other five were identified as glucosamine, galactosamine, galactose, glucose and a pentose. Analysis of the pentose spot by the method of Whistler and Wofform (1952) suggested that it was ribose. The R_f value of this spot also corresponded to the R_f fo a ribose standard.

D. Label in cellular DNA and amino acids.

DNA was isolated by the method of Marmur (1961) and was shown to contain 0.6% of the total radioactivity. Attempts were also made to identify various cellular amino acids which had been derived from the substrate lactate-U-^{14}C. A portion of the cell fraction (P_1 - Fig. 3.2) was hydrolyzed with 6 N HCl at 121 C for 24 hours. Following removal of the HCl by evaporation, the hydrolysate was subjected to paper chromatography as described in Methods. The developed chromatogram was

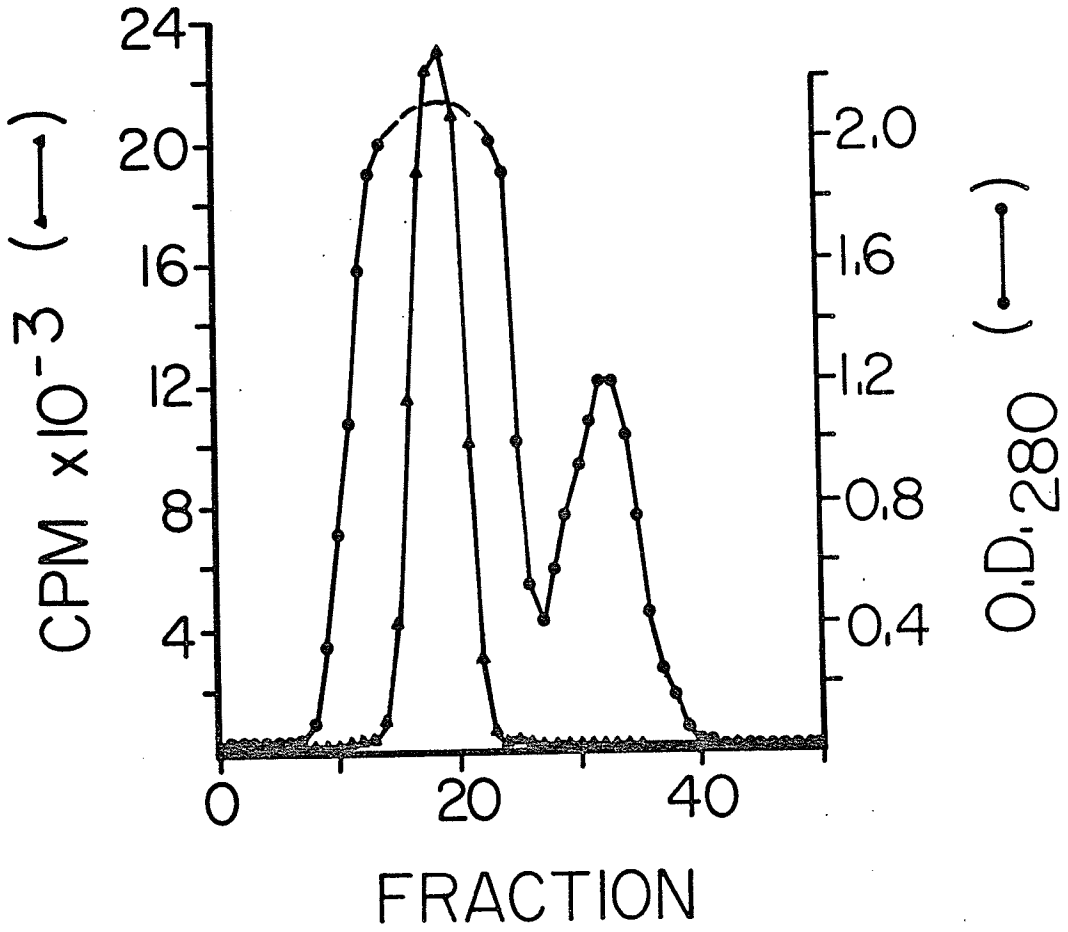


Fig. 3.4. Elution profile of the LPS_2 fraction from a column of Sephadex G-50. The 280 mμ scan above 2.0 was drawn arbitrarily.

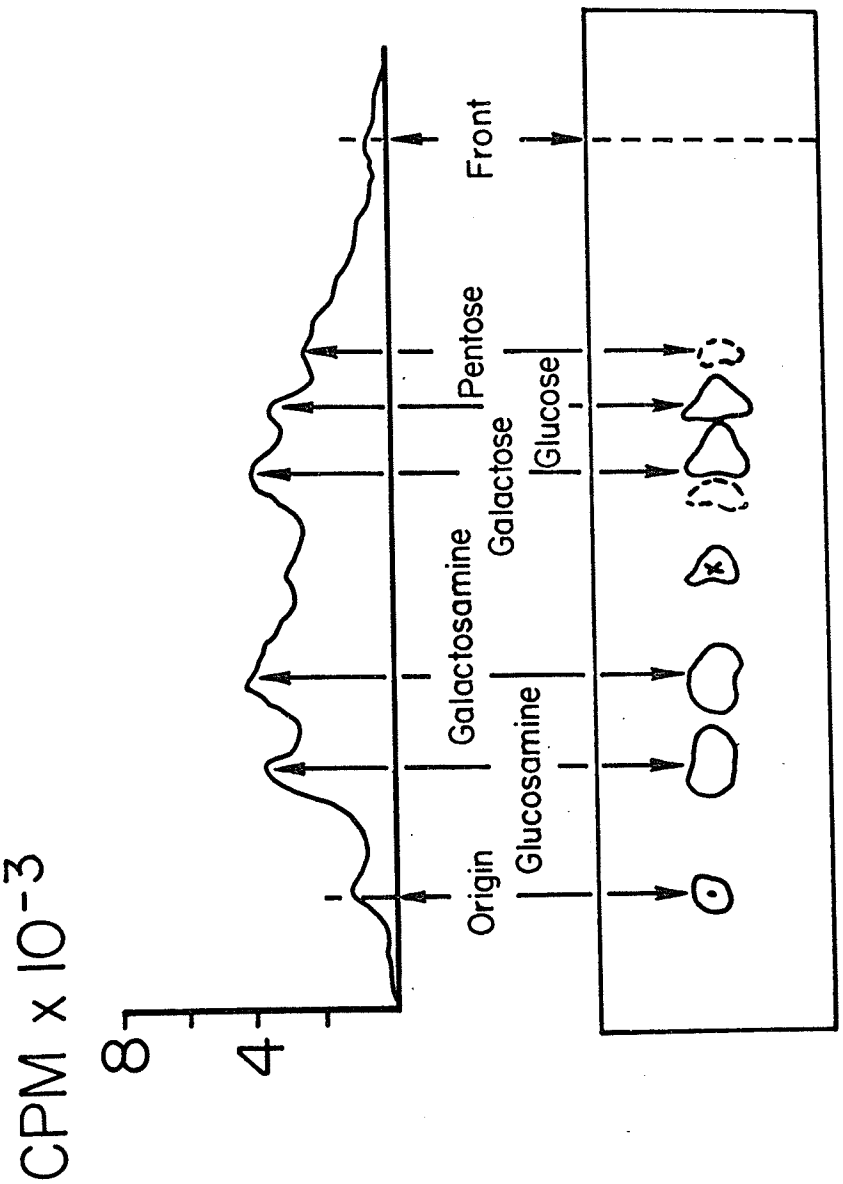


Fig. 3.5. Thin layer chromatogram and radioactive profile of the LPS_2 fraction isolated from V. parvula M_4.

then exposed to X-ray film for 15 days to locate the labelled amino acids. The chromatogram showing the distribution of the ^{14}C (shaded areas) in the various ninhydrin-positive spots is shown in Fig. 3.6. This chromatogram shows that the growth of V. parvula M_4 in broth with lactate-U-^{14}C resulted in the incorporation of ^{14}C into arginine, aspartate, glutamate, alanine, serine and an unidentified spot.

IV. DISCUSSION

Veillonella species ferment lactate to propionate, acetate, carbon dioxide and hydrogen (Foubert and Douglas, 1948; Johns, 1951a; Rogosa, 1964; Ng and Hamilton, 1971). The pathway for product formation by V. parvula M_4 is outlined in Fig. 3.7 (Ng, 1968) and is similar to that proposed by Johns (1951) for V. alcalescens. Of the intermediates, oxaloacetate, pyruvate, malate, fumarate and succinate can be utilized by species of Veillonella for growth (Johns, 1951b; Rogosa, 1964) while citrate, isocitrate and malonate are not attacked (Rogosa, 1964). Similar results have been found with V. parvula M_4 (Table 3.1). It can be seen from this table that the growth rate constant and yield decreased the further the intermediate was removed from pyruvate or lactate in the scheme indicating that these latter compounds are the most useful substrates for gluconeogenesis. However, the utilization of lactate for biosynthetic purposes occurs only during growth since previous studies with resting cells of V. parvula (Ng and Hamilton, 1971) demonstrated

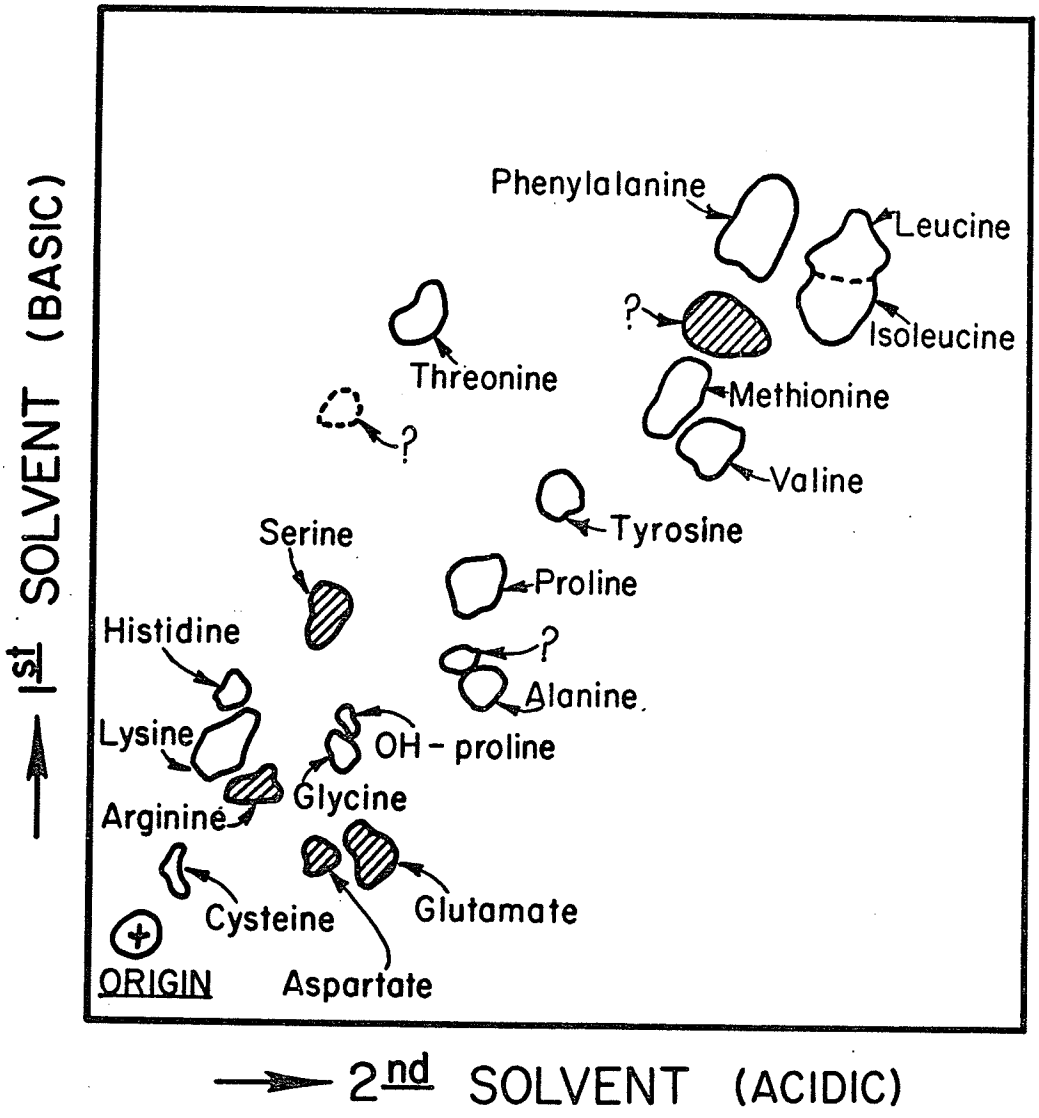


Fig. 3.6. Two-dimensional paper chromatogram of the amino acid hydrolysate from cells of V. parvula M_4 grown in Rogosa's medium with lactate-U-^{14}C.

Shaded areas contain ^{14}C.

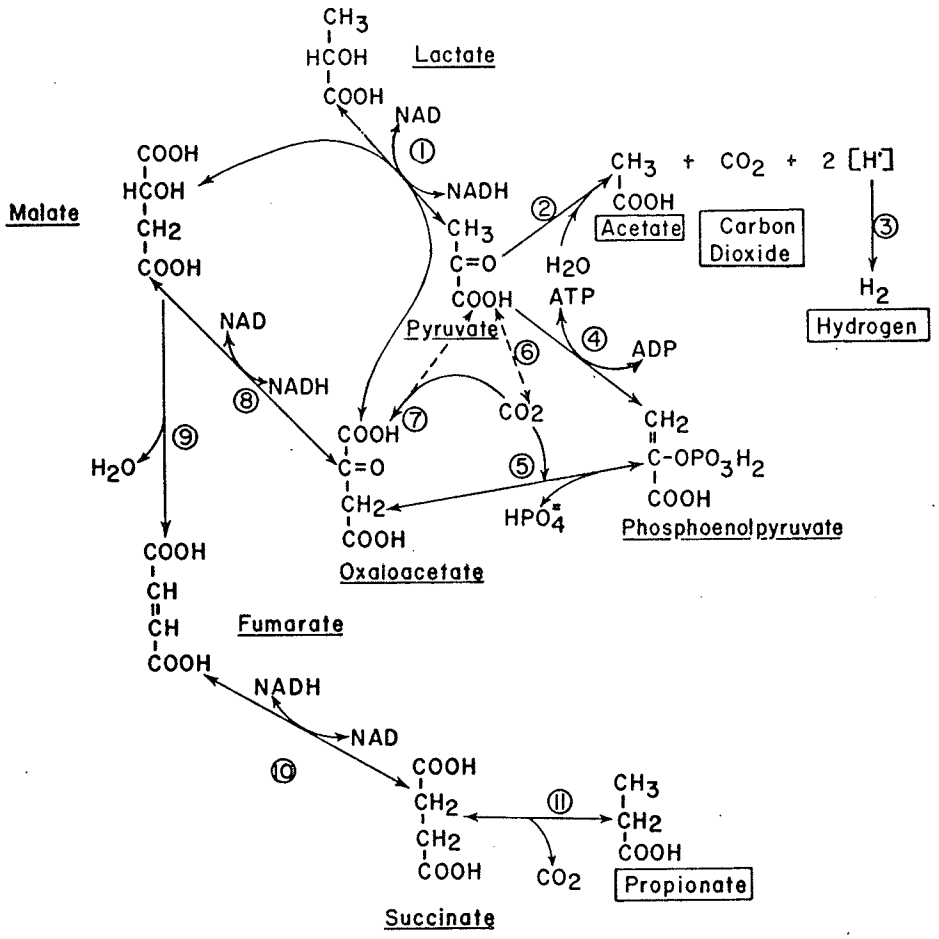


Fig. 3.7. Pathway of lactate metabolism in V. parvula M_4.

(1) malic-lactic transhydrogenase; (2) enzyme complex for the pyruvate phosphoroclastic reaction; (3) hydrogen: ferredoxin oxidoreductase; (4) ATP: pyruvate phosphotransferase; (5) pyrophosphate: oxaloacetate carboxyl-lyase; (6) enzyme(s) involved in the carbon dioxide exchange reaction; (7) oxaloacetate carboxyl-lyase; (8) L-malate: NAD oxidoreductase; (9) L-malate hydro-lyase; (10) succinate: (acceptor) oxidoreductase; (11) enzyme complex for succinate decarboxylation.

that all of the lactate-U-^{14}C was recovered in the catabolic end-products: propionate, acetate and carbon dioxide. On the other hand, with growing cells only 69% of the lactate-^{14}C label appeared in the catabolic end-products, while 25% of the recovered ^{14}C was distributed amongst the various biosynthetic products.

While this distribution (Table 3.2) is presumptive evidence for gluconeogenesis in V. parvula, the analysis of the LPS_1 (Fig. 3.3), LPS_2 (Fig. 3.5) and cellular fractions (Fig. 3.6) confirmed this conclusion. The presence of lactate-^{14}C carbon in the amino acids, aspartate, glutamate, arginine, serine and alanine, albeit a small fraction of the total, was significant. Assuming that the biosynthetic pathways for these amino acids in V. parvula M_4 are the same as those of other bacteria (Umbarger and Davis, 1962; Thimann, 1964; Greenberg, 1969), alanine would be synthesized from pyruvate, aspartate from oxaloacetate and glutamate from α-keto-glutarate (Whiteley and Ordal, 1957). In addition, the radioactive arginine could be derived from the labelled glutamate, while serine could be produced from 3-P-glycerate in the gluconeogenic pathway. How α-ketoglutarate is synthesized in this organism is unknown.

Probably the most convincing evidence for gluconeogenesis in V. parvula M_4 was the detection of the radioactivity from lactate-U-^{14}C in glucose, galactose, glucosamine, galactosamine and ribose (LPS_1 and LPS_2 fractions). The presence of label in the ribose suggests that the radioactivity observed in DNA is contained in the ribose moiety. Similar results were observed by Michaud (1968), who observed that growing cells of V. alcalescens incorporated ^{14}C from lactate-1-^{14}C into the pyrimidine nucleotides, uridylic acid, cytidylic acid, deoxycytidylic acid, as well

as into ribose. Whether the lactate carbon also participated in the synthesis of cellular polysaccharides was not determined.

In this same study, Michaud also reported the incorporation of ribose-1-^{14}C into the cellular material of V. alcalescens. This result is surprising since a long standing taxonomic characteristic of Veillonella species is their inability to metabolize any type of carbohydrate (Rogosa, 1965). This strain of V. alcalescens was also reported to be capable of incorporating glucose carbon into nucleic acids (Michaud and Delwiche, 1967 and Michaud, 1968). However, the validity of these reports must be questioned since the amount of glucose-1-^{14}C incorporated was only 1.3% of the total and this carbon source was autoclaved with the growth medium. This sterilization process could have resulted in the thermal degradation of the glucose-^{14}C into another smaller radioactive compounds, which were then incorporated and contributing the small amount of label observed in the nucleic acids.

Recently, however, Kafkewitz and Delwiche (1972) confirmed the assimilation of ribose into growing cells of V. parvula (ATCC 10790) and strains of V. alcalescens isolated from sheep rumen and human saliva. These workers indicated that the ribose was not degraded catabolically but was rather incorporated into cellular nucleotides. Kafkewitz and Delwiche further showed that the enzymes necessary for phosphorylating ribose-^{14}C and dephosphorylating ^{14}C-ribose-5-P were present in V. alcalescens and suggested that the nonoxidative pentose phosphate pathway, reported by Michaud, Carron and Delwiche (1970), was involved in the assimilation of this pentose. This pathway is thought to function when non-carbohydrate substrates for sugar synthesis are provided although

the exact nature of these substrates is not known. The assimilation of glucose by a similar system has not been reported.

CHAPTER 4

INITIAL STAGES OF GLUCONEOGENESIS

I. INTRODUCTION

Gluconeogenesis is a complex sequence of reactions involving many intermediate stages. It is now generally accepted that this process involves the reversal of most of the reactions in glycolysis with the addition of three major steps involved in bypassing energy barriers. As mentioned in Chapter 2, these barriers occur between pyruvate and P-enolpyruvate (PEP), between fructose-1,6-P_2 (FDP) and fructose-6-P (F6P), and between glucose-6-P (G6P) and glucose. Of these, the conversion of pyruvate to P-enolpyruvate is particularly important for microorganisms since major gluconeogenic precursors, such as lactate and pyruvate, enter at this point. Bacteria possess two basic pathways for the conversion of pyruvate to PEP: (a) the "indirect" pathway involving the formation of the intermediate, oxaloacetate, which is also present in animal systems, and (b) the "direct" conversion, which is present only in microorganisms.

A. Indirect synthesis of PEP.

The indirect pathway of PEP formation involves two main reactions: (a) the synthesis of oxaloacetate from pyruvate and CO_2 and (b) the decarboxylation and phosphorylation of oxaloacetate to form PEP. The most direct route of PEP formation via oxaloacetate involves the enzymes,

pyruvate carboxylase (reaction 4.8) and P-enolpyruvate carboxykinase (reaction 4.9) and involves the net utilization of two molecules of ATP (4.10).

$$\text{Pyruvate} + HCO_3 + \text{ATP} \longrightarrow \text{Oxaloacetate} + \text{ADP} \qquad (4.8)$$

$$\text{Oxaloacetate} + \text{ATP} \longrightarrow \text{P-enolpyruvate} + \text{ADP} + HCO_3 \qquad (4.9)$$

$$\text{Net: Pyruvate} + 2\ \text{ATP} \longrightarrow \text{P-enolpyruvate} + 2\ \text{ADP} \qquad (4.10)$$

The pyruvate carboxylase reaction, which catalyzes the formation of oxaloacetate by CO_2-fixation with pyruvate in the presence of ATP, was first observed by Wood and Werkman (1935,1936,1938) during studies on glycerol fermentation by the propionic acid bacteria. The original proof of this reaction involved the quantitative determination of all the products of fermentation and the calculation of the carbon and oxidation-reduction balances as initially proposed by Johnson, Peterson and Fred (1931). These latter calculations indicated that CO_2 was actually assimilated by these bacteria. Wood et al (1941) later showed, with the aid of isotopic CO_2 that the mechanism of carbon dioxide fixation was as follows (reaction 4.11):

$$C^*O_2 + CH_3\text{-}CO\text{-}COO \longrightarrow HOOC^*\text{-}CH_2\text{-}CO\text{-}COOH \qquad (4.11)$$

pyruvate — oxaloacetate

With the propionibacteria, the oxaloacetate thus formed was further degraded to propionic acid via succinic acid (Krebs and Eggleston, 1941). The value of this carbon dioxide fixation reaction in gluconeogenesis, however, was not recognized until Solomon et al (1940) first demonstrated

the incorporation of $^{13}CO_2$ into liver glycogen. The isotope distribution in the glucosyl-residues of glycogen observed under such conditions further indicated the presence of a symmetrical four-carbon intermediate in the pathway of incorporation (Lorber et al, 1950; Topper and Hastings, 1949).

Although oxaloacetate formation from pyruvate and CO_2 had been long established, pyruvate carboxylase was not isolated until 1960 when it was purified by Utter and Keech from liver mitochondria. Since then, a number of investigations have been carried out with microorganisms, the enzyme being isolated from Saccharomyces cerevisiae (Ruiz-Amil et al, 1965), Pseudomonas citronellolis (Seubert and Remberger, 1961), Aspergillus niger (Woronick and Johnson, 1960), Rizopus nigrican (Overman and Romano, 1969) and Arthrobacter globiformis (Bridgeland and Jones, 1967). In all cases, pyruvate carboxylase was shown to be a biotin-enzyme having an absolute requirement for ATP.

The second reaction in the indirect synthesis of PEP is the conversion of oxaloacetate to PEP (equation 4.9). Although this reaction has been written in the direction of oxaloacetate decarboxylation, it has been most extensively studied as a CO_2-fixing process. For example, the reaction was first described by Utter and Kurahashi (1953a,b) while studying the reversible formation of oxaloacetate and ATP from PEP, ADP and HCO_3^- in the presence of an enzyme from pigeon liver. In the same year, Bandurski, Greiner and Bonner (1953) and Bandurski and Greiner (1953) described another process, which would form oxaloacetate from PEP and HCO_3^- in the absence of ADP (equation 4.12). Confusion then

$$PEP + HCO_3^- \longrightarrow \text{oxaloacetate} + P_i \qquad (4.12)$$

arose as to whether these reactions (equation 4.9 and 4.12) were actually catalyzed by the same enzyme with the ADP acting solely to stimulate the reaction. Subsequently, Utter and Kurahashi (1954b) following the study of kidney preparations gave the name, oxaloacetate carboxylase, to the enzyme catalyzing the formation of PEP from oxaloacetate in the presence of ATP. When the reaction was first proposed by Utter and Kurahashi (1954a), it was believed that ATP was the only active nucleotide. However, further investigation by Utter and his co-workers showed that ITP (Utter, Kurahashi and Rose, 1954) and GTP (Kurahashi, Pennington and Utter, 1957) were the active nucleotides for the avian liver enzyme and not ATP. From these latter studies it was also apparent that reactions (4.9) and (4.12) were catalyzed by two individual enzymes; these enzymes were named PEP carboxykinase and PEP carboxylase, respectively, the latter replacing the name, oxaloacetate carboxylase. This proposal was supported by the results of Graves et al (1956).

PEP carboxykinase has been generally accepted as the enzyme unique to gluconeogenesis as it has been shown to play no role in the introduction of three-carbon compounds into the tricarboxylic acid cycle (Theodore and Englesberg, 1964). The enzyme has been isolated from Thiobacillus thiooxidans (Suzuki and Werkman, 1958), Pseudomonas oxaluticus (Quayle and Keech, 1959), Escherichia coli (Hsie and Rickenberg, 1966), Salmonella typhimurium (Theodore and Englesberg, 1964), Neurospora crassa (Flavell and Fincham, 1968), yeast (Cannata and Stoppani, 1959), and Tetrahymena pyriformis (Shrago, Breech and Templeton, 1967).

PEP carboxylase, on the other hand, is not considered to be a gluconeogenic enzyme as it is apparently concerned solely with the formation of oxaloacetate from PEP and CO_2 (Kornberg, 1965; Scrutton and Utter, 1968). In addition to its presence in mammalian tissue, this enzyme has been isolated from Sal. typhimurium (Maeba and Sanwal, 1965), Thio. thiooxidans (Suzuki and Werkman, 1958) and E. coli (Amarsingham, 1959).

In addition to the above two enzymes, Siu, Wood and Stjernholm (1961) and Siu and Wood (1962) discovered, in Propionibacterium shermanii, a third enzyme catalyzing the oxaloacetate-PEP interconversion; this reaction is catalyzed by the enzyme, PEP carboxytransphosphorylase (equation 4.13). Thus far, this enzyme appears to be unique to the

$$PEP + HCO_3^- + P_i \longrightarrow \text{Oxaloacetate} + PP_i \qquad (4.13)$$

propionibacteria since it has not been reported in other microorganisms.

It should be kept in mind throughout any discussion of the indirect pathway of PEP formation that this compound can also be synthesized from pyruvate via the malic enzyme. This enzyme, first isolated by Ochoa, Mehler and Kornberg (1948) from pigeon liver, was shown to require NADPH for the fixation of CO_2 (equation 4.14). Since then the NADP-specific

$$\text{Pyruvate} + HCO_3^- + \text{NADPH} \longrightarrow \text{Malate} + \text{NADP} \qquad (4.14)$$

enzyme has been isolated from pigeon liver (Veiga, Salles and Ochoa, 1950; Rutter and Lardy, 1958; Stickland, 1958a,b), wheat germ (Harary, Korey and Ochoa, 1953) and also from the microorganisms, Lactobacillus arabinosus (Korkes, Del Campillo and Ochoa, 1950; Kaufmann, Korkes and

Del Campillo, 1951), Mycobacterium 607 (Parvin, Pande and Venkitasubramanian, 1964) and Escherichia coli (Ashworth, Kornberg and Wood,1965; Sanwal and Smando, 1969).

Following the discovery of the NADP-specific malic enzyme, Duerre and Lichstein (1961) observed an NAD-specific malic enzyme in L. delbrueckii, L. casei, Leuconostoc mesentoroides and Streptococcus faecalis, when grown on malate. This enzyme was also isolated from Shizosaccharomyces pombe (Temperali et al. 1965) and E. coli (Kutsuki et al, 1960). Thus, PEP can be formed from either the NADP-specific or NAD-specific malate enzyme coupled to malate dehydrogenase (equation 4.15) and PEP carboxykinase.

$$\text{Pyruvate} + HCO_3^- + \text{NADH (NADPH)} \longrightarrow \text{Malate} + \text{NAD (NADP)} \qquad (4.14)$$

$$\text{Malate} + \text{NAD} \longrightarrow \text{Oxaloacetate} + \text{NADH} \qquad (4.15)$$

$$\text{Oxaloacetate} + \text{ATP} \longrightarrow \text{PEP} + HCO_3^- + \text{ADP} \qquad (4.9)$$

$$\text{net: Pyruvate} + \text{ATP} \longrightarrow \text{PEP} + \text{ADP} \qquad (4.10)$$

B. Direct synthesis of PEP.

Two enzymes have been discovered in bacteria which are capable of catalyzing the formation of PEP directly from pyruvate: PEP synthetase (equation 4.16) and pyruvate, phosphate dikinase (equation 4.17):

$$\text{Pyruvate} + \text{ATP} \longrightarrow \text{PEP} + \text{AMP} + P_i \qquad (4.16)$$

$$\text{Pyruvate} + \text{ATP} + P_i \longrightarrow \text{PEP} + \text{AMP} + PP_i \qquad (4.17)$$

PEP synthetase was first discovered by Cooper and Kornberg (1965;

1967) in E. coli; the enzyme catalyzes the direct formation of PEP from pyruvate and ATP with the concomitant formation of AMP and inorganic phosphate. In 1967, Cooper and Kornberg purified this enzyme (80-fold) and observed that PEP synthesis proceeded most rapidly at pH 8 - 8.5. At pH values between 6.2 and 7.5, the enzyme catalyzed the formation of ATP and pyruvate from PEP, AMP and P_i; if arsenate was used instead of phosphate, pyruvate and ADP were produced instead. Further studies demonstrated the physiological role of the enzyme with mutants of E. coli devoid of the enzyme. In contrast to the wild-type strain, these mutants would neither grow in pyruvate, lactate or alanine, nor form glycogen from lactate, indicating that PEP synthetase plays an important role during growth on gluconeogenic substrates.

Pyruvate, phosphate dikinase has been observed in the propionic acid bacteria by Evans and Wood (1968a,b), in the leaves of tropical grasses by Hatch and Slack (1968) and in the parasitic amoeba, Entamoeba histolytica, by Reeves (1968). The presence of enzyme in the plant, Amaranthus palmeri (Slack, 1968), and in Bacteroides symbiosus (Reeves et al, 1968) has also been reported. Hatch and Slack (1968) proposed that the enzyme functions in tropical grasses in the direction of PEP synthesis in order to provide precursors for CO_2-fixation. However, the opposite conversion i.e., PEP to pyruvate, appears to occur in Ent. histolytica and Bact. symbiosus since these organisms lack pyruvate kinase (Reeves, 1968; Reeves, Menzie and Hsu, 1968).

C. PEP formation by Veillonella species.

Since gluconeogenesis occurs in the obligate anaerobe, V. parvula

M_4, the question now remains as to the biochemical reactions involved. Furthermore, how are these reactions regulated in an organism such as V. parvula which depends on lactate as an energy source?

One can assume that gluconeogenesis proceeds in Veillonella species by the reversal of, at least part of, the glycolytic pathway, since an impaired glycolytic scheme is known to exist in V. alcalescens (Rogosa, Krichevsky and Bishop, 1965; Michaud and Delwiche, 1970). Probably the most crucial step in this process is the initial conversion of lactate to PEP since a delicate balance must be struck between PEP formation and lactate catabolism. Of the possible enzymes involved in the lactate-PEP conversions, only one has been demonstrated in the veillonella: that involved in the conversion of lactate to pyruvate. These bacteria do not possess a conventional lactate dehydrogenase but rather convert lactate to pyruvate by the enzyme, malate-lactate transhydrogenase (equation 4.18). This enzyme was first demonstrated

$$\text{Lactate}^{*} + \text{Oxaloacetate} \longrightarrow \text{Pyruvate}^{*} + \text{Malate} \qquad (4.18)$$

in V. alcalescens by Dolin, Phares and Long (1964,1965), and subsequently purified and characterized by Allen and Galivan (1965) and Allen (1966). The enzyme contains a bound pyridine nucleotide (Dolin, Phares and Long, 1964,1965) and has an equilibrium constant of 1.8 favoring pyruvate formation from lactate (Allen, 1966).

Once the pyruvate has been formed by the transhydrogenase, PEP may be formed either by the 'direct' or 'indirect' pathways mentioned previously. The formation of oxaloacetate from pyruvate and CO_2 by crude extracts of V. parvula M_4 has been demonstrated (Ng, 1968) suggesting that the

'indirect' pathway may function in this organism. No information is available regarding the direct synthesis of PEP from pyruvate in Veillonella species.

With this in mind, the main concern of the present Chapter was to study the possible enzymes present in V. parvula M_4 which may participate in the initial reactions of gluconeogenesis, i.e., the conversions from pyruvate to P-enolpyruvate. These reactions are outlined in Fig. 4.8.

II. METHODS

A. Preparation of cell-free extracts.

Cells of V. parvula M_4 were grown in Rogosa's lactate (1%) broth (Rogosa, 1964) and harvested in the late exponential phase by centrifugation at 35,000 g for 20 min at 4 C. The cells were washed three times with phosphate buffer (pH 6.5, 50 mM) containing 20 mM β-mercaptoethanol and stored at - 20 C until used. Cell-free extracts were obtained from these cells, following thawing, by sonic disruption in a Branson Sonifier (Heat Systems Ultrasonics Inc., Plainview, N.Y.) for 20 min at 4 C in a nitrogen atmosphere. The supernatant, obtained by centrifugation at 45,000 g for 20 min, was used as the enzyme source following overnight dialysis against the above phosphate buffer.

B. Spectrophotometric Enzyme Assays.

Crude extracts of V. parvula M_4 were assayed for pyruvate kinase, PEP-synthetase, pyruvate carboxylase, PEP carboxykinase, PEP carboxylase,

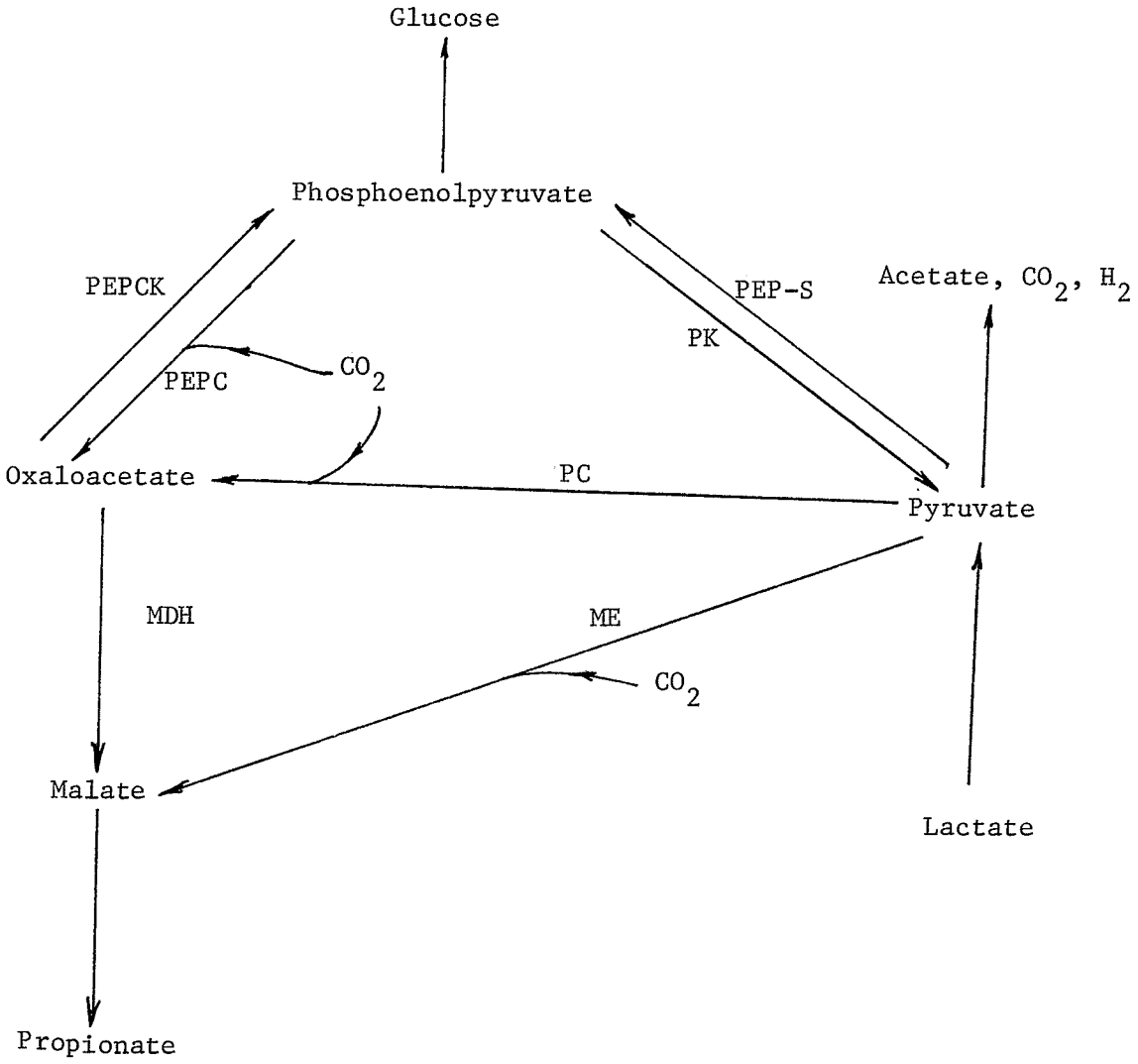
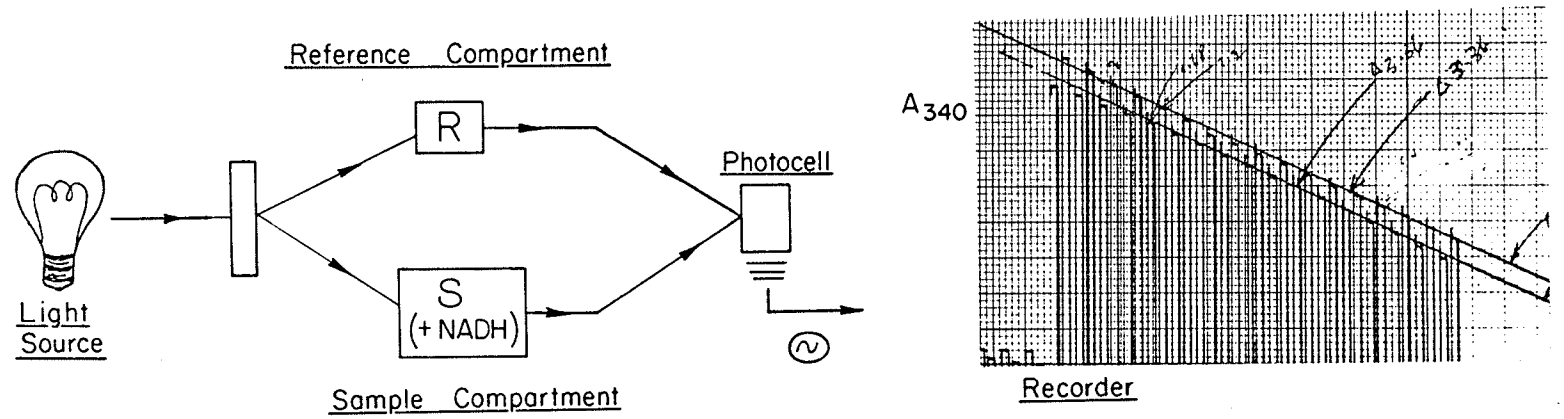


Fig. 4.8. Possible reactions for the conversion of pyruvate to P-enolpyruvate. (PEP-S) PEP synthetase.

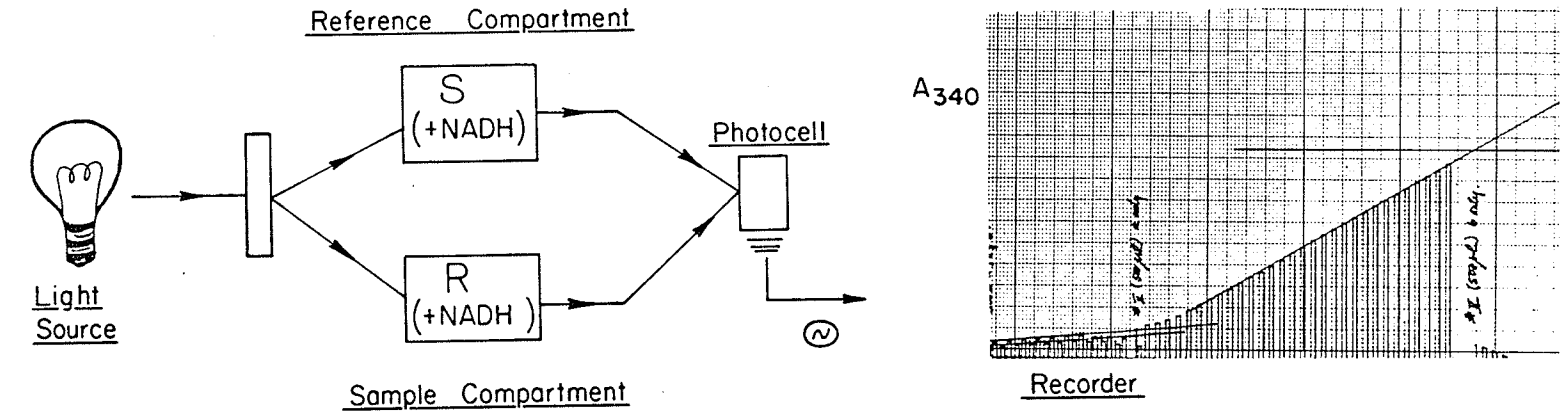
NAD-specific and NADP-specific malic enzymes by spectrophotometric methods.

a) General spectrophotometric procedures. In all cases, enzyme activity was assayed by coupling the reaction to the oxidation of NADH and measuring the absorbance change spectrophotometrically at 340 nm in a Unicam SP 800 double-beam recording spectrophotometer. The temperature of each reaction was controlled at 37 C by circulating a methanol-water mixture (50:50) through a jacketed cuvette holder with a Lauda constant emperature apparatus (Brinkman Instruments, Westbury, N.Y.). Each reaction was initiated, following a 5-10 min equilibration period, by the addition of either the enzyme or the substrate to the cuvette. The cuvette was then rapidly covered with parafilm and the contents mixed by inverting several times. The time required for complete mixing was approximately 5 seconds.

Freshly prepared cell-free extracts of V. parvula M_4 exhibited high endogenous NADH oxidizing activity (e.g. 0.7 µmoles NADH oxidized/mg/min). To circumvent this problem, a modified method was used with the double-beam spectrophotometer to measure the desired coupled reactions. Since a double-beam spectrophotometer measures the difference in light intensity emitted from the sample (S) and reference (R) cuvettes, conventional assays measuring NADH oxidation have the NADH present only in the sample cuvette, the activity being measured by a decrease in the 340 nm absorbance of the sample cuvette (Fig. 4.9) (A)). In our modified assay, the NADH and extract preparation were present in both the S and R cuvettes such that any endogenous NADH oxidizing activity in these cuvettes would be cancelled out. In addition to this, the position of the cuvettes was changed, i.e., the S cuvette to the reference compartment and the R



A.)- CONVENTIONAL METHOD



B.)-METHOD USED

Fig. 4.9. The spectrophotometric method employed in the continuous measurement of enzyme activity in the crude extracts of V. parvula M_4.

(control) cuvette to sample compartment. The reaction was started by the addition of the substrate or enzyme to the S cuvette in the reference compartment. In this manner, as the NADH in the S cuvette was being oxidized by the reaction being measured it became "lighter" than the control in the R cuvette resulting in an increase in the optical density of the system at a rate proportional to the net rate of NADH oxidation (Fig. 4.9 (B)). This method was used both with crude extracts and with purified preparations, even if the latter contained no endogenous NADH oxidizing activity, since the method had a number of advantages over the conventional method: (a) the reactions can be followed to completion, (b) a wider range of NADH concentrations can be employed (e.g. 0.2 mM for the conventional method vs. 2 mM for the modified method), (c) the reference or control cuvette (R) (Fig. 4.9 (A and B)) contained all of the reaction ingredients except either the substrate for the reaction or the appropriate dehydrogenase. In all assays, the concentration of NADH was determined from a standard curve (Fig. 4.10) prepared with precise amounts of the disodium salt prepared in phosphate buffer (50 mM, pH 6.5). The values obtained coincided with those calculated from the extinction coefficient (6.22×10^6 cm^2 /mole).

b) Specific assays.

i) Pyruvate kinase. Pyruvate kinase (PK) activity (equation 2.5) in cell-free extracts of V. parvula M_4 was measured by converting the pyruvate formed from PEP and ADP to lactate with lactic dehydrogenase (LDH) and NADH (equation 4.19).

$$\text{PEP} + \text{ADP} \xrightarrow{\text{PK}} \text{Pyruvate} + \text{ATP} \qquad (2.5)$$

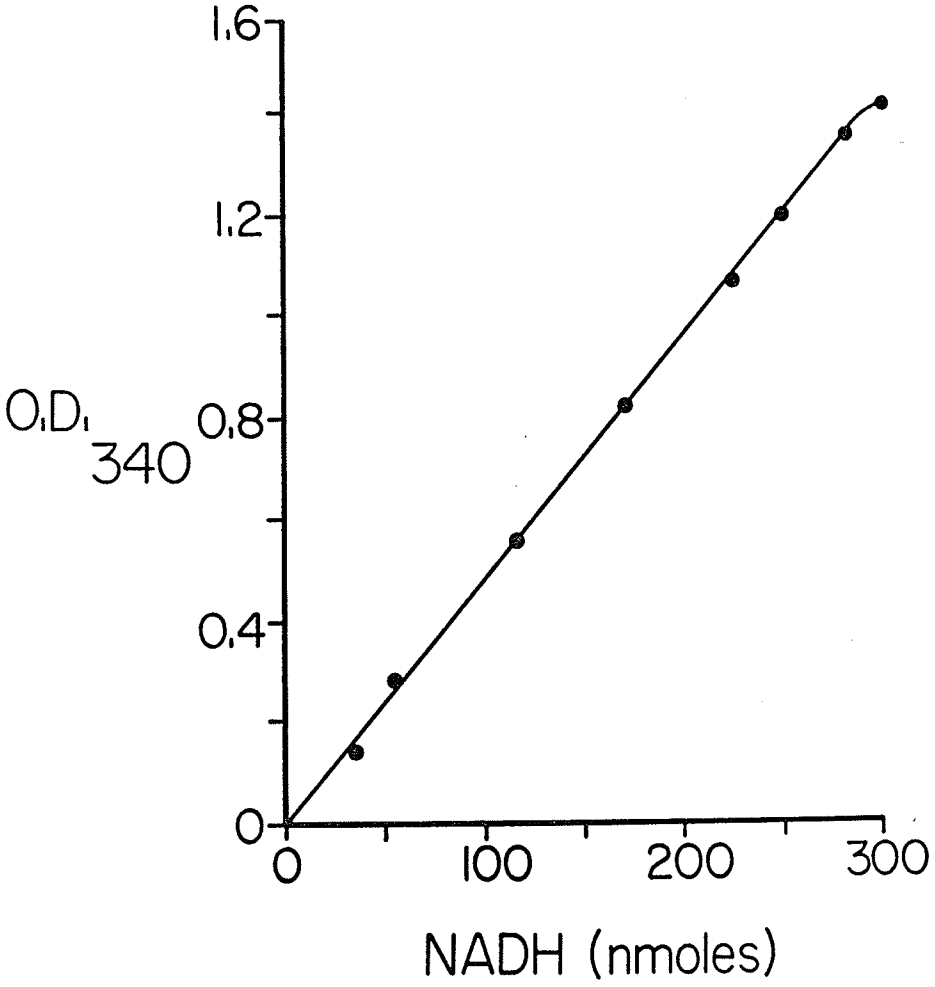


Fig. 4.10. Standard curve for the change in the absorbance at 340 nm versus the NADH concentration.

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD} \qquad (4.19)$$

The reaction mixture contained (in mM): PEP, 5; ADP, 5; $MgSO_4$, 10; NADH, 0.5; sodium arsenate, 5; phosphate buffer (pH 6.5), 50 and 2 mg extract in a volume of 1 ml. The reaction was initiated by adding 20 µl of lactic dehydrogenase. The arsenite was added to prevent the degradation of pyruvate to acetate and CO_2 by pyruvate dehydrogenase present in the crude extracts (Ng and Hamilton, 1971).

Attempts were made to assay pyruvate kinase activity by measuring the disappearance of PEP at 240 nm by the direct spectrophotometric method (Allen, 1966; Grisola, 1962). However, this method was not appropriate since pyruvate (Fig. 4.11), as well as extract protein interfered with the estimation. Furthermore, PEP carboxylase and PEP carboxykinase could not be measured by this method either because oxaloacetate also absorbed significantly at 240 nm. None of the above compounds interfered with the oxidation of NADH at 340 nm.

ii) PEP synthetase. P-enolpyruvate synthetase (PEP-S), which catalyzes the synthesis of PEP directly from pyruvate and ATP (equation 4.20),was assayed by the method of Czok and Eckert (1963). In this method, the PEP formed was converted to D-glyceraldehyde-3-P (GAP) by reactions (4.20) to (4.25) which were catalyzed by commercial enzymes. The amount of PEP

$$\text{Pyruvate} + \text{ATP} \xrightarrow{\text{PEP-S}} \text{PEP} + \text{AMP} + P_i \qquad (4.16)$$

$$\text{PEP} \xrightarrow{\text{enolase}} \text{2-P-glycerate (2-PGA)} \qquad (4.20)$$

$$\text{2-PGA} \xrightarrow{\text{PGM}} \text{3-P-glycerate (3-PGA)} \qquad (4.21)$$

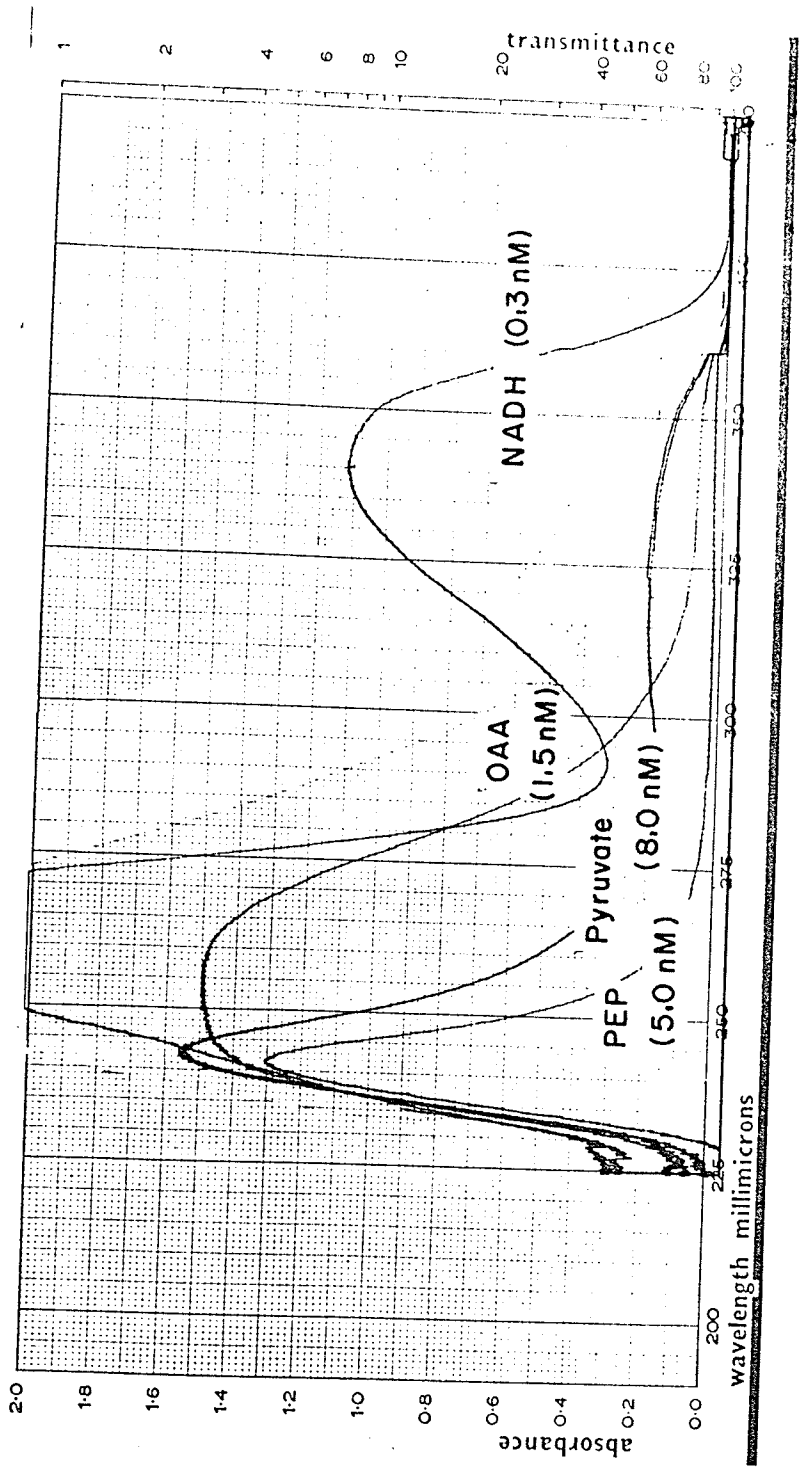


Fig. 4.11. Spectrophotometric scan of pyruvate, P-enolpyruvate, oxaloacetate and NADH.

$$3\ PGA + ATP \xrightarrow{PGK} 2,3\ Diphosphoglycerate\ (1,3\ DPGA) \qquad (4.22)$$

$$1,3\ DPGA + NADH \xrightarrow{GAPDH} Glyceraldehyde\text{-}3\text{-}P\ (GAP) + NAD + P_i \qquad (4.23)$$

formed in reaction (4.16) was thus proportional to the amount of NADH oxidized in reaction (4.24). The reaction mixture contained (in mM): pyruvate, 10; ATP, 10; sodium arsenite, 5; NADH, 1; $MgSO_4$, 10; and 2,3-diphosphoglycerate (2,3 DPGA), 10; phosphate buffer (pH 6.5), 50 and the following enzymes (in µg): enolase, 100; phosphoglyceromutase (PGM), 20; phosphoglycerokinase (PGK), 20; and glyceraldehyde-3-P dehydrogenase (GAPDH), 400, and crude extract in a volume of 1 ml. The 2,3 DPGA was required as a cofactor for the conversion of 2 PGA to 3 PGA (Krimsky, 1963). This system will be referred to as the "GAPDH assay system".

iii) Pyruvate carboxylase. Pyruvate carboxylase (PC) (equation 4.8) was assayed by converting the oxaloacetate formed to malate with commercial malic dehydrogenase (MDH) and NADH (equation 4.15).

$$Pyruvate + ATP + HCO_3^- \xrightarrow{PC} Oxaloacetate + ADP \qquad (4.8)$$

$$Oxaloacetate + NADH \xrightarrow{MDH} Malate + NAD \qquad (4.15)$$

The assay system contained (in mM): pyruvate, 10; Na bicarbonate, 10; NADH, 1; $MgSO_4$, 10; phosphate buffer (pH 6.5) 50; Na arsenite, 5; sodium malonate, 10; malate dehydrogenase (1.5 µg) and crude extract in a volume of 1 ml. The sodium malonate was added to prevent the degradation of malate to succinate (Ng and Hamilton, 1971).

iv) PEP carboxykinase (PEPCK) and PEP carboxylase (PEPC). Since cell-free extracts of V. parvula M_4 contain an active malate dehydrogenase (Ng and Hamilton, 1971) PEPCK activity could not be measured readily with oxaloacetate as the substrate. Therefore, PEPCK was assayed with PEP as the substrate (equation 4.9) and the oxaloacetate formed converted to malate in the presence of NADH and commercial MDH.

$$PEP + ADP + HCO_3^- \xrightarrow{PEPCK} Oxaloacetate + ATP \qquad (4.9)$$

$$Oxaloacetate + NADH \xrightarrow{MDH} Malate + NAD \qquad (4.15)$$

The reaction mixture contained (in mM): PEP, 10; ADP, 10; Na bicarbonate, 10; NADH, 1; $MgSO_4$, 10; phosphate buffer (pH 6.5), 50; malate dehydrogenase (1.5 µg) and crude extract in a volume of 1 ml. PEP carboxylase was measured in the same assay without the ADP.

v) Malic enzyme (NAD- and NADP-dependent). Malic enzyme in crude extracts of V. parvula was assayed by monitoring the oxidation of either NADH or NADPH at 340 nm.

$$Pyruvate + HCO_3^- + NADH \xrightarrow{NAD\text{-}ME} Malate + NAD \qquad (4.24)$$

$$Pyruvate + HCO_3^- + NADPH \xrightarrow{NADP\text{-}ME} Malate + NADP \qquad (4.14)$$

The assay contained (in mM): pyruvate, 10; NADH or NADPH, 1; $MgSO_4$, 10; phosphate buffer (pH 7.5), 50 and 2 mg of crude extract in a volume of 1 ml.

c) General procedures. The cuvettes in the above procedures were washed well between assays with tap water, distilled water, deionized water and

acetone, and finally dried in a stream of air. With the exception of the buffer, the enzyme preparations and reagents were kept at 0 C in ice water at all times. Assays were designed such that the volume of the buffer solution was the largest of all the components in the reaction mixture and hence the time required for temperature equilibration was minimized.

One unit of enzyme activity corresponds to the oxidation of 1 μmole of NADH/mg protein/min.

C. Radioactive Enzyme Assays.

Radioactive assays were used to confirm the presence of pyruvate carboxylase, PEP carboxykinase, PEP carboxylase and malic enzyme in crude extracts of V. parvula M_4.

a) Specific assays.

i) Pyruvate carboxylase. Preliminary studies measuring pyruvate carboxylase activity in the cell-free extracts of V. parvula M_4 employed assays containing either Na bicarbonate-^{14}C (1.4×10^5 dpm/μmole) or pyruvate-3-^{14}C (2×10^5 cpm/μmole). The assay system contained (in mM): $NaHCO_3$, 10; pyruvate, 9.1; ATP, 10; phosphate buffer (pH 6.5), 50 and 3 mg crude extract in a final volume of 1 ml. Malonate (10 mM) and arsenite (5 mM) were added to the system to inhibit malate and pyruvate dehydrogenase, respectively.

ii) PEP carboxykinase (PEPCK) and PEP carboxylase (PEPC). PEPCK and PEPC were assayed by two radioactive methods. The first method (I) was the direct fixation of $H^{14}CO_3^-$ with PEP to form oxaloacetate-^{14}C in the presence and absence of nucleoside diphosphates. The assay system contained (in mM) PEP, 10: $NaH^{14}CO_3$ (1×10^5 dpm), 10; $MgSO_4$, 10; sodium malonate, 10; phosphate buffer (pH 7.0) 50, and 3 mg crude extract in a final volume

of 1 ml. ADP, GDP and IDP were added where indicated at a concentration of 10 mM.

The second method (II) employed was the $^{14}CO_2$-oxaloacetate exchange method of Hsie and Rickenberg (1966). This assay contained (in mM): oxaloacetate, 10; ATP, 10; sodim malonate, 10; $NaH^{14}CO_3$ (1.25×10^5 dpm/μmole), 10; and $MgSO_4$, 10 in Tris-HCl buffer (pH 6.5, 50 mM) in a volume of 1 ml.

In both cases, the crude extracts were dialyzed against the appropriate buffer for 12 hrs to remove the endogenous nucleotides prior to the assay. PEP carboxylase activity was measured in the same assay in the absence of nucleotides.

iii) Malic enzyme. The NAD- or NADP-dependent malic enzymes were assayed with pyruvate-3-^{14}C in the presence of sodium bicarbonate and either NADH or NADPH. The reaction mixture contained (in mM): pyruvate-3-^{14}C (5.50×10^4 dpm/μmole), 10; $NaHCO_3$, 10; NADH or NADPH, 1; $MgSO_4$, 10; phosphate buffer (pH 7.5), 50 and 20 mg of crude extracts in a 1 ml volume.

In all of the above radioactive assays, the reactions were stopped by the addition of an equal volume of 2%, 2,4 dinitrophenylhydrazine (DNPH) in 17.6 N H_2SO_4, the tubes sealed and allowed to stand at 4 C for 6 hr to ensure complete precipitation of the hydrazone material in the reaction mixture. Following this, the mixture was centrifuged and the supernatant, containing (where appropriate) the malate-1-^{14}C, transferred to another tube. The pellet was washed twice with the DNPH solution and then ethyl-acetate (0.5 ml) was added to the pellet to dissolve the precipitated hydrazones. The total radioactivity was determined by liquid scintillation counting with an aliquot of the resultant solution. Samples of the radio-

active solutions, together with their unlabelled analogues, were then spotted individually on Whatman No. 1 paper and the chromatograms developed either with n-butanol-ethanol-0.5 M ammonium hydroxide (70:10:20) (El Hawari and Thompson, 1953) or with isopropanol-water-ammonia (200:20:10) (Smith and Smith, 1960). Following development, the chromatograms were air-dried and the hydrazone spots identified by U.V.-light. The radioactive spots on the individual strips were located by radiochromatogram scanning and their location compared to their unlabelled counterparts. The standard unlabelled malate spot was located with bromcresol green reagent (Nordmann and Nordmann, 1960). The R_f of this spot was then used to locate the radioactive malate peak in the other chromatogram strips. The total radioactive count of the respective spots was determined by cutting them out and counting them by the liquid scintillation procedure. Counting efficiencies were 20-30%. The basic solvent system of El Hawari and Thompson (1953) was the most useful, readily separating the two pyruvate hydrazones (R_fs = 0.43 and 0.67) from the oxaloacetate hydrazone (0.27) and from malate (0.15) (Fig. 4.12). The tendency to form more than one pyruvate hydrazone is well-established (Schwartz and Brewington, 1967) and is increased by the use of acidic solvent systems.

D. Preparation of labelled compounds.

a) Sodium bicarbonate-^{14}C. In most cases, radioactive sodium bicarbonate (mc/mM) was prepared from $Ba^{14}CO_3$ by the method of Aronoff (1967) just prior to each experiment.

b) Oxaloacetate-^{14}C. Since radioactive oxaloacetate was not commercially available, a standard labelled oxaloacetate solution was prepared for

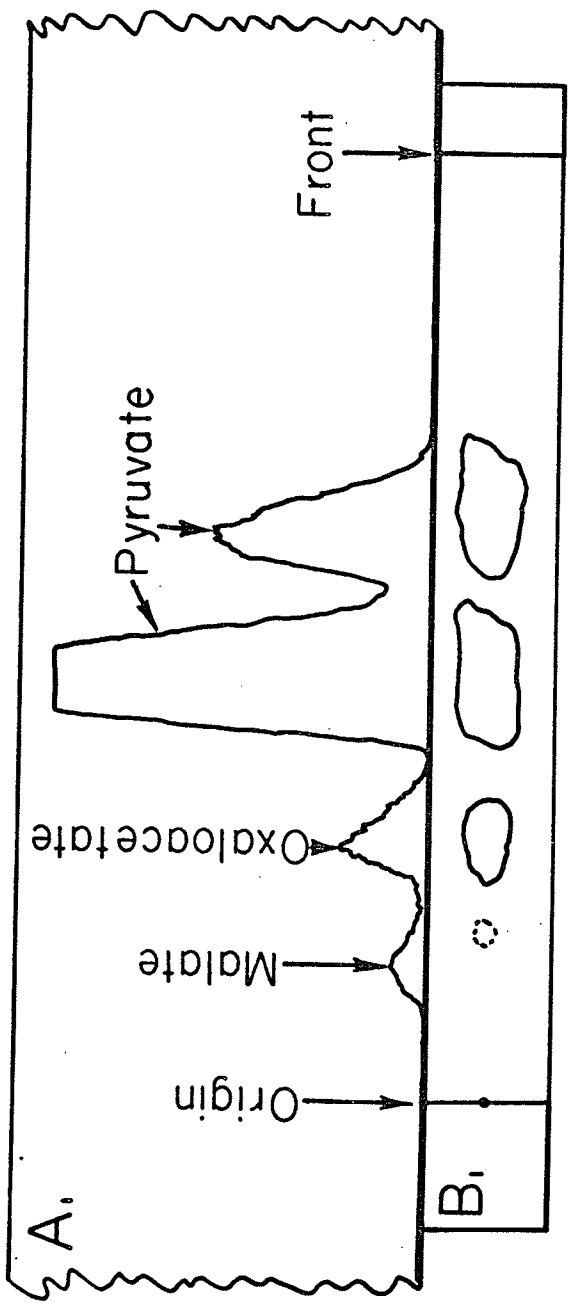


Fig. 4.12. Paper chromatogram and radiochromatogram scan of the oxaloacetate-^{14}C and pyruvate-^{14}C hydrazones, and malate-^{14}C after development by the basic solvent system of El Hawari and Thompson (1953).

chromatographic purposes. For this, oxaloacetate-1-^{14}C was generated from aspartate-1-^{14}C with commercial glutamate-oxaloacetate transaminase. The reaction mixture contained (in mM): sodium aspartate-1-^{14}C (20 μc/mM) 5; α-ketoglutarate, 5; $MgSO_4$, 10; Tris buffer (pH 7.0), 50 and 100 μl of the transaminase enzyme in 1 ml. The addition of NADH (1 mM) and malate dehydrogenase (11 μg) to the assay mixture permitted the preparation of malate-1-^{14}C from the oxaloacetate-1-^{14}C formed. The reaction mixture was contained in a cuvette and the reaction followed at 340 nm at 37 C for 10 min at which time 0.5 ml of 0.1% 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCL was added to stop the reaction. Under these conditions only a portion of the oxaloacetate-^{14}C was converted to malate-^{14}C. The contents of the cuvette were then transferred to a tube, the tube sealed and allowed to stand for 6 hr to complete the precipitation of the hydrazone material. The labelled compounds were isolated by paper chromatography employing the basic solvent system of El Hawari and Thompson (1953) as previously described.

E. Materials.

All radioactive materials were purchased either from NEN Canada Ltd., (Montreal) or from the Radiochemical Centre (Amersham, England). The commercial enzymes were obtained from Boehringer-Mannheim Corp. (New York).

III. RESULTS

The main object of the work in this Chapter was to indicate, in a preliminary way, the enzymes present in dialyzed crude extracts of V. parvula M_4 capable of converting pyruvate to P-enolpyruvate. For the purposes of the discussion, the following spectrophotometric results have been divided into two sections: (1) the enzymes catalyzing the formation and utilization of P-enolpyruvate, and (2) those enzymes involved in the fixation of CO_2 with pyruvate. The activity of various enzymes observed by this procedure was then studied with radiochemical assays.

A. Spectrophotometric Assays.

a) Reactions involving P-enolpyruvate. When crude dialyzed extracts were incubated with PEP, ADP and lactic dehydrogenase, rapid oxidation of NADH (72 units) was observed indicating the presence of an active pyruvate kinase in V. parvula M_4 (Table 4.3). This result, therefore, sets V. parvula M_4 apart from V. alcalescens, which has been shown recently to be devoid of pyruvate kinase (Michaud and Delwiche, 1971). It should be noted that the rate of 72 units in Table 4.3 represents the net rate of PEP utilization since the reference or control cuvette contained all of the ingredients except the substrate, PEP.

The incubation of the crude extract with pyruvate, ATP and the GAPDH assay system (Methods) resulted in a net oxidation rate of 3 units. Since this assay measured the conversion of pyruvate to PEP in the absence of bicarbonate, this result suggested the possible presence of PEP synthetase (Cooper and Kornberg, 1967) in V. parvula M_4. PEP carboxykinase (PEPCK) and

TABLE 4.3

Apparent enzyme activities in crude extracts of
V. parvula M_4 related to the formation or utilization of PEP

Enzyme[a]	Enzyme activity[b]
Pyruvate kinase	72
PEP-synthetase	3
PEP-carboxykinase (ATP)	8
PEP-carboxylase	4
Enolase	38

[a] Enzymes assayed as described in Methods. In all cases, the control or reference cuvette contained no substrate.

[b] µmoles NADH oxidized/mg protein/min.

PEP carboxylase (PEPC) activity was assayed by measuring the conversion of PEP and bicarbonate to oxaloacetate with malic dehydrogenase and NADH in the presence and absence of ATP. With ATP, a net oxidation rate of 8 units (Table 4.3) was observed suggesting the presence of PEP carboxykinase in V. parvula M_4. In the absence of ATP, however, the NADH oxidation rate decreased to 4 units. This 'residual' activity suggested that PEP carboxylase might also be present in this organism. Since both the PEPCK and PEPC enzyme activities were probably measured in the assay containing ATP, the actual PEPCK activity would be only 4 units (i.e., 8-4 = 4) instead of the 8 units shown in Table 4.3.

During the course of this investigation, it was observed that the crude extracts contained high enolase activity (38 units). This obviated the necessity of adding commercial enolase to the GADPH assay system. Furthermore, in testing the operation of the GAPDH assay system, it was also observed that the enzyme phosphoglyceromutase was absent in the crude dialyzed extracts of V. parvula M_4.

b) Reactions involving CO_2-fixation with pyruvate. As shown in Fig. 4.8, CO_2-fixing reactions involving pyruvate can synthesize oxaloacetate directly via pyruvate carboxylase or indirectly via malic enzyme.

i) Pyruvate carboxylase (PC). Pyruvate carboxylase activity was demonstrated in crude dialyzed extracts by the rapid formation of oxaloacetate in the presence of pyruvate, NADH, malic dehydrogenase, Mg^{+2}, HCO_3^- and ATP (91 units) (Table 4.4). Surprisingly, when ATP was omitted from the assay a net rate of 72 units of 'pyruvate carboxylase' activity was observed. As will be confirmed later in Chapter 6, this ATP-independent (IPC) activity constitutes an enzymatic function distinct from that of the conventional or

TABLE 4.4

Enzyme activity in crude extracts of *V. parvula* M_4 catalyzing CO_2-fixation with pyruvate

Enzyme[a]	Enzyme activity[b]
Pyruvate carboxylase	
+ ATP	91
- ATP	72
Malic enzyme	
(Pyr ⟶ malate)	
+ NADH	10
+ NADPH	18
- $NaHCO_3$ + NADPH	4
(Malate ⟶ Pyr)	
+ NAD	0
+ NADP	11

[a] Enzyme assays as described in Methods. Reference (control) cuvettes contained no pyruvate.

[b] μmoles NADH oxidized/mg protein/min.

ATP-dependent pyruvate carboxylase (DPC). Using the same rationale as employed with PEPCK and PEPC, the net rate of 91 units observed with ATP would constitute the total of the DPC and IPC activities. If such were the case, the DPC activity produced a net rate of only 19 μmoles NADH oxidized/mg protein/min. However, as will be discussed in Chapter 6, this is an over-simplification.

ii) Malic enzyme. This incubation of crude dialyzed extracts with pyruvate, bicarbonate and NADH resulted in a net oxidation rate of 10 units indicating the presence of NADH-dependent malic enzyme activity in V. parvula M_4. Surprisingly, the substitution of NADPH for NADH resulted in a net rate of 18 units suggesting the possible presence of a separate NADPH-specific malic enzyme in the organism. Some endogenous CO_2-fixing activity (4 units) was observed in the absence of $NaHCO_3$, probably due to the presence of some dissolved carbon dioxide in the buffer.

By using the conventional spectrophotometric method and assaying malic enzyme activity in the direction of pyruvate formation with NADP or NAD, some clarification of the situation was possible. For example, when crude extracts (2 mg) were incubated with malate (10 mM), $MgSO_4$ (10 mM), NADP (1 mM) and malonate (10 mM) (to inhibit endogenous malate dehydrogenase), 11 μmoles of NADP were reduced/mg protein/min. However, when NAD was used in the assay, no reduction of the NAD was observed. This suggested that a NADP-specific malic enzyme was present in the crude extracts of V. parvula M_4, but not the NAD-specific enzyme as previously indicated. However, results obtained with the radiochemical assay for malic enzyme (Table 4.9) will show that the NAD(H) concentration is an important factor in this assay.

B. Radioactive Assays.

Traditionally, radioactive bicarbonate has been employed to demonstrate the formation of oxaloacetate by carbon dioxide fixation with either pyruvate or P-enolpyruvate, the measure of this fixation being the amount of label in the total 2,4 dinitrophenylhydrazone precipitate. However, with extracts of Veillonella sp, this procedure is not satisfactory since rapid CO_2-pyruvate exchange activity has been observed in crude extracts of V. alcalescens (Whiteley and McCormick, 1963) and V. parvula M_4 (Ng and Hamilton, 1971). It was, therefore, necessary to substantiate any results obtained with labelled bicarbonate by the isolation of the radioactive products of the assay, or by the use of labelled pyruvate (e.g. pyruvate-3-C^{14}). With this in mind, radioactive assays were employed to measure the presence of pyruvate carboxylase, PEP carboxylase, PEP carboxykinase and malic enzyme in crude dialyzed extracts of V. parvula M_4.

a) Pyruvate carboxylase. The presence of pyruvate carboxylase activity in V. parvula M_4, as indicated from the spectrophotometric results (Table 4.4), was confirmed by the formation of oxaloacetate-^{14}C by dialyzed crude extracts of V. parvula in the presence of pyruvate, ATP and bicarbonate-^{14}C (Table 4.5). With the complete assay, 24.5% of the original radioactivity was incorporated into the total hydrazones precipitated by DNPH. The majority of those counts (88%) were present in pyruvate as a result of the pyruvate-CO_2 exchange reaction, while 4% (14,460 cpm) was present in oxaloacetate. The incorporation of the bicarbonate-label into oxaloacetate was greatly reduced in the absence of 10 mM sodium arsenite because of the presence of an active pyruvate dehydrogenase in extracts of V. parvula M_4 (Ng and Hamilton, 1971). Furthermore, the total incorporation of ^{14}C

TABLE 4.5

Formation of oxaloacetate-^{14}C from pyruvate and sodium bicarbonate-^{14}C by dialyzed crude extracts of V. parvula M_4

Assay conditions	Net ^{14}C incorporation (cpm)		
	Total[a]	Pyruvate	Oxaloacetate
Basic system[b]	344,528	303,170	14,460
+ Arsenite	216,453	200,092	2,024
- ATP, - Arsenite	157,352	148,553	1,012

[a] Radioactivity in total hydrazone precipitate.

[b] Assay, which contained 10 mM $NaH^{14}CO_3$ (1.4×10^5 dpm/μmole), was carried out as described in Methods (Radioactive-pyruvate carboxylase).

dropped 37% (to 216,453 cpm) in the absence of this inhibitor. With the further omission of ATP, the total hydrazone radioactivity again decreased to only 54% (157,352) of the radioactivity obtained with the complete system. This decrease was accompanied by a further decrease in the oxaloacetate radioactivity (to 0.6%). The comparative contribution of the ATP-dependent and ATP-independent activities to the oxaloacetate radioactivity in the complete system was not assessed here, but will be examined in greater detail in Chapter 6.

b) PEP carboxylase and PEP carboxykinase. The results shown in Table 4.3 indicated that both PEP carboxykinase and PEP carboxylase enzymes were present in the crude extracts of V. parvula M_4. Hsie and Rickenberg (1966) have utilized an oxaloacetate-$^{14}CO_2$ exchange reaction to demonstrate both PEPC and PEPCK activity in the AB257 and AB257^{suc-} mutants of E. coli K12. This method was employed in this investigation using both dialyzed and undialyzed extracts of V. parvula M_4 and the results are shown in Table 4.6. With the basic system, which contained no nucleotides (exp. 1), 22 and 15% of total $H^{14}CO_3^-$ radioactivity was incorporated into the hydrazone precipitate by the undialyzed and dialyzed extracts, respectively. The higher activity observed with the undialyzed extract was undoubtedly due to the presence of nucleoside triphosphates in the preparation. The significant incorporation of radioactivity into hydrazone material by the dialyzed extract incubated in the absence of nucleotides (15%-exp. 1) indicated the presence of PEP carboxylase activity. This was probably not residual PEPCK activity since the addition of ATP to the dialyzed extract (exp. 2) brought about a greater than 2-fold (35 vs 15%) increase in activity, whereas no increase in the incorporation of radioactivity was

TABLE 4.6

Apparent PEP carboxykinase and PEP carboxylase activity in dialyzed and undialyzed extracts of V. parvula M_4

Exp.	Assay conditions	Radioactivity incorporated into hydrazone material			
		Undialyzed		Dialyzed	
		cpm	%	cpm	%
1.	Basic system[a]	27,538	22[b]	18,804	15
2.	+ ATP	27,795	22	43,831	35
3.	+ GTP	18,721	15	35,018	28
4.	+ UTP	28,848	23	30,052	24
5.	+ ITP	20,005	16	21,254	17

[a] Assay II for PEPCK and PEPC was used with 10 mM $NaH^{14}CO_3$ (1.25×10^4 cpm/μmole) without added nucleotides.

[b] % of total radioactivity.

observed with the undialyzed preparation. These results suggest that the latter extract contained components inhibiting PEPCK activity. The nucleoside triphosphates of guanosine, uridine and inosine also stimulated the exchange reaction with the dialyzed preparation, whereas both GTP and ITP inhibited enzyme activity with the undialyzed extract.

The above results (Table 4.6), indicating the presence of PEP carboxykinase and PEP carboxylase activity in extract preparations of V. parvula M_4, was further examined by measuring the formation of oxaloacetate-^{14}C following the incubation of dialyzed extracts with bicarbonate-^{14}C and PEP (Table 4.7). In the absence of nucleotides (exp. 1), the radioactivity incorporated into the total hydrazone material (11,950 cpm) was distributed between pyruvate and oxaloacetate, with the pyruvate hydrazone possessing the highest radioactivity (11 vs 1%). This was possibly due to the decarboxylation of oxaloacetate formed in the reaction and the subsequent exchange of the bicarbonate-^{14}C with the resultant pyruvate, since pyruvate kinase should not have been active under these conditions. Crude extracts of V. parvula M_4 have been shown to decarboxylate oxaloacetate to pyruvate and CO_2 (Ng, 1968). The addition of ADP (exp. 2) stimulated oxaloacetate production 6-fold, while GDP (exp. 3) had only a minor effect (2-fold). On the other hand, oxaloacetate-^{14}C formation was completely inhibited by the addition of 10 mM IDP (exp. 4). These results, although suggesting the presence of PEPCK and PEPC in extracts of V. parvula M_4, also indicated that the radioactivity in the hydrazone material in Table 4.6 was probably distributed between the pyruvate- and the oxaloacetate-hydrazones.

To determine whether the above suggestion was correct, the

TABLE 4.7

Effect of nucleoside phosphates on the fixation of sodium bicarbonate-^{14}C with PEP by dialyzed crude extracts of V. parvula M_4

Exp.	Assay conditions	Distribution of radioactivity					
		Total[a]		Pyruvate		Oxaloacetate	
		cpm	%	cpm	%	cpm	%
1.	Basic system[b]	11,950	12[c]	10,924	11	1,013	1
2.	+ ADP	19,923	20	13,931	14	5,976	6
3.	+ GDP	11,927	12	10,005	10	1,992	2
4.	+ IDP	6,971	7	6,897	7	-	-

a Radioactivity incorporated into 2,4 dinitrophenylhydrazone material.

b Assayed with Method I (PEPCK-PEPC), which contained 10 mM sodium bicarbonate-^{14}C (1.0×10^5 cpm), but without added nucleotides.

c % of total radioactivity.

α-ketoacid hydrazone precipitate obtained from the experiment shown in Table 4.6 was subjected to paper chromatography to separate the various components. As shown in Table 4.8, the largest radioactive component obtained in the oxaloacetate-$^{14}CO_2$ exchange assay, without added nucleotides (exp. 1), was pyruvate (14%). The small amount of label in oxaloacetate may have resulted from PEP carboxylase activity. A substantial increase in the production of oxaloacetate-^{14}C was observed when ATP was added to the assay (exp. 2), with less synthesis observed with GTP and UTP. No labelled oxaloacetate was formed in the presence of ITP (exp. 4).

While the combined radioactive (Tables 4.6 and 4.8) and spectrophotometric (Table 4.3) results present reasonable evidence for PEP carboxykinase activity in crude extracts of V. parvula M_4, the position of PEP carboxylase is less clear. As mentioned previously, although little oxaloacetate-^{14}C was formed in the absence of nucleotides, the presence of label in pyruvate (exp. 1 - Table 4.7) is presumptive evidence for the presence of this enzyme in V. parvula extracts. PEPCK activity has also been observed in extracts of V. alcalescens (Michaud, 1968).

c) Malic enzyme. When assayed in the direction of malate formation, the spectrophotometric assay for malic enzyme (Table 4.4) indicated that crude extracts of V. parvula M_4 contained both a NAD- and a NADP-specific malic enzyme. However, when assayed in the opposite direction, only activity for the latter enzyme was detected. To clarify these results, dialyzed crude extracts were incubated with pyruvate-3-^{14}C, $NaHCO_3$, NADH or NADPH and the products of the reaction separated by paper chromatography; the radioactivity in these products was then determined by liquid scintillation

TABLE 4.8

The distribution of label from sodium bicarbonate-^{14}C into oxaloacetate and pyruvate by dialyzed extracts of V. parvula M_4 under conditions of the oxaloacetate-$^{14}CO_2$ exchange assay.

Exp.	Assay condition	Distribution of radioactivity			
		Pyruvate		Oxaloacetate	
		cpm	%	cpm	%
1.	Basic system[a]	17,448	14[b]	885	0.7
2.	+ ATP	31,205	25	12,495	10
3.	+ GTP	27,499	22	6,297	6
4.	+ UTP	25,000	20	4,998	4
5.	+ ITP	21,296	17	-	-

[a] Conditions as for Table 4.6.

[b] % of total radioactivity.

counting procedures. In the presence of $NaHCO_3$ and 1 mM NADH, the extract converted a small amount of the pyruvate to oxaloacetate and malate (exp. 1 - Table 4.9); no CO_2-fixation was observed in the absence of bicarbonate (exp. 2). The conversion observed in exp. 1 was increased by adding an additional amount (0.5 mM) of NADH, the majority of the radioactivity (22%) appearing in malate (exp. 3). With the further addition of 0.5 mM NADPH (exp. 4), the formation of labelled malate increased 2-fold (22 to 45%) over that with only NADH. The fact that increased amounts of malate-^{14}C were formed from pyruvate-3-^{14}C by the addition of NADPH in the latter experiment further suggested the presence of both a NADH- and a NADPH-specific malic enzyme in V. parvula M_4. However, since significant fixation was apparent only at increased levels of NADH (exp. 1 vs exp. 3), the NADH-specific enzyme may be of lesser physiological importance.

IV. DISCUSSION

The results obtained with both the spectrophotometric and the radioactive assays have indicated that crude extracts of V. parvula M_4 contained enzymatic activity for ATP-dependent pyruvate carboxylase, ATP-independent pyruvate carboxylase, PEP carboxykinase, NADH-specific malic enzyme, NADP-specific malic enzyme, pyruvate kinase, enolase and possibly PEP carboxylase (PEP carboxytransphosphorylase) and PEP synthetase. However, it is clear that the results presented in this Chapter provide only presumptive evidence for the presence of these enzymes due to the complexity of the metabolic interconversions (Fig. 4.8). However, even

TABLE 4.9

Conversion of pyruvate-3-^{14}C to oxaloacetate-^{14}C and malate-^{14}C by crude dialyzed extracts of V. parvula M_4 in the presence of bicarbonate and reduced pyridine nucleotides.

Exp.	Assay conditions	Distribution of radioactivity					
		Pyruvate		Oxaloacetate		Malate	
		cpm	%	cpm	%	cpm	%
1.	Basic system[a]	520,368	94.5[b]	1,052	2	12,448	4
2.	- $NaHCO_3$	549,720	99.7	-	-	439	-
3.	+ NADH (0.5 mM)	420,130	76.4	538	1	121,062	22
4.	+ NADH + NADPH (both 0.5 mM)	301,341	54.7	2,472	4	245,517	45

a The basic assay contained 10 mM pyruvate-3-^{14}C (5.51×10^5 cpm) and 1 mM NADH as described in Methods.

b % of original pyruvate-3-^{14}C radioactivity.

within the limitations of the assays, as will be indicated below, the results were valuable in directing the course of future research.

A. Pyruvate kinase.

Although, only assayed spectrophotometrically, the results indicated that crude extracts of V. parvula M_4 contained a very active pyruvate kinase. As mentioned previously, this observation differs from the recent results obtained with V. alcalescens by Michaud and Delwiche (1970), who were unable to detect pyruvate kinase activity in that organism. However, Rogosa, Krichevsky and Bishop (1965) earlier observed that the incubation of crude extracts from V. alcalescens (strain VH-11) with fructose-6-P and hexose diphosphates, in the presence of commercial lactic dehydrogenase, resulted in the production of lactic acid. This suggested that all of the glycolytic enzymes were present in this organism for the conversion of fructose-6-P to pyruvate, including pyruvate kinase. In their later work, Michaud and Delwiche (1970) proposed that this earlier observation by Rogosa's group did not actually indicate the presence of pyruvate kinase, but rather indicated a reaction which effectively circumvented it. These results, together with those from V. parvula M_4 in this study, indicate that the presence of pyruvate kinase in Veillonella sp is a controversial one. Certainly, the function of this enzyme would be particularly important to the over-all metabolism of these bacteria since they utilize short chain acids, such as lactate and pyruvate, for biosynthesis (Chap. 2). Since an active pyruvate kinase in V. parvula M_4 would readily convert PEP to pyruvate, one would imagine that it must be effectively controlled to permit gluconeogenesis to occur. The purification and characterization of

pyruvate kinase from V. parvula M_4 will be the subject of the next Chapter.

B. PEP synthetase.

With the presence of pyruvate kinase in V. parvula M_4, it was important to see whether PEP synthetase was present in the organism since these two enzymes catalyzed opposite reactions between PEP and pyruvate. The spectrophotometric data in Table 4.3, obtained with the GADPH assay system, indicated that PEP was synthesized from pyruvate with a net oxidation rate of 3 units. This low activity suggested that a PEPS enzyme might be present in V. parvula M_4. While the indirect GADPH assay system was employed to measure the formation of PEP from pyruvate, the complexity of this assay made it less desireable than a direct assay for PEP. However, the direct assay method proposed by Grisolia (1962), which monitors the appearance of PEP at 240 nm, proved to be unsatisfactory because V. parvula crude extracts absorb very strongly at this wavelength and the PEP absorbance could not be detected above this 'noise'. One advantage of the GADPH system was that, in addition to suggesting the presence of PEPS activity, it also indicated the existence of an active enolase and the absence of phosphoglyceromutase in the crude extracts of the organism. The possible existence of PEP synthetase in this organism will be examined in more detail in Chapter 5.

C. PEP carboxykinase and PEP-carboxylase.

The results presented in Tables 4.3 and 4.6 to 4.8 have indicated that PEP carboxykinase, and possibly PEP carboxylase, were present in the crude extracts of V. parvula M_4. PEPCK activity in extracts of V. alcalescens

has also been reported by Michaud (1968). In this latter study, Michaud used the OAA-$^{14}CO_2$ exchange assay to test for the enzyme and failed to detect any ATP-dependent activity with untreated crude extracts in the presence of 4.5 mM ATP. In fact, the omission of ATP from the reaction mixture resulted in increased $^{14}CO_2$ exchange. Michaud (1968) attributed this observation to the presence of catalytic levels of ATP in the untreated extracts and that the further addition of ATP resulted in inhibitory levels of this cofactor. The data obtained with crude extracts of V. parvula M_4 (Table 4.6), while not showing inhibition by ATP with the undialyzed extract, did show increased activity with the dialyzed preparation in the presence of ATP suggesting the existence of inhibiting compounds in the undialyzed extract. Furthermore, activity in the latter extract was inhibited by GTP and ITP.

It is unfortunate that the exchange assay was the only method employed by Michaud in the V. alcalescens study since his conclusions were based solely on the amount of radioactivity present in the total 2,4 dinitrophenylhydrazone precipitate. Comparison of Tables 4.6 and 4.8 in this study indicates that during this assay the radioactivity is distributed both into oxaloacetate and into pyruvate. For this reason, further studies on CO_2-fixation with extract preparations of V. parvula M_4 (Chap. 6) employed pyruvate-3-^{14}C as the radioactive tracer. However, it was still necessary to isolate the individual radioactive end-products.

Although Michaud (1968) gave no indication as to the presence or absence of PEP carboxylase in crude extracts of V. alcalescens, his results with the oxaloacetate-$^{14}CO_2$ exchange reaction are worth noting here. With Dowex-1-treated extracts, more $^{14}CO_2$ was incorporated into the hydrazone

precipitate with inorganic phosphate (4748 cpm) than with ATP (1491 cpm). Although these results were thought to represent a reaction other than that catalyzed by PEP carboxylase, there was no valid evidence to suggest that this activity was not due to such an enzyme. The reaction would not have been catalyzed by PEP carboxytransphosphorylase (equation 4.13) since the addition of pyrophosphate drastically inhibited the exchange activity by the V. alcalescens extract.

As mentioned previously, the presence of PEP carboxylase in V. parvula M_4 is based on the assumption that oxaloacetate was synthesized in this organism from PEP and bicarbonate-^{14}C, in the absence of nucleotides, and then was decarboxylated to pyruvate, the pyruvate then participating in the exchange reaction with the labelled bicarbonate. This hypothesis further assumes that the PEP was not converted to pyruvate directly by pyruvate kinase since ADP should not have been present in the system. Furthermore, the appearance of ^{14}C in the pyruvate isolated in the above assay (Table 4.7) could have arisen, in part, through the action of PEP carboxytransphosphorylase, since the assay was carried out in phosphate buffer. Obviously, the question as to whether a PEP carboxylase or a PEP carboxytransphosphorylase is present in V. parvula M_4 must await more detailed studies with purified enzyme preparations.

D. Pyruvate carboxylase.

The spectrophotometric assay results in Table 4.4 have demonstrated the presence of a conventional pyruvate carboxylase (ATP-dependent) in the dialyzed extracts of V. parvula M_4. However, the significant formation of oxaloacetate in the absence of ATP (72 units vs 91 units with ATP) was an

unusual finding, which could be readily reproduced with all extracts tested. Further investigation of this ATP-independent activity with crude and purified enzyme preparations (Chap. 6) will show that V. parvula M_4 contains two enzymes capable of forming oxaloacetate from pyruvate and CO_2: one requiring ATP (DPC) and one active without ATP (IPC).

E. Malic enzymes.

To date, malic enzyme has not been reported in species of the genus, Veillonella. Although the presence of both a NAD- and a NADP-specific malic enzyme in V. parvula M_4 was suggested from Tables 4.4 and 4.9, the results could have occurred through the action of other enzymes which may have been present in the extracts and which do not catalyze the direct conversion of pyruvate to malate in the presence of pyridine nucleotides. For example, if V. parvula M_4 contained a NADP transhydrogenase (NADP-TH) similar to that observed in Pseudomonas fluorescens (Colowick et al, 1952; Kaplan, Colowick and Neufeld, 1952), the utilization of NADPH during the formation of malate could be explained by coupling this reaction to oxaloacetate formation by the apparent ATP-independent 'pyruvate carboxylase' (IPC-equation 4.11) and to the conversion of this oxaloacetate to malate by malate dehydrogenase (MDH-equation 4.15):

$$\text{Pyruvate} + HCO_3^- \xrightarrow{\text{IPC}} \text{Oxaloacetate} \qquad (4.11)$$

$$\text{Oxaloacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD} \qquad (4.15)$$

$$NADPH + NAD \xrightarrow{NADP\text{-}TH} NADH + NADP \qquad (4.25)$$

$$\text{net: } Pyruvate + HCO_3^- + NADPH \longrightarrow Malate + NADP \qquad (4.14)$$

Similarly, the results suggesting the presence of the NAD-specific enzyme could have resulted from the combination of equations 4.11 and 4.15 above and given equally spurious results.

The results in this Chapter suggest that crude extracts of V. parvula M_4 probably contain a variety of enzymes capable of the initial reactions in gluconeogenesis. It is also apparent that the conclusions reached in this crude extract study are limited and must await the purification of the respective enzymes. This aspect forms the main theme of the second section of this thesis, which is concerned with the purification and characterization of two enzymes observed in the preliminary study outlined in this Chapter: pyruvate kinase and the unusual ATP-independent 'pyruvate carboxylase'.

SECTION II.

This section contains detailed information on the purification, characteristics and factors regulating the activity of pyruvate kinase (Chapter 5) and ATP-independent 'pyruvate carboxylase' (Chapter 6) from *V. parvula* M_4.

CHAPTER 5

PYRUVATE - P-ENOLPYRUVATE INTERCONVERSIONS: PYRUVATE KINASE

I. INTRODUCTION

The cellular concentration of P-enolpyruvate is an important factor regulating the process of gluconeogenesis in a variety of systems (Krebs, 1954; Kornberg, 1966). P-enolpyruvate (PEP) was discovered in 1934 by Lohmann and Meyerhof, who showed that this compound was an intermediate in the transfer of phosphate from phosphoglycerate to the adenylic acid system (equations 4.20 and 2.5):

$$2\text{-P-glycerate} \longrightarrow \text{P-enolpyruvate} \qquad (4.20)$$

$$\text{P-enolpyruvate} + \text{ADP} \longrightarrow \text{Pyruvate} + \text{ATP} \qquad (2.5)$$

The conversion of 2-P-glycerate to PEP (4.20) is catalyzed by enolase, a fluoride-sensitive enzyme (Lohmann and Meyerhof, 1934), while the conversion of PEP to pyruvate is catalyzed by pyruvate kinase (Parnas, Ostern and Mann (1934a).

A. Pyruvate kinase.

a) Animal systems. The exact nature of the reaction catalyzed by pyruvate kinase has been a controversial one since its discovery by Lohmann and Meyerhof, particularly in relation to the role of adenine nucleotides in catalysis. Lohmann and Meyerhof (1934) regarded the role

of the adenine nucleotides as that of a coenzyme for the dephosphorylation of PEP to give orthophosphate (P_i) and pyruvate (equation 5.25).

$$\text{P-enolpyruvate} \longrightarrow \text{Pyruvate} + P_i \qquad (5.25)$$

This theory was also supported by Parnas, Ostern and Mann, 1934(a), who also observed that the breakdown of phosphoglycerate to pyruvate led to the phosphorylation of creatine. This observation led these workers to regard creatine as the acceptor of the phosphoryl group from PEP (Parnas, Ostern and Mann, 1934(b). However, Lohmann (1935), Lutwak-Mann and Mann (1935), and Needham and van Heyningen (1935) subsequently presented clear evidence for the phosphorylation of AMP by P-enolpyruvate with the resultant formation of ATP and pyruvate. This phosphorylation was later shown to result from the action of two enzymes: pyruvate kinase and adenylate kinase (Meyerhof and Junowicz-Kocholaty, 1942; Boyer, Lardy and Philips, 1942,1943; Colowick and Kalckar, 1943; Colowick, 1951, 1955).

For many years it was believed that the pyruvate kinase reaction would not proceed to any measureable degree in direction of P-enolpyruvate formation. This conclusion was based on the failure by Meyerhof et al, (1938) to observe the incorporation of (^{32}P) from (^{32}P)ATP into P-enolpyruvate in a muscle extract, the (^{32}P)ATP being continuously generated from ($^{32}P_i$) by the reactions in glycolysis. This led to speculation concerning al alternate pathway of P-enolpyruvate formation, in which it was assumed that pyruvate was converted,via CO_2 fixation, to malate and that oxidation of the latter gave rise to P-enolpyruvate by the reactions proposed by Kalckar (1941). This scheme involved the

formation of the intermediates fumarate, phosphomalate and phosphooxaloacetate.

However, Lipmann (1941,1946) calculated that the direct phosphorylation of pyruvate by ATP should take place to a measureable extent since the ΔG^{o} for pyruvate formation was only -4 Kcal. In this same period, Lardy and Ziegler (1945) demonstrated the incorporation of ($^{32}P_i$) into PEP under the same conditions used by Meyerhof and coworkers, but with the addition of potassium ions. Boyer, Lardy and Philips (1942) had previously shown that K^{+} and Mg^{2+} ions were essential for the pyruvate kinase reaction in muscle extracts. These results were later confirmed by Meyerhof and Oesper (1949).

Despite these earlier findings, however, PEP formation from pyruvate by the reversal of the pyruvate kinase reaction is now considered to be of little phisiological importance since the K_{eq} is too far in the direction of pyruvate formation (Krebs, 1954; Krebs and Kornberg, 1957). Thus, this enzyme is probably involved only in the formation of ATP and pyruvate. Nevertheless, pyruvate kinase is in a position to play an important role in the regulation of gluconeogenesis through its action on PEP.

b) Microorganisms. Abundant information is available on pyruvate kinase activity in carbohydrate-fermenting microorganisms, such as yeast (Seits, 1949; Washio and Mano, 1960), Escherichia coli (Maeba and Sanwal, 1968; Waygood and Sanwal, 1972), Brevibacterium flavum (Ozaki and Shiio, 1969), Azotobacter vinelandii (Liao and Atkinson, 1971) Acetobacter xylinum (Bensiman, 1969) and Bacillus licheniformis (Tuominen and Bernlohr, 1971a,b). However, less information is available on the enzyme in Veillonella species,

organisms shown to be incapable of metabolizing carbohydrates as an energy source (Foubert and Douglas, 1948; Johns, 1951b; Rogosa, 1965; Ng and Hamilton, 1971). Rogosa, Krichevsky and Bishop (1965) showed that V. alcalescens VH-11, and V. parvula BYR-2 were unable to ferment carbohydrates because they lacked hexokinase and hence were incapable of converting hexoses to hexose phosphates. However, when the extracts of V. alcalescens VH-11 were supplemented with yeast hexokinase, pyruvate-^{14}C was produced from glucose-^{14}C suggesting that these extracts contained all of the remaining enzymes in the glycolytic pathway, including pyruvate kinase. This was also confirmed with hexose phosphates and commercial lactic dehydrogenase as mentioned previously. Contrary to these findings are the recent results obtained with strain (C1) of V. alcalescens, obtained from sheep rumen (Michaud and Delwiche, 1970). This strain, besides lacking hexokinase, was also devoid of phsopho-glyceromutase and pyruvate kinase activity.

While mammalian tissues are unable to form PEP from pyruvate directly, microorganisms are more versatile and possess two enzymes capable of direct PEP-pyruvate interconversions: PEP synthetase and pyruvate, phosphate dikinase.

B. PEP synthetase.

The microorganisms, E. coli and Salmonella typhimurium, have been shown to be devoid of the enzyme, pyruvate carboxylase, which catalyzes the formation of oxaloacetate from pyruvate and CO_2. In these bacteria, oxaloacetate is formed by the carboxylation of PEP through the action of

PEP carboxykinase (equation 4.9) (Theodore and Engelberg, 1964; Ashworth, Kornberg and Ward, 1965; Asworth and Kornberg, 1966).

$$\text{P-enolpyruvate} + HCO_3^- + \text{ADP} \longrightarrow \text{Oxaloacetate} + \text{ATP} \qquad (4.9)$$

This observation led to the obvious question: how do these bacteria form PEP when growing in a pyruvate medium keeping in mind the irreversible nature of the pyruvate kinase reaction? This question was answered when Cooper and Kornberg (1968) isolated an enzyme from a mutant (B_m) of E. coli B, which catalyzed the direct synthesis of PEP from pyruvate and ATP; AMP and P_i were the other products of the reaction (equation 4.16). This enzyme was called PEP synthetase (PEP-S) and required Mg^{2+} for activity. While acetyl-phosphate could substitute for

$$\text{Pyruvate} + \text{ATP} \xrightarrow{\text{PEP-S}} \text{P-enolpyruvate} + \text{AMP} + P_i \qquad (4.16$$

ATP with crude extracts, only ATP could serve as the phosphate donor with the purified enzyme. The role of acetyl-phosphate was postulated to facilitate the synthesis of PEP indirectly by the conversion of the small amounts of ADP present in the crude extracts to ATP by acetokinase.

The enzyme from E. coli was studied more recently by Berman and Cohn (1970a,b). The enzyme was reversible having a pH optimum of 8.4 for PEP synthesis and an optimum at pH 6.8 for the reverse reaction. CTP, GTP and UTP could not replace ATP for activity. Although the enzyme was cold-labile, losing 50% of the activity in crude extracts in about 4 hours when stored in ice, it was stable at room temperature in Tris-HCl buffer ranging from pH 5.8 to 6.8. Enzymatic activity was rapidly lost at pH values above 7.0. To date, PEP synthetase activity has been

observed in S. typimurium, E. coli strain B (Cooper and Kornberg, 1965), Acetobacter xylinum (Benziman, Eisen and Palgi, 1969) and in Tetrahymena pyriformis (Liang, 1970).

The mechanism of the PEP synthetase reaction was earlier investigated by Cooper and Kornberg (1967a,b, 1969) and by Berman, Itada and Cohn (1967), who presented evidence for the following partial reactions:

$$\text{Enzyme} + \text{ATP} \longrightarrow \text{Enzyme} - \overset{\beta\gamma}{\text{PP}} + \text{AMP} \qquad (5.26)$$

$$\text{Enzyme} - \overset{\beta\gamma}{\text{PP}} + \text{H}_2\text{O} \longrightarrow \text{Enzyme} - \overset{\beta}{\text{P}} + \overset{\gamma}{\text{P}}_i \qquad (5.27)$$

$$\text{Enzyme} - \overset{\beta}{\text{P}} + \text{Pyruvate} \longrightarrow \text{Enzyme} + \overset{\beta}{\text{P}}\text{-enolpyruvate} \qquad (5.28)$$

$$\text{Sum: Pyruvate} + \text{ATP} + \text{H}_2\text{O} \longrightarrow \overset{\beta}{\text{P}}\text{-enolpyruvate} + \text{AMP} + \text{P}_i \qquad (4.16)$$

C. Pyruvate, phosphate dikinase.

a) Non-bacterial sources. In 1967, Hatch and Slack, studying P-enolpyryvate formation in tropical grass, observed that leaf extracts possessed an enzyme similar to the PEP synthetase reported by Cooper and Kornberg(1968). Further investigation by Hatch and Slack (1968), however, indicated that the enzyme in question was not PEP synthetase but rather a new enzyme catalyzing the following reversible reaction (equation 4.17):

$$\text{Pyruvate} + \text{ATP} + \text{P}_i \longrightarrow \text{P-enolpyruvate} + \text{AMP} + \text{PP}_i \qquad (4.17)$$

In the same year, Reeves (1968) reported a similar enzyme in extracts of

the amoeba, Entamoeba histolytica. However, this enzyme was postulated to function in a manner similar to a conventional pyruvate kinase, which is absent in this amoeba, and was called, pyruvate-phosphate ligase (AMP).

b) Bacterial sources. Using the anaerobe, Bactoroides symbiosus, Reeves, Menzies and Hsu (1968) purified an enzyme similar to that isolated by Hatch and Slack (1968). The equilibrium constant for the bacterial enzyme was directly dependent upon the square of the hydrogen ion concentration. For example, at pH 7.0, the equilibrium constant was 1140 in favor of pyruvate formation, while at pH 8.0, it was only 10. These authors named the enzyme, ATP:pyruvate, phosphate diphosphotransferase, with the trival name, pyruvate, phosphate dikinase (PPD).

Evans and Wood (1968) isolated a similar enzyme from Propionibacterium shermanii with a reaction mechanism somewhat similar to that of PEP synthetase, except for the partial reaction (5.29):

$$\text{Enzyme} + \text{ATP} \longrightarrow \text{Enzyme} - \overset{\beta\gamma}{\text{P}}\text{P} + \text{AMP} \qquad (5.26)$$

$$\text{Enzyme} - \overset{\beta\gamma}{\text{P}}\text{P} + \text{P}_i \longrightarrow \text{Enzyme} - \overset{\beta}{\text{P}} + \text{PP}_i \qquad (5.29)$$

$$\text{Enzyme} - \overset{\beta}{\text{P}} + \text{pyruvate} \longrightarrow \text{Enzyme} + \overset{\beta}{\text{P}}\text{-enolpyruvate} \qquad (5.28)$$

$$\text{net: Pyruvate} + \text{ATP} + \text{P}_i \longrightarrow \overset{\beta}{\text{P}}\text{-enolpyruvate} + \text{AMP} + \text{PP}_i \qquad (4.17)$$

Hatch and Slack (1969) and Andrews and Hatch (1969) studied the purified enzyme from the leaves of maize and the plant, Amaranthus palmeri, and suggested a two-step reaction mechanism instead of the

three-step one as shown above. However, Evans and Wood (1971) substantiated their original postulation following further purification of their bacterial enzyme (300-fold).

An enzyme similar to pyruvate, phosphate dikinase was also indicated in crude extracts of Aceto. xylinum by Benziman and Eizen (1971), who suggested that this enzyme might be involved in the gluconeogenic processes of the organism.

D. Rationale for present studies on the possible interconversion of PEP and pyruvate with V. parvula M_4.

The preliminary results presented in Chapter 4 indicated that pyruvate kinase, and possibly PEP synthetase, were present in crude extracts of V. parvula M_4 (Table 4.3). However, the presence of both enzymes in the organism would suggest a paradoxical situation since the latter enzyme would facilitate the formation of P-enolpyruvate for gluconeogenesis, while pyruvate kinase would inevitably counteract this by decreasing the intracellular concentration of PEP.

In carbohydrate fermenters, such as E. coli, the pyruvate kinase reaction is used during glycolysis for ATP formation and is controlled by metabolites of energy metabolism, such as fructose-1,6-P_2 and AMP (Sanwal, 1970). However, in non-carbohydrate fermenters, such as V. parvula M_4, which degrades short chain acids for energy, the enzyme would presumably not carry the same function. In fact, one would imagine that PEP synthetase would be a more useful enzyme for this organism than pyruvate kinase.

Since both pyruvate kinase and PEP synthetase are important enzymes

to the function and control of gluconeogenesis, a more detailed investigation was undertaken to determine whether one or both of these enzymes was present in V. parvula M_4. Evidence will be given for the presence of only pyruvate kinase in the organism. The purification and characterization of the enzyme will be outlined, as well as the factors regulating its activity.

II. METHODS

A. Crude extract preparation.

Cells of V. parvula M_4 was grown anaerobically and harvested in as previously described (Ng and Hamilton, 1971). Crude extracts were obtained by sonication as described in Chapter 4.

B. Enzymatic assays.

a) Pyruvate kinase. The formation of pyruvate from PEP was assayed by four methods: (i) colorimetrically with 2,4 dinitrophenylhydrazine (DNPH), (ii) with NADH and lactic dehydrogenase in reactions stopped with ethanolic-NaOH, (iii) by the continuous recording of NADH oxidization and (iv) by the use of radioactive ATP labelled in either the α or γ position with (^{32}P). All of these assays were carried out at 37 C for specific time intervals.

(i) DNPH method. This method measured pyruvate kinase activity by estimating the amount of pyruvate formed by the 2,4 dinitrophenylhydrazine

method of Hamilton, Burris and Wilson (1965). The assay contained (in mM): PEP, 5; ADP, 5; $MgSO_4$, 10; phosphate buffer (pH 6.5), 50 and 20 mg of crude extract in a volume of 1 ml. The assay mixture was incubated for 1 hour at which time the reaction was stopped by the addition of 0.2 ml of 0.1% 2,4 dinitrophenylhydrazine in 2 N HCl. After 30 min at room temperature, NaOH was added and the contents centrifuged at 10,000 g for 15 mins at 4 C. The supernatant fraction was then read at 520nm in a Unicam SP 500 spectrophotometer along with freshly prepared pyruvate standards. When necessary the reaction mixture was diluted to approximately 10 µg per ml of pyruvate before the addition of the NADH. Enzyme activity had been previously shown to be linear during the one hour incubation period.

(ii) The NADH-ethanolic NaOH method. This method was developed to assay for pyruvate kinase activity in fractions eluted from chromatography columns, and in experiments not requiring continuous monitoring of NADH oxidation. This method involved the conversion of the pyruvate formed from PEP to lactate in the presence of commercial lactic dehydrogenase and NADH. The reaction mixture used in this method was similar to that employed in the DNPH Method, except for the addition of 10 µg lactic dehydrogenase and 5 mM NADH. Incubation was for 1 hour and the reaction was stopped by adding equal volume of 0.2 N NaOH in 50% ethanol. The mixture was then covered and incubated at 4 C for 30 min. Following centrifugation at 10,000 g for 15 min, the supernatant was read at 340 nm.

Ethanolic-NaOH was used to stop the reaction by precipitating the protein present. Furthermore, Lowry, Passonneau and Bock (1961) have shown that NADH at room temperature is very stable at high pH (12),

while NAD is completely degraded. Thus, the reaction mixture obtained after centrifugation will contain only NADH. The loss in the NADH content of the samples, compared to the zero time control, was the measure of enzyme activity.

(iii) Continuous spectrophotometric method. This method coupled the formation of pyruvate from PEP by pyruvate kinase to the reduction of pyruvate to lactate in the presence of commercial lactic dehydrogenase and NADH. The oxidation of NADH was monitored continuously at 340 nm in a recording spectrophotometer. The details of this assay have been described in Chapter 4. This method was used to study the kinetics of pyruvate kinase.

(iv) Radioactive methods involving α- and γ-labelled (^{32}P)ATP. This method was employed in PEP-pyruvate conversions to differentiate between pyruvate kinase, pyruvate, phosphate dikinase and PEP synthetase. This assay was based on the fact that pyruvate kinase produces ADP from ATP in the conversion of pyruvate to PEP, whereas both pyruvate, phosphate dikinase and PEP synthetase produce AMP instead. The initial concern in the present study was to determine whether crude extracts of V. parvula M_4 could convert pyruvate to PEP in the presence of ATP.

1) Method I ((α-^{32}P)ATP): In this method, pyruvate kinase activity, in the direction of PEP synthesis, was assayed by determining the incorporation of (^{32}P) from (α-^{32}P)ATP into ADP. The assay contained (in mM): sodium pyruvate, 20, (α-^{32}P)ATP (1.05×10^5 dpm), 20; sodium malonate, 10; sodium arsenite, 10 and 0.2 mg crude extract in 1 ml of phosphate buffer (50 mM, pH 7.0). The reaction was stopped by the addition of 20 µl of 5 N perchloric acid and the tubes immediately mixed, covered and placed

in ice-water for 30 min. Following this, samples were neutralized with 5 N NaOH, centrifuged and aliquots of the supernatant spotted on Whatman 3 MM paper. The nucleotides were isolated by two dimensional chromatography, employing first the solvent system of Krebs and Hems (1953), which consisted of isobutyric acid - 1 N ammonium hydroxide - 0.1 M EDTA (100:60:1.6). The second solvent system was isobutyric acid - 0.5 M ammonium hydroxide (5:3) (Stakiw, 1971). Each solvent system took 16-18 hours to run on 28 x 28 cm paper. The spots for ATP, ADP and AMP were located by UV light and the (^{32}P) located by radiochromatogram scanning. The radioactivity in the spots was counted by the liquid scintillation procedures previously described.

2) Method II ((γ-^{32}P)ATP): Pyruvate kinase activity was determined in this method by measuring the incorporation of (^{32}P from (γ-^{32}P)ATP into PEP in the presence of pyruvate. The (^{32}P) PEP formed was isolated and identified by paper chromatography. The reaction mixture contained (in mM): potassium pyruvate, 30; (γ-^{32}P)ATP (4 x 10^4 cpm/μmole), 30 and extract preparation in 1 ml of buffer solution. Sodium arsenate and sodium malonate (10 mM each) were added to the assay to inhibit pyruvate dehydrogenase and malate dehydrogenase activity, respectively (Ng and Hamilton, 1971). Since the pH optimum for the conversion of pyruvate to PEP was unknown, Tris-HCl buffers (50 mM) were employed between pH 6-8.

The above assays were initiated by the addition of 20 μg of crude extract to the reaction mixture equilibrated at 37 C. The reaction was stopped with 0.5 ml of 2 N NaOH, the mixture well-mixed and then incubated (covered) at 0 C for 15 min. Following this, the assay mixture was

centrifuged at 30,000 g for 10 min at 4 C and the resultant supernatant subjected to paper chromatographic analysis.

Paper chromatography was carried out by the method of Bandurski and Axelrod (1951) on Whatman 3 MM paper and run unidirectionally with methanol - ammonium: hydroxide-water (60:10:30). The chromatograms were developed for 5 - 7 hours at room temperature and always included standards for ^{32}P-ATP, PEP and inorganic phosphate. Phosphate compounds were located by spraying with the Hanes-Isherwood reagent (1949), which consisted of 5 ml of 60% perchloric acid, 25 ml of 4% ammonium molybdate, 10 ml of 1 N HCl, and 60 ml of H_2O. The phosphate spots were readily visible in UV light after drying at 85 C for 1 min and the (^{32}P) was located by scanning with a radiochromatogram scanner. The radioactive spots were cut into 2 x 4 cm strips and the radioactivity quantitated by liquid scintillation counting; counting efficiency was determined by the channels ratio method.

b) PEP synthetase and Pyruvate, phosphate dikinase. The presence of synthetase and dikinase activity in the extracts of V. parvula M_4 was assayed with (α-^{32}P)ATP as described in Method II for pyruvate kinase. Since AMP, and not ADP, are the end-products of these two enzymes (equations 4.16 and 4.17), the extent of the ^{32}P-label in AMP is a measure of their activity.

c) Enolase. The presence of enolase activity in purified pyruvate kinase preparations was assayed by determining the increased oxidation of NADH in the pyruvate kinase-lactic dehydrogenase assay system (methods ii and iii) upon the addition of 2-P-glycerate. The reaction mixture contained (in mM): 2-P-glycerate, 5; lactic dehydrogenase, 2 µg; ADP, 5; $MgSO_4$,

5 and 20 µg of the purified V. parvula enzyme preparation in 1 ml of phosphate buffer (50 mM, pH 7.0).

d) PEP carboxykinase and PEP carboxylase. These enzymes were assayed by converting the oxaloacetate formed from PEP to malate via commercial malate dehydrogenase and NADH. The reaction medium for PEP carboxykinase contained (in mM): PEP, 5; ADP, 5; NADH, 0.5; sodium bicarbonate, 5; $MgSO_4$, 5: 1.5 µg malate dehydrogenase and 20 µg of V. parvula enzyme preparation. To assay PEP carboxylase, ADP was omitted from the reaction mixture.

e) Pyruvate carboxylase. Pyruvate carboxylase was assayed by converting the oxaloacetate formed from radioactive pyruvate to malate and isolating the labelled malate by paper chromatography. The assay contained (in mM): sodium pyruvate-3-^{14}C (5.4×10^4 dpm), 5; ATP, 5; NADH, 0.5; sodium bicarbonate, 5; $MgSO_4$, 5; 5 µg commercial malate dehydrogenase and enzyme preparation in 1 ml of phosphate buffer (50 mM, pH 7.0). The reaction was stopped by adding 0.5 ml of 0.2% 2,4 dinitrophenylhydrazine in 2 N HCl. The α-keto acid hydrazone precipitate was sedimented by centrifugation at 35,000 g for 15 min at 4 C and the radioactive malate-^{14}C isolated from the supernatant by paper chromatography as previously described (Chapter 4).

f) Pyruvate dehydrogenase. The presence of pyruvate dehydrogenase activity in purified preparations of V. parvula pyruvate kinase was determined by measuring the evolution of $^{14}CO_2$ from pyruvate-1-^{14}C. The reaction mixture contained (in mM): sodium pyruvate-1-^{14}C (5.8×10^4 dpm), 5; $MgSO_4$, 5 and 20 g of isolated enzyme in 1 ml of phosphate buffer (50 mM, pH 7.0). The assay was carried out in a centrifuged tube containing a tight fitting serum stopper through which was suspended a polyethylene

cup. The reaction was allowed to proceed at 37 C for 30 min and the reaction stopped by the addition of 0.2 ml of 4 N HCl to the tube by syringe. Following an additional 30 min incubation period, 0.2 ml of 1 N hyamine hydroxide was added by syringe to the polyethylene cup to absorb any $^{14}CO_2$ produced in the reaction. The tube was again incubated for 30 min, the contents of the cup then washed into a counting vial with 3 ml methanol and 10 ml of scintillation liquid and the radioactivity determined.

C. Chromatographic procedures.

Sephadex columns (G-200 and G-100) were used to purify pyruvate kinase from extracts of V. parvula M_4 and were prepared according to the techniques outlined by the Pharmacia Company. The swollen gels (Fine grade) were packed into a 5 x 100 cm column with the operating pressure maintained at 10% of the gel height. The operation of all columns was carried out at 4 C and the void volume of each column determined with 1 ml of 0.2% Blue dextran. The column was connected to a UV monitor and fraction collecter as shown in Fig. 5.13. All samples were applied through the sample tube (A) at the bottom of the column by clamping off the Mariotte bottle and operating the pump. The pumping rate was set at the flow rate obtained with the normal operating pressure (P = 6 ml/hr). When the sample had been pumped into the column, the sample tube was washed twice with 5-6 mls of phosphate buffer (50 mM, pH 7.0), the sample tube clamped off and buffer from the Mariotte bottle was allowed to flow into the column. The pump was operated until the sample was in the gel matrix and then shut off. From this point, the column was operated with

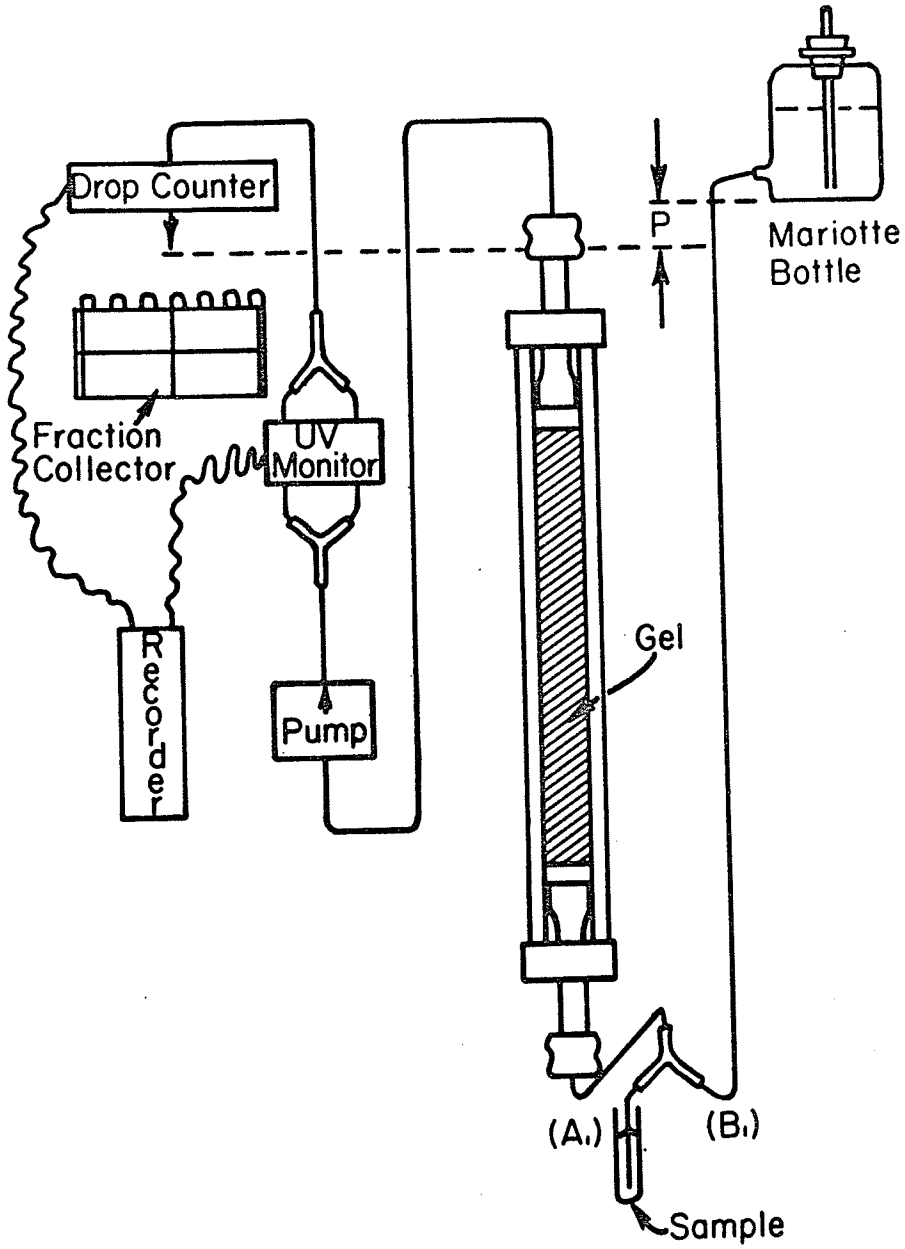


Fig. 5.13. Diagram of the apparatus employed to column chromatography during the purification of the pyruvate kinase from V. parvula M_4.

the pressure head between the Mariotte bottle and the tubing outlet over the drop counter. The elution of protein from the column was monitored by dual channel UV monitor (ISCO, Lincoln, Neb.) set at 280 and 254 nm, respectively. The fractions (3 ml) containing enzyme activity were pooled and concentrated by the "Diaflo" ultrafiltration method (Model 52, Amicon Ultrafiltration, Lexington, Ky.).

D. Acrylamide disc gel electrophoresis.

Acrylamide gel electrophoresis was carried by the procedures outlined by Davis and Ornstein (1964). The gels were stained for 1 hour in a solution of 1% Amido Swartz stain in 7% acetic acid.

E. Analyses.

Protein was assayed by the methods of Lowry et al (1951) and Layne (1951). Lowry's method was employed with samples devoid of β-mercaptoethanol because this reducing agent is known to interfere with color development. The spectrophotometric method of Layne was used with samples containing the reducing agent since interference by this compound could be eliminated by its presence in the reference cuvette at the same concentration as that present in the sample.

F. Data processing.

The data presented in this chapter are mean values obtained from 3-4 separate experiments.

G. Materials.

The Sephadex column materials were purchased from Pharmacia (Montreal). (α-^{32}P)ATP and (γ-^{32}P)ATP were obtained from New England Nuclear (Canada) Ltd.(Montreal).

III. RESULTS

Part A - Crude Extract Studies

a) Metabolism of (α-^{32}P)ATP. Since the spectrophotometric data in Chapter 4 (Table 4.3) had suggested that crude extracts of V. parvula contained both pyruvate kinase and PEP synthetase, additional experiments were undertaken to substantiate the presence of these enzymes in this organism. Crude dialyzed extracts were incubated with pyruvate and (α-^{32}P)ATP, and the nucleotides formed in the assay isolated and counted. The presence of (^{32}P) in ADP would indicate pyruvate kinase activity, while (^{32}P) in AMP would suggest the presence of the synthetase or pyruvate, phosphate dikinase activity. As shown in Fig. 5.14, (^{32}P)ADP was rapidly formed by the dialyzed extracts in the presence of pyruvate and (α-^{32}P)ATP. The production of (α-^{32}P)ADP reached a maximum in 10 min and then declined. During this period, a small but gradual amount of (^{32}P)AMP was being produced from the (α-^{32}P)ATP. While the small amount of (^{32}P)AMP formation might suggest low PEP synthetase or dikinase activity in the extracts, the decline in the observed (^{32}P)ADP concentration between 10-20 min suggested that adenylate kinase was present in the preparations. This latter activity could account for the formation of the (^{32}P)AMP.

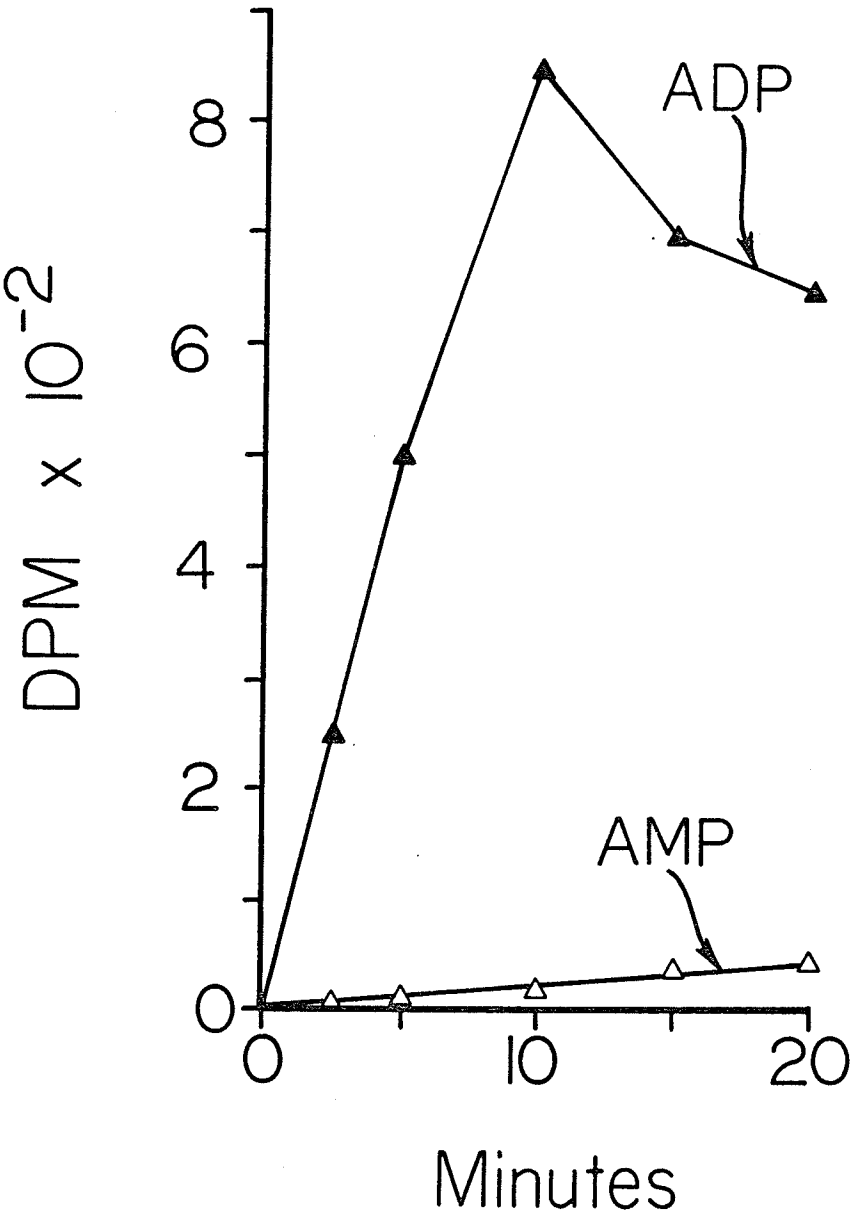


Fig. 5.14. Incorporation of ^{32}P from (α-^{32}P) ATP into ADP and AMP by crude extracts of V. parvula M_4 incubated with unlabelled pyruvate.

b) Metabolism of (γ-^{32}P)ATP.

The presence of pyruvate kinase in crude extracts of V. parvula was further confirmed by the use of (γ-^{32}P)ATP and unlabelled pyruvate. Since pyruvate kinase will incorporate the γ-phosphate of ATP into PEP, incubations with (γ-^{32}P)ATP and pyruvate will result in the formation of (^{32}P) P-enolpyruvate. As shown in Fig. 5.15(A), the total utilization of (γ-^{32}P)ATP by the crude extracts was optimal at pH 7.0 with linear rate for 30 min. One can see also that the formation (^{32}P)PEP was optimal at the same pH (Fig. 5.15 (B)). By quantitating the distribution of (^{32}P), it can be seen that much more (γ-^{32}P)ATP was utilized than (^{32}P)PEP formed (Table 5.10). At the pH optimum, approximately 17% of the (^{32}P)ATP was converted to PEP by the crude extracts during the 60 min incubation period.

c) pH optimum for pyruvate kinase. The above data, together with that presented previously, indicates that an active pyruvate kinase is present in V. parvula M_4. Since this enzyme has not been observed in V. alcalescens (Michaud and Delwiche, 1970), and since its presence in a non-glycolytic organism such as V. parvula M_4 raises questions as to its regulation during gluconeogenesis, purification of the enzyme was undertaken. However, to facilitate the purification process, the optimal pH for pyruvate kinase activity in the direction of pyruvate formation was investigated with crude extracts. Two assay methods were used for this purpose: (1) the 2,4 dinitrophenylhydrazine method and (2) the lactic dehydrogenase-NADH-ethanolic NaOH method. As demonstrated in Fig. 5.16(A), pyruvate formation from PEP was optimal at pH 7.0 when measured by the enzymatic assay. The same results were obtained whether phthalate, Tris-HCl or phosphate buffers were employed. This similarity in activity between the non-phosphate and

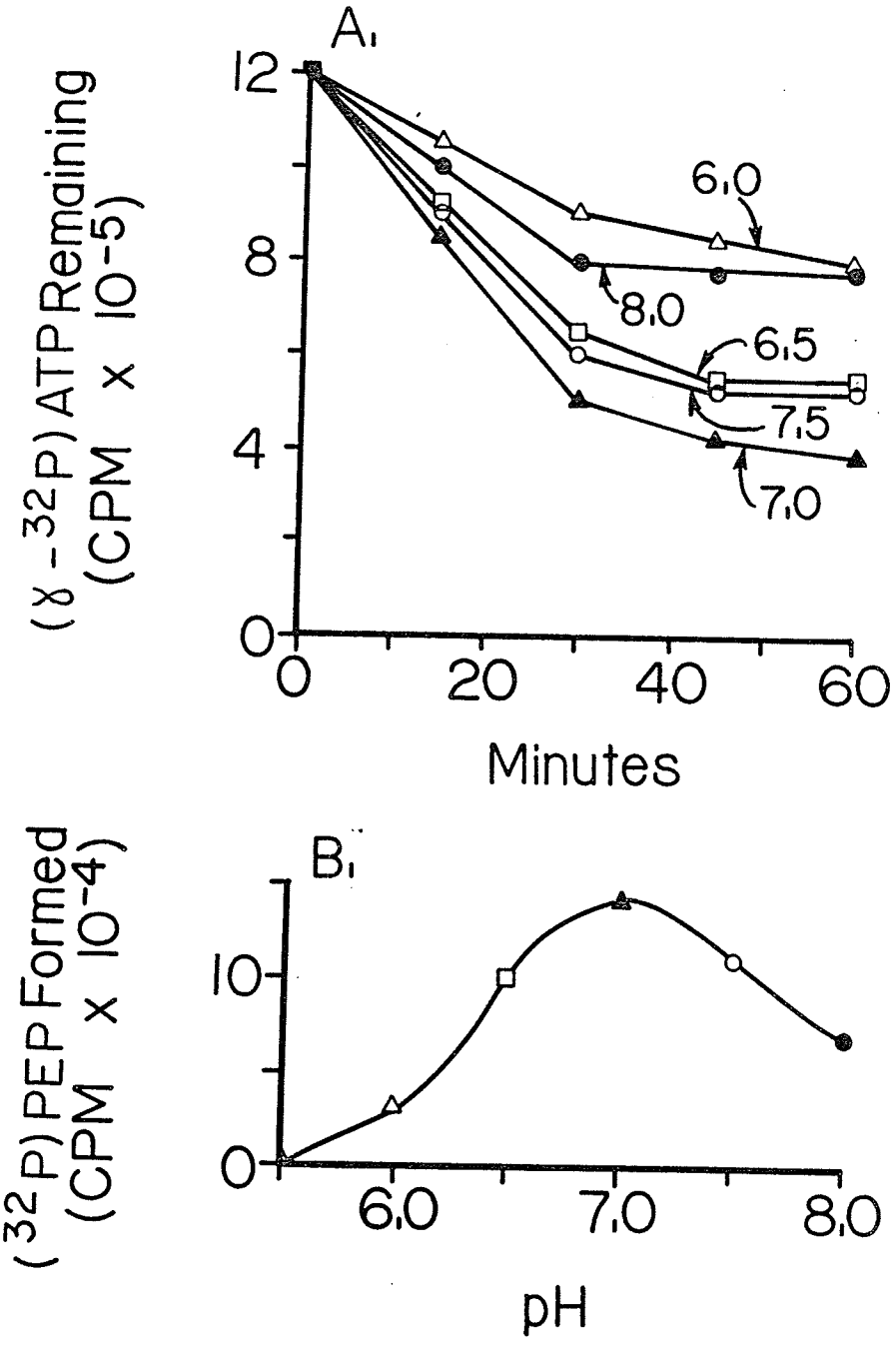


Fig. 5.15. Effect of pH on the metabolism of (γ-^{32}P) ATP and the production of (^{32}P) P-enolpyruvate by crude dialyzed extracts of V. parvula M_4 (A) Total utilization of (γ-^{32}P) ATP. (B) (^{32}P) PEP formation.

TABLE 5.10

Comparison between the utilization of (γ-^{32}P) ATP and the production of (^{32}P) PEP by crude dialyzed extracts of *V. parvula* M_4[a].

pH	(γ-^{32}P) ATP utilized	(^{32}P) PEP formed	Percentage of total
6.0	40.0[b]	3.0[b]	7.5
6.5	65.1	10.0	15.1
7.0	83.0	14.3	17.2
7.5	67.0	11.1	16.4
8.0	42.0	8.0	19.1

[a] Incubation was for 60 min.

[b] cpm x 10^{-4}.

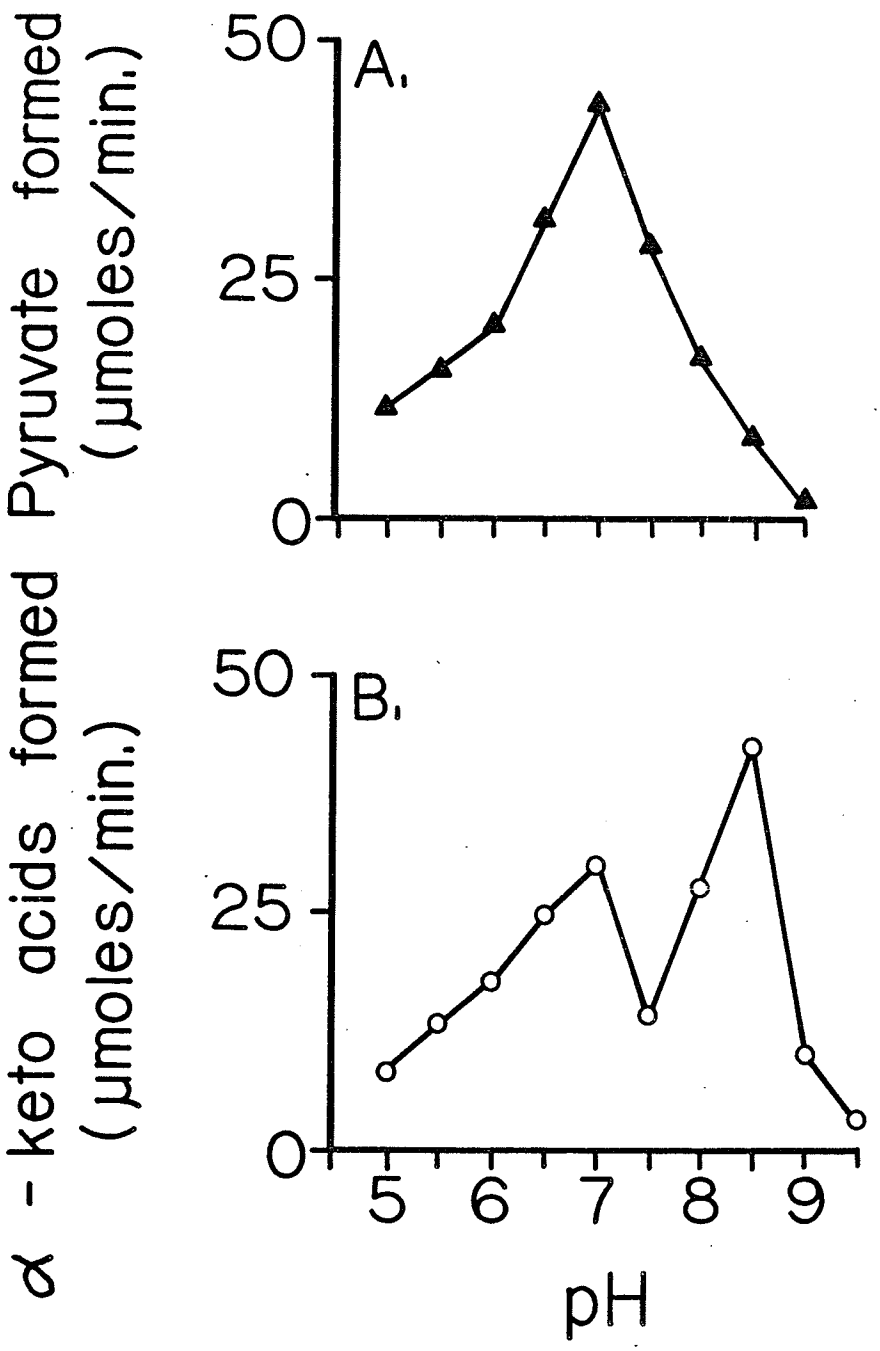


Fig. 5.16. Effect of pH on the production of pyruvate from PEP as assayed by (A) the lactic dehydrogenase and (B) the 2,4 dinitrophenylhydrazine methods.

phosphate buffers indicated that P_i played no part in the conversion of PEP to pyruvate. These results also show that the pH optimum for pyruvate formation was the same as that for the reverse reaction (Fig. 5.15).

The results obtained with the hydrazine method, unlike that shown in Fig. 5.16(A), indicated two peaks of activity (7.0 and 8.5) for the conversion of PEP to pyruvate (Fig. 5.16(B)). Since the DNPH assay is specific for all α-keto acids, these results probably indicate that, in addition to pyruvate, oxaloacetate was being formed from PEP in the assay. The fact that crude extracts appear to possess activity for PEP carboxykinase (Chap. 4) supports this suggestion. As the reaction mixtures in the assays were not enclosed, sufficient dissolved CO_2 may have been present to permit the reaction to function. Because of the non-specific nature of the hydrazone method, it was used in future assays only with purified enzyme preparations.

d) Enzyme stability.

Crude extracts of V. parvula obtained by sonic disruption were dark reddish-brown in colour, which, on storage in the absence of a reducing agent rapidly faded to a light pink colour. This loss of reddish-brown colour correlated with the loss of pyruvate kinase activity, as well as the activity for PEP carboxylase, PEP carboxykinase, pyruvate dehydrogenase, pyruvate carboxylase and malate dehydrogenase. However, if the extracts were stored under nitrogen at 4 C in phosphate buffer (50 mM, pH 6.5) with 20 mM β-mercaptoethanol, at a minimum concentration of 50 mg/ml, activity was preserved for at least two months (Table 5.11). It can be seen that only 5.1% of the original pyruvate kinase activity in the crude extracts was lost in 8 weeks under these conditions. However,

TABLE 5.11

Effect of storage under nitrogen in a reduced buffer on pyruvate kinase activity in the crude extracts of V. parvula M_4[a].

Time (weeks)	Enzyme activity	Percent loss of activity[b]
0	5.7	0
1	5.7	0.6
2	5.7	1.4
3	5.6	1.8
4	5.6	2.3
6	5.5	4.1
8	5.4	5.1

a Extracts were stored in phosphate buffer (50 mM, pH 6.5) containing 20 mM mercaptoethanol.

b Per cent loss compared to the freshly prepared crude extract.

c µmoles of NADH oxidized/mg protein/min. Enzyme activity was assayed by the continuous recording method.

freezing and thawing always resulted in a rapid decrease in enzyme activity.

Part B - Purification of Pyruvate Kinase

a) Crude extracts. Purification of pyruvate kinase from V. parvula M_4 was undertaken with cells harvested in the exponential phase of growth and washed with phosphate buffer (50 mM, pH 6.5) containing 20 mM mercaptoethanol. All suspensions (70-25 mg dry weight per ml) were disrupted anaerobically in a Branson Sonifier for 20 min and the supernatant, obtained after centrifugation at 45,000 g for 20 min, dialyzed overnight at 4 C against 2 L of the above buffer. The dialyzed extracts were then concentrated to 5 ml by the Diaflo ultrafiltration process using a PM 30 filter with pure nitrogen gas (65 p.s.i.). The concentrated extract was then made to 50-100 mg/ml with 100 mM phosphate buffer (pH 6.5) containing 20 mM β-mercaptoethanol and stored at 4 C under nitrogen until used.

b) Protamine sulphate treatment. Nucleic acids present in the crude extract were precipitated by the addition of 2.0% (w/v) solid protamine sulphate to the cell-free preparation. Following mixing for 30 min at 4 C, the suspension was centrifuged at 35,000 g for 20 min and the supernatant further purified. Attempts were made to eliminate DNA by incubating the extract with crystalline DNAse (final concentration, 1 µg/ml) for 1 hour at 37 C, however, pyruvate kinase activity was lost during this procedure. Protamine sulphate was used in subsequent purification processes. As can be seen in Table 5.13, this procedure also removed about 40% of the total protein, while retaining 86% of the total

activity.

c) Ammonium sulphate fractionation. The supernatant obtained following the protamine sulphate treatment was subjected to ammonium sulphate fractionation. In order to determine the appropriate amount of ammonium sulphate to use, 10% increments of the salt were added to the enzyme preparation, and the pyruvate kinase activity and protein concentration in precipitate determined. Before the enzyme assay, however, each precipitate, dissolved in minimal amount Tris buffer (50 mM, pH 7.5), was dialyzed against 2 L of the same buffer to which had been added 20 mM mercaptoethanol. As shown in Table 5.12, the largest amount of protein was precipitated at 30%, while the major part of the pyruvate kinase was observed in the 10-20% fraction. Since more than 80% of the pyruvate kinase activity was precipitated between 10 and 30% ammonium sulphate while removing 50% of the total proteins, this salt concentration was used during purification. This procedure resulted in almost a 2-fold increase in specific activity (Table 5.13).

d) Sephadex G-200 chromatography. Following ammonium sulphate fractionation, the 10-30% fraction was subjected to column (50 x 100 cm) chromatography on Sephadex G-200; the protein was eluted from the column with 50 mM Tris-HCl (pH 7.0) buffer. As shown in Fig. 5.17, following the elution of the void volume, which was not collected, the protein was eluted from the column in four major peaks. The pyruvate kinase activity was found in the second peak which merged with the protein in the third peak. The fractions in the second peak were pooled according to the presence of enzyme activity and concentrated by the Diaflo procedure. At this stage, the enzyme had been purified 60-fold while retaining 65%

TABLE 5.12

Precipitation of pyruvate kinase activity from protamine sulphate-treated extracts of V. parvula M_4 by various concentrations of ammonium sulphate.

$(NH_4)_2SO_4$ (%)	Protein concentration (mg)	Specific activity	Total activity
0	5.5	8.0[a]	44
10	0.9	6.0	5.4
20	1.2	22.4	26.9
30	1.5	3.9	5.8
40	0.9	0.1	0.1
50	0.7	-	-

[a] µmoles NADH oxidized/mg protein/min.

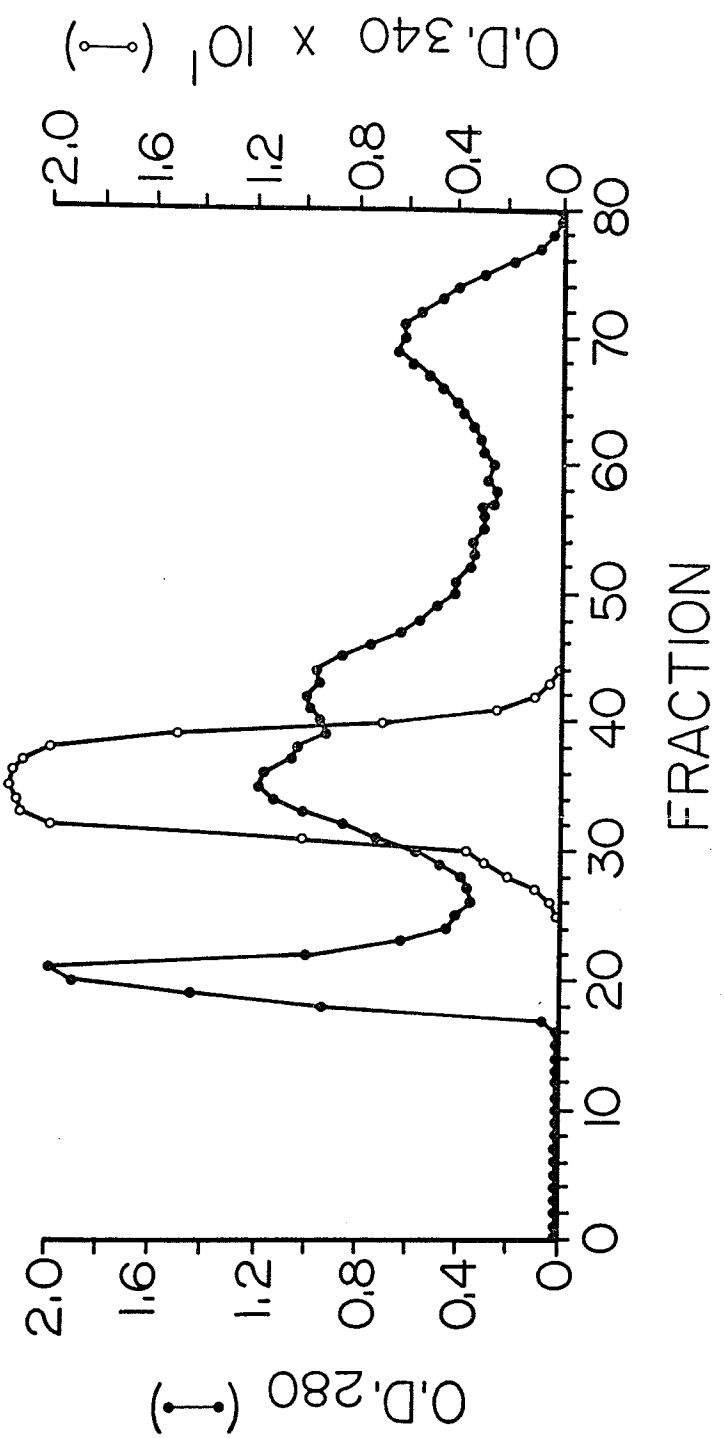


Fig. 5.17. Profile of the gel filtration of the partially purified pyruvate kinase from V. parvula M4 on Sephadex G-200. Void volume had been eluted prior to collection of fractions. Assay was by the NADH-LDH ethanolic-NaOH method.

of the total enzyme activity (Table 5.13).

e) Sephadex G-100 chromatography. Further purification of the enzyme was carried out by Sephadex G-100 column chromatography. A smaller column (1.5 x 90 cm) was used for this purpose and the protein eluted with 50 mM phosphate buffer (pH 6.5). As demonstrated in Fig. 5.18, the protein in the pooled G-200 fractions was separated into three peaks on G-100 with the kinase activity appearing in the first peak, which was eluted immediately after the void volume. No protein was observed in the void volume. The fractions from the first peak were pooled and concentrated as previously described. Pyruvate kinase activity in this pooled, concentrated fraction had been purified 126-fold (Table 5.13). This fraction will be known as the purified enzyme.

The elution of the pyruvate kinase activity from the Sephadex G-100 column immediately after the void volume suggested that the molecular weight of the enzyme was about 150,000.

f) Disc gel electrophoresis. Polyacrylamide disc gel electrophoresis was carried out on all fractions obtained during the purification process, except the Sephadex G-200 fraction. Fig. 5.19 shows the four gel patterns obtained with the crude extract, protamine sulphate, ammonium sulphate and Sephadex G-100 fractions. It can be seen that the preparation obtained following Sephadex G-100 column chromatography contains only one major protein component.

g) Absence of other enzymes in the purified pyruvate kinase preparation. Before studying the kinetics and characteristics of the V. parvula M_4 purified enzyme, and despite the apparent purity of the preparation as seen by disc gel electrophoresis (Fig. 5.19), assays were carried out to

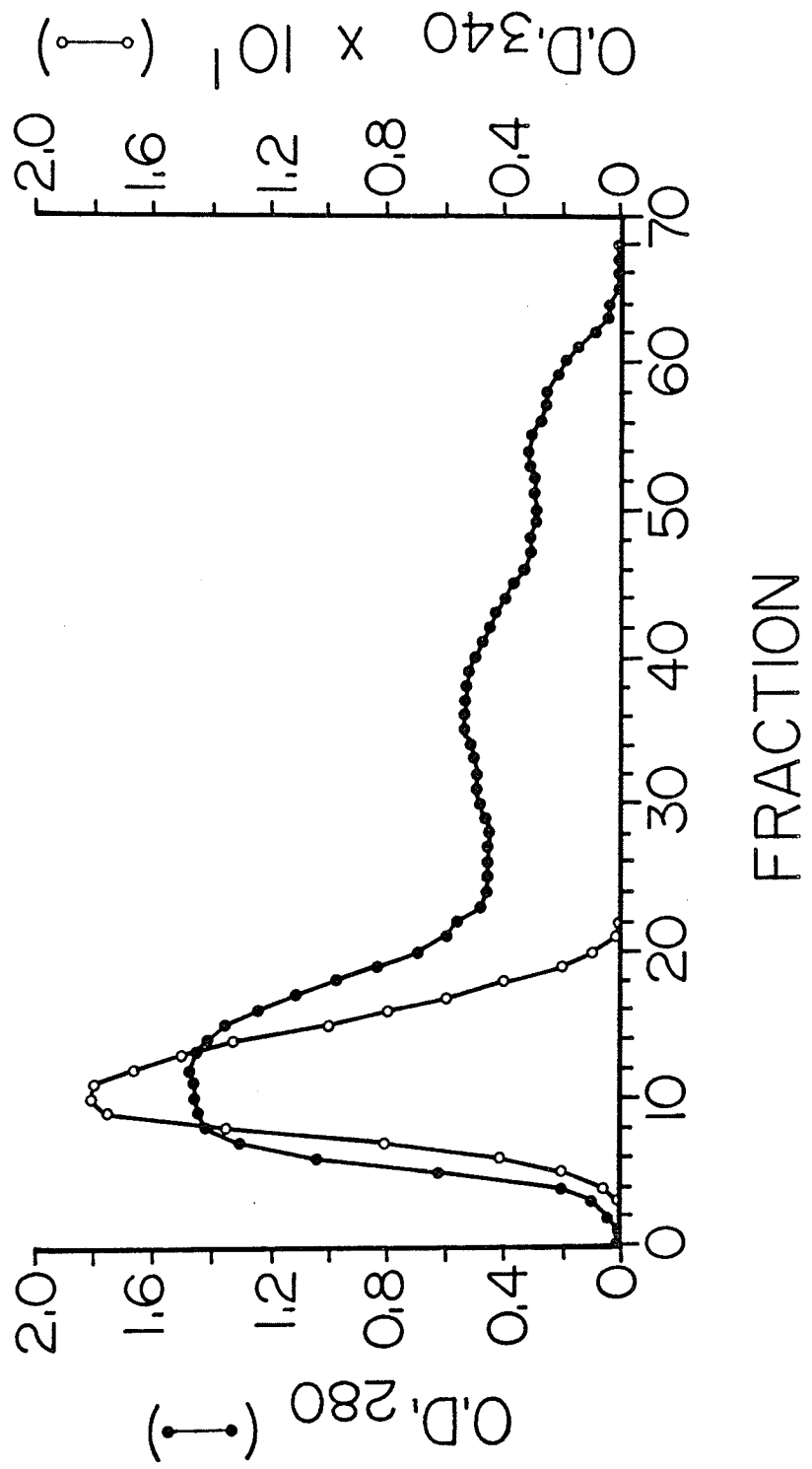


Fig. 5.18. Profile of the gel filtration of the partially purified pyruvate kinase from V. parvula M_4 on Sephadex G-100. Void volume was not collected. Assayed as in Fig. 5.17.

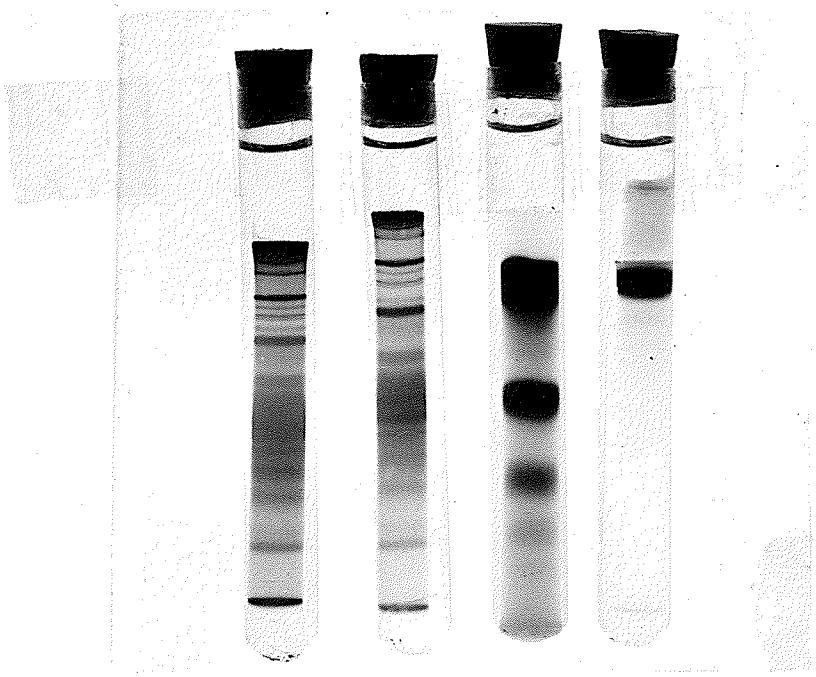
TABLE 5.13

Purification of pyruvate kinase from sonically prepared extracts of *V. parvula* M_4.

Step	Protein (mg)	Specific activity	Fold purification	Total activity
				($x10^{-3}$)
1. Crude extracts (40,000 xg)	12807	5.7[a]	1	73
2. Protamine sulphate	7758	8.0	1.4	63
3. Ammonium sulphate (10-30%)	3495	13.8	2.4	48
4. Sephadex G-200	139	344	60	48
5. Sephadex G-100	70	720	126	50

[a] µmoles NADH oxidized/mg protein

Fig. 5.19. Disc gel electrophoresis profiles of the (1) crude, (2) protamine sulphate, (3) ammonium sulphate and (4) Sephadex G-100 fractions, obtained during the purification of pyruvate kinase from V. parvula M_4.



I II III IV

TABLE 5.14

Activity of various enzymes, associated with pyruvate or P-enolpyruvate metabolism, in the purified *V. parvula* M_4 pyruvate kinase preparation[a].

Enzyme assayed	Enzyme activity
Pyruvate kinase	720[b]
PEP-synthetase	0
Pyruvate, phosphate dikinase	0
Enolase	0.3
PEP carboxylase	0
PEP carboxykinase	0.1
ATP-dependent pyruvate carboxylase	0.1
ATP-independent pyruvate carboxylase	0
Pyruvate dehydrogenase	0.01

[a] All assays contained 1 mg of the 126-fold purified enzyme.

[b] µmoles NADH oxidized/mg protein/min.

determine whether contaminating or competing enzymes were present. Table 5.14 shows that negligible activity was observed in the purified pyruvate kinase preparation for 8 enzymes associated with pyruvate or P-enolpyruvate metabolism.

Part C - Characteristics of the Purified Pyruvate Kinase

a) pH optimum.

(i) "Downward" direction. The activity of the purified V. parvula M_4 pyruvate kinase was tested in the direction of pyruvate and ATP formation at pH values between 5.0 and 8.5. Fig. 5.20(A) shows that maximum activity was observed at pH 7.0 confirming the early data obtained with crude extracts (Fig. 5.15 and 5.16). By plotting log against pH, one could obtain the PK's of the ionizing groups in the ES complex i.e., the groups which either form part of the active centre of the enzyme or were closely associated with it (Massey and Alberty, 1954). Such a plot shows that for the reaction converting PEP to pyruvate, the enzyme-PEP complex had pK's of 6.2 and 7.4, respectively (Fig. 5.20(B)).

(ii) "Upward" direction. Fig. 5.21(A) shows the effect of pH on the activity of pyruvate kinase in the direction of PEP formation at pH values between 5.5 and 9.0. The optimum pH was 8.0 with a rate of 5 µmoles PEP formed/mg protein/min. This optimum is one pH unit higher than that observed with crude extracts for the same reaction (Fig. 5.10). By plotting the logarithm of the velocity against pH, the pK values for the charged groups in the enzyme catalyzing the upward direction were shown to be 7.5 and 8.5 (Fig. 5.21(B)).

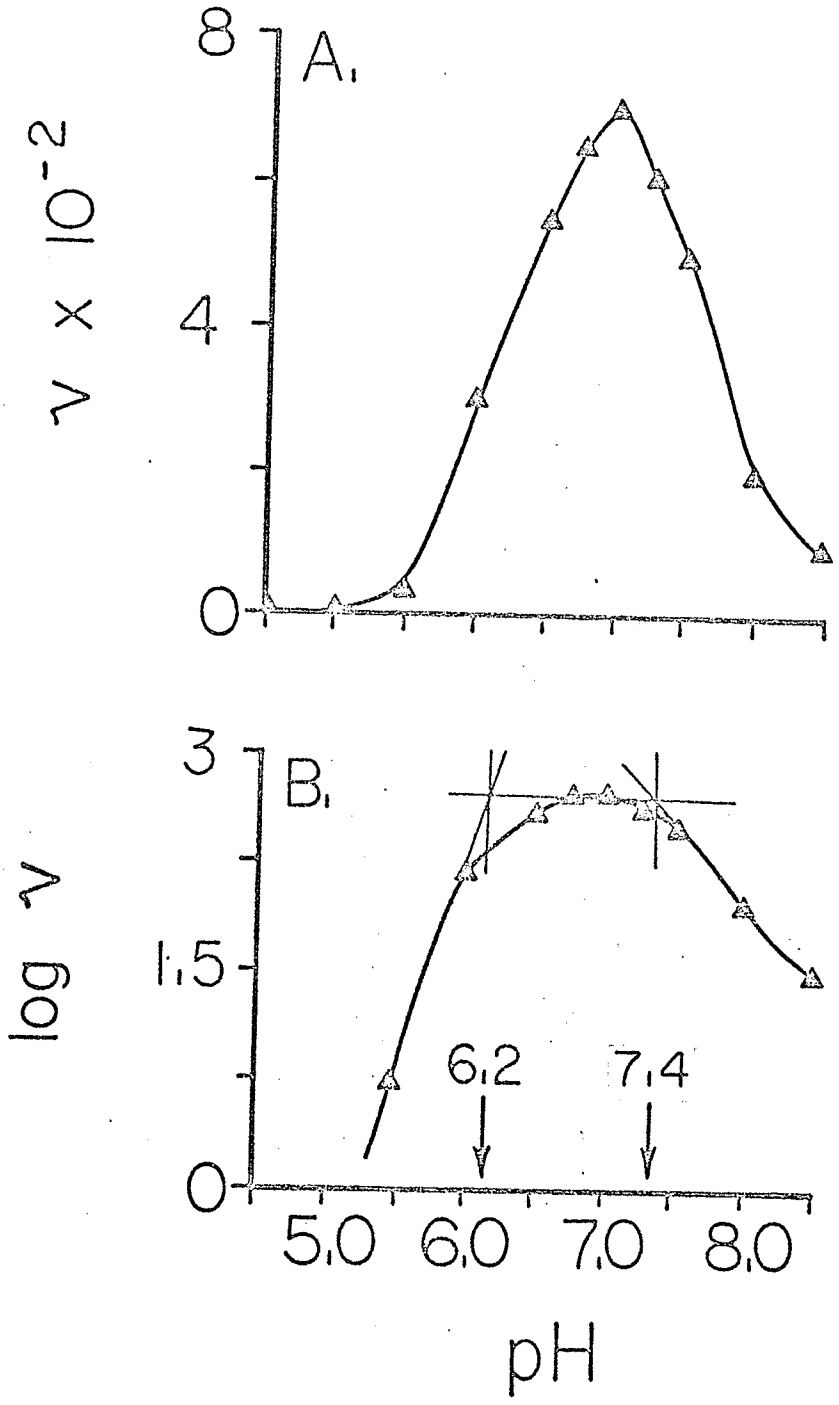


Fig. 5.20. The effect of pH on the activity of the purified pyruvate kinase of V. parvula M_4 in the direction of pyruvate and ATP formation. Assayed as in Fig. 5.17.

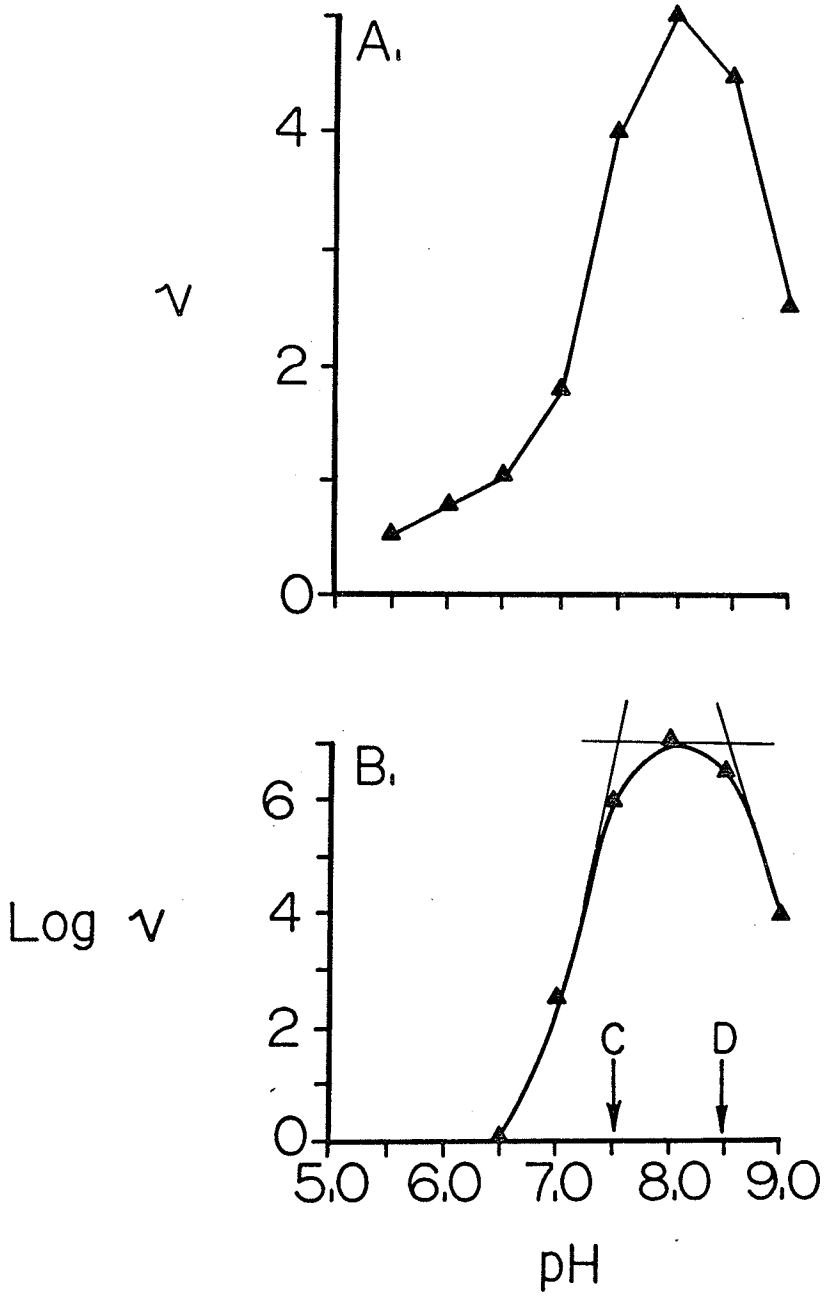


Fig. 5.21. The effect of pH on the activity of the purified pyruvate kinase of V. parvula M_4 in the direction of PEP and ADP formation. Assayed as in Fig. 5.17.

b) Effect of time and enzyme concentration. Following the establishment of the optimal pH enzyme activity, the effect of time and enzyme concentration on the conversion of PEP to pyruvate was studied. At pH 7.0 with 4 mM PEP, the rate of PEP utilization was linear for at least 50 min and stopped only when the substrate became exhausted (Fig. 5.22(B)). It can be seen that linear kinetics were obtained with enzyme concentrations up to 200 µg (Fig. 5.22(B)).

c) Substrate concentration. The activity of the purified pyruvate kinase was assayed at PEP concentrations as high as 10 mM. By plotting the initial rate (v) against the PEP concentration, a sigmoidal curve was obtained indicating that the V. parvula M_4 enzyme is a allosteric protein (Fig. 5.23). For reasons unknown, the activity curve of the enzyme could not be extrapolated to zero in the absence of PEP. The maximum velocity (V_{max}) of the enzyme (710 units) was observed at PEP concentrations between 2 and 4 mM; at higher concentrations progressive substrate inhibition was observed. This inhibition limited the range of substrate concentrations that could be used in kinetic studies. For most of these experiments, 4 mM PEP was employed with 20 µg of the purified enzyme.

Since a sigmoidal plot was also obtained with the Lineweaver-Burk double reciprocal plot (Fig. 5.23(B)), it was not possible to obtain an accurate Michaelis-Menten constant (K_m) for the enzyme. However, by observation of the v vs (PEP) curve in Fig. 5.23(A), a K_m value of 1.2 mM can be obtained for PEP.

d) ADP concentration. Since ADP is one of the substrates for the enzyme, the activity of the enzyme was tested with 4 mM PEP at various concentrations of ADP. Like those for P-enolpyruvate, the ADP concentration curves

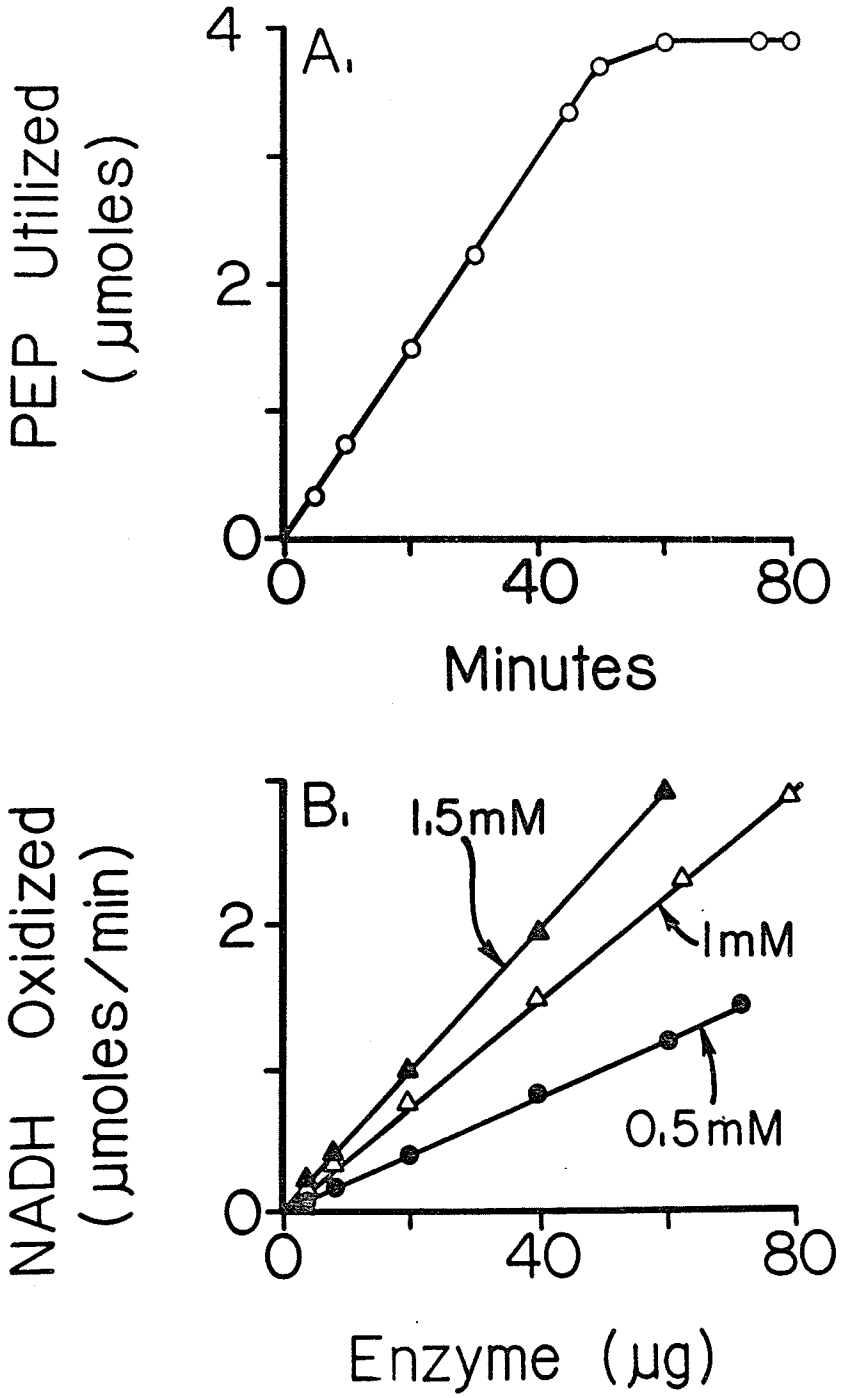


Fig. 5.22. Effect of (A) time and (B) enzyme concentration on the activity of the purified pyruvate kinase of V. parvula M_4 when assayed with PEP and ADP. Assay was by the NADH-LDH continuous method.

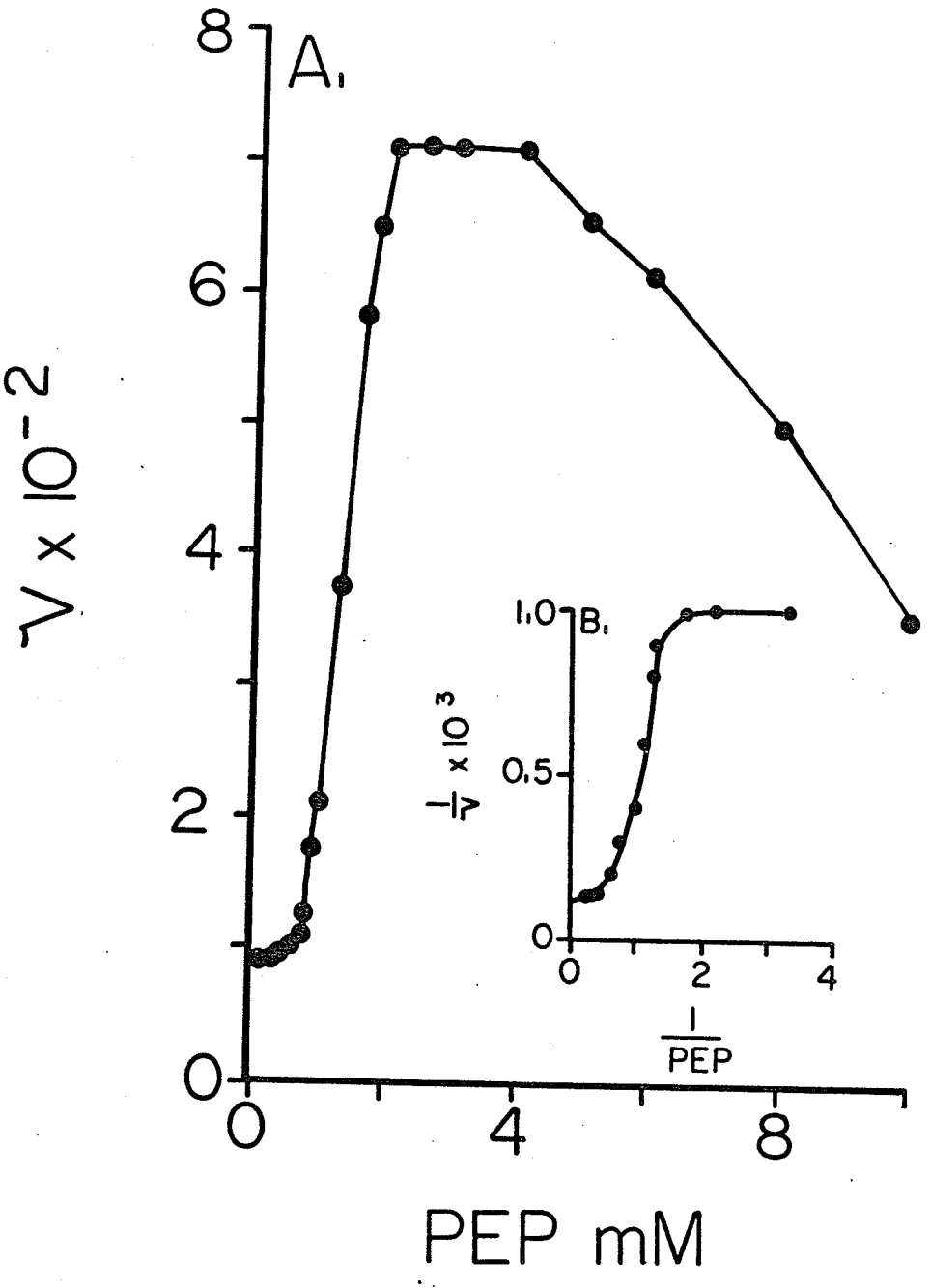


Fig. 5.23. The effect of PEP concentration on the activity of the purified pyruvate kinase from V. parvula M_4 in the presence of ADP and Mg^{2+}. The assay was as in Fig. 5.22.

were also sigmoidal (Fig. 5.24 (A) and (B) and could not be extrapolated to zero in the absence of ADP. The observed maximum velocity was 910 units of ADP concentrations between 3.5 and 4.0 mM, while the apparent K_m for ADP was 3.5 mM.

e) Magnesium concentration. Lohmann and Meyerhof (1934), investigated the pyruvate kinase in rabbit muscle, were the first to observe that Mg^{2+} ions were essential for enzyme activity. Hence, the effect of various concentrations of magnesium on the activity of the purified pyruvate kinase from V. parvula M_4 was tested with the standard LDH-NADH continuous assay system. As was the case for PEP and ADP, increasing concentrations of magnesium produced sigmoid kinetics (Fig. 5.25(A)), although the sigmoidal nature of the curve was not as pronounced as that for PEP and ADP. The V_{max} (830 units) occurred at Mg^{2+} concentrations between 6 and 10 mM, with slight inhibition occurring at concentrations above 10 mM.

From the above results, the optimum conditions for the purified V. parvula pyruvate kinase were with 4 mM PEP, 4 mM ADP and 6 mM Mg^{2+} in 50 mM phosphate buffer (pH 7.0).

f) Effect of temperature. The enzymic studies to this point were carried out at 37 C. To determine the optimum temperature for enzyme activity, the initial velocity (in the direction of pyruvate formation) was determined between 0 - 50 C. As seen in Fig. 5.26(A) the activity increased with increasing temperatures to 35 C. By plotting the logarithm of the initial velocity against the reciprocal of the absolute temperature (Stearn, 1949), it was possible to obtain the energy of activation (E) and also the temperature at which the (E) value changed (Fig. 5.26(B)). The plot shows that 32 C was a transitional temperature when the value of E changed. At

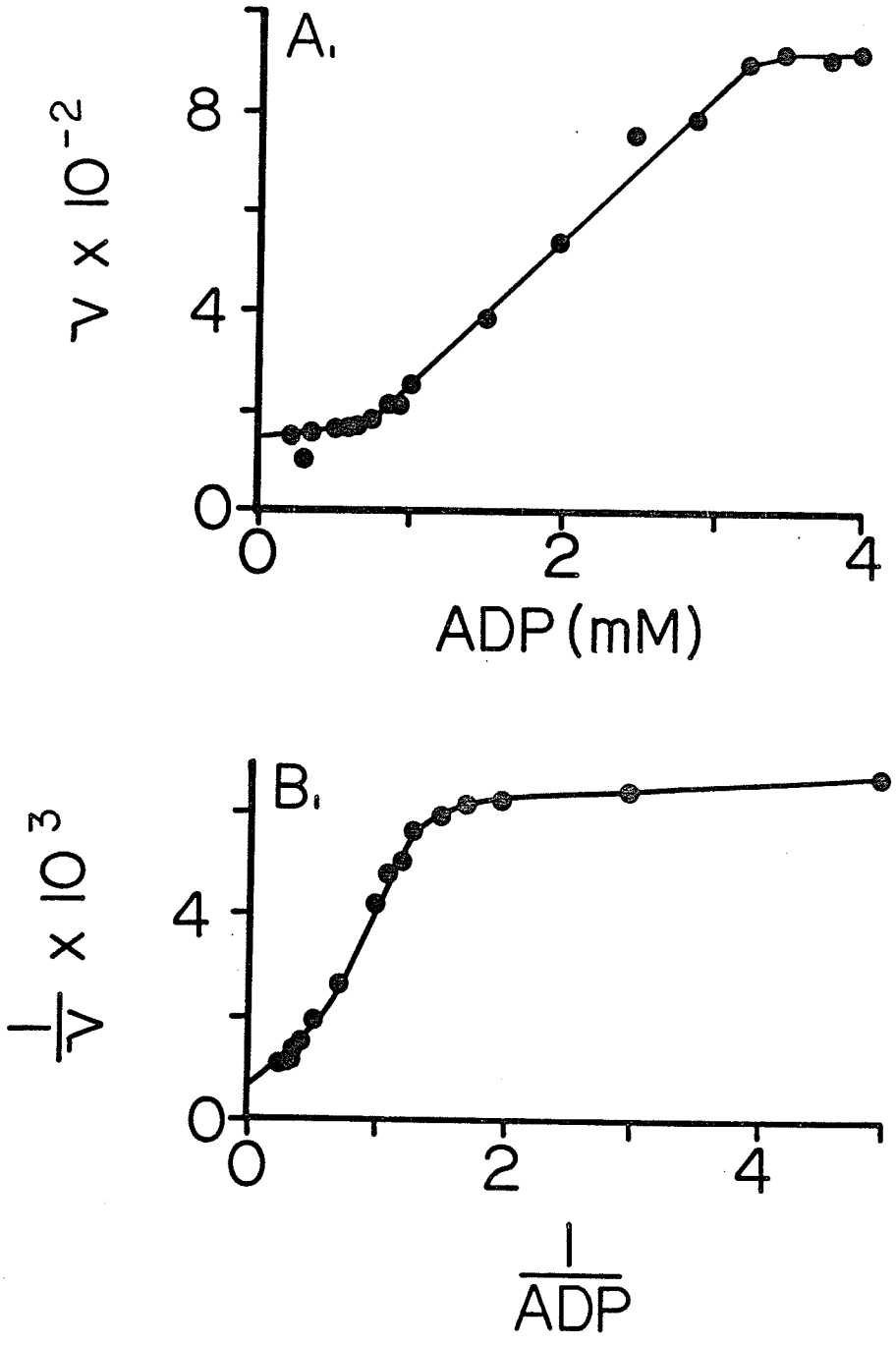


Fig. 5.24. The effect of ADP concentration on the activity of the purified pyruvate kinase from V. parvula M_4 in the presence of PEP and Mg^{2+}. The assay was as in Fig. 5.22.

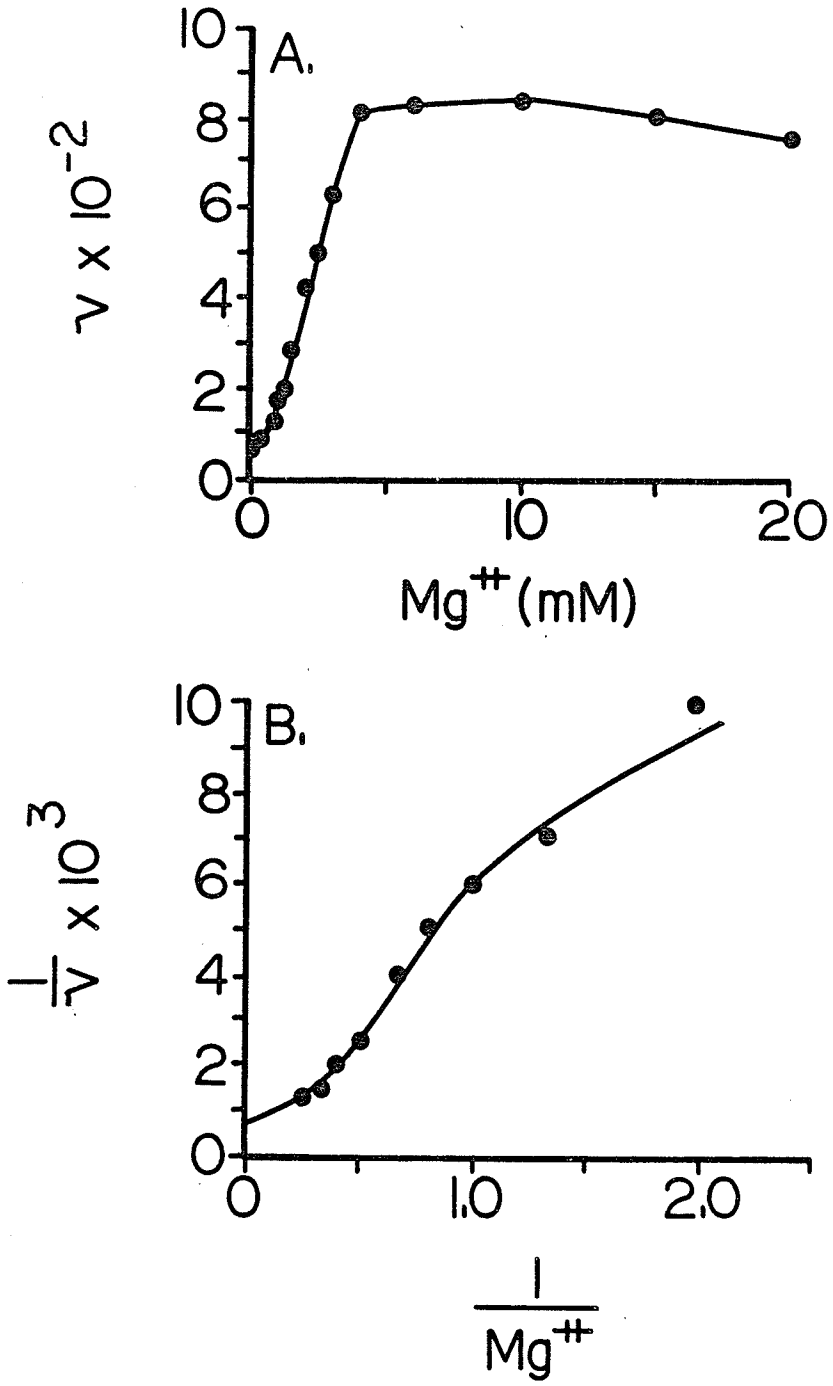


Fig. 5.25. The effect of magnesium concentration on the activity of the purified pyruvate kinase from V. parvula M_4 in the presence of optimal concentrations of PEP and ADP. The assay was as in Fig. 5.22.

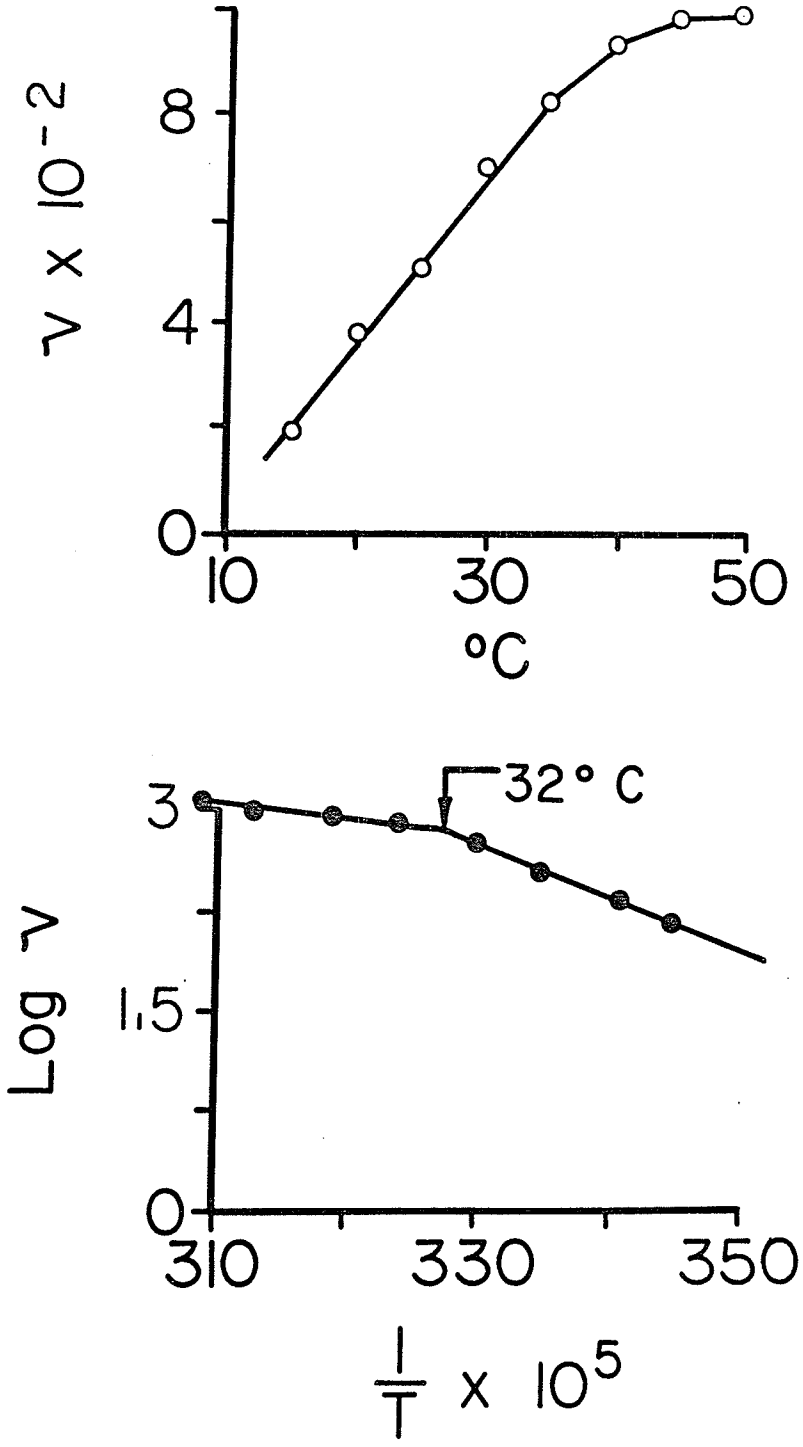


Fig. 5.26. The effect of temperature on the activity of the purified pyruvate kinase from V. parvula M_4. The assay was as in Fig. 5.22.

temperatures lower than 32 C, the energy of activation was 9,495 calories, while at higher temperatures E decreased to 3,652 calories. Subsequent kinetic studies were carried out at 33 C.

g) Hill plots for PEP, ADP and Mg^{2+}. The aforementioned results indicated that the purified pyruvate kinase from V. parvula M_4 was an allosteric enzyme. To study the co-operativity of the interacting sites for PEP, ADP and Mg^{2+}, Hill plots (Brown and Hill, 1923) were made (Fig. 5.27).

The Hill coefficient (n) for PEP was 4, while a value of 2 was obtained for both ADP and Mg^{2+}. The positive nature of these Hill coefficients suggested that there was a high degree of co-operativity with the enzyme possessing at least 4 interacting sites for PEP but only 2 for ADP and Mg^{2+}. It can also be seen that on the negative side of the substrate axis, the Hill coefficients for all three reactants changed to value of one.

h) Equilibrium constant for the pyruvate kinase reaction. The equilibrium constant (Keq) of the pyruvate kinase reaction was determined in two directions: (i) 'upward' from pyruvate to PEP and (ii) 'downward' from PEP to pyruvate. The downward reaction was carried out in pH 7.0 phosphate buffer, (50 mM), while the upward reaction was carried out at pH 8.0. Table 5.15 shows the concentrations of the reactants and products at equilibirium. The Keq calculated from the experimental data for the conversion of PEP to pyruvate ('downward') was 828, while with pyruvate and ATP in the 'upward' direction, a Keq of 1.21×10^{-3} was obtained, the reciprocal of which is 827. As expected, pyruvate and ATP formation from PEP and ADP was greatly favoured giving a ΔG^{o} of -4.1 Kcal. It can be seen in Table 5.15 that stoichiometric amounts of ADP were not obtained. The reason for this discrepancy is not known, but it could be attributed

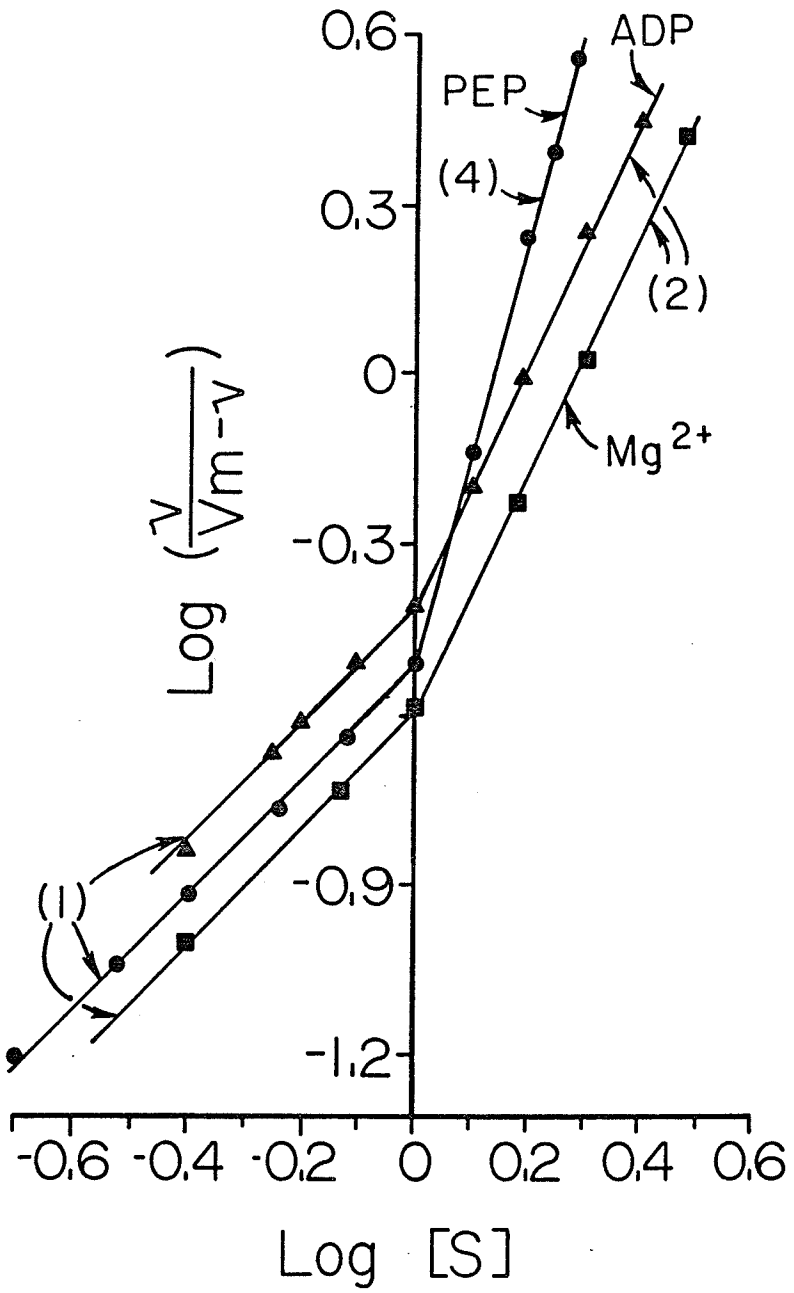


Fig. 5.27. Hill plots of the data from Figs. 23, 24 and 25.

TABLE 5.15

Concentrations of the reactants and products at equilibrium following catalysis by the pyruvate kinase of V. parvula M_4.

Reactants	Concentrations at equilibrium	
	PEP → Pyr[a]	Pyr → PEP[b]
Pyruvate	2.52[c]	3.34
PEP	0.45	0.53
ATP	2.96	3.94
ADP	0.02	0.03
Keq (PEP → Pyr)		
Experimental	828	827
Theoretical	37	50

[a] In this assay system (PEP to pyruvate), the reaction mixture contained (in mM): PEP, 3; ADP, 3; $MgSO_4$, 6; phosphate buffer (pH 7.0); 50 with 30 µg of the purified PK in a volume of 1 ml. The reaction was incubated at 37 C for 2 hours at which time 0.1 ml of IN perchloric acid was added to stop the reaction. The mixture was then neutralized and centrifuged at 30,000 g for 10 min at 4 C. The pyruvate formed was assayed by the continuous spectrophotometric method as mentioned in Chapter 4, while the remaining PEP was assayed with commercial pyruvate kinase. The ATP formed was assayed by the hexokinase and glucose-6-phosphate dehydrogenase method of Lamprecht and Trautschold (1963).

[b] For the reaction from pyruvate to PEP, the assay mixture was the same as that mentioned above, except pyruvate-3-^{14}C and a labelled ATP-8-^{14}C (1.8×10^4 dpm/mM) both at 4 mM, were employed. The pyruvate and PEP were assayed as above, while the adenosine phosphate concentrations were determined following paper chromatography as described in Methods for (α-^{32}P) ATP (Method 1).

[c] mM.

to the recovery of radioactivity from the paper chromatograms. The amount of radioactivity obtained in PEP or ADP was very low due to the quenching of the ^{14}C-label by the paper. Since the Keq values in Table 5.15 were calculated from the experimental data, the values (i.e., 828 and 826) are probably too high. If, however, stoichiometric amounts of PEP and ADP (i.e., 0.45 and 0.53, respectively) were used in the calculation, theoretical Keq values of 37 and 50 are obtained for the downward and upward methods, respectively. Even with this lower theoretical value, it is obvious that the V. parvula M_4 pyruvate kinase will readily convert PEP to pyruvate in the presence of ADP and magnesium ions.

Part D - Effectors of Pyruvate Kinase

a) Inhibitors.

(i) Effects of ATP. During the conversion of PEP to pyruvate in the presence of ADP, ATP is a product of the pyruvate kinase reaction. Thus, it was of interest to see if ATP had any effect on the activity of the enzyme. By varying the concentration of ATP from 0 to 4 mM, at various concentrations of PEP, non-competitive inhibition was observed (Fig. 5.28(A)). The non-competitive nature of the inhibition was confirmed by plotting s/v_i (i.e., PEP/inhibited velocity) versus the PEP concentration, at various ATP concentrations (Webb, 1963). The curves obtained by this method were sigmoidal and converged on the abscissa (Fig. 5.28 (B)). The non-convergence of these lines on the ordinate indicated that the inhibition was non-competitive. No alteration in the K_m value for PEP was observed in the presence of the nucleotide.

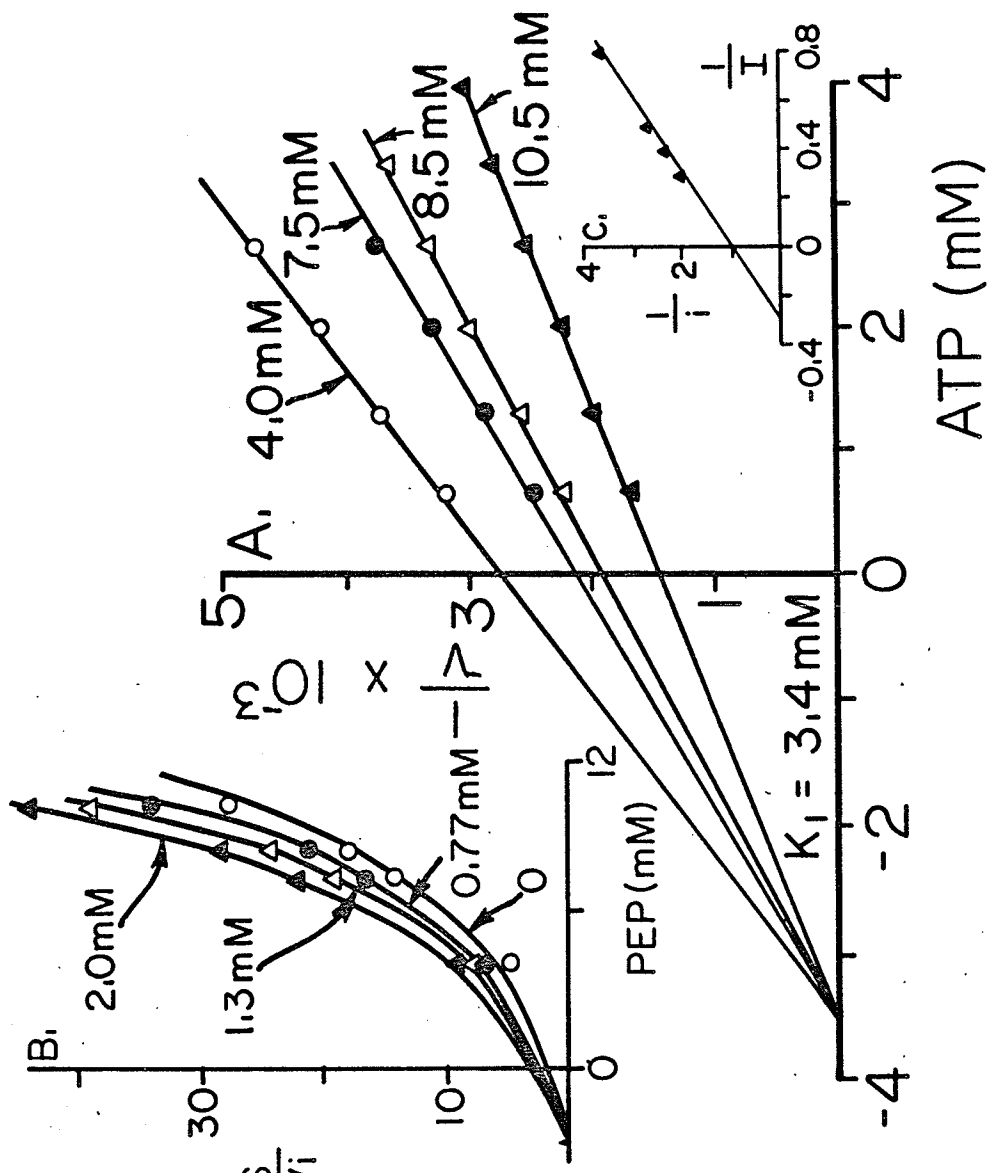


Fig. 5.28. The inhibitory effect of ATP on the activity of the purified pyruvate kinase from V. parvula M_4. (A) Effect of varying concentration of ATP at various fixed concentrations of PEP. (B) Effect of varying concentrations of ATP. (C) Webb plot.

The inhibition constant (K_i), obtained in plot 5.28(A) was 3.4 mM. This was confirmed by plotting 1/i vs 1/I (Fig. 5.28(C)) according to the method of Webb (1963). This plot gave a straight line with the intersection on the abscissa (i.e., -0.29) giving a K_i value of 3.45 mM, while the slope gave a value of 3.3 mM. The average of these K_i values was 3.4 mM.

(ii) Effect of magnesium on ATP inhibition. Holmsen and Storm (1969) have observed that Mg^{2+} salts at concentrations above 4 mM abolished the ATP inhibition of rabbit skeletal muscle pyruvate kinase. Therefore, similar experiments were undertaken with the purified pyruvate kinase from V. parvula M_4.

Table 5.16 shows the effect of increasing concentrations of $MgSO_4$ on the activity of pyruvate kinase in a reaction mixture containing 4 mM PEP and 1.3 mM ATP. In the absence of ATP, but with 6 mM Mg^{2+}, the rate was 714 units or 1.86 times the activity of the enzyme in the presence of 1.3 mM ATP (383 units). With increasing Mg^{2+} concentrations up to 12 mM, the rate of enzyme activity increased to 448 units, which was 1.17 times the activity with 6 mM Mg^{2+}. Above 12 mM Mg^{2+}, however, the reaction rates decreased progressively such that above 20 mM Mg^{2+} the rates were less than that with 6 mM Mg^{2+}. These results indicate that Mg^{2+} ions, at concentrations between 6 and 18 mM, had only a slight effect on reversing the ATP inhibition of the V. parvula enzyme. Furthermore, the inhibition of enzyme activity by Mg^{2+} at concentrations above 20 mM, in the presence of 1.3 mM ATP, was more drastic than that observed with Mg^{2+} alone (Fig. 5.25(A)).

(iii) Inhibition by 3-P-glycerate. Increasing inhibition of V. parvula M_4

TABLE 5.16

Effect of magnesium on the inhibition of the purified pyruvate kinase of *V. parvula* M_4 by 1.32 mM ATP.

Magnesium concentration (mM)	Rate	Relative activity[a]
6 (-ATP)	714[b]	1.86
6	383	1.00
10	395	1.03
12	448	1.17
15	436	1.14
18	389	1.02
20	359	0.94
30	269	0.70

[a] Activity relative to that obtained with 1.3 mM ATP and 6 mM $MgSO_4$ (line 2).

[b] µmoles NADH oxidized/mg protein/min.

pyruvate kinase was observed in the presence of 3-P-glycerate (3-PGA) at concentrations above 1 mM (Fig. 5.29(A)). At an inhibitor concentration of 12 mM, the 'α' ($\alpha = v_i/v_o$) value was o.71 or 71 per cent of the original activity.

With inhibition kinetics, it is essential to state the type of inhibition exhibited by an inhibitor. The usual procedure is to present the data graphically by a double reciprocal Lineweaver-Burk plot. However, this method could not be employed with the V. parvula M_4 pyruvate kinase because of the sigmoid nature of the enzyme (Fig. 5.22). Therefore, the Hunter and Downs (1945) method, which plots ($i\ (\alpha/1-\alpha)$) vs the substrate (PEP) concentration, was used (i = the concentration of the inhibitor). As shown in Fig. 5.29(B), a straight line was obtained below 12 mM PEP with a slope value of + 2.3 indicating that 3-PGA was a competitive inhibitor of the enzyme. By extrapolating the line to the ordinate (PEP concentration = 0), an inhibition constant (K_i) of 4.4 mM was obtained. From this graph it was also possible to obtain the K_m for PEP in the presence of the inhibitor since the slope of the line in this plot is K_i/K_m. Accordingly, a K_m value of 1.9 mM for PEP was obtained by this procedure.

(iv) Inhibition by 2,3 diphosphoglycerate. Although 2,3 diphosphoglycerate (2,3 DPGA) is not an intermediate of the glycolytic process, it s a cofactor involved in conversion of 3-P-glycerate to 2-P-glycerate by phosphoglycerate mutase (Krimsky, 1963). Therefore, the effect of 2,3 DPGA on purified pyruvate kinase from V. parvula M_4 was studied.

Kinase activity was progressively inhibited by concentrations of 2,3 DPGA above 1 mM Fig. 5.30(A). The K_i, obtained by the Hunter and

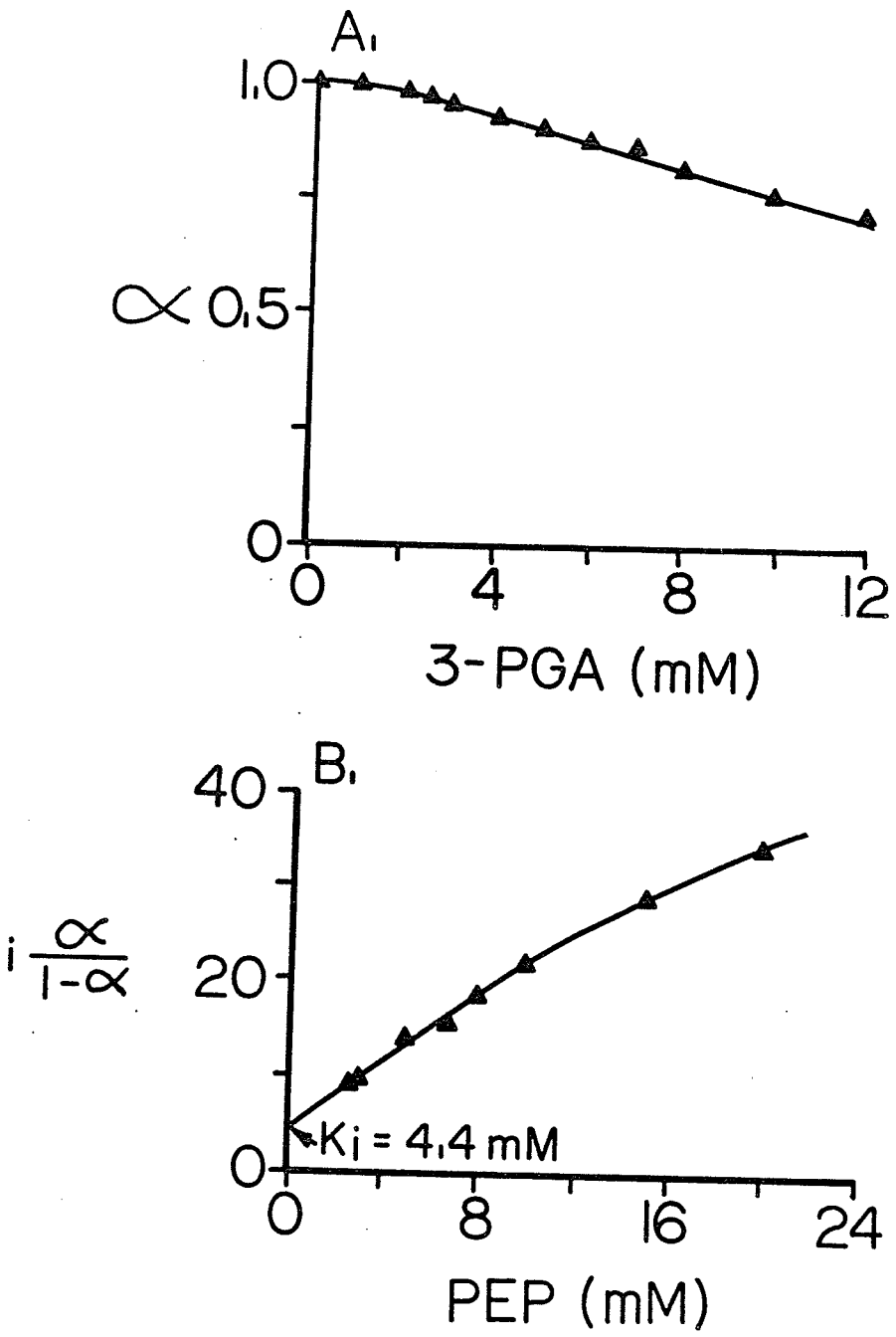


Fig. 5.29. The inhibitory effect of 3-P-glycerate (3 PGA) on the activity of the purified pyruvate kinase from V. parvula M_4. Activity was assayed by the NADH-LDH continuous method with (A) 4 mM PEP and (B) 5 mM 3-PGA. $\alpha = v_i/v_o$ (inhibited velocity/control velocity).

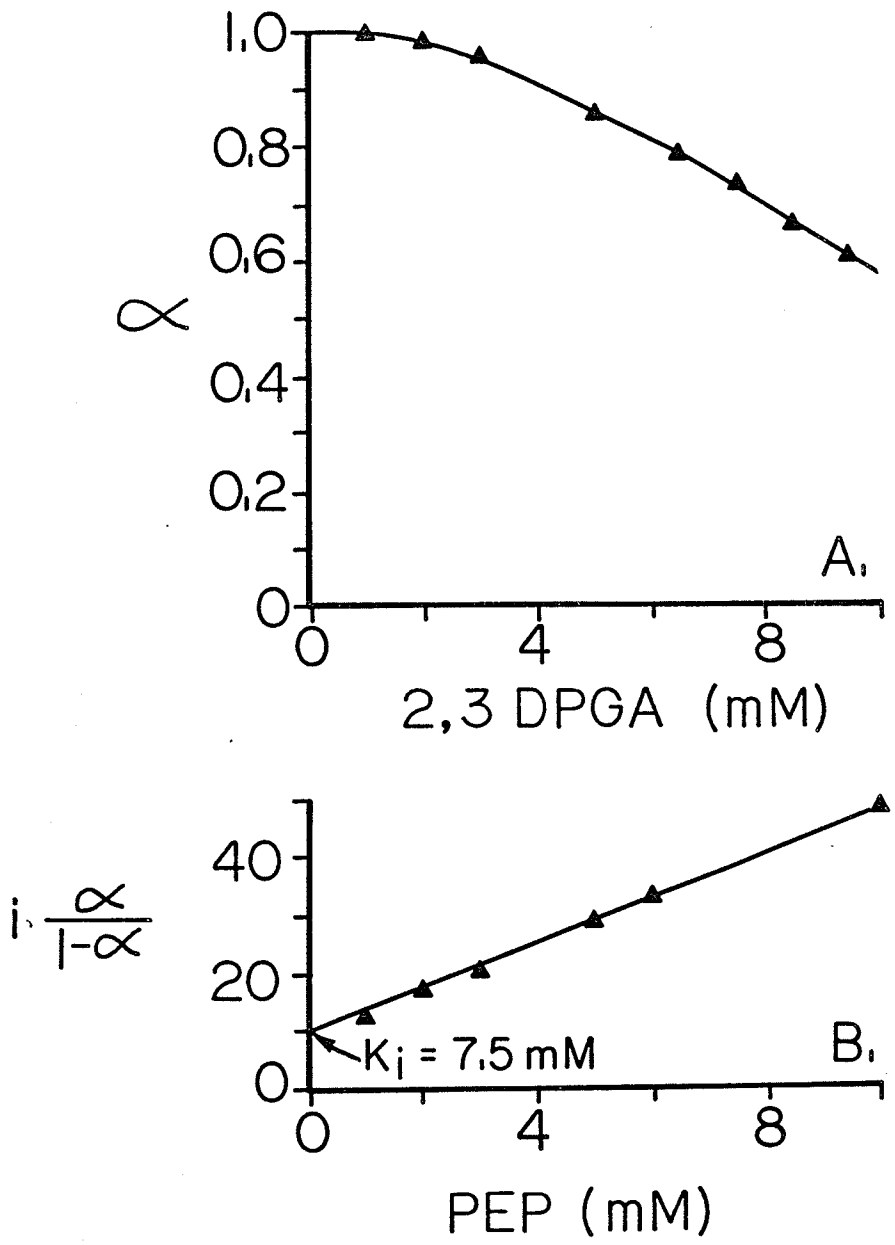


Fig. 5.30. The inhibitory effect of 2,3 diphosphoglycerate (2,3 DPGA) on the activity of the purified pyruvate kinase from V. parvula M_4. Activity was assayed as in Fig. 5.29 with (A) 4 mM PEP and (B) 5 mM 2,3 DPGA.

Downs method (1945), was 7.5 mM and the inhibition was competitive Fig. 5.30(B). The slope of the line in the latter graph gave a K_m of 1.8 for PEP in the presence of 2,3 DPGA.

v) Inhibition by malate. Malate was a potent inhibitor of the V. parvula pyruvate kinase. With inhibition by this compound, like that for 2,3 DPGA, non-linear with respect to the concentration of malate (Fig. 5.31(A)). Inhibition by malate was competitive with a K_i value of 5.5 mM (Fig. 5.31(B)); the slope value (3.3) gave a K_m value of 1.7 mM for PEP.

(vi) Summary: Inhibitors. Table 5.17 summarizes the effects of the inhibitors on the activity of the V. parvula M_4 pyruvate kinase. Without inhibitors, the enzyme exhibited a K_m of 1.2 mM of PEP (Fig. 5.23), while with 3-PGA, 2,3 DPGA and malate, the K_m was increased slightly. Although ATP had no effect on the K_m for PEP, it had the greatest affinity for the enzyme of all the inhibitors tested.

b) Activators. A variety of metabolites are known to activate pyruvate kinase from various sources (Sanwal, 1970). The purified pyruvate kinase from V. parvula M_4 was also activated by various intermediates of the glycolytic pathway.

(i) Activation by glucose-6-P. As shown previously, the incubation of the V. parvula pyruvate kinase with PEP, ADP and Mg^{2+}, produced sigmoid kinetics (Fig. 5.23 to 5.25). However, as shown in Fig. 5.32(A), the addition of glucose-6-P (G6P) to the assay resulted in hyperbolic kinetics and activation of the enzyme at PEP concentrations less than 8 mM. Above 8 mM PEP, glucose-6-P inhibited the enzyme. By inspection, it can be seen that the V_{max} increased from 700 to 940 units in the presence of G6P,

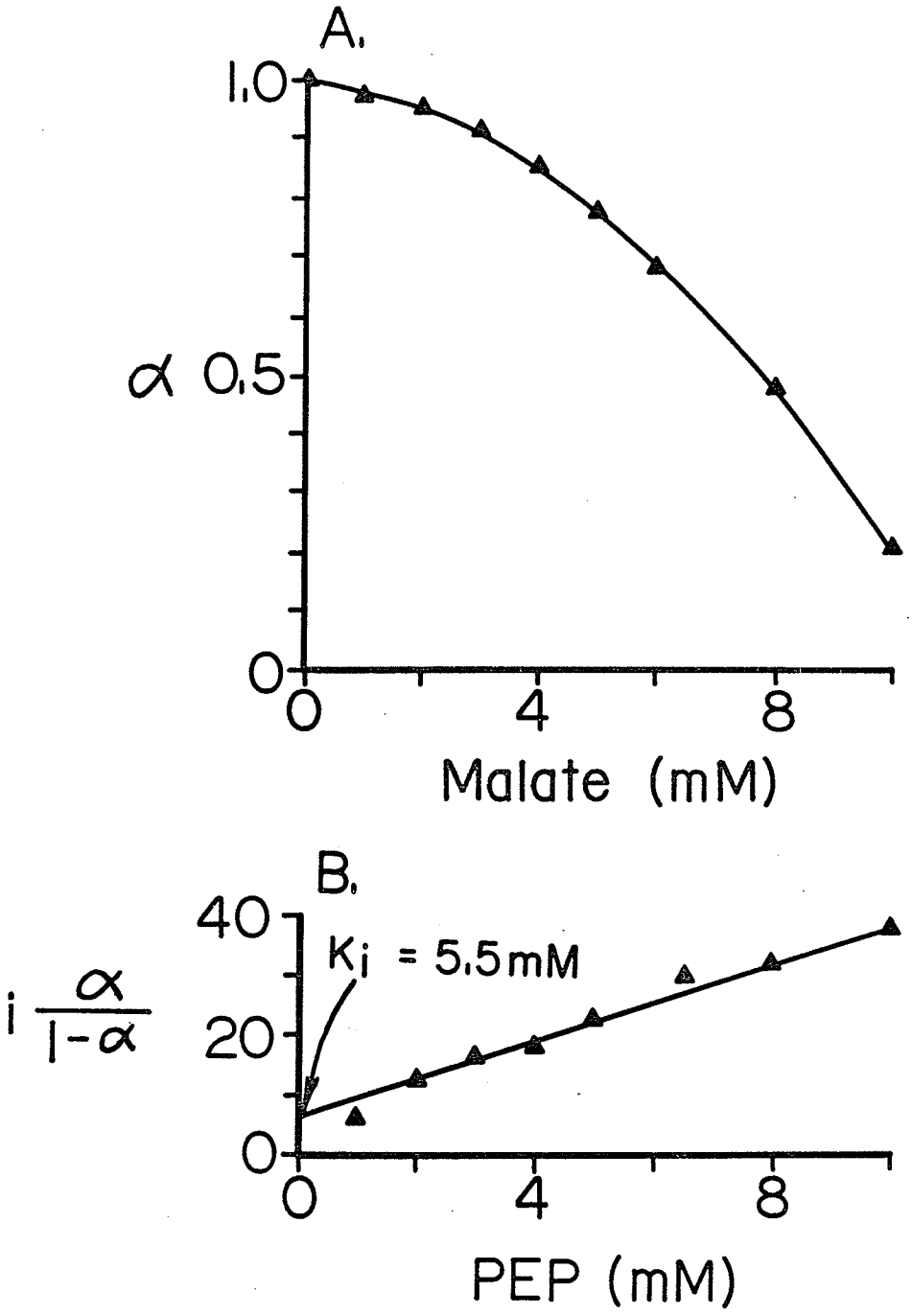


Fig. 5.31. The inhibitory effect of malate on the activity of the purified pyruvate kinase from V. parvula M_4. Activity was assayed as in Fig. 5.29 with (A) 4 mM PEP and (B) 5 mM malate.

TABLE 5.17

Summary table of the inhibitors of the purified pyruvate kinase from *V. parvula* M_4.

Inhibitor	K_m (mM)	K_i (mM)	Type of inhibition
ATP	1.2	3.4	non-competitive
3-PGA	1.9	4.4	competitive
2,3 DPGA	1.8	7.5	competitive
Malate	1.7	5.5	competitive
No inhibitor	1.2	-	-

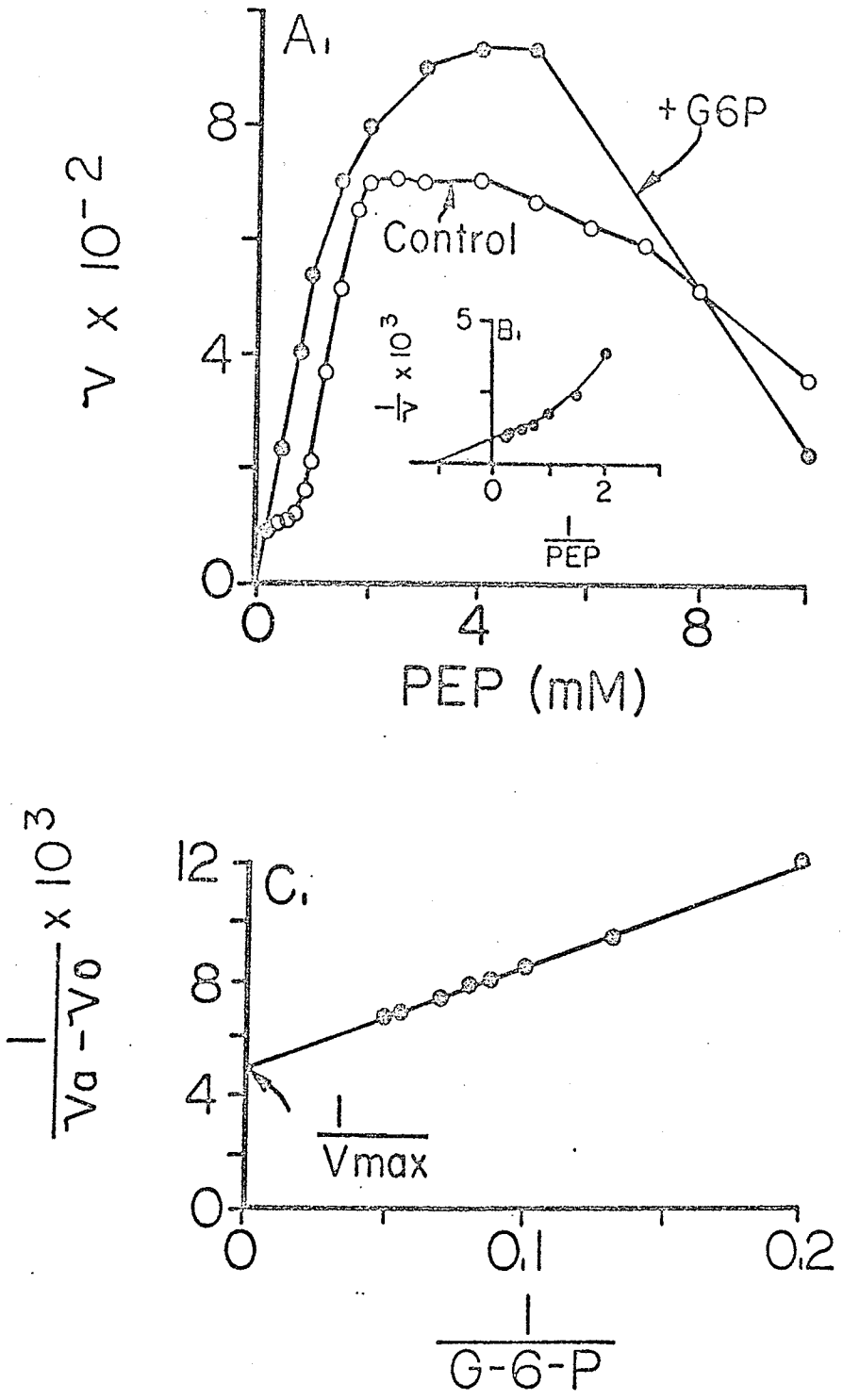


Fig. 5.32. The activation of the purified pyruvate kinase from V. parvula M_4 by glucose-6-P (G6P). Activity was assayed as in Fig. 5.29 with (A) 5 mM glucose-6-P and (B) 4 mM PEP.

while the apparent K_m was 0.91 mM (Fig. 5.32(B)). By plotting $(1/v_a - v_o)$ against (1/G6P) (Dixon and Webb, 1964) with 4 mM PEP, a straight line was obtained which, when extrapolated to the abscissa (not shown), gave a value of 5.3 mM for the activation constant (K_a) for glucose-6-P. The intersection of the line with the ordinate gave a value of 210 units for the difference in the V_{max} (ΔV_{max}) with and without the activator. This was close to that observed by the inspection of Fig. 5.32(A), (i.e. 940 - 700 = 240).

(ii) Activation by fructose-6-P. As shown in Fig. 5.33(A), 5 mM fructose-6-P (F6P) also produced hyperbolic kinetics and slight activation of kinase activity. Unlike glucose-6-P, little effect of F6P was observed until the PEP concentration was at least 3 mM, with maximum stimulation occurring between 4 and 5 mM PEP. Like glucose-6-P, this activator did not prevent substrate inhibition and was slightly inhibitory above 8.0 mM PEP. The observed V_{max} change (ΔV_{max}) due to the activation was 110 units, while the apparent K_m in the presence of fructose-6-P was 1.43 mM (Fig. 5.33(B)).

(iii) Activation by fructose-1,6-P_2. Hyperbolic kinetics and activation of the V. parvula pyruvate kinase were also observed by the addition of 5 mM fructose-1,6-P_2 (FDP) to the assay at increasing concentrations of PEP (Fig. 5.34(A)). However, the activation by FDP was different from that observed with glucose-6-P since FDP was inhibitory at low concentrations of PEP (below 3.3 mM). Above 3.3 mM PEP, FDP activated the enzyme and abolished the observed substrate inhibition even at PEP concentrations as high as 10 mM.

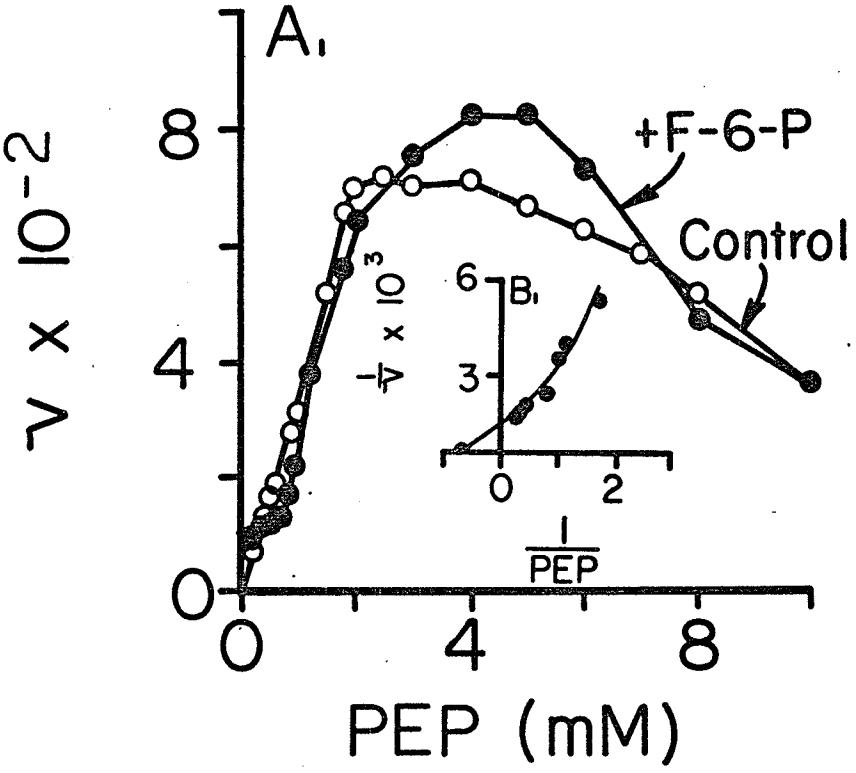


Fig. 5.33. The activation of the purified pyruvate kinase from V. parvula M_4 by fructose-6-P. Activity was assayed as in Fig. 5.29 with 5 mM fructose-6-P (F6P).

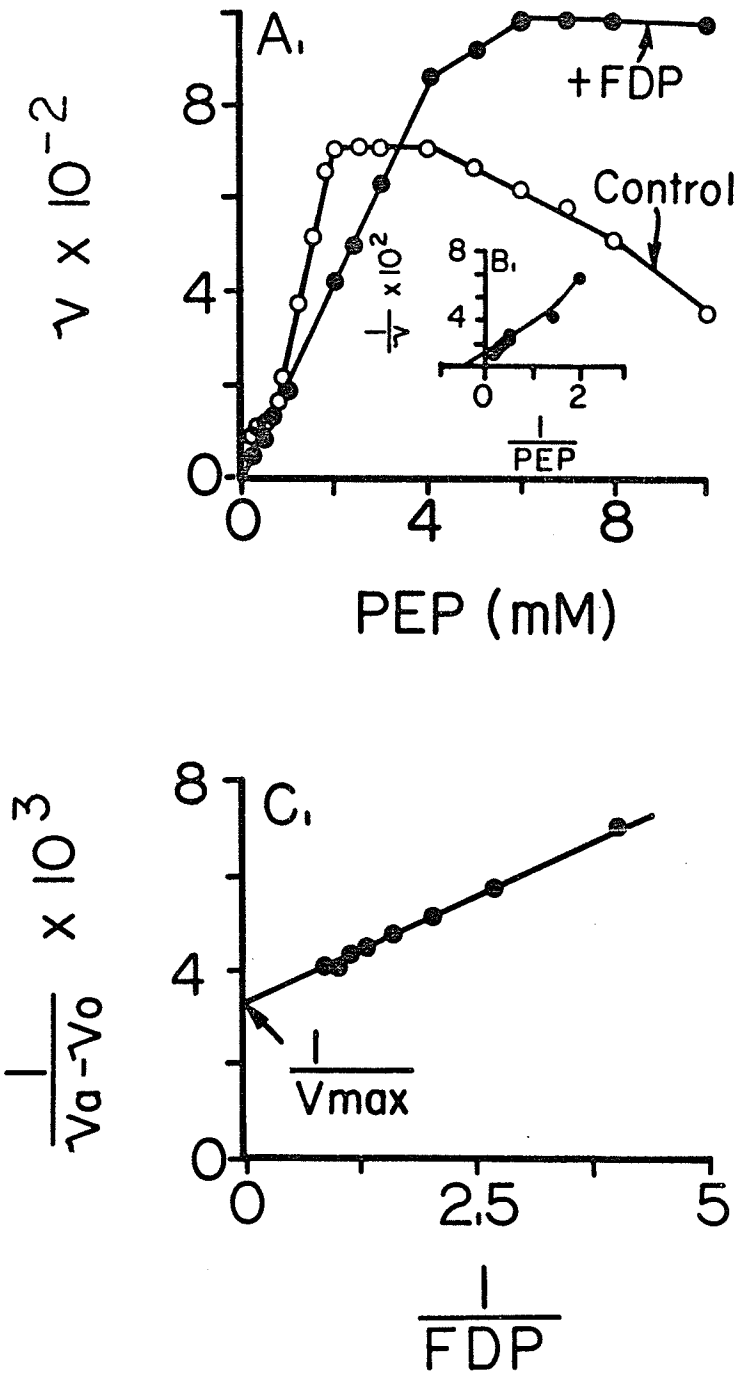


Fig. 5.34. The activation of the purified pyruvate kinase from V. parvula M_4 by fructose-1,6-P_2 (FDP). Activity was assayed as in Fig. 5.29 with (A) 5 mM fructose-1,6-P_2 and (B) 4 mM PEP.

In the presence of FDP, the V_{max} was observed to be 985 units compared to 705 units without the activator. The apparent K_m for PEP in the presence of FDP was 2.5 mM (Fig. 5.34(B)). The Lineweaver-Burk plot of $(1/v_a - v_0)$ against (1/FDP) (Fig. 5.34(C)) indicated that the activation by FDP was proportional to the increasing concentration of FDP. Extrapolation of this line to the abscissa gave a K_a of 0.32 mM, while the ΔV_{max} was 310 units compared to 280 units obtained by inspection of Fig. 5.34(A).

(iv) Activation by dehydroxyacetone-P. Dihydroxyacetone-P (DHAP) was an effective activator of the V. parvula pyruvate kinase and also produced hyperbolic kinetics (Fig. 5.35). With 5 mM DHAP, the observed V_{max} was 950 units between 4 and 8 mM PEP, while drastic inhibition was observed above 8 mM PEP. The apparent K_m, as determined from Fig. 5.35(B), was 1.1 mM.

(v) Activation by AMP. Of the compounds tested, AMP was the most potent activator of the V. parvula purified pyruvate kinase (Fig. 5.36(A)). Maximum activity of 1140 μmoles/mg protein/min was observed with 0.5 mM ATP at PEP concentrations of 2 - 2.5 mM with inhibition occurring at higher substrate levels. The K_m in the presence of AMP was 0.5 mM (Fig. 5.36(B)). The K_a for AMP, as determined from Fig. 5.36(C), was 1.1 mM, while the ΔV_{max} was 480 units and similar to that obtained by inspection of Fig. 5.36(A).

(vi) Hill plots for activators. The respective Hill coefficients for glucose-6-P, fructose-6-P and fructose-1,6-P_2 were 2.1, 2.5 and 1.8 (Fig. 5.37), while those for AMP and DHAP were 2.9 and 1.8 (Fig. 5.38) respectively. These positive coefficient values indicate that positive

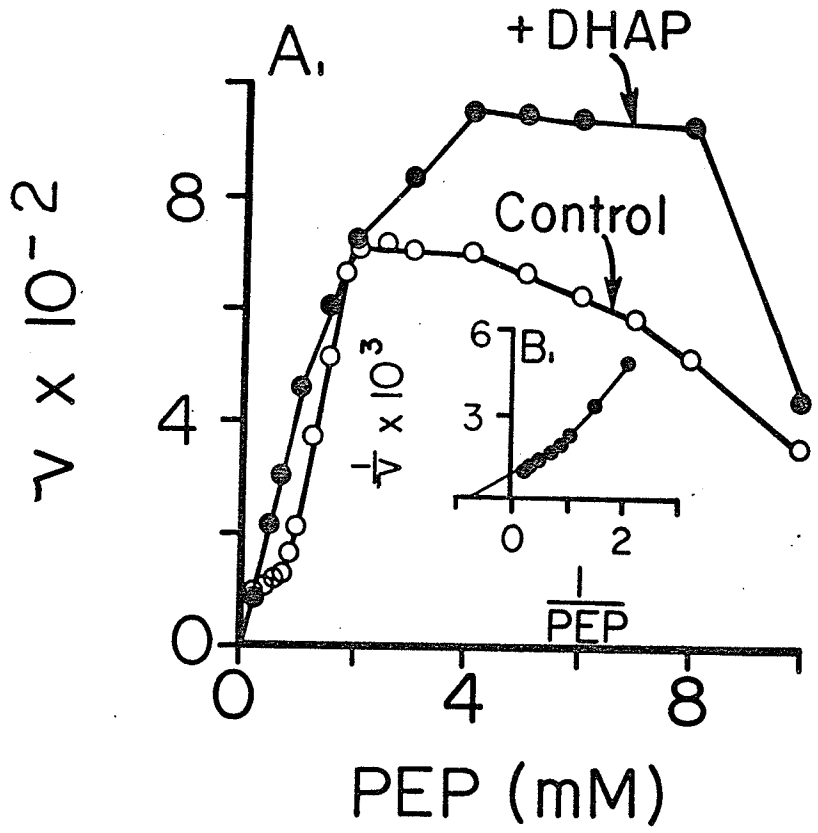


Fig. 5.35. The activation of the purified pyruvate kinase from V. parvula M_4 by dihydroxyacetone-P (DHAP). Activity was assayed in Fig. 5.29 with 5 mM dihydroxyacetone-P.

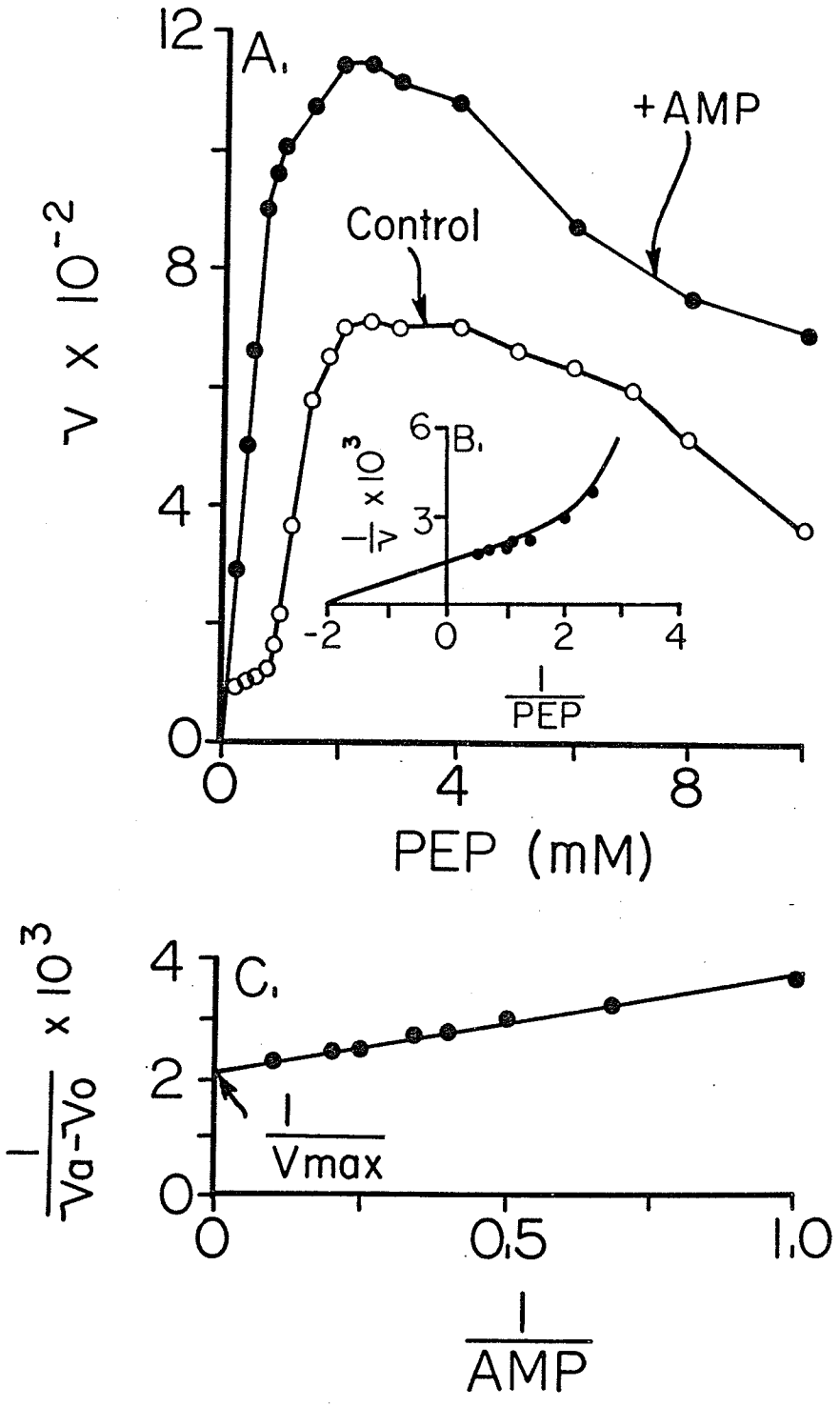


Fig. 5.36. The activation of the purified pyruvate kinase from V. parvula M_4 by AMP. Activity was assayed as in Fig. 5.29 with (A) 0.5 mM AMP and (B) 4 mM PEP.

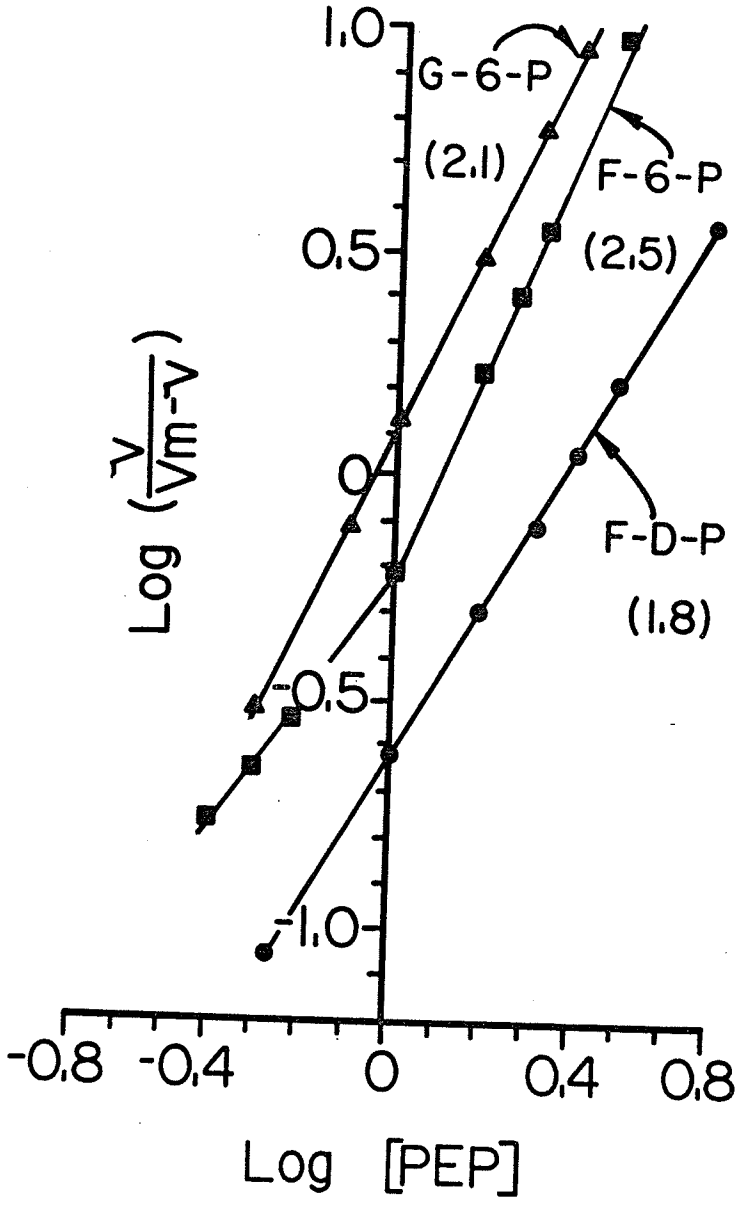


Fig. 5.37. Hill plots of the kinetic data in Figures 5.32 - 5.34.

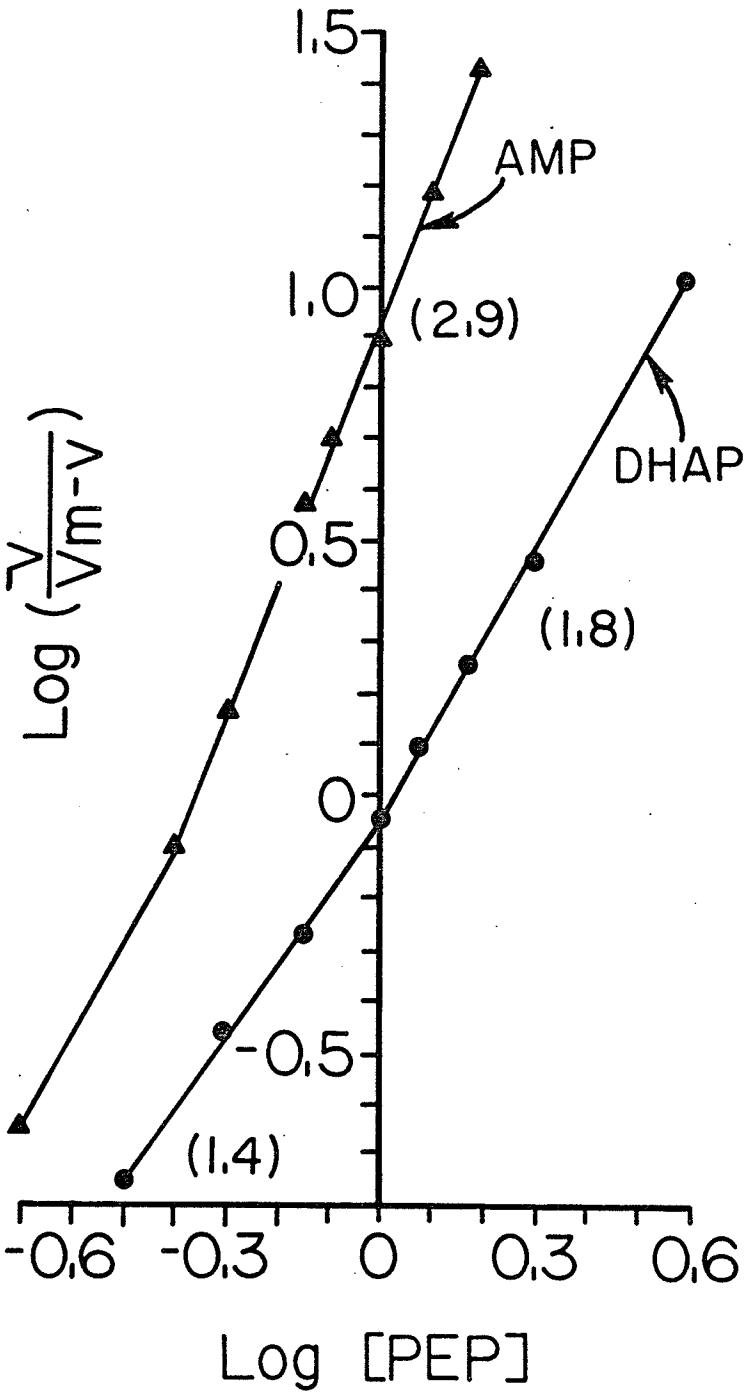


Fig. 5.38. Hill plots of the kinetic data in Figures 5.35 and 5.36.

co-operativity occurs during the activation of the V. parvula pyruvate kinase and that AMP had the most molecules binding the enzyme during this process, followed by fructose-6-P and then fructose-1,6-P_2 and dihydroxyacetone-P.

(vii) Summary: Activators. Table 5.18, summarizes the effect of the various activators of the V. parvula M_4 pyruvate kinase. Without activators, the K_m was 1.2 mM and this constant was not greatly affected by the presence of glucose-6-P, fructose-6-P and dijydroxyacetone-P (i.e. ± 0.3 mM). However, while the K_m value in the presence of fructose-1,6-P_2 was twice that of the control, the low K_a for FDP (0.3 mM) suggested that this metabolite had a high affinity for the enzyme. On the other hand, AMP not only had high affinity for the enzyme (K_a=1.1 mM) but also lowered the K_m for PEP from 1.2 to 0.5 mM. Furthermore, the addition of this compound to the assay system resulted in a 63% increase in the V_{max}.

c) Metabolites having no effect. Table 5.19 summarizes the effect of the various metabolites on the activity of the V. parvula M_4 pyruvate kinase. The kinetic constants in (A) and (B) have been taken from Tables 5.17 and 5.18. Not mentioned previously are those metabolites which had no effect on the enzyme. As shown in (C), the activity of the enzyme was not significantly affected by glucose, glyceraldehyde-3-P, 2-P-glycerate, lactate, fumarate, succinate and cyclic AMP.

TABLE 5.18

Summary table of the activators of the purified pyruvate kinase from *V. parvula* M_4.

Activator	K_m (mM)	K_a[b] (mM)	V_{max}
Glucose-6-P	0.9	5.3	940[a]
Fructose-6-P	1.4	-	820
Fructose-1,6-P_2	2.5	0.3	985
Dihydroxyacetone-P	1.1	-	950
AMP	0.5	1.1	1140
No activator	1.2	-	720

[a] Units = µmoles NADH oxidized/mg protein/min.

[b] Activation constant.

TABLE 5.19

Summary table of the metabolites inhibiting, activating and having no effect on the activity of the purified pyruvate kinase from V. parvula M_4.

A. Inhibitors[a]	K_i (mM)
Adenosine triphosphate	3.4
3-P-glycerate	4.4
malate	5.5
2,3 Diphosphoglycerate	7.5

B. Activators[a]	K_a (mM)
Fructose-1,6-P_2	0.3
Adenosine monophosphate	1.1
Glucose-6-P	5.3
Fructose-6-P	-
Dihydroxyacetone-P	-

C. No Effect	Relative activity[b]
Glucose	0.99
Glyceraldehyde-3-P	1.00
2-P-glycerate	1.01
Lactate	1.01
Fumarate	1.00
Succinate	1.00
Cyclic adenosine monophosphate	1.00

[a] Metabolite concentration = 5 mM (except ATP = 0.5 mM). Substrate (PEP) concentration = 4 mM.

[b] Velocity relative to enzyme without added metabolite.

IV. DISCUSSION

The main purpose of the research outlined in this chapter was to answer two questions: (1) what enzyme or enzymes are responsible for the interconversions between PEP and pyruvate, and (2) what type of cellular control regulates this enzymatic activity? The results presented indicate that the only conversion between PEP and pyruvate V. parvula is catalyzed by an active pyruvate kinase and that this enzyme is regulated by a variety of cellular metabolites.

A. Evidence against PEP synthetase.

As outlined previously, the preliminary evidence in Chapter 4 indicated that crude extracts of V. parvula contained pyruvate kinase and PEP-synthetase or pyruvate, phosphate dikinase. However, the results obtained in this chapter with γ and α-labelled (^{32}P) ATP indicate that only pyruvate kinase is present. This conclusion is made possible because pyruvate kinase can convert pyruvate to PEP in the presence of ATP with the γ-phosphate of ATP being transferred to PEP with resultant production of ADP. With either the synthetase or the dikinase, however, the β-phosphate of ATP is transferred to PEP and AMP is the end-product. The presence of pyruvate kinase was indicated by the formation of (^{32}P) PEP following the incubation of crude extracts with pyruvate and (γ-^{32}P)-ATP (Table 5.10). Nevertheless, this experiment did not exclude the presence of synthetase and dikinase activity since only the γ-phosphate transfer was observed. Some of the ATP utilized in the experiment may have been converted to PEP by these enzymes thereby reducing the specific activity

of the (^{32}P) PEP formed. However, because of the small amount of PEP formed from pyruvate during this experiment, it was not possible to test for this accurately.

To circumvent this problem (α-^{32}P) ATP and unlabelled pyruvate were incubated with crude extracts. The analysis of the distribution of (^{32}P) into the various adenine nucleotides demonstrated the rapid production of (^{32}P) ADP, the product of the pyruvate kinase reaction (Fig. 5.14). (^{32}P) AMP, the product of either the synthetase or dikinase reaction, was produced at a very slow rate. Since the concentration of (^{32}P) ADP decreased after 10 mins, the presence of adenylate kinase in the extract preparation was indicated and could have resulted in the slow rate of (^{32}P) AMP formation. Furthermore, the inability of crude extracts to form ATP and pyruvate from AMP, PEP and P_i would substantiate the conclusion that, while pyruvate kinase is present, PEP synthetase and pyruvate, phosphate dikinase are absent in V. parvula M_4.

B. Properties of the V. parvula pyruvate kinase.

The 126-fold purified pyruvate kinase from V. parvula M_4 required only ADP, Mg^{2+} ions and PEP for pyruvate and ATP formation and, in this respect, is similar to enzymes from Brev. flavum (Ozaki and Shiio, 1969) and E. coli (Maeba and Sanwal, 1968). The enzyme purified from yeast, however, has been shown to require NH_4^+ ions for activity (Washio and Mano, 1960; Hunsley and Suelter, 1969). Further comparison shows that the pH for optimum activity (7.0) of the V. parvula enzyme was similar to that observed for the enzyme from Acet. xylinum (Benziman, 1969), E. coli (Maeba and Sanwal, 1968) and Bac. licheniformis (Tuominen and

Bernlohr, 1971b). However, it differed from the optimum obtained with the kinase from Brev. flavum (6.5) (Ozaki and Shiio, 1969), Azoto. vinelandii (6.3 - 6.8) (Liao and Atkinson, 1971) and Baker's yeast 15.8) (Washio and Mauo, 1960).

The allosteric nature of the V. parvula pyruvate kinase was apparent from the sigmoidal nature of the kinetic curves for PEP, ADP and Mg^{2+} (Fig. 5.23 to 5.25). Sigmoidal kinetics is a general property of many pyruvate kinases, having been observed with the enzyme from rat liver (Taylor and Bailey, 1967; Tanaka et al, 1967), yeast (Hess, Haeckel and Brand, 1966), E. coli (Maeba and Sanwal, 1968), Brev. flavum (Ozaki and Shiio, 1969) and Azoto. vinelandii (Liao and Atkinson, 1971). However, the V. parvula M_4 enzyme exhibited a much higher Hill coefficient for PEP (4.0) than that observed with the enzyme from yeast (2.0), E. coli (1.3) Brev. flavum (3.0) and Azoto. vinelandii (1.8). Strong positive co-operativity was also observed with Mg^{2+} ions and ADP in the present study, each exhibiting a Hill coefficient of 2.0. While similar values are not available for the enzyme from other microbial sources, hyperbolic kinetics was obtained with the enzyme from Azoto. vinelandii (Liao and Atkinson, 1971) and Brev. flavum (Ozaki and Shiio, 1969) at varying concentrations of ADP, and with the Bac. licheniformis enzyme, when Mn^{2+} ions were substituted for Mg^{2+} ions (Tuominen and Bernlohr, 1971b).

C. Effectors of the enzyme.

Unlike the enzyme from Azoto. vinelandii (Liao and Atkinson, 1971) and Saccharomyces cerevisiae (Barwell, Woodword and Brant, 1971), the pyruvate kinases from B. licheniformis (Tuominen and Bernlohr, 1971)

Brev. flavum (Ozaki and Shiio, 1969), Acet. xylinum (Benzimen, 1969) and V. parvula M_4 (Fig. 5.28), were inhibited by ATP. However, with these latter enzymes, the mode of ATP inhibition varied. The enzyme from V. parvula M_4, Acet.xylinum and Brev. flavum was non-competitively inhibited by the nucleotide, whereas the inhibition of the Bac. licheniformis enzyme was competitive with respect to PEP. Furthermore, the enzyme from Acet. xylinum was also non-competitively inhibited by ATP when tested at increasing concentrations of ADP.

Wood (1968) has suggested that the competitive ATP inhibition of muscle pyruvate kinase was due to the binding of Mg^{2+} ions by ATP and thus made them unavailable to ADP, which is of course, necessary for enzyme activity. This situation does not appear to be applicable to the V. parvula pyruvate kinase since ATP inhibition was not significantly reversed by Mg^{2+} ions up to 12 mM (Table 5.16). One might have predicted this from the fact that the ATP inhibition of the latter enzyme was non-competitive although a clearer picture of the mechanism of ATP inhibition would have been gained by observing the effect of ATP at varying concentrations of ADP.

Liao and Atkinson (1971) have reported that the activity of pyruvate kinase from Azoto. vinelandii was activated by glucose-6-P, fructose-6-P and fructose-1,6-P_2, as well as by 3-P-glycerate. The fact that 3-P-glycerate, 2,3 diphosphoglycerate and malate competitively inhibited the enzyme from V. parvula M_4 (Fig. 5.29 - 5.31) further differentiates this enzyme from that in Azoto. vinelandii. The enzyme from Brev. flavum was also competitively inhibited by malate (Ozaki and Shiio, 1969). The competition of 3-P-glycerate, 2,3 diphosphoglycerate

for the active site of the V. parvula enzyme can be readily visualized since the structure of these compounds is somewhat similar to that of PEP. However, since the structure of malate differs significantly from that of PEP, malate may alter the affinity of the enzyme for PEP, but bind at a different site.

The inhibition of the V. parvula enzyme by this latter compound indicates that it may play a part in the regulation of gluconeogenesis in this organism since inhibition of pyruvate kinase must occur before gluconeogenesis can proceed. The possible presence of malic enzyme in the organism (Table 4.4, Chap. 4) suggests that during gluconeogenesis pyruvate could be converted directly to malate by CO_2-fixation with the resultant malate inhibiting pyruvate kinase. This assumes that malate can accumulate to the appropriate intracellular concentration to cause inhibition. This may be the limiting factor in such a regulatory mechanism since this compound is also an intermediate in the pathway of energy metabolism in this organism (Ng and Hamilton, 1971).

Of the compounds inhibiting the V. parvula M_4 pyruvate kinase, ATP and malate would probably be the most readily available and thus exert the greatest effect on the enzyme under in vivo conditions. Opposing the effect of these compounds would be activating effects of AMP, glucose-6-P, fructose-6-P, fructose-1,6-P_2 and dihydroxyacetone-P, the steady state concentration of which must be kept low for gluconeogenesis to occur. All of these positive modifiers altered the sigmoid shape of the PEP response curve to that of a hyperbola, and all increased the V_{max} of the enzyme at saturating PEP concentrations. Furthermore, all of these compounds, except FDP and AMP, had little or no effect on the affinity of

the enzyme for PEP. With fructose-1,6-P_2, the affinity was decreased, while with AMP it was increased (Table 5.18). Although the sigmoidal nature of the curves was changed by these effectors, positive co-operativity still existed since the Hill coefficients were between 1.8 and 2.9 (Fig. 5.37 and 5.38).

AMP and the hexose phosphates had a somewhat different effect on the kinetic constants of the pyruvate kinase from Azoto. vinelandii (Liao and Atkinson, 1971). Although these effectors (with the exception of FDP) altered the sigmoid nature of the PEP response curve, as they did with the V. parvula enzyme, the V_{max} was not affected, but the affinity of the enzyme for PEP was increased. The pyruvate kinase from E. coli (Maeba and Sanwal, 1968) was also shown to be activated by FDP and AMP, while the enzyme from Acet. xylinum was insensitive to both these metabolites (Benziman, 1969). Different results have been observed with the pyruvate kinase from Brev. flavum (Ozaki and Shiio, 1969). This latter enzyme was not activated by FDP, but was activated by AMP in a manner which did not alter the sigmoidal kinetics observed in the absence of AMP.

Recently, Liao and Atkinson(1971) have shown that the properties of the Azoto. vinelandii pyruvate kinase are dependent on interaction between the energy charge of the cell (Atkinson, 1969) and the concentration of hexose phosphates. Such a control system appears also to apply to the regulation of the V. parvula M_4 pyruvate kinase since the enzyme is inhibited by ATP (and malate) and activated by AMP and various hexose phosphates. Probably of crucial importance to the regulatory process in this organism is the regulation of ATP production because the organism

being a strict anaerobe cannot generate ATP by oxidative phosphorylation and cannot metabolize carbohydrates. Therefore, with the growth of the organism on lactate or pyruvate, a delicate balance must be struck between catabolism and biosynthesis, particularly that occurring during gluconeogenesis via amphibolic pathways. This balance is probably affected in V. parvula M_4 by the control of critical enzymes, such as pyruvate kinase, which must partition P-enolpyruvate between synthesis and energy metabolism. With excess energy charge (high ATP), gluconeogenesis would be allowed to proceed because of the inhibition of pyruvate kinase by ATP, while, on the other hand, at low energy charge (high AMP), synthesis of hexoses and other compounds would be slowed since any PEP formed undoubtedly would be converted to pyruvate and ATP by the AMP-activated enzyme. Thus, ATP would be a major cellular signal for gluconeogenesis to occur, while AMP and hexose phosphates can be seen as the primary signal responsible for stopping this process.

Superimposed on the ATP effect would be the response of the enzyme to malate. As the cell approaches high energy charge, malate should accumulate since its conversion to propionate (Fig. 6.34) would decline. This cellular increase would then reinforce the inhibition of the enzyme by ATP. In the reverse situation of low energy charge, the cellular content of malate would be kept at low level because of its rapid conversion to propionate.

CHAPTER 6

ATP - INDEPENDENT 'PYRUVATE CARBOXYLASE'

I. INTRODUCTION

The biosynthesis of dicarboxylic acids by carbon dioxide fixation with pyruvate was discovered in 1938 by Wood and Werkman (1938, 1940), who showed that CO_2 was utilized for the synthesis of succinic acid during the fermentation of glycerol by propionic acid bacteria. As a result of this observation, these workers proposed the reactions shown in Fig. 6.39 to explain succinate synthesis and later confirmed this scheme by demonstrating the incorporation of CO_2 into the carboxyl group of succinic acid (Wood *et al*, 1941). These findings clarified the previous observations of Elsden (1938) which demonstrated the rate of succinate formation in *E. coli* was dependent on the partial pressure of carbon dioxide.

The pathway shown in Fig. 6.39 was first extrapolated to *Veillonella* sp by Johns (1961b) to explain the production of propionate by *V. alcalescens* metabolizing lactate. In John's proposal, lactate was thought to be converted to propionate by the formation and decarboxylation of succinate. Johns assumed that CO_2 fixation had occurred in *V. alcalescens* after demonstrating the incorporation of $^{13}CO_2$ into propionate. To date, this has been the extent of the information available on carbon dioxide fixation by members of the genus *Veillonella*.

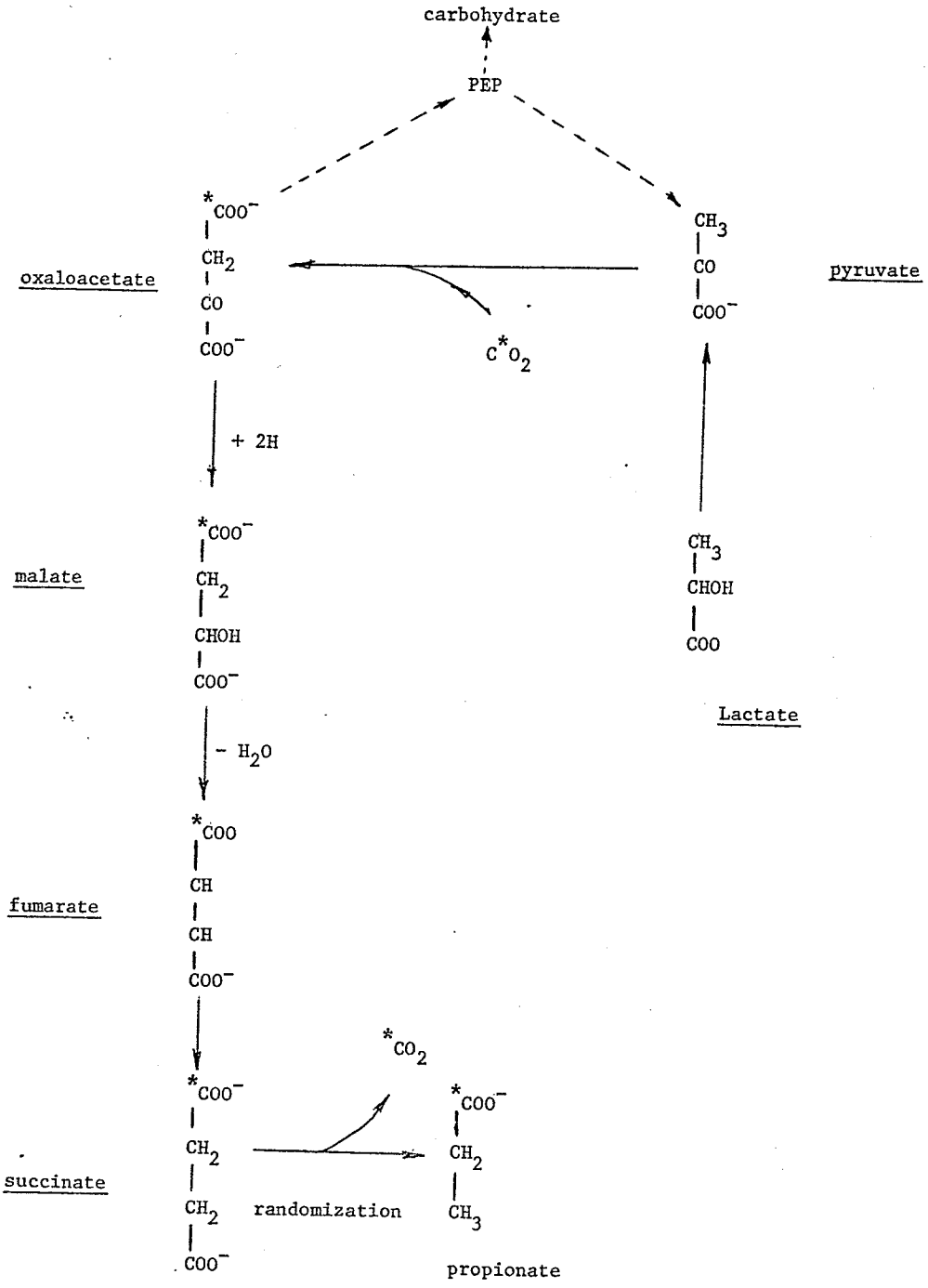


Fig. 6.39. Pathway for the formation of succinate from pyruvate by the propionic acid bacteria and propionate from lactate by Veillonella sp.

A. Primary reactions of CO_2 fixation.

Primary CO_2 fixation was defined by Wood and Stjernholm in 1962 as a reaction by which CO_2 is combined with some compound to form a new carbon-carbon bond resulting in the net fixation of CO_2. By applying this definition in retrospect, primary fixation was not clearly demonstrated until 1945 when Ochoa described the synthesis of isocitrate from α-ketoglutarate, CO_2 and TPNH by the 'isocitric' enzyme. A second primary fixation reaction was reported in 1948 with the discovery of the "malic enzyme" (Ochoa, Mehler and Kornberg). Subsequently, numerous additions have been made to the list of primary reactions and, as proposed by Calvin and Pon (1959), these can be classified into three categories: (a) reactions requiring reduced pyridine nucleotide as a source of energy, e.g. NADPH- and NADH-specific malic enzyme (Suz and Hubbard, 1957; Ochoa, Mehler and Kornberg, 1948; Sanwal, 1970; Sanwal and Smando, 1969 a,b,c) and isocitric dehydrogenase (Ochoa and Weiz-Tabori, 1948; Kornberg and Pricer, 1951), (b) reactions requiring no apparent extra energy source, e.g. PEP carboxylase (Bandurski and Greiner, 1953: Suzuki and Werkman, 1958), PEP carboxykinase (Utter and Kurahashi, 1953; Suzuki and Werkman, 1958; Quayle and Keech, 1959), and PEP carboxytransphosphorylase (Siu, Wood and Stjernholm, 1961; Tchen and Vennesland, 1955), and (c) reactions requiring ATP as a source of energy, e.g. propionyl carboxylase (Flavin, Ortiz and Ochoa, 1955; Kaziro et al, 1961) and pyruvate carboxylase (Utter and Keech, 1960; Seubert and Remberger, 1961).

The preliminary data presented in Chapter 4 has suggested that of the above enzymes, NADPH- and NADH-specific malic enzyme, PEP

carboxylase, PEP carboxykinase and pyruvate carboxylase are possibly present in crude extracts of V. parvula M_4. The primary concern of this chapter will be the fixation of CO_2 by 'pyruvate carboxylase' in enzyme preparations of V. parvula M_4.

B. Pyruvate carboxylase.

a) Historical development. As mentioned previously, a role for carbon dioxide fixation in the pathway of gluconeogenesis was first indicated when the incorporation of $^{13}CO_2$ into liver glycogen was demonstrated by Solomon et al (1940). It was later shown from the distribution of the isotope in glycogen that oxaloacetate was an intermediate on the pathway of synthesis (Lorber et al, 1950; Topper and Hastings, 1949). These observations, together with the discovery of malic enzyme (Ochoa et al, 1947) and PEP carboxykinase (Utter and Kurahashi, 1954a), led to the proposal that PEP formation from pyruvate occurred in gluconeogenic tissues by a pathway involving malate and oxaloacetate as intermediates (Fig. 6.40) (Krebs, 1954; Utter and Kurahashi, 1954b). This pathway was considered at the time to be more thermodynamically feasible than the direct reversal of the pyruvate kinase reaction proposed by Lardy and Ziegler (1945).

A closer examination of this latter pathway by Utter (1959), however, revealed that the equilibrium of the overall system was no more favourable to PEP synthesis than the reversal of the pyruvate kinase reaction. Furthermore, evidence against the participation of malate in PEP synthesis was obtained from the examination of the kinetic propertiess, tissue distribution and the maximal capacity of the enzymes involved in

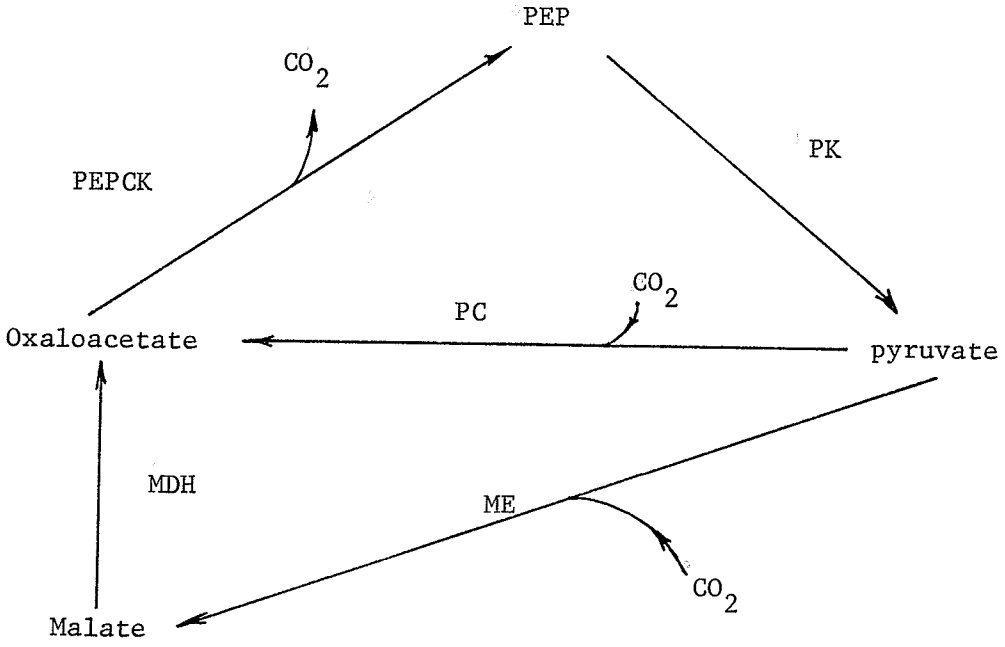


Fig. 6.40. Indirect pathways of P-enolpyruvate synthesis from pyruvate.

(PC), pyruvate carboxylase,(ME) malic enzyme, (MDH) malate dehydrogenase (PEPCK) PEP carboxykinase, (PK) pyruvate kinase.

response to variations in the gluconeogenic flux induced by starvation and refeeding, adrenolectomy, and other conditions (Shrago et al, 1963; Utter, 1959). In most instances, PEP carboxykinase exhibited all of the properties expected of an enzyme involved in gluconeogenesis, while malic enzyme did not. In fact, the evidence suggested the participation of this latter enzyme, not in gluconeogenesis, but in the production of NADPH for fatty acid synthesis. Finally, when it was demonstrated that chicken liver mitochondria, although devoid of malic enzyme, could form PEP from pyruvate, an obligatory role for malic enzyme in PEP synthesis became untenable (Utter, 1963).

An alternative mechanism for the synthesis of PEP from pyruvate was suggested from the earlier discovery of the enzyme, pyruvate carboxylase (PC), which catalyzes the ATP-dependent fixation of CO_2 with pyruvate to yield oxaloacetate (equation 4.8) (Utter and Keech, 1960). This enzyme, coupled with PEP carboxykinase (PEPCK) (equation 4.9), provided a pathway for PEP synthesis, which, because of the utilization of two equivalents of ATP, had an overall equilibrium far in the direction of PEP synthesis.

$$\text{Pyruvate} + \text{ATP} + CO_2 \xrightarrow{\text{PC}} \text{Oxaloacetate} + \text{ADP} + P_i \qquad (4.8)$$

$$\text{Oxaloacetate} + \text{ATP } (\text{GTP}^*) \xrightarrow{\text{PEPCK}} \text{PEP} + CO_2 + \text{ADP } (\text{GDP}) \qquad (4.9)$$

$$\text{net: Pyruvate} + 2\ \text{ATP} \longrightarrow \text{PEP} + \text{ADP} + P_i \qquad (4.10)$$

(*GTP is used instead of ATP in animal systems.)

b) Pyruvate carboxylase in microorganism. Since the discovery of pyruvate carboxylase in animal tissues, the enzyme has been observed in Psendomonas citronellolis (Seubert and Remberger, 1961), yeast (Losada, Canovas and Ruiz-Amil, 1964), Aspergillus niger (Bloom and Johnson, 1962), Arthrobacter globiformis (Bridgeland and Jones, 1967), Bacillus coagulans (Cazzulo and Suderman, 1969) and Rhodopseudomonas spheroides (Payne and Morris, 1969). The purified P. citronellolis enzyme was shown to differ from the liver enzyme by not requiring acetyl CoA or other CoA esters as cofactors (Seubert and Remberger, 1961). On the other hand, the pyruvate carboxylase from yeast was shown to be partially dependent on the acetyl-CoA for activity (Losada, Canovas and Ruiz-Amil, 1964), while the enzyme from Arth. globiformis and Bac. coagulans had an absolute requirement for this compound. As with the animal pyruvate carboxylases, all of these microbial enzymes required ATP for oxaloacetate formation.

c) Reaction mechanism for pyruvate carboxylase. All of the pyruvate carboxylases of animal and microbial origin have been rapidly inactivated by avidin indicating a critical role for biotin in the catalytic mechanism. This has been confirmed recently by the investigations of Scrutton and Mildvan (1968), Scrutton and Utter (1965) and Young et al (1968), who isolated protein-bound biotin residues from the pyruvate.

The involvement of biotin in CO_2-fixation became apparent originally from the observed metabolic relationship in many living forms between biotin and aspartate. Burk, Winzler and du Vigneaud (1941,1944) observed that either biotin or aspartate was capable of stimulating the fermentation and nitrogen assimilation of biotin-deficient yeast (Sacch. cerevisiae) in media containing ammonia. Independently, Koser,

Wright and Dorfman (1942) found that aspartate partially replaced biotin for the growth of Torula cremoris. In 1947, Larsen and Gunness presented evidence that biotin, in some manner, promoted the synthesis of aspartate by several bacterial species, e.g. Strep. durans 984, Strep. zymogenes 5 C1, Lact. casei LD5 and Leuconostoc mesenteroides P-60. Pursuing this further, Lardy, Potter and Elvenhjem (1947) found that bicarbonate greatly stimulated the growth of Lact. arabinosus in aspartate-free media if biotin was present, but not if biotin was absent from the medium. Furthermore, oxaloacetate could partially replace aspartate in promoting the growth of biotin-deficient cultures. Thus, when Lyman et al (1947) demonstrated the connection between carbon dioxide assimilation and aspartate synthesis, it was then assumed that biotin exerted its influence on aspartate synthesis by way of the Wood-Werkman reaction (1942), i.e., the condensation of pyruvate and CO_2 to yield oxaloacetate. To further substantiate this assumption, Lardy, Potter and Burris (1949) demonstrated that the amount of $NaH^{14}CO_3$ assimilated into cellular aspartate by Lact. arabinosus was related to the biotin concentration in the growth medium, e.g. a low concentration of biotin = low incorporation of $^{14}CO_2$ into aspartate. In addition, biotin analogs, such as homobiotin and homobiotin sulfone, inhibited the fixation of CO_2.

In the intervening years, the role of biotin in CO_2-fixation was established, and in 1962 Kaziro et al finally postulated the mechanism of action of biotin in the pyruvate kinase reaction (equation 6.30 and 6.31):

$$\text{E-biotin} + \text{ATP} + HCO_3^- \xrightarrow{Mg^{2+}} \text{E-biotin} \sim CO_2 + \text{ADP} + P_i \qquad (6.30)$$

$$\text{E-biotin} \sim CO_2 + \text{pyruvate} \longrightarrow \text{E-biotin} + \text{oxaloacetate} \qquad (6.31)$$

In this mechanism, the CO_2 is initially activated by ATP and bound to the I-N position of the biotin molecule (equation 6.30). This biotin-complexed CO_2 is then subsequently transferred to the pyruvate molecule to form oxaloacetate (equation 6.31). This mechanism was later confirmed by Kaziro and Ochoa (1964) and Scrutton, Keech and Utter (1965). It is now known that the first partial reaction (equation 6.30) is typical of all carboxylases, such as propionyl-CoA carboxylase, methylmalonyl-CoA transcarboxylase and acetyl-CoA carboxylases. The relationship of biotin to the CO_2-fixation process had been thoroughly reviewed by Wood and Stjernholm (1962), Utter (1968), Utter and Scrutton (1969) and Knappe (1970).

C. Enzymatic formation of oxaloacetate from pyruvate and CO_2 in the absence of ATP.

The original carbon dioxide fixation reaction proposed by Wood and Werkman in 1938, to account for the utilization of CO_2 by the propionic acid bacteria, did not include a high energy cofactor, such as ATP (equation 4.11).

$$\text{Pyruvate} + CO_2 \longrightarrow \text{Oxaloacetate} \qquad (4.11)$$

However, at the time, the presence of oxaloacetate was not clearly demonstrated since it was assumed that the equilibrium of this reaction

was in the direction of decarboxylation, and since oxaloacetate was an unstable compound, the formation of oxaloacetate from pyruvate and CO_2 could not be demonstrated. However, a variety of investigators (Krampitz, Wood and Werkman, 1943; Utter and Wood, 1946; Vennesland, Evans and Altman, 1947) subsequently showed that during the decarboxylation of oxaloacetate in the presence of $^{14}CO_2$ some exchange took place, with the tracer being located exclusively in the carboxyl adjacent to the methylene group of oxaloacetate. Furthermore, Kalnitsky and Werkman (1944) had demonstrated that a small amount of oxaloacetate was formed from pyruvate and CO_2 by the crude extracts of E. coli in the absence of ATP. Pursuing this aspect further, Kaltenbach and Kalnitsky (1951a,b) observed that relatively large amounts of oxaloacetate were produced from pyruvate and carbon dioxide by crude extracts of E. coli and Proteus morganii incubated in the presence of high concentrations of bicarbonate; the oxaloacetate formed was identified by manometric, chromatographic and isotopic techniques. The enzyme catalyzing this reaction was named, oxaloacetate decarboxylase (Kaltenbach and Kalnitsky, 1951a,b).

Since little information is available concerning this enzyme, it is difficult to generalize as to the basic characteristics of the enzyme. For example, the oxaloacetate decarboxylase from P. morganii was stimulated by the addition of biotin and magnesium ions (Kaltenbach and Kalnitsky, 1951b), while the same enzyme from Micrococcus lysodeikticus was inhibited by avidin (Herbert, 1951). The latter enzyme readily decarboxylated oxaloacetate with a pH optimum at 5.4 and was highly specific for oxaloacetate being unable to decarboxylate pyruvate,

α-ketoglutarate, acetoacetate, oxalo-succinate, acetone dicarboxylate, dihydroxymalate or dihydroxyacetone (Herbert, 1955). Horton and Kornberg (1964) more recently demonstrated that the oxaloacetate decarboxylase from Pseudomonas ovalis was unaffected by avidin, but was completely inhibited by acyl CoA derivatives, such as succinyl CoA, propionyl CoA and acetyl CoA.

D. 'Pyruvate carboxylase' in V. parvula M_4.

From the equilibrium data in the previous chapter, it is apparent that the V. parvula M_4 pyruvate kinase will readily convert PEP to pyruvate under normal physiological conditions. This data, coupled with the apparent absence of PEP synthetase or pyruvate, phosphate dikinase in V. parvula M_4 has indicated that there is no direct metabolic pathway converting pyruvate to PEP. The preliminary results in Chapter 4 have indicated two possible indirect pathways of PEP synthesis from pyruvate in V. parvula M_4. The shortest of these involves the formation of oxaloacetate directly from pyruvate and carbon dioxide by pyruvate carboxylase, with the subsequent decarboxylation of the oxaloacetate to PEP by PEP carboxykinase (Fig. 6.40). The second pathway involves three enzymes: malic enzyme for the conversion of pyruvate and CO_2 to malate, malate dehydrogenase for the formation of oxaloacetate from malate and finally PEP carboxykinase for the decarboxylation of the oxaloacetate to PEP (Fig. 6.40).

Our interest in the first of these pathways was aroused by the observation (Chap. 4 - Table 4.4) that significant oxaloacetate could be synthesized from pyruvate and CO_2 in the absence of ATP. This finding

suggested the presence of 'ATP-independent'pyruvate carboxylase activity in V. parvula M_4. In order to confirm these preliminary findings, a study was undertaken to examine in detail the ability of the extracts of V. parvula M_4 to form oxaloacetate from pyruvate and CO_2 in the absence of ATP. The results in this chapter will demonstrate the presence of an ATP-independent 'pyruvate carboxylase' (IPC) in this organism and outline the purification and characteristics of the enzyme.

II. METHODS

A. Crude extracts.

Cells of V. parvula M_4 were grown, harvested and crude extracts prepared as outlined in Chapter 4. In all crude extracts studies, the supernatant obtained after centrifugation at 45,000 g for 25 min was treated immediately with protamine sulphate (0.2% final concentration) for 30 min and this mixture centrifuged at 40,000 g for 15 min. Unless otherwise specified, the supernatant was then treated with 0.2% (w/v) activated charcoal (Norit A) for 30 min and again centrifuged at 45,000 g to remove the fine charcoal powder. This procedure was followed, in most cases, by overnight dialysis against phosphate buffer (50 mM, pH 6.5) containing 20 mM β-mercaptoethanol. All procedures were carried out at 4 C.

B. Enzyme Assays.

a) ATP-independent 'pyruvate carboxylase' (IPC). This activity was assayed by two methods outlined previously in Chapter 4: (i) the NADH-MDH spectrophotometric procedure, which measure the oxidation of NADH resulting from the conversion of the oxaloacetate formed to malate with commercial malate dehydrogenase (MDH) and (ii) the radioactive assay, employing either $NaH^{14}CO$, or pyruvate-3-^{14}C as the labelled substrate. All assays were carried out at 37 C.

(1) Spectrophotometric Methods.

(i) Continuous assay. The continuous assay for the IPC enzyme was carried out as described in Chapter 4. The assay system contained (in mM): pyruvate, 20; Na bicarbonate, 10; NADH, 0.5; $MgSO_4$, 10; phosphate buffer (pH 7.0), 260; malate dehydrogenase (2 µg) and extract in a volume of 1 ml. All assays were carried out with a Unicam SP 800 recording spectrophotometer at 340 nm as previously described. When this method was used with crude extracts, protamine sulphate or ammonium sulphate-treated preparations, 10 mM sodium arsenite was added to the assay mixture to inhibit the activity of pyruvate dehydrogenase in these fractions.

(2) Non-continuous assay. IPC activity was, in some instances, assayed by the NADH-malate dehydrogenase assay procedure for a predetermined time. The assay mixture was the same as that for the continuous assay except that the pyruvate concentration was 4 mM, and the reaction was terminated by the addition of 1 ml of 0.5 N NaOH in 50% ethanol following a 30 min incubation period. The assay tubes were then sealed and incubated for a further 30 min followed by centrifuged at 10,000 g at 4 C. The amount of

NADH remaining in the supernatant was determined at 340 nm and compared to the control containing no protein.

(ii) Radioactive methods. The radioactive assay was used in two ways: (1) to measure the actual amount of labelled oxaloacetate formed from either pyruvate-3-^{14}C for ^{14}C-bicarbonate, and (2) to measure the amount of ^{14}C-malate formed from ^{14}C-oxaloacetate in the presence of excess NADH and malate dehydrogenase. For the latter assay, the method outlined for the non-continuous spectrophotometric method above was used, while procedure (1) employed this assay without the NADH and malate dehydrogenase. Sodium malonate (10 mM) was added to each assay to inhibit endogenous malate and succinate dehydrogenase activity (Ng and Hamilton, 1971). In both cases, the reaction was stopped by the addition to each assay tube of 50 µl of 2% 2,4 dinitrophenylhydrazine in 17.6% H_2SO_4, the contents mixed and allowed to stand at 4 C for 1 hour to permit complete precipitation of the α-keto acid hydrazones. The mixture was then centrifuged at 30,000 g for 15 min at the same temperature and the supernatant transferred to another tube. The hydrazone precipitate was washed twice with 0.5 ml of 2% 2,4 DNPH and the supernatant fractions, obtained after centrifugation, pooled with the original supernatant fraction. The radioactive α-keto acid hydrazones in the pellet and the malate-^{14}C in the supernatant were analyzed as described in Chapter 5.

B. Malate dehydrogenase.

The presence of malate dehydrogenase activity (MDH) in preparations of the ATP-independent pyruvate carboxylase were determined by measuring NADH oxidation in the presence of oxaloacetate. The reaction

contained (in mM): oxaloacetate, 3; NADH, 0.5; phosphate buffer (pH 7.0), 50 and 0.67 µg of the IPC enzyme preparation in a volume of 1 ml.

b) Malate-lactate transhydrogenase. Malate-lactate transhydrogenase (MLTH) is unique to Veillonella sp. (Phares and Long, 1956; Ng and Hamilton, 1971), readily and reversibly converting lactate to pyruvate in the presence of catalytic amounts of oxaloacetate (equation 4.18):

$$^{*}\text{Lactate} + \text{oxaloacetate} \longrightarrow {}^{*}\text{Pyruvate} + \text{malate} \qquad (4.18)$$

The assay for this enzyme contained (in mM): pyruvate, 4; malate, 4; Tris-HCl (pH 7.5), 50 with 0.6 µg of the IPC preparation in a total volume of 1 ml. The reaction was stopped by the addition of NaOH-glycine buffer (pH 10.5) containing NAD and commercial lactic dehydrogenase. At this pH, the lactate formed by the transhydrogenase was converted back to pyruvate and the reduction of NAD at 340 nm measured.

c). ATP-dependent pyruvate carboxylase. ATP-dependent pyruvate carboxylase (DPC) was assayed by the spectrophotometric and radioactive methods outlined previously for the ATP-independent enzyme, except that 5 mM ATP was added to the assay mixture.

d) Other enzymes. The NADP-specific malic enzyme, pyruvate dehydrogenase, PEP carboxylase and PEP carboxykinase were assayed as described in Chapter 4.

C. Disc gel electrophoresis.

Disc gel electrophoresis was carried out by the method recommended

by R.A. Kapitany and E.J. Zebrowski (personal communication). This method differed from that mentioned in the previous chapter in that the concentration of the acrylamide gel was 7.5% and the buffer system employed during electrophoresis was not uniform in the upper and lower chambers. Instead, a gradient of pH 8.95 (upper chamber) to pH 8.07 (lower chamber) was employed. The other procedures and ingredients were as previously mentioned.

III. RESULTS

Part A - Crude Extract Studies

As mentioned previously, the results in Tables 4.4 and 4.5 (Chap. 4) have indicated that crude dialyzed extracts of *V. parvula* M_4 were capable of forming oxaloacetate from pyruvate and CO_2, both in the presence and absence of ATP. These results prompted a further detailed investigation of this activity in cell-free preparations of *V. parvula* M_4. However, to ensure that endogenous cofactors were not responsible for the observed results, all crude extracts were initially treated with 0.2% protamine sulphate to remove nucleic acid material as described in Methods. As will be outlined in the first series of experiments, the CO_2-fixing activity in the extract preparations was then tested under various conditions to confirm the previously observed ATP-dependent and ATP-independent activity.

a) Spectrophotometric data. In the first series of experiments, oxaloacetate formation, as assayed by the malate dehydrogenase-NADH continuous method, was measured with crude extracts receiving various dialysis treatments (Table 6.20). Treatment A consisted of dialyzing the extracts overnight against 50 mM phosphate buffer (pH 7.0) containing 20 mM β-mercaptoethanol, while treatment B involved the same dialysis but with previous treatment of the extract with 0.2% activated charcoal. Treatment C was dialysis against 50 mM Tris-HCl buffer (pH 7.0), also containing 20 mM mercaptoethanol.

In all cases, it can be seen that in the absence of ATP (exp. 2), substantial quantities of oxaloacetate were formed from pyruvate and CO_2 confirming the previous suggestion that V. parvula M_4 contained an ATP-independent 'pyruvate carboxylase' (IPC). The increased rate of NADH oxidation in the presence of ATP (exp. 1) also confirmed the presence of ATP-dependent pyruvate carboxylase (DPC) activity in these extracts. Both bicarbonate (exp. 3) and Mg^{2+} (exp. 5) were required for the IPC and DPC activity, although the omission of Mg^{2+} was not as crucial as the omission of bicarbonate with dialysis treatment A, suggesting the presence of protein-bound Mg^{2+} in the extract preparation.

Analysis of the three pre-treatment procedures demonstrated that dialysis against Tris-HCl was deleterious to both the IPC and DPC activity. Activity in the presence of ATP was reduced 69%, while the activity in the absence of ATP was reduced 63%. The addition of 5 mM inorganic phosphate resulted in some recovery of the original DPC (exp. 7) and IPC (exp. 8) activity. These results suggest the importance of phosphate ions for the observed CO_2-fixing activity.

TABLE 6.20

Oxaloacetate formation from pyruvate and CO_2 in the presence and absence of ATP by crude extracts of *V. parvula* M_4.

Exp.	Assay conditions	Extract pretreatment[a]		
		A	B	C
1	Basic assay[b]	91[c]	90	28
2	- ATP	72	72	26
3	- HCO_3^-	4	-	-
4	- ATP, - HCO_3^-	-	3	-
5	- Mg^{2+}	14	-	-
6	- ATP, - Mg^{2+}	-	15	-
7	+ P_i	-	-	32
8	- ATP, + P_i	-	-	30

[a] Treatment A: overnight dialysis against indicated 50 mM phosphate buffer (pH 7.0) containing 20 mM mercaptoethanol. Treatment B: dialysis as in A followed by treatment with activated charcoal (Norit A). Treatment C: dialysis against 50 mM Tris-HCl (pH 7.0) containing 20 mM MSH.

[b] Basic assay contained (in mM): pyruvate, 30; HCO_3^-, 20; Mg^{2+}, 10; arsenate, 10; ATP, 10; NADH, 0.5; malate dehydrogenase, 2 µg and 4.4 mg of crude extract treated as indicated. Where indicated, P_i was added at a concentration of 5 mM.

[c] µmole NADH oxidized/mg protein/min.

While 10 mM malonate was normally added to crude extract preparations to inhibit succinate dehydrogenase, this compound could not be used in the assay since it inhibited the commercial malate dehydrogenase, which was used to convert oxaloacetate to malate. Therefore, this inhibitor was left out of all assays employing malate dehydrogenase.

The stability of the ATP-dependent and ATP-independent activities was tested in extracts dialyzed against phosphate buffer and subsequently treated with and without charcoal (Table 6.21). It can be seen from this table that charcoal treatment protected both activities over a 24 hour period. Without charcoal treatment, 62% of the DPC and 65% of the IPC activity was lost within 24 hours, while only 17 and 8% of the respective DPC and IPC activity was lost in the same period after charcoal treatment.

b) Radioactive data. The presence of the ATP-dependent and ATP-independent activity in treated extracts of V. parvula M_4, as observed spectrophotometrically, was further confirmed with the use of the radioactive tracers: sodium bicarbonate-^{14}C and pyruvate-3-^{14}C. However, in these and subsequent crude extract studies, the cell-free preparations were treated with protamine sulphate and then treated with charcoal as previously described. The supernatant resulting from this procedure was then dialyzed overnight against 50 mM phosphate buffer (pH 7.0). This method of extract treatment, as shown above, produced relatively stable preparations, particularly with regard to IPC activity.

(i) $NaH^{14}CO_3$. Carbon dioxide fixation was tested initially by measuring the (^{14}C) incorporated into total α-keto acid hydrazones, as well as into

TABLE 6.21

Stability of the DPC and IPC activity in dialyzed extracts of *V. parvula* M_4 treated with and without charcoal.[a]

Enzyme activity	Time (hrs)	Residual enzyme activity	
		Treatment A	Treatment B
ATP-dependent activity (DPC)	0	91[b]	90
	12	47	80
	24	35	75
ATP-independent activity (IPC	0	72	71
	12	36	69
	24	25	65

[a] Treatment A and B as outlined in Table 6.20. Following these treatments, the crude extracts were stored at 4 C under nitrogen. At the times indicated, aliquots were removed and assayed with (DPC) and without (IPC) ATP as outlined in Fig. 6.20. Each assay contained 4.4 mg protein.

[b] µmoles NADH oxidized/mg protein/min.

pyruvate and oxaloacetate, following the incubation of the treated extracts with sodium bicarbonate-^{14}C and pyruvate (Table 6.22). While $NaH^{14}CO_3$ is not a desirable tracer for CO_2-fixing studies with V. parvula M_4, for the reasons discussed in Chapter 4, it did provide an opportunity to test both for CO_2-fixation, as well as for the pyruvate-CO_2 exchange reaction with the protamine sulphate-charcoal treated dialyzed (PCD) extract. As shown in Table 6.22, treatment of the extract in this manner did not appreciably effect the exchange reaction since about 94% of the total radioactivity in the hydrazone precipitate was present in pyruvate (exp. 1). Furthermore, without arsenite in the system to inhibit pyruvate dehydrogenase, the total (^{14}C) incorporated was reduced 22%. Nevertheless, significant oxaloacetate formation was observed both with (exp. 2) and without (exp. 3) ATP in the absence of arsenite. In the experiment without ATP, the activity was 74% of that obtained with the nucleotide, which is similar to that observed previously with the spectrophotometric assay (Table 4.4 and 6.20). Subsequently, additional radiochemical assays were carried out with the more specific substrate: pyruvate-3-^{14}C.

(ii) Pyruvate-3-^{14}C. The use of pyruvate-3-^{14}C and unlabelled bicarbonate as substrates permitted an accurate determination of the amount of pyruvate converted to oxaloacetate during CO_2-fixation in the presence of pyruvate-CO_2 exchange activity. As shown in Table 6.23, when pyruvate-3-^{14}C was the labelled component in the assay, the formation of oxaloacetate-^{14}C in the absence of ATP again occurred to about the same extent (85%) as that previously observed with the spectrophotometric assay (80% - Table 6.20) when compared to the assay containing ATP. No activity was observed in the absence of Mg^{2+} ions, while significant activity was observed without

TABLE 6.22

Incorporation of $^{14}CO_2$ into oxaloacetate and pyruvate following the incubation of crude PCD-treated extracts of V. parvula M_4 with pyruvate and $NaH^{14}CO_3$.[a]

Exp.	Assay condition	Net incorporation		
		Total	Pyruvate	Oxaloacetate
1	Basic assay[b]	403[c]	380	22
2	- arsenite	316	287	19
3	- arsenite, - ATP	275	250	14

[a] PCD-treated extracts = crude extracts treated with protamine sulphate, activated charcoal, and dialyzed overnight against 50 mM phosphate buffer (pH 7.0).

[b] Assay system (in mM): pyruvate, 30; $NaH^{14}CO_3$ (2×10^5 dpm/μmole), 10; ATP, 20; Na arsenite, 10; $MgSO_4$, 10 and 4.4 mg of crude extract in 1 ml phosphate buffer, (50 mM, pH 7.0). Incubation was for 30 min.

[c] dpm/mg protein $\times 10^{-3}$. The control value for the complete system + DNPH at zero time has been subtracted.

TABLE 6.23

Oxaloacetate-^{14}C formation from pyruvate-3-^{14}C and unlabelled bicarbonate by PCD-treated crude extracts of V. parvula M_4.

Assay condition	Oxaloacetate-^{14}C formed
Basic assay[a]	173[b]
- ATP	147
- $NaHCO_3$	42
- Mg^{2+}	0

[a] Basic assay (in mM): pyruvate-3-^{14}C (6.7×10^4 cpm/μmole), 30; $NaHCO_3$, 20; $MgSO_4$, 10; Na arsenite, 10; phosphate buffer (pH 7.0), 50 with 4.4 mg crude extract (dialyzed charcoal treated) in a volume of 1 ml. Incubation was for 30 min.

[b] cpm $\times 10^{-3}$.

bicarbonate suggesting that the buffer may have contained dissolved CO_2.

c) Factors affecting oxaloacetate formation. Having thus confirmed ATP-independent CO_2 fixation in the PCD-treated extracts of V. parvula M_4, oxaloacetate formation was tested in the presence of various compounds known to affect pyruvate carboxylase activity. Table 6.24 summarizes the effect of these compounds when assayed both by the spectrophotometric (malate dehydrogenase-NADH) and radioactive (pyruvate-3-^{14}C) methods. With the former assay, activity was measured with and without ATP, while the radioactive assay employed only ATP.

According to the radioactive assay, oxaloacetate formation in the presence of ATP was stimulated by aspartate (21%), biotin (19%) and coenzyme A (10%), while 2 units of avidin inhibited activity 26%. Conflicting results were obtained in the spectrophotometric assay with ATP, which indicated that biotin had no effect on the assay, while both coenzyme A (27%) and avidin (20 and 23%) were inhibitory, as was acetyl CoA (11 and 31%). When the latter assay was used without ATP, aspartate was shown to be slightly stimulatory (20%), while biotin and avidin had relatively little effect on activity. On the other hand, significant inhibition was observed with coenzyme A (61%), and 0.2 mM acetyl CoA (56%). The above compounds had no detectable effect on the commercial malate dehydrogenase employed in the assay.

The results presented with the PCD-treated crude extracts provide evidence for both ATP-dependent and ATP-independent pyruvate carboxylase activity in cells of V. parvula M_4. Because of its unusual ability, the purification of the ATP-independent activity was undertaken.

TABLE 6.24

Effect of various compounds on the formation of oxaloacetate from CO_2 and pyruvate by PCD-treated crude extracts of *V. parvula* M_4 as assayed by the radioactive and spectrophotometric methods.

Exp.	Assay condition	Enzyme activity		
		Radioactive assay[a]	Spectrophotometric assay[b]	
		+ ATP	+ ATP	- ATP
1	Basic assay	173[c]	90[d]	72[d]
2	+ aspartate (10 mM)	210	-	86
3	+ biotin (8 uM)	206	90	70
4	+ CoA (0.2 mM)	191	66	28
5	+ avidin (2 units)	127	72	72
6	+ avidin (4 units)	-	69	68
7	+ acetyl CoA (0,2 mM)	-	80	32
8	+ acetyl CoA (0.4 mM)	-	62	-

[a] Assay conditions as in Table 6.23.

[b] Assay conditions as in Table 6.20.

[c] Oxaloacetate-^{14}C formed (cpm x 10^{-3}).

[d] µmoles NADH oxidized/mg protein/min.

Part B - Purification of the IPC Enzyme

a) Protamine sulphate treatment. Purification of the ATP-independent 'pyruvate carboxylase' from V. parvula M_4 was carried out with crude extracts obtained by the sonic disruption of exponential-phase cells. As the initial step, the nucleic acid material in the extracts was precipitated with protamine sulphate (0.2%) during a 30 min incubation period at 4 C and then treated with activated charcoal as previously described.

b) Ammonium sulphate fractionation. Following protamine sulphate treatment, the extract was subjected to ammonium sulphate fractionation in increments of 10% saturation (Table 6.25). It can be seen that the majority of the IPC activity was precipitated between the 30 and 40% ammonium sulphate levels, while most of the DPC and malate dehydrogenase activity was precipitated at the 60 and 20% concentrations, respectively. With these data in mind, further purification of the IPC enzyme was undertaken with the protein precipitating between 30 and 50% saturation. This procedure resulted in a modest increase in specific activity (Table 6.26).

c) Sephadex G-100 column chromatography. The ATP-independent enzyme was further purified by applying the 30-50% ammonium sulphate fraction to a column (2.5 x 100 cm) of Sephadex G-100 and eluting with phosphate buffer (50 mM, pH 7.0) containing 20 mM β-mercaptoethanol. As shown in Fig. 6.41(A), the IPC and DPC activity was separated by this procedure with the DPC activity appearing in fractions 1 - 3, while the IPC activity

TABLE 6.25

Activity of the ATP-independent (IPC) and ATP-dependent (DPC) pyruvate carboxylases, and malate dehydrogenase (MDH), in various fractions following ammonium sulphate precipitation.

Ammonium sulphate (%)	Protein (mg)	Total Enzyme Activity[a]		
		IPC	DPC	MDH
0	50	3950[b]	2750	3100
10	2	0	0	116
20	12	1.2	0	1044
30	23	18	69	1426
40	6	3720	90	60
50	4.3	18	215	0
60	2.1	0.4	882	0
Supernatant	0.5	0	977	0

a Assayed spectrophotometrically as described in Methods.

b µmoles NADH oxidized/min.

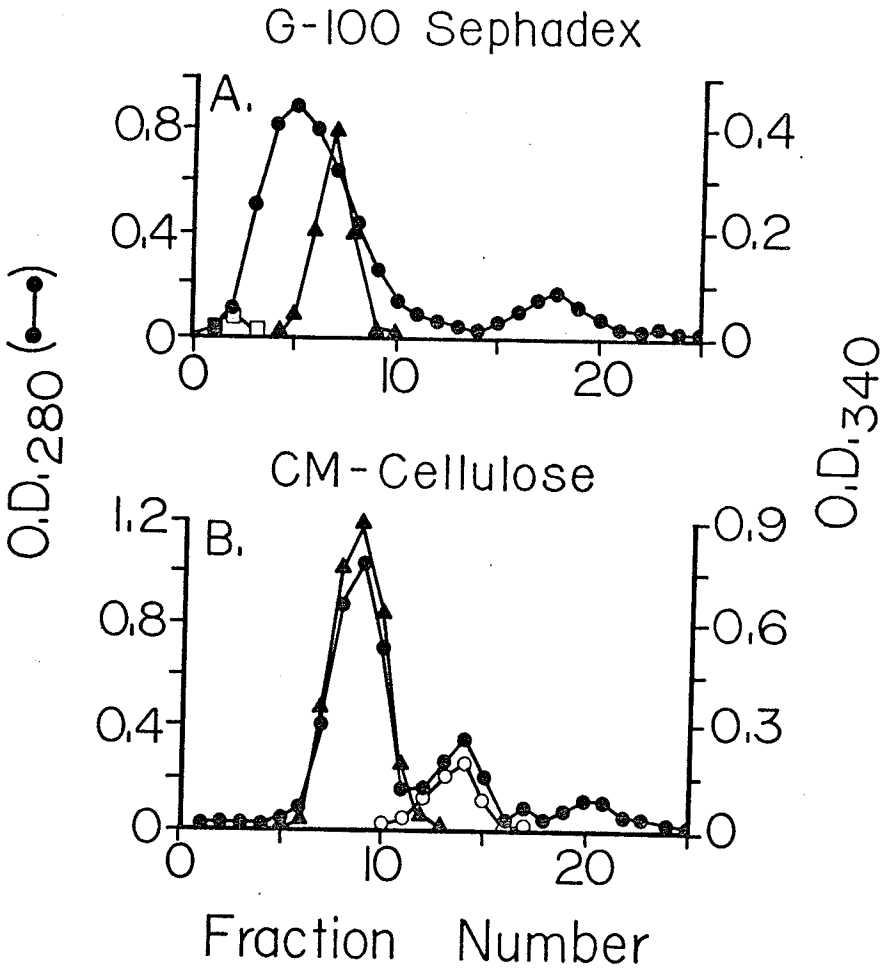


Fig. 6.41. Elution patterns obtained during the column chromatography of the IPC preparations on (A) Sephadex G-100 and (B) CM-cellulose.

● , protein
□ , ATP-dependent PC
▲ , ATP-independent PC
○ , malate dehydrogenase

was present in fractions 4-10. Following Sephadex G-100 chromatography, the fractions containing the IPC activity were pooled and concentrated by the Diaflo procedure. A dramatic increase in specific activity (i.e., 1.6 to 92 units) was observed following this procedure.

d) CM-cellulose column chromatography. The concentrated fraction from Sephadex G-100 column was then applied to a column (2.5 x 45 cm) of CM-cellulose and eluted with 0 - 0.3 M gradient of NaCl in phosphate buffer (50 mM, pH 7.0). Although not shown in Fig. 6.41(A), the IPC peak eluting from the Sephadex G-100 column was contaminated with a small amount of malate dehydrogenase activity; most of this activity was separated from the IPC enzyme during chromatography on CM-cellulose at NaCl concentrations above 0.1 M (Fig. 6.41(B)). Attempts were made to completely remove the MDH activity from the IPC fraction by applying a pooled and concentrated fraction from the CM-cellulose column to second CM-cellulose column and employing a lower NaCl gradient of 0.1 - 0.2 M. However, since this procedure resulted in the complete loss of IPC activity in fractions 5-10 of the first CM-cellulose column was concentrated by the Diaflo procedure and constituted the purified (135-fold, Table 6.26) ATP-independent 'pyruvate carboxylase'.

e) Purification summary. Table 6.26 summarizes the purification of the ATP-independent 'pyruvate carboxylase' from crude extracts of V. parvula M_4. This procedure, utilizing protamine sulphate and ammonium sulphate treatment followed by column chromatography on Sephadex G-100 and CM-cellulose, purified the enzyme 135-fold and was relatively efficient, losing only 35% of the initial total activity. The single most useful step was column chromatography on Sephadex G-100, which increased the

TABLE 6.26

Purification of ATP-independent 'pyruvate carboxylase' from *V. parvula* M_4.[a]

	Protein (mg)	Specific activity	Fold purification	Total activity ($x10^3$)
1. Crude extract	4800	72[b]	1.0	346
2. Protamine sulphate	4318	79	1.1	341
3. Ammonium sulphate (30 to 50%)	2875	113	1.6	325
4. Sephadex G-100	46	6590	92	303
5. CM-cellulose	24	9700	135	233

[a] Assayed by the NADH-MDH continuous method as outlined in Methods.

[b] µmoles NADH oxidized/mg protein/min.

specific activity 57 times. The 135-fold purified enzyme, concentrated by the diaflo procedure, was routinely stored at 4 C under nitrogen at a concentration of 20 mg/ml; in this condition it was stable for at least 3 months.

f) Purity of the enzyme. Throughout the entire purification procedure, the activity of malate dehydrogenase and the ATP-dependent pyruvate carboxylase was monitored in the fractions along with the activity of the ATP-independent enzyme (Table 6.27). As can be seen, while the IPC specific activity increased at each step of the process, activity for the DPC enzyme was eliminated after Sephadex G-100 column chromatography. Furthermore, only about 0.04% of the malate dehydrogenase activity was present in the final purified preparation, obtained after chromatography on CM-cellulose.

The purity of the purified IPC preparation was further tested by determining the distribution of label following the incubation of the enzyme with unlabelled bicarbonate and pyruvate-3-^{14}C (and Mg^{2+} ions). Table 6.28 shows that under these conditions the enzyme formed only oxaloacetate-^{14}C with 19% of the substrate pyruvate-3-^{14}C being utilized. In the presence of 5 mM ATP, however, less oxaloacetate-^{14}C was formed (12%) indicating that ATP inhibited the enzyme. In the presence of 5 mM NADH, 0.04% of the (^{14}C) appeared in malate confirming the presence of a small amount of MDH activity in the purified IPC preparation. This small amount of MDH contamination was considered insignificant for the purposes of this study since, in most cases, further studies employed the NADH-MDH assay method. Unlike the malate-lactate transhydrogenase from Veillonella sp. (Phares and Long, 1956), the purified IPC enzyme

TABLE 6.27

Presence of ATP-dependent pyruvate carboxylase and malate dehydrogenase activity during the purification of the ATP-independent pyruvate carboxylase.

	Purification	Specific activities		
		IPC	DPC	MDH
1.	Crude extract	72[a]	91	80
2.	Protamine sulphate	79	55	62
3.	Ammonium sulphate (30 to 50%)	113	60	72
4.	Sephadex G-100	6590	-	77
5.	CM-cellulose	9700	-	3.8

[a] µmole NADH oxidized/mg protein/min.

TABLE 6.28

Metabolism of pyruvate-3-^{14}C and unlabelled bicarbonate by the purified ATP-independent 'pyruvate carboxylase' of V. parvula M_4.

Addition to basic system[a]	Radioactivity (cpm)			
	Pyruvate-3-^{14}C	Oxaloacetate	Malate	Recovery %
Control (0 time)	989,675	-	-	-
None	805,374	189,352	-	99
+ ATP (5 mM)	778,596	122,431	-	90
+ NADH (5 mM)	879,428	121,420	438	100

[a] Basic system contained (in mM): pyruvate-3-^{14}C (4.9×10^5 cpm/μmole), 2.0; HCO_3^-, 10; $MgSO_4$, 5; phosphate buffer (pH 7.0), 50 and 0.67 μg of IPC enzyme in a volume of 1 ml. The reaction was stopped by 2,4 DNPH and the labelled components isolated by paper chromotography as described in Methods.

did not contain tightly-bound pyridine nucleotide, which might participate in the reaction, since the UV spectrum of the enzyme contained no absorbance peak at 340 nm (Appendix 1, p.255).

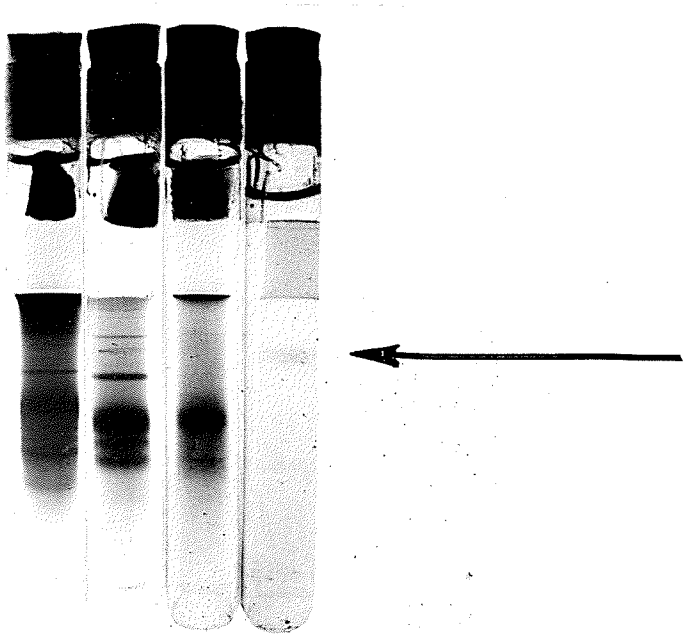
Additional testing showed that, in addition to DPC, the purified IPC preparation was completely free of activity for pyruvate dehydrogenase, malate-lactate transhydrogenase, NADP-specific malic enzyme, PEP carboxykinase and PEP carboxylase.

Fig. 6.42 shows the disc gel electrophoresis patterns of the enzyme preparation at various stages of the purification process. The type of gel and pH gradient used in this case was slightly different from that used with pyruvate kinase and permitted the clearer separation of the bands in the early fractions (tubes 1-3) and yet prevented the purified IPC enzyme from running with the indicator band (tube 4). This procedure, however, excluded larger protein molecules, as shown by the heavy stain in the sample gel in tubes 1 - 3. Fortunately, the stacking gel in tube 4 was free of stain, indicating that the final IPC preparation did not contain a large molecular weight component and was essentially devoid of other contaminating protein.

Part C - Characteristics of the IPC Enzyme

a) Stability of the enzyme. As observed previously (Table 6.20), the ATP-independent enzyme lost activity rapidly upon dialysis against 50 mM Tris-HCl buffer (pH 7.0) containing 20 mM β-mercaptoethanol. However, dialysis against phosphate buffer followed by charcoal treatment (Table 6.21) stabilized the enzyme.

Fig. 6.42. Disc gel electrophoresis patterns of the various fractions obtained during the purification of the ATP-independent 'pyruvate carboxylase' from V. parvula M_4. (1) crude extract, (2) following protamine sulphate treatment, (3) the 30-50% ammonium sulphate fraction and (4) the purified enzyme obtained following CM-cellulose column chromatography (Table 6.26).



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With this in mind, the stability of the purified IPC enzyme was tested following dialysis against various buffers (pH 7.0) e.g. phosphate, Tris, TES, HEPES, MES and arsenate (Table 6.29). The buffers were at a concentration of 50 mM and contained 20 mM mercaptoethanol. It can be seen that the enzyme was relatively stable in phosphate buffer for a period of 24 hours (i.e., 5% loss), but lost activity during dialysis and storage with Tris buffer confirming the earlier results (Table 6.20). With the HEPES, MES and TES buffers, enzyme stability was better than that observed with Tris but less than that obtained with phosphate buffer. Furthermore, the initial activity was approximately 10% less than that with the phosphate or arsenate buffers. With this latter buffer, no loss of IPC activity was observed on dialysis and storage for 24 hours, in fact, the enzyme was stable for more than 6 months when stored with this compound. As will be seen later (Fig. 6.48), concentrations above that used in this experiment (0.5 mM), inhibited enzyme activity. The presence of significant IPC activity in all of the non-phosphate buffers, except Tris-HCl, demonstrated that phosphate was not involved in the catalytic activity of the enzyme as originally thought (Table 6.20). Enzyme activity was destroyed by heating at 100C for 10 mins

b) Optimal conditions.

(i) pH optimum. Prior to more detail kinetic studies with the purified ATP-independent 'pyruvate carboxylase', the optimal conditions were established. As shown in Fig. 6.43 (A), the pH optimum for oxaloacetate formation by the IPC enzyme was 7.0. By plotting the log of the velocity against pH, it was possible to obtain the pK's of the ionizable groups in the ES complex, for the reaction converting bicarbonate and pyruvate

TABLE 6.29

Effect of dialysis in various buffers on the activity and stability of the purified IPC enzyme from V. parvula M_4.

Buffer[a]	Time (hours)		
	0	12	24
Phosphate	97[b]	95	93
Tris	26	11	3
HEPES[c]	93	80	72
MES[d]	92	84	70
TES[e]	90	79	70
Arsenate (0.5 mM)	97	97	97

a Enzyme was dialyzed onvernight in 2 litres of 50 mM (pH 7.0) buffer (except arsenate) containing 20 mM β-mercaptoethanol. The continuous spectrophotometric assay method was used.

b µmole NADH oxidized/mg protein/min x 10^{-2}.

c HEPES = N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid.

d MES = 2-(N-Morpholino)ethanesulfonic acid.

e TES = N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

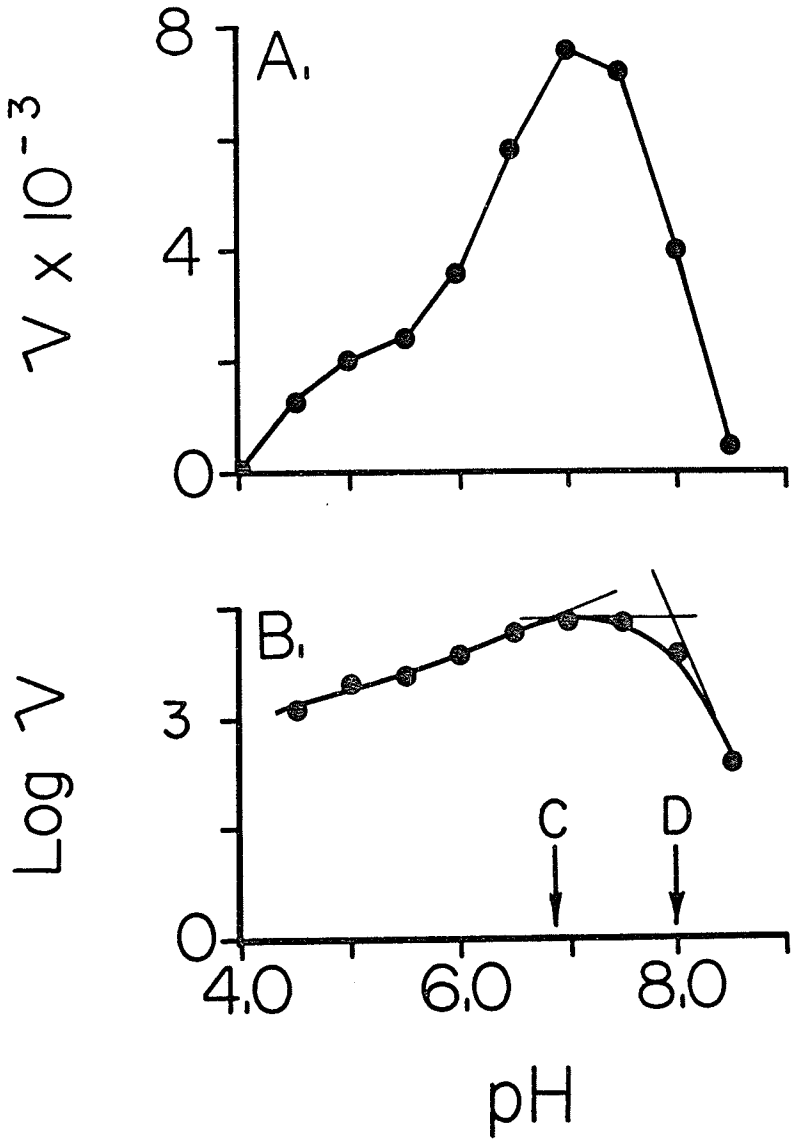

Fig. 6.43. Effect of pH on the activity of the purified IPC enzyme from V. parvula M_4.
Activity assayed by the NADH-MDH non-continuous method.

to oxaloacetate; pK values of 6.0 and 8.0 were obtained (Fig. 6.43(B)).

(ii) Pyruvate concentration. When tested at pH 7.0 and at saturating concentrations of Mg^{2+} and bicarbonate, the rate response curve for pyruvate was hyperbolic (Fig. 6.44(A)). The double reciprocal plot of the curve in (Fig. 6.44(A)) was a straight line which gave a K_m of 3.3 mM for pyruvate (Fig. 6.44(B)). The V_{max}, obtained from the same plot, was 9.3 mmoles NADH oxidized/mg protein/min, which was slightly higher than that obtained by inspection of Fig. 6.44(A) (8.0 mmoles/mg/min); maximum velocity was reached at a concentration of 20 mM pyruvate.

(iii) Bicarbonate concentration. The optimal bicarbonate concentration was obtained by varying the bicarbonate concentration in the presence of 20 mM pyruvate and 10 mM $MgSO_4$. A hyperbolic rate curve was obtained in this experiment (Fig. 6.45(A)), with the rate increasing to 10 mM bicarbonate and then levelling off. The double reciprocal Lineweaver-Burk plot gave a straight line (Fig. 6.45(B)) from which were obtained V_{max} and K_m values of 9.4 mmoles/mg/min and 1.74 mM, respectively. A concentration of 10 mM bicarbonate was used in future studies.

(iv) Magnesium concentration. When the Mg^{2+} ion concentration was varied in the presence of 20 mM pyruvate and 10 mM bicarbonate, a hyperbolic rate curve was obtained (Fig. 6.46(A)). The rate of IPC activity increased to 12 mM Mg^{2+} and then levelled off at an observed V_{max} of 10 mmoles/mg/min. A K_m of 1.85 mM was obtained for Mg^{2+} from the double reciprocal (Fig. 6.46(B)), with a V_{max} identical to that observed by inspection of Fig. 6.46(A). Thus, the optimum conditions for the formation of oxaloacetate by the ATP-independent 'pyruvate carboxylase' from V. parvula M_4 was with 20 mM pyruvate, 10 mM bicarbonate and 12 mM Mg^{2+} at a pH of 7.0.

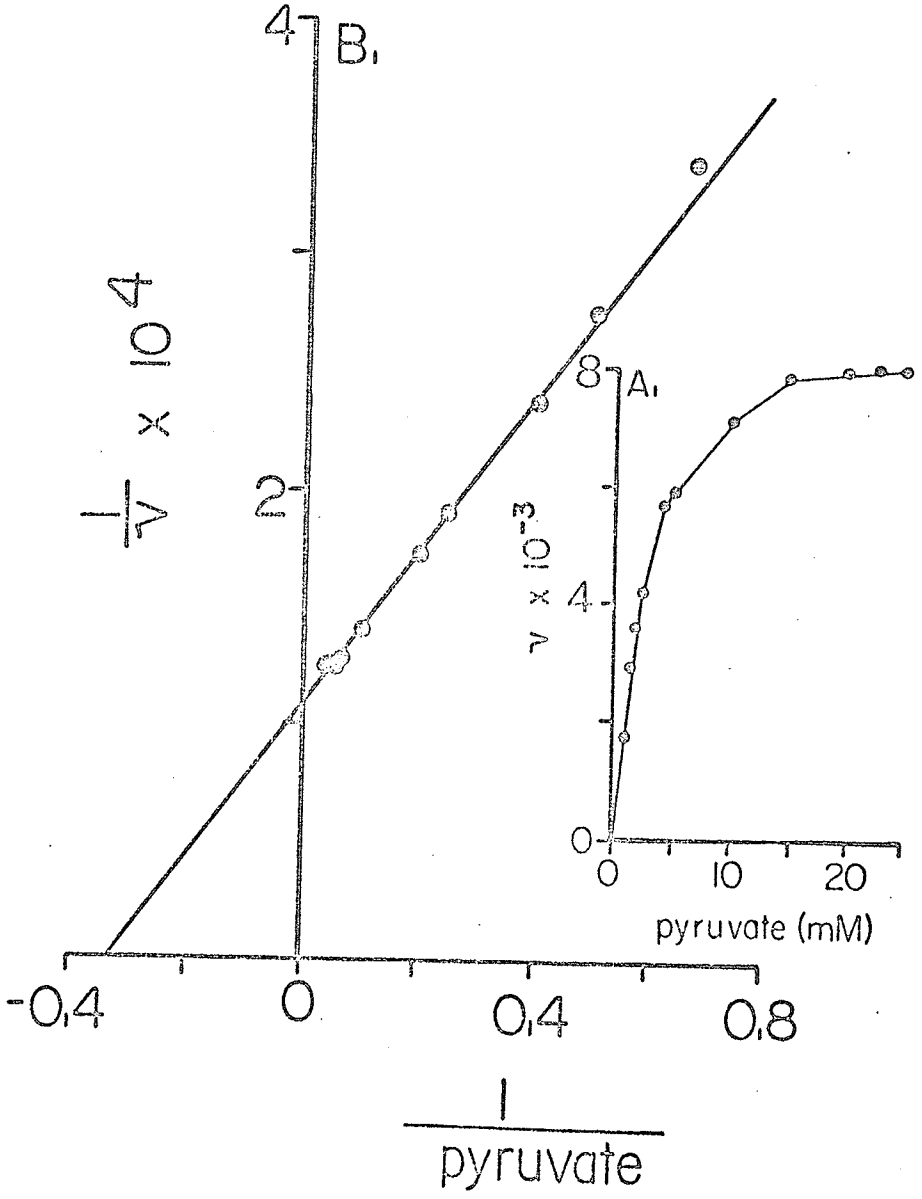

Fig. 6.44. Effect of pyruvate concentration on the activity of the purified IPC enzyme from V. parvula M_4.
Activity was assayed by the NADH-MDH continuous method with 10 mM sodium bicarbonate and 12 mM Mg^{2+}.

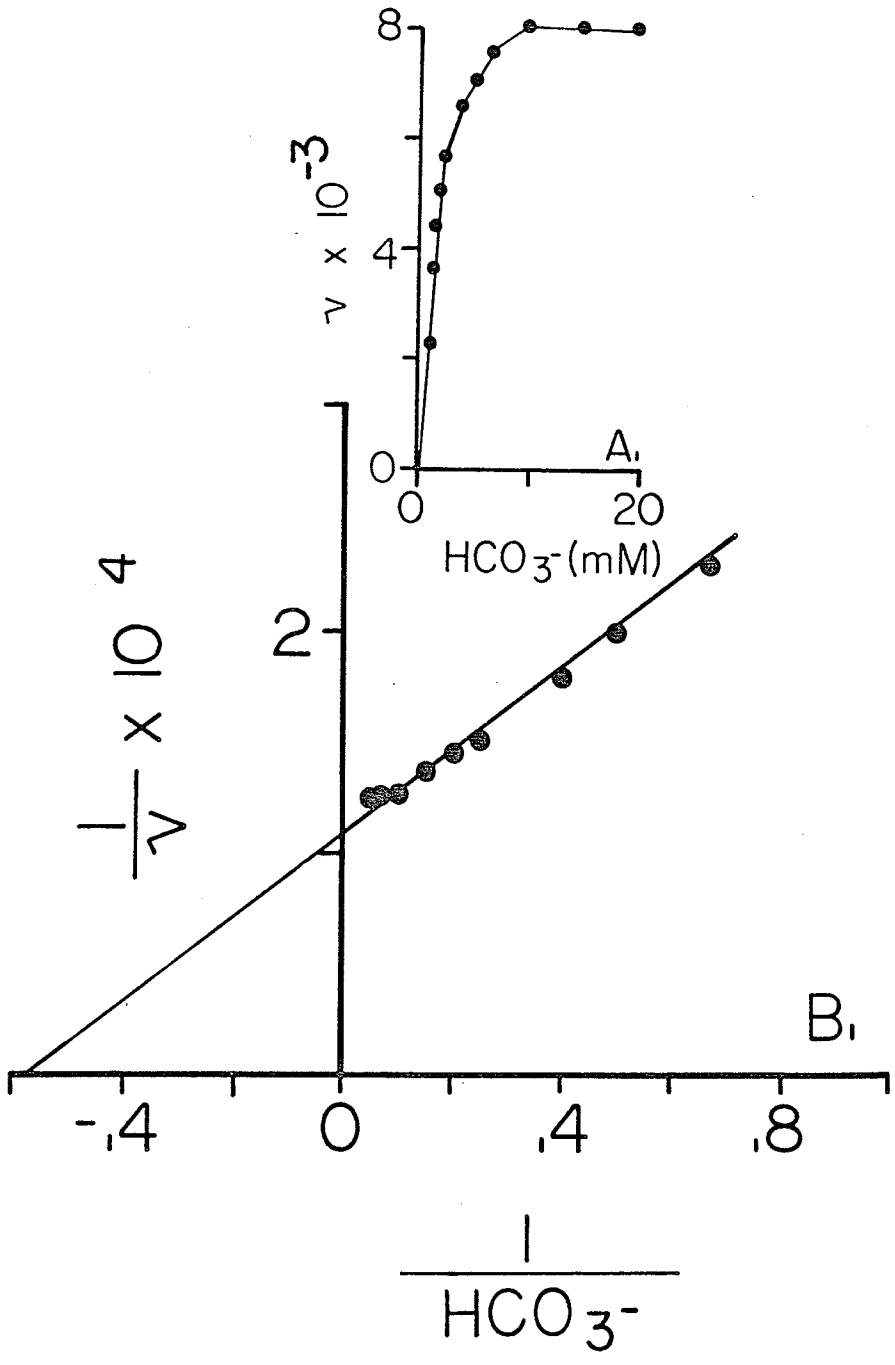

Fig. 6.45. Effect of sodium bicarbonate concentration on the activity of the purified IPC enzyme from V. parvula M_4. Activity was assayed as in Fig. 6.44 with 20 mM pyruvate and 10 mM Mg^{2+}.

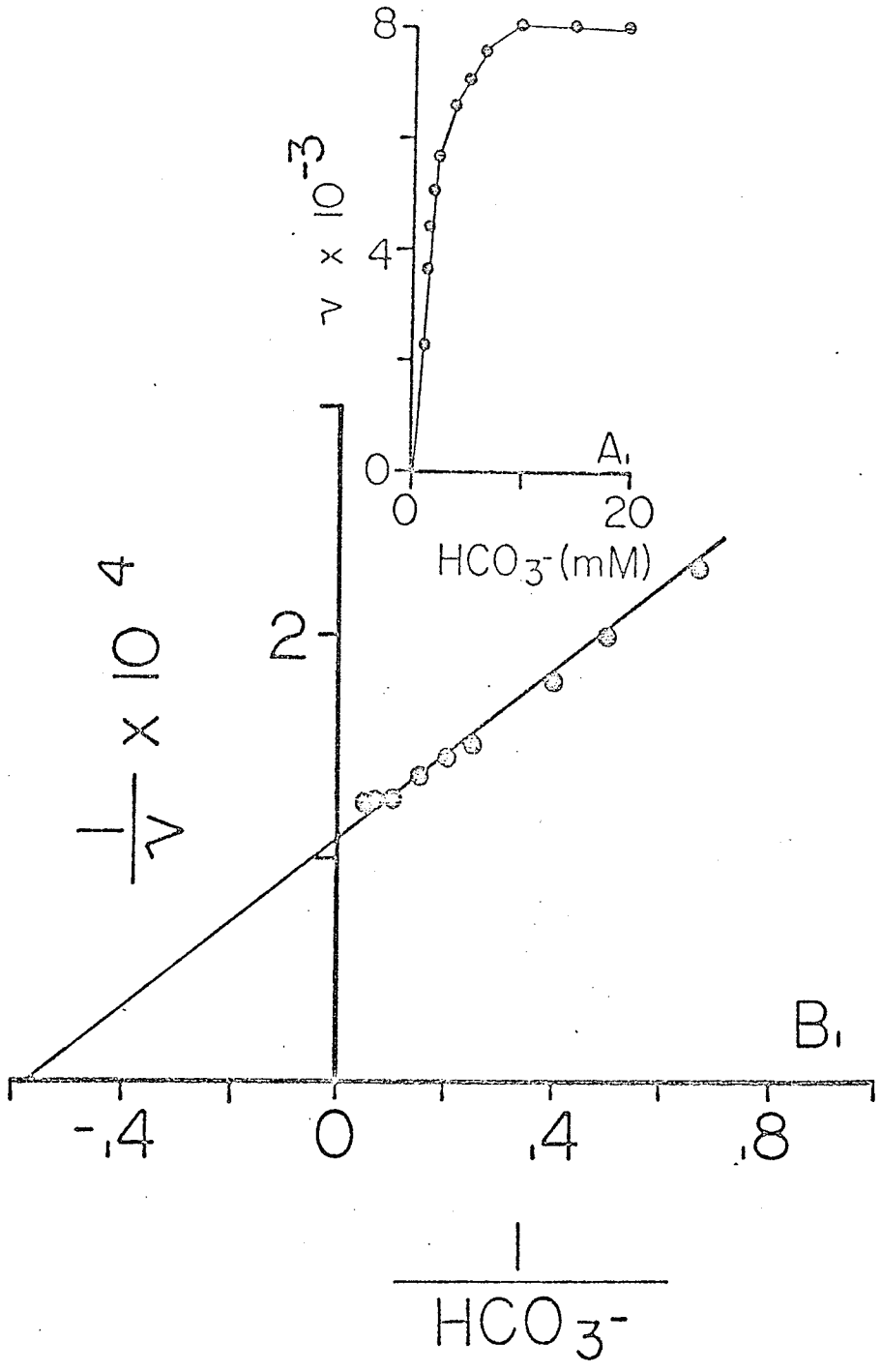

Fig. 6.45. Effect of sodium bicarbonate concentration on the activity of the purified IPC enzyme from V. parvula M_4. Activity was assayed as in Fig. 6.44 with 20 mM pyruvate, 10 mM Mg^{2+} in degassed buffer in sealed cuvettes.

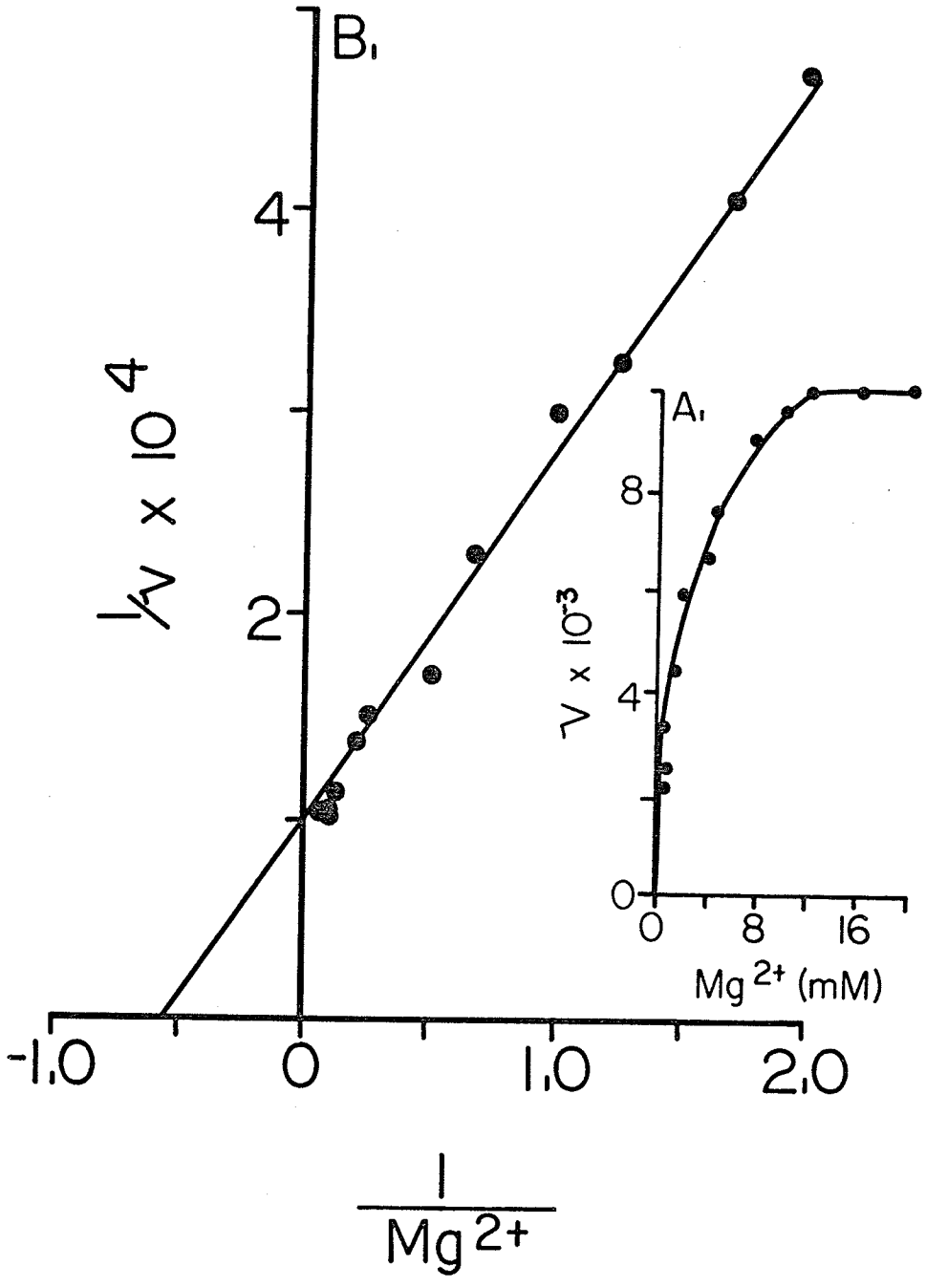

Fig. 6.46. Effect of magnesium concentration on the activity of the purified IPC enzyme from V. parvula M_4. Activity was assayed as in Fig. 6.44 with 20 mM pyruvate and 10 mM sodium bicarbonate.

Part D - Effectors for the IPC Enzyme

a) Preliminary data with the purified enzyme. As outlined previously (Table 6.24), ATP-independent 'pyruvate carboxylase' activity in the crude extracts was activated by aspartate and inhibited by acetyl CoA, coenzyme A and slightly inhibited by avidin. The effect of these and other compounds on the purified (135-fold) IPC enzyme was tested (Table 6.30) with the radioactive assay method, i.e., oxaloacetate-^{14}C was isolated following incubation with pyruvate-3-^{14}C and unlabelled bicarbonate. This assay was chosen over the MDH-NADH spectrophtometric method to eliminate the possible effects of the added compounds on malate dehydrogenase in the latter assay, these compounds were later shown, however, to have no effect on MDH. Despite the previous observations with crude extracts (Table 6.24), biotin, avidin, coenzyme A, CoA) and acetyl CoA, as well as ADP (exp. 2-6), had virtually no effect on the IPC enzyme. However, the enzyme was significantly inhibited (25%) by 5 mM ATP (exp. 8) and activated (1.4 times) by 10 mM aspartate (exp. 9). As 0.5 mM NADH (exp. 7) also inhibited IPC activity slightly, it was important that the NADH concentration not exceed this value in the NADH-malate dehydrogenase assay. Fortunately, the concentration of NADH used in previous NADH-MDH assays was 0.5 mM; subsequent spectrophotometric assays employed 0.2 mM NADH.

b) Inhibition by ATP. The inhibition of the ATP-independent enzyme by ATP, as demonstrated Table 6.30 , was examined in more detail at increasing ATP and pyruvate concentrations in the presence of excess bicarbonate and Mg^{2+}. By plotting the reciprocal of the velocity

TABLE 6.30

Effect of various compounds on the formation of oxaloacetate-^{14}C from pyruvate-3-^{14}C and unlabelled bicarbonate by the purified IPC from V. parvula M_4.

Exp.	Additions to basic system[a]	Concentration (mM)	Oxaloacetate formed (cpm x 10^{-2})	Relative activity[b]
1	None	-	325	1.0
2	+ biotin	8	318	0.98
3	+ avidin	2 (units)	324	0.99
4	+ CoA	0.2	315	1.01
5	+ acetyl CoA	0.2	327	1.01
6	+ ADP	5	317	0.97
7	+ NADH	0.5	295	0.91
8	+ ATP	5 mM	243	0.75
9	+ aspartate	10	447	1.38

a Basic system and analysis was as in Fig. 6.23, except that pyruvate was 1.5 x 10^5 cpm/µmole and incubation was for 45 min.

b Activity relative to activity with no additions.

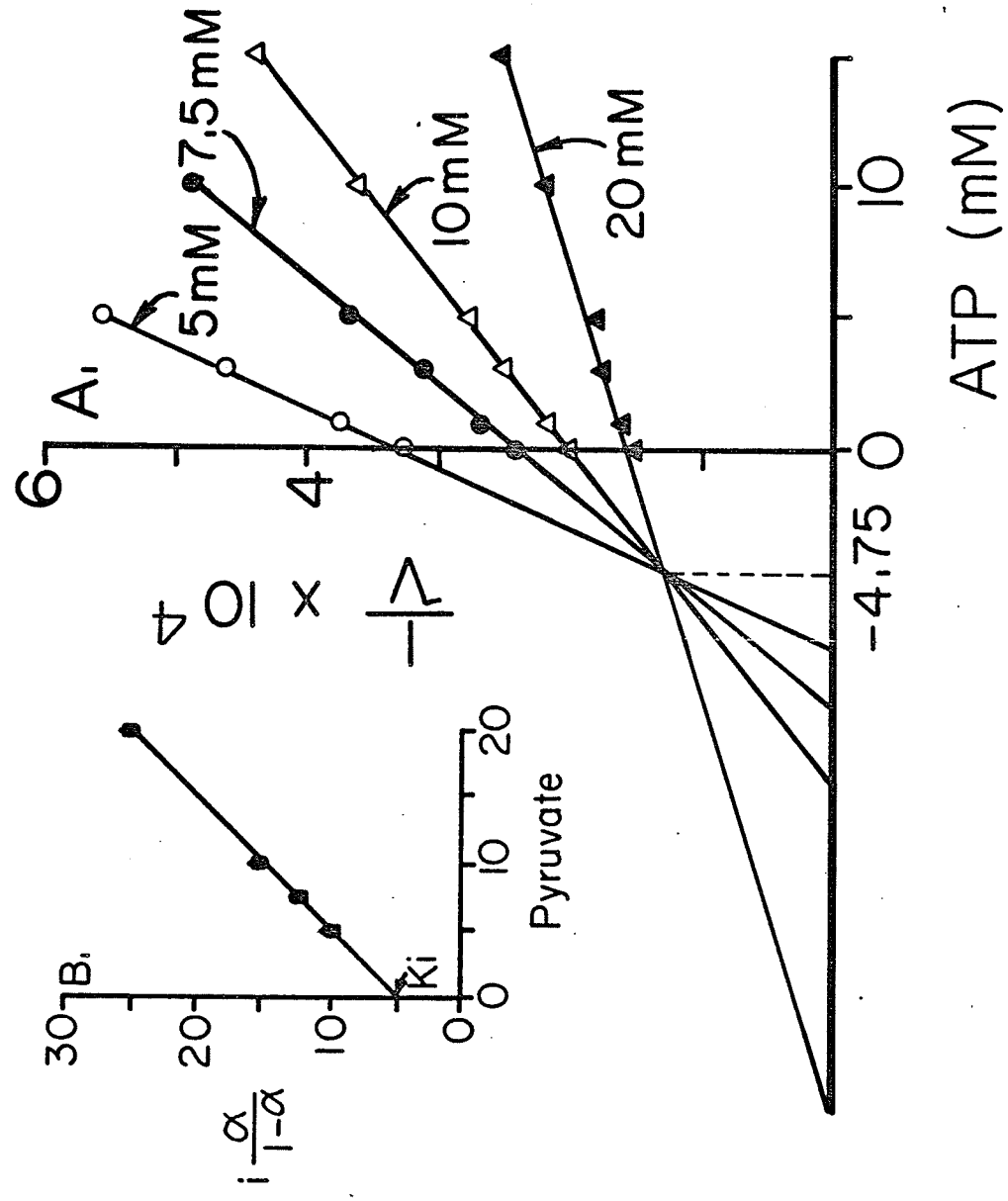

Fig. 6.47. Inhibition of the purified IPC enzyme from V. parvula M_4 vy ATP. Activity assayed as in Fig. 6.44.

against the ATP concentration (Webb, 1963), it can be seen that the inhibition was competitive with respect to the pyruvate (Fig. 6.47(A)); an inhibition constant (K_i) of 4.75 mM was obtained. This value confirmed by plotting the data ccording to the method of Hunter and Downs (Fig. 6.47(B)).

c) Inhibition by other compounds. In addition to ATP, adenosine monophosphate (AMP), cyclic adenosine 3',5'-monophosphate (cyclic AMP) and arsenate also inhibited the activity of the V. parvula ATP-independent 'pyruvate carboxylase'. With AMP, rapid and progressive inhibition was observed to a concentration of 5 mM when inhibition had reached 50%; higher AMP concentrations had no additional inhibitory effect. On the other hand, the inhibition by cyclic AMP was progressive up to a concentration of 15 mM when only 11% of the original IPC activity remained. It can also be seen that cyclic AMP was a more potent inhibitor than ATP. Probably the most surprising result was the inhibition by arsenate since dialysis against 9.5 mM arsenate had been previously shown to stabilize the enzyme (Table 6.29). With this compound, progressive inhibition was observed to 10 mM (50% inhibition), but higher concentrations (to 70 mM) had no further effect on enzyme activity. Obviously, the concentration of arsenate used for the stability test outlined in Table 6.29 (0.5 mM) was not high enough to cause inhibition of the enzyme.

d) Activation by aspartate. As shown in Table 6.30, aspartate was slight activator of the ATP-independent enzyme. This activation was studied further by observing the effect of 10 mM aspartate on enzyme activity at increasing concentrations of pyruvate (Fig. 6.49(A)). Aspartate did not activate the enzyme below 2.3 mM pyruvate but did produce progressive

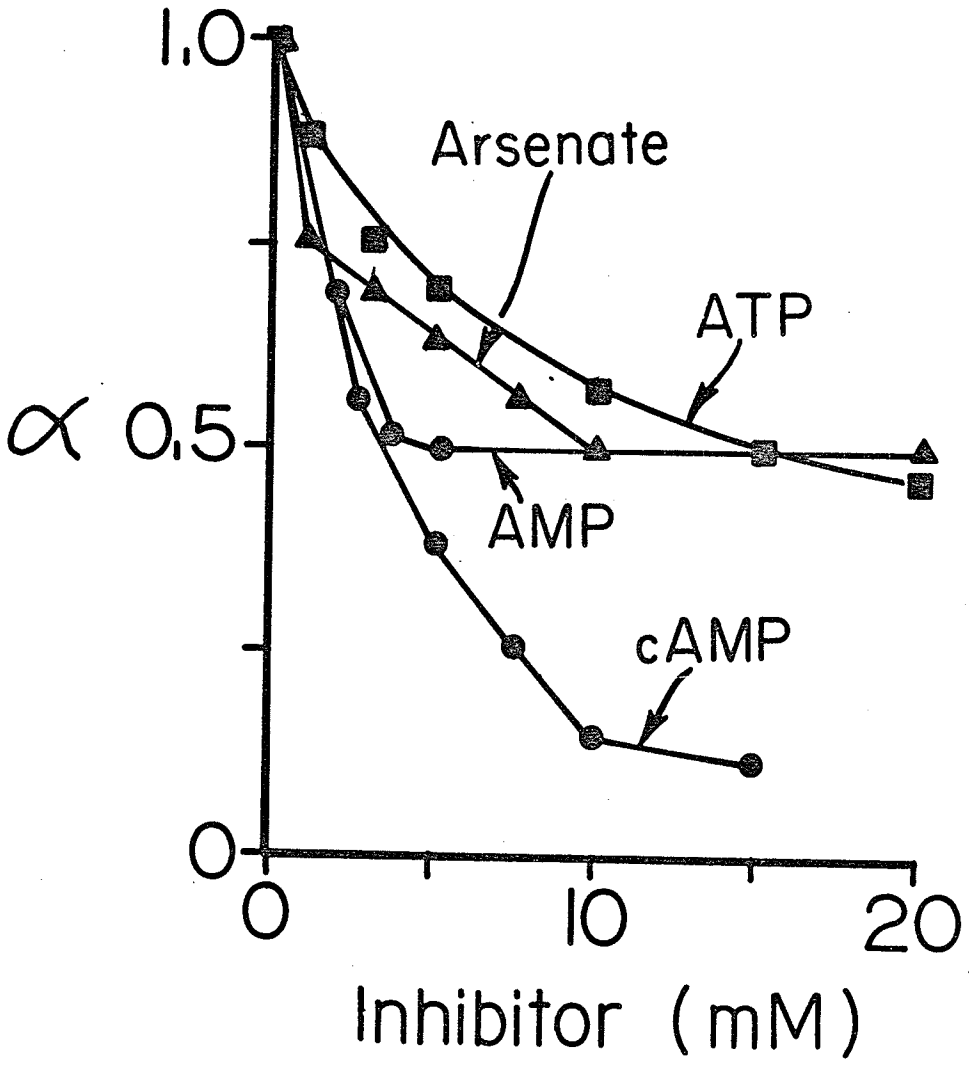

Fig. 6.48. Inhibition of the purified IPC enzyme from V. parvula M_4 by arsenate, ATP, AMP and cyclic AMP.
Activity assayed as in Fig. 6.44.

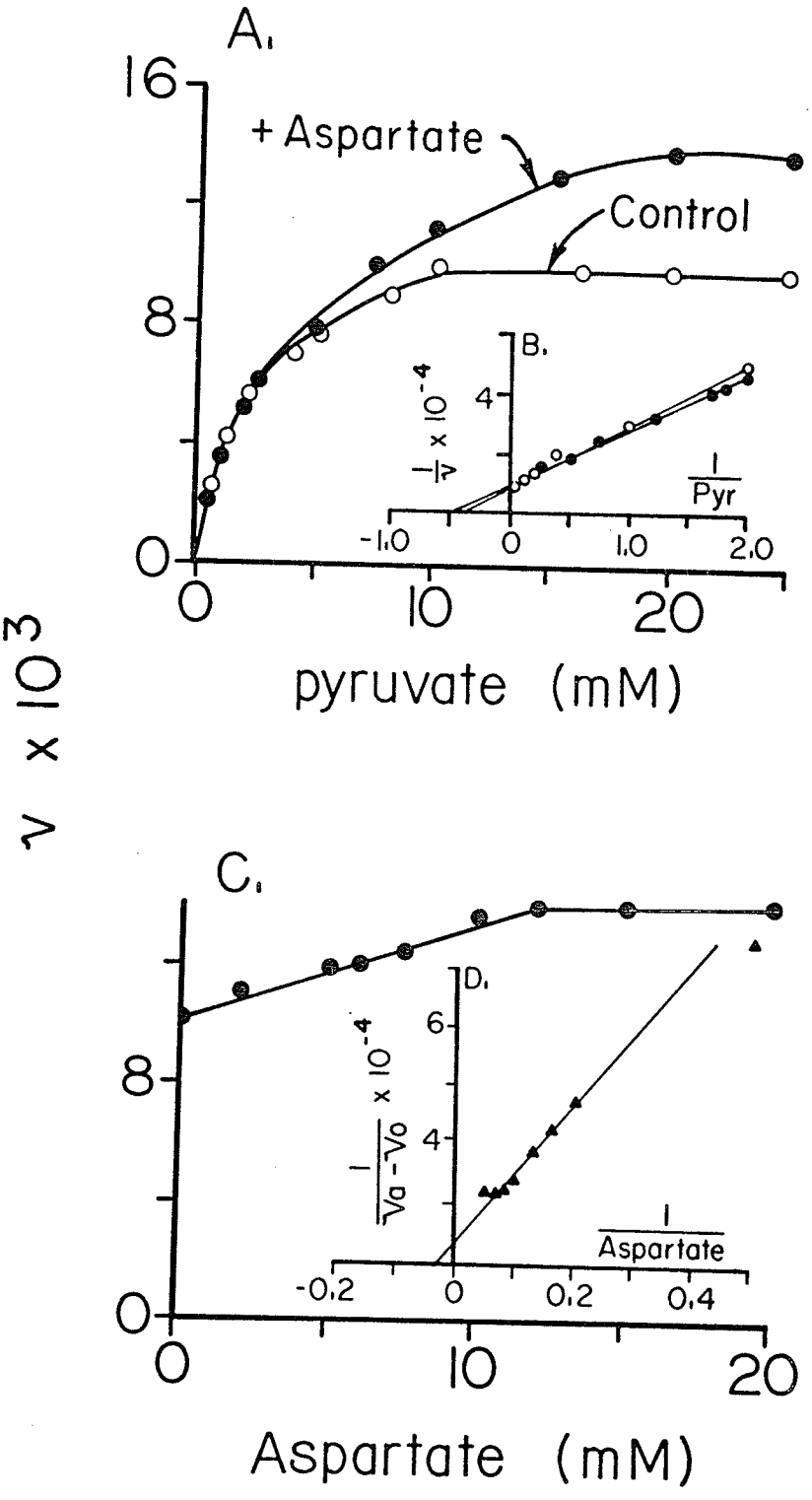

Fig. 6.49. Activation of the IPC enzyme from V. parvula M_4 by aspartate.

activation at higher concentrations of pyruvate. At the 20-25 mM pyruvate, the observed increase in V_{max} was approximately 40% (10 to 14 mmoles/mg protein/min), while the double reciprocal plot (Fig. 6.49(B)) showed that in the presence of aspartate the K_m for pyruvate decreased slightly from 3.3 to 2.2mM

By varying the aspartate concentration of 20 mM pyruvate, the activation by aspartate was found to be proportional to the aspartate concentration up to 12 mM (Fig. 6.49(C)); above this concentration no further activation was observed. By replotting the data in Fig. 6.49(C),with $(1/v_a - v_o)$ versus (1/aspartate) (Dixon and Webb, 1964), the activation constant (K_a) for aspartate (33 mM) was obtained (Fig. 6.49(D)). While this small aspartate activation may not have any physiological significant, it does differentiate the V. parvula IPC enzyme from the conventional pyruvate carboxylase which are inhibited by aspartate.

The Hill plot of the data in Fig. 6.49(A) gave a Hill coefficient of 1.0 with pyruvate alone, suggesting only one binding site for pyruvate on the enzyme (Fig. 6.30). On the other hand, the addition of 10 mM aspartate gave a curved line with a value of 0.9 between 0.5 - 5.0 mM pyruvate. Beyond this pyruvate concentration, the curve tended to increase exponentially and gave a value of 2.0 from a best-fitting straight line through the curve. Thus, it appears that above 8.0 mM pyruvate, the co-operativity between the ATP-independent enzyme and pyruvate increased in the presence of aspartate although it may have been due solely to the high levels of pyruvate in the assay.

c) Equilibrium constant for the IPC enzyme. Investigators working with carbon dioxide fixation reactions in microorganisms generally agree that some form of high energy compound must be available for fixation to occur. Thus, the data presented in this chapter, demonstrating the formation of oxaloacetate by a purified protein fraction from V. parvula M_4 in the

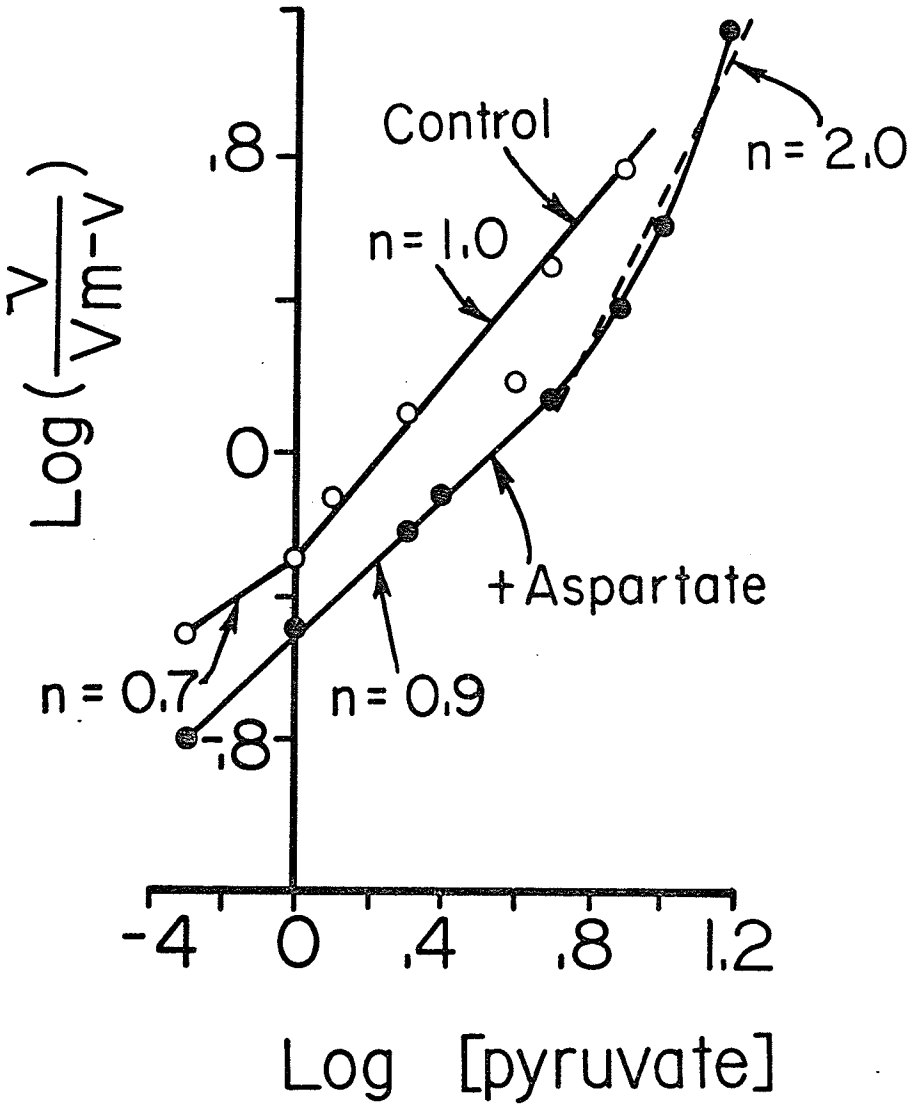

Fig. 6.50. Hill plot of the data in Fig. 6.49.

presence of only pyruvate, bicarbonate Mg^{2+} ions, is contrary to this general concept. The question remains as to whether this reaction is thermodynamically favourable in the direction of oxaloacetate synthesis. In an attempt to answer this question, the equilibrium constant (Keq) for the IPC reaction was determined at various temperatures (Fig. 6.51) and the standard free energy change (ΔG^{o}) calculated. To ensure that the reaction at each temperature had reached equilibrium, the assays were incubated for 90, 120 and 150 min and the concentration of oxaloacetate, pyruvate determined; in all cases, equilibrium was reached in 120 min. A Q_{10} value of 1.6 was obtained from Fig. 6.51.

The respective concentrations of the substrates (pyruvate and bicarbonate) and product (oxaloacetate) at equilibrium (25 C) are shown in Table 6.31. From these values, an equilibrium constant (Keq) of 2.13, in the direction of oxaloacetate formation was obtained, which resulted in a calculated ΔG^{o} of -0.44 Kcal/mole. Although this standard free-energy change is small, it indicates that oxaloacetate formation from pyruvate and CO_2 by the ATP-independent enzyme is thermodynamically feasible, particularly if coupled to another, more negative, exergonic reaction.

IV. DISCUSSION

The role of carbon dioxide fixation in the degradation of lactate to propionate by members of the Veillonella was suggested by Johns as

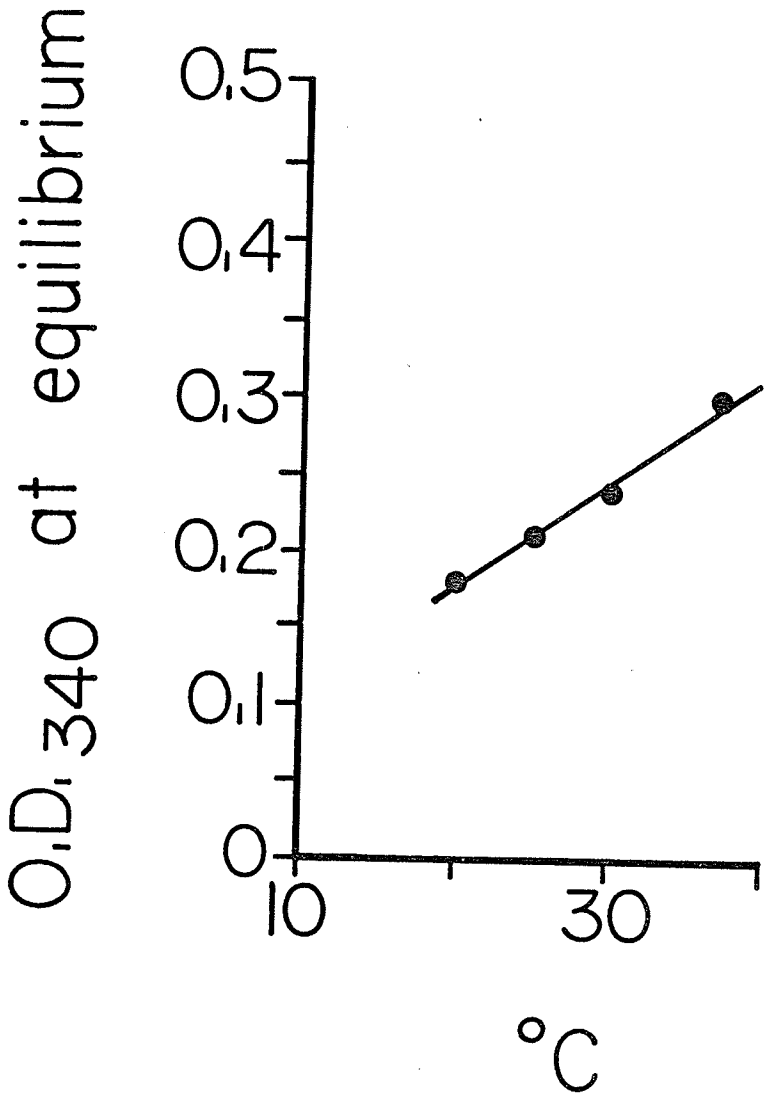

Fig. 6.51. Effect of temperature on the equilibrium concentration of oxaloacetate synthesized by the purified IPC enzyme from V. parvula M_4.

TABLE 6.31

The concentration of pyruvate, bicarbonate and oxaloacetate at equilibrium following catalysis by the purified ATP-independent 'pyruvate carboxylase' from V. parvula M_4.

Compounds	Concentrations at 25 C[a] (mM)
Pyruvate	19.6
Bicarbonate	9.6
Oxaloacetate	0.4
K_{eq} (PYR + CO_2 ⟶ OAA)	2.13
ΔG^o	-0.447 Kcalories

[a] The reaction mixture (in mM): pyruvate, 20; HCO_3^-, 10; Mg^{2+}, 10; phosphate buffer(pH 7.0), 50 and 0.67 µg of IPC enzyme in 1 ml. At equilibrium (120 min), 0.2 ml of 5 N NaOH was added to stop the reaction and then the contents centrifuged at 35,000 g at 4 C for 15 min following neutralization. The supernatant was divided into two portions: one portion was assayed for oxaloacetate by the malate dehydrogenase assay, while the second assayed the remaining pyruvate by the lactic dehydrogenase method. The amount of HCO_3^- remaining at equilibrium was calculated from the amount of oxaloacetate formed.

[b] $$K_{eq} = \frac{(OAA)}{(pyruvate)\ (HCO_3^-)}$$

early as 1951. Although CO_2-fixation was clearly demonstrated with crude extracts of V. parvula M_4 (Ng, 1968), the enzymes responsible for this fixation in this genus had not been elucidation.

The results in this chapter indicate that two separable enzyme activities are capable of forming oxaloacetate from pyruvate and CO_2: one requiring ATP and the other active without ATP. The first enzyme is clearly a conventional pyruvate carboxylase (Wood and Stjernholm, 1962), although its properties were not studied. The second enzyme has been called ATP-independent 'pyruvate carboxylase' for the want of a better name. It might be argued that this latter enzyme is really an oxaloacetate decarboxylase, however, the ability of this enzyme to synthesize net quantities of oxaloacetate suggested that this latter name was not appropriate. In order to determine whether the ATP-independent enzyme is more related to the conventional ATP-dependent pyruvate carboxylase (DPC) or to oxaloacetate decarboxylase (OAA-DC), the characteristics of the V. parvula enzyme was compared to the DPC from Sacch. cerevisiae (Ruiz-Amil et al, 1965) and the decarboxylases from M. lysodeikticus (Herbert, 1950, 1955) and Azoto. vinelandii (Plant and Lardy, 1949) (Table 6.32). The activity of both the ATP-independent and ATP-dependent enzymes was measured by formation of oxaloacetate from pyruvate and bicarbonate, while the decarboxylases were assayed in the opposite direction.

While the kinetic constants and pH optima for these enzymes varied considerably, as might be expected, the aspects which most clearly differentiated them were their cofactor requirements. The activity of V. parvula IPC enzyme was not affected by biotin and acetyl CoA

TABLE 6.32

Comparison of the characteristics of the V. parvula M_4 ATP-independent pyruvate carboxylase with the (ATP-dependent) pyruvate carboxylase from Sacch. cerevisiae and the oxaloacetate decarboxylases from Micro. lysodeikticus and Azoto. vinelandii.

	IPC	DPC[a]	OAA-DC[b]
K_m values			
Pyruvate	3.3	0.8	
Bicarbonate	1.74	2.7	
Oxaloacetate			2.0
Magnesium (Mn^{2+})	1.3	4.2	0.2
Optimum pH	7.0	8.4	5.4
K_{eq} (for OAA synthesis)	2.13		1.1×10^{-3}
Cofactors			
Acetyl CoA or CoA derivatives	NR	R	(-)
Biotin	NR	R	NR
Effectors			
Oxalate		(-)	
Arsenate	(-)		
ATP	(-)	(+)	(-)[c]
ADP	(0)		
AMP	(-)		
cAMP	(-)		
NADH	(-)		
L-aspartate	(+)	(-)	
P_i	(0)		(-)[c]
Avidin	(0)	(-)	

[a] Pyruvate carboxylase (ATP-dependent) from Sacch. cerevisiae (Ruiz-Amil et al, 1965).

[b] OAA-decarboxylase from Micro. lysodeikticus (Herbert, 1950, 1955) unless otherwise specified.

[c] OAA-decarboxylase from Azoto. vinelandii (Plant and Lardy, 1949).

R = required; NR = not required; (0) = no effect; (-) = inhibitor; (+) = activator.

(Table 6.30), while both of these compounds were required by the ATP-dependent pyruvate carboxylase from yeast. On the other hand, acyl CoA derivatives inhibited the OAA decarboxylases from M. lysodeikticus (Herbert, 1951) and Pseud. ovalis (Horton and Kornberg, 1964). The enzyme from M. lysodeikticus (Herbert, 1950), like the V. parvula IPC enzyme, was not affected by biotin.

Of the three enzymes listed in Table 6.32, pyruvate carboxylase (ATP-dependent) has been the most intensively studied. As mentioned previously, all pyruvate carboxylases require biotin for activity, the biotin being tightly-bound to the enzyme and not really dialyzable. As a consequence of the biotin requirement, the enzyme is readily inhibited by avidin.

Considerable information is now available on pyruvate carboxylase purified from Sacch. cerevisiae. This enzyme has been shown to catalyze pyruvate carboxylation, oxaloacetate decarboxylation, P_i-ATP exchange and pyruvate-oxaloacetate exchange reactions (Cazzulo and Stoppani, 1967). The rates of oxaloacetate decarboxylation and P_i-ATP exchange were approximately 2% of the rate of pyruvate carboxylation, but all of these reactions were inhibited by avidin to the same extent. The enzyme was strictly dependent on Mg^{2+} (or Mn^{2+}) ions and was inhibited by Ca^{2+} and other divalent cations. K^+, Rb^+ and NH_4^+ were activators, whereas Na^+ and Li^+ ions were inhibitors. This enzyme also required acetyl CoA, Mg^{2+} and K^+ for the activation reaction.

A similar requirement for acetyl CoA was seen with the pyruvate carboxylase isolated from Rhizopus nigricans (Overman and Romano, 1969) and Neocosmospora vasinfecta (Budd, 1971). On the other hand, the

carboxylases purified from Pseud. citronellolis (Seubert and Remberger, 1961) and Aspergillus niger (Woronick and Johnson, 1960) were not activated by acetyl CoA while the enzyme from Chromatium was only partially activated by this compound (Fuller et al, 1961). All these enzymes, however, were inhibited by avidin.

While the lack of a requirement for biotin and CoA derivatives differentiates the V. parvula IPC enzyme from the 'conventional' pyruvate carboxylase, other differing characteristics can be found. For example, aspartate is an allosteric inhibitor of the pyruvate carboxylase from Sacch. cerevisiae (Palacian, de Torrontegui and Losada, 1966), with inhibition competitive with respect to acetyl-CoA (Cazzulo and Stoppani, 1967). However, the inhibition of the enzyme from Asper. niger by aspartate was simple, linear non-competitive inhibition (Feir and Suzuki, 1969); the difference between these latter two enzymes was attributed to the fact that acetyl -CoA had no effect on the Asper. niger enzyme. In direct contrast to these results, was the observation in this work that the ATP-independent carboxylase from V. parvula M_4 was activated by aspartate (Fig. 6.49) with a K_a of 33 mM.

Probably the most important characteristic differentiating the IPC enzyme from the ATP-dependent carboxylases was the effect of ATP. For these latter enzymes, ATP is a mandatory requirement for CO_2-fixation, while with the V. parvula IPC enzyme, this nucleotide was competitive inhibitor (K_i = 4.75 mM). Thus, it is apparent that, the so-called ATP-independent 'pyruvate carboxylase' from V. parvula M_4 is not a 'conventional' pyruvate carboxylase.

The characteristics differentiating the IPC enzyme from the

oxaloacetate dicarboxylases are not as numerous since relatively little research has been carried on the latter enzyme. This probably resulted from the conclusion in early 1960's that net carbon dioxide fixation would not occur in the absence of ATP (Wood and Stjernholm, 1962), and therefore the enzyme was of less significance in the overall formation of dicarboxylic acids. This conclusion was reached despite the observation by Herbert (1951) that the formation of oxaloacetate, or oxaloacetate-like material, would occur by means of a pyruvate/CO_2-fixation reaction catalyzed by the purified oxaloacetate decarboxylase of M. lysodeikticus coupled to the malate dehydrogenase-NADH system. This observation, as well as that by Kaltenbach and Kalnitsky (1951) demonstrating the formation of oxaloacetate by extracts of E. coli and Proteus morganii by CO_2-fixation in the absence of ATP, confirmed the earlier results of Krampitz, Werkman and Wood (1943) with M. lysodeikticus.

In addition to the previously mentioned differences in cofactor requirements, the oxaloacetate decarboxylase from Azoto. vinelandii was inhibited by inorganic phosphate (Plant and Lardy, 1949), while the V. parvula IPC enzyme was somewhat more active with P_i and was also stabilized by this compound (Table 6.29). The single most important factor differentiating the IPC enzyme from the decarboxylase from M. lysodeikticus (Herbert, 1950, 1955) was the equilibrium of the reaction. The Keq (890) for the latter enzyme at pH 5.4 greatly favoured oxaloacetate decarboxylation, while that for the IPC enzyme (2.14), albeit low, favoured oxaloacetate formation. Oxaloacetate decarboxylation by the V. parvula was not actively pursued because of the difficulty in obtaining 'pure' oxaloacetate and lactic dehydrogenase free of malic

dehydrogenase. Since chemical decarboxylation of oxaloacetate is well-known (Krebs, 1942), it was hoped that oxaloacetate decarboxylation could be monitored by converting the pyruvate formed to lactate with LDH and NADH. A variety of experiments were undertaken to test for decarboxylation, but the small amount of MDH activity, both in the commercial LDH and in the IPC enzyme preparations, made this assay unfeasible.

With the foregoing comparison, it is apparent that the purified ATP-independent enzyme from V. parvula M_4 is a unique enzyme. In terms of oxaloacetate formation it is somewhat akin to the ATP-dependent pyruvate carboxylases, yet is not affected by biotin and avidin, and is inhibited by ATP. On the other hand, the Keq for the enzyme and the effects of acyl CoA derivatives and P_i differentiate it from the oxaloacetate decarboxylases.

Evidence for ATP-independent CO_2-fixing activity in other Veillonella sp.

Michaud (1968), in studies with untreated and Dowex-treated extracts of V. alcalescens, suggested that carbon dioxide fixation by this organism was carried out by PEP carboxykinase and pyruvate carboxylase. Of interest to the present investigation with V. parvula are his observations relative to pyruvate carboxylase, which appeared in his Ph.D. Thesis (Table 7, p. 131). These results have been reproduced here for ease of discussion (Table 6.33). Michaud's experiments 1 - 3, 6 and 9 were assays designed to demonstrate pyruvate carboxylase (ATP-dependent) activity in V. alcalescens. The assay consisted of measuring the presence of (^{14}C) from bicarbonate-^{14}C in the hydrazone precipitate,

TABLE 6.33

Assay[a] of pyruvate carboxylase activity in extracts of Veillonella alcalescens ^{14}C as determined by Michaud[b]

Exp.	Components added[c]	Radioactivity fixed in hydrazone precipitate (net cpm/0.1 ml)
1	Pyruvate, Mg^{2+}, ATP	1,832
2	Pyruvate (½X), Mg^{2+}, ATP	1,648
3	Pyruvate (2X), Mg^{2+}, ATP	2,262
4	Pyruvate, ATP	870
5	Pyruvate, Mg^{2+}	224
6	Pyruvate, Mn^{2+}, ATP	1,313
7	Pyruvate, Mg^{2+}, ATP, ADP	2,905
8	Pyruvate, Mg^{2+}, P_i	6,810
9	Pyruvate, Mg^{2+}, ATP, P_i	7,200

[a] Basic system (in µmoles): Tris-HCl (pH 7.6),150; GSH, 2.4; NaF, 15; β-mercaptoethanol, 30; $NaHCO_3$, 49; $NaH^{14}CO_3$, 9.52×10^5 cpm; extract Dowex-1 treated 3.18 mg; and water to 1.5 ml. Temperature, 37 C; gas phase 100% N_2; incubation time 7 min.

[b] Results taken from Table 7 of the Ph.D. Thesis of R.N. Michaud, Cornell University, p. 131 (1968).

[c] Components added (expressed as µmoles): pyruvate, 20 (except exp. 2, 10 µmoles and exp. 3, 40 µmoles); $MgCL_2$, 7.5; $MnCl_2$, 7.5; ATP, 5; ADP, 10; P_i, 10.

'tentatively identified' as the oxaloacetate hydrazone. Of particular interest were the results in experiment 8, where 6,810 cpm appeared in the hydrazone precipitate following incubation of the Dowex-treated extract with only pyruvate, $HC^{14}O_3^-$, Mg^{2+} and P_i. This observation suggested that an ATP-independent enzyme, similar to the V. parvula M_4 enzyme, was also present in V. alcalescens. Unfortunately, Michaud did not clearly identify the radioactive hydrazones formed and it is likely that a portion of the label in the hydrazone material may be radioactive pyruvate, formed by the CO_2-pyruvate exchange reaction. Although the exact distribution of the (^{14}C) between pyruvate and oxaloacetate was not measured, a significant fraction of the label could have been in oxaloacetate since Whiteley and McCormick (1963) have shown that extracts of Micrococcus lactilyticus (V. alcalescens) rapidly lost their ability to carry out the CO_2-pyruvate exchange reaction following passage of the extracts through Dowex-1. Thus, it is conceivable that all species of this genus, Veillonella, contain the unusual ATP-independent 'pyruvate carboxylase'.

CHAPTER 7

GENERAL DISCUSSION

The results obtained in the present study clearly show that lactate carbon is utilized by Veillonella parvula M_4 during gluconeogenesis for the synthesis of glucose, glucosamine, galactose, galactosamine and ribose, as well as other cellular constituents. Furthermore, the enzymatic studies have now given some indication as to how the inital reactions of lactate metabolism are related to the synthesis of these compounds.

Coupled with data obtained previously (Ng, 1968, Ng and Hamilton, 1971), V. parvula M_4 has been shown to contain the following enzymes: malate-lactate transhydrogenase, pyruvate dehydrogenase, malate dehydrogenase, pyruvate kinase, pyruvate carboxylase (ATP-dependent), 'pyruvate carboxylase' (ATP-independent), enolase, along with an indication of activity for PEP carboxykinase, NADP-specific and NAD-specific malic enzymes, and possibly PEP carboxylase. While the presence of activity for the latter enzymes was observed in crude extracts, the ability to observe activity both by the spectrophotometric method and by radiochemical means, substantiated their presence in V. parvula M_4. In addition to the above, evidence was presented against the presence of PEP synthetase or pyruvate, phosphate dikinase in the organism.

The possible relationship of these enzymes to the metabolism of lactate by V. parvula M_4 is depicted in Fig. 7.52. As can be seen,

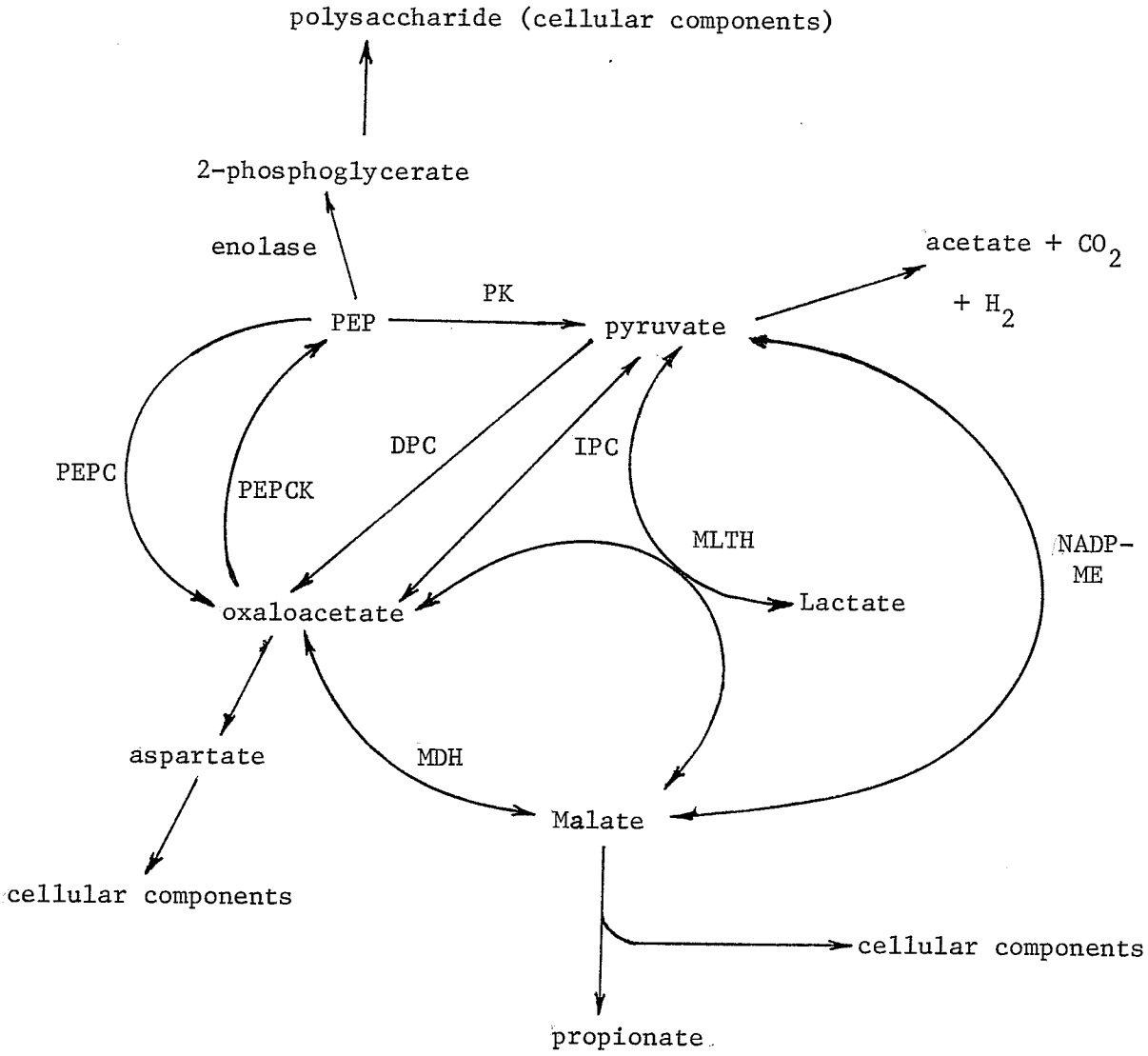

Fig. 7.52. Possible catabolic and anabolic reactions occurring during lactate metabolism by V. parvula M_4.

(NADP-ME), NADP-specific malic enzyme; (MLTH), malate-lactate transhydrogenase, PEP-S), P-enolpyruvate synthetase; (PK), pyruvate kinase; (IPC), ATP-independent pyruvate carboxylase; (DPC), ATP-dependent pyruvate carboxylase; (PEPCK),P-enol-pyruvate carboxykinase; (PEPC), P-enolpyruvate carboxylase; (MDH), malate dehydrogenase.

the reactions associated with the formation of PEP from lactate form a complex picture. The complexity of this scheme is directly related to the paucity of information available on the function and regulation of many of the enzymes shown.

The first step in PEP formation is the conversion of lactate and oxaloacetate to pyruvate and malate by the malate-lactate transhydrogenase (Ng and Hamilton, 1971). Once pyruvate is formed, it is either degraded to acetate, CO_2 and hydrogen by pyruvate dehydrogenase and hydrogenase, or it is converted to dicarboxylic acids for anabolic and catabolic purposes by the process of CO_2 fixation. Since pyruvate kinase is the only apparent enzyme catalysing PEP-pyruvate interconversions in the organism, pyruvate must proceed to PEP via the 'indirect' route, which involves the formation of oxaloacetate. In examining the formation of oxaloacetate one is immediately struck by the seemingly superfluous CO_2-fixing activity in cell-free preparations of V. parvula M_4 in the form of ATP-dependent and ATP-independent pyruvate carboxylases and the two malic enzymes.

Kornberg (1965) has regarded the primary role of pyruvate carboxylase in microorganisms to be anaplerotic, i.e., the oxaloacetate produced is used to maintain the levels of the tricarboxylic acid cycle intermediate for biosynthetic purposes. However, the function of pyruvate carboxylase in V. parvula M_4 is not restricted to biosynthesis since this compound is also in an intermediate in the pathway of energy metabolism which results in the formation of propionate (Johns, 1951; Ng and Hamilton, 1971). Furthermore, as seen above, oxaloacetate is required also for the initial conversion of lactate to pyruvate. Therefore, since

the presence of oxaloacetate is a critical factor in the overall survival of the cells, the presence of two carboxylases for the direct conversion of pyruvate and CO_2 to oxaloacetate is perhaps understandable. Not as easily understood, however, are the relative roles that these two enzymes play during lactate metabolism. Presumably, the differing effects of ATP on these two enzymes is the key to the regulaton of oxaloacetate formation directly from pyruvate and CO_2. The ATP-dependent enzyme would be active at high ATP concentration during gluconeogenesis, while the ATP-independent enzyme would be inhibited by this nucleotide under these conditions.

Since exogenous lactate cannot be degraded by malate-lactate transhydrogenase without stoichiometric quantities of oxaloacetate, the cell must be prepared to synthesize this compound under conditions of high and low energy charge (i.e., high and low ATP). The particular value of the IPC enzyme would be its ability to form oxaloacetate under conditions of low ATP concentration when the ATP-dependent enzyme would be less active. For example, upon the addition of lactate to cells at low ATP levels, the IPC enzyme would initiate the formation of the oxaloacetate required for the transhydrogenase reaction from 'residual' pyruvate and CO_2, which, in turn would convert the oxaloacetate and lactate to malate and pyruvate, respectively. The resulting metabolism would increase the celluar ATP content permitting the ATP-dependent pyruvate carboxylase enzyme to function. Since V. parvula M_4 is a non-glycolytic organism, it is probably not capable of synthetizing much energy storage material (i.e., glycogen), therefore, this mechanism would ensure that the metabolism of exogenous lactate could be initiated.

The position of the malic enzymes is less clear. There is general agreement that the NADP-malic enzyme, in both bacterial and mammalian systems, is concerned only with the generation of pyruvate and reducing power for lipogenesis thus functioning in a decarboxylating role rather than a CO_2-fixing role (Wood and Stjernholm, 1962; Jacobson, Bartholomans and Gunsalus, 1966, Sanwal, 1970). This has been confirmed with Salmonella typhimurium and E. coli (Theodore and Englesberg, 1964; Canovas and Kornberg, 1965; Nishikido et al, 1968), while Matula, McDonald and Martin (1969) have shown that in a Micrococcus sp the NADP malic enzyme was used for CO_2 fixation.

Cells of V. parvula M_4 could profitably employ the malic enzymes in either direction since malate formation from pyruvate could be used for energy purposes, while the reverse reaction could be employed to regenerate oxaloacetate (Ng and Hamilton, 1971). Fermentations studies has shown that this latter process was necessary since the amount of oxaloacetate required for the transhydrogenase reaction exceeded the amount of propionate formed during lactate degradation. Clearly, further speculation as to the role of these enzymes must await their purification and characterization.

It is apparent from the previous discussion that considerable competition exists in cells of V. parvula M_4 for oxaloacetate since this compound is required for the formation of PEP, aspartate and propionate, as well as for the transhydrogenase reaction. Thus, for gluconeogenesis to occur successfully, the diversion of this oxaloacetate to PEP must be an efficient process and is generally assumed to be catalyzed by PEP carboxykinase (Scrutton and Utter, 1968; Sanwal,

1970). Although the properties of this enzyme in V. parvula M_4 are unknown, one would imagine that it would be subject to allosteric regulation as is the enzyme from E. coli (Wright and Sanwal, 1969). Since its function would appear to be purely gluconeogenic in nature, the regulation of this enzyme must be controlled in a manner opposite to that observed for pyruvate kinase.

It will be recalled that the V. parvula pyruvate kinase was inhibited primarily by malate and ATP, and activated by AMP and various hexose phosphates. Obviously, under conditions of high energy charge, gluconeogenesis would proceed via PEP carboxykinase with pyruvate kinase subject to ATP (and possible malate) inhibition. At high AMP levels, this process would be reversed, with PEP being preferentially converted to pyruvate by the AMP-stimulated pyruvate kinase. This regulation, however, does not take into account the function of the hexose phosphates, which have been shown to activate the enzyme. Liao and Atkinson (1971) have suggested that cellular hexose phosphate concentrations interact with the adenylate pools to regulate the activity of the Azoto. vinelandii pyruvate kinase. The concept of cellular energy charge control of enzymatic activity is now well-known (Atkinson, 1969). On the other hand, the hexose phosphates, particularly fructose-1,6-P_2, were considered to be the most important factors for the activity of the enzyme from Saccharomyces cerevisiae (Barwell, Woodward and Brunt, 1971).

It is difficult to see how the hexose phosphates, or other glycolytic intermediates, would play a significant role in regulating the activity of the pyruvate kinase in V. parvula M_4, since the organism

is non-glycolytic, being unable to degrade glucose. In fact, the only mechanism for the synthesis of these compounds would be through the formation of PEP during gluconeogenesis. They might, however, function as "feedback activators" of the enzyme if these intermediates were allowed to accumulate within the cells through a reduction in the rate of macromolecular synthesis. Of particular importance in this regard was the observed effect of fructose-1,6-P_2 on pyruvate kinase activity. As shown in Fig. 5.34, FDP inhibited pyruvate kinase at low PEP concentrations, while activating the enzyme at concentrations above 3.3 mM PEP. Thus, this effect of FDP would be conducive to the operation of gluconeogenesis at low cellular concentrations of PEP.

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APPENDIX 1.

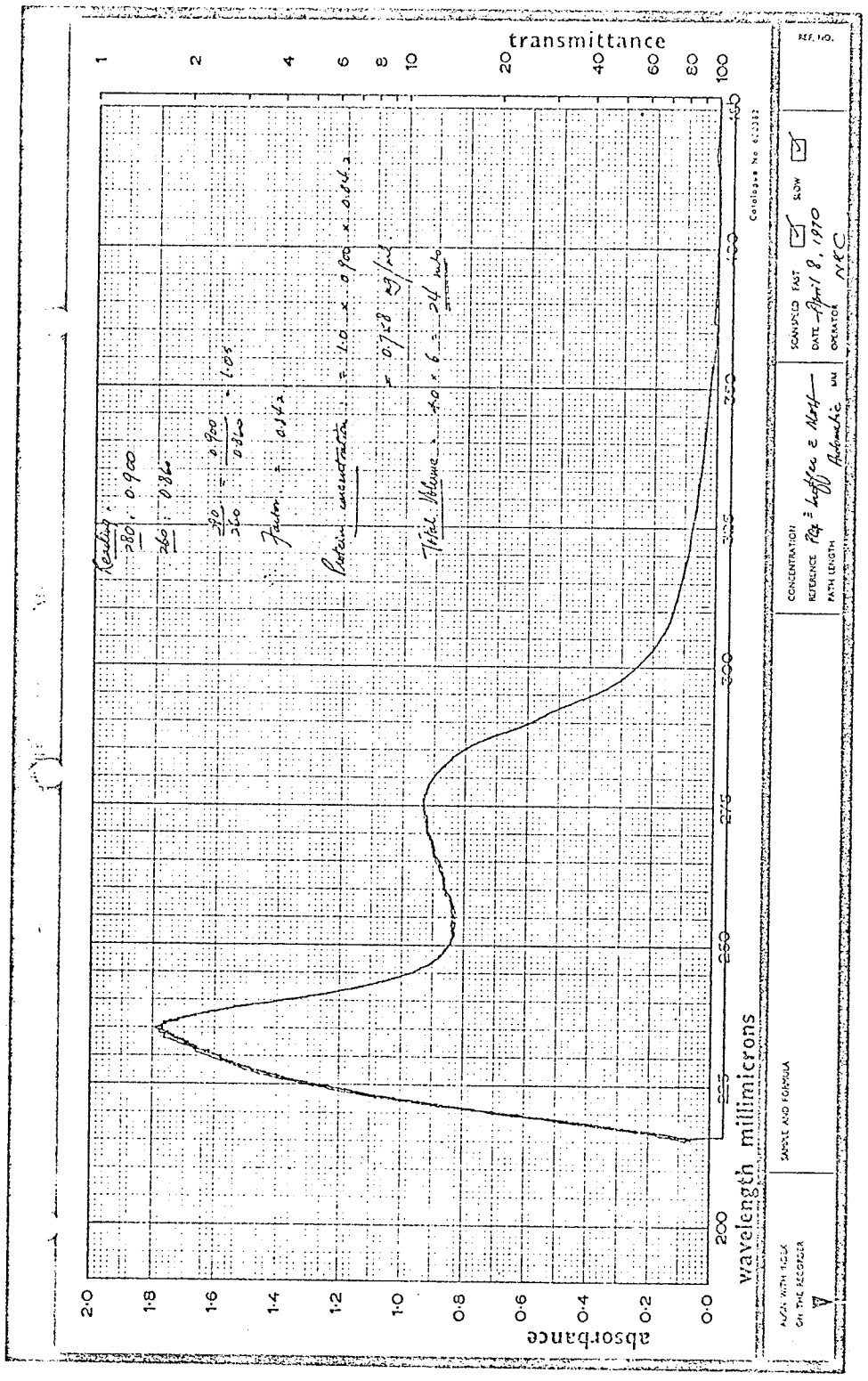

Fig. A.53. Spectrophotometric scan of the ATP-independent "pyruvate carboxylase" isolated from V. parvula M_4.