PURIFICATION AND STUDY OF ACETOLACTATE SYNTHETASE, AND NADH-SPECIFIC LACTATE DEHYDROGENASE FROM AEROBACTER AEROGENES

by

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To My Parents

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ABSTRACT

(Acetolactate Synthetase)

ABSTRACT

Acetolactate synthetase, the enzyme which catalyzes the condensation of pyruvic acid to acetolactic acid was extracted and partially purified from Aerobacter aerogenes. For kinetic studies the enzyme was assayed using a spectrophotometric coupled assay procedure. At pH 5.7, the optimum pH, the enzyme displayed a sigmoid rate saturation curve when assayed in phosphate buffer. When assayed in distilled water or in the presence of acetate, propionate, or formate, normal Michaelis-Menten type kinetics were observed. Acetate activated enzyme activity to a maximum at 0.1 M, after which inhibition was observed. Propionate and formate also activated enzyme activity over that observed in distilled water. This activating effect by these monocarboxylic acid buffers could not be duplicated by other carboxylic acid buffers (succinate, fumarate, citrate, and lactate). Double reciprocal plots of enzyme activity and pyruvate concentration were still non-linear in these buffers, and inhibition was still observed but to a lesser degree than in phosphate buffer.

Acetolactate synthetase is strongly influenced by its ionic environment. The inhibiting effectiveness of monovalent anions on the activity of the enzyme followed the Hofmeister lyotropic series. In addition these anions exhibited stimulation of enzyme activity at low anion concentrations. A model to account for these ionic effects has been proposed.

ABSTRACT

(Lactate Dehydrogenase)

ABSTRACT

An NADH-specific lactate dehydrogenase was extracted and purified from a laboratory strain of Aerobacter aerogenes. The enzyme gave one protein peak at pH 8.0 in a Spinco Model E analytical centrifuge.

The enzyme displayed normal Michaelis-Menten type kinetics at pH 5.7 where the rate saturation curve was hyperbolic. A marked deviation from Michaelis-Menten kinetics was observed when the enzyme was assayed at pH 6.5 and greater. Under these conditions the rate of NADH oxidation has been shown to be a sigmoid function of pyruvate concentration. Although the affinity of the enzyme for pyruvate decreased with increasing pH, the maximum velocity was unaffected by pH changes.

The presence of α-ketobutyrate at a concentration of 20 mM transformed the sigmoid rate curve to a rectangular hyperbola. This effect could not be duplicated by α-ketoglutarate or oxamate, although these compounds inhibited enzyme activity at both pH 5.7 and 7.0.

ATP inhibited enzyme activity competitively with respect to the coenzyme NADH, and non-competitively with respect to pyruvate. No evidence was obtained to suggest that ATP was an allosteric effector.

The inhibiting effectiveness of various anions on lactate dehydrogenase activity followed the Hofmeister series. SCN^-, Cl^-, and $SO^=_4$ inhibited enzyme activity competitively with respect to pyruvate and non-competitively with respect to the coenzyme NADH.

The minimal subunit molecular weight of lactate dehydrogenase determined using SDS polyacrylamide gel electrophoresis was estimated to be 36,000. The molecular weight of the enzyme was estimated to be 145,000, and varied with the presence of a number of cofactors, and enzyme concentration, and pH.

At enzyme concentrations approaching the concentration existing in cells, lactate dehydrogenase displayed normal Michaelis-Menten type kinetics at pH 7.0.

A model for the enzyme has been proposed as an aid in the discussion of the results obtained in this study.

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ABBREVIATIONS

AMP	adenosine-5'-monophosphate
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphospate
GTP	guanosine-5'-triphosphate
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide phosphate (reduced)
TDP	thiamine diphosphate (cocarboxylase)
SDS	sodium dodecyl sulfate
DEAE-cellulose	diethylaminoethyl-cellulose
DEAE-sephadex	diethylaminoethyl-sephadex

Part I ACETOLACTATE SYNTHETASE

INTRODUCTION

INTRODUCTION

Purification of acetolactate synthetase from Aerobacter aerogenes was carried out by Juni (1952). Using manometric techniques, a hyperbolic relationship was observed between enzyme activity and pyruvate concentration. The effect of thiamine diphosphate and manganese on enzyme activity were observed by Juni (1952), but it was Krampitz et al (1961) who studied the role of thiamine in catalysis and showed that the initial attack of pyruvate was by the carbon at position 2 of the thiazole ring of thiamine to form hydroxyethyl thiamine, which condensed with the second molecule of pyruvate to form α-acetolactic acid.

Evidence was presented for the existence of two distinct α-acetolactate-forming enzymes in Aerobacter aerogenes (Halpern and Umbarger, 1959). One enzyme, a biosynthetic enzyme involved in the biosynthesis of valine and isoleucine displayed a pH optimum of 8.0, and the other enzyme not involved in valine and isoleucine biosynthesis displayed a pH optimum of 6.0.

The pH 6.0 enzyme from Aerobacter aerogenes has been purified and crystallized, and some of its kinetic, molecular, and biochemical properties have been studied (Störmer, 1967; Störmer, 1968; Störmer et al, 1969; Tveitt and Störmer, 1969).

The kinetic behaviour of acetolactate synthetase is strongly influenced by its ionic environment. In the present study the effect of carboxylic acid buffers, and mono and

divalent anions was undertaken. In addition a model is proposed to account for the observed results.

HISTORICAL

HISTORICAL

Voges and Proskauer (1893) described the formation of an eosin-like coloration in glucose peptone cultures of certain bacteria to which had been added a 10% solution of potassium hydroxide. The reaction, termed the Voges-Proskauer reaction, depends on the formation of acetylmethylcarbinol (acetoin). Hardin and Norris (1912) explained the production of acetoin by assuming a condensation of intermediately formed acetaldehyde. Subsequently, Neuberg and Reinfurth (1923) concluded that one molecule of naturally formed acetaldehyde condensed with one molecule of added acetaldehyde to form one molecule of acetoin. The reaction was termed "acyloin condensation" and the enzyme responsible was called carboligase.

$$2\ CH_3CHO \longrightarrow CH_3\ CHOHCOCH_3$$

Starting from pyruvic acid, the sequence of reaction is:

$$2\ CH_3COCOOH \longrightarrow 2CH_3CHO + 2CO_2$$

$$2\ CH_3CHO \longrightarrow CH_3CHOHCOCH_3$$

A number of investigators have suggested that the mechanism of acetoin formation involves the condensation of acetaldehyde and either pyruvic acid, or a derivative in the case of animal and yeast preparations (Dirscherl, 1931; Green et al, 1942). It was generally accepted that in the formation of acetoin from pyruvic acid by such bacteria as Aerobacter aerogenes, the pyruvic acid must first be decarboxylated to acetaldehyde. No evidence has been presented that Aerobacter

aerogenes could carry out such a decarboxylation. The mechanism of formation of acetoin may differ in different organisms and tissues as evidenced by the fact that addition of acetaldehyde to bacterial juices of Aerobacter aerogenes does not increase the yield of acetoin, whereas the addition of acetaldehyde to yeast juices does yield an increase in acetoin (Green et al, 1942; Silverman and Werkman, 1941; Gross and Werkman, 1947). Similar results have been obtained by Lemoigne et al (1949) with Bacillus subtilis. If acetaldehyde is an intermediate in the bacterial system, only the biologically active form is utilized by the enzyme.

By grinding cells with powdered glass, Silverman and Werkman (1941) extracted an enzyme preparation from cells of Aerobacter aerogenes capable of converting pyruvic acid to acetoin and CO_2. In their studies on pyruvic acid metabolism of bacteria, Watt and Krampitz (1947) suggested that a new intermediate, α-acetolactic acid, $CH_3COH(COCH_3)\ COOH$, probably formed by condensation of acetaldehyde and pyruvic acid, might be involved in the formation of acetoin. Krampitz (1948) synthesized α-acetolactic acid and has shown that a bacterial preparation capable of forming acetoin from pyruvic acid is also capable of decarboxylating the synthetic α-acetolactic acid rapidly.

Based on these observations, Juni (1952) undertook a study of the mechanism of acetoin formation and resolved the enzyme system of Aerobacter aerogenes into two components.

The first of these components acts on pyruvic acid to produce α-acetolactic acid.

$$2CH_3COCOOH \longrightarrow CH_3COH(COCH_3)COOH + CO_2$$

The enzyme was termed acetolactate synthetase. The second component decarboxylated α-acetolactic acid to yield acetoin.

$$CH_3COH(COCH_3)COOH \longrightarrow CH_3COCHOCH_3 + CO_2$$

The enzyme was termed α-acetolactate decarboxylase. Both enzyme components were found to have a pH optimum of 6.0 and acetolactate synthetase was stimulated by cocarboxylase (thiamine diphosphate). Juni (1952) presented evidence that in contrast to the bacterial system, the formation of acetoin by yeast and pig heart tissue preparations involves the condensation of an acetaldehyde to a pyruvic acid and does not involve α-acetolactic acid as an intermediate; whereas acetaldehyde plays no role in bacterial systems. Evidence now shows that there are at least three different mechanisms for the formation of acetoin; the four-carbon ketol originating from pyruvic acid in bacteria (Silverman and Werkman, 1941; Lemoigne et al, 1949), from pyruvic acid and acetaldehyde in yeast (Neuberg and Simon, 1925) and animal tissue (Green et al, 1942) and from acetaldehyde alone in plants (Tomiyasu, 1937).

Carboxylase in yeast (Neuberg and Karozag, 1911) brings about the following reaction:

$$CH_3COCOOH \longrightarrow CH_3CHO + CO_2$$

Auhagen (1933) was able to split carboxylase into a protein

and a prosthetic group. The latter was recognized as thiamine diphosphate by Lohmann and Schuster (1937). Because of the similarity in mechanism between yeast carboxylase and bacterial systems forming acetoin, the effect of thiamine diphosphate on acetoin production in bacteria was studied by Silverman and Werkman (1941) who found that the cell-free enzyme preparation acting on pyruvic acid was stimulated 50% by the addition of cocarboxylase (thiamine diphosphate). Juni (1952) working with the purified enzyme system, found 100% stimulation by the addition of thiamine diphosphate.

Several theories have been advanced to explain the role of thiamine diphosphate and its mechanims of action in the formation of acetoin. Langenbeck (1933) proposed a Schiff's base intermediate between pyruvate and the amino group at position 4 of the pyrimidine moiety of thiamine diphosphate. Lipmann (1936) suggested an oxidation-reduction mechanism involving the quaternary nitrogen of the thiazole moiety. Ingraham and Westheimer (1956) as well as Breslow (1956), proposed that carbanion formation on the N-methylene group of thiamine was involved. None of these mechanisms have been experimentally verified.

Two important observations made with a non-enzymatic system contributed to a rational approach to elucidation of the mode of action of the coenzyme. Mizuhara et al (1951, 1954) observed that thiamine and pyruvate, when adjusted to pH 8.8

formed acetoin and that thiamine was acting catalytically. This non-enzymatic system is remarkably similar to bacterial systems which form acetoin from pyruvic acid, with the exception that the rate of reaction in the model system is much slower. α-Acetolactic acid has been shown to be an intermediate in the formation of acetoin from pyruvic acid by both systems, while acetaldehyde cannot be detected (Juni, 1952; Koffler and Krampitz, 1955; Yatoo-Manzo et al, 1959). The following equations have been employed to describe the formation of acetoin from pyruvic acid:

$$CH_3COCOOH + TDP \longrightarrow CH_3CHO\text{-}TDP + CO_2$$

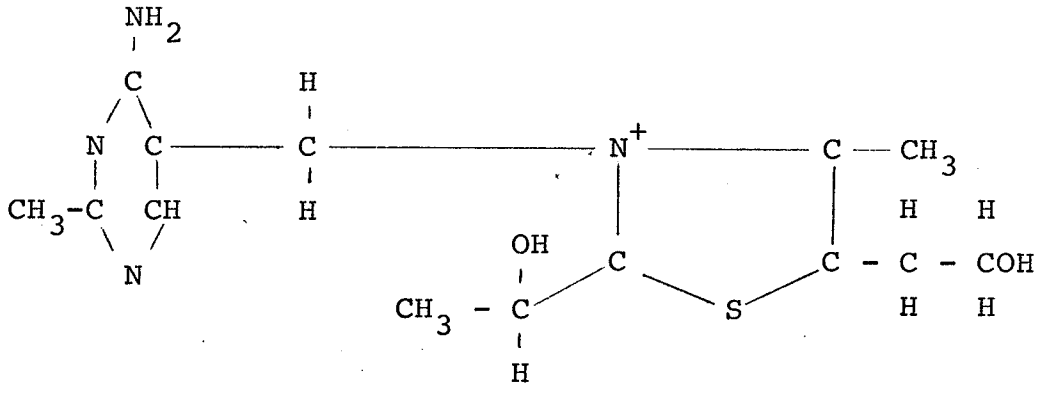
$$CH_3CHO\text{-}TDP + CH_3COCOOH \longrightarrow CH_3\underset{\displaystyle OH}{\overset{\displaystyle CH_3}{COCCOOH}} + TDP$$

$$CH_3\underset{\displaystyle OH}{\overset{\displaystyle CH_3}{COCCOOH}} \longrightarrow CH_3COCHOHCH_3 + CO_2$$

In contrast to the enzyme systems obtained from bacterial sources, the non-enzymatic system will also form acetoin from acetaldehyde. α-Acetolactic acid is not an intermediate under these conditions.

The second important observation was made by Breslow (1957) who found that the hydrogen at position 2 of the

thiazole ring exchanged with deuterium of deuterium oxide under conditions of the non-enzymatic system (pH 8.8). He proposed that the initial attack of pyruvic acid was on the carbanion formed by the dissociation of the hydrogen atom at position 2 of the thiazole ring of thiamine, resulting in the formation of a thiamine-acetaldehyde complex (active acetaldehyde). Krampitz et al (1958) synthesized DL-3-(2-methyl-4-amino-5-pyrimidyl)methyl-2-(1-hydroxyl)-4-methyl-5-(2-hydroxyethyl)-thiazolium chloride hydrogen chloride, abbreviated as α-hydroxyethylthiamine (HET), the proposed intermediate in acetoin formation from pyruvic acid.



(HET)

It was established that this intermediate replaced thiamine in microbial nutrition experiments and substituted for thiamine diphosphate in yeast carboxylase preparations containing ATP and thiaminokinase (Krampitz et al, 1961). Krampitz et al (1959) showed that HET and acetaldehyde, adjusted to pH 8.5, formed acetoin non-enzymatically. While these results strongly

indicated that HET is the active intermediate in acetoin formation, the chemical models did not provide proof for the enzymatic process. Proof of the intermediate role of an α-hydroxyethyl substitution at the position 2 of the thiazole ring depended upon the synthesis of α-hydroxy ethyl thaimine diphosphate (HETDP) and the conversion of substrate quantities of this compound to specific end products by various enzymes which catalyze pyruvic reactions. HETDP was prepared (Krampitz et al, 1961) and shown to be an intermediate in the conversion of pyruvic acid to acetoin. The enzyme complex from Aerobacter aerogenes, which forms acetoin from pyruvic acid with α-acetolactic acid as an intermediate, resembles in some detail the non-enzymatic system. The reaction sequence for the system can be written as follows:

$$E + TDP \longrightarrow E\text{-}TDP$$

$$E\text{-}TDP + \text{pyruvate} \longrightarrow E\text{-}TDP\text{-pyruvate}$$

$$E\text{-}TDP\text{-pyruvate} \longrightarrow E\text{-}HETDP + CO_2$$

$$E\text{-}HETDP + \text{pyruvate} \longrightarrow E\text{-acetolactic-}TDP$$

$$D\text{-acetolactic-}TDP \longrightarrow \text{acetolactate} + E\text{-}TDP$$

$$\text{acetolactate} \longrightarrow \text{acetoin} + CO_2$$

where E represents acetolactate synthetase.

Umbarger and Brown (1958) showed that extracts of E. coli have an acetolactate-forming enzyme and that this enzyme is involved in the biosynthesis of valine and isoleucine, catalyzing the synthesis of α-acetolactate and α-acetohydroxy-

butyrate (Umbarger and Brown, 1957), the first five and six-carbon precursors of valine and isoleucine respectively. It has been shown both in E. coli strains W and K12 (Leavitt and Umbarger, 1962) and in Salmonella typhimurium (Bauerle et al, 1964) that this enzyme shows strong end product inhibition by valine. The enzyme in these bacteria has a pH optimum of 8.0 (Umbarger and Brown, 1958) in contrast to the enzyme from Aerobacter aerogenes, studied by Juni, which has a pH optimum of 6.0 and is not inhibited by valine. It was later shown by Halpern and Umbarger (1959) that Aerobacter aerogenes possessed two different enzymes that catalyze the formation of α-acetolactic acid from pyruvate, one enzyme being that studied extensively by Juni (1952). This enzyme catalyzed the formation of α-acetolactic acid only at acid pH (6.0). The other acetolactic acid-forming enzyme is similar to that which was observed in E. coli (Umbarger and Brown, 1958) and Salmonella typhimurium (Bauerle et al, 1964) having a pH optimum of 8.0, and cannot function at low pH. Since this system active at pH 8.0 can only function in the absence, and appears only in the absence of exogenous valine, its only function is in the biosynthesis of valine. It is interesting that Aerobacter aerogenes grows well in minimal media at low pH in spite of the fact that under these conditions no significant activity of the pH 8.0 enzyme can be detected, suggesting that the enzyme active at pH 5.7 can perform a biosynthetic role in the formation of valine, as well as in the diversion

of glucose metabolism from acidic to neutral products (Halpern and Umbarger, 1959). In studies of the biosynthetic enzyme (pH 8.0) from Salmonella typhimurium, normal Michaelis-Menten type kinetics were observed (Baurle et al, 1964). Both the E. coli and Salmonella typhimurium pH 8.0 enzyme are inhibited by valine and display a requirement for FAD (Buarle et al, 1964; Stormer and Umbarger, 1964; Desai, et al, 1965). The pH 6.0 enzyme from Aerobacter aerogenes is not inhibited by valine, and does not require FAD for activity (Sawula, 1966; Stormer, 1968). Early kinetic studies of acetlactate synthetase from Aerobacter aerogenes using the manometric technique revealed a hyperbolic rate saturation curve (Juni, 1952; Buarle, et al, 1964). This enzyme has now been shown to deviate from normal Michaelis-Menten type kinetics and displays a sigmoid rate saturation curve when assayed in a variety of buffers (Sawula, 1966; Störmer, 1968) but displays normal kinetics in the presence of acetate, propionate, or formate. The enzyme has been crystallized and some of its kinetic, molecular, and biochemical properties have been studied (Störmer, 1967; Störmer et al, 1969; Tveit, and Störmer, 1969).

METHODS AND MATERIALS

(Acetalactate Synthetase and Lactate Dehydrogenase)

METHODS AND MATERIALS

GROWTH OF BACTERIAL CULTURE

A laboratory strain of Aerobacter aerogenes was grown in a medium consisting of 1.0% glucose, 0.3% proteose peptone, and 0.8% K_2HPO_4. The proteose peptone and K_2HPO_4 were dissolved in 1500 ml of water in a 3 liter Fernbach culture flask, stoppered with a cotton plug, and autoclaved for 20 min at 20°C. The glucose was dissolved in 500 ml of water and autoclaved separately. The solutions were then mixed by asceptic addition of the glucose solution to the peptone-K_2HPO_4 solution. The medium was incubated at room temperature until adequate temperature equilibrium was achieved, then innoculated with 35 ml of an 18 hour culture of Aerobacter aerogenes, and incubated at 28°C without shaking or aeration for 18 hours. The cells were harvested by centrifugation in a sharples centrifuge at 50,000 rpm, washed in 0.1 M potassium phosphate buffer of pH 5.7, and centrifuged at 50,000 x g for 10 min. The high speed was necessary as a large amount of slime was produced by the cells, preventing them from packing tightly. The cell paste was stored in a freezer at -20°C.

PREPARATION OF DEAE-CELLULOSE

DEAE-cellulose (medium mesh) obtained from Sigma Chemicals Co. was washed with distilled water, then twice with 0.5 N NaOH, then again with distilled water until the

pH was about 9.0. The DEAE-cellulose suspension was adjusted to pH 6.5 with concentrated phosphoric acid, filtered, washed with 0.05 M potassium phosphate buffer of pH 6.2, and finally suspended in 0.05 M potassium phosphate buffer of the same pH.

PREPARATION OF DEAE-SEPHADEX

DEAE-sepahdex, obtained from Pharmacia Fine Chemicals, was allowed to swell in water, then washed repreatedly with 0.5 N NaOH on a Buchner funnel until free of chloride. The excess NaOH was removed by repeated rinsing with distilled water until the pH of the slurry was approximately 9.0. The pH was adjusted to 7.0 using concentrated phosphoric acid. The slurry was then suspended in 0.05 M potassium phosphate buffer of pH 7.0.

PREPARATION OF CRUDE EXTRACT

The cell paste was homogenized in 0.05 M potassium phosphate buffer of pH 7.0 containing 10.0 mM dithiothreitol, using 2 ml of buffer for each gram of cell paste. Dithiothreitol was replaced with 0.01 M mercaptoethanol when acetolactate synthetase was being prepared. The homogenized suspension was treated in a Virtis 23 homogenizer for one minute at medium speed to remove the slime capsule, then sonicated in 50 ml portions in a 10-KC Raytheon oscillator for 20 min,

followed by centrifugation at 80,000 x g for 30 min.

PURIFICATION OF ACETOLACTATE SYNTHETASE

First ethanol precipitation

To the slightly turbid cell-free extract was added thiamine diphosphate (TDP), and $MnCl_2$ to give a final concentration of 0.1 mM of the former and 0.5 mM of the latter. To this, cold absolute ethanol was added dropwise with constant stirring until the alcohol concentration was 45%. The cell-free extract was kept in a salt-ice bath and the temperature of the extract was gradually lowered to -15°C. The mixture was placed in a freezer at -20°C for 12 hours to allow maximum precipitation to take place. The precipitate was collected by centrifugation at 30,000 x g for one hour at -15°C, homogenized in 0.05 M potassium phosphate buffer of pH 7.0 containing 0.1 mM TDP, 0.5 mM $MnCl_2$, and 10 mM mercaptoethanol, using one half the volume as that of the cell-free extract, and centrifuged at 30,000 x g for one hour. The supernatant contained the enzyme activity. This ethanol precipitation step gave 3 fold purification.

Protamine sulfate treatment

To the slightly turbid supernatant was added 0.05 ml of 0.5% protamine sulfate of pH 7.0 for each mg of protein. The mixture was stirred in an ice bath for 20 min, then

centrifuged at 30,000 x g for one hour. The ratio of the optical densities at 280 mμ and 260 mμ increased from 0.5 to 0.7 or 0.8 during this procedure due to the removal of nucleic acid.

Ammonium sulfate precipitation

To the protamine sulfate supernatant, ammonium sulfate was slowly added while stirring until the concentration was 65% saturation. The mixture was allowed to stir in an ice bath for 30 min, then centrifuged at 30,000 x g for 20 min. When precipitation did not occur immediately the mixture was placed in the freezer at -20°C until precipitation took place. After centrifugation the precipitate was dissolved in 0.05 M potassium phosphate buffer of pH 7.0 containing 0.1 mM TDP, 0.5 mM $MnCl_2$, and 10 mM mercaptoethanol, using one quarter the volume of the volume of the original cell-free extract. The precipitated enzyme gave a clear yellowish solution when dissolved in the buffer. The preparation still contained active acetolactate decarboxylase.

Second ethanol precipitation

To the dissolved ammonium sulfate precipitate, cold absolute ethanol was added dropwise with constant stirring to a final concentration of 60%. The mixture was kept in a salt-ice bath at 0°C and the temperature was gradually lowered

to -15°C by the addition of more salt as the ethanol concentration increased. The mixture was allowed to precipitate at -20°C for 2-12 hours, or until large aggregates appeared. The precipitate was collected by centrifugation at 30,000 x g for one hour at -15°C and homogenized in 0.05 M potassium phosphate buffer of pH 7.0 containing 0.1 mM TDP, 0.5 mM $MnCl_2$, and 0.1 mM dithiothreitol. The turbid solution was centrifuged for 20 min to remove any insoluble protein. The supernatant at this stage was clear and colorless.

DEAE-sephadex chromatography

A DEAE-sephadex column (2.5 cm x 50 cm) was packed and equilibrated with one liter of 0.05 M potassium phosphate buffer of pH 7.0 containing 5 mM sodium pyruvate, 0.1 mM TDP, 0.5 mM $MnCl_2$ and 0.1 mM dithiothreitol. The enzyme was placed on the column, allowed to adsorb, and eluted with a linear gradient of 0.05 M to 0.5 M potassium phosphate buffer of pH 7.0 containing 5.0 mM pyruvate, 0.1 mM TDP, 0.5 mM $MnCl_2$, and 0.1 mM dithiothreitol. Fractions of 70 drops per tube were collected at a rate of one drop every four seconds using a Gilson automatic fraction collector. The fractions were assayed for enzyme activity using the colorimetric assay procedure. Those fractions containing enzyme activity were pooled and brought to 80% ammonium sulfate saturation. The precipitate was collected by

centrifugation at 30,000 x g for 20 min. The precipitate was then dissolved in 0.05 M potassium phosphate buffer of pH 7.0 containing 0.1 mM TDP, 0.5 mM $MnCl_2$, and 0.1 mM dithiothreitol.

PURIFICATION OF 2,3-BUTANEDIOL DEHYDROGENASE

To 300 ml of the 45% ethanol supernatant from the purification of acetolactate synthetase, absolute ethanol was added to a final concentration of 60%. The process was carried out in a salt-ice bath and the temperature was gradually lowered to -15°C by the addition of more salt as the alcohol concentration increased. The mixture was stored at -20°C for 12 hours to allow maximum precipitation to take place. The precipitate was collected by centrifugation at -15°C, at 30,000 x g for 30 min, homogenized in 10 ml of 0.05 M potassium phosphate buffer of pH 7.0 containing 0.1 mM dithiothreitol, then centrifuged to remove any insoluble protein. The clear enzyme solution was then placed on a DEAE-cellulose column (2.5 cm x 50 cm) which was previously equilibrated with one liter of 0.05 M potassium phosphate of pH 6.5 containing 0.1 mM dithiothreitol. The enzyme was eluted with 500 ml of the same fresh buffer. Fractions of 2.0 ml were collected using an RSCo (Research Specialties Co.) automatic fraction collector. An enzyme activity peak was located by assaying every third fraction as follows:

To 2.7 ml of 0.13 M potassium phosphate buffer of pH 5.7 was added 0.1 ml of 0.5 M acetoin (50 μmole), 0.1 ml of 3 mM NADH, and 0.1 ml of eluent respectively. The rate of oxidation of NADH was observed at 340 mμ on a Unicam SP-700 spectrophotometer. Those fractions showing enzyme activity were pooled and made 80% saturated with ammonium sulfate. The precipitate was recovered by centrifugation at 30,000 x g for 20 min, suspended in 5 ml of 0.05 M potassium phosphate buffer of pH 7.0 containing 0.1 mM dithiothreitol, and dialyzed against the same fresh buffer for 4 hours. This preparation was free of lactate dehydrogenase activity. One unit is defined as the amount of enzyme which oxidizes 1 μmole of NADH per minute.

PREPARATION OF ACETOLACTATE DECARBOXYLASE

This method is based on a method by Juni (1952). A preparation active for the decarboxylation of α-acetolactic acid, but completely inactive on pyruvic acid was obtained by heating 25 ml of crude extract in a 100 ml Erlenmyer flask at 70°C for 3 min in a water bath, and removing the heat-denatured protein by centrifugation. Ammonium sulfate was added to 80% saturation and the mixture was allowed to stir for 30 min at 4°C. The precipitate was collected by centrifugation at 20,000 x g for 20 min, suspended in 15 ml of 0.05 M potassium phosphate buffer of pH 7.0 and dialyzed

for 4 hours against 2 liters of the same buffer and again for 2 hours against 2 liters of fresh buffer. This preparation was free of lactate dehydrogenase activity.

PROTEIN DETERMINATIONS

Protein was determed by two methods.

(a) the method of Lowry, Rosenbrough, Farr, and Randall (1951), using bovine albumin as a standard. To 10 ml of sample containing 5 to 100 µg of protein, 5 ml of alkaline copper solution (50 ml of 2% $NaCO_3$ in 0.1 N NaOH + 1.0 ml of 0.5% $CuSO_4$ in 1.0% sodium potassium tartrate) was added and mixed well. After 10 min, 0.5 ml of 1.0 N Folin reagent was added with immediate mixing. After 30 min the extent of color formation was read in a Klett-Summerson photo - electric colorimeter using a 66 K.S. (red) filter.

(b) the method of Warburg and Christian (1941), from the ratio of the absorptions at 280 mµ and 260 mµ.

DETERMINATION OF ACETOLACTATE SYNTHETASE ACTIVITY

Method (a), colorimetric assay procedure

To 0.70 ml of 0.13 M potassium phosphate buffer of pH 5.7 was added 0.05 ml of acetolactate synthetase, 0.1 ml of acetolactate decarboxylase (in excess), and 0.1 ml of 200 µg/ml thiamine diphosphate solution. The reaction was initiated by the addition of 0.1 ml of 1.0 M sodium pyruvate. The mixture

was allowed to incubate for 20 min, after which time the reaction was stopped by the addition of 0.15 ml of 5 N H_2SO_4. The amount of acetoin formed was then determined by the method of Westerfeld (1945) as follows. To 5 ml of sample containing 1.0 to 12 µg of acetoin, was added consecutively, 1.0 ml of 0.5% creatine, and 0.1 ml of 0.5% α-napthol in 2.5 N NaOH. The color was allowed to develop for one hour, after which time the samples were read in a Klett-Summerson photoelectric colorimeter using a 54 K.S. (green) filter. One unit is defined as the amount of enzyme which forms 1.0 µmole of acetoin per minute. The enzyme was assayed using this method during purification procedures and when otherwise stated.

Method (b), spectrophotometric assay procedure

To 2.25 ml of 0.13 M potassium phosphate buffer in a silica cuvette of 1 cm light path was added:

0.1 ml of a 200 µg per ml thiamine diphosphate solution,

0.1 ml of distilled water,

0.1 ml of acetolactate decarboxylase (in excess),

0.1 ml of 2,3-butanediol dehydrogenase (22.0 units),

0.1 ml of acetolactate synthetase (30 to 60 µg),

0.15 ml of 3.0 mM NADH.

The reaction as initiated by the addition of 0.1 ml of 0.5 M sodium pyruvate. The sample was mixed by inversion and placed

in a Unicam SP-700 spectrophotometer. Enzyme activity was measured by the rate of NADH oxidation at 340 mμ. The enzyme activity was determined by this method for kinetic and other studies unless otherwise stated.

DETERMINATION OF NADH-SPECIFIC LACTATE DEHYDROGENASE ACTIVITY

Routine spectrophotometric assay

The NADH-specific lactate dehydrogenase catalyzing the reduction of pyruvate to lactate was assayed by following the rate of conversion of NADH to NAD^+ at 340 mμ on a Unicam SP-700 spectrophotometer or on a Gilford Multiple Sample Absorbance Recorder. Assays were carried out in silica cuvettes of 1 cm light path. Routinely the reaction mixture contained in a final volume of 3.0 ml, 0.1 M potassium phosphate buffer of pH 5.7, 5.0 mM potassium pyruvate, and 0.15 mM NADH. The reaction was initiated by the addition of enzyme. A unit of lactate dehydrogenase activity was defined as the amount of enzyme that oxidized 1 μmole of NADH per minute.

Stopped flow spectrophotometric assay

For the determination of velocities at high enzyme concentrations, a Durrum-Gibson stopped flow spectrophotometer was used. The reaction was initiated by mixing a solution of pyruvate and NADH with an enzyme solution. Both solutions were prepared in 0.1 M potassium phosphate buffer (pH 7.0).

The stopped flow cuvette held 0.15 ml of reaction mixture, and had a light path of 2.0 cm. The change in transmittancy was recorded on a Tektronix Storage Oscilloscope, having an attached polaroid camera, and converted to absorbancy change.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Disc electrophoresis was used to study the purity of enzyme preparations. Electrophoresis was performed according to the method described by Ornstein (1964) and Davis (1964). The runs were made in an electrophoresis apparatus obtained commercially from Buchler Instruments Co., using 3 milliamps per gel. Bromophenol Blue was used as a tracking dye. After the runs, the gels were stained in a staining solution of 0.025% Commassie Blue in 10% trichloracetic acid for 1 hour and destained for 1 to 2 hours in 10% trichloracetic acid.

MOLECULAR WEIGHT DETERMINATIONS

Molecular weight determinations were carried out using sucrose gradient techniques described by Martin and Ames (1961). Sucrose gradients ranging from 4% to 20% in 0.1 M potassium phosphate buffer of specified pH were prepared in cellulose nitrate centrifuge tubes to a volume of 4.8 ml. Two-tenths of a milliliter of a mixture of enzyme and hemoglobin were carefully layered on top of each gradient. The gradients were centrifuged at 50,000 rpm for 8 to 10 hours in a SW-50

rotor in a Beckman L2-65B ultracentrifuge at 4°C. At the end of the run the rotor was allowed to deccelerate with the brake off, and the tubes were removed from the rotor. The bottom of each tube was punctured with a fine needle and ten drop fractions were collected using a Buchler fraction collecting apparatus. Each fraction was assayed for enzyme activity. A hemoglobin peak was located by assaying a diluted portion of each fraction on a Gilford Multiple Sample Absorbance Recorder at 406 $m\mu$. The molecular weight was estimated using the following equation.

$$S_1/S_2 = (MW_1/MW_2)^{2/3}$$

where

S_1 = the distance travelled from the meniscus by the enzyme

S_2 = the distance travelled from the meniscus by the hemoglobin

MW_1 = molecular weight of the enzyme

MW_2 = molecular weight of the hemoglobin (68,000)

SUB-UNIT MOLECULAR WEIGHT DETERMINATION OF LACTATE DEHYDROGENASE

Electrophoresis in polyacrylamide gels in the presence of the anionic detergent sodium dodecyl sulfate (SDS) has proven to be a useful tool for the separation and identification of polypeptide chains (Maizel, 1966; Shapiro et al, 1966; Vinuela et al, 1967).

The same technique may be used for the rapid and simple estimation of the molecular weights of proteins and

their subunits (Shapiro et al, 1967). The subunit molecular weight of Aerobacter aerogenes lactate dehydrogenase was determined by SDS-polyacrylamide gel electrophoresis techniques described by Weber et al (1969), with slight modification as follows.

Preparation of protein solutions

Horse heart cytochrome c, hemoglobin, trypsin, phosphoglyceraldehyde dehydrogenase, alcohol dehydrogenase (liver), aldolase, glutamate dehydrogenase, catalase, and bovine serum albumin, and lactate dehydrogenase (Aerobacter aerogenes) were individually incubated at 37°C for two hours in 0.01 M sodium phosphate buffer, pH 7.0, 1% sodium dodecyl sulfate, and 1% mercaptoethanol. The protein concentration was 1.0 mg per ml. After incubation the protein solutions were dialyzed for 3-4 hours at room temperature against one liter of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate and 1% mercaptoethanol.

Prior to the above treatment the sample of Aerobacter aerogenes lactate dehydrogenase was dialyzed against 0.01 M sodium phosphate buffer to remove any potassium present in the enzyme solution. This is necessary because sodium dodecyl sulfate precipitates in the presence of even small amounts of potassium.

Preparation of gels

A gel buffer containing 3.9 grams of $NaH_2PO_4.H_2O$, 19.3 grams of $Na_2HPO_4.7H_2O$, and 2 grams of sodium dodecyl sulfate per liter was prepared. A 10% acrylamide solution was prepared by dissolving 22.2 grams of acrylamide and 0.6 grams of methylenebisacrylamide in water to give a 100 ml of solution. Glass tubes of 10 cm length and 6 mm inner diameter were thoroughly cleaned and dried. For a typical run of 12 gels, 15 ml of gel buffer were mixed with 13.5 ml of 10% acrylamide solution. To this mixture was added 1.5 ml of freshly prepared ammonium persulfate solution (15 mg per ml) and 0.045 ml of N,N,N',N'-tetramethylenediamine. After mixing, 2.5 ml of the mixture was pipetted into each glass tube. Before the gel hardened a few drops of water were layered on top of each gel solution. After 20 minutes an interface could be seen, indicating that the gels had hardened, forming a flat surface. The water was removed prior to placing the sample on the gel.

Electrophoresis

Ten micrograms of each protein were layered on individual gels. To each gel was added 10 microliters of mercaptoethanol, 10 microliters of bromophenol blue (0.5% in water), and one drop of glycerol. The gels were swirled to ensure even mixing of the solutions. Gel buffer, diluted 1:1 with water was carefully layered on top of each sample to

fill the tubes. The tubes were then placed in an electrophoresis apparatus. The upper and lower compartments were filled with gel buffer diluted 1:1 with water. Electrophoresis was carried out at a constant current of 8.0 milliamps per gel with the positive electrode in the bottom chamber. Under these conditions the marker dye moved three quarters down the gel in approximately 6 hours. The gels were removed from the tubes and the gel length and the distance moved by the marker dye were measured and recorded. Each gel was placed in a 6 inch test tube containing a staining solution of 0.05% Coomassie Blue prepared by dissolving 0.2 grams of Coomassie Blue in a mixture of 450 ml of 50% methanol and 50 ml of glacial acetic acid. The gels were stained at room temperature for 6-12 hours, then rinsed in distilled water and placed in a destaining solution (75 ml of acetic acid, 50 ml of methanol, and 875 ml of water) for 24 hours. The length of the gels and the position of the blue protein bands were measured and recorded. The mobility of each protein was calculated as follows.

$$\text{mobility} = \frac{D_1}{L_2} \times \frac{L_1}{D_2}$$

where D_1 = distance of protein migration

D_2 = distance of dye migration

L_1 = length of gel before staining

L_2 = length of gel after staining

The mobilities were plotted against the known molecular

weights expressed on a semi-logrithmic scale.

SEDIMENTATION VELOCITY STUDIES

Sedimentation velocity studies were performed with a Spinco Model E analytical centrifuge equipped with a Schlieren double sector optical system. The runs were made at 5°C in 0.05 M potassium phosphate buffer of pH 5.7 and pH 8.0, containing 0.1 mM dithiothreitol. All S values reported have been corrected for viscosity to $S_{20,W}$.

CHEMICALS

The following chemicals were obtained from commercial sources:

ATP (disodium, crystalline	Sigma Chemical Co.
Cocarboxylase (thiamine diphosphate chloride)	Sigma Chemical Co.
DEAE-cellulose (medium mesh)	Sigma Chemical Co.
α-ketobutyric acid (sodium salt)	Sigma Chemical Co.
α-ketoglutaric acid (sodium salt)	Sigma Chemical Co.
D(-)lactic acid (lithium salt)	Sigma Chemical Co.
NADH (disodium)	Sigma Chemical Co.
Oxamic acid (sodium salt)	Sigma Chemical Co.
Folin reagent	Fisher Scientific Co.
Fumaric acid	Fisher Scientific Co.
lactic acid	Fisher Scientific Co.

Manganese chloride	Fisher Scientific Co.
α-napthol	Fisher Scientific Co.
Potassium acetate	Fisher Scientific Co.
Potassium phosphate (mono and dibasic)	Fisher Scientific Co.
Propionic acid	Fisher Scientific Co.
Sodium dodecyl sulfate	Fisher Scientific Co.
Sodium potassium tartrate	Fisher Scientific Co.
DEAE-sephadex	Pharmacia Fine Chemicals
Sephadex G-200	Pharmacia Fine Chemicals
Dithiothreitol (Clelands reagent)	Calbiochem
Fluoropyruvic acid (sodium salt)	Calbiochem
Succinic acid	Nutritional Biochemicals Corp.
Creatine	Nutritional Biochemicals Corp.
Sodium citrate	Matheson, Colemand and Bell
Acrylamide reagents	Canalco

RESULTS

RESULTS

PURIFICATION OF ACETOLACTATE SYNTHETASE

A summary of the purification of acetolactate synthetase is given in Table 1 , and represents an overall purification of 28-fold. Occasionally a large amount of activity was lost during ammonium sulfate fractionation. Also in many trials, the protein did not precipitate during this step until the mixture was kept at -20°C for several hours. Because of the great loss in activity during DEAE-Sephadex Chromatography various cofactors and protecting agents were added to both the wash buffer, and eluting buffers in an attempt to protect the enzyme during chromatography. Thiamine diphosphate and $MnCl_2$ were essential cofactors during chromatography, their absence resulting in complete loss of enzyme activity. No marked protection was observed during DEAE-sephadex chromatography by the addition of sodium pyruvate to the buffers. Addition of dithiothreitol to the buffers resulted in a 5-fold increase in enzyme recovery. Subsequently, all buffers used for DEAE-cellulose chromatography were prepared containing 0.1 mM thiamine diphosphate, 0.5 mM $MnCl_2$, 0.1 mM dithiothreitol, and 5.0 mM sodium pyruvate. The enzyme was eluted at approximately 0.1 M phosphate concentration.

In many cases enzyme recovery was extremely low. All attempts to purify the enzyme were unsuccessful, since the

Table I

Summary of acetalactate synthetase purification

Fraction	Total Protein (mg)	Total Units	Specific Activity	% Recovery
Crude extract	960	1660	1.73	100
0-45% Ethanol	230	1325	5.72	80
Protamine Sulfate	182	1110	6.04	70
0-65% Sat. $(NH_4)_2SO_4$	88	957	10.9	58
0-60% Ethanol	24	719	29.8	43.5
DEAE-cellulose	4	152	48.0	12

enzyme was extremely labile under the conditions of this study. Replacing phosphate by acetate in the purification procedure did not improve enzyme recovery, rather in most cases decreased the recovery of enzyme.

EFFECT OF ACETATE AND PHOSPHATE ON THE KINETIC BEHAVIOUR OF ACETOLACTATE SYNTHETASE

Fig. 1 shows the effect of 0.1 M potassium acetate and 0.1 M potassium phosphate on the kinetic behaviour of acetolactate synthetase at pH 5.7. In the presence of 0.1 M phosphate the saturation rate curve for the enzyme was distinctly sigmoidal and a double reciprocal plot of enzyme activity against pyruvate concentration was non-linear, (Fig. 2). When the enzyme was assayed in 0.1 M potassium acetate buffer the saturation rate curve was hyperbolic, and the double reciprocal plot was linear. In the presence of both 0.1 M phosphate and 0.1 M acetate the saturation rate curve was a hyperbolia. In addition the reaction in phosphate buffer alone and in phosphate buffer and acetate, is catalyzed at a lower maximal rate than in acetate alone. Hill plots of the data from Fig. 1 are shown in Fig. 3 . In the absence of acetate the slope of the Hill plot is 2.4, while in the presence of acetate the slope value is 1.0.

During the early investigation of this enzyme (Sawula, 1966) it was observed that an initial lag in the enzyme reaction was present. This lag was shortened with increasing

enzyme concentration but was always present. This lag was observed when the colorimetric and spectrophotometric assay procedure was used to follow the course of the enzyme reaction. This lag was not removed by the presence of acetate.

To determine whether the enzyme was actually being inhibited by phosphate or activated by acetate, or both, the enzyme was assayed in water, acetate buffer, and phosphate buffer at pH 5.7. All reagents were adjusted to pH 5.7 for the enzyme assays. As shown in Fig. 4 , double reciprocal plots of enzyme activity and pyruvate concentration showed that the enzyme was both activated by acetate and inhibited by phosphate.

EFFECT OF ACETATE CONCENTRATION

Fig. 5 shows the effect of acetate concentration on the activity of acetolactate synthetase. Activity was stimulated to a maximum at 0.1 M acetate when compared to the activity in distilled water, after which activity decreased with increasing acetate concentration. Inhibition was non-competitive with respect to pyruvate, indicating that it binds at a site other than the pyruvate-binding site. An activity profile is shown in the insert of Fig. 5 where it can be clearly seen that maximum stimulation was observed at 0.1 M acetate. Replots of the slopes and intercepts against acetate concentration

Figure 1. Effect of acetate and phosphate on the kinetic behaviour of acetalactate synthetase at pH 5.7. The spectrophotometric assay procedure was used as described in METHODS. Reactions were carried out in the following buffers:

A = 0.1 M acetate

B - 0.1 M acetate and 0.1 M phosphate

C = 0.1 M phosphate

$v = \Delta A340\ m\mu/min$

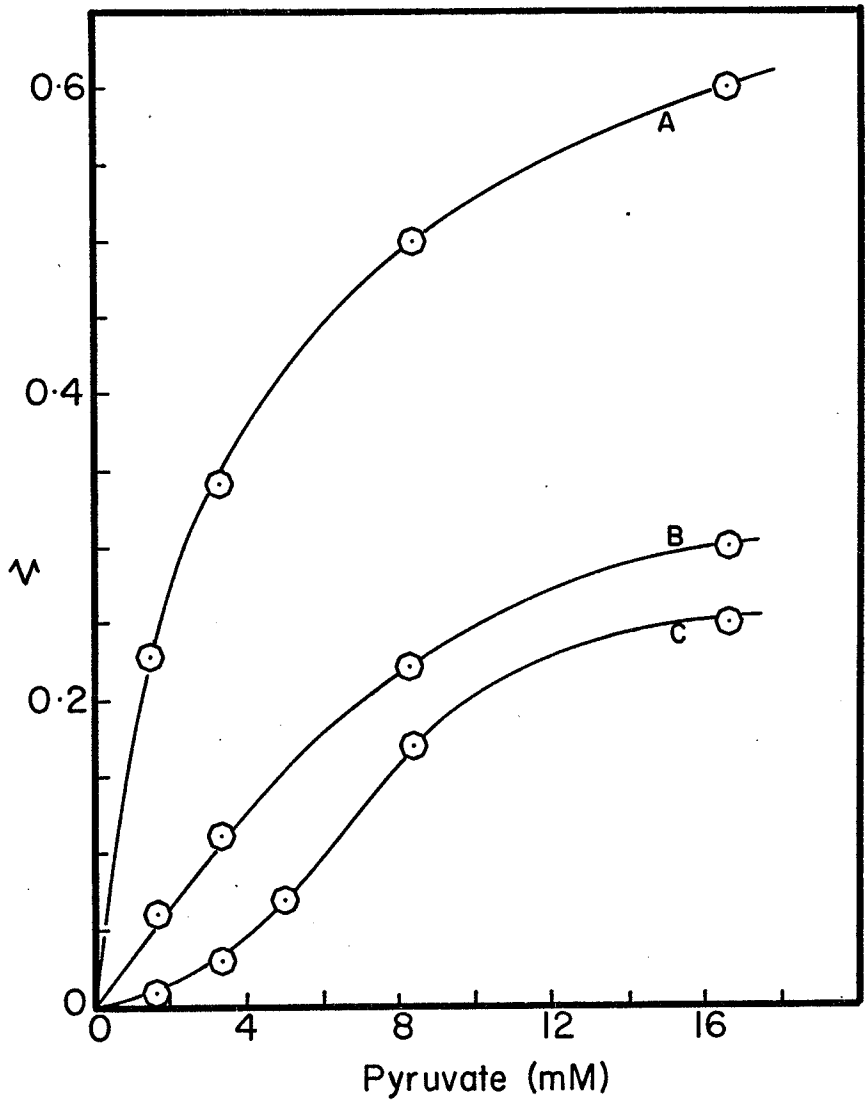

0·6
0·4
0·2
0
v
A
B
C
0
4
8
12
16
Pyruvate (mM)

Figure 2. Double reciprocal plots of velocity against pyruvate concentration at pH 5.7. The spectrophotometric assay procedure was used as described in METHODS. Reactions were carried out in the following buffers:

A = 0.1 M phosphate

B = 0.1 M acetate and 0.1 M phosphate

C = 0.1 M acetate

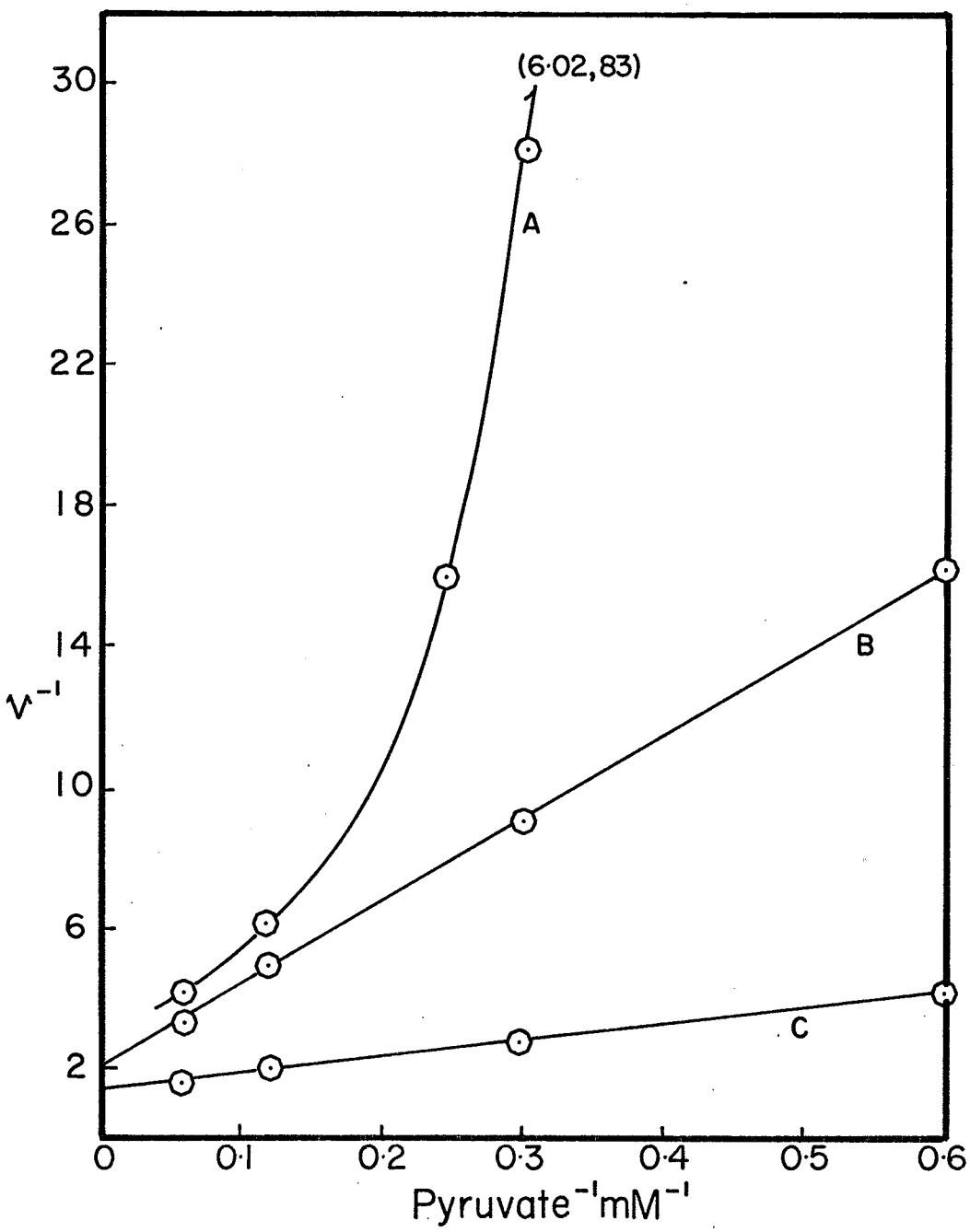

(6·02,83)
A
B
C
30
26
22
18
14
10
6
2
V⁻¹
0
0·1
0·2
0·3
0·4
0·5
0·6
Pyruvate⁻¹mM⁻¹

Figure 3. Plots of v/(V-v) against pyruvate concentration at pH 5.7, of the data from Fig. 1 expressed logrithmically.

A = 0.1 M acetate and 0.1 M phosphate

B = 0.1 M phosphate

C = 0.1 M acetate

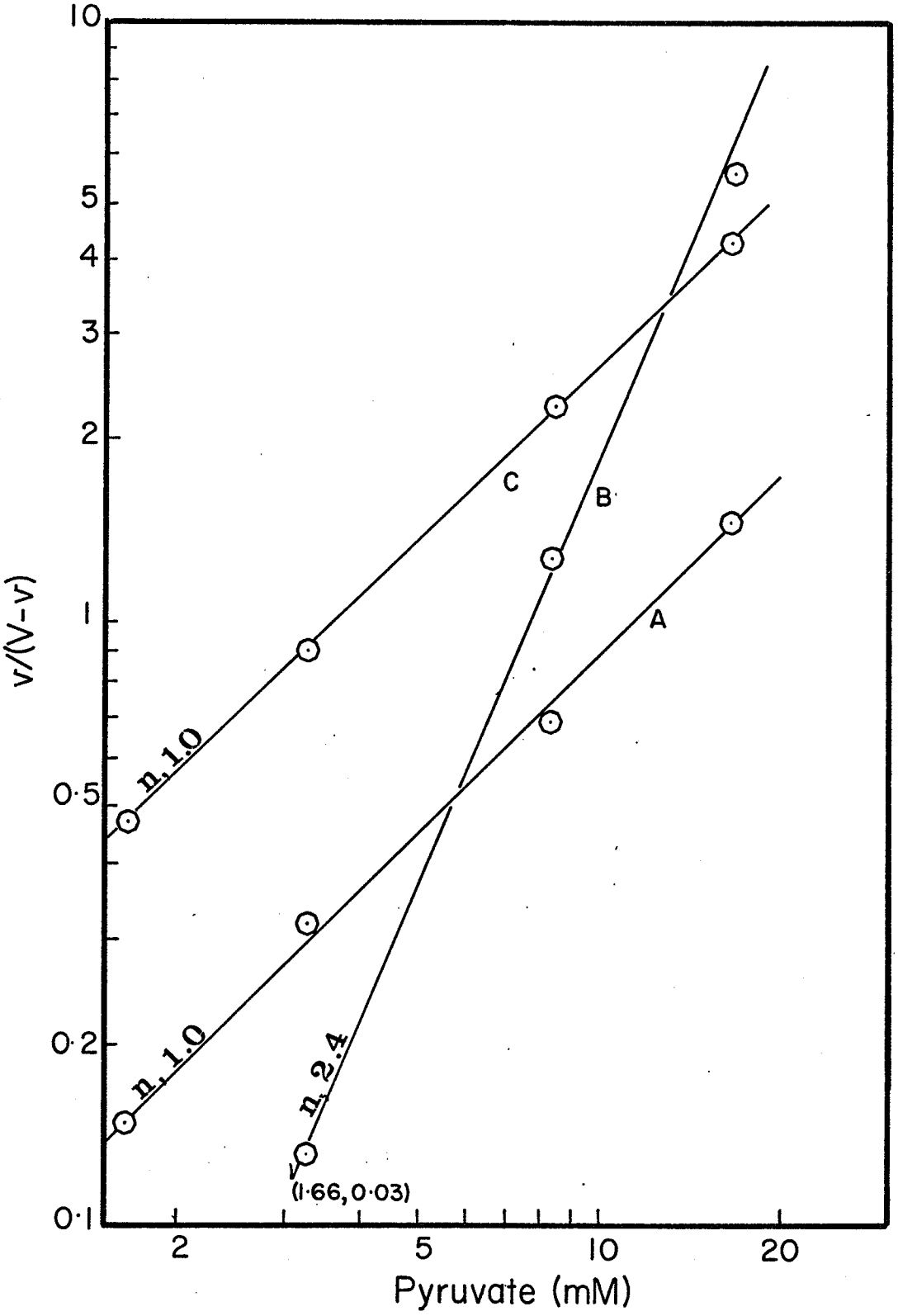

10
5
4
3
2
1
0·5
0·2
0·1
v/(V-v)
n, 1.0
n, 1.0
n, 2.4
(1·66, 0·03)
C
B
A
2
5
10
20
Pyruvate (mM)

Figure 4. Double reciprocal plots of velocity against pyruvate concentration at pH 5.7. The spectrophotometric assay procedure was used as described in METHODS. Reactions were carried out in the following buffers:

A = 0.1 M acetate

B = distilled water

C = 0.1 M phosphate

All reagents were adjusted to pH 5.7 prior to assay.

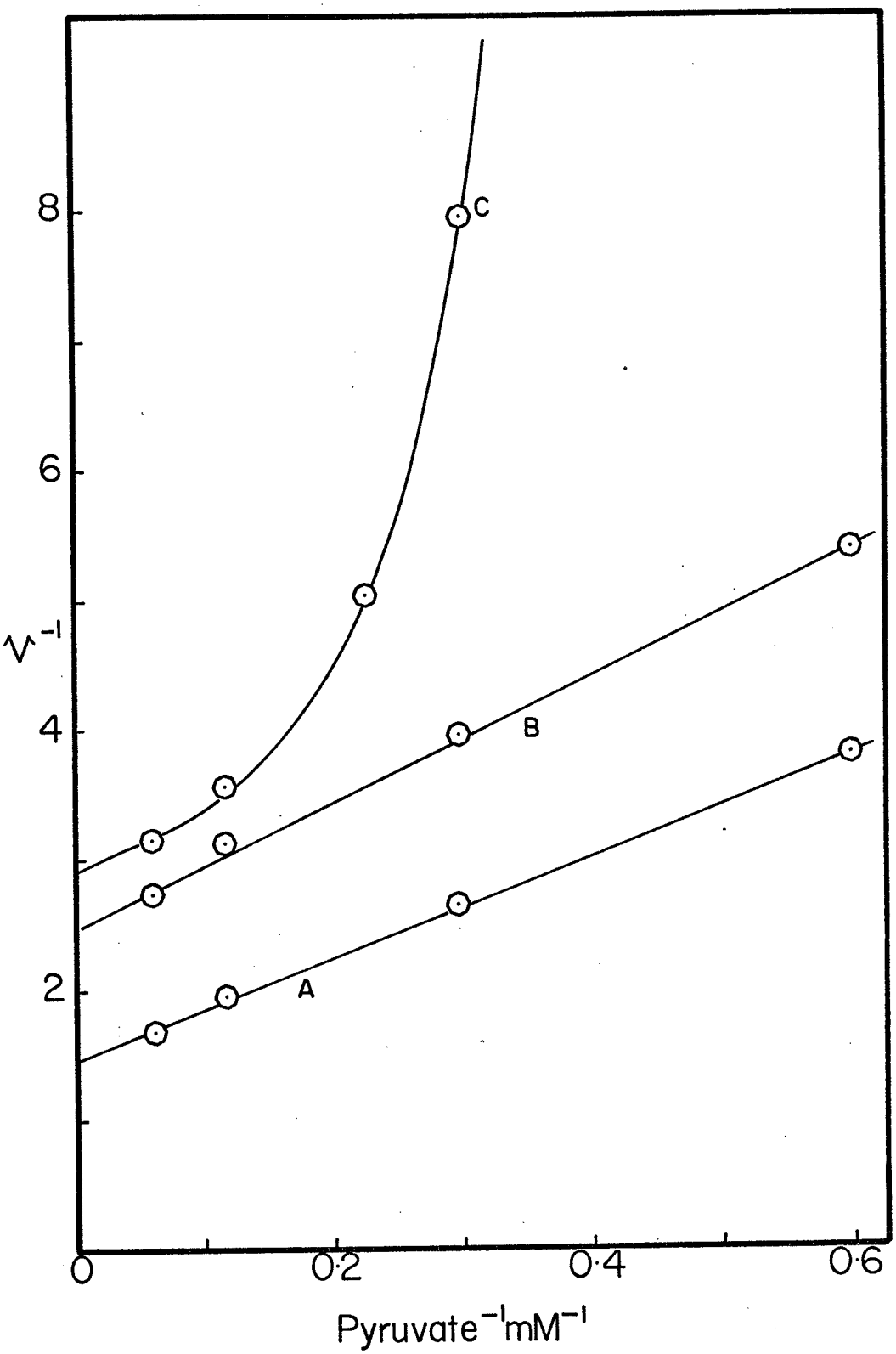

8
6
4
2
v^{-1}
C
B
A
0
0·2
0·4
0·6
Pyruvate^{-1}mM^{-1}

Figure 5. Effect of acetate concentration on activity of acetolactate synthetase. Assays were carried out in acetate buffer using the spectrophotometric assay procedure as described in METHODS. The following concentrations of acetate were used:

A = 0.1 M

B = 0.138

C = 0.231

D = nil (assayed in distilled water)

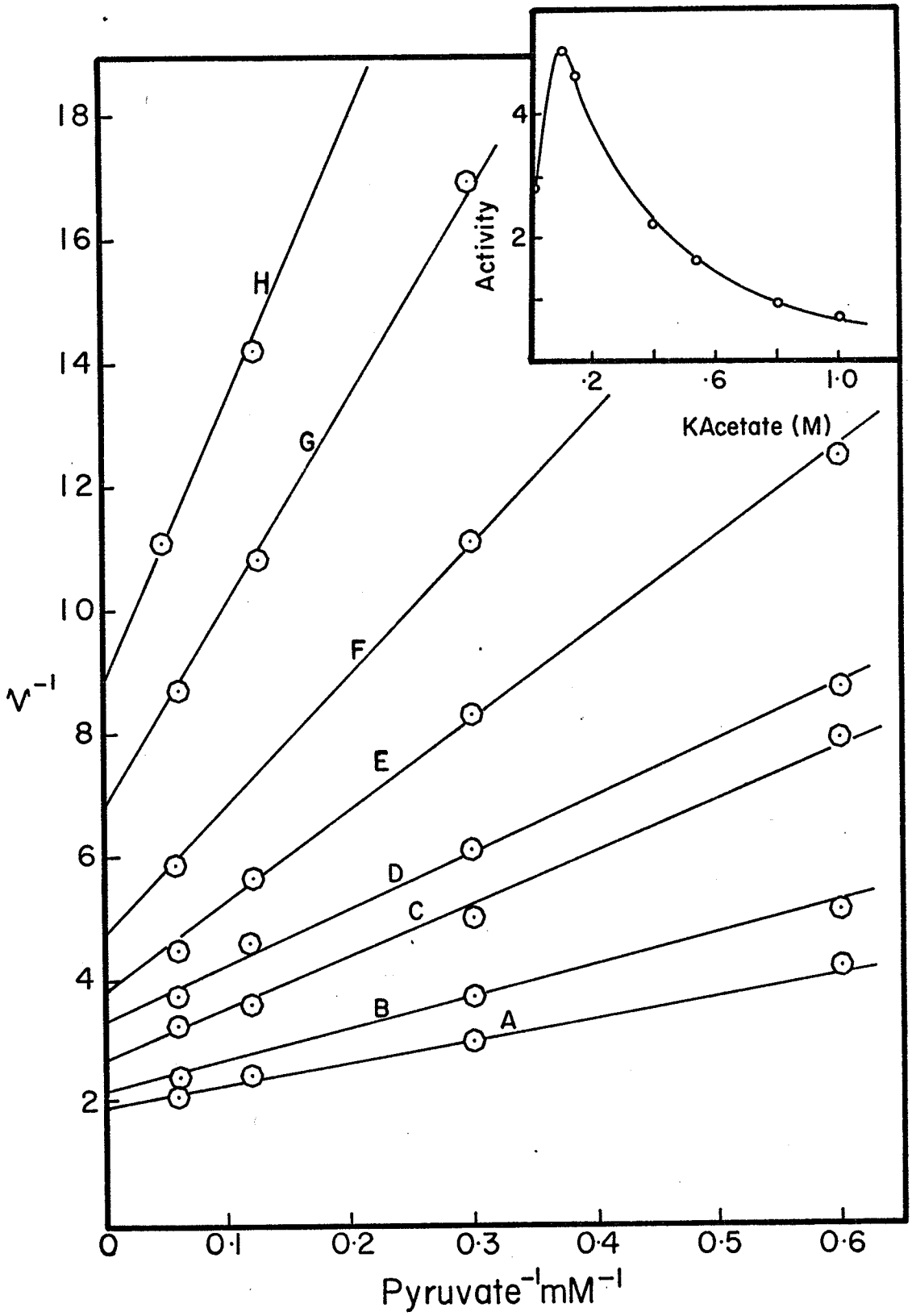
E = 0.385 M

F = 0.54 M

G = 0.8 M

H = 1.0 M

The insert shows an activity profile with increasing acetate concentration.


18
16
14
12
10
8
6
4
2
v^{-1}
H
G
F
E
D
C
B
A
0
0·1
0·2
0·3
0·4
0·5
0·6
Pyruvate^{-1}mM^{-1}
Activity
4
2
·2
·6
1·0
KAcetate (M)

are shown in Fig. 6 where it can be seen that the relationship is non-linear.

INHIBITION BY PHOSPHATE

Using the spectrophotometric assay system the enzyme was assayed in 0.1 M potassium acetate buffer of pH 5.7 at several fixed concentrations of potassium phosphate. The results are shown in Fig. 7 in the double reciprocal form. Phosphate inhibited enzyme activity non-competitively with respect to pyruvate.

EFFECT OF OTHER MONO-CARBOXYLIC ACID BUFFERS

Linear double reciprocal plots of enzyme activity and pyruvate concentration were also observed in 0.1 M potassium formate and 0.1 M potassium propionate buffers, the activity in propionate buffer being equivalent to that in acetate. The activity in formate buffer was less than in acetate, but greater than in phosphate, or when assayed in distilled water. These results are shown in Fig. 8 . Both the maximal activity and the affinity of the enzyme for pyruvate over that observed in the presence of phosphate was increased in the presence of acetate and formate buffers.

Although these carboxylic acid buffers were effective in transforming the double reciprocal plots from curved to linear, they did not succeed in removing the initial

Figure 6. Replots of slopes and intercepts against acetate concentration from the data in Figure 5 .

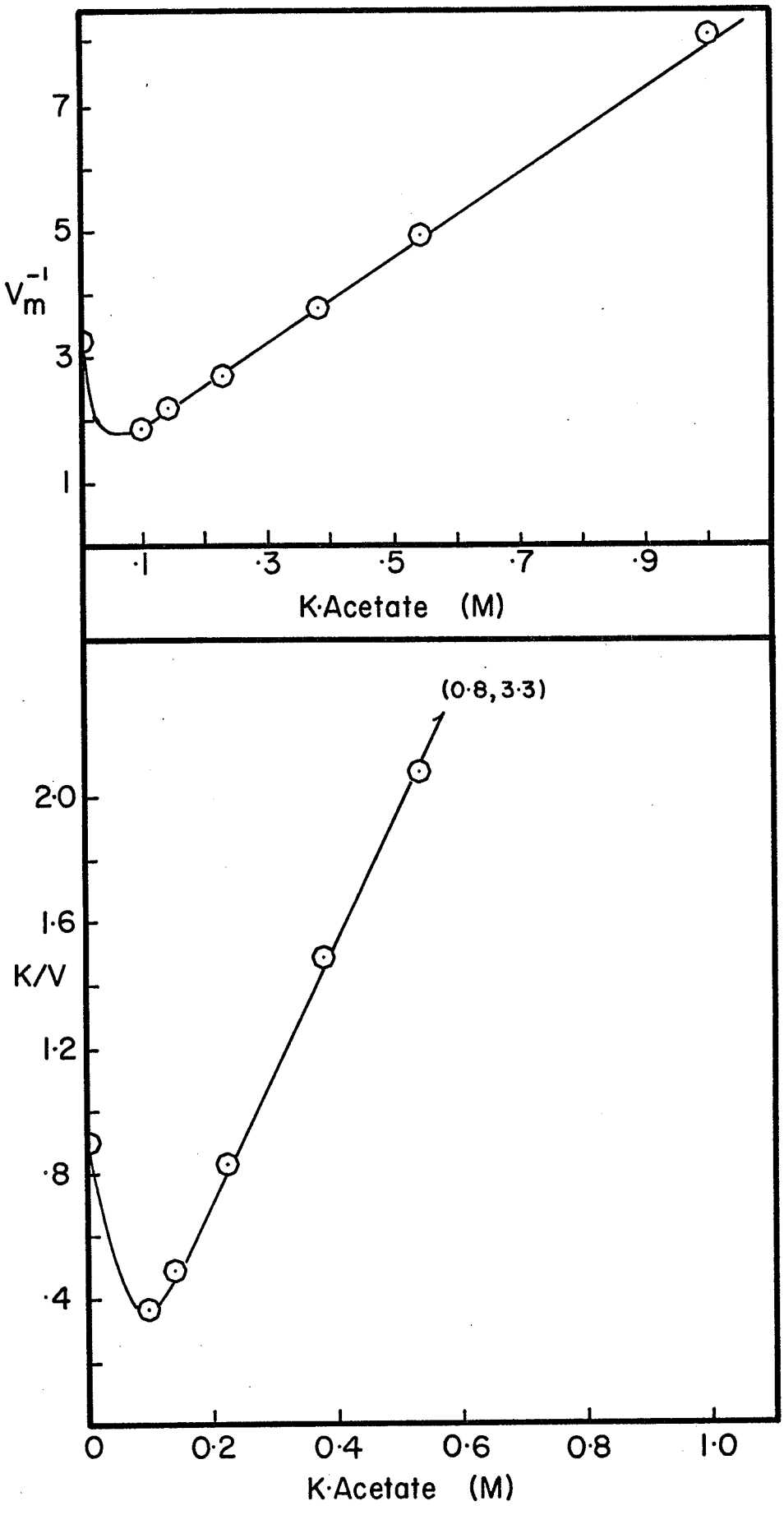

7
5
V_m^{-1}
3
1
·1
·3
·5
·7
·9
K·Acetate (M)
(0·8, 3·3)
2·0
1·6
K/V
1·2
·8
·4
0
0·2
0·4
0·6
0·8
1·0
K·Acetate (M)

Figure 7. Inhibition of acetolactate synthetase by phosphate at pH 5.7. Assays were carried out using the spectrophotometric assay procedure. Reactions were carried out in 0.1 M acetate buffer in the presence of the following concentrations of potassium phosphate (adjusted to pH 5.7):

A = nil

B = 25 mM

C = 50 mM

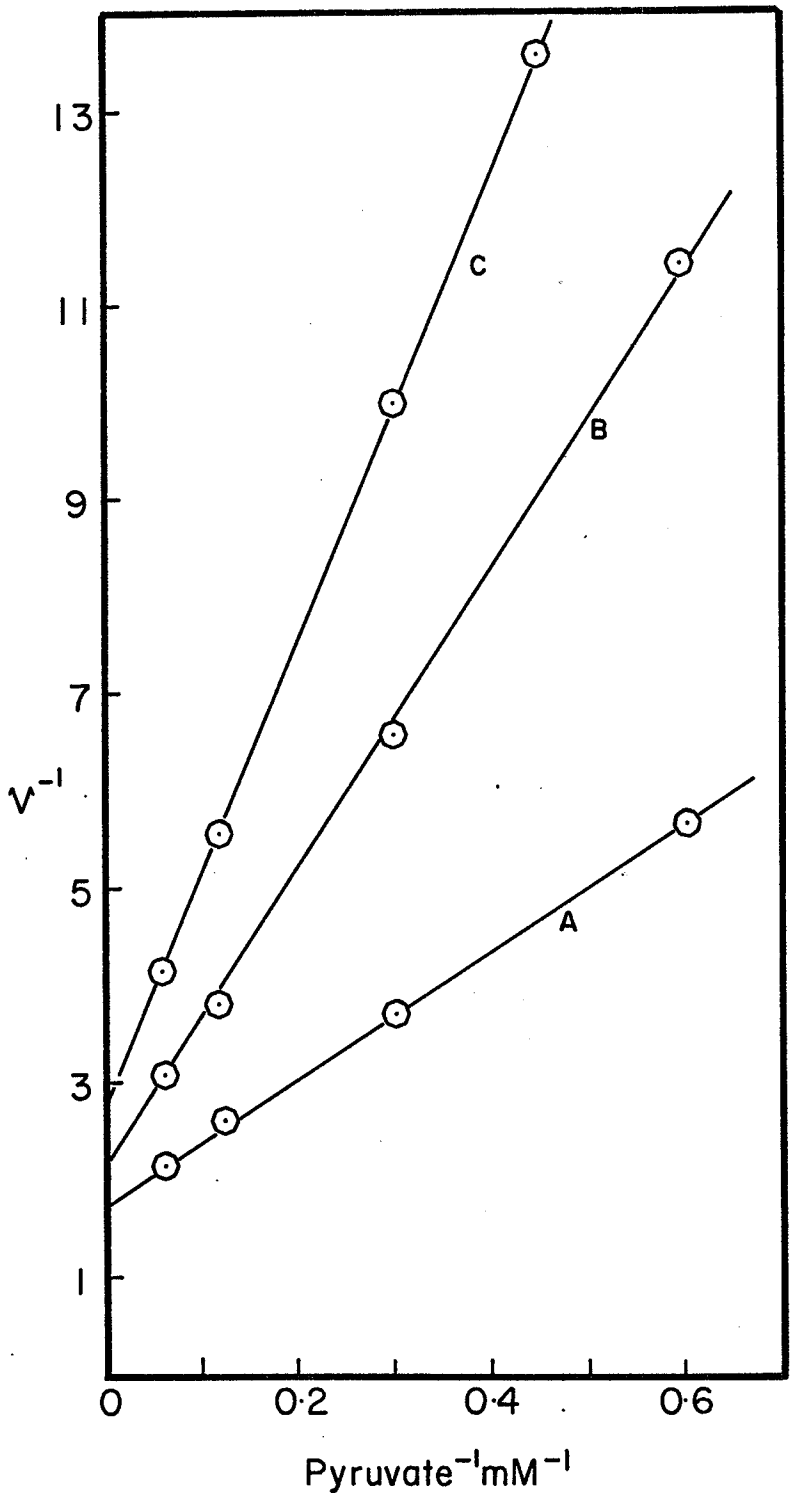

13
11
9
7
5
3
1
v^{-1}
C
B
A
0
0·2
0·4
0·6
Pyruvate^{-1}mM^{-1}

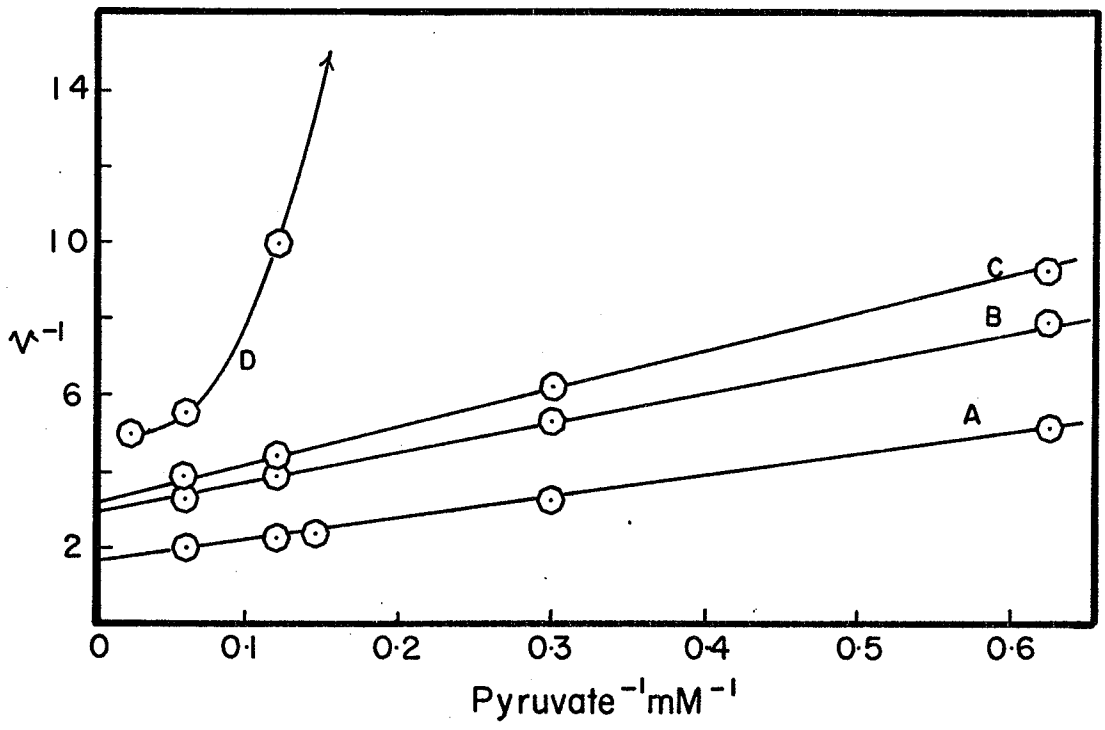
Figure 8. Effect of other monocarboxylic acid buffers on kinetic behaviour of acetolactate synthetase at pH 5.7. Assays were carried out using the spectrophotometric assay procedure. Reactions were carried out in 0.1 M of the following buffers:

A = acetate, or propionate

B = formate

C = distilled water

D = phosphate


14
10
6
2
v⁻¹
D
C
B
A
0
0·1
0·2
0·3
0·4
0·5
0·6
Pyruvate⁻¹mM⁻¹

lag from the enzyme reaction.

When the enzyme was assayed in 0.1 M lactate buffer at pH 5.7, double reciprocal plots of enzyme activity against pyruvate concentration, were linear up to 3.3 mM pyruvate, and became non-linear at lower pyruvate concentrations. Nevertheless, activity in lactate buffer was greater than that observed in phosphate buffer, but much lower than observed in phosphate buffer (Fig. 9).

EFFECT OF DI AND TRICARBOXYLIC ACID BUFFERS

The enzyme displayed sigmoidal rate saturation curves and non-linear double reciprocal plots of enzyme activity against pyruvate concentration when assayed in 0.1 M succinate, fumarate, and citrate buffers. Activity in these carboxylic acid buffers was less than that observed in acetate buffer but greater than the activity observed in phosphate buffer (Fig. 10). A detailed study of the effect of these carboxylic acid buffers was not undertaken.

EFFECT OF INORGANIC IONS

Neutral salts at high concentrations (0.3 M to 3.0 M) inhibit the activity of widely different enzymes in an order of increasing effectiveness for anions (Warren et al, 1966), $Ac^- < Cl^- < NO_3^- < Br^- < I^- < SCN^- \quad ClO_4$. When the enzyme was assayed in 0.1 M potassium phosphate buffer at pH 5.7

Figure 9. Effect of lactic acid buffer on the kinetic behaviour of acetolactate synthetase at pH 5.7 as compared to the kinetic behaviour in other monocarboxylic acid buffers. Assays were carried out using the spectrophotometric assay procedure. Reactions were carried out in 0.1 M of the following buffers:

A = acetate, or priopionate

B = formate

C = lactate

D = phosphate

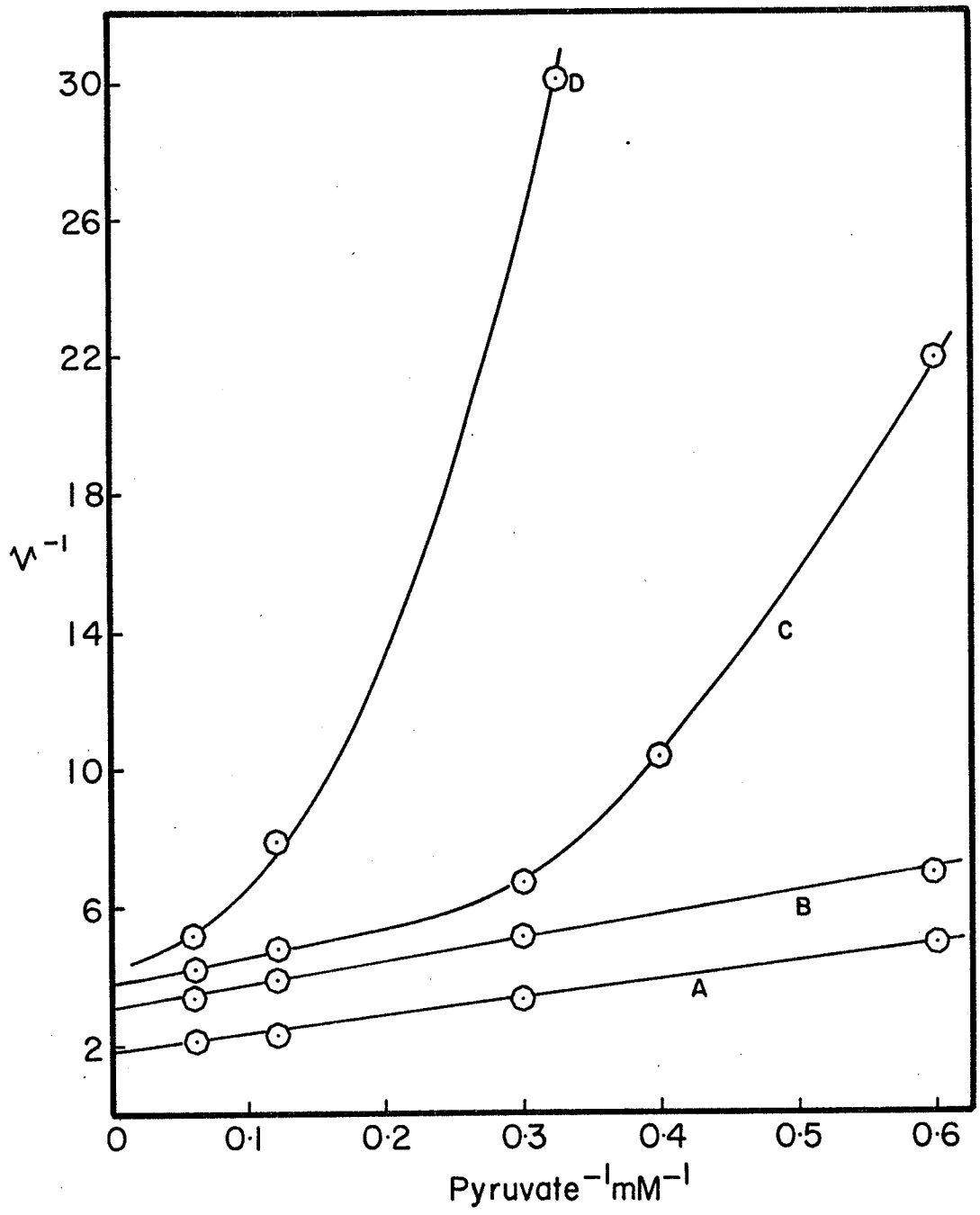

30
26
22
18
14
10
6
2
v^{-1}
D
C
B
A
0
0·1
0·2
0·3
0·4
0·5
0·6
Pyruvate^{-1}mM^{-1}

Figure 10. Effect of di- and tricarboxylic acid buffers on acetolactate synthetase, shown in the double reciprocal form of velocity against pyruvate concentration. Assays were carried out using the spectrophotometric assay procedure. Reactions were carried out in 0.1 M of the following buffers:

A = acetate

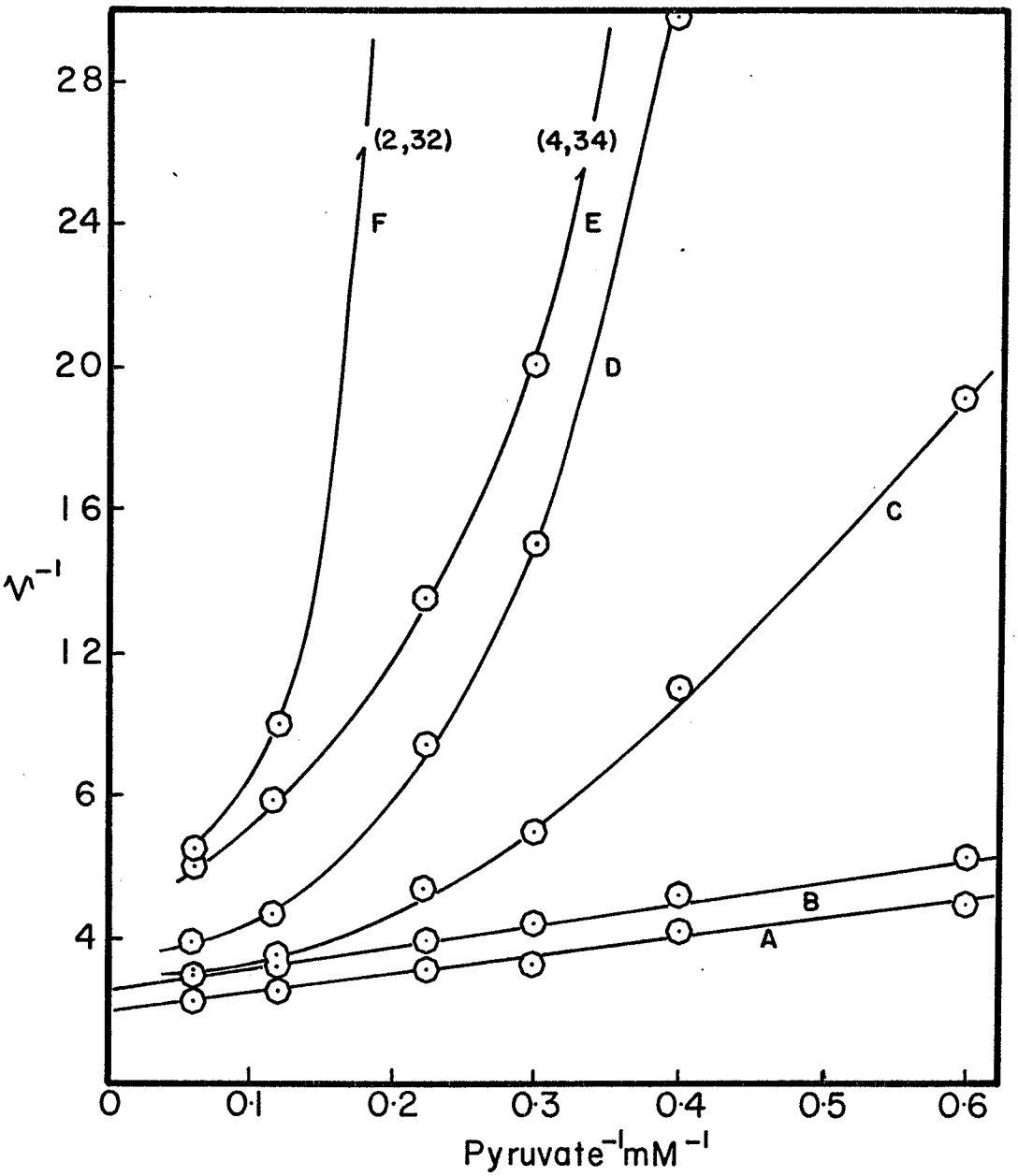
B = distilled water

C = succinate

D = citrate

E = fumarate

F = phosphate


28
24
20
16
12
6
4
v^{-1}
(2,32)
(4,34)
F
E
D
C
B
A
0
0·1
0·2
0·3
0·4
0·5
0·6
Pyruvate^{-1}mM^{-1}

in the presence of 8.33 mM pyruvate, the order of increasing inhibiting effectiveness for the above anions was: Cl^- < Br^- < I^- < SCN^- < ClO_4^- at a final anion concentration of 0.1 M, and so appeared to follow the anionic series observed for other enzymes. Acetate at 0.1 M did not inhibit the enzyme, but rather activated the enzyme. The same order of effectiveness was observed when the enzyme was assayed in acetate buffer and in distilled water, in the presence of added ions.

Inhibition of enzyme activity was not only observed at high anion concentrations, but at concentrations as low as 0.025 M. A detailed study of anionic effects on enzyme activity was undertaken.

EFFECT OF HALIDES

When the enzyme was assayed in the absence of acetate or phosphate (assayed in distilled water) at pH 5.7, inhibition by KCl (0.1 M), KBr (0.05 M), and KI (0.025 M) was competitive with respect to pyruvate as shown in Fig. 11. When the effect of KCl was studied at various anion concentrations it was observed that at low concentrations of KCl the enzyme was activated, and at higher KCl concentrations enzyme activity was inhibited. These results are shown in Fig. 12 in the double reciprocal form. From the graph it can be seen that the enzyme was stimulated up to 0.01 M KCl, and the degree of stimulation decreased as the anion concentration increased, until at KCl

Figure 11. Double reciprocal plots of velocity against pyruvate concentration at pH 5.7 in the presence of KCl, KBr, and KI in the concentrations indicated. Assays were carried out in 0.1 M acetate buffer using the spectrophotometric assay procedure.

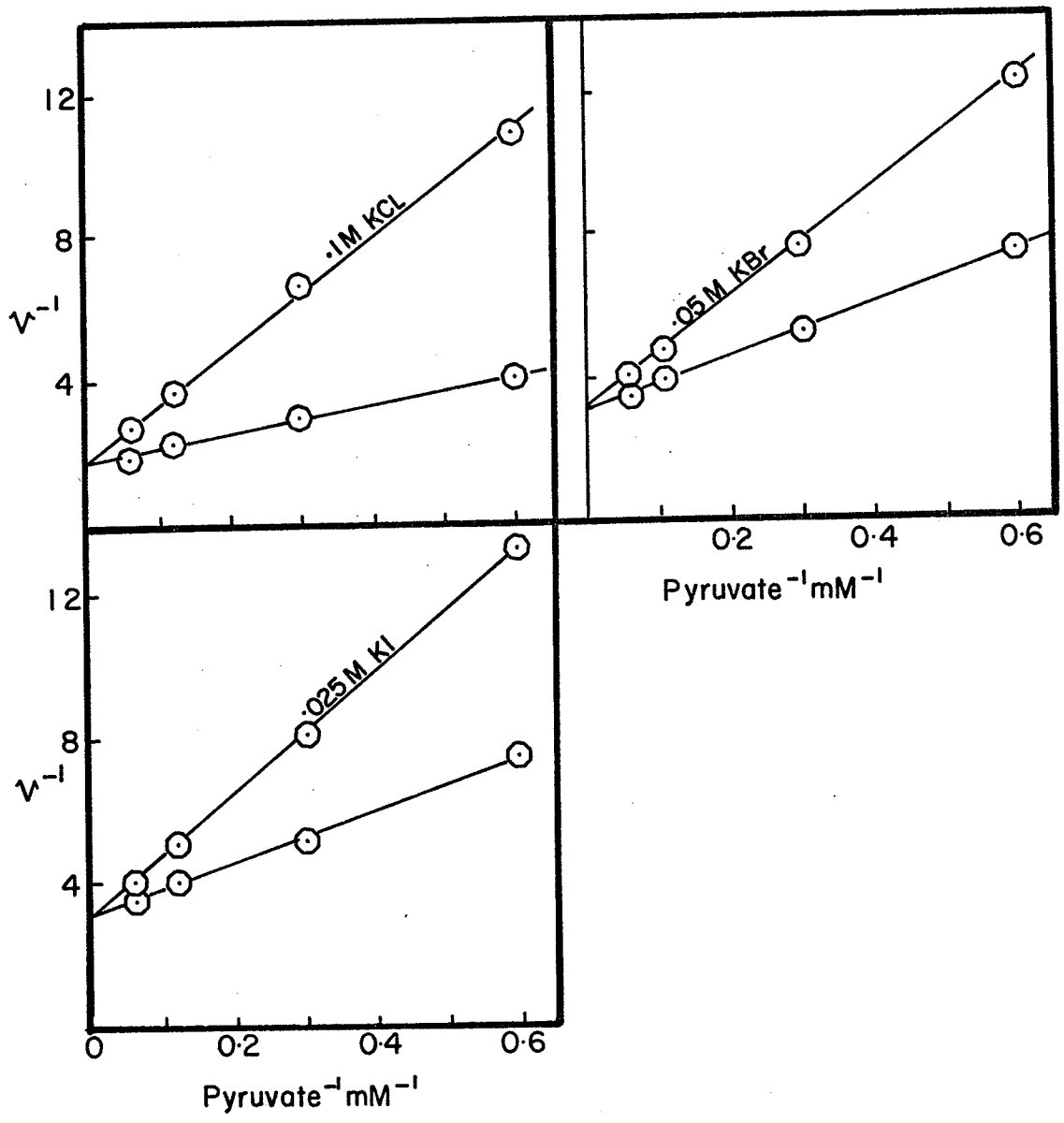

12
8
4
v⁻¹
·1M KCL
·05M KBr
0·2
0·4
0·6
Pyruvate⁻¹mM⁻¹
·025M KI
0
0·2
0·4
0·6
Pyruvate⁻¹mM⁻¹

Figure 12. Double reciprocal plots of velocity against pyruvate concentration in various concentrations of KCl at pH 5.7. Assays were carried out in distilled water at pH 5.7 using the spectrophotometric assay procedure, in the presence of KCl in the following concentrations:

A = 0.01 M

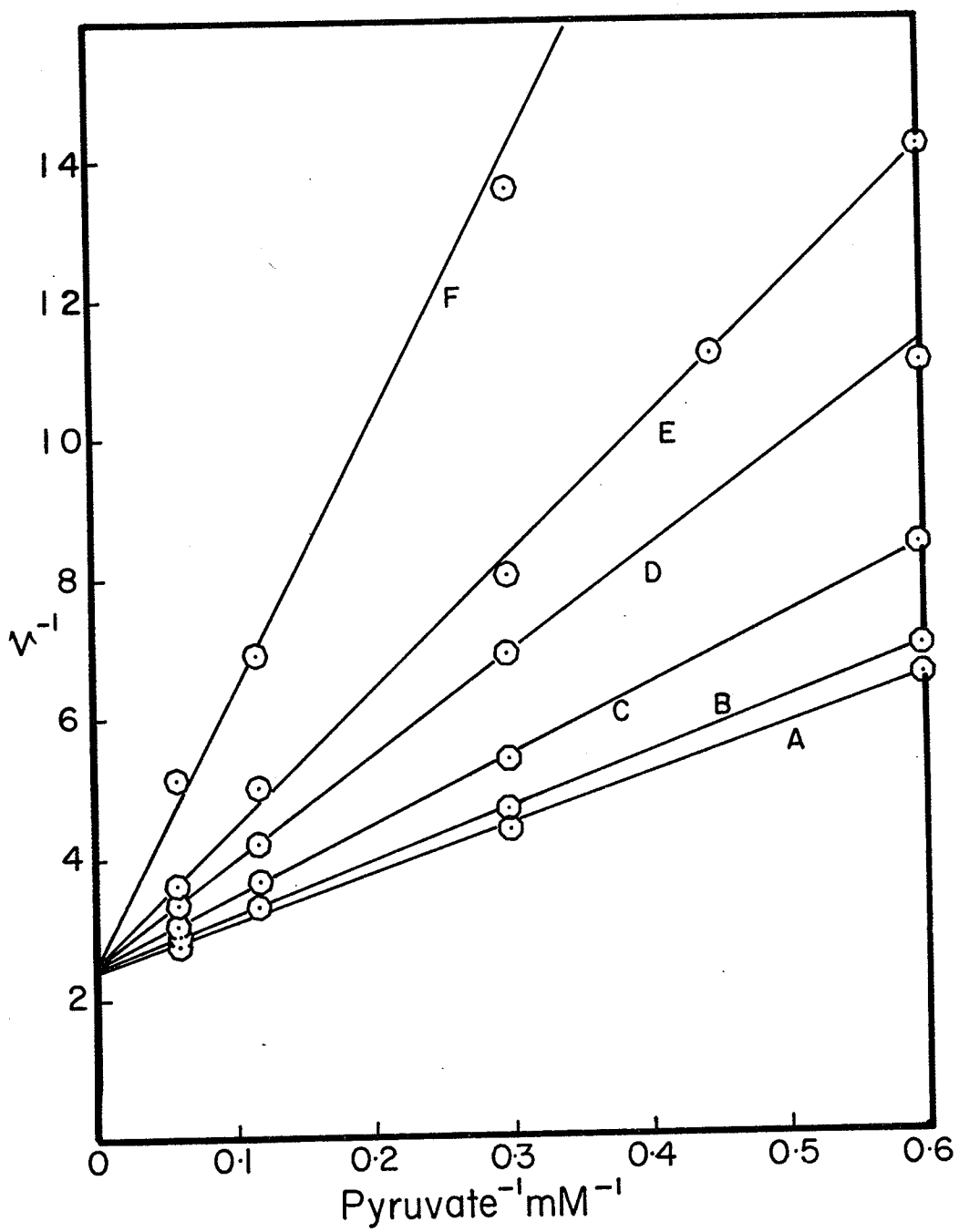
B = 0.025 M

C = nil

D = 0.08 M

E = 0.1 M

F = 0.15 M


14
12
10
8
6
4
2
v^{-1}
F
E
D
C
B
A
0
0·1
0·2
0·3
0·4
0·5
0·6
Pyruvate^{-1}mM^{-1}

concentrations greater than 0.03 M inhibition was observed.

A replot of slopes against KCl concentrations is shown in Fig. 13 , and is represented by a curved line.

In the presence of 0.1 M potassium acetate at pH 5.7 inhibition by KCl, KBr, and KI was non-competitive with respect to pyruvate. Double reciprocal plots of enzyme activity against pyruvate concentration in the presence of changing fixed KCl concentrations is shown in Fig.14 . Activation by low KCl concentrations was not observed under these conditions, as was observed when the enzyme was assayed in water.

A replot of the slopes against KCl concentration yielded a straight line as shown in Fig. 14.

EFFECT OF KSCN

The effect of KSCN on enzyme activity was similar to that of KCl and the other halides. When assayed in water enzyme activity was stimulated to a maximum at 0.005 M KSCN after which activity decreased, and inhibition was observed. The inhibition was competitive with respect to pyruvate. These results are shown in Fig. 15. A replot of the slopes from Fig.15 against KCN concentration gave a non-linear relationship, as seen in the insert of Fig. 15. When the enzyme was assayed in acetate inhibition by KSCN was non-competitive with respect to pyruvate as shown in Fig. 16.

Figure 13. Replot of slopes against acetate concentration from Fig. 12.

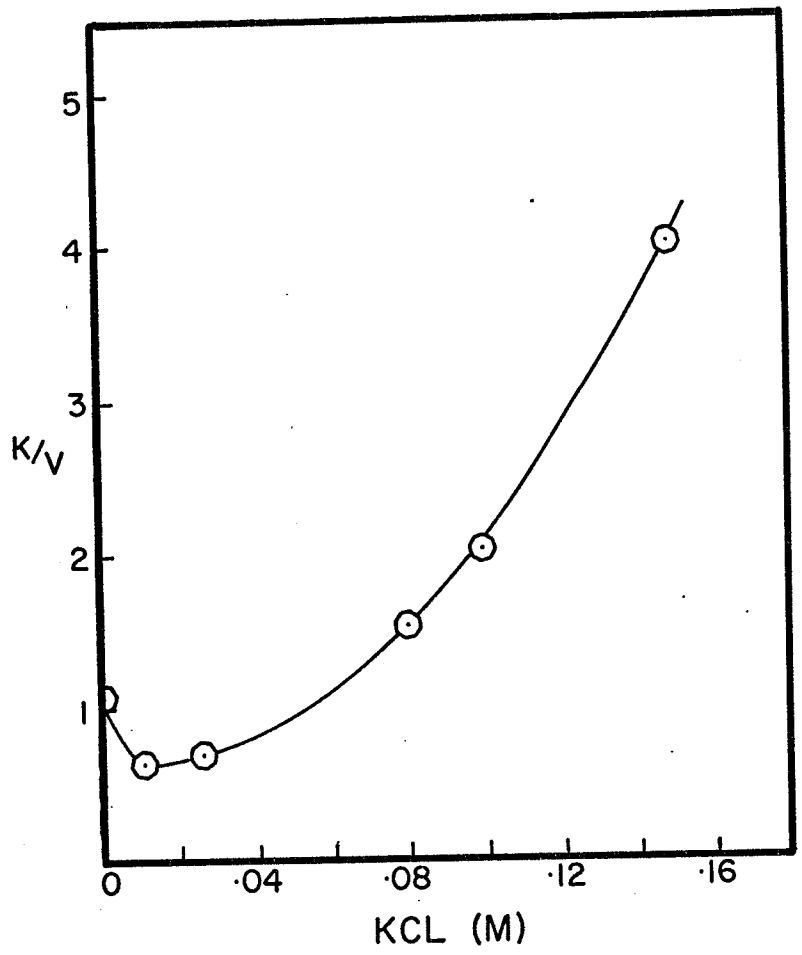

5
4
3
K/V
2
1
0
·04
·08
·12
·16
KCL (M)

Figure 14. Double reciprocal plots of velocity against pyruvate concentration at different concentrations of KCl at pH 5.7. Assays were carried out in acetate buffer using the spectrophotometric assay procedure in the following concentrations of KCl:

A = nil

B = 0.01 M

C = 0.025 M

D = 0.05 M

E = 0.1 M

F = 0.15 M

The replot of slopes against KCl concentration is shown in the insert.

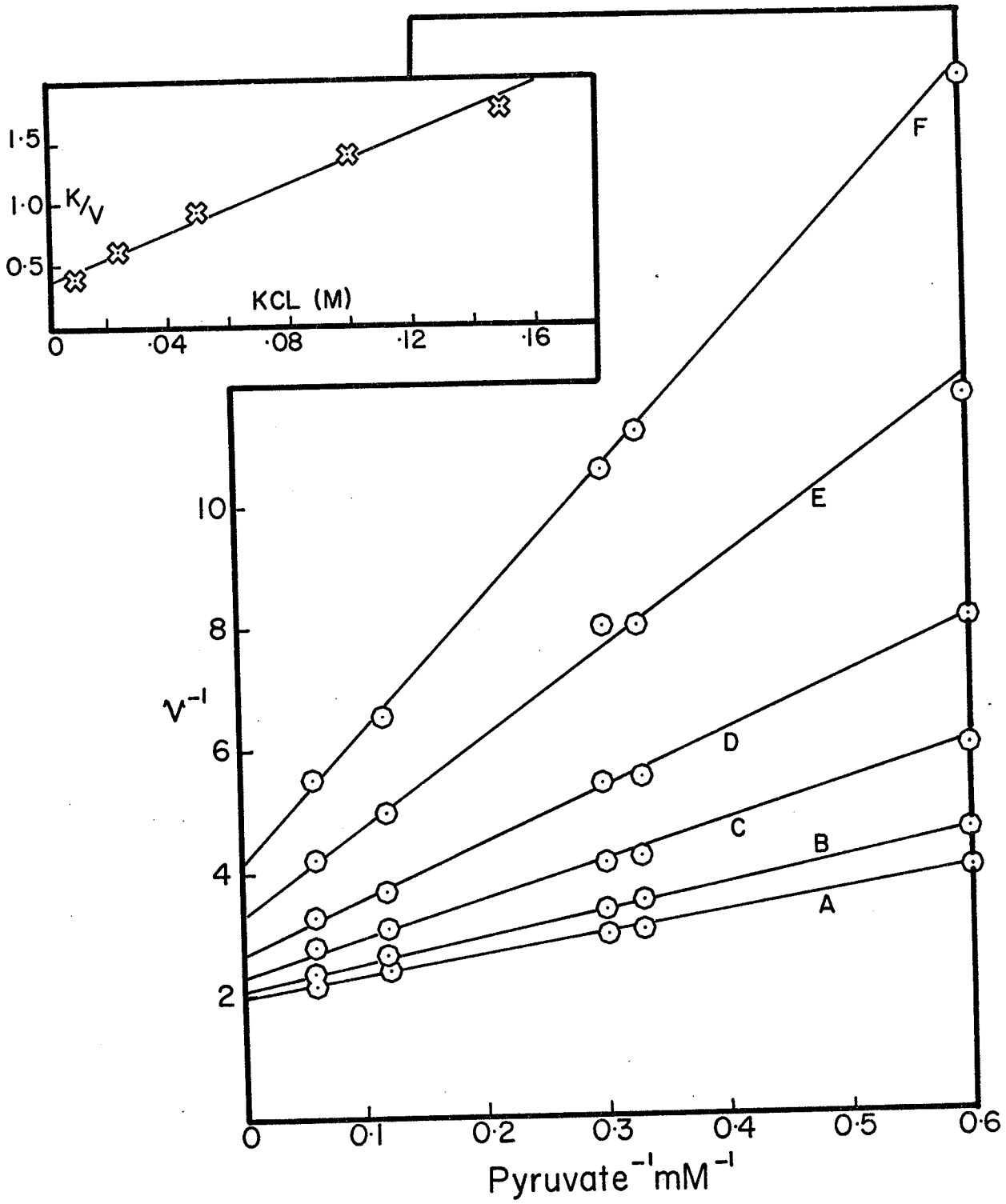

1·5
1·0
0·5
K/V
KCL (M)
0
·04
·08
·12
·16
10
8
6
4
2
V⁻¹
F
E
D
C
B
A
0
0·1
0·2
0·3
0·4
0·5
0·6
Pyruvate⁻¹mM⁻¹

Figure 15. Double reciprocal plots of velocity against pyruvate concentration at different concentrations of KSCN. Assays were carried out in distilled water using the spectrophotometric assay procedure, in the following concentrations of KSCN:

A = 0.005 M

B = 0.0025

C = nil

D = 0.03 M

The replot of slopes against KSCN concentration is shown in the insert.

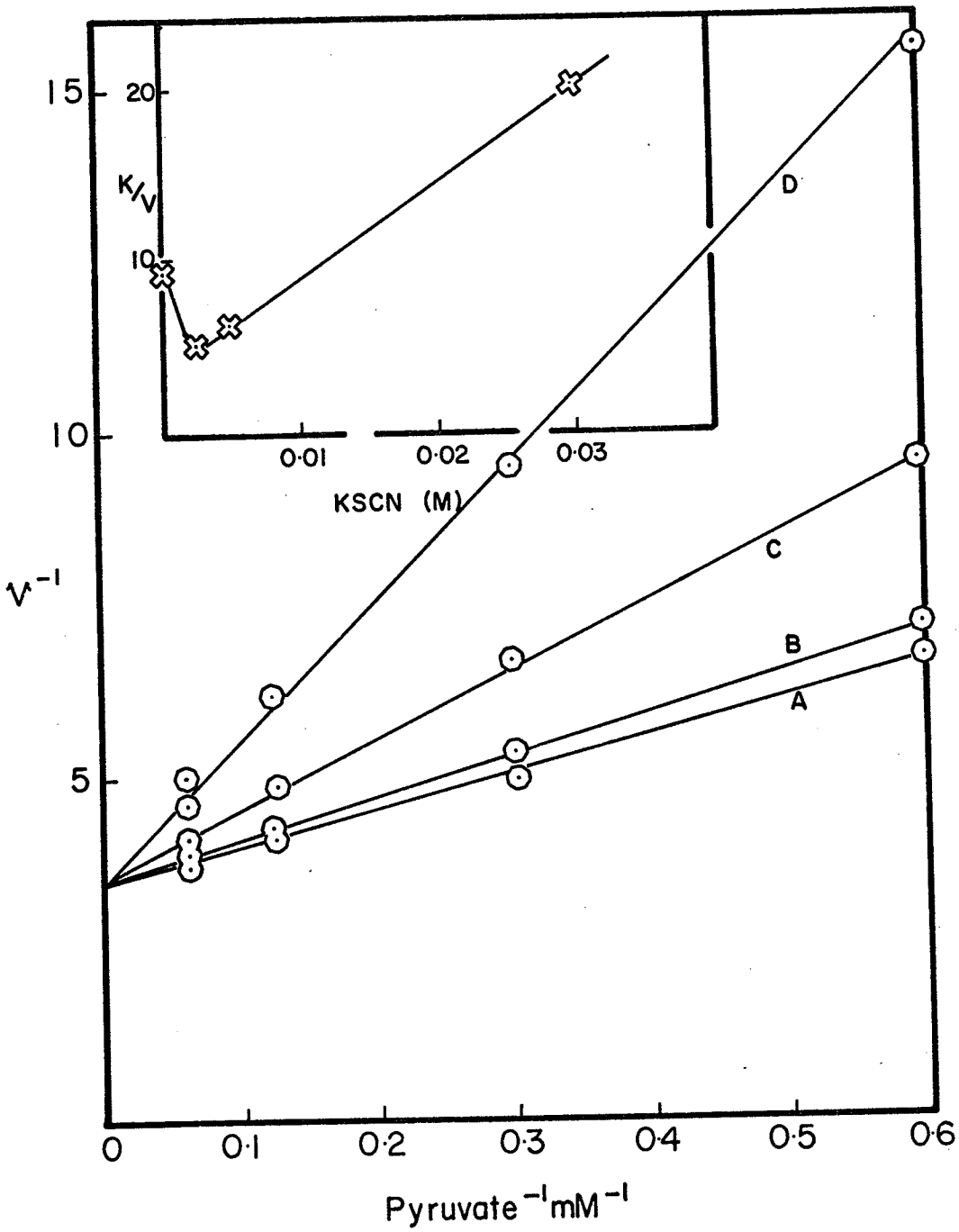
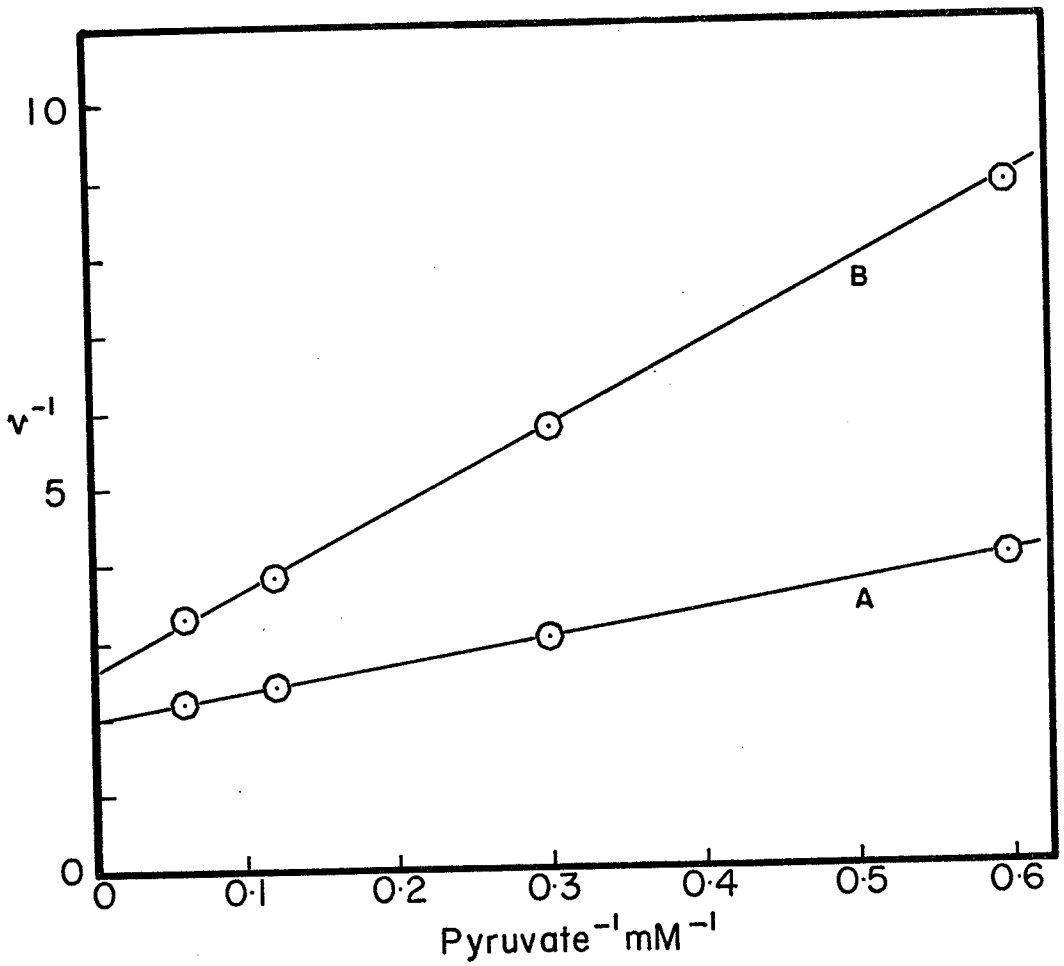

15
10
5
V⁻¹
20
K/V
10
0·01
0·02
0·03
KSCN (M)
D
C
B
A
0
0·1
0·2
0·3
0·4
0·5
0·6
Pyruvate⁻¹mM⁻¹

Figure 16. Double reciprocal plots of velocity against pyruvate concentration at different KSCN concentrations at pH 5.7. Assays were carried out in acetate buffer using the spectrophotometric assay procedure, in the following concentrations of KSCN:

A = nil

B = 0.025 M


10
5
0
v^{-1}
B
A
0
0·1
0·2
0·3
0·4
0·5
0·6
Pyruvate^{-1} mM^{-1}

EFFECT OF SULFATE ON ENZYME ACTIVITY

As an analogue of phosphate, the effect of sulfate was tested in the presence of 0.1 M potassium acetate buffer at pH 5.7. The results are shown in Fig. 17 in the double reciprocal form. At 0.4 mM, 1.0 mM, 2.0 mM, and 4.0 mM Na_2SO_4, inhibition was competitive with respect to pyruvate, whereas at high phosphate concentration, inhibition by phosphate was non-competitive. In addition, the enzyme was much more sensitive to sulfate inhibition than to phosphate inhibition.

Double reciprocal plots of enzyme activity and pyruvate concentration were linear in the presence of 0.4 mM and 1.0 mM sulfate, but became non-linear at low pyruvate concentrations in the presence of 2.0 mM and 4.0 mM sulfate.

A replot of slopes against sulfate concentration yielded a straight line as shown in the insert of Fig. 17.

EFFECT OF FLUOROPYRUVATE

Fluoropyruvate is a powerful inhibitor of acetolactate synthetase. At a final concentration of 1.0 mM fluoropyruvate inhibited enzyme activity by approximately 30% at 16 mM pyruvate. Inhibition was competitive with respect to pyruvate (Fig. 18).

EFFECT OF 1-FLUORO,2,4-DINITROBENZENE

The arylation of amino acids with 1-fluoro,2,4-dinitrobenzene is of great importance in structural protein chemistry. This

Figure 17. Double reciprocal plots of velocity against pyruvate concentration at different Na_2SO_4 concentrations at pH 5.7. Assays were carried out in acetate buffer using the spectrophotometric assay procedure in the following concentrations of Na_2SO_4:

A = nil

B = 0.4 mM

C = 1.0 mM

D = 2.0 mM

E = 4.0 mM

The insert shows a replot of slopes against Na_2SO_4 concentration.

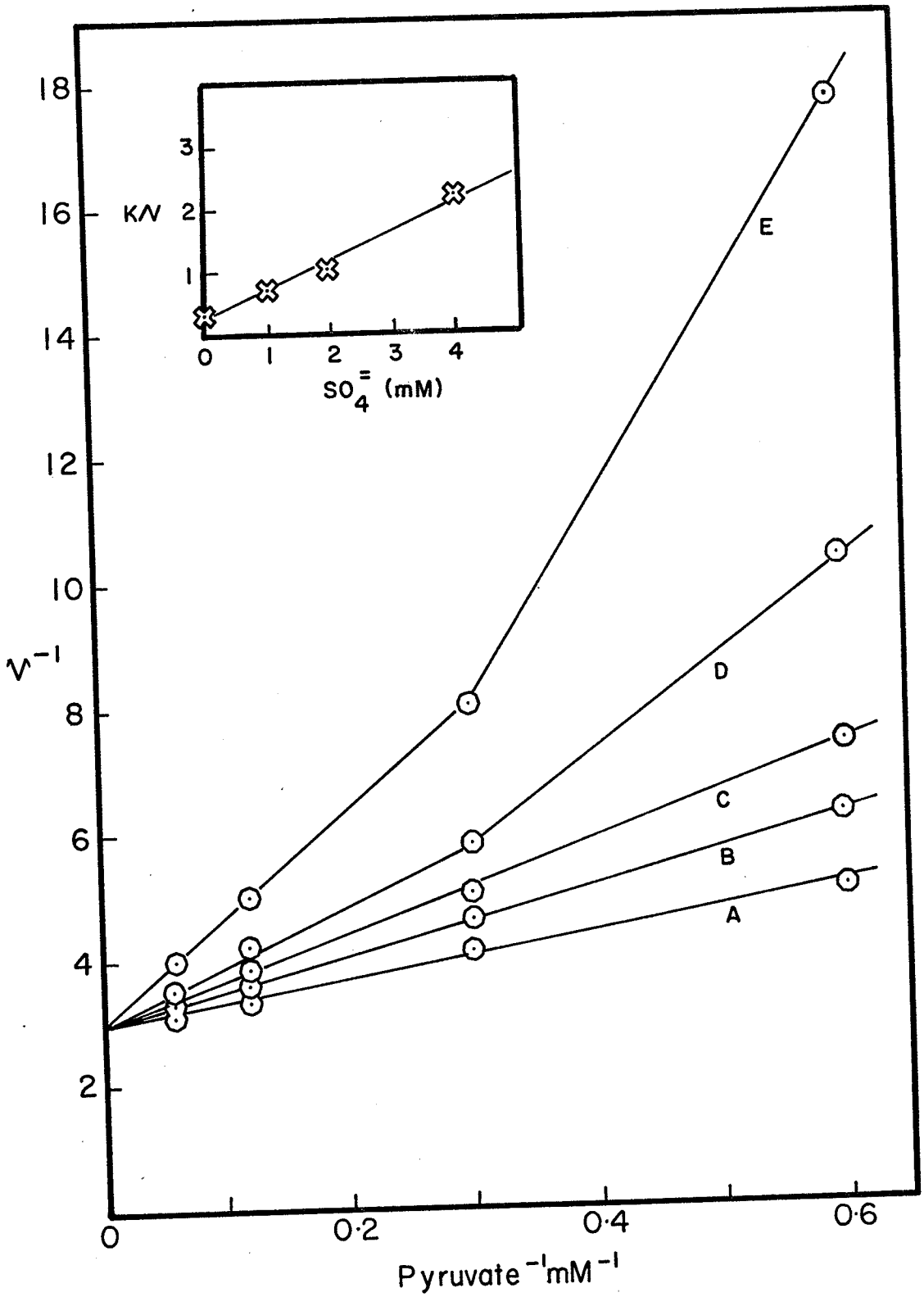
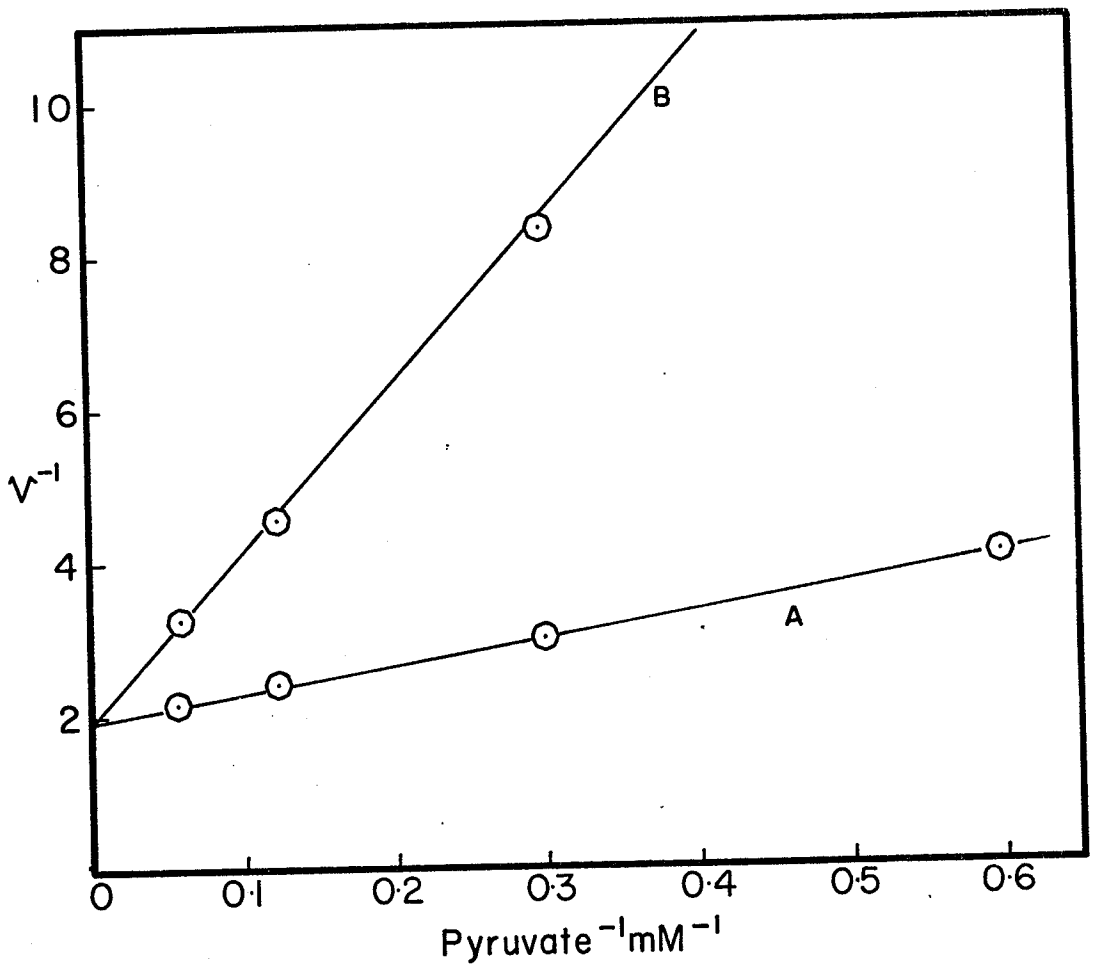

18
16
14
12
10
8
6
4
2
0
0·2
0·4
0·6
v⁻¹
Pyruvate⁻¹mM⁻¹
A
B
C
D
E
3
2
1
0
1
2
3
4
KV
SO4= (mM)

Figure 18. Double reciprocal plots of velocity against pyruvate concentration at different fluoropyruvate concentrations at pH 5.7. Assays were carried out in acetate buffer using the spectrophotometric assay procedure in the following concentrations of fluoropyruvate:

A = nil

B = 1.0 mM


10
8
6
4
2
V⁻¹
B
A
0
0·1
0·2
0·3
0·4
0·5
0·6
Pyruvate⁻¹mM⁻¹

compound reacts with the ε amino group of lysine, masking it but not modifying it. To determine if lysine residues were involved at the active site of the enzyme, the enzyme was incubated in 1.0 M 1-flouro,2,4-dinitrobenzene for periods up to 60 min, both in the absence and presence of 0.03 M pyruvate at pH 5.7. After incubation enzyme activity in all samples was equivalent to a control containing no 1-flouro,2,4-dinitrobenzene, indicating no effect of this compound on enzyme activity, and suggesting that lysine residues are not involved at the active site. These results support previous studies in which it was found that shiff's base formation is not involved in the formation of α-acetolactate by acetolactate synthetase (Sawula, 1966).

DISCUSSION

DISCUSSION

In the course of this study of acetolactate synthetase from Aerobacter aerogenes it was observed that acetate, propionate, and to a lesser degree formate buffers act as an activator for the enzyme, and that normal Michaelis-Menten type kinetics were displayed only in these buffers, or when assayed in distilled water. Other buffers caused sigmoidal kinetics and inhibited enzyme activity. For example, the enzyme exhibited apparent Michaelis-Menten kinetics in water and in acetate buffer at pH 5.7, but showed cooperative effects in phosphate buffer at the same pH with a marked decrease in its maximum velocity, and a decrease in its affinity for pyruvate. From further studies it became apparent that the enzyme is strongly influenced by various anions, both organic and inorganic.

When assayed in distilled water at pH 5.7 chloride, bromide, iodide, and thiocyanate at low concentrations activated the enzyme activity, but at high concentrations inhibited the activity competitively with respect to pyruvate. In the presence of acetate however, inhibition by these anions was non-competitive with respect to pyruvate, and no activation at low anion concentration was observed. In the presence of acetate alone enzyme activity was stimulated up to 0.1 M acetate, beyond which stimulation decreased and inhibition was observed. The nature of this inhibition was non-competitive with respect to pyruvate. The effect of these

monovalent inorganic anions followed the order expected from the Hofmeister lyotropic series.

The reaction catalyzed by acetolactate synthetase is regarded as a two substrate reaction. The formation of α-acetolactate is believed to take place as follows:

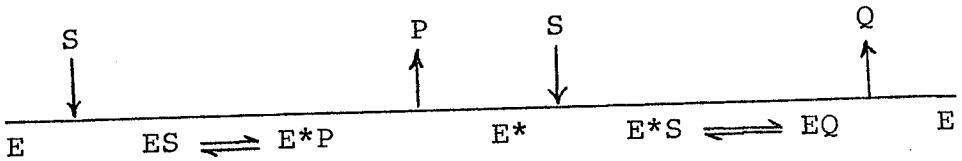
$$E + S \longleftrightarrow ES \longleftrightarrow E^*P \longleftrightarrow E^* + P$$

$$E^* + S \longleftrightarrow E^*S \longleftrightarrow EQ \longleftrightarrow E + Q$$

where E is enzyme-TDP, S is pyruvate, E* is enzyme-HETDP (enzyme-hydroxyethylthiamine diphosphate), P is CO_2, and Q is α-acetolactate.

In this mechanism the first pyruvate binds the enzyme-TDP complex (E) and is decarboxylated to form the enzyme-HETDP complex (E*) and CO_2. The second pyruvate then binds E* and condenses with the hydroxyethyl group to form α-acetolactate and the original enzyme-TDP complex (E).

In the short-hand expression of Cleland (1963) the mechanism is expressed as follows:



The initial velocity equation for this mechanism in the absence of products is:

$$v = VS/(Ka + Kb) + S$$

where v is initial velocity, V is maximal velocity, S is pyruvate concentration, Ka is the Michaelis constant for

pyruvate binding E, and Kb is the Michaelis constant for pyruvate binding E*. The Km for pyruvate determined in the Lineweaver-Burk plots is Ka + Kb.

This is essentially a Ping Pong Bi Bi reaction (Cleland 1963) with two substrates being identical (pyruvate). This mechanism is supported by linear double reciprocal activity-pyruvate concentration plots obtained in distilled water or acetate buffer. Nonlinear plots would be expected of other mechanisms such as an Ordered Bi Bi reaction.

For the purpose of this discussion the first pyruvate-binding site (on E) and the second binding site (on E*) will be referred to as site A and site B, respectively. It is assumed that sites A and B can bind anions with different affinities. Site A has a high affinity for monovalent inorganic anions and can be referred to as an activator site for these anions resulting in lowering Ka upon anion binding to this site. The second site, site B, has a lower affinity for anions and acts as an inhibitor site for all anions. With this model in mind the kinetic behaviour of acetolactate synthetase can be discussed.

In the absence of acetate or phosphate (when the enzyme is assayed in distilled water), low concentrations of monovalent inorganic anions (KCl, KBr, KI, KSCN) stimulate enzyme activity by binding to site A. Because this site has a high affinity for these anions all the anion will bind

exclusively to this site. The presence of anion at this site facilitates in some way the binding of pyruvate, accounting for activation. As the anion concentration is increased the anions bind to both site A and site B. As a result, with increasing anion concentration, activation followed by inhibition is observed since Ka may decrease but Kb will increase leading to higher Km for pyruvate. This inhibition is competitive since both pyruvate and anion bind to the same enzyme form (E*). At saturating pyruvate concentrations both activation and inhibition are removed.

In the presence of acetate the effect of added inorganic monovalent anions is quite different. In this case inhibition was non-competitive with respect to pyruvate and activation at low anion concentrations was not observed. The simplest explanation is to suggest that the acetate anion binds preferentially at a distinct site (site C) and activates the enzyme. Once acetate is bound the substrate binding sites are modified losing their affinity for anions such that these anions cannot bind at the first pyruvate site (A) and can bind the second pyruvate site (B) only after the binding of pyruvate to that site. Since pyruvate and anion bind to different enzyme forms (E* and E*S respectively) non-competitive inhibition is expected.

Inhibition by phosphate in the presence of acetate was non-competitive with respect to pyruvate. In this case

phosphate behaves in a manner similar to monovalent anions in that it can bind to site B only after pyruvate has been bound to the site. Phosphate and pyruvate bind to different enzyme forms resulting in non-competitive inhibition.

The behaviour of the enzyme in phosphate buffer alone is very interesting. The rate saturation curve is sigmoidal, and double reciprocal plots of enzyme activity and pyruvate concentration are non-linear. The degree of non-linearity of the double reciprocal plots is high and is probably represented by a cubic function or higher. Phosphate may be binding to all three sites, site A, site B, and site C, the acetate site, as inhibitor. In this case the mechanism becomes very complex and non-linear double reciprocal plots are expected.

The effect of acetate concentration is shown in Fig. 5 where it is observed that both activation and inhibition are displayed in the presence of the acetate ion. It should be noticed that both Vmax and Km change either in activation or inhibition. It is important to stress that acetate binds preferentially at a distinct site (C) and activates the enzyme increasing its affinity for pyruvate as well as the maximal velocity. Activation increases with increasing acetate concentration to a maximum at 0.1 M acetate. Once this acetate site is saturated, acetate can then bind at site B, the low anion affinity site, which acts as an

inhibitor site. It was previously stated that once acetate binds to the enzyme at its distinct site (C), the pyruvate sites are modified so that anions cannot be bound at site A and can be bound at site B only after pyruvate binding. It is expected that acetate binding at site B is weak because of its position with respect to other anions in the Hofmeister series. Almost a ten-fold greater concentration of acetate was required to cause the degree of inhibition equivalent to that displayed by 0.1 M KCl, the next anion in the Hofmeister anionic series. Since acetate and pyruvate bind to different forms of the enzyme (E*S and E*), the expected inhibition with respect to pyruvate is non-competitive.

The fact that propionate can activate the enzyme equally as well as acetate, and also formate to a lesser degree suggests that all three compounds can bind at the distinct activating site (C). There appears to be some structural specificity required for the activator. Although acetate, propionate and formate had a similar activating effect, resulting in normal Michaelis-Menten type kinetics, other carboxylic acid buffers (lactate, fumarate, citrate, and succinate) failed to display any activating effect. Enzyme activity in these latter buffers was inhibited but to a lesser degree than in phosphate buffer, and double reciprocal plots of enzyme activity and pyruvate concentration were still non-linear. Since the effect of these buffers is similar to

phosphate, it is probable that the non-linear kinetics is due to these compounds binding at all sites (A, B and C) as inhibitors.

Sulfate inhibited enzyme activity competitively with respect to pyruvate in the presence of 0.1 M acetate. This was unexpected since sulfate being an analogue of phosphate was expected to display the same type of inhibition as phosphate. It appears that in the presence of acetate, sulfate can still bind site B competing with pyruvate. At increasing sulfate concentrations, the double reciprocal plots became curved at low pyruvate concentrations, indicating that sulfate may be binding to sites A and C also as inhibitor. At a lower acetate concentration (50 mM) inhibition by sulfate is more pronounced and double reciprocal plots are more non-linear (Störmer, 1968) indicating that sulfate may be occupying the acetate site (C). In this respect the sulfate effect seems to be similar to the phosphate effect except that sulfate is a stronger inhibitor.

In studies with the pyruvate analogue, fluoropyruvate, in the presence of acetate, inhibition with respect to pyruvate was competitive.

Part II Lactate Dehydrogenase

INTRODUCTION

INTRODUCTION

Early kinetic studies of NAD^+-specific lactate dehydrogenases from various sources indicated that the reaction followed classic Michaelis-Menten kinetics and had a compulsory order of binding of substrates (Chance and Neilands, 1952; Takinaka and Schwert, 1956). Recent reports, however, have indicated the existence of several NAD^+-specific lactate dehydrogenases whose kinetics deviate from those described by Michaelis. Lactate dehydrogenases from Escherichia coli (Tarmy and Kaplan, 1965) and Butyribacterium rettgeri (Wittenberger, 1966) and isozyme 5 from rabbit muscle (Fritz, 1965) have been reported to be characterized by a sigmoid rate concentration curve with respect to pyruvate rather than the usual hyperbolic curve noted for other lactate dehydrogenases. The Aerobacter aerogenes enzyme has been shown to share this property with the E. coli and Butyribacterium rettgeri enzyme (Hollier, 1967). Kinetic studies at pH 5.2 (Hollier, 1967) indicated that the mechanism of the reaction catalyzed by the Aerobacter enzyme was an ordered one with NADH and NAD^+ binding to the free enzyme.

The E. coli and Butyribacterium rettgeri enzyme, as well as that from Aerobacter aerogenes have been shown to be essentially unidirectional enzymes, catalyzing the reduction of pyruvate. This is in marked contrast to the lactate dehydrogenases from most animal sources which have been

reported to have a rate of lactate oxidation of the order of 10-20% that of pyruvate reduction.

It has been postulated that these lactate dehydrogenases are allosteric, being negatively effected by ATP in the case of the E. coli and Butyribacterium enzyme, and being positively effected by oxalacetate for isozyme 5 of rabbit muscle. All three enzymes show sigmoidal kinetics at alkaline pH, but the sigmoidal character of the substrate saturation curves become hyperbolic by a lowering of pH, in the case of the Aerobacter aerogenes (Hollier, 1967) and the Butyribacterium enzyme (Wittenberger et al, 1967).

The lactate dehydrogenase from E. coli has been purified (Tarmy et al, 1968) and some of its molecular, biochemical, and kinetic properties have been studied. Since E. coli and Aerobacter aerogenes are both members of the family Entero-bacteriaceae, it was of interest to determine whether the Aerobacter enzyme displayed the same properties as the E. coli lactate dehydrogenase.

It has been recently demonstrated (Warren et al,1966) the neutral salts at high concentrations (0.3 to 3.0 M) inhibit the activity of widely different enzymes in the order of increasing effectiveness for anions, $Acetate^- < Cl^- < NO^-_3 < Br^- < I^- < SCN^- < ClO^-_4$. The effect of these anions on lactate dehydrogenase activity was studied.

Kinetic studies of mammalian lactate dehydrogenase by Wuntch et al (1970) have shown that kinetic properties determined at highly dilute concentrations in vitro may be quite different from kinetic properties that exist in vivo. The effect of high enzyme concentrations on the pattern of the substrate saturation curve at pH 7.0 was studied to determine if the kinetic properties at this pH were the same, or different at dilute and near physiological enzyme concentrations.

This thesis represents the purification of lactate dehydrogenase from Aerobacter aerogenes; the results of the above mentiond studies; somes of its kinetic and physical properties and a comparison of the enzyme with those of other NAD^+-specific dehydrogenases. A model for the enzyme is proposed.

HISTORICAL

HISTORICAL

Interest in lactate dehydrogenase dates back to almost half a century ago when Thunberg in 1920 recognized an enzyme in tissues which oxidized lactate, and depended on a water-soluble cofactor for activity. In animal cells, the enzyme is linked to the pyridine nucleotide, NAD^+ and catalyzes the following reaction.

$$\text{lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+$$

The reaction is reversible, the rate of lactate oxidation being about 20% the rate of pyruvate reduction. The lactate dehydrogenase from yeast is a flavoprotein and is coupled to the cytochrome system (Appleby and Morton, 1954). Many bacteria seem to contain two types of lactate dehydrogenase, one which is NAD^+ specific, and the other which can only be coupled to oxidation-reduction dyes and is presumably a flavoprotein.

The enzyme was first purified from beef heart in 1940 by Straub, and the beef muscle enzyme was crystallized and studied by Kubowitz, and Ott in 1943. Since then lactate dehydrogenase from a wide variety of sources has been crystallized.

Meister in 1950, and later Neilands (1952) demonstrated that the beef heart lactate dehydrogenase when subjected to high voltage electrophoresis, showed two catalytically active components. Wieland and his co-workers (1957), and Markert

and Møller (1959) demonstrated by paper, and starch gel electrophoresis, that there were five forms of lactate dehydrogenase in animal tissues. These forms were called isozymes, and were designated as LDH_1, ------, LDH_5. The relative amounts of the five isozymes were found to vary in different tissues of the same animal.

The nature of the multiple forms has now been resolved from studies by Markert (1959), Wieland (1957), and Kaplan (1964). There appears to be two major types of LDH, each consisting of four identical subunits, and are referred to as the H (heart) and M (muscle) types. When these two types are present in one cell they can form hybrids consisting of subunits of both types, producing five molecular species (HHHH, HHHM, HHMM, HMMM, MMMM) all possessing lactate dehydrogenase activity. The two parent forms are under the control of separate genes, and are immunologically distinct.

Isozymes analogous to those found in higher organisms have not been observed in bacteria. The properties of bacterial lactate dehydrogenases appear to be considerably more diversified than those of vertebrates and invertebrates, even aside from the numerous enzymes not dependent on pyridine nucleotide, that catalyze the oxidation of lactate to pyruvate.

Although the reaction catalyzed by the mammalian lactate dehydrogenases are readily reversible, this characteristic is not shared by all bacterial lactate dehydrogenases. The

E. coli and Butyribacterium rettgeri lactate dehydrogenases are unidirectional enzymes catalyzing the reduction of pyruvate with NADH (Tarmy and Kaplan, 1968; and Wittenberger, 1966), although lactate is oxidized by the 3-acetylpyridine analogue of NAD^+ to a slight degree. The enzyme from Lactobacillus plantarum (Dennis et al, 1960), does catalyze lactate oxidation with NAD^+. Irreversibility has been demonstrated for the Aerobacter aerogenes enzyme, but no oxidation of lactate with the 3-acetylpyridine analogue of NAD^+ could be demonstrated (Hollier, 1967).

All bacterial lactate dehydrogenases do not catalize the formation of the same isomer of lactate. In at least one bacterium, Lactobacillus plantarum two distinct lactate dehydrogenases are present; one catalyzing the oxidation of the L(+) isomer of lactate, and the other catalyzing the oxidation of the D(-) isomer (Dennis et al, 1960). Other lactate dehydrogenases, from E. coli (Tarmy, et al, 1968) and Butyribacterium rettgeri (Wittenberger, 1966) have been found to be specific for the D(-) isomer of lactic acid.

Although the muscle and heart lactate dehydrogenases are very different in many properties they have very similar molecular weights. All the L(+) lactate specific NAD^+ linked lactate dehydrogenases examined appear to fall in the molecular weight range between 140,000 and 150,000, and all appear to have four sub-units (Kaplan, 1964; Kaloustian et al, 1969). The molecular weight of bacterial lactate

dehydrogenases appears to be much smaller than those reported for the vertibrate and invertebrate types. The Lactobacillus plantarum D(-) lactate dehydrogenase appears to have a molecular weight of 60,000 to 70,000, estimated from the sedimentation of a partly purified preparation (Fondy and Kaplan, 1965). The enzyme from Leuconostoc mesenteroides has a molecular weight of about 80,000 measured by gel filtration of a crude extract by sephadex G-100 (Kaplan, 1964). The molecular weight of lactate dehydrogenase from E. coli is considerably larger than for other D(-) lactate dehydrogenases for which molecular weights have been estimated. This enzyme has an apparent molecular weight of 115,000 (Tarmy and Kaplan, 1968).

Many of the lactate dehydrogenases appear to be sulfhydryl enzymes. The bovine heart enzyme (Takenaka and Schwert, 1956) has been shown to be slowly inactivated by P-hydroxymercuribenzoate with reversal of this inhibition by cysteine and glutathione. It has been suggested by these workers that for bovine heart muscle lactate dehydrogenase, the sulfhydryl groups participate in the coenzyme binding, since NADH seems to have a protective effect. Studies on the sulfhydral groups of the E. coli enzyme indicate they must be fully reduced in order that the enzyme maintains full enzymatic activity (Tarmy and Kaplan, 1968). The effect of mercurials has also been shown for the Aerobacter aerogenes enzyme by Hollier (1967).

Early studies of the kinetics of the reaction catalyzed by lactate dehydrogenase were consistent with the hypothesis that coenzyme and substrate were bound at independent sites on the enzyme surface (Socquet and Laidler, 1951). Bovine Heart (Hakala et al, 1956) and crystalline rat liver (Gibson et al, 1953) lactate dehydrogenases followed classical Michaelis-Menten kinetics. Equations derived by Hakala et al (1956) suggested that the reaction was either an ordered one with a compulsory order of binding of substrates, or a Theorell-Chance mechanism (Theorell and Chance 1951) in which there is a compulsory sequence of interaction of enzyme with reactants, but in which ternary complexes are so short lived as to be without kinetic significance. Chance and Neilands (1952) observed a spectrophotometrically detectable NADH-enzyme complex. Takenaka and Schwert (1956) studying bovine heart lactate dehydrogenase found that approximately four moles of NAD were bound by the enzyme, but that neither lactate nor pyruvate were measurably bound.

In 1965 and 1967 Fritz reported that isozyme 5, the MMMM tetramer of rabbit muscle lactate dehydrogenase did not follow the classical Michaelis-Menten kinetics, but rather exhibited a sigmoid shaped pyruvate saturation curve which became hyperbolic when the hydrogen ion concentration was lowered from pH 7.8 to pH 6.5. A similar deviation from Michaelis-Menten kinetics was observed for the D(-) enzyme of E. coli (Tarmy and Kaplan 1965, 1968). The enzyme was

characterized by a markedly sigmoidal pyruvate saturation curve, and was irreversible at pH 7.5. In 1966 Wittenberger reported that an NADH-linked D(-) specific lactate dehydrogenase from Butyribacterium rettgeri demonstrated kinetic properties very similar to those of the E. coli enzyme.

Studies of the Aerobacter aerogenes D(-) lactate dehydrogenase by Hollier (1967) have revealed some unusual kinetic properties similar to those displayed by the E. coli and Butyribacterium rettgeri enzyme. The Aerobacter aerogenes lactate dehydrogenase displays a pH optimum of 5.5, and displays typical Michaelis-Menten kinetics as pH 5.2. However, at pH 6.5 the hyperbolic pyruvate saturation curve becomes distinctly sigmoidal, indicating that the enzyme reacts in some manner with more than one molecule of the substrate.

Sigmoid curves of rate as displayed by these lactate dehydrogenases are characteristic of a class of enzymes which serve regulatory functions. Such enzymes have also been found to be modulated by the concentration of one or more specific metabolites or effectors which are often neither direct participants in the reaction nor structural analogues of the substrate. These enzymes were designated as "allosteric" by Monod et al (1963).

With recognition that allosteric enzymes are endowed with unusual kinetic and structural characteristics, various

proposals have been made to explain their regulatory mechanism. Of particular interest is the hypothesis of Monod, Wyman, and Changeaux (1965) which directs attention to the subunit structure of allosteric enzymes as a characteristic of singular importance in accounting for the unique kinetic behaviour of these enzymes. In essence, the model is based on the assumptions; (1) that all allosteric enzymes are polymers, composed of two or more identical subunits, and that they are capable of existing in at least two different conformational states; (2) that each of the identical subunits possesses a catalytic site specific for the substrate, and a separate allosteric site specific for the allosteric effector; (3) that for each conformational state the catalytic sites and the allosteric sites have equal affinities for their respective ligands; (4) that the various conformational states are in dynamic equilibrium with one another; and (5) that the transition from one state to the other involves simultaneous changes in all the identical subunits within a given molecule. For those situations in which the affinity of a given ligand for one conformational state is greater than it is for another, the addition of that ligand will result, at low ligand concentrations, in preferential binding of a single ligand molecule to one subunit of the enzyme species for which it has the greatest affinity. This

will cause the displacement of the equilibrium in favor of that enzyme species having the greater affinity for the ligand, and, concomitantly, will facilitate the subsequent binding of additional ligands, owing to the simultaneous formation of more than one additional reaction site. It is evident that the sigmoid response of activity to increasing substrate, and, or allosteric effector concentrations is readily explained by this model. The most attractive feature of this model is the fact that it takes into special account the unique subunit structural characteristics of most allosteric enzymes, and provides an elegant explanation for the observed susceptibility of allosteric enzymes to association and dissociation in response to the substrate and allosteric agents, and to changing environmental conditions such as pH and temperature.

Other investigators have proposed alternate theories that embrace concepts of flexibility of binding sites, as proposed by Koshland (1958, 1963, 1964).

The model of Koshland postulates that hybrid conformational states of the protein can exist, i.e., that one subunit can change conformation without necessarily causing an equal change in all other sub-units, whereas the Monod-Wyman-Changeux model predicts that the transition from one state to another involves simultaneous changes in all the identical subunits within a molecule.

As well as being affected by certain compounds several allosteric enzymes have been found to have their rate saturation curves changed from sigmoidal to hyperbolic by pH. These enzymes include, among others previously mentioned, threonine deaminase (Changeaux, 1963), aspartate transcarbamylase from E. coli (Gerhardt and Pardee, 1962, 1963), and isocitric dehydrogenase from Neurospora (Sanwal et al, 1964). The reason for the loss of the allosteric site are not known, but may be due to the ionization of some critical binding groups, or may be connected by conformational changes induced by a change in hydrogen ion concentration. In this case, pH would seem to have the same effect as an allosteric activator.

A detailed kinetic study of the Aerobacter aerogenes lactate dehydrogenase at pH 5.2 suggested that the data fitted the equation pertaining to all sequential mechanisms involving two substrates. Although the same equation applies to Ordered, Rapid Random Equilibrium, and Theorell-Chance mechanisms, the product inhibition patterns obtained at pH 5.2 indicated that the reaction was an ordered one with NADH and NAD^+ binding to the free enzyme (Hollier 1967, Cleland, 1963).

A large variety of bacterial lactate dehydrogenases is known to exist (Dennis et al, 1959 and 1962; Pascal et al, 1966; Yoshida et al, 1965; Wolin, 1964; Wittenberger, 1966; Wittenberger et al, 1967). With the exception of the L-lactate dehydrogenase from Lactobacillus plantarum, none of the

bacterial enzymes had been extensively purified, partly because these enzymes tend to become very labile during the course of fractionation. Recently, the lactate dehydrogenases from Butyribacterium rettgeri and E. coli have been purified (Wittenberger, 1966; Tarmy and Kaplan, 1968), and their chemical, molecular, and kinetic properties have been studied.

RESULTS .

RESULTS

PURIFICATION OF NADH-SPECIFIC LACTATE DEHYDROGENASE

Heat precipitation

Crude extract, obtained from 1,000 grams of cell paste was placed in a 3 liter flask and heated in a water bath. The temperature of the extract was kept at 45°C for 20 min, during which time the flask was swirled at one minute intervals. After heating the flask was immersed in an ice bath. When the temperature of the extract reached room temperature, the extract was centrifuged for 20 min at 80,000 x g to remove the denatured protein.

Ammonium sulfate fractionation

To the clarified solution from the previous step ammonium sulfate was added to a final concentration of 40% saturation. The solution was allowed to stir for 30 min at 4°C in the cold room. The precipitate was removed by centrifugation at 80,000 x g for 20 min. The supernatant had a yellow color. Ammonium sulfate was added to the supernatant to a final concentration of 60% saturation. The suspension was allowed to stir for 30 min at 4°C, the pH being maintained between 6.2 and 6.5. The precipitate was recovered by centrifugation and dissolved in 0.05 M potassium phosphate buffer of pH 6.2 containing 0.01 M dithiothreitol. The volume was one-fifth that of the original extract. The enzyme solution at this stage had an opalescent amber color. The enzyme solution was dialyzed at 4°C against

6 liters of 0.05 M potassium phosphate buffer of pH 6.2 containing 1.0 mM dithiothreitol for 24 hours. The dialysis buffer was changed every 6 hours. The precipitate formed during dialysis was removed by centrifugation and discarded.

First DEAE-cellulose column

The enzyme solution was placed on a DEAE-cellulose column (5 cm x 100 cm), previously equilibrated with 0.05 M potassium phosphate buffer of pH 6.2 containing 1.0 mM dithiothreitol. The protein was eluted with a linear potassium phosphate gradient of pH 6.2, containing 1.0 mM dithiothreitol, extending between 0.05 M and 1.0 M potassium phosphate in a total volume of 5 liters. The flow rate was adjusted to one drop every 2 seconds, and 10 ml fractions were collected. Fractions containing lactate dehydrogenase activity were pooled, and dialyzed against 6 liters of low molarity potassium phosphate buffer of pH 6.2 containing 1.0 mM dithiothreitol, for at least 12 hours to equilibrate the enzyme solution to 0.05 M potassium phosphate. The slight precipitate formed was removed by centrifugation and discarded.

Second DEAE-cellulose column

The clear colorless enzyme from the above step was placed on a second DEAE-cellulose column (2.5 cm x 50 cm), previously equilibrated as mentioned above. The protein was eluted using a linear gradient extending from 0.2 M to 1.0 M

potassium phosphate buffer of pH 6.2 containing 1.0 mM dithiothreitol in a total volume of 800 ml. The flow rate was adjusted to one drop every 4 seconds. Fractions of approximately 5 ml were collected. Those fraction containing lactate dehydrogenase activity were pooled and dialyzed against 6 liters of low molarity potassium phosphate buffer of pH 6.2 containing 1.0 mM dithiothreitol for 6 hours. Usually no precipitation occurred during this dialysis.

Sephadex G-200 column

The clear enzyme solution (100 to 150 ml) was placed in a celophane dialysis sack and concentrated against a fan at 4°C to a volume of 15 ml. The concentrated solution was then placed on a sephadex G-200 column (2.5 cm x 100 cm). The protein was eluted with 0.05 M potassium phosphate buffer of pH 6.2 containing 1.0 mM dithiothreitol. Three milliliter fractions were collected and assayed. Those fractions containing lactate dehydrogenase activity were pooled and stored at 4°C.

General observations

A summary of the purification of lactate dehydrogenase is presented in Table II . It represents an overall purification of greater than 1,000-fold with 36% recovery of the enzyme. The enzyme was stable to heating at 45°C for 20 min. In most cases no activity was lost. The precipitate was a translucent

Table II

Summary of purification procedure for NADH-specific lactate dehydrogenase.

Fraction	Volume	Units per ml	Total units	Protein (mg/ml)	Specific Activity	Purification	% Recovery
Crude	2440	13	31,500	15	0.87	1	100
Heat	2420	12.5	30,250	12	1.04	1.21	93
40-60% $(NH_3)_2SO_4$ fractionation	375	63	23,600	10	6.3	7.24	75
DEAE-cellulose	150	200	30,000	0.5	400	462	93
Air dialysis and Sephadex G-200	45	250	11,250	0.225	1111.0	1310.0	36

slimy pellicle, probably consisting of polysacharide and ribosomes. The enzyme began to precipitate between 35% to 40% ammonium sulfate saturation. In some purification procedures almost 50% of the enzyme precipitated before 40% saturation was reached. In most cases very little or no enzyme precipitated before 40% ammonium sulfate saturation was attained.

The enzyme binds very tightly to DEAE-cellulose. Volumes of enzyme solution as large as 500 ml were placed on the first DEAE-cellulose column (5 cm x 100 cm) and when eluted with a volume of 5 liters of buffer the enzyme was always eluted between 0.7 M and 0.8 M potassium phosphate buffer as a sharp peak resulting in considerable concentration (Fig. 19). Two or more contaminating protein peaks usually preceeded the enzyme peak. These faster moving peaks usually had a deep amber color and sometimes exhibited a degree of opalescence. The enzyme fractions were free of NADH oxidase activity. The second DEAE-cellulose column removed more contaminating protein and also concentrated the enzyme volume to one quarter of that placed on the column. Again the enzyme was eluted between 0.7 M and 0.8 M potassium phosphate. The enzyme was eluted from sephadex G-200 as a single peak (Fig. 20).

Figure 19. DEAE-cellulose chromatography of NADH-specific lactate dehydrogenase. Protein was eluted at pH 6.2 with a potassium phosphate gradient between 0.05 M and 1.0 M using a final volume of 5 liters of buffer containing 1.0 mM dithiothreitol.

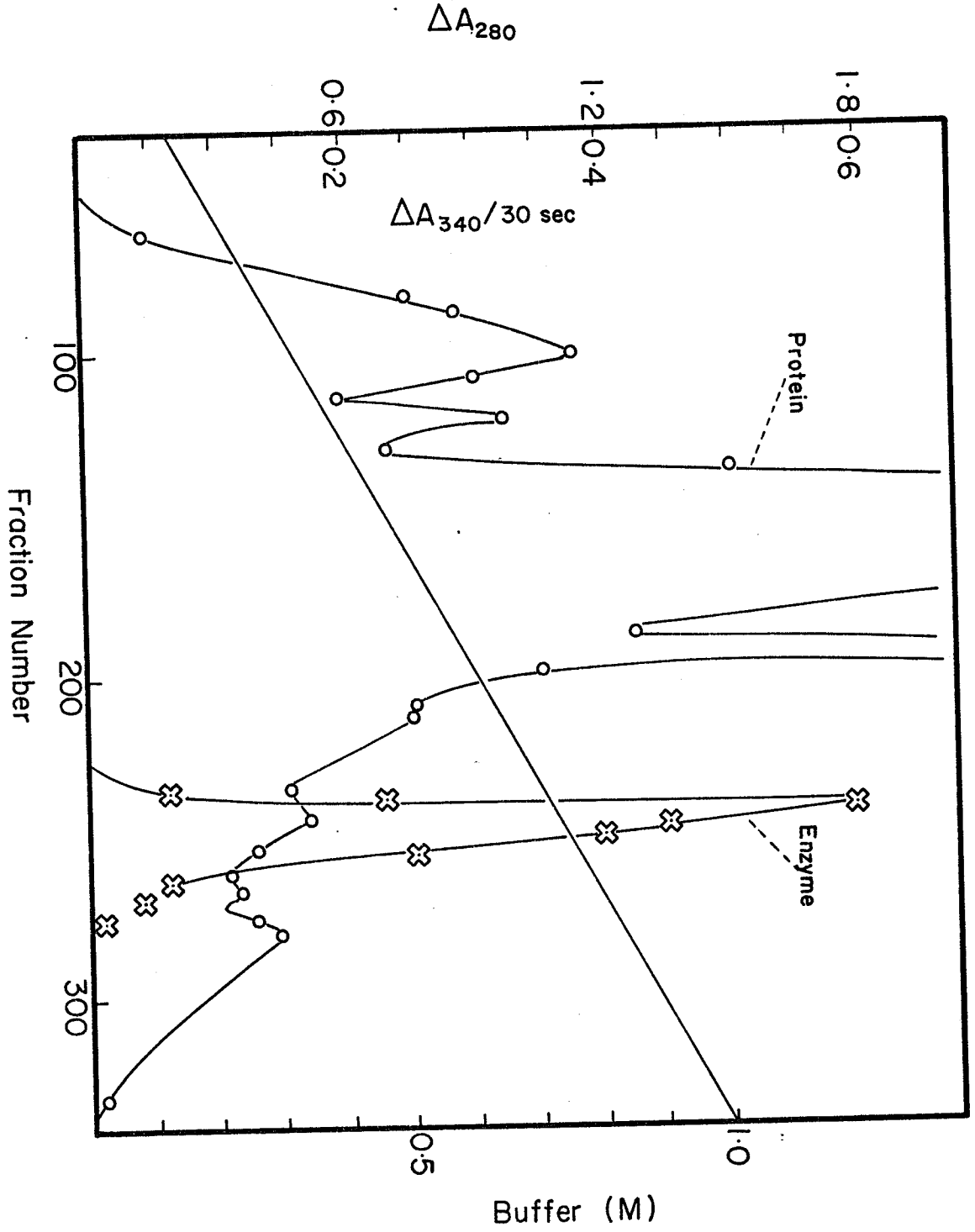
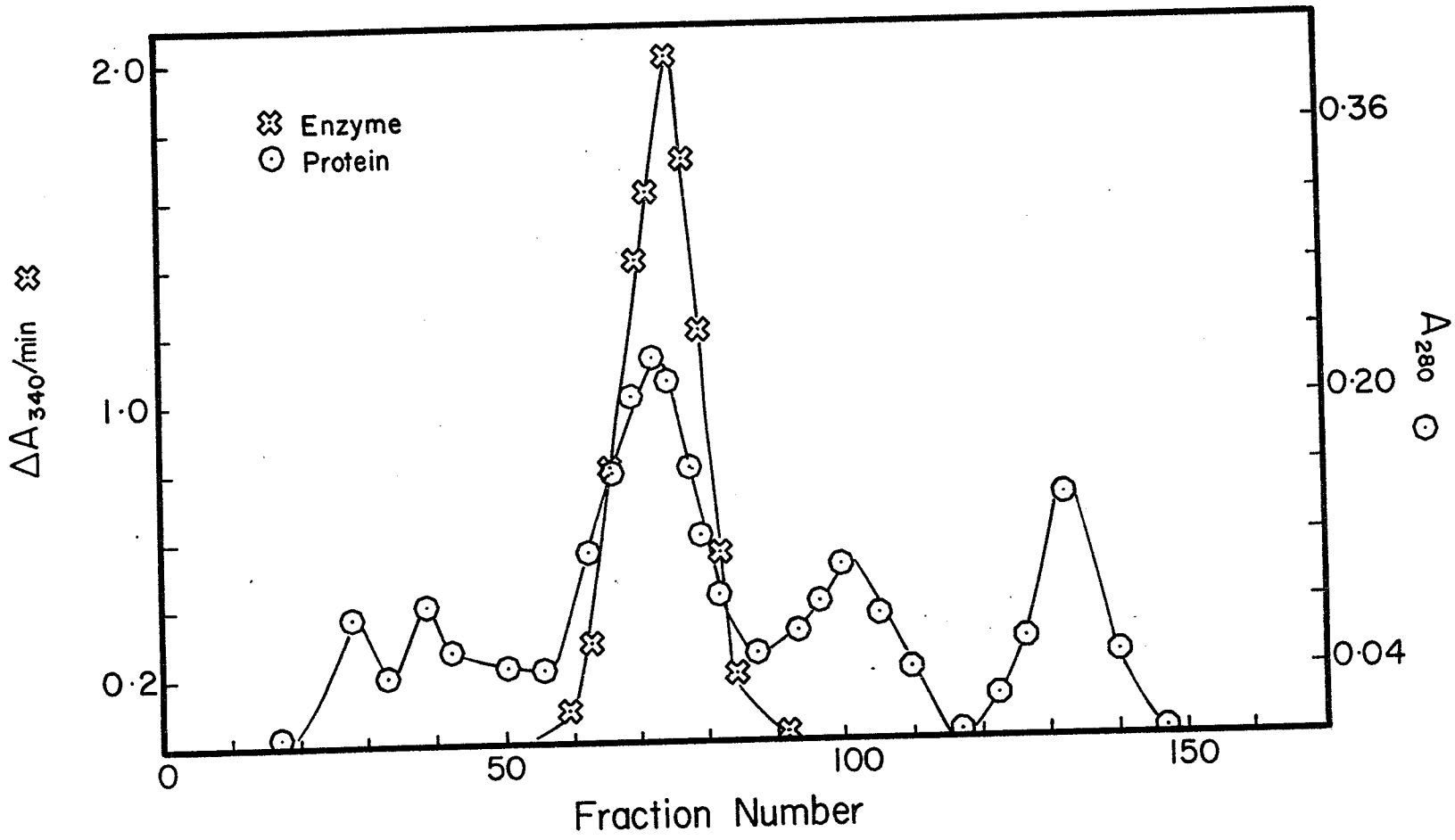

ΔA280
0·6
0·2
1·2
0·4
1·8
0·6
ΔA340/30 sec
Protein
Enzyme
100
200
300
Fraction Number
0·5
1·0
Buffer (M)

Figure 20. Sephadex G-200 chromatography of NADH-specific lactate dehydrogenase. Protein was eluted with 0.05 M potassium phosphate buffer of pH 6.2 containing 1.0 mM dithiothreitol.


Enzyme
Protein
2·0
1·0
0·2
0·36
0·20
0·04
0
50
100
150
Fraction Number
ΔA340/min
A280

CRITERIA OF PURITY

When the purified enzyme was placed on a small DEAE-cellulose column (1.0 cm x 30 cm) and a sephadex G-200 column (2.5 cm x 50 cm) only one symmetrical protein peak, the peak corresponding to enzyme activity was observed.

The enzyme preparation was also tested for purity by observing its sedimentation pattern in a Spinco Model E analytical centrifuge equipped with a Schlierin double sector optical system. At pH 8.0, a single protein peak was observed. However when the same enzyme preparation was analyzed for purity by polyacrylamide gel electrophoresis at pH 8.9 one major band accompanied by 3 to 4 minor light bands were observed. A possible reason for the presence of these minor bands in the gel will be discussed later.

KINETIC BEHAVIOUR OF LACTATE DEHYDROGENASE

Effect of pyruvate concentration on activity at various pH's

The effect of pyruvate concentration on the activity of lactate dehydrogenase is shown in Fig.21, 22. At pH 5.7 the enzyme displayed normal Michaelis-Menten type kinetics with respect to pyruvate concentration. The rate saturation curve was hyperbolic and the double reciprocal plot of enzyme activity and pyruvate concentration was linear.

At pH 6.5 and 7.0 the rate saturation curve deviated from normal kinetics, displaying a sigmoid curve. Double reciprocal plots were non-linear, the degree of non-linearity increased with increasing pH.

Figure 21. Effect of pH on the rate response of lactate dehydrogenase to increasing concentrations of pyruvate. Assays were carried out in the presence of 0.1 M potassium phosphate buffer, 0.15 mM NADH, and pyruvate as indicated, at the following pH:

A = pH 7.0

B = pH 6.5

C = pH 5.7

Reactions were initiated by the addition of enzyme.

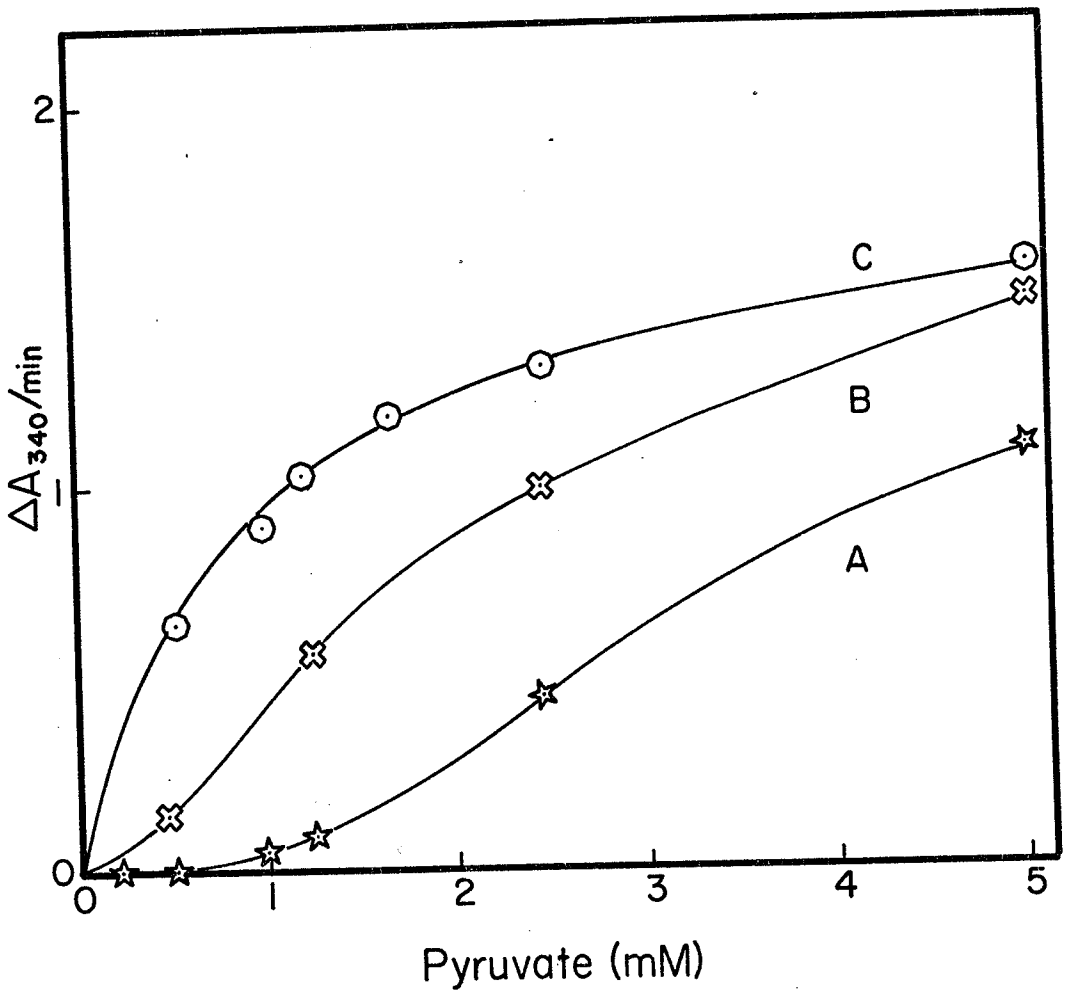

2
1
0
ΔA_{340}/min
C
B
A
0
1
2
3
4
5
Pyruvate (mM)

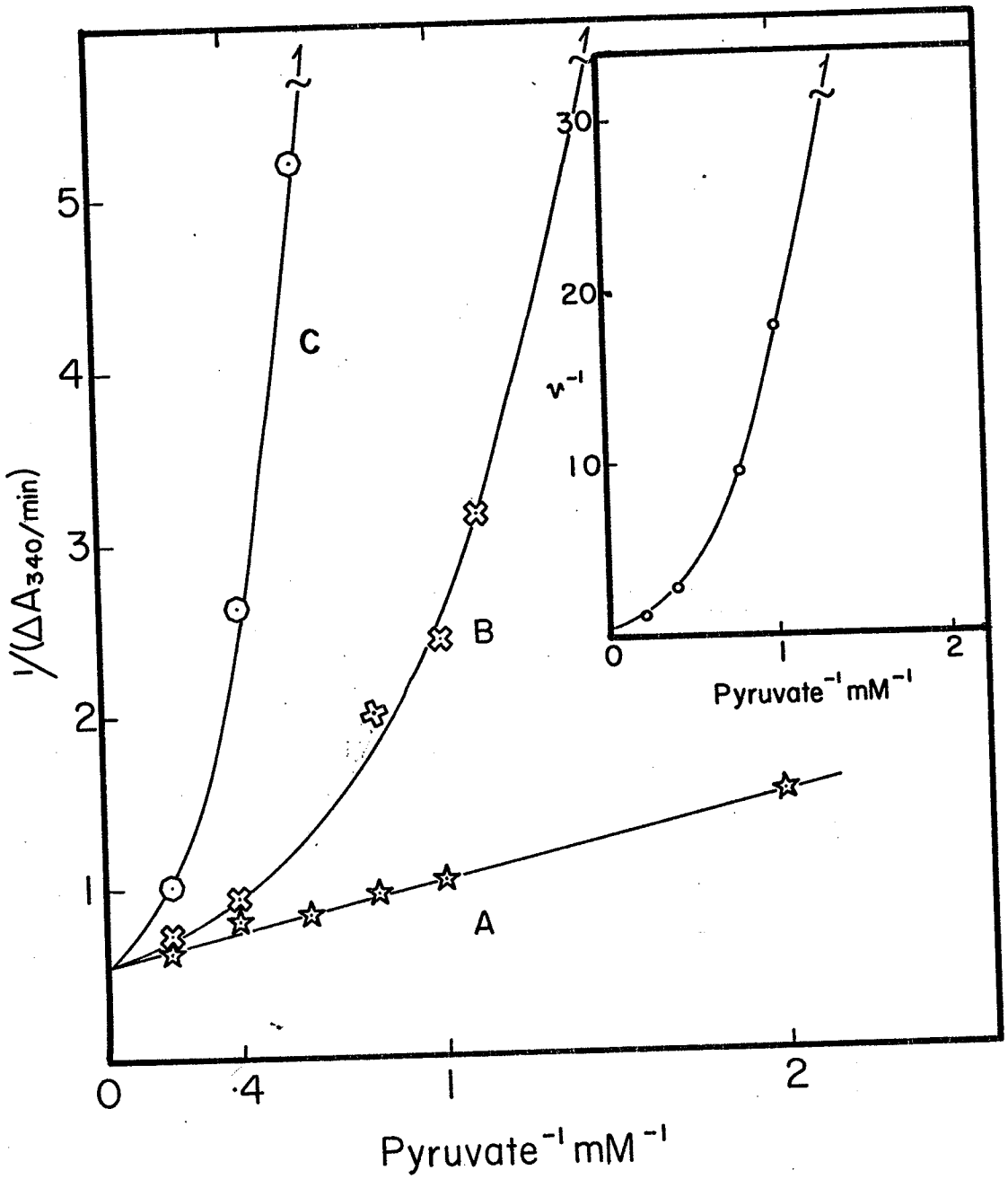
Figure 22. Double reciprocal plots of velocity against pyruvate concentration at various pH. Assays were carried out in the presence of 0.1 M phosphate, 0.15 mM NADH, and pyruvate as indicated, at the following pH:

A = pH 5.7

B = pH 6.5

C = pH 7.0

The insert shows a more detailed plot of the curve at pH 7.0. Reactions were initiated by the addition of enzyme.

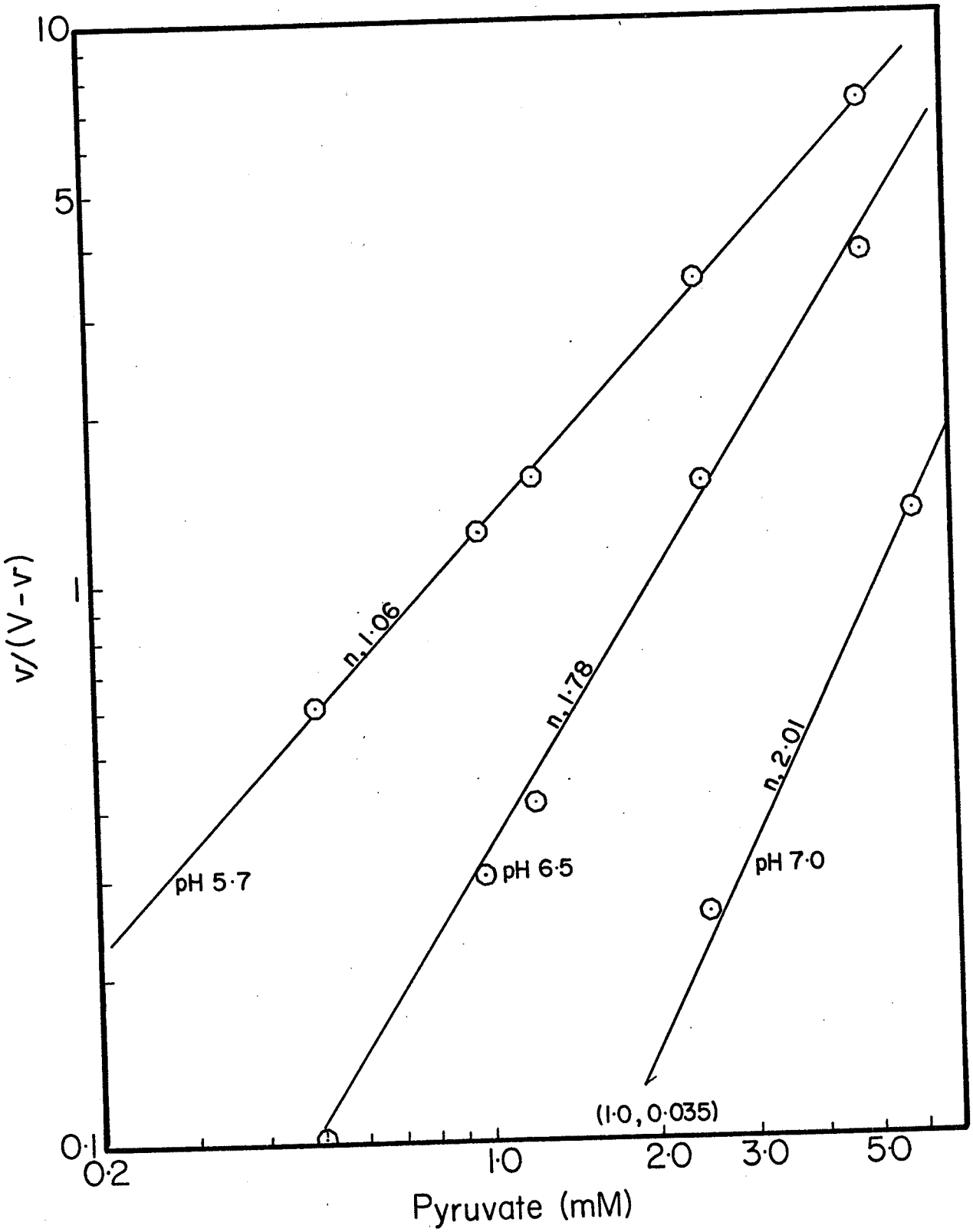

1/(ΔA340/min)
Pyruvate⁻¹ mM⁻¹
C
B
A
v⁻¹
Pyruvate⁻¹ mM⁻¹

A sigmoid curve of rate as a function of substrate concentration indicates that the enzyme acts in some manner with more than one molecule of substrate. Hill plots of the data from Fig. 21 show an increase in the slope value "n" (Fig. 23) with increasing pH. At pH 5.7 where normal kinetics was observed an "n" value of 1.06 was calculated. At pH 6.5 and pH 7.0 where kinetics are non-linear in the double reciprocal form "n" values of 1.78 and 2.01 respectively were observed. An "n" value of approximately 2 would indicate that the enzyme possesses at least two binding sites for pyruvate. This data does not indicate the nature of these sites, whether they are catalytic or allosteric. Although the affinity of the enzyme for pyruvate decreases with increasing pH, the maximum velocity is not affected by pH changes. In the course of this investigation no initial lag in the enzyme reaction was observed at pH 5.7 or pH 7.0. Even at pH 7.5 under assay conditions employed for the E. coli enzyme for which a lag has been reported (Tarmy and Kaplan, 1968), no lag could be demonstrated for the Aerobacter aerogenes enzyme. However at pH 8.0 an initial lag was observed, which could be eliminated by prior incubation in 5.0 mM pyruvate.

Effect of acetate and phosphate

Acetolactate synthetase has been shown to be inhibited by 0.1 M potassium phosphate as compared to its activity in

Figure 23. Plots of v/(V-v) against pyruvate concentration from the data in Fig. 20 expressed logrithmically.


10
5
1
0·1
v/(V-v)
0·2
1·0
2·0
3·0
5·0
Pyruvate (mM)
n, 1·06
n, 1·78
n, 2·01
pH 5·7
pH 6·5
pH 7·0
(1·0, 0·035)

distilled water. In addition acetolactate synthetase displayed a sigmoid rate saturation curve when assayed in phosphate, but the sigmoid curve was transformed to a rectangular hyperbola in water or in the presence of 0.1 M acetate. Since both acetolactate synthetase and lactate dehydrogenase occupy key positions in the metabolism of pyruvate, both metabolizing the same substrate, pyruvate, it was of interest to see if acetate or water alone could transform the sigmoid rate saturation curve observed for lactate dehydrogenase at pH 7.0 to a rectangular hyperbola. The results of this study are shown in Fig. 24 in the double reciprocal form. As shown in the graph, at pH 7.0 acetate activated lactate dehydrogenase relative to its activity in water, or phosphate buffer. Although the double reciprocal plots of enzyme activity and pyruvate concentration displayed a lesser degree of curvature in acetate, the plots being almost linear between 5.0 mM and 1.25 mM pyruvate and curving at lower pyruvate concentrations, acetate did not succeed in converting the double reciprocal plots to a straight line. It can be seen that lactate dehydrogenase is more active in 0.05 M acetate than in 0.1 M acetate.

An activity profile in the presence of varying acetate concentration is shown in Fig. 25 where it can be seen that acetate activated enzyme activity over that observed in water alone to a maximum at 0.05 M acetate, after which activity decreased, until at 1.0 M acetate no enzyme activity could be detected.

Figure 24. The effect of acetate and phosphate concentration on activity of lactate dehydrogenase at pH 7.0 shown in the double reciprocal form. Assays were carried out in the presence of 0.15 mM NADH, pyruvate as indicated, and the following buffers:

A = 0.05 M acetate

B = 0.1 M acetate

C = 0.05 M phosphate

D = 0.1 M phosphate

E = assayed in distilled water

Reactions were carried out by the addition of enzyme.

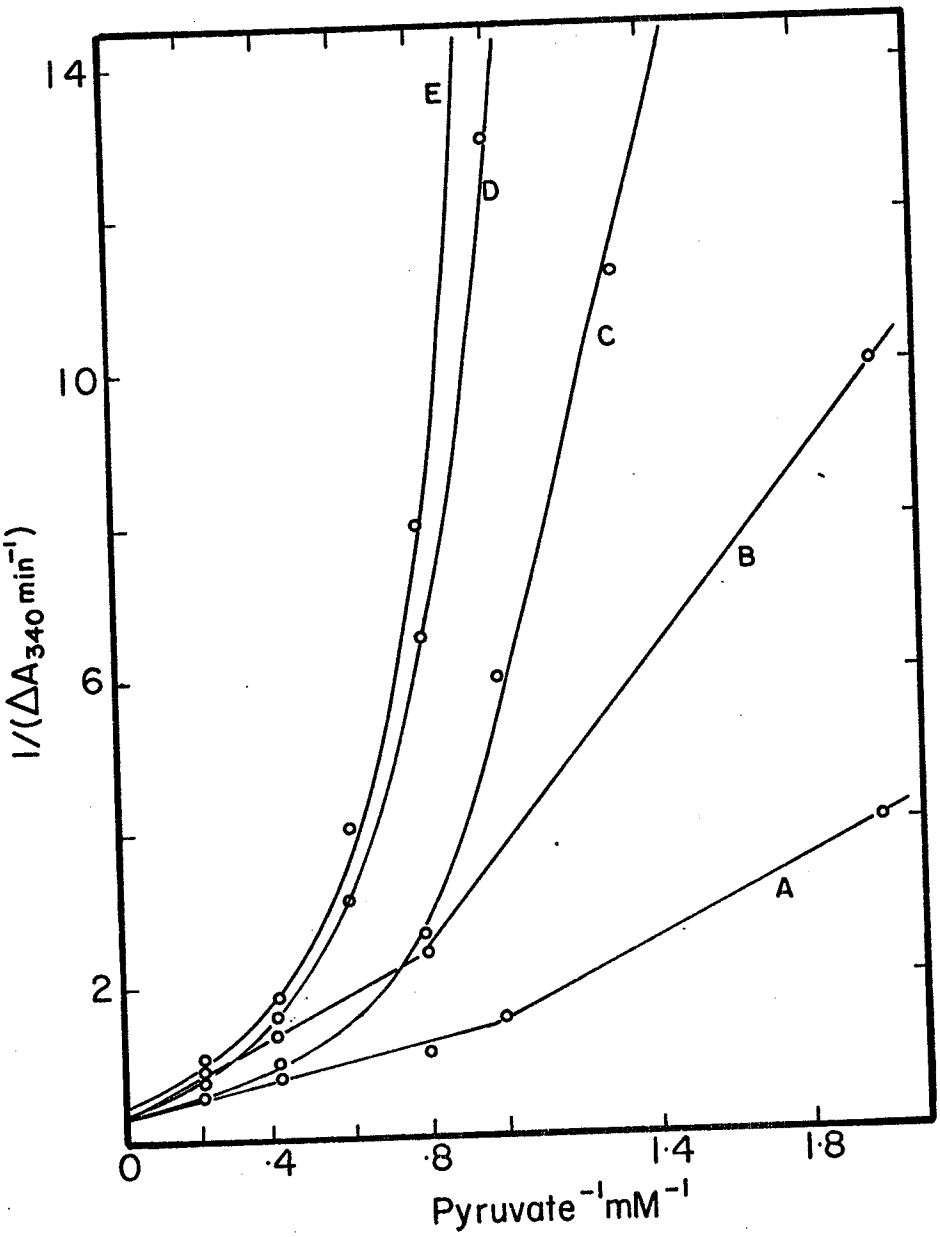
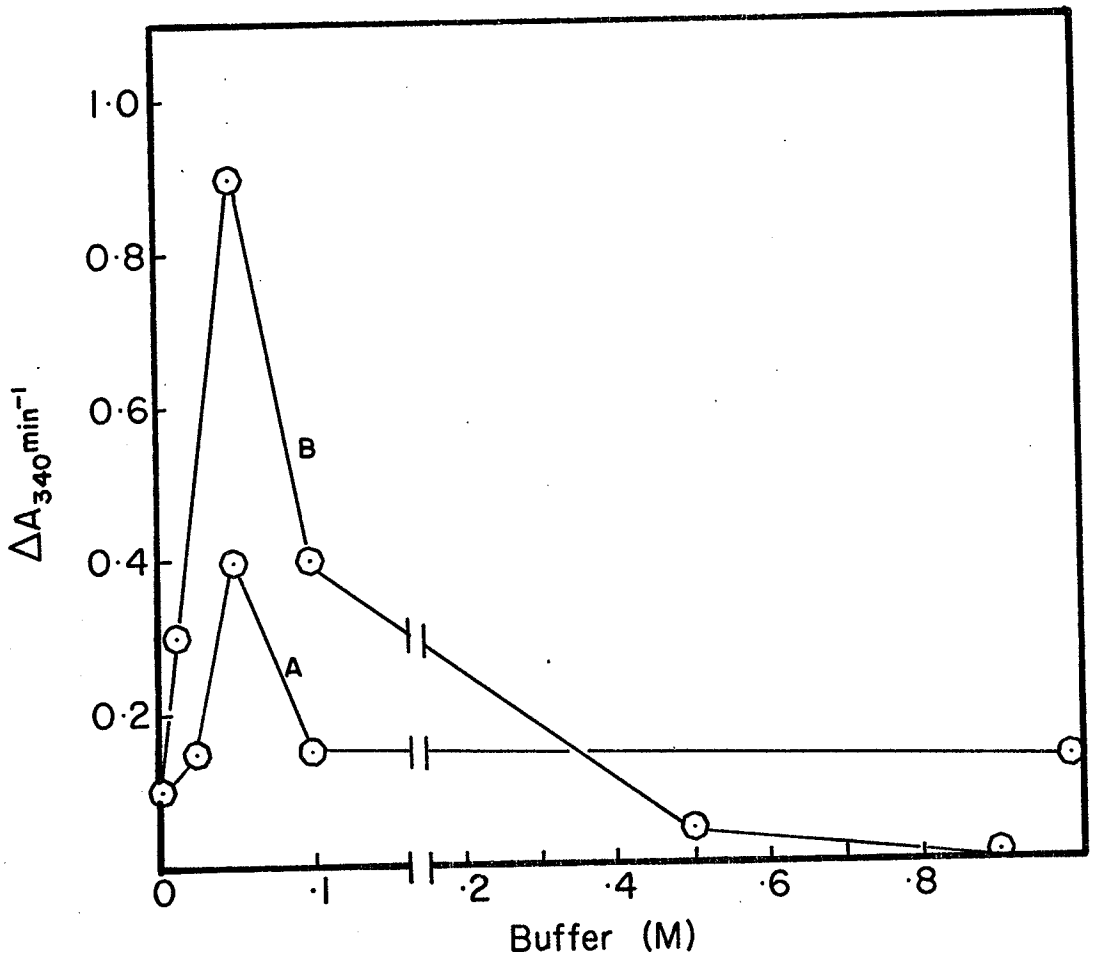

14
10
6
2
0
·4
·8
1·4
1·8
E
D
C
B
A
1/(ΔA340 min⁻¹)
Pyruvate⁻¹mM⁻¹

Figure 25. Enzyme activity profile at pH 7.0 in the presence of (A) phosphate and (B) acetate buffer. Assays were carried out in the presence of 0.15 mM NADH and 2.5 mM pyruvate. Reactions were initiated by the addition of enzyme.


1·0
0·8
0·6
0·4
0·2
$\Delta A_{340}min^{-1}$
B
A
0
·1
·2
·4
·6
·8
Buffer (M)

Phosphate also showed a similar ionic effect (Fig. 24 and 25), stimulating enzyme activity to a maximum at 0.05 M phosphate and decreasing in stimulation until a phosphate concentration of 0.1 M was attained. Between 0.1 M and 1.0 M phosphate, the enzyme maintained the same activity. At all concentrations of phosphate employed double reciprocal plots were distinctly non-linear.

At pH 5.7 the effect of acetate and phosphate was not as pronounced as it was at pH 7.0. No difference in activity was observed when the enzyme was assayed in acetate or phosphate buffer at 0.025 M, 0,05 M, and 0.1 M, over a range of 0.5 mM to 5.0 mM pyruvate. Double reciprocal plots yielded the same straight line for both buffers at the molarities mentioned. At 1.0 M of buffer activity in phosphate was approximately 75% that observed in 0.1 M phosphate, and activity in acetate was 25% that observed in 0.1 M acetate. It appears that the effect of these anions at Ph 5.7 was not as drastic and pronounced as at pH 7.0. At pH 7.0 where a cooperative pyruvate effect is observed, the enzyme is much more sensitive to its ionic environment.

Substrate Inhibition

Substrate inhibition was displayed by the enzyme for both pyruvate and NADH at pH 5.7 and pH 7.0.

The degree of inhibition by pyruvate varied with pH

as shown in Fig. 26 . At pH 5.7 under normal assay conditions the maximum velocity was reached at approximately 10.0 mM pyruvate, with 50% inhibition at 50 mM pyruvate. Inhibition by pyruvate was not as marked at pH 7.0 where the maximum velocity reached was at 35 mM pyruvate and approximately 50% inhibition was observed at 100 mM pyruvate.

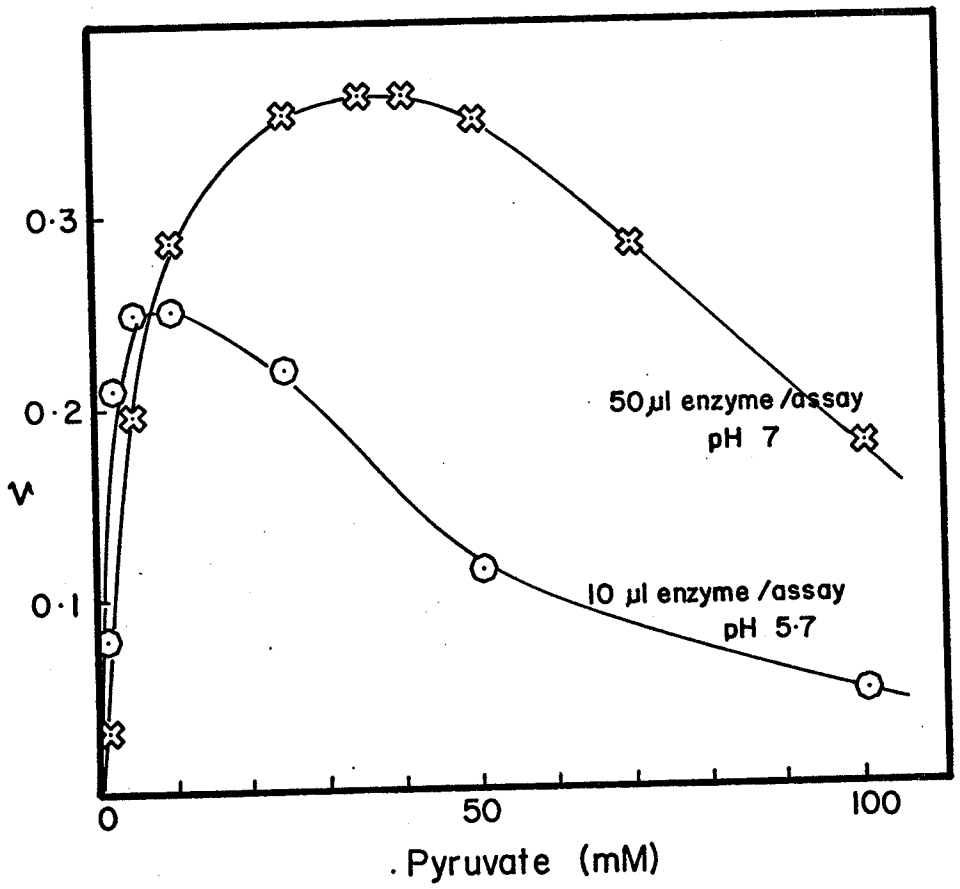
Wuntch et al (1969) have suggested that an abortive ternary complex of enzyme (lactate dehydrogenase), pyruvate, and NAD^+ may be responsible for the pyruvate inhibition of dilute concentrations of rabbit lactate dehydrogenase isozymes 1 and 5. In studies with lactate dehydrogenase isozyme 1, Gutfreund et al (1968) observed that the rate of abortive complex formation was inversly related to pyruvate concentration but independent of NAD^+ concentrations above 0.2 mM.

Inhibition of E. coli lactate dehydrogenase by a binary complex of pyruvate and NAD^+ has been reported by Tarmy and Kaplan (1968). This binary complex is presumed to mimic the observed substrate inhibition. The Aerobacter aerogenes enzyme was not inhibited when preincubated in the presence of 5.0 mM pyruvate and 0.4 mM NAD^+ for up to a period of 15 min in contrast to the E. coli enzyme (Tarmy and Kaplan, 1968). Either inactive complex formation does not take place, or once formed has no effect on enzyme activity.

Rate concentration plots of the Aerobacter aerogenes enzyme for NADH are rectangular hyperbolas (Hollier, 1967) except for the substrate inhibition at high NADH concentrations.

Figure 26. Effect of pyruvate concentration on activity of lactate dehydrogenase. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 0.15 mM NADH at the pH indicated. Reactions were initiated by the addition of enzyme.

$v = \Delta A340m\mu/min.$


0·3
0·2
0·1
v
50 µl enzyme /assay
pH 7
10 µl enzyme /assay
pH 5·7
0
50
100
. Pyruvate (mM)

As shown in Fig. 27 NADH inhibited enzyme activity at concentrations greater than 0.3 mM at pH 5.7. At NADH concentrations greater than 0.3 mM, an initial lag in the enzyme reaction was observed. The length of the lag increased with NADH concentration. In addition preincubation with NADH for several minutes increased the length of the lag and the degree of inhibition.

Effect of α-ketobutyrate

It has been indicated that the Aerobacter aerogenes lactate dehydrogenase possesses at least two binding sites for pyruvate and that the binding of substrate at one site facilitiates the binding of the substrate at the other site. It was of interest to determine what effect α-ketobutyrate, an analogue of pyruvate but a poor substrate for the enzyme (the rate of reduction being 1.0% or less than the reduction of pyruvate) would have on the interaction between these substrate binding sites. As shown in Fig. 27, at pH 6.5, 20 mM α-ketobutyrate actually stimulated enzyme activity at a limiting concentration of pyruvate, although it acts as an inhibitor of the reaction at saturating concentrations of the substrate. This kinetic response of the Aerobacter aerogenes enzyme is analogous to the response observed with aspartate transcarbamylase in the presence of the aspartate analogue maleate (Gerhart and Pardee, 1963, 1964). The addition of

Figure 27. Effect of NADH concentration on activity of lactate dehydrogenase at pH 5.7. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 5.0 mM NADH and the NADH concentrations as indicated. Reactions were initiated by the addition of enzyme.

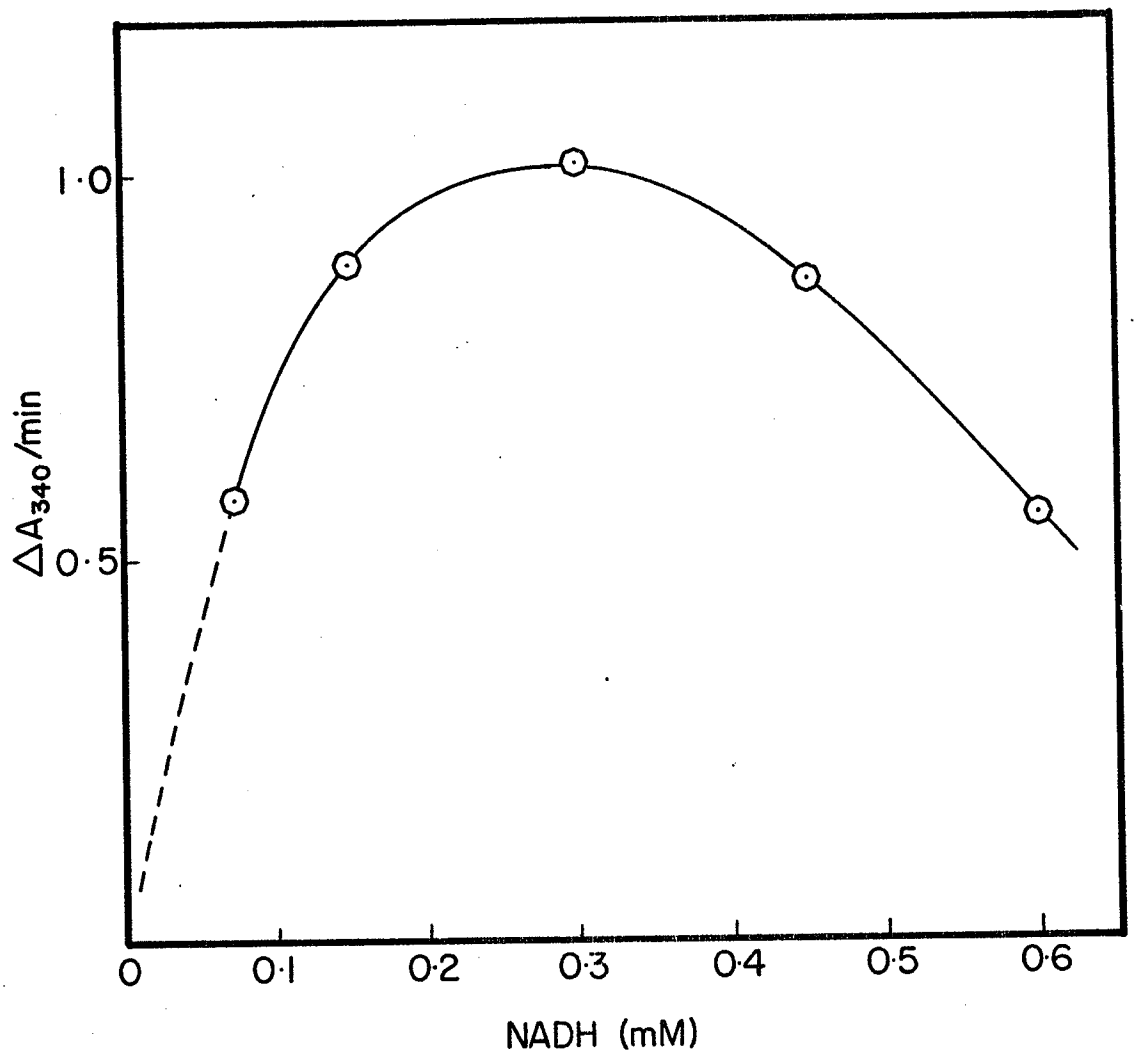
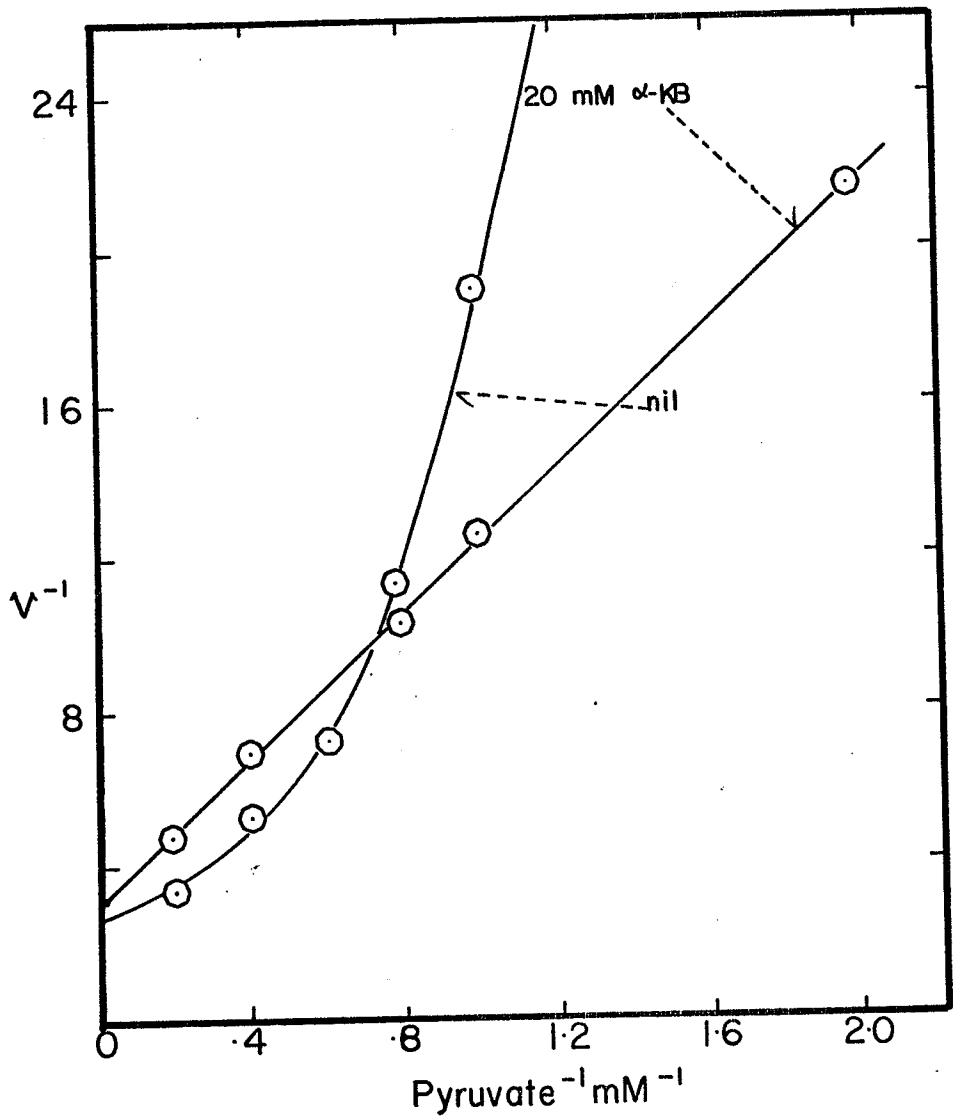

1·0
0·5
ΔA340/min
0
0·1
0·2
0·3
0·4
0·5
0·6
NADH (mM)

Figure 28. Double reciprocal plots of velocity against pyruvate concentration at pH 6.5 in the absence and presence of 20 mM α-ketobutyrate. Assays were carried out in the 0.1 M potassium phosphate buffer in the presence of 0.15 mM NADH, varying concentrations of pyruvate, and 20 mM α-ketobutyrate where indicated. Reactions were initiated by the addition of enzyme.

$v = \Delta A340\ m\mu/min.$


24
16
8
V⁻¹
20 mM α-KB
nil
0
·4
·8
1·2
1·6
2·0
Pyruvate⁻¹ mM⁻¹

α-ketobutyrate to the NADH pyruvate assay system at pH 6.5 resulted in a transposition of the curve of the rate with respect to substrate concentration, from a sigmoid to a rectangular hyperbola (Fig. 29). When the data was replotted in the form log v = nlog S - log K, it was observed that α-ketobutyrate changed the apparent kinetic order of the reaction with respect to pyruvate from second to first (Fig. 30). Slopes of Hill plots changed from 1.9 in the absence of α-ketobutyrate to approximately 1.0 in the presence of α-ketobutyrate as shown in Fig. 30 .

It appeared that α-ketobutyrate not only increased the affinity of the enzyme for pyruvate at low concentrations of the substrate but also altered the number of available pyruvate binding sites, or the interaction strength between these sites, or both.

At pH 5.7 α-ketobutyrate inhibited the enzyme at both low and saturating concentrations of pyruvate (Fig. 31), the inhibition being non-competitive with respect to pyruvate.

Effect of α-ketoglutarate

At pH 6.5 and pH 7.0 the activating effect of α-ketobutyrate could not be duplicated by α-ketoglutarate. At pH 6.5 and pH 7.0 inhibition was observed resulting in a greater degree of nonlinearity in double reciprocal plots of substrate concentration against enzyme activity, but a transition from second order to first order kinetics was not observed.

Figure 29. Effect of α-ketobutyrate on the pyruvate-saturation curve at pH 6.5. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 0.15 mM NADH, pyruvate as indicated, and 20 mM α-ketobutyrate were indicated. Reactions were initiated by the addition of enzyme.

$v = \Delta A340$ mμ/min.

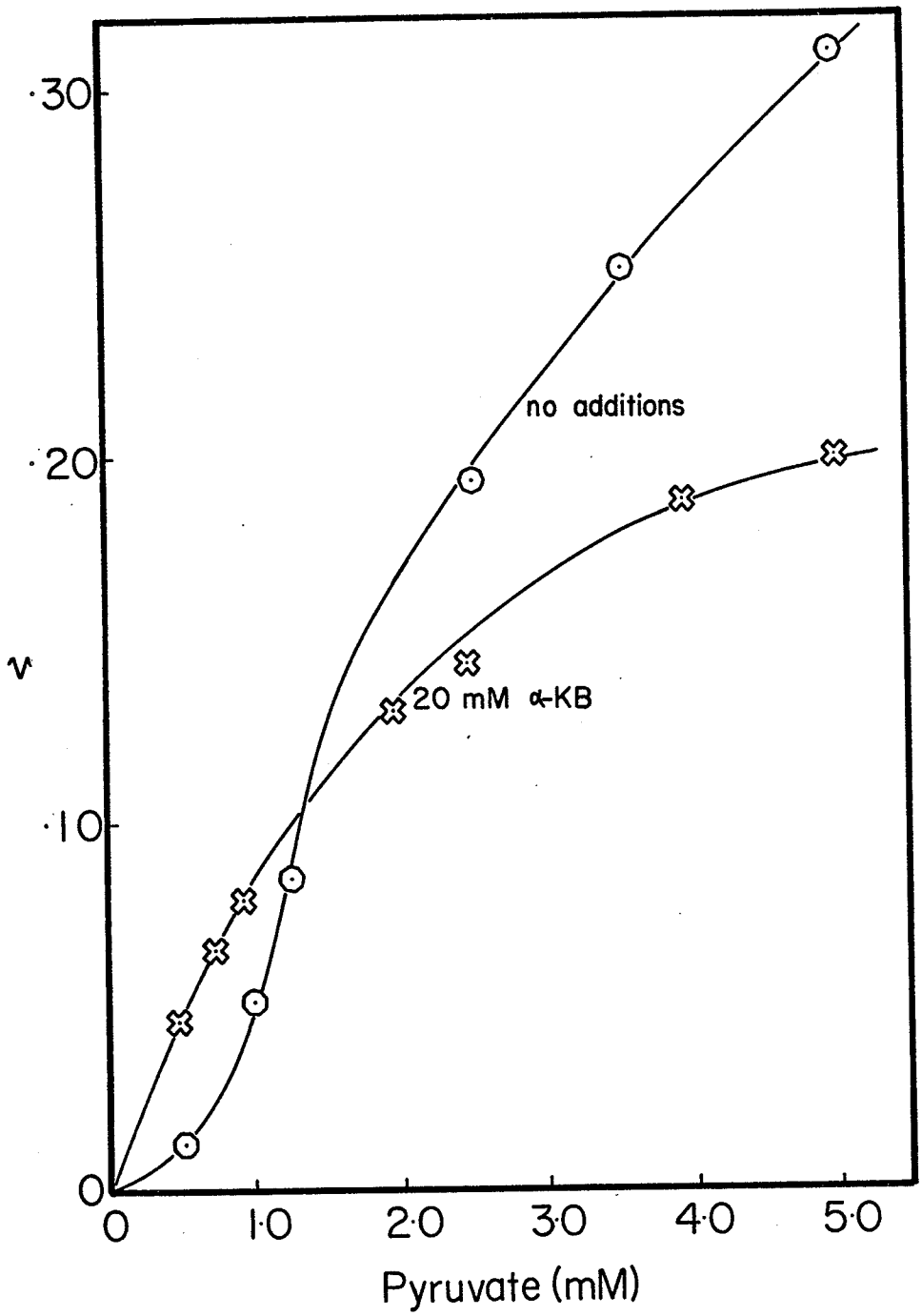

·30
·20
·10
0
v
no additions
20 mM α-KB
0
1·0
2·0
3·0
4·0
5·0
Pyruvate (mM)

Figure 30. Plots of v/(V-v) against pyruvate concentration of the data from Fig. 28 expressed logrithmically.

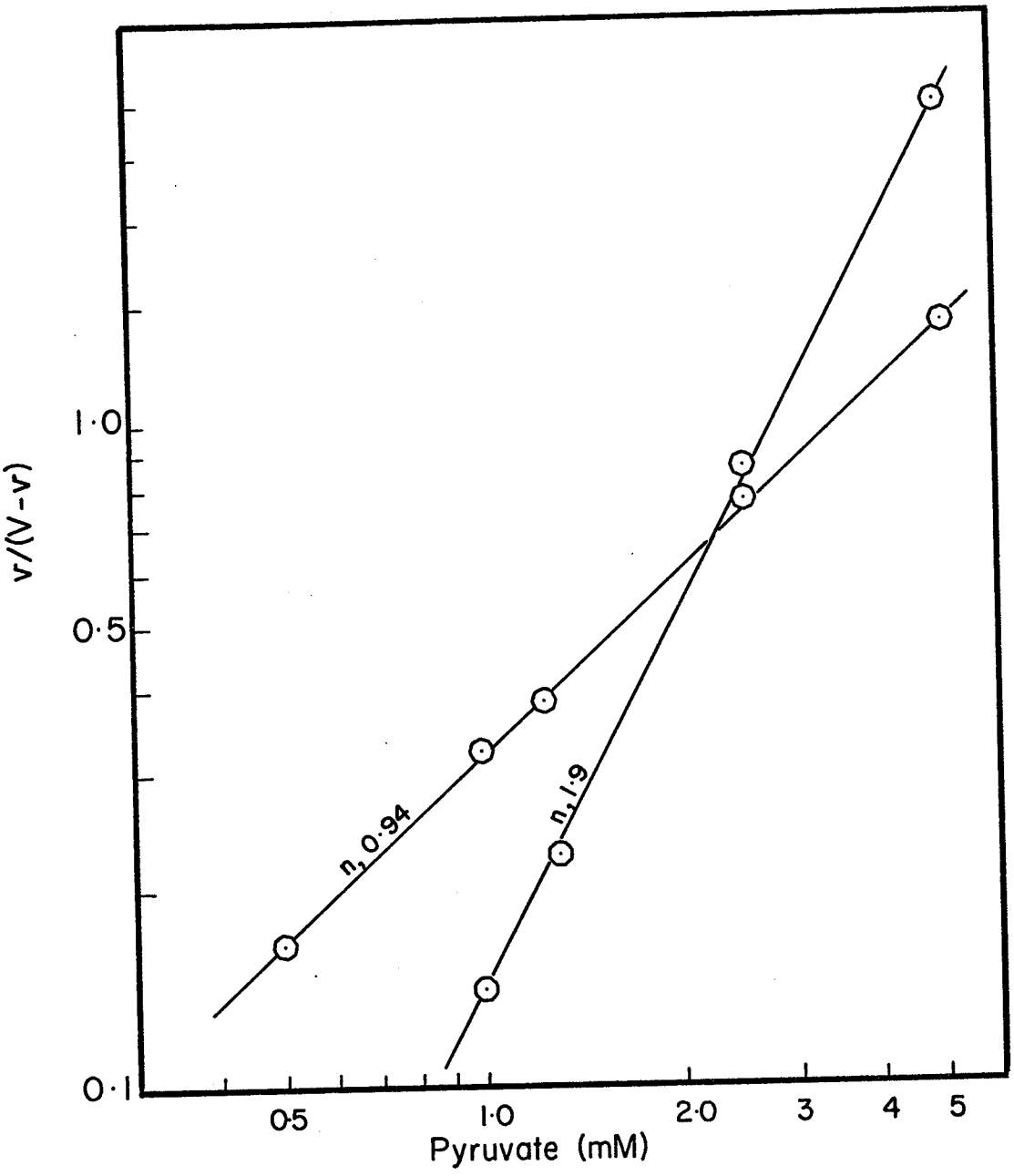
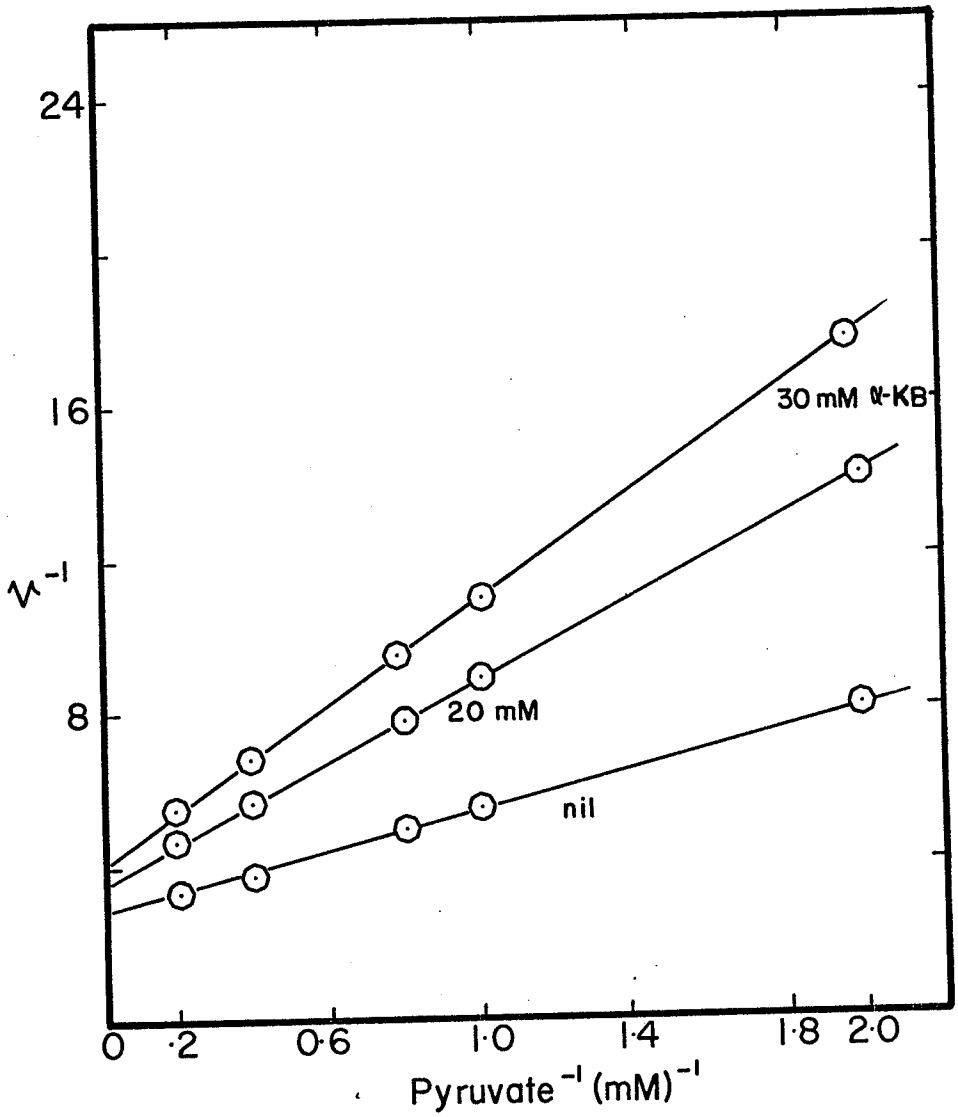

v/(V-v)
1·0
0·5
0·1
n, 0·94
n, 1·9
0·5
1·0
2·0
3
4
5
Pyruvate (mM)

Figure 31. Double reciprocal plots of velocity against pyruvate concentration at different concentrations of α-ketobutyrate at pH 5.7. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 0.15 mM NADH, pyruvate as indicated, and α-ketobutyrate in the concentrations shown. Reactions were initiated by the addition of enzyme.

$v = \Delta A340$ mμ/min.


24
16
8
v^{-1}
30 mM α-KB
20 mM
nil
0
·2
0·6
1·0
1·4
1·8
2·0
Pyruvate^{-1} (mM)$^{-1}$

At pH 5.7 α-ketoglutarate inhibited enzyme activity competitively with respect to pyruvate as shown in Fig. 32. The K_i from Fig. 32 was approximately 10 mM.

Effect of oxamate

Oxamate, another analogue of pyruvate, did not replace α-ketobutyrate in the activation process. As in the case of animal (Novoa et al, 1959) and E. coli lactate dehydrogenase (Tarmy and Kaplan, 1968), oxamate was found to be an inhibitor of the Aerobacter aerogenes enzyme. Inhibition was non-competitive with respect to pyruvate (Fig. 33). The assays were carried out in 0.1 M potassium phosphate containing 0.30 mM NADH. The Ki_I and Ki_S for oxamate were determined to be approximately 28 mM and 17 mM respectively, as compared to a Ki of 18 mM for the E. coli enzyme (Tarmy and Kaplan 1968).

Effect of ATP

At pH 5.7 when the pyruvate concentration was varied between 0.5 mM and 5.0 mM, ATP inhibited enzyme activity non-competitively with respect to pyruvate when the enzyme was assayed in 0.1 M potassium phosphate buffer in the presence of 0.15 mM NADH as shown in Fig. 34. The double reciprocal plots were linear at all levels of ATP. Inhibition by ATP was strong, the enzyme being inhibited 50% by 1.25 mM ATP, and 96% by 5.0 mM ATP at 5.0 mM pyruvate. The E. coli enzyme was inhibited less strongly by ATP (Tarmy and Kaplan, 1968).

Figure 32. Double reciprocal plots of velocity against pyruvate concentration at different concentrations of α-ketoglutarate at pH 5.7. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 0.15 mM NADH, varying concentrations of pyruvate, and α-ketoglutarate in the concentrations indicated. Reactions were initiated by the addition of enzyme.

$v = \Delta A340$ mμ/min

The insert shows a replot of slopes against α-ketoglutarate concentration.

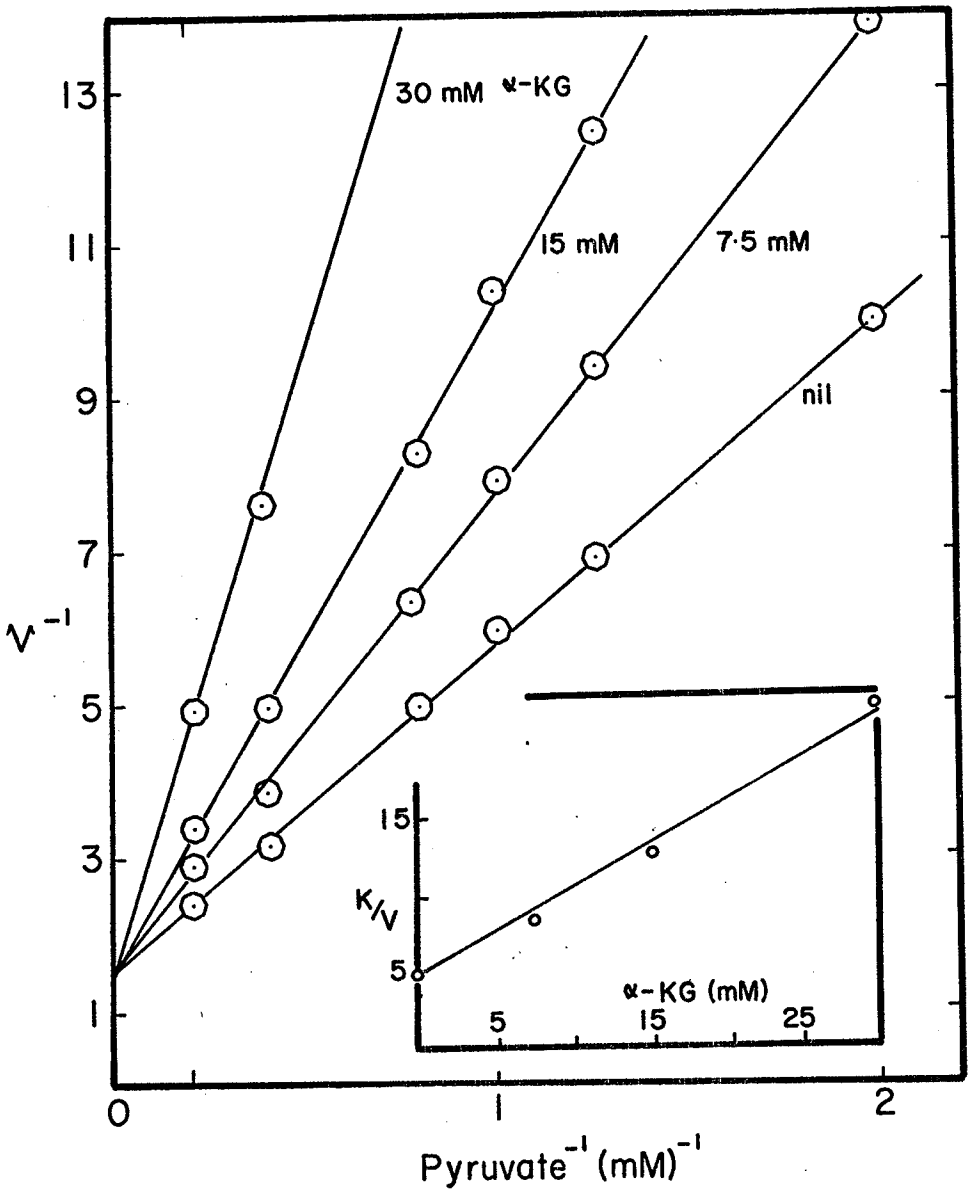

30 mM α-KG
15 mM
7·5 mM
nil
13
11
9
7
5
3
1
0
1
2
V⁻¹
Pyruvate⁻¹ (mM)⁻¹
15
K/V
5
α-KG (mM)
5
15
25

Figure 33. Double reciprocal plots of velocity against pyruvate concentration at different concentrations of oxamate. Assays were carried out in 0.1 M potassium phosphate buffer, in the presence of 0.3 mM NADH, varying concentrations of pyruvate, and oxamate in the following concentrations:

A = nil

B = 6 mM

C = 15 mM

D = 30 mM

E = 60 mM

Reactions were initiated by the addition of enzyme.

$v = \Delta A340$ mμ/min.

The insert shows replots of slopes and intercepts against oxamate concentration.

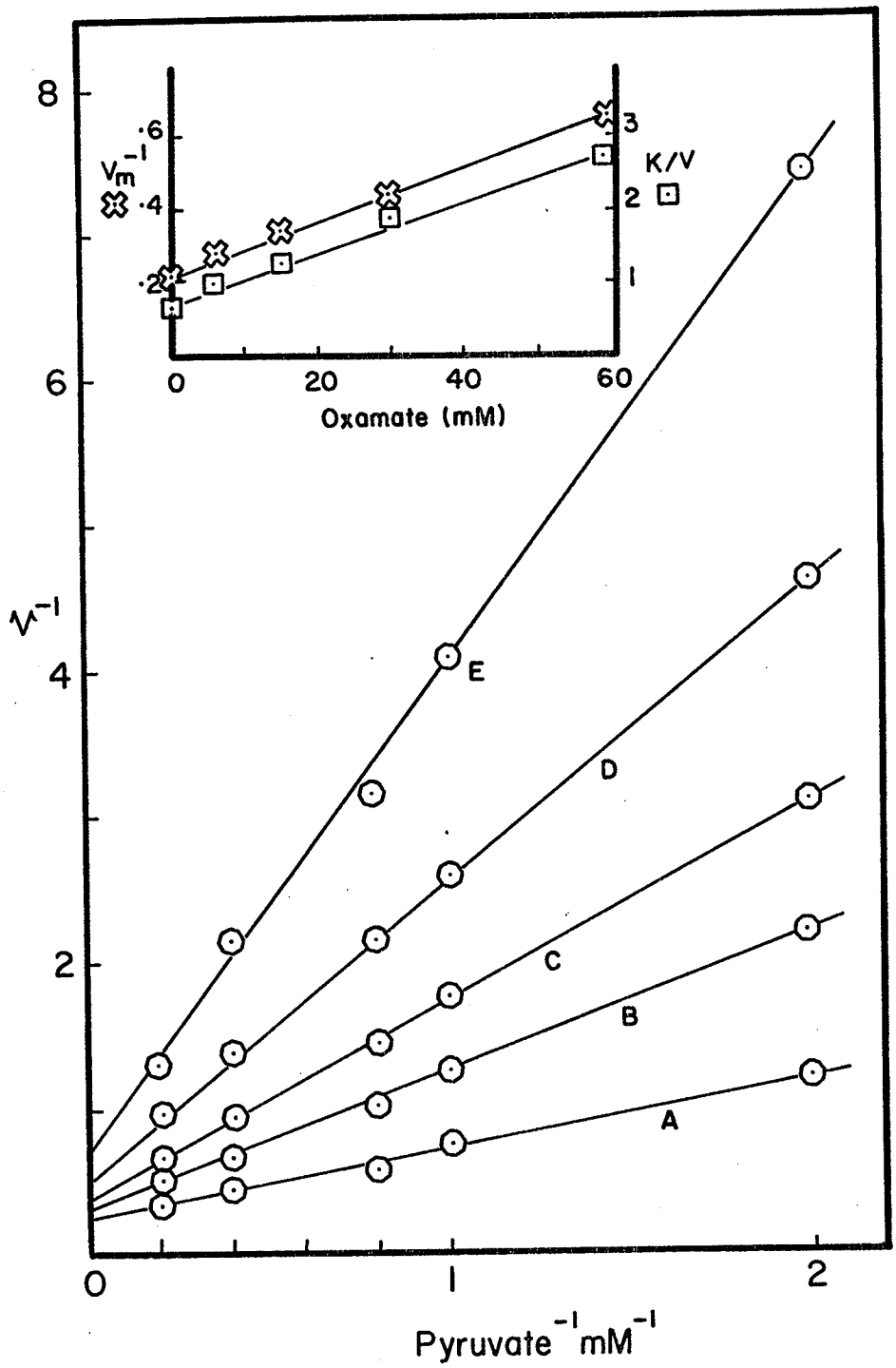

8
6
4
2
0
1
2
V^{-1}
Pyruvate^{-1} mM^{-1}
E
D
C
B
A
V_m^{-1}
·6
·4
·2
3
2
1
K/V
0
20
40
60
Oxamate (mM)

Figure 34. Double reciprocal plots of velocity against pyruvate concentration at pH 5.7 at different concentrations of ATP. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 0.15 mM NADH, varying concentrations of pyruvate as indicated, and ATP in the following concentrations:

A = nil

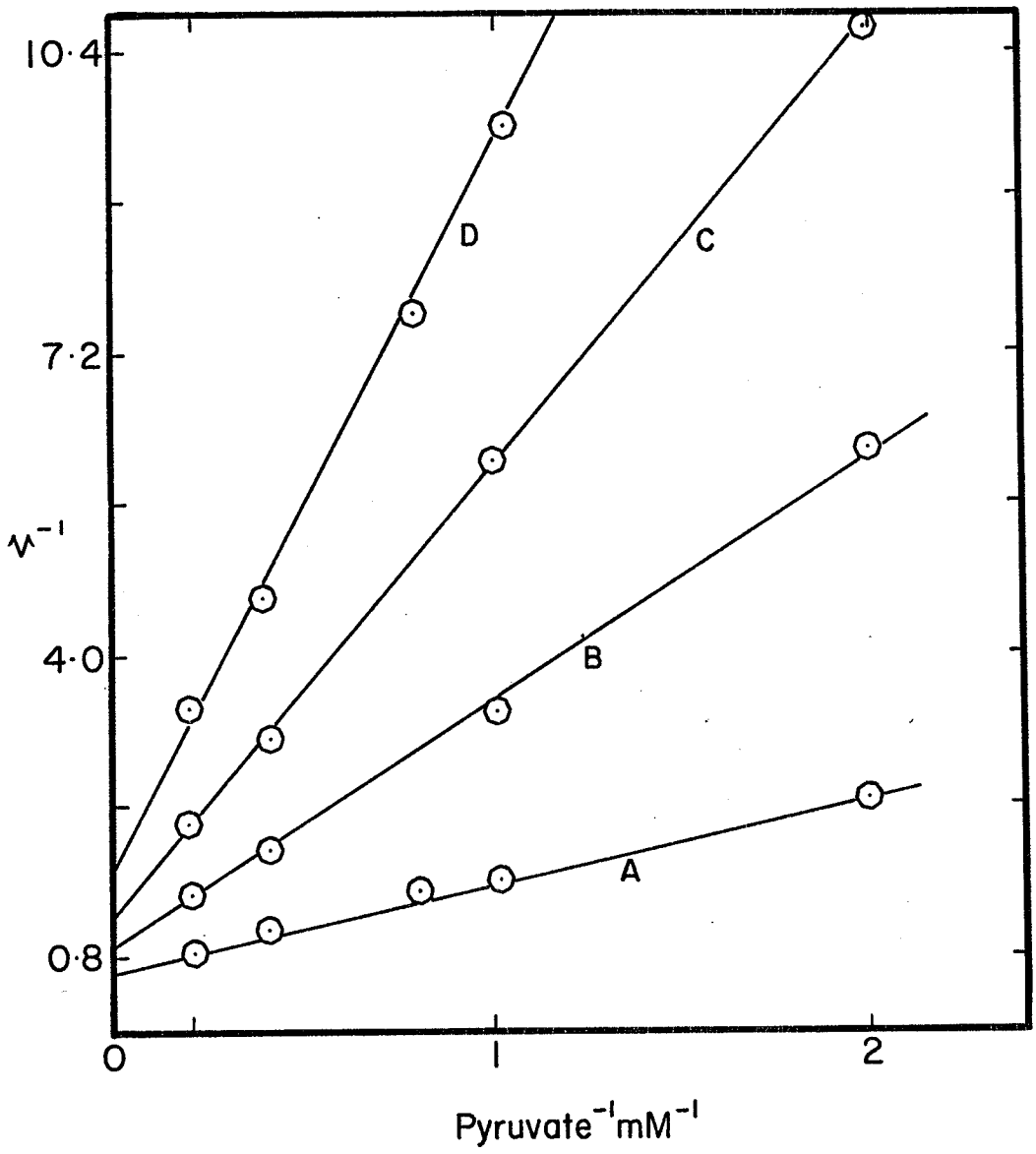
B = 0.75 mM

C = 1.25 mM

D = 2.5 mM

Reactions were assayed by the addition of enzyme.

$v = \Delta A340\ m\mu/min.$


10·4
7·2
4·0
0·8
v⁻¹
0
1
2
Pyruvate⁻¹mM⁻¹
A
B
C
D

A replot of intercepts and slopes against ATP concentration yielded straight lines (Fig. 35) with a Ki_I of 1.5 mM and a Ki_S of 0.3 mM for ATP.

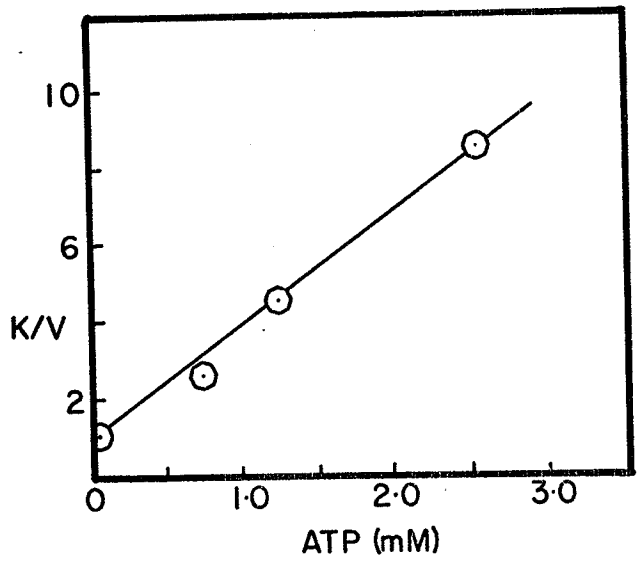
The non-competitive nature of inhibition by ATP was also observed when the enzyme was assayed in 0.1 M potassium acetate buffer (Fig.36A). Replots of the intercepts and slopes against ATP concentration yielded straight lines (Fig.36B,C) with a Ki_I of 0.75 mM and a Ki_S of 1.0 mM for ATP.

When NADH was varied between 0.03 mM and 0.3 mM at a fixed concentration of pyruvate (5.0 mM) inhibition by ATP was competitive with respect to NADH at pH 5.7 as shown in Fig. 37. When the slopes of the double reciprocal plots were plotted against ATP concentration a straight line was obtained with a Ki value for ATP of 0.4 mM.

At pH 7.0 ATP was again a potent inhibitor. Double reciprocal plots of enzyme activity and pyruvate concentration remained non-linear, the degree of non-linearity increasing with increasing ATP concentration (Fig. 38). Hill plots of the data from Fig. 38 are shown in Fig. 39 . The lines are essentially parallel with a slope of approximately 2.0.

An interesting observation was that at pH 5.7 and pH 7.0 with 0.15 mM NADH where no initial lag in the enzyme reaction was observed during routine assays, a pronounced lag was observed in the presence of ATP but not in its absence.

Figure 35. Replots of slopes and intercepts against ATP concentration from the data in Fig. 34.


10
6
K/V
2
0
1·0
2·0
3·0
ATP (mM)

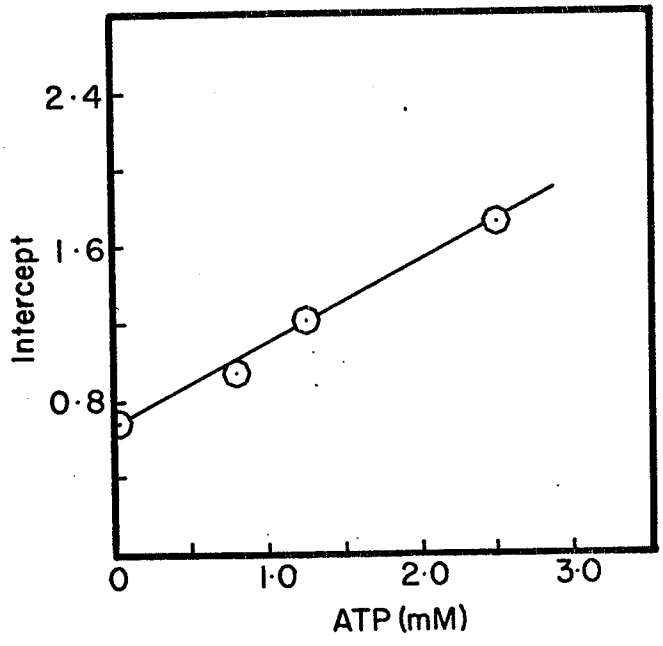

2·4
1·6
Intercept
0·8
0
1·0
2·0
3·0
ATP (mM)

Figure 36A. Double reciprocal plots of velocity and pyruvate concentration at pH 5.7 at different ATP concentrations. Assays were carried out in 0.1 M potassium acetate buffer in the presence of 0.15 mM NADH, varying concentrations of pyruvate as indicated, and ATP in the following concentrations:

i = nil

ii = 0.75 mM

iii = 1.25 mM

iv = 2.5 mM

Reactions were initiated by the addition of enzyme.

v = A340 m /min.

Figure 36B. Replot of intercepts against ATP concentration from the data in Fig. 36A.

Figure 36C. Replot of slopes against ATP concentration from the data in Fig. 36A.

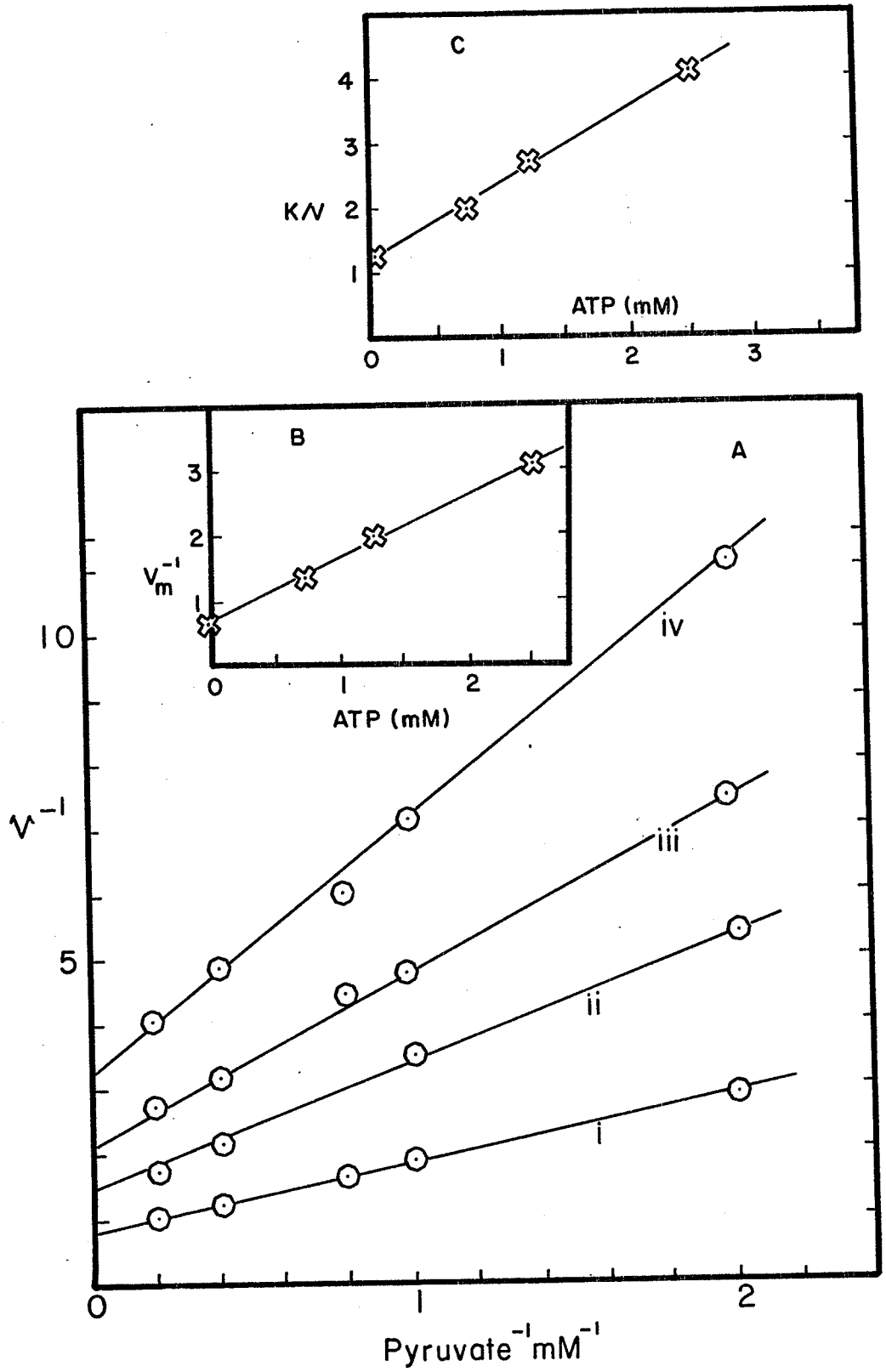

C
K/V
ATP (mM)
B
V_m^{-1}
ATP (mM)
A
iv
iii
ii
i
V^{-1}
Pyruvate^{-1}mM^{-1}

Figure 37. Double reciprocal plots of velocity against NADH concentration at pH 5.7 at a fixed concentration of pyruvate at different concentrations of ATP. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 5 mM pyruvate, varying concentrations of NADH as indicated, and ATP in the following concentrations:

A = nil

B = 0.25 mM

C = 0.5 mM

D = 1.0 mM

E = 2.5 mM

Reactions were initiated by the addition of enzyme.

v = A340 mμ/min.

The insert is a replot of slopes against ATP concentration.

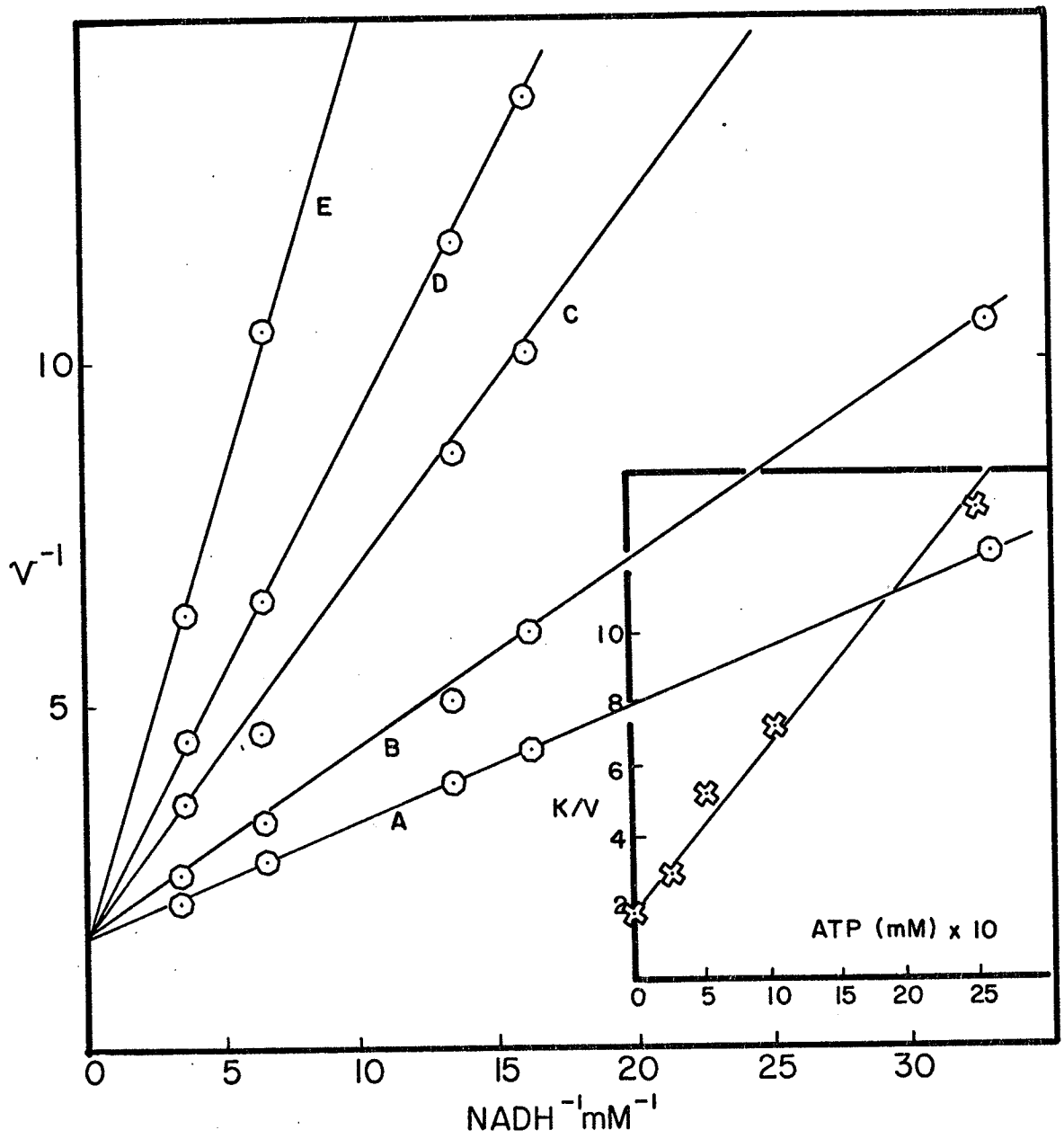

E
D
C
B
A
10
5
V^{-1}
0
5
10
15
20
25
30
$NADH^{-1}mM^{-1}$
K/V
10
8
6
4
2
ATP (mM) x 10
0
5
10
15
20
25

Figure 38. Double reciprocal plots at pH 7.0 of velocity against pyruvate concentration at different concentrations of ATP. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 0.15 mM NADH, varying concentrations of pyruvate as indicated and ATP in the following concentrations:

A = nil

B = 1.25 mM

C = 2.5 mM

Reactions were initiated by the addition of enzyme.

$v = \Delta A340\ m\mu/min.$

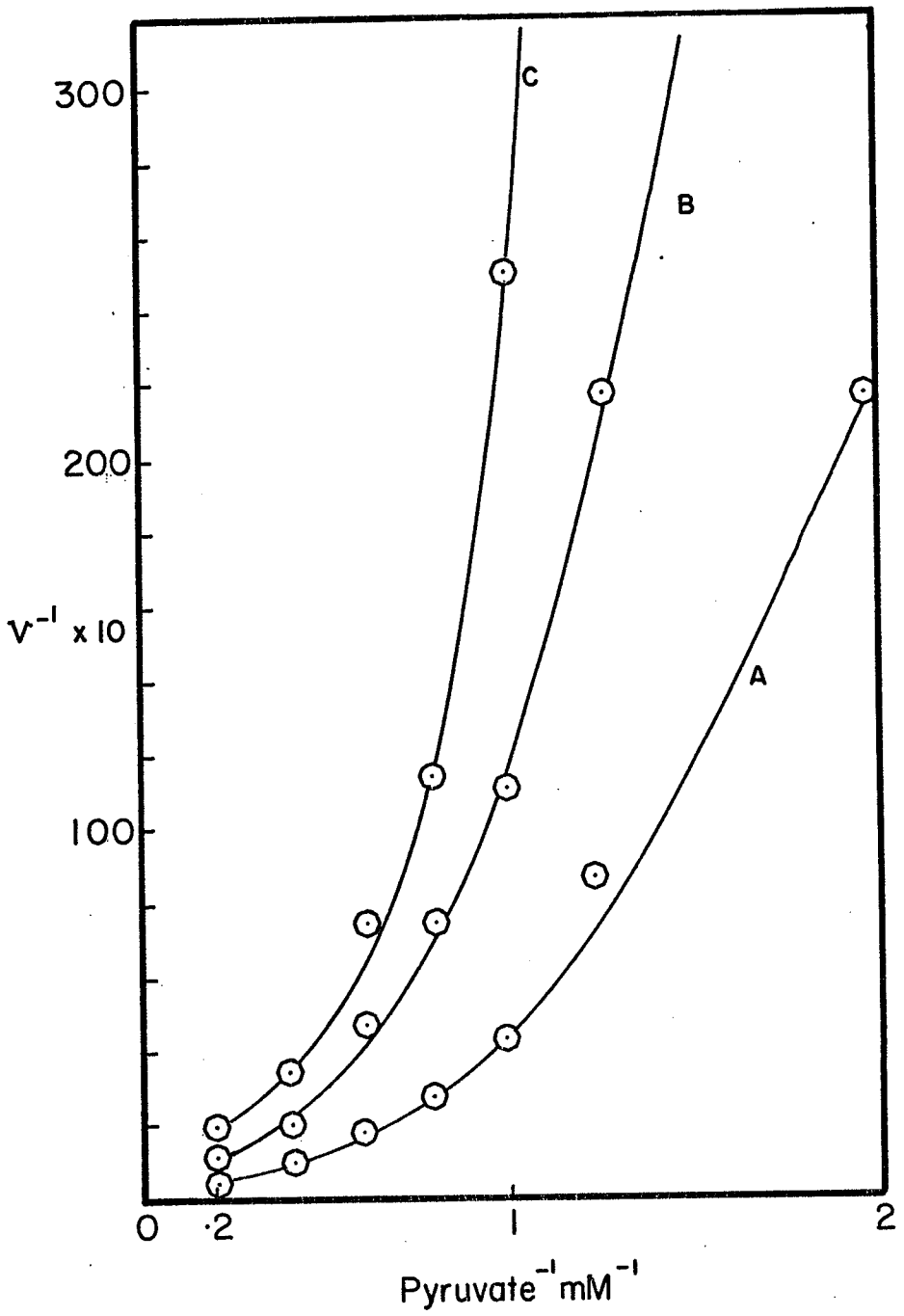
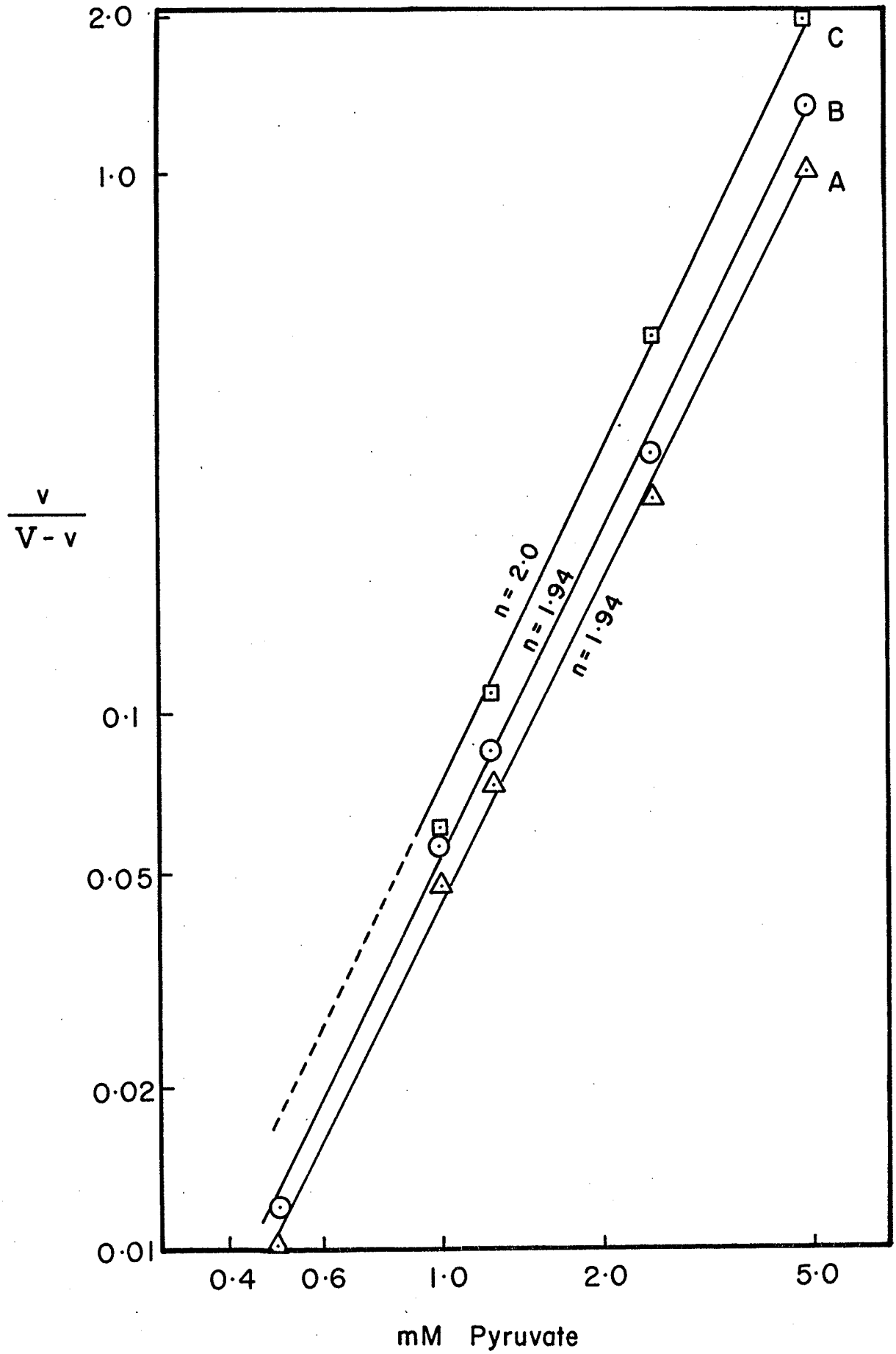

300
200
100
V^{-1} x 10
C
B
A
0
·2
1
2
Pyruvate^{-1} mM^{-1}

Figure 39. Plots of v/(V-v) against pyruvate concentration expressed logrithmically from the data in Fig. 38.

A = no additions

B = 1.25 mM ATP

C = 2.5 mM ATP


2·0
1·0
0·1
0·05
0·02
0·01
v / V - v
0·4
0·6
1·0
2·0
5·0
mM Pyruvate
C
B
A
n = 2·0
n = 1·94
n = 1·94

This lag was eliminated by preincubation of the enzyme with pyruvate in the absence of NADH. Although the lag was eliminated, inhibition by ATP was still observed.

Effect of other nucleotides

Other nucleotides such as ADP, AMP, GTP and GDP had no effect on enzyme activity even at concentrations as high as 4.0 mM at both pH 5.7 and 7.0. AMP at concentrations ranging from 1.0 mM to 4.0 mM did not remove the inhibition by ATP. Cyclic 3',5'-AMP which is an inhibitor of the malic enzyme from E. coli (Sanwal et al, 1969) did not inhibit lactate dehydrogenase.

Effect of enzyme concentration

Kinetic studies of enzymes are limited by the type of equipment used for measurement of activity. The use of spectrophotometers which are used for the determination of enzyme activity, automatically places restrictions on the upper and lower limits of enzyme concentration as well as substrate concentration. Srere (1967) pointed out that many enzymes are present in animal tissues and mitochondria at concentrations thousands-fold higher than those used for in vitro study conditions and may therefore have different properties from those observed in vitro.

Wuntch et al (1970) reported that substrate inhibition of mammalian lactate dehydrogenase observed at low enzyme concentrations, was removed when the enzyme concentration was raised to the level existing in the cells. It was of interest to determine what effect enzyme concentration had on the kinetic behaviour of A. aerogenes lactate dehydrogenase. As mentioned previously, at pH 7.0 the enzyme does not display classical Michaelis-Menten type kinetics. When the enzyme was assayed on a Unicam SP-700 spectrophotometer double reciprocal plots of enzyme activity and pyruvate concentration became increasingly less curved as the enzyme concentration was increased (Fig. 40), until at an enzyme concentration of 0.1 μM, using a stopped flow spectrophotometer the double reciprocal plot became perfectly linear (Fig. 41). Further increase in enzyme concentration by 10-fold still resulted in Michaelis-Menten type kinetics.

Hill plots for the enzyme activity are shown in Fig. 42 and Fig. 43 . At low enzyme concentration (0.0025 μM) the Hill plot showed a slope value of 2.6 (not shown). The slope value decreased with increasing enzyme concentration such that at 0.025 μM a slope of 1.7 was calculated when the enzyme was assayed on the Unicam SP-700 spectrophotometer, and a slope value of 1.5 was calculated when the enzyme was assayed using a stopped flow spectrophotometer. At 0.1 μM enzyme the Hill plot gave a slope of 1.0.

Figure 40. Effect of pyruvate concentration on D-lactate dehydrogenase of A. aerogenes at different enzyme concentrations. Reaction velocities (V_A at pH 7.0 and V_B at pH 5.7) were determined as absorbancy change at 340 mμ per minute in a Unicam SP-700 spectrophotometer (1.0 cm light path). The reaction mixture contained in a total volume of 3.0 ml: 0.1 M potassium phosphate buffer (A: pH 7.0 and B: pH 5.7), potassium pyruvate, 0.15 mM NADH and enzyme.

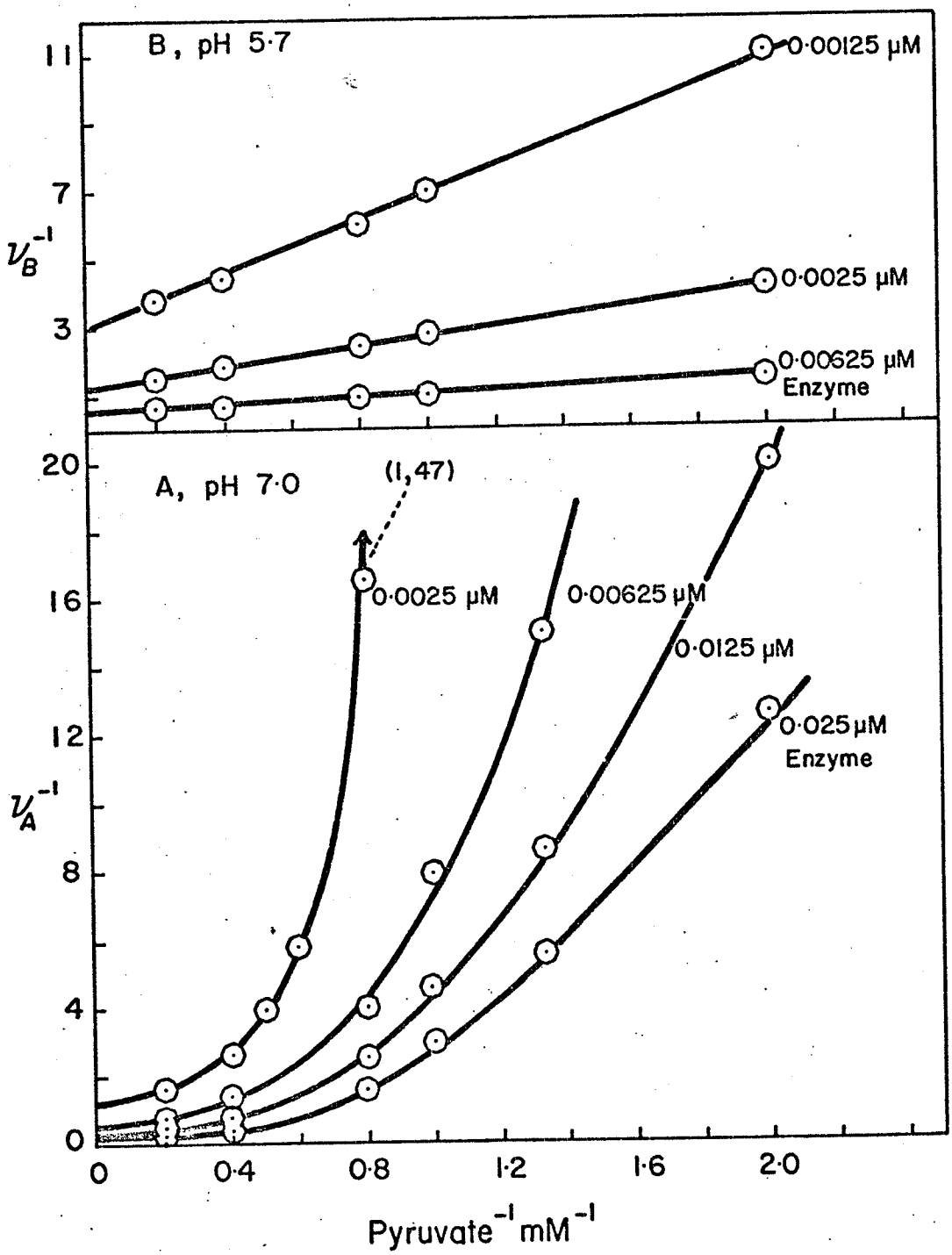

B, pH 5·7
0·00125 μM
0·0025 μM
0·00625 μM
Enzyme
11
7
3
v_B^{-1}
A, pH 7·0
(1,47)
0·0025 μM
0·00625 μM
0·0125 μM
0·025 μM
Enzyme
20
16
12
8
4
0
v_A^{-1}
0
0·4
0·8
1·2
1·6
2·0
Pyruvate^{-1} mM^{-1}

Figure 41. Effect of high enzyme concentration on the Lineweaver-Burk plot of velocity versus pyruvate concentration. Reaction velocities (V_A with 0.025 μM enzyme and V_B with 0.1 μM enzyme) were determined in a Durrum-Gibson Stopped flow spectrophotometer at 340 mμ as described in Materials and Methods and expressed as absorbancy change per second. NADH, 0.15 mM; pH, 7.0 (0.1 M potassium phosphate); enzyme and pyruvate, as indicated.

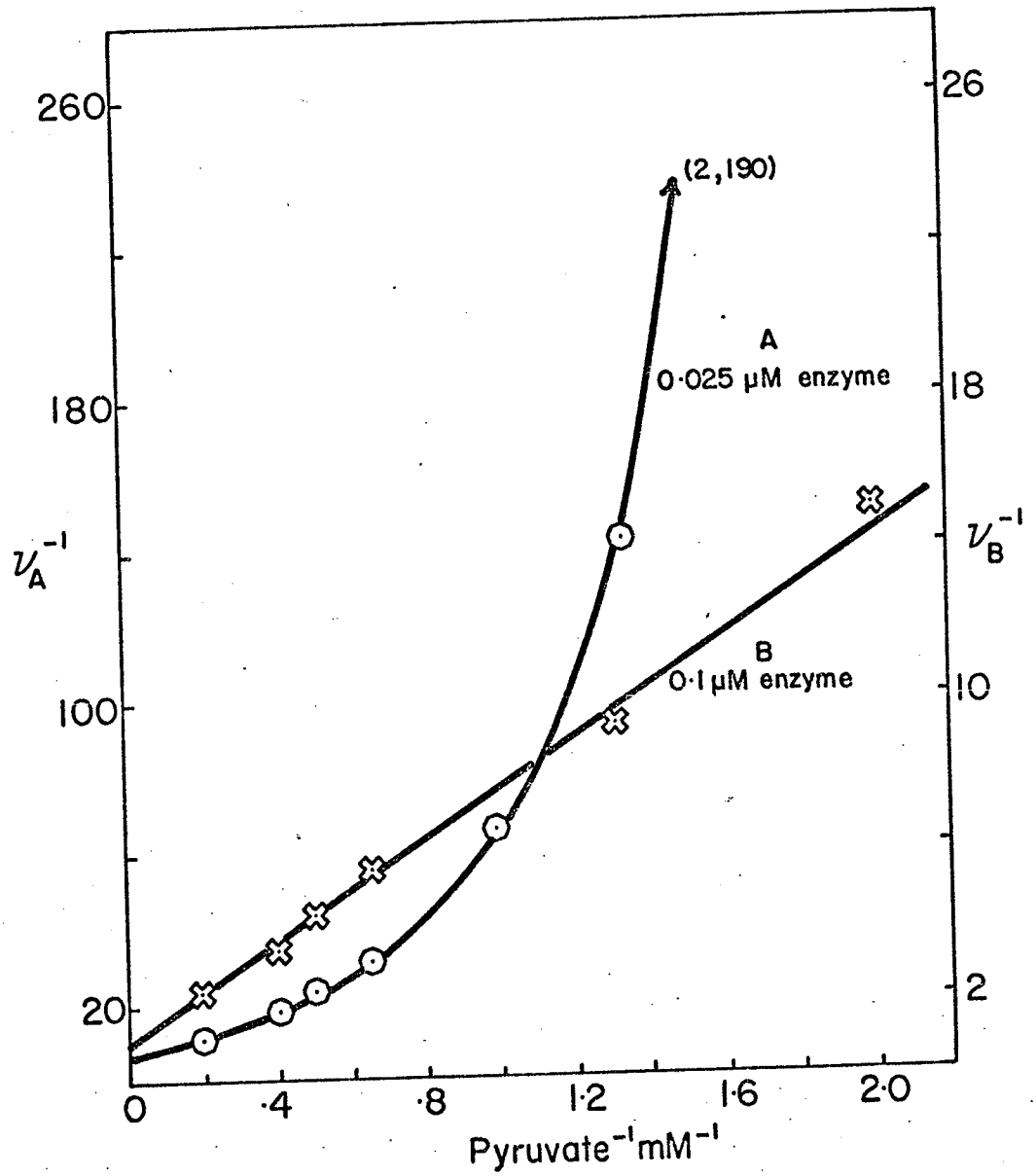

260
180
100
20
26
18
10
2
(2,190)
A
0·025 μM enzyme
B
0·1 μM enzyme
v_A^{-1}
v_B^{-1}
0
·4
·8
1·2
1·6
2·0
Pyruvate^{-1}mM^{-1}

Figure 42. Plots of v/(V-v) against pyruvate concentration expressed logrithmically from the data in Fig. 40 at the enzyme concentrations indicated.

At 0.0025 μM enzyme, n = 2.6 (not shown).

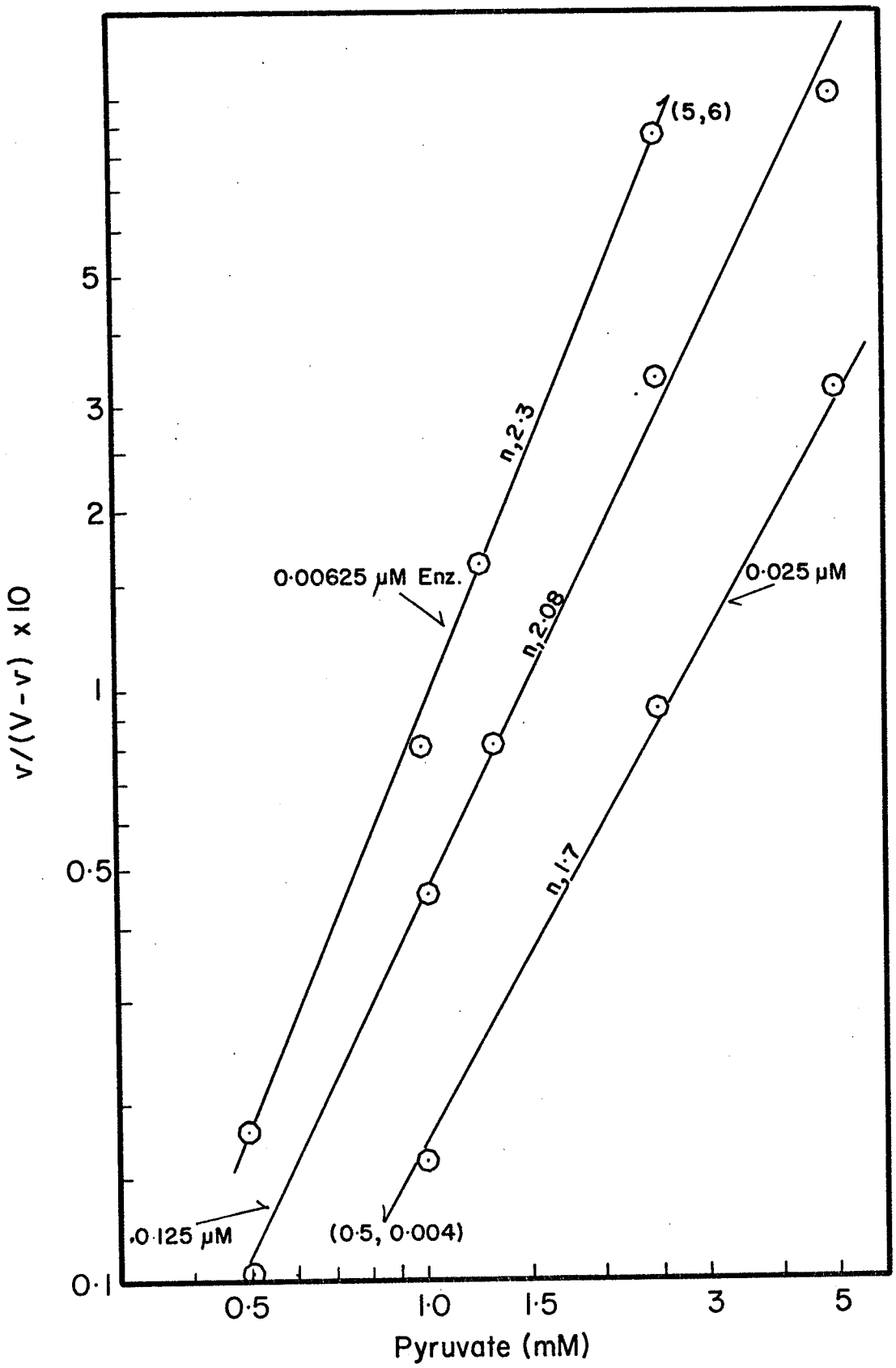
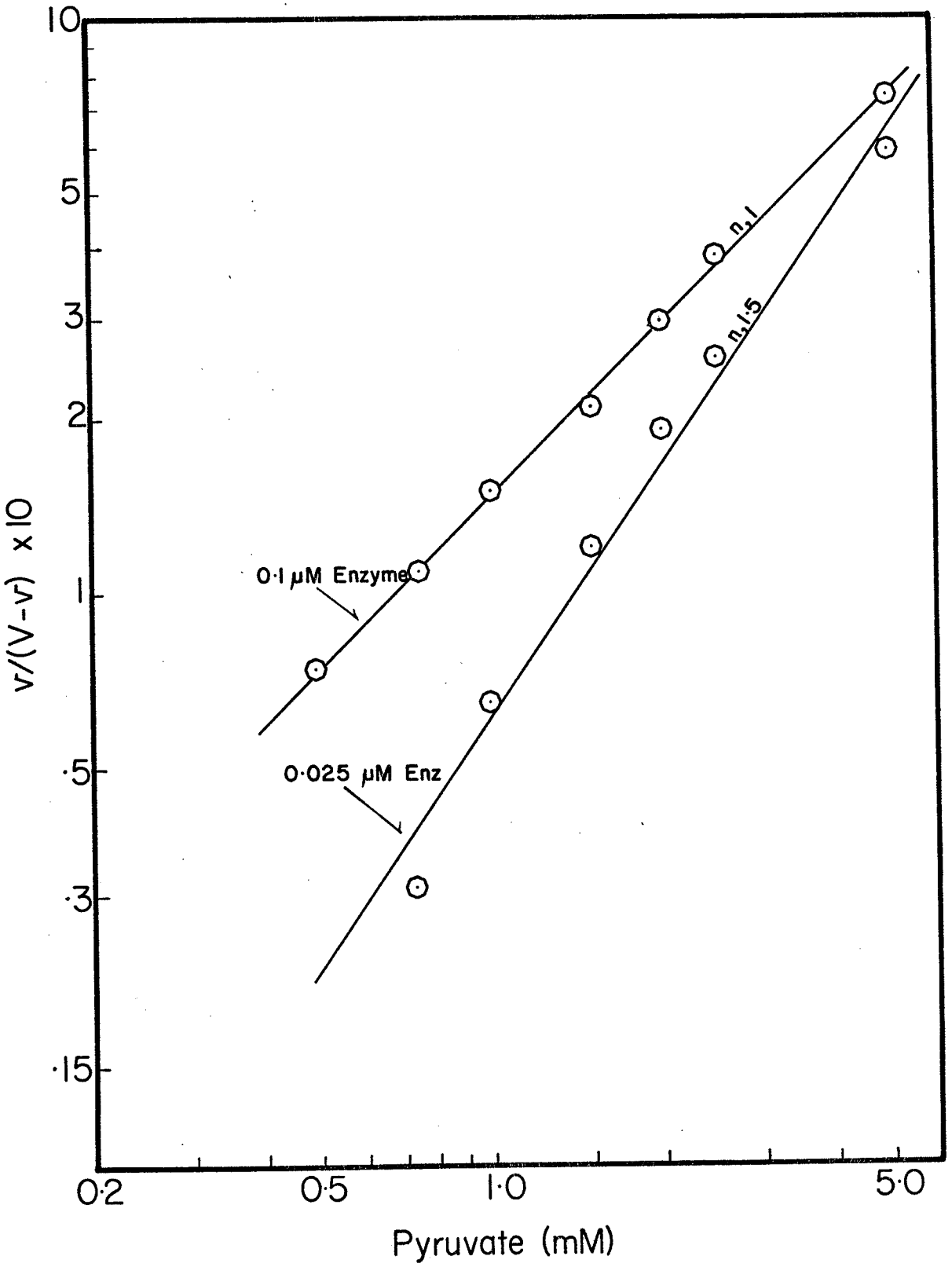

(5,6)
n,2·3
0·00625 μM Enz.
n,2·08
0·025 μM
n,1·7
0·125 μM
(0·5, 0·004)
v/(V - v) x 10
Pyruvate (mM)
0·1
0·5
1
2
3
5
0·5
1·0
1·5
3
5

Figure 43. Plots of v/(V-v) against pyruvate concentration expressed logrithmically from the data in Fig. 41 at the enzyme concentrations indicated.

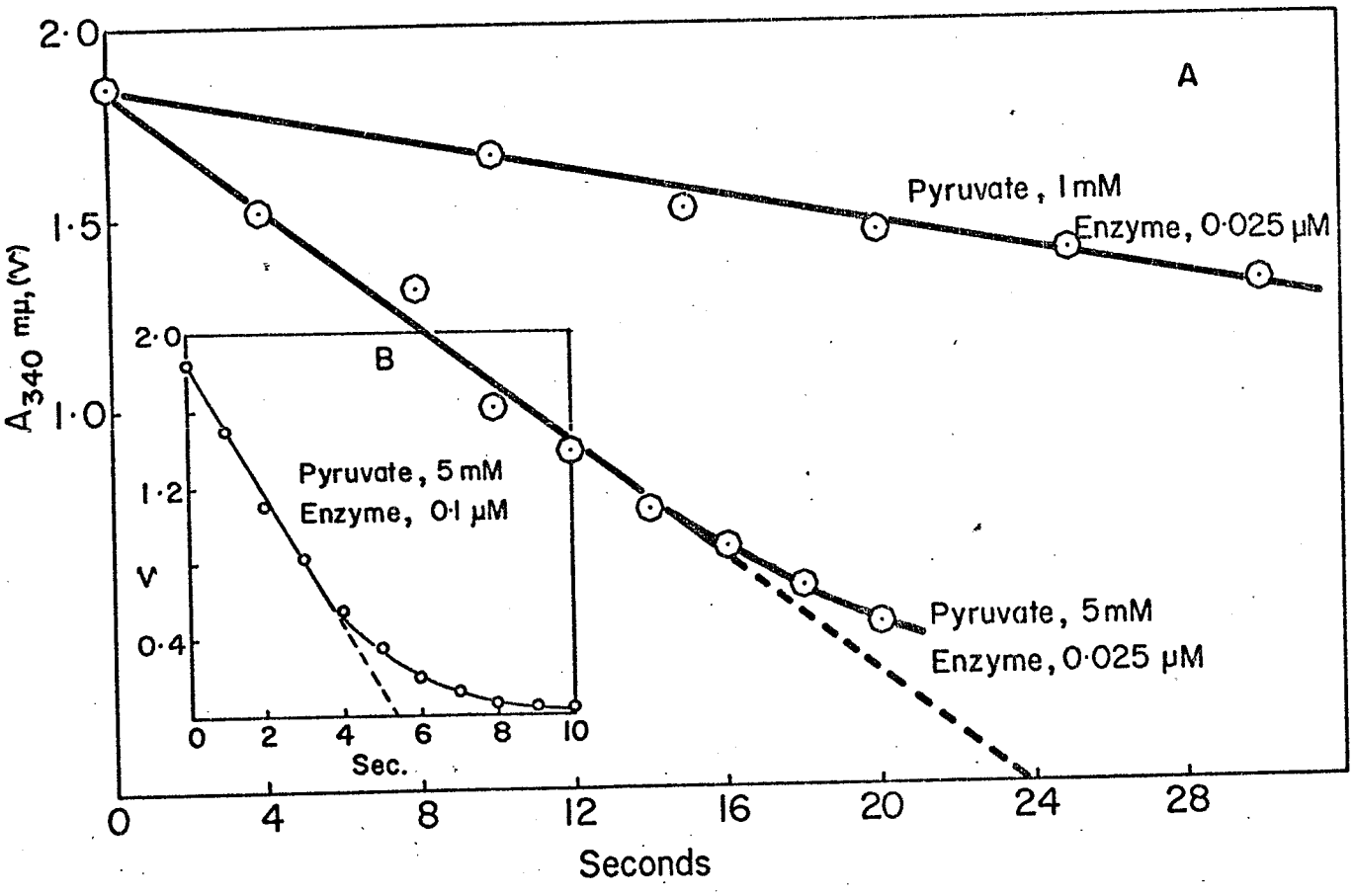

10
5
3
2
1
·5
·3
·15
v/(V-v) x10
0·2
0·5
1·0
5·0
Pyruvate (mM)
0·1 μM Enzyme
0·025 μM Enz
n, 1
n, 1·5

Under the conditions of these experiments there was no initial lag of reaction (Fig. 44) in contrast to the E. coli enzyme (Tarmy and Kaplan 1968).

Desensitization

Most allosteric enzymes can be desensitized by heat, mercurials, temperature, pH, or the addition of specific anions. Under these conditions double reciprocal plots are transformed from non-linear to linear, or lose their property of being influenced by allosteric effectors. Attempts to desensitize lactate dehydrogenase to the effect of pyruvate by means of heat treatment, urea, Ag^+, Hg^+, and pHMB by Hollier (1966) were unsuccessful. However in the course of this investigation, quite by accident, the desensitization of lactate dehydrogenase was achieved. An enzyme preparation recovered from the stopped flow experiments was treated as follows: The enzyme had been incubated in 2.5 mM pyruvate, 0.15 mM NADH, and 0.1 M potassium phosphate buffer of pH 7.0 for a period of two weeks at 4°C, then dialyzed against 0.1 M potassium phosphate buffer of pH 7.0 containing 1.0 mM dithiothreitol. This preparation was then frozen for one month. When the enzyme solution was thawed and assayed at pH 7.0 in 0.1 M potassium phosphate buffer over a range of 0.5 mM to 5.0 mM pyruvate, in the presence of 0.15 mM NADH, double reciprocal plots of enzyme activity and pyruvate concentration were linear. Linear double reciprocal plots were also obtained when the

Figure 44. Linearity of lactate dehydrogenase reaction with time in stopped flow experiments. The conditions were the same as in Fig. 41.


2·0
1·5
1·0
A
Pyruvate, 1 mM
Enzyme, 0·025 μM
Pyruvate, 5 mM
Enzyme, 0·025 μM
B
Pyruvate, 5 mM
Enzyme, 0·1 μM
V
1·2
0·4
0
2
4
6
8
10
Sec.
0
4
8
12
16
20
24
28
Seconds
A340 mμ, (V)

enzyme was assayed in distilled water at pH 7.0 and 0.001 M TES [N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffered at pH 7.0. These results are shown in Fig. 45 .

A study was undertaken to determine the exact source or cause of desensitization. Samples of crude extract (pH 7.0) containing 10 mM dithiothreitol were incubated in the presence of (a) 5.0 mM lactate

(b) 1.0 mM NAD^+

(c) 5.0 mM lactate + 1.0 mM NAD^+

(d) 50 mM pyruvate + 0.15 mM NADH

(e) 5 mM pyruvate previously adjusted to pH 10.0 and heated in a boiling water bath for 20 min.

(f) 5 mM acetoin

(g) 5 mM diacetyl

at 4°C, 28°C, and 40°C. Unfortunately, incubation in these compounds was not successful in desensitizing the enzyme.

INHIBITION OF LACTATE DEHYDROGENASE BY INORGANIC ANIONS

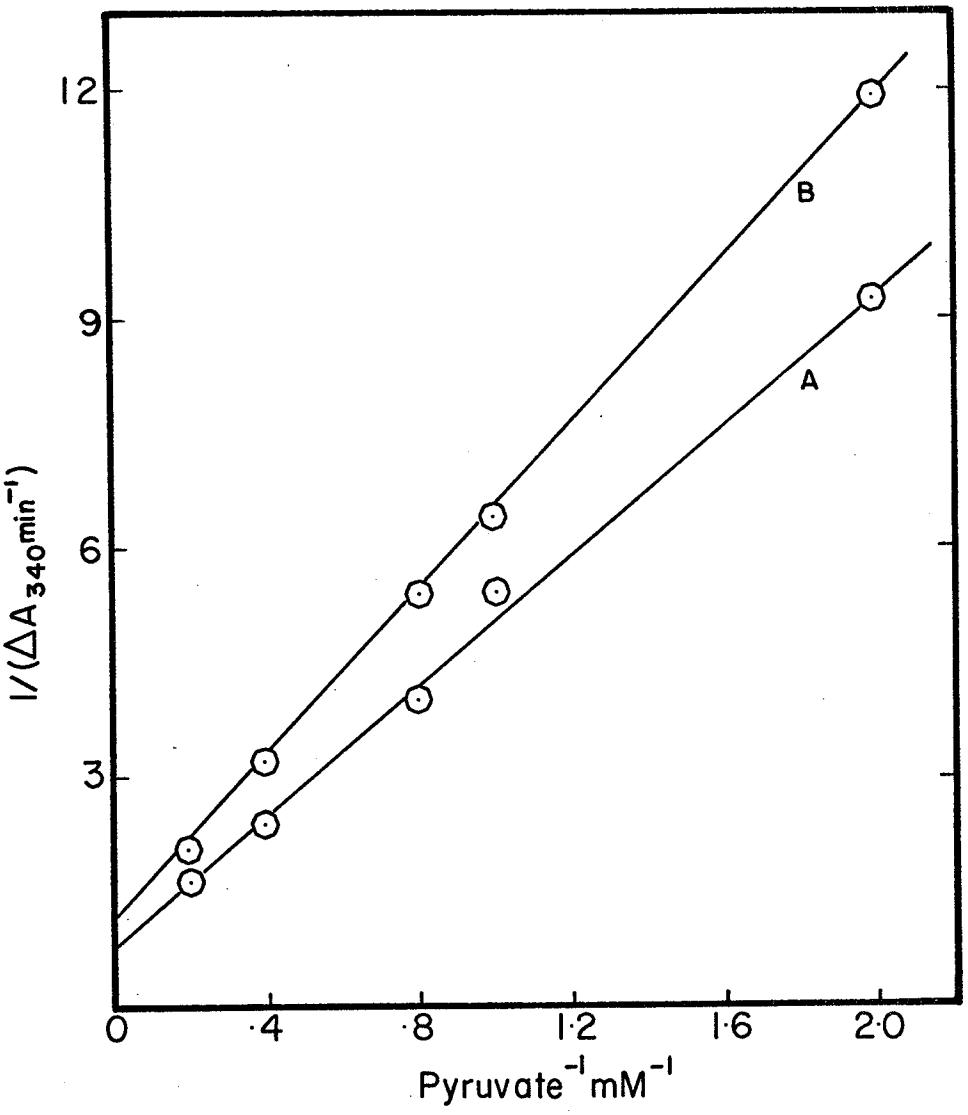
As stated earlier the enzyme was inhibited by high buffer concentrations, both phosphate and acetate at pH 7.0. At pH 5.7 there was no difference in activity in these buffers at 0.025 - 0.1 M concentration range.

It has been recently demonstrated (Warren et al, 1966) that neutral salts at high concentrations (0.3 M to 3.0 M) inhibit the activity of widely different enzymes in an order of increasing effectiveness for anions, $Ac^- < Cl^- < NO_3^- < Br^- < I^- < SCN^- < CLO_4^- <$. When the enzyme was assayed in 0.1 M

Figure 45. Desensitization of lactate dehydrogenase. Reactions were carried out at pH 7.0 in the following buffers:

A = 0.1 M potassium phosphate

B = 0.001 TES


12
9
6
3
0
·4
·8
1·2
1·6
2·0
B
A
1/(ΔA340min⁻¹)
Pyruvate⁻¹mM⁻¹

potassium phosphate buffer at pH 5.7 in the presence of 5.0 mM pyruvate, the order of increasing effectiveness for the above ions at a final concentration of 0.1 M was as follows: $Ac^- < Cl^- < Br^- < NO_3^- < SCN^- < I^- < ClO_4^-$, and so appeared to follow the anionic series observed for other enzymes. The same order of effectiveness was observed when the enzyme was assayed in acetate buffer.

Inhibition by KCl

When assayed in potassium phosphate buffer at pH 5.7, KCl inhibited the enzyme competitively with respect to pyruvate as shown in Fig. 46. A replot of slopes and KCl concentration yielded a straight line with a Ki value for KCl of 10 mM (Fig. 47). The same inhibition pattern was observed when the enzyme was assayed in acetate buffer. The effect of the other halide anions Br^- and I^- was similar to that exhibited by Cl^-, inhibiting competitively with respect to pyruvate.

At pH 7.0 inhibition by KCl was still observed but the nature of inhibition was difficult to predict because of the non-linearity of the double reciprocal plots (Fig. 48). Hill plots of data from Fig. 48 are shown in Fig. 49. Slopes are essentially parallel showing an "n" value of 1.8 and 1.90.

When NADH was varied and the pyruvate concentration was fixed at 5.0 mM, inhibition by KCl with respect to NADH was non-competitive as shown in Fig. 49. Replots of slopes and intercept against KCl concentration were linear (Fig. 50),

Figure 46. Double reciprocal plots of velocity against pyruvate concentration at pH 5.7 at different concentrations of KCl. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 0.15 mM NADH, varying concentrations of pyruvate as indicated, and KCl in the following concentrations:

A = nil

B = 0.025 M

C = 0.05 M

D = 0.1 M

Reactions were initiated by the addition of enzyme.

$v = \Delta A340$ mμ/min.

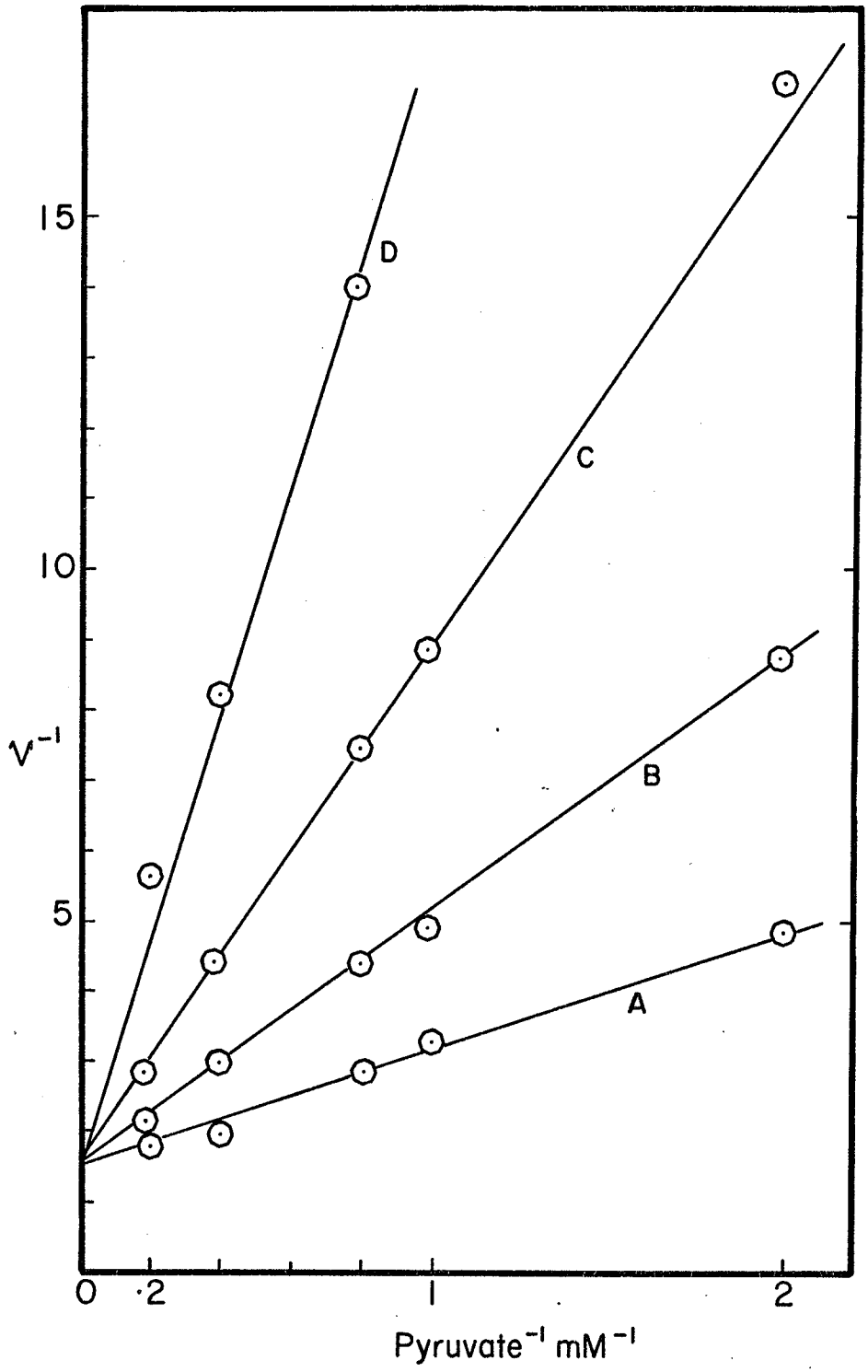

15
10
5
V^{-1}
D
C
B
A
0
·2
1
2
Pyruvate^{-1} mM^{-1}

Figure 47. Replots of slopes from Fig. 46 against KCl concentration.

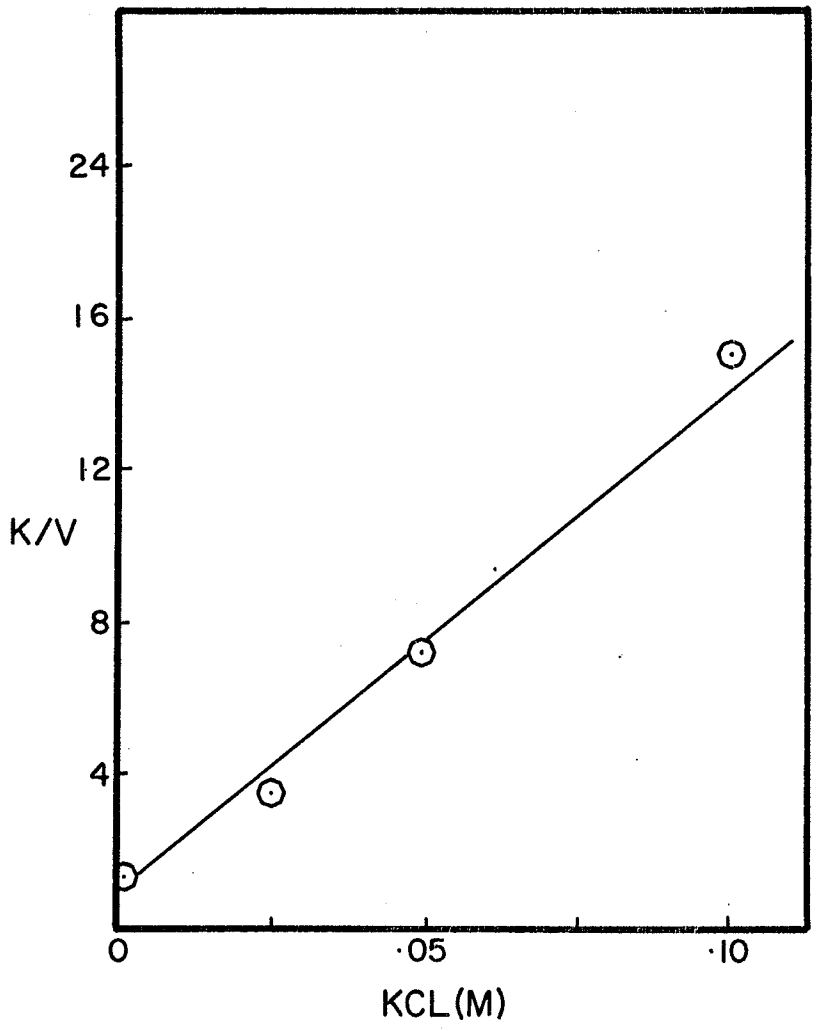

24
16
12
K/V
8
4
0
·05
·10
KCL(M)

Figure 48. Double reciprocal plots of velocity against pyruvate concentration at pH 7.0 at different concentrations of KCl. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 0.15 mM NADH, varying pyruvate as indicated, and KCl in the following concentrations:

A = nil

B = 0.005 M

C = 0.025 M

D = 0.05 M

Reactions were initiated by the addition of enzyme.

$v = \Delta A340\ m\mu/min.$

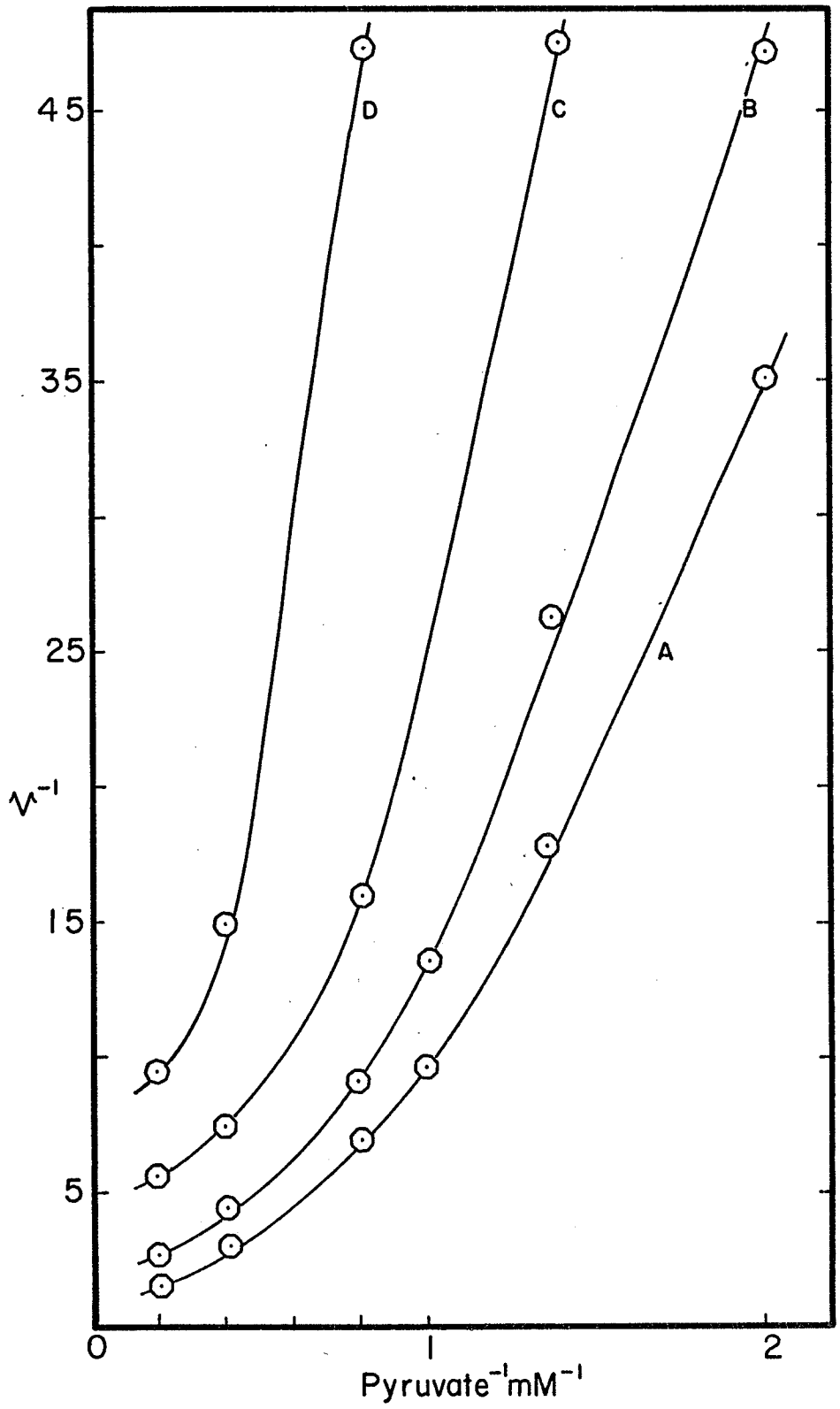

45
35
25
15
5
v^{-1}
D
C
B
A
0
1
2
Pyruvate^{-1}mM^{-1}

Figure 49. Plots of v/(V-v) against pyruvate concentration expressed logrithmically from the data in Fig. 48.

A = nil

B = 0.005 M KCl

C = 0.025 M KCl

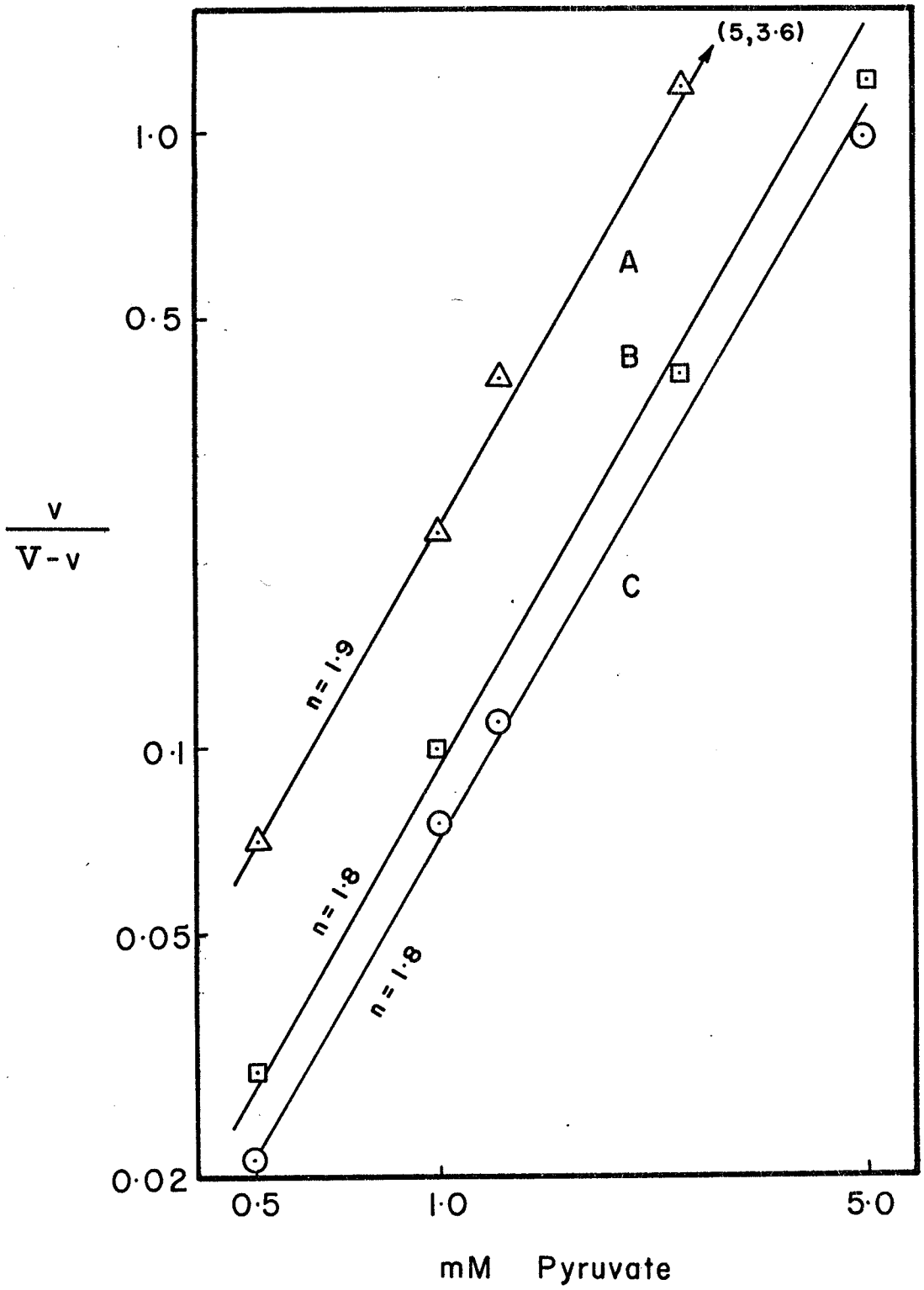

(5,3·6)
1·0
0·5
0·1
0·05
0·02
v / V-v
A
B
C
n=1·9
n=1·8
n=1·8
0·5
1·0
5·0
mM Pyruvate

Figure 50. Double reciprocal plots of velocity against NADH concentration at pH 5.7 in the presence of a fixed concentration of pyruvate, at different concentrations of KCl. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 5.0 mM pyruvate, varying NADH as indicated, and KCl in the following concentrations:

A = nil

B = 0.01 M

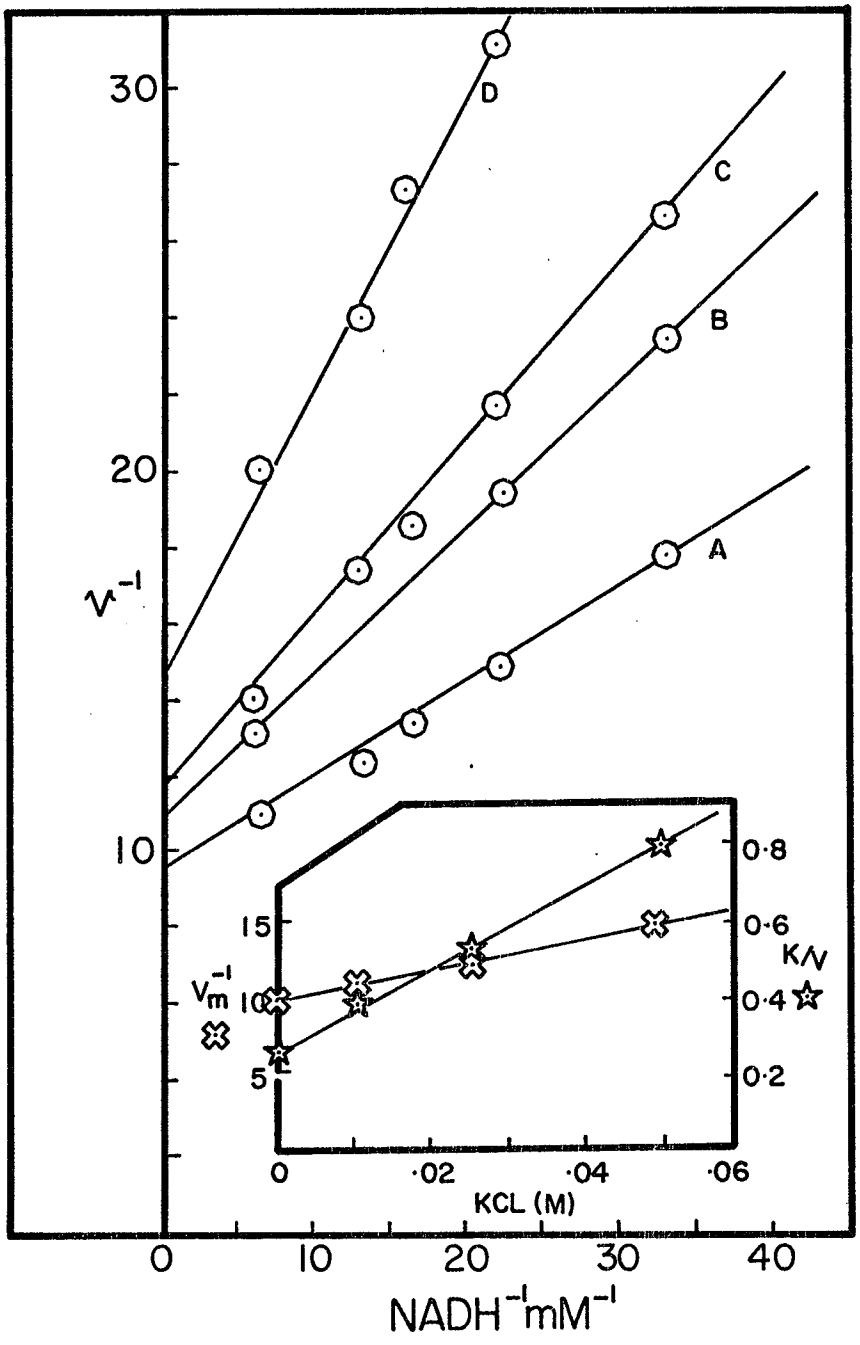
C = 0.025 M

D = 0.05 M

Reactions were initiated by the addition of enzyme.

$v = \Delta A340$ mμ/min.

The insert shows a replot of slopes and intercepts against KCl concentration.


30
20
10
V^{-1}
D
C
B
A
0
10
20
30
40
$NADH^{-1}mM^{-1}$
15
10
5
V_m^{-1}
0·8
0·6
0·4
0·2
K/V
0
·02
·04
·06
KCL (M)

with a Ki_I of 90 mM and a Ki_S of 25 mM for KCl.

Inhibition by KSCN

As shown in Fig. 51 inhibition by KSCN at pH 5.7 was competitive with respect to pyruvate. A replot of the slopes against inhibitor concentration yielded a straight line with a Ki value for KSCN of 5.0 mM, (Fig. 52).

Inhibition by K_2SO_4

At pH 5.7 K_2SO_4 inhibited lactate dehydrogenase in a competitive manner with respect to pyruvate. At 0.5 mM pyruvate in the presence of 0.15 mM NADH the enzyme was inhibited approximately 30% by 0.016 M K_2SO_4 (Fig. 53). A replot of the slopes against inhibitor concentration yielded a straight line with a Ki for K_2SO_4 of 20 mM as shown in Fig. 54.

EFFECT OF METAL IONS

The enzyme was assayed in the presence of the following metal ions (chloride salts) at a final concentration of 0.1 mM and 1.0 mM to determine their effect on enzyme activity: Ni^{++}, K^{+}, Li^{+}, Co^{++}, Zn^{++}, Mn^{++}, Mg^{++}, Ba^{++}, Cu^{+}, Cu^{++}, Fe^{++}, and Ca^{++}. Of these cations tested only Cu^{+} and Cu^{++} had any effect. Cu^{++} inhibited enzyme activity by approximately 80% at a final concentration of 0.1 mM in the presence of 2.5 mM pyruvate, and Cu^{+} inhibited enzyme activity by 30% under the same conditions.

Figure 51. Double reciprocal plots of velocity against pyruvate concentration at pH 5.7 at different concentrations of KSCN. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 0.15 mM NADH, varying pyruvate as indicated, and KSCN in the following concentrations:

A = nil

B = 0.0025 M

C = 0.005 M

D = 0.01 M

Reactions were initiated by the addition of enzyme.

$v = \Delta A340\ m\mu/min.$

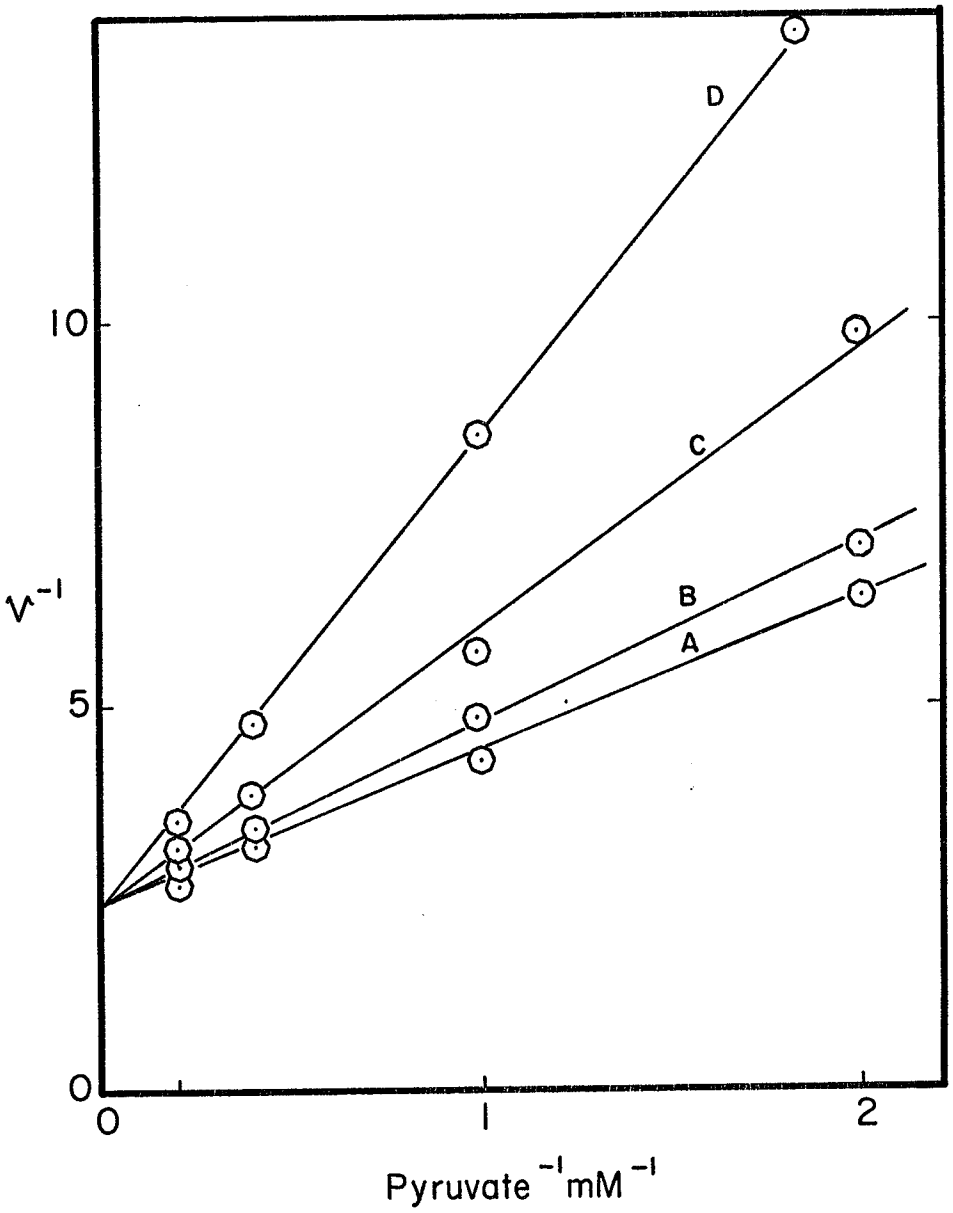

D
C
B
A
10
5
0
0
1
2
V^{-1}
Pyruvate $^{-1}$ mM $^{-1}$

Figure 52. Replot of the slopes from Fig. 51 against KSCN concentration.

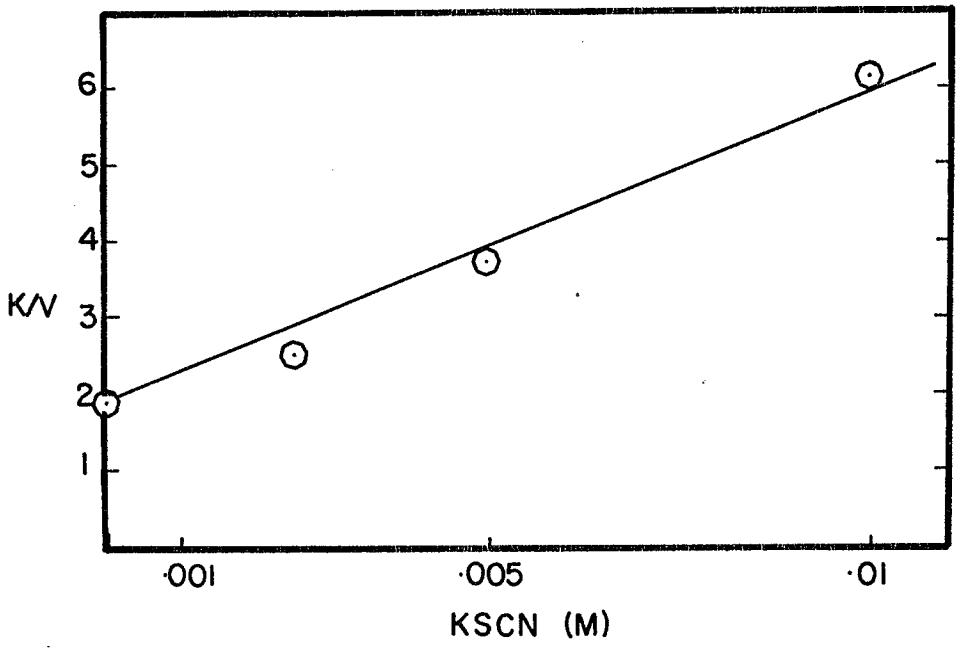

6
5
4
K/V 3
2
1
·001
·005
·01
KSCN (M)

Figure 53. Double reciprocal plots of velocity and pyruvate concentration at pH 5.7, at different concentrations of Na_2SO_4. Assays were carried out in 0.1 M potassium phosphate buffer in the presence of 0.15 mM NADH, varying pyruvate as indicated, and Na_2SO_4 in the following concentrations:

A = nil

B = 0.0165 M

C = 0.033 M

D = 0.066 M

Reactions were initiated by the addition of enzyme.

v = ΔA340 mμ/min.

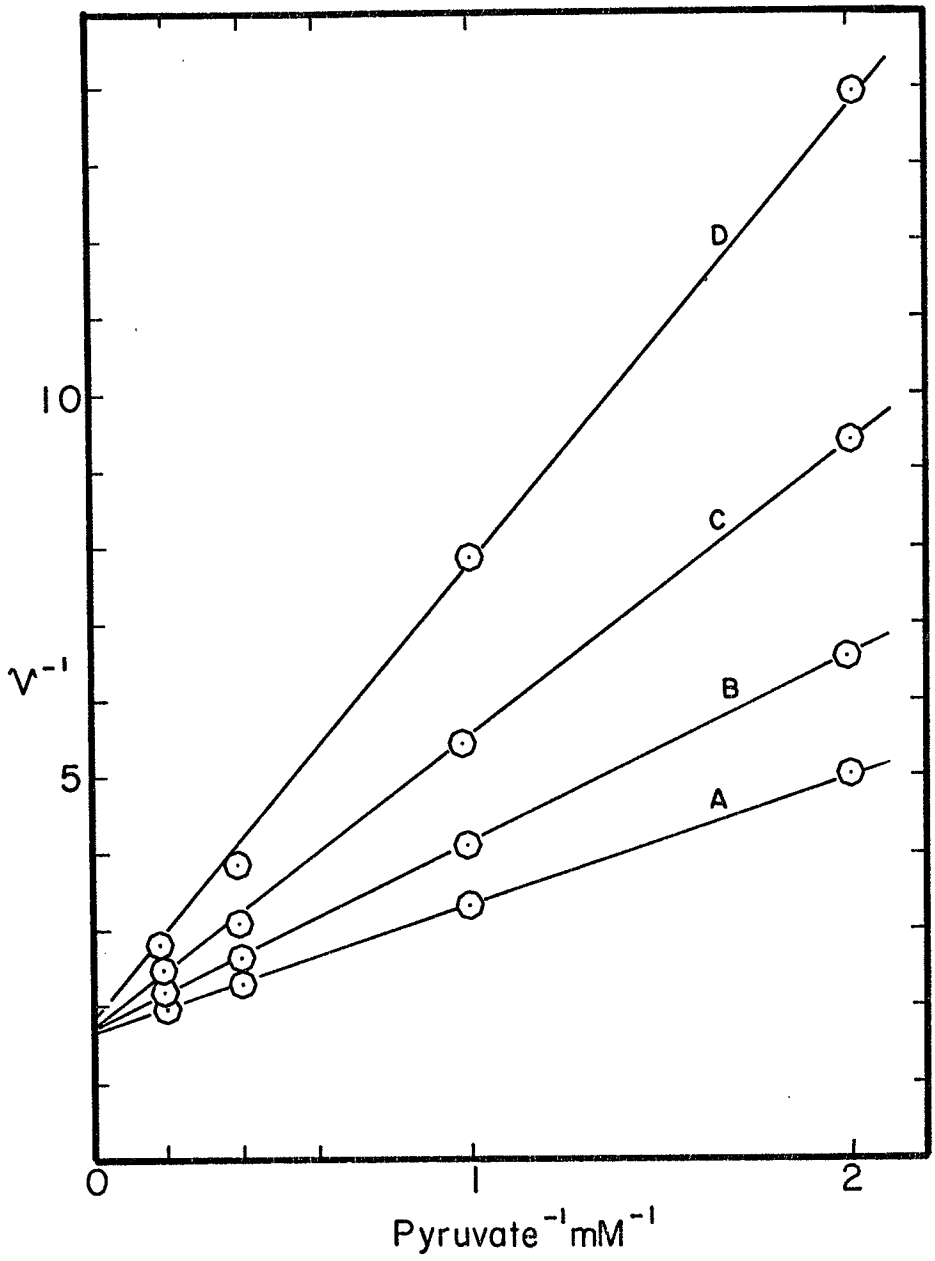
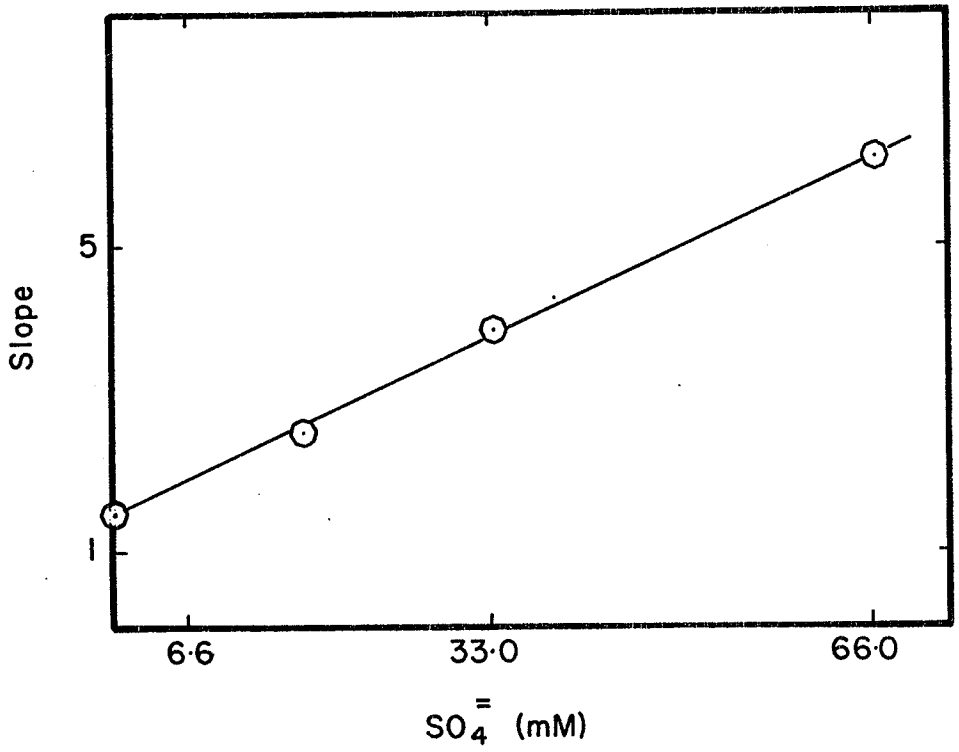

D
C
B
A
10
5
0
1
2
v^{-1}
Pyruvate^{-1}mM^{-1}

Figure 54. Replot of the slopes from Fig. 53 against Na_2SO_4 concentration.


Slope
5
1
6·6
33·0
66·0
$SO_4^{=}$ (mM)

SUBSTRATE SPECIFICITY

Glyoxylate

The reduction of glyoxylate to glycollate by mammalian lactate dehydrogenase has been reported by several authors (Meister 1952, Sawaki and Yamada 1966, Banner and Rosalki 1967). Sawaki *et al* (1966, 1967) have briefly reported that lactate dehydrogenases from several sources will reduce NAD^+ using glyoxylate as the other substrate. The oxidation of glyoxylate to oxalate has also been demonstrated for crystalline rabbit muscle lactate dehydrogenase (Duncan *et al*, 1969). When the *Aerobacter aerogenes* enzyme was assayed in the presence of 2.0 mM glyoxylate and 0.15 mM NADH in potassium phosphate buffer of pH 5.7, the rate of reduction of glyoxylate was about 3% the rate observed when pyruvate was the substrate. The same reaction rate was observed for 2.0 mM and 5.0 mM glyoxylate. No reduction of NAD^+ was observed when the enzyme was assayed in the presence of 0.10 mM NAD^+ and glyoxylate (2 mM and 4 mM) in Tris-HCl buffer or potassium pyrophosphate buffer at pH 9.0. Since the *Aerobacter aerogenes* lactate dehydrogenase is essentially a unidirectional enzyme no oxidation of glyoxylate was expected. It appeared that the enzyme could not utilize glyoxylate as a substrate as do lactate dehydrogenases from other sources.

Fluoropyruvate and phenyl pyruvate

The enzyme was assayed at pH 5.7 using fluoropyruvate and phenyl pyruvate in place of pyruvate to determine if these compounds could act as substrates. Fluoropyruvate was reduced by lactate dehydrogenase, but the rate of reduction was only 60% of that observed when the same concentration of pyruvate (5 mM) was used as the substrate.

Phenyl pyruvate (5.0 mM) was not reduced by the enzyme, but when it was added to the normal assay mixture in the presence of 5.0 mM pyruvate, 30% inhibition was observed. The kinetics of this inhibition were not investigated.

MOLECULAR WEIGHT STUDIES OF LACTATE DEHYDROGENASE

Sedimentation velocity

Sedimentation velocity studies were conducted with a Spinco Model E analytical ultracentrifuge equipped with a Schlieren double sector optical system. The runs were made in the presence of 0.05 M potassium phosphate buffer of pH 5.7 and 8.0 containing 1.0 mM dithiothreitol, at a protein concentration of 3.5 to 4.0 mg per ml.

It was observed that the sedimentation pattern of the enzyme was influenced by pH. At pH 5.7 one major peak with a small faster-moving shoulder was observed. After dialysis against potassium phosphate buffer (0.05 M) of pH 8.0 containing

1.0 mM dithiothreitol, the enzyme gave a single moving peak. The $S_{w,20}$ value of this single peak was calculated to be 13.5. In another identical experiment the $S_{w,20}$ value was 13.1. At pH 5.7 the $S_{2,20}$ value of the major peak was calculated to be 13.1 similar to the $S_{w,20}$ value observed at pH 8.0. The smaller faster-moving shoulder showed an $S_{w,20}$ value of 17.7.

Sucrose gradient centrifugation

Molecular weight determinations were carried out according to the technique described by Martin and Ames (1961). Sucrose gradients (4% to 20%) were prepared in phosphate buffer (0.1 M) of specified pH containing 1.0 mM dithiothreitol. The molecular weight of lactate dehydrogenase varied with pH, enzyme concentration, and the presence of added cofactors.

At pH 5.7 the estimated molecular weight using 3 to 5 units of enzyme was calculated to be 105,000 $\pm$ 5,000 from several trials. It was observed that the peak of enzyme activity was unsymmetrical. As shown in Fig.55 a faster moving small shoulder preceeded the major enzyme peak of 105,000 molecular weight, suggesting the presence of a heavy and a lighter species of enzyme. In the presence of 5.0 mM pyruvate, the estimated molecular weight of the enzyme in several trials increased to 145,000 $\pm$ 5,000 for the major peak. As shown in Fig. 56 two molecular species were observed, the major enzyme peak corresponding to a molecular weight of approximately

Figure 55. Molecular weight determinations. 4-20% sucrose gradients were prepared in 0.1 M potassium phosphate buffer of indicated pH. Hemoglobin (68,000) was used as a reference.

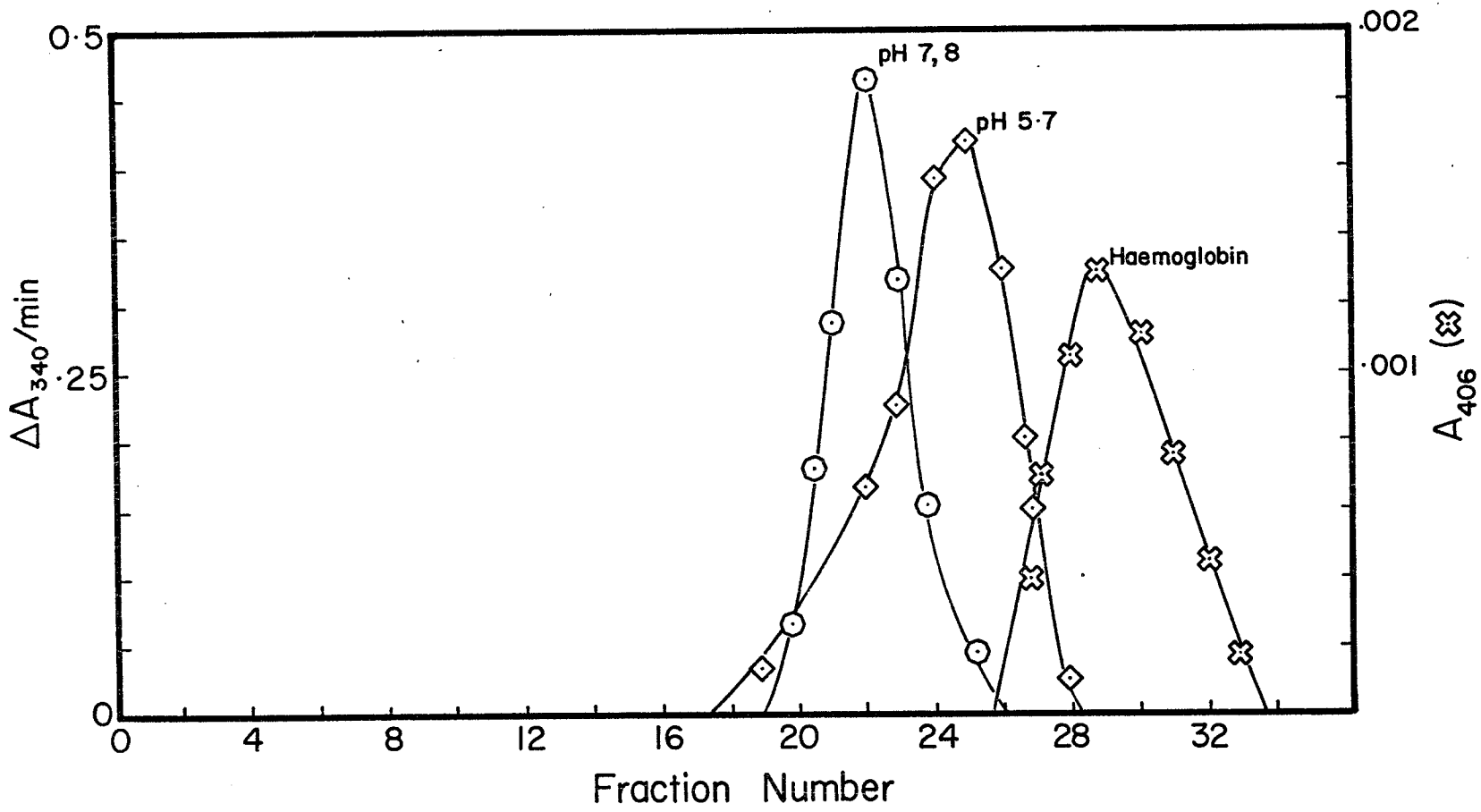
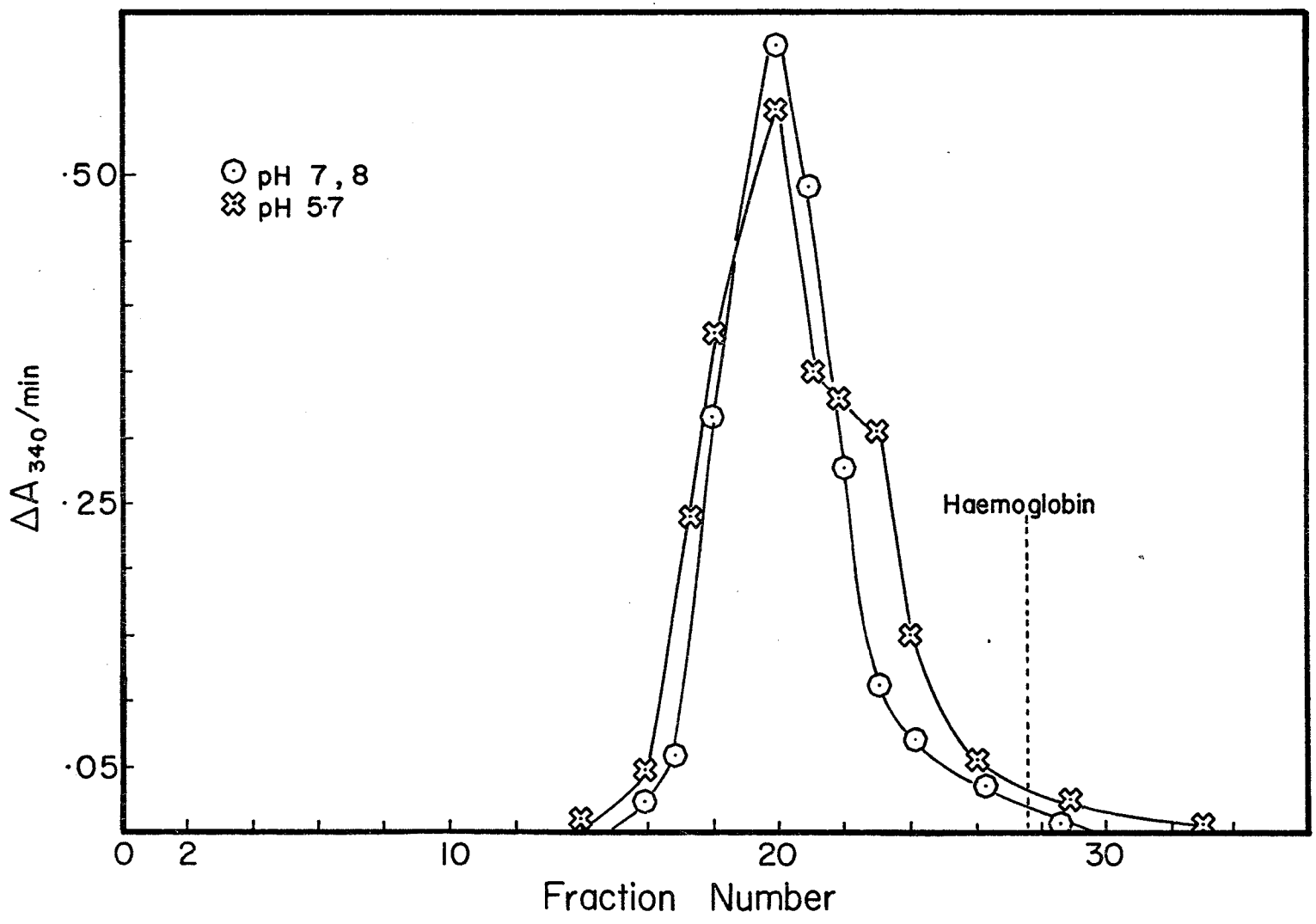

0·5
·25
0
ΔA340/min
.002
·001
A406 (⊠)
pH 7, 8
pH 5·7
Haemoglobin
0
4
8
12
16
20
24
28
32
Fraction Number

Figure 56. Molecular weight determinations. 4-20% sucrose gradients were prepared in 0.1 M potassium phosphate buffer of indicated pH. Each gradient contained 5 mM pyruvate. Hemoglobin (68,000) was used as a reference.


pH 7, 8
pH 5·7
·50
·25
·05
ΔA340/min
Haemoglobin
0
2
10
20
30
Fraction Number

145,000, and a lighter species represented by the following shoulder. At lower enzyme concentrations (0.5 to 1 unit) a molecular weight of 140,000 to 150,000 was still observed in the presence of 5.0 mM pyruvate, but no enzyme activity could be detected in the gradients which did not contain pyruvate.

At pH 7.0 and pH 8.0 the molecular weight using 3 to 5 units of enzyme was estimated to be 145,000 $\pm$ 6,000. No change in the molecular weight was observed in the presence of 5.0 mM pyruvate (Fig. 55,5 6). At lower enzyme concentrations (0.5 to 1.0 unit) the molecular weight at pH 7.0 did not change from the above value, but the molecular weight at pH 8.0 and pH 9.0 decreased to 80,000 $\pm$ 4,000. Under these latter conditions, however, the presence of 5.0 mM pyruvate increased the molecular weight of enzyme to 120,000 $\pm$ 6,000.

In the presence of high concentrations of NADH (6.5 mM) at pH 7.0 the molecular weight was estimated to be 77,000 $\pm$ 4,000 using 0.5 - 1 units of enzyme. At lower NADH concentrations (0.15 mM) the molecular weight of 140,000 $\pm$ 5,000 was estimated from several trials. The presence of ATP (10 mM) in the sucrose gradients showed no marked changes in molecular weight at pH 5.7 (0.5-1 or 3-5 units enzyme) or pH 7.0 and pH 8.0 with 3-5 units of enzyme.

The effect of anions, SCN^-, Cl^-, and $SO_4^=$, on molecular weight was studied at pH 5.7 and pH 7.0. In the presence of 0.1 M anion at pH 5.7 the molecular weight was estimated from several trials to be 100,000 $\pm$ 6,000 in the presence of KSCN,

110,000 ± 5,000 in the presence of KCl, and 110,000 ± 5,000 in the presence of Na_2SO_4 when 3 to 5 units of enzyme were used. These molecular weights are in close agreement with the value observed in the absence of anions at pH 5.7. At pH 7.0, again no difference in molecular weight was observed in the presence and absence of anions.

At low concentration of enzyme (0.5 to 1.0 unit) 20 mM α-ketobutyrate decreased the molecular weight from its estimated average of 145,000 to 110,000 ± 5,000 at pH 7.0. In the presence of 30 mM α-ketoglutarate at pH 7.0 the molecular weight was reduced to 80,000 ± 4,000. The implication of these molecular weight changes under various conditions will be discussed later.

Effect of guanidine hydrochloride

It has been amply demonstrated (Gordon and Jenks, 1963; Tanford et al, 1966; Nozaki and Tanford, 1967) that guanidine hydrochloride containing mercaptoethanol is one of the most effective reagents for the dissociation of polymerized proteins. Its effect on lactate dehydrogenase was investigated.

When the partially purified enzyme was frozen at 20°C and thawed, it was often observed that all the enzyme protein had precipitated, no activity being detected in the clear supernatant. This fine white precipitate was insoluble in 0.05 M potassium phosphate buffer of pH 7.0. When suspended

in 6.0 M guanidine hydrochloride prepared in 0.05 M potassium phosphate buffer of pH 7.0, the enzyme dissolved readily but no enzyme activity could be detected due to inhibition by guanidine hydrochloride. Upon dialysis against 0.05 M potassium phosphate buffer of pH 7.0 containing 1.0 mM dithiothreitol for several hours to remove the guanidine hydrochloride, precipitation occurred. The precipitate showed no activity when suspended in phosphate buffer but the supernatant contained up to 30.0% of the original enzyme activity. The guanidine hydrochloride treatment was repeated on the insoluble white precipitate, again with recovery of 1% of the original enzyme activity. When the reactivated enzyme was frozen and thawed, precipitation and complete loss of activity again resulted. The insoluble precipitate, after guanidine hydrochloride treatment, regained up to 75% of the activity previously displayed by the reactivated enzyme.

When a sample of crude extract (15 mg per ml) was denatured by heating at 60°C for 25 min, only about 1% of the enzyme activity could be recovered by guanidine hydrochloride treatment.

Sub-unit molecular weight

Determination of the molecular weights of polypeptide chains in oligomeric proteins is an important problem. The most frequently employed physicochemical method is equilibrium

centrifugation in guanidine hydrochloride solution (Ullmann et al, 1968; Kawahara, 1969). For many purposes a procedure which is experimentally less demanding, but still yields reliable molecular weights is of great value. The determination of the sub-unit molecular weight of lactate dehydrogenase employing sucrose gradient technique in the presence of 6.0 M guanidine hydrochloride was not possible because of failure of sucrose solutions to form a gradient due to decrease in viscosity. Sephadex gel filtration was not practical due to the strongly inhibitory effect of guanidine hydrochloride on enzyme activity hampering the detection of lactate dehydrogenase sub-unit.

Electrophoresis in polyacrylamide gels in the presence of the anionic detergent, sodium dodecyl sulfate (SDS), has proven to be a useful tool for the rapid and simple estimation of the molecular weight of proteins and their sub-units (Shapiro et al, 1967).

The minimal molecular weight of lactate dehydrogenase was determined according to the techniques described by Weber and Osborne (1967) with some modification. Fig. 57 shows the relationship between the molecular weight and electrophoretic mobility for various proteins. The sub-unit molecular weight of the various reference proteins used are listed in Table III. The sub-unit molecular weight of lactate dehydrogenase was determined from Fig. 57 as 36,000.

Figure 57. Determination of the molecular weight of the sub-unit of NADH-specific lactate dehydrogenase from a set of eight individual standard gels. The reference proteins used are indicated.

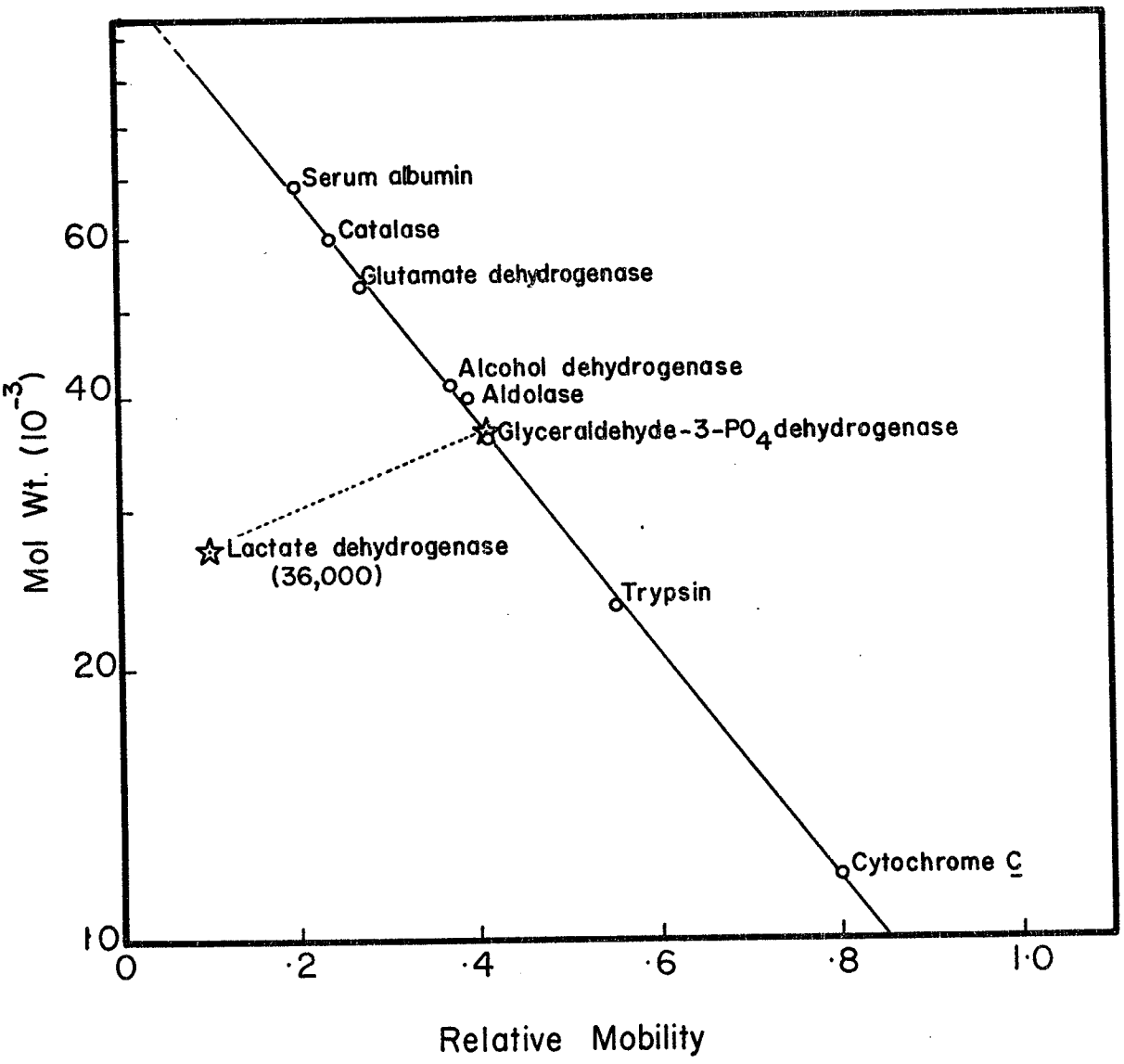

Serum albumin
Catalase
Glutamate dehydrogenase
Alcohol dehydrogenase
Aldolase
Glyceraldehyde-3-PO4 dehydrogenase
Lactate dehydrogenase
(36,000)
Trypsin
Cytochrome C
Mol Wt. (10-3)
60
40
20
10
0
·2
·4
·6
·8
1·0
Relative Mobility

Table III

Subunit molecular weight of reference proteins.

Protein	Mol. wt. of polypeptide chain
cytochrome c	11,700
trypsin	23,300
glyceraldehyde 3,P-dehydrogenase	36,000
aldolase	40,000
alcohol dehydrogenase (liver)	41,000
glutamate dehydrogenase	53,000
catalase	60,000
bovine serum albumin	68,000

DISCUSSION

DISCUSSION

Although the Aerobacter aerogenes lactate dehydrogenase shares similar properties with the E. coli enzyme, some degree of difference within these properties are observed. Both enzymes are inhibited by ATP, but the Aerobacter aerogenes enzyme is more sensitive to this inhibitor, being strongly inhibited by 1.0 mM ATP. Although an initial lag in the enzyme reaction was reported for the E. coli enzyme at pH 7.5, no lag in the enzyme reaction was observed for the Aerobacter aerogenes enzyme until pH values of 8.0 and higher were used for assay. Even in the presence of 0.3 mM NADH a lag could not be demonstrated at pH 5.7-7.5 at both high (5.0 mM) and low (1.0 mM) pyruvate. This lag at pH 8.0 was removed by preincubation of the enzyme with 5.0 mM pyruvate indicating that pyruvate interacts with the enzyme in some manner converting it to a more active form. The Aerobacter aerogenes lactate dehydrogenes is considerably larger than the other D-lactate dehydrogenases for which molecular weights have been determined. The lactobacillus plantarum enzyme appears to have a molecular weight of 60,000 to 70,000, and the enzyme from Leuconostoc mesenteroides has a molecular weight of 80,000. A molecular weight of 105,000 to 145,000 estimated for the Aerobacter aerogenes enzyme is in closer agreement to that from E. coli which has a molecular weight of 112,000 to 130,000 depending on the method employed to determine the molecular weight.

It has not been possible to crystallize the Aerobacter aerogenes enzyme to date. In the absence of dithiothreitol the enzyme is extremely labile. It was only when high concentrations of dithiothreitol were employed during purification procedures that any success in obtaining reasonable yields of purified enzyme was obtained. This requirement for thiol compounds is shared by the E. coli enzyme (Tarmy and Kaplan, 1968) for which it is indicated that the sulfhydryl groups of the enzyme must be fully reduced in order that the enzyme maintain full activity. Because of the low amount of enzyme protein obtained, purification procedures were cumbersome, since large volumes of crude extract were required. In addition centrifugations were tedious and time consuming due to the presence of slime which prevented the denatured protein from sedimenting at a favorable rate. Centrifugation speeds of 80,000 x g were required for at least 20 min in order for the suspended protein to pack sufficiently. Failure to obtain enzyme in large quantities made it impossible to perform binding studies and physical studies which require large amounts of enzyme.

The enzyme was judged pure by its sedimentation pattern in a Spinco Model E analytical centrifuge, and by polyacrylamide gel electrophoresis. At pH 5.7 in the Model E a major peak was observed with a small leading heavier shoulder. At pH 8.0 a single peak was observed with an $S_{w,20}$ equivalent to the

$S_{w,20}$ value of the major peak at pH 5.7. It appears that at very high enzyme concentration at acidic pH some degree of polymerization could be taking place as evidenced by the heavier leading shoulder in the sedimentation pattern. This heavier aggregating species was not detected in DEAE-cellulose or Sephadex G-200 chromatography due to dilution on the column. Zone centrifugation studies in 4 to 20% sucrose gradients at pH 5.7 in the absence of pyruvate showed an unsymmetrical enzyme activity profile indicating a light and heavy species. It appears that at these lower enzyme concentrations dissociation is occurring, and at higher concentrations (3 to 5 mg per ml) as employed in the Model E aggregation occurs. At alkaline pH this aggregation to polymers is either very slow so as not be be noticeable during the time course of the Model E run or does not occur at all. The appearance of several minor bands at pH 8.9 during polyacrylamide gel electrophoresis was unexpected since at alkaline pH (8.0) in the Model E only one protein peak was observed. It is possible that some degree of aggregation or dissociation is taking place due to protein-polyacrylamide interactions as the protein migrates through the polyacrylamide matrix. In most cases the minor bands followed the major band indicating protein association. Another interesting observation encountered with polyacrylamide gel electrophoresis was that sometimes during purification procedures the number of observed protein bands increased after

additional purification steps suggesting that there was some degree of interaction between the enzyme protein and the polyacrylamide matrix, which increased with increasing degree of purification.

One of the unusual properties exhibited by the D-lactate dehydrogenase from Aerobacter aerogenes is its inability to catalyze the reduction of NAD^+ with lactate (Hollier, 1967). This property is shared by the E. coli enzyme (Tarmy and Kaplan, 1968), and the Butyribacterium rettgeri enzyme (Wittenberger et al, 1967). In these organisms it appears that the regulation of the pyruvate-lactate interconversion is carried out by totally distinct enzymes; a D-lactate dehydrogenase which acts essentially as a pyruvate reductase, and by flavin-linked lactate oxidases which appear to be inducible enzymes. Although slight oxidation of the acetyl pyridine nucleotide analogue of the coenzyme NAD^+ could be demonstrated for the E. coli enzyme (Tarmy and Kaplan, 1968), and the Butyribacterium rettgeri enzyme (Wittenberger et al, 1967) no oxidation of the coenzyme analogue could be demonstrated for the Aerobacter aerogenes enzyme (Hollier, 1967).

The simplest and most likely explanation for the kinetic data reported here is that the Aerobacter aerogenes lactate dehydrogenase possesses at least two binding sites for pyruvate which interact with one another in a cooperative manner. This would seem to characterize it as an allosteric enzyme since such homotropic interactions appear to be

characteristic for all allosteric proteins. The kinetic behaviour of NAD^+ specific D-lactate dehydrogenase from Aerobacter aerogenes is quite similar to that observed for the enzyme from E. coli, Butyribacterium rettgeri, and rabbit muscle isozyme 5. In the acid pH range (5.2 to 5.7) the enzyme activity showed normal Michaelis-Menten type kinetics with respect to pyruvate concentration, while at pH 6.5 and higher the activity-pyruvate plots deviated from the normal kinetics, resulting in curved double reciprocal plots. The activity-NADH plots were always linear in the double reciprocal form (Hollier, 1967). In addition the enzyme showed a marked decrease in affinity for its substrate with increases in pH, although V_{max} appeared unaffected by pH changes. This property is shared with Butyribacterium rettgeri enzyme (Wittenberger et al, 1967) but not with the E. coli enzyme (Tarmy and Kaplan, 1968).

Until recently haemoglobin appeared as an almost unique example of a protein endowed with the property of mediating such indirect interactions between distinct specific binding sites. Following the pioneer work of Helmerich and Cori (1964) it became clear, especially during the past few years, that in bacteria as well as in higher organisms many enzymes are endowed with specific functions of metabolic regulation. A systematic comparative analysis of the properties of these proteins has lead to the conclusion that in most if not all of the indirect interactions between distinct specific binding sites (allosteric effects) are responsible for the performance of their regulatory function. By their

very nature, allosteric effects cannot be interpreted in terms of the classical theories of enzyme action. It must be assumed that these interactions are mediated by some kind of molecular transition (allosteric transition) which is induced or stabilized in the protein when it binds an allosteric ligand. It is clear from a multitude of recent studies that many enzymes display kinetic properties of allosteric enzymes such that the velocity as a function of substrate concentration shows a sigmoidal dependence, and that such behaviour may be affected by a modifier. A number of mechanisms are available for the interpretation of sigmoid kinetic data, the most restrictive one being that proposed by Monod, Wyman, and Changeaux (1965). The postulation of alternate or modified models to explain the data obtained in any given set of experiments becomes necessary when it is felt that the model proposed by Monod et al is not adequate. It has become evident that there are many enzymes which undergo a reversible association-dissociation reaction, i.e., specific polymerization, and that some of the kinetic properties of these enzymes may depend upon the particular molecular weight species. To date no definite model has been proposed for the mechanism of bacterial lactate dehydrogenases. Based on the kinetic results and some of the physical properties of A. aerogenes lactate dehydrogenase observed in the course of this investigation a model for the enzyme is proposed.

The proposal is made that subunits can interact to form a number of polymeric structures depending on various parameters in the environment. A schematic diagram is shown in Fig. 58. It should be emphasized that this model is to be considered as a guide in the discussion of the observed results obtained in this study rather than an actual interpretation of the molecular structure which would require a further study on the physical properties.

The model postulates that:

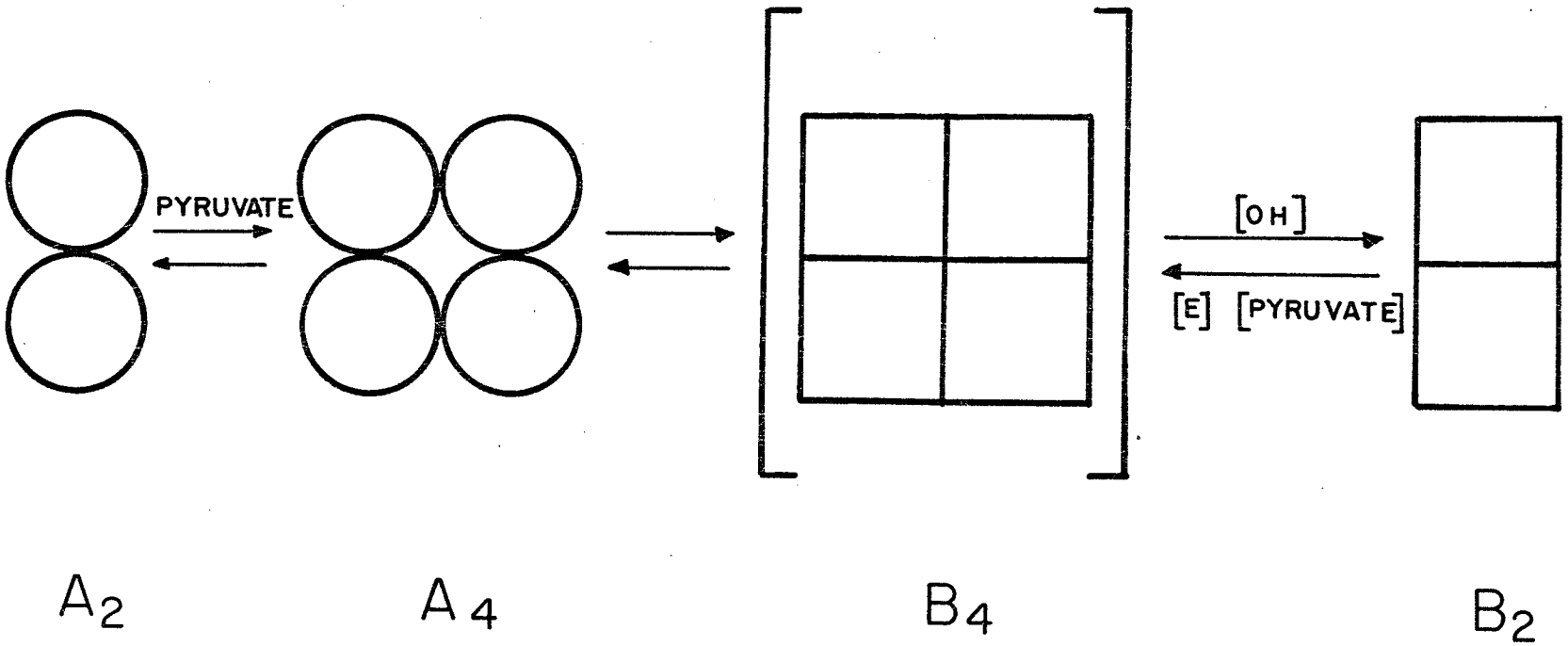
(a) the enzyme exists in two basic conformational states, an active state A (Monod's R state) and an inactive state B (T state). Both states exist in dimeric and tetrameric forms. These forms are designated A_2, A_4, B_2, and B_4.

(b) A_2 and A_4 are equally active.

(c) The conversion between form A and B is dependent upon pH, and pyruvate and enzyme concentrations.

It is also assumed in the model that the equilibrium constant for $A_4 \rightleftharpoons B_4$ transition favors the formation of A_4, i.e., $K = B_4/A_4 << 1$ and the constant for $B_4 \rightleftharpoons 2B_2$ dissociation favors the dissociation, i.e., $K' = B_2^2/B_4 >> 1$. Under these conditions B_4 is not present at any appreciable concentration and the transition from A_4 to B_2 is governed by a constant, $L = K.K' = B_2^2/A_4$. Since it is assumed that K' increases with increasing pH, L can have a large value at high pH's. Following the model by Monod *et al*, if only the

Fig. 58. A schematic model for the Aerobacter aerogenes NADH-specific lactate dehydrogenase.


PYRUVATE
[OH]
[E] [PYRUVATE]
A2
A4
B4
B2

active A state can bind pyruvate (one pyruvate per monomer), then sigmoid activity-pyruvate concentration plots will be expected at high pH's. When the enzyme concentration is raised it is obvious that A_4/B_2 ratio will increase and the sigmoidicity of the activity-pyruvate plots will decrease.

Using this model the results obtained in this work will be discussed.

MOLECULAR WEIGHT

At acidic pH values in the range 5.2 to 5.7 the enzyme exists as an active dimer and active tetramer, A_2 and A_4. Under assay conditions (in the presence of pyruvate) the active dimer polymerizes to the active tetramer, so that the A_4 species is predominant during assay procedures at pH 5.7. These proposals are supported by molecular weight studies. At pH 5.7 the molecular weight of lactate dehydrogenase was estimated to be approximately 100,000 in the absence of pyruvate, and 145,000 in the presence of pyruvate. The subunit molecular weight of the enzyme determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate is 36,000. This subunit molecular weight of 36,000 validates the postulation of a tetrameric species at pH 5.7. Although a molecular weight of 100,000 is slightly higher than would be predicted for a dimer, it should be stressed that the molecular weight obtained from sucrose

gradient analysis in the absence of pyruvate was estimated from the peak of the enzyme activity profile, and that the entire profile represents a heavier (probably tetramer) and lighter species (mixture of dimer and tetramer). Activity profiles were not symmetrical indicating non-homogeneity of molecular species both in the presence and absence of pyruvate. With this in mind it seems reasonable to postulate the existence of a dimer and a tetramer.

At pH values of 6.5 and greater the active tetramer A_4 is converted to the inactive dimer B_2. The equilibrium between the two forms is a function of pH and pyruvate and enzyme concentrations. This is postulated since at alkaline pH enzyme activity deviates from normal kinetics and shows a sigmoid rate saturation curve. Since the V_{max} is not affected by pH changes, at saturating pyruvate concentrations B_2 is completely converted to the active A_4 tetramer. Evidence for the tetrameric species in this pH range at high enzyme concentrations is supported by sucrose gradient molecular weight studies in which at pH 7.0 and pH 8.0 molecular weights of 145,000 were estimated in both the absence and presence of pyruvate. In addition the $S_{w,20}$ value at pH 5.7 and pH 8.0 were equal at high enzyme concentration suggesting that at pH 5.7 and pH 8.0 a tetrameric species exists. The inactive tetramer B_4 is dissociated to the inactive dimer B_2. The equilibrium of this dissociation-association is dependent

upon pH, enzyme concentration, and pyruvate as shown in the model. Evidence to support the dissociation of the tetramer to the dimer at alkaline pH is that at pH 8.0 and pH 9.0, at low enzyme concentration (0.5 to 1.0 units) molecular weights of 80,000 were estimated. Although no change in molecular weight from 145,000 was observed at pH 7.0 at low enzyme concentration, it is assumed that lower enzyme concentrations than those used in sucrose gradients are required to display an observed decrease in molecular weight. Since at pH 8.0 and pH 9.0 dissociation was noticeably observed, it is concluded that at alkaline pH the enzyme probably exists as a dimer under dilute conditions as in assay procedures. It is well documented that enzymes classified as allosteric are composed of subunits, and some of these enzymes do show a tendency to dissociate to a smaller molecular weight form at very low enzyme concentrations. It seems probable that under the dilute conditions of assay, these enzymes may dissociate to a molecular weight species having different binding or activity characteristics. At increasing enzyme concentrations molecular weights at alkaline pH increased from 80,000 to 145,000. In addition at low enzyme concentration at alkaline pH the molecular weight increased from 80,000 to 110,000 $\pm$ 10,000 in the presence of pyruvate. The polymerized enzyme is dissociated to the monomer by guanidine hydrochloride and can be reassociated to the active tetramer by dialysis and

removal of guanidine hydrochloride, showing that the dissociation-association phenomenon is a reversible process. For the E. coli enzyme no evidence has been obtained to suggest that the enzyme is dissociated into subunits at low concentrations of protein at pH 7.5 (Tarmy and Kaplan, 1968). Molecular weight studies of the E. coli enzyme indicated that pyruvate had no effect on the molecular weight (130,000 $\pm$ 15,000),(Tarmy and Kaplan, 1968), in contrast to the Aerobacter aerogenes enzyme which shows aggregation in the presence of pyruvate. Failure to observe any effect by pyruvate on the molecular weight of the E. coli enzyme may be due to the pH at which the study was undertaken. The optimum pH for the E. coli enzyme is approximately 6.5, in contrast to the Aerobacter aerogenes enzyme which displays a pH optimum of 5.5 (Hollier, 1967). In the case of the E. coli enzyme, it is possible that higher pH values that 7.5 are required to observe changes in molecular weight in the presence of pyruvate, or dissociation at lower enzyme concentrations.

KINETIC BEHAVIOUR

It appears that the Aerobacter aerogenes lactate dehydrogenase depends on a complex conformational change involving association-dissociation equilibrium. At acidic pH values in the range 5.2 to 5.7, under assay conditions the enzyme exists as the active A_2 and A_4 species, but predominantly as the A_4 species since in the presence of pyruvate the equili-

brium between the A_2 and the A_4 species is shifted in favor of the active tetramer A_4. In this pH range kinetics displayed by the enzyme are of the Michaelis-Menten type. The rate saturation curves are hyperbolic and double reciprocal plots of enzyme activity and pyruvate concentration are linear. Hill plots showed a slope value of 1.0 indicating that the reaction is first order with respect to pyruvate. At more alkaline pH values (pH 6.5 and greater) kinetics deviate from the normal Michaelis-Menten kinetics. The rate saturation curve is sigmoidal indicating cooperativity in substrate binding. It appears that hydroxyl ions as ligands can play an important role in the activity of this enzyme. This cooperativity with increasing pH can be explained according to the proposed model as follows. Increasing pH (OH^- ions) brings about a conformational change in the enzyme structure such that the active tetramer A_4 is converted to the inactive dimer, B_2. It is assumed that at low enzyme concentration as would be present in a cuvette during assay procedures at pH 6.5 and greater the enzyme exists predominantly in the B_2 state. In the presence of pyruvate the B_2 dimer polymerizes to the B_4 tetramer. Since the equilibrium between A_4 and B_4 is always in favor of A_4 formation, the formation of B_4 immediately increases the amount of A_4 tetramer. With increasing concentrations of pyruvate the amount of active A_4 tetramer increases so that observed activity increases. This explains the

observed cooperative effects at pH 6.5 and greater. Since at saturating pyruvate concentration the predominating species is the active A_4 tetramer, the V_{max} remains the same at pH 6.5 and greater as the value at pH 5.2 to 5.7. A sigmoid curve of rate as a function of substrate concentration indicates that the enzyme interacts in some way with more than one molecule of substrate. The simplest assumption is that each enzyme molecule contains several catalytically active sites, although as has been pointed out (Atkinson et al, 1965) this is not a necessary condition for kinetics of order higher than one. Hill plots at alkaline pH show a slope value of approximately 2.0 indicating the reaction at this pH is second order with respect to pyruvate. This slope value "n" is a function of the number of interacting binding sites per enzyme molecule and the strength of interaction. When the interactions are strong the slope will be numerically equal to "n", the number of sites. If the interactions become weakened, no matter what the number of binding sites is the slope "n" decreases to a value of one. From Fig. 23 it is observed that "n" increased from 1.06 to 2.01 as the pH was raised from 5.7 to 7.0. At pH 7.0 at very low enzyme concentrations "n" values as high as 2.6 at 0.0025 μM enzyme were observed (Fig. 42). This latter detail will be discussed later. It appears therefore that the enzyme possesses at least two or possibly three pyruvate binding

sites and that the interaction between these sites increases with increasing pH. Although these data do not indicate the nature of these sites, whether they are catalytic or allosteric, or the exact nature of the interactions between these sites, they do not contradict with the proposed model since the maximum number of pyruvate binding sites in A_4 should be 4.

Since desensitization of the enzyme from the stopped flow experiments was achieved with a highly purified enzyme preparation failure to permanently desensitize lactate dehydrogenase from the crude extract may be due to the presence of some unidentified factor(s) present in the crude extract preventing desensitization. Further work will have to be carried out to resolve this problem.

EFFECT OF ENZYME CONCENTRATION

Many enzyme exist intracellularly in such high concentrations that dilution of cell homogenates several hundred fold is required for spectrophotometric assay. Sigmoid rate saturation curves observed using dilute lactate dehydrogenase preparations at pH 7.0 were converted to normal Michaelis-Menten type kinetics at high enzyme concentrations (0.1 μM or higher). These results indicate that the kinetic properties of the enzyme determined at highly dilute concentrations in vitro are quite different from the observed kinetic properties at much higher concentrations that exist

in vivo. The purified D-lactate dehydrogenase from Aerobacter aerogenes has a specific activity of 1000 units per mg protein. Since 1000 grams of packed wet cells after sonication released 31,500 units of the enzyme, the concentration of the enzyme in Aerobacter aerogenes is higher than 31.5 mg per liter, the volume calculated assuming that 1000 grams of cells occupy one liter of volume. The molecular weight of the enzyme determined by sucrose gradient centrifugation was approximately 140,000. The minimum concentration of enzyme in the cells is calculated as $31.5 \times 10^{-3}/140,000$, i.e., 2.25×10^{-7} M or 0.225 μM. It is therefore concluded that the lactate dehydrogenase of Aerobacter aerogenes shows normal Michaelis-Menten kinetics with respect to pyruvate when the enzyme concentration approaches the concentration in the cells.

The model proposed here can reasonably account for this observed effect of high enzyme concentration on the kinetic behaviour of lactate dehydrogenase. Because the equilibrium between the inactive tetramer B_4 and the inactive dimer B_2 is affected by enzyme concentration (dissociation occuring at low enzyme concentration at alkaline pH) as supported by molecular weight studies, the model predicts that in the cuvette under assay procedures, the enzyme exists as the inactive dimer B_2. As the enzyme concentration is increased the equilibrium between B_4 and B_2 is shifted to the left

favoring formation of the B_4 tetramer. It is important to stress again that the equilibrium between the A_4 tetramer and the B_4 tetramer is strongly in favor of A_4 formation. As the B_4 increases with increasing enzyme concentration, it is immediately converted to the A_4 tetramer, until at very high enzyme concentration all enzyme is in the A_4 state resulting in normal Michaelis-Menten type kinetics. This is highlighted by the observation in Fig. 40 where double reciprocal plots of enzyme activity and pyruvate concentration became less curved as enzyme concentration increased from 0.0025 μM to 0.025 μM. As the enzyme concentration is increased further, the double reciprocal plot became perfectly linear at 0.1 μM enzyme (Fig. 41). Linear double reciprocal plots were still observed at enzyme concentrations as high as 1.0 μM. The slope value "n" from the Hill plots in Fig. 42 and 43 decreased from 2.6 at 0.0025 μM enzyme to an average of 1.6 at 0.025 μM enzyme, and decreased further to a numerical value of one at 0.1 μM enzyme.

EFFECT OF SUBSTRATE ANALOGUES

Significantly the *Aerobacter aerogenes* lactate dehydrogenase shares certain kinetic properties with the *E. coli* and the *Butyribacterium rettgeri* enzyme, and with such well studied enzymes as aspartate transcarbamylase and yeast NAD^+ isocitric dehydrogenase. The effect of α-ketobutyrate on

enzyme activity is similar to that observed for the E. coli (Tarmy and Kaplan, 1968) and the Butyribacterium rettgeri (Wittenberger et al, 1967) enzyme. At alkaline pH (6.5 and greater) where sigmoid rate saturation curves were observed, α-ketobutyrate actually stimulated the reaction rate at a limiting concentration of pyruvate, although it acted as an inhibitor of the reaction at a saturating concentration of pyruvate. This kinetic response of lactate dehydrogenase is analagous to that observed with aspartate transcarbamylase in the presence of the aspartate analogue maleate (Gerhart and Pardee 1963, 1964) and also to that exhibited by yeast NAD^+ isocitrate dehydrogenase in the presence of the isocitrate analogue citrate (Hathaway et al, 1963). The addition of 20 mM α-ketobutyrate to the standard NADH-pyruvate assay system resulted in a transposition of the curve of the rate with respect to pyruvate concentration from a sigmoid to rectangular hyperbola. It appears that at alkaline pH α-ketobutyrate not only increases the affinity of the enzyme for pyruvate at low substrate concentrations but also alters the interaction between binding sites. At pH 7.0 the molecular weight of lactate dehydrogenase was estimated to be 110,000 in the presence of 20 mM α-ketobutyrate, suggesting dissociation from the tetrameric species. The assumption is made that in the presence of saturating α-ketobutyrate at both acidic and alkaline pH the enzyme exists in a completely new dimer state,

C_2, which is active but less active than the active A_2 dimer and A_4 tetramer, and is completely desensitized to the cooperative effect of pyruvate. This conversion to the less active C_2 dimer accounts for the observed inhibition at pH 5.7 in the presence of 20 mM α-ketobutyrate and the activation at alkaline pH. At alkaline pH (6.5 and greater) the interconversion between the B_2 dimer and the A_4 tetramer is no longer in effect in the presence of saturating α-ketobutyrate, and sigmoid kinetics are abolished since all the enzyme is in the C_2 form. The postulation of this C_2 species accounts for the observed inhibition by α-ketobutyrate at saturating pyruvate at pH 6.5 and greater. According to the model, at saturating pyruvate concentrations at pH 6.5 in the absence of α-ketobutyrate the enzyme is in the active A_4 tetrameric state. In the presence of 20 mM α-ketobutyrate the enzyme exists in the C_2 dimer state which is less active than the A_4 tetramer, but nevertheless is still active. Obviously, at saturating pyruvate in the presence of α-ketobutyrate inhibition occurs over that observed in the absence of α-ketobutyrate because of the lower activity possessed by the C_2 dimer. At low pyruvate concentration α-ketobutyrate activates the enzyme because the C_2 dimer is active and B_2 dimer is inactive. At pH 5.7 inhibition by α-ketobutyrate is non-competitive. This is interesting because it was unexpected that a substrate analogue should show non-competitive inhibition rather than the expected competitive type. Oxamate

another analogue of pyruvate was a noncompetitive inhibitor with respect to pyruvate at acidic pH (5.7), but could not duplicate the desensitizing effect of α-ketobutyrate at pH 6.5 or 7.0. The same was true of α-ketoglutarate, a competitive inhibitor with respect to pyruvate at pH 5.7. At pH 7.0 inhibition was still observed in the presence of α-ketoglutarate but double reciprocal plots of enzyme activity and pyruvate concentration were still non-linear. The molecular weight of lactate dehydrogenase at pH 7.0 in the presence of 30 mM α-ketoglutarate was estimated to be 80,000. The implication of this molecular weight change cannot be explained as sufficient data is not available. Further studies will have to be carried out to reveal the significance of this observation.

EFFECT OF ATP

ATP does not appear to be an allosteric effector of the Aerobacter aerogenes lactate dehydrogenes. Inhibition was non-competitive with respect to pyruvate at pH 5.7 and replots of slopes and intercepts against ATP concentration were linear and not curved. Inhibition by ATP was still observed at pH 7.0. The slope of the Hill plots (Fig. 39) remained essentially the same in the absence and presence of ATP indicating that ATP does not affect the interaction between enzyme and substrate. Inhibition by ATP with respect to the coenzyme NADH was competitive at pH 5.7 and a replot of the slope against ATP concentration was linear.

Since no appreciable molecular weight change was observed in sucrose gradients in the presence of 10.0 mM ATP it appears that the observed inhibition is not associated with association or dissociation. It appears that ATP merely competes with NADH for the nucleotide site and acts as a simple competitive inhibitor. No evidence has been acquired to suggest that ATP binds at a distinct site as proposed for the Butyribacterium rettgeri enzyme (Wittenberger, 1968).

The failure of other nucleotides, ADP, GTP, and GDP to inhibit enzyme activity suggests that the NADH site cannot be occupied by these nucleotides, and that the NADH site is specific for NADH and ATP only.

The initial lag in the enzyme reaction in the presence of ATP further supports the suggestion that ATP and NADH occupy the same site. At NADH concentrations of 0.45 mM and greater an initial lag in the enzyme reaction was observed. The length of this lag was related to the NADH concentration. In addition enzyme activity was inhibited at NADH concentrations greater than 0.30 mM. The inhibition and the length of the lag were augmented when the enzyme was preincubated in the presence of high NADH for several minutes. If ATP and NADH are binding at a common site the lag observed in the presence of ATP could be due to ATP binding at the NADH site and mimicking NADH to produce an initial lag.

Under the partly anaerobic conditions employed for the growth of cells in this investigation, a major portion of energy is being generated during the breakdown of glucose to pyruvate. The inhibition of lactate dehydrogenase by ATP makes it possible for the cell to regulate the rate of glycolysis when ATP generation exceeds the need for biosynthetic reactions. Thus, ATP is probably acting as an inhibitor in controlling the rate of glycolysis. When the ATP level is high glycolysis is proceeding at a rate faster than that required by the cell, and so ATP inhibits lactate dehydrogenase, resulting in a reduction of NAD^+ production. Since NAD^+ is a rate limiting factor in the triose phosphate oxidation by triose phosphate dehydrogenase, glycolysis is slowed down.

EFFECT OF NEUTRAL SALTS

When anions or cations are observed to affect enzymatic activity, it is natural to think they do so because of their binding near the active site where they exert local effects on the electric field directly involved in the enzymatic process. When the ion concentrations are low (0.1 M or less) the foregoing explanation may undoubtedly be correct. On the other hand, at considerably higher salt concentrations (1 to 3 M) it is possible that activity effects arise because ions are disrupting organized structures in the enzyme which are necessary to carry on catalysis, and in this way may indirectly alter the enzymatic process.

It has been recently demonstrated (Warren et al, 1966) that neutral salts at high concentrations inhibit the activity of widely different enzymes in an order of increasing effectiveness for anions ($Ac^- < Cl^- < NO_3^- < Br^- < I^- < SCN^- < ClO_4^-$). Because the order is similar to that in which these ions disrupt the structure of diverse macromolecules as determined by physical methods (Von Hippel and Wong, 1964), it seems plausible that the observed inhibition may result at least in part from the disruption of organized enzyme structure. Since the anion concentrations employed in this study were 0.1 M and lower it is difficult to speculate whether or not the observed inhibition is due to structural dissruption. The anions studied had no affect on molecular weights. However, this does not rule out the possibility of structural changes since molecular weight changes merely indicate the state of aggregation of polypeptide chains and do not indicate any structural change in the individual polypeptide itself.

Because the anions employed in this study (Cl^-, SCN^-, and $SO_4^=$) inhibited enzyme activity competitively with respect to pyruvate it seems reasonable to assume that the observed inhibition is a result of the competition of these anions with pyruvate for the substrate site. The mechanism by which an inorganic ion can competitively inhibit the association of an enzyme with its substrate is not readily apparent, but several hypothesis may be developed to explain this observation.

A possible mechanism is that pyruvate could be attached to the active enzyme-coenzyme complex by electrostatic forces. The introduction of ionic compounds into the reaction mixture could possibly prevent the association of pyruvate with the complex. Assuming the ionic form of pyruvate is the substrate for lactate dehydrogenase, then the inorganic components would be competing with pyruvate for the active site on the enzyme.

Since replots of slopes against anion concentration were linear with all anions tested allosteric effects by anions were ruled out. Slopes of the Hill plots at pH 7.0 did not change in the presence of KCl (Fig. 49) indicating that KCl did not affect the interactions between enzyme and substrate.

The failure of acetate and phosphate to display inhibition at concentrations between 0.025 M and 0.1 M at pH 5.7 was surprising. The inhibiting effectiveness of acetate according to the Hofmeister series is the weakest with respect to the other anions in the series. On this account the failure to observe inhibition by 0.1 M acetate can be dismissed. However, phosphate was expected to display some inhibition since sulfate, an analogue of phosphate, was a strong inhibitor at concentrations as low as 0.016 M. This phenomenon may be a reflection of the stereospecificity of the enzyme for charged groups. Another possible explanation for the difference in inhibiting effectiveness between sulfate and phosphate may lie in the relative charge of the anionic species at pH 5.7.

At this pH phosphate is predominantly in the monovalent state, whereas sulfate is in the divalent state. This increased charge on the sulfate ion may account for the difference in inhibiting effectiveness between phosphate and sulfate.

This same explanation could account for the observed inhibition by phosphate with respect to acetate at pH 7.0 where acetate is monovalent and phosphate is a mixture of mono and divalent forms. The observed activation by acetate and phosphate to a maximum at 0.05 M anion concentration followed by inhibition at higher salt concentrations suggests that at alkaline pH where allosteric effects are observed, the enzyme is more sensitive to changes in ionic environment than at acidic pH.

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