A STUDY OF PHOSPHOENOLPYRUVATE CARBOXYLASE FROM THIOBACILLUS THIOOXIDANS

A Thesis

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To My Parents

ACKNOWLEDGEMENTS

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ABSTRACT

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The enzyme, phosphoenolpyruvate (PEP) carboxylase (orthophosphate: oxalacetate carboxylase (phosphorylating) EC 4.1.1.31) was extracted and purified 19-fold from Thiobacillus thiooxidans by a combination of ultracentrifugation, acid precipitation, DEAE-cellulose column chromatography and $(NH_4)_2SO_4$ precipitation. Enzyme activity was found to be dependent on culture age. No enzyme activity could be obtained from frozen cell suspensions regardless of culture age.

The 19-fold purified enzyme was used for all kinetic studies. The pH optimum of the enzyme was determined to be 7.9 - 8.0. All kinetic studies were carried out at pH 7.0 and 8.0. Apparent Michaelis constants were determined for the substrates involved in this reaction; phosphoenolpyruvate (1.4, 1.5 mM), bicarbonate (0.4, 1.1 mM) and magnesium (1.1, 0.8 mM) at pH 7.0 and 8.0 respectively. Acetyl coenzyme A was found to be a powerful activator of this enzyme, with the amount of activation being dependent on the pH. The Ka for acetyl coenzyme A, however, remained fairly constant and low, being 1.2 and 1.0 μM at pH 7.0 and 8.0 respectively. L-aspartate and L-malate were strong

inhibitors of enzyme activity. In the presence of aspartate at pH 7.0 the double reciprocal activity-PEP plots became non-linear characteristic of negative cooperativity. At pH 8.0, the same plots were linear with L-aspartate acting as a competitive inhibitor of PEP. All the other effectors of PEP carboxylase from Salmonella typhimurium and Excherichia coli were found to be ineffective towards the enzyme from T. thiooxidans.

ABBREVIATIONS

The following abbreviations have been used:

NADH	- β-dihydronicotinamide adenine dinuclectide
AMP	- adenosine-5'-monophosphate
ATP	- adenosine-5'-triphosphate
PEP	- phosphoenolpyruvate
DEAE	- diethylaminoethyl cellulose
FDP	- fructose-1,6-diphosphate
Bicine	- N,N-bis(2-hydroxyethyl) glycine
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MES	- 2-(N-morpholino) ethanesulfonic acid
MOPS	- morpholinopropane sulfonic acid
Tris	- Tris(hydroxymethyl)aminomethane
Acetyl CoA	- acetyl coenzyme A
TCA	- tricarboxylic acid
GTP	- guanosine triphosphate
UTP	- uracil triphosphate
CDP	- cytosine diphosphate
MDH	- malate dehydrogenase; L-malate:NAD oxidoreductase, EC 1.1.1.27

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INTRODUCTION

INTRODUCTION

In 1887 Winogradsky established the fundamental principles of autotrophy. The main principles of his work: (a) that carbon dioxide is the only carbon source utilized for cell synthesis, and (b) the sole source of energy is derived from inorganic material, have been modified slightly, but the overall concept remains intact. Starkey (1925b) found that Thiobacillus thiooxidans, a chemoautotroph, utilized glucose in the prescence of sulfur, but could not grow using the organic compound in the absence of sulfur. The inability of T. thiooxidans to grow using an organic compound as the sole source of energy is a characteristic of the chemoautotrophs.

T. thiooxidans was first isolated by Waksman and Joffe (1922) from composts of soil, sulfur and rock phosphate. The organism derives its energy from cell synthesis by the oxidation of inorganic sulfur to sulfuric acid, and utilizes atmospheric CO_2 as its sole source of cellular carbon. The oxidation of sulfur with the production of sulfuric acid produces an environment where the hydrogen ion concentration reaches a pH of 1.0 or less, and the organism grows at a pH lower than any other bacterium. The acidic environment at which T. thiooxidans

is able to grow differentiates it from similar organisms of the same genus. The optimum growth temperature was 28 - 30° C with the optimum pH 2.0 - 2.8. The unique position of the chemoautotrophs with respect to their carbon and energy source provide an interesting system on which to study.

The enzyme phosphoenolpyruvate carboxylase which snythesizes oxalacetate from phosphoenolpyruvate and CO_2 has been under intensive study in the enteric bacteria from the standpoint of metabolic control. This enzyme termed "anaplerotic" by Kornberg (1965) is primarily responsible for replenishing oxalacetate which is drained away from the tricarboxylic acid cycle for synthetic purposes. The importance of biosynthesis (and thus PEP carboxylase) in the chemoautotrophs in which the sole source of cellular carbon is atmospheric carbon dioxide laid the foundation for a study of the kinetic and control properties of this enzyme from T. thiooxidans.

HISTORICAL

HISTORICAL

The ability of autotrophic microorganisms to fix CO_2 has been well known for a long time (Winogradsky, 1887). The first demonstration of CO_2 fixation by heterotrophic organisms (Wood and Werkman, 1935) renewed interest in autotrophic carbon dioxide fixation and its mechanism. These workers had shown that carbon dioxide was fixed by heterotrophic, non-photosynthetic bacteria in the fermentation of glycerol by the propionic acid bacteria. Results obtained in 1938 led these workers to suggest that succinic acid, a 4-carbon compound, was formed from a 3-carbon compound through the addition of carbon dioxide. Since that time much evidence has been produced to show that several enzymes are able to catalyse the direct carboxylation of a three carbon compound to yield a 4-carbon compound.

The enzymes involved in the carboxylation of a 3-carbon compound are phosphoenolpyruvate carboxykinase, pyruvate carboxylase, phosphoenolpyruvate carboxytransphosphorylase, phosphoenolpyruvate carboxylase, and the malic enzyme.

The enzyme phosphoenolpyruvate carboxykinase, first isolated by Utter and Kurahashi (1954) from chicken liver, catalysed the following reversible reaction where IDP is

inosine diphosphate and ITP is inosine triphosphate (1):

$$CH_2{:}C(OPO_3)\cdot COOH + CO_2 + IDP \rightleftharpoons COOH\cdot CH_2\cdot CO\cdot COOH + ITP \quad (1)$$

Pyruvate carboxylase was discovered in avian and beef liver by Utter and Keech (1960) and in Aspergillus niger by Woronick and Johnson (1960). It catalysed the following reaction (2):

$$CH_3\cdot CO\cdot COOH + CO_2 + ATP \rightleftharpoons COOH\cdot CH_2\cdot CO.COOH + ADP + Pi \quad (2)$$

The enzyme PEP carboxytransphosphorylase found in propionic acid bacteria (Siu et al, 1961) catalysed the following reaction (3):

$$CH_2{:}C(OPO_3)\cdot COOH + CO_2 + P \rightleftharpoons COOH\cdot CH_2\cdot CO\cdot COOH + PPi \quad (3)$$

Bandurski and Greiner (1953) found in spinach leaves an enzyme which synthesized oxalacetate from phosphoenolpyruvate and CO_2 according to the following irreversible reaction (4):

$$CH_2{:}C(OPO_3)\cdot COOH + CO_2 \longrightarrow COOH\cdot CH_2\cdot CO\cdot COOH + Pi \quad (4)$$

The last enzyme, the malic enzyme, first isolated by Ochoa et al (1948) catalysed the reversible reaction (5):

$$CH_3\cdot CO\cdot COOH + CO_2 \overset{NADP \quad NADPH + H^+}{\rightleftharpoons} COOH\cdot CH_2\cdot CO\cdot COOH \quad (5)$$

The role of these enzymes in the organisms in which

they function has been under study for a long time. The fixation of CO_2, the so-called Wood-Werkman reaction (Wood and Werkman, 1938), has long been considered a key reaction in the tricarboxylic acid cycle, and hence in the formation of many amino acids. A considerable amount of evidence exists that, of the five enzymes previously mentioned that can synthesize C_4-acids from C_3-compounds, phosphoenol-pyruvate carboxylase is the enzyme that serves an "anaplerotic" function in the Enterobacteriaceae, (Kornberg, 1966) i.e., it replenishes the supply of oxalacetate drained away for the synthesis of proteins from the tricarboxylic acid cycle. The evidence rests primarily on the demonstration that mutants of Escherichia coli (Amarsingham, 1959; Ashworth and Kornberg, 1963) and Salmonella typhimurium (Theodore and Englesberg, 1964) which lack phosphoenolpyruvate carboxylase fail to grow on pyruvate or its precursors unless TCA cycle intermediates are also added to the growth medium.

Although most work on pyruvate carboxylase has made use of chicken livers as the source, this enzyme has also been isolated from Pseudomonas citronellis (Seubert and Remberger, 1961) and A. niger (Bloom and Johnson, 1962). It appears that pyruvate carboxylase, which catalyses the carboxylation of pyruvate to oxalacetate and the simultaneous conversion of ATP to ADP, fullfills the anaplerotic function in these cases (Keech and Utter,

1963; Utter, Keech and Scrutton, 1964). This enzyme is also thought to serve an anaplerotic function in the bacilli (Wood and Utter, 1965; Sundaram, Cazzulo and Kornberg, 1969; Cazzulo, Sundaram and Kornberg, 1969).

The enzyme PEP carboxylase originally reported by Bandurski and Greiner (1953) in spinach leaves was subsequently found in wheat germ (Tchen and Vennesland, 1955), peanut cotyledons (Maruyama and Lane, 1963), Ps. strain AM1 (Large, Peele and Quale, 1962), Thiobacillus thiooxidans (Suzuki and Werkman, 1958a), E. coli (Cánovas and Kornberg, 1965), and Ferrobacillus ferrooxidans (Din et al, 1967).

In T. thiooxidans carboxydismutase and PEP carboxylase were considered responsible for the rapid labeling of phosphoglyceric acid (carboxyl group) and aspartic acid (β-carboxyl group), respectively, using radioactive carbon dioxide (Suzuki and Werkman, 1958b). PEP carboxylase, in keeping with its important metabolic role is subject to a multiplicity of controls and belongs, therefore, to a category of enzymes termed "allosteric" by Monod, Changeux and Jacob (1963).

Since allosterism has been discussed at great length by the pioneers in the field (Monod et al, 1963; Monod, Wyman and Changeux, 1965; and Koshland et al, 1966), it will not be discussed in detail here.

The allosteric proteins (enzymes) located at key

(branching) points in the metabolic scheme are controlled by certain metabolites (effectors) acting as signals. These effectors bind to the enzyme molecule and either activate or inhibit the enzyme involved, thus favoring one or the other pathway in a branched scheme (Monod, Changeux and Jacob, 1963). The effector is often the end product of one of the pathways. According to the theory postulated by Monod et al. (1963), allosteric enzymes possess at least two distinct, non-overlapping receptor sites. One of these, the active or catalytic site, binds the substrate and is responsible for the activity of the enzyme. The other site, the allosteric site, is complementary to the structure of another metabolite (allosteric effector) which it binds specifically and reversibly. The allosteric effector binds to the subunit (all allosteric enzymes are polymers consisting of two or more identical subunits) at a site physically separate from the substrate binding site. The binding of the effector usually changes the affinity of the enzyme for its substrate (Monod, Wyman and Changeux, 1965) and it is assumed only to bring about a discrete reversible molecular transition of the protein (allosteric transition) which modifies the properties of the active site (Monod et al, 1963). The possible nature of this change in the enzyme molecule has been discussed at length in papers by Monod, Wyman and Changeux (1965). A recent review on "Allosteric Regulation of Enzyme Activity" has

been published by E. R. Stadtman (1966).

Since PEP carboxylase occupies such a key position within the metabolic pattern of the bacterial cell and functions as an anaplerotic enzyme, its allosteric effectors and control mechanisms have been intensively studied.

Activators: Cánovas and Kornberg (1965) have shown that PEP carboxylase of E. coli was strongly activated by acetyl Coenzyme A. The same phenomenon has also been reported in S. typhimurium by Maeba and Sanwal (1969) and in the autotroph F. ferrooxidans by Din, Suzuki and Lees (1967). The purpose of this activation seems to be to catalyse the formation of oxalacetate required for the oxidation of acetyl CoA. The operation of such a regulatory system ensures that, during growth on glucose or three carbon compounds, the necessary balance between catabolic and anaplerotic reactions may be maintained (Cánovas and Kornberg, 1965).

Sanwal and Maeba (1966a) demonstrated that PEP carboxylase of S. typhimurium is markedly activated by fructose 1,6-diphosphate (FDP). Since the functioning of PEP carboxylase is absolutely necessary when the organism is growing on glucose (Kornberg, 1965), this mechanism may ensure an uninterrupted supply of oxalacetate. The observation that an adequate supply of oxalacetate is already ensured by acetyl CoA activation of the enzyme

(Utter and Keech, 1963; Kornberg, 1965) implies that a simple interpretation, precursor activation, may not be correct. It has been suggested that FDP activation is related to the energy metabolism of the cell (Atkinson, 1965). The ATP/AMP ratio is the controlling factor for a large number of enzymes involved in carbohydrate metabolism (Krebs, 1964). Changing ATP/AMP ratios and its effect on the enzyme phosphofructokinase cause varying levels of FDP which may in turn effect PEP carboxylase in an allosteric manner.

Sanwal and Maeba (1966b) have shown that a variety of nucleotides, notably cytosine diphosphate and guanosine triphosphate, activate PEP carboxylase in S. typhimurium. They suggested that these nucleotides which are feedback inhibitors of aspartate transcarboxylase (Gerhart and Pardee, 1962) would lead to accumulation of aspartate, which in turn would inhibit PEP carboxylase. A compensatory feedback activation of PEP carboxylase by these nucleotides would restore the original levels of oxalacetate required for the running of the tricarboxylic acid cycle.

Sanwal, Maeba and Cook (1966) have demonstrated activation of PEP carboxylase in S. typhimurium with various macroions and dioxane. These workers found that poly-l-lysine had the most pronounced effect (activation) on the enzyme although other compounds functioned in the same manner to a lesser extent. A considerable number of enzymes are known to be either activated or inhibited by

polyamino acids, but the actual mechanisms by which the modifications of activity are brought about are uncertain (Katchalski et al, 1964). The possibility exists that charge interactions cause an alteration in the geometery of the catalytic site as a result of some conformaticnal change of the enzyme induced by the binding of these molecules. Dioxane activated PEP carboxylase and was also effective in preventing inhibition by aspartate (Sanwal et al, 1966). These authors concluded that the enzyme had been desensitized to the inhibitor. Corwin and Fanning (1968) did a similar work with the E. coli enzyme and proposed that dioxane may be acting like acetyl CoA in preventing inhibition and may be binding at the same site.

Inhibitors: Inhibition of PEP carboxylase by aspartate was demonstrated in S. typhimurium (Maeba and Sanwal, 1965) and in E. coli (Corwin and Fanning, 1968). Corwin and Fanning (1968) also showed that the same enzyme was inhibited by malate. Since oxalacetate is removed from the tricarboxylic acid cycle for the synthesis of aspartate via aspartate-glutamate transaminase, PEP carboxylase could be considered as the first enzyme of the pathway leading to the end product, aspartate. This being the case it is easy to understand that aspartate should act as a feedback inhibitor of PEP carboxylase. Inhibition by malate is much more difficult to explain. Malate and aspartate are interconvertible through the transamination reaction so that

inhibition by malate seems to be a redundant control of the enzyme. Maeba, (doctoral dissertation, 1968) taking into account work by Roberts et al (1955) which indicates that malate is a very poor inhibitor of *in vivo* CO_2 fixation in *E. Coli*, suggested that allosteric inhibition by malate may be physiologically non-functional. On the other hand, inhibition by malate would allow direct control of TCA cycle acid formation by a TCA cycle acid itself.

In the classical allosterism discussed earlier they normally assume positive cooperativity, in which the first molecule of ligand makes it successively easier for the next molecule to bind. Many enzymes have been observed to follow this type of cooperativity since its initial discovery in hemoglobin (Bohr et al, 1904). It was assumed to be the only type of cooperativity between like ligands which would be of any advantage to an organism since the operation of this phenomenon would make the regulation of certain key control enzymes more sensitive to environmental change.

Recently, (Conway and Koshland, 1968) a new phenomenon has been proposed in which the binding of a ligand to the enzyme molecule *decreases* the affinity for the binding of subsequent molecules of that ligand at the vacant sites of neighbouring subunits. This effect ("negative cooperativity") would have the biological advantage of making some enzymes less sensitive to

environmental changes and could then act complementarily to positive cooperativity. This negative cooperativity appears to be strong support in favour of the "sequential" changes of subunit conformation induced by the binding of a ligand as proposed by Koshland et al (1966).

Levitzki and Koshland (1969) have proposed a series of diagnostic procedures for negative cooperativity. Since negative cooperativity tends to insulate the enzyme from changes in metabolite concentration, this decreased sensitivity must also be advantageous to the system.

It may be important that some enzymes of major metabolic pathways should be of constant activity despite fluctuations in metabolite concentrations. Levitzki and Koshland (1969) cited D-glyceraldehyde 3-phosphate dehydrogenase and enzymes involved in nerve conduction as examples of enzymes which might need to be desensitized from environmental changes. It has been shown that glyceraldehyde 3-phosphate dehydrogenase (Conway and Koshland, 1968) and PEP carboxylase (Corwin and Fanning, 1968) exhibit negative cooperativity. Recently (Levitzki and Koshland, 1969) negative cooperativity has been observed in CTP synthetase. This enzyme exhibits negative cooperativity for GTP (an effector) and glutamate (substrate) and positive cooperativity for ATP and UTP (both substrates).

METHODS AND MATERIALS

METHODS AND MATERIALS

Organisms

Thiobacillus thiooxidans, obtained from the American Type Culture Collection (ATCC No. 8085), was used throughout this study. Elemental sulfur was used as the energy source for growth. Cultures of *Thiobacillus thioparus* (ATCC No. 8158), *Thiobacillus novellus* (ATCC No. 8093), and *Nitrosomonas europeae* (ATCC No. 221) were also grown for comparative studies.

Growth Medium

T. thiooxidans was grown in 2800 ml Fernbach flasks as described by Suzuki (1965). Each flask contained 1 liter of Starkey's medium (1925) of the following composition; 0.3 g $(NH_4)_2SO_4$, 3.5 g KH_2PO_4, 0.5 g $MgSO_4$, 0.25 g $CaCl_2$, 0.02 g $FeSO_4 \cdot 7H_2O$, 50 g powdered sulfur, and 1000 ml of distilled water. The pH of the medium was 4.5. The sulfur was spread on the surface of the medium after inoculation (10% v/v) of an active culture. The flasks were incubated at 28°C for five days without shaking.

T. thioparus and *T. novellus* were grown on Starkey's medium (R. L. Starkey, 1934) and *N. europeae* was grown on ATCC Medium No. 221 with aeration.

Cell Preparation

At the end of the incubation period the sulfur was removed by filtration through a Whatman No. 1 filter paper under suction. The pH of the media after this period was 1.5. The cells were then harvested in a Sharples Super Centrifuge and washed twice with distilled water. The yield was approximately 6.0 g wet weight cells 20 l of medium.

Preparation of Cell-free Extracts

The washed cells were resistant to breakage by sonic oscillation when suspended in distilled water or buffer. Breaking the cells was achieved according to Suzuki (1958): 5 g wet weight of cells were suspended in 20 ml of distilled water, then 5 g DOWEX 50 - X8 (H^+ form) and 5 g DOWEX 1 - X8 (OH^- form) resins were added. The mixture was then shaken vigorously at 28°C for 15 minutes on a rotory shaker. The resins were then removed by filtration through a Pharmacia column (1.8 x 30 cms) under pressure, and the cells were harvested by centrifugation at 24,000 x g for 10 minutes. The treated cells were then suspended in 10 ml of 0.2 M Tris(hydroxymethylaminomethane)-HCl buffer (pH 8.0 in 10^{-2} M 2-mercaptoethanol) and sonicated in a Raytheon Magnetostrictive Oscillator for 20 minutes under a nitrogen atmosphere. The cell debris was removed by centrifugation at 40,000 x g for 30 minutes in a Sorvall refrigerated centrifuge.

Crude extracts of T. thioparus, T. novellus, and N. europeae were prepared in the following manner. The cells were harvested in a Sharples Super Centrifuge and washed 3 times with 0.1 M potassium phosphate buffer (pH 7.5). A suspension of cells (10% w/v T. thioparus and T. novellus, 50% w/v N. europeae) was made in 0.1 M phosphate buffer and sonicated for 20 minutes under a nitrogen atmosphere. The cell debris was removed from the extracts by centrifugation at 40,000 x g for 15 minutes.

Reagents

The following chemicals were obtained from the Sigma Chemical Company:

β-Diphosphopyridine Nucleotide (Nicotinamide Adenine Dinucleotide; β-DPN; β-NAD) from yeast, disodium salt, Grade III

β-Diphosphopyridine Nucleotide, reduced form (β-Dihydronicotinamide Adenine Dinucleotide; β-DPNH; β-NADH) from yeast, disodium salt, Grade III

Adenosine-5'-Monophosphoric acid (AMP), from yeast, sodium salt, Type II

Adenosine-5'-Triphosphoric acid (ATP), from equine muscle, disodium salt, crystalline

Poly-l-Lysine

D,L-Isocitrate, trisodium salt, Type I

Pyruvic acid, sodium salt, Type III

Phosphoenolpyruvic acid (PEP), tricyclohexylamine salt

Diethylaminoethyl cellulose (DEAE)

L-Aspartic acid

Fructose-1,6-Diphosphate (FDP)

Calbiochem:

Morpholinopropane Sulfonic Acid (MOPS), A grade

2-(N-Morpholino)ethanesulfonic Acid (MES), A grade

N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES), A grade

N,N-Bis(2-Hydroxyethyl) glysine (Bicine), A grade

P-L Biochemicals, Inc.:

Acetyl Coenzyme A (Acetyl CoA), lithium salt

Boerhinger Mannheim Corp:

Malate Dehydrogenase (MDH), from pig heart, specific activity approximately 720 units/mg

Enzyme Assay

The enzymatic activity of phosphoenolpyruvate carboxylase was measured by following the rate of oxidation of NADH spectrophotometrically at 340 mμ by coupling the PEP carboxylase reaction with malic dehydrogenase. All assays were done with a Gilford Model 2000 recording spectrophotometer attached to a Beckman DU monochromater in silica cuvettes of 1 cm light path. The standard assay contained: 5.0 mM PEP, 10.0 mM $NaHCO_3$, 5.0 mM $MgCl_2$,

0.1 mM NADH, 10.0 µg malic dehydrogenase, 50.0 mM HEPES buffer (pH 7.0), and 0.2 ml of enzyme in a volume of 3.0 ml.

Activities of other enzymes that could possibly interfere with the assay of PEP carboxylase were determined as follows.

Pyruvate Carboxylase: the assay mixture contained: 40.0 mM HEPES buffer (pH 8.0), 5.0 mM sodium pyruvate, 3.3 mM $MgCl_2$, 1.25 mM ATP, 33.3 mM KCl, 0.12 mM NADH, 10 µg MDH, and enzyme.

Phosphoenolpyruvate Carboxytransphosphorylase: the assay mixture contained: 10.0 mM potassium phosphate buffer (pH 7.0), 2.0 mM PEP, 30.0 mM $KHCO_3$, 12.0 mM $MgCl_2$, 0.1 mM $CoCl_2$, 0.125 mM NADH, 1.0 mM 2-mercaptoethanol, 10.0 µg MDH, and enzyme.

NAD-specific isocitric dehydrogenase: the assay mixture contained: 200.0 mM Tris-acetate buffer (pH 7.5), 0.5 mM NAD, 1.0 mM AMP, 3.0 mM $MgCl_2$, 1.0 mM isocitrate, and enzyme.

All assays were carried out in a total volume of 3.0 ml. The optical density was measured at 340 mµ with a Gilford Model 2000 spectrophotometer.

Definition of Unit and Specific Activity

One unit of activity is defined as that amount of enzyme which oxidizes 1 µmole of NADH per minute under the standard assay conditions. Specific activity is expressed

per mg protein. Protein was determined by the method of Lowry et al (1951). For all the kinetic studies of PEP carboxylase 320 µg of 40% ammonium sulfate precipitate was used.

Purification of PEP Carboxylase from T. thiooxidans

All steps in the purification of the enzyme were carried out at 4°C. After the cell debris was removed by centrifugation at 40,000 x g for 30 minutes, the crude extract was centrifuged at 149,000 x g for 1 hour, and the supernatant fluid decanted from the pellet. The pellet was discarded, and 1.0 M acetic acid was added (with stirring) to the supernatant solution until pH 5.4 was reached. The mixture was then centrifuged at 40,000 x g for 10 minutes. The pellet was again discarded, and the supernatant was adjusted to pH 7.5 with 1.0 M Tris-HCl buffer (pH 9.0). The concentration of 2-mercaptoethanol was kept at approximately 0.01 M throughout the purification by subsequent additions after the various steps. The enzyme up to this point was very stable at -4°C for long periods of time. A DEAE-cellulose column (1.8 x 30 cm) was equilibrated in 0.1 M phosphate buffer (pH 7.5) with 0.01 M 2-mercaptoethanol. The acid-treated extract was placed on the DEAE column and the enzyme was eluted with a linear gradient of 0.1 - 0.4 M phosphate buffer in 0.01 M 2-mercaptoethanol. A Gilson Model VL fraction collector

was used to collect 5 ml fractions. Tubes 27 - 42 containing the active enzyme were pooled and solid $(NH_4)_2SO_4$ was slowly added with gentle stirring until a 40% saturation level was reached. After stirring for 30 minutes the mixture was centrifuged at 40,000 x g for 10 minutes. The supernant fluid was discarded, and the pellet containing the purified enzyme was redisolved in 15 ml of 0.075 M HEPES buffer (pH 7.5). The 40% $(NH_4)_2SO_4$ precipitate was the enzyme form used in all physical and kinetic studies.

RESULTS

RESULTS

Dependence of Enzyme Level on Culture Age

Thiobacillus thiooxidans (No. 8085) was grown at 28°C on Starkey's medium for a period of five to seven days. Cells harvested after a seven day growth period demonstrated no phosphoenolpyruvate carboxylase activity. Cell mass continued to increase from five to seven days, but the enzyme level dropped drastically during this period. It was also found that cells grown under standard conditions for five days and then stored at -4° C produced inactive extracts under various conditions of preparation (combinations of Clelands reagent, EDTA, and mercaptoethanol in varying concentrations, and varying sonication times).

Purification of Phosphoenolpyruvate Carboxylase

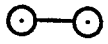
A 19-fold purification of PEP carboxylase was obtained with a combination of ultracentrifugation, acid precipitation, DEAE-cellulose column chromatography, and $(NH_4)_2SO_4$ precipitation. Table I presents the purification steps of phosphoenolpyruvate carboxylase from *T. thiooxidans*.

TABLE I

PURIFICATION OF PEP CARBOXYLASE FROM T. THIOOXIDANS

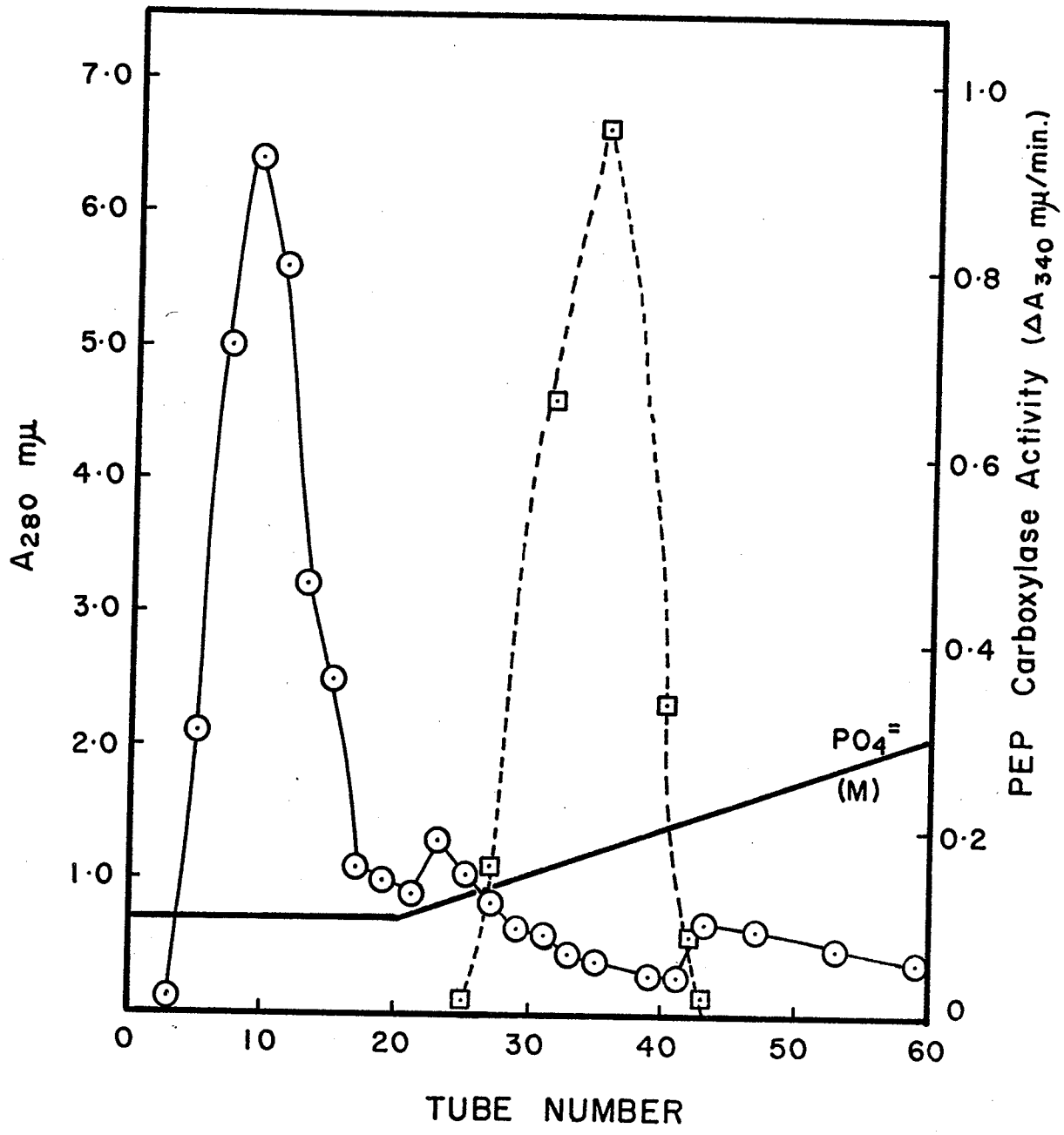
Stage	Total Protein (mg)	Total Activity (units)	Recovery (%)	Specific Activity (units/mg)
I. Crude Extract	1786	.808	100	21.91
II. 149,000 x g Supernatant	1049	.545	67.5	25.20
III. pH 5.4 Supernatant	636	.615	76.1	46.95
IV. DEAE fractions	94.6	.416	51.5	228.60
V. $(NH_4)_2SO_4$ precipitate 0-40%	48	.403	49.8	406.3

FIGURE 1. Elution of PEP carboxylase from a DEAE-cellulose column (1.8 x 30 cms).

 - optical density at 280

 - PEP carboxylase activity/ml of eluate

---- - $PO_4^{=}$ buffer concentration


7·0
6·0
5·0
4·0
3·0
2·0
1·0
A280 mμ
1·0
0·8
0·6
0·4
0·2
0
PEP Carboxylase Activity (ΔA340 mμ/min.)
PO4=
(M)
0
10
20
30
40
50
60
TUBE NUMBER

Storage of Phosphoenolpyruvate Carboxylase

Storage of the enzyme was most successful after the acid precipitation step of purification. The extract at this stage could be frozen indefinitely without appreciable loss of activity. When the purified form was obtained, work with it was completed as soon as possible. The purified enzyme was stable at 4°C for 36 hours without loss of activity. Storage for more than 36 hours at 4°C resulted in loss of activity (50% in 72 hours). All attempts to stabilize the enzyme in the purified form for more than 36 hours failed. Freezing the purified enzyme resulted in a 25% loss of activity and a very rapid decrease in activity after thawing when kept at 4°C.

pH Optimum

The pH optimum of PEP carboxylase was pH 8.0 - 8.1 (Figure 2). This curve was determined using a series of different buffers. Four of the new zwitterionic buffers described by Good et al (1966), MES, MOPS, HEPES, and Bicine were used. Tris-HCl, a common laboratory buffer, was also used. The zwitterionic buffers showed a higher activity than did Tris-HCl in the range of pH 7.0 - 9.0. HEPES buffer was chosen for all subsequent studies.

All buffers were used at a concentration of 0.15 M and were brought to the desired pH with NaOH.

FIGURE 2. The effect of hydrogen ion concentration (pH) on PEP carboxylase activity. Assay mixtures were prepared as described in Methods and Materials with different buffers as noted.

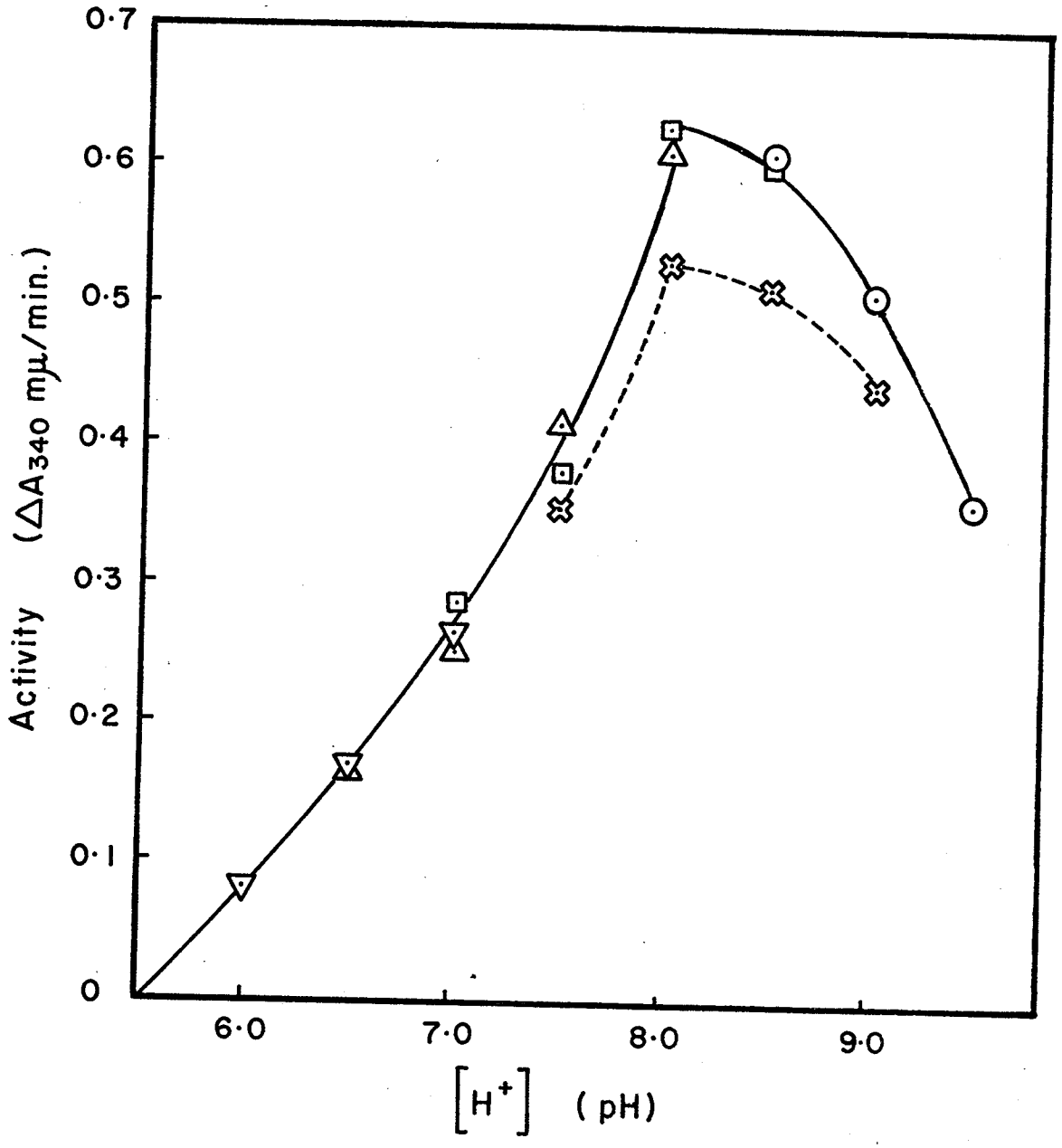
▽ - MES buffer

△ - MOPS buffer

⊡ - HEPES buffer

✣ - Tris-HCl buffer

⊙ - Bicine buffer


0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
Activity (ΔA340 mμ/min.)
6·0
7·0
8·0
9·0
[H+] (pH)

Kinetic Studies of PEP Carboxylase from T. thiooxidans

The enzyme catalyses the reaction:

$$\text{phosphoenolpyruvate} + HCO_3^- \xrightarrow{Mg^{++}} \text{oxalacetate} + Pi$$

The reaction is essentially irreversible and can only be measured in the direction of oxalacetate formation. Magnesium ion is required as a cofactor.

The initial velocity data were plotted in the double reciprocal form (1/v versus 1/s) according to Lineweaver and Burk (1934) where v is the initial velocity and s is the variable substrate concentration. The concentrations of substrates other than the variable substrate were those of the standard conditions.

Determination of Km Values for Reaction Components

The apparent Km values for various substrates were calculated from the intercepts at the horizontal axis of double reciprocal plots.

The values were calculated for the substrates at pH 7.0 and 8.0 since experimental work was done in both ranges.

1. Km for Phosphoenolpyruvate

The plots for 1/v versus 1/s, for pH 7.0 and 8.0 are shown in Figures 3 and 4. The apparent Km's were calculated to be 1.4 and 1.5 mM for pH 7.0 and 8.0 respectively.

FIGURE 3. The effect of phosphoenolpyruvate concentration at pH 7.0 plotted in the double reciprocal form. Reaction mixtures were prepared as described in Methods and Materials. PEP concentration was varied as indicated.

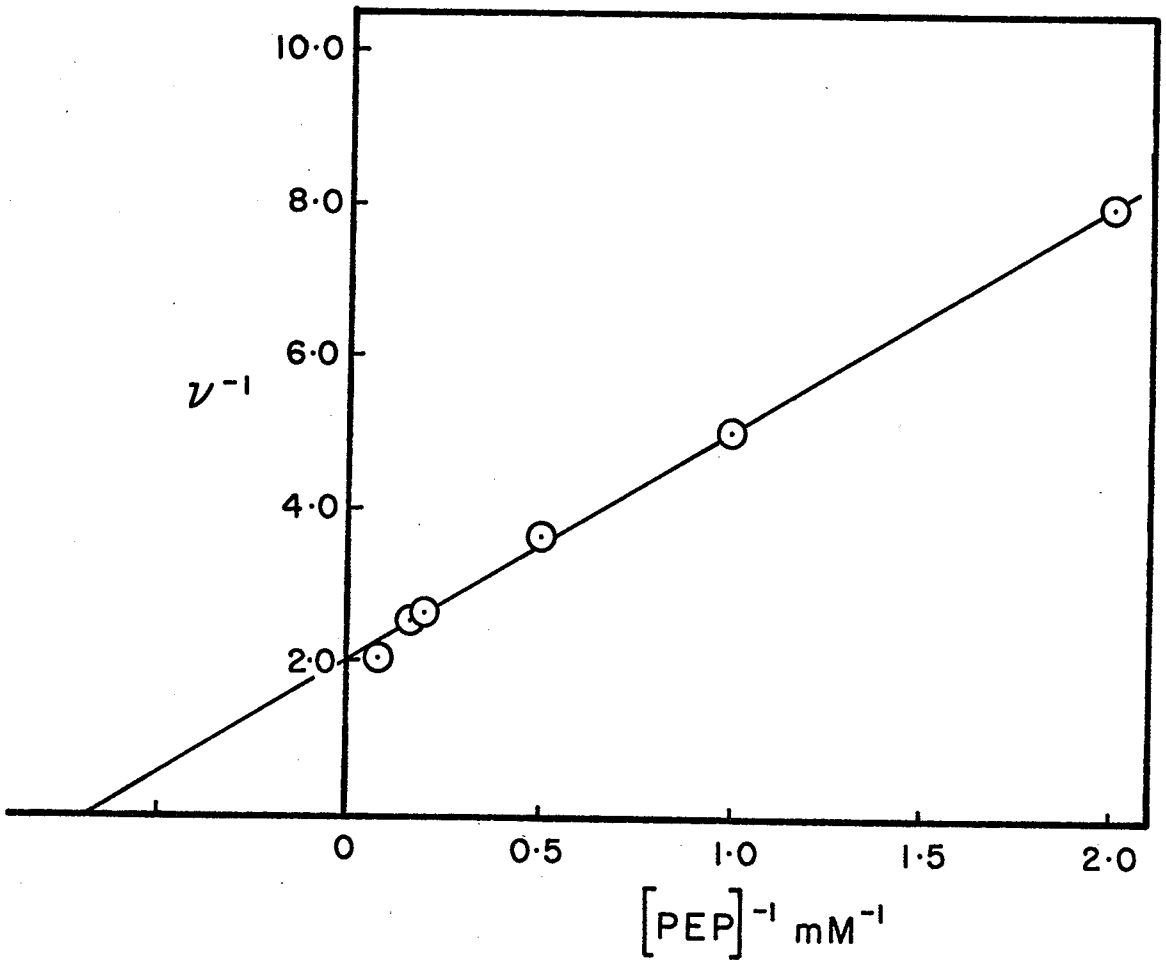
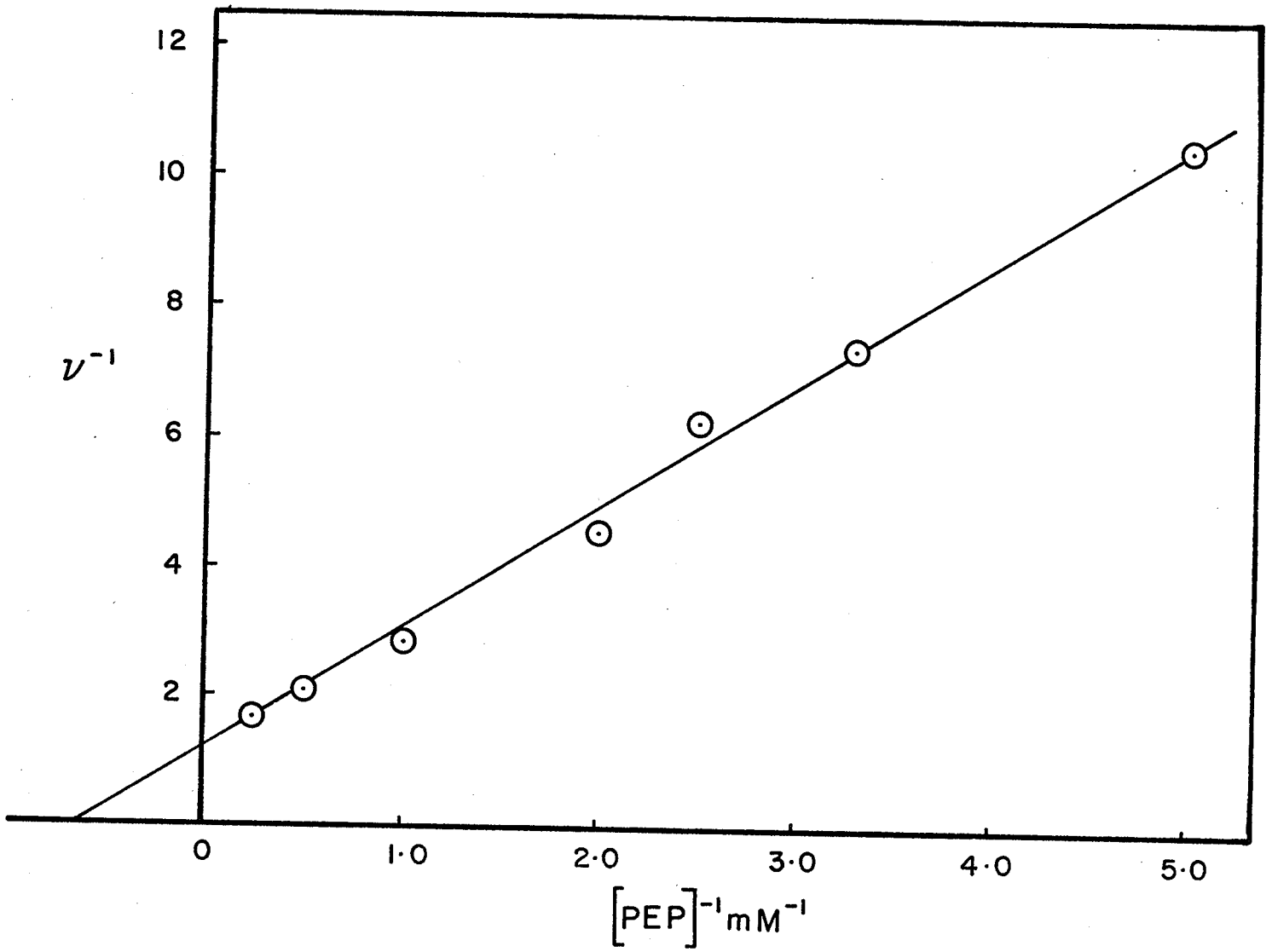

10·0
8·0
6·0
4·0
2·0
v^{-1}
0
0·5
1·0
1·5
2·0
$[PEP]^{-1}$ mM^{-1}

FIGURE 4. The effect of phosphoenolpyruvate concentration at pH 8.0 plotted in the double reciprocal form. Reaction mixtures were prepared as described in Methods and Materials except that the pH of HEPES buffer used was 8.0 and the PEP concentration was varied as indicated.


12
10
8
6
4
2
v^{-1}
0
1·0
2·0
3·0
4·0
5·0
$[PEP]^{-1} mM^{-1}$

2. Km for Magnesium

The plots for 1/v versus 1/s for magnesium are shown in Figures 5 and 6. The apparent Km's were calculated to be 1.1 mM and 0.8 mM for pH 7.0 and 8.0 respectively.

3. Km for Bicarbonate

The plots of 1/v versus 1/s for bicarbonate are shown in Figures 7 and 8. The endogenous bicarbonate at pH 7.0 was determined to be 0.2 mM with PEP carboxylase according to Feir and Suzuki (1969). The endogenous bicarbonate at pH 8.0 was similarly determined to be 0.8 mM. The apparent Km's were calculated to be 0.4 mM and 1.1 mM at pH 7.0 and 8.0 respectively.

Effectors of PEP Carboxylase

The enzyme PEP carboxylase, occupies a key position in the metabolic pattern within the bacterial cell, and it is not surprising that its activity is subject to control. This enzyme is particularly important in autotrophic organisms since fixation of atmospheric carbon dioxide is the sole source of carbon. It was shown by Maeba and Sanwal (1965) and Corwin and Fanning (1968), that aspartate and malate are powerful inhibitors of PEP carboxylase in S. typhimurium and E. coli. Acetyl CoA (Cánovas and Kornberg, 1965; Sanwal and Maeba, 1966a; and Corwin and Fanning, 1968), fructose diphosphate (Sanwal and Maeba, 1966b), a variety of nucleotides (Sanwal and Maeba, 1966b),

FIGURE 5. The effect of Mg^{++} concentration at pH 7.0 plotted in the double reciprocal form. The reaction mixtures were prepared as described in Methods and Materials. Mg^{++} concentration was varied as indicated.

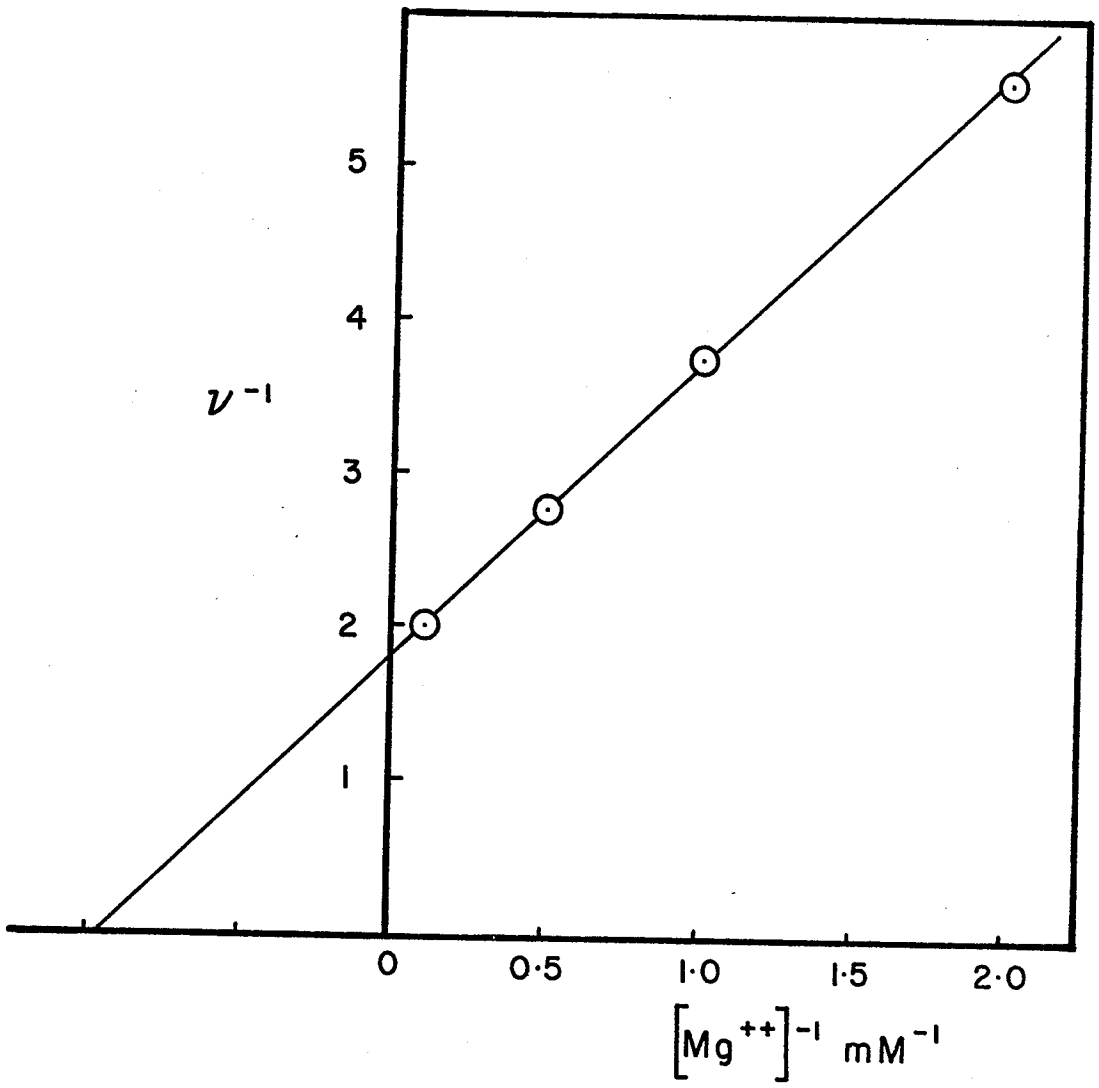

5
4
3
2
1
ν^{-1}
0
0·5
1·0
1·5
2·0
$[Mg^{++}]^{-1}$ mM^{-1}

FIGURE 6. The effect of Mg^{++} concentration at pH 8.0 plotted in the double reciprocal form. Reaction mixtures were prepared as described in Methods and Materials except that the pH of HEPES buffer used was 8.0 and the Mg^{++} concentration was varied as indicated.

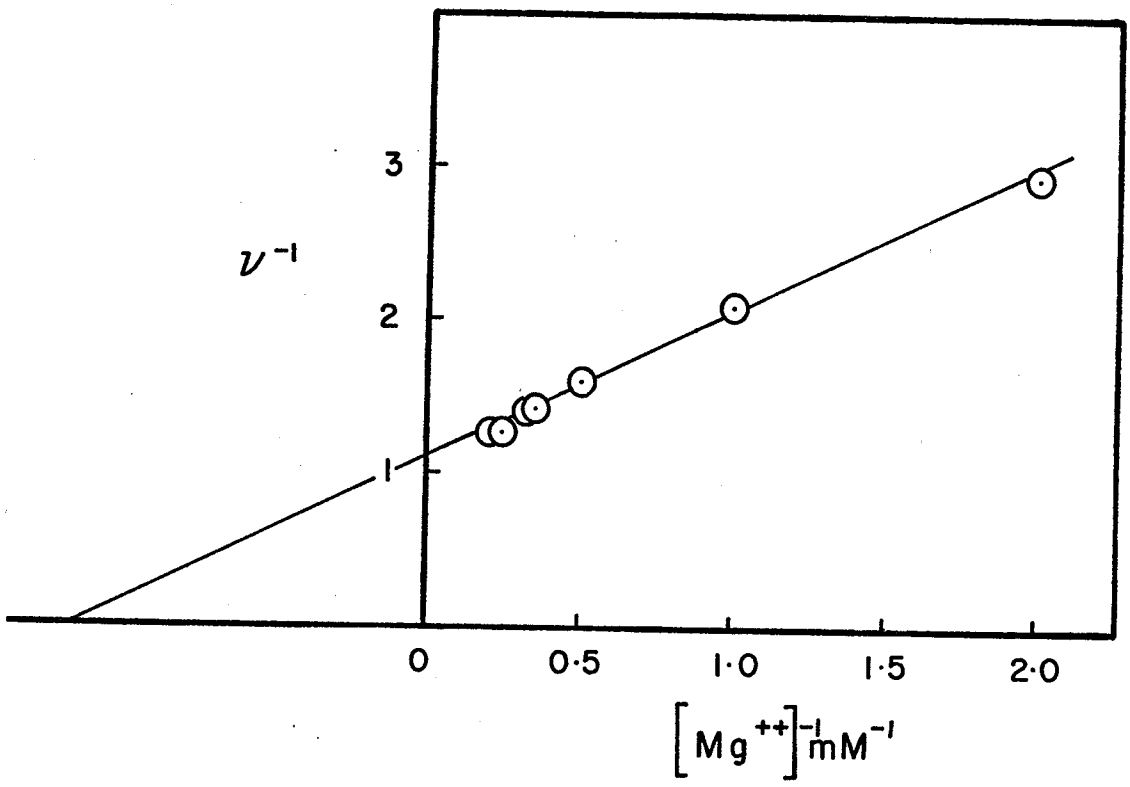

v^{-1}
3
2
1
0
0·5
1·0
1·5
2·0
$[Mg^{++}]^{-1} mM^{-1}$

FIGURE 7. The effect of bicarbonate concentration at pH 7.0 plotted in the double reciprocal form. Reaction mixtures were prepared as described in Methods and Materials except that the bicarbonate concentration was varied as indicated. The endogenous bicarbonate was determined to be 0.2 mM as described in the text.

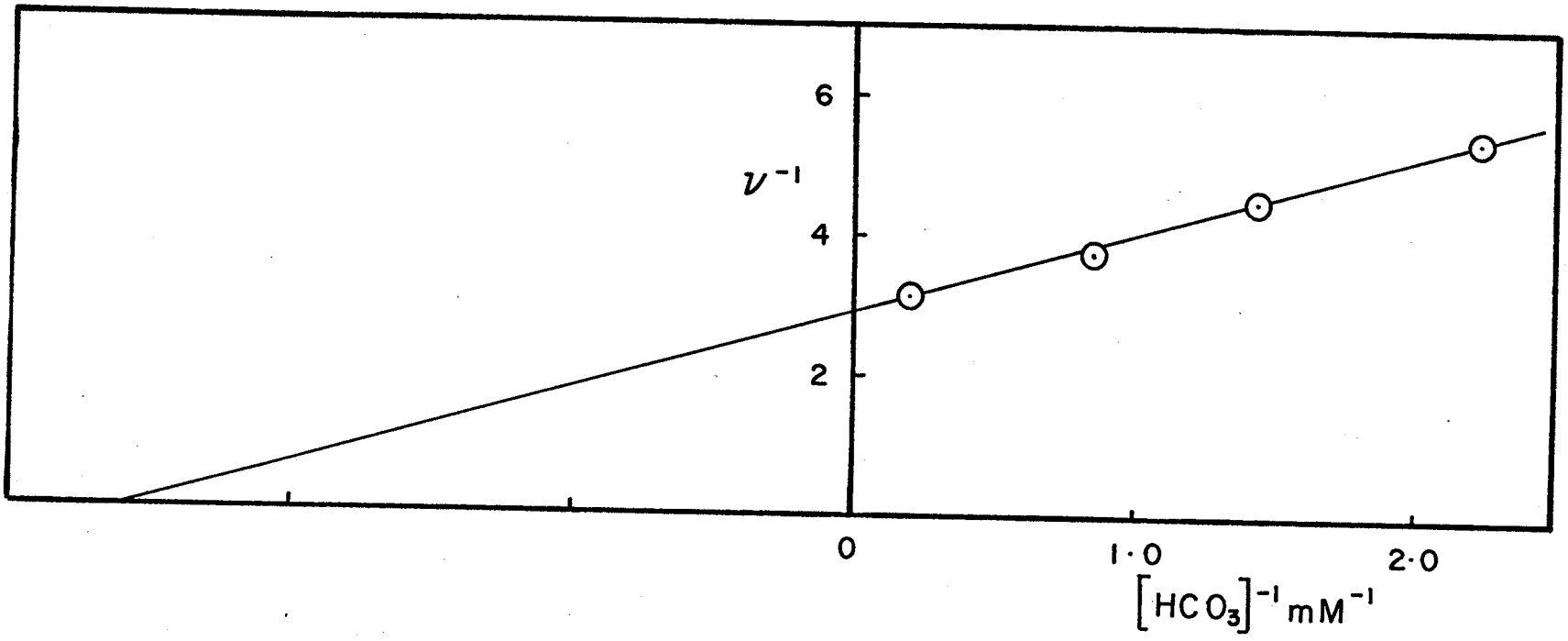
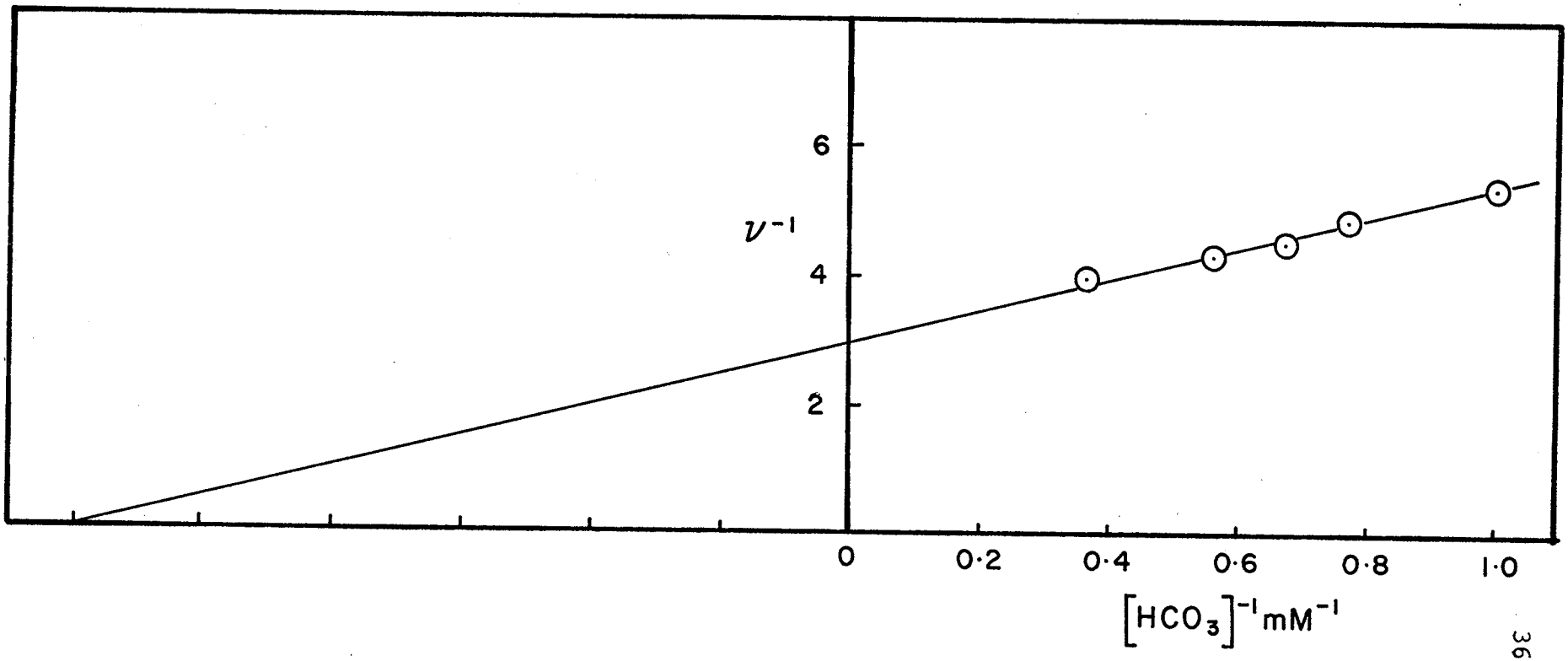

6
ν^{-1}
4
2
0
1·0
2·0
$[HCO_3]^{-1}\,mM^{-1}$

FIGURE 8. The effect of bicarbonate concentration at pH 8.0 plotted in the double reciprocal form. Reaction mixtures were prepared as described in Methods and Materials except that bicarbonate concentration was varied as indicated and the pH of HEPES buffer used was 8.0. The endogenous bicarbonate was determined to be 0.8 mM as described in the text.


6
v^{-1}
4
2
0
0·2
0·4
0·6
0·8
1·0
$[HCO_3]^{-1}mM^{-1}$

macroions and dioxane (Sanwal, Maeba and Cook, 1966) are able to activate this enzyme in S. typhimurium and E. coli. Activation by acetyl CoA and inhibition by aspartate have also been demonstrated in the autotroph F. ferrooxidans by Din et al (1967). To this date there has been no study preformed on a purified form of PEP carboxylase in autotrophic bacteria.

Activation by acetyl CoA: Cánovas and Kornberg (1965) and Corwin and Fanning (1968) have shown that PEP carboxylase of E. coli was strongly activated by acetyl CoA. Sanwal and Maeba (1966a) demonstrated the same effect in S. typhimurium. Both bacteria are heterotrophic organisms. Din et al (1967) showed the same effect in a crude extract of F. ferrooxidans, an autotroph. It was not surprising therefore that acetyl CoA also activated PEP carboxylase in T. thiooxidans. All attempts to find activators other than acetyl CoA were unsuccessful.

Using the standard conditions described in "Methods and Materials" with varied concentrations of acetyl CoA, and PEP constant at 2.0 mM, the concentration of activator required to produce half-maximal velocity, or Ka, was found to be 1.2 and 1.0 μM at pH 7.0 and 8.0 respectively (Figures 9 and 10). The Ka was calculated from the intercepts at the horizontal axis of 1/v - Vo versus 1/a plots where Vo is the velocity in the absence and v is that in

FIGURE 9. The effect of acetyl CoA at pH 7.0. Standard conditions were used for the velocity determination in the absence of acetyl CoA (v_o). v was determined in the presence of varied concentrations of acetyl CoA.

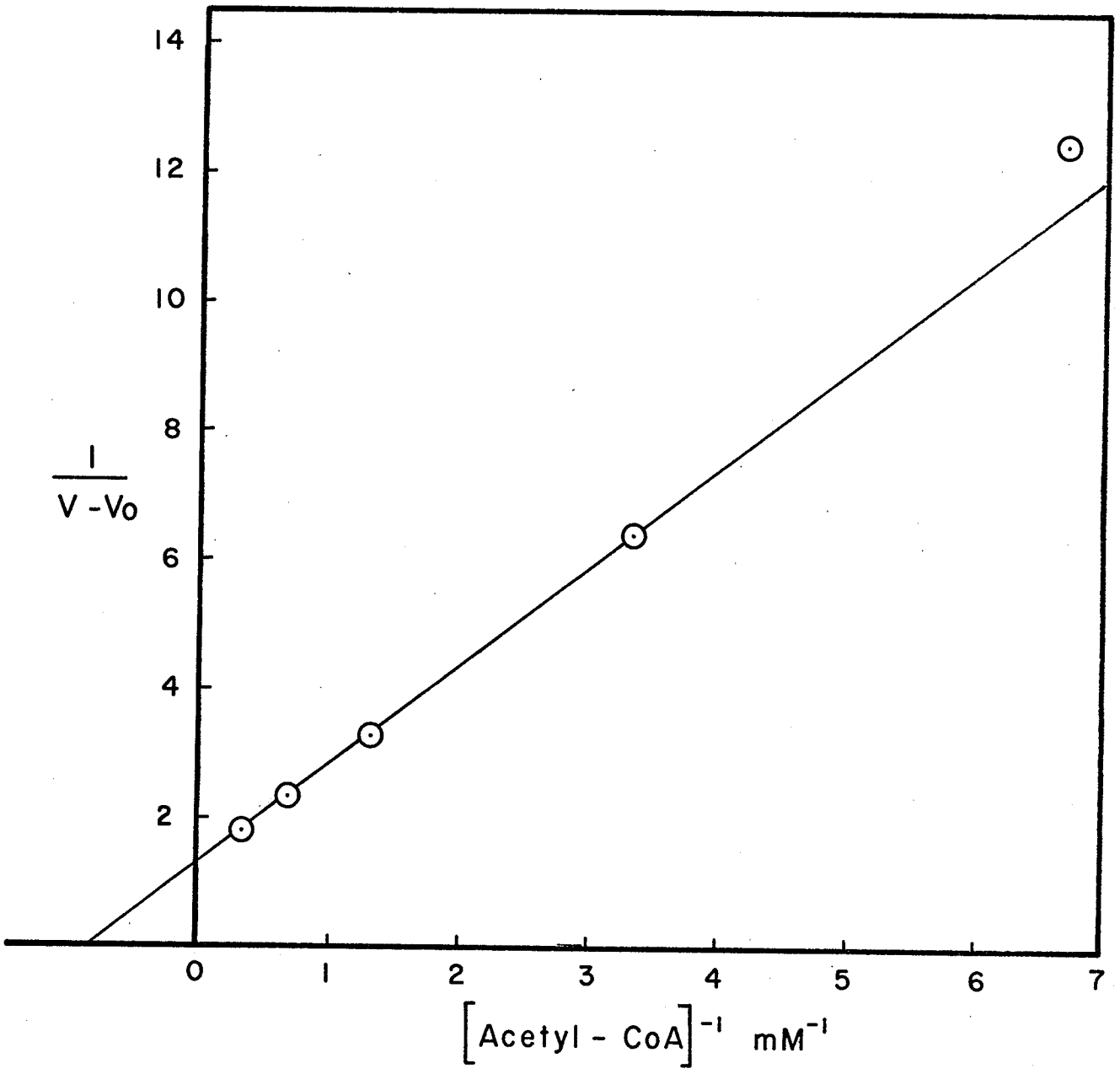
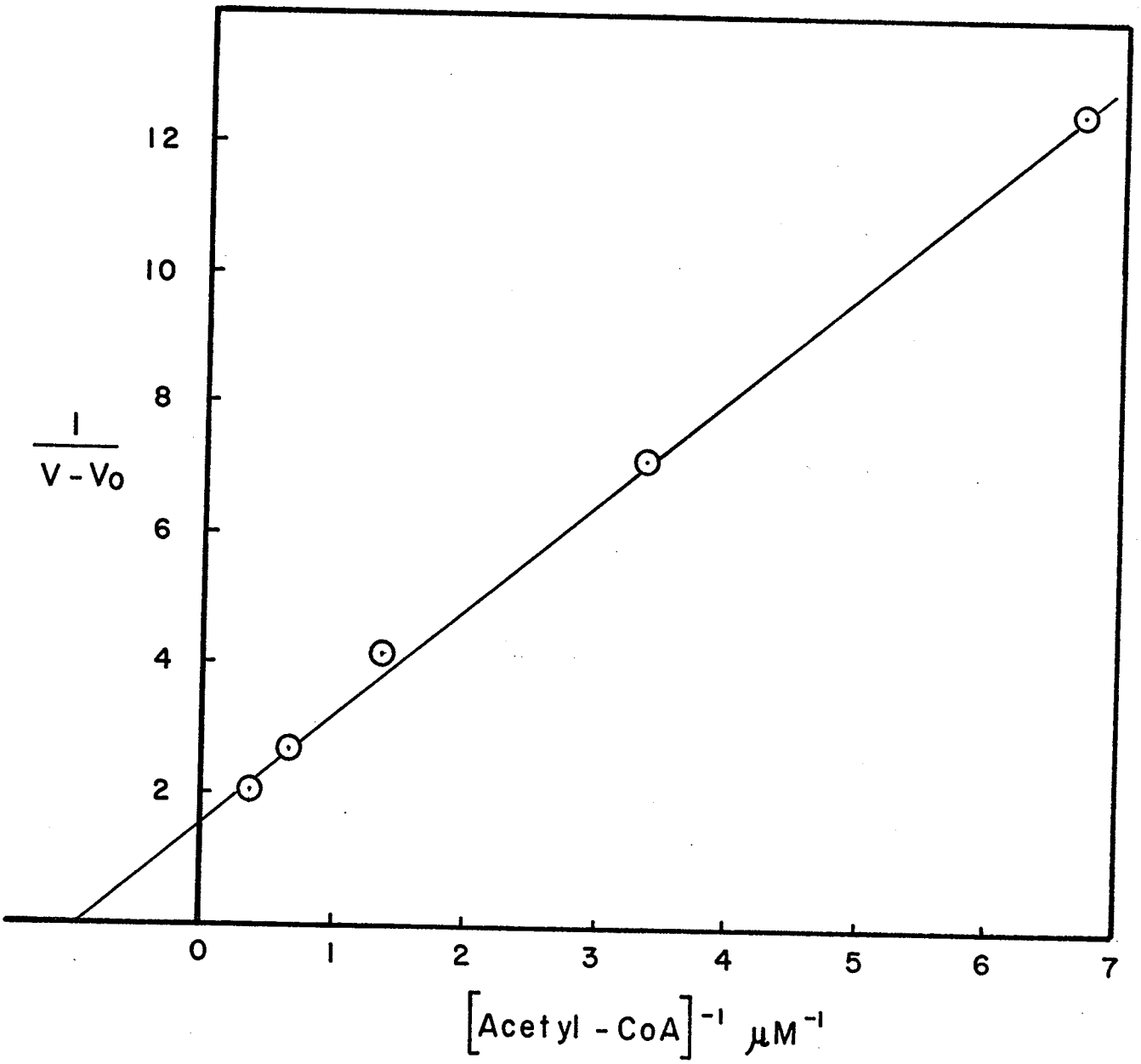

14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
1
V - Vo
[Acetyl - CoA]⁻¹ mM⁻¹

FIGURE 10. The effect of acetyl CoA at pH 8.0. Standard conditions were used for the velocity determination in the absence of acetyl CoA (v_o). v was determined in the presence of varied concentrations of acetyl CoA. The pH of HEPES buffer used was 8.0.


12
10
8
6
4
2
1/(V - V0)
0
1
2
3
4
5
6
7
[Acetyl - CoA]^-1 μM^-1

the presence of acetyl CoA.

It was found that acetyl CoA was also a strong activator of PEP carboxylase in crude extracts of T. novellus and N. europeae. The crude extract of T. thioparus was not activated by acetyl CoA when the same assay procedures were used (Methds and Materials). PEP carboxylase activity was much higher in this extract (T. thioparus) than the extracts of T. thiooxidans, T. novellus and N. europeae when these extracts were assayed in the absence of acetyl CoA.

Another pH curve under the same conditions as the previous experiment (Figure 2) was done with the addition of 30.0 μM acetyl CoA (Figure 15). A sharp drop in activity below pH 8.0 observed in Figure 2 is now replaced by a broad optimum around pH 7.5. Figure 16 shows a replot of v/Vo versus pH, in which Vo is the velocity in the absence and v is that in the presence of acetyl CoA. It is obvious that acetyl CoA is a better activator on the acid side of neutrality.

Inhibition by aspartate and malate: L-aspartate and L-malate are strong inhibitors of PEP carboxylase activity from T. thiooxidans. At pH 7.0, a 5.0 mM concentration of L-aspartate and L-malate produced 90% and 65% inhibition respectively. L-aspartate exhibited stronger inhibition than L-malate in both pH ranges studied, and the effect of

FIGURE 11. Plot of velocity versus varying PEP concentration with acetyl CoA as activator at pH 7.0. Acetyl CoA concentrations:

▽ - 0.0 μM

⊙ - 0.75 μM

△ - 3.0 μM

⊡ - 30.0 μM

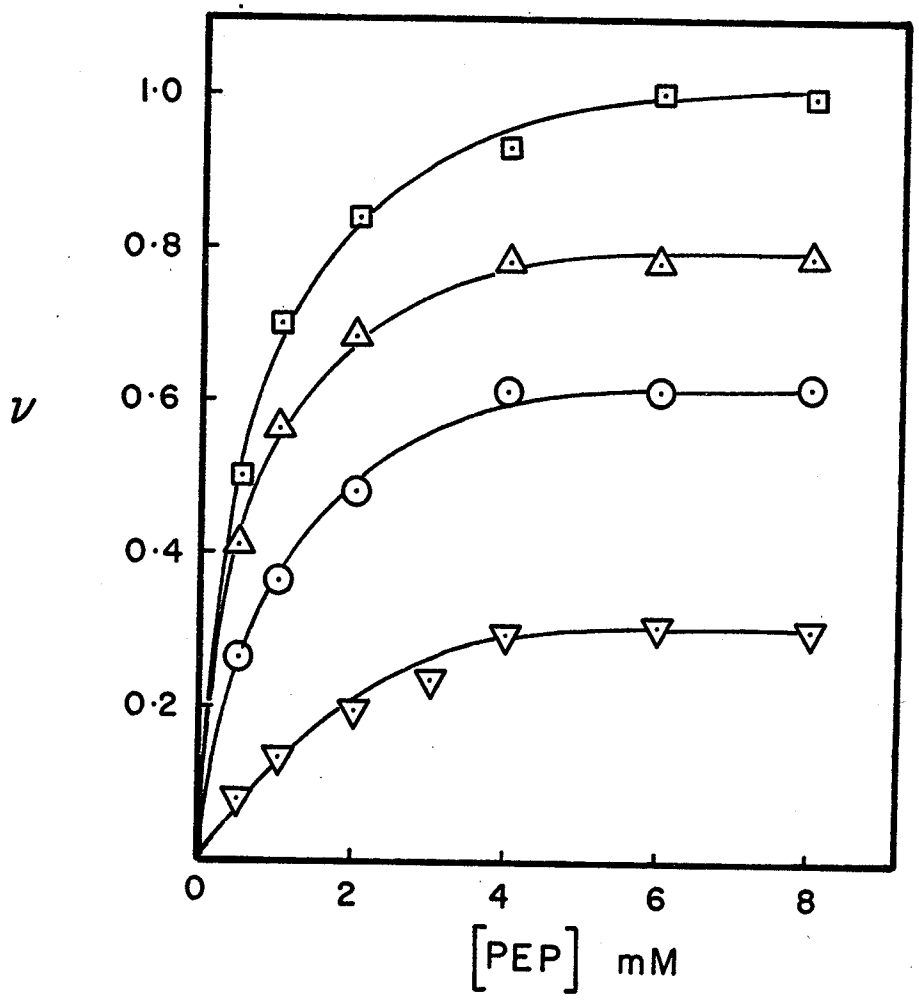

1·0
0·8
0·6
0·4
0·2
ν
0
2
4
6
8
[PEP] mM

FIGURE 12. The double reciprocal plot of Figure 11. PEP was the variable substrate with acetyl CoA as activator at pH 7.0.

Acetyl CoA concentrations:

▽ - 0.0 μM

⊙ - 0.75 μM

△ - 3.0 μM

⊡ - 30.0 μM

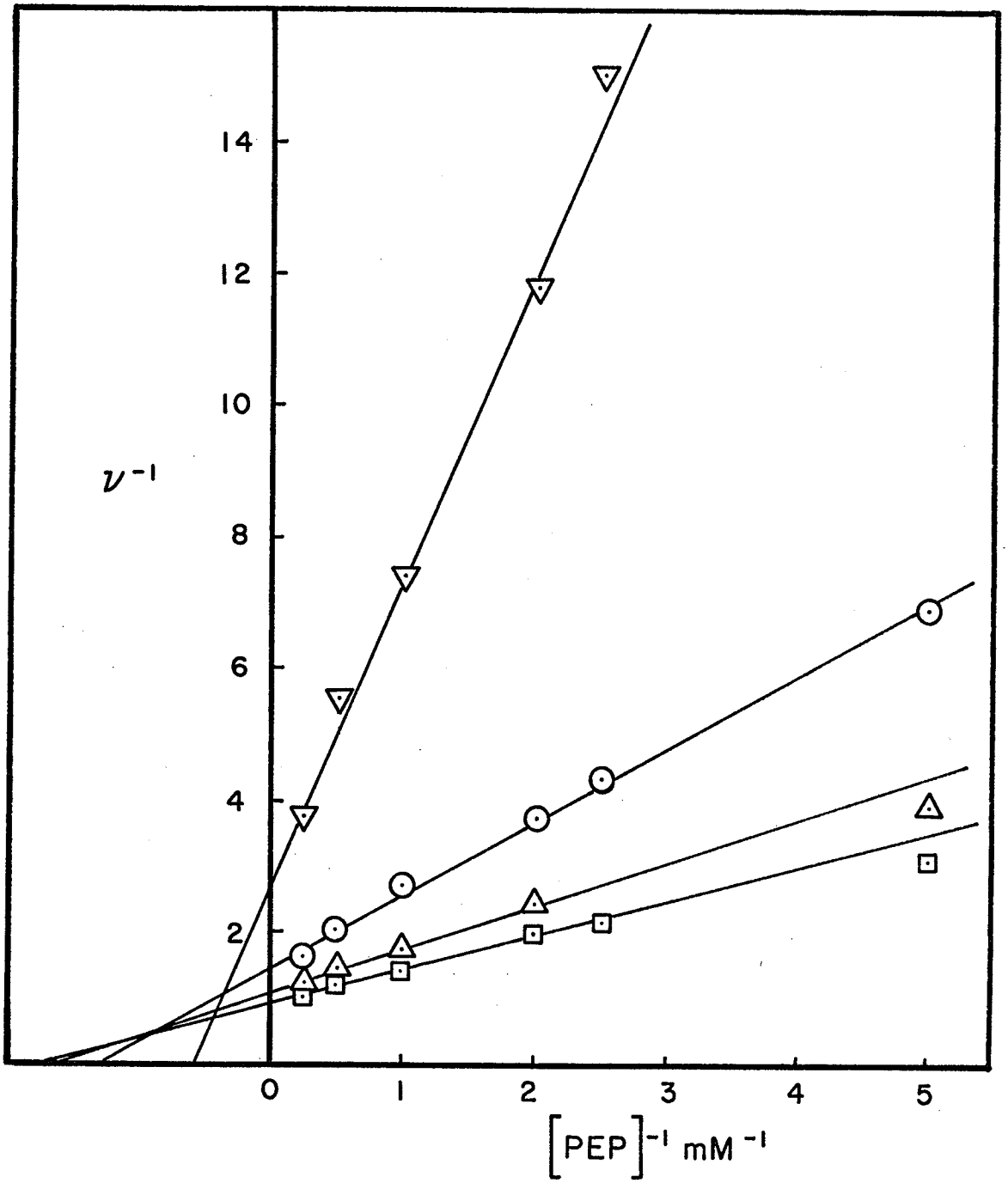

14
12
10
8
6
4
2
0
1
2
3
4
5
ν^{-1}
$[PEP]^{-1}$ mM $^{-1}$

FIGURE 13. Plot of velocity versus varying PEP concentration with acetyl CoA as activator at pH 8.0. The pH of HEPES buffer used was 8.0.

Acetyl CoA concentrations:

▽ - 0.0 μM

⊙ - 0.3 μM

△ - 3.0 μM

⊡ - 30.0 μM

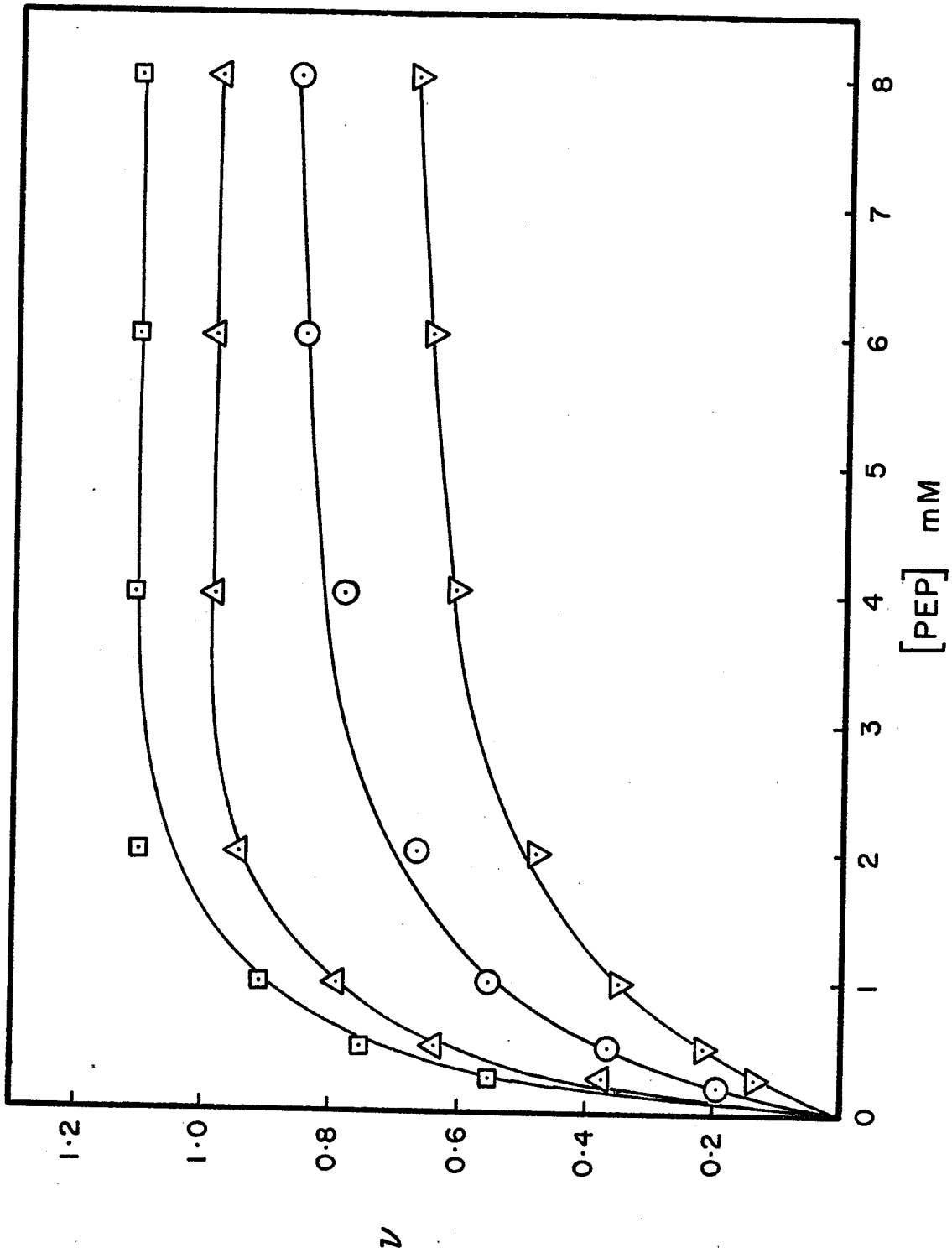

1·2
1·0
0·8
0·6
0·4
0·2
ν
0
1
2
3
4
5
6
7
8
[PEP] mM

FIGURE 14. The double reciprocal plot of Fig. 13. PEP was the variable substrate with acetyl CoA as activator at pH 8.0.

Acetyl CoA concentrations:

▽ - 0.0 μM

⊙ - 0.75 μM

△ - 3.0 μM

⊡ - 30.0 μM

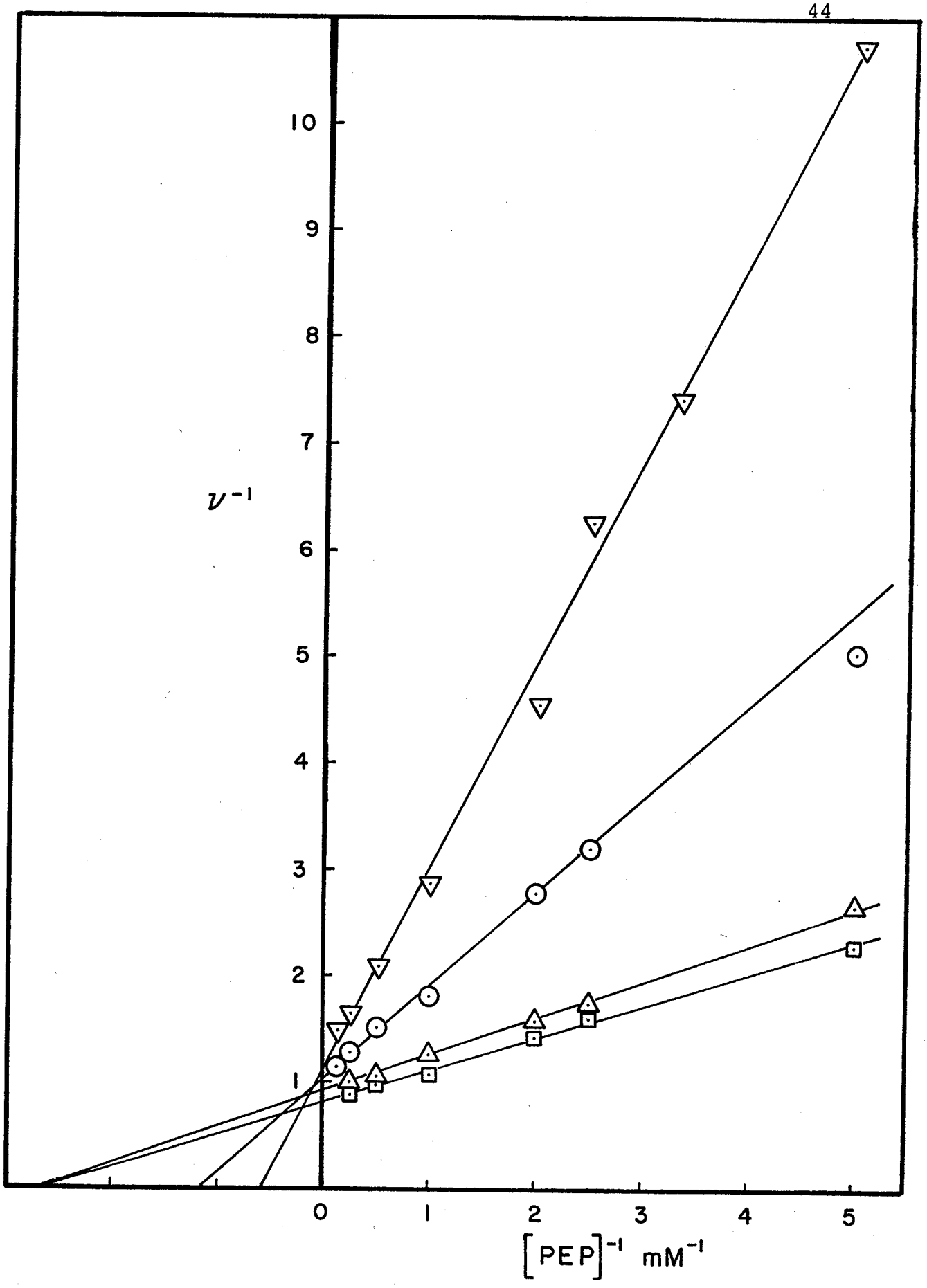

10
9
8
7
6
5
4
3
2
1
ν^{-1}
0
1
2
3
4
5
$[PEP]^{-1}$ mM^{-1}

FIGURE 15. Effect of the hydrogen ion concentration on PEP carboxylase activity in the presence of 30.0 μM acetyl CoA (activator). Assay mixtures were prepared as described in Methods and Materials with different buffers as noted.

Buffers:

▽ - MES buffer

△ - MOPS buffer

⊡ - HEPES buffer

⊙ - Bicine buffer

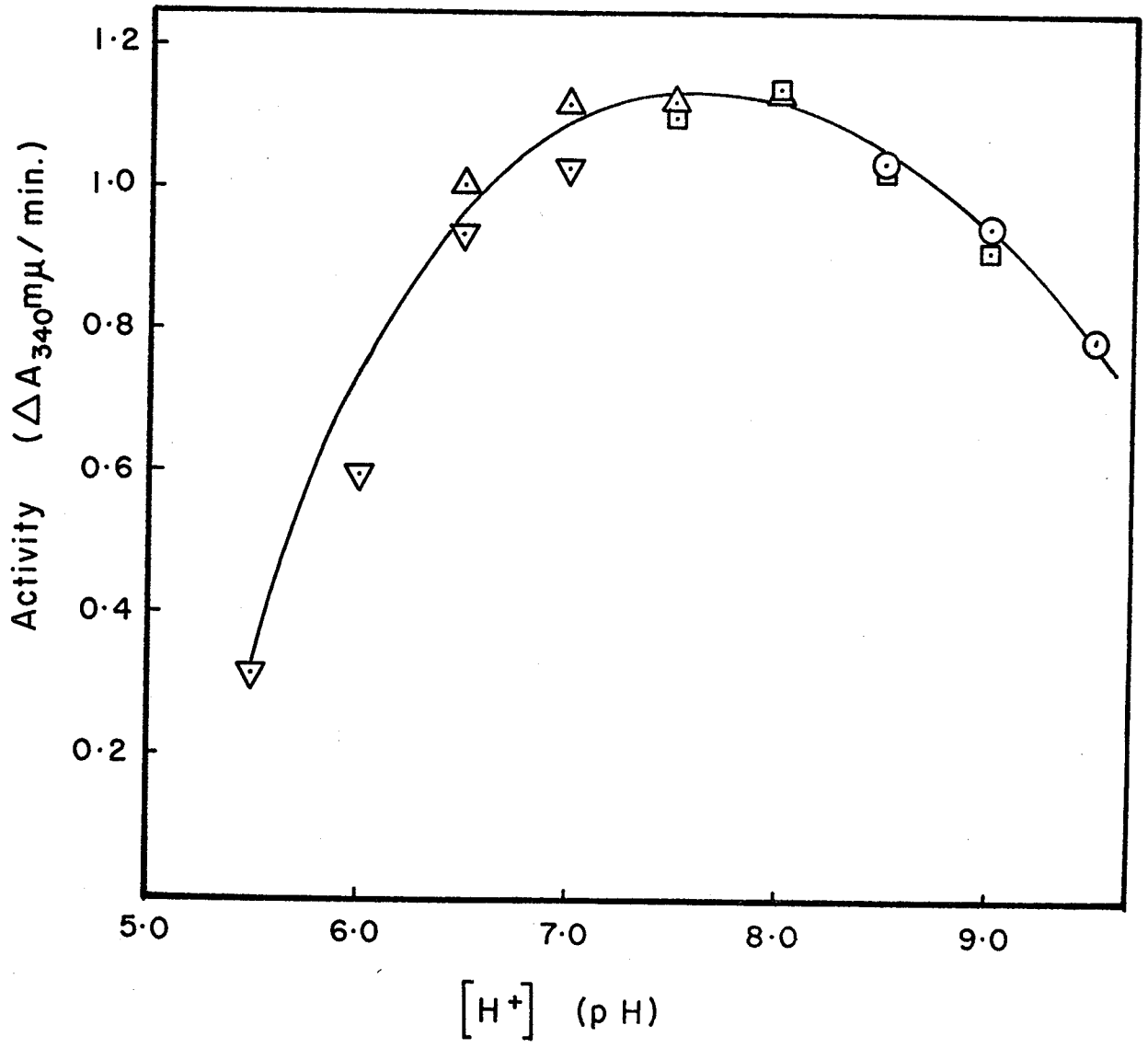
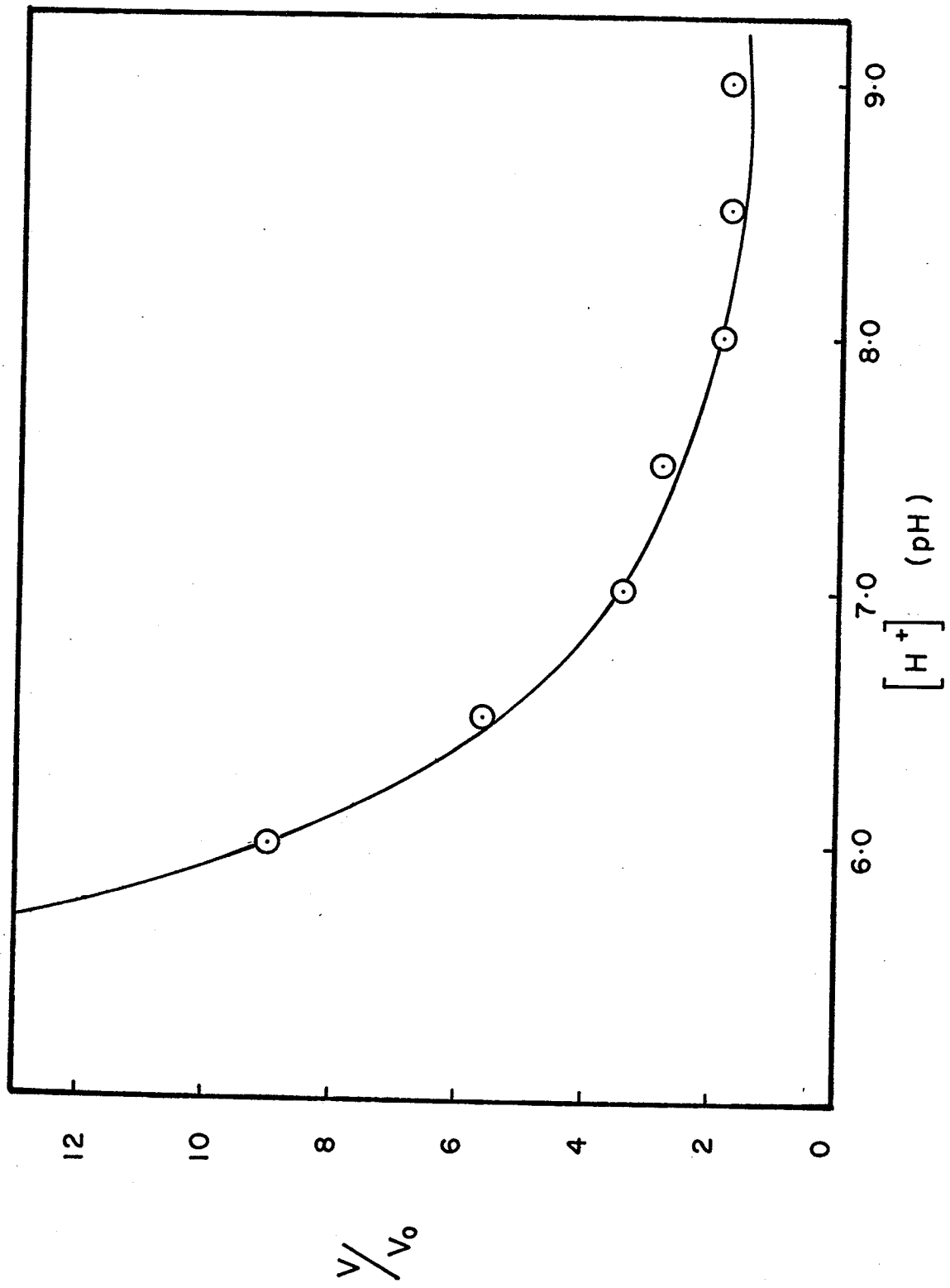

1·2
1·0
0·8
0·6
0·4
0·2
Activity (ΔA340mμ / min.)
5·0
6·0
7·0
8·0
9·0
[H+] (p H)

FIGURE 16. Replot of Figure 15 in the form v/v_o versus the hydrogen ion concentration. Standard conditions were used for the velocity determination in the absence of acetyl CoA (v_o). v was determined in the presence of 30.0 μM acetyl CoA.


V/V0
0
2
4
6
8
10
12
6·0
7·0
8·0
9·0
[H+] (pH)

aspartate was chosen for a detailed study. At pH 7.0 the reaction does not follow the classic Michaelis-Menton kinetics when PEP was used as the variable substrate in the presence of aspartate (Figure 17). In Figure 18, the data was plotted in the double reciprocal form. Instead of a straight line indicative of a Michaelis-Menton type reaction, the plot was non-linear. In order to establish the exact shape of the curved lines, another experiment was done using a large number of substrate concentrations (Figures 19 and 20). At pH 8.0, the data plotted in the same form resulted in straight lines and indicates competitive inhibition (Figure 21).

It was found that aspartate was also a powerful inhibitor of PEP carboxylase in crude extracts of T. thioparus, T. novellus and N. europeae.

It was of interest to determine the effect of L-aspartate in the presence of the activator, acetyle CoA. Plots were done using 30.0 μM acetyl CoA in the presence of aspartate, with PEP as the variable substrate. Under these conditions the non-linear double reciprocal plot of PEP with aspartate (Figure 18) became linear (Figure 22).

Other effectors: The literature cites a number of nucleotides (Sanwal and Maeba, 1966b), intermediates of the glycolytic cycle (Sanwal and Maeba, 1966b), dioxane and various macroions (Sanwal, Maeba and Cook, 1966) as

FIGURE 17. Plot of velocity versus varying PEP concentration with L-aspartate as inhibitor at pH 7.0.

L-aspartate concentrations:

▽ - 0.0 mM

⊙ - 0.25 mM

△ - 0.50 mM

⊡ - 1.0 mM

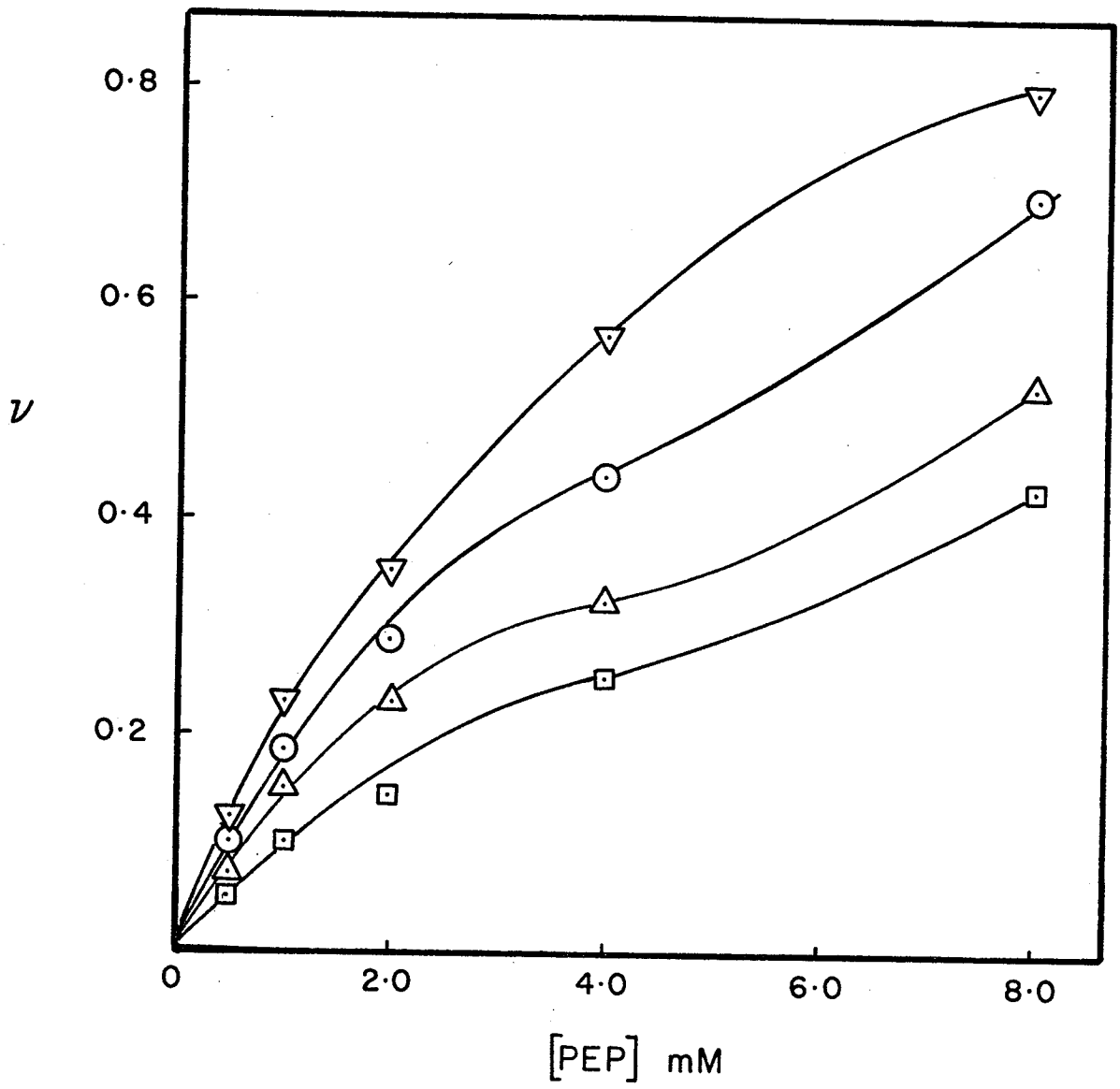

0·8
0·6
0·4
0·2
ν
0
2·0
4·0
6·0
8·0
[PEP] mM

FIGURE 18. Replot of Figure 17 in the double reciprocal form in which PEP was the variable substrate and L-aspartate the inhibitor.

L-aspartate concentrations:

▽ - 0.0 mM

⊙ - 0.25 mM

△ - 0.50 mM

⊡ - 1.0 mM

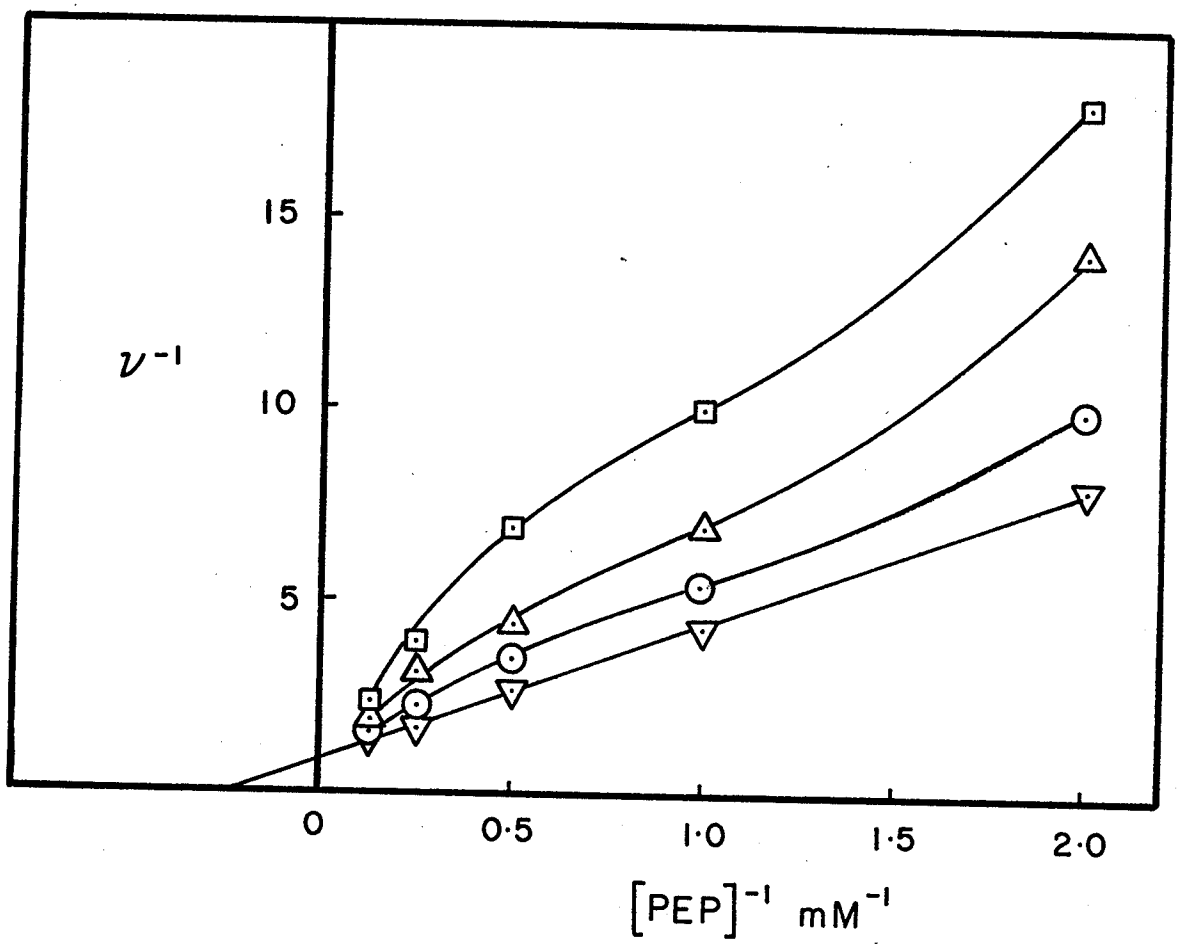

15
10
5
v^{-1}
0
0·5
1·0
1·5
2·0
$[PEP]^{-1}$ mM^{-1}

FIGURE 19. Plot of velocity versus varying PEP concentration with L-aspartate as inhibitor at pH 7.0.

L-aspartate concentrations:

⊙ - 0.0 mM

⊡ - 1.0 mM

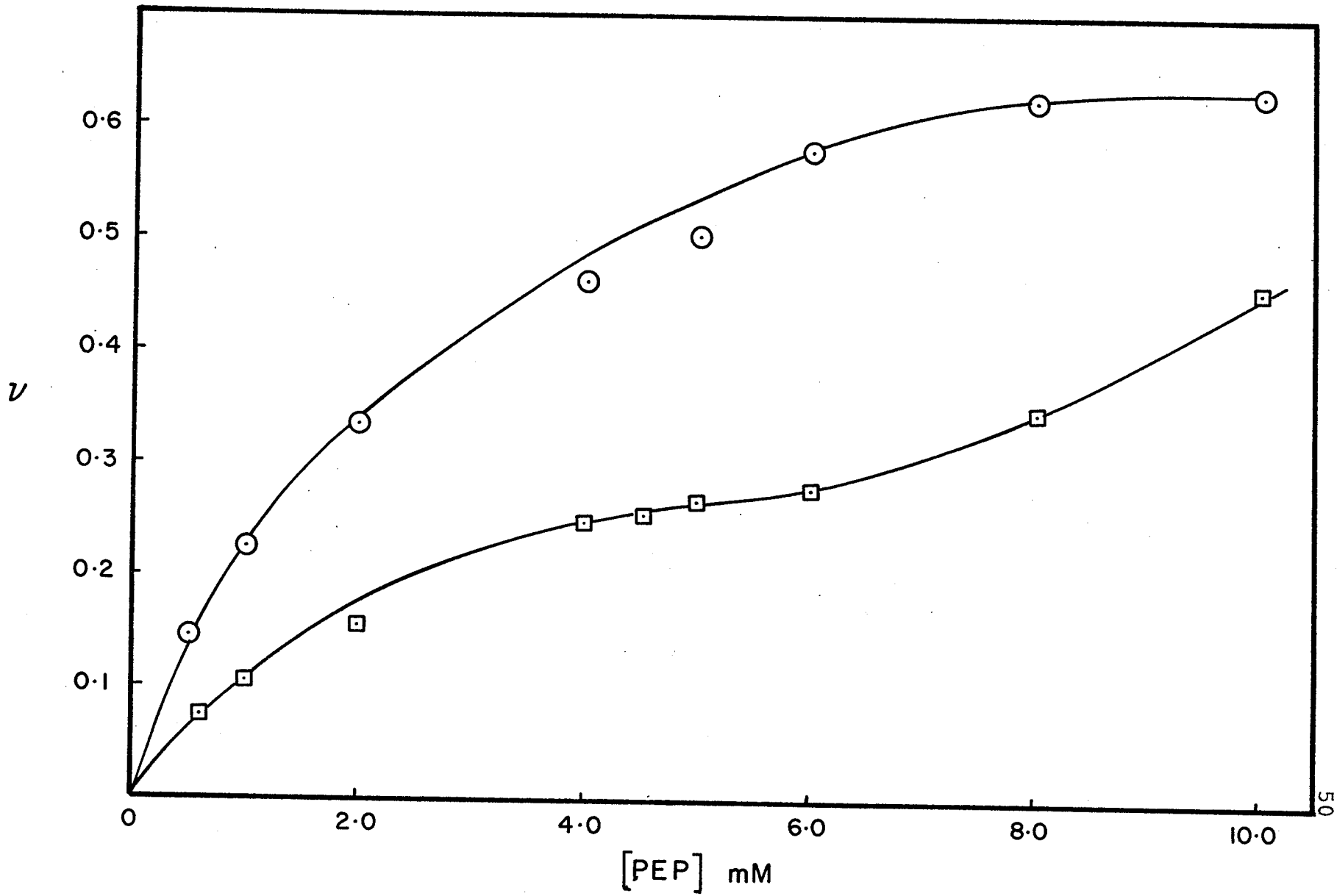

0·6
0·5
0·4
0·3
0·2
0·1
v
0
2·0
4·0
6·0
8·0
10·0
[PEP] mM

FIGURE 20. Replot of Figure 19 in the double reciprocal form in which PEP was the variable substrate and L-aspartate the inhibitor.

L-aspartate concentrations:

⊙ - 0.0 mM

⊡ - 1.0 mM

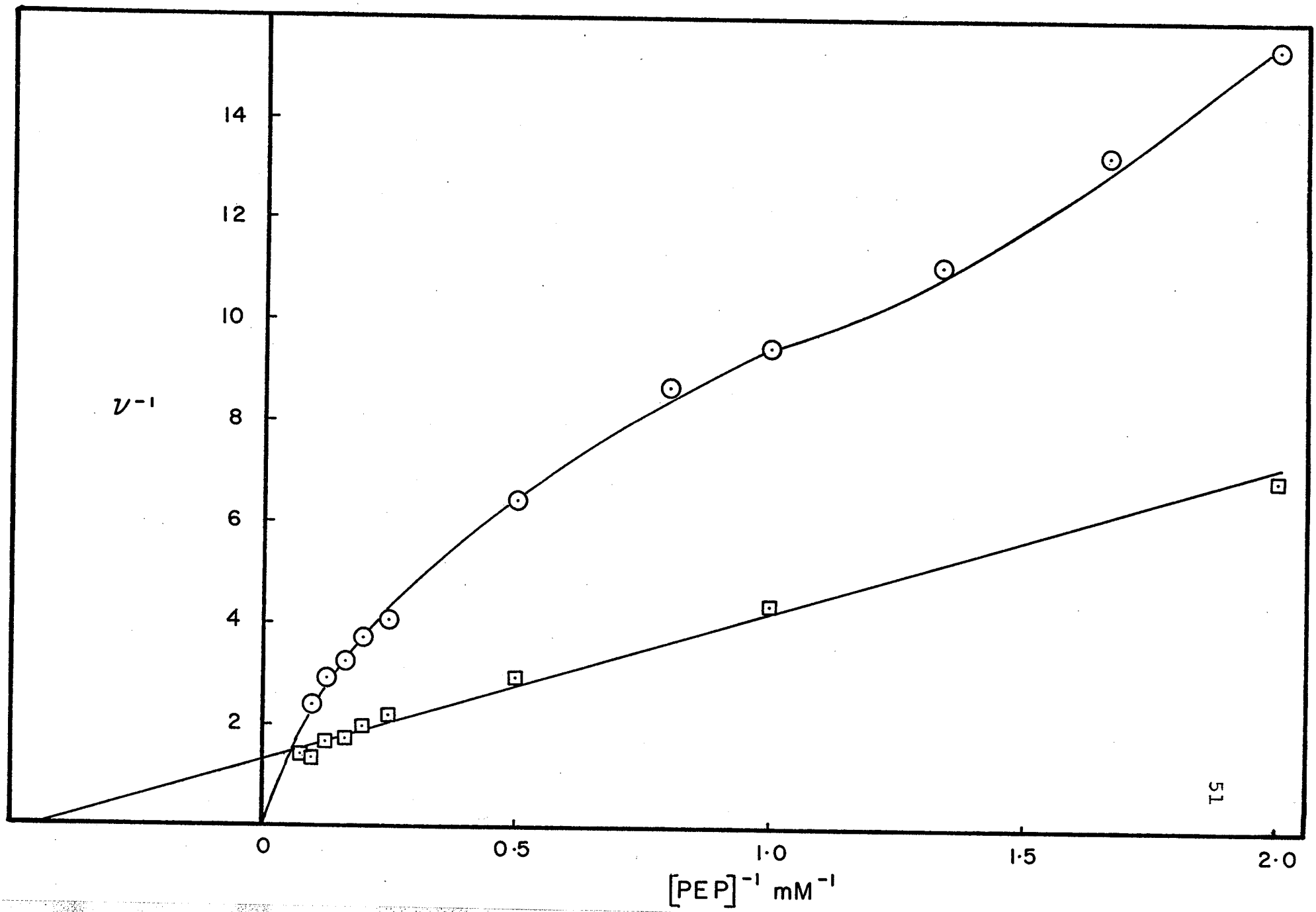

14
12
10
8
6
4
2
ν^{-1}
0
0·5
1·0
1·5
2·0
$[PEP]^{-1}$ mM^{-1}
51

FIGURE 21. The double reciprocal plot of velocity versus PEP concentration with L-aspartate as inhibitor at pH 8.0.

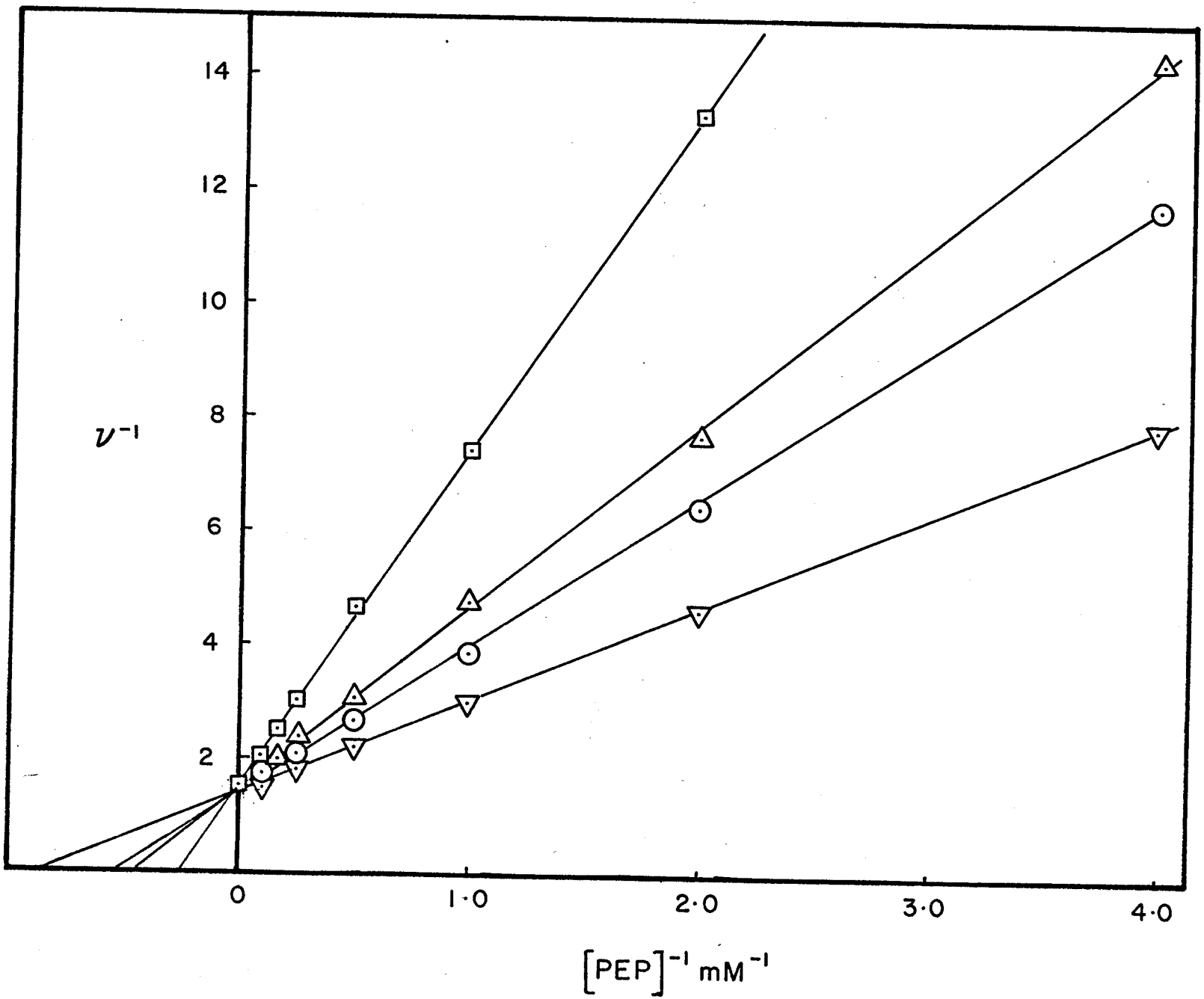
L-aspartate concentration:

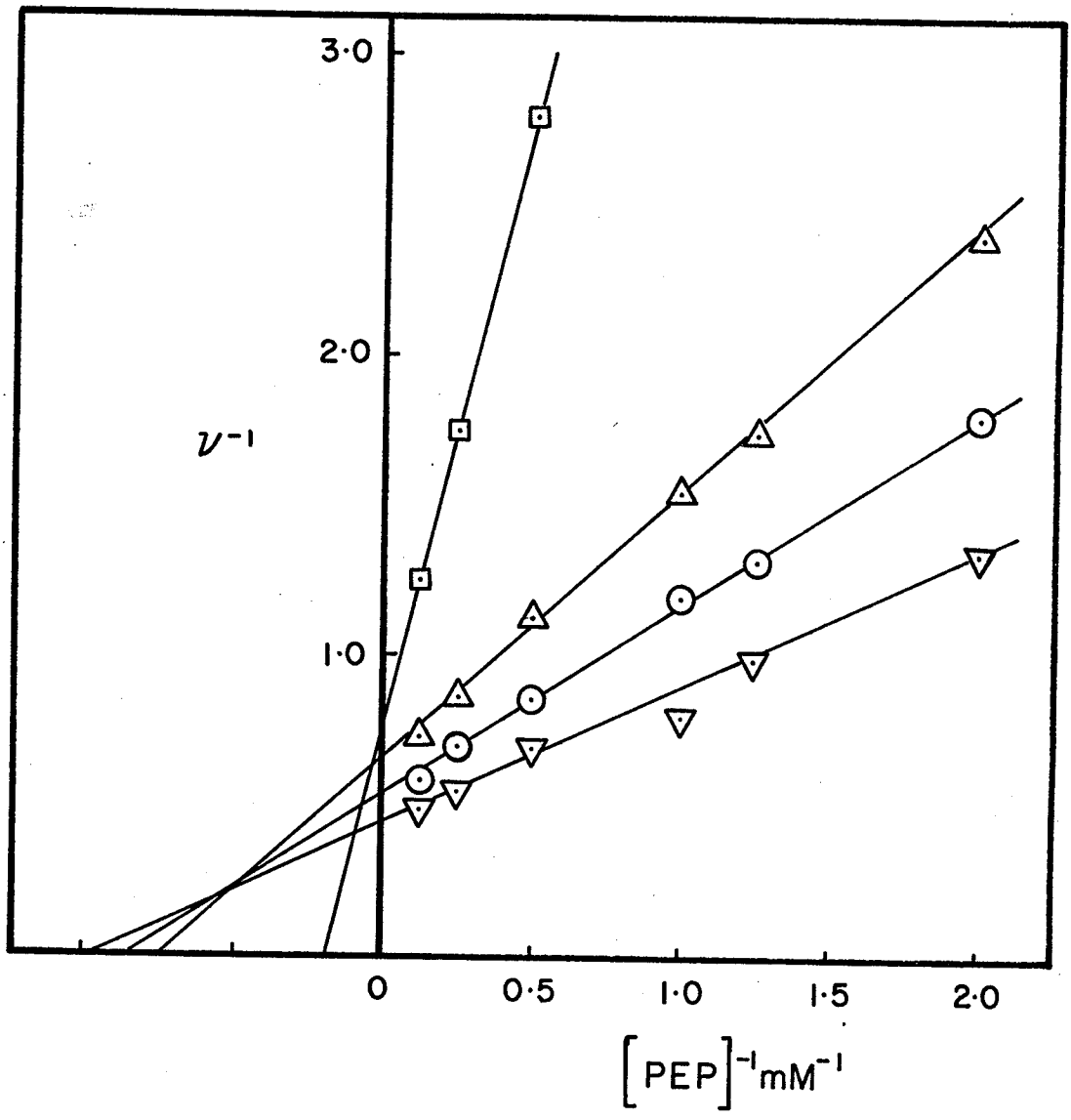
▽ - 0.0 mM

⊙ - 3.0 mM

△ - 5.0 mM

⊡ - 10.0 mM


14
12
10
8
6
4
2
v^{-1}
0
1·0
2·0
3·0
4·0
$[PEP]^{-1}$ mM^{-1}


3·0
2·0
1·0
v^{-1}
0
0·5
1·0
1·5
2·0
$[PEP]^{-1} mM^{-1}$

effectors of PEP carboxylase in the heterotrophic bacteria. The effects of many of these compounds were tested with the same enzyme from T. thiooxidans. All those compounds except those previously mentioned (acetyl CoA, aspartate and malate) failed to yield any positive results. Further investigation of this enzyme may provide evidence of other controls which this research did not uncover.

DISCUSSION

DISCUSSION

General

This study was initiated to elucidate the role of PEP carboxylase in autotrophic bacteria. As discussed earlier (see Historical), evidence has been accumulated indicating that in the enteric bacteria (heterotrophs) PEP carboxylase, an allosteric enzyme, has an anaplerotic function. It was our desire to obtain enough kinetic data on PEP carboxylase from T. thiooxidans in order to compare and contrast it to the same enzyme obtained from a heterotrophic source. For a study of this nature, the enzyme must be isolated and purified to the extent that contaminating protein (enzymes) which may influence the kinetic results of the enzyme (PEP carboxylase) are removed, and sufficient activity of the enzyme be obtained to ensure a high reliability of the data accumulated.

Effect of Incubation Time on PEP Carboxylase Activity

An important factor in obtaining maximum activity of PEP carboxylase from crude extracts of T. thiooxidans was the length of incubation time. The dependence of enzyme level on culture age is evident in T. thiooxidans. Although the yield (wet weight of cells) increased with an extension of the incubation period from five to seven days,

the level of PEP carboxylase activity dropped drastically during this two day period.

After five days, the organism may not require PEP carboxylase for protein biosynthesis, but the production of carbohydrate continues with a corresponding increase in yield (wet weight). The PEP carboxylase activity decreases since its production is no longer necessary for cell growth.

Effect of Freezing Whole Cells on PEP Carboxylase Activity

As previously mentioned (see Results), PEP carboxylase activity could not be found in extracts prepared from frozen cells regardless of culture age. As a result of this discovery, all extracts were prepared immediately after harvesting. The loss of activity upon freezing could be due to a result of structural changes in the cell membrane caused by the lowering of the temperature. There is no direct evidence as to the location of PEP carboxylase within the cell, but if the enzyme is associated with the cell membrane, structural changes of the cell wall induced by freezing could trap the enzyme and prevent its release when the cells are disrupted.

Detergents (Triton-X, sodium dodecyl sulfate) were tested to facilitate the release of the enzyme from frozen cells during sonication, both of which failed to yield PEP carboxylase activity.

Kinetic Constants of PEP Carboxylase

The results of initial velocity studies of PEP carboxylase yielded apparent Km's determined from the double reciprocal rate-concentration plots (Lineweaver and Burk, 1934) for Mg^{++}, HCO_3^- and PEP at pH 7.0 and 8.0. A comparison of Michaelis constants for PEP carboxylase from T. thiooxidans as determined in this study with those from the S. typhimurium enzyme has been presented in Table II. The results for the enzyme from S. typhimurium were those of Maeba (Doctoral Dissertation, 1965). It can be seen that the values are of the same order of magnitude with the exception of the Km for PEP from S. typhimurium which is much larger. With the Salmonella enzyme, however, the double reciprocal plot for PEP was non-linear unlike the T. thiooxidans enzyme where the plot became non-linear only in the presence of aspartate, an allosteric inhibitor. The Km for PEP from S. typhimurium was determined from half-maximal velocity.

Effect of pH on PEP Carboxylase Activity

As indicated (Figure 2) the pH optimum of PEP carboxylase was 7.9 - 8.0 in the absence of allosteric effectors. The S. typhimurium enzyme shows a pH optimum of 8.6 - 9.2 (Sanwal and Maeba, 1966b). A marked change occurs when the effect of hydrogen ion concentration is studied in the presence of the activator acetyl CoA (Figure 15). The

TABLE II

COMPARISON OF MICHAELIS CONSTANTS FOR

PEP CARBOXYLASE FROM SEVERAL SOURCES

	T. thiooxidans (pH 7.0)	*T. thiooxidans* (pH 8.0)	*S. typhimurium*
Michaelis constants (mM)			
magnesium	1.1	0.8	1.0
bicarbonate	0.4	1.1	2.0
phosphoenolpyruvate	1.4	1.5	10.0

results of this study will be discussed later.

Activation by Acetyl CoA

PEP carboxylase isolated from T. thiooxidans is strongly activated by acetyl CoA (Figures 12 and 14). This result conforms with those obtained by Cánovas and Kornberg (1965) in E. coli, Sanwal and Maeba (1965) in S. typhimurium and Din et al (1967) in F. ferrooxidans. The concentration of activator required to produce half-maximal activation, or Ka, was determined to be 1.2 and 1.0 μM (Figures 9 and 10) at pH 7.0 and 8.0 respectively. The purpose of this activation seems to be to catalyse the formation of oxalacetate required for the oxidation of acetyl CoA. This mechanism ensures that a balance between catabolic and anaplerotic reactions in vivo are maintained. The Ka (acetyl CoA) of PEP carboxylase from T. thiooxidans is much smaller than those obtained from a heterotrophic source (0.5 mM in S. typhimurium, Sanwal and Maeba, 1966; 0.17 mM in E. coli, Smith, 1968). Atmospheric CO_2 being the sole source of carbon in autotrophic organisms, an extremely efficient control over biosynthesis is necessary. The low Ka for acetyl CoA demonstrated by T. thiooxidans would provide this very fine control of the "anaplerotic" PEP carboxylase.

It should be noted that the effect of hydrogen ion concentration is greatly effected by the presence of

acetyl CoA (Figure 15). The pH curve in the presence of acetyl CoA levels off between pH 6.5 - 8.5 and does not show a singular optimum pH as shown in the absence of this effector (Figure 2). A plot of activation versus hydrogen ion concentration (Figure 16) shows the enzyme is activated more at low pH with the magnitude of activation increasing as the hydrogen ion concentration is increased. Since the optimum pH for growth of T. thiooxidans is 2.0 - 2.8, the physiological pH could be much lower than organisms which have a higher pH optimum. This being the case, the enzyme would be in a natural conformation at low pH and more susceptible to its controls. As the pH rises these controls would gradually be lost and the conformation of the enzyme would become more rigid and densensitized to its effectors.

Inhibition by L-Aspartate

It has already been demonstrated that L-aspartate serves as a powerful inhibitor of PEP carboxylase (see Historical) in E. coli, S. typhimurium and F. ferrooxidans. It is not unexpected that L-aspartate should also be a feedback inhibitor of PEP carboxylase in T. thiooxidans, since aspartate is one of the major products synthesized through the action of this enzyme.

The kinetics of inhibition by L-aspartate were very interesting. The double reciprocal plot of PEP in the

presence of L-aspartate (Figure 17) at pH 7.0 was non-linear, and the nature of the curves seems to indicate that the phenomenon of negative cooperativity is occurring. The same plot at pH 8.0 (Figure 21) is linear and indicates competitive inhibition. As in the case of acetyl CoA, the effect of aspartate increases as the pH drops. The concentration of aspartate required to produce significant inhibition is much higher at pH 8.0 than at 7.0.

The non-linear double reciprocal plot of PEP in the presence of L-aspartate at pH 7.0 (Figure 18) became linear with the addition of 30.0 μM acetyl CoA (Figure 22). The addition of acetyl CoA may desensitize the enzyme to the allosteric inhibitor (aspartate) due to a conformational change of the enzyme brought about by the addition of the allosteric activator (acetyl CoA). Aspartate demonstrates non-competitive inhibition in this case (Figure 22).

Inhibition by L-malate

L-malate was found to be a potent inhibitor of PEP carboxylase from T. thiooxidans, as it is in E. coli (Corwin and Fanning, 1968) and S. typhimurium (Maeba and Sanwal, 1969).

Since the major function of this enzyme is to furnish oxalacetate to replace the Krebs cycle acids used up in biosynthesis, it is important that the cells recognize the over-accumulation of these acids. This inhibition by

malate allows the direct control of Krebs cycle acid formation by a Krebs cycle acid itself.

Lack of Other Effectors

A number of other effectors have been demonstrated for PEP carboxylase from S. typhimurium (Sanwal and Maeba, 1966a,b; Sanwal, Maeba and Cook, 1966). A number of these compounds were tested on the same enzyme from T. thiooxidans. Those tested (FDP, dioxane, GTP and CDP) showed no effect on this enzyme.

Since chemoautotrophic bacteria derive energy from the oxidation of inorganic compounds, the role of the tricarboxylic acid cycle is presumably mainly biosynthetic rather than energy-generating unlike the cycle for the heterotrophic Enterobacteriaceae. This being the case, those controls which operate on PEP carboxylase to regulate energy production of the tricarboxylic acid cycle would be unnecessary since this cycle functions mainly for biosynthesis in T. thicoxidans. Fructose diphosphate, an activator of PEP carboxylase from S. typhimurium (Sanwal and Maeba, 1966), is proposed to be related to the energy metabolism of the cell (Atkinson, 1965). It is therefore not unexpected that this control should be absent in T. thiooxidans.

The absence of other controls may reflect the basic difference between heterotrophic and autotrophic organisms.

Further investigation would be required to determine whether or not other control mechanisms are operating in T. thiooxidans.

Table III is a summary of effectors of PEP carboxylase determined for a number of autotrophic organisms compared to those of the heterotroph S. typhimurium. The results for the enzyme from S. typhimurium are those of Sanwal and Maeba (1968). Extracts were assayed in the crude, with the exception of T. thiooxidans and S. typhimurium.

The fact that PEP carboxylase from T. thioparus was not affected in the presence of acetyl CoA may be due to already saturating amounts of this activator present in the crude system. It has been noted that the Ka for acetyl CoA in T. thiooxidans is approximately 1.0 μM. If the Ka for acetyl CoA in T. thioparus is of the same order of magnitude, it is possible that the crude preparation may contain saturating amounts of this compound. This theory is supported by the fact that extracts prepared from this source (T. thioparus) contained PEP carboxylase of much higher activity than did those extracts prepared from other autotrophic organisms. Crude extracts of T. novellus and N. europeae were also activited by acetyl CoA and inhibited by aspartate.

TABLE III

COMPARISON OF EFFECTORS OF PEP

CARBOXYLASE FROM SEVERAL SOURCES

	T. Thiooxidans	*T. Thioparus*	*F. Ferrooxidans*	*S. Typhimurium*
Effector				
acetyl CoA	+	NE	+	+
FDP	NE	NE	NE	+
aspartate	-	-	-	-
malate	-	-	-	-

+ = activator; - = inhibitor; NE = no effect

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