PURIFICATION AND PROPERTIES OF ACETOLACTATE SYNTHETASE OF *AEROBACTER AEROGENES*

by

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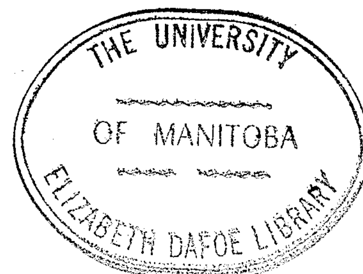

TO MY NEPHEW KENNETH MICHAEL

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ABSTRACT

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Acetolactate synthetase, the enzyme which catalyzes the condensation of pyruvic acid to α-acetolactic acid, was extracted and purified from *Aerobacter aerogenes*. In order that a detailed kinetic study of the enzyme could be carried out, a spectrophotometric assay procedure was developed. It was found that the enzyme reaction had an initial lag, after which the reaction became linear with respect to time. All attempts to remove the lag were unsuccessful. The enzyme yielded a non-linear plot when the reciprocals of enzyme activity were plotted against the reciprocals of pyruvate concentration. Various metabolic intermediates, and compounds structurally related to pyruvic acid did not change the nature of the non-linear kinetics. Isocitric acid inhibited enzyme activity. The molecular weight of the enzyme was estimated to be 242,000 in the absence of sodium pyruvate, and 168,000 in the pressence of sodium pyruvate. No evidence could be found for Schiff's base formation.

ABBREVIATIONS

DPNH - diphosphopyridine nucleotide (reduced)

DEAE-cellulose - diethyl amino ethyl cellulose

TDP - thiamine diphosphate

ATP - adenosine triphosphate

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INTRODUCTION

INTRODUCTION

Purification of acetolactate synthetase from *Aerobacter aerogenes* was first carried out by Juni (1952). Using manometric techniques, a linear relationship was obtained 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 α-hydroxy ethyl thiamine, which condensed with the second molecule of pyruvate to form α- acetolactic acid.

Evidence was presented for the existance 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, showed a pH optimum of 8.0 and the other enzyme not involved in valine or isoleucine biosynthesis, showed a pH optimum of 6.0. This pH 8.0 enzyme was also found in *Escherichia coli* (Umbarger and Brown, 1958) and in *Salmonella typhimurium* (Bauerle *et al* 1964).

In studies of the biosynthetic enzyme from *Salmonella*

typhimurium, double reciprocal plots of enzyme activity and pyruvate concentration yielded a straight line when the colorimetric techniques were used to follow the course of the enzyme reaction. This enzyme was inhibited by valine and showed requirement for flavine adenine dinucleotide (Bauerle et al, 1964; Stormer and Umbarger, 1964).

Recently it has been shown that pyruvic acid can form a Schiff's base with the ϵ-amino group of lysine in 4-hydroxyketoglutaric aldolase (Rosso and Adams, 1966) and 2-keto-3-deoxy-6-phosphogluconate aldolase (Grazi et al, 1963). It appears that Schiff's base formation is a general phenomenon in biological aldol condensation reactions.

In the present study a spectrophotometric assay method for the enzyme was developed, and used to study the kinetics in physiological concentrations of pyruvate, since the previous linear Lineweaver-Burk plots were obtained using very high concentrations of pyruvate. A kinetic study of the enzyme was undertaken to confirm the chemical mechanism of α-acetolactate syntheses proposed by Krampitz et al (1961).

A possibility of Schiff's base formation during the enzyme reaction was examined in order to compare aldol condensation reactions involving thiamine diphos-

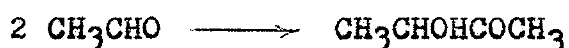
phate with those not involving thiamine diphosphate.

The properties of the enzyme under study were compared with those of the biosynthetic enzyme studied by Bauerle *et al* (1964) and Halpern and Umbarger (1959).

HISTORICAL

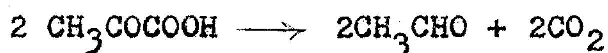
HISTORICAL

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.



$$2\ CH_3CHO \longrightarrow CH_3CHOHCOCH_3$$

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, 1947a). 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.

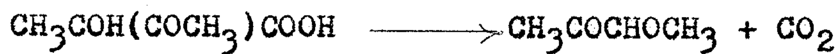
By grinding cells with powdered glass, Silverman and Werkman (1941) extracted an enzyme preparation from cells of Aerobacter aerogenes capable of converting pyruvic acid to acetoin and CO_2. In their studies on pyruvic acid metabolism of bacteria, Watt and Krampitz (1947) suggested that a new intermediate, 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.

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

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



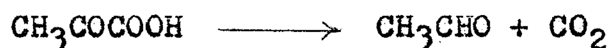
$$CH_3COH(COCH_3)COOH \longrightarrow CH_3COCHOCH_3 + CO_2$$

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 Karczog, 1911) brings about the following reaction:



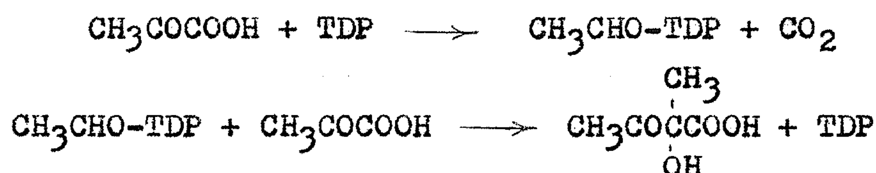
$$CH_3COCOOH \longrightarrow CH_3CHO + CO_2$$

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 mechanism 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

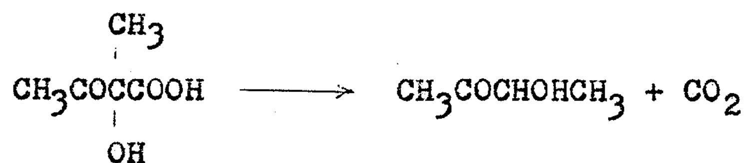
diphosphate. Lipmann (1936) suggested an oxidation-reduction mechanism involving the quaternary nitrogen of the thiazole moiety. Ingraham and Westheimer (1956) as well as Breslow (1956), proposed that carbanion formation on the N-methylene group of thiamine was involved. None of these mechanisms have 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; Yatco-Manzo _et al_, 1959). The following equations have been employed to describe the formation of acetoin from pyruvic acid:



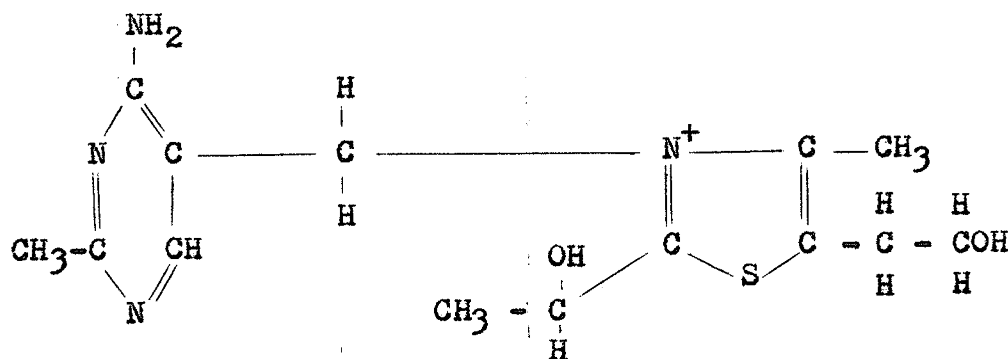
$$CH_3COCOOH + TDP \longrightarrow CH_3CHO\text{-}TDP + CO_2$$

$$CH_3CHO\text{-}TDP + CH_3COCOOH \longrightarrow CH_3CO\underset{OH}{\overset{CH_3}{C}}COOH + TDP$$



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.

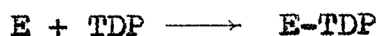
The second important observation was made by Breslow (1959) who found that the hydrogen at position 2 of the thiazole ring exchanged with deuterium of deuterium oxide under conditions of the non-enzymatic system (pH 8.8). He proposed that the initial attack of pyruvic acid was on the carbanion formed 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.

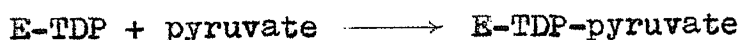


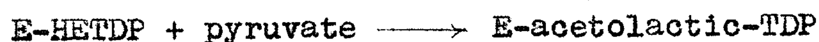
(HET)

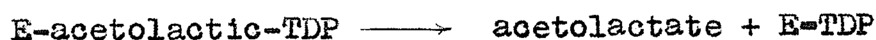
It was established that this intermediate replaced thiamine in microbial nutrition experiments and substituted for thiamine diphosphate in yeast carboxylase preparations containing ATP and thiaminokinase (Krampitz et al, 1961). Krampitz et al (1959) showed that HET and acetaldehyde, adjusted to pH 8.5,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

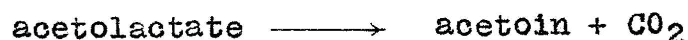
acid to acetoin. The enzyme complex from Aerobacter aerogenes, which forms acetoin from pyruvic acid with α-acetolactic acid as an intermediate, resembles in some detail the non-enzymatic system. The reaction sequence for the system can be written as follows:











where E represents acetolactate synthtase.

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-acetohydroxybutyrate (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 Umbarger and Halpern (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 recently found in E. coli (Umbarger and Brown, 1958) and Salmonella typhimurium (Bauerle et al, 1964) having a pH optimum of 8.0.

Evidence for the existence of a covalent complex between the enzyme fructose diphosphate aldolase and one of its substrates, dihydroxyacetone phosphate, has been presented by a number of workers (Rose and Reider, 1955; Bloom and Topper, 1956; Rose and Reider, 1958; Rutter and Ling, 1958). The aldol condensation reactions catalyzed by fructose diphosphate aldolase and transaldolase have been shown to involve Schiff's base formation with the substrate carbonyl groups (Horecker et al, 1961). The active site of the enzyme responsible for the formation of the active intermediate has been identified with both aldolases as ϵ-amino group of lysine (Grazi et al, 1962a). When the Schiff's base:enzyme complex is treated with boro-

hydride, it is reduced to a stable derivative and at the same time the active site is blocked and enzyme activity is lost (Grazi et al, 1963). The results with transaldolase and fructose diphosphate aldolase suggest that similar reducible linkages may be formed with aliphatic carbonyl groups and that the borohydride technique can be extended to other enzymes in order to bring about stabilization of complexes involving the active site of the enzyme. Recently evidence has been obtained for Schiff's base formation with 2-keto-3-deoxy-6-phosphogluconate aldolase (Grazi et al, 1963), suggesting that an azomethene group is formed between an amino group, presumably the ϵ-amino group of lysine, and the carbonyl group of pyruvic acid. It has also been shown that pyruvic acid can form a Schiff's base complex with the ϵ-amino group of one or more lysines in 4-hydroxyketoglutaric aldolase (Rosso and Adams, 1966).

These findings provide support for the hypothesis that Schiff's base formation is a general phenomenon in biological aldol condensation reactions. Since the formation of α-acetolactic acid from pyruvic acid is essentially an aldol condensation reaction, it is possible that a Schiff's base intermediate may be involved in the reaction.

METHODS AND MATERIALS

METHODS AND MATERIALS

GROWTH OF BACTERIAL CULTURE

A laboratory strain of *Aerobacter aerogenes 28-A* 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 distilled water in a 3 liter Fernbach culture flask, stoppered with a cotton plug, and autoclaved for 20 min. at 120°C. The glucose was dissolved in 500 ml. of distilled 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 equilibrium was achieved, then innoculated with a loopfull of a culture of *Aerobacter aerogenes* from a nutrient slant, and incubated at 28°C without shaking or aeration for 18-24 hours. The cells were harvested by centrifugation in a Sharples centrifuge at 50,000 r.p.m., washed in 0.13 M potassium phosphate buffer, of pH 5.7, and centrifuged at 30,000 x g for 1 hour. The high speed and long time were 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.

CENTRIFUGATION

All centrifugations were done at 0-4°C. in a Servall RC-2 refrigerated centrifuge, unless otherwise stated.

PREPARATION OF CRUDE EXTRACT

The cell paste was homogenized in 0.05 M potassium buffer of pH 7.0 containing 0.01 M mercaptoethanol, using 2 ml. of buffer for each gram of cell paste. The homogenized suspension was sonicated in 50 ml. portions in a 10-KC Ratheon oscillator for 20 min., followed by centrifugation at 30,000 x g for 1 hour.

PURIFICATION OF ACETOLACTATE SYNTHETASE

First Ethanol Precipitation

To the slightly turbid cell-free extract was added thiamine diphosphate 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 at 0°C. and the temperature was gradually lowered to -15°C. by the addition of more salt as the alcohol concentration increased. The mixture was then placed in a freezer at

$-20^{o}C$. for 12 hours to allow maximum precipitation to take place. The precipitate was collected by centrifugation at 30,000 x g for 1 hour, at $-15^{o}C$, homogenized in 0.05 M potassium phosphate buffer of pH 7.0 containing 0.1 mM thiamine diphosphate, 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 1 hour. The supernatant contained all 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 1 hour. The ratio of the optical density at 280 mμ/the optical density at 260 mμ increased from 0.55 to 0.7-0.8 during this procedure due to the removal of nucleic acid.

Ammonium Sulfate Precipitation

To the protamine sulfate supernatant, ammonium sulfate was slowly added with 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 1 hour. When precipitation did not occur immediately the mixture was placed in the freezer at $-20^{o}C$. until pre-

cipitation took place. After centrifugation the precipitate was dissolved in 0.05 M potassium phosphate buffer of pH 7.0 containing 0.1 mM thiamine diphosphate, 0.5 mM $MnCl_2$, and 10 mM mercaptoethanol, using one quarter the volume as that of the original cell-free extract. The precipitated enzyme made a clear yellowish solution when dissolved in the buffer. This 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 1 hour at -15°C. and homogenized in 0.05 M potassium phosphate buffer of pH 7.0 containing 0.1 mM thiamine diphosphate, 0.5 mM $MnCl_2$, and 0.1 mM dithiothreitol. The turbid solution was centrifuged for 30 min. to remove any insoluble protein. The supernatant at this point was clear and colorless.

DEAE-Cellulose Chromatography

DEAE-cellulose (medium mesh) was washed with distilled water, then twice with 0.5 N NaOH, then again with distilled water until the pH was about 10.0. 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 6.5, and finally suspended in potassium phosphate buffer of the same molarity and pH. The DEAE-cellulose was packed in a column (1.5 cm. diameter) to a height of 25 or 30 cm., and equilibrated with 1 liter of 0.05 M potassium phosphate buffer of pH 6.5 containing 0.1 mM thiamine diphosphate, 0.5 mM $MnCl_2$, and 0.1 mM dithiothreitol. This column was used for the chromatography of 5.0 mg. of protein or less. For more than 5.0 mg. of protein a larger column (3 cm. x 30 cm.) was used. The enzyme was gently placed on the column, and eluted with a batchwise elution method using 0.05 M, 0.1 M, 0.15 M, 0.2 M potassium phosphate buffer of pH 6.5 containing 0.1 mM thiamine diphosphate, 0.5 mM $MnCl_2$, and 0.1 mM dithiothreitol. The eluent was collected in 2 ml. fractions using an RSCO (Research Specialities Company) automatic fraction collector. An enzyme peak was located by assaying every third tube as follows:

One tenth of a milliliter of eluent was incubated in 0.475 ml. of 0.13 M potassium phosphate buffer of pH 5.7,

0.1 ml. of acetolactate decarboxylase, and 0.025 ml. of 1.0 M sodium pyruvate (25 μmole) for 20 min. at room temperature. The reaction was stopped by the addition of 0.2 ml. of 5.0 N H_2SO_4, and the amount of acetoin was determined.

The contents of those tubes which showed enzyme activity were pooled, made 80% saturated with ammonium sulfate, and the precipitate was collected by centrifugation at 30,000 x g for 30 min. The precipitate was then dissolved in 0.05 M potassium phosphate buffer of pH 7.0 containing 0.1 mM thiamine diphosphate, 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, and centrifuged to remove any insoluble protein. The clear enzyme solution was then placed on a DEAE-cellulose column (3 cm. x 30 cm.) which was previously equilibrated with 1 liter of 0.05 M potassium phosphate buffer 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 ESCO automatic fraction collector. An enzyme peak was located by assaying every third tube for enzyme activity 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 DPNH, and 0.1 ml. of eluent respectively. The rate of oxidation of DPNH was observed at 340 mμ on a Unicam SP.700 spectrophotometer. Those tubes 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 lactic dehydrogenase. One unit is defined as the amount of enzyme which brings about 1.0 optical density change per minute.

PREPARATION OF ACETOLACTATE DECARBOXYLASE

This preparation 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 then added to 80% saturation and the mixture allowed to stir for 30 min. in the cold. 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 against 1 liter of the same fresh buffer for 2 hours, and again for 2 hours against 1 liter of fresh buffer. This preparation was free of lactic dehydrogenase.

PROTEIN DETERMINATION

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951), using crystalline bovine albumin as a standard.

To 1.0 ml. of sample containing 5 to 100 µg. of protein, 5 ml. of alkaline copper solution (50 ml. of 2% Na_2CO_3 in 0.1 N NaOH + 1 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 photoelectric colorimeter using a 66 K.S. (red) filter.

DETERMINATION OF ACETOLACTATE SYNTHETASE ACTIVITY

Method A. Colorimetric Assay Procedure

To 0.75 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, and 0.1 ml. of a 200 μg./ml. thiamine diphosphate solution. The reaction was started 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 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 1 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 glass cuvette 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,

0.1 ml. of 2,3-butanediol dehydrogenase (4.5 units),

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

0.15 ml. of 3.0 mM DPNH.

The reaction was started 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 oxidation of DPNH at 340 mµ. The enzyme activity was determined by this method for kinetic and other studies unless otherwise stated.

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

1.0 M potassium phosphate buffer of pH 5.7, containing 0.1 mM thiamine diphosphate, and 0.5 mM $MnCl_2$ were made in lusteroid centrifuge tubes to a volume of 4.8 ml. One tenth of a milliliter of acetolactate synthetase (0.037 mg. protein) plus 0.1 ml. of a hemoglobin solution were premixed, then gently placed on top of the gradient using a micro pipette. The gradients were centrifuged at 39,000 r.p.m. for 10 hours in a Model L Spinco centrifuge, using a SW-39 swinging bucket rotor. At the end of the run the rotor was allowed to decelerate with the brake off. The tubes were removed from the rotor, and the bottom of each tube was punctured with a fine needle. Two-drop fractions were collected, and each fraction was assayed colorimetrically for enzyme activity. An enzyme peak was located by the method described for assay after DEAE-cellulose chromatography, using 10 ul. of eluent and incubating for 10 min. A hemoglobin peak was located by bringing the volume of each fraction to 2 ml. with distilled water, and measuring the amount of absorption at 405 mμ on a Gilford Multiple Sample Absorbance Recorder. The molecular weight was estimated using the following equation:

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

S_1 = the distance travelled from the meniscus by the enzyme,

S_2 = the distance travelled from the meniscus by the reference protein,

MW_1= the molecular weight of the enzyme,

MW_2= the molecular weight of the reference protein (hemoglobin = 67,000)

TREATMENT OF ACETOLACTATE SYNTHETASE WITH KBH_4

Enzyme was incubated in 2.25 ml. of 0.13 M potassium phosphate buffer (pH 5.7), 0.1 ml. thiamine diphosphate (20 µg.) and 0.1 ml. of 0.5 M sodium pyruvate, in a final volume of 2.50 ml, for 4 min., after which time 3 µmoles of KBH_4 were added to the reaction mixture. A control was carried out exactly as above except water was added in place of KBH_4. The reaction mixtures were then dialyzed separately against 1 liter of 0.05 M potassium phosphate buffer of pH 7.0 containing 0.1 mM thiamine diphosphate, 0.5 mM $MnCl_2$, and 0.1 mM dithiothreitol for 2 hours. A second control, containing KBH_4, but without dialyzing was also carried out. After dialysis the reaction mixtures were assayed for residual activity spectrophotometrically. Fifty micromoles of sodium pyruvate were added to initiate the reaction.

CHEMICALS

The following chemicals were obtained from commercial

sources:

Sodium pyruvate	Sigma Chemical Co.
DPNH (di sodium)	"
α-ketobutyrate	"
cis-Aconitic acid	"
Glucose 6-phosphate	"
Fructose 6-phosphate	"
Fructose 1,6-diphosphate	"
2-Phosphoglyceric acid d(+)	"
3-Phosphoglyceric acid (D(-)	"
Phosphoenol pyruvic acid	"
L-Glutamic acid	"
L-Aspartic acid	"
L-Valine	"
L-Isoleucine	"
Thiamine diphosphate (cocarboxylase)	"
Manganese chloride	Fisher Scientific Co.
α-Napthol	"
Dioxane	"
Fumaric acid	"
Sodium citrate	"
Potassium phosphate (mono. and dibasic)	"
Sodium potassium tartrate	"
Folin reagent	"

Sodium phenyl pyruvate	Nutritional Biochemicals Corp.
Creatine	"
Succinic acid	"
Malic acid	"
Salicylaldehyde	Matheson, Coleman, and Bell

RESULTS

RESULTS

PURIFICATION OF ACETOLACTATE SYNTHETASE

A summary of the purification of acetolactate synthetase is given in Table I 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. After this ammonium sulfate fractionation step, freezing of the recovered enzyme often resulted in as much as a 50% increase in enzyme activity.

Because of the great loss of activity during DEAE-cellulose chromatography, various cofactors and protecting agents were added to both the wash buffer and the elution 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-cellulose chromatography by the addition of sodium pyruvate to the buffers. Addition of dithiothreitol to the wash and elution buffers resulted in a 5 fold increase in enzyme recovery. Subsequently all buffers used for DEAE-cellulose chromatography were made containing 0.1 mM thiamine diphosphate, 0.5 mM

TABLE I

SUMMARY OF ACETOLACTATE SYNTHETASE PURIFICATION

Fraction	Total Protein (mg.)	Total Units	Specific Activity	Recovery %
Crude Extract	960	1660	1.73	100
0-45% Ethanol	232	1327	5.72	80
Protamine Sulfate	182	1110	6.04	70
0-65% $(NH_4)_2SO_4$	88	957	10.9	58
0-65% Ethanol	24	719	29.8	43.5
DEAE-cellulose	4.0	197	48.0	12

$MnCl_2$, and 0.1 mM dithiothreitol. Figure I shows the results of DEAE-cellulose chromatography on enzyme purification. Two enzyme peaks were observed, the first peak eluted immediately after the dead volume of the column, the second and major peak eluted between 0.1 M and 0.15 M potassium phosphate buffer. Occasionally only one peak was observed, this peak being eluted at 0.1 M potassium phosphate buffer (Fig. 2).

SPECTROPHOTOMETRIC ASSAY PROCEDURE

Although the colorimetric assay procedure was a reliable one, and gave a linear relationship between enzyme concentration and enzyme activity, it was a tedious one and not suitable for detailed kinetic study of the enzyme, using low concentrations of pyruvate. A spectrophotometric assay procedure was therefore developed, coupling the production of acetoin to DPNH oxidation using 2,3-butanediol dehydrogenase purified from *Aerobacter aerogenes.* From the double reciprocal plot of 2,3-butanediol dehydrogenase activity and acetoin concentration, shown in Figure 3, the Km for acetoin was calculated as 1.25 mM (Lineweaver and Burk, 1934). In the presence of excess acetolactate decarboxylase and 2,3-butenediol dehydrogenase the rate of DPNH oxidation by pyruvate was proportional to

FIGURE 1. DEAE-Cellulose Chromatography of Acetolactate Synthetase.

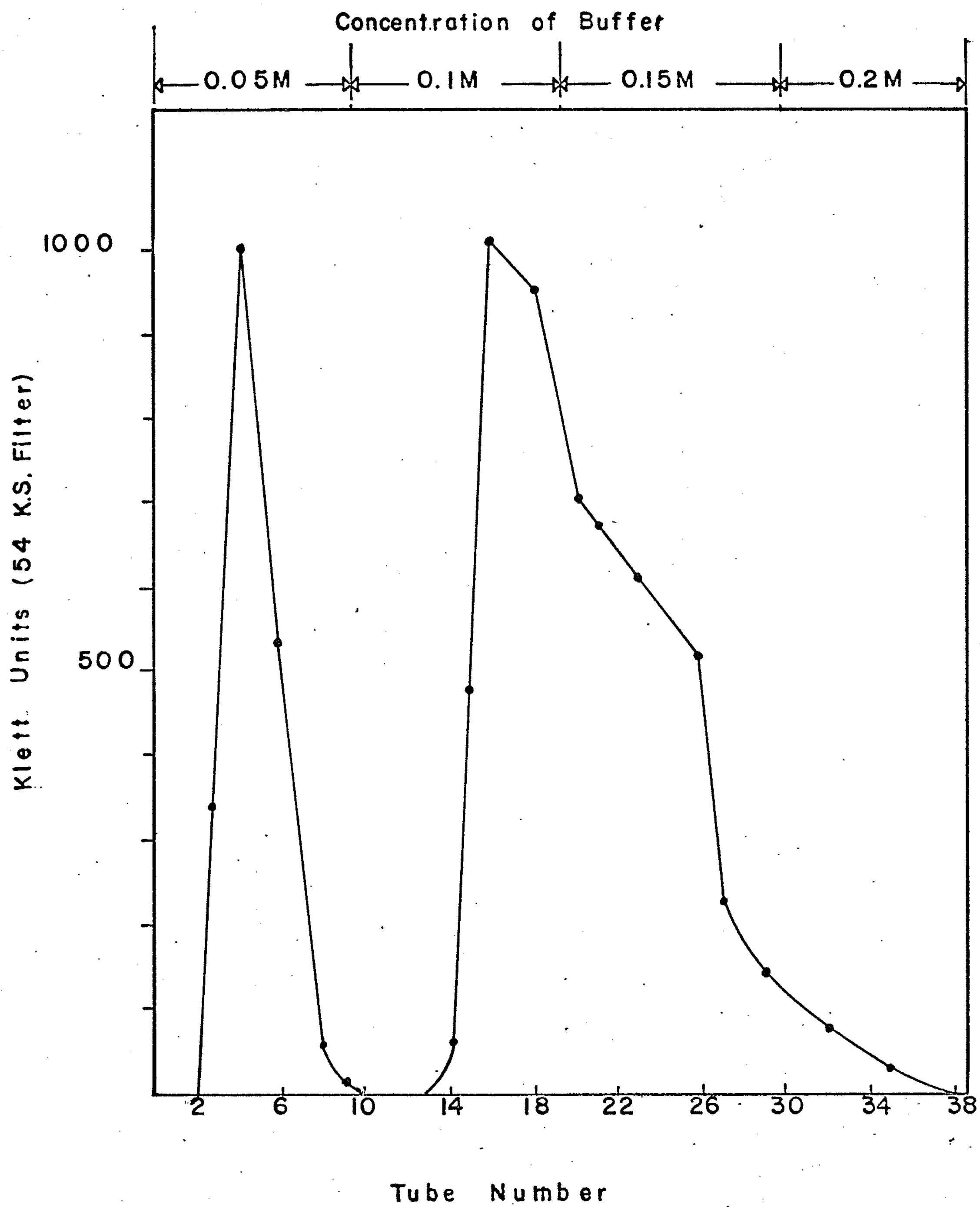

Concentration of Buffer
0.05M
0.1M
0.15M
0.2M
1000
500
Klett Units (54 K.S. Filter)
2
6
10
14
18
22
26
30
34
38
Tube Number

FIGURE 2. DEAE-Cellulose Chromatography of Acetolactate Synthetase.

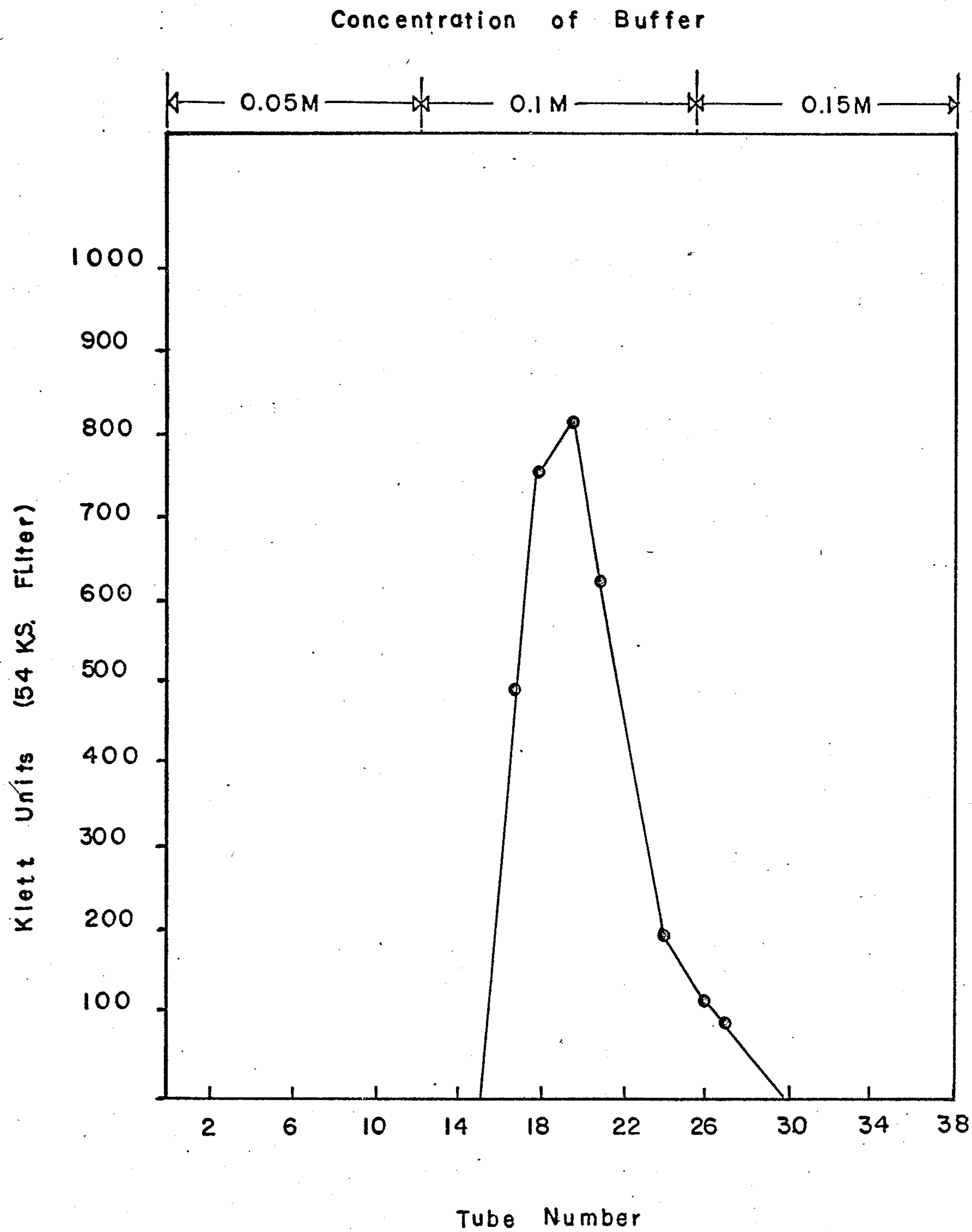
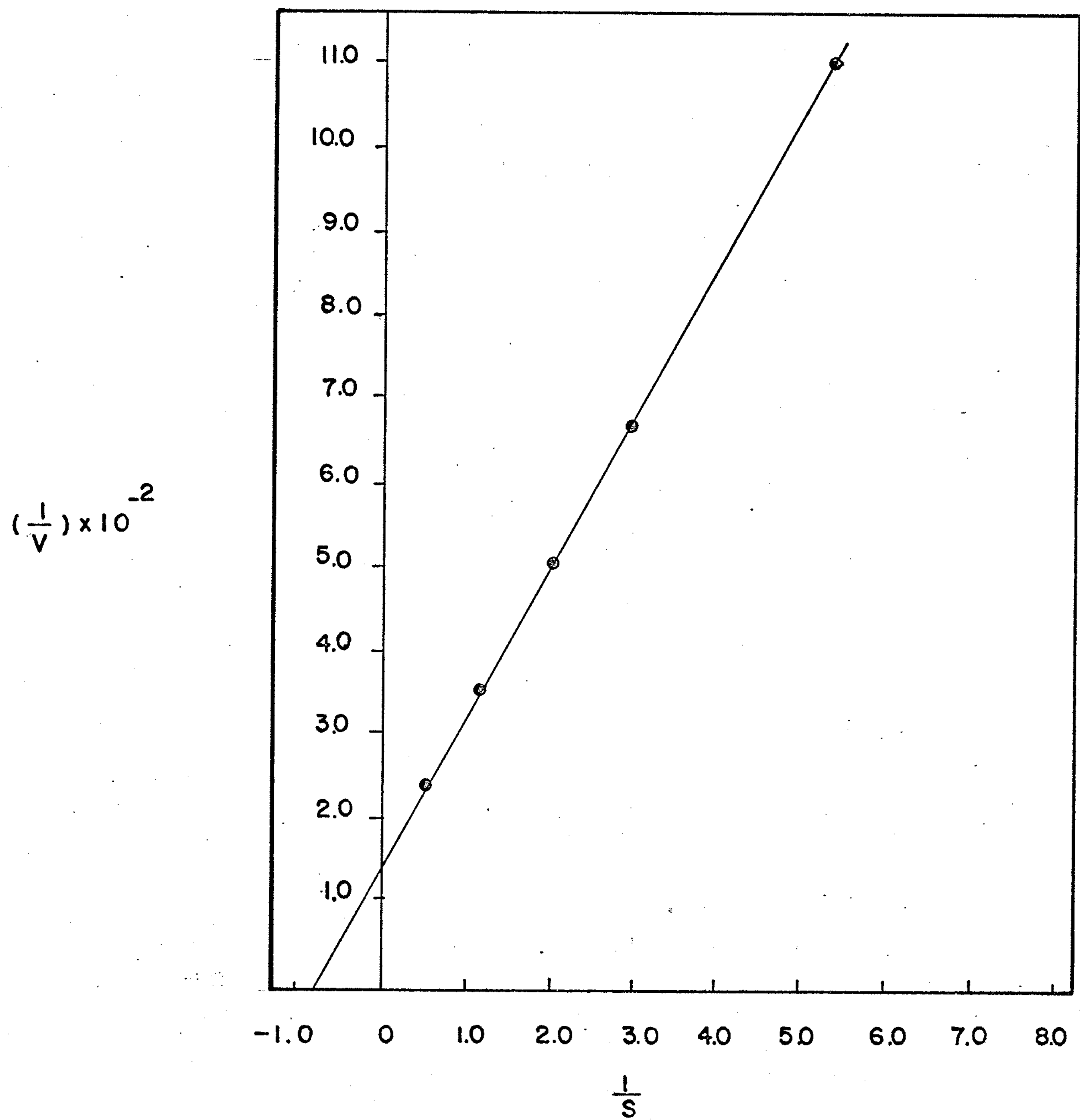

Concentration of Buffer
0.05M
0.1 M
0.15M
Klett Units (54 KS. Filter)
1000
900
800
700
600
500
400
300
200
100
2
6
10
14
18
22
26
30
34
38
Tube Number

FIGURE 3. Effect of Acetoin Concentration on 2,3-Butanediol Dehydrogenase Activity.

V = initial velocity of DPNH oxidation (optical density change per second at 340 mμ)

S = acetoin concentration (mM)


11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
$(\frac{1}{V}) \times 10^{-2}$
−1.0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
$\frac{1}{S}$

the amount of acetolactate synthetase present (Fig.4). 2,3-Butanediol dehydrogenase was inhibited by a high concentration of pyruvate (Table II). The spectrophotometric assay procedure for acetolactate synthetase, therefore, could not be used at a concentration of pyruvate greater than 50 μmoles per 3 ml.

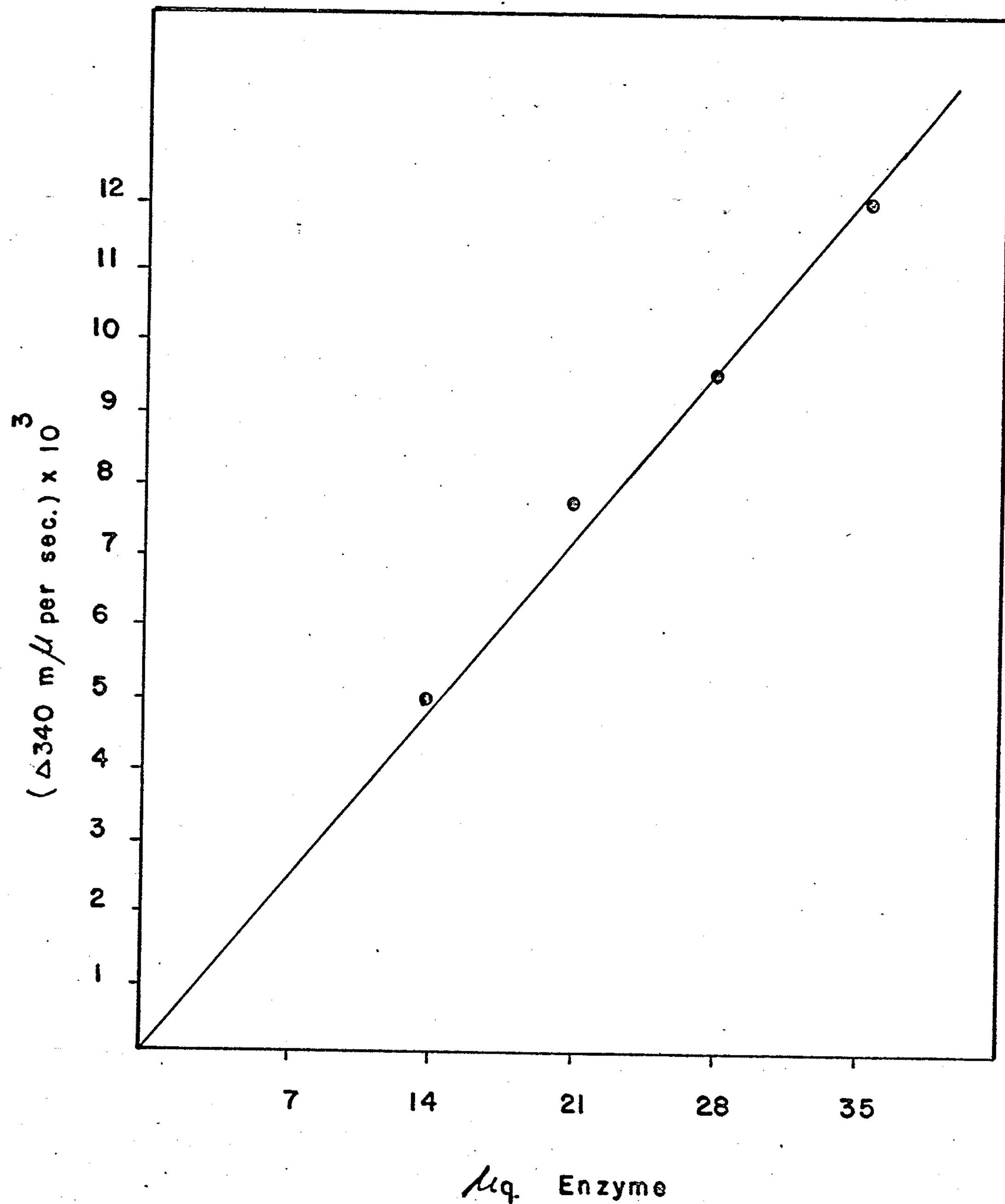
ENZYME ACTIVITY AND INCUBATION TIME

In a study of the relationship between enzyme activity and incubation time, a peculiar curve was obtained. As shown in Figure 5, an initial lag was observed, after which the reaction became linear. The lag was shortened with increasing enzyme concentration, but was always present. A similar lag period was also observed when the colorimetric assay procedure was used to follow the course of the enzyme reaction.

ENZYME ACTIVITY AND PYRUVATE CONCENTRATION

After measuring the linear portion of the enzyme reaction, double reciprocal plots of enzyme activity and pyruvate concentration yielded a non-linear Lineweaver-Burk plot (Fig.6). This non-linear plot was also obtained when the standard colorimetric techniques were used to follow the enzyme reaction (Fig.7).

FIGURE 4. Effect of Enzyme Concentration on the Spectrophotometric Assay of Acetolactate Synthetase Activity.



12
11
10
9
8
7
6
5
4
3
2
1
(Δ340 mμ per sec.) x 10^3
7
14
21
28
35
μg. Enzyme

TABLE II

EFFECT OF PYRUVATE CONCENTRATION ON

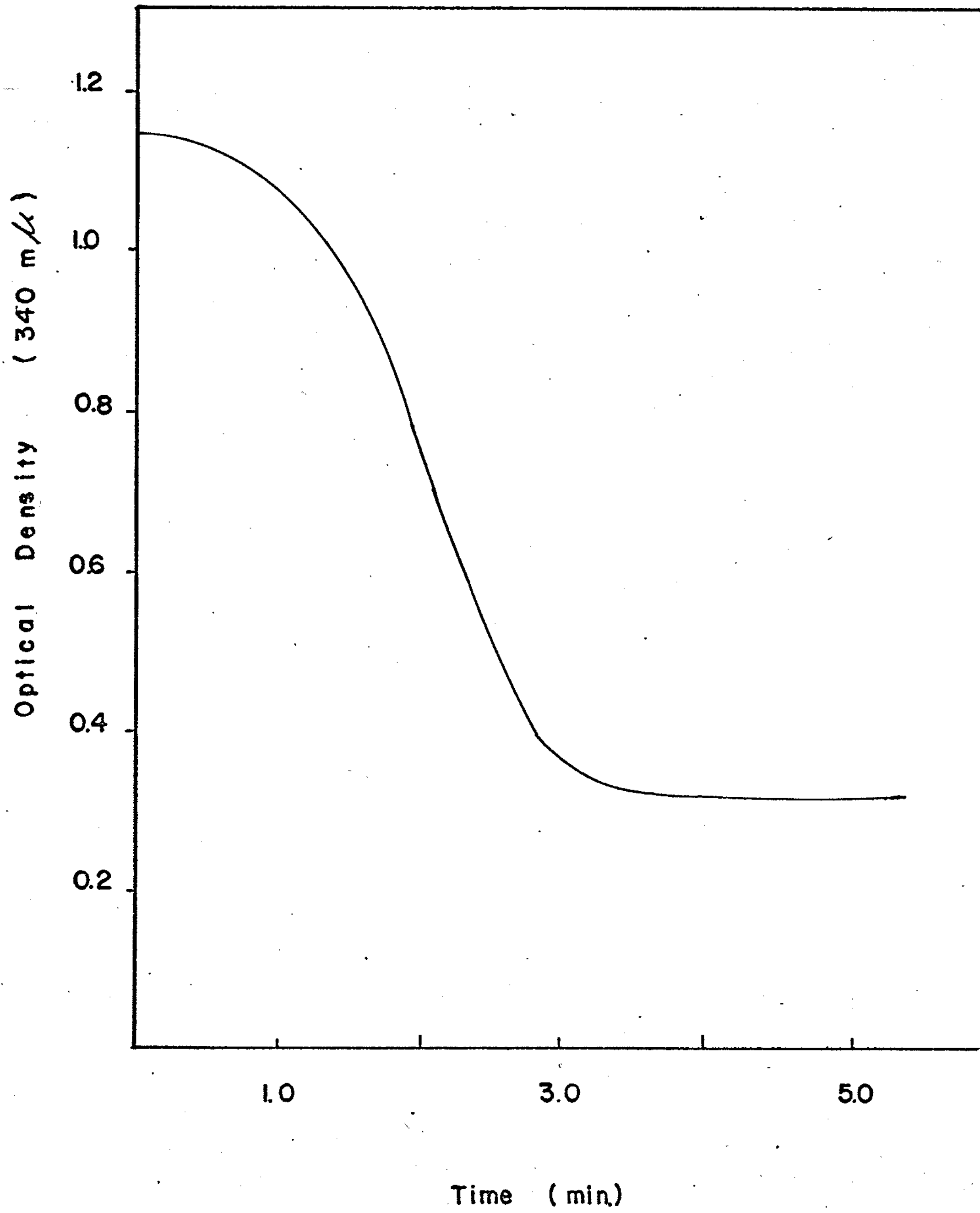
2,3-BUTANEDIOL DEHYDROGENASE

μmoles Pyruvate	Activity %
0.0	100
30	80
50	80
75	32
100	28

Activity was measured spectrophotometrically as described in Materials and Methods.

FIGURE 5. Acetolactate Synthetase Activity and Incubation Time.

Activity was measured spectrophotometrically.

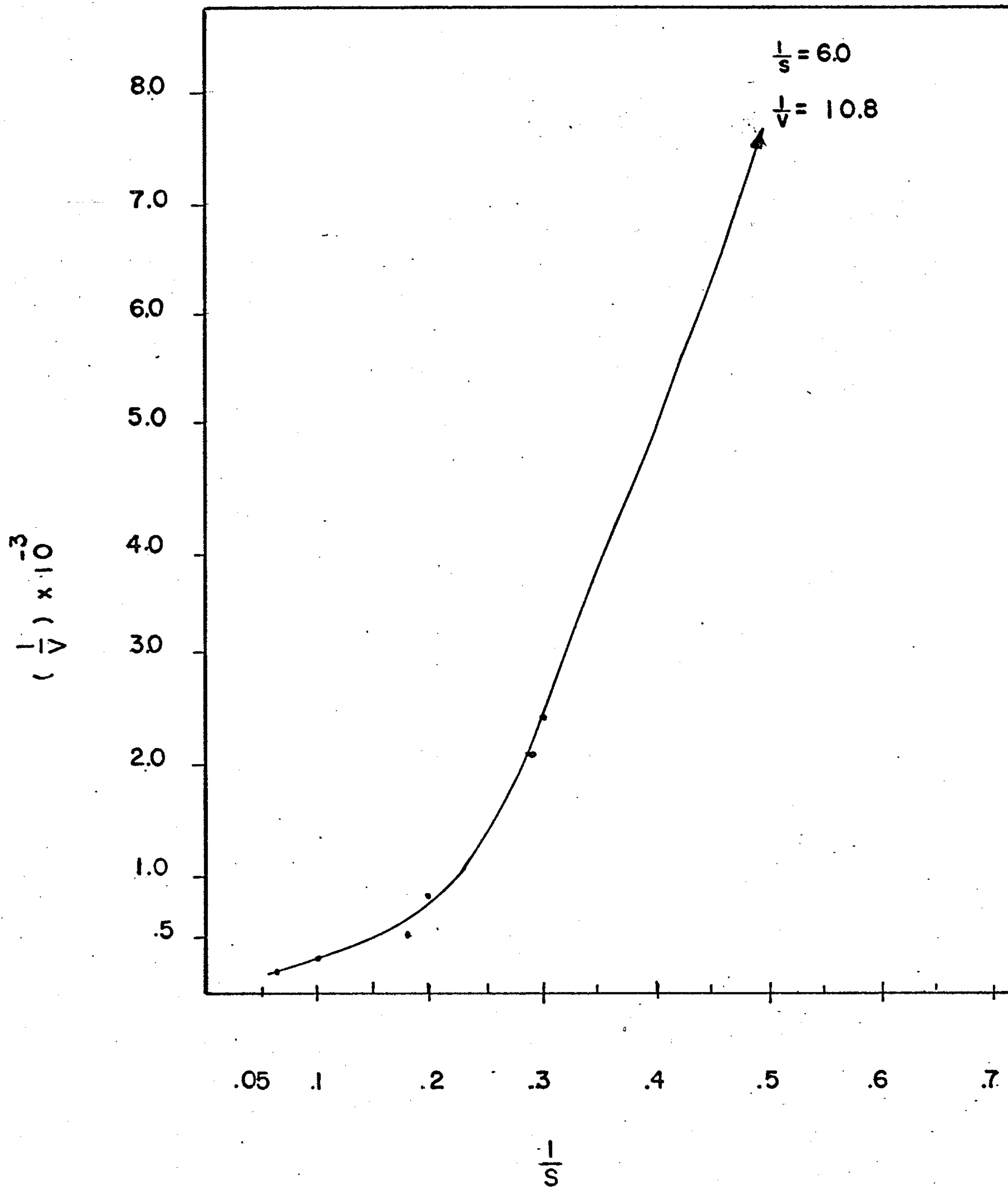


Optical Density (340 mμ)
1.2
1.0
0.8
0.6
0.4
0.2
1.0
3.0
5.0
Time (min.)

FIGURE 6. Effect of Pyruvate Concentration on Acetolactate Synthetase Activity. (Spectrophotometric Assay).

V = change in optical density per second at 340 mμ.

S = pyruvate concentration (mM)

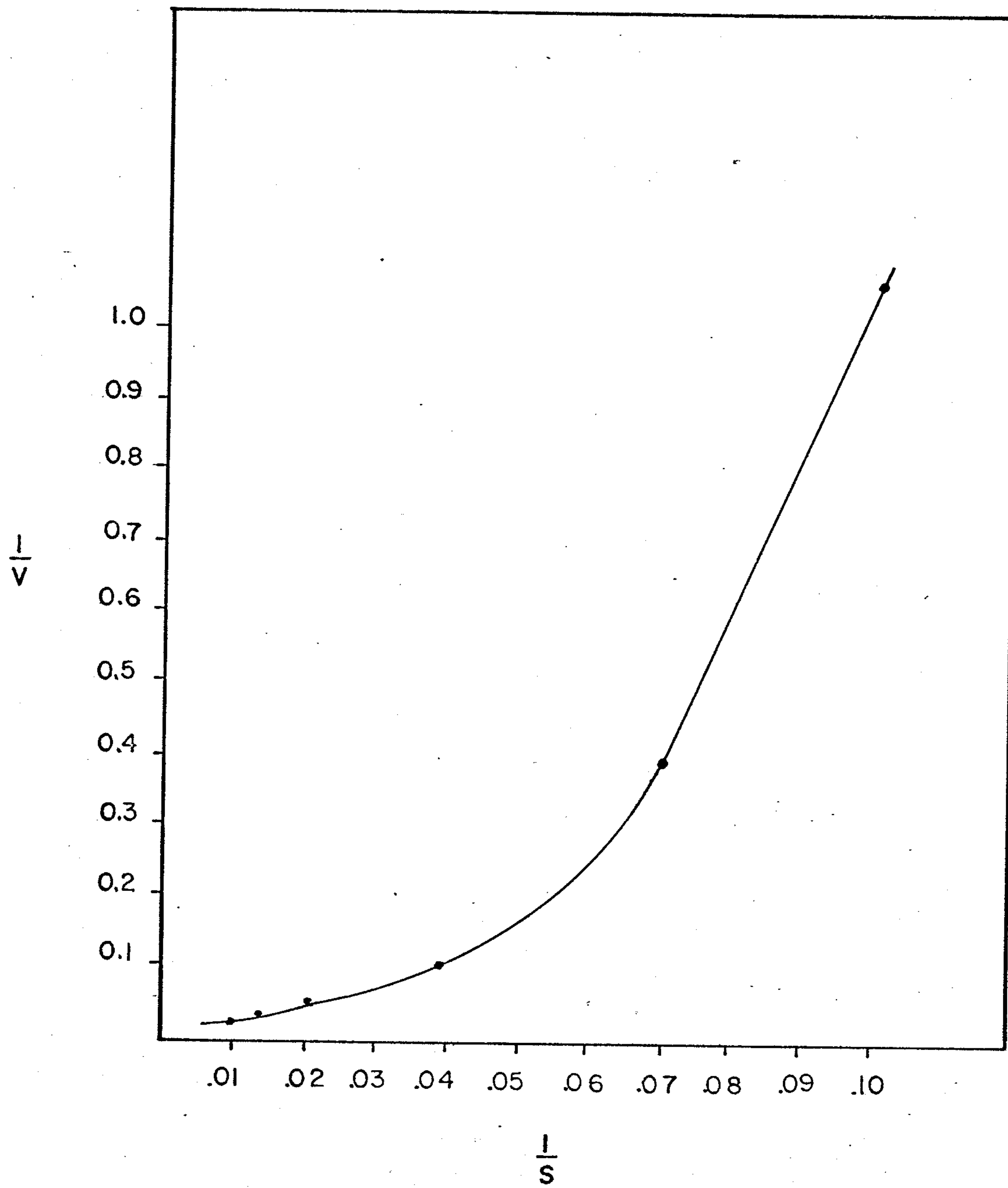


$\frac{1}{s}$ = 6.0
$\frac{1}{v}$ = 10.8
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
.5
$(\frac{1}{v}) \times 10^{-3}$
.05
.1
.2
.3
.4
.5
.6
.7
$\frac{1}{S}$

FIGURE 7. Effect of Pyruvate Concentration on Acetolactate Synthetase Activity. (Colorimetric Assay).

V = µmoles acetoin produced in 10 min.

S = pyruvate concentration (mM).



1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1/V
.01
.02
.03
.04
.05
.06
.07
.08
.09
.10
1/S

ACTIVITY OF CRUDE EXTRACT AND PYRUVATE CONCENTRATION

As shown in Figure 8, the crude extract yielded a non-linear plot when the reciprocal of enzyme activity was plotted against the reciprocal of pyruvate concentration. This result was consistent with that obtained for the purified enzyme.

STABILITY OF ACETOLACTATE SYNTHETASE

In the absence of thiamine diphosphate and $MnCl_2$, all the enzyme activity was destroyed after heating at 55°C., for 3 min. In the presence of these cofactors 70% of the activity remained after the same treatment, and 30% of the activity remained after heating at 60°C. for 3 min. In the presence of thiamine diphosphate and $MnCl_2$, pyruvate and mercaptoethanol gave further protection to the enzyme during heating as shown in Table III.

The enzyme was stable to freezing at -20° C. for at least several months. Occasionally freezing of the purified enzyme resulted in up to a 10 fold increase in enzyme activity.

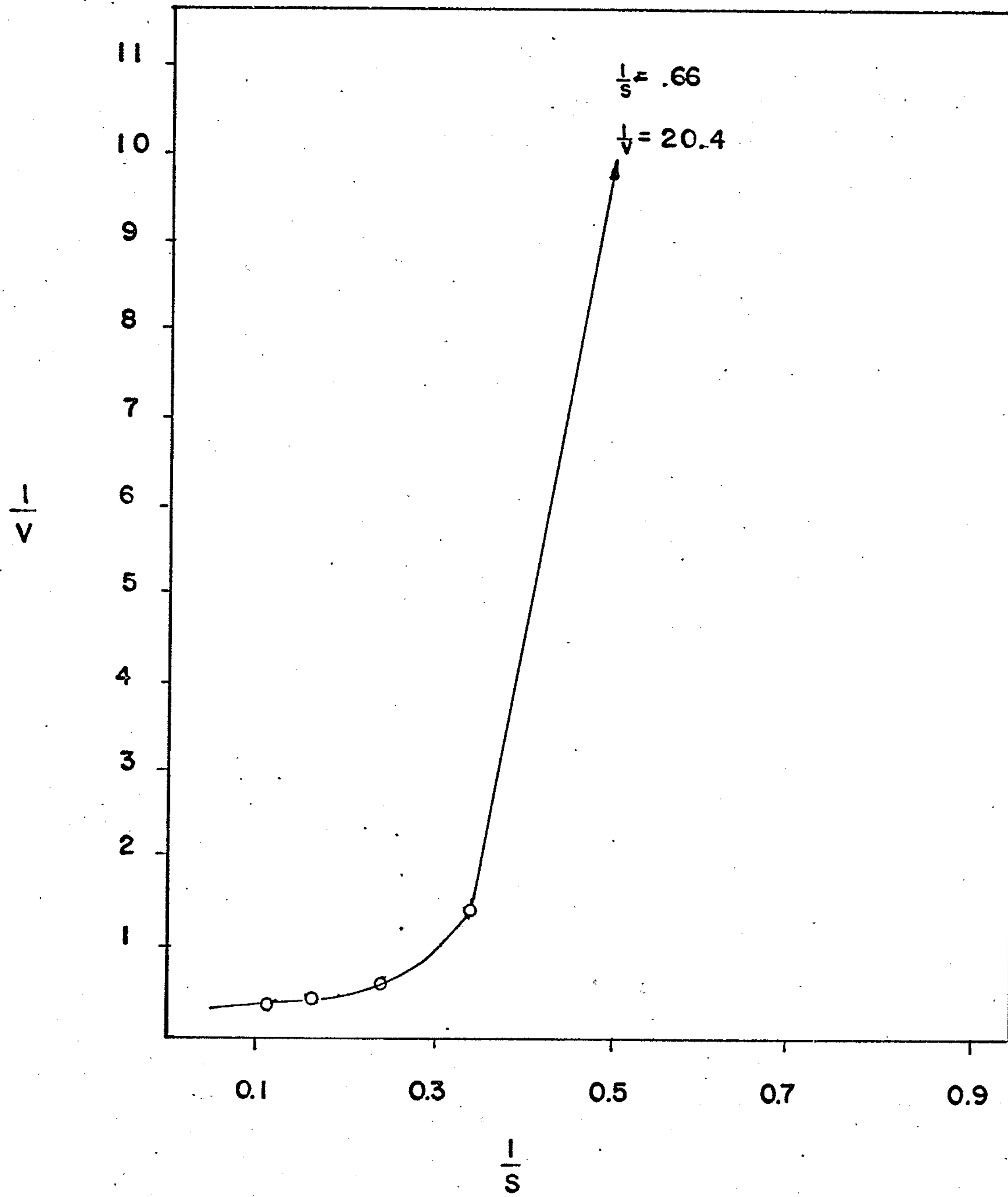
EFFECT OF PREINCUBATION WITH PYRUVATE

Because of the initial lag in the enzyme reaction and the non-linear Lineweaver-Burk plot, it was difficult to

FIGURE 8. Effect of Pyruvate Concentration on Acetolactate Synthetase Activity in Crude Extract (Colorimetric Assay).

V = umoles acetoin produced in 10 min.

S = pyruvate concentration (mM).



1/s = .66
1/v = 20.4
1/v
1/s
11
10
9
8
7
6
5
4
3
2
1
0.1
0.3
0.5
0.7
0.9

TABLE III

EFFECT OF PYRUVATE AND MERCAPTOETHANOL ON THE HEAT STABILITY OF ACETOLACTATE SYNTHETASE

	Reactants	Activity %
I.	Complete system	100
II.	Complete system minus pyruvate	42.5
III.	Complete system minus mercaptoethanol	64
IV.	Complete system minus pyruvate and mercaptoethanol	42
V.	Unheated enzyme	100

The complete system contained, in a total volume of 0.22 ml., 0.1 μmole of mercaptoethanol, 1.0 μmole of sodium pyruvate, 20 μg. of thiamine diphosphate, and 0.1 ml. of enzyme (60 μg.) previously dialyzed for 2 hours against 1 liter of 0.05 M potassium phosphate buffer of pH 7.0, containing 0.1 mM thiamine diphosphate and 0.5 mM $MnCl_2$, to remove dithiothreitol. The mixtures were heated for 3 min. at 55°C., cooled in an ice bath, and then assayed for residual activity.

carry out kinetic studies on the enzyme. It was desirable to remove this lag in the hope that a linear reaction rate would result in a linear Lineweaver-Burk plot. The enzyme was preincubated with a small amount of pyruvate in an effort to remove the lag. As shown in Table IV, the treatment activated the enzyme, but the initial lag was not removed. Dialysis of the enzyme with sodium pyruvate (100 μmole/ml. of enzyme) resulted in 56% greater activity than enzyme dialyzed without pyruvate, but again the lag was not removed. The double reciprocal plots of enzyme activity and pyruvate concentration were not affected by this treatment.

EFFECT OF COMPOUNDS STRUCTURALLY RELATED TO PYRUVATE

Various compounds which have a structure similar to that of pyruvic acid were employed in an attempt to remove the initial lag from the enzyme reaction. Incubation of the enzyme with sodium phenyl pyruvate (10 mM final conc.), α-ketoglutarate (10 mM final conc.), and α-ketobutyrate (30 mM final conc., assayed colorimetrically) exhibited 53%, 96% and 36% inhibition of enzyme activity respectively. No difference in enzyme activity was observed with 0.1 mM (final conc.) of α-ketoglutarate. Dialysis of the enzyme with salicylaldehyde (10 mM final conc.) against

TABLE IV

EFFECT OF PYRUVATE ON ENZYME ACTIVITY DURING PREINCUBATION AT 28°C.

	Reactants	Activity %
I.	Complete system	267
II.	Complete system minus pyruvate	100
III.	Complete system minus mercaptoethanol	170
IV.	Complete system minus pyruvate and mercaptoethanol	100

The complete reaction system contained in a volume of 0.12 ml., 0.1 μmole of mercaptoethanol, 1.0 μmole of sodium pyruvate, and 0.1 ml.(60 μg.) of enzyme previously dialyzed for 2 hours against 1 liter of 0.05 M potassium phosphate buffer of pH 7.0, containing 0.1 mM thiamine diphosphate, and 0.5 mM $MnCl_2$ to remove dithiothreitol. The mixtures were incubated at 28°C. for 30 min., then assayed for residual activity.

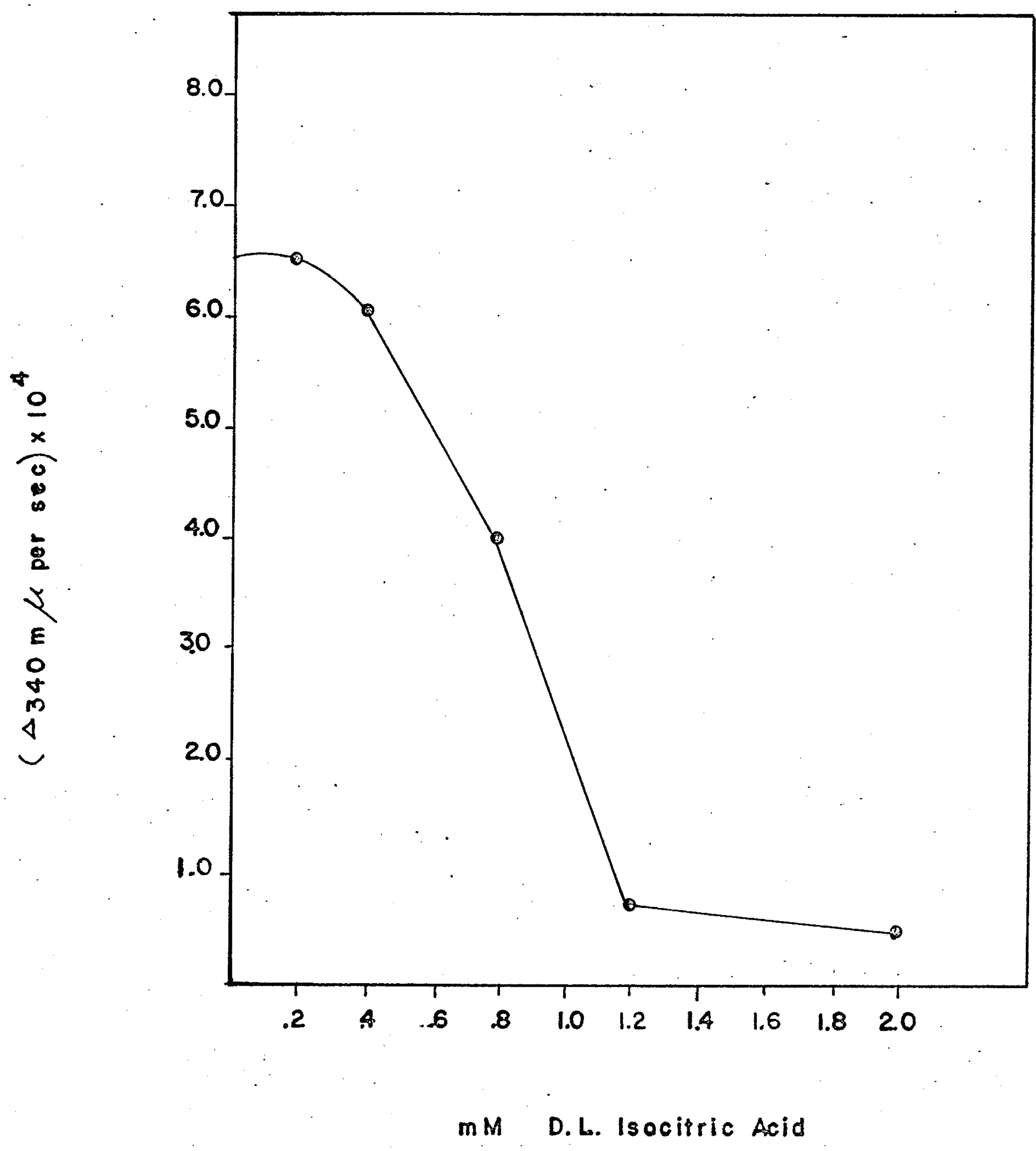
0.05 M potassium phosphate buffer of pH 7.0 for 2 hours resulted in 40% inhibition of enzyme activity. Addition of acetaldehyde to the assay system (10 mM final conc.) resulted in a 25% increase in enzyme activity. None of the compounds mentioned had any effect on removing the lag from the enzyme reaction.

EFFECT OF METABOLIC INTERMEDIATES ON ENZYME ACTIVITY

Glucose, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, 2-phosphoglyceric acid, 3-phosphoglyceric acid, phosphoenol pyruvic acid, and acetyl CoA were shown to have no effect on enzyme activity or on the removal of the initial lag when added to the assay mixture. The final concentration of each compound was 1.0 mM, except acetyl CoA which was 0.1 mM. Results with sodium citrate, cis-aconitate, succinate, fumarate, and malate, (all 1.0 mM final conc.) were inconsistent, all showing slight inhibition at times and at other times showing no effect. Enzyme activity was measured by both the colorimetric and the spectrophotometric procedures using 10 μmoles of sodium pyruvate per 3 ml. assay mixture. Isocitric acid was the only intermediate tested which showed consistent inhibition of enzyme activity, inhibition being complete at 2.0 mM (final conc.) D-L isocitric acid. At 0.2 mM (final conc.) no effect was observed (Fig.9). Addition

FIGURE 9. Effect of Isocitric Acid on Acetolactate Synthetase Activity.

Activity was measured spectrophotometrically under standard conditions.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
(Δ340 mμ per sec) x 10⁴
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
mM D. L. Isocitric Acid

of D-L isocitric acid (2.0 mM final conc.) to the assay mixture during the linear portion of the enzyme reaction resulted in immediate and complete inhibition of enzyme activity (Fig.10).

ISOCITRIC ACID AND PYRUVATE CONCENTRATION

Double reciprocal plots of enzyme activity and sodium pyruvate concentration in the presence of 2.0 mM D-L isocitric acid (final conc.) are shown in Figure 11. Increasing concentrations of pyruvate reduced the inhibitory action of isocitric acid.

EFFECT OF AMINO ACIDS

L-valine, L-isoleucine, L-glutamic acid, and L-aspartic acid showed no effect on enzyme activity at a final concentration of 1.0 mM. Activity was measured spectrophotometrically using 10 µmoles of sodium pyruvate.

EFFECT OF PH ON ENZYME ACTIVITY

As shown in Figure 12, the enzyme showed a pH optimum around 6.0. There was a marked decrease in enzyme activity at pH 7.0 where 19% of the activity at pH 6.0 was observed. The decrease was less marked on the acid side of the optimum, where 76% of the activity was observed at pH 4.5.

FIGURE 10. Effect of Addition of Isocitric Acid During the Linear Portion of the Enzyme Reaction.

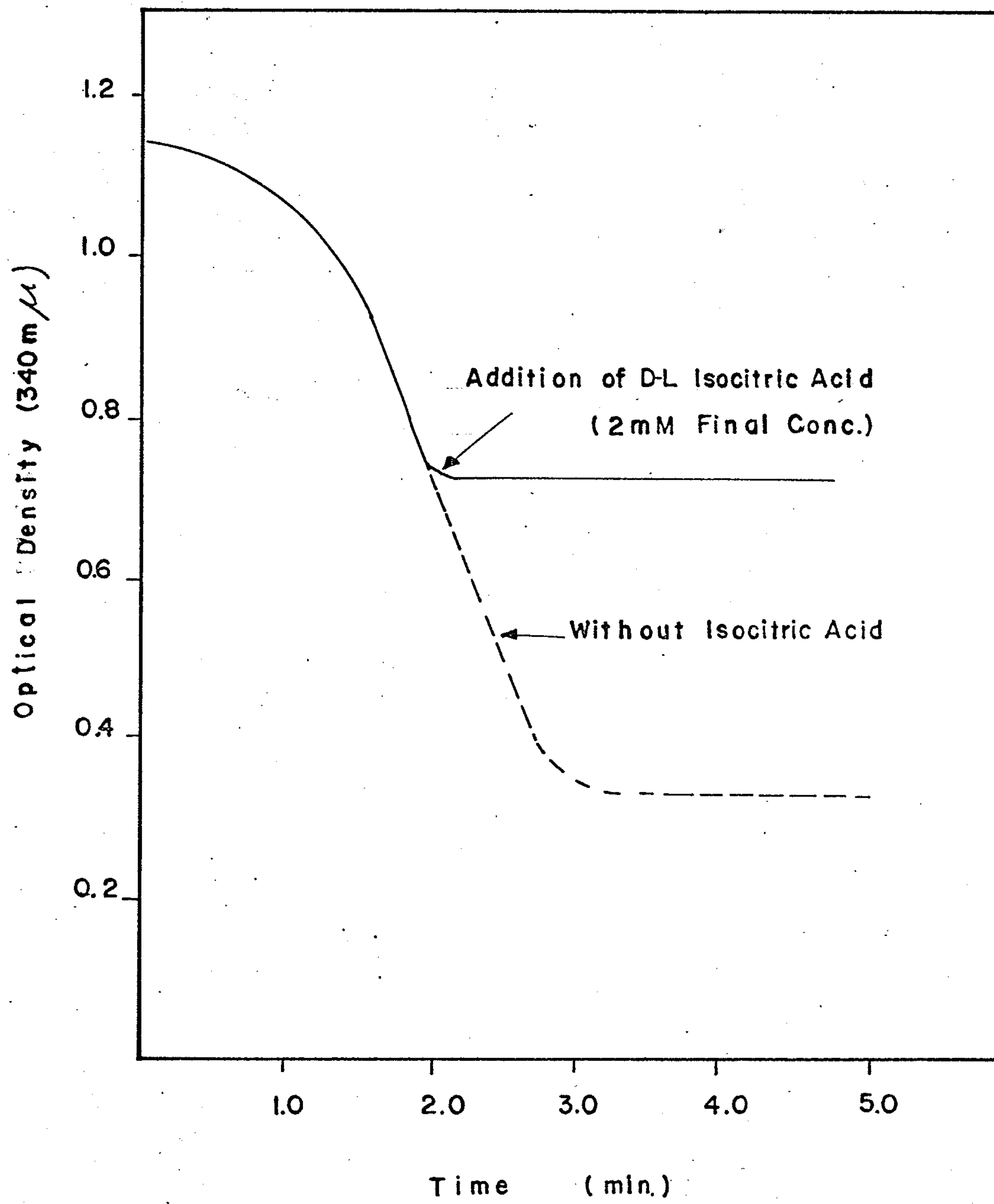

Optical Density (340 mμ)
1.2
1.0
0.8
0.6
0.4
0.2
Addition of D-L Isocitric Acid
(2mM Final Conc.)
Without Isocitric Acid
1.0
2.0
3.0
4.0
5.0
Time (min.)

FIGURE 11. Effect of Isocitric Acid on Acetolactate Synthetase Activity at Various Concentrations of Pyruvate.

V = change in optical density per second at 340 mμ.

S = concentration of pyruvate (mM)

A = no isocitric acid

B = 1.0 mM isocitric acid

C = 1.6 mM isocitric acid.

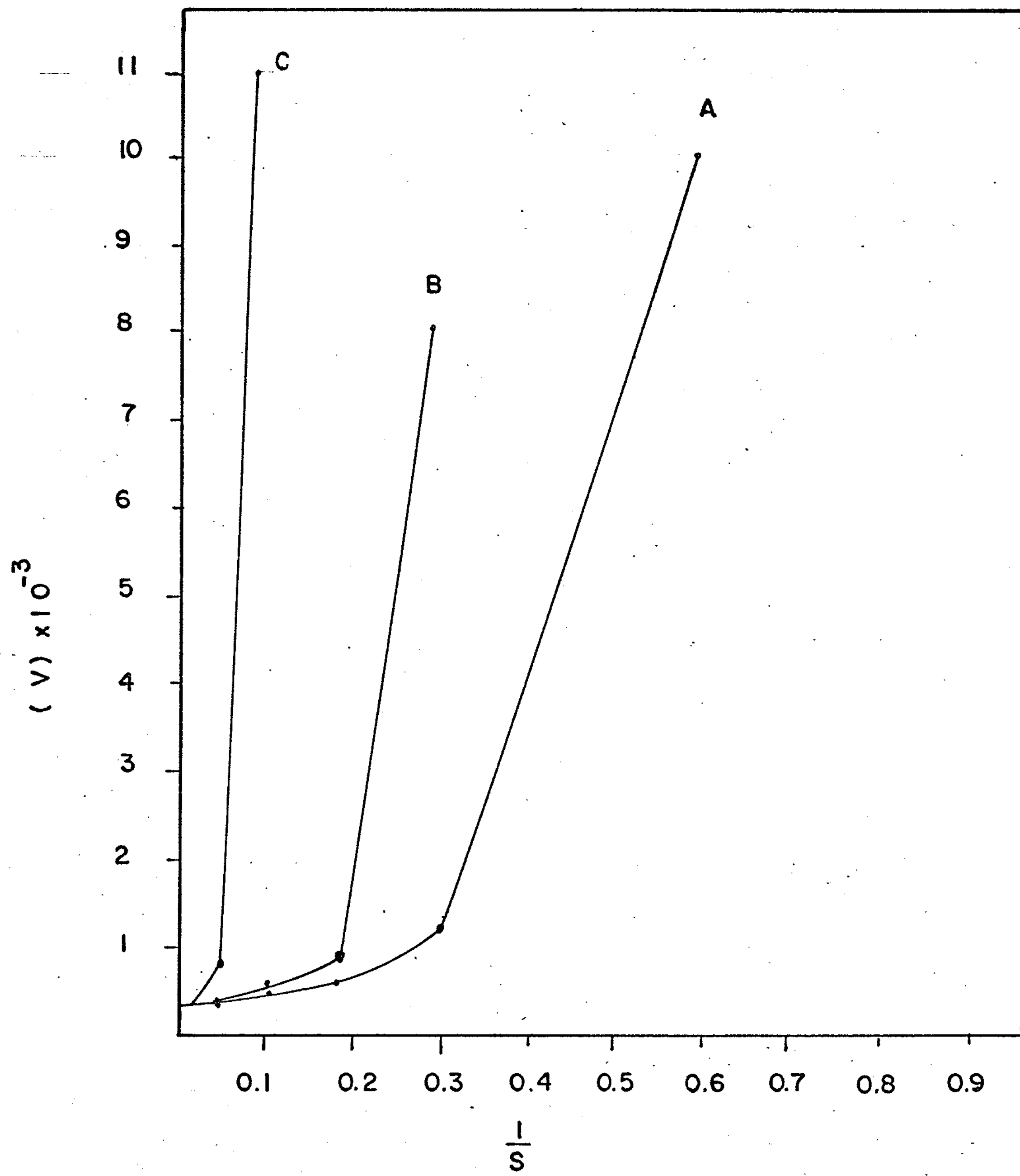
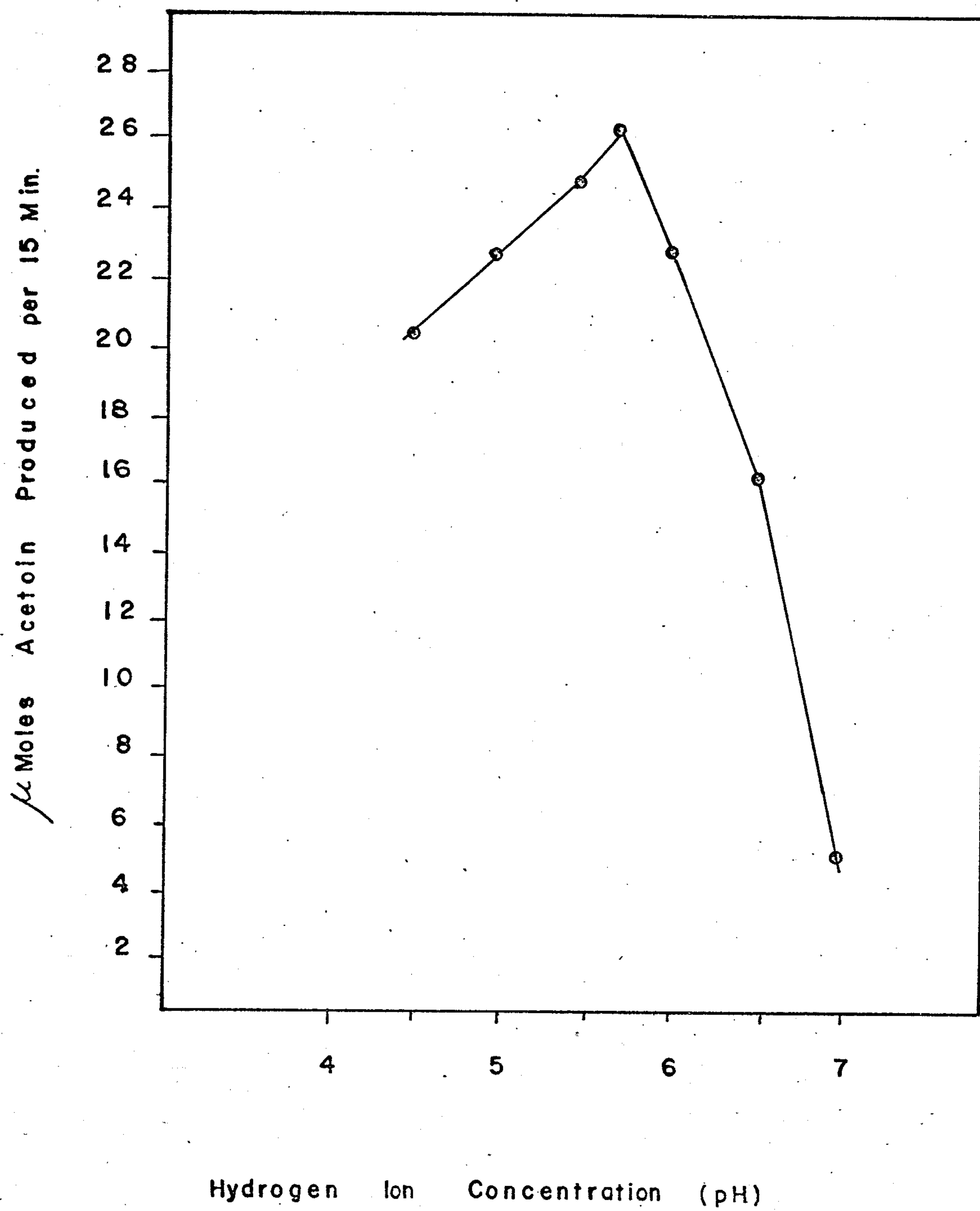

C
A
B
11
10
9
8
7
6
5
4
3
2
1
(V) x 10^{-3}
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1/S

FIGURE 12. Effect of Hydrogen Ion Concentration on Acetolactate Synthetase Activity.

The colorimetric assay procedure was used.


µ Moles Acetoin Produced per 15 Min.
28
26
24
22
20
18
16
14
12
10
8
6
4
2
4
5
6
7
Hydrogen Ion Concentration (pH)

EFFECT OF INORGANIC SALTS

No difference in enzyme activity was observed when the enzyme was assayed colorimetrically in 0.02 M, and in 0.1 M potassium phosphate buffer, indicating that phosphate concentration does not affect enzyme activity.

Addition of 0.015 ml. of a saturated ammonium sulfate solution to the enzyme assay mixture resulted in 88% inhibition of enzyme activity.

EFFECT OF ORGANIC SOLVENTS

Ethanol (3% final conc.) showed no effect on enzyme activity. Dioxane (10% final conc.) exhibited 44% inhibition of enzyme activity.

EFFECT OF UREA

Addition of urea (1.0 M final conc.) to the assay mixture resulted in complete inhibition of enzyme activity.

MOLECULAR WEIGHT OF ACETOLACTATE SYNTHETASE

Three trials of sucrose gradient centrifugation to estimate the molecular weight of acetolactate synthetase gave the values 238,000, 249,000, 239,600 (average = 242,000). With sodium pyruvate (10.0 mM final conc.) in the gradient

the molecular weight was estimated from two trials as 186,750, and 149,400 (average = 168,000). With isocitrate (1.0 mM final conc.) present the enzyme sedimented to the bottom of the tubes during centrifugation, with complete loss of activity.

TREATMENT WITH KBH_4

Treatment of the enzyme with KBH_4 (1 μmole/ml. of assay mixture), followed by dialysis, showed no effect on enzyme activity when the enzyme was assayed spectrophotometrically. There was formation of acetoin in the undialyzed reaction mixture, but the enzyme activity remained unchanged by the borohydride treatment. In order to examine the effect of a large excess of KBH_4 the colorimetric assay procedure was employed, adding 0.5-1.0 mg. of KBH_4 in small quantities to the reaction mixture immediately after addition of pyruvate. Because of the inhibition of color formation by KBH_4 in the colorimetric assay it was not possible to determine the residual activity without the removal of KBH_4. After dialysis of the reaction mixture for 2 hours against 0.13 M potassium phosphate buffer of pH 5.7 containing 0.1 mM thiamine diphosphate, 0.5 mM $MnCl_2$, and 0.1 mM dithiothreitol, 25% of the activity was observed.

EFFECT OF QUINACRINE DIHYDROXYCHLORIDE

No difference in activity was observed when acetolactate synthetase was assayed in the presence of quinacrine dihydrochloride (0.1 mM final conc.).

EFFECT OF BOILED EXTRACT

To determine whether there were any soluble, heat stable cofactors present in boiled extract which might affect the activity of acetolactate synthetase, 0.1 ml. of boiled extract of *Aerobacter aerogenes* was added to the enzyme assay mixture. No difference in activity was observed.

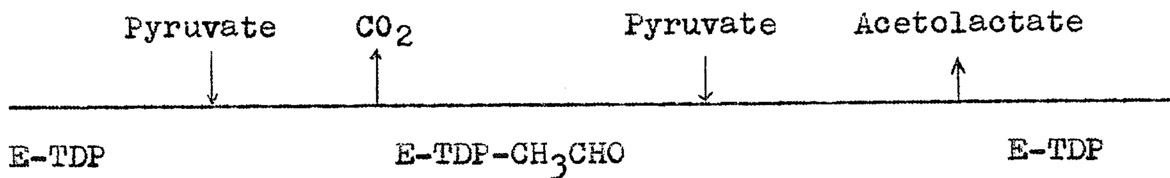
DISCUSSION

DISCUSSION

Thiamine diphosphate, $MnCl_2$, and sulfahydryl reagents protected acetolactate synthetase during purification procedures. The observation of two enzyme peaks during DEAE-cellulose chromatography was not due to overloading of the column, or to incomplete removal of salt, but must have been due to intrinsic properties of the enzyme. In view of the fact that the results of sucrose density gradient centrifugation studies suggest a possibility of different degrees of aggregation of the enzyme, the initial peak in DEAE-cellulose chromatography may have been due to greater aggregation of the enzyme than the enzyme eluted in the second peak. This idea is supported by an increased ratio of the first peak to the second peak when the enzyme protein concentration of the sample placed on the column was increased. Occasionally the first peak eluate came out as an opalescent solution indicating a high degree of aggregation.

The new spectrophotometric method developed for the determination of enzyme activity is particularly suitable for kinetic studies using physiological concentrations of pyruvate. Previous studies of acetolactate synthetase using either the manometric, or the colorimetric method (Juni, 1952, Baurle _et al_, 1964) gave a linear Lineweaver-Burk plot, since the pyruvate concentrations used were 0.1 M or even higher.

In the present study the Lineweaver-Burk plot gave a non-linear curve which was particularly obvious at lower pyruvate ranges. The results were unexpected since the mechanism of acetolactate formation proposed by Krampitz <u>et al</u> (1961) should give Ping-Pong kinetics (Cleland,1963) as shown below (E:enzyme).



In the Ping-Pong Bi Bi reaction (Cleland, 1963) involving substrates A and B, the initial velocity, V, is given by the following equation:

$$V = \frac{K_1 AB}{K_2 A + K_3 B + K_4 AB} \qquad (K_1,\ K_2,\ K_3,\ K_4\text{: constants})$$

When A = B = pyruvate,

$$V = \frac{K_1 A^2}{(K_2 + K_3)A + K_4 A^2} \qquad \frac{K_1 A}{K_2 + K_3 + K_4 A}$$

The reciprocal form is:

$$\frac{1}{V} = \frac{K_4}{K_1} + \frac{K_2 + K_3}{K_1} \left(\frac{1}{A}\right)$$

which should give a linear double reciprocal plot of enzyme activity and pyruvate concentration.

Since the Lineweaver-Burk plot was non-linear, the mechanism of acetolactate synthetase must be different from the simple Ping-Pong reaction.

An ordered Bi Bi reaction (Cleland, 1963) gives the following initial velocity equation:

$$V = \frac{K_1AB}{K_2 + K_3A + K_4B + K_5AB} \quad (K_1, K_2, K_3, K_4, K_5\text{:constants})$$

When A = B = pyruvate.

$$V = \frac{K_1A^2}{K_2 + (K_3 + K_4)A + K_5A^2}$$

which is a parabolic function in the double reciprocal form.

A preliminary effort to fit the data to a parabola using an IBM 1620 digital computer (Maeba and Sanwal, 1966) was unsuccessful. From the steep rise of the double reciprocal plot at lower pyruvate concentrations it is obvious that the function is more complicated than a simple parabola. It is probably a cubic function or higher, involving at least one activation process by pyruvate. A more detailed kinetic study is required in order to clarify this situation.

Every effort to remove the initial lag period from

the enzyme reaction was unsuccessful. Since the lag period was also observed when the colorimetric procedure was used to follow the course of the enzyme reaction, it was not due to the coupled assay system. Also, since the only compound which removed the lag was the substrate, pyruvate, it is believed that pyruvate converts the enzyme to an active form. The sucrose density gradient centrifugation studies indicate a change in the molecular weight of the enzyme in the presence of pyruvate. Without pyruvate the molecular weight of the enzyme was found to be 242,000, while with pyruvate it was found to be 168,000. These results may indicate that the enzyme consists of three subunits, each with a molecular weight of 80,000. In the presence of pyruvate the enzyme may change to an active form which consists of two subunits. These hypotheses will have to be tested in further studies.

Failure to find activators which can replace pyruvate in removing the lag period from the enzyme reaction, or straighten out the non-linear Lineweaver-Burk plot was very disappointing from the standpoint of clear-cut kinetic analyses. None of the compounds tested, either structurally or metabolically related to pyruvate removed the initial lag, or activated the enzyme, except acetaldehyde which showed some activation.

Inhibition of acetolactate synthetase by isocitrate seemed to be competitive with pyruvate since the inhibition was removed at high concentrations of pyruvate. It is, however, difficult to make a definite conclusion because the non-linear Lineweaver-Burk plot makes the analysis difficult. When the enzyme was centrifuged in a sucrose gradient in the presence of 1.0 mM isocitrate, all the enzyme protein was found at the bottom of the centrifuge tube, but no enzyme activity was recovered. Thus it seems that isocitrate inhibits the enzyme activity by forming inactive aggregates.

The concentration of KBH_4 used by other workers for the reduction of Schiff's base intermediate is less than 1.0 µmole per ml. of reaction mixture, the concentration used in the present study. Thus the results obtained with the spectrophotometric assay clearly indicate that Schiff's base formation is not involved in the formation of α-acetolactic acid by acetolactate synthetase. It appears that the thiamine-pyruvate interaction obviates the Schiff's base formation, which is necessary for aldol-type condensation reactions not involving thiamine diphosphate. The inhibition of enzyme activity by a large excess of KBH_4 may have been due to a secondary effect on the enzyme rather than the reduction of a Schiff's base.

Even after such a drastic treatment 25% of the enzyme activity remained, contrary to Schiff's base mechanism.

Since the addition of boiled extract to the enzyme assay mixture did not yield an increase in enzyme activity, it appears that there is no heat-stable cofactor requirement by this enzyme, in contrast to the biosynthetic acetolactate synthetase which requires flavine adenine dinucleotide (Stormer, and Umbarger, 1964). Failure of quinacrine dihydrochloride to inhibit enzyme activity at 0.1 mM further supports the conclusion that flavine is not required by the enzyme under study. These observations and the fact that the enzyme showed a pH optimum around 6.0 and was not inhibited by either valine or isoleucine make it apparent that the enzyme under study is distinct from the biosynthetic enzyme, which has a pH optimum of 8.0 and is inhibited by valine (Umbarger, and Brown, 1958; Bauerle et al, 1964; Halpern and Umbarger, 1959).

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